Modulation of nuclear REST by alternative splicing: a potential therapeutic target for Huntington’s disease

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Abstract

Huntington’s disease (HD) is caused by a genetically mutated huntingtin (mHtt) protein with expanded polyQ stretch, which impairs cytosolic sequestration of the repressor element-1 silencing transcription factor (REST), resulting in excessive nuclear REST and subsequent repression of neuronal genes. We recently demonstrated that REST undergoes extensive, context-dependent alternative splicing, of which exon-3 skipping (ΔE3)—a common event in human and nonhuman primates—causes loss of a motif critical for REST nuclear targeting. This study aimed to determine whether ΔE3 can be targeted to reduce nuclear REST and rescue neuronal gene expression in mouse striatal-derived, mHtt-expressing STHdh Q111/Q111 cells—a well-established cellular model of HD. We designed two morpholino antisense oligos (ASOs) targeting the splice sites of Rest E3 and examined their effects on ΔE3, nuclear Rest accumulation and Rest-controlled gene expression in STHdh Q111/Q111 cells. We found that (1) the ASOs treatment significantly induced ΔE3, reduced nuclear Rest, and rescued transcription and/or mis-splicing of specific neuronal genes (e.g. Syn1 and Stmn2) in STHdh Q111/Q111 cells; and (2) the ASOs-induced transcriptional regulation was dependent on ΔE3 induction and mimicked by siRNA-mediated knock-down of Rest expression. Our findings demonstrate modulation of nuclear REST by ΔE3 and its potential as a new therapeutic target for HD and provide new insights into environmental regulation of genome function and pathogenesis of HD. As ΔE3 is modulated by cellular signalling and linked to various types of cancer, we anticipate that ΔE3 contributes to environmentally tuned REST function and may have a broad range of clinical implications.

Keywords: REST/NRSF • alternative splicing • nuclear translocation • gene therapy • Huntington’s disease • antisense oligos • Syn-1 • Stmn2 • PPARγ

Introduction

Originally identified as a transcriptional repressor of neuronal genes, the repressor element-1 silencing transcription factor (REST, also named NRSF for neuron-restrictive silencing factor) is now recognized as a coordinate transcriptional and epigenetic regulator that orchestrates cellular epigenome [1,2]. REST contains a DNA-binding domain consisting of eight zinc fingers (ZFs), which is sandwiched by two repression domains capable of recruiting numerous transcriptional and epigenetic cofactors, through which REST promotes dynamic, context-dependent chromatin remodelling and repression or activation of thousands of genes [2,3]. As such, REST controls many cellular processes fundamental to normal physiology and pathological conditions and is implicated in a wide range of human diseases including cancer and neurodegenerative diseases.

The physical separation of the genome from cytoplasm by nuclear envelope in eukaryotic cells requires translocation of REST from cytoplasm to nucleus to modulate genome function. It was documented...
that ZF-5 is critical for REST nuclear targeting, and that altered nuclear REST is implicated in adenovirus-induced cell transformation, ageing and neurodegenerative diseases including Huntington’s and Alzheimer’s diseases (HD and AD). Notably, a protein named huntingtin (Htt) associates with REST through a cytoskeletal complex which prevents nuclear translocation of REST; however, Htt is mutated in HD, leading to impaired cytosolic sequestration of REST and excessive accumulation of nuclear REST, which in turn represses neuronal genes important for the maintenance and function of specific neurons [8–10]. Accordingly, rescue of neuronal gene expression through modulation of REST activity has been suggested as a therapeutic strategy for HD [11,12].

As a multi-exonic gene, REST undergoes alternative splicing, a cotranscriptional process which enables a single gene to produce multiple mRNA and protein variants, with an N-terminal REST4 isoform having been well documented [8,13]. Recently, we identified 45 REST mRNA splice variants with highly context-dependent expression, suggesting an underappreciated role of alternative splicing in modulation of REST function [14]. Particularly, skipping of exon-3 (ΔE3), a common splicing event which eliminates ZF-5 critical for REST nuclear targeting, is linked to cancer and modulated by pioglitazone—a highly selective activator of the peroxisome proliferator-activated receptor gamma (PPARγ) exerting biological actions overlapping with REST [15–17]. Hence, we suggested that ΔE3 may act as an endogenous, manipulable modulator of REST activity, and it may be targeted to treat HD related to REST dysfunction.

To test this hypothesis, we determined whether manipulation of ΔE3 alters nuclear REST and neuronal gene expression in a cell model of HD. We designed two antisense oligos (ASOs) targeting the splice sites of Rest ΔE3 and examined their effects on ΔE3, nuclear Rest and neuronal gene expression in STHdhQ111/Q111 cells. We demonstrated that treatment of STHdhQ111/Q111 cells with the ASOs significantly induced ΔE3, reduced nuclear Rest and rescued transcription and/or mis-splicing of specific neuronal genes and that the ASOs-induced transcriptional regulation was dependent on ΔE3 induction while mimicked by siRNA knock-down of Rest. Our findings validate ΔE3 as a modulator of nuclear REST and a potential therapeutic target for HD and provide new insights into HD pathogenesis.

Immunofluorescence confocal microscopy and image analysis

Two widely used anti-REST antibodies, sc-25398 (Santa Cruz, Dallas, TX, USA) and ab21635 (Abcam, Cambridge, MA, USA), were employed to perform immunocytochemistry (ICC). Briefly, cells cultured on poly-D-lysine-coated coverslips were treated with ASOs (I2E3 + E3I3) or a control oligo. After 48-hr incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and incubated with sc-25398 (1:100) or ab21635 (1:200), followed by incubation with a goat anti-rabbit secondary antibody conjugated with Alexa dye (1:500, Invitrogen). Nuclei were stained with Hoechst-33342 (Thermo Scientific, Waltham, MA, USA), and cells were mounted on glass slides.

For confocal microscopy, image stacks along the z-axis were acquired using a Leica TCS SP5 Spectral Confocal Microscope (Leica Microsystems, Cambridge, UK). Image acquisition settings were kept the same for all scans in the same experiment group when fluorescence intensity was compared. Images were analysed using ImageJ program (NIH). Fluorescence intensities measured as integrated pixel intensities were determined for the entire cell and its nucleus area, respectively. Nucleus percentage of fluorescence intensity was calculated for each cell, and values of 100 cells in each group were averaged and presented as Mean ± S.E.M. All groups to be compared were run simultaneously using cells from the same culture preparations.

RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol® reagent (Invitrogen) and reverse transcribed into cDNA using Superscript™ III reverse transcriptase and oligo-dTs (Invitrogen). To avoid DNA contamination, samples were treated with RNase-free DNase I (Promega, Madison, WI, USA) for 1 hr at 37°C. Synthesized cDNA was diluted to 50 ng/μl for use.

Polymerase chain reaction (PCR)

For detection of ΔE3, nested PCR was performed as previously described [14] in a MJ Research PTC-200 Peltier Thermal Cycler using Rest-E1F2/E4R3 and Rest-E2F2/E4R1 (or -E1F1/E4R1) (Table 1) as the primer set for 1st- and 2nd-round amplification, respectively. PCR with Syn1-E1F2/E4R1 was performed to verify Syn1 variants with/-out intron-8 (I8). PCR products were electrophoresed on 2% agarose gel, excised, purified and sequence verified. PCR sequencing was serviced by Functional Biosciences (Madison, WI, USA).

Quantitative real-time PCR (qRT-PCR)

SYBR Green qRT-PCR for Bdnf, Stmn2, Syn1, Gapdh, Nmnat2, Lrp11, Rtn2 and specific Rest variants was performed using primers listed in...
Table 1 Oligos used for PCR, ΔE3 manipulation and RNAi

| Name        | Sequence (5’ to 3’) | Note                        |
|-------------|---------------------|-----------------------------|
| Regular PCR |                     |                             |
| Rest-E1F2   | CCTGACGCCCAACTTTTCC |                             |
| Rest-E2R3   | CACATAATTGCATGATCACTTTA |                         |
| Rest-E1F1   | CGAACAATCCGAGAAAGA   |                             |
| Rest-E2F2   | CACTCGAGCAATGCAACTTTA |                             |
| Rest-E1R1   | CTGCACGTGACATTTAAATG |                             |
| Syn1-E1F1   | CTAGCCCTTATTGAGTCTGCT |                      |
| Syn1-E2R1   | CACAGGCAGATGCTCAAGTC |                             |
| gRT-PCR (SYBR Green I) |               |                             |
| Rest-E2/F1  | ACCACTGGAGAAGAACACTGT | For E2/E3 (ΔE3) variant          |
| Rest-E2/R1  | TTAAATGCTTACACTGTGCT |                             |
| Rest-E3F1   | AACTCATACGAGGAAGCC   | Paired with Rest-E1R1        |
| Syn1-E3F1   | GAGCAATGCTCCATGCTGCA | Paired with Syn1-E2R1        |
| Syn1-E3R1   | GCCAATGGTGGATTCTCTGT | For global Syn1              |
| Syn1-E3R1   | CAGCCCAATGACAAACTCTC |                             |
| Syn1-E3F2   | TGTCGGTTAACTGGAAAGACC | For E3/I3 variant            |
| Syn1-E3R1   | GCTTGATTGCTTGGCTGCTA |                             |
| Bdnf-F1     | TGAGCTTGGTTAGCAGAGCA |                             |
| Bdnf-R1     | TTGGTAAACGGCACAAAACA |                             |
| Stmn2-F1    | GCAATGGCCTACAAGGAAAAA |                          |
| Stmn2-R1    | GTGGCTCTGAGATGCTGCTA |                             |
| Gapdh-F3    | GAACGGATTTGGCCGTACGGG |                          |
| Gapdh-R3    | TGGCTTCTGAAGATGCGTGT |                             |
| Rtn2-F1     | GACTCGGAGACGACAGAGACAC |                    |
| Rtn2-R1     | TGCAAGATGCTAAGCGTTG |                             |
| Lrp11-F1    | AGGCACACTACCAACAACAC |                             |
| Lrp11-R1    | AAAACAGTGATGCGCAAGACC |                             |
| Nmnat2-F1   | ACCATGGGAAGATGCTATACAG |                      |
| Nmnat2-R1   | CAGGTGCTGAGAAGGTTG |                             |
| Morpholino oligos |                   |                             |
| I2E3        | GGGTTTCTCTGCAAAGTCTAAAT |                     |
| E3I3        | CGACGTACACCTTACACACCGAA |                     |

Table 1. Continued

| Name        | Sequence (5’ to 3’) | Note                        |
|-------------|---------------------|-----------------------------|
| Control     | CCTTTAATCTTACATAATTTA |                             |
| Trilencer-27 siRNA duplex |     |                             |
| siRNA-1     | rCCrGrArUrArArUrArUrGrArArUrUrGrUrCCT | Target Rest E3 |
| siRNA-2     | rArGrArCrArGrArUrArUrGrArArUrUrGrUrG | Target Rest 3’-UTR |

PCR, polymerase chain reaction. (1) Rest-E1F2 paired with -E3R3 were used for 1st round of nested PCR, while REST-E4R1 paired with -E3F2 or -E3F2 were used for 2nd round of nested PCR; (2) Syn1-E7F1 paired with -E9R1 were used for verification of I8 retention in mRNA.

Transcriptional profiling

Three total RNA samples of high quality (RNA integrity number: 8.6–9.4) isolated from cells treated with ASOs (I2E3 + E3I3) (Q111-ASOs) or a control oligo (Q111- and Q7-Control) were transcriptionally profiled by the Affymetrix’s GeneChip Mouse Gene 2.0 ST Array serviced by EpigenDx (Hopkinton, MA, USA). Pairwise comparisons of expression between the samples were performed by analysis of variance (ANOVA) using the Partek Genomic Suite software (St. Louis, MO, USA). The heat map was generated by hierarchical clustering of expression values using the genes generated by ANOVA comparisons between the samples. The mean expression is shifted to 0 and scaled to a S.D. of 1.

Western blotting for Stmn2

Cytoplasmic protein fraction was extracted using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific), and protein concentration was determined by Bradford assay. The same amount (40 μg) of protein was boiled with 2× Laemmll buffer, electrophoresed on a 10% SDS-PAGE gel and electrotranslocated onto an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA) presoaked in methanol. The membrane was blocked with 5% non-fat milk and incubated with a goat anti-STMN2 (SAB2500997) (Sigma-Aldrich, St Louis, MO, USA) at 1:500 overnight at 4°C, followed by incubation with a rabbit anti-goat IgG (A5420; Sigma-Aldrich). Immunoreactive signals were detected using the VisiGlo Select HRP Chemiluminescent Substrate Kit (Amresco, Solon, OH, USA) with an ECL-based LAS-3000 image system (Fujifilm, Life Science, New Haven, CT, USA). Densitometric analysis was carried out using Image-Gauge (Fujifilm). Assays were performed on three independent occasions.
ELISA for Bdnf

Cells were lysed using the lysis buffer consisting of 10% glycerol, 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1 mM EGTA supplemented with 1:100 Halt™ Protease Inhibitor cocktail (Thermo Scientific). Samples were homogenized, sonicated and centrifuged at maximum speed for 10 min. at 4°C, and the supernatants were collected and stored at −80°C. Samples were assayed using a mouse Bdnf ELISA Kit (Boster, Fremont, CA, USA). Assays were performed in triple on three independent occasions.

Bioinformatics and data analysis

Bioinformatics were performed by checking specific tracks provided by the UCSC Genome Browser (http://genome.ucsc.edu). Statistics were carried out using the SAS Software Version 9.4 (SAS Institute Inc., Cary, NC, USA). Comparisons of nuclear REST percentage and mRNA/protein expression level between different groups were performed by appropriate ANOVA or Student’s t-test.

Results

Induction of ΔE3 by ASOs targeting the splice sites of E3

We designed two ASOs (ΔE3+ and ΔE3−, Fig. 1A) targeting the splice acceptor and donor sites of Rest E3, respectively. As shown in Figure 1B, both ASOs induced ΔE3 in STHDhQ7/Q7 and STHDhQ111/Q111 cells, as indicated by formation and up-regulation of the E3/E4 (ΔE3) and E3/E4 (ΔE2 + ΔE3) variants, respectively, as well as apparent reduction of E3-retained variants (E3/E3/E4 and E3/E3/E4). In both STHDhQ7/Q7 and STHDhQ111/Q111 cells, based on the ratio of variants with/-out ΔE3, ΔE3+ induced more ΔE3 than ΔE3− while combination of both STOs yielded the most ΔE3 induction, such is validated by qRT-PCR-assyayed expression of the E3/E4 variant (Fig. 1C) using primer set Rest-E3E4F1/R1 listed in Table 1. The ASOs also induced ΔE3 in mouse primary hippocampal neurons (implying a potential in vivo effect), along with cells of other species including human NCCIT and rat RN46A cells (Fig. S1A), which may be explained by the highly conserved intron–exon junctions of E3 as indicated by the ‘Vertebrate Multiz Alignment & Conservation’ track from the UCSC Genome Browser (data not shown).

ASOs-induced reduction of nuclear Rest in STHDhQ111/Q111 cells

To examine whether the ASOs reduced nuclear Rest in STHDhQ111/Q111 cells, we performed ICC with two antibodies (sc-25398 and ab21635) against the N- and C-terminal of REST, respectively. As shown in Figure 2 and Figure S2, regardless of the antibody, accumulation of nuclear Rest was significantly higher in STHDhQ111/Q111 than that in STHDhQ7/Q7, while ASOs treatment (ΔE3+ + ΔE3−) significantly reduced nuclear Rest in STHDhQ111/Q111 cells. ASOs-induced reduction in nuclear Rest was also observed in RN46A as indicated by ICC (Fig. S1B).

ASOs-induced transcriptional derepression of Rest target genes in STHDhQ111/Q111 cells

We then determined the effect of ASOs treatment on transcription of three well-documented REST target genes, Bdnf, Syn1 and Stmn2 (also named Scg10), in STHDhQ111/Q111 cells. To evaluate the relevance of ASOs-induced transcriptional regulation to altered Rest activity, we also examined regulation of the genes by two Rest siRNAs. As shown in Figure 3A, we found that (1) transcription of the three genes (especially Stmn2) was repressed in STHDhQ111/Q111 compared with STHDhQ7/Q7; (2) the ASOs treatment increased transcription of the genes depending on ΔE3 induction (ΔE3+/ΔE3− < ΔE3+/ΔE3− and ΔE3+/ΔE3−); that is, ΔE3 exerts a dose-dependent rescue of gene transcription; (3) ASOs-induced transcriptional regulation was mimicked by different two siRNAs which reduced Rest mRNA expression by ~70% as assayed by qRT-PCR with Rest-E3E4F1/E4R1; and (4) compared with Bdnf, Stmn2 and Syn1 showed greater repression in STHDhQ111/Q111 and more derepression by ASOs/siRNAs.

Unexpectedly, we identified a new Syn1 splice variant (Syn1-S, GenBank Accession No. KJ174470) with I2 retention during verification of qRT-PCR product for Syn1-E3E4F1/E4R1. As shown in Figure 3B and C, we found that Syn1-S was abundantly expressed in STHDhQ111/Q111 but barely in STHDhQ7/Q7, while its expression in STHDhQ111/Q111 was decreased by ASOs treatment in relation to ΔE3 induction and that the ASOs-induced rescue of Syn1 mis-splicing was mimicked by Rest siRNAs. Notably, I2 retention is predicted to introduce a pre-mature stop codon and therefore predictive of a truncated N-terminal Syn1-S protein isoform missing partial domains and phosphorylation sites.

To determine whether additional genes were transcriptionally derepressed by the ASOs, we performed gene expression array to compare global transcription between three representative samples (Q111-Control, Q111-ASOs and Q7-Control; GEO Accession No. GSE7194). As shown in Figure 3D and Table 2, global transcription differed significantly between STHDhQ7/Q7 and STHDhQ111/Q111 cells, while repression of numerous genes in STHDhQ111/Q111 was derepressed, at least in part, by ASOs treatment (ΔE3+/ΔE3−). With a two-fold change as the criteria for significance, the transcriptional data well supported the qRT-PCR assayed Stmn2 and Syn1 expression change and extended ASOs-induced transcriptional derepression to another 15 genes (Sarm1, Nmnat2, Kcnk3, Stmn3, Gnp1g, H2-T23, Ppm1e, Fbxl16, Lrp11, Ina, Gdap1 l1, Unc13a, Vgf, Rundc3a and Rtn2), of which Nmnat2, Lrp11 and Rtn2 were selectively confirmed by qRT-PCR with additional samples (data not shown).

ASOs-induced protein expression change of Rest target genes in STHDhQ111/Q111 cells

We also performed Western blotting and ELISA to evaluate ASOs-induced regulation of Stmn2 and Bdnf protein expression,
Fig. 1 Induction of Rest ΔE3 by specific ASOs in STHdh<sup>Q7/Q7</sup> and STHdh<sup>Q111/Q111</sup> cells. (A) Schematic structure of Rest gene and targeted splicing sites of the two ASOs (l<sub>E2</sub>E3 and l<sub>E3</sub>I<sub>3</sub>). Primers used for polymerase chain reaction (PCR) detection of ΔE3 are shown by arrows. (B) PCR verification of ASOs-induced ΔE3. Cells treated with the ASOs (l<sub>E2</sub>E3 and/or l<sub>E3</sub>I<sub>3</sub> 3 μM of each) or a control oligo were harvested for RNA isolation at 48 hrs post-treatment. Nested PCR was performed with E<sub>1</sub>F<sub>1</sub>/E<sub>4</sub>R<sub>3</sub> and E<sub>2</sub>F<sub>2</sub>/E<sub>4</sub>R<sub>1</sub> (or E<sub>2</sub>F<sub>2</sub>/E<sub>4</sub>R<sub>1</sub>) as the primer set for the 1st- and 2nd-round amplification, respectively. Amplicons were sequence verified, and ratio of the variants with/-out ΔE3 for each lane was analysed by GeneTools software (Syngene, Cambridge, UK). (C) Relative expression of the E<sub>2</sub>/E<sub>4</sub> (ΔE3) mRNA assayed by qRT-PCR with E<sub>2</sub>F<sub>2</sub>/E<sub>4</sub>R<sub>1</sub>. The E<sub>2</sub>/E<sub>4</sub> mRNA levels were expressed as folds over the control using Gapdh as the reference. Data are shown as Mean ± S.E.M. ANOVA: F = 165.54, P < 0.0001 for STHdh<sup>Q7/Q7</sup>; F = 32.76, P = 0.0006 for STHdh<sup>Q111/Q111</sup>. **P < 0.01, ***P < 0.001 compared with I<sub>E2</sub>E3 group; #P < 0.05 compared with I<sub>E2</sub>E3 + I<sub>E3</sub>I<sub>3</sub> group.

Fig. 2 Immunofluorescence analysis of STHdh<sup>Q7/Q7</sup> and STHdh<sup>Q111/Q111</sup> cells with or without ASOs treatment. ICC was performed on with P3 and P6 cells using the antibody sc-25398 (A) and ab21635 (B) against N- and C-terminal of REST, respectively. Note that the two ASOs were combined for the treatment. Percentage of nuclear REST was analysed by ImageJ, and values of 100 cells were averaged for each group and shown as Mean ± S.E.M. ***P < 0.001 compared with the control STHdh<sup>Q111/Q111</sup> group by Student’s t-test.
respectively. As shown in Figure 4A and B, compared with STHdhQ7/Q7, STHdhQ111/Q111 expressed significantly lower levels of Stmn2 and BDNF, of which Stmn2 protein expression was significantly increased by ASOs treatment (E3I3), while there is a tendency for the BDNF protein expression to be increased by the ASOs.

Discussion

As a major orchestrator of the cellular epigenome, REST governs the dynamic, context-dependent expression of a huge gene network; however, it is poorly understood how REST function is environmentally tuned to moderate the dynamic, context-dependent genome function. Emerging evidence indicates that pre-mRNA splicing is environmentally regulated through epigenetic mechanisms [23,24], suggesting a role of alternative splicing in environmentally tuned genome function fundamental to all aspects of cellular processes. In support of this notion, a shift from the full-length REST to a truncated REST4, which is caused by inclusion of an extra exon (N3c) introducing a premature stop codon, contributes to neurogenesis—a process for neural stem/progenitor cells to differentiate into neurons [25]. Recently, we demonstrated that REST undergoes extensive, context-dependent alternative splicing, suggesting a major role of alternative splicing in environmental modulation of REST function; however, functionalities of most splicing events and mRNA variants are yet to be determined albeit somewhat predictable. Using specific ASOs and a cellular model of HD, our present study validates mechanism by which a common splicing (ΔE3) modulates REST activity, as well as its potential as a therapeutic target for HD—a neurodegenerative disease associated with excessive nuclear REST.

ASOs are widely used for study of alternative splicing and may be utilized for gene therapy [26–28]. Just as expected, the two ASOs targeting RestE3 significantly induced ΔE3, reduced nuclear Rest and rescued neuronal gene expression in a cellular model of HD, while the
ASOs-induced transcriptional regulation was mimicked by siRNAs which considerably down-regulate Rest mRNA expression, suggesting a causative link from ASOs-induced ΔE3 to Rest activity. As ΔE3 does not occur naturally in rodents, our findings were unlikely confounded by endogenous ΔE3. So, as illustrated in Figure 5, this study provides evidence for ΔE3 as an endogenous modulator of REST activity as well as a potential therapeutic avenue for HD. As REST controls many cellular processes fundamental to normal physiology and disease aetiology, ΔE3 manipulation may have a broad range of clinical implications (e.g., cancer therapy and stem cell engineering). Notably, while the ASOs induced ΔE3 in both STHdhQ7/Q7 and STHdhQ111/Q111 cells, their effects on nuclear REST and gene transcription were only pronounced in STHdhQ111/Q111 but not in STHdhQ7/Q7 cells (data not shown), presumably due to a low level of basal nuclear Rest maintained by the normal nucleocytoplasmic shuttling in STHdhQ7/Q7 cells. Also, it should be pointed out that although both ΔE3 induction and RNAi down-regulate Rest function, their effects are mediated by different mechanisms. While RNAi reduces global expression of Rest isoforms including the full-length Rest, ΔE3 induction does not affect total Rest expression but alters ratio of two types Rest isoforms discriminated by the capability of being transported into nucleus. Hence, a specific Rest isoform (e.g. the full-length Rest) might be differentially

| Symbol | Fold change | Gene function |
|--------|-------------|---------------|
| Sarm1  | 2.1/2.2     | Negative regulator of MYD88/TRIF-dependent Toll-like receptor signalling pathway which plays a pivotal role in activating axonal degeneration following injury; Also involved in immune response. |
| Nmnat2 | 6.3/2.2     | Catalyses an essential step in NAD (NADP) biosynthetic pathway |
| Kcnk3  | 4.0/2.2     | An outwardly rectifying channel sensitive to changes in extracellular pH |
| Stmn2  | 27.3/2.2    | A member of the stathmin family of phosphoproteins; Regulator of microtubule stability and neuronal growth. |
| Gnptg  | 2.3/2.3     | Catalyses the first step in synthesis of a mannose 6-phosphate lysosomal recognition marker; Necessary for targeting of lysosomal hydrolases to the lysosome. |
| Stmn3  | 7.4/2.3     | A member of the stathmin protein family which form a complex with tubulins; Involved in microtubule formation and function. |
| H2-T23 | 2.3/2.3     | Involved in immune response |
| Ppm1e  | 3.9/2.4     | D Dephosphorylates and inactivates multiple substrates including serine/threonine-protein kinase 1, AMPK and the multifunctional calcium/calmodulin-dependent protein kinases |
| Fbxl16 | 3.9/2.5     | Functions in protein ubiquitination |
| Gdap1  | 4.1/2.7     | Likely functions in neuron differentiation; Associated with neuroblastoma. |
| Syn1   | 6.7/3.0     | Plays a role in regulation of axonogenesis, synaptogenesis and neurotransmitter release |
| Ina    | 2.4/3.1     | Facilitates axonal neurite elongation in neuroblastoma cells; Involved in morphogenesis of neurons |
| Lrp11  | 3.2/3.5     | Involved in multacellular organismal response to stress |
| Unc13a | 6.8/3.6     | Binds to phorbol esters and diacylglycerol; Plays a role in neurotransmitter release at synapses |
| Vgf    | 6.4/3.7     | Plays a role in maintenance of organismal energy balance and hippocampal synaptic activity |
| Rundc3a| 9.3/3.9     | Regulator of guanylate cyclase activity |
| Rtn2   | 10.0/6.1    | Plays a role in generation of tubular endoplasmic reticulum and intracellular vesicular transport |

Q7/Q111—relative mRNA expression in STHdhQ7/Q7 over STHdhQ111/Q111 (both were treated with a control oligo). ASOs/Ctrl—relative mRNA expression in ASOs-treated STHdhQ111/Q111 over control STHdhQ111/Q111.
influenced by the two approaches. For this reason, and considering the potential absence of correlation between expression levels of mRNA and protein [30], the effects of ΔE3 induction and RNAi on Rest target gene transcription may not be simply determined by the observed alteration in Rest expression. However, based on the ΔE3-dependent effects of the ASOs on specific gene transcription (Fig. 3), a threshold along with a plateau likely exists for the transcriptional response to down-regulation of Rest function. Presumably, once the plateau (i.e. maximum derepression) is achieved, further down-regulation of Rest function will no longer increase transcriptional response, such may explain our observation of a similar transcriptional change yielded by ΔE3 induction and RNAi regardless of the different down-regulation of REST by the two approaches.

Although reduced BDNF expression has been documented in HD [31], it was reported that BDNF levels in human blood are not informative nor reliable as HD biomarkers [32]. In accordance, we found that transcription of Stmn2 and Syn1, but not Bdnf, was quite vulnerable to altered REST activity (i.e. up-regulation by mHtt and down-regulation by ASOs/siRNA), suggesting that Stmn2 and Syn1 may have advantage over Bdnf as potential HD biomarkers. Syn1 controls synapse function which is dysregulated in HD [33,34], with abnormal phosphorylation of Syn1 being implicated in neurotransmission impairment in R6/2 HD mice [35]. By identifying a REST-controlled Syn1-S isoform predictive of loss-of-function, this study provides a new mechanism underlying synapse dysfunction in HD. As alternative splicing is epigenetically regulated while REST orchestrates cellular epigenome, it is not surprising that pre-mRNA splicing of Syn1 (and probably other genes) is modulated by REST. Stmn2 functions in microtubule stability and neuronal growth, and given that decreased Stmn2 expression has been implicated in injury-induced axonal degeneration [36], Down’s syndrome [37], and AD [38], the striking repression of Stmn2 in HD cells suggests a common Stmn2 dysfunctions in a certain number of neurodegenerative diseases.

Besides Bdnf, Stmn2 and Syn1, transcriptional profiling revealed ASOs-induced derepression of other genes in STHdhQ111/Q111 cells. Despite the limited sample size, reliability of the transcriptional data was supported by several lines of evidence: (1) It is in good consistence with qRT-PCR data for the tested genes; (2) bioinformatics indicates that almost all the transcriptionally regulated genes harbour at least one RE-1 element; and (3) majority of the transcriptionally regulated genes are reportedly modulated by REST [39–41] and have been implicated in neurodegenerative diseases. For example, Sar1 and Nmnat2 contribute to axonal degeneration—a critical, early event in neurodegenerative diseases [42,43], while Unc13a, Vgf, Rtn2, Lrp11 and Rundc3a have been implicated in amyotrophic lateral sclerosis [44], frontotemporal dementia [45], hereditary spastic paraplegias [46], and Parkinson’s disease and AD [47–49]. Thus, dysfunction of numerous specific genes is shared by neurodegenerative diseases, which may be explained by common pathogenic processes (e.g. axonal degeneration, endoplasmic reticulum stress and apoptosis) in such diseases. It should be pointed out that while only three samples were transcriptionally profiled, just a single dose and treatment duration of ASOs was examined in this study, making it possible that transcriptional regulation of some REST target genes cannot be observed in this study. Nevertheless, we demonstrate that repression of specific neuronal genes in STHdhQ111/Q111, which is presumably caused by mHtt-induced excessive nuclear REST, can be rescued by ASOs-induced ΔE3 which reduces nuclear REST.

While increased nuclear REST and its neurotoxicity in HD were well documented [8–10] and supported by this study, Lu et al. [7] recently reported reduced nuclear REST in AD and neuroprotection of REST in ageing brain. This discrepancy may reflect the complexity of REST function under distinct pathophysiological conditions; however, considering the extensive alternative REST splicing, variable assays of REST might be attributable. Of the 45 REST mRNA variants we
previously identified, many are predictive of truncated protein products, of which REST4 is predicted by multiple mRNA variants [14], just like the case in rat [50]. Notably, it is recently reported that (1) for E2-lacking variants (e.g. E1a/E3/E4), an in-frame AUG in E3 may initiate translation of a C-terminal REST C isoform (XP_005265817) [51]; and (2) previously annotated noncoding RNAs with short ORF can encode small peptides [52,53], such might be the case for numerous REST variants (e.g. JX896962, JX896965 and JX896967). Hence, REST protein isoforms might be much more complex than we expected; however, not all the predicted REST protein isoforms have been experimentally verified, and due to post-translational modifications, they may not be observed as the predicted size by Western, making it challenging to determine whether an unexpected immunoreactive band is non-specific or a REST isoform. Due to the extensive alternative REST splicing, it can be inferred that assay of REST by different primers/probes and antibodies may target different REST isoforms and therefore yield variable results. In support of this notion, the two anti-REST antibodies used in this study yielded distinct immunostaining profiles (Fig. 2 and Fig. S1) and different immunoreactive bands in accordance with the manufacturer’s instruction (data not shown). In the study of Lu et al. [7], only the full-length REST was actually considered; however, qRT-PCR with four primer sets targeting different exons of REST yielded different expression levels, providing evidence for the existence of alternative REST splicing [54]. Notably, Lu et al. performed immunostaining with multiple anti-REST antibodies without considering differences between the antibodies and disclosing detailed usage of the antibodies, making it possible that nuclear REST differences between the experimental groups might be confounded by biased usage of the antibodies for samples of different groups [54].

RESTΔ: REST isoform missing ZF-5
MeCP2: CpG methyl DNA binding protein
Sin3a: SIN3 transcription regulator family member A

RE1: cis-element for REST
HDAC: histone deacetylase
CoREST: REST corepressor

Fig. 5 Illustration of ΔE3 as an endogenous, manipulable modulator of REST function. The splicing event ΔE3, which is common in human and non-human primates, results in RESTΔ protein isoform(s) missing ZF-5 critical for nuclear targeting and therefore modulates nuclear REST levels and genome function. Note that (1) ΔE3 is modulated by PPARγ in a cell-dependent manner, presumably through a cis-element in E3; and (2) inclusion of E5 as the last exon, which is mutually exclusive to E4, was only observed in human but not rodents and non-human primates [14].
In summary, using specific ASOs which induces a common alternative splicing (ΔE3), we demonstrate that ΔE3 modulates nuclear REST and its gene regulation function in a cellular module of HD, and it thus represents a potential therapeutic target for HD. Our findings, which may extend to in vivo due to the ASOs-induced ΔE3 in mouse primary neurons, highlight the role of alternative splicing in modulation of REST function and provide new insights into environmental regulation of genome function as well as the pathogenesis of HD.

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Conflict of interest

All of the authors do not have any financial disclosures to report.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 The effect of ASOs on ΔE3 in additional cells (A) and nuclear REST in RN46A cells (B).

Fig. S2 Expanded view of immunofluorescence analysis of STHdhQ7/ Q7 and STHdhQ11/Q11 cells with/-out ASOs treatment.

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