NRSF/REST lies at the intersection between epigenetic regulation, miRNA-mediated gene control and neurodevelopmental pathways associated with Intellectual disability (ID) and Schizophrenia

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Genetic evidence indicates disrupted epigenetic regulation as a major risk factor for psychiatric disorders, but the molecular mechanisms that drive this association remain to be determined. EHMT1 is an epigenetic repressor that is causal for Kleefstra Syndrome (KS), a genetic disorder linked with neurodevelopmental disorders and associated with schizophrenia. Here, we show that reduced EHMT1 activity decreases NRSF/REST protein leading to abnormal neuronal gene expression and progression of neurodevelopment in human iPSC. We further show that EHMT1 regulates NRSF/REST indirectly via repression of miRNA and leads to aberrant neuronal gene regulation and neurodevelopment timing. Expression of a NRSF/REST mRNA that lacks the miRNA-binding sites restores neuronal gene regulation to EHMT1 deficient cells. Significantly, the EHMT1-regulated miRNA gene set not only controls NRSF/REST but is enriched for association for Intellectual Disability (ID) and schizophrenia. This reveals a broad molecular interaction between H3K9 demethylation, NSRF/REST regulation and risk for ID and Schizophrenia.

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INTRODUCTION

Genetic evidence points to an association of chromatin remodelers, mediators of epigenetic regulation, as a substantial risk factor for many common psychiatric disorders [1, 2]. Genome-wide association studies (GWAS) have revealed multiple risk loci for neurodevelopmental disorders (NDD) that are associated with genes encoding epigenetic regulators. These often encompass more than one condition, including Intellectual Disability (ID), autism spectrum disorders (ASD) and schizophrenia. Accordingly, alleles affecting epigenetic regulatory mechanisms are associated with a range of psychiatric symptoms, including cognitive deficits, autistic traits, and psychosis. Epigenetic-related risk alleles are linked with biological pathways that converge on chromatin regulation via control of nucleosome positioning and histone methylation, leading to altered gene transcription [3, 4].

Studies on high risk, loss of function (LOF) gene variants associated with NDD reinforce this view. Disruption of the SNF-2 family chromatin re-modellers CHD7 and CHD8 are strongly associated with ID and along with CHD2 confer risk for ASD [5]. Histone lysine methyltransferases (KMT) are key epigenetic regulators, and many are associated with NDD and psychiatric disorders. LOF mutations of MLL3 (KMT2C), MLL5 (KMT2E), ASH1L (KMT2H), SUV420H1 (KMT5B) and histone lysine demethylases (KDM), KDM5B and KDM6B are all associated with ASD [6–8]. LOF variants of the H3K4 methyltransferase SETD1A are associated with schizophrenia, developmental delay (DD) and ID [9]. Other KMT are linked to severe neurodevelopmental disruption and ID associated genetic syndromes; KMT2A with Wiedemann-Steiner Syndrome, KMT2D with Kabuki Syndrome and KMT1D with Kleefstra syndrome (KS). The latter is subject of this study and here referred by its common name, Euchromatic Histone-Lysine N-Methyltransferase 1 or EHMT1. KS is associated with autistic features, psychosis, and schizophrenia [10]. A recent study indicates an association of de novo postzygotic EHMT1 mutation and an ASD and neurocognitive dysfunctions in adults [11]. Although the genetic case for epigenetic regulation is well established, there is little knowledge of the downstream molecular mechanisms that link their actions to the underlying pathophysiology of the NDD. To address this question, we have investigated the mechanism by which reduced EHMT1 activity leads to an
altered neurodevelopmental programme in both isogenic cell models of Kleefstra syndrome and patient-derived iPSCs.

Canonical Kleefstra syndrome (KS) arises from a sub-telomeric microdeletion at 9q34, resulting in a heterozygous deletion of approximately ~700 kb [12]. This region contains at least five genes, including ZMYND19, ARRD1C, C9ORF37, EHMT1, and CACNA1B [12, 13], however, the core clinical phenotypes are driven by haploinsufficiency of EHMT1 [12]. Consistent with an epigenetic origin of KS, a broader Kleefstra syndrome phenotypic spectrum (KSS) is also associated with other chromatin modifiers, including MLML3 (KMT2C) [14]. EHMT1 is the primary enzyme for dimethylation of histone H3 at Lys9 residues (H3K9me2) [15] and is generally associated with transcriptional gene silencing [16]. Genetic manipulation of EHMT1 has been studied in Drosophila and in rodent models at molecular, cellular, and behavioural levels. Drosophila ehm1 mutants exhibit decreased dendrite branching of sensory neurons and impaired short and long-term memory that is reversed by restoring EHMT expression [17]. EHMT1−/− mice demonstrate cranial abnormalities, hypotonia and delayed postnatal growth [18]. Functionally they show deficits in fear extinction and novel object recognition [18–21]. In rodent primary neuron cultures EHMT1 regulates the dynamics of multiple neural processes, including synaptic scaling and response to addiction and stress [20, 22] and knockdown of Ehmt1 or the other KSS associated genes alter synaptic gene regulation and neuronal excitability. [23, 24]. To date, studies on KS patient-derived iPSC have focussed on differentiated, mature neurons, and like mouse studies they show altered neuronal activity, synaptic signalling, and network properties [25]. Here we identify a molecular biological pathway that is disrupted during early differentiation of human cellular models of KS. We show that EHMT1 regulates the transcriptional repressor NRSF/REST, a neuron-specific gene regulator [26–28]. This occurs via up-regulation of microRNA (miRNA)-mediated suppression of NRSF/REST protein synthesis. Analysis of the EHMT1-regulated miRNA expression profile, including miR-153, miR-26a and miR-142, indicates a broader association between EHMT1 regulated miRNA, NRSF/REST and both ID and schizophrenia. These gene regulatory abnormalities have substantial effects on neuronal gene expression and in vitro neurodevelopment, causing premature neurodifferntiation and neuronal dysfunction.

MATERIALS AND METHODS

Human iPSC culture and neuronal differentiation

The IB4 human iPSC line derived from the BJ fibroblast cell line (ATCC; CRL-2522) was used, unless indicated. HiPSC were grown on matrigel (Corning) in Essential 8™ medium (ThermoFisher scientific) at 37 °C, 5% CO2 [29]. Medium was changed every day and cells were passaged using gentle cell dissociation reagent (Stemcell Technologies) or singularised using 0.05% trypsin (Invitrogen). Two KS patients were selected: (i) a 13-year-old male patient, (FSIQ 78) diagnosed with KS, AS and anxiety disorder; and (ii) 20-year-old female patient, (FSIQ 78) diagnosed with KS, AS, an anxiety disorder and depression; (iii) 20-year-old female patient, (FSIQ 78) diagnosed with ASD, specific phobia, psychotic symptoms, hypotonia, unprovoked seizures, and cardiac, mitral valve insufficiency. The participants were recruited as part of a research cohort on neurodevelopmental copy number variants at Cardiff University (the Defining Endophenotypes from Integrated Neuroscience (DEFINE) Study). Procedures included clinical and cognitive testing, where possible, and blood sampling for generation of iPSCs and were approved by the South-East Wales Research Ethics Committee. Where participants did not have capacity to consent, as in this case, a representative (next of kin) provided written informed consent on their behalf. Peripheral blood mononuclear cells (PBMCs) from each donor were reprogrammed using a CytoTune-iPS 2.0 Sendai reprogramming kit (A16317, ThermoFisher scientific) [31]. Karyotype analysis showed 46, XX normal diploid female karyotype (ISCN classification) and possessed a 9q34 deletion (Fig. S1B).

Mouse ES cells culture and neuronal differentiation. Analysis used two independent clonal populations of mESC Ehm1+/−/− cells line mutant mouse ES cells (mESCs), obtained from the European Mouse Mutant Cell Repository Centre (EuMMCR); each had a single copy of a ‘knockout first’ conditional allele Ehmt1loxLacZ/loxLacZ (Addgene) inserted into the ES genome [32]. Control (Ehm1fl) clones were generated using the flip-allelo to restore a wild type gene. mESCs were grown on gelatin-coated plates in knockout DMEM (Gibco), supplemented with ESC certified FBS (Invitrogen), L-Glutamine (Gibco), 2-mercaptoethanol (Sigma) and ESGRO leukemia inhibitory factor (LIF) (Chemicon) at 37 °C. Feed-er-free neuronal differentiation was initiated in media lacking LIF for 4 days and 5 µM Retinoic acid was added to the culture until day 8. At this stage, cells were dissociated with 0.05% trypsin (Sigma) and seeded at 1.5 ×104 cm−2 density on Poly-D-ornithine (Sigma)/laminin (Roche) in N2 medium (Sigma) [33].

Molecular genetic manipulation of hiPSC lines. CRISPR-mediated mutagenesis of the ehm1 gene used a modified IB4 cell line, possessing the patchy integrase (Addgene)-inserted into the ASPN locus (Addgene) inserted into the ASPN safe harbour locus. This contains a Tet-inducible Cas9-nuclease, which was induced by 2 µg/ml Doxycycline (Dox) 24 h day before transfection with 10 pmol each of two ehm1-specific synthetic gRNA (SynRGs, crRNA and tracrRNA using Lipofectamine CRISPRMAX (Life Technologies). Successful editing was confirmed by PCR amplification using the flanking primers 5′-AGCACTCTCACGTTTGGT-3′ and 5′-CTTTTCAGGGAGCACTGG-3′, size-separated by electrophoresis on a 4% agarose gel. The open CRISPR design tool (Sigma) was used to predict four potential off-target sites, which shared 3 base mismatches in the guide RNA (no 1 or 2 base mismatches were identified). PCR analysis demonstrated to be unmaturated in our hiPSC lines (product sizes are shown in Table S2). A Tet-inducible REST gene was created by replacing the Cas9 gene of pAAV5-POI-CRISPRn with a REST cDNA sequence missing the 3′UTR (GenBank BC132859.1). A synthetic NRSF/REST fragment (IDT Inc.) was subcloned into the linearised plasmid backbone vector in an isothetical Gibson assembly reaction (Gibson Assembly® Cloning Kit, New England Biolabs); (Fig. SSC) [34]. Successful assembly was verified by sequencing, and by PCR where the custom construct was digested with AflII and Agel to release REST cDNA insert of ~3200 bp (Fig. S5D).

Expression Analysis

qR-PCR: Cells were lysed with QIAzol Lysis Reagent (Qiagen) and total RNA was extracted using the miRNasy mini kit (reference 217004, Qiagen, Germany). For each sample, 1 µg of total RNA was reverse transcribed using the miScript II Kit RT Kit (Qiagen). qR-PCR analysis of miRNA used miScript SYBR Green PCR kit (218073; Qiagen, Germany). All qR-PCR reactions were performed in triplicate on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and relative expression calculated using the 2−ΔΔCT method [35] with data normalized to GAPDH and Cdh143 (see Table S3 for primer sequences).

Western analysis: Cells were washed with ice-cold PBS and lysed in ice-cold RIPA buffer (Sigma) and protease inhibitor cocktail (Sigma) or 30 min at 4 °C. Cell supernatants were collected by centrifugation at 21000 rcf at 4 °C, LDS sample buffer (NuPAGE) and sample reducing agent (NuPAGE) added at 4 °C. Cell supernatants were separated by electrophoresis on 4–12% Bis-Tris Plus Gels (Life Technologies), transferred to nitrocellulose, blocked solution 5% (w/v) powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) for 60 min at RT and incubated overnight at 4 °C with primary antibody against REST (1:5000) (ab75785, Abcam), H3K9me2 (1:500) (ab1220, Abcam), MAP2 (1:750) (MAB8304, R&D Systems, Minneapolis, MN) or Caspase-3 (1:500) (9662, Cell Signaling Technology, USA) diluted in the blocking buffer. After washing in TBST, blots were incubated with an appropriate IRDye®-conjugated secondary antibody (LI-COR) and visualised/quantified with a Licor Odyssey infrared imaging imaging system ( Biosciences, Biotechnology). All data normalization was GAPDH.
miRNA-seq and analysis

The method used to amplify RNA was adapted from Abruzzi et al. [36] and was performed using TruSeq® Small RNA Library Prep kit (Illumina, USA). Total RNA was extracted from the iBJ4 line with or without UNC0638 treatment (untreated control is treated with the same volume of DMSO as treated sample) and ligated to 3’ polyadenylated and 5’-adaptors, followed by reverse transcription, PCR amplification (Fig. S5C) and size selection on 3% certified™ low range ultra-agarose (Bio-Rad Laboratories Ltd) in TBE buffer. Quality of purified miRNA libraries (QIAquick Gel Extraction Kit; Qiagen, Germany) were confirmed by Bioanalyzer (Agilent Technologies) and by Qubit (Thermo Fisher Scientificc) (Fig. S4B) and sequenced on an Illumina HiSeq 4000 using single-end 50 bp pair reads to deliver a minimum of 35 million mapped reads (PCR-amplified libraries). Single-end reads were trimmed with Trimmomatic [37], assessed for quality using FastQC and mapped to the human GRCh38 reference genome using STAR [38]. Counts were assigned to miRBase miRNAs using featureCounts [39] and the GRCm38.84 Ensembl gene build GTF. Differential gene expression (DEG) analyses used the DESeq2 package [40] and miRNA expression was FPKM-normalised. Hierarchical miRNAs lacking significant differences between control and UNC0638-treatment at ≥2 fold change cut-off threshold (significance: adj. p-value <0.05, Benjamini-Hochberg correction for multiple testing). miRNA-seq data have been deposited to ArrayExpress under accession number E-MTAB-10480.

A crossover analysis between miRNA gene targets and disease-associated genes was performed for Intellectual Disability, Schizophrenia and Autism Spectrum Disorder (ASD). Genes associated with Intellectual Disability and ASD (HP:0001249 and HP:0000717 respectively) were shortlisted from the DECIPHER database (DDG2P - V1.2), whilst Schizophrenia associated genes were shortlisted from the GWAS Catalog - EMBL-EBI (V1.0.2). Predicted mRNA target genes were determined using the mirDB database (V6.0). To account for miRNA target selectivity, gene crossover probability was calculated for each miRNA in ‘R’ (V4.0.4), using noncentral hypergeometric distribution, with P < 0.05 considered statistically significant. Crossover probability between disease-associated miRNAs and REST targeting miRNAs was assessed for each disorder by calculating hypergeometric probability, with P < 0.05 considered statistically significant.

Chromatin immunoprecipitation (ChIP)-qRT-PCR

Chromatin immunoprecipitation was performed as previously described [41]. Briefly, hiPSC suspensions c in serum-free media were crosslinked in 1% Formaldehyde (Sigma) for 10 min at RT and quenched by 0.125 M 200 y, hiPSC suspensions c in serum-free media were crosslinked in 1% Formaldehyde (Sigma) for 10 min at RT and quenched by 0.125 M

PBS-T with 0.3% Triton-X-100 (PBS-T) and 5% donkey serum for 1 h, before incubation with primary antibodies in PBS-T with 5% donkey serum overnight at 4 °C. Secondary antibodies were applied in PBS-T for 1.5 h at RT, counterstained with DAPI (Molecular Probes) and mounted in DAKO fluorescent mountant (Life Technologies). Samples were imaged on a Leica DMi6000b fluorescent microscope or analysed using a CX7 High-Content Screening (HCS) Platform (Thermo Fisher Scientific). Primary antibodies were as follows: Nanog (1:200, 4903, Cell Signaling Technology, USA), Oct-4 (1:200, 2750, Cell Signaling Technology, USA), Sox2 (1:200, 3579, Cell Signaling Technology, USA), NeuN (1:250, MAB377, Sigma). Secondary antibodies used were: Alexa 594-conjugated donkey anti-rabbit (1:1000, Invitrogen, A21207), Alexa 488-conjugated donkey anti-rabbit (1:1000, Invitrogen, A21206) and Alexa 488-conjugated donkey anti-mouse (1:1000, Invitrogen, A21202). Cells were viewed using a Zeiss Axio Observer inverted microscope (40x objective) using Zeiss Zen software. Region of interests (ROIs) were recorded for 5 min per experiment at a frame rate of 10 Hz and 1024 x 1024 pixel resolution. Image stacks were analysed by Fiji [43], NeuroCa [44], and FluoSNAAP [45] software packages. Results were imported into Prism 7.0 for statistical evaluation.

Statistical analysis

Prism 7.0 (GraphPad Software) was used for the statistical analysis. Data shown are the mean ± SEM. with P < 0.05 considered statistically significant. Two-tailed unpaired t-tests were used for comparisons between two groups. Group differences were analysed with one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. Data distribution was assumed to be normal, but this was not formally tested.

RESULTS

Loss of Ehmt1 reduces expression of NRSF/REST and increases expression of REST-target genes

We initially examined the effect of Ehmt1 hemizygosity on neurodevelopment-specific gene expression in mouse embryonic stem cells (mESC). mESC Ehmt1+/− and Ehmt1−/− control cell lines were cultured to neural progenitor cell stage (NPC) and expression profiles of 65 genes examined using qRT-PCR. A notable feature of the resulting data was a greater than sixfold decrease of REST mRNA, one of only 3 genes showing a decrease in mESC Ehmt1+/− compared to control lines (Fig. 1A; Table S1). This was accompanied by significant increased expression of 10 out of 13 Nrsf/Rest-repressed genes present in the study. Based on these data we proposed that Ehmt1 may regulate neuronal gene transcription via control of Nrsf/Rest. To pursue the hypothesis, we treated wild type mESC with UNC0638, a selective inhibitor of EHMT histone methyltransferases [46]. As Nrsf/Rest is expressed in pluripotent stem cells, we tested mESC in the pluripotent state by treatment for 48 h with UNC0638. Western blot analysis showed a dose-dependent decrease of H3K9meth2, with a half-maximal change at 200 nM (Fig. 1B). This was accompanied by a similar dose-dependent decrease in NRSF/REST protein.

To establish whether this observation is common to KS patients, we generated hiPSCs from two patients (Fig. S1). Both patient lines showed an approx. Twofold decrease in H3K9m2, accompanied by an equivalent decrease of NRSF/REST protein (Fig. 1C). This is likely to be solely due to loss of EHMT1 activity, as EHMT2 protein expression is unaltered (Fig. S1C). As the microdeletion in KS patients ablates multiple genes, we employed CRISPR-Cas9 to introduce a 56-bp deletion in exon 12 of Ehmt1 (Fig. S1D). This would be isogenic with the parental wild type control cells. This deletion caused a 50% loss of EHMT1 protein (Fig. S2) and an accompanying reduction of H3K9me2 expression (Fig. 1C). As for KS patient iPSC and mESC, reduced EHMT1 activity again led to a significant reduction in the expression of NRSF/REST protein in EHMT1 wild type cells. Finally, UNC0638 treatment of wild-type hiPSCs caused a
dose-dependent reduction of NRSF/REST protein, with half-
maximal change at 250 nM (Fig. 1D).

To confirm that changes to NRSF/REST protein lead to altered
gene transcription, we examined expression in undifferentiated,
pluripotent cells of the NRSF/REST target genes NRNX3, Calbindin
and L1CAM [47–49], which initiate their expression during early
stages of neurodevelopment, but are not reported to be present
in pluripotent cells. Increased gene expression was induced in
non-patient hiPSC following 72 h of 250 nM UNC0638 treatment
(Fig. 1E). Furthermore, hiPSC lines from both KS-patients showed a
5-fold or greater expression of these genes than control cultures
(Fig. 1F). Collectively, these mouse and human data indicate that
EHMT1 regulates the level of NRSF/REST of pluripotent stem cells,
and when reduced directly elevates the expression of its down-
stream target genes.

EHMT1 regulates NRSF/REST via suppression of miRNA-
associated that are associated with psychiatric disorders

Conventionally, H3K9me2 is considered to be a transcriptional
repressor [50], yet we observed that reduced EHMT1 activity leads
to decreased NRSF/REST protein. We found no evidence for
H3K9me2 at the NRSF/REST promoter using ChIP-qRT-PCR (Figs. 2A
and S3), arguing against direct regulation by EHMT1 of NRSF/REST
gene transcription. We therefore considered the potential of a de-
pression mechanism acting via suppression of an intermediate
NRSF/REST repressor. MicroRNAs (miRNAs) are ~22-nt noncoding
RNAs expressed in a wide range of eukaryotic organisms and play
a critical role in the regulation of gene expression at the post-
transcriptional level. They have crucial roles at key stages in the
development of the nervous system [51] and several brain-related
miRNAs, including mir-142, mir-153 and mir-9 have been shown
to target NRSF/REST mRNA [49]. This offers a mechanism to
connect repressive EHMT1 histone methylation at the genome
level to control of NRSF/REST protein and its subsequent
regulation of neuronal gene expression.

We conducted an unbiased search for miRNAs regulated by
EHMT1 by miRNA-seq of wild-type hiPSC in the presence or
absence of UNC0638. We detected 56 miRNAs with greater than a
2.5-fold increase of expression when EHMT1 is inhibited (Fig. S4).
11 of these miRNAs were predicted to target NRSF/REST mRNA
based on miRDB database, and 9 replicated by qRT-PCR analysis
of UNC0638-treated hiPSC. Among these miRNAs and EHMT1 activity
association between EHMT1 activity and miRNA expression was
examined in mESCs and 4.62 ± 0.35-fold, respectively; Fig. 2B). To validate this further
we examined miR-142, miR-153-1, miR-26a-2 in our KS-patient
hiPSC, and found up-regulation of all three miRNA, with the
exception of miR-142 in KS patient 1 (Fig. 2C). Furthermore, the
association between EHMT1 activity and miRNA expression was
conserved in mESC as expression of miR-142, miR-153-1, miR-26a-2,
and miR-769 were all elevated after UNC0638 treatment (Fig. 2D).
To investigate direct linkage between these NRSF/REST-regulating
miRNAs and EHMT1 activity we examined their association with
H3K9me2-modified chromatin, we further re-probed our
H3K9me2-ChIP to show that in contrast to the NRSF/REST gene
itself, H3K9 dimethylation was present at TSS of miR-142, miR-153
and miR-26a miRNA genes (Fig. 2A). Our combined gene expression
and ChIP analysis supports an indirect regulation of
EHMT1 on NRSF/REST via suppression of miRNA transcription.

To test our mechanistic hypothesis, we introduced a
doxycycline-inducible ("Tet-on") expression plasmid into the
AAVS1 safe harbour site of wild-type hiPSC that expresses a
recombinant NRSF/REST cDNA (REST

Translational Psychiatry (2022) 12:438
NRSF/REST expression from its miRNA regulators is sufficient to overcome the effects of reduced EHMT1 activity. Given the potential association between EHMT1 activity and patient diagnosis that extends across a range of NDD, we examined the relationship between the up-regulated miRNA gene set due to reduced EHMT1 activity and GWAS data for ID, schizophrenia and ASD. Remarkably, 43 and 34 of the 56 up-regulated miRNA have genetic association with ID and schizophrenia respectively (Table S5). In contrast, only 15 ASD-associated miRNA were up-regulated, all of which overlapped with the ID gene set and 13 of which were also associated with schizophrenia (Fig. S6). Of the up-regulated miRNA gene sets that are known NRSF/REST regulators, there was significant enrichment for those associated with ID (all 11 miRNA) and schizophrenia (10 of 11 miRNA), but not ASD (5 of 11 miRNA) (Figs. 2F and S6). Furthermore, the five NRSF/REST targeting miRNA associated with ASD were present in both ID and schizophrenia overlaps (Fig. 2F), giving a core miRNA gene set of miR-26a, miR-26b, miR-153, miR-181a and miR-548. This analysis suggests a broad association of a miRNA-mediated NRSF/REST regulatory pathway and psychiatric risk, particularly for a diagnosis of ID and schizophrenia.

**Reduced EHMT1 activity accelerates neuronal differentiation**

We investigated whether the relationship between reduced EHMT1 activity and NRSF/REST protein is maintained beyond the pluripotent cell state and persists into neurodevelopment. Mutant EHMT1+/− hiPSCs and their isogenic wild-type controls were differentiated into neurons using a standard dual-SMAD inhibition protocol [30] and NRSF/REST protein levels sampled at time points that span neuronal differentiation (Fig. 3A). NRSF/REST protein was significantly lower during differentiation of EHMT1+/− mutant cells compared to isogenic controls. This indicates that the effect of EHMT1 on NRSF/REST protein persists from early NPC stage into neuronal differentiation. The NRSF/REST reduction in differentiating EHMT1+/− iPSC was accompanied by increased expression of the human orthologues of the target genes MASH1 and NGN2, which are first expressed in NPC [48, 52] (Fig. 3B). Expression of MASH1 in control hiPSCs derived NPCs was also increased in the presence of UNC0638 and suppressed by doxycycline-mediated induction of RESTΔUTR mRNA (Fig. 3C). These results suggest that EHMT1 acts via NRSF/REST as a negative regulator of neuronal gene expression to prevent premature neurodevelopment. To confirm that dysregulation of NRSF/REST-mediated gene repression subsequently impacts on neurodifferentiation in general, we investigated the non-NRSF/REST target genes, PAX6 and Nestin [53, 54]. These genes were upregulated at Day 10 and 20 of neuronal differentiation as EHMT1+/− cells transit from the NPC stage (Fig. 4A, B). Likewise, cell staining of differentiated iPSC demonstrated an increase in PAX6 and Nestin in KS-patients (Fig. 4C). To probe neurodifferentiation further, we examined the expression of NCAM and MAP2 (microtubule-associated protein 2) proteins, which are expressed in all neurons [55]. Both genes were up-regulated during the early stages of neuronal differentiation in EHMT1+/− hiPSCs and their isogenic wild-type controls.
Fig. 3  Reduced EHMT1 activity elevates REST-target gene expression during neuronal development.  

A Western blot analysis of NRSF/REST protein expression in hiPSCs-derived neurons at Day 20 (NPC stage) and maturing neurons at Days 35, 40 and 45 of differentiation. NRSF/REST protein was decreased in EHMT1<sup>−/−</sup>-derived neurons. Quantification of Western blot analysis was performed by normalization to GAPDH. A representative image from at least three independent experiments is shown, with all values shown on the graph above. 

B Time-course qRT-PCR analysis at Days 15 and 20 of differentiation to examine changes in the expression of lineage-specific REST-target genes NGN2 and MASH1 in EHMT1<sup>−/−</sup>-derived neurons compared to their isogenic control hiPSCs. Relative changes is expressed as mean fold change over pluripotent cells, n ≥ 3 independent experiments. 

C Relative mRNA abundance measured by qRT-PCR of MASH1 in wild type hiPSCs treated with UNC0638. Induction of REST<sup>Δ</sup>UTR with doxycycline (DOX) suppresses the UNC0638-induced MASH1 gene expression. Data were presented as Mean±SEM and analysed by student’s t-test or One-way ANOVA with post hoc comparisons using Dunnett’s multiple comparisons test comparing to control samples.

Fig. 4  Reduced EHMT1 activity results in accelerated neuronal development.  

A qRT-PCR analysis at days 10 and 20 of differentiation to examine the expression of the neural progenitor markers, (A) Nestin and (B) PAX6 in EHMT1<sup>−/−</sup>-derived NPC relative to expression in the isogenic control. 

B qRT-PCR analysis of NCAM at days 20 and 25 of early stage differentiated neurons in EHMT1<sup>−/−</sup>-derived relative to expression in the isogenic control.

C qRT-PCR analysis at days 15, 20 and 40 of differentiation to examine changes in the expression of the neuronal marker MAP2 in EHMT1<sup>−/−</sup>-derived neurons relative to their expression in the isogenic control neurons. 

D UNC0638 (250 nM) induced expression of MAP2 in differentiating wild-type hiPSC-derived neurons, monitored by qRT-PCR analysis over a time course of 5, 10, 30 and 40 days of treatment. 

E Relative mRNA abundance measured by qRT-PCR of Nestin and MAP2 in wild-type hiPSCs treated with UNC0638. Induction of REST<sup>Δ</sup>UTR with doxycycline (DOX) suppresses the UNC0638-induced MASH1 gene expression. n ≥ 3 independent experiments. Data were presented as Mean±SEM and analysed by One-way ANOVA with post hoc comparisons using Dunnett’s multiple comparisons test comparing to control samples.
cells during NPC stages, indicative of a rapid transition through the progenitor cell state and into full neuronal differentiation (Fig. 4D, E). As also seen with other genes, elevated MAP2 expression was induced in differentiated wild-type hiPSC by UNC0638 inhibition of EHMT1 (Fig. 4F). Finally, elevated expression of both Nestin and MAP2 was suppressed by doxycycline-induced RESTΔUTR expression demonstrating NRSF/REST dependency (Fig. 4G). These results are indicative of indicate accelerated neuronal differentiation with reduced EHMT1 activity, commencing as cells leave the pluripotent cell state and continuing during the formation of mature neurons.

Reduced EHMT1 activity is associated with increased apoptosis and aberrant neuronal function

Although we observed a consistent pattern of rapid neurodevelopment in cells with reduced EHMT1 activity in early developmental stages by Day 40 these differences appeared to be lost (Figs. 4D, E). Previous reports described mice lacking NRSF/REST as having a transient increase in neurogenesis but eventually decreased of neuronal numbers [56]. We therefore investigated the impact of the prolonged inhibition of EHMT1 activity on neuronal differentiation, monitoring neuronal cell number using cell staining with the nuclear protein, NeuN, a marker of mature neurons. Wild-type cells were differentiated to neurons in the presence and absence of UNC0638 and sampled at Days 25, 30, 35 and 45 (Fig. 5A). At Day 35, we observed an elevation level of NeuN protein in stained cells Fig. 5A), consistent with our observations for NPC and early-stage neurons, however, cultures tracked over an extended induction period showed a progressive decrease in the proportion of NeuN-positive cells in the treated cells compared to untreated controls (Fig. 5B). By Day 45, UNC0638-treated cell cultures had 50% of the number of NeuN positive cells compared to untreated controls. This may explain the levelling of MAP2 expression observed between control and cells with reduced EHMT1 at later developmental time points, as the expression per cell may be higher, but the overall number of cells less (Fig. 4E, F).

During neurodevelopment, elevated rates of neurogenesis can be balanced by decreased neuronal cell survival [57]. To determine the mechanism underlying the neuronal loss, we examined Caspase-3 activation in UNC0638-treated cells in relation to the control. At days 25 and 30 of neuronal differentiation, which are the time points preceding the reduction in cell number, we detected an elevated Caspase-3 cleavage to the activated form in UNC0638-treated cells (Fig. 5C). The increase in Caspase-3 activation was accompanied by increased Caspase-3 gene expression in control neurons treated with UNC0638 (Fig. 5D). These observations support the hypothesis that in EHMT1−/− cells the reduction in the number of cells stained with NeuN and the neuronal gene expression may be due to an induction of programmed cell death.

We next examined whether the abnormal developmental programme seen in EHMT1−/− mutant cells altered neuronal function. To investigate neuronal activity, but minimise the impact of early neurodevelopmental deficits, differentiated wild-type cultures were switched BrainPhys medium [58] at Day 35 as they begin to form neurons to in the presence or absence of 250 nM UNC0638. Spontaneous calcium influx was measured two weeks later when the neurons typically begin to exhibit
DISCUSSION

Here, we report a regulatory pathway that connects the molecular lesion in EHMT1 activity to altered neuronal cell development and neuronal function in human iPSC-derived neurons. The key mechanistic components of the pathway are the transcriptional regulator NRSF/REST and its control via miRNA, with reduced EHMT1-mediated H3K9me2 resulting in elevated miRNA transcription. As a consequence, gene expression of both NRSF/REST-regulated and general neuronal specific markers is elevated with lower levels of EHMT1 activity but can be reduced to control levels by expression of a NRSF/REST miRNA that lacks the miRNA regulation sites. Although KS presents a strong genetically penetrant case for this pathway, our genetic association analysis suggests a broader association for ID and schizophrenia, with implications for therapeutic intervention.

NRSF/REST plays a key role in repression of neuronal gene expression to maintain stem cells in the undifferentiated state [28, 48], making it a major regulator of neurogenesis and neural differentiation [61]. Here, we can explain the in vitro cell phenotype seen in KS patient cells as due to reduced NRSF/REST protein. Our observations fit with the previous investigation of NRSF/REST hypofunction in neurodevelopment [28]. In the mouse brain, conditional NRSF/REST knockout mice show rapid neuronal differentiation of hippocampal neural stem cells and elevation in the expression of pro-neuronal genes, NeuroD1, Tuj1, and DCX [56]. In the study reported here, we show that reduced NRSF/REST expression in EHMT1−/− cells is associated with elevated expression of the human pro-neural transcription factors Mash1 and Ngn2.

The linkage between NRSF/REST and mental health is not well explored, but there are some mechanistic observations reported. In human neuronal culture, decreased nuclear NRSF/REST has been observed in neuronal cultures derived from sporadic Alzheimer’s Disease (AD) patient cells and again leads to accelerated neural differentiation and increased excitability, which can be reversed by exogenous NRSF/REST expression [62]. In the context of NDD, Down’s Syndrome cells have increased expression of Dyrk1A which leads to reduced NRSF/REST and misregulation of neurodevelopmental genes [55].
Likewise, suppression of Chromodomain helicase DNA-binding protein 2 (CHD2), associated with a range of NDD, including ASD and ID, was shown to inhibit the self-renewal of radial glial cells and increase the generation of neural progenitors and neurons and this phenotype was attributed to the reduced expression of the neuronal regulator NRSF/REST [63]. The exact mechanism leading to cell death of EHMT1+/− hiPSC at these later stages of neurodifferentiation is unclear, but it is noteworthy that NRSF/REST-suppressed genes include cell death-inducing genes that may directly induce apoptosis [64]. We also note that reports in the mouse brain studies indicate that loss of ehm t1 [65] or rest genes increase cell proliferation and adult neurogenesis, but the prolonged loss of NRSF/REST leads to a functional depletion of the adult neuronal stem cells and decreased granule neuron production [56]. Finally, analysis of post-mortem AD, where NRSF/REST is reduced compared to age-matched controls, show elevation of NRSF/REST targets, including genes encoding pro-apoptotic signalling components, associated with neurodegeneration [64].

Nonetheless, GWAS has not strongly associated NRSF/REST with psychiatric disorders. This may be because of a combination of network robustness that protects against minor fluctuations of its upstream regulatory pathway, and the severity caused by major changes in NRSF/REST expression, as reported with its association with dementias (AD, Huntington’s Disease and Parkinson’s Disease), ischemic shock and some NDD. Our cross-disorder analysis of the miRNA under EHMT1-regulation and their association with GWAS-significant miRNA genes suggests hitherto unexplored linkage with NRSF/REST not only for KS but across a broader range of ID and schizophrenia cases and may offer significant insights for alternative therapeutic approaches.

In summary, this study identifies a mechanism that couples EHMT1 activity to the neuronal regulator NRSF/REST through miRNA-dependent pathway, which leads to altered neurodevelopment. It suggests NRSF/REST as a key node within a miRNA-mediated gene regulatory network and offers a mechanism for the specific case of KS. Importantly it also reveals the presence of a more extensive pathway centred around NRSF/REST regulation of the neurodevelopmental gene regulation programme, which has broader significance for neurodevelopmental and psychiatric disorders, such as ID and schizophrenia.

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