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ORIGINAL ARTICLE

Translation of TNFAIP2 is tightly controlled by upstream open reading frames

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Abstract

Translation is a highly regulated process, both at the global as well as on a transcript-specific level. Regulatory upstream open reading frames (uORFs) represent a mode to alter cap-dependent translation efficiency in a transcript-specific manner and are found in numerous mRNAs. In the majority of cases, uORFs inhibit the translation of their associated main ORFs. Consequently, their inactivation results in enhanced translation of the main ORF, a phenomenon best characterized in the context of the integrated stress response. In the present study, we identified potent translation-inhibitory uORFs in the transcript leader sequence (TLS) of tumor necrosis factor alpha induced protein 2 (TNFAIP2). The initial description of the uORFs was based on the observation that despite a massive induction of TNFAIP2 mRNA expression in response to interleukin 1β (IL1β), TNFAIP2 protein levels remained low in MCF7 cells. While we were able to characterize the uORFs with respect to their exact size and sequential requirements in this cellular context, only TPA stimulation partially overcame the translation-inhibitory activity of the TNFAIP2 uORFs. Characterization of TNFAIP2 translation in the context of monocyte-to-macrophage differentiation suggested that, while the uORFs efficiently block TNFAIP2 protein synthesis in monocytes, they are inactivated in mature macrophages, thus allowing for a massive increase in TNFAIP2 protein expression. In summary, we establish TNFAIP2 as a novel target of uORF-mediated translational regulation. Furthermore, our findings suggest that during macrophage differentiation a major uORF-dependent translational switch occurs.

Keywords Translation · Upstream open reading frame · Macrophage · Differentiation · Inflammation

Abbreviations

CDS	Coding sequence
CHX	Cycloheximide
DMSO	Dimethyl sulfoxide
IL1β	Interleukin 1β
ISR	Integrated stress response
MΦ	Macrophages
MO	Monocytes
RPF	Ribosome-protected fragments
TLS	Transcript leader sequence
TNFAIP2	Tumor necrosis factor α induced protein 2
TPA	12-*O*-tetradecanoylphorbol-13-acetate
uORF	Upstream open reading frame
UTR	Untranslated region

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Introduction

As translation, i.e. the biosynthesis of proteins, is among the most energy-demanding intracellular processes, it is tightly regulated already at its initiation step [1]. Along these lines, cap-dependent translation initiation is controlled by the mTOR kinase, which serves as a key node to integrate the cellular energy status [2]. In addition to such global, i.e. non transcript-specific, modes of regulation, the translation of various mRNAs can further be regulated specifically either via cap-independent translation initiation mechanisms (e.g. internal ribosome entry site (IRES)-dependent) [3] or by adding additional layers of regulation to the cap-dependent initiation [e.g. by upstream open reading frames (uORFs)] [4]. Many of these alternative modes of regulation of translation initiation are encoded within the transcript leader sequence (TLS) of mRNAs [5]. Furthermore, such

regulatory principles are especially important in proteins involved in stress responses. In this context it is interesting to note that IRESs appear to be enriched in proto-oncogenes, ensuring their sustained expression under otherwise translation-inhibitory conditions [6]. In contrast, uORFs typically act as translation inhibitors of their associated main ORFs [7]. As implied by their name, uORFs are localized upstream of the main ORFs, i.e. within the TLS, but they can also overlap with the latter [8].

uORF-dependent regulation of translation is best characterized for the integrated stress response (ISR) [9]. The ISR is induced by various stress stimuli, including heat stress, viral infection, amino acid deprivation, and most prominently the unfolded protein response, i.e. ER stress, and is characterized by the elevated phosphorylation of the eukaryotic initiation factor 2α (eIF2α) [10]. While this results in a reduction of global translation, a number of uORF-containing transcripts show enhanced translation under such conditions, many of which are associated with stress adaptive processes (e.g. ATF4, CHOP, ATF5, GADD34) [10, 11]. Yet, only a subset of uORF-containing transcripts is translationally induced by the ISR, indicating that uORF-mediated translational regulation is highly specific for each transcript [12]. Moreover, commonly single transcripts contain more than one uORF, each of which may be preferentially translated depending on the environmental conditions, thus allowing for the sophisticated regulation of the main ORF [13, 14]. For example, the translation of thrombopoietin (TPO) is usually strongly repressed due to seven uORFs present within the TLS, the seventh of which exerting the strongest inhibitory effect. Mutations which inactivate uORF 7 have been shown to result in higher TPO protein levels, thereby contributing to hereditary thrombocytosis [15]. Recently, the analysis of mutations in uORFs in various human malignancies further revealed that loss-of-function mutations of specific uORFs result in overexpression of oncoproteins, thereby contributing to tumor development [16].

In the present study, we found indications for the presence of inhibitory uORFs in the TLS of tumor necrosis factor alpha-induced protein 2 (TNFAIP2) in a ribosome profiling data set of human MCF7 breast cancer cells treated with interleukin 1β (IL1β). TNFAIP2 is a primary response gene of TNFα, previously shown to be expressed in various cell types including endothelial, lymphoid, and myeloid cells [17, 18], which responds to inflammatory stimuli such as TNFα, IL1β, lipopolysaccharide (LPS), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [19, 20]. Upon initial validation and characterization of the TNFAIP2 uORFs, the translation regulatory properties were assessed in the context of myeloid cells, where complete translational repression of TNFAIP2 in monocytes was efficiently relieved upon differentiation to macrophages. Since TNFAIP2 mRNA expression remained unaltered between monocytes and macrophages, translational regulation of TNFAIP2 appears to be of major importance in controlling TNFAIP2 expression during myeloid differentiation.

Materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich (Munich, Germany), if not stated otherwise. Antibodies were purchased from the following companies: anti-TNFAIP2 (NBP1-33480) from Novus Biologicals (Wiesbaden, Germany); anti-β-actin (A2066) and anti-β-tubulin (T4026) from Sigma-Aldrich; anti-*renilla* luciferase (EPR17791) from Abcam (Cambridge, UK); IRDyes 800CW (925–32212; 925–32213) and IRDyes 680RD secondary antibodies (925-68070) from LI-COR Biosciences GmbH (Bad Homburg, Germany).

Cell culture

MCF7 cells were purchased from ATCC-LGC Standards GmbH (Wesel, Germany) and maintained in RPMI 1640 GlutaMAX medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. THP1 cells were purchased from ATCC-LGC Standards GmbH and maintained in DMEM high glucose medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Medium was obtained from Thermo Fisher Scientific (Schwerte, Germany), FBS from Capricorn Scientific (Elbsdorfer Grund, Germany), and supplements from Sigma-Aldrich.

Isolation of $CD14^+$ human primary monocytes and differentiation to macrophages

Peripheral blood mononuclear cells (PBMCs) were prepared from human buffy coats (DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt, Germany) using Bicoll Separating Solution (Biochrom GmbH, Berlin, Germany). Subsequently, $CD14^+$ cells, i.e. monocytes (MO), were isolated by magnetic cell sorting using microbeads for human CD14 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were differentiated to macrophages (MΦ) over a period of 7 days using Macrophage-SFM medium (Thermo Fisher Scientific) supplemented with 20 ng/ml macrophage-colony stimulating factor (M-CSF) (ImmunoTools GmbH, Friesoythe, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin.

mRNA analysis

RNA was isolated using the PeqGold RNAPure Kit (PeqLab Biotechnology, Erlangen, Germany). RNA was reverse transcribed with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). mRNA levels of TNFAIP2 and GAPDH were analyzed by qPCR using the iQ SYBR Green Supermix (BioRad, München, Germany) and primers for human TNFAIP2 (for: 5′-TAC GCT GGC CGA GAT CAT TC-3′; rev: 5′-TCC CCT TGA TGG CCA GGA TA-3′) or human GAPDH (for: 5′-TGC ACC ACC AAC TGC TTA GC-3′; rev: 5′-GGC ATG GAC TGT GGT CAT GAG-3′).

Protein analysis

For the analysis of protein changes, cells were lysed in lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 1× protease inhibitor and 1× phosphatase inhibitor mix (Roche Diagnostics, Mannheim, Germany)] and snap frozen. 40 µg protein was separated via SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Chalfont St Giles, UK). Proteins were detected using specific antibodies and appropriate secondary antibodies and visualized and quantified on an Odyssey infrared imaging system (LI-COR Biosciences GmbH).

Ribosome profiling

Ribosome profiling was carried out using the TruSeq® Ribo Profile (Mammalian) Library Prep Kit (Illumina, San Diego, USA) according to the manufacturer's protocol. Briefly, 5×10^6 MCF7 cells were seeded in 10 cm dishes 24 h prior to cultivation with or without 50 ng/ml IL1β for 4 h. Cycloheximide (CHX, 100 µg/ml) was added to the cells for the last 10 min of the stimulation to stall translation. RNA was isolated according to the protocol and samples were split into two to serve as ribosome footprint and total RNA control samples. The ribosome footprint samples were treated with RNase and RNA-ribosome complexes, i.e. ribosome protected fragments (RPFs), were isolated using Microspin™ S-400 HR columns (Thermo Fisher Scientific). rRNA was removed from total RNA and ribosome footprint samples using the RiboZero Gold rRNA Removal Kit (Human/Mouse/Rat, Illumina). The sequencing libraries were prepared according to the protocol. The libraries were sequenced on a NextSeq 500 instrument (Illumina). Subsequent data processing was performed using cutadapt for adapter and quality trimming [21], bowtie2 for rRNA removal [22], and STAR for mapping of the samples to the human genome (hg38) [23].

Plasmid construction

For cloning purposes, the psiCHECK-2 plasmid (Promega, Mannheim, Germany) was digested with *Nhe*I (New England Biolabs, Frankfurt, Germany). The transcript leader sequence (TLS) of TNFAIP2 (131 bp) was amplified from human cDNA using the primers TNFAIP2_psi_for and TNFAIP2_psi_rev. The PCR product was purified via agarose gel (1%) separation and extraction of the expected fragment using the NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel, Dueren, Germany). The fragment was inserted into the linearized vector with the In-Fusion HD Cloning Kit (Takara, Frankfurt, Germany) according to the manufacturer's protocol. Nine plasmid bases between the 3′-end of the insert and the luciferase gene were removed, ATGs were mutated to TTGs, and the respective TAG was mutated to TCG using the QuikChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, USA) according to manufacturer's instructions. All primers are listed in Supplementary Table S1.

Transient transfection and luciferase reporter assay

6×10^5 MCF-7 cells were seeded in 24-well plates 24 h prior to transfection with 500 ng plasmid DNA per well using the Roti-Fect transfection reagent (Roth, Karlsruhe, Germany) according to the manufacturer's protocol. After 24 h, cells were either harvested immediately or subjected to stimulation with either 50 ng/ml IL1β, 10 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or dimethyl sulfoxide (DMSO) for additional 24 h. Cells were lysed in 100 µl Passive Lysis Buffer (Promega) and snap frozen. *Firefly* and *renilla* luciferase activities were determined using the Dual Luciferase kit assay (Promega) on a TriStar^2 Multimode Reader LB 942 (Berthold Technologies, Bad Wildbad, Germany). *Firefly* luciferase activity served as internal transfection control.

Polysomal fractionation

$CD14^+$ MO or MΦ were subjected to polysomal fractionation as described previously [24]. Briefly, cells were incubated with 100 µg/ml CHX for 10 min, washed with PBS/CHX (100 µg/ml), and lysed in 750 µl polysome lysis buffer (140 mM KCl, 20 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 0.5% NP40, 0.5 mg/ml heparin, 1 mM DTT, 100 U/ml RNasin (Promega), 100 µg/ml CHX). After pelleting the cell debris, 600 µl of the cell lysates was layered onto 11 ml 10–50% continuous sucrose gradients. 100 µl of the lysates was kept for total RNA extraction. The sucrose gradients were subjected to ultra-centrifugation at 35,000 rpm for 2 h at 4 °C without break using an SW40 Ti rotor (Beckman Coulter, Brea, USA). Afterwards the gradient was separated into 1-ml fractions using a Gradient Station (BioComp Instruments,

Fredericton, Canada). UV-absorbance was measured at 254 nm. RNA was precipitated using sodium-acetate (3 M, 1/10 of total volume) and isopropyl alcohol. RNA was further purified using the NucleoSpin RNA kit (Macherey–Nagel) according to the manufacturer's manual. mRNA obtained from polysomal fractions or total RNA extracted from the lysates was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit. TNFAIP2 mRNA was subsequently quantified by qPCR using the iQ SYBR Green Supermix.

Statistical analyses

Data are reported as mean ± SEM of at least three independent experiments and analyzed using a two-tailed *t* test unless otherwise stated.

Results

IL1β induces transcription but not translation of TNFAIP2 in MCF7 cells

As tumors commonly develop within an inflammatory microenvironment, we exposed MCF7 breast carcinoma cells to the pro-inflammatory cytokine interleukin 1β (IL1β) (50 ng/ml) for 4 h to assess translational changes occurring under such conditions. Using ribosome profiling, we observed that the mRNA expression of tumor necrosis factor alpha-induced protein 2 (TNFAIP2) (NM_006291.2) was strongly induced by IL1β (Fig. 1a, upper panels), which was paralleled by an increase in the abundance of ribosome protected fragments (RPFs) (Fig. 1a, lower panels). While we were able to validate the massive induction of TNFAIP2 mRNA expression in response to IL1β (64.8 ± 17.6 fold) via qPCR (Fig. 1b), much to our surprise, TNFAIP2 protein expression remained unaltered, at a very low expression level after IL1β treatment (Fig. 1c). This unexpected discrepancy led us to revisit the ribo-seq results. Upon closer inspection, we found that while RPFs were present across the entire coding sequence (CDS) of the TNFAIP2 transcript, the strongest peak was observed within the transcript leader sequence (TLS) (Fig. 1d). Per definition, RPFs can only be found in actively translated regions of a transcript and, therefore, are almost exclusively restricted to the CDS of mRNAs. Yet, certain TLS contain short, peptide-coding upstream open reading frames (uORFs), which appear also in ribo-seq experiments and are considered as translational control elements to regulate the translation of the associated main ORFs. Indeed, uORF-Tools, a recently developed software for the identification of actively translated uORFs [25], predicted a uORF within the TLS of TNFAIP2, spanning a total of 36 nucleotides (Fig. 1d, lower panel). Taken together, we observed that TNFAIP2 mRNA expression is strongly induced in MCF7 breast cancer cells by the pro-inflammatory stimulus IL1β, whereas the translation of TNFAIP2 remained almost completely suppressed, bearing indications of a translation-repressive uORF.

The TNFAIP2 TLS contains translation-inhibitory uORFs

To determine, if the predicted uORF might contribute to the translational regulation of TNFAIP2, we introduced the TLS of TNFAIP2 (131 nucleotides) into the psiCHECK-2 reporter vector in front of the *renilla* luciferase coding sequence (Fig. 2a). Interestingly, the bioinformatically predicted uORF contained 3 ATG start codons all in frame with the same stop codon (TAG). Based on the definition of uORFs, as upstream start codons followed by in-frame stop codons, the predicted single uORF likely reflects three overlapping, in-frame uORFs. To validate if the predicted uORFs indeed regulate the translation of the main ORF, we mutated all 3 ATGs to TTGs. Combined mutation of the three start codons resulted in a significant increase in the *renilla* luciferase activity compared to the intact TLS (mut ATG 1 + 2+3: 1.90 ± 0.19) (Fig. 2b), supporting the translation inhibitory function of the uORFs. To determine which uORF conveys the translation repressive properties of the TLS, we next mutated each of the ATGs separately to TTGs in the luciferase reporter vector containing the entire TLS. Surprisingly, similar to the intact TLS, the luciferase activity was reduced in all three single mutants when compared to the triple mutant (Fig. 2b). Therefore, we next assessed if the overlapping uORFs might act in concert to control the translation of the TNFAIP2 main ORF and mutated the ATGs in all possible combinations. Interestingly, only the mutant containing changes of the first and the second ATG showed a strongly elevated luciferase activity compared to the intact TLS (mut ATG 1 + 2: 2.11 ± 0.11), similar to the triple mutant (Fig. 2c). In contrast, if either the first or the second ATG remained intact, the inhibitory capacity of the TLS was only minimally reduced.

To verify that the uORFs are indeed actively translated, we next mutated the shared uORF stop codon [mut. TAG (TAG → TCG)]. Since the uORF start codons are also in frame with the coding sequence of the main ORF, inactivation of the uORF stop codon was predicted to result in the production of an elongated *renilla* protein. Interestingly, the mutation of the uORF stop codon further reduced the *renilla* luciferase activity (0.36 ± 0.04) as compared to the vector containing the complete, intact TLS. In contrast, the mutation of the stop codon did not alter the luciferase activity in the vector in which the three uORF ATGs were also mutated (mut. 3xATG: 1.42 ± 0.15; mut. 3xATG + TAG: 1.75 ± 0.15) (Fig. 2d). Importantly,

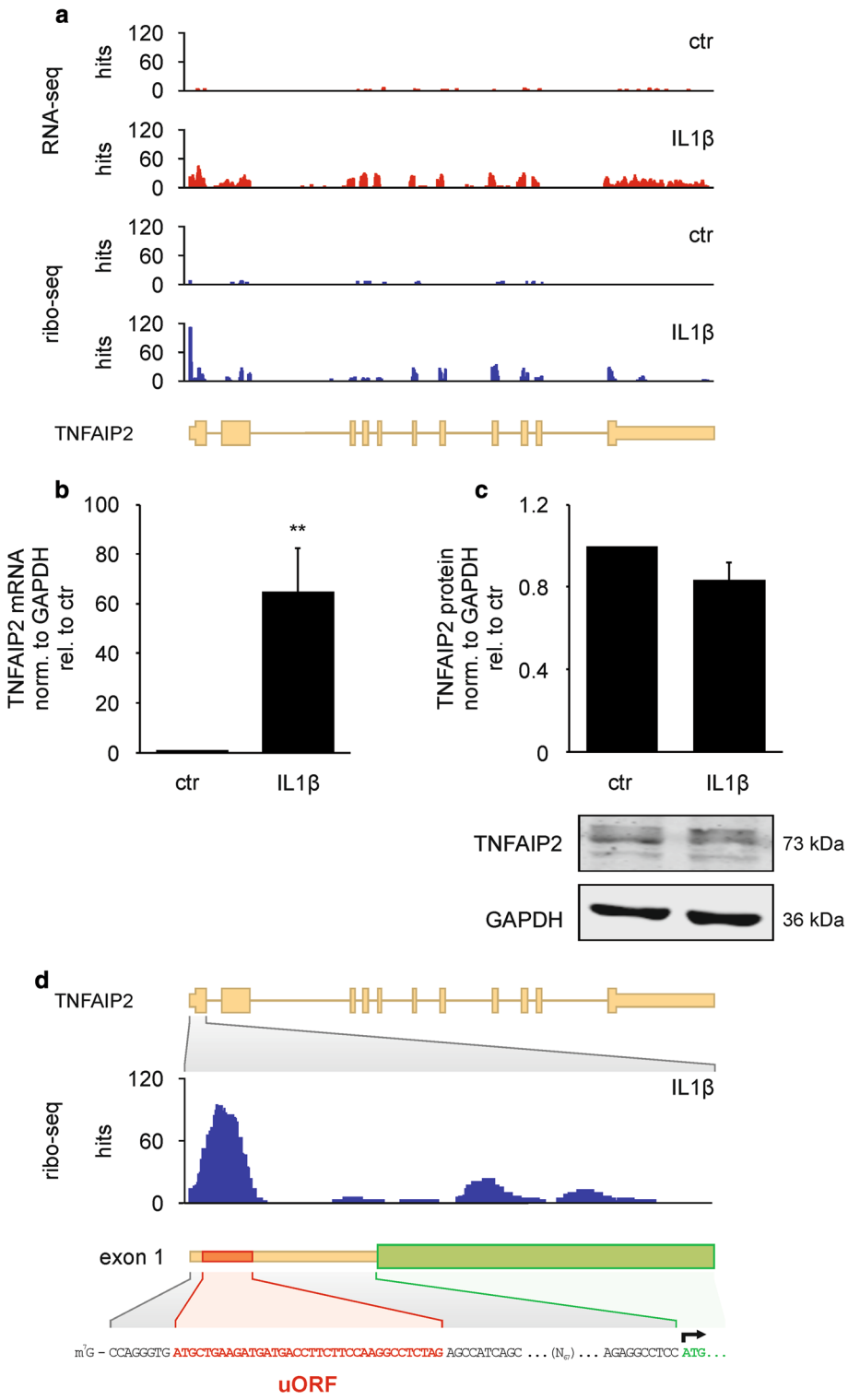

Fig. 1 IL1β induces TNFAIP2 transcription but not translation. MCF7 cells were stimulated with IL1β (50 ng/ml) for 4 h. **a** Ribosome profiling total mRNA (red) and ribosome footprint (blue) reads mapped to the TNFAIP2 transcript. Depicted are the reads of an untreated control (ctr) and an IL1β-treated sample. **b** mRNA expression of TNFAIP2 was analyzed by RT-qPCR and normalized to GAPDH expression. Data are presented relative to the untreated control (ctr) as mean ± SEM ($n \geq 4$; $^*p < 0.05$). **c** Protein expression of TNFAIP2 was analyzed by Western blot analysis. Blots are representative of at least three independent experiments. Data are presented relative to the untreated control (ctr) as mean ± SEM ($n \geq 4$; $^*p < 0.05$). **d** Zoom-in on exon 1 of TNFAIP2 and the associated ribosome footprints of the IL1β-stimulated sample (from **a**). The location of the uORF is marked in red, the main ORF in green

Western blot analyses of *renilla* luciferase protein revealed a single band in MCF7 cells transfected with the TLS containing reporter vector, while two larger proteins appeared when the stop codon was mutated (Fig. 2e). In line with the distance between the uORF start codons and the main ORF start codon [uORF1: 123 nt (41 amino acids); uORF2: 114 nt (38 amino acids)] the additional *renilla* luciferase antibody reactive proteins were approximately 4 kDa larger than the *renilla* luciferase protein in cells transfected with either the empty vector or the vector containing the unaltered TNFAIP2 TLS with the intact uORF stop codon. If the uORF start codons were mutated in addition to the

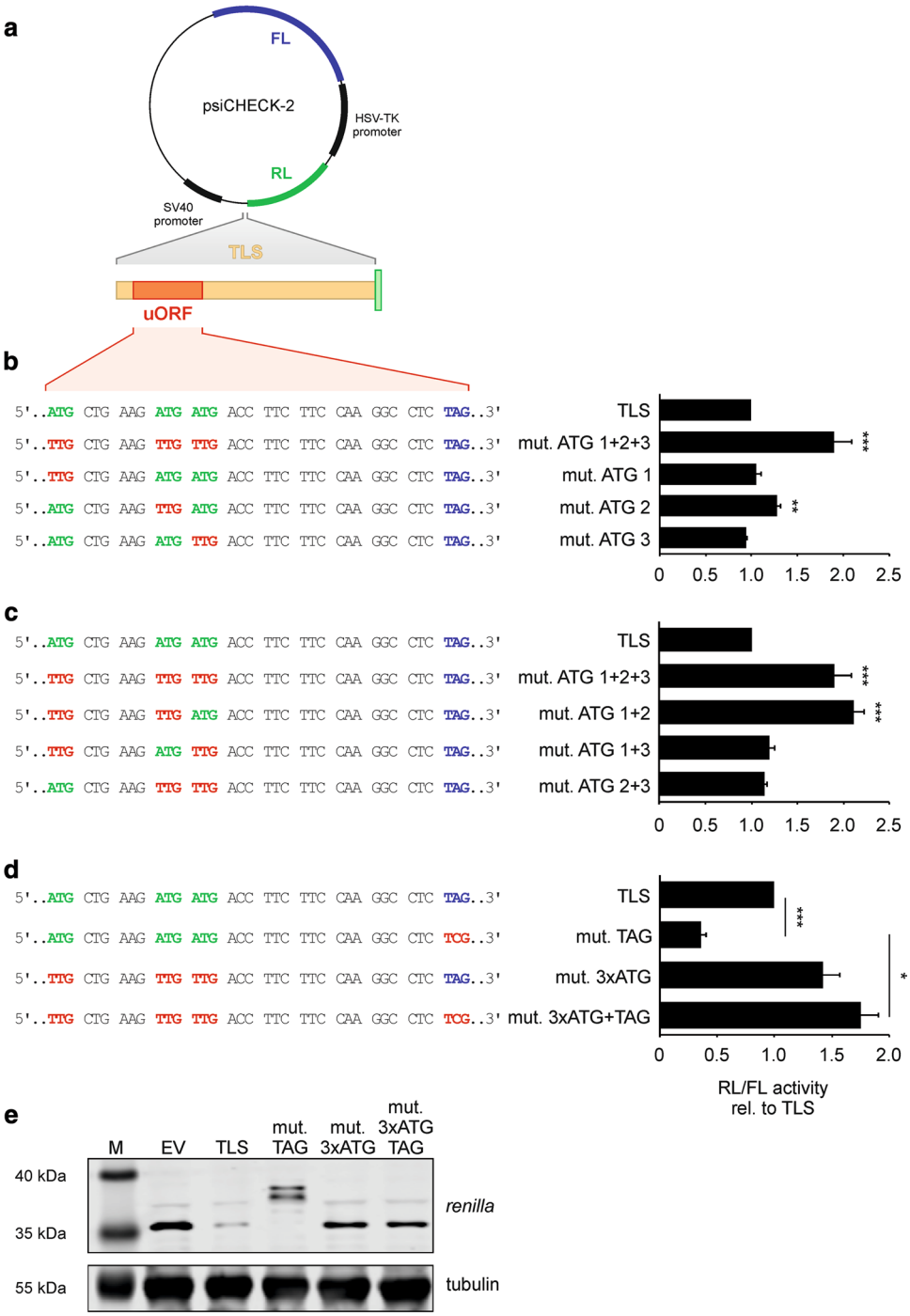

Fig. 2 The TNFAIP2 transcript leader sequence (TLS) contains inhibitory uORFs. **a** The TNFAIP2 TLS was inserted into the psiCHECK-2 vector in front of the *renilla* luciferase coding sequence, while *firefly* luciferase expression served as normalization control. **b**, **c**, **d** MCF7 cells were transfected with the psiCHECK-2 vector containing either the empty vector, the intact TNFAIP2 TLS, or the TLS containing specifically introduced mutations. *Renilla* (RL) and *firefly* luciferase (FL) activities were determined 24 h after transfection, luciferase activities of cells transfected with the vector containing the intact TLS served as control. **b** Combined mutation of all three uORF start codons and individual mutations of the three uORF start codons [ATG (green) to TTG (red)] were compared to the intact TLS-containing vector. **c** Mutations of various combinations of the three uORF start codons [ATG (green) to TTG (red)] were compared to the intact TLS-containing vector. **d**, **e** Mutation of the shared uORF stop codon [TGA (blue) to TCG (red)] were compared to the intact TLS-containing vector in the presence and absence of the mutations of the three uORF start codons [ATG (green) to TTG (red)] on luciferase activity (**d**) and protein level (**e**). All luciferase data are normalized to the vector containing the intact TNFAIP2 TLS and are shown as mean ± SEM ($n \geq 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). *Renilla* protein was analyzed by Western blot analysis and a representative blot is shown

stop codon mutation, the larger *renilla* luciferase-fusion proteins disappeared and the regular-sized *renilla* protein emerged again.

Thus, our data provide evidence for the presence of translation-inhibitory, overlapping uORFs within the TLS of TNFAIP2. Moreover, we provide evidence that the overlapping uORFs in fact are able to initiate translation and transmit the potent translation-inhibitory activity with respect to the TNFAIP2 main ORF.

The translation-inhibitory activity of the TNFAIP2 uORFs is attenuated by TPA

In a next step, we aimed to evaluate the impact of different stimuli on the extremely potent translation-inhibitory TNFAIP2 uORFs. Therefore, we transfected MCF7 breast cancer cells with the TLS containing vector and treated the cells with different stimuli. In line with the observation that IL1β did not induce TNFAIP2 protein expression in MCF7

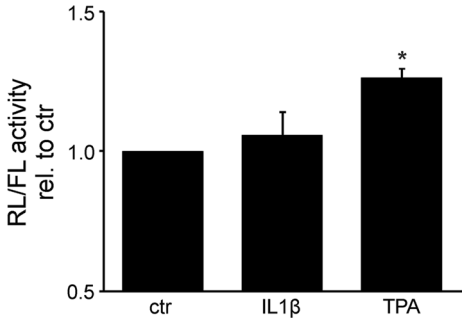

Fig. 3 The translation-inhibitory activity of the TNFAIP2 uORFs is attenuated by TPA. MCF7 cells were transfected with the psiCHECK-2 vector containing the TNFAIP2 TLS. Subsequently, the transfected cells were treated with IL1β (50 ng/ml) or TPA (10 nM), or the appropriate vehicle controls (water or DMSO, respectively) for 24 h, before *renilla* (RL) and *firefly* luciferase (FL) activities were determined. Data are normalized to the respective controls (ctr) and are shown as mean ± SEM ($n \geq 3$; $^*p < 0.05$)

cells, IL1β did not affect the inhibitory uORF activity in transfected cells (Fig. 3). Similarly, none of the other inflammatory stimuli tested, i.e. lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and interferon γ (IFNγ), relieved the uORF-mediated translational repression (Supplementary Fig. S1). In contrast, stimulation of transfected MCF7 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) significantly increased the *renilla* luciferase activity of the uORF-bearing vector (Fig. 3). Thus, TPA appears to at least partially overcome the inhibitory activity of the TNFAIP2 uORFs.

TPA induces protein expression of TNFAIP2 in THP1 cells

Since TNFAIP2 was previously reported to be predominantly expressed in myeloid cells, e.g. in response to pro-inflammatory stimuli [19, 26], we next aimed at evaluating the impact of the TNFAIP2 uORFs in monocytic THP1 cells. To determine, whether the TPA effect on the uORF activity in the reporter vector translates to a differential expression of endogenous TNFAIP2 protein, we assessed both mRNA and protein levels in THP1 cells upon stimulation with TPA. While TPA only slightly induced TNFAIP2 mRNA expression (Fig. 4a), it significantly enhanced TNFAIP2 protein expression (1.70 ± 0.06) (Fig. 4b), consequently resulting in an enhanced translational activity of TNFAIP2 as determined by calculation of relative TNFAIP2 protein to mRNA ratios (Fig. 4c). Of note, both TNFAIP2 protein and mRNA appeared to be present at much higher levels in THP1 cells compared to MCF7 cells (Supplementary Fig. S2).

Taken together, monocytic THP1 cells appear to express much higher levels of TNFAIP2 than MCF7 breast tumor cells both at the mRNA and protein level. Furthermore, TPA

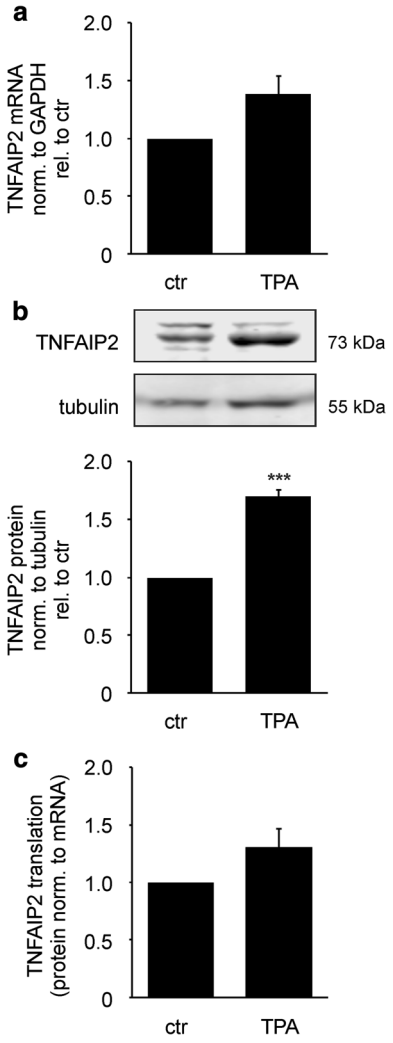

Fig. 4 TPA induces protein expression of TNFAIP2 in THP1 cells. THP1 cells were stimulated with TPA (10 nM) or the vehicle control (DMSO) for 24 h. **a** mRNA expression of TNFAIP2 was analyzed by RT-qPCR and normalized to GAPDH expression. **b** Protein expression of TNFAIP2 was analyzed by Western blot analysis. Blots are representative of at least three independent experiments. **c** TNFAIP2 translational activity was determined as relative TNFAIP2 protein expression normalized to relative TNFAIP2 mRNA expression. Data are presented relative to the vehicle control (ctr) as mean ± SEM ($n \geq 4$; $^*p < 0.05$)

was able to induce TNFAIP2 protein expression in THP1 cells only, however, to a rather small extent.

Translation of TNFAIP2 is induced during the differentiation of primary human macrophages

As TNFAIP2 protein was previously shown to be differentially expressed during the course of mouse embryonic development [17] as well as in response to the myeloid differentiation inducer retinoic acid [27], and considering

that the well-established monocyte-to-macrophage differentiation stimulus TPA [28] appeared to limit the activity of the TNFAIP2 uORF, we next questioned if the uORF-dependent regulation of TNFAIP2 translation might be more pronounced in the context of myeloid differentiation. Therefore, we isolated $CD14^+$ monocytes (MO) from human buffy coats and differentiated them to macrophages (MΦ) over a period of 7 days in the presence of macrophage colony-stimulating factor (M-CSF). In line with the observations in THP1 cells, the expression of TNFAIP2 mRNA was only marginally elevated in MΦ as compared to MO (Fig. 5a), yet both expressed much higher basal levels of TNFAIP2 mRNA than MCF7 cells (Supplementary Fig. S3). Strikingly, while TNFAIP2 protein was not detectable in MO, it was highly abundant in MΦ (Fig. 5b). To investigate whether the differences in protein expression were due to changes in translation, we determined the translational status of TNFAIP2 mRNA by polysomal fractionation analyses. While total RNA showed similar distribution across the gradients in MO and MΦ as seen in the UV profile of the fractionation (Fig. 5c, upper panel), the specific distribution of TNFAIP2 mRNA appeared to be massively altered between MO and MΦ (Fig. 5c, lower panel). Specifically, MO (black line) displayed significantly higher relative TNFAIP2 mRNA abundance in fraction 5, whereas fractions 8 and 9 contained significantly higher TNFAIP2 mRNA levels in MΦ (blue line). To allow for quantitative assessment, we compared the ratios of TNFAIP2 mRNA distributions between MO and MΦ within the early polysomes (EP), i.e. fractions 4–6, with mRNAs covered by < 5 ribosomes, and the late polysomes (LP), i.e. fractions 7–9, containing mRNAs bound by > 5 ribosomes, according to the UV profile (Fig. 5c, upper panel). MΦ-to-MO ratios were 0.70 ± 0.05 for the EP and 2.25 ± 0.28 for the LP (Fig. 5d), supporting more efficient translation in the MΦ. When comparing LP/EP distributions within the single cellular populations instead, the TNFAIP2 mRNA distribution in MO was skewed towards the EP (LP/EP = 0.46 ± 0.01) suggesting limited TNFAIP2 translation efficiency for the MO (Fig. 5e). In contrast, in MΦ the distribution was strongly biased toward the LP (LP/EP = 1.39 ± 0.06) indicative for efficient TNFAIP2 translation in MΦ.

The observation that in MO TNFAIP2 mRNA was highly abundant in EPs, while no TNFAIP2 protein was produced (Fig. 5b), suggests that the association of the TNFAIP2 transcript with single or few ribosomes only might not translate into active translation. Strong skewing of the TNFAIP2 transcript distribution to the EPs might be indicative of the translation of a short uORF instead, which is expected to allow for the recruitment of only a limited number of ribosomes per transcript. To validate this concept, we analyzed a publically available ribosome profiling dataset of $CD14^+$ cells isolated from human buffy coats, which were differentiated to MΦ for 24 h in the presence of M-CSF (GSE66810) [29]. While abundant RPF reads were detected across the entire TNFAIP2 transcript (Fig. 5f, upper panel), the uORF also contained an appreciable number of RPF reads (Fig. 5f, lower panel). Comparison of the RPF distributions between the main ORF of TNFAIP2 and its regulatory uORF as indicator of altered translation further suggested that TNFAIP2 translation was largely inhibited in MCF7 cells (ratio main ORF-to-uORF = 0.12), whereas in MΦ TNFAIP2 appeared to be much more efficiently translated (ratio main ORF-to-uORF = 0.82) (Fig. 5g).

Taken together, our data suggest that TNFAIP2 translation is tightly controlled by uORFs within its TLS. Moreover, while TNFAIP2 translation appears to be efficiently inhibited in MCF7 breast cancer cells, the uORF-dependent translational repression appears to be abolished during the differentiation of MO to MΦ, resulting in pronounced TNFAIP2 protein production.

Discussion

In this study, we characterized so far unknown, overlapping uORFs in the TLS of TNFAIP2, which exert strong inhibitory functions towards the translation of the TNFAIP2 main ORF. We identified the uORFs in breast tumor cells, where they prevented TNFAIP2 protein synthesis almost completely even when the transcription was strongly induced in response to inflammatory conditions. Similarly, the TNFAIP2 uORFs effectively suppressed protein expression in monocytes, while they appeared to be inactivated during the differentiation to macrophages, consequently allowing for high TNFAIP2 protein levels in mature macrophages.

uORFs have been shown to play an important regulatory role in response to various stress conditions [7]. Most prominently, uORFs have been characterized in the context of the integrated stress response (ISR) as induced, e.g. by the unfolded protein response [30]. During the ISR the translation initiation factor eIF2α is phosphorylated and inactivated by various kinases, which reduces the formation of the ternary complex. The consequences are well-characterized for certain mRNAs such as ATF4, which is controlled by the coordinated activity of two uORFs [31].

We initially identified the TNFAIP2 uORF in breast tumor cells exposed to IL1β. In line with previous reports showing elevated TNFAIP2 transcription in response to inflammatory stimuli including TNFα, LPS, and IL1β in endothelial cells [19], we observed strong TNFAIP2 mRNA induction upon IL1β stimulation. Surprisingly though, TNFAIP2 protein expression remained unchanged and almost undetectable. We attributed this apparently efficient translational repression to the presence of highly potent uORFs within the TLS of TNFAIP2, the activity of which was not affected by any of

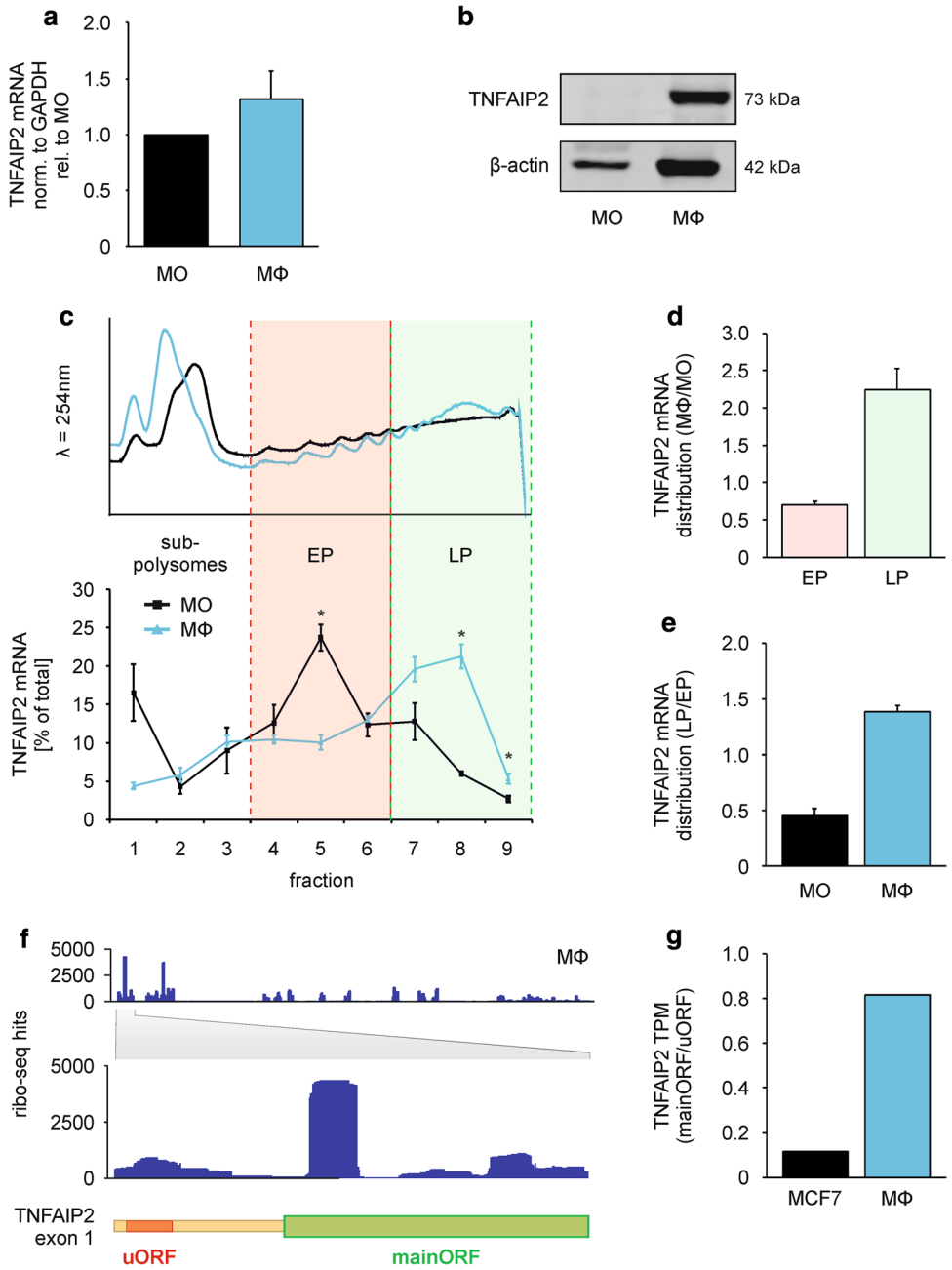

Fig. 5 Translation of TNFAIP2 is induced during the differentiation of primary human macrophages. $CD14^+$ monocytes (MO) were isolated from human buffy coats and differentiated to macrophages (MΦ) over a period of 7 days using M-CSF (20 ng/ml). **a** mRNA expression of TNFAIP2 in MO (black bar) and MΦ (blue bar) was analyzed by RT-qPCR and normalized to GAPDH expression. Data are presented relative to MO expression as mean ± SEM ($n = 7$). **b** Protein expression of TNFAIP2 in MO and MΦ was analyzed by Western blot analysis. Blots are representative of seven independent experiments. **c** Translational status of TNFAIP2 in MO (black lines) and MΦ (blue lines) was assessed by polysomal fractionation analysis. UV profiles obtained for the sucrose gradients during fractionation for MO and MΦ representative of three replicates are shown (upper panel). The distribution of TNFAIP2 mRNA across the gradients for MO and MΦ was analyzed by RT-qPCR. Data are presented as mean ± SEM ($n = 3$; $^*p < 0.05$). Early polysomal fractions (EP, red) and late polysomal fractions (LP, green) are highlighted. **d** Relative TNFAIP2 mRNA distribution for MΦ vs. MO was compared for EP (fractions 4–6, red) and LP (fraction 7–9, green). **e** Relative TNFAIP2 mRNA distribution between LP and EP was compared for MO (black bar) and MΦ (blue bar). **f** Ribosome profiling reads mapped to the TNFAIP2 transcript and zoom-in on exon 1 in human MΦ based on a previously published data set (GSE66810) [29]. Depicted are the tracks of one replicate. **g** Comparison of the distribution of normalized ribosome profiling reads (transcripts per million, TPM) between the main ORF and the uORFs of TNFAIP2 for MCF7 cells and MΦ

the inflammatory stimuli. In line with the very low TNFAIP2 protein expression in MCF7 cells, TNFAIP2 was recently shown to be highly expressed at both mRNA and protein levels in triple negative breast cancer cell lines as compared to estrogen receptor positive breast cancer cells [20]. While the authors nicely established transcriptional regulation of TNFAIP2 by the transcription factor Kruppel-like factor 5 (KLF5), it will be interesting to see in future studies how exactly the translation-inhibitory activity of the TNFAIP2 uORFs might be overcome in these cells. In addition to cell-type specific uORF-activity modulating signals, uORF-inactivating mutations might be envisioned. Along these lines, Schulz and co-workers recently showed that loss-of-function mutations in uORFs can lead to overexpression of certain oncogenes, thus contributing to cancer progression [16].

TNFAIP2 was previously described to be expressed in myeloid cells both in response to inflammatory stimuli [32], but importantly also during developmental processes [17, 27, 33]. In contrast to most of these studies, we did not find differences in TNFAIP2 mRNA expression between MO and MΦ, while a massive induction in TNFAIP2 protein levels was observed upon differentiation of MO to MΦ. This corroborates the earlier finding that TNFAIP2 protein is present in differentiated dendritic cells and MΦ [34]. Surprisingly, while no protein was detectable in MO, TNFAIP2 mRNA was highly abundant within the early polysome-associated fractions, which should be translationally active. Taking the length of the two regulatory TNFAIP2 uORFs (36 and 27 nucleotides) into account, 1 to maximally 2 ribosomes should be able to bind within the respective uORFs at the same time. The fact that TNFAIP2 mRNA was enriched in the early polysomal fractions (4 + 5) containing mRNAs associated with 1 or 2 functional ribosomes only, might, therefore, point to an active uORF. Therefore, we conclude that in the case of TNFAIP2, association with up to 2 ribosomes, is an indicator of translation of the uORFs rather than of the main ORF. The observation that TNFAIP2 mRNA moved to the highly translated fractions (late polysomal fractions) upon release of the uORF-dependent repression, suggests that, aside from the uORFs, TNFAIP2 translation is very efficient, at least in myeloid cells. The finding that ample amounts of TNFAIP2 mRNA are produced even when the translation is completely inhibited in MO, indicates that it might be absolutely crucial to keep TNFAIP2 protein levels low in MO. Nevertheless, the constant availability of TNFAIP2 mRNA allows for rapid regulation of the protein, once needed, e.g. during the differentiation to MΦ. As a side note, the concept that the TNFAIP2 uORFs might be functionally important is further supported by the observation that the almost complete inhibitory activity of the uORFs is retained when either one of the three potential start codons is mutated individually, requiring the loss of at least two ATGs for inactivation. Efficient translation of at least two of the overlapping uORFs was further substantiated by the detection of two larger sized *renilla* luciferase fusion-proteins when the shared uORF stop codon was mutated. As additional uORFs were previously identified during monocyte differentiation [35], this regulatory principle might prove to be of general importance in this context. Interestingly, the induction of the unfolded protein response was previously shown during monocyte-to-macrophage differentiation [36]. As the unfolded protein response is a classical stimulus inactivating translation-repressive uORFs, it might also contribute to the translational derepression of TNFAIP2. Along similar lines, strong uORFs have also been shown to control protein expression of key differentiation regulators like Nanog, POU5F3, and Smad7 during zebrafish development [37].

In conclusion, our study identified so far unknown overlapping uORFs with strong translation regulatory properties in the TLS of TNFAIP2, which appeared to be of major importance in myeloid cell differentiation. Furthermore, our observation that polysomal fractionation analyses might allow for the differentiation between uORF and main ORF translation, at least for transcripts containing short uORFs only, could open new possibilities for the characterization of the impact of such uORFs.

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