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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. However, the molecular consequences of reduced *PHF21A* expression remain elusive. 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 Cyclic Adenosine Monophosphate (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. *This article is part of a Special Issue entitled: [SI: Molecules & Cognition].*

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 (Stickens et al., 1996; Wu et al., 2000; Mavrogiannis et al., 2001; Wakui et al., 2005). *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 Speci-

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Abbreviations: cAMP, Cyclic Adenosine Monophosphate; CRE, cAMP-Responsive Elements; DE-genes, Differentially Expressed genes; H3K4me, Histone H3 Lysine 4 methylation; IEG, Immediate Early Gene; NDD, neurodevelopmental disorder; PSS, Potocki–Shaffer Syndrome; RNA-Seq, RNA Sequencing; Sc, Scramble.

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fic 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, 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 (Hakimi et al., 2002; Bruce et al., 2004). Previous work has shown that loss of *PHF21A* leads to the de-repression of REST target genes in non-neuronal cells (Lan et al., 2007; Klajn et al., 2009). *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 two mismatches and only uniquely mapped reads were analyzed further. Aligned reads of technical replicates from each individual were merged using SAM tools 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 ($p\_average = p1 * p2 * [1 - \log(p1 * p2)]$). 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 h. 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.

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