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Human accelerated region 1 noncoding RNA is repressed by REST in Huntington's disease

Rory Johnson,[1] Nadine Richter,[1] Ralf Jauch,[2] Philip M. Gaughwin,[1] Chiara Zuccato,[3] Elena Cattaneo,[3] and Lawrence W. Stanton[1]

[1]*Stem Cell and Developmental Biology Group,* [2]*Laboratory of Structural Biochemistry, Genome Institute of Singapore, Singapore; and* [3]*Department of Pharmacological Sciences and Centre for Stem Cell Research, University of Milan, Milan, Italy*

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Johnson R, Richter N, Jauch R, Gaughwin PM, Zuccato C, Cattaneo E, Stanton LW. Human accelerated region 1 noncoding RNA is repressed by REST in Huntington's disease. *Physiol Genomics* 41: 269–274, 2010. First published February 23, 2010; doi:10.1152/physiolgenomics.00019.2010.—In the neurons of Huntington's disease (HD) patients, gene regulatory networks are disrupted by aberrant nuclear localization of the master transcriptional repressor REST. Emerging evidence suggests that, in addition to protein-coding genes, noncoding RNAs (ncRNAs) may also contribute to neurodegenerative processes. To discover ncRNAs that are involved in HD, we screened genome-wide data for novel, noncoding targets of REST. This identified human accelerated region 1 (HAR1), a rapidly evolving cis-antisense locus that is specifically transcribed in the nervous system. We show that REST is targeted to the HAR1 locus by specific DNA regulatory motifs, resulting in potent transcriptional repression. Consistent with other REST target genes, HAR1 levels are significantly lower in the striatum of HD patients compared with unaffected individuals. These data represent further evidence that noncoding gene expression changes accompany neurodegeneration in Huntington's disease.

neurodegeneration; regulation; RE1-silencing transcription factor; neuron-restrictive silencing factor; long noncoding RNA

THE FATAL AND INCURABLE NEURODEGENERATIVE condition Huntington's disease (HD) is caused by a trinucleotide expansion in the first exon of the ubiquitously expressed gene encoding huntingtin (Htt). The resultant mutant protein, mutHtt, carries a polyglutamine expansion and is neurotoxic. Intriguingly, in affected individuals the presence of mutHtt leads to specific death of medium spiny neurons in the striatum, although other brain regions and neurons are also affected (30). At a molecular level, HD brains are characterized by widespread changes in gene expression caused by several transcriptional mechanisms (8). In particular, diseased neurons suffer from widespread depression in neuronal-specific gene transcription caused by a mutHtt-dependent relocation of the transcriptional repressor REST (RE1-silencing transcription factor) into the nucleus (31). REST is a master regulator of neuronal gene expression programs: >1,000 genes are associated with genomic REST binding sites (9). One key target is the gene encoding the neurotrophic peptide BDNF, whose repression in the presence of mutHtt is likely to be one of the central pathological pathways underlying neurodegeneration (26, 30). However, many other REST target genes are similarly affected, and it is possible that they also contribute to HD pathology (31).

Address for reprint requests and other correspondence: L. W. Stanton, Genome Inst. of Singapore, 60 Biopolis St., Singapore 138672, Singapore (e-mail: stantonl@gis.a-star.edu.sg).

Numerous and diverse noncoding RNAs are expressed in the mammalian nervous system (19). Among the best understood class is the microRNAs, small 21 nt single-stranded transcripts that target protein-coding mRNAs for posttranscriptional repression (1). We previously employed genomic maps of REST binding sites to predict new gene dysregulation pathways in HD: we identified at least seven neuronal microRNAs that are targets of REST and that are consequently repressed in HD-affected striatum of both humans and mouse models (10, 11, 22). More recently, it has been shown that extensive transcription of poorly understood long mRNA-like transcripts with no protein-coding potential termed "long noncoding RNAs" (lncRNAs) takes place in the brain (20, 21). Evidence is emerging for potent functionality of such RNAs: *Evf2* was recently shown to be essential for correct development of hippocampal GABAergic neurons in mouse (3), while animals deficient for the dendritic ncRNA *BC1* have impaired survival in the wild (17). It has been proposed that lncRNAs are important, unexplored mediators of neurological disease (20). For example, it was recently shown that an lncRNA antisense gene regulates BACE1 levels in Alzheimer's disease patients (6).

In the present study, we use a genomic strategy to search for candidate lncRNAs in HD. We show that the neural RNA human accelerated region 1 (HAR1) is repressed by REST, and its level is significantly reduced in the brains of human patients. This implicates neural lncRNAs in HD for the first time.

MATERIALS AND METHODS

Human brain samples. The following tissue from normal and HD subjects cortex (Brodman area 7, 9) and striatum was analyzed in this study.

1) New York Brain Bank, Columbia University (New York, NY); all cortex [Brodman area (BA) indicated]. Controls: T-99 (BA9), T-111 (BA9), T-145 (BA9), T-168 (BA9); HD (grade 4): T-128 (BA9), T-310 (BA9), T-329 (BA9), T-354 (BA9).

2) Harvard Brain Tissue Resource Center (Belmont, MA); all striatum, except where indicated. Controls: 6182, 6142, 6002, 5959, 5936, 5919; HD (grade 3): 5570, 6010, 6183, 5576 (cortex, BA7); HD (grade 4): 5507, 6062.

3) Massachusetts General Hospital (Charlestown, MA); cortex, BA7. Control: 3932.

Genomic analysis. Human REST chromatin immunoprecipitation (ChIP)-seq data were obtained from Ref. 9. Mouse REST ChIP-PET data were those for mouse embryonic stem cells, mapped in Ref. 15. Repressor element 1 (RE1) motifs were identified in genomic DNA using the position weight matrix method described previously (13). Multispecies sequence alignments were obtained for a 200 bp window centered on each RE1 using the Galaxy web server (7) and then realigned using ClustalW2 from the EBI web server (http://www.ebi.ac.uk/Tools/clustalw2/).

Cell culture and gene knockdown. HEK293, HeLa, and 3T3 cells were cultured at 37°C in 5% CO_2, in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% nonessential amino acids. SiRNA knockdown was carried out as described in Ref. 14. Subconfluent cells were grown in 24-well plates and transfected 24 h after seeding with 0.05 nMol of REST Smartpool siRNA or nontargeting, scrambled siRNA (Dharmacon) using Dharmafect 2 reagent. Cells were harvested 48 h after transfection. To check the effectiveness of siRNA, we measured the levels of REST mRNA, as well as that of known target genes such as *Celsr3*, and observed decrease/increase respectively.

NS5 cells were grown as described in Ref. 15. The DN:REST transgene was introduced into the cells by means of an adenovirus (Ad DN:REST), as described in Ref. 29. Infection efficiency was monitored by green fluorescent protein (GFP) fluorescence from a second transgene carried by the adenovirus, and control cells were infected with adenovirus expressing GFP alone (Ad). RNA was harvested from cells 48 h following infection.

Mouse embryonic neural precursor cells (NPCs) were cultured according to a standard protocol as described in Ref. 16. Brains of embryonic day 13.5 embryos were dissected and cultured in nondifferentiating media: DMEM FF12, 5 U/ml penicillin/streptomycin, 1× nonessential amino acids, 1% (vol/vol) B27, 1% (vol/vol) N2, 20 ng/ml EGF, 20 ng/ml bFGF, 5 μg/ml heparin. After 3 days of culture, NPCs were transfected with siRNAs using the Amaxa system, according to manufacturer's standard nucleofection protocol for mouse neural stem cells (protocol DPG-1004 vs. 06-2004). Cells were divided into aliquots of five million cells each. Four aliquots each were transfected with 50 pMol of siREST (Smartpool, Dharmacon) or scrambled nontargeting sequence. Following transfection, cells were cultured for a subsequent 72 h prior to RNA harvesting.

Luciferase reporter assay. We synthesized a 50 bp double-stranded DNA containing each RE1 and cloned it into the pGL4 reporter plasmid (Promega) upstream of the constitutive thymidine kinase promoter driving luciferase expression [a kind gift of David Rodda, Genome Institute of Singapore (GIS)] (14). Each construct was verified by sequencing. HEK293 cells were seeded at low density into 24-well plates. The following day cells were at ~40% confluence, and 0.5 μg of reporter plasmid and 5 ng of the control Renilla-expressing plasmid, pSV40-Ren, were transfected in each well using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's protocol. Where indicated, we additionally transfected 0.5 μg of control GFP (pAd Track) or dominant-negative REST (pAdEASY DN:REST) expressing plasmids, by mixing with the reporter plasmids and transfecting with appropriately greater amounts of lipofection reagents. All transfection conditions were repeated for three independent cell wells. Forty-eight hours after transfection there was no evidence of cell death. Cells were treated using the Dual Luciferase Kit (Promega) according to manufacturer's protocol. Each biological replicate was divided and measured as two separate technical replicates in the luminometer. For each luminometer reading, the luciferase intensity was divided by that of renilla. The mean of pairs of technical replicates was calculated. For each triplicate of biological replicates mean and standard error were calculated. For each RE1 sequence tested, the fold repression was calculated with reference to a control plasmid lacking any RE1 sequence.

ChIP. ChIP was carried out according to the protocol described in Refs. 5, 14 using the Upstate anti-REST antibody (catalog: 07-579). ChIP enrichments were detected using real-time quantitative PCR using the following primers: human HAR1 RE1 (CAGAAGCTGGGTTTCATCTG,TTGGGGGATAAAGCACTCTG), mouse HAR1 RE1 (GATGGGGTCTCTTCCCTCTT, GACAGATGGTGTCCCAGTCA), mouse *Erich1* RE1 (ACCAAGGACAGCACCAGAAG, AATGTTCCCTGAAGGCACTG). Data were normalized to non-RE1 containing loci using the delta-delta Ct method (18).

Reverse transcriptase PCR. Complementary DNA was synthesized from 0.5 μg of total RNA using random primers. Control "No RT" samples were prepared by omitting reverse transcriptase enzyme. cDNA was purified using a PCR purification kit (Qiagen), eluted in 100 μl of water.

For the conventional quantitative PCR, we used the touchdown PCR method: DNA melting at 94°C for 15 s, primers annealing at a starting temperature of 70°C, polymerase extension at 68°C. The annealing temperature was decreased by 0.5°C each cycle for 30 cycles until it reached 55°C. Subsequently, a variable number of cycles was used with a constant annealing temperature of 55°C (the exact number of cycles used is indicated below). We prepared 50 μl reactions using Accuprime Taq (Invitrogen) according to manufacturer's protocol, with 1 μl of cDNA template. PCR product was purified using Microcon columns (Millipore) and eluted in 20 μl of water, 10 μl of which was run on 2% agarose gel. The primer sequences used were: hHAR1F (GGCCTGGAAACCCTCTAAAA, AAACGGGACACACCAGAGTC) (30 cycles), hHAR1R in striatum (CAGGAGGCAGCAGTGTAGACCA, ACGTCTCCTCCGTTTCATGCTC)(22 cycles), hHAR1R in cortex (GCTGCCTCCTGGAACCA, CGGGAGCTTCCGAGTGA)(14 cycles). The ability of each primer set to amplify the appropriate region was validated by sequencing.

For quantitative gene expression measurements, Fast Taqman assay (ABI) was used with the following custom sequences (forward primer, probe, reverse primer): hHAR1F (AGACCATGTAATTAAGCTGCGTTCT, ACTCTTGCCCTGGCTGC, GGTGCGGGAAGATGTTCAGA), hHAR1R (GCTGCCTCCTGGAACCA, CCACTCTGCAACCAGC, CGGGAGCTTCCGAGTGA), mHAR1F (GGCTTTAATTGCAGGCTTACTAGGA, TCCCCGTGTTTCTCG, TTAGGAAGGAGAGAGACCCTCAAG), mHAR1R (GGCACCAGTCACCCATGT, TTCCCCAGGATTTTC, CAGTTTGCATGCAGCTTGAATG). The mHAR1F primers may detect some splice isoforms of mHAR1R, but this did not affect the interpretation of our data. Taqman probes were used for all HAR1 measurements, except in mouse NPCs where the primers described by Pollard et al. (23) were used (TGTGGGTGTGTTTGAGTGTG, TGCAGATGCAAGTCTGAACC). Coding gene primer sequences are available upon request. Transcripts were considered to be not detectable ("N.D.") if PCR amplification was detected in <50% of replicates. Error bars represent the standard error of the mean, and statistical significance was assessed using the unpaired, one-sided Student's *t*-test.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were carried out essentially as described in Ref. 12. Unlabeled 40 bp dsDNA competitor sequences were used to compete for the binding of recombinant REST DNA-binding domain, against a labeled strong RE1 sequence. The 40 bp RE1 element probe (GGGTTTCAGCACCATGGACAGCGCCAGAGT), adapted from the rat *Scn2a2* gene RE1, was modified with 5′ Cy5 on both strands. Control RE1 competitor sequences were adapted from the rat *Chrm4* gene: a high-affinity RE1, "Ideal" (TCAGCCTCTCTTCAGCACCTCGGACAGCTCCGGACCTCTC), and a mutated version with strongly-reduced affinity, "Mutant" (TCAGCCTCTCTTCAGCAGGTCCCACAGCTCCGGACCTCTC). HAR1 RE1 competitor sequences were the following: human (GGAGATGCCGTTCAGAACCTCGGACAGCGCCGCGGCCCAT), macaque (GGAGAGGCCATTCAGCACCTCGGACAGCGCCACGGCCCAT), dog (TGGGAGGCGGCTCAGCACCTCGGCAGCGGTCCGTGGTCTC), armadillo (AGAGGCACCGTTCAGCACCCTGGAGAGCGGCAGCCAGCTC), mouse (TGATGAAAGAGTCAGCACCTCGGATAGAGCCACAGTGGTC). The probe and competitor were premixed at 4°C for 1 h, and then a master mix containing the HisMBP-tagged REST protein was added. The reaction mixture contained EMSA buffer (2 mM β-mercaptoethanol, 10 mM Tris·HCl pH 8.0, 100 mM KCl, 50 μM $ZnCl_2$, 10% Ultrapure Glycerol, 0.1% NP-40, and 0.1 mg/ml bovine serum albumin), and final concentrations of 0.5 nM probe, 40 nM protein, and various concentrations of unlabeled competitor RE1 DNA. This mixture was incubated at 4°C for 1 h. The bound and unbound probes were subsequently separated at 4°C on a prerun Tris-glycine 12% polyacrylamide gel for 30 min at 200 V. Gels were scanned immediately using a Typhoon 9140 PhosphorImager. Densitometry was carried out using ImageQuant 5.2 software (Am-

ersham Biosciences). For each lane, the fraction of REST-bound probe was calculated as the total intensity of the bound probe band, divided by the total combined intensity of bound and unbound probe bands. We call this measure "fraction bound," where a value of 1 would indicate no binding, and a value of 0 would indicate complete binding of all available labeled probe. All RE1 sequences were tested by running three independent reaction mixes on the same gel.

RESULTS

To discover new disease pathways in HD, we employed whole genome chromatin immunoprecipitation sequencing (ChIP-Seq) data to predict lncRNAs that are targeted by REST (9). This screen identified the locus, HAR1, a deeply conserved genomic region consisting of a *cis*-antisense pair of structured lncRNAs (*HAR1F* and *HAR1R*) (2, 23) located ~80 kb from the neural microRNA, miR-124-3. HAR1 shows evidence of rapid positive evolutionary selection in recent human evolution and is specifically transcribed in developing cortical neurons (23). Genome-wide ChIP-Seq maps predict three regions of REST binding in the vicinity of human HAR1, two of which (*peaks A* and *B*) can be explained by high-quality RE1 DNA motifs (Fig. 1*A*).

We investigated the basis of REST binding to the strongest RE1 at *peak A*. This genomic sequence is evolutionarily conserved in multiple other placental mammals, although not in rodents: there is no homologous region in mouse, while in rat the RE1 motif is disrupted by insertions and deletions of nucleotides. The human RE1 motif was capable of binding REST with high affinity in competition EMSA (Fig. 1*B*). In HEK293 cells expressing high endogenous levels of REST, the same RE1 motif acted as a potent transcriptional repressor when cloned upstream of a luciferase reporter gene (Fig. 1*C*). Expression of a REST dominant-negative construct ("DN: REST") - lacking NH_2- and COOH-terminal repressor domains - cancelled this repressive activity. The DNA sequence from orthologous regions of macaque and armadillo genomes also displayed high affinity for REST and were capable of strong transcriptional repression in luciferase assays, indicating that the function of this sequence is deeply conserved in mammals, like the *HAR1* RNA itself (23). Surprisingly, while the dog RE1 sequence bound REST only slightly better than a mutated RE1 sequence in EMSA, it was capable of repressing transcription of a reporter gene construct. Using chromatin immunoprecipitation, we found that the human binding site A recruits REST in both nonneural (HEK293) and neural (U373) human cell lines (Fig. 1*D*). Thus REST is specifically recruited to the HAR1 locus by deeply conserved, high-affinity DNA motifs.

We next confirmed that recruitment of REST to HAR1 indeed serves to regulate its transcription, since it is possible that other nearby genes are in fact the true target. REST mRNA was knocked down by siRNA in both nonneural HEK293 and neural U373 cell lines (Fig. 1*E*). Loss of REST resulted in the induction of *HAR1F* and *HAR1R* levels in both, consistent with their being repressed through genomic recruitment of REST. Surprisingly, this phenomenon is not conserved in mouse: we could find no evidence that disruption of REST function resulted in derepression of HAR1 in mouse (Supplementary Data).[1] This may be due to loss of REST binding sites in the mouse HAR1 locus, where REST is absent (Supplementary Data). This is consistent with our recent findings that extensive evolutionary remodeling of REST targeting has taken place since divergence of human and mouse (12).

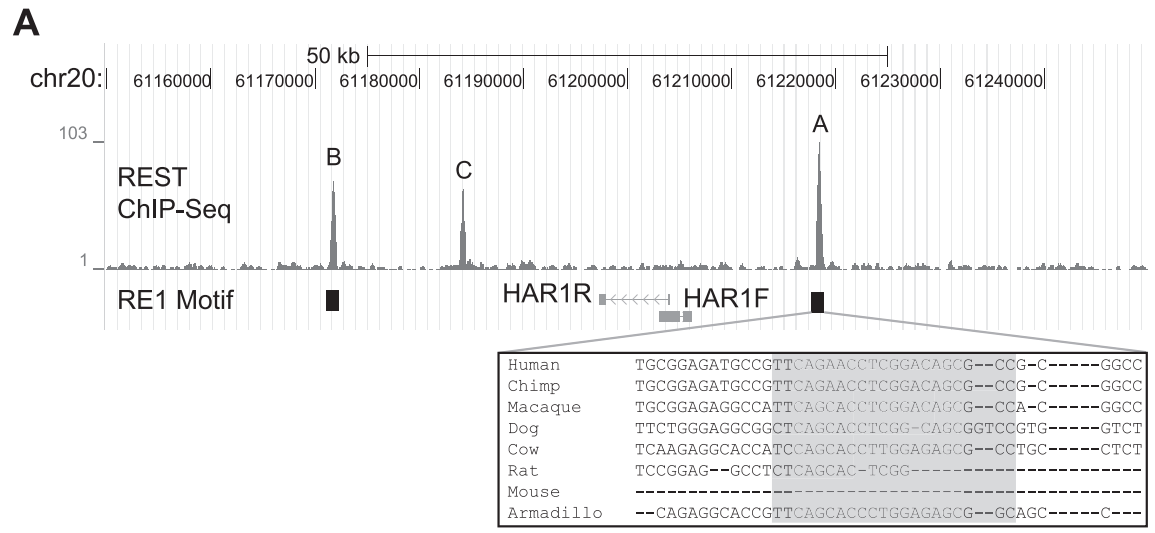
We have previously shown that many REST target genes, both coding and noncoding, are repressed in HD patients (11, 31). We hypothesized that, as a REST target, HAR1 would be similarly dysregulated in HD. Both *HAR1F* and *HAR1R* could be detected by RT-PCR in postmortem samples of normal human striatum and cortex, where neurodegeneration is most pronounced in HD (Fig. 2*A*). We investigated RNA differences between control and HD postmortem samples from two brain regions: cortex (Brodman area 7/9) and striatum (details of samples can be found in MATERIALS AND METHODS). While HD is reflected in molecular changes in multiple subregions of the brain, the striatum is the most profoundly affected (30). Both *HAR1F* and *HAR1R* transcripts could be detected in these samples (Fig. 2*A*). Using the sensitive Taqman assay, we compared their levels in normal and HD brains. We observed significantly lower levels of both *HAR1F* and *HAR1R* in striatum of HD patients, but no significant difference in cortex from Brodman area 7/9 (Fig. 2, *B* and *C*). Thus, in common with protein-coding target genes of REST, the lncRNAs from the HAR1 locus are downregulated in the brains of HD patients.

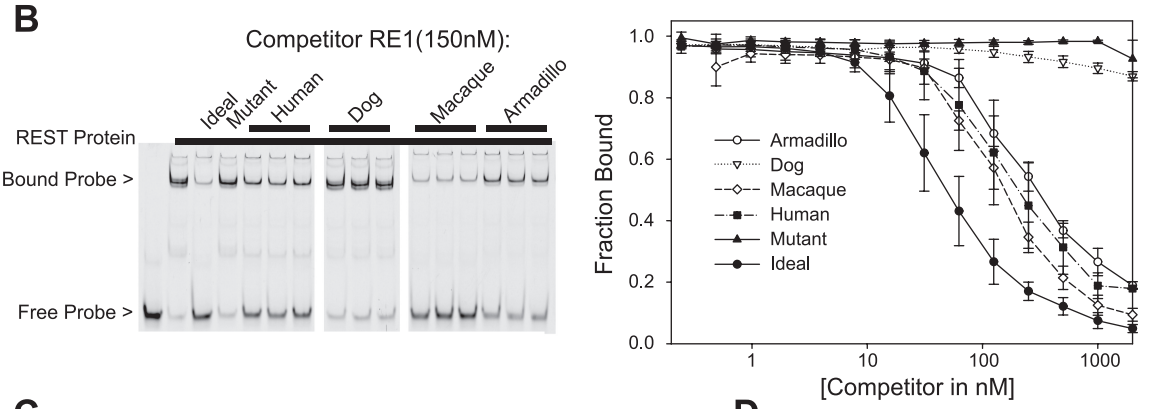
DISCUSSION

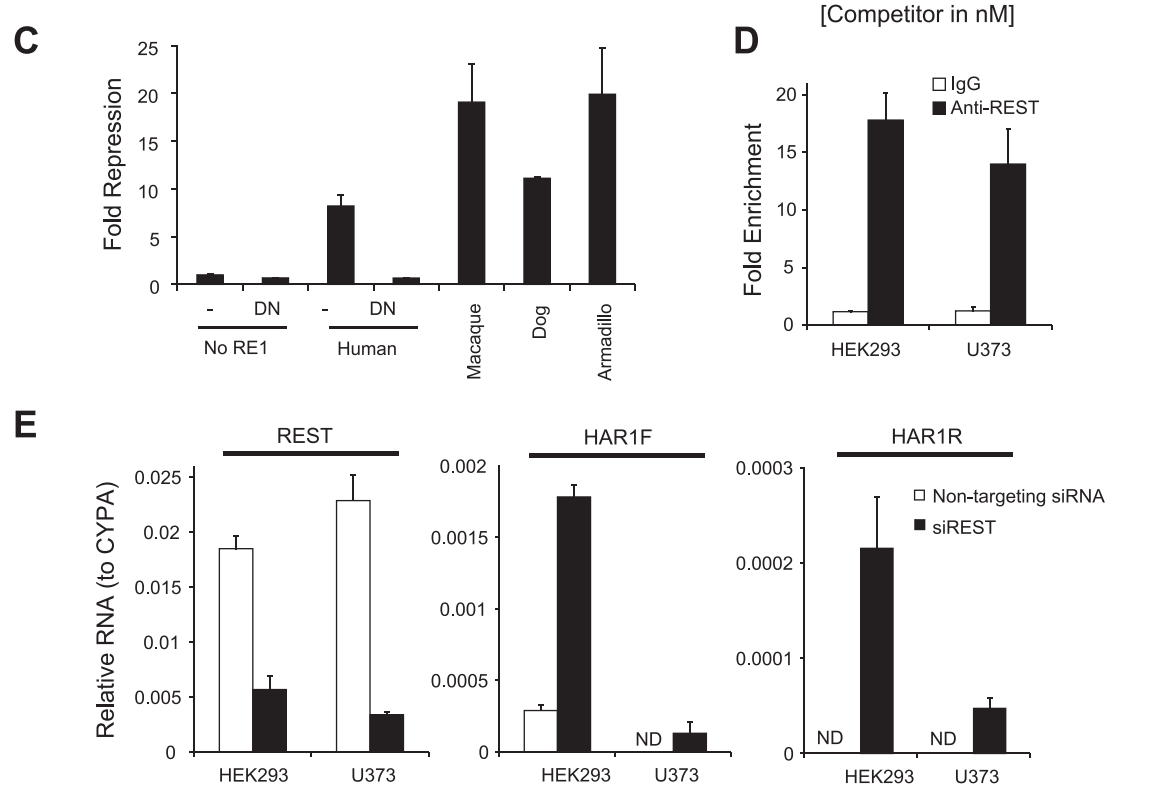
Deciphering the programs of gene expression that are switched on and off in neurons affected by HD offers hope of developing effective therapies to this common, fatal condition. By employing bioinformatic and experimental genomic methods to examine gene regulation on a global scale, we have recently been able to gain new insights into the changes that take place in diseased brain (10, 11).

In the present study, we have shown that the HAR1 noncoding locus is a direct target of the master neural gene regulator REST, and this is likely to cause both forward and reverse HAR1 transcripts to be downregulated in the striatum of HD patients. This is the first indication that the abundant, but poorly understood, class of lncRNAs are part of the widespread transcriptional dysfunction caused by HD. The range and number of transcripts affected in HD are likely to be much greater than previously thought: current estimates have lncRNAs outnumbering protein-coding genes by an order of magnitude (4). While it is possible that many lncRNAs represent nonfunctional transcriptional noise, the growing number of functionally validated examples would seem to suggest that lncRNAs represent a rich, undiscovered source of candidates involved in health and disease of the nervous system (21, 24). In future, it will be important to apply new genomic technologies - principally gene expression measurement by next-generation sequencing [RNASeq (27)] - to discover those that are affected by HD. Given the emerging roles of neural lncRNAs in neurodevelopment (3), neurodegeneration (6), behavior (17), nuclear matrix structure (25), and neuronal protein-coding gene regulation (24), it is reasonable to expect

[1] The online version of this article contains supplemental material.







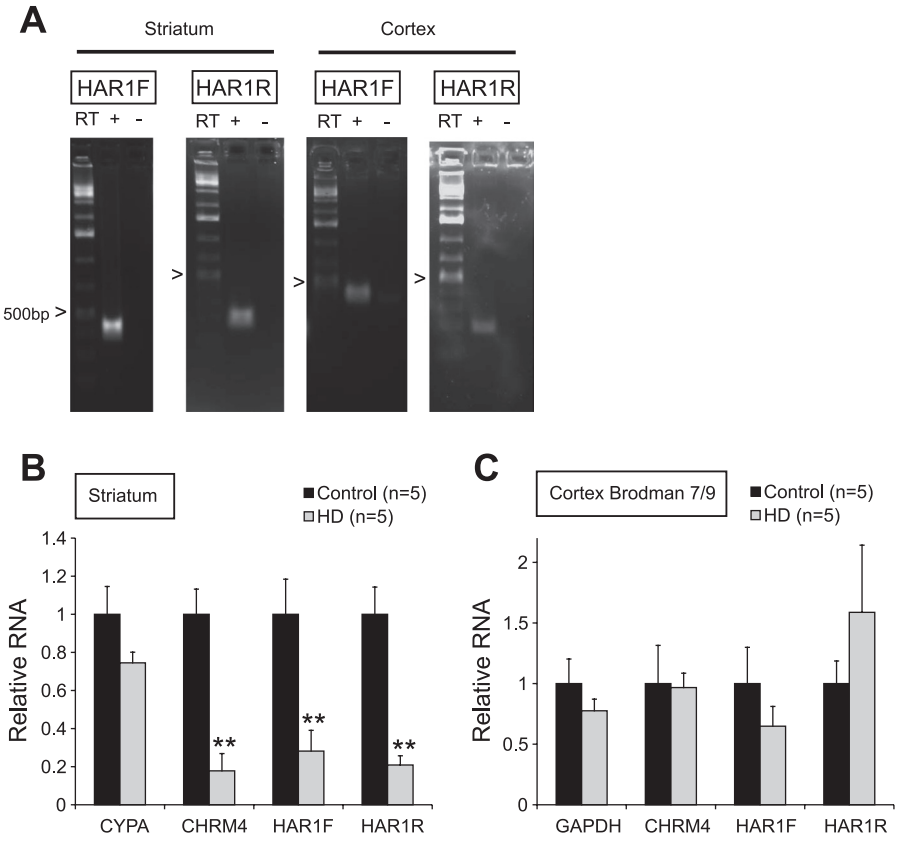


Fig. 2. Repression of HAR1 transcripts in Huntington's disease (HD). *A*: *HAR1F* and *HAR1R* were detected in normal human postmortem brain using RT-PCR. RT, reverse transcriptase; +lane containing cDNA that has been reverse transcribed in the presence of RT enzyme. To control for possible genomic DNA contamination, RNA was mock reverse-transcribed in the absence of RT enzyme, and run in the lane marked "−". The position of the arrowhead ">" corresponds on the gel to DNA of 500 bp. *B, C*: *HAR1F* and *HAR1R* were quantitated using Taqman assay in postmortem brain samples from unaffected (Control) and Huntington's disease sufferers (HD). Statistical significance was estimated using Student's *t*-test; $^{**}P < 0.01$. Levels were normalized to the housekeeping gene *ACTB*. *CYPA* and *GAPDH* are housekeeping genes. *CHRM4* is a known REST target gene (28).

that additional lncRNAs will be discovered that play a role in HD and other neurodegenerative disorders.

It is difficult to assess the functional implications of the loss of HAR1 expression that we observe in HD striatum, since we currently have little functional or mechanistic information about this RNA. However, several lines of evidence make it highly likely that HAR1 does play important roles in the nervous system: *1*) it displays strong and ancient sequence conservation from human to chicken (23); *2*) there is evidence that this sequence underwent a recent spurt of positive selection since humans/chimpanzee divergence (23); *3*) the RNA is highly structured (2); *4*) HAR1 is specifically transcribed during neural development (23). Its transcriptional regulation by REST may now be added as further support to this list. Thus, we may suppose that the loss of as important a molecule as HAR1 from the brain in HD has important consequences, which will become clearer once further studies are carried out on this intriguing molecule.

A surprising aspect of our findings is that repression of HAR1 by REST is not conserved in mouse. Loss of particular RE1 motifs in rodents appears to have removed the basis for REST targeting to the HAR1 locus, where no proximal recruitment of REST can be observed (Supplementary Data). In support of this, knockdown of REST in three mouse cell lines failed to provide any evidence for repression of the locus. In fact, these experiments suggest that REST may activate *HAR1F* and *HAR1R*, likely through an indirect pathway. In support of these conclusions, a recent genome-wide survey of evolutionary conservation of REST targeting showed that gain and loss of REST binding sites have been common during vertebrate evolution (12). Thus, in addition to rapid sequence evolution, the HAR1 locus has also experienced dramatic

Fig. 1. Repressor element 1-silencing transcription factor (REST) targets the human accelerated region 1 (HAR1) locus. *A*: chromatin immunoprecipitation sequencing (ChIP-Seq) read density is shown in *top* panel and predicted RE1 motifs are shown as black boxes. The repressor element 1 (RE1) motif of *peak A* is expanded and aligned to other species. *B*: in electrophoretic mobility shift assay (EMSA), recombinant REST DNA binding domain was mixed with a Cy5-labeled idealized RE1 DNA and electrophoresed, resulting in a protein:DNA complex ("bound probe"). The ability of various species' RE1 motifs to compete this interaction was tested. On the *left* is shown an example EMSA gel, where competitor DNA was at 150 nM (i.e., 300-fold excess of unlabeled competitor over labeled probe); in the *right-hand* panel are shown the summarized results of a titration series where competitor DNA concentration was varied. Experiments were performed in triplicate. The error bars represent standard deviation (*right* panel). An idealized RE1 motif and mutated RE1 were used as control competitors. See MATERIALS AND METHODS for further explanation of EMSA quantitation. *C*: luciferase reporter gene assay was used to measure the effect of RE1 motifs on gene transcription. HAR1 RE1s were cloned upstream of the constitutive thymidine kinase promoter driving firefly luciferase transcription, and transfected into HEK293 cells. Data are normalized to a plasmid carrying no RE1. In the case of the empty (non-RE1) plasmid and the human HAR1 RE1 plasmid, specificity was confirmed by cotransfecting a plasmid expressing a dominant-negative REST construct, which binds to RE1 sites via its DNA binding domain but lacks both NH_2- and COOH-terminal repressor domains (DN), or a control plasmid expressing GFP (−). *D*: ChIP was carried out on the human cell lines indicated, using nonspecific IgG or anti-REST antibodies. Resultant ChIP DNA was amplified by quantitative PCR, and normalized to a non-REST binding locus. *E*: cells were transfected with siRNA targeting REST mRNA, or scrambled siRNA. REST and HAR1 levels were measured using Taqman assay. N.D., transcripts were not detected in >50% of samples. For each sample, 5 independent biological replicates were measured.

regulatory evolution since human and mouse diverged, highlighting the limitations imposed by genomic evolution on the use of mouse models of human disease.

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Present addresses: R. Johnson, Center for Genomic Regulation, Barcelona, Spain; P. M. Gaughwin, Neuronal Survival Unit, Lund University, 221-84 Lund, Sweden.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

1. **Bartel DP.** MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215–233, 2009.
2. **Beniaminov A, Westhof E, Krol A.** Distinctive structures between chimpanzee and human in a brain noncoding RNA. *RNA* 14: 1270–1275, 2008.
3. **Bond AM, Vangompel MJ, Sametsky EA, Clark MF, Savage JC, Disterhoft JF, Kohtz JD.** Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat Neurosci* 12: 1020–1027, 2009.
4. **Carninci P, et al.; FANTOM Consortium; RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group).** The transcriptional landscape of the mammalian genome. *Science* 309: 1559–1563, 2005.
5. **ENCODE**. *Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project*. *Nature* 447: 799–816, 2007.
6. **Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE, Finch CE, St. Laurent G 3rd, Kenny PJ, Wahlestedt C.** Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat Med* 14: 723–730, 2008.
7. **Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A.** Galaxy: a platform for interactive large-scale genome analysis. *Genome Res* 15: 1451–1455, 2005.
8. **Hodges A, Strand AD, Aragaki AK, Kuhn A, Sengstag T, Hughes G, Elliston LA, Hartog C, Goldstein DR, Thu D, Hollingsworth ZR, Collin F, Synek B, Holmans PA, Young AB, Wexler NS, Delorenzi M, Kooperberg C, Augood SJ, Faull RL, Olson JM, Jones L, Luthi-Carter R.** Regional and cellular gene expression changes in human Huntington's disease brain. *Hum Mol Genet* 15: 965–977, 2006.
9. **Johnson D, Mortazavi A, Myers R, Wold B.** Genome-wide mapping of in vivo protein-DNA interactions. *Science* 316: 1497–1502, 2007.
10. **Johnson R, Buckley NJ.** Gene dysregulation in Huntington's disease: REST, microRNAs and beyond. *Neuromolecular Med* 11: 183–199, 2009.
11. **Johnson R, Zuccato C, Belyaev ND, Guest DJ, Cattaneo E, Buckley NJ.** A microRNA-based gene dysregulation pathway in Huntington's disease. *Neurobiol Dis* 29: 438–445, 2008.
12. **Johnson R, Samuel J, Ng CK, Jauch R, Stanton LW, Wood IC.** Evolution of the vertebrate gene regulatory network controlled by the transcriptional repressor REST. *Mol Biol Evol* 26: 1491–1507, 2009.
13. **Johnson R, Gamblin RJ, Ooi L, Bruce AW, Donaldson IJ, Westhead DR, Wood IC, Jackson RM, Buckley NJ.** Identification of the REST regulon reveals extensive transposable element-mediated binding site duplication. *Nucl Acids Res* 34: 3862–3877, 2006.
14. **Johnson R, Teh CH, Jia H, Vanisri RR, Pandey T, Lu ZH, Buckley NJ, Stanton LW, Lipovich L.** Regulation of neural macroRNAs by the transcriptional repressor REST. *RNA* 15: 85–96, 2009.
15. **Johnson R, Teh CH, Kunarso G, Wong KY, Srinivasan G, Cooper ML, Volta M, Chan SS, Lipovich L, Pollard SM, Karuturi RKM, Wei CL, Buckley NJ, Stanton LW.** REST regulates distinct transcriptional networks in embryonic and neural stem cells. *PLoS Biol* 6: e256, 2008.
16. **Kelly CM, Tyers P, Borg MT, Svendsen CN, Dunnett SB, Rosser AE.** EGF and FGF-2 responsiveness of rat and mouse neural precursors derived from the embryonic CNS. *Brain Res Bull* 68: 83–94, 2005.
17. **Lewejohann L, Skryabin BV, Sachser N, Prehn C, Heiduschka P, Thanos S, Jordan U, Dell'Omo G, Vyssotski AL, Pleskacheva MG, Lipp HP, Tiedge H, Brosius J, Prior H.** Role of a neuronal small non-messenger RNA: behavioural alterations in BC1 RNA-deleted mice. *Behav Brain Res* 154: 273–289, 2004.
18. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 25: 402–408, 2001.
19. **Mehler MF, Mattick JS.** Non-coding RNAs in the nervous system. *J Physiol* 575: 333–341, 2006.
20. **Mehler MF, Mattick JS.** Noncoding RNAs and RNA editing in brain development, functional diversification, and neurological disease. *Physiol Rev* 87: 799–823, 2007.
21. **Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS.** Specific expression of long noncoding RNAs in the mouse brain. *Proc Natl Acad Sci USA* 105: 716–721, 2008.
22. **Packer AN, Xing Y, Harper SQ, Jones L, Davidson BL.** The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. *J Neurosci* 28: 14341–14346, 2008.
23. **Pollard KS, Salama SR, Lambert N, Lambot MA, Coppens S, Pedersen JS, Katzman S, King B, Onodera C, Siepel A, Kern AD, Dehay C, Igel H, Ares M, Vanderhaeghen P, Haussler D.** An RNA gene expressed during cortical development evolved rapidly in humans. *Nature* 443: 167–172, 2006.
24. **Ponjavic J, Oliver PL, Lunter G, Ponting CP.** Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. *PLoS Genet* 5: e1000617, 2009.
25. **Sone M, Hayashi T, Tarui H, Agata K, Takeichi M, Nakagawa S.** The mRNA-like noncoding RNA Gomafu constitutes a novel nuclear domain in a subset of neurons. *J Cell Sci* 120: 2498–2506, 2007.
26. **Strand AD, Baquet ZC, Aragaki AK, Holmans P, Yang L, Cleren C, Beal MF, Jones L, Kooperberg C, Olson JM, Jones KR.** Expression profiling of Huntington's disease models suggests that brain-derived neurotrophic factor depletion plays a major role in striatal degeneration. *J Neurosci* 27: 11758–11768, 2007.
27. **Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, Schmidt D, O'Keeffe S, Haas S, Vingron M, Lehrach H, Yaspo ML.** A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science* 321: 956–960, 2008.
28. **Wood IC, Roopra A, Buckley NJ.** Neural specific expression of the m4 muscarinic acetylcholine receptor gene is mediated by a RE1/NRSE-type silencing element. *J Biol Chem* 271: 14221–14225, 1996.
29. **Wood IC, Belyaev ND, Bruce AW, Jones C, Mistry M, Roopra A, Buckley NJ.** Interaction of the repressor element 1-silencing transcription factor (REST) with target genes. *J Mol Biol* 334: 863–874, 2003.
30. **Zuccato C, Cattaneo E.** Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat Rev Neurol* 5: 311–322, 2009.
31. **Zuccato C, Belyaev N, Conforti P, Ooi L, Tartari M, Papadimou E, MacDonald M, Fossale E, Zeitlin S, Buckley N, Cattaneo E.** Widespread disruption of repressor element-1 silencing transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. *J Neurosci* 27: 6972–6983, 2007.