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碩士論文

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Master Thesis

Tristetraprolin家族蛋白在小鼠巨噬細胞功能之研究

Functional Characterization of Tristetraprolin Family Proteins in Mouse Macrophages

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中文摘要

先天免疫反應的基因表達受到嚴密調控。絲裂原活化蛋白激酶（MAPK）在先天免疫反應中扮演關鍵腳色，它透過磷酸化下游轉錄因子和 RNA 結合蛋白，可活化炎性細胞因子（proinflammatory cytokines）的合成。MAPK 磷酸酶（MKP）藉由去磷酸化作用使 MAPK 失去活性。目前研究指出 Mkp-1 是一個重要的負調控因子，它可以關閉炎性細胞因子的生產。我們已經證明 Tristetraprolin（Ttp）可以藉由與具有特殊的多腺嘌呤-尿嘧啶序列（AU-rich element, ARE）的 *Mkp-1* mRNA 結合，並透過後轉錄機制將 *Mkp-1* mRNA 降解。TTP 家族包含三個主要成員，Ttp，Zfp36l1 和 Zfp36l2。本論文的目標即在了解其他 TTP 家族蛋白在先天免疫反應中扮演的角色。首先我們觀察 TTP 家族蛋白的 mRNA 與蛋白質表達。在受到脂多醣（Lipopolyssacharide, LPS）刺激的老鼠巨噬細胞 RAW264.7 中，TTP 的 mRNA 和蛋白質被高度誘導，但 *Zfp36l1* 和 *Zfp36l2* 的 mRNA 表現降低，而蛋白質表現則保持一致。利用 Zfp36l1 和 Zfp36l2 的 knockdown 分析，我們發現兩個受到 Zfp36l1 和 Zfp36l2 調控的目標 mRNA：*Mkp-1* 和 *Cox-2*。減少 Zfp36l1 和 Zfp36l2 的表現，可延長目標 mRNA 的半衰期，進而使目標 mRNA 增加。當細胞內 Mkp-1 的表達增加時，會抑制 p38 MAPK 的活性，使老鼠巨噬細胞對 LPS 刺激的敏感度下降。此外，我們還發現高度磷酸化的 Zfp36l1 會與支架蛋白 14-3-3 結合，並使 *Mkp-1* mRNA 不被降解。綜合上述，我們的研究結果顯示 Zfp36l1 和 Zfp36l2 的表現與磷酸化修飾，可調控老鼠巨噬細胞 RAW264.7 受脂多醣刺激時，*Mkp-1* mRNA 的表現，這也說明 Zfp36l1 和 Zfp36l2 是先天免疫反應中重要的調控因子。

關鍵字

mRNA 穩定性、Tristetraprolin、先天免疫反應、磷酸化、絲裂原活化蛋白激酶

Abstract

Gene expressions are tightly controlled in the innate immune response. Mitogen-activated protein kinases (MAPKs) play critical roles in the innate immune response through phosphorylating downstream transcription factors and RNA binding proteins to elicit the biosynthesis of proinflammatory cytokines. Inactivation of MAPKs is done by MAPK phosphatases (MKPs) through dephosphorylation. The previous studies strongly suggested that Mkp-1 was a critical negative regulator for switching off the production of proinflammatory cytokines. We had demonstrated that *Mkp-1* mRNA containing AU-rich element (ARE) was post-transcriptionally regulated by an ARE-binding protein Tristetraprolin (Ttp). The TTP family contains three major members, Ttp, Zfp36l1 and Zfp36l2. To examine whether other family proteins also play roles in the innate immune response, their expression profiles were determined. The mRNA and protein of Ttp were highly induced by Lipopolyssacharide (LPS) in mouse macrophage RAW264.7 cells, whereas the mRNAs of *Zfp36l1* and *Zfp36l2* were down-regulated and their proteins were maintained in the consistent levels in the period of LPS-stimulation. By knockdown analysis, we found that *Mkp-1* and *Cyclooxygenase-2* (*Cox-2*) were the mRNA targets of Zfp36l1 and Zfp36l2 in the resting condition. Knockdown of Zfp36l1 and Zfp36l2 increased the basal levels of

target mRNAs by prolonging their half-lives. Increasing the expression of Mkp-1 repressed the activity of p38 MAPK, and the sensitivity to LPS-stimulation was decreased. Furthermore, we found that hyper-phosphorylation of Zfp36l1 stabilized Mkp-1 expression by forming a complex with adapter protein 14-3-3. Our findings imply the expression and phosphorylation of Zfp36l1 and Zfp36l2 might play roles in modulating the mRNA level of *Mkp-1* to control p38 MAPK activity in LPS-stimulation, and both Zfp36l1 and Zfp36l2 are important regulators in the innate immune response.

Keywords：

mRNA stability、Tristetraprolin、Innate immune response、Phosphorylation、Mitogen-activated protein kinase

Contents

Abbreviations

3'UTR	3'-untranslated region
ARE	Adenylate/uridylate-rich element
CCL-2	Chemokine (C-C motif) ligand 2
CCR4	Carbohydrate catabolism repression 4
cIAP2	Cellular inhibitor of apoptosis 2
CIP	Calf intestinal alkaline phosphatase
COX-2	Cyclooxygenase-2
DCP	Decapping enzyme
DEPC	Diethyl pyrocarbonate
DTT	Dithiothreitol
EDC3	Enhancer of mRNA decapping 3
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GST	Glutathione S-transferase
HDAC	Histone deacetylases
Hedls	Human enhancer of decapping large subunit
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
HNSCC	Human head and neck squamous cell carcinoma
HuR	Hu antigen R
I-cam1	Intercellular adhesion molecule 1
IEG	Immediate early gene
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun amino-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MK2	Mitogen-activated protein kinase-activated protein kinase 2
MKP	MAPK phosphatase
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
PARN	Poly(A)-specific ribonuclease
P-body	Processing body
PBS	Phosphate buffered saline

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
T-ALL	T-cell acute lymphoblastic leukemia
Tnf-α	Tumor necrosis factor-α
TPA	12-O-tetradecannoylphorbol-13-acetate
TTP	Tristetraprolin
TZF	Tandem zinc finger
Xrn1	5'-3' exoribonuclease 1
Zfp36l1	Zinc finger protein 36, C3H type-like 1
Zfp36l2	Zinc finger protein 36, C3H type-like 2
Zfp36l3	Zinc finger protein 36, C3H type-like 3

1. Introduction

1.1 TTP Family Proteins

1.1.1 Identification of TTP Family Proteins

The sequence of *Tristetraprolin* (*Ttp*; also known as *Zfp36, Tis11*) was first confirmed from the cDNA of TPA-treated (12-O-tetradecannoylphorbol-13-acetate) Swiss 3T3 cells [1], and the expression of Ttp was transiently activated by insulin or growth factor [2,3]. The characteristics of amino acid sequence of Ttp are three Pro-Pro-Pro-Pro repeats [2] and two tandem zinc finger (TZF; also known as CCCH domain) domains which are required for RNA binding [1].

Zfp36l1 (Tis11b, Brf1, cMG1, Erf1, and Berg36) and Zfp36l2 (Tis11d, Brf2, and Erf1) which belong to TTP family proteins were also identified by Dr. Blackshear group [1] . It was reported that the TZF domains of Zfp36l1 and Zfp36l2 were more than 70% identical to Ttp. Although their sequences are similar, their responses to TPA stimulation are different. Zfp36l1 and Zfp36l2 are detectable without TPA stimulation; however, Ttp is undetectable in Swiss 3T3 cells without TPA stimulation [1].

The fourth protein of TTP family, Zfp36l3, is located on the X chromosome of mouse and rat, but it is not found in human [4]. It suggests that Zfp36l3 may play a unique role in rodent, but the function of Zfp36l3 in non-rodent may be replaced by

other TTP family proteins.

1.1.2 Function of TTP Family Proteins

The *Ttp* knockout mice develop complex syndromes of inflammatory arthritis, dermatitis, autoimmunity, cachexia, and myeloid hyperplasia. Those symptoms can be recovered by using Tumor necrosis factor-α (Tnf-α) antibodies [5]. The amounts of Tnf-α which secreted by bone marrow-derived macrophages from the *Ttp* knockout mice are about 5 times than control cells [6]. Those studies indicate that Ttp is involved in the regulation of Tnf-α *in vivo*. Ttp binds to mRNA 3'-untranslated region (3' UTR) of *Tnf-α* containing adenylate/uridylate rich element (ARE) and down-regulates the expression of Tnf-α in the post-transcriptional level [7]. Ttp has been confirmed to modulate the expressions of mRNAs containing ARE [8]. As a result, Ttp participates in many cellular processes, including immune response, cell cycle, carcinogenesis, angiogenesis, development, and protein glycosylation (Appendix 1) [8].

The *Zfp36l1* knockout mice are failed to pass chorioallantoic fusion and die at embryonic day 10.5 [9]. The *Zfp36l2* knockout mice die after 14 days of birth because the formation of hematopoietic stem cells are failed [10]. The ova of female mice with N-terminal truncated 29 amino acids (the first exon) of *Zfp36l2* can be fertilized, but the

embryos die in two-cell stage of development [11]. The conditional knockout mice of *Zfp36l1* and *Zfp36l2* will both bother the development of thymus and develop T-cell acute lymphoblastic leukemia (T-ALL) [12]. The RNA binding abilities of Zfp36l1 and Zfp36l2 are similar to Ttp due to the conserved TZF domains of those proteins. *Tnf-α* mRNA are also destabilized by the overexpression of Zfp36l1 and Zfp36l2 [13], and the other mRNA targets of Zfp36l1 and Zfp36l2 are listed in Table 1.

1.1.3 TTP Family Proteins Mediate ARE-containing mRNA Decay

Ttp associates with ARE-containing mRNAs and results in mRNAs decay. The sequences of ARE motifs are highly conserved in those mRNA. In the statistics, 96% of ARE motifs are AUUUA (pentamers), and 44% are UUAUUUAUU (nonamers) [14].

There are three major deadenylation complexes: Ccr4/Caf1/Not, poly-A specific ribonuclease (Parn), and Pan2/Pan3. They initiate mRNA degradation through removing the poly-A tail of mRNA. Using co-immunoprecipitation experiments, Ttp and Zfp36l1 interacte with Ccr4 [15], and Ttp can also associate with Caf1 through Not1 [16]. However, Ttp cannot interact with Parn [15].

After the deadenylation of mRNA, there are two pathways of mRNA degradation. The first one is mRNA decapping followed by 5’→3’ exonucleolytic decay in p-bodies,

and the second is 3'→5' exonucleolytic decay. Ttp existes in mRNA decapping complexes, involving Edc3 and Hedls (enhancers of decapping enzymes), Dcp1 and Dcp2 (decapping enzymes) [15,17]. After the decapping of mRNA, the exposed 5' end of mRNA is digested by Xrn-1 (5'-exoribonuclease) which is recruited by Ttp [15]. The N-terminal domain of Ttp is necessary for associating with Dcp1, Dcp2, and Xrn-1 [15]. For 3'→5' exonucleolytic decay, Ttp is also required for recruiting exosome components, including Rrp4, Rrp40, Rrp41, Rrp42, Rrp43, Rrp46, hCsl4, Mtr3, and PM-Scl75 [15,18]. Both dacay pathways are shown in Appendix 2 [19].

In addition, Ttp interacts with Ago2 and Ago4, and it helps microRNA 16 (containing an UAAAUAUU sequence which is complementary to the ARE sequence) targeted to ARE-containing mRNA [20].

1.1.4 Ttp in the Transcriptional Regulation

Ttp serves as a repressor in NF-κB-dependent transcription by associating with different proteins. It was observed that the amount of nuclear p65, one subunit of NF-κB, was increased in the mouse embryonic fibroblasts from the *Ttp* knockout mice [21]. Loss of mRNA binding ability, Ttp still impairs the nuclear import of p65, and the activity of NF-κB is repressed [21]. Another study indicates that Ttp also associates with

histone deacetylases (HDACs) which are transcriptional co-repressors, such as HDAC-1, HDAC-3, and HDAC-7. The abolished effect of Ttp is recovered in the knockdown of *HDAC-1*, *HDAC-3*, and *DHAC-7* or the treatment of histone deacetylase inhibitors [22].

1.1.5 The Phosphorylation Modification of TTP Family Proteins

The modification of phosphorylation alters the functions, subcellular localizations, protein stability of TTP family proteins. Ser52, Ser80, Ser82, Ser178, Ser249, Ser250, and Ser264 of Ttp are phosphorylated by mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) [23]. Ttp is stabilized and locates in the cytoplasm when Ser52 and Ser178 are phosphorylated; moreover, phosphorylation of Ser52 and Ser178 is necessary for Ttp associating with adapter protein 14-3-3. The complex of 14-3-3 and Ttp abolishes the recruitment of deadenylase complex [24]; therefore, TTP cannot destabilize the mRNA targets [25].

Similarly, Ser54, Ser92, and Ser203 of Zfp36l1 are the phosphorylation sites of MK2, and the mRNA destabilizing ability of Zfp36l1 is repressed. Phosphorylated Zfp36l1 can form the complex with 14-3-3, but the association with AREs, Ccr4, and Dcp2 is still remained [26]. These results indicate that the modification of phosphorylation of Zfp36l1 regulates its mRNA decay activity by the steps after

recruiting mRNA decay enzymes. Other studies showed that Ser92 and Ser203 are phosphorylated by Protein kinase B (also known as AKT) [23,27]. Phosphorylated Ser92 and Ser203 of Zfp36l1 binds to 14-3-3 and abolishes its mRNA decay activity; moreover, the protein stability of Zfp36l1 is up-regulated by the phosphorylation of those two sites [23]. The phosphorylation of Zfp36l2 is unclear until now.

1.1.6 TTP family Proteins in Cancers

The accumulation of ARE-containing mRNA is observed in the early stage of tumorigenesis. Abnormal expressions of ARE-binding-proteins may result in the loss of ARE-mediated mRNA decay [28]. The low expression of TTP had been examined in breast, colon, cervix, prostate, and lung cancer [8], whereas the expression of HuR (mRNA stabilizing factor which also associating with AREs) was high in colon carcinogenesis [29]. Human head and neck squamous cell carcinoma (HNSCC) with low expression level of ZFP36L1 are insensitive to cisplatin (a common chemotherapeutic drug) because the decrease of ZFP36L1-mediated destabilization of *cellular inhibitor of apoptosis 2 (cIAP2)* mRNA [30].

1.2 Innate Immune Response

1.2.1 Mitogen-activated Protein Kinase (MAPK) in the Innate Immune Response

There are three major MAPKs signaling pathways in mammals: p38 MAPK, extracellular signal-regulated kinase (ERK), and the c-Jun amino-terminal kinases (JNK) (Appendix 3) [31]. MAPKs are the important regulators in differentiation, survival, apoptosis, and production of inflammatory mediators [31,32].

The activation of MKK3-p38 pathway induced the expression of inflammatory mediator, Interleukin-12 (IL-12) [33]. Another inflammatory mediators, Tnf-α, is up-regulated by p38 MAPK and ERK. p38 MAPK induces the transcriptional increase of *Tnf-α* mRNA through NF-κB [34], and the activation of p38 MAPK pathway up-regulates the translation of *Tnf-α* mRNA [35]. In addition, TPL2-ERK pathway is required for the translocation of *Tnf-α* mRNA from nuclear to cytoplasm [36].

1.2.2 Mitogen-activated Protein Kinase Phosphatases (MKPs) in the Innate Immune Response

Mitogen-activated protein kinase phosphatases (MKPs) are negative regulators of MAPKs through de-phosphorylation at both threonine and tyrosine residues. Many studies reported that the substrate specificity of MKPs was different [37] .

MKP-1 is one of the most commonly studied MKP in the innate immune response. Mkp-1 can be activated by ERK signaling pathway [38]. Overexpression of Mkp-1 may inactivate Jnk and p38, and then inhibits the expressions of Tnf-α and IL-6 [38]. Therefore, inducers of MKP-1 are potential drugs for autoimmune diseases [38].

1.2.3 TTP Family Proteins in the Innate Immune Response

According to the study from Dr. Baltimore [39], there are three types of gene expression profiles after inflammatory stimuli. The expressions of type I genes are rapidly increased and decreased, and they are also known as immediate early gene. The expressions of type II genes are continuously increased until 2 hour after Tnf-α treatment and then their expressions are sustained. Last, the expressions of type III genes are continuously increased, and they do not reach the peak until 24 hour after Tnf-α treatment (Appendix 5). The quality and timing of those genes expressions are controlled by mRNA stability. For example, the transcripts of type I genes are unstable in the resting condition, but they were stable after Tnf-α treatment.

In mouse macrophages RAW264.7 cells, immune signals such as Lipopolysaccharide (LPS) from gram-negative bacteria and Tnf-α activate MAPK signaling pathways [40]. The expression of Ttp is also induced after LPS-stimulation

[41], and it serves as an anti-inflammatory role through rapidly destabilizing mRNAs of pro-inflammatory mediators which containing AREs. The mRNA decay of one third unstable transcripts depends on Ttp, and the decay timing was controlled by the activity of p38 MAPK [42]. In addition, Ttp is able to interact with mRNA 3'UTR of itself, and the auto-regulation of Ttp can limit its own expression [41,43].

As mentioned before, many diseases related to the innate immune response are developed in the *Ttp* knockout mice due to the expression of Tnf-α is increased. Those results also indicate that Ttp may be a negative regulator in the innate immune response. Moreover, *Tnf-α* mRNA is down-regulated by overexpression of Zfp36l1 and Zfp36l2 in HEK 293 cells [13]. However, the effects of protein overexpression may be different with the real effects, and the identified mRNA targets may be considered as "non-physiological targets". It is better to analysis the functions and mRNA targets of TTP family proteins in the knockout/knockdown mice or cells than in the overexpression systems. Hence, we study the detail mechanisms of Zfp36l1 and Zfp36l2 in the innate immune response in their knockdown cells.

2. Materials and Methods

2.1 Plasmid Constructs

To generate Flag-tagged mouse Ttp, Zfp36l1, and Zfp36l2, oligonucleotides for *Ttp*, *Zfp36l1*, and *Zfp36l2* coding sequences were amplified from 2 hours LPS-treated RAW264.7 cDNA. The sequences of primers used in PCR were listed in Table 1. The PCR fragments were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). After sequences were confirmed, recombinant genes were then subcloned into mammalian cells expression vector pCMV-Tag-2B (Stratagene, La Jolla, CA, USA) via *Eco*RI sites.

For preparing riboprobes for RNA pull-down assay, the 3'UTR of *Mkp-1* was PCR amplified from 1 hour differentiation-triggered 3T3-L1 cDNA. The sequences of primers used in PCR were listed in Table 1. The PCR fragment was cloned into pCRII-TOPO vector, and sequence was confirmed. For luciferase reporter constructs, *Mkp-1* 3′UTR was subcloned into pCMV-Tag-2C-Luciferase (Stratagene) reporter using the *Apa*I and *Kpn*I sites.

To generate Flag-tagged and Myc-tagged mouse Caf1a (Ccr4-Associated Factor; CCR4-NOT transcription complex, subunit 7 (Cnot7)), the coding sequence of *Caf1a* was amplified from 2 hours LPS-treated RAW264.7 cDNA. The sequences of primers

used in PCR were listed in Table 1. The PCR fragments were cloned into pCRII-TOPO vector. After sequence were confirmed, recombinant gene was then subcloned into mammalian cells expression vector pCMV-Tag-2B and pCMV-Tag-3 via *Hind*III and *BamH*1 sites.

For glutathione-S-transferase (GST) fusion protein mouse 14-3-3 zeta construct, *14-3-3 zeta* coding region were PCR-amplified from 2 hour LPS-treated RAW264.7 cDNA. The sequences of primers used in PCR were listed in Table 1. The PCR fragment was cloned into pCRII-TOPO vector. After sequences were confirmed, *14-3-3zeta* was subcloned into *E.coli* expression vector pGEX-3 (GE Healthcare BioScience, Chalfont St. Giles, UK).

2.2 Cell Culture

RAW264.7 cells (mouse macrophage) were grown in Dulbecco's modified Eagle medium (Gibco) containing 1.5 g/L sodium bicarbonate, and supplemented with 10% fetal bovine serum (Characterized; Hyclone or 12003C; SAFC), 2mM L-glutamine (Gibco). Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL) containing 3.7 g/L sodium bicarbonate, and

supplemented with 10% fetal bovine serum (Qualified, Gibco). Both RAW264.7 and HEK 293T cells were cultured in the 37℃, humidified, 5% CO_2 incubator.

2.3 Preparation Whole Cell Extracts and Cytoplasmic/Nuclear Extracts

Cells in a 10-cm dish were washed once with PBS and then harvested. For preparing whole cell extracts, the harvested cells were lysed in 400 μL of whole cell extract buffer (25 mM HEPES pH7.7, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 0.3 M NaCl, protease inhibitor cocktail (Sigma) and phosphatase inhibitor containing 0.01M beta-glycerol phosphate, 0.1 mM Na_2MoO_4, 0.1 mM Na_3VO_4 pH10, 0.01M NaF). The cell lysates were shaken at 4℃ for 30 minutes, and next they were centrifuged for 5 minutes at 13,000 rpm, 4℃. The supernatant was collected as a whole cell extract. For preparing cytoplasmic/nuclear extracts, harvested cells were lysed in 400 μL hypotonic buffer (10 mM HEPES pH7.5, 10 mM KOAc, 2.5 mM DTT, 0.05% NP-40, protease inhibitor and phosphatase inhibitor). The cell lysates were shaken at 4 ℃ for 30 minutes, and then they were centrifuged for 30 seconds at 9,000 rpm, 4℃. The supernatant was collected in as a cytosolic extract. The pellets were washed once with hypotonic buffer, and then they were re-suspended in 50 μL of buffer C (20 mM HEPES pH7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease

inhibitor and phosphatase inhibitor). The cell suspension was shaken at 4℃ for 30 minutes, and then it was centrifuged for 5 minutes at 13,000 rpm, 4℃. The supernatant was collected as a nuclear extract.

2.4 Alkaline Phosphatase, Calf Intestinal (CIP) Treatment

The total proteins from LPS-stimulated RAW264.7 cells were extracted by hypotonic buffer. Taking 100 μg of total proteins incubated with 1μL of CIP (NEB; M0290s) in 37℃ for 30 minutes.

2.5 Western Blot Assay and Antibodies

4X SDS-PAGE Sample buffer (200 mM Tris pH 6.8, 8% SDS, 0.4% bromopheol blue, 40% glycerol, 400 mM β-mercaptoethanol) was added to the sample till final concentration was 1X, and then heated at 100℃ for 5 minutes. Proteins were separated on 10% polyacrylamide gels and transferred onto 0.45 μm-pore-size PVDF membrane (Millipore) for Western blotting. The specific antibodies were used including anti-BRF1/2 (Cell signaling; 2119S), anti-hnRNPC1/C2 (Santa Cruz; sc-10037), anti-MKP-1 (Santa Cruz; sc-1102), anti-COX-2 (Santa Cruz; sc-1745), anti-p-p38

MAPK T180/Y182 (Cell signaling; 9211S), anti-total-p38 (Sigma), anti-p-p44/42 MAPK (Cell signaling; 9101S), anti-ERK1 (Santa Cruz; sc-093), anti- p-JNK (Sigma), anti-JNK1 (Santa Cruz; sc-571), anti-Myc (Genscrip; A00172), anti-Flag M2 (Sigma; F1804), anti-Ttp, anti-Zfp36l2, anti-beta-tubulin for 1 hour at room temperature. After washing with PBST (PBS containing 0.1% Tween 20) for an appropriate time, horseradish peroxidase-conjugated secondary antibodies, goat anti-rabbit IgG (KPL; 474-1516), goat anti-mouse IgG (KPL; 474-1806), or rabbit anti-goat (Sigma) was incubated for 1 hour at room temperature. Western Lightning enhanced chemiluminescemce substrate (Perkin Elmer) were used for detection.

2.6 RNA Extraction and Reverse-transcription

RNA isolation was performed with TRIzol reagent (Invitrogen) according to the suggested procedures of manufacturer. Cells in a six-well plastic culture plate were washed once with PBS and directly lysed in a well with 1ml TRIzol. The samples were incubated at room temperature for 5 minutes, and next they were added with 200 μL of chloroform and vortexed for 15 seconds. After incubated at room temperature for 3 minutes, the samples were centrifuged for 15 minutes at 13,000 rpm, 4℃. The upper layer (colorless) was transferred to a new tube, and then it was add with equal volume

of isopropanol and mixed well. After incubated at room temperature for 10 minutes, the mixture was centrifuged for 15 minutes at 13,000 rpm, 4℃. The white RNA pellet was washed by 75% ethanol, and then it was centrifuged for 10 minutes at 13,000 rpm, 4℃. After the pellet was air-dried for 10 minutes, it was dissolved with 20-30 μL DEPC H_2O. In order to dissolve RNA entirely, the sample was incubated twice at 60 ℃ for 10 minutes. After quantitated by A_{260}/A_{280} measurements, 5 μg RNA was taken for reverse-transcription. 5 μg total RNAs and 0.5 μg oligo dT were annealed at 70 ℃ for 5 minutes, and then the samples were cooled down on ice for 2 minutes. cDNAs were produced following the recommended procedures of M-MLV reverse transcriptase (Promega).

2.7 Real-time PCR

RNA quantitative assay was preform with the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems) in total volume of 20 μL, containing 10 μL FastStart Universal SYBR Green Master (Rox) (Roche; 04913914001), 4 μL of 10-times diluted cDNA, 0.4 μL of 5-20 μM forward and reverse primer (see table 2), and 5.6 μL DEPC H_2O. The result was normalized with beta-actin according to $2^{-\Delta\Delta Ct}$ relative quantitation method on the manual of manufacturer.

2.8 RNA Pull-down Assay

Cytoplasmic extracts from LPS-stimulated RAW264.7 cells were collected as previously described. Potassium acetate was adjusted to 90 mM, and 0.1 U/μL RNasin (Promega) and 20 μg/μL yeast tRNA were add to the lysates. In order to remove the non-specific binding, heparin-agarose (Sigma-Aldrich) was incubated with the lysates for 15 minutes at 4℃ and then centrifuged for 1 minute at 8,000 rpm, 4℃. The supernatant was further cleaned with Streptavidin Sepharose (Invetrogen) for 1 hour at 4 ℃, and then centrifuged for 1 minute at 8000 rpm, 4℃. The supernatant was incubated with biotinylated *Mkp-1* 3'UTR or negative control 18S RNA which made by T7-MEGA shortscript™, High Yield Transcription Kit (Ambion) for 1 hour at 4 ℃. Next, Streptavidin Sepharose was added to pulled down biotinylated RNA complex for 2 hours at 4℃. The pulled down complexes were washed four times with binding buffer (hypotonic buffer containing 90 mM potassium acetate). Last, the RNA complexes were separated by 10% SDS-PAGE and detected by Western blotting.

2.9. Dual Luciferase Reporter Assay

HEK293T cells were seeded in six-well plastic culture plate, and they were transfected using calcium phosphate precipitation method with different plasmids (containing 0.5 μg *Renilla* luciferase expression vector as a control of transfection rate) at 30% confluency. At 24 hours post-transfection, the cells were harvested and lysed in 50 μL of passive lysis buffer (Promega). The samples were shaken for 30 minutes at 4℃ and centrifuged for 5 minutes at 13000 rpm, 4℃. The supernatant was transferred to a well of black 96-well plate. The firefly luciferase activities were measured by luminometer (Packard, Downer Grove) after adding 50 μL of LAR II. The renilla luciferase activities were measured sequentially after adding 50 μL of Stop & Glo® Reagent. Each treatment group contained duplicate cultures, and each experiment was repeated more than 3 times. The firefly luciferase activities were normalized with the renilla luciferase activities. The relative luciferase activity represented the luciferase activity of reporter carrying 3'UTR normalized with that of reporter alone.

2.10 Short-hairpin RNA (shRNA)

Lentivirus vectors encoding shRNA targeted to mouse *Zfp36l1* and *Zfp36l2* were purchased from National RNAi core facility (Academia Sinica). TRCN0000123469

clone (5’-CCACATACAATATCTGTGTAA-3’) for mouse *Zfp36l1* knockdown, TRCN0000173172 clone (5’-CCAAACACTTAGGTCTCAGAT-3’) for mouse *Zfp36l2* knockdown, TRCN0000072243 clone (5′-CTTCGAAATGTCCGTTCGGTT-3′) for *Luciferase* knockdown. Both of TRCN0000123469 and TRCN0000173172 clones targeted to the 3’UTR of mRNA.

2.11. Lentivirus Knockdown

HEK293T cells were seeded in a 10-cm dish for transfection (calcium phosphate precipitation method) of virus production vectors, 14 μg of CMV ΔR8.9.1, 2 μg of pMD.G, and 14 μg of specific shRNA sequence bearing-pLKO.1 plasmids. 8 hours after transfection, the culture medium was replaced with fresh medium for RAW264.7 cells. 24 and 48 hours later, the virus-containing medium was collected for primary-infection and super-infection for RAW264.7 cells. 24 hours after super-infection, virus-containing medium was replaced with fresh medium for RAW264.7 cells. For generation of stable knockdown clones, puromycin (3 μg/mL) was added and GFP signal was served as a selection marker. After puromycin-selection for one week, cells were harvested and analyzed by Western blotting in order to check knockdown efficiency.

2.12. Co-immunoprecipitaion (Co-IP)

24 hours after transfection, HEK293T cells from 10-cm dishes were harvested and re-suspended in NET buffer (50 mM Tris pH 7.4, 350 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, protease inhibitor, phosphatase inhibitor). The cell lysates were shaken at 4℃ for 30 minutes, and then they were centrifuged for 5 minutes at 13,000 rpm, 4℃. The supernatant was collected for total proteins measurement using Bradford assay (Bio-Rad). 20 μL of 30% anti-FLAG M2 affinity agarose beads (Sigma) were added for 2-hour incubation, and then washed 3 times with NET buffer. The protein complexes were separated by 10% SDS-PAGE, and then they were detected by Western blotting.

2.13. GST Fusion Protein Production and GST Pull-down Assay

pGEX-2T or pGEX-3-14-3-3 zeta plasmid was transformed to *E.coli* (DH5α), and then *E.coli* (DH5α) were grown in 5 mL of LB (1% tryptone, 0.5% yeast extract, 1% NaCl) overnight in the 37℃ incubator. The broth was seeded to 100 ml of LB and cultured for 2 hours in the 37℃ incubator. 0.1 mM IPTG was added to induce production of GST fusion protein (GST only or GST-14-3-3) for 2 hours, and then cells

were harvested and re-suspended in PBST (1X PBS containing 1% triton X-100). The cell suspension was frozen (liquid nitrogen) and thawed (37℃ water bath) 4 times. The cell suspension was lysed completely using Bioruptor® (Diagenode; UCD-200) with high voltage for 10 minutes. Last, the cell suspension was centrifuged for 20 minutes at 13,000 rpm, 4℃. The supernatant was collected and purified with Glutathione Sepharose 4B (Amersham Biosciences, GE Healthcare) by incubating at 4℃ for 1 hour and washing 3 times with PBST. The pulled down complex was analyzed by 10% SDS-PAGE and Coomassie blue staining to check the expression of GST-tagged protein.

LPS-stimulated RAW264.7 cells from 10-cm dishes were harvested and lysed in LSBT buffer (20 mM HEPES pH 7.9, 100mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1.5% Triton X-100, 0.05% NP-40, protease inhibitor, phosphatase inhibitor). The cell lysates were shaken at 4℃ for 30 minutes, and then centrifuged for 5 minutes at 13,000 rpm, 4℃. The lysates of GST-fusion protein were incubated with glutathione beads at 4 ℃ for 1 hour. The beads were washed two times with PBST and then once with LBST buffer. The beads conjugated with GST fusion protein were incubated with the lysates form LPS-stimulated RAW264.7 cells at 4℃ for 2 hours, and then the beads were washed three times with LBST buffer. The protein complexes were separated by 10%

SDS-PAGE and analyzed by Western blotting.

2.14 Statistical Analysis

All of the results were presented as the mean ± SD of at least three independent experiments. The statistically significant values were calculated by one-tailed Student's t-test. One asterisk indicated p-value < 0.05, and two asterisks indicated p-value < 0.01.

3. Specific Aims

1.Analysis of expression kinetics of TTP family proteins in the period of LPS-stimulation in RAW264.7 cells.

2. Identification of novel Zfp36l1 and Zfp36l2-targeted mRNAs.

3. Molecular mechanism of which Zfp36l1 and Zfp36l2 regulate Mkp-1 mRNA stability in LPS-stimulation.

4. Functional mechanism of Zfp36l1 and Zfp36l2 in the innate immune response.

4. Results

4.1 The Consistent Expression and Protein Phosphorylation of Zfp36l1 and Zfp36l2 in the Period of LPS-stimulation in RAW264.7 Cells.

The TTP family contains four major members, Ttp, Zfp36l1, Zfp36l2, and rodent specific Zfp36l3. It is well known that Ttp plays a key role in innate immune response. The mRNA and protein of Ttp were highly induced by LPS (Figure 1A), and it was suggested to destabilize mRNA targets rapidly. To investigate the roles of other two TTP family proteins, Zfp36l1 and Zfp36l2, in the innate immune response, we first examined their RNA and protein expression profiles in LPS-stimulated RAW264.7 cells. The proteins of Zfp36l1 and Zfp36l2 were maintained in the consistent level in cytoplasm (Figure 1A), whereas their mRNA expression levels were down-regulated in the period of LPS-stimulation (Figure 1B). Multiple forms of Zfp36l1 and Zfp36l2 were detected in Western blotting and LPS treatment resulted in their band shifts (Figure 1A). When the cytosolic extracts (100μg) from 120 minutes of LPS-stimulation were treated with alkaline phosphatase (CIP) for 30 minutes, the higher protein bands of Zfp36l1 and Zfp36l2 were back to the lower positions (Figure 1C), which might indicate a status of protein hypo-phosphorylation. These observations suggested that the protein levels of Zfp36l1 and Zfp36l2 were maintained constantly and were phosphorylated under

LPS-stimulation.

4.2 Zfp36l1 and Zfp36l2 Destabilize MAPK Phosphotase-1 (*Mkp-1*) and Cyclooxygenase-2 (*Cox-2*) mRNAs in Resting RAW264.7 Cells.

Since Zfp36l1 and Zfp36l2 were constitutively expressed in macrophages, we inferred that they play roles to control mRNA stability in the resting condition. The strategy to identify the mRNA targets of Zfp36l1 and Zfp36l2 was knockdown of Zfp36l1 and Zfp36l2 using Lentivirus-carrying shRNAs in RAW264.7 cells. As shown in Figure 2A, the knockdown efficiency of shRNA specific to *Zfp36l1* and *Zfp36l2* was confirmed by Western blotting. ARE-containing immediate early genes as well as inflammatory mediator genes were target candidates of Zfp36l1 and Zfp36l2, such as *Ttp*, *Mkp-1*, *Tnf-α*, *Cox-2*, *Ccl-2*, and *Icam-1*. The RNA and protein expression of these candidates were examined by real-time PCR and Western blotting in different knockdown cells, including Zfp36l1 knockdown, Zfp36l2 knockdown, and dual knockdown cells. We predicted that the mRNA targets of Zfp36l1 and Zfp36l2 would be increased in knockdown cells because Zfp36l1 and Zfp36l2 function in mRNA destabilizing. We found that the RNA (Figure 2B) and protein expressions (Figure 2A) of Mkp-1 were significantly increased in all knockdown cells, whereas the RNA (Figure

2C) and protein expressions (Figure 2A) of Cox-2 were significantly increased in Zfp3l2 knockdown cells and dual knockdown cells but not in Zfp36l1 knockdown cells. On the contrary, mRNA levels of *Ttp*, *Tnf-α*, *Ccl-2*, and *Icam-1* were decreased in all knockdown cells (Figure 2F).

Mkp-1 and *Cox-2* mRNAs were possible targets of Zfp36l1 and Zfp36l2 in the resting condition of RAW264.7 cells. To further elucidate whether *Mkp-1* and *Cox-2* mRNAs were post-transcriptionally regulated by Zfp36l1 and Zfp36l2, we determined the mRNA half-lives of *Mkp-1* and *Cox-2* in different knockdown cells. As shown in Figure 2D, half-lives of *Mkp-1* mRNA increased from 15.2 minutes in control cells (Luciferase knockdown cells) to at least 41.6 minutes in all knockdown cells. Furthermore, Figure 2E showed that half-lives of *Cox-2* mRNA increased from 15.8 minutes in control cells (Luciferase knockdown cells) to at least 55 minutes in the cells which containing Zfp36l2 knockdown. Collectively, *Mkp-1* mRNA stability was down-regulated by both Zfp36l1 and Zfp36l2, and *Cox-2* mRNA stability was only down-regulated by Zfp36l2 in the resting macrophages.

4.3 Zfp36l1 and Zfp36l2 Down-regulate the *Mkp-1* and *Cox-2* 3'UTR-mediated Luciferase Reporter Activity and Interact with

Deadenylase Caf1a.

To delineate the molecular mechanism underlying Zfp36l- and Zfp36l2-regulated *Mkp-1* mRNA stability, the full-length 3'UTR of *Mkp-1* mRNA was cloned into downstream of the luciferase reporter gene for co-transfection analysis. The *Mkp-1* 3'UTR derived luciferase activity was reduced when co-transfected with Zfp36l1 or Zfp36l2 expression plasmid in HEK293T cells which did not constitutively express human ZFP36L1 or ZFP36L2 (Figure 3A). Similarity, the 3'UTR (1-1477 base pairs) of *Cox-2* mRNA was cloned into downstream of the luciferase reporter gene, and luciferase activity was also decreased when co-transfected with Zpf36l1 and Zfp36l2 expression plasmid in HEK293T cells (Figure 3B). The associated proteins of *Cox-2* 3'UTR were analyzed by the RNA pull-down. The *Cox-2* 3'UTR was separated into four fragments (Figure 3C, lower panel). Four biotinylated *Cox-2* 3'UTR fragments were incubated with cytosolic lysates from RAW264.7 cells. The streptavidin sepharoses were used to precipitate the ribonucleoprotein complexes. The complexes were analyzed by Western blotting with anti-Zfp36l1 and anti-Zfp36l2 (Figure 3C, upper). Both Zfp36l1 and Zfp36l2 could be pulled down by ARE-rich fragment 1 of *Cox-2* 3'UTR.

Deadenylase Caf1a was recruited by Ttp to result in destabilization of target

mRNAs [16]. To understand whether Caf1a associated with Zfp36l1 and Zfp36l2 for their mRNA destabilizing activity, Flag-tagged Zfp36l1or Zfp36l2 was co-expressed with myc-tagged Caf1a in HEK293T cells. In order to rule out the RNA mediated interactions, RNase A and RNase T1 were added to the lysates and they were incubated in 30℃ for 10 minutes. The lysates were precipitated by anti-Flag M2 affinity agarose beads. Ttp severed as a positive control, and mock (Flag only) severed as a negative control. The protein samples were detected by anti-Myc and anti-Flag. According to the results of Figure 3D, the interaction between TTP family proteins and Caf1a was RNA-independent. These results suggested that through ARE-containing 3'UTR, Zfp36l1 and Zfp36l2 could down-regulate *Mkp-1* and *Cox-2* mRNA expressions, which might be due to the recruitment of deadenylase Caf1a.

4.4 Regulation of *Mkp-1* mRNA Stability by Phosphorylation of Zfp36l1.

To further investigate the regulation of *Mkp-1* mRNA during the early LPS-stimulation in RAW264.7 cells, we examined its RNA expression profiles in LPS-stimulated RAW264.7 cells (Figure 4A). *Mkp-1* mRNA level was increased significantly after LPS-stimulation from 15 to 30 minutes. However, *Mkp-1* mRNA

level was decreased rapidly after LPS-stimulation from 45 to 60 minutes. In order to demonstrate that the rise of *Mkp-1* mRNA level at the early LPS-stimulation was regulated by mRNA stability, we examined the mRNA half-lives of *Mkp-1* in the resting condition and LPS-stimulation for 25 minutes. In Figure 4B, half-lives of *Mkp-1* mRNA increased from 15.3 minutes in the resting condition to 54.7 minutes LPS-stimulation for 25 minutes. This result suggested that the increase of *Mkp-1* mRNA in the early LPS-stimulation was regulated in part post-transcriptionally.

To understand how Zfp36l1 and Zfp36l2 regulate *Mkp-1* mRNA stability during LPS-stimulation, firstly, the RNA pull-down assay was performed to examine the RNA-protein interaction. The biotinylated *Mkp-1* 3'UTR was incubated with cytosolic lysates from LPS-stimulated RAW264.7 cells. The ribonucleoprotein complexes were precipitated by the streptavidin sepharoses and then subjected to SDS-PAGE for Western blotting with anti-Zfp36l1 and anti-Zfp36l2 (Figure 4C). The interaction of Zfp36l1 and *Mkp-1* 3′UTR was constant during LPS-stimulation, whatever Zfp36l1 was phosphorylated or not. However, the interaction of Zfp36l2 and *Mkp-1* 3′UTR was changeable, and it was depended on the protein amount of Zfp36l2 (Figure 4C). In addition, the amount of deadenylase Caf1a in the ribonucleoprotein complexes was decrease in the early stage of LPS-stimulation, and this change might stabilize *Mkp-1*

mRNA.

It had been reported that the interaction between phosphorylated Ser92 of Zfp36l1 and 14-3-3 protein could inhibit the mRNA decay activity of Zfp36l1 after insulin-stimulation [27,44]. Since LPS-induced Zfp36l1 phosphorylation did not affect its RNA binding activity (Figure 4C), GST pull-down assay was performed to study whether hyper-phosphorylated Zfp36l1 in the period of LPS-stimulation interacted with 14-3-3. As shown in Figure 4D, only hyper-phosphorylated Zfp36l1 formed the complex with 14-3-3. This complex might repress the mRNA destabilization function of Zfp36l1.

4.5 The Induction of *Mkp-1* mRNA in Early LPS-stimulation is Post-transcriptionally Modulated by Zfp36l1 and Zfp36l2.

To verify the functions of Zfp36l1 and Zfp36l2 in the initiation stage of *Mkp-1* expression, we examined the activation levels of *Mkp-1* mRNA in different knockdown cells after LPS-stimulation for 15 minutes. A marked rise of the activation levels in all knockdown cells compared with control knockdown cells implied that the decline of Zfp36l1 and Zfp36l2 protein expressions facilitated the mRNA expression of *Mkp-1*

(Figure 5A); what is more, half-lives of *Mkp-1* mRNA in control, Zfp36l1, Zfp36l2, or dual knockdown cells were 15.2, 41.6, > 60, and > 60, respectively (Figure 5B). This result indicated that knockdown of Zfp36l1, Zfp36l2, or dual proteins could stabilize *Mkp-1* mRNA in early LPS-stimulation.

4.6 p38 MAPK Activity is Regulated by Zfp36l1 and Zfp36l2 Through Mkp-1.

Inflammatory stimuli such as LPS activate the three major MAPKs pathways in mammals: p38 pathways, ERK, and JNK. MAPK pathways can activate innate immune response through the transcriptional induction and the post-transcriptional modulation [37,45]. Inactivation of MAPKs mediated by the de-phosphorylation activity of Mkp-1 [46]. MAPKs was inactivated by Mkp-1 through de-phosphorylation [46]. We examined the three MAPK activities in different knockdown cells. In the resting condition, p38 activity was down-regulated by the increase of Mkp-1 in all knockdown cells, but the activities of the other two MAPKs were consistent (Figure 6A). The time courses of Mkp-1 expression showed that Mkp-1 expression was detected earlier in all knockdown cells than in control cells in LPS-stimulation, and it was correlated with p38 activity (Figure 6B).

Furthermore, the activation of p38 pathway is required for the mRNA inductions of *Ttp* and *Tnf-α* in LPS stimulated RAW264.7 cells [47,34]. The mRNA expression levels of *Ttp* and *Tnf-α* were reduced in all knockdown cells in LPS-stimulation due to decrease of p38 activity by Mkp-1 (Figure 6C). In the other word, Zfp36l1 and Zfp36l2 are necessary for macrophages in the initiation of the innate immune response.

5. Discussion

The activities of MAPKs are necessary for the initiation of the innate immune response. Both the activation of transcription factors by phosphorylation and the stabilization of ARE-containing mRNA can help cells to produce pro-inflammatory mediators or cytokines. The activations of MAPKs are turned off within several minutes, and then the mRNAs of those pro-inflammatory mediators or cytokines will be degraded rapidly. Some negative regulators such as Mkp-1 (phosphatase) and Ttp (mRNA decay mediator) can help cells to return to the resting condition. Hence, the gene expressions should be tightly controlled in both transcription and post-transcription levels in the innate immune response.

We are the first to observe the protein expression profiles of endogenous TTP family proteins during the period of LPS-stimulation in mouse macrophage RAW264.7 cells. There are two interesting observations. One is the protein expression profiles of these three TTP family members were different, and the other is the uncorrelated mRNA and protein expression profiles of Zfp36l1 and Zfp36l2.

Ttp was induced after LPS-stimulation, and Zfp36l1 and Zfp36l2 were constitutive expressions (Figure 1A). This observation indicates that Zfp36l1 and Zfp36l2 may play important roles in the resting condition, and the low expressions of pro-inflammatory

mediators or cytokines are maintained by the mRNA destabilized abilities of Zfp36l1 and Zfp36l2.

As shown in Figure 1B, the mRNA expressions of Zfp36l1 and Zfp36l2 were down-regulated after LPS-stimulation, but the protein expressions of Zfp36l1 and Zfp36l2 were consistent. The mRNA expression profiles of Zfp36l1 and Zfp36l2 were uncorrelated with their protein profiles after LPS-stimulation (Figure 1B). Some similar observations of mRNA expression profiles of Zfp36l1 and Zfp36l2 were also reported by Liang *et al.* [48] and Cao *et al.* [49]. This uncorrelated relationship implys the expressions of Zfp36l1 and Zfp36l2 are regulated in the translational and post-translational levels. The polysome analysis of their mRNA during LPS-stimulation can be performed to verify the translational regulation. Furthermore, the post-translational modifications such as phosphorylation (Figure 1C) may alter protein stability [25]. Zfp36l1 and Zfp36l2 may be more stable in the hyper-phosphorylated forms than the hypo-phosphorylated forms. Thus, their protein expression levels are near consistent, even their mRNAs are decreased after LPS-stimulation.

To identify the possible mRNA targets of Zfp36l1 and Zfp36l2 in resting macrophages, we knocked down Zfp36l1 and Zfp36l2 by using Lentivirus-carrying shRNA. Based on previous reports, the candidates of mRNA targets are chosen by the

ARE number in their 3'UTR, the associations with Ttp, and their mRNA expression profiles which were categorized to the immediate-early genes [39,50].

In our study, the RNA and protein levels of Mkp-1 were increased in Zfp36l1, Zfp36l2, and dual knockdown cells (Figure 2A, 2B). In addition, the RNA and protein levels of Cox-2 were increased in the cells which Zfp36l2 was knocked down (Figure 2A, 2D). The mRNA expression levels of *Mkp-1* and *Cox-2* were increased through the mRNA stabilities when Zfp36l1 and Zfp36l2 were knocked down, which were correlated with the typical function of Zfp36l1 and Zfp36l2 in mRNA destabilization (Figure 2C, 2E).

Tnf-α is one of the well-known targets of TTP family proteins. Much to our surprise, the mRNA expressions of *Tnf-α* were decreased in all knockdown cells (Figure 2F). This result may be due to the importance of the transcription regulation of *Tnf-α* mRNA controlled by activation of p38 MAPK [35]. Therefore, the increase of Mkp-1 expression in Zfp36l1 and Zfp36l2 knockdown cells repressed the activity of p38 MAPK (Figure 6A), which down-regulated the *Tnf-α* mRNA expression. Similarly, the mRNA expression of *Ccl-2* is also activated by p38 MAPK [51]. However, the mRNA expression of *Ttp* and *Icam-1* were no significant differences whether Zfp36l1 and Zfp36l2 were knockdown or not (Figure 2F). The possible explanation is that the

expressions of their mRNAs are controlled equally in both transcriptional and post-transcriptional levels.

The mRNA targets of TTP family proteins are not all the same [52], although their RNA binding domains are highly conserved. In Figure 2A and 2D, *Cox-2* was the Zfp36l2-specific mRNA target. However, the activity of luciferase containing *Cox-2* 3'UTR was down-regulated by both over-expressed Zfp36l1 and Zfp36l2 (Figure 3B). This result indicates that both of Zfp36l1 and Zfp36l2 can recognize and destabilize the *Cox-2* mRNA in HEK 293T cells. However, overexpression of Zfp36l1 and Zfp36l2 in HEK 293T cells may not reflect their "real" functions and the "real" conditions in RAW264.7 cells. The RNA pull-down assay was shown that both of Zfp36l1 and Zfp36l2 could associate with *Cox-2* 3'UTR in RAW264.7 cells (Figure 3C). Both of Zfp36l1 and Zfp36l2 do not have enzyme activity of mRNA decay, so the difference of their associated proteins may alter their functions. Mass spectrometry may be used to identify the associated proteins of Zfp36l1 and Zfp36l2, and the detailed regulation can be further studied. In conclusions, the mRNA expression of *Cox-2* is Zfp36l2-specific regulation in the post-transcriptional level.

The rapid mRNA activation of *Mkp-1* after the LPS-stimulation was regulated through mRNA stability (Figure 4A, 4B). In Figure 4C, the interaction between Zfp36l1

and *Mkp-1* 3'UTR was consistent during LPS-stimulation, but the interaction between Zfp36l2 and *Mkp-1* 3'UTR was variable with the protein expression of Zfp36l2. This result suggests that the decline of Zfp36l2 on *Mkp-1* 3'UTR stabilizes *Mkp-1* mRNA. Knockdown of Zfp36l1 and Zfp36l2 increased the mRNA expression of *Mkp-1* through RNA stability in the early stage of LPS-stimulation (Figure 5A, 5B). This result confirms that the mRNA activation of Mkp-1 is regulated by Zfp36l1 and Zfp36l2 after LPS-stimulation.

In addition, the activation of Mkp-1 is also regulated by MAPKs in both transcriptional and post-translational levels. The activation of ERK signaling pathway increases the transcription of *Mkp-1* [53], and the activation of ERK and p38 MAPK signal pathway can increase the protein stability of Mkp-1 by phosphorylation [54,55].

Associated proteins may change the functions of Zfp36l1 and Zfp36l2. We were the first to observe that TTP family proteins, Zfp36l1 and Zfp36l2, could associate with deadenylase Caf1a and degrade mRNA targets by removing poly-A tails (Figure 4C). However, Zfp36l1 could be phosphorylated after LPS-stimulation and form the complex with 14-3-3 (Figure 4D). This complex may repress the function of Zfp36l1 [26], and *Mkp-1* mRNA can be stabilized.

Knockdown of Zfp36l1 and Zfp36l2 increased the protein expression of Mkp-1 (Figure 6B), and the activity of p38 MAPK was down-regulated in the resting condition (Figure 6A). Therefore, p-38 mediated mRNA expressions of *Ttp* and *Tnf-α* were repressed (Figure 6C). According to the results, we proposed the following model for the mechanism (Figure 7). In the resting condition, Zfp36l1 and Zfp36l2 destabilize mRNA of *Mkp-1*, and the cells are sensitive to the stimuli such as LPS under the low expression of *Mkp-1*. In LPS-stimulation, the induction of *Mkp-1* mRNA is done by hyper-phosphorylated Zfp36l1 which losing its ability of mRNA decay and decreasing the expression of Zfp36l2.

A number of important ARE-containing genes are commonly involved in inflammation and cancer [45]. Both MKP-1 and COX-2 have been reported playing important roles in human cancers. MKP-1 is over-expressed in many human cancer cell lines, including breast, lung, prostate, ovarian, pancreatic, liver, and gastric cancer [56], and due to MKP-1 expression, the lung and ovarian cell lines are resistant to chemotherapy such as cisplatin [57]. TTP mRNA and protein levels are significantly decreased in tumors of the thyroid, lung, ovary, uterus, and breast compared to non-transformed tissues [58,59]. Another study showed that the mRNA expression levels of TTP family proteins were repressed in lung and ovarian cancers [60]. However,

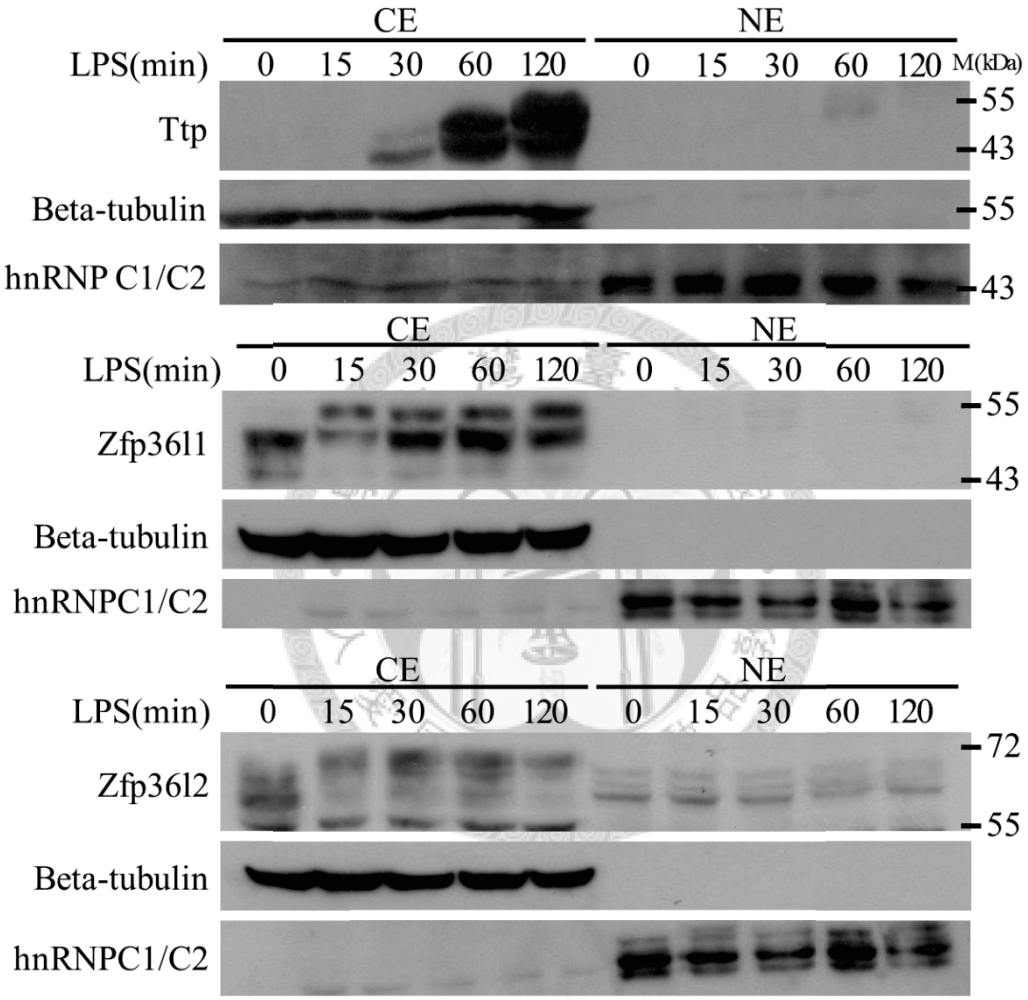
rare studies clearly elucidate the expression and function of three TTP family members in a cancer cell. Combining previous researches with our observations, the possible explanation of MKP-1 overexpression is losing of ZFP36L1 and ZPL36L2 in cancer cells, and the detailed mechanism can be further investigated.

The abnormal expression of COX-2 in breast cancer cells is reported in many studies[61,62], and it may involve in tumorigenesis and angiogenesis in the breast cancers [63,64]. The relationship between COX-2 and TTP is confirmed [65], but whether the overexpression of COX-2 is regulated by ZFP36L2 in breast cancer cells is unclear.

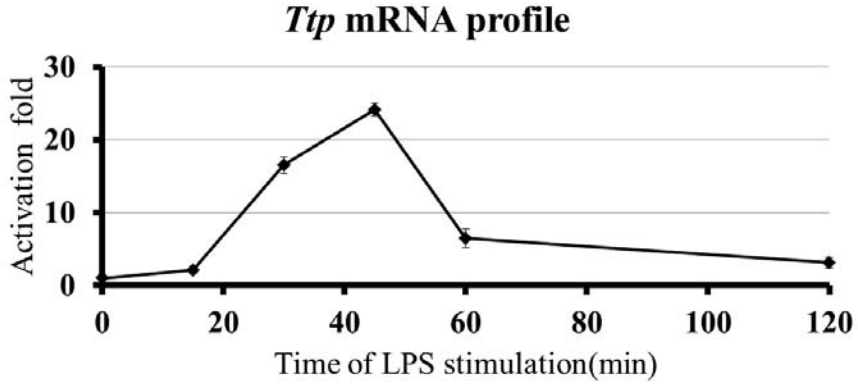
6. Figures

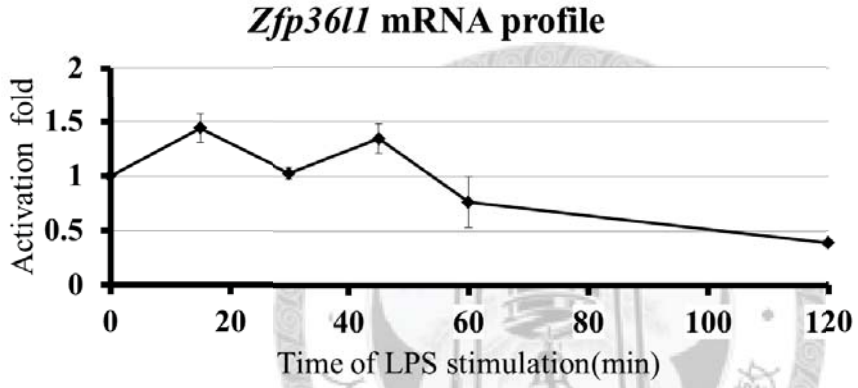
Figure 1.

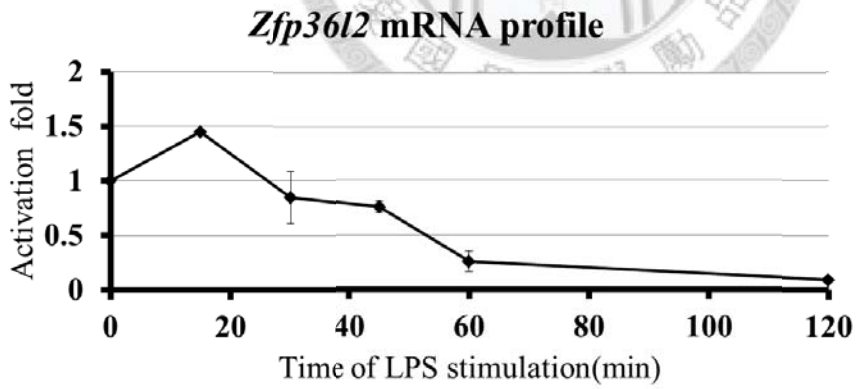
A



B


Ttp mRNA profile
Activation fold
30
20
10
0
0
20
40
60
80
100
120
Time of LPS stimulation(min)


Zfp36l1 mRNA profile
Activation fold
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Time of LPS stimulation(min)


Zfp36l2 mRNA profile
Activation fold
2
1.5
1
0.5
0
0
20
40
60
80
100
120
Time of LPS stimulation(min)

C

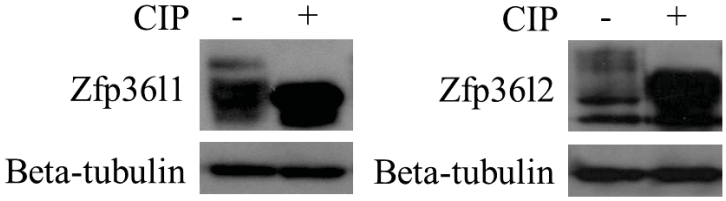
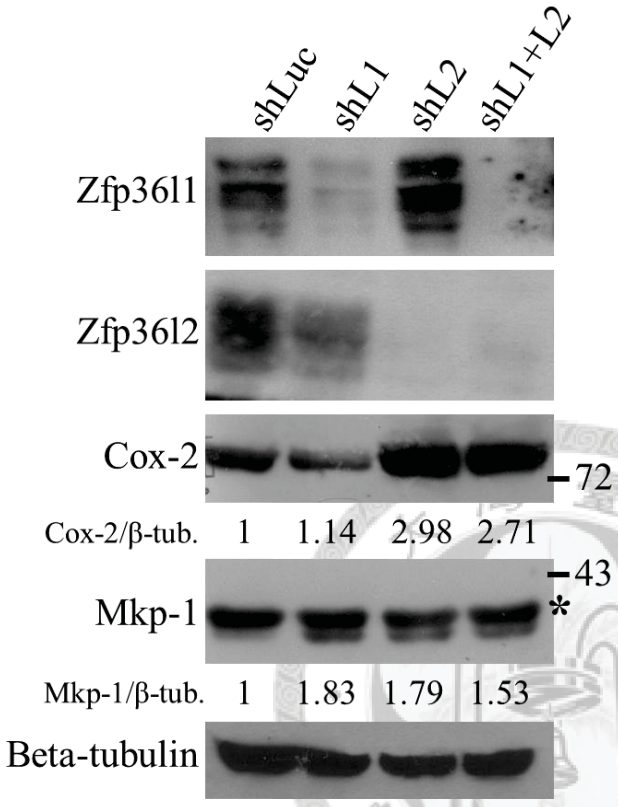

CIP - +
Zfp36l1
Beta-tubulin
CIP - +
Zfp36l2
Beta-tubulin

Figure 1. The consistent expression and protein phosphorylation of Zfp36l1 and Zfp36l2 in the period of LPS-stimulation in RAW264.7 cells.

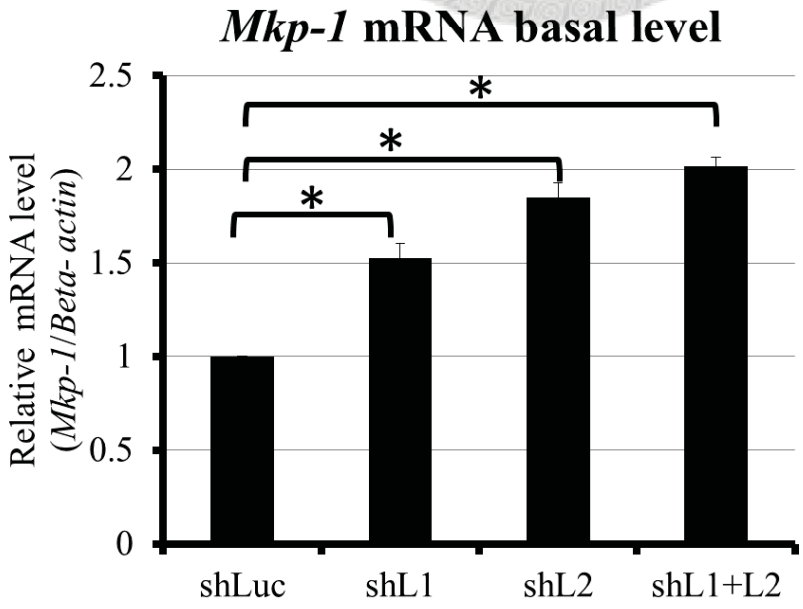
(A) Protein expression profiles of TTP Family members in LPS-stimulated RAW264.7 cells for 0, 15, 30, 60, 120 minutes. Beta-tubulin was a positive control for the cytosolic extract (CE), and hnRNPC1/C2 was a positive control for the nuclear extract (NE). **(B)** mRNA expression profiles of TTP Family members in LPS-stimulated RAW264.7 cells for 0, 15, 30, 60, 120 minutes. **(C)** The cytosolic extracts from LPS-stimulated RAW264.7 cells for 120 minutes were treated with alkaline phosphatase (CIP) for 30 minutes. The data indicated both Zfp36l1 and Zfp36l2 were phosphorylated after LPS-stimulation.

Figure 2.

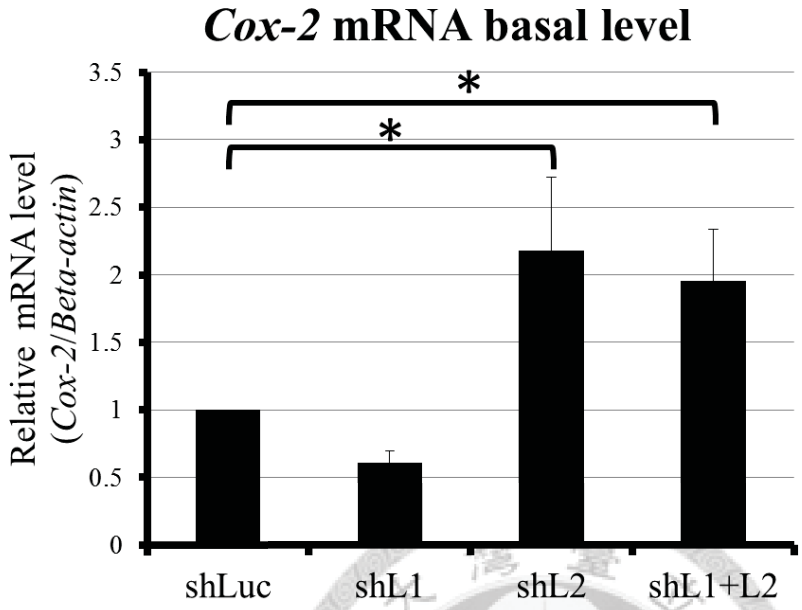
A


shLuc
shL1
shL2
shL1+L2
Zfp36l1
Zfp36l2
Cox-2
72
Cox-2/β-tub. 1 1.14 2.98 2.71
43
Mkp-1
*
Mkp-1/β-tub. 1 1.83 1.79 1.53
Beta-tubulin

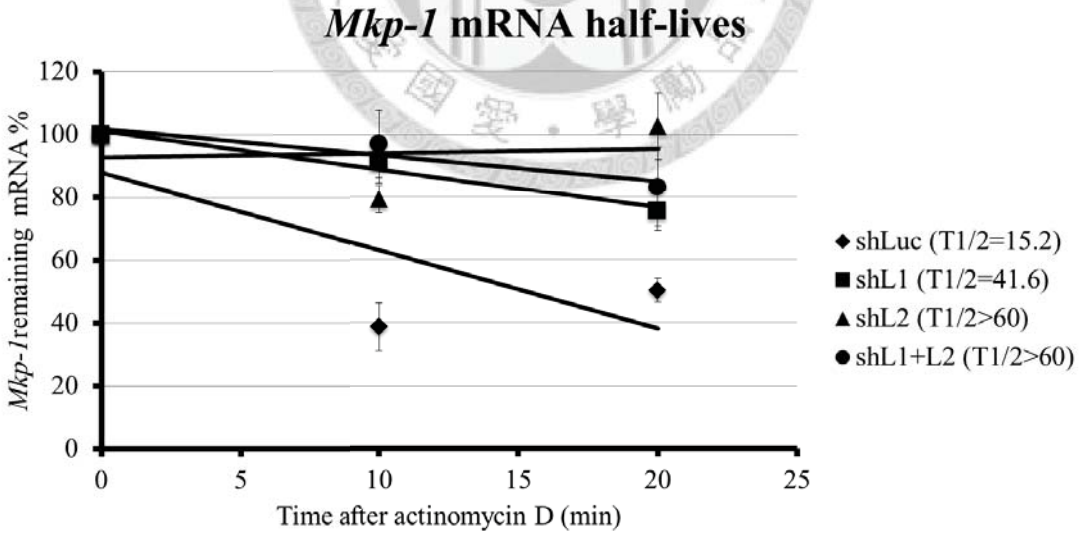
B


Mkp-1 mRNA basal level
Relative mRNA level (Mkp-1/Beta-actin)
2.5
2
1.5
1
0.5
0
*
*
*
shLuc
shL1
shL2
shL1+L2

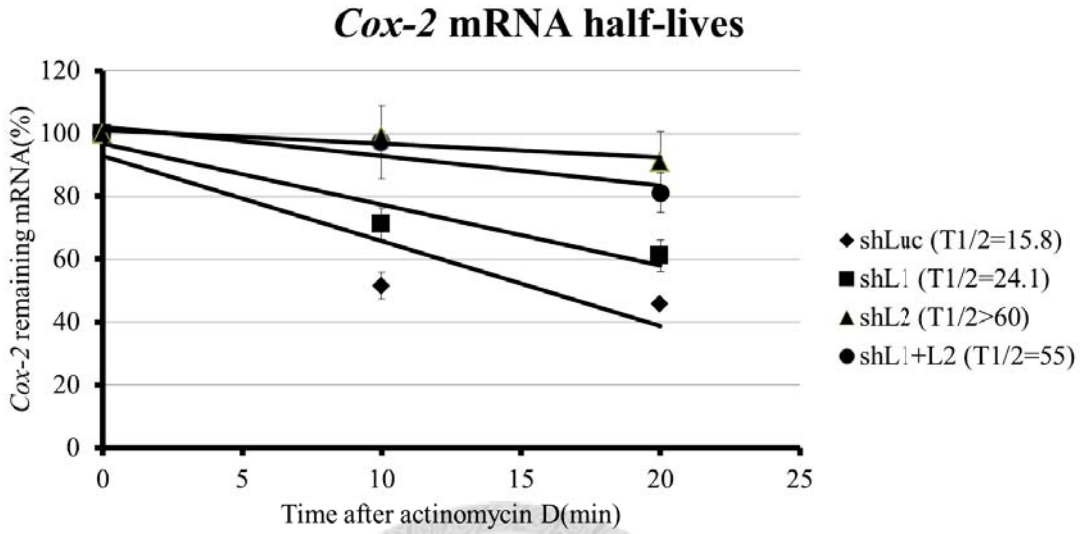
C


Cox-2 mRNA basal level
Relative mRNA level (*Cox-2*/*Beta-actin*)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
shLuc
shL1
shL2
shL1+L2

D


Mkp-1 mRNA half-lives
Mkp-1 remaining mRNA %
120
100
80
60
40
20
0
0
5
10
15
20
25
Time after actinomycin D (min)
shLuc (T1/2=15.2)
shL1 (T1/2=41.6)
shL2 (T1/2>60)
shL1+L2 (T1/2>60)

E



F

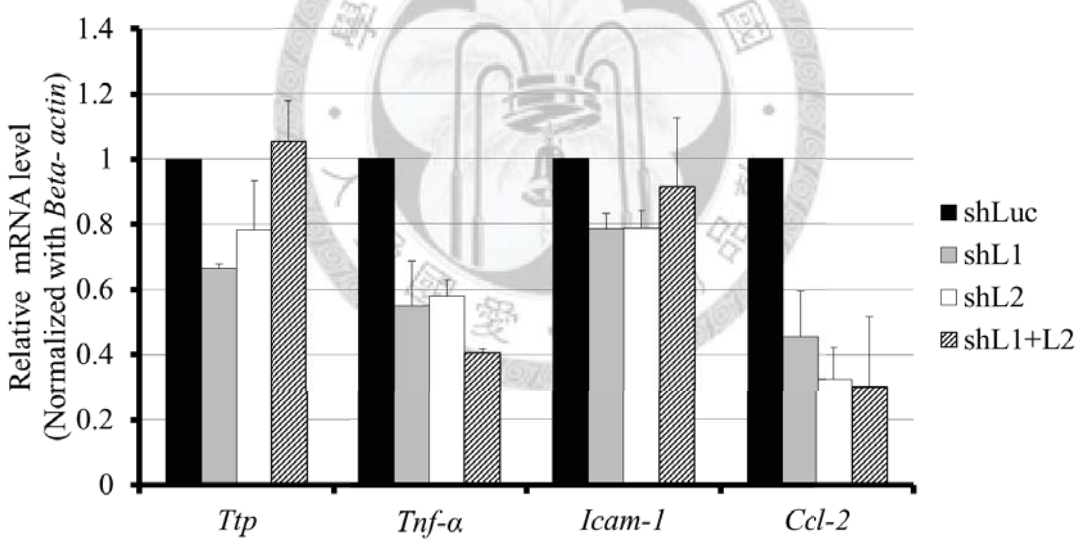


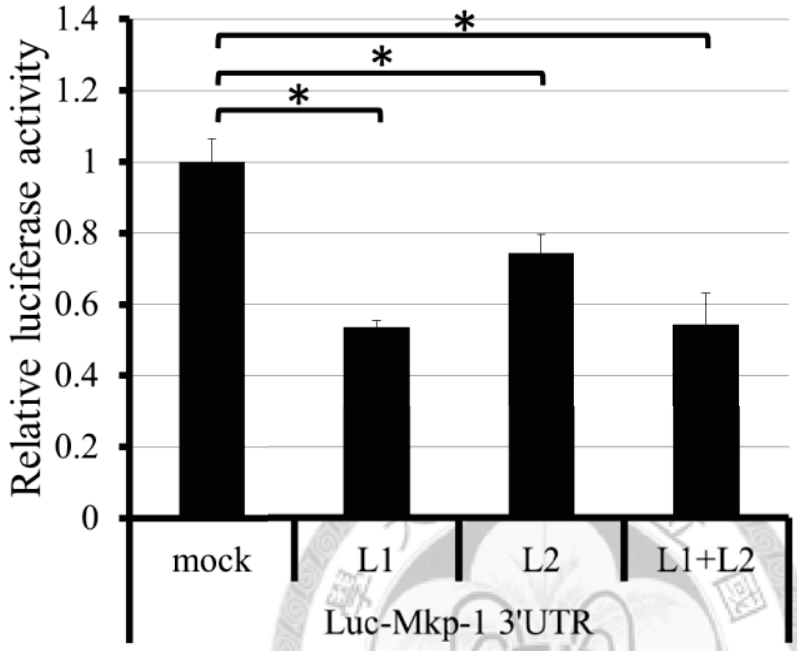
Figure 2. Zfp36l1 and Zfp36l2 destabilize MAPK phosphotase-1 (*Mkp-1*) and cyclooxygenase-2 (*Cox-2*) mRNAs in resting RAW264.7 cells.

(A) shLuc, shL1, shL2, shL1+L2 represented Luciferase knockdown cells, Zfp36l1 knockdown cells, Zfp36l2 knockdown cells, and dual Zfp36l1 and Zfp36l2 knockdown

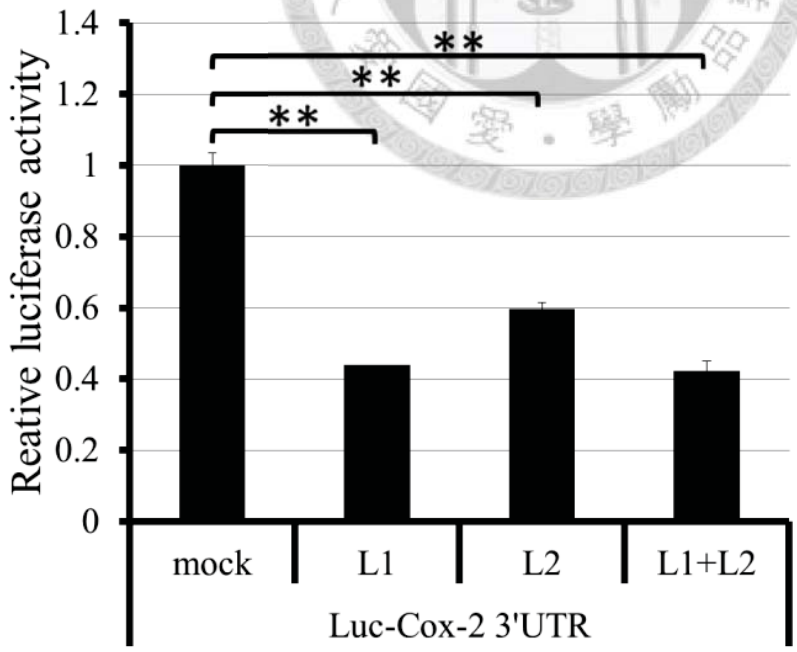
cells, respectively. The upper two panels showed the efficiency of knockdown. The third and the forth panels showed the protein levels of Mkp-1 and Cox-2 in different knockdown cells. Beta-tubulin was a loading control. shLuc cells were used as reference sample to calculate the relative expression which indicated in the panels. The asterisk indicated the Mkp-2. **(B)** The basal mRNA levels of *Mkp-1* (detected by quantitative PCR) in different knockdown cells. The asterisk indicated p-value < 0.05. **(C)** The basal mRNA levels of *Cox-2* (detected by quantitative PCR) in different knockdown cells. The asterisk indicated p-value < 0.05. **(D)** Analysis of *Mkp-1* mRNA half-lives in different knockdown cells. The 10 μg/mL of actinomycin D was added to stop transcription for 0, 10, and 20 minutes. Remaining mRNA was detected by quantitative PCR. *Mkp-1* mRNA half-lives was calculated by linear regression, and the half-life of shLuc, shL1, shL2, shL1+L2 were 15.2 minutes, 41.6 minutes, > 60 minutes, and > 60 minutes, respectively. **(E)** Analysis of *Cox-2* mRNA half-lives in different knockdown cells. *Cox-2* mRNA half-lives were calculated by linear regression, and the half-lives of shLuc, shL1, shL2, shL1+L2 were 15.8 minutes, 24.1 minutes, > 60 minutes, and 55 minutes, respectively. **(F)** The basal mRNA levels of *Ttp*, *Tnf-α*, *Icam-1*, and *Ccl-2* (detected by quantitative PCR) in different knockdown cells.

Figure 3.

A


Relative luciferase activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
mock
L1
L2
L1+L2
Luc-Mkp-1 3'UTR

B


Reative luciferase activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
mock
L1
L2
L1+L2
Luc-Cox-2 3'UTR

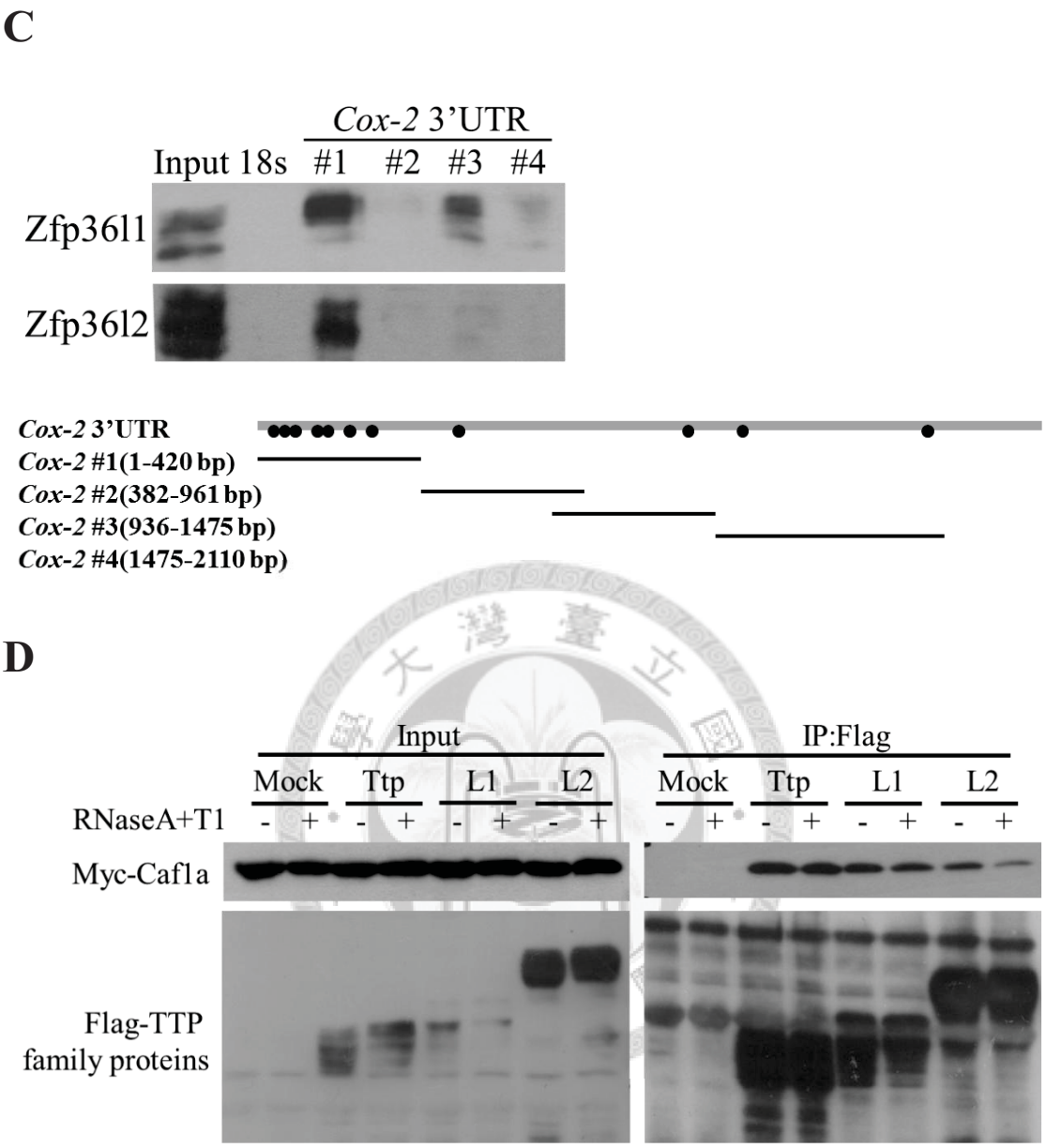


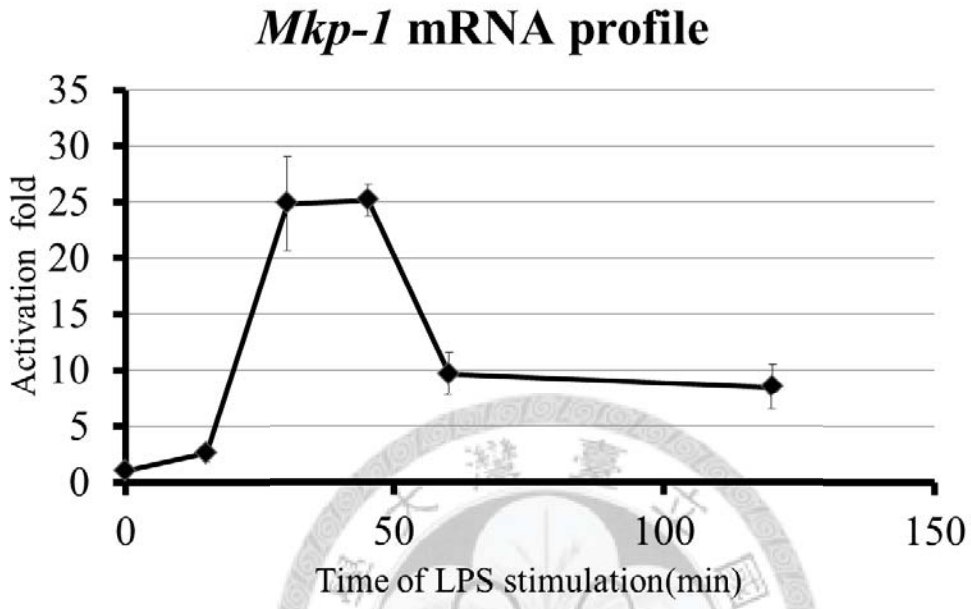
Figure 3. Zfp36l1 and Zfp36l2 down-regulate the *Mkp-1* and *Cox-2* 3'UTR -mediated luciferase reporter activity and interact with deadenylase Caf1a.

(A)(B) Luciferase reporter assay. HEK293T cells were co-transfected with pCMV-Flag-Zfp36l1 (L1) or pCMV-Flag-Zfp36l2 (L2) or both together (L1+L2) and pCMV-Luciferase reporter construct containing *Mkp-1* 3'UTR or *Cox-2* 3'UTR. The firefly

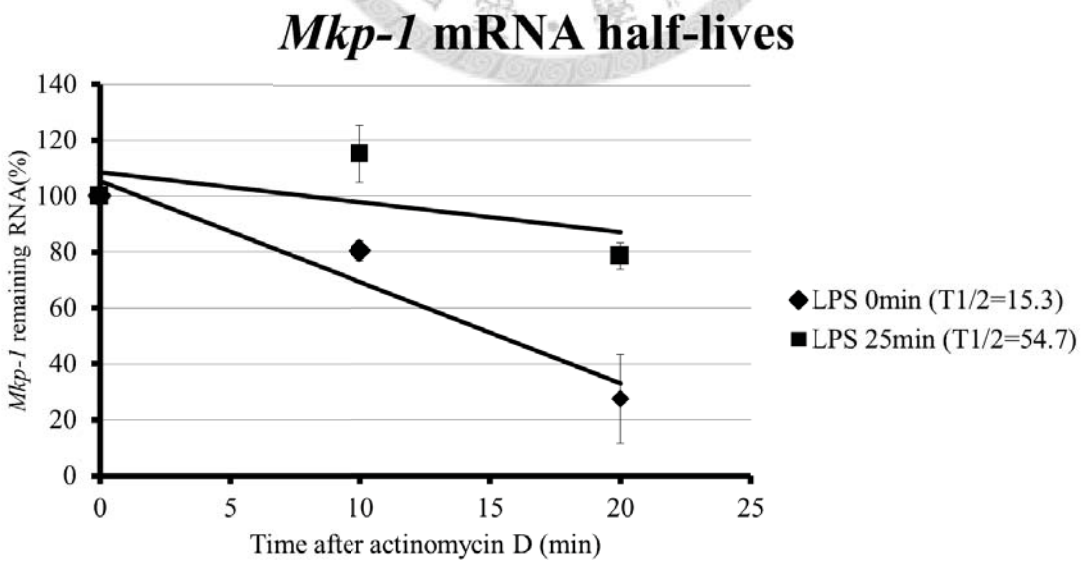
luciferase reporter activities were normalized with relina luciferase activity to correct for the transfection efficiency. The relative luciferase activity represents the luciferase activity of reporter carrying 3'UTR normalized with that of reporter alone. One asterisk indicated p-value < 0.05, and two asterisks indicated p-value < 0.01. **(C)** RNA pull-down assay. *Cox-2* 3'UTR was separated into four fragments as indicated. Four Biotinylated-*Cox2* 3'UTR fragments were incubated with cytosolic extracts from RAW264.7 cells. The biotinylated RNAs and associated proteins were precipitated by strepavidin beads. Biotinylated 18s rRNA was a negative control. The lower panel show the schematics RNA probes of the *Cox-2* 3' UTR and the dots indicate AREs. **(D)** Co-immunoprecipitation. Flag-tagged TTP family proteins and Myc-tagged Caf1a were overexpressed in HEK293T cells, and Flag-tagged TTP family proteins were precipitated by anti-Flag M2 affinity agarose beads. RNase A and RNase T1 were added to digest RNA. The Co-immunoprecipitated Myc-tagged Caf1a was detected by anti-Myc. The interaction between TTP family proteins and Caf1a was RNA-independent.

Figure 4.

A


Mkp-1 mRNA profile
Activation fold
35
30
25
20
15
10
5
0
0
50
100
150
Time of LPS stimulation(min)

B


Mkp-1 mRNA half-lives
Mkp-1 remaining RNA(%)
140
120
100
80
60
40
20
0
0
5
10
15
20
25
Time after actinomycin D (min)
LPS 0min (T1/2=15.3)
LPS 25min (T1/2=54.7)

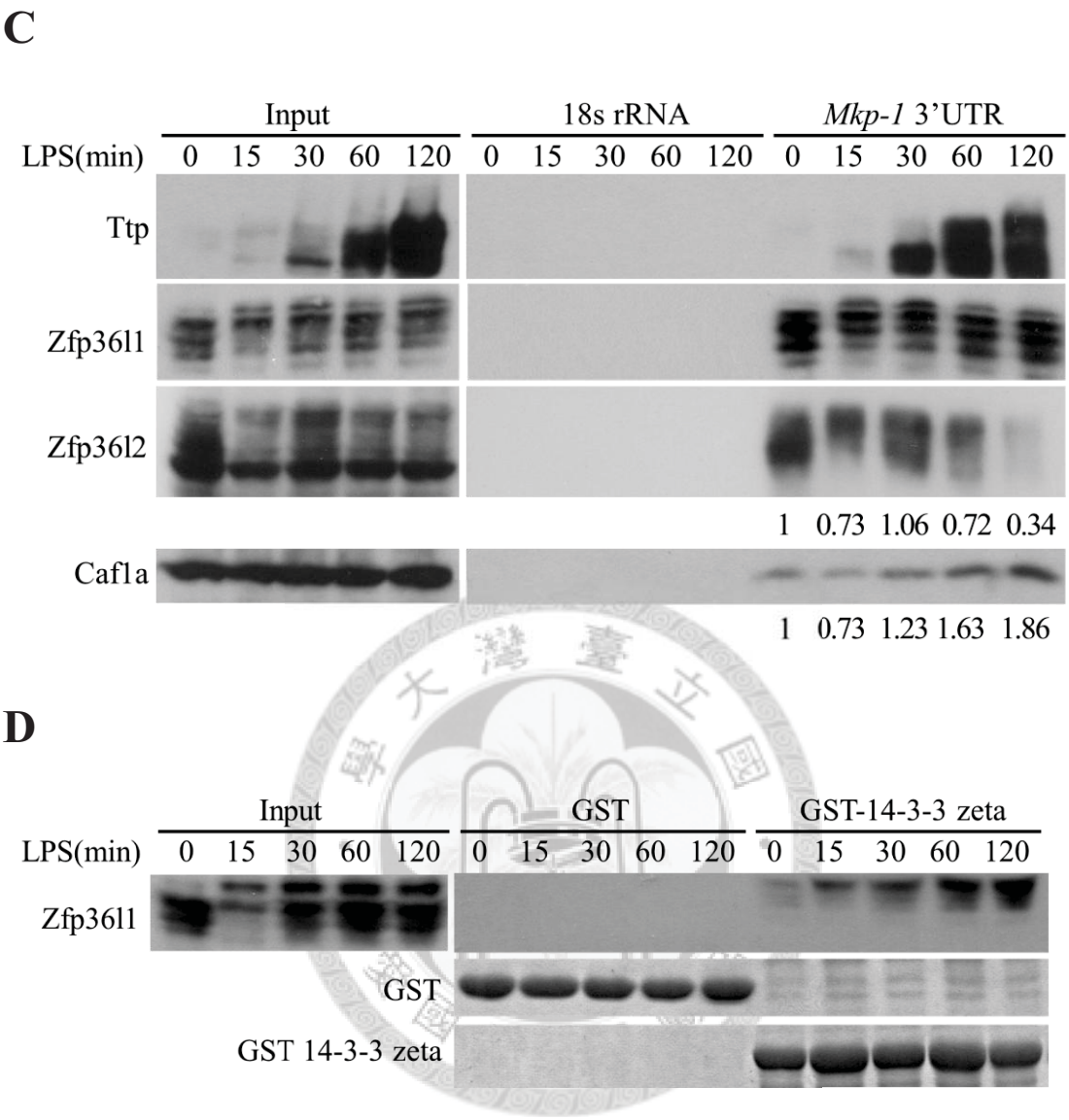


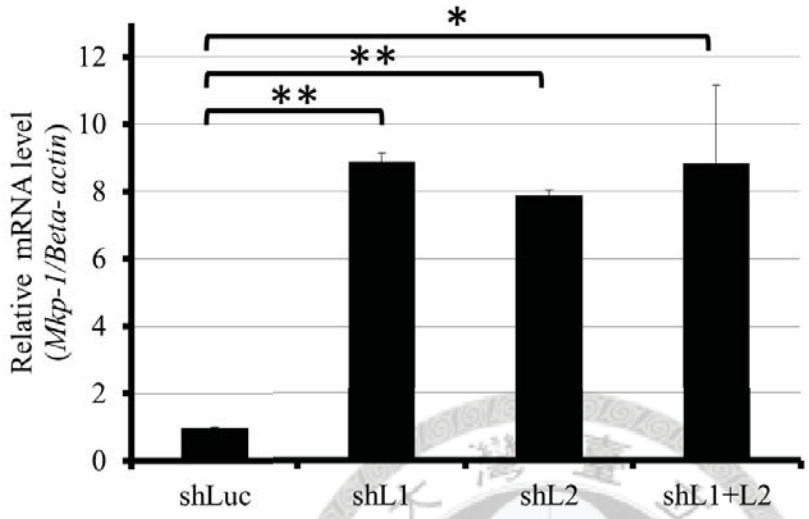
Figure 4. Regulation of *Mkp-1* mRNA stability by phosphorylation of Zfp36l1.

(A) mRNA expression profile of *Mkp-1* in LPS-stimulated RAW264.7 cells for 0, 15, 30, 45, 60, 120minutes. **(B)** Analysis of *Mkp-1* mRNA half-lives in LPS-stimulated RAW264.7 cells for 0 minute and 25 minutes. The 10 μg/mL of actinomycin D was added to stop transcription for 0, 10, and 20 minutes. Remaining mRNA was detected by quantitative PCR. *Mkp-1* mRNA half-lives was calculated by linear regression, and

the half-lives of LPS-stimulated RAW264.7 cells for 0minute and 25 minute were 15.3 minutes, 54.7 minutes, respectively. **(C)** RNA pull-down assay. Biotinylated-*Mkp-1* 3'UTR was incubated with cytosolic extracts from LPS-stimulated RAW264.7 cells for 0, 15, 30, 60, 120 minutes. The biotinylated RNA and associated proteins were precipitated by strepavidin beads. Biotinylated 18s rRNA was a negative control. The resting cells were used as reference sample to calculate the relative quantity which indicated in the panels. **(D)** *In vitro* GST pull-down assay. *E.coli* expressed GST-14-3-3 zeta or GST were bound on Glutathione Sepharose 4B. The beads were incubated with the cell lysates from LPS-stimulated RAW264.7 cells for 0, 15, 30, 60, 120 minutes. The pulled down protein complexes were analyzed by Western blotting with anti-Zfp36l1

Figure 5. .

A



B

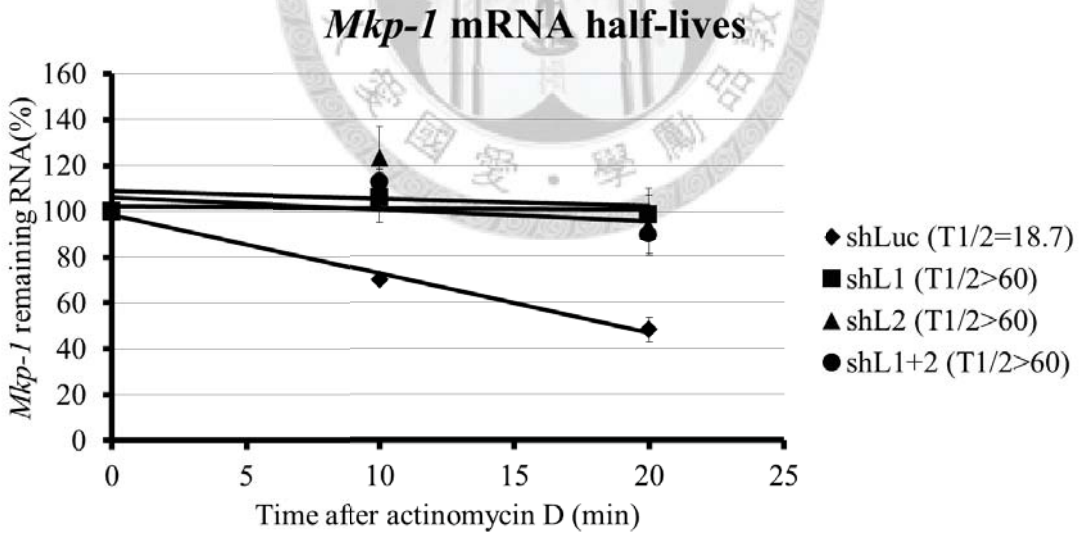
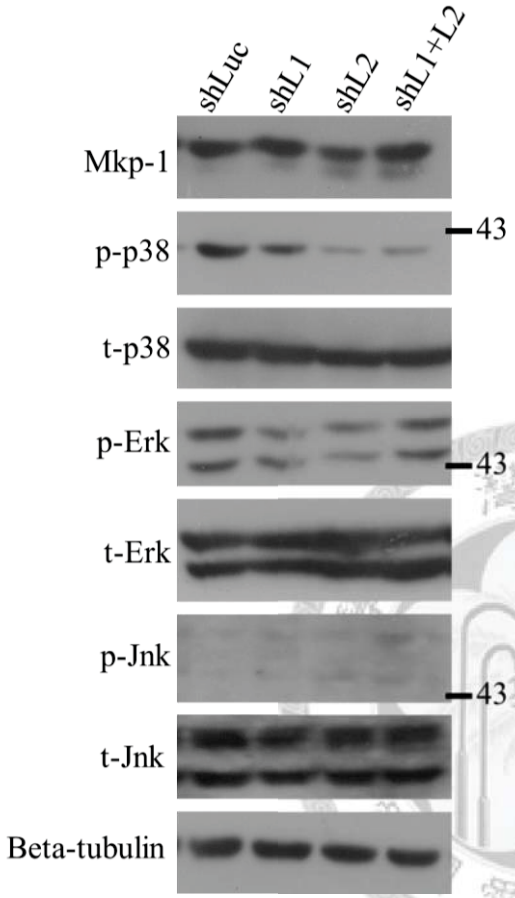


Figure 5. The induction of *Mkp-1* mRNA in early LPS-stimulation is post-transcriptionally modulated by Zfp36l1 and Zfp36l2.

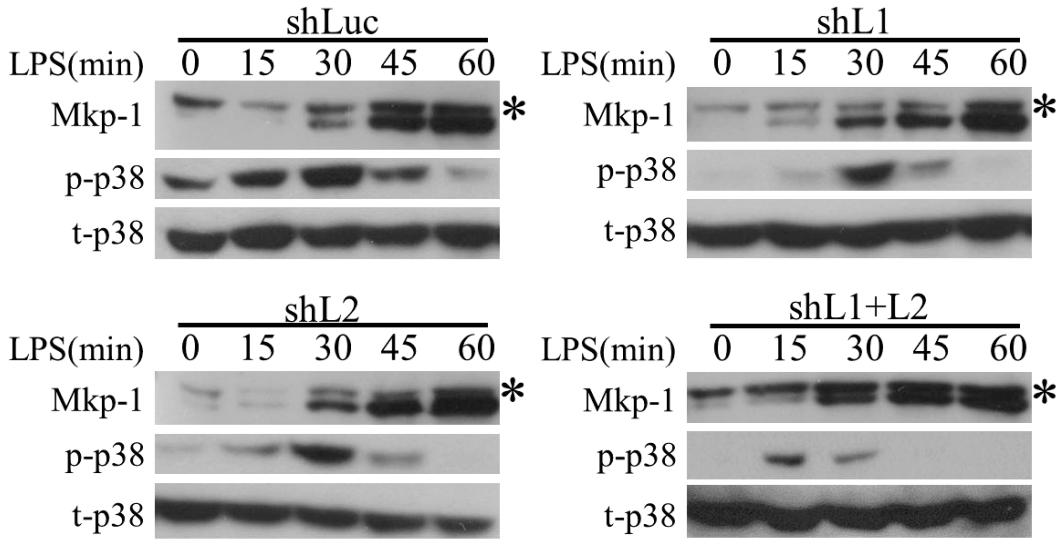
(A) The mRNA levels of *Mkp-1* (detected by quantitative PCR) in different knockdown cells after LPS-stimulation for 15minutes. One asterisk indicated p-value < 0.05, and two asterisks indicated p-value < 0.01. **(B)** Analysis of *Mkp-1* mRNA half-lives in different knockdown cells after LPS-stimulation for 15minutes. The 10 μg/mL of actinomycin D was added to stop transcription for 0, 10, and 20 minutes. Remaining mRNA was detected by quantitative PCR. *Mkp-1* mRNA half-lives was calculated by linear regression, and the half-lives of shLuc, shL1, shL2, shL1+L2 were 18.7 minutes, > 60 minutes, > 60 minutes, and > 60 minutes, respectively.

Figure 6.

A


shLuc
shL1
shL2
shL1+L2
Mkp-1
p-p38
43
t-p38
p-Erk
43
t-Erk
p-Jnk
43
t-Jnk
Beta-tubulin

B


shLuc
LPS(min) 0 15 30 45 60
Mkp-1
p-p38
t-p38
shL1
LPS(min) 0 15 30 45 60
Mkp-1
p-p38
t-p38
shL2
LPS(min) 0 15 30 45 60
Mkp-1
p-p38
t-p38
shL1+L2
LPS(min) 0 15 30 45 60
Mkp-1
p-p38
t-p38

C

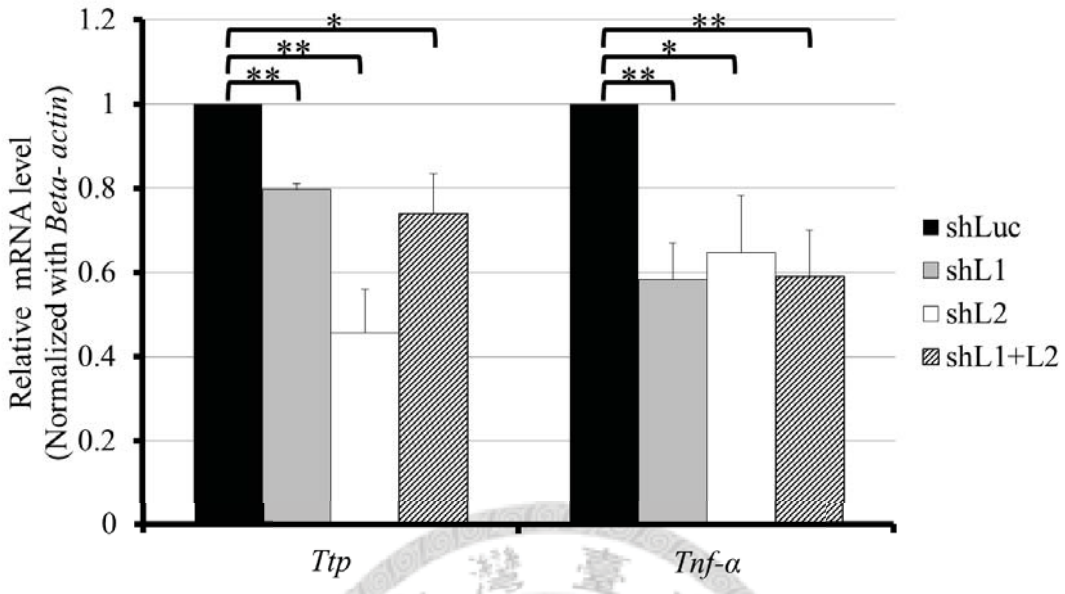


Figure 6. p38 MAPK activity is regulated by Zfp36l1 and Zfp36l2 through Mkp-1.

(A) Basal activities of three MAPKs in different knockdown cells. Whole cell extracts were isolated from RAW264.7 cells with different knockdown as indicated. Western blotting was performed to detect the expressions of MAPKs and phosphor-MAPKs. The asterisk indicated the Mkp-2. **(B)** Mkp-1 expression profiles in different knockdown cells stimulated with LPS for 0, 15, 30, 45, 60 minutes. The activity of p38 was correlated to the expression of Mkp-1. The asterisk indicated the Mkp-2. **(C)** The relative expression levels of *Ttp* and *Tnf-α* mRNA (detected by quantitative PCR) in different knockdown cells after LPS-stimulation for 15 minutes. One asterisk indicated p-value < 0.05, and two asterisks indicated p-value < 0.01.

Figure 7.

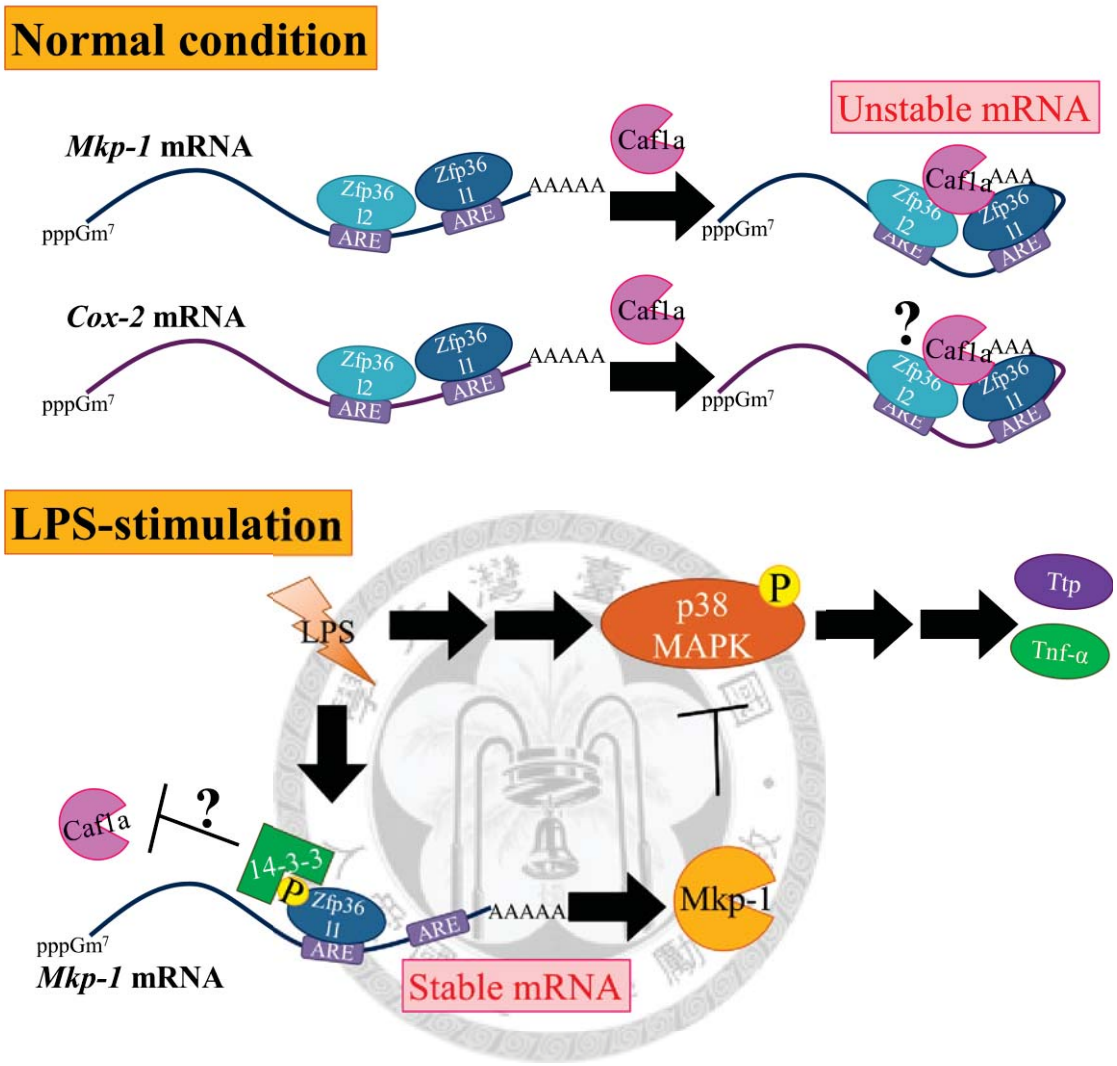


Figure 7. Hypothesized regulatory networks between Zfp36l1, Zfp36l2, Mkp-1, and p38 MAPK in RAW264.7 cells.

7. Tables

Table 1. Primers for PCR

gene	primers
Caf1a	F: 5'-CGGATCCATGCCAGCAGCAACCGTAGATC-3'
	R: 5'-CGAAGCTTTCATGACTGCTTGCTGGCTTC-3'
Ttp	F: 5'-CTCAGAGACAGAGATACGATTG-3'
	R: 5'-ATGGATCTCGCCATCTAC-3''
Mkp-1	F: 5'-AGGTGTGGAGTTTCACTTGCC-3'
	R: 5'-CCCAGTAACAAAATGTCTTCAC-3'
14-3-3 zeta	F: 5'-ATGGATAAAAATGAGCTGG-3'
	R: 5'-CCAGCTCATTTTTATCCAT-3'
Zfp36l1	F: 5'-ATGACCACCACCCTCGTGTC-3'
	R: 5'-TTAGTCATCTGAGATGGAGAG-3
Zfp36l2	F: 5'-ATGTCGACCACACTTCTGTCAC-3'
	R: 5'-TCAGTCGTCGGAGATGGAGAGGCG-3'

Table 2. Primers for real-time PCR

gene	primers
Cox-2	F: 5’-TGGAGGCGAAGTGGGTTTTA-3’
	R: 5’-GTTTTGGTAGGCTGTGGATCTTG-3’
Ttp	F: 5’-GGATCTCTCTGCCATCTACGA-3’
	R: 5’-CAGTCAGGCGAGAGGTGAC-3’
Mkp-1	F: 5’-TAGACTCCATCAAGGATGCTGG-3’
	R: 5’-GCAGCTTGGAGAGGTGGTGAT-3’
Tnf-α	F: 5'-GACCCTCACAC TCAGATCATCTTCT-3'
	R: 5'-CCTCCACTTGGTGGTTTGCT-3'
Ccl-2	F: 5’-CTGAAGACCTTAGGGCAGAT-3’
	R: 5’-AAGGAATGGGTCCAGACATAC-3’
Icam-1	F: 5’-TGTCAGCCACTGCCTTGGTA-3’
	R: 5’-CAGGATCTGGTCCGCTAGCT-3’
Beta-actin	F: 5’-TCCTTCCTGGGCATGGAGTC-3’
	R: 5’-ACTCATCATACTCCTGCTTG-3’
Zfp36l1	F: 5’-TGAGCGAAGTTTTATGCAAGGG-3’
	R: 5’-GCTGGGCAGAGTGACCGAG-3’
Zfp36l2	F: 5’-GATGTCGACTTGTTGTGCAAGACG-3’
	R: 5’-GCGTCCCTACCGCCTTCT-3’

8. Appendix

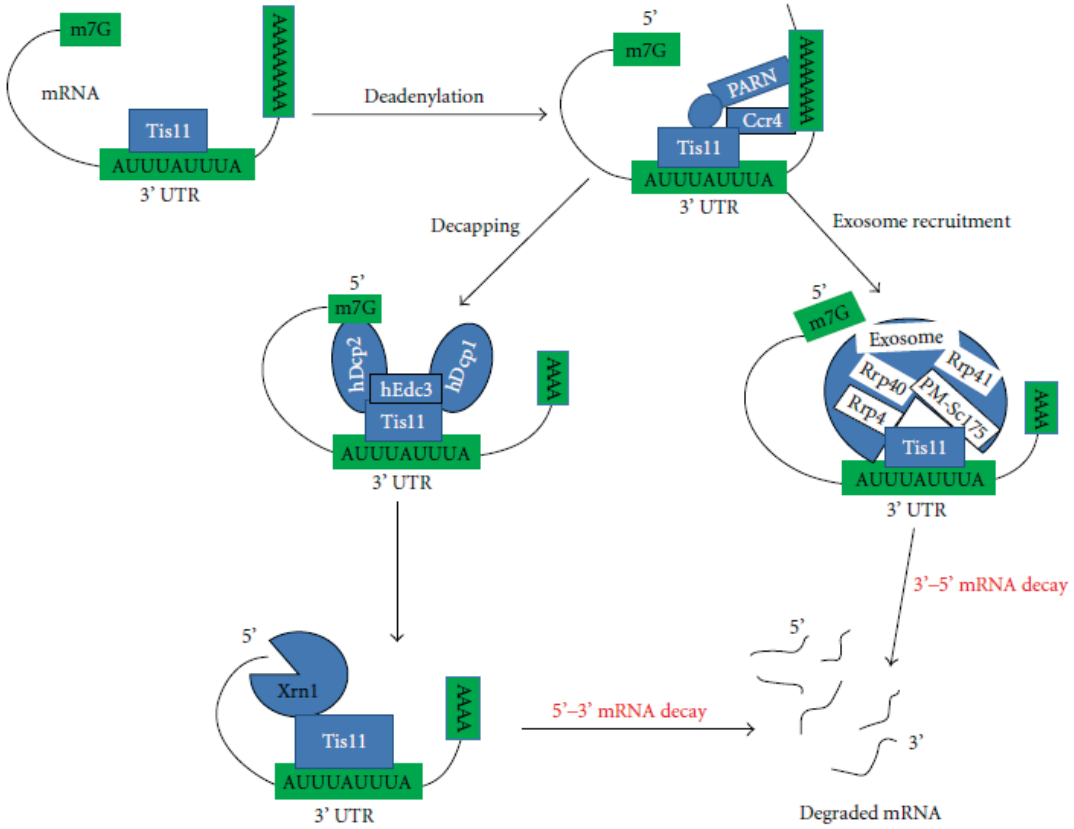
Appendix 1. mRNA targets of human TTP family proteins.

[8]

TTP	Cellular Process	mRNA Targets
	Immune response	Ccl2, Ccl3 CD86 COX-2 DUSP1 (MKP-1) E47 GM-CSF2 IDO Interleukins(IL-2, IL-32, IL-8, IL-10, IL-12) IFN-γ MHC (Class 1B and F) PAI-2 SOD2 TNF-α TIS11(TTP)
	Cell cycle	Cyclin D1 MIP-2 p21 Plk3
	Carcinogenesis	c-myc, c-fos E6-AP
	Angiogenesis	VEGF1
	Development	PITX2
	Protein glycosylation	1,4-galactosyltransferase
ZFP36L1	Immune response	TNF-α
	Angiogenesis	VEGF1
	Hormone activated steroidogenesis	STAR
ZFP36L2	Immune response	TNF-α

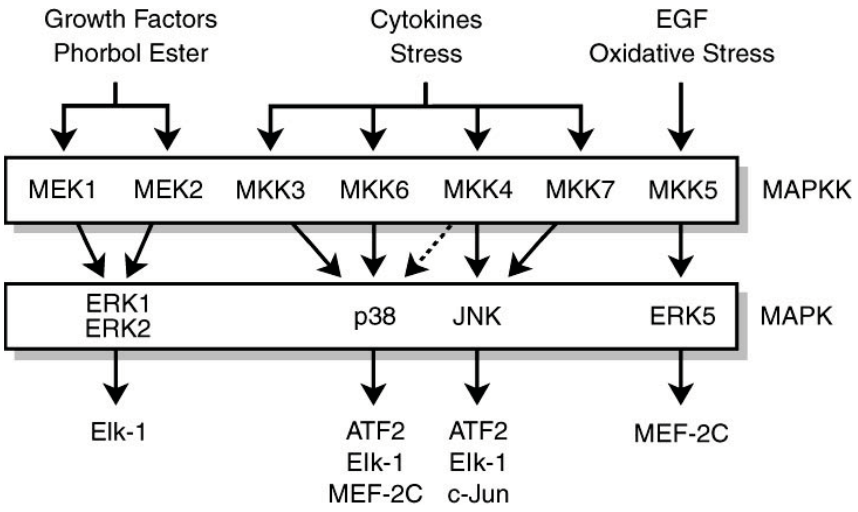
Appendix 2. The pathway of ARE-mediated mRNA decay.

[19]



Appendix 3. Mammalian MAP kinase pathways.

[31]



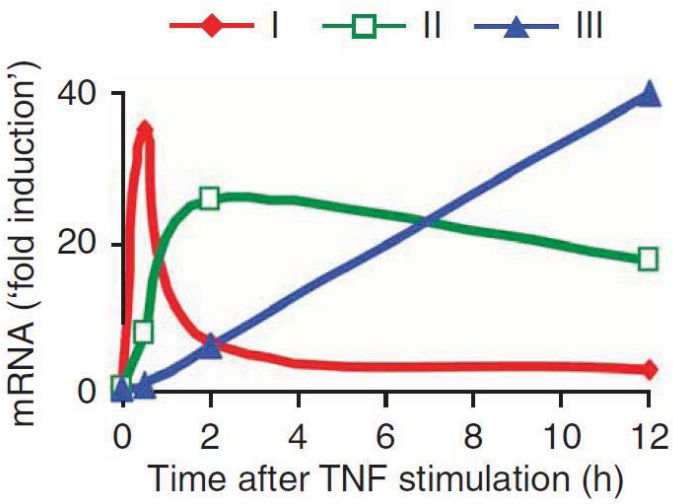
Appendix 4. Classification of MAPK phosphatases

[37]

MKP	Species orthologues	Substrate specificity	Subcellular localization	Immediate-early gene
MKP1	DUSP1, CL100, HVH1, 3CH134, ERP	p38 ~ JNK >> ERK	Nuclear	Yes
MKP2	DUSP4, HVH2, TYP1	ERK ~ JNK >> p38	Nuclear	Yes
MKP3	DUSP6, PYST1, RVH6	ERK >> JNK ~ p38	Cytosolic	No
MKP4	DUSP9, PYST3	ERK > p38 > JNK	Nuclear and cytosolic	No
MKP5	DUSP10	p38 ~ JNK >> ERK	Nuclear and cytosolic	No
MKP7	MKPM, DUSP16	JNK ~ p38 >> ERK	Cytosolic	No
MKPX	DUSP7, B59, PYST2	ERK >> JNK ~ p38	Cytosolic	No
DUSP2	PAC1	ERK ~ p38 >> JNK*	Nuclear	Yes
HVH3	DUSP5, B23	ERK	Nuclear	Yes
HVH5	DUSP8, M3/M6	JNK ~ p38 >> ERK	Nuclear and cytosolic	No

Appendix 5. The three types of gene expression profiles in Tnf-α activated genes.

[39]



9. References

[1] B. Varnum, Q. Ma, T. Chi, and B. Fletcher, “The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat.,” *Mol Cell Biol.*, vol. 11, no. 3, pp. 1754-1758, 1991.

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