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IFNγ induces DNA methylation-silenced GPR109A expression via pSTAT1/p300 and H3K18 acetylation in colon cancer

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Abstract

Short-chain fatty acids, metabolites produced by colonic microbiota from fermentation of dietary fiber, act as anti-inflammatory agents in the intestinal tract to suppress proinflammatory diseases. GPR109A is the receptor for short-chain fatty acids. The functions of GPR109A has been the subject of extensive studies, however, the molecular mechanisms underlying GPR109A expression is largely unknown. We show that GPR109A is highly expressed in normal human colon tissues, but is silenced in human colon carcinoma cells. The *GPR109A* promoter DNA is methylated in human colon carcinoma. Strikingly, we observed that IFNγ, a cytokine secreted by activated T cells, activates *GPR109A* transcription without altering its promoter DNA methylation. Colon carcinoma grows significantly faster in IFNγ-deficient mice than in wildtype mice in an orthotopic colon cancer mouse model. A positive correlation was observed between GPR109A protein level and tumor-infiltrating T cells in human colon carcinoma specimens, and IFNγ expression level is higher in human colon carcinoma tissues than in normal colon tissues. We further demonstrated that IFNγ rapidly activates pSTAT1 that binds to the promoter of *p300* to activate its transcription. p300 then binds to the *GPR109A* promoters to induce H3K18 hyperacetylation, resulting in chromatin remodeling in the methylated *GPR109A* promoter. The IFNγ-activated pSTAT1 then directly binds to the methylated but hyperacetylated *GPR109* promoters to activate its transcription. Overall, our data indicate that GPR109A acts as a tumor suppressor in colon cancer and the host immune system might use IFNγ to counteract DNA methylation-mediated *GPR109A* silencing as a mechanism to suppress tumor development.

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Keywords

IFNγ; p300; STAT1; GPR109A; acetylated H3K18; DNA methylation

Introduction

Short-chain fatty acids, metabolites produced by colonic bacteria from fermentation of dietary fiber, are the essential energy source for colonocytes. Without these short-chain fatty acids for energy, colonocytes undergo autophagy and die. In addition, short-chain fatty acids, such as butyrate, also act as a histone deacetylase (HDAC) inhibitor that mediates both normal and cancerous colonocyte proliferation (1), and anti-inflammatory response in the intestinal tract (2). GPR109A is a G-protein-coupled receptor for short-chain fatty acids (2–6). It has been shown that inhibition of DNA methylation increases GPR109A expression in human colon carcinoma cells, suggesting that *GPR109A* is silenced by DNA methylation (2). Although the function of GPR109A has been extensively studied in normal cells and cancer cells, the molecular mechanisms underlying GPR109A expression is unknown.

IFNγ is a proinflammatory cytokine secreted primarily by activated T cells (7). IFNγ functions through signal transducer and activator of transcription 1 (STAT1) to regulate the expression of its target genes. It has been reported that IFNγ secretion is elevated in the peripheral blood (8) and IFNγ expression level is increased in the inflamed colonic mucosa tissues in patients with ulcerative colitis (UC) (9). The expression and activation level of STAT1 is also significantly increased in colonic tissues of UC patients (10). Furthermore, chronic IFNγ signaling increases Cox-2 and iNOS expression to promote inflammation-dependent spontaneous colon cancer development (11). These observations thus suggest that chronic IFNγ signaling plays a key role in human UC pathogenesis and in inflammation-dependent spontaneous colorectal cancer development (8, 9, 11–13). However, it is apparent that the IFNγ signaling pathway is a two-edged sword. Although chronic IFNγ signaling promotes inflammation-dependent colon cancer development, the best known function of IFNγ in the tumor microenvironment is tumor suppression (7, 14–16).

We report here a novel mechanism underlying the regulation of *GPR109A* expression in colon cancer cells. Our data reveal that although the *GPR109* promoter is methylated in human colon carcinoma cells, exposure of tumor cells to IFNγ reverses DNA methylation-mediated *GPR109A* silencing both *in vitro* and *in vivo* without altering the methylation status of the *GPR109A* promoter.

Materials and Methods

Human cell lines and tissue specimens

Human colon cancer cell lines SW480, SW620, SW116 and T84, and mouse colon carcinoma cell line CT26 were obtained from American Type Culture Collection (ATCC) (Manassas, VA). ATCC has characterized these cells by morphology, immunology, DNA fingerprint, and cytogenetics. De-identified human colon carcinoma specimens were obtained from the Georgia Regents University Medical Center and University Hospital with

approval by the Georgia Regents University and University Hospital Human Assurance Committees.

Mouse tumor model

IFNγ KO mice (129S7(B6)-Ifngtm1Ts/J) and age-matched WT control mice (BALB/cJ) were obtained from the Jackson Laboratory. Mice were anesthetized under constant flow of oxygen and isofluorane. A small abdominal incision was made to expose the cecum. Tumor cells ($1x10^4$ cells in 20 μl saline) were injected into the cecal wall on the serosal side. The wound was sealed with a wound clip. The use of mice and surgery procedures was approved by Georgia Regents University Institutional Animal Care and Use Committee.

RT-PCR analysis

Total RNA was isolated from cells using Trizol (Invitrogen, San Diego, CA) according to the manufacturer's instructions, and used for the first strand cDNA synthesis using the MMLV reverse transcriptase (Promega, Madison, WI). The cDNA was then used as the template for PCR amplification. RT-PCR was conducted as previously described (17). The sequences of primers are listed in Table S1.

Analysis of gene expression with RT-PCR array

Total RNA was isolated from freshly dissected human colon carcinoma specimens and matched adjacent normal colon tissues. RNAs from 3 patients were pooled and used for cDNA probe preparation using the RT2 First Strand Kit (Cat# 330401 Qiagen). The Human Inflammasomes PCR Arrays (Qiagen, Cat# PAHS-097Z) were used to analyze the inflammation-related gene expression using real-time RT-PCR according to the manufacturer's instructions.

Western blot analysis

Western blotting analysis was performed as previously described (16). The blot was probed with antibodies specific for pSTAT1 (Cat# 612133, BD Biosciences, San Diego, CA), H3K9ac (Cat# 9649, Cell Signaling, Danvers, MA) H3K18Ac (Cat# 9675, Cell Signaling), H3K27ac (Cat#4753, Cell signaling), H3 (Cat#4499, Cell Signaling), p300 (Cat# sc-584, Santa Cruz Biotech, Santa Cruz, CA), GPR109A (Cat#sc-134583, Santa Cruz Biotech) and β-actin (Cat# A1978, Sigma-Aldrich, St Louis, MO).

Cell treatment

For demethylation of DNA, cells were treated for 3 days with 5′-aza-deoxycytidine (Sigma) at a final concentration of 1 μg/ml. For IFNγ treatment, cells were cultured in the presence of recombinant IFNγ (R & D Systems, Minneapolis, MN) at a final concentration of 100 U/ml.

DNA methylation analysis

Genomic DNA was purified using DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Sodium bisulfite treatment of genomic DNA was carried out using DNA Modification Kit (Zymo Research) according to the manufacturer's instructions.

Methylation-sensitive PCR (MS-PCR) and DNA sequencing were carried out as previously described (18). For DNA sequencing, the bisulfite-modified genomic DNA was used as the template for PCR amplification of the human *GPR109A* promoter region. The amplified DNA fragments were cloned into pCR2.1 vector (Invitrogen), and individual clones were sequenced. DNA methylation was analyzed using QUMA program as previously described (19). All primer sequences are listed in Table S1.

Immunohistochemistry

Immunohistochemical staining was performed at the Georgia Pathology Service. CD4– and CD8-specific antibodies were obtained from Dako (Cat# IS649 and IS623, DAKO, Carpinteria, CA). GPR109A-specific antibody has been previously described (20). Stained tissue specimens were evaluated independently by two pathologists (CMH and JRL). For the CD4 and CD8 immunohistochemical slides, each pathologist scored the density of peritumoral lymphocytes In addition, the percentage of tumor staining was documented.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using anti-pSTAT1 antibody (Cat#sc-8394, Santa Cruz), anti-HDAC1 (Cat#sc-7872, Sant Cruz) and protein A-agarose beads (Millipore) as previously described (16). The *GPR109A* promoter DNA was detected by PCR using gene-specific primers (Table S1).

Protein-DNA interaction assay

DNA-protein interaction was determined by electrophoresis mobility shift assay (EMSA) as previously described (19). The probe sequences are listed in Table S1.

Gene silencing

T84 cells were transiently transfected with scrambled siRNA (Dharmacon, Lafayette, CO), p300-specific siRNA (Cat# sc-29431, Santa Cruz), and STAT1-specific siRNA (Qiagen, cat# SI02662324, SI02662884) respectively, using lipofectamine 2000 (Invitrogen) overnight.

Chromatin remodeling assay

Cells ($5x10^6$) were harvested and washed in cold PBS, and then re-suspended in nuclear extraction buffer (25 mM Tris-HCl pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 8% glycerol, 0.5% NP-40, and protease/phosphatase inhibitors) and incubated on ice for 10 min. Then the cells were homogenized in a glass homogenizer, and spun down at 1000g for 5 min. Cell pellets were washed once with DNaseI buffer, spun down at 1000g for 5 min and then washed once with nucleus storage buffer (NSB: 50% glycerol, 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 2.5 mM DTT, 0.1 mM EDTA, Protease/phosphatase inhibitors). Endonuclease digestion was performed in a reaction mixture containing 320 μl of nuclei in NSB, 480 μl of 2x Nuclease buffer (30 mM NaCl, 8 mM $CaCl_2$), 160 μl Tris buffer (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$) and DNaseI (0.5 Unit/ml) (Roche Applied Science). Before the addition of DNaseI, an aliquot was removed from the reaction mixture for the zero time point. After addition of DNaseI, the digestion mixture was incubated at 37°C. Aliquots were taken out of the

digestion mixture at 3, 6, 9 and 12 min after DNaseI addition, mixed immediately with 500 μl of chilled phenol:choloroform:isoamyl alcohol (25:24:1) mixture, vortexed and spun down at 14000 rpm for 10 min. DNA was collected from the top aqueous layer, purified, and analyzed by PCR. Human *GPR109A* promoter-specific primers were used to amplify the purified genomic DNA.

Statistical analysis

Statistical analysis was performed using Student's *t* test. A $P < 0.05$ was taken as statistically significant.

Results

GPR109A is silenced by DNA methylation in human colon carcinoma

Analysis of GPR109A mRNA levels from matched normal colon tissues and colon carcinoma tissues indicates that *GPR109A* is highly expressed in normal human colon epithelial cells but is silenced in human colon carcinoma cells. GPR109A expression is approximately 1.4-fold to 150-fold higher in the normal colon tissues as compared to that in the matched colon carcinoma tissues among the six matched pairs of tissues analyzed (Fig 1A). To determine whether the *GPR109A* promoter is methylated *in vivo* in colon carcinoma, genomic DNA was isolated from human colon carcinoma tissues of five colon cancer patients. MethPrimer program was used to design MS-PCR primers and MS-PCR analysis of the bisulfite-modified genomic DNA indicated that the *GPR109A* promoter is methylated in all 5 colon cancer specimens examined (Fig. 1B). To determine whether *GPR109A* expression can be re-activated by inhibition of DNA methylation, tumor cells were treated with 5′-aza-deoxylcytidine (Aza-dC) and analyzed for *GPR109A* expression. Consistent with the heavy DNA methylation level, Aza-dC treatment dramatically increased *GPR109A* expression in human colon carcinoma cells in a dose-dependent manner (Fig. 1C).

Inhibition of DNA methylation up-regulates *GPR109A* expression in human colon carcinoma cells (2). However, DNA sequence analysis indicates that there are no classical CpG islands in the human *GPR109A* gene promoter region (Fig. 1D). To determine whether DNA methylation directly regulates *GPR109A* expression, we analyzed the methylation level of the *GPR109A* promoter. Genomic DNA was isolated from human colon carcinoma SW116 and T84 cells and modified with bisulfite. Bisulfite sequencing PCR primers were designed using the MethPrimer program and were used to amplify one region of the *GPR109A* promoter. The amplified region was cloned. Single clones were then sequenced and examined for DNA methylation level. It is clear that the majority of cytosines of the CpG dinucleotides in this region are methylated in SW116 and T84 cells (Fig. 1D). Taken together, these data demonstrate that *GPR109A* is silenced in human colon carcinoma cells by methylation of its promoter DNA.

STAT1 directly binds to the *GPR109A* promoter region to activate transcription from the methylated promoter

While analyzing the inflammation-related gene expression in colon carcinoma cells, we observed, surprisingly, that *GPR109A* was up-regulated by IFNγ, a proinflammatory cytokine secreted by activated T cells. RT-PCR analysis of four human colon carcinoma cell lines revealed that *GPR109A* was dramatically up-regulated by IFNγ despite its promoter DNA being methylated (Fig. 2A). Analysis of *GPR109A* expression kinetics revealed that *GPR109A* mRNA starts to increase 3–6 h after IFNγ treatment and continued to increase even 24 h after the treatment (Fig. 2A). Furthermore, IFNγ at a concentration as low as 10 u/ml can re-regulate GPR109A expression (Fig. 2A). Flow cytometry analysis revealed that IFNγR is expressed on the cell surface of all 4 cancer cell lines examined (Fig. 2B). Western blotting analysis indicated that pSTAT1, the key mediator of the IFNγ signaling pathway, is rapidly activated by IFNγ through phosphorylation in human colon carcinoma cells. pSTAT1 was detected as early as 10 min after IFNγ treatment (Fig. 2C). Analysis of the human *GPR109A* gene promoter revealed the presence of two gamma activation sequence (GAS) elements in the region (Fig. 2D). ChIP analysis with pSTAT1-specific mAb showed that IFNγ-activated pSTAT1 is associated with both GAS sites in the *GPR109A* promoter chromatin (Fig. 2D). Furthermore, EMSA indicated that pSTAT1 binds to the GAS DNA element directly (Fig. 2E). IFNγ functions through both STAT1-dependent and STAT1-independentl signaling pathways (21). To determine whether IFNγ-induced GPR109A expression depends on STAT1, STAT1 was silenced using specific siRNAs in human colon carcinoma T84 cells. Analysis of GPR109A expression revealed that silencing STAT1 diminished IFNγ-induced GPR109A expression (Fig. S1). These observations thus indicate that IFNγ up-regulates *GPR109A* expression through direct binding of pSTAT1 to the methylated *GPR109A* promoter DNA to activate its transcription.

To determine whether other cytokines induce GPR109A expression, T84 cells were treated with IFNα, IFNβ, TNFa, IL23 and GM-CSF for 24h, and analyzed for GPR109A expression. None of these cytokines up-regulate GPR109A in human colon carcinoma cells (Fig. S2).

IFNγ regulates *GPR109A* expression in colon carcinoma cells *in vivo*

IFNγ is primarily produced by activated T cells *in vivo*. To determine the expression levels of *GPR109A* and T-cell infiltration in human colon carcinoma, we analyzed 18 human colon carcinoma specimens using immunohistochemical staining with GPR109A-, CD4-, and CD8-specific antibodies. The stained tissues were then analyzed by two pathologists (CMH and JRL). The images are presented in Figure 3 and Figures S3–5. The staining scores are presented in Table S2. Four colon carcinoma specimens (Patients 7, 9, 10 and 13) showed no detectable GPR109A protein level. Tumor tissues from two of these four patients (Patients 7 and 9) also had minimal to no $CD4^+$ and $CD8^+$ T-cell infiltrations. However, tumor specimen from patient 10 had moderate to abundant T-cell filtration, but GPR109 protein levels were low to non-detectable in the tumor cells. In the tumor tissues from the remaining fourteen patients, either or both $CD4^+$ and $CD8^+$ T cells were present inside the tumors, and GPR109A protein was detectable in the tumor cells. These observations indicate that although the *GPR109A* promoter DNAs is methylated in human colon carcinoma cells

(Figs. 1), GPR109A is expressed in most of the human colon carcinoma cells (with the exception of samples from Patients 10 and 13) if $CD4^+$ or $CD8^+$ T cells are present in the tumor microenvironment (Table S2).

To determine the inflammation gene expression profiles, we analyzed the expression levels of inflammation-related genes in human colon carcinomas and matched adjacent normal colon tissues using OCR arrays. Real-time RT-PCR analysis identified 41 inflammation-related genes that are differentially expressed in normal colon tissues and in colon carcinoma tissues. Among these 41 genes, the expression level of IFNγ is about 4 times higher in carcinoma tissues than in the adjacent normal colon tissues (Table S3).

To determine functionally whether IFNγ activates *GPR109A* transcription from the methylated promoter under pathophysiologic conditions, we established mouse colon carcinoma CT26 cell orthotopic transplant models in WT and IFNγ KO mice. The *gpr109a* promoter is methylated in CT26 cells (Fig. 4A); however, IFNγ treatment dramatically increased *gpr109a* mRNA levels in CT26 cells *in vitro* (Fig. 4A). A low-dose of CT26 ($1x10^4$ cells/mouse) and a short tumor growth time (21 days) were used in this study to unmask the difference between wt and IFNγ KO mice. When surgically implanted into mouse cecum, CT26 tumors grew much faster in IFNγ KO mice than in WT mice (Fig. 4B). Analysis of the dissected tumor tissues revealed that mRNA levels of *gpr109a* is significantly higher in tumors grown in WT mice than that in IFNγ KO mice (Fig. 4C). Our data thus demonstrate that IFNγ activates *gpr109a* transcription from their methylated promoters *in vivo*.

IFNγ does not alter *GPR109A* promoter DNA methylation

IFNγ has been shown to induce DNA demethylation (22). To determine whether IFNγ up-regulates *GPR109A* expression through inhibition of DNA methylation, human colon carcinoma cells were cultured in the absence or presence of IFNγ for 24 h and the DNA methylation level in the pSTAT1-binding consensus regions (Fig. 2D) of the *GPR109A* promoter was analyzed. DNA sequencing analysis indicated that the *GPR109A* promoter DNA is still methylated after IFNγ treatment in human colon carcinoma cells (Fig. 5).

Inhibition of HDACs enhances IFNγ-mediated *GPR109A* up-regulation

Promoter DNA methylation of CpG islands often causes chromatin condensation to block transcription factor binding to the DNA, thereby silencing gene expression (23). However, histone acetylation may mediate the switch between repressive and permissive chromatin and thus dictate the functional state of genes (24–26). Our above observations that IFNγ up-regulates *GPR109A* expression from the methylated *GPR109A* promoter without altering the promoter DNA methylation level suggest that histone acetylation might play a role in IFNγ-mediated GPR109A expression. Therefore, we hypothesized that inhibition of HDAC activity may enhance *GPR109A* transcription activation by IFNγ. To test this hypothesis, human colon carcinoma cells were treated with either TSA or IFNγ, or both TSA and IFNγ together, and analyzed for *GPR109A* expression. RT-PCR analysis revealed that either TSA or IFNγ treatment dramatically increased *GPR109A* expression (Fig. 6A). However,

combined TSA and IFNγ treatment resulted in an even greater up-regulation of *GPR109A* than either agent alone (Fig. 6A).

It is known that HDAC1 mediates histone acetylation level in many gene promoters (27). Our observation that inhibition of HDAC activity dramatically increased IFNγ-induced *GPR109A* expression in human colon carcinoma cells led us to reason that IFNγ might regulate *GPR109A* expression by repressing HDAC1 expression. To test this hypothesis, we analyzed HDAC1 association with the *GPR109A* promoter. ChIP analysis showed that HDAC1 is associated with the chromatin at one of the two regions analyzed at the *GPR109A* promoter region (Fig. 6A). Consistent with the observation that HDAC1 expression is not regulated by IFNγ, which also did not alter HDAC1 association with the *GPR109A* promoter chromatin (Fig. 6A).

p300 is the immediate early target of the IFNγ signaling pathway

Histone acetyltransferases (HAT) and HDACs antagonize each other through modification of the lysine side chains (27, 28). p300 is a HAT that often acts as a transcription co-factor (29–33). For example, p300 can mediate H3K18/27 acetylation (34–37). Therefore, we sought to determine whether IFNγ mediates p300 expression to regulate GPR109A expression. RT-PCR analysis revealed that IFNγ treatment indeed rapidly up-regulates p300 expression level in human colon carcinoma cells. The increase in p300 mRNA and protein levels started 10 min after IFNγ treatment and reached a plateau at 6 h (Fig. 6B). Analysis of the human *p300* gene promoter region identified two GAS sites (Fig. 6C), and ChIP analysis indicted that IFN-γ-activated pSTAT1 is associated with one of the two GAS sites at the *p300* promoter region in human colon carcinoma cells (Fig. 6C). Consistent with the ChIP results, EMSA indicated that pSTAT1 directly and specifically binds to the GAS DNA of the *p300* promoter (Fig. 6D). Taken together, our data indicated that *p300* is an immediate early target of the IFNγ signaling pathway in human colon cancer cells.

IFNγ activates *GPR109A* transcription through p300 association with its promoter

To determine whether p300 directly mediates IFNγ regulation of *GPR109A* transcription, we first analyzed p300 association with the *GPR109A* promoter chromatin. ChIP analysis revealed that IFNγ treatment dramatically increased p300 association with the *GPR109A* promoter chromatin (Fig. 6E). Next, we sought to determine whether p300 mediates *GPR109* expression. *p300* was silenced with *p300*-specific siRNA in human colon carcinoma cells, and *GPR109A* expression levels were then analyzed. Real-time RT-PCR analysis revealed that silencing *p300* diminished IFNγ-induced *GPR109A* (Fig. 6F). Thus, we conclude that p300 directly mediates pSTAT1-activated *GPR109A* transcription in human colon carcinoma cells.

IFNγ increases p300 expression to increase H3K18 hyperacetylation

Although p300 acetylates multiple lysine residues in both H3 and H4 (37), p300 has been shown to specifically mediate H3K18/27 acetylation (34, 36). We therefore analyzed the effects of IFNγ on H3K18/27 acetylation. H3K9ac was also included as a positive control. IFNγ treatment increased the global acetylation levels of H3K9, H3K18 and H3K27 in human colon cancer cells (Fig. 7A). However, repeated ChIP analysis revealed that H3K27

is not acetylated in the *GPR109A* promoter regions and that IFNγ treatment does not induce acetylation of H3K27 in the *GPR109A* promoter region in human colon cancer cells (Fig. 7B). H3K9 is constitutively acetylated in the *GPR109A* promoter region in human colon cancer cells, and IFNγ increases H3K9 acetylation and induces H3K18 acetylation in the *GPR109A* promoter region in human colon cancer cells. To determine whether IFNγ induces H3K18 hyperacetylation specifically through p300, p300 was silenced with p300-specific siRNA. Silencing p300 diminished the IFNγ-induced acetylation in both H3K9 and H3K18 in the *GPR109A* promoter region in human colon cancer cells (Fig. 7C). Therefore, our data indicated that H3K9 is constitutively acetylated in the *GPR109A* promoter region, and IFNγ signaling activates p300 to induce hyperacetylation of H3K18 in the *GPR109A* promoter region in human colon cancer cells.

IFNγ induces *GPR109A* promoter chromatin remodeling

Our above findings raise the possibility that IFNγ induces chromatin remodeling at the *GPR109A* promoter region to convert a methylated DNA-mediated repressive chromatin conformation to a hyperacetylated and transcriptionally permissive one to facilitate pSTAT1 binding to activate *GPR109A* transcription. To test this hypothesis, we used limited DNaseI digestion to detect chromatin remodeling at the *GPR109A* promoter region. Human colon carcinoma cells were treated with IFNγ. Nuclei were then isolated from the cells and incubated with DNase I for various time periods. Genomic DNA was purified from the nuclei and used as the template for PCR amplification using the *GPR109A* promoter DNA-specific primers. Comparison of the PCR-amplified DNA fragment levels showed that the *GPR109A* promoter chromatin regions in IFNγ-treated cells are significantly more accessible to DNaseI than their counterparts in the untreated cells (Fig. S6). Thus, our data indicate that IFNγ induces p300-mediated H3K18 hyperacetylation and resultant chromatin remodeling to create a transcriptionally permissible chromatin conformation at the methylated *GPR109A* promoter regions, which facilitates IFNγ-activated pSTAT1 binding to the GAS sites to activate *GPR109A* transcription.

Discussion

In colorectal cancer, genome-wide analysis showed that many of the methylated genes have known or predicted function in the suppression of tumorigenesis (38). Therefore, colon cancer cells use DNA methylation as a mechanism to silence tumor suppressor genes to advance the disease (39). Although there are no classical CpG islands in the human *GPR109A* gene promoter region, we identified several CpG diculeotides in the region, and observed that cytosines in these dinucleotides are methylated in human colon carcinoma cells. Analysis of six matched pairs of normal human colon tissues and colon carcinoma tissues revealed that GPR109A expression is indeed dramatically lower in the tumor tissues as compared to the adjacent normal colon tissues, which is consistent with the *GPR109A* promoter DNA methylation status. These observations thus indicate that *GPR109A* is silenced by its promoter DNA methylation in human colon carcinoma cells *in vitro* and in colon carcinoma tissues *in vivo*.

GPR109A is the receptor in colonocytes for the gut commensal bacterial metabolite butyrate (2), and mediates the butyrate-dependent anti-inflammatory effects to suppress colonic inflammation and inflammation-dependent colon cancer (2, 13). Therefore, it is not surprising that the *GPR109A* gene is silenced in human colon carcinoma cells. However, although the *GPR109A* promoter is methylated, GPR109A is expressed to some degree in a majority of colon carcinoma specimens examined, suggesting the presence of a novel mechanism that activates *GPR109A* transcription in the tumor microenvironment in spite of the methylation of its promoter. IFNγ is a proinflammatory cytokine secreted by activated T cells that functions as a key component of the host cancer immune surveillance system (40). IFNγ exerts its function through activation of STAT1 that acts as a master transcription factor to regulate IFNγ target gene transcription. Our observations that T cells extensively infiltrate colon cancer tissues and that high expression level of GPR109A is present in colon carcinoma cells in WT but not in IFNγ KO mice suggest that IFNγ is responsible, at least in part, for *GPR109A* expression in colon carcinoma *in vivo*. Because IFNγ is a key component of the host cancer immune surveillance system (40–42), our data thus suggest that the immune system might counteract tumor cell-induced and DNA methylation-mediated silencing of *GPR109A* expression through IFNγ-mediated reactivation of *GPR109A* transcription in tumor cells.

It has been shown that DNA methylation may render tumor cells resistant to IFN-induced apoptosis and that IFN-stimulated gene expression determines tumor cell sensitivity to DNA demethylation-induced apoptosis (43, 44). Strikingly, in the present study we observed that IFNγ can override the silencing effects of DNA methylation to activate *GPR109A* transcription without obvious demethylation of the *GPR109A* promoter DNA in human colon carcinoma cells. Current approach to re-activate DNA methylation-silenced tumor suppressor genes primarily relies on chemical inhibitors, mainly 5′-aza-dC that inhibits DNA (cytosine-5)-methyltransferase (DNMT) activity (43, 45). This type of DNA methylation inhibitors are highly cytotoxic, and their use as chemotherapeutic agents in cancer therapy is associated with extensive toxicity (45). Our data suggest that immunotherapeutic approach, such as adoptive or active CTL immunotherapy (46), might be an alternative and yet effective and less toxic approach to activate DNA methylation-silenced expression of genes in cancer tissues. In addition, 5′-aza-dC is a general DNA methylation inhibitor that causes global DNA demethylation. In contrast, IFNγ-activated pSTAT1 only binds to specific DNA sequences (GAS element) to activate specific gene transcription. Therefore, IFNγ-activated expression of genes from DNA-methylation-silenced promoters is gene-specific; this approach is thus likely to be associated with low toxicity.

p300 is a histone acetyltransferase that also functions as a master transcriptional mediator in mammalian cells (27, 34, 47–49). In this study, we demonstrate that p300 is an immediate early target of the IFNγ signaling pathway. p300 transcription activation was detected 10 min after IFNγ treatment. STAT1 was also activated within 10 min after IFNγ treatment. Although it has been shown that pSTAT1 and p300 directly interact with each other (50), we observed that pSTAT1 physically binds to the *p300* promoter region. Therefore, the likely signaling transduction cascade is that pSTAT1 is activated first, and it then binds to the *p300*

promoter to activate *p300* transcription. Elevation of p300 enhances its association with the *GPR109A* promoters to induce H3K18 hyperacetylation, and chromatin remodeling, resulting in a permissive chromatin conformation at the *GPR109A* promoter to facilitate pSTAT1 binding to activate *GPR109A* transcription.

Based on the activation and expression kinetics of pSTAT1, p300, and GPR109A, we propose a model to illustrate IFNγ-induced *GPR109A* transcription activation from their methylated promoters. We propose that the *GPR109A* promoter is methylated and thus transcriptionally inactive in human colon carcinoma cells. Exposure of human colon carcinoma cells to IFNγ induces rapid STAT1 activation. Activated STAT1 directly binds to the *p300* promoter region to rapidly activate *p300* transcription. P300 then binds to the *GPR109A* promoters to induce H3K18 hyperacetylation and resultant chromatin remodeling to create a transcriptionally permissive chromatin at the *GPR109A* promoter regions without an obvious change in the methylation status of the promoter DNA. A transcriptionally permissive chromatin structure allows pSTAT1 binding to activate *GPR109A* transcription despite DNA methylation (Fig. S7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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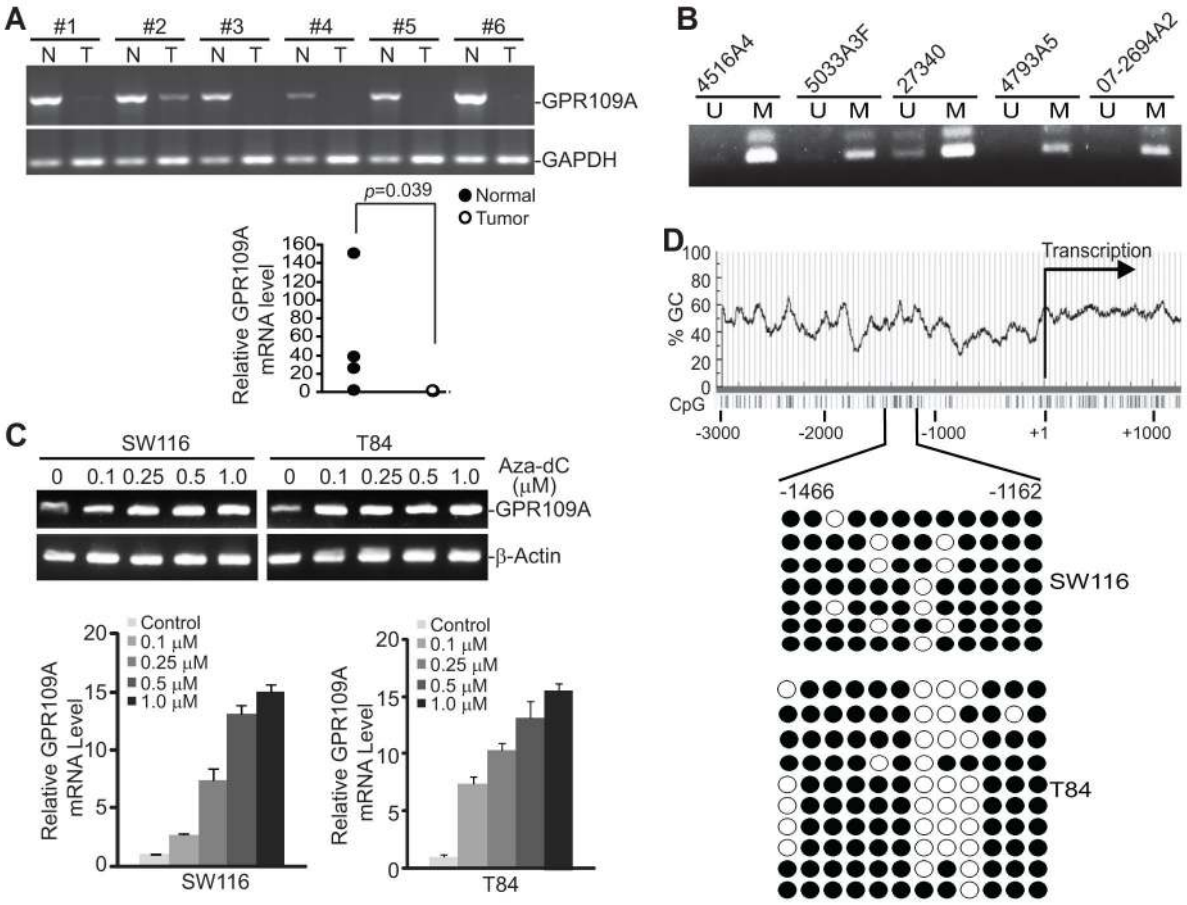

Figure 1. The human *GPR109A* promoter is methylated and GPR109A expression is silenced in human colon carcinoma cells

A. GPR109A expression level in matched pairs of human normal colon and colon carcinoma tissues. Colon carcinoma tissues and adjacent normal tissues were collected from 6 patients, and analyzed for GPR109A expression by RT-PCR. GAPDH was used as normalization control. Bottom panel: the GPR109A levels were quantified using the NIH J program. The ratio of GPR109A vs GAPDH in patient #1 was arbitrarily set at 1. The GPR109A expression levels of the remaining five specimens were then normalized based on patient #1. **B**. Methylation status of the *GPR109A* gene promoter in human colon carcinoma specimens. Genomic DNA was isolated from colon carcinoma specimens of 5 colon cancer patients and modified with bisulfate. The modified DNA was then analyzed by MS-PCR (U, unmethylated; M, methylated). Numbers above the figure are patient codes. **C**. Inhibition of DNA methylation increases GPR109A expression. SW116 and T84 cells were treated with Aza-dC for 3 days at the indicated doses and analyzed for GPR109A expression level by semi-quantitative RT-PCR (top panel) and real-time RT-PCR (bottom panel). The GPR109A expression levels of untreated cells were arbitrarily set at 1. *Column*: mean, *bar*: SD. **D**. Methylation level of the human *GPR109A* gene promoter in human colon carcinoma cell lines. The human *GPR109A* gene DNA sequence was exported from the human genome database and analyzed for CpG islands using MethyPrimer computer program. Top panel: the human *GPR109A* gene promoter structure. Vertical bars under the line indicate location

of CpG dinucleotides, and the number under the line indicates nucleotide number relative to *GPR109A* transcription initiation site (+1). Bottom panel: methylation level of the *GPR109A* gene promoter in the indicated cell lines. Genomic DNA was modified with bisulfite. The indicated DNA fragment was amplified by PCR and cloned into pCR2.1 vector. Individual clones for each cell line were sequenced and the methylation level of the cytosine in the CpGs was analyzed using QUMA computer program (open circle, unmethylated CpG; closed circle, methylated CpG).

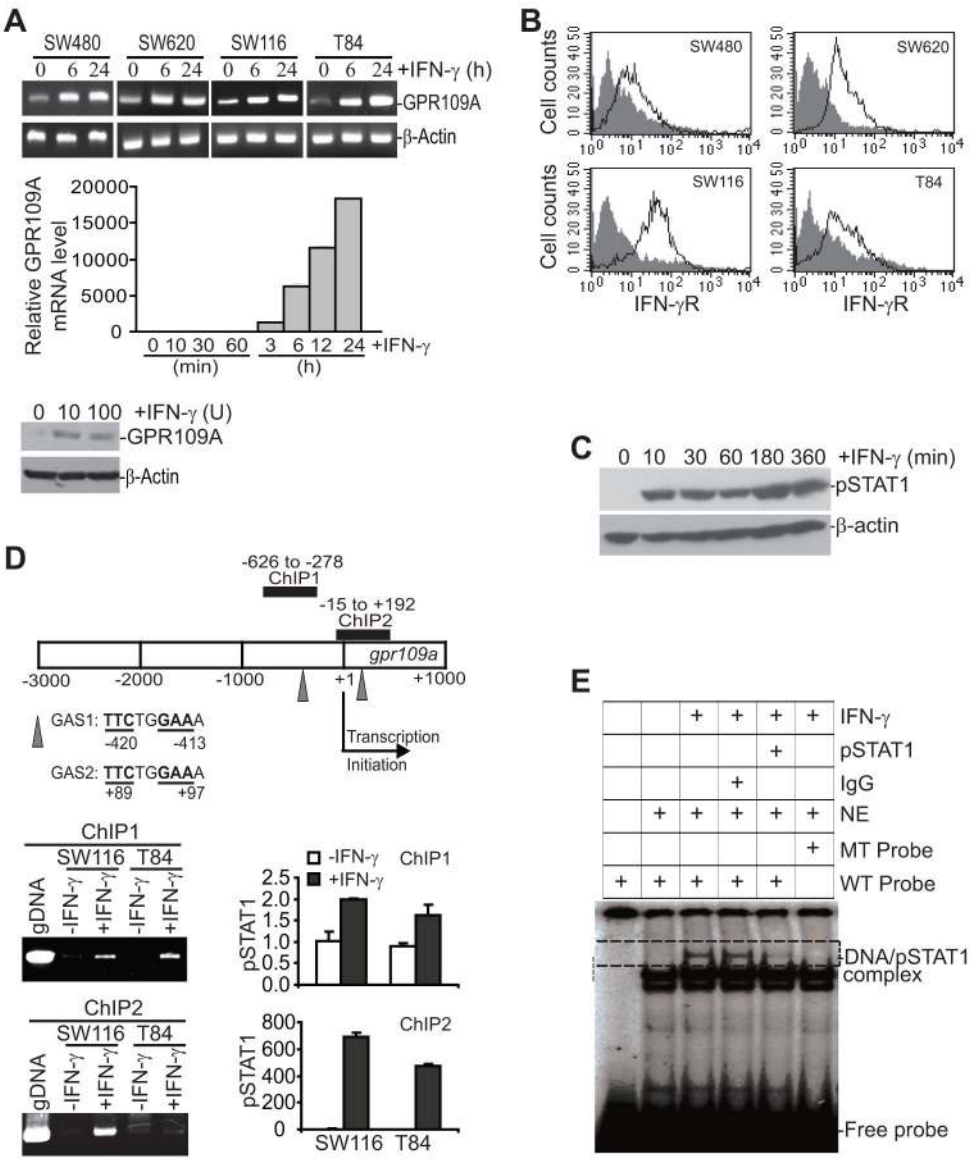

Figure 2. IFNγ activates *GPR109A* transcription from the methylated *GPR109A* promoter

A. Tumor cells were treated with IFNγ and analyzed for GPR109A expression level by RT-PCR (top panel). The GPR109A expression in T84 cells were also analyzed by real-time PCR (middle panel). *Column*: mean, *Bar*: SD. Bottom panel: SW116 cells were treated with IFN-γ at 10 and 100 U/ml for 24h and analyzed by Western blotting analysis. **B**. Tumor cells were stained with IFN-γR mAb and analyzed for cell surface IFN-γR protein level. Grey area: IgG isotype control; solid line: IFN-γR-specific staining. **C**. T84 cells were treated with IFN-γ for the indicated time and analyzed for pSTAT1 protein level by Western blotting. **D**. The human *GPR109A* promoter structure. The GAS element locations and consensus sequences are shown under the bar. The ChIP PCR amplified regions are indicated above the bar. Bottom panel: SW116 and T84 cells were either untreated (–IFN-γ) or treated with IFN-γ (+IFN-γ) for 6 h and analyzed by ChIP using pSTAT1-specific mAb. Purified genomic DNA (gDNA) was used as a positive control for the PCR (left panel). The

ChIP DNA was then analyzed by real time PCR (right panel). **E**. T84 cells were either untreated or treated with IFN-γ (+IFN-γ) for 6 h and used for nuclear extract preparation. The nuclear extracts were incubated with the GAS element-containing DNA probe in the absence or presence of IgG or pSTAT1 mAb and then analyzed for protein-DNA interaction by EMSA. A mutant GAS DNA probe (MT probe) was used as negative control.

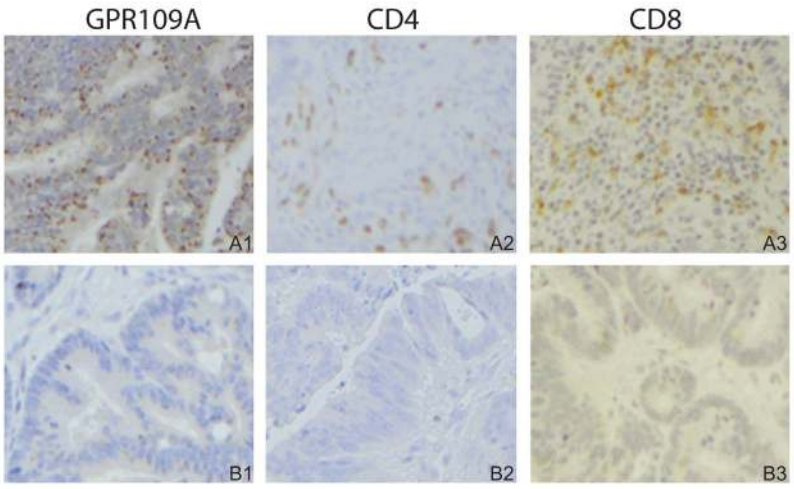

Figure 3. GPR109A protein level and T-cell infiltration level in human colon carcinoma tissues

Human colon carcinoma specimens were stained with GPR109A-, CD4-, and CD8-specific antibodies. Shown are images from two patients with high (A, patient 3 as in Fig. S2–4) and low (B, patient 9 as in Fig. S2–4) GPR109A, CD4 and CD8 staining, respectively.

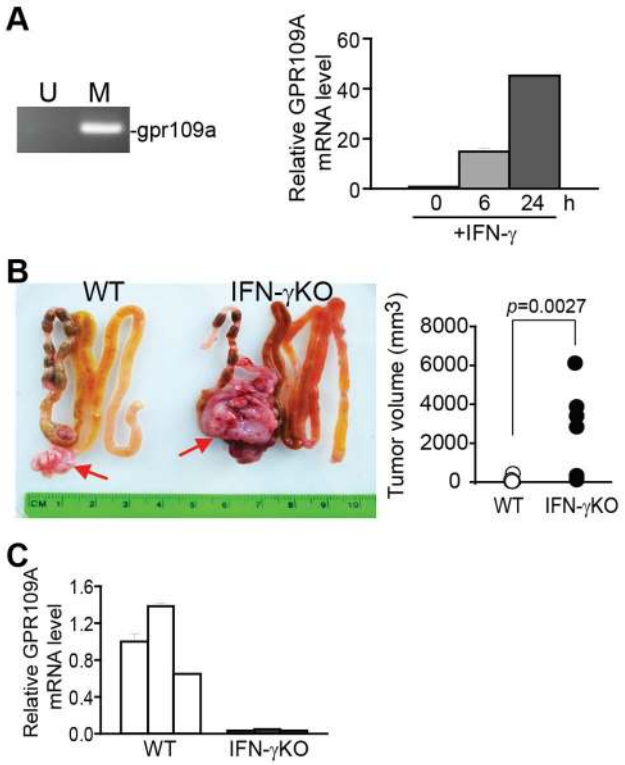

Figure 4. IFNγ activates *gpr109a* transcription from their methylated promoters *in vivo*

A. Genomic DNA was isolated from mouse colon carcinoma cell line CT26 (left bottom panel), and analyzed for *gpr109a* promoter DNA methylation by MS-PCR. U: unmethylated, M: methylated. CT26 cells were also treated with IFN-γ (100U/ml) and analyzed for GPR109A expression level by real-time RT-PCR (right panel). *Column*: mean, *Bar*: SD. **B**. CT26 cells ($1x10^4$/mouse) were surgically implanted into the cecal wall of WT (n=9) and IFN-γ KO (n=7) mice. Tumor growth on the colon tissues were analyzed 21 days after tumor transplant. Left panel: representative image of WT and IFN-γKO mouse colon tissues showing colon tumor development (red arrows). The tumor volumes were quantified and presented in the right panel. **C**. Colon carcinoma tissues as shown in B were dissected from the colon tissues of three WT and three IFN-γKO mice, respectively, and analyzed by real-time RT-PCR for GPR109A expression levels. Each column represents relative GPR109A expression level in one mouse.

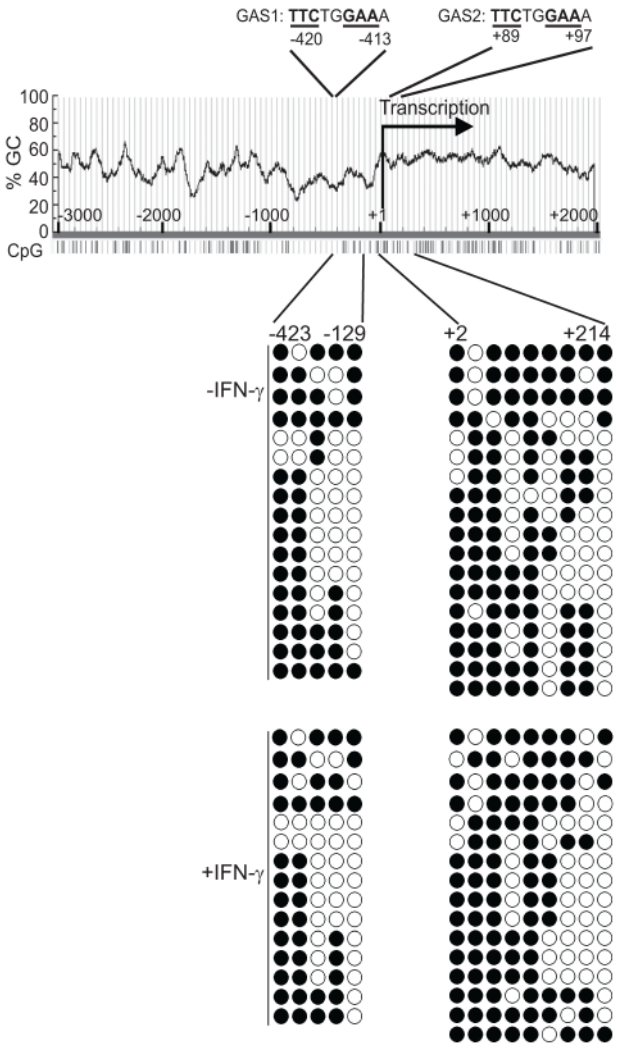

Figure 5. IFNγ does not alter the methylation level of the GPR109 Apromoter DNAs in human colon carcinoma cells

T84 cells were cultured in the absence or presence of IFN-γ for 24 h. Genomic DNA was isolated from the cells and analyzed for the *GPR109A* promoter DNA methylation level in the 2 GAS-containing *GPR109A* promoter regions as shown. Open circle: Unmethylated CpG; Closed circle: Methylated CpG.

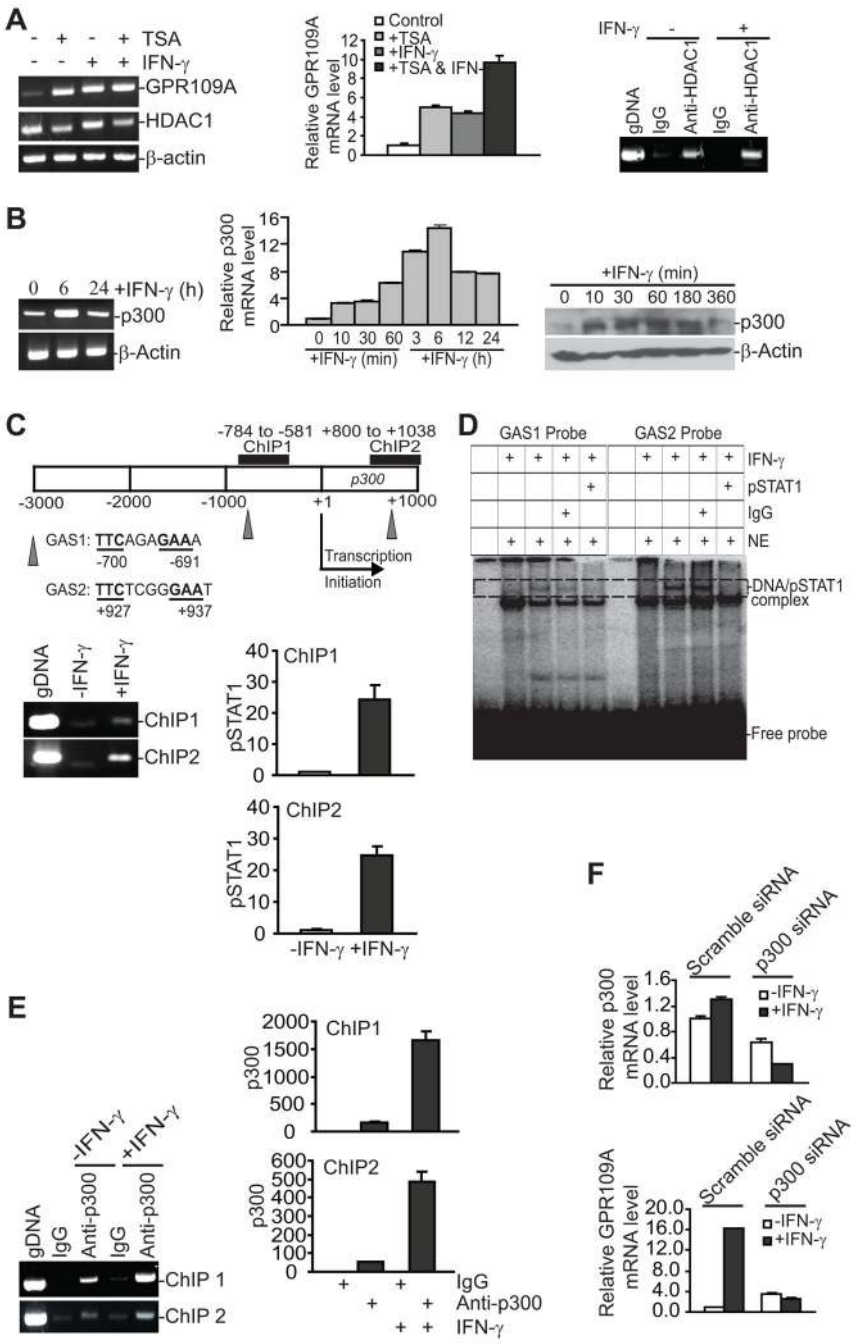

Figure 6. IFNγ regulates GPR109A expression through direct regulation of *p300* expression

A. T84 cells were treated with TSA (200 nM) or IFN-γ (100U/ml) for 24 h and analyzed for the expression of the indicated genes by RT-PCR (left panel) and real-time RT-PCR (middle panel). The untreated (–IFN-γ) or treated cells (+IFN-γ) were also analyzed by ChIP using HDAC1-specific antibody for HDAC1 association with the *GPR109A* promoter region (right panel). **B**. T84 cells were treated with IFN-γ for the indicated time and analyzed by RT-PCR for p300 expression level by semi-quantitative PCR (left panel) and real-time PCR (middle panel). p300 protein level in T84 cells was analyzed by Western blotting (right

panel). **C**. Top panel: The human *p300* promoter structure. The ChIP PCR-amplified regions are indicated above the bar. The GAS element locations and consensus sequences are shown under the bar. Bottom panel: T84 cells were either untreated (−IFN-γ) or treated with IFN-γ (+IFN-γ) for 6 h and analyzed by ChIP using pSTAT1-specific mAb for pSTAT1 association with the *p300* promoter region. Left panel: semi-quantitative PCR. Right panel: real-time PCR. The value of untreated cells was set at 1. **D**: EMSA of pSTAT1 binding to the GAS element-containing *p300* promoter DNA. T84 cells were either untreated or treated with IFNγ (+IFN-γ) for 6 h and used for nuclear extract preparation. The nuclear extracts were incubated with the GAS element-containing DNA probe in the absence or presence of IgG or pSTAT1 mAb and then analyzed for protein-DNA interactions. **E**. T84 cells were cultured in the absence (−IFN-γ) or presence (+IFN-γ) of IFNγ for 6 h and then analyzed by ChIP using p300-specific antibody to detect p300 association with the *GPR109A* promoter chromatin. Left panel: semi-quantitative PCR. Right panel: real-time PCR. The value of IgG of untreated cells was set at 1. **F**. T84 cells were transiently transfected with scrambled or p300-specific siRNAs overnight, and then treated with IFNγ for 8 h. p300 and GPR109A expression levels were then analyzed by real-time RT-PCR.

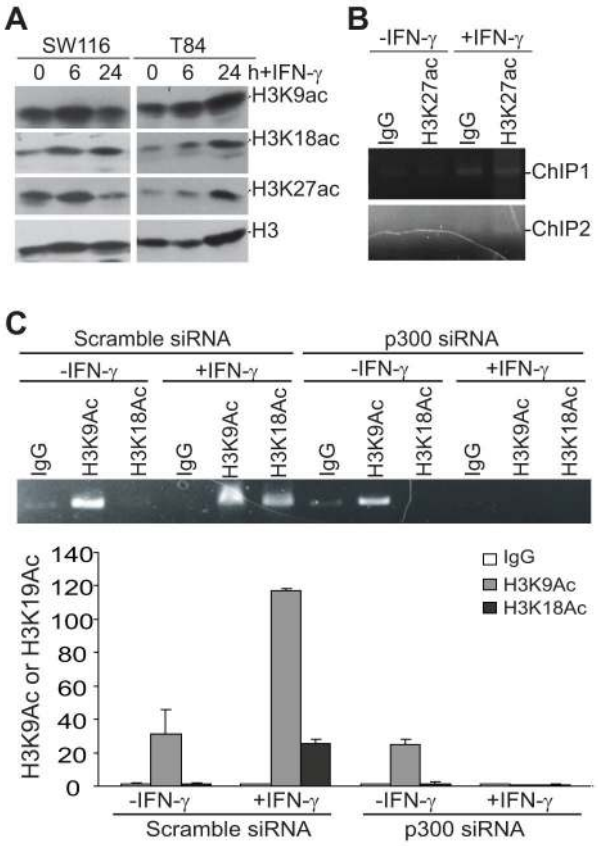

Figure 7. IFNγ up-regulates p300 expression to mediate H3K18 acetylation in the *GPR109A* promoter region

A. IFN-γ induces global acetylation of H3K9, H3K18 and H3K27. SW116 and T84 cells were treated with IFN-γ. Histone acidic extracts were prepared at the indicated time points from the cells and analyzed for the indicated acetylated lysine residues of H3 by Western blotting. **B**. H3K27 is not acetylated in the *GPR109A* promoter regions. T84 cells were treated with IFN-γ overnight and analyzed for acetylated H3K27 in the *GPR109A* promoter regions by ChIP. **C**. p300 mediates H3K18 acetylation in the *GPR109A* promoter region. T84 cells were transiently transfected with either scramble siRNA or p300-specific siRNA overnight, followed by treatment with IFN-γ for 8h. Cells were then analyzed by ChIP for acetylated H3K9 and H3K18 levels in the *GPR109A* promoter region. Top panel: semi-quantitative PCR. Bottom panel: real-time PCR. The value of IgG of untreated cells was set at 1.