Published in final edited form as:

Mol Carcinog. 2015 August ; 54(8): 642–653. doi:10.1002/mc.22132.

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

Ronald Bozeman^{1,2}, Erika L. Abel^{4,5}, Everardo Macias², Tianyi Cheng², Linda Beltran², and John DiGiovanni^{2,3}

¹Graduate School of Biomedical Sciences, the University of Texas MD Anderson Cancer Center, Houston, Texas, USA

²Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, Texas, USA

³Department of Nutritional Sciences, College of Natural Sciences, The University of Texas at Austin, Austin, Texas, USA

⁴Department of Carcinogenesis, Science Park-Research Division, the University of Texas MD Anderson Cancer Center, Smithville, Texas, 78957, USA

Abstract

The current study was designed to explore the role of signal transducer and activator of transcription 1 (Stat1) during tumor promotion using the mouse skin multistage carcinogenesis model. Topical treatment with both 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 3-methyl-1,8-dihydroxy-9-anthrone (chrysarobin or CHRY) led to rapid phosphorylation of Stat1 on both tyrosine (Y701) and serine (S727) residues in epidermis. CHRY treatment also led to upregulation of unphosphorylated Stat1 (uStat1) at later time points. CHRY treatment also led to upregulation of interferon regulatory factor 1 (IRF-1) mRNA and protein, which was dependent on Stat1. Further analyses demonstrated that topical treatment with CHRY but not TPA upregulated interferon-gamma (IFN γ) mRNA in the epidermis and that the induction of both IRF-1 and uStat1 was dependent on IFN γ 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. 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. These studies define a novel mechanism associated with skin tumor promotion by the anthrone class of tumor promoters involving upregulation of IFN γ signaling in the epidermis and downstream signaling through activated (phosphorylated) Stat1, IRF-1 and uStat1.

Correspondence: John DiGiovanni, Ph.D., The University of Texas at Austin, Dell Pediatric Research Institute, 1400 Barbara Jordan Blvd, Austin, TX 78723, USA. Phone: 512-495-4726, john.digiovanni@austin.utexas.edu.

⁵Current address: Department of Biology, Baylor University, Waco, Texas, 76798, USA.

Keywords

Stat1; IRF-1; IFN γ ; tumor promotion; inflammation; epithelial tumorigenesis

Introduction

Signal transducers and activators of transcription (Stats) are a family of latent cytoplasmic transcription factors that transduce extracellular signals from the cell membrane to the nucleus [1]. Stats are comprised of seven family members consisting of Stat1, Stat2, Stat3, Stat4, Stat5A/B and Stat6 that are involved in many normal physiological processes including differentiation, cell growth, proliferation and apoptosis [2-5]. Stats are activated by a variety of factors such as cytokines, growth factors, hormones and oncogenic signals. Stats can be activated by receptor tyrosine kinases such as the epidermal growth factor receptor which possess intrinsic tyrosine kinase activity [2]. Stats can also be activated by receptors that lack intrinsic tyrosine kinase activity such as cytokine receptors [e.g. interferon gamma receptor (IFN γ R), interleukin-6 receptor (IL-6R)] [3]. These receptors activate Stat proteins by means of a class of receptor-associated kinases called Janus kinases (JAKs) [6-8]. Stats are constitutively activated in various mouse models of tumorigenesis, human cancer cell lines, solid neoplasms, and human blood malignancies [4,9-14]. Stat family members 1, 3, and 5 have been found most frequently to be constitutively activated in human tumors [4,15].

A critical role for Stat3 in epithelial multistage carcinogenesis in mouse skin has been established [10,16,17]. In this regard, Stat3 was shown to be important during the initiation phase of epithelial carcinogenesis by regulating keratinocyte survival, including survival of bulge-region keratinocyte stem cells [18,19] and for clonal expansion of initiated cells during tumor promotion [10,18,19]. Stat3 also appears to play an important role in the progression of skin tumors in this model system [10,20]. The role of other Stat family members, such as Stat1, in epithelial multistage carcinogenesis remains to be elucidated. Stat1, which is activated via IFN signaling [21-23], has been associated with anti-tumorigenic actions by modulating key components of immune tumor surveillance [24-26], inducing pro-apoptotic regulators such as Fas/FasL and caspases, and regulating negative cell cycle regulatory proteins such as p21 and p27 [27]. In contrast, other studies have suggested a pro-tumorigenic action of Stat1. In this regard, activation of Stat1 and/or overexpression of IFN-related genes in breast cancer have been associated with poor prognosis and resistance to radiation and adjuvant cancer therapy [28,29]. In addition, a role for activated Stat1 in promotion of leukemia development has also been reported [30]. A pro-tumorigenic role of IFN γ -Stat1 signaling has been suggested in melanoma [31,32]. Collectively, the available data suggest that Stat1 may exhibit both tumor suppressive effects as well as pro-tumorigenic effects depending on the tissue and/or cellular context.

The current study was undertaken to examine the role of Stat1 in skin tumor promotion using the mouse skin model of multistage carcinogenesis [33]. During the course of these studies, it was discovered that skin tumor promotion by the anthrone tumor promoter, chrysarobin (CHRY), is mediated by a novel signaling pathway involving Stat1 activation

(phosphorylation) and upregulation of interferon regulatory factor-1 (IRF-1) via upregulation of IFN γ and IFN γ receptor (IFN γ R) signaling in the epidermis. This pathway is not activated by the phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Thus, under certain conditions Stat1 may have a pro-tumorigenic role during the promotion stage of epithelial carcinogenesis.

Materials and Methods

Chemicals and Reagents

7,12-dimethylbenz(a)anthracene (DMBA), bromodeoxyuridine (BrdU), proteinase inhibitor cocktails, phosphatase inhibitor cocktails, anti-actin and anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). TPA was purchased from LC Laboratories (Woburn, MA, USA). CHRY was prepared from chrysophanic acid (Sigma-Aldrich) according to a published method [34]. All procedures with CHRY were performed under low intensity yellow lights. Antibodies against phosphorylated Stat1 (Y701 or S727) and total Stat1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-IRF-1 and IFN γ R β antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Cox-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). Chemiluminescence detection kits were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The recombinant IFN γ was purchased from BD Bioscience (Franklin Lakes, NJ, USA).

Animals

Female FVB/N mice were purchased from the National Cancer Institute and used when 7-9 weeks of age. All mice were group-housed and all experiments were conducted in accordance with NIH and institutional guidelines. Stat1^{-/-} mice were a generous gift from Dr. David Levy from the Kaplan Cancer Center, New York School of Medicine. Stat1^{-/-} mice were originally generated on a mixed C57Bl/6 genetic background [35] and were backcrossed for at least five generations onto the FVB/N background before use. Heterozygous knockout (KO) mice were then mated to generate Stat1^{-/-} and Stat1^{+/+} littermate control mice. Genotypes were determined by PCR analysis of DNA isolated from tail snips using the following primers: (ST11) 5'-GATATAATTACAAAATCAGAGAG-3', (ST12) 5'-CTGATCCAGGCAGGCGTTG-3', (ST13) 5'-TAATGTTTCATAGTTGGATATCAT-3' in a multiplex reaction utilizing 0.2 mM dNTPs and AmpliTaq DNA polymerase with Buffer I (Life Technologies, Grand Island, NY, USA) with an annealing temperature of 55°C and 33 amplification cycles to detect both wild-type and mutant products (450 and 150 bp, respectively). IFN γ R1 KO mice and C57BL/6 wild-type control mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

Preparation of Epidermal Protein Lysates and Western Blot Analyses

Groups of 3-4 mice were shaved on the dorsal skin and treated a total of four times with acetone vehicle (0.2 mL), 6.8 nmol of TPA (2x/week for 2 weeks) or 220 nmol CHRY (1x/week for 4 weeks) and then sacrificed at the indicated times. Preparation of epidermal protein lysates and Western blot analyses were performed as previously described [36,37].

Histological Analyses

To evaluate epidermal hyperplasia and LI, the dorsal skin of mice (3–4 mice per group) was shaved, and then topically treated with either 0.2 ml of acetone (vehicle), 6.8 nmol of TPA (2x/week for 2 weeks) or 220 nmol CHRY (1x for 4 weeks). Mice were sacrificed 48 hours after the last treatment. Thirty min prior to sacrifice, mice received an i.p. injection of BrdU (100 µg/g body weight) in phosphate-buffered saline. Dorsal skin samples were fixed in formalin, embedded in paraffin, and sectioned. Sections were cut and stained with hematoxylin and eosin or anti-BrdU antibody (Sigma-Aldrich). Epidermal thickness and labeling index (LI) were determined as described previously [36,38].

Two-Stage Skin Carcinogenesis

Groups of mice for each genotype were used for the two-stage skin carcinogenesis studies as follows: Wild-type, TPA (n=29); Stat1^{-/-}, TPA (n=24); wild-type, CHRY (n=24) and Stat1^{-/-}, CHRY (n=19). For initiation, the dorsal skin of mice was shaved and 48 hours later, all mice received a 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. The incidence and multiplicity of papillomas was recorded weekly until the average number of papillomas reached a plateau. Squamous cell carcinomas (SCCs) were recorded beginning at the time the first SCC was noted. All SCCs were histologically verified at the end of the experiment as described [33,39,40].

Adult Primary Keratinocyte Culture

Preparation and culture of adult mouse primary keratinocytes from Stat1^{-/-} and Stat1^{+/+} mice was performed as previously described [37,41]. Primary keratinocytes were serum starved for 24 hours prior to treatment with rIFN γ (100 ng/ml).

Nuclear and Cytoplasmic Fractionation

Primary keratinocytes from Stat1^{+/+} mice were serum starved for 24 hours, stimulated with rIFN γ (100 ng/ml) and subjected to cellular fractionation using an NE-PER Nuclear and Cytoplasmic Extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific).

Total RNA Isolation and Real-Time Quantitative PCR (RT-qPCR)

Epidermal scrapings from the dorsal skin of sacrificed mice were submerged in RNeasy lysis buffer and stored at 4°C for a minimum of 48 hours. Total RNA was isolated using the QIAGEN RNeasy RNA Isolation kit according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Life Technologies) and first strand synthesis using random hexamer primers (Invitrogen, Life Technologies) was used for cDNA preparation. RT-qPCR using SYBR Green dye PCR product incorporation and the primers listed in Supplemental Table 1 was performed on an Applied Biosystems Viia7 instrument (Applied Biosystems, Austin, TX, USA).

Statistical Analyses

For comparisons of quantitative Western blot data, epidermal thickness, epidermal LI and tumor multiplicity, the Mann-Whitney U-test was used ($p < 0.05$). For comparison of tumor incidence the Chi square (χ^2) test was used ($p < 0.05$).

Results

Examination of Stat1 Activation in Mouse Epidermis following Treatment with Tumor Promoters

We previously reported that treatment of mouse epidermis with TPA led to activation (i.e., tyrosine phosphorylation) of Stat1 [42]. To further evaluate Stat1 activity in response to treatment with diverse classes of tumor promoters, total Stat1 and Stat1 phosphorylation status was assessed following treatment with both TPA and CHRY. For these experiments, the dorsal skins of FVB/N mice were treated topically with either 0.2 mL acetone (vehicle), TPA (6.8 nmol) given 2x/week for 2 weeks, or CHRY (220 nmol) given 1x/week for 4 weeks. Mice were then sacrificed at various time points following the last treatment. As shown in Figure 1, topical application of mouse epidermis with both TPA and CHRY induced phosphorylation of Stat1 on both tyrosine (Y701) and serine (S727) residues (pStat1^{Y701} and pStat1^{S727}, respectively) at the earliest time point examined (6 hours) as well as later time points. 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 (compare Figures 1A and 1B) with a peak occurring approximately 48 hours after the last CHRY treatment. This increase in Stat1 protein level occurred at a time when little or no phosphorylation could be detected at either site. Thus, CHRY treatment led to the induction of unphosphorylated Stat1 (uStat1). No activation of Stat1 was observed in epidermis of Stat1^{-/-} mice as expected.

Further analyses revealed that the increase in uStat1 protein was due to an increase in Stat1 mRNA as shown in Supplemental Figure 1. Stat1 mRNA levels increased as early as 6 hours (the earliest time point examined) after treatment with CHRY with a peak at 12 hours.

Examination of IRF-1 Expression following Treatment with Tumor Promoters

In light of the data in Figure 1 showing induction of uStat1 following treatment of mouse epidermis with CHRY we examined the status of IRF-1. IRF-1 is an IFN γ /pStat1 responsive transcription factor that is known to regulate expression of a variety of genes, including uStat1 [43]. As shown in Figure 2A, treatment with CHRY but not TPA led to an increase in IRF-1 expression (both mRNA and protein). In addition, examination of Stat1^{-/-} mice showed that they were resistant to the induction of IRF-1 mRNA and protein following treatment with CHRY compared to wild-type FVB/N controls. Notably, treatment with TPA had no effect on the levels of IRF-1 mRNA and protein regardless of the genotype of the mice.

Role of IFN γ in the Upregulation of IRF-1 and uStat1 by CHRY

The data shown in Figures 1 and 2A suggested that CHRY treatment led to tyrosine phosphorylation of Stat1 via a different signaling pathway compared to TPA, possibly

involving IFN γ signaling. To further explore this hypothesis, we examined the expression of IFN γ following topical treatment with both tumor promoters. For these experiments, FVB/N mice were again treated using a multiple treatment protocol. Epidermal lysates were prepared and RNA isolated for qPCR as described in the Materials and Methods. As shown in Figure 2B, treatment with TPA did not induce epidermal IFN γ mRNA and, in fact, led to a statistically significant decrease at all time points examined. In contrast, treatment with CHRY led to a significant increase in epidermal IFN γ mRNA at 3, 6 and 12 hours after treatment with a peak at ~3 hours after the last treatment (~8-fold increase compared to the control mice treated with acetone).

As shown in Figure 2C, topical treatment with TPA did not induce IRF-1 mRNA in either wild-type or IFN γ R1 KO mice whereas treatment with CHRY induced a significant increase in epidermal IRF-1 mRNA in wild-type mice but not in epidermis of IFN γ R1 KO mice. Thus, CHRY treatment leads to activation of IFN γ signaling in the epidermis whereas this does not occur following treatment with TPA. We also examined the level of Stat1 mRNA following treatment with either promoter and found an induction of Stat1 mRNA in wild-type epidermis by 24 hours after CHRY treatment (Figure 2C). However, no statistically significant increase in Stat1 mRNA was observed in the IFN γ R1 KO mice at any of the time points examined after treatment with CHRY.

IFN γ Induces IRF-1 and uStat1 in Mouse Primary Keratinocytes

To demonstrate that mouse keratinocytes have an intact IFN γ R signaling pathway, primary mouse keratinocytes were treated with rIFN γ . Treatment of wild-type keratinocytes with rIFN γ (Figure 3A) led to a rapid increase in pStat1^{Y701} as early as 15 min following stimulation. rIFN γ stimulation also caused an increase in IRF-1 protein that was first evident at 30 min and persisted until the end of the experiment (24 hours). Total Stat1 (uStat1) also increased beginning as early as 3 hours and persisted throughout the duration of the experiment. In contrast, no upregulation of either IRF-1 or uStat1 protein was observed in primary keratinocytes from Stat1^{-/-} mice treated with rIFN γ as expected. As shown in Figure 3B, rIFN γ stimulation of wild-type keratinocytes led to an increase in nuclear pStat1^{Y701} at 30 min, which subsided but was still evident at the 18-hour time point. Interestingly, uStat1 remained localized to the nucleus 18 hours after stimulation. In addition, nuclear IRF-1 protein levels increased 30 min following treatment and remained localized in the nucleus at 18 hours.

Effect of Stat1 Deficiency on Tumor Promotion and Epithelial Carcinogenesis

To further determine the role of Stat1 signaling in tumor promotion and epithelial carcinogenesis, we compared wild-type mice with Stat1^{-/-} mice for their susceptibility to two-stage skin carcinogenesis using either TPA or CHRY as promoters. Groups of wild-type and Stat1^{-/-} mice were initiated with 25 nmol of DMBA and two weeks later promoted either twice-weekly with topical applications of 6.8 nmol TPA or once-weekly with topical applications of 220 nmol CHRY. As shown in Figure 4, Stat1 deficiency had no significant effect on two-stage skin carcinogenesis when TPA was used as the promoting agent. In this regard, there was no significant difference in the average number of papillomas or SCCs per

mouse (panels A and B, respectively) or tumor incidence (data not shown) in Stat1^{-/-} mice compared to wild-type controls ($p>0.05$; Mann-Whitney U-test).

In contrast to the results obtained with TPA as the promoter, Stat1^{-/-} mice were highly resistant to two-stage skin carcinogenesis when CHRY was used as the tumor promoter. In this regard, Stat1^{-/-} mice developed far fewer papillomas compared to wild-type controls (Figure 4). By week 31, Stat1^{-/-} mice had only developed an average of 0.16 papillomas/mouse compared to 4.52 papillomas/mouse in the control group. This difference was highly statistically significant ($p<0.0001$, Mann Whitney U-test). At the time of study termination, the papillomas that had developed in Stat1^{-/-} mice had not converted to SCCs, while the control mice had 0.66 SCCs per mouse (see again Figure 4).

We also compared the epidermal proliferative response following treatment with both tumor promoters in wild-type and Stat1^{-/-} mice. The proliferative response was assessed through measuring epidermal thickness and LI 48 hours after the last treatment with either TPA or CHRY as previously described [36,38]. As shown in Supplemental Figure 2, there were no significant differences in the proliferative response following treatment with TPA between wild-type and Stat1^{-/-} mice. In contrast, Stat1^{-/-} mice had a reduced proliferative response as seen in the reduced LI following treatment with CHRY at all three doses used (100, 220 and 440 nmol) and a reduction in epidermal thickness at the highest dose tested (see Supplemental Figure 3).

Effect of Stat1 Deficiency on Cyclooxygenase-2 (Cox-2) Expression and NF κ B Activation following Topical Treatment with TPA and CHRY

To further explore potential mechanisms whereby IFN γ /pStat1/IRF-1 signaling mediates skin tumor promotion by CHRY but not TPA, we examined the status of Cox-2 expression (both mRNA and protein) following topical treatment with TPA and CHRY in both wild-type and Stat1^{-/-} mice. As shown in Figure 5a, topical treatment with TPA led to an increase in Cox-2 expression (both mRNA and protein) as early as 6 hours after the last treatment that was similar in both wild-type and Stat1^{-/-} mice. In contrast, 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-hour time point compared to wild-type controls treated with CHRY (Figure 5B). The observed difference of Cox-2 levels in Stat1^{-/-} mice persisted until levels reached baseline values at 48 hours following treatment with CHRY. Experiments evaluating Cox-2 expression levels with both tumor promoters were repeated with very similar results (see Supplemental Figure 4A). As expected, Stat1^{-/-} mice treated with CHRY exhibited a decrease in epidermal prostaglandin E₂ (PGE₂) levels compared to wild-type mice, whereas no differences in epidermal PGE₂ levels were observed between the two genotypes treated with TPA (see Supplemental Figure 4B).

As shown in panel C of Figure 5, the reduced expression of Cox-2 in epidermis of Stat1^{-/-} mice observed following treatment with CHRY occurred in the presence of apparently normal NF κ B signaling as seen by similar levels of pNF κ B^{S536} in both genotypes. We also evaluated the expression of inducible nitric oxide synthase (iNos). As shown in Supplemental Figure 5A, topical treatment with TPA led to an increase in iNos mRNA levels at 6 hours, which quickly returned to basal levels at 12 hours following treatment. No

differences were seen in this response to TPA in Stat1^{-/-} mice compared to wild-type mice. In contrast, treatment with CHRY led to a significant increase in iNos mRNA levels in wild-type but not Stat1^{-/-} mice (Supplemental Figure 5B).

Discussion

This paper represents the first report that topical treatment with CHRY leads to upregulation of IFN γ and IFN γ R signaling in mouse epidermis. There are many different types of mouse skin tumor promoters and the similarities and differences between the different types and classes have been reviewed extensively [44-46]. A major difference between the anthrone type tumor promoters and the phorbol ester tumor promoters such as TPA is that the latter directly activate PKC isozymes. In contrast, anthrone tumor promoters such as CHRY do not directly activate PKC and undergo autooxidation to generate both reactive oxygen species (ROS) as well as carbon centered radicals as part of their mechanism of action [44,47]. Collectively, the current data demonstrate a novel mechanism of skin tumor promotion involving IFN γ /pStat1/IRF-1 signaling and upregulation of uStat1 in keratinocytes for anthrone skin tumor promoters of which CHRY is the most potent and prototypical member [48,49]. Notably, this pathway does not appear to play a major role in skin tumor promotion by TPA.

The possible role of IFN γ in skin tumor promotion by TPA has previously been explored with conflicting results. In earlier studies, Reiners and colleagues [50] reported that IFN γ acted as a co-promoter when injected i.p. together with topical application of TPA. IFN γ given alone (via i.p. injection) did not promote skin tumors in mice initiated with DMBA in these earlier studies. More recently, the role of IFN γ signaling in tumor promotion by TPA has been explored more directly using genetically engineered mouse models. In this regard, Xiao *et al.* reported that IFN 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 [51]. In addition, these authors reported that Stat1 was activated in protein lysates isolated from whole skin 24 hours following the last TPA treatment. In this same study, administration of an anti-IFN γ antibody or using IFN 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.* [52] reported that IFN γ ^{-/-} mice had a 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 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 of IFN γ as well as previous data showing that low doses of CHRY could also act as a co-promoter when given together with TPA [48]. Further work in this latter area would seem warranted.

As noted in the Introduction, several reports have indicated a possible pro-tumorigenic role for Stat1 [30,32,53] although other studies have supported a tumor suppressor role for this signaling molecule. In a previous study, Schreiber *et al.* reported that IFN γ R^{-/-} and Stat1^{-/-} mice were highly susceptible to tumor formation compared to 129/Sv control mice following a single subcutaneous injection of the carcinogen 3-methylcholanthrene (MCA)

[54]. In this study the authors also reported that Stat1^{-/-}/p53^{-/-} double KO mice developed tumors more rapidly and with greater frequency than p53^{-/-} mice, when challenged with MCA. Other studies have also shown that IFN γ plays an important role in immune surveillance for chemically induced tumors, including skin tumors [55-57]. In contrast, Hanada *et al.* [58] reported that mice deficient in suppressor of cytokine signaling-1 (SOCS1), a negative regulator of STAT signaling, developed spontaneous colorectal carcinomas dependent on IFN γ . In this study, IFN γ ^{-/-}/SOCS1^{-/-} mice failed to develop tumors regardless of the upregulation of Stat3 responsive genes such as Bcl-X_L and c-myc when compared to the SOCS1^{-/-} deficient mice. These and additional data suggested a critical role for the IFN γ /Stat1 signaling in the development of colorectal tumors in this mouse model. The current data clearly demonstrate an important role for Stat1 activation via IFN γ signaling in epidermis in mediating skin tumor promotion by CHRY. Perhaps Stat1 activation via IFN γ 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 have developed. Further ongoing studies using conditional Stat1 KO mice will help to address these ideas in more detail. In addition, identification of the cells in the epidermis that produce IFN γ in response to CHRY treatment is currently being pursued.

The mechanism(s) for how Stat1 mediates skin tumor promotion by CHRY remain to be fully determined. As shown in Supplemental Figure 3, Stat1^{-/-} 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 4). Stat1 has been shown to regulate the production of pro-inflammatory molecules such as iNos and Cox-2 [59,60]. Furthermore, IFN γ is also known to upregulate a variety of inflammatory mediators including tumor necrosis factor alpha [61], interleukin-12 [62], gp91^{phox} [63] and iNos [64,65]. 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) [60,66-68]. As shown in Figure 2, treatment with CHRY led to upregulation of IRF-1 that was blocked in both Stat1^{-/-} and IFN γ R1^{-/-} mice (Figures 2A and 2C, respectively). Both TPA and CHRY treatment led to upregulation of epidermal Cox-2 expression (both mRNA and protein; Figure 5). 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 (Supplemental Figure 5). 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 κ B (Figure 5C) 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 PGE₂ represent important events in the process of skin tumor promotion [69-71]. Therefore, pStat1/IRF-1 regulation of the induction of Cox-2 and 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 1). These results are consistent with recent studies showing that Stats 1 and 3 can function to regulate gene expression in the absence of tyrosine phosphorylation including regulating their own expression [72,73]. As a result, activation of Stat1 or Stat3 can lead to the induction and accumulation of uStats 1 and 3, which may persist for days after pStat levels have subsided [73]. In addition, induction of the Stat1 target gene, IRF-1, aids in the continued accumulation of uStat1 in response to IFN 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 pStats [72,74,75]. 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 [76]. Disruption of IRF-8 in soft tissue sarcoma cells leads to the accumulation of uStat1 and promotes sarcoma cell metastasis by regulating transcription of apoptosis regulators Fas and Bad [77]. Future studies are aimed at defining the role of uStat1 in skin tumor promotion by CHRY.

In conclusion, the current data identify a novel mechanism for skin tumor promotion involving activation of IFN γ signaling via pStat1 and IRF-1 that is required for the skin tumor promoting activity of CHRY, a member of the anthrone class of skin tumor promoters [44,49,78]. This mechanism does not appear to play a major role in skin tumor promotion by the phorbol ester, TPA. The mechanism(s) may involve the canonical pathway involving formation of pStat1 homodimers, which leads to direct transcriptional regulation of IRF-1 and uStat1. Alternatively, or in addition, this could involve a recently identified non-canonical pathway involving the translocation of a complex containing IFN γ R1-JAKS1/2-pStat1 to the nucleus where it binds to the IRF-1 promoter and induces transcription of IRF-1 [79,80]. 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 pStat1 and IRF-1 have been shown to interact with NF κ B [81,82], which may also play a role in altered expression of some genes. Figure 6 proposes a working model for the role of IFN γ /pStat1/IRF-1 signaling and uStat1 in skin tumor promotion by CHRY encompassing the various aspects discussed above. 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. Because the generation of ROS plays a significant role in ultraviolet radiation-mediated skin carcinogenesis [83-85] current studies are now focused on determining the possible involvement of this novel pathway in the promotion stage associated with UV-mediated non-melanoma skin carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Cancer Institute grant CA076520 (J. DiGiovanni) and the University of Texas M.D. Anderson Cancer Center Support grant CA016672.

References

1. Darnell JE Jr. STATs and gene regulation. *Science*. 1997; 277(5332):1630–1635. [PubMed: 9287210]
2. Catlett-Falcone R, Dalton WS, Jove R. STAT proteins as novel targets for cancer therapy. Signal transducer and activator of transcription Curr Opin Oncol. 1999; 11(6):490–496. [PubMed: 10550013]
3. Calo V, Migliavacca M, Bazan V, et al. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol*. 2003; 197(2):157–168. [PubMed: 14502555]
4. Bromberg J. Stat proteins and oncogenesis. *J Clin Invest*. 2002; 109(9):1139–1142. [PubMed: 11994401]
5. Akira S. Functional roles of STAT family proteins: lessons from knockout mice. *Stem Cells*. 1999; 17(3):138–146. [PubMed: 10342556]
6. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. *The Journal of biological chemistry*. 2007; 282(28):20059–20063. [PubMed: 17502367]
7. Kiu H, Nicholson SE. Biology and significance of the JAK/STAT signalling pathways. *Growth Factors*. 2012; 30(2):88–106. [PubMed: 22339650]
8. Pellegrini S, Dusanter-Fourt I. The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). *European journal of biochemistry / FEBS*. 1997; 248(3):615–633. [PubMed: 9342212]
9. Johnston PA, Grandis JR. STAT3 signaling: anticancer strategies and challenges. *Molecular interventions*. 2011; 11(1):18–26. [PubMed: 21441118]
10. Macias E, Rao D, Digiovanni J. Role of stat3 in skin carcinogenesis: insights gained from relevant mouse models. *Journal of skin cancer*. 2013; 2013 684050.
11. Greten FR, Weber CK, Greten TF, et al. Stat3 and NF-kappaB activation prevents apoptosis in pancreatic carcinogenesis. *Gastroenterology*. 2002; 123(6):2052–2063. [PubMed: 12454861]
12. Blando JM, Carbajal S, Abel E, et al. Cooperation between Stat3 and Akt signaling leads to prostate tumor development in transgenic mice. *Neoplasia*. 2011; 13(3):254–265. [PubMed: 21390188]
13. Benavides F, Blando J, Perez CJ, et al. Transgenic overexpression of PKCepsilon in the mouse prostate induces preneoplastic lesions. *Cell Cycle*. 2011; 10(2):268–277. [PubMed: 21224724]
14. Saini MK, Vaish V, Sanyal SN. Role of cytokines and Jak3/Stat3 signaling in the 1,2-dimethylhydrazine dihydrochloride-induced rat model of colon carcinogenesis: early target in the anticancer strategy. *Eur J Cancer Prev*. 2013; 22(3):215–228. [PubMed: 23514809]
15. O'Shea JJ, Holland SM, Staudt LM. JAKs and STATs in immunity, immunodeficiency, and cancer. *The New England journal of medicine*. 2013; 368(2):161–170. [PubMed: 23301733]
16. Kim DJ, Chan KS, Sano S, Digiovanni J. Signal transducer and activator of transcription 3 (Stat3) in epithelial carcinogenesis. *Mol Carcinog*. 2007; 46(8):725–731. [PubMed: 17610223]
17. Sano S, Chan KS, DiGiovanni J. Impact of Stat3 activation upon skin biology: a dichotomy of its role between homeostasis and diseases. *Journal of dermatological science*. 2008; 50(1):1–14. [PubMed: 17601706]
18. Chan KS, Sano S, Kiguchi K, et al. Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. *J Clin Invest*. 2004; 114(5):720–728. [PubMed: 15343391]
19. Kim DJ, Kataoka K, Rao D, Kiguchi K, Cotsarelis G, Digiovanni J. Targeted disruption of stat3 reveals a major role for follicular stem cells in skin tumor initiation. *Cancer Res*. 2009; 69(19):7587–7594. [PubMed: 19738054]

20. Chan KS, Sano S, Kataoka K, et al. Forced expression of a constitutively active form of Stat3 in mouse epidermis enhances malignant progression of skin tumors induced by two-stage carcinogenesis. *Oncogene*. 2008; 27(8):1087–1094. [PubMed: 17700521]
21. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004; 75(2):163–189. [PubMed: 14525967]
22. Gough DJ, Levy DE, Johnstone RW, Clarke CJ. IFN γ signaling-does it mean JAK-STAT? Cytokine & growth factor reviews. 2008; 19(5-6):383–394. [PubMed: 18929502]
23. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 2005; 5(5):375–386. [PubMed: 15864272]
24. Kamiya S, Owaki T, Morishima N, Fukai F, Mizuguchi J, Yoshimoto T. An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4⁺ T cells. *J Immunol*. 2004; 173(6):3871–3877. [PubMed: 15356135]
25. Lee CK, Gimeno R, Levy DE. Differential regulation of constitutive major histocompatibility complex class I expression in T and B lymphocytes. *J Exp Med*. 1999; 190(10):1451–1464. [PubMed: 10562320]
26. Meraz MA, White JM, Sheehan KC, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell*. 1996; 84(3):431–442. [PubMed: 8608597]
27. Kim HS, Lee MS. STAT1 as a key modulator of cell death. *Cellular signalling*. 2007; 19(3):454–465. [PubMed: 17085014]
28. Magkou C, Giannopoulou I, Theohari I, et al. Prognostic significance of phosphorylated STAT-1 expression in premenopausal and postmenopausal patients with invasive breast cancer. *Histopathology*. 2012; 60(7):1125–1132. [PubMed: 22320867]
29. Weichselbaum RR, Ishwaran H, Yoon T, et al. An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. *Proc Natl Acad Sci U S A*. 2008; 105(47):18490–18495. [PubMed: 19001271]
30. Kovacic B, Stoiber D, Moriggl R, et al. STAT1 acts as a tumor promoter for leukemia development. *Cancer cell*. 2006; 10(1):77–87. [PubMed: 16843267]
31. Zaidi MR, Merlino G. The two faces of interferon-gamma in cancer. *Clin Cancer Res*. 2011; 17(19):6118–6124. [PubMed: 21705455]
32. Schultz J, Koczan D, Schmitz U, et al. Tumor-promoting role of signal transducer and activator of transcription (Stat)1 in late-stage melanoma growth. *Clin Exp Metastasis*. 2010; 27(3):133–140. [PubMed: 20180146]
33. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc*. 2009; 4(9):1350–1362. [PubMed: 19713956]
34. Auterhoff H, Scherff FC. Dianthrone of pharmacologically important hydroxyanthraquinones. *Arch Pharm*. 1960; 293/65:918–925. [PubMed: 13685223]
35. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*. 1996; 84(3):443–450. [PubMed: 8608598]
36. Checkley LA, Rho O, Moore T, Hursting S, DiGiovanni J. Rapamycin is a potent inhibitor of skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Prev Res (Phila)*. 2011; 4(7):1011–1020. [PubMed: 21733825]
37. Kim DJ, Tremblay ML, Digiovanni J. Protein tyrosine phosphatases, TC-PTP, SHP1, and SHP2, cooperate in rapid dephosphorylation of Stat3 in keratinocytes following UVB irradiation. *PLoS One*. 2010; 5(4):e10290. [PubMed: 20421975]
38. Moore T, Beltran L, Carbajal S, Hursting SD, Digiovanni J. Energy balance modulates mouse skin tumor promotion through altered IGF-1R and EGFR crosstalk. *Cancer Prev Res (Phila)*. 2012; 5(10):1236–1246. [PubMed: 22896210]
39. Aldaz CM, Conti CJ, Chen A, Bianchi A, Walker SB, DiGiovanni J. Promoter independence as a feature of most skin papillomas in SENCAR mice. *Cancer Res*. 1991; 51(3):1045–1050. [PubMed: 1899044]

40. Kim DJ, Angel JM, Sano S, DiGiovanni J. Constitutive activation and targeted disruption of signal transducer and activator of transcription 3 (Stat3) in mouse epidermis reveal its critical role in UVB-induced skin carcinogenesis. *Oncogene*. 2009; 28(7):950–960. [PubMed: 19137019]
41. Lu J, Rho O, Wilker E, Beltran L, Digiovanni J. Activation of epidermal akt by diverse mouse skin tumor promoters. *Mol Cancer Res*. 2007; 5(12):1342–1352. [PubMed: 18171992]
42. Chan KS, Carbajal S, Kiguchi K, Clifford J, Sano S, DiGiovanni J. Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res*. 2004; 64(7):2382–2389. [PubMed: 15059889]
43. Nguyen H, Lin R, Hiscott J. Activation of multiple growth regulatory genes following inducible expression of IRF-1 or IRF/RelA fusion proteins. *Oncogene*. 1997; 15(12):1425–1435. [PubMed: 9333018]
44. DiGiovanni J. Multistage carcinogenesis in mouse skin. *Pharmacol Ther*. 1992; 54(1):63–128. [PubMed: 1528955]
45. Fischer, S. Chemical carcinogens and anticarcinogens. In: Bowden, GT.; Fischer, SM., editors. *Comprehensive Toxicology*. Vol. 12. New York: Elsevier Science; 1997. p. 349-381.
46. Rundhaug JE, Fischer SM. Molecular Mechanisms of Mouse Skin Tumor Promotion. *Cancers (Basel)*. 2010; 2(2):436–482. [PubMed: 21297902]
47. DiGiovanni J, Kruszewski FH, Coombs MM, Bhatt TS, Pezeshk A. Structure-activity relationships for epidermal ornithine decarboxylase induction and skin tumor promotion by anthrones. *Carcinogenesis*. 1988; 9(8):1437–1443. [PubMed: 3402040]
48. DiGiovanni J, Boutwell RK. Tumor promoting activity of 1,8-dihydroxy-3-methyl-9-anthrone (Chrysarobin) in female SENCAR mice. *Carcinogenesis*. 1983; 4:281–284. [PubMed: 6831635]
49. Kruszewski FH, Conti CJ, DiGiovanni J. Characterization of skin tumor promotion and progression by chrysarobin in SENCAR mice. *Cancer Res*. 1987; 47(14):3783–3790. [PubMed: 3109733]
50. Reiners JJ Jr, Rupp T, Colby A, Cantu AR, Pavone A. Tumor copromoting activity of gamma-interferon in the murine skin multistage carcinogenesis model. *Cancer Res*. 1989; 49(5):1202–1206. [PubMed: 2492903]
51. Xiao M, Wang C, Zhang J, Li Z, Zhao X, Qin Z. IFN γ promotes papilloma development by up-regulating Th17-associated inflammation. *Cancer Res*. 2009; 69(5):2010–2017. [PubMed: 19244111]
52. Wang L, Yi T, Zhang W, Pardoll DM, Yu H. IL-17 enhances tumor development in carcinogen-induced skin cancer. *Cancer Res*. 2010; 70(24):10112–10120. [PubMed: 21159633]
53. Timofeeva OA, Plisov S, Evseev AA, et al. Serine-phosphorylated STAT1 is a prosurvival factor in Wilms' tumor pathogenesis. *Oncogene*. 2006; 25(58):7555–7564. [PubMed: 16799645]
54. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 1998; 95(13):7556–7561. [PubMed: 9636188]
55. Shankaran V, Ikeda H, Bruce AT, et al. IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001; 410(6832):1107–1111. [PubMed: 11323675]
56. Mumm JB, Emmerich J, Zhang X, et al. IL-10 elicits IFN γ -dependent tumor immune surveillance. *Cancer cell*. 2011; 20(6):781–796. [PubMed: 22172723]
57. Wakita D, Chamoto K, Ohkuri T, et al. IFN- γ -dependent type 1 immunity is crucial for immunosurveillance against squamous cell carcinoma in a novel mouse carcinogenesis model. *Carcinogenesis*. 2009; 30(8):1408–1415. [PubMed: 19509217]
58. Hanada T, Kobayashi T, Chinen T, et al. IFN γ -dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. *J Exp Med*. 2006; 203(6):1391–1397. [PubMed: 16717119]
59. Ganster RW, Taylor BS, Shao L, Geller DA. Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF- κ B. *Proc Natl Acad Sci U S A*. 2001; 98(15):8638–8643. [PubMed: 11438703]

60. Blanco JC, Contursi C, Salkowski CA, DeWitt DL, Ozato K, Vogel SN. Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expression. *J Exp Med*. 2000; 191(12):2131–2144. [PubMed: 10859338]
61. Berner MD, Sura ME, Alves BN, Hunter KW Jr. IFN-gamma primes macrophages for enhanced TNF-alpha expression in response to stimulatory and non-stimulatory amounts of microparticulate beta-glucan. *Immunol Lett*. 2005; 98(1):115–122. [PubMed: 15790516]
62. Yoshida A, Koide Y, Uchijima M, Yoshida TO. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochem Biophys Res Commun*. 1994; 198(3):857–861. [PubMed: 7906942]
63. Almeida AC, Rehder J, Severino SD, Martins-Filho J, Newburger PE, Condino-Neto A. The effect of IFN-gamma and TNF-alpha on the NADPH oxidase system of human colostrum macrophages, blood monocytes, and THP-1 cells. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 2005; 25(9):540–546.
64. Coccia EM, Stellacci E, Marziali G, Weiss G, Battistini A. IFN-gamma and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. *International immunology*. 2000; 12(7):977–985. [PubMed: 10882409]
65. Chan ED, Riches DW. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol Cell Physiol*. 2001; 280(3):C441–450. [PubMed: 11171562]
66. Martin E, Nathan C, Xie QW. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J Exp Med*. 1994; 180(3):977–984. [PubMed: 7520478]
67. Leppanen T, Korhonen R, Laavola M, Nieminen R, Tuominen RK, Moilanen E. Down-regulation of protein kinase Cdelta inhibits inducible nitric oxide synthase expression through IRF1. *PLoS One*. 2013; 8(1):e52741. [PubMed: 23326354]
68. Fritsche G, Dlaska M, Barton H, Theurl I, Garimorth K, Weiss G. Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression. *J Immunol*. 2003; 171(4):1994–1998. [PubMed: 12902503]
69. Yamamoto S, Jiang H, Otsuka C, Kato R. Involvement of prostaglandin E2 in ornithine decarboxylase induction by a tumor-promoting agent, 7-bromomethylbenz[a]anthracene, in mouse epidermis. *Carcinogenesis*. 1992; 13(5):905–906. [PubMed: 1587007]
70. Fischer SM, Pavone A, Mikulec C, Langenbach R, Rundhaug JE. Cyclooxygenase-2 expression is critical for chronic UV-induced murine skin carcinogenesis. *Mol Carcinog*. 2007; 46(5):363–371. [PubMed: 17219415]
71. Ansari KM, Rundhaug JE, Fischer SM. Multiple signaling pathways are responsible for prostaglandin E2-induced murine keratinocyte proliferation. *Mol Cancer Res*. 2008; 6(6):1003–1016. [PubMed: 18567804]
72. Yang J, Stark GR. Roles of unphosphorylated STATs in signaling. *Cell research*. 2008; 18(4):443–451. [PubMed: 18364677]
73. Cheon H, Yang J, Stark GR. The functions of signal transducers and activators of transcriptions 1 and 3 as cytokine-inducible proteins. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 2011; 31(1):33–40.
74. Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J*. 2000; 19(15):4111–4122. [PubMed: 10921891]
75. Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. *Genes Dev*. 2007; 21(11):1396–1408. [PubMed: 17510282]
76. Cheon H, Stark GR. Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes. *Proc Natl Acad Sci U S A*. 2009; 106(23):9373–9378. [PubMed: 19478064]
77. Zimmerman MA, Rahman NT, Yang D, et al. Unphosphorylated STAT1 promotes sarcoma development through repressing expression of Fas and bad and conferring apoptotic resistance. *Cancer Res*. 2012; 72(18):4724–4732. [PubMed: 22805310]

78. DiGiovanni J, Boutwell RK. Tumor promoting activity of 1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin) in female SENCAR mice. *Carcinogenesis*. 1983; 4(3):281–284. [PubMed: 6831635]
79. Ahmed CM, Johnson HM. IFN-gamma and its receptor subunit IFNGR1 are recruited to the IFN-gamma-activated sequence element at the promoter site of IFN-gamma-activated genes: evidence of transactivational activity in IFNGR1. *J Immunol*. 2006; 177(1):315–321. [PubMed: 16785527]
80. Johnson HM, Noon-Song EN, Kemppainen K, Ahmed CM. Steroid-like signalling by interferons: making sense of specific gene activation by cytokines. *Biochem J*. 2012; 443(2):329–338. [PubMed: 22452815]
81. Sgarbanti M, Remoli AL, Marsili G, et al. IRF-1 is required for full NF-kappaB transcriptional activity at the human immunodeficiency virus type 1 long terminal repeat enhancer. *Journal of virology*. 2008; 82(7):3632–3641. [PubMed: 18216101]
82. Ohmori Y, Schreiber RD, Hamilton TA. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem*. 1997; 272(23):14899–14907. [PubMed: 9169460]
83. Nishigori C. Cellular aspects of photocarcinogenesis. *Photochem Photobiol Sci*. 2006; 5(2):208–214. [PubMed: 16465307]
84. Nishigori C, Hattori Y, Toyokuni S. Role of reactive oxygen species in skin carcinogenesis. *Antioxidants & redox signaling*. 2004; 6(3):561–570. [PubMed: 15130282]
85. Chen AC, Halliday GM, Damian DL. Non-melanoma skin cancer: carcinogenesis and chemoprevention. *Pathology*. 2013; 45(3):331–341. [PubMed: 23478234]

Abbreviations

IFNγ	interferon gamma
Stat1	signal transducer and activator of transcription-1
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
CHRY	(chrysarobin), 3-methyl-1,8-dihydroxy-9-anthrone
uStat1	unphosphorylated Stat1
IRF-1	interferon regulatory factor 1
DMBA	7,12-dimethylbenz(a)anthracene
BrdU	bromodeoxyuridine
rIFNγ	recombinant IFN γ
KO	knockout
LI	labeling index
SCC(s)	squamous cell carcinomas
Cox-2	cyclooxygenase-2
PGE₂	prostaglandin E ₂
iNos	inducible nitric oxide synthase
MCA	3-methylcholanthrene
SOCS1	suppressor of cytokine signaling-1

Figure 1. Analysis of Stat1 phosphorylation and protein level following tumor promoter treatment

Stat1^{+/+} or 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). Epidermal lysates were prepared at the indicated time points for protein analysis by Western blot using antibodies specific for the indicated proteins. Protein level was quantitated by densitometry. β-Actin was used to normalize protein loading. Western blots in panels A and B are from a single representative experiment using pooled protein samples from groups of 3-5 mice. All

quantitative values (given as mean $\pm$ SEM) are relative to the acetone control group (given a value of 1). For some time points where error bars are not shown, the value represents an average of only two independent experiments whereas all time points with error bars represent an average from at least three independent experiments. * Indicates value was significantly different from the acetone control group (Mann-Whitney U-test, $p < 0.05$).

Figure 2. Examination of the IFN γ /pStat1/IRF-1 signaling axis in response to tumor promoter treatment

Stat1^{+/+} or Stat1^{-/-} mice received either TPA (6.8 nmol 2x/week for 2 weeks) or 220 nmol CHRY (1x/week for 4 weeks) in 0.2 mL acetone (Ace). (A) 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. β -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 is presented as mean $\pm$ SD and was obtained from individual mice (n=3-5/group).

*Indicates values between Stat1^{+/+} and Stat1^{-/-} groups were significantly different (Mann-Whitney U-test, $p < 0.05$). (B) IFN γ mRNA levels following topical application of TPA and CHRY to FVB/N wild-type mice. Values shown represent the mean $\pm$ SEM. † Indicates value was significantly different than that of the acetone control group. (C) Epidermal IRF-1 and Stat1 mRNA levels following topical application of TPA and CHRY to IFN γ R1 KO and wild-type mice. Values shown are the mean $\pm$ SD. †Indicates values significantly different from the acetone control group ($p < 0.05$); *indicates values between KO and wild-type mice were significantly different (Mann-Whitney U-test, $p < 0.05$).

Figure 3. rIFN γ induces IRF-1 and uStat1 in primary keratinocytes

Primary keratinocytes were isolated from adult Stat1^{+/+} and Stat1^{-/-} mice as described in the Materials and Methods section. **(A)** Serum starved primary keratinocytes were stimulated with rIFN γ (100 ng/ml) and protein lysates were prepared for Western Blot analysis at the indicated time points. **(B)** Stat1^{+/+} primary keratinocytes were treated with rIFN γ as above and cellular fractionation was conducted as described in the Materials and Methods section. Western blot analyses were performed on protein lysates prepared from cytoplasmic and nuclear fractions. Lamin A/C and GAPDH were used as nuclear and cytoplasmic loading controls, respectively.

Figure 4. Impact of Stat1 deficiency on skin tumor promotion

Groups of mice of each genotype (Stat1^{+/+} or Stat1^{-/-}) were initiated with 25 nmol DMBA; two weeks after initiation, mice were treated topically 2x/week with 6.8 nmol TPA or 1x/week with 220 nmol CHRY until the tumor response reached a plateau. Significant differences in tumor multiplicity between groups were analyzed by the Mann-Whitney U-test. *Indicates value is significantly different than acetone control (p<0.05).

Figure 5. Impact of Stat1 deficiency on Cox-2 induction following treatment with TPA and CHRY

Stat1^{+/+} or Stat1^{-/-} mice received topical applications of either acetone (Ace) or 6.8 nmol TPA given 2x/week for 2 weeks, or 220 nmol CHRY given 1x/week for 4 weeks and were sacrificed at the indicated time points. Epidermal lysates were prepared for both Western blot and qPCR analysis. (A) Cox-2 expression following TPA treatment. (B) Cox-2 expression following CHRY treatment. (C) NFκB status following treatment with CHRY. Western blot data are from a single experiment (pooled protein samples) that has been

repeated with very similar results. The mRNA data is presented as the average $\pm$ SD from individual mice (n=5/group). *Indicates values between Stat1^{+/+} and Stat1^{-/-} groups were significantly different by Mann-Whitney U-test (p<0.05).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Working model for the role of IFN γ /pStat1/IRF-1 signaling and uStat1 in skin tumor promotion by CHRY

Treatment of mouse skin with CHRY leads to the upregulation of IFN γ in the epidermis. The actual cellular source of the IFN γ produced in the epidermis after treatment with CHRY is not known at the present time. Increased levels of IFN γ lead to activation of its receptor on keratinocytes and subsequent downstream signaling events. Because both TPA and CHRY cause an initial activation (i.e., phosphorylation) of Stat1, the subsequent induction of IRF-1 and uStat1 must occur through a process unique to this pathway. Several mechanisms could explain this difference and may involve both the canonical (pStat1

homodimers) as well as a novel non-canonical pathway involving the translocation of a complex containing IFN γ R1/Jak2/pStat1 to the nucleus where it binds to the IRF-1 promoter and induces transcription of IRF-1. 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. Both pStat1 and IRF-1 have been shown to interact with NF κ B, which may also play a role in altered expression of some genes.