REST Negatively and ISGF3 Positively Regulate the Human STAT1 Gene in Melanoma

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Abstract

STAT1 plays a pivotal role in signal transduction and transcriptional activation in response to type I and II IFNs. Regulation of STAT1 expression has significant consequences in human cancer cells, where STAT1 deficiencies have been associated with cellular resistance to type I IFN. Distinct promoter, enhancer, and repressor regions have previously been described in the regulatory part of the human STAT1 gene extending as far as the second intron. A putative IFN-stimulated response element sequence in the STAT1 promoter is inducible by type I IFN and binds the IFN-α/β-induced complex, ISGF3. Together with the previously characterized IRF-E/GAS/IRF-E (IGD) motif, these positive regulatory elements provide a means for intracellular amplification of STAT1 expression, which is necessary for increasing cell responsiveness to the IFNs. In contrast, the transcriptional repressor REST binds to an RE-1 element in the STAT1 repressor region and in doing so represses transcription from the STAT1 gene regulatory region in melanoma cell lines. Repression significantly decreased in a REST-null cell line. Altering REST function from a transcriptional repressor into an activator as REST-VP16 increased expression from RE-1–targeted reporters. RNA expression of 65 melanoma cell lines by microarray and selected lines with known IFN responsiveness showed significant inverse correlations between STAT1/REST that were related to cellular responses to IFN. Thus REST, through the intronic RE-1 element, provides a means for downregulating STAT1 expression, affecting melanoma responsiveness to IFN. Intracellular levels of REST may be a useful marker to test for IFN resistance and as a novel therapeutic target in IFN-resistant melanomas. Mol Cancer Ther; 12(7); 1288–98. ©2013 AACR.

Introduction

STAT1 has a central role in the JAK/STAT signaling used by type I and II IFNs (reviewed in refs. 1, 2). The transcriptional activation complexes, ISGF3 (comprising phospho-STAT1, phospho-STAT2, and IRF-9) and GAF (a homodimer of phospho-STAT1), which assemble in response to type I and II IFNs, respectively, translocate to the cell nucleus and bind to their signature DNA sequences called IFN-stimulated response elements (ISRE) and IFN-γ–activated sites (GAS). ISRE sequences usually comprise repeats of either 5’T-TTT-C-3’ or its complement, 5’-GAAA-3’, separated by 2 nucleotides. In addition to the ISGF3 complex, ISREs have been shown to bind IRF-1, IRF-2, and IRF-3 (3, 4), and with lower affinity, IRF-8, Blimp-1, and a trimeric complex of STAT1 homodimer/IRF-9 (5–7). Recently, ISREs were also shown to bind an ISGF3 variant called ISGF3g, containing unphosphorylated STAT2, in response to IFN-γ (8). The GAS elements that show greatest affinity for phospho-STAT1 homodimers comprising GAF complexes share the consensus sequence TTCCNN(G/T)AA (9). Sequences similar to GAS elements that can bind a range of STAT homo- and heterodimers activated by various cytokines and growth factors are often referred instead as STAT-binding elements (SBE). SBEs bind STAT dimers with varying affinities and kinetics and usually comprise 2 half sites (TTIC/A) and (G/T)AA), or close variations of these separated by 2 to 4 bases (9, 10).

Binding of ISGF3 and GAF to their respective DNA-binding motifs activates expression of downstream IFN-stimulated genes (ISG). STAT1-dependent activation of ISG transcription is responsible for the range of antiviral, immunomodulatory, antiproliferative, and proapoptotic functions of the IFNs (11). STAT1 expression in tumor cells has been shown to be important for tumor elimination by immunosurveillance mechanisms (12). However, STAT1 expression is deficient in several different human cancers resistant to the antiproliferative and antiviral effects of type I IFNs (13–16). In malignant melanoma, loss of responsiveness to IFN-α was shown to correlate with decreased STAT1 protein and tyrosine-phosphorylated STAT1 (13, 16–18). Resistance to IFN-α presents a
major problem for the adjuvant treatment of high-risk patients with melanoma (those with stage IIB disease or higher), for whom response rates are typically less than 20% (19).

Previously, we identified a combined IRF-E/GAS/IRF-E (IGI) enhancer motif at the intron 1/exon 2 boundary of the human STAT1 gene (20). The IGI motif binds a high-molecular-weight complex containing IRF-1 and CBP in cells treated with either type I or II IFNs. A separate region containing a consensus ISRE sequence was identified in tissues (21) but was not characterized. Here, for the first time, we show binding of the ISGF3 complex to the STAT1 ISRE and show its inducibility by type I IFN.

Repressor element-1 silencing transcription factor (REST), or neuron-restrictive silencer factor, is a master DNA-binding protein whose critical function is to repress expression of neuron-specific genes in non-neuronal cells (22). However, REST acts as an oncogene in the development of brain tumors and other cancers and increased REST has been found in a range of neural derived cancers (reviewed in ref. 23). The Kruppel-like zinc finger domain of REST recognizes a 21-base repressor element-1 (RE-1) with consensus sequence 5'-NGT(G/C)AGA(C/A)(G/GCC-NNA/GG(A/G)(C/G)AGNNNC-3' (24). Upon binding to DNA, REST recruits the co-repressors Co-REST and mSin3A, which in turn recruit histone deacetylases and histone methylases to repress gene transcription (25, 26). Splice variants of REST are expressed at low levels in some tissues (27, 28) and are thought to antagonize the function of full-length REST (29).

Our previous analysis of the STAT1 gene recognized a region downstream of the promoter and enhancer regions strongly repressing transcription of luciferase gene reporters (20). In the present study, we define an RE-1 element within this repressor region, to which REST bound and repressed transcription. In the absence of REST, transcriptional repression of the STAT1 gene was dramatically reduced. Analysis of REST and STAT1 expression levels in human melanoma cells showed that REST is commonly expressed in melanomas and, together with transcriptional activation and chromatin immunoprecipitation (ChIP) assay, shows that REST negatively regulates STAT1 expression, contributing to the nonresponsiveness of melanomas to IFN.

Materials and Methods

Cell culture

All cell lines were obtained and cultured as described previously (13, 30). The rat insulinoma cell line, INS-1 (obtained from Dr. Trevor J. Biden, Garvan Institute of Medical Research, Sydney, Australia) was cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS, 1% Glutamax (Life Technologies), and 1.9 mmol/L sodium pyruvate. SK-MEL-28 and MM96 human melanoma cell lines used in this study were authenticated by their IFN responsiveness and relative content of STAT1 and INS-1 by its deficient level of REST expression (31, 32). Where indicated, cells were stimulated with 1,000 IU/mL human IFN-α2b (Hoffmann-La Roche), 1,000 IU/mL human IFN-β (Biogen), or 1,000 IU/mL human recombinant IFN-γ (Hoffmann-La Roche).

Electromobility shift assays

Whole-cell extracts were prepared and assayed using methods described (33). For more detail, also see Supplementary Methods and Materials.

Transient transfection and dual-luciferase reporter assays

The construction of pGL3-STAT1 reporter vectors is outlined in the Supplementary Methods and Materials. Subconfluent melanoma cell cultures were prepared in antibiotic-free medium supplemented with 10% FBS. Unless otherwise indicated, cells were cotransfected with pGL3-STAT1 promoter construct vector DNA (1 µg) and phRL-SV40 (10 ng) using 2.5 µL GenJet transfection reagent (SignaGen Laboratories) according to the manufacturer’s protocol. PhRL-SV40, encoding luciferase from Renilla reniformis, was used as an internal calibration control for normalization of firefly luciferase levels. Dual-luciferase assays were carried out using previously described methods (34). Luciferase activity was detected using a FLUOstarOPTIMA (BMG Labtech).

Chromatin immunoprecipitation assay

The specific method used for chromatin immunoprecipitation (ChIP) is outlined in the Supplementary Methods and Materials. About 2 µL ChIP samples and 0.5 µL input samples were used for PCR amplification. Quantitative PCR (Q-PCR) analyses were carried out in 20 µL reactions containing 10 µL PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences) and 500 nmol/L forward and reverse primers. Primers used for ChIP assays were: STAT1 (-RE-1 region)_fwd: 5'-GACTCACACTTGTGAGATCA-3', STAT1 (-RE-1 region)_rev: 5'-CTTGTGATGCCTCCAGAGTC-3', STAT1 (exon 3)_fwd: 5'-CATATAGGATGTCATGCAGG-3', STAT1 (exon 3)_rev: 5'-ACTCTCTCTTCTGTCTAGTG-3', SCG10 (-RE-1 region)_fwd: 5'-CCAGTAGCATCTCATACGT-3', and SCG10 (-RE-1 region)_rev: 5'-CTCCAGGAACATACAGC-3'. PCR amplifications were carried out using an iCycler (Bio-Rad) with cycling parameters: 3 minutes at 95°C, then 43 cycles of: 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds. Endpoint analysis and PCR product specificity were confirmed by electrophoresis on 2% agarose gels. Results obtained by Q-PCR were represented as a percentage of input DNA by subtracting the mean cycle threshold (Ct) of the respective input to obtain ΔCt values. % input was calculated by 100/2ΔCt.

Gene expression

For reverse transcriptase PCR (RT-PCR) data, total RNA was extracted from 1 × 10^6 cells and isolated using the High Pure RNA Isolation Kit (Hoffmann-La Roche) according to the manufacturer’s protocol. About 1 µg of RNA was used for cDNA synthesis using the Transcripter.
First Strand cDNA Synthesis Kit (Hoffmann-La Roche) according to the manufacturer’s protocol. A total of 0.5 μL of the cDNA reaction was used for Q-PCR amplification of specific target genes in 20 μL reactions containing 10 μL PerfeCta SYBR Green FastMix for IQ and 500 nmol/L forward and reverse primers. Primers used for Q-PCR were: STAT1_fwd: 5'-GGCAGAAGGATGCTGA-3', STAT1_rev: 5'-GTCAGGACATCAGCAAC-3', REST_fwd: 5'-GTAGGCGATCATCAGTGG-3', REST_rev: 5'-CCCATTGTAACCTGTCTGC-3' (specific for full-length REST mRNA), 18S_rRNA_fwd: 5'-CTTAGAGGGACAAGTGGCG-3' and 18S_rRNA_rev: 5'-ACGCTGAGCCAGTCAGTGTA-3'. PCR amplifications were carried out using an IQ iCycler with cycling parameters: 3 minutes at 95°C, then 40 cycles of: 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds. For the microarray expression data analysis, the data sets used are indicated in the figure legends. Data for the mRNA analysis of levels of REST versus STAT1 in 65 different melanoma cell lines were obtained from A. Pritchard and N. Hayward (personal communication/unpublished data, QIMR, Australia). Cancer Cell Line Encyclopedia (CCLE) data were obtained from the Broad Institute CCLE website (http://www.broadinstitute.org/ccle/home), accession number GSE36133. Supplementary Tables S1 to S4 show the raw data according to MIAME guidelines.

Western blotting
Proteins were separated on an SDS-PAGE gel and transferred onto BioTrace PVDF membrane (Pall Corporation). Membranes were blocked and incubated with primary and secondary antibodies in 5% (w/v) skim milk powder, PBS, and 0.1% Tween-20. Following antibody incubations, membranes were washed 3 times in copious volumes of PBS containing 0.1% Tween-20. Immunodetection was carried out using SuperSignal West Pico ECL substrate (Thermo Scientific), detected on a Gel Doc 2000 system (Bio-Rad) and analyzed using Quantity One software (Bio-Rad). Membranes were stripped at 60°C for 20 minutes using a buffer containing 62.5 mmol/L Tris-Cl, pH 6.8, 2% (w/v) SDS, and 100 mmol/L β-mercaptoethanol. Primary antibodies used for immunoblotting were rabbit polyclonal anti-REST (Millipore; cat# 07-579), specific for the full-length isoform, mouse monoclonal anti-STAT1 (Cell Signaling Technology; cat# 9176) and mouse monoclonal anti-α-tubulin (DMIA; Sigma-Aldrich; cat# T92026). Secondary antibodies used for immunoblotting were horseradish peroxidase-conjugated sheep anti-mouse IgG (Chemicon; cat# AP326P) and horseradish peroxidase-conjugated sheep anti-rabbit IgG (Cell Signaling Technology, cat# 7074).

REST-VP16 cloning
The NheI/NcoI fragment of pcDNA3.1-REST-VP16 (35) was subcloned into NheI/NcoI-digested plasmid, pEFRES-Puro. The complete sequence of the REST-VP16 insert was verified by sequencing a selected clone.

Statistical analysis
To facilitate statistical analysis, experiments were carried out using no fewer than triplicate reactions. The differences between control and test were determined by independent samples t test (α = 0.05). Significance for Pearson correlation coefficient (r) was determined by P value (one-tailed). All luciferase assays, electromobility shift assays (EMSA), ChIP assays, Western blots, and RT-PCR assays were reproduced in at least 2 independent experiments.

Supplementary data
Full-length, uncropped gel images from Supplementary Figures S1A, S4A, S5A, S6B, S6D, and S7 are presented in the Supplementary Data, which also includes Supplementary Tables S1 to S4 containing the microarray gene expression results used to obtain Fig. 7B and D–F.

Results
Characterization of an ISRE in the human STAT1 gene promoter
A consensus ISRE sequence was previously identified in the STAT1 promoter (20) immediately upstream of the first exon (Supplementary Fig. S1A). The sequence of this motif is fully conserved between human and chimpanzee and highly conserved among less related mammalian species, although the spacing of the GAAA repeats has changed (Supplementary Fig. S1B). To confirm that the human sequence functions as an ISRE, EMSA was conducted using oligonucleotides spanning the ISRE with 10 bases flanking either side, plus overhangs for radiolabeling and cloning purposes.

As previously described (36), SK-Mel-28 melanoma cells were primed overnight with IFN-γ before treatment with IFN-β to enhance the assembly of ISGF3 complexes. The results showed binding of an IFN-β-dependent complex with the same mobility as that bound to the confirmed ISRE from the ISG15 gene but with less mobility than the STAT1 homodimer complex that binds to the sis-inducible element (SIE) from the c-fos gene (m67 mutation) in response to IFN-γ (Fig. 1A; ref. 37). The complex bound to the ISRE of the STAT1 gene was not detectable in the presence of anti-STAT2 and anti-IRF-9 antibodies, confirming the identity of the complex as ISGF3. The STAT1 ISRE oligonucleotides cloned into the basal luciferase reporter vector, pGL3-basic, were shown to be inducible in SK-Mel-28 cells by IFN-α2b (2.0-fold, P < 0.001) and IFN-β (2.6-fold, P < 0.001), but not by IFN-γ (Fig. 1B). Upon mutating the ISRE, it was no longer inducible by type I IFN.

Determining the location of a repressor element in the STAT1 gene
A region downstream of base +498 in the STAT1 promoter and enhancer was previously shown to possess potent repressor activity (20). To determine the precise location of the repressor site, vectors were constructed...
using different regions of the STAT1 gene extending further downstream than the promoter/enhancer regions, which were cloned into the pGL3-basic (Fig. 2A). The constructs were transiently transfected into melanoma cell lines, SK-Mel-28 and MM96, and analyzed for constitutive luciferase activity. Constructs extending as far as base +1,033 showed a high constitutive level of luciferase activity. The addition of bases +1,034 to +1,156 reduced the constitutive luciferase activity by more than 99% and 98% in SK-Mel-28 (P < 0.01) and MM96 (P < 0.01) cells, respectively (Fig. 2B and C).

Identification of an RE-1 site in the STAT1 repressor region

Scrutiny of the STAT1 region containing repressor activity identified a sequence sharing close homology to the consensus sequence of an RE-1 element (24) to which the known transcriptional repressor, REST, binds. The putative STAT1 RE-1 element matched the consensus RE-1 sequence at 18 of 21 bases (Fig. 3A), which represents considerable homology given that some mismatching can be tolerated in RE-1 elements while still maintaining binding of REST (22). Although the sequence of this motif is fully conserved between human and chimpanzee, it is not well-conserved among less related mammalian species (Fig. 3B).

To determine whether the putative RE-1 element mediated the repression observed from the whole repressor region, oligonucleotides containing only the 21-bp sequence were ligated onto the 3'-end of the STAT1prom/enh insert (~388, +1,033), generating the construct pGL3:STAT1-RE-1 (Fig. 3C). To provide additional confirmation of the repression of transcriptional activity by the RE-1 element, luciferase constructs containing tandem copies of the putative RE-1 sequence were prepared, including both STAT1-2`RE-1 with 2 copies and STAT1-4`RE-1 with 4 copies. As a control for REST activity, pGL3:STAT1/SCG10-RE-1 was generated, where-in oligonucleotides containing the well-characterized RE-1 element from the REST-repressible neuronal gene, SCG10 (22), were ligated onto the 3'-end of the STAT1prom/enh insert.

Transient transfection of these constructs showed that a single copy of the putative RE-1 sequence from the STAT1 gene was sufficient to significantly reduce the luciferase expression when cloned downstream of the STAT1prom/enh insert [by 70% of the inhibitory levels mediated with the full STAT1 gene repressor region in SK-Mel-28 cells (P < 0.0001; Fig. 3D) compared with 78% of the total repression in MM96 cells (P < 0.0001; Fig. 3E)]. Both cell lines showed cumulative repression of the STAT1prom/enh as multiple copies of the RE-1 sequence were cloned in tandem (Fig. 3D and E), confirming this motif as the predominant repressor element within the repressor region of the human STAT1 gene.

REST binds to the STAT1 RE-1 site in melanoma cell lines

To show the binding of REST to the RE-1 element in the STAT1 gene in the context of chromatin in live melanoma cell lines, ChIP assays were conducted using a 211-bp ampiclon containing the RE-1 element. As a positive control, a 153-bp region of the SCG10 gene containing a previously validated RE-1 element was amplified whereas a 167-bp region of STAT1 encompassing part of the third exon was used as a negative control to validate the efficiency of chromatin shearing. Using chromatin from the melanoma cell line MM96, the region of STAT1 containing the RE-1 sequence was amplified following ChIP with an anti-REST antibody, as was the RE-1 region from...
SCG10 (Fig. 4A; for complete gel, refer to Supplementary Fig. S2). However, the region encompassing the third exon of STAT1 was not amplified.

To determine the extent of specificity of the REST immunoprecipitation and to enable a quantitative comparison between 2 different cell lines, quantitative ChIP was carried out using chromatin extracts from MM96 and SK-Mel-28 cells. Indeed, as expected, amplification using mouse whole IgG was negligible, whereas the binding of REST to the RE-1 elements was highly specific (Fig. 4B and C). Furthermore, the binding activity of REST to the STAT1 RE-1 element was slightly lower in SK-Mel-28 cells (11% of input) than in MM96 cells (13% of input). REST was shown to bind more avidly to the SCG10 RE-1 element; however, a similar pattern between the cell lines was observed (25% in SK-Mel-28 cells vs. 33% in MM96 cells).

Transcriptional repression of the STAT1 gene is decreased in a REST-null cell line

Previous studies of REST have used the rat insulinoma cell line, INS-1, as a REST knockdown because expression of REST in these cells was undetectable (31, 32). Similarly, we did not detect REST mRNA in INS-1 cells by RT-PCR (Fig. 5A; for complete gel, refer to Supplementary Fig. S3), nor was REST protein detectable by Western blotting (Fig. 5B; for complete uncropped gel, refer to Supplementary Fig. S4) and, hence, INS-1 is the equivalent of a REST genetic knockdown cell.

Given that REST was shown to be responsible for repressing transcription of the STAT1 gene through the RE-1 element (Fig. 3D and E), the same constructs were tested by transiently transfecting into untreated SK-Mel-28 cells (B) and MM96 cells (C). Luciferase reporter analysis was carried out using these constructs transfected into untreated SK-Mel-28 cells (B) and MM96 cells (C). Data are shown as mean RLU ± SE. #, P < 0.01; **, P < 0.0001 by 2-tailed t test, with respect to the previous construct.
region (STAT1prom/enh/repr) was used (90% reduction in INS-1 compared with nearly complete inhibition in SK-Mel-28 and MM96 cells, \( P < 0.0001 \)).

Altering REST function from transcriptional repressor into an activator as REST-VP16 induces expression from the STAT1 gene promoter region. Thus, a construct was tested where the recombinant transcriptional activator, REST-VP16, was generated by replacing the 2 repressor domains of REST with the activation domain from the herpes simplex virus (HSV) protein VP16 (Fig. 6A; ref. 35). By retaining the DNA-binding domain of REST, REST-VP16 is able to bind to RE-1 elements, competing with endogenous REST, but now switches from repressor to an activator of expression from REST target genes. To maximize the expression of the REST-VP16 protein, the REST-VP16 gene was cloned into the expression vector pEFires-Puro, placing REST-VP16 under the transcriptional control of the highly active EF-1a promoter. Cotransfection of pEFires-Puro:REST-VP16 into SK-Mel-28 cells with the previously described luciferase vectors resulted in a consistent induction compared with controls and significantly lowered levels of any RE-1–mediated repression (Fig. 6B).

**REST expression is inversely correlated with STAT1 expression in melanoma cells**

To determine whether REST-mediated repression might affect STAT1 expression in melanoma cells, RNA and protein were extracted from SK-Mel-28 and MM96 cells. Comparison of full-length REST mRNA by RT-PCR showed levels 21-fold higher in MM96 cells than in SK-Mel-28 cells (Fig. 7A; for complete blot, refer to Figure 3.)

**Figure 3.** Repression of the STAT1 promoter and enhancer regions by an RE-1 element within the STAT1 repressor region. A, alignment of the RE-1 element in the human STAT1 gene with the sequence for a canonical RE-1 element. B, alignment of the RE-1 element in STAT1 with the orthologous sequences in chimpanzee, mouse, rat, and dog. C, map showing the constructs containing none, single and tandem STAT1 RE-1 elements, and the SCG10 canonical RE-1 element. D and E, luciferase assay results in SK-Mel-28 cells (D) and MM96 cells (E). Data are shown as the mean RLU \( \pm \) SE. **, \( P < 0.0001 \) by 2-tailed t test, with respect to STAT1prom/enh.
were carried out in triplicate and the data are shown as the mean ChIP products are shown as the percentage of input DNA. Reactions MM96 (B) and SK-Mel-28 (C) cells. The relative abundances of indicated, ampli from MM96 cells were sheared, immunoprecipitated by the antibodies from IM96, real-time PCR was carried out on the immunoprecipitates from element in melanoma cell lines. A, cross-linked chromatin extracts of REST to the RE-1 (Fig. 1C and D), confirming this element as a bona fide ISRE. Although IRF-1 has also been shown to bind to ISREs, we did not observe binding using the time course described here, probably due to the requirement for de novo protein synthesis of IRF-1 following IFN treatment (3). A functional ISRE element in the promoter of the

Figure 4. ChIP assay shows binding of REST to the STAT1 RE-1 element in melanoma cell lines. A, cross-linked chromatin extracts from MM96 cells were sheared, immunoprecipitated by the antibodies indicated, amplified by PCR, and separated on an agarose gel. B and C, real-time PCR was carried out on the immunoprecipitates from MM96 (B) and SK-Mel-28 (C) cells. The relative abundances of ChIP products are shown as the percentage of input DNA. Reactions were carried out in triplicate and the data are shown as the mean ± SE. *, P < 0.001; **, P < 0.0001 by 2-tailed t test, with respect to the nonspecific IgG control.

Discussion

Resistance to IFN-α presents a significant challenge for the adjuvant treatment of high-risk melanoma, with response rates typically less than 20% (19). This loss of responsiveness to IFN-α has been shown to correlate with reduced levels of STAT1 expression (13, 16). Previously, an IGI enhancer motif at the intron 1/exon 2 boundary of the STAT1 gene was characterized and found to bind a complex containing IRF-1 and CBP. Interestingly, despite the presence of an SBE sequence within the IGI, only low-level binding of STAT1 was observed in EMSA reactions and only when recombinant STAT1 was used in the place of extracts from IFN-treated MCF-7 cells (20). A consensus GAS element in the IRF1 gene was also previously shown to bind STAT1 (40), suggesting the existence of an IFN-inducible or virus-inducible intracellular amplifier circuit involving the IRF1 and STAT1 genes and the proteins they encode. We have shown here that an ISRE exists imme-

Supplementary Fig. S5) whereas protein levels of the full-length REST were 7-fold higher in MM96 cells as determined by Western blotting (Fig. 7B; for complete blots, refer to Supplementary Figs. S6 and S7). Conversely, STAT1 expression was higher in SK-Mel-28 cells as determined by analysis of protein levels (Fig. 7B; for complete blots, refer to Supplementary Figs. S6 and S7) and previously by analysis of mRNA levels (13). RT-PCR analysis was then carried out on nine melanoma cell lines. Quantitative analysis determined that a significant inverse correlation ($r = -0.6286, P < 0.05$) existed between the levels of REST and STAT1 mRNA expression (Fig. 7C; for data used, refer to Supplementary Table S1). Moreover, the 2 cell lines, SK-MEL-28 (S1) and MM96 (R1) in Fig. 7C, were at either extreme of the graph, corresponding with their IFN sensitivity and IFN resistance, respectively.

For further validation of this correlation, expression microarray data from 65 melanoma cell lines, 26 B-cell and 18 T-cell lymphoma lines, were analyzed. Again a significant inverse correlation ($r = -0.2352, P < 0.05$) existed between STAT1 and REST in the large group of melanoma lines (Fig. 7D; for data used, refer to Supplementary Table S2). Also, SK-Mel-5 was at one extreme, with high REST/low STAT1 levels consistent with it being very resistant to IFN (38, 39). However, no relationship was apparent for REST and STAT1 when similar array data from B or T cell lines was analyzed (Fig. 7E and F; for data used, refer to Supplementary Tables S3 and S4).
STAT1 gene provides the potential for an additional positive feedback loop for STAT1 expression following viral or IFN stimulation. Such an autoregulatory loop, combined with the STAT1/IRF-1–positive feedback loop, described before may be responsible for the surge in expression of unphosphorylated STAT1 observed in many cell types in response to type I and II IFNs (41).

An RE-1 element that repressed transcription of luciferase from gene reporter constructs was identified in the second intron of the human STAT1 gene (Fig. 3C–E) and shown by ChIP assays binding the REST repressor protein in melanoma cells (Fig. 4A–C). The STAT1 RE-1 element differs at three of the 21 bases from the consensus RE-1 element (Fig. 3A) and, unlike most RE-1 elements, is not evolutionarily conserved (Fig. 3B). RE-1 elements that differ from the consensus sequence at 3 or more bases are common and they can differ by as many as 5 bases while still being able to bind REST (22). For this reason, attempts to mutate the STAT1 RE-1 element by as many as 4 bases were unsuccessful at altering its repressive activity (results not shown). Repression activity in REST-null INS-1 cells was reduced by 78% when the RE-1 element alone was fused to the promoter/enhancer construct and by 10% in the context of the full “repressor region”, when compared with melanoma cell lines (Fig. 5C). In addition, RE-1–mediated repression could be overcome in REST-positive SK-Mel-28 cells when they were cotransfected with the RE-1–targeted transcriptional activator, REST-VP16 by changing REST function from repressor to activator (Fig. 6B). Together these findings provide evidence of a regulatory function for REST on the expression of the human STAT1 gene.

RE-1 elements are common in the regulatory regions of genes involved in the terminal differentiation of neurons.
Genes containing RE-1 elements are repressed in non-neuronal tissues due to the abundant expression of REST, which is abundantly expressed in blastocysts, embryonic stem cells, and early neural stem cells, in which it functions to maintain self-renewal and pluripotency (42). The low level of REST protein observed in normal melanocytes (results not shown) most likely reflects the shared neuroectodermal and Schwann cell precursor origins of these cells (43). Oncogenic roles for REST have recently been established in a range of neural-derived cancers including medulloblastoma, neuroblastoma, and glioblastoma multiforme (23). We can now add melanoma to this list, which reflects their common neural crest–derived embryonic origin. A role for REST in promoting genomic instability has been proposed (44), although in many other cancer cell types such as colon and mammary epithelial cancer, REST has a tumor suppressor role whose function is disrupted (45, 46). Recently, RE-1 elements have been identified in genes encoding proteins involved in immune and inflammatory responses such as complement proteins, CCR7, IL17c, MyD88, TNF superfamily members (47), and STAT1 can now be added to this expanding list.

Using 2 melanoma cell lines (SK-MEL-28 and MM96), well-characterized for their IFN responsiveness, and 7 additional cell lines established at low passage from melanoma patient biopsies, comparing expression of full-length REST versus STAT1 by qPCR showed a significant inverse correlation ($r = -0.6286$, $P = 0.035$; Fig. 7B). In addition, SK-Mel-28 (S1) and MM96 (R1), with low and high levels of REST respectively, represent the extreme ranges of REST expression among the cell lines and also express significantly different levels of STAT1 (high and low, respectively; Fig. 7A and B). These 2 also represent extreme cases of melanoma cell responsiveness to the growth-inhibitory effects of IFN with SK-Mel-28 very sensitive whereas MM96 is resistant to the IFNs (13, 36). Recruitment of histone deacetylases by REST
would provide an efficient mechanism to negate the activity of histone acetylases recruited to the ICI motif (20), or vice versa. Given that reduced levels of STAT1 protein are associated with a loss of responsiveness to IFN in melanomas (13, 16), if this is correlated with high REST expression levels, the ratio of REST/STAT1 may prove to be a useful predictor for the nonresponsiveness of such cells to IFN.

In both the SK-MEL-28 and MM96 melanoma cell lines tested here, the magnitude of repression by the RE-1 element alone constituted only 70% to 80% of the total repression mediated by the complete STAT1 gene "repressor region" (Fig. 3D and E), suggesting that one or more additional elements within this region also contributes toward the repression. This is also supported by the results of the REST-1–deficient INS-1 cell line (Fig. 5) where the STAT1 prom/enh/repr reporter construct was still repressed compared with the STAT1 prom/enh vector.

In summary, we define 2 novel regulatory elements within the human STAT1 gene, one a stimulatory and the other a repressor site. Whilst the ISRE element provides for autoregulation by IFN activating STAT1 expression, the intrinsic RE-1 element, most likely cooperating with additional repressor elements, provides a suppressive mechanism to reduce basal STAT1 expression. These results have implications in the development of various malignancies, particularly neuronal-derived cancers where REST expression is involved. Through binding to the downstream RE-1 element, inhibiting STAT1 expression, REST could be a useful marker for IFN-resistance of cancers and provides a novel therapeutic target in IFN-resistant melanomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Amalraj, S.J. Cutler, I. Ghazawi, S.J. Ralph
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