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TNIP1 reduction of HSPA6 gene expression occurs in promoter regions lacking binding sites for known TNIP1-repressed transcription factors

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Abstract

TNF α -induced protein 3-interacting protein 1 (TNIP1) represses signaling pathways initiated by specific nuclear and transmembrane receptors. This effect results in reduced activity of distinct transcription factors such as retinoic acid receptors (RAR), peroxisome-proliferator-activated receptors (PPAR), and NF κ B. TNIP1-null and TNIP1-knockin defective for ubiquitin-binding mice show increased liver apoptosis, and enlarged spleen and lymph nodes, respectively. To complement current knowledge of TNIP1's broad physiologic functions as interpreted from *in vivo* studies and specific expression consequences from transcription factor repression, we determined effects of excess TNIP1 on global gene regulation. Following experimentally increased expression of TNIP1 in cultured keratinocytes, our gene expression microarray analysis not only confirmed TNIP1's association in previously known pathways and functions but also found a novel TNIP1-regulated pathway – the cell stress response. Under standard culture conditions, expression of several heat shock proteins, including HSPA1A, HSPA6, DNAJA1 and DNAJB1, was reduced. In heat-stressed conditions, differential regulation of HSPA1A and HSPA6 was observed, where only HSPA6 expression was reduced after heat-shock. Using HSPA6 as a model to elucidate the mechanism of the TNIP1-mediated HSP repression, we determined TNIP1 likely represses HSPs through factors other than RAR, PPAR or NF κ B despite presence of these factors' binding sites in the HSPA6 promoter. These results indicate that regulation of HSPs may be through a yet unknown TNIP1-associated pathway. Additionally, these results suggest TNIP1's reduction of HSP expression levels could negatively impact HSP chaperone capacity or their participation in the cell stress response.

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Keywords

TNIP1; ABIN1; HSP; HSPA6

1. Introduction

Tumor necrosis factor (TNF) α -induced protein 3-interacting protein 1 (TNIP1) regulates signaling initiated by several transmembrane and nuclear receptors (NR) ([1] for review). TNIP1 (alias ABIN-1 for A20 binding and inhibitor of NF κ B 1) overexpression represses signaling downstream of TNF α receptor (TNF α -R) activation [2] by interacting with NEMO (IKK γ), reducing IKK complex activation, thus decreasing NF κ B translocation to the nucleus [3] and ultimately reducing NF κ B-dependent gene expression [4]. Additionally, TNIP1 represses signaling cascades initiated by epidermal growth factor receptor (EGF-R) [5] and toll-like receptor (TLR) [6, 7] resulting in reduced activation of transcription factors Elk-2 and C/EBP β , respectively. We previously reported TNIP1 is a ligand-dependent corepressor for PPAR [8] and RAR [9] likely via competition with coactivators otherwise binding to and stimulating agonist-bound, NR-dependent transcription. Additionally, TNIP1 binding with the HIV proteins nef (alias Naf, nef-associated factor) [10] or matrix (alias VAN, virion-associated matrix-interacting protein) [11] extends its potential interacting partners to exogenous proteins and emphasizes the opportunities presented by its cytoplasmic and nuclear location [12] to control numerous signaling events. As expected from these characteristics, experimental genomic alterations of TNIP1 coding sequences have pervasive effects. Germline knockout results in smaller mice with high fetal and neonatal lethality due to liver apoptosis and anemia [13]. Knockin [6] of TNIP1 coding sequences altered to prevent its interaction with NEMO leads to development of lupus-like autoimmune pathologies in spleen and lymph nodes. Thus, the accumulated aliases for TNIP1 and the dramatic consequences from its loss or altered function demonstrate its important contribution to several complex cell and organ level physiological endpoints.

To date, most studies complementing TNIP1 knockout or altered sequences by expressing increased TNIP1 levels have focused on consequences to specific signaling pathways (NF κ B- or NR-dependent gene expression [2, 4, 8, 9]) as might be expected from its interaction partners (A20 or NEMO and PPAR or RAR). To recognize potential new TNIP1-targeted pathways in a relevant cell type, we examined human keratinocyte (KC) gene expression via microarray following TNIP1 overexpression. We originally isolated TNIP1 from a human KC cDNA library as an NR interacting protein [8, 9] and subsequently established endogenous TNIP1 expression in multiple cell and tissue types including stratified squamous epithelia such as skin [12]. Unexpectedly, increasing TNIP1 in cultured keratinocytes showed reduced expression of several heat shock protein (HSP) genes, HSPA6, HSPA1A, HSPA1B, DNAJA1, and DNAJB1, members of the HSP70 and HSP40 families, respectively. HSPs are a superfamily of proteins well known for protecting cells from numerous stress events, such as exposure to heat, chemicals, and inflammatory signals. Characteristic increased HSP expression follows stress events leading to improved refolding of denatured proteins thus blocking protein aggregation and preventing cell death. However, there is also an important role from constitutive expression of several HSP family members

as “house-keeping” chaperone proteins [14]. Both constitutively and inducibly expressed HSP are important for cell homeostasis. Knockdown of HSPA1A in unstressed conditions leads to reduced cell growth and differentiation [15]. Preventing HSPA1A and HSPA6 induction under stress conditions results in reduced cell survival providing evidence for HSP roles in cell protection and recovery [16].

In the present study, we examined global changes following increased TNIP1 levels to complement earlier work done on specific NFκB- and NR-mediated pathways highlighting the effect on biological function and changes on transcript expression. Gene clustering analysis not only confirmed TNIP1’s regulation over TNFα-R and NR targeted genes but it also revealed a novel pathway involved in a possible role for TNIP1 — the cell stress response centering on HSPs. As a model to determine the means for the repression of HSP expression by TNIP1, we studied expression from the endogenous gene and cloned HSPA6 promoter under control and excess TNIP1 conditions. *In silico* promoter analyses of HSPA6 and other HSP promoters found predicted binding sites for NFκB, PPAR and RAR, suggesting TNIP1 could regulate these genes through these transcription factors. Surprisingly, our results indicate that TNIP1 does not repress HSPA6 through known TNIP1-targeted transcription factors. These data indicate a novel TNIP1-repressible pathway leading to reduced gene expression of HSP family members and by extension, suggest a possible TNIP1 effect on signal mediators other than could be inferred from previous studies centering on NFκB [2, 4, 8, 9] and NR [2, 4, 8, 9] regulated gene expression.

2. Materials and methods

2.1 Cell Culture

HaCaT KCs (kindly provided by Dr. N Fusenig [17]) were cultured in a 37°C humidified incubator with 5% CO₂ in a 3:1 DMEM/F12 media containing 10% FBS (Thermo Scientific HyClone, Logan, UT), 100U/ml penicillin, and 100 µg/ml streptomycin. The cells were plated on 6- or 24-well plates at a density of 6.8×10^5 or 1.5×10^5 cells per well, respectively. Twenty-four hours after plating, cells were infected with an adenovirus construct expressing TNIP1 (Ad-TNIP1) or LacZ as a control (Ad-LacZ) at a multiplicity of infection (MOI) of 500 using 8 µg/ml Polybrene (Millipore, Billerica, MA) [18] in DMEM/F12 media containing 2% FBS. Sixteen hours post-infection, the viral mixture was aspirated and media replaced. Twenty-four hours post-infection, cells were collected for isolation of total RNA using RNeasy (Qiagen, Valencia, CA) or protein using RIPA lysis buffer (10 mM Tris, 150 mM NaCl, 1% deoxycholic acid, 1% Triton, 0.1% SDS). Parallel cultures were used for thermal stress assays. Thermal stress was induced for 1 hour at 42°C (water bath) while control cells were transferred to a 37°C water bath for the same amount of time. Cells were allowed various recovery times in a 37°C humidified incubator, as indicated.

Normal human epidermal keratinocytes (NHEK) were cultured in a 37°C humidified incubator with 5% CO₂ in KBM-Gold supplemented with KBM-Gold Bullet Kit (cells and media, Lonza Biologics Inc, Hopkinton, MA) at passage numbers 2–4. On day 0, cells were plated at 9.5×10^4 cells per well on 6-well plates with low calcium-containing media. Two

KC culture conditions were used for NHEK experiments: the first generating undifferentiated and the second generating differentiated KCs. KC differentiation can be induced by switching the media calcium content from a 0.1 mM (low) to 1.2 mM (high) concentration [19]. On day 2, cells to be collected from low calcium media were infected with Ad-TNIP1 or Ad-LacZ (control) at an MOI of 50 using 8 µg/ml Polybrene. On day 3, the cells cultured in low calcium medium were collected for total RNA. Cultures to generate differentiated KCs were plated on day 0 at 9.5×10^4 cells per well on 6-well plates under low calcium-containing media. On day 3, growth medium was replaced with fresh media supplemented to a final concentration of 1.2 mM calcium. On day 5, cells were infected with Ad-TNIP1 or Ad-LacZ (control) at an MOI of 50 using Polybrene and collected for total RNA on day 6.

2.2 Microarray

Total RNA isolated from triplicate cultures of LacZ- or TNIP1-expressing adenovirus-infected cells was provided to the University of Connecticut Health Center Molecular Core Facility for microarray analysis using Illumina Beadarray Human WG-6 3.0 microarrays. Primary analysis was carried out using the GenomeStudio software (Illumina, San Diego, CA). Significantly altered genes were determined using a fold change of 2 and a p-value 0.05 (DiffScore = 16). Functional grouping and analysis was performed using Ingenuity Pathway Analysis (IPA) online software (Ingenuity Systems, www.ingenuity.com) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) web-accessible program [20, 21].

2.3 Real-Time quantitative PCR analysis

Reverse transcription was performed using aliquots from total RNA used for the microarray using iScript reverse transcriptase (BioRad, Hercules, CA). Gene expression changes were analyzed using POWER SYBR green master mix (Life Technologies, Grand Island, NY). Real-time PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR system. Data analysis was carried out on ABI 7500 software using the $\Delta\Delta C_T$ method. All data was normalized to the ribosomal protein L13a (RPL13a) transcript [22].

2.4 Immunoblot analysis

Whole-cell lysates were prepared in RIPA buffer and the protein concentration was determined using the Pierce 660 nm Protein Assay (Thermo Scientific Pierce, Rockford, IL). Ten micrograms of protein were separated by SDS/PAGE, transferred to nitrocellulose membranes, rinsed with nanopure water and treated with Qentix (Thermo Scientific Pierce). Blots were incubated in blocking buffer consisting of 5% (w/v) non-fat dried milk, phosphate-buffered saline, and 0.1% Tween 20, then probed with HSP70B' (HSPA6) antibody (ADI-SPA-754) at 1:1000 dilution (Enzo Life Sciences, Farmingdale, NY) or anti-HSPA1A antibody (ADI-SPA-810) at 1:1000 dilution (Enzo Life Sciences) [23, 24] followed by HRP (horseradish peroxidase)-conjugated secondary goat anti-mouse antibody at 1:10,000 dilution (PerkinElmer, Branford, CT). Blots were subsequently probed with β -actin antibody (ab8227) at 1:5,000 dilution (Abcam, Cambridge, MA) followed by HRP-conjugated secondary goat anti-rabbit antibody at 1:20,000 dilution (PerkinElmer). Between probing steps, blots were washed with 0.2% Tween 20 in phosphate-buffered saline.

Detection of binding was determined with enhanced chemiluminescence reagents (Thermo Scientific Pierce). Band signals were digitally captured and analyzed using Kodak image station CCF and Carestream molecular imaging software. Fold changes for TNIP1 and HSP were determined relative to β -actin.

2.5 Generation of luciferase constructs

The HSPA6 promoter containing the -2962 to +48 bp sequence was PCR amplified from human genomic DNA (cat# 636401) (BD Biosciences, San Jose, CA) as described in [25] using forward: 5'-GAT GGG TAC CTC ATC TTG AAT TCC CAC AAC ACA TGG-3' and reverse: 5'-GGC TGA AGC TTA GTG AGG CTC TCC CTG CGG TTT CTC T-3' with added *KpnI* and *HindIII* cloning sites (underlined), respectively for insertion into the promoterless vector pGL4.10 (Promega, Madison, WI). Numbering is based on +1 as the transcription start site as previously described [26, 27]. Five-prime promoter truncations (-1230, -647 and -70 -luc) were performed by using the inserted *KpnI* site within the forward primer and native restriction enzyme sites *BglII*, *EcoRI* and *NruI* within the promoter, respectively. Digested sites were blunted and ligated. Constructs A, B, C, and D were generated using the -647 luc construct. Internal promoter deletions and fragment G site-specific mutants were generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA). Candidate transcription factor-binding sites were determined using NHR Scan [28], Nubiscan [29] and MatInspector [30] web-based software. All HSPA6 3kb isolate and truncation constructs were confirmed by sequencing (University of Connecticut Biotech Center).

2.6 Plasmid transfections

HaCaT KCs were plated a density of 1.5×10^5 cells per well using 24-well plates. Twenty-four hours after plating, cells were infected with Ad-TNIP1 or Ad-LacZ at a MOI of 500 using Polybrene in DMEM/F12 media supplemented with 2% FBS [18]. Eight hours later, the appropriate HSPA6 promoter pGL4.10 plasmid (200 ng) and pCMV- β galactosidase (100 ng) was transfected using Fugene6 (Promega) using 100 μ L serum-free media. Twenty-four hours later, cells were collected and assayed for the luciferase activity (Promega) and protein concentration (Pierce).

2.7 Statistical analysis

All data were analyzed using Prism Software version 5 (GraphPad, La Jolla, CA). Student's t-test was used to compare between paired results. Statistical significance was defined as shown in the figure legends.

3. Results

3.1 Functional classification of the TNIP1-affected genes

To determine the effects of increased TNIP1 levels in KC, recombinant TNIP1 was overexpressed in HaCaT cells, a human immortalized, but non-tumorigenic KC cell line which retains KC differentiation properties [17]. Using an adenoviral vector expressing either TNIP1 (Ad-TNIP1) or control β -galactosidase (Ad-LacZ), an ~11-fold increase of TNIP1 protein was observed twenty-four hours after adenoviral infection (Fig 1a). Gene

expression microarray analysis showed 139 genes were significantly regulated using cutoffs of 2-fold change and a p-value ≤ 0.05 (Fig 1.b). Within that group, expression for 135 genes was reduced (see Table 1 for top 10 reduced genes) and expression for 4 genes was increased (Table 2). RT-qPCR analysis for several genes representing targets of NF κ B and NR regulation validated the microarray results (Fig 1c)

To determine the biological processes most affected by TNIP1 overexpression, we used two pathway analyses and clustering web-accessible programs, IPA and DAVID. IPA analysis groups significantly altered gene expression by biological function and affected toxicological pathways. The biological functions most affected by increased TNIP1 levels include cancer, cell death, inflammatory diseases and gene expression (Fig 2a). IPA clustering showed that signaling involving PPAR and RAR activation were affected (Fig 2b). Prominent pathways previously unknown to be regulated by TNIP1 included general and Nrf2-mediated oxidative stress pathways. To increase statistical significance for predictive pathway analysis by IPA, we examined the population of regulated genes additionally using the functional annotation software, DAVID (Fig 2c). Consistently, classes demonstrating significant enrichment of regulated genes included cell death and transcriptional regulation and we conclude that the functional classes identified by the IPA and DAVID software packages are highly similar, increasing confidence in the predictive analyses. From both the IPA and DAVID analyses, the unexpected biological function affected by TNIP1 was the stress response pathway.

3.2 Heat shock response gene expression is repressed by TNIP1

We examined expression of specific genes within the stress response pathway to better characterize this potentially novel consequence of TNIP1 excess. The microarray and cluster analyses results showed reduced gene expression of several stress response proteins, including five known HSP family members (HSPA6, DNAJB1, HSPA1B, HSPA1A and DNAJA1) repressed by 20-, 5-, 3.2-, 3- and 2-fold, respectively, and one predicted HSP gene (HSP70B) reduced by 5-fold. HSPA6 expression was most repressed by TNIP1 in our analysis. HSP gene expression results were validated via qPCR (Fig 3a). Since the role of the HSPA family is well characterized in KC biology, we further assessed whether TNIP1's inhibitory effect on this family extended to altered protein expression. We observed reduced protein expression of both HSPA1A and HSPA6 (21- and 45-fold, respectively) in response to increased TNIP1 protein expression (Fig 3b).

In addition to using HaCaT KCs, we also examined the transcript expression levels of HSPA1A and HSPA6 in normal human epidermal keratinocytes (NHEK) following TNIP1 overexpression. NHEK differentiation can be induced by a switch from low to high calcium-concentration media (see Materials and Methods). We confirmed that differentiation occurred by examining the mRNA levels of two KC differentiation markers [31], keratin 1 (K1) and involucrin (IVLN). Control cells (LacZ-expressing, Fig 3c & d, gray bars) show increased expression of K1 and IVLN (38- and 13-fold), respectively, indicating KC differentiation is occurring following the media change to high calcium. Both HSPA1A and HSPA6 increased 3- and 23-fold, respectively at the transcript level in differentiated cells infected with the Ad- LacZ vector (Fig 3e & f, gray bars). However, in parallel

differentiated cultures overexpressing TNIP1, transcription of these HSPs was repressed (Fig 3e & f, high Ca^{2+} gray versus black bars). HSP expression repression did not occur in undifferentiated KCs (Fig 3e & f, low Ca^{2+} conditions, gray versus black bars).

3.3 Repression of HSPA1A and HSPA6 gene expression by TNIP1 differs following heat stress

Since TNIP1 over-expression represses HSPA1A and HSPA6 in non-stressed conditions, we tested whether TNIP1 excess would also prevent the stress-inducibility of these two genes. In LacZ-expressing HaCaT KCs, the stress-induced expression profile was similar for both HSPA1A and A6 (Fig. 4a and b, respectively, open gray circles). However, HSPA6 had a greater degree of induction at 300-fold compared to HSPA1A's 10-fold induction. Consistent with results above from standard 37°C culture conditions, TNIP1 over-expression reduced unstressed expression of both HSPs (Fig 4a and b, filled black circles, $t = 0$ hours of heat stress). The repression under stressed conditions was different for HSPA1A and HSPA6. After the 1 hour heat shock ($t = 1$), HSPA1A expression levels during the recovery period ($t = 2, 3, 4, 6, 8, 12$ and 24 hours) overlapped for control LacZ- and TNIP1-expressing cells (Fig 4a). Conversely, HSPA6 expression was decreased immediately after cell stress and up to 12-hours post heat shock (Fig 4b). By 24 hours following heat shock, both HSPA1A and HSPA6 expression levels were similar in LacZ- and TNIP1-expressing cells.

3.4 TNIP1 does not regulate HSPA6 through known TNIP1-responsive transcription factors

Since TNIP1 reduces both HSPA1A and HSPA6 transcript levels in unstressed conditions, we sought to determine the possible mechanism of TNIP1's repression on this gene family. We hypothesized that the TNIP1 repression of transcriptional activation by PPAR and RAR nuclear receptors or NFκB [8, 9, 32, 33] could be responsible for the reduced HSP expression. We performed an *in silico* analysis of HSPA6 human gene for potential sites for these transcription factors and identified several potential NFκB sites, retinoic acid response elements (RARE), and peroxisome proliferator response elements (PPRE) (Fig 5a). To test TNIP1-associated repression throughout this extended promoter length, we cloned a region of the HSPA6 gene spanning ~3kb (−2962 to +48 bp relative to the transcription start site) containing its promoter. Predicted sites from genome databases were confirmed by our own sequencing: the distal promoter region (−2962 to −1230 bp) contains two NFκB sites, two RAREs and five PPREs; two predicted NFκB sites were found within the central promoter region (−1230 to −647 bp). The proximal (−647 to −70 bp) and minimal (−70 to +48 bp) regions did not contain any binding sites for predicted TNIP1-targeted transcription factor. To localize the TNIP1-responsive region, we used the −2962-luc construct and generated three 5'-truncation constructs (−1230, −647 and −70-luc) using restriction enzyme sites native to the promoter (Fig 5a). Luciferase levels were unaffected by TNIP over-expression for the minimal −70-luc construct (Fig 5b) indicating that the core promoter is not sensitive to TNIP mediated regulation. However, we observed a 60% reduction in promoter activity following TNIP1 overexpression when the reporter construct contained the −647 to −70 bp upstream sequence. Promoter activity for the −1230 and −2962-luc constructs was not further repressed following TNIP1 overexpression, suggesting the −647 to −70 bp fragment of the HSPA6 promoter contains the sequence information encoding the TNIP responsive

region (Fig 5b). Intriguingly, no sites for known TNIP1-repressed transcription factors are located within this sequence segment indicating that HSPA6 down-regulation does not occur through PPAR, RAR or NF κ B.

To identify the TNIP1 responsive region more accurately within -647 to -70 bp DNA stretch, a series of internal deletion promoter constructs was generated each removing ~150 bp within the -647-luc construct. Four constructs were generated: A- (deleting the region between -647 to -478 bp), B- (-478 to -346 bp), C- (-346 to -216 bp) and D-luc (-216 to -70 bp) (Fig 5c). Consistent with its use in independent experiments (Fig. 5b), the -647-luc construct promoter activity was reduced by 65% compared to the LacZ control (Fig. 5d). Individual deletion of fragments A, B or C relieved the TNIP1-mediated repression (Fig 5d). Removal of region D not only negated the repressive effect of TNIP1 but also increased it beyond that of the LacZ control (Fig 5d). *In silico* promoter analysis of the proximal (-647 to -70 bp) region (Table 3) indicated the presence of multiple transcription factor binding sites; however no known TNIP1-repressed transcription factors were among them. Although we broadened our search to other TNIP1-repressed factors, including C/EBP [6, 7] and Elk-1 [5], predicted binding sites for these transcription factors were also not found. This suggests that TNIP1 may repress HSPs through a novel, as yet uncharacterized TNIP1-repressible factor and pathway.

4. Discussion

Although TNIP1 represses certain receptor-mediated signaling pathways [2–4, 6, 8, 12] and has links to specific inflammatory diseases [34–37] (refer to [1] for review), the wider consequences of altered TNIP1 levels are still being determined. We report here microarray results from KCs with acutely increased levels of TNIP1 showing increased expression for 4 genes and decreased expression for 135 genes. Consistent with published TNIP1 repressive effects [2, 8, 9], genes with reduced expression included those transcriptionally activated by NF κ B and NR such as interleukin 6 NF κ B inhibitor alpha and retinoic acid receptor responder 3. Additionally, pathway clustering analyses supported TNIP1 association with immunological and inflammatory diseases. Unexpectedly, this approach also revealed a previously unknown TNIP1-associated effect – the possible regulation of chaperone function and cell stress response through repressed expression of several HSPs (HSPA6, HSPA1A, HSPA1B, DNAJA1, and DNAJB1).

In addition to their widely-appreciated role in protein stabilization following thermal or chemical stress, several HSPs, through their constitutive expression, provide ongoing support to cells by assisting protein folding and shuttling, which is critical for cellular homeostasis [14, 38–40]. While increased TNIP1 repressed constitutive expression of HSP70 gene family members HSPA1A and HSPA6, it was only the latter that showed continued reduction under stress conditions. How the stress-induced transcriptional increase of HSPA1A enabled expression to overcome the TNIP1-associated repression remains to be fully determined. Several expression regulation scenarios are possible, ranging from repression mechanisms similar for HSPA1A and HSPA6 under constitutive expression conditions to repression unique to each promoter under both constitutive and stress-induced conditions. Events such as stress induction may involve binding of multiple transcription

factors; we have recently reported that maximal stress induction of the HSPA6 promoter relies not only on heat shock response elements, but also sites for basal transcription factors [25]. Any of these factors, or possibly others, could contribute to the TNIP1-repression on these HSPA6, but not HSPA1A. Additionally, several reports [41–44] have established that transcription factor binding can be mutually exclusive and competition may occur for same or nearby sites changing transcriptional regulation of the target gene. During a stress response, different regulatory elements or different spacing of shared elements within the HSPA1A and HSPA6 promoters, such as binding of heat shock factors, may impact promoter conformation, which affects access for any TNIP1-regulated factor and ultimately HSP expression. As demonstrated by Noonan and colleagues, the cell's ability to recover and survive following exposure to stress is dependent on cell type, availability of multiple HSPs, and the type of stressor. Additionally, their work showed that both HSPA1A and HSPA6 are needed for maximal survival following heat or proteasome inhibitor exposure [16]. Thus, the full impact of TNIP1-reduced HSPA6 expression will likely depend on other events affecting cell homeostasis including the availability of other HSP.

TNIP1 inhibition of NF κ B- and NR-mediated transcription is well established [2, 4, 8, 9]. However, despite the presence of consensus binding sites for NF κ B, PPAR, and RAR throughout 3kb of the HSPA6 promoter, experimental deletion of these areas with these does not relieve sensitivity to TNIP1 suggesting repression is occurring via as yet unrecognized pathways. Following localization of TNIP1 repression to –647 to –70 bp of the HSPA6 promoter, we observed that individual deletion of three contiguous subregions (A – C) each returned reporter promoter activity to that seen with the LacZ control. Unexpectedly, removal of fragment D from the –647-luc construct increased expression beyond the control level. To account for the increased activity of the D-luc reporter, TNIP1 may have a positive effect on the remaining sub regions A – C seen only after removal of the dominant repression-mediating site(s) in region D. However, we consider any such possible positive consequence of TNIP1 on the HSPA6 promoter to be very minimal since there is a pronounced repressive effect on the –647-luc construct, which contains all sub regions (A – D). Furthermore, promoter constructs including upstream sequences up to –1230 and –2962 bp show a similar degree of repression (see Fig 5a and b). *In silico* promoter analysis of the –647 to –70 bp region did not recognize any TNIP1-repressed transcription factors even after extending our search to include Elk2 [5] and C/EBP [45], the known TNIP1-inhibited downstream targets of EGFR and TLR, respectively. Further analysis does not reveal any prime candidate as a transcription factor binding site mediating the TNIP1 effect within all four fragments. Using a stringent cutoff (i.e., threshold score 0.95), no one site was in common to all fragments (Table 3, 14 unique and repeated sites), the loss of which might alleviate its cognate factor's repression by TNIP1. This distinction among fragments holds even using a relaxed cutoff (i.e., threshold score 0.90 yielding 37 unique and repeated sites, not shown) suggesting that extensive further physical testing (promoter reporter mutagenesis, DNA-protein binding assays, etc.) of one or possibly several sites within a fragment will be needed to determine which is (are) responsible for relief from TNIP1 repression. This consideration further points to transcription activity control by TNIP1 beyond that previously known via NF κ B and NR pathways.

In the present study, we performed a gene microarray analysis to broadly examine gene expression and signaling altered by increased TNIP1 levels in KCs. The results confirmed known TNIP1 associations with specific signaling pathways and diseases and also discovered a novel cellular target, the cell stress response. Reduced expression levels of several HSPs were observed and detailed analysis of the HSPA6 promoter suggested TNIP1 can repress transcription activation outside of known NF κ B- and NR-mediated signals. The potential to identify other TNIP1 signaling targets and possible long-term effects of TNIP1 on HSP-mediated chaperoning or survival following heat or chemical exposure warrant further study.

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Abbreviations

ABIN1	A20 binding inhibitor of NF κ B
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNAJA1	DnaJ (HSP40) homolog, subfamily A, member 1
DNAJB1	DnaJ (HSP40) homolog, subfamily B, member 1
HSPA1A	heat shock 70kDa protein 1A
HSPA6	heat shock 70kDa protein 6
IPA	Ingenuity pathway analysis
IL6	Interleukin 6
KC	keratinocyte
MAPK13	Mitogen activated protein kinase 13
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
NHEK	normal human epidermal keratinocytes
PPRE	Peroxisome proliferator activated receptor response element
RARE	Retinoic acid response element
RARRES3	Retinoic acid responder gene 3
RNASE7	RNase A family 7
TNIP1	TNF α -induced protein 3-interacting protein 1

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Highlights

- Overexpression of TNIP1 results in repression of a majority of gene expression.
- Transcription of several heat shock protein family members is blocked by TNIP1.
- TNIP1 repression of HSPs is through a novel, as yet uncharacterized mechanism.

Fig. 1. TNIP1 overexpression microarray results. **a.** Expression and quantitation of TNIP1 protein from control- (Ad-LacZ) or TNIP1-overexpressing (Ad-TNIP1) HaCaT KCs. Representative western blot shown. Statistical significance was determined using Student's t-test, ** $p < 0.01$. Bars are mean + SEM from experimental triplicates. β -actin is used as a loading control. **b.** Volcano plot analysis of microarray results. Each point represents one gene. **c.** Validation of microarray using qPCR analysis of several genes.

Fig. 2. Cluster analysis of genes with significantly altered expression. **a.** IPA biological function analysis of the top affected clusters. **b.** IPA pathway cluster of the top affected pathways. **c.** DAVID biological function analysis of the top affected clusters.

Fig. 3. Effect of TNIP1 overexpression on HSPs. **a.** Microarray and qPCR analysis of HSP genes in HaCaT KCs after TNIP1 overexpression. **b.** Western blot analysis of HSPA1A and HSPA6 in HaCaT KCs. β-actin is used as a loading control. Quantitative PCR analysis of **c.** keratin 1 (K1) **d.** involucrin (IVLN) **e.** HSPA1A and **f.** HSPA6 normalized to the ribosomal protein L13a transcript in undifferentiated (low Ca²⁺) or differentiated (high Ca²⁺) NHEKs infected with Ad-LacZ (control) or Ad-TNIP1. Statistical significance was determined using Student's t-test, * $p < 0.05$. Bars are mean + SEM from experimental triplicates.

Fig. 4.

Effect of TNIP1 overexpression on HSP transcription levels in heat stressed HaCaT KCs. Quantitative PCR of **a.** HSPA1A and **b.** HSPA6 before ($t = 0$ hour) or after ($t = 1, 2, 3, 4, 6, 8, 12$ and 24 hours) a 1 hour heat stress at 42°C . Statistical significance was determined using Student's t -test, LacZ vs. TNIP1: $**=p<0.01$; $*=p<0.05$; $\dagger=p<0.1$; $\ddagger=p<0.12$. Bars are mean + SEM from experimental triplicates.

Fig. 5. HSPA6 promoter luciferase reporter construct analysis. **a.** Diagram of the HSPA6 5' promoter truncation constructs. The triangles, arrows and diamonds denote predicted PPRE, RARE and NFκB binding sites, respectively. **c.** Diagram of the HSPA6 internal promoter deletions within -647-luc construct. Deletions are shown as the dotted line. Open box indicates the start of the luciferase reporter gene. Graphs shown as percent promoter response to increased TNIP1 compared to LacZ control of the HSPA6 promoter truncations (**b**) or internal deletions (**d**). Letters denote statistical significance between Ad-LacZ vs. Ad-TNIP1 infected cells $p < 0.05$, using Student's t-test. Bars are mean + SEM from $n=3$, experimental replicates.

Table 1

List of top 10 genes reduced following TNIP1 overexpression

Gene Symbol	Gene Name	Fold Change
HSPA6	Heat Shock 70kDa protein 6 (HSP70B')	-20.00
IL6	Interleukin 6 (interferon, beta 2)	-10.00
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	-8.33
ZC3HAV1	Zinc finger CCCH-type, antiviral 1	-6.25
EGR1	Early growth response 1	-5.56
DNAJB1	DnaJ (HSP40) homolog, subfamily B, member 1	-5.00
LOC652878	Predicted: Heat shock 70kDa protein 6 (HSP70B)	-5.00
ARID5B	AT rich interactive domain 5B(MRF1-like)	-4.76
HERC5	Hect domain and RLD5	-4.35
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78	-4.17

Table 2

List of genes increased following TNIP1 overexpression.

Gene Symbol	Gene Name	Fold Change
MAPK13	Mitogen Activated Protein Kinase 13	5.63
UAP1L1	UDP-N-acetylglucosamine pyrophosphorylase 1-like 1	2.14
RNASE7	RNase A family, 7	2.07
LTB4R2	Leukotriene B4 receptor 2	2.04

Table 3

List of predicted transcription factor binding sites located within –647 to –70 bp using MatInspector threshold score 0.95.

Transcription Factor Binding Site	Frag A	Frag B	Frag C	Frag D
Activator Protein 1	✓		✓	
Activator Protein 2		✓		
CCAAT Binding Factors		✓		
GATA Binding Factors		✓		
Heat Shock Factors	✓	✓		✓
Human and Murine ETS1 Factors		✓		
MAF and AP1 Related Factors	✓		✓	
Mouse Krueppel like Factor		✓		
Myeloid Zinc Finger 1 Factors		✓		
Neuron-specific Olfactory Factor		✓		
Odd-Skipped Related Factors		✓		
RNA Pol II Transcription Factor II B		✓		
Winged Helix Binding Sites		✓		
Zfx- and Zfy-Transcription Factors		✓		