A long noncoding RNA signature for ulcerative colitis identifies IFNG-AS1 as an enhancer of inflammation

A long noncoding RNA signature in Ulcerative Colitis

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Running title: IFNG-AS1 lncRNA regulates inflammation

New and Noteworthy: Long noncoding RNA (lncRNA) biology represents an exciting new field with implications in gene regulation. This field has only recently been investigated in the pathogenesis of ulcerative colitis. By utilizing high-throughput technologies on clinical samples, a lncRNA signature was generated and the lncRNA IFNG-AS1 was shown to regulate the important inflammatory cytokine interferon-γ. This work represents one of the first mechanistic studies of lncRNA biology in inflammatory bowel disease.

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Abstract:

High-throughput technologies revealed new categories of genes, including the long noncoding RNAs (lncRNAs), involved in the pathogenesis of human disease; however, the role of lncRNAs in the ulcerative colitis (UC) has not been evaluated. Gene expression profiling was used to develop lncRNA signatures in UC samples. Jurkat T cells were activated by PMA/ionomycin subsequently interferon-γ (IFNG) and tumor necrosis factor (TNF)-α protein levels were assessed by ELISA. Anti-sense molecules were designed to block IFNG-AS1 expression. A unique set of lncRNAs was differentially expressed between UC and control samples. Of these, IFNG-AS1 was among the highest statistically significant lncRNAs (fold change: 5.27, *P* value: 7.07E-06). Bioinformatic analysis showed that IFNG-AS1 was associated with the IBD susceptibility loci SNP rs7134599 and its genomic location is adjacent to the inflammatory cytokine IFNG. In mouse models of colitis, active colitis samples had increased colonic expression of this lncRNA. Utilizing the Jurkat T cell model, we found IFNG-AS1 to positively regulate IFNG expression. Novel lncRNA signatures differentiate UC patients with active disease, patients in remission, and control subjects. A subset of these lncRNAs was found to be associated with the clinically validated IBD susceptibility loci. IFNG-AS1 was one of these differentially expressed lncRNAs in UC patients and found to regulate the key inflammatory cytokine, IFNG, in CD4 T cells. Taking these findings together, our study revealed novel lncRNA signatures deregulated in UC and identified IFNG-AS1 as a novel regulator of IFNG inflammatory responses, suggesting the potential importance of noncoding RNA mechanisms on regulation of inflammatory bowel disease-related inflammatory responses.

Keywords: inflammatory bowel disease; ulcerative colitis; lncRNA; inflammation; Interferon-γ

Introduction

INFLAMMATORY BOWEL DISEASES (IBDs) are common gastrointestinal disorders that are clinically comprised of ulcerative colitis (UC) and Crohn's disease (CD) (14, 24). These diseases have a devastating toll on a patient's morbidity and quality of life (27). The etiology of IBD, however, still remains elusive. Studies have shown that IBD may result from an inappropriate inflammatory response to intestinal microbes and foreign antigens in genetically susceptible hosts (8, 40). Key mediators of this inflammatory process such as TNF-α, IL-23, IL-12, and interferon-γ (IFNG) have been implicated in the pathogenesis of UC (1, 6, 10, 20, 25, 26). Therapies directed against these cytokines have been relatively successful in treating UC symptoms; however, a subpopulation of patients may fail to respond to these treatments (32, 33, 35). In addition to these cytokines, researchers have also focused on the genetic underpinnings of the disease. Familial clustering and twin studies have shown a role for genetic factors for UC (3, 14). Indeed, meta-analyses of multiple genome-wide association studies have implicated 163 genetic loci in both CD and UC pathogenesis (14). However, the mechanisms of action for many of the genes in close association with these loci have yet to be determined.

The IBD susceptibility loci point to key genomic positions and highlight areas of the genome for further mechanistic studies. In addition to the associated protein-coding transcripts previously reported, noncoding transcripts may be associated with these loci. Recently, investigators have begun to address the noncoding transcripts and their role in disease pathogenesis. One such category of noncoding transcript are the long noncoding RNAs (lncRNAs) (21). The central dogma, wherein "DNA makes RNA makes protein," has long relegated RNA as an intermediary between gene and protein. Recent research has challenged the notion that the noncoding RNAs are innocent bystanders and may indeed be critical to gene regulation (30, 31). Long noncoding RNAs are defined as sequences of greater than 200 nucleotides in length. They can express introns and exons, which can be alternatively spliced and generally lack open reading frames for protein translation (29). lncRNAs appear to have a diverse set of functions in chromatin remodeling, telomere activity, and subcellular structural organization (4). They can mediate epigenetic changes by recruiting chromatin-remodeling complexes to specific loci in the genome. As an example, the lncRNA HOTAIR has been implicated in inducing a repressive chromatin state by recruiting the Polycomb chromatin remodeling complex PRC2 (12). In addition, lncRNAs can regulate transcription by altering RNA polymerase II activity and thereby influence promoter activation (30). Finally, lncRNAs have also been implicated in various steps in the posttranscriptional processing of messenger RNAs, including splicing, editing, transport, translation, and degradation (21).

Based on these above findings, it has been suggested that noncoding RNAs play a role in the pathogenesis of UC (15, 16, 28). In addition, lncRNAs have been shown to participate in multiple

inflammatory processes and contribute to the pathogenesis of autoimmune and immune-related disorders; however, their role in UC has yet to be fully determined (2, 13, 18, 23). To this end, we have profiled the expression of both coding and noncoding transcripts in UC patients. By comparing their expression to those of control patients, we have identified a set of differentially expressed lncRNAs in adult UC patients. To evaluate the relationship of this set of lncRNAs with known IBD genomic alterations, we compared the chromosomal location of the lncRNAs with those of the IBD susceptibility loci. The lncRNA IFNG-AS1 was associated with the IBD susceptibility loci SNP rs7134599 and is in close proximity to the inflammatory cytokine IFNG. It was found to be among the most statistically significant differentially expressed lncRNAs. Given the close proximity to IFNG within the human genome, we investigated its cellular mechanism of action and found a positive regulatory mechanism between IFNG-AS1 and IFNG expression.

Materials and Methods

Human tissue collection and RNA extraction

Colonic tissues were obtained from two sources. For the microarray analysis, RNA extracted from colonic surgical samples were obtained from Origene. A total of 50 anonymized samples, 19 for the initial profiling experiment and 31 for the validation cohort, were included in this study. For the profiling experiment, 7 control patients, 8 UC-active (UCA), and 4 UC-inactive (UCI) were collected. Diagnoses were confirmed by an independent set of pathologists associated with Origene. The samples were obtained through institutional review board (IRB) protocols and with documented patient consent, all from accredited U.S.-based medical institutions (www.origene.com). A second set of colonic samples was obtained from colonic surgeries performed at Cedars-Sinai Medical Center, Los Angeles, CA. These samples were collected under an IRB-approved protocol. Patients that participated in the IRB tissue recruitment were undergoing colonic resections as part of their regular medical care. A total of 31 anonymized patient samples were included in this part of the study, 16 control patients and 15 UC patients. A segment of the colon resection was flash frozen in liquid nitrogen and stored at -80°C. The diagnoses were confirmed on histopathology. Available patient information is summarized in Table 1.

RNA extraction

Total RNA was extracted from frozen colonic tissues and cells in culture using TRIzol (Qiagen, Valencia, CA; cat. no. 79306) and miRNeasy Mini Kit (Qiagen; cat. no. 217004) following the product instructions and utilizing DNase I digestion (cat. no. 79254). RNA was stored at _80°C and measured with a Nano-Drop 1000 Spectrophotometer (Thermo Scientific). The integrity of the RNA

submitted for microarray analysis was assessed with either the Agilent Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies) or gel analysis.

Microarray analysis for coding and non-coding transcripts

For microarray analysis, the RNA samples were submitted to Arraystar for coding and noncoding RNA expression profiling. An Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). The labeled cRNAs were hybridized onto the Human LncRNA Array v3.0 (8 X 60 K, Arraystar). After slides were washed, the arrays were scanned by the Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that at least 19 samples have flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs were identified using Student's t-test between two groups with a fold change cut-off >2 and $P < 0.05$. Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed mRNAs played in these biological pathways or GO terms. Finally, Hierarchical Clustering was performed to show the distinguishable lncRNAs and mRNAs expression patterns among samples.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (9) and are accessible through GEO Series accession number GSE77013.

Quantitative-Real-time PCR for lncRNA and mRNA

To analyze human gene expression, 500 ng of RNA was converted to Cdna using iScript cDNA synthesis kit (Bio-Rad; cat. no. 1708891). Real-time PCR was performed in triplicate using the SYBR Green mastermix (Bio-Rad; cat. no. 203450). qPCR primers were designed for the reference genes ACTIN and GAPDH, as well as IFNG-AS1, and IFNG. The SYBR-Green primers were ordered from Integrated DNA Technologies. The sequences are *ACTIN-F*: 5'-cccagcacaatgaagatcaa-3'; *ACTIN-R*: 5'-acatctgctggaaggtggac-3'; *GAPDH-F*: 5'-

atgttcgtcatgggtgtgaa-3'; *GAPDH-R*: 5'-ggtgctaagcagttggtggt-3'; *IFNG-AS1-F*: 5'-tgcctaggtgctctctgatg-3'; *IFNG-AS1-R*: 5'-ggcacaacccatggaagac-3'; *IFNG-F*: 5'-gagtgtggagaccatcaagga-3'; *IFNG-R*: 5'-gtattgctttgcgttggaca-3'. The qPCRs were performed in a CFX384 Real Time PCR detection system (Bio-Rad). For gene expression of mouse genes 18S and Tmevpg1, TaqMan master mix (Applied Biosystems; cat. no. 4352042) was used and primers were ordered from Applied Biosystems (18S:Mm03928990_g1; Tmevpg1: Mm01161206_m1).

Cell culture

Jurkat CD4 T cells were generously provided by Dr. Otto Yang's laboratory at UCLA. The cells were grown in RPMI+10%FBS+1% penicillin+1% streptomycin. Experiments were performed in 24-well plates with 1 million cells plated in 1 ml of media. Activation of T cells was achieved by 6 h incubation at 37°C with phorbol-12-myristate-13-acetate (PMA) at a final concentration of 50 ng/ml and ionomycin at 1 µM concentration (PMA: Sigma-Aldrich; cat. no. P8139. Ionomycin: Sigma-Aldrich; cat. no. I3909).

Peripheral mononuclear blood cells were obtained from blood draws at Cedars-Sinai Medical Center, Los Angeles, CA. The samples were obtained from control patients under the IRB no. 23705. CD4 cells were isolated using the EasySep Human CD4+ T cell enrichment kit (Stemcell; cat. no. 19052).

Knockdown and overexpression of lncRNA IFNG-AS1

Reductions in *IFNG-AS1* lncRNA expression were achieved using two custom-made siRNA constructs: siIFNG-AS1#1 sense, caaucuuaaggauacagaatt; siIFNG-AS1#2 sense, caaugcauuuauuaauucutt (Ambion). siRNA control was transfected for comparison (Ambion; cat. no. AM4613). Transfections were performed using Lipofectamine RNAiMax (Invitrogen; cat. no. 13778-075) following the product instructions. Briefly, 5 µl of a 20 µM concentration of siRNA was mixed with 7.5 µl of Lipofectamine RNAiMax in 200 µl of Optimem media (Thermo Scientific; cat. no. 31985-062). The sample was incubated for 15 min and then added to 1 million Jurkat cells for 24 h. Overexpression of the lncRNA was achieved by transfecting 4 µg of pCMV6-AC-IRES expression vector with *IFNG-AS1* (Blue Heron). Control cells were transfected with the expression vector alone without insert.

Protein analysis

IFNG protein production was quantified using the Quantikine ELISA (R&D Systems; cat. no. DIF50). TNF-α protein production was assayed using the Milliplex Human High Sensitivity T cell Magnetic Bed Panel (Millipore; cat. no. HSTCMAG28SPMX13). Jurkat cells were grown in 24-well plates in 1 ml of volume. The cells were activated with PMA/ionomycin for 6 h and medium was collected 24 h after activation. The medium was centrifuged to remove excess cells. Manufacturer's instructions were followed for quantification of protein using both kits. The quantity of protein expression was normalized to cell numbers at the time of collection.

Mouse models of colitis

Colitis was induced in mice via trinitrobenzenesulfonic acid (TNBS) treatment with modifications of previously described protocols (17). All animal studies were approved by the IACUC. Total RNA from the colonic tissues of the TNBS model was purified by use of the miReasy Mini Kit (Qiagen). Tissue sections were scored for histopathology analyses in a double-blinded manner, as previously reported (17). Animals were maintained at University of California-Los Angeles animal research facility and received standard pelleted chow and water ad libitum. The 6- to 8-wk-old male C57BL/6J mice ($n = 8$ per group) received a 50μl intracolonic injection of 40 mg/kg TNBS (Fluka) in 30% ethanol, using a 1-ml syringe (Becton Dickinson) fitted with a polyethylene cannula (Intramedic PE-20 tubing). The control groups were injected with 50 μl of 30% ethanol alone into the colon. The mice were held head down for 1 min after the enema administration to ensure the containment of the TNBS solution into the colon. The colons were fixed in 10% buffered formalin for histological analysis. Paraffin sections were stained with hematoxylin/eosin.

IL10-/- C57BL/6 male ($n = 8$) and female ($n = 8$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were kept under specific pathogen-free conditions and fed a standard diet and water ad libitum, under the approval by the institutional animal care and use committee (IACUC) at the Department of Veteran Affairs. Mice were weighed weekly and monitored for appearance of diarrhea and blood in the stools. Mice that developed severe rectal prolapse that was evident at all times were euthanized. Control mice were of the same background and euthanized at the same age. After euthanasia, the entire colon was excised and a portion of the proximal and distal colon was fixed in 10% buffered formalin for histological analysis. Paraffin sections were stained with hematoxylin/eosin.

Experimental statistical analysis

The Student's *t*-test for two-group comparisons was performed to determine any statistically significant differences between the experimental groups. *P* < 0.05 was considered statistically significant. All results are expressed as means ± standard error on the mean.

Results

lncRNA signatures in UC colonic tissues.

Clinical samples from a total of 19 patients consisting of colon resections from 8 UCA, 4 UCI, and 7 control patients (Table 1), were used to perform lncRNA expression profiling analysis. The lncRNA expression profiling was performed using the Arraystar lncRNA microarray V3.0, which consists of ~30,600 lncRNAs. This analysis (>2-fold and *P* < 0.05) revealed that there were 1,931 differentially expressed lncRNAs between UCA and control, while 1,361 lcnRNAs were differentially expressed between UCA and UCI colonic tissues (Figs. 1A and 2). Furthermore, 287 lncRNAs were found to be differentially expressed in UCI relative to control tissues (Fig. 2).

A focus was placed on the comparison between UCA vs. control samples due to the robust statistical differences seen between these groups as well as access to a separate cohort of UC and control samples for validation studies. As a representative sample of these lncRNAs, Fig. 1, B and C demonstrates the top 15 most statistically significant upregulated and 15 downregulated lncRNAs differentially expressed between the UCA and control samples. As shown, the expression of these 30 lncRNAs enables the clustering of the UCA samples away from the control samples, indicating that these IBD samples express a unique lncRNA expression signature that is distinct from control samples. Figure 1C identifies the 30 differentially expressed lncRNAs and shows the range of expression changes from over 20-fold upregulated to over 20-fold downregulated between the UCA and control patients. These results demonstrate that lncRNAs are significantly differentially expressed between UCA samples and controls and that the identity of the majority of these differentially expressed lncRNAs is unique between these comparisons. Taken together, these analyses show novel lncRNA signatures that were deregulated between UCA, UCI, and control colonic tissues.

Differentially expressed lncRNAs are associated with known IBD loci.

The list of lncRNAs differentially expressed in UCA samples compared with control samples spans the human genome. Previous research has identified 163 distinct single nucleotide polymorphism (SNP) loci as IBD susceptibility loci (14). A comparison of the chromosomal locations was per formed between the known IBD susceptibility loci and a list of lncRNAs differentially expressed in IBD samples. A list of lncRNAs was generated to compare the common differentially expressed lncRNAs between the UCA samples and the controls as well as the UCA and the inactive UC

samples. This comparison was performed to assess for lncRNAs that were associated with active inflammation in UC samples. The analysis revealed that, of the 767 lncRNAs evaluated, only 31 lncRNAs were found to be within 250 kilobases from the IBD loci (Fig. 3). The 250-kb distance was selected based on the analysis performed by Jostins et al. (14) where a locus was defined as at 500-kb region with the SNP at its center. This comparison demonstrates that a portion of the lncRNAs identified as differentially expressed transcripts between IBD samples and control samples are closely associated with clinically validated IBD susceptibility loci.

Validating lncRNA expression in a second cohort of UC patient samples.

The lncRNA expression differences seen on the microarray analysis showed robust expression changes between the comparison groups. To validate these findings, a separate second cohort of UC samples was assayed for lncRNA expression. The samples consisted of 15 UCA samples and 16 control colonic biopsy samples (Table 2). Total RNA from these samples was extracted and the level of expression for a select number of lncRNAs was performed using quantitative real-time polymerase chain reaction (qRT-PCR). We chose to validate the lncRNAs that were associated with the IBD susceptibility loci and were among the most statistically significant differentially expressed lncRNAs (Fig. 3). The lncRNAs *RP11-81H14.2* and *IFNG-AS1* were selected for validation (Fig. 4, A and B). RP11-81H14.2 expression analysis did not reveal a statistically significant difference between UC and control samples (Fig. 4D). *IFNG-AS1*, however, was found to be statistically different between the two sample cohorts and was chosen as a lncRNA of interest for further analysis (Fig. 4C).

lncRNA-associated gene pathway analysis.

To understand how changes in lncRNA expression may affect phenotypic changes in UC, an analysis of the changes in coding transcript expression was performed. It was hypothesized that changes in long noncoding RNA expression may affect coding transcript expression and as a result alter distinct gene pathways. To address this, the coding transcripts of each of the UC samples and control samples were assayed using the microarray platform from Arraystar. Nineteen patient samples were then divided into two groups: those expressing relatively high quantities of *IFNG-AS1* and those expressing relatively low amounts *IFNG-AS1* as determined by their expression level on the microarray analysis (Fig. 5). In dividing the clinical samples into these two groups, the coding transcripts could then be assessed for differences in expression between the two groups. A threshold of a fold change of 1.5 upregulated and 2 downregulated was used to generate the coding transcript list. A total of 426 coding transcripts were differentially expressed using these cutoffs. By averaging the expression of the differentially expressed coding transcripts, a gene signature

associated with relative *IFNG-AS1* expression could be generated. Taking this list of differentially expressed coding transcripts, a bioinformatics gene pathway analysis was performed using Ingenuity Pathway Analysis software. Based on this analysis, several upstream regulators were shown to be activated in the *IFNG-AS1* high samples including IFNG, IL1, IL6, and TNF-α (Fig. 5). Of particular interest, was the enhancement of IFNG signaling in the group that expresses higher levels of *IFNG-AS1* as this had the lowest associated *P* value (Fig. 5).

IFNG-AS1 expression in CD4 T cells.

The lncRNA *IFNGAS1* has previously been implicated in immune cell biology in both mouse and human studies (11, 39). In an attempt to identify a target cell for our selected lncRNA, we first examined peripheral CD4 T cells isolated from control patients. The expression of *IFNG-AS1* was compared in the total peripheral mononuclear cells with the selected CD4 T subpopulation. Figure 6A shows qRT-PCR data showing that CD4 T subpopulations have enriched expression of *IFNG-AS1* consistent with recently published data that *IFNG-AS1* is expressed in the Th1 immune cells (39).

Given these results, we chose to study the function of *IFNG-AS1* using the Jurkat immune T cell model. Jurkat CD4 T cells can be activated via PMA and ionomycin stimulation and can be induced to secrete a variety of immune mediators such as *IFNG* and *TNF-α* (Fig. 6, B and C). Analyzing the RNA expression, PMA/ionomycin was found to induce *IFNG-AS1* as well as *IFNG* and *TNF-α* (Fig. 6D). Figure 7 highlights the time course of *IFNG-AS1* and *IFNG* expression with *IFNG-AS1* reaching maximal induction 24 h after stimulation with PMA/ionomycin.

IFNG-AS1 regulates IFNG expression at the RNA and protein level.

Previous studies in mouse immune cells have suggested that *Ifng-as1* can directly regulate the expression of its genomic neighbor, *Ifng* (11, 36). These studies reveal a pattern of histone methyltransferase recruitment to the *Ifng* promoter resulting in positive regulation of Ifng expression. We sought to show that the human ortholog, *IFNG-AS1*, could regulate *IFNG* production in a similar positive relationship. Using two siRNA constructs, we transiently transfected Jurkat T cells and assessed the expression of *IFNG-AS1* and *IFNG* in the context of T cell activation via PMA/ionomycin. The induction of *IFNG-AS1* was abrogated in both siRNAs by ~30-40% and resulted in reduced expression of IFNG RNA (Fig. 8, A and B).

Protein expression was then analyzed with an IFNG-specific ELISA kit. IFNG protein levels were similarly abrogated when IFNG-AS1 expression was reduced (Fig. 8, C and D).

In addition to the knockdown studies, we generated an IFNG-AS1 overexpression construct and transiently transfected it to Jurkat T cells. Figure 9A demonstrates the level of overexpression of IFNG-AS1 achieved with this construct. When transfected into cells and induced with PMA/ionomycin, there was an increase in IFNG RNA levels as early as 4 h of PMA/ionomycin induction (Fig. 9B). Under these conditions, protein expression of IFNG was increased by an average of 45% (Fig. 9C). These data suggest that IFNG-AS1 has a direct relationship with the expression of IFNG within immune cells.

Mouse models of colitis have increased Ifng-as1 levels.

We then assessed whether colitis models would show a similar increase in the expression of the mouse ortholog *Ifng-as1*. We utilized two mouse models of colitis, the acute TNBS colitis model at 48 h post-TNBS administration as well as the IL10 knockout mouse model of spontaneous colitis at age 12 wk. Colonic tissues were obtained from each mouse model and compared with controls. Expression of the lncRNA was then assessed by qRT-PCR. In both models, the mouse ortholog *Ifng-as1* was found to be significantly elevated in the colitis setting compared with control (Fig. 10, A and B). These results are consistent with our human clinical data showing increased *IFNG-AS1* expression in colitis tissues.

Discussion

The diagnosis and treatment of IBD faces many challenges that can only be addressed by gaining a deeper understanding of the molecular mechanisms involved in the pathogenesis of the disease. Noncoding RNA biology has emerged as a new and potent form of gene regulation and has only recently began to be evaluated in IBD (7, 42). In the past year, Mirza et al. (22) performed the first transcriptomic profiling of lncRNAs in IBD patients and revealed hundreds of differentially expressed lncRNAs between UC, CD, and control patients. Additionally, Wu et al. (41) also reported a profiling analysis of lncRNAs differentially expressed in UC patients vs. controls. They evaluated over 60 patients with and without IBD and identified 455 lncRNAs that were differentially expressed. This group focused on one of the differentially expressed lncRNAs, BC012900, and showed that it can regulate epithelial cell apoptosis. Our IBD studies investigated differentially expressed lncRNAs among UC patients and in addition uncovered new potential mechanisms of action. Through a noncoding RNA expression analysis, we identified a set of lncRNAs that are differentially expressed between UC samples and controls. By focusing on those lncRNAs differentially expressed in UC patients, we then filtered our list to identify lncRNAs that were associated with the clinically validated IBD susceptibility loci. We were able to validate the

differential expression of one of these lncRNAs, *IFNG-AS1*, with the use of a separate clinical cohort of colonic samples. Of note, Mirza et al. also found this lncRNA to be differentially expressed in their IBD patient samples, thereby providing further validation (22). Given its discovery on two independent analyses, *IFNG-AS1* presents itself as an ideal candidate for further study. As one of the first mechanistic studies for lncRNAs in the IBD field, this work focused on *IFNG-AS1*. Future studies will be needed to investigate additional lncRNAs involved in IBD pathophysiology.

IFNG-AS1 is a noncoding transcript located on chromosome 12 and adjacent to the *IFNG* gene. Prior studies have suggested that *IFNG-AS1* can positively regulate *IFNG* expression; however, much of the data implies function from the mouse ortholog *ifng-as1/NeST/tmevpg1*. These previous investigations have shown that *ifng-as1* is expressed in immune cells such as CD8 and CD4 T cells (5, 11, 37). The prior studies highlighted a mechanism wherein *ifng-as1* recruits a subunit of MLL/SET1 H3K4 methyltransferase complex to the *ifng* promoter and lays down an activating histone methylation signal that promotes expression of *ifng* in mouse immune cells (11). This mechanism, however, has yet to be validated in the human context. Indeed, the human studies of *IFNG-AS1* by Collier et al. (5) in the context of general immune cell biology and Wang et al. (39) in the context of Sjogren syndrome have shown expression of this noncoding transcript within CD4+ T cells. Our own studies show that a site of expression of *IFNG-AS1* is within these CD4+ cells. Using knockdown and overexpression constructs in human T cells, we directly show that *IFNG-AS1* expression can positively regulate IFNG production. We suspect the relatively low abundance of the IFNG-AS1 transcript results in modest knockdown efficiencies seen in our model system. However, our data suggest that within immune cells there is a mechanistic link between the overexpression of this noncoding RNA in colitis samples and the production of a key inflammatory cytokine. In addition, lncRNAs have also been implicated in the stability of mRNA transcripts, thereby providing additional modes by which IFNG-AS1 could potentially regulate its targets (38).

Through this work, we have identified a novel set of lncRNAs implicated in UC pathophysiology. Additional studies that are focused not only on *IFNG-AS1* but also on the other noted lncRNAs presented in this work will be essential in expanding our knowledge of UC pathogenesis. lncRNAs have served as novel targets in preclinical studies from other fields of medicine such as cancer biology and may serve as important therapeutic targets in UC (19). Additionally, lncRNAs could also serve as important biomarkers with a range of uses (34). lncRNA biology is a nascent field in IBD and much work is needed to expand its potential applications. Future studies evaluating the effects of the IBD susceptibility SNPs on expression of lncRNAs will help to link the genotypic changes seen in patients with specific SNPs with expression changes in SNP-associated genes. Additionally, mouse models of colitis will need to be employed to better define the mechanisms for lncRNA biology. To this end, this study has identified that the murine ortholog *ifgn-as1* is also

overexpressed in the context of mouse models of colitis. This opens up additional avenues of research to carefully dissect other mechanisms of action for this noncoding RNA. The generation of *ifng-as1* knockout and transgenic animals provides additional tools to investigate its function (11). All told, the lncRNA biology serves as an additional layer of gene regulation adding to the complexity of IBD pathophysiology but also providing new tissue-specific targets.

Acknowledgements

Figures

Figure 1.

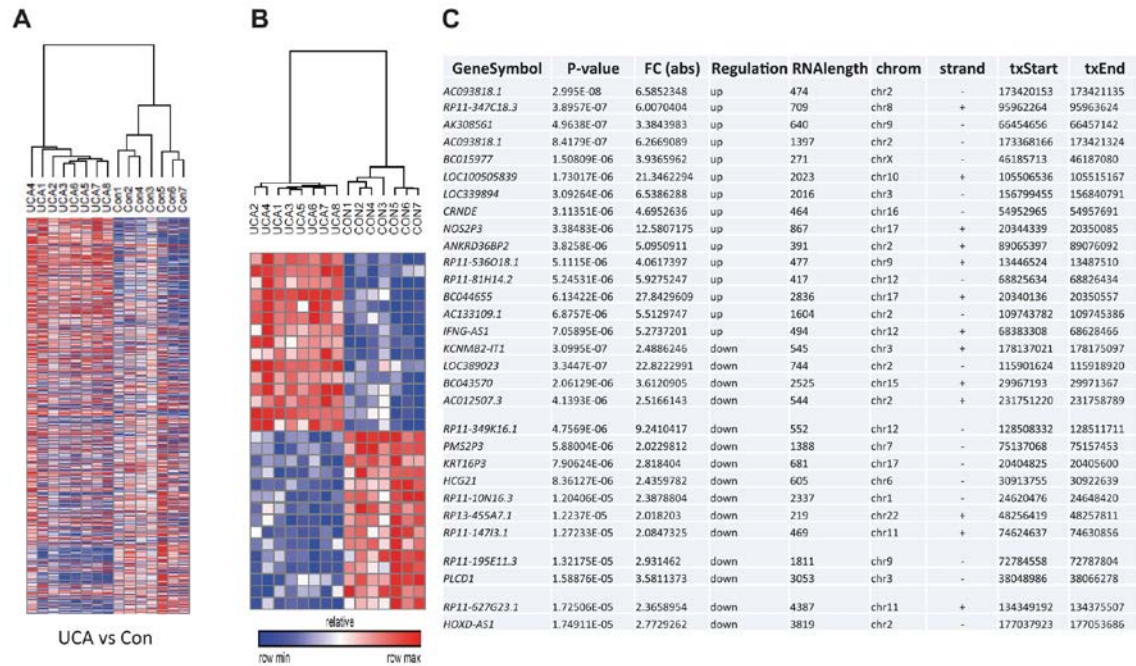| GeneSymbol	P-value	FC (abs)	Regulation	RNAlength	chrom	strand	txStart	txEnd
AC093818.1	2.995E-08	6.5852348	up	474	chr2	-	173420153	173421135
RP11-347C18.3	3.8957E-07	6.0070404	up	709	chr8	+	95962264	95963624
AK308561	4.9638E-07	3.3843983	up	640	chr9	-	66454656	66457142
AC093818.1	8.4179E-07	6.2669089	up	1397	chr2	-	173368166	173421324
BC015977	1.50809E-06	3.9365962	up	271	chrX	-	46185713	46187080
LOC100505839	1.73017E-06	21.3462294	up	2023	chr10	+	105506536	105515167
LOC339894	3.09264E-06	6.5386288	up	2016	chr3	-	156799455	156840791
CRNDE	3.11351E-06	4.6952636	up	464	chr16	-	54952965	54957691
NOS2P3	3.38483E-06	12.5807175	up	867	chr17	+	20344339	20350085
ANKRD36BP2	3.8258E-06	5.0950911	up	391	chr2	+	89065397	89076092
RP11-536O18.1	5.1115E-06	4.0617397	up	477	chr9	+	13446524	13487510
RP11-81H14.2	5.24531E-06	5.9275247	up	417	chr12	-	68825634	68826434
BC044655	6.13422E-06	27.8429609	up	2836	chr17	+	20340136	20350557
AC133109.1	6.8757E-06	5.5129747	up	1604	chr2	-	109743782	109745386
IFNG-AS1	7.05895E-06	5.2737201	up	494	chr12	+	68383308	68628466
KCNMB2-IT1	3.0995E-07	2.4886246	down	545	chr3	+	178137021	178175097
LOC389023	3.3447E-07	22.8222991	down	744	chr2	-	115901624	115918920
BC043570	2.06129E-06	3.6120905	down	2525	chr15	+	29967193	29971367
AC012507.3	4.1393E-06	2.5166143	down	544	chr2	+	231751220	231758789
RP11-349K16.1	4.7569E-06	9.2410417	down	552	chr12	-	128508332	128511711
PMS2P3	5.88004E-06	2.0229812	down	1388	chr7	-	75137068	75157453
KRT16P3	7.90624E-06	2.818404	down	681	chr17	-	20404825	20405600
HCG21	8.36127E-06	2.4359782	down	605	chr6	-	30913755	30922639
RP11-10N16.3	1.20406E-05	2.3878804	down	2337	chr1	-	24620476	24648420
RP13-455A7.1	1.2237E-05	2.018203	down	219	chr22	+	48256419	48257811
RP11-147I3.1	1.27233E-05	2.0847325	down	469	chr11	+	74624637	74630856
RP11-195E11.3	1.32175E-05	2.931462	down	1811	chr9	-	72784558	72787804
PLCD1	1.58876E-05	3.5811373	down	3053	chr3	-	38048986	38066278
RP11-627G23.1	1.72506E-05	2.3658954	down	4387	chr11	+	134349192	134375507
HOXD-AS1	1.74911E-05	2.7729262	down	3819	chr2	-	177037923	177053686

Fig. 1. A: heat map representation of expression changes between UCA and control (Con) groups with a fold change greater than twofold and *P* value less than 0.05. B: heat map representation of the top 15 upregulated and 15 downregulated lncRNAs in the UCA vs. Con comparison. C: table shows the identity of the lncRNAs along with their associated *P* value, fold change, direction of regulation, RNA length, chromosomal location (chrom), strand location (strand), and transcriptional start (txStart) and end (txEnd) positions. Row min and max refer to the minimum and maximum expression levels.
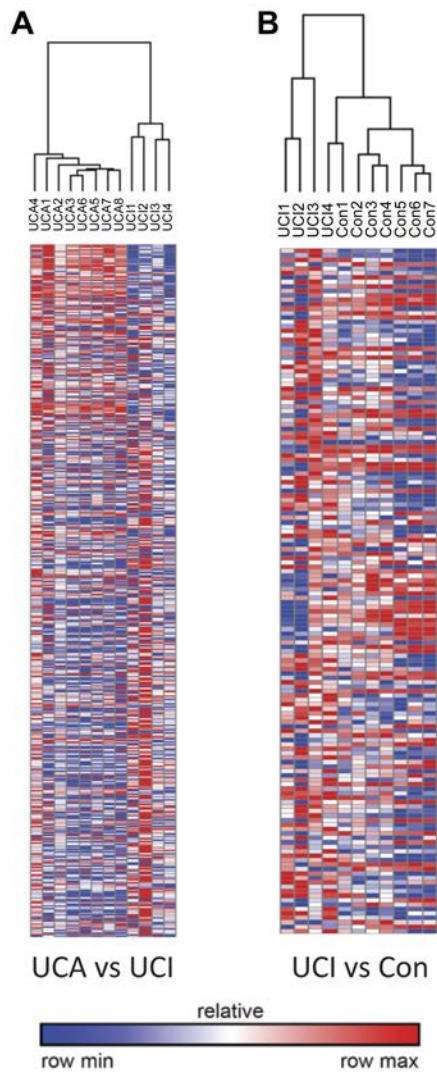
Figure 2.

Fig. 2. Heat map representations of expression changes in lncRNA transcripts between UCA vs. UCI, UCI vs. Con. The number of differentially expression lncRNAs with a fold change cutoff of 2 and a *P* value cutoff of 0.05.
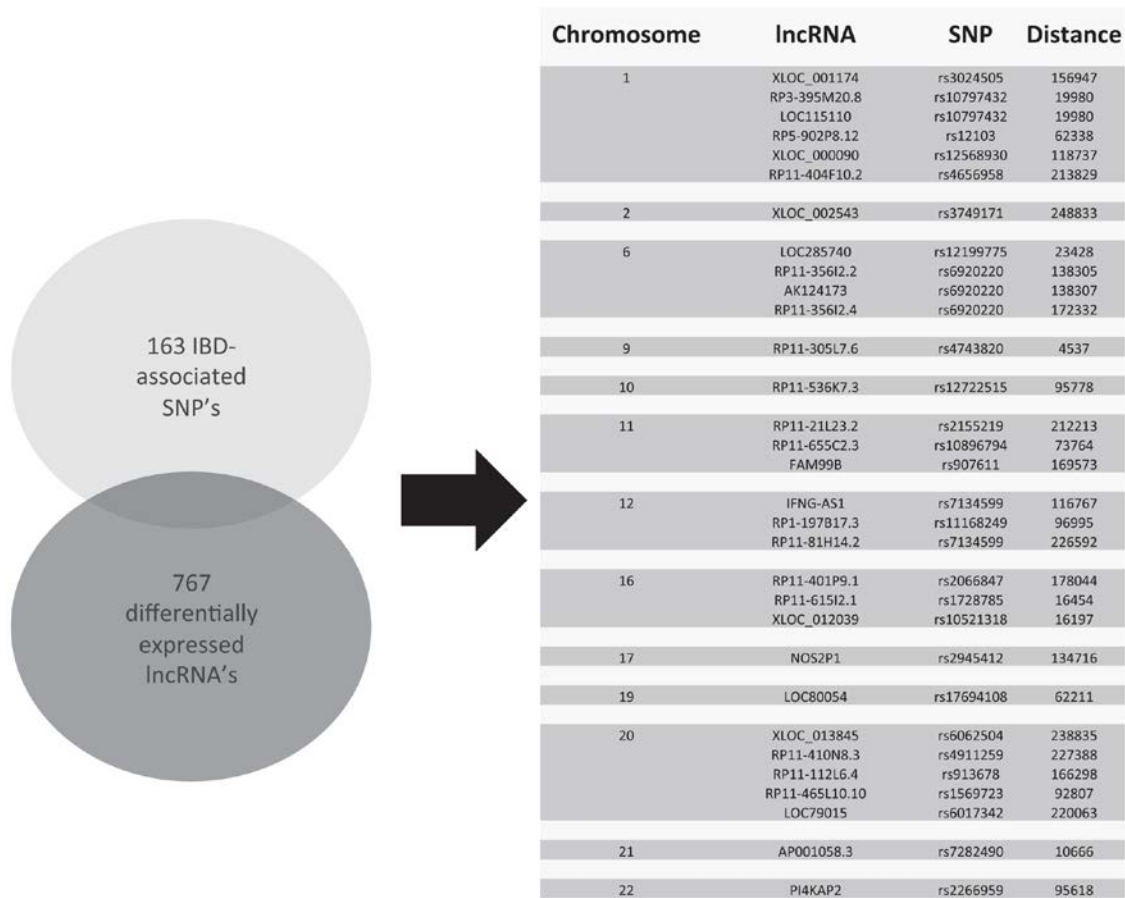
Figure 3.

Chromosome	lncRNA	SNP	Distance
1	XLOC_001174	rs3024505	156947
	RP3-395M20.8	rs10797432	19980
	LOC115110	rs10797432	19980
	RP5-902P8.12	rs12103	62338
	XLOC_000090	rs12568930	118737
	RP11-404F10.2	rs4656958	213829
2	XLOC_002543	rs3749171	248833
6	LOC285740	rs12199775	23428
	RP11-356I2.2	rs6920220	138305
	AK124173	rs6920220	138307
	RP11-356I2.4	rs6920220	172332
9	RP11-305L7.6	rs4743820	4537
10	RP11-536K7.3	rs12722515	95778
11	RP11-21L23.2	rs2155219	212213
	RP11-655C2.3	rs10896794	73764
	FAM99B	rs907611	169573
12	IFNG-AS1	rs7134599	116767
	RP1-197B17.3	rs11168249	96995
	RP11-81H14.2	rs7134599	226592
16	RP11-401P9.1	rs2066847	178044
	RP11-615I2.1	rs1728785	16454
	XLOC_012039	rs10521318	16197
17	NOS2P1	rs2945412	134716
19	LOC80054	rs17694108	62211
20	XLOC_013845	rs6062504	238835
	RP11-410N8.3	rs4911259	227388
	RP11-112L6.4	rs913678	166298
	RP11-465L10.10	rs1569723	92807
	LOC79015	rs6017342	220063
21	AP001058.3	rs7282490	10666
22	PI4KAP2	rs2266959	95618

Fig. 3. Graphical representation of the overlap between the 163 IBD associated SNPs and the lncRNAs commonly differentially expressed between UCA vs. Con and UCA vs. UCI. The chromosomal location, lncRNA identity, associated SNP, and genomic distance from the SNPs are presented.
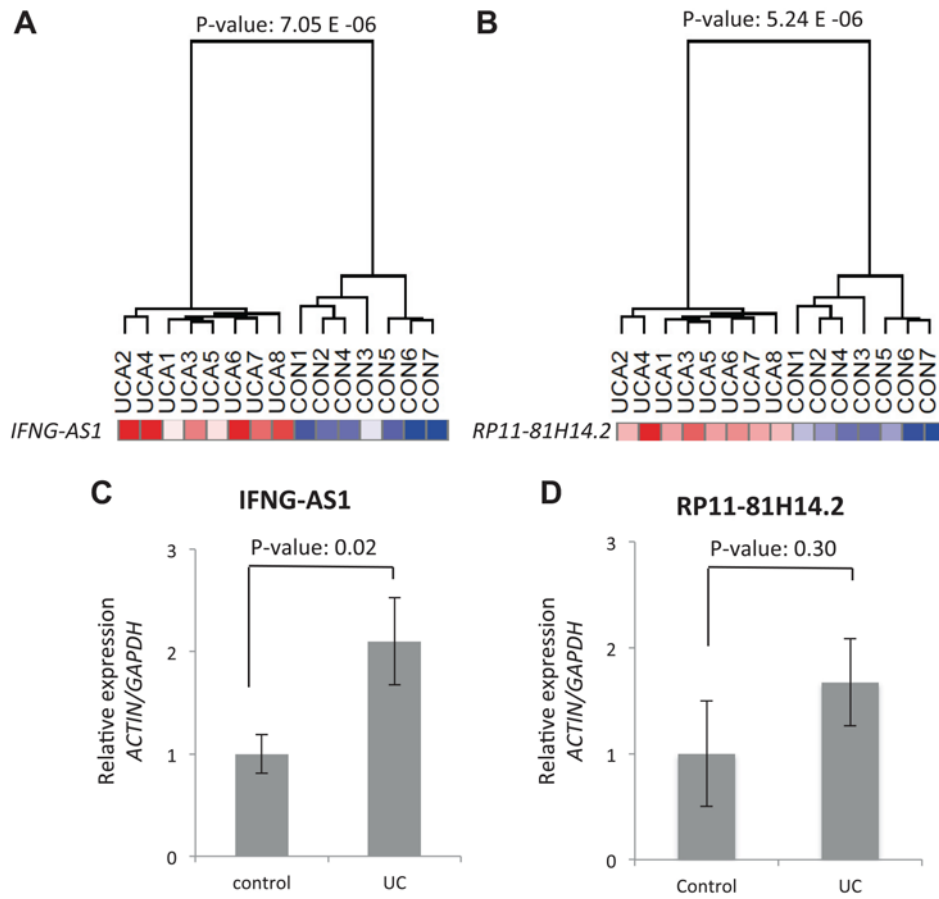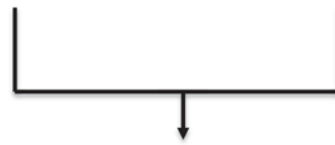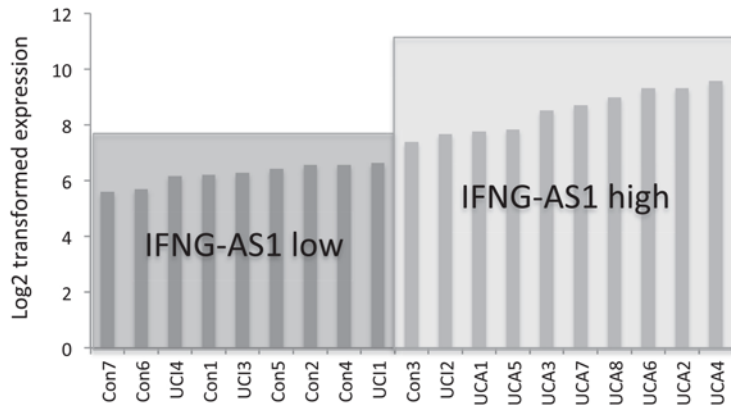
Figure 4.

Fig. 4. A and B: microarray expression data for the lncRNAs IFNG-AS1 and RP11-81H14.2 in the analysis comparing UCA vs. Con. C and D: qPCR expression for IFNG-AS1 and RP11- 81H14.2 in the validation cohort (control N = 16, UC N = 15). ACTIN and GAPDH were used as control housekeeping genes. Relative expression was to the average expression in the control samples.

Figure 5.

Upstream Regulator	Molecule Type	Predicted Activation State	p-value
TNF	cytokine	Activated	9.53E-14
IL1B	cytokine	Activated	1.59E-11
IFNG	cytokine	Activated	3.03E-17
IL1	group	Activated	1.96E-15
IL6	cytokine	Activated	1.55E-14

Fig. 5. Expression of IFNG-AS1 based on microarray expression across all UC and control samples. Two groups were created showing relative high and low levels of IFNG-AS1. Bioinformatic analysis was performed on the associated coding transcript expression changes between the two groups. A list of upstream regulators found to be activated in the IFNG-AS1 high group are listed along with the predicated activation state and the associated P value.
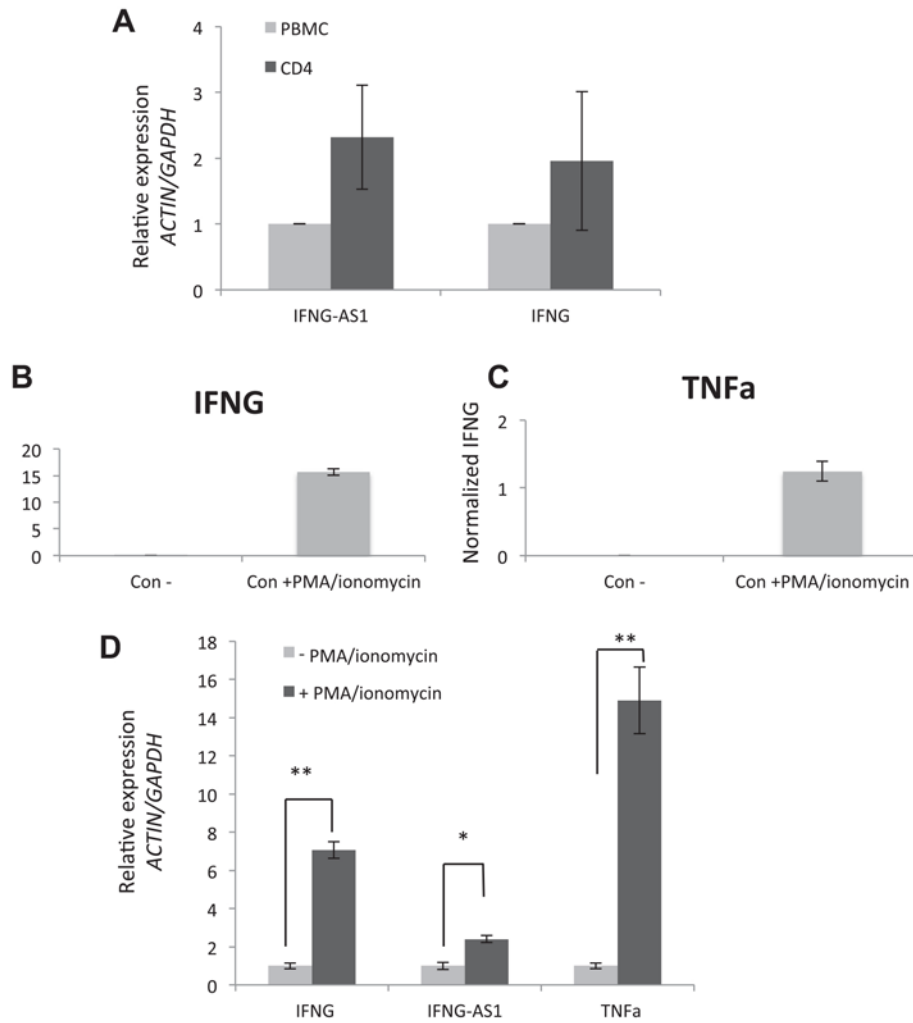
Figure 6.

Fig. 6. A: qPCR expression of IFNG-AS1 and IFGN among CD4 cells and total PBMC ($N = 4$). B and C: Jurkat T cells activated with PMA/ionomycin for 6 h and cultured media collected after 24 h show inductions of IFNG and TNF-α protein, respectively. D: Jurkat T cells activated with PMA/ionomycin for 6 h; qPCR analysis of IFNG, IFNG-AS1, and TNF-α. *P value < 0.05, **P value < 0.01; $n = 3$.
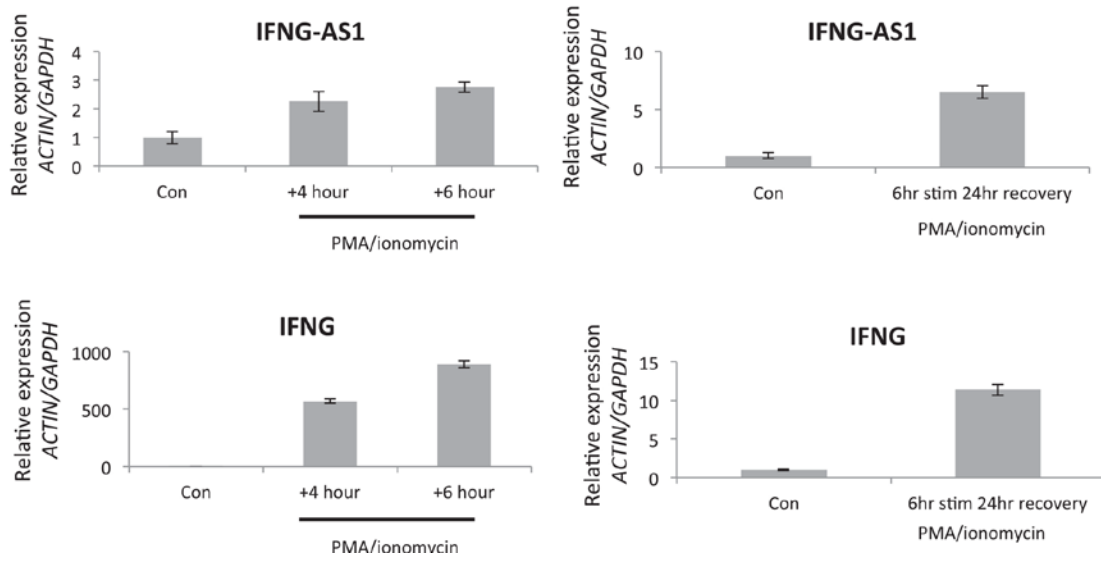
Figure 7.

Fig. 7. qPCR expression for a time course evaluating the expression IFNG-AS1 and IFNG in Jurkat T cells.
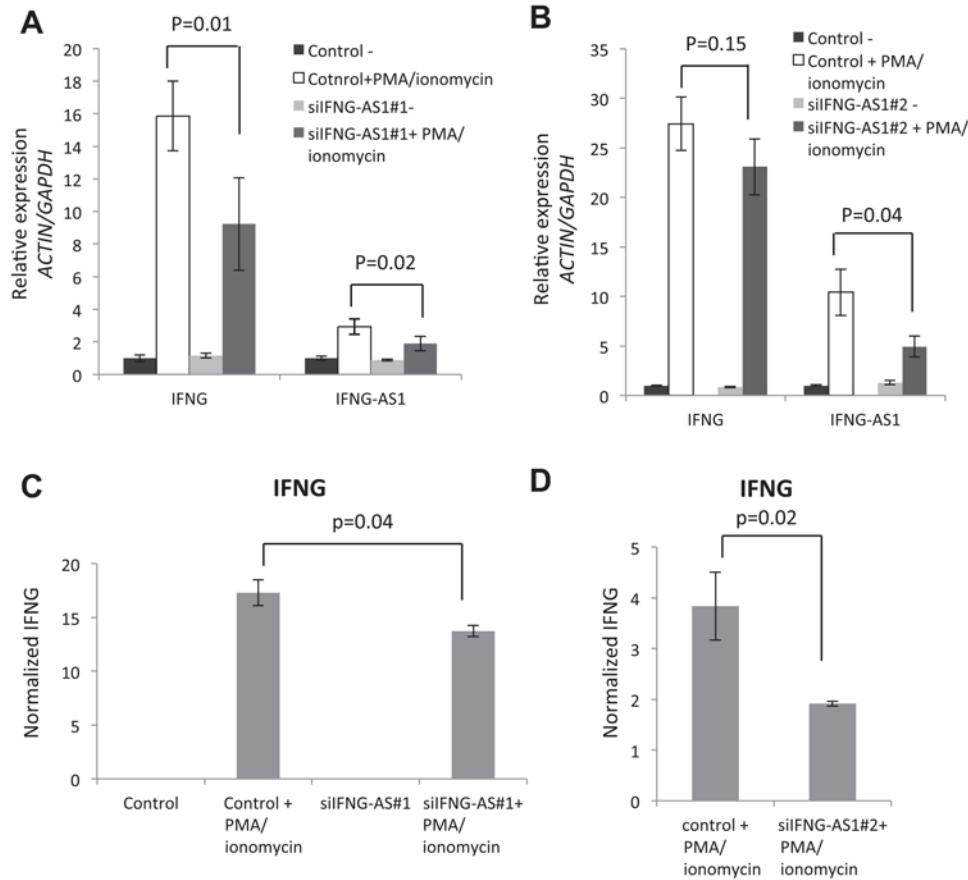
Figure 8.

Fig. 8. *A* and *B*: qPCR analysis of Jurkat T cells treated with PMA/ionomycin for 6 h followed by a 24-h recovery period. Cells were treated with 2 independent antisense siRNAs against IFNG-AS1 (*n* = 3). *C* and *D*: protein analysis for IFNG was assessed by measuring the culture medium (*n* = 3). The siRNAs refer to small interfering RNAs with siIFNG-AS1#1 and siIFNG-AS1#2 referring to two independent small interfering RNAs directed against IFNG-AS1.
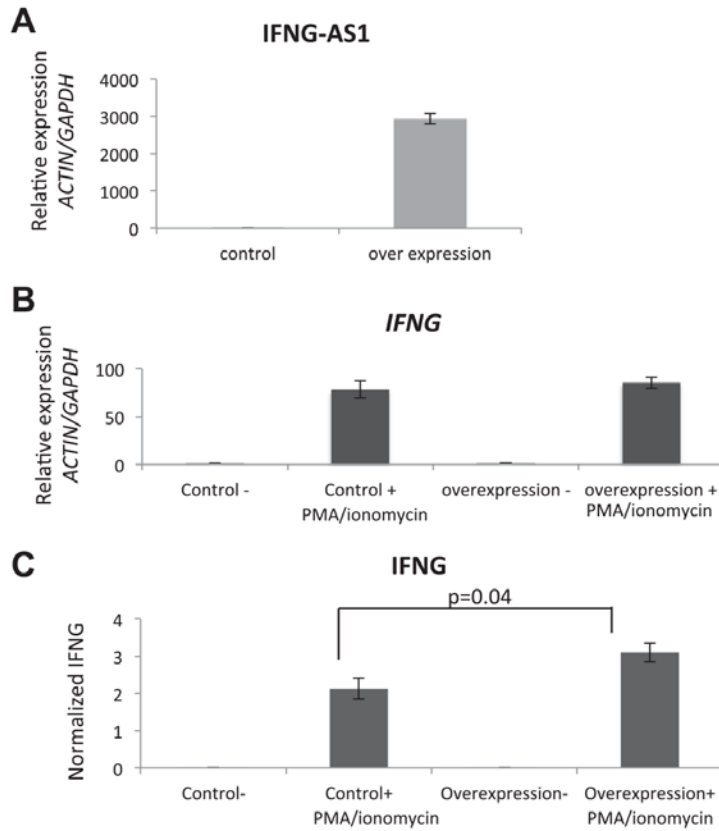
Figure 9.

Fig. 9. A: qPCR expression of Jurkat T cells transiently overexpressing IFNG-AS1. B: qPCR analysis of Jurkat T cells with and without transient transfection of an IFNG-AS1-overexpressing construct. Cells were activated with PMA/ionomycin for 4 h ($n = 3$). C: IFNG protein was measured from cultured media ($n = 3$).
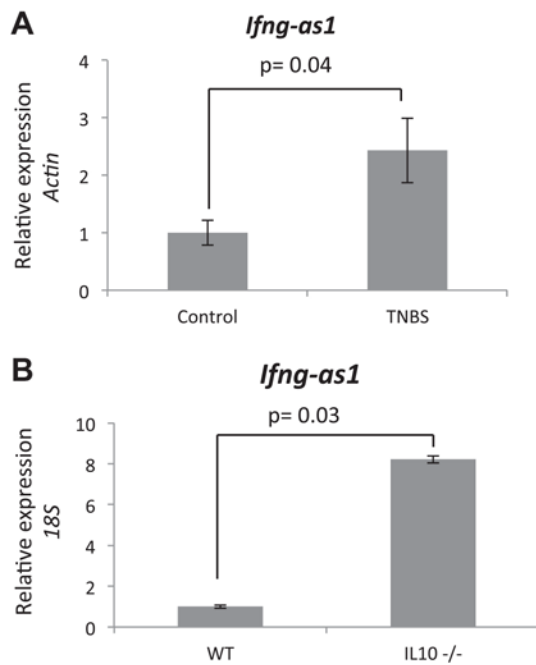
Figure 10.

Fig. 10. *A*: Ifgn-as1 qPCR expression analysis. Whole colon samples from TNBS-treated animals vs. controls (*N* = 5) were assays for Ifng-as1 expression with actin as the control gene. *B*: Ifng-as1 qPCR analysis from colon tissue derived from IL10 knockout animals compared with controls (*N* = 4).

Tables

Table 1. Patient information

Sample	Age	Gender	Location
Con1	31	Male	Colon
Con2	45	Female	colon
Con3	26	Male	Colon: sigmoid
Con4	46	Male	Colon
Con5	50	Female	Colon
Con6	60	Female	Colon
Con7	56	Male	Colon
UCA1	30	Female	Colon
UCA2	56	Female	Colon: left
UCA3	26	Female	Colon
UCA4	46	Female	Colon
UCA5	29	Male	Colon
UCA6	24	Male	Colon
UCA7	45	Female	Colon
UCA8	26	Female	Colon
UCI1	31	Male	Colon
UCI2	71	Female	Colon
UCI3	51	Male	Colon
UCI4	33	Female	Colon

Table 2. Ulcerative colitis and control samples

Control	Disease	Age	Gender	Pathology
1	Diverticulosis	n/a	n/a	n/a
2	rectal cancer	50	F	benign colonic tissue

3	rectal villous adenoma	68	F	non-dysplastic mucosa
4	metastatic colon cancer	53	M	colonic tissue with no histopathologic abnormality
5	colon cancer, cecum	76	F	invasive adenocarcinoma of cecum with adajcent normal colon
6	colon cancer, rectum	70	M	colonic adenocarcinoma with adjacent normal colon
7	colon-normal	n/a	n/a	n/a
8	colon-normal	n/a	n/a	n/a
9	colon-normal	n/a	n/a	n/a
10	colon-normal	n/a	n/a	n/a
11	colon-normal	n/a	n/a	n/a
12	colon-normal	42	F	foci of endometriosis, large intestine colon without significant change
13	colon-normal	n/a	n/a	n/a
14	colon cancer	n/a	n/a	n/a
15	perforation	n/a	n/a	n/a
16	vaginal prolapse	n/a	n/a	n/a
UC				
1	ulcerative colitis	61	F	moderate toseverely active ulcerative colitis, neg for dysplasia
2	ulcerative colitis	49	M	chronic pancolitis with severe activity, extensive ulcerations
3	ulcerative colitis	52	F	moderate to severe diffuse active pancolitis neg for dysplasia
4	ulcerative colitis	35	M	moderate to severe activity, chronic pancolitis, neg dysplasia
5	ulcerative colitis	68	F	severely active ulcerative colitis
6	ulcerative colitis	31	M	chronic active colitis with foci of ulceration
7	ulcerative colitis	43	M	n/a
8	ulcerative colitis	57	M	chronic colitis with moderate to severe activity and extensive ulcerations

9	ulcerative colitis	39	F	moderately active ulcerative colitis, neg dysplasia
10	ulcerative colitis	30	F	colon with moderate chronic colitis, no dysplasia
11	ulcerative colitis	36	M	chronic pancolitis with moderate to severe activity, ujlcration and inflammatory polyps
12	ulcerative colitis	49	F	n/a
13	ulcerative colitis	64	M	severe inflammatory bowel disease type indeterminate
14	ulcerative colitis	34	F	Rectosigmoid, Involved
15	ulcerative colitis	n/a	n/a	n/a

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