Regulation of RE1 Protein Silencing Transcription Factor (REST) Expression by HIP1 Protein Interactor (HIPPI)*

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Background: HIPPI, along with its molecular partner HIP1, can regulate transcription of the caspase-1 gene.

Results: HIPPI binds to the promoter of REST and increases its expression in neuronal and non-neuronal cells and in a Huntington disease cell model.

Conclusion: HIPPI is a novel transcription regulator of REST.

Significance: This study provides a novel mechanistic interpretation of HD pathogenesis through HIPPI-mediated transcriptional regulation of REST.

Earlier we have shown that the proapoptotic protein HIPPI (huntingtin interacting protein 1 (HIP1) protein interactor) along with its molecular partner HIP1 could regulate transcription of the caspase-1 gene. Here we report that RE1-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) is a new transcriptional target of HIPPI. HIPPI could bind to the promoter of REST and increase its expression in neuronal as well as non-neuronal cells. Such activation of REST downstream-regulated expression of REST target genes, such as brain-derived neurotrophic factor (BDNF) or proenkephalin (PENK). The ability of HIPPI to activate REST gene transcription was dependent on HIP1, the nuclear transporter of HIPPI. Using a Huntington disease cell model, we have demonstrated that feasible interaction of HIP1 with mutant huntingtin protein resulted in increased nuclear accumulation of HIPPI and HIP1, leading to higher occupancy of HIPPI at the REST promoter, triggering its transcriptional activation and consequent repression of REST target genes. This novel transcription regulatory mechanism of REST by HIPPI may contribute to the deregulation of transcription observed in the cell model of Huntington disease.

Huntington disease is an autosomal dominant progressive neurodegenerative disorder of the central nervous system (CNS) characterized by uncontrolled movement, psychiatric abnormalities, and cognitive deficits (1). The molecular basis of the disease lies in the expansion of CAG repeats in the first exon of the huntingtin gene (HTT) (2). The mutated protein contains an abnormally long polyglutamine (poly(Q)) stretch at the N terminus, which is highly self-associative and forms intracellular aggregates (3, 4). Mutant HTT aggregates as well as the soluble form of the protein are believed to be toxic and deregulate various cellular processes and ultimately induce death of the striatal neurons possibly by apoptosis (5, 6).

The exact mechanism by which mutant HTT gives rise to the pathogenic condition is not clearly known. The dominant pattern of inheritance of the disease suggests a toxic gain of function of the mutant protein (7). However, loss of wild type protein function has also been implicated in the disease (8). In vitro and in vivo studies indicate that wild type HTT has a prosurvival role. Overexpression of wild type HTT ameliorates mutant HTT-induced toxicity in cells (9, 10), whereas depletion of wild type HTT renders the cells more sensitive to apoptotic insults (11). Direct evidence of wild type HTT loss of function comes from the work of Zuccato et al. (12), who demonstrated that wild type HTT but not the mutant protein promotes transcription of BDNF, a neurotrophin required for survival and function of cortico-striatal neurons. Expression of BDNF has been reported to be down-regulated in neurons affected with HD (12). Subsequent studies have established that BDNF transcription is negatively regulated by REST, a transcriptional repressor that binds to the repressor element 1 (RE1) site present in the BDNF promoter (13). Wild type HTT sequesters REST in the cytoplasm by forming a multiprotein complex with RILP (REST/NRSF-interacting LIM domain protein), HAP1 (huntingtin-associated protein 1), and dynactin p150<sup>Glued</sup> (14), leading to normal BDNF transcription. Mutation in HTT, however, disrupts this complex, resulting in increased nuclear accumulation of REST and down-regulation of BDNF expression along with other essential neuronal genes (13, 15).

HIPPI-HIP1-mediated aberrant activation of the apoptotic cascade provides another instance of loss of function of wild type HTT (16). HIP1 is a proapoptotic protein that preferentially interacts with wild type HTT (17). Under diseased conditions when HTT is mutated, HIP1 dissociates from mutant HTT and interacts with HIPPI, a novel pseudo-death effector domain (pDED)-containing protein. HIPPI-HIP1 heterodimer then recruits procaspase-8, followed by its activation and induction of the downstream apoptotic cascade (16, 18).

While investigating the HIPPI-mediated apoptotic pathway in detail, Majumder et al. (18) observed that HIPPI positively...
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regulates the expression of several caspases, including caspase-1, -3, -7, and -8 in a cell model. Hippi directly interacts in vitro and in vivo with a specific 9-bp DNA sequence, AAGACATG, present at the putative promoter of the caspase-1 gene (19, 20). The protein lacks any conventional nuclear localization signal (NLS) and is carried to the nucleus by the NLS present at the C terminus of HIP1 (21). The Hippi-Hippi heterodimeric complex is then recruited to the promoter of caspase-1 to regulate its transcription (21).

The emergence of Hippi as a new transcription regulator for caspase-1 motivated us to investigate the global change in gene expression brought about by Hippi. Transcriptional profiling of HeLa cells in the presence of exogenous Hippi revealed elevated expression of REST together with other genes. In our present study, we report that Hippi could directly bind to the upstream sequence of REST to increase its expression in neuronal and non-neuronal cells. We also have shown that, due to lower affinity of HIP1 for mutant HTT, Hippi and HIP1 predominantly localized to the nucleus in STHdhQ111/Q111 cells, a cell model of HD (22), compared with STHdhQ7/Q7 cells. Finally, we have demonstrated that occupancy of HIPPI in the REST promoter was higher in STHdhQ111/Q111 cells compared with STHdhQ7/Q7 cells, which led to increased expression of REST and a consequent up-regulation of REST-mediated neuronal gene repression in STHdhQ111/Q111 cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals—**Geneticin, Hygromycin, and anti-β-actin antibody (A2228, clone AC-74, lot number 107K4791) were obtained from Sigma. The anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase and protein A-agarose beads were purchased from Bangalore Genei (India). Anti-HIP1 antibody was purchased from Novus Biologicals (NB300-204, IB11, lot number A), anti-HIPPI (ab5205-100, lot number 63362), and anti-lamin B antibodies (ab16048-25, lot number 393854) were purchased from Abcam. Anti-REST antibody (sc-25398) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immobilon-P Transfer membrane was from Millipore; and restriction enzymes (BamHI, SalI, HindIII, and XbaI) were obtained from BioLine; and restriction enzymes (BamHI, Sall, HindIII, BglII, and KpnI) were from Promega. Protease inhibitor mixture was purchased from Roche Applied Science. TRIZol reagent was obtained from Invitrogen. Other molecular biology grade fine chemicals were procured locally.

**Construction of Plasmids—**Constructions of GFP-Hippi, GFP-wpDED (coding for amino acids 335–429 of full-length Hippi) and GFP-mpDED (R393E) clones have been described earlier (21). Construction of wild type huntingtin exon 1 cloned in DsRedC1 vector (DsRed-16Q) was described earlier (23). The HIPPI-HIP1 heterodimeric complex is then recruited to the promoter of the caspase-1 gene (19, 20). The protein lacks any conventional nuclear localization signal (NLS) and is carried to the nucleus by the NLS present at the C terminus of HIP1 (21). The Hippi-Hippi heterodimeric complex is then recruited to the promoter of caspase-1 to regulate its transcription (21).

**Cell Culture and Transfection—**HeLa and Neuro2A cells were routinely grown in minimal essential medium (Himedia, Mumbai, India) supplemented with 10% fetal bovine serum (Biowest) at 37 °C in 5% CO2 atmosphere under humidified conditions. Immortalized striatal HD cell lines, STHdhQ111/Q111 and STHdhQ7/Q7 cells (22) were grown in DMEM (Himedia) supplemented with 10% FBS and 400 µg/ml G418 (Invitrogen) at 33 °C in humidified conditions and 5% CO2. Transfection of cells was performed using Lipofectamine 2000 (Invitrogen). Unless otherwise mentioned, for the single transfection experiment, 2.5 µg (60-mm plate) or 5 µg (100-mm plate) of DNA constructs as well as 5 or 10 µl of Lipofectamine 2000, respectively, were used. After 24 h, transiently transfected cells were checked for transfection efficiency by monitoring either GFP or DsRed expression under a fluorescence microscope and were used for experiments. Transfection efficiency varied from 70 to 90%.

**Knockdown of HIP1 and HIPPI by siRNA—**Knockdown of HIP1 by gene-specific siRNA in HeLa and Neuro2A cells was described earlier (21).

For siRNA mediated knockdown of Hippi in Neuro2A and STHdhQ111/Q111 cells, the mouse Hippi cDNA sequence was submitted to the online GenScript siRNA designing tool. Among the various sequences retrieved by the software for siRNA, we choose the sequence 537-GTACGAGTTAGAATTAGACA-519. The scrambled sequence (5’-GACCGTGTACACAGATTAT-3’) for the siRNA was also designed using the GenScript sequence scrambler tool. The complete sequence that was inserted into the expression vector pRNA-U61Hygro (Genescript) was 5’-GTACGAGTTAGAATTAGACAATTCAAGAGATGTCAGTCCTAATCGTGAC-3’ (designated “HippiSi”) with a termination signal and appropriate restriction site linkers (BamHI and HindIII; not shown) and an insert for loop formation (underlined). The entire sequence for the scramble siRNA was 5’-GACCGGTGACACAGATTATTTGATATCCGATAATCTTGGTCACGCGTC-3’ (designated “HippiScr”). Both HippiSi and HippiScr were cloned in our laboratory using the restriction enzymes BamHI and HindIII.

HippiSi and HippiScr clones were transfected in Neuro2A and STHdhQ111/Q111 cells using Lipofectamine 2000 (Invitrogen) using a protocol provided by the manufacturer. Transfected cells were selected for hygromycin resistance. Knockdown of Hippi was confirmed by RT-PCR using sequence-specific primers for Hippi. A list of various cell lines generated either by stable or transient transfection is given in [supplemental Table S1](#).

**Subcellular Fractionation and Western Blot Analysis—**Methods for subcellular fractionation and Western blot were essentially the same as described previously (21). Briefly, cells grown in 100-mm Petri dishes were washed with ice-cold PBS and harvested at 300 × g for 3 min at 4 °C. Cytosol was extracted using cytosol extraction buffer (50 mM Tris-HCl, pHi 7.5, 10 mM NaCl, 2 mM EDTA, 1 mM PMSF, and 1% protease inhibitor (encompassing the Hippi binding site from position −4430 to −4422) in pGLO3 basic vector (designated as Luc-RESTups) between the restriction sites of BglII and KpnI. The primer sequences used for constructing this clone are given in [supplemental Table S1](#).

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mixture, 0.25% Nonidet P-40). The nuclear pellet was suspended in nuclear extraction buffer (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 2 mM EDTA, 1 mM PMSF, and 1× protease inhibitor mixture) and kept on ice for 40 min followed by centrifugation at 13,000 × g for 20 min at 4 °C. The supernatant was kept as nuclear extract. For preparing whole cell extracts, cell lysis was carried out using lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, and PMSF with 100 µg/ml final concentrations). Protein concentration was measured by Bradford assay. The samples were boiled with SDS gel loading buffer, run on SDS-polyacrylamide gel, transferred to membrane, and probed with antibodies. β-Actin was used as an internal control for cytoplasmic and whole cell extracts, whereas lamin B was used as loading controls for nuclear fractions. Each experiment was repeated three times.

Integrated optical density (IOD) of each band was calculated using Image Master VDS software (Amersham Biosciences). Whenever necessary, IOD was normalized with that of the loading control.

**Chromatin Immunoprecipitation (ChIP) Assay—Methods**

used for the ChIP experiments were described earlier (21). In brief, STHdhQ111/Q111 and STHdhQ7/Q7 cells expressing endogenous HIPPI and HeLa and Neuro2A cells expressing exogenous GFP-Hippi or GFP-wpDED or GFP-mpDED were cross-linked with 1.1% formaldehyde for 10 min at room temperature. This cross-linking reaction was stopped using 125 mM glycine. Cells were washed with ice-cold PBS and harvested at 300 × g for 3 min at 4 °C. Cytosol was extracted with Buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 1 mM PMSF). Nuclei were harvested at 13,000 × g for 10 min at 4 °C, and the pellet was resuspended in breaking buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS, and 2% Triton X-100) and sonicated twice (two pulses of 10 s each). Contents were then centrifuged. Triton buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100) was added to the nuclear extract. The immunoprecipitation reaction was carried out with either anti-HIPPI antibody (for STHdhQ111/Q111 and STHdhQ7/Q7 cells and cells expressing GFP-Hippi) or anti-GFP antibody (for GFP-wpDED- or GFP-mpDED-transfected Neuro2A cells) followed by the addition of BSA-soaked Protein G-agarose beads. The immunoprecipitated complex was washed, followed by decross-linking, phenol chloroform extraction, and ethanol precipitation of the DNA. PCR amplification of the eluted DNA was carried out using sequence-specific primers for REST. The primer sequences are given in supplemental Table S1.

**Semi-quantitative RT-PCR—Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Two µg RNA was reverse transcribed using random hexamer primer (Fermentas) and murine leukemia virus reverse transcriptase (Fermentas). Semi-quantitative RT-PCR was carried out using Red TaqDNA polymerase (Bioline) for 35 cycles. Expression of β-actin was taken as endogenous control. The densitometry of the bands was carried out using Image Master VDS software (Amersham Biosciences).**

The gene-specific primers used for RT-PCR were designed using Primer Express software (Applied Biosystems). Primer sequences used for amplification of the target genes are given in supplemental Table S1.

**Luciferase Assay**—The method for the luciferase assay was described previously (24). Briefly, cells grown in 35-mm plates were transfected with 500 ng of pGL3 construct (empty pGL3 vector orLuc-RestTups construct) and GFP construct (empty GFP vector, GFP-Hippi, GFP-wpDED, or GFP-mpDED). Twenty-four h after transfection, the luciferase assay was carried out using the luciferase reporter assay system (Promega) according to the manufacturer’s protocols and detected by a Sirius Luminometer (Berthold Detection Systems). Five µg of protein was used for each assay. Transfection efficiency was normalized by measuring GFP fluorescence at 510 nm (Fluoromax-3, Jobin Yvon Horiba).

**Statistical Analysis**—For statistical analysis, an unpaired t test was carried out to compare the means of two experimental groups using the on-line software GraphPad QuickCalc.

**RESULTS**

**Induction of REST Gene Transcription by HIPPI in HeLa Cells—**

From microarray experiments, we observed that exogenous expression of HIPPI increased REST expression together with that of many other genes in HeLa cells. To validate this observation, we measured REST expression in GFP-Hippi-expressing HeLa cells by semi-quantitative RT-PCR (Fig. 1, A and B). REST expression was 2-fold increased (p = 0.0002, n = 3) in GFP-Hippi-transfected HeLa cells (supplemental Table S2, Hippi) compared with the parental HeLa cells (HeLa). Our earlier work has established HIP1 as the nuclear translocator of HIPPI (21). We, therefore, checked the expression of REST in the presence of HIPPI in HIP1-knocked down HeLa cells (Hip1SiHi). Generation of HIP1-knocked down HeLa cells has already been reported (21). HIP1 knockdown prevented nuclear entry of HIPPI, and as a result, HIPPI-mediated up-regulation of REST was lost in Hip1SiHi cells (Fig. 1, A and B). Our result, thus, suggests that up-regulation of REST by HIPPI was dependent on the presence of HIP1. To address the functional consequence of such HIPPI-mediated up-regulation of the REST gene in HeLa cells, we measured the expression level of PENK, a gene negatively regulated by REST (15). REST expression was down-regulated (p = 0.002, n = 3) in GFP-Hippi-transfected HeLa cells compared with parental HeLa cells (Fig. 1, A and B). On the contrary, PENK expression in Hip1SiHi cells did not alter significantly from the corresponding untransfected cells (Hip1Si). Thus, HIPPI-mediated REST up-regulation resulted in repression of REST downstream target gene in HeLa cells. Further analysis of the REST promoter region revealed the presence of a putative 9-bp HIPPI binding site (20) from position −1264 to −1256 in the upstream promoter of the human REST gene (Fig. 1C). To test whether this binding site was functional, we carried out chromatin immunoprecipitation from GFP-Hippi-expressing HeLa cells. The result showed that HIPPI could bind to this site (Fig. 1D, n = 3), indicating that REST could be a direct transcriptional target of HIPPI in HeLa cells.

**HIPPI Enhances REST Gene Transcription by Binding to Its Upstream Promoter in Neuro2A Cells—**

To investigate the effect of HIPPI on neuronal REST expression, we transfected GFP-
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Hippi in the mouse neuroblastoma cell line, Neuro2A (N2A). REST expression was 2-fold higher \( (p = 0.0001, n = 3) \) in HIPPI-transfected N2A cells (N2A Hippi) compared with the parental N2A cells (Fig. 2, A and B). Because REST is a transcription repressor, such up-regulation of REST expression by HIPPI would alter the expression of its downstream targets. Therefore, we measured the expression of BDNF, a RE1-containing gene known to be targeted by REST (15). REST regulates BDNF expression from BDNF promoter II (13). We observed that BDNF expression was reduced \( (p = 0.02, n = 3) \) in N2A Hippi cells compared with N2A (Fig. 2, A and B). However, HIPPI was unable to increase REST expression in HIPPI-knocked down N2A cells (Fig. 2, A and B, N2AHIP1SiHi). Knockdown of HIPPI in Neuro2A cells has already been shown (21).

To address this further, we knocked down endogenous HIPPI in Neuro2A cells using specific siRNA. Knockdown of HIPPI was confirmed by RT-PCR, and specificity of knockdown was checked by using scrambled siRNA control (supplementary Fig. S1, A and B, N2Asi and N2AsiSc). HIPPI knockdown also resulted in down-regulation \( (p = 0.006, n = 3) \) of endogenous REST (supplementary Fig. S1, A and C) and consequent up-regulation of BDNF \( (p = 0.02, n = 3) \). Next, we attempted to recover HIPPI expression in HIPPI-knocked down cells by transiently transfecting GFP-Hippi in N2Asi cells. HIPPI expression recovery also recovered REST expression \( (p = 0.0003, n = 3) \), suggesting a specific nature of regulation (supplementary Fig. S1, A and C, N2ASiHi). We then searched the upstream promoter of the mouse REST gene for the presence of the HIPPI binding motif. A putative 9-bp HIPPI binding sequence, 5’-AAAGACATT-3’, was present at positions −4430 to −4422 (Fig. 2C). Chromatin immunoprecipitation from GFP-Hippi-transfected N2A cells indicated association of HIPPI with this promoter (Fig. 2D, \( n = 3 \)). To investigate it further, we determined the level of REST and BDNF expressions at different time points after HIPPI transfection in N2A cells. The rationale behind this is that if REST is a direct target of HIPPI, then one would expect REST up-regulation to occur at a much earlier time point compared with BDNF down-regulation, which is downstream of REST up-regulation. Results showed that REST was significantly up-regulated \( (p = 0.02, n = 4) \) within 3 h of GFP-Hippi transfection (Fig. 2E), reached its peak within 6 h \( (p = 0.001, n = 4) \), and remained almost at the same level until 24 h of transfection \( (p = 0.0001, n = 4) \). BDNF expression, on the other hand, was unaltered until 12 h post-transfection, but significant reduction was observed after 14 h \( (p = 0.006, n = 4) \) and at 24 h \( (p = 0.02, n = 4) \) of transfection.

Similar early response (peaks within 3–6 h) was obtained with caspase-1 gene expression (supplemental Fig. S2), an already reported direct transcriptional target of HIPPI (21). Thus, REST up-regulation by HIPPI was an early event indicating that REST could be a direct transcriptional target of HIPPI.

Effect of HIP1 and HIPPI Mutations on REST and BDNF Expression in Neuro2A Cells—It has been reported earlier that HIPPI does not contain an NLS, and its nuclear entry is mediated by HIP1 (21). A mutation (R1005E) in the NLS domain of HIP1 prevents nuclear entry of HIPPI, and consequently HIPPI-driven transcription regulation of caspase-1 is blocked (21). To check whether this could be true for REST also, we first measured the level of REST and BDNF expressions in N2A cells stably overexpressing wild type HIP1 protein (N2A HIP1). Overexpression of wild type HIP1 increased nuclear accumulation of HIPPI (21), and as a result, REST expression was elevated \( (p = 0.001, n = 3) \), and BDNF expression was reduced \( (p = 0.02, n = 3) \) in N2A HIP1 cells compared with the parental N2A (Fig.
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3A). However, cells stably overexpressing the NLS mutant of HIP1 (N2A 1005E) showed significant lowering of REST expression ($p = 0.0008$, $n = 3$) and consequently BDNF up-regulation ($p = 0.01$, $n = 3$) compared with N2A cells (Fig. 3A). Therefore, the presence of intact NLS in HIP1 was a prerequisite of HIPPI-mediated up-regulation of REST.

HIPPI interacts with DNA through its pDED (21). A mutation in the pDED replacing an arginine residue at position 393 of the full-length protein by glutamic acid (R393E) abolishes the DNA binding activity of HIPPI (21). To check the effect of such a mutation on REST expression, we transiently transfected GFP-tagged wild type pDED of HIPPI (N2A wpDED) and R393E mutant pDED of HIPPI (N2A mpDED) in N2A cells. Expression of wild type pDED was as effective as the full-length HIPPI protein in increasing REST expression ($p = 0.03$, $n = 3$) and BDNF down-regulation ($p = 0.0004$, $n = 3$) in N2A cells (Fig. 3B). On the contrary, expression of the mutant pDED was unable to alter either of the genes (Fig. 3B). A similar trend was observed in the protein level expression of REST, as detected by Western blot analysis (Fig. 3, C and D). Wild type HIP1, HIPPI, and wpDED of HIPPI were able to increase REST expression significantly in Neuro2A cells (designated as “Hip1,” “Hippi,” and “wpDED,” respectively), whereas the mutant pDED (mpDED) could not. The NLS mutant of HIP1 (1005E), on the other hand, showed significant reduction in REST expression compared with either parental N2A cells or N2A cells overexpressing wild type HIP1. Next to see the DNA binding of these two proteins (wpDED and mpDED), we carried out luciferase reporter assay with the upstream sequence of the REST gene encompassing the HIPPI binding site (Luc-RESTups). Expression of full-length GFP-HIPPI in N2A cells (N2A Hippi) increased luciferase activity by ~2-fold ($p = 0.01$, $n = 4$) compared with empty GFP-transfected N2A cells (Fig. 3E, N2A GFP), indicating the interaction of HIPPI with REST upstream sequence followed by transcriptional activation. A similar increase in luciferase activity ($p = 0.002$, $n = 4$) was observed with wild type pDED of HIPPI (Fig. 3E), reflecting its intact DNA binding and transactivation ability. Mutant pDED HIPPI, on the other hand, was compromised in its ability to interact with REST upstream, and consequently luciferase activity remained at the level of N2A GFP cells. This was also evident from the chromatin immunoprecipitation result (Fig. 3F, $n = 3$). The ChIP experiment showed that wild type pDED HIPPI (wpDED) could immunoprecipitate REST upstream DNA from N2A cells where as mutant pDED (mpDED) could not.

FIGURE 2. HIPPI-mediated REST up-regulation and its interaction with REST promoter in Neuro2A cells. A, gel image representative of three ($n = 3$) independent experiments for sqRT-PCR of REST and BDNF expressions in parental Neuro2A cells (N2A), GFP-Hippi-transfected Neuro2A cells (N2AHippi), HIP1-knocked down Neuro2A cells (N2AHIP1Si), and GFP-Hippi-transfected HIP1-knocked down Neuro2A cells (N2AHIP1Sih). Expression of β-actin was taken as endogenous control. B, bar graph representing the mean IOD of bands obtained in A. The expression levels of REST and BDNF in a sample were normalized by the corresponding β-actin expression level. -Fold change was calculated by considering the relative expression level of target genes in N2A to be 1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). C, presence of the HIPPI binding site in the upstream sequence of the mouse REST gene (ENSMUSG00000029249). Sequence was retrieved from ENSEMBL Biomart. Putative 9-bp HIPPI binding sequence (~4430 to ~4422) is underlined. D, in vivo interaction of HIPPI with the REST promoter in Neuro2A cells detected by ChIP assay. Immunoprecipitation was carried out with anti-HIPPI antibody, and precipitated DNA was PCR-amplified using REST upstream sequence-specific primers. The lane markings are as described in the legend to Fig. 1D. E, sqRT-PCR ($n = 3$) for REST and BDNF expression in GFP-Hippi-transfected Neuro2A cells at different time points after transfection. β-Actin expression level was considered as endogenous control. -Fold change was calculated by considering the relative expression level of REST and BDNF in control untransfected N2A cells (cont) to be 1. Error bars, S.D.
Increased Nuclear Abundance of Endogenous HIPPI and HIP1 Increases REST Expression in STHdhQ111/Q111 Cells—To check whether feeble interaction between HIP1 and mutant HTT might result in increased nuclear accumulation of HIPPI-HIP1, we measured the cytoplasmic and nuclear content of the two proteins in STHdhQ111/Q111 and STHdhQ7/Q7 cells. The relative expression levels of HIPPI and HIP1 did not vary significantly in the two cell lines (supplemental Fig. S3). However, it was observed that nuclear fraction of HIP1 was increased (p < 0.02, n = 3) from 40% in STHdhQ7/Q7 cells to 60% in STHdhQ111/Q111 cells (Fig. 4, A (left) and B). Similarly, nuclear HIPPI content increased (p < 0.02, n = 3) to 57% in STHdhQ111/Q111 cells from 43% in STHdhQ7/Q7 cells (Fig. 4, A (left) and C). To test whether expression of wild type HTT could prevent such increased nuclear localization of HIPPI and HIP1 in STHdhQ111/Q111 cells, we exogenously transfected DsRed-16Q in both of the cell lines. In STHdhQ7/Q7 cells, nuclear localization of HIPPI and HIP1 decreased in the presence of DsRed-16Q (Fig. 4, A–C) compared with the parental STHdhQ7/Q7 cells, but the change was not statistically significant (p < 0.07, n = 3). This could be because of the fact that in presence of wild type HTT in STHdhQ7/Q7 cells, HIP1 remained mostly cytoplasmic; the addition of more wild type HTT fragments therefore exhibited only a small change in HIP1 subcellular distribution. However, in STHdhQ111/Q111 cells, there was a significant accumulation of both HIP1 (p = 0.001, n = 3) and HIPPI (p = 0.008, n = 3) in the cytoplasm following DsRed-16Q expression (Fig. 4, A–C).
Thus, although the expression levels of HIPPI and HIP1 remained unchanged in STHdh<sup>Q111/Q111</sup> and STHdh<sup>Q7/Q7</sup> cells, subcellular distribution of the two proteins altered significantly in the two cell lines, nuclear content of both the proteins being greater in STHdh<sup>Q111/Q111</sup> cells. This indicated that HIPPI-mediated transcriptional activation would be greater in STHdh<sup>Q111/Q111</sup> cells. To investigate that possibility, we measured the relative transcript levels of REST and BDNF in the two cell lines. REST expression was ∼2.5-fold higher (p = 0.0008, n = 5) in STHdh<sup>Q111/Q111</sup> cells (Q111/111) compared with STHdh<sup>Q7/Q7</sup> cells (Q7/7), whereas BDNF expression was reduced by ∼40% in STHdh<sup>Q111/Q111</sup> cells (p = 0.01, n = 4).

At the protein level, REST expression was increased 2-fold in Q7/7 cells compared with Q7/7 cells (Q7/7Hi), whereas BDNF expression was reduced by 40% in STHdh<sup>Q111/Q111</sup> cells (Q7/7Hi). Due to increased nuclear localization of REST in STHdh<sup>Q111/Q111</sup> cells. Our data suggest that increased expression of REST along with its increased nuclear localization might contribute to REST-mediated down-regulation of BDNF in STHdh<sup>Q111/Q111</sup> cells. Ectopic expression of HIPPI in STHdh<sup>Q7/Q7</sup> cells (Q7/7Hi) increased REST expression, but the change was not significant (Fig. 4D). This could be due to the unavailability of HIP1 to transport excess overexpressed HIPPI.
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FIGURE 5. Relative occupancy of HIPPI in the REST promoter is greater in STdhQ111/Q111 cells. A, luciferase reporter assay (n = 4) of the REST upstream cloned in pGL3 vector in empty GFP vector-transfected STdhQ7/Q7 (Q7/Q7) cells, GFP-Hippi-transfected STdhQ7/Q7 (Q7/Hippi) cells, empty GFP vector-transfected STdhQ7/Q7 (Q7/H11011) cells, and GFP-Hippi transfected STdhQ7/Q7 (Q7/H1111) cells. Luciferase activity of the above cells was normalized by the luciferase activity of the corresponding empty pGL3 vector-transfected cells. Error bars, S.D. The statistical significance level between various experimental pairs is shown (*, p < 0.05; **, p < 0.01; ***, p < 0.001). B, comparative ChIP analysis showing relative occupancy of endogenous HIPPI in the REST promoter was greater in STdhQ111/Q111 cells compared with STdhQ7/Q7 cells. Immunoprecipitation was carried out with anti-HIPPI antibody, and the immunoprecipitated DNA was PCR-amplified using primers specific for REST upstream sequence. The lane markings are as described earlier. C, bar graph representing the mean IOD (n = 2) of PCR bands obtained in B. The IOD for the −Ab lane was normalized by the IOD of the corresponding input (in) lane. Fold change was calculated by considering the normalized IOD of STdhQ7/Q7 cells as 1.

DISCUSSION

In the present study, we have identified HIPPI as a novel transcription regulator for REST. Using both neuronal and non-neuronal cell lines, we have demonstrated that exogenous expression of HIPPI increased expression of REST by binding to the upstream promoter of the gene. The ability of HIPPI to regulate REST expression was dependent on HIP1 because HIPPI-mediated REST up-regulation was lost in HIP1-knockdown down cells or cells expressing the NLS mutant of HIP1. We also report that altered nuclear and cytoplasmic distribution of endogenous HIPPI and HIP1 in STdhQ111/Q111 cells led to higher occupancy of HIPPI in the REST promoter and consequently increased its expression in these cells. Expressions of two known targets of REST (viz. PENK and BDNF) were also decreased in the presence of HIPPI in our study, as an effect of REST up-regulation.

The transcription repressor REST regulates myriads of protein coding and noncoding genes (26–29). Involvement of REST in neuronal development is well studied (30, 31). REST expression remains high in neuronal progenitor cells, leading to repression of neuronal genes. However, during terminal differentiation of progenitor cells to mature neurons, REST expression is turned off, allowing expression of the essential neuronal genes (32). Recent work has demonstrated the role of the functional REST regulatory network in maintaining pluripotency of embryonic stem cells (28). REST is, therefore, a master regulator that maintains cells in an undifferentiated state. Although a large number of studies have been carried out to elucidate REST function, studies depicting control of REST gene expression are limited. In neuronal cells, REST expression is activated by YY1, DRYK1A, and Wnt/Tcf signaling (33–35). Recently, transcription factor Sp1 has been shown to regulate REST expression in undifferentiated NG108 cells as well as in a mouse model of HD (36). Additionally, a double negative feedback loop involving REST-dependent expression of microRNA 9 and
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9e controls REST expression in HD (37). Our initial observation that exogenous expression of HIPPI increased REST expression in HeLa cells in a microarray study prompted us to investigate whether HIPPI could act as a transcription regulator of REST. Overexpression of HIPPI in both HeLa and Neuro2A cells upregulated REST expression in these cells. Knockdown of HIP1, the nuclear transporter of HIPPI, or overexpression of NLS-mutated HIP1 prevented this activation, indicating that nuclear translocation of HIPPI was essential for HIPPI-mediated REST up-regulation. Analysis of REST promoter sequence revealed the presence of a 9-bp HIPPI binding site in the upstream promoter of the REST gene. Using a luciferase reporter assay and chromatin immunoprecipitation, we confirmed that HIPPI could bind to this site and induce REST gene transcription. To address the functional consequence of this regulation, we measured expression of two REST target genes (viz. PENK and BDNF) under different experimental conditions. A consistent negative correlation between REST and PENK/BDNF expression was observed in the study, suggesting that HIPPI-mediated transcription activation of REST could affect the downstream cellular processes.

Involvement of transcription deregulation is well documented in HD. Several mechanisms for transcription deregulation have been elucidated, which include sequestration of cellular transcription factors and co-factors in mutant HTT aggregates, nuclear translocation, and aberrant nonspecific DNA binding of pathogenic N-terminal fragment of mutant HTT and also loss of wild type HTT-mediated transcriptional control (38). Involvement of REST in HD falls in the last category (12). The interaction of wild type HTT with REST through RILP, HAP1 (huntingtin-associated protein 1), and dynactin p150Glued sequesters the complex in the cytoplasm (14), allowing expression of essential neuronal genes that are otherwise targets of REST-mediated repression (12, 13). Mutation in HTT, however, disrupts this interaction, which facilitates nuclear entry of REST and formation of the repressor complex on the RE1 site followed by target gene silencing. Many of the REST target genes have been shown to be down-regulated in HD (15). Moreover, there are reports indicating an overall increase in REST expression in various models of HD (25, 36, 39). The importance of REST in regulating HD pathogenesis inspired us to investigate whether HIPPI-mediated up-regulation of REST plays any role in HD. Feeble interaction of HIP1 with mutant HTT results in increased heterodimerization of HIP1 with HIPPI (16). Keeping this fact in mind, we hypothesized that increased heterodimerization of HIPPI-HIP1 might increase the nuclear content of the two proteins, which in turn could activate REST transcription in HD. Using STHdhQ111/Q111 cells, we observed that it was indeed the case. Nuclear localization of both HIPPI and HIP1 was significantly enhanced in STHdhQ111/Q111 cells compared with the wild type STHdhQ7/Q7 cells. As a result, occupancy of HIPPI in the REST promoter was increased 2-fold, resulting in induction of REST gene expression. The observation could be reversed by overexpressing wild type HTT exon 1 (DsRed16Q) in STHdhQ111/Q111 cells, where nuclear fractions of HIPPI and HIP1 were significantly reduced compared with untransfected STHdhQ111/Q111 cells. Wild type HTT sequesters HIP1 in the cytoplasm, preventing its nuclear entry, along with HIPPI, which in turn repressed REST expression. On the other hand, expression of HYPK (huntingtin-interacting protein K), a chaperone-like protein capable of reducing mutant HTT aggregates (23), was unable to reduce REST expression in STHdhQ111/Q111 cells (data not shown), indicating the process to be independent of mutant HTT aggregation. Therefore, our results demonstrate that HIPPI-mediated aberrant transcriptional activation of REST in STHdhQ111/Q111 cells could be

FIGURE 6. Schematic representation of the involvement of HIPPI-HIP1 in the pathogenesis of HD. A, the stronger interaction between wild type HTT and HIP1 prevents formation of HIPPI-HIP1 heterodimer, thereby blocking nuclear localization of HIPPI. REST expression remains in the basal level. Also wild type HTT can sequester REST protein in the cytoplasm. This leads to activation of REST target genes like BDNF, which is essential for the survival of striatal cells. Additionally, the absence of HIPPI-HIP1 heterodimer blocks caspase-8-mediated apoptosis induction, leading to cell survival. B, mutation in HTT favors release of HIP1 from the HTT-HIP1 complex and facilitates formation of HIPPI-HIP1 heterodimer. The heterodimer enters into the nucleus, where HIPPI interacts with the REST promoter to increase its expression. Unlike wild type HTT, mutant HTT cannot sequester REST protein in the cytoplasm. Thus, increased expression along with increased nuclear localization of REST causes repression of the BDNF gene. Also, formation of HIPPI-HIP1 heterodimer triggers activation of caspase-8-mediated apoptosis, leading to cell death.
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attributable to the loss of wild type HTT function in these cells. This was further corroborated by the observation that overexpression of HIPPI in the wild type STHdhQ7/Q7 cells could not activate REST transcription significantly. Retention of HIPPI in the cytoplasm by wild type HTT created a deficiency of nuclear transporter for HIPPI in STHdhQ7/Q7 cells. Overexpression of HIPPI therefore exhibited no effect on REST expression.

Down-regulation of BDNF in cell and animal models of HD resulting from increased nuclear localization of REST has been reported previously by Zuccato et al. (12, 13). Our results predict an additional mechanism for REST activation and BDNF down-regulation in the HD cell model. The complete loss of wild type HTT-mediated sequestration of HIPPI in STHdhQ111/Q111 cells caused redistribution of HIPPI and HIP1 in the nucleus, leading to induction of REST gene expression. The elevated level of REST protein in the cytoplasm could not be sequestered by mutant HTT, as depicted by Zuccato et al. (13), resulting in increased nuclear localization of REST, which in turn could reduce BDNF expression in STHdhQ111/Q111 cells.

HIPPI may therefore participate in HD pathogenesis by two mechanisms (Fig. 6). First, increased HIPPI-HIP1 heterodimerization in the presence of mutant HTT may induce caspase-8-mediated apoptosis as reported by Gervais et al. (16). Second, the heterodimer may translocate to the nucleus, leading to activation of REST transcription, which in turn may repress prosurvival genes like BDNF and induce neuronal death (Fig. 6).

Based on our results, we show, for the first time, the HIPPI-mediated transcriptional regulation of the REST gene and establish its connection with HD pathogenesis. In addition, the work of Houde et al. (40) has shown that HIPPI knock-out mice are embryonic lethal and exhibit defects in the Sonic hedgehog (Shh) pathway. Shh is a trophic factor that promotes neurogenesis from adult mouse stem cells (41). It remains to be determined whether aberrant interaction of HIPPI and HIP1 in HD sequesters HIPPI from its normal function, resulting in alteration of the Shh pathway (40). Additionally, increased HIPPI-HIP1 interaction may induce transcriptional activation of the REST gene, leading to down-regulation of neuronal gene expression. Taken together, this study sheds light on a novel mechanistic interpretation of HD pathogenesis through HIPPI-mediated transcriptional regulation of REST.

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