Structural basis for the recruitment of the human CCR4–NOT deadenylase complex by tristetraprolin

Marc R Fabian1,2,6, Filipp Frank3,4,6, Christopher Rouya3,4,6, Nadeem Siddiqui3,4, Wi S Lai5, Alexey Karetnikov3,4, Perry J Blackshear2, Bhushan Nagar3,4 & Nahum Sonenberg3,4

TRISTETRAPROLIN (TTP) is an RNA-binding protein that controls the inflammatory response by limiting the expression of several proinflammatory cytokines. TTP post-transcriptionally represses gene expression by interacting with AU-rich elements (AREs) in 3’ untranslated regions of target mRNAs and subsequently engenders their deadenylation and decay. TTP accomplishes these tasks, at least in part, by recruiting the multisubunit CCR4–NOT deadenylase complex to the mRNA. Here we identify an evolutionarily conserved C-terminal motif in human TTP that directly binds a central domain of CNOT1, a core subunit of the CCR4–NOT complex. A high-resolution crystal structure of the TTP–CNOT1 complex was determined, providing the first structural insight, to our knowledge, into an ARE-binding protein bound to the CCR4–NOT complex. Mutations at the CNOT1–TTP interface impair TTP-mediated deadenylation, demonstrating the significance of this interaction in TTP-mediated gene silencing.

Mammalian gene expression is tightly regulated by an array of post-transcriptional control programs, including mRNA translation and stability. The mRNA 3’ untranslated region (3’ UTR) plays a critical part in post-transcriptional control by cis-acting elements, such as micro RNA (miRNA)-target sites and AREs1,2. These RNA elements are targeted by miRNAs and ARE-binding proteins (ARE-BPs). TTP is an ARE-BP, which represents the prototypical member of the 12-O-tetradecanoylphorbol-13-acetate (TPA)-inducible sequence-11 (TIS11) family of RNA-binding proteins that include the TTP-related proteins butyrate response factors BRF-1 and BRF-2 (refs. 3–5). TTP is an important regulator of the inflammatory response and a b Raf–MEK–ERK pathway, and subsequent decay by recruiting the CCR4–NOT complex11. The N-terminal and C-terminal domains of TTP effect deadenylation through the CCR4–NOT complex11,12. However, controversy exists with respect to how TTP interacts with the deadenylase machinery. One report concluded that components of the CCR4–NOT complex interact only with the TTP N-terminal domain11, whereas another only documented interactions with the TTP C-terminal domain12. Thus, it is currently unclear how TTP physically interacts with the CCR4–NOT complex to bring about deadenylation.

CCR4–NOT is a multisubunit protein complex originally described in Saccharomyces cerevisiae as a global regulator of transcription and the cell cycle13. However, it has since gained prominence as a master regulator of mRNA stability that interfaces with several key post-transcriptional control programs, including those mediated by miRNAs and by TTP-family proteins14. The mammalian CCR4–NOT complex comprises multiple proteins, termed CCR4–NOT (CNOT) subunits. The CNOT1 subunit acts as a scaffold for other CCR4–NOT components, including the CNOT6 (CCR4) and CNOT7 (CAF1) deadenylases. To determine the mechanism of TTP-mediated deadenylation, we have identified a new motif in TTP that binds CNOT1, elucidated the structural basis for the TTP–CNOT1 interaction and established its significance for TTP-mediated gene silencing.

RESULTS

TTP directly binds CNOT1

The human CNOT1 subunit is a large scaffolding protein that associates with TTP through unknown contacts12. To demonstrate that CNOT1 directly interacts with TTP, as recently postulated12, we performed in vitro co-precipitation experiments using recombinant maltose-binding protein (MBP)-fused TTP (Fig. 1a) and a series of glutathione S-transferase (GST)-fused CNOT1 fragments covering the entire CNOT1 isoform C (2,371-residue) protein (Fig. 1b). A CNOT1 fragment encompassing residues 727–1266 efficiently bound MBP-TTP, whereas all other CNOT1 fragments did not (Supplementary Fig. 1). We subsequently mapped the region of CNOT1 that binds TTP to residues 800–1015 (Fig. 1c and Supplementary Fig. 1).

Next, we set out to identify what region of TTP binds the CNOT1800–1015 fragment. To this end, we generated MBP-fused TTP fragments containing either the TTP N-terminal (residues 1–99) or C-terminal (residues 174–326) regions (Fig. 1a), which have been reported to associate with the CCR4–NOT complex11,12. GST-tagged CNOT1800–1015 did not interact with the TTP N-terminal domain in

1Lady Davis Institute for Medical Research, Jewish General Hospital, Montréal, Québec, Canada. 2Department of Oncology, McGill University, Montréal, Québec, Canada. 3Department of Biochemistry, McGill University, Montréal, Québec, Canada. 4Goodman Cancer Centre, McGill University, Montréal, Québec, Canada. 5Laboratory of Signal Transduction, National Institute of Environmental Health Science, Research Triangle Park, North Carolina, USA. 6These authors contributed equally to this work. Correspondence should be addressed to M.R.F. (marc.fabian@mcgill.ca), B.N. (bhushan.nagar@mcgill.ca) or N.S. (nahum.sonenberg@mcgill.ca).

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Figure 1 Human TTP directly binds a central fragment of CNOT1. (a) Schematic diagram of full-length TTP (TTP-FL) and TTP fragments used in co-precipitation experiments in c. Dashed lines indicate the region required for CNOT1 binding in c. (b) Schematic diagram of full-length CNOT1 and CNOT1 fragments used in co-precipitation experiments (in Fig. 1c and Supplementary Fig. 1). Positions are indicated at the left of each fragment. Dashed lines indicate the region required for TTP binding in c. (c) Coomassie-stained SDS-PAGE gel of input (left) and GST pulldown (right) of proteins by recombinant GST-tagged CNOT1 800–1015 immobilized on glutathione-Sepharose beads incubated with MBP-tagged full-length TTP or TTP fragments. MW, molecular weight.

(d) Sequence alignment of conserved amino acids within the TTP C terminus that binds CNOT1 of human (Hs) TTP, BRF-1 and BRF-2, X. tropicalis (Xt) TTP, D. melanogaster (Dm) Tis11 and C. elegans (Ce) CCCH1. Highlighted are amino acids identical in 100% of proteins (red) or conservative substitutions by related amino acids (blue).

binding assays (Fig. 1c). In contrast, it bound the TTP C-terminal domain very efficiently (~50% of input) (Fig. 1c). Sequence alignment of the TTP C-terminal domain (CTD) and its related proteins BRF-1 and BRF-2 revealed two conserved patches of amino acids in the TTP-CTD: residues 179–192 and 314–326 (Supplementary Fig. 2). GST-CNOT1 800–1015 bound MBP-TTP lacking residues 179–192 (TTP-MUT1; Fig. 1a,c). However, MBP-TTP lacking the terminal 13 amino acids (aa) (TTP-MUT2) failed to bind GST-CNOT1 800–1015 (Fig. 1a,c). Notably, the TTP C-terminal amino acid sequence is highly conserved in Tis11 homologs, including in Xenopus tropicalis, Drosophila melanogaster and Caenorhabditis elegans (Fig. 1d). Taken together, these results demonstrate that CNOT1 interacts with an evolutionarily conserved amino acid motif at the C terminus of TTP.

Structural determination of the TTP-CNOT1 interaction

To gain structural insight into the CNOT1-TTP interaction, we solved the crystal structure of a TTP-interacting fragment of CNOT1 (residues 800–999) in complex with a peptide covering residues Ala312 to Glu326 of human TTP (hTTP; Fig. 1d) at 1.5-Å resolution (Fig. 2a and Table 1). The N-terminal 20 residues (800–819) of the CNOT1 fragment are disordered, whereas TTP could be modeled from residues Arg314 to Ser325. CNOT1 820–999 consists of eight α-helices stacked as a series of helix-turn-helix motifs in an arrangement similar to that of the HEAT repeats of the middle domain of initiation factor 4G (MIF4G) (Supplementary Fig. 3a). The MIF4G domain is generally used as a protein–protein interaction domain and is found in numerous scaffolding proteins involved in the regulation of translation and RNA metabolism. The main difference between the CNOT1 domain and the MIF4G domain resides at the N-terminal region, where the MIF4G domain forms the first HEAT repeat. In CNOT1, this region forms a long loop held in place by Trp828, which folds over α3 and contributes to a highly acidic surface adjacent to the TTP-binding site (not shown in figure).

The TTP-binding site is located close to the N terminus of CNOT1 820–999 and is formed by a highly conserved hydrophobic groove between helices α1 and α3 that is flanked by negatively charged patches of amino acids (Fig. 2b). The central portion of the TTP peptide forms a short, two-turn amphipathic α-helix resulting in the insertion of Leu316, Ile318, Phe319 and Ile322 into the hydrophobic groove of CNOT1 (Fig. 3a). The bottom of the groove is lined with aromatic residues Phe847, Tyr851 and Tyr900 (Fig. 3b). A network of electrostatic interactions between several polar residues located at the termini of the TTP peptide and the surrounding negatively charged residues in CNOT1 is also formed. The most prominent of these interactions is a salt bridge between Glu893 of CNOT1 and Arg315 of GST–CNOT1 880–1015 complex. (a) Cartoon representation of the crystal structure of human CNOT1 820–999 (gray) in complex with TTP peptide (purple). The eight CNOT1 helices are numbered starting from the N terminus. (b) Left, surface representation of CNOT1 820–999. The bound TTP 314–325 peptide (purple) is shown as a cartoon representation. Right, magnification of TTP-binding site on CNOT1 820–999, colored according to electrostatic potential from dark red (~5 kT/e) to white (0 kT/e) to dark blue (+5 kT/e).
TTP at the edge of the groove (Fig. 3b). Hydrogen-bonding between Glu893 and Tyr900 of CNOT1 with residues Arg315 and Ser323 of TTP, respectively, generates a stable closed-loop conformation in the TTP-CNOT1 complex (Fig. 3b). Additional contacts stabilizing the complex are provided by hydrogen bonds on the solvent-exposed side of the TTP helix between residues Pro317 and Arg321 of TTP with Asn844 and Gln848 of CNOT1, respectively (Fig. 3b). The sum of these interactions confers an affinity (Kₐ) of ~2 μM between the TTP peptide and CNOT1₈₀₀–₉₉₉ as measured by isothermal titration calorimetry (ITC) experiments (Supplementary Fig. 3).

We generated mutants of TTP to biochemically validate the structure. Alanine substitutions of Arg315, which makes a salt bridge with Glu893 of CNOT1, and Phe319, which is at the center of the hydrophobic interaction site and forms van der Waals contacts with the CNOT1 surface (Supplementary Fig. 4a), in the TTP-CTD abolished its interaction with CNOT1₈₀₀–₁₀₁₅ in vitro (Fig. 3c). In addition, mutating Phe319 to alanine in the TTP peptide disrupted its binding to CNOT1₈₀₀–₉₉₉ (Kₐ = ~7 mM; Supplementary Fig. 3c). Mutation of Asn320, which does not make contact with CNOT1, to alanine failed to disrupt the TTP-CNOT1 interaction (Fig. 3c). Taken together, these data demonstrate that the TTP-CNOT1 interaction requires several invariant residues in the C terminus of TTP, a sequence we now refer to as a TTP-CCR4-NOT interaction motif (TTP-CIM).

**TTP-CNOT1 contact promotes mRNA deadenylation in vitro**

TTP requires its CTD to effect maximal deadenylation of target mRNAs¹¹,¹². To examine the functional significance of the interaction between CNOT1 and TTP, we established an in vitro assay for TTP-mediated deadenylation. Using the AN-BoxB system⁷, we artificially tethered recombinant TTP proteins to an RNA reporter containing five BoxB stem loops and a 98-nucleotide poly(A) tail (5-BoxB-pA), which was added to a cell-free extract derived from mouse Krebs-2 ascites (termed Krebs extract; Fig. 4a). This strategy has been used successfully to biochemically dissect the mechanics of miRNA-mediated deadenylation by the CCR4–NOT complex.¹⁸ Recombinant wild-type GST-TTP was unable to mediate deadenylation, whereas wild-type GST-λNHA–TTP engendered complete deadenylation of 5-BoxB-pA RNA in the Krebs extract within 2 h (Fig. 4b). In contrast, a TTP mutant that could not bind CNOT1 (TTP-F319A) displayed severely impaired deadenylation, with deadenylation barely detectable by 2 h (Fig. 4b). Similar results were obtained when using a polyadenylated reporter containing two TTP-binding sites derived from the tumor necrosis factor 3′ UTR (TNF-ARE-pA), a bona fide TTP mRNA target (Fig. 4c). Wild-type GST-TTP engendered complete deadenylation of TNF-ARE-pA by 2 h, whereas GST-TTP-F319A displayed partial and incomplete deadenylation (Fig. 4d). Taken together, these results demonstrate that TTP-mediated deadenylation in vitro requires the TTP-CTD–CNOT1 interaction, irrespective of whether TTP is directly binding or being artificially tethered to a target RNA.

**Figure 3** Analysis of TTP-CNOT1 interaction. (a) Electron density (gray mesh) of the TTP₃₁₄–₃₂₅ peptide (purple sticks). Density (F₀ – Fᵡ) of a map calculated by omitting CNOT1₉₂₀–₉₉₉ is contoured at 2.5σ. (b) View of the interface between the CNOT1 domain (gray) and TTP peptide (purple). Interacting side chains of CNOT1 (blue) and TTP (purple) are shown as sticks. The backbone amide of Pro317 is shown as a blue sphere. Dashed lines indicate hydrogen-bonding interactions. (c) Coomassie-stained SDS-PAGE gel of GST pulldown of proteins by GST alone (negative control) or recombinant GST-tagged CNOT1₈₀₀–₁₀₁₅ immobilized on glutathione-Sepharose beads and incubated with MBP-tagged wild-type (WT) or mutant TTP-CTD.
TTP-CNOT1 contact promotes mRNA decay in vivo

We also examined the significance of the TTP-CNOT1 interaction on the stability of a TTP-targeted reporter mRNA in vivo. We used a plasmid containing the mouse MARCKS-like protein (MLP) promoter and coding region that is fused to the TTP-targeted TNFα 3′ UTR (MLP-TNF3′), which has previously served as a reporter to assay TTP function in cell cultures. HEK-293 cells were co-transfected with the MLP-TNF3′ reporter plasmid along with a plasmid coding GFP or TTP protein constructs. Blots were probed with a 32P-labeled GFP and TTP protein constructs. Blots were probed with a 32P-labeled MLP probe (top) or 32P-labeled GFP and TTP probes (bottom). Transcripts of endogenous MLP (endo-MLP), the reporter MLP-TNF3′, MLP-TNF3′, and GFP WT are indicated. (f) Quantification of MLP-TNF3′ RNA levels in GFP-expressing cells, in the presence of GFP, wild-type TTP, TTP1-322 and TTP1-313. Mean values ± s.e.m. from seven independent experiments are shown. (g) Model for structural organization of mRNA-bound TTP in complex with CNOT1 and CAF1 and CCR4 deadenylases. The cartoon summarizes structural data reported in this manuscript combined with data from crystal structures of yeast20 and human21 NOT1 proteins in complex with CAF1 and CCR4 deadenylases. TTP and CAF1-binding domains in CNOT1 (820–999 and 1088–1312, respectively) refer to coordinates of human CNOT1 isoform c. Other CCR4–NOT subunits (that is, CNOT2, 3, 9 and 10) are not shown.

DISCUSSION

We have identified a CIM at the C terminus of human TTP. The TTP-CIM directly binds the CNOT1 subunit of the CCR4–NOT complex. Notably, this represents the first structure, to our knowledge, of the interface between an ARE-BP and the CCR4–NOT complex. Furthermore, our data demonstrate that optimal TTP-mediated deadenylation requires the TTP-CIM to be bound to CNOT1, as disrupting this interaction impairs, but does not completely abolish, deadenylation in vitro (Fig. 4a) and mRNA decay in vivo (Fig. 4c).

These data fit well with previous studies demonstrating that the TTP N-terminal domain facilitated deadenylation of target mRNAs in a manner dependent on the CCR4–NOT complex. How the deadenylase machinery interacts with the TTP N-terminal domain remains to be determined. Notably, the TTP-binding domain of CNOT1 is N-terminally adjacent to the CNOT1 domain that binds the CAF1 deadenylase20,21. It is therefore plausible that TTP binding next to CAF1 on CNOT1 may help position the CAF1 deadenylase so that it has unimpeded access to the mRNA poly(A) tail (Fig. 4g).

TTP residues that interact with CNOT1 are phylogenetically conserved in Tis11 proteins, including the D. melanogaster Tis11 homolog and the C. elegans protein CCCH–1 (Fig. 1d). Moreover, CNOT1 residues that form a groove, which contains amino acids that interact with the TTP-CIM (Phe847, Tyr851, Glu893 and Tyr900), are highly conserved across these species as well (Supplementary Fig. 4b). A recent study reported the crystal structure of the yeast Not1 N-terminal region (residues 154–753)20. It consists of 13 HEAT repeats, of which repeats 10–13 (residues 571–746) correspond to the four HEAT repeats found in human CNOT1800–999. Although yeast Not1 and human CNOT1 proteins share only 35% sequence identity in this region, the structures are very similar, with a Cα r.m.s. deviation of 1.19 Å (Supplementary Figs. 4c,d). Notably, yeast Not1 HEAT repeats 10–13 form a separate unit within the protein in that they are arranged in a parallel fashion while there is a rotation by 90° with respect to repeats 1–9 (ref. 20). This suggests that HEAT repeats 10–13 form a separate domain. Our results confirm this notion because we show that this domain is stable and can carry out its function of TTP recruitment in the absence of the more N-terminal HEAT repeats. The residues in human CNOT1 that form the TTP-docking site are also conserved in yeast Not1, which suggests that they may also act as a protein-binding site. However, the yeast Tis11 homolog CTH1 (cysteine three histidine 1) does not contain a sequence that shares any detectable homology to the hTTP-CIM. Nevertheless, it is conceivable...
that ancient NOT1 proteins maintained this protein-interaction groove, which was exploited during the course of eukaryotic evolution by proteins such as TTP. NOT1 binds the miRNA-associated GW182 protein through two conserved CIDs and additional tryptophan-containing sequences18,22. However, the TTP-CID does not share homology with the GW182 CIDs, which suggests that TTP and GW182 bind different regions of NOT1.

Mouse TTP activity is controlled by phosphorylation on a number of residues, including Ser52 and Ser178 (Ser60 and Ser186 in human TTP, respectively) by the p38 MAPK-activated protein kinase 2 (MK2) (ref. 23). Ser52 and Ser178, when phosphorylated, act as substrates for 14-3-3 adapter proteins that bind and stabilize TTP23,24. Phosphorylation of TTP by MK2 also impairs TTP recruitment of the CCR4–NOT complex and TTP-mediated deadenylation25,26. On the basis of these data, it has been postulated that 14-3-3 binding to TTP impairs deadenylase recruitment26. However, our data demonstrate that 14-3-3-binding sites (Ser52 and Ser178) do not overlap with the C-terminal TTP-CID. Notwithstanding this, MK2-induced TTP phosphorylation partially inhibits association with CCR4–NOT even when these serines are mutated in tandem to alanines, thereby abolishing 14-3-3 binding26. It has therefore been suggested that additional phosphorylation sites act to inhibit deadenylase recruitment to TTP28. Notably, MK2 has also been reported to phosphorylate TTP and BRF-1 on a highly conserved serine residue residing within the TTP-CID (Ser323 in hTTP; Fig. 1d)23. Our structural data indicate that Ser323 forms multiple contacts at the TTP-CNOT1 interface, both within the TTP peptide and with CNOT1 (Fig. 1b). Thus, the addition of a phosphate group to Ser323 would probably perturb TTP-CNOT1 binding. Indeed, a TTP-CID peptide containing a phosphorylated Ser323 binds with a markedly lower affinity to CNOT1 (K_d = 625 µM; Supplementary Fig. 3d) as compared to wild type. Taken together, these data suggest that the ability of the TTP-CID to bind CNOT1 is regulated through the p38 signaling pathway. This model may help explain how MK2 phosphorylation regulates TTP recruitment of the CCR4–NOT complex. It is possible that 14-3-3 proteins and the CCR4–NOT complex compete for TTP binding. Phosphorylation of Ser323 may therefore aid the TTP transition from being bound to the CCR4–NOT complex to interacting with 14-3-3 proteins. Whether this post-translational modification has a role in regulating TTP-mediated silencing, however, remains to be determined.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession code. Structure factors and coordinates for the TTP–CNOT1 complex have been deposited in the Protein Data Bank under accession code 4J8S.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.R.F, F.F, C.R. and N. Siddiqui designed experiments. M.R.F. and C.R. performed binding assays and in vitro deadenylation assays. N. Siddiqui performed ITC experiments. F.F. crystallized the CNOT1–TTP complex, and B.N. and F.F. determined its structure. W.S.L. and P.J.B. performed in vivo stability assays. A.K. provided technical support. M.R.F, F.F, B.N. and N. Sonenberg wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Recombinant protein expression and purification. His-tagged recombinant proteins were expressed in Rosetta-2(DE3) E. coli cells (EMD Biosciences) and purified by using Ni-NTA agarose (Qiagen). CNOT1800–999 was subsequently treated with Up1 to cleave the SUMO-His tag and dialyzed into buffer containing 25 mM Tris-HCl, pH 8.0, 100 mM NaCl. The sample was diluted twice to obtain a final NaCl concentration of 50 mM. The sample was loaded onto a Mono Q (GE Healthcare) anion-exchange column. Fractions containing the CNOT1 protein were collected, concentrated and further purified over a Superdex-75 size-exclusion column in 25 mM MES-HCl, pH 6.5, and 20 mM NaCl.

GST pulldown experiments. Various MBP-TTP fragments (35 pmol) were incubated with 20 µl of packed glutathione Sepharose-4B and 35 pmol of GST or various recombinant GST-CNOT1 fragments in binding buffer (25 mM Tris, pH 7.5, 150 mM NaCl and 0.1% NP-40) for 2 h at 4 °C with gentle rocking. Beads were then washed five times with 1 ml of binding buffer, and proteins were eluted by boiling the beads with 40 µl of Laemmli sample buffer at 95 °C for 8 min. Proteins were separated by SDS-PAGE and visualized by Coomassie staining.

Peptide synthesis and purification. Peptides corresponding to the wild-type (312-APRRLPIFNRISVSE-326) and F319A mutant (312-APRRLPIANRISVSE-326) CNOT1-binding site in TTP (GI:136471) were synthesized by Fmoc-(9-fluorenyl)-methoxycarbonyl solid-phase peptide synthesis (Sheldon Biotechnology Center) and purified by reverse-phase chromatography on a Vydac C18 column. Peptides were verified by ion-spray quadrupole MS. The pSer323 peptide (312-APRRLPIFNRIpSVSE-326) was generated and purified by Canpeptide.

Crystallization, data collection and structure determination. TTP peptide (concentration ~7 mg/ml in buffer containing 25 mM MES-HCl, pH 6.5, 200 mM NaCl) was mixed with CNOT1800–999 (concentration 15 mg/ml) at a 1:51 molar ratio of peptide to protein, and crystals were grown by hanging-drop vapor diffusion. The protein–peptide complex was mixed 1:1 with well solution containing 0.2 M MgCl2, 0.1 M Bis-Tris, pH 5.5, and 25% PEG 3350 at room temperature. Crystals diffracting to 1.53 Å with excellent quality appeared after 7 d. Native data for 360° collected in 0.5° images on a rotating anode Cu Kα source (λ = 1.54 Å) at 100 K were processed with HKL2000 (ref. 30). CNOT1 complex with TTP peptide crystallized in space group P21 with cell dimensions a = b = c = 80.07 Å. The structure of CNOT1800–999 was solved by using sulfur-single wavelength anomalous dispersion (S-SAD). The protein contains nine sulfur atoms (six from methionine and three from cysteine). The sulfur substructure was solved by SHELXD31, which found eight of nine sites. Data up to 2.5 Å were used for the search where the signal-to-noise ratio was ~55σ in that shell. Phases were calculated by using SHELXE with the e−1.0 flag (free lunch algorithm32) for data up to 1.55 Å, which produced electron density maps of extremely high quality that were almost completely traced by Arp/Warp33, including the ligand peptide. The structure was further refined by using PHENIX34. Statistics are described in Table 1. In the Ramachandran plot, 99.5% of residues were found in favored regions, and 0% outliers were observed.

In vitro degradylation assays. S-Boxx-pA and TNF-pA RNA transcripts were generated from a PCR product derived from RL-5-Boxx-pA and RL-TNF-pA, respectively. PCR product was digested with AgeI (New England Biolabs). To synthesize radiolabeled mRNA, [α-32P]UTP (800 Ci/mmol, 10 mCi/ml; PerkinElmer) was used according to the manufacturer's protocol. The mRNA was loaded on a mini Quick Spin RNA Column (Roche) to remove unincorporated nucleotides. In vitro degradylation assays were carried out as described18 in the presence of 172 mM GST, GST-TTP and GST-ANHA–TTP proteins.

In vivo mRNA stability assays. HEK-293 cells were transiently transfected with CMV-mTNP7 or other constructs in calcium-phosphate precipitates as described previously27. Each 100-mm dish of cells (0.8 × 10⁶) was co-transfected with 3 µg of plasmid TNF-expression vector CMV-BGH3 together with 5 ng of vector CMV.BGH3/B5 as a control. Vector plasmid (B5+) was added to make the total amount of co-transfected DNA 5 µg per dish in all cases. In every experiment, three dishes of cells were pooled for each co-transfection condition. Total cellular RNA was harvested from the cells by using the illustra RNA spin Mini RNA Isolation Kit (GE Healthcare). Each gel lane for northern blotting contained 10 µg of RNA. Northern blots were hybridized to a random-primed, α-32P-labeled MLP Mscf fragment or to a mouse TTP cDNA together with the EGFP-coding cDNA19,29.

Isothermal titration calorimetry. Software for data acquisition and instrument control was from MicroCal. The ITC buffer used contained 20 mM Tris, pH 7.2, 150 mM NaCl and 200 µM TCEP. The reaction cell contained 1.8 ml of 25–30 mM CNOT1800–999 at 20 °C. The sample cell was titrated with 29 injections of 10 µl peptide (300 µM–1 mM) with a 4-min time interval between injections. The isotherm was fitted with a 1:1 binding model to determine the thermodynamic binding constant and stoichiometry. Experiments were carried out on a MicroCal VP-ITC titration calorimeter, using the VPViewer software for data acquisition and instrument control (MicroCal). The ITC buffer contained 20 mM Tris, pH 7.2, 150 mM NaCl and 200 µM TCEP. The reaction cell contained 1.8 ml of 25–30 µM CNOT1800–999 and was thermostated to 20 °C. The sample cell was titrated with 29 injections of 10 µl peptide (300 µM–1 mM) with a 4-min time interval between injections. The isotherm for the wild-type titration was fitted with a 1:1 binding model to determine the thermodynamic binding constant and stoichiometry. The number of binding sites was set to 1 for calculating the binding constants of the mutant and phosphorylated peptides.

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