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Tristetraprolin as a Negative Feedback Regulator in the Control of Chronic and Acute Inflammation

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning!!
Logic will get you from A to B but imagination will take you everywhere."

Albert Einstein

Many Thanks To…

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TABLE OF CONTENTS

1. ABSTRACT

Tristetraprolin (TTP) is a prototypic family member of the cystein-cystein-cystein-histidin (CCCH)-type tandem zinc-finger (TZF) domain proteins that regulate mRNA stability in eukaryotic cells. TTP specifically binds to AU-rich elements (AREs) in the 3'-untranslated region (UTR) of certain mRNAs exhibiting high turnover rates (e.g. cytokines, growth factors or proto-oncogenes), recruits components of the RNA-decay machinery via its N- and C-terminal domains and thereby facilitates the rapid degradation of the transcripts. Described targets that undergo this so called ARE-mediated decay (AMD) include tumor necrosis factor α (TNFα), granulocyte macrophage colony-stimulating factor (GM-CSF) and immediate early response 3 (Ier3).

TTP is an inducible, hyperphosphorylated protein. Phosphorylation by the mitogen-activated-protein-kinase (MAPK) p38-MK2 leads to its stabilization and accumulation in the cell, but at the same time to the inactivation of its mRNA degrading function. In endothelial cells, expression of TTP is up-regulated by a variety of agents including inflammatory mediators such as TNFα. TNF receptor (TNFR) engagement leads to the concomitant activation of MAPKs and IKKs (inhibitor of κB kinase) resulting in the onset of p38, JNK (c-Jun N-terminal kinase) and nuclear factor kappa B (NF-κB) signaling cascades. These trigger biological responses reaching from proliferation and differentiation to inflammatory responses and apoptosis.

We describe here a novel, AMD-independent function of TTP in regard to the transcription factor NF-κB: TTP suppresses the transcriptional activity of NF-κB dependent promoters independent of its mRNA destabilizing property. This is evidenced by several gain- and loss-of-function experiments, e.g. in TTP knock out (k.o) mouse embryonic fibroblasts (MEF), lack of TTP leads to enhanced nuclear p65 levels and activity, which are associated with the upregulation of certain ARE-less NF-κB target genes. The attenuation of NF-κB activity is at least in part due to an interference of TTP with the nuclear import of the p65 subunit of the transcription factor.

In addition the presented data suggest that the MAP3K MEKK1 phosphorylates TTP leading to its K63-linked polyubiquitination, which impacts cell viability in HEK 293 cells. This is associated with strongly impaired JNK activity in TTP k.o MEF. In human umbilical vein endothelial cells (HUVEC) TTP was found to be involved in the onset of apoptosis in a TNFα independent and dependent manner.

This novel inhibitory function of TTP towards NF-κB may synergize with its mRNA degrading ability to contribute to the coordinated crosstalk between NF-κB and MAPK cascades, facilitating a flexible response to incoming signals and cellular requirements.

1. ZUSAMMENFASSUNG

Tristetraprolin (TTP) ist ein klassischer Vertreter einer Proteinfamilie, die als typisches Merkmal sogenannte Tandem-Zinkfinger-Strukturen aufweisen. Diese Motive bestehen jeweils aus drei Cysteinen und einem Histidin (CCCH) und sind maßgeblich an der mRNA Degradierung in eukaryotischen Zellen beteiligt. TTP bindet spezifisch an sogenannte AU-reiche Elemente (ARE), die sich hauptsächlich in der 3'-untranslatierten Region von mRNAs befinden, welche eine hohe Turnover-Rate aufweisen, wie zum Beispiel Cytokine, Wachstumsfaktoren oder auch Proto-Onkogene. In der Folge rekrutiert TTP mit Hilfe seiner N- und C-terminalen Proteindomänen Faktoren der RNA-Degradierungsmaschinerie und führt so zum schnellen Abbau der Transkripte. Typische mRNAs, die über diesen sogenannten ARE-gesteuerten Abbau (AMD) degradiert werden, sind unter anderem TNFα, GM-CSF und Ier3.

TTP ist ein durch verschiedene Faktoren induzierbares, hyperphosphoryliertes Protein, wobei die Phosphorylierung durch die Mitogen-aktivierte-Proteinkinase (MAPK) p38-MK2 einerseits zur Stabilisierung und Akkumulation von TTP, und andererseits zur Inaktivierung der mRNA-Degradierungsfunktion führt. In Endothelzellen wird die Expression von TTP durch verschiedenste Stimuli hochreguliert, zum Beispiel durch das proinflammatorische Protein TNFα, welches ein typisches Mitglied der TNF-Familie darstellt. Die Bindung eines Liganden an einen entsprechenden Rezeptor der TNFR Familie führt immer zur gleichzeitigen Aktivierung von MAPKs und IKKs (Inhibitor der κB Kinase), welche folglich p38, JNK und NF-κB Signalkaskaden aktivieren. Dies führt zur Expression von Genen, die verschiedenste biologische Prozesse wie Proliferation, Zelldifferenzierung, inflammatorische Antworten aber auch Apoptose, kontrollieren.

Wir beschreiben hier eine neue, AMD-unabhängige Funktion von TTP: es inhibiert NF-κB-abhängige Promotoren unabhängig von seiner Funktion mRNAs zu degradieren. TTP knock out MEFs zum Beispiel zeigen erhöhte, nukleäre p65-Levels, die weiters mit der Hochregulation von spezifischen, ARE-losen NF-κB-target Genen einhergehen. Im Rahmen dieser Arbeit wird auch gezeigt, dass der zugrunde liegende Mechanismus, zumindest zum Teil, eine Blockade des nukleären Imports der NF-κB-Untereinheit p65 durch TTP ist. Weiters konnte gezeigt werden, dass die Phosphorylierung von TTP, hervorgerufen durch die MAP3K MEKK1, zur K63-verknüpften Ubiquitinylierung des Proteins führt und die Viabilität von HEK 293 Zellen stark beeinflusst. Dies unterstützt die Tatsache, dass TTP knock out MEF eine auffallend schwache JNK Aktivierung zeigen, sowie dass TTP an der TNFα unabhängigen, und auch abhängigen Apoptose in HUVEC beteiligt ist. Diese neue regulatorische Rolle von TTP könnte in Kombination mit der bereits beschriebenen mRNA-Destabilisierungsfunktion zur verbesserten Koordination von NF-κB und MAPK Signaltransduktion beitragen und dadurch die Grundlage für eine angemessene und umfassende Antwort einer Zelle auf vielerlei unterschiedliche Signale und Bedingungen bilden.

2. INTRODUCTION

2.1. Inflammation

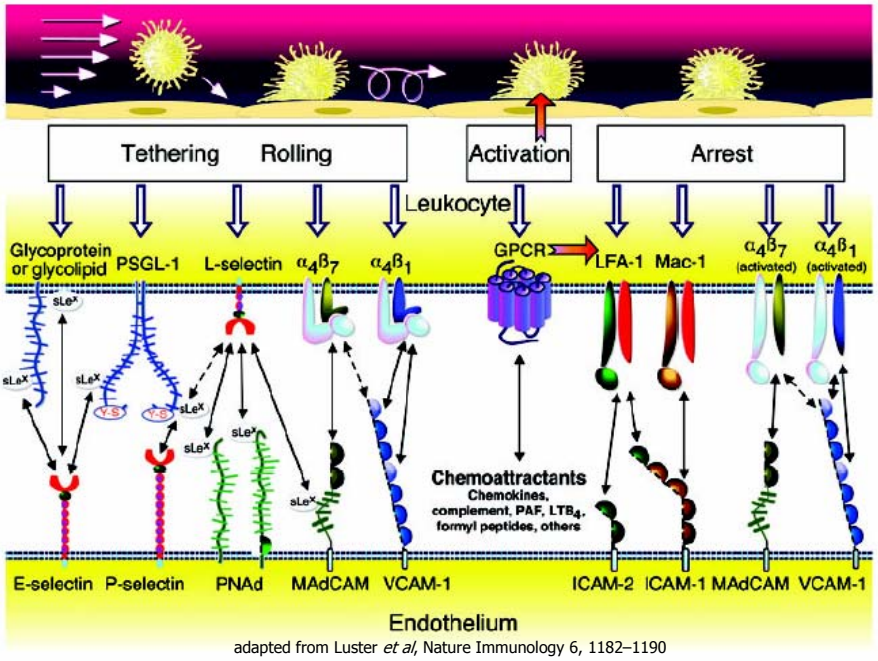
Inflammation is defined as a complex biological process that constitutes a protective mechanism to defend an organism against injurious triggers, prevents tissue destruction, initiates healing and is therefore the main actor in maintaining tissue homeostasis. Inflammation is classified in acute and chronic response and whereas the former brings benefit to the organism the latter causes detrimental effects which are reflected by a variety of diseases including e.g. atherosclerosis.

Acute inflammation is known as the initial, immediate response to injury and is characterized by the increased movement of leukocytes and plasma from the blood into the harmed tissue, resulting in the onset of repair mechanisms. These facilitate the removal of damaged cells, killing of invaders and finally promote cell replacement. In principle, acute inflammation is divided into three main phases, namely the inductive or initiation phase, which is followed by a sustained response that finally becomes terminated when triggers of the initial response are resolved [1, 2]. At the onset of inflammation cells of the innate immune system, mainly resident macrophages and dendritic cells, undergo activation leading to the release of so-called inflammatory mediators which are indispensable for the appropriate progression of the response.

2.1.1. Activation of the inflammatory response

Classical signs of inflammatory activation are increased blood flow causing extensive redness and increased heat, as well as enhanced permeability of blood vessels leading to the leakage of plasma proteins into the tissue and resulting in swelling. One of the most important steps in the initiation and maintenance of inflammation is the migration of leukocytes from the blood stream through the vessel wall to the site of injured tissue. Coordinated migration of mainly neutrophils is thereby achieved by a chemotactic gradient [3] and can be devided into different steps: recruitment and adhesion to the endothelium, transmigration via diapedesis and finally movement within the tissue via chemotaxis. Recruitment of leukocytes is mainly a receptor mediated process whereby proinflammatory mediators such as histamines, TNFα, Interleukin-1 (IL-1) or Lipopolysaccharide (LPS) trigger the expression of different receptors, including P- and E-selectin, on the endothelial cell surface. These are quite weakly bound by ligands on the surface of leukocytes (e.g. L-selectin, PSGL-1 or α4-integrins) which forces them

to slow down and "roll" along the endothelial surface by means of alternating bonding and release [4]. Further activation of leukocytes through the release of chemokines finally attaches them to the endothelium (Fig. 1T). Once adhered, chemokine gradients induce leukocyte movement through the basement membrane into the tissue and further assure the directed movement towards the source of inflammation. Based on the above described process it gets obvious that the endothelium plays an



adapted from Luster *et al*, Nature Immunology 6, 1182–1190

Fig. 1T: Leukocyte-adhesion is a multistep cascade. Leukocytes undergo four adhesion steps to accumulate at a specific site within the blood vessel: tethering, rolling, activation and arrest (top). Thereby they express trafficking mediators specific for each step which act as ligands for their endothelial counter-receptors (bottom). Leukocyte-receptors such as L-selectin, PSGL-1 and α4-integrins mediate tethering and rolling and interact with ligands on inflamed endothelial cells as E-selectin, P-selectin and peripheral node addressin (PNAd). Thereby L- and P-selectin mediate rolling whereas E-selctin and α_4-integrins act as rolling-stabilizers. Of equal importance are the G-protein-coupled receptors (GPCR) on rolling cells. These receptors become engaged by e.g. chemokines which finally leads to an intracellular signal transmission and the rapid activation of β_2 and α_4-integrins. This results in a tight fixation to the endothelial surface by interaction with molecules of the endothelial immunoglobulin superfamily as ICAMs, VCAMs or MAdCAM. Interactions of molecules with their binding partner(s) are visualized by arrows. (PAF, platelet-activating factor; LTB_4, 5-lipoxygenase pathway producto leukotriene B_4; Mac-1, myeloid cell-associated marker).

important role in the regulation and control of inflammatory processes. As a normally nonadhesive barrier between blood stream and tissues, basically impermeable to macromolecules, it undergoes diverse changes in permeability and adhesiveness, processes regulated by a complex interplay between incoming inflammatory signals, release of mediators that lead to the engagement of receptors, and the final activation of a variety of cellular responses. Main proinflammatory mediators include cytokines such as IL-1 or TNFα, which promote the progression of the response and trigger the coordinated interplay of different signaling cascades. One of the activated key regulators in cytokine-initiated proinflammatory signaling is the dimeric transcription factor NF-κB that accounts for a highly selective regulation of different sets of genes. These include proinflammatory cytokines, adhesion molecules (E-selectin, vascular

cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM)-1), chemokines, inducible enzymes such as cyclooxygenase 2 (Cox-2) and inducible nitric oxide synthase (iNos), and growth factors [5, 6].

2.1.2. Resolution of Inflammation

Beside the importance of a tightly regulated initation of inflammation its termination is of equal importance since the persistent or prolonged exposure to injurious agents leads to the permanent presence of proinflammatory mediators. This results in chronic inflammation which is a hallmark of various diseases and autoimmunity.

A prerequisite for resolution is the effective elimination of all "activating" contributors, including the removal of the initial stimuli, downregulation of proinflammatory gene expression, and the effective elimination of activated neutrophils. The latter is accomplished by the onset of programmed cell death leading to their specific recognition and clearance by macrophages [7]. Additionally important is the clearance of other inflammatory cells as well as excess resident tissue cells that have been driven to proliferate in response to repair mechanisms [8], making the process of apoptosis an indispensable event in the termination of inflammatory responses.

In terms of inter- and intracellular signaling, the resolution phase is characterized mainly by the release of anti-inflammatory agents, including cytokines such as IL-4, IL-10, IL-13, Interferon-α (IFNα), as well as transforming growth factor β (TGFβ). At the beginning of the 21st century it has been shown that the transcription factor NF-κB is not only a potent initiator of inflammation but also plays an essential role in the resolution phase [9]. While its activation during the initiation of inflammation triggers pro-inflammatory gene expression, its onset during resolution is associated with the expression of anti-inflammatory genes. Hence, it is quite clear that the tight control of NF-κB signaling and the balance between pro-inflammatory genes and survival of cells, and the expression of anti-inflammatory genes are the main determinants for an appropriate cellular response to a diversity of challenging invaders.

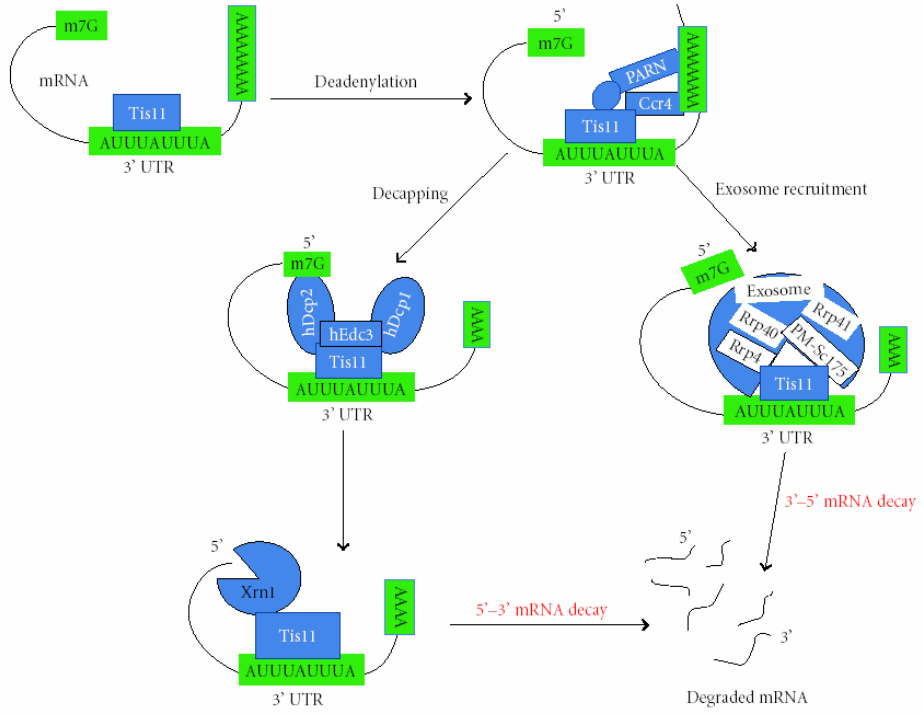
2.1.2.1. Role of negative feedback regulators in the resolution of inflammation

Whereas the activation of inflammation is quite well understood its resolution and the involved regulatory molecules remain largely elusive. Meanwhile it is commonly accepted that the triggers of resolution are already set within the early stages of inflammatory processes. These include so called negative feedback loops that occur post induction and allow an unhindered first wave of activity before ensuring the organized termination of the NF-κB response [10, 11]. Well described negative feedback regulators

include the inhibitor of kappa B (IκB) and the de- and ubiqutinating enzyme A20 [12-14]. Feedback loops occur at all levels of signal transduction: at the receptor level, affecting all downstream events, at the level of signal transduction and individual transcription factors, leading to the selective silencing of genes, and also at the posttranscriptional level, preventing translation and promoting degradation of transcripts. The latter depends on the accessibility of cis-acting AREs situated in their 3'-UTR [15-17]. These elements account for effective destabilization and translational block of proinflammatory mediators regulated by specific ARE binding proteins. Generally, ARE-binding proteins either act in RNA stabilization, as it is the case for HuR [18], or in degradation, as already demonstrated for BRF1 and TTP [17, 19].

2.2. Tristetraprolin: an mRNA destabilizing protein

TTP is the best studied member of a small family of proteins, the 12-O-tetradecanoylphorbol-13-acetate (TPA) inducible sequence 11 (TIS11) proteins. All TIS11-proteins function in the posttranscriptional regulation of genes exhibiting high turnover rates as cytokines, cell cycle regulators or transcription factors. The TIS-family consists of four members: TTP (also known as TIS11, ZFP36, GOS24 or Nup475), TIS11b (Berg36, ERF-1, BRF-1, ZFP36L1), TIS11d (ERF-2, BRF-2, ZFP36L2) and the fourth member



adapted from Baou *et al.*, Journal of Biomedicine and Biotechnology, 2009

Fig. 2T: ARE-mediated mRNA decay by TIS11 proteins. Binding of TIS-proteins to ARE motifs in the transcript-3'UTR leads to the recruitment of deadenylases (hCcr4, PARN). Deadenylation subsequently results in either the recruitment by the exosome or decapping by hDcp1 and hDcp2. The exosome constitutes a multiproteincomplex containing exonucleases as Rrp4, Rrp40, Rrp41 and PM-Scl75, and triggers the 3'-5' directed RNA-decay. Conversely, decapping by hDcp1 and hDcp2 is followed by exunocleolytic 5'-3' decay facilitated by Xrn1.

ZFP36L3, whose expression was described in mouse placenta but was not detected in human tissues [20]. All these proteins have been found to contribute to mRNA destabilization by either the exosome mediating 3'-5' RNA decay, or by exonculease activity, where XRN1 triggers the 5'-3' degradation of the transcript [21]. Mode of action is thereby the binding of TIS-proteins to specific AREs in the 3'-UTR of the target mRNAs (Fig. 2T). ARE-binding is accomplished by a tandem-zinc-finger-structure, which appears to be the common feature of TIS-family proteins [22], since the spacing as well as the length of the linker between the tandem CCCH-zinc fingers is highly conserved. Although the analogy of the zinc-binding motifs accounts for a similarity in function, they exhibit differences in target affinity as well as a cell type specific regulation.

TTP was first identified in the late 1980ies as an immediate early gene rapidly induced by phorbol 12-myristate 13-acetate (PMA) [23-25], and a variety of other stimuli including serum, insulin, and growth factors [26-29] in different cell types, including fibroblasts, murine as well as human macrophages but also in monocytes, neutrophils, myeloid as well as in T-cells. The initial characterization of a tandem zinc-finger structure, as well as its reported nuclear localization, initially led to the assumption that it might function as a transcriptional regulator [30-32]. However, in the meanwhile it is well established that TTP promotes AMD. TTP was shown to bind to a subset of AREs, and the nonamer UUAUUUAUU was identified as the minimal complete binding site [33]. The motif is recognized by the tandem CCCH-type zinc-finger domain of TTP, and the integrity of this domain has been shown to be indispensable for mRNA degradation, since a single mutation within either zinc-finger abolishes the ARE binding activity [34]. TTP exhibits no enzymatic activity itself but recruits various components of the basic mRNA decay machinery via its N- and C-terminal domains (Table 1T) [35, 36]. TTP interacts with decapping-complex-members as Dcp2, Dcp1a, Edc3 or Hedls via its N-terminal domain [36, 37]. In addition, TTP binds to Xrn1, a well described 5'-3' exonuclease [36] as well as to Ago2 and Ago4, which comprise key regulators of the RNA-induced silencing complex [38]. All these components colocalize together with TTP at so called "processing-bodies" (P-bodies), small cytoplasmic foci where specific mRNAs are recruited for either translational silencing by micro RNAs or mRNA decay triggered by AREs. A classical P-body-associated protein that binds to TTP is the deadenylase Ccr4. Moreover, it is reported that the enzyme Dicer, normally involved in the processing of miRNAs, is involved in TTP-mediated mRNA degradation, in addition to miR16 which contributes to mRNA destabilization through association with argonaute family members [38]. Thus, TTP and miRNAs codependently mediate RNA-decay by partly sharing the same processing pathways. Furthermore, TTP was found to be a component of the RNAi pathway in drosophila melanogaster [39]. Apart from that, TTP facilitates the 3'-5'-directed degradation of mRNAs by interacting with the exosomal subunits PM-Scl75 and Rrp4 [40], two of at least ten exonucleases that form, together with RNA binding proteins, the exosom-multiprotein-complex which accounts for coordinated processing and degradation of RNAs.

Table 1T: TTP-interacting proteins

Partner	Function
14-3-3	Family of adaptor proteins: binds to phosphoserine containing motifs (Ser^{52} and Ser^{178} in mous inhibits TTP activity stabilizes TTP protein enhances cytoplasmic localization of TTP prevents TTP from associating with stress granules
MK2	MAPKAP kinase-2: directly phosphorylates TTP at Ser^{52} and Ser^{178} (mouse TTP)
PP2A	Protein phosphatase 2A, heterotrimer: competes with 14-3-3 protein dephosphorylates TTP at Ser^{178} (mouse TTP) activates TTP
PM-Scl75	Subunit of the exosome (3′–5′-exoribonuclease complex): contains RNase PH domain
Rrp4	Subunit of the exosome (3′–5′-exoribonuclease complex): contains KH and S1 RNA-binding domains
Ccr4	Deadenylase, subunit of the Ccr4–Caf1–Not complex: contains exonuclease III domain P-body component
Dcp2	Subunit of the decapping complex: catalytically active decapping enzyme P-body component
Dcp1a	Subunit of the decapping complex: enhancer of decapping P-body component
Edc3	Subunit of the decapping complex: enhancer of decapping P-body component
Hedls	Subunit of the decapping complex: enhancer of decapping P-body component
Xrn1	5′–3′-exoribonuclease: P-body component
Ago2, Ago4	Argonaute 2 and 4, subunits of the RNA-induced silencing complex: P-body component
KSRP	KH-type splicing regulatory protein: binds to and destabilizes ARE-containing mRNAs TTP proposed to inhibit iNOS mRNA decay through binding to KSRP
Nup214	Nuclear pore protein: interacts with TTP through the FG repeat-containing C-terminus
Tax	Retroviral oncoprotein: TTP inhibits the ability of Tax to transactivate LTR promoters Tax interferes with TTP activity

adapted from Sandler & Stoecklin, Biochem. Soc. Trans; 2008

2.2.1. Importance of TTP phosphorylation

ARE-motifs are not only important elements in the regulation of transcript decay, they also contribute to the stabilization of mRNAs when desired. This can be achieved by the competitive binding of RNA-stabilizing and destabilizing proteins as HuR and TTP, respectively. Whereas destabilizers such as TTP account for the degradation of targets in unstimulated cells, they are squeezed out by stabilizing proteins and "inactivated" through posttranslational modifications to allow the rapid accumulation of mRNAs upon stimulation. The inhibition of AMD is controlled by various signaling pathways including the JNK, p38-MAPK and PI3K (phosphoinositide-3-kinase) cascades [41-44]. It was described recently [45, 46] that p38-MAPK-MK2 signaling is of particular interest in terms of cytokine stabilization. MK-2 was shown to directly phosphorylate TTP at serine residues Ser^{52} and Ser^{178} after LPS stimulation in mouse macrophages [47], which allows binding of the adaptor protein 14-3-3 and further reduces the ability of TTP to destabilize mRNAs [48, 49]. In line with that, $MK2^{-/-}$ mice exhibit strongly reduced levels of known TTP mRNA targets such as IL-1, IL-6 and IFNγ [45, 46]. Furthermore, the double-knockout of MK2 and TTP results in the high TNFα levels observed in $TTP^{-/-}$ mice [50]. It is suggested that the phosphorylation mediated by MK-2 reduces the ARE-binding affinity of TTP and affects protein-protein interactions important for the recruitment of components of the RNA-decay machinery. While TTP "inhibition" is triggered by phosphorylation, dephosphorylation by the protein phosphatase 2A (PP2A) results in reactivation of its destabilizing ability. MK-2 seems to be counterbalanced by PP2A which is believed to compete with the adaptor 14-3-3 for binding to TTP (Fig. 3T) [51]. In addition to p38-MK2 various

adapted from Sandler & Stoecklin, Biochem. Soc. Trans; 2008

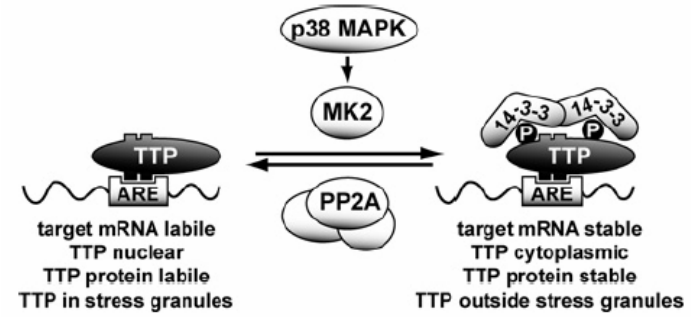


Fig. 3T: TTP controls ARE-mediated decay. TTP binds to ARE-motifs in the uninduced state and facilitates mRNA degradation. Upon extracellular stimulation, the onset of signaling cascades as the p38-MAPK-MK2, trigger the phosphorylation of TTP resulting in binding of the adaptor protein 14-3-3 and stabilization of target mRNAs. In addition, phosphorylation stabilizes TTP itself, leading to its accumulation in the cytoplasm and its exclusion from stress granules. Finally, the phosphatase PP2A squeezes 14-3-3 out and causes dephosphorylation of TTP restoring the initial state.

other kinases have been shown to phosphorylate TTP, but their functions remain largely elusive yet. Those kinases include the p42 MAPK, which accounts for posphorylation of Ser^{220} in mouse TTP [52] and the p38α and -β isoforms which are involved in multiple phosphorylation events [53]. In addition,

MALDI/MS analysis revealed various phosphorylation sites of human TTP including Ser^{66}, Ser^{88}, Thr^{92}, Ser^{169}, Ser^{186}, Ser^{197}, Ser^{218}, Ser^{276}, Ser^{296} [54]. The related kinases such as protein kinase A (PKA), protein kinase C alpha (PKCα), protein kinase C beta (PKCβ), protein kinase C gamma (PKCγ), protein kinase C zeta (PKCζ) and inhibitor of kappa B kinase 1 (IKK1), glycogen synthase kinase-3 (GSK-3) as well as extracellular-signal-regulated kinase 1 (ERK1) were predicted bioinformatically. However, not all TTP-kinases alter its function as a destabilizing protein as supported by a study using a series of TTP-mutants, wherein the single mutations of serines and/or threonines showed that phosphorylation events must not necessarily impact its mRNA-degrading function [55].

Initially, the biological function of Tristetraprolin was uncovered by the creation and analysis of TTP knock out mice. They exhibit classical inflammatory signs as spontaneous arthritis, cachexia, neutrophilia and dermatitis [56]. Careful analysis of these mice and isolated cells revealed that these symptoms are caused by the extensive overproduction of the cytokine TNFα, a principal mediator of inflammation in acute and chronic inflammatory diseases [57]. Likewise, mutations in TTP have been associated with autoimmune diseases in humans [58]. While the transcription of TNFα is not affected in macrophages obtained from TTP k.o mice its mRNA half-life is prolonged, which was the initial observation leading to the identification of the first TTP-mRNA target [19]. Beside TNFα, the turnover of several other mRNAs is controlled by TTP, including GM-CSF, Cox-2, Ier3 as well as the interleukins -2, -3, -6 and -10 [19, 59-61].

2.3. NF-κB signaling

One of the main signaling pathways triggered by e.g. the proinflammatory cytokine TNFα is the NF-κB pathway. The complexity of the NF-κB response is reflected by a great diversity of different effects of NF-κB activation, which is due to the fact that varying heterodimer combinations of the transcription factor subunits can regulate different sets of genes. NF-κB target genes can be grouped into different classes and regulate diverse biological responses. The most important role of NF-κB is its function in innate and adaptive immunity, including not only the proinflammatory gene expression of cytokines, chemokines and related receptors but also the regulation of antiapoptotic genes. Beside the general accepted role NF-κB plays in the balance between cell survival and cell death, it additionally accounts for the expression of genes which are important regulators of differentiation and proliferation. Since during the NF-κB activation cascade various stimuli trigger effectors that signal to one single transcription factor, dysregulation of its function is implicated in several pathophysiological situations (e.g. [62-64]).

2.3.1. Canonical versus non-canonical NF-κB signaling

Generally, two signaling pathways lead to the activation of the transcription factor NF-κB, the classical or canonical pathway on the one hand, and the alternative, also called the non-canonical pathway, on the other hand. In both cases signaling events start with receptor engagement leading to the onset of receptor-specific proximal cascades that finally converge at the inhibitor of kappa B (IKK)-signalosome. Activation of the latter triggers the release and subsequent activation of NF-κB subunits. These consist of either hetero- or homodimers of different combinations of the five members of the NF-κB-transcription factor family, p50, p52, p65/RelA, c-Rel, and RelB. During the onset of the canonical pathway (Fig. 4T, left), receptor ligation leads to the recruitment of adaptor proteins such as TNF receptor-associated factors (TRAFs; see 2.3.3) which in turn activate the IKK-complex leading to phosphorylation and subsequent degradation of the inhibitory protein IκBα. The IKK-multiprotein-complex contains two kinase subunits, namely IKKα (IKK1) and IKKβ (IKK2) as well as the non-catalytic regulator subunit IKKγ (NEMO) (Fig. 5T, bottom) [65]. Activated through upstream incoming signals, mainly IKKβ accounts for phosphorylation of IκBα on Ser^{32} and Ser^{36} and of Ser^{19} and Ser^{23} of IκBβ. Phosphorylation is a prerequisite for K48-linked polyubiquitination, which targets the IκB protein for proteasomal degradation. This results in released NF-κB dimers, which subsequently translocate to the nucleus. The classical pathway leads to the activation of NF-κB dimers consisting of different combinations of the subunits p65, p50, c-Rel and RelB. However, the most common and best studied combination constitutes the p65/p50 heterodimer. Its activation is achieved by a variety of proinflammatory signals including IL-1, TNFα and LPS and leads to the expression of genes encoding cytokines, adhesion molecules, enzymes as well as antiapoptotic mediators [66].

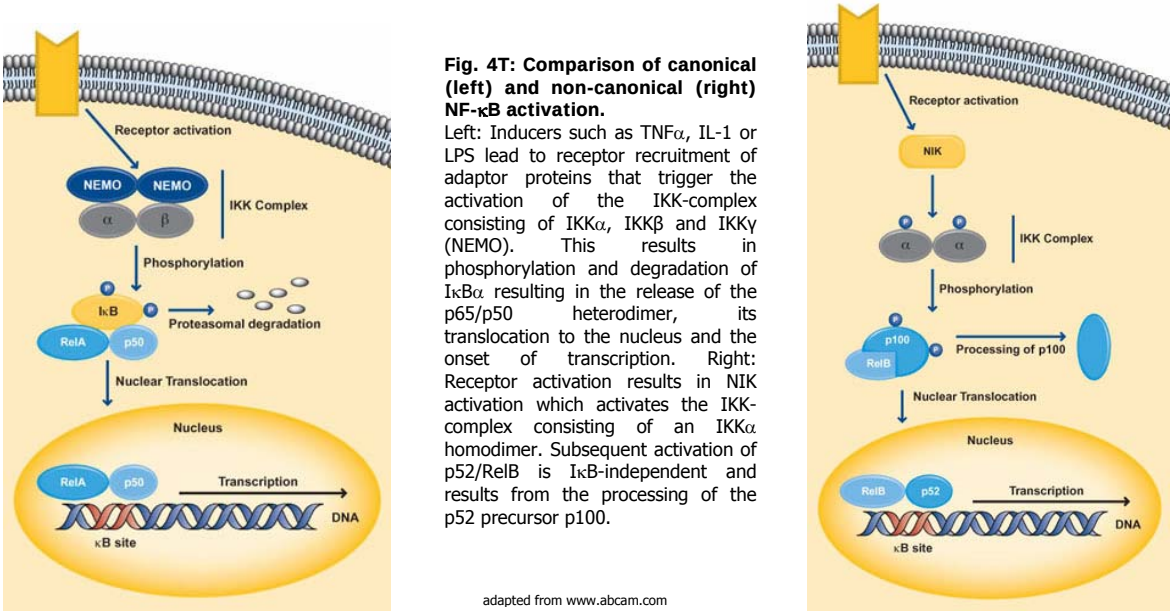


Fig. 4T: Comparison of canonical (left) and non-canonical (right) NF-κB activation.
Left: Inducers such as TNFα, IL-1 or LPS lead to receptor recruitment of adaptor proteins that trigger the activation of the IKK-complex consisting of IKKα, IKKβ and IKKγ (NEMO). This results in phosphorylation and degradation of IκBα resulting in the release of the p65/p50 heterodimer, its translocation to the nucleus and the onset of transcription. Right: Receptor activation results in NIK activation which activates the IKK-complex consisting of an IKKα homodimer. Subsequent activation of p52/RelB is IκB-independent and results from the processing of the p52 precursor p100.

adapted from www.abcam.com

The alternative pathway is induced by factors such as lymphotoxin B and the B cell activating factor (BAFF) that activate the NF-κB-inducing kinase (NIK). This results in the phosphorylation of an IKK-complex that comprises solely two IKKα subunits in this case (Fig. 4T, right). Activation of the p52/RelB heterodimer is IκB-independent and achieved through phosphorylation and processing of the p52 precursor p100 [67, 68].

All NF-κB-family members comprise an N-terminal Rel-homology domain (RHD), responsible for homo- and heterodimerization and DNA-binding. NF-κB dimers bind to κB binding motifs in various promoters, recruit either coactivators or corepressors and specifically regulate gene expression. A main structural difference between the five members is the C-terminal regulatory domain. While p65, RelB, as well as c-Rel own transcriptional activation domains (TADs), responsible for the positive regulation of transcription, the subunits p50 and p52 lack TADs and act in the inhibition of promoter activity unless they become associated with a TAD-containing NF-κB-subunit (Fig. 5T, left). The three typical IκB proteins IκBα, IκBβ, IκBε, characterized by so called ankyrin-repeat domains (Fig. 5T, right), are crucial for keeping NF-κB dimers inactively in the cytoplasm. The prototypic inhibitor IκBα for example, masks

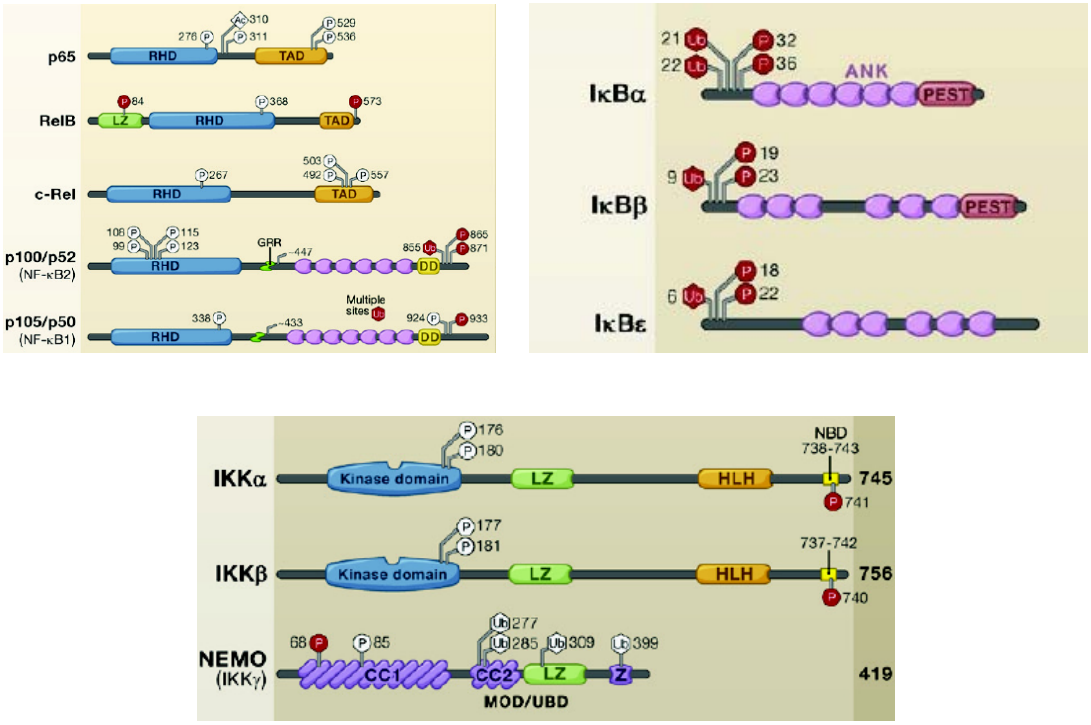


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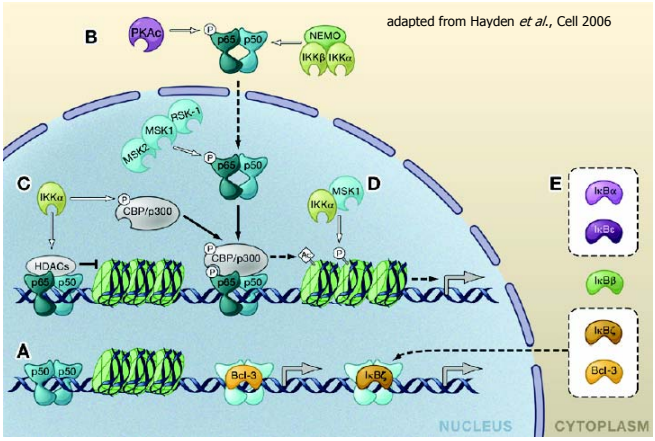
Fig. 5T: Schematic representation of the NF-κB (left), IκB (right) and IKK (bottom) protein families. Posttranslational modifications are indicated with P (phosphorylation), Ac (acetylation), Ub (ubiquitination); inhibitory events are represented by red colour. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine-rich region; DD, death domain; HLH, helix-loop-helix domain; Z, zinc finger; NBD, NEMO-binding domain; CC1/2, coiled coil domains; MOD/UBD, minimal oligomerization domain and ubiquitin-binding domain.

the nuclear localization signal (NLS) of p65 whereas the NLS of the p50 subunit is left exposed resulting in the constitutive nuclear-cytoplasmatic shuttling [69, 70] of the IκBα/NF-κB complex. Finally, degradation of IκBα leads to unmasking of the p65 NLS, enabling its nuclear translocation. p65 contains a classical monopartite NLS, characterized by a stretch of 4-6 basic amino acid residues, and it has been shown that the karyopherin importin α3 specifically binds the p65-NLS [71], whereas importin β is responsible for docking to the nuclear pore complex (NPC). Nevertheless, it is not well established so far how nuclear shuttling of the known diversity of transcription factors is selectively timed and regulated. However, the variety of differently structured NLS' is known to contribute to the specific recognition by various karyopherins [72, 73]. The nuclear localization and accumulation of NF-κB dimers is prerequisite for transcriptional activation: they bind to the κB consensus sequence 5'GGGRNWYYCC3' (N-any base; R-purine; W-adenine or thymine; Y-purimidine) [74] of promoters and the high sequence variability of the κB binding motif in combination with differences of NF-κB-dimer affinities is one of the main explanations for the high diversity of NF-κB regulated genes [75, 76].

2.3.2. Role of posttranslational modifications in the activation of NF-κB

Beside the tight regulation of p65 nuclear import, further activation of NF-κB is achieved through post translational modifications, recruitment of coactivator proteins, increased stabilization and the coordinated interaction with the basal transcriptional apparatus as well as the epigenetically regulated accessibility of promoter binding sites (Fig. 6T). Various kinases have been described to phosphorylate p65 at different serine residues present in the RHD and TADs of the transcription factor (Fig. 6T, right). PKA for example, mediates phosphorylation of Ser^{276} of p65 promoting its interaction with the coactivating histone-acteyl-transferases CBP (cyclic adenosine monophosphate responsive element-binding protein (CREB)-binding-protein) and p300 [77, 78]. In addition, the nuclear kinases MSK1 and MSK2, which are activated by multiple pathways containing components as the MAPKs ERK and p38, account for Ser^{276} phosphorylation and also contribute to enhanced transcriptional activity [79]. Furthermore PKCζ phosphorylates Ser^{311} of p65 also mediating the interaction with CBP, [80] and CKII inducibly phosphorylates Ser^{529} but it is still unclear whether this modification has an effect on transcription [81, 82]. Beside phosphorylation, also other posttranslational modifications are indispensable for full activation of NF-κB-mediated transcription. CBP/p300 mediated acetylation of p65 at Lys^{310} leads to highly increased transcriptional activity [83] and depends on the prior phosphorylation of Ser^{276} [77, 84]. The IKK-signalosome members IKKα and IKKβ play an interesting role in the p65 phosphorylation-acetylation process. They have been shown to directly phosphorylate Ser^{536} of p65, which significantly enhances p65 Lys^{310} acetylation [77, 84, 85]. Ser^{536} phohsphorylation is believed to alter the interaction of p65 with the SMRT (silencing mediator of retinoic



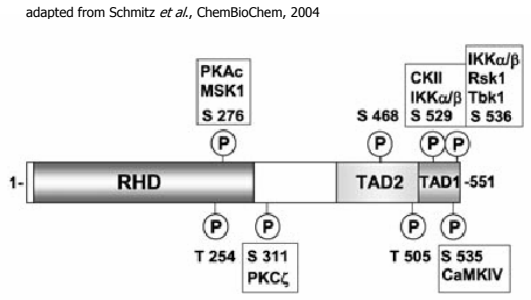


Fig. 6T: Posttranslational modifications regulate NF-κB transcriptional activity. Left: A) in the uninduced state, κB elements are occupied by p50 or p52 homodimers, transcription is repressed. B) stimulation leads to release of NF-κB dimers, phosphorylation of p65 by various kinases and the recruitment of the the CBP/p300 coactivator complex which facilitates p65 acetylation and promotes target gene expression. C) IKKα catalyzes the exchange of corepressor – coactivator complexes, by phosphorylation of both the coactivator complex CBP/p300 as well as corepressor complexes leading to HDAC release. D) MSK and IKKα promote transcriptional activation through histone phosphorylation. E) IκB proteins are involved in both positive and negative regulatory mechanisms: Bcl-3 and IκBζ promote or repress transcription by interacting with p50- and p52-containing complexes. IκBα and IκBε sequester NF-κB dimers away from DNA and act as negative regulators. Right: Summary of inducible phosphorylation sites within p65, related kinases as indicated.

acid and thyroid hormone receptor)-complex resulting in decreased levels of histone-deacteylases as HDAC3, finally promoting an interaction with CBP/p300. An additional important aspect is that IKKα not only acts on p65 but also directly phosphorylates the SMRT-itself [86], is associated with κB sites in promoters of diverse NF-κB-target genes, and is involved in the phosphorylation of histone H3 on Ser^{10} [87, 88]. Furthermore, IKKα has been associated with phosphorylation of the CBP/p300 coactivator complex [89]. Although phosphorylation events resulting in the recruitment of nuclear coactivators as CBP/p300 are the most critical step in building an "enhanceosome" to modulate general levels of gene expression, other signaling pathways and transcription factors, such as activator protein 1 (AP-1, composed of Fos (c-Fos, FosB, Fra-1, Fra-2) and Jun (c-Jun, JunB, JunD) family members [90], CREB or CCAAT enhancer binding protein (C/EBP) act in cooperation with NF-κB to facilitate a tightly regulated response to the diversity of incoming signals [91].

2.3.3. Interplay of NF-κB and MAPK signaling and the importance of Ubiquitination

Since various stimuli trigger the activation of NF-κB, there is a remarkable diversity of protein components of different pathways triggering signals that finally culminate on the level of the IKK-signalsome. However, several activating components upstream of the IKK-complex are shared by these signaling pathways. Two main actors are the receptor-interacting proteins (RIPs) and TRAF protein families that efficiently activate IKKs (Fig. 7T). RIPs are classical adaptor proteins that interact with other adaptors as TRADD (TNF receptor-associated death domain) and FADD (fas-associated death domain) at the cytoplasmic part of the receptor. RIP proteins furthermore build a complex with TRAF-proteins, which is prerequisite for the onset of downstream kinases and successful IKK activation. RIP family members are a

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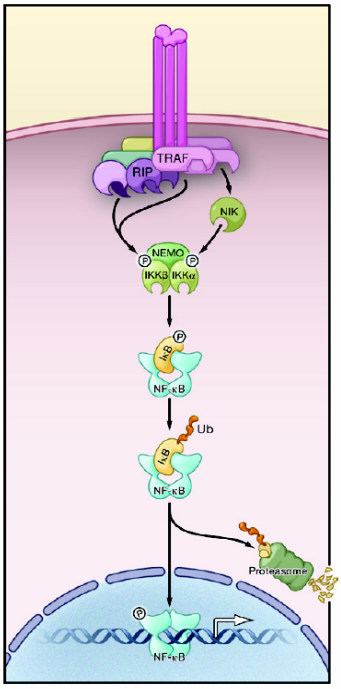

Fig. 7T: Simplified TNFR-activated NF-κB signaling. Receptor ligation leads to recruitment of TRAF/RIP complexes which facilitate activation of the IKK-signalosome, resulting in phosphorylation and degradation of IκBα und subsequent release of p65/p50 and its translocation into the nucleus.

general hallmark of TRAF-dependent pathways as exemplified by the TNFR superfamily signaling cascades (Fig. 7T). Seven different TRAF proteins have been identified so far. They share a C-terminal TRAF-domain, mediating protein-protein interactions, and all of them, with the exception of TRAF1, contain N-terminal RING finger domains that function as E3 ubiquitin ligases catalyzing the ubiquitin-transfer to target proteins. Ubiquitination is a process where ubiquitin is first activated by an activating enzyme (E1) that forms an intermediate complex with a ubiquitin conjugating enzyme (E2). Activated ubiquitin-chains are finally transferred onto lysine residues of target proteins by E3-ubiquitin-ligases. The ubiquitination-process is an essential regulatory, posttranslation event that accounts for the tightly coordinated and timed transmission of signals between proteins. Whereas lysin-48 (K48)-linked ubiquitination is responsible for 26S-proteasomal degradation, the lysine-63 (K63)-linked ubiquitination of various signaling mediators has been implicated in the specific regulation of signal transmission leading to either activation or inhibition of NF-κB [92, 93]. Whereas for example the de- and ubiquitinase A20 acts, beside IκBα, as the most prominent negative feedback inhibitor of NF-κB, TRAF-E3-Ligase activity plays an important role in the activation of the IKK complex [92]. Importantly, TRAFs are also indispensable for regulating other pathways, as the JNK cascade leading to AP-1 activation, and are therefore important mediators for the crosstalk of various signaling cascades activated by TNFR superfamily members. In the case of TNFR1 engagement, TRAF2 interacts with TRADD [94] at the receptor and facilitates, through recruitment of receptor-interacting protein (RIP) and TGFβ activated kinase 1 (TAK1) the activation of the IKK-complex. Interestingly, $TRAF2^{-/-}$ cells exhibit normal TNF mediated activation of NF-κB but exert deficiencies in AP-1 activation [95] pointing at the importance of TRAF2 in the regulation of signals that lead not only to NF-κB, but also to the activation of AP-1.

MAPKs and IKK-Ks (inhibitor of κB kinase kinase) are the commonly known upstream kinases that account for AP-1 and NF-κB activation, respectively. After TNFR stimulation, these pathways are concomitantly activated and share common regulatory proteins until the level of MAP3Ks but at this point they diverge (Fig. 8T). The outcome of receptor engagement on the biological level ranges from cell survival to cell death and includes influences on gene expression, differentiation, adhesion, cell metabolism and motility [90]. Importantly, activation of IKK proceeds faster than the onset of MAPK, which accounts for the tight temporal separation of IKK and MAPK signaling and is therefore the basis for the ability to control the life-or-death decision in TNFα induced cells. Two of the main MAP3Ks that account for JNK activation are TAK1 and MEKK1. TAK1 is a MAP3K that was initially found activated upon TGFβ treatment [96] and

studies of TAK1$^{-/-}$ fibroblasts revealed defective NF-κB, JNK as well as p38 activation after TNFα stimulation [97]. In addition TAK1$^{-/-}$ B-cells show reduced proliferation, and ablated NF-κB activation after Toll-like-receptor (TLR) stimulation [97], TAK1-deficient T-cells exhibit impaired JNK as well as NF-κB signaling, but neither B nor T-cells show any response after TNFR family member-activation [98]. Based on this, the role of TAK1 is receptor as well as cell-type dependent. On the molecular level, TAK1 interacts with the TAK-binding proteins 1 and 2 (TAB1/2) [99, 100], which facilitate binding to TRAF6, finally allowing the recruitment to the receptor complex via interaction with RIP.

MEKK1 is a 196kD protein with dual function. It displays catalytic activity, which is attributed to the kinase domain on its C-terminus, as well as E3-Ligase activity, due to a ring finger motif at the N-terminus. The kinase activity is directed against downstream targets as MKK 3, 4, 6, and 7, MAP2Ks that mediate p38 and JNK activation [101]. The ring finger domain of MEKK1 mediates K-63-linked autoubiquitination of the protein after CD40 receptor stimulation in B-cells, stabilizing its interaction with other proteins [102]. Intriguingly, self-ubiquitination of MEKK1 strongly depends on an intact kinase domain and on the presence of TRAF2 as well as the E2-Ligase Ubc13 [102, 103]. It has to be noted that the recruitment of MEKK1 to the membrane-associated TNFR-superfamily-member-CD40-receptor complex is dependent on IKKγ but not on autoubiquitination [103]. In this case K63-linked ubiquitination is subsequent to the dissociation of the protein from the receptor complex. Basically, MEKK1 is implicated in TNF-mediated signaling to JNK which was unraveled by the careful analysis of cells obtained from MEKK1$^{-/-}$ mice, showing that MEKK1 plays an important role in TNF signaling, as activator of both p38 and JNK, but has no effect on IKK and NF-κB activation [104-107]. The signaling mechanism that forms the basis for the concomitant onset of MAPK and IKK-Ks, was well described recently by a study carried out in CD40 induced B-cells (Fig. 9T) [103]. Both, CD40 as well as TNFR1, belong to TNFR superfamily of proteins that transmit signals by utilizing TRAF proteins as intermediate adaptors to phosphorylate downstream effectors. Upon CD40 receptor engagement the TRAF-proteins TRAF2 and TRAF6 undergo K63-linked autoubiquitination, which promotes the recruitment of the E3-ligases cIAP1/2, as well as TRAF-3 and the MAP3Ks MEKK1 and TAK1, resulting in a receptor-associated signaling complex. Both TRAF2 and TRAF6 exhibit E3-Ligase activity and mediate the K63-linked ubiquitination of cIAP1/2 resulting in their activation and the subsequent K48-linked ubiquitination of TRAF3 targeting this adaptor for proteasomal degradation. This destroys the "brake" and results in the release of the signaling complex into the cytosol.

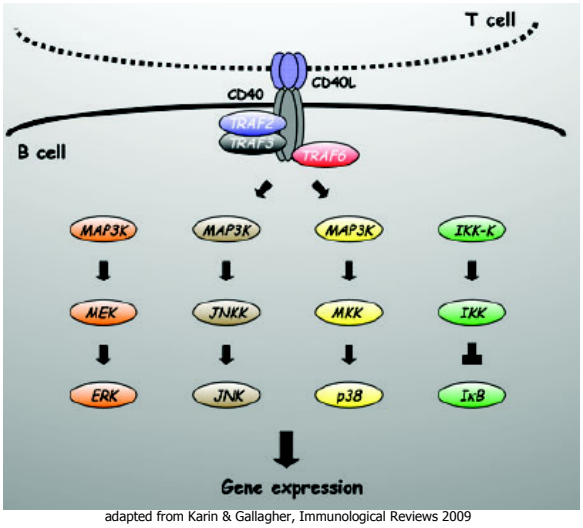

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Fig. 8T: TNFR-induced, concomitant activation of MAPK and NF-κB signaling. Receptor engagement leads to TRAF-recruitment and facilitates activation of MAP3Ks. Subsequent phosphorylation events result in either MAPK or IKK activation.

The "translocation"-step is prerequisite for MEKK1 and TAK1 autophosphorylation leading to JNK and p38 MAPK activation. This so called "two-stage-activation mechanism" is described as specific feature of MAPK activation and does not hold true for the onset of the IKK complex. The signalosome is activated as soon as receptor recruitment occurs and is released to the cytosol independent of cIAP1/2 and the proteasome [103]. This timed difference between a "two-step MAPK" activation, which occurs with much slower kinetics compared to the rapid induction of the IKK-NF-κB pathway, ensures in the first instance the onset of a "cell-survival-programme" and concomitantly prevents prolonged p38 and JNK activation which promote the "cell-death-programme". Therefore, the tightly controlled balance between NF-κB and MAPK signaling is indispensable for the requested response to TNFR stimulation. TNFα represents therefore a proinflammatory cytokine with the additional optional capacity to induce cell death.

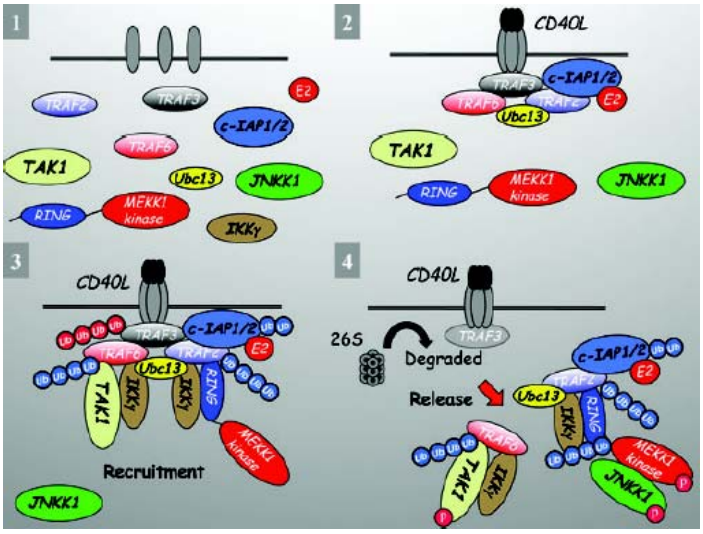

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Fig. 9T: CD40-receptor engagement induces "two-stage" MAPK signaling. 1) MAPK-signaling members in the uninduced state. 2) TNFR ligation recruits TRAF2, 3, 6 to the receptor. 3) TRAF2 and TRAF6 are autoubiquitinated resulting in cIAP1/2 ubiquitination and activation. MEKK1 and TAK1 are recruited via the inhibitor of nuclear factor κB kinase γ (IKKγ); TRAF3 acts as "brake". 4) cIAP1/2 mediate K48-linked ubiquitination of TRAF3 leading to its degradation and the release of TRAF2-MEKK1 and TRAF6-TAK1 signaling complexes to the cytosol. Complex transclocation is prerequisite for proper activation of signaling cascades leading to the onset of p38 and JNK.

Here we show that TTP attenuates NF-κB activity independently from its ability to destabilize mRNAs in human and murine cells. We demonstrate that TTP interferes with NF-κB activation at least in part by impairing nuclear translocation of p65 leading to the selective regulation of NF-κB dependent gene expression. Furthermore, we find TTP implicated in the TNFα induced onset of apoptosis, which is most probably regulated by the interplay with MEKK1 and the JNK signaling cascade. These findings reveal a previously unknown "dual-function" of TTP and suggest that this protein might play a broader role in the regulation of diverse cellular processes.

3. RESULTS

3.1. Tristetraprolin is an immediate early inducible, hyperphosphorylated, cytoplasmic protein in HUVEC

The endothelium acts as a critical barrier between blood stream containing immune cells and the underlying tissues and therefore constitutes an important hallmark in the regulation and control of inflammatory processes. The disturbance of its functional integrity in response to mechanical, chemical or immunologic injuries promotes both local as well as systemic inflammation. Therefore, the tight genetic and molecular regulation is of great importance to facilitate a quick and appropriate response to injury.
In search of potential molecular contributors to the regulation of inflammation in HUVEC, we found that the mRNA binding and degrading protein Tristetraprolin is induced by the proinflammatory agents LPS, IL-1 as well as TNFα in endothelial cells (Fig. 10T). LPS treatment of HUVECs resulted in the accumulation of TTP mRNA reaching a maximum 90 minutes after stimulation (Fig. 10T, left) whereas IL-1 as well as TNFα induction lead to a quite rapid response within 30 minutes, followed by the fast drop of TTP mRNA levels reaching the uninduced basal levels after around 150 minutes of cytokine-treatment (Fig. 10T, middle and right).

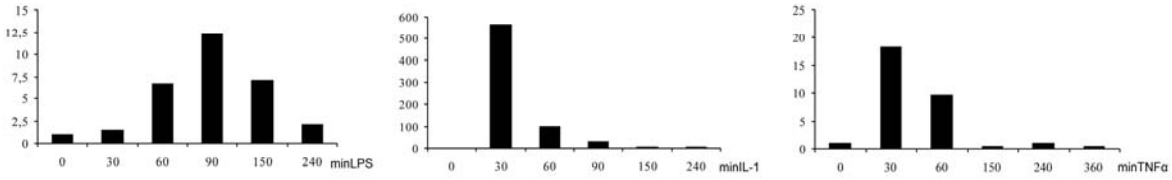

Fig. 10T: Tristetraprolin exhibits immediate early kinetics induced by proinflammatory agents in HUVECs. Real-time PCR analysis of LPS (left), IL-1 (middle) and TNFα (right) induced HUVECs after indicated time points is shown. Depicted are relative TTP expression levels normalized to β2-microglobulin.

Subsequently, we analyzed TTP protein levels in TNFα induced HUVECs and found that it mimics mRNA behaviour in terms of rapid immediate induction. Interestingly, whereas TTP mRNA levels declined quite fast, the protein accumulated over a period of around 3 hours (Fig. 11T, A). Importantly, we observed a molecular weight shift of the protein on the Western Blot which has been previously attributed to phosphorylation. Obviously, this posttranslational modification is associated with the accumulation of TTP in HUVEC, which could be indicative of stabilization (Fig. 11T, A, bottom panel). Interestingly, protein levels started to decrease after 3 hours of induction and decreased below detection limit after 6 hours;

this decline could be prevented by the addition of the proteasome inhibitor MG132 (Fig. 11T, B, 180 min time point). To determine the subcellular localization of TTP and therefore its potential site of action we analyzed cytoplasmic and nuclear fractions of HUVECs after TNFα stimulation. We could not detect any TTP protein in the nucleus, neither under unstimulated nor under induced conditions (Fig. 11T, C).

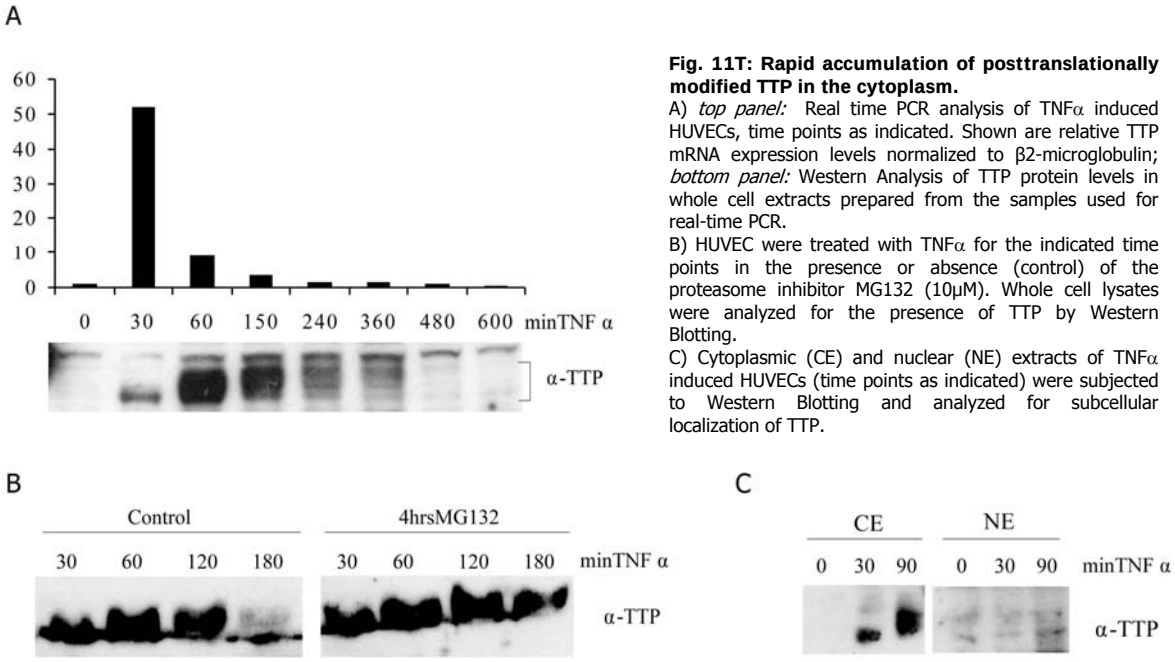

Fig. 11T: Rapid accumulation of posttranslationally modified TTP in the cytoplasm.
A) *top panel:* Real time PCR analysis of TNFα induced HUVECs, time points as indicated. Shown are relative TTP mRNA expression levels normalized to β2-microglobulin; *bottom panel:* Western Analysis of TTP protein levels in whole cell extracts prepared from the samples used for real-time PCR.
B) HUVEC were treated with TNFα for the indicated time points in the presence or absence (control) of the proteasome inhibitor MG132 (10μM). Whole cell lysates were analyzed for the presence of TTP by Western Blotting.
C) Cytoplasmic (CE) and nuclear (NE) extracts of TNFα induced HUVECs (time points as indicated) were subjected to Western Blotting and analyzed for subcellular localization of TTP.

So-called immediate early response genes (IEGs) are generally known to be activated transiently and rapidly in response to a wide variety of cellular stimuli and were found to be regulators of different cellular processes including cell growth and differentiation. Examples include transcription factors, secreted proteins, cytoskeletal proteins as well as receptor subunits.

In the context of inflammation the early synthesis of proteins functions as gateway to the activation of late response genes, which is indispensable for a proper, timed resolution of the process in order to prohibit prolonged, chronical inflammation.

Since most proinflammatory signals activate cascades leading to the onset of the transcription factor NF-κB we aimed to determine whether the immediate early gene TTP is able to act as a negative feedback regulator of the TNFα mediated NF-κB-response.

3.2. Tristetraprolin impairs NF-κB/p65 nuclear translocation [108]

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Tristetraprolin impairs NF-κB/p65 nuclear translocation*

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Running head: TTP impairs p65 nuclear translocation

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Tristetraprolin (TTP) is a prototypic family member of CCCH-type tandem zinc-finger (TZF) domain proteins that regulate mRNA destabilization in eukaryotic cells. TTP binds to AU-rich elements (AREs) in the 3´-UTR of certain mRNAs, including TNFα, GM-CSF and Ier3, thereby facilitating their ARE-mediated decay (AMD). Expression of TTP is upregulated by a variety of agents including inflammatory mediators such as TNFα, a prominent activator of the nuclear factor (NF)-kappaB (NF-κB) family of transcription factors. Accordingly, TTP is involved in the negative feedback regulation of NF-κB through promoting mRNA degradation. We describe here a novel, AMD-independent function of TTP on the termination of NF-κB response: TTP suppresses the transcriptional activity of NF-κB dependent promoters independent of its mRNA destabilizing property. In TTP knock out (k.o) mouse embryonic fibroblasts (MEF) lack of TTP leads to enhanced nuclear p65 levels, which is associated with the upregulation of specific, ARE-less NF-κB target genes. We find that attenuation of NF-κB activity is at least in part due to an interference of TTP with the nuclear import of the p65 subunit of the transcription factor. This novel role of TTP may synergize with its mRNA degrading function to contribute to the efficient regulation of proinflammatory gene expression.

Tristetraprolin (TTP) was first identified in the late 1980ies as an immediate early gene rapidly induced by phorbol 12-myristate 13-acetate (PMA) (1-3), and a variety of other stimuli including serum, insulin, and growth factors (4-7). The finding that this phosphoprotein is composed of a tandem zinc-finger structure, as well as its reported nuclear localization, initially led to the assumption that it might function as a transcriptional regulator (8-10). However, investigations over the last decade have demonstrated that TTP binds to and accelerates the degradation of mRNAs exhibiting high turnover rates (e.g. cytokines, growth factors or proto-oncogenes) (11,12). These mRNAs are characterized by AU-rich element (ARE) motifs in their 3'-UTRs that serve as docking sites for RNA binding proteins. TTP was shown to bind a subset of these AREs, and the nonamer UUAUUUAUU was identified as the minimal complete binding site (13). The motif is recognized by the tandem CCCH-type zinc-finger domain of TTP, and the integrity of this domain has been shown to be indispensable for mRNA degradation, because a single mutation within either zinc-finger abolishes the ARE binding activity (14). TTP exhibits no enzymatic activity itself but recruits various components of the basic RNA decay machinery via its N- and C-terminal domains (15,16). The degradation of ARE containing mRNAs is regulated mainly by extracellular cues. Typically, TTP target mRNAs are rapidly degraded in unstimulated cells, but become transiently stabilized in response to activation. Several signaling pathways were shown to be involved in the control of AMD including p38-MAPK-MK2, which accounts for direct phosphorylation of mouse TTP in response to LPS. Importantly, this phosphorylation stabilizes the TTP protein but abrogates its ability to degrade mRNAs, resulting in the accumulation of the known target TNFα (17-19). TNFα, a principal mediator of inflammation in acute and chronic inflammatory diseases (20), is upregulated in TTP k.o mice, leading to a complex syndrome of inflammatory arthritis (21). Likewise, mutations in TTP have been associated with autoimmune diseases in humans (22). In addition to TNFα, the turnover of several other mRNAs is controlled by TTP, including GM-CSF, Cox-2, Ier3 as well as the interleukins -2, -3, -6 and -10 (11,12,23,24). One of the main signaling pathways triggered by TNFα is the NF-κB pathway. NF-κB comprises a family of transcription factors (p65 (RelA), c-Rel, RelB, p50 (NF-κB1), p52 (NF-κB2)) that form homo- and heterodimers, as well as the inhibitory subunits IκBα, -β, -ε. Engagement of TNF receptor superfamily members leads to

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

the activation of the classical NF-κB signaling cascade via recruitment of adaptor proteins (FADD, TRADD and TRAFs) to the cytoplasmic part of the receptor complex (25). The TRAF/RIP complex subsequently recruits and activates TAK1, leading to further activation of the IκB kinase (IKK) complex (26). IKKs in turn phosphorylate IκBs, thus promoting their ubiquitination and subsequent degradation by the 26S proteasome. This results in unmasking of the NF-κB/p65 nuclear localization signal (NLS), enabling its nuclear translocation. p65 contains a classical monopartite NLS, which is characterized by 4-6 basic amino acid residues, and it has been shown that the karyopherin importin α3 specifically binds the p65-NLS (27), whereas importin β is responsible for docking to the nuclear pore complex (NPC). Nevertheless, it is not well established so far how nuclear shuttling of the known diversity of transcription factors is selectively timed and regulated, but the variety of differently structured nuclear localization signals is known to contribute to the specific recognition by various karyopherins (28,29).

Beside the tight regulation of p65 nuclear import, further activation of NF-κB is achieved through post translational modifications. For example, phosphorylation of serine-threonine residues in either the N-terminal Rel homology domain (RHD) of p65, which accounts for dimerization and DNA binding, or the C-terminal transcriptional activation domains (TA1 and TA2), is linked to enhanced binding of co-activator proteins, increased nuclear localization and stability, as well as to supporting the connection with the basal transcriptional apparatus (reviewed in (30)). In addition, other signaling pathways and transcription factors, such as activator protein-1 (AP-1), cyclic adenosine monophosphate responsive element-binding protein (CREB) or CCAAT enhancer binding protein (C/EBP) often act in cooperation with NF-κB to contribute to the expression of inflammatory genes. Furthermore, the recruitment of nuclear co-activators, mainly acting on chromatin relaxation (e.g. the histone acetyltransferases CREB binding protein (CBP) and p300 and the nuclear kinase MSK1), is a critical step in building an "enhanceosome" to modulate general levels of gene expression (31). We show here that TTP attenuates NF-κB activity independently from its ability to destabilize mRNAs in human and murine cells. Further we demonstrate that TTP interferes with NF-κB activation at least in part by impairing nuclear translocation of p65 leading to the selective regulation of NF-κB dependent gene expression. These findings reveal a previously unknown function of TTP and suggest that this protein might play a broader role in the regulation of diverse cellular processes.

Experimental Procedures

Plasmid Constructs - Reporter plasmids 5xNF-κB-luc, p53-luc, NFAT-luc, pFR-luc were obtained from Stratagene (PathDetect). Il-8-luc, the truncated version of the IL-8 promoter, TF-luc as well as expression vectors for p65, IKK2 and TAK1 have been described elsewhere (32-34). TAB1 was purchased from Invitrogen. The Gal4-p65 mutant constructs (p65*: p65 aa286-551; M1: p65 aa286-520, M2: p65 aa521-551; M3: p65 aa450-520) were kind gifts from L. Schmitz (35) as well as the pRC/CMVp65 plasmid used for generation of p53NLSΔNLSp65: briefly, a synthetic p53NLS linker (5'*HindIII*-3'*NcoI*) was fused to the N-terminus of p65. The p65 NLS sequence was excised by *BstEII-AleI* restriction and exchanged with the corresponding fragment from the pCDNA3.1/Myc-Hisp65NLSmut vector kindly provided by R. Fagerlund (27). The expression plasmids for SV40NLSp65 and SV40NLSΔNESp65 were gifts from Shao-Cong Sun (36). Human mycTTP was generated by PCR from the RZPD clone IRAK p961M0614Q2 and ligated into the *EcoRI/SalI* sites of pCMV-myc (Clontech). MycTZF (aa97-173) and mycC124R were generated by subcloning and in vitro mutagenesis, respectively. Oligonucleotide sequences are shown in Supplementary Table 1. The integrity of all constructs generated by PCR was confirmed by sequencing. Expression plasmids for TRAF2, CBP/p300 and pRSGFP-C1-NFATc were kindly provided by H. Wajant, C.Brostjan and L.Gerace, respectively.

Cell culture and transfections - HEK 293 cells and HeLa cells were obtained from ATCC, wild-type (wt) and TTP k.o MEF were kindly provided by PJ Blackshear. Cells were cultured in DMEM (Bio-Whittaker) supplemented with 10% FCS (Sigma), 2mM L-glutamine (Sigma) penicillin (100units/ml) and streptomycin (100µg/ml). Human umbilical vein endothelial cells were isolated from umbilical cords as described (37) and maintained in M199 medium (Lonza) supplemented with 20% FCS (Sigma), 2mM L-glutamine (Sigma), penicillin (100U/ml), streptomycin (100µg/ml) and 0.25µg/ml fungizone, 5U/ml Heparin and 25µg/ml ECGS (Promocell). HEK 293 cells were transfected by the calcium phosphate method as described (38). MEF as well as HUVEC were transfected using the polyethylenimine (PEI) method (39).

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

siRNA mediated knock down of TTP was performed using the Stealth® custom siRNA pool for TTP (Invitrogen). The sequences are given in Supplementary Table 1.

Reporter Gene Assays - HEK 293 cells were grown in 24-well plates, MEF and HUVEC in 12-well plates. Cells were transfected as described with the indicated reporter and/or expression plasmids using 4µg total DNA. Cells were stimulated with TNFα (10ng/ml) for 16hrs prior to harvesting. All experiments were performed in triplicates, and are representative for at least three independent experiments. Luciferase values were normalized for cotransfected β-gal or GFP and are illustrated as mean fold induction. Error bars represent standard deviation of the mean.

Statistical Significance Calculations - Differences between samples were analyzed using the paired Student's T-Test. Two-tailed probability values of <0.05 and <0.01 were considered significant and highly significant, respectively. p-values are given within the figure legends.

Cell extracts, Western Blotting & Densitometry - For p65 nuclear translocation and IκBα degradation, cells were grown and transfected in 6cm (HEK 293) or 10cm dishes (MEF). 48hrs post transfection cells were stimulated with 10ng/ml TNFα (mouse or human rTNFα, R&D systems) for the indicated time points. Cells were harvested and nuclear extracts were prepared as described elsewhere (40). For determination of total p65, cells were lysed in 1xpassive lysis buffer (Promega). Proteins were separated by 10% SDS-PAGE, transferred electrophoretically onto a nitrocellulose membrane (Hybond-C, Amersham) and blocked in 5% non fat dry milk in PBS/0,1% Tween-20 for 1h at room temperature before incubation with the first antibody over night (4C). The following antibodies were used: α-myc (sc-40), α-β-actin (sc-1616), α-p65 (sc-109) α-IκBα (sc-203), and α-SP1 (sc-59), all obtained from Santa Cruz Biotechnology, α-GAPDH was from Chemicon (MAB 374). The α-TNFα blocking antibody (R&D systems, AF-410-NA) was used at a concentration of 5µg/ml. The α-TTP antibody was kindly provided by P. Kovarik. HRP-conjugated antibodies α-rabbit IgG, α-mouse IgG (Amersham) and α-goat IgG (Santa Cruz) were used as secondary reagents. Blots were developed using chemiluminescence (West Pico, Pierce & ECL Detection Reagents, Amersham). Densitometric analysis of nuclear p65 levels was done using the background-corrected integrated densities of the p65 bands normalized to the SP-1 bands assessed by the ImageJ software freely available at http://rsb.info.nih.gov/ij/.

Immunocytochemistry - Cells were seeded onto fibronectin-coated Lab Tek II Chamber Slides (Nunc), transfected with either empty vector or mycTTP and 24hrs later stimulated with TNFα (20ng/ml) for the indicated times. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining of p65 was done using the rabbit-α-p65 antibody (sc-109; Santa Cruz Technology; 1:500), mycTTP was detected with mouse-α-myc antibody (sc-40, Santa Cruz) followed by goat-α-rabbit Alexa 488 (molecular probes) and APC goat-α-mouse (BD Biosciences) secondary reagents at dilutions of 1:5000 and 1:500, respectively. For NFAT translocation, cells were transfected with GFP-NFATc and mycTTP expression vectors as described above and stimulated with Ionomycin (500mM final conc.; Sigma).

Real time PCR **-** Total RNA was isolated from either HEK 293 cells or MEF grown in 6-well plates using the Roche High Pure RNA Isolation Kit, according to the manufacter's protocol. 1µg RNA was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems) with random hexamers and MuLV reverse transcriptase following the manufacter's recommendations. Real time PCR was performed in a LightCycler (Roche) using SYBR green for detection. Experiments were done in triplicates, the relative amount of mRNA was calculated using the Pfaffl method and normalization to β2-micoglobulin. Error bars represent standard deviation of the mean. Primer sequences are given in Supplementary Table 1.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

Results

TTP specifically attenuates NF-κB dependent transcription. Our investigations of negative feedback regulators and potential contributors to the resolution phase of the inflammatory process resulted in the observation that TTP has the capacity to suppress the transcriptional activity of different promoters of inflammatory genes. TTP reduced basal as well as TNFα-stimulated luciferase expression driven by the human IL-8 (Fig. 1A) or Tissue Factor (TF) promoter (Fig. 1B) in HEK 293 cells. These promoters contain binding sites for various transcription factors including C/EBP, AP-1, SP-1 and NF-κB. In order to gain mechanistic insight into which transcription factor or signaling pathway was affected by TTP, we used a truncated version of the IL-8 promoter containing only one NF-κB site ~100bp upstream of the transcriptional start. This modified promoter responded to TTP similarly as did the full length promoters (Fig. 1C), suggesting that the NF-κB site accounted for TTP sensitivity. Consequently, we analyzed a minimal artificial promoter containing a multimerized NF-κB binding site and observed that it was also potently inhibited by TTP (Fig. 2A). These findings could be reproduced in other cell types such as HeLa and human umbilical vein endothelial cells (HUVEC; data not shown). Conversely, the lack of TTP generates conditions of a "pre-activated" state in knock out fibroblasts, reflected by a ~10 fold higher basal NF-κB level in TTP deficient cells (Fig. 2B, left). Furthermore, knock-down of endogenous TTP in HUVEC by RNA interference enhanced NF-κB activity (Fig. 2B, right) recapitulating the situation observed in TTP k.o MEF. Similar results were obtained in HEK 293 cells (Sup. Fig. 1A). The functionality of the siRNA was verified on the mRNA (Fig. 2C) and protein level (Sup. Fig. 1A, bottom panels). In addition, reconstitution of TTP expression by transient transfection into TTP k.o cells resulted in a 5xNF-κB activity that was comparable to that observed in WT cells (Sup. Fig. 1B). Importantly, TTP did not decrease the basal and induced transcriptional activity of either minimal SP-1 or p53 or NFAT dependent promoters, as shown in Fig. 2D, demonstrating specificity and ruling out possible effects on the reporter plasmids. Additionally, we did not observe any influence of TTP on the degradation of luciferase mRNA (Sup. Fig. 1C). Together, this demonstrates that TTP specifically interferes with transcriptional activation triggered by the transcription factor NF-κB.

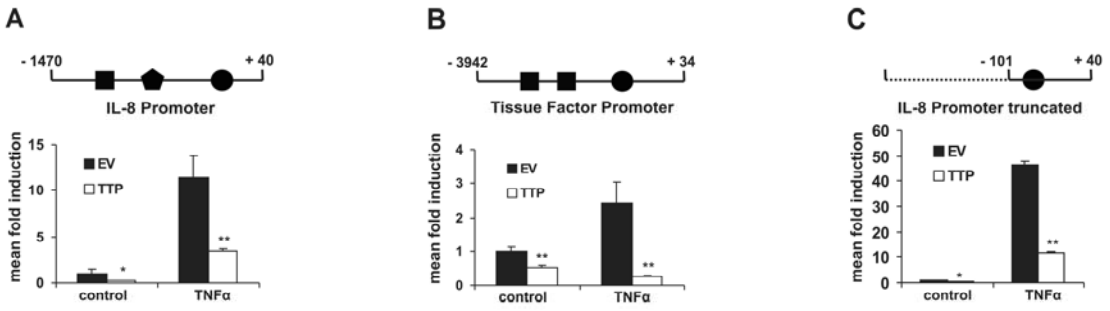

Fig. 1: TTP attenuates NF-κB activity. HEK 293 cells were transfected with luciferase reporter genes driven by either the IL-8 promoter (A), the Tissue Factor promoter (B), or a truncated version of the IL-8 promoter (C) as shown schematically on top (● represents NF-κB ■ represents AP-1 and ⬟ represents C/EBP transcription factor binding sites). Luciferase activity was analyzed before (control) and after TNFα stimulation (10ng/ml, 16hrs) in the absence (EV, empty vector) or presence of coexpressed TTP and normalized to the activity of cotransfected β-Galactosidase (β-Gal). Mean fold induction was calculated in relation to unstimulated conditions (control). * P<0.05, ** P<0.01, compared to the respective EV sample.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

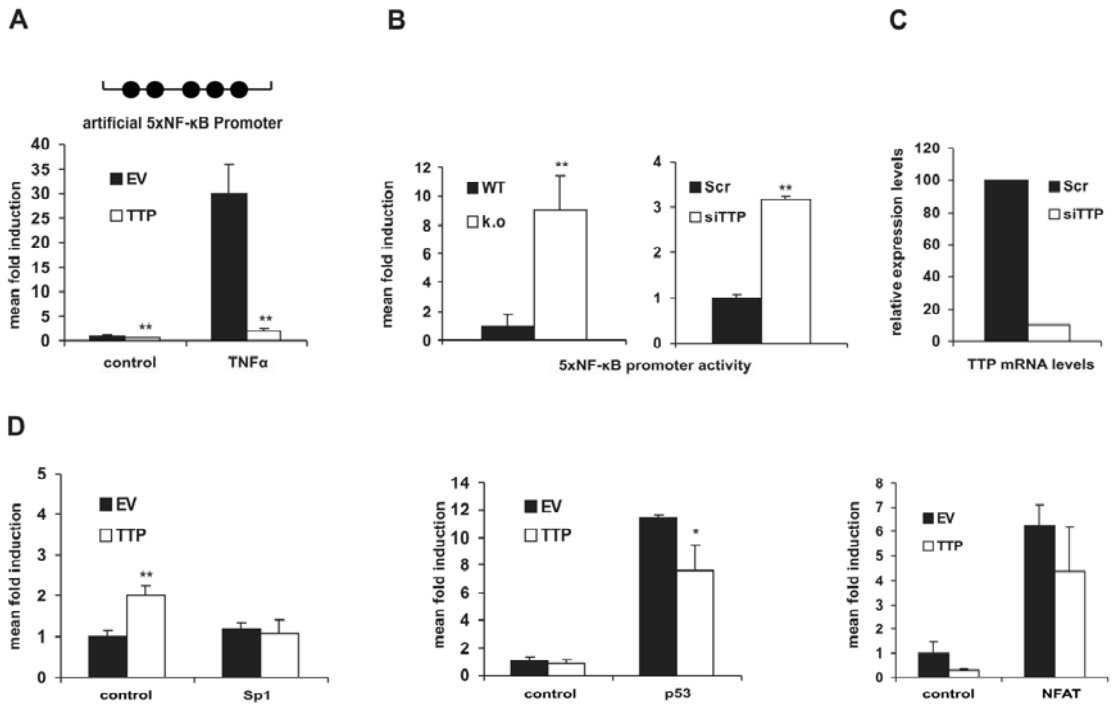

Fig. 2: Specificity towards NF-κB activity. A) HEK 293 cells were transfected with a luciferase reporter gene driven by a minimal NF-κB promoter containing 5 NF-κB binding sites (5xNF-κB-luc; scheme on top) and luciferase activity was analyzed as described in Fig. 1. ** P<0.01, compared to the respective EV samples. B) Lack of TTP triggers NF-κB activity. Left: 5xNF-κB promoter activity was analyzed in wild type (wt) versus TTP knock out (k.o) MEF. Luciferase levels were normalized to cotransfected β-Gal. Right: human umbilical vein endothelial cells (HUVEC) were transfected with the 5xNF-κB-luc reporter along with a siRNA directed against TTP (siTTP) or a scrambled control (Scr) and luciferase activity normalized to coexpressed GFP was assessed 48hrs post transfection. ** P<0.01, compared to wt (left) or the scrambled control (right). C) Real time PCR analysis of TTP mRNA levels normalized to β2-microglobulin in HUVEC 48hrs post transfection with either siTTP or the scrambled control (Scr) used in (B). A representative experiment is shown. D) TTP does not bias NF-κB independent promoters. HEK 293 cells were transfected with either a SP-1 (left), a p53 (middle) or a NFAT promoter-dependent (right) luciferase reporter gene in the presence (TTP) or absence of TTP (EV). Luciferase activities were analyzed in the absence (control) or presence of cotransfected SP-1, p53, or NFAT expression plasmids, respectively. * P<0.05, ** P<0.01, compared to the respective EV samples.

TTP interferes with NF-κB signaling downstream of the IKK signalosome, at the level of p65. Based on the results above we sought to examine whether TTP interferes with NF-κB signaling up- or downstream of the IKK signalosome. Activation of NF-κB by TRAF2 or TAK1/TAB1, acting upstream of the IKK complex, did not result in the abrogation of TTP mediated NF-κB inhibition (Fig. 3A). Moreover, TTP also potently inhibited IKK2- and even NF-κB/p65-mediated activation of the 5xNF-κB reporter in HEK 293 cells (Fig. 3B), suggesting it acts downstream or at the level of p65. In addition, overexpression of p65 did not impede TTP action in WT nor in reconstituted k.o MEF (Sup. Fig. 2A). Further examination of TTP effects on p65 induced IL-8 promoter activity (Sup. Fig. 2B) confirmed the results obtained for the artificial NF-κB promoter. Increasing amounts of p65 expression levels could not overcome the TTP-mediated inhibition (Fig. 3C) and in addition, attenuation of NF-κB activity occurred in a dose dependent manner (Fig. 3D). We observed 50% inhibition of p65-mediated luciferase activity at approximately 7ng cotransfected TTP. Since overexpression of p65 might bypass its natural regulation through the IκBs, we examined a possible influence of TTP on IκBα degradation. We found that TTP did not prevent TNFα induced IκBα degradation, neither in HEK 293 cells after overexpression (Fig. 3E, top), nor was it altered in TTP k.o compared with WT MEF (Fig. 3E, bottom).

Another important step prior to transcriptional activation by NF-κB is the recruitment of nuclear coactivators such as CBP/p300, or activating kinases like MSK1, into the transcriptional activation complex. Analysis of these nuclear signaling components in transient transfection experiments, using the respective expression vectors, revealed that neither of them could dampen the inhibitory TTP effect (Fig. 3F). To exclude that TTP affects p65 mRNA or protein, we analyzed these levels in HEK 293 cells after TTP overexpression (Sup. Fig. 2C, left) and in TTP k.o MEF (Sup. Fig. 2C, right) and could not detect significant differences.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

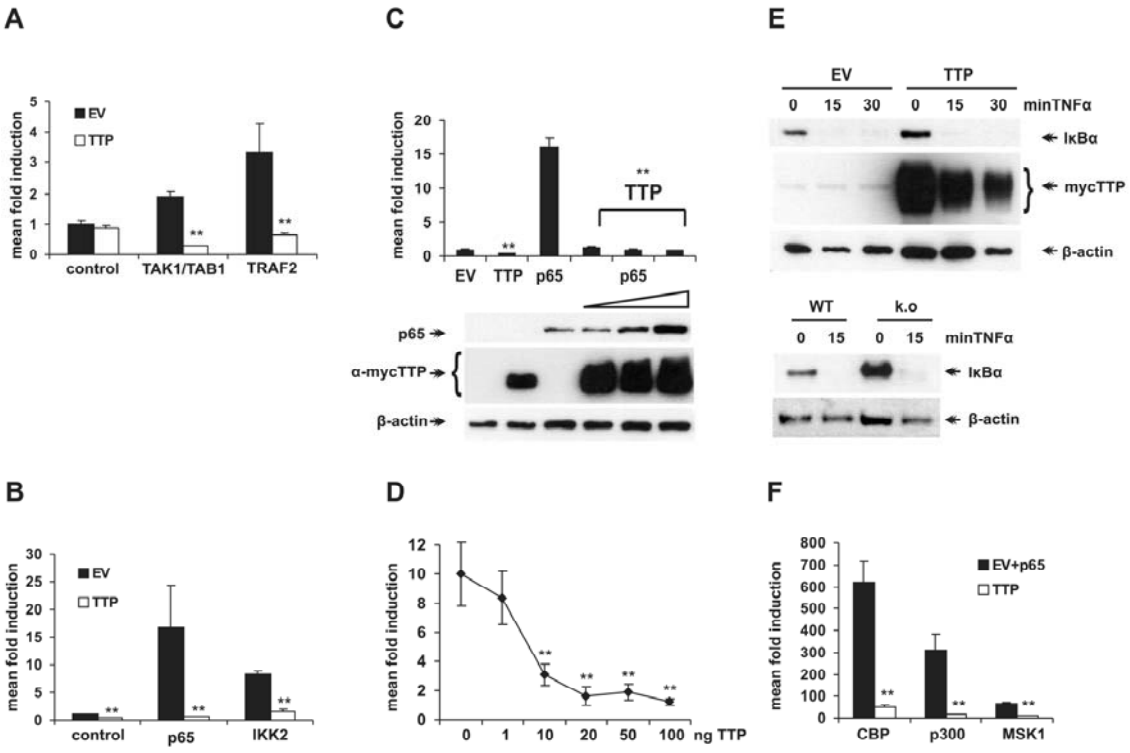

Fig. 3: TTP interferes with NF-κB signaling downstream of p65 transactivation. HEK 293 cells were transfected with 5xNF-κB-luc alone (control) or in combination with TAB1/TAK1, TRAF2 (A), p65 or IKK2 expression plasmids (B) and luciferase activities were analyzed in the absence (EV) or presence of coexpressed TTP. Mean fold induction was calculated in relation to basal 5xNF-κB activity (control). ** P<0.01, compared to the respective EV samples. C) Increased p65 levels do not compensate for the inhibition of NF-κB by TTP. HEK 293 cells were transfected with 5xNF-κB-luc either in the absence (EV) and presence of TTP (TTP) (** P<0.01, compared to the EV sample) or together with increasing amounts of p65 (0.1-0.4μg) and a constant amount of TTP (100ng) expression plasmids, as indicated. (** P<0.01, compared to the p65 induced sample). 5xNF-κB promoter activity was analyzed as above. Protein levels of p65 as well as myc-tagged TTP were assessed by Western Blotting, β-actin represents the loading control (middle panels). D) TTP reduces NF-κB activity in a dose-dependent manner. HEK 293 cells were transfected with increasing amounts of TTP as indicated (1-100ng) in combination with a constant amount (0.1μg) of p65 and 5xNF-κB activity was determined as described above. ** P<0.01, compared to the sample without TTP coexpression. E) IκBα degradation is not affected by TTP. Top: HEK 293 cells were transfected with either empty vector (EV) or myc-tagged TTP and stimulated with TNFα (10ng/ml) for the indicated time points followed by preparation of cytoplasmic extracts. IκBα degradation (upper panel) as well as ectopic TTP expression (middle panel) was assessed by Western Blotting. β-actin was used as loading control (lower panel). Bottom: Wild type (wt) and knock out (k.o) MEF were stimulated with TNFα (10ng/ml) for 15 minutes and cytoplasmic extracts were subjected to Western Blot analysis for IκBα degradation. β-actin served as loading control (lower panel). F) Nuclear p65-coactivators can not counteract TTP. HEK 293 cells were transfected with a 5xNF-κB reporter in combination with p65 (EV+p65), along with CBP, p300 or MSK1 expression vectors. Luciferase activities were analyzed in the presence (TTP) and absence of coexpressed TTP. Fold induction was calculated in relation to the basal reporter activity (not displayed). ** P<0.01, compared to the respective p65-induced EV samples.

The mRNA binding ability of TTP is dispensable for its inhibitory effect on NF-κB promoter activity. One simple explanation of our results could have been that TTP affects the stability of an ARE-containing mRNA that encodes a protein necessary for full NF-κB activation. Therefore we took advantage of the TTP mutant C124R (Fig. 4A), which is deficient in ARE binding and therefore cannot destabilize e.g. TNFα mRNA ((41-43) and Sup. Fig. 3A). TTP-C124R retained the capacity to inhibit 5xNF-κB activity, both after stimulation with cotransfected p65 (Fig. 4B), as well as under basal conditions (Fig. 4C). In contrast, the TTP construct comprising the zinc-finger domain only (TZF; Fig. 4A), which binds mRNAs efficiently (44) but fails to induce degradation (Sup. Fig. 3A), displayed no influence on luciferase activity (Fig. 4C).

This suggests that the ability of TTP to inhibit NF-κB is independent of its mRNA binding capacity, but requires the presence of the N- and/or C-terminal domains. As already observed for TTP (Fig. 2D), the C124R mutant did not affect SP-1- or p53-dependent promoter activities (Sup. Fig. 3B).

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

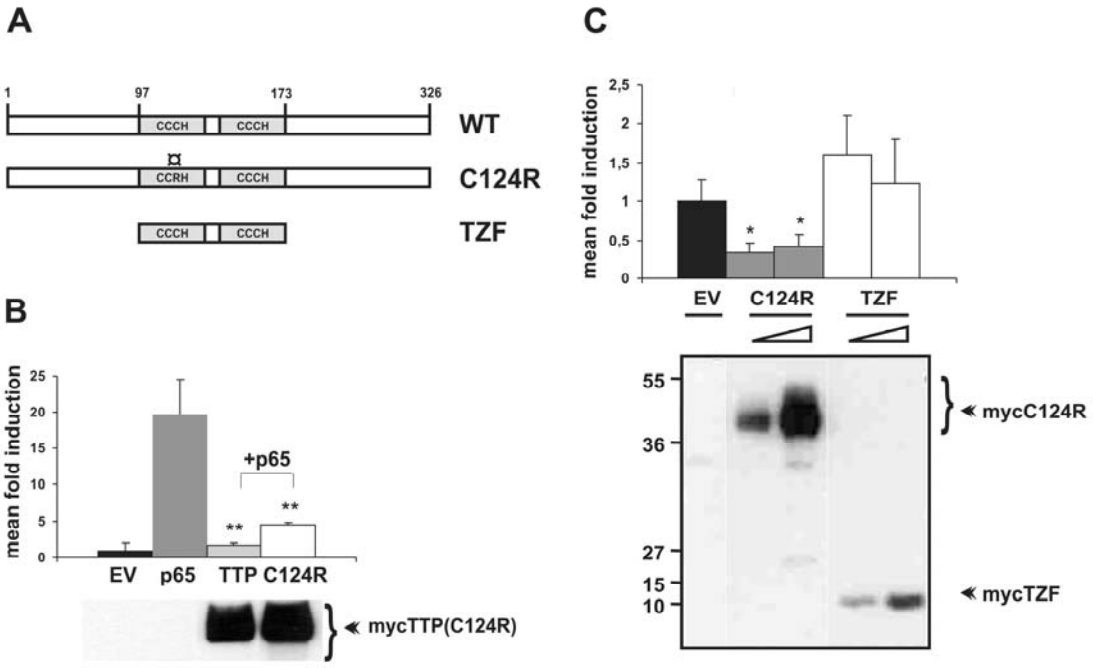

Fig. 4: Attenuation of NF-κB activity is independent of the mRNA binding ability of TTP. A) Schematic depiction of TTP constructs used in reporter gene assays. Numbers represent amino acid residues of human TTP. A single amino acid mutation within the first zinc-finger (C124R) completely abolishes RNA binding. TZF: tandem zing-finger. CCCH: cysteine-cysteine-cysteine-histidine zinc-finger structure; all proteins are N terminally linked to a myc-tag. B) HEK 293 cells were transfected with 5xNF-κB-luc alone (EV) or in combination with a p65 expression vector as indicated. The effect of coexpression of either TTP or the RNA binding mutant C124R on p65 stimulated 5xNF-κB promoter activity was monitored by analysis of luciferase expression levels as described above. Mean fold induction was calculated in relation to the empty vector sample. The bottom panel represents Western analysis of the same cell lysates and shows equal protein expression levels of both TTP and C124R by detection with a α-myc antibody. ** P<0.01, compared to the p65-induced sample. C) HEK 293 cells were transfected with 5xNF-κB-luc alone (EV) or in combination with increasing amounts of TTP-C124R or TTP-TZF expression plasmids. Luciferase activities were analyzed, and cell lysates subjected to Western Blotting (bottom panel) to verify expression of TTP constructs as described in (B). Numbers at the left side represent molecular weight in kDa. * P<0.05, compared to the EV sample.

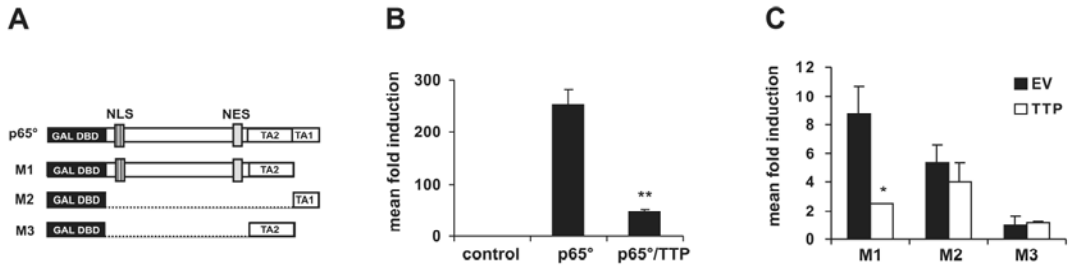

Fig. 5: The p65 region containing the NLS/NES motifs accounts for TTP sensitivity. A) Schematic representation of expression constructs containing different p65 domains fused to the yeast Gal4 DNA binding domain (Gal4DBD: Gal4 DNA binding domain; NLS: nuclear localization signal; NES: nuclear export signal; TA1 & TA2: transactivation domain 1 & 2; for detailed description see Experimental Procedures and (35). B) HEK 293 cells were transfected with the yeast based Gal4 reporter plasmid pFR-luc (control) together with p65° (see scheme (A)) to enable promoter activity, and the effect of TTP coexpression on p65° induced luciferase expression was analyzed (p65°/TTP). The impact of TTP coexpression on promoter activity induced by the p65 mutants M1-M3 (see scheme (A)) is depicted in (C). * P<0.05 compared to the respective EV sample, ** P<0.01, compared to the p65° induced sample.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

TTP impairs cytoplasmic-nuclear transition of p65. Having established that TTP inhibits NF-κB downstream or at the level of p65 we sought to gain further insight into its mode of action. Since one of the additional regulatory steps of NF-κB activation occurs at the level of transactivation, we utilized different hybrid constructs where parts of p65 are fused to the yeast Gal4 DNA binding domain (Fig. 5A). When we tested the ability of these constructs to influence Gal4 dependent luciferase expression we found that the exchange of the N-terminal Rel homology domain with the yeast Gal4 DNA binding domain (DBD) retained the inhibitory effect of TTP (Fig. 5B), indicating that neither p65 dimerization nor DNA binding was impaired. Further truncations of the p65 protein revealed that TTP could not suppress the activity of each of the two transactivation domains when they were fused directly to the Gal4 DBD. The only region of p65 that was sensitive to TTP was the central part, which contains the NLS as well as the nuclear export sequence (NES), suggesting that TTP may prevent import or expedite export of the transcription factor (Fig. 5C). Comparable results were obtained in HeLa cells (data not shown). To substantiate these data, we investigated the possible influence of TTP on p65 subcellular localization. Therefore we analyzed p65 protein levels in cytoplasmic and nuclear fractions of TNFα induced HEK 293 cells and found that the overexpression of TTP impaired nuclear accumulation of p65 (Fig. 6A, top). Additionally, and in line with previous reporter results (Fig. 2B, left; Sup. Fig. 1B), the nuclear localization of p65 was enhanced in TTP k.o MEF (Fig. 6B, top) supporting the notion of a "pre-stimulated" scenario. Although these cells do not express detectable amounts of TNFα ((43) and real-time PCR data not shown) we wanted to rule out that potential minute amounts of TNFα are the cause for enhanced NF-κB activity in k.o cells. Therefore, we performed this experiment in the presence of a neutralizing α-TNFα antibody. Furthermore, immunocytochemical analysis of HUVEC, transiently transfected with TTP, showed reduced translocation of endogenous p65 after TNFα stimulation (Fig. 6C; Sup. Fig. 4A); in contrast, shuttling of NFAT was not affected (Sup. Fig. 4B).

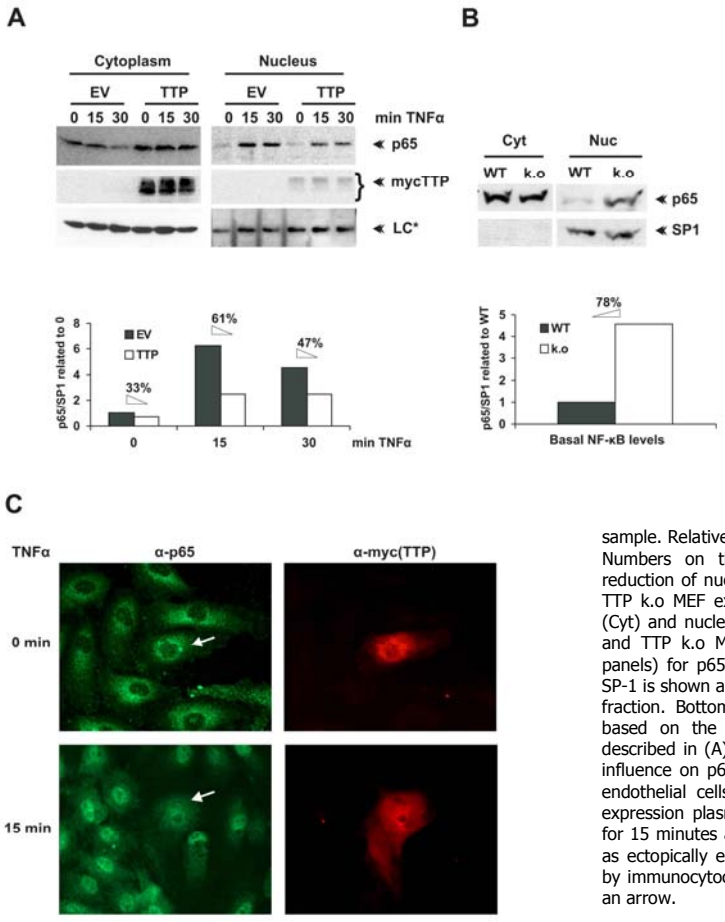

Fig. 6: TTP impairs p65 cytoplasmic-nuclear shuttling. A) Top: Western Blot analysis of endogenous p65 and myc-tagged TTP in cytoplasmic and nuclear extracts prepared from HEK 293 cells stimulated with TNFα for the indicated time points, 48hrs after transfection with empty vector (EV) or TTP (mycTTP expression plasmid); LC*: loading control, β-actin was used for the cytoplasmic and SP-1 for the nuclear fraction. Bottom: Densitometric analysis of nuclear p65 levels based on the Western Blot shown in the upper panel using the ImageJ software (for detailed information see Experimental Procedures). p65 levels were normalized to SP1 levels in the same sample. Relative p65 levels are shown in relation to time point 0. Numbers on the top of the bars represent the percental reduction of nuclear p65 levels in the presence of TTP. B) Top: TTP k.o MEF exhibit increased nuclear p65 levels. Cytoplasmic (Cyt) and nuclear (Nuc) extracts prepared from wild type (WT) and TTP k.o MEF were analyzed by Western Blotting (upper panels) for p65 levels in the presence of an α-TNFα antibody. SP-1 is shown as loading control and for the purity of the nuclear fraction. Bottom: Densitometric analysis of nuclear p65 levels based on the Western Blot on top. Analysis was done as described in (A). C) Immunocytochemical analysis showing TTP influence on p65 nuclear translocation in human umbilical vein endothelial cells (HUVEC). Cells were transfected with a TTP expression plasmid (mycTTP), stimulated with TNFα (20ng/ml) for 15 minutes and endogenous p65 (green, left panels) as well as ectopically expressed TTP (red, right panels) were detected by immunocytochemistry. TTP-transfected cells are indicated by an arrow.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

TTP impairs p65 nuclear import and affects the transcriptional onset of specific NF-kB target genes in an ARE independent manner. NF-κB/p65 was shown to constitutively shuttle between cytoplasm and nucleus under basal conditions (45). The abundance of regulatory proteins like IκBα, in addition to the appropriate composition of the nuclear pore complex, as well as the accessibility of NLS and NES, are indispensable for the proper timing of import and export. The NLS of p65 belongs to the monopartite class of leader-motifs (46,47) that further includes those of the SV40 T antigen and the human c-Myc. Recently it was described by Li and colleagues (48) that the transcription factor p53 shuttles through a specific nuclear import pathway that seems to be selective for the bipartite p53-NLS and different from the pathway used by the monopartite SV40 T antigen. Accordingly, we tested different p65-NLS/NES mutants, as summarized schematically in Fig. 7A, in reporter gene assays. The N-terminal fusion of the SV40NLS (PKKKRKV) to wild type p65 resulted in activation of 5xNF-κB activity similar in strength as the induction achieved with the WT protein, and in both cases TTP had a comparable inhibitory effect (Fig. 7B). In contrast, the fusion of the bipartite nuclear leader of p53 (see scheme Fig. 7A), that is distinguished from the monopartite class of p65 and SV40 nuclear localization signals, significantly abrogated the inhibitory effect of TTP (Fig. 7C). Mutation of the nuclear export signal of p65 (ΔNES) resulted in strongly enhanced promoter activity, but could nevertheless be suppressed by TTP (Fig. 7B). This suggests that TTP does not act via enhancing p65 export, but through regulation of nuclear import that is dependent on the nature of the p65 NLS. To study the biological consequences of altered NF-κB activity, we analyzed the levels of NF-κB dependent target genes in WT and TTP k.o MEF. To exclude any contribution of AMD, NF-κB target genes were selected with regard to the absence of any ARE in their respective mRNAs (adapted from http://people.bu.edu/gilmore/nfkb/target/index.html). Six NF-κB dependent genes, belonging to diverse groups of NF-κB targets were chosen (Table 1) and the absence of any AU-cluster was confirmed bioinformatically. They were analyzed by real time PCR for their relative expression levels in WT compared to TTP deficient MEF (Fig. 8). While MCP-1, also known as the macrophage chemotactic protein, as well as the antioxidant genes FTH1 and TXN2 were unaffected (Fig. 8, left graph), the chemokine RANTES as well as Gadd45β and TRAF1 were substantially upregulated in TTP k.o cells (Fig. 8, right graph).

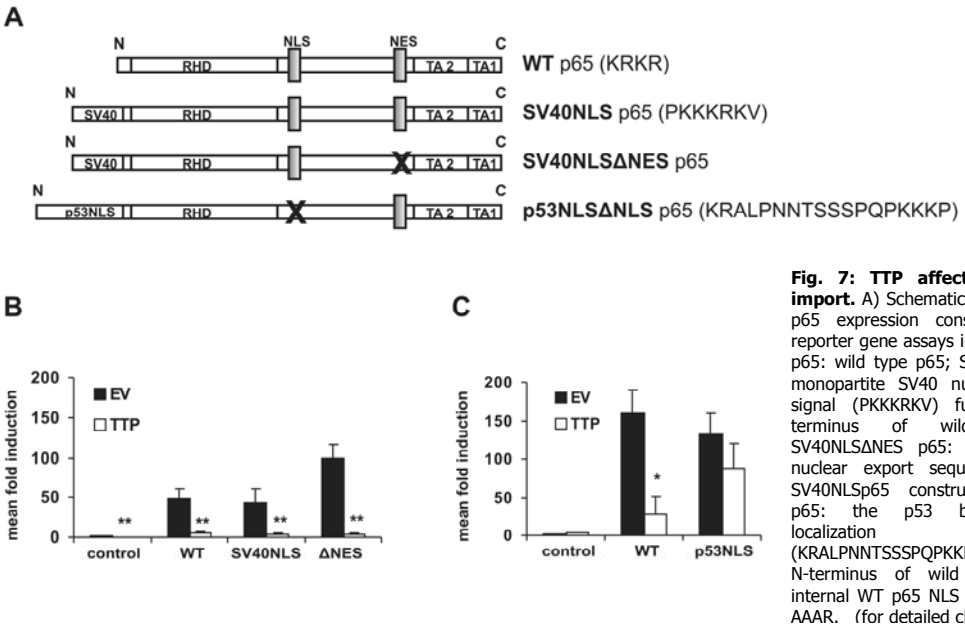

Fig. 7: TTP affects p65 nuclear import. A) Schematic representation of p65 expression constructs used for reporter gene assays in (B) and (C). WT p65: wild type p65; SV40NLS p65: the monopartite SV40 nuclear localization signal (PKKKRKV) fused to the N-terminus of wild type p65; SV40NLSΔNES p65: mutation of the nuclear export sequence within the SV40NLSp65 construct; p53NLSΔNLS p65: the p53 bipartite nuclear localization signal (KRALPNNTSSSPQPKKKP) fused to the N-terminus of wild type p65, the internal WT p65 NLS KRKR mutated to AAAR. (for detailed cloning information see Experimental Procedures).B & C) HEK 293 cells were transfected with 5xNF-κB-luc alone (control) or in combination with either wt p65 (wt), SV40NLSp65 (SV40NLS) and SV40NLSΔNESp65 (ΔNES) (B) or wt p65 (wt) and p53NLSΔNLSp65 (p53NLS) (C). Luciferase activities were analyzed in the absence (EV) or presence of coexpressed TTP. Fold induction was calculated in relation to the basal reporter activity (control). * P<0.05, ** P<0.01, compared to the respective EV samples.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

Table 1
Bioinformatic analysis of selected NF-κB target genes. NF-κB dependent target genes, involved in a variety of different cellular functions, were preselected from http://people.bu.edu/gilmore/nf-kb/target/index.html and analyzed for the absence of an AU-rich element (ARE) in the 3'-UTR of the respective mRNAs using the ARED database at http://brp.kfshrc.edu.sa/ARED/. Targets lacking an ARE-motif were further analyzed for the presence/absence of the minimal AU-pentamer AUUUA.
* mus musculus RefSeq; **ARED: AU-rich element database; the ARED organism search engine was used for preanalysis; *** AUUUA: minimal pentamer, core sequence of classI/II/III ARE clusters.

Gene	Human Gene Name	Function	RefSeq*	ARED**	AUUUA***
CYTOKINES & MODULATORS					
MCP-1 RANTES	CCL2 CCL5	macrophage chemotactic protein regulated upon activation normal t lymphocyte exressed and secreted	NM_011333 NM_013653	no no	1 pentamer no
STRESS RESPONSE GENES					
Ferritin heavy chain Thioredoxin	FTH1 TXN2	antioxidant redox-active protein/antioxidant	NM_010239 NM_019913	no no	no no
REGULATORS OF APOPTOSIS					
TRAF-1	TRAF1	TNF-receptor associated factor 1	NM_009421	no	1 pentamer
MISCELLANEOUS					
Gadd45beta	GADD45B	DNA repair/cell cycle control	NM_008655	no	no

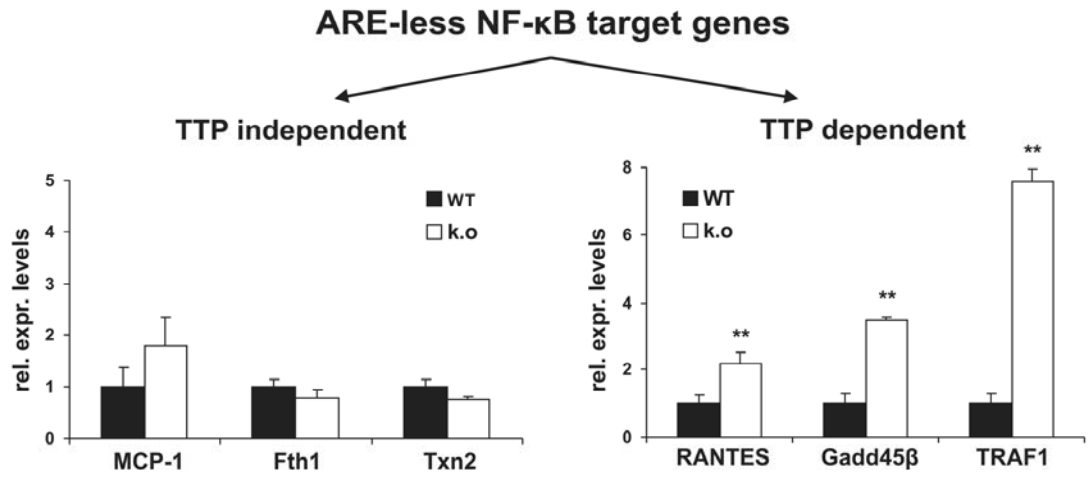

Fig. 8: TTP controls the expression of ARE-less NF-κB target genes. Real time PCR analysis of selected, ARE-less NF-κB dependent genes (Table 1) in wt and TTP k.o MEF. Relative expression levels were normalized to β2-microglobulin, the results shown are representatives of two independent experiments done in triplicates. ** P<0.01, compared to the respective wt samples.

Discussion

The notion that the zinc-finger protein Tristetraprolin might act as a negative regulator of transcription initially emerged more than 10 years ago, when the first suppressive effects on promoters were described (11). Nevertheless, this view has taken a back seat by the concomitant finding that TTP acts as mRNA destabilizing protein. However, evidence has accumulated that TTP might have a broader function. Examples include the influence on other RNA binding proteins, e.g. the KH-type splicing regulatory protein (KSRP) (49), as well as the proposed multifunctional activity in the control of glucocorticoid mediated gene expression (50). We describe here that TTP is involved in the negative transcriptional

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

regulation of promoters containing NF-κB binding sites in different cell types. TTP inhibits the induced activity of these promoters about 70-95% while the influence on the basal levels fluctuates below 50%. This can be explained by the fact that NF-κB constitutively shuttles between cytoplasm and nucleus (45) leading to a basal promoter activity with its magnitude being determined by the actual situation and condition of the cell. The negative regulatory role of TTP was further supported by the analysis of TTP deficient mouse embryonic fibroblasts as well as by siRNA mediated knock down of endogenous TTP in HUVEC.

TTP exhibited a dose dependent effect on NF-κB with only ~7ng of transfected TTP expression plasmid resulting in half-maximal reduction of promoter activity, an amount that was below our detection limit on Western Blots (whereas endogenous TTP could be detected at least after stimulation in other cell types; data not shown). This indicates that the transfected amounts are well below the induced, endogenous TTP levels of other cell types. Lai and colleagues have shown previously (14) that the overexpression of human TTP exhibits different effects on the degradation of TNFα mRNA dependent on the expression levels: while low levels of TTP efficiently destabilize the TNFα message, they observed that higher levels lead to the stabilization of the mRNA, which is explained by the possibility that TTP might form inactive dimers at higher concentrations or sequester components of the RNA degradation machinery. We initially hypothesized that TTP might trigger the down-regulation of the expression of one ore more components of the NF-κB signaling pathway through its known mRNA destabilizing activity. However, the use of TTP constructs, which either carry a mutation in the zinc-finger domain (C124R) that impairs ARE binding and mRNA degradation, or lack the N- and C-terminal domains (TZF), provided evidence that TTP inhibits NF-κB independent of its well-described mRNA destabilizing function. The fact that the RNA binding mutant C124R could not diminish, but rather augment TNFα mRNA levels has already been observed by Lai and colleagues and was confirmed in our experiments (Sup. Fig. 3A). It can be explained by the inhibition of mRNA turnover due to dominant-negative function of this mutant (41). Further examination of the mechanism of TTP action unveiled that TTP influences the activation of NF-κB at the level of p65: no upstream mediator of NF-κB signaling (TRAF2, TAK1/TAB1, IKK2) could overcome the strong, inhibitory TTP effect, IκBα degradation was not affected and overexpression of the known p65 nuclear co-activators CBP, p300 and MSK1 did not prohibit TTP action. Besides, neither total p65 protein amounts nor mRNA levels were influenced and no physical interaction of TTP with p65 could be demonstrated by coimmunoprecipitation (data not shown). However, the results of follow-up experiments revealed that the nuclear import of NF-κB/p65 was affected by TTP: biochemical analysis demonstrated that p65 appeared in the nucleus 15 minutes post TNFα stimulation in control cells, whereas TTP transfected cells showed a severely impaired nuclear translocation. Densitometric analysis of nuclear p65 levels revealed that already under unstimulated conditions less p65 was present in the nucleus and the difference became even more evident after stimulation with TNFα, in agreement with the results obtained by the reporter gene assays. These findings were further confirmed at the single-cell level by immunofluorescent analysis of endogenous p65 in primary HUVEC. In line with the promoter studies performed in TTP k.o MEF, we found that p65 exhibited increased nuclear localization under unstimulated conditions in these cells. Here, the lack of TTP seems to allow enhanced nuclear shuttling of NF-κB, leading to a "pre-stimulated" situation that is reflected by the upregulation of certain NF-κB target genes. It has to be pointed out that the target genes analyzed in untreated TTP k.o cells provide an insight into the prestimulated situation generated by TTP deficiency. It is in all probability that under e.g. TNFα stimulated conditions other and/or additional target genes might be differentially regulated. The fact that only a subset of NF-κB regulated genes is affected in our experiments can be explained by the complexity of NF-κB activation: the selective transcriptional onset of specifically required target genes is dependent on the formation of distinct NF-κB dimer/heterodimer combinations, the abundance and distribution of essential cofactors as well as the epigenetic regulation of the accessibility of the promoters. The proper timing and interplay of these components may facilitate the differential expression of a plethora of NF-κB dependent genes. Our analysis revealed the ARE-less NF-κB targets RANTES, Gadd45β and TRAF1 being upregulated in TTP k.o MEF. RANTES is a chemokine that appears chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. TRAF1 is a

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

member of the TNFR2 signaling complex, which exerts multiple biological effects on cells such as proliferation, cytokine production, and cell death. Gadd45β in turn is known to be involved in the regulation of DNA repair and cell cycle control. In the light of the fact that TTP k.o cells grow somewhat faster than WT MEF, it appears interesting that all these genes have been connected to the regulation of cell proliferation or apoptosis, a decision that is predominantly controlled by the crosstalk of NF-κB with JNK signaling. The elucidation of a possible influence of TTP in this crosstalk will be subject of further studies.

Concerning the mechanism of TTP mediated inhibition of the nuclear import of NF-κB it is interesting to note that in drosophila, nup214, a component of the nuclear pore complex (NPC), has been implicated in the modulation of NF-κB activation. Nup214 is a member of the so called FG-Nups that provide specificity and abundance of transport factor binding sites at the NPC and have been shown to facilitate the karyopherin-cargo movement across the nuclear pore. Nup214 mutants impaired the nuclear accumulation of the p65/p50 homologues Dorsal and Dif after bacterial challenge (51). A separate study showed that TTP was able to interact with nup214 in THP-1 cells (52). It is therefore tempting to speculate that the mechanism of TTP action might involve the selective prevention of NF-κB import via regulation of a Nup214-containing nuclear pore. Selectivity could thereby be achieved through the nature of the monopartite p65 NLS as opposed to e.g. the bipartite leader of p53 which, when exchanged, abrogated the inhibitory function of TTP (Fig. 7). Taken together, these observations reveal an additional, and, to our knowledge, novel activity for TTP. To date we can not completely exclude that TTP triggers the activation or repression of other ARE binding proteins through protein-protein interactions, or that the loss of function mutation in the zinc-finger concerns only a subset of ARE containing targets. In this regard a recent finding revealed that TTP does not exclusively bind to ARE sequences, but additionally recognizes a non-ARE cis element in the MHC class I mRNA (53). However, 3'-UTR analysis of this mRNA demonstrated a binding deficiency of the zinc-finger mutant as well, suggesting that the loss of function is not restricted to a certain ARE motif.

In conclusion, TTP contributes to the regulation of NF-κB-mediated inflammatory responses at least on two levels: on the one hand through the destabilization of cytokine mRNAs (e.g. TNFα), and on the other hand through the impairment of NF-κB/p65 nuclear translocation. It thus controls the selective, transcriptional onset of a subset of NF-κB-dependent genes and acts as a negative feedback regulator during a variety of NF-κB - dependent pathophysiological situations.

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FOOTNOTES

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#these authors contributed equally to this work

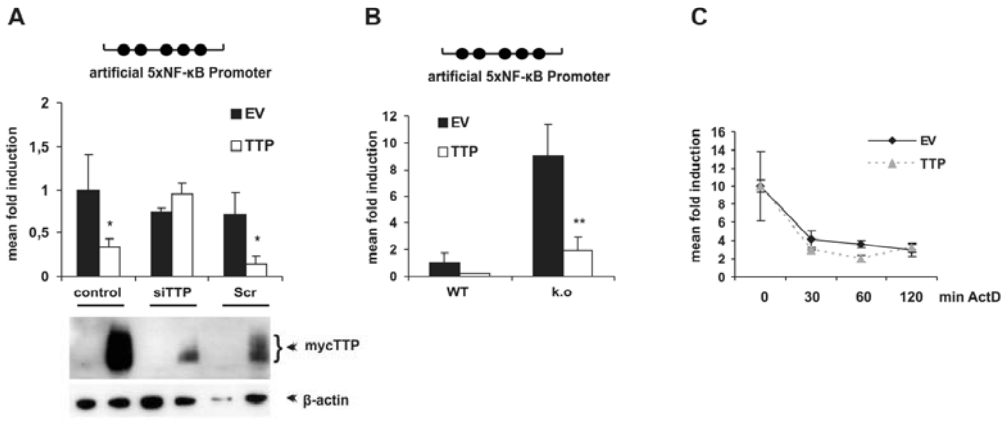
The abbreviations used are: CCCH, cysteine-cysteine-cysteine-histidine; Cox-2, Cyclooxygenase-2; DMEM, Dulbecco's modified eagle medium; ECGS, endothelial cell growth supplement; FADD, Fas-associated death domain; FCS, fetal calf serum; GFP, green fluorescent protein; GM-CSF, granulocyte macrophage colony stimulating factor; HRP, horseradish peroxidase; Ier3: Immediate early response 3; IκBα, inhibitor of kappa B alpha; MHC, major histocompatibility complex; MuLV, murine leukemia virus; MSK-1, mitogen- and stress-activated protein kinase 1; NFAT, nuclear factor of activated T cells; PBS, phosphate buffered saline; RIP, receptor interacting protein; TAB, transforming growth factor activated protein kinase 1 binding protein; TAK1, transforming growth factor beta activated kinase 1; TNFα, tumor necrosis factor alpha; TRADD, TNF receptor-associated death domain; TRAF, tumor necrosis factor receptor associated factor; UTR, untranslated region;

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

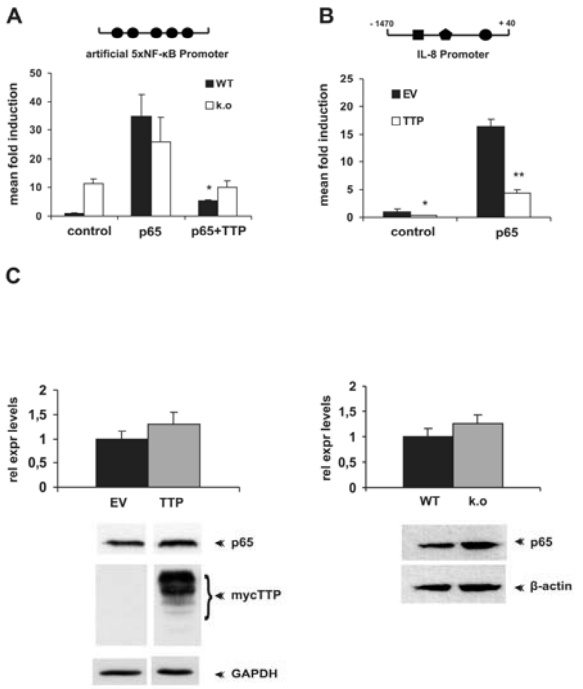
3.2.1. Supplemental Material

Supplementary Fig. 1:

A) HEK 293 cells were transfected with 5xNF-κB-luc alone (EV) or in combination with TTP, along with a siRNA directed against TTP (siTTP) or a scrambled control (Scr). Luciferase activity was assessed 48hrs post transfection and normalized to coexpressed βGal. Mean fold induction was calculated in respect to the basal promoter activity (control). Knock-down efficiency of ectopically expressed TTP was confirmed by Western Blotting (lower panel) β-actin was used as loading control. B) 5xNF-κB promoter activity was analyzed in wild type (wt) versus TTP knock out (k.o) MEF before (EV) and after reconstitution of TTP expression. Luciferase levels were normalized to cotransfected β-Gal. * P<0.05, ** P<0.01, compared to the respective EV samples. C) TTP does not impact luciferase mRNA degradation. HEK 293 cells were transfected with 5xNF-κB-luc alone (EV) or in combination with a TTP expression vector (TTP). Cells were treated with ActinomycinD (5µg/ml) as indicated, and the degradation of luciferase mRNA was analyzed by real-time PCR as described in Experimental Procedures. Values obtained for the untreated samples were set to 10 and the experiment was done in triplicates. Error bars represent standard deviation of the mean.



Supplementary Fig. 2:

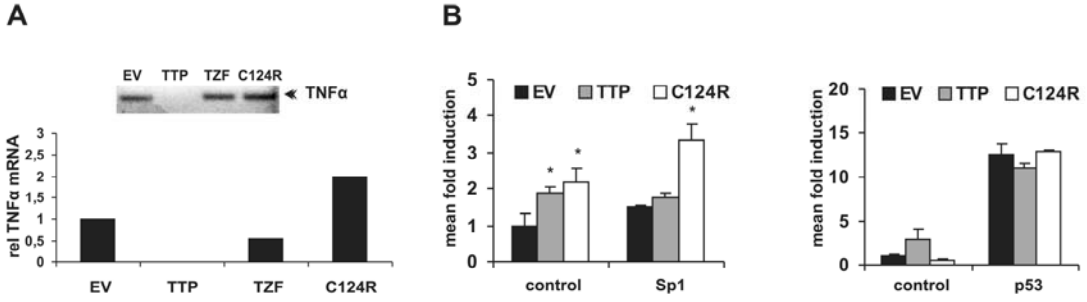


A) Lack of TTP mimics "pre-stimulation". 5xNF-κB promoter activity was analyzed in wild type (wt) versus TTP knock out (k.o) MEF before (control) and after stimulation with p65 (p65) as well as in the presence of cotransfected TTP (p65+TTP). Luciferase levels were normalized to cotransfected β-Gal. * P<0.05 compared to the respective p65 induced sample. B) TTP dampens p65 induced IL-8 promoter activity. HEK 293 cells were transfected with a reporter gene construct driven by the IL-8 promoter (scheme as described in Fig. 1) alone (control) or stimulated by a cotransfected p65 expression plasmid. Luciferase expression levels were assessed in the presence (TTP) and absence (EV) of coexpressed TTP and normalized to the activity of cotransfected β-Gal. Mean fold induction was calculated in relation to unstimulated conditions (control). * P<0.05, ** P<0.01, compared to the respective EV samples. C) Total p65 expression levels are not influenced by TTP. Left: Endogenous p65 mRNA levels assessed by real time PCR (upper graph) or Western Blot analysis (lower panel) in control (EV) or TTP transfected (TTP) HEK 293 cells. Relative mRNA levels were normalized to β2-microglobulin and expression of ectopic TTP as well as equal protein loading was confirmed by α-myc (TTP) and GAPDH antibodies, respectively. Right: Expression levels of endogenous p65 in wild type (wt) and TTP k.o MEF determined by real time PCR (top) and Western Blot analysis (bottom). β-actin represents the loading control. Results are representatives of three independent experiments done in triplicates.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

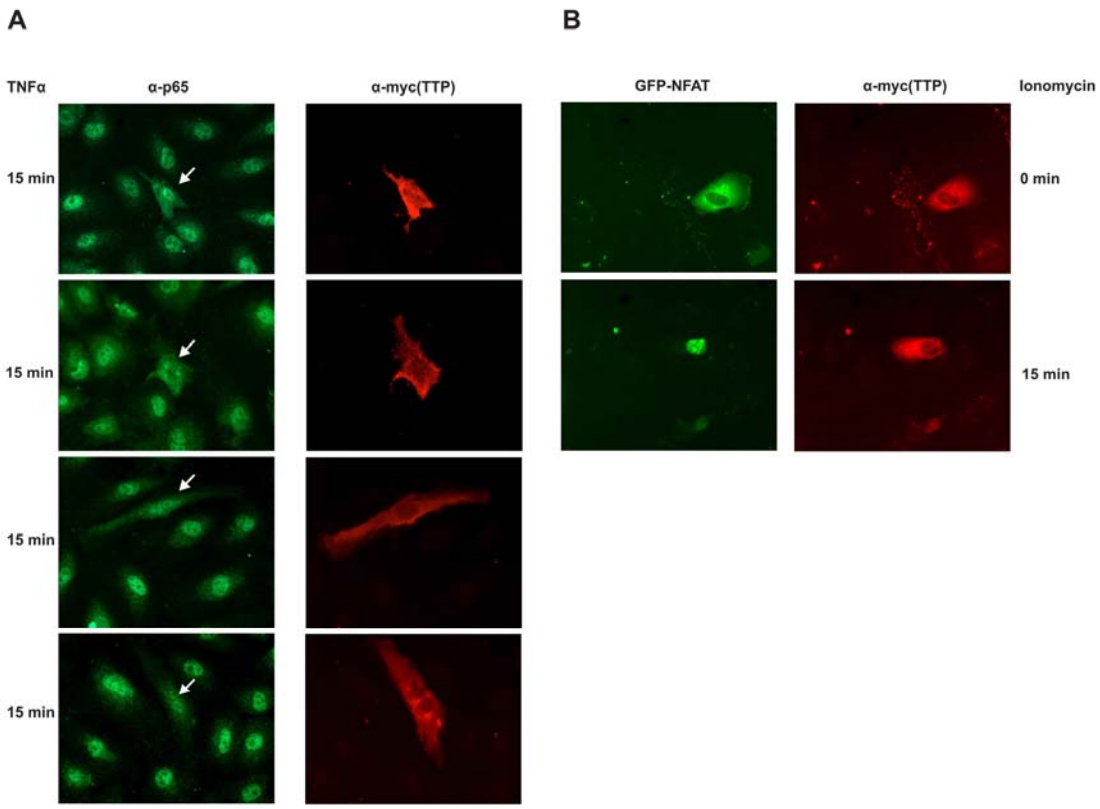
Supplementary Fig. 3:

A) TTP-C124R does not initiate degradation of TNFα mRNA. HEK 293 cells were transfected with either empty vector (EV), myc-tagged TTP (TTP), TZF (TZF) or the RNA-binding mutant C124R (C124R) (see scheme Fig. 4) and relative TNFα mRNA levels were determined by real time PCR 24hrs post transfection. Expression levels were normalized to β2-microglobulin and PCR samples were subjected to a control 2% agarose gel (top insert). B) TTP-C124R does not affect SP-1 and p53 dependent promoters. HEK 293 cells were transfected with either a SP-1 (left) or a p53 (right) promoter dependent luciferase reporter plasmid. The influence of cotransfected TTP or C124R was analyzed under unstimulated conditions (control) as well as after activation with cotransfected SP-1 and p53 expression plasmids, respectively. * P<0.05 compared to the respective EV samples.



Supplementary Fig. 4:

TTP impairs p65 but not NFAT nuclear translocation. A) Human umbilical vein endothelial cells (HUVEC) were transfected with a TTP expression vector (mycTTP), stimulated with TNFα (10ng/ml) for 15 minutes and endogenous p65 (green, left panels) and ectopically expressed TTP (red, right panels) were detected by immunocytochemistry. TTP-transfected cells are indicated by an arrow. B) HUVEC were transfected with expression vectors for TTP (mycTTP) and a NFAT-GFP fusion construct (GFP-NFATc), and nuclear translocation of NFAT was analyzed after stimulation with Ionomycin (500nM) for 10 minutes. (NFAT: green; TTP: red).



THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 43, pp. 29571–29581, October 23, 2009

Supplementary Table 1:

Application	Sequence 5'-3'
RNA interference	
Zfp36_1*	ACA AGA CUG AGC UAU GUC GGA CCU U
Zfp36_2*	UGG AUC UGA CUG CCA UCU ACG AGA G
Zfp36_3*	CCU GGG AUC CGA CCC UGA UGA AUA U
scrambled control	ACA AGA GUG UGC AAU CUC GGA GCU U
Real time PCR	
human p65 fwd	CTA CGA CCT GAA TGC TGT GC
human p65 rev	GCC AGA GTT TCG GTT CAC TC
mouse p65 fwd	GAG CCC ATG GAG TTC CAG TA
mouse p65 rev	CAA AGT TGA TGG TGC TGA GG
human b2mg fwd	GAT GAG TAT GCC TGC CGT GTG
human b2mg rev	CAA TCC AAA TGC GGC ATC T
mouse b2mg fwd	ATT CAC CCC CAC TGA GAC TG
mouse b2mg rev	TGC TAT TTC TTT CTG CGT GC
human TTP fwd	GGA TCT GAC TGC CAT CTA CGA
human TTP rev	CGG GAG GTG ACC CCA GAC
human TNFα fwd	CCC CAG GGA CCT CTC TCT AA
human TNFα rev	GCT TGA GGG TTT GCT ACA ACA
mouse MCP-1 fwd	AGG TCC CTG TCA TGC TTC TG
mouse MCP-1 rev	TCT GGA CCC ATT CCT TCT TG
mouse Fth1 fwd	CCT GCA GGA TAT AAA GAA ACC A
mouse Fth1 rev	GAA GTC ACA TAA GTG GGG ATC A
mouse Txn2 fwd	TGG ACT TTC ATG CAC AGT GG
mouse Txn2 rev	GGC ACA GCT GAC ACC TCA TA
mouse RANTES fwd	GTG CCC ACG TCA AGG AGT AT
mouse RANTES rev	GGG AAG CGT ATA CAG GGT CA
mouse Gadd45β fwd	CCT GGC CAT AGA CGA AGA AG
mouse Gadd45β rev	TGA CAG TTC GTG ACC AGG AG
mouse TRAF1 fwd	CCT GAG AGA TGA TGA GGA TCG
mouse TRAF1 rev	GCT CCC CTT GAA GGA ACA G
TTP subcloning	
TTP fwd	CGG AAT TCC CAT GGA TCT GAC
TTP rev	CGG TCG ACT CAC TCA GAA ACA G
p53NLS cloning	
p53NLS sense	AGC TTC CAC CAT GAA GCG AGC ACT GCC CAA CAA CAC CAG CTC CTC TCC CCA GCC AAA GAA GAA ACC AGC
p53NLS antisense	CAT GGC TGG TTT CTT CTT TGG CTG GGG AGA GGA GCT GGT GTT GTT GGG CAG TGC TCG CTT CAT GGT GGA
p53NLS fwd	CCA ACA ACA CCA GCT CCT CT
p53NLSp65 rev	GCT GCT CAA TGA TCT CCA CA

* pool of 3 oligos

3.3. TTP as dual-functional balancer between NF-κB and MAPK signaling

TTP is described as hyperphosphorylated protein in both murine and human cells. TTP phosphorylation has been studied by several laboratories, and mass spectrometry as well as site directed mutagenesis have identified a number of potential phosphorylation sites and predicted kinases. However, the most extensively studied kinase that was shown to phosphorylate and inactivate TTP's mRNA binding and degrading ability remains the MAPK p38α whereas the function of other predicted kinases is still a matter of research.

In respect to these observations we decided to examine whether any of these predicted kinases can rescue the effect of TTP mediated downregulation of NF-κB. We tested IKK1, PKA, PKCα, PKCβ, PKCγ and PKCζ in NF-κB-dependent reporter gene assays and could not detect any significant differences in respect to inhibition of TTP function (data not shown). Strikingly, and although not predicted as a possible kinase yet, we found that the MAP3K MEKK1 was able to prevent TTP action whereas the p38α- as well as the MEKK1-p38α-induced NF-κB activity was effectively downregulated by TTP (Fig. 12T, A). Interestingly,

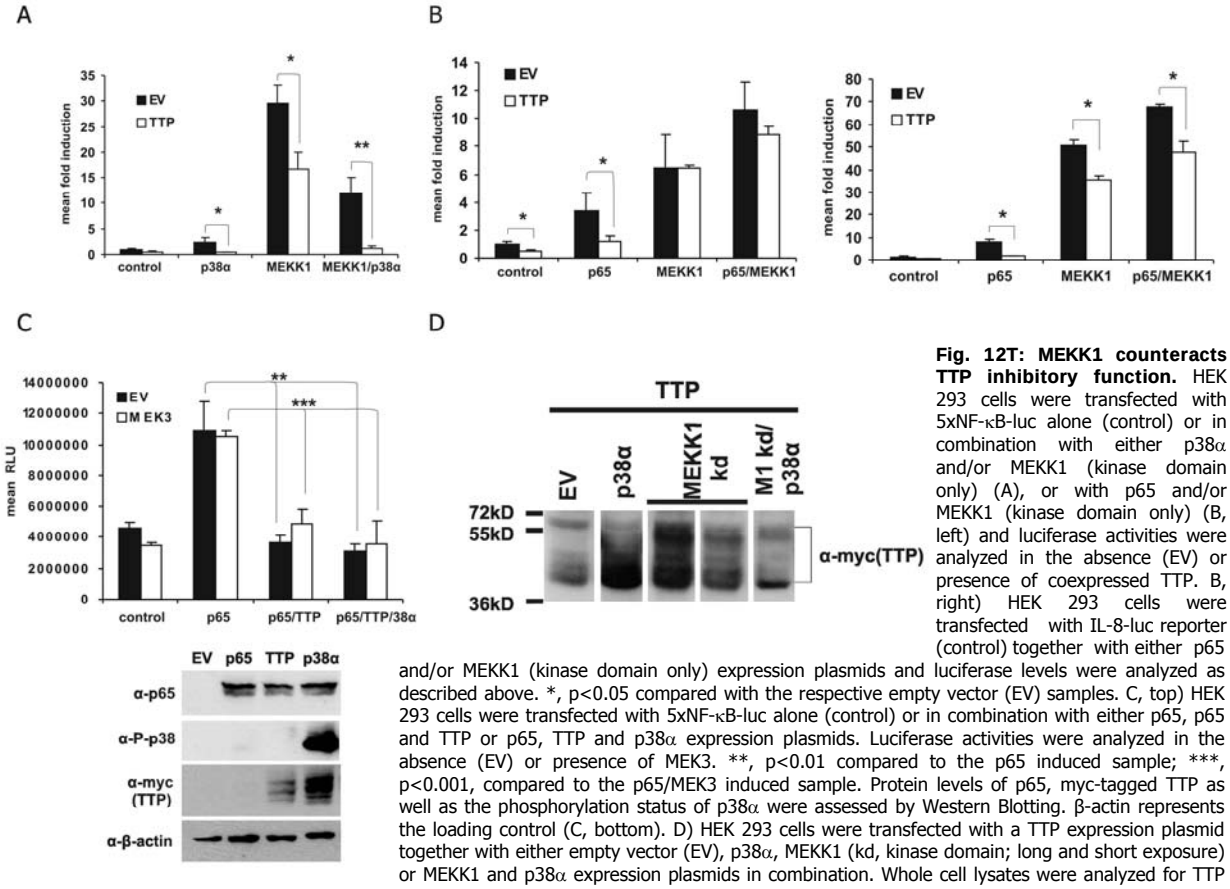


Fig. 12T: MEKK1 counteracts TTP inhibitory function. HEK 293 cells were transfected with 5xNF-κB-luc alone (control) or in combination with either p38α and/or MEKK1 (kinase domain only) (A), or with p65 and/or MEKK1 (kinase domain only) (B, left) and luciferase activities were analyzed in the absence (EV) or presence of coexpressed TTP. B, right) HEK 293 cells were transfected with IL-8-luc reporter (control) together with either p65 and/or MEKK1 (kinase domain only) expression plasmids and luciferase levels were analyzed as described above. *, $p<0.05$ compared with the respective empty vector (EV) samples. C, top) HEK 293 cells were transfected with 5xNF-κB-luc alone (control) or in combination with either p65, p65 and TTP or p65, TTP and p38α expression plasmids. Luciferase activities were analyzed in the absence (EV) or presence of MEK3. **, $p<0.01$ compared to the p65 induced sample; ***, $p<0.001$, compared to the p65/MEK3 induced sample. Protein levels of p65, myc-tagged TTP as well as the phosphorylation status of p38α were assessed by Western Blotting. β-actin represents the loading control (C, bottom). D) HEK 293 cells were transfected with a TTP expression plasmid together with either empty vector (EV), p38α, MEKK1 (kd, kinase domain; long and short exposure) or MEKK1 and p38α expression plasmids in combination. Whole cell lysates were analyzed for TTP expression and "patterning" by application on a 5-12% SDS-gradient gel and Western Blotting.

MEKK1 also prohibited the negative effect of TTP on the artificial 5xNF-κB as well as the IL-8 promoter when induced with p65 (Fig. 12T, B), whereas co-expression of p38α as well as the additional coexpression of the p38α upstream kinase MEK3 did not result in any changes (Fig. 12T, C). Based on this result and the fact that p38α mediated TTP phosphorylation results in a well described electrophoretic shift of the protein we analyzed the "molecular patterning" of TTP after coexpression with either p38α or MEKK1 or both by Western Blotting. As shown by the Blot in Fig. 12T (D) p38α mediated phosphorylation of TTP led to a different molecular shift as compared to the modification obtained by coexpression of MEKK1. Interestingly, the combined overexpression of MEKK1 and p38α led to a molecular appearance of TTP comparable to that of the empty vector (EV) control (Fig. 12T, D, left panel).

MEKK1 is a 195kDa protein containing a C-terminal kinase domain that facilitates the phosphorylation of several different MEKs, with the strongest activity towards MKK4, the upstream kinase for JNKs. Its N-terminal domain contains a plant homeodomain (PHD) that has a RING finger like structure and exhibits E3 Ubiquitin Ligase activity.

To substantiate our findings and gain further insight into MEKK1 mediated TTP inhibition, we analyzed the effect of coexpression of full length MEKK1 compared to its kinase domain only on TTP mediated NF-κB inhibition. We observed that while the kinase domain effectively prohibited TTP function, full length MEKK1 exhibited a weaker effect (Fig. 13T, A). Moreover, coexpression of a MEKK1-kinase-deficient construct (D1369A) led to the complete reconstitution of the TTP-mediated NF-κB inhibition, and mutation of the PHD domain slightly attenuated TTP activity (Fig. 13T, B). In order to exclude the possibility that

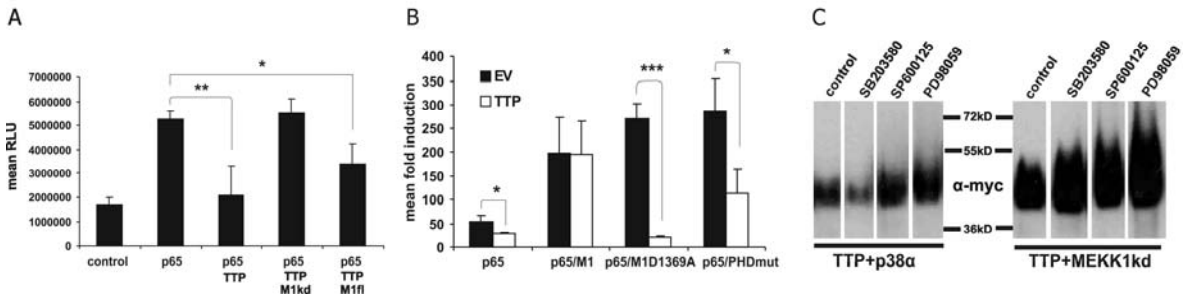


Fig. 13T: Kinase domain of MEKK1 accounts for TTP blockage. A) HEK 293 cells were transfected with 5xNF-κB-luc alone (control) or in combination with p65, TTP, MEKK1kd (kinase domain) or MEKK1fl (full length) expression plasmids as indicated and luciferase expression levels were analyzed. *, p<0.05; **, p<0.01, compared to the p65 induced sample; B) HEK 293 cells were transfected with 5xNF-κB-luc together with p65 and in combination with either MEKK1 D1369A (kinase domain mutant) or MEKK1 PHDmut (E3 ligase domain mutant) expression plasmids. Luciferase activities were assessed in the absence (EV) and presence of TTP. *, p<0.05; ***, p<0.001, compared to the respective empty vector (EV) samples. C) HEK 293 cells were transfected with expression plasmids for TTP and p38α (left panel) or TTP and MEKK1kd (kinase domain; right panel) and either left untreated (control) or treated with the MAPK inhibitors SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) or PD98059 (MEK1 inhibitor) for 1 hour. Whole cell lysates were analyzed for TTP expression and "patterning" by Western blotting.

downstream kinases such as ERK, JNK or p38α account for the indirect MEKK1 triggered TTP inhibition, we analyzed the p38α and MEKK1 mediated TTP modifications and resulting molecular patterning in the

presence of ERK, JNK and p38 inhibitors. As shown by the Western Blot in Fig. 13T (C) the p38 inhibitor SB203580 had a dampening effect on p38α-mediated TTP phosphorylation (left, second lane) while the inhibitors for JNK (SP600125) and ERK (PD98059) showed no effect, supporting the specificity of the inhibitors. Moreover, none of the inhibitors applied could prohibit the strong molecular shift of TTP triggered by the coexpression of the MEKK1 kinase domain (Fig. 13T, panels, right side). Based on the above results we hypothesized that MEKK1 as well as p38α physically interact with TTP which was

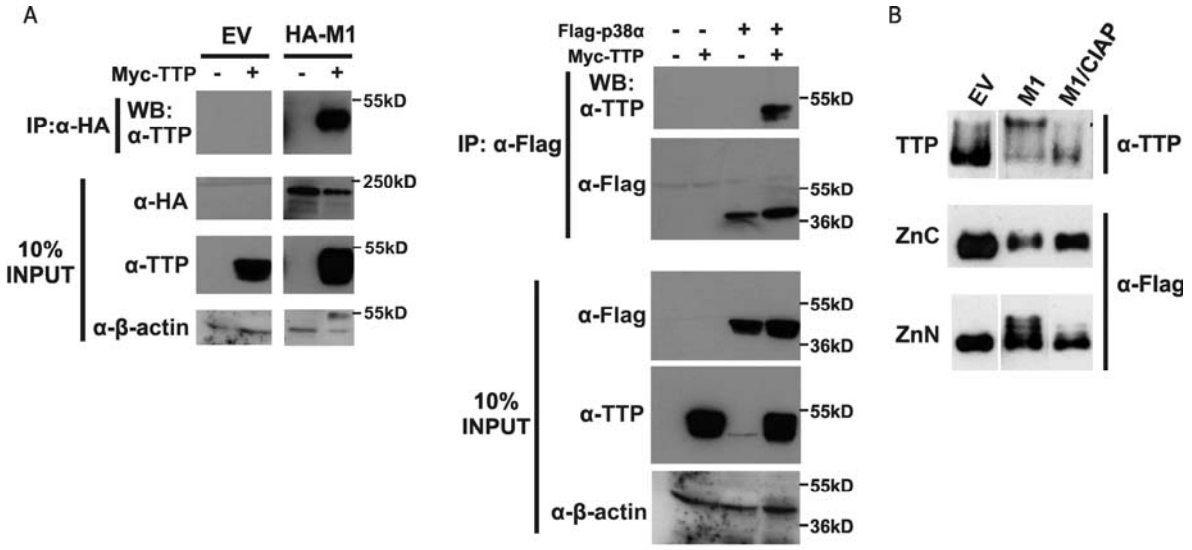


Fig. 14T: MEKK1 physically interacts with TTP and phosphorylates its N-terminus. A, left) HEK 293 cells were transfected with Myc-TTP in combination with either empty vector (EV) or HA-tagged MEKK1 and MEKK1 was immunoprecipitated. Co-precipitated TTP was analyzed by Western Blotting (upper panels). The lower three panels represent the input control. A, right) HEK 293 cells were transfected with Myc-TTP in combination with Flag-p38α what was immunoprecipitated using an anti-Flag matrix. Coimmunoprecipitation of TTP as well as the amount of precipitated p38α was assessed by Western Blotting (upper two panels), the input control is shown in the lower three panels. B) HEK 293 cells were transfected with TTP, TTP_{ZnC} or TTP_{ZnN} expression plasmids in combination with either empty vector (EV) or M1 (MEKK1 kinase domain only) vectors. Cell lysates of samples coexpressing MEKK1kd were devided and one half was treated with calf intestine alkaline phosphatase (CIAP) for 15 minutes. Expression of TTP and of TTP_{ZnC} and TTP_{ZnN} as well as their molecular shift was analyzed by 5-12% gradient SDS-PAGE and Western Blotting.

subsequently shown by coimmunoprecipitation in HEK 293 cells (Fig. 14T, A). To further determine whether phosphorylation is responsible for the special molecular patterning of TTP, we analyzed the molecular shift of the protein before and after treatment with calf intestine alkaline phosphatase (CIAP). As shown by the Immunoblot in Fig. 14T (B, top panel), phosphatase treatment reversed the molecular weight shift of TTP, indicating that it is indeed due to phosphorylation. Furthermore, the same molecular weight shift and CIAP-mediated reversion could be observed when the TTP_{ZnN} construct was used instead of full length TTP, whereas MEKK1 did not affect the TTP_{ZnC} mutant construct (Fig. 14T, B, bottom panels), indicating that MEKK1 posphorylates the N-terminal part of TTP.

The initial step in TNFR signaling is the assembly of a multicomponent receptor-associated cytoplasmic signaling complex immediately after TNFR engagement. Key players within this multiproteincomplex are

the TRAFs serving as E3 ubiquitin ligases and adaptors that recruit and activate protein kinases such as TAK1 and MEKK1, which in turn activate IKKs and JNK, respectively. In this context it is important to note that TNFR1-induced JNK activation strongly depends on activated MEKK1 which is facilitated by TRAF2 mediated K63-linked ubiquitination.

Based on this as well as our finding that MEKK1 and TTP physically interact, we aimed to examine whether TRAF2 is capable to form a complex with TTP. Coimmunoprecipitation in HEK 293 cells unveiled that TTP indeed coprecipated with TRAF2 (Fig. 15T, A). When we tested whether TRAF2 is able to act as

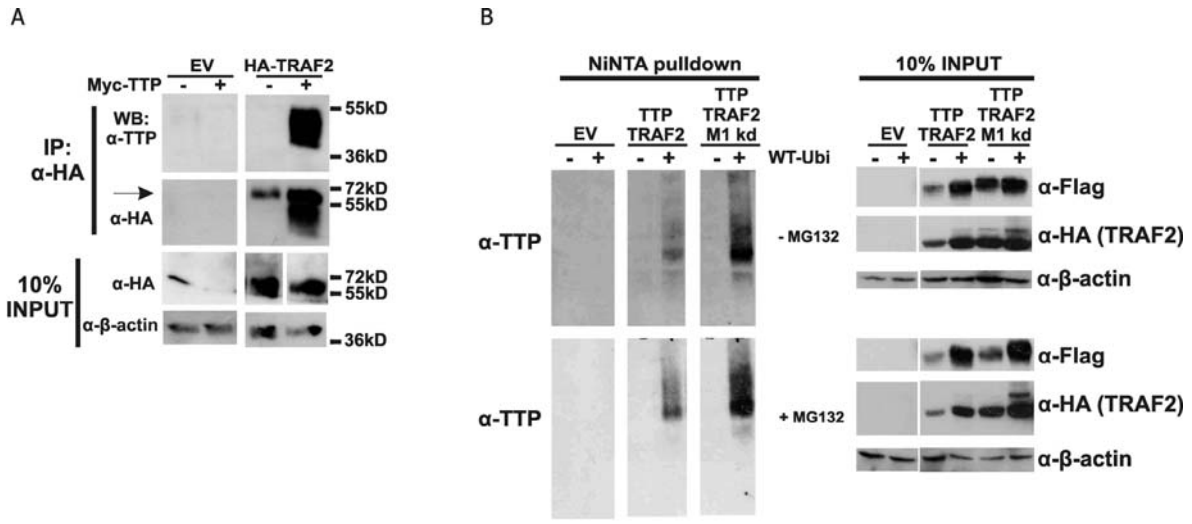


Fig. 15T: TRAF2 physically interacts with TTP and triggers its ubiquitination which is enhanced in the presence of MEKK1. A) HEK 293 cells were transfected with Myc-TTP in combination with either empty vector (EV) or HA-tagged TRAF2 which was precipitated using an α–HA matrix. Amounts of TRAF2 as well as coprecipitated TTP were analyzed by Western Blotting (upper two panels), the input control is shown in the lower two panels. B) HEK 293 cells were transfected with Flag-TTP, HA-TRAF2 and M1 (MEKK1 kinase domain) expression plasmids in the presence (+) or absence (-) of a wild type-ubiquitin (wt-Ubi) expression vector as indicated. Ubiquitinated proteins were precipitated under denaturating conditions using Ni-NTA-beads and the presence of polyubiquitinated TTP was assessed before (-MG132) and after treatment with MG132 (6 hours) by immunoblot analysis (left panels). The corresponding input controls are depicted within the right panels.

an E3 ubiquitin ligase towards TTP, we found that TRAF2 can facilitate polyubiquitination of TTP, which is further enhanced upon coexpression of the MEKK1 kinase domain (Fig. 15T, B). Furthermore, we observed that proteasome inhibition by MG132 had no effect on TTP-ubiquitination, raising the possibility that attached ubiquitin chains might be K63-linked (Fig. 15T, B, left, bottom panels).

To follow up on the nature of ubiquitination we utilized mutated versions of His-tagged ubiquitin (K48R, K63R) and tested their potential to ubiquitinate different TTP mutants in the presence or absence of TRAF2 and MEKK1. Whereas no ubiquitination took place in the absence of TRAF2 and MEKK1 (Fig. 16T, left panel), the full length TTP protein, as well as the TTP mutant TTP_{ZnN} underwent polyubiquitination when MEKK1 and TRAF2 were coexpressed. Interestingly, only the K48R mutant triggered ubiquitination, whereas the TTP mutant TTP_{ZnC} was not ubiquitinated (Fig. 16T, right panel).

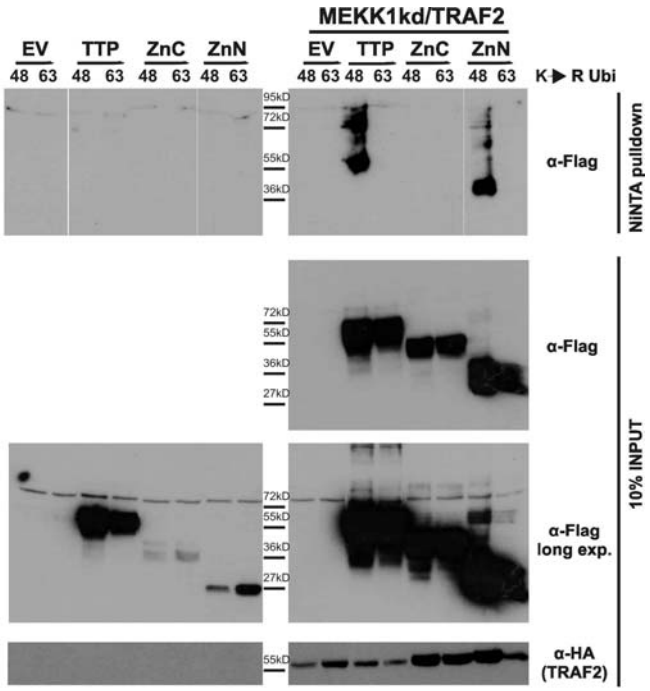


Fig. 16T: TTP-N-terminal phosphorylation by MEKK1 is a prerequisite for K63-linked, TRAF2-mediated ubiquitination of its zinc-finger. HEK 293 cells were transfected with empty vector (EV), Flag-TTP or the TTP mutants Flag-TTP_{ZnC} and Flag-TTP_{ZnN} together with mutated version of His-tagged ubiquitin (K48R or K63R) as indicated and either without (left panels) or with (right panels) expression plasmids for MEKK1kd (kinase domain) and HA-TRAF2. Ubiquitinated proteins were precipitated under denaturating conditions using Ni-NTA-beads, and their presence analyzed by Western Blotting. The input control is shown in the lower three panels.

In the course of our experimental set-up for *in vivo* ubiquitination assays we observed that ectopic expression of different combinations of TTP, MEKK1 and TRAF2 variably influenced cell morphology and growth in HEK 293 cells. As shown in Fig. 17T (A), the combined ectopic expression of TTP, TRAF2 and the kinase domain of MEKK1 alleviated cell growth which was further enhanced upon additional overexpression of wt-ubiquitin (Fig. 17T, A, bottom row, last pictures). Note, that when the MEKK1 kinase domain construct was exchanged with full length MEKK1, cell growth was completely unaffected. In addition, only the TTP_{ZnN} mutant construct but not the TTP-N-terminus-only had an effect on cell growth and morphology, which resembled the appearance of cells undergoing apoptotis (Fig. 17T, B). Cell growth, differentiation and apopotsis are processes that are mainly modified by the accurately timed interplay between NF-κB and JNK signaling. Onset of the JNK signaling cascade through e.g. the activation of MEKK1 results in the phosphorylation of JNK, finally leading to AP-1 mediated target gene expression. When we analyzed p-JNK levels after ectopic expression of TTP in HEK 293 cells we found that the activity of JNK was enhanced by elevated levels of TTP, whereas total JNK levels were not affected (Fig. 18T, A). This observation was further supported by AP-1-dependent reporter gene assays, where TTP overexpression led to upregulation of AP-1 dependent luciferase expression in a dose dependent manner (Fig. 18T, B). Conversely, TTP k.o MEF displayed strongly impaired JNK activation at basal as well as under TNFα stimulated conditions while, comparable to HEK 293 cells, the total JNK levels were not influenced (Fig. 18T, C). In addition, AP-1 dependent reporter analysis in MEFs supported the prior findings: ectopic TTP expression led to enhanced AP-1 promoter activity in both wt as well as TTP k.o MEF, whereas overall luciferase levels were significantly lower in TTP k.o compared to wt MEF (Fig. 18T, D).

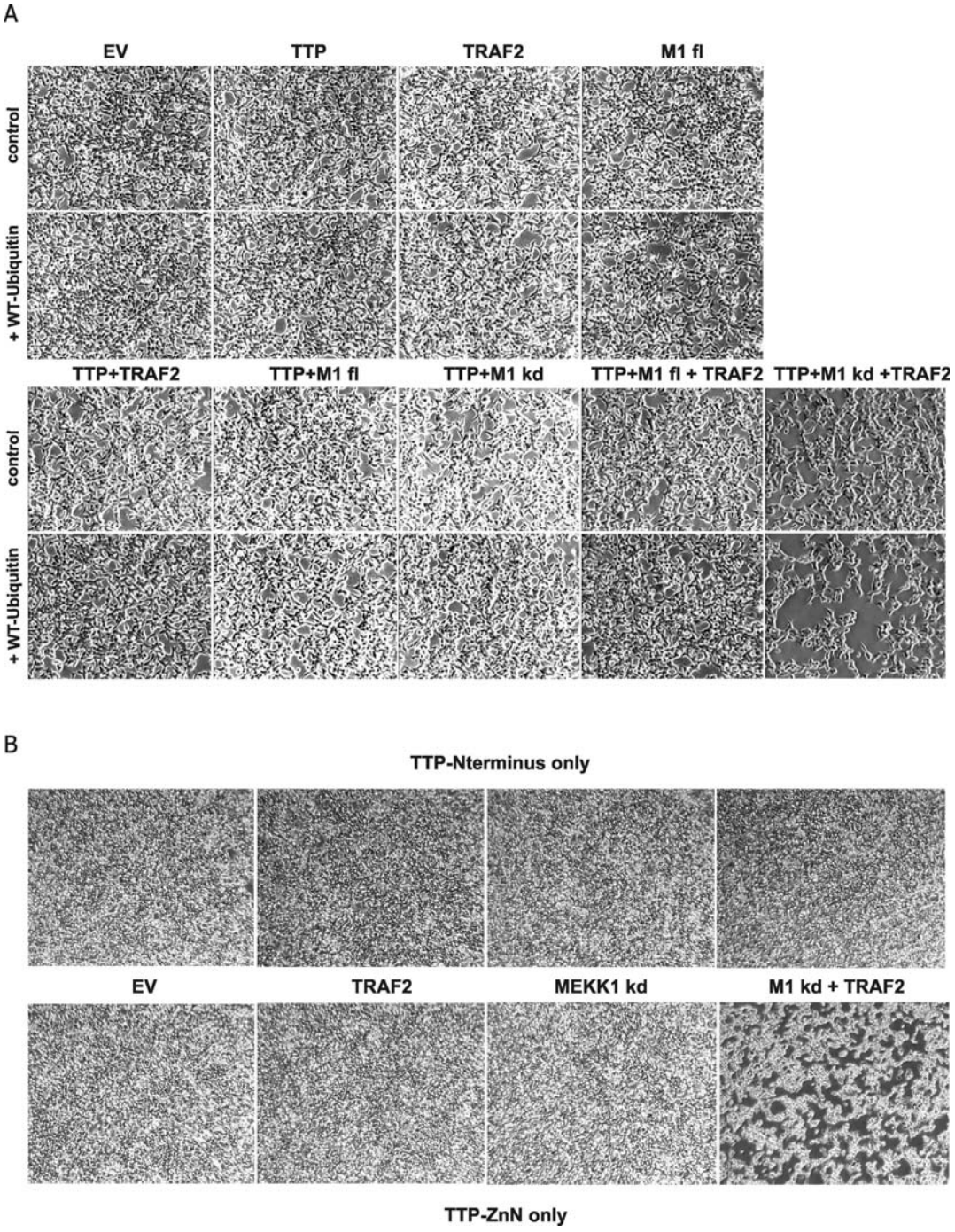


Fig. 17T: Coexpression of TTP, MEKK1 kd (kinase domain) and TRAF2 influences cell growth in HEK 293 cells. A) HEK 293 cells were transfected with either empty vector (EV), TTP, TRAF2, MEKK1 full length (M1 fl), MEKK1 kinase domain only (M1 kd) or with different combinations of these expression plasmids, without (control) or together with an expression vector for wt-ubiquitin, as indicated. B) HEK 293 cells were transfected with the TTP-constructs TTP-Nterminus only (top pictures) or TTP_{ZnN} (bottom pictures) together with either empty vector (EV), TRAF2, MEKK1 kd (kinase domain; M1 kd) or MEKK1 kd and TRAF2 expression plasmids in combination. Phase contrast pictures were taken 24 hours post transfection.

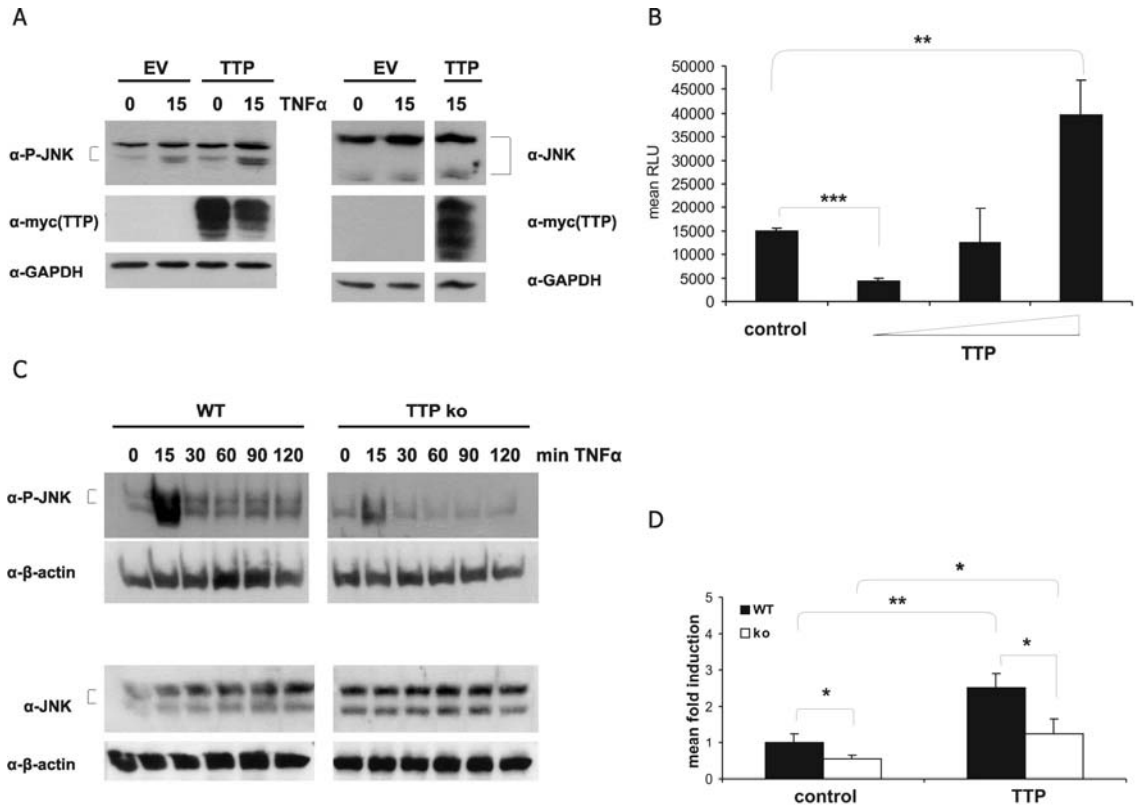


Fig. 18T: TTP influences JNK and AP-1 activity. A) HEK 293 were transfected with either empty vector (EV) or a TTP expression plasmid and stimulated with TNFα for the indicated time points. Whole cell extracts were analyzed for activation of JNK (P-JNK, left panel) as well as for total JNK levels (right panels) and the presence of transfected TTP (α-myc) by Western Bloting. GAPDH is shown as loading control. B) HEK 293 cells were transfected with an AP1-luc reporter alone (control) or in combination with increasing amounts of a TTP expression plasmid. Depicted are mean relative luciferase levels normalized to cotransfected β-galactosidase. **, p<0.01; ***, p<0.001, compared to the control sample. C) wt and TTP k.o MEF were stimulated with TNFα for the indicated time points and whole cell lysates were subjected to Western Bloting and analyzed for activated JNK (P-JNK; top panels) as well as for total JNK-levels (bottom panels). β-actin represents the loading control. D) wt and TTP k.o MEF were transfected with an AP1-luc reporter alone (control) or in combination with a TTP expression plasmid. Luciferase expression was normalized to cotransfected GFP. *, p<0.05; **, p<0.01.

In the light of the above results we analyzed the potential of TTP to influence cell growth and/or apoptosis in primary HUVEC. We generated a recombinant adenovirus for expression of myc-tagged TTP under the control of the tetracycline responsive promoter (tet-OFF system, where expression of the transgene can be suppressed in the presence of doxycyclin [109]). Adenoviral-mediated overexpression of TTP did not influence HUVEC viability at low protein levels (Fig. 19T, part AdV 1/100). Interestingly, the concomitant stimulation of the cells with TNFα led to programmed cell death reflected by a high percentage of Annexin V positive cells (Fig. 19T, AdV 1/100, 16 hours time point; protein levels checked by Western Blot are shown in the insert). Note that TNFα stimulation in controls (cells infected with adenovirus where TTP expression was suppressed by doxycycline) did not result in any changes (Fig. 19T, part AdV 1/100, right three bars). Importantly, when TTP expression levels were increased, apoptosis

occurred completely independent of TNFα costimulation (Fig. 19T, part AdV 1/10, left three bars, corresponding TTP expression levels shown in the insert).

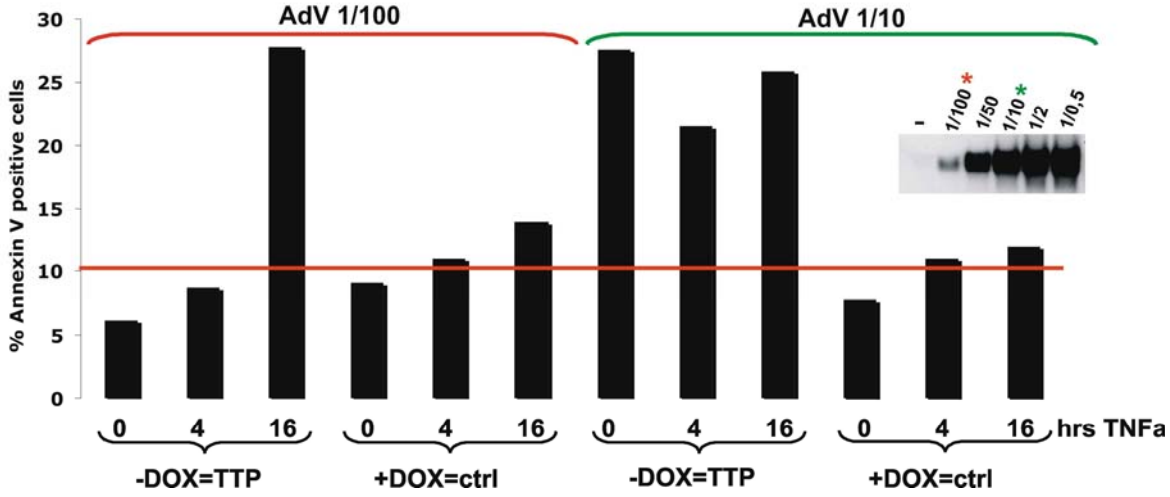


Fig. 19T: TNFα and TTP synergistically induce apoptosis in primary HUVECs. HUVECs were infected with increasing amounts of TTP-adenovirus (AdV 1/100 and AdV 1/10) and grown for 24 hours in medium containing no (-Dox = cells express ectopic TTP) or 4nM Doxycycline (+Dox = cells do not express ectopic TTP). Cells were stimulated with TNFα for the indicated time points and Annexin V positive cells were assessed by FACS analysis. TTP expression levels achieved by adenoviral transduction were analyzed by Western Blotting and are depicted in the insert. The red line represents the threshold of the background settings. Note that low TTP expression levels induce apoptosis only in synergism with 16 hours of TNFα stimulation.

In summary, the presented data suggest that TTP has at least two functions: First, TTP mediates degradation of messenger RNAs, ensuring the rapid and accurate downregulation of proinflammatory mediators; second, TTP is involved in the inhibition of the prosurvival pathway NF-κB through impairing p65 nuclear translocation, and furthermore it acts as a trigger in the onset of apoptosis. This new mRNA-decay-independent function of TTP is due to expression levels and posttranslational modifications. These modifications might differentially regulate its involvement in the crosstalk between NF-κB and MAPK signaling, thus constituting a trigger that ensures the functional switch from the RNA-destabilizer to the signaling-balancer (Fig. 20T).

4. DISCUSSION

In the last decade substantial progress has been made towards elucidating negative regulatory mechanisms that function in the appropriate termination of the inflammatory response. Evidence has accumulated that resolution is already set early in the activation phase of the response, by so called immediate-early and early genes that act as gateways for the subsequent downregulation of proinflammatory gene expression. Elucidation of these feedback mechanisms and its contributors is of great importance for the understanding of acute versus chronic inflammation and for novel strategies of therapeutic intervention.

In the course of our search of immediate early response genes with potential negative regulatory function in HUVEC we found Tristetraprolin. It is the prototype of a class of CCCH-zinc finger proteins and is rapidly induced by the proinflammatory agents LPS, IL-1 and TNFα. Its expression pattern exhibits classical features of an immediate early gene in HUVECs. Its mRNA levels accumulate within 30 minutes of cytokine induction and drop to undinduced levels approximately within the same time period. Remarkably, induction by IL-1 and TNFα leads to comparable upregulation and kinetics of TTP mRNA while treatment with LPS results in a much slower response with mRNA levels peaking 90 minutes after induction, where TTP levels have already dropped to a minimum upon cytokine treatment. A possible explanation for this diverging kinetics might be the fact that, although LPS, IL-1 as well as TNFα induced signaling cascades converge on a common IκB kinase complex that finally activates NF-κB, the upstream components are mainly receptor specific. This leads to a timed and signal specific activation of target gene expression dependent on the cell's environmental demands.

Another important aspect in the initial analysis of TTP expression in HUVECs was the matter of induced protein accumulation and stabilization. Detectable protein levels emerge shortly after accumulation of the TTP transcript. Important to note is that the protein does not undergo such a rapid turnover compared to its mRNA. Conversely, the protein is stabilized and accumulates over around 3-4 hours of TNFα stimulation, which is most likely facilitated by posttranslational modifications, since the protein undergoes a heavy shift in its molecular weight. This is further supported by earlier studies in various murine as well as human cell types, were it was shown that the molecular weight shift results from hyperphosphorylation of the protein [47, 50]. Nevertheless, after this period of stabilization, TTP protein levels drop until the initial undetectable state is reached. This decrease is due to proteasomal degradation since its inhibition by MG132 effectively blocks its degradation. Interestingly, the addition of MG132 does not lead to an accumulation of TTP upon uninduced conditions (data not shown), which is either due to the lack of active kinases that account for hyperphosphorylation and stabilization, or to an extremely high TTP mRNA turnover preventing translation. The notion that Tristetraprolin might function as a negative feedback

regulator emerged concomitantly with the initial finding that TTP regulates the levels of TNFα mRNA posttranscriptionally, and that its biosynthesis is induced by the same agents that stimulate TNFα production itself [19]. Nevertheless, evidence has accumulated that TTP might have a broader function. Examples include the influence on other RNA binding proteins, e.g. the KH-type splicing regulatory protein (KSRP) [110], as well as the proposed multifunctional activity in the control of glucocorticoid mediated gene expression [111].

Here we show that TTP is involved in the negative transcriptional regulation of promoters containing NF-κB binding sites in different cell types. TTP inhibits the induced activity of these promoters about 70-95% while the influence on the basal levels fluctuates below 50%. This might be explained by the fact that NF-κB constitutively shuttles between cytoplasm and nucleus [69] leading to a basal promoter activity with its magnitude being determined by the actual situation and condition of the cell. The negative regulatory role of TTP is further supported by the analysis of TTP deficient mouse embryonic fibroblasts as well as by siRNA mediated knock down of endogenous TTP in HUVEC.

TTP exhibits a dose dependent effect on NF-κB, with only ~7ng of transfected TTP expression plasmid resulting in half-maximal reduction of promoter activity, an amount that was below our detection limit on Western Blots (whereas endogenous TTP could be detected at least after stimulation in other cell types; data not shown). This indicates that the transfected amounts are well below the induced, endogenous TTP levels of other cell types. Lai and colleagues have shown previously [34] that the overexpression of human TTP exhibits different effects on the degradation of TNFα mRNA, dependent on its expression levels: while low levels of TTP efficiently destabilize the TNFα message, they observed that higher levels lead to the stabilization of the mRNA, which is explained by the possibility that TTP might form inactive dimers at higher concentrations or sequester components of the RNA degradation machinery.

Initially we hypothesized that TTP might trigger the down-regulation of the expression of one ore more components of the NF-κB signaling pathway through its known mRNA destabilizing activity. However, the use of TTP constructs, which either carry a mutation in the zinc-finger domain (C124R) that impairs ARE binding and mRNA degradation, or lack the N- and C-terminal domains (TZF), provided evidence that TTP inhibits NF-κB independent of its well-described mRNA destabilizing function. The fact that the RNA binding mutant C124R does not diminish, but rather augments TNFα mRNA levels has already been observed by Lai and colleagues and is further confirmed in our experiments. It can be explained by the inhibition of mRNA turnover due to dominant-negative functions of this mutant [112]. Further examination of the mechanism of TTP action revealed that TTP influences the activation of NF-κB at the level of p65: no upstream mediator of NF-κB signaling (TRAF2, TAK1/TAB1, IKK2) overcomes this strong, inhibitory TTP effect, IκBα degradation is not affected and overexpression of the known p65 nuclear co-activators CBP, p300 and MSK1 does not prohibit TTP action. Besides, neither total p65 protein

amounts nor mRNA levels are influenced and no physical interaction of TTP with p65 can be demonstrated by coimmunoprecipitation (data not shown). However, the results of follow-up experiments uncovered that the nuclear import of NF-κB/p65 is affected by TTP: biochemical analysis demonstrated that p65 appears in the nucleus 15 minutes post TNFα stimulation in control cells, whereas TTP transfected cells show a severely impaired nuclear translocation. Densitometric analysis of nuclear p65 levels revealed that already under unstimulated conditions less p65 is present in the nucleus and the difference gets even more evident after stimulation with TNFα, in agreement with the results obtained by reporter gene assays. These findings were further confirmed at the single-cell level by immunofluorescent analysis of endogenous p65 in primary HUVEC.

In line with the promoter studies performed in TTP k.o MEF, we found that p65 exhibits increased nuclear localization under unstimulated conditions in these cells. Here, the lack of TTP seems to allow enhanced nuclear shuttling of NF-κB, leading to a "pre-stimulated" situation that is reflected by the upregulation of certain NF-κB target genes. It has to be pointed out that the target genes analyzed in untreated TTP k.o cells provide an insight into the prestimulated situation generated by TTP deficiency. It is in all probability that under e.g. TNFα stimulated conditions other and/or additional target genes might be differentially regulated. The fact that only a subset of NF-κB regulated genes is affected in our experiments might be explained by the complexity of NF-κB activation: the selective transcriptional onset of specifically required target genes is dependent on the formation of distinct NF-κB dimer/heterodimer combinations, the abundance and distribution of essential cofactors as well as the epigenetic regulation of the accessibility of the promoters. The proper timing and interplay of these components may facilitate the differential expression of a plethora of NF-κB dependent genes.

Our analysis revealed that the "ARE-less" NF-κB targets RANTES, Gadd45β and TRAF1 are upregulated in TTP k.o MEF. RANTES, a chemokine that appears chemotactic for T cells, eosinophils, and basophils, plays an active role in recruiting leukocytes to inflammatory sites. TRAF1 is a member of the TNFR2 signaling complex, which exerts multiple biological effects on cells such as proliferation, cytokine production, and cell death. Gadd45β in turn is known to be involved in the regulation of DNA repair and cell cycle control. In the light of the fact that TTP k.o cells grow somewhat faster than wt MEF, it appears interesting that all these genes have been connected to the regulation of cell proliferation or apoptosis, a decision that is predominantly controlled by the crosstalk of NF-κB with JNK signaling, which will be discussed later.

Concerning the mechanism of TTP mediated inhibition of the nuclear import of NF-κB it is interesting to note that in drosophila, nup214, a component of the nuclear pore complex (NPC), has been implicated in the modulation of NF-κB activation. Nup214 is a member of the so called FG-Nups that provide specificity and abundance of transport factor binding sites at the NPC and have been shown to facilitate the karyopherin-cargo movement across the nuclear pore. Nup214 mutants impaired the nuclear

accumulation of the p65/p50 homologues Dorsal and Dif after bacterial challenge [113]. A separate study showed that TTP was able to interact with nup214 in THP-1 cells [114]. It is therefore tempting to speculate that the mechanism of TTP action might involve the selective prevention of NF-κB import via regulation of a Nup214-containing nuclear pore. Thereby selectivity might be achieved through the nature of the monopartite p65 NLS as opposed to e.g. the bipartite leader of p53 which, if exchanged, abrogates the inhibitory function of TTP.

The aim of further investigations was to identify potential upstream triggers that control TTP function. Meanwhile it is quite well established that hyperphosphorylation of TTP occurs at multiple sites which were determined by site directed mutagenesis and mass spectometry [54]. In addition, different kinases have been shown to phosphorylate TTP in intact cells and/or in cell-free systems, including the MAPKs ERK, p38 as well as JNK [52, 53, 115, 116]. However, effects of phosphorylation on TTP function(s) remained largely elusive compared to its number of phospho-sites and predicted kinases. The only kinase which has been elucidated in detail so far is p38α-MK2, which mediates phosphorylation of TTP and contributes to LPS induced accumulation of TTP in murine macrophages [45-47]. Furthermore it has been shown that dephosphorylated TTP binds more tightly to AU-rich elements than phosphorylated TTP [116]. In our studies predicted TTP kinases as IKK1, PKA, PKCα, PKCβ, PKCγ and PKCζ were tested for their potential to compensate the inhibitory TTP effect, but none of these kinases prohibits TTP action in respect to NF-κB activity (data not shown). However, we found that the MAP3K MEKK1, which has not been associated with TTP phosphorylation yet, exhibits the ability to prevent TTP from dampening NF-κB activity. MEKK1 is an upstream kinase of p38α, which was shown to phosphorylate TTP and counteract its RNA-destabilizing function. For this reason we tested p38α for its potential to compensate for TTP activity and observed that it can not prevent TTP from inhibiting NF-κB. Together with the fact that the RNA binding deficient TTP mutant C124R inhibits promoter activity as well, this substantiates the possibility that TTP has a function that differs from its transcript-degrading ability. Furthermore this indicates that MEKK1 mediated TTP control is not due to signaling via p38α. This is further supported by the fact that also co-expression of the p38α upstream kinase MEK3 does not result in any changes of the TTP effect. In addition, both p38α as well as MEKK1 lead to an electrophoretic shift of the protein but with a completely differential patterning of molecular weight species. While the addition of a p38 inhibitor leads to the ablation of p38α induced TTP-phosphorylation it does not alter the molecular pattern of the MEKK1-mediated protein-shift, nor do ERK or JNK inhibitors, supporting the idea of a direct MEKK1-TTP interaction.

Together these findings indicate that TTP acts as inhibitor of NF-κB signaling at the transcription factor level, while the MAP3K MEKK1 seems to act as upstream trigger that "pulls TTP out of its inhibitory scenario". Surprisingly, the combined expression of p38α and MEKK1 does not alter TTP inhibitory function at all, which might be explained by the fact that upon overexpression, MEKK1 predominantly and

possibly with higher affinity, phosphorylates its direct downstream target p38α that in rapid succession leads to phosphorylation of TTP resulting in the TTP effect seen for p38α coexpression. Besides, it might be possible that p38α mediated TTP phosphorylation prevents the interaction with MEKK1. MEKK1 is a 195kD protein containing a C-terminal kinase domain as well as a N-terminal PHD domain that acts as E3 ligase. The observation that only the kinase domain of MEKK1 efficiently blocks the inhibitory TTP effect might be due to the fact that full length MEKK1 undergoes autoubiquitination dependent on its kinase domain, which finally leads to the inactivation of its catalytic function [117]. The further finding that a kinase-dead MEKK1 mutant is completely inactive in regard to TTP proves that the kinase domain is sufficient to account for TTP control. Furthermore, the physical interaction of TTP and MEKK1 as well as the fact that phosphatase treatment leads to a complete reversion of its electrophoretic mobility shift, confirms the assumption that MEKK1 phosphorylates TTP. MEKK1 is a multifunctional protein containing several domains including the above described PHD and catalytic domains. Additionally important regions include the activation loop comprising phsophorylation sites, two ubiquitin interacting motifs (UIMs), binding sites for e.g. the MAPK ERK as well as a caspase cleavage site. These are indispensable and account for the multifunctionality of the protein. This might additionally explain, why full length MEKK1 as well as full length MEKK1 with a mutated PHD domain, show a weaker effect on TTP. Interestingly, MEKK1-mediated phosphorylation occurs at the N-terminal part of the TTP protein, a region that is not conserved among the members of the well described TIS-family of proteins which all exhibit the ability to destabilize mRNAs. This might be an indication for a TTP specific function, acquired in the course of evolution where TTP may have structurally and functionally diverged from an initially common ancestor.

Regarding TNFR engagement, it is known that MEKK1 activation as well as its specific function are not only dependent on autophosphorylation and autoubiquitination but are strongly context dependent and rely on the formation of different complexes as well as the abundance of cofactors. These include TRAF2, 3, and 6 which are recruited to the receptor and facilitate the subsequent activation of the MAP3 kinases MEKK1 and TAK1 that culminate in MAPK and IKK activation. Specific activation of the latter is achieved through K63-linked autoubiquitination of TRAF2 and TRAF6, the degradation of inhibitory proteins and the appropriately timed release and activation of distinct signaling complexes, containing TAK1-TRAF6 or MEKK1-TRAF2, which trigger the onset of p38 or JNK activity leading to programmed cell death [118]. In this context it is important that MEKK1 is known to activate various downstream MAP2Ks leading to either cell proliferation or apoptotic signaling. Whereas the promotion of cell survival depends on the intact full length MEKK1 protein that is associated with the membrane-bound receptor complex, apoptotic signaling is dependent on caspase cleavage of MEKK1 resulting in a COOH-terminal catalytic MEKK1-fragment that localizes to the cytosol and promotes caspase and JNK activation. Therefore, our observation that the kinase domain of MEKK1 sufficiently accounts for the phosphorylation of TTP, which is a prerequisite for its K63-linked-TRAF2-mediated polyubiquitination, makes it tempting to speculate that TTP might, under specific circumstances, be a "multicomplex" member and contributes to the regulated balance between

NF-κB and JNK signaling. TTP inhibits not only NF-κB-survival signals by blocking p65 translocation but also interacts with its regulatory cofactors MEKK1 and TRAF2 to jointly facilitate the onset of proapoptotic JNK signaling. Overexpression of TTP clearly enhances phospho-JNK levels and AP-1 promoter activity in HEK 293 cells and the absence of TTP leads to the converse scenario in TTP k.o MEF: JNK activation is strongly impaired in these cells, which is reflected by the attenuation of AP-1 promoter activity. Together with our finding that p65 levels are elevated in these cells, this supports the notion that the absence of TTP might lead to a shift in the balance for the benefit of prosurvival signaling. This is supported by the fact that TTP k.o MEF grow faster compared to wt cells, as well as by the specific upregulation of a subset of NF-κB dependent genes that are all connected to the regulation of cell proliferation or apoptosis, as discussed above.

The proapoptotic activity of TTP was already described earlier [119, 120] as well as the potential of the TTP_{ZnN} domain to induce programmed cell death synergistically with TNFα in a variety of cell types but the underlying regulatory mechanism(s) remained elusive. Interestingly, we find that MEKK1-TRAF2 mediated posttranslational modifications of TTP occur in its ZnN-terminal region, which further lead to an obvious decrease of cell viability in HEK 293 cells, supporting the idea of a specific TTP-MEKK1-TRAF2 triggered onset of proapoptotic events. Furthermore, high levels of ectopically expressed TTP lead to TNFα independent apoptosis in primary HUVEC, whereas low levels of TTP induce apoptosis exclusively in synergism with TNFα, which is in addition strongly dependent on the duration of TNFα induction. Together this seems suggestive of a scenario where TTP might be tightly regulated by MEKK1-TRAF2 controlled posttranslational modifications that determine at least in part its proapoptotic activity.

Taken together, these observations reveal an additional, and, to our knowledge, novel activity for TTP. To date we can not completely exclude that TTP triggers the activation or repression of other ARE binding proteins through protein-protein interactions, or that the loss of function mutation in the zinc-finger concerns only a subset of ARE containing targets. In this regard a recent finding revealed that TTP does not exclusively bind to ARE sequences, but additionally recognizes a non-ARE cis element in the MHC class I mRNA [121]. However, 3'-UTR analysis of this mRNA demonstrated a binding deficiency of the zinc-finger mutant as well, suggesting that the loss of function is not restricted to a certain ARE motif.

In addition, the finding that the MAP3K MEKK1 leads to direct or indirect phosphorylation of TTP promoting its subsequent K63-linked polybuiquitination, finally affecting cell viability, could be important *in vivo* in multiple ways. Together with its well-described mRNA destabilizing function these complex relationships suggest that TTP may contribute to the regulation of NF-κB-mediated responses at least on two levels: through the destabilization of cytokine mRNAs (e.g. TNFα), and through acting as specific "balancer" between the choice of either NF-κB dependent survival and differentiation, or death via TNFα induced apoptosis (Fig. 20T). Therefore TTP might be indispensable for the appropriately regulated

negative feedback control during a variety of NF-κB-dependent pathophysiological situations and serves as a perfect target for specific therapeutic interventions.

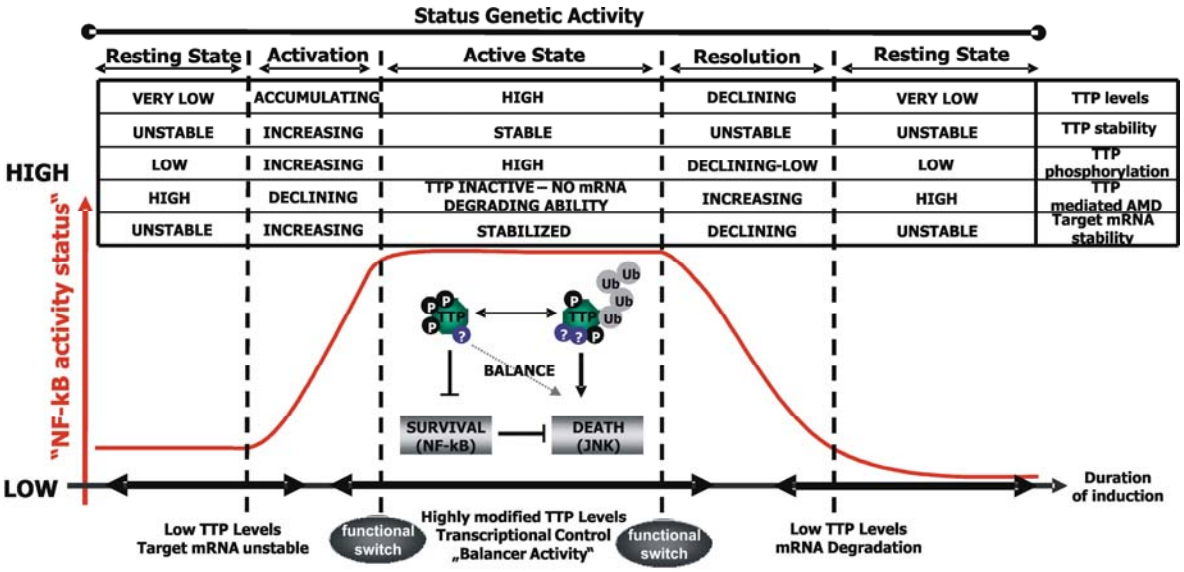


Fig. 20T: Model: TTP as "bifunctional" balancer in the TNFα induced NF-κB response. NF-κB activity can be separated into different activation states upon cytokine induction (red line), as well as in different TTP-regulated phases (Table on top). In the uninduced state the basal levels of gene expression as well as TTP itself are kept low. Upon TNFα stimulation NF-κB -mediated transcription is enhanced, mRNAs are stabilized which is achieved through the activation of kinases as e.g p38α that mediate phosphorylation of TTP leading to a block of its RNA degrading ability. Hyperphosphorylated TTP accumulates, the protein is stabilized and NF-κB-target genes are unaffectedly expressed, mediating an appropriate cellular response. At this phase a TTP-functional switch might occur, at least partly regulated by posttranslation modifications as ubiquitination and/or other modifications, resulting in the transcriptional control of NF-κB and its appropriate crosstalk with apoptotic JNK signals. This controlled balance might constitute a "basic-support-mechanism" during the active state of inflammation and might finally facilitate the unhindered, correct termination of the response. In the end the removal of TTP-modifications, through for example phosphatases like PP2A [51], results in a functional reversion and in high levels of "unmodified" TTP that finally, following the "back-to-the-RNA-degrading-roots"-principle, facilitate the rapid decrease of proinflammatory mRNAs leading to the resolution of the response reconstituting the initial, basal state of the healthy cell.

5. MATERIALS AND METHODS

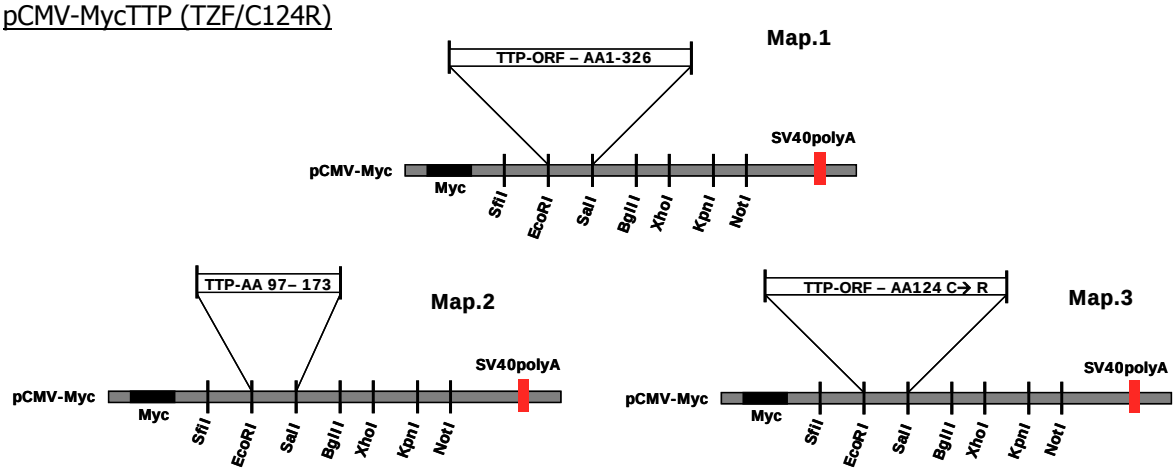
5.1. Plasmids & Cloning

5.1.1. Plasmids

Reporter plasmids 5xNF-κB-luc, p53-luc, NFAT-luc, pFR-luc as well as pFc-MEKK were obtained from Stratagene (PathDetect). IL-8-luc, the truncated version of the IL-8 promoter, TF-luc as well as expression vectors for p65, IKK2 and TAK1 have been described elsewhere [122-124]. TAB1 was purchased from Invitrogen. The Gal4-p65 mutant constructs (p65*: p65 aa286-551; M1: p65 aa286-520, M2: p65 aa521-551; M3: p65 aa450-520) were kind gifts from L. Schmitz [125] as well as the pRC/CMVp65 plasmid used for generation of p53NLSΔNLSp65. SV40NLSp65 and SV40NLSΔNESp65 were gifts from Shao-Cong Sun [126]. Expression plasmids for TRAF2 and CBP/p300 were kindly provided by H. Wajant and C.Brostjan, respectively. The wt-HA-MEKK1 expression vector as well as mutant constructs of MEKK1 (pCEP4 HA-MEKK1 D1369A and HA-MEKK1 C433A/487A) were obtained from Tony Hunter's lab [127], IKKγ was provided by S. Gosh and the pMT123 HA-Ubiquitin-Octamer and His-tagged-ubiquitin expression vectors were made available by D. Bohmann and are described elsewhere [128]. The basic vectors pAdEasyTetOFFrev as well as pShuttle TripTet-Lac-BamHI, used for the generation of the TTP-Adenovirus, were kindly provided by G. Akusjärvi [109].

5.1.2. Cloning and Maps

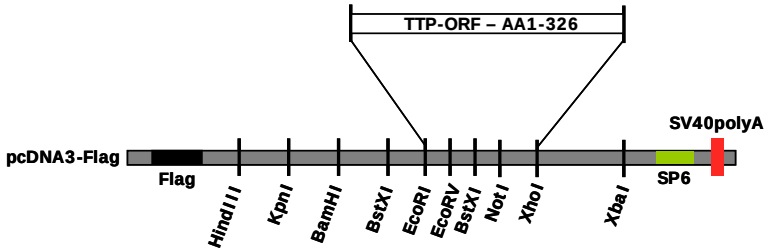
pCMV-MycTTP (TZF/C124R)



pCMV-MycTTP (Map.1) was generated by PCR from the RZPD clone IRAK p961M0614Q2 and ligated into

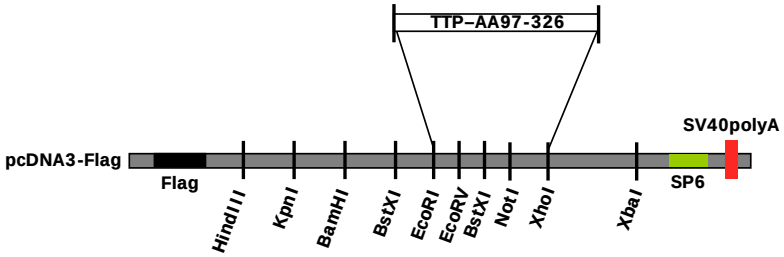
the *EcoRI/SalI* sites of pCMV-myc (Clontech). pCMV-MycTZF (aa97-173; Map.2) and pCMV-MycC124R (Map.3) were generated by subcloning and in vitro mutagenesis, respectively.

pCDNA3-Flag-TTP **(Map.4)**



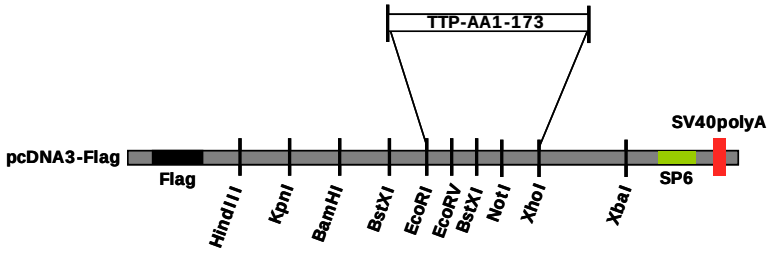
The coding sequence of human TTP, corresponding to AA 1-326, was amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP 1-326 fwd/rev (see 5.1.3). The 926bp PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

pCDNA3-Flag-TTP_{ZnC} **(Map.5)**



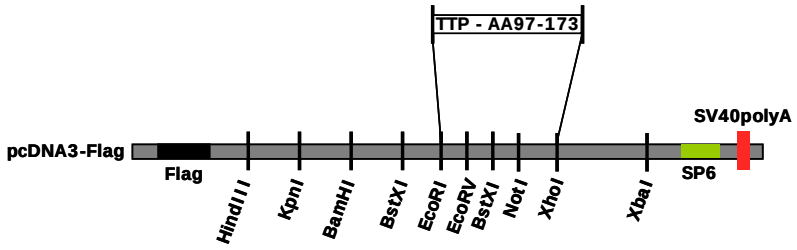
Amino Acids 97-326 of the human TTP coding sequence were amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP 289-981 fwd/rev (see 5.1.3). The ~700bp PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

pCDNA3-Flag-TTP$_{ZnN}$ **(Map.6)**



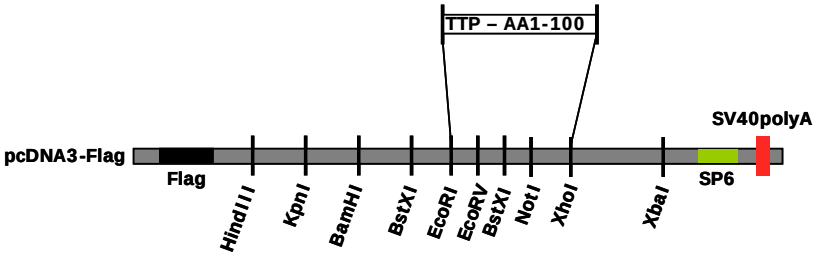
Amino Acids 1-173 of the human TTP coding sequence were amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP 1-519 fwd/rev (see 5.1.3). The ~500bp PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

pCDNA3-Flag-TTP$_{TZF}$ **(Map.7)**



Amino Acids 97-173 of the human TTP coding sequence were amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP-TZF fwd/rev (see 5.1.3). The ~230bp PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

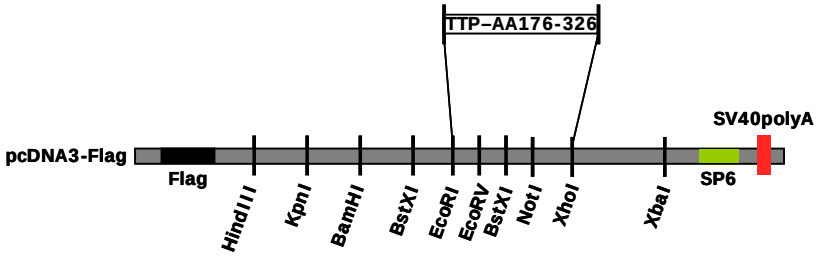
pCDNA3-Flag-TTP$_{Nterm}$ **(Map.8)**



Amino Acids 1-100 of the human TTP coding sequence were amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP-Nterm fwd/rev (see 5.1.3). The ~300bp PCR product was ligated

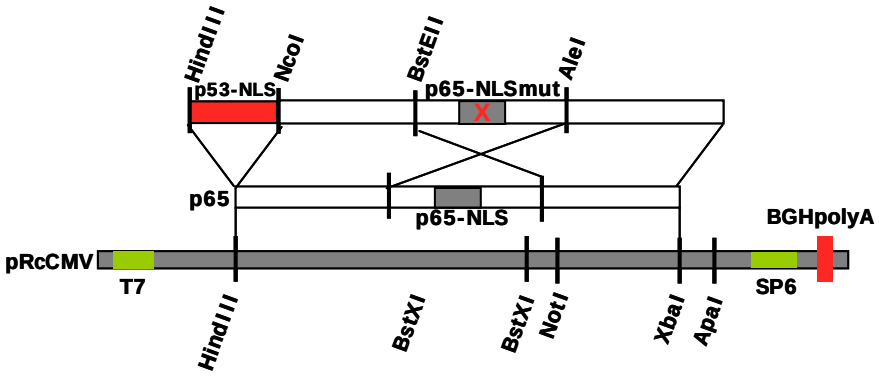
into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

pCDNA3-Flag-TTP$_{Cterm}$ **(Map.9)**



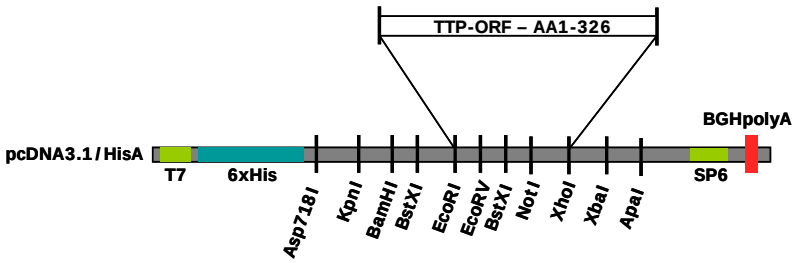
Amino Acids 176-326 of the human TTP coding sequence were amplified by PCR from the vector pCMV-MycTTP (Map.1) using the primers TTP-Cterm fwd/rev (see 5.1.3). The ~470bp PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *EcoRI* and *XhoI* and ligation into the respective sites of pcDNA3-Flag.

pRC_CMVp53NLSΔNLSp65 **(Map.10)**



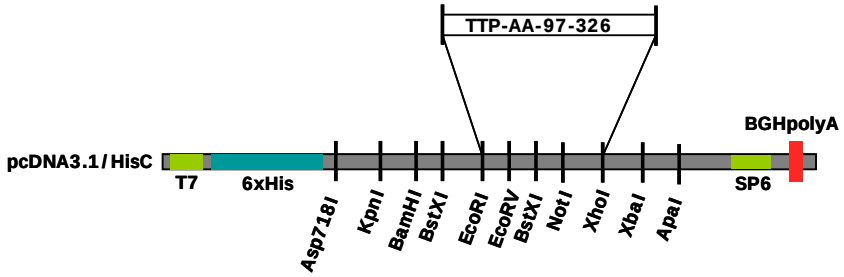
A synthetic p53NLS linker (*5'HindIII-3'NcoI*; see 5.1.3) was fused to the N-terminus of p65. The p65 NLS sequence was cut out by *BstEII-AleI* restriction and exchanged with the same fragment from the pCDNA3.1/Myc-Hisp65NLSmut vector kindly provided by R. Fagerlund [71].

pCDNA3.1-6xHis-TTP **(Map.11)**



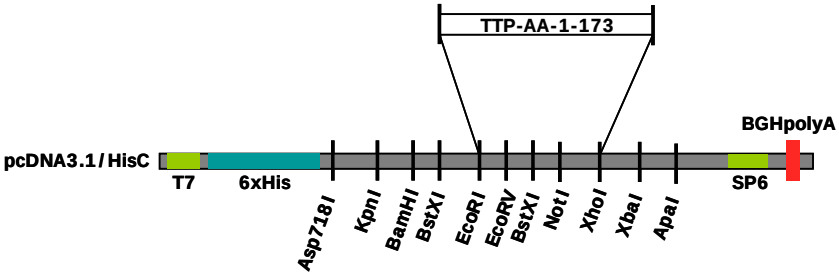
The human TTP coding sequence was obtained by *EcoRI* and *XhoI* restriction of the vector pCMV-MycTTP (Map.1) and ligated into the respective sites of the pCDNA3.1-HisA vector purchased from Invitrogen.

pCDNA3.1-6xHis-TTP_{ZnC} **(Map.12)**



Amino Acids 97-326 of the human TTP coding sequence were cut out of the vector pCDNA3-Flag-TTP_{ZnC} (Map.5) using *EcoRI/XhoI* restriction. The ~700bp fragment was ligated into the respective sites of the pcDNA3.1-HisC vetor (Invitrogen).

pCDNA3.1-6xHis-TTP_{ZnN} **(Map.13)**



Amino Acids 1-173 of the human TTP coding sequence were cut out of the vector pCDNA3-Flag-TTP_{ZnN} (Map.6) using *EcoRI/XhoI* restriction. The ~500bp fragment was ligated into the respective sites of the pcDNA3.1-HisC vector (Invitrogen).

5.1.3. Primer Sequences

TTP 1-326 fwd (*EcoRI*)	5'-CGA ATT CAT GGA TCT GAC TGC-3'
TTP 1-326 rev (*XhoI*)	5'-CGC TCG AGT CAC TCA GAA A-3'
TTP 289-981 fwd (*EcoRI*)	5'-GAA TTC ACC TCC ACC ACC C-3'
TTP 289-981 rev (*XhoI*)	5'-CTC GAG TCA CTC AGA AAC AGA-3'
TTP 1-519 fwd (*EcoRI*)	5'-GAA TTC ATG GAT CTG ACT GCC ATC-3'
TTP 1-519 frev (*XhoI*)	5'-CTC GAG TTA CGC CAG GTC TTC G-3'
TTP-TZF fwd (*EcoRI*)	5'-GAA TTC ACC TCC ACC ACC CCC T-3'
TTP-TZF rev (*XhoI*)	5'-CTC GAG TTA CGC CAG GTC TTC GC-3'
TTP-Nterm fwd (*EcoRI*)	5'-GGA ATT CAT GGA TCT GAC TGC C-3'
TTP-Nterm rev (*XhoI*)	5'-CGC TCG AGT CAG GTG GTG-3'
TTP-Cterm fwd (*EcoRI*)	5'-GAA TTC GGC CAC CCT CCT-3'
TTP-Cterm rev (*XhoI*)	5'-CGC TCG AGT CAC TCA GAA AC-3'

p53NLS linker

p53NLS sense:

5'- AGC TTC CAC CAT GAA GCG AGC ACT GCC CAA CAA CAC CAG CTC CTC TCC CCA GCC AAA GAA GAA ACC AGC - 3'

p53NLS antisense:

5'- CAT GGC TGG TTT CTT CTT TGG CTG GGG AGA GGA GCT GGT GTT GTT GGG CAG TGC TCG CTT CAT GGT GGA - 3'

5.2. Cell Culture

5.2.1. Cell maintainance and Media

HEK 293 cells and HeLa cells were obtained from ATCC, wild-type (wt) and TTP k.o MEF were kindly provided by PJ Blackshear. Cells were cultured in DMEM (Bio-Whittaker) supplemented with 10% FCS (Sigma), 2mM L-glutamine (Sigma), penicillin (100units/ml), and streptomycin (100µg/ml). Human

umbilical vein endothelial cells were isolated from umbilical cords as described [129] and maintained in M199 medium (Lonza) supplemented with 20% FCS (Sigma), 2mM L-glutamine (Sigma), penicillin (100U/ml), streptomycin (100µg/ml), and 0.25µg/ml fungizone, 5U/ml Heparin, and 25µg/ml ECGS (Promocell).

5.2.2. Cytokines and Inhibitors

Recombinant human and mouse TNFα were purchased from R&D systems and Biosource respectively. They were stored as 10 µg/ml stock in phosphate buffered saline (PBS) with 0.1% bovine serum albumine (BSA, Sigma) and used at a final concentration of 10ng/ml. IL-1 and LPS were stored in PBS/0.1%BSA and used at final concentrations of 10µg/µl and 500µg/ml respectively. The α-TNFα blocking antibody (R&D systems, AF-410-NA) was used at a concentration of 5µg/ml.
The proteasome inhibitor MG132 (Affinity) was stored in DMSO as 10mM stock solution and used at a final concentration of 10µM. The MAPK inhibitors SB203580 (specific inhibitor of p38 MAPK), SP600125 (selective JNK inhibitor) and PD98059 (specific inhibitor of MAP2K MEK1) were obtained from Tocris Bioscience. They were kept as 10mM stock solutions in DMSO and used at final concentrations of 10-30µM as indicated. The transcriptional inhibitor ActinomycinD was stored as 1mg/ml stock in DMSO and used at a final concentration of 10µg/ml.

5.2.3. Transfection Methods and Reporter Gene Analysis

HEK 293 cells were transfected by the calcium phosphate method as described [130]. MEF as well as HUVEC were transfected using the polyethylenimine (PEI) method [131].
For reporter gene assays, HEK 293 cells were grown in 24-well plates, MEF and HUVEC in 12-well plates. Cells were transfected as described with the indicated reporter and/or expression plasmids using 4µg total DNA. Cells were stimulated with TNFα (10ng/ml) for 16 hours.
Cells were lysed 45-48 hours post transfection in 1xPassive Lysis buffer (Promega) for 30-45' at RT. Lysates were analyzed for luciferase activity, which was normalized to β-galactosidase or GFP expression. The reaction mix for luciferase measurements contained 25mM Glycin-Glycin pH7.8, 10mM $MgSO_4$, 2mM ATP, the injection mix was comprised of 25mM Glycin-Glycin pH7.8 and 0.2mM D-Luciferin (Roche) and measurements were accomplished using a Luminometer (Lumat, Berthold). β-galactosidase activity was determined by mixing the cell lysate with chlorophenolred-β-D-galactopyranoside (CPRG, Roche, 0.05%

w/v in PBS with 0.1% w/v BSA) followed by absorbance-measurement at 570nm in an ELISA reader (SLT 340ATCC). GFP levels were determined by excitation-emission measurements (485nm/535nm) in the Wallac Victor^2 1420 Multilabel Counter.

All experiments were performed in triplicates, and are representative for at least three independent experiments. Luciferase values were normalized for cotransfected β-gal or GFP and are illustrated as mean fold induction. Error bars represent standard deviation of the mean.

5.2.4. siRNA mediated knock down

To elucidate the effect of diminished TTP levels on NF-κB promoter activity in HUVEC, we have used RNA interference. Therefore cells were grown in 12-well plates and transfected with the indicated reporter plasmid together with the stealth custom siRNA pool* for Zfp36 (Invitrogen, see Table I for sequences) using the PEI method.

Table I: sequences of siRNA-oligos targeting human Zfp36

Zfp36_1*	5'-ACA AGA CUG AGC UAU GUC GGA CCU U-3'
Zfp36_2*	5'-UGG AUC UGA CUG CCA UCU ACG AGA G-3'
Zfp36_3*	5'-CCU GGG AUC CGA CCC UGA UGA AUA U-3'
scrambled control	5'-ACA AGA GUG UGC AAU CUC GGA GCU U-3'

*pool of 3 oligos

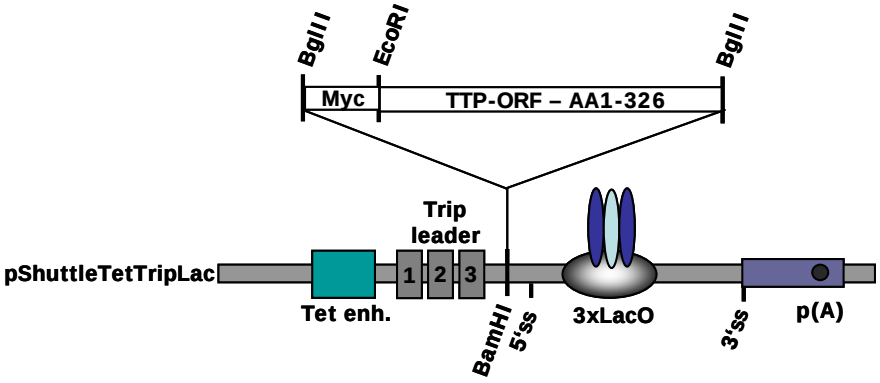
Cells were harvested 48 hours post transfection and lysed in 1xPassive Lysis buffer (Promega) followed by analysis of Luciferase and GFP activities as described in 5.2.3.

Luciferase values were normalized for cotransfected GFP and are illustrated as mean fold induction. Error bars represent standard deviation of the mean.

5.3. Recombinant TTP-Adenovirus

5.3.1. Cloning and Recombination

Cloning of pShuttleTripTetlac-MycTTP vector **(Map.14)**



The human TTP coding sequence, N-terminally fused to a Myc-Tag, was amplified by PCR out of the vector pCMV-MycTTP (Map.1) using the primers *BglII*-TTPfwd (5'-CGA GAT CTC CAT GGC ATC AA-3') and *BglII*-TTPrev (5'-GCA GAT CTT CAC TCA GAA ACA GA-3'). The ~1kb PCR product was ligated into pCRII-Topo (Invitrogen) followed by restriction with *BglII* and ligation into the *BamHI* site of the pShuttleTripTetlac-BamHI vector.

Generation of recombinant AdEasyTetOFF-MycTTP

To produce the recombinant AdEasyTetOFF-MycTTP plasmid, the pShuttleTripTetlac-MycTTP (Map.14) vector was linearized with *PmeI* and cotransformed with the pAdEasyTetOFFrev (Berenjian, 2006) plasmid into BJ5183 bacterial cells via electroporation using the Biorad gene pulser. Settings were as follows: 200Ω, 2.5kV,25µF. Freshly electroporated cells were incubated in 1ml LB medium at 37°C while shaking at 250rpm for 1 hour followed by plating onto LB-Kanamycin (50µg/ml) plates. Positive Clones were confirmed by *PacI* restriction digest and sequencing.

5.3.2. Virus propagation in HEK 293lac cells

HEK 293lac cells [109] were grown in 6-well plates to 50% confluency and transfected with *PacI* linearized AdEasyTetOFF-MycTTP using the PEI transfection method. Cells were grown in the presence of 4nM Doxycycline until plaque formation. To obtain a crude virus-lysate, cells were collected, washed with PBS and the cell pellet was resuspended in 1xHE buffer followed by 3x freeze-thawing in liquid nitrogen. The intact TTP sequence was confirmed at this stage by direct PCR on the crude virus lysate. For generation of the "concentrated, high titer" – adenovirus stock, HEK 293lac cells were grown on 40x 15cm dishes in the presence of 4nM Doxycycline to ~70% confluency. Cells were infected with 2µl of crude viral-lysate/dish and left until plaque formation. Pure virus was prepared as follows: infected cells were harvested in PBS, washed once and resuspended in 1xHE buffer. The cell-suspension was subjected to 3 freeze-thaw cycles followed by a 1.45g/cm³-1,33g/cm³ CsCl step gradient centrifugation as described elsewhere [132, 133]. Subsequently, the accumulated virus was collected and dialysed twice against 0.5l of 20mM HEPES/50mM NaCl/40% glycerol at 4°C. The purified virus-stock was stored in aliquots at -80°C.

1xHE-buffer:
10mM HEPES
1mM EDTA
pH 7.5

CsCl-gradient:
1.33g/cm³ CsCl in 1xHE
1.45g/cm³ CsCl in 1xHE

5.3.3. Adenoviral Infection of HUVEC and FACS analysis

To study a potential effect of TTP overexpression on programmed cell death in primary HUVECs, cells were grown on 6-well plates to ~90% confluency and infected with the AdEasyTetOFF-MycTTP Adenovirus (MOI=250). Infection was performed in M199 complete medium (see 5.2.1) for 4-6 hours in the presence or absence of 4nM doxycycline. Infected cells were stimulated with hrTNFα (10ng/ml final concentration) for the indicated time points and harvested at the latest 48 hours post infection. After trypsinization, the pellet was washed once followed by resuspension in 1xAnnexin binding buffer.
For the assessment of apoptosis the resuspended cells were double-stained using 10 µL Annexin V-PE (BD Pharmingen; 1:10 diluted in 1xAnnexin Binding Buffer) and 5µl 7-AAD (eBioscience). Subsequently, the

samples were incubated for 15 minutes in the dark prior to analysis in the flow cytometer (BD - Excalibur). Data were analyzed using the CellQuestPro program.

10xAnnexin binding buffer:

0.1 M Hepes
140mM NaCl
25mM $CaCl_2$
pH=7.4

5.4. Cell Extracts

5.4.1. Total Protein Purification

Generation of cell lysates using 1xPassive Lysis Buffer

Cells were rinsed in PBS and collected by centrifugation, the pellet was resuspended in 1xPassive Lysis Buffer (Promega) supplemented with protease inhibitors (Complete Protease Inhibitor cocktail tablets, Roche) and incubated for 30 min on ice. Subsequently, insoluble fractions were removed by centrifugation at 13000rpm for 3 minutes at 4°C. 5xLämmli loading buffer was added to supernatants prior to loading on sodium dodecyl sulfate-polyacrylamide-gels and Western Blotting.

Generation of cell lysates using Lämmli sample buffer

Cells were rinsed in PBS, the pellet was resuspended in 2xLämmli Buffer and subjected to 3 freeze-thaw cycles (liquid nitrogen, 95°C) prior to loading on sodium dodecyl sulfate-polyacrylamide-gels and Western Blotting.

5xLämmli buffer
50% (v/v) Glycerin
10% (w/v) sodium dodecyl sulphate
0.4M Tris-HCl pH 6.8
5mg Bromphenol blue
10% (v/v) β-mercaptoethanol (Fluka) was freshly added before use

5.4.2. Cell Fractionation

For p65 nuclear translocation and IκBα degradation, cells were grown and transfected in 6cm (HEK 293) or 10cm dishes (MEF). 48 hours post transfection cells were stimulated with 10ng/ml TNFα (mouse or human rTNFα, R&D systems) for the indicated time points. Cells were harvested and cytoplasmic and nuclear extracts were prepared as follows: cells were washed once with PBS followed by centrifugation at 3000rpm for 3 minutes. Pellets were rinsed with ice cold buffer A prior to lysis in buffer A for 10 minutes on ice. Subsequently, cell membranes and nuclei were pelleted by centrifugation at 7000rpm for 10 minutes. Supernatants (cytosolic extracts) were mixed with 5xLämmli buffer and heated 5 minutes to 95°C prior to loading on sodium dodecyl sulfate-polyacrylamide-gels. Remaining pellets were washed with PBS and buffer A followed by extraction of nuclear proteins with buffer C 30 minutes on ice. After centrifugation at 14000rpm for 15 minutes the supernatants (nuclear extracts) were supplemented with 5xLämmli buffer and treated as described for cytoplasmic extracts.

Buffer A

10mM Hepes, pH 7.9
1.5mM $MgCl_2$
10mM KCl
1mM DTT
Complete Protease Inhibitor Tablet (Roche)
1mM Na_3VO_4

Buffer C

20mM Hepes, pH 7.9
0.42M NaCl
1.5mM $MgCl_2$
0.2mM EDTA
1mM DTT
25% glycerol
1mM Na_3VO_4

5.4.3. Alkaline phosphatase treatment of Cell Lysates

For *in vitro* dephosphorylation of TTP cells were grown on 12-well plates and transfected with a total amount of 2µg DNA/well. 24 hours post transfection cells were harvested in PBS and lysed in 50µl 1xPassive Lysis Buffer (Promega) for 30 minutes on ice. Lysates were divided and one half was treated with 20U calf intestine alkaline phosphatase (Roche) for 15 minutes at 37°C. Subsequently, 5xLämmli loading dye was added to all lysates before heating to 95°C and loading on 5-12.5% sodium dodecyl sulfate-polyacrylamide-gradient gels.

5.5. Western Analysis and Densitometry

Purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Hybond-C, Amersham) or PVDF (Millipore) membranes by semidry blotting (Peqlab Semidry Blotter) (for conditions see Table II). After blotting, membranes were stained with PonceauS solution (Serva) to evaluate loading and subsequently blocked in 5% non fat dry milk in PBS/0.1% Tween-20 (Amersham) for 1h at room temperature before incubation with the first antibody (in blocking solution; see Table III) over night at 4°C. Secondary antibodies (HRPO-labeled, Amersham; see Table IV) were incubated in 1% non fat dry milk in PBS/0.1% Tween-20 for 1 hour at room temperature after washing (3x 5 minutes at room temperature) in PBS/0.1% Tween-20. For phospho-specific antibodies blocking- as well as incubation-solutions contained tris-buffered saline (TBS) instead of PBS.
Visualization of Immunocomplexes was carried out by chemiluminescence (Super Signal West Pico, Pierce or ECL Detection Reagents, Amersham) on SuperRX films (Fujifilm).

Table II: SDS-PA gel conditions

Resolution gel	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**7.5% gradient to 15%***	
Aqua des.	5.75ml	4,95ml	4.1ml	3.3ml	2.45ml	2.5ml	1.25ml
Solution A	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	1.25ml	1.25ml
Solution B	1.7ml	2.5ml	3.4ml	4.2ml	5.0ml	1.25ml	2.5ml
APS 10%	50µl	50µl	50µl	50µl	50µl	25µl	25µl
TEMED	10µl	10µl	10µl	10µl	10µl	5µl	5µl

* gradient gels were prepared using a gradient gel mixer

Stacking Gel	**4%**
Aqua dest.	3.1ml
Solution Ast	1.25ml
Solution B	0.65ml
APS 10%	50µl
TEMED	10µl

Solution A
1.5M Tris-HCl
0.4% SDS
pH 8.8

Solution Ast
0.5M Tris-HCl
0.4% SDS
pH 6.8

Solution B
Acrylamide/Bis-Acrylamide
(37.5 :1; Serva)

APS

10% (w/v) Ammoniumperoxydisulphate (Sigma) in aqua dest.

TEMED

N, N, N', N' – Tetramethylen-ethylendiamine (Fluka)

10xRunning buffer

250mM Tris-Base

2M Glycine

1% SDS

1xTowbin Blotting Buffer

25mM Tris-Base

192mM Glycine

1% SDS

pH 8.3

5.5.1. Primary (Table III) and Secondary (Table IV) Antibodies

Table III: Primary Antibodies used for Western Analysis

Specificity	Provider	Cat. Number	source	dilution
TTP	Pavel Kovarik	[134]	rabbit	1:2000
TTP N term	Santa Cruz	sc-8458	goat	1:1000
IκBα (C-15)	Santa Cruz	sc-203	rabbit	1:1000
p65 (A)	Santa Cruz	sc-109	rabbit	1:1000
SP1 (PEP2)	Santa Cruz	sc-59	rabbit	1:500
β-Actin (I-19)	Santa Cruz	sc-1616	goat	1:1000
HA-Tag (probe Y-11)	Santa Cruz	sc-805	rabbit	1:1000
Flag-Tag (M1)	Sigma	F3165-1M6	mouse	1:2000
Myc-Tag (9E10)	Santa Cruz	sc-40	mouse	1:1000
His-Tag (Pentamer)	Qiagen	#34660	mouse	1:2000
Phospho- IκBα (Ser32)	Cell Signaling	#9241	rabbit	1:1000
Phospho-p38 (Thr180/Tyr182)	Cell Signaling	#9211	rabbit	1:1000
Phospho-JNK (Thr183/Tyr185)	Cell Signaling	#9251	rabbit	1:1000
JNK	Cell Signaling	#9252	rabbit	1:1000
MEKK1 (43-Y)	Santa Cruz	sc-437	rabbit	1:500
p42/44 MAPK (ERK)	Cell Signaling	#9102	rabbit	1:1000
Phospho-ERK1/2 (Thr 202/ Tyr 204)	Cell Signaling	#9106	mouse	1:1000
GAPDH	Chemicon	MAB 374	mouse	1:500 000

Table IV: Secondary Antibodies used for Western Analysis

Specificity	Provider	Cat. Number	source	dilution
α-goat IgG-HRP	Santa cruz	sc-2020	donkey	1:5000
α-rabbit IgG-HRP	GE Healthcare	NA934V	donkey	1:5000
α-mouse IgG-HRP	GE Healthcare	NA931V	sheep	1:5000

5.5.2. Densitometry

Densitometric analysis of nuclear p65 levels was done using the background-corrected integrated densities of the p65 bands normalized to the SP-1 bands assessed by the ImageJ software freely available at http://rsb.info.nih.gov/ij/.

5.6. Immunocytochemistry

Cells were seeded onto fibronectin-coated Lab Tek II Chamber Slides (Nunc), transfected with either empty vector or mycTTP and 24 hours later stimulated with TNFα (20ng/ml) for the indicated times. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma). Immunostaining of p65 was done using the rabbit-α-p65 antibody (sc-109; Santa Cruz Technology; 1:500), mycTTP was detected with mouse-α-myc antibody (sc-40, Santa Cruz) followed by goat-α-rabbit Alexa 488 (molecular probes) and APC goat-α-mouse (BD Biosciences) secondary reagents at dilutions of 1:5000 and 1:500, respectively. For translocation of NFAT, cells were transfected with GFP-NFATc and mycTTP as described above and stimulated with Ionomycin (500mM final conc.; Sigma).

5.7. *In vivo* ubiquitination Assay

To detect ubiquitinated proteins in mammalian cells, HEK 293 cells were grown in 6-well plates to a confluency of ~80%. They were transfected using the calcium-phosphate method with 1µg His-tagged Ubiquitin (wild type, K48R or K63R mutants) together with 1µg Flag-tagged TTP or 1.5µg of the Flag-tagged constructs TTP_{ZnC} and TTP_{ZnN} , and pFcMEKK (plasmid containing the MEKK1 kinase domain; Stratagene, PathDetect – *trans* reporting systems) and HA-tagged TRAF2 and filled up with empty vector

to 4µg of total DNA. 24-36 hours post transfection cells were harvested and ubiquitinated proteins were detected according to the Tansey lab protocol available at http://tanseylab.cshl.edu/protocols.html. Briefly, cells were harvested in PBS and 10% of the cell suspension was separated and mixed with 5xLämmli loading dye to serve as Input control. The remaining cells were resuspended in 1ml of denaturating buffer A, mixed carefully and subjected to sonication for 15 seconds at maximal intensity. After centrifugation, the supernatant was incubated with 50µl of Ni-NTA agarose (equilibrated in buffer A; Qiagen) for 3 hours at room temperature while constantly rotating. Subsequently, beads were washed two times with buffer A, two times with buffer A/TI and once with buffer TI. Finally, the supernatant was removed carefully and beads were resuspended in 40µl 2xLämmli loading buffer containing 0.5M Imidazol, heated for 10 minutes at 95°C and 30µl were loaded on SDS-Polyacrylamide gels.

Buffer A	Buffer TI	Buffer A/TI
6M Guanidine Hydrochloride	25mM Tris-HCl	1 vol. Buffer A
0.1M Na_2HPO_4/NaH_2PO_4	20mM Imidazole	3 vol. buffer TI
10mM Imidazole	pH 6.8	
pH 8.0		

5.8. Co-Immunoprecipitation

For the elucidation of protein-protein interactions, HEK 293 cells were grown in 6-well plates and transfected with expression plasmids containing the tagged genes of interest at a confluency of ~80%. 24-48 hours post transfection, cells were resuspended in 1xLysis Buffer containing 300mM NaCl, 20mM Tris-HCl pH 8.0, 1mM EDTA, 0.5% Triton X-100, 0.3% NP-40 as well as protease inhibitors (Complete Cocktail, Roche) for 30 minutes on ice. 5-10% of the cell lysates were mixed with 5xLämmli buffer and served as Input control later on. Remaining lysates were centrifuged for 15 minutes at full speed at 4°C, followed by the over night incubation of the supernatants with the respective antibodies prebound (4 hours at 4°C) to Protein A/G sepharose (Santa Cruz) according to the manufacter's instructions. Subsequently, sepharose beads were washed three times with lysis buffer, the supernatant was removed carefully, remaining beads were resuspended in 2xLämmli buffer and finally subjected to electrophoresis on SDS-polyacrylamide-gels.

5.9. Gene expression Analysis

5.9.1. Preparation of total RNA

Total RNA was purified from cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. Briefly, cells were harvested in PBS and mixed with Lysis/-Binding buffer. Subsequently, the cell lysates were applied to High Pure Filter tubes followed by a centrifugation step at 8000rpm and a direct "on-column" DNAse digestion. The filter tube was washed once with wash buffer I and two times with wash buffer II followed by the elution of total RNA with nuclease-free, sterile, double distilled water.

5.9.2. cDNA Synthesis

Total RNA was subjected to reverse transcription using the GeneAmp RNA PCR kit (Applied Biosystems). Routinely, 1µg of RNA was reverse transcribed in a total volume of 20µl using MuLV reverse transcriptase and random hexamers as primers.

5.9.3. Real-time PCR analysis

For quantitation of gene expression, total RNA was isolated from either HEK 293 cells or MEFs grown in 6-well plates as described under 5.9.1. 1µg RNA was reverse transcribed and real time PCR was performed using 2.5µl of 1:10 diluted cDNA (corresponding to 12,5ng) as template. The PCR was performed using the Roche LightCycler utilizing the SYBR green detection method. Experiments were done in triplicates, the relative amount of mRNA was calculated using the Pfaffl method and normalization to β2-microglobulin. Error bars represent standard deviation of the mean. Primer sequences are given in Table V.

Table V: Real-time PCR primer-sequences

qPCR primer	Sequence 5'-3'
human p65 fwd	CTA CGA CCT GAA TGC TGT GC
human p65 rev	GCC AGA GTT TCG GTT CAC TC
mouse p65 fwd	GAG CCC ATG GAG TTC CAG TA
mouse p65 rev	CAA AGT TGA TGG TGC TGA GG
human b2mg fwd	GAT GAG TAT GCC TGC CGT GTG
human b2mg rev	CAA TCC AAA TGC GGC ATC T
mouse b2mg fwd	ATT CAC CCC CAC TGA GAC TG
mouse b2mg rev	TGC TAT TTC TTT CTG CGT GC
human TTP fwd	GGA TCT GAC TGC CAT CTA CGA
human TTP rev	CGG GAG GTG ACC CCA GAC
human TNFα fwd	CCC CAG GGA CCT CTC TCT AA
human TNFα rev	GCT TGA GGG TTT GCT ACA ACA
mouse MCP-1 fwd	AGG TCC CTG TCA TGC TTC TG
mouse MCP-1 rev	TCT GGA CCC ATT CCT TCT TG
mouse Fth1 fwd	CCT GCA GGA TAT AAA GAA ACC A
mouse Fth1 rev	GAA GTC ACA TAA GTG GGG ATC A
mouse Txn2 fwd	TGG ACT TTC ATG CAC AGT GG
mouse Txn2 rev	GGC ACA GCT GAC ACC TCA TA
mouse RANTES fwd	GTG CCC ACG TCA AGG AGT AT
mouse RANTES rev	GGG AAG CGT ATA CAG GGT CA
mouse Gadd45β fwd	CCT GGC CAT AGA CGA AGA AG
mouse Gadd45β rev	TGA CAG TTC GTG ACC AGG AG
mouse TRAF1 fwd	CCT GAG AGA TGA TGA GGA TCG
mouse TRAF1 rev	GCT CCC CTT GAA GGA ACA G

6. ABBREVIATIONS

AdV	adenovirus
AMD	ARE-mediated-decay
AP-1	activator protein 1
ARE	AU-rich elements
BAFF	B cell activating factor
CBP	CREB-binding-protein
CCCH	cystein-cystein-cystein-histidin
C/EBP	CCAAT enhancer binding protein
CIAP	calf intestine alkaline phosphatase
Cox-2	cycloocygenase 2
CREB	cyclic adenosine monophosphate responsive element binding protein
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FADD	fas-associated death domain
GM-CSF	granulocyte macrophage colony stimulating factor
GPCR	G-protein-coupled receptor
GSK-3	glycogen synthase kinase-3
HDAC	histon deacetylase
HEK	human embryonic kidney
HUVEC	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis
ICAM	intercellular adhesion molecule
IEG	immediate early gene
IER3	immediate early response 3
IFN	Interferon
IκB	inhibitor of κB proteins
IKK1	inhibitor of kappa B kinase 1
IKK-K	inhibitor of kappa B kinase kinase
IL	Interleukin
iNos	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
K.o	knock out
LPS	Lipopolysaccharide
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblast
MEKK1	mitogen-activated protein kinase extracellular signal-regulated kinase kinase kinase-1
MHC	major histocompatibility complex
NES	nuclear export signal
NF-κB	nuclar factor-kappaB
NIK	NF-κB - inducing kinase
NLS	nuclear localization signal
NPC	nuclear pore complex
PHD	plant homeodomain
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKCα	protein kinase C alpha
PKCβ	protein kinase C beta

PKCγ	protein kinase C gamma
PKCζ	protein kinase C zeta
PP2A	protein phosphatase 2A
RHD	Rel-homology domain
RIP	receptor-interacting protein
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
TAB1/2	TAK-binding proteins 1/2
TAD	transcriptional activation domain
TAK1	TGFβ activated kinase 1
TGFβ	transforming growth factor β
Tis	TPA inducible sequence 11
TNFα	tumor necrosis factor
TNFR	TNF-receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRADD	TNF receptor-associated death domain
TRAF	TNF-receptor-associated factor
TTP	Tristetraprolin
TZF	tandem zinc finger
UIM	ubiquitin interacting motifs
UTR	untranslated region
VCAM	vascular cell adhesion molecule
wt	wild type

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Publications

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