ROLE OF THE TTRAP ADAPTOR MOLECULE IN THE SIGNALING MECHANISMS OF TGF-β FAMILY LIGANDS

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PUBLICATIONS

In the two-year period (between 2009 April - 2011 March), during which the laboratory received support from the NNF78805 grant, we published our results in the following forms.

Attended conferences:

Károly Fátyol - poster presentation
March 23 - March 28, 2010
Integration of Developmental Signaling Pathways (Z2), Keystone Research Conference, Victoria, British Columbia

Conference abstracts:

Integration of Developmental Signaling Pathways (Z2)
Dates: March 23 - March 28, 2010
Location: Fairmont Empress Victoria, Victoria, British Columbia
Poster Number: 113

Károly Fátyol, Ying E. Zhang: ***Identification of a novel component of the non-canonical TGF-β signaling pathway.***, Integration of Developmental Signaling Pathways, Keystone Research Conference, 2010

Scientific papers:

Károly Fátyol, György Várady, Zhihong Zeng, Keith W. Caldecott, Balázs Sarkadi Ying E. Zhang ***TTRAP a novel component of the non-canonical TGF-β signaling pathway.*** Molecular and Cellular Biology, submitted, 2011, IF: 6.057

I. INTRODUCTION

TGF-β has pervasive and diverse effects on cell physiology as well as it acts as a potent anticancer agent that prohibits uncontrolled cell proliferation. The most prevalent model for the signaling mechanism of the cytokines belonging to the TGF-β family presents a linear pathway from the type II to the type I receptor kinase to Smad activation, resulting in ligand-induced transcription. It is increasingly apparent however, that TGF-β can also mobilize several intracellular signal transducers in Smad-independent manner as well. These non-canonical, non-Smad pathways are also activated directly by ligand-occupied receptors to reinforce, attenuate, or otherwise modulate downstream cellular responses. The non-Smad pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, phosphatidylinositol-3-kinase/AKT pathways and more. Such alternative signal transducers often regulate the Smad pathway itself and represent extensive opportunities for crosstalk with other signaling routes contributing to the surprising diversity of TGF-β responses.

Perhaps one of the most important non-Smad pathways is the p38/JNK MAPK signaling cascade. This signaling route functions in conjunction with the Smad-dependent pathway to regulate such cellular responses as programmed cell death (apoptosis) and eptithelial-to-mesenchymal transition (EMT). Despite the obvious biological importance of the mentioned processes, we still have serious caveats in understanding the mechanisms by which TGF-β governs them. The need to fill out these gaps is further underscored by several recent observations suggesting that imbalances arising between the Smad-dependent and the p38/JNK MAPK signaling branches during tumorigenesis may contribute to the conversion of TGF-β from a suppressor to a promoter of cancer growth.

Previous genetic studies could only place the TGF-β-activated kinase 1 (TAK1) in the TGF-β mediated p38/JNK activation pathway however, the link between TAK1 and the activated receptor complex had been lacking. Recently, we have demonstrated that the E3 ubiquitin ligase, TRAF6 is one of the missing pieces. The molecule physically interacts with the TGF-β receptor complex and is required for Smad-independent activation of the JNK and p38 MAP kinases. TGF-β promotes association between TRAF6 and TAK1 resulting in K63-linked ubiquitylation and subsequent activation of TAK1. Interestingly, the TRAF6-TAK1 signaling module is also employed by a number of different signaling routes such as those emanating from the IL-1β receptor, Toll-like receptors, T-cell receptor etc. Considering the numerous divergent stimuli capable of modulating TAK1 activity, the

cell has to apply elaborate mechanisms to achieve sufficient signaling specificity. It is believed that this goal is attained at least partly, by the selective use of adaptor proteins indigenous to a given signaling route and/or the employment of unique combinations of more common ones. Regardless, the identification of these adaptor proteins and the elucidation of their complex interactions are essential.

In my laboratory we are studying one such adaptor molecule, TTRAP that may ensure the specificity of TAK1 activation in response to TGF-β. TTRAP (TRAF and TNF receptor-associated protein) was originally reported to interact with members of the TNF receptor family and the TRAF adaptor proteins, exhibiting the highest affinity for TRAF6. TTRAP was also identified as an ETS1-associated protein II (EAPII), which negatively modulates ETS1 transcriptional activity and attenuates synergistic transactivation by ETS1 and AP-1. The zebrafish ortholog of the protein was shown to associate with ALK4- (the type I Nodal/activin receptor) and Smad3, thereby controlling gastrulation movements and left-right axis determination. Recently, human TTRAP has also been demonstrated to possess 5' tyrosyl phosphodiesterase activity and repairs topoisomerase mediated DNA damage. In summary, the above observations define TTRAP as a multifunctional protein, playing important roles in such diverse processes as transcription, DNA repair and signal transduction.
Now - with the support of the NNF78805 grant - we demonstrate that TTRAP is an integral component of the TGF-β signaling pathway. Our main findings are the following:

(**1.**) Modulation of TTRAP level affects cell viability in the presence of TGF-β, indicating that the protein is an important component of the TGF-β induced apoptotic process.

(**2.**) TTRAP is involved in TGF-β dependent EMT.

(**3.**) TTRAP associates with components of the TGF-β receptor-TRAF6-TAK1 signaling axis.

(**4.**) TTRAP promotes the formation of the TAK1 signaling complex and also help its recruitment to TGF-β receptors.

(**5.**) TTRAP is involved in the Smad-dependent canonical and the Smad-independent non-canonical branches of TGF-β signaling.

(**6.**) TTRAP is a subject of various post-translational modifications including phosphorylation, ubiquitylation and sumoylation.

(**7.**) The ubiquitin proteasome pathway plays an important role in the regulation of TTRAP

In the next section I discuss our findings in greater details.

II. RESULTS

1. TTRAP plays a role in TGF-β induced apoptosis

Based on earlier results implicating TTRAP in the signaling processes of Nodal/activin ligands, we explored the potential involvement of TTRAP in TGF-β signaling in mammalian cells. As an initial approach, we wanted to test TTRAP's role in a well-defined biological test system for TGF-β induced responses. We chose the NMuMG mammary epithelial cell line because, it has been a widely employed and extensively characterized model system for TGF-β mediated apoptosis and EMT. In NMuMG cells both of these processes are regulated by TGF-β both in Smad-dependent and -independent manner, with the Smad-independent component predominantly involving the p38MAPK pathway. We started with the establishment of stable NMuMG cell populations expressing the EGFP-tagged full-length TTRAP molecule (EGFP-TTRAP cells). As control, cells were also produced expressing EGFP alone (EGFP cells) or the EGFP tagged N-terminal 123aa segment of TTRAP (EGFP-N-TTRAP cells) (Figure 1)

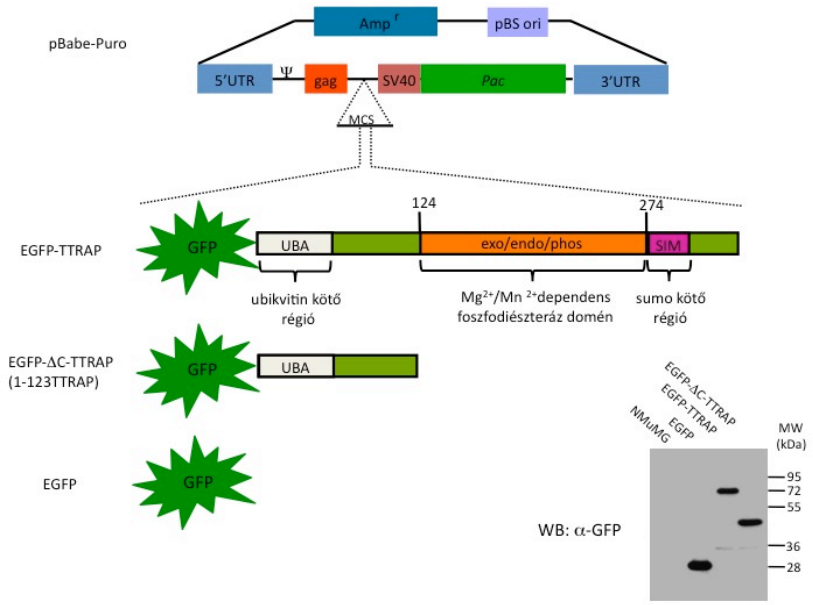
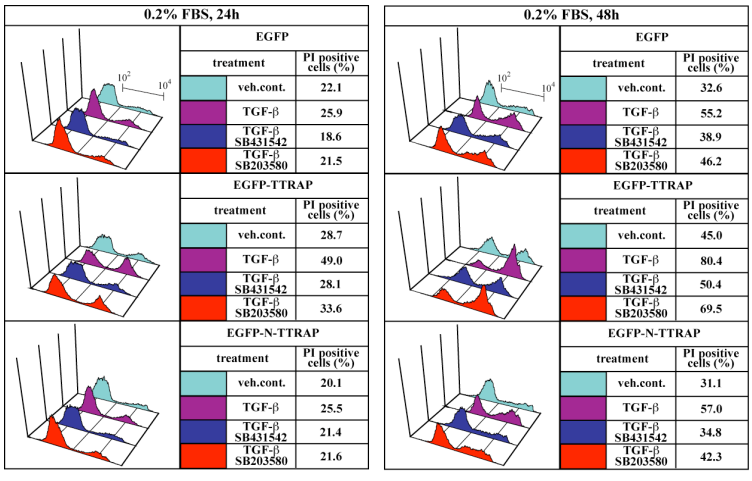


Figure 1. NMuMG stable model cell lines created for studying the biological effects of TGF-β.

We treated these cells with TGF-β under various conditions and their viability was measured by propidium iodide uptake using fluorescence activated cell sorting (FACS) (Figure 2A-D). In accordance with published data, TGF-β elicited only weak apoptosis in the control cell populations after 24h under both high- and low-serum culture conditions. In contrast, at the same time point EGFP-TTRAP cells exhibited robust TGF-β induced apoptotic response (~50%) in the presence of 0.2% FBS. Importantly, the TGF-β dependent apoptosis was completely preventable by the TβRI receptor kinase inhibitor (SB431542) and the p38 MAPK inhibitor (SB203580) also provided strong protection. In 10% FBS medium TGF-β was not able to elicit significant apoptosis in the control EGFP and EGFP-N-TTRAP cells even after 48h. Again, under the same conditions, the EGFP-TTRAP cells died at a much higher rate (>50%) and whereas the TβRI inhibitor was still able to prevent apoptosis, the p38 MAPK inhibitor lost its protective effect. TGF-β can induce not only p38 MAPK but JNK as well. JNK has been implicated in TGF-β dependent apoptosis in some cell types and similarly to p38MAPK, it is also activated by TAK1. Significantly, a small molecule inhibitor of TAK1 ((5Z)-7-oxozeaenol) could not rescue the cells from TGF-β induced apoptosis in the presence of 10% FBS indicating that under such conditions the Smad-dependent pathway plays a decisive role and the involvement of TAK1 regulated kinase cascades is negligible (Figure 2F). Using MTT assay to assess cell viability, we obtained essentially the same results detailed above (Figure 2E, G). We showed that the EGFP-TTRAP cells exhibit similar sensitivity to other apoptotic signals (staurosporine, MG132) as the control EGFP cells, emphasizing the specific role TTRAP plays in TGF-β induced apoptosis (Figure 2H).



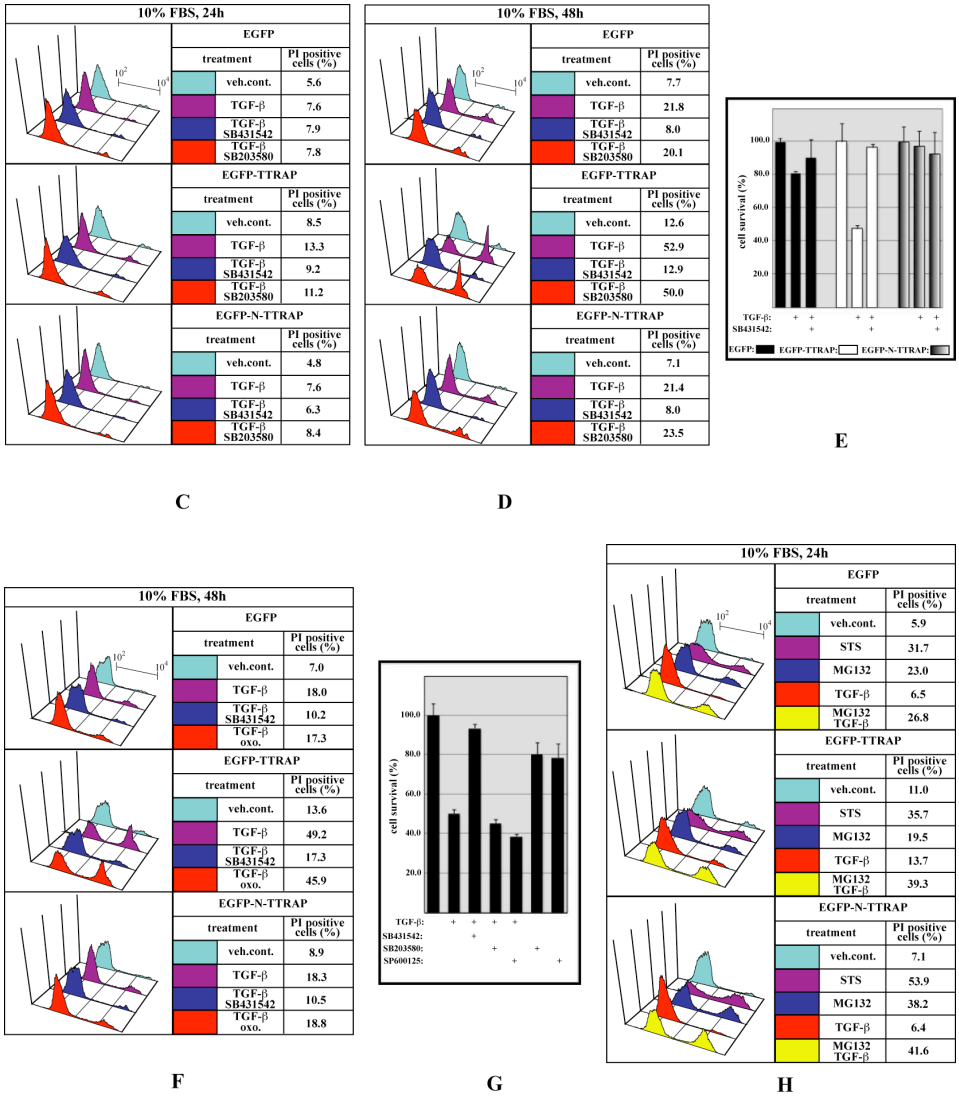


Figure 2. TTRAP sensitizes mammary epithelial cells to TGF-β mediated cell killing both in Smad-dependent and -independent manner

Finally, the effect of TTRAP deficiency on TGF-β induced apoptosis was examined in two different cell types. For one, we chose AML12 normal murine hepatocytes because, they exhibit more robust TGF-β-dependent apoptotic response than NMuMG cells, and beside the Smad pathway, they also utilize the TGF-β receptor-TRAF6-TAK1-p38 route, allowing one to appraise TTRAP's involvement in both the canonical and non-canonical TGF-β signaling branches. Ectopic expression of EGFP-TTRAP increased TGF-β induced cell death in these cells, similarly to NMuMG cells (data not shown). Endogenous expression of TTRAP was knocked down by lentiviruses expressing shRNAs specific for the murine TTRAP mRNA. Contrary to our expectations, reduction of endogenous TTRAP level also sensitized the cells to TGF-β mediated cell killing under both high- and low-serum growth conditions (Fig. 3A). To confirm the above result in a different cell type, TGF-β induced apoptosis was also measured in genetically modified DT40 chicken lymphoblasts. While in wild type DT40 cells TGF-β induced no significant cell death after 24h, in their TTRAP knockout counterparts the same treatment resulted in a ~3-fold increase in apoptosis. Significantly, stable expression of the human TTRAP protein in the TTRAP knockout DT40 cells endowed them with partial resistance against TGF-β induced cell killing (Fig. 3B). In summary, the functional data described above indicate that TTRAP is involved in TGF-β induced apoptosis. The importance of its role in the above process is underscored by the observation that either increasing or decreasing of TTRAP protein level result in diminished cell survival upon TGF-β treatment.

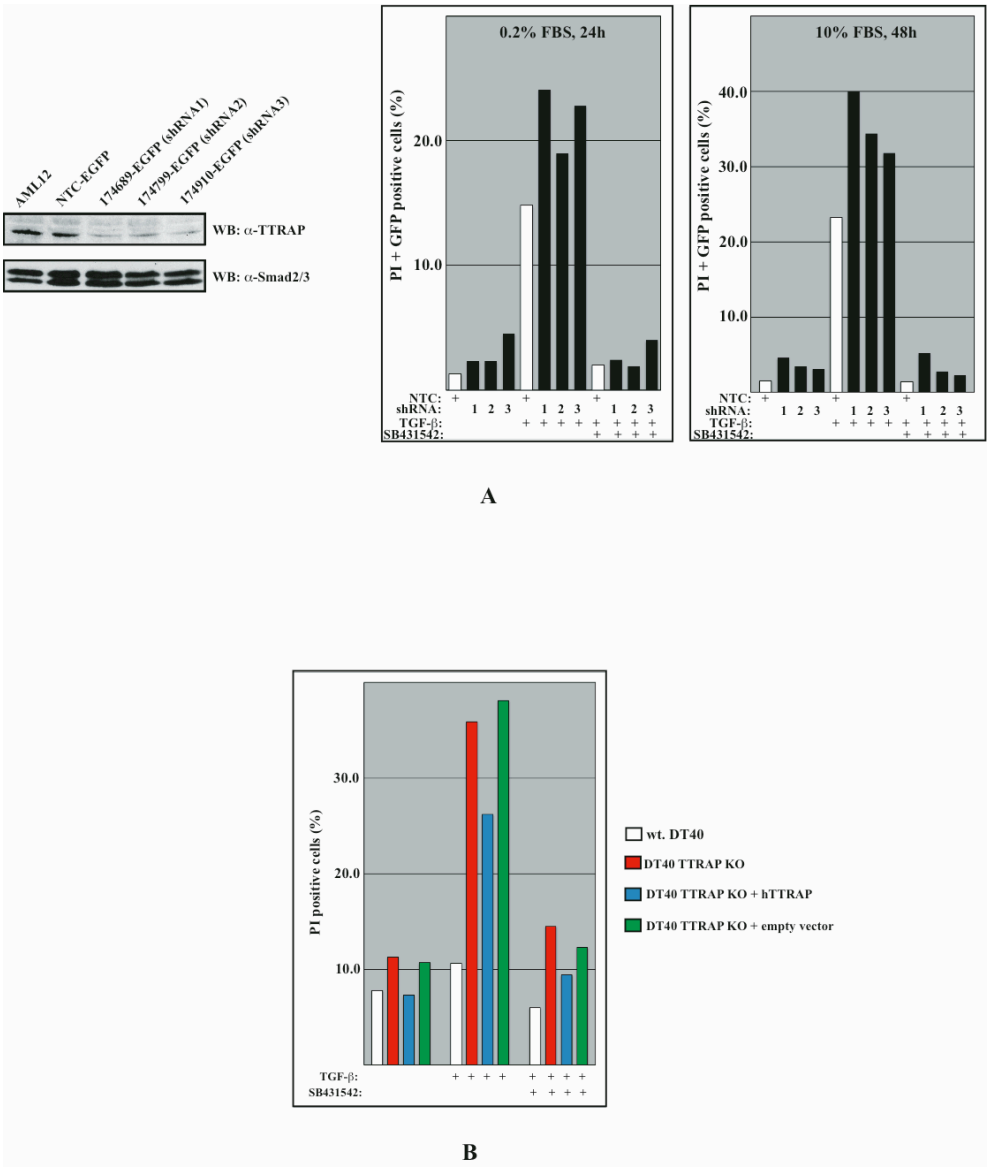


Figure 3. Reduction of endogenous TTRAP levels increases TGF-β induced apoptosis

2. TTRAP associates with the TGF-β receptor complex

Since zebrafish TTRAP has been shown to bind component of the Nodal/activin pathway (Alk4 receptor, Smad3), we studied the possible interactions of mammalian TTRAP with elements of the TGF-β signaling machinery by different protein-protein interaction techniques. Using co-immunoprecipitation, we demonstrated that TTRAP associates with both the type I and type II TGF-β receptors (TβRI and TβRII), however it does not bind to Smad2, 3 or 4 (Figure 4A). TTRAP exhibits higher affinity toward the catalytically inactive TβRI-KR receptor mutant compared to the constitutively active TβRI-TD form, suggesting that TTRAP may be a substrate for TβRI (see below) (Figure 4B). In GST-pull-down, *in vitro* translated TTRAP protein binds with the purified cytoplasmic domains of both TβRI and TβRII indicating that the TTRAP-TGF-β receptor interaction is direct (Figure 4C). TTRAP also associates with ^{125}I-TGF-β ligand occupied TGF-β receptor complexes on the cells surface providing further support for the physiological relevance of the interaction (Figure 4D). Subcellular distribution of EGFP-tagged TTRAP molecules was studied as well. TTRAP was present both in the cytoplasm and the nucleus, consistently with the described nuclear/cytoplasmic functions of the protein. In the cytoplasm TTRAP exhibited partial co-localization with TβRI in juxta-membrane foci (Figure 4E). Finally, using C-terminally truncated TTRAP molecules, we mapped the TGF-β receptor interacting region between amino acids 123 and 274. Intriguingly, this 150aa segment exhibits strong resemblance to the evolutionary conserved functional domains of Mg^{2+}/Mn^{2+} dependent phosphodiesterases (Figure 4F).

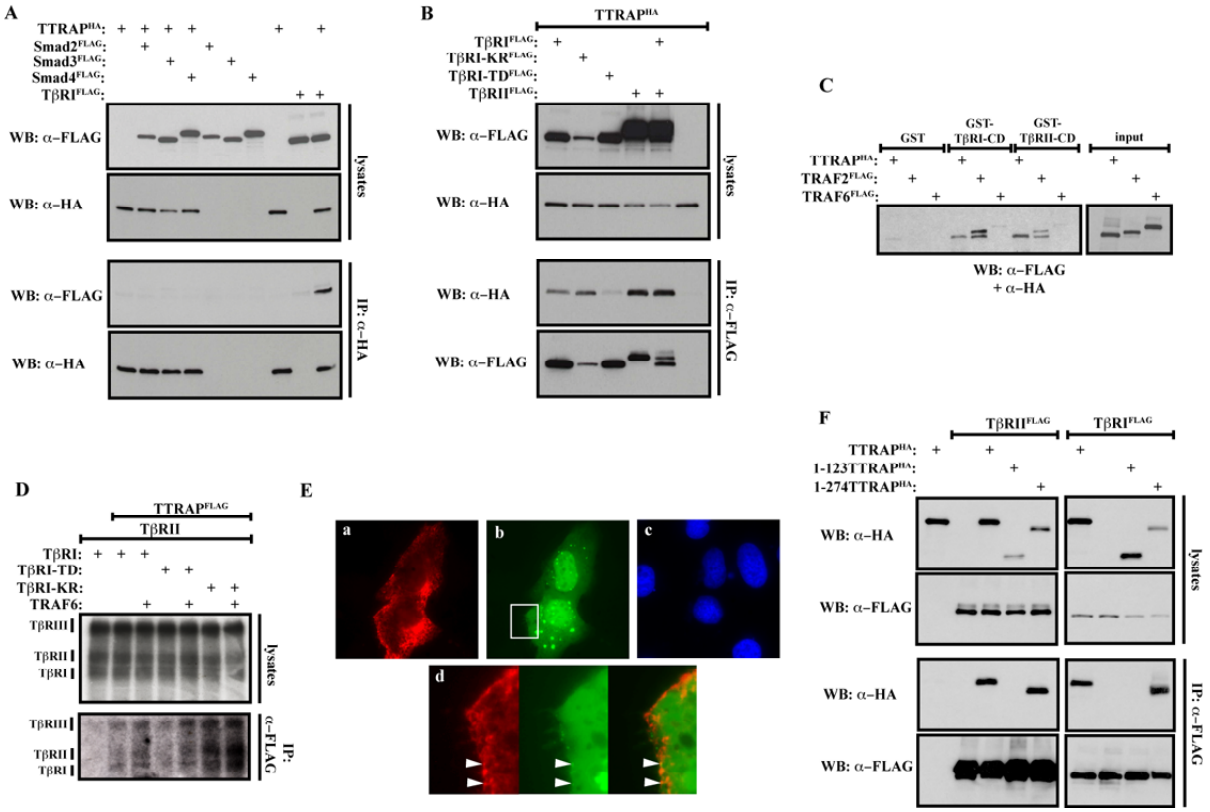


Figure 4. TTRAP associates with the TGF-β receptor complex

3. Analysis of post-translational modifications of TTRAP

In eukaryotes most proteins are the subjects of post-translational modifications. These modifications usually have profound effects on the functions of proteins and thus represent one of the most important layers of biological regulation. Therefore, we decided to analyse TTRAP's post-translational modifications mostly focusing our attention on phosphorylation, ubiquitylation and sumoylation. These studies will provide the basis for better understanding TTRAP's cellular functions.

3. 1. TTRAP is phosphorylated by the TGF-β receptors

The TGF-β receptors are transmembrane serine/threonine kinases. The constitutively active type II receptor's main function is to phosphorylate and thereby activate the type I receptor, while the primary substrates of the type I receptor are the R-Smads (Smad2 and -3). It has been known for some time however, that both receptors have additional substrates. The observation that TTRAP binds more avidly to the catalytically inactive TβRI-KR receptor form compared to the constitutively active TβRI-TD raises the intriguing possibility that TTRAP is a substrate for TβRI. To explore this possibility, first we tested whether TTRAP is a phosphoprotein. To this end mammalian cells were cultured in the presence of ^{32}P inorganic phosphate and subsequently TTRAP was affinity isolated from them. The purified protein incorporated significant amount of radioactive phosphate proving that TTRAP is a phosphoprotein. Importantly, TGF-β treatment also led to increased TTRAP phosphorylation (Figure 5A). Next, we proved that in immune complex kinase assays both TβRI and TβRII could phosphorylate TTRAP (Figure 5B). Finally, we found that TβRI phosphorylated purified recombinant TTRAP *in vitro,* while other TGF-β regulated kinases (p38, JNK and TAK1) were unable to do so (Figure 5C).

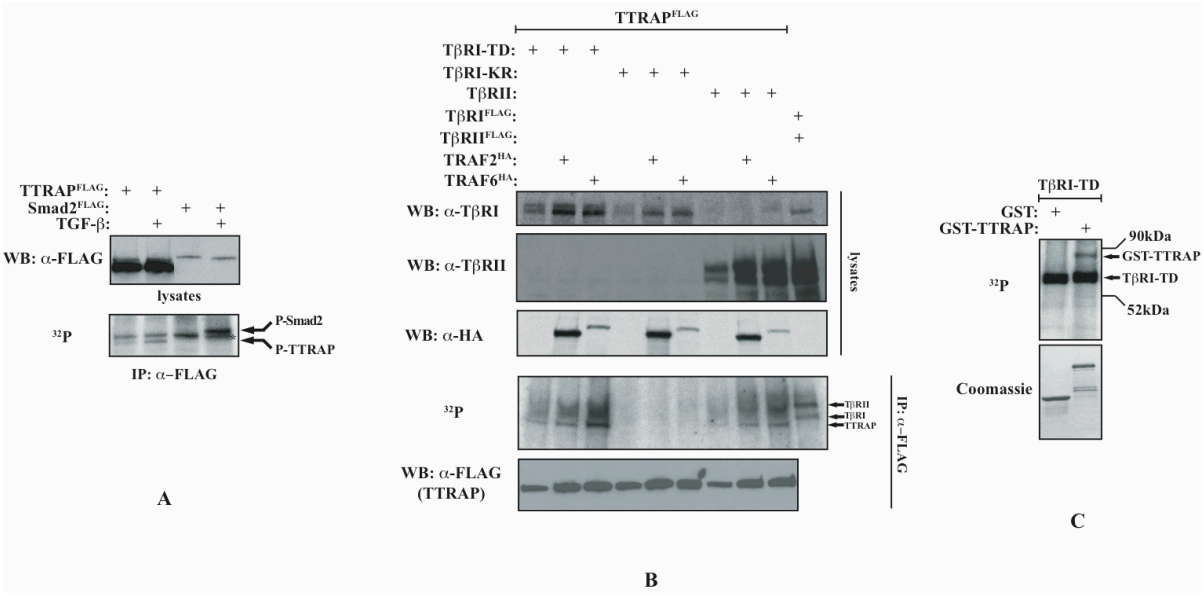


Figure 5. TTRAP is phosphorylated by the TGF-β receptors

3. 2. TTRAP is ubiquitylated in a TRAF6 dependent manner

TTRAP has been shown to associate with members of the TRAF adaptor protein family, exhibiting the highest affinity for TRAF6. TRAF6 is an E3 ubiqutin ligase capable of catalyzing the formation of lysine 63-linked (K63) polyubiquitin chains. Unlike the polyubiquitin chains formed via lysine-48 (K48), K63-linked polyubiquitin chains do not target proteins for proteasomal degradation, rather they provide scaffold for the assembly of protein kinase complexes thereby contributing to their activation. In light of these observations, we examined the possibility whether TTRAP is ubiquitylated by TRAF6. First, we confirmed that TTRAP binds to TRAF6 and also showed that the interaction is mediated by the Mg^{2+}/Mn^{2+} dependent phosphodiesterase domain of TTRAP (Figure 6A, B). In an *in vivo* ubiquitylation assay we demonstrated that TRAF6 was capable of ubiquitylating TTRAP and the reaction was dependent on the intact RING domain of TRAF6 (Figure 6C). Next, we mapped the lysine residues in TTRAP serving as acceptor sites for the attachment of polyubiquitin chains. A series of lysine to arginine mutants were generated by site-directed mutagenesis. These mutants were then used in *in vivo* ubiquitylation assays. As shown in Figure 6D mutation of lysines in the 172-202aa segment of TTRAP strongly diminished ubiquitylation. This indicates that most or all of the ubiquitin acceptor lysisnes are located in this region of TTRAP. Finally, we also examined the effect of TβRI on TRAF6 mediated TTRAP ubiquitylation. The catalytically inactive TβRI-TD receptor form inhibited TRAF6 mediated TTRAP ubiquitylation in a dominant negative fashion, consistently with the binding of both proteins to the same region of TTRAP (Figure 6E). This observation may have important functional implications regarding the activation of the TRAF6-TAK1 signaling axis upon ligand engagement of the TGF-β receptor complex (see later).

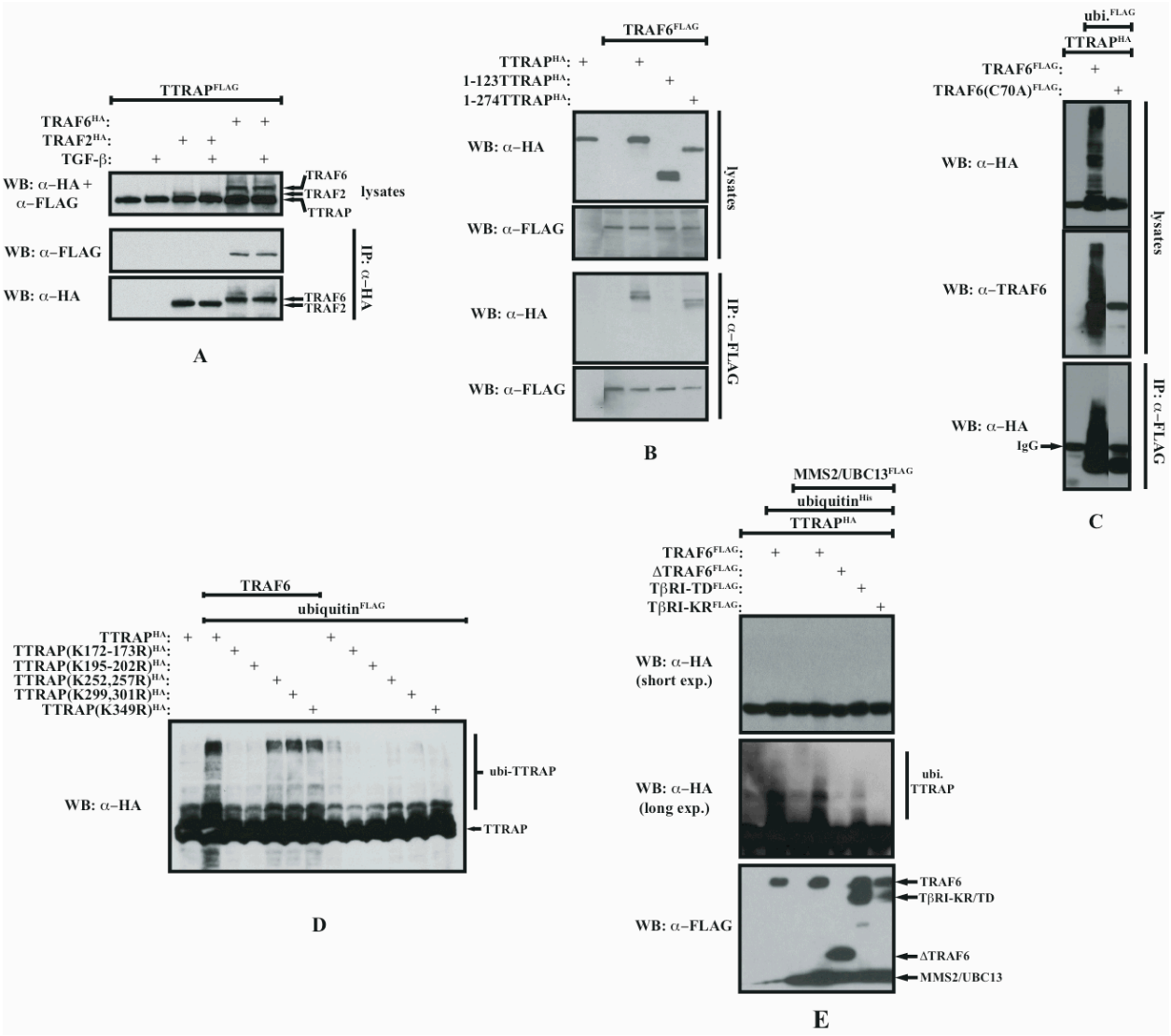


Figure 6. TTRAP is ubiquitylated by TRAF6

3. 3. TTRAP is modified by sumo1

Sumoylation, the covalent attachment of a small ubiquitin-like modifier (SUMO), regulates the functions of proteins engaged in diverse processes. Sumoylation of TβRI enhances receptor function by facilitating the recruitment and phosphorylation of Smad3. Interestingly, in zebrasfish TTRAP was also shown to modulate Smad3-dependent signaling. Combined these data raises the exciting possibility that SUMO modification plays an important role in the TTRAP-TβRI interaction. TTRAP was shown to contain a SUMO interaction motif (SIM). Since SUMO binding proteins themselves are frequently substrates for sumoylation, we examined whether TTRAP itself is sumoylated. Indeed, in *in vivo* sumoylation assay we demonstrated that TTRAP is modified by sumo 1 but not sumo 2 or -3 (Figure 7A). We also mapped the sumoylation site of TTRAP between 195-202aa (Figure 7B). Finally, we reconstituted TTRAP sumoylation *in vitro* from purified components and determined that the E3 sumo ligase capable of promoting the sumoylation of TTRAP is most likely PIASX (Figure 7C).

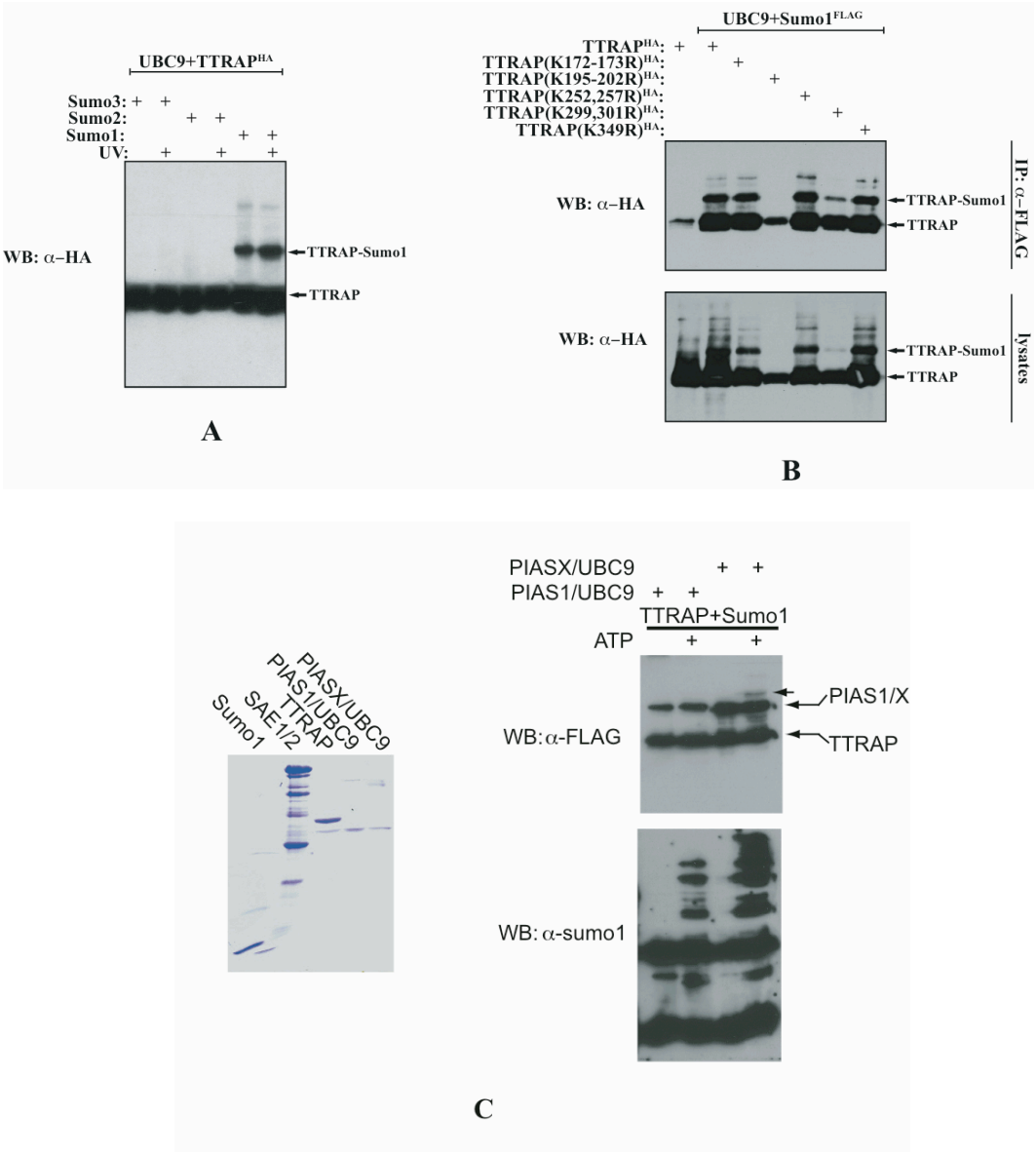


Figure 6. Analysis of the sumoylation of TTRAP

4. TTRAP interacts with the TAK1 complex

TTRAP was identified as a TRAF6 interacting protein. Since TRAF6 plays a crucial role in TGF-β induced p38 activation, we examined TTRAP's interaction with additional components of the TGF-β receptor-TRAF6- TAK1-p38 pathway by co-immunoprecipitation. As seen in Figure 7A TTRAP interacted with active TAK1 molecules, but exhibited no significant affinity toward the ATPase deficient or activation loop mutant forms. Endogenous TAK1 also associated with TTRAP in a TGF-β inducible manner (Figuer 7B). The N-terminal 1-123aa segment of TTRAP was sufficient for the interaction with TAK1 (Figure 7C). Since this region is distinct from the one used by the molecule to bind TRAF6 (124-274aa), it is possible that TTRAP can interact with TAK1 and TRAF6 simultaneously in a ternary comlex. Indeed, using sequential immunoprecipitations we were able to prove the existence of such protein complexes (Figure 7D). Finally, we examined TTRAP's interaction with known components of the TAK1 complex (Figure 7E). We detected strong association of TTRAP with the ubiquitin adaptor molecule TAB2. TAB1, another TAK1 binding protein did not interact with TTRAP, neither did the MAP2 kinase, MKK6 nor Smad7, an inhibitory Smad which was previously implicated in TGF-β dependent p38 activation. Interestingly, we were also able to demonstrate the TRAF6 dependent oligomerization of TTRAP.

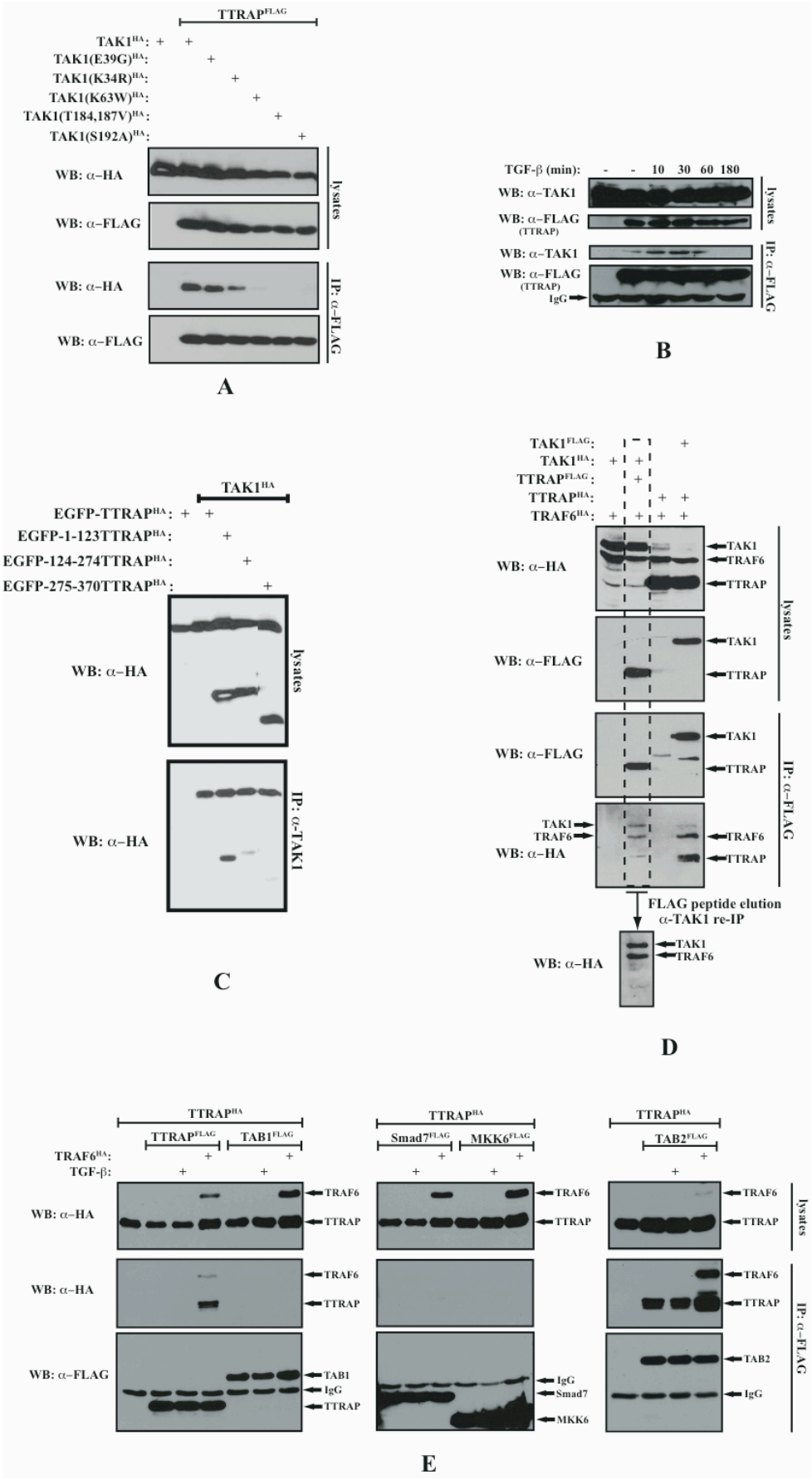


Figure 7. TTRAP forms ternary complexes with TAK1 and TRAF6

5. The TTRAP-TRAF6-TAK1 ternary complex is stabilized by ubiquitylation

Members of the TRAF adaptor protein family display significant similarity to each other and are all involved in cellular signaling. It has been reported that in some signaling pathways they may even be sharing similar functions and act redundantly. For instance, in the CD40 pathway TRAF2 and TRAF6 are closely collaborating with each other and perhaps perform partially overlapping tasks. Therefore, we tested whether TRAF2 can replace TRAF6 in the protein complexes described above. Interestingly, we found that unlike TRAF6, TRAF2 did not display significant affinity for TAK1 and furthermore it was able to disrupt the TTRAP-TAK1 complexes (Figure 8A). These data underscore the specificity of the TTRAP-TRAF6-TAK1 interactions and raises the possibility that TRAF2 interferes with the signaling events in which the above complex is involved. TRAF6 has been shown to promote the formation of signaling complexes, by at least partly depending on its ubiquitin ligase activity. Akin to this, we found that he TTRAP-TRAF6-TAK1 complex is stabilized by TRAF6 mediated ubiquitylation (Figure 8B, C). Finally, we demonstrated that TTRAP promotes the ubiquitylation of TAK1 by TRAF6 Figure 8D).

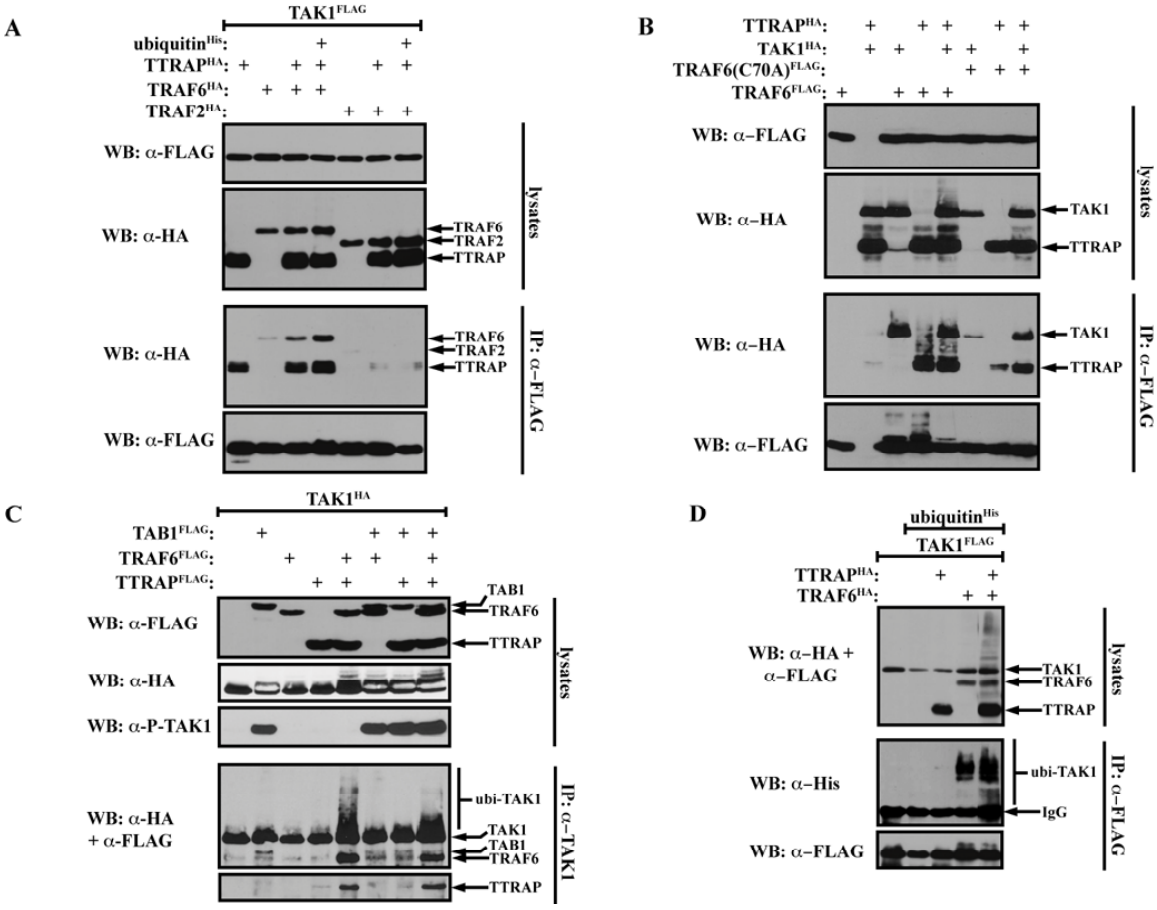


Figure 8. The TTRAP-TRAF6-TAK1 ternary complex is stabilized by ubiquitylation

6. The TTRAP-TRAF6-TAK1 complex interacts with the TGF-βreceptor complex

We have reported previously that TRAF6 links the TAK1-MKK3/6-p38MAPK cascade to the TGF-β receptor complex. We have also demonstrated physical association between TRAF6 and the TGF-β receptors (Figure 9A). Building on these data, we tested the possible effect of TRAF6 on the TTRAP-TGF-β receptor interactions. Interestingly, we found that TRAF6 greatly enhances the binding of TTRAP to TβRI (Figure 9B). Furthermore, TTRAP, TRAF6 and TAK1 together associated more efficiently with TβRI, compared to their binding in separation (Figure 9C). In summary, our results suggest that the TTRAP-TRAF6-TAK1 complex can stably associate with the TGF-β receptor complex.

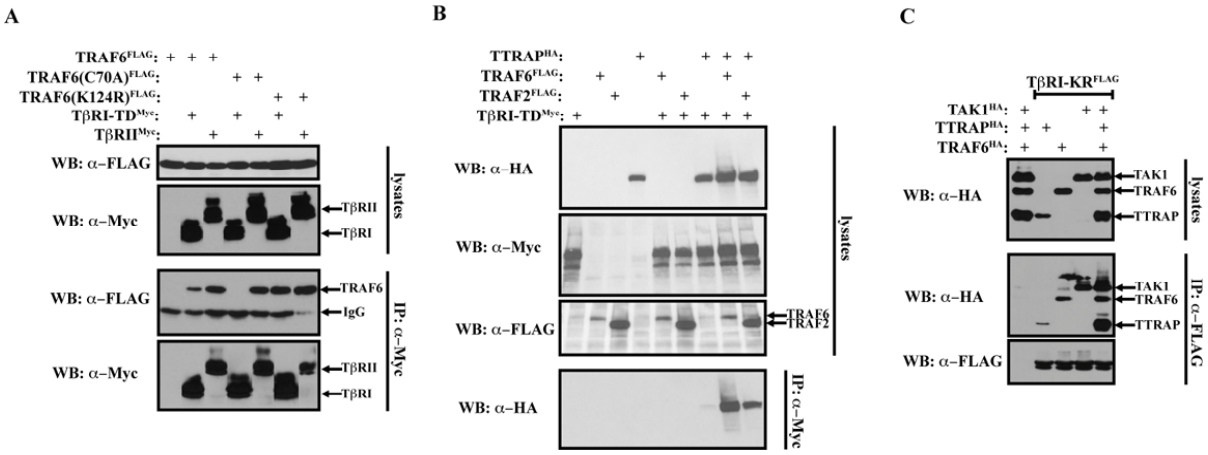


Figure 9. The TTRAP-TRAF6-TAK1 complex interacts with the TGF-βreceptor complex

7. TTRAP is involved in both the Smad-dependent and -independent branches of TGF-β signaling

Our observations discussed above suggested that TTRAP plays a role both in Smad-dependent and -independent TGF-β signaling. To confirm this hypothesis, we tested the effect of TTRAP on the activity of different reporter plasmids in transient transfection assays. We found that TTRAP over-expression significantly inhibited the activity of the Smad responsive 4XSBE-luc (Figure 10A) and 3TP-lux (data not shown) reporter plasmids and in agreement with earlier observations negatively affected NF-κB activity as well. TTRAP over-expression did not have an effect on Smad2 phosphorylation by TβRI, indicating that TTRAP may exert its inhibitory influence on the Smad-pathway at one of the receptor distal steps (Figure 10B). TTRAP's ability to promote TGF-β induced apoptosis suggested that the protein might also be involved in p38 MAPK regulation. Indeed we found that TTRAP over-expression activated p38 even in the absence of TGF-β (Figure 10C). Additionally, the N-terminal 1-123aa region of TTRAP, which does not interact with TGF-β receptors inhibited TβRI dependent p38 activation in a dominant negative fashion (Figure 10D). TGF-β has been proposed to activate p38 by at least two different mechanisms. Delayed p38 activation requires Smad-dependent expression of the GADD45β adaptor protein, which subsequently binds the MTK1 kinase thereby setting off the MTK1-MKK3/6-p38 cascade. A second mechanism, described by us and others, uses the TRAF6 E3 ubiquitin ligase to ubiquitylate and thus activate TAK1 in a Smad-independent fashion, resulting in rapid p38 phosphorylation. The fact that TTRAP interacts with not only the TGF-β receptors but TRAF6 and components of the TAK1 complex, strongly suggests that the protein is involved in the rapid p38 activation pathway. Indeed, shRNA mediated knockdown of endogenous TTRAP expression strongly inhibited the TGF-β dependent early p38 phosphorylation in AML12 cells, while the delayed p38 activation and Smad2 phosphorylation remained unaffected (Figure 10E).
In summary, these data strongly suggest that in addition to its involvement in the canonical Smad-dependent signaling events, TTRAP is also an important component of the TGF-β induced p38 pathway.

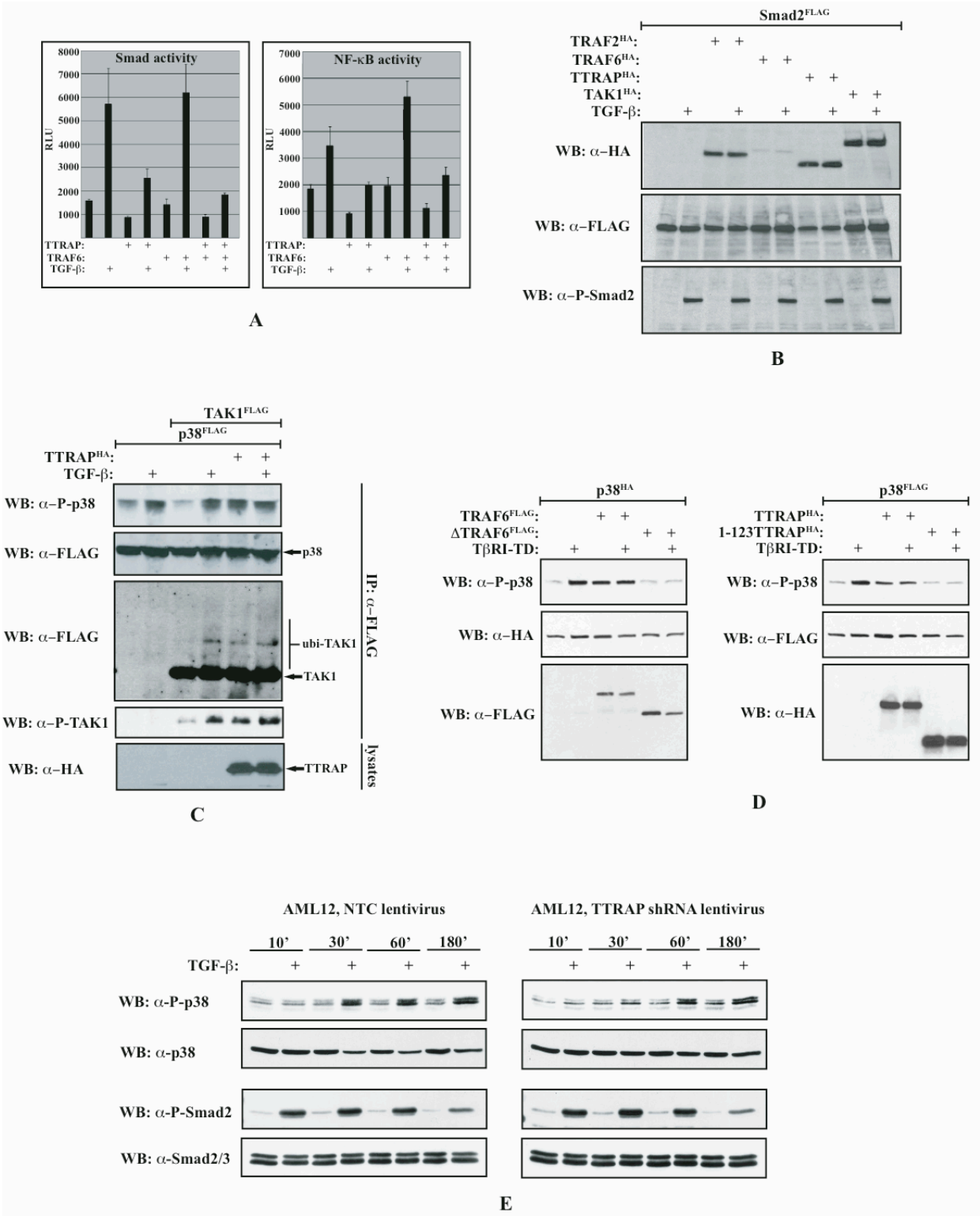


Figure 10. TTRAP is involved in both the Smad-dependent and -independent branches of TGF-β signaling

8. TTRAP plays a role in TGF-β induced EMT

TGF-β regulates epithelial cell polarity and differentiation by inducing epithelial-mesenchymal transition (EMT). The murine mammary epithelial cell line, NMuMG is a well-established and widely used model system for TGF-β induced EMT. In the absence of TGF-β, NMuMG cells appear as typical epithelial cells with actin cytoskeleton and E-cadherin arranged in a cortical pattern at cell-cell junctions. Following 24-36h of TGF-β treatment, this regular, cobblestone-like shape gives way to a

more elongated mesenchymal morphology, with formation of actin stress fibers. Previous data indicated that in NMuMG cells TGF-β regulates EMT by both the Smad pathway and the p38 kinase cascade. Having established that TTRAP is an important regulator of the TGF-β activated p38 pathway, we examined TTRAP's involvement in TGF-β dependent EMT. NMuMG cell lines stably expressing different forms of TTRAP were established (Figure 11A). These cells were treated with TGF-β and the progression of EMT was monitored by staining of the actin fibers with FITC-labeled phalloidin. As seen in Figure 11B stable expression of full-length TTRAP did not have an effect on TGF-β induced EMT. In contrast, the N-terminal 123aa fragment of TTRAP (N-TTRAP) significantly accelerated the progression of EMT in NMuMG cells (compare the much more organized actin stress fibers in N-TTRAP cells to that of the control cells). These data suggest that TTRAP is involved in this TGF-β induced response, an observation, which definitely warrants further studies.

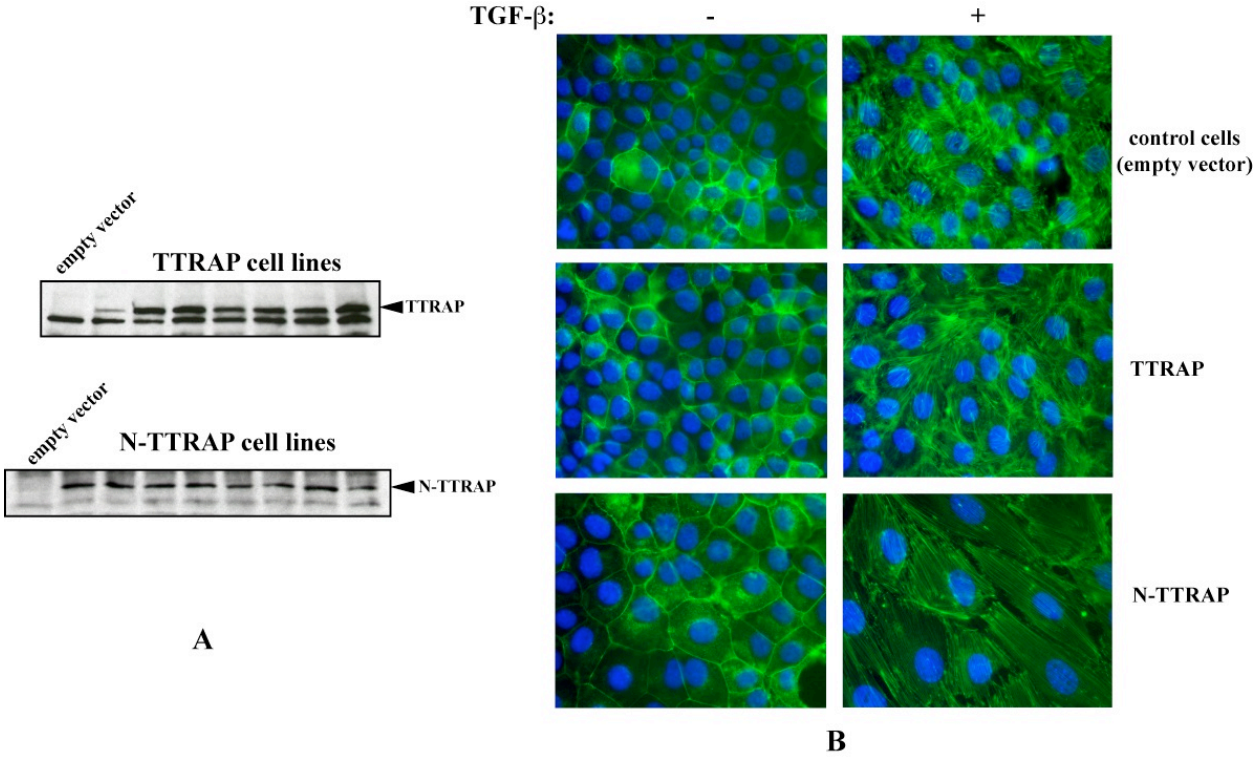


Figure 11. TTRAP is involved in TGF-β induced EMT

9. TGF-β induces proteasomal degradation of TTRAP

As discussed above TTRAP interacts with the E3 ubiquitin ligase TRAF6. Additionally, we also demonstrated that TRAF6 promotes the ubiquitylation of TTRAP. So far, TRAF6 has only been shown to promote the formation of K63-linked polyubiquitin chains, which are normally not proteasomal substrates. Therefore, it was surprising to see that TGF-β treatment of NMuMG cells resulted in decreased TTRAP protein level. The decrease was preventable by both the TGF-β receptor inhibitor, SB431542 and the proteasome inhibitor, MG132 (Figure 12). These results suggest that besides the TRAF6 mediated K63-linked polyubiquitination, TTRAP is also a target for K48-linked polyubiquitylation triggered by a TGF-β receptor-dependent phosphorylation step. Thorough understanding of this process will require significant research effort, but will undoubtedly contribute for the better understanding of TTRAP's role in TGF-β signaling.

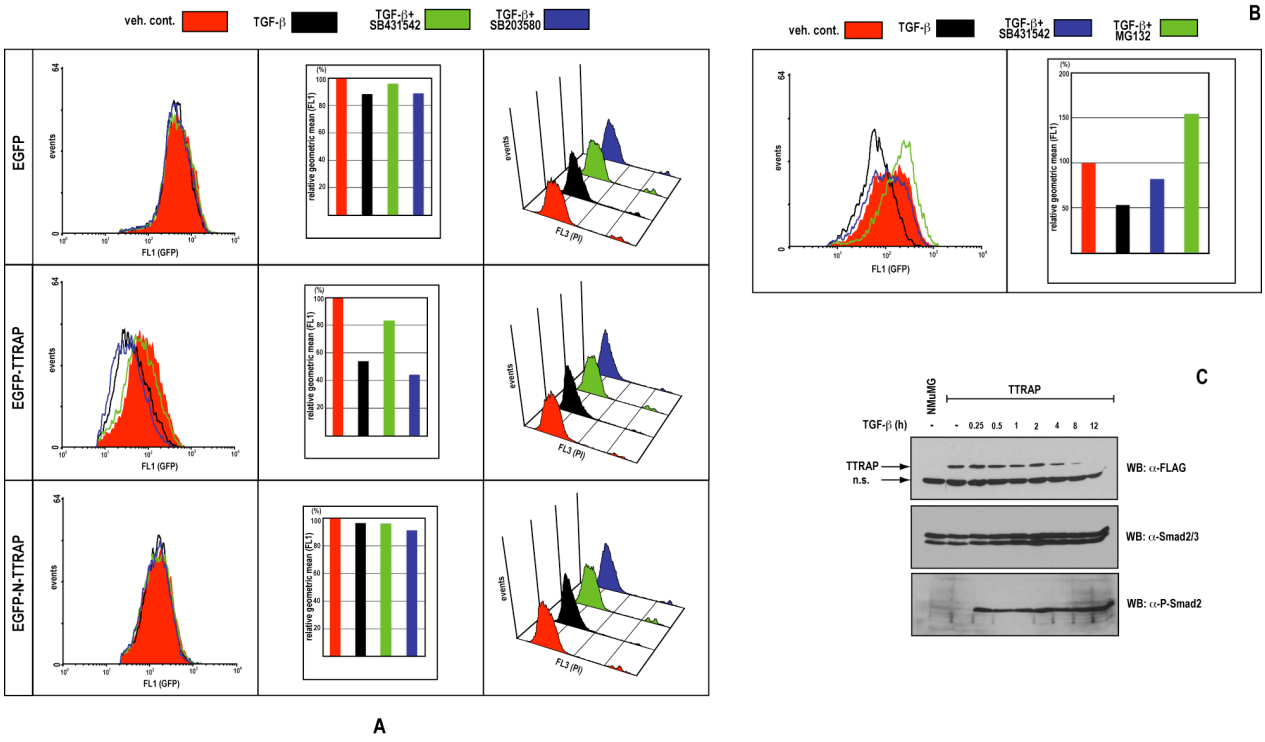


Figure 12. TGF-β induces TTRAP degradation.

III. SUMMARY

Based on our observations and the data published by other laboratories we can formulate the following model for the Smad-independent activation of the TAK1 signaling route by TGF-β (Figure 13). In the non-stimulated basal state, TTRAP associates with both TβRI and TβRII. Upon ligand engagement TβRI phosphorylates TTRAP, which subsequently helps the recruitment of TRAF6 and the TAK1 complex to the active TGF-β receptors. The resulting signaling complex is stabilized by TRAF6 mediated ubiquitylation of itself, TTRAP, TAK1 and possibly other components. In the receptor associated complex, TAK1 becomes activated by auto-phosphorylation and subsequently activates the AP1 (ATF-2, cJun) and NF-κβ transcription factors. Although not included in the current model, our observations also indicate that TTRAP negatively influence the Smad-dependent branch of TGF-β signaling. R-Smad phosphorylation is not affected by TTRAP suggesting that the protein acts at one of the receptor-distal steps.

Recent studies suggest that imbalances arising during tumor progression between the different branches of TGF-β signaling, may conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth. Thus, one may hypothesize that restoration of this equilibrium could be of great therapeutic value. Targeted augmentation of the tumor suppressing ability of TGF-β at the expense of its pro-oncogenic effects, could be an especially prolific treatment strategy. From this perspective, the TGF-β receptor-TRAF6-TAK1 signaling module could be a unique and very attractive target for intervention. This module is a point of convergence for the pro-apoptotic p38/JNK MAP kinase cascades and the pro-survival NF-κB and PI3K/Akt pathways. TGF-β has been shown to be able set off any of these signaling routes yet, exactly what determines the choice of pathway in a given cell type is still far from completely understood. The TTRAP adaptor molecule described here could be an important player of the TAK1 dependent signaling events mentioned above. It interacts with all key components of the TGF-β receptor-TRAF6-TAK1 signaling module and also involved in TGF-β dependent apoptosis. Thus, thorough understanding of TTRAP's cellular functions, may also help us formulate strategies for steering the TGF-β pathway in different directions, favoring either survival or apoptosis. The importance of TTRAP in tumorigenesis is also underscored by recent epidemiological studies indentifying the molecule as a putative human tumor suppressor involved in various malignancies.

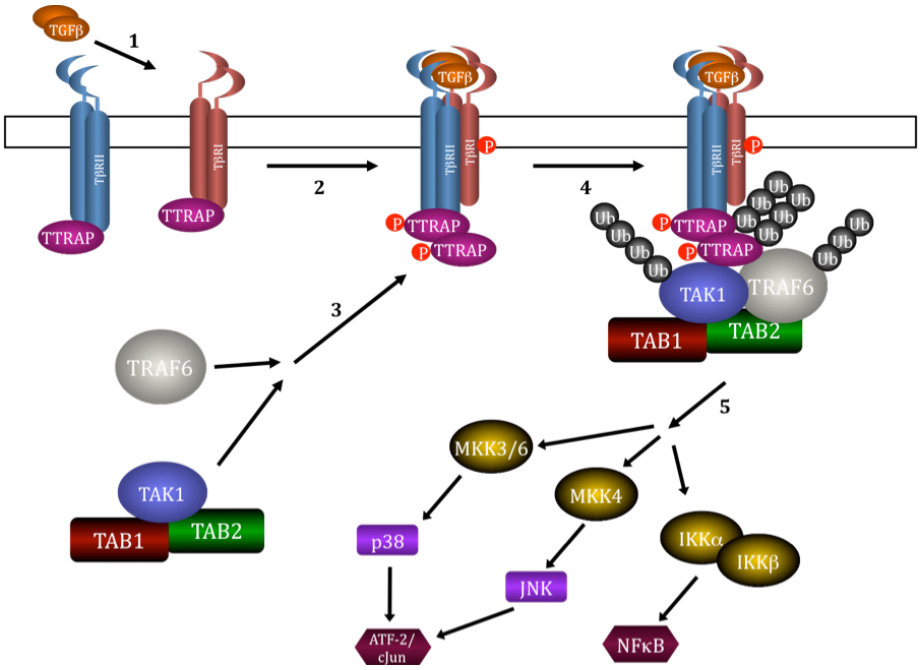


Figure 13. A model for Smad-independent activation of the TAK1 signaling route by TGF-β