



TPL2 kinase expression is regulated by the p38 γ /p38 δ -dependent association of aconitase-1 with TPL2 mRNA

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p38 γ and p38 δ (p38 γ /p38 δ) regulate inflammation, in part by controlling tumor progression locus 2 (TPL2) expression in myeloid cells. Here, we demonstrate that TPL2 protein levels are dramatically reduced in p38 γ /p38 δ -deficient (p38 γ / δ ^{-/-}) cells and tissues without affecting TPL2 messenger ribonucleic acid (mRNA) expression. We show that p38 γ /p38 δ posttranscriptionally regulates the TPL2 amount at two different levels. p38 γ /p38 δ interacts with the TPL2/A20 Binding Inhibitor of NF- κ B2 (ABIN2)/Nuclear Factor κ B1p105 (NF- κ B1p105) complex, increasing TPL2 protein stability. Additionally, p38 γ /p38 δ regulates TPL2 mRNA translation by modulating the repressor function of TPL2 3' Untranslated region (UTR) mediated by its association with aconitase-1 (ACO1). ACO1 overexpression in wild-type cells increases the translational repression induced by TPL2 3'UTR and severely decreases TPL2 protein levels. p38 δ binds to ACO1, and p38 δ expression in p38 γ / δ ^{-/-} cells fully restores TPL2 protein to wild-type levels by reducing the translational repression of TPL2 mRNA. This study reveals a unique mechanism of posttranscriptional regulation of TPL2 expression, which given its central role in innate immune response, likely has great relevance in physiopathology.

p38 γ /p38 δ -MAPK | TPL2 | ACO1 | 3'UTR | mRNA translation

p38 mitogen-activated protein kinase (p38MAPK) signaling pathways are central in the immune and inflammatory response. In addition, they help in the adaptation of cells to a wide range of environmental changes (1, 2). The p38MAPK group belongs to the mitogen-activated protein kinase family and is composed by four isoforms, p38 α , p38 β , p38 γ , and p38 δ , which are encoded by different genes. p38MAPKs are activated by dual phosphorylation mediated by the mitogen-activated protein kinase kinases (MAP2K) MKK3, MKK6, and MKK4, in response to inflammatory cytokines and to cellular stresses, among other stimuli (1, 3). p38 γ and p38 δ , also known as alternative p38MAPKs (1, 4), are very similar proteins that have overlapping functions, although some specific roles have been described for each of them (1). Recent studies show the importance of p38 γ and p38 δ for inflammatory response. The use of mice lacking both p38 γ and p38 δ has provided solid evidence of the implication of these kinases in different inflammation-related pathologies, such as sepsis, colitis, arthritis, or colon cancer associated with inflammation (1, 5). Several studies have described the role of p38 γ and p38 δ in myeloid cells, particularly in bone marrow-derived macrophages (BMDMs), where they control the expression of multiple genes encoding for proteins implicated in the activation or recruitment of immune cells (e.g., cytokines or chemokines) or the killing of pathogens (e.g., inducible nitric oxide synthase (NOS)) (5, 6). This is essential for a proper innate inflammatory response to fight infections and a correct subsequent adaptive response. In macrophages, the combined lack of p38 γ and p38 δ reduces the production of inflammatory molecules, particularly cytokines and chemokines, triggered by the activation of Dectin-1 and Toll-like receptors (TLRs) (5, 6). This effect is not as marked in either p38 γ or p38 δ single-knockout cells. This could be due to the fact that only the combined deletion of the two alternative p38MAPKs causes a substantial decrease in the steady-state levels of the protein tumor progression locus 2 (TPL2; MAP3K8) in BMDMs and dendritic cells (DCs) (6).

TPL2 is the MAPK3K that in macrophages, mediates the TLR activation of Extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 α by directly phosphorylating and activating the upstream MAP2Ks: MKK1, -2, -3, and -6 (7–9). The activation of ERK1/2 and p38 α pathways is essential for cytokine production (1, 6, 7). In resting macrophages, TPL2 is bound to A20 binding inhibitor of NF- κ B 2 (ABIN2) and NF- κ B1 p105 (p105), forming a complex, which is needed to maintain the stability of TPL2 protein (8, 10). TPL2 expression is required for an efficient immune and inflammatory

Significance

p38 γ and p38 δ (p38 γ /p38 δ) are implicated in different inflammation-related pathologies by regulating the immune response. We have shown that p38 γ /p38 δ deletion in myeloid cells decreased tumor progression locus 2 (TPL2) steady-state levels. TPL2 kinase mediates activation of mitogen-activated protein kinases, which are essential for cytokine production and an efficient inflammatory response. The molecular mechanism by which p38 γ /p38 δ regulates TPL2 expression is not known. Here, we show multitier posttranscriptional control of TPL2 protein levels by p38 γ /p38 δ , independent of their kinase activity. p38 γ /p38 δ stabilizes TPL2 protein by direct interaction. p38 γ /p38 δ also regulates TPL2 messenger ribonucleic acid (mRNA) translation by modulating the association of aconitase-1 with TPL2 mRNA. This work unveils a mechanism of the mRNA-specific modulation of translation by p38 γ /p38 δ .

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The authors declare no competing interest.

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response, and some of the effects observed in p38 γ /p38 δ -deficient (p38 γ / $\delta^{-/-}$) macrophages could be due to the severely reduced levels of TPL2 in these cells. In fact, the production of the cytokine tumor necrosis factor- α (TNF α) induced by lipopolysaccharide (LPS) in p38 γ / $\delta^{-/-}$ macrophages phenocopies the effect of TPL2 deficiency (6, 11). Moreover, the expression of TPL2 in p38 γ / $\delta^{-/-}$ restores LPS-induced ERK1/2 activation and TNF α production (6). However, the molecular mechanism by which p38 γ and p38 δ regulate TPL2 expression is unknown.

Here, we demonstrate that alternative p38MAPKs control TPL2 protein levels not only in BMDMs or DCs but also, in different mouse tissues and other cell types. We show evidence that TPL2 stabilization is mediated by the interaction of p38 γ and p38 δ with the TPL2/ABIN2/p105 complex. Also, p38 γ and p38 δ modulate TPL2 mRNA translation by regulating the binding of the iron-responsive cytoplasmic aconitase-1 (ACO1) enzyme to the 3'UTR of TPL2 mRNA. ACO1 is an RNA binding protein (RBP), also known as iron regulatory protein-1, that interacts with conserved RNA structures, called iron response elements, present in the 5'- and 3'UTRs of target genes, controlling the translation or stability of their mRNAs (12). Our results reveal the multitier posttranscriptional control of TPL2 protein levels by alternative p38MAPKs, independently of their kinase activity, by stabilizing TPL2 protein and modulating TPL2 mRNA translation through the association with ACO1.

Results

p38 γ and p38 δ Regulate TPL2 Protein Levels by Modulating Its Protein Stability. Lack of p38 γ and p38 δ (p38 γ /p38 δ) causes a decrease in the levels of TPL2 protein in mouse tissues as well as in peritoneal macrophages and mouse embryonic fibroblasts (MEFs) (Fig. 1 *A* and *B*). Consistently, in p38 γ / $\delta^{-/-}$ cells, ERK1/2 activation was impaired in response to TPL2-dependent stimuli, such as LPS or TNF α , but not in response to the TPL2-independent stimulus 12-*O*-tetradecanoylphorbol-13-acetate, which is dependent on Raf-1 activation in MEFs (13) (*SI Appendix, Fig. S1A*). These results indicate that the regulation of TPL2

protein levels by p38 γ /p38 δ is a general process in various tissues and cells.

Notably, total TPL2 mRNA levels were similar in p38 γ / $\delta^{-/-}$ and wild type (WT) mouse tissues, MEFs, and peritoneal macrophages (Fig. 1 *C* and *D*). Subcellular fractionation experiments also showed that the levels of TPL2 mRNA were similar in the cytoplasm of WT and p38 γ / $\delta^{-/-}$ cells, although TPL2 mRNA levels were higher in the nucleus of p38 γ / $\delta^{-/-}$ compared with WT cells (Fig. 1 *E* and *SI Appendix, Fig. S1B*). These results show that p38 γ /p38 δ does not impair TPL2 gene expression and suggest that the effect of p38 γ /p38 δ deficiency on TPL2 protein levels resulted from posttranscriptional modulation.

Since the steady-state protein level of TPL2 is reduced without decreasing its mRNA level, it is possible that p38 γ and/or p38 δ control the stability of TPL2 by interacting with the TPL2/ABIN2/p105 complex. We performed gel filtration experiments using Raw 264.7 macrophages extracts and found that p38 δ and the ternary complex TPL2, ABIN2, and p105 (14) coeluted together above the 669-kDa fraction. In contrast, p38 γ mainly eluted at \sim 40 kDa, which correlates with its monomeric molecular weight (*SI Appendix, Fig. S2A*). This result indicates that p38 δ can interact with the Tpl2/ABIN2/p105 complex, although we cannot exclude a possible association of p38 γ with this complex below the detection level of our experiment. Additionally, gel filtration experiments using hepatocyte extracts from WT and tumor progression locus 2-deficient (TPL2 $^{-/-}$) mice confirmed that p38 δ and TPL2 coelute in WT cells, whereas p38 δ eluted in fractions correlating with its monomeric molecular weight in TPL2 $^{-/-}$ cells (*SI Appendix, Fig. S2B*). p38 γ also coeluted with p38 δ and TPL2 in WT hepatocytes; however, its elution pattern in TPL2 $^{-/-}$ hepatocytes was similar to that in WT cells (*SI Appendix, Fig. S2B*), suggesting that p38 γ may interact with more than one protein complex, including the Tpl2/ABIN2/p105 complex. Indeed, Glutathione S-transferase (GST) pull-down assays in cotransfection experiments demonstrated that both p38 γ and p38 δ interacted with the TPL2/ABIN2/p105 complex and with TPL2 alone (Fig. 2*A*). p38 γ and p38 δ interactions with TPL2 were also confirmed by

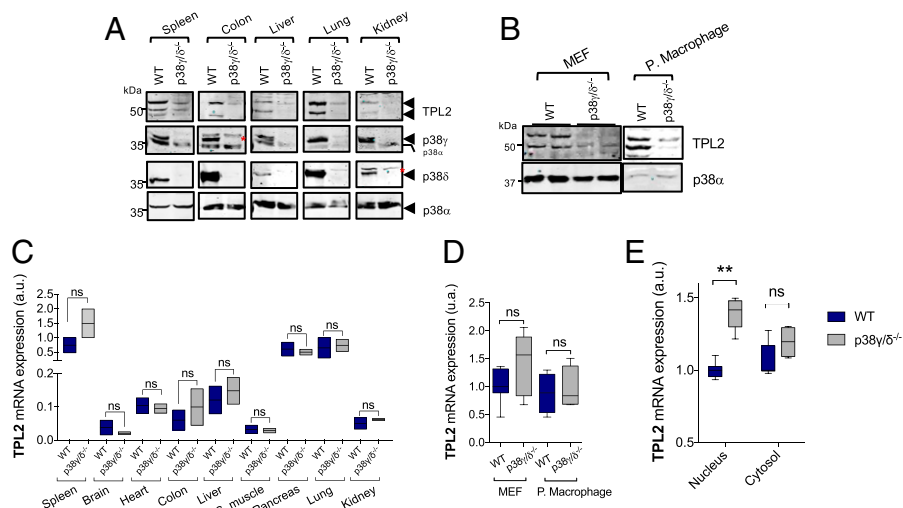


Fig. 1. p38 γ and p38 δ regulate TPL2 protein levels. (*A*) Lysates of the indicated tissues from WT or p38 γ / $\delta^{-/-}$ mice were immunoblotted with anti-TPL2, -p38 α , -p38 γ , or -p38 δ antibodies. p38 α expression was used as the loading control. The red asterisks indicate nonspecific protein bands. (*B*) MEFs and peritoneal macrophage lysates from WT or p38 γ / $\delta^{-/-}$ mice were immunoblotted with anti-TPL2 or -p38 α . (*C*) qPCR of TPL2 mRNA from indicated WT or p38 γ / $\delta^{-/-}$ tissues. Results were normalized to GAPDH mRNA expression. Data show mean \pm SEM ($n = 3$) from one representative experiment of two with similar results. (*D*) qPCR of TPL2 mRNA from indicated WT or p38 γ / $\delta^{-/-}$ cells. Results were normalized to GAPDH mRNA expression. (*E*) qPCR of TPL2 mRNA in cytosolic or nuclear RNA from WT or p38 γ / $\delta^{-/-}$ MEFs. Results were normalized to GAPDH mRNA expression. Data show mean \pm SEM from one representative experiment of two with similar results. ns, not significant; *** $P \leq 0.001$ relative to WT.

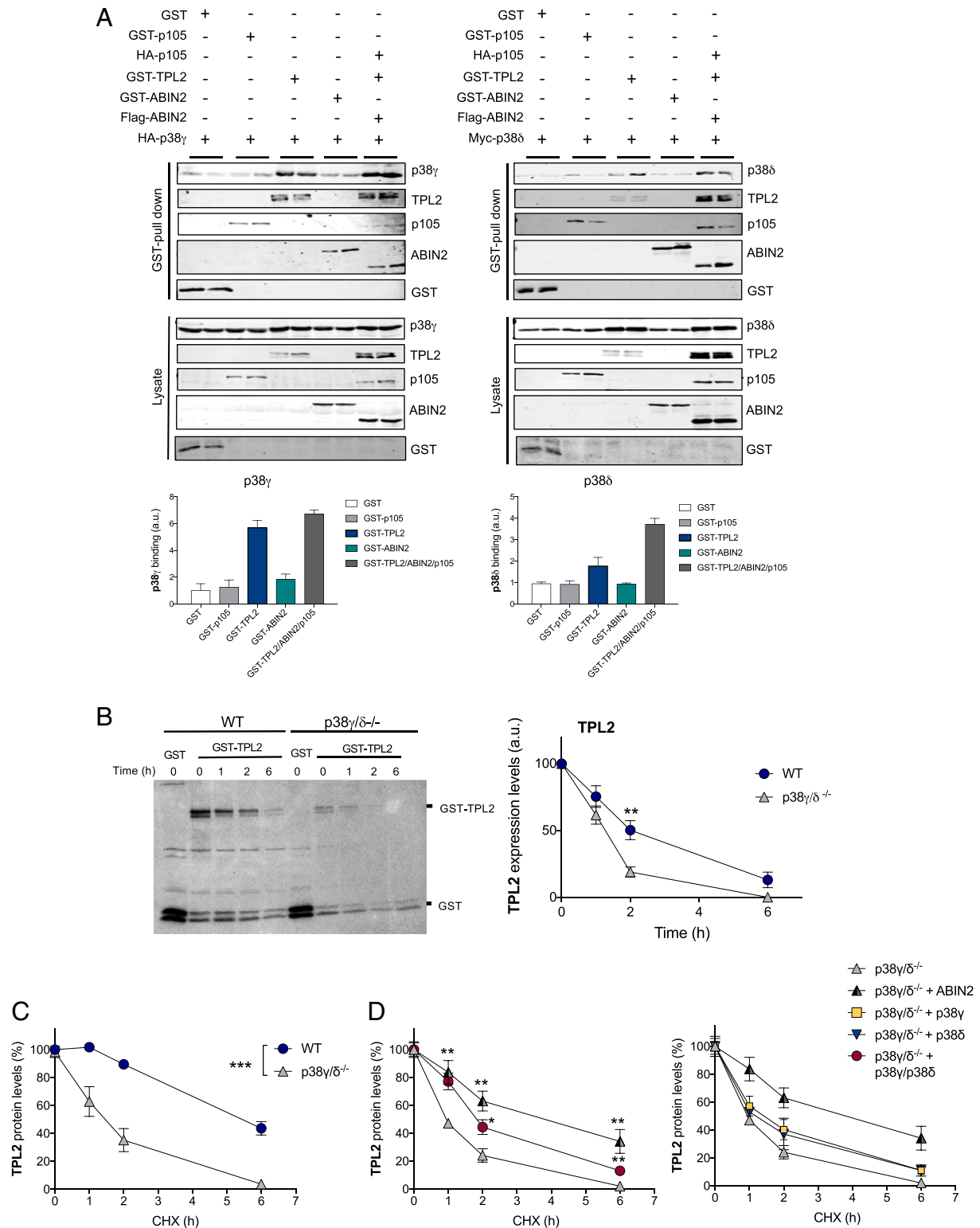


Fig. 2. p38 γ and p38 δ regulate TPL2 protein levels by modulating its stability. (A) HEK293 cells were transfected with plasmids encoding the indicated proteins. After transfection, cells were lysed, pull downs of GST proteins were performed, and pellets were immunoblotted with the indicated antibodies. Total lysates were immunoblotted with the indicated antibodies to examine protein expression. (A, Lower) p38 γ and p38 δ band intensities in pellets were quantified using the Fiji program. Histogram values are means \pm SEM of two independent experiments in duplicate. (B) Metabolic labeling and pulse-chase analysis. WT or p38 $\gamma/\delta^{-/-}$ MEFs transfected with plasmids encoding GST-TPL2 or GST alone as a control were pulse labeled with ^{35}S -Met/Cys and lysed. (B, Left) Pull downs of GST proteins were performed after the times of chase indicated. TPL2 was separated by SDS-PAGE and revealed by autoradiography. (B, Right) TPL2 bands were quantified using the Fiji program, and data show mean \pm SEM from two experiments in duplicate. $***P \leq 0.01$ relative to WT. (C) CHX-chase analysis. WT or p38 $\gamma/\delta^{-/-}$ MEFs were transfected with a plasmid encoding GST-TPL2 and incubated with 100 $\mu\text{g}/\text{mL}$ CHX for the indicated times before lysis. Following cell lysis and SDS-PAGE, immunoblotting was carried out with antitotal TPL2 (SI Appendix, Fig. S2). TPL2 bands were quantified using the Odyssey infrared imaging system, and data show mean \pm SEM from two experiments in duplicate. $***P \leq 0.001$ relative to WT. (D) p38 $\gamma/\delta^{-/-}$ MEFs were transfected with plasmids encoding GST-TPL2 alone or with Flag-ABIN2, hemagglutinin (HA)-p38 γ , myc-p38 δ , or HA-p38 γ plus myc-p38 δ and treated with CHX as in C. Following cell lysis, SDS-PAGE, and immunoblotting with antitotal TPL2 (SI Appendix, Fig. S2), TPL2 bands were quantified. Data show mean \pm SEM from two experiments in duplicate. $*P \leq 0.05$ relative to p38 $\gamma/\delta^{-/-}$ MEFs transfected with GST-TPL2 alone; $**P \leq 0.01$ relative to p38 $\gamma/\delta^{-/-}$ MEFs transfected with GST-TPL2 alone.

coimmunoprecipitation assays using Flag-TPL2 (*SI Appendix, Fig. S3*). p38 γ and p38 δ did not interact with either ABIN2 or p105 alone (Fig. 2*A*). These results suggest that the interaction of p38 γ and p38 δ with the TPL2/ABIN2/p105 complex may either increase TPL2 stability or decrease TPL2 proteolysis. Since TPL2 is proteolyzed in macrophages after LPS stimulation (15, 16), we then investigated if the reduced levels of this protein in p38 $\gamma/\delta^{-/-}$ cells could result from changes in lysosome- or proteasome-mediated proteolysis. Incubation of MEFs with the lysosome-mediated proteolysis inhibitor leupeptin did not change TPL2 levels in WT or p38 $\gamma/\delta^{-/-}$ MEFs (*SI Appendix, Fig. S4A*), whereas the proteasome inhibitor MG132 caused a slight but significant increase in TPL2 protein levels in p38 $\gamma/\delta^{-/-}$ cells only (*SI Appendix, Fig. S4B*). Transferrin receptor and serum- and glucocorticoid-induced protein kinase 1 levels (17) were measured as a control for lysosome inhibition by leupeptin and proteasome inhibition by MG132, respectively (*SI Appendix, Fig. S4C*). These data suggest that degradation mediated by the proteasome contributes only marginally to the low TPL2 levels in p38 $\gamma/\delta^{-/-}$ cells and that other proteasome- and lysosome- independent proteolytic mechanisms might be involved in TPL2 turnover.

To study if p38 γ and p38 δ affected TPL2 stability, we performed pulse-chase analysis comparing WT and p38 $\gamma/\delta^{-/-}$ MEFs. Since the low level of endogenous TPL2 in p38 $\gamma/\delta^{-/-}$ cells prevented its correct detection in pulse-chase experiments, we examined TPL2 protein stability by overexpressing GST-TPL2 or GST-ABIN2 as control in WT and p38 $\gamma/\delta^{-/-}$ MEFs. Endogenous ABIN2 protein and mRNA levels were similar in WT and p38 $\gamma/\delta^{-/-}$ MEFs (*SI Appendix, Fig. S4D*). After metabolic labeling with ³⁵S-Met/Cys, we found that in TPL2 (Fig. 2*B*) but not ABIN2 (used as the control) (*SI Appendix, Fig. S4E*), stability was affected by the lack of p38 γ and p38 δ . TPL2 degradation was significantly increased in p38 $\gamma/\delta^{-/-}$ MEFs compared with WT, decreasing its half-life from roughly 2 to ~1 h (Fig. 2*B*). We confirmed these results by incubating the cells with the protein synthesis inhibitor cycloheximide (CHX) and performing CHX-chase analysis of overexpressed TPL2 and ABIN2 as control (Fig. 2*C* and *SI Appendix, Fig. S4F and G*). TPL2 half-life was shortened from ~5.5 h in WT to 1 to 1.5 h in p38 $\gamma/\delta^{-/-}$ MEFs compared with WT (Fig. 2*C*), whereas ABIN2 degradation was not affected (*SI Appendix, Fig. S4G*). Since GST-tag proteins can form dimers and this could affect the results, we also analyzed the stability of endogenous TPL2 by performing CHX-chase analysis. We confirmed that TPL2 stability was decreased in p38 $\gamma/\delta^{-/-}$ MEFs (*SI Appendix, Fig. S4H*). Endogenous TPL2 half-life was shortened from ~6 h in WT to ~2.5 h in p38 $\gamma/\delta^{-/-}$ MEFs (*SI Appendix, Fig. S4H*).

We then examined if coexpression of TPL2 with p38 γ , p38 δ , or both increased TPL2 stability in p38 $\gamma/\delta^{-/-}$ MEFs. Since it has been shown that ABIN2 stabilizes TPL2 (10, 18), we used ABIN2-TPL2 coexpression as a positive control. TPL2 stability was significantly increased when coexpressed with both p38 γ and p38 δ , although to a lesser degree than when coexpressed with ABIN2 (Fig. 2*D* and *SI Appendix, Fig. S4I*). Coexpression with either p38 γ and p38 δ did not significantly increase TPL2 stability (Fig. 2*D* and *SI Appendix, Fig. S4I*). These data indicate that both p38 γ and p38 δ regulate TPL2 protein levels by partially controlling TPL2 protein stabilization through their association with the TPL2/ABIN2/p105 complex. However, the effect of p38 γ and p38 δ on TPL2 stability does not fully explain the low-TPL2 steady-state level in p38 $\gamma/\delta^{-/-}$ cells, suggesting the presence of other posttranscriptional

mechanisms (e.g., translational control of *TPL2* mRNA) by p38 γ and p38 δ .

p38 γ and p38 δ Modulate *TPL2* mRNA Translation through *TPL2* 3'UTR. To examine if p38 γ and p38 δ regulate *TPL2* mRNA translation, we first analyzed polysome profiling of WT and p38 $\gamma/\delta^{-/-}$ MEFs by sucrose gradient. Polysome profiling separates ribosomal subunits and free ribosomes from polysomes, which are translating ribosomes. Notably, we found that the polysome profile was altered in p38 $\gamma/\delta^{-/-}$ MEFs compared with WT MEFs (Fig. 3*A*). The lack of p38 $\gamma/\delta^{-/-}$ induced a decrease in total RNA absorbance in polysome fractions and an increase in 40S and 60S fractions compared with WT (Fig. 3*A*). A similar observation was made in human p38 $\gamma/\delta^{-/-}$ -HEK293 cells (*SI Appendix, Fig. S5 A and B*). The ratio between polysomes and monosomes was lower in p38 $\gamma/\delta^{-/-}$ MEFs than in WT (*SI Appendix, Fig. S5C*). These results indicate that p38 γ and p38 δ are involved in the regulation of mRNA translation and suggest the translational repression of certain mRNAs in p38 γ - and p38 δ -deficient cells.

To examine if p38 γ and p38 δ regulate the specific translation of *TPL2* mRNA, we used a qRT-PCR assay to analyze the distribution of *TPL2* mRNA as well as *ABIN2* and β -actin mRNAs (as control) from polysome profiling fractions. While distribution of *ABIN2* and β -actin mRNA along the sucrose gradient was similar in p38 $\gamma/\delta^{-/-}$ and WT MEFs, the *TPL2* mRNA content was markedly shifted from heavy polysome fractions toward 60S and 80S fractions in p38 $\gamma/\delta^{-/-}$ cells (Fig. 3*B*), suggesting that *TPL2* translation is repressed in the absence of p38 γ and p38 δ .

We next investigated the possible molecular mechanism by which p38 γ and p38 δ may be regulating *TPL2* mRNA translation. For this, we used the *TPL2* mRNA sequence (ENSMUST0000025078.8 mouse gene Map3K8) to interrogate different databases in order to identify RBPs (rbpdb.ccb.utoronto.ca) and upstream open reading frames (uORFs; <https://useast.ensembl.org/index.html>) that could control *TPL2* mRNA translation (Fig. 3*C*). uORFs are located at the mRNA 5'UTR and repress normal translation of the protein coding sequence in stress conditions where the global mRNA translation initiation is reduced (19). We did not find any canonical uORF at the *TPL2* 5'UTR mRNA. However, we found several predicted RBP binding sites in the *TPL2* mRNA sequence, most of which were within the *TPL2* mRNA 3'UTR (*SI Appendix, Table S1*). We thus investigated the role of the *TPL2* 3'UTR in the translational repression regulated by p38 γ and p38 δ . For this, *TPL2* 3'UTR and also, *ABIN2* 3'UTR as a control were cloned into the psiCHECK-2 *Renilla* luciferase reporter vector (Fig. 3*C and D*) and transfected into WT and p38 $\gamma/\delta^{-/-}$ MEFs. In WT MEFs, *TPL2* 3'UTR caused an approximately threefold repression of *Renilla* expression compared with the empty psiCHECK-2 vector, whereas in p38 $\gamma/\delta^{-/-}$ MEFs, the repression increased approximately sixfold (Fig. 3*E*). In contrast, there was no significant effect of *ABIN2* 3'UTR in the expression of *Renilla*, which was also similar in WT and in p38 $\gamma/\delta^{-/-}$ MEFs (Fig. 3*E*). These results indicated a *TPL2* 3'UTR-specific repression that is regulated by p38 γ and p38 δ .

p38 γ and p38 δ Regulate Binding of ACO1 to *TPL2* mRNA 3'UTR. To elucidate the mechanism by which p38 γ and p38 δ control the *TPL2* mRNA 3'UTR-induced repression, we reasoned that p38 γ and p38 δ likely regulate the function of certain RBPs that interact and control *TPL2* mRNA 3'UTR. Therefore, we

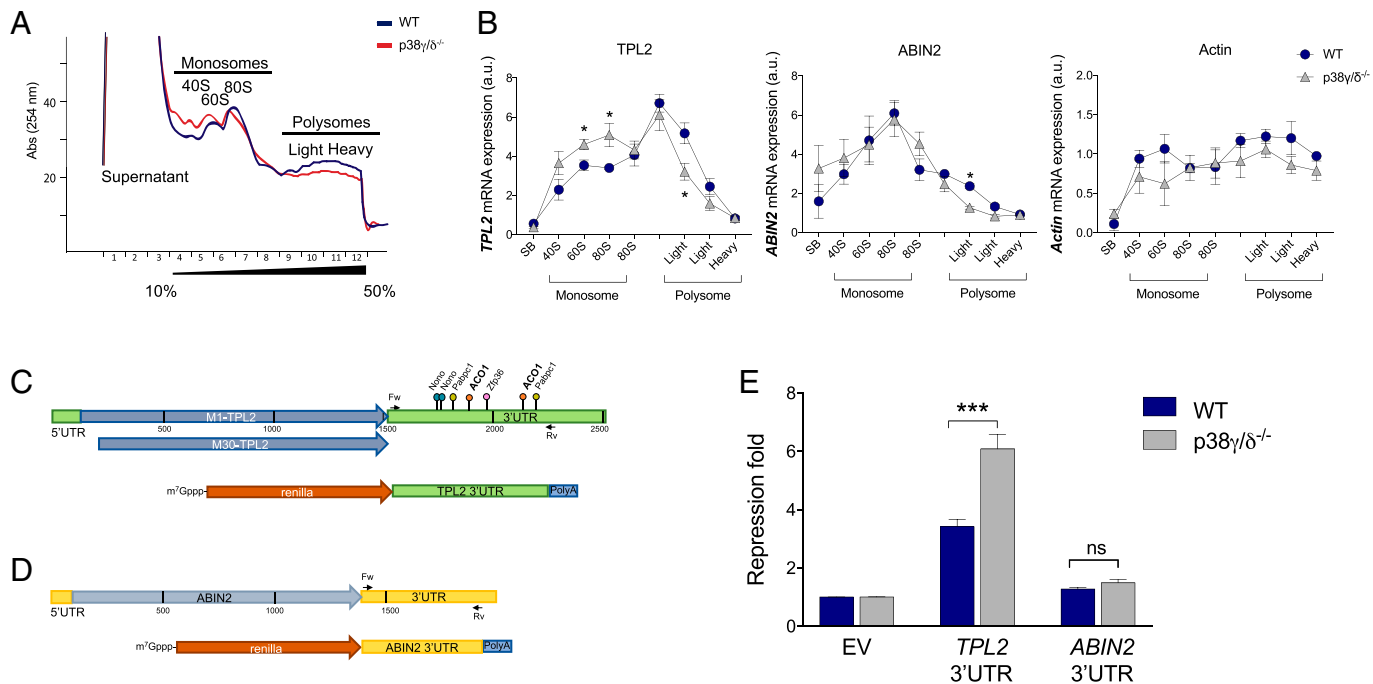


Fig. 3. p38 γ and p38 δ regulate TPL2 translation. (A) Polysome profiling (10 to 50% sucrose gradient) of total WT (dark blue) and p38 $\gamma/\delta^{-/-}$ (red) MEF lysates. The figure shows one representative profile ($n = 3$). (B) qPCR of *TPL2*, *ABIN2*, and β -actin mRNA from polysome profile fractions. Results were normalized to *GAPDH* mRNA expression. Data show mean \pm SEM from one representative experiment in triplicate. $*P < 0.05$ relative to WT MEFs. (C, Upper and D, Upper) Schematic representations of mouse *TPL2* mRNA (C) and *ABIN2* mRNA (D). *TPL2* is expressed as two isoforms due to the alternative translational initiation at methionine 1 (M1) or methionine 30 (M30) (27). Representations show 5'UTR, coding regions (M1-Tpl2, M30-Tpl2, and *ABIN2*), and 3'UTR. Cloning sites for psiCHECK2 plasmids are shown on the 3'UTRs, with forward (Fw) and reverse (Rv) primers indicated. Some binding sites for the RBPs (ACO1, NonO, PABPC1, and ZFP36) at the *TPL2* 3'UTR are indicated (C, Upper). (C, Lower) Schematic representation of the psiCHECK2-Renilla-*TPL2* 3'UTR and psiCHECK2-Renilla-*ABIN2* 3'UTR (*ABIN2* 3'UTR). (E) WT and p38 $\gamma/\delta^{-/-}$ MEFs were transfected with luciferase plasmids psiCHECK2 as empty vector (EV) or containing *TPL2* 3'UTR or *ABIN2* 3'UTR. Renilla values were normalized against Firefly levels, and repression fold was calculated for the *TPL2* 3'UTR or the *ABIN2* 3'UTR reporter relative to EV for each condition. Data are mean \pm SEM from one representative experiment in triplicate. ns, not significant. $***P \leq 0.001$ relative to WT.

performed RNA affinity purification assays to identify proteins in which binding to *TPL2* 3'UTR was regulated by p38 γ and p38 δ using the peritoneal macrophage. WT and p38 $\gamma/\delta^{-/-}$ macrophage lysates were incubated with in vitro-transcribed *TPL2* 3'UTR-1sm RNA bound to streptavidin-conjugated magnetic beads (Fig. 4A). We found that the lack of p38 γ and p38 δ did not affect the global level of protein binding to *TPL2* 3'UTR (Fig. 4B). We next identified the bound proteins by liquid chromatography–tandem mass spectrometry. In WT and p38 $\gamma/\delta^{-/-}$ macrophage extracts, 164 and 193 proteins were identified, respectively (SI Appendix, Tables S2 and S3) (only proteins identified with greater than or equal to three peptides in the two replicas were considered). Venn diagram analysis revealed that 80% of the proteins bound to *TPL2* 3'UTR in WT extracts were also present in p38 $\gamma/\delta^{-/-}$ macrophage pull downs (Fig. 4C). Functional classification analyses of *TPL2* 3'UTR-bound proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic resources through classification into Gene Ontology (GO) categories based on biological processes and on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways showed that proteins involved in translation and the ribosome pathway represented the largest groups with high significance in the two genotypes (Fig. 4D and E and SI Appendix, Fig. S6A).

As mentioned, the number of proteins bound to *TPL2* 3'UTR in p38 $\gamma/\delta^{-/-}$ macrophage pull downs was larger than in WT (133 common proteins, 60 exclusive to p38 $\gamma/\delta^{-/-}$, and 31 exclusive to WT macrophages) (Fig. 4C). To narrow down the search for the protein(s) implicated in p38 $\gamma/\delta^{-/-}$ -regulated translational repression of *TPL2* mRNA, we compared proteins bound to *TPL2* 3'UTR in WT and p38 $\gamma/\delta^{-/-}$ macrophage

extracts (SI Appendix, Tables S2 and S3) with the list of RBPs with predicted binding sites in the *TPL2* mRNA (SI Appendix, Table S1). Venn diagram analysis showed that only one protein, ACO1, has several predicted binding sequences at 3'UTR of *TPL2* mRNA (Fig. 3C) and is bound to *TPL2* 3'UTR in p38 $\gamma/\delta^{-/-}$ macrophage extracts but not in WT (Fig. 4F and SI Appendix, Tables S2 and S3). ACO1 is a bifunctional cytoplasmic protein, which is involved in the isomerization of citrate into isocitrate and also, functions as an RBP, controlling the iron-dependent translation of proteins involved in iron metabolism (20).

To verify that ACO1 binds to *TPL2* mRNA, we performed RNA immunoprecipitation (RIP) with anti-ACO1 antibody in WT and p38 $\gamma/\delta^{-/-}$ MEFs. First, we confirmed that ACO1 protein levels were similar in WT and p38 $\gamma/\delta^{-/-}$ MEFs and that immunoprecipitation with anti-ACO1 antibody resulted in specific recovery of ACO1 (SI Appendix, Fig. S6B). After immunoprecipitation, we examined the amount of ACO1-bound *TPL2* mRNA by qRT-PCR and found that this was significantly enriched in ACO1 RIP in p38 $\gamma/\delta^{-/-}$ cells in comparison with WT (Fig. 4G), confirming that ACO1 binds to *TPL2* mRNA and indicating that the binding can be regulated by p38 $\gamma/\delta^{-/-}$. To determine if the increase in ACO1-bound *TPL2* mRNA in p38 $\gamma/\delta^{-/-}$ cells was due to the lack of p38 $\gamma/\delta^{-/-}$, we reconstituted p38 γ , p38 δ , or p38 $\gamma/\delta^{-/-}$ MEFs by transient transfection (SI Appendix, Fig. S6B). We found that ACO1-*TPL2* mRNA binding decreased in all conditions, although p38 δ or p38 $\gamma/\delta^{-/-}$ expression had a more pronounced effect than p38 γ expression alone (Fig. 4H). All these data suggest that p38 δ and to a lesser extent, also p38 γ impair the interaction of ACO1 with the 3'UTR of *TPL2* mRNA.

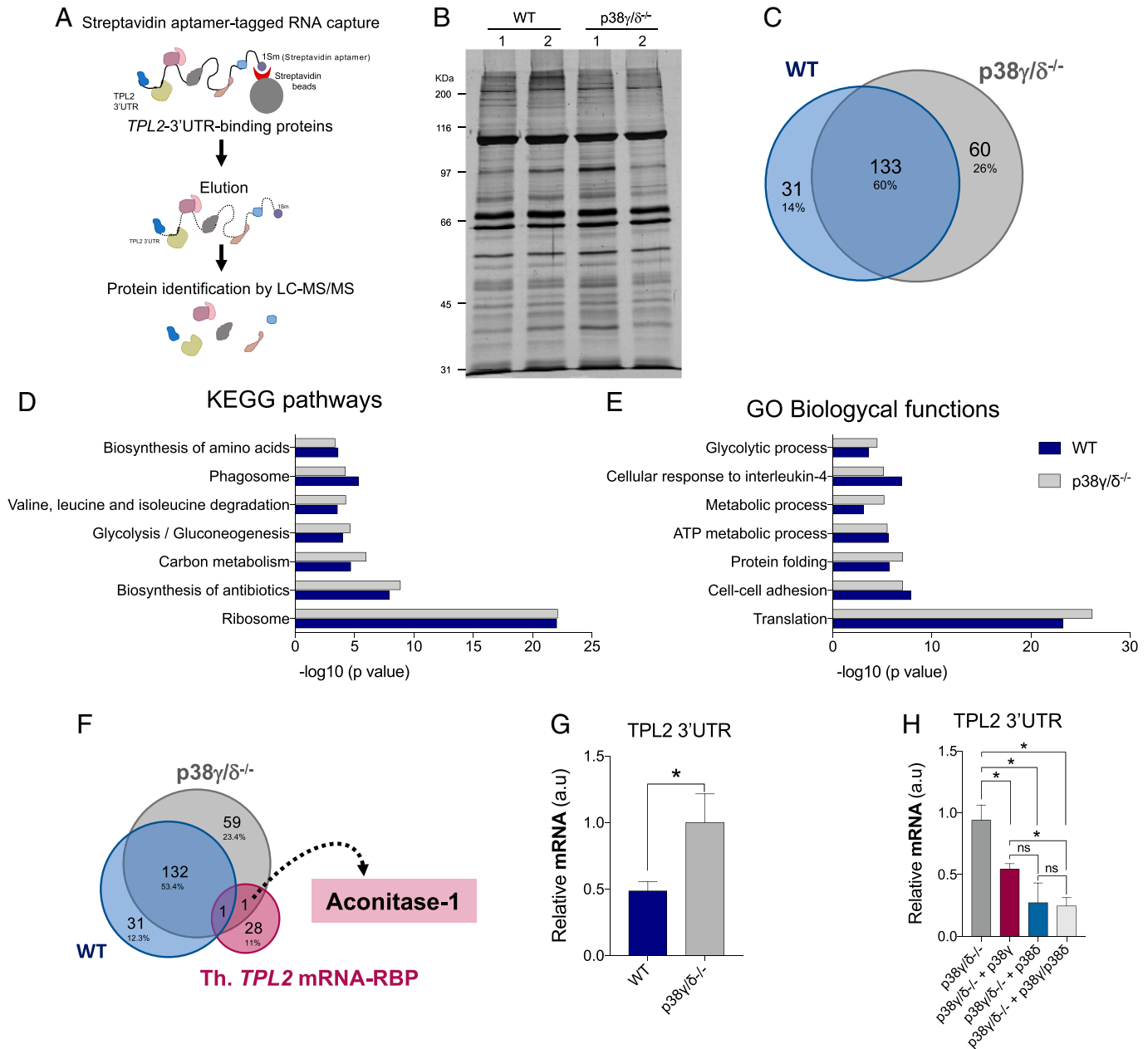


Fig. 4. Analysis of the proteins bound to the 3'UTR of *TPL2* mRNA. (A) Schematic representation of the streptavidin aptamer (15m)-tagged RNA-protein capture methodology and identification of *TPL2* 3'UTR-bound proteins. LC-MS/MS, liquid chromatography-tandem mass spectrometry. (B) SDS-PAGE and silver staining of proteins bound to 3'UTR of *TPL2* mRNA from WT and p38 $\gamma/\delta^{-/-}$ macrophage extracts. Lanes 1 and 2 are biological replicas. (C) Venn diagrams (<https://bioinfogp.cnb.csic.es/tools/venny/>) showing the number of common and specific proteins bound to 3'UTR of *TPL2* mRNA from WT and p38 $\gamma/\delta^{-/-}$ cell extracts. (D and E) Enrichment analysis of KEGG pathways (D) and GO biological processes (E) of the proteins bound to 3'UTR of *TPL2* mRNA from WT and p38 $\gamma/\delta^{-/-}$ cell extracts. (F) Venn diagrams showing the number of common and specific proteins bound to 3'UTR of *TPL2* mRNA from WT and p38 $\gamma/\delta^{-/-}$ cells and RBPs containing binding sites in the *TPL2* mRNA. Th. *TPL2* mRNA-RBP, proteins containing predicted binding sites in *TPL2* mRNA 3'UTR. (G) Endogenous ACO1 was immunoprecipitated from WT and p38 $\gamma/\delta^{-/-}$ MEFs lysates under Ribonuclease (RNase)-free conditions. RNA was isolated from pellets and retro-transcribed to complementary DNA (cDNA) for qPCR analysis. *TPL2* 3'UTR RNA levels were quantified and normalized to 18S RNA levels and to each input control. Data show mean \pm SEM from two experiments in duplicate. * $P \leq 0.05$ relative to p38 $\gamma/\delta^{-/-}$ MEFs. (H) p38 $\gamma/\delta^{-/-}$ MEFs were transfected with empty HA vector (p38 $\gamma/\delta^{-/-}$) or with plasmids encoding HA-p38 γ (p38 $\gamma/\delta^{-/-}$ + p38 γ), HA-p38 δ (p38 $\gamma/\delta^{-/-}$ + p38 δ), or HA-p38 γ plus HA-p38 δ (p38 $\gamma/\delta^{-/-}$ + p38 $\gamma/p38\delta$), and endogenous ACO1 was immunoprecipitated as described in G. *TPL2* 3'UTR RNA levels were quantified and normalized to 18S RNA levels and to each input control. Data show mean \pm SEM from two experiments in duplicate. ns, not significant. * $P \leq 0.05$ relative to p38 $\gamma/\delta^{-/-}$ MEFs.

ACO1 Controls *TPL2* Expression and Interacts with p38 $\gamma/p38\delta$.

To check if ACO1 had a role in *TPL2* expression, we overexpressed ACO1 in WT and p38 $\gamma/\delta^{-/-}$ cells. We observed that this caused a down-regulation of *TPL2* protein levels in WT MEFs to levels similar to p38 $\gamma/\delta^{-/-}$ MEFs (Fig. 5A). ACO1 overexpression had no significant effect on *TPL2* protein levels in p38 $\gamma/\delta^{-/-}$ cells (Fig. 5A), likely due to the repression of the *TPL2* mRNA by the endogenous ACO1 protein. Additionally,

overexpression of ACO1 in WT MEFs significantly increased the repression of *Renilla* expression caused by *TPL2* 3'UTR to levels similar to p38 $\gamma/\delta^{-/-}$ MEFs (Fig. 5B). Again, ACO1 overexpression did not have a significant effect on the repression of the *TPL2* 3'UTR reporter in p38 $\gamma/\delta^{-/-}$ cells (Fig. 5B). These results support the idea that ACO1 overexpression results in an increase in ACO1-*TPL2* 3'UTR association, repressing the translation of *TPL2* mRNA, and that this is modulated by p38 $\gamma/p38\delta$.

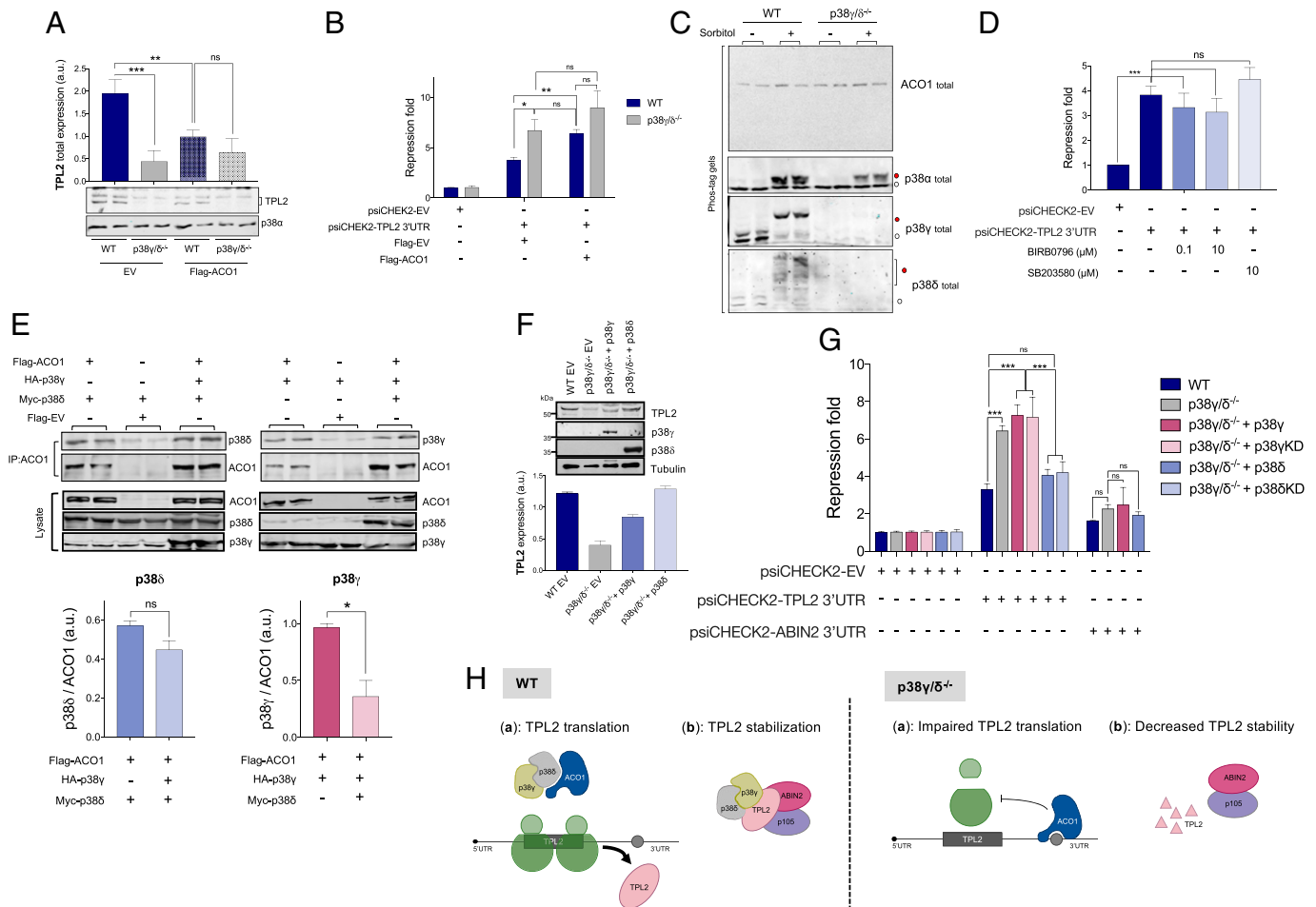


Fig. 5. Alternative p38MAPKs interact with ACO1, and p38 δ expression increases TPL2 protein levels. (A) WT and p38 $\gamma/\delta^{-/-}$ MEFs cells were transfected with plasmid encoding FLAG-ACO1 or with empty vector (EV). After transfection, cell lysates were immunoblotted with anti-TPL2 or -p38 α antibodies (A, Lower). The intensity of TPL2 bands was quantified using Fiji program. Histogram values are means \pm SEM of two independent experiments in duplicate. (B) WT and p38 $\gamma/\delta^{-/-}$ MEFs were transfected with plasmids encoding either FLAG-ACO1 or with EV (Flag-EV), and luciferase plasmid psiCHECK2-TPL2 3'UTR or luciferase plasmid psiCHECK2-EV was used as a control. Renilla luciferase luminescence values were measured and normalized against Firefly luciferase levels. Repression fold was calculated as described in Fig. 3. Data are mean \pm SEM from one representative experiment in triplicate. * $P \leq 0.05$ relative to WT; ** $P \leq 0.01$ relative to WT. (C) WT and p38 $\gamma/\delta^{-/-}$ MEFs were stimulated with 0.5 M sorbitol for 20 min and lysed. Lysates were run in a Phos-tag gel and immunoblotted with the indicated antibodies. White and red circles indicate unphosphorylated and phosphorylated proteins, respectively. (D) WT MEFs were transfected with luciferase plasmids psiCHECK2-EV or with psiCHECK2-TPL2 3'UTR or psiCHECK2-ABIN2 3'UTR, and with plasmids encoding HA-p38 γ , or HA-p38 δ or GFP-p38 γ or HA-p38 δ KD as indicated. Renilla luciferase values were normalized against Firefly luciferase levels, and repression fold was calculated for the TPL2 3'UTR or the ABIN2 3'UTR reporter relative to EV for each condition. Data are mean \pm SEM from one representative experiment in triplicate. ns, not significant; *** $P \leq 0.001$. (E) HEK293 cells were transfected with plasmids encoding the indicated proteins. After transfection, cells were lysed, and immunoprecipitation of ACO1 with anti-Flag antibody was performed. Pellets were immunoblotted with the indicated antibodies (panels immunoprecipitation (IP); ACO1). Total lysates were immunoblotted with the indicated antibodies to examine protein expression (panels Lysate). (E, Lower) p38 γ , p38 δ , and ACO1 band intensities in pellets were quantified using the Fiji program. p38 γ and p38 δ intensity bands were normalized to immunoprecipitated ACO1. Histogram values are means \pm SEM of two independent experiments in duplicate. (F) WT MEFs or p38 $\gamma/\delta^{-/-}$ MEFs stably expressing p38 γ , p38 δ , or EV as control were lysed, and 50 μ g of total lysate protein was immunoblotted with the indicated antibodies. Representative blots are shown. In F, Lower, the intensity of TPL2 bands was quantified using the Fiji program. Histogram values are means \pm SEM of three independent experiments. (G) MEFs were transfected with luciferase plasmids psiCHECK2-EV or with psiCHECK2-TPL2 3'UTR or psiCHECK2-ABIN2 3'UTR, and with plasmids encoding HA-p38 γ , or HA-p38 δ or GFP-p38 γ or HA-p38 δ KD as indicated. Renilla luciferase values were normalized against Firefly luciferase levels, and repression fold was calculated for the TPL2 3'UTR or the ABIN2 3'UTR reporter relative to EV for each condition. Data are mean \pm SEM from one representative experiment in triplicate. ns, not significant; *** $P \leq 0.001$. (H) Proposed model for the regulation of TPL2 protein levels by p38 γ and p38 δ . In WT cells, p38 γ and p38 δ associate with ACO1 (Left panel (a)) and also, with the TPL2/ABIN2/p105 complex (Left panel (b)). In WT cells, the p38 γ /p38 δ /ACO1 complex prevents ACO1 from binding to TPL2 3'UTR, and TPL2 mRNA is translated (Left panel (a)). In p38 $\gamma/\delta^{-/-}$ cells, free ACO1 binds to TPL2 3'UTR and impairs TPL2 mRNA translation by a yet unknown mechanism (Right panel (a)). In WT cells, the p38 γ /p38 δ /TPL2/ABIN2/p105 complex stabilizes TPL2 protein (Left panel (b)), whereas p38 γ /p38 δ absence decreases TPL2 stability and increases its degradation (Right panel (b)). ns, not significant; * $P \leq 0.05$.

To determine how p38 γ and p38 δ modulate ACO1-TPL2 3'UTR interaction, we first examined if the phosphorylation of ACO1 was affected by the lack of p38 γ and p38 δ . ACO1 protein contains at least six Ser or Thr followed by Pro, which are p38MAPK phosphorylation sites (2). The phosphorylation state of ACO1 was examined by immunoblotting using the Phos-tag sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which can resolve phosphorylated and unphosphorylated proteins (21). The mobility of ACO1

bands was the same in WT and p38 $\gamma/\delta^{-/-}$ MEFs, even after sorbitol stimulation that caused the strong phosphorylation and activation of p38MAPK (Fig. 5C). These results suggest that the state of phosphorylation of ACO1 is not affected by p38 γ and p38 δ . In addition, the incubation of WT MEFs with the p38MAPK inhibitors (BIRB0796, which inhibits p38 α and p38 β at 0.1 μ M and p38 γ and p38 δ at 10 μ M, or SB203580, which only inhibits p38 α and p38 β) did not affect the repression of *Renilla* expression caused by TPL2 3'UTR compared

with untreated cells (Fig. 5D). All these data indicate that p38 γ and p38 δ regulate TPL2 protein levels by a mechanism independent of their kinase activity.

We then examined if p38 γ and/or p38 δ bind to ACO1 in cells by performing coimmunoprecipitation experiments with endogenous ACO1, p38 γ , and p38 δ (SI Appendix, Fig. S6D) and also, in HEK293 cells cotransfected with Flag-ACO1 and HA-p38 γ , myc-p38 δ , or both HA-p38 γ and myc-p38 δ together (Fig. 5E). Under these experimental conditions, ACO1 coimmunoprecipitated with p38 γ and with p38 δ (Fig. 5E and SI Appendix, Fig. S6D). However, the binding of HA-p38 γ to Flag-ACO1 was reduced when cotransfected with myc-p38 δ , whereas the myc-p38 δ /Flag-ACO1 binding was not affected by cotransfection with HA-p38 γ (Fig. 5E). These results suggest that ACO1 preferentially binds to p38 δ , which would compete with p38 γ for the same binding site.

p38 δ Regulates TPL2 mRNA Translation and TPL2 Protein Level. We then performed rescue experiments in which either p38 γ or p38 δ was stably expressed in p38 γ / δ ^{-/-} MEFs. First, we checked by immunoblotting that exogenous p38 γ or p38 δ was detected in p38 γ / δ ^{-/-} MEFs after infection with viral vectors expressing p38 γ or p38 δ and then, if TPL2 protein expression was rescued in those p38 γ / δ ^{-/-} MEFs (Fig. 5F). We found that TPL2 level was fully restored by p38 δ expression and partially restored by p38 γ (Fig. 5F). We also examined the effect of transient expression of HA-p38 γ or HA-p38 δ on TPL2 expression in p38 γ / δ ^{-/-} MEFs (SI Appendix, Fig. S6E). We confirmed that the TPL2 level was fully restored by p38 δ expression, but only partially by p38 γ , in cells expressing similar amounts of HA-p38 γ or HA-p38 δ (SI Appendix, Fig. S6E).

In addition, expression of p38 δ , but not the control empty vector or p38 γ , was associated with a reduction of TPL2 3'UTR translational repression of the reporter gene (Fig. 5G). We have also analyzed the effect of p38 δ and p38 γ inactive mutants (p38 δ Kinase dead (KD) and p38 γ KD) and obtained the same results than with the WT version of p38 δ and p38 γ (Fig. 5G and SI Appendix, Fig. S6F). These results suggest that the main contributor regulating the specific translation of TPL2 was p38 δ and supporting the idea that p38 δ activity is not required to rescue TPL2 translation.

Discussion

This study shows the regulation of TPL2 expression by p38 γ and p38 δ in various cells and tissue types. TPL2 expression is essential for the activation of the ERK1/2 pathway following TLR and TNF receptor stimulation and therefore, for cytokine and chemokine production in myeloid cells. The combined lack of p38 γ and p38 δ causes a substantial reduction in the steady-state levels of TPL2 protein in BMDMs and DCs. This effect is not observed in single p38 γ or p38 δ knockout cells, indicating that they compensate for each other (5, 6). Here, we further demonstrate that the levels of TPL2 protein are severely decreased in other p38 γ / δ ^{-/-} cell types and in different tissues from p38 γ / δ ^{-/-} mice and that stable expression of exogenous p38 γ or p38 δ in p38 γ / δ ^{-/-} cells restores TPL2 expression. We show that these two kinases are central for posttranscriptional regulation of TPL2 protein levels and that they have substantially more complex functions than previously anticipated.

We have addressed the molecular mechanism by which both p38 γ and p38 δ are necessary to maintain TPL2 protein levels in cells and found that these alternative p38MAPKs act at different stages of posttranscriptional regulation. We show that

optimal TPL2 stability in cells requires the presence of p38 γ and p38 δ and present evidence that these p38MAPKs associate with the TPL2/ABIN2/p105 complex through direct interaction with TPL2. It has been described that most TPL2 and ABIN2 proteins form a complex with p105 in the cell and that maximum TPL2 stability requires the binding to ABIN2 and p105 (10, 16, 18). Our results strongly suggest the presence of a TPL2 protein complex formed by TPL2/ABIN2/p105/p38 γ /p38 δ in cells and that this is essential for the stability of TPL2 protein (Fig. 5H). Thus, our data point to an additional level of regulation of TPL2 expression by alternative p38MAPKs. We have shown that proteasome marginally contributes to the low TPL2 levels in p38 γ / δ ^{-/-} cells and that lysosome-mediated degradation is not involved in TPL2 turnover, which raises the question of how p38 γ /p38 δ regulates TPL2 proteolysis. The precise mechanism of TPL2 stabilization by p38 γ /p38 δ needs to be further investigated by structural analysis.

We also demonstrate the role of p38 γ /p38 δ in translational regulation of TPL2 mRNA. Comparative polysome profile analyses suggested a decrease in protein synthesis in cells lacking p38 γ and p38 δ . This decrease could be due in part to the low TPL2 expression in these cells, since it has been described the positive implication of TPL2 in the activation of Cap-dependent translation in TLR-stimulated macrophages (22). Also, it has been described that p38 γ /p38 δ controls the protein synthesis rate by regulating the mammalian target of rapamycin (mTOR) pathway in the heart (1). On the other hand, the increased content of TPL2 mRNA in light monosome fractions vs. heavy polysome fractions in p38 γ / δ ^{-/-} cells indicates that TPL2 mRNA translation is specifically diminished in those cells compared with wild-type cells. Further experiments, such as comparative proteome analysis, need to be carried out to elucidate the mechanism by which p38 γ /p38 δ regulates the translation of specific mRNAs. RBP that recognize and bind to certain elements present in the target mRNA play a key role in the regulation of stability and/or translation efficiency of specific mRNAs (23). Using a bicistronic reporter system, we demonstrate that translational repression of a reporter mRNA fused to the TPL2 3'UTR is exacerbated in the absence of p38 γ /p38 δ . These results suggest that p38 γ and p38 δ could regulate TPL2 mRNA translation by specifically modifying the interactome of mRNA regulatory sequences, particularly of the TPL2 mRNA 3'UTR. Mass spectrometry and RIP analyses revealed the interaction of TPL2 mRNA with the cytoplasmic protein ACO1 in a p38 γ /p38 δ -dependent manner. ACO1 is a metabolic enzyme that catalyzes the conversion of citrate to isocitrate in the cytoplasm. Over the past decades, different studies have shown that metabolic enzymes can act as RBPs and regulate the expression of specific target mRNAs (24, 25). ACO1 plays a central role in the control of iron levels in the cells by regulating the expression of proteins of the iron metabolism, such as ferritin or transferrin receptor. For example, when cellular iron levels are low, ACO1 interacts with the 5'UTR of *Ferritin* mRNA, repressing its translation and increasing free iron, and with the 3'UTR of *Transferrin Receptor* mRNA, increasing its stability and promoting iron uptake (12). Here, we show evidence of ACO1 being a TPL2 3'UTR binding protein that represses the translation of TPL2 mRNA. Indeed, overexpression of ACO1 in WT cells causes a decrease in endogenous TPL2 protein expression and also, increased repression of *Renilla* reporter caused by TPL2 3'UTR to similar levels as those in p38 γ / δ ^{-/-} cells. Although we provide evidence that ACO1-TPL2 3'UTR binding could be directly regulated by p38 γ /p38 δ , full details of the molecular mechanism by which p38 γ and p38 δ regulate the ACO1-TPL2 mRNA 3'UTR interaction remain to be elucidated.

Our data indicate that the regulation of ACO1-mediated repression of *TPL2* mRNA translation by alternative p38MAPK is independent of their kinase activity. Incubation with the pan-p38MAPK inhibitor, BIRB0796, does not affect the repression of *Renilla* expression caused by *TPL2* 3'UTR in WT cells. Moreover, the state of phosphorylation of ACO1 is similar in p38 γ / $\delta^{-/-}$ and WT cells, and it does not seem to be affected under stress conditions, where p38 γ and p38 δ are fully activated. It should be noted that p38 δ and p38 γ associate with endogenous ACO1 when overexpressed alone. However, when p38 δ and p38 γ are overexpressed together, p38 δ displaces p38 γ from ACO1, indicating that ACO1 preferentially binds to p38 δ , which might compete with p38 γ . This is supported by the observation that the expression of p38 δ (WT or KD version), but not p38 γ , in p38 γ / $\delta^{-/-}$ cells reverts the repression of *Renilla* expression caused by *TPL2* 3'UTR and fully restores TPL2 protein levels. Kinase-independent activity of alternative p38MAPK has been reported previously. For instance, we have demonstrated that p38 γ regulates the nuclear protein complex formed by the human discs large protein (hDlg), the polypyrimidine tract binding protein-associated splicing factor (PSF), and various RNAs. Under hyperosmotic conditions, p38 γ moves from the cytosol to the nucleus, where it interacts with hDlg and displaces PSF RNAs (1, 26). These results highlight the importance of p38MAPK functions independent of their kinase activity, which may have relevant cellular and physiological implications.

In conclusion, we propose a model in which p38 γ and p38 δ act simultaneously at distinct levels to control TPL2 protein expression by 1) regulating TPL2 stability mediated by the direct interaction with the TPL2/ABIN2/p105 complex and 2) indirectly modulating *TPL2* mRNA translation through association with the RBP ACO1. The binding of ACO1 to p38 δ and also, p38 γ would prevent its interaction with the *TPL2* 3'UTR, resulting in a more efficient translation of *TPL2* mRNA (Fig. 5H). This is an instance of a description of mRNA-specific modulation of translation by alternative p38MAPKd. Thus, our study has the potential to inaugurate an unexplored mechanism of posttranscriptional regulation of gene expression. This will likely have great significance in various fields, including the control of the innate immune response, given the central role of TPL2 in regulating TLR and Tumor Necrosis factor

receptor (TNFR) signaling in myeloid cells after either pathogen infection or exposure to other inflammatory agents.

Materials and Methods

All materials and methods, cell culture, generation of reporter plasmids, immunoblot analysis, pulse-chase metabolic labeling, Phos-tag SDS-PAGE, RIP, polysome profiling, gene expression analysis, affinity purification of protein complexes and of *TPL2* 3'UTR-bound protein complexes, luciferase reporter assay, and statistical analyses are described in *SI Appendix, SI Materials and Methods*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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