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LARP4 Is Regulated by Tumor Necrosis Factor Alpha in a Tristetraprolin-Dependent Manner

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LARP4 is a protein with unknown function that independently binds to poly(A) RNA, RACK1, and the poly(A)-binding protein (PABPC1). Here, we report on its regulation. We found a conserved AU-rich element (ARE) in the human LARP4 mRNA 3′ untranslated region (UTR). This ARE, but not its antisense version or a point-mutated version, significantly decreased the stability of β-globin reporter mRNA. We found that overexpression of tristetraprolin (TTP), but not its RNA binding mutant or the other ARE-binding proteins tested, decreased cellular LARP4 levels. RNA coimmunoprecipitation showed that TTP specifically associated with LARP4 mRNA *in vivo*. Consistent with this, mouse LARP4 accumulated to higher levels in TTP gene knockout (KO) cells than in control cells. Stimulation of WT cells with tumor necrosis factor alpha (TNF-α), which rapidly induces TTP, robustly decreased LARP4 with a coincident time course but had no such effect on LARP4B or La protein or on LARP4 in the TTP KO cells. The TNF-α-induced TTP pulse was followed by a transient decrease in LARP4 mRNA that was quickly followed by a subsequent transient decrease in LARP4 protein. Involvement of LARP4 as a target of TNF-α–TTP regulation provides a clue as to how its functional activity may be used in a physiologic pathway.

La-related protein 4 (LARP4), a member of the La family of proteins (1, 2), is a multimodular protein involved in mRNA metabolism. Unlike La protein, the most ancient member of the family, which binds most avidly to 3′-terminal oligo(U) tracts found on nascent transcripts and processing intermediates of small nuclear RNAs, LARP4 is predominantly cytoplasmic and binds preferentially to poly(A) RNA (2). LARP4 interacts with the MLLE domain of the cytoplasmic poly(A)-binding protein (PABPC1) via a well-characterized PAM2 motif near its N terminus (2). LARP4 independently interacts with the receptor for activated C kinase (RACK1), which associates with mRNAs and the 40S ribosome (2). Small interfering RNA (siRNA)-mediated knockdown of LARP4 decreased general protein synthesis (2). Modest overexpression of LARP4 conferred stability on some mRNAs more than others (2).

LARP4 underwent a gene duplication event in an ancient vertebrate that gave rise to LARP4B, which has been independently maintained thereafter (3). The two genes are located on different human chromosomes and produce different proteins that each bind to PABP and RACK1 but likely exhibit different sequence-specific RNA binding (1, 2, 4). LARP4B promotes mRNA accumulation and translation by binding 3′ untranslated region (UTR) AU-rich sequences of a set of mRNAs (4, 5). The data suggest that LARP4 and -4B modulate mRNA stability and translation but that they may target different sets of mRNAs.

AU-rich elements (AREs) are a specific subset of AU-rich sequences found in mammalian *cis*-acting tracts of 50 to 150 nucleotides (nt) that in humans reside within the 3′ UTRs of 5 to 8% of mRNAs (6) and that consist of AUUUA pentamers, either overlapping or near other AU-rich sequences (7). ARE-containing mRNAs mostly encode proteins that are tightly regulated, e.g., transcription factors, cytokines, and cell-cycle-regulatory genes. Several ARE-binding proteins exist and can have different effects on ARE-containing mRNAs. Examples of these are HuR, AUF1, and tristetraprolin (TTP) (also known as ZFP36), the last of which contains two CCCH zinc fingers necessary for ARE binding (8, 9). TTP-responsive AREs contain a TTP binding site, UAUUUAU, and similar motifs (10, 11). TTP binding often leads to recruitment of poly(A) deadenylases, followed by degradation of the target mRNAs (12–16; reviewed in reference 15).

The best-studied and most physiologically relevant mRNA decay substrate of TTP in mice is the tumor necrosis factor alpha (TNF-α) mRNA. TTP knockout (KO) mice develop debilitating generalized inflammation associated with high levels of TNF-α, the symptoms of which are reversed by treatment with antibodies against TNF-α (17).

TTP is induced by serum mitogens and cytokines, including TNF-α (18). A cycle of TTP regulation that has been proposed is as follows. Upon stimulation of the p38 mitogen-activated protein kinase/MK2 protein kinase, TTP becomes phosphorylated, which recruits 14-3-3 proteins that in turn inhibit association of TTP with the deadenylases. Accordingly, this leads to increased levels of the TTP target mRNAs that would otherwise be substrates for decay. MK2 phosphorylation of TTP also protects it from degradation by the proteasome, which allows its levels to increase (19–21). Protein phosphatase 2A (PP2A) dephosphorylates TTP; this reactivates it for mRNA decay and makes it susceptible to degradation (22). It is notable that part of the regulatory cycle is TNF-α protein induction of TTP, which itself targets TNF-α mRNA for decay.

While investigating features of LARP4, we subjected the 3′

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UTR of its mRNA to the ARE database search engine (23), which predicted an ARE. Phylogenetic sequence alignment of LARP4 3′ UTRs revealed that the predicted ARE includes an evolutionarily conserved UAUUUAU sequence and related AU-rich sequences. Inserting the putative ARE in the 3′ UTR of a β-globin mRNA reporter caused instability, while point mutations to the UAU UUAU motifs reversed this destabilizing effect. We show that TTP binds to LARP4 mRNA *in vivo* and that overexpression of wild-type TTP but not a previously characterized RNA binding mutant, TTP-C147R, decreases LARP4 levels in HeLa cells. Consistent with this, mouse LARP4 levels are higher in TTP gene KO cells than in the wild-type (WT) control cells. Upon stimulation of the WT cells with TNF-α to induce TTP, LARP4 is downregulated, coincident with TTP induction, but is unaffected in the TTP KO cells. These data show that accumulation of a LARP is regulated by TTP. Moreover, the time course of the response reveals that LARP4 responds as an ARE-regulated mRNA, which results in quick decrease in LARP4 protein levels. More significantly, the results indicate that LARP4 is a target of TNF-α–TTP-mediated regulation and may therefore provide clues about physiologic function.

MATERIALS AND METHODS

Cell culture and reagents. HeLa Tet-Off cells (Clontech) were maintained in Dulbecco's modified Eagle's medium (DMEM) plus Glutamax (Gibco) supplemented with 100 μg/ml G418 and 10% heat-inactivated fetal bovine serum (FBS) in a humidified 37°C, 5% CO_2 incubator. TTP WT (+/+) and TTP KO (−/−) mouse embryonic fibroblast (MEF) cell line numbers 67 and 66, respectively were kind gifts from P. Blackshear (10) and were cultured in DMEM plus Glutamax with 10% heat-inactivated FBS. Doxycycline was from Sigma.

Antibodies. Anti-FLAG (Sigma; F1804), anti-green fluorescent protein (anti-GFP) (Santa Cruz Biotechnology; sc-8334), and anti-LARP4 have been described previously (2). Rabbit anti-LARP4B was generated using the LARP4B C-terminal sequence KEEKKLAEPAERYREPPALK as an antigen and affinity purified using the same peptide (Open Biosystems). Anti-MBP TTP (number 1192) was a kind gift from P. Blackshear (24). To detect human and mouse La, anti-La (Go serum) and anti-mouse La were used (25, 26).

DNA constructs. pTRERβ-wt, encoding rabbit β-globin under the transcriptional control of a tetracycline-responsive promoter, and pTRERβ-TNF-α-ARE (containing the 38-nt ARE from TNF-α in the β-globin 3′ UTR) were kind gifts from G. Wilson (27). pTRERβ-wt contains a unique BglII site located after the stop codon of β-globin. Human genomic DNA was used as a template to amplify a 317-nt AU-rich fragment from the 3′ UTR of LARP4 using primers BglII-LARP4-ARE Fwd (5′-GCCCCCAGATCTCCCATTTGATGGCATGTATAG-3′) and BglII-LARP4-ARE Rev (5′-GCCGCCAGATCTATACCTACTTTTAGCCTCC AAC-3′). The resulting PCR product was cloned into the BglII site of pTRERβ-wt to generate the constructs pTRERβ-LARP4-317nt-ARE sense and pTRERβ-LARP4-317nt-ARE antisense. Mutants in 317nt-ARE sense were obtained from Eurofins and subcloned into the BglII site of pTRERβ.

pTRERβ-LARP4-317nt-ARE was used as a template for PCR to generate a shorter, 184-nt fragment of the ARE, both wild type and mutant, using the primers L4-ARE-wt-Fwd (5′-GCCGCCAGATCTTGTGTGTA ATATTTATATATATTCACAGTATG-3′) with L4-ARE-wt-Rev (5′-GC CGCCAGATCTCCATCTAAAGAACCAAATAAATAAACT ACAATG-3′) and L4-ARE-mutant-Fwd (5′-GCCGCCAGATCTTGTGTGTAATA GGTATATATATTCACAGTATG-3′) with L4-ARE-mutant-Rev (5′-GCCGCCAGATCTCCATCTAAAGAACCAAATCCATAAACTACA ATG-3′). The resulting PCR products were cloned into the BglII site of pTRERβ-wt.

Constructs for overexpression of FLAG-tagged wild-type TTP and the RNA binding mutant TTP C147R were a kind gift from Gerald Wilson. The construct used to overexpress FLAG-tagged HuR was a gift from M. Gorospe (28), and the plasmids for overexpression of the 4 isoforms of AUF1 (p37, p40, p42, and p45) were a gift from R. J. Schneider (29).

mRNA decay measurements. HeLa Tet-Off cells (5.5×10^5 per well) were seeded in 6-well plates 1 day prior to transfection with Lipofectamine 2000 (Invitrogen). The pTRERβ plasmid (100 ng) containing a Tet-responsive promoter and 100 ng of GFP control plasmid containing a conventional cytomegalovirus (CMV) promoter were cotransfected according to the manufacturer's instructions. Twenty-four hours posttransfection, the cells were divided into multiple 6-well plates. The next day, the medium was replaced with medium containing 2 μg/ml doxycycline. At various times thereafter, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed by adding TRIzol (Invitrogen), and total RNA was isolated, analyzed by Northern blotting (see below), quantified with a phosphorimager, and plotted using the statistical software package Prism (GraphPad, San Diego, CA) (30).

Northern blotting. Total RNA was separated on 1.8% agarose-formaldehyde gels, transferred to a GeneScreen PlusR membrane (PerkinElmer) overnight, and subsequently UV cross-linked. The blot was then baked under vacuum for 2 h at 80°C. The blot was prehybridized for 2 h in 15 ml hybridization buffer A (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% SDS, 2× Denhardt's solution [catalog number 750018; Life Technologies], 100 μg/ml *Saccharomyces cerevisiae* RNA) at the incubation temperature (T_i) [$T_i = T_m$ (melting temperature) − 15°C, where T_m is equal to $16.6 \log(M) + 0.41(P_{gc}) + 81.5 - P_m - (B/L)$, where M is the molar salt concentration up to a maximum of 0.5; P_{gc} is the percent G+C content in the oligonucleotide DNA probe; P_m is the percentage of mismatched bases, if any; B is 675; and L is the oligonucleotide DNA probe length]. Antisense oligonucleotides were 5′ labeled with ^{32}P and added to the hybridization buffer at 2×10^6 cpm/ml. Hybridization was done overnight. Blots were washed 4 times in 2× SSC plus 0.1% SDS for 15 min at room temperature before being exposed to a phosphorimager screen. Multiple oligonucleotides were mixed to detect one target (Table 1). After exposure, the blots were stripped at 95°C in 0.1× SSC plus 0.1% SDS and subsequently probed.

To detect human LARP4 (hLARP4), a random-primed ^{32}P-labeled probe made from human LARP4 cDNA was hybridized overnight at 44°C in hybridization buffer B (4× SSC, 5× Denhardt's solution, 1% SDS, 5 mM EDTA, 100 μg/ml yeast RNA, and 50% formamide). The blot was washed 3 times for 10 min each time at 55°C.

RNA coimmunoprecipitation (co-IP) and reverse transcription (RT)-PCR. HeLa Tet-Off cells in 6-well plates were transfected with 1 μg of empty pCMV2 vector or expression vector FLAG-TTP or FLAG-TTP-C147R mutant per well using Lipofectamine 2000. Twenty-four hours posttransfection, cells were harvested in lysis buffer (100 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7, 0.5% NP-40, 1 mM dithiothreitol [DTT], 100 U/ml Superasin [Life Technologies], and protease inhibitor cocktail [Roche]). The lysate was centrifuged for 30 min at 13,000 rpm at 4°C, and the supernatant was isolated for further analysis.

cDNA was generated from 0.3 μg total RNA using the SuperScript III first-strand synthesis system and oligo(dT) (Life Technologies). The primers used were as follows: LARP4 RT-PCR Fwd, 5′-CCTAATTCAGG TGTCCTTTGCATCTC-3′; LARP4 RT-PCR Rev, 5′-CCCATAGCACCT TGGCGATGTTG-3′; LARP4B RT-PCR Fwd, 5′-CACATCACCGGCTG TCACTGG-3′; LARP4B RT-PCR Rev, 5′-CTCGAGGAGCGACCACAT GACC-3′; GAPDH RT-PCR Fwd, 5′-CCACCCATGGCAAATTCCATG GCA-3′; GAPDH RT-PCR Rev, 5′-TCTAGACGGCAGGTCAGGTCCA CC-3′. The PCR conditions were annealing at 60°C (35 cycles for LARP4 and LARP4B and 27 cycles for GAPDH [glyceraldehyde-3-phosphate dehydrogenase]).

Polysome profile analysis. Polysome fractionations were done by standard methods (31), as previously reported (2), using a programmable density gradient fractionation system spectrophotometer (model Foxy Jr.;

TABLE 1 Oligonucleotide DNA sequences of probes used

Target and probe	Sequence (5′→3′)
GFP	
GFP-1	CGTGCTGCTTCATGTGGTC
GFP-2	GTTCACCAGGGTGTCGCC
GFP-3	GGTCACGAACTCCAGCAGG
β-Globin	
β-globin-1	CACCAGCCACCACCTTCTG
β-globin-2	GGCAGCCTGCACCTGAGG
β-globin-3	GCACCTTCTTGCCATGAGCC
Mouse RppH1	
mRppH1-1	TGATGAGCTTCCTCCGCCCACT
mRppH1-2	GAGTGACACGCACTCAGCACGT
mRppH1-3	CCCAAGTCTCAGACCTTCCCAG
Human La	
hLa-1	GTCCCGTGGCAAATTGAAGTCGCC
hLa-2	CCATGAGTTCTGCCTTGGATTTGCTC
hLa-3	CACTTCAGGTAGGGGTTTGCTTGGAG
hLa-4	GTTTCTTTGTACTTCTGGCCAGGGGTC
hLa-5	TTGGCTTTACCCAATGCTTCCTTGGC
Mouse La	
mLa-1	TTGTCTCGTGGCAAATTGAAGTCTC
mLa-2	CAAAGGTACCCAGCCTTCATCC
mLa-3	GTCAGCCGGTTTAGCCTGTTG
mLa-4	GATTTGCTCAGTGCTTGCACAATTAC
mLa-5	GTCACTTCAGGGAGTGGTCTG
Mouse LARP4	
mLARP4-1	GCCTTTAGATGTCACCTCAACG
mLARP4-2	CCAGAAACATCATAGAGTTGGTAAC
mLARP4-3	CCTTTTCTCCCTTCTCATCAAC
mLARP4-4	GAGAATCACAATACATCGCTTATGAC
mLARP4-5	TTATCACTTTGGGGCAGTTTTC
mLARP4-6	TGTGTCTGACTGGAAAGTGATG
mLARP4-7	GTGGTGTTTCAAAGTAAGGTGC
mLARP4-8	CAAAACTACCATTGGGAAAGGG
mLARP4-9	ACGATTTTTTGGGAATGGTCGG
mLARP4-10	CCACTGGATGACCTAAAATGAG
mLARP4-11	CTGTTACTGTTGGGTTATGCCG
mLARP4-12	AGATAAGTTTGTTCCTGCTGCC
mLARP4-13	GCAAGCAAATCAAACTTCGGAG
mLARP4-14	TGTTCTCCAAACCAAGTTCATC
mLARP4-15	CTTCTGGCACACTTCAGCATAG
mLARP4-16	CTTCATTTCTGGTGGGGGATAC
mLARP4-17	CTGTTCCCTGATTTTTCCAGCC

Teledyne Isco, Lincoln, NE). TTP WT and KO MEFs were each seeded in 5- by 15-cm culture plates so that the next day they were 60 to 70% confluent. Fresh sucrose solutions (47% and 7% sucrose [lsqb[wt/vol]) in 10 mM HEPES, pH 7.3, 150 mM KCl, 20 mM $MgCl_2$, and 1 mM DTT were prepared and filter sterilized. Sucrose gradients (7% to 47% sucrose [wt/vol]) were prepared using a Gradient Master (Biocomp). The cell growth medium was replaced 4 h before addition of cycloheximide (Chx) to the cells at a final concentration of 100 μg/ml (from freshly made 10-mg/ml stock in water). After 10 min at 37°C, the cells were washed once with prewarmed PBS plus 100 μg/ml Chx and once with ice-cold PBS plus 100 μg/ml Chx and moved to ice. Five milliliters of ice-cold PBS plus 100 μg/ml Chx was added per plate, and the cells were scraped and added to an ice-cold tube. The cell suspension was centrifuged for 3 min at 1,200 rpm at 4°C. The cell pellet was taken up in 300 μl lysis buffer (10 mM HEPES, pH 7.3, 150 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, 2% NP-40, 2 mM phenylmethylsulfonyl fluoride [PMSF], 20 μg/ml aprotinin, 20 μg/ml leupeptin, 100 μg/ml Chx, and 40 U/ml Superasin [Life Technologies]) and kept on ice for 2 min with occasional flicking. The lysate was cleared by centrifugation at 13,000 rpm for 5 min at 4°C. Four hundred microliters of the gradient was removed, and the lysate was carefully loaded on top. The gradients were spun in an ultracentrifuge (Beckman SW41 rotor) at 38,000 rpm for 2.5 h at 4°C. Fractions of 1 ml were collected, and RNA was purified from 500 μl of each fraction using the Maxwell 16 LEV simplyRNA kit with the Maxwell 16 instrument, which includes treatment with DNase I (Promega).

TTP induction by TNF-α. TTP WT (150,000 cells/well) and TTP KO (100,000 cells/well) MEFs were seeded in 6-well plates. The next day the growth medium was replaced with 2 ml DMEM plus 10% charcoal-stripped FBS (Life Technologies). Twenty-four hours later, the medium was replaced with medium containing recombinant mouse TNF-α (amino acids [aa] 84 to 235; R&D Systems, Minneapolis, MN) at a final concentration of 10 ng/ml. At various times thereafter, cells were harvested for RNA and protein isolation. For RNA, cells were washed with ice-cold PBS, lysed in Tripure (Roche), and purified according to the manufacturer's instructions. For protein, cells were washed with ice-cold PBS and directly lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) plus protease inhibitors (Roche).

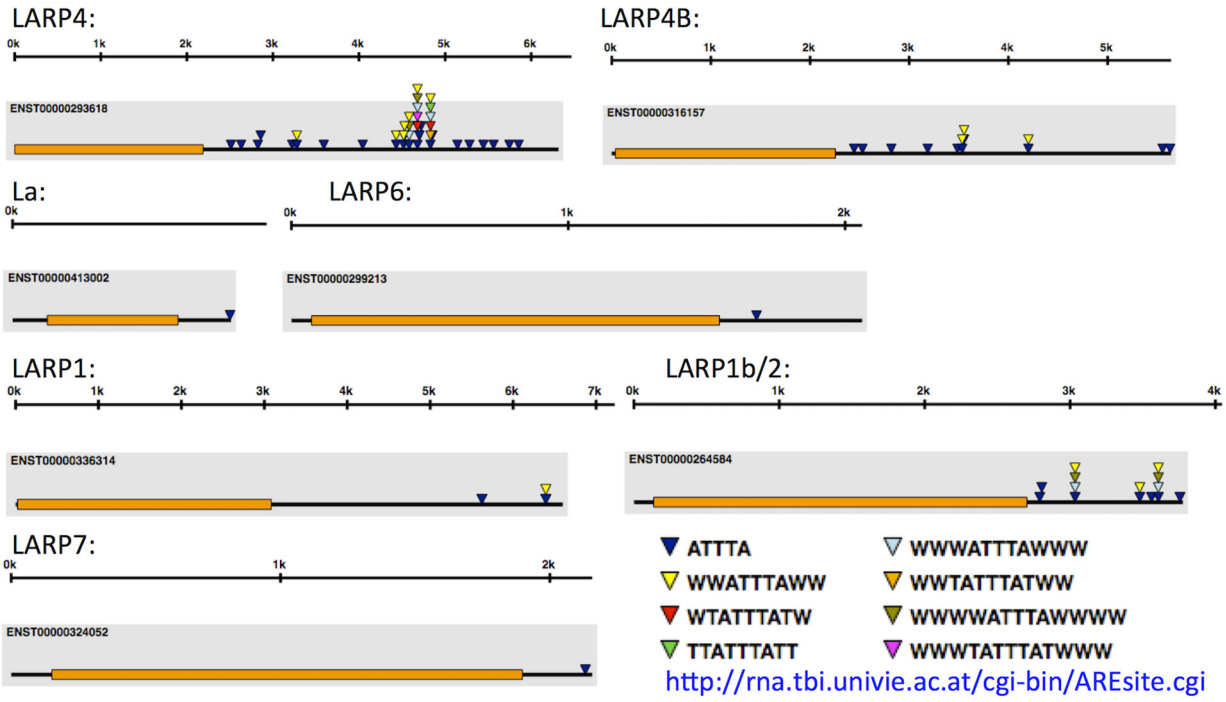
Protein half-life measurements. TTP WT and KO MEFs were seeded in 10-cm plates so that they were 60% confluent the next day. The cells were washed with 5 ml PBS and incubated with 1 ml of cysteine- and methionine-free DMEM containing 10% dialyzed fetal calf serum (FCS) (Life Sciences) at 37°C for 1 h with gentle rocking so that the cells were fully bathed in medium. EasyTag Express ^{35}S protein labeling mix (200 μCi; PerkinElmer) was added to each plate. After 30 min, the ^{35}S medium was removed, and the cells were washed with 5 ml prewarmed PBS. For the zero time point, the cells were immediately harvested, and extract was prepared. For other time points, 3 ml chase medium (DMEM containing 10% FCS, 3 mM L-methionine, and 1 mM cysteine) was added, and the cells were incubated as before. For harvesting, the medium was removed, and the cells were washed with 3 ml PBS. Lysis was performed by adding 300 μl ice-cold RIPA buffer (Pierce) plus protease inhibitors (Roche) with sonication (3 times for 30 s each time) in a Bioruptor (Diagenode). After clearing the lysate by centrifugation, the protein concentration of each extract was determined. Three hundred fifty micrograms of total protein of each lysate was precleared with 20 μl of protein A-Sepharose (GE Healthcare). For the IP, 5 μl of antibody to LARP4, LARP4B, La, or normal rabbit IgG was incubated with 20 μl of protein A-Sepharose (GE Healthcare) and washed extensively. The washed beads were incubated with 125 μg precleared lysate plus ice-cold RIPA buffer containing protease inhibitors in a final volume of 500 μl in Eppendorf tubes on a rotation mixer at 4°C overnight. The beads were then washed 3 times with 1 ml RIPA plus protease inhibitors and resuspended in 2× SDS-PAGE buffer containing fresh 4% β-mercaptoethanol. After heating and centrifugation, the supernatants were fractionated by SDS-PAGE and transferred to a membrane. Visualization and quantitation were performed using a phosphorimager.

RESULTS

LARP4 mRNA contains an evolutionarily conserved ARE that confers instability on a reporter mRNA. The ARE database (ARED) search engine was used to analyze the 3′ UTR of human LARP4 mRNA. It found what is referred to as a cluster V ARE (6, 23) sequence residing within a larger AU-rich tract in the LARP4 3′ UTR that contains additional AUUUA pentamers. In contrast, the ARED search engine did not predict an ARE in LARP4B in any organism.

We also analyzed the human LARP family member mRNA 3′ UTRs using the AREsite (32; http://nibiru.tbi.univie.ac.at/AREsite2/welcome) algorithm (Fig. 1A). LARP4 was the most

A)

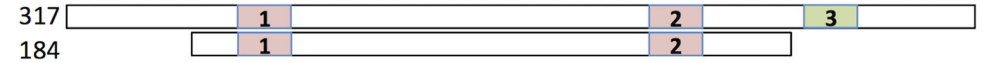


B)

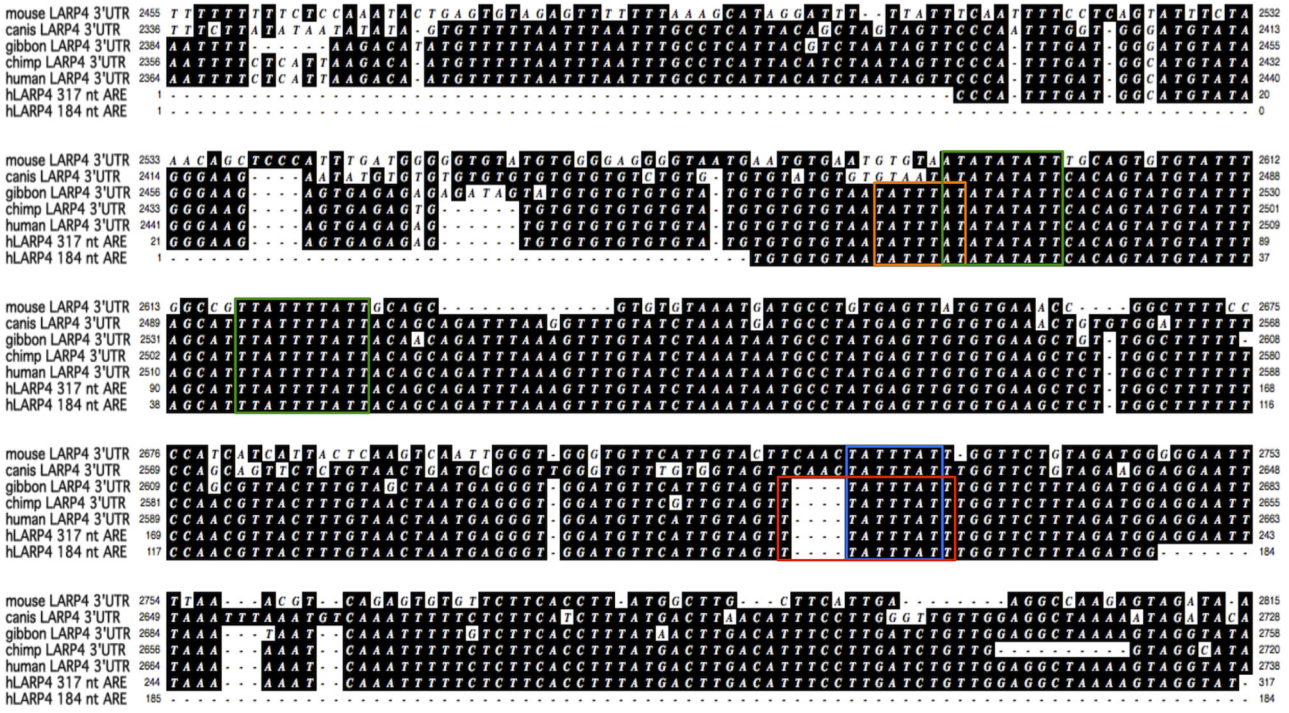
human LARP4 ARE:

CCCAUUUGAUGGCAUGUAUAGGGAAGAGUGAGAGAGUGUGUGUGUGUGUAUGUGUGUGUAAUAUUUAUAUAUAUUCACAG
UAUGUAUUUAGCAUUUAUUUUAUUACAGCAGAUUUAAAGUUUGUAUCUAAAUAAUGCCUAUGAGUUGUGUGAAGCUCUUG
GCUUUUUUCCAACGUUACUUUGUAACUAAUGAGGGUGGAUGUUCAUUGUAGUUUAUUUAUUUGGUUCUUUAGAUGGAGGA
AUUUAAAAAAUCAAAUUUUUCUCUUCACCUUUAUGACUUGACAUUUCCUUGAUCUGUUGGAGGCUAAAAGUAGGUAU

C)



D)



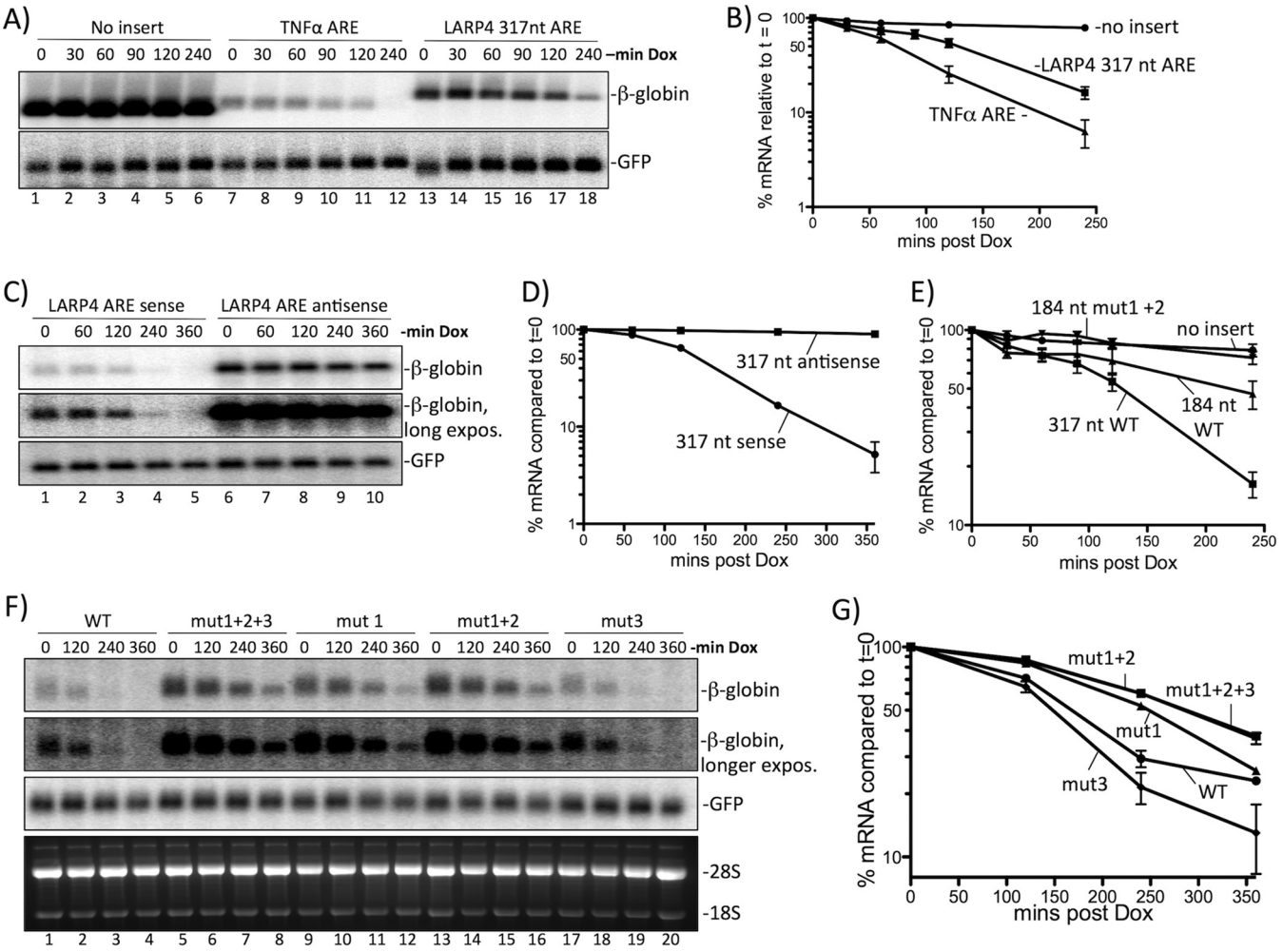


FIG 2 The predicted LARP4 ARE confers instability on β-globin reporter mRNA. HeLa Tet-Off cells were cotransfected with a vector containing GFP under the control of a constitutive CMV promoter, along with β-globin constructs under the control of a doxycycline (Dox)-repressible promoter. Forty-eight hours after transfection, Dox was added, and cells were harvested at the indicated times thereafter. (A) Total RNA was analyzed by Northern blotting using ^{32}P-labeled oligonucleotide probes against β-globin (top) and GFP (bottom). (B) Decay analysis using data from panel A quantified with a phosphorimager. The constructs examined were no insert, LARP4 317-nt ARE, and TNF-α ARE, as indicated. For all of them, the β-globin signal was normalized to GFP, and the amount of mRNA at time zero was set to 100%. (C and D) Constructs were analyzed as for panels A and B, i.e., the LARP4 317-nt ARE in the sense or antisense direction. (E) Constructs examined were no insert, LARP4 317-nt WT ARE, and LARP4 184-nt WT ARE and 184-nt mutants 1 plus 2 (mut1+2). (F) Constructs examined were LARP4 317-nt ARE (WT), the triple mutant (mut1+2+3), single mutant 1 (mut1), combination mutant 1 plus 2 (mut1+2), and single mutant 3 (mut3). An ethidium-stained agarose gel that was used for the Northern blot is also shown. (G) Quantitation was as described above. For all the graphs, the error bars represent the standard errors of the mean (SEM) of at least 3 independent experiments.

highly enriched in predicted AREs, even relative to its close homolog LARP4B (Fig. 1A). The AREsite found multiple AU-rich motifs that overlapped with the cluster V ARE found by ARED (Fig. 1A and data not shown). As discussed below, they include three UAUUUAU sequences that correspond to minimal binding sites for the ARE-binding protein TTP (two of which are expanded sequences with predicted higher affinity for TTP). In contrast, the hLARP4B 3′ UTR contained no UAUUUAU sequence tracts corresponding to a minimal TTP binding site, although it contained other AU-rich sequences (see Discussion).

FIG 1 Overview of predicted AREs in LARP family mRNAs. (A) AREsite was used to predict AREs in human LARP family members. W represents A or T. The red triangles reflect minimal TTP binding sites. (B) The sequence designated the 317-nt LARP4 ARE. The fragment designated the 184-nt ARE is in blue. Sequences corresponding to high-affinity TTP binding sites, UAUUUAU, are in red. AUUUA pentamers are in green. The UU→GG point-mutated residues characterized in Fig. 2 are underlined. (C) Schematic of the 317-nt and 184-nt fragments containing the red and green mutated underlined elements (numbered 1, 2, and 3) in panel B, analyzed in Fig. 2. (D) Conservation of ARE elements in mammalian LARP4 3′ UTRs. Shown is a sequence alignment of a segment of the larger ~4-kb 3′ UTRs of five mammalian LARP4 mRNA sequences, in addition to the 317- and 184-nt fragments of the human LARP4 ARE characterized in Fig. 2. A UAUUUAU heptamer that represents a conserved TTP binding site, corresponding to element 2 in panel C, is boxed in blue. Note that comparison of the blue and red boxes shows that this element has been expanded in primates to a higher-affinity TTP binding site, UUAUUUAUU (reviewed in reference 15). Another conserved AU-rich sequence that represents a reasonably high-affinity TTP binding site is boxed in green. UAUUUAU, corresponding to element 1 in panel C, is boxed in orange.

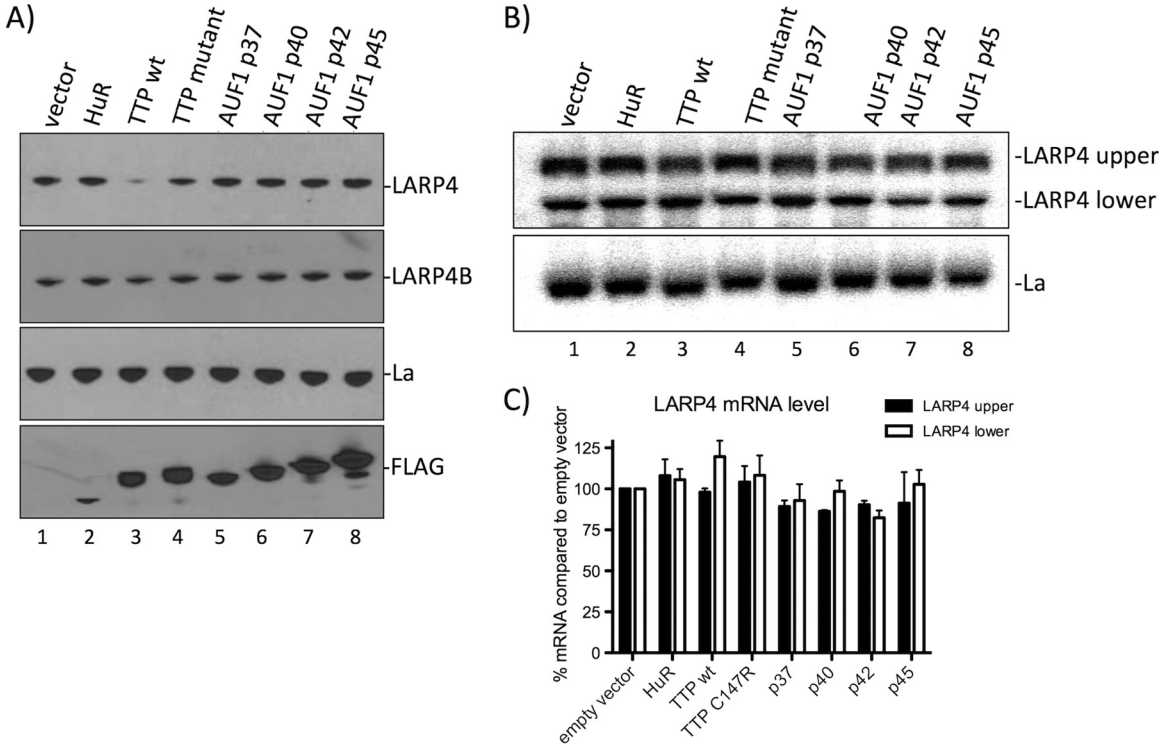


FIG 3 TTP overexpression decreases LARP4 levels. HeLa cells were transfected with empty pCMV2 vector or plasmids encoding FLAG-tagged versions of HuR, TTP wild type, or the RNA binding mutant TTP-C147R and the AUF1 isoforms p37, p40, p42, and p45. Forty-eight hours after transfection, cells were harvested, and lysates were prepared for protein and RNA analyses. (A) Proteins were analyzed by immunoblotting using anti-La, anti-LARP4, anti-LARP4B, and anti-FLAG antibodies as indicated on the right. (B) Total RNA was analyzed by Northern blotting, which detected two LARP4 mRNA bands (see the text). The blot was stripped and then hybridized with ^{32}P-labeled oligonucleotide probes against human La mRNA. (C) Quantitation of Northern blot signals was performed using a phosphorimager. The LARP4 mRNA signals were normalized to the La mRNA signals. The error bars represent the SEM of 3 independent experiments.

Figure 1A also shows that LARP4 has a unusually long 3′ UTR (≥4 kb) (33), suggesting negative regulation (reference 34 and references therein). The LARP4 3′ UTRs from mouse, dog, and primates span a size range of 4,135 to 4,180 nt. In contrast, La mRNA has a short 3′ UTR (Fig. 1A) and encodes a ubiquitously abundant housekeeping protein (35).

A 317-nt region encompassing the cluster V ARE is shown in Fig. 1B, with some of the individual sequence motifs tested for mRNA destabilization highlighted below, and is schematized in Fig. 1C. Sequence alignment of the region around the 317 nt of several mammalian LARP4 3′ UTRs is shown in Fig. 1D. This revealed perfect conservation of a UAUUUAU motif from mouse to human (Fig. 1D, blue box), representing a high-affinity TTP recognition site (reviewed in reference 15), as well as other AU-rich sequences. For example, the mouse LARP4 mRNA full 3′ UTR contains five UAUUUA hexamers, two AUUUAU hexamers, and 11 AUUUA pentamers (not shown).

To test if the ARE predicted in LARP4 might have functional potential, we used a popular β-globin mRNA reporter assay system (30, 36). The predicted 317-nt ARE (shown in Fig. 1B) was cloned into the β-globin reporter 3′ UTR in the pTRERβ vector under the control of a Tet-responsive promoter, allowing transcription to be switched off upon addition of doxycycline (36). We used the 38-nt-long ARE from TNF-α as a positive control (27). A GFP expression vector was cotransfected for normalization of the β-globin mRNA signal. After 48 h, doxycycline was added (time zero); samples were removed at various times thereafter, and RNA was isolated. It may be helpful to note that this assay reflects mRNA stability in two different ways; (i) differences in mRNA levels at time zero, which is reflective of the net accumulation for the preceding 48 h, during which ongoing transcription and decay collectively contribute, and (ii) the decay rate itself after transcription is switched off, i.e., beginning at time zero.

While the β-globin reporter without an insert in its 3′ UTR produced high levels of mRNA at time zero and was stable for 240 min (Fig. 2A, lanes 1 to 6), insertion of the 317-nt LARP4 ARE dramatically decreased the mRNA levels at time zero and the half-life relative to GFP mRNA (Fig. 2A, lanes 13 to 18). The 38-nt ARE from TNF-α caused dramatic instability (Fig. 2A, lanes 7 to 12), consistent with the reported results (27). Quantitations of triplicate experimental data, including those in Fig. 2A, are shown in Fig. 2B.

To examine specificity, we tested the 317-nt LARP4 ARE in antisense orientation. Indeed, the antisense ARE yielded higher RNA levels at time zero and thereafter (Fig. 2C) with no apparent decay relative to GFP mRNA (Fig. 2D).

To further map the human LARP4 ARE, an internal 184-nt fragment containing the longest AU-rich stretches (Fig. 1B) was cloned into the β-globin 3′ UTR. Decreasing the length of the 317-nt ARE to 184 nt changed the half-life of the reporter mRNA from 2 to 4 h (Fig. 2E), suggesting that an instability determinant lies within a part(s) of the 317-nt fragment that is not present in

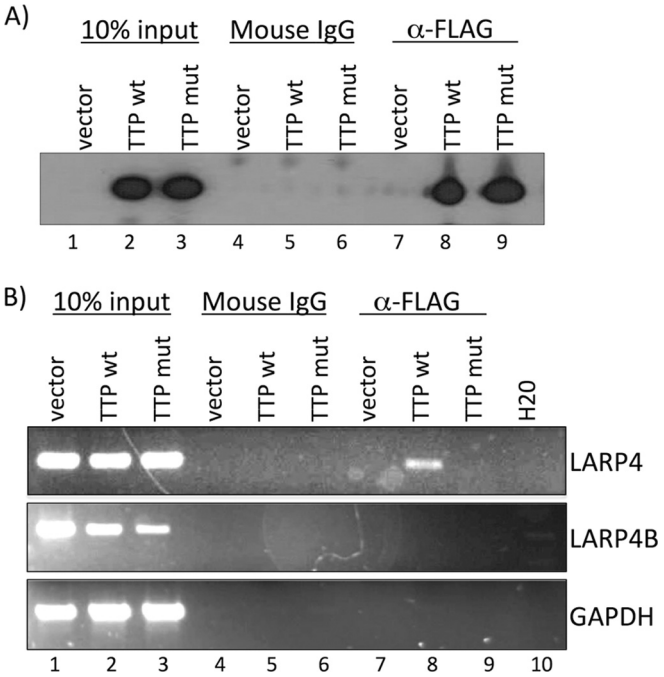


FIG 4 TTP, but not its RNA binding mutant C147R, associates with LARP4 mRNA. HeLa cells were transfected with empty pCMV2 vector, FLAG-tagged TTP wild type, or the RNA binding mutant TTP-C147R. Twenty-four hours after transfection, immunoprecipitation was performed using anti-FLAG antibody-coupled protein A-Sepharose (PAS) beads or beads coupled with control mouse IgG. After IP, the beads were separated into aliquots for protein and RNA analyses. (A) Immunoblot with anti-FLAG (α-FLAG). (B) RNA isolated from the PAS and cell extract (input), DNase I treated, and reverse transcribed with oligo(dT) into cDNA. Subsequently, PCR was performed using gene-specific primers for LARP4, LARP4B, and GAPDH. The products were analyzed on agarose gels containing ethidium bromide.

the 184-nt fragment. The data also indicate that the 184-nt fragment harbors significant destabilizing activity. Therefore, a point-mutated version of the 184-nt fragment was made in which two pairs of Us were changed to Gs in the two UAUUUAU motifs (Fig. 1B and C, elements 1 and 2). This rendered the 184-nt fragment as stable as the no-insert construct (Fig. 2E), indicating that one or both of the UAUUUAU motifs are active for reporter mRNA destabilization.

We also examined the same point mutations in the context of the 317-nt ARE, as well as an additional AUUUA pentamer (Fig. 1B and C, element 3), alone and in combinations. A representative blot is shown in Fig. 2F and quantitation of data from 3 independent experiments in Fig. 2G. Mutation of the first UAUUUAU element alone reversed some of the destabilizing effect, whereas mutating both UAUUUAU elements 1 and 2 reversed the destabilizing effect more (Fig. 2G, WT versus mut1 versus mut1+2). These results and data not shown indicate that both UAUUUAU elements 1 and 2 have destabilizing activity in this reporter assay. Mutating element 3, an AUUUA pentamer, in combination with 2 and 3, or alone, did not reverse destabilization of the reporter (Fig. 2F and G, WT versus mut1+2 versus mut1+2+3 versus mut3). Collectively, the results argue that the ARE in human LARP4 that was predicted to be complex is indeed functional in a standard reporter mRNA assay and that it contains multiple functional components, including two UAUUUAU motifs.

TTP overexpression decreases LARP4 levels. To investigate if an ARE-binding protein might be involved in regulating LARP4 expression, FLAG-tagged versions of HuR; the TTP wild type; its RNA binding mutant, TTP-C147R; and the AUF1 isoforms p37, p40, p42, and p45 were overexpressed in HeLa cells. Forty-eight hours after transfection, cells, protein, and RNA were isolated. LARP4 protein levels were reproducibly decreased by TTP overexpression compared to TTP-C147R and the other proteins, while LARP4B and La levels were relatively unchanged (Fig. 3A and data not shown). With the exception of HuR, the proteins, including TTP and TTP-C147R, accumulated to similar levels, as detected by anti-FLAG antibody (Fig. 3A, bottom).

LARP4 mRNA was examined by Northern blotting. Two bands were detected for human LARP4 (Fig. 3B, top and bottom), likely reflective of alternative polyadenylation, both of which could be depleted by siRNA treatment against LARP4 mRNA (data not shown). Quantification of triplicate experiments revealed that LARP4 mRNA levels were not convincingly altered by this experimental approach (Fig. 3C) (see Discussion).

TTP binds cellular LARP4 mRNA. RNA immunoprecipitation was used to test if TTP binds endogenous LARP4 mRNA. FLAG-tagged TTP- and TTP-C147R-binding mutants were overexpressed in HeLa cells and immunoprecipitated using anti-FLAG antibody. One-third of the IP was used to check protein levels by immunoblotting with anti-FLAG or with mouse IgG as a negative control (Fig. 4A). The remainder of the IP was processed for RNA isolation. After reverse transcription with oligo(dT), gene-specific PCR with primers for LARP4, LARP4B, and GAPDH was carried out to detect coimmunoprecipitated RNA. LARP4 mRNA was reproducibly coimmunoprecipitated with wild-type TTP but not with the TTP-C147R-binding mutant (Fig. 4B and data not shown). Further specificity was revealed by absence of GAPDH and LARP4B mRNA signals in the IP (Fig. 4B).

Mouse LARP4 is regulated by TNF-α in a TTP-dependent manner. As noted in the introduction, TTP has been well characterized in mouse, prompting us to examine its relationship to LARP4 using an established mouse cell system. We obtained previously characterized MEFs with TTP genetically deleted and control wild-type MEFs (10). Immunoblot analysis revealed that LARP4 levels were significantly higher in the TTP KO cells than in the WT cells, while La levels were more similar (Fig. 5A). Significantly higher levels of LARP4 in TTP KO versus WT cells were observed in many independent experiments. Next, we analyzed LARP4 mRNA levels by Northern blotting (Fig. 5B and C). In accordance with the protein levels, LARP4 mRNA levels were higher in TTP KO versus WT cells.

We examined the distribution of LARP4 mRNA on sucrose gradient-sedimented polysomes isolated from TTP KO and WT cells. The optical density at 254 nm (OD_{254}) tracing of a typical polysome fractionation of the KO cell lysate, using a highly standardized Teledyne Isco system, is shown at the top of Fig. 5D. After parallel gradient sedimentations of KO and WT lysates, total RNA was prepared from the fractions and subjected to Northern blot analysis for LARP4 mRNA (Fig. 5D). This reproducibly revealed that LARP4 mRNA was similarly distributed in TTP KO and WT cells, toward the bottom of the gradient, associated with heavy polysomes in both cell types (Fig. 5D and data not shown).

We next wanted to use these KO and WT cells to compare the abilities of their LARP4 proteins to respond to a signal pathway that involves TTP induction by TNF-α. After growth in medium

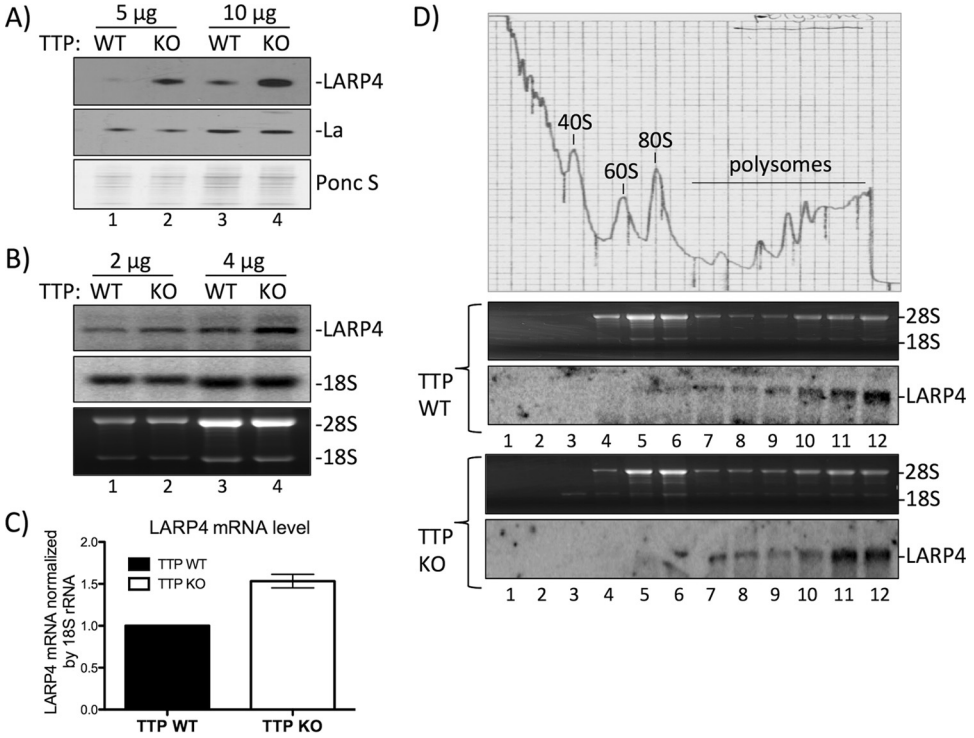


FIG 5 Mouse LARP4 levels are increased in the absence of TTP. TTP WT and TTP KO MEFs were analyzed. (A) Immunoblot analysis of LARP4 and La from 5 μg and 10 μg of total protein extracts. Ponceau S (Ponc S) staining of the membrane is also shown. (B) Northern blot analysis of LARP4 mRNA and 18S rRNA from 2 μg and 4 μg of total RNA. Ethidium bromide staining of the gel used to generate the Northern blot is also shown. (C) Quantitation using data from panel B. LARP4 mRNA signal was normalized to 18S rRNA, and this was set to 1 for wild-type cells. The error bars represent the SEM of 3 independent extracts. (D) Sucrose gradient sedimentation analysis of mRNA distribution on polysomes from TTP KO and WT MEFs. (Top) A typical OD_{254} tracing; the vertical lines indicate fractions, numbered below the gels. Total RNAs were isolated from the fractions from the WT and KO cell profiles, separated on agarose gels, stained with ethidium for 28S and 18S rRNAs, transferred to nylon membranes, and processed for LARP4 mRNA using ^{32}P-labeled probes.

lacking growth factors and cytokines for 24 h, recombinant mouse TNF-α was added, and cells were harvested for protein and RNA at multiple times up to 10 h thereafter. Immunoblots showed that TTP expression was pulsatile, as expected, becoming reproducibly detectable at 1 h, sharply rising to a peak at 2 h, and declining thereafter (Fig. 6A and data not shown). In this and similar experiments, we could observe two electrophoretic isoforms of TTP, with the lower band appearing early followed by an upper band that presumably represents a phosphorylated form (20, 22). With remarkable coincidence, the maximal decrease in LARP4 was inversely mirrored by TTP expression in the WT MEFs, with the lowest level at 2 h, increasing to the highest level at 6 h, and then stabilizing (Fig. 6A, lanes 1 to 7). In contrast, TNF-α had no such effects in the TTP KO cells (Fig. 6A, lanes 8 to 14). The same blot was subsequently processed for LARP4B and La proteins, which showed little if any response to TNF-α in the WT or KO cells (Fig. 6A). Staining with Ponceau S prior to processing showed nearly equal loading (Fig. 6A, bottom). This and similar experiments reproducibly showed that LARP4 is negatively regulated by TNF-α in a TTP-dependent manner.

Northern analysis was performed on the RNA isolated from the samples analyzed in Fig. 6A. We note that, unlike human LARP4 mRNA, mouse LARP4 mRNA appears as a single band. The LARP4 mRNA was decreased in WT TTP MEFs at 1 h relative to time zero after TNF-α addition (Fig. 6B, lanes 1 and 2), notably followed by the maximal decrease in LARP4 protein levels at 2 h (Fig. 6A, lane 3). The LARP4 mRNA then increased maximally at 4 h (Fig. 6B, lane 4), followed by the maximal increase in LARP4 protein at 6 h (Fig. 6A, lane 5). In contrast to both LARP4 and its mRNA in the TTP WT cells, both were relatively unchanged in the TTP KO cells throughout the TNF-α time course (Fig. 6A and B, lanes 8 to 14). For quantification, LARP4 mRNA levels were normalized to RppH1 RNA (nonpolyadenylated RNA polymerase III transcript), and the data from duplicate biological experiments, including those shown in Fig. 6A and B, were plotted in Fig. 6C. These data show a fairly quick, tight, and robust negative response of LARP4 levels to TNF-α–TTP in the WT MEFs but no significant changes in the KO MEFs.

The time course response of LARP4 mRNA and LARP4 to TNF-α–TTP would be explained most simply by a short half-life of LARP4 protein relative to the time intervals of the experiment. By this model, a TTP-mediated decrease in mRNA levels alone would be quickly followed by a decrease in LARP4 protein levels. Indeed, a relatively short half-life of LARP4 protein was confirmed by a pulse-chase ^{35}S label and decay experiment (Fig. 6D). For this, growing cells were pulsed with ^{35}S labeling mix for 30 min and then incubated with unlabeled cysteine, methionine, and serum for the chase period, and extracts were prepared at various times thereafter. Each extract was subjected to IPs for LARP4, La, and LARP4B and analyzed by gel electrophoresis and autoradiography. Initial experiments revealed that LARP4 was barely detectable after only 30 min (data not shown). Examination at earlier

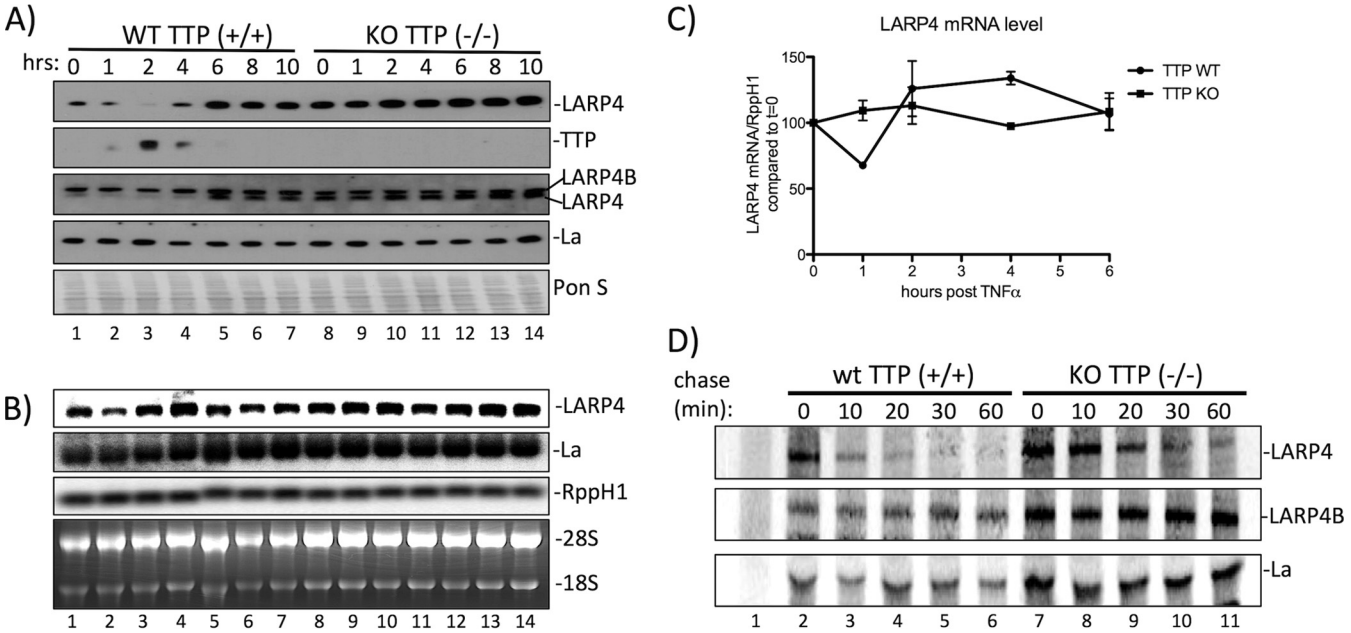


FIG 6 LARP4 is regulated by TNF-α, dependent on TTP in MEFs. TTP WT (+/+) and KO (−/−) MEFs were stimulated with recombinant TNF-α and then harvested for protein and RNA at the times indicated above the lanes. (A) Immunoblot. The top 4 panels represent the blot after staining with Ponceau S (Pon S) (bottom), washing, and processing with antibodies against LARP4, LARP4B, TTP, and mouse La. (B) Northern blot. ^{32}P-labeled probes against mouse LARP4, mouse La, and mouse RppH1 RNA were used. At the bottom is shown an ethidium bromide-stained gel used to generate the blots above it. (C) Quantitation using data from panel B. The LARP4 mRNA signal was normalized to RppH1 RNA, and the amount at time zero was set to 100%. The error bars represent the standard deviations (SD) of 2 independent experiments at all time points. (D) Pulse-chase ^{35}S-labeling experiment to monitor the time course of LARP4 decay in TTP WT and TTP KO cells. After a short pulse incubation with ^{35}S protein-labeling mix, cells were washed and chased for the times indicated above the lanes, and extracts were made. Parallel IPs using antibodies to the proteins indicated on the right were performed, and the products were analyzed by SDS-PAGE and autoradiography.

times revealed that newly synthesized LARP4 protein exhibited similarly short half-lives in both the TTP WT and KO cells, with most of the protein gone by 30 min (Fig. 6D). In contrast, LARP4B was stable, as was La, the latter consistent with a half-life turnover for human La protein of 12 h (37). We also observed about 50% reduction in LARP4 levels after ~30 min of treatment of TTP KO and WT cells with cycloheximide followed by immunoblotting, while La and actin levels were unchanged (data not shown). All of the time course data in Fig. 6 are consistent with a pathway in which TNF-α induces TTP, which transiently and robustly downregulates LARP4 mRNA, followed by a decrease in LARP4 protein levels.

DISCUSSION

The major conclusion that can be drawn from this study is that the LARP4 mRNA 3′ UTR has an ARE with sequence motifs corresponding to high-affinity binding sites for the ARE-binding protein, TTP, and is regulated by TTP. The LARP4 mRNA ARE contains a conserved UAUUUAU tract and related sequences that represent known sequence-specific motifs to which TTP binds. Inserting a 317-nt fragment representing the ARE into the 3′ UTR of a widely used reporter of mRNA stability significantly decreased the half-life of the reporter mRNA. Point mutations that changed UU (underlined) to GG in two of the UAUUUAU tracts in the ARE significantly reversed the instability. We showed that the LARP4 mRNA was specifically coimmunoprecipitated with TTP from HeLa cells. Overexpression of wild-type TTP, but not its RNA binding mutant or other ARE-binding proteins, decreased LARP4 levels in HeLa cells.

The pulsatile nature of TTP observed in the MEFs and consequent quick decrease and recoil of LARP4 may be accounted for by the phosphorylation- and dephosphorylation-mediated regulation of TTP (19–22; reviewed in reference 15). We found no decrease in LARP4 mRNA to match the decreased LARP4 protein levels 48 h after TTP transfection of HeLa cells. While other possibilities exist, we suspect that this approach is not optimal for observing a tight relationship between TTP and LARP4, as was revealed by TNF-α-induced TTP, in which a sharp (pulsatile) increase in TTP was followed by a decrease of LARP4 mRNA and a subsequent decrease in LARP4 protein. We are left to presume that the transfected TTP decreased LARP4 mRNA, which led to decreased LARP4 protein levels, and that while the mRNA levels recovered by the end of the experiment, the protein levels remained low. Although no TNF-α treatment was used in the HeLa transfection system, we note that we have not examined LARP4 levels beyond 10 hours after TNF-α treatment of the MEFs. Development of a more appropriate human cell system may address this discrepancy in the future.

For LARP4B, there is no canonical minimal TTP binding site (UAUUUAU) present in its 3′UTR (Fig. 1A and data not shown), while human LARP4 has 2 within about 150 nt, one of which is part of a sequence that corresponds to the highest-affinity binding site known for TTP, UUAUUUAUU (Fig. 1D, red rectangle) (reviewed in reference 15). This difference presumably explains why TTP and TNF-α did not have the same effect on LARP4B as on LARP4. However, the LARP4B 3′ UTR does contain multiple AUUUA pentamers, in addition to longer AU-rich motifs

(Fig. 1A), leaving open the likely possibility that its stability is regulated by another mechanism(s).

The destabilizing effect of the LARP4 317-nt ARE is sequence specific, since it had no effect on reporter mRNA stability in the antisense direction. Because the 317-nt fragment rendered the mRNA reporter more unstable than the 184-nt fragment, there is likely an additional destabilizing element in the longer version. However, this appears not to be due to the AUUUA pentamer downstream of the 184-nt fragment. This was not surprising, because, lacking U residues immediately flanking it, the AUUUA pentamer in its context would not be expected to be a good binding substrate for TTP (reviewed in reference 15).

By using previously characterized TTP KO MEFs, we found that LARP4 levels were significantly higher than in the control WT MEFs, consistent with the corresponding LARP4 mRNA levels in these cells. We then used an established system of TTP induction, treatment of the cells with recombinant TNF-α. When TTP expression was induced this way in the WT MEFs, LARP4 protein levels decreased with a time course coincident with TTP induction while there was no change in LARP4 in the absence of TTP in the TTP KO MEFs. A TTP-induced decrease in LARP4 mRNA levels preceded and could account for the decrease in LARP4 protein. As TTP levels then receded, LARP4 mRNA and protein returned to higher levels. We conclude that in this cytokine signaling cell culture system, TNF-α regulates LARP4 levels in a TTP-dependent manner.

It is remarkable that LARP4 was so strongly and quickly downregulated by TTP in the MEFs, which should be possible by altering mRNA levels only if the LARP4 half-life is short. This was confirmed for LARP4 by ^{35}S pulse-chase experiments, while two other proteins examined in the same extracts exhibited significantly longer half-lives. While this suggests that LARP4 levels can be quickly managed by controlling its mRNA levels, it also raises interesting questions. What are the determinants of LARP4 fast turnover, and do they vary under different conditions? Our unpublished data indicate that differences in LARP4 levels, which vary considerably in different cell lines, are not always linearly concordant with mRNA levels in the same cells. These data suggest that regulation of LARP4 may be multifactorial and complex.

The fact that LARP4 expression is regulated by TTP, a master cytokine regulator, may provide an important clue as to what the function of LARP4 may be. We should therefore expect that LARP4 is somehow involved in the inflammatory response controlled by TNF-α. Previous evidence has shown that LARP4 binds to the 40S ribosome-associated RACK1, as well as with PABPC1 on translating ribosomes (2). Evidence that LARP4 can promote the stability of certain mRNAs is consistent with its interaction with the MLLE domain of PABPC1 via its conserved PAM2 motif (2). Control of inflammatory gene expression occurs to a significant extent at the posttranscriptional level (reviewed in reference 38). The cumulative data suggest that LARP4 might be involved in mRNA metabolism during inflammatory response involving TNF-α and TTP.

We believe it is noteworthy that this study shows that LARP4 is regulated differently than LARP4B, especially with regard to TNF-α and TTP. This is interesting, because these proteins have strikingly similar architectures, both interact with PABPC1 and RACK1, and their activities appear to be directed at linking mRNA stability and translation. However, as noted previously, they likely have different RNA binding specificities. The data reported here suggest that they also function in different ways, at least in the context of TNF-α- and TTP-related pathways.

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