A Novel Mechanism of Skin Tumor Promotion Involving Interferon-gamma (IFNγ)/Signal Transducer and Activator of Transcription-1 (Stat1) Signaling in Epidermis

Ronald Bozeman

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A Novel Mechanism of Skin Tumor Promotion Involving Interferon-gamma (IFNγ)/Signal Transducer and Activator of Transcription-1 (Stat1) Signaling in Epidermis

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A

DISSERTATION

Presented to the Faculty of The
University of Texas Health Science Center at Houston
and
The University of Texas M.D. Anderson Cancer Center Graduate School of
Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY
by
Ronald Bozeman M.S.
Houston, Texas
May 2013
Dedication

This thesis is dedicated to my family, for which without them none of this would be possible. First I would like to thank my mother, Ms. Barbara Bozeman, who sacrificed so much so that I may never have to go without. She has been my backbone and my source of inspiration my entire life and has supported me in every endeavor. I would also like to dedicate this project to my older brother Tilford T. Bozeman, for who has always been there for me and taught me life lessons to make me a better man. Lastly, I would like to dedicate this dissertation to my maternal grandmother, Mrs. Sophia Whitney Bozeman. Thank you for being the inspiration to our family. I love you more than words could express.
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I would also like to thank my outstanding committee members. Drs. Sue Fischer, Dean Tang, Gary Johanning, Mark Bedford, and David Johnson. I want to especially thank Dr. David Johnson who has served as a second mentor in many ways. I want to thank you all for your guidance and continued support. I also want to thank Becky Brooks who has served as a second mom to many of us graduate students during our time away from home.

I would like to give a heartfelt thank you to all the members of the DiGiovanni Lab past and present. I would like to thank past lab members Drs. Tricia Moore, Dae Joon Kim, and Erika Abel. I would like to especially thank Dr. Abel, who provided me with invaluable mentorship early on in my academic career. Without her guidance it is unimaginable to think I will be where I am today. I would also like to thank current members of the lab, Drs. Everado Macias, Okkyung Rho, Joe Angel, Karou Kiguchi, Jorge Blando, Achinto Saha, and Jaya Srivastava. I would also like to thank the DiGiovanni support staff, Alex McClellan, Steve Carbajal and Linda Beltran. Because of their tireless dedication to the lab we’re able to focus on science. Last but not least, I would like to thank
former graduate students Drs. Allyson Checkley and Dharanija Rao. Over the past few years we had become known as the three musketeers and I could always count on you guys both personally and professionally.
The JAK-STAT pathway is a major signaling pathway involved in many biological processes including proliferation, apoptosis, and differentiation. Aberrant expression of STATs has been reported in multiple human cancers and murine mouse models of tumorigenesis. Previous studies from our lab and others have established a critical role for Stat3 in epithelial tumorigenesis, but the role of Stat1 is largely unknown. The current study was designed to explore the role of Stat1 during multistage skin carcinogenesis. Topical treatment with both TPA and the anthrone derivative chrysarobin (CHRY) led to rapid phosphorylation of Stat1 on both tyrosine (Tyr701) and serine (Ser727) residues in epidermis. CHRY treatment also led to upregulation of unphosphorylated Stat1 (uStat1) at later time points. In addition, CHRY treatment also led to upregulation of IRF-1 mRNA and protein which was dependent on Stat1. Further analyses demonstrated that topical treatment with CHRY but not TPA upregulated interferon-gamma (IFNγ) mRNA in the epidermis and that the induction of both IRF-1 and uStat1 was dependent on IFNγ signaling. Stat1 deficient (Stat1\(^{-/-}\)) mice were highly resistant to skin tumor promotion by CHRY. In contrast, the tumor response (in terms of both papillomas and squamous cell carcinomas) was similar in Stat1\(^{-/-}\) mice and wild-type littermates with TPA as the promoter.

Histological evaluation of the proliferative response confirmed the data obtained
from the tumor study for both TPA and CHRY. In addition, maximal induction of both cyclooxygenase-2 and inducible nitric oxide synthase in epidermis following treatment with CHRY was also dependent on the presence of functional Stat1. Following CHRY treatment, Stat1\(^{-/-}\) mice exhibited reduced macrophage infiltration and reduced production of many immune cell derived chemokines/cytokines. These studies define a novel mechanism associated with skin tumor promotion by the anthrone class of tumor promoters involving upregulation of IFN\(\gamma\) signaling in the epidermis and downstream signaling through activated (phosphorylated) Stat1 and subsequent upregulation of IRF-1 and uStat1.
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Chapter 1: Introduction

1.1 Signal Transducer and Activators of Transcription

Signal Transducers and Activators of Transcription (STATs) represent a family of conserved transcription factors that transduce extracellular signals from the cellular membrane of the cell to the nucleus. These latent cytoplasmic transcription factors consist of seven members: Stat1α/β, Stat2, Stat3α/β, Stat 4, Stat5A, Stat5B and Stat6, 7(1). STATs can generally be classified into two groups according to their function. One group consists of Stat2, Stat4 and Stat6, which are activated by various cytokines and play distinct roles in T-cell development and interferon signaling. The other group includes Stat1, Stat3 and Stat5, which regulate important aspects of cellular growth, proliferation and apoptosis (2).

Our understanding of STAT signaling began over 40 years ago when Alick Isaacs and Jean Lindenmann et al reported on a phenomenon in the field of “virus interference,” that described a condition that disrupted virus formation (3) (4). Over the years the understanding of this pathway has grown to encompass all aspects of its function and can be seen in the nomenclature itself. The aptly named STATs represent a class of molecules with dual functionality. Stat proteins not only provide a means to transmit signals from the exterior of the cell to the nucleus but they also directly participate in gene regulation by binding to DNA.

1.2 Structure of STATs

STATs are proteins composed of approximately 850 amino acids, with the exception of Stat2 and Stat6, which have between 750 and 800 amino acids and range
in size from 90-115kDa (5). STATs share several highly conserved domains, which are important for their respective functions. STATs contain an N-terminal oligomerization domain, which is involved in stabilizing protein-protein interactions. This domain is also responsible for dimer-dimer interactions that facilitate the formation of STAT tetramers or oligomers. STATs contain a DNA-binding domain (DBD) that confers specificity of binding to cognate DNA sequences of target gene promoters. A linker domain resides between the DBD and the SRC homology-2 (SH2) domain. The function of this domain has not been fully characterized, although mutations within this domain affect the stability of DNA binding (6). The SH2 domain contains the critical tyrosine residues (Y701 for Stat1, Y690 for Stat2, Y705 for Stat3, Y693 for Stat4, Y695 for Stat5, and Y641 for Stat6) that are required for Stat recruitment to the cytoplasmic tail of the phosphorylated receptor. Phosphorylation of this site leads to activation (dimerization) of STATs. Differences in the SH2 domain confer selectivity to different cytokine receptors of each respective STAT protein [reviewed in (7)]. The transactivation domain (TAD) of each STAT is located in the carboxyl terminal region. The TAD domains contain the serine phosphorylation site that is responsible for maximal transcriptional activation (Bowman Oncogene 2000). Some STATs, in particular Stat1 and Stat3, have naturally occurring splice variants (Stat1β and Stat3β), which lack a portion of the C-terminal transactivation domain. These variants can potentially act as dominant-negative forms (8, 9). Figure 1 shows the structure of a representative STAT (Stat1) dimer bound to DNA (panel a) as well as a diagram of the various structural domains (panel b).
Figure 1. The Core Structure of a STAT1 Dimer Bound to DNA.  
(a) The core structure (amino acids 130–712) shows binding of a STAT1 dimer to DNA and the location of binding sites of various proteins in various domains. STATs share several highly conserved domains that are important for their respective function.  
1.3 Activation of STATs

STATs are activated by a variety of stimuli, including cytokines, growth factors, and hormones. Cytokines represent a large number of soluble molecules that regulate cellular growth, differentiation, and immune responses. Cytokines are classified as Type I (i.e. IL-2, IL-3, IL-4) and Type II (interferons and IL-10) and signal via cytokine receptors that contain no intrinsic enzymatic kinase activity, but accomplish phosphorylation of STATs via receptor associated Janus Kinases (JAKs). The JAK family of receptor associated tyrosine kinases consist of four members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2), which are activated by receptor dimerization after ligand binding (10).

STATs are also activated by growth factor receptors such as epidermal growth factor (EGF) receptor, insulin-like growth factor (IGF)-1 receptor, platelet-derived growth factor (PDGF) receptor, hepatocyte growth factor (HGF) receptor, and colony-stimulating factor- 1 (CSF-1) receptor, all of which possess an intrinsic tyrosine kinase activity (11). In addition to cytokine and growth factor receptor activation, STATs can also be activated by non-receptor tyrosine kinases such as SRC and ABL. STATs can also be activated by seven transmembrane domain receptors, such as angiotatin II, serotonin and α-melatonin (12).

Following ligand binding, a conformational change occurs on the cytoplasmic tail of the receptor inducing autophosphorylation and transphosphorylation of the associated JAK. This is followed by phosphorylation of the cytoplasmic tail of the receptor, which provides docking sites for the recruitment of STAT molecules that recognize phosphotyrosine via their phosphotyrosine binding domain (PTB) or SH2
domain (13). Once recruited to the SH2 domain of the receptor the STAT molecules are subsequently phosphorylated on tyrosine residues (14). Tyrosine phosphorylated STATs subsequently form homo/hetero dimers or heterotrimeric complexes that translocate to the nucleus where they bind to cognate DNA sequences and activate gene transcription. Figure 2 is a schematic depicting activation of STATs via receptors such as growth factor receptors, cytokine receptors and G-protein coupled receptors.
Figure 2. Activation of the JAK-STAT Pathway via Receptor Signaling. A conceptual diagram of the multiple mechanisms for tyrosine phosphorylation of STATs by Janus kinases (JAKs) or other protein tyrosine kinases (PTKs) that are intrinsic to receptors or that are present in the cytoplasm or nucleus. Tyrosine phosphorylation generates STAT dimers that can bind specific DNA targets. The JAK-STAT pathway is activated by a variety of stimuli. STATs can be activated by growth factor receptors that possess intrinsic kinase activity. They may also be activated by cytokine receptors which employ the use of a family of receptor associated kinases called Jaks (Jak1, Jak2, Jak3, Tyk2). STATs may also be activated by non-receptor tyrosine kinases such as Src and ABL. Reproduced with the permission of the journal Nature Reviews: Immunology. Reich NC and Liu L. (2006) “Tracking STAT nuclear traffic.” Nature Reviews Immunology 6, 602-612.
Previous studies also reported that STATs undergo cycles of activation-inactivation. STAT activity is coupled with nuclear-cytoplasmic shuttling and regulated by posttranslational modifications such as phosphorylation and acetylation. Nuclear retention of STATs is dependent upon their phosphorylation status. For example, phosphorylation of Stat1 on tyrosine 701 results in nuclear translocation, whereas acetylation of lysine residues 410 and 413 leads to increased dephosphorylation. Thus, acetylation results in Stat1 inactivation (15). After binding to DNA and activating gene transcription, STAT inactivation occurs rapidly and the unphosphorylated STAT molecule is exported out of the nucleus.

1.4 Negative Control of STATs

STAT activation is a transient process. Within hours the activating signals subside and STATs are again in an inactive state. The JAK-STAT signaling pathway is negatively regulated by two nuclear regulators, protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTPs) and one cytoplasmic regulator, suppressor of cytokine signaling (SOCS). There is data to suggest that PTPs also act at the membrane level by dephosphorylating activated JAKs at the receptor. PIAS interact exclusively with tyrosine-phosphorylated forms of Stat proteins, thereby preventing DNA binding. PTPs, which contain SH2 domains, negatively regulate activated STATs by interacting and directly dephosphorylating STATs in the nucleus. SOCS are cytokine-induced proteins that are recruited to activated receptor complexes to regulate signaling. SOCS proteins inhibit signaling by directly binding to activated JAKS, thereby competing for STAT binding and/or by targeting activated JAKs for ubiquitin-mediated proteasomal degradation (16). Figure 3 shows the major pathways involved in negative regulation of STAT signaling.
Figure 3. Negative Regulators of STAT Signaling. Stat activation is a transient process and therefore requires negative regulation. The Janus kinase (JAK)—signal transducer and activator of transcription (STAT) pathway is regulated at many levels. Two nuclear regulators, protein inhibitors of activated STATs (PIAS), protein tyrosine phosphatases (PTP) and one cytoplasmic regulator, suppressor of cytokine signaling (SOCS), negatively regulate the JAK-STAT signaling pathway. PIAS bind directly to activated STATs whereby they block the transcription of downstream target genes. PTP dephosphorylate activated STATs thereby facilitating their export out of the nucleus. The SOCS class of proteins acts in a classical negative feedback loop. They are transcriptionally upregulated after JAK-STAT pathway activation. SOCS negatively regulate STAT signaling by binding to activated JAKs, thereby blocking JAK activity. Reproduced with the permission of the journal Nature Reviews: Immunology. Shuai K, and Liu B. (2003) “Regulation of JAK-STAT signaling in the immune system.” Nature Reviews Immunology 3, 900-911.
1.5 Interferon Signaling

Isaacs and Lindenmann identified interferon (IFN), the first cytokine discovered, during their seminal studies over 50 years ago (3, 4). IFNs are a family of multifunctional proteins that were first discovered due to their ability to interfere with viral replication. IFNs are historically classified as Type I and Type II, based on receptor specificity and sequence homology. Type I IFNs are comprised of multiple interferon-alpha (\(\alpha\)) subtypes (14-20 depending species), interferon beta (\(\beta\)), interferon omega (\(\omega\)) and interferon tau (\(\tau\)). All Type I IFNs are structurally related and bind to a heterodimERIC receptor, IFNAR (comprised of IFNAR1 and IFNAR2).

Type II IFNs are represented by only one cytokine, which is IFN gamma (\(\gamma\))(IFN\(\gamma\)). IFN\(\gamma\) binds to a different receptor than that of Type I IFNs. The IFN\(\gamma\)R is comprised of two ligand-binding IFN\(\gamma\)R1 chains that are associated with two signal-transducing IFN\(\gamma\)R2 chains (17). IFN receptors, like other cytokine receptors, possess no intrinsic kinase activity and therefore depend on receptor-associated kinases to phosphorylate their substrates. IFNs also employ JAK family tyrosine kinases as described above (17).

Binding of the Type I IFNs, such as IFN\(\alpha\), to its receptor induces dimerization of the two subunits and leads to the auto and transphosphorylation of TYK2 and Jak1. Activated JAKs then phosphorylate the receptor cytoplasmic domain on tyrosine residues, which provides a docking site for Stat1 or Stat2. Stat1 or Stat2 is then able to bind and is subsequently phosphorylated on tyrosine (Y701 for Stat1; Y690 for Stat2) by JAKs, which in turn allows the recruitment of Stat1. Stat1 is subsequently phosphorylated on tyrosine, which allows the release of Stat1/Stat2 heterodimer. The
Stat1/Stat2 heterodimer is now available to form a heterotrimeric complex containing p48 (also known as ISGF3 or IRF9), thus forming the ISGF3 complex. The ISGF3 transcription factor complex translocates to the nucleus where it binds to DNA containing the ISRE (IFNα-stimulated gene response element) sequence in the promoter region of its target genes(18).

Upon binding of IFNγ to its receptor, IFNγR1 and IFNγR2 dimerize which leads to the auto and transphosphorylation of JAK1 and JAK2. The JAKs in turn cause the phosphorylation of the cytoplasmic domain of the IFNγR1 tail, which provides SH2 docking sites for STAT1. Once phosphorylated, Stat1 homodimers translocate to the nucleus and bind to DNA containing the GAS (IFNγ-activated site) sequences. Figure 4 illustrates the canonical signaling pathways for both Type I and Type II IFNs.
Figure 4. Interferon Receptor Activation by Classical JAK-STAT signaling. IFNs are historically classified as Type I and Type II, which is based on receptor specificity and sequence homology. Type I IFNs are comprised of multiple interferon-alpha (α) subtypes, interferon beta (β), interferon omega (ω), and interferon tau (τ). Type II IFNs are represented by only one cytokine, IFNγ. JAK1 and TYK2 activation is associated with the type I IFN receptor activation. Which results in tyrosine phosphorylation of STAT2 (signal transducer and activator of transcription 2) and STAT1; which results in the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) complexes. IFNs also induce the formation of Stat1 homodimers that bind to IFN-gamma activated sequences (GAS) elements located in the promoter region ISGs, thereby initiating the transcription of these genes. Reproduced with the permission of the journal Nature Reviews: Immunology. Platanias LC. (2005) “Mechanisms of type-I- and type-II-interferon-mediated signaling.” Nature Reviews Immunology 5, 375-386.
1.6 Stat1 and Immune Function

Stat1 was the first member of the STAT family of proteins discovered. Stat1 has been shown to be the principal transducer of Type I (α and β) and Type II (γ) IFNs. The IFNγ/Stat1 axis has been shown to regulate many biological processes such as cellular growth, immune response, and even inflammation [reviewed in (5)]. Type I and Type II IFNs play a complementary and non-redundant role in defense against a broad spectrum of viral and bacterial pathogens. IFNs play a vital role in both the innate and humoral immune response. Various immunocytes such as lymphocytes, dendritic cells and macrophages produce IFNs in response to invading pathogens (19).

Activation of the IFNγ/Stat1 pathway is critical to processing and presentation of tumor antigens. Stat1 regulates key components of the immunoediting machinery such as major histocompatibility complex (MHC) class I and II (20). MHC class proteins are necessary for immune cells such as lymphocytes to display phagocytosed tumor antigens on their cell surface. Dysregulation of Stat1-dependent expression of MHC and other components such as the MHC class II transactivator CIITA, leads to defective antigen presentation (21).

1.7 Stat1 and Growth Arrest/Growth Inhibition

Stat1 also plays a role in cellular growth, as its activation generally evokes an anti-proliferative program. Stat1 has the ability to regulate the cell cycle at various points of its progression. Previous reports have shown that Stat1 activation may inhibit cell cycle progression by blocking oncogenic signals such as c-Myc and various cyclins (A, B, D2, D3 and E) (22). Stat1 has also been shown to negatively affect the cell cycle by up-regulating various tumor suppressor proteins, such as the cyclin-dependent
kinase inhibitors (CKIs), p21 WAF1/CIP1 and p27/KIP1 [reviewed in(23)]. The activation of these CKIs ultimately leads to G₀/G₁ cell cycle arrest. In the prostatic cancer cell lines DU145, LNCaP, and PC-3 it was reported that treatment with IFNγ resulted in the down-regulation of the proto-oncogene (Her)-2/neu, which was shown to be Stat1-dependent (24).

1.8 Stat1 and Apoptosis

The importance of Stat1 in the apoptotic response has been supported by the fact that pro-apoptotic properties of IFNs are largely mediated by STAT signaling. Stat1 has the ability to promote apoptosis by regulating both transcription-dependent and independent mechanisms. Stat1 promotes apoptosis by inducing both initiator and effector caspases. Stat1 can induce the expression of caspases 1 and 11, which are required for activation of the effector caspases, 3 and 7, (25, 26). Activation of Stat1 via IFNγ signaling induces the expression of several surface cell death receptors and their ligands, such as Fas/FasL, TRAIL and its receptor Killer/DR5, (27-29). Stat1 can also regulate other pro-apoptotic genes such as XIAP-associated factor- (XAF)-1, (30) and IFN-induced transmembrane protein 1 (IFITM1), (31). It is noteworthy that Stat1 can also negatively regulate the expression of pro-survival genes such as Bcl-2 and Bcl-xl (32).

Stat1 can serve as a co-activator of pro-apoptotic signaling. In this regard, Stat1 acts as a co-activator by interacting with tumor necrosis factor (TNF)-α receptor 1 (TNFR1) and TNFR1-associated death domain protein (TRADD). Acting as a component in this complex, Stat1 serves to inhibit NF-κB-mediated pro-survival signaling (33). Stat1 is required for DNA damage-induced apoptosis through its direct interaction with p53 (34).
In addition Stat1 can also act on the negative regulator of p53, Mdm2, by targeting it for proteosomal degradation (34).

1.9 Stat1 and Angiogenesis

Angiogenesis plays an important role in the development of cancer (35). Neovascularization is vital to the progression of tumor development, and may also serve as a prognostic indicator of metastatic potential. Stat1 has been shown to inhibit vascular endothelial growth factor (VEGF), a potent signaling molecule that promotes the formation of new blood vessels to tissues with inadequate oxygen supply. Stat1 inhibits the action of VEGF by inhibiting factors required for VEGF-induced tube formation, including urokinase plasminogen activator, angiopoetin-2, tissue inhibitor of matrix metalloproteinase (TIMP)-1, and VEGFR-2 (36). In addition, Stat1 can activate the production of the CXC chemokine family member, interferon-inducible protein IP10 (CXCL10). IP-10, a known anti-angiogenic gene, blocks the production of fibroblast growth factor (FGF)-β, (37) and inhibits endothelial cell motility (38). Endothelial cell motility is a critical component of the angiogenic response.

1.10 Potential Role of Unphosphorylated Stat1 (uStat1)

Recently, it has been found that Stats 1 and 3 (and possibly other Stats) may play important roles in mediating gene expression in the absence of tyrosine phosphorylation (39, 40). Stat1 and Stat3 genes are targets of activated (phosphorylated) Stat1 and Stat3 proteins, respectively (41). As a result, cytokine activation of Stat1 or Stat3 (e.g., IFNγ or IL-6, respectively), leads to the induction and
accumulation of uStats 1 and 3, which may persist for days after p-Stat levels have subsided (40). In addition, induction of the Stat1 target gene, IRF-1, aids in the continued accumulation of uStat1 in response to IFNγ. It is well-documented that uStats 1 and 3 can act as transcription factors to regulate a subset of genes that are different from those regulated by p-STATs (31, 39, 42). Together these data suggest that uStat1 may be transcriptionally active and may play a significant role in various cellular responses. Transcriptional profiling has shown that the majority of uStat1 target genes are antiviral immune response genes, however, uStat1 also induces a subset of genes implicated in radio- and chemo-resistance in cancer cells (42) (43). Disruption of IFN effector molecule 8 (IRF8) in soft tissue sarcoma cells leads to the accumulation of uStat1 (44) and promotes sarcoma cell metastasis by regulating gene transcription of apoptosis regulators Fas and Bad (44). Overall, the role and mechanism(s) by which uStat1 mediate cellular responses and possible protumorigenic effects are largely unknown.
1.11 Evidence for a Pro-Tumorigenic Role of Stat1

As previously stated, Stat1 is widely considered to possess tumor suppressive properties. There is emerging data that Stat1 may influence tumorigenesis by modulating several different aspects of tumor development. Utilizing large-scale gene expression studies from melanoma patients, Stat1 was shown to be elevated at the peripheral edge (invasion front) compared to central areas of the tumor. In addition, stable knockdown of Stat1 in metastatic melanoma cells significantly impaired their migratory and invasive capacity in matrigel and wounding assays (45).

Stat1 may also influence tumorigenesis by suppressing the apoptotic response although in many instances Stat1 has a pro-apoptotic function. For example, overexpression or constitutive activation of Stat1 may provide resistance to genotoxic stress including chemotherapy and ionizing radiation (IR). Utilizing a squamous cell carcinoma cell line (SCC61), which was serially passaged to create a radioresistant cell line (nu61), Khodarev et al. reported that nu61 cells were unresponsive to cytotoxic signals by radiation or IFNs, but suppression of Stat1 by short hairpin RNA rendered nu61 cells radiosensitive to IR. They proposed a model where transient induction of Stat1 activates cytotoxic genes, which results in cell death. Overexpression of Stat1 led to the suppression of the cytotoxic response and induced the expression of pro-survival genes (MCL-1, IFITM1, and USP18) that render the cells resistant to apoptosis (46).

As previously stated, Stat1 may also influence tumor development by regulating key components of the antigen presentation machinery, such as MHCI and II. Kovacic et al. found that Stat1 deficiency inhibited the development of leukemia in Stat1−/− mice
Loss of Stat1 resulted in low levels of MHC class I proteins, which apparently enabled efficient NK cell lysis, and enhanced tumor clearance. These findings define Stat1 as a tumor promoter in terms of leukemia development, since the upregulation of MHC I provides a mechanism by which newly formed tumors escape elimination by immune surveillance.

Over the years it has been suggested that Stat1 has a role in the induction of a pro-inflammatory signaling cascade. Stat1 has been found to be associated with several human pathological conditions associated with chronic inflammation including rheumatoid arthritis (48), pulmonary fibrosis (49), and Alzheimer’s disease (50). Stat1 influences the inflammatory response by inducing key components such iNOS (51) and IRF-1 (52). Utilizing models of inflammation-associated cancers, several groups have demonstrated the importance of Stat1 in inflammation. Stat1 was shown to provide a pro-inflammatory signal in ConA-induced hepatitis by driving the expression of several chemoattractant chemokines via IRF-1 (53). In addition, utilizing a model for gastric cancer, Ernest et al reported that loss of Stat1 reduced gastric cancer associated inflammation by reducing the pro-inflammatory interleukin, IL-11. Collectively these findings demonstrate the complexity of Stat1 signaling, whereby Stat1 has a pro-tumorigenic function by desensitizing cells to apoptosis, increasing metastatic potential, decreasing host recognition capacity and increasing the pro-inflammatory program. Table 1 summarizes Stat1 responsive genes involved in the process of tumorigenesis.
Table 1. Summary of Stat1 responsive genes that are involved in the process of tumorigenesis

<table>
<thead>
<tr>
<th>Cell Cycle</th>
<th>Angiogenesis</th>
<th>Apoptosis</th>
<th>Immune Surveillance</th>
<th>Inflammation</th>
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<tbody>
<tr>
<td>C-MYC</td>
<td>bFGF</td>
<td>Caspases (1,3,7,8)</td>
<td>MHC I and II</td>
<td>iNOS</td>
</tr>
<tr>
<td>p21</td>
<td>MMP9</td>
<td>Bcl-xl</td>
<td>CIITA</td>
<td>VCAM</td>
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<tr>
<td>p27</td>
<td>MMP2</td>
<td>Bcl-2</td>
<td>LMP2/7</td>
<td>ICAM</td>
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<tr>
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<td>Fas/FasL</td>
<td>TAP1/2</td>
<td>IRF-1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IP-10</td>
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1.12 Multistage Epithelial Carcinogenesis in Mouse Skin

Skin carcinogenesis in mice can be accomplished by utilizing either complete or initiation-promotion protocols (54). The complete carcinogenesis protocol involves the topical application of a single large dose or repeated applications of a smaller dose of a carcinogen [i.e. 7,12-Dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene (MCA)]. The initiation-promotion protocol involves the application of a single subcarcinogenic dose of a carcinogen followed by repeated applications of a tumor promoter. This well-established protocol in mouse skin recapitulates many aspects of human epithelial cancer (54). Cancer development is a complex process whereby a normal cell undergoes genetic alterations that results in an altered phenotype that is characterized by a selective growth advantage. Multiple lines of evidence suggest that these alterations occur in a multi-step process (55). The complete carcinogenesis model does not allow interpretation of different stages of the tumorigenic process, whereas the initiation-promotion protocol allows for observable delineation. In the initiation-promotion protocol three distinct mechanistic stages can be identified. Initiation involves a DNA damaging event leading to mutation(s) in critical target cells (i.e. stem cells). Promotion involves increased proliferation and altered cell behavior, which allows for clonal expansion of initiated cells into clonal outgrowths called papillomas. Finally, tumor progression is characterized by the conversion of papillomas to SCCs.

The first stage in chemically induced skin carcinogenesis is referred to as “initiation” and involves a DNA damaging event to genes in epidermal keratinocytes. The most commonly used initiating agent is the polycyclic aromatic hydrocarbon (PAH), DMBA. DMBA causes a specific A to T (182) transversion mutation in codon 61 of the
Hras1 gene, although Kras mutations have also been documented in lesions initiated with DMBA [reviewed in (54)]. Mice are treated topically with a sub-carcinogenic dose of DMBA that primarily targets keratinocyte stem cells, which are found at the base of the epidermal proliferative units and in the bulge region of the hair follicle [reviewed in (54, 56)].

During the process of tumor promotion, initiated stem cells undergo clonal expansion. The promotion stage is characterized by repeated applications of chemical agents called tumor promoters (e.g. TPA, chrysarobin (CHRY), okadaic acid, and others) or wounding leading to sustained epidermal hyperplasia and epidermal proliferation. Initiated stem cells that harbor Ras mutations are believed to have a selective growth advantage during tumor promoter-induced epidermal proliferation. Classical tumor promoters are not intrinsically mutagenic but alter the expression of genes that are associated with tissue remodeling, hyperplasia, and inflammation (57). Repeated exposure to a given tumor promoter may increase mitogenic factors, increase inflammatory mediators and inhibit anti-apoptotic molecules. The end result of the promotion stage is characterized by the development of pre-malignant outgrowths in the skin called papillomas.

The progression stage of multistage carcinogenesis results in further genetic alterations such as loss of heterozygosity, aneuploidy and trisomy leading to the conversion of pre-malignant papillomas to malignant SCCs (54). A typical initiation-promotion protocol using DMBA and TPA leads to the robust formation of both papillomas and SCCs. The frequency of malignant conversion is dependent on many factors such as genetic background, dose of initiator and type of promoter used (54).
Figure 6. Multistage Carcinogenesis in Mouse Skin. The initiation-protocol protocol involves the application of a subcarcinogenic dose of a carcinogen (DMBA, MCA, MNNG) followed by repeated applications of a tumor promoter (TPA, UV, CHRY, wounding). The model is divided into three distinct mechanistic stages: initiation, promotion and progression. The initiation phase is characterized by an irreversible DNA damaging event in the target cells of the epidermis. The second stage, promotion, involves repeated applications of a tumor promoter, which lead to the clonal expansion of initiated cells. During the promotion stage, benign pre-malignant outgrowths called papillomas develop. In the final stage, cells undergo further genetic alterations leading to increased genomic instability. During this final stage, papillomas convert to invasive squamous cell carcinomas (SCC). Figure adapted from Abel et al. 2009 Nature Protocols.
1.13 Mechanisms of Skin Tumor Promotion

While the initiation stage of mouse multi-stage skin carcinogenesis is relatively well understood in terms of molecular mechanism, the mechanisms underlying the promotion stage are less well defined. Tumor promotion is characterized by the development of a dramatic increase in epidermal cell proliferation and by significant dermal changes characterized by inflammation (58-62). Processes altered during tumor promotion include increased DNA synthesis, increased ODC activity, increased growth factor production, altered redox status and increased prostaglandin synthesis [reviewed in (63)]. The effects observed during tumor promotion are largely due to promoter-induced alterations in gene expression and signaling molecules (58-60, 64, 65). In general, many genes that encode growth regulatory molecules are up-regulated (mRNA and protein) or enzymatic activities are stimulated in response to exposure of mouse skin to tumor promoting stimuli (63, 66). A number of changes in growth regulatory proteins and molecules occur during tumor promotion in the mouse skin model that are thought to stimulate a cascade of cell signaling events that alter cell proliferation and/or differentiation. Some of these proteins/molecules include: protein kinase C (PKC), EGFR, transforming growth factor alpha (TGF\(\alpha\)), transforming growth factor beta 1 (TGF\(\beta\)); and prostaglandins. Downstream mediators of these regulatory molecules include but are not limited to: protein kinase B (AKT), c-myc proto-oncogene (MYC), FBJ osteosarcoma oncogene (c-FOS), E2F transcription factor 1 (E2F-1), transformation related protein 63 (p63), mitogen activated protein kinase (MAPK),
phosphatidylinositol 3-kinase (PI3K), cyclin D1 (CCND1), and STAT3.

For the phorbol ester type tumor promoters (includes TPA, teleocidins and alysiatoxins) the cellular receptor that initiates their actions is PKC (67). For compounds such as OA and calyculin A, the cellular receptors are protein phosphatases 1 and 2A (68). For many other skin tumor promoters, the existence of cellular receptors is not known and for compounds that break down to form reactive oxygen species and other types of radical intermediates such as the anthrones (e.g., anthralin, CHRY) and the organic peroxides [e.g., benzoyl peroxide (BzPo)] it is believed that they work by inducing oxidative stress that can activate multiple signaling pathways associated with skin tumor promotion (69).

1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin or CHRY) is a prototypical anthrone derivative that represents a class of skin tumor promoters that work through a mechanism that is different than the phorbol esters. CHRY does not directly interact with the PKC receptor suggesting that its activity is independent of the PKC signaling pathway (70). CHRY is very similar to another 9-anthrone derivative, anthralin. Anthralin was first shown to be an effective tumor promoter by Bock and Burns in ICR Swiss mice over 40 years ago (Bock and Burns 1963) and subsequently CHRY was shown to be approximately 2-fold more active than anthralin when applied at equimolar doses (71). Anthrones such as CHRY and anthralin undergo auto-oxidation in biological fluids producing reactive oxygen species and anthrone intermediate radicals. Utilizing electron spin resonance,
anthralin has been shown to produce several oxidized products in mouse skin, including singlet oxygen and superoxide anion radicals (72, 73). Structure-activity studies for tumor promoting activity with anthrone derivatives have shown that oxidation at C$_{10}$ of the molecule and subsequent generation of ROS is critical for their tumor promoter activity (74).

Despite the known differences in mechanism between TPA and CHRY, both compounds cause several similar histological and biochemical alterations such as ornithine decarboxylase (ODC) induction and increased polyamine synthesis, increased epidermal DNA synthesis, edema, and hyperplasia following topical treatment. ODC is the rate-limiting step in the synthesis of the polyamines. At optimal promoting doses, induction of ODC and subsequent polyamine synthesis by CHRY exhibited differences in time course and magnitude compared to TPA (75, 76). Epidermal DNA synthesis as well as epidermal hyperplasia of mice skin treated topically with CHRY exhibited a delayed peak compared to TPA (76) (77). The results from these early studies has shown that hyperplasia produced in mice treated with TPA reached a maximum level at 48 hrs compared to mice treated with CHRY, which peaked at 96 hrs following treatment. Collectively, these findings have suggested that although TPA and CHRY ultimately produce similar effects (epidermal hyperproliferation, inflammation, and tumor promotion) their initial biochemical/molecular mechanism is different.

1.14 Role of Inflammation in Cancer and Tumor Promotion
Calor Dolor Rubor and Tumor translates to Heat, Pain, Redness and Swelling which represents the four classical signs of inflammation as was originally described by the First century encyclopedist Celsus AC., (Celsus AC., De medicina. Self published; A.D. 25). The functional relationship between inflammation and cancer is not new. There is overwhelming experimental and epidemiological evidence that illustrates the importance of inflammation during the process of cancer development (78). Prostaglandin endoperoxide H synthases, also known as cyclooxygenase (Cox) is a key enzyme involved in this complex signaling cascade (79).

Cox enzymes catalyze the synthesis of prostaglandins from arachidonic acid (AA). Cox enzymes exist in predominately two major isoforms, Cox-1 and Cox-2. Cox-1 is constitutively expressed in most cells and is responsible for tissue homeostasis, such as maintenance of gut mucosa and renal blood flow [reviewed in (80)]. Cox-2, on the other hand, is an inducible protein that exists at low levels in mammalian cells devoid of stimulation. Cox-2 can be activated by a variety of stimuli including bacterial endotoxins, cytokines, growth factors and hormones. There is overwhelming evidence linking Cox-2 expression to the development of cancer. Cox-2 has been shown to be overexpressed in various pre-malignant and malignant tissues, such as actinic keratosis/SCCs, adenoma/adenocarcinoma and bile duct hyperplasia/cholangiocarcinoma, [reviewed in (78)].
There are also several lines of experimental data that support the role of Cox-2 in the process of tumorigenesis. Female transgenic mice that overexpress Cox-2 in mammary tissue developed focal mammary gland hyperplasia, dysplasia and metastatic tumors (81). Lastly, in addition to data from epidemiological and genetic studies, there are also numerous pharmacological studies that indicate that Cox-2 is a suitable therapeutic target. Treatment with a selective Cox-2 inhibitor reduces the formation of tumors in inflammation-associated malignancies such as intestinal (82), colon (83), bladder (84), and skin tumors (85) in animals.

Growth factors, cytokines and tumor promoters stimulate Cox-2 expression by multiple signaling pathways including PKC and Ras-mediated signaling [reviewed in (78)]. Tumor promoters such as UV and TPA are known to induce Cox-2 expression in epidermis following exposure (86, 87). It has been shown that both Cox isoforms have a role in keratinocyte differentiation. Utilizing the two-stage skin model, it was reported that tumor development in Cox-1 and Cox-2 null mice were reduced compared to wild-type mice and was associated with premature keratinocyte differentiation (88). Further studies have revealed that the impact of Cox-2 on epithelial tumorigenesis is context dependent. Transgenic mice that overexpress Cox-2 under the control of the keratin 14 promoter (K14), referred to as K14.Cox-2 mice, were shown to be resistant to skin tumor formation using the two-stage model when TPA was used as the promoting agent. The decrease in tumor development was attributed to reduced
ODC induction, reduced interleukin-1α, and reduced TNFα in these K14.Cox-2 mice. In the same study, it was also shown that K14.Cox-2 transgenic mice when promoted with anthralin developed 6 times more tumors than control mice. Additionally, K14.Cox-2 mice treated only with DMBA developed 3.5 times more tumors compared to wild-type control mice (89). Collectively, these results demonstrated the Cox-2 expression was important for epithelial tumor development and its role is context dependent.

The biological effects of Cox-2 induction are largely mediated via the actions of a class of signaling molecules called prostaglandins (PGs). Prostaglandins have several different isoforms that include PGE₂, PGD₂, PGI₂, and PGF₂. PGE₂ represents the most abundant isoform produced in the skin. PGE₂ induces keratinocyte proliferation via multiple pathways including EGFR and PI3K (90). PGs bind to four G-protein linked membrane receptors referred to EP1, EP2, EP3, and EP4 (91). Mouse epidermis treated with TPA increase EP1 and EP2 expression and both are upregulated in DMBA/TPA-induced papillomas and carcinomas (92). Taken together these findings suggest Cox-2 expression and subsequent PGE₂ induction are important signaling events in epithelial tumorigenesis.
Chapter 2: Rationale, Hypothesis and Specific Aims

Rationale

The primary goal of this research project is to understand the role of Stat1 in multistage epithelial carcinogenesis and tumor promotion using the multistage mouse skin model. Aberrant activation of STAT signaling has been associated with various pathological events, including cellular transformation and oncogenesis. Of all the STAT family members Stat1, 3 and 5 are most commonly associated with cell growth, proliferation, and apoptosis in cancer. Previous work performed in our laboratory has established a critical role for Stat3 in epithelial tumorigenesis. However, the role of Stat1 in epithelial tumorigenesis has not been well defined. Traditionally, Stat1 activity has been most commonly associated with anti-tumorigenic properties through modulating key components of immune tumor surveillance, inducing pro-apoptotic regulators such as Fas/FasL, caspases, and regulating cell cycle regulating genes as p21 and p27. Stat1 is also considered an indispensible upstream regulator of interferon signaling, as Stat1 deficient cells are unresponsive to both Type I and Type II interferons. The overall contribution of the IFN\(\gamma\)/Stat1 axis to the tumorigenic process is poorly understood and recent evidence suggests a pro-tumorigenic role for this pathway in several cancers.

Recently, we have found that Stat1 is absolutely required for skin tumor promotion by the non-phorbol ester tumor promoter, CHRY, but not for tumor promotion by the classical phorbol ester tumor promoter, TPA. This requirement
for Stat1 in tumor promotion by CHRY involves IFN\(_\gamma\) signaling and the induction of IRF-1 and unphosphorylated Stat1 (uStat1) in the epidermis.

**Hypothesis**

The hypothesis to be tested is that Stat1 plays a critical role in skin tumor promotion by CHRY but not TPA. An additional hypothesis to be tested is that activation of Stat1 by CHRY occurs via IFN\(_\gamma\) signaling.

**Specific Aims**

**Specific Aim 1:** To determine the effect of treatment with mechanistically distinct tumor promoters on Stat1 activation in mouse epidermis. In this aim, we evaluated STAT1 activation following treatment with diverse tumor promoting agents in dorsal mouse skin. Utilizing Western blot analysis, we evaluated the activation status of Stat1 proteins following treatment with single and multiple applications of mechanistically distinct tumor promoters.

**Specific Aim 2:** To determine the effect of Stat1 deficiency on epithelial tumorigenesis and investigate the underlying mechanism of CHRY-mediated tumor promotion. Utilizing the two-stage skin carcinogenesis model, we evaluated the impact of Stat1 deficiency on epithelial tumorigenesis using both TPA and CHRY. In addition, we evaluated the impact of Stat1 deletion on short-term markers of tumorigenesis such as the proliferative response, apoptosis and differentiation.

**Specific Aim 3:** To determine the role of IFN\(_\gamma\) signaling in activation and up-regulation of Stat1. In this aim, we investigated the IFN\(_\gamma\)/Stat1/IRF-1 signaling
axis following multiple treatments with either TPA or CHRY. In addition, utilizing loss of function mouse models we also investigated the impact of Stat1 and IFNγR loss on interferon-mediated signaling following treatment with both CHRY and TPA.

Specific Aim 4: To determine the role of IFNγ/pStat1/IRF-1 signaling on inflammatory signaling induced by CHRY. In this aim, we examined the impact of Stat1 deficiency on both TPA- and CHRY-mediated Cox-2 expression. The status of several other signaling pathways following topical application of either TPA or CHRY in both wild-type and Stat1 deficient (Stat1−/−) mice were also investigated. Utilizing Western blot and qPCR analysis, we evaluated the inflammatory response following treatment with both TPA and CHRY.
Chapter 3: Materials and Methods

Animals, Antibodies, Chemicals and Reagents

Animals

Stat1\(^{-/-}\) mice were a generous gift from Dr. David Levy (Kaplan Cancer Center, New York School of Medicine, New York, NY). A functionally null Stat1 allele was generated by deleting a portion of the protein-coding region. Using gene-targeting techniques, 5.7 kb of genomic DNA was deleted. The DNA that was excised contained three complete exons and a portion of a fourth that encode amino acids 221-365. The deletion results in the loss of a portion of the DNA-binding Domain (DBD), which produces a functionally inert protein (93). Stat1\(^{-/-}\) knockout progeny were born at the expected frequencies from heterozygous mating, and exhibited no gross developmental abnormalities described by Durbin et al (93). Even though Stat1 mice developed normally they were extremely sensitive to microbial challenge, (93, 94). Stat1\(^{-/-}\) mice were originally generated on a mixed C57Bl/6 genetic background. These mice were backcrossed for 5 generations onto the FVB/N background. Heterozygous (Stat\(^{+/+}\)) mice were then mated to generate Stat1\(^{-/-}\) and Stat1\(^{+/+}\) littermate control mice. IFN\(\gamma\)R1 deficient (IFN\(\gamma\)R1\(^{/-}\)) mice were purchased from The Jackson Laboratory (Bay Harbor, MA).
Antibodies and Reagents

Polyclonal rabbit anti-Cox-2 was purchased from Cayman Chemical Co. (Ann Arbor, MI); Rabbit anti-IRF-1 from Santa Cruz Biotechnology (Santa Cruz, CA) and Antibodies against phospho-Stat1 Y701, phospho-Stat1 S727 and total Stat1 were purchased Cell Signaling Technologies (Danvers, MA).

Chemiluminescence detection kits were purchased from Pierce (Rockford, IL). 7, 12 dimethylbenz(a)anthracene (DMBA), 5-bromo 2-deoxyuridine (Brdu), protease inhibitor cocktails, phosphatase inhibitor cocktails, mouse and rabbit secondary antibodies, and beta-actin were purchased from Sigma-Aldrich (St. Louis, MO). 12-O-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Alexis Biochemicals (Plymouth Meeting, PA). Chrysarobin was synthesized in house from chrysaphanic acid as previously described (95). Mouse recombinant interferon gamma rIFNγ was purchased from BD Bioscience and used at 250 ng/ml in primary cultures as previously described(96).

Short-term promotion experiments

For experiments were only Stat1 wild-type mice were used, female FVB/N mice (7-9 weeks of age) were purchased from the National Cancer Institute (Fredrick, MD) and group housed for the duration of the study in all experiments. In all other instances, Stat1+/− and Stat1+/+ mice were obtained from in house breeders as described above. The dorsal skin was shaved 48 hrs prior to treatment with the indicated tumor promoter. Mice were treated with either
acetone vehicle (0.2 ml), TPA (3.4, 6.8, 13.6 nmol) twice weekly for two weeks or
with chrysarobin (100, 220, 440 nmol) once weekly for four weeks. Mice were
sacrificed at the indicated time point(s) following the last treatment.

For the analysis of epidermal proliferation and thickness, mice were
injected intraperitoneal (i.p.) with 5-bromo-2'-deoxyuridine (BrdU) in PBS (100
µg/g body weight) 30 min. prior to sacrifice. Excised dorsal skin sections were
fixed in 10% neutral buffered formalin for 24-48 hrs and embedded in paraffin.
Sections were either stained with, S100A8, K1, K5, K10, loricrin, K6, Hemotoxylin
and eosin (H&E), anti-BrdU antibody or anti-caspase-3 by the histopathology
core at UT MD Anderson Cancer Center-Smithville Science Park Division.
Epidermal index was calculated as the percentage of BrdU positive cells; 600
basal cells were counted from 3-4 skin sections for each mouse. For epidermal
thickness, 20 measurements were taken for each of 3-4 skin sections per mouse
and the average thickness was calculated (97). Apoptotic keratinocytes were
counted microscopically and the index was calculated from the total number of
caspase-3 positive basal cells in the entire skin sections from 3-4 individual mice
from each group.

**Two-Stage Skin Carcinogenesis model**

Groups of 15-29 Stat1+/+ and Stat1−/− age matched females were used per
group. Forty-eight hours prior to initiation the dorsal skin of was shaved. Mice
were initiated with 25nmol DMBA dissolved in 0.2ml acetone. Two-weeks after
initiation mice received topical application of specified tumor promoter. Due to mechanistic differences we used two different treatment regimens for promotion with TPA and CHRY. Two weeks after initiation, mice were treated topically with either 0.2ml acetone, 6.8nmol TPA, or 220nmol CHRY in 0.2 ml acetone. Mice were treated twice weekly with TPA or treated once weekly with CHRY. Mice received tumor promoter treatments until tumor multiplicity plateaued. Tumor incidence (percentage of mice with papilloma) and tumor multiplicity (average number papilloma per mouse) was recorded weekly for the duration of the studies.

**Epidermal Lysate preparation**

After euthanization, the depilatory agent Nair was applied to the dorsal skin of mice for 30-45 sec and removed under running water. The skin was then excised and the epidermal layer was removed by scraping with a razor blade into chilled lysis buffer (1M Tris HCL pH 7.4, 3M NaCl, 0.5M EDTA pH 8.0, 10% Triton X-100, protease inhibitor, phosphatase inhibitor cocktail 1 and 2) and then homogenized using an 18-gauge needle. Epidermal lysates were centrifuged at 14,000 RPM for 15min, and supernatant was collected. Protein concentration was determined and supernatant was snap frozen using liquid nitrogen and stored at -80°C until further analysis.
**Adult Primary Keratinocyte Culture**

Primary mouse keratinocytes were isolated from 6-8 week old Stat1 wild-type and Stat1\(^{-/-}\) mice as previously described (98). Briefly, mice were shaved, treated with a hair depilatory agent, after which dorsal skin was excised and floated on 0.25% trypsin for 4 hrs. The epidermal cell layer was separated from dermis, minced and stirred in growth media supplemented with 10% fetal bovine serum for 30 min. The cell suspension was filtered through a 70 \(\mu\)m cell strainer. Cells were counted using a hemocytometer and plated on collagen coated plates and maintained in complete keratinocytes growth medium made up of Eagle’s minimal essential base medium without Ca\(^{2+}\) supplemented as previously described (98). Primary keratinocytes were serum starved for 24 hrs prior to treatment.

**Western Blot Analysis**

Protein concentration was determined using Lowry protein assay according to manufacturer instructions. Fifty micrograms of protein lysate per lane was electrophoresed in 4-15% SDS-Page gradient gels and then transferred onto 0.45 um nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked for 1 hour in 5% bovine serum albumin (BSA) in TBS with 0.1% tween (TBST) and incubated overnight at 4\(^\circ\)C with specified primary antibody. The membranes were washed 3 times for 15 min each in TBST. Membranes were then incubated for 45 min in corresponding horseradish peroxidase-
conjugated secondary antibodies in 3% non-fat dry milk (NFDM). Membranes were then washed 3 times for 15 min. Protein bands were visualized using a chemiluminescence detection kit (Pierce ECL Western Blotting Substrate, Rockford, IL). Quantitation of total protein, and relative phosphorylation levels were calculated by densitometric analysis. Actin was used to normalize protein loading and relative phosphorylation is calculated as a percentage of total protein.

**mRNA analysis by Real-Time PCR.**

Epidermal scrapings from the dorsal skin of sacrificed mice was submerge into RNA Later and placed at 4C for a minimal of 48 hrs. Total RNA was isolated by using the QIAGEN RNeasy RNA Isolation kits (74104) according to manufactures instructions. First strand cDNA synthesis kit using random hexamer primers (Invitrogen) was used for cDNA preparation. SYBR Green was used for quantitative real-time PCR, which was performed on the Applied Biosystems RT-PCR system (Applied Biosystems Viia 7). Relative gene induction was calculated using Viia 7 software using the comparative Ct method ($\Delta\Delta$CT).

**Quantitation of Cytokines and Chemokines**

RT2 Profiler PCR array mouse inflammatory cytokines and receptors (PAMN-011Z) ($n = 4$) were used to analyze the expression of a focused panel of genes. Data analysis was performed using the $\Delta\Delta$CT method according to the manufacturer’s protocol (SABiosciences).
**Prostaglandin E\(_2\) Assay**

Dorsal epidermis was chipped from frozen skin into ice-cold Eicosanoid Affinity Column Buffer (0.5 M phosphate solution, pH 7.0, containing 2.5 M sodium chloride and 0.25% sodium azide) containing 10 \(\mu\)mol indomethacin. After homogenization sample was centrifuged at 8,000X g for 10 min. Supernatant was collected. Samples of mouse origin may contain antibodies, which interfere with the assay by binding to the goat anti-mouse plate. Samples were purified prior to assay. PGE\(_2\) was purified by passing clear supernatant through prostaglandin E\(_2\) affinity columns (Caymen chemical), the eluate was dried under a steady stream of nitrogen. For assay, PGE\(_2\) was reconstituted in enzyme immunoassay (EIA) buffer (Cayman Chemical Company, Ann Arbor, MI) and assayed according to the manufacturer’s instruction.

**Statistical Analyses.**

To compare epidermal thickness (\(\mu\)m) and labeling index (LI) (% BrdU positive cells) data were presented as the mean ± standard error of the mean (SEM). For comparisons of epidermal thickness and LI, the Mann-Whitney \(U\)-test was used (significance set at \(p\leq0.05\)). For comparison of tumor incidence the Chi-square (\(\chi^2\)) test was used (significance set at \(p\leq0.05\)). For comparison of tumor multiplicity data, the Mann-Whitney \(U\)-test was used (significance set at \(p\leq0.05\)). Statistical analysis was conducted using GraphPad Prism 4 software (San Diego, CA).
## Antibody List

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Chapter 4: Effect of treatment with mechanistically distinct tumor promoters on Stat1 activation in mouse epidermis

Rationale

As stated in the introduction, STAT activation proceeds by a process of phosphorylation on conserved tyrosine and serine residues. Phosphorylation of these conserved residues is critical for STAT transcriptional activity, but accumulating data has established a mechanism whereby uSTATs may also affect gene expression and contribute to the overall effects associated with this transcription factor. In previous studies, it was shown that a variety of tumor promoters including the commonly used phorbol ester TPA, the phosphatase inhibitor okadaic acid, and the anthrone derivative CHRY activated epidermal STATs, following topical treatment. All these tumor promoters were found to activate Stat3 in epidermis and in addition TPA was found to activate Stat1 and Stat5 (99). The aim of this project was to further expand our knowledge of Stat1 activity/phosphorylation following treatment with various tumor promoters. By utilizing mechanistically distinct tumor promoters the goal was to determine whether activation of Stat1 was a common mechanism in tumor promotion.

4.1 Effect of a single topical application of mechanistically distinct tumor promoters on Stat1 activation in epidermis

For these experiments, groups of 3-4 female FVB mice (6-8 weeks of age) received a single topical application with either acetone vehicle or 6.8 nmol TPA, 7.5
nmol okadaic acid or 220 nmol CHRY. Mice were sacrificed at the indicated time points and epidermal lysates were prepared as previously described. As shown in Figure 7, topical application of TPA caused an increase in Stat1 phosphorylation. In this regard, following TPA treatment there was a significant increase in serine 727 (S727) phosphorylation as early as 3 hrs, and phosphorylation remained elevated for at least 18 hrs following treatment. Tyrosine phosphorylation of Stat1 was observed to be slightly elevated at 6 hrs following treatment. TPA treatment also resulted in an approximate 1.5 fold increase of total Stat1 protein 18 hours following treatment. Topical treatment with CHRY also led to an increase in phosphorylation of Stat1 on both tyrosine and serine residues compared to the acetone control group. In addition, CHRY treatment also led to an approximate 2-fold increase in total Stat1 as early as 18 hrs following treatment. At the time points evaluated, there were no significant changes in Stat1 phosphorylation compared to the acetone controls following okadaic acid treatment. These data suggested that activation/phosphorylation represents an early signaling event following topical treatment with both TPA and CHRY. The increased tyrosine phosphorylation of Stat1 following both TPA and CHRY appeared to be due primarily to increases in Stat1 protein. The increased phosphorylation of Stat1 on S727 by CHRY also appeared to be due to increased Stat1 protein whereas TPA treatment led to a significant increased phosphorylation at S727 (see quantitation in Figure 7). Because the process of tumor promotion involves repeated treatments with tumor promoters, the next set of experiments involved using a multiple treatment regimen using both TPA and CHRY.
Figure 7. Effect of a single topical application of TPA, CHRY, and okadaic acid on epidermal Stat1. FVB mice (3-4/group) received a single topical application of either 6.8 nmol TPA, 7.5 nmol okadaic acid (OA), or 220 nmol CHRY in 0.2 ml acetone (Ace) and were sacrificed at the indicated timepoints. A) Mice were sacrificed at various time points and epidermal protein lysates were prepared for Western blot analysis using antibodies specific for the indicated proteins. B) Quantitation of total protein, normalized phospho-Stat1(S727) and normalized phospho-Stat1(Y701) were determined by densitometric analysis. Actin was used to normalize protein loading and normalized phosphorylation is calculated as a percentage of total protein. Values represent the average of three experiments ± SEM.
4.2 Effect of Multiple treatments with TPA and CHRY on Epidermal Stat1

To better understand Stat1 signaling following tumor promoter treatment, we used a multiple treatment regimen for both TPA and CHRY and established a time course for Stat1 activation/phosphorylation. Stat1−/− mice and wild-type controls were treated topically using a multiple treatment regimen involving 4 treatments with either acetone, 6.8nmol TPA (2x/week for two weeks) or 220nmol CHRY (1x/week for four weeks). Mice were sacrificed at the specified time points and epidermal lysates were prepared for protein analysis by Western blot.

As shown in Figure 8, topical application with both TPA and CHRY induced rapid phosphorylation of epidermal Stat1 on both tyrosine (Y701) and serine (S727). In this regard, phosphorylation at both Y701 and S727 occurred as early as 6 hrs following the last treatment with either promoter. Phosphorylation at S727 persisted for up to 24-48 hrs after treatment whereas phosphorylation at Y701 was more transient. During the course of these experiments, we observed that Stat1 protein levels were consistently increased at later time points following treatment with CHRY but not TPA (see again Figure 8) with a peak occurring approximately 48 hrs after treatment. This increase in Stat1 protein level occurred at a time when little or no phosphorylation could be detected. Thus, CHRY treatment led to the induction of uStat1. To determine the mechanism for increased uStat1, we measured epidermal Stat1 mRNA levels following a single application of CHRY. As shown in Figure 9, CHRY treatment led to a significant increase in Stat1 mRNA. Thus, the increase in uStat1 protein was due to an
increase in Stat1 mRNA. As shown if Figure 9, Stat1 mRNA levels increased as early as 6 hrs (the earliest time point examined) and reached peak levels at 12 hrs before returning to basal levels 24 hrs after treatment. Collectively, the data in Figures 8 and 9, demonstrate that treatment with both tumor promoters led to rapid activation of Stat1 (i.e. phosphorylation on both tyrosine and serine residues). However, only CHRY led to induction of uStat1.
Figure 8. Effect of multiple topical applications with TPA and CHRY Stat1 on phosphorylation/activation. Stat1 wild-type (Stat1+/+) or Stat1 Knock-out (Stat1−/−) mice received four topical applications of either 6.8 nmol TPA, or 220nmol CHRY in 0.2 ml acetone (Ace). Epidermal lysates were collected for protein analysis by Western blot. Mice were sacrificed at the indicated timepoints following last treatment and probed using antibodies specific for indicated protein. Quantitation of total protein levels was assessed by densitometric analysis. Actin was used to normalize protein loading. A) Western blot analysis of Stat1 following topical application of TPA; B) Western blot analysis of Stat1 following topical application of CHRY.
Figure 9. A single application of CHRY led to de novo synthesis of Stat1 mRNA. Wild-type (FVB) mice 6-8 weeks of age, received a single topical application of 220 nmol CHRY. Mice were sacrificed at the indicated time points for analysis of mRNA expression of Stat1 by RT-PCR. * Represents significant differences from acetone control (P-value ≤ 0.05)
4.3 uStat1 is elevated and detected in nuclei of epidermal keratinocytes

As previously stated in the Introduction, STATs undergo cycles of cytoplasmic-nuclear shuttling. Although uSTATs have been reported to shuttle between the nucleus and cytoplasm in un-stimulated cells the general consensus remains that STAT molecules require tyrosine phosphorylation for nuclear localization to induce subsequent gene expression (100). Accumulating data has established a role for unphosphorylated STATs at the transcriptional level (39, 40, 43). To investigate whether uStat1 could affect transcription, we evaluated uStat1 nuclear localization. As shown in Figure 10, uStat1 was elevated in epidermis as assessed by immunostaining at 48 hrs following treatment with CHRY. Some nuclei appeared to be stained for uStat1. Additional confocal microscopic analysis (Figure 11) revealed that following multiple applications of CHRY, uStat1 could be detected in the nuclei of keratinocytes 48 hrs after the last treatment. It should be noted that there was no detectable phosphorylation of Stat1 at this time point (see again Figure 8). These data confirm that CHRY treatment leads to upregulation of uStat1 and that uStat1 may have a function in regulating gene expression as has been recently proposed (39, 40, 42).
Figure 10. IHC analysis of Stat1 in mouse skin of Stat1 wild-type and Stat1 deficient mice following multiple treatments with CHRY. Female FVB (3-4 mice/group) mice were treated with either acetone or 440 nmol CHRY once weekly for four weeks and sacrificed 48 hrs following final treatment. Skin sections were fixed in formalin, paraffin embedded, and immunostained for Stat1 alpha.
Figure 11. Multiple applications of CHRY led to an increase in uStat1 nuclear localization 48hrs after treatment.  Wild-type FVB mice received either acetone vehicle or 220nmol CHRY in 0.2 ml acetone (once weekly for four weeks). Whole skin sections were excised, fixed in formalin and paraffin embedded. Skin sections were immunostained with Stat1 and observed using confocal microscopy.
4.4 Topical application of $\alpha$-Tocopherol acetate ($\alpha$TA), inhibits epidermal proliferation and Stat1 Y701 phosphorylation by CHRY

As noted in the Introduction, anthrones such as CHRY undergo auto-oxidation to generate free radicals that are involved in their tumor promoting action. Early studies from Fushs et al reported that treatment with the antioxidant $\alpha$-tocopherol acetate ($\alpha$-TA) inhibited ODC induction by anthralin, (72). In early studies performed in our laboratory, it was shown that $\alpha$-TA could partially block the effect of CHRY on EGF binding in vitro (101). In addition, early studies by Battalora and DiGiovanni, (102) also showed that topical application of $\alpha$-TA effectively inhibited CHRY mediated ODC induction, hyperplasia and tumor promotion. The aim of this study was to evaluate the impact of $\alpha$-TA on the epidermal proliferative response following treatment with CHRY and to evaluate its effect on CHRY-induced Stat1 Y701 phosphorylation. For these experiments, groups of 3-4 female FVB mice received topical application of either acetone (vehicle), $\alpha$-TA only, CHRY only or $\alpha$-TA plus CHRY. Antioxidant treatment was administered topically 5 min prior to receiving a topical dose of 220 nmol CHRY.

$\alpha$-TA treatment resulted in a decrease in the proliferative response as measured by both BrdU incorporation and epidermal thickness (Figure 12). Mouse skin pre-treated with 10 and 40 $\mu$mol of $\alpha$-TA exhibited an approximate 22% reduction in epidermal thickness compared to the CHRY treated group (46.4 microns CHRY treated group versus 35.75 microns for both does of $\alpha$-TA) (Figure 12). $\alpha$-TA treatment also led to a statistically significant reduction in
epidermal labeling index (LI) induced by CHRY (Figure 13). Compared to the CHRY treated group, $\alpha$-TA pretreatment resulted in an approximately 29% and 23% decrease for 10 and 40 $\mu$mol, respectively (20.77% labeled in the CHRY treated group versus 14.74% and 15.94, respectively)(Figure 13).

As shown in Figure 14, $\alpha$-TA pretreatment reduced total Stat1 protein levels at 18 hrs after treatment with CHRY. In this regard, $\alpha$-TA pretreatment resulted in an approximate 1.6 and 1.3-fold (10 and 40 $\mu$mol) decrease in total Stat1 induction compared to the CHRY only treated group. $\alpha$-TA also caused an approximate 1.8 and 4.2-fold decrease in Y701 phosphorylation compared to the CHRY only treated group, at the 10 $\mu$mol and 40 $\mu$mol doses, respectively. After normalization, $\alpha$-TA effectively inhibited CHRY-mediated Stat1 Y701 phosphorylation by decreasing Stat1 activation 3.0 and 8.3-fold (10 $\mu$mol and 40 $\mu$mol doses, respectively) compared to the CHRY only treated group (see again Figure 14). Collectively, these data indicate that the activation of Stat1 Y701 phosphorylation requires the generation of free radicals from CHRY.
Figure 12. Topical application of α-tocopherol caused a significant reduction in epidermal thickness. FVB mice (3-4/group) received a topical application of either acetone, α-TA only, or α-TA 5 min prior to receiving an application of 220 nmol CHRY in 0.2 ml acetone. Mice were treated once weekly for four weeks and sacrificed 18 hrs following final treatment. Representative sections of whole skin were stained with hematoxylin are shown.
Figure 13. Topical application of α-TA caused a significant reduction in BrdU incorporation. FVB mice (3-4/group) received a topical application of either acetone, α-TA only, or α-TA 5 min prior to receiving an application of 220 nmol CHRY in 0.2 ml acetone. Mice were treated once weekly for four weeks and sacrificed 18 hrs following final treatment. Representative sections of whole skin were stained with 5-bromo-2-deoxyuridine (BrdU) are shown.
Figure 14. Topical application of α-tocopherol caused a significant reduction in Stat1 Y701 phosphorylation. FVB mice (3-4/group) received a topical application of either acetone, α-TA only, or α-TA 5 min. prior to receiving an application of 220 nmol CHRY in 0.2 ml acetone. Mice were sacrificed at 18 hrs after the last treatment with CHRY and epidermal protein lysates were prepared for Western blot analysis using antibodies specific for the indicated proteins Normalization performed by densitometric analysis. Actin was used to normalize protein loading and relative phosphorylation is calculated as a percentage of total protein.
Chapter 5: Stat1 is critical for CHRY Skin Tumor Promotion

Rationale

STATs are involved in many normal physiological processes but their aberrant activation has been associated with many pathological conditions including oncogenesis. Previous work performed in our lab, demonstrated that Stat1 phosphorylation was induced by TPA and was elevated in DMBA/TPA-induced papillomas compared to control skin, (99). Furthermore, the data in Figures 8-11 suggested the possibility that activated Stat1, including uStat1 may play an important role in skin tumor promotion by CHRY and that activated Stat1 may play an important role in skin tumor promotion by TPA. To further explore this possibility, Stat1^{+/−} mice (93) were used to evaluate their susceptibility to skin tumor promotion by both TPA and CHRY.

5.1 Impact of Stat1 deletion on susceptibility to skin tumor promotion by TPA and CHRY

To evaluate the impact of Stat1 deletion on skin tumor promotion by TPA and CHRY, groups of 19-29 mice for each genotype were used for two-stage skin carcinogenesis studies. The dorsal skin of mice was shaved 48 hours prior to initiation with DMBA. All mice received topical application of 25 nmol of DMBA in 0.2 ml acetone to the shaved dorsal skin. Two weeks after initiation, mice were treated topically with either 6.8 nmol TPA twice/weekly or 220 nmol CHRY once/weekly in 0.2 ml acetone. Mice were treated topically until tumor multiplicity reached a plateau. The incidence of papillomas (percentage of mice with papillomas) and papilloma multiplicity (average number of papillomas per mouse) were tabulated and recorded weekly until the
papilloma response reached a plateau. SCCs (both incidence and number) were recorded beginning at the time the first SCC appeared. As shown in Figure 15, Stat1 deficiency had no significant effect on skin tumor development when TPA was used as the promoting agent. In this regard, there was no significant difference in formation of papillomas or SCCs in Stat1 deficient mice compared to wild-type controls. By week 23, Stat1 deficient mice had developed an average of 13.14 papillomas/mouse compared to 15.25 papillomas/mouse in the wild-type control group. In addition, there were no observed differences in tumor latency between the two genotypes. Both Stat1 deficient mice and wild-type control mice reached 100% tumor incidence by week 10. Furthermore, there were no statistically significant differences observed in the malignant conversion between the two genotypes (Figure 15).

In contrast to the results with TPA, mice deficient in Stat1, where highly resistant to papilloma development when CHRY was used as the promoting agent. Stat1 deficient mice developed far fewer papillomas compared to wild-type controls (Figure 16). By week 31, Stat1 deficient mice had only developed an average of only 0.16 papillomas/mouse compared to 4.52 papillomas/mouse in the wild-type control group. Additionally, Stat1 deficient mice reached approximately 17% tumor incidence compared to 89% incidence in the wild-type control group. At the time of study termination, the papillomas that developed in Stat1 deficient mice had not converted to squamous cell carcinomas, while Stat1 wild-type mice had 0.7 SCCs per mouse and an SCC incidence of 79%. All SCCs were confirmed histologically in mice treated with both TPA and CHRY. Due to the unusual findings with CHRY, a repeat two-stage skin carcinogenesis experiment was performed using groups of Stat1\(^{-/-}\) (n=15) and wild-type
mice (n=19). In this experiment, Stat1\textsuperscript{−/−} mice again were highly resistant to skin tumor development with CHRY. In this second experiment, wild-type mice had developed an average of 2.5 papillomas/mouse compared to 0.14 papillomas/mouse in the Stat1\textsuperscript{−/−} group by week 29. In addition, Stat1 wild-type mice had reached approximately 83% tumor incidence compared to 7.14% in the Stat1\textsuperscript{−/−} group).
Figure 15. Stat1 deficiency had no significant effect on tumor incidence and tumor multiplicity in the two-stage skin carcinogenesis model when TPA was used as the promoting agent. Groups of mice of each genotype, (Stat1^{+/+} or Stat1^{-/-}) were initiated with 25 nmol DMBA; two weeks after initiation mice were treated topically with 6.8nmol TPA twice per week until the tumor response reached a plateau; wild-type mice (n=29): Stat1^{-/-} mice (n=24). A) Papilloma multiplicity and incidence B) Carcinoma multiplicity and incidence. Significant differences in tumor incidence between groups were analyzed by Chi-square (χ²) and tumor multiplicity was analyzed by Mann-Whitney U. *(p < 0.05, Mann Whitney U).
Figure 16. Stat1 deficiency resulted in a significant reduction in tumor incidence and tumor multiplicity in the two-stage skin carcinogenesis model when CHRY was used as the promoting agent. Groups of mice of each genotype, (Stat1\textsuperscript{+/+} or Stat1\textsuperscript{−/−}) were initiated with 25 nmol DMBA; two weeks after initiation mice were treated topically with 6.8 with 220 nmol CHRY once per week until the tumor response reached a plateau. ; wild-type mice (n=24); Stat1\textsuperscript{−/−} mice (n=19). A) Papilloma multiplicity and incidence B) Carcinoma multiplicity and incidence. Significant differences in tumor incidence between groups were analyzed by Chi-square (χ\textsuperscript{2}) and tumor multiplicity was analyzed by Mann-Whitney U. *(p < 0.05, Mann Whitney U).
5.2 Impact of Stat1 deletion on DMBA-induced epidermal apoptosis

To determine the impact of Stat1 deletion on epidermal apoptosis following treatment with DMBA, Stat1<sup>-/-</sup> mice and wild-type controls were treated with DMBA and the apoptotic response was evaluated by examining epidermal caspase-3 staining. Groups of 3-4 wild-type control and Stat1<sup>-/-</sup> mice were treated once topically with acetone vehicle or DMBA (200 nmol and 1000 nmol). The apoptotic response was assessed by caspase-3 activation as described in the “Material and Methods” section. Topical application of DMBA resulted in an increase in caspase-3 positive cells in the epidermis compared to the acetone control, but there were no observable differences between the response in Stat1<sup>-/-</sup> mice and the wild-type control mice at either dose of DMBA (Figure 17). These results confirmed that Stat1 does not play a significant role in survival of DNA damaged cells or apoptosis during initiation with DMBA. These data also confirm that Stat1 plays a major role in tumor promotion by CHRY as indicated by the data in Figures 15 and 16.
Figure 17. Stat1 deficiency had no effect on epidermal apoptosis induced by DMBA. FVB mice (3-4 mice/group) 6-9 weeks of age received a single application of acetone, 200 nmol, or 1000 nmol of DMBA. Mice were sacrificed 48hrs following treatment. Skin sections were collected and fixed in formalin and embedded in paraffin. Whole skin sections were stained with anti-caspase-3 and caspase-3 positive cells were accessed.
5.3 Impact of Stat1 deletion on epidermal hyperproliferation induced by TPA and CHRY

Earlier studies have established a good correlation between the tumor promoting ability of a compound with the induction of short term biomarkers such as induction of ODC, increase polyamine synthesis, inflammation and the ability to induce sustained epidermal hyperplasia (103). To further explore the mechanism for the dramatic differences in response of Stat1−/− mice to TPA vs CHRY, we examined the impact of Stat1 deficiency on epidermal hyperplasia by evaluating the proliferative response as measured by BrdU incorporation and epidermal thickness. The proliferative response was evaluated after treatment with both tumor promoters. Mice were again treated using a multiple treatment regimen and sacrificed 48 hrs following the last treatment. Epidermal thickness and LI were determined as previously described in the “Materials and Methods” section. Stat1−/− and wild-type control mice were treated with either acetone vehicle or 3.4, 6.8, and 13.6 nmol TPA. Following TPA treatment, there were no significant differences in BrdU incorporation or epidermal thickness at all doses tested between Stat1−/− and wild-type control mice. Figure 18 shows representative H&E and BrdU stained skin sections from TPA treated mice as well as quantitation of epidermal thickness and LI at all TPA doses tested.

A parallel study was conducted to evaluate the proliferative response following CHRY treatment. Stat1−/− and wild-type control mice were treated with either acetone vehicle or 110, 220, or 440 nmol CHRY using a multiple treatment regimen. Histological evaluation showed that Stat1−/− mice treated with CHRY displayed a reduced
proliferative response. In this regard, Stat1<sup>−/−</sup> mice exhibited a reduced LI at all three doses of CHRY tested and reduced epidermal thickness at the highest dose tested (440 nmol). Figure 19 displays representative H&E and BrdU stained skin sections from CHRY mice as well as quantitative analyses of both epidermal thickness and LI following treatment with CHRY.
Figure 18. Stat1 deficiency had no significant effect on the epidermal proliferative response following topical application of TPA. Wild-type (Stat1^{+/+}) or knockout (Stat1^{-/-}) mice received either acetone (vehicle) or TPA (3.4, 6.8, 13.6nmol) twice weekly for two weeks. Forty-eight hours following the last treatment the dorsal skin sections were excised and fixed in formalin, embedded in paraffin, and then sectioned. Sections were cut and stained with hematoxylin & eosin or anti-BrdU. Epidermal thickness and labeling index (LI) were determined as described previously.
Figure 19. Stat1 deficiency resulted in a significant reduction in the epidermal proliferative response following topical application of CHRY. Wild-type (Stat1^{+/+}) or knockout (Stat1^{-/-}) mice received either acetone vehicle or CHRY (100, 220, 440nmol) once weekly for four weeks. Forty-eight hours following the last treatment the dorsal skin sections were excised and fixed in formalin, embedded in paraffin, and then sectioned. Sections were cut and stained with hematoxylin & eosin or anti-BrdU. Epidermal thickness and labeling index (LI) were determined as described previously.
5.4 Impact of Stat1 deletion on epidermal differentiation markers following treatment with CHRY.

Epidermal differentiation plays an important role in the process of tumor development. Differentiation is characterized by quiescence at the G1 phase of the cell cycle (104) therefore differentiation represses the cells ability to respond to growth factors (105). Based on the data that Stat1\(^{-/-}\) mice were highly resistant to tumor formation by CHRY and the data in Figure 19 showing reduced proliferation, Stat1\(^{-/-}\) mice were compared with wild-type mice for any changes in differentiation marker expression following treatment with this tumor promoter. To evaluate epidermal differentiation, Stat1 wild-type and Stat1\(^{-/-}\) mice were treated once topically with 220 nmol CHRY and sacrificed 18 hrs following treatment. Whole skin sections were excised then fixed in formalin and paraffin embedded. To assess keratinocyte differentiation we evaluated several common epidermal markers of differentiation including K5 (basal), K1 (suprabasal), K10 (suprabasal), loricrin (late state differentiation) and K6 (proliferation/hair follicle). As shown in Figure 20, the absence of Stat1 did not appear to significantly affect expression of the selected differentiation markers following treatment with CHRY.
Figure 20. Stat1 deficiency had no significant effect on epidermal differentiation following topical application of CHRY compared to controls. Stat1 KO (Stat1−/−) mice and wild-type mice 6-8 weeks of age received either a single topical application of acetone or 220nmol CHRY in 0.2 ml. Mice were sacrificed at various time points and whole skin sections were prepared to assess epidermal differentiation. Representative sections of whole skin sections stained with K5, K1, K10, Loricrin and K6 are shown.
Chapter 6 Role of IFNγ Signaling in Activation and upregulation of Stat1

Rationale

Type I and II IFNs represent a class of pleiotropic cytokines with diverse functions that are context and cell specific. IFNs play a vital role in the innate and humoral immune response to deal with invading pathogens. IFNs are activated by a variety of stimuli and once activated they drive the expression of genes important for clearance of pathogens (e.g. IRF-1, OAS, iNOS). The expression of IFNs in response to tumor promoter treatment is not well established. The aim of this study was to examine IFN signaling following treatment with both TPA and CHRY and determine its role in activation of Stat1 observed following treatment with these promoters.

6.1 Examination of IRF1 expression following treatment with diverse tumor promoters.

In light of the data in Figure 8 showing induction of uStat1 following treatment of mouse epidermis with CHRY, we examined the status of IRF-1. IRF-1 is an IFN-γ/p-Stat1 responsive transcription factor that is known to regulate expression of a variety of genes, including uStat1 (106). Stat1−/− mice and wild-type controls were treated topically using a multiple treatment regimen involving 4 treatments with either acetone, 6.8 nmol TPA (2x/week for two weeks) or 220 nmol CHRY (1x/week for four weeks) as described above. Mice were sacrificed at the specified time points and epidermal lysates were prepared for protein analysis by Western blot or RNA analysis by q-RT-PCR as described in the Materials and Methods section. As shown in Figure 21, IRF-1 protein levels decreased in both wild-type and Stat1−/− epidermal lysates following TPA
treatment. In addition, IRF-1 mRNA levels were not induced in response to TPA in epidermis of either genotype. On the other hand, CHRY treatment led to an increase in IRF-1 expression at both mRNA and protein levels (Figure 21B). Quantitation of IRF-1 protein shows an approximate 3-fold increase of IRF-1 in wild-type controls as early as 6 hours following treatment with CHRY. Consistently, an increase in IRF-1 mRNA was observed by q-RT-PCR, peaking at 6 hrs and tapering off 24 hrs after treatment with CHRY. Interestingly, Stat1^{−/−} mice were highly resistant to IRF-1 induction by CHRY. Examination of epidermal lysates from Stat1^{−/−} mice showed that loss of Stat1 abrogated the induction of IRF-1 mRNA and protein following treatment with CHRY as observed in wild-type controls (panel B).
Figure 21. CHRY treatment led to an increase in IRF-1 expression, whereas TPA decreased IRF-1 levels. Wild-type (Stat1+/+) or knockout (Stat1−/−) mice received either A) TPA (6.8 nmol 2x/week for 2 weeks) or B) 220 nmol CHRY (1x/week for 4 weeks) in 0.2 mL acetone (Ace). Mice were sacrificed at the indicated time points and epidermal lysates prepared for IRF-1 protein (Western blot) and mRNA expression (qPCR) analyses. Total protein levels were quantitated by densitometry. Actin was used to normalize protein loading. Western blot data are from a single experiment (pooled protein samples) that has been repeated with similar results. The mRNA data was obtained from individual mice (n=3-5/group) allowing statistical analysis. * indicates values between Stat1+/+ and Stat1−/− groups were significantly different (Mann Whitney U, p≤0.05).
6.2 Examine the role of IFN signaling on Stat1 activation following treatment with TPA and CHRY.

The data shown in Figure 8 suggested that CHRY treatment influenced Stat1 signaling via a different pathway than TPA, possibly involving IFNγ signaling. This was substantiated by examining IRF-1 status as shown in Figure 21. Collectively these data suggested that CHRY upregulated IFNγ signaling. In light of these findings, we sought to determine how these two mechanistically different tumor promoters influence production of both Type I and II IFNs. Therefore, the expression of IFNα, IFNβ, and IFNγ was examined following treatment with both TPA and CHRY. For these experiments, Stat1−/− and wild-type mice were again treated using a multiple treatment protocol as described above. As shown in Figure 22, TPA treatment did not induce epidermal IFNγ mRNA expression and, in fact, a statistically significant decrease was observed at all time points examined (panel A). In contrast, a significant increase in IFNγ mRNA levels at 3, 6 and 12 hours after treatment with CHRY, with a peak at approximately 3 hrs after the last treatment (~8-fold increase compared to acetone treated control mice) (Figure 22 panel B). Thus, induction of IFNγ was observed by treatment with CHRY but not following treatment with TPA.
Next we wanted to examine the levels of Type I IFNs, to determine if they played a role in tumor promotion following tumor promoter treatment. As shown in Figure 23, there were no significant increases in the Type I (α and β) IFNs following treatment with TPA and again like IFNγ the mRNAs for these two IFNs actually decreased after treatment. Following treatment with CHRY, there was a slight increase in IFNα mRNA at 6 hrs (p<0.05) but not at other time points and there were no increases seen in IFNβ mRNA. Collectively, these data indicate the Stat1 activation and upregulation of IRF-1 in epidermis is associated with significant upregulation of IFNγ mRNA and together these data demonstrate that CHRY activates IFNγ signaling whereas TPA does not.

Based on the data obtained in Figures 22 and 23, we hypothesized that CHRY-mediated tumor promotion was dependent on a IFNγ/p-Stat1/IRF-1 signaling pathway, via induction of IFNγ ligand and activation of IFNγR1, whereas this pathway is not involved in skin tumor promotion by TPA. To further test this hypothesis we employed the use of IFNγR1 knockout mice (IFNγR1−/−). IFNγR1−/− and control mice were treated using a multiple treatment regimen as described above. As shown in Figure 24, topical treatment with TPA did not induce IRF-1 mRNA in either wild-type or IFNγR1−/− mice. In contrast, a significant increase in IRF-1 mRNA levels was observed in wild-type mice but not in IFNγR1−/− mice following treatment with CHRY. Furthermore, topical treatment with TPA did not increase Stat1 mRNA levels in either wild-type or IFNγR1−/− mice. However, CHRY treatment caused an approximate 2-fold increase in Stat1 mRNA 24 hrs following treatment. These data demonstrate that induction of IFNγ in the epidermis by CHRY leads to induction of IRF-1 and uStat1 via signaling through
IFNγR1.

To further validate our findings, we used Stat1\(^{-/-}\) primary keratinocytes and recombinant mouse IFNγrIFNγ as a surrogate for CHRY treatment in these cells. Primary keratinocytes were harvested as described in the Material and Methods section and placed in starvation media for 24 hrs prior to stimulation. As shown in Figure 25, treatment of wild-type keratinocytes with rIFNγ and TPA increased phosphorylation of Stat1\(^{Y701}\) Phosphorylation of Stat1 occurred rapidly in response to rIFNγ treatment compared to TPA. In addition, stimulation of primary keratinocytes with rIFNγ resulted in a significant increase in IRF-1 protein levels within 3 hours following treatment in wild-type, but not Stat1\(^{-/-}\) keratinocytes. In contrast, IRF-1 expression was unaffected in both wild-type and Stat1\(^{-/-}\) keratinocytes, following treatment with TPA, in spite of increased phosphorylation of Stat1\(^{Y701}\) in wild-type keratinocytes. In addition, we observed an increase in total Stat1 protein levels in rIFNγ but not TPA-treated wild-type keratinocytes. These data confirm that keratinocytes respond to rIFNγ and TPA treatment in a manner similar to that observed in mouse epidermis in vivo and support the hypothesis that CHRY works via upregulation of the IFNγ signaling pathway.
Figure 22. CHRY treatment led to increased levels of IFNγ whereas TPA resulted in a decrease. Wild-type mice 6-8 weeks of age received topical applications of acetone (Ace), TPA (6.8 nmol 2x/week for 2 weeks) or CHRY (220 nmol 1x/week for 4 weeks). Mice were sacrificed at the indicated time points and epidermal mRNA expression was analyzed by qPCR. A) IFNγ mRNA levels following TPA and B) IFNγ mRNA levels following CHRY treatment †indicates values significantly different from the Ace control;†(Mann Whitney U, p≤0.05.)
Figure 23. Following tumor promoter treatment Type I (α and β) Interferons levels decreased compared to acetone controls. Wild-type mice 6-8 weeks of age received topical applications of acetone (Ace), TPA (6.8 nmol 2x/week for 2 weeks) or CHRY (220 nmol 1x/week for 4 weeks). Mice were sacrificed at the indicated time points and epidermal mRNA expression was analyzed by qPCR. A) Type I IFN mRNA levels following TPA treatment; B) Type I IFN mRNA levels following CHRY treatment * indicates values significantly different from the Ace control; (Mann Whitney U, p≤0.05).
Figure 24. CHRY treatment led to activation of the IFNγ-Stat1 signaling axis whereas TPA resulted in a decrease. Wild-type and IFNγR1 knock-out mice 6-8 weeks of age received topical applications of acetone (Ace), TPA (6.8 nmol 2x/week for 2 weeks) or CHRY (220 nmol 1x/week for 4 weeks). Mice were sacrificed at the indicated time points and epidermal mRNA expression was analyzed by qPCR. A) IRF-1 mRNA levels following TPA and CHRY treatment; B) Stat1 mRNA levels following TPA and CHRY treatment. + indicates values significantly different from the Ace control; * indicates values between KO and wild-type mice were significantly different (Mann Whitney U, p≤0.05).
Figure 25. Primary keratinocytes stimulated with IFNγ led to an induction of IRF-1. Primary keratinocytes were isolated from Stat1 wild-type and Stat1−/− mice and cultured by methods described in the Material and Method section. Primary keratinocytes were stimulated with either TPA (680 nM) or IFNγ (250 ng/ml). Untreated cultured primary keratinocytes served as control. Keratinocytes were stimulated for the indicated time point and harvested. Protein lysates were prepared for Western Blot analysis.
Chapter 7. IFNγ/pStat1/IRF-1 signaling axis regulates inflammation and inflammatory signaling induced by CHRY

Rationale

As noted in the Introduction inflammation has long been associated with cancer development. The JAK-STAT pathway is indispensable for innate as well as adaptive immunity, but persistent activation of these immunological response pathways may lead to chronic inflammation. During a state of chronic inflammation in the skin, activated immunocytes are recruited to the area where they produce copious amounts of pro-inflammatory cytokines such as IL-6 [reviewed in (107)], TNFα [reviewed in (108)] and IFNγ [reviewed in (109)]. Based on the data presented in Chapters 5 and 6, a role for the IFNγ/pStat1/IRF-1 signaling axis in CHRY-mediated tumor promotion has been presented. The aim of the studies in this chapter was to evaluate the potential role of this signaling pathway in inflammation and inflammatory signaling pathways associated with skin tumor promotion by CHRY. For these studies we again used Stat1−/− mice.

7.1. Impact of Stat1 deficiency on NF-κB and Cox-2 following treatment with TPA and CHRY

To further explore potential mechanism(s) whereby IFNγ/pStat1/IRF-1 signaling mediates skin tumor promotion by CHRY, we examined the impact of Stat1 deficiency on NF-κB activation and Cox-2 expression following treatment with TPA and CHRY. For these experiments, wild-type and Stat1−/− mice were again treated using a multiple treatment protocol with either 6.8 nmol TPA or 220 nmol CHRY as described above.
Mice were sacrificed at the indicated time points following the last treatment. Epidermal lysates were collected for protein analysis by Western blot and analysis of mRNA by q-RT-PCR. As shown in Figure 26, topical treatment with TPA led to an increase in Cox-2 expression (both mRNA and protein) as early as 6 hrs after the last treatment in both wild-type and Stat1\(^{-/-}\) mice. In contrast, Stat1\(^{-/-}\) mice exhibited a significant reduction in Cox-2 expression compared to the wild-type controls following treatment with CHRY. In this regard, Stat1\(^{-/-}\) mice exhibited an approximate 3-fold reduction in Cox-2 protein levels and an even greater reduction in Cox-2 mRNA at the 12 hr time point compared to wild-type controls treated with CHRY (panel B). The observed reduction in levels of Cox-2 in Stat1\(^{-/-}\) mice persisted until levels reached baseline values at 48 hrs following treatment with CHRY. Consistent with significant reductions in Cox-2 protein levels, Stat1\(^{-/-}\) mice treated with CHRY exhibited a decrease in epidermal PGE\(_2\) levels compared to wild-type mice, whereas no differences in epidermal PGE\(_2\) levels were observed between the two genotypes treated with TPA (Figure 27). Since NF-\(\kappa\)B has previously been linked to Cox-2 expression in mouse epidermis, we evaluated whether the reduction in Cox-2 expression was associated with reduced NF-\(\kappa\)B signaling after CHRY treatment. Interestingly, activation of NF-\(\kappa\)B signaling (measured by phosphorylation at Ser536) was similar in wild-type and Stat1\(^{-/-}\) mice after treatment with CHRY. As shown in Figure 26 panel C, the reduced expression of Cox-2 in epidermis of Stat1\(^{-/-}\) mice seen following treatment with CHRY occurred in the presence of normal NF-\(\kappa\)B signaling.
Figure 26. Impact of Stat1 deficiency on the promoter-induced Cox-2 and NF-κB expression. Wild-type (Stat1+/+) or Stat1 knock-out (Stat1−/−) mice received 4 topical applications of either acetone (Ace), 6.8 nmol TPA, or 220 nmol CHRY and were sacrificed at the indicated time points. Epidermal lysates were prepared for protein analysis by Western blot and mRNA expression was examined by qPCR. Cox-2 expression following A) TPA; and B) CHRY treatment; C) NF-κB signaling following CHRY treatment. Western blot data are from a single experiment (pooled protein samples) that has been repeated with very similar results. The mRNA data was obtained from individual mice (n=5/group) allowing statistical analyses. * indicates values between Stat1+/+ and Stat1−/− groups were significantly different by Mann-Whitney U (p≤0.05).
Figure 27. Effect of Stat1 deficiency on prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) synthesis following tumor promoter treatment. Wild-type (Stat1\textsuperscript{+/+}) or knockout (Stat1\textsuperscript{-/-}) mice received either a single 220 nmol dose of chrysarobin CHRY in 0.2 mL acetone (Ace) or four applications of TPA (6.8 nmol 2x/week for 2 weeks). Following indicated time after last treatment the dorsal skin was excised and snap frozen with liquid nitrogen. Pooled frozen epidermis from 3-4 mice was chipped into PGE\textsubscript{2} lysis buffer. PGE\textsubscript{2} was eluted and assayed using manufacturer instructions.
7.2 Effect of Stat1 deficiency on iNOS expression and following topical treatment with TPA and CHRY.

iNOS is a Stat1 responsive gene that is responsible for the production of the reactive oxygen radical nitrous oxide (NO). iNOS overproduction and the production of NO is associated with pathological conditions of inflammation (110) (111, 112) and tumor development (113). Therefore, we also determined the impact of Stat1 deficiency on the expression of iNOS in response to treatment with TPA and CHRY. As shown in Figure 28 (panel A), topical treatment with TPA led to an increase in iNOS mRNA levels at 6 hrs, which quickly returned to basal levels at 18 hrs following treatment. Furthermore, no differences were observed in the response of Stat1\(^{-/-}\) mice compared to wild-type control mice following treatment with TPA. In contrast, treatment with CHRY led to a significant increase in iNOS mRNA levels in wild-type mice, but Stat1\(^{-/-}\) mice were highly resistant to iNOS induction (Figure 28, panel B). CHRY treatment led to a rapid increase in mRNA levels of iNOS as early as 6 hrs that remained elevated until iNOS reached basal levels at 48 hrs following treatment in wild-type mice. iNOS mRNA levels peaked around 12 hrs following treatment with CHRY: There was an approximate 10-fold increase in iNOS mRNA at this time point in wild-type compared to Stat1\(^{-/-}\) mice.
Figure 28. Stat1 deficient mice were resistant to iNOS induction following CHRY treatment, whereas TPA-mediated induction of iNOS occurs independent of Stat1. Wild-type (Stat1\textsuperscript{+/+}) or knockout (Stat1\textsuperscript{-/-}) mice received either A) TPA (6.8 nmol 2x/week for 2 weeks) or B) 220 nmol CHRY (1x/week for 4 weeks) in 0.2 mL acetone (Ace). Epidermal lysates were prepared at the indicated time points for mRNA analysis by qPCR. The mRNA data was obtained from individual mice (n=3-5/group) allowing statistical analysis. * indicates value was significantly different from control (Mann Whitney U, p≤0.05).
7.3 Stat1 deficiency results in attenuated dermal infiltration of macrophages following treatment with CHRY

Chronic inflammation is characterized by the infiltration of immunocytes. This influx of immune cells represents a double-edge sword in that it has anti-tumorigenic as well as pro-tumorigenic effects. As mentioned previously, Stat1 has been shown to be a vital player in the immune response, representing the major transducer of both Type 1 and Type II IFNs. Stat1 is rapidly activated in macrophages in response to treatment with lipopolysaccharide (LPS), a bacterial cell wall component that is a potent activator of immunocytes (114). In addition, Stat1 regulates the expression of many chemoattractant chemokine/cytokines such as monokine induced by IFNγ (MIG), CC chemokine ligand-20 (CCL-20), epithelial cell- derived neutrophil-activating peptide (ENA-78) and IFN-inducible T cell-chemoattractant (I-TAC) (53). To examine the impact of Stat1 deletion on immune cell infiltration, Stat1−/− and wild-type control mice were evaluated for immune cell influx of various immunocytes by immunohistochemistry (leukocytes, mast cells, neutrophils, and macrophages). Groups of 3-4 mice of each genotype were treated once with 220 nmol CHRY and sacrificed at the indicated time points. Qualitative immunohistochemistry studies revealed that Stat1 deficiency resulted in a decrease of macrophage infiltration into the dermal compartment of Stat1−/− mouse skin after treatment with CHRY compared to wild-type control mice (see Figure 29). The differences in macrophage influx were most evident during the earlier time points examined (6 and 12 hour). There were no noticeable differences seen in the infiltration of Tcells, mast cells or neutrophils (data not shown).
Figure 29. Stat1 deficient mice displayed a reduced dermal inflammatory response (decreased macrophages) following topical application of CHRY compared to controls. Stat1 KO (Stat1\(^{-/-}\)) mice and wild-type (Stat1\(^{+/+}\)) mice 6-8 weeks of age received either a single topical application of acetone or 220nmol CHRY in 0.2 ml. Mice were sacrificed at the indicated time points and whole skin sections were prepared to assess immune cell infiltration. Representative sections of whole skin sections were stained with S100A8 are shown.
7.4 Stat1 is necessary for expression of inflammatory chemokine/cytokines induced by CHRY

Chemokines/cytokines play a vital role in tumor development. Chronic inflammation promotes tumor development by inducing a cascade whereby both tumor cells and stromal cells produce various chemokines and cytokines [reviewed in (115, 116)]. These various chemokines and cytokines may act in an autocrine or paracrine fashion to sustain tumor growth, induce angiogenesis, or facilitate evasion of immune surveillance by immunoediting. Chemokines are classified into four groups, CXC, CC, CX3C and C, which is based on the positioning of the conserved two N-terminal cysteine residues (117). To further evaluate the role of Stat1 in the production of chemokines and cytokines induced by CHRY, we utilized a real-time based inflammatory panel assay described in the Materials and Methods section and, mRNA isolated 6 hrs after the last of 4 treatments with CHRY. As shown in Figure 30, the absence of Stat1 led to a significant reduction in expression of several inflammatory-associated chemokines/cytokines (>2-fold) reduction compared to wild-type control mice following treatment with CHRY.
Figure 30. Stat1 deficiency caused a significant decrease in several inflammatory mediators following CHRY treatment. Four to five Stat1^{+/+} (solid black) or Stat1^{-/-} (white) mice received 4 topical applications of 220nmol CHRY and were sacrificed 6 hrs following the last treatment. RNA was isolated from epidermal lysates as previously described in the Material and Methods section. mRNA expression was analyzed by RT^{2} Profiler inflammatory cytokine/chemokine array (SA Bioscience) using manufacturer instructions.
Chapter 8: Ongoing and Future studies

8.1 Stat1 and UV carcinogenesis

In terms of human relevance, UV radiation (UVR) represents the major risk factor for skin cancer. Acute exposure to UVR induces cell damage and induces DNA repair pathways. Chronic exposure leads to increased epidermal damage, increased hyperplasia, and inflammation and skin cancer. Skin cancer represents the most common cancer in humans today, with steadily increasing rates in new cases. UVR is subdivided into UVA (315-400nm), UVB (290-315nm) and UVC (100-280nm). The solar output that reaches the earth’s surface is approximately 95-98% UVA and 2-5% UVB, while UVC is completely absorbed by the stratospheric layer of the ozone. Although UVA is much more abundant, several studies have determined that wavelengths in the range of 295 nm to 305nm (UVB) are the most important in influencing tumor development. [reviewed in(118)]. However UVA has been shown to posses both weak complete carcinogenicity and moderate skin tumor promotion ability [reviewed in(119)]. It should also be noted that solar radiation is more effective at inducing skin tumors in experimental animals than UVB alone further implicating a role for UVA in human skin cancer.

Stat3 has been shown to play an important role in UVB-mediated skin carcinogenesis by modulating cell proliferation and apoptosis [reviewed in (118, 120)]. In contrast, the role of Stat1 during UVB-mediated skin carcinogenesis
remains largely unknown. Studies by Zhang et al report that following UVB irradiation, Stat1 is phosphorylated on its serine 727 residue, and not its Y701 residue (121). As shown in Figure 31, preliminary data has confirmed that following a single exposure to 350 mJ/cm\(^2\) UVB, Stat1 S727 phosphorylation occurs as early as 3 hours in epidermis of FVB/N wild-type mice. In contrast, there was no detectable phosphorylation of Stat1 on Tyr701 (see again Figure 31). Since serine phosphorylation is necessary for maximal transcriptional activity of STATs, it will be interesting to examine the transcriptional activity of Stat1 in the absence of tyrosine phosphorylation. Studies are underway to fully characterize the role Stat1 in response to UVB irradiation.

In addition to investigating UVB, we also plan to investigate UVA. Data in the literature indicate that UVA exposure alone can induce skin papillomas and SCCs in mouse skin although with weaker activity that UVB (122). Additional studies with UVA suggest that it produces biochemical and molecular changes in mouse skin similar to other types of tumor promoters (123) and that it possess tumor promoting activity in vivo (124). A previous study reported that UVA (320-400 nm) but not UVB (280-320 nm) irradiation of mouse skin led to the induction of IFN\(_\gamma\) in epidermis of hairless mice (125). Furthermore, low dose UVA irradiation of cultured human keratinocytes led to activation (DNA binding activity and tyrosine phosphorylation) of Stat1 (126). These data suggest the possibility that UVA exposure may activate a similar signaling pathway involving IFN\(_\gamma\)/Stat-1/IRF-1 that may contribute to its tumor promoting activity. Studies are currently underway to investigate the role of UVA irradiation on the IFN\(_\gamma\)/pStat1/IRF-1
signaling axis. For these experiments Stat1 wild-type and Stat1\textsuperscript{-/-} will be
irradiated with UV (UVA or UVB) and the impact of Stat1 deletion on signaling
pathways, apoptosis, proliferation, and tumor development will be examined.
Figure 31. Effect of UVB exposure on Stat1. FVB/N wild-type mice 6-8 weeks of age were exposed to 350mJ/cm² of UVB irradiation. Mice were sacrificed at the indicated timepoint following irradiation. Untreated mice served as the control. Mice were sacrificed at the indicated time points and epidermal lysates were prepared for protein analysis by Western blot.
8.2 Possible role of IFN\(_\gamma\)/pStat1/IRF-1 signaling in tumor promotion by other non-phorbol ester promoters

Benzoyl Peroxide (BzPo) is an organic peroxide that is commonly used in cosmetic and pharmaceutical industries, in particular in the form of acne medication. BzPo is another non-phorbol ester skin tumor promoter that forms radicals upon breakdown (69, 127). The generation of such radicals is believed to be critical to its mechanism of skin tumor promoting action (128-130). BzPo induces short-term biomarkers commonly associated with tumor development such as ODC induction and increased epidermal hyperplasia (131). Thus, BzPo and CHRY share some similarities in this regard. Preliminary data from our lab (see Figure 32), show that a single topical application of BzPo (20 mg/mouse) led to activation of Stat1 (phosphorylation at both Tyr\(^{701}\) and Ser\(^{727}\)) at several time points after treatment. In addition, Stat1 protein levels were significantly elevated at later time points (48, 72 and 96 hrs) after treatment. These results are very similar to those obtained with CHRY suggesting that BzPo may work, at least in part through activation of the IFN\(_\gamma\)/pStat1/IRF-1 signaling pathway. We are currently determining the extent to which this pathway applies to the promoting action of BzPo and possibly other free radical generating skin tumor promoters.
Figure 32. Effect of BzPo treatment on Stat1 phosphorylation/induction in mouse epidermis. Wild-type FVB/N mice 6-8 weeks of age received a single application of acetone (Ace) or 20 mg BzPo. Mice were sacrificed at the indicated time points and epidermal lysates were prepared for protein analysis by Western blot. Protein levels were quantitated by densitometry. Actin was used to normalize protein loading. phospho-Stat levels were normalized to total Stat1.
8.3 Cellular source of IFNγ production following treatment with CHRY

As shown in Figure 22, treatment with CHRY caused a rapid increase in IFNγ mRNA in the epidermis. At the present time we are unsure of the source of the IFNγ production. Although keratinocytes express the IFNγ receptor it is not clear whether keratinocytes are capable of producing IFNγ. The majority of IFNγ is produced by the activated immunocytes [reviewed in(132)]. Based on the findings that IFNγ mRNA is induced rapidly, it is likely that it is resident immunocytes of the epidermis. Cells that reside in the epidermis include langerhans cells (LCs) and dendritic cells, especially γδ T cells(133). γδ T cells are known to produce IFNγ (134) and it is possible that these cells or other resident T-cells are responsible for the increase in IFNγ mRNA seen in epidermal RNA samples following treatment with CHRY. Utilizing flow cytometry methods, cells will be sorted based on immune subtype specific surface markers. Once pure populations of the various cell types are acquired, the IFNγ production will be evaluated by intracellular cytokine staining.

8.4 Studies using epidermis specific Stat1 deficient mice

To date all experiments regarding Stat1 have been performed utilizing mice with deletion of Stat1 in all cells of the body (93). Thus, some of the effects of Stat1 deletion on skin tumor promotion by CHRY could be due to loss of Stat1 in other cells, e.g. immune/inflammatory cells. Stat1−/− mice are known to have some defects in immune response (94) as well as defects in cytokine production (51). In future studies, BK5.Cre x Stat1flox/flox mice will be used to evaluate the
impact of Stat1 deletion on epidermal proliferation and skin inflammation induced by CHRY. Keratinocyte specific deletion of Stat1 will allow us to determine if the effects of Stat1 are cell autonomous to keratinocytes or are a result of the resident populations of immunocytes.
Chapter 9: Discussion

In the current study, we examined the role of Stat1 in tumor promotion and epithelial carcinogenesis utilizing the well-established two-stage skin carcinogenesis model (54). The results demonstrate that topical application of both TPA and CHRY led to activation of Stat1 (phosphorylation at both Y701 and S727). Furthermore, topical application of CHRY caused a significant increase in total uStat1 protein level whereas TPA did not. Further mechanistic studies revealed that treatment with CHRY led to upregulation of signaling via IFNγ and that this was responsible for a significant portion of epidermal Stat1 activation as well as upregulation of uStat1 following treatment with this tumor promoter. Utilizing the two-stage skin carcinogenesis model, the importance of this signaling pathway in skin tumor promotion by CHRY was further demonstrated using Stat1−/− mice. Stat1−/− mice were highly resistant to skin tumor promotion by CHRY but not TPA, indicating an absolute requirement for this pathway for tumor promotion by anthrone tumor promoters. Collectively, the current data demonstrate a novel mechanism of skin tumor promotion involving IFNγ-pStat1-IRF-1 signaling and upregulation of uStat1 in keratinocytes for the anthrone class of skin tumor promoters of which CHRY is the most potent member (135). This pathway does not play a major role in skin tumor promotion by the phorbol ester, TPA.
In earlier studies from our laboratory, we reported that Stat1 activation (Y701 phosphorylation) along with Stat3 activation (Y705 phosphorylation) occurred in epidermis following topical treatment with TPA (99). It was also shown that Stat1 phosphorylation was elevated in papillomas generated by a standard two-stage DMBA/TPA protocol (99). The activation of Stat3 in keratinocytes was shown to be dependent on signaling through the EGFR although the mechanism for activation of Stat1 by TPA was not further investigated. As shown in Figure 8, treatment with both TPA and CHRY led to rapid activation of Stat1 in epidermis. However, treatment with CHRY also led to a significant upregulation of uSTAT1 suggesting that there might be fundamental differences in the mechanism of Stat1 activation between the two compounds. This hypothesis was borne out by further analyzing the potential role of IFN\(\gamma\) signaling following treatment with both types of promoters. As noted in the introduction, a major pathway for activation of Stat1 in various cells, including keratinocytes, is via IFN\(\gamma\) receptor signaling (136, 137). As shown in Figures 21 and 24, treatment with CHRY but not TPA led to upregulation of IRF-1 and this was dependent on IFN\(\gamma\) receptor signaling via Stat1.

The data in the current paper represent the first report that topical treatment with CHRY leads to upregulation of IFN\(\gamma\) and IFN\(\gamma\)-receptor signaling in epidermis. The possible role of IFN\(\gamma\) in skin tumor promotion by TPA has previously been explored. In earlier studies, Reiners and colleagues reported
that IFN\textsubscript{γ} could act as a co-promoter when injected i.p. together with topical application of TPA (138). IFN\textsubscript{γ} given alone did not promote skin tumors in mice initiated with DMBA in these earlier studies. More recently, the role of IFN\textsubscript{γ} signaling in tumor promotion by TPA has been explored more directly using genetically engineered mouse models with conflicting results. In this regard, Xiao et al reported that IFN\textsubscript{γ} mRNA levels were elevated in RNA samples isolated from whole skin following treatment with DMBA and either a single or multiple treatments with TPA (139). In addition, these authors reported that Stat1 was activated in protein lysates isolated from whole skin 24 hrs following the last TPA treatment. These authors also reported that administration of an anti-IFN\textsubscript{γ} antibody or using of IFN\textsubscript{γ} receptor deficient mice reduced the number of papillomas but had no effect on the incidence of SCCs in a two-stage carcinogenesis protocol. In contrast to these data, Wang et al (137) reported that IFN\textsubscript{γ}/− mice had nearly identical tumor response (both the percentage of mice with papillomas and papillomas per mouse) when compared with wild-type mice undergoing a two-stage (DMBA-TPA) protocol. Our current data support the conclusion that IFN\textsubscript{γ} signaling via Stat1 and IRF-1 is not involved in skin tumor promotion by TPA. However, the current data may explain the co-promoting effects if IFN\textsubscript{γ} as well as previous data showing that low doses of CHRY could also act as a co-promoter when given together with TPA (135).
As noted in the Introduction, several reports have indicated a possible pro-tumorigenic role for Stat1 (45, 47, 140) although other studies have supported a tumor suppressor role for this signaling molecule. In a previous study, Schreiber et al. reported that following a single subcutaneous injection of the carcinogen methylcholanthrene (MCA), IFNγR−/− and Stat1−/− mice were highly susceptible to tumor formation compared to 129/Sv controls (141). In this study they also report that Stat1 and p53 double knockout mice developed tumors more rapidly and with greater frequency then p53 single knockout, when challenged with MCA. Other studies have also shown that IFNγ plays an important role in immune surveillance for chemically-induced tumors, including skin tumors (141). In contrast, Hanada et al. (142) reported that mice deficient in Suppressor of Cytokine Signaling-1 (SOCS1), a negative regulator of STAT signaling, developed spontaneous colorectal carcinomas and their development was IFNγ-dependent. In this study, IFNγ−/−SOCS1−/− mice failed to develop tumors regardless of the upregulation of Stat3 responsive genes such as Bcl-xL and c-myc when compared to the SOCS1−/− deficient mice. These results suggested a critical role for IFNγ/Stat1 signaling in the development of colorectal tumors in this mouse model. The current data clearly demonstrate an important role for Stat1 activation via IFNγ signaling in epidermis in mediating skin tumor promotion by CHRY. Perhaps Stat1 activation via IFNγ signaling has opposing roles during epithelial carcinogenesis in mouse skin: a pro-tumorigenic role during the early tumor promotion stage and an immune surveillance function once tumors are
developed. Further ongoing studies using conditional Stat1 knockout mice will help to address these ideas in more detail.

The mechanism(s) for how Stat1 mediates skin tumor promotion by CHRY remain to be fully determined. As shown in Figure 19, Stat1 deficient mice treated with CHRY had a reduced proliferative response as measured by BrdU incorporation and epidermal thickness. However, the decrease in epidermal proliferation seen in Stat1−/− mice in response to treatment with CHRY did not appear sufficient to explain the dramatic inhibition of skin tumor promotion by CHRY seen in these mice (Figure 16). Stat1 has been shown to regulate the production of pro-inflammatory molecules such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2)(52, 143). Furthermore, IFNγ is also known to upregulate a variety of inflammatory mediators including tumor necrosis factor alpha (TNFα) (144), interleukin-12 (IL-12) (145), gp91phox (146) and iNOS, (147). IRF-1 is a Stat1 responsive transcription factor that acts as a secondary response to activate other downstream targets (i.e., iNOS and Cox-2) (52, 148-150). As shown in Figure 21, treatment with CHRY also led to upregulation of IRF-1 that was blocked in both Stat1−/− and IFNγR1−/− mice (Figures 21 and 24). Both TPA and CHRY treatment led to upregulation of epidermal Cox-2 expression (both mRNA and protein) (Figure 26). Furthermore, the upregulation of Cox-2 (and PGE2) by CHRY but not TPA was dependent on activation of Stat1. Similar results were obtained for the upregulation of iNOS by both compounds (Figure 28). The reduction in Cox-2 and iNOS expression in Stat1−/− mice
compared to wild-type mice following treatment with CHRY occurred in the presence of apparently normal activation of NF-κB (Figure 26, panel C) further demonstrating the importance of Stat1 signaling in mediating skin tumor promotion by CHRY. The induction of Cox-2 and the increased production of prostaglandins such as PGE\textsubscript{2} represent important events in the process of skin tumor promotion (90, 151, 152). Therefore, Stat1/IRF-1 regulation of the induction of Cox-2 (and possibly iNOS) by CHRY may explain, at least in part, some of the mechanism associated with skin tumor promotion by this compound.

Another interesting observation in the current study was the induction of uStat1 by CHRY but not TPA (Figure 8). Recently, it was found that Stats 1 and 3 (and possibly other Stats) also play important roles in mediating gene expression in the absence of tyrosine phosphorylation (39, 40), Stat1 and Stat3 genes are targets of activated (phosphorylated) Stat1 and Stat3 proteins, respectively (41). As a result, cytokine activation of Stat1 or Stat3 (e.g., IFN\textsubscript{γ} or IL-6, respectively), leads to the induction and accumulation of uStats 1 and 3, which may persist for days after p-Stat levels have subsided (40). In addition, induction of the Stat1 target gene, IRF-1, aids in the continued accumulation of uStat1 in response to IFN\textsubscript{γ}. It is well documented that uStats 1 and 3 can act as transcription factors and regulate a subset of genes that are different from those regulated by p-Stats (39, 42, 153). Together these data suggest that uStat1 may be transcriptionally active and play a significant role in skin tumor promotion by CHRY. Transcriptional profiling has shown that the majority of uStat1 target genes are
antiviral immune response genes, however uStat1 also induces a subset of
genes implicated in radio- and chemo-resistance in cancer cells (42, 43).
Disruption of interferon effector molecule 8 (IRF8) in soft tissue sarcoma cells
leads to the accumulation of uStat1 (44) and promotes sarcoma cell metastasis
by regulating gene transcription of apoptosis regulators Fas and Bad (44).
However, the role and mechanism(s) by which uStat1 mediates a pro-
tumorigenic effect are largely unknown. Future studies are aimed at defining the
role of uStat1 in skin tumor promotion by CHRY and possibly other skin tumor
promoters.

An additional noteworthy aspect of the current studies involves the
potential cellular source of epidermal IFNγ induced by treatment with CHRY.
Although keratinocytes are known to express IFNγ receptors(154), there is little
evidence in the literature suggesting that these cells produce IFNγ. Other cells
that reside in the epidermis include Langerhans cells (LCs) and dendritic cells,
especially γδT cells. γδT cells are known to produce IFNγ (155, 156) and it is
possible that these cells or other resident or recruited T cells may be responsible
for the increase in IFNγ mRNA seen in epidermal RNA samples following
treatment with CHRY. Ongoing studies are aimed at determining the cells in the
epidermis responsible for production of IFNγ during tumor promotion by CHRY.
In conclusion, the current data identify a novel mechanism for skin tumor promotion involving activation of IFNγ signaling via p-Stat1 and IRF-1 that is required for the skin tumor promoting activity of CHRY, a member of the anthrone class of skin tumor promoters. This mechanism does not appear to play a major role in skin tumor promotion by the phorbol ester, TPA. The mechanisms may involve the canonical pathway involving formation of p-Stat1 homodimers, which lead to direct transcriptional regulation of IRF-1 and uStat1. Alternatively, or in addition, this could involve a non-canonical pathway involving the translocation of a complex containing IFNγR1-JAKS1/2-p-Stat1 to the nucleus where it binds to the IRF-1 promoter and induces transcription of IRF-1 (157, 158). Upregulated IRF-1 then leads to increased transcription of a number of genes, including uStat1, iNOS and Cox-2 as well as others that ultimately contribute to the skin tumor promoting action of CHRY. Furthermore, both p-Stat1 and IRF-1 have been shown to interact with NF-κB (159), and that may also play a role in altered expression of some genes. Figure 33 proposes a working model for the role of IFNγ/p-Stat1/IRF-1 signaling in skin tumor promotion by CHRY encompassing these various aspects discussed above.

The current data also support other emerging data in the literature that, under certain circumstances, Stat1 can have a pro-tumorigenic function. In this regard, the current studies demonstrate that Stat1 influences epithelial multistage carcinogenesis early during the process of tumor promotion with certain types of
chemical tumor promoters. The extent to which this mechanism applies to other types of chemical tumor promoters, especially those that work primarily through the generation of free radicals (e.g., BzPo) is currently under investigation. Further understanding of the downstream effectors of this novel skin tumor promotion pathway will aid in our understanding of the process of tumor promotion in general and in the identification of novel targets for cancer prevention.
Figure 33. Working Model For the Role of IFNγ/p-Stat1/IRF-1 Signaling in Skin Tumor Promotion by CHRY
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Vita

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