Published in final edited form as:

Expert Rev Proteomics. 2007 December ; 4(6): 711–726. doi:10.1586/14789450.4.6.711.

Phosphorylation site analysis of the anti-inflammatory and mRNA destabilizing protein tristetraprolin

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Abstract

Tristetraprolin (TTP) is a member of the CCCH zinc finger proteins and is an anti-inflammatory protein. Mice deficient in TTP develop a profound inflammatory syndrome with erosive arthritis, autoimmunity and myeloid hyperplasia. TTP binds to mRNA AU-rich elements with high affinity for UUAUUUAUU nucleotides and causes destabilization of those mRNA molecules. TTP is phosphorylated extensively *in vivo* and is a substrate for multiple protein kinases *in vitro*. A number of approaches have been used to identify its phosphorylation sites. This article highlights the recent progress and different approaches utilized for the identification of phosphorylation sites in mammalian TTP. Important but limited results are obtained using traditional methods including *in vivo* labeling, site-directed mutagenesis, phosphopeptide mapping and protein sequencing. Mass spectrometry including MALDI/MS, MALDI/MS/MS, LC/MS/MS, IMAC/MALDI/MS/MS and multidimensional protein identification technology (MudPIT) has led the way in identifying TTP phosphorylation sites. The combination of these approaches has identified multiple phosphorylation sites in mammalian TTP, some of which are predicted by motif scanning to be phosphorylated by several protein kinases. This information should provide the molecular basis for future investigation of TTP's regulatory functions in controlling pro-inflammatory cytokines.

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Financial & competing interests disclosure

This work was supported in part by USDA-ARS Human Nutrition Research Program and the Intramural Research Program of the NIH, NIEHS. The authors have no other relevant affiliation or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those discussed.

No writing assistance was utilized in the production of this manuscript.

Keywords

Cytokine; *in vivo* radiolabeling; inflammation; mass spectrometry; mRNA instability; phosphorylation site; protein; sequencing; site-directed; mutagenesis; tristetraprolin; zinc finger protein

Introduction

Tristetraprolin (TTP), a member of CCCH tandem zinc finger proteins (ZFP), is involved in the regulation of inflammatory responses at the post-transcriptional level [1]. TTP binds to AU-rich elements (AREs) with high affinity for UUAUUUAUU nucleotides within mRNA sequences [2–7]. The specific binding of TTP to AREs causes destabilization of mRNA molecules encoding proteins such as tumor necrosis factor- α (TNF α) [3,8–10], granulocyte-macrophage colony-stimulating factor (GM-CSF) [11,12], cyclooxygenase 2 (COX2) [13,14], interleukin 2 (IL2) [15] and transcription factor E47 [16]. The mRNAs encoding TNF α and GM-CSF are stabilized in TTP-deficient mice and in cells derived from them [9,11]. Excessive levels of these cytokines in TTP knockout mice result in a severe systemic inflammatory response including arthritis, autoimmunity and myeloid hyperplasia [17,18]. On the other hand, up-regulation of TTP reduces inflammatory responses in macrophages [19]. These lines of evidence support the conclusion that TTP is an anti-inflammatory protein and arthritis suppressor [1,20–23].

TTP plays other important roles in normal physiology and disease development. Recently, Lai et al showed that immediate-early response gene 3 (Ier3/IEX-1/gly96) mRNA turnover is decreased in cells derived from TTP knockout mice [24]. Thalmeier et al demonstrated that TTP gene expression is reduced by approximately two-fold in post-mortem orbitofrontal cortex of violent suicide victims [25]. TTP expression is also reduced by approximately four-fold in people with metabolic syndrome [26]. These new studies suggest that, in addition to its role in inflammatory responses, TTP is a promising candidate gene for the physiological control of blood pressure and for the prevention of suicide and obesity-associated metabolic disorders.

TTP, the best-studied TTP/ZFP36 family member, is the product of the *Zfp36* in mouse and *ZFP36* in humans [27–29]. TTP cDNA clones were initially isolated by three laboratories based on its rapid and dramatic transcriptional induction in fibroblasts in response to insulin, phorbol esters and serum [27,28,30]. TTP is so-named [27] because the deduced amino acid sequences of TTP from mammals contain three repeats with four consecutive proline residues in each repeat (Figure 1). The TTP family proteins consist of three well-known members found in mammals (TTP, ZFP36L1 and ZFP36L2) and a fourth member found in mouse and rat, but not in humans (ZFP36L3) [1,31]. TTP is also known as ZFP36, TIS11, G0S24 and NUP475; ZFP36L1 is also known as TIS11B, cMG1, ERF1, BRF1 and Berg36; and ZFP36L2 is also known as TIS11D, ERF2 and BRF2 [1]. BLAST search of the non-redundant protein sequences using human TTP as the query sequence has identified similar protein sequences in many species, ranging from human through yeasts and plants. This group of proteins can be divided into several subfamilies by phylogenetic analysis, including TTP/TIS11/ZFP36, TIS11B/ZFP36L1, TIS11D/ZFP36L2, Yeast, Plant, and other CCCH subfamilies (Figure 2). The tandem CCCH zinc finger sequences are highly conserved among the diverse species (Figure 3).

TTP is a very low abundance protein predominantly localized in the cytosol, but its gene expression is rapidly induced by many agents, such as lipopolysaccharide (LPS) [32–37], fetal calf serum (FCS) [33], insulin [27,38,39], glucocorticoids [40], zinc [41], cinnamon [42], green tea [43] and herpes simplex virus 1 [44]. Unlike its labile mRNA [27], TTP protein is relatively

stable once induced in cells [33]. In addition, TTP in normal tissues and in stimulated cells exhibits a larger molecular mass on SDS gels than the predicted size due to extensive phosphorylation [33]. Dephosphorylation of TTP from transfected human embryonic kidney (HEK) 293 cells and LPS-stimulated mouse RAW264.7 cells results in a marked reduction of apparent TTP size on SDS gels, resulting in an apparent size close to that of TTP from over-expressed *E. coli* [3,12,33].

TTP is known to be hyperphosphorylated *in vivo*. *In vitro* phosphorylation assays show that TTP is phosphorylated by a number of protein kinases. These include p42 mitogen-activated protein (MAP) kinase (ERK2) [3,8,45], p38 MAP kinase [3,8,12,37], c-Jun N-terminal kinase (JNK) [8], MAP kinase-activated protein kinase 2 (MAPKAP kinase 2, or MK2) [36,46–48], glycogen synthase kinase 3 (GSK3 β) [49], protein kinase A (PKA) [49], protein kinase B (PKB α) [49] and protein kinase C (PKC μ) [49]. Several investigations have studied the effect of phosphorylation on TTP function(s). For example, mouse TTP (mTTP) is phosphorylated at S²²⁰ *in vivo* and *in vitro* [45] but phosphorylation at this serine residue does not affect the protein shuttling from the nucleus to the cytoplasm [50]. Phosphorylation of S¹⁷⁸ of mTTP increases its binding to the multifunctional 14-3-3 proteins and provides one of the mechanisms targeting TTP in the cytoplasm [51]. Similarly, dephosphorylation of mTTP by calf intestinal alkaline phosphatase (CIAP) prevents its binding to 14-3-3 proteins [51]. Human TTP (hTTP) expressed in HEK293 cells and then dephosphorylated by CIAP is able to bind GM-CSF mRNA ARE sequence more tightly than phosphorylated hTTP [12]. Finally, recombinant hTTP purified from *E. coli* binds to TNF mRNA ARE sequence with higher affinity than hTTP purified from mammalian cells [3]. Recent studies have demonstrated the importance of MK2 phosphorylation on TTP stability and function. MK2 is essential for the stabilization of TTP mRNA, and phosphorylation by MK2 leads to increased TTP protein stability but reduced ARE affinity [52]. In addition, the regulation of both subcellular localization and protein stability of mTTP is dependent on MK2 and on the integrity of S⁵² and S¹⁷⁸ [53]. Finally, mutation of S⁵² to A⁵² in mTTP weakly reduces the assembly of TTP-14-3-3, whereas mutation of S¹⁷⁸ to A¹⁷⁸ and of S^{52/178} to A^{52/178} substantially reduces the association of TTP with 14-3-3 [54].

This article highlights the recent progress and different approaches utilized for the identification of phosphorylation sites in mammalian TTP. A few phosphorylation sites have been determined using traditional methods, including *in vivo* labeling with [³²P]-orthophosphate, site-directed mutagenesis of putative phosphorylation sites, and phosphopeptide mapping in combination with Edman sequencing. Mass spectrometric (MS) analyses utilizing matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) as well as these ionization techniques in combination with immobilized metal ion affinity chromatography (IMAC), high performance liquid chromatography (HPLC), and multidimensional protein identification technology (MudPIT) have provided significant insights into the identification of TTP phosphorylation sites. These combined efforts have successfully identified many sites of phosphorylation in mammalian TTP. This information should provide the molecular basis for future investigation of TTP's regulatory functions in controlling pro-inflammatory cytokines.

***In vivo* radiolabeling**

In vivo radiolabeling is a widely used method for direct demonstration of protein phosphorylation. With this method, proteins are globally labeled with [³²P]-orthophosphate in living cells [45]. Cells are lysed in a hypotonic buffer containing detergent and inhibitors for proteases and phosphatases. Proteins of interest in the lysate can be purified using either affinity-tags (e.g., purification of His-tagged hTTP with Ni-NTA beads) or immunoprecipitation with antibodies (e.g., purification of hTTP with an anti-MBP-hTTP

antibody) [3]. Proteins are typically separated by SDS-PAGE, and the phosphorylated proteins can be visualized by autoradiography. The major drawbacks of this approach are the requirement of handling large quantities of radioactive materials and the inability to identify specific phosphorylation sites. Using this method, we have shown His-tagged hTTP is extensively labeled with ^{32}P in transfected HEK293 cells. Following *in vivo* radiolabeling, hTTP was purified and eluted from Ni-NTA affinity beads with varying concentrations of imidazole solutions. The phosphorylated hTTP could then be detected by autoradiography and identified by immunoblot with anti-MBP-hTTP antibody. These results show that the purified human TTP is highly phosphorylated in HEK293 cells and that [^{32}P]-labeled TTP is essentially the only labeled phosphoprotein recovered in the purified protein fraction [55].

In an attempt to identify the phosphorylation sites in TTP, a number of constructs encoding truncated hTTP protein fragments were made. HEK293 cells were transfected with the truncated plasmids and labeled with [^{32}P]-orthophosphate. Autoradiography and immunoblot results indicate that the truncated hTTP fragments were differentially labeled and expressed at different levels in HEK293 cells [55]. Comparison of the autoradiogram and corresponding immunoblot indicate that multiple phosphorylation sites were present in the N-terminal and the C-terminal regions of hTTP [55]. These analyses provided additional evidence for hyperphosphorylation of human TTP in normal cells [55]. Using this approach, however, the determination of a specific amino acid residue as being phosphorylated could not be determined.

Site-directed mutagenesis

Site-directed mutagenesis coupled with the *in vivo* radiolabeling approach can be a powerful tool for identifying phosphorylation sites if the protein of interest has only a few potential phosphorylation sites. This approach, however, has very limited utility if the protein of interest has multiple phosphorylation sites. Site-directed mutagenesis has shown that S²²⁰ of mouse TTP is a major phosphorylation site [45]. The utility of this approach for the identification of human TTP phosphorylation sites was investigated. The putative phosphorylation sites were selected based on sequence alignment of proteins from mammalian species, including full-length amino acid sequences of TTP from human, mouse, rat, cow and sheep; and partial amino acid sequences deduced from EST and genomic sequences from chimpanzee, dog, horse and pig [55]. Dozens of serine/threonine to alanine mutant plasmids were constructed to contain a single site mutation or 2-, 3-, 4-, 5-, 6-, 7- or 8-site mutations (Figure 4). These plasmids were transfected into HEK293 cells labeled with [^{32}P]-orthophosphate. The expressed wild-type and mutant human TTP proteins were purified from the supernatant using Ni-NTA affinity beads and by immunoprecipitation with anti-MBP-hTTP antibody. Following separation by SDS-PAGE, the autoradiogram shows that all of the hTTP proteins appear to be ^{32}P -labeled to a similar amount, despite the presence of extensive mutations in some forms of hTTP [55]. Similarities in the degree of phosphorylation as determined from the autoradiogram may possibly be accounted for by increased levels of expression of some forms of the mutant proteins, as shown by immunoblot. It is also possible that mutations of hTTP at the major phosphorylation sites expose other minor sites to more extensive phosphorylation than in the wild-type protein. Nevertheless, these *in vivo* radiolabeling studies of mutant TTP provide evidence for human TTP as a hyperphosphorylated protein. Due to the highly phosphorylated nature of human TTP in these cells, this method, however, does not provide precise information about the phosphorylation of specific amino acid residues.

The combination of site-directed mutagenesis and immunoblotting has also been used for the analysis of TTP phosphorylation sites. Previous studies have demonstrated that decreases in the electrophoretic mobility of proteins on SDS-PAGE can serve as an indicator of stoichiometric phosphorylation in some proteins [56,57]. Similar to these reports, *in vitro*

phosphorylation of TTP using protein kinases results in a decrease in mobility on SDS-PAGE [3]. On the other hand, dephosphorylation of TTP from mammalian cells significantly increases its electrophoretic mobility on SDS-PAGE [3,12,33,45]. Under this paradigm, mutation of a specific phosphorylation site to alanine in TTP should abrogate the retarded mobility on a SDS gel. Silver staining and immunoblotting have shown that hTTP purified from over-expressed *E. coli* migrates as a single, narrow band, whereas wild-type hTTP migrates as a wide band or series of bands [3]. S220A mutation in mTTP (corresponding to S228A mutation in hTTP) profoundly affects electrophoretic mobility of the protein when expressed in NIH3T3 cells [45]. We recently expanded this approach with extensive site-directed mutants of hTTP. Our results demonstrated that S197A, S218A and S228A mutants had a major effect on the electrophoretic mobility of hTTP. Human TTP protein with a single mutation at S¹⁹⁷ migrated faster than the wild-type protein. Human TTP protein with double mutations at S²²⁸ and S¹⁹⁷ sites resulted in even faster migration than the mutated protein at S¹⁹⁷ alone. Furthermore, human TTP protein with triple mutations at S¹⁹⁷, S²¹⁸ and S²²⁸ sites migrated more rapidly than the protein with the double mutations. There was no further significant increase in migration using human TTP proteins with additional mutant sites, including S²⁹⁶, S⁸⁸, S¹⁸⁶, T²⁷¹, or S⁹⁰ plus S⁹³ [55]. However, the wild-type protein, as well as all of the nine mutant hTTP proteins tested in our experiment, migrated more slowly than hTTP purified from *E. coli*. The mutant proteins were subjected to “global” dephosphorylation with CIAP. CIAP treatment of all of these mutant proteins increased their migration [55], suggesting the presence of additional phosphorylation sites in these mutant proteins in intact cells. *In vivo* labeling results have supported the above findings that S¹⁹⁷, S²¹⁸ and S²²⁸ are three major sites affecting the electrophoretic mobility of hTTP, but that even the most extensively mutated proteins are still phosphorylated in intact cells [55].

Phosphopeptide mapping & protein sequencing

Phosphopeptide mapping allows for an estimation of the number of phosphorylated peptides present in a protein. Typically, the proteins are *in vivo* radiolabeled, purified and digested with an enzyme, and the resulting peptides separated and analyzed for ³²P-containing peptides. This approach was investigated to determine its utility for the identification of hTTP phosphorylation sites. Phosphopeptide mapping of wild-type and site-directed mutant hTTP proteins was performed in an effort to uncover potential phosphorylation sites. Transfected HEK293 cells were labeled with [³²P]-orthophosphate, and wild-type and mutant hTTP proteins were purified using Ni-NTA affinity beads, and digested with either trypsin or lysyl endopeptidase. The resulting peptides were separated by reverse-phase HPLC and the amount of radioactivity in each fraction was determined. Surprisingly, the phosphopeptide profiles showed that the wild-type and the mutant hTTP proteins contained a similar number of phosphopeptide-containing fractions, although some minor differences in retention time of phosphopeptides among these proteins were observed (unpublished results).

The position of the phosphorylated residue within a phosphopeptide can be determined by Edman degradation. To investigate this technique, the HPLC fractions containing ³²P-labeled human TTP phosphopeptides were subjected to sequential Edman degradation. An increase in the amount of [³²P] released during one amino acid cycle over the previous cycle during the Edman degradation process indicates that the amino acid residue released in that cycle is phosphorylated. One hTTP phosphorylation site was successfully identified at S⁸⁸ using this methodology (unpublished results). This finding is consistent with the MS/MS results showing that S⁸⁸ of hTTP is phosphorylated in the transfected HEK293 cells [55]. Although one phosphorylation site could be identified using Edman sequencing, this method is tedious, time- and sample-consuming, and yielded limited information on hTTP phosphorylation sites.

Mass Spectrometry

Mass spectrometry (MS) has proven to be an important analytical technique in the characterization of phosphorylated proteins. The increased sensitivity and high mass accuracy of MS has provided for the rapid analysis and identification of many sites of phosphorylation. A number of various mass spectrometric-based techniques were investigated to determine the sites of phosphorylation in human TTP.

MALDI/MS—MALDI/MS is a very effective tool for the detection and determination of the number of phosphate groups in a phosphopeptide. This method is based on the observation of the addition of 80 Da per phosphate group to an ion, which corresponds in mass to the nonphosphorylated peptides. As a first step in MS-based TTP phosphorylation site analysis, MALDI/MS analyses were performed on a tryptic digest of hTTP purified from HEK293 cells. The hTTP protein band on an SDS-PAGE gel was identified by immunoblot, the band was excised from the gel and the protein digested with sequencing grade trypsin using an Investigator™ Progest Protein Digestion Station (Genomic Solutions, Ann Arbor, MI). The tryptic peptide mixtures were analyzed by MALDI/MS using a Voyager Super DE STR (Applied Biosystems, Framingham, MA) delayed-extraction time-of-flight mass spectrometer. Most of observed ions correspond in mass to the predicted nonphosphorylated tryptic peptides of hTTP. However, the MALDI/MS data suggested the presence of several monophosphorylated peptides (tryptic peptide T21 and T1) and the presence of multiple phosphorylation sites in tryptic peptide T26 (amino acids 271–314) and tryptic peptide T20–21/T21–22 (amino acids 195–242/196–243) [55]. From the MALDI data, the relative abundance of the ions corresponding in mass to nonphosphorylated T21 (amino acids 196–242) and T20–21/T21–22 peptides were observed at <8% of the relative abundance of the phosphorylated T21 and T20–21/T21–22 peptides (normalized to 100%). These data suggest that at least one phosphorylation site in tryptic peptides T21 and T20–21/T21–22 is normally present [55]. MALDI/MS, however, is not considered a very quantitative technique for phosphopeptide analysis. The addition of negatively charged modifications, such as phosphorylation, to peptides can reduce the sensitivity of the peptide under the positive ion conditions used in the mass spectrometer.

Tandem MS—Peptide sequencing by tandem MS is extremely useful for the identification of specific phosphorylation sites in proteins. For MS/MS analyses, a parent ion is selected with the first MS and transmitted into a collision cell for fragmentation. The resulting fragment ions are detected with the second MS. In this type of experiment, only ions resulting from fragmentation of the selected parent ion are observed. hTTP phosphorylation sites have been analyzed by both MALDI (IMAC/MALDI/MS/MS) and ESI (LC/ESI/MS/MS) in combination with tandem MS [55]. Prior to the LC/ESI/MS/MS analyses, the proteins (purified by Ni-NTA beads) from HEK293 cells were further purified by HPLC to remove imidazole using a Zorbax C4 reversed phase column (Amersham Biosciences, Piscataway, NJ) and an Agilent (Palo Alto, CA) Cap1100 HPLC system consisting of an autosampler and binary pumps for delivering the gradients. The hTTP containing fraction(s) was determined by analyzing all of the HPLC fractions by MALDI/MS. It was determined that only a minor HPLC peak contained the hTTP, thereby, indicating a poor recovery of hTTP protein following HPLC separation [3,8]. The HPLC-purified hTTP was then digested with trypsin, and the resulting tryptic peptides were analyzed by LC/ESI/MS/MS using a Micromass Q-ToF Ultima Global (Waters/Micromass, Milford, MA) hybrid tandem mass spectrometer. From these analyses, a triply charged ion of m/z 796.4, which corresponds in mass to monophosphorylated tryptic peptide T18 (amino acids 162–182), was observed. Upon MS/MS fragmentation, the ion showed an abundant fragment ion corresponding to the loss of HPO_3 from the molecular ion. The observation of this fragment ion indicates the presence of a phosphate group in the T18 peptide. In addition, a number of

other fragment ions were observed suggesting that S¹⁶⁹ in tryptic peptide T18 (amino acids 162–182) of hTTP is phosphorylated [55].

MALDI/MS/MS was also used to identify phosphorylation sites in hTTP from HEK293 cells following enrichment of phosphopeptides with immobilized metal ion affinity chromatography (IMAC). IMAC has been shown to be a useful method to selectively isolate and enrich phosphopeptides from a peptide mixture prior to MS analyses [58]. After trypsin digestion, the hTTP peptide mixtures were loaded onto an Fe³⁺-IMAC column. The column was then washed to remove any unbound peptides. The IMAC resin with bound phosphopeptides was directly analyzed by MALDI/MS and MALDI/MS/MS. MALDI/MS/MS analyses were performed with either a Micromass Q-ToF Ultima Global (Waters/Micromass, Milford, MA) hybrid tandem mass spectrometer equipped with a MALDI source or a Voyager Proteomics 4700 (Applied Biosystems, Framingham, MA). Both instruments are equipped with a nitrogen laser (337 nm) to desorb and ionize the samples and utilize time-of-flight mass spectrometers as the mass analyzer. These tandem MS analyses have identified phosphorylation sites at S⁶⁶ in tryptic peptide T4, S⁸⁸ and T⁹² in tryptic peptide T5, S¹⁶⁹ in tryptic peptide T18, and S¹⁸⁶ in tryptic peptide T19 of hTTP [55].

MudPIT—Site-directed mutagenesis coupled with *in vivo* radiolabeling and immunoblot identified S¹⁹⁷, S²¹⁸ and S²²⁸ as three major sites affecting the electrophoretic mobility of hTTP, implying stoichiometric phosphorylation [55]. The tandem MS analyses, however, did not identify these phosphorylation sites in peptides at the carboxyl terminus of hTTP, probably due to unfavorable sizes of the tryptic peptides for the MS/MS analyses as previously suggested [46]. Therefore, MudPIT, a 2D liquid chromatographic separation technique, [58,59], was investigated in an effort to determine these and any additional phosphorylation sites in hTTP. Purified hTTP was digested separately with three different enzymes, including trypsin, subtilisin, and elastase, according to a triple digest protocol [59]. The three digestion mixtures were pooled together and separated by a triphasic microcapillary column containing 7 cm of 5- μ m Polaris C18-A material (Metachem, Ventura, CA), 6 cm of 5- μ m Partisphere strong cation exchanger (Whatman) and 3 cm of 5- μ m hydrophilic interaction chromatography material (PolyLC). Peptides were analyzed by MS and tandem MS as described [60]. The use of the triple digestion protocol resulted in 96–97% of hTTP sequence coverage. The resulting MS/MS data were initially analyzed to identify spectra containing a prominent 98-Da ($-H_3PO_4$) neutral loss (or 49 loss from a plus 2 charge state or 33 loss from a plus 3 charge state) from the precursor. In addition, the spectra were searched against the protein database using SEQUEST software to identify the peptide. The resulting SEQUEST output files were filtered for modifications. The spectra from three datasets containing the prominent 98-Da neutral loss were further analyzed for unique MS/MS fragmentation patterns of phosphopeptides using SEQUEST-PHOS, a modified SEQUEST program to model the neutral loss (i.e., -33 , -49 , -98) in the theoretical fragment ion spectrum [59,60]. The masses of phosphorylated peptides were identified in this manner and matched against predicted proteolytic peptides.

Using this approach, a number of phosphorylated peptides were identified based on the observation of loss of H₃PO₄ from the protonated molecule. Specific sites of phosphorylation were assigned on the basis of biological predictions. Based on this combined information, the following sites were assigned as major phosphorylation sites in hTTP from HEK293 cells: S¹⁹⁷, S²²⁸, S²⁷⁶ and S²⁹⁶ [55]. The multiple unique phosphopeptides recovered by the MudPIT analyses containing these amino acids indicate that these residues are probably the major phosphorylation sites in hTTP. In addition, numerous other phosphorylation sites, including the previously found sites at S⁶⁶, S⁸⁸, T⁹², S¹⁶⁹ and S¹⁸⁶, were assigned with lower degrees of confidence by this method. These results are consistent with the MALDI/MS data, where up to three sites of phosphorylation are observed in tryptic peptide T20–21/T21–22 (amino acids

195–242/196–243) and up to two sites of phosphorylation are observed in tryptic peptide T26 (amino acids 271–314) in hTTP.

Computational analysis

Computer programs can also be used to predict potential phosphorylation sites in proteins. A motif scanning program (<http://scansite.mit.edu>) [61,62] was used to predict hTTP phosphorylation sites and potential protein kinases that could phosphorylate TTP. The results of this software program have suggested that hTTP is potentially phosphorylated at multiple sites by a variety of protein kinases, including: 1) ERK1; 2) p38 MAPK; 3) Cdc2; 4) GSK3; 5) PKA; 6) PKC μ and 7) PKC ξ (Table 1). Some of the phosphorylation sites identified from the MS-based analyses, i.e. S¹⁹⁷, S²¹⁸ and S²²⁸ are predicted by motif scanning programs to be potential sites for protein kinase A (S¹⁹⁷), extracellular-signal-regulated kinase 1 (both S²¹⁸ and S²²⁸) and glycogen synthase kinase-3 (S²²⁸), respectively (Table 1). The Pile-up program of the GCG software (Accelrys, San Diego, CA) was also used to analyze the conservation of TTP phosphorylation sites identified by various approaches as described above and in other studies. Interestingly, almost all of the phosphorylation sites identified in hTTP are conserved in mammalian TTP proteins from various species, including human, mouse, rat, bovine, sheep, pig, horse, chimpanzee and dog (Figure 1). Of these, S⁶⁶, S⁸⁸, S⁹⁰, S⁹³, S¹⁹⁷, S²¹⁸, S²²⁸, S²⁵², T²⁷¹ and S²⁹⁶ sites in hTTP are conserved in all mammalian orthologues of TTP identified so far, along with their corresponding surrounding sequence motifs (Figure 1). Some of the phosphorylation sites reported here are located in relatively conserved sequence blocks among all three proteins of the TTP family (TTP, ZFP36L1 and ZFP36L2), including T¹⁰⁶, Y¹⁵⁸, S¹⁸⁴, S¹⁸⁶, S²¹⁷, S²¹⁸, S²⁷³, S²⁷⁶, Y²⁸⁴, S²⁹⁴ and S²⁹⁶ in hTTP (Figure 4).

In summary, both experimental and computational analyses described above indicate that TTP is a hyperphosphorylated protein in normal cells and is potentially phosphorylated at multiple sites by different protein kinases, and that almost all of the phosphorylation sites reported here is conserved in TTP from various mammalian species. LC/ESI/MS/MS and MALDI/MS/MS have identified phosphorylation sites at S⁶⁶, S⁸⁸, T⁹², S¹⁶⁹ and S¹⁸⁶ in various tryptic peptides of hTTP, and direct protein sequencing has confirmed a phosphorylation site at S⁸⁸ in hTTP. MudPIT analysis has identified phosphopeptides with numerous phosphorylation sites in hTTP; these phosphopeptides contain the five sites listed above as well as other high probability sites (those detected in multiple unique phosphopeptides) at S¹⁹⁷, S²¹⁸, S²²⁸, S²⁷⁶, S²⁹⁶ and numerous lower probability sites. Site-directed mutagenesis has shown that alanine mutations of hTTP at S¹⁹⁷, S²¹⁸ and S²²⁸ increase the electrophoretic mobility of the mutant proteins on SDS-PAGE with a tris/glycine gel buffer system, an indication of stoichiometric changes in phosphorylation [56,57,63]. Computer programs predict that TTP is potentially phosphorylated by a variety of protein kinases. *In vitro* phosphorylation assays have demonstrated that TTP is phosphorylated by a number of protein kinases, including p42 MAP kinase (ERK2), p38 MAP kinase, JNK, MAPKAP kinase 2 (MK2), GSK3 β , PKA, PKB α and PKC μ . All of the sites reported here are conserved in TTP from various mammalian species. Some of the phosphorylation sites in TTP reported in our study and other studies are conserved in TTP homologues.

Expert commentary & five-year view

To increase our understanding of the function of TTP and the role of phosphorylation, additional function/structure studies are necessary. The first major task in the future is to analyze phosphorylation sites in TTP from other cell lines and tissues from various physiological conditions, since the goal of our previous work was to investigate the phosphorylation sites in hTTP expressed in HEK293 cells. It is possible that the major phosphorylation sites and the total number of phosphorylation sites will be different in different cell lines under normal physiological circumstances. This possibility is supported by the

observed differences between our study and other studies published in the literature as highlighted below. First, previous studies have suggested that S⁵², S¹⁷⁸ and S²²⁰ of mTTP (corresponding to S⁶⁰, S¹⁸⁶ and S²²⁸ of hTTP) are the major phosphorylation sites in mTTP [36,46,51]. Based on the techniques described above, S⁶⁰ was not observed as a major site in hTTP; however, S¹⁸⁶ and S²²⁸ were confirmed as major phosphorylation sites in hTTP [55]. In addition, S⁶⁶, S⁸⁸, T⁹², S¹⁶⁹, S¹⁹⁷, S²¹⁸, S²⁷⁶ and S²⁹⁶ are predicted as high probability sites in hTTP [55]. Second, some phosphorylation sites described in our study have not been reported previously, including the high probability sites based on site-directed mutagenesis and MS analyses at S⁸⁸, T⁹², S¹⁶⁹, S¹⁹⁷, S²¹⁸, S²⁷⁶ and S²⁹⁶, and the lower probability predicted sites based on MudPIT at S¹², S²¹, S⁴¹, S⁴³, S⁴⁶, S⁴⁸, S⁹⁰, S⁹³, T⁹⁵, T¹⁰⁶, T¹¹¹, Y¹⁵⁸, S¹⁶⁰, S¹⁸⁸, T¹⁹⁶, S²⁰⁷, S²¹⁰, S²¹⁷, S²³⁰, S²³³, T²³⁸, S²⁵², S²⁷³, S²⁷⁹, Y²⁸⁴ and S²⁹⁴ in hTTP [55]. Finally, although previous studies have shown that S⁵², S¹⁰⁵ and S³¹⁶ of mTTP are phosphorylated in intact cells [46], the equivalent sites in hTTP, S⁶⁰, S¹¹³ and S³²³, respectively, were not observed as phosphorylated [55]. These observed differences may be due to the differences in cell cultures and/or analytical methods used by the two groups.

The second major task in future research will be to investigate the functional consequence(s) of phosphorylation. It has been shown that hTTP expressed in HEK293 cells and then dephosphorylated by CIAP binds to GM-CSF mRNA ARE more tightly than native, phosphorylated hTTP [12]. Human TTP purified after expression in *E. coli* exhibits approximately a two-fold greater affinity for TNF mRNA ARE than the hTTP protein purified from transfected HEK293 cells [3]. However, mRNA binding activity of TTP is not significantly affected by phosphorylation with p42 MAP kinase/ERK2, p38 MAP kinase, JNK [8] or MK2 [46]. The hTTP mutants we studied exhibit ARE-binding activity using the electrophoretic mobility shift assay, and several of the mutants exhibit activity in the *in vitro* deadenylation assay [55]. Confocal microscopy following immunostaining has shown that the subcellular localization of hTTP is not apparently altered by the S186A, S(197, 218, 228)A or the S(88, 90, 93, 186, 214, 218, 228, 296)A mutations [55]. Additional studies with pure mutant proteins would be necessary to determine the precise relationship between the status of phosphorylation at individual sites and ARE-binding activity.

In addition to the effect of phosphorylation on mRNA ARE binding activity of TTP [3,12, 52], phosphorylation may have other functional consequences on the protein, including TTP's stability [33,52,53], autoregulation [32,64], and subcellular localization [51,53]. Phosphorylation of TTP may also affect its association with exosome [65], stress granule [36], nucleoporin CAN/Nup214 [66], and retroviral Tax oncoproteins [67]. Phosphorylation may play a role in TTP-mediated delivery of ARE-mRNAs to processing bodies for translational silencing and mRNA decay since ARE-mRNA localization in processing bodies is mediated by the phosphorylation site-rich N- and C-termini of TTP [68]. TTP was shown to interact through association with Ago/eIF2C family members to complex with miR16 (a human miRNA containing an UAAAUAAU sequence that is complementary to the ARE sequence) and assist in the targeting of TNF ARE [69]. Cth2 (a TTP homologue in yeast) was shown to regulate mRNA degradation in response to iron deficiency [70]. Significant progress has been made on the effects of phosphorylation at S⁵² and S¹⁷⁸ in mouse TTP (corresponding to S⁶⁰ and S¹⁸⁶ in human TTP) on TTP's stability, subcellular localization, binding to ARE sequences, and binding 14-3-3 proteins [36,46,51–53]. However, much needs to be learned about the effects of other phosphorylation sites on TTP functions and interactions with other partners under physiological conditions.

The biological significance of the phosphorylation sites identified in the zinc finger motifs is still not clear. Four phosphorylation sites are located in the highly conserved zinc finger domains in human TTP: T¹⁰⁶, T¹¹¹, Y¹⁵⁸, and S¹⁶⁰ (Figure 1). One additional site at S¹¹³ in human TTP is potentially phosphorylated since the corresponding site at S¹⁰⁵ in mouse TTP

is identified as a phosphorylation site [46]. However, they are probably minor phosphorylation sites since truncated TTP fragments are not significantly phosphorylated in transfected cells by *in vivo* radioactive labeling [55]. The zinc finger domains synthesized chemically or expressed in *E. coli* [2] can bind to the same ARE as the recombinant full-length TTP with similar binding affinity by the electrophoretic mobility shift assay [3]. However, it is impossible to rule out minor effects of phosphorylation at these sites since the binding assay is a semi-quantitative method. It may still be possible to reveal the minor difference of binding affinity by directly comparing the RNA binding affinity between the phosphorylated and unphosphorylated zinc finger domains in the future.

The third major task in future studies is to understand the mechanisms of regulation of TTP phosphorylation, including the types of protein kinases and their target sites in TTP. Twelve of the phosphorylation sites reported in hTTP, including S⁴¹, S⁴⁶, S⁸⁸, S⁹⁰, S⁹³, S¹⁹⁷, S²¹⁸, S²²⁸, T²³⁸, T²⁵⁷, T²⁷¹ and S²⁹⁶ (Figure 1), are potential sites for proline-directed protein kinases [55]. Motif scanning (<http://scansite.mit.edu>) [61,62] suggests that hTTP is a potential substrate for a variety of protein kinases, including: 1) ERK1; 2) p38 MAPK; 3) Cdc2; 4) GSK3; 5) PKA; 6) PKC μ and 7) PKC ξ (Table 1). S¹⁹⁷, S²¹⁸ and S²²⁸ are predicted by motif scanning programs to be potential sites for protein kinase A (S¹⁹⁷), extracellular-signal-regulated kinase 1 (both S²¹⁸ and S²²⁸) and glycogen synthase kinase-3 (S²²⁸), respectively (Table 1). The ligands that induce TTP expression are quite diverse from pro-inflammatory stimuli like LPS to anti-inflammatory stimuli contained in tea and cinnamon. What regulates phosphorylation events that are appropriate for the activity of TTP as a regulator of mRNA? Understanding the importance of these potential phosphorylation reactions will require further studies using a variety of techniques to discover the types of protein kinases and their target sites in TTP.

The RNA ARE sequences bound by TTP were characterized recently. The nonamer 5'-UUAUUUAUU-3' was identified as the optimal site from a random collection of oligonucleotides using the SELEX procedure and the recombinant full-length TTP protein [4]. The identical nonamer sequence was also determined to be the preferred binding sequence for the synthetic TTP zinc finger domains with binding affinities in the low nanomolar range by gel-shift analysis [2]. Nuclear magnetic resonance analysis has shown that the nonamer 5'-UUAUUUAUU-3' is the minimal complete binding site for the TTP peptide and that several tandem molecules of the TTP peptide could bind to a single RNA ARE containing several repeats of this sequence, such as those found in the TNF- α and GM-CSF AREs [2]. Meanwhile, the first zinc finger domain binds to UUAUUUU and both zinc finger domains bind to UUAUUUAUUU with micromolar affinity [7,71]. Finally, Hudson and colleagues determined the structure of the tandem zinc domain of TTP homologue ZFP36L2 (TIS11D) in complex with 5'-UUAUUUAUU-3' [72]. Each of the two zinc fingers forms very similar structures with 5'-UAUU-3'. The 'lead-in' motifs R(K)YKTEL of each finger also participate through hydrogen-bonding interactions with the 5'-most bases on each 5'-UAUU-3'. It was suggested that a similar model for interaction between TTP and 5'-UUAUUUAUU-3' exist [22] since the zinc finger sequences between TTP and TIS11D are highly conserved (Figure 4). All of the above studies employed nonphosphorylated TTP peptide, therefore, it is unknown if the preferred ARE sequence 5'-UUAUUUAUU-3' is the same if using phosphorylated TTP peptide.

Finally, it will be informative to identify all of the phosphorylation sites in the other TTP family members, as well as the potential remaining sites in TTP, and to determine the roles that these phosphorylation events play in the regulation of mRNA metabolism by these proteins. There are two TTP-related proteins in human cells, ZFP36L1 and ZFP36L2 [1]. A third related protein (ZFP36L3) was recently identified that seems to be expressed only in rodents as a placenta-specific protein [31]. Recent studies have shown that mice deficient in ZFP36L1 develop chorioallantoic fusion defects and embryonic lethality [73], and mice with decreased levels of

an amino-terminal truncated form of ZFP36L2 exhibit female infertility and disrupted early embryonic development [74]. Some of the phosphorylation sites reported here are located in relatively conserved sequence blocks among all three proteins of the TTP family, including T¹⁰⁶, Y¹⁵⁸, S¹⁸⁴, S¹⁸⁶, S²¹⁷, S²¹⁸, S²⁷³, S²⁷⁶, Y²⁸⁴, S²⁹⁴ and S²⁹⁶ in hTTP (Figure 4). The phosphorylation site at S³¹⁶ reported in mTTP [46], corresponding to S³²³ in hTTP, is localized at the carboxyl terminus and is conserved in all of the sequences in the TTP family (Figure 4). Furthermore, T¹⁰⁶ and Y¹⁵⁸ in hTTP are conserved in the two TTP homologues, which are located close to the termini of the two zinc finger binding motifs (Figure 4). These two sites in ZFP36L2/TIS11D (T¹⁵⁶ and Y²⁰⁸ in mouse TIS11D) are closely associated with the bound RNA sequence 5'-UUAUUUAUU-3' [72]. The interaction between TTP and 5'-UUAUUUAUU-3' was also proposed to be similar based on the TIS11D model and the sequence identity between TTP and TIS11D [22]. The structural analyses suggest potential effects of the two phosphorylation sites at T¹⁵⁶ and Y²⁰⁸ in mouse TIS11D (T¹⁰⁶ and Y¹⁵⁸ in human TTP) on the binding of ARE sequences, although sufficient evidence is lacking. Finally, both TTP and TIS11B/BRF1 are phosphorylated by protein kinase B/AKT1 [49,75,76]. On the other hand, some of the major phosphorylation sites identified in hTTP (S⁶⁶, S⁸⁸, T⁹², S¹⁶⁹, S¹⁸⁶, S¹⁹⁷ and S²²⁸) and mTTP (S⁵², S¹⁷⁸ and S²²⁰) appear to be specific to TTP as these sites are not present in the other two members of TTP family proteins (Figure 4). Therefore, it will be important to identify the phosphorylation sites in the other family members, and the remaining sites in TTP before one can fully understand the different roles of TTP family proteins in the regulation of mRNA metabolism under physiological conditions. Understanding the dynamics and complexity of rapid and heavy phosphorylation of TTP family proteins in responding to numerous cellular and environmental signals could serve as a model for other hyperphosphorylated proteins involved in mammalian posttranscriptional regulatory mechanisms.

Key issues

- *In vivo* radiolabeling continues to be a useful method for the study of TTP phosphorylation sites.
- Site-directed mutagenesis coupled with *in vivo* labeling and immunoblot has proven to be a useful approach for identifying TTP phosphorylation sites.
- Matrix-assisted laser desorption/ionization mass spectrometry (MS) is a very effective method for the initial determination of phosphopeptides of TTP.
- Tandem MS analyses are a powerful tool for the identification of specific phosphorylation sites in TTP.
- Immobilized metal ion affinity chromatography in combination with MS allows for the selective enrichment and detection of phosphopeptides in TTP.
- Multidimensional protein identification technology in combination with MS is a useful approach for the analysis of phosphorylation sites in TTP.
- Multiple approaches are required to determine and localize the sites of phosphorylation in proteins, especially if the protein of interest has multiple phosphorylation sites such as TTP.

Acknowledgements

We greatly appreciate Dr Joseph F Urban Jr (USDA-ARS) and Dr Kenneth B Tomer (NIH-NIEHS) for their strong support. We also thank Dr John D Venable in Dr John R Yates III's Laboratory (Scripps Research Institute) for the collaboration on the MudPIT MS analyses, and Dr Kiran S Panickar (USDA-ARS) and Dr Joseph F Urban Jr for their helpful comments on the manuscript.

Abbreviations not used in the final publication

TTP	tristetraprolin
hTTP	human TTP
mTTP	mouse TTP
ARE	AU-rich element
CIAP	calf intestine alkaline phosphatase
COX2	cyclooxygenase 2
ERK2	p42 MAP kinase
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK	glycogen synthase kinase
HEK	human embryonic kidney
IL	interleukin
IMAC	immobilized metal ion affinity chromatography
JNK	c-Jun-N-terminal kinase
LC	liquid chromatography
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption ionization
MAP	mitogen-activated protein
MK2	MAP kinase-activated protein kinase 2

MS	mass spectrometry
MudPIT	multidimensional protein identification technology
PK	protein kinase
TNFα	tumor necrosis factor-alpha
ZFP	zinc finger protein

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• of interest

• • of considerable interest

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Species	Alignment of amino acid sequences of mammalian TTP. Potential phosphorylation sites in human TTP are indicated on top and those previously identified in mouse TTP at the bottom. The differences in amino acid sequences between human and pig TTP are underlined in pig sequence.
hTTP site	S ¹² S ²¹ S ⁴¹ S ⁴³ S ⁴⁶ S ⁴⁸ S ⁶⁶ S ⁸⁸ S ⁹⁰ T ⁹² S ⁹³ T ⁹⁵
Human	MDLTAIYESLLS ¹² SPDVPVPS ²¹ DHGGTESSPGWGSSGSPWSL ⁴¹ SP ⁴³ SDSS ⁴⁶ SPSGVTSRLPGRSTSLVEGRSCGWVPPPPGFAPLAPRLGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Pig	MDLTAIYESLLS ¹² SPD.LPSSDHGGTESSPGWATAGLWSLNS ⁴¹ SDSS ⁴³ SPAGVAARLPGRSTSLVEGRSCGWVPPPPGFAPLAPRGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Mouse	MDLSAIYESLQSM ¹² SHD..LSSDHGGTES....LGGLWNIN.SDSIP ⁴¹ SGVTSRLTGRSTSLVEGRSCGWVPPPPGFAPLAPRGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Rat	MDLSAIYESLMS ¹² SHD..LSPDHGGTES....SGGLWNINS ⁴¹ SDSIP ⁴³ SGVTSRLTGRSTSLVEGRSCSWVPPPPGFAPLAPRGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Bovine	MDLAAIYKSLLS ¹² SPD..LP ²¹ SDLGETESSTWASSGSPWSL ⁴¹ SS ⁴³ SDSLPEVAARLPGRSTSLVEGRSCGWVPPPPGFAPLAPRPS ⁶⁶ SDWSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Sheep	MDLAAIYKSLLS ¹² SPD..LP ²¹ SDLGETESSTWASSGSPWSL ⁴¹ SS ⁴³ SDSLPEAAARLPGRSTSLVEGRSCGWVPPPPGFAPLAPRPS ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Chimpanzee	...NWETVLSLLS ¹² SPDVPVPS ²¹ DHGGTESSPGWGSSGSPWSL ⁴¹ SP ⁴³ SDSS ⁴⁶ SPSGVTSRLPGRSTSLVEGRSCGWVPPPPGFAPLAPRLGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Dog	.....QSLLS ¹² SPD..MP ²¹ SDHGDTESSIPGWPPSGLWSL ⁴¹ SS ⁴³ SDSS ⁴⁶ SPVGVAAARLPGRSTSLVEGRSCGWVPPPPGFAPLAPRGP ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
Horse	MDLTAIYESLLS ¹² SPD..LP ²¹ SDHGGTESSPGWGSSGLWSL ⁴¹ SS ⁴³ SDSS ⁴⁶ SPVGVAAARLPGRSTSLVEGRSCGWVPPPPGFAPLAPRPS ⁶⁶ ELSP ⁸⁸ SP ⁹⁰ TS ⁹² PT ⁹⁵
mTTP site	S ⁵² S ⁵⁸
hTTP site	T ¹⁰⁶ T ¹¹¹ Y ¹⁵⁸ S ¹⁶⁰ S ¹⁶⁹ S ¹⁸⁴ S ¹⁸⁶ S ¹⁸⁸ T ¹⁹⁶ S ¹⁹⁷ S ²⁰⁷ S ²¹⁰
Human	PSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Pig	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Mouse	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Rat	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Bovine	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Sheep	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Chimpanzee	PSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Dog	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
Horse	SSRYKTEL ¹⁰⁶ CR ¹¹¹ TFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLGQRC ¹⁵⁸ PYGSRCHFI ¹⁶⁰ HNPS ¹⁶⁹ EDLAPGHPVLRQ ¹⁸⁴ SI ¹⁸⁶ FS ¹⁸⁸ GLPSGRR ¹⁹⁶ TS ¹⁹⁷ PPPLAGP ²⁰⁷ SL ²¹⁰ SSSF
mTTP site	S ¹⁰⁵ S ¹⁷⁶ S ¹⁷⁸
hTTP site	S ²¹⁷ S ²¹⁸ S ²²⁸ S ²³⁰ S ²³³ T ²³⁸ S ²⁵² T ²⁵⁷ T ²⁷¹ S ²⁷³ S ²⁷⁶ S ²⁷⁹ Y ²⁸⁴ S ²⁹⁴ S ²⁹⁶
Human	SPSSSPPPP.GDLPLSPSAFSAAPGTPLARRDPTPVCCPSCRRRA.TPISVWGVLGGLVLRTPSVQSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Pig	SPSSSPPPP.GDLPLSPSAFSAAPGTPLARRDPTPVCCPSCRRRA.TPSSIWGVLGGLPRSPSAHSLGSDPDEYASSGSSLGGSDSPVFDAGVLGPQPTAVPRRLPIFNRI ²¹⁷ SVSE
Mouse	SPSSSPPPP.GDLPLSPSAFSAAPGTPVTRRDNPQACCPSCRRSTTPSTIWGVLGGLARSPSAHSLGSDPDDYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Rat	SPSSSPPPP.GDLPLSPSAFSAAPGTPVSRDDPTPVCCPSCRRSTTPSTIWGVLGGLARSPSAHSLGSDPDDYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Bovine	SPSSSPPPP.GDLPLSPSAFSAAPG.HLCRRDPTPVCCPSCRRRA.TPNSVWGVLGGLARSPSAHSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Sheep	SPSSSPPPP.GDLPLSPSAFSAAPGTPVSRDDPTPVCCPSCRRRA.TPNSVWGVLGGLARSPSAHSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Chimpanzee	SPSSSPPPP.GDLPLSPSAFSAAPGTPLARRDPTPVCCPSCRRRA.TPISVWGVLGGLVLRTPSVQSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Dog	SPSSSPPPP.GDLPLSPSAFSAAPGTPVARRDPTPVCCPSCRRRA.TPSSVWGVLGGLARSPSAHSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
Horse	.....S ²²⁰ .....CRRA.TPSSVWGVLGGLARSPSAHSLGSDPDEYASSGSSLGGSDSPVFEAGVFAPPQVAPARRLPPIFNRI ²¹⁷ SVSE
mTTP site	S ²²⁰ T ²³⁰ S ²⁶⁴ S ³¹⁶

Figure 1. Tristetraprolin (TTP) is so-named because the deduced amino acid sequences of TTP from mammals contain three repeats with four consecutive proline residues in each repeat
The amino acid sequences used in the alignment include human (NP_003398) [77], mouse (NP_035886) [27], rat (P47973) [78], bovine (P53781) [79], sheep (AY462109) [67], pig (AJ943797, CB288050, CB286240, DY416794 and DY419026) [80], horse (CD536573 and CD536523), chimpanzee (CR555169 and XM_001136016) and dog (AAEX01054372.1 and AAEX01054371.1) [81]. The sequences were aligned with the PILEUP program from GCG. There are 180 amino acid residues in chimpanzee TTP N-terminal region (not shown). The gaps in the horse TTP sequence represent incomplete sequence and it is probably highly similar to the other mammalian TTP sequences. The four proline residues in the three repeats are underlined. The bold residues within the sequence alignment are the phosphorylation sites identified in our study and in other studies and the CCCH residues in the tandem zinc-finger binding motifs. The conserved serine, threonine and tyrosine residues, which correspond to the phosphorylation sites in hTTP identified in our report, are indicated at the top of the sequence alignment. Those corresponding to the previously identified sites in mTTP are indicated at the bottom of the sequence alignment (updated from supplemental table 2 [55]).

Figure 2. Tristetraprolin (TTP) related proteins have been found in many species, ranging from human through yeasts and plants

Sequence identity relative to human proteins: human TTP (100%); mouse TTP (86.2%); rat TTP (86.5%); bovine TTP (87.3%); frog TTP (53.0%); human TIS11B (100%); mouse TIS11B (98.5%); rat TIS11B (98.5%); frog TIS11B (81.8%); human TIS11D (100%); mouse TIS11D (99.6%); frog TIS11D (74.4%).

At: *Arabidopsis thaliana* (weed); Bt: Bovine; Cc: Carp; Ce: *Caenorhabditis elegans*; Cv: Oyster; Dm: *Drosophila*; Dr: Zebra fish; Hs: Human; Mm: Mouse; Rn: Norway rat; Rr: Black rat; Sc: Baker's yeast; Sp: Fission yeast; Xl: Frog.

Species	Protein	GenBank number	Amino acid residues of tandem CCCH zinc finger motifs and surrounding regions
At		AAF26498	YVRGGKKEDQEEIEVEVYNQGMT Y TELC N KNQ E TG T C P YGD H C Q FA H G I KELRPVIRH P K Y TE V CR M VL A GD N C P Y G H R C H F R H S L S E Q E K L V A G F
Bt	TTP	P53781	DW...SPSPSTPTATPT...TSSRY Y TELC R TFSESG R C R YGA K C Q FA H GLGELRQASRH P K Y TE L CH K F Y L Q GR C P Y GS R C H F I HN P SEDLAA.PGHP
Cc	CTH1	CAA71245	ALLPLVESPPMTPWL...CSTRY Y TELC S RYA E TG T C K YAE R C Q FA H GLHDLHVP S R H P K Y T E L CR T Y H T A G C V Y GT R CL F V H N L KE R P V R Q RC R
Ce		T21954	PQLTPMATPNGQAPPPK...NP K LY Y TELC R SWMDH G R C N Y GE R C Q Y A H G ELEKRPV R H P K Y TE A C Q S F H Q S G Y C P Y GP R C H F I HN...EPPSAQ S Q S
Ce		T21955	PQLTPMATPNGQAPPPK...NP K LY Y TELC R SWMDH G R C N Y GE R C Q Y A H G ELEKRPV R H P K Y TE A C Q S F H Q S G Y C P Y GP R C H F I HN...EPPSAQ S Q S
Cv	TIS11	AAB69448	LSSAASRQ N Q...NV...NSSR Y TELC R PFEE S GH C K Y GD K C Q FA H GAHELRLN R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN D EERN Q N.VN.K
Dm	CG4070-PA	AAF48194	RKLERTQSEPLPPQ Q PM...NTSR Y TELC R PFEE E AG C K Y GE K C Q FA H GSHELRLNV R H P K Y TE L CR T F H SV G F C P Y GP R C H F I HN A DEARA Q .QAAQ
Dm	TIScc1	CAA57066	RKLERTQSEPLPPQ Q PM...NTSR Y TELC R PFEE E AG C K Y GE K C Q FA H GSHELRLNV R H P K Y TE L CR T F H SV G F C P Y GP R C H F I HN A DEARA Q .QAAQ
Dr	ZF-CCCH	CAB55775	ALLPLVESPPMTPWL...CSTRY Y TELC S RYA E TG T C K YAE R C Q FA H GLHDLHVP S R H P K Y T E L CR T Y H T A G C V Y GT R CL F V H N L KE R P I R R R
Hs	TTP	NP_003398	EL...SPSPSTPTATST...TPSR Y TELC R TFSESG R C R YGA K C Q FA H GLGELRQAN R H P K Y TE L CH K F Y L Q GR C P Y GS R C H F I HN P SEDLAA.PGHP
Hs	TIS11B	Q07352	LL...PTQ K Q P GGG Q V...NSSR Y TELC R PFEE E AG C K Y GD K C Q FA H GIHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERRAL.AGAR
Hs	TIS11D	P47974	LLHL Q . Q Q K GGG S Q I ...NSTRY Y TELC R PFEE S GT C K Y GE K C Q FA H GFHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERR P A.PS..
Mm	TTP	NP_035886	EL...SPSPSTPTATPT...TSSR Y TELC R TYSESG R C R YGA K C Q FA H GLGELRQAN R H P K Y TE L CH K F Y L Q GR C P Y GS R C H F I HN P TEDLAL.PGQP
Mm	TIS11B	NP_031590	LL...PTQ K Q P GGG Q V...NSSR Y TELC R PFEE E AG C K Y GD K C Q FA H GIHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERRAL.AGGR
Mm	TIS11D	B39590	LL...PTQ K Q P GGG Q V...NSSR Y TELC R PFEE E AG C K Y GD K C Q FA H GIHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERRAL.AGGR
Mm	ZFP36L3	NM_001009549	EHS L Q K PK Q K V SGSSSLAT S ERY Y TELC R PFEE S GI C K Y GH K C Q FA H GYELR T L S R H P K Y T E P CR T F H SV G F C P Y GT R C H F I HN Q PE Q PV L SE T
Os	ZF-CCCH	BAB40156	KKGDE K QA P ADSGAELE V YNQ G MF Y TELC N KW E ETG D C P YGD Q C Q FA H GVTELR P V I R H P Y K T AV C RM V L A GD V C P Y G H R C H F R H S L T PAER L LL R S
Rn	TTP	P47973	EL...SPSPSTPTATPT...TSSR Y TELC R TYSESG R C R YGA K C Q FA H GPGLRQAN R H P K Y TE L CH K F Y L Q GR C P Y GS R C H F I HN P TEDLAL.PGQP
Rn	TIS11B	NP_058868	LL...PTQ K Q P GGG Q V...NSSR Y TELC R PFEE E AG C K Y GD K C Q FA H GIHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERRAL.AGGR
Rn	TIS11D	NM_001036626	RSQ L HL L Q Q Q K GGSG S Q I NSTRY Y TELC R PFEE S GT C K Y GE K C Q FA H GFHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERR P AP S GG A
Rn	ZFP36L3	XM_288661	EHS L Q K PK Q K V SGSSSSQAT S ERY Y TELC R PFEE E NG T C R YGN K C Q FA H GYELR T L S R H P K Y T E P CR T F H SV G F C P Y GS R C H F I HN Q PE Q L P ML P GS
Sc	CTH1	NP_010435	QKT T LE P CR R AP L Q L PQ V N K T L Y Y TELC E S F T I K G Y C K Y GN K C Q FA H GLNEL K F K KS N N Y R T K P C I N W S K L G Y C P Y G R C C F K H G DD K D V E I Y Q N A
Sc	CTH2/TIS11	NP_013237	FSSEK E SSA Q PK V K S Q V Q E TP K L Y TELC E S F T L K G S C P Y GS K C Q FA H GLGEL K V K K S C K N F T K P C V N H V L G Y C P Y G R C C F K H G DD N D I AV V V K A G
Sp	ZFS1	P47979	.....GSSNGV A PN G K...R A LY K TE P C K N Q W I SG T C R YGS K C Q FA H G N Q E L K EP P R H P K Y K S E R S C S FM M Y G Y C P Y GL R C C FL H D...ESNAQ S ...A
Xl	TTP	AAD24207	.....KTAL P AL P A...PS R Y Y TELC R TFSE T GT C K Y GA K C Q FA H G K IEL R EP N R H P K Y Y TE L CH K F Y L Y GE C P Y GS R C N F I HP R EQ...G T S Q
Xl	TIS11B	AAD24208	LL.....Q K P G . G Q V ...NSSR Y TELC R PFEE E NG S C K YGD K C Q FA H GIHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERR..L.VSGR
Xl	TIS11D	AAD24209	LLHLQ Q Q Q Q Q K A Q V ...NSTRY Y TELC R PFEE E AG C K Y GE K C Q FA H GFHELRL S L R H P K Y TE L CR T F H T I G F C P Y G P R C H F I HN A ERR.....
Xl	C3H-4	AAD24210	ETPL P S F S A PP K HL S L...SS R Y Y TELC S RYA E SG F C A Y R N R C Q FA H GLR P V Q H P K Y TE L CR S F H LV G T C N Y L G R C L F I S SP Q ER R EP P V L P D
			Consensus sequence of the tandem CCCH zinc finger sequences: YKTELC(X₈)C(X₃)C(X₃)H(X₁₃)YKTELC(X₈)C(X₃)C(X₃)H

Figure 3. The tandem CCCH zinc finger sequences are highly conserved among the diverse species
The sequences were aligned with the PILEUP program of GCG software. The vertical shading lines represent the consensus sequence of the tandem CCCH zinc finger sequences as shown at the bottom of the sequence alignment. The bold amino acid residues within the sequence alignment are the CCCH residues in the tandem zinc-finger binding motifs. The amino acid residues in human TTP and homologues are also bolded. The Ce sequences are not used in the alignment because the amino acid residues between the two zinc fingers and within the fingers are relatively divergent from the sequences presented in the figure. A number of CCCH homologues are present in Ce, including PIE-1 (GenBank accession number: AAB17868, germline specification [82]), MEX-5 (GenBank accession number: Q9XUB2, soma/germline asymmetry [83]), MEX-6 (GenBank accession number: Q09436, soma/germline asymmetry [83]), and POS-1 (GenBank accession number: NP_505172): At: *Arabidopsis thaliana* (weed); Bt: Bovine; Cc: Carp; Ce: *Caenorhabditis elegans*; Cv: Oyster; Dm: *Drosophila*; Dr: Zebra fish; Hs: Human; Mm: Mouse; Os: *Oryza sativa* (rice); Rn: Norway rat; Sc: Baker's yeast; Sp, Fission yeast; Xl, Frog.

Species	Alignment of amino acid sequences of mammalian TTP and its related two members: ZFP36L1 and ZFP36L2. Potential phosphorylation sites in TTP are indicated on top.
TTP (hTTP site)	Human: MDLTAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Mouse: MDLSAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Rat: MDLSAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Bovine: MDLAAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Sheep: MDLAAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Chimpanzee: MDLAAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Dog: MDLAAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Horse: MDLTAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Pig: MDLTAIYESI...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95}
ZFP36L1	Human: MTTTLVSATFDLSEV...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Mouse: MTTTLVSATFDLSEV...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Rat: MTTTLVSATFDLSEV...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95}
ZFP36L2	Human: MSTTLLSAFYVDVDF...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95} Mouse: MSTTLLSAFYVDVDF...^{S12}...^{S21}...^{S41}...^{S43}...^{S46}...^{S60}...^{S66}...^{S88}...^{S90}...^{S92}...^{S93}...^{S95}
TTP (hTTP site)	Human: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Rat: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Bovine: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Sheep: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Chimpanzee: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Dog: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Horse: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Pig: PSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}
ZFP36L1	Human: SSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: SSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Rat: SSRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}
ZFP36L2	Human: STRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: STRYKTELC...^{S106}...^{T111}...^{Y158}...^{S160}...^{S169}...^{S184}...^{S188}...^{T196}...^{S197}...^{S207}...^{S210}...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}
TTP (hTTP site)	Human: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Rat: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Bovine: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Sheep: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Chimpanzee: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Dog: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Horse: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Pig: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}
ZFP36L1	Human: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Rat: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}
ZFP36L2	Human: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323} Mouse: SPSSSSPPPP...^{S217}...^{S218}...^{S228}...^{S230}...^{S233}...^{T238}...^{S252}...^{T257}...^{T271}...^{S273}...^{S276}...^{S279}...^{Y284}...^{S294}...^{S296}...^{S323}

Figure 4. Alignment of amino acid sequences of mammalian TTP (ZFP36), ZFP36L1 (TIS11B), ZFP36L2 (TIS11D), site-directed mutants of human TTP used in our study and the phosphorylation sites identified in our study and in other studies

The amino acid sequences used in the alignment include full-length sequences of TTP from human (NP_003398), mouse (NP_035886), rat (P47973), bovine (P53781) and sheep (AY462109), and from partial amino terminal sequences deduced from the EST sequences of pig (AJ943797, CB288050, CB286240, DY416794 and DY419026), horse (CD536573 and CD536523), chimpanzee (CR555169 and XM_001136016), and dog (BQ172881); full-length sequences of ZFP36L1 (TIS11B) from human (Q07352), mouse (B39590) and rat (NP_058868); and full-length sequences of ZFP36L2 (TIS11D) from human (P47974) and mouse (NP_008818). The sequences were aligned with the PILEUP program of GCG software. The gaps in the horse TTP sequence represent incomplete sequence and it is probably highly similar to the other mammalian TTP sequences. The conserved sequence motifs between the subgroups are boxed and linked with a line between the corresponding boxes. The underlined amino acid residues are the four-proline residues in the three repeats of mammalian TTP. The bold amino acid residues within the sequence alignment are the CCCH residues in the tandem zinc-finger binding motifs; and the conserved serine threonine and tyrosine residues, which correspond to the phosphorylation sites in hTTP identified in our report (indicated at the top of the sequence alignment: S¹², S²¹, S⁴¹, S⁴³, S⁴⁶, S⁴⁸, S⁶⁶, S⁸⁸, S⁹⁰, T⁹², S⁹³, T⁹⁵, T¹⁰⁶, T¹¹¹, Y¹⁵⁸, S¹⁶⁰, S¹⁶⁹, S¹⁸⁴, S¹⁸⁸, T¹⁹⁶, S¹⁹⁷, S²⁰⁷, S²¹⁰, S²¹⁷, S²¹⁸, S²²⁸, S²³⁰, S²³³, T²³⁸, S²⁵², T²⁵⁷, T²⁷¹, S²⁷³, S²⁷⁶, S²⁷⁹, and Y²⁸⁴, S²⁹⁴ and S²⁹⁶); and the three additional phosphorylation sites in mTTP identified previously (also indicated at the top of the sequence alignment: S⁶⁰, S¹¹³ and S³²³ in hTTP, corresponding to S⁵², S¹⁰⁵ and S³¹⁶ in mTTP). The bold phosphorylation sites at the top of the alignment are the conserved phosphorylation sites in TTP, ZFP36L1 and ZFP36L2; which are linked with a line between the two conserved phosphorylation sites. A total of 40 site-directed mutants of His-hTTP were used in our study

[55], including 1-site mutation (10 mutants): S88A, S90A, S93A, S186A, S197A, S214A, S218A, S228A, S296A, T271A; 2-site mutation (8 mutants): S(214,218)A, S(214,228)A, S(214,228)A, S(197, 214)A, S(197, 218)A, S(197,228)A, S(197, 296)A, S(93, 197)A; 3-site mutation (5 mutants): S(88,90,93)A, S(197,214,218)A, S(197,214,228)A, S(197,218,228)A, S(214,218,228)A; 4-site mutation (3 mutants): S(214,218,228,296)A, S(88,90,93,197)A, S(197,214,218,228)A; 5-site mutation (5 mutants): S(88,90,93,214,218)A, S(88,90,93,214,228)A, S(88,90,93,218,228)A, S(197,214,218,228,296)A, S(88,197,214,218,228)A; 6-site mutation (4 mutants): S(88,90,93,214,218,228)A, S(88,197,214,218,228,296)A, S(88,186,197,214,218,228)A, S(88,197,214,218,228)AT271A; 7-site mutation (4 mutants): S(88,186,197,214,218,228,296)A, S(88,197,214,218,228,296)T271A, S(88,90,93,214,218,228,296)A, S(88,90,93,197,214,218,228)A; 8-site mutation (1 mutant): S(88,90,93,197,214,218,228,296)A.

Table 1

TTP is predicted to be phosphorylated at multiple sites by various protein kinases.

Predicted protein kinase	Predicted phosphorylation site position	Predicted peptide sequence with phosphorylation site in bold letter	Phosphorylation sites observed by MS analysis*
ERK1	41	SSGPW S LSPSDSSPS	S41
	88	PRLGPE L SPSPTSPT	S88
	214	SLSSSF S PSSPPP	
	218	SSFSPSS S PPPGDL	S218
	228	PPGDLPL S PSAFSAA	S228
P38	93	ELSP S PTSPTATSTT	S93
	238	AFSAAPG T PLARRDP	T238
GSK3	35	SSPGW G SSGPW S LSP	
	39	WGSSGPW S LSPSDSS	
	52	SSPSGV T SRLPGRST	
	214	SLSSSF S PSSPPP	
	218	SSFSPSS S PPPGDL	S218
PKA	257	CPSCRRAT P ISVWGP	T257
	197	LPSGRRT S PPPPGLA	S197
PKB/Akt	60	RLPGR S TS L VEGRSC	
	113	TELCRT F SESGRCRY	
PKC-a/b/g -epsilon -mu -zeta	252	PTPVCC P SCRRAT P I	S252
	111	YKTELCRT F SESGRC	T111
	66	TSLVEGR S CGWVPPP	S66
	144	NRHPKY K TELCHKFY	
Cdc2/Cdk5	238	AFSAAPG T PLARRDP	T238
	100	SPTATST T PSRYKTE	

from [55]

(Adopted from [49]).