Target Genes of Neuron-Restrictive Silencer Factor Are Abnormally Up-Regulated in Human Myotilinopathy

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Myotilinopathy is a subgroup of myofibrillar myopathies caused by mutations in the myotilin gene in which there is aggregation of abnormal cytoskeletal proteins and ubiquitin. We report here on the accumulation of neuron-related proteins such as ubiquitin carboxy-terminal hydrolase L1 (UCHL1), synaptosomal-associated protein 25, synaptophysin, and α -internexin in aberrant protein aggregates in myotilinopathy. We have determined that the neuron-restrictive silencer factor (NRSF)/RE1 silencing transcription factor (REST), a transcription factor expressed in non-neuronal tissues repressing the expression of several neuronal genes, is reduced in myotilinopathies. Moreover, NRSF transfection reduces UCHL1, synaptosomal-associated protein 25, synaptophysin, and α -internexin mRNA levels in DMS53 cells, whereas short interfering NRSF transfection increases UCHL1 and synaptophysin mRNA levels in U87-MG cells. Chromatin immunoprecipitation assays have shown that NRSF interacts with the UCHL1 promoter in U87-MG and HeLa cells. *In silico* analysis of the UCHL1 gene promoter sequence using the MatInspector software has predicted three potential neuron-restrictive silencer elements (NRSEs): NRSE1 located in the complementary DNA chain and NRSE2 and NRSE3 in intron 1, in the coding and complementary chains, respectively. Together, these findings show, for the first time, abnormal regulation of NRSF/REST as a mechanism associated with the aberrant expression of selected neuron-related proteins, which in turn accumulate in abnormal protein aggregates, in myotilinopathy. (Am J Pathol 2007; 171:1312–1323; DOI: 10.2353/ajpath.2007.070520)

Myofibrillar myopathies (MFMs) are a clinically and genetically heterogeneous group of inherited or sporadic muscle diseases characterized morphologically by the presence of nonhyaline structures corresponding to foci of dissolution of myofibrils and hyaline lesions composed of protein aggregates. Immunohistochemical studies have demonstrated intracytoplasmic accumulation of several muscle-related proteins, including cytoskeletal and myofibrillary proteins, as well as α B-crystallin and ubiquitin.^{1–8} Mutations in several genes have been identified as causing MFMs: desmin, α B-crystallin, selenoprotein N, myotilin, ZASP, and filamin C.^{5,7,9–17} The causes of protein aggregation in MFMs are not fully understood, but our previous studies have shown that impaired protein degradation probably plays a crucial role, as suggested by abnormal expression levels and aberrant localization of several subunits of the proteasome 19S and 26S and by the up-regulation of immunoproteasomal subunits in muscle fibers containing abnormal protein deposits.¹⁸ Moreover, protein accumulations are enriched in clusterin and γ -tubulin, whereas p62 and mutant ubiquitin colocalize with protein aggregates, thus suggesting p62 involvement in protein aggregation and mutant ubiquitin in protein degradation in MFMs.^{19,20}

Preliminary work in our laboratory identified the presence of ubiquitin carboxy-terminal hydrolase L3 (UCHL3) in normal and diseased muscle, but UCHL1 was also present in the setting of abnormal protein deposits in MFMs. This was unexpected, because UCHL1 is abundant in brain and testis, whereas other members of the UCHL family, but not UCHL1, are expressed in other organs.^{21–23} UCHLs are enzymes involved in the hydrolysis of polyubiquitin chains to increase the availability of free monomeric ubiquitin to the ubiquitin-proteasome

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system favoring protein degradation.²⁴ In the nervous system, UCHL1 associates with ubiquitin and maintains free ubiquitin levels in neurons. Loss of UCHL1 reduces free ubiquitin and leads to inadequate ubiquitylation and protein accumulation in neurons.²⁵

Based on these findings, the present work was designed to study the localization and distribution of UCHL1 in MFMs, the expression of other neuronal proteins in MFMs that are not normally expressed in adult muscle fibers, and the mechanisms that modulate the abnormal expression of neuronal proteins in MFMs. For practical purposes, the study was focused on the MFM subgroup of myotilinopathies, disorders associated with mutations in the myotilin gene.

We have determined that neuron-restrictive silencer factor (NRSF)/RE1 silencing transcription factor (REST), a transcription factor expressed in non-neuronal tissues repressing the expression of several neuronal genes, is reduced in myotilinopathies.^{26,27} This reduction is accompanied by aberrant expression of the neuronal proteins synaptosomal-associated protein 25 (SNAP25) and synaptophysin, which are encoded by NRSF/REST target genes, and α -internexin.²⁸ Finally, we have shown that NRSF/REST is also involved in the regulation of UCHL1 and α -internexin gene expression.

Materials and Methods

Muscle Biopsies

Muscle biopsies from 10 patients with myotilinopathy were included in the present study. In addition, muscle samples from five age-matched patients who were considered to be free of any neuromuscular disease after detailed clinical and pathological studies were used as controls. A detailed description of the clinical, pathological, and molecular studies of seven of the cases here included has been presented elsewhere.¹⁵ A summary of

the patients included in the present study is shown in Table 1.

Immunohistochemistry and Double-Labeling Immunofluorescence and Confocal Microscopy

For immunohistochemical studies, 6- μ m-thick cryostat sections were stained with hematoxylin and eosin (H&E) and modified Gomori trichrome and processed for myotilin, ubiquitin, UCHL1, SNAP25, synaptophysin, and α -internexin immunohistochemistry. Briefly, cryostat sections were incubated with 1% hydrogen peroxide and 10% methanol for 30 minutes at room temperature, followed by 5% normal serum for 2 hours, and then incubated overnight with one of the primary antibodies. Mouse monoclonal anti-myotilin (Novocastra, Newcastle, UK), anti-synaptophysin (Dako, Glostrup, Denmark), anti-SNAP25 (Chemicon, Temecula, CA), and anti- α -internexin (Zymed, South San Francisco, CA) antibodies were used at dilutions of 1:200, 1:100, 1:2000, and 1:100, respectively. Rabbit polyclonal anti-ubiquitin (Dako) and anti-UCHL1 (Abcam, Cambridge, UK) antibodies were used at dilutions of 1:100 and 1:200, respectively. After washing, the sections were processed with the streptavidin-biotin Super Sensitive IHC detection system (BioGenex, San Ramon, CA). The peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxide in phosphate-buffered saline (PBS).

For double-labeling immunofluorescence, 6- μ m-thick cryostat sections were stained with a saturated solution of Sudan black B (Merck, Darmstadt, Germany) for 30 minutes to block autofluorescence of lipofuscin granules, rinsed in 70% ethanol, and washed in distilled water. Sections were incubated at 4°C overnight with mouse monoclonal anti-myotilin antibody (Novocastra) at a dilution of 1:200 and rabbit polyclonal anti-UCHL1 antibody (Abcam) at a dilution of 1:200 in PBS. Secondary antibodies were Alexa488 anti-mouse (green) and Alexa546 anti-rabbit (red) (Molecular Probes, Leiden, The Netherlands), used at a dilution of 1:400. After washing with PBS, the sections were incubated in a cocktail of secondary antibodies in the same vehicle solution for 3 hours at room temperature. After washing in PBS, the sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals, Solon, CA), sealed, and dried overnight. Nuclei were stained with TO-PRO-3-iodide (Molecular Probes) diluted 1:1000. Sections incubated with the secondary antibodies only were used as controls. Samples were examined under a Leica TCS-SL confocal microscope (Leica, Wetzlar, Germany).

Gel Electrophoresis and Western Blotting

Frozen muscles were directly homogenized in a final volume of 1:20 (w/v) in lysis buffer (75 mmol/L Tris-HCl, pH 6.8, 0.001% bromophenol blue, 15% sodium dodecyl sulfate, 20% glycerol, 5% β -mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin, leupeptin, and pepstatin). Samples were boiled at 95°C for 3 minutes and centrifuged at 9500 rpm for 5 minutes at room temperature. Samples were loaded in sodium dode-

Table 1. Summary of the Main Clinical Findings in the Present Series

Case	Age/ gender	Age at onset	Site of muscle biopsy	Mutation
Patients				
1	52/M	50	Lateral gastrocnemius	S55F
2	56/M	52	Soleus	S55F
3	70/M	58	Vastus lateralis	S60C
4	69/F	62	Deltoid	S60C
5	80/M	70	Deltoid	S60F
6	82/M	62	Vastus lateralis	S60C
7	73/F	69	Deltoid	S55F
8	80/F	77	Vastus lateralis	K36E
9	60/F	50	Deltoid	S60C
10	54/M	50	Lateral gastrocnemius	S55F
Controls				
11	75/M		Deltoid	
12	64/M		Deltoid	
13	65/F		Vastus lateralis	
14	80/M		Vastus lateralis	
15	58/F		Lateral gastrocnemius	

M, male; F, female.

cyl sulfate-polyacrylamide gels with Tris-glycine running buffer. Proteins were electrophoresed using a miniprotein system (Bio-Rad, Alcobendas, Spain) and transferred to nitrocellulose membranes with a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) for 45 minutes at 40 mA. Cell lines grown in six-well plates were homogenized with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Lysates were maintained in agitation for 30 minutes at 4°C and then centrifuged at 12,000 rpm for 20 minutes at 4°C. Protein concentration was determined with BCA (Pierce, Woburn, MA) method. Twenty micrograms of total protein was boiled at 95°C for 3 minutes and loaded in sodium dodecyl sulfate-polyacrylamide gels with Tris-glycine running buffer. Proteins were electrophoresed using a miniprotein system (Bio-Rad) and transferred to nitrocellulose membranes with a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad) for 1 hour at 100 V.

Nitrocellulose membranes were blocked with PBS containing 5% skim milk for 30 minutes. Subsequently, the membranes were incubated at 4°C overnight with one of the primary antibodies in PBS containing 5% skim milk. The following antibodies were used: mouse monoclonal myotilin (Novocastra) used at a dilution of 1:10,000, rabbit polyclonal UCHL1 (Chemicon) diluted 1:500, anti-REST (Abcam) used at a dilution of 1:500 for muscle biopsies and diluted 1:250 for cell lines, anti- α -internexin (Zymed) at a dilution of 1:500, and mouse monoclonal anti- β -actin (clone AC-74; Sigma) diluted 1:30,000. After primary antibody incubation, the membranes were washed three times with PBS containing 5% skim milk for 5 minutes at room temperature and then incubated with the corresponding anti-rabbit or anti-mouse antibodies labeled with horseradish peroxidase (Dako) at a dilution of 1:1000 for 1 hour at room temperature. The membranes were subsequently washed four times for 5 minutes each with PBS at room temperature and developed with the chemiluminescence ECL Western blotting system (Amersham/Pharmacia, Buckinghamshire, UK) followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL; Amersham). Control of protein loading was tested with Coomassie blue on the band of 205 kd corresponding to myosin.

Cell Culture

The selection of cell lines was based on preliminary studies in our lab directed to sample cells with different basal expression of UCH-L1 and NRSF/REST. The cell lines selected on this basis were as follows. Small cell lung cancer DMS53 (American Type Culture Collection no. CRL-2062; Manassas, VA) cells were maintained in Waymouth's MB 752/1 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. HeLa cells were maintained in Dulbecco's minimal essential medium (Invitrogen, El Prat de Llobregat, Spain) supplemented with 10% fetal bovine serum. U87-MG cells (American Type Culture Collection no. HTB-14) were maintained in

minimal essential medium (Eagle's) with 2 mmol/L L-glutamine and supplemented with 10% fetal bovine serum and 1 mmol/L sodium pyruvate. Human neuroblastoma SH-SY5Y cell line was maintained in Dulbecco's minimal essential medium (Invitrogen) (European Collection of Cell Cultures no. 94030304; Salisbury, UK) supplemented with 10% fetal bovine serum. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂.

NRSF/REST Transfection

DMS53 and SH-SY5Y cells were plated in six-well dishes at a concentration of 10⁵ cells/well and cultured overnight before transfection. One microgram of *REEX1* vector (kindly provided by Dr. Gail Mandel, Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY) was transfected using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. After 5 hours of transfection, the medium was replaced by fresh medium.

NRSF/REST siRNA Transfection

U87-MG cells were plated in six-well dishes at a concentration of 50,000 cells/well and cultured overnight before transfection. Following the instructions of the manufacturers, 100 nmol/L NRSF/REST short interfering (si)RNA 1 (5'-GCUUAUUAUGCUGGCAAATT-3'; Ambion, Madrid, Spain), NRSF/REST siRNA 2 (5'-GC-CUUCUAAUAAUGUGUCATT-3'; Ambion), and a negative control or scramble siRNA (Ambion) were transfected using Lipofectamine 2000 (Invitrogen). After 5 hours of transfection, the medium was replaced by fresh medium. The analysis of siRNA was performed 48 hours later.

mRNA Isolation

RNA obtained from muscle biopsies was purified as previously described.²⁹ The purification of RNA from cell lines was performed with RNeasy Midi kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. The concentration of each sample was obtained from A₂₆₀ measurements. RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA).

cDNA Synthesis

The retrotranscriptase reaction (100 ng RNA/ μ l) was performed using the High capacity cDNA Archive kit (Applied Biosystems, Madrid, Spain) following the protocol provided by the supplier. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

TaqMan Polymerase Chain Reaction

The NRSF/REST TaqMan assay (Hs00194498_m1, TaqMan probe 5'-AGGAAGGCCGAATACAGTTATGGCC-3') (Applied Biosystems) generates an amplicon of 79 bp and is located at position 341 between the 1 and 2 exon boundary of NM_005612.3 transcript sequence. The TaqMan assay for UCHL1 (Hs00188233_m1, TaqMan probe 5'-CCTGCTGAAGGACGCTGCCAAGGTC-3') (Applied Biosystems) is located at position 648 between the 8 and 9 exon boundary of NM_004181.3 transcript sequence. It generates an amplicon of 100 bp. The TaqMan assay for Synaptophysin (Hs00300531_m1, TaqMan probe 5'-CGAGTACCCCTCAGGCTGCACCAA-3') (Applied Biosystems) generates an amplicon of 63 bp and is located at position 241 of NM_003179.2 transcript sequence. The TaqMan assay for α -internexin (Hs00190771_m1, TaqMan probe 5'-AGCAGCTTACAGGAACTGCTGGAA-3') (Applied Biosystems) generates an amplicon of 92 bp and is located at position 1240 of NM_032727.2 transcript sequence. The TaqMan assay for SNAP25 (Hs00268296_m1, TaqMan probe 5'-GAAGCCCAGGTCCAGAGCCAAACCC-3') (Applied Biosystems) generates an amplicon of 63 bp and is located at position 153 of NM_003081.2 transcript sequence.

TaqMan polymerase chain reaction (PCR) assays for all genes analyzed were performed in duplicate on cDNA samples in 96-well optical plates using an ABI Prism 7900 Sequence Detection system (Applied Biosystems). The plates were capped using optical caps (Applied Biosystems). For each 20- μ l TaqMan reaction, 9 μ l of cDNA (diluted 1/50 for studies with DMS53 and 1/10 for muscle biopsies) was mixed with 1 μ l of 20 $\times$ TaqMan Gene Expression Assays and 10 μ l of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were performed with β -glucuronidase (GUSB) (Hs99999908_m1, TaqMan probe 5'-GACTGACAGTCACCGACGAGAGTG-3'), for normalization. The reactions were performed using the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were prepared for all genes analyzed using serial dilutions of cDNA from DMS53 cells, U87-MG cells, and human control muscle biopsies. Finally, all TaqMan PCR data were captured using the Sequence Detector Software (SDS version 1.9; Applied Biosystems). For each experimental sample, the amount of tar-

get and endogenous reference (GUSB) was determined from the appropriate standard curve, which was plotted showing the cycle threshold (y) versus the log of nanograms of total control RNA. Then the amount of each target gene was divided by the endogenous reference GUSB amount to obtain a normalized target value, permitting determination of the relative mRNA levels of every gene in normal muscle and in myotilinopathy. TaqMan PCR assays for α -internexin and SNAP25 from human muscle biopsies were performed using the TaqMan Pre-Amp Master Kit (Applied Biosystems).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's protocol (Upstate, Madrid, Spain) using 10⁶ U87-MG, HeLa, and DMS53 cells. Ten micrograms of anti-NRSF/REST (P-18X, sc-15118X; Santa Cruz Biotechnology, Santa Cruz, CA) and 10 μ g of anti-acetylated H3 (residue Lys9; Cell Signaling, Danvers, MA) were used for immunoprecipitation. Purified DNA was resuspended in 20 μ l of DNase-free water, and 2 μ l was used as a template in 25 μ l of PCR reaction using GoTaq Flexi DNA Polymerase (Promega, Madrid, Spain). Primer concentration was 200 nmol/L. PCR primers were 5'-ACAAATCCCgTCTCCACAAC-3' and 5'-GCCTAGGGAAGACGAAAAACA-3' for the amplification of neuron-restrictive silencing element 1 (NRSE1) sequence of the UCHL1 gene promoter. The reaction was performed using the following parameters: 95°C for 2 minutes and 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and a last hold of 72°C for 5 minutes.

Results

General Aspects

Muscle biopsies were characterized by the presence of nonhyaline and hyaline polymorphic inclusions of varying shapes and sizes. Hyaline inclusions stained bright pink with hematoxylin and eosin and blue-red or red-purple with the modified trichrome Gomori stain. Rimmed and unrimmed vacuoles were found in every case. Cytoplasmic bodies and collections of dense spheroid bodies

Figure 1. Muscle pathology in myotilinopathy. In addition to marked variation in the fiber size and increased numbers of internal nuclei, several muscle fibers contain eosinophilic hyaline inclusions on H&E stain (**A**) that look blue-red with modified Gomori (**B**). Some vacuolated fibers are seen in **B**. Several muscle fibers containing prominent protein aggregates show strong myotilin immunoreactivity (**C**). Bar = 100 μ m.

were observed in the majority of cases. Immunohistochemical studies showed strong myotilin (Figure 1) and ubiquitin immunoreactivity in hyaline inclusions.

Abnormal Expression of Neuronal Proteins in Myotilinopathies

Additional immunohistochemical studies demonstrated the presence of UCHL1, α -internexin, synaptophysin, and SNAP25 in parallel with myotilin aggregates in abnormal muscle fibers in myotilinopathy (Figure 2).

Colocalization of UCHL1 and Myotilin in Abnormal Fibers in Myotilinopathy

Double-labeling immunofluorescence and confocal microscopy disclosed UCHL1 immunoreactivity in dam-

aged muscle fibers in myotilinopathy, whereas no traces of UCHL1 occurred in control muscles. Aberrant UCHL1 deposition colocalized with myotilin aggregates in damaged fibers. These changes were specific, because no immunoreaction was elicited after incubation with the secondary antibodies alone (Figure 3).

Inverse Relationship between UCHL1 and NRSF/REST Expression Levels in Myotilinopathy

Synaptophysin and SNAP25 have been described as being regulated by NRSF/REST.²⁸ Following up on these data, we tested whether the expression levels of REST were modified in diseased muscles. Protein levels were analyzed by gel electrophoresis and Western blotting. Analysis of protein levels and densitometric quantifica-

Figure 2. Immunohistochemistry reveals an abnormal expression of neuronal proteins in myotilinopathy. Two sets of serial sections of a muscle biopsy from a patient with myotilinopathy reveal the presence of myotilin (A), α -internexin (B), SNAP25 (C), UCHL-1 (D), α -internexin (E), and synaptophysin (F). All muscle samples listed in Table 1 were analyzed. Bar = 100 μ m.

Figure 3. Double-labeling immunofluorescence and confocal microscopy reveal colocalization of UCHL1 and myotilin aggregates in myotilinopathy. **A–I:** UCHL1 deposition (green) colocalizes with myotilin aggregates (red) in damaged fibers (merge, yellow). **J–L:** These changes are specific because no immunoreaction is obtained after incubation with the secondary antibodies alone. Nuclei are visualized in blue with TO-PRO-3 iodide. All muscle samples listed in Table 1 were analyzed.

tions are shown in Figure 4. UCHL1 protein levels were significantly increased in myotilinopathies [$P < 0.01$, analysis of variance with post hoc least significant difference (LSD) test], whereas NRSF/REST protein levels were reduced in diseased muscles ($P < 0.05$, analysis of variance with post hoc LSD test). Although the modifications in UCHL1 and REST levels were variable from one case to another, the density of the bands of UCHL1 inversely correlated with REST expression in every individual case. Interestingly, the higher expression of UCHL1 was associated with the higher deposition of myotilin (Figure 4).

Abnormal Expression of Neuronal Proteins Is Associated with Up-Regulation of the Corresponding mRNA Levels and Down-Regulation of REST mRNA Levels

A significant increase in UCHL1 and SNAP25 mRNA levels was found in myotilinopathy when compared with control muscles ($P < 0.05$, analysis of variance with post hoc LSD test). This was associated with a parallel decrease in REST mRNA expression levels in diseased muscles (Figure 5A). α -Internexin mRNA levels were un-

Figure 4. Inverse relation between NRSF/REST and UCHL1 protein levels in myotilinopathy. **A:** NRSF/REST (121 kd) and UCHL1 (25 kd) protein levels are detected by Western blot in muscle biopsies of patients suffering from myotilinopathy and age-matched controls. An inverse relationship is observed between UCHL1 and NRSF/REST in every individual case. Also note increased myotilin (57 kd) levels in diseased muscles. Myosin (205 kd), as seen with Coomassie blue, is shown as a control of protein loading. The image is representative of all of the cases listed in Table 1. **B:** Densitometric analysis of myotilin, UCHL1, and NRSF/REST protein levels of all samples listed in Table 1 is shown (mean $\pm$ SD). AU, arbitrary units; C, control; M, myotilinopathy. * $P < 0.05$; ** $P < 0.01$ compared with age-matched control muscle biopsies (analysis of variance with post hoc LSD test).

Figure 5. TaqMan PCR assays reveal abnormal up-regulation of neuronal genes and down-regulation of NRSF/REST in myotilinopathy. **A:** NRSF/REST, UCHL1, and SNAP25 mRNA levels (mean $\pm$ SD) normalized with GUSB in muscle biopsies of patients suffering myotilinopathy compared with age-matched controls listed in Table 1. Determination of arbitrary units is indicated in Materials and Methods. **B:** TaqMan PCR amplification plot for α -internexin mRNA levels in muscle biopsies of patients suffering myotilinopathy and age-matched controls. The cycle threshold values for control samples are below the threshold lane (red line). AU, arbitrary units; C, control; M, myotilinopathy. * $P < 0.05$ compared with age-matched control muscle biopsies (analysis of variance with post hoc LSD test).

detected in normal muscles, but they were clearly expressed in myotilinopathy (Figure 5B).

NRSF Transfection Down-Regulates SNAP25, Synaptophysin, UCHL1, and α -Internexin Genes in DMS53 Cells

As mentioned above, some of the up-regulated neuronal proteins detected in myotilinopathy have been described as being NRSF/REST target genes.²⁸ Therefore, we tested whether NRSF/REST overexpression reduced the mRNA levels of known target genes SNAP25 and synap-

Figure 6. NRSF/REST overexpression reduces α -internexin, SNAP25, synaptophysin, and UCHL1 mRNA levels in the DMS53 cell line. **A:** One μ g of REEX1 vector, which encodes human full-length NRSF cDNA, was transfected in DMS53 cells using Lipofectamine 2000. NRSF/REST mRNA and protein levels are increased after 48 hours of REEX1 vector transfection. The protein levels were detected by Western blot. β -Actin (45 kd) was blotted to control protein loading. The image is representative of three independent experiments. **B:** Overexpression of NRSF/REST transcription factor reduces endogenous α -internexin, SNAP25, synaptophysin, and UCHL1 mRNA levels. REEX1 overexpression was performed in triplicate (six-well plates) in three independent experiments. The mRNA levels of all of the analyzed genes were detected by TaqMan PCR, and the endogenous control used was GUSB. AU, arbitrary units (mean $\pm$ SD). * $P < 0.05$ and ** $P < 0.01$ compared with nontransfected cells (analysis of variance with post hoc LSD test).

tophylin and the products of putative target genes UCHL1 and α -internexin. For this purpose, a human lung carcinoma DMS53 cell line was transiently transfected with the REEX1 vector, coding for human full-length NRSF cDNA. Endogenous NRSF/REST protein levels were increased at 48 hours in DMS53 cells (Figure 6A). This was accompanied by a reduction of 27% in the expression levels of UCHL1 mRNA ($P < 0.01$, analysis of variance with post hoc LSD test) (Figure 6B). As to positive controls, we detected that NRSF overexpression reduced synaptophysin mRNA levels by 35% ($P < 0.01$, analysis of variance with post hoc LSD test) and SNAP25 mRNA levels by 25% ($P < 0.05$, analysis of variance with post hoc LSD test) (Figure 6B), as previously described.^{28,30} Finally, α -internexin mRNA levels were reduced by 17% at 48 hours after NRSF transfection ($P < 0.05$, analysis of variance with post hoc LSD test).

NRSF siRNAs Increase UCHL1 and Synaptophysin mRNA Levels in U87-MG Cells

We also tested the effect of NRSF siRNA transfection in U87-MG cells. As shown in Figure 7, A and B, endog-

Figure 7. NRSF/REST siRNA transfection increases UCHL1 mRNA levels in U87-MG cell line. **A:** NRSF/REST protein levels, as detected by Western blot, are decreased after 48 hours of NRSF/REST siRNA transfection (siRNA 1 and siRNA 2). The scramble siRNA transfection does not modify the expression of endogenous NRSF/REST levels. β -Actin (45 kd) was blotted to control protein loading. **B:** Similarly, NRSF/REST mRNA levels are reduced after siRNA 1 and siRNA 2 transfection. Reduction of NRSF/REST transcription factor increases endogenous UCHL1 (**C**) and synaptophysin (**D**) mRNA levels. siRNA transfection was performed in triplicate (six-well plates) in three independent experiments. The mRNA levels of all of the analyzed genes were detected by TaqMan PCR, and the endogenous control used was GUSB. AU, arbitrary units (mean $\pm$ SD). * $P < 0.01$ and ** $P < 0.001$ compared with nontransfected cells (analysis of variance with post hoc LSD test).

enous NRSF protein and mRNA levels were reduced after transfection with NRSF siRNA 1 and siRNA 2 but remained unchanged with scramble siRNA ($P < 0.01$, analysis of variance with post hoc LSD test). Reduction of NRSF expression was accompanied by increased

expression of endogenous UCHL1 mRNA levels ($P < 0.01$, analysis of variance with post hoc LSD test). No changes were found after transfection of scramble siRNA (Figure 7B). Transfection of siRNA 1 also up-regulated endogenous synaptophysin mRNA levels ($P < 0.001$, analysis of variance with post hoc LSD test) (Figure 7C).

NRSF Interacts Directly with the UCHL1 Promoter

In silico analysis of UCHL1 gene promoter sequence was examined using the MatInspector software.³¹ The analysis predicted three potential functional NRSEs, which are the binding sites for NRSF. NRSE1 was located in the complementary DNA chain, upstream from the transcription start site between positions -121 and -101 bp (Figure 8A). NRSE2 and NRSE3 were located in the intron 1, in the coding and complementary chains, respectively. Both NRSEs were located very close together, separated only by 11 bp. The sequence of all NRSEs and the consensus sequence are shown in Figure 8B. As the figure shows, NRSE1 and NRSE3 have a very high homology with the murine corresponding sequence.

To examine the interaction of NRSF with the UCHL1 gene promoter ChIP, assays were performed in U87-MG, HeLa, and DMS53 cells. After cross-linking of proteins and DNA with formaldehyde, sonicated cell lysates from each cell line were subjected to immunoprecipitation with the goat polyclonal anti-NRSF antibody. The precipitated DNA fragments were amplified with a set of primers that spanned a 247-bp region covering the NRSE1 site of the UCHL1 gene promoter. In U87-MG and HeLa cells, ChIP PCR products were detected with the NRSF antibody but not with a goat serum used as a negative control (Figure 8C, lanes 4, 5, and 7). These results show that NRSF binds to the NRSE regions of UCHL1 gene promoter in both cell lines. The same analysis performed in DMS53 cells revealed the absence of binding of NRSF to NRSE1 of UCHL1 gene promoter, because no PCR amplification was obtained using the same set of primers in the anti-NRSF DNA immunoprecipitate (Figure 8C, lane 6). To assure that ChIP analysis was performed in DMS53 cells, the acetyl-histone 3 antibody was used as positive control (Figure 8D). These findings correlate with NRSF protein levels found in each of the cell lines. NRSF protein levels were absent in DMS53 cells but high in U87-MG and even higher in HeLa cells (Figure 8E). In accordance with previous observations described in this study, UCHL1 mRNA levels were very high in NRSF-negative DMS53 cells, lower in U87-MG, and undetectable in NRSF-positive HeLa cells (Figure 8F).

NRSF Reduces α -Internexin Protein Levels

A similar *in silico* analysis approach was not possible for α -internexin, because the sequence of human α -internexin promoter is not available in GenBank. However, transient overexpression of NRSF in human neuroblastoma SH-SY5Y cells was accompanied by a significant reduction in α -internexin protein levels ($P <$

0.05, analysis of variance with post hoc LSD test) (Figure 9). Therefore, these preliminary data suggest that NRSF regulates α -internexin expression.

Discussion

NRSF/REST is a Kruppel-type zinc-finger transcription factor that binds to a 21- to 23-bp consensus DNA se-

Figure 9. NRSF/REST overexpression reduces endogenous levels of neuronal α -internexin in human SH-SY5Y cells. **A:** One μ g of REEX1 vector was transfected in SH-SY5Y cells using Lipofectamine 2000. NRSF/REST protein levels were increased after 48 hours of REEX1 vector transfection as shown by Western blot. β -Actin was detected to control the protein loading. Overexpression of NRSF/REST transcription factor reduces endogenous α -internexin protein levels. **B:** Densitometric analysis of NRSF/REST and α -internexin protein levels (mean $\pm$ SD). REEX1 overexpression was performed in triplicate (six-well plates) in three independent experiments. AU, arbitrary units. * $P < 0.01$ compared with nontransfected cells (analysis of variance with post hoc LSD test).

quence called the *NRSE*. NRSF/REST was originally identified as a regulator of the sodium channel type 2 and SCG10 genes; it is defined as a neuronal gene repressor

Figure 8. NRSF/REST binds to a NRSE binding site located at the minimal UCHL1 gene promoter in HeLa and U87-MG cell lines. **A:** Schematic representation of UCHL1 gene promoter. White boxes represent three putative DNA-binding sites for NRSF/REST (NRSE) detected by MatInspector software. NRSE1 and NRSE3 are located in the complementary DNA chain (-) and the NRSE2 in the positive DNA chain (+). Exons 1 and 2 are indicated as dashed boxes and intron 1 corresponds to gray boxes. The location of NRSE1, NRSE2, and NRSE3 sites is indicated relative to TATA signal located at position 268 of the sequence with GenBank no. X17377. The transcription start site is shown with an arrow. **B:** The consensus sequence for NRSE site is indicated. DNA sequences for the three NRSE sites found in UCHL1 gene promoter are indicated as well as their alignment with the murine homolog. Nucleotides conserved in relation to consensus NRSEs are underlined, and those conserved in relation to the murine homolog are dashed with a gray box. **C:** ChIP assay was performed in U87-MG, HeLa, and DMS53 cells using a goat anti-human NRSF/REST antibody and with a goat serum as a negative control. Immunoprecipitated DNA was analyzed by PCR using a set of primers that amplified a 247-bp region of the UCHL1 gene promoter that encompasses an NRSE site (NRSE1). **Lane 1**, input, U87-MG cells; **lane 2**, input, HeLa cells; **lane 3**, input, DMS53 cells; **lane 4**, ChIP with anti-NRSF/REST, U87-MG; **lane 5**, ChIP with anti-NRSF/REST, HeLa; **lane 6**, ChIP with anti-NRSF/REST, DMS53; and **lane 7**, ChIP goat serum, U87-MG. Input refers to DNA chromatin not immunoprecipitated with the specific antibody. ChIP refers to DNA chromatin immunoprecipitated with the specific antibody. **M**, 100-bp DNA ladder marker. **D:** ChIP assay was performed in DMS53 cells using a rabbit polyclonal anti-human acetyl-histone 3 (Lys9) antibody as positive control. **Lane 1**, input; **lane 2**, ChIP. **E:** NRSF/REST and UCHL1 protein levels detected by Western blotting in DMS53 (**lane 1**), U87-MG (**lane 2**), and HeLa (**lane 3**) cell lines. The figure shows the densitometric analysis of NRSF/REST protein levels normalized with β -actin. AU, arbitrary units. **F:** UCHL1 mRNA levels normalized with GUSB. The detection was performed with TaqMan PCR as indicated in Materials and Methods. The results are representative of three independent experiments.

in non-neuronal cells.^{26,27} NRSF/REST mediates active repression via two independent domains, one encompassed by the N-terminal 83 residues and the other by the C-terminal zinc finger.³² The N-terminal domain recruits the mSin3A/B histone deacetylase complex, and the C-terminal domain recruits a distinct HDAC complex via its interaction with REST cofactor (CoREST).^{33,34} The role of NRSF in nervous system development is closely related to its expression, which is lower in neural stem/progenitor cells than in pluripotent stem cells and which becomes minimal in postmitotic neurons, whereas it is highly expressed in adult non-neuronal tissues.³⁵ NRSF/REST knockout mice show precocious neuronal differentiation.³⁶ Conversely, overexpression of NRSF/REST in developing chick spinal cord neurons causes repression of neuronal-specific gene expression and significantly increases the frequency of axon guidance errors.³⁷ NRSF/REST binds to a large number of genes encoding fundamental neuronal traits such as ion channels, synaptic vesicle proteins, and neurotransmitter receptors.³⁸ In this line, previous studies have shown that synaptophysin and SNAP25 are NRSF/REST target genes.^{28,30,39} Functional assays performed in the present study have shown that up-regulation of NRSF/REST correlates with down-regulation of SNAP25 and synaptophysin and that down-regulation of NRSF after siRNA transfection is associated with increased synaptophysin expression in cell lines.

The present study has shown that mRNA and protein NRSF/REST levels are reduced in myotilinopathy muscles when compared with control muscles and that this is accompanied by aberrant expression levels of several neuronal proteins such as UCHL1, SNAP25, synaptophysin, and α -internexin.^{21,22,40,41} Abnormal expression of neuronal genes is manifested as increased mRNA levels, increased protein levels, and occurrence of abnormal protein aggregates, which colocalize with certain muscle protein aggregates as myotilin deposits. Together, these findings show an inverse relation between NRSF/REST and neuron-related proteins and suggest that increased expression of certain noncanonical proteins in myotilinopathy is the result of abnormal NRSF/REST regulation.

The mechanisms that modulate *UCHL1* gene transcription are poorly understood. The gene is located at chromosome 4, spans 10 kb of genomic DNA, and consists of nine exons. The transcription start site is located 29 nucleotides downstream from the TATA box.^{42,43} Two conserved evolutionary sequences in the 5'-untranscribed region functioning as potential regulatory elements have been reported, whereas another study has demonstrated that the transcription factor B-Myb stimulates the expression of murine *UCHL1* gene in cultured cells and *in vivo*.^{44,45} Finally, 35 CpG sites have been shown to be present in the *UCHL1* gene promoter, spanning the putative transcription start site and exons 1 and 2; the gene promoter was fully methylated in non-*UCHL1*-expressed HeLa cells.⁴⁶

Although several genome-wide analyses of *NRSE* sites have been performed without identifying any *NRSE*s in the *UCHL1* gene promoter, we have identified three putative *NRSE* sites after *in silico* analysis of the minimal

UCHL1 gene promoter.^{28,38,47–49} After an orthologous alignment, we have shown that the three putative *NRSE* sites identified by MatInspector software in the *UCHL1* promoter present a different percentage of homology with the murine homolog sequence, with the *NRSE1* being the most conserved. It is worth stressing that a segment of *NRSE1* has previously been described as the PSN element in the *UCHL1* promoter region⁴⁴ and that a similar 12-bp sequence appears in the human and rat synapsin I and neuron-specific enolase gene promoters.^{50,51} Analysis of the *UCHL1* gene promoter by CAT assay and electrophoretic mobility shift assay have shown that the PSN element has a functional role regulating the expression of *UCHL1* gene contributing to neuron-restricted transcription.⁵² Alignment of the PSN element and *NRSE1* shows that PSN perfectly matches part of the *NRSE1* site identified here.

Interestingly, a recent analysis has revealed that among the 895 candidate genes to contain *NRSE* sites, more than 60% have *NRSE* sites within 20 kb of their transcriptional starts, whereas a few are located more than 150 kb away from genes.⁴⁸ We have identified one *NRSE* site around the first 100 bp from the transcription start site, whereas the other two *NRSE* sites are located in the first intron of *UCHL1* gene.

In the present study, we have demonstrated that NRSF/REST regulates the expression of UCHL1 by several functional assays. Transient overexpression of NRSF reduces endogenous levels of UCHL1 in DMS53 cells, whereas NRSF siRNA transfection increases UCHL1 expression in U87-MG. Furthermore, ChIP analysis has revealed NRSF interaction with *UCHL1* gene promoter in UCHL1-expressing U87-MG cells. Based on these findings, it can be suggested that NRSF acts as a repressor, not as a silencer, of *UCHL1* gene expression, as has been previously suggested.⁵³ However, further analysis, such as electrophoretic mobility shift assays, must be performed to demonstrate fully that the three *NRSE* sites identified in the *UCHL1* minimal promoter are functional.

In relation to α -internexin, we have here described for the first time how overexpression of NRSF/REST in DMS53 and SH-SY5Y cells reduces the expression levels of α -internexin. However, further studies must be done to determine whether NRSF regulates α -internexin expression by a direct interaction with its promoter or by an indirect pathway.

Knowledge of the mechanisms leading to NRSF/REST deregulation in myotilinopathy seems crucial in this setting. It has been suggested that a set of brain-related miRNAs are likely under the control of NRSF/REST, and there is evidence for a double-negative feedback loop between NRSF/REST complex and brain-related micro RNAs.^{48,49,54} Future studies are necessary to gain understanding about the role of micro RNA in relation to aberrant NRSF/REST signaling in muscles of patients suffering from myotilinopathy. Whether abnormal expression of neuronal proteins contributes to protein aggregation in myotilinopathies deserves further work.

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