A post-transcriptional mechanism pacing expression of neural genes with precursor cell differentiation status

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Nervous system (NS) development relies on coherent upregulation of extensive sets of genes in a precise spatiotemporal manner. How such transcriptome-wide effects are orchestrated at the molecular level remains an open question. Here we show that 3’-untranslated regions (3’ UTRs) of multiple neural transcripts contain AU-rich cis-elements (AREs) recognized by tristetraprolin (TTP/Zfp36), an RNA-binding protein previously implicated in regulation of mRNA stability. We further demonstrate that the efficiency of ARE-dependent mRNA degradation declines in the neural lineage because of a decrease in the TTP protein expression mediated by the NS-enriched microRNA miR-9. Importantly, TTP downregulation in this context is essential for proper neuronal differentiation. On the other hand, inactivation of TTP in non-neuronal cells leads to dramatic upregulation of multiple NS-specific genes. We conclude that the newly identified miR-9/TTP circuitry limits unscheduled accumulation of neuronal mRNAs in non-neuronal cells and ensures coordinated upregulation of these transcripts in neurons.
ukaryotic gene expression is an intricate balancing act between transcription and post-transcriptional steps of RNA metabolism. Developmental readjustment of this balance allows large cohorts of genes to be expressed in cell- and tissue-specific manner. Among other regulators, RNA-binding proteins (RBPs) provide an important means for modulating RNA processing and turnover in the context of cellular differentiation. For example, downregulation of the ubiquitously expressed RBP Ptbp1/PTB/hnRNAP I in the developing brain stimulates neuron-specific alternative pre-mRNA splicing patterns and stabilizes a subset of neuronal transcripts.

We have previously shown that Ptbp1 levels are dampened in developing the nervous system (NS) by the microRNA miR-124, a non-coding molecule base pairing with partially complementary sites in the Ptbp1 mRNA. Notably, Ptbp1 knockdown is sufficient to induce morphological and functional neuron-like differentiation in non-neuronal cells. Several important targets have been described for another brain-enriched microRNA, miR-9 (refs 10,11). However, it is unknown whether the transcriptome-wide effects of miR-9 might be amplified through post-transcriptional mechanisms similar to those implemented in the miR-124/Ptbp1 circuitry.

In contrast to Ptbp1, many RBPs are enriched in the NS. Of these, the Hu/Elav-like (for example, HuB/Elavl2, HuC/Elavl3 and Hud/Elavl4) and Nova (for example, Novаль and Nova2) protein families are essential for proper brain development and function. NS-specific Hu/Elav proteins stabilize important neuronal mRNAs including that encoding the axonal Gap43 protein and additionally regulate several neuron-specific pre-mRNA-processing reactions. Besides their other functions, Nova proteins control a large fraction of neuron-specific alternative splicing events thus diversifying the proteome and modulating steady-state levels of a subset of mRNAs. Mechanisms ensuring elevated expression of these RBPs in the NS are poorly understood.

A considerable fraction of mammalian transcrisps contains 3′ untranslated region (3′ UTR)-localized AU-rich cis-elements (AREs). These sequences often diminish mRNA stability by recruiting corresponding trans-acting RBPs. Tristetraprolin (TTP/Zip36), a zinc-finger protein interacting with AUUUA motifs typically within a longer AU-rich context (for example, AUUUAU), provides an important example of this RBP motifs within a longer AU-rich context (for example, TTP/Zip36)-dependent ARE function. Interestingly, TTP has been implicated in the regulation of HuR/Elavl1, a ubiquitously expressed paralogue of HuB, HuC and HuD 28,29. A recent transcriptome-wide analysis revealed that ARE-containing transcripts might undergo coordinated upregulation during brain development.

Results

AU-enriched mRNAs tend to accumulate in the neural lineage. We examined previously published microarray data and detected a significant over-representation of A- an U-rich pentamers in predicted 3′ UTRs of genes upregulated during embryoid body/retinoic acid (EB/RA)-induced neural differentiation of mouse P19 cells (Supplementary Fig. 1a). Interestingly, the top hits included the three pentamers, AUUUU and AUUUA (Supplementary Fig. 1a and Supplementary Data 1), overlapping with the AUUUAU motif known to function as a tristetraprolin (TTP/Zip36)-dependent ARE. Moreover, mRNAs containing one or several ARE cores, AUUUA were more frequently upregulated in differentiated cultures compared with their AUUUA-less counterparts and this effect was especially pronounced for mRNAs with six or more AUUUA pentamers (Supplementary Fig. 1b,c).

Neural differentiation involves dramatic changes in mRNA 3′ UTR lengths triggered by globally altered patterns of pre-mRNA cleavage and polyadenylation. Since it is difficult to distinguish between APA isoforms using microarrays, we analysed transcriptomes of undifferentiated and EB/RA-differentiated P19 cells by 3′ READS procedure recently developed by our group. The newly acquired 3′ READS data showed a good correlation with the microarray results (Pearson’s correlation coefficient r = 0.72, P < 2 × 10−15; Supplementary Fig. 1d). Importantly, 3′ UTRs of 3′ READS-deduced transcripts up-regulated in differentiated P19 cells often contained one or several AUUUA motifs (Fig. 1a,b). Overall, these analyses suggested that ARE-containing transcripts might undergo coordinated upregulation during neural differentiation.

Many neural mRNAs are tristetraprolin targets. Important examples of mRNAs upregulated in differentiated P19 cells on the basis of the microarray are the 3′ READS data contained two or more AUUUAU motifs in their 3′ UTRs and encoded neuronal markers (for example, Tubb3, Eno2 and Pcdh19), brain-enriched RBPs (for example, HuB/Elavl2, HuC/Elavl3, Hud/Elavl4 and Novalsa) along with neurotrophic receptors and their ligands (for example, Ntrk2 and Bdnf; Fig. 1c and Supplementary Fig. 2). In most of these cases, AUUUAU motif occurred within a longer stretch of A/U nucleotides (Fig. 1c and Supplementary Fig. 2), a characteristic feature of bona fide AREs. Our reverse transcription–quantitative PCR (RT–qPCR) analyses with open reading frame-specific primers confirmed that these mRNAs were indeed dramatically upregulated in EB/RA-differentiated P19 cells (Fig. 1c and Supplementary Fig. 2; Fu/Ru and F/R pairs, respectively).

To account for differentiation-induced changes in APA patterns, we re-analysed the above P19 samples using RT–qPCR with primer pairs designed towards downstream 3′ UTR sequences (Fig. 1c; Fd/Rd pairs). For genes with most AUUUAU sequences preceding a single constitutive cleavage/polyadenylation site (pA; Tubb3) or several alternative pAs (Eno2 and HuB), upregulation effects detected using this assay were largely similar to the above Fu/Ru RT–qPCR data (Fig. 1c). However, when AUUUAU repeats followed proximal
alternative pA’s, Fd/Rd targets were upregulated to a significantly larger extent than Fu/Ru ones (HuR/Elavl1, Nova1 and Ntrk2; Fig. 1c). Of note, the UAUUUAU motifs occurring in the 3′-terminal extension of the long, brain-enriched APA isoform of the HuR mRNA have been previously implicated in TTP-dependent destabilization.28,29,37

Notably, when we analysed a subset of the above mRNAs (Tubb3, HuB, HuC, HuD and Nova1) expressed in undifferentiated P19 cells using ultraviolet crosslinking/immunoprecipitation (CLIP) with TTP-specific antibodies, TTP–mRNA interaction was detected for Tubb3, HuB, HuC and Nova1 (Fig. 2a,b). We could not obtain conclusive HuD-specific data likely because of the extremely low expression of this mRNA in undifferentiated P19 cells. As expected24, the anti-TTP antibody generated a robust CLIP signal for the HuR mRNA control (Fig. 2b). On the other hand, no significant TTP binding was detected for the Alas1 mRNA lacking UAUUUAU sequences (Fig. 2b).

Overall, these data indicated that at least a fraction of ARE-containing and TTP-interacting mRNAs was upregulated during neuronal differentiation. Paradoxically, long alternatively cleaved and polyadenylated isoforms containing a larger number

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**Figure 1** ARE-containing transcripts are frequently upregulated during neural differentiation. (a) Empirical cumulative distribution function (ECDF) plots for 3′READS-deduced expression changes in P19 cells undergoing neural differentiation. Individual curves correspond to groups of transcripts with specified numbers of AUUUA motifs within the 3′ UTR. (b) Comparison of the transcript groups in a using two-sided KS test suggests that mRNAs containing one or several AUUUA motifs are more frequently upregulated than their AUUUA-less counterparts. (c) Changes in the expression levels of ARE-containing mRNAs in P19 cells after 3.5 days of EB/RA-induced neural differentiation. Top, 3′ UTR diagrams showing positions of pA sites, AREs and primers used for RT-qPCR analyses. Long black ticks, canonical UAUUAA and UAUUAAA pA hexamers occurring within 10–30 nt upstream of the 3′ end in at least one cDNA or EST clone (UCSC Genome Browser) or one-nucleotide modifications of these hexamers used as pA sites in at least five cDNA/EST clones. Short black ticks, AAUAAA and AUUAAA pA hexamers not associated with available cDNA clones. Red ovals, UAUUUAU motifs occurring as a part of at least one cDNA or EST clone (UCSC Genome Browser) or one-nucleotide modifications of these hexamers used as pA sites in at least five cDNA/EST clones. Red ticks, canonical AAUAAA and AUUAAA pA hexamers occurring within 10–30 nt upstream of the 3′ UTR. (c) 0–2006

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of these motifs appeared to be upregulated to a greater extent than their shorter variants with fewer UAUUUAUs.

The TTP pathway is inactivated during neural differentiation. A parsimonious explanation for the above results would involve a decrease in the efficiency of TTP/ARE-dependent RNA degradation in differentiated P19 cells. To explore this possibility, we generated two dTomato cassettes containing HuR 3′ UTRs with mutated pA2, a major pA utilized in proliferating cells28 (Fig. 2c). One of these constructs (dTom-3′HuR-pA2mut) contained the WT ARE repeat previously shown to be targeted by TTP 28, whereas the other one (dTom-3′HuR-pA2mut/ΔARE) had this element deleted (Fig. 2c). Both plasmid-encoded transcripts were expected to terminate predominantly at the downstream pA4-pA7 sites, thus generating long mRNA products with (dTom-3′HuR-pA2mut) or without AREs (dTom-3′HuR-pA2mut/ΔARE)28.

The two constructs were then used to generate corresponding single-copy transgenic P19 cells using a previously described procedure29. As expected, when we analysed dTomato expression in these cells using flow cytometry, undifferentiated dTom-3′HuR-pA2mut/ΔARE samples showed noticeably higher dTomato levels than undifferentiated dTom-3′HuR-pA2mut cells (Fig. 2d; P = 4.7 × 10−203, Wilcoxon rank-sum test). However, dTomato expression levels in the two transgenic populations became virtually indistinguishable following EB/RA differentiation (Fig. 2d; P = 0.060, Wilcoxon rank-sum test). We also failed to detect any difference in dTomato expression between neuron-like fractions of the two differentiated cultures positive for neuronal tubulin βIII (Tubb3) marker, a product of the Tubb3 gene mentioned above (Supplementary Fig. 3; P = 0.24, Wilcoxon rank-sum test).

Since the above results suggested that the efficiency of ARE-mediated RNA destabilization could be reduced during neuronal differentiation, we introduced the above dTom-3′HuR-pA2mut and dTom-3′HuR-pA2mut/ΔARE transgenes into a primary postnatal cortical neurons from E15.5 mouse embryos using a transient magnetofection protocol (Supplementary Fig. 4; see Methods for details). Subsequent flow cytometry analyses showed that both neuronal populations expressed dTomato protein at statistically indistinguishable levels (P = 0.76, Wilcoxon rank-sum test). On the other hand, magnetically transfected primary cortical neurons expressed significantly larger amounts of dTomato from dTom-3′HuR-pA2mut/ΔARE than from dTom-3′HuR-pA2mut (Supplementary Fig. 4; P = 1.8 × 10−180, Wilcoxon rank-sum test). We concluded that the UAUUUAU-dependent branch of the RNA decay pathway was largely inactive in neurons.

TTP levels decrease as a result of miR-9 upregulation. To test whether reduced efficiency of ARE-dependent mRNA decay in developing neurons could be because of corresponding changes in the TTP expression, we analysed P19 cells undergoing EB/RA differentiation by immunoblotting with a TTP-specific antibody (Fig. 3a). TTP protein levels progressively diminished as a function of differentiation time (Fig. 3a). Immunoblot analysis of mouse embryonic stem cells, neural stem cells and primary cortical neurons further suggested that that protein levels of both TTP and its closely related paralogue BRF1/Zfp36L1 were also dramatically reduced during neurogenesis in vivo (Supplementary Fig. 5). Interestingly, TTP appeared to be downregulated at an earlier developmental point than BRF1 (Supplementary Fig. 5). We noticed that the 3′ UTR of TTP–mRNA contained an evolutionarily conserved sequence complimentary to the seed region of microRNA miR-9 (Fig. 3b) known to be expressed in neuronal progenitors and neurons11. As expected39, mature miR-9 levels increased markedly in differentiating P19 cultures (Fig. 3c), thus suggesting a possible mechanism for TTP downregulation.
Figure 3 | MicroRNA miR-9 reduces TTP expression in neural cells. (a) Immunoblot analysis demonstrating a decrease in TTP expression in P19 cells undergoing neural differentiation. Antibody against β-tubulin (Tubβ) is used to control lane loading. (b) Top, interaction between miR-9 and the cognate target sequence in the mouse TTP 3’ UTR predicted using RNAhybrid62. Middle, interspecies alignment of the target sequences with invariant nucleotides shown in upper case Nucleotides mutagenized to inactivate miR-9-binding are marked by asterisks. Bottom, PhastCons score reflecting probability of sequence conservation across vertebrates63. (c) Northern blot showing that mature miR-9 levels dramatically increase in P19 cells following EB/RA-induced neural differentiation. U6 RNA is used as a loading control. (d) RLuc-3’UTR-wt and RLuc-3’UTR-miR9TSmut Renilla luciferase reporter constructs used in this study. (e) HEK293T cells were co-transfected with RLuc-3’UTR-wt or RLuc-3’UTR-miR9TSmut and miR-9 expression plasmid or the corresponding empty vector. Firefly luciferase plasmid pEM231 (ref. 7) was included as a normalization control. Luciferase expression was assayed 24 h post transfection using the Dual-Glo kit (Promega) and the data were processed as recommended. Note that miR-9 dramatically inhibits the RLuc-3’UTR-wt expression while having a scientifically lesser effect on the RLuc-3’UTR-miR9TSmut construct lacking the conserved miR-9 target site. Expression levels of the corresponding miR-vector samples were set to 1. Data are averaged from three experiments ± s.d. and compared by t-test. (f) Following the EB/RA steps, differentiating P19 cells were plated for 12 h and then transfected with a 2’OMe-RNA antisense oligonucleotide against miR-9. Samples were collected 48 h post transfection and the effects of the antisense treatment on the levels of mature miR-9 were analysed with northern blot analysis using U6 RNA as a loading control. (g) Immunoblot analysis showing that knockdown of miR-9 carried out as described in f leads to noticeable upregulation of the TTP protein.

To assess whether miR-9 could directly target the predicted site, we fused a Renilla luciferase (RLuc) reporter gene with the TTP 3’ UTR and co-transfected HEK293T cells with this construct (RLuc-3’UTR-wt) and either a miR-9 expression plasmid or the corresponding empty vector (Fig. 3d,e). Satisfyingly, miR-9 reduced RLuc expression ~2.9-fold \((P = 4.7 \times 10^{-5}; \ t\text{-test})\) and mutation of the putative miR-9 target site (RLuc-3’UTR-miR9TSmut) rescued RLuc expression \((P = 5.5 \times 10^{-5}; \ t\text{-test}; \text{Fig. 3e})\). A similar rescue effect was observed when we treated HEK293T cells co-transfected with RLuc-3’UTR-wt and miR-9 expression plasmid with a target protector oligonucleotide shielding the miR-9 target site in the TTP 3’ UTR (Supplementary Fig. 6a).

To ensure that naturally occurring miR-9 levels were sufficient for the regulation, we transfected differentiated P19 cells with an miR-9-specific antisense 2’-O-meRNA oligonucleotide (Fig. 3f). As expected40, this reduced mature miR-9 levels as compared with control-treated cells (Fig. 3f). Notably, miR-9 downregulation resulted in increased TTP protein levels (Fig. 3g). TTP protein levels also noticeably increased in EB/RA-treated P19 cells in response to TTP-specific miR-9 target site protector (Supplementary Fig. 6b). We concluded that TTP expression is reduced during neuronal differentiation and that this effect is at least in part mediated by miR-9.

TTP downregulation is required for neuronal differentiation. To determine whether TTP downregulation was required for neuronal differentiation, we prepared P19 cells containing a single-copy HA-tagged TTP transgene driven by a doxycycline (Dox)-inducible promoter and lacking its natural 3’ UTR (TRE-TTP; Fig. 4a). Following the EB/RA induction, TTP expression was induced by Dox and the cells were analysed by RT–qPCR, immunoblotting and immunofluorescence. The RT–qPCR assay suggested that expression of the miR-9-resistant TRE-TTP transgene in differentiated P19 cells led to a significant
downregulation of neuronal genes both containing and lacking UAUUUAU repeats in their 3' UTRs (Fig. 4b and Supplementary Fig. 7a). The immunoblot analysis confirmed Dox-inducible expression of transgenic TTP and showed that this lowered protein levels of two TTP targets, HuB and TubβIII (Fig. 4c), as well as neuronal marker Map2 not predicted to be targeted by TTP (Supplementary Fig. 7b). We also detected a significant decrease in the number of TubβIII-positive cells in EB/RA-differentiated P19 cultures expressing transgenic TTP (Fig. 4d,e).

To address functional significance of reduced TTP expression during neuronal development in vivo, we transiently magnetically transfected primary cortical neurons from E15.5 embryos with a plasmid expressing recombinant TTP from a constitutive promoter (pBS-CMV-TTP; Supplementary Fig. 8a,b). Subsequent RT-qPCR analyses showed that this treatment significantly reduced steady-state levels of UAUUUAU-containing neuronal mRNAs (Tubb3 and HuB) while having no detectable effect on neuronal mRNA lacking UAUUUAU motifs (Map2 and L1cam; S8c-d). Taken together, these results suggested that TTP downregulation in the context of neurogenesis was required for establishing a proper neuronal gene expression programme.

**TTP dampens neuronal mRNAs in neuroblastoma cells.** To test whether reduced TTP expression was sufficient for upregulation of ARE-containing neuronal mRNAs, we knocked down TTP expression in an easy-to-transfect mouse neuroblastoma cell line, Neuro2a, with a corresponding small interfering (si) RNA (siTTP; Fig. 5a). Compared with cells treated with siControl, treatment with siTTP led to significant upregulation of predicted TTP targets, Tubb3, HuB, HuC, HuD and Nova1, at the mRNA level (Fig. 5a), as well as noticeable accumulation of the HuB and TubβIII proteins (Fig. 5b). Consistent with these data, our IF staining showed that cultures treated with siTTP contained a significantly larger fraction of TubβIII-positive cells than the control-treated ones (Fig. 5c,d). Conversely, overexpression of TTP-TRE in Dox-treated single-copy transgenic Neuro2a cells resulted in a modest but significant decrease in the basal expression levels of Tubb3, HuB, HuC, HuD and Nova1 mRNAs (Supplementary Fig. 9). Thus, TTP limits expression levels of ARE-containing neuronal mRNAs in a transformed non-neuronal cell line.

**TTP KO reprogrammes embryonic fibroblast transcriptome.** To find out whether TTP could repress neural genes in primary
non-neural cells, we turned to the TTP/Zfp36 KO mouse model\(^26\). Gene expression patterns of MEFs from the KO (TTP \(-/-\)) animals and their WT (TTP \(+/-\)) littermates have been previously compared using a microarray approach\(^41\). The authors treated first-starved-then-serum-stimulated cells expressing TTP at an elevated level with RNA polymerase inhibitor actinomycin D and focused on a subset of transcripts with increased half-lives in the KO cells as compared with the WT. This resulted in identification of 33 TTP targets representing a range of functional categories\(^41\).

Since the bioinformatics pipeline used in this study could not detect transcripts destabilized by TTP to undetectably low steady-state levels in the WT background but rescued in the KO, we reanalysed the data focusing on genes consistently showing significant expression differences between corresponding KO and WT samples. This yielded 80 genes represented by 100 distinct probes sets that were significantly upregulated and 95 genes represented by 131 probes set that were downregulated across all KO samples (\(>10\)-fold effects; Benjamini–Hochberg-corrected \(P<0.05\)). Strikingly, the upregulated set was significantly enriched for neuron-specific Gene Ontology processes (Supplementary Data 2) and contained Tubb3 and HuB along with several other NS-enriched transcripts (Supplementary Data 3). The upregulated genes tended to be expressed at a level reaching the median in the KO cells (Supplementary Fig. 10a). Notably, cerebral cortex rank distribution plotted for upregulated genes as compared with the whole array (Fig. 6a and Supplementary Fig. 10b; \(P = 4.23 \times 10^{-9}\); one-sided Kolmogorov–Smirnov (KS) test). On the other hand, downregulated genes were statistically indistinguishable from the whole array (Fig. 6a and Supplementary Fig. 10b; \(P = 0.976\); one-sided KS test). When we repeated this procedure for the rest of the tissues/cell types represented in the atlas, all NS-specific distributions showed significant right shifts for genes upregulated in KO MEFs (Fig. 6b and Supplementary Fig. 10c). NS-specific \(P\) values were significantly lower than their non-NS counterparts for upregulated (inset in Fig. 6b) but not downregulated genes (Supplementary Fig. 10d). A similar trend was apparent when we analysed changes in the NS and non-NS rank medians (Supplementary Fig. 10e). Taken together, these analyses suggested that TTP repressed a large cohort of neural transcripts in stimulated MEFs.

To examine whether basal TTP expression levels could dampen expression of neural transcripts in non-stimulated fibroblasts, we propagated the WT and KO MEFs kindly provided by the Blackshear laboratory in the presence of 10% of fetal bovine serum (FBS) for several days and analysed the microarray hits using RT–qPCR and immunoblotting. Both Tubb3 and HuB mRNAs were dramatically upregulated in the KO fibroblasts (Supplementary Fig. 10f).
tissues/cells. (Fig. 5a) RT–qPCR analysis showing significantly elevated relative expression of TTP targets in untreated TTP KO MEFs compared with similarly prepared WT control. (d) RT–qPCR suggesting that the loss of TTP in the KO MEFs leads to accumulation of the long, ARE-containing form of the HuR mRNA. Data in c,d are averaged from three experiments ± s.d. and compared by t-test. WT expression levels are set to 1 (see Fig. 1c for further details). (e) Immunoblot analysis showing drastic upregulation of TubβIII and HuB proteins in TTP KO MEFs.

Although HuC, HuD and Nova1 were not shortlisted with our microarray analysis algorithm, RT–qPCR quantitation showed that these UAUAUAU-containing genes were also significantly upregulated in non-stimulated KO MEFs (Fig. 6c). TTP KO MEFs also expressed increased amounts of the long alternatively cleaved/polyadenylated isoform of the HuR mRNA (Fig. 6d). On the other hand, TTP KO had no effect on the expression levels on the Alas1 ‘housekeeping’ mRNA lacking UAUAUAU motifs (Supplementary Fig. 11c). We concluded that one of the TTP functions in non-neural cells in vivo could be dampening steady-state expression of multiple neural transcripts.

**Discussion**

Our study suggests that, in addition to its well-documented role in destabilizing mRNAs encoding cytokines, growth factors and proto-oncogenes, TTP limits steady-state abundance of a
A considerable number of ARE-containing neuronal mRNAs in non-neuronal cells (Fig. 7). These transcripts are upregulated in cells undergoing neural differentiation since TTP protein expression is diminished in this context by the brain-enriched microRNA miR-9 (Fig. 7). The experiments with transgenic P19 cells and primary neurons suggest that the newly identified post-transcriptional circuitry is essential for proper neuronal differentiation (Fig. 4 and Supplementary Fig. 8). Further underscoring importance of this mechanism, the newly identified TTP targets include mRNAs encoding RBPs from the Hu/Elavl and Nova families known for their critical contributions to NS development and function as well as mRNAs of essential neuronal markers (for example, TubIII)13-15,45.

Data presented in this study (for example, Figs 1c and 6d) further indicate that reduced expression of TTP during neural differentiation may enable expression of 3'-elongated mRNA APA isoforms appearing as a result of frequent skipping of open reading frame-proximal alternative pA sites in the NS33-35. Given the major role of the 3' UTR in defining mRNA translational efficiency, stability and intracellular localization, the impact of the TTP dynamics on the cellular repertoire of APA isoforms warrants further systematic investigation.

The newly identified regulation circuitry is evocative of the previously described miR-124/Pbp1 switch regulating the choice between non-neuronal and neuronal splicing and mRNA stability patterns7. In both cases, a post-transcriptional repressor of neuronal genes is placed under a negative control of a NS-enriched microRNA. This underscores the role of post-transcriptional regulation in differentiating cells and suggests that gene expression changes in this context rely on an extensive crosstalk between microRNA- and RBP-mediated pathways. Interestingly, another well-described example of such double negative regulation logic is provided by the REST/NRSF complex,34 which essentially depends on the newly identified mechanisms including the microRNA pathways10,46,47.

Earlier studies have demonstrated that miR-9 and miR-124 might stimulate neurogenesis in a synergistic manner. Indeed, combined expression of these two microRNAs promotes neuronal fate in differentiating mouse ES cell cultures31,34 and triggered detectable trans-differentiation of embryonic fibroblasts into neurons49. By identifying a global repressor of neuronal mRNA stability as one of the miR-9 targets, our work sheds new light on molecular mechanisms underlying pro-neural activity of this microRNA in mammals.

Although TTP is a critical component of the ARE-dependent mRNA-destabilization machinery, several other RBPs controlling mRNA stability and translation also interact with AREs51. Some of these proteins, including TTP paralogues BRF1/Zip36l1 and BRF2/Zfp36l2, are thought to interact with TTP-specific AREs52. Although BRF2 protein was undetectable in cells undergoing neurogenesis, BRF1 was expressed at readily detectable levels early in neurogenesis and dramatically downregulated in neurons (Supplementary Fig. 5). Therefore, it might be interesting—as one of the future directions—to examine possible contribution of BRF1 to post-transcriptional regulation of neuronal genes.

Yet another line of further studies should focus on the Hu/Elavl protein functions. All four genes encoding mammalian Hu/Elavl paralogues contain 3'-terminal AREs (Fig. 1c and Supplementary Fig. 1) and the steady-state levels of HuB, HuC and HuC, increase markedly on TTP downregulation (Figs 5 and 6). Importantly, Hu proteins are known to interact with U-rich sequences that include but are not limited to AREs recognized by TTP and its paralogues13,30,50. This interaction often stabilizes mRNA targets possibly by minimizing their interaction with repressive RBPs31,52. Therefore, even partial stabilization of the HuB, HuC and HuD mRNAs triggered by reduced TTP expression may initiate a positive reinforcement mechanism further increasing stability of ARE-containing mRNAs in a Hu/Elavl-dependent manner.

Hu/Elavl protein accumulation would potentially explain the robust upregulation of the Gap43 mRNA in the TTP KO MEFs (Supplementary Fig. 1b). Indeed, this important mRNA is known to be stabilized in neurons through a 3' UTR U-rich cis-element distinct from a TTP-specific ARE but capable of recruiting HuR43,44. Similarly, initial upregulation of Noval protein might account19 for the increase in the steady-state level of the Gria3 mRNA lacking discernible AREs (Supplementary Fig. 1b and Supplementary Data 3).

Intriguingly, human Hu/Elavl and Nova have been originally identified as auto-antigens associated with paraneoplastic neurological disorders and members of these protein families are consistently overexpressed in several types of cancers53. Moreover, elevated expression of the neural enolase subunit encoded by the Eno2 gene (Fig. 1c) is commonly used as a neuroendocrine tumour marker54. Since human orthologues of mouse HuB, HuC, HuD, Noval and Eno2 genes contain readily discernable AREs and TTP is often downregulated in tumours27,55,56, it will be interesting to examine whether ectopic expression of HuB/C/D, Noval and other NS-specific antigens in cancer cells might be triggered by aberrantly low TTP levels. Notably, TTP knockdown was sufficient for HuB/C/D and Noval upregulation in Neuro2a neuroblastoma cells (Fig. 5).

In conclusion, our work uncovers a post-transcriptional circuitry dampening expression of multiple neuronal genes in non-neuronal cells and allowing their coordinated upregulation in neurons. This finding may open up new possibilities for improved conversion of non-neuronal cells into neurons for research and therapeutic applications and inform further studies of mechanisms driving overexpression of onconeural antigens by tumour cells.

**Methods**

**Plasmids.** Mouse TTP-HA expression plasmid (pBS-CMV-TTP) was a gift from Perry Blackshear and pBS-vector control (pBluscriptR) was from RIKEN BioResource Center. New constructs were generated using standard molecular cloning techniques57 as outlined in Supplementary Data 4. Site-specific mutations were introduced using modified QuickChange site-directed mutagenesis protocol (Stratagene) using corresponding mutagenic primers (Supplementary Data 5) and KAPA HiFi DNA polymerase (KAPA Biosystems). All primers used in this study are listed in Supplementary Data 5 and plasmid maps and sequences are available on request.

**Cell lines.** P19 cells (ATCC) were routinely propagated in P19 growth medium (P19GM) containing z-MEM (Hyclone), 10% FBS (Hyclone, characterized grade), 100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Life Technologies). HEK293T and Neuro2a cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) containing 10% FBS, 100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Life Technologies). The cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Mouse TTP was knocked down in cell lines using corresponding ON-TARGETplus mixture containing four proprietary siRNAs designed by Dharmacon/Thermo Scientific. Non-targeting control ON-TARGETplus siRNA was also from Dharmacon/Thermo Scientific. miR-9 was inactivated using either an anti-miR-9 antisense 2O-Me-RNA oligonucleotide (5'-UCAAUCAGCGAACAUUAAACAAAG-3'; Dharmacon/Thermo Scientific) or a target protector (Qiagen) against the predicted miR-9-binding sequence within mouse TTP 3' UTR (5'-CCUCUCAAGGCAUUAGCGCAUG-3'). Transfections were carried out using Lipofectamine 2,000 (Life Technologies) as recommended. To transfet cells cultured in a 60-mm dish (4 ml medium), we typically combined 200-500 pmol of an appropriate siRNA or an oligonucleotide with 10 μl of Lipofectamine 2000 pre-diluted with 250 μl of Opti-MEM 1 reduced serum medium (Life Technologies).

**EB/RA differentiation of P19 cells.** To initiate neural differentiation7, P19 cells were plated at 1 × 10⁶ cell ml⁻¹ into bacterial-grade dishes in P19 induction medium (P19IM) containing 2'Z-MEM (Hyclone), 5% FBS (Hyclone, characterized grade)
and 1 μM of all-trans-RA (Sigma). Two days post plating, P19IM was replaced and the EBs were cultured for another 2 days. The EBs harvested from a single 10 cm dish were washed with 1 × PBS and dissociated in 2 ml of 0.25% trypsin-EDTA (Life Technologies) supplemented with 100 μg/ml RNase I (Roche) for 10 min at 37 °C. The cell suspensions were passed through 100-μm strainers (BD Biosciences) and plated into poly-D-lysine (Sigma)-treated dishes in P19IM lacking RA. Twelve hours after plating, the medium was changed to NeuroBasal (Life Technologies) supplemented with 2 mM Glutamax (Life Technologies) and the cells were allowed to undergo neural differentiation for up to six more days.

Primary cells. Mouse embryonic neural stem cells were isolated from E14 cortices and cultured essentially as in ref. 58, except no astroglial feeders were used for cultures maintained ≤5 days in vitro (DIV). Neurons were transfected using NeuroMag reagent (Ox Biosciences) as recommended. Briefly, 2.4 × 10⁶ cortical neurons were plated per 60-mm dish (Corning) pretreated with poly-D-lysine (Sigma). At DIV2-DIV5, 0.4 μl of TTP expression plasmid (pc85-PVMT-TTP) or corresponding vector control or 4 μg of a dTomato reporter plasmid (dTom-t3His-pA2mut or dTom-t3His-pA2mut/ΔARE) was transfected with 6 μl NeuroMag beads in 150 μl Opti-MEM I for 20 min and the mixture was added dropwise to the dish. The dishes were then incubated on top of a magnetic plate (Ox Biosciences) for 40 min and incubated for another 40–48 h at 37 °C and 5% CO₂ before subsequent analyses.

Flow cytometry. We used natural dTomato fluorescence to sort transgenic P19 cultures and transfected neurons and MEFs. For intracellular staining, cells were trypsinized, washed and fixed at 2 × 10⁶ cells/ml in 1 × PBS containing 2% paraformaldehyde for 15 min at room temperature. Fixed cells were washed twice with 1 × PBS additionally containing 1% BSA. This was followed by incubation with blocking/permeabilization buffer (1 × PBS, 10% horse serum, 1% BSA and 0.15% saponin) for 1 h at room temperature. The cells were then incubated with a rabbit anti-Tubb3 (Tuj1) primary antibody diluted in blocking/permeabilization buffer at 4 °C for 1 h. Cells were washed twice with 1 × PBS additionally containing 1% BSA and 0.15% saponin and incubated with a corresponding Alexa-488-conjugated secondary antibody (Life Technologies) diluted 1:10,000 in blocking/permeabilization buffer at 37 °C for 45 min at 4 °C. The cells were finally washed twice with 1 × PBS additionally containing 1% BSA and 0.15% saponin and analysed using a FACS Calibur flow cytometer (BD).

Routine molecular biology procedures. Isolation of total RNA, mRNA northern blot analysis, RT-qPCR, immunoblotting, immunofluorescence and luciferase assays were carried out using standard protocols28. MicroRNA northern blot analysis59 was carried out using an appropriate 5'-[32P]-labelled antisense DNA oligonucleotide probes (Supplementary Data 5). RT-qPCR signals obtained using gene-specific primers were normalized to either glyceraldehyde-3-phosphate dehydrogenase or 18S ribosomal RNA levels represented as reads per million of total PASS reads. The log2-based reads per million ratios between 3.5-day differentiated and undifferentiated samples were used to assess neural differentiation-induced changes in gene expression levels.

Data analyses. Statistical analyses were performed using Excel and R (http://www.R-project.org/). Images were quantified in ImageJ (http://imagej.nih.gov/ij/). Unless indicated otherwise, data sets were compared using two-tailed t-test assuming unequal variance. Published microarray comparison of gene expression in undifferentiated and EA/R155-differentiated neuron-like P19 cells (7 days post EB/RA induction) was downloaded from GEO (accession number GSE23710; ref. 31). To analyse motif enrichment in this data set, we used 3′-extended RefSeq gene models assembled as previously described32. Enriched motifs were identified using our previously published computer programme PROBE.32. Differentiation-induced expression changes in gene populations containing specified numbers of AUAA motifs were compared using two-sided K-S test. Flow cytometry data were analysed using the flowCore Bioconductor package (http://www.bioconductor.org/). MicroRNA target sites were predicted using Microcosm Targets resource (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets_v5/search.pl). Genes showing consistent expression changes in TTP KO MEFs compared with the corresponding WT littermate controls were identified by re-analysing previously published Affymetrix microarray results (ref. 41; GEO accession number GSE53324) using gc.rma, genefilter and limma Bioconductor packages. Tissue and cell type-specific gene ranks33 were computed using the 81 experimentally naive samples from a published gc.rma-normalized Affymetrix gene expression atlas (ref. 61; GEO accession number GSE10246).

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