

# Molecular mechanisms of phosphorylation-regulated TTP (tristetraprolin) action and screening for further TTP-interacting proteins

Christopher Tiedje, Alexey Kotlyarov and Matthias Gaestel<sup>1</sup>

Institute of Biochemistry, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

## Abstract

TTP (tristetraprolin) is an RNA-binding protein which regulates mRNA stability or translation or both. The molecular mechanisms which are responsible and which discriminate between regulation of mRNA stability and translation are not completely understood so far, but are clearly dependent on p38 MAPK (mitogen-activated protein kinase)/MK (MAPK-activated protein kinase) 2/3-mediated phosphorylation of TTP. To learn more about these mechanisms, phosphorylation-dependent TTP-interacting proteins could be of great interest. Many interacting partners, which belong to the mRNA-processing and -regulating machinery, have been identified by hypothesis-driven co-immunoprecipitation and in the classical Y2H (yeast two-hybrid) approach, where TTP was identified as prey, and are summarized in the present paper. However, because of transactivating properties of TTP, an unbiased Y2H approach using TTP as bait was hindered. Since novel methods for the identification of phosphorylation-dependent interaction partners and of interactors of full-length auto-activating proteins in eukaryotic systems have evolved in the last few years, these methods should be applied to screen for additional phosphorylation-dependent interaction partners of TTP and could lead towards a complete understanding of TTP function at the molecular level.

## Regulation of TTP (tristetraprolin) by phosphorylation

TTP is one of four members of the TIS11 [PMA ('TPA')-inducible sequence 11] protein family known as post-transcriptional regulator of mRNA stability and translation. TTP binds to AREs (AU-rich elements) in the 3'-UTRs (untranslated regions) of target mRNAs [1]. As physiological targets for TTP regulation, mRNAs of TNF (tumour necrosis factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), IL (interleukin)-2, IL-3, IL-10, Ier (immediate early regulated protein) 3, Plk (Polo-like kinase) and VEGF (vascular endothelial growth factor) have been identified (reviewed in [2]), whereas, for other members of the TIS11 family, further interesting targets, such as the oncogenic transcription factor Notch 1, have been described recently [3]. So far, the molecular mechanism of the TTP effects on mRNA stability and translation are not completely understood and it is also not clear whether TTP could regulate mRNA stability and translation independently by different mechanisms. However, in the last few years, it has become increasingly clear that these mechanisms depend on stress-

and LPS (lipopolysaccharide)-induced p38 MAPK (mitogen-activated protein kinase)/MK (MAPK-activated protein kinase) 2-dependent phosphorylation of TTP (reviewed in [4,5]). The current understanding of phosphorylation-dependent regulation of TTP function is summarized in Figure 1. In the non-phosphorylated state, soluble TTP protein and its target mRNA are unstable, probably due to co-degradation that proceeds in the exosome in the nucleus of the cell, and some insoluble TTP can also be detected in cytoplasmic stress granules or P-bodies (processing bodies) [6–9]. The low stability of TTP lacking the MK2/3-catalysed phosphorylation at Ser<sup>52</sup> and Ser<sup>178</sup> is documented by the strongly decreased TTP level detected in MK2-deficient macrophages and by the almost undetectable TTP expression in MK2/3 double-deficient macrophages even upon LPS stimulation [10]. Phosphorylated TTP protein is soluble, stable and mainly localized in the cytoplasm [9]. Furthermore, it does not significantly destabilize its target mRNAs, since, as shown very recently, phosphorylation of TTP inhibits its interaction with and recruitment of specific deadenylases to the target mRNAs [11]. In addition, phosphorylated TTP is believed to facilitate translation of the target mRNAs by recruiting them to subcellular compartments of high ribosomal activity. MK2/3 phosphorylation of TTP creates high-affinity 14–3–3-binding sites [7], which could be involved in this recruitment.

To learn more about the phosphorylation-dependent function of TTP, several attempts to identify TTP-interacting proteins and to characterize their phosphorylation-dependent

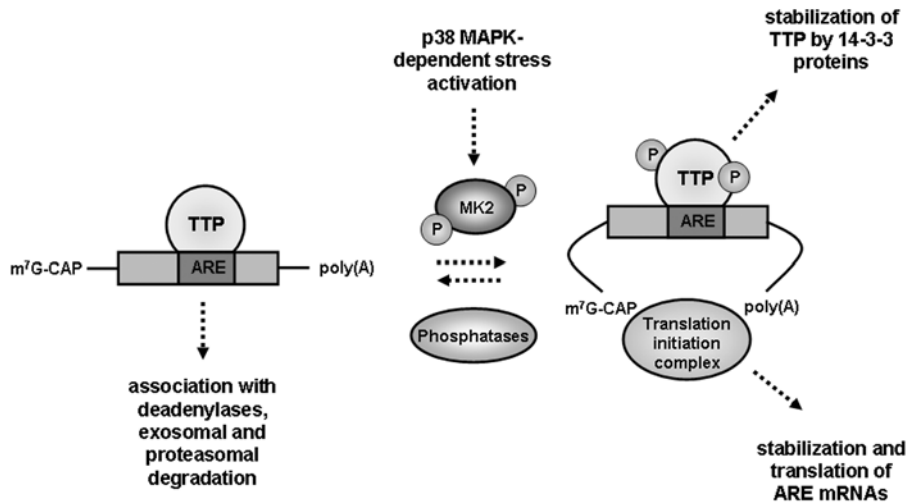
**Key words:** mRNA stability, phosphorylation, protein–protein interaction, translation, tristetraprolin (TTP), yeast two-hybrid screen.

**Abbreviations used:** ARE, AU-rich element; IL, interleukin; IP, immunoprecipitation; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MK, MAPK-activated protein kinase; PABP1, poly(A)-binding protein 1; P-body, processing body; PP2A-C, catalytic subunit of protein phosphatase 2A; TIS11, PMA ('TPA')-inducible sequence 11; TNF, tumour necrosis factor; TTP, tristetraprolin; UTR, untranslated region; Y2H, yeast two-hybrid.

<sup>1</sup>To whom correspondence should be addressed (email [gaestel.matthias@mh-hannover.de](mailto:gaestel.matthias@mh-hannover.de)).

**Figure 1** | Schematic representation of phosphorylation-dependent regulation of TTP function

For details, see the text.



binding were undertaken. Some interacting proteins identified are components of the mRNA-processing and -regulating machinery (reviewed in [4] and summarized in Table 1) and clearly link TTP function to mRNA metabolism and translation. However, because of experimental hindrance, such as auto-activating properties of full-length TTP in the classical Y2H (yeast two-hybrid) screens, the detailed picture of TTP interactions and their action on post-transcriptional regulation of gene expression is far from being complete. New approaches to characterize further interacting partners, such as recently undertaken for TTP fragments [12], and to describe TTP interactions, which depend on specific post-translational modifications, will be necessary to draw a conclusive picture of TTP's functions. In particular, it would be extremely interesting to learn which interaction partners of TTP after mRNA storage/repression make the decision between P-body-mediated mRNA degradation and P-body exit for mRNA derepression/re-initiation of translation and how these interactions are regulated by phosphorylation.

### Transient interactions of enzymes covalently modifying TTP

Since the introduction of the lock and key principle by Emil Fischer in 1894, it is obvious that enzymatic modification of substrates requires transient enzyme-substrate interaction. Hence, TTP transiently interacts with its modifying enzymes MK2, MK3 and PP2A-C (catalytic subunit of protein phosphatase 2A) (see Table 1 for detailed information). Phosphorylation of TTP at the two MK2/3 sites, Ser<sup>52</sup> and Ser<sup>178</sup>, generates two binding sites for stable interactions with 14-3-3 proteins [7,13,14]. 14-3-3 binding competes with binding of PP2A-C to TTP and prevents its dephosphorylation [15]. When overexpressed in HEK (human embryonic kidney)-293 cells, TTP is highly phosphorylated even under non-stimulated conditions, and various phosphorylation sites

have been identified [16]. Many of these sites are not similar to the consensus motif of MK2/3 phosphorylation, indicating transient interactions of TTP with other protein kinases. *In vitro* phosphorylation of TTP by various proline-directed kinases, such as ERKs (extracellular-signal-regulated kinases), JNKs (c-Jun N-terminal kinases) and p38 MAPK [17,18], indicates a transient interaction of TTP with these protein kinases as well.

### Identification of TTP interacting proteins by co-IP (immunoprecipitation)

Protein-protein interactions can be monitored by co-IP of proteins from cell lysates, often reflecting their interaction in the living cell. However, to identify the co-immunoprecipitated proteins, their specific detection in Western blot experiments is necessary and hence requires a bias towards specific interaction partners. Proteins of mRNA-processing and -degrading machinery in P-bodies and of the exosome were tested in several studies, and interactions of endogenous TTP with endogenous PM/ScI-75 (polymyositis/scleroderma autoantigen 75 kDa), KSRP (K homology-type splicing regulatory protein), hDcp1a (human decapping enzyme 1a) and endonuclease hXrn1 were demonstrated (IP in Table 1). Often, epitope-tagged TTP or potential interaction partners were overexpressed in the cells of interest and co-immunoprecipitated in pull-down experiments (OE-IP in Table 1). In one study, the interacting proteins isolated from BHK (baby-hamster kidney) and NIH 3T3 cells were analysed by tandem MS sequencing. This resulted in the identification of a significant number of different mRNA-associated proteins [19]. The successful pull-down of various proteins together with TTP may not always reflect interactions of endogenous proteins, especially if no RNase was used to treat the lysates before the binding reactions. The use of RNase shows the RNA-dependency

**Table 1 | Interaction partners of TTP**

The proteins identified are listed in specific functional groups and the methods applied are indicated (IP, co-immunoprecipitation; OE-IP, co-immunoprecipitation or pull-down where at least one protein is overexpressed or epitope-tagged; KA, kinase assay; KO, knockout; Y2H, yeast two-hybrid assay). Where analysed, the region and sites of TTP necessary for the interaction are given (NTD, N-terminal domain; CTD, C-terminal domain; RBD, RNA-binding domain; pS, phosphoserine; RD, RNA-dependent; RID, RNA-independent; RDND, RNA-dependency not determined).

Function	Interacting partners	Method and reference	TTP part involved
Exosome-related	hRrp4 (S1/KH RBD)/EXOSC4	OE-IP, RID [20]	NTD
	PM/Scl-75	IP, RID [20]	
	KSRP	IP, RID [34]	
	Nucleolin	Y2H, OE-IP, RID [12]	
	EXOSC6	Y2H [21]	
P-body-related	EXOSC8	Y2H [21]	RBD
	hDcp1a	IP, RID [20]	
	hDcp2	OE-IP, RID [20,35]	
	hXrn1	Y2H [21]; IP, RID [20]	
	hCcr4	OE-IP, RID [20]	
	hEdc3	OE-IP, RID [35]	
	Hedls/hEdc4	OE-IP, RID [35]	
	hAgo2/eIF2C2	OE-IP, RDND [36]	
mRNA-binding	hAgo4/eIF2C4	OE-IP, RDND [36]	RBD
	p72/DDX17	Y2H [37]; OE-IP, RDND [19]	
	p68/DDX5	OE-IP, RDND [19]	
	NDH II/DHX9	OE-IP, RDND [19]	
	CRD-BP/IGF2BP1	OE-IP, RDND [19]	
	PABPC1/PABP1	Y2H, OE-IP, RID [12]; OE-IP, RDND [19]; OE-IP, RD [20]; OE-IP, RD [11]	
	PABP4	OE-IP, RDND [19]	
	HuR	OE-IP, RDND [19]	
	AUF1	OE-IP, RDND [19]	
	TIAR	OE-IP, RDND [19]	
	CAF1a	OE-IP, RID [11]	
	CAF1b	OE-IP, RID [11]	
	hnRNP A1	OE-IP, RD [20]	
	hnRNPs A/B, A1, A2/B, A3, F, G, H, H', K, L, M, R and U	all OE-IP, RDND [19]	
	Phosphorylation-related	MK2	
MK3		Mouse-KO [10]	
ERK2		<i>In vitro</i> KA [18]	
JNK		<i>In vitro</i> KA [17]	
p38		<i>In vitro</i> KA [17]	
PP2A-C		OE-IP, RDND [15]	
14-3-3 $\beta$		OE-IP, RDND [14]	
14-3-3 $\beta$		OE-IP, RDND [13]	
14-3-3 $\beta$		OE-IP, RDND [7]	
14-3-3 $\eta$		Y2H, OE-IP, RDND [14]	
14-3-3 $\gamma$ , $\epsilon$ , $\zeta$ , $\sigma$ and $\tau$		OE-IP, RDND [14]	
Proteasome-related	Hsp70	Y2H, OE-IP, RID [12]	RBD
	Miscellaneous		CTD
Miscellaneous	Cbl-interacting protein 85	Y2H, OE-IP, RID [12]	CTD
	Nup214	Y2H, OE-IP, RDND [38]	
	Tax/p40	Y2H, OE-IP, RDND [39]	
	DIPA/Ccdc85B	Y2H [40]	
	Upf1	Y2H [21]	
	Upf2	Y2H [21]	
	HmgB1	OE-IP, RDND [41]	
	Stat2	Y2H [42]	

of putative interactions and reduces the number of artificial interactions that occur just because of the RNA-binding properties of the proteins involved. Different results in terms of RNA-dependency were obtained for PABP1 [poly(A)-binding protein 1]. The interaction of PABP1 with TTP was identified by several groups [11,12,19,20], described as RNA-independent in one set of experiments [12], but displaying clear RNA-dependency in other experiments [11,20]. The reasons for these differences can be found in the characteristics of the RNases used. One study exclusively used benzonase, an unspecific artificial endonuclease that also cleaves double-stranded RNAs and DNAs [11], but in the other experiments sequence- and single-strand-specific RNases, such as RNase A and RNase T<sub>1</sub>, have been applied [12,20]. The latter allow the formation of mRNA pieces that can still act as binding platforms for the interacting proteins. Regardless of these differences, the various interactions identified by co-IP contribute further to the picture that TTP associates with P-bodies, the exosome and other mRNA-binding proteins.

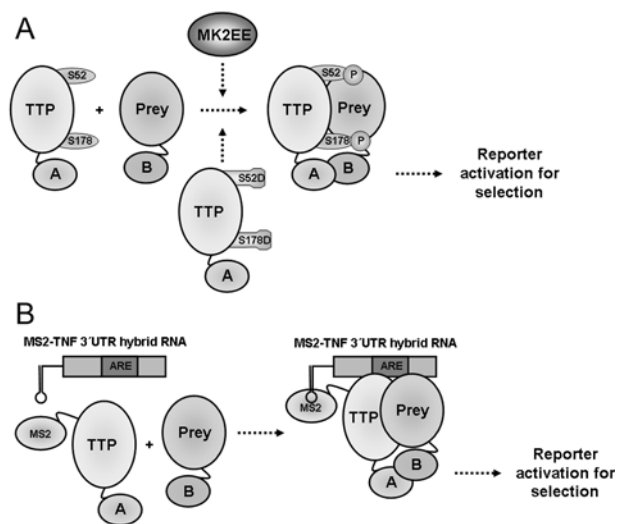
## Unbiased screening for and verification of unknown interactions

Full-length TTP was used for a Y2H screen only in one study, which reveals 14-3-3 $\eta$  as an interacting partner [14]. In some cases, TTP was received as a prey for proteins involved in mRNA degradation [21]. Because of a significant degree of auto-activation by TTP in the Y2H system, TTP fragments were preferred in the classical Y2H screens so far. However, as a result of interaction, the two halves of ubiquitin can form a reconstituted ubiquitin due to the fact that both moieties are brought together in close proximity. Intracellular UBPs (ubiquitin-specific proteases) then recognize the reconstituted 'split-ubiquitin', cleave off the reporter protein and allow the selection of potential interactors. The original system [22] is a pure cytosolic screening, whereas the improved versions anchor even cytosolic bait proteins to the membrane of the endoplasmic reticulum and, in addition, allow screening of all kinds of membrane-associated proteins [23–26]. The successful use of all methods using yeast, plant and mammalian bait proteins over the last few years is documented [27–30].

With regard to the detection of phospho-specific interactions in this system, one could benefit from such developments for the classical Y2H system, where the presence of post-translational modifications is a prerequisite for a positive interplay of two proteins [31,32]. When a specific bait-modifying protein, such as a bait-kinase, is introduced into the yeast cells, the commonly used screening procedure is carried out and results in the identification of additional phosphorylation-specific interactions [29,31–33]. For TTP, one could imagine the following scenario (Figure 2A). In combination with the split-ubiquitin system, one could induce the expression of constitutively active MK2 in the yeast cells, which phosphorylates TTP at Ser<sup>52</sup> and

## Figure 2 | Identification of TTP interactors in a phosphorylation-specific and RNA-dependent fashion

TTP and the prey library are expressed as fusions with the protein domains A and B respectively. A and B may represent DNA-binding and *trans*-activating domains of transcription factors, such as gal4 or lexA (classical Y2H), or N- and C-terminal domains of ubiquitin (split-ubiquitin system). (A) Phospho-specific interaction. Expression of a constitutively active MK2 mutant {e.g. MK2-T205E/T317E (MK2EE) [43]} results in the phosphorylation of TTP on Ser<sup>52</sup> and Ser<sup>178</sup>. This should allow the detection of phosphorylation-specific TTP-interacting proteins even in the split-ubiquitin system. The use of a TTP phosphomimetic mutant (TTP-S52D,S178D) could lead to similar results. (B) RNA-dependent interaction. Identification of TTP interactors in a RNA-dependent fashion. Based on the work of SenGupta et al. [33], an MS2-TNF 3'-UTR hybrid RNA serves as a binding platform for TTP that is expressed as an MS2 coat protein fusion. Putative interactors of TTP that only interact with mRNA-bound TTP could be detected. A combination with the phosphomimetic mutants should be possible.



Ser<sup>178</sup> [7,13,14]. Alternatively, a phosphomimetic mutant, such as TTP-S52D/S178D, could be used. Only proteins that recognize the TTP phosphorylation pattern should now be able to interact.

To investigate the influence of mRNAs, especially ARE mRNAs, on the interactions of and with TTP, a different approach would be preferable. With the use of a method to detect RNA-protein interaction based on a bacteriophage MS2-RNA hybrid [33], it could be possible to generate an MS2-TNF 3'-UTR hybrid that serves as a binding platform for TTP. When TTP is expressed as an MS2 coat protein fusion together with its putative interactors, interaction will lead to the liberation of a reporter protein (Figure 2B). With this method, it should be possible to detect interactions of ARE mRNA-bound TTP to certain proteins and even to detect interaction of proteins bound to ARE mRNAs with TTP. In addition, a combination with the phospho-specific techniques seems to be possible.

## Conclusions

A complete picture of molecular interactions of TTP seems necessary to understand its mRNA-modulating functions. New methods for screening protein-interacting partners have been developed in the last few years. These methods enable us to identify even partners of proteins which display transactivating properties in the classical Y2H method, such as TTP, and to screen for interactions which depend on specific phosphorylations of the bait. In the future, these methods will facilitate the detection of new TTP-interaction partners previously missed in screening with fragments of TTP, completing our picture of the molecular mechanisms of TTP action on mRNA stability and translation.

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