TNIP1 [TNFα (tumour necrosis factor α)-induced protein 3-interacting protein 1] is a co-repressor of RAR (retinoic acid receptor) and PPAR (peroxisome-proliferator-activated receptor). Additionally, it can reduce signalling stemming from cell membrane receptors such as those for TNFα and EGF (epidermal growth factor). Consequently, it influences a variety of receptor-mediated events as diverse as transcription, programmed cell death and cell cycling. Thus changes in TNIP1 expression levels are likely to affect multiple important biological end points. TNIP1 expression level changes have been linked to psoriasis and systemic sclerosis. As such, it is crucial to determine what controls its expression levels, starting with constitutive control of its promoter. Our analysis of the TNIP1 promoter revealed multiple transcription start sites in its GC-rich proximal regions along with two transcriptionally active Sp (specificity protein) sites, responsive to both Sp1 and Sp3. EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation) demonstrated physical binding between Sp1 and Sp3 at these sites. A decrease in Sp1 protein levels via siRNA (short interfering RNA) or diminished Sp1 DNA binding by mithramycin decreased TNIP1 mRNA levels. This Sp-binding GC-rich region of the TNIP1 promoter also participates in transcriptional activation by ligand-bound RAR. Together, these results demonstrate newly identified regulators of TNIP1 expression and suggest possible transcription factor targets which in turn control TNIP1-related biological end points ranging from apoptosis to inflammatory diseases.

Key words: nuclear receptor, retinoic acid receptor (RAR), specificity protein 1 (Sp1), specificity protein 3 (Sp3), tumour necrosis factor α-inducible protein 3-interacting protein 1 (TNIP1).

INTRODUCTION

TNIP1 [TNFα (tumour necrosis factor α)-induced protein 3-interacting protein 1] controls signalling in diverse receptor pathways [1]. Under its alias of ABIN-1 [A20-binding inhibitor of NF-κB (nuclear factor κB) activation 1], TNIP1 has been demonstrated to reduce signalling downstream of TNFα receptor activation [2]. Separate studies have shown TNIP1 also dampens post-EGF (epidermal growth factor) receptor signalling, by preventing ERK2 (extracellular-signal-regulated kinase 2) nuclear translocation [3]. We have found previously that TNIP1 interacts with PPAR (peroxisome-proliferator-activated receptor) and RAR (retinoic acid receptor) in an agonist-dependent fashion, but reduces their transcriptional activity, placing TNIP1 in a small but iconiclastic set of nuclear receptor co-regulators, the co-repressors of agonist-bound receptors, which also includes LCoR (ligand-dependent co-repressor) and PRAME (preferentially expressed antigen of melanoma) [4]. Beyond these receptor pathways, early work with TNIP1 demonstrated its interaction with the HIV proteins Nef and matrix, suggesting a possible role in viral pathogenesis [5]. These diverse roles suggest that changes in TNIP1 expression levels could have dramatic and wide-ranging consequences. For TNFα signalling, this has been borne out in an experimental system by genomic knockout of the mouse Tnip1 gene [6]. Loss of TNIP1 leads to reduced body weight of those few live born pups and otherwise to high rates of fetal death accompanied by liver apoptosis and reduced haemopoiesis; the TNIP1 phenotype is rescued by knockout of TNFα. Whereas organ-level consequences of the absence of TNIP1 were strikingly seen in liver, cultured Tnip1-null embryonic fibroblasts were also more sensitive to TNFα-induced apoptosis. TNIP1 expression is found in many non-hepatic tissues and experimentally delivered TNIP1 is protective against allergen-induced lung inflammation [7]. These genetic knockout and recombinant overexpression studies suggest that expression level changes of the endogenous gene could have biological importance in a number of diverse tissues.

TNIP1 has also garnered clinical attention. Through associated SNPs (single nucleotide polymorphisms), gene sequence variations or mRNA levels, several studies have linked TNIP1 with psoriasis, systemic lupus erythematous, systemic sclerosis and rheumatoid arthritis [8–14]. Increased TNIP1 expression has been found in biopsy samples from the inflammatory diseases psoriasis and rheumatoid arthritis, a finding particularly intriguing given demonstration of NF-κB sites in the human TNIP1 proximal and distal promoter [15,16]. Beyaert and colleagues have reviewed expression patterns and splicing of TNIP1 [17]. They note that levels of transcript variants, especially those from the difference in use of exons mirroring the protein’s C-terminus, vary in normal and transformed haemopoietic cells. However, as they conclude, it is unknown what impact these differences may have in normal or malignant cell physiology. Additionally, TNIP1 as a cause of, or target of, aberrant receptor signalling in such diseases has yet to be resolved. Elevated levels of TNIP1...
may quench the anti-inflammatory activity of nuclear receptors such as PPAR [18] and RAR [19]. Thus, whereas reports of TNIP1 expression increases following TNFα exposure have helped us to understand its induction above baseline levels, no study of control over its constitutive expression has been available. In the present study, we have experimentally mapped the TSS (transcriptional start site) and identified nearby sequences expected to contribute to constitutive expression. Specifically, these sites bind Sp (specificity protein) 1 and 3 and possibly coordinate the recruitment of other transcription factors contributing to inducible TNIP1 expression.

Sp factors function by binding GC boxes in the promoters of target genes [20] where, especially for GC-rich regions as occur in the human TNIP1 promoter, they may help to maintain expression by protecting CpG islands from methylation. The Sp family of transcription factors is ubiquitously expressed and often involved in expression of housekeeping genes. However, Sp proteins are subject to post-translational modification and capable of interaction with inducible transcription factors, thus extending their role in expression control. Sp1 and Sp3 share 90% sequence homology in the zinc-finger-binding domain [21–23] and exhibit very similar DNA-binding specificities and affinities. However, despite the structural similarities between the two, their functional comparison reveals Sp1 as a transcriptional activator, with Sp3 as either a gene expression activator or repressor depending on promoter structure or cell type [24,25]. Consistent with this, a comparison of Sp1- and Sp3-knockout phenotypes show that Sp1 and Sp3 have distinct functions in vivo; genomic loss of Sp1 results in lethality at day 10 of embryogenesis, whereas Sp3-null mice are live-born, but die very shortly thereafter due to respiratory abnormalities. Thus, despite their shared binding site, and sometimes shared function as a transcriptional activator, Sp1 and Sp3 are not functionally equivalent.

The present study is the first to examine constitutive regulation of the human TNIP1 gene promoter. We have identified GC boxes near experimentally defined TSSs for the human TNIP1 promoter and their function as Sp1 and Sp3 sites through in silico sequence analysis, in vivo and in vitro protein–DNA interaction assays, reporter assays with the cloned wild-type and Sp-site mutant promoter, pharmacological inhibition of GC box occupation, and siRNA (short interfering RNA) interference of Sp production. Our identification of two functional GC boxes and multiple start sites are consistent with the GC-rich nature of the TATA-less TNIP1 promoter. Although these two Sp-binding sites differ by only one nucleotide from each other, they do not function similarly for binding or activation by Sp, suggesting that the sequence of nucleotides surrounding them may affect their function. Lastly, the GC boxes may serve not only as the Sp sites we have demonstrated, but also as intermediates for transcription factors beyond Sp1.

**MATERIALS AND METHODS**

**Plasmids**

Promoter luciferase constructs used in the present study contain the −549 to +111 (hereafter referred to as −549-luc) and −5887 to +111 (−6kb-luc) nucleotides of the human TNIP1 promoter/gene, using previously established numbering [15,16] for the TSS, inserted into the promoterless vector pGL4.10 (Promega). Predicted Sp sites at −151 and −130 were examined as either wild-type or mutant sequences, the latter generated using a site-directed mutagenesis kit (Stratagene). Mutants were generated by altering the core Sp sequence from GGTCCC to GGTCAC for the −151 site and CCCGC to CCCCTC for the −130 site. All plasmids were sequenced (University of Connecticut Biotech Center) to confirm base-pair substitutions. *Drosophila* actin promoter-driven pPac0 (plasmid 12094) and pPacSp1 (plasmid 12095) were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). pPacSp3 was obtained from Dr Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Marburg, Germany). Overlap extension PCR [26] was used to generate pPac expression vectors containing RARγ and RXR (retinoid X receptor)α.

**Cell culture and transfection**

HeLa cells (A.T.C.C.) were cultured at 37°C with 5% CO2 in DMEM ( Dulbecco’s modified Eagle’s medium)/Ham’s F12 (3:1, v/v) containing 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin (media and antibiotics were from Invitrogen). HeLa cells were seeded in 24-well dishes at a density of 1.5×104 cells/well with 0.5 ml of medium/well 24 h before transfection with FuGENE® (Roche) according to the manufacturer’s instructions. At 48 h after transfection, cells were harvested using 100 μl of Passive Lysis Buffer (Promega) per well by shaking at room temperature (22°C) for 20 min to achieve cell lysis. Mithramycin (MP Biomedicals) exposure was started 24 h before harvesting. Lysates were collected, centrifuged at 14 000 g for 10 min and stored at −80°C until assayed. SL2 cells (A.T.C.C.) were cultured at 24°C in Schneider’s *Drosophila* Medium (Invitrogen) containing 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. For transfections, SL2 cells were plated as described for HeLa cells and transfected using FuGENE® with amounts of pPacSp1 or pPacSp3 vectors as indicated in the Figure legends. The copy number of the total expression construct was standardized by inclusion of empty vector if necessary. After 16 h, transfection medium was removed and replaced with fresh medium. At 48 h after transfection, cells were lysed as described for HeLa cells with the extraction time extended to 30–40 min at room temperature. Luciferase activities produced by TNIP1-luc constructs were measured with an LMaxII luminometer (Molecular Devices) and normalized as described in [27].

**5’ RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends)**

Total RNA was extracted from Caco-2, HaCaT, HeLa, HepG2, Jurkat, MCF7, SCC13, SCC25, normal dermal foreskin fibroblasts and scleroderma explant fibroblast human cell cultures using RNasy (Qiagen). 5’ RLM-RACE was performed with the GeneRacer system (Invitrogen). Briefly, 1–2 μg of total RNA was treated with calf intestinal phosphatase and then tobacco acid pyrophosphatase, ligated to the GeneRacer RNA oligonucleotide sequence, and then reverse-transcribed using SuperScript III reverse transcriptase. PCR amplification of the resulting cDNA was performed using two sets of primers (Table 1). PCR products were cloned into pCR4-TOPO vector and sequenced.

---

**Table 1 Primers used in the present study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
</tr>
</thead>
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<tr>
<td>Outer</td>
<td>CGACUGGAGCCACGAGGACUGAAGG</td>
<td>GGAGGCAGCTGGGAGCAACAG</td>
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<tr>
<td>Nested</td>
<td>GGGCGACUGCAAUGGACGAGGAUGA</td>
<td>ACCCGGGGCAGGACGAGGAAGT</td>
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<tr>
<td>ChIP DHFR</td>
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</tbody>
</table>

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Table 2  Probes used for EMSAs

<table>
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<th>Probe name</th>
<th>Sequence (5′−3′)</th>
</tr>
</thead>
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<td>Sp 1−151</td>
<td>CTAGCTGGCTGGCTCGG</td>
</tr>
<tr>
<td>Sp 1−130</td>
<td>CTAGCTGGCTCGGCTCGG</td>
</tr>
<tr>
<td>Sp consensus</td>
<td>CTAGATTCGATCGGGCGG</td>
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<tr>
<td>Dbl Sp WT forward</td>
<td>CTAGCTGGCTCGGCTCGG</td>
</tr>
<tr>
<td>Dbl Sp WT reverse</td>
<td>GCGAGC</td>
</tr>
<tr>
<td>Dbl Sp 151 mutant</td>
<td>CTAGCTGGCTGGCTCGG</td>
</tr>
<tr>
<td>Dbl Sp 130 mutant</td>
<td>CTAGCTGGCTGGCTCGG</td>
</tr>
</tbody>
</table>

siRNA knockdown and qPCR (quantitative real-time PCR)

RNA interference was performed with human Sp1 and control non-targeting siRNAs (Dharmacon) in HeLa cells. Cells were seeded in 96-well plates at a density of 10^4 cells per well and 24 h later were transfected using 100 nM targeting or non-targeting control siRNA with DharmaFECT1 (Dharmacon) transfection reagent. Cells were collected at 72 h using the Taqman Gene Expression Cells-to-CT lysis kit (Applied Biosystems). qPCR was performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. Probes were used for quantitative real-time PCR analysis. Primers were designed using the ABI 7500 version 2.0.1 software using the ΔΔCt method.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from HeLa cells as described previously [28]. Protein concentrations were determined using the BCA (bicinchoninic acid) protein assay (Pierce). Oligonucleotides (Integrated DNA Technology) were annealed and end-labelled with [32P]ATP (PerkinElmer) (Table 2) as described in [16,29]. Nuclear extracts were incubated with radiolabelled DNA probes as described in [29]. For oligomer competition and antibody supershift experiments, binding reactions were incubated with unlabelled double-stranded oligonucleotides or specific antibodies for 20 min before the addition of the radiolabelled oligonucleotide. Anti-Sp1 (sc-59x) and Sp3 (sc-644x) antibodies were obtained from Santa Cruz Biotechnology. Samples were run on 7% polyacrylamide gels and electrophoresed at 200 V in 0.5× TBE (22.5 mM Tris/borate and 0.5 mM EDTA) buffer for 13 h at 4 °C as described in [29]. Gels were dried and exposed to Amersham Hyperfilm MP (GE Healthcare) for at least 16 h at −80 °C with intensifying screens. Films were developed using a Kodak X-Omat 2000.

ChIP (chromatin immunoprecipitation)

HeLa cells were seeded at 5×10^4 per 10-cm-diameter plate and 16 h later were processed with a two-step fixation protocol utilizing first disuccinimidyl glutarate and then formaldehyde [30]. Subsequent steps were as described in [30] with DNA−protein complexes being precipitated with 4 μg of rabbit polyclonal normal IgG (Millipore), rabbit polyclonal anti-Sp1 antibody CS200631 (Millipore) or rabbit polyclonal anti-Sp3 antibody D-20X (sc-644) (Santa Cruz Biotechnology). PCR primers are listed in Table 1. Cycling conditions were 94 °C for 3 min, 32 cycles of 94 °C for 20 s, 51 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 2 min. Amplicons were resolved on an ethidium bromide-stained 4% agarose gel. For re-ChIP, complexes were washed in TE (Tris/EDTA) then eluted in 300 μl of 10% (w/v) SDS, 1 M DTT (dithiothreitol) and TE at 37 °C for 30 min twice. Then, 4 μg of specific re-ChIP antibodies (against RARγ, RARx and Sp1) were each mixed with the eluate in low ionic dilution buffer with 40 μl of Dynabeads and incubated for 4 h. Cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 59 °C for 1 min and 72 °C for 30 s, with a final extension of 72 °C for 5 min. Amplicons were resolved on an ethidium bromide-stained 3% agarose gel.

Immunoblot analysis

Whole-cell lysates were prepared in RIPA buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1% sodium deoxycholate and 0.1% SDS) and, following determination of protein concentration, equal amounts of protein were separated by SDS/PAGE, transferred on to nitrocellulose membranes, rinsed with nanopure water and treated with Qentix (Pierce). Blots were incubated in 5% (w/v) non-fat dried milk, TBS (Tris-buffered saline) and 0.1% Tween 20, and then subsequently probed with anti-Sp1 antibody (Upstate Biotechnology) or anti-Sp3 antibody (Santa Cruz Biotechnology) followed by HRP (horseradish peroxidase)-conjugated secondary antibody (PerkinElmer). Detection of binding was determined with enhanced chemiluminescence reagents (Pierce). Band signals were digitally captured and analysed using a Kodak image station CCF and Carestream molecular imaging software.

Statistical analysis

Data were analysed with Prism software (GraphPad) using one-way ANOVA followed by Dunnett’s post-hoc test to compare all groups to control or Tukey’s post-hoc test to compare all groups. Statistical significance was defined as P < 0.05.

RESULTS

Mapping the TSSs of TNIP1

The TNIP1 gene sequence is increasingly GC-rich and without a recognizable TATA box resembling the cluster of expected TSSs defined by CAGE (cap-analysis of gene expression) database extractions [16,31]. These characteristics are consistent with this region being a dispersed rather than focused type of promoter and furthermore predict multiple TSSs over a 50–100-nt region [32]. Because of its reliance on 5′-capped mRNA, the RLM-RACE approach is advantageous in determining bona fide TSSs. Using it and RNA from human cell cultures, we mapped multiple TSSs to the 5′ region of the TNIP1 gene (Figure 1A). All but one of the TSSs reside in either of two alternative first exons [33,34] (Figures 1B and 1C).

A TSS in exon 2 (Figure 1D) is previously unknown. Among the cell types examined, it is characteristic of MCF-7 cells; no other cell type’s TSSs mapped to this point in exon 2, and neither did MCF-7 cells produce mRNA from either of the two major TSS clusters. Within each TSS cluster, the actual start sites spanned lengths of ∼30 or ∼90 nt. Both of these clusters experimentally validate our CAGE-mapped TSSs [16]. TSSs in the 90-nt span overlap the point previously used as +1 in numbering ∼600 bp [15] and ∼6000 bp [16] promoter clones and are associated with long exon 1. For consistency with these previous papers, we have retained such numbering in the present study (+1, open arrow, Figure 1C). The other TSS cluster is ∼6000 nt upstream of this region suggestive of
an alternative promoter. Interestingly, whereas TSSs for some cells (normal dermal fibroblasts, immortalized non-tumorigenic HaCaT keratinocytes, malignant keratinocyte lines SCC13 or SCC25, Caco2 and adenocarcinoma HeLa cells) mapped to both regions, for other cells (HepG2 and Jurkat), the TSS cluster mapped to only the upstream region. We surveyed several cell types used in the TSS mapping on the basis of their subsequent use in Sp reporter assays and the shared or distinctive localization of their TSSs (Figures 1E and 1F). The Sp1 and Sp3 levels varied across all cells and were not determinant of the TSSs. Fibroblasts and epithelial cells, with low and high relative levels of Sp factors respectively, shared TSSs, e.g. fibroblasts and HeLa or SCC13 TSSs mapped to long exon 1 and fibroblasts and SCC13 or HeLa TSSs mapped to short exon 1. Overall, the cells containing the most Sp factors were of epithelial origin.

Sp1 and Sp3 bind the TNIP1 promoter

In silico analysis of the GC-rich sequence immediately upstream of the long exon 1 TSS cluster revealed two predicted binding sequences for the Sp family of transcription factors (Figure 2) based on a minimum match to a core sequence of 5′-GGG\(^3\)/GG-3′ [20]. Using the numbering schema described in Figure 1, these are positioned at \(-151\) and \(-130\) of the long exon 1 TSS in the human TNIP1 gene. Examining orthologous TNIP1 sequences with the promoter alignment tool ConTra [35] revealed that the \(-151\) site, but not the \(-130\) site, is conserved across primate and rodent species. For the human sequence, these possible Sp sites are in a predicted \(~200\)-nt CpG island where the GC content is 76%. To test for physical association of Sp factors with the TNIP1 promoter in vivo, we performed ChIP from HeLa cells with anti-Sp1 and anti-Sp3 antibodies (Figure 3). Also included in the ChIP experimental analysis were primers for Sp site-containing (positive) and Sp site-devoid (negative) control genes DHFR (dihydrofolate reductase) and p21 respectively. Predicted Sp-binding regions of the TNIP1 promoter were successfully immunoprecipitated with anti-Sp1 and anti-Sp3 antibodies, indicating that Sp1 and Sp3 were associated physically with the TNIP1 promoter in vivo. One limitation of this ChIP, however, was that the \(<20\) nucleotides separating the two predicted sites did not allow for determining whether either or both sites were binding Sp factors. To test whether Sp1 or Sp3 could bind these individual sites, EMSAs were carried out. Radiolabelled oligomers containing either of the predicted Sp sites at \(-151\) or \(-130\) in the TNIP1 promoter were used as probes along with HeLa nuclear extract. One complex was formed with each of the probes. With the \(-151\) probe, there was a disruption of the complex using anti-Sp1, but not anti-Sp3, antibody (Figures 4A and 4C). Unexpectedly, even with its better match to the core Sp site consensus, the protein(s) associating with the \(-130\) probe showed no binding disruptions or supershifts with either the Sp1- or Sp3-specific antibodies (Figures 4B and 4D). This result and the lack of detectable Sp3 binding to the \(-130\) EMSA probe, despite the ChIP results detecting Sp3 binding in the native promoter, led us to consider possible neighbouring effects on Sp binding to TNIP1 promoter DNA. That is, TNIP1 Sp sites isolated as individual sequences in the EMSA may not present an equivalent target as the two adjacent sites in the endogenous gene, as captured in the ChIP. Such adjacent sites are known to stabilize Sp3–DNA interactions and facilitate EMSA capture of the complexes [36,37]. EMSAs were conducted with the adjacent sites (\(-151\) and \(-130\)) in a longer inclusive
Sp sites were based on a match to a minimum core sequence of 5′-GGGgc/GG-3′. The top strand sequence is shown; numbering is based on human promoter sites. Full species names are Homo sapiens, Pan troglodytes, Pongo pygmaeus, Macaca mulatta, Mus musculus and Rattus norvegicus. Asterisks (*) indicate positions ten bases apart.

![Figure 2](image)

**Figure 2** Sp site predictions (core sequence in bold) and species alignment of orthologous TNIP1 promoter regions

Sp sites were based on a match to a minimum core sequence of 5′-GGGgc/GG-3′. The top strand sequence is shown; numbering is based on human promoter sites. Full species names are Homo sapiens, Pan troglodytes, Pongo pygmaeus, Macaca mulatta, Mus musculus and Rattus norvegicus. Asterisks (*) indicate positions ten bases apart.

![Figure 3](image)

**Figure 3** ChIP assay demonstrating Sp1 and Sp3 association with the TNIP1 promoter in vivo

Sheared chromatin from HeLa cells was immunoprecipitated with antibody (control IgG, anti-Sp1 and anti-Sp3) and bound DNA was isolated. The immunoprecipitated DNA was used as template in PCR using various primer sets amplifying TNIP1, p21 (negative control) or DHFR (positive control).

probe, which resulted in three band species, two of which were disrupted by anti-Sp3 antibody (Figure 4E) concurrent with the generation of a supershifted complex. All three band species were competed with unlabelled self and consensus Sp site oligomers, but much less so by an excess of mutated Sp site oligomer. Thus we were able to detect Sp3 binding to TNIP1-specific Sp sites in vitro. However, although the presence of the two sites allows for Sp3 binding, we were not able to distinguish occupation of one site from the other with this approach. Therefore, to determine to which of the two sites Sp3 may be binding, each of the single sites was mutated in the context of the longer double-site probe and tested in EMSAs. Radiolabelled oligomers spanning both predicted Sp sites with either the 151 site, or the 130 site, or the double mutant showed 50, 80 or 90% reductions respectively in relative luciferase activity compared with wild-type TNIP1 proximal promoter or a version containing mutations in either or both of the Sp sites (Figures 5C and 5D) was assayed from transfected SL2 cells. Separately, the mutated 151 site, or the 130 site, or the double mutant showed 50, 80 or 90% reductions respectively in relative luciferase activity compared with wild-type TNIP1 promoter. Neither the Sp double-site wild-type nor the Sp single-site mutants responded to the lower Sp1 levels despite transfection into the Sp-null SL2 system. Critical or threshold levels of expression-controlling factors sufficient for assembly of active transcription complexes have long been implicated in the regulation of promoter activity [39].

**Sp1 and Sp3 activate the TNIP1 promoter in SL2 cells**

To complement the Sp physical binding assays, we tested Sp functional activation of the cloned human TNIP1 promoter in driving expression from a luciferase reporter plasmid in SL2 cells, which lack all Sp proteins [38]. Two lengths of the human TNIP1 promoter were tested with and without co-expression of recombinant Sp factors. One TNIP1-luc reporter (−6kb-luc) contains a sequence (denoted as the broken line over the promoter diagram in Figure 1) overlapping the TSS cluster mapped for long exon 1 and continues for ∼6 kb up to, but not including, the upstream TSS cluster of short exon 1. A shorter construct (−549-luc) contains the same TSS cluster from long exon 1 and the two adjacent Sp sites. SL2 cells were co-transfected with either the −6kb-luc or −549-luc TNIP1 promoter constructs and pPac expression vectors for either Sp1 or Sp3 (Figures 5A and 5B). Co-transfection of pPacSp1 with −549-luc resulted in an up to 17-fold increase in this proximal promoter’s activity for the highest amount of Sp1 compared with SL2 cells transfected with the control empty pPac expression vector (pPac0) (Figure 5A). Co-transfection of pPacSp1 with −6kb-luc resulted in a 10-fold increase in this full-length promoter’s activity for the highest amount of Sp1 compared with SL2 cells transfected with pPac0. Sp3 co-transfected with either TNIP1 promoter construct also increased reporter activity compared with empty vector; however, the fold increase was much less than for Sp1. For the −549-luc reporter, Sp3 fold activation was roughly one-third that of Sp1 for the highest amount used (Figure 5B).

We examined the contribution of the Sp factors to activating the GC-rich TNIP1 proximal promoter (represented by −549-luc) by introducing site-directed mutations within the Sp site core sequence and comparing mutant and wild-type reporter responses to co-expressed Sp protein. We focused on the possible loss of the ability of Sp1 to activate expression due to the Sp site mutations because it was several times more effective than Sp3 in activating the TNIP1 reporters. Sp1 induction of the wild-type −549-luc TNIP1 proximal promoter or a version containing mutations in either or both of the Sp sites (Figures 5C and 5D) was assayed from transfected SL2 cells. Separately, the mutated 151 site, or the 130 site, or the double mutant showed 50, 80 or 90% reductions respectively in relative luciferase activity compared with wild-type TNIP1 promoter. Neither the Sp double-site wild-type nor the Sp single-site mutants responded to the lower Sp1 levels despite transfection into the Sp-null SL2 system. Critical or threshold levels of expression-controlling factors sufficient for assembly of active transcription complexes have long been implicated in the regulation of promoter activity [39].

**Sp1 modulates TNIP1 promoter activity**

The ChIP, EMSA and promoter reporter mutagenesis results are consistent with Sp-binding and -activating sites within the proximal human TNIP1 promoter. To examine further the effects of endogenous Sp factors on TNIP1 promoter activity, we utilized the −549-luc reporter plasmid in HeLa cells treated with mithramycin post-transfection. This drug binds GC-rich sites impeding endogenous Sp-dependent expression through
Figure 4  EMSAs demonstrating that Sp1 and Sp3 bind TNIP1 Sp sites

(A–D) EMSAs using 15 μg of nuclear extract (NE) from HeLa cells and TNIP1 radiolabelled Sp1 site probes — 151 (A and C) or — 130 (B and D). As indicated above lanes, some binding reactions received unlabelled TNIP1 Sp site oligomers for non-radiolabelled self competition (Comp, Self at 25× or 100×) or antibodies (Ab) against Sp1 or Sp3 or non-specific IgG. Each panel came from the same gel, although some lanes have been rearranged for presentation purposes. (E) EMSAs using TNIP1 radiolabelled double-site-containing probe were incubated with 15 μg of HeLa nuclear extract with or without non-radiolabelled self competition (25× and 100×), IgG (control), non-radiolabelled consensus Sp oligonucleotide (25× and 100x), non-radiolabelled mutant consensus oligonucleotide (25× and 100×) or anti-Sp3 antibody, and were subjected to gel electrophoresis. (F and G) EMSAs using TNIP1 radiolabelled double-site-containing probe were incubated with 15 μg of HeLa nuclear extract with or without non-radiolabelled self competition (25× and 100×), IgG (1× and 2×) (control), non-radiolabelled consensus Sp oligonucleotide, wild-type double-site TNIP1 probe, anti-Sp1 (1× and 2×), and anti-Sp3 (1× and 2×) antibody and subjected to gel-electrophoresis. Lanes in the images from (E) have been rearranged from the originals for presentation purposes. The closed arrowhead indicates major Sp-binding species. The open arrowhead indicates minor Sp-binding species. The asterisks (*) indicate twice the amount of antibody.

steric hindrance [40]. Mithramycin significantly decreased the activity of the −549-luc reporter at all concentrations tested (Figure 6A). Others have also reported that a reduction in Sp-mediated transcription is not necessarily proportionate to mithramycin concentration [41].

Similarly, compared with the wild-type −549-luc reporter constitutive activity in HeLa cells of the Sp double-site mutant was decreased presumably through loss of endogenous Sp binding to the construct. This reduction, however, occurred to different extents in two distinct cell types: HeLa and HaCaT. Interestingly, when only one of the two sites was mutated, there was no loss of activity in HaCaT cells (Figure 6B), but a significant increase in activity in HeLa cells (Figure 6C) when compared with the wild-type −549-luc response. An increased responsiveness of a single-site mutant in the presence of Sp has been documented previously [41–43]. Activation in HaCaT cells was reduced...
Sp sites contribute to basal and inducible expression of human TNIP1 promoter

Figure 5 Effect of transfected Sp on human TNIP1 promoter in Drosophila SL2 cells

(A) and (B) Activation of TNIP1 promoter–luciferase constructs with increasing amounts (25–250 ng) of Sp1 (A) and Sp3 (B). Note for (A) and (B) that −549 and −6 kb are each normalized to their own pPac0 control. *P < 0.05 compared with control (pPac0 transfected with either −549- or 6 kb-luc) using ANOVA with Dunnett’s post-hoc test. (C and D) Mutational analysis of the Sp-binding sites in the TNIP1 SL2 cells were transfected with wild-type TNIP1-luc, the −130 mutated site, the −151 mutated site or the double mutant (Dbl mut) and then transfected with pPac0 or 25 ng or 250 ng of pPacSp1. *P < 0.01 compared with wild-type (wt) TNIP1 in the presence of high Sp1 using Student’s t test. Results are means ± S.E.M. for three independent experiments with each sample transfected in triplicate.

Figure 6 Endogenous Sp factors control expression from the TNIP1 reporter construct (A–C) and the endogenous TNIP1 gene (D and E)

(A) Effect of different amounts of mithramycin on the activation of −549-luc in HeLa cells. *P < 0.05 compared with control (0 nM mithramycin) using ANOVA with Dunnett’s post-hoc test. (B and C) Effect of Sp site mutagenesis on −549-luc reporter luc activity in HaCaT (B) and HeLa cells (C). *P < 0.05 compared with control. Dbl mt, double mutant. (D) Mithramycin (Mith) treatment (24 h) of HeLa cells reduces TNIP1 mRNA. UT, untreated. (E) Sp1-targeting siRNA reduces TNIP1 mRNA levels by ∼85% compared with non-targeting (NT) control siRNA. Inset: Sp1-targeting siRNA reduces Sp1 protein by >60% compared with NT siRNA. Bands were normalized to actin as loading control. Results are means ± S.D. for triplicate samples, representative of three independent experiments.
by approximately 60%, whereas activation in HeLa cells was reduced by approximately 25% when using the TNIP1 luciferase reporter constructs with both sites mutated (Figures 6B and 6C). Testing expression from the endogenous TNIP1 gene, we found that mithramycin treatment caused a 3-fold decrease in TNIP1 mRNA (Figure 6D). Although mithramycin may prevent GC-binding proteins in addition to Sp from activating gene promoters, we found that Sp1-targeting siRNA caused a decrease in endogenous TNIP1 expression similar to mithramycin. TNIP1 mRNA levels were measured via qPCR 72 h after transfection of HeLa cells with 100 nM Sp1-targeting or non-targeting siRNA. At 72 h after Sp1 siRNA transfection, Sp1 protein levels were decreased by >60% compared with non-targeting control siRNA. Coincident with the Sp1 protein reduction, TNIP1 mRNA levels were significantly decreased (by ~85%) (Figure 6E).

**Sp1 sites may allow other transcription factors to contribute to TNIP1 promoter activity**

In studies concurrent with the present study, we noted activation of TNIP1 transcription by retinoic acid via response elements in the distal region of the promoter. Intriguingly, the Sp-regulated proximal TNIP1 region described in the present paper has no recognizable retinoic acid-response elements, but is also inducible by liganded RAR (Figure 7A). Given that Sp can serve as a docking site for other expression-activating factors in a transcription complex [44,45], we tested the effect of mutating the Sp-binding sites within the proximal TNIP1 promoter on its response to transactivation by retinoid receptors. Retinoid receptor transactivation of wild-type TNIP1 proximal promoter was ligand- and Sp site-dependent. Induction of the Sp double-site mutant was significantly reduced compared with the wild-type proximal construct when the heterodimer pair RARγ–RXRα was co-expressed (Figure 7A).

Possible interaction of endogenous retinoid receptors and Sp in the proximal promoter region of the TNIP1 gene was tested via ChIP from HeLa cells cultured with either vehicle (DMSO) or retinoic acid (Figure 7B). Consistent with the results in Figure 3, anti-Sp1 antibody successfully captured the TNIP1 proximal region from chromatin prepared under either growth condition. RARα- and RARγ-specific antibodies showed that predicted TNIP1 Sp-binding regions were successfully amplified and immunoprecipitated with anti-Sp1 antibody. However, re-ChIP did not indicate binding of RARγ or RARα to TNIP1 Sp-binding regions. ChIP from retinoic acid-treated HeLa cells showed that predicted TNIP1 Sp-binding regions were successfully amplified and immunoprecipitated with anti-Sp1 antibody. Furthermore, retinoic acid treatment and subsequent re-ChIP of TNIP1 Sp-binding regions with anti-RARα antibody showed that RARα and Sp1 were both associated with the predicted Sp-binding regions. These results indicate that both Sp1 and RARα are found in the same immunoprecipitated
complex binding to the endogenous TNIP1 promoter. However, these results do not indicate whether RARα is interacting directly or indirectly with Sp1 and its binding sites.

To extend the understanding of how the interaction between Sp1, RAR and TNIP1 promoter may be occurring, we generated plasmid constructs for expression of RAR and RXR in SL2 cells. In this way, we would be able to test whether or not Sp1 was forming a scaffold for RAR for interaction with TNIP1. Results from this experiment showed that, in the absence of Sp1, RAR was still able to transactivate TNIP1 in the presence of TTNPB \((\{E\}-4\{2\-(5\-,6\-,7\-,8\-tetrahydro\-5\-,5\-,8\-,8\-tetramethyl\-2\-naphthyl\)enyl\)-1\-propanyl\} benzoic acid)\) (Figure 7C). In addition, when the Sp double-site mutant was used, the ability of RAR to transactivate TNIP1 in the presence of TTNPB was attenuated. Furthermore, when RAR and RXR were co-transfected with Sp1, there was an attenuated response following ligand treatment, indicating that when these factors are in the same cellular context, they may compete for occupation of the same binding site on the TNIP1 promoter.

**DISCUSSION**

Human TNIP1 is expressed in normal tissues and cells cultured under control conditions and is inducible by TNFα as mediated by NF-κB sites [16,46]. Although characterized to have alternative first exons, experimentally defined TSSs and what might contribute to constitutive expression were not investigated before the present study. We have performed an analysis of the human TNIP1 promoter to identify TSSs and transcription factors that regulate constitutive expression of this gene. Cloning and sequencing of capped TNIP1 mRNA revealed multiple TSSs in various cell types. The present study shows that the alternative first exons of TNIP1 identified previously by others [33] each have a cluster of TSSs associated with them rather than any one single TSS. Certain TSSs from either exon were shared between various cell types, irrespective of cell derivation. In contrast, other TSSs were cell-specific, including the lone TSS in exon 2, which was only observed in MCF-7 cells. The TSS cluster associated with short exon 1 originates ∼100 bp upstream of the most-distal point of our cloned 6000 bp promoter. It is likely that an alternative promoter regulates transcription, initiating with this alternative exon. In contrast, the TSS cluster associated with long exon 1 (Figure 1A) lies ∼40 bp downstream of a NF-κB-inducible element [15,16]. To complement these studies on inducible expression from this promoter region, we focused the present study on elements expected to contribute to as yet unstudied constitutive TNIP1 promoter activity. Specifically, nearby this proven NF-κB site and ∼80 bp upstream of the TSS associated with long exon 1, we found GC boxes conserved across several primate (but not rodent) species. These GC sequences had core consensus matches to Sp sites and were tested for Sp binding in vivo, in vitro and by drug inhibition and mutagenesis for mediating transcriptional activation and thus contributing to constitutive TNIP1 expression.

We identified critical roles for promoter sequences −151 and −130 nt relative to the TSS cluster of long exon 1. These sites provided positive transcriptional regulation by the Sp family members Sp1 and Sp3 and, surprisingly, RAR. ChIP with Sp1- and Sp3-specific antibodies captured this region, indicating interaction on native chromatinized DNA in vivo; however, the close proximity of the −151 and −130 sites and the length of the ∼200–500 bp of DNA in the sheared chromatin did not allow for conclusive interpretation on which of the two sites were being bound by Sp1 and Sp3. In order to tease out which site (s) were bound by Sp1 and Sp3, we went back to in vitro DNA–protein interactions as visualized by EMSA. As representatives of the individual sites, EMSA oligomers for −151 could capture Sp1, but not Sp3; the −130 probe could not retrieve either Sp1 nor Sp3. To better recapitulate the endogenous promoter and capture Sp3 binding in vitro, an extended EMSA oligomer containing both the −151 and −130 sites was generated. Within the context of the wild-type double-site probe, Sp3 binding was observed. In an attempt to distinguish which site was binding Sp3, we utilized versions of the double-site oligomers with either the −151 or −130 sites mutated. Interestingly, the results showed that Sp1 can interact with both the −151 and −130 sites in the context of the double-site oligomer in vitro. Furthermore, the addition of the anti-Sp3 antibody disrupted a minor band species formed with the −151 mutant double-site oligomer. Although the double-site −151 mutant probe indicated a disruption in a minor complex by anti-Sp3 antibody, there is a limit to the ability of the in vitro system to recapitulate the cellular environment (i.e. native binding conditions) necessary for optimal binding of Sp3 to either the −151 or −130 sites. As such, although it could not distinguish individual site contribution for Sp binding, the ChIP assay results may best represent the in vivo Sp–TNIP1 promoter physical interactions, consistent with our functional assays showing a positive Sp contribution to human TNIP1 promoter activity.

Mutagenesis of these sites in the context of the cloned TNIP1 promoter demonstrated positive regulation by Sp1 when both Sp sites were mutated and a negative regulation by Sp1 when only one of the two sites were mutated. These results suggest that when both sites are available for binding, repressive transcription factors are able to complex with the bound Sp proteins; however, when only one of the sites is bound by Sp, the repressive factors are not able to complex with the bound Sp protein leading to a more active transcriptional complex. Decreased genomic TNIP1 expression subsequent to siRNA knockdown of Sp1 demonstrated direct involvement of Sp1 in activating the endogenous TNIP1 promoter. Furthermore, RARα was shown to be associated with the same GC-rich regions within TNIP1 as Sp1, as indicated by ChIP–re-ChIP analysis. Although we cannot rule out a role for other GC-binding transcription factors, mutational analysis of the TNIP1 promoter at the −130 or −151, or both sites suggests that Sp1 is a key player in the constitutive regulation of the proximal region of the TNIP1 promoter. Also, this same mutation of the two Sp sites within the context of the proximal promoter in the presence of transfected heterodimer receptor resulted in an attenuated response to ligand by the proximal promoter. This indicates that GC boxes characterized as Sp-binding sites either directly or indirectly mediate the RAR-responsiveness of the proximal promoter [44,47–50]. The decrease in the retinoic acid responsiveness of the proximal promoter upon mutation of the GC boxes is consistent with the interpretation that an uncharacterized RARE overlaps them. Last but certainly not least, we saw that endogenous TNIP1 mRNA was significantly decreased in response to both mithramycin treatment and Sp1-targeting siRNA.

The presence of GC-rich sequences within the proximal human TNIP1 promoter region is a feature of TATA-less genes, and the expression of such genes is often regulated by members of the Sp family [51,52]. It has been proposed that one or more Sp1 molecules may bind TATA-less and/or GC-rich promoters to recruit specific co-factors, such as TATA-binding protein-associated factors, which subsequently interact with other transcriptional machinery, such as TFIIID (transcription factor IID), to initiate transcription [53]. Since Sp1 and Sp3 are ubiquitously expressed and are at various amounts in different cell types, there may be cell-specific
regulation of TNIP1 by these factors. Sp and other transcription factors activity, or epigenetic modifications of the TNIP1 promoter, may also be important for constitutive expression of this gene. Additionally, post-translational modifications, such as phosphorylation, glycosylation, ubiquitylation, acetylation, ribosylation and SUMOylation [51,52] may alter Sp1 and Sp3 gene regulation of TNIP1 in various cell types.

In conclusion, we have demonstrated the importance of the Sp family of transcription factors as regulators of TNIP1 constitutive expression, particularly Sp1 and Sp3. Sp1 activates TNIP1 through the −151 site and Sp3 activates TNIP1 through both the −151 and −130 sites in the proximal region of the TNIP1 promoter, constitutively activating TNIP1. Whereas other factors such as the nuclear receptors RAR and PPAR [16,54] or transcription factors such as NF-κB further modulate the expression of TNIP1 beyond that of Sp1 or Sp3. Therefore Sp1 or Sp3 modulation of the expression of TNIP1 may ultimately serve as a defined target which may be able to regulate the expression of TNIP1, and depending on the disease state increase or decrease TNIP1 transcription. Finally, if the expression of TNIP1 could be increased further by inducible factors such as NF-κB, beyond the constitutive activity of Sp1 or Sp3, TNIP1 may become a new target in the regulation of nuclear receptors and various inflammatory diseases.

AUTHOR CONTRIBUTIONS
Priscilla Encarnacao designed, executed and interpreted Sp binding, reporter activation, reporter mutagenesis and cell transfection experiments and drafted most of the paper and Figure legends. Vincent Ramirez designed, executed and interpreted TSS experiments and clone sequencing. Carmen Zhang designed, performed and participated in analysis of ChIP work and siRNA experiments. Brian Aaneskievich oversaw all experiments and data interpretation, participated in drafting of the Figures and text, and contributed to writing the final paper.

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