Identification of a Major Phosphopeptide in Human Tristetraprolin by Phosphopeptide Mapping and Mass Spectrometry

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Abstract

Tristetraprolin/zinc finger protein 36 (TTP/ZFP36) binds and destabilizes some pro-inflammatory cytokine mRNAs. TTP-deficient mice develop a profound inflammatory syndrome due to excessive production of pro-inflammatory cytokines. TTP expression is induced by various factors including insulin and extracts from cinnamon and green tea. TTP is highly phosphorylated in vivo and is a substrate for several protein kinases. Multiple phosphorylation sites are identified in human TTP, but it is difficult to assign major vs. minor phosphorylation sites. This study aimed to generate additional information on TTP phosphorylation using phosphopeptide mapping and mass spectrometry (MS). Wild-type and site-directed mutant TTP proteins were expressed in transfected human cells followed by in vivo radiolabeling with [32P]-orthophosphate. Histidine-tagged TTP proteins were purified with Ni-NTA affinity beads and digested with trypsin and lysyl endopeptidase. The digested peptides were separated by C18 column with high performance liquid chromatography. Wild-type and all mutant TTP proteins were localized in the cytosol, phosphorylated extensively in vivo and capable of binding to ARE-containing RNA probes. Mutant TTP with S90 and S93 mutations resulted in the disappearance of a major phosphopeptide peak. Mutant TTP with an S197 mutation resulted in another major phosphopeptide peak being eluted earlier than the wild-type. Additional mutations at S186, S296 and T271 exhibited little effect on phosphopeptide profiles. MS analysis identified the peptide that was missing in the S90 and S93 mutant protein as LGPELSPSPTSPTATSTTPSR (corresponding to amino acid residues 83–103 of human TTP). MS also identified a major phosphopeptide associated with the first zinc-finger region. These analyses suggest that the tryptic peptide containing S90 and S93 is a major phosphopeptide in human TTP.

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Introduction

Tristetraprolin (TTP) is the prototypic member of a small family of tandem C8C5C3H zinc finger proteins (ZFP). Similar tandem C8C5C3H zinc finger sequences have been found in many species, ranging from human through yeasts and plants [1–3]. The TTP protein family consists of three members common to mammals (ZFP36 or TTP, ZFP56L1 or TIS11B and ZFP36L2 or TIS11D) and a fourth member in mouse and rat but not in humans (ZFP36L3) [1,4]. TTP family proteins bind to AU-rich elements (ARE) within single stranded RNAs [5–11] and promote the deadenylation and subsequent destruction of those transcripts [12,13]. TTP-deficiency in knockout mice causes a severe inflammatory syndrome with erosive arthritis, autoimmunity and myeloid hyperplasia [10,14]. This is largely due to excessive production of pro-inflammatory cytokines including tumor necrosis factor alpha (TNFα) and granulocyte-macrophage colony-stimulating factor, whose mRNAs are direct targets of TTP but are stabilized in TTP knockout mice cells [8,12,15]. TTP is therefore regarded as an anti-inflammatory protein.

TTP protein expression is induced in various cell types by a number of factors including insulin [16], lipopolysaccharide (LPS) [17] and cinnamon polyphenolic extract [12,18–20]. TTP is highly phosphorylated in intact cells and in cell-free systems [9,12–20–23]. TTP is a substrate for a number of protein kinases such as p42 mitogen-activated protein (MAP) kinase (ERK2) [6,7,23], p38 MAP kinase [6,7,9,24], c-Jun N-terminal kinase (JNK) [6], MAP kinase-activated protein kinase 2 (MAPKAP kinase 2 or MK2) [25–28], glycogen synthase kinase-3β [29] and protein kinases A, B and C [29]. Mass spectrometry (MS) and site-directed mutagenesis have identified a number of phosphorylation sites in human and mouse TTP (hTTP and mTTP) [3,21,23,25]. However, it is puzzling that mutant TTP with extensive mutations is still phosphorylated extensively in vivo [21,30]. Recent studies
have shown that mutant hTTP with some phosphorylation sites mutated could be a potent inhibitor of malignant glioma cell growth [31]. Therefore, it is important to understand the structure-function relationships of TTP phosphorylation.

Several approaches have been used to identify TTP phosphorylation sites including in vivo labeling, site-directed mutagenesis, mass spectrometry and computational analysis. One major problem is that the major phosphorylation sites identified by mass spectrometry are not necessarily in agreement among different laboratories [3,21,25]. For example, S52, S178 and S220 of mTTP (corresponding to S60, S186 and S228 of hTTP) are the major phosphorylation sites in mTTP, but the human equivalent S60 is not identified as a major site in hTTP [21,25,28,32]. Another example is that S105 and S316 of mTTP are phosphorylated in intact cells [25] but the equivalent sites at S113 or S323 of hTTP are not confirmed in transfected human cells [21].

In this study, we extended our investigation on the identification of potential phosphorylation sites by phosphopeptide mapping, site-directed mutagenesis and mass spectrometry. Our results demonstrated that mutations at both S90 and S93 in hTTP resulted in the disappearance of a major phosphopeptide peak in the HPLC chromatogram. The missing phosphopeptide identified by MS contained both S90 and S93, suggesting that the tryptic peptide is a major phosphopeptide in hTTP from transfected human cells.

Results

Expression of TTP Proteins in Transfected Human Cells

HEK293 cells were transfected with pHis-hTTP plasmids encoding wild-type and mutant proteins with serine and threonine to alanine mutation(s) in hTTP. Immunoblotting showed that all His-hTTP proteins were expressed in the transfected cells (Figure 1A). Immunostaining with TTP antibodies showed that endogenous TTP was undetectable in HEK293 cells transfected only with the pBS+ carrier plasmid (Figure 1B). Wild-type TTP was overexpressed and mainly localized in the cytosol of HEK293 cells transfected with wild-type pHis-hTTP (Figure 1B). Mutant TTP proteins were also expressed and primarily localized in the cytosol of transfected HEK293 cells in patterns similar to those of the wild-type TTP (Figure 1B).

Multiple Distinctive Species of TTP Proteins from Transfected Human Cells

Wild-type and a number of mutant His-hTTP proteins were purified by Ni-NTA beads and eluted with an imidazole solution. The purified proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB) and silver reagents for observing the electrophoretic mobility of the proteins. CBB staining is less sensitive so that minor size differences between TTP protein bands could be seen on the gel. Silver staining is more sensitive, which could easily obscure the neighboring TTP bands, resulting in a smear or fat band instead of distinctive bands. CBB staining showed that the wild-type and mutant hTTP proteins with 1–4 mutations exhibited multiple distinctive bands on protein gels (Figure 2A). Silver staining showed that the wild-type and mutant hTTP proteins with 1–4 mutations exhibited multiple distinctive bands on protein gels (Figure 2B, lanes 2, 6, 7, 8, and 10 in Figure 2A). Mutations at additional sites in hTTP resulted in apparently single band or sharp bands on the protein gel (Figure 2B, lanes 12–15). These results suggest that mutations

Figure 1. Expression and localization of the wild-type and mutant hTTP in transfected human cells. (A) Immunoblotting. HEK293 cells were transfected with pHis-hTTP plasmids. Proteins in the soluble extracts (10,000g, 10 μg/lane) were separated by SDS-PAGE (10% Tris-glycine gel) and transferred onto nitrocellulose membrane. The membrane was incubated in anti-MBP-hTTP serum (1:10,000 dilution, 2 h) followed by secondary antibodies (1:10,000 dilution, 1 h). The blot was incubated in Super Signal for 5 min and exposed to X-ray film for 5 sec. The underlined numbers in the plasmids 1–9 below the gel represent the sites of serine/threonine residues mutated to alanine residues in addition to the mutations of hTTP in the preceding plasmid. (B) Immunostaining. HEK293 cells were transfected with pBS+ control plasmid and pHis-hTTP plasmids encoding wild-type His-hTTP and mutant His-hTTP with S(214,218,228)A and S(88, 90, 93, 197, 214, 218, 228, 296)A mutations. The cells were stained with anti-MBP-hTTP antibodies (1:5,000 dilution, overnight) and labeled with goat anti-rabbit Alexa Fluor 488 (1:1,000 dilution, 1 h). Immunofluorescence was recorded by confocal microscopy.
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Tristetraprolin Phosphopeptide Mapping
at multiple potential phosphorylation sites can have a large effect on the electrophoretic mobility of the human TTP protein.

**Phosphopeptide Mapping of Wild-type TTP**

Previous in vivo labeling studies showed that TTP was highly phosphorylated [21]. To further investigate TTP phosphorylation in the cells, we labeled HEK293 cells with [32P]-orthophosphate following transfection with the wild-type plasmid pHis-hTTP. The wild-type protein was purified from the 10,000 g supernatant by Ni-NTA affinity beads. SDS-PAGE followed by autoradiography showed that hTTP was essentially the only phosphoprotein purified by this procedure (Figure 3A, lane 1). The purified proteins were completely digested for extended time into smaller fragments by trypsin and lysyl endopeptidase (Figure 3A, lanes 2–3). These peptides were separated by reverse-phase HPLC and radioactivity in each fraction was counted. Phosphopeptide mapping showed that several radioactive peaks were present in the trypsin and lysyl endopeptidase digests, and the first small peak of radioactivity was washed off the column (Figure 3B). These results are in agreement with a previous report that hTTP is phosphorylated at multiple sites in intact cells [21].

**Phosphopeptide Mapping of TTP with Mutations at S214, S218 and S228**

S218 and S228 are two of the major phosphorylation sites in hTTP [21]. To investigate if TTP with these two mutations lacks any phosphopeptide, we labeled HEK293 cells transfected with plasmids encoding hTTP with double mutations at the S218 and S228 sites and hTTP with triple mutations at the S214, S218 and S228 sites. The proteins were purified by Ni-NTA beads and digested with trypsin. Autoradiography showed that the full-length wild-type hTTP and the two hTTP mutant proteins were digested by trypsin, resulting in smaller size bands on SDS-PAGE (Figure 4A). Surprisingly, phosphopeptide profiles showed that the wild-type and the mutant hTTP proteins contained the same numbers of phosphopeptide peaks, although there were some minor differences in retention time of phosphopeptides among the three proteins (Figure 4B).

**Phosphopeptide Mapping of TTP with More Mutations**

HEK293 cells were transfected with pHis-hTTP plasmids encoding wild-type and nine mutant hTTP proteins. HEK293 cells were then labeled with [32P]-orthophosphate. The wild-type and mutant His-hTTP proteins were purified from the 10,000 g supernatant by Ni-NTA affinity beads. Autoradiography showed that the proteins appeared to be labeled to similar extents, despite...
their extensive mutations (Figure 5). The radiolabeled proteins were digested to completion with TPCK-treated trypsin, as judged by SDS-PAGE and autoradiography (Figure 5).

The digested peptides were separated by reverse-phase HPLC through a C18 column and the radioactivity in each fraction was counted. The phosphopeptides from mutant hTTP contained more radioactivity than those from the wild-type hTTP (Table 1).

The selected profiles of phosphopeptide mapping comparisons are shown in Figures 6–8. A comparison of phosphopeptide maps between wild-type and S197A mutant hTTP is shown in Figure 6A. The overall phosphopeptide maps were similar between these two proteins. The most striking difference between these two profiles was that the phosphopeptide peaks of the mutant protein were eluted earlier than those of the wild-type protein.

Figure 3. Phosphopeptide mapping of the wild-type hTTP protein from transfected human cells. HEK293 cells were transfected with the wild-type pHis-hTTP plasmid followed by in vivo radiolabeling with [32P]-orthophosphate. Proteins in the soluble extracts were bound to Ni-NTA beads. The bound proteins were eluted with 250 mM imidazole solution. Proteins were digested overnight with trypsin and lysyl endopeptidase. (A) Autoradiography. The undigested protein and digested peptides were separated by SDS-PAGE (4–20% Tris-glycine gel). The gel was dried and exposed to X-ray film. (B) HPLC separation. The digested peptides were separated by reverse phase HPLC and eluted from the column. The radioactivity of every fraction was counted and plotted.

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Figure 4. Phosphopeptide mapping of the wild-type and mutant hTTP protein with S214, S218 and S228 mutations from transfected human cells. HEK293 cells were transfected with the wild-type and mutant plasmids with S(218,228)A and S(214,218,228)A mutations followed by in vivo radiolabeling with [32P]orthophosphate. Proteins in the soluble extracts were bound to Ni-NTA beads and eluted with 250 mM imidazole solution. Proteins were digested overnight with trypsin followed by HPLC separation. (A) Autoradiography. The undigested protein and digested peptides were separated by SDS-PAGE (8–16% Tris-glycine gel). The gel was dried and exposed to X-ray film. (B) HPLC separation. The trypsin-digested peptides were separated by reverse phase HPLC and eluted from the column. The radioactivity of every fraction was counted and plotted.

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The elution profiles of phosphopeptides of hTTP with an alanine mutation at S 197 plus additional mutations at S 228 (Figure 6B), S(218,228) (Figure 6C), S(214,218,228) (Figure 6D), or S(214,218,228,296) (Figure 7A) were similar to that of hTTP with the only mutation at S197 (Figure 6B). Additional mutation at S188 in addition to S197, 214,218,228, 296 clearly changed the elution pattern by increasing the retention time of the phosphopeptides (Figure 7B), but more mutations at S186 (Figure 7C) or T271

Figure 5. Trypsin digestion of the wild-type and mutant hTTP proteins from transfected human cells. HEK293 cells were transfected with the wild-type and 9 mutant plasmids followed by in vivo radiolabeling with [32P]-orthophosphate. Proteins in the soluble extracts were bound to Ni-NTA beads and eluted with imidazole solution. Proteins were digested with trypsin. The undigested protein and digested peptides were separated by SDS-PAGE (8–16% Tris-glycine gel). The gel was dried and exposed to X-ray film. The underlined numbers in the plasmids 1–10 below the gel represent the sites of serine/threonine residues mutated to alanine residues in addition to the mutations of hTTP in the preceding plasmid.

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Table 1. HPLC recovery of radioactivity.

| Plasmid no. | Construct (pHis-hTTP) | Radioactivity before separation (cpm) | Radioactivity in the pellet (cpm) | Radioactivity recovered in the HPLC fractions (cpm) | HPLC recovery (%) | Total recovery (%) |
|-------------|-----------------------|--------------------------------------|---------------------------------|---------------------------------------------------|------------------|--------------------|
| 1           | Wild-type             | 42341                                | 955                             | 12455                                             | 30.1             | 31.7               |
| 2           | S197A                 | 128820                               | 2010                            | 58776                                             | 46.3             | 47.2               |
| 3           | S(197,228)A           | 192986                               | 9872                            | 87160                                             | 47.6             | 50.3               |
| 4           | S(197,218,228)A       | 80527                                | 2916                            | 42395                                             | 54.6             | 56.3               |
| 5           | S(197,218,214)A       | 124994                               | 5617                            | 57760                                             | 48.4             | 50.7               |
| 6           | S(197,218,214,296)A   | 123763                               | 4042                            | 49752                                             | 41.6             | 43.5               |
| 7           | S(197,218,214,296,88)A| 147327                              | 4000                            | 61284                                             | 42.8             | 44.3               |
| 8           | S(197,218,214,288,88,186)A | 150917                           | 8920                            | 56555                                             | 39.8             | 43.4               |
| 9           | S(197,218,214,296,88,T271)A | 114453                          | 3318                            | 51259                                             | 46.1             | 47.7               |
| 10          | S(197,218,214,296,88,90,93)A | 145495                          | 3914                            | 58768                                             | 41.5             | 43.1               |

1The recovery of radioactivity of HPLC fractions from every mutant hTTP protein was higher than that from the wild-type protein.
2The underlined numbers in the plasmids 1–10 below represent the sites of serine/threonine residues mutated to alanine residues in addition to the mutations of hTTP in the preceding plasmid.

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Figure 7D and Figure 8A did not exhibit significant effects on the phosphopeptide elution patterns.

The major effects of mutations on phosphopeptide mapping were observed when hTTP was mutated at S(90,93). Two phosphopeptide peaks in fractions 37–43 disappeared in hTTP with these two mutations in addition to those mutations at S(88, 197, 214,218,228, 296) (Figure 8B) or S(88, 197, 214,218,228, 296)T271 (Figure 8C). The two missing peaks in fractions 37–43 from hTTP containing S90 and S93 mutations corresponded to one major and one minor peak of radioactivity from the wild-type protein (Figure 8D).

MALDI-MS Analysis of Phosphopeptides of TTP

To identify the phosphopeptides eluted from HPLC columns, we used MS methods to analyze the HPLC fractions with high levels of radioactivity from the wild-type His-hTTP (Figure 6A).

The first major peak of radioactivity (Figure 6A, peak 1) washed off the column contained a phosphopeptide with the amino acid sequence CHFlHNPSELDLAPGHPPVLR (Table 2). This peptide corresponds to amino acid residues 162–182 of hTTP (T18 in Figure 9). The underlined S169 site is the only phosphorylation site identified previously [21].

The second peak of radioactivity (Figure 6A, peak 2) from the wild-type hTTP that disappeared in mutant hTTP with S90 and S93 mutations was determined to contain a phosphopeptide with the amino acid sequence LGPELSSPTSPTATSTTPSR (Table 2). This peptide sequence corresponds to amino acid residues 83–103 of hTTP (T5 in Figure 9). This peptide contained five potential phosphorylation sites as previously identified by MS analyses (S88, S90, T92, S93 and T95 sites) (Figure 9).

The third major peak of radioactivity (Figure 6A, peak 3) contained a phosphopeptide with the amino acid sequence RDPTPVCCPSR (Table 2) (corresponding to amino acid residues 243–255 of hTTP) (T22–24 in Figure 9). The S252 site is the only phosphorylation site identified previously [21]. This fraction might also contain a non-phosphorylated peptide with the amino acid sequence YGAKCQFAHGLGELR (corresponding to amino acid residues 120–134 of hTTP) (T10–11 in Figure 9) since this peptide has the same molecular mass.

The fourth major peak of radioactivity (Figure 6A, peak 4) contained a phosphopeptide with the amino acid sequence YKTELCRFTSESGR (Table 2) (corresponding to amino acid residues 104–117 of hTTP) (T6–8 in Figure 9). It is notable that this peptide is located in the first C8C5C3H zinc-finger region with three potential phosphorylation sites at T106, T111 and S113 in hTTP (Figure 9).

The last major peak of radioactivity (Figure 6A, peak 5) contained a phosphopeptide with the amino acid sequence FYLQGRCPYGSR (Table 2) (corresponding to amino acid residues 150–161 of hTTP) (T16–17 in Figure 9). The Y158 and S160 sites are phosphorylation sites identified previously [21].

Figure 6. Phosphopeptide mapping of the wild-type and mutant hTTP proteins from transfected human cells. Wild-type and mutant hTTP proteins were labeled with [32P]-orthophosphate in HEK293 cells. His-hTTP was purified and digested by trypsin as described in Figure 5 legend. The trypsin-digested peptides were separated by reverse phase HPLC and eluted from the column at 0.5 mL/min with 20-bed volume of a linear gradient. The fractions were collected at 0.25 mL/well in 96-well plates. The radioactivity of every fraction was counted and plotted. The radioactivity in the plots from the wild-type proteins was five times of the actual radioactivity for visual comparisons of its phosphopeptides to the mutant ones because of the low recovery of phosphopeptides of the wild-type protein in HPLC fractions (Table 1). The radioactivity in the plots from the mutant proteins was the actual radioactivity. The phosphopeptide profiles in each pair of hTTP proteins are (A) Wild-type vs. S197A, (B) S197A vs. S(197, 228)A, (C) S(197,228)A vs. S(197,218,228)A, and (D) S(197,218,228)A vs. S(197,214,218,228)A.
RNA-binding Activity of Wild-type and Mutant TTP

RNA gel mobility shift assays were used to evaluate the effect of mutations on TTP’s ability to bind to TNF mRNA ARE. This method showed that both the wild-type and mutant His-hTTP proteins containing one or multiple alanine mutations were all capable of binding to the RNA ARE probes, resulting in accumulation of high molecular size TTP-ARE complexes and disappearance of ARE fragment 2 of the radiolabeled mRNA ARE probes (Figure 10). These results suggest that all mutant proteins tested possess the essential structures for ARE binding under these assay conditions.

Discussion

TTP is a highly phosphorylated protein in intact cells [1]. Mass spectrometry and site-directed mutagenesis have identified a number of phosphorylation sites in hTTP, including S66, S88, T92, S169, S186, S197, S218, S228, S276 and S296, as well as 29 other potential phosphorylation sites [3,21]. To better understand TTP phosphorylation, we aimed to uncover additional phosphorylation sites by phosphopeptide mapping coupled with in vivo labeling, site-directed mutagenesis and mass spectrometry.

The major finding in this report is that the tryptic peptide containing S90 and S93 is a major phosphopeptide in hTTP. The S90A and S93A mutations in hTTP resulted in the disappearance of a major phosphopeptide with the amino acid sequence LGPELSPSPTSPTATSTTPSR (corresponding to the amino acid residues 83–103 of hTTP) (Figure 9). Mutation at S197 resulted in another major phosphopeptide peak being eluted earlier than the wild-type, in agreement with the finding that mutation at this site increases the electrophoretic mobility on SDS-PAGE [21]. Additional mutations at S106, S296 and T271 exhibited little effect on the phosphopeptide mapping profiles. The individual contributions of S80 and S93 phosphorylation to the overall phosphorylation status of TTP require further investigation. Results from single or double mutations at S80 and S93 should be able to determine if mutations in these two sites are sufficient for the disappearance of the major radioactivity peak. There is a complete lack of information on the physiological significance of phosphorylation at S80 and S93 sites in human TTP. Previously, S80 was shown to be a potential site for the p38 MAP kinase [3]. The host cells (HEK293 cells) do not express TTP under our culture conditions (Figure 1B) and do not respond to various stimuli. These technical difficulties prevent us from performing experiments to stimulate TTP expression using the constructs in HEK293 cells or other types of cells.

Remarkably, one of the major phosphopeptides was identified in the first C8C5C3H zinc-finger region, and contained three potential phosphorylation sites at T106, T111 and S113 in hTTP. Five potential phosphorylation sites are located in the highly conserved zinc finger domains of hTTP: T106, T111 and S113 in the first zinc-finger region, and Y158 and S160 in the second zinc-finger region (Figure 9). The biological significance of the potential phosphorylation sites identified in the zinc finger motifs is not clear. The zinc finger domains synthesized chemically or expressed in E. coli [5] can bind to the same ARE as the recombinant full-length TTP with similar binding affinity by the electrophoretic mobility shift assay [7]. It is difficult to evaluate the precise effects of phosphorylation at these sites since the binding assay is a semi-quantitative method. It will be important to compare the RNA
binding affinity between the phosphorylated and unphosphorylated zinc finger domains in the future.

It is interesting to note that mutation at S186 in hTTP (corresponding to S178 in mTTP) exhibited little effect on the phosphopeptide mapping profiles. S186 in mTTP was shown to be one of the major sites phosphorylated by MK2 in vivo and in vitro [25–28]. MK2 phosphorylation increased TTP protein stability but reduced ARE affinity [33]. The regulation of subcellular localization and protein stability of mTTP is dependent on MK2 and on the integrity of S52 and S178 [34]. Phosphorylation of mTTP at S178 increases the relative ratio of TTP protein in the cytoplasm [32]. Mutation of S52 to A52 in mTTP weakly reduces the assembly of TTP-14-3-3 protein complex, whereas mutation of S178 to A178 and of S52/178 to A52/178 substantially reduces the association of mTTP with 14-3-3 protein complex [35]. Therefore, it will be a great challenge to correlate the phosphorylation sites and the functional consequence in future studies.

It is still difficult to assign the relative contributions of individual phosphorylation sites in TTP. The fact that S90A and S93A mutations in hTTP caused the disappearance of major phosphorylation sites suggests that mass spectrometry alone has limitations on assigning major vs. minor phosphorylation sites. In previous MS analysis, it was suggested that both S90 and S93 sites in hTTP were minor phosphorylation sites because fewer unique phosphopeptides containing both sites were observed by MudPIT [21]. Instead, S66, S88, T92, S169, S186, S218, and S296 were proposed as major sites in hTTP from transfected human cells for a number of reasons [21]: 1) phosphopeptides containing S66, S88, T92, S169 and S186 in hTTP observed by MudPIT were confirmed by LC/MS/MS, MALDI/MS/MS, or protein sequencing; 2) S197, S218, and S220 strongly affected the electrophoretic mobility of hTTP; 3) More than four copies of phosphopeptides containing S197, S218, S220, and S296 were identified from triple digested hTTP by MudPIT; 4) [32P]-labeling studies showed that the truncated hTTP peptides containing S210 and S220 were highly phosphorylated in intact cells; 5) the MALDI MS analysis of the in-gel tryptic digest of hTTP showed ions corresponding in mass to tryptic peptide T20–21/T21–22 (aa 195–242/196–243) plus the addition of one, two, and three phosphate groups; 6) all of these sites were conserved in TTP from various mammalian species; and 7) S90, S176, S178, S218, T250 and S264 of mTTP were phosphorylated in vivo and/or in vitro [25]; these sites corresponded to S66, S184, S186, S220, T257, and T271 of hTTP. More studies are needed to address the question of relevant contributions of various phosphorylation sites towards the total TTP phosphorylation status in intact cells.

It is thought that phosphorylation of TTP in cells can decrease its mRNA binding activity. For example, TTP expressed in human HEK293 cells and then dephosphorylated by CIAP was able to bind more tightly to an GM-CSF mRNA ARE probe than native, phosphorylated TTP [9]. TTP purified from overexpressed E. coli exhibits approximately 2-fold greater affinity for the TNF mRNA ARE than the protein purified from transfected human HEK293 cells [7]. In the present study, wild-type and mutant His-hTTP proteins containing one or multiple phosphorylation site mutations were all capable of binding to ARE-containing RNA probes, resulting in accumulation of high molecular size complexes and disappearance of radiolabeled mRNA ARE probes. These results

Figure 8. Phosphopeptide mapping of the wild-type and mutant hTTP proteins from transfected human cells. The methods for generating the phosphopeptide mapping profiles were identical to those described in Figure 6 legend. The phosphopeptide profiles in each pair of hTTP proteins are (A) S(88,186,197,214,228,296)A vs. S(88,197,214,218,228,296)A, (B) S(88,197,214,218,228,296)A vs. S(88,90,93,197,214,218,228,296)A, (C) S(88,197,214,228,296)T271A vs. S(88,90,93,197,214,218,228,296)A, and (D) Wild-type vs. S(88,90,93,197,214,218,228,296)A.

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suggest that the mutant proteins possess the essential structures for ARE binding under these assay conditions. However, quantitative evaluation of TTP-mRNA ARE binding affinity is limited by several factors: 1) It is difficult to normalize the amount of TTP in each protein sample because of other contaminating proteins in the Ni-NTA purified samples; 2) The levels of protein expression are still too low to generate sufficient amounts of purified proteins for quantitative assays; 3) The RNA-gel mobility shift assay is only semi-quantitative, and cannot distinguish minor differences of binding affinity among the TTP forms [7]. Therefore, it is impractical to perform these types of experiments under the current assay conditions. Adaption of other methods after overcoming these technical difficulties is beyond the scope of this study with its focus on identifying major phosphopeptides. Nevertheless, given the potential importance of TTP in cancer progression and inflammation [31,36,37], it is important to understand the precise nature and functions of the phosphorylation sites of this important protein in the future.

### Materials and Methods

#### Expression Plasmids

The plasmids (pHis-hTTP) coded for recombinant His-hTTP proteins containing six histidine residues at the N-terminus of the full-length hTTP (GenBank accession no. NP_003398) [40]. The site-directed mutant plasmids contained single or multiple alanine mutation(s) at serine and threonine positions in hTTP. The plasmids coding for ten His-TTP proteins were 1) WT, 2) S197A, 3) S(197,228)A, 4) S(197,218,228)A, 5) S(197,214,218,228)A, 6) S(197,214,218,228,296)A, 7) S(88,197,214,218,228,296)A, 8) S(88,186,197,214,218,228,296)A, 9) S(88,197,214,218,228,296)T271A and 10) S(88,90,93,197,214,218,228,296)A [21].

#### Transfection of Human HEK293 Cells

HEK293 cells were transiently transfected with pHis-hTTP plasmids using the calcium phosphate precipitation method [7,12]. HEK293 cells were grown at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin,
### Table 2. MAIDI-MS analysis of HPLC fractions with major radioactivity.

| Radioactivity peak | Differential mass (Da) | Unmodified mass (Da) | Observed mass (Da) | Corresponding region in HTTP | Amino acid sequence in hTTP (potential phosphorylation site underlined) |
|--------------------|------------------------|----------------------|-------------------|-----------------------------|-------------------------------------------------------------------|
| 1                  | 2307.24                | 80.99               | 2307.24           | T18 162–182 (R) CHFIHNP      |
|                    |                        |                     |                   |                             | SEDLAAPGHPPVLR (Q) S 169                                           |
| 2                  | 2165.25                | 81.13               | 2165.25           | T5 83–103 (R) LGPELP         |
|                    |                        |                     |                   |                             | P         |
|                    |                        |                     |                   |                             | S         |
|                    |                        |                     |                   |                             | P               |
|                    |                        |                     |                   |                             | T         |
|                    |                        |                     |                   |                             | ATSTTPSR (Y) S 88,S 90,T 92,S 93 and T95                         |
| 3                  | 1757.12                | 80.31               | 1757.12           | T6–8 104–117 (R) YKELCR     |
|                    |                        |                     |                   |                             | T         |
|                    |                        |                     |                   |                             | F         |
|                    |                        |                     |                   |                             | S               |
|                    |                        |                     |                   |                             | ESGR (C) T 105,T 111 and S113                                    |
| 4                  | 1526.92                | 80.22               | 1526.92           | T16–17 150–161 (K) FYLQGRCP |
|                    |                        |                     |                   |                             | G         |
|                    |                        |                     |                   |                             | R( C ) Y 158 and S160                                            |

1. The wild-type hTTP protein was purified from transfected human cells after in vivo radiolabeling with [32P]-orthophosphate. The protein was purified and digested by trypsin to completion. The phosphopeptides were identified by radioactivity peak on HPLC chromatogram (Figure 6A).
2. The observed peptide mass of [M + H]+ ion was obtained after phosphopeptides were sequenced by MAIDI-MS.
3. The unmodified peptide mass of [M + H]+ ion was obtained after theoretical digestion of His-hTTP with trypsin.
4. The differential mass was obtained by subtraction the unmodified ion mass from the observed ion pass. Phosphorylation results in a peptide ion with a +80 Da mass increase compared to the unmodified peptide for each phosphorylated Ser, Thr or Tyr residue (HPO3).

In Vivo Phosphate Radiolabeling

The transfected HEK293 cells were washed after being cultured overnight and incubated in fresh medium under the same conditions for 24 h. The old medium was removed from culture dish followed by washing with no-phosphate DMEM. To the dish was added no-phosphate DMEM plus 1% FCS (approximately 15 µM phosphate in the medium) and incubated at 37°C with 5% CO2 for 5 h. The medium was then aspirated off. DMEM (4 mL, no phosphate, no serum) with [32P]-orthophosphate (0.1 mCi/mL) was added to each dish [21]. The cells were labeled at 37°C with 5% CO2 for 90 min.

Cell Lysis

The [32P]-orthophosphate-medium was removed after in vivo radiolabeling. The cells were washed with PBS and lysed directly in the plate at 4°C for 1 h with 0.6 mL His-tag purification buffer (50 mM NaH2PO4, 250 mM NaCl, 50 mM NaF, 1 mM PMSF, 1 µg/mL leupeptin, 0.5% NP-40) plus 10 mM imidazole. The lysate was transferred into microcentrifuge tubes and centrifuged at 10,000g for 10 min. Protein concentrations were determined with the Bio-Rad Dye assay kit (Bio-Rad Laboratories) and BSA as standards [7].

His-tag Purification

The 10,000 g supernatants from transfected HEK293 cells were transferred to 13-mL Falcon tubes and mixed with 5% Ni-NTA beads (Qiagen, Valencia, CA). The mixtures were rotated at 4°C for 2 h and then transferred into a Cytospin column followed by centrifugation at 1000 g for 2 min. The beads were washed with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 50 mM NaF, 0.05% Tween-20, pH 8.0) plus 10 mM imidazole. The lysate was transferred into microcentrifuge tubes and centrifuged at 10,000g for 2 min. The bound proteins were eluted with 100, 200 and 250 mM imidazole in wash buffer by centrifugation at 1000 g for 2 min. The eluted proteins and the remaining beads were stored at −20°C.

Digestion of TTP

His-hTTP proteins eluted from Ni-NTA beads as described above (100 µL) were digested directly for 70 h (extended time for complete digestion) at 37°C with 6 µg of modified trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) (Sigma, St. Louis, MO). The protein was also digested with lysyl endopeptidase (Sigma, St. Louis, MO) under the same conditions.

HPLC Separation of TTP Peptides

The trypsin-digested peptide mixtures were adjusted to 0.065% TFA by addition of 10% trifluoroacetic acid (TFA). The mixture was set at room temperature for 30 min followed by centrifugation at 10,000 g for 10 min. The supernatant was manually injected into a 100 µL loop. The peptide fragments were separated by reverse phase chromatography through a Sephasil Peptide C18 5 µ ST 4.6/100 column (GE Healthcare Life Sciences, Piscataway, NJ) using an AKTAbasic system equipped with UNICORN 3.10 version (GE Healthcare Life Sciences, Piscataway, NJ). The peptide mixtures were loaded onto the column from the sample loop with 500 µL of buffer A (0.065% TFA in 2% acetonitrile). The column was washed with 5-bed volumes of buffer A. The peptides were eluted from the column at 0.5 mL/min with a 20-
bed volume of a linear gradient from 100% buffer A to 100% of buffer B (0.05% TFA in 100% acetonitrile). The fractions were collected at 0.25 mL/well in 96-well plates with Frac-950 fraction collector (GE Healthcare Life Sciences, Piscataway, NJ). The radioactivity of every fraction was counted using MicroBeta JET (PerkinElmer Life Sciences, Gaithersburg, MD).

MALDI-MS (matrix-assisted laser-desorption–ionization MS) Analysis

HPLC fractions corresponding to the phosphopeptide peaks from the wild-type protein were analyzed by MALDI MS. The tryptic peptide mixtures were freeze-dried and reconstituted in 5 μL of acetonitrile/water (1:1, v/v) with 0.1% formic acid. The tryptic peptide solution (0.5 μL) was mixed with 0.5 μL of MALDI matrix consisting of a saturated solution of a-cyano-4-hydroxycinamic acid in ethanol/water/formic acid (45:45:10, v/v). MALDI analyses were performed as described [38] using 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA).

SDS-polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting

SDS-PAGE and immunoblotting followed described procedures [39]. The primary antibodies were anti-MBP-hTTP sera raised in New Zealand white rabbits against the purified MBP-TTP fusion protein [17]. The secondary antibodies were affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate with human IgG absorbed (Bio-Rad Laboratories).

 Autoradiography

The undigested His-hTTP proteins and trypsin-digested His-hTTP peptides from radiolabeled cells were separated by SDS-PAGE (Norvex precast, 8–16% Tris-glycine gel). The gel was dried and exposed to X-ray film (Eastman Kodak, Rochester, NY).

Immunostaining and Confocal Microscopy

HEK293 cells were grown overnight on glass coverslips in tissue culture plate (Becton Dickinson and Company, Lincoln Park, NJ). The cells were transfected with pHis-TTP (50 ng DNA/1 mL/well) and incubated overnight. After another 24-h incubation, the

Figure 10. RNA Binding Activity Assay. TNF mRNA ARE binding activity of the wild-type and mutant TTP was evaluated with the RNA gel mobility shift assay. (A) Electrophoretic mobility shift assay. The binding reaction mixtures were incubated for 30 min followed by RNase T1 digestion. The TTP–ARE complexes and free probes were separated by 6% native PAGE and detected by autoradiography on X-ray film. (B) TNF mRNA ARE probe. The RNA probe was transcribed from mouse TNF mRNA ARE region with the following sequence: 5′-CUCUAUUUAUUUG|CA-CUUAUUAUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUU
cells were used for immunocytochemistry with anti-MBP-hTTP antibodies using a similar procedure as described [7]. The slides were examined and imaged with an LSM510 UV confocal microscope (Zeiss, Thornwood, NY).

RNA Binding Activity Assay

TNF mRNA ARE binding activity was evaluated with the RNA gel mobility shift assay (GMSA) according to a previous procedure [7] using a TNF mRNA ARE probe (100000–200000 cpm/reaction) with the sequence shown in Figure 7B. The RNA probe was transcribed from mouse TNF mRNA ARE region (nucleotides 1281–1350 of GenBank accession no. X02611) using [32P]UTP (NEN Life Sciences, Boston, MA) and T7 RNA polymerase with Promega's RiboProbe 100 units of RNase T1 (Epicentre, Madison, WI) for 15 min at 30°C. The binding reaction mixtures were incubated for 30 min at room temperature before digestion with 100 units of RNase T1 (Epicentre, Madison, WI) for 15 min at 30°C. The TTP–probe complexes and free probes were separated by 6% native PAGE and detected by autoradiography on X-ray film.

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Author Contributions

Conceived and designed the experiments: HC. Performed the experiments: HC. LJD. Analyzed the data: HC. LJJD PJB. Contributed reagents/materials/analysis tools: HC. PJB. Contributed to the writing of the manuscript: HC.
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