The fructose-2,6-bisphosphatase TIGAR suppresses NF-κB signaling by directly inhibiting the linear ubiquitin assembly complex LUBAC

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Abstract

The systems integration of whole-body metabolism and immune signaling are central homeostatic mechanisms necessary for maintenance of normal physiology, and dysregulation of these processes leads to a variety of chronic disorders. However, the intracellular mechanisms responsible for cell-autonomous cross talk between the inflammatory signaling pathways and metabolic flux have remained enigmatic. In this study, we discovered that the fructose-2,6-bisphosphatase TIGAR (Tp53-induced glycolysis and apoptosis regulator) critically regulates NF-κB activation. We found that TIGAR potently inhibits NF-κB-dependent gene expression by suppressing the upstream activation of IKKβ phosphorylation and kinase activation. This inhibition occurred through a direct binding competition between NEMO and TIGAR for association with the linear ubiquitination assembly complex (LUBAC). This competition prevented linear ubiquitination of NEMO, which is required for activation of IKKβ and other downstream targets. Furthermore, a TIGAR phosphatase activity-deficient mutant was equally effective as wildtype TIGAR in inhibiting NEMO linear ubiquitination, IKKβ phosphorylation/activation, and NF-κB signaling, indicating that TIGAR’s effect on NF-κB signaling is due to its interaction with LUBAC. Physiologically, TIGAR knockout mice displayed enhanced adipose tissue NF-κB signaling whereas adipocyte-specific over expression of TIGAR suppressed adipose tissue NF-κB signaling. Together, these results demonstrate that TIGAR has a non-enzymatic molecular function that modulates the NF-κB signaling pathway by directly inhibiting the E3 ligase activity of LUBAC.

Introduction

TIGAR (Tp53-induced Glycolysis and Apoptosis Regulator) is a 270 amino acid protein that was originally identified as a p53-inducible protein that functions as a fructose-2,6-bisphosphatase but subsequently has been shown to have
phosphatase activities for 2,3-bisphosphoglycerate (23BPG), 2-phosphoglycerate, phosphoglycolate and phosphoenolpyruvate (1-5). Fructose-2,6-bisphosphate (F26P) is an allosteric activator of 6-phosphofructo-1-kinase (PFK1) and a negative regulator of fructose-1,6-bisphosphatase (FBP) that are key regulatory steps controlling glycolysis and gluconeogenesis, respectively (6,7). By reducing the levels of F26P, TIGAR was found to suppress glycolysis and the subsequent accumulation of glucose-6-phosphate was diverted into the pentose phosphate pathway to generate nucleotides, NADPH and antioxidants such as reduced glutathione (5,8). In contrast, TIGAR enzymatic activity was also reported to be 400-fold greater for 23BPG than F26P, generating 3-phosphoglycerate (9). Although an increase in 3-phosphoglycerate would be expected to increase glycolysis, the proximal substrate for the formation of phosphoenolpyruvate is 2-phosphoglycerate. 2-phosphoglycerate is generated from 3-bisphosphoglycerate by phosphoglycerate mutase (PGM), which is allosterically activated by 23BPG. Thus, whether TIGAR functions to inhibit or activate glycolysis is dependent upon the relative contributions of PFK1, FBP and PGM and their allosteric regulation by F26P and 23BPG. Through these mechanisms, TIGAR can modulate glucose metabolism for energy production and macromolecular synthesis as well as cellular redox state in different ways depending on the combination of several cell context modulators.

Physiologically, consistent with an overall increase in glycolysis and decrease in pentose flux, TIGAR deficiency was found to increase oxidative stress and cardiac damage during cardiac ischemic injury (10). TIGAR deficiency was also found to correlate with increased oxidative stress during Alzheimer’s disease progression (11). Similarly, in some tumor cell models, TIGAR was found to contribute to the anti-tumor promoting activity of p53 by suppressing aerobic glycolysis and cellular survival (5,12). However, TIGAR was over expressed in several other tumor cell types suggesting that TIGAR may also function to promote rather than inhibit cancer development (4,13,14). For example, TIGAR deficiency in a mouse intestinal tumor model was shown to increase animal survival with decreased tumor burden whereas increased TIGAR expression enhanced tumor progression (13). In these systems, TIGAR appears to promote cell survival and expansion by decreasing oxidative stress and increasing production of ribose for DNA and RNA synthesis. Moreover, TIGAR was reported to protect cells from genotoxic drug induced DNA damage partly through the regulation of pentose phosphate pathway products (NADPH and ribose) and reduction of reactive oxygen species (15). Thus, the growth promoting and/or growth inhibitory effects of TIGAR also appear to be cell context dependent suggesting the influence of additional signaling/metabolic events that determine the biological outcome of TIGAR function.

In this regard, there is mutual crosstalk and complex entanglement between metabolic flux, cellular oxidative stress, intracellular signal transduction and inflammatory signaling cascades, such that changes in one of these pathways can have multiple effects on any or all of these other pathways. During our studies of insulin resistance and inflammatory signaling of adipocytes, we examined the effect of TIGAR deficiency and over expression in the differentiated 3T3-L1 adipocyte cell line. Surprisingly, independent to its role as a regulator of glucose flux, we have found that TIGAR is also a potent negative regulator of NF-κB signaling and inflammatory cytokine production. Molecular analyses of the NF-κB activation pathway revealed that TIGAR suppresses the activation of IKKβ and IKKβ-dependent phosphorylation of downstream substrate targets. Moreover, the ability of TIGAR to prevent NF-κB activation is independent of TIGAR’s phosphatase activity that results from a direct binding
interaction with the HOIP subunit of the linear ubiquitination assembly complex (LUBAC). TIGAR competes for NEMO binding to HOIP and thereby suppresses NEMO linear ubiquitination necessary for IKKβ activation and activation of downstream targets.

Results

TIGAR suppresses NF-κB signaling in a phosphatase activity independent manner

Adipocytes play a central role in the integrative normal and pathophysiologic regulation of appetite, adiposity, energy balance and insulin responsiveness, including glucose and lipid metabolism (16-20). To assess the potential role of TIGAR in adipocytes, we generated control empty lentivirus (CTL), TIGAR shRNA knockdown lentivirus (TKD) and TIGAR cDNA overexpressing lentivirus (TOE) infected 3T3-L1 murine adipocyte cell lines. Examination of several signaling pathways in the differentiated adipocytes revealed that the TKD cells displayed increased TNF-α-stimulated serine 32/36 IkBα and serine 536 p65 (RelA) phosphorylation (Fig. 1A). In contrast, the TOE cells had reduced IkBα and p65 phosphorylation following TNF-α stimulation. In addition to IkBα and p65, CYLD is also a direct substrate of the IKKβ kinase whose phosphorylation on serine residue 418 suppresses its deubiquitinase activity (21,22). Similar to IkBα and p65, TNF-α-stimulated CYLD phosphorylation was increased in the TKD and suppressed in the TOE adipocytes. Consistent with TIGAR regulating the NF-κB signal transduction pathway, TNF-α increased Ccl2 and A20 gene expression in the TKD adipocytes that was suppressed in the TOE cells (Fig. 1B and C). Essentially identical results were obtained from multiple independently generated CTL, TKD and TOE 3T3-L1 adipocyte cell lines (data not shown).

To further confirm specificity of the TIGAR knockdown shRNA and in parallel the necessity of the TIGAR phosphatase activity in the 3T3-L1 adipocyte cell context of TIGAR knockdown (TKD), we next re-expressed a shRNA-resistant wild type TIGAR (TWT) and the phosphatase activity defective TIGAR mutant (TMU) in which the three catalytic pocket residues (H11, E102 and H198) were mutated to alanine (5). As expected, re-expression of wild type TIGAR in the cell context of TIGAR deficiency (TWT) resulted in suppression of TNF-α-stimulated IkBα, p65 and CYLD phosphorylation (Fig. 1D). The ability of the phosphatase defective mutant to inhibit these IKKβ-dependent phosphorylation events was also reflective in the suppression of TNF-α-stimulated gene expression (Fig. 1E and 1F). To directly demonstrate that the TIGAR inhibition of ligand-stimulated gene expression was specific to the NF-κB pathway and not due to potential regulation of other signaling pathways (i.e.: MAP kinase, PI3 kinase and protein kinase C), HEK293T cells were co-transfected with a specific NF-κB driven luciferase gene reporter with either empty vector, TWT or TMU cDNAs and then subsequently treated with TNF-α (Fig. 2A). As is apparent, both TWT and TMU were effective suppressors of TNF-α-stimulated luciferase activity. Together, these data demonstrate that TIGAR has a unique function to inhibit the canonical NF-κB signaling pathway that is independent of the TIGAR phosphatase activity.

TIGAR suppresses IKKβ activation by competing for NEMO

To address the mechanism(s) that could account for the apparent non-enzymatic function of TIGAR to suppress NF-κB signaling, we first examined the effect of TIGAR in cells over expressing IKKβ. Previous studies have observed that over expression of IKKβ results in its
spontaneous phosphorylation and activation of NF-κB signaling (23,24). Consistent with these findings, we observed that over expression of IKKβ resulted in the marked increase of Ccl2 (Fig. 2B) and A20 (Fig. 2C) mRNA levels. However, co-expression with TWT or the TMU cDNAs also substantially suppressed the Ccl2 and A20 gene expression. Moreover, over expression of IKKβ resulted in a robust level of IKKβ phosphorylation that was reduced when co-expressed with either TWT or TMU (Fig. 2D). These data suggest that TIGAR inhibits the function of IKKβ either directly or indirectly by inhibiting an upstream pathway.

To determine if TIGAR directly inhibited IKKβ, we expressed IKKβ alone or in combination with TWT or TMU and examined the in vitro kinase activity of IKKβ in cell extracts (Fig. 2E). Consistent with the inhibition of IKKβ phosphorylation and NF-κB dependent gene expression, co-expression of either TWT or TMU inhibited the in vitro kinase activity of IKKβ (Fig. 2E). We next mixed the cell extracts containing IKKβ with cell extracts containing TWT followed by determination of in vitro IKKβ kinase activity (Fig. 2F). Under these conditions, TIGAR was completely ineffective in altering the IKKβ kinase activity. These data indicate that TIGAR blocks the activation of IKKβ indirectly and not through a direct interaction with IKKβ.

Since the in vitro kinase assay used IκBα as the substrate, it remained possible that TIGAR interacted with the IκBα/NF-κB complex preventing IκBα accessibility as a substrate. To examine this, we co-expressed TIGAR with IKKβ and examined the phosphorylation of the IKKβ substrate CYLD (Fig. 3A). Over expression of IKKβ resulted in the phosphorylation of the activation site serine residues 177/181 in IKKβ as well as the phosphorylation of serine 418 in CYLD (Fig. 3A, lane 3). It should be noted that unlike 3T3-L1 adipocytes in which there is only a single band detected with the pS418-CYLD antibody (Fig. 1A and 1D), in HEK293T cells several bands are detected with only one corresponding to the molecular weight of the CYLD protein (depicted by arrow). The presence of these additional bands is probably due to the overexpression of IKKβ as only a single band at the expected molecular is observed when HEK293T cells are stimulated with TNF-α (data not shown). In any case, over expression of TIGAR alone (Fig. 3A, lane 2) or NEMO alone (Fig. 3A, lane 4) had no effect on endogenous CYLD phosphorylation and was essentially identical to vector transfectected cells (Fig. 3A, lane 1). In contrast, co-expression of IKKβ with TIGAR markedly suppressed IKKβ and endogenous CYLD phosphorylation (Fig. 3A, compare lane 3 with lane 5) whereas co-expression of IKKβ with NEMO resulted in the full extent of substrate phosphorylation (Fig. 3A, lane 6). These data demonstrate that TIGAR suppresses IKKβ-mediated phosphorylation of both expressed IKKβ and endogenous CYLD supporting an inhibition of IKKβ activation rather than an effect on substrate accessibility. Interestingly, expression of NEMO with TIGAR partially restored IKKβ and CYLD phosphorylation (Fig. 3A, compare lane 5 with lane 8). These data further suggest that TIGAR and NEMO compete at a common site responsible for IKKβ activation.

To test this prediction, we first determined the dose-dependent effect of TIGAR on IKKβ and CYLD phosphorylation (Fig. 3B). At a fixed IKKβ expression level (Fig. 3B, lane 2) TIGAR in a dose-dependent manner suppressed S177/181-IKKβ and S418-CYLD phosphorylation (Fig. 3B, lanes 3-6). Quantification of the TIGAR dose-dependent inhibition of IKKβ and CYLD phosphorylation is shown in Figure 3C and 3D. Conversely, we determined the ability of NEMO to reverse the TIGAR inhibition of IKKβ activation (Fig. 4A). At a fixed concentration of IKKβ and TIGAR that results in a marked inhibition of IKKβ and CYLD phosphorylation (Fig. 4A, lane 2 versus lane 3), NEMO in a dose-dependent manner reversed the TIGAR inhibition of IKKβ and CYLD phosphorylation (Fig. 4A,
lanes 4-7). It should be noted that with increasing amounts of expressed NEMO protein levels, the levels of the endogenous CYLD protein decreased. Whether this results from the activation of the MALT1-dependent proteolytic cleavage or ubiquitination and proteasome-mediated degradation of CYLD remains to be determined (25,26). Nevertheless, quantification of the NEMO dose-dependent reversal of TIGAR mediated inhibition of IKKβ and CYLD phosphorylation is shown in Figure 4B and 4C. In parallel, we also demonstrated the ability of NEMO to reverse the TIGAR inhibition of IKKβ stimulated Ccl2 gene expression (Fig. 4D). Together these data are consistent with a direct competition of TIGAR and NEMO at a common upstream site necessary for IKKβ activation and NF-κB signaling.

**TIGAR directly binds to the linear ubiquitination assembly complex (LUBAC) and inhibits NEMO linear ubiquitination**

Multiple analyses of endogenous and transfected TIGAR co-immunoprecipitation experiments failed to demonstrate any specific association of TIGAR with IKKα, IKKβ or NEMO (data not shown). Although negative, this suggests that the site(s) of TIGAR action is not through direct interaction with the IKK complex, consistent with the data presented in Figure 2. Furthermore, we have been unable to detect a specific interaction of TIGAR with the scaffolding proteins TRAF2 (TNF-α receptor) or TRAF6 (IL1β receptor) (data not shown). We then examined the interaction of TIGAR with the linear ubiquitination assembly complex (LUBAC) in cells over expressing TIGAR and three individual LUBAC subunit complex proteins, HOIP, HOIL-1L and SHARPIN (Fig. 5A). Although neither HOIL-1L nor SHARPIN displayed any specific interaction with TIGAR, immunoprecipitation of HOIP clearly demonstrated a specific co-precipitation with TIGAR (Fig. 5B).

Based upon the finding that TIGAR and NEMO compete for IKKβ activation, we examined the NEMO competition for TIGAR binding to HOIP. Cells were transfected with empty vector, HOIP, TIGAR and NEMO cDNAs separately or HOIP+TIGAR and HOIP+NEMO cDNAs together (Fig. 6A, lanes 1-6). In addition, HOIP and TIGAR were co-expressed with increasing amounts of NEMO cDNA (Fig. 6A, lanes 7-10). HOIP immunoprecipitation of the cells expressing HOIP+TIGAR demonstrated the co-immunoprecipitation of TIGAR and similarly cells expressing HOIP+NEMO demonstrated the co-immunoprecipitation of NEMO (Fig. 6B, lanes 5 and 6). Increasing levels of NEMO expression resulted in increased amounts of the NEMO protein co-immunoprecipitated with HOIP (Fig. 6B, lanes 7-10). In parallel, the amount of TIGAR co-immunoprecipitated with HOIP progressively decreased. These data directly demonstrate that TIGAR and NEMO compete for binding to the HOIP subunit of LUBAC.

HOIP is composed of several modular domains including a ZF (zinc finger), two NZF (Npl4 zinc finger), UBA (ubiquitin-associated), two R (ring fingers), RBR (ring between ring fingers) and LDD (linear ubiquitin chain determining domain) (27-30), schematically represented in Figure 7A. Previous studies have demonstrated that the NEMO binding specifically maps to the HOIP NZF1 domain (27). Consistent with these previous studies, immunoprecipitation of a HOIP NZF1 deletion mutant substantially reduced the amount of co-immunoprecipitated NEMO protein compared to wild type HOIP (Fig. 7B, lanes 6 and 7). In contrast, deletion of the NZF1 domain had no effect on the ability of HOIP to co-immunoprecipitate TIGAR (Fig. 7B, lanes 8 and 9). In contrast, deletion of the ZF domain reduced TIGAR binding by 20% whereas deletion of the NZF2 domain reduced TIGAR binding by approximately 60% (Fig. 7C, lanes 3 and 5). Deletion of the NZF1 had no effect on HOIP binding to TIGAR (Fig. 7C, lane 4). Moreover, deletion of the UBA resulted in an apparent increase in binding TIGAR binding (Fig. 7C, lane 6).
As the only domain that apparently resulted in a decrement in TIGAR binding, albeit relatively small was the NZF2 domain, we next analyzed an amino terminal HOIP deletion set (Fig. 7D). Progressive deletions of the HOIP amino terminus resulted in a decrease in the binding of TIGAR to HOIP when residues between 175 and 526 were deleted (Fig. 7D, lanes 2-5). Analyses of a carboxyl deletion set demonstrated loss of residues from 679-1029 had little effect on HOIP binding to TIGAR (Fig. 7E, lanes 2-5). However, TIGAR binding was almost completely abrogated upon further deletion to eliminate the UBA domain (Fig. 7E, lane 6). These data suggest that the HOIP amino acid region required for TIGAR binding primarily lies between residues 175-679 which includes the ZF, NZF1, NZF2 and UBA domain.

Quantification of all these binding data are presented as the percent of TIGAR immunoprecipitated with full length HOIP binding (denoted by the parenthesis) immediately next to the schematic HOIP structures shown in Figure 7A. Taken together, these data indicate that TIGAR does not directly compete for NEMO at the NZF1 HOIP binding site and therefore probably suppresses NEMO binding through steric hindrance and/or via induction of a mutually exclusive conformational change. In addition, TIGAR apparently interacts with HOIP through multiple contact sites most likely requiring the overall folding/conformational state of HOIP.

As HOIP is an essential component of the E3 linear ubiquitination activity of LUBAC, we hypothesized that TIGAR might suppress NF-κB activation by inhibiting NEMO M1 linear ubiquitination. As shown in Figure 8A, we expressed the LUBAC components (HOIP and SHARPIN) along with the E2 conjugating enzyme UBE2L3. This resulted in a substantial increase in total cellular protein linear ubiquitination that was prevented by the co-expression of both wild type (TWT) and the phosphatase defective (TMU) TIGAR proteins. Similarly, the expression of LUBAC with UBE2L3 resulted in a NEMO specific increase in M1 linear ubiquitination as observed in NEMO immunoprecipitates (Fig. 8B, lane 4). As observed in the whole cell lysates, expression of TWT or TMU blocked NEMO specific M1 linear ubiquitination (Fig. 8B, lanes 5 and 6). Furthermore, consistent with TIGAR and NEMO competing for IKKβ activation and HOIP binding, over expression of NEMO reversed the TIGAR inhibition of NEMO linear ubiquitination (Fig. 9A and 9B). In addition, immunoprecipitation of NEMO resulted in the co-immunoprecipitation of HOIP that was decreased in the presence of over expressed TWT or TMU (Fig. 8B, lanes 4-6).

Since these data are based upon over expression of LUBAC or IKKβ, we examined TNF-α-stimulated NEMO M1 linear ubiquitination in TIGAR knockout (TKD) and over expressing (TOE) cells. As previously observed in Figure 1, TNF-α stimulation increased IkBα and p65 phosphorylation in control 3T3-L1 adipocytes (NT) (Fig. 8C). TIGAR knockdown cells (TKD) displayed enhanced whereas TIGAR over expressing cells (TOE) had reduced TNF-α-stimulated IkBα, p65 and CYLD phosphorylation. In parallel, TNF-α treatment of control cells increased NEMO M1 linear ubiquitination that was further increased in the TKD cells and suppressed in the TOE cells. These data demonstrate that endogenous TNF-α receptor signal transduction results in NEMO M1 linear ubiquitination that is suppressed by the TIGAR protein.

**TIGAR regulates NF-κB signaling in adipose tissue in vivo**

To examine a potential role of TIGAR in adipocyte biology in vivo, we first determined the basal and TNF-α stimulation of NF-κB signaling in epididymal adipose tissue from wild type (WT) and whole body TIGAR knockout (TKO) mice. Intraperitoneal injection of TNF-α for 15 min resulted in a small increase in in serine32/36-IκB, serine536-Rel A and serine418-CYLD phosphorylation (Fig. 10A). In the TKO mice,
the vehicle treated adipose tissue displayed an increase in serine^{32/36}-IkB, serine^{536}-Rel A and serine^{416}-CYLD phosphorylation, similar to that of the TNF-α treated WT mice. However, TNF-α stimulation in the TKO mice resulted in significantly greater extent of IkB, RelA and CYLD phosphorylation. It should be noted that as the extent of IkB phosphorylation increased there was a concomitant decrease in IkB mobility most likely due to an increase in IkB ubiquitination (31).

If TIGAR deficiency increased NF-κB signaling, then we would expect that increased TIGAR levels should decrease NF-κB signaling. To test this in vivo, we generated mice with TIGAR cDNA knockin Rosa26-floxed stop mice that were crossed with Adiponectin-Cre (TGRS) mice to specifically increase TIGAR expression in adipocytes (32). Under these conditions, the TIGAR protein was increased approximately 1.7±0.3-fold, p<0.01. In the basal state, there was a small but not significant decrease in the extent of adipose tissue CYLD phosphorylation and no discernible change in IkB or RelA phosphorylation (Fig. 10B). However, following acute TNF-α stimulation the adipocytes from the TGRS mice displayed a marked blunting of IkB, RelA and CYLD phosphorylation compared to adipose tissue of control mice. In parallel, the basal adipose tissue Ccl2 gene expression was elevated in the TKO mice and suppressed in the TGRS mice. Moreover, TIGAR deficiency enhanced whereas TIGAR over expression suppressed TNF-α stimulation of Ccl2 mRNA levels (Fig. 10C). It should be noted that acute (15 min) of TNF-α stimulation was insufficient to significantly increase the mRNA levels of Ccl5, IL-1β, IL-13, A20 or TNF-α itself (data not shown). Together these data demonstrate that TIGAR suppresses NF-κB signaling in adipocytes in vitro as well as adipose tissue in vivo.

**Discussion**

Detailed molecular and cellular analyses have defined a complex set of intracellular signal transduction events that controls the activation of the NF-κB signaling pathway. NF-κB is a transcription factor composed of homo- or heterodimers of Rel domain homology proteins including p65/RelA, RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) (33,34). The dimeric NF-κB complexes are localized to the cytosol through interaction with IkBa, and following canonical signaling activation, for example by TNF-α, results in TNF-α receptor activation of RIP1, TRAF2 and NEMO K63-linked ubiquitination (35,36). Ubiquitated TRAF2 also provides a scaffold for the recruitment to the TAK1/TAB1/TAB2/3 complex and K63 ubiquitination recruits the IKK complex so that the TAK1 complex can phosphorylate and activate IKKβ leading to subsequent IkBα phosphorylation and IkBα K48 ubiquitination and subsequent IkBα degradation (37,38). The phosphorylation-dependent decrease in IkBα then releases the NF-κB dimer (i.e.: p65/p50) that translocates to the nucleus and activates NF-κB dependent target genes.

In addition to multi-site ubiquitination, recent studies have demonstrated that NEMO undergoes M1 linear ubiquitination by the linear ubiquitin chain assembly complex (LUBAC) composed of catalytically active HOIP associated with HOIL-1L and/or SHARPIN assembled as either HOIP/HOIL-IL or HOIP/SHARPIN dimeric and/or HOIL-IL/HOIP/SHARPIN trimeric complexes (35,36,39-42). Inhibition of LUBAC function substantially reduced NEMO linear ubiquitination, suppressed NF-κB activation and downstream signaling events in cell context dependent manner (27,36,39,40).

Numerous studies have demonstrated an intimate and co-dependent relationship between metabolism and NF-κB regulated inflammatory signaling. For example, endoplasmic reticulum and oxidative stress activate NF-κB signaling and conversely, NF-κB activation has also been reported to induce oxidative stress (43-45). In both humans and animal models
of insulin resistance, diet-induced obesity results in marked activation of adipose tissue inflammation, NF-κB signaling, endoplasmic reticulum and oxidative stress (16,17,44,46,47). Relieving of any of these events has been shown to improve metabolic function and to restore insulin sensitivity.

One important pathway controlling cellular redox state is the bifurcation of glucose catabolism through the glycolytic or pentose phosphate pathways that increases or decreases the cellular oxidation state, respectively. As TIGAR was identified as an important control enzyme regulating carbon flux through the glycolytic and pentose phosphate pathways (5,8), we undertook an analysis of TIGAR’s metabolic function in cultured 3T3-L1 adipocytes. To our surprise, we noticed that TIGAR deficiency resulted in an enhanced TNF-α activation of NF-κB signaling and NF-κB dependent gene expression. In this regard, TIGAR was causally linked, albeit indirectly, to the regulation of the NF-κB inflammatory signaling pathways in aging and cancer (48-51). TIGAR was also found in the connectome associated with the E2 ubiquitin-conjugating enzyme UBE2L3 that provides ubiquitin to LUBAC for linear chain ubiquitination (52). As adipocytes express relatively high levels of the TIGAR protein, we speculated that the metabolic regulatory functions of TIGAR might also intersect with the NF-κB inflammatory signaling pathway. The data presented in this study demonstrate that TIGAR, independent of its phosphatase activity, suppresses NF-κB activation by preventing NEMO linear ubiquitination through a direct binding to the LUBAC subunit HOIP. Since TIGAR suppresses the E3 conjugating ligase activity of the LUBAC complex and co-immunoprecipitated with HOIP in the absence of either SHARPIN or HOIL-1L, it is unlikely that TIGAR binds to the HOIL-1L or SHARPIN subunits. Consistent with this conclusion we were unable to detect any direct interaction of TIGAR with HOIL-1L or SHARPIN.

Several studies have identified the structural domains of HOIP, HOIL-1L and SHARPIN and their interactions responsible for LUBAC assembly and linear ubiquitination activity (27-30). Although the molecular basis for NEMO recognition has not been fully elucidated, NEMO has been reported to bind to the NZF1 domain of HOIP (27). The ability of NEMO in a dose-dependent manner to reverse TIGAR inhibition of IKKβ activation and conversely TIGAR in a dose-dependent to inhibit NEMO binding to HOIP strongly argues that TIGAR and NEMO compete with each other for binding to LUBAC. Although we confirmed the requirement for the HOIP NZF1 domain for NEMO binding, ablation of the NZF1 domain had no significant effect on TIGAR binding. Moreover, we were unable to detect any specific HOIP motif required for TIGAR binding, and both amino and carboxyl terminal deletions suggest that the TIGAR binding requires multiple contacts across the conformational state of HOIP. This conclusion is consistent with an allosteric NEMO and TIGAR induced conformational change in HOIP that is responsible for the mutually exclusive binding properties rather than through a direct competition at a unique amino acid domain. Although the molecular structure of the HOIP Ring Between Ring (UBA-RING1-IBR-RINF2-LDD) domain has recently been defined (53), structural analysis of full length HOIP with TIGAR remains necessary to define our understanding of LUBAC-NEMO interactions.

Currently, it is generally thought that over expressing IKKβ can result in the autophosphorylation and activation of IKKβ kinase activity independent of upstream activators, although it has also been reported that the TAK1 kinase complex (TAK1/TAB2/3) can phosphorylate IKKβ at a priming site (S177) that then allows for IKKβ autophosphorylation at S181 necessary for full activation (24). Nevertheless, our data clearly demonstrate that TIGAR expression inhibited the autophosphorylation of over expressed
IKKβ, proximal IKKβ downstream substrates as well as NF-κB target gene expression. These findings further indicate that over expressed IKKβ does not simply auto-activate itself but is also dependent upon LUBAC function for activation. In this regard, recent studies have suggested that the IKK complex when recruited to LUBAC results in the linear ubiquitination of NEMO (27,36). In turn, NEMO also contains an ubiquitin-binding domain such that NEMO in a second IKK complex is then recruited to the previous ubiquitinated IKK complex bound to LUBAC (36,37). The assembly of multimeric IKK complexes on LUBAC as proposed by Iwai and colleagues (54) then allows for the trans-autophosphorylation and activation of IKKβ.

In addition to these new mechanistic finding, our data also suggests and important physiologic role for TIGAR function in regulating adipose tissue inflammation. In vivo analysis of TIGAR knockout and adipocyte-specific TIGAR over expressing mice recapitulated the enhanced and suppressed TNF-α stimulation of NF-κB signaling, respectively, that occurred in cultured 3T3-L1 adipocytes. We also observed that high fat diet induced obesity, a well-established inducer of adipose tissue inflammation (16), also results in the down regulation of adipocyte TIGAR protein and mRNA levels (unpublished results). Although the major contribution to adipose tissue inflammation is the recruitment/activation of immune cell infiltrates, our data suggests that the enhancement of intrinsic adipocyte NF-κB signaling is, at least, partly due to the down regulation of the TIGAR protein. Further studies are now needed to determine the mechanism(s) responsible for the adipocyte-specific diet-induced down regulation of TIGAR.

In summary, the data presented in this manuscript demonstrate a novel cross talk between an important metabolic regulator, TIGAR, and the major signaling pathway controlling innate and adaptive immune responses, NF-κB. TIGAR, independent of its phosphatase activity, inhibits NEMO linear ubiquitination through a direct binding interaction with LUBAC. This results in a suppression of NF-κB downstream inflammatory signaling and may provide novel targets for the development of agents to modulate these signaling events in states of dysregulated metabolism and inflammation.

Experimental Procedures

TIGAR knockout/over expressing and adiponectin-Cre mice

Adiponectin-Cre mice maintained on the C56Bl/6/J background for greater than 10 generations were a kind gift from Dr. Evan Rosen (Beth Israel Deaconess Medical Center). TIGAR conventional knockout (deletion) ES cells were purchased from UCDAVIS KOMP Repository KNOCKOUT MOUSE PROJECT (Project ID: VG19113). ES cells were cultured and the B6 chimera mice were produced by the Gene Targeting Facility, Albert Einstein College of Medicine, Bronx, NY. TIGAR deficient mice (TKO) were backcrossed eight generations in the C57BL/6/J strain (000664, Jackson Laboratory). TIGAR tissue-specific over expressing mice was generated by Applied StemCell, Inc. (Milpitas, California). Briefly, the TIGAR mouse cDNA plasmid (9630033f20rik) was ligated into plasmid pCAG-loxP-stop-loxP-attB (pBT378-CAG-LSL). This plasmid and integrase mRNA for attP integration was injected into pronucleus of zygotes and the recombinant positive F1 germline transmitted mice were shipped to Albert Einstein College of Medicine. These mice were then backcrossed with C57BL/6J more than seven times before mating with the Adiponectin-Cre mice to generate adipocyte-specific TIGAR over expressing (TGRS) mice.

Mice husbandry

Wild type, TKO and TGRS mice were housed in a facility equipped with a 12 hr light/dark cycle. Animals were fed either a normal chow diet (NCD) that...
contains 62.3% (kcal) carbohydrates, 24.5% protein, and 13.1% fat (5053, LabDiet). Male mice at 12 weeks of age were given an intraperitoneal injection with either 100 µl of saline or 100 µl of TNF-α (10 µg TNF-α per kilogram body weight) in saline solution for 15 min. The mice were killed and the tissues were collected and snap frozen in liquid nitrogen and stored in -80 °C freezer. All studies were approved by and performed in compliance with the guidelines of the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Method Details

Cell Lines

Murine 3T3-L1 preadipocyte and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1X Penicillin-Streptomycin. Cell lines were maintained in a 5% CO2 incubator at 37°C. Cell lines were routinely tested to exclude Mycoplasma contaminations.

3T3-L1 adipocyte differentiation

3T3-L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured at 37°C in an 8% CO2 atmosphere in DMEM containing 25mM glucose and 10% calf serum. Confluent cultures were induced to differentiate by incubation of the cells with DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 µg of insulin per ml, 1 µM dexamethasone, 0.5 mM isobutyl-1-methylxanthine, and 1 µM rosiglitazone. After 3 days, the medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 1 µg of insulin per ml, and the incubation was continued for an additional 2 days. The medium was then changed to DMEM containing 25 mM glucose and 10% fetal bovine serum. Under these conditions, more than 95% of the cell population morphologically differentiated into adipocytes. The adipocytes were maintained for an additional 3 to 5 days prior to use. Fully differentiated 3T3-L1 adipocytes were placed over night in DMEM in the presence of 10% serum and then incubated with 10 ng/ml of mouse TNF-α for the time indicated.

Lentivirus short hairpin RNA knockdown in 3T3-L1 preadipocytes

MISSION lentiviral short hairpin RNA (shRNA) Bacterial Glycerol Stocks for mouse TIGAR shRNA and Non-Target control shRNA plasmids were obtained from Sigma-Aldrich (St. Louis, MO). The lentiviral human TIGAR plasmid DNA was obtained from GeneCopoeia (Rockville, MD). The plasmid DNAs were transformed and amplified in Mix & Go Competent Cells-Strain HB 101 (Zymo Research, Cat#T3013, Irvine, CA) and purified using the PowerPrep HP Plasmid Maxiprep Kits with Prefilters (Origene, Rockville, MD) and were transfected into human embryonic kidney 293T cells along with Lentiviral Packaging Mix (Sigma-Aldrich) to produce lentiviruses per the manufacturer’s instruction. 3T3-L1 preadipocytes (80% confluence) were infected with the Non-Target, mouse TIGAR shRNA, and human TIGAR overexpression lentivirus, respectively, selected by puromycin, and subjected to standard adipocyte differentiation. The TKD cells were further infected with human wild type TIGAR or enzymatic inactive mutant TIGAR lentiviruses and selected by hygromycin to produce TKD rescue TWT and TMU cells.

Total RNA extraction and quantitative RT-PCR

Cellular total RNA was extracted using QIAzol Lysis Reagent and RNeasy Mini Kit (Qiagen, Germantown, MD). First-strand cDNA was synthesized using SuperScript® VILO cDNA Synthesis Kit (ThermoFisher Scientific Invitrogen, Grand Island, NY). TaqMan RT-PCR was performed for measurement of mRNA using ΔΔCt method. Gene expression was adjusted by comparison with Rpl7 expression. The quantitative RT-PCR results were analyzed by RG Manager 1.2.1 (Applied Biosystems). Primer-probe mixture for Rpl7 was
customized, and other primer-probe mixtures were obtained from Thermo Fisher Applied Biosystems.

**Wild type and mutant human TIGAR lentiviral plasmid construction**

The full-length human TIGAR cDNA (SC320794, Origene) was used as a template to produce DNA fragment with addition of BamHI and XbaI sites, which were then cloned into a pCR-Blunt II-TOPO vector (Life Technologies Corporation, Grand Island, NY). The resulting constructs were sequenced and were used as templates to perform wild type and site-directed mutagenesis to change histidine\(^{11}\), glutamic acid\(^{102}\), and histidine\(^{198}\) to alanines simultaneously using the QuikChange II XL Site-Directed Mutagenesis kit (StrataGene, San Diego, CA) according to the manufacturer’s instructions. The primers used for specific histidine and glutamic acid to alanine mutations are listed in Key Resources Table. The fragments for wild type and multiple mutated TIGAR were then sub-cloned into the lentiviral empty vector (CMV/TO promoter, hygromycin selectable, Addgene plasmid #17484) using BamHI and XbaI restriction sites. The full-length wild type and mutated human TIGAR in mammalian expression lentiviral vectors were confirmed by sequencing. qRT-PCR and Western blotting were also used to confirm the TWT and TMU for mRNA and protein expression. Plasmids used in this study are reported in the Key Resources Table.

**Immunoblotting**

Culture cells were washed with cold PBS and scraped and homogenized using Ceria Stabilized Zirconium Oxide Beads (MidSci, Valley Park, MO) in a RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology) containing Halt Protease and Phosphatase Inhibitor Cocktail (Cat#78442, ThermoFisher Scientific), 50 µM MG132, 50 µM ALLN, and 50 µM PR-619 (EMD Millipore, Darmstadt, Germany). Homogenates were centrifuged for 15 min at 21,000 x g at 4 °C and supernatants were collected for protein assay. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane using iBlot Blotting System (Thermo Fisher Scientific). The immunoblot membrane was blocked with Pierce Protein-Free T20 (TBS) Blocking Buffer (Prod # 37571, Thermo Fisher Scientific) and incubated with the first antibody indicated in the blocking buffer. Blots were washed in Tris-buffered saline with Tween 20 (TBST) and incubated with either IRDye 800CW secondary antibody (Licor) or HRP-conjugated secondary antibody in blocking buffer (listed in Key Resource Table). The Membrane was washed with TBST and visualized either by Odyssey Imaging System (Licor, Lincoln, NE) or enhanced chemiluminescence (ECL) (Thermo Fisher Scientific Pierce) method. The ImageJ was used to quantify protein bands on the membrane.

**Co-immunoprecipitation**

The antibody for immunoprecipitation (IP) was diluted in 250 µl of Pierce Protein-Free T20 (TBS) Blocking Buffer and incubated with magnetic Dynabeads Protein G (Cat# 10004D, Thermo Fisher Scientific Novex) for 10 minutes at room temperature. The cleared cellular lysate was then incubated with the antibody-beads complex for 15 minutes at RT and washed 5 times with IP lysis buffer (Cat# 87788, Thermo Fisher Scientific Pierce). Immunoprecipitated proteins were eluted from beads by boiling samples for 5 min at 95°C in 2X SDS loading sample buffer and...
analyzed by western blots. For linear ubiquitinated NEMO blot, 1.2X SDS loading sample buffer was used to elute the denatured immunoprecipitated proteins and 7.5% polyacrylamide gel was used for protein separation.

**Plasmid DNA transfection**

HEK293T cells were transfected when 80% confluent in 60 mm dishes except otherwise indicated with the various plasmid DNAs with transfection reagents Lipofectamine 2000 (Thermo Fisher), GenJet II (SignaGen Laboratories, Rockville, MD), or BioT (Morganville Scientific, Morganville, NJ) per manufacturer’s instructions. The transfected cells were in culture for 24 hours and were used for further experiments.

**HOIP deletion Constructs**

HOIP deletion mutants were generated from N-terminal Flag tagged human HOIP (EX-Z1067-M12, GeneCopoeia). Deletions of N-terminal Flag tagged human HOIP were made using QuikChange II XL Site-Directed Mutagenesis kit (StrataGene, San Diego, CA) according to the manufacture protocol and verified by sequencing.

**Luciferase activity assay**

The NF-κB specific luciferase reporter construct (0.8 µg) plus renilla luciferase (0.2 µg) and either empty vector (2 µg), TWT (2 µg) or TMU (2 µg) plasmid cDNAs were mixed in 100 µl of DMEM medium and 3 µl of BioT were added into the DNA/DMEM solution to make the DNA/transfection reagent complex by standing at room temperature for 5 minutes. The entire complex was added directly to the HEK293T cells carefully that were tilted back and forth a few times to mix the complex into the medium and the cells return it to the CO2 incubator for 24 hours. The transfected cells were either left untreated or stimulated with 20 ng/ml human TNF-α (PeproTech, Rocky Hill, NJ) for 24 hours. The cells were lysed in 0.5 ml of passive lysis buffer and the cleared lysates were used for luciferase assay using Dual-Luciferase Reporter Assay System and Glomax 96-microplate Luminometer (Promega, Madison, WI) with the company’s protocol.

**IKKβ in vitro kinase assay**

HEK293T cells were cultured on 6-well plates for 24h and transfected with IKKβ and TIGAR expression plasmids. The cells were collected and lysed with 150 µl lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40 and protease inhibitor cocktails) after 18 h transfection. The cell extracts were prepared and used (10 µl) for IKKβ kinase assay according to manufacture’s protocol. IκBα was used as a substrate.

**Quantification and statistical analyses**

The number of independent experimental replications and the average with standard deviation (SD) are reported in the figure legends. The data were analyzed by one-way ANOVA using Prism 7 software (GraphPad, La Jolla, CA) for comparison of multiple groups, or unpaired-t-test for two groups. The statistical analyses were made at a significance level of ns (not statistically significant), *p < 0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

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Author Contributions: YT, HK and BN designed and conducted experiments, compiled/analyzed and interpretation of data, prepared figures, writing and editing manuscript. MKM conducted experiments that provided the basis for the data presented, assisted in experimental design and editing manuscript. JBP assisted in experimental design, compiled/analyzed data, writing and editing manuscript. EY prepared plasmids, assisted in experimental design, compiled/analyzed data, writing and editing manuscript. JEP was responsible for the overall directions, experimental design, analyses and interpretation of data, writing and editing manuscript.

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Figure 1. TIGAR regulates canonical NF-κB signaling. Non-Target (NT), TIGAR knockdown (TKD) and TIGAR over expressing (TOE) 3T3-L1 pre-adipocytes were generated as described under Method Details. A) 3T3-L1 adipocytes were either left untreated (Basal) or stimulated with 10ng/ml TNF-α for 5 min (TNF-α). Cell lysates were prepared and immunobotted for the indicated proteins. These are representative immunoblots independently performed five times. B and C) The adipocytes were either left untreated or stimulated with 10 ng/ml TNF-α for four hours and the expression of Ccl2 (B) and A20 (C) mRNAs were determined by qRT-PCR. These data represent the average of five independent determinations ± standard deviation of the mean. D) The TKD 3T3-L1 cells were stably infected with shRNA resistant human wild type (TWT) and phosphatase defective mutant (TMU) lentiviruses as described under Method Details. The 3T3-L1 TKD, TWT and TMU pre-adipocytes were differentiated into adipocytes either left untreated (Basal) or stimulated with 10ng/ml TNF-α for 5 min (TNF-α). Cell lysate were prepared and immunobotted for the indicated proteins. These are representative immunoblots independently performed three times. E and F) The TKD, TWT and TMU adipocytes were treated with vehicle or 10ng/ml TNF-α for four hours and the expression of Ccl2 (E) and A20 (F) mRNAs were determined by qRT-PCR. These data represent the average of three independent determinations ± standard deviation of the mean. *p < 0.05, ****p<0.0001.
Figure 2. TIGAR wild type (TWT) and phosphatase defective mutant (TMU) inhibit NF-κB pro-inflammatory gene expression, IKKβ phosphorylation and IKKβ enzymatic activity. A) HEK293T cells were seeded in a 6-well plate (0.8 X10^6 cells/well) in 2 ml of growth medium (10% fetal bovine serum with antibiotics) for six to eight hours. Following transfection with the NF-κB luciferase reporter gene and relative basal and TNF-α-stimulated levels of luciferase activity were determined as described under Method Details. These data are the average of three independent determinations ± standard deviation of the mean. B and C) HEK293T cells were transfected with cDNA for IKKβ alone or in combination with TWT or TMU cDNAs as described under Method Details. Ccl2 (B) and A20 (C) mRNA levels were determined by qRT-PCR and these data represent the average of three independent determinations ± standard deviation of the mean. D) The HEK293T cells were transfected with the indicated combinations of cDNAs (20 µg total/100 mm dish) for 24 h followed by immunoblotting of cell lysates for the indicated proteins as described under Method Details. Lane 1: 20 µg pcDNA, lane 2: 10 µg TWT, lane 3: 10 µg TMU, lane 4: 5 µg IKKβ, lane 5: 10 µg IKKβ plus 10 µg TWT, lane 6: 10 µg IKKβ plus 10 µg TMU cDNA plus various amounts of empty vector for at total of 20 µg DNA. The extracts were then immunoblotted for the various proteins indicated. These are representative immunoblots independently performed three times. E) HEK293T cells 6-well plate (0.8 X10^6 cells/well) were transfected with 2.5 µg empty vector (Ctrl), 1 µg IKKβ, 1.5 µg TWT, 1.5 µg TMU, 2.5 µg IKKβ plus TWT and IKKβ plus TMU cDNAs for 24 h. Cell extracts were prepared and IKKβ kinase activity in vitro was determined using IκBα as substrate as described under Method Details. F) Cell extracts were prepared and equal amount of IKKβ and TWT cell extracts were pre-mixed (IKKβ + TWT) for 0.5 h before determination of IKKβ kinase activity. The data are the average ± standard error of the mean from four independent experiments each performed in duplicate.
Figure 3. TIGAR inhibits IKKβ dependent phosphorylation of several direct IKKβ cellular substrate targets. A) HEK293T cells were transfected with the indicated combinations of cDNAs for 24 h followed by immunoblotting of cell lysates for the indicated proteins as described under Method Details. Lane 1: 4 μg empty vector, lane 2: 2 μg TIGAR, lane 3: 1 μg IKKβ, lane 4: 1 μg NEMO, lane 5: 2 μg TIGAR plus 1 μg IKKβ, lane 6: 1 μg IKKβ plus 1 μg NEMO, lane 7: 2 μg TIGAR plus 1 μg of NEMO and lane 8: 2 μg TIGAR plus 1 μg IKKβ plus 1 μg NEMO cDNA plus various amounts of pcDNA for at total of 4 μg DNA/60 mm dish for 24 h. These are representative immunoblots independently performed 3-5 times. B) HEK293T cells were transfected with 3 μg empty vector (lane 1), 1 μg IKKβ (lane 2), 1 μg IKKβ (lane 3) plus increasing amounts of TIGAR cDNA 0.25 μg (lane 3), 0.5 μg (lane 4), 1 μg (lane 5) and 2 μg (lane 6) plus various amounts of empty vector for at total of 3 μg/60 mm dish DNA. Twenty-four h later cell extracts were prepared immunobloted for the indicated proteins. These are representative immunoblots independently performed 3-5 times. C) The TIGAR dose-dependent inhibition of IKKβ phosphorylation from the data obtained in panel B were quantified by ImageJ densitometry +/- standard deviation of the mean as described under Method Details. D) The TIGAR dose-dependent inhibition of CYLD phosphorylation (band indicated by arrow) from the data obtained in Figure 3B were quantified by ImageJ densitometry +/- standard deviation of the mean described under Method Details.
Figure 4. NEMO rescues the TIGAR inhibition of IKKβ dependent signaling. A) HEK293T cells were transfected with 4 µg empty vector (lane 1), 1 µg IKKβ (lane 2), 1 µg IKKβ plus 2 µg TIGAR (lane 3) with increasing amounts 0.125 µg (lane 4), 0.25 µg (lane 5), 0.5 µg (lane 6) and 1 µg (lane 7) NEMO plus various amounts of empty vector for a total of 4 µg/60 mm dish DNA. Twenty-four h later cell extracts were prepared immunoblotted for the indicated proteins. This is a representative immunoblot independently performed 3-5 times. B) The NEMO dose-dependent rescue of TWT inhibition of IKKβ phosphorylation from the data obtained in panel A were quantified by ImageJ densitometry +/- standard deviation of the mean. C) The NEMO dose-dependent rescue of TWT inhibition of CYLD phosphorylation from the data obtained in panel A were quantified by ImageJ densitometry +/- standard deviation of the mean. D) The NEMO dose-dependent rescue of TWT inhibition of Ccl2 gene expression from the cells transfected with cDNA as performed in Figure 3C. Ccl2 mRNA was determined by qRT-PCR from two independent experiments. The data are presented as the average +/- standard deviation of the mean. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 comparing IKKβ vs IKKβ + TIGAR. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 comparing IKKβ + TIGAR versus increasing amounts of NEMO.
Figure 5. TIGAR specifically co-immunoprecipitates with HOIP, the E3 ligase subunit of LUBAC. A) HEK293T cells were transfected with 3 μg TIGAR or 3 μg TIGAR plus 3 μg Flag-HOIP, 3 μg TIGAR plus 3 μg Flag-HOIL-1L or 3 μg TIGAR plus 3 μg Flag-SHARPIN cDNAs on 100mm plates for 18 h. Aliquots of the cell lysates were immunoblotted with the Flag and TIGAR antibodies. B) The cell lysates in panel A were immunoprecipitated with the Flag antibody and immunoblotted for Flag and TIGAR. These are representative immunoblots independently performed 2 times.
Figure 6. TIGAR and NEMO compete for HOIP binding.  A) HEK293T cells were transfected with the indicated combinations of cDNAs (8 µg total/100 mm dish) 16 h followed by immunoblotting of cell lysates for the indicated proteins. Lane 1: 8 µg empty vector, lane 2: 3 µg of HOIP, lane 3: 2 µg of TIGAR, lane 4: 1 µg of the NEMO, lane 5: 3 µg HOIP plus 2 µg TIGAR, lane 6: 3 µg HOIP plus 1 µg NEMO, lanes 7-9: 3 µg HOIP plus 2 µg TIGAR with increasing amounts of NEMO (0.1, 0.3, 1 and 3 µg, respectively). B) The cell lysates from panel A were immunoprecipitated with a Flag antibody and immunoblotted for the indicated proteins. These are representative immunoblots independently performed 2 times.
Figure 7. Identification of the HOIP amino acid sequences responsible for TIGAR binding.

A) Schematic representation of the HOIP linear amino acid sequence with known protein interaction domains and various deletion mutants generated. The values to the right of each model structure reflect the percent HOIP mutant binding to TIGAR compared to full length HOIP determined as described below. The percent of TIGAR binding was normalized for the relative levels of HOIP expression.

B) HEK293T cells were transfected with TIGAR and HA-NEMO with full length and NZF1 domain deleted Flag-HOIP cDNAs as indicated. The cell lysates were immunoblotted for Flag-HOIP, HA-NEMO and TIGAR (left panel, lanes 1-9). The cell lysates were immunoprecipitated with the Flag antibody and these immunoprecipitates were immunoblotted for Flag-HOIP, HA-NEMO and TIGAR (right panel, lanes 1-9). These are representative immunoblots independently performed 2-3 times.

C) HEK293T cells were transfected with TIGAR and the ZF, NZF1, NZF2 and UBA domain deleted Flag-HOIP cDNAs as indicated. The cell lysates were immunoblotted for Flag-HOIP and TIGAR (left panel, lanes 1-6). The cell lysates were immunoprecipitated with the Flag antibody and these immunoprecipitates were immunoblotted for Flag-HOIP and TIGAR (right panel, lanes 1-6). These are representative immunoblots independently performed 3 times.

D) Cells were transfected with TIGAR and various amino terminal deletion Flag-HOIP cDNAs as indicated. The cell lysates were immunoblotted for Flag-HOIP and TIGAR (left panel, lanes 1-5). The cell lysates were immunoprecipitated with the Flag antibody and these immunoprecipitates were immunoblotted for Flag-HOIP and TIGAR (right panel, lanes 1-5). These are representative immunoblots independently performed 3 times.

E) Cells were transfected with TIGAR and various carboxyl terminal deletion Flag-HOIP cDNAs as indicated. The cell lysates were immunoblotted for Flag-HOIP and TIGAR (left panel, lanes 1-6). The cell lysates were immunoprecipitated with the Flag antibody and these immunoprecipitates were immunoblotted for Flag-HOIP and TIGAR (right panel, lanes 1-6). These are representative immunoblots independently performed 3 times.
Figure 8. TIGAR suppresses LUBAC induced NEMO linear ubiquitination. A) HEK293T cells were transfected with HOIP-Flag, HOIL-1L-Flag, SHARPIN-Flag, UBE2L3-Flag (2.5 µg/each), and either TWT or TMU (10 µg/each) plus various amounts of empty vector for a total of 20 µg cDNA/100 mm dish. Twenty-four h later cell lysates were prepared and immunoblotted for the indicated proteins and total M1 linear ubiquitinated proteins as described under Method Details. Lane 1: pcDNA, lane 2: TWT, lane 3: TMU, lane 4: LUBAC plus UBE2L3, lane 5: LUBAC plus UBE2L3 and TWT, lane 6: LUBAC plus UBE2L3 and TMU cDNAs. These are representative immunoblots independently performed three times. B) The lysates (500 µg) from panel A were immunoprecipitated with a NEMO specific antibody (5 µg) and immunoblotted with the linear ubiquitination specific (M1 Ub), NEMO and HOIP antibodies. These are representative immunoblots independently performed three times. C) 3T3-L1 NT, TKD, and TOE adipocytes were either left untreated (Basal) or stimulated with 10 ng/ml TNF-α for 5 min. Cell lysates were prepared and immunoblotted for the indicated proteins. The cell lysates (800 µg) were also immunoprecipitated with NEMO specific antibody and the NEMO immunoprecipitates immunoblotted with the linear ubiquitination specific (M1 Ub) and NEMO antibody. These are representative immunoblots independently performed 3 times.
Figure 9. NEMO over expression rescues the TIGAR inhibition of linear ubiquitination. A) HEK293T cells were transfected with the various indicated cDNAs composed of HOIP, SHARPIN, HOIL-1L, UBE3L3 cDNAs (LUBAC), TWT, TMU, and NEMO cDNAs (4 µg each) plus various amounts of empty vector for at a total of 20 µg/100 mm dish for 24 h. Cell lysates were immunoblotted for the indicated proteins as described in Methods Details. B) The cell lysates in panel A were immunoblotted with the M1 linear ubiquitination (M1 Ub) antibody. These are representative immunoblots independently performed two times.
Figure 10. TIGAR regulates adipose tissue NF-κB signaling in vivo. C57Bl6/J male wildtype (WT), whole body TIGAR knockout (TKO) and adipose tissue-specific over expressing TIGAR protein (TGRS) mice at 12 weeks of age were maintained on a low fat diet as described under Materials and Methods. A) Two independent control wild type (WT) and TIGAR knockout (TKO) male mice were given an intraperitoneal injection of vehicle or TNF-α (10 µg/kg) for 15 min. The epididymal adipose tissue was extracted and immunoblotted for TIGAR, total IκB, pS32/36-IκBα, p536-RelA, total RelA, pS418-CYLD, total CYLD and actin as loading control. These are represented immunoblots independently performed three times. B) Two independent control wild type (WT) and adipocyte-specific TIGAR over expressing mice (TGRS) were given an intraperitoneal injection of vehicle or TNF-α for 15 min. The epididymal adipose tissue was extracted and immunoblotted for TIGAR, total IκB, pS32/36-IκBα, p536-RelA, total RelA, pS418-CYLD, total CYLD and actin as loading control. These are represented immunoblots independently performed two times. C) Epididymal adipose tissue from control wild type (WT), TKO and TGRS mice treated with and without TNF-α for 15 min were subjected to qRT-PCR for Ccl2 mRNA expression. These data are the average of four independent determinations ± standard deviation of the mean. ****p < 0.0001, TKO-TNF vs WT-TNF and TGRS-TNF vs WT-TNF.
The fructose-2,6-bisphosphatase TIGAR suppresses NF-κB signaling by directly inhibiting the linear ubiquitin assembly complex LUBAC

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