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Neurobiology of Disease

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Neurobiology of Disease 29 (2008) 438–445

A microRNA-based gene dysregulation pathway in Huntington's disease

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Received 18 May 2007; revised 7 October 2007; accepted 5 November 2007
Available online 13 November 2007

Huntington's disease (HD) is a dominantly-inherited neurodegenerative disorder which is incurable and ultimately fatal. HD is characterised by widespread mRNA dysregulation, particularly in neurons of the forebrain, by mechanisms which are not fully understood. Such dysregulation has been demonstrated to result, in part, from aberrant nuclear localisation of the transcriptional repressor, REST. Here, we show that expression of a number of neuronal-specific microRNAs is also dysregulated in HD tissues, probably as a result of increased repression by REST. This phenomenon is observed in both murine models of HD and in the brains of human HD sufferers. MicroRNA loss is reflected in increased levels of a number of target messenger RNAs. These data are the first to demonstrate a role for microRNAs in HD, and indicate that the molecular aetiology of HD is reflected in a loss of neuronal identity, caused in part by dysregulation of both transcriptional and post-transcriptional mechanisms.

Keywords: miRNA; REST; Huntington's disease; Neurodegeneration

Introduction

Huntington's disease (HD) is a fatal, incurable neurodegenerative disease caused by a CAG expansion in the gene encoding the protein huntingtin (Htt). The disease manifests in cognitive defects, motor control impairment, and ultimately death, symptoms that result from a neuronal dysfunction characterised by progressive loss of cortical and striatal neurons. This neuronal death appears to be due to a combination of the toxicity of the mutant huntingtin and loss of the neuroprotective effects of the wild type protein (Cattaneo et al., 2005).

However many aspects of HD pathology remain unexplained. In particular, a number of studies have observed widespread differences in mRNA levels between brains of HD sufferers and normal adults with one study reporting at least ~100 mRNAs significantly upregulated, and slightly fewer significantly downregulated (Hodges et al., 2006). The degree of this difference varies with disease severity, both in terms of the disease progression and in the brain region examined. These findings are recapitulated in similar experiments on mouse models (Crocker et al., 2006; Luthi-Carter et al., 2002; Sipione et al., 2002). Htt interacts with several transcriptional regulators including CBP (Steffan et al., 2000; Nucifora et al., 2001), p53 (Steffan et al., 2000; Bae et al., 2005) Sp1 (Dunah et al., 2002), TAFII130 (Dunah et al., 2002) and TBP (Huang et al., 1998) and this interaction is frequently disrupted in the presence of mutant Htt (Rubinsztein, 2003). In previous studies, we have shown that Htt interacts with the essential transcriptional repressor, REST (Repressor Element 1 Silencing Transcription Factor, also known as NRSF, Neuron-Restrictive Silencing Factor) in neurons (Zuccato et al., 2003; Ooi and Wood, 2007). In normal individuals, wild-type huntingtin sequesters REST in the cytoplasm of neurons; in the case of HD, the polyglutamine expansion of mutant huntingtin inhibits this interaction, allowing aberrantly high levels of REST to accumulate in the nucleus of HD neurons and leading to increased transcriptional repression of *BDNF*, a REST target gene (Zuccato et al., 2003). Reduced levels of BDNF consequently lead to reduced survival of striatal neurons.

We have shown that REST can potentially interact with more than 1300 sites in the human and murine genomes and many putative target genes encode proteins regulating neuronal function, survival and differentiation (Bruce et al., 2004; Johnson et al., 2006). Furthermore, we have shown that REST occupancy of

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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.nbd.2007.11.001

multiple target genes is increased in the presence of mutant Htt and in the absence of wild type Htt and this widespread disruption of the REST regulon may contribute to HD pathology (Zuccato et al., 2007).

Recently, a large class of non-coding regulatory RNAs, microRNAs (miRNAs), have been described in multiple metazoan species including human and mouse (Bartel, 2003). miRNAs play important roles in organogenesis, metabolism and neuronal development through degradation and translational repression of mRNAs. Many miRNAs are selectively expressed in the neurons and glia of the brain (for review, see (Kosik, 2006)) and appear to regulate fundamental neuronal processes, including elaboration of the neuronal transcriptome (Lim et al., 2005) and dendrite growth (Vo et al., 2005). Recently, it was shown that REST can regulate the expression of a number of neuronal miRNAs (Conaco et al., 2006), suggesting that miRNA expression in both development and disease, may be controlled by similar mechanisms as protein-coding genes.

The profound importance of miRNAs in gene regulation, and the large numbers of genes they probably regulate (predicted to be >30% of all mRNAs) have led many to speculate on the potential role of miRNAs in human disease. Consistent with this, miRNAs have highly specific expression patterns in certain cancers (Esquela-Kerscher and Slack, 2006). Nevertheless, the role of miRNAs in disease, including neurodegenerative disorders is only now coming to light. Bilen et al. have shown that the miRNA processing pathway has a broadly protective effect against the toxicity of the Ataxin-3 polyglutamine-expansion mutant (Bilen et al., 2006). This effect was observed in both flies and human cell lines, suggesting that one or more microRNAs have conserved, neuroprotective functions. The authors identified at least one of these to be the miRNA, *ban*. More recently, a regulatory network containing the miRNA mir-133b and the transcription factor Pitx3 was shown to control the development and identity of midbrain dopaminergic neurons (Kim et al., 2007). Importantly, the same study found that mir-133b is amongst a small set of miRNAs which display significantly reduced expression in the brains of Parkinson's Disease sufferers. These studies suggest that loss of expression of certain miRNAs may be a general feature in the pathogenesis of both polyglutamine and non-polyglutamine neurodegenerative diseases.

In the present study, we show that REST can regulate a number of brain-specific miRNAs in vivo and further, we show that a number of these miRNAs have significantly altered expression levels in HD. Finally, we demonstrate that the reduction in expression of two miRNAs, mir-124a and mir-132, leads to increases in the levels of their target mRNAs. This study is the first indication of the potential involvement of miRNAs in HD, and suggests that REST may play an important role in this process.

Results

Identification of miRNA targets of REST

We hypothesised that REST might regulate the expression of brain-restricted miRNA genes. Therefore, the position data from our previous search of the human genome for binding sites of REST (known as Repressor Element-1 sites, or RE1s) (Johnson et al., 2006) was compared to the positions of known miRNA genes (Griffiths-Jones, 2004), with the search criteria that either the miRNA gene resides within 100 kb of an RE1, or that the miRNA resides in the intron of a gene within 100 kb of an RE1. This analysis uncovered 17 likely miRNA targets of REST, 13 of which have an orthologous miRNA-RE1 pair in mouse (see Table 1). Most have a neuron-specific or brain-specific expression pattern, while mir-1d and mir-133a expression is restricted to heart and skeletal muscle, patterns consistent with REST's known role of regulating gene expression in neural and cardiovascular tissue (Palm et al., 1998; Cheong et al., 2005; Bingham et al., 2007). Some miRNAs are encoded by more

Table 1
Identification of REST–target miRNAs in the human genome

miRNA	Expression	Host gene	RE1 ID	RE1 PSSM score (Johnson et al., 2006)	RE1 distance (kb)	Conserved in mouse?
1-d	Heart/muscle (C)	*C20orf166*	hum42172	0.9398	35	Yes
133a-2	Heart/muscle (C)	*C20orf166*	hum42172	0.9398	35	Yes
9-1	Brain (S)	*CROC4*	hum2610	0.9628	5	Yes
9-3	Brain (S)		hum33584	0.9426	3	Yes
29a	Brain (S)		hum18483	0.9364	20	Yes
29b-1	Brain (S)		hum18483	0.9364	20	Yes
124a-1	Brain (S)		hum19259	0.9237	20	IG
124a-2	Brain (S)		hum19995	0.981	0.8	No
124a-3	Brain (S)		hum42331	0.9583	0.5	Yes
132	Brain (S)		hum35996	0.9574	0.2	Yes
135b	Brain (S)		hum3303	0.9721	10	Yes
139	Brain (S)	*PDE2A*	hum27012	0.9507	2	Yes
203			hum32236	0.9506	15	No
204		*Q9HOX2*	hum21795	0.913	600	Yes
212			hum35996	0.9574	200	Yes
330	Brain (S)	*EML2*	hum40445	0.9134	40	IG
346	Brain (S)	*GRID1*	hum24546	0.9337	40	Yes

A bioinformatic search for RE1 sites in the human genome identified a number of miRNA genes as likely targets of REST (Johnson et al., 2006). The RE1 ID can be used to access relevant data from the RE1 Database [http://www.bioinformatics.leeds.ac.uk/RE1db_mkII/]. RE1 Position-Specific Scoring Matrix (PSSM) score reflects the similarity of the sequence to the RE1 motif, where a score >0.91 indicates that the sequence is likely to be a REST binding site. RE1 distance refers to the genomic distance between the RE1 and corresponding miRNA gene in human. Instances where the orthologous miRNA gene in mouse is also proximal to an RE1, are defined to be conserved targets in mouse. IG: intervening gene, i.e. in mouse another annotated gene lies between the orthologous miRNA/RE1 pair. C: Expression data from Chen et al. (2006); S: Expression data from Sempere et al. (2004).

than one gene and for three of the miRNAs identified in this study, multiple genes encoding the same or similar miRNAs all have RE1s, including both mir-29a and 29b-1, all three copies of the gene encoding mir-124a, and two out of three encoding mir-9.

Validation of RE1s

Having identified miRNA genes putatively regulated by REST, we next proceeded to verify that the RE1 sequences identified proximal to miRNAs were capable of binding REST in vitro. Fig. 1 shows that unlabelled competitor DNAs of all the human miRNA RE1s can compete with REST binding to a radiolabelled consensus RE1 probe in EMSA (Electrophoretic Mobility Shift Assay). Furthermore, the supershift caused by addition of an anti-REST antibody confirmed the identity of the specific REST:radioprobe complex. It was of note that the miRNA RE1s have a range of affinities for REST in this assay; for example, a 10–100 fold greater concentration of mir-124a-1 RE1 DNA compared to that of mir-124a-2 is required to inhibit REST binding. Although the relationship of in vitro affinity to in vivo recruitment is unclear, these data suggest that the RE1s of miRNA genes may differentially recruit REST in vivo.

In order to examine recruitment of REST to the RE1 of endogenous miRNA genes we carried out a chromatin immunoprecipitation assay (ChIP). Chromatin was extracted from the mouse neural stem cell line NS5 (Conti et al., 2005), precipitated with anti-REST antibodies and the resultant DNA was interrogated by quantitative real-time PCR (qPCR) using primers flanking each of the mouse miRNA RE1s (Fig. 2). All sites tested were strongly and specifically occupied by REST with relative enrichments ranging from 4- to 30-fold (for comparison, enrichment at the tandem RE1 of the *Syt7* gene, a known REST target was about 50 fold in these cells). Collectively, these data show that miRNA RE1s interact with REST both in vitro and in vivo.

REST represses miRNA expression in cultured neural cells

The presence of functional RE1s in genomic proximity to neural and cardiac miRNAs strongly suggested that they are targets of REST. To test this, we investigated the regulation of these miRNAs in a REST-expressing mouse cell line derived from embryonic striatum, $Hdh^{7/7}$ (Zuccato et al., 2003). Endogenous REST function was reduced by infecting the cells with recombinant adenovirus expressing a dominant-negative REST construct (Ad DN:REST) (Fig. 3). Resulting changes in miRNA gene expression were measured using a modified RT-PCR technique (see Materials and Methods). Comparison to control adenovirally treated cells (Ad) showed that mir-29a, mir-29b-1, mir-132 and mir-135b were significantly upregulated upon loss of REST function. Other miRNAs were either unaffected (mir-133a and the non-target control, mir-21), or silent in these cells. Therefore, our bioinformatic method correctly predicted that 4/5 detected miRNAs in these cells are REST targets.

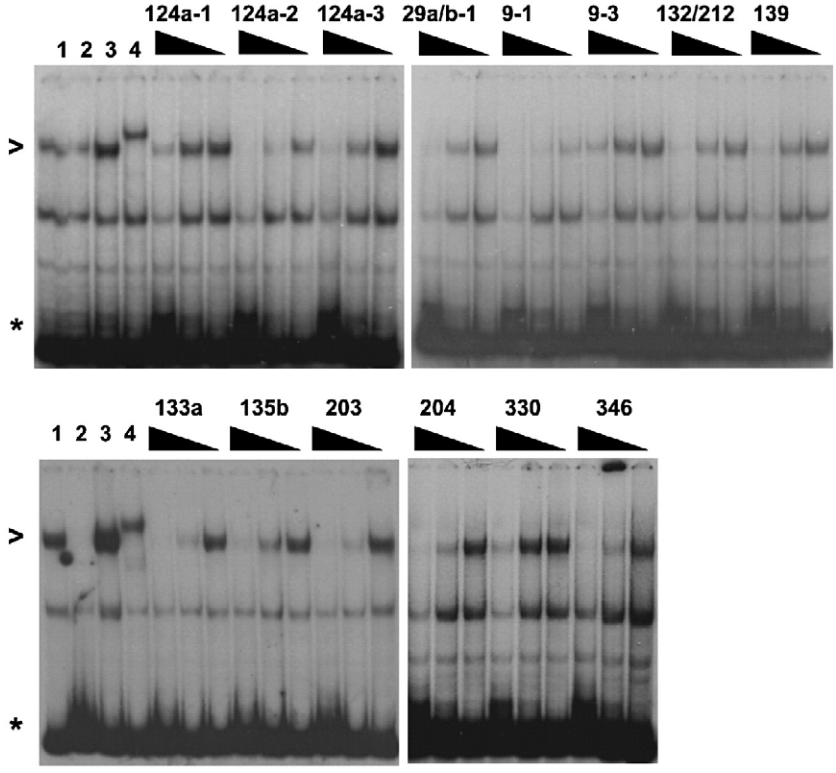

Fig. 1. REST can bind miRNA RE1s in vitro. RE1s identified proximal to miRNA genes were tested for the ability to compete for REST binding with a radiolabelled RE1 in EMSA. Decreasing relative concentrations (100:1, 10:1, 1:1) of unlabelled test sequences were competed against radiolabelled rat *NaV1.2* promoter RE1. The loss of specific REST:radioprobe complex is indicative of binding to test competitor RE1. The putative target miRNA of each RE1 is indicated above the image. > indicates the REST:radioprobe complex, * indicates unbound radioprobe. Controls: 1—No competitor; 2—*Chrm4* RE1 unlabelled competitor oligonucleotide; 3—non-specific unlabelled competitor oligonucleotide; 4—anti-REST antibody (P18, Santa Cruz). Note that the additional band shift induced by addition of REST antibody in Lane 4 identifies the specific REST:probe complex.

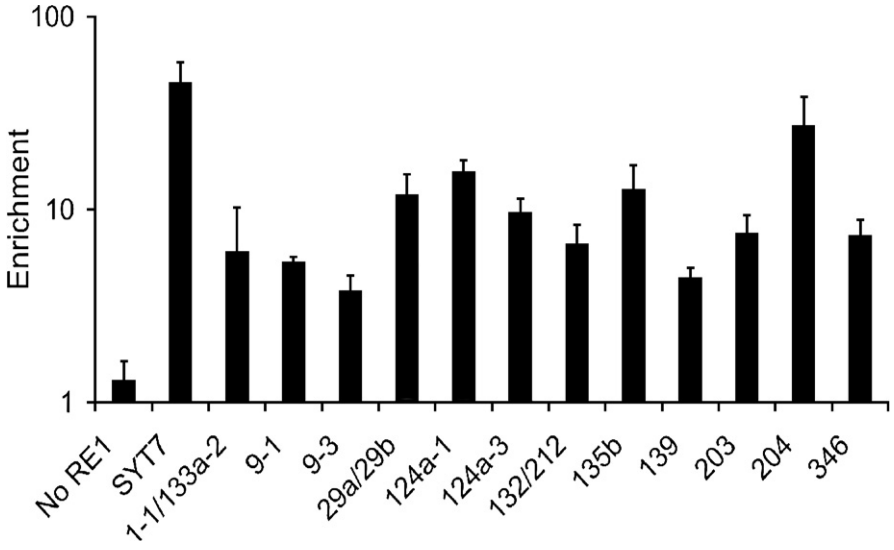

Fig. 2. REST is recruited to miRNA genes in vivo. Chromatin immunoprecipitation was carried out on chromatin extracted from the mouse neural stem cell line, NS5, using the anti-REST serum, R2174. Purified ChIP DNA was interrogated in qPCR with primers flanking the RE1s of the miRNAs indicated. Data are expressed as fold enrichments of R2174 immunoprecipitation over an appropriate pre-immune serum. Error bars indicate standard error of the mean for at least three independent biological replicates. All enrichment values were statistically significantly different ($P<0.05$) from the background enrichment measured for a coding region of the *Chrm4* gene ('No RE1') according to Student's *t* test. As a positive control, the enrichment at the known target *SYT7* RE1 was measured.

Dysregulation of neuronal miRNAs in a mouse model of HD

REST target genes are aberrantly regulated in the brains of mouse models of HD and in post-mortem tissue of HD sufferers (Zuccato et al., 2003, 2007). Specifically, the CAG expansion in the mutant huntingtin gene impairs Htt protein's ability to sequester REST in the cytoplasm of affected neurons, thereby increasing the levels of nuclear REST and depressing levels of REST target gene expression, including *BDNF*. Accordingly, we hypothesised that the aberrant repression of miRNAs by REST in the presence of mutant Htt, may consequently lead to upregulation of miRNA–target mRNA expression. Such regulatory events could contribute to the widespread abnormalities in mRNA profiles of HD brains (Hodges et al., 2006).

To test this hypothesis, we began by measuring the levels of REST target miRNAs in the cortex of the mouse model of HD, R6/2. These mice express the human huntingtin exon 1 fragment bearing a 150 glutamine expansion and recapitulate many features of HD (Mangiarini et al., 1996). Total RNA from dissected cortex samples from symptomatic R6/2 mice at 12 weeks of age, as well as control littermates, was reverse-transcribed and interrogated for miRNA expression levels by qPCR (Fig. 4). Of the seven target miRNAs which were found to be expressed in these samples after 40 cycles of qPCR, four displayed significantly reduced expression in R6/2 samples: mir-29a, mir-124a, mir-132 and mir-135b. In addition,

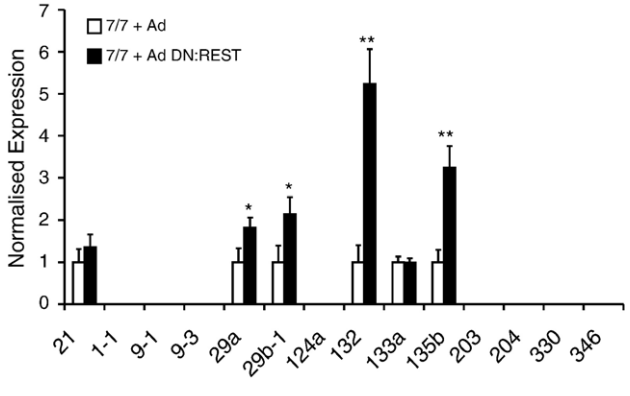

Fig. 3. REST regulates miRNA levels in $Hdh^{7/7}$. The concentrations of putative REST target miRNAs were measured by modified RT-PCR using cDNA prepared from the neural cell line, $Hdh^{7/7}$. Cells were infected with either a control adenovirus (Ad), or a recombinant adenovirus expressing a dominant-negative REST construct (Ad DN:REST) (Zuccato et al., 2007). Expression levels were normalised to the small RNA, U6, then normalised to the level of Ad-treated sample. Data represent the mean of at least three biological replicates, while error bars denote the standard error of the mean. *$P<0.05$ and **$P<0.01$ by Student's t test.

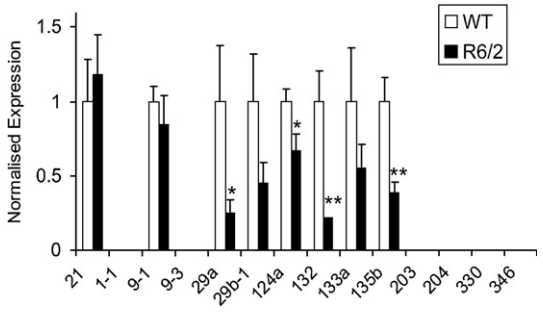

Fig. 4. miRNAs are differentially expressed in the cortex of the mouse HD model, R6/2. The concentrations of miRNAs were measured in RNA extracted from the dissected cortices of 8-week-old R6/2 and wild-type littermate mice. miRNA expression levels were calculated relative to the small RNA, U6, then normalised to the level in wild-type cortex. Error bars indicate the standard error of the mean for at least three independent biological replicates. *, $P<0.05$ and **, $P<0.01$ by Student's *t* test.

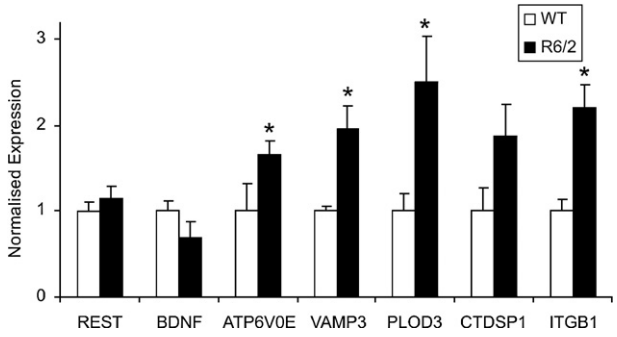

Fig. 5. Increased levels of mir-124a–target mRNAs in R6/2 cortex. Conventional RT-PCR was used to measure the concentrations of mRNAs in the same RNA samples as Fig. 4. cDNAs were interrogated in qPCR using intron-spanning primers to *Atp6v0e*, *Vamp3*, *Plod3*, *Ctdsp1* and *Itgb1*, all reported mRNA targets of mir-124a (Kosik, 2006). Expression levels were calculated relative to the housekeeping gene, cyclophilin, then normalised to the level of wild-type cortex. Error bars indicate the standard error of the mean for at least three independent biological replicates. *, $P<0.05$ by Student's *t* test.

mir-29b and mir-133a were reduced but at levels which are not statistically significant with the number of experimental samples tested. None of the miRNAs measured displayed increased expression in R6/2, while a control miRNA, mir-21 that does not bear any RE1 site, showed no difference in expression between control and R6/2 cortices. Similar differences in miRNA expression were observed in the hippocampi of the same animals (see Supplementary data). Together these data indicate that a large proportion of REST–target miRNAs display altered expression levels in cortex and hippocampus of the R6/2 HD model. These dysregulated miRNAs include the important neuronal-specific miRNAs mir-124a and mir-132. Thus, widespread and significant dysregulation of REST–target miRNAs takes place in the brains of R6/2 animals.

To confirm the observed dysregulation of miRNAs in R6/2 brain and to investigate the consequent downstream effects of this, we sought to measure the levels of target mRNAs of dysregulated miRNAs in the same cortex samples as described above. The identification of mRNA targets of miRNAs is a major challenge, and so far bioinformatic target prediction for most miRNAs remains untested. Fortunately, mir-124a is one of the best studied miRNAs and has many experimentally-verified target mRNAs (Lim et al., 2005; Vo et al., 2005; Conaco et al., 2006). Therefore we selected five such target mRNAs and assayed their levels in the same R6/2 cortex samples discussed above. The selected target mRNAs are: *Atp6v0e*, a vacuolar proton pump subunit; *Vamp3*, a vesicle associated membrane protein; *Plod3*, a lysyl hydroxylase; *Ctdsp1*, a regulatory RNA polymerase II phosphatase; and *Itgb1*, a fibronectin receptor subunit. Consistent with the idea that mir-124a depresses levels of non-neuronal transcripts in neurons, none of the five target mRNAs display neuronal-specific expression profiles and most are ubiquitous. These mRNAs all have complementary seed recognition sequences for mir-124a in their 3' UTR, and have been shown to be reduced in response to introduction of mir-124a into HeLa cells (Lim et al., 2005). We found that of the five mRNAs tested, all showed increased levels in R6/2 cortex over control, of which four are statistically significant (Fig. 5). No such change was observed for REST mRNA. These data are consistent with the observed decrease in mir-124a levels in R6/2 cortex.

Dysregulation of miRNAs in human HD cortex

Data from the mouse HD model R6/2 suggested that miRNAs are dysregulated in the HD state, leading in turn to altered levels of their target mRNAs. We next wished to perform similar experiments in the cortex of human HD sufferers, in order to ask whether miRNAs are dysregulated in HD and if so, whether they are the same as those observed to be dysregulated in the mouse model.

RNA was extracted from tissue samples of human unaffected individuals ('WT') and HD-sufferers ('HD'), and miRNA precursor levels measured (Fig. 6). Of the putative REST–target miRNAs, mir-29a, mir-124a, mir-132 and mir-330 could be detected. Interestingly, mir-330 was not detected in mouse cortex, while mir-133a was detected in mouse but not human. Comparison of human WT and HD individuals shows that significant differences in miRNA levels exist: mir-132 levels were significantly lower in the HD samples, indicating that this miRNA is down-regulated in HD. In contrast, mir-29a and mir-330 were significantly higher in HD samples. This contrasts with the equivalent R6/2 data, where mir-29a is significantly downregulated while mir-330 is not detected. As expected, no significant difference in expression of the control mir-21 was observed.

The human tissue sample represents tissue derived from Brodman Area 4, while the mouse sample was taken from the whole cortex. Therefore these data suggest that while region-specific miRNA expression might occur in the cortex, miRNA expression profiles differ in a species-specific manner. Furthermore, the mir-29a expression profiles suggest that the R6/2 model has important differences in miRNA gene expression patterns to those seen in human HD. Nevertheless, the data confirm that neuronal miRNAs are dysregulated in the brains of HD sufferers. The nature of expression changes in miRNAs (both up- and down-regulation) suggest that other factors, in addition to increased nuclear REST, might contribute to this effect.

We next asked whether the observed changes in miRNA expression in human HD cortex leads to altered levels of their mRNA targets. While no experimentally-validated target mRNAs have been reported for mir-29a or mir-330, the work of Vo et al. identified the mRNA of *p250GAP* to be a target of mir-132 (Vo et al., 2005). Therefore, an increase in *p250GAP* mRNA would lend support to our observation of decreased mir-132 in human HD

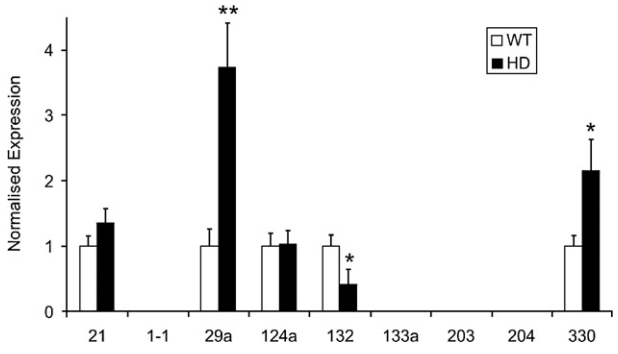

Fig. 6. The cortices of human HD sufferers have altered miRNA levels. RT-PCR was carried out on RNA extracted from postmortem cortex samples of human HD sufferers ('HD') and unaffected individuals ('WT'). miRNA levels were compared to the small RNA, U6, and normalised to the WT level. Error bars indicate the standard error of the mean for at least three independent biological replicates. *, $P<0.05$ and **, $P<0.01$ by Student's *t* test.

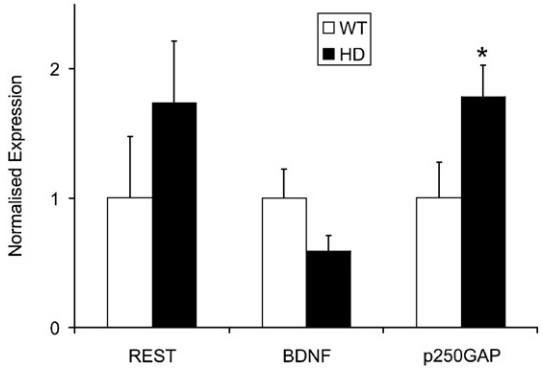

Fig. 7. Human HD cortices have increased levels of the mir-132–target mRNA, *p250GAP*. Conventional RT-PCR was used to measure the concentrations of mRNAs in RNA extracted from the postmortem cortex samples of human HD sufferers ('HD') and unaffected individuals ('WT'). cDNAs were interrogated in qPCR using intron-spanning primers to *p250GAP*, a target of mir-132 (Vo et al., 2005). Expression levels were calculated relative to the housekeeping gene, Cyclophilin A, then normalised to the level of wild-type cortex. Error bars indicate the standard error of the mean for at least three independent biological replicates. *, $P<0.05$ by Student's *t* test.

cortex. We found that the level of *p250GAP* mRNA in human HD cortex was significantly higher than control samples (Fig. 7), supporting our hypothesis. No significant change in REST mRNA was observed, while a decrease in *BDNF* mRNA was observed, consistent with previous findings (Zuccato et al., 2003). Therefore, transcriptional dysregulation of miRNAs in human HD cortex is reflected in altered mRNA levels of an important neuronal gene.

Discussion

Since the discovery of miRNAs, a pressing concern has been to explain how their highly specific expression patterns are achieved. As miRNAs have been shown to be transcribed by RNA Polymerase II, it was natural to expect that their transcription might be regulated by similar mechanisms as protein-coding genes, i.e. recruitment of sequence-specific transcription factors to miRNA genes to alter their transcriptional rate. In 2005, O'Donnell et al. confirmed this by showing that c-Myc activates the expression of a cluster of miRNAs, containing mir-17-5p, mir-18, mir-19, mir-20, mir-92 and mir-106 (O'Donnell et al., 2005). Therefore, miRNA transcription can be regulated by similar mechanisms to that of protein-coding genes.

Given that aberrant gene expression underlies many human diseases, one might expect that miRNAs play a role in disease. In the present study, we have presented evidence that miRNA dysregulation accompanies the neurodegenerative disorder, Huntington's disease. Furthermore, these results coupled with a number of other findings (Zuccato et al., 2003, 2007) suggest a model where enhanced levels of nuclear REST in HD neurons leads to changes in the neuronal transcriptome both directly, via repression of target gene expression, and indirectly, via regulation of neuronal miRNA expression (Fig. 8).

miRNAs and gene dysregulation in Huntington's disease

HD is characterised by widespread changes in neuronal gene expression (Hodges et al., 2006; Crocker et al., 2006; Luthi-Carter et al., 2002; Sipione et al., 2002; Borovecki et al., 2005). The affected mRNAs belong to a large number of functional categories, and approximately similar numbers are observed to have increased and decreased expression. Together, these data suggest that mutant huntingtin mediates widespread dysregulation of both transcriptional activation and repression pathways.

Consistent with this, a number of transcriptional activators have been shown to be affected by mutant huntingtin (Bae et al., 2005), in addition to repressors including REST (Zuccato et al., 2003). The results presented here suggest that dysregulation of neuronal miRNAs may represent an additional, hitherto unappreciated, cause of HD pathology. We have observed statistically significant differences in miRNA precursor levels in the brain of a mouse HD model and in the cortices of human HD patients. This phenotype is observed at symptomatic stages in both mouse and human. Interestingly, distinct but overlapping changes in miRNA expression occur in the cortices of mouse and human, perhaps reflecting species-specific or region-specific differences or indeed, limitations of the veracity of the mouse models of HD. For the majority of miRNAs, we do not know the identity of their target genes, but for mir-124a and mir-132, two cases where experimentally-validated target mRNAs exist, we verified that dysregulation of miRNA leads to reciprocal changes in target mRNA in the same samples.

Both mir-124a and mir-132 are known REST target genes (Conaco et al., 2006), and both have been the subject of experiments employing exogenous miRNA constructs to dissect their regulatory role. mir-124a is highly and specifically expressed in neurons (Smirnova et al., 2005). In a seminal paper, Lim et al. showed that by introducing mir-124a into HeLa cells the transcriptome of the cell is shifted towards that of a neuron (Lim et al., 2005). The large number of mRNAs affected, and their highly significant over-enrichment for mRNAs with low expression levels in neurons, suggests that mir-124a may be a potent high-level regulator of neuronal identity. Bioinformatic analyses suggest that this and other miRNAs have large-scale effects on mRNA expression of tissue specific miRNAs, in effect defining the tissue-specific transcriptome of cells (Sood et al., 2006). The finding that miRNAs are dysregulated in R6/2 suggests that HD may reflect a fundamental loss of neuronal identity through the dysregulation of many non-neuronal mRNA transcripts. The pathway by which this loss leads to neuronal death remains unclear, and must be a focus of future work.

Our findings on mir-132 suggest a means by which neurite growth and, presumably, neuronal connectivity may be aberrantly regulated in HD (Vo et al., 2005). In cultured neurons, mir-132 expression is induced by BDNF via recruitment of CREB to an upstream CRE. mir-132 promotes neurite sprouting by inhibiting translation of *p250GAP*, a member of the Rac/Rho family of GAPs, which is an inhibitor of neurite growth. In this manner, the coordinated interplay of aberrant transcriptional and post-transcriptional events in HD pathology may be inferred (Fig. 8).

Integration of miRNA and transcriptional regulation

Since REST is an essential transcriptional repressor that regulates expression of many neuron-specific genes, we hypothesised that REST might also repress transcription of miRNAs. Inspection of whole-genome REST binding site prediction indicated that around 17 miRNA genes, encoding 13 miRNAs, are likely target genes based on their proximity to predicted RE1s (Johnson et al., 2006). The neuron-specific expression profiles of most of the novel miRNA targets lend further support to these genes being REST targets and include mir-124a and mir-9 as described previously by Conaco et al. using ChIP-SACO

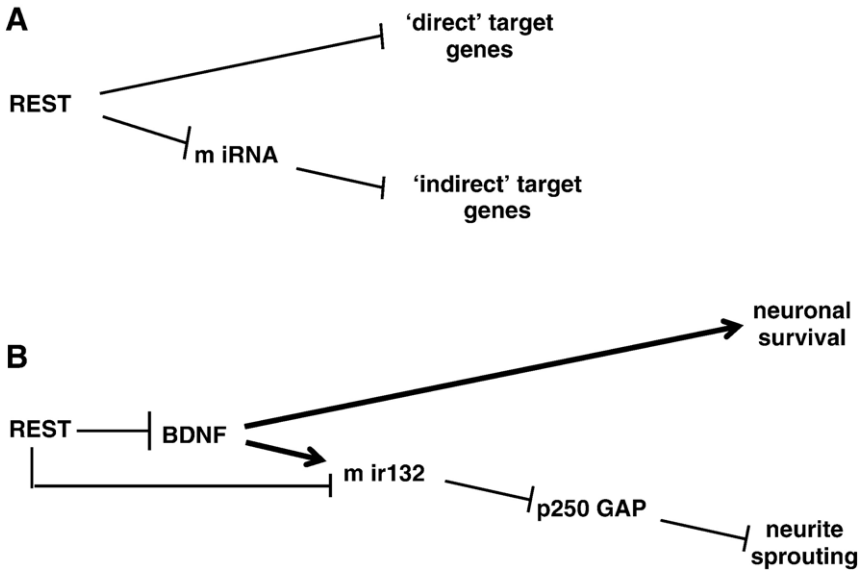

Fig. 8. An example of how aberrant transcriptional and post-transcriptional processes might converge to lead to a loss of neuronal identity in HD. (a) Increased REST expression leads to a direct repression of target gene transcription and an indirect activation of gene expression via repression of an intermediate miRNA. (b) REST decreases *BDNF* transcription and consequent neuronal survival via a direct transcriptional repression of the *BDNF* gene. Decreased BDNF transcription leads to a decrease in mir-132 transcription consequently leading to increased translation of *p250GAP* and inhibition of neurite outgrowth. The same end-point is reached via direct REST inhibition of mir-132 transcription.

(Conaco et al., 2006). As mentioned above, little is presently known about the biological roles of most of these miRNAs; mir-124a is very highly and specifically expressed in neurons (Smirnova et al., 2005), and transfection into non-neuronal cells drastically alters the transcriptome of those cells (Lim et al., 2005). Exogenous mir-132 expression in neurons has been shown to promote dendrite growth (Vo et al., 2005), while mir-133a has been shown to promote proliferation of myoblasts (Chen et al., 2006). Most of the RE1s we identified are conserved in their sequence and location relative to the miRNA gene in question. All the RE1s we identified in human could interact with REST in vitro; intriguingly, this assay suggested that miRNA RE1s have a range of affinities for REST, perhaps reflecting different levels of REST recruitment in vivo. This observation is most strikingly found in the three RE1s regulating mir-124a genes, which display highly divergent affinities for REST. Finally, REST is robustly recruited to all the endogenous RE1s identified in this study. Together, these data indicate that REST is an important regulator of multiple neuron-specific miRNAs.

There is no doubt that the multiple highly connected networks governing gene regulation at both transcriptional and post-transcriptional levels must be interconnected. By showing how miRNA expression is regulated by transcription factors such as REST, we are beginning to understand how post-transcriptional gene regulatory systems, mediated by small RNAs, and transcriptional regulatory systems mediated by transcription factors 'talk' to each other in both normal and dysfunctional states.

In summary, our data suggest a dual action of REST in HD. We propose that increased levels of nuclear REST in HD leads to both a direct repression of REST target genes such as *BDNF* and to an indirect activation of gene expression by increased repression of miRNA expression The latter is exemplified by loss of mir-132, in turn leading to higher levels of *p250GAP* mRNA. The future discovery of other target mRNAs of mir-132 and other miRNAs discussed in this study will lead to insights into the molecular pathology of HD.

Materials and methods

Identification of REST–target miRNAs

RE1s identified in the human genome by position-specific scoring matrix search (Johnson et al., 2006) were manually cross-referenced to locations of known miRNA genes contained in the *MicroRNA Registry* (Griffiths-Jones, 2004).

Cell lines and HD mouse tissue

NS5 were cultured as described in Conti et al. (2005). Frozen brain tissues were obtained from R6/2 transgenic mice (Mangiarini et al., 1996). $Hdh^{7/7}$ cells were cultured and infected with recombinant adenovirus as described in Zuccato et al. (2007).

Human brain tissue

Human post-mortem brain tissues (parietal cortical tissues) were obtained from the Massachusetts General Hospital (MGH; Charleston, MA) and from the Harvard Brain Tissue Resource Centre (HBTRC; Belmont, MA).

Chromatin immunoprecipitation (ChIP)

ChIP DNA was prepared from cultured NS5 as described in Greenway et al. (2007). Immunoprecipitation was carried out using the R2174 anti-REST serum, as well as an appropriate pre-immune serum. Purified anti-REST and control ChIP DNA was interrogated by the quantitative real time PCR (qPCR) on the iCycler system (Biorad), using primers designed to flank the indicated RE1s within 600 bp. DNA concentrations were assessed by reference to a standard curve of serial dilutions of genomic DNA. ChIP enrichments

were calculated for each sample by dividing the R2174 immunoprecipitation by the equivalent pre-immune value.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described in Johnson et al. (2006), using serial dilutions of unlabelled competitor custom-synthesised oligodeoxynucleotides representing the RE1s indicated.

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from dissected tissue by crushing in liquid nitrogen, followed by purification using Tri-Reagent (Sigma) according to manufacturer's protocols. Subsequently, distinct protocols were used to measure levels of mRNAs and miRNAs. For mRNA quantitation, 1 μg of total RNA was reverse transcribed using MMLV RTase (Promega) according to manufacturer's protocols, using oligo dT and random hexamers. Resulting cDNA was purified and interrogated in qPCR for 40 cycles using intron-spanning primers. mRNA levels were evaluated by the ΔC_t method relative to the housekeeping gene, cyclophilin.

miRNAs were quantitated by the technique of Schmittgen et al. (2004). 1 μg of total RNA was reverse transcribed at 60 °C using Transcriptor RTase (Roche) according to manufacturer's protocol, with a cocktail of primers designed antisense to the stem loop of pre-miRNAs under study (primers were designed using the criteria described in Schmittgen et al., 2004). Resulting cDNA was purified and interrogated in qPCR for 40 cycles using primers designed sense and antisense to the pre-miRNA stem loop, with the PCR cycle as described by Schmittgen et al. miRNA levels were evaluated by the ΔC_t method relative to the small RNA, U6.

Acknowledgments

This work was supported by the Wellcome Trust and RJ was a Wellcome Trust PhD student.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2007.11.001.

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