Novel Phosphorylation-dependent Ubiquitination of Tristetraprolin by Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase Kinase 1 (MEKK1) and Tumor Necrosis Factor Receptor-associated Factor 2 (TRAF2)*\footnote{This work was funded by Austrian Science Foundation Project TS11-B20 (to Y. M. S.) and Project P19217-B13 (to R. d. M.) and European Commission XENOME Project LSHB-CT-2006-037377 (to R. d. M.). The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Fig. S1. 1 To whom correspondence should be addressed: Dept. of Vascular Biology and Thrombosis Research, Center for Physiology and Pharmacology, Medical University of Vienna, Lazarettgasse 19, 1090 Vienna, Austria. Tel.: 43-1-40160-33123; Fax: 43-1-40160-933100; E-mail: Yvonne.Schichl@meduniwien.ac.at or yvonne.schichl@gmx.net.}

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**Background:** During inflammation, tristetraprolin displays a dual function as mRNA destabilizer and regulator of NF-κB signaling.

**Results:** Tristetraprolin is inducibly hypermodified by phosphorylation and ubiquitination, affecting cell viability.

**Conclusion:** Tristetraprolin hypermodification provokes its functional shift toward the activation of JNK signaling.

**Significance:** The NF-κB/JNK cross-talk is central for our understanding of chronic inflammatory diseases and cancer development.

Acute versus chronic inflammation is controlled by the accurate activation and regulation of interdependent signaling cascades. TNF-receptor 1 engagement concomitantly activates NF-κB and JNK signaling. The correctly timed activation of these pathways is the key to account for the balance between NF-κB-mediated cell survival and cell death, the latter fostered by prolonged JNK activation. Tristetraprolin (TTP), initially described as an mRNA destabilizing protein, acts as negative feedback regulator of the inflammatory response: it destabilizes cytokine-mRNAs but also acts as an NF-κB inhibitor by interfering with the p65/RelA nuclear import pathway. Our biochemical studies provide evidence that TTP contributes to the NF-κB/JNK balance. We find that the MAP 3-kinase MEKK1 acts as a novel TTP kinase that, together with the TNF receptor-associated factor 2 (TRAF2), constitutes not only a main determinate of the NF-κB-JNK cross-talk but also facilitates “TTP hypermodification”: MEKK1 triggers TTP phosphorylation as prerequisite for its Lys-63-linked, TRAF2-mediated ubiquitination. Consequently, TTP no longer affects NF-κB activity but promotes the activation of JNK. Based on our data, we suggest a model where upon TNFα induction, TTP transits a hypo- to hypermodified state, thereby contributing to the molecular regulation of NF-κB versus JNK signaling cascades.

Inflammation constitutes a complex process involving different cell types that release proinflammatory mediators that in turn engage cell surface receptors, leading to activation of intracellular signaling cascades and diverse cellular responses. TNFα is a pleiotropic proinflammatory cytokine that elicits proliferation, differentiation, or cell death. Binding of TNFα to its receptor, TNFR1\(^2\), initiates the recruitment of various adaptors including TRAF proteins, which trigger the onset of receptor specific proximal cascades that finally converge at the inhibitor of κB kinase (IKK) signalosome. The multiprotein complex consisting of the inhibitor of κB kinases α and β (IKK α/β) and the regulatory subunit IKKγ (NEMO) phosphorylates the inhibitor of κB α (IκBα) protein, leading to its proteosomal degradation. This results in nuclear translocation of the transcription factor NF-κB/p65 and the expression of genes encoding pro- as well as anti-inflammatory cytokines, adhesion molecules, enzymes, and antiapoptotic mediators (1). In addition to proinflammatory signaling and cell survival, NF-κB also controls the timed cross-talk with proapoptotic pathways, e.g. activation of the MAPK JNK.

One important aspect during TNFR1 signaling is the kinetic of IKK and MAPK activation. Mechanistically, the TRAF proteins, constituting E3 ubiquitin ligases, mediate the ubiquitination of other TNFR1 complex members, thereby facilitating both NF-κB and JNK activation (2). Although these cascades are concomitantly activated and share common regulatory proteins up to the level of MAP3Ks, the onset of IKKs proceeds faster than the activation of MAPKs, resulting in a temporal.

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\(\ast\) The abbreviations used are: TNFR1, TNF receptor 1; HMW, high molecular weight; HUVEC, human umbilical vein endothelial cells; IKK, inhibitor of κB kinase; LMW, low molecular weight; TIS, 12-O-tetradecanoylphorbol-13-acetate-inducible sequence 11; TRAF2, TNF receptor-associated factor 2; TTP, tristetraprolin; IKK, inhibitor of κB kinase; NEM, N-ethylmaleimide; MEF, mouse embryonic fibroblast; M1 Kin, MEKK1 kinase domain only; PHD, plant homeo domain.
separation of these signaling events (3). Accordingly, immediate early expressed NF-κB target genes constitute essential inhibitory feedback mediators as exemplified by GADD45β, which blocks the activated JNK cascade at the level of the MAP2Ks MKK4/7, thereby allowing an unhindered first wave of NF-κB activity (4, 5).

The main MAP3K that accounts for JNK activation in this context is MEKK1, a 196-kDa protein with dual function: it displays phospho-kinase activity, which is attributed to the kinase domain at its C terminus, and E3 ligase activity, because of the PHD domain at its N terminus. The PHD domain of MEKK1 mediates not only its Lys-63-linked autoubiquitination, leading to a stabilized interaction with other proteins (6), but also ERK1/2 polyubiquitination and degradation in response to stress stimuli (7). Autoubiquitination of MEKK1 strongly depends on its kinase activity, as well as the presence of TRAF2. Importantly, it is the kinase domain that accounts for TNFα-induced signaling to JNK as demonstrated by the analysis of cells obtained from MEKK1−/− mice (8, 9).

Inflammation is in principal a beneficial reaction that only becomes detrimental when it occurs in an excessive manner or is not appropriately terminated. Therefore, its resolution has to be correctly timed and controlled. Importantly, NF-κB is not only a potent initiator of inflammation but also plays an essential role during its resolution (10, 11), because it controls the expression of a set of terminators acting at different levels: at the receptor level, affecting all downstream events (12), at the level of signal transduction (13, 14) and individual transcription factors (15–17), as well as at the post-transcriptional level, preventing translation and promoting degradation of transcripts. One example for the latter is TTP, an mRNA destabilizing protein, which is involved in the degradation of proinflammatory mediators including TNFα, various interleukins, as well as enzymes, phosphatases, and adhesion molecules. It belongs to the 12-O-tetradecanoylphorbol-13-acetate-inducible sequence 11 (TIS11) family of proteins also including TIS11b, TIS11d, and ZFP36L3. All of these members have been found to contribute to mRNA destabilization. They act by binding to specific AU-rich elements in the 3′-UTR of the target mRNAs through a tandem zinc finger structure, which appears highly conserved among all TIS proteins (18, 19). TTP itself is induced by a variety of stimuli including proinflammatory as well as anti-inflammatory cytokines, serum, insulin, and growth factors and appears abundant in many different cell types, including fibroblasts, monocytes, macrophages, neutrophils, T-cells, and endothelial cells (20–23). TTP knock-out mice exhibit a classical inflammatory phenotype (24), which is mainly caused by the extensive overproduction of the cytokine TNFα. However, beside TNFα, the turnover of several other mRNAs is controlled by TTP in a stimulus a cell type-specific manner, including inflammatory mediators as interleukin-1β, -2, -3, and -6, Ccl2 and -3, Cox-2, as well as IFNγ or IL-10 (25–30). Removal of these “inflammatory mRNAs” occurs with a specific time delay indicating that TTP activity is specifically regulated. In this regard, an important aspect appears to be TTP hyperphosphorylation; however, its functional relevance remained largely elusive up to now (31). The most extensively studied TTP kinase is p38α: p38-MAPK-MK2 signaling is of particular interest in terms of cytokine stabilization, because MK2 was shown to directly phosphorylate TTP after LPS stimulation in mouse macrophages, reducing its ability to destabilize mRNAs (32–36). Vice versa, dephosphorylation by protein phosphatase 2A results in reactivation of its destabilizing ability (37). In addition to p38α, various other kinases have been shown to phosphorylate TTP in vitro and in vivo (38). These include the MAPK ERK1/2, JNK, the protein kinase family members PAK and PKC, as well as IKK1 and glykogen synthase kinase-3 (39). However, not all TTP kinases alter the function of TTP as a destabilizing protein as indicated by a study using a series of TTP mutants, wherein the single mutations of serines and/or threonines showed that phosphorylation does not necessarily impact its mRNA-degrading function (40). Recently, we and others (41) have shown that TTP does not solely act as an mRNA destabilizing protein, it also impairs NF-κB transcriptional activity by specifically interfering with the p65 nuclear import pathway and p65 transactivation, respectively. Targeting the NF-κB cascade at the level of the transcription factor itself extends its activity to selectively influence subsets of NF-κB target genes that do not contain an AU-rich element, most notably the JNK blocker GADD45β (42). Because our earlier results indicated that TTP also contributes to the onset of signals leading to JNK activation, we aimed to gain deeper insight into its role in the NF-κB-JNK signaling cross-talk. We found that the ability of TTP to differentially affect both cascades depends on specific post-translational modifications: phosphorylation by MEKK1 and subsequent TRAF2-mediated, Lys-63-linked ubiquitination resulted in a TTP functional switch away from NF-κB inhibition toward the onset of JNK signaling. This finding provides novel insight into the regulated balance between NF-κB and JNK signaling cascades on the molecular level.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cloning**—Expression vectors for FLAG-TTP, -ZnN, and -ZnC were generated using the Expand High Fidelity PCR system (Roche Applied Science). PCR fragments were subcloned into pcDNA3.1-FLAG. For other plasmids, general vector information, and primer sequences, see supplemental Tables S1 and S2.

**Generation and Propagation of TTP Adenovirus**—For TTP adenovirus generation, the human TTP coding sequence was amplified from pCMV-MycTTP using the primers BglII/MycTTP fwd/rev. The PCR fragment was subcloned into the BamHI site of pShuttleTripTetlac-BamHI. To produce the recombinant AdEasy TetOFF-MycTTP plasmid, pShuttleTripTetlac-MycTTP was linearized (PmeI) and cotransformed with pAdEasy TetOFF Frev into BJ5183 bacterial cells (43). The resulting AdEasy TetOFF-MycTTP was, after quality control, linearized with PacI, transfected into HEK 293 lac cells, and grown in the presence of 4 nM doxycycline until plaque formation. A high titer adenoviral stock was obtained as described previously (44).

**Reporter Gene Assay**—Cells were transfected with the indicated reporter and/or expression plasmids using 4 μg of total DNA/6-well. The experiments were performed in triplicate, and luciferase values were normalized to cotransfected β-galac-
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Cell Culture and Transfections—HEK 293 cells and HeLa cells were obtained from ATCC; WT, TTP KO, and MEKK1 ΔKD MEF kindly provided by P. J. Blakshewar and Ying Xia (45), respectively. The cells were cultured in DMEM (Bio-Whit-taker) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml). HUVEC were isolated from umbilical cords as described (46) and maintained in M199 medium (Lonza) supplemented with 20% FCS (Sigma), 2 mM L-glutamine (Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml), 5 units/ml heparin, and 25 μg/ml ECGS (Promocell). HEK 293 cells were transfected using calcium phosphate (47), MEF using polyethylenimine (48). For TTP adenoviral transduction, the cells were infected with AdTTP adenosivirus (multiplicity of infection = 250) for 4–6 h and analyzed 24 h after infection. For lentiviral, shRNA-mediated MEKK1 knockdown in HUVEC, 1 × 10⁵ cells were seeded and transduced with 3 × 10⁵ transducing units of MISSION lentiviral transduction particles (Sigma-Aldrich, Clone ID TRCN0000006162). 48 h post-transduction cells were induced and analyzed as described. Recombinant human (R & D systems) and mouse TNFα (BIOSOURCE) were used at a final concentration of 10 ng/ml, the proteasome inhibitor MG132 (Affinity) and the MAPK inhibitors SB203580, (R & D systems) and mouse TNFα (BIOSOURCE) were used at 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton; protease/phosphatase inhibitors, 1 mM NEM) containing 6 M urea, RNase A (3 μg/μl; Qiagen), Benzonase® (1 units/μl; Merck), and 1.5 mM MgCl₂ (denaturing lysis buffer) for 15 min at 4 °C. The lysates were diluted with lysis buffer 1/25 and precleared (1 h, protein A/G-agarose) prior to immunoprecipitation with TTP antibody prebound to protein A/G-agarose for 2 h at 4 °C. Precipitates were washed three times with lysis buffer, resuspended in denaturing buffer again, and finally diluted with Laemmli buffer prior to loading on 7.5% SDS-polyacrylamide gels.

Data Analysis—Statistical differences between samples were analyzed using the paired Student’s t test. Two-tailed probability values of <0.05, <0.01, and <0.001 were considered significant and highly significant, respectively. The p values are given within the figure legends.

RESULTS

MEKK1 Constitutes a Novel TTP Kinase—As we have shown previously, TTP impairs NF-κB activity in an AU-rich element-mediated decay-independent manner (42). The MAPK p38α induces TTP phosphorylation, traceable by a “molecular weight shift” of the protein in Western blots, inactivating TTP mRNA binding and degrading ability, but in contrast, p38α did not block TTP function toward NF-κB (supplemental Fig. S1A, left panel). When testing a set of other kinases, we found that coexpression of MEKK1, which has not been described as TTP kinase yet, could even counteract TTP inhibition of p65-included NF-κB promoter activity (Fig. 1A, left panel, and supple-mental Fig. S1A, right panel). As shown in Fig. 1B, the kinase domain of MEKK1 appeared sufficient for the rescue, whereas a kinase-deficient MEKK1 mutant led to the complete reconstitution of TTP-mediated NF-κB inhibition. Mutation of the PHD domain slightly attenuated TTP activity. Of note, p38α even counteracted MEKK1 function toward TTP (Fig. 1A and supplemental Fig. S1A), and we noticed a substantial difference in TTP protein “patterning” on Western blots after coexpression of either p38α or MEKK1. Whereas p38α expression resulted in the molecular weight shift of TTP already described earlier (49, 50), coexpression of MEKK1 led to an increase of “higher molecular weight species” (50–72 kDa, termed HMW) of TTP, accompanied by a different patterning (Fig. 1A, right panel). In line with that, the kinase-dead MEKK1 mutant inhibi-
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FIGURE 1. MEKK1 but not p38α abrogates TTP-mediated down-regulation of NF-κB activity. A, left panel, HEK 293 cells were transfected with a 5× NF-κB-luc reporter plasmid (ctrl) together with either p65 alone (−) or in combination with TTP; TTP and p38α; TTP and M1 Kin; or TTP, p38α, and M1 Kin expression plasmids as indicated. Analyses of luciferase expression revealed that, in contrast to p38α, MEKK1 reverted the inhibitory TTP effect toward NF-κB. Right panel, Western blot depicting a varying TTP pattern (α-Myc) in HEK 293 cells transfected with TTP only (−) or with different combinations of expression plasmids as indicated. UB, unspecific band serving as loading control. B, left panel, HEK 293 cells were transfected with a 5× NF-κB-luc reporter plasmid together with p65 and in combination with either M1 Kin, MEKK1 dominant negative kinase activity (M1 dnKin), or M1 dominant negative PHD (MEKK1, dominant negative E3 ligase activity). Luciferase expression was analyzed in the absence (EV, empty vector, black bars) or presence of TTP (white bars). Right panel, Western blot of transfected HEK 293 cells (ectopically expressed proteins as indicated on top) showing that MEKK1 dominant negative kinase activity (M1 dnKin) reverted the TTP electrophoretic shift resulting in LMW-species. *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, HUVECs were treated with TNFα for the indicated periods of time and analyzed for endogenous TTP expression and "patterning" revealing both LMW and HMW species of TTP (top panels). GA, grayscale adjustment for single lanes was done to highlight differences in the molecular appearance of the protein. LC, loading control.

Importantly, we observed a similar patterning of endogenous TTP after TNFα stimulation in human umbilical vein endothelial cells (HUVEC): “lower molecular weight” species (~36–50 kDa, termed LMW), for example as produced by p38α, and becomes phosphorylated by the cytosolic kinase fragment of MEKK1. To further confirm that the observed HMW species are the result of MEKK1-mediated phosphorylation, we utilized phosphatase treatment, resulting in a complete reversal of the molecular shift and reappearance of TTP comparable with the control sample (Fig. 2D). To confine the localization of the MEKK1-mediated TTP phosphorylation, we used TTP truncation constructs (scheme in Fig. 2D). The molecular weight of a ZnC-TTP-mutant was not influenced by MEKK1 coexpression, whereas the ZnN-TTP mutant had similar “shifting” characteristics as the full-length protein (Fig. 2D, middle and bottom panels, respectively). The differences in band strength and distribution can be attributed to the varying composition of the gradient polyacrylamide gels. Together, our data suggest that MEKK1 physically interacts with TTP to phosphorylate its N terminus, thereby abrogating TTP function toward NF-κB.

MEKK1-mediated Phosphorylation Serves as Basis for TRAF2-directed, Lys-63-linked TTP Ubiquitination—MEKK1 is a key player of the TNFR1-associated cytoplasmic signaling complex and acts in concert with TRAF2 to trigger activation of the JNK signaling cascade (53). Thereby, TRAF2-mediated ubiquitination processes provide the basis for stabilization and functionality of the JNK-activating complex (54). Using in vivo ubiquitin assays, we investigated whether TTP was also part of these ubiquitination events. We observed that not only TTP, but also its mRNA binding-deficient mutant (C124R), as well as the ZnN-TTP mutant were ubiquitinated when phosphorylated by MEKK1, whereas the ZnC-TTP mutant was not (Fig. 3A). Moreover, TTP ubiquitination was confirmed endogenously in MEF and HUVEC (Fig. 3B). In line with the observed MEKK1-TTP interaction, TTP polyubiquitination was detected only after TNFα stimulation in the presence of the deubiquitinase inhibitor NEM.

Because the MEKK1 mutant M1 Kin exhibits no E3 ligase activity, we investigated whether the MEKK1 interacting adap-
tor protein TRAF2 could function as E3 ligase in this context. TRAF2 coexpression indeed resulted in a strongly enhanced TTP ubiquitination that was independent of proteasome inhibition by MG132 (Fig. 3C). This suggested that TRAF2 attached ubiquitin chains were Lys-63-linked, and moreover, phosphorylation by MEKK1 was important for effective ubiquitination. These observations were further supported by the use of dominant negative MEKK1, as well as TRAF2 mutants: mutation of either MEKK1 or TRAF2 impaired whereas mutation of both completely abolished TTP ubiquitination (Fig. 3D). The expression of dominant negative MEKK1 could not be detected in the input when dominant negative TRAF2 was coexpressed (Fig. 3D, bottom panels, M1dnKin/T2dn), explained by the fact that MEKK1 stabilization depends on an intact kinase domain as well as an intact TRAF2 E3 ligase domain (3, 6, 53). The use of ubiquitin mutants additionally validated the TRAF2-mediated, Lys-63-linked TTP ubiquitination (Fig. 4, A and B). Over and above, we also found Lys-63-linked ubiquitin chains attached to endogenous TTP after TNFα stimulation in HUVEC. In line with the above results, MG132 treatment had no effect, whereas the addition of NEM strongly enhanced Lys-63-linked TTP ubiquitination (Fig. 4C). The latter can be attributed to the zinc finger domain of TTP, because required ubiquitin target lysine residues occur only in this region. Further, the loss of MEKK1 function, achieved either by lentiviral, shRNA-mediated MEKK1 knockdown in HUVEC (Fig. 4D, left panel) or by the
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FIGURE 3. TTP ubiquitination depends on MEKK1 kinase activity and TRAF2. A, ubiquitination assay in HEK 293 cells transfected with M1 Kin together with FLAG-tagged TTP, its mutants C124R (mRNA binding deficient), ZnN or ZnC in the absence or presence of His-tagged WT ubiquitin (WT-Ubi) using nickel-nitriilotriacetic acid (Ni-NTA) beads. Precipitation of ubiquitinated proteins under denaturating conditions revealed that TTP ubiquitination occurred independent of its mRNA binding ability (compare second and third lanes) but that the N-terminal phosphorylation by MEKK1 coexpression (M1 Kin) was indispensable for TTP ubiquitination (compare fourth and fifth lanes). Precipitated proteins were analyzed by 5–12% gradient SDS-PAGE and Western blotting (WB) using α-TTP or α-FLAG antibodies (top panels). The lower panels represent the input controls. B, MEF (left panels) or HUVEC (right panels) were treated with TNFα for 90 min in the absence or presence (90 μM) of NEM (10 μM, added 30 min after TNF treatment). TTP was immunoprecipitated (IP) under denaturing conditions. Western blot analyses show endogenous TTP ubiquitination occurring after TNFα stimulation in the presence of the deubiquitinase inhibitor NEM (upper panels). The lower panels represent the input and loading controls. C, HEK 293 cells were transfected with combinations of FLAG-TTP, HA-tagged TRAF2 (T2), and M1 Kin expression plasmids in the absence or presence of a His-tagged WT ubiquitin (WT-Ubi) as indicated. Precipitation and analysis of ubiquitinated proteins was done as in A. Ubiquitination of TTP appeared dependent on the E3 ligase TRAF2 and was strongly enhanced by coexpression of M1 Kin (compare second and third panels). Ubiquitination was not affected by MG132 treatment (bottom panels). The corresponding input controls are depicted within the right panels. D, HEK 293 cells were transfected with FLAG-TTP and His-tagged WT ubiquitin in combination with MEKK1 and TRAF2 expression vectors as indicated (M1 Kin; M1 dnKin, dominant negative MEKK1; T2, TRAF2; T2dn, dominant negative TRAF2 lacking RING finger domain). Ubiquitinated TTP was analyzed as in A and appeared dependent on both a functional MEKK1 kinase domain (compare fourth lane with sixth and seventh lanes) as well as an intact TRAF2 (compare second through fifth lanes). Input and loading control are shown in the lower panels. ctrl, control.

Use of MEF deficient in the MEKK1 kinase domain (Fig. 4D, right panel) confirmed that endogenous TTP ubiquitination depended on phosphorylation by MEKK1. Importantly, the E3 ligase activity of MEKK1 was negligible in terms of TTP ubiquitination, proven by the respective MEKK1 mutant (data not shown), as well as the use of ΔKD MEF (Fig. 4D, right panel). Our results indicate that TTP is covalently modified by ubiquitin in vivo (although we cannot formally exclude that other proteins bound to TTP complexes are not dissociated by 6 M urea) and that attached polyubiquitin chains are predominantly Lys-63-linked. In summary, MEKK1 mediated, N-terminal TTP phosphorylation appears to be prerequisite for TRAF2-mediated, Lys-63-linked TTP polyubiquitination.

The molecular cross-talk between NF-κB and JNK signaling cascades is the key to account for the timed regulation of biological processes as cell growth, differentiation, and apoptosis. TNFR1-induced prolonged JNK activation results not only from blocking NF-κB activity but depends on TRAF2-MEKK1 complex formation leading to JNK and subsequent AP-1 activation (8, 53, 55). When we analyzed TTP KO MEF for TNFα-induced JNK activation, we observed that the lack of TTP caused an impairment of p-JNK levels under stimulated conditions, comparable with the situation observed in MEKK1 ΔKD MEF, whereas total JNK levels were not affected (Fig. 5A). When analyzing TTP expression in WT MEF (Fig. 5A, middle panel), we noticed that forced, prolonged JNK activation (achieved through specific IKK2 inhibition by BMS; Fig. 5A, 90°–180°) goes in line with the appearance of HMW forms of TTP (see Fig. 7, top blot). In contrast, TTP was generally less abundant in ΔKD MEF, and although HMW-TTP was somewhat detectable, which can also be attributed to phosphoryla-
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FIGURE 4. MEKK1 mediated N-terminal TTP phosphorylation is prerequisite for nondegradative TTP ubiquitination of its zinc finger. A, the type of polyubiquitin chains (Lys-48-linked versus Lys-63-linked) of TTP and its mutants ZnN and ZnC were analyzed in transfected HEK 293 cells, in the presence (right panels) and absence (control (ctrl), left panels) of M1 Kin and TRAF2 (T2). Analysis of ubiquitinated proteins (done as in Fig. 3) revealed Lys-63-linked ubiquitination of full-length TTP and its ZnN mutant in the presence of M1 Kin and TRAF2 (right panel, third, fourth, seventh, and eighth lanes). Note that the mutation of ubiquitin-lysine 48 (Lys-48 to Arg-48) allowed TTP ubiquitination, whereas mutation of lysine 63 (Lys-63 to Arg-63) abolished TTP ubiquitination. The lower three panels show the input control. TTP as well as its mutants were detected by a FLAG antibody. B, HEK293 cells were transfected with the FLAG-tagged TTP mutant ZnN in combination with M1 Kin and TRAF2 either in the absence (−) or the presence of the His-tagged ubiquitin mutants K48R or K63R. The cells were treated with NEM 30 min before harvesting. FLAG-ZnN was analyzed by immunoprecipitation (IP) and Western blotting (WB) using a specific antibody for detection of Lys-63-linked ubiquitin chains (α-Ub K63) revealed TTP ubiquitination when lysine 48 was mutated (K48R). The lower panels represent the input controls. C, HUVEC were induced with TNFα (90 min) in the absence or presence of either MG132 (90 min) or MG132 and NEM (90 min), added 30 min after TNFα stimulation, and TTP was immunoprecipitated under denaturing conditions. Western blot analysis of Lys-63-linked ubiquitin (α-Ub K63) as well as total ubiquitin (α-Ub) revealed that MG132 had no influence on TTP ubiquitination, whereas NEM strongly enhanced the signal. The bottom panels show input controls (LC', loading control GAPDH). D, left panel, HUVEC were transfected with either an shRNA lentivirus to knockdown MEKK1 (shM1) or a scrambled control virus (shCtrl), 48 h later, the cells were induced with TNFα for 90 min. NEM was added to all samples 45 min after TNFα treatment. TTP was immunoprecipitated as described in C. Western blot analysis of Lys-63-linked ubiquitin (α-Ub K63) as well as total ubiquitin (α-Ub) uncovered that TTP ubiquitination was dependent on TNFα stimulation and MEKK1 (top panels). The lower panels represent the input controls. Note that knockdown efficiency of MEKK1 after 0 and 90 min of TNFα varied between 95 and 70%, respectively, as calculated by densitometric analysis (data not shown). LE', long exposure of TTP showing that virus infection induced TTP expression in HUVEC. Right panel, WT and MEKK1ΔKD MEF were induced with TNFα (90 min; −), NEM added after 30 min of TNFα treatment, and TTP immunoprecipitation was performed under denaturing conditions. Specific detection of Lys-63-linked ubiquitin chains (α-Ub K63) occurred in WT MEF, whereas TTP ubiquitination was impaired in cells lacking the MEKK1 kinase domain (ΔKD). Note that detection of total ubiquitin (α-Ub) appears equal in both cell types. The lower panels represent the inputs (β-galactosidase detection used as genotype control, because ΔKD cells express an MEKK1-β-galactosidase fusion protein.

AP-1 activity was also affected by TTP; whereas TTP increased AP-1 promoter activity in a dose-dependent manner in HEK 293 cells (Fig. 5C, top panel), AP-1 activity was diminished in
KO MEF and could be restored to WT levels after TTP reconstitution (Fig. 5, bottom panel). This is in line with our previous findings where TTP down-regulated NF-κB promoter activity dose-dependently in HEK 293 cells, whereas MEF lacking TTP displayed a much higher basal NF-κB promoter activity (42).

In light of the results above, we analyzed the potential of TTP to influence cell growth and/or apoptosis. We generated a recombinant adenovirus for expression of Myc-tagged TTP under the control of a tetracycline responsive promoter (Tet-OFF-system). This allowed the controlled expression of TTP in terms of time and expression levels. Via titration experiments, we assured that viral infection does not affect cell viability (data not shown). Under the established conditions, we tested the impact of TTP on cell viability: HeLa cells ectopically expressing TTP (Fig. 6A, verified by Western blotting) proliferated significantly slower as compared with non-TTP-expressing control cells over a time period of 72 h. Because doxycycline itself affected the cells between 48 and 72 h of treatment (data not shown) only effects within the first 48 h were further studied (Fig. 6A, bottom graphs). Uninfected and control adenovirus-infected cells without or treated with doxycycline exhibited a doubling in cell number (100%) during the first 24 h. The cells that ectopically expressed TTP reached a cell number of 60% compared with the above mentioned controls after 24 h (42).

**FIGURE 5. TTP is involved in TNFα-induced phosphorylation of JNK.** A, WT, TTP KO, and MEKK1 ΔKD MEF were stimulated with TNFα as indicated (+, BMS added 30 min after TNFα treatment). Western blot analysis of whole cell lysates showed that p-JNK levels (top panels) were comparably diminished in TTP KO and MEKK1 ΔKD MEF, whereas total JNK levels were not affected (third row panels). Note that TNFα-induced TTP expression levels, as well as TTP patterning indicating phosphorylation, were different in WT and MEKK1 ΔKD cells (second panels). GAPDH represents the loading controls (bottom panels). M, molecular weight marker loaded. B, left panel, WT and TTP KO MEF were transduced with an adenovirus expressing TTP (AdTTP, right panels). Analysis of p-JNK levels (top panels) revealed restored p-JNK2 in KO cells after reconstitution of TTP expression in comparison to control cells (ctrl, left panels). Ponceau S staining served as loading control. Right panels, densitometric analysis of p-JNK1 (bottom right panel) and p-JNK2 (top right panel) levels based on the Western blots depicted on the left side. Mean intensities of bands were normalized to the loading control (*). C, top panel, AP-1-driven luciferase reporter assay in HEK 293 cells revealed that increasing levels of ectopically expressed TTP successively increased AP-1-driven promoter activity. Mean relative luciferase levels were normalized to cotransfected β-galactosidase. ctrl, AP-1 reporter plasmid only. Bottom panel, AP-1 reporter assay in WT (black bars) compared with TTP KO MEF showed a decreased AP-1 promoter activity in KO MEF (white bars). Note that reconstituted TTP expression (TTP) restored AP-1 activity to levels observed in WT MEF (compare first and fourth bars). Luciferase levels are depicted as mean fold induction and were normalized to cotransfected GFP. ctrl, AP-1 reporter plasmid only;
Together, our data provide evidence that TTP is hypermodified by the combined action of MEKK1 and the E3 ligase TRAF2 (Fig. 6D). Thus we hypothesize that the ability of TTP to affect cell viability is strongly dependent on an accurately timed, post-translational control of the protein through p38α on the one hand and MEKK1 on the other. The expression of TTP in combination with appropriately directed hypermodification events differentially regulate its involvement in the cross-talk

**Figures and Captions**

**FIGURE 6.** TTP hypermodification affects cell viability. A, HeLa cells were either left untreated (ctrl) or transduced with a TET-responsive TTP adenovirus (AdTTP, 2 h after seeding). The cells were grown in the presence (+) or absence (−) of doxycycline (−Dox, ectopic TTP expression; +Dox, no ectopic TTP expression); harvested after 24, 48, and 72 h; and counted to determine mean cell numbers (see timeline and bottom panels), which were negatively influenced by ectopic TTP expression (bottom graphs). TTP expression levels were verified by Western blotting. One sample (48 h) was erroneously treated with a lower Dox concentration resulting in ectopic TTP expression (Western blot, #) and a spontaneous reduction of the mean cell number (graph 2, #). Duplicates of cells harvested from two technical replicates were counted for each time point, and the error bars represent the standard deviations of the mean. B, HEK293 cells were transfected with TTP and WT ubiquitin in the absence (−) or presence of expression plasmids for MEKK1 and TRAF2 as indicated (−, empty vector control; M1 Kin, MEKK1 kinase domain only; T2, TRAF2; M1dnKin, dominant negative MEKK1; T2dn, dominant negative TRAF2 lacking RING finger domain). 24 h post-transfection, the cells were analyzed by phase contrast microscopy. The images are representatives of three independent experiments. C, HEK293 cells were transfected with M1 Kin (kinase domain only) and TRAF2 (T2) in the absence (−) or presence of the indicated expression plasmids (K63 Ubi, Lys-63-linked ubiquitin chains only; TTP-ZnN mutant). The cells were analyzed as in B. D, scheme for TTP hypermodification. TNFα induces complex formation of TRAF2 and MEKK1, which targets TTP. MEKK1 phosphorylates TTP at its N-terminal domain and the proximity of TRAF2 leads to the subsequent polyubiquitination of the TTP zinc finger.
between NF-κB and JNK signaling. Therefore, we propose that TTP acts as “balancer” in the coordinated control of survival-versus death-promoting signaling cascades (Fig. 7).

**DISCUSSION**

NF-κB constitutes the key transcription factor in the regulation of inflammatory processes. It concomitantly controls cell survival by initiating the transcription of antipapoptotic and antioxidative genes, as well as the timed cross-talk with “pro-apoptotic” pathways, represented mainly by the MAPK cascade leading to the activation of JNK. In this context, our novel finding that MEKK1, an upstream JNK-activating kinase, counteracts TTP-mediated inhibition of NF-κB promoter activity, provides further insight into the interdependent regulation of NF-κB and JNK signaling cascades. *In vitro*, the observed reversal occurred with canonical 5× NF-κB promoters, as well as with the more physiologic human IL-8 or tissue factor promoters (data not shown), both containing NF-κB-binding sites, and was dependent on MEKK1 kinase activity.

Remarkably, p38α, which has already been shown to abolish TTP-mediated mRNA degradation, had no effect on TTP-mediated NF-κB inhibition, it rather counteracted MEKK1 function in our settings. Additionally, we found distinct “TTP forms” bound to these two proteins: p38α coprecipitated with LMW-TTP, whereas MEKK1 interacted with the HMW species. Interesting enough, these different TTP species resembled the appearance of endogenous TTP in HUVEC and MEF, where TTP appears in a LMW form in the beginning of TNFα stimulation and gradually adopts the HMW form later on.

After TNFR1 ligation, MEKK1 acts in concert with TRAF2 to activate JNK signals, and our observation of TRAF2-MEKK1-mediated ubiquitination suggested a role for TTP in this scenario. TTP indeed promoted JNK activity mainly affecting MEKK1 kinase cascade ending at NF-κB activation, thereby affecting cell proliferation and transformation or inducing apoptosis in a cell type- and stimulus-dependent manner. JNK2 exerts higher binding affinities for c-Jun than JNK1, and fibroblasts lacking JNK2 exhibit a proliferation advantage (56, 57), which is in line with the fact that TTP KO MEF grow faster than WT MEF.

Strikingly, also AP-1 activity was influenced by TTP: low doses of TTP inhibited, whereas increasing amounts enhanced AP-1 activity in reporter gene assays. Interestingly, the opposite is true for the effect of TTP on NF-κB promoters: increasing TTP levels successively decrease its activity (42).
Relevance of TTP Hypermodification

Moreover, MEKK1-mediated phosphorylation occurs at the N terminus of TTP, a region that is not conserved among the members of the well described TIS family of proteins, all of which 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. This is supported by the described functions of homologous proteins: in Saccharomyces pombe, a TTP-related protein accounts for the transmission of a pheromone-induced Ras/mitogen-activated protein kinase signal (58). In Saccharomyces cerevisiae, a similar cysteine-cysteine-cysteine-histidine-type tandem zinc finger protein is involved in metabolism and retards cell growth when overexpressed. In addition, a TIS11 homologue was found implicated in cell cycle control in Drosophila melanogaster (59). Interestingly, in Caenorhabditis elegans, a related cysteine-cysteine-cysteine-histidine tandem zinc finger protein termed PIE-1 was described as bifunctional protein, involved in the control of mRNA stability as well as in the inhibition of transcription (60).

Additionally, it is important to note that also the expression of human TTP is not restricted to pro- and anti-inflammatory mediators and their mRNA degradation: TTP is induced in response to the breast cancer susceptibility protein BRCA1 (61), as well as upon TGFβ stimulation in human T-cells. Interestingly, TGFβ controls cell proliferation and differentiation and signals through the SMAD-dependent pathway to induce apoptosis by the activation of JNK. Additionally, TGFβ acts on the control of NF-κB: TNFα stimulation of TGFβ pretreated cells results in restricted NF-κB target gene expression caused by impaired p65 nuclear translocation (62), reminiscent of our previous findings. Furthermore, TGFβ stimulated T-cells up-regulate TTP via heterodimeric SMAD3/4 transcription factors, and TGFβ as well as TGFβ-deficient mice exhibit overlapping phenotypes (63). Thus, it is tempting to implicate TTP into TGFβ-triggered, JNK-mediated apoptosis.

Moreover, a study by Johnson and colleagues (64, 65) unraveled that continuous expression of TTP/TIS11 proteins induces various primary and transformed cell types to undergo apoptosis; however, the mechanism remained elusive. In their study, moderate expression levels of TTP were insufficient to cause widespread cell death, but they induced cell death upon concomitant TNFα treatment. It is striking, therefore, that neither TIS11b nor TIS11d acted in synergy with TNFα, which is the only functional difference that has been identified among TTP/TIS11 proteins so far. In addition, they determine the ZnN region of TTP as being responsible for the onset of cell death, reflecting a specific functional divergence between TTP and other TIS11 proteins (64, 65). This supports our hypothesis that TTP, once hypermodified in its ZnN region, actively contributes to cell death-promoting signaling cascades.

In summary, it is well established that TTP regulates a variety of biological processes. Its described role in inflammatory as well as tumorigenic diseases clearly shows that TTP is functionally much more complex than previously appreciated. Our findings suggest a model wherein TTP contributes to the balance of NF-κB versus JNK signaling, which depends on a series of time-dependent, alternating post-translational hypermodifications, including phosphorylation and regulatory ubiquitination. These contribute to a functional switch of TTP: upon the course of a TNFα induced response the “early TTP” (probably LMW species) might interfere with NF-κB signaling, whereas the time-dependent accumulation of hypermodified HMW-TTP is associated with the onset of JNK signaling (model in Fig. 7). Future studies will provide more mechanistic insight into TTP-mediated JNK activation and include analyses of these modifications on the endogenous level, investigation of MEKK1-specific target phospho-sites as well as lysine residues, which are targeted for ubiquitination.

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