Regulation of tristetraprolin during differentiation of 3T3-L1 preadipocytes

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Tristetraprolin is a zinc-finger-containing RNA-binding protein. Tristetraprolin binds to AU-rich elements of target mRNAs such as proto-oncogenes, cytokines and growth factors, and then induces mRNA rapid degradation. It was observed as an immediate-early gene that was induced in response to several kinds of stimulus, such as insulin and other growth factors and stimulators of innate immunity such as lipopolysaccharides. We observed that tristetraprolin was briefly expressed during a 1–8 h period after induction of differentiation in 3T3-L1 preadipocytes. Detailed analysis showed that tristetraprolin mRNA expression was stimulated by fetal bovine serum and differentiation inducers, and was followed by rapid degradation. The 3′UTR of tristetraprolin mRNAs contain adenine- and uridine-rich elements. Biochemical analyses using RNA pull-down, RNA immunoprecipitation and gel shift experiments demonstrated that adenine- and uridine-rich element-binding proteins, HuR and tristetraprolin itself, were associated with tristetraprolin adenine- and uridine-rich elements. Functional characterization confirmed that tristetraprolin negatively regulated its own expression. Thus, our results indicated that the tight autoregulation of tristetraprolin expression correlated with its critical functional role in 3T3-L1 differentiation.

Post-transcriptional processes such as RNA splicing, and mRNA export, turnover and translation, are important mechanisms of gene regulation in mammalian cells. The mRNAs of many regulatory proteins, including proto-oncoproteins, cytokines and growth factors, bear adenine- and uridine-rich elements (AU-rich elements, or AREs) in their 3′UTR in order to govern their half-life and translation rate [1–5]. AREs vary in size, generally contain one or more copies of the pentameric sequence AUUUA, and have been divided into classes I, II, and III, according to their sequence characteristics [6]. To date, many ARE-binding proteins have been shown to be involved in regulating mRNA turnover and translation *in vivo*, such as the ARE- and poly(U)-binding and degradation factor AUF1/hnRNP D [7], tristetraprolin (TTP)

[8], HuR [9], and T-cell-restricted intracellular antigen 1 [10]. T-cell-restricted intracellular antigen 1 has been observed to act as a translational repressor in response to environmental stress agents [11]. HuR is a ubiquitous member of the embryonic lethal abnormal vision family of RNA-binding proteins [12]. Overexpression of HuR in transiently transfected mammalian cells can stabilize short-lived ARE-containing mRNAs [13] or can induce the translation silencing of specific mRNA targets [14,15]. In contrast, TTP is important for the destabilization of tumor necrosis factor-α (TNF-α) and granulocyte–macrophage colony-stimulating factor mRNAs, as shown in knockout mice [8,16] and in tissue culture by ectopic-overexpression studies [17]. TTP, also named G0S24, Nup475, TIS11, or Zfp36, is an RNA-binding protein containing two CCCH zinc fingers and three tetraproline motifs. It binds AREs of target mRNAs and induces deadenylation [17,18], or directs them to the exosome [19–21], or associates with the RNA-induced silencing complex (RISC)–microRNA complexes [22] for rapid degradation of target mRNAs. It was observed as an immediate-early gene that was induced in response to several kinds of stimulus, such as growth factors and mitogens [23,24]. The serum-responsive elements have been identified in the 5′-regulatory region of the TTP gene [25,26]. In macrophages, the expression of TTP and TNF-α can both be activated by lipopolysaccharide, and the feedback inhibition of TTP, in turn, downregulates the production of TNF-α [27]. In addition to its involvement in post-transcriptional regulation in the immune system, TTP was found to regulate cellular metabolism during iron deficiency [28] and to respond to muscle damage [29].

White adipose tissue is mainly composed of adipocytes, which are cells that store energy in the form of triglycerides in times of nutritional adequacy and release free fatty acids during nutritional deprivation. The established preadipocyte cell line 3T3-L1 has been used to examine the process of adipogenesis *in vitro* [30–32]. When treated with an empirically derived pro-differentiative regimen that includes cAMP, insulin and glucocorticoids in the presence of fetal bovine serum, the cells undergo differentiation to mature fat cells over a 4–6 day period. Numerous investigators have demonstrated that, during adipocyte differentiation, many genes are regulated in a differentiation-dependent manner. The first step in the process of adipogenesis is the re-entry of growth-arrested preadipocytes into the cell cycle and the completion of several rounds of cell division known as clonal expansion [33–35]. Several transcriptional factors are expressed coordinately to cause terminal differentiation. C/EBPβ, C/EBPδ and ADD-1/ SREBP-1 are active early during the differentiation process [36,37]. These earlier events are followed by the accumulation of PPARγ and C/EBPα, which causes cell division to stop [38,39]. This triggers the expression of adipocyte-specific genes, giving rise to the adipocyte phenotype and increased delivery of energy to the cells.

Recently, C/EBPβ was reported to be a ligand for HuR, and the depletion of HuR by small interfering RNA (siRNA) attenuated the differentiation process in 3T3-L1 cells [40]. The other ARE-binding protein, TTP, was briefly induced in 3T3-L1 after adipogenic induction [24]. In the current study, we showed that TTP could be induced during 3T3-L1 differentiation and that its expression was controlled by negative autoregulation.

Results

TTP expression during 3T3-L1 differentiation

To study post-transcriptional regulation during 3T3-L1 differentiation, 3T3-L1 preadipocytes were treated with a cocktail of fetal bovine serum, 1-methyl-3-isobutylmethylxanthine (MIX), dexamethasone and insulin (MDI) to induce their differentiation. Light microscopy observations showed that massive amounts of triglyceride accumulated in the cytoplasm, indicating that the 3T3-L1 cells differentiated normally (data not shown). RNAs were isolated for semiquantitative RT-PCR analysis. After hormone stimulation, the expression level of TTP mRNA did not change significantly from day 1 to day 9 when compared to the levels of the internal control, actin mRNA (Fig. 1A). Because the TTP gene was an immediate-early gene, we investigated whether it could be rapidly induced in 3T3-L1 cells. Both semiquantitative PCR and real-time PCR showed that its mRNAs reached their highest level at 1 h and then decreased dramatically (Fig. 1B). TTP protein was expressed only in the cytoplasm, reaching its highest levels 4 h after induction of differentiation, and then gradually returning to its original level (Fig. 1C). The multiple bands of TTP proteins on SDS/PAGE were caused by protein phosphorylation (data not shown). Another ARE-binding protein, HuR, was detected in both the nucleus and cytosolic fractions, and its expression level was consistent in the period of early differentiation.

To further verify which components in the hormone cocktail directly trigger the expression of TTP, we monitored the effect of each component. Quantitative PCR demonstrated that the TTP mRNA expression
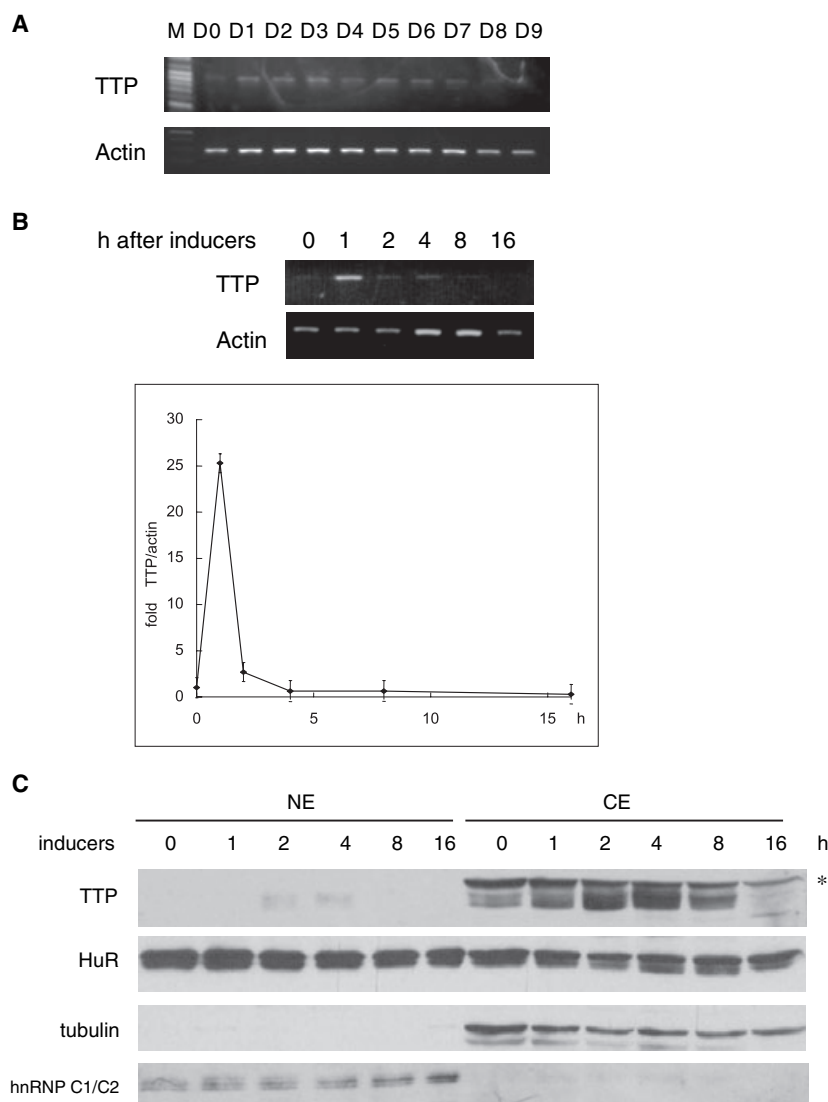
A

M D0 D1 D2 D3 D4 D5 D6 D7 D8 D9

TTP

Actin

B

h after inducers   0   1   2   4   8   16

TTP

Actin

Fig. 1. Expression of TTP mRNA and protein during differentiation of 3T3-L1 preadipocytes. (A) Semiquantitative RT-PCR of TTP. Two-day postconfluent 3T3-L1 preadipocytes were induced to differentiate with MDI and 10% fetal bovine serum. RNAs were prepared at day 0 to day 9 at 1-day intervals, and RT-PCR was performed with TTP-specific and actin-specific (internal control) primers. (B) 3T3-L1 cells were induced to differentiate for 0, 1, 2, 4, 8 and 16 h, and RNAs were isolated for semiquantitative RT-PCR (upper) and real-time PCR (lower). (C) Cytosolic and nuclear proteins were isolated for western blotting using TTP- and HuR-specific antibodies; tubulin- and hnRNP C1/C1 antibodies served as markers of cytosolic and nuclear fractions, respectively. The star sign indicates the nonspecific band recognized by anti-TTP serum.

C

NE   CE

inducers   0   1   2   4   8   16   0   1   2   4   8   16   h

TTP   *

HuR

tubulin

hnRNP C1/C2

level after 1 h of treatment with either one of dexamethasone, MIX or insulin in the presence of fetal bovine serum was lower than after treatment with all three combined (Fig. 2A). Without fetal bovine serum, the MDI mixture induced TTP mRNA expression 7.5-fold, but the TTP protein levels were very low under these conditions (Fig. 2B). Maximum induction of TTP mRNA and protein required the presence of MDI and fetal bovine serum. This result indicates that fetal bovine serum and each of the MDI components contribute to TTP mRNA expression.

Rapid degradation of TTP mRNAs

TTP expression in response to 3T3-L1 differentiation rapidly increased and then rapidly decreased. We were interested in the post-transcriptional regulation of TTP

mRNAs. The half-life of TTP mRNAs was determined after hormone induction for 0.5, 1 and 2 h (Fig. 3). Intriguingly, the results showed that the mRNA half-life was more than 30 min at 30 min of induction and that it was less than 15 min at 1 h and 2 h of induction. TTP mRNA expression thus appeared to be short-lived. Thus, TTP mRNA expression in 3T3-L1 differentiation was controlled by rapid RNA degradation.

Negative autoregulation of TTP expression in 3T3-L1 differentiation

AREs could be found in the 3′UTR of TTP mRNAs [41,42] (Fig. 4A). To investigate the mechanism of its rapid degradation during 3T3-L1 differentiation, we analyzed proteins that were possibly associated with TTP AREs. The biotinylated TTP AREs were incubated with
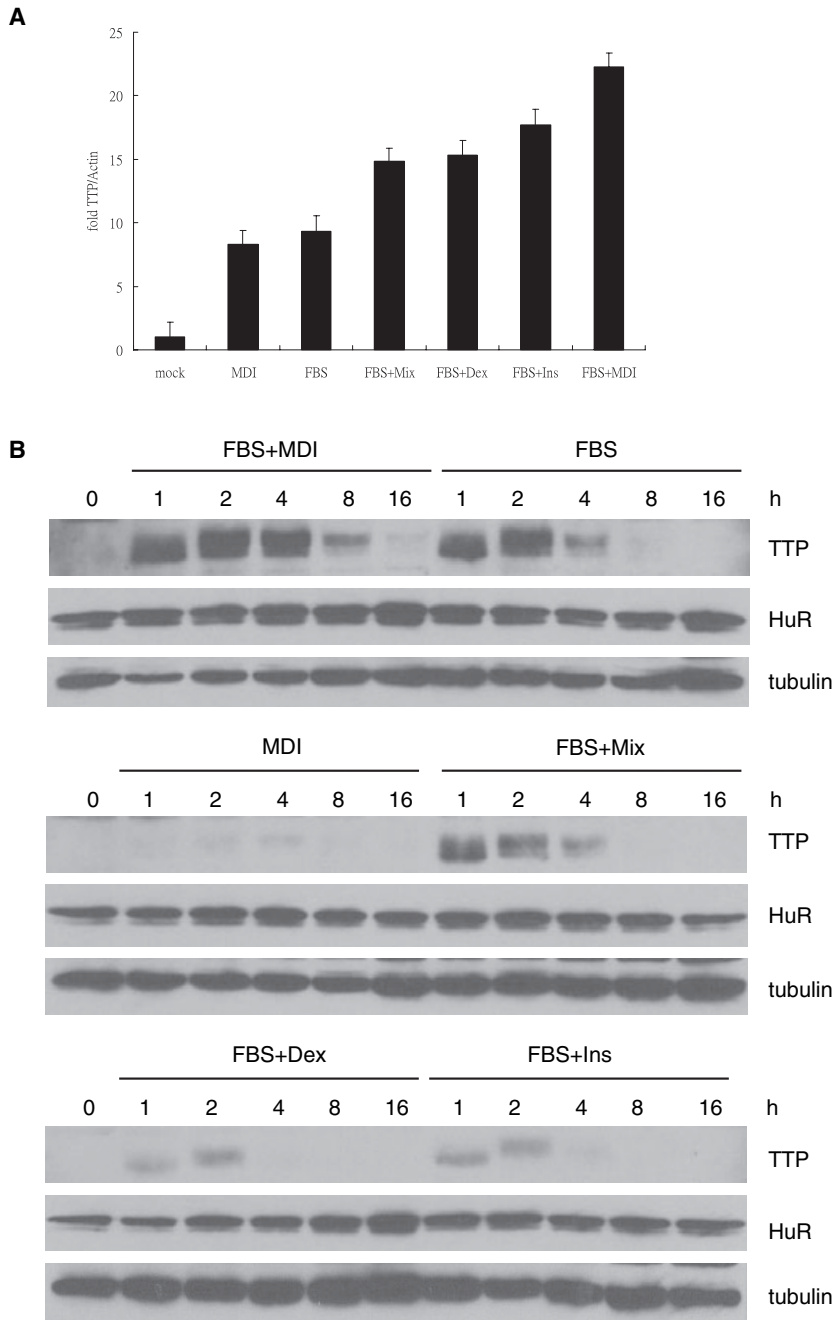
A

B

Fig. 2. mRNA and protein expression of TTP in 3T3-L1 cells. (A) Two-day postconfluent 3T3-L1 preadipocytes were treated with MDI, fetal bovine serum, fetal bovine serum + MIX, fetal bovine serum + dexamethasone, fetal bovine serum + insulin or fetal bovine serum + MDI for 1 h, and RNAs were isolated for quantitative real-time RT-PCR using TTP-specific and actin-specific primers. (B) Treatments were similar to those in (A) for 0, 1, 2, 4, 8 and 16 h, and cytosolic extracts were isolated for western blotting using TTP-, HuR- and tubulin specific antibodies.

cytoplasmic extracts from untreated 3T3-L1 or hormone-stimulated 3T3-L1 cells to precipitate the ARE-interacting proteins. After identification by western blotting using antibodies against HuR, TTP and hnRNP A1, the known ARE-binding proteins, it was observed that TTP and HuR could be precipitated by TTP AREs (Fig. 4B). HuR was consistently bound to TTP AREs either predifferentiation or postdifferentiation, whereas TTP was expressed and bound to TTP AREs only after stimulation. The other ARE-binding

protein, hnRNP A1, was not precipitated by TTP AREs in our experiment (data not shown). Figure 4C shows that antibodies to HuR and TTP could also precipitate TTP mRNAs. Furthermore, in vitro transcribed TTP AREs were incubated with recombinant glutathione-S-transferase (GST)–HuR or GST–TTP for gel shift assay (Fig. 4D). The lowest dose of HuR totally occupied the ARE probe. When increasing amounts of GST–TTP were incubated with ARE probes, the RNA–protein complexes gradually became larger
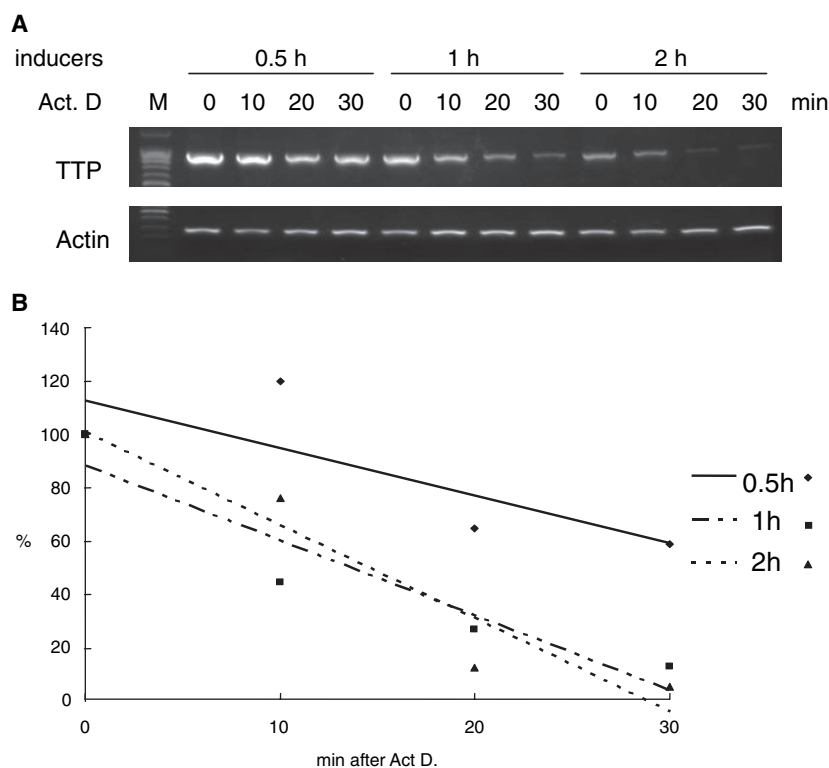
A

B

Fig. 3. Half-life determination of TTP mRNAs. Two-day postconfluent 3T3-L1 preadipocytes were treated with MDI and 10% fetal bovine serum for 0.5, 1 and 2 h. Transcription was then stopped by adding 10 $\mu g \cdot mL^{-1}$ of actinomycin D (Act. D) for 10, 20 and 30 min. The total RNAs were isolated and reverse-transcribed to produce cDNAs for semiquantitative PCR and quantitative PCR. (A) Semiquantitative RT-PCR with specific primers for TTP and actin. (B) Quantitative result of real-time PCR. Levels of TTP RNAs were normalized to those of actin in each sample, and then were plotted as a percentage of the initial value against Act. D incubating time.

(Fig. 4D, left, lanes 5–7). The addition of increasing levels of TTP in the presence of a constant HuR concentration led to the formation of larger complexes in the gel shift assay (Fig. 4D, left, lanes 8–10). This observation suggests that TTP and HuR could bind to TTP AREs simultaneously. The TTP AREs contain three potential HuR- or TTP-binding sites (Fig. 4A, underlined sequence). The RNA electrophoretic mobility shift assay (REMSA) showed that the mutated TTP AREs could not bind to recombinant TTP or HuR (Fig. 4D, right panel).

To further explore the functional effects of HuR and TTP binding on TTP AREs, we constructed a luciferase reporter containing TTP AREs for reporter assays. Figure 5A,B shows the results when 293T cells were co-transfected with increasing amounts of TTP or HuR expression plasmids, and a reporter encoding luciferase was fused to TTP AREs. When compared to the control luciferase reporter, HuR had almost no effect on the TTP ARE-containing luciferase activity. In contrast, TTP diminished the ARE-containing reporter activity. In the presence of HuR, TTP also had suppressive activity (Fig. 5C). Although the activities of both the control reporter and the TTP ARE-containing reporter in the absence of expression plasmid were represented as 100, the control reporter actually had 10-fold higher activity than the TTP ARE-containing reporter. This result implied that TTP could downregulate its own expres-

sion through AREs to limit its production during 3T3-L1 differentiation. Analysis of RNA using northern blot gave similar results to those shown in Fig. 5A, in that the expression of TTP protein decreased the mRNA level of the TTP ARE-containing reporter (Fig. 5D). Taken together, these results suggest that the TTP AREs may modulate gene expression post-transcriptionally, and that the binding of TTP protein destabilized the mRNA, resulting in downregulation of reporter activity.
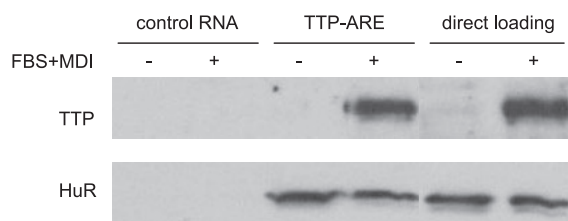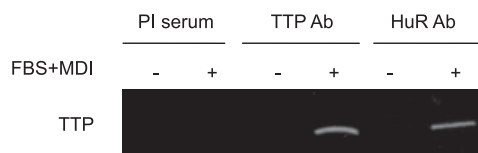
Discussion

Our results showed that the RNA-binding protein TTP could be induced by fetal bovine serum and MDI during 3T3-L1 differentiation. TTP mRNA was briefly expressed because of its short half-life. The AREs in the 3′UTR of TTP mRNAs could regulate its gene expression post-transcriptionally. Moreover, the physical interactions in the RNA pull-down assay, RNA immunoprecipitation assay and REMSA showed that TTP and HuR protein associated with wild-type TTP AREs but not with mutated AREs. The ectopic expression of TTP decreased the ARE-mediated luciferase mRNA level and the reporter activity. These results indicate that TTP may bind to its AREs to destabilize the mRNA and downregulate its own protein expression during 3T3-L1 early differentiation.

A

ARE of TTP mRNA

1529UUGCCAAAUCCCUUCUCCCCCAACCAGUGGGCCCUU<u>UAUUUAU</u>GACGACU<u>UUAUUUAUU</u>GU

AUUAAGAUUUUAUAG<u>UAUUUAU</u>AUAUAUUGGGUCGUCUACUCCGUUUUUCUUUUUGUAAUGUUA

AAACUGAUACUGUAUUAAGUAUAUGCUAUAAUAUAUUAAUAUAUUGCUACCGUACAAGUCUA1715

B

C

D

Fig. 4. Interaction between TTP and HuR proteins and TTP AREs. (A) Nucleotide sequences of TTP AREs. The potential AREs are underlined. (B) RNA pull-down assay. The biotinylated TTP ARE or control 18S RNA was incubated with cytoplasmic extracts from 3T3-L1 cells treated (+) or not treated (–) with MDI and fetal bovine serum. The RNA–protein complexes were precipitated with streptavidin Sepharose and subjected to SDS/PAGE for western blotting. TTP- and HuR-specific antibodies were used. The amount of protein in the lanes with direct loading was 1/10 of the protein used in the pull-down assay. (C) RNA immunoprecipitation analysis. The cytoplasmic extracts from MDI and fetal bovine serum-treated (+) or nontreated (–) 3T3-L1 cells were immunoprecipitated using preimmune serum, or anti-TTP or anti-HuR sera After extensive washes, the protein-associated RNAs were extracted for RT-PCR with TTP specific primers. (D) REMSA. The ribo-probes were generated as described in Experimental procedures. Left panel: 1 pmol of radiolabeled TTP ARE (nucleotides 1529–1715) was incubated with increasing amounts of GST–HuR (lanes 2–4, 1, 2 and 4 pmol) or GST–TTP (lanes 5–7), or 2 pmol of GST–HuR and increasing amounts of GST–TTP (lanes 8–10). Right panel: radio-labeled mutated TTP ARE was incubated with GST–HuR (lane 2) or GST–TTP (lane 3). Controls were wild-type TTP ARE (nucleotides1557–1621) incubated with GST (lane 5) or GST–HuR (lane 6).

In addition to TTP itself, HuR was seen to bind to TTP AREs (Fig. 5). HuR is predominantly localized in the nucleus and shuttles between the nucleus and cytoplasm by means of a nuclear-cytoplasmic shuttling sequence in HuR (HNS) [43]. It could serve as an adaptor for mRNA export through the CRM1 route or through transportin-2 [44,45]. Thus, the functional role of HuR in TTP expression may be to enhance TTP mRNA export from the nucleus to the cytoplasm. Some extracellular stimuli have been reported that could cause the redistribution of HuR between the nucleus and cytoplasm [46–48]. Gantt *et al.* reported that, within 30 min of initiation of 3T3-L1 differentiation, the HuR cytosolic content increased by 30% [40]. In our experiments, HuR was consistently detected in both the nucleus and cytoplasm of 3T3-L1 cells, with no significant alteration in the distribution after

exposure to the differentiation stimulus. The binding assay showed that HuR and TTP could occupy TTP AREs concurrently. The functional reporter assay also showed that the ectopic expression of HuR did not have a prominent effect on TTP ARE-mediated luciferase mRNA expression and protein activity. As shown in Fig. 5A,C, either in the absence or presence of HuR expression vector, the low dosage of TTP could result in a decrease of reporter gene expression. These results may be explained by the existence of high amounts of endogenous HuR.

Our results showed that small amounts of TTP could mediate a significant decrease in TTP ARE-dependent luciferase expression, and the higher doses of TTP did not lead to a greater inhibitory effect. In previous reports, the higher expression levels of TTP appeared to cause a slight increase in TNF-α ARE-mediated gene
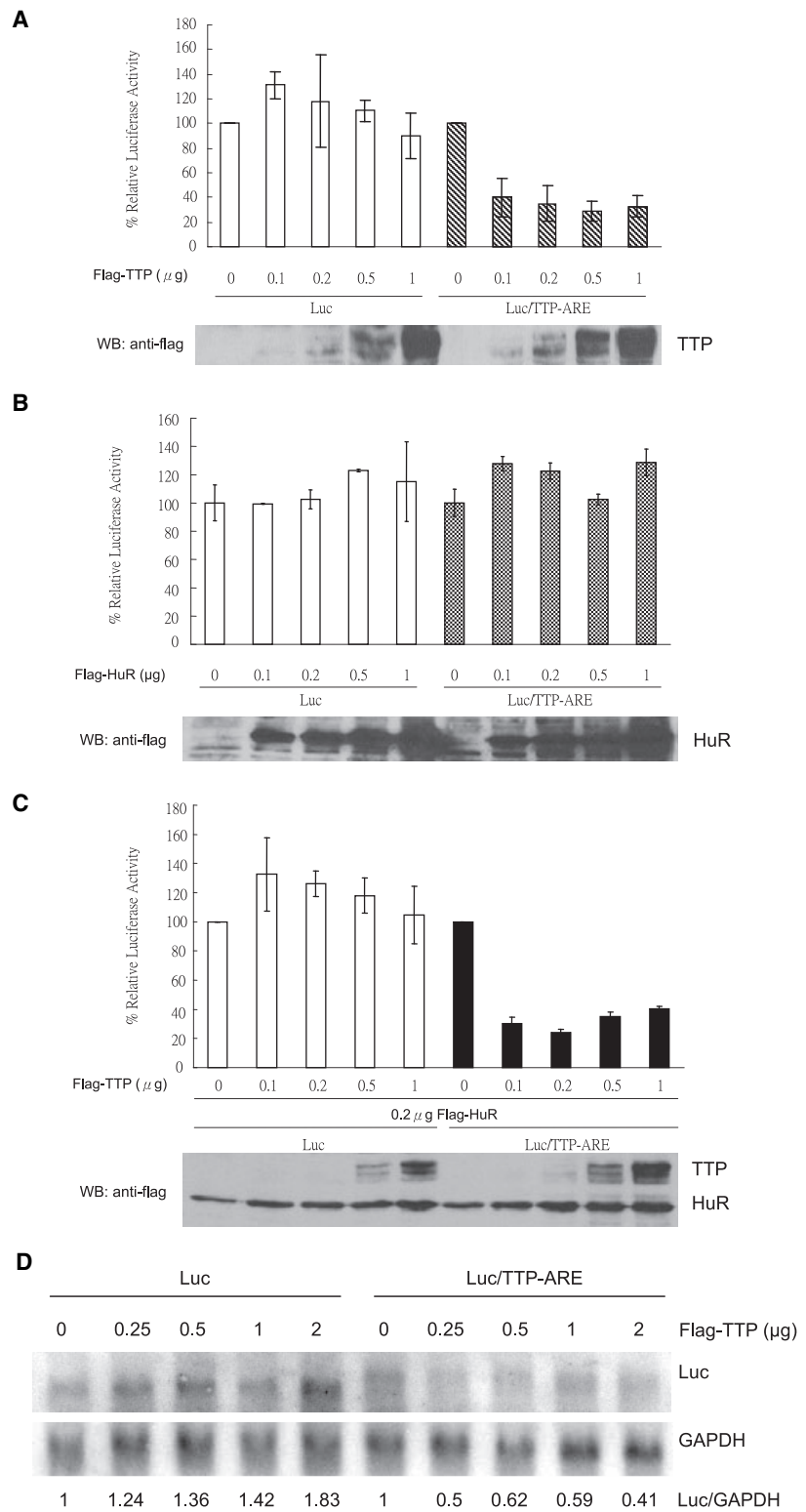
Fig. 5. Functional characterization of TTP- and HuR-mediated ARE-containing TTP mRNA expression. 293T cells were cotransfected with 0, 0.1, 0.2, 0.5 and 1 μg of pCMV-Flag-TTP (A) or pCMV-Flag-HuR (B) and in the presence of 0.2 μg of pCMV-Flag-HuR (C) together with 0.5 μg of reporter pLuc-TTP-ARE or control vector pLuc and 1 μg of pSV-β-galactosidase. The results are presented with the percentage of luciferase activity normalized with β-galactosidase activity. The activity of the reporter alone is shown as 100%. The lower panel shows the amounts of transfected Flag-TTP and Flag-HuR expressed in each experiment. (D) Northern blot analysis of experiment (A) targeting luciferase mRNA. glyceraldehyde-3-phosphate dehydrogenase mRNAs served as internal control.

expression [17,49]. Our protein-binding assay provided a possible explanation: TTP at a high dose could form a large protein complex with TTP ARE, which could block TTP interactions with other proteins that are essential for facilitating post-transcriptional regulation. The other explanation is that TTP was highly phosphor-

ylated during the high-level protein expression and this may cause alteration of its function. Recent reports demonstrated that TTP phosphorylation by the p38 MAPK pathway could lead to increased TTP protein stability but reduced ARE affinity [50,51]. Our study also showed that TTP could differentially regulate cytokine mRNA expression by phosphorylation [52]. The effect of the p38 MAPK pathway on TTP negative autoregulation will be investigated further.

TTP mRNA expression was activated by fetal bovine serum and the components of MDI. Insulin and serum have been reported to activate TTP expression transcriptionally [23,25]. Some DNA elements that responded to MIX and dexamethasone were also found in the TTP promoter. Interestingly, without fetal bovine serum, TTP protein expression appeared to be blocked. The possible mechanisms are that fetal bovine serum triggers TTP protein synthesis or enhances TTP protein stability. During adipogenesis, replacement of fetal bovine serum with calf serum markedly retarded and decreased the extent of differentiation (data not shown) [53]. Thus, fetal bovine serum is required for the rapid and complete acquisition of adipocyte characteristics, and this might be due to the requirement for fetal bovine serum-mediated TTP expression.

Extracellular stimuli regulate a spectrum of cellular events, such as cell growth, differentiation and death, by altering the gene expression profile. The resulting induction of immediate-early genes then triggers subsequent expression cascades. TTP is induced as an immediate-early gene in 3T3-L1 preadipocytes, and little is known about its biological function in adipogenesis. To demonstrate the implication of endogenous TTP in adipogenesis, we knocked down its expression in 3T3-L1 preadipocytes using siRNA, and observed a 40% reduction of TTP expression, associated with partial inhibition of adipogenesis (Fig. 1). According to the timing of TTP expression during adipogenesis, we predict that TTP may control the gene expression profile that is involved in cell cycle progression in order to regulate mitotic clonal expansion. Retinoblastoma protein and transcription factor E2F have been reported to be involved in this cell cycle progression [54]. Many cell cycle-associated proteins, such as cyclin-dependent kinases (CDKs) and their inhibitors, p18, p21 and p27, also play crucial roles during cell cycle progression [39,55], and some of these cell cycle-associated proteins are encoded by ARE-containing mRNAs. Compared to the plentiful knowledge of transcriptional regulation accumulated through the study of differentiation of 3T3-L1 preadipocytes, our understanding of post-transcriptional control during adipogenesis is very poor. The mRNA targets of TTP during 3T3-L1 differenti-

ation will be investigated to improve our understanding of the onset of adipogenesis through regulation of some ARE-containing transcripts.

Experimental procedures

Cell culture

3T3-L1 cells were grown in DMEM (Gibco-BRL, Grand Island, NY, USA) containing 1.5 g·L^{-1} NaHCO$_3$ and supplemented with 10% bovine serum (Gibco-BRL), 100 U·mL^{-1} penicillin, and 100 mg·mL^{-1} streptomycin (Gibco-BRL) in a 5% CO$_2$ humidified atmosphere (37 °C). Two-day postconfluent cells (day 0) were stimulated to differentiate by changing to fresh medium containing 10% fetal bovine serum (Characterized; Hyclone Laboratories, Logan, UT, USA) and addition of hormone cocktail [5 μM dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 1.7 μM insulin (bovine; Sigma-Aldrich), and 0.5 mM MIX (Sigma-Aldrich)].

Plasmid constructs and protein purification

The cDNAs of HuR and TTP were PCR synthesized by using primers 5′-ATGTCTAATGGTTATGAAGAC-3′ and 5′-ATGAGCGAGTTATTTGTGGG-3′ for HuR, primers 5′-CTCAGAGACAGAGATACGATTG-3′ and 5′-ATG GATCTCGCCATCTAC-3′ for TTP, and the 2 h LPS-treated RAW264.7 cDNA as templates. The PCR fragments were ligated into pGEM-Teazy vector (Promega, Madison, WI, USA). After DNA sequence confirmation, the *Eco*RI fragment was further cloned into both bacterial expression vector pGEX (Amersham Pharmacia, Uppsala, Sweden) and mammalian cell expression vector pCMV-Tag2 (Stratagene, La Jolla, CA, USA). The 3′-ARE of TTP (from cDNA nucleotides 1529–1715) was PCR cloned by using primers 5′-TTGCCAAATCCCTTCTC-3′ and 5′-TAGACT TGTACGGTAGC-3′. The PCR fragment was cloned into pGEM-Teazy vector (Promega) to prepare the riboprobe. For the heterologous 3′UTR assay, this ARE fragment was inserted into the 3′-end of the cytomegalovirus (CMV)-driven luciferase gene (Stratagene). The GST fusion proteins were induced and purified followed the manufacture's procedure (Amersham Pharmacia).

RNA isolation and RT-PCR

Total RNAs were extracted from the cell cultures by using Blue extract reagent (LTK, Inc., Taoyuan, Taiwan), following the procedures recommended by the manufacturer. Five micrograms of total RNAs extracted from 3T3-L1 cells treated with differentiation inducers for different time intervals was reverse-transcribed to produce cDNA using reverse transcriptase and oligo dT (Promega) as a primer. The specific

cDNA was amplified using 5% of the reverse transcription reaction mixture in 20 μL containing 10 pmol of forward primer, 10 pmol of reverse primer, and lypholized *Taq* DNA polymerase, buffer and dNTPs (LTK, Inc.). The sequences of the primers used for TTP and actin were: 5′-CTCAGAGACAGAGATACGATTG-3′ and 5′-ATGG ATCTCGCCATCTAC-3′ for TTP; and 5′-TCCTTCCTG GGCATGGAGTC-3′ and 5′-ACTCATCATACTCCTGCT TG-3′ for actin. The expected size of the PCR product was 957 bp for TTP and 300 bp for actin. The semiquantitative PCR was performed in a Robocycler gradient 96 PCR thermal machine (Stratagene), using the following conditions: 94 °C (3 min) for one cycle, 94 °C (40 s), 55 °C (40 s), 72 °C (depending on the product length, 1 min per 1 kb) for 20–25 cycles, and a final incubation at 72 °C for 3 min. The PCR products were separated in agarose gel and quantitated by UVP (Upland, CA, USA) LABWORK 4.5 software.

Real-time PCR

Real-time PCR was performed with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μL. Expression of TTP was analyzed using FastStart TaqMan Probe Master (Rox) (Roche Molecular Biochemicals, Mannheim, Germany) containing 50 ng of cDNA, 100 nM probe (Universal Probelibrary probe no. 58, 5′-CTCCATCC-3′, Roche) and 200 nM both forward and reverse primers (5′-GGAT CTCTCTGCCATCTACGA-3′ and 5′-CAGTCAGGCGA GAGGTGAC-3′ respectively). Expression of actin was analyzed using SYBR Green PCR Master Mix (Applied Biosystems) containing 50 ng of cDNA and 160 nM each primer (the primers were identical to those used in semiquantitative RT-PCR). The real-time PCR amplification conditions were 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ relative quantitation method, according to the manufacturer's directions.

Preparation of cytoplasmic and nuclear extracts and western blotting assay

To prepare the cell extract, 5×10^6 cells were resuspended in 400 μL of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM dithiothreitol, 1 μg·mL^{-1} leupeptin, 1 μg·mL^{-1} papstatin A, 100 μg·mL^{-1} phenylmethanesulfonyl fluoride, and phosphatase inhibitors). The cell suspension was kept on ice for 15 min, and then 25 μL of 10% NP-40 was added; this was followed by vortexing for 10 s. After centrifugation at 10 000 *g* for 30 s using Heraeus Biofuge fresco fixed-angle rotor 7500 3325 (Kendro Laboratory Products, Hanau, Germany), the supernatant was collected as a cytoplasmic extract. The nuclear pellets were resuspended in 100 μL of buffer C (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM

dithiothreitol, 1 μg·mL^{-1} leupeptin, 1 μg·mL^{-1} papstatin A, 100 μg·mL^{-1} phenylmethanesulfonyl fluoride, and phosphatase inhibitors) and rocked on ice for 20 min. After centrifugation using Heraeus Biofuge fresco fixed-angle rotor 7500 3325 (Kendro Laboratory Products) at top speed for 10 min, the supernatant was collected as a nuclear extract. The samples were then aliquoted and stored at −80 °C for further assays. The proteins separated by SDS/PAGE were transferred to poly(vinylidene fluoride) membranes (Millipore, Billerica, MA, USA), and western blotting was done using Flag- (Sigma-Aldrich), HuR- (Santa Cruz, Santa Cruz, CA, USA), TTP- and α-tubulin-specific antibodies [52].

Immunopreciptation assays

Cytoplasmic extracts (1 mg) from 3T3-L1 cells in 25 mM Hepes were adjusted to 25 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol and 1 U·μL^{-1} of RNase inhibitor (Promega), and were precleaned with protein A Sepharose (Amershan Pharmacia) for 1 h. After centrifugation at 8000 *g* for 1 min using Heraeus fresco fixed-angle rotor 7500 3325 (Kendro Laboratory Products), the supernatants were added with preimmune serum or HuR or TTP sera and protein A Sepharose at 4 °C rotated for 2 h. Beads were washed three times with NT2 buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl$_2$, 0.05% NP-40) [56]. The beads were then incubated with 100 μL NT2 buffer containing 5 U of RNase-free Dnase I (Ambion, Austin, TX, USA) for 15 min at 30 °C, washed with NT2 buffer, and further incubated in 100 μL of NT2 buffer containing 0.1% SDS and 0.5 mg·mL^{-1} proteinase K at 55 °C for 15 min. RNA was extracted with blue extract reagent as described above for RT-PCR analysis.

RNA pull-down assay

Cytoplasmic extracts from 10^7 3T3-L1 cells were precleaned by centrifugation, and the potassium acetate concentration was adjusted to 90 mM. After addition of RNase inhibitor (Promega) (0.1 U·μL^{-1}) and yeast tRNA (20 μg·mL^{-1}) (Ambion), cytoplasmic extracts were absorbed with heparin-agarose (Sigma-Aldrich) at 4 °C for 15 min. After centrifugation at 8000 *g* for 1 min using Heraeus fresco fixed-angle rotor 7500 3325 (Kendro Laboratory Products), the supernatant was further cleaned with 20 μL of streptavidin Sepharose (Invitrogen, San Diego, CA, USA) for 1 h at 4 °C with rotation. After centrifugation at 8000 *g* for 1 min using Heraeus fresco fixed-angle rotor 7500 3325 (Kendro Laboratory Products), the supernatants were added with 4 μg of *in vitro* transcribed biotinylated TTP ARE or control 18S RNA (T7-MEGA shortscript^{TM}, Ambion) was added to the supernatant and the mixture was incubated for 1 h at 4 °C. The protein and biotinylated RNA

complexes were recovered by addition of 12 μL of strept-avidin Sepharose at 4 °C for 2 h with rotation. The precipitated complexes were washed five times with binding buffer (10 mM Hepes, pH 7.5, 90 mM potassium acetate, 1.5 mM MgCl₂, 2.5 mM dithiothreitol, 0.05% NP40, protease and phosphatase inhibitor cocktail, 0.5 mM phenylmethanesulfonyl fluoride), boiled in SDS/PAGE sample buffer, and resolved by gel electrophoresis followed by western blotting with anti-HuR and anti-TTP sera.

REMSA

TTP ARE (nucleotides 1529–1715) in pGEM-Teazy was linearized with *Sal*I and *in vitro* transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP for REMSA (Fig. 4D, left panel). To generate the wild-type and mutant TTP ARE probes, antisense oligonucleotides containing a T7 promoter were synthesized: 5′-CCCAATATATATAAATACTATAAA ATCTTAATACAATAAATAAAGTCGTCATAAATAA AGGGCCCTATAGTGAGTCGTATTA-3′ for the wild-type (nucleotides 1557–1621); and 5′-CCCAATATATAT<u>CCCT</u> ACTATAAAATCTTAATACAAT<u>CCC</u>TAAAGTCGTC ATCCCTAAAGGGCCCTATAGTGAGTCGTATTA-3′ for the mutant (underlining indicates the mutation introduced). These oligonucleotides were hybridized with a sense T7 primer (5′-TAATACGACTCACTATAG-3′) and extended to fill-in the probes by PCR (95 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, 35 cycles). PCR fragments of 0.5 μg were *in vitro* transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP. Radiolabeled probe (1 pmol) was incubated with the indicated recombinant proteins at room temperature for 40 min in a final volume of 10 μL containing 15 mM Hepes (pH 7.9), 10 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.2 mM dithiothreitol, 0.5 μg heparin sulfate, and 5 μg of yeast total RNA. Binding mixtures were then loaded onto native 5% polyacryamide gel (acryl/bis = 40 : 1) containing 2.5% glycerol in 0.25 × Tris/borate/EDTA buffer. After electrophoresis at 15 V·cm^{-1} for 60 min, the gel was dried and exposed to Kodak XAR film (Rochester, NY, USA) at − 70 °C for the appropriate time.

Transfection, luciferase and β-galactosidase assay, northern blotting

The HEK293T cells (2 × 10⁵) were seeded in each well of a six-well plastic culture plate. Cells were transfected using the calcium phosphate precipitation method with 0.5 μg of the indicated luciferase constructs, 1 μg of SV40–β-galactosidase plasmid (Promega), and TTP or HuR expression vector. After 24 h, cells were harvested, and the cell lysates were assayed for luciferase and β-galactosidase activity, and western blotting of ectopic expressed proteins was performed, or RNAs were isolated 12 h after transfection for northern blotting analysis. Luciferase activity was determined in a luminometer (Packard, Downers Grove, IL,

USA) with Promega luciferin as substrate. β-galactosidase activity was determined with a standard colorimetric assay using 2-nitrophenyl β–D-galactopyranoside (Sigma-Aldrich) as substrate. The luciferase assay results were normalized to β-galactosidase activity to correct for variations in transfection efficiency. Each treatment group contained duplicate cultures, and each experiment was repeated three times. Relative luciferase activity, defined as luciferase light units/β-galactosidase activity, is presented as means ± SE. After digestion by RNase-free DNaseI, the isolated RNAs were subjected to 1.4% formaldehyde agarose gel electrophoresis and then capillary-transferred onto a nylon membrane (Hybond-N; Amersham Pharmacia).^{32} P-Labeled cDNA probes against the luciferase and glyceraldehyde-3-phosphate dehydrogenase coding regions were synthesized using Ready-to-Go DNA labeling Beads (Amersham Biosciences). After hybridization, the membranes were visualized and quantified by phosphoimaging (BAS-1000 and Image Gauge; Fuji, Tokyo, Japan).

Acknowledgements

References

1 Guhaniyogi J & Brewer G (2001) Regulation of mRNA stability in mammalian cells. *Gene* **265**, 11–23.

2 Bevilacqua A, Ceriani MC, Capaccioli S & Nicolin A (2003) Post-transcriptional regulation of gene expression by degradation of messenger RNAs. *J Cell Physiol* **195**, 356–372.

3 Kracht M & Saklatvala J (2002) Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine* **20**, 91–106.

4 Zhang T, Kruys V, Huez G & Gueydan C (2002) AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. *Biochem Soc Trans* **30**, 952–958.

5 Espel E (2005) The role of the AU-rich elements of mRNAs in controlling translation. *Semin Cell Dev Biol* **16**, 59–67.

6 Chen CY & Shyu AB (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* **20**, 465–470.

7 Zhang W, Wagner BJ, Ehrenman K, Schaefer AW, DeMaria CT, Crater D, DeHaven K, Long L & Brewer G (1993) Purification, characterization and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. *Mol Cell Biol* **13**, 7652–7665.

8 Carballo E, Lai WS & Blackshear PJ (2000) Evidence that tristetraprolin is a physiological regulator of

granulocyte–macrophage colony stimulating factor messenger RNA deadenylation and stability. *Blood* **95**, 1891–1899.

9 Ma WJ, Cheng S, Campbell C, Wright A & Furneaux H (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J Biol Chem* **271**, 8144–8151.

10 Dember LM, Kim ND, Liu K-Q & Anderson P (1996) Individual RNA recognition motifs of TIA-1 and TIAR have differential RNA binding specificities. *J Biol Chem* **271**, 2783–2788.

11 Piecyk M, Wax S, Beck AR, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Kruys V, Streuli M *et al.* (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNFα. *EMBO J* **19**, 4154–4163.

12 Keene JD (1999) Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. *Proc Natl Acad Sci USA* **96**, 5–7.

13 Peng SS, Chen CY, Xu N & Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J* **17**, 3461–3470.

14 Katsanou V, Papadaki O, Milatos S, Blacksheer PJ, Anderson P, Kollias G & Kontoyiannis DL (2005) HuR as a negative posttranscriptional modulator in inflammation. *Mol Cell* **19**, 777–789.

15 Kawai T, Lal A, Yang X, Galban S, Mazan-Mamczarz K & Gorospe M (2006) Translational control of cytochrome c by RNA-binding proteins TIA1 and HuR. *Mol Cell Biol* **26**, 3295–3307.

16 Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF *et al.* (1996) A pathogenic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* **4**, 445–454.

17 Lai WS, Carballo E, Strum JR, Kennington EA, Philips RS & Blackshear PJ (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Mol Cell Biol* **19**, 4311–4323.

18 Lai WS & Blackshear PJ (2001) Interactions of CCCH zinc finger protein with mRNA: tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. *J Biol Chem* **276**, 23144–23154.

19 Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJM, Stoecklin G, Moroni C, Mann M & Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**, 451–464.

20 Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS & Wilusz J (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J* **21**, 165–174.

21 Lykke-Andersen J & Wagner E (2005) Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev* **19**, 351–361.

22 Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Padiva FD, Lin S-C, Gram H & Han J (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120**, 623–634.

23 Lai WS, Stumpo DJ & Blackshear PJ (1990) Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. *J Biol Chem* **265**, 16556–16563.

24 Inuzuka H, Nanbu-Wakao R, Masuho Y, Muramatsu M, Tojo H & Wakao H (1999) Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. *Biochem Biophys Res Commun* **265**, 664–668.

25 Lai WS, Thompson MJ, Taylor GA, Liu Y & Blackshear PJ (1995) Promoter analysis of *Zfp-36*, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. *J Biol Chem* **270**, 25266–25272.

26 Lai WS, Thompson MJ & Blackshear PJ (1998) Characteristics of the intron involvement in the mitogen-induced expression of *Zfp-36*. *J Biol Chem* **273**, 506–517.

27 Carballo E, Lai WS & Blackshear PJ (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science* **281**, 1001–1005.

28 Puig S, Askeland E & Thiele DJ (2005) Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. *Cell* **120**, 99–110.

29 Sachidanandan C, Sambasivan R & Dhawan J (2002) Tristetraprolin and LPS-inducible CXC chemokine are rapidly induced in presumptive satellite cells in response to skeletal muscle injury. *J Cell Sci* **115**, 2701–2712.

30 Green H & Kehinds O (1974) Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1**, 113–116.

31 Green H & Kehinds O (1975) An established cell line and its differentiation in culture II. Factors affecting adipose conversion. *Cell* **5**, 19–27.

32 Green H & Kehinds O (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* **7**, 105–113.

33 MacDougald OA & Lane MD (1995) Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem* **64**, 345–373.

34 Rosen ED & Spiegelman BM (2000) Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* **16**, 145–171.

35 Rosen ED, Walkey CJ, Puigserver P & Spiegelman BM (2000) Transcriptional regulation of adipogenesis. *Genes Dev* **14**, 1293–1307.

36 Darlington GJ, Ross SE & MacDougald OA (1998) The role of C/EBP genes in adipocyte differentiation. *J Biol Chem* **273**, 30057–30060.

37 Kim JB & Spiegelman BM (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev* **10**, 1096–1107.

38 Shao D & Lazar MA (1997) Peroxisome proliferator activated receptor gamma, CCAAT/enhancer-binding protein alpha, and cell cycle status regulate the commitment to adipocyte differentiation. *J Biol Chem* **272**, 21473–21478.

39 Morrison RF & Farmer SR (1999) Role of PPARγ in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18 (INK4c), and p21 (Waf1/Cip1), during adipogenesis. *J Biol Chem* **274**, 17088–17097.

40 Gantt K, Cherry J, Tenney R, Karschner V & Pekala PH (2005) An early event in adipogenesis, the nuclear selection of the CCAAT enhancer-binding protein β (C/EBPβ) mRNA by HuR and its translocation to the cytosol. *J Biol Chem* **280**, 24768–24774.

41 Brooks SA, Connolly JE & Rigby WF (2004) The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent. Autoregulatory pathway. *J Immun* **172**, 7264–7271.

42 Tchen CR, Brook M, Saklatvala J & Clark AR (2004) The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 by tristetraprolin itself. *J Biol Chem* **279**, 32393–32400.

43 Fan XC & Steitz JA (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J* **17**, 3448–3460.

44 Brennan CM, Gallouzi IE & Steitz JA (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J Cell Biol* **151**, 1–14.

45 Gallouzi IE & Steitz JA (2001) Delineation of mRNA export pathways by the use of cell permeable peptides. *Science* **294**, 1895–1901.

46 Atasoy U, Watson J, Patel D & Keene JD (1998) ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. *J Cell Sci* **111**, 3145–3156.

47 Wang W, Furneaux H, Cheng H, Caldwell MC, Hunter D, Liu Y, Holbrook NJ & Gorospe M (2000) HuR regulates p21 mRNA stabilization by UV light. *Mol Cell Biol* **20**, 760–769.

48 Li H, Park S, Kilburn B, Jelinek MA, Henschen-Edman A, Aswad DW, Stallcup MR & Laird-Offringa IA (2002) Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. *J Biol Chem* **277**, 44623–44630.

49 Rigby WFC, Roy K, Collins J, Rigby S, Connolly JE, Bloch DB & Brooks SA (2005) Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function. *J Immun* **174**, 7883–7893.

50 Brook M, Tchen CR, Santalucia T, McIlrath J, Arthur JS, Saklatvala J & Clark AR (2006) Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Mol Cell Biol* **26**, 2408–2418.

51 Hitti E, Iakovleva T, Brook M, Deppenmeier S, Gruber AD, Radzioch D, Clark AR, Blackshear PJ, Kotlyarov A & Gaestel M (2006) Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. *Mol Cell Biol* **26**, 2399–2407.

52 Chen YL, Huang YL, Lin NY, Chen HC, Chiu WC & Chang CJ (2006) Differential regulation of ARE-mediated TNFα and IL-1β mRNA stability by lipopolysaccharide in RAW264.7 cells. *Biochem Biophys Res Commun* **346**, 160–168.

53 Huang H, Lane MD & Tang QQ (2005) Effect of serum on the down-regulation of CHOP-10 during differentiation of 3T3-L1 preadipocytes. *Biochem Biophys Res Commun* **338**, 1185–1188.

54 Rinchon VM, Lyle RE & McGehee RE Jr (1997) Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. *J Biol Chem* **272**, 10117–10124.

55 Reichert M & Eick D (1999) Analysis of cell cycle arrest in adipocyte differentiation. *Oncogene* **18**, 459–466.

56 Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL & Gorospe M (2004) Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. *EMBO J* **23**, 3092–3102.

Supplementary material

The following supplementary material is available online:

Fig. S1. RNA interference with TTP expression during 3T3-L1 differentiation.

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