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Transcriptome analysis revealed impaired cAMP responsiveness in *PHF21A*-deficient human cells

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Abstract

Potocki-Shaffer Syndrome is a rare neurodevelopmental syndrome associated with microdeletion of a region of Chromosome 11p11.2. Genetic evidence has implicated haploinsufficiency of *PHF21A*, a gene that encodes a histone-binding protein, as the likely cause of intellectual disability and craniofacial abnormalities in Potocki-Shaffer Syndrome. Previous work, however, has not investigated the molecular consequences of reduced *PHF21A* expression. In this study, we analyzed by RNA-Sequencing (RNA-Seq) two patient-derived cell lines with heterozygous loss of *PHF21A* compared to unaffected individuals and identified 1,885 genes that were commonly misregulated. The patient cells displayed down-regulation of key pathways relevant to learning and memory, including cAMP-signaling pathway genes. We found that *PHF21A* is required for full induction of a luciferase reporter carrying cAMP-responsive elements (CRE) following stimulation by the cAMP analog, forskolin. Finally, *PHF21A*-deficient patient-derived cells exhibited a delayed induction of immediate early genes following forskolin stimulation. These results suggest that an impaired response to cAMP-signaling might be involved in the pathology of *PHF21A* deficiency.

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Author Contributions

RP, HK, SI designed the experiments. RP, HN, YN, and HK performed the experiments. RP analyzed the RNA-Seq data. RP and SI wrote the manuscript. All authors approved and edited the manuscript.

Ethics Approval

This study was approved by the Institutional Review Board of Augusta University.

Competing financial interests

The authors declare no competing financial interests.

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Keywords

RNA-Sequencing; Neurodevelopmental Disorders; Chromatin; Histone Methylation; cAMP signaling; Potocki-Shaffer Syndrome

Introduction

Recent genome-wide studies that have sought the genetic basis for neurodevelopmental disorders, such as intellectual disability and autism, have implicated a large number of histone methylation regulating genes (De Rubeis et al., 2014; Iossifov et al., 2014). Histone H3 Lysine 4 methylation (H3K4me) is a histone modification associated with areas of open chromatin and is one of the most extensively regulated histone modifications in higher eukaryotes, by seven writer enzymes, six eraser enzymes, and a number of reader proteins that recognize this modification and recruit effectors (Vallianatos and Iwase, 2015; Zhou et al., 2016). Mutation in 8 out of these 13 H3K4me writers and erasers and multiple H3K4me readers leads to neurodevelopmental disorders (Vallianatos and Iwase, 2015), indicating that correct dynamic regulation of histone H3K4 methylation is critical for proper brain development and cognitive function. However, little is known about the molecular mechanisms that underlie the dynamics of histone methylation and how their function contributes to proper neurodevelopment.

PHF21A is a histone-binding protein that is associated with Potocki-Shaffer Syndrome (PSS, OMIM: 601224). PSS is a rare, congenital disorder resulting from a deletion in chromosomal region 11p11.2 (Potocki and Shaffer, 1996). PSS is characterized by intellectual disability, craniofacial abnormalities, and two bone phenotypes: multiple exostoses and parietal foramina. The original genetic lesion identified in PSS was a 2.1 Mb microdeletion, which leads to the heterozygous loss of 13 genes (Potocki and Shaffer, 1996). Within this chromosomal region, *EXT2* and *ALX4* have been identified as the genes responsible for the bone phenotypes of PSS (Mavrogiannis et al., 2001; Stickens et al., 1996; Wakui et al., 2005; Wu et al., 2000). *PHF21A*, however, has been specifically associated to the intellectual disability and craniofacial abnormality phenotypes, since patients with genetic alterations only in *PHF21A* do not exhibit the characteristic bone malformations (Kim et al., 2012; Labonne et al., 2015; McCool et al., 2017). Although the genetic evidence linking *PHF21A* in intellectual disability and craniofacial abnormalities is compelling, the molecular mechanism by which *PHF21A* loss leads to these phenotypes has not been previously determined.

The *PHF21A* gene encodes a histone-binding protein that recognizes the absence of post-translational modifications (i.e. the lack of methylation) on histone 3 lysine 4 (H3K4me0) through its PHD finger domain (Lan et al., 2007). PHF21A is a component of the Lysine Specific Demethylase 1, Corepressor of REST (LSD1-CoREST) complex. LSD1 (also known as KDM1A) demethylates mono- or di-methylated histone 3 lysine 4 (H3K4me1/2) to repress gene transcription (Shi et al., 2004; Shi et al., 2005). PHF21A therefore binds to the reaction product of LSD1-mediated H3K4 demethylation. The LSD1-CoREST corepressor complex is recruited to the neuron-restrictive silencer element (RE-1, or NRSE)

via REST and is important for mediating repression of neuron-specific genes in non-neuronal cells (Bruce et al., 2004; Hakimi et al., 2002). Previous work has shown that loss of *PHF21A* leads to the de-repression of REST target genes in non-neuronal cells (Klajn et al., 2009; Lan et al., 2007). *PHF21A* is expressed ubiquitously, but expression is highest in the brain and the testes, implicating specialized roles of PHF21A in these two tissues (Iwase et al., 2004). A mouse model of *Phf21a* homozygous loss led to neonatal lethality due to a defect in suckling (Iwase et al., 2006); however, structural and/or cytoarchitectural abnormalities have yet to be identified in the brain. It remains elusive if PHF21A plays any roles outside the repression of neuron-specific genes in non-neuronal cells.

In this study, we performed RNA-Sequencing (RNA-Seq) of *PHF21A*-deficient patient-derived cells to probe the molecular dysfunction associated with heterozygous loss of *PHF21A* in an unbiased manner. Our bioinformatic analyses and reporter assays identified cAMP signaling as an impaired molecular pathway in the *PHF21A*-deficient patient cells, thereby providing insights into the cellular role of PHF21A and how *PHF21A* loss may contribute to cognitive defects.

Experimental Procedures

Patient-Derived Cell Lines

Patient blood samples were collected from the individuals as described previously (Kim et al., 2012; Labonne et al., 2015). Lymphocytes were harvested and then transformed by Epstein-Barr Virus into lymphoblastoid cell lines as described previously (Nishimoto et al., 2014). Lymphoblast cell lines were maintained in RPMI medium 1640 (Gibco) containing 10% FBS, 1x GlutaMax (Gibco), and 1% penicillin and streptomycin (Gibco).

RNA-Sequencing

RNA was isolated from lymphoblast cell lines in technical duplicates, where each cell line was collected from two separate culture dishes, using the RNA purification kit from Life Technologies. Poly-A mRNA was separated from total RNA with the NEB Magnetic mRNA Isolation Kit. Libraries were prepared using Direct Ligation of Adapters to First-strand cDNA as previously described (Agarwal et al., 2015). Multiplexed libraries were pooled in approximately equimolar ratios and were purified from a 1.8% TBE-agarose gel. The libraries were sequenced to a length of single-end 50 bases using an Illumina HiSeq 2000 according to standard procedures. Reads were mapped to the human genomes (hg19) using bowtie2, allowing up to 2 mismatches and only uniquely mapped reads were analyzed further. Aligned reads of technical replicates from each individual were merged using samtools for all subsequent analyses. Merged files were then converted to bigwig files for visualization in the Integrated Genome Viewer. Merged files were also analyzed for differential expression using DESeq between sex-matched patient and controls (Anders and Huber, 2010). Genes called as significantly misregulated were calculated by creating a merged differential expression file that averaged the fold change and calculated an average p-value using Fisher's method ($p_{\text{average}} = p_1 * p_2 * [1 - \log(p_1 * p_2)]$). Genes that were up-regulated in one comparison but down-regulated in the other comparison were excluded. Pathway analysis was run using LR-Path (Sartor et al., 2009). Network analysis was carried

out by separating down- and up-regulated GO terms and using REVIGO (Reduce and Visualize Gene Ontology) (Supek et al., 2011), and network representation was generated using Cytoscape (Cline et al., 2007).

shRNA-mediated *PHF21A* knockdown

Lentiviral packaging plasmids and shRNA constructs were transfected into 293T cells using Transit293 transfection reagent. shRNA plasmids, including a scramble and three *PHF21A* targeting shRNAs (V2LHS_135304, V2LHS_135309, V2LHS_162572) were obtained from the pGIPZ microRNA-adapted shRNA library (GE Life Sciences). Viral supernatants were collected and concentrated using LentiX (ClonTech). Control male lymphoblast cells were transduced with the lentiviruses containing scramble or *PHF21A* shRNA constructs, and cell lines were then selected with 1 µg/ml puromycin for 4 days. RNAs were harvested from cells using PureLink RNA Mini Kit from Ambion. cDNAs were prepared by RevertAid RT Reverse Transcription Kit (Thermo Scientific) and then were analyzed by qPCR (Applied Biosystems 7500 Instrument). The oligonucleotide sequences used for qPCR are available upon request.

Luciferase Assays

Three CRE sequences (both the consensus sequence: TGACGTCA, as well as a mutated sequence: TGATATCA) were tandemly inserted upstream of the HSV-TK promoter in a pGL3-based Luciferase plasmid using Gibson assembly. 10,000 HEK293T cells in a 96-well dish were transfected by Lipofectamine 2000 (Thermo Fisher) with 100 ng of the CRE-TK-Luciferase constructs, 1 ng of a CMV-Renilla construct, and 100 ng of either scramble or *PHF21A* shRNA. Two days later, cells were exposed to 30 µM Forskolin or an equal volume of DMSO for 8 hours. Then, cells were harvested for Luciferase analysis using the Promega Dual Luciferase Assay System. The ratio between luciferase and renilla expression was normalized to the empty plasmid (TK-Luciferase only), and then Luciferase expression was reported relative to the DMSO, scramble shRNA condition.

Stimulation of patient derived cells

The two patient derived lymphoblast cell lines, as described above, and one control lymphoblast cell line were incubated with 30 µM Forskolin or an equal volume of DMSO for 0 minutes, 30 minutes, 2 hours, or 6 hours. Then, cells were harvested for RNA extraction and qRT-PCR as described above. mRNA expression was reported relative to the untreated cells (0 minutes), and mRNA expression from forskolin treated cells was normalized to DMSO treated cells.

Results

Transcriptome analysis of patient derived cells with *PHF21A* alterations

To interrogate the genome-wide gene expression changes in *PHF21A* deficiency, we performed RNA-Seq analysis on two patients with *PHF21A* alterations and two unaffected controls. The male patient, DGDP262, was recently published as one of the smallest microdeletion cases of PSS-related developmental delay (Labonne et al., 2015). The female patient, MCN1762, has a balanced translocation with the breakpoint within the *PHF21A*

gene leading to a truncation of 13 out of 18 *PHF21A* coding exons (Kim et al., 2012) (Fig. 1A). Lymphoblasts were derived from these two *PHF21A* haploinsufficient patients and two unaffected, unrelated individuals. Then, cDNA libraries of poly-adenylated mRNAs were prepared and subjected to high-throughput sequencing. At least 17 million uniquely-mapped reads were obtained per sample and inter-replicate variability was low (Fig. 1B) indicating the sufficient coverage and reproducibility of sequencing data.

Consistent with the heterozygous *PHF21A* alterations, 58.4% and 49.7% of *PHF21A* expression was observed in the male and female patient cells compared to the control cells (Fig. 1A and 1C). The male patient cells have a ~234 kb microdeletion that encompasses five genes, including *PHF21A* (Labonne et al., 2015). Our RNA-Seq data confirmed this finding with an approximately 50% reduction of mRNA levels of genes mapped to this microdeletion region (Fig 1D). The female patient cells contain a translocation in the fifth intron of *PHF21A* (Chr. 11) with Chromosome 1 (t(1:11)(p21.1; p11.2)) (Kim et al., 2012). The translocation breakpoint on Chromosome 1 is located within a gene desert, mostly composed of LINE-1 elements, with the nearest gene 635 kb downstream (*PRMT6*). We did find ectopic transcripts generated from the Chr. 1 translocation breakpoint, which is likely attributed to read through of RNA polymerase II from the translocated *PHF21A* promoter (Fig. 1E). Our data corroborate the previously-reported genetic lesions in the patient cells and found local and patient-specific changes in transcripts, which are associated with each genetic alteration.

We next sought to identify commonly misregulated genes in the two patient lymphoblast cell lines. Differentially expressed genes (DE genes) were determined by DESeq (Anders and Huber, 2010) for each patient lymphoblast line compared to the sex-matched control. We found 1,885 genes (7% of 26,463 total annotated genes) that were misregulated in both of the two patient cell lines with a p-value < 0.05 (Fig. 2A).

Given that PHF21A is a component of the LSD1-CoREST corepressor complex, we speculated that PHF21A haploinsufficiency may lead to up-regulation of gene transcription. However, we found that roughly an equal number of genes are up- (49.4%) and down-regulated (50.6%) in patient lymphoblasts (Fig. 2B). The magnitude of misregulation, judged by median fold change, of up-regulation (log2-fold change: 0.715) was slightly higher than the magnitude of down-regulation (log2-fold change: -0.549, Fig. 2C). DE-genes were distributed uniformly throughout lowly- and highly- expressed genes (Fig. 2D). We then asked if the published set of 2,172 REST-target genes (Bruce et al., 2004) are significantly misregulated in the patient lymphoblasts. We found that although REST-target genes were over-represented in the set of misregulated genes (chi-square test, p-value = 1.06×10^{-5}), these REST-target genes were equally distributed between up- and down-regulated (Fig. 2E) similar to the total DE-genes. Overall these data suggest that PHF21A has bidirectional roles in maintaining expression levels of REST-target genes and a large number of non-REST targets.

Pathway analysis suggests down-regulation of cAMP-signaling genes

To obtain insights into biological processes influenced by *PHF21A* haploinsufficiency, we applied our RNA-Seq data to LR-Path, a gene set enrichment ontology program that takes

into account statistical significance and direction of differential expression in the entire RNA-Seq data set (Sartor et al., 2009). Interestingly, although we analyzed lymphoblasts, the most significantly downregulated pathways were relevant to neural development and function, such as “cerebral cortex neuron differentiation”, “chemical synaptic transmission, postsynaptic”, “visual learning”, and “cortical actin cytoskeletal organization” (Table 1 and Fig. 3A–B). At a FDR<0.05, many more pathways were found to be significantly downregulated than up-regulated (Fig. 3A–B). LR-Path provides signature genes that have been well-characterized in their roles in a given biological process, thereby contributing to the significant enrichment. An example of signature down-regulated genes in our analysis are Amyloid Precursor Protein (*APP*) and Neuropilin and Tolloid-like 1 (*NETO1*) which have been shown to play important roles in NMDA-receptor trafficking required for synaptic plasticity and learning (Cousins et al., 2013; Ng et al., 2009). *APP* and *NETO1* were down-regulated 4-fold and 37-fold on average, respectively, in the patient cells compared to the control cells (Fig. 2A).

It is noteworthy that two cyclic AMP- (cAMP) related pathways were among the top down-regulated categories because cAMP signaling plays pivotal roles in broad physiological processes including learning and memory (Kandel, 2001; West et al., 2002). cAMP signaling elicited by external stimuli ultimately leads to phosphorylation of the transcription factor, CREB, and the expression of CREB target genes that carry out cellular responses to these stimuli (Mayr and Montminy, 2001; Zhang et al., 2005). In the patient cells, key genes, whose products mediate cAMP signaling, were down-regulated. For example, the down-regulated genes *CRTC3* (CREB regulated transcription coactivator 3) and *FOSL1* (Fos-like 1 subunit) have well characterized roles as a CREB co-activator (Conkright et al., 2003) and a CREB-responsive inducible gene (Benito and Barco, 2015), respectively. Thus, we next sought to determine if the CREB target genes, which are down-stream targets of cAMP signaling, are misregulated in our RNA-Seq data sets. We first obtained 4,084 putative CREB target genes that were identified in a previous study based upon the presence of evolutionarily-conserved CRE sequences in proximal gene promoters (Zhang et al., 2005). We found that the putative CREB target genes were over-represented in our set of DE-genes in patient cells (chi-square test, p-value = 2.96×10^{-10}). Slightly more genes were down-regulated (53.2%) than up-regulated (46.8%) in the patient lymphoblasts (Fig. 3C). These results suggest that *PHF21A* haploinsufficiency leads to reduced expression of genes that mediate cAMP signaling, yet the ultimate consequence in the expression of CREB-target genes can be bidirectional in unstimulated lymphoblasts.

***PHF21A* knockdown in lymphoblasts recapitulates the gene expression changes in the patient-derived cells**

We next sought to confirm that the key gene expression changes we observed in our RNA-Seq analysis were due to reduced expression of *PHF21A* rather than any other confounding factors such as inter-person variation. To this end, we quantified expression of selected DE-genes upon shRNA-mediated knockdown of *PHF21A* in the control lymphoblast cells derived from an unaffected individual. Male control lymphoblast cells were transduced with lentivirus carrying either scramble (sc) shRNA or one of three independent shRNAs against *PHF21A*. After establishing cell lines that stably express the shRNAs, we harvested RNA

and performed qRT-PCR analysis. We first validated efficient knockdown, yielding 8%–38% of *PHF21A* expression compared to the sc-transduced cells. We found that several of the most significantly misregulated genes in the patient cells show similar changes, including *APP* and *OXR*, upon *PHF21A* RNAi by the three independent shRNAs (Fig. 4A). We also analyzed a number of genes that mediate cAMP-signaling, namely *NETO1*, *CRTC3*, and *FOSL1*, and found that they were all down-regulated upon *PHF21A* knockdown (Fig. 4A). Finally, we chose two REST target genes, one that was down-regulated in the patient RNA-Seq data (*SCN3A*) and one that was up-regulated in the RNA-Seq data (*MAP1B*). In line with previous work on the effect of *PHF21A* knockdown in cancer cell lines and non-neuronal tissues (Iwase et al., 2006; Klajn et al., 2009; Lan et al., 2007), both genes were up-regulated upon *PHF21A* RNAi (Fig. 4B). While patient lymphoblasts lack half *PHF21A* expression constitutively, the RNAi knockdown depletes *PHF21A* in a relatively short time window; therefore, down-regulation of some REST-targets, including *SCN3A*, in the patient lymphocytes may reflect an indirect consequence of long-term *PHF21A* deficiency throughout development. These data provide support to the role of *PHF21A* in promoting expression of cAMP-signaling genes and also suggest that some gene misregulation in the patient lymphoblasts could be the result of indirect effects.

PHF21A is required for the optimal transcriptional response mediated by cAMP-signaling

The down-regulation of cAMP-signaling mediators (Table 1) and misregulation of CREB-target genes in patient cells (Fig. 3C) prompted us to test whether the induction of the cAMP-mediated gene transcription is altered by *PHF21A* deficiency. To do this, we designed a reporter plasmid with three cyclic-AMP responsive elements (CRE: TGACGTCA) upstream of the firefly *luciferase* gene, which is linked to the HSV *thymidine kinase* (TK) promoter. As a negative control, we mutated two critical nucleotides in the CRE sequence (mCRE: TGATATCA) (Tinti et al., 1997). We transfected these luciferase constructs and scramble or *PHF21A* shRNAs into HEK293T cells and then elicited cAMP signaling by treatment of forskolin, a cAMP analog (Zhang et al., 2005). In this luciferase reporter assay, *PHF21A* knockdown did not change the basal activity of the CRE-Luciferase construct (Fig. 5A). However, upon forskolin stimulation, cells with two independent *PHF21A* shRNAs were not able to induce expression of CRE-luciferase as highly as the scramble shRNA-treated cells ($p < 0.05$, ANOVA, Fig. 5A), and another shRNA showed a similar trend (shRNA-3). The construct with the mutated CRE sequence did not respond to forskolin nor did it change upon *PHF21A* knockdown, demonstrating that this effect is cAMP/CREB-dependent (Fig. 5B). These results demonstrate that *PHF21A* is required for the transcriptional response to cAMP-mediated signaling.

Our RNA-Seq analysis of *PHF21A*-deficient patient cells captured a snapshot of the steady-state transcriptome of these cells. Given our luciferase data following forskolin stimulation, we evaluated the response kinetics to cAMP signaling in the patient cells. We exposed control and *PHF21A*-deficient patient cells to forskolin, collected cells at different time points, and measured mRNA levels of two immediate early genes (IEGs) by qRT-PCR. *c-FOS* is a classic IEG that exhibits rapid transcriptional induction following stimulation with a peak response between 30–60 minutes (Sheng and Greenberg, 1990), whereas *FOSB* is a related gene that shows a delayed response (Kovacs, 1998). Whereas control cells displayed

the peak of *c-FOS* expression 30 minutes after treatment, patient cells were unable to increase transcription of *c-FOS* until two hours (Fig. 5C). The delayed response gene, *FOSB*, rose and fell in expression gradually in the control cells, but only began to rise in mRNA level at six hours in the patient cells (Fig. 5D). These data suggest that PHF21A is required to elicit a rapid transcriptional response to cAMP signaling.

Discussion

Genetic evidence has associated *PHF21A* with the pathogenesis of the intellectual disability and craniofacial abnormalities in Potocki-Shaffer Syndrome (PSS), but previous work has not studied the mechanism or molecular pathogenesis underlying these phenotypes. The present study is the first to describe the molecular dysfunction associated with heterozygous loss of the histone-binding protein, PHF21A, which is implicated in PSS-related cognitive deficit. Through an RNA-Seq study of *PHF21A*-deficient patient-derived cells, we found that pathways relevant to brain development and learning, including the cAMP-signaling pathway, were downregulated. Moreover, we demonstrated that *PHF21A* is required for full induction of a CRE-Luciferase reporter gene and that *PHF21A*-deficient cells exhibit a delayed transcriptional response to cAMP signaling. Altogether, these results suggest that *PHF21A*-deficient cells are unable to mount the proper response to external stimuli.

cAMP-mediated signaling is one of the primary pathways utilized by cells in response to extracellular stimuli, such as depolarization of neurons by afferent sensory inputs (Kandel, 2001). The experiments carried out in this study used non-neuronal cells, but it is tempting to speculate that these findings may extend to neurons. Recent literature has implicated activity-dependent signaling in the pathogenesis of neurodevelopmental disorders (NDDs) (Chahrour et al., 2012; Cohen et al., 2011; Ebert and Greenberg, 2013; Morrow et al., 2008); a common feature of NDDs may therefore involve an inability for neurons to respond to sensory inputs and form the proper neural networks during brain development. cAMP signaling is a major pathway in neurons that establishes memory, which involves long-term potentiation of synaptic efficacy (Benito and Barco, 2015; Bourtchuladze et al., 1994; Kandel, 2001; West et al., 2002). Down-regulation of cAMP-mediated signaling pathways could partially explain reduced expression of genes relevant to neuronal signaling, such as the CREB-target gene, *NETO1*. Our data suggest that PHF21A is required for maintaining expression of cAMP signaling molecules, thereby ensuring the proper response to extracellular stimuli.

LSD1, a histone demethylase, which physically associates with PHF21A and generates a binding substrate for PHF21A has been found to directly interact with CREB and other transcription factors that are necessary for neuronal activity-response pathways, such as SRF (Rusconi et al., 2016; Wang et al., 2015). PHF21A, together with LSD1, may therefore be directly engaged in transcription of neuronal activity-dependent genes. Importantly, mutations in LSD1 have also been reported in a rare NDD. Loss-of-function missense mutations of *LSD1* lead to a Kabuki-like syndrome characterized by intellectual disability, thinning of the corpus callosum, hypotonia, and craniofacial dysmorphisms (Chong et al., 2016; Pilotto et al., 2016; Tunovic et al., 2014). Thus, future investigations should address

whether cognitive deficits associated with *PHF21A* and *LSD1* share impaired response to neuronal activity via cAMP signaling as a core mechanism.

There is controversy in the literature regarding the role of *PHF21A* as a coactivator or corepressor of transcription. *PHF21A* knockdown in cells causes de-repression of REST target genes, in line with the role of PHF21A as part of the repressive LSD1-CoREST complex (Klajn et al., 2009; Lan et al., 2007). On the other hand, biochemical studies have shown that PHF21A inhibits LSD1-mediated demethylation of H3K4me2 *in vitro*, suggesting that PHF21A may dampen the repressive function of the LSD1-CoREST complex (Shi et al., 2005). A previous genetic screen in *C. elegans* identified CoREST and LSD1 orthologues, *spr-1* and *spr-5*, respectively, as suppressors of the developmental defects observed in the *sel-12*/presenilin mutant worms (Eimer et al., 2002; Jarriault and Greenwald, 2002). In humans, mutations in the presenilin genes, *PSEN1* and *PSEN2*, encoding intramembrane proteases, and their substrate, amyloid precursor protein (*APP*) gene are the major genetic basis for the severe cognitive decline in familial Alzheimer's disease (Brunkan and Goate, 2005). The LSD1-CoREST complex may therefore play evolutionarily-conserved roles in repressing presenilin/APP pathway genes, thereby contributing to normal cognitive development and function. Contrary to the repressive roles of LSD1 and CoREST on presenilin expression, our patient RNA-Seq data showed significant down-regulation of both *APP* and the human presenilin, *PSEN2* upon reduction of PHF21A level. Of note, invertebrates, including *C. elegans*, do not appear to carry an orthologue of *PHF21A* (Lakowski et al., 2006), while *PHF21A* orthologues are present broadly among vertebrates. These observations lead us to speculate that PHF21A may have evolved in vertebrates as a new component of the LSD1-CoREST complex to negatively tune the action of that complex, thereby promoting transcription of specific genes.

In conclusion, this work identified reduced expression of cAMP signaling genes as a molecular signature in PSS-related developmental delay and provided insights into the etiology of this condition. Modulation of cAMP-signaling could be a novel therapeutic target for the cognitive disorders stemming from dysfunction of PHF21A and LSD1, and more broadly, dynamic regulation of H3K4 methylation.

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Abbreviations

PSS	Potocki-Shaffer Syndrome
H3K4me	Histone H3 Lysine 4 methylation
cAMP	Cyclic Adenosine Monophosphate
CRE	cAMP Responsive Elements

RNA-Seq	RNA Sequencing
DE-genes	Differentially Expressed genes
Sc	Scramble
NDD	Neurodevelopmental Disorder
IEG	Immediate Early Gene

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Highlights

- *PHF21A* is genetically associated with Potocki Shaffer Syndrome, but the molecular basis of cognitive deficits is unknown
- RNA-Seq of *PHF21A*-deficient patient cells revealed 1,885 commonly misregulated genes
- Pathway analysis showed downregulation of pathways relevant to learning and memory, including cAMP-mediated signaling
- Reporter assays showed *PHF21A* is required for full induction of the cAMP-mediated transcriptional response
- *PHF21A*-deficient patient cells exhibited delayed transcription of immediate early genes following cAMP signaling

Figure 1.

RNA-Seq analysis of *PHF21A*-deficient patient-derived lymphoblasts. **A.** Genome browser shot of the *PHF21A* locus shows decreased read density from the male patient (heterozygous deletion) and the female patient (translocation) compared to the unaffected individuals. The gold bar represents the microdeletion in the male patient. Arrow: translocation breakpoint. **B.** PCA plot showing the clustering of each of the samples with technical duplicates along two principle components. Each technical duplicate clusters tightly with the other. Male vs. female and WT vs. *PHF21A*-deficient also cluster together in the plot. **C.** Relative gene read counts of *PHF21A* calculated by DESeq. **D.** Relative gene read counts of genes in the microdeletion region for the male patient calculated by DESeq. **E.** The gene desert region on Chromosome 1 is the recipient region of the translocation found in the female patient. Ectopic transcripts are detected likely due to the *PHF21A* promoter being fused to this region.

Figure 2.

Commonly misregulated genes in *PHF21A*-deficient cells. **A.** DESeq reveals 1,885 misregulated genes (significantly misregulated genes defined as a p -value < 0.05 , after determining average non-adjusted p -value through Fisher's method). Volcano plot profiles – $\log_{10} p$ -value and $\log_2$ fold change of gene expression between *PHF21A*-deficient vs. control samples. **B.** and **C.** A similar number of genes (**B.**) are up-regulated and down-regulated with similar magnitude of change (**C.**). In the box plot, whiskers represent 1.5 times the interquartile range (IQR) with the median. **D.** Scatter plot of gene expression changes in the *PHF21A*-deficient cells. Average $\log_2$ read counts per gene of the two *PHF21A*-deficient samples and the two unaffected samples are plotted on the Y- and X-axes respectively. Differentially expressed genes with a p -value < 0.05 by DESeq are in red. **E.** 213 REST target genes are misregulated with a similar number up- and down-regulated.

Figure 3.

Pathway analysis shows down-regulation of processes important to neurodevelopment and function. **A and B.** Cytoscape network representations (Cline et al., 2007) of significantly down-regulated (A) and up-regulated (B) pathways (FDR<0.05) and their connections based upon commonly-shared signature genes. The significance of the GO term misregulation is denoted by color. The size of each circle represents the number of genes in the ontology term (e.g. a large circle denotes a GO term with many genes, whereas a small circle denotes a more specific GO term with fewer genes). The larger terms in boxes represent main clusters identified by REVIGO (Supek et al., 2011). **C.** 395 CREB target genes are misregulated with slightly more of them being down-regulated.

Figure 4.

qRT-PCR analysis of *PHF21A* shRNA knockdown in lymphoblasts. **A.** *PHF21A* shRNA leads to downregulation of *APP* and *OXTR*, two genes shown to be down-regulated in the RNA-Seq analysis. Several genes relevant to cAMP signaling are also downregulated upon *PHF21A* knockdown using three independent shRNAs. **B.** Expression of two REST-target genes, *SCN3A* and *MAP1B*, upon *PHF21A* knockdown. Error bars represent $\pm$ SEM of a technical triplicate (N=3) of qRT-PCR analysis. *p-value < 0.05, **p-value < 0.01. Significance was calculated using ANOVA with Tukey post-hoc analysis between the scramble shRNA samples and each *PHF21A* shRNA sample.

Figure 5.

Luciferase reporter assays and transcription of IEGs following forskolin stimulation demonstrate PHF21A's roles in the transcriptional response to the cAMP-mediated signaling pathway. **A.** HEK293T cells were co-transfected with a luciferase construct with CRE sequences inserted upstream of the HSV-TK promoter and expression plasmids for *PHF21A*- or scramble shRNAs. After 8 hours of 30 μ M forskolin treatment, the luciferase activity was lower upon *PHF21A* knockdown compared to scramble shRNA (*p-value < 0.05; **p-value < 0.01 as calculated using ANOVA with Tukey post-hoc analysis between the scramble shRNA samples and each *PHF21A* shRNA sample). **B.** The same experiment was performed using the mutated (mCRE) reporter. Forskolin treatment nor *PHF21A* shRNA knockdown induced increased expression of the mutated CRE-luciferase construct. Error bars represent $\pm$ SEM of a biological triplicate (N=3) obtained from cells grown in independent wells. **C and D.** Patient-derived lymphoblastoid cells were stimulated with forskolin or DMSO for 30 minutes, 2 hours, or 6 hours and *c-FOS* (C) and *FOSB* (D) mRNA levels were quantified by qRT-PCR. Shown is mRNA induction by forskolin normalized to DMSO relative to unstimulated cells (zero hour time point). Error bars represent the range of data from two independent experiments.

Table 1

LR-Path Analysis of *PHF21A*-deficient patient RNA-Seq compared to control samples (called by Gene Ontology- Biological Processes terms). Shown are the top 14 most significantly misregulated pathways, ranked by most significant p-value, as reported by LR-Path

GO-Biological Processes Pathway	P-Value	Direction of misregulation	Signature Genes
Visual Learning	7.49×10^{-9}	Down	APP, NETO1
Visual Behavior	1.43×10^{-8}	Down	APP, HOXA1, NETO1
Myelin Maintenance	2.89×10^{-8}	Down	MYRF, EPB41L3
cAMP-Mediated Signaling	7.52×10^{-8}	Down	PCLO, CRT3, RIMS2
Associative Learning	1.38×10^{-7}	Down	APP, NETO1
Cortical Actin Cytoskeletal Organization	2.75×10^{-7}	Down	EPB41L3, FMNL2
Regulation of cAMP-Mediated Signaling	3.09×10^{-7}	Down	CRT3, PEX5, RGS2
Positive Regulation of G2/M Transition of Mitotic Cell Cycle	4.25×10^{-7}	Down	APP, CCND1
Protein Localization to Synapse	6.20×10^{-7}	Down	NETO1
Learning	6.28×10^{-7}	Down	FOSL1, NETO1, APP
Endoplasmic Reticulum Calcium Ion Homeostasis	1.05×10^{-6}	Down	PSEN2, CAMK2D, APP
Suckling Behavior	1.13×10^{-6}	Down	OXTR, APP, PHF21A
Cyclic-Nucleotide-Mediated Signaling	1.37×10^{-6}	Down	CRT3, RIMS2, PEX5L
Regulation of Voltage-Gated Calcium Channel Activity	1.54×10^{-6}	Up	AHNAK