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Human TNF α -induced protein 3-interacting protein 1 (TNIP1) promoter activation is regulated by retinoic acid receptors

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Abstract

Coregulator proteins play key roles in transcriptional control by members of the nuclear receptor superfamily. Previously, we demonstrated that tumor necrosis factor α (TNF α)-induced protein 3-interacting protein 1 (TNIP1) is a corepressor of agonist-bound retinoic acid receptors (RARs). Additionally, TNIP1 has been shown to repress peroxisome proliferator-activated receptors (PPAR) and NF- κ B activity and interact with HIV proteins nef and matrix. *TNIP1* transcriptional regulation, however, is under studied. Here we show that under permissive epigenetic conditions, *TNIP1* expression is induced by all *trans* retinoic acid (ATRA). Within a 6000bp region of the human TNIP1 promoter we cloned, both proximal and distal promoter regions are RAR responsive with the latter having RA response elements (RAREs) recognizable by their sequence and functionality in native promoter and synthetic RARE luciferase constructs, EMSA, and ChIP assays. These findings suggest a feedback loop whereby RARs activate expression of TNIP1, which then attenuates their activity. Together with anticipated constitutive transcription factors and the previously described NF- κ B-responsiveness of the proximal TNIP1 promoter, the expected combinatorial control of *TNIP1* expression could likely modulate TNIP1's impact in any of its target pathways. The degree of control by RARs or other transcription factors would in turn depend on their cell-specific level of expression and/or activation from signals in the environment such as ATRA and TNF α .

Keywords

TNIP1; promoter; RAR; nuclear receptor

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1. Introduction

Nuclear receptor (NR) coregulator proteins modify chromatin structure or interact with the components of cellular transcription machinery to facilitate or inhibit transcription of NR target genes (Lonard and O'Malley B, 2007). In doing so, they help execute the transcriptional activation or repression due to ligand presence or absence, respectively. Given the importance of coregulators in mediating NR functions, we previously conducted a yeast two-hybrid screen of a human epidermal keratinocyte cDNA library using the DEF domains of peroxisome proliferator-activator receptor α (PPAR α) seeking novel NR coregulator proteins. Among the proteins isolated in this screen was tumor necrosis factor α (TNF α)-induced protein 3-interacting protein 1 (TNIP1). Our study of TNIP1 revealed it interacts with agonist bound PPARs and RARs but represses their activity (Flores et al., 2011; Gurevich and Aneskievich, 2009). In earlier reports of its isolation (Fukushi et al., 1999; Gupta et al., 2000), TNIP1 (also known as Naf and VAN) was shown to associate with the HIV proteins nef and matrix with a functional role in shuttling HIV particles between the cytoplasm and the nucleus. Endogenous TNIP1 protein is incorporated into progeny virions but experimentally-elevated TNIP1 inhibits HIV replication (Gupta et al., 2000). Beyond its association with HIV proteins, TNIP1 represses the activity of nuclear factor κ B (NF- κ B) (Cohen et al., 2009; Heyninck et al., 1999; Mauro et al., 2006; Oshima et al., 2009; Tian et al., 2005). This repression is mediated through TNIP1 interaction with the components of the inhibitor of NF- κ B (I κ B) kinase (IKK) complex (Heyninck et al., 2003; Mauro et al., 2006). Additionally, inhibition by TNIP1 of p105 processing to p50, one of the components of the p50/p65 heterodimer that constitutes NF- κ B, is also partly responsible for TNIP1 repression of NF- κ B (Cohen et al., 2009). Finally, associative links between increased *TNIP1* gene expression or sequence variations and the inflammatory diseases psoriasis, rheumatoid arthritis, and systemic lupus erythematosus have been demonstrated (Ramirez et al., 2012).

The effects of experimentally increased amounts of TNIP1 protein on NR, NF- κ B signaling, and HIV replication, as well as TNIP1 expression changes in inflammatory disease pathologies suggested to us the importance of identifying mechanisms controlling its expression levels. To this end, we cloned 6kb of the human *TNIP1* gene promoter and examined it for potential transcription factor binding sites. A previous study on regulation of *TNIP1* expression demonstrated the TNF α responsiveness of the gene and identified a NF- κ B binding site within a 626bp cloned fragment of the promoter (Tian et al., 2005). Our ongoing *in silico* and *in vivo* examination of this larger promoter region revealed additional NF- κ B sites (Gurevich et al., 2011), as well as a GC-rich region near the transcription start site containing previously uncharacterized expression control elements (Encarnacao and Aneskievich, in preparation). Relevant to our previous finding of TNIP1 protein repressing retinoic acid receptor (RAR) activity (Gurevich and Aneskievich, 2009) was the *in silico* prediction of retinoic acid response elements (RAREs) in the 6kb promoter region. The studies detailed here show that the transcription of *TNIP1* gene is regulated in part by ATRA and RAR. RAR regulation can be mapped to multiple locations including two RAREs located in the distal promoter region. Our results predict the existence of a negative feedback loop wherein RARs up-regulate TNIP1 expression, and TNIP1, in turn, represses RAR activity.

2. Materials and methods

2.1. Cloning and algorithm analysis of the human TNIP1 promoter

The -5887 to +111 and -3106 to +111 fragments of the of human *TNIP1* gene promoter were generated by PCR from a bacterial artificial chromosome (BAC) CTB-35A8

(Invitrogen, Carlsbad, CA) as described previously (Gurevich et al., 2011). Numbering is based on the overlap with the 626bp promoter fragment previously described in the reports by Brasier and colleagues (Shiote et al., 2006; Tian et al., 2005). *In silico* analysis of the promoter for potential transcription factor binding sites was carried out using public domain algorithms NHR-scan (Sandelin and Wasserman, 2005) and NUBIScan (Podvinec et al., 2002) as well as Transfac database (Biobase, Beverly, MA).

2.2. Generation of the luciferase reporter plasmids and transient transfections

The −5887 to +111 (6kb) and −3106 to +111 (proximal 3kb) fragments were moved as *Sall*/*XhoI* and *NheI*/*XhoI* PCR amplicons into the corresponding sites of pGL4.10 vector (Promega, Madison, WI). The latter fragment contained the promoter's naturally occurring *NheI* site internal to the PCR amplicon. The −5887 to −3106 (distal 3kb) fragment was subcloned from the 6kb-luc construct into the *NheI* site of the tk-luc vector (pGL4.10 with thymidine kinase minimal promoter). Other distal promoter fragments were subcloned into the tk-luc vector using existing restriction enzyme sites, or as PCR amplicons using primers described previously (Gurevich et al., 2011). The mutant DR5 constructs (see Fig. 2C) were prepared using the QuikChange XL kit (Stratagene, LaJolla, CA) according to manufacturer instructions with PCR primers shown in Table 1. The 3xDR5-tk-luc 3xDR2-tk-luc were prepared using oligomers shown in Table 1. All constructs were confirmed by sequencing at the University of Connecticut DNA Biotechnology Facility. Transient transfections using these vectors, luciferase reporter assays, protein assays and data analyses were carried out in HeLa cells or HaCaT keratinocytes, as previously described (Gurevich and Aneskievich, 2009). Cells were treated with either vehicle (0.1% DMSO) or 1μM ATRA. When reporters containing differing lengths of the promoter were used, the transfected construct amounts were normalized to keep the copy numbers in different transfections equal.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

Single stranded RARE-containing oligomers (IDT, Coralville, IA) were annealed according to manufacturer instructions. Sequences of scrambled oligomers were generated using the Shuffle DNA program within the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/shuffle_dna.html). In all cases, restriction fragments or oligomers were designed to produce a thymidine-containing overhang on each end, which were labeled with α³²P-dATP (PerkinElmer, Waltham, MA) using T4 DNA polymerase (Promega) according to manufacturer instructions. Nuclear extracts were prepared as previously described (Lee et al., 1988) from COS-7 cells transiently transfected with pSG5-RARγ or pSG5-RXRα. Reactions were performed as previously described using 6μg each of RAR- and RXR-enriched nuclear extract (Gurevich et al., 2010). To confirm specificity of binding, the experiments were conducted with the addition of 30- or 90-fold molar excess of unlabeled oligomers. Supershift assays were performed with specific RAR or RXR antibodies or non-specific IgG (all from Santa Cruz Biotechnologies, Santa Cruz, CA) which were added to the reaction 5 minutes after incubation of nuclear extracts with radiolabeled probes.

2.4. Quantitative real-time PCR (qPCR)

HeLa cells were treated with either vehicle (0.1%DMSO), 1μM ATRA, 100nM trichostatin A (TSA) or ATRA+TSA for 1h before harvest. qPCR was performed using TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems, Foster City, CA) according to the manufacturer instructions on ABI 7500 Fast Real Time PCR System (Applied Biosystems). TNIP1 expression was normalized to RPLP0. Probes used were Hs00374581_m1 for TNIP1 and 4326314E for RPLP0. Data analyses were carried out on ABI 7500 v2.0.1 software using ΔΔC_T method.

2.5. Western blotting

HeLa cells were seeded, allowed to grow for 24h, and then exposed to 1 μ M ATRA+ 100nM TSA for 0, 3, 6, 12, or 24h before harvest. Treatments were timed so that all the samples were collected at the same time to have cultures of the same age but differing duration of ATRA+TSA exposure. Western blotting was performed as previously described (Gurevich et al., 2011) using rabbit polyclonal TNIP1 antibody (Gurevich et al., 2011) and, to confirm equal loading, β -actin antibody (Abcam, Cambridge, MA). Images were digitally captured using Kodak IS440CF imager (Kodak, Rochester, NY) and densitometry analyses were performed using Kodak Image Station software.

2.6. Chromatin immunoprecipitation (ChIP) assays

RAR ChIP assays were performed with chromatin isolated from HaCaT keratinocytes processed with a two-step fixation protocol utilizing first disuccinimidyl glutarate and then formaldehyde (Nowak et al., 2005). HaCaT keratinocytes were seeded at 5×10^6 per 10cm diameter plate and 16 hours later were treated with vehicle (0.1% DMSO) or 2 μ M ATRA for 1hr prior to fixation. Subsequent steps were as described (Nowak et al., 2005). DNA/protein complexes were precipitated with 4 μ g of normal rabbit IgG (sc-2027) or rabbit polyclonal (sc-550) anti RAR α antibody (both from Santa Cruz). PCR primers were: 5'-ACTTCCTGAGAGGCTGGGCC-3' forward and 5'-GGCCCCTCCCACAGTTCCTA-3' reverse. Cycling conditions were: 94°C for 3min; 32 cycles of 94°C for 20sec, 64°C for 30sec, 72°C for 30sec; with a final extension of 72°C for 2min. Amplicons were resolved on an ethidium bromide stained 2% agarose gel.

For H3K4me3 ChIP assay, the chromatin was prepared as described above and precipitated using normal rabbit IgG or rabbit polyclonal H3K4me3 antibody (Abcam). SYBR Green qPCR was performed using previously described primers (Gurevich et al., 2011) and analyzed using ABI 7500 v2.0.1 software.

3. Results

3.1. The human TNIP1 promoter is ATRA and holo-RAR responsive

The human *TNIP1* gene has a TATA-less promoter that is increasingly GC rich near the transcription start site (Gurevich et al., 2011). *In silico* analysis for potential transcription factor binding sites revealed numerous putative constitutive and inducible elements suggesting *TNIP1* expression is likely to be controlled by an interplay of transcription factors and vary depending on cell type and environment. In light of our previous finding that TNIP1 represses RAR activity (Gurevich and Aneskievich, 2009), we were intrigued by prediction (Table 2) of three potential direct repeat spaced by 5 nucleotides (DR5) RAREs, the most preferred RARE architecture (Balmer and Blomhoff, 2005) and a potential direct repeat spaced by 2 nucleotides (DR2), another common RARE conformation (Aranda and Pascual, 2001). The retinoid responsiveness of the cloned promoter sequence was tested in the context of a luciferase reporter driven by the -5887 to +111 (here referred to as 6kb) promoter (Fig. 1A) in transient transfections of HeLa cells. Expression from the TNIP1 promoter construct was activated by 1 μ M ATRA and further enhanced by transfection of either RAR α or RAR γ together with their heterodimer partner RXR α (Fig. 1B). This recombinant DNA study demonstrated retinoid responsiveness of the cloned promoter, lent credence to algorithm prediction of RAREs, and led us to test the retinoid responsiveness of the endogenous *TNIP1* gene. Surprisingly, qPCR analysis showed no difference in *TNIP1* expression in HeLa cells treated with ATRA compared to control (Fig. 1C) suggesting some repressive modification of the endogenous promoter. Epigenetic silencing of retinoid-responsive genes can be alleviated through HDAC inhibition (Epping et al., 2007) and consistent with this premise, treatment with the HDAC inhibitor TSA and ATRA together

led to a significant increase in the *TNIP1* mRNA level; TSA alone had no effect on *TNIP1* expression (Fig. 1C). Further, ATRA+TSA treatment of HeLa cells resulted in a gradual increase of TNIP1 protein with 2.2-fold higher levels after 24h of treatment compared to vehicle control (Fig. 1D).

To demonstrate RAR occupancy of its putative response elements sites within the native distal *TNIP1* promoter, we performed chromatin immunoprecipitation (ChIP). While endogenous levels of HeLa cell RAR levels were sufficient to detect an ATRA-induced activation of the *TNIP1* reporters (Fig. 1B) that could be augmented with recombinant RAR, for the ChIP assay we sought to examine RAR-*TNIP1* promoter interaction in cells where RAR levels are not as limiting as in HeLa cells. Our earlier studies demonstrated higher endogenous levels of RAR α , RAR γ and *TNIP1* proteins in HaCaT keratinocytes than in HeLa cells (Gurevich et al., 2011). The results of the ChIP assay (Fig. 1E) from HaCaT keratinocytes demonstrate the capture of endogenous RAR α on the distal portion of the *TNIP1* promoter containing the algorithm-predicted RAREs. As RARs occupy their target response elements regardless of ligand presence (Gillespie and Gudas, 2007; Privalsky, 2004), we saw no difference in signal between vehicle and ATRA treated cells. To further address ATRA induction of *TNIP1* gene expression, we performed a ChIP assay against trimethylated lysine 4 residue on histone 3 (H3K4me3), a highly conserved and well characterized mark of active chromatin near the transcription start site (TSS) of a gene (Eissenberg and Shilatifard, 2010). Because of the established association of H3K4me3 with the gene TSS (Eissenberg and Shilatifard, 2010), we employed primers located in the proximal *TNIP1* promoter. Our results (Fig. 1F) indicate that ATRA treatment of HaCaT keratinocytes does indeed lead to the increased enrichment of H3K4me3 over control IgG, further suggesting ATRA responsiveness of the gene.

3.2. Dissecting *TNIP1* promoter responsiveness to RAR α and γ

Subcloned fragments of the *TNIP1* 6kb promoter were examined for their regional responsiveness to ATRA and RARs. Because algorithmic analysis did not predict any potential RAREs in the proximal 3kb fragment of the promoter (−3106 to +111), it was somewhat surprising to find activation of this reporter by ATRA and RAR α and γ (Fig. 2A). With multiple predicted RAREs, the distal 3kb region was tested *in toto* and as subdivisions driving the minimal tk promoter (tk-luc). Activation of the reporter containing the distal 3kb portion (−5887 to −3106) with all three predicted DR5 RAREs (−4546, −3743, −3564) and the predicted DR2 RARE (−4716) was increased over the tk-luc reporter in the presence of either RAR α or RAR γ (Fig. 2A) by about 6–8-fold. However, the −4396 to −3106 reporter, which contains the two more-downstream predicted DR5 RAREs at −3564 and −3743, had no more response to ATRA or RAR α or γ than the base tk-luc construct (Fig. 2A) and was eliminated from further consideration. Conversely, activation within the 3kb distal portion could be entirely localized to the −5887 to −4396 fragment, containing the putative DR5 RARE at −4546 and the −4716 DR2. Subsequent studies (Fig. 2B) refined RAR response in the distal promoter to −5206 to −4396, since its activation by holo-RAR was similar to that of the reporter driven by the entire 3kb distal promoter. The DR5 in this fragment, as wild-type and mutated sequences, was tested for its function as a RARE (Fig. 2C) in the context of the smallest RAR-responsive fragment of the distal promoter (−5206 to −4396). For mutants in the −5206 to −4396 fragment (M1, M2, or del), nucleotides within a half site of the DR5 were altered or the entire DR5 element was deleted. Expression from mutant reporters was reduced by ~50%, a significant activity loss compared to wild-type for both ligand-activated RAR α and RAR γ (Fig. 2C). Using radiolabeled oligomer containing the −4546 DR5 with 6 flanking nucleotides on each side as probe, we found a complex forming in the presence of RAR γ and RXR α that was disrupted by the addition of RAR γ antibody but not control IgG (Fig. 2D, top panel). Furthermore, in

competition assays, co-incubation with excess unlabeled –4546 or the well-characterized *RARβ2* promoter RARE (de The et al., 1990), eliminated the shifted complex, whereas the scrambled –4546 probe had a very limited effect and only when added at 90-fold molar excess (Fig. 2D, bottom panel). These results, taken together, show that RARs bind –4546 DR5 and it does play a role in the RAR responsiveness of *TNIP1* promoter.

3.3. RAR binding and activation of distal *TNIP1* promoter direct repeats

While the above results are consistent with the –4546 DR5 conferring transcriptional responsiveness to RAR, mutation of the element did not eliminate all RAR activation (Fig. 2C), leading us to hypothesize that the predicted –4716 DR2 plays a role in the RAR-mediated promoter activation. To examine this possibility, we first prepared four luciferase reporter constructs, each fusing approximately one quarter of the –5206 to –4396 *TNIP1* promoter fragment (Fig. 3) to tk-luc, and examined the ability of these constructs to be activated by RARs. Indeed, the majority of the responsiveness contained within parental construct stemmed from the –4828 to –4599 region containing the –4716 DR2. Notably though, as an internal reference, the –4624 to –4396-tk-luc reporter was prepared with the –4546 DR5 deleted (Δ –4624 to –4396, similar to del construct in Fig. 2C), and included in the experiment. This reporter did exhibit significantly less activation in response to RAR than did the wild type (Fig. 3), confirming our earlier observations that DR5 –4546 does play a role in the RAR responsiveness of the promoter.

To further investigate RAR responsiveness of the –4828 to –4599 fragment, we used double stranded oligomers each covering about a third of the that region as EMSA probes. Only the central third, containing the presumptive DR2 element at –4716, formed a RAR-dependent complex that could be supershifted by RAR γ or RXR α antibodies (Fig. 4A), whereas only non-specific complexes were observed for each of the other two thirds (not shown). These results were confirmed by EMSA restricting the oligomer probe to the –4716 DR2 with minimal flanking sequences (6 base pairs up- and down-stream of the DR2) (Fig. 4B). Consistent with the larger promoter fragment, a complex formed with nuclear extract was disrupted by RAR γ antibody but not non-specific IgG. In competition assays, this DR2-nuclear extract complex was disrupted by excess unlabeled probe or unlabeled β RARE oligomer but not by scrambled β RARE oligomer. The functionality of the –4546 DR5 and the –4716 DR2 (Fig. 4C and D, respectively) was demonstrated by their conferring RAR α and γ and further ATRA responsiveness to a basal minimal tk-luc reporter.

4. Discussion

Our studies of retinoid responsiveness of the cloned human *TNIP1* promoter showed endogenous RAR levels present in HeLa epithelial cells are sufficient to activate the reporter construct driven by the entire 6kb fragment. Activation in the presence of ATRA is significantly enhanced when RAR is recombinantly expressed in HeLa cells. Furthermore, we demonstrated under permissive epigenetic conditions, i.e., in the presence of HDAC inhibitor TSA, *TNIP1* gene expression is ATRA responsive, confirmed by ATRA-induced increase in the levels of marker of active chromatin H3K4me3. Silencing of *TNIP1* retinoid responsiveness may parallel that seen for other retinoid responsive genes such as the extensively-studied *RARβ* promoter, especially its reduced or lack of ATRA response in malignant cells (Epping et al., 2007). Notably through, silencing does not necessarily equate with lack of RAR interaction with the identified RAREs. We detected endogenous RAR α binding to the *TNIP1* promoter independent of experimental ligand. These results parallel observations by Gudas and colleagues of constitutive receptor occupancy of the RAR β RARE (Gillespie and Gudas, 2007) and more recent observations from the same laboratory that RARs participate in epigenetic regulation of target genes in the absence of ATRA (Laursen et al., 2012), supporting the interpretation that RARs bind to their cognate response

elements regardless of ligand presence and only the composition of transcription-regulating complexes associated with the receptor changes depending on the presence or absence of agonist (Gillespie and Gudas, 2007; Privalsky, 2004). Which cells routinely are capable of *TNIP1* retinoid responsiveness may need to be determined empirically given recent results for nuclear receptor PPAR γ occupancy of a specific genomic peroxisome proliferator response element in preadipocytes versus macrophages. For these two different cell types, only a 28% overlap of loci bound by PPAR γ after agonist treatment was found (Lefterova et al., 2010), indicating cell-specific limits on what response elements may be utilized for NR-mediated transcriptional activation of *TNIP1* or other genes.

The functional dissection of the *TNIP1* promoter indicated that the RAR-driven induction may stem not only from two distinct distal RAREs (–4546 DR5 and –4716 DR2), but also from as yet uncharacterized retinoid-responsive region or regions within the proximal portion of the promoter. This region may contain RAR binding sites with atypical architecture especially given the numerous confirmed RAREs of non-DR5 or non-DR2 conformation (Germain et al., 2006; Moutier et al., 2012; Wu et al., 2009).

It should be kept in mind that RARs are but one of several transcription factors involved in activation of the *TNIP1* promoter. Previous work demonstrated NF- κ B induction of the proximal promoter (Tian et al., 2005) and our ongoing work expanded that by demonstrating NF- κ B and PPAR regulation of the distal promoter (Gurevich et al., 2011). It is likely that these inducible as well as constitutive transcription factors, depending on their cell-specific amounts, contribute to overall control of *TNIP1* gene expression. Like *TNIP1*, two other genes, hyaluronan synthase 2 (*HAS2*) and immediate early response 3 (*IER3* also known as *DIF2*) have been recently characterized as responsive to both NR ligands and TNF α (Makkonen et al., 2009; Saavalainen et al., 2007; Witcher et al., 2008). For instance, the human *HAS2* promoter is coordinately regulated by RAR, NF- κ B, cAMP response element binding (CREB), epidermal growth factor, and Sp1 in cell-dependent manners. For *HAS2* expression in HaCaT keratinocytes, Sp1 played a comparatively minor role but was the predominant regulator in HEK293 cells (Makkonen et al., 2009). Moreover, the content of transcriptional complexes assembled on the different control elements of the *HAS2* promoter varied greatly depending on whether the cells were treated with ATRA or TNF α as well as the duration of the treatment (Saavalainen et al., 2007).

It is more likely the rule rather than the exception for genes such as *HAS2*, *DIF*, and *TNIP1*, that final levels of their expression are derived from combinatorial effects of transcription factors (e.g., RAR and NF- κ B) as well as the relative amounts and duration of their cognate inducing soluble factors (e.g., NR and TNFR ligands). Intriguingly, in addition to this, the activation of the *TNIP1* promoter by multiple factors including RAR (this work), PPARs (Gurevich et al., 2011) and NF- κ B (Gurevich et al., 2011; Tian et al., 2005) may set the stage for negative feedback loops whereby increased expression of *TNIP1* leads to its repression of RAR (Gurevich and Aneskievich, 2009), PPAR (Flores et al., 2011), and NF- κ B (Heyninck et al., 2003) transcriptional activity. Likewise, expression of RIP140, the archetypical corepressor of agonist-bound NRs, is activated by the receptors it targets (White et al., 2003). Regulatory feedback loops between corepressors and their target signaling pathways may serve to buffer cells against extremes of hormone- or cytokine-regulated signaling, whether due to excess or inappropriately timed exposure, as well as participate in routine fine control of transcription as opposed to all-on or completely-repressed scenarios.

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Abbreviations

HIV	human immunodeficiency virus
NR	nuclear receptor
TNFα	tumor necrosis factor α
TNIP1	TNF α -induced protein 3-interacting-protein 1
ATRA	all <i>trans</i> retinoic acid
RIP140	receptor interacting protein 140
NF-κB	nuclear factor κ B
Sp1	specificity protein 1
RAR	retinoic acid receptor
EMSA	electrophoretic mobility shift assay
ChIP	chromatin immunoprecipitation
RARE	retinoic acid response element
PPAR	peroxisome proliferator-activated receptor
RXR	retinoid X receptor
DR5	direct repeat spaced by 5 nucleotides
DR2	direct repeat spaced by 2 nucleotides
HDAC	histone deacetylase
TSA	trichostatin A
qPCR	quantitative real-time PCR

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Highlights

- Putative RAR binding sites were identified in 6kb promoter of human *TNIP1* gene
- The cloned *TNIP1* promoter is ATRA and RAR responsive
- The *TNIP1* gene is ATRA inducible under permissive epigenetic conditions
- Two distinct functional RAREs were confirmed in *TNIP1* promoter
- Control over *TNIP1* expression depends on complex transcription factor interplay

Fig. 1.

TNIP1 promoter is ATRA responsive and *TNIP1* expression is ATRA-inducible following HDAC inhibition. (A) Schematic of the luciferase reporter with the putative RARE starting positions relative to the transcription start site marked (DR5 in gray, DR2 in black). (B) Activation of the -5887 to +111 TNIP1 luciferase (Luc) reporter in HeLa cells transfected as indicated. *:P<0.05 vs. vehicle (Student's t-test); *: P<0.05 vs. empty vector + ATRA (ANOVA with Newman-Keuls *post hoc* test). (C) RT qPCR of TNIP1 expression normalized to RPLP0 expression from HeLa cells treated as indicated. *:P<0.05 vs. all other conditions (ANOVA with Newman-Keuls *post hoc* test). (D) Western blots for TNIP1 and β -actin (loading control) from HeLa cells treated with 1 μ M ATRA and 100 nM TSA for indicated time periods. (E) ChIP assay for endogenous RAR α from vehicle (0.1% DMSO) or ATRA-treated HaCaT keratinocytes with PCR primers localized to the RARE-containing distal portion of the *TNIP1* promoter. (F) ChIP assay for H3K4me3 analyzed by qPCR with primers localized near *TNIP1* transcription start site and chromatin from HaCaT keratinocytes as indicated in E.

Fig. 2. RAR responsiveness of the TNIP1 promoter stems partially from -4546 DR5. (A) Activation of the luciferase reporters driven by TNIP1 promoter fragments shown on the left with the putative RARE sites marked (DR5 in gray, DR2 in black). (B) A distal promoter fragment containing the -4546 and -4716 RAREs but not other putative RAREs provides reporter activation similar to the entire distal 3kb region. (C) Mutation of the DR5 within -5206 to -4396 reduces reporter activity in HeLa cells compared to wild-type (WT) sequence. RARE DR5 half-site mutations (M1 and M2) in italics or crossed-out sequence indicating deletion (del). *: $P < 0.05$ vs WT + ATRA (ANOVA with Dunnett's *post hoc* test). (D) *Top*: EMSA with -4546 oligomer as probe. NE, nuclear extract. Arrowhead indicates receptor-specific complex disrupted by RAR γ antibody (0.3, 0.6 or 1.2 μ g) but not control IgG (0.6 or 1.2 μ g). NS, non-specific complex. *Bottom*: Competition for binding to the -4546 RARE from inclusion of unlabeled oligomers representing S, self; β R, β RARE; or sS, scrambled self nucleotide sequences.

Fig. 3.

RAR responsiveness maps to the DR2 -4716 segment of the -5206 to -4396 region in addition to the -4546 DR5. All promoter subsections were prepared to share a 25bp overlap with adjacent sections and subcloned into the tk-luc reporter. Reporter activation in HeLa cells cotransfected with RARγ and RXRα expression plasmids is expressed as % of activation seen with the -5206 to -4396-tk-luc treated with ATRA. *P<0.05 vs. each other (Student's t-test).

Fig. 4. RARs bind to the -4716 DR2 and both the -4546 DR5 and -4716 DR2 are RAR inducible. (A) EMSA using TNIP1 promoter fragment -4771 to -4700 as probe. (B) EMSA with -4716 oligomer as probe. In A and B, NE: RAR γ and RXR α containing nuclear extract, IgG: normal rabbit IgG, γ : RAR γ antibody, α : RXR α antibody. In A, supershifted complexes are denoted by asterisks. In B, S: self; β R: β RARE; s β R: scrambled β RARE; NS: non-specific complex; the RAR/DR2 complex is denoted by arrowhead. (C and D) Activation in HeLa cells of tk-luc reporter or tk-luc reporter containing 3 copies of -4546 DR5 (C) or -4716 DR2 (D). *P<0.05 vs. tk-luc+ATRA (Student's t-test).

Table 1

Primers used in generation of TNIP1 promoter constructs with mutation sites underlined and core RARE sequences bolded.

Construct		Primer sequence
3xDR5	For	5'-CTA GTT GTA ACA GGC CAG CCC GAG GAC ACA GGG AGG-3'
	Rev	5'-CTA GCC TCC CTG TGT CCT CGG GCT GGC CTG TTA CAA-3'
3xDR2	For	5'-CTA GTC CAG CTT GAC ACC CTG ACC TGG AGA CG-3'
	Rev	5'-CTA GCG TCT CCA GGT CAG GGT GTC AAG CTG GA-3'
M1	For	5'-CCC TAG AAA CTG AAG TGT AAC <u>TAA</u> CCA GCC CAG GGA CAC AGG G-3'
	Rev	5'-CCC TGT GTC CCT GGG CTG <u>GTT AGT</u> TAC ACT TCA GTT TCT AGG G-3'
M2	For	5'-GAA GTG TAA CAG GCC AGC CCA <u>TTT</u> ACA CAG GGA AAA TAG TTG GG-3'
	Rev	5'-CCC AAC TAT TTT CCC TGT <u>GTA AAT</u> GGG CTG GCC TGT TAC ACT TC-3'
del	For	5'-TAG AAA CTG AAG TGT AAC CAG GGA AAA TAG TTG GGA-3'
	Rev	5'-TCC CAA CTA TTT TCC CTG GTT ACA CTT CAG TTT CTA-3'

Table 2

Algorithm predicted RAREs in the promoter of *TNIP1* gene with half sites in capital letters.

Element	Sequence
DR5 -3564	CACAAAtttagcCGTAAG
DR5 -3716	AGTTCAAtttagcTGCTCC
DR5 -4546	AGGCCAgcccaGGGACA
DR2 -4716	TGACACccTGACCT