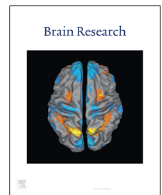
Brain Research 1647 (2016) 50–56

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres



Research Report

TBPH/TDP-43 modulates translation of Drosophila *futsch* mRNA through an UG-rich sequence within its 5′UTR

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ARTICLE INFO

Article history:
Received 27 November 2015
Received in revised form
5 February 2016
Accepted 11 February 2016
Available online 18 February 2016

Keywords:
Drosophila
TBPH
TDP-43
Futsch
MAP1B
Neurodegeneration

ABSTRACT

Nuclear factor TDP-43 is an evolutionarily conserved multifunctional RNA-binding protein associated with frontotemporal lobar degeneration (*FTLD*) and amyotrophic lateral sclerosis (ALS). In recent years, Drosophila models of ALS based on TDP-43 knockdown/overexpression have allowed to find several connections with disease. Among these, we have previously described that silencing the expression of its fly ortholog (TBPH) can alter the expression of the neuronal microtubule-associated protein Futsch leading to alterations of neuromuscular junction (NMJ) organization. In particular, TBPH knocked out flies displayed a significant reduction of Futsch protein levels, although minimal variation in the *futsch mRNA* content was observed. These conclusions were recently validated in an independent study. Together, these observations strongly support the hypothesis that TBPH might regulate the translation of *futsch* mRNA. However, the mechanism of TBPH interference in *futsch* mRNA translation is still unknown. In this work, we use EMSA experiments coupled with RNA-protein co-immunprecipitations and luciferase assays to show that TBPH interacts with a stretch of UG within the 5′UTR of *futsch* mRNA and translation is positively modulated by this binding. Most importantly, this function is also conserved in human TDP-43. This result can therefore represent the first step in elucidating the relationship between TDP-43, protein translation, and eventual disease onset or progression.

This article is part of a Special Issue entitled SI:RNA Metabolism in Disease.

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1. Introduction

Transactive response DNA binding protein 43 kDa (TDP-43) is a multifunctional nuclear factor that regulates expression, splicing, transport, and mRNA stability of numerous cellular genes, including its own transcript (Ayala et al., 2011; Buratti and Baralle, 2012).

In human disease, TDP-43 aggregation and/or the presence of mutations in the TAR DNA binding protein (TARDBP) gene have been associated with the development of different neurodegenerative disorders, principally Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTLD) (Arai et al., 2006; Buratti, 2015; Janssens and Van Broeckhoven, 2013; Kabashi et al., 2008; Neumann et al., 2006; Polymenidou et al., 2011; Tollervey et al., 2011).

Since this discovery, many animal models have been developed that aim to reproduce at least some of the aspects of these diseases by overexpressing either the wild type or mutated forms of this protein.

Importantly, all these studies have taken advantage of the fact that TDP-43 structure and function is highly conserved through evolution (Ayala et al., 2005). Accordingly, several animal models show that both knockdown and overexpression of this factor recapitulate different aspects of ALS pathology such as neuronal degeneration and locomotor alterations (Belzil et al., 2013; Lee et al., 2012; Liu et al., 2013; Wegorzewska and Baloh, 2011).

In particular, several studies have used Drosophila as a model system, because the fly TDP-43 ortholog (TBPH) is structurally and functionally homolog to the human nuclear factor (Ayala et al., 2005; Buratti et al., 2004; Romano et al., 2014b). In general, therefore, Drosophila TDP-43-related models suggest that TBPH controls phenotypes that can be associated with human ALS pathology (Romano et al., 2012).

For this reason, characterization of TBPH physiological and pathological functions can provide important insights into ALS pathophysiology. In particular, among the Drosophila genes whose expression is regulated by TBPH, there are several genes known to code for different pre-synaptic proteins. Specifically, in the presynaptic compartment the expression levels of Futsch, Syntaxin 1A

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http://dx.doi.org/10.1016/j.brainres.2016.02.022
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(syx), Synapsin (syn) and Cysteine string protein (csp) proteins become downregulated after neuronal suppression of TBPH. This suggests that TBPH can influence synaptic transmission through direct or indirect modulation of proteins controlling microtubule network or synaptogenesis (Feiguin et al., 2009; Romano et al., 2014a).

Among these factors, the *futsch* gene (CG34387; FBgn0259108) was the first gene identified as mis-regulated in *TBPH*-null flies (Feiguin et al., 2009). In addition to the fact that *futsch* is the fly homolog of human MAP1B, this gene was particularly interesting because *TBPH*-null flies showed a significant reduction in futsch protein expression without modification of *futsch* mRNA levels. However, co-immunopreciptation assays demonstrated that TBPH protein can strongly interact with *futsch* mRNA (Feiguin et al., 2009; Godena et al., 2011). These observations suggested a direct role of TBPH in the post-transcriptional regulation of *futsch* expression. In this case, therefore, TBPH might be acting similarly to what demonstrated for the Drosophila Fragile X-related protein, another RNA binding protein, that works as a translational repressor of *futsch* to finely tune synaptic growth (Zhang et al., 2001). In addition to these data, a recent report has further supported a role of TBPH in *futsch* protein translation (Coyne et al., 2014). Interestingly, this study confirmed that TDP-43 interacts *in vivo* with *futsch* mRNA and showed that this interaction modulates the intra-cellular transport/localization and translation of this mRNA in fly motor neurons (Coyne et al., 2014). This study also suggested that TBPH might sequester *futsch* mRNA into RNP complexes and regulate its expression in motor neuron cell bodies through its controlled release (Coyne et al., 2014). Notwithstanding these concordant lines of evidence connecting TBPH/TDP-43 with the translation process, it is not yet clear whether this action is direct or not. Therefore, the aim of this work was to better characterize this process by mapping the yet unknown binding site of TBPH/TDP-43 to the *futsch* mRNA and validating its functional importance in the translation process.

2. Results

2.1. Mapping in vitro the interaction of TBPH/TDP-43 with the 5′UTR of futsch mRNA

Considering the importance of 5′UTRs sequences for translational regulation of gene expression (Araujo et al., 2012), it was very likely that TBPH could play a role in the translation of the *futsch* mRNA by binding to this particular region.

More specifically, we observed that futsch mRNA 5′UTR contains a stretch of UGs (Fig. 1A). Considering that both TBPH and TDP-43 share the ability to bind to UG-rich sequences with high affinity, we then tested whether TBPH/TDP-43 could bind this region. To this end, we performed EMSA analysis (Electrophoretic Mobility Shift Assay) using a labeled probe containing this sequence and purified recombinant TBPH/TDP-43. This experiment clearly showed that both the fly and human TDP-43 proteins can

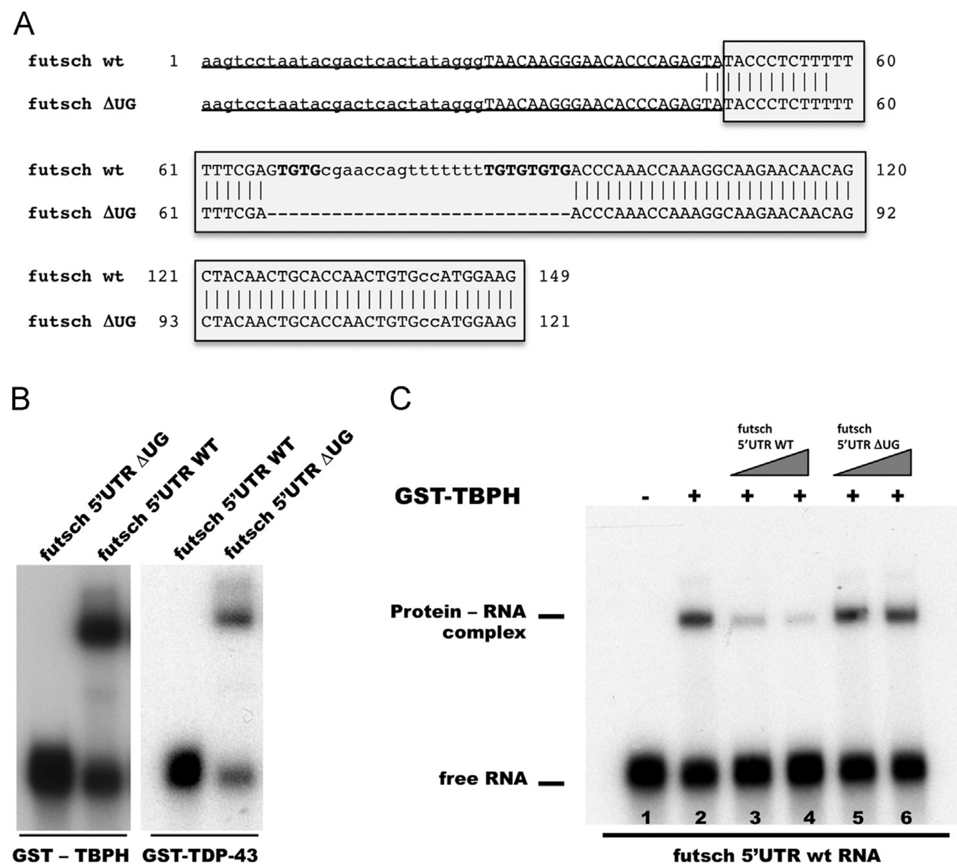

Fig. 1. TDP-43 binds to UGs within the futsch 5′UTR region. (A) Sequence of the *in vitro* transcribed RNA. Both wild type and ΔUG *futsch* 5′-UTRs including the UG-rich tract (boldfaced) were amplified by PCR (gray shaded sequence). The sequence of the forward oligo (underlined) includes the T7 promoter used for either the cold or ^{32}P-labeled RNA transcription. (B) EMSA analysis with both recombinant GST-TDP-43 and GST–TBPH proteins shows that the proteins can bind UG-rich RNAs at comparable levels. (C) TDP-43 binds specifically the UG-rich-tract within the 5′UTR *futsch* RNA. The interaction of GST-TDP-43 with the ^{32}P-labeled UG-sequence of *futsch* 5′UTR (lane 2) can be successfully competed with cold wild type *futsch* 5′UTR RNA (lanes 3 and 4), but not by cold ΔUG *futsch* 5′UTR RNA (lanes 5 and 6). (C) Co-immunoprecipitation of the 5′UTR *futsch* RNA by TDP-43. This experiment shows the RT-qPCR analysis of 5′UTR *futsch* RNA immunoprecipitated by Flag-tagged TDP-43. The enrichment-fold is referred to an unrelated protein (EGFP) or to the mutant TDP-43 F/L that is unable to bind RNA (TDP-43 F/L). Significant levels of enrichment were observed for the wild type but not for ΔUG *futsch* 5′UTR RNA. This analysis confirms the interaction of TDP-43 with the UG-rich tract of *futsch* 5′UTR mRNA.

bind the 5′UTR *futsch* RNA probe, and that such an interaction was abolished by the deletion of the UG-rich tract (Fig. 1B).

The binding specificity of TBPH for the 5′UTR of futsch was also confirmed by competition studies where increasing amounts of unlabeled wild type or ΔUG 5′UTR *futsch* RNA were used to compete with radioactive wild type 5′UTR *futsch* RNA for GST–TBPH binding (Fig. 1C). As shown in this figure, increasing amounts of wild type cold 5′UTR RNA were able to effectively interfere with the interaction between TBPH and the labeled probe. However, when ΔUG cold 5′UTR *futsch* RNA was used as a competitor, the band intensity corresponding to the complex formed by the GST–TBPH protein and the labeled wild type 5′UTR *futsch* RNA did not decrease (Fig. 1C).

Taken together, these experiments support the hypothesis that the interaction between TBPH/TDP-43 and the 5′UTR *futsch* RNA is specific occurs through this UG-rich sequence.

2.2. RNA-protein pull down

In order to verify the interaction between both fly and human TDP-43 orthologs with *futsch* mRNA 5′UTR, we then performed RNA-protein pull down assays.

In the first experiment, GST–TBPH or GST proteins were initially incubated with Glutathione resin. Then, *in vitro* transcribed wild type 5′UTR (*futsch* 5′UTR wt) or ΔUG 5′UTR (*futsch* 5′UTR ΔUG) RNAs were added. After several washes followed by RNA purification, RT-qPCR was used to quantify the amount of wild type or ΔUG 5′UTR RNA pulled down by TBPH. The enrichment-fold was normalized to the GST protein. As shown in Fig. 2A, significant levels of enrichment (30x) were observed for the wild type RNA sequence and not for ΔUG RNA sequence.

Subsequently, this specific interaction was also tested using the human TDP-43 ortholog (Fig. 2B, upper panel). In this experiment, HEK293 cells were transfected with pFLAG-TDP43, pFLAG-TDP43 F/L or pEGFP-N1 vectors. Then, cell extracts expressing comparable levels of recombinant proteins were used for RNA-protein co-immunoprecipitation assays (Fig. 2B, lower panel). In this case, the enrichment-fold was referred to an unrelated protein (EGFP) or to the mutant TDP-43 F/L (TDP-43 F/L). This mutant was used because it carries two F/L substitutions (F147L/F149L) into the first RNA recognition motif (RRM1) that abolish the TDP-43 RNA-binding ability (Buratti and Baralle, 2001). Also in this case, significant levels of enrichment were observed for only the wild type 5′UTR RNA sequence and not for the ΔUG 5′UTR RNA (Fig. 2B). As expected, no enrichment was observed for an unrelated gene (RPL13a mRNA). On the other hand, when the preferred TDP-43/TBPH $(UG)_9$ RNA sequence was added to extract samples a high level of enrichment was observed for the TDP-43 wild type protein as opposed to the TDP-43 F/L mutant (data not shown).

Taken together, these experiments support the hypothesis that both Drosophila and human TDP-43 orthologs interact specifically with the UG-rich tract of the *futsch* 5′UTR RNA.

2.3. Futsch 5′UTR modulates translational efficiency

Next, we sought to understand whether the 5′UTR UG-rich tract of *futsch* might directly influence translational efficiency. This was performed using a cell-based luciferase assay. To set this up, we cloned in the inducible pGL4.11 vector the *Drosophila melanogaster Hsp70Ba* gene promoter (in order to get a controlled and uniform luciferase expression) with the *futsch* 5′UTR sequence cloned immediately upstream of the luciferase ATG-start codon. As part of

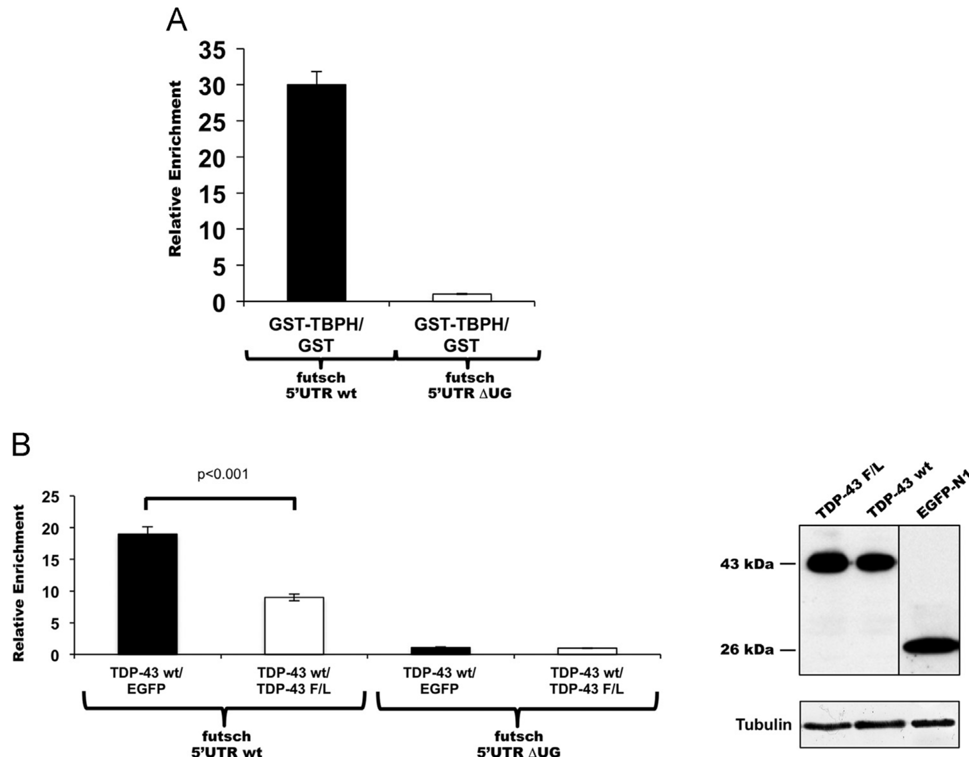

Fig. 2. TBPH interacts with the *futsch* 5′UTR. (A) RNA pull-down of the 5′UTR *futsch* RNA by GST–TBPH recombinant protein. This experiment shows the RT-qPCR analysis of *futsch* 5′UTR pulled down by GST–TBPH. The enrichment-fold is referred to the GST protein used as a negative control. Significant levels of enrichment were observed for the wild type but not for ΔUG *futsch* 5′UTR RNA. (B) Co-immunoprecipitation of the *futsch* 5′UTR RNA by TDP-43. Left panel: RT-qPCR analysis of *futsch* 5′UTR RNA immunoprecipitated by Flag-tagged TDP-43. The enrichment-fold is referred to an unrelated protein (EGFP) or to the mutant TDP-43 F/L (TDP-43 F/L). Significant levels of enrichment were observed for the wild type but not for ΔUG *futsch* 5′UTR RNA. This analysis confirms the interaction of TDP-43 with the UG-rich tract of the 5′UTR *futsch* mRNA. Right panel: the levels of proteins used for co-immunoprecipitation experiments were evaluated in cell extracts of HEK293 transfected with pFLAG-TDP43, pFLAG-TDP43 F/L and empty pEGFP-N1 vectors. Western blot analysis was carried out using anti-FLAG (TDP-43 wt and TDP-43 F/L), anti-GFP, and anti-Tubulin antibodies.

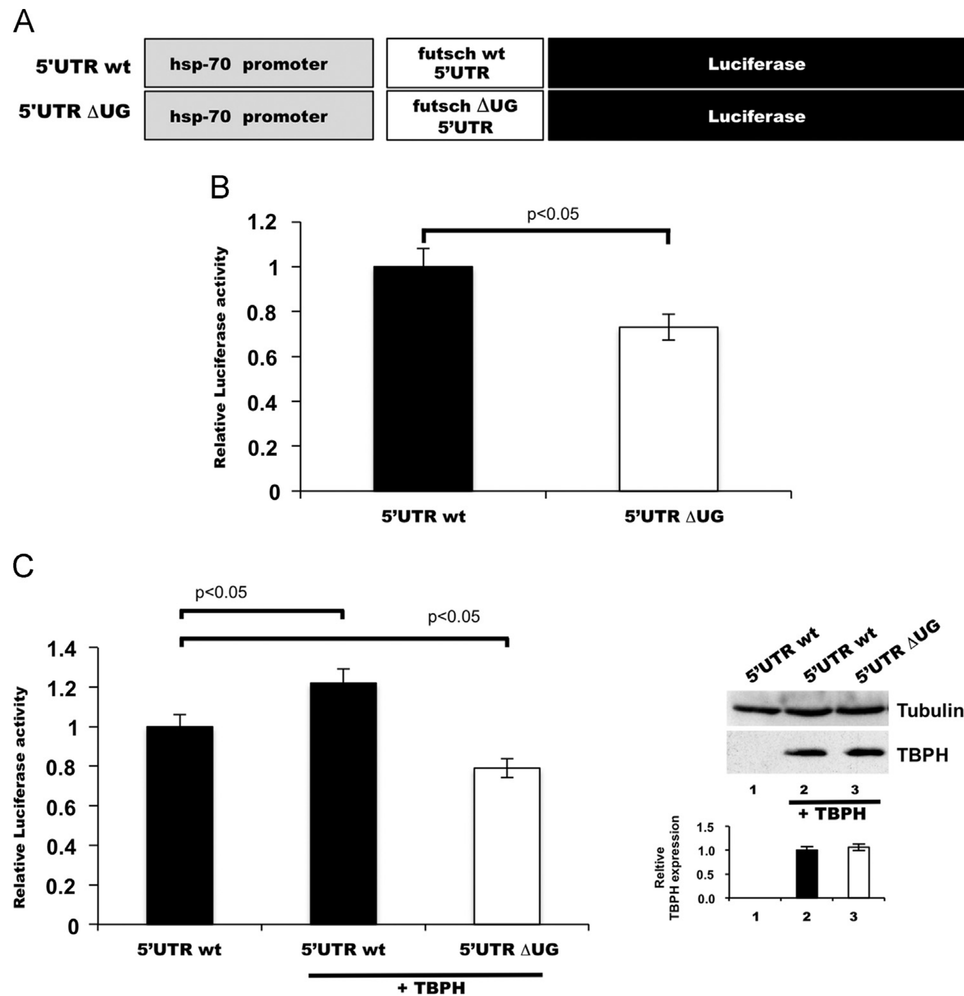

Fig. 3. Luciferase translational efficiency mediated by the *futsch 5′UTR*. (A) Structure of the expression vector showing the inserted sequence that included either the wild type and ΔUG *futsch* 5′-UTRs sequences. (B) Luciferase activity of these vectors following transfection in Drosophila S2 cells. The relative ratio of firefly/renilla luciferase activity determined from cells transfected with the plasmid carrying the wild type *futsch* 5′UTR was set at 1 to normalize results. The relative ratio of firefly/renilla luciferase activity was significantly lower ($p<0.05$) for the ΔUG than the wild type *futsch* 5′UTR RNA. (C) Luciferase activity in HeLa cells. Left panel: Wild type and ΔUG 5′UTR constructs were transfected into HeLa cells, along with a TBPH-wild type cDNA pFLAG-expression vector. 24 h after transfection, cells were heat shocked for 1 h at 42 °C and the luciferase activities were measured after 6 h-restore at 37 °C. The relative ratio of firefly/renilla luciferase activity determined from cells transfected with the plasmid carrying the wild type *futsch* 5′UTR sequence was set at 1 to normalize results. All experiments were performed in triplicate. Right panel: Western blot to determine flag-TBPH overexpression. Anti-Tubulin (Tubulin) and anti-Flag (TBPH) antibodies were used to verify the levels of TBPH overexpression in cells cotransfected with wild type and the ΔUG *futsch* 5′UTR constructs (left panel). Optical densitometry (ratio TBPH/Tubulin bands) was used to compare the levels of TBPH expression. All experiments were performed in triplicate.

this system, we generated two vectors carrying either the wild type or the ΔUG 5′UTR sequence, as negative control (Fig. 3A).

Initially, these constructs were transfected in the S2 Drosophila cell line and preliminary setup experiments showed that the peak of *Hsp70Ba* promoter activity was observed 6 h after thermal shock (not shown). Therefore, after transfection, the induction of luciferase expression was obtained by exposing the cells to thermal shock (20 min at 37 °C) followed by a 6 h restoration period at 25 °C, before assaying luciferase activity. First of all, the results of the initial transfection showed that luciferase activity of the *futsch* 5′-UTR ΔUG construct resulted significantly lower (27%) of that from the 5′-UTR wt (Fig. 3B). This finding suggested that the (UG)-rich tract could influence the translational efficiency of the downstream open reading frame. Subsequently, the wild type 5′ UTR and the ΔUG 5′UTR *futsch* constructs were transfected into HeLa cells along with a TBPH-wild type cDNA pFLAG-expression vector. Then, 24 h after the end of the transfection cells were heat-shocked for 1 h at 42 °C and firefly/renilla luciferase activities were measured after 6 h, as previously described. In this experiment, the relative ratio of firefly/renilla luciferase activity determined from cells transfected with the plasmid carrying the wild type futsch 5′UTR was set at 1 for normalization purposes. As shown in Fig. 3C, TBPH overexpression induced a statistically significant increase (22%) in the luciferase activity, and this occurred only in presence of the wild type 5′UTR futsch sequence. In fact, TBPH overexpression did not influence luciferase activity of the ΔUG 5′ UTR *futsch* construct (that remained significantly lower than that the wild type 5′UTR construct without TBPH overexpression, Fig. 3C).

3. Discussion

In this work, we report that both fly and human TDP-43 orthologs interact specifically with an UG-rich sequence within the *futsch* 5′ UTR mRNA and that overexpression of Drosophila TBPH can positively modulate the translational efficiency only in presence of this UG-rich sequence. In fact, using an inducible promoter (in order to modulate gene expression more finely compared to constitutive promoters), we have found that *futsch* 5′UTR

can positively regulate the expression the reporter gene only if the UG-rich sequence is present.

Therefore, our data confirm and extend the hypothesis of the TBPH-dependent translational regulation of *futsch* gene expression and define the sequence context required to affect the translational efficiency of *futsch* mRNA. With respect to the human homolog of *futsch*, MAP1B gene, it has to be noted that the UG sequence is not conserved. However, MAP1B has been found to be associated with TDP-43-containing RNP complexes in mouse models (Sephton et al., 2011), suggesting that TBPH/TDP-43 might also play a role in the translational regulation of this transcript (although further work will be required to clarify this issue).

Most importantly, however, this property of TBPH/TDP-43 is probably not confined to just this transcript and it is very likely that several additional targets exist which might be affected at the translational level. For example, at least another presynaptic protein, Cysteine string protein (csp) (Dawson-Scully et al., 2007), might be regulated in this manner. In fact, in *TBPH* null flies, this synaptic marker has been found to be downregulated only at the protein level and *csp* mRNA levels were not modified notwithstanding its co-immunopreciptation by TBPH (Romano et al., 2014a).

Also in this respect, a recent report has identified TDP-43 as a general component of mRNP transport granules in neurons, with its involvement in the anterograde axonal transport of target mRNAs from the soma to distal axonal compartments, such as NMJ (Alami et al., 2014). This finding, along with observation that some TDP-43 mutations can alter trafficking of TDP-43 cognate mRNAs in ALS patients, has suggested that TDP-43 might support spatially appropriate translation of target mRNAs and that the alteration of this TDP-43 activity might contribute to neurodegeneration (Alami et al., 2014). In summary, altogether these observations support the hypothesis that TDP-43 might play a role in translation regulation, that is conserved through evolution and whose alteration might contribute to the ALS pathogenesis. Consistently, futsch/MAP1B localization seems to be altered in the spinal cord of motor neurons of ALS patients (Coyne et al., 2014).

Therefore, further studies with patients affected by TDP-43 proteinopathy will be useful to better define the contribution of the translational properties of this factor in ALS pathogenesis and to test whether this activity can be modulated by TDP-43 disease-causing mutations.

4. Experimental procedure

4.1. Constructs

In order to generate an inducible Drosophila *hsp70* plasmid (pGL4–hsp70-Luc), the *Hsp70Ba* gene promoter from W1118 genomic DNA was amplified using the following primers: hsp70_Kpn_s: 5′-ACTGGTACCTTATAAAGAAATTTCCAAAATAA-3′ and hsp70_HindIII_as: 5′-ATGAAGCTTTGCACTTTACTGCAGATTGTTTAGCTT-3′. Subsequently, the *futsch* 5′UTR sequence was cloned in between the *Hsp70Ba* promoter and the luciferase ATG-start codon, creating two vectors with the wild type (5′UTR wt) and ΔUG (5′UTR ΔUG) *futsch* 5′UTR. To this aim, the *futsch* 5′UTR sequence was amplified from W1118 genomic DNA with the following primer couple (5′UTR *futsch* _HindIII_S: 5′-CCAAGCTTATTAAACAAAACCAAAAACCG-3′ and 5′UTR *futsch* NcoI_AS: 5′-CTTCCATGGCACAGTTGGTGCAGTTGTAGCTGT-3′) or this couple of primers to delete the UG-rich sequence (5′UTR *futsch* ΔUG_S: 5′-GTATACCCTCTTTTTTTTCGAA-3′ and 5′UTR *futsch* ΔUG_AS:-GTTCTTGCCTTTGGTTTGGGT-3′).

All PCR amplicons were initially cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and fully sequenced.

4.2. Electrophoretic mobility shift assay (EMSA).

The wild type and ΔUG *futsch* 5′UTR RNAs were *in vitro* transcribed using a PCR product carrying the T7 promoter (5′-TAATACGACTCACTATAGGG-3′). The amplification was obtained using *futsch* -T7_2: 5′-TAATACGACTCACTATAGGGTAACAAGGGAACACCCAGAGTA-3′ and *futsch* 5′UTR +127: 5′-TGTTCTTGCCTTTGGTTTG-3′. The production of recombinant GST proteins and EMSA were performed as previously described (Ayala et al., 2005; Buratti and Baralle, 2001; D'Ambrogio et al., 2009).

4.3. RNA-GST pull down

In vitro transcribed wild type or ΔUG *futsch* 5′UTR RNA (0.1ng) was added to total W1118 fly RNA (1 μg) in 500 μl of HEGN buffer (20 mM Hepes pH 7.7, 150 mM NaCl, 0.5 mM EDTA, 10% Glycerol, 0.1% Triton X-100, 1 mM DTT). In a parallel experiment, 0.1 ng of (UG_9) *in vitro* transcribed RNA was used as positive control.

The RNA mix was incubated 1 h at 4 °C, and then 30 μl of Glutathione-Superflow resin (Clontech, Mountain View, CA). After 4 wash cycles (each 10 min at 4 °C in rotation) with 1 ml of wash buffer (HEGN buffer with 0.2% Deoxycholic acid, DOC, and 0.5 M urea), RNA was extracted with Trifast reagent (Euroclone, Milan, Italy), according to manufacturer's instruction. Reverse transcription was performed using M-MLV Reverse Transcriptase (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) according to manufacturer's protocol with an oligo specific for the *futsch* 5′UTR construct.

4.4. Cell cultures, transfections and luciferase assays

D. melanogaster S2 cells were maintained in Schneider's Drosophila Medium containing 10% FBS and 1x Antibiotic Antimycotic Solution (Sigma, St. Louis, MO, USA) at 25 °C. Transfections of 2×10^5 S2 cells in 24-well dishes were carried out with 1.1 μg of DNA (pGL4 vectors 1000 ng of Renilla vector and 100 ng of pRenilla) using Effectene reagent (Qiagen, Valencia, CA, USA). At about 24 h after the end of transfections, S2 cells were heat shocked for 20 min at 37 °C and then restored for 6 h at 25 °C, before undergoing luciferase assays performed with the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

Human Embryonic Kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium-Glutamax-I (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) containing 10% fetal bovine serum (Gibco-BRL, Life Technologies Inc., Frederick, MD, USA) and 1x Antibiotic Antimycotic Solution (Sigma, St. Louis, MO, USA). Co-transfections of 2×10^5 HEK-293 cells in 6-well dishes were carried out with 500 ng of pFLAG-TBPH (Ayala et al., 2005; Ayala et al., 2011; D'Ambrogio et al., 2009) or empty pFLAG constructs and pGL4-hsp70-futsch 5′UTR (300 ng)/pRenilla (20 ng) vectors using Effectene reagent. At about 24 h after the end of transfections, HEK-293 cells were heat shocked for 1 h at 42 °C and then restored for 6 h at 37 °C before undergoing luciferase assays, performed with the Dual-Luciferase Reporter Assay system.

4.5. RNA-protein co-immunoprecipitations

Cells transfected with the constructs pFLAG-TDP43 wt, pFLAG-TDPΔFL, pEGFP-N1 were harvested 48 h after the end of transfections, washed with PBS and resuspended in 500 μl of HEGN buffer (20 mM Hepes pH 7.7, 150 mM NaCl, 0,5 mM EDTA, 10% Glycerol, 0.1% Triton X-100, 1 mM DTT) containing 2x Cocktail Protein Inhibitors (Roche Diagnostic GmbH, Mannheim, Germany) and 5 μl of RNAse inhibitor (Ambion, Austin, TX, USA, 40 U/μl). After sonication, each sample was divided in aliquots and *in vitro* transcribed wild type or ΔUG 5′UTR futsch RNAs (0.1 ng) or 0.1 ng

of UG_9 RNA were added. Samples (150 μl) were incubated 2 h at 4 °C with 0.5 μg/ml of anti-FLAG M2 mouse monoclonal or monoclonal Anti-Green Fluorescent Protein (Sigma, St. Louis, MO, USA) antibody in PBS containing 0.1% Tween 20 and 3% Bovine Serum Albumin (Sigma, St. Louis, MO, USA). Then, 30 μl of A/G Plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to each co-immunoprecipitation sample (2 h at 4 °C in rotating wheel). After this incubation, five wash cycles (5 min/each at 4 °C in rotating wheel; centrifugation at 5000 × g for 10 min) were carried with HEGN buffer containing 0.2% DOC and 0.5 M urea. RNA extraction and reverse transcription were carried out as described in the RNA-GST pull down section.

4.6. Quantitative real-time PCR analysis

All quantitative PCRs were performed on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), with primers specific for *futsch* 5′UTR or UG_9 amplicons (Godena et al., 2011). All amplifications were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA). In order to calculate the enrichment fold, all data were initially normalized to the respective inputs. The signal was measured and represented according to fold increases compared to the control signal. The enrichment-fold is referred to pull down experiments with GST protein. The results derived from three independent immunoprecipitation experiments and error bars were used to calculate standard deviations on the normalized ratios.

4.7. Western blots

Cell pellets were resuspended in lysis buffer (10 mM Tris–HCl, pH 7,4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X100) containing 1x Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and were sonicated with Bioruptor Sonication System (Diagenode, Liege, Belgium). Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Clifton, NJ, USA), blocked overnight in 3% BSA, and probed with the following primary antibodies: mouse anti-FLAG (1:2000, SigmaAldrich, St. Louis, MO, USA) and mouse anti-Tubulin (1:10,000, Calbiochem, San Diego, CA, USA). Membranes were then incubated with HRP-labeled anti-mouse (Dako, Heverlee, Belgium, 1:2000) secondary antibody. Finally, protein detection was assessed with ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA). Protein expression was quantified with the NIH ImageJ software (Schneider et al., 2012) and normalized versus Tubulin. Histograms are representative of 3 independent experiments.

Contributors

Authors M. Romano and E. Buratti designed the study. Author M. Romano wrote the protocols and carried out the experiments. Authors M. Romano, E. Buratti and F. Feiguin performed the literature searches and analyzes. Authors M. Romano, E. Buratti and F. Feiguin wrote the manuscript. All authors contributed to and have approved the final manuscript.

Acknowledgments

This work was supported by Thierry Latran Fondation (REHNPALS), AriSLA (CHRONOS), and the EU Joint Programme-Neurodegenerative Diseases JPND (RiMod-FTD, Italy, Ministero della Sanità).

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