p38 Mitogen-activated protein kinase stabilizes SMN mRNA through RNA binding protein HuR

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Spinal muscle atrophy (SMA) is an autosomal recessive neurodegenerative disease which is characterized by the loss of α motor neurons resulting in progressive muscle atrophy. Reduced amount of functional survival motor neuron (SMN) protein due to mutations or deletion in the SMN1 gene is the cause of SMA. A potential treatment strategy for SMA is to upregulate levels of SMN protein originating from the SMN2 gene compensating in part for the absence of functional SMN1 gene. Although there exists a sizeable literature on SMN2 inducing compounds, there is comparatively less known about the signaling pathways which modulate SMN levels. Here, we report a significant induction in SMN mRNA and protein following p38 activation by Anisomycin. We demonstrate that Anisomycin activation of p38 causes a rapid cytoplasmic accumulation of HuR, a RNA binding protein which binds to and stabilizes the AU-rich element within the SMN transcript. The stabilization of SMN mRNA, rather than transcriptional induction results in an increase in SMN protein. Our demonstration of SMN protein regulation through the p38 pathway and the role of HuR in this modulation may help in the identification and characterization of p38 pathway activators as potential therapeutic compounds for the treatment of SMA.

INTRODUCTION

Spinal muscle atrophy (SMA) is a comparatively common autosomal recessive neurodegenerative disease, characterized by the loss of α motor neurons from the anterior horn of spinal cord resulting in progressive muscle atrophy (1). It has a prevalence of one in 10 000 live births and a carrier frequency of one in 50 (2). Reduced amount of functional survival motor neuron (SMN) protein due to mutations or, most commonly, deletion of the SMN1 gene is the cause of SMA (3).

SMN, a 294 amino acid ubiquitously expressed protein is a key component of the SMN complex central to the biogenesis of spliceosomal small nuclear ribonucleoproteins including the removal of introns during pre-mRNA splicing (4). SMN also has diverse functions in the assembly, metabolism and transport of other ribonucleoproteins (5–14). Humans have two nearly identical SMN genes, SMN1 and SMN2 due to an evolutionary recent duplication event on chromosome 5 (15,16). SMN1 gene produces full-length functional SMN protein, whereas SMN2 due to a C to T transition at position 6 of exon 7 (one of five non-SMN1–SMN2 nucleotide differences) produces mostly aberrantly spliced mRNA and produces only 10% of the full-length functional SMN protein (3,17,18).

A significant majority of SMA patients lack the functional SMN1 gene due to homozygous deletion (3). Although the lack of functional full-length SMN protein ultimately leads to an apoptotic death to a subset of motor neurons (19), the precise mechanism of neuronal dysfunction remains unclear. One model suggests a reduced pre-synaptic transcriptome leading to anomalous neuromuscular junction (NMJ) architecture and functionality (19,20). All SMA patients have at least one copy of the SMN2 gene which produces low levels of functional SMN protein. An inverse correlation is seen between the SMN2 gene copy number and SMA severity as higher SMN2 copy numbers produce more functional SMN protein attenuating disease severity (21,22). Presently, there is neither cure nor a particularly effective therapy for SMA. Potential treatment strategies for SMA include the induction of SMN2 gene, the modulation of splicing of SMN2 transcripts and the stabilization of either SMN mRNA and/or protein all

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of which might compensate in part for the absence of functional SMN gene.

The p38 pathway comprises mitogen-activated protein kinases (MAPK) which are activated by a number of external stimuli such as UV light, osmotic shock, heat, growth factors and inflammatory cytokines (23–32). These stimuli activate two main upstream kinases, the MAPK kinases MKK3 and MKK6 which in turn phosphorylate and activate p38. The p38 pathway modulates a number of cellular responses including cell differentiation and apoptosis. One mechanism by which p38 works is the modulation of stability of various AU-rich element (ARE) containing mRNAs which comprise of as many as 4000 genes. The pentameric AUUUUA instability motif in the 3′-UTR region of these transcripts marks them through specific ARE-binding proteins (ARE-BPs) (33–37) for a rapid degradation which, at least in part, is modulated by p38. Conversely, post-transcriptional stabilization of various ARE-rich transcripts through ARE-BPs is an important step in modulating the protein levels. For example, HuR [a member of embryonic lethal abnormal vision/Hu family of proteins] is a ubiquitously expressed ARE-BP that has a constitutive nuclear localization (38). Upon stimulation, HuR accumulates in the cytoplasm where it binds to the 3′-UTR of a variety of ARE containing cellular transcripts contributing to their stabilization (39). It has been shown that Anisomycin, which is a known MAPK (p38, c-JNK) activator, can cause rapid nuclear to cytoplasmic shuttling of HuR protein (40). Interestingly, SMN mRNA is classified as ARE containing mRNA (37) and contains a putative HuR binding motif (39).

Following preliminary data indicating p38 in SMN modulation in both cellular and in vivo models, we hypothesized that the activation of p38 pathway might stabilize SMN mRNA thus increasing functional SMN protein as a result of complex formation between 3′-UTR of SMN mRNA and HuR protein. P38 and HuR mediated regulation of SMN mRNA and protein level is demonstrated in this study representing novel avenues both for SMN regulation as well as SMA therapeutics.

RESULTS

We have mined the Johnson & Johnson Pharmaceutical Research and Development archived gene expression profiles for SMN levels under different treatment conditions and with different compounds. SMN was found to be up-regulated in both cell lines and human white blood cells after systemic administration of lipopolysaccharide (LPS). The up-regulation of peripheral leukocyte SMN in response to LPS in human volunteers could be completely inhibited by a compound that inhibits p38 (unpublished data), indicating that the induction of SMN is downstream to the p3′ MAPK pathway. Consonant with this interpretation, analysis of Connectivity Map data (41), revealed that of 1309 small molecules assayed, the p38 inducing antibiotic Anisomycin was the most effective at upregulating SMN mRNA in three independent cell lines (Justin Lamb, pers commun).

Anisomycin treatment induces SMN gene expression

To investigate a potential p38 role in SMN gene regulation, Human neuron-committed teratocarcinoma (NT2) cells were treated with Anisomycin. SMN transcript levels were found to be increased significantly (3–4-fold) in NT2 cells upon treatment with Anisomycin (Fig. 1A). NT2 or motor neuron derived (MN-1) cells were then treated with Anisomycin and then harvested for western blot analysis at 1 h intervals revealing a time-dependent increase of SMN protein in both NT2 and MN-1 cells (Fig. 1B and C). Taken together these results demonstrate that Anisomycin treatment causes an increase in SMN steady-state mRNA and protein levels in neuronal-cell lines.

Activation of p38 pathway causes upregulation of SMN protein and is responsible for anisomycin conferred SMN protein increase

Anisomycin activates both the c-JNK and p38 pathways. To directly assay for the role of the p38 pathway in the regulation of SMN levels, MN-1 cells were transfected with either pcdNA3 or p38+MKK6 overexpression plasmid which constitutively phosphorylates and activates the p38 pathway. Cells were harvested for western blot analysis after 24 h of transfection. Overexpression of p38+MKK6 was found to increase the level of SMN protein by ~2-fold compared with pcDNA3 vector control (Fig. 2A). We next pre-treated NT2 cells with c-JNK inhibitor SP-600125 or p38 inhibitor SB-239580 for 1 h and then treated the cells with Anisomycin for 4 h. Western blot analysis revealed that treatment with c-JNK inhibitor had no effect on Anisomycin-induced increase in SMN steady-state mRNA and protein (Fig. 2B). To further confirm the role of p38 in Anisomycin-induced increase in SMN protein, NT2 cells were transfected with p38-specific siRNA or non-targeting control siRNA for 48 h, and then treated with Anisomycin for 4 h. Cells were harvested and western blot analysis was conducted. siRNA-mediated abrogation of p38 expression blocked Anisomycin-induced increase in SMN protein.
These results strongly suggest that activation of p38 but not c-JNK pathway causes Anisomycin-mediated upregulation of SMN protein.

SMN mRNA stabilization by anisomycin in NT2 cells

To determine whether the increase in steady-state levels of SMN mRNA by p38 activation is through mRNA stabilization or by transcriptional upregulation, NT2 cells were treated with Anisomycin in the presence or absence of transcriptional inhibitors Actinomycin D or 1-b-D-ribobenzimidazole (DRB) and total RNA was harvested at different time points (0, 2 and 4 h) for RT–PCR. Activation of p38 by Anisomycin increased the SMN mRNA content in the presence of both transcriptional inhibitors, suggesting that Anisomycin treatment does not increase SMN transcription, but most likely stabilizes SMN mRNA (Fig. 3A and Supplementary Material, Fig. S2a). In contrast, levels of Caspase-3 and TNFα, two mRNA’s which are known to be unstable genes and served as controls for transcriptional inhibition, decreased rapidly upon treatment with Actinomycin D (Fig. 3B and Supplementary Material, Fig. S2b).

RNA binding protein HuR interacts with SMN mRNA

Trans-acting factors, such as HuR, AUF-1 and others have been shown to play an important role in the regulation and fate of an ARE-rich transcripts. It has been suggested previously that the 3’-UTR of SMN transcript contains an ARE-motif (37) and a sequence that resembles a HuR binding site (42). To assess whether endogenous HuR protein interacts with and potentially stabilizes endogenous SMN transcript, RNA–protein complexes were cross linked using formaldehyde in both NT2 and MN-1 cells. RNA–protein complexes were then immunoprecipitated with IgG or HuR antibody after cellular lysis. After cross-linking reversal, cDNA was reverse transcribed from RNA isolated from the immunoprecipitate followed by PCR amplification with SMN primers. In this fashion, we were able to RT–PCR amplify SMN mRNA from immunoprecipitated HuR but not with the IgG confirming that endogenous HuR associates with endogenous SMN mRNA in cells (Fig. 4A and Supplementary Material, Fig. S3). To determine if HuR binds directly to the 3’-UTR of SMN mRNA, we performed a UV cross-linking experiment using a purified recombinant GST-HuR protein and radiolabeled probe of 3’-UTR of SMN mRNA. GST or GST-HuR were incubated with 32P-labeled 3’-UTR of SMN mRNA probe followed by UV cross-linking and separation by SDS–PAGE. We found by autoradiography that 3’-UTR of SMN mRNA was crosslinked to purified recombinant GST-HuR protein in vitro, suggesting that HuR binds directly to the 3’-UTR of SMN mRNA (Fig. 4B).
Anisomycin causes p38-dependent cytoplasmic accumulation of HuR

Although HuR is primarily localized in nucleus, it has been reported previously that in DDT1-MN2 cells, Anisomycin can induce shuttling of HuR from nucleus to cytoplasm (38). To investigate whether there was a similar nuclear to cytoplasmic shuttling of HuR upon Anisomycin treatment and if so, whether it was p38 dependent, MN-1 cells were treated with Anisomycin for 4 h with or without 1 h pretreatment with p38 inhibitor SB-203580. Immunohistochemistry results were consistent with previous reports showing that in untreated cells, HuR is primarily localized in the nucleus (Fig. 5C), whereas some relocalization of HuR from nucleus to cytoplasm is observed after 4 h Anisomycin treatment (Fig. 5F). Importantly, HuR persists in the nucleus when cells are treated with the p38 inhibitor SB-203580 prior to Anisomycin treatment. Thus Anisomycin-induced relocalization of HuR from nucleus to cytoplasm is effectively inhibited by p38 inhibition (Fig. 5I).

HuR is required for p38-mediated upregulation of SMN protein

To further elaborate the role of HuR in SMN transcript regulation, MN-1 cells were transfected with either empty pcDNA3 or HuR expressing plasmid. Cells were harvested for western blot analysis after 24 h of transfection. Overexpression of HuR protein (which increased both nuclear and cytoplasmic HuR content; data not shown) increased the level of SMN protein by ~2-fold compared with empty pcDNA3 vector control (Fig. 6A). To directly show that the increase in SMN protein levels mediated by Anisomycin or p38+MKK6 overexpression plasmid is through the HuR-mediated stabilization of SMN mRNA, NT2 cells were transfected with HuR siRNA or non-targeting control siRNA for 48 h and then treated with Anisomycin for 4 h. siRNA-mediated abrogation of HuR expression completely blocked the Anisomycin-induced increase in SMN protein (Fig. 6B). Similarly, in MN-1 cells, HuR knockdown followed by overexpression of p38+MKK6 blocked the increase of SMN protein by p38 pathway activation. These results clearly indicate that HuR is required for p38-mediated upregulation of SMN protein (Fig. 6C).

Role of 3′-UTR of SMN mRNA in Anisomycin-induced increase in SMN gene expression

The 3′-UTR of an mRNA can play an important role in modulating transcript stability. The p38 pathway stabilizes various ARE containing mRNA species through binding of ARE-BPs

Figure 3. SMN mRNA stabilization by Anisomycin in NT2 cells. Anisomycin-induced increase in SMN mRNA content is independent of transcriptional upregulation. (A) NT2 cells were treated with Anisomycin (75 nm) with/without transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 and 4 h) and then harvested for RT–PCR. Quantification of SMN mRNA relative to β-actin. Mean ± SD (bars) of three independent experiments performed in triplicate. (B) NT2 cells were treated with transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 and 4 h) and then harvested for RT–PCR. Quantification of Caspase-3 mRNA relative to β-actin. Mean ± SD (bars) of three independent experiments performed in triplicate.

Figure 4. HuR interacts with SMN mRNA. (A) Endogenous HuR interacts with SMN mRNA in vivo. In NT2 cells, RNA–protein complexes were cross-linked with formaldehyde and immunoprecipitated after cell lysis using antibodies against HuR and IgG. Following crosslink reversal the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by PCR amplification with SMN and Tubulin primers. A representative agarose gel of three independent experiments is shown. (B) HuR binds directly to the 3′-UTR of SMN mRNA in vitro. GST (lane 1) or GST-HuR (lane 2) were incubated with 32P-labeled 3′-UTR of SMN mRNA probe, UV cross-linked and then separated by SDS–PAGE and visualized by autoradiography. As a negative control, GST-HuR (lane 3) was incubated with 32P-labeled 3′-UTR of SMN mRNA probe without UV cross-linking.

Figure 5. Anisomycin induces cytoplasmic relocalization of HuR. (A) HeLa cells were treated with Anisomycin (75 nm) for 4 h and then harvested for immunohistochemistry. Representative images from three independent experiments are shown. (B) Quantification of HuR localization in HeLa cells treated with Anisomycin (75 nm) for 4 h. Mean ± SD (bars) of three independent experiments performed in triplicate.

Figure 6. HuR binds and stabilizes SMN mRNA. (A) Intracellular localization of HuR is increased by overexpression of HuR. MN-1 cells were transfected with HuR expressing plasmid or empty vector. After 24 h, total RNA was extracted and subjected to RT–PCR. Quantification of SMN mRNA relative to β-actin. Mean ± SD (bars) of three independent experiments performed in triplicate. (B) HuR binds directly to the 3′-UTR of SMN mRNA in vitro. GST (lane 1) or GST-HuR (lane 2) were incubated with 32P-labeled 3′-UTR of SMN mRNA probe, UV cross-linked and then separated by SDS–PAGE and visualized by autoradiography. As a negative control, GST-HuR (lane 3) was incubated with 32P-labeled 3′-UTR of SMN mRNA probe without UV cross-linking.
at the 3'-UTR of the mRNA. We have shown that HuR is involved in Anisomycin-induced increase in SMN; HuR is a RNA binding protein (RBP) which is known to interact with 3'-UTR of its target mRNAs. The fact that SMN transcript also contains ARE element in its 3'-UTR region prompted us to explore the role of 3'-UTR of SMN mRNA in Anisomycin-induced increase in SMN transcript. MN-1 cells were transfected with the different Chloramphenicol acetyltransferase (CAT) reporter plasmids containing either the full-length 3'-UTR of SMN mRNA (SMN 3'-UTR), empty vector (pMcpA, control) or full-length 3'-UTR of β-globin mRNA (which has no ARE sequence and serves as negative control) with/without Anisomycin treatment (Fig. 7A). CAT and Neo-mycin (that is expressed from an independent promoter on the same plasmid and was used as transfection efficiency control) expressions were assessed by RT–PCR and ELISA, respectively. An increase in the CAT expression in MN-1 cells transfected with SMN 3'-UTR CAT reporter plasmid upon treatment with Anisomycin was observed at both mRNA and protein level. No increase in CAT expression was seen in MN-1 cells transfected with either pMcpA or β-globin upon treatment with Anisomycin. These results demonstrate that the SMN 3'-UTR reporter construct mimics endogenous SMN mRNA behavior and that 3'-UTR of SMN mRNA is required for Anisomycin-induced increase in SMN mRNA.

**DISCUSSION**

The p38 MAPK pathway which in addition to its role in the regulation of cell responses such as cell cycle arrest and apoptosis also plays an additional important role in the post-transcriptional regulation of gene expression. P38 has been found to regulate the stability of various ARE-containing mRNAs such as COX-2 and TNFα. The antibiotic Anisomycin has been shown to activate MAPK (p38, c-JNK) stabilizing some ARE-containing mRNA such as β2-adrenergic receptor mRNA (43). The p38 pathway is activated by Anisomycin in an RAS-independent manner and has been shown to mediate the activation of several early response genes known to be induced Anisomycin. It should be noted that the low concentration of Anisomycin used does not appear to inhibit protein synthesis, affect cell growth or cause other toxic effects on cells (44). In this study, we found that when NT2 and MN-1 cells were treated with Anisomycin, there was a significant time-dependent increase in the levels of SMN mRNA and protein levels. SMN mRNA has been suggested as an ARE-rich transcript that contains an AUUUA motif in its 3'-UTR. We have demonstrated that an activation of p38 regulates SMN mRNA and protein by relocating HuR protein from nucleus to cytoplasm whereafter it binds SMN mRNA 3'-UTR thereby stabilizing the SMN transcript. Previously,
Singh et al. have shown that C5-substituted quinazolines inhibit DcpS decapping activity by interacting and opening the enzyme in a catalytically incompetent conformation thereby inhibiting SMN2 mRNA decay notionally increasing SMN protein levels. These results along with those presented here suggest that post-transcriptional modulation of SMN mRNA may be a productive means of modulating SMN levels and may play a significant impact on SMN levels.

The ARE motifs in 3′-UTR are well-established targets of ARE-BPs regulating the post-transcriptional expression levels of diverse mRNAs by either promoting or suppressing their stabilization. HuR is well-characterized ARE-BP which is known to bind and stabilize various p38-regulated ARE containing mRNAs (33,39,46,47). Here we report that HuR directly binds to SMN transcript in the 3′-UTR region of the mRNA (Fig. 4). Our findings are consistent with the previous reports that Anisomycin can cause rapid accumulation of HuR protein in the cytoplasm (40). However, blocking of p38 pathway using specific p38 inhibitor prevents the shuttling of HuR protein from nucleus to cytoplasm after Anisomycin treatment, which indicates that activation of p38 pathway is necessary for Anisomycin-induced nuclear to cytoplasmic movement of HuR protein in NT2 cells (Fig. 5). Overexpression of HuR protein, even in the absence of additional triggers, is sufficient to increase SMN protein level which is consistent with the previous reports that HuR, through transcript stabilization, can increase the levels of various p38 regulated proteins (33,39,46,47). The p38-mediated upregulation of SMN protein levels by Anisomycin or p38 overexpression plasmid is abrogated when HuR protein is knocked down using siRNA, indicating that HuR protein is required for p38-mediated induction in SMN protein (Fig. 6).

We have shown that the 3′-UTR of SMN mRNA is critical in Anisomycin-induced increase in SMN expression. The exact binding motif for HuR in ARE transcripts has not been characterized yet. It has been suggested by Silanes et al. that Hu binding sequences comprise short stretches of U-rich nucleotides which also contains A and G nucleotides (48). These sequences are present in 3′-UTR regions of both human SMN1 and mouse Smn mRNA which we have shown to be required for Anisomycin-induced increase in SMN induction, an observation which is consistent with HuR binding to this region of SMN mRNA. The most parsimonious model developing from our data is that Anisomycin activates p38 pathway which in turn relocates HuR from nucleus to cytoplasm. In the cytoplasm, HuR binds to 3′-UTR of SMN mRNA thus stabilizing SMN transcript resulting in increased protein levels (Fig. 8).
Interestingly, HuR has been shown to be present in murine motor neurons and to undergo translocation to cytoplasm in the presence of ALS causing SOD1 mutation (49). In SMA, anomalous NMJ structure and function is observed, possibly compounded by a loss of axonal ability to regenerate and ultimately a loss of motor neurons. In this regard, it has been shown that the p38 pathway is required for efficient growth cone regeneration after axon injury (50, 51), suggesting that activation of p38 pathway is required for the maintenance of motor neuron function and its integrity. A recent study showed an under-expression of p38 pathway in SMA I muscles, suggesting that p38 may be regulator of protein synthesis in SMA I (52).

If one is to realistically consider p38 activation as an SMN enhancing SMA therapeutic there are a number of issues which need to be addressed. First, the in vivo recapitulation of our in vitro observations shall be critical; this is underway in our laboratory. There is no clearly defined time that SMN absence causes SMA or conversely when SMN repletion may ameliorate SMA. Extensive analysis of the robust SMA mouse models and electrophysiological analysis of clinical SMA cases appears to point to a dysfunction which antecedes actual denervation and ultimately cell loss that occurs early in the lifespan even in the milder forms of SMA. A picture of a window of motor neuron vulnerability which may span the antenatal period to the early weeks and months in mice and years in human emerges. This coincides with the greatest level of SMN expression in both humans and mice. There is the risk that the SMN induction achieved both in this work and others is to some extent mimicking what already happens physiologically early in development; induction achieved so readily in these systems may not be so readily attained in the disease setting. However, if the high levels of SMN observed developmentally are a result of transcriptional induction, then the HuR mediated stabilizing a pool of transcripts appears to be a more realizable goal.

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The other issue is that of p38 activation itself. Although p38 is involved in a large number of biological phenomena, in the

Figure 7. The 3'-UTR of SMN mRNA is required for Anisomycin-induced increase in SMN mRNA and protein. MN-1 cells were transfected with CAT reporter plasmids containing either the full-length 3'-UTR of human SMN mRNA (SMN UTR), α-globin mRNA (GLOBIN 3'-UTR) or an empty vector (pMcpA) with/without Anisomycin treatment (75 nM). CAT and Neomycin (used as transfection control) expression was assessed by RT–PCR and ELISA. Mean ± SD (bars) of three independent experiments performed in triplicate.

Figure 8. Proposed model for Anisomycin-mediated induction of SMN. Anisomycin treatment causes phosphorylation and activation of p38 which results in accumulation of HuR in the cytoplasm where it is able to interact with the 3'-UTR of SMN transcript. This binding stabilizes SMN mRNA which results in an increase in SMN steady-state mRNA levels and ultimately increases SMN protein expression.
majority of cases its stimulation appears to be driven by<br>cellular stress. Assuming that the motor neuron of a child<br>with SMA has a pool of SMN mRNA that are less than<br>100% occupied by HuR, achieving an innocuous means of<br>upregulating p38 to activate/translocate more HuR may<br>represent a challenge.

In conclusion, we present here direct mechanism for SMN<br>induction; p38 pathway activation triggering HuR protein<br>shuttling resulting in SMN mRNA stabilization and SMN<br>protein level increase. This may lead to novel treatments<br>for SMA.

MATERIALS AND METHODS

Reagents

Anisomycin was purchased from Alomone laboratories and<br>c-JNK inhibitor SP-600125, p38 inhibitor SB-239580, Actino-<br>mycin D and DRB were from Sigma. Non-silencing siRNA<br>control, p38 siRNA and HuR siRNA were supplied by Qiagen,<br>Cell Signaling and Dharmacon, respectively. CAT ELISA kit<br>was from Roche and NEO ELISA kit was supplied by Agdia.<br>The antibodies used in this study are SMN (BD Transduction<br>Laboratories), p38 (Cell Signaling), Phospho-c-JNK (Cell<br>Signaling) and HuR (Santa Cruz).

Primer sequences

SMN—Human
Forward: 5'-GCTATCATACTGATACTGGCTATTATA<br>Reverse: 5'-CTCTAACAGCTTACATCCAGATCTG
SMN (3'-UTR) Human
Forward: 5'-ATGGATCCCGAGAAATGTTCATGAG<br>Reverse: 5'-ATTCTAGACAGTACAATGACACTCAG
SMN—Mouse
Forward: 5'-ATGGATCCCGAGAAATGTTCATGAG
Reverse: 5'-ATTCTAGACTGATACAGTACAATGACACTCAG
Actin
Forward: 5'-CTGGAACGCTGGAAGGTGCAC
Reverse: 5'-AAAGGACTTCTGTGAACAATGCA
Tubulin—Human
Forward: 5'-ATGGCCAGATGCAGTCAGTGAAT
Reverse: 5'-TACCGAGGTGTCGCTGAAT
Tubulin—Mouse
Forward: 5'-ATGGCCAGATGCAGTCAGTGAAT
Reverse: 5'-TACCGAGGTGTCGCTGAAT
CAT
Forward: 5'-GCCTGTTACGGTCGAACCT
Reverse: 5'-GGCGGGAAGCCTGGTTACGG
NEO
Forward: 5'-TGAATGAACTGACGGAGAG
Reverse: 5'-CATCTAGACGCGTTCTTC
Caspases-3 (Hs_CASP3_1_SG QuantiTect Primer Asssay,<br>Qiagen).

Cell culture and drug treatment conditions

NT2 or MN-1 cells were maintained in standard conditions<br>(37°C in a 5% CO2 humidified atmosphere) in Dulbecco’s<br>modified Eagle’s medium (DMEM) supplemented with 10%<br>fetal calf serum (FCS), 1% antibiotics (100 U/ml penicillin–<br>streptomycin) and 2 mM glutamate.

NT2 or MN-1 cells were seeded in 12-well plates (2.5 ×<br>105 cells/well) and treated 24 h later with Anisomycin<br>(75 nM) for up to 4 h. For c-JNK and p38 inhibitor treatment,<br>NT2 cells were seeded in 12-well plates (2.5 × 105 cells/well)<br>and treated 24 h later with c-JNK inhibitor SP-600125 (10 μM)<br>or p38 inhibitor SB-239580 (3 μM) for 1 h followed by Aniso-<br>mycin (75 nM) treatment for up to 4 h. For transcriptional<br>inhibitor treatment, NT2 cells were seeded in 12-well plates<br>(2.5 × 105 cells/well) and treated 24 h later with Actinomycin<br>D (2.5 μg/ml) or DRB (100 nM) for up to 4 h.

Transfection

MN-1 cells were seeded in 12-well plates (2.5 × 105 cells/br>well) and transfected on the following day in serum-free<br>DMEM with 2 μg of DNA per well using LipofectAMINE<br>2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).<br>The transfection mixture was supplemented 3 h later with<br>1 ml DMEM containing 10% FCS, antibiotics and glutamate.<br>Cells were harvested 24 h after transfection for analysis.

For siRNA transfections, NT2 or MN-1 cells were seeded in<br>12-well plates (2.0 × 105 cells/well) and transfected on the<br>following day in serum-free DMEM with p38 siRNA(25 nM)<br>or HuR siRNA (100 nM) or non-silencing control siRNA (25<br>or 100 nM), using LipofectAMINE 2000 transfection reagent<br>for 48 h.

Western blot analysis

Cells were washed two times with 1 ml PBS (1 ×) and lysed<br>in 75 μl RIPA buffer containing 10 mg/ml each of aprotin-<br>tin, PMSF and leupeptin (all from Sigma), 5 mM β-Glycerylphosphate, 50 mM NaF and 0.2 μM sodium ortho-<br>nadate for 30 min at 4°C, followed by centrifugation at<br>13 000g for 15 min and supernatants were collected and kept<br>frozen at −20°C. Protein concentrations were determined<br>by Bradford protein assay using a Bio-Rad protein assay kit<br>(Richmond, CA, USA). For western blot analysis, protein<br>samples were boiled for 5 min and equal amounts of protein<br>extract were separated by 10% SDS–PAGE. Proteins were<br>subsequently transferred onto nitrocellulose membrane and<br>the membrane was incubated in blocking solution (PBS, 5%<br>non-fat milk, 0.2% Tween-20) for 1 h at room temperature<br>followed by overnight incubation with primary antibody at 4°C<br>at the dilution prescribed by the manufacturer. Membranes<br>were washed with PBS-T (PBS and 0.2% Tween-20) two times<br>followed by incubation with secondary antibody (anti-mouse<br>or rabbit, Cell Signaling) for 1 h at room temperature. Antibody<br>complexes were visualized by autoradiography using the<br>ECL Plus and ECL western blotting detection systems (GE<br>Healthcare). Quantification was performed by scanning the<br>autoradiographs and signal intensities were determined by<br>densitometry analysis using Odyssey v1.1 program.
Quantitative RT–PCR

Total RNA was isolated according to the protocol provided by the manufacturer using the RNAeasy kit (Qiagen). For quantitative RT–PCR, cDNA was reverse transcribed from isolated RNA with oligo dT<sub>18</sub> primer using First-Strand cDNA Synthesis kit from GE Healthcare following manufacturer’s instructions. The synthesized c-DNA template was used for quantitative PCR employing the QuantiTect SYBR Green PCR kit (Qiagen) and analysed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCR was carried out to detect SMN, Caspase-3, Actin, CAT and NEO genes using primers listed above.

**In vivo RNA–protein complex immunoprecipitation**

In vivo RNA–protein complex cross-linking and coprecipitation was performed as described previously (53). RNA–protein complexes were immunoprecipitated with IgG or HuR antibody at 1:50 dilution after cellular lysis. After cross-linking reversal, RNA was isolated from the immunoprecipitate using a Stratagene kit. cDNA was reverse transcribed from isolated RNA with oligo dT<sub>18</sub> primer using a First-Strand cDNA Synthesis kit from GE Healthcare according to the protocol provided by the manufacturer. The partial sequence of SMN and Tubulin was PCR amplified with the cDNA using SMN and Tubulin primers. PCR products were run on 1% agarose gel and visualized by ethidium bromide staining.

**UV cross-linking of RNA–protein complexes**

UV cross-linking of 3′-UTR of SMN mRNA with HuR was performed as previously described (54).

**Immunohistochemistry**

Cells were washed two times with 1 ml PBS (1 ×) and fixed with 4% PFA for 10 min. The cells were incubated in blocking solution (PBS, 10% normal goat serum, 0.3% Triton X-100) for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C. Cells were washed three times with PBS (1 ×) followed by incubation with fluorescence dye conjugated secondary antibody (Alexa Fluor 546, anti-mouse, Invitrogen) for 1 h at room temperature. 4′,6-diamidino-2-phenylindole was used for nuclear staining.

**Construct preparation and CAT analysis**

The CAT reporter plasmids pMcpA was described previously (55). The 3′-UTR of α-globin and SMN was generated by PCR amplification from cDNA from HeLa and NT2 cells, respectively, and insertion of the 3′-UTR downstream of CAT in a pMcpA construct. Transiently transfected cells were washed with 1 ml PBS (1 ×) followed by lysis with CAT ELISA kit lysis buffer. CAT ELISA kit was used according to the manufacturer’s protocol to determine CAT levels in the cell lysate. NEO levels were determined also using NEO ELISA kit according to the protocol provided by the manufacturer.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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