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Elucidation of Mechanisms Underlying Metastatic Melanoma Immune Escape via Suppression of Major Histocompatibility Complex (MHC) II through Dysregulation of the JAK/STAT Pathway

Jodi Osborn

ELUCIDATION OF MECHANISMS UNDERLYING METASTATIC MELANOMA IMMUNE ESCAPE VIA SUPPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) II THROUGH DYSREGULATION OF THE JAK/STAT PATHWAY

by

JODI LYNN OSBORN

Under the Direction of Susanna F. Greer, PhD

ABSTRACT

Transcriptional activation of Major Histocompatability Complex (MHC) I and II molecules by the cytokine interferon gamma (IFN-γ) is a key step in cell-mediated immunity against pathogens and tumors. Following IFN-γ induction, JAK/STAT signaling triggers activation of MHC genes. Recent evidence suggests suppression of MHC I and II expression on multiple tumor types plays important roles in tumor immunoevasion. One such tumor is malignant

melanoma, the leading cause of skin cancer related deaths. Despite awareness of MHC expression defects, the molecular mechanisms by which melanoma cells suppress MHC and escape from immune-mediated destruction remain unknown. Here we analyze dysregulation of the JAK/STAT pathway and its role in suppression of MHC II in melanoma cell lines at the Radial Growth Phase (RGP), the Vertical Growth Phase (VGP) and the Metastatic Phase (MET). RGP and VGP cells express both MHC II and the MHC master regulator, the Class II Transactivator (CIITA). MET cells lack not only MHC II and CIITA, but also both STAT 1 and the STAT 1 coactivator, the Interferon Response Factor (IRF) 1. Our studies have implicated that the suppression of MHCII on the cell surface of metastatic melanoma is due to silencing at the level of STAT1 transcription. Furthermore, we determined that silencing of STAT1 is, in part, due to hemi-methylation of the STAT1 promoter.

INDEX WORDS: Metastatic Melanoma, Tumor Immune Escape, MHC II, STAT1, Promoter Methylation, JAK/STAT Signaling

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JODI LYNN OSBORN

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by

JODI LYNN OSBORN

Committee Chair: Susanna F. Greer

Committee: Ritu Aneja

Charlese Garnett-Benson

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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DEDICATION

This dissertation is dedicated to my amazing parents, Jerry and Deby Osborn. They have been amazingly supportive throughout this LONG, HARD road. Without their support, both financial and emotional, I would not have made it this far. I love you both very much!

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1 INTRODUCTION

Transcription is a highly regulated cellular process in which even slight dysfunction can lead to disease. One level of regulation is chromatin structure which protects promoters from transcription factor binding (Petesch and Lis 2012). To circumvent this blockade, proteins known as histone chaperones aid in displacement of nucleosomes (Eitoku, Sato et al. 2008). In particular, the histone chaperone complex HUCA, consisting of Hira, Ubn1, Cabin1, and ASF1a, replaces histone variant H3.1 with H3.3 in front of actively transcribing RNA Polymerase II (Elsaesser and Allis 2010). The 26S proteasome is the major degrader of proteins within the cell and plays both proteolytic and non-proteolytic roles in transcriptional regulation (Bhat and Greer 2011). One major role is the degradation of irreversibly arrested RNAPII (Wilson, Harreman et al. 2013). Several interactions between HUCA, the 26S proteasome, and RNAPII have been characterized individually. However, the importance of the observed interactions and the consequences of dysregulation have not been fully elucidated. The loss of the interaction between the HUCA complex, the 26S proteasome and RNAPII may lead to transcriptional defects, aberrant protein expression, and disease. Dysregulation of transcription is an underlying mechanism in both tumorigenesis and altered immune responses.

Transcriptional activation of Major Histocompatability Complex (MHC) I and II molecules by the cytokine interferon gamma (IFN-γ) is a key step in cell-mediated immunity against pathogens and tumors (Sartoris, Scupoli et al. 1990). Following IFN-γ

induction, JAK/STAT signaling triggers activation of MHC genes (Igarashi, Garotta et al. 1994). Recent evidence suggests suppression of MHC I and II expression on multiple tumor types plays important roles in tumor immunoevasion (Garrido, Cabrera et al. 2010). One such tumor is malignant melanoma, the leading cause of skin cancer related deaths (Serrano, Tanzarella et al. 2001). Despite awareness of MHC expression defects, the molecular mechanisms by which melanoma cells suppress MHC and escape from immune-mediated destruction remain unknown. The aforementioned topics will be described in detail below. Interactions between HUCA and the 26S proteasome and silencing of MHC II in metastatic melanoma will be the focus of subsequent chapters.

1.1 TRANSCRIPTIONAL REGULATION

Every cell in the human body contains the same genetic material, yet each cell functions to allow the human body to work practically seamless as a whole. This is achieved through orchestrated regulation of gene expression. Genes are regulated at the level of transcription and are either permanently silenced, constitutively or facultatively transcribed, or activated by stimulation via signaling molecules.

The major mechanism by which genes are silenced during development is methylation of CpG sites which consist of cytosines followed by guanines connected by a phosphate bond (Dobrovic, Gareau et al. 1988, Hershkovitz, Gruenbaum et al. 1990). Many promoters are rich in CpG sites which are called CpG islands (Gardiner-Garden

and Frommer 1987). During cell differentiation, genes that are superfluous to a specific cell type are silenced via methylation at CpG islands (Monk 1995). The addition of methyl groups at CpGs by DNA methyltransferases forms 5-methylcytosine (Vanyushin, Mazin et al. 1973). Generally, the formation of 5-methylcytosine silences genes by physically blocking binding of transcription factors (Bednarik, Duckett et al. 1991). Another mechanism by which the formation of 5-methylcytosine silences genes is by recruiting proteins that either assist in blocking binding of transcription factors or by physically altering chromatin (Jones, Veenstra et al. 1998). Genes that are rarely needed in a particular cell type are super silenced, meaning they are both silenced at the level of promoter methylation and contained in heterochromatin (Surani 1991). In contrast, genes that are indispensable for cellular function are constitutively transcribed (Su, Strand et al. 1988). Constitutively expressed genes are usually considered housekeeping genes which are used in cell structure, metabolism, and basic signaling molecules such as transcription factors. Genes that are needed only occasionally can be turned on facultatively, for example during mitosis (Delcuve, Rastegar et al. 2009). Genes that are needed in specific situations can be induced by environmental cues, paracrine, autocrine, or endocrine signaling (Maniatis, Goodbourn et al. 1987)

1.2 TRANSCRIPTIONAL MACHINERY

All eukaryotic genes encoding proteins are transcribed into mRNA by the RNA Polymerase II complex which is approximately 550 KDa and consists of 12 subunits, RPB 1-12 (Ghosh and Van Duyne 1996). The core enzyme and largest subunit, RPB1,

contains 52 repeats of the sequence YSPTSPS at its C terminal domain (CTD) which are modified to regulate transcription (Barron-Casella and Corden 1992). The serines within this heptapeptide repeat are phosphorylated and govern activity of the RNA Pol II complex. Serine 5 phosphorylation leads to activation of the complex, while serine 2 phosphorylation initiates transcript elongation (Heidemann, Hintermair et al. 2013). RPB2, the second largest subunit, assists in maintaining contact with the template (Kershnar, Wu et al. 1998). The third largest subunit, RPB3, serves to open and close the cleft at the transcription site in conjunction with RPB2 (Kershnar, Wu et al. 1998, De Angelis, Iezzi et al. 2003). The individual roles of the remaining subunits, RBP4-12, are not yet fully understood.

The 12 subunits of the RNA Pol II complex along with the transcription factors TFIIA, TFIIB, TFIID, TFIIE, and TFIIH from the preinitiation complex (PIC) (**Figure 1.1**) (Cox, Kays et al. 1998). TFIIA is a heterodimer consisting of TFIIAα/β and TFIIAγ (Mitsiou and Stunnenberg 2000). TFIIA interacts with the TATA-binding Protein (TBP) subunit of TFIID as well as TFIIB (Nikolov, Chen et al. 1995, Solow, Salunek et al. 2001). TFIIB plays a role in recognition of transcription start sites (TSS) (Lagrange, Kapanidis et al. 1998, Lee and Young 2000). TFIID consists of TBP and at least 16 TBP-associated factors (TAFs) (Timmers, Meyers et al. 1992). TFIID, in complex with TFIIA and TFIIB, bind at the TATA-box at the promoter of genes (Lee and Young 2000). TFIIE binds single stranded DNA and has been posited to assist in maintaining the transcription bubble possibly through direct DNA melting (Okamoto, Yamamoto et al. 1998). TFIIF both stabilizes the PIC as well as prevents binding outside of promoters

(Tan, Conaway et al. 1995). TFIIH is a ten subunit complex which includes Cyclin dependent kinase (CDK) 7 and cyclin H responsible for phosphorylation of serine residues with the CTD of RPB1 (Kershnar, Wu et al. 1998, Lee and Young 2000, Zurita and Merino 2003). TFIIH also assists in maintenance of the transcription bubble (Kim, Ebright et al. 2000).

1.3 THE THREE STATES OF RNA POL II

In general the RNA Pol II complex exists in three different states: poised, stalled, or arrested. RNA Pol II is *paused* at the promoters of other stimulus-responsive genes where it is found 40-60 nt upstream of the TSS (Muse, Gilchrist et al. 2007). The two major causes of RNAPII *stalling* are lesions in the DNA template and nucleotide misincorporation (Donahue, Yin et al. 1994). Once the event leading to RNA Pol II stalling is corrected, elongation resumes (Tornaletti and Hanawalt 1999). However, in the event that stalled RNAPII cannot resume elongation, RNAPII becomes irreversibly *arrested* and is poly-ubiquitinated and tagged for degradation by the 26S proteasome (Somesh, Reid et al. 2005).

1.4 CHROMATIN: ROADBLOCK TO TRANSCRIPTION

Chromatin is a multi-subunit complex of DNA and histone proteins (Luger, Mader et al. 1997, Petesch and Lis 2012). The core nucleosome octamer consists of two each of histones H2A, H2B, H3 and H4 (Klug and Butler 1983). Approximately 147 base pairs of

DNA wraps around the histone octamer twice and is stabilized by the H1 linker protein (Luger, Mader et al. 1997).

Chromatin serves to compact two meters of DNA into the 6 µm nucleus. In addition to compacting DNA, chromatin also serves as a regulator of transcription. There are two major types of chromatin: heterochromatin and euchromatin. Heterochromatin is super condensed and consists of genes that are not transcribed (Grewal and Jia 2007). Euchromatin consists of genes that are either constitutively transcribed, facultatively transcribed, or are inducibly expressed (Ghirlando, Giles et al. 2012).

1.5 OVERCOMING THE ROADBLOCK

Chromatin aids in compacting DNA into a small space and, as a consequence of that function, also prevents transcription by blocking binding of transcription factors. There are two major mechanisms by which the chromatin structure can be altered to allow for transcription: via histone modifications and histone chaperones (Eitoku, Sato et al. 2008, Bannister and Kouzarides 2011).

Histone modifications include the covalent addition of functional groups to the N-terminal tail of histones (Delcuve, Rastegar et al. 2009, Bannister and Kouzarides 2011). The majority of these covalent modifications occur on lysine, arginine, serine, and threonine residues. The most prevalent modifications are methylation and acetylation (Ito 2007). Methylation generally occurs on lysine residues and is most often

a silencing modification. The addition of a methyl group alters the electrostatic charge of the chromatin, thus “tightening” it and blocking binding of transcription factors (Zhang and Reinberg 2001). On the other hand, acetylation “loosens” chromatin structure allowing binding of transcription factors to occur (Verdone, Caserta et al. 2005). Other epigenetic modifications of chromatin include phosphorylation, ubiquitination, and sumoylation. Phosphorylation often occurs on serine and threonine residues and usually in the context of DNA damage (Perez-Cadahia, Drobic et al. 2010). Ubiquitination of histones has not yet been fully characterized but recent studies implicate this post-translational modification in both activation and silencing of transcription in the context of H2A and H2B respectively (Higashi, Inoue et al. 2010, Cao and Yan 2012). Sumoylation of histones indirectly controls transcriptional silencing via recruitment of histone deacetylases (Shiio and Eisenman 2003). The aforementioned modifications work in concert to orchestrate transcription in what has been dubbed the “histone code” (Strahl and Allis 2000).

In addition to modifications of the canonical histones (H2A, H2B, H3, and H4) some histones can be replaced with variants which differ in either their C-terminus or N-terminus (Ausio, Abbott et al. 2001). The variants of H2A are H2A.X and H2A.Z which vary primarily at their C-terminus and affect binding to other histones (Redon, Pilch et al. 2002). H3 exists as H3.1, H3.2, or H3.3 which vary from one another by 5 amino acids (Ahmad and Henikoff 2002). Many consider H3.1 and H3.2 as one in the same due to similar expression patterns and transcriptional regulation roles. H3.3 is considered the true variant and replaces H3.1 in actively transcribed genes with the aid

of its histone chaperone HIRA (Ray-Gallet, Quivy et al. 2002, Tagami, Ray-Gallet et al. 2004, Szenker, Ray-Gallet et al. 2011).

Histone chaperones are histone-dependent, ATP-independent proteins which assist in transcriptional regulation (Eitoku, Sato et al. 2008). Once thought of as simply accessory proteins, histone chaperones are now known to play important roles in histone assembly and disassembly during transcription (Burgess and Zhang 2010, Petesch and Lis 2012). Histone chaperones are either H2A-H2B binding or H3-H4 binding and function in three broad contexts: transport of histones to and from the nucleus, maintaining a pool of histones in the cytoplasm, and physically aiding in nucleosome assembly or disassembly during transcription (Gurard-Levin, Quivy et al. 2014).

1.3 CONSEQUENCES OF DYSREGULATION OF TRANSCRIPTION

Transcription is a meticulously orchestrated series of events involving numerous proteins and enzymes in which the slightest mishap can result in severe consequences. While it is generally thought that errors in transcription are not as devastating as errors in replication, there are many diseases which can result from transcriptional dysregulation. Transcriptional dysregulation has been implicated in muscle malformations, oncogenesis, and many neurological disorders such as Huntington's Disease (Padberg and van Engelen 2009, Jin and Johnson 2010, Bywater, Pearson et al. 2013). Huntington's results from repeats of glutamate in the protein Huntingtin which

plays important roles in transcriptional regulation (Kumar, Vaish et al. 2014). One possible cause of these polyglutamate repeats in Huntingtin is insufficient degradation of arrested RNA Pol II.

1.4 THE 26S PROTEASOME

The major degrader of proteins in mammalian cells is the 26S Proteasome which consists of a 20S catalytic core and a 19S Regulatory Lid (**Figure 1.2**) (Hershko and Ciechanover 1986, Bedford, Paine et al. 2010). The catalytic core recognizes proteins which have been poly-ubiquitinated and thus targeted for degradation (Pickart 1997). The 19S regulatory particle assists in feeding these proteins into the catalytic core with the help of a hexameric ring of ATPases (Bajorek and Glickman 2004). The hexameric ring of ATPases is a trimer of the dimers S4-S7, S6a-S10b, and S6b-SUG1 (Tomko and Hochstrasser 2011).

The most well characterized role of the 26S proteasome is degradation of proteins to either control for protein concentration or rid the cell of misfolded proteins. Recent evidence suggests that the 26S proteasome also has roles outside of degradation (Kodadek 2010, Bhat and Greer 2011, Durairaj and Kaiser 2014). The Ubiquitin Proteasome System (UPS) has been shown to have roles in many processes varying from DNA double stranded break repair to cell cycle regulation (Genschik, Marrocco et al. 2014, van Cuijk, Vermeulen et al. 2014). Understandably, dysregulation of the UPS

can lead to tumors and neurodegenerative diseases (Ding, Xiao et al. 2014, Jansen, Reits et al. 2014).

1.5 THE IMMUNE SYSTEM

The 26S proteasome also plays a major regulatory role in the immune system. A variant known as the Immunoproteasome is induced by inflammatory cytokines and play major roles in antigen presentation (Basler, Kirk et al. 2013). There are two arms of the immune system in higher eukaryotes: innate and adaptive.

Innate immunity is the first line of defense against invading pathogens. It consists of resident immune effector cells including macrophages, monocytes, eiosinophils, basophils, and natural killer cells (Medzhitov and Janeway 2000, Vivier, Raulet et al. 2011). The complement cascade and inflammatory cytokines also assist in defending against invading pathogens (Trouw and Daha 2011, Striz, Brabcova et al. 2014).

Adaptive immunity evolved in higher eukaryotes to add specificity to the immune response. Through antigen presentation, B cells and T cells are able to respond to specific pathogens and develop immune memory. Antigens are presented to T cells through Major Histocompatability Complex Class I (MHC I) or MHC II (Braciale, Morrison et al. 1987). MHC I is constitutively expressed on all nucleated cells and presents intracellular pathogens to $CD8^+$ T cells (Agrawal and Kishore 2000). MHC II is constitutively expressed on professional antigen presenting cells and thymic stromal

cells and is interferon inducible on all nucleated cells and presents extracellular antigens to $CD4^+$ T cells (Boss 1997, Boss 1999).

1.6 JAK/STAT SIGNALING

The JAK/STAT cascades are a diverse group of mix and match signaling cascades involved in signal transduction of both Type I and Type II interferons. Each JAK/STAT pathway contains a receptor, a Janus Kinase (JAK) and at least one signal transducer and activator of transcription (STAT) (Aaronson and Horvath 2002).

The IFN-γ receptor (IFNGR) is a heterodimer of IFNGR1 and IFNGR2, however these proteins are not associated with one another prior to interferon stimulation (Schroder, Hertzog et al. 2004). Upon stimulation, dimerization occurs and the cytoplasmic tails of the receptor are phoshphorylated at tyrosine residues by Janus Kinases forming a docking site for STAT1 (Igarashi, Garotta et al. 1994, Sakatsume, Igarashi et al. 1995).

Janus Kinases (JAKs) are non-receptor tyrosine kinases initially identified in a large PCR screen and were later shown to both negatively and positively regulate signaling cascades (Wilks 1989). There are four members of the JAK family, JAK1-3 as well as TYK2 (Kiu and Nicholson 2012). JAK1 and JAK2 are involved in MHC II transcription (Igarashi, Garotta et al. 1994).

Signal Transducers and Activators of Transcription (STATs) are a diverse family of molecules involved in an array of signaling cascades. In the context of MHC II transcription, STAT1 binds to phosphorylated docking sites on the IFNGR and is phosphorylated (Igarashi, Garotta et al. 1994). Phospho-STAT1 forms a homodimer via Src-Homology (SH2) domains (Gupta, Yan et al. 1996). These homodimers are known as Gamma Activated Factor (GAF). GAF is an integral part in MHC II transcription as it is required for transcription of both IRF1 and CIITA (Morris, Beresford et al. 2002).

Interferon Regulatory Factor (IRF1) is a transcription factor required for CIITA transcription (Morris, Beresford et al. 2002). IRF-1 is a 36 KDa protein that is located solely in the nucleus (Kroger, Koster et al. 2002). It is involved in all cytokine signaling and in the context of IFN- γ signaling, forms a heterodimer with IRF2 (Morris, Beresford et al. 2002). Dysregulation of IRF1 has been implicated in many tumor types including but not limited to ovarian cancer, breast cancer, and myeloid leukemia (Perambakam, Li et al. 2001, Connett, Badri et al. 2005, Pavan, Olivero et al. 2013).

CIITA, the Class II Transactivator, is required for but not sufficient enough for transcription of MHC II (Chang, Fontes et al. 1994, Muhlethaler-Mottet, Otten et al. 1997). There are four promoters that encode CIITA, PI-PIV. PI is constitutively expressed in dendritic cells and macrophages. The role of PII is not well defined. PIII is primarily associated with CIITA expression in B cells. PIV is inducible in nucleated cells (Chang, Fontes et al. 1994, Muhlethaler-Mottet, Otten et al. 1997, LeibundGut-Landmann, Waldburger et al. 2004). Upon transcription and translation, CIITA binds the

enhanceosome complex at the promoter of MHC II, allowing for binding of the Pol II Preinitiation complex, recruitment of transcription elongation factors, and transcription of MHC II (Boss 1997, Morris, Beresford et al. 2002).

1.7 JAK/STAT SIGNALING IN MHC II TRANSCRIPTION

MHC II is inducible by the Type II interferon IFN-γ through the JAK/STAT signaling cascade (**Figure 1.3**). IFN-γ binds to its receptor on the cell surface leading to dimerization of IFNγR1 and IFNγR2 (Greenlund, Schreiber et al. 1993). Upon dimerization of the IFNγ receptor, Janus Kinase (JAK) 1 and JAK2 bind and phosphorylate the cytoplasmic tails of the receptor as well as cross-phosphorylate one another (Sakatsume, Igarashi et al. 1995). Phosphorylation of the cytoplasmic tail of the IFNγ receptor creates a docking site for Signal Transducer and Activator of Transcription (STAT) 1 (Igarashi, Garotta et al. 1994). STAT1 binds and is phosphorylated by JAK2. Gamma Activation Factor (GAF) is formed by homodimerization of pSTAT1 and translocates to the nucleus where it binds to the Gamma Activation Sequence (GAS box) at the promoter of Interferon Response Factor (IRF) 1 (Shuai, Horvath et al. 1994). IRF1 forms a heterodimer with IRF2 and both the GAF and IRF heterodimer bind at the promoter of the Class II Transactivator (CIITA) (Nikcevich, Piskurich et al. 1999). CIITA is known as the master regulator of MHC II and is required for, but not sufficient enough, for MHC II transcription to occur. Once CIITA is transcribed and translated, it binds at the enhanceosome complex at the MHC II

promoter, allowing for the RNA Pol II complex to bind and initiate transcription (Boss 1997, Morris, Beresford et al. 2002).

1.8 TUMOR IMMUNOSURVEILLANCE

Tumor immunosurveillance refers to the phenomena by which the immune system is able to detect, and subsequently respond to neoplastic cells (Mlecnik, Bindea et al. 2011). In this context, Tumor Associated Antigens (TAAs) are expressed on either MHC I or MHC II to CD8+ and CD4+ T cells, respectively (**Figure 1.4**) (Gerloni and Zanetti 2005). The immune system can then see the tumor as a danger signal, and thus, eliminate it. While tumor immunosurveillance can be a positive thing, it can also lead to tumor immunoediting. Tumor immunoediting is the process by which the immune system is able to eliminate a majority of tumor cells, and those it cannot eliminate, propagate stronger tumors (Dunn, Bruce et al. 2002, Reiman, Kmieciak et al. 2007). Those it cannot eliminate are usually those it cannot detect.

1.9 METASTATIC MELANOMA

One tumor which has been shown to evade immune detection is metastatic melanoma (Degenhardt, Huang et al. 2010). Melanoma progresses through distinct phases from healthy melanocyte to dysplastic nevi, radial growth phase, vertical growth phase, and finally metastasis (Chin 2003). Healthy melanocytes exist in a fixed ratio to keratinocytes and produce melanin (Cichorek, Wachulska et al. 2013). Dysplastic nevi,

while not necessarily cancerous, are often identified by over-pigmentation (Skender-Kalnenas, English et al. 1995). Radial Growth Phase (RGP) melanomas are marked by over-proliferation but, in general, show normal responsiveness to paracrine growth inhibition (Ciarletta, Foret et al. 2011). Vertical Growth Phase (VGP) melanomas are able to proliferate throughout skin layers and no longer respond to anti-proliferative cytokines (Laga and Murphy 2010). Metastatic melanomas (MET) are able to break through the basement membrane, enter circulation, and establish tumors elsewhere in the body (Liotta, Guirguis et al. 1987).

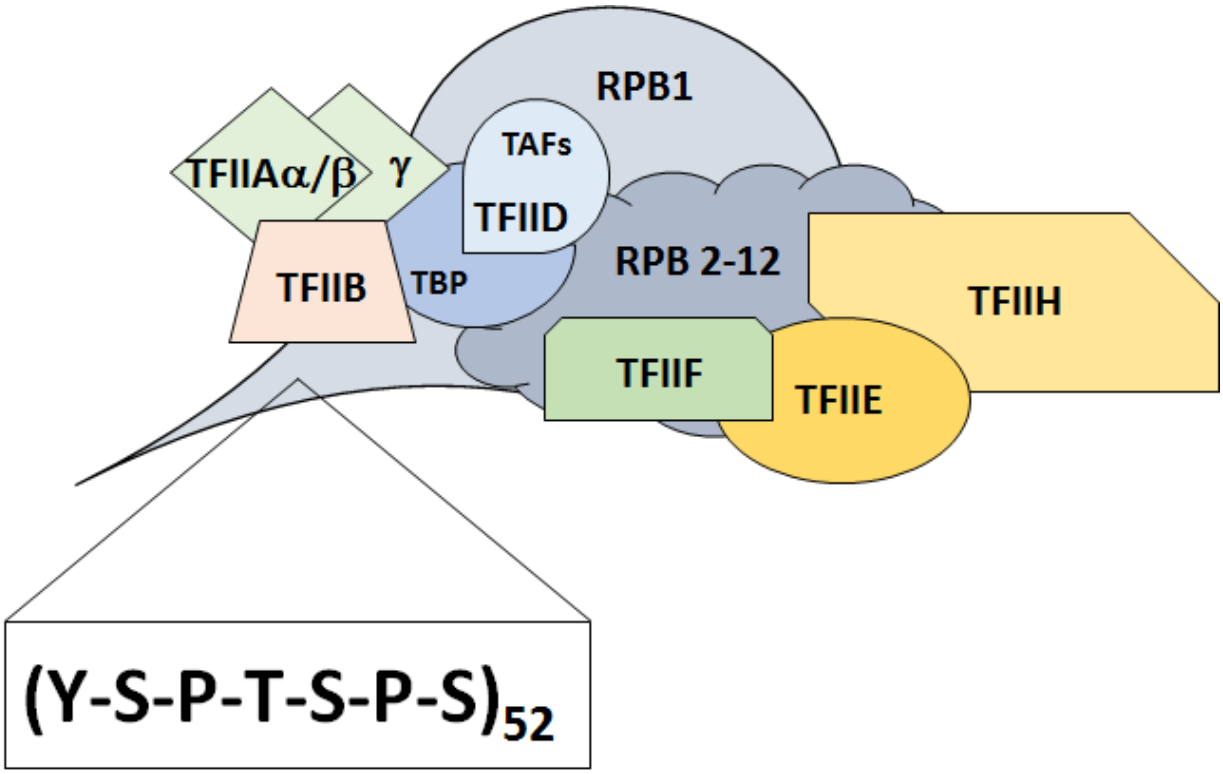


Figure 1.1 Preinitiation Complex

RNA Pol II is made up of 12 RPB subunits. The largest subunit, RPB1 has a C-terminal domain consisting of 52 repeats of the sequence Y-S-P-T-S-P-S. TFIID is comprised of TATA-binding protein (TBP) and a host of TBP-associated factors (TAFs). TFIID forms a complex with both TFIIA and TFIIB. TFIIF, TFIIE, and the ten subunit complex TFIIH are also a part of the preinitiation complex.

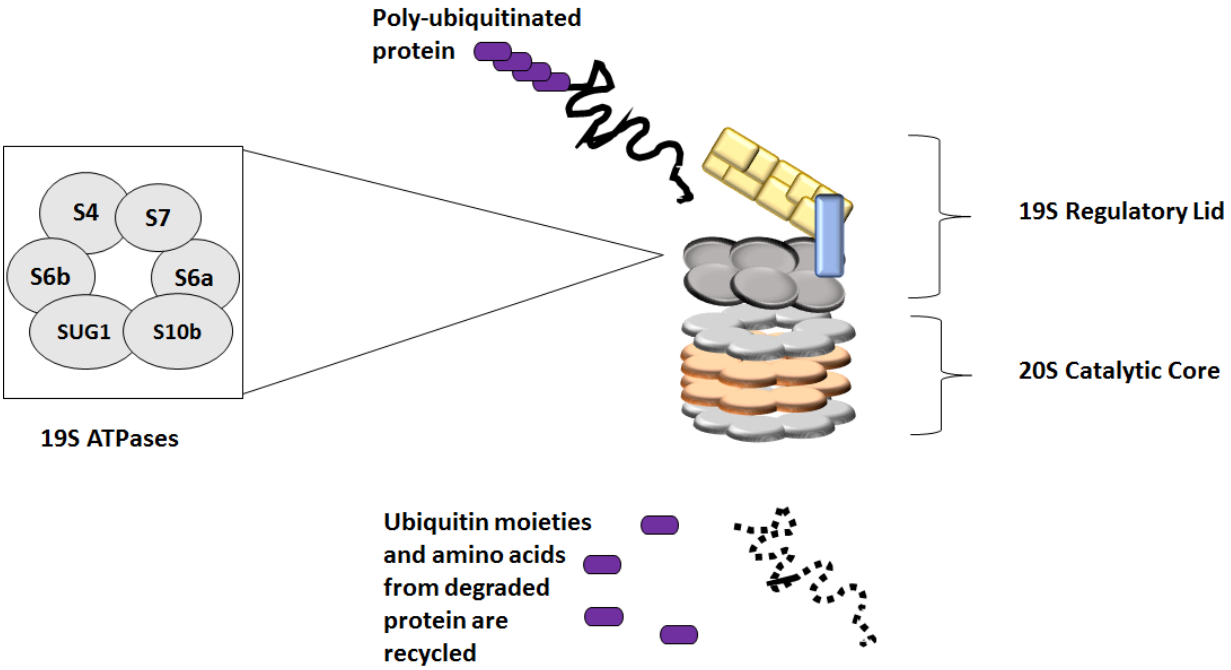


Figure 1.2 The 26S Proteasome

The 26S Proteasome consists of the 20S catalytic core and the 19S Regulatory Lid. Within the regulatory lid are a trimer of dimers of the ATPases S4-S7, S6a-S10b, and S6b-SUG1. Poly-ubiquitinated proteins are targeted for degradation.

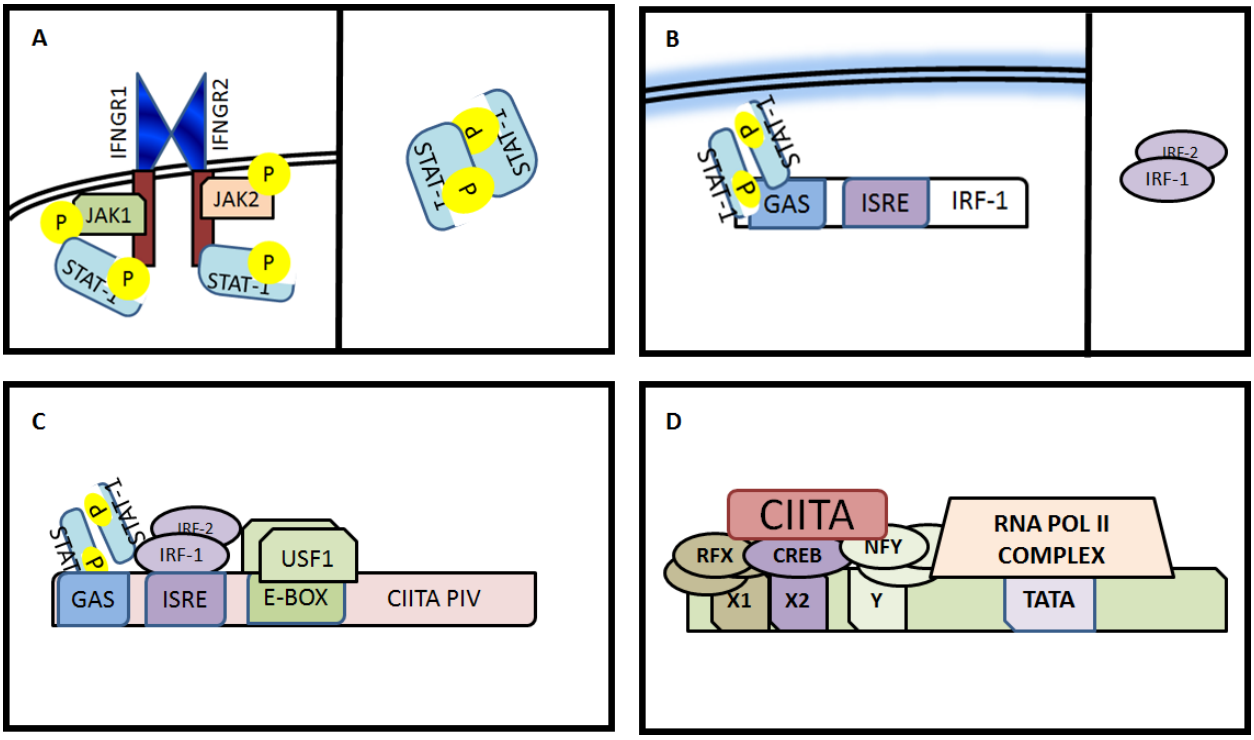


Figure 1.3 MHC II Transcription

A. Upon interferon stimulation, JAK1 and JAK2 cross phosphorylate each other as well as the cytoplasmic tails of the interferon receptor providing a docking site for STAT1. STAT1 is phosphorylated by JAK2 and forms the homodimer shown at left. **B.** The STAT1 homodimer translocates to the nucleus and binds at the Gamma-Activation Sequence on the IRF-1 promoter (left). IRF1 forms a heterodimer with IRF2 (right). **C.** The STAT1 homodimer and the IRF1/2 heterodimer bind at the promoter for the Class II Transactivator, CIITA. **D.** Once CIITA is transcribed and translated, it binds at the enhanceosome at the MHC II promoter.

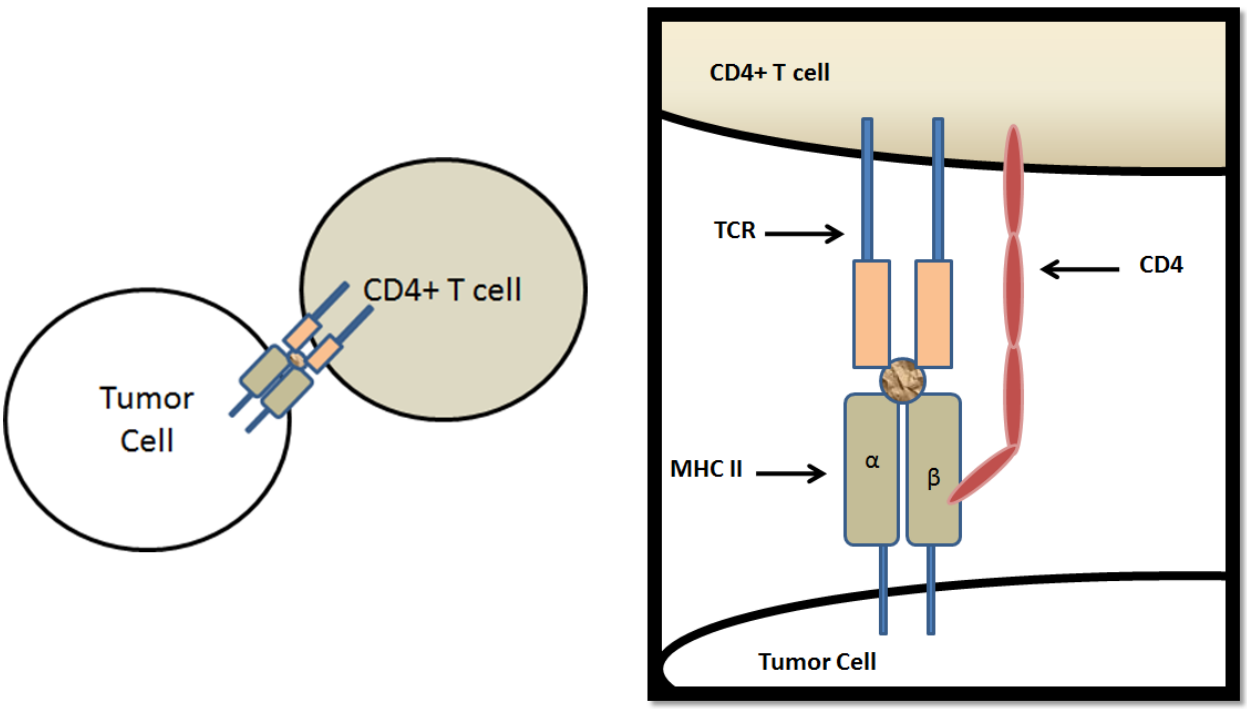


Figure 1.4 TAA Presentation to MHC II

Tumor associated antigens are presented to $CD4^+$ T-cells via MHC II on the tumor cell. Recognition of the tumor antigen by $CD4^+$ T-cells leads to a tumor-specific immune response.

2 A ROLE FOR HISTONE CHAPERONES IN REGULATING RNA POLYMERASE II (RNA POL II)

Cellular function is highly dependent on regulated gene expression which is controlled by the coordinated efforts of thousands of proteins including transcription factors, cofactors, and chromatin regulators. The coordinated efforts and interactions of these proteins control cell fate and cellular responses to physiological or environmental changes. One level at which protein interaction, and thus gene expression, is ordered is control of transcription factor accessibility to DNA by chromatin (Reviewed in (Petesch and Lis 2012)).

Despite over half a century of research in transcriptional regulation, the intricate details of the system have not been fully elucidated. Our proposed model addresses the regulatory interactions between RNA Polymerase II, the histone chaperone complex HUCA, and the 26S Proteasome. We hypothesize that the histone chaperone HUCA tethers the 26S proteasome to elongating RNAPII to facilitate rapid degradation in the case of irreversibly arrested RNAPII. A schematic of the proposed mechanism is provided in **Figure 2.1**. Disassembly of RNAPII elongation complexes has previously been linked to the ATPase Cdc48 with proposed roles for ubiquitination in dissociating Rpb1 from the complex (Verma, Oania et al. 2011). The 26S proteasome binds transcribing genes at locations enriched with RNAPII (Auld, Brown et al. 2006), 19S and 20S subunits of the proteasome bind promoters, ORFs, and termination regions (Gillette, Gonzalez et al. 2004, Krogan, Lam et al. 2004, Auld, Brown et al. 2006, Collins

and Tansey 2006). In *C. elegans*, RNAPII is found localized in “degradation centers” where blocked transcription initiates proteasome mediated degradation (Scharf, Grozdanov et al. 2011). Thus, components of the proteasome are linked with chromatin in multiple and overlapping contexts. Each of these observations suggests that removal of proteins from chromatin and degradation of at least some of these proteins, is likely to be a common theme in the regulation of gene expression.

2.1 CHROMATIN ORGANIZATION AND HISTONE MODIFICATIONS

The basic unit of chromatin is the nucleosome which consists of 147 base pairs of DNA wrapped around a histone octamer of two dimers each of canonical histones H2A, H2B, H3, and H4 as well as the linker histone H1 (Luger, Mader et al. 1997). Nucleosomes are packaged into higher order chromatin structures; modulation of which influences transcriptional outcomes. The N-terminal tails of histones undergo multiple covalent modifications including acetylation, methylation, sumoylation, ADP-ribosylation, and ubiquitination which can positively or negatively affect protein binding to DNA (Bannister and Kouzarides 2011). Different combinations of these histone marks dictate whether the chromatin is relaxed, allowing access by transcription factors, or closed, preventing transcription (Reviewed in (Zentner and Henikoff 2013)).

In addition to the impact of histone modifications on chromatin structure, nucleosome positioning poses an inherent block to elongating RNA Polymerase II (RNAPII) (Kireeva, Walter et al. 2002). Nucleosomes are arranged in regularly spaced

intervals, with the length of intervening linker regions between nucleosomes varying between species and cell types (Kelly, Liu et al. 2012). In general, nucleosomes are depleted at many enhancers and promoters and occupy preferred positions within genes (Fenouil, Cauchy et al. 2012). Nucleosome occupancy differs in that it is a reflection of the depletion of nucleosomes from functional regions of genes and is critical to transcriptional outcomes. Access of RNAPII and other DNA-binding proteins to DNA is inhibited by nucleosomes and RNAPII is unable to mediate nucleosome eviction on its own (Izban and Luse 1992, Chang and Luse 1997). To assist RNAPII passage, chromatin remodelers and histone chaperones aid in removal of the nucleosome blockade. Chromatin remodelers unwind DNA from nucleosomes in an ATP-dependent manner (Hota and Bartholomew 2011) whereas histone chaperones are enzymes that facilitate exchange of histone variants during transcription in a histone-dependent, ATP-independent manner (Eitoku, Sato et al. 2008). In addition to histone modifications and chromatin remodeling, replacement of canonical core histones with specialized variants assists in regulation of the open or closed states of chromatin (Tagami, Ray-Gallet et al. 2004, Szenker, Ray-Gallet et al. 2011).

2.2 HISTONE CHAPERONES: MULTI-FACETED ROLES

Because nucleosome assembly and disassembly regulate chromatin dynamics, histone exchange is coordinated by multiple histone chaperones and their many functional partners. Histone chaperones form multi-subunit complexes and perform a multitude of functions depending on binding partners (Avvakumov, Nourani et al. 2011).

While their main role is to facilitate nucleosome disassembly and reassembly, their secondary roles lie in a variety of processes such as transcriptional regulation, cellular senescence, histone variant deposition, cell cycle regulation, DNA replication and repair, mRNA processing, and gene silencing (Eitoku, Sato et al. 2008, Burgess and Zhang 2010). The dynamic role of chromatin itself highlights the need for histone chaperones to serve versatile roles in modulating the many complexes that regulate chromatin dynamics and subsequent pathways. One such example of a multi-faceted histone chaperone is the HUCA complex. HUCA is known to be critical in chromatin reorganization; we now further postulate HUCA to be of equal importance in regulating RNAPII processivity and arrest.

2.3 HUCA COMPLEX: AN IMPORTANT BUT NOT FULLY UNDERSTOOD HISTONE CHAPERONE COMPLEX

Histone chaperones work in concert with one another and other proteins to perform a multitude of cellular functions (Avvakumov, Nourani et al. 2011). One such histone chaperone complex is HUCA, which consists of the histone chaperone Histone Regulatory A (HIRA), Ubinuclein 1 (UBN1), Calcineurin binding 1 (CABIN1), and the histone chaperone Anti-silencing factor 1a (ASF1a) (Tang, Poustovoitov et al. 2006, Rai, Puri et al. 2011, Tang, Puri et al. 2012). Human HIRA is an evolutionarily conserved fusion of Hir1 and Hir2 which form a nucleosome remodeling complex in lower eukaryotes (Lamour, Lecluse et al. 1995). The N-terminal Hir1-like domain consists of seven WD40 repeats (Lorain, Demczuk et al. 1996). Immediately following

the di-peptide WD repeats is a 37 amino acid domain known as the B-domain (Tang, Poustovoitov et al. 2006). The C-terminal Hir2-like domain consists of a leucine zipper (LXXLL motif) (Magnaghi, Roberts et al. 1998). It is via these functional domains that HIRA acts as a scaffold around which other complex subunits form (Tang, Poustovoitov et al. 2006, Banumathy, Somaiah et al. 2009). UBN1 is an ortholog of the yeast protein Histone promoter control 2 (Hpc2) and contains an evolutionarily conserved Hpc2-related domain (HRD) (Banumathy, Somaiah et al. 2009). The N-terminal region of UBN1 binds the WD40 repeats of HIRA as well as histone tails (Tang, Puri et al. 2012). CABIN1 is a functional ortholog of Hir3p but the only structural similarity is 30 tetratricopeptide repeats (TPR) at the N-terminus (Tagami, Ray-Gallet et al. 2004). The TPR domain of CABIN1 interacts with the C-terminus of HIRA (Rai, Puri et al. 2011). ASF1a has an evolutionarily conserved immunoglobulin fold at its N-terminus and a divergent, species specific, C-terminus (Daganzo, Erzberger et al. 2003). ASF1a binds HIRA via interaction of its N-terminal sequence with the B domain of HIRA (Tang, Poustovoitov et al. 2006). For a schematic of the HUCA complex and its components, see **Figure 2.2**. HIRA binds the promoters of histone genes and prevents their transcription outside of S-phase (Osley and Lycan 1987, Prochasson, Florens et al. 2005). The mechanism of histone promoter silencing is through blockade of the SWI/SNF complex which is required for transcription of histone genes during S-phase (Dimova, Nackerdien et al. 1999). This was initially shown for histones H2A and H2B but is not the case for histone H3.3 which is transcribed throughout the entire cell cycle (Wunsch and Lough 1987). HIRA simultaneously binds DNA and RNAPII while depositing histone H3.3 during transcription (Ray-Gallet, Quivy et al. 2002). The ability

of HIRA to bind DNA non-specifically has been implicated in prevention of cryptic transcription in *Schizosaccharomyces pombe* (Anderson, Wardle et al. 2009). Heterogenous HIRA expression leads to DiGeorge Syndrome in humans and has been implicated in developmental impediments in *Drosophila melanogaster*, *Gallus gallus domesticus,* and *Mus musculus* (Llevadot, Scambler et al. 1996, Roberts, Sutherland et al. 2002, Loppin, Bonnefoy et al. 2005). Complete knockout of HIRA is lethal as early as embryonic development day 10 (Roberts, Sutherland et al. 2002).

In addition to its role in the histone chaperone HUCA complex, ubinuclein 1 has been posited to be a histone chaperone in its own right (Balaji, Iyer et al. 2009). UBN1 has also been shown to act as a scaffold protein and to play a role in cell-cell adhesion in canine kidney cells (Aho, Lupo et al. 2009, Gruffat, Lupo et al. 2011). CABIN1 has recently been classified as a member of the HUCA complex and implicates regulation of this histone chaperone complex by intracellular calcium (Elsaesser and Allis 2010, Rai, Puri et al. 2011). ASF1a, which is also a histone chaperone, binds H3.1, H3.2, and H3.3 and plays roles in nucleosome eviction in both DNA-replication and transcription (Schwabish and Struhl 2006, Tang, Poustovoitov et al. 2006). In addition to its role in nucleosome eviction, ASF1 has been shown to play roles in regulating acetylation in both histones H3 and H4 in *Saccharomyces cerivisiae* (Avvakumov, Nourani et al. 2011). Despite the elucidation of various roles of HUCA components, specific regulatory roles of the complex as a whole are not understood.

2.4 TRANSCRIPTIONAL ELONGATION BY RNA POLYMERASE II

RNAPII transcribes mRNA from a DNA template in all protein-encoding genes. Transcription relies on a coordinated network of histone modifying enzymes and histone chaperones to allow elongating RNAPII to traverse the chromatin landscape. In particular, HUCA deposits the histone variant H3.3 in front of elongating RNPII to facilitate transcription through nucleosome complexes (Ray-Gallet, Quivy et al. 2002). Transcription proceeds in three distinct stages: initiation, elongation, and termination (Shandilya and Roberts 2012). The pre-initiation complex (PIC) which forms at the gene promoter, contains RBP1 and the general transcription factors (GTFs) TFIIB, TFIID, TFIIE, and TFIIH (Orphanides, Lagrange et al. 1996). Other factors are also present including DRB Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF) which positively (Wada, Takagi et al. 1998) and negatively (Yamaguchi, Takagi et al. 1999) regulate RNAPII, respectively. The C-terminal domain (CTD) of RBP1 consists of 52 repeats of the heptapeptide Y-S_2-P-S_5-T-P-S (Corden 1990). Phosphorylation status of the serine residues within the CTD correlates with transcriptional activity. Serine 5 phosphorylation (Ser5ph) by the cyclin dependent kinase 7 (cdk7) subunit of TFIIH leads to transcriptional initiation by recruitment of factors that facilitate escape from the promoter (Ohkuma and Roeder 1994). Initial transcription can be non-productive until the formation of an 8 to 9 bp hybrid of the DNA template and the nascent mRNA (Liu, Bushnell et al. 2011). Serine 2 is phosphorylated (ser2ph) by the cdk9 subunit of Positive Transcription Elongation Factor b (P-TEFb)

which stimulates productive elongation (Peterlin and Price 2006), in part by triggering the release of NELF (Ivanov, Kwak et al. 2000).

2.5 THE THREE STATES OF RNA POL II: PAUSED, STALLED, AND ARRESTED

Regulation of chromatin states by histone remodelers and histone chaperones is only one level at which transcription elongation can be controlled. In addition to blocking RNAPII access to DNA, RNAPII processivity regulates transcription. Early studies of heat shock proteins showed that RNAPII is present at the promoter prior to induction (Lis 1998). Subsequent studies have shown that RNAPII is *paused* at the promoters of other stimulus-responsive genes (Gilchrist, Fromm et al. 2012). Pausing is mediated by DRB-Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF). NELF preferentially binds hypophosphorylated RNAPII and is required for promoter-proximal pausing (Muse, Gilchrist et al. 2007). The two major causes of RNAPII *stalling* are lesions in the DNA template and nucleotide misincorporation. Lesions in the template cause RNAPII to stall until transcription-coupled DNA repair can occur (Donahue, Yin et al. 1994). The stalled RNAPII remains bound to the DNA template and once the lesion has been excised and repaired, elongation can resume (Tornaletti and Hanawalt 1999, Toulme, Guerin et al. 1999). Nucleotide misincorporation stalls RNAPII by causing it to backtrack one base pair (Zenkin, Yuzenkova et al. 2006, Nudler 2012). TFIIS stimulates RNAPII to hydrolytically cleave the misincorporated nucleotide (Jeon and Agarwal 1996). After cleavage, the newly formed 3’ end is prepared for the addition of the appropriate nucleotide, a process which occurs rapidly and has been termed “kinetic

proofreading" (Larson, Zhou et al. 2012). Pausing and stalling of RNAPII are mediated by both histone chaperones and the 26S proteasome. In the event that stalled RNAPII cannot resume elongation, RNAPII becomes irreversibly *arrested* and is poly-ubiquitinated and tagged for degradation by the 26S proteasome (Ratner, Balasubramanian et al. 1998, Somesh, Reid et al. 2005).

2.6 UBIQUITINATION OF RNA POL II AND DEGRADATION BY THE 26S PROTEASOME

Arrested RNAPII is tagged for degradation by the addition of four ubiquitin moieties linked by lysine 48 (K48-linkage) (Hershko and Ciechanover 1998). Poly-ubiquitination is the concerted effort of three types of enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligase (E3) (Hershko and Ciechanover 1998). RNAPII poly-ubiquitination is unique in that it occurs in a sequential manner involving two different E3 ligases. Neural precursor cell Expressed Developmentally Downregulated 4 (NEDD4) mono-ubiquitinates RNAPII (Anindya, Aygun et al. 2007) and then Elongin/Cullin converts this mono-ubiquitination to poly-ubquitination (Yasukawa, Kamura et al. 2008, Harreman, Taschner et al. 2009). Ubiquitination of RNAPII occurs on its largest subunit Rpb1 and only occurs when the polymerase is irreversibly arrested (reviewed in (Wilson, Harreman et al. 2013)).

2.7 THE 26S PROTEASOME: DEGRADER OF PROTEINS

The 26S proteasome is the major non-lysosomal player in degradation of damaged or unnecessary proteins within the cell including RNAPII (Hershko and Ciechanover 1998). The 26S proteasome also plays roles in transcription initiation by controlling the location and quantity of available transcription factors (Kwak, Workman et al. 2011). The 26S proteasome is composed of three distinct sub-complexes: the 19S regulatory particle (RP) lid, the 19S RP base, and the 20S catalytic particle (CP) (Bedford, Paine et al. 2010). The 19S RP lid is composed of 9 unique subunits: Regulatory Particle Non-ATPase 3 (Rpn3), Rpn5-9, Rpn11-12 and Rpn15 (Bedford, Paine et al. 2010). The 19S base consists of a hexameric ring of AAA ATPases (RPT1-6) and three non-ATPase subunits and is connected to the 19S RP lid by the linker protein Rpn10 (Glickman, Rubin et al. 1998, Bedford, Paine et al. 2010). The 20S catalytic particle (CP) is a barrel-shaped complex composed of four heptameric rings composed of either α or β subunits stacked in an αββα conformation (Bedford, Paine et al. 2010).

2.8 INTERACTIONS BETWEEN HUCA, THE 26S PROTEASOME, AND RNAPII

Several interactions between HUCA, the 26S proteasome, and RNAPII have been characterized. Liquid chromatography tandem mass spectrometry revealed that all six ATPase subunits of the 19S RP base co-purify with HIRA in *S. pombe* (Anderson, Kagansky et al. 2010). The interaction between the 19S ATPases and HIRA is thought

to counteract the repressive functions of HIRA on histone genes, but this role has yet to be further studied (Anderson, Kagansky et al. 2010). We have further verified interactions between the 19S ATPases and HIRA in co-immunoprecipitation assays in mammalian cells (**Figure 2.3**). ChIP-seq density profiles followed by co-immunoprecipitation show that multiple subunits of the HUCA complex bind to both the initiating and elongating forms of RNAPII (Ray-Gallet, Woolfe et al. 2011). The importance of the interaction between HUCA and RNAPII is underscored by the fact that in yeast cells depleted of HIRA, RNAPII recruitment is impaired (Chujo, Tarumoto et al. 2012). HIRA binds to both subunits of DSIF (DeSilva, Lee et al. 1998) which travels throughout the coding region along with RNAPII (Andrulis, Guzman et al. 2000). Additionally, HIRA interacts directly with the elongation factors SPT6 and SPT16 (DeSilva, Lee et al. 1998, Formosa, Ruone et al. 2002). Finally, the ASF1a subunit of HUCA interacts directly with the bromodomain of CCG1, the largest subunit of TFIID (Chimura, Kuzuhara et al. 2002).

Recent studies from our lab and others are the first to directly associate elongating RNAPII with the degradation machinery through observations of the involvement of the HUCA chaperone complex. We propose that the HUCA complex tethers the 26S Proteasome to elongating RNAPII to enable rapid recognition and degradation of RNAPII by the proteasome. In addition to non-proteolytic roles in transcription (reviewed in (Bhat and Greer 2011)), tethering of the 26S proteasome to elongating RNAPII would facilitate its rapid degradation upon arrest. Recent observations of a novel Ubiquitin-independent proteasome pathway suggest that

elongating RNAPII stalls at Topoisomerase IIβ-DNA cleavage complexes where blocked RNAPII serves as a damage signal and associated 19S ATPases are activated as an early event during the encounter for detecting the Topoisomerase IIβ roadblocks (Ishizuka, Satoh et al. 2001, Satoh, Ishizuka et al. 2009). The 19S ATPases have also been proposed to unfold non-covalent protein-DNA nucleosome complexes during elongation; we suggest the ATPases and HUCA serve these and much earlier roles in allowing consistent tethering of RNAPII to the degradation machinery.

2.9 CONCLUDING REMARKS

While the molecular basis for interactions among stalled elongation, proteasomal degradation, and HUCA remain to be elucidated, understanding roles for 19S ATPases in mediating degradation of RNAPII will contribute therapeutically to multiple disease scenarios. In Cockayne syndrome, irreversibly arrested RNAPII leads to transcription anomalies resulting in phenotypes ranging from subtle to severe (Dianov, Houle et al. 1997). Additionally CAG repeat diseases such as Huntington disease and Spinocerebellar Ataxias are caused, in part, by repeated transcription by arrested RNAPII (Salinas-Rios, Belotserkovskii et al. 2011). While diseases such as Cockayne Syndrome and CAG repeat disorders have transcriptional arrest as an underlying cause, the molecular mechanisms of decreased transcript fidelity leading to disease manifestation have not been further investigated. In addition, several cancer therapeutics including etoposide/VP-16 and doxorubicin stabilize topoisomerase II□-DNA cleavage complexes and trigger degradation of topoisomerase IIβ, the large

subunit of RNAPII, and exposure of DNA damage (Lyu, Kerrigan et al. 2007, Zhang, Liu et al. 2012). These drugs and the DNA damage they cause are associated with severe side effects; better understanding of the mechanisms by which these drugs initiate degradation will contribute to their enhanced clinical application.

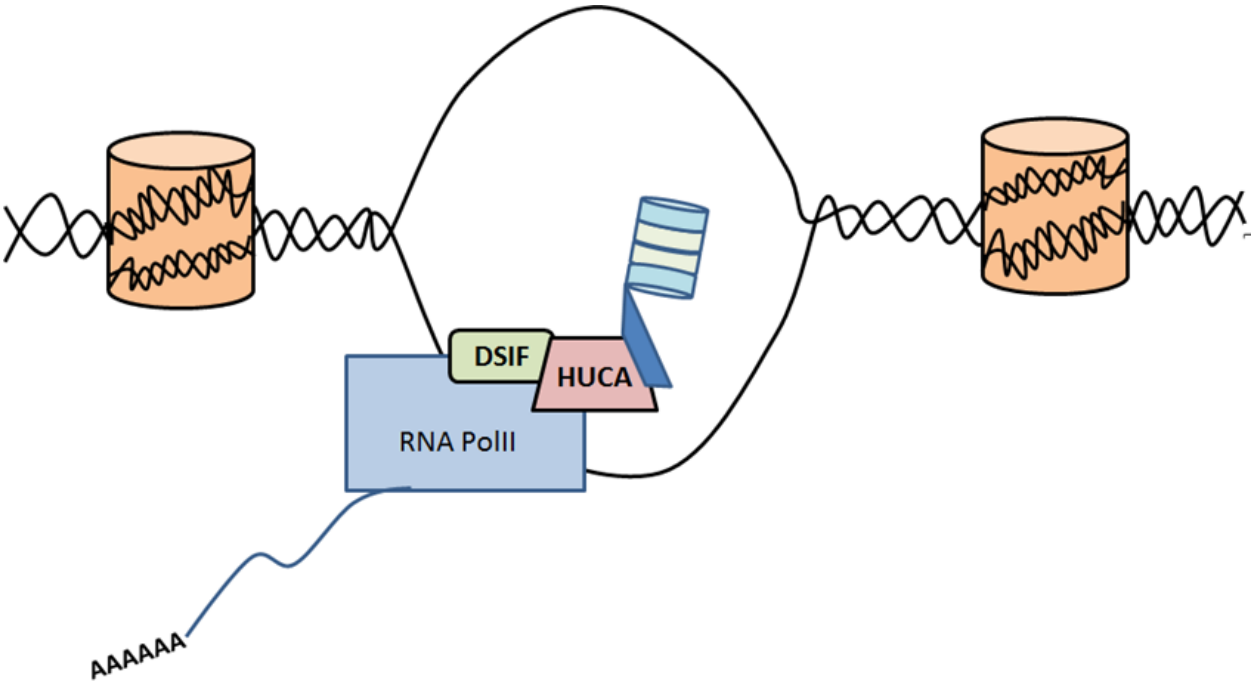


Figure 2.1 Proposed Mechanism

We hypothesize that the histone chaperone HUCA tethers the 26S proteasome to elongating RNA Polymerase II to facilitate rapid degradation in the case of irreversible stalling of the RNAPolII elongation complex. By keeping the degradation machinery of the cell in close proximity, stalled RNAPolII can be quickly degraded, thus avoiding continual transcription of the same nucleotides which can lead to abnormal protein accumulation within the cell.

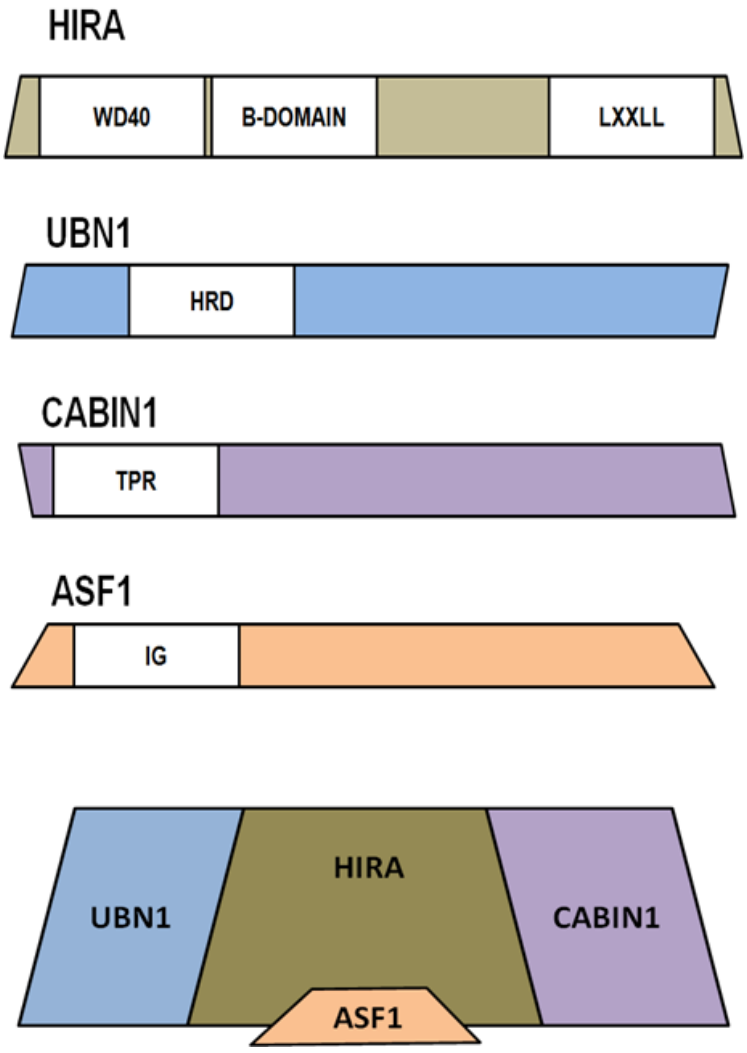


Figure 2.2 HUCA Complex

HUCA is composed of the histone chaperone Histone Regulatory A (HIRA), Ubinuclein 1 (UBN1), calcinurin binding 1 (CABIN1), and the histone chaperone Anti-silencing Factor 1 (ASF1). HIRA acts as a scaffold around which other complex subunits form. The N-terminal domain of UBN1 binds the N-terminal WD repeats of HIRA. The TPR domain of CABIN1 interacts with the C-terminus of HIRA. ASF1 binds the B-domain of HIRA.

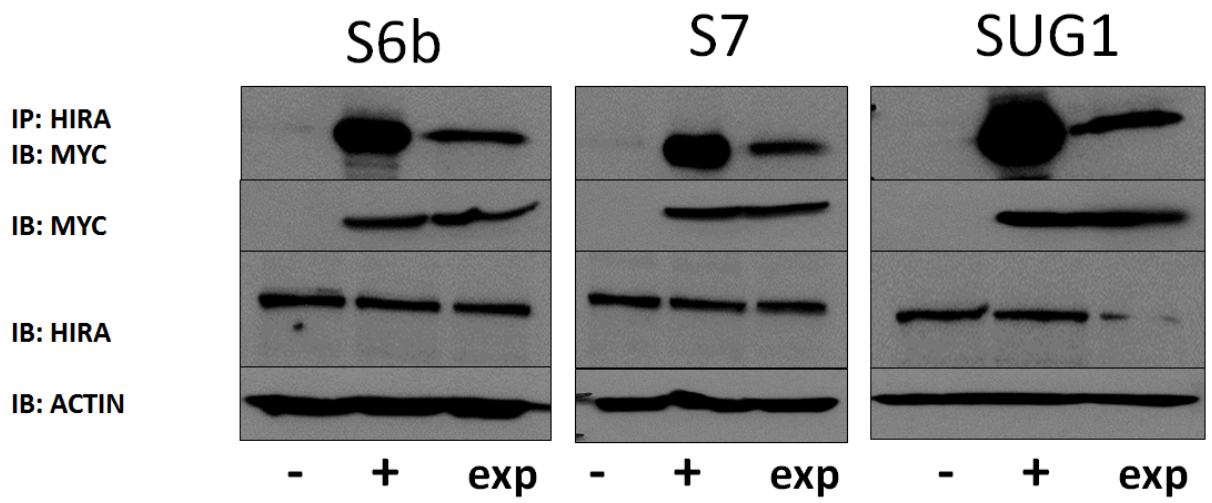


Figure 2.3 Co-immunoprecipitation of HIRA with the ATPases S6b, S7, and SUG1

Hela cells were transfected with 5μg of plasmid encoding a myc-tagged ATPase. Cells were harvested after 24 hours, lysed, and incubated overnight with antibodies against HIRA. Lysates were blotted for a myc-tagged ATPase. Lysates showing equal expression of myc-ATPase, HIRA, or actin are shown as controls.

3 METASTATIC MELANOMA CELLS EVADE IMMUNE DETECTION BY SILENCING STAT1

Skin cancer is the most common form of cancer diagnosed in the United States. Despite comprising only 5% of skin cancers, malignant melanoma is the leading cause of skin cancer related deaths annually. Melanoma progresses through stages, from radial growth phase (RGP) to vertical growth phase (VGP) to metastatic, with distinct morphologic phenotypes, however the molecular changes associated with these transitions are not well defined (Clark, Ainsworth et al. 1975). Healthy melanocytes exist in a fixed ratio to keratinocytes in a heterogeneous environment that includes fibroblasts, endothelial cells, and resident immune effector cells (**Figure 3.1 A**) (Cichorek, Wachulska et al. 2013). Initial gene dysregulation leads to the development of dysplastic nevi which exhibit phenotypes including, but not limited to, over-production of melanin (Crutcher 1987). RGP melanomas are characterized by uncontrolled cellular division as well as the ability to spread within the epidermis, but generally resemble healthy melanocytes at the molecular level (**Figure 3.1 B**) (Herlyn, Thurin et al. 1985, Ciarletta, Foret et al. 2011). VGP melanomas are capable of spreading throughout skin layers and develop resistance to paracrine growth inhibition via cytokines secreted by surrounding endothelial cells (**Figure 3.1 C**) (Rak, Hegmann et al. 1994, Laga and Murphy 2010). Finally, metastatic (MET) melanomas develop the ability to intravasate, allowing them to establish secondary tumors elsewhere in the body (**Figure 3.1 D**) (Liotta, Guirguis et al. 1987). While the mechanisms underlying the transition between RGP, VGP, and MET are not fully understood, the genes involved in the transition and

their contributions to the deadly nature of metastatic melanoma are beginning to be elucidated.

The immune system is able to specifically detect and target neoplastic cells through the process of immunosurveillance (Mlecnik, Bindea et al. 2011). The promise of cancer immunotherapy is to use immunosurveillance to eliminate cancer cells without harming normal tissues, and thus with fewer side effects. Immunotherapy approaches have been tested against malignant melanoma, and while detectable outcomes have been induced, the clinical results have largely been disappointing (Srivastava and McDermott 2014). Escape from tumor immunosurveillance is a major mechanism leading to the lethality of metastatic melanomas (Gajewski 2006). A first step in immunosurveillance is the recognition of tumor peptide antigens by T cells of the immune system. Tumor antigens are presented by cell-surface glycoproteins termed Major Histocompatibility Complex (MHC) molecules I and II (Braciale, Morrison et al. 1987). MHC I molecules present intracellular peptides and are expressed on nucleated cells whereas MHC II molecules present extracellular peptides constitutively on professional antigen presenting cells and inducibly on nucleated cells (Braciale, Morrison et al. 1987).

In the case of tumors, MHC I molecules present tumor associated antigens (TAA) to $CD8^+$ cytotoxic T cells, and MHC II molecules present TAAs to $CD4^+$ helper T cells, thus facilitating an anti-tumor immune response (Gerloni and Zanetti 2005). In these contexts, T cells recognize dysplastic cells as “altered self” and eradicate the tumor (Mlecnik, Bindea et al. 2011). Traditionally, $CD8^+$ T cells have been considered the

major mediators of effective anti-tumor immune responses and suppression of MHC I in tumors is well studied. Decreases in MHC I expression, and thus antigen presentation to $CD8^+$ T cells, negatively impacts tumor prognosis in numerous cancer types (Garrido, Cabrera et al. 2010). The observation that antigen presentation via MHC I is critical in tumors is supported by exomic sequencing studies identifying that presentation of mutated TAAs on MHC I led to increased tumor burden in mice (Matsushita, Vesely et al. 2012). However, a growing number of studies indicate limited anti-tumor activity of $CD8^+$ T cells alone (Nishimura, Iwakabe et al. 1999, Gao, Khammanivong et al. 2002, Janssen, Lemmens et al. 2003, Antony, Piccirillo et al. 2005). The helper function of anti-tumor $CD4^+$ T cells improves the efficacy of anti-tumor $CD8^+$ T cells; seen early on in studies where transfecting tumor cells with MHC class II genes resulted in increased anti-tumor immune responses (Ostrand-Rosenberg, Thakur et al. 1990, Ostrand-Rosenberg, Roby et al. 1991). Multiple studies now indicate $CD4^+$ T cells enhance the anti-tumor response of $CD8^+$ T cells (Corthay, Skovseth et al. 2005, Muranski, Boni et al. 2008, Corthay, Lundin et al. 2009, Quezada, Simpson et al. 2010, Xie, Akpinarli et al. 2010, Haabeth, Lorvik et al. 2011). Indeed, $CD4^+$ T cells can also eliminate tumor cells in the absence of $CD8^+$ T cells (Fujiwara, Fukuzawa et al. 1984, Greenberg, Kern et al. 1985, Lauritzsen, Weiss et al. 1994, Perez-Diez, Joncker et al. 2007). Collectively, these findings suggest $CD4^+$ T cells are powerful antitumor effector cells.

$CD4^+$ T cells recognize peptides bound to the groove of MHC II molecules (Rudensky, Preston-Hurlburt et al. 1991). As MHC II cell surface expression can be induced in nucleated cells by the cytokine Interferon Gamma (IFN-γ) through the

JAK/STAT signaling cascade (Sartoris, Scupoli et al. 1990), the molecular mechanisms regulating the MHC II status of tumor cells is of clear importance. It has been well established that IFN-γ plays substantial roles in both anti-viral and pro-immune responses (Billiau and Matthys 2009). Both MHC I and MHC II are IFN-γ inducible, further emphasizing the significance of this Type II interferon in immunosurveillance. In the context of MHC II transcription, IFN-γ binds to IFN-γ Receptor 1 (IFN γR1) leading to dimerization of IFN-γR1 and IFN γR2 (Schroder, Hertzog et al. 2004). The cytoplasmic tails of the hetero-dimeric IFN-γ Rs are cross-phosphorylated, allowing binding of Janus Kinase (JAK) 1 and JAK2 (Igarashi, Garotta et al. 1994). JAK1 and JAK2 are subsequently phosphorylated, leading to recruitment and binding of Signal Transducer and Activator of Transcription 1 (STAT1) (Sakatsume, Igarashi et al. 1995). Phosphorylated STAT1 then forms a homodimer known as Gamma-Activated Factor (GAF) (Shuai, Horvath et al. 1994). GAF next translocates to the nucleus where it binds Gamma-Activation Sequence (GAS) on the promoters of Interferon Response Element (IRF) 1 and IRF2 (Nguyen, Hiscott et al. 1997). An IRF1 and IRF2 heterodimer along with GAF binds promoter IV of the Class II Transactivator (CIITA). CIITA is necessary but not sufficient for transcription of MHC II and lacks intrinsic DNA binding capabilities (Masternak, Muhlethaler-Mottet et al. 2000). MHC II transcription requires the presence of the enhanceosome complex to which CIITA binds (Masternak, Muhlethaler-Mottet et al. 2000). The enhanceosome is comprised of Regulatory Factor X (RFX), Nuclear Factor Y (NFY), and cAMP Regulatory Element Binding protein (CREB) (Zika, Greer et al. 2003). CIITA binding at the enhanceosome allows binding of RNA Polymerase II leading to transcription of MHCII (Spilianakis, Kretsovali et al. 2003). It has previously

been reported that metastatic melanoma lacks cell surface expression of MHCII which leads to tumor escape from immunosurveillance (Degenhardt, Huang et al. 2010). We therefore sought to determine the mechanisms underlying silencing of MHCII in metastatic melanoma.

3.1 RESULTS

3.1.1 MHCII IS INCREASINGLY SUPPRESSED IN RGP, VGP, AND MET CELLS

Despite the ability of the immune system to detect and eradicate tumor cells, tumor immunosurveillance often fails (Groth, Kloss et al. 2011). Selective pressure leads to tumor cells evolving to down regulate immunomodulatory molecules, a phenomenon known as tumor immunoediting (Enderling, Hlatky et al. 2012). While metastatic melanoma cells often lack MHC II surface expression, the mechanisms supporting MHC II suppression by metastatic melanoma remain unknown (Degenhardt, Huang et al. 2010). We used flow cytometry to determine the expression levels of MHC II during progression of melanoma. Radial Growth Phase (**Figure 3.2 - RGP**) cells express basal levels (blue) of MHC II as compared to unstained control (shaded) with MHC II increasing upon stimulation. Relative fluorescence intensity (x-axis) representing APC-MHCII complexes increases after 18 hours (orange) and continues to increase throughout 24 (green), 48 (brown) and 72 (mauve) hours of IFN-γ stimulation. In comparison, vertical growth phase (**Figure 3.2 - VGP**) cells express lower basal MHCII with a majority of cells being APC-MHC II negative in the absence of IFN- γ stimulation (blue). Similar to RGP cells, VGP cells demonstrate increased MHC II cell surface

expression throughout 72 hours of stimulation; albeit to a lesser extent. In contrast to both RGP and VGP, metastatic (**Figure 3.2 - MET**) cells lack constitutive or inducible cell surface expression of MHC II. These data implicate that MHC II cell surface expression decreases as melanoma cells progress through RGP and VGP stages and confirm previously reported findings of MET cells lacking cell surface MHC II (Degenhardt, Huang et al. 2010).

3.1.2 MELANOMA CELLS REMAIN IFN-γ RESPONSIVE THROUGHOUT DISEASE PROGRESSION

As cell surface expression of MHCII on nucleated cells requires stimulation with the pro-inflammatory cytokine IFN-γ, we questioned whether the MET cell line was capable of responding to stimulation (Boss 1997). We performed flow cytometry experiments to determine expression levels of this cell surface receptor, focusing on IFNγR1 because it is the heterodimer constituent to which IFN-γ binds prior to heterodimerization (Schroder, Hertzog et al. 2004). Furthermore, mutations in IFNγR2 slightly decrease cell surface expression attenuation of signaling, but do not impart a total loss of IFN-γ responsiveness (de Paus, Kilic et al. 2011, Gros, Petzold et al. 2011). As expected, RGP cells express high levels of cell surface IFNγR1 with no significant change in receptor expression during cytokine stimulation (**Figure 3.3 – RGP**). VGP cells express moderate levels of IFNγR, and also show no change during stimulation (**Figure 3.3 – VGP**). MET cells express moderate to low levels of IFNγR1 (**Figure 3.3 - MET**), where expression levels varied throughout stimulation, but not to a significant extent. Together,

these data indicate RGP, VGP and MET cells are each capable of responding to interferon stimulus.

3.1.3 *MET CELLS EXPRESS BOTH JAK1 AND JAK2*

MHCII transcription is a product of the Janus Kinase (JAK) signaling cascade (Igarashi, Garotta et al. 1994). Upon IFN-γ binding to the IFN-γ receptor on the cell surface, JAK1 and JAK2 bind the cross linked receptor and cross-phosphorylate one another, leading to STAT1 activation (Sakatsume, Igarashi et al. 1995). The fact that both JAK1 and JAK2 are imperative in the signaling cascade required for MHCII cell surface expression is well established, and their abrogation leading to decreased MHCII has been seen in certain bacterial infections (Srisatjaluk, Kotwal et al. 2002). Therefore, we performed western blots to determine the expression levels of JAK1 and JAK2 during the course of melanoma development. We observed that in all three cell lines JAK1 and JAK2 are expressed in the presence or absence of IFN-γ stimulation (**Figure 3.4**). These data show that JAK1 and JAK2 are intact in metastatic melanoma and are not the underlying cause of MHC II silencing in these cells.

3.1.4 *METASTATIC MELANOMA CELLS LACK THE INTERFERON RESPONSE FACTOR IRF-1*

Downstream from JAK1 and JAK2 and following IFN-γ stimulation, IRF-1 forms a heterodimer with IRF-2 and binds CIITA PIV leading to transcription of the Class II Transactivator (Morris, Beresford et al. 2002). Because IRF-1 is necessary for CIITA transcription, we investigated the expression of IRF-1 with and without interferon stimulation (**Figure 3.5**). We and others have determined that IRF-1 is expressed at its maximum level after 4 h of stimulation in near normal cells (Reinsbach, Nazarov et al. 2012, Truax, Thakkar et al. 2012). As expected, IRF-1 is expressed following four hours of IFN-γ stimulation in RGP and VGP cells. However, MET cells lack IRF-1 expression despite interferon stimulation. These data implicate that silencing of MHCII in metastatic melanoma is due to silencing of IRF-1.

3.1.5 *SILENCING OF MHCII IN METASTATIC MELANOMA IS THE RESULT OF DYSREGULATION OF BASAL STAT1α EXPRESSION*

IRF-1 is required for CIITA and thus MHCII expression following stimulation with the pro-inflammatory cytokine IFN-γ. The Gamma Activated Sequence (comprised of a homodimer of STAT1) is required for both IRF-1 and CIITA expression. We hypothesized that dysregulated STAT1 activation was the cause of the silencing of both IRF-1 and CIITA leading to the lack of MHCII cell surface expression. Western blot analysis verified that STAT1 is constitutively expressed and is inducibly phosphorylated upon interferon stimulation in RGP and VGP cells. In contrast, MET cells lack not only phosphorylated STAT1, but also lack constitutive STAT1 protein expression.

Conversely, re-introduction of STAT1 into MET cells restores cell surface expression of MHCII. (**Figure 3.6**).

3.1.6 *STAT1 EXPRESSION IS DECREASED IN PATIENT METASTATIC MELANOMA SAMPLES*

To investigate if observations seen *in vitro* mirror metastatic melanoma samples, we examined STAT1 expression levels in patient tumor samples. In normal melanocytes from a benign nevus, STAT1 is expressed throughout the cell (**Figure 3.7 A**). RGP lesions show a visual decrease in STAT1 expression. Melanoma cells are identified by up-regulation of biomarkers as seen in green. (**Figure 3.7 B**). VGP lesions show further decrease in STAT1 expression which correlates with an increase in melanoma biomarker expression (**Figure 3.7 C**). Metastatic lesions from lymph nodes (**Figure 3.7 D**) and bone (**Figure 3.7 E**) both show a marked decrease in STAT1 expression. Data is representative of over 150 samples. These data show that in clinical samples, STAT1 expression has an inverse correlation with upregulation of biomarkers for metastatic melanoma.

3.2 DISCUSSION

Tumor immunosurveillance refers to the ability of the immune system to detect, and ideally, respond to neoplastic cells. Successful immunosurveillance depends on the presentation of TAAs to $CD8^+$ cytotoxic T cells and to $CD4^+$ T-helper cells via MHC I and

MHC II respectively. The suppression of either one or both of these Major Histocompatibility Complexes is a common mechanism by which tumor cells avoid immunosurveillance. Numerous silencing mechanisms have been elucidated, including epigenetic silencing and genomic deletion of key mediators involved in MHC cell surface expression (Serrano, Tanzarella et al. 2001, Radosevich, Song et al. 2004, Rodriguez, Mendez et al. 2007, Respa, Bukur et al. 2011)

The goal of this study was to determine mechanisms underlying previous observations of silencing of IFN-γ inducible MHC II expression in metastatic melanoma. We report here that MHC II is suppressed as melanoma cell lines evolve from RGP to VGP to MET. We further determined that despite gradual suppression of MHC II, each melanoma cell line remained IFN-γ responsive throughout simulated disease progression. Continued cell surface expression of the IFN-γ receptor led us to inspect the remaining components of the IFN signaling cascade leading to MHC II cell surface expression. Following IFN-γ stimulation, JAK1 and JAK2 bind to the intercellular domains of the IFN-γ receptor (Igarashi, Garotta et al. 1994). RGP, VGP and MET cell lines all express both JAK1 and JAK2, further indicating that the major components of the MHC II signaling pathway are intact regardless of stage of melanoma progression. Upon further investigation of the JAK/STAT pathway, we found that metastatic melanoma cells lack the interferon response factor IRF-1. IRF-1 requires activated STAT1 (phosphorylated at Y701 to form a homodimer) for transcriptional activation. MET cells not only lack phosphorylated STAT1, but lack basal expression of unphosphorylated STAT1 as well. From these results, we conclude that suppression of

MHC II on the cell surface of these MET melanoma cell lines is due to silencing of STAT1. To determine if a similar phenomenon is present in patient samples, we compared STAT1 expression levels in metastatic and non-metastatic melanocytic lesions. Within primary tumors and secondary metastases, we see significant decreases in STAT1 expression as cells gain metastatic ability. We are currently investigating the molecular mechanisms by which STAT1 is suppressed in metastatic melanoma.

Studies over the past decade have led to the discovery of varying causal mechanisms of melanoma immune escape centered on suppression of antigen presenting molecules. The majority of these studies have focused on MHC I due to its importance in activation of cytotoxic T cells. Rodriguez and colleagues investigated cell lines from the European Searchable Tumor Line Database (ESTDAB) and found multiple metastatic melanoma cell lines that suppress MHC I with differences in mechanisms of MHC I suppression (Rodriguez, Mendez et al. 2007). The melanoma cell line ESTDAB-004 expresses STAT1, but the STAT1 expressed lacks phosphorylation at Y701. In contrast, lack of MHC I in ESTDAB-159 (GR-mel-3) cells was due to promoter methylation of IRF-1. Methylation was also found to be the cause of MHC I silencing in MSR3-mel cells, but in this case hypermethylation occurred at the MHC I promoter itself (Serrano, Tanzarella et al. 2001). Additionally, in metastatic melanoma Colo857 cells, MHC is absent due to a genomic deletion of JAK2 (Respa, Bukur et al. 2011).

Silencing of MHC I molecules has been observed at nearly every level of the JAK/STAT signaling cascade. Because STAT1 is involved in both MHC I and MHC II expression, it is likely that the observed effects of STAT1 silencing in MHC I impairment are mirrored in impairment of MHC II. Few reports are available that investigate the silencing of MHC II in metastatic melanoma. One early study shows that MHC II silencing in the ocular melanoma cell line Mel202 is the result of epigenetic silencing of CIITA (Radosevich, Song et al. 2004). In this study, ectopic expression of CIITA restored MHC II cell surface expression. Aside from the report indicating that the metastatic melanoma cell line 1205lu do not express cell-surface MHC II (Degenhardt, Huang et al. 2010), little has been reported in regard to the expression of MHC II in skin melanomas. In concurrent studies with our own, it was recently shown that STAT1 is silenced in two additional metastatic melanoma cell lines, SK-Mel-28 and MM96 (Amalraj, Cutler et al. 2013). The RE-1 Silencing Transcription (REST) complex was shown to regulate STAT1 in this study. REST is responsible for silencing neuronal genes in non-neuronal cells, but has also been shown to play a role in some genetic disorders (Schoenherr, Paquette et al. 1996). Interestingly, STAT1 contains an RE1 binding site and it was shown that this is the mechanism by which REST silences STAT1. These findings revealed a previously undocumented mechanism of down regulation of STAT1 in melanoma. The above observations underscore the variation in underlying causes of MHC silencing in metastatic melanoma. In contrast to the Wistar melanoma cell lines utilized in our study, the aforementioned cell lines are not categorized by progression status. To our knowledge, our study is the first to compare MHC suppression between RGP, VGP, and MET melanoma cell lines.

Silencing of STAT1 may be one mechanism by which melanoma can evade immune detection and thus increase its metastatic potential. By investigating STAT1 expression in patient tumors, oncologists may be able to employ targeted immunotherapies, thus increasing a patient's immune response to metastatic melanoma. The ability to introduce plasmid DNA (pDNA) into human subjects has become an increasingly common part of clinical trials. STAT1-encoding pDNA can be introduced into tumors via electroporation (Cemazar, Golzio et al. 2009, Lin, Shen et al. 2012). Additionally, techniques are in development to use mRNA in lieu of pDNA to augment gene expression in tumors (Rejman, Tavernier et al. 2010). By developing mechanisms to introduce STAT1 DNA into tumors lacking the protein, and by treating the patient with a course of interferon, it may be possible to induce MHC I and MHC II expression on the cell surface of melanoma, leading to an increased response by the patient's own immune system. Immunotherapy has gained notoriety in recent years because it is less toxic to patients than chemotherapy and irradiation. The timing of immunotherapeutic intervention has been shown to drastically affect patient outcomes. Observations of the importance of timely intervention with immunotherapy have led to the establishment of the Copenhagen Prospective Personalized Oncology (CoPPO) program in which samples from newly diagnosed patients undergo multiple tests including expression arrays, RNA sequencing, and SNP analysis to determine expression levels of therapeutic targets. Knowing the mutations leading to neoplasms will allow clinicians to tailor treatment and, ideally, improve patient prognosis (Tuxen, Jonson et al. 2014). Consequently, determining the multitude of underlying causes of tumors is imperative to improving personalized medicine.

3.3 METHODS

3.3.1 CELL LINES

Wistar Melanoma (WM) cell lines representing Radial Growth Phase (WM-35), Vertical Growth Phase (WM-1366), and metastasis (1205Lu) were purchased from the Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, NJ). Cell lines were maintained in 4:1 MDB153: Liebowitz L-15 Media (Sigma-Aldrich, St. Louis, MO) and 2% FBS (Atlas Biologicals, Fort Collins, CO). Cells were cultured at 37℃ in 5% CO_2.

3.3.2 WESTERN BLOTS

Cells were treated with 50 U/mL IFN-γ (Peprotech, Rocky Hill, NJ) for 0 hours, 0.5 hours, or 4 hours. Post-stimulation, cells were lysed in NP-40 lysis buffer (20 mM Tris pH 8.0, 0.14 M KCl, 10% Nonadet P-40, 5 mM EDTA, 20 mM NaCl, 1 mM DTT) supplemented with eComplete Protease Inhibitors (Roche Applied Science, Indianapolis, IN). Clarified lysates were normalized for protein concentration, separated via SDS-PAGE, transferred to nitrocellulose and blotted for protein of interest with specific antibodies as follows: IRF-1, STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT1 (Y701) (BD Biosciences, San Jose, CA), and JAK1, JAK2 (Cell Signaling Technology, Boston, MA). Blots were incubated with appropriate secondary antibodies conjugated to HRP and developed with Hyglo™ (Denville Scientific, South

Plainfield, NJ) per manufacturer's instructions. HRP conjugated β-actin antibodies (Cell Signaling Technology, Boston, MA) were used as loading controls.

3.3.3 FLOW CYTOMETRY

Cells were treated with 0, 18, 24, 48, or 72 hours of IFN-γ at a concentration of 50 U/mL. Post-stimulation, cells were harvested with Accutase (EMD Millipore, Billerica, MA). After washing with PBS, cells were stained with fluorophore conjugated specific antibodies as follows: PE-CD119 (IFN- γR1) or APC-HLADR (MHC II) (Biolegend, San Diego, CA). Cells were then fixed in 2% paraformaldehyde and analyzed on an LSRFortessa Flow Cytometer (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo Software (Tree Star, Ashland, OR).

3.3.4 IMMUNOFLUORESCENCE

Formalin-Fixed Paraffin Embedded (FFPE) tissue microarrays (Biomax, Rockville, MD) were dewaxed in two 20 minute washes with xylenes. Microarrays were rehydrated and blocked as previously described (Robertson, Savage et al. 2008). Slides were incubated with primary antibodies for STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA) or the metastatic melanoma biomarkers HMB45 and MART-1 (Abcam, Cambridge, MA) for one hour at room temperature. After staining with fluorophore-conjugated secondary antibodies, nuclei were stained with NucBlue® per manufacturer's instructions (Life Technologies, Grand Island, NY). Cover slips were mounted using

Vectasheld® (Vector Labs, Burlingame, CA), allowed to harden overnight at 4C and then sealed. Images were acquired on a Zeiss LSM700 confocal microscope (Carl Zeiss, Germany) and analyzed with ImageJ software (NIH, Bethesda, MD). Microarrays analyzed via immunofluorescence included tumor samples (1 mm x 5 μm) of melanomas ranging from benign nevi, RGP, VGP, and distant metastases (lymph nodes, bone, and spleen).

3.3.5 OVEREXPRESSION ASSAYS

MET cells were transfected with Flag-STAT1 plasmid (Addgene, Cambridge, MA) with Avalanche®-Omni (EZ Biosystems, College Park, MD) per manufacturer's instructions. After 24 hours of incubation, cells were stimulated with 50 U/mL of IFN-γ for 48 hours. Cells were harvested and analyzed as described in sections 3.3.2 and 3.3.3.

3.3.6 STATISTICAL ANALYSES

All western blots were quantified with Un-Scan-It Gel™ analysis software (Silk Scientific, Orem, UT). Unpaired student's t-tests were used to determine significance (NS $p>0.05$, * $p<0.05$, ** $p<0.005$, *** $p<0.001$, **** $p<0.0001$) between treated and untreated samples.

3.4 CONCLUDING REMARKS

Despite many studies reporting downregulation of Major Histocompatibility Molecules in melanoma, the mechanisms underlying the silencing have not been elucidated. Our study is the first to investigate mechanisms of MHC silencing during disease progression from Radial Growth Phase (RGP), Vertical Growth Phase (VGP), and metastatic melanomas (MET). Our data will impact personalized medicine by expanding the database of therapeutic targets for diagnosis and treatment of metastatic melanoma.

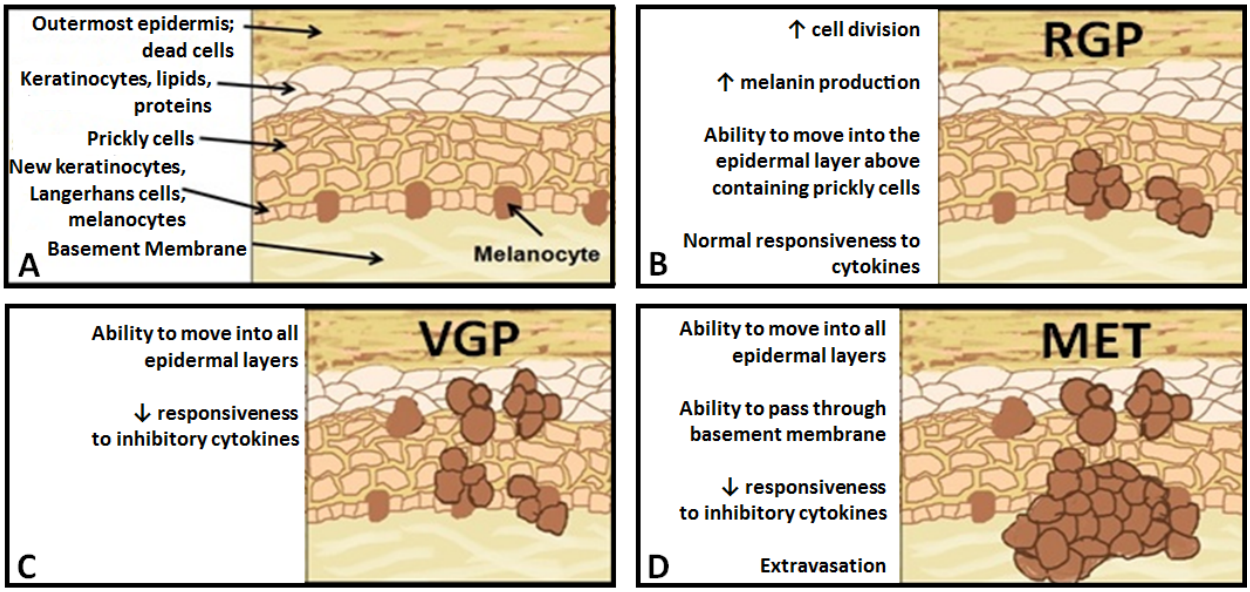


Figure 3.1 Representation of the progression from healthy melanocyte, to RGP, to VGP, to MET melanoma

(**A**). Healthy melanocytes exist in a fixed ratio to keratinocytes in the basal layer of the epidermis. (**B**). RGP cells exhibit uncontrolled cell division and over-production of melanin. (**C**). VGP cells can spread throughout the epidermis and no longer respond to proliferation inhibiting cytokines. (**D**). MET cells are able to break through the basement membrane and enter the circulation.

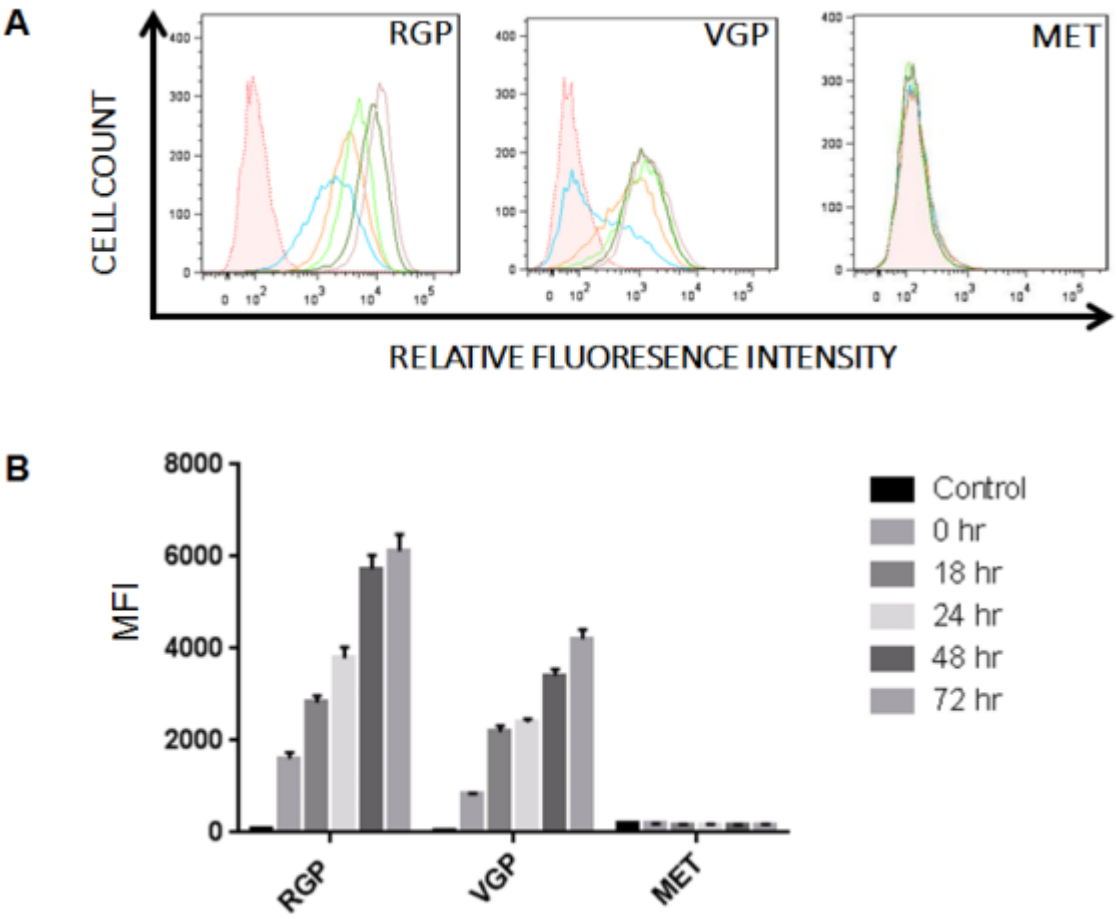


Figure 3.2 Flow cytometric analysis of MHC II cell surface expression in RGP, VGP, and MET cells

(**A**). Cells were treated with IFN-γ for 0 hours (blue), 18 hours (orange), 24 hours (lime green), 48 hours (dark green) or 72 hours (mauve). Unstained cells (shaded histogram) are shown as a control. Live cells were stained with APC conjugated antibody against HLA-DR and were fixed in 2% paraformaldehyde and were then analyzed on a Fortessa flow cytometer. RGP cells demonstrate basal expression of MHC II that increases following IFN-γ stimulation. VGP cells demonstrate less

basal expression of MHC II with an increase upon IFN-γ stimulation comparable to basal expression seen in RGP cells. MET cells lack cell surface expression of MHC II with, or without, stimulation with IFN-γ. Data shown is representative of a minimum of three experimental replicates. (**B**). Mean fluorescence intensity of histograms seen in A. normalized to isotype control. Data is the average of three experimental replicates.

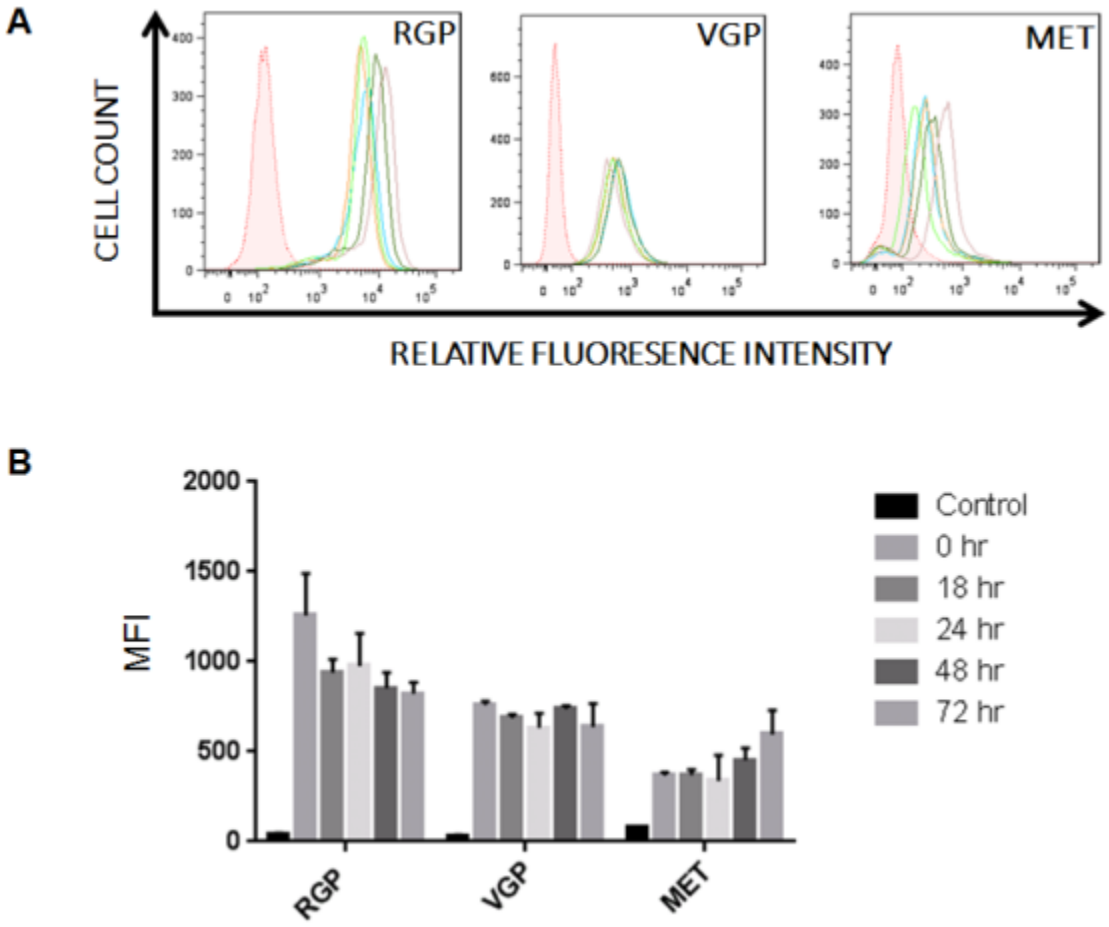


Figure 3.3 Flow cytometric analysis of IFN-γ receptor expression.

(**A**). Cells were treated with IFN-γ for 0 h (blue), 18 h (orange), 24 h (lime green), 48 h (dark green) or 72 h (mauve). Unstained cells (shaded histogram) are shown as a control. Live cells were stained with PE conjugated antibody against CD119 (IFNγR1) and were fixed in 2% paraformaldehyde and were then analyzed on a Fortessa flow cytometer. RGP cells express high levels of the IFN-γ receptor as compared to unstained control. VGP cells express low levels of IFN-γ receptor with no change throughout 72 h of stimulation. MET cells express varying amounts of

IFN-γ receptor throughout 72 h of stimulation. Changes in receptor expression between timepoints is not statistically significant ($p>0.05$). Data shown is representative of a minimum of three experimental replicates. (**B**) Mean fluorescence intensity of histograms seen in (A) normalized to isotype control. Data shown is the average of three experimental replicates.

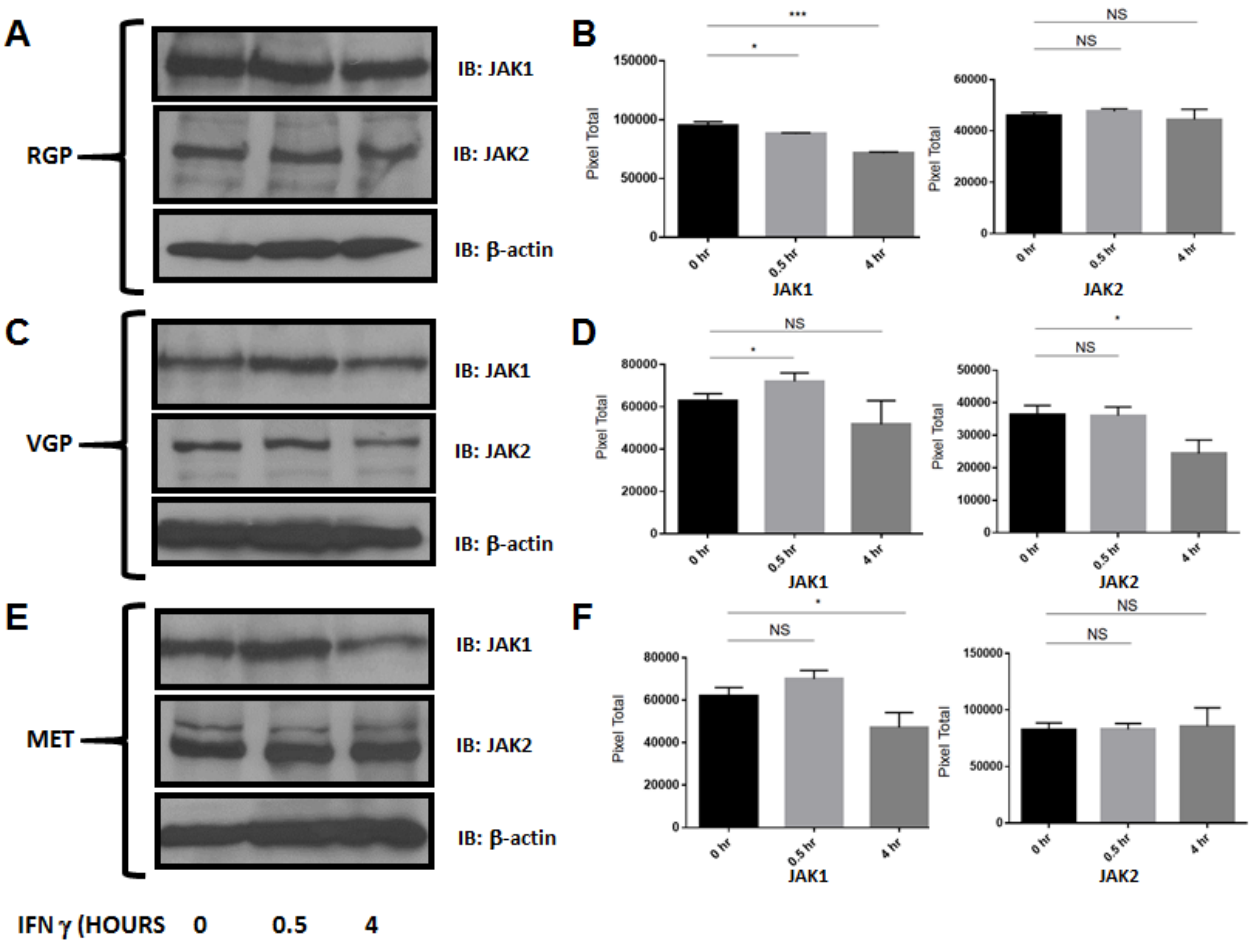


Figure 3.4 Western blot analysis of JAK1 and JAK2 expression.

Cells were stimulated with IFN-γ for 0, 0.5 or 4 h. Lysates were cleared of cellular debris and equal concentrations of protein were separated via SDS-PAGE. Proteins were identified by incubating nitrocellulose with antibodies against JAK1 (RGP, VGP, MET, top panel) or JAK2 (RGP, VGP, MET, middle panel). β-Actin was used as a loading control (RGP, VGP, MET, bottom panel). (**A**–**F**) JAK1 and

JAK2 are constitutively expressed in RGP, VGP, and MET cells. Data shown is representative of a minimum of three experimental replicates.

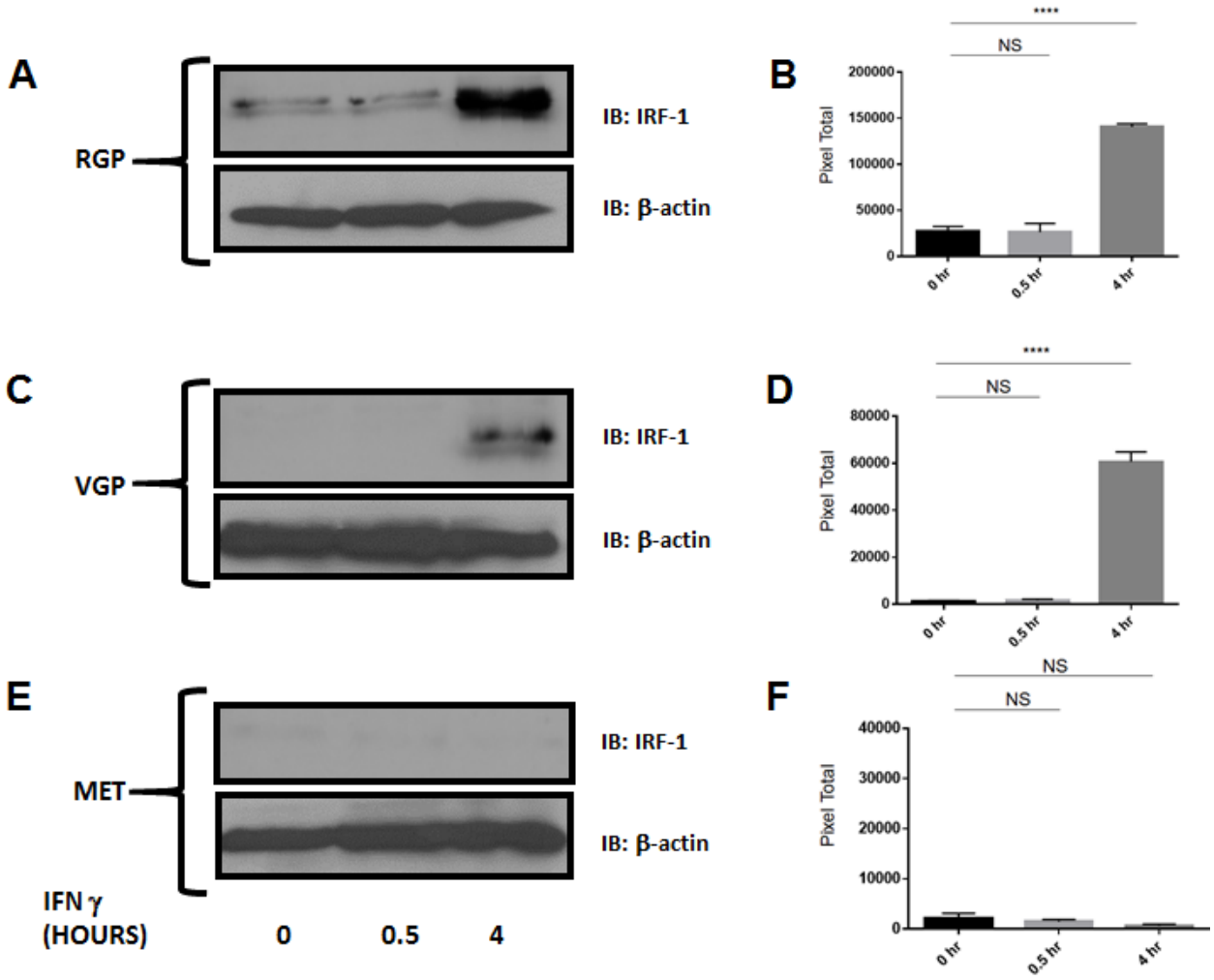
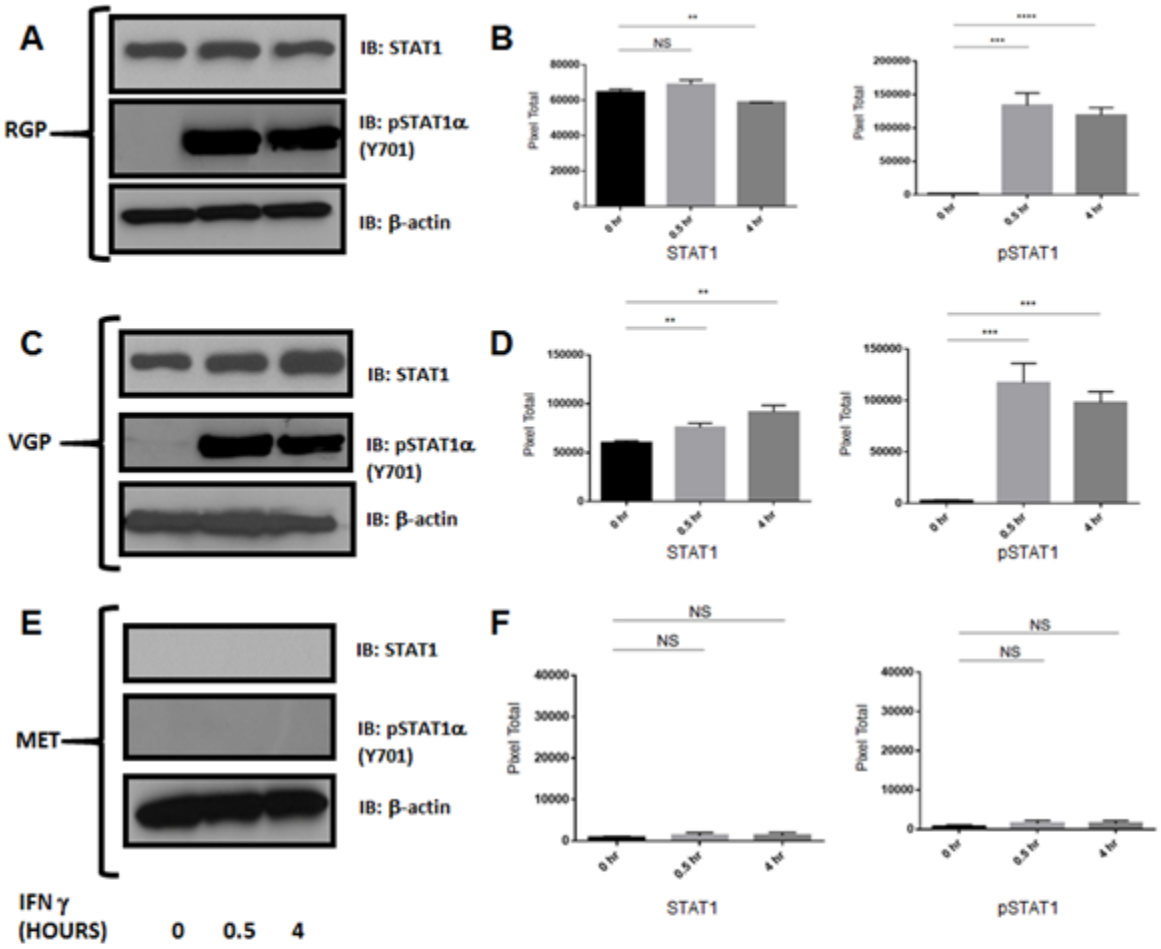


Figure 3.5 Western blot analysis of IRF-1 Expression.

Cells were stimulated with IFN-γ for 0, 0.5, or 4 h. Lysates were cleared of cellular debris and equal concentrations of protein were separated via SDS-PAGE. Proteins were identified by incubating nitrocellulose with antibodies against IRF-1 (RGP, VGP,

MET, top panel). β-Actin was used as a loading control (RGP, VGP, MET, bottom panel). (**A**,**B**) IRF-1 is expressed in RGP cells following four hours of IFN-γ stimulation. (**C**,**D**) In VGP cells, IRF-1 is expressed to a greater extent after four hours of stimulation as compared to RGP. (**E**,**F**). MET cells lack IRF-1 expression following four hours of IFN-γ stimulation.


A
RGP
IB: STAT1
IB: pSTAT1α (Y701)
IB: β-actin
B
NS
**
Pixel Total
STAT1
pSTAT1
***
****
C
VGP
D
E
MET
F
IFN γ (HOURS) 0 0.5 4

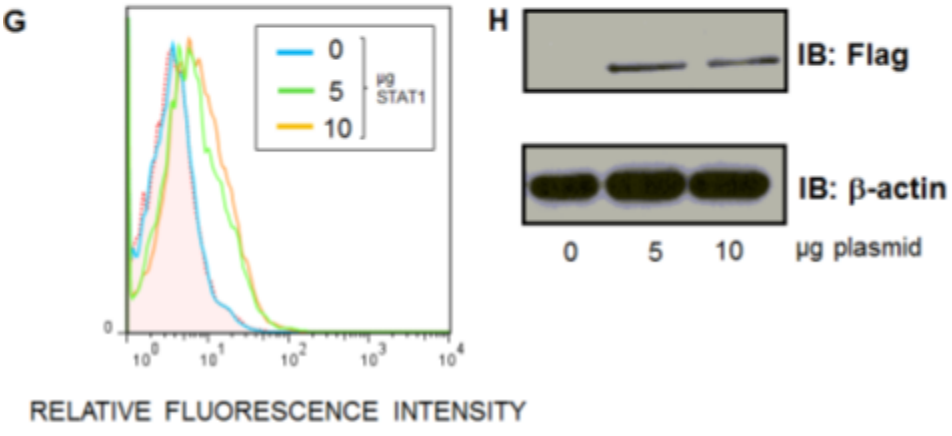

G
0
5
10
μg STAT1
RELATIVE FLUORESCENCE INTENSITY
H
IB: Flag
IB: β-actin
0 5 10 μg plasmid

Figure 3.6 Western blot analysis of STAT1 expression and phosphorylation.

Cells were stimulated with IFN-γ for 0, 0.5, or 4 h. Lysates were cleared of cellular debris and equal concentrations of protein were separated via SDS-PAGE. Proteins were identified by incubating nitrocellulose with antibodies against STAT1 (RGP, VGP, MET, top panel) or pSTAT1 (RGP, VGP, MET, middle panel). β-Actin was used as a loading control (RGP, VGP, MET, bottom panel). (**A**,**B**) STAT1 is constitutively expressed and phosphorylated at Tyrosine 701 (Y701) following 30 minutes of IFN-γ stimulation in RGP cells. (**C**,**D**) STAT1 expression and phosphorylation in VGP cells is similar to RGP. (**E**,**F**) STAT1 expression is absent in MET cells. Data shown is representative of at least 3 experimental replicates. (**G)**. MET cells + STAT1. MET cells were transfected with 0, 5, 10 µg of Flag-STAT1 for 24 h followed by 48 h of IFN-γ stimulation. Cell surface expression of MHCII was analyzed via flow cytometry. H. Expression of Flag-STAT1 following 24 h incubation with transfection reagent:plasmid complexes. Both 5µg (light green) and 10µg (orange) of STAT1 led to restoration of MHCII on the cell surface as compared to non-transfected cells (light blue) and unstained control (shaded).

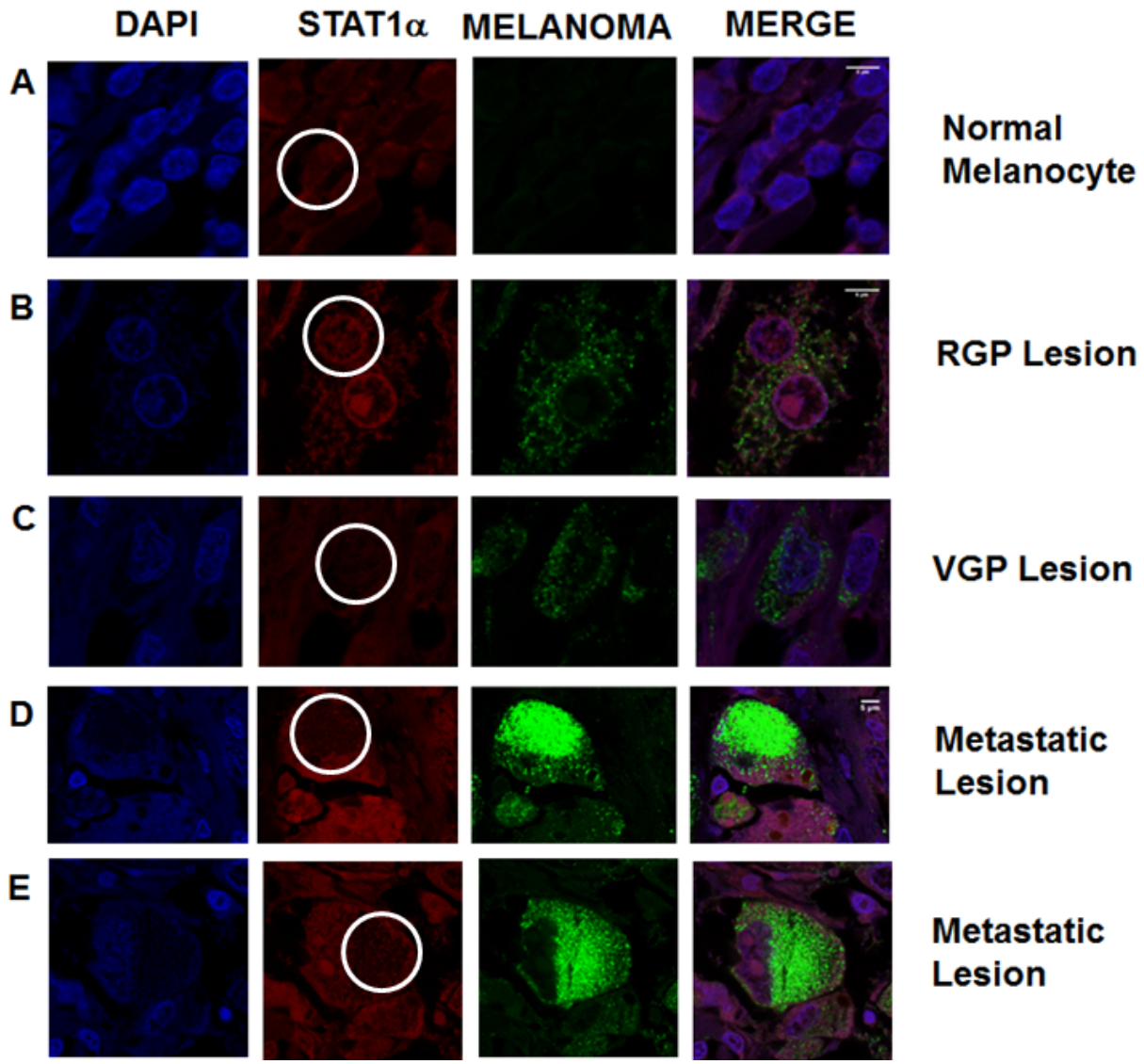


Figure 3.7 Immunofluorescence staining of STAT1 expression in non-metastatic and metastatic melanocytic lesions from patient samples.

Tissue microarrays of patient samples were de-waxed, rehydrated, and stained for the expression of STAT1 (red) and metastatic melanoma biomarkers (green). Cell nuclei are stained with DAPI (blue). Regions of interest are circled in white on the

STAT1 panels. (**A**) Normal melanocytes show constitutive STAT1 expression. (**B**). Primary tumors characterized as RGP show a slight decrease in STAT1 expression. (**C**). VGP melanoma lesions show an increase in melanoma biomarkers as well as a decrease in STAT1. (**D**,**E**). Metastatic melanoma lesions show a marked increase in metastatic melanoma biomarkers which correlates with a decrease in STAT1. These data show correlation between biomarkers of metastasis and decrease in STAT1 expression in patient tumor samples. Data are representative of images taken from approximately 150 patient samples.

4 STAT1 SILENCING IN METASTATIC MELANOMA IS DUE TO HEMI-METHYLATION AT THE STAT1 PROMOTER

Tumor immunosurveillance describes the phenomenon by which the immune system detects neoplastic cells as danger signals and responds accordingly (Mlecnik, Bindea et al. 2011). The recognition of "altered self" is in the context of tumor associated antigens (TAAs) presented on either Major Histocompatibility Complex (MHC) Class I or MHC II (Mlecnik, Bindea et al. 2011). Recognition of TAAs presented by MHCI elicit a $CD8^+$ cytotoxic T cell response, whereas TAAs presented in the context of MHCII triggers activation of $CD4^+$ T helper cells (Corthay, Skovseth et al. 2005, Groth, Kloss et al. 2011). However, many tumors evade immune detection by changing MHC cell surface protein expression (Dunn, Bruce et al. 2002, Reiman, Kmieciak et al. 2007, Groth, Kloss et al. 2011). Metastatic melanoma is one such tumor that downregulates MHC cell surface proteins in order to escape detection by the adaptive immune system (Hsu, Meier et al. 2002, Gajewski 2006).

Metastatic melanoma downregulates cell surface expression of MHCII (Degenhardt, Huang et al. 2010, Osborn and Greer 2015). By silencing MHCII, melanoma cells remain undetected by $CD4^+$ T cells. MHCII expression is a product of the interferon gamma (IFN γ) inducible JAK/STAT signaling cascade (Morris, Beresford et al. 2002). Upon binding to its receptor, JAK1 and JAK2 bind and cross-phosphorylate one another as well as the cytoplasmic tails of the interferon receptor (Sakatsume, Igarashi et al. 1995). Phosphorylation of the interferon- γ receptor creates a docking site

for Signal Transducer and Activator of Transcription (STAT) 1 (Igarashi, Garotta et al. 1994). Upon docking, STAT1 is phosphorylated by JAK2 and subsequently forms a homodimer known as the Gamma Activated Factor (GAF) (Shuai, Schindler et al. 1992, Shuai, Horvath et al. 1994). GAF then translocates to the nucleus and binds at the Gamma Activated Sequence (GAS) at the promoter of Interferon Response Factor (IRF) 1. IRF1 is transcribed and translated and forms a heterodimer with IRF2 (Hobart, Ramassar et al. 1997). The GAF and IRF heterodimer then bind at the promoter of the master regulator of MHC II, the Class II Transactivator, CIITA (Lee and Benveniste 1996, Hobart, Ramassar et al. 1997). Upon transcription and translation, CIITA binds an enhanceosome which assembles at the MHCII promoter (Jabrane-Ferrat, Nekrep et al. 2003, Drozina, Kohoutek et al. 2005).

Cancer is a heterogenous disease and there are multiple mechanisms by which interactions with T cells are circumvented in metastatic melanoma. Decreased interaction with Natural Killer cells was seen in both uveal and malignant melanoma due to a decrease in cell surface molecules required to interact with NKG2D (Vetter, Lieb et al. 2004, Schwinn, Vokhminova et al. 2009). Multiple mechanisms underlying silencing of MHCI have been shown *in vitro* and *in vivo* including defects in antigen loading, loss of heavy chain expression, or a gradual inability to express MHCI-restricted antigens (Jager, Ringhoffer et al. 1997, Seliger, Ritz et al. 2001, Garrido, Algarra et al. 2010). It has been reported that the loss of MHCII in cell lines is often at the level of silencing of CIITA and reinstatement of CIITA expression restores MHCII on the cell surface (Radosevich, Song et al. 2004, Le, Zhang et al. 2005, Radosevich, Jager et al. 2007).

Regardless of the mechanisms behind decreasing interaction with T cells, many studies show that an increase in Tumor Infiltrating Lymphocytes positively correlates with patient outcome (Bernsen, Hakansson et al. 2003, van Houdt, Sluijter et al. 2008, Fujisawa, Nabekura et al. 2009)

In 2010, the Weber group at Novartus published that the metastatic melanoma cell line 1205lu lacks cell surface expression of MHCII (Degenhardt, Huang et al. 2010). We recently reported that 1205lu also lacks both IRF1 and STAT1 (Osborn and Greer 2015). Because IRF1 is dependent upon the transcription factor STAT1, we conclude that the lack of STAT1 is responsible for the absence of MHCII on the cell surface. In this study, we set out to determine the mechanisms underlying silencing of STAT1 in metastatic melanoma.

4.1 RESULTS

4.1.1 THE ABSENCE OF STAT1 IN METASTATIC MELANOMA CELLS IS NOT DUE TO ABERRANT PROTEASOMAL DEGRADATION

One mechanism by which protein expression can be diminished in tumor cells is via abberant proteasomal degradation (Bashir and Pagano 2003) . Proteins are tagged for degradation by the addition of four or more ubiquitin moieties (Hershko and Ciechanover 1986, Hershko and Ciechanover 1998). The poly-ubiquitin tag is recognized by the 26S proteasome, which subsequently recycles the amino acids which

comprise the tagged protein (Pickart 1997, Bajorek and Glickman 2004). To determine if STAT1 was being abbarently degraded in MET cells, we blocked proteasomal degradation with the FDA-approved drug Velcade™ (Bortezomib) (Einsele 2014). Despite varying concentrations of Bortezomib treatment, STAT1 protein expression was not restored in MET cells (**Figure 4.1**). RGP cells, which constitutively express STAT1, were used as a positive control to verify STAT1 staining.

4.1.2 STAT1 IS SILENCED AT THE LEVEL OF TRANSCRIPTION IN METASTATIC MELANOMA CELLS

After determining that dysregulated protein turnover is not responsible for the loss of STAT1 expression in the MET cells, we next turned our attention to the possibility of STAT1 silencing at the level of transcription. To determine if STAT1 is being transcribed, we performed message level experiments which measure mRNA expression in cells. Following interferon-γ stimulation, cDNA specific for either STAT1 or beta actin was generated and amplified via PCR. Following amplification, PCR products were resolved on 1% agarose gels. As expected, RGP cells express basal levels of STAT1 which increase in expression following stimulation (**Figure 4.2A**). A similar trend is seen in VGP cells with basal expression of STAT1 which increases after stimulation (**Figure 4.2B**). In contrast, MET cells show no STAT1 mRNA expression (**Figure 4.2C**). We therefore conclude that STAT1 is silenced at the level of transcription in MET cells.

4.1.3 ***THE STAT1 PROMOTER IS HEMI-METHYLATED IN RGP, VGP, AND MET CELL LINES***

Promoter methylation is often seen in tumors that silence constitutively expressed genes (Akhavan-Niaki and Samadani 2013, Xiang, Yuan et al. 2013). The addition of methyl groups to CpG residues within the promoter blocks binding of transcription factors (Bednarik, Duckett et al. 1991, Fukushige and Horii 2013). Methylation at promoters can also silence genes by recruiting chromatin remodeling complexes (Jones, Veenstra et al. 1998). To determine whether the STAT1 promoter is methylated in melanoma cells, we utilized methylation specific PCR. We observed two distinct populations of methylated and unmethylated STAT1 promoter in RGP cells (**Figure 4.3A**). Because STAT1 is constitutively expressed in RGP cells, we expected to see completely unmethylated STAT1 promoter in this cell line. VGP cells also show both methylated and unmethylated STAT1 (**Figure 4.3B**). Unexpectedly, the STAT1 promoter in MET cells is incompletely methylated (**Figure 4.3C**). While dysregulation of promoter methylation has been shown to contribute to silencing of proteins in many cancers (Maeda, Ando et al. 2006), hemi-methylation is not sufficient alone to continue suppression (Braga Lda, Silva et al. 2014).

4.1.4 ***TREATMENT WITH 5-AZA-DEOXYCITIDINE DOES NOT RESTORE STAT1 EXPRESSION IN MET CELLS***

To determine if hemi-methylation contributes to silencing of STAT1 in MET cells, we treated the MET cells with 5-aza-deoxycitidine (5-aza). The mechanism of methylation reversal by 5-aza is prevention of *de novo* methylation by binding to DNMT3 (Lavelle, Saunthararajah et al. 2008). Following 48 hours of 5-aza treatment, the levels of STAT1 protein expression were measured via western blot. Treatment with 5-aza did not restore levels of STAT1 protein in MET cells (**Figure 4.4**). Therefore we conclude that promoter methylation contributes to, but is insufficient alone, for silencing of STAT1 in MET cells.

4.2 DISCUSSION

Tumor immunosurveillance refers to the ability of the immune system to recognize and respond to cells displaying cell surface tumor associated antigens via major histocompatibility complex molecules (Mlecnik, Bindea et al. 2011). Downregulation of MHC molecules leads to immune evasion and plays roles in morbidity and mortality in cancers including metastatic melanoma (Seliger, Ritz et al. 2001). We recently reported that metastatic melanoma silences MHC II, and thus TAA expression to $CD4^+$ T cells, by silencing the transcription factor STAT1 (Osborn and Greer 2015). The variety of mechanisms by which tumor cells evade immune detection underscores the importance of determining the underlying cause of suppression in each tumor type, thus contributing to personalized medicine and improved patient outcomes.

The goal of this study was to determine mechanisms by which STAT1 is silenced in melanoma cells. Inhibition of the 26S proteasome did not restore STAT1 protein

expression in MET cells. Next, we investigated whether STAT1 was being transcribed and were unable to detect STAT1 specific transcripts in MET cells. To determine if methylation at the STAT1 promoter led to silencing of the gene, we preformed methylations specific PCR. Surprisingly, we found populations of both methylated and unmethylated STAT1 promoters in RGP, VGP, and MET cell lines. Therefore, we concluded that silencing of STAT1 is not due to aberrant proteasomal degradation, but rather hemi-methylation of the STAT1 promoter.

4.3 METHODS

4.3.1 CELL CULTURE

Wistar melanoma cell lines representing the Radial Growth phase (WM35), Vertical Growth Phase (WM1366) and Metastatic (1205lu) phases of melanoma were obtained from the Coriell Repository (Camden, NJ). Cells were grown in MCDB153:Liebowitz-15 4:1 media (Sigma-Aldrich, St. Louis, MO) at 37° C in 5% CO_2.

4.3.2 PROTEASOME INHIBITION

Cells were plated at a density of 8 x 10^5 in 10 cm^2 plates. Following adhesion, cells were treated with 5, 10, 15, or 20 nM Bortezomib (LC Laboratories, Woburn, MA) for 48 hours. Following treatment, cells were lysed in NP40 buffer (1% NP40, 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1 mM DTT). Clarified lysates were resolved on

10 % polyacrylamide gels, transferred to nitrocellulose, and blotted for STAT1 or β-actin (Cell Signaling, Danvers, MA).

4.3.3 ***POLYMERASE CHAIN REACTION***

Cells were plated at a density of 7 x 10^5 in 10 cm^2 plates. The following day, cells were treated with 50 U/mL of Interferon gamma (IFN-γ) for 4 hours or 30 minutes. Total mRNA was extracted via Qiazol according to the manufacturer's protocol (Qiagen, Valencia, CA).

4.3.4 ***METHYLATION SPECIFIC POLYMERASE CHAIN REACTION***

Cells were plated at a density of 7 x 10^5 in 10 cm^2 plates. The following day, cells were treated with 50 U/mL of Interferon gamma (IFN-γ) for 4 hours or 30 minutes. Genomic DNA was extracted using the E.Z.N.A. kit according to the manufacturer's protocol. Using the EZ-DNA Methylation-Direct ™ Kit (Zymo Research, Irvine, CA), 500 ng of DNA was bisulfite converted per the manufacturer's instructions. Methylation specific PCR was carried out with previously described primers (Chang, Chiang et al. 2012)

4.3.5 ***5-AZA TREATMENT***

Cells were grown in T175 flasks in the presence of 5, 10, or 15 μM 5-aza-deoxycytidine (Sigma-Aldrich, St. Louis, MO). After 48 hours, cells were harvested and subjected to western blot, PCR, or methylation specific PCR as described above.

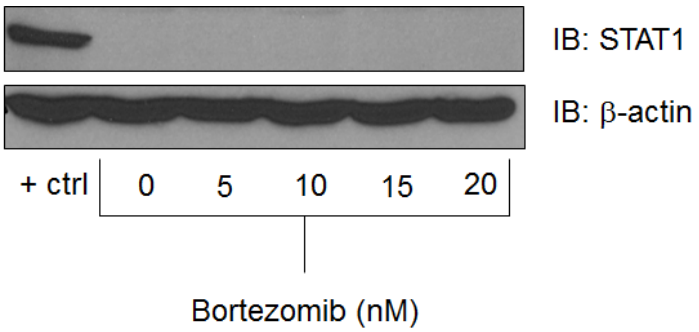


Figure 4.1 Western blot analysis of STAT1 expression following inhibition of the 26S proteasome.

Cells were treated with 0, 5, 10, 15, or 20 nM Bortezomib for 48 hours. Equal concentrations of protein from clarified lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with antibody specific for STAT1 (Top Panel). RGP cells, which constitutively express STAT1, were used as a positive control. β-actin was used as a loading control (Bottom Panel). Data shown is representative of at least 3 independent experiments.

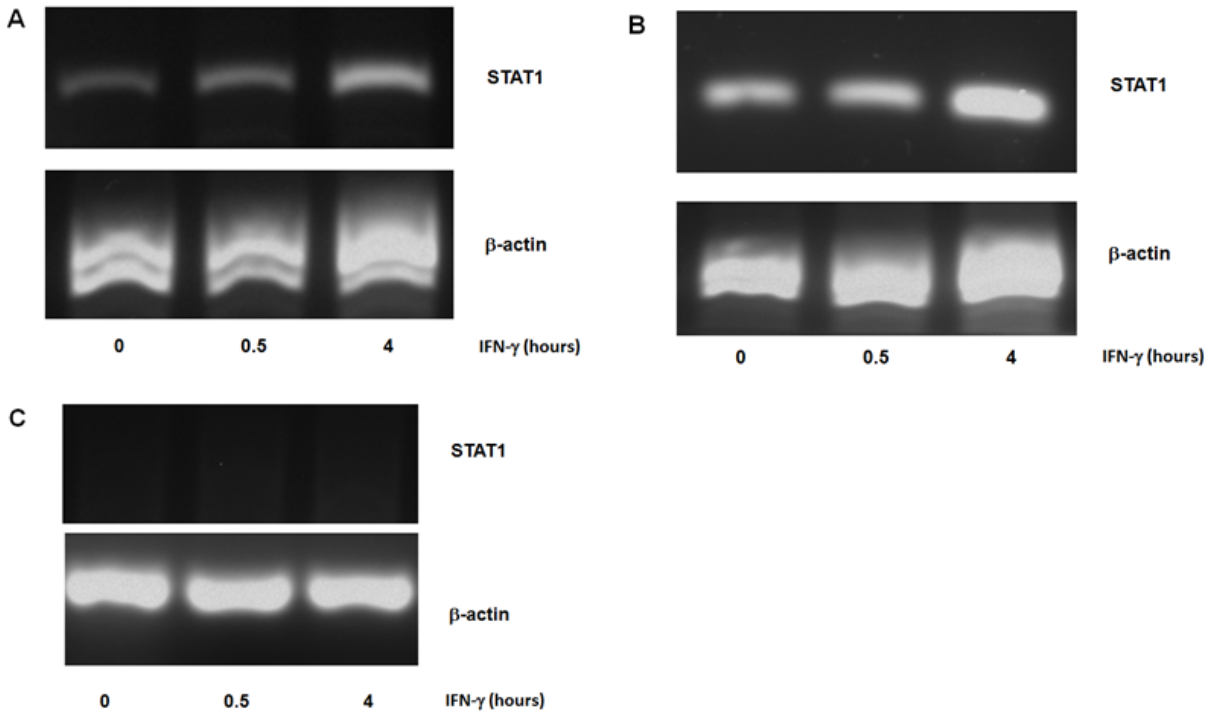


Figure 4.2 PCR analysis of STAT1 transcript expression in RGP, VGP, and MET cells

Cells were stimulated with IFN-γ for 4 hours or 30 minutes. Following stimulation, total RNA was isolated from cells using Qiazol® extraction reagent. Then, 2 μg of RNA was converted to cDNA and amplified via PCR with primers specific for either STAT1 or β-actin. Data shown is representative of at least 3 independent experiments.

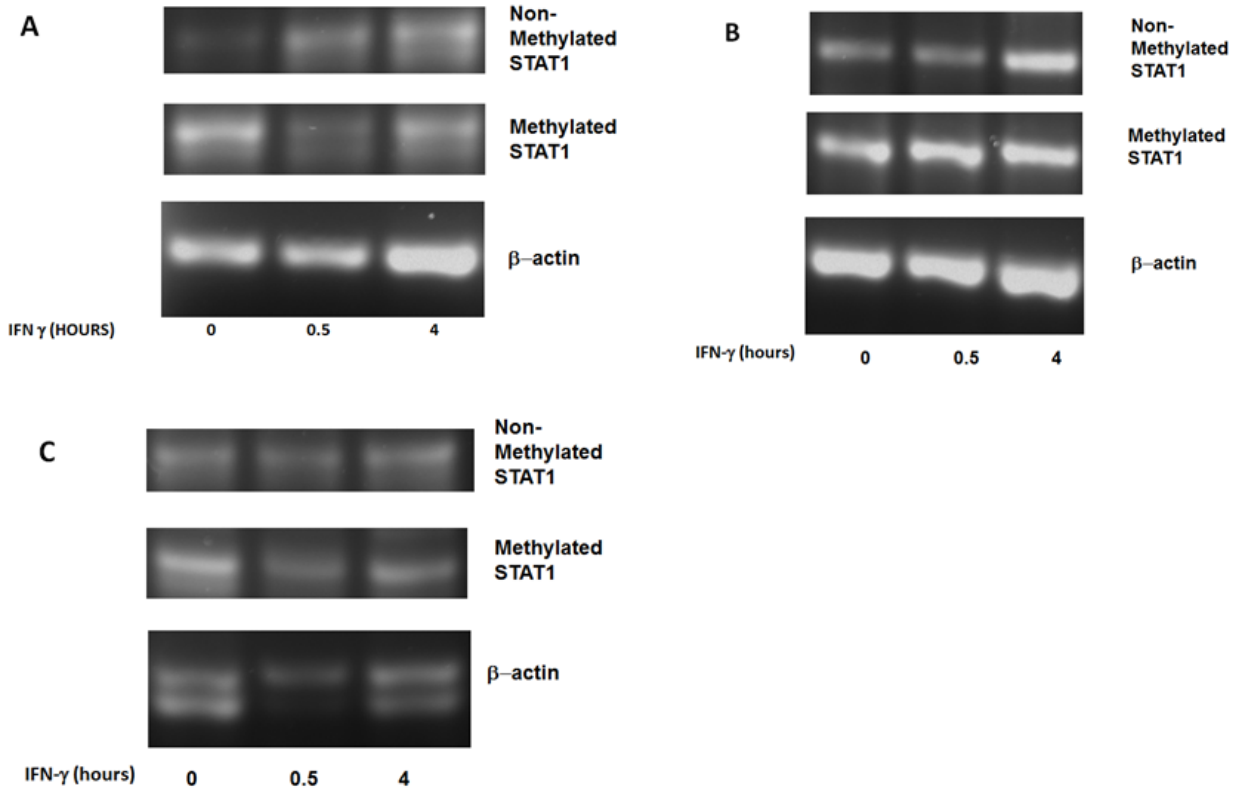


Figure 4.3 Methylation specific PCR analysis of methylation at the STAT1 promoter in RGP, VGP, and MET cell lines.

Genomic DNA was isolated from cells following a course of IFN stimulation. gDNA was bisulfite converted and amplified in PCR with primers specific for non-methylated STAT1, methylated STAT1, or actin. **A.** RGP cells exhibit low levels of non-methylated STAT1 which, as expected, increases upon stimulation. An inverse trend is seen for methylated STAT1, which decreases after stimulation, but does not completely disappear. **B.** Interestingly, VGP cells show basal levels of non-methylated STAT1 with no increase upon stimulation. Similar to non-methylated STAT1, methylated STAT1 does not change throughout 4 hours of stimulation. **C.** Unexpectedly, MET cells show both non-methylated and methylated STAT1 with no change throughout stimulation.

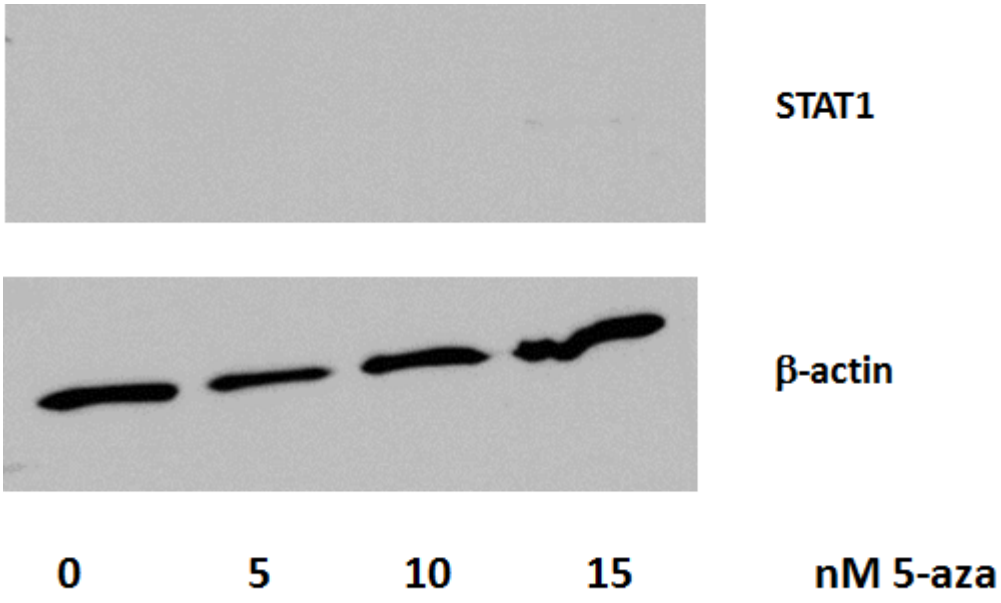


Figure 4.4 Western blot analysis of STAT1 expression following treatment with 5-aza.

Cells were grown in T175 flasks in the absence or presence of 5, 10, or 15 nM 5-aza. After 48 hours, cells were harvested and lysed. Clarified lysates were resolved on 10% polyacrylamide gel, transferred to nitrocellulose, and blotted for either STAT1 or actin. Despite increasing concentrations of 5-aza, STAT1 expression was not restored.

5 CONCLUSIONS

Transcriptional regulation refers to the orchestrated interactions of transcription factors, transcriptional machinery, histones, histone chaperones, and the target genes which are being transcribed. Dysregulation at the level of transcriptional regulation therefore results in a myriad of malformations and disease. Our initial studies revealed possible interactions between the 26S proteasome and histone chaperones, further implicating the 26S proteasome in transcriptional regulation. Determining the role of the interactions between the 26S proteasome, the HUCA complex, and elongating RNA Polymerase II will lead to better understanding of diseases caused by transcriptional dysregulation.

Transcriptional regulation is further important in the immune response against tumors. Inducible expression of MHC II aids in presentation of tumor associated antigens to $CD4^+$ T cells, triggering an immune response and, ideally, elimination of neoplastic cells. However, many tumors downregulate MHC molecules and are thus able to evade detection. One such tumor that downregulates MHC II is metastatic melanoma. We investigated the mechanisms by which MHC II is silenced in metastatic melanoma cells.

We initially confirmed the observations by Degenhardt et al. that the metastatic melanoma cell line 1205lu lacks cell surface expression of MHC II (Degenhardt, Huang et al. 2010, Osborn and Greer 2015). We determined that 1205lu cells are able to

respond to IFN-γ due to constitutive expression of the IFNGR1, the portion of the receptor heterodimer to which IFN-γ binds. Next, we explored the expression levels of the Janus Kinases JAK1 and JAK2 which are required for downstream signaling upon IFN-γ stimulation. All three cell lines investigated,representing the stages of melanoma development through RGP, VGP and MET, constitutively expressed both JAK1 and JAK2. As expected, RGP cells expressed the inducible protein IRF-1 after 4 hours of IFN-γ stimulation. This pattern was seen in VGP cells as well, but with less expression at 4 hours. Conversely, MET cells lack IRF-1 expression despite 4 hours of stimulation.

IRF-1 expression requires the Gamma Activated Factor (GAF) formed by the homodimer of STAT1, we therefore investigated the expression and activation of STAT1. In RGP cells, constitutive STAT1 expression was observed with phosphorylation of Tyrosine 701 seen as early as 30 minutes. The same expression and activation pattern was seen in VGP cells. MET cells showed no expression of STAT1 and thus no phosphorylation after stimulation. Because STAT1 is required for both IRF-1 and CIITA expression, it is indispensable for MHC II transcription. We therefore concluded that the lack of MHC II in MET cells is due to silencing of the transcription factor STAT1.

Our next studies sought to determine the mechanisms underlying silencing of STAT1 in metastatic melanoma cells. We first suspected that dysregulation of protein turnover may be responsible for a lack of STAT1 protein expression. To investigate, we inhibited the 26S proteasome with the FDA-approved drug Bortezomib. Despite 72

hours of treatment with varying concentrations of Bortezomib, we saw no reinstatement of STAT1 protein expression. Therefore, we concluded that STAT1 silencing is not at the level of translation or protein turnover.

Next, we investigated whether or not STAT1 was being transcribed in MET cells. Using primers specific or either STAT1 or actin, we performed PCR on total mRNA from RGP, VGP, and MET cells treated with a course of IFN-γ. As expected we saw both basal and IFN-γ inducible STAT1 in both RGP and VGP cells. MET cells showed essentially no transcripts for STAT1 compared to constitutive actin expression. Therefore, we concluded that STAT1 is silenced at the level of transcription in MET cells.

Promoter methylation is a common mechanism by which neoplastic cells silence proteins. The addition of a methyl group on CpG residues within the promoter of a gene prevents binding of transcription factors. To determine whether or not promoter methylation is responsible for the silencing of STAT1 in MET cells, we performed methylation specific PCR (MS-PCR). MS-PCR uses primers specific to either methylated or unmethylated promoters to amplify DNA. Unexpectedly we saw both methylated and unmethylated STAT1 promoters in both RGP and VGP cells which constitutively express STAT1. A similar expression pattern was seen in MET cells which showed both unmethylated and methylated STAT1 promoters. To determine if hemi-methylation is responsibe for silencing of STAT1 in MET cells, we treated with a course of 5-aza-2'-deoxycytidine (5-aza). 5-aza prevents methylation in daughter cells, and

ideally re-instates protein expression when silencing is caused by aberrant promoter methylation. Despite 2 days of 5-aza treatment, we saw no reinstatement of STAT1 expression in MET cells. We concluded that STAT1 is likely super-silenced in MET cells. Super silencing is a combination of promoter methylation and a dysregulation of epigenetic modifications of the histones surrounding a gene. Our findings suggest super silencing of STAT1 is one mechanism by which metastatic melanoma circumvents detection by the immune system, andthus increasing its lethality.

Determining the mechanism underlying carcinogenesis in individual tumors can serve to improve individualized medicine. By targeting the specific mutation(s) leading to immune evasion, clinicians can direct the patient's own immune system to target their tumor thus increasing patient outcomes.

Our studies implicate STAT1 hemi-methylation in silencing of MHC II in metastatic melanoma. Because demethylation of the STAT1 promoter did not restore STAT1 protein expression, it is likely that STAT1 is super-silenced, and thus histone modifications are also dysregulated in metastatic melanoma. Investigation of histone modifications is imperative in determining treatment routes. For example, our lab has shown that RGS10 is silenced in ovarian cancer by a combination of promoter methylation and aberrant acetylation which leads to chemoresistance (Ali, Cacan et al. 2013, Cacan, Ali et al. 2014). In patients with this pattern of gene dysregulation, the addition of epigenetic modifying drugs could improve patient prognosis. Many FDA-

approved drugs are undergoing clinical trials for use in additional maladies (Nebbioso, Carafa et al. 2012)

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