

# A Role for the Chromatin-Remodeling Factor *BAZ1A* in Neurodevelopment

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**ABSTRACT:** Chromatin-remodeling factors are required for a wide range of cellular and biological processes including development and cognition, mainly by regulating gene expression. As these functions would predict, deregulation of chromatin-remodeling factors causes various disease syndromes, including neurodevelopmental disorders. Recent reports have linked mutations in several genes coding for chromatin-remodeling factors to intellectual disability (ID). Here, we used exome sequencing and identified a nonsynonymous de novo mutation in *BAZ1A* (NM\_182648.2:c.4043T > G, p.Phe1348Cys), encoding the ATP-utilizing chromatin assembly and remodeling factor 1 (ACF1), in a patient with unexplained ID. ACF1 has been previously reported to bind to the promoter of the vitamin D receptor (VDR)-regulated genes and suppress their expression. Our results show that the patient displays decreased binding of ACF1 to the promoter of the VDR-regulated gene *CYP24A1*. Using RNA sequencing, we find that the mutation affects the expression of genes involved in several pathways including vitamin D metabolism, Wnt signaling and synaptic formation. RNA sequencing of *BAZ1A* knockdown cells and *Baz1a* knockout mice revealed that *BAZ1A* carry out distinctive functions in different tissues. We also demonstrate that *BAZ1A* depletion influence the expression of genes important for nervous system development and function. Our data point to an important role for *BAZ1A* in neurodevelopment, and highlight a possible link for *BAZ1A* to ID.

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**KEY WORDS:** ACF1; *BAZ1A*; *CYP24A1*; epilepsy; intellectual disability; neurodevelopment; vitamin D metabolism; Wnt signaling

## Introduction

Chromatin organization is a dynamic and regulated process that allows differential accessibility of DNA to different regulatory proteins to activate or repress transcription. The establishment of different chromatin states is enabled by a group of ATP-dependent chromatin-remodeling complexes that utilize ATP hydrolysis to alter the state, placement, or position of the nucleosomes, thereby regulating transcription [Hargreaves and Crabtree, 2011]. Together with other chromatin organizers, they represent the first layer of regulatory control, which ascertains a stable and correct temporal and spatial gene expression [Zentner and Scacheri, 2012]. Most ATP-dependent chromatin remodelers are multisubunit complexes and share a conserved catalytic ATPase subunit, which contains an ATPase domain and other specialized domains related to their mode of action. Additionally, they harbor several noncatalytic proteins, also known as accessory subunits, required to regulate the activity of the catalytic subunit and to ensure accurate recruitment of chromatin remodelers to their target genomic regions [Flaus et al., 2006]. Chromatin-remodeling complexes can be divided into four families based on the composition of their ATPase subunit. These families are referred to as the SWI/SNF, ISWI, CHD, and INO80 families of remodelers [Becker and Horz, 2002]. Several studies have illustrated a fundamental role of chromatin-remodeling factors during development, differentiation, and homeostasis [Day and Sweatt, 2011; Ronan et al., 2013]. In line with these observations, dysfunction of chromatin remodelers has been predicted to cause multisystem disorders.

ISWI, also known as the imitation switch, is among the most conserved chromatin remodeling families [Li and Reinberg, 2011]. In humans, complexes belonging to ISWI possess one of two possible ATPase catalytic subunits, Snf2H and Snf2L, and various noncatalytic subunits [Mohrmann and Verrijzer, 2005]. The differential incorporation of the several accessory units with the two ATPases in ISWI leads to the formation of complexes with distinct functions and tissue specificity [Lessard et al., 2007; He et al., 2008; Ho et al., 2009]. ISWI is involved in various biological functions, including transcriptional activation and repression, chromatin assembly and nucleosome spacing, and DNA replication [Langst and Becker, 2001; Narlikar et al., 2002; Eberharter and Becker, 2004; Banting et al., 2005]. Recently, several studies highlighted essential roles for members of ISWI during embryogenesis and neurodevelopment and for cognition [Strohner et al., 2001; Stopka and Skoultchi, 2003; Dirscherl et al., 2005; Yoshimura et al., 2009; Alvarez-Saavedra et al., 2014]. Knockout mice for components of this complex displays growth and cell proliferation abnormalities, cognitive defects, neurodevelopmental deficits, and in some cases compromise mice viability [Stopka and Skoultchi, 2003;

Additional Supporting Information may be found in the online version of this article.

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Landry et al., 2008; Alvarez-Saavedra et al., 2014]. In line with this, dysfunction of members of ISWI has been predicted to cause cancer and developmental disorders such as intellectual disability (ID) [Perez Jurado, 2003; Ye et al., 2009; Buganim et al., 2010; Langst and Manelyte, 2015].

ID is a common neurodevelopmental condition that affects around 1%–3% of the population [Leonard and Wen, 2002; Shevell et al., 2003]. It is generally characterized by reduced intellectual and behavioral abilities, which imposes a significant burden on patients and their families. ID is a complex condition and considered to be clinically and genetically heterogeneous. Several human exome sequencing studies have linked mutations in genes encoding chromatin-remodeling complexes to general ID, to distinct ID disorders such as Coffin-Siris syndrome (CSS) and to autism spectrum disorder [Lopez and Wood, 2015]. Although the majority of these mutations have been found in the *ARID* and *SMARC* gene families, it is likely that more mutations will be identified in other genes coding for chromatin-remodeling factors [Hargreaves and Crabtree, 2011; Wilson and Roberts, 2011; Santen et al., 2012; Santen et al., 2013].

Using exome sequencing of a trio family with two healthy parents and a child suffering from ID, epilepsy, ataxia, and hyper mobile joints, we identified a single nonsynonymous de novo variant in the gene *BAZ1A* (see Supp. Methods for detailed phenotypic description of the patient). *BAZ1A* (NM\_182648, OMIM 605680) codes for the chromatin-remodeling factor ACF1, a member of the ISWI chromatin-remodeling complexes ACF and CHRAC. ACF1 has been implicated in different functions including chromatin assembly and remodeling, and in double-strand DNA damage repair [Ito et al., 1999; Lan et al., 2010]. It has also been shown to have a function in transcriptional repression of vitamin D3 receptor (VDR)-regulated genes by blocking the accessibility of the transcription factors to VDR in the absence of vitamin D3 (VD3) [Ewing et al., 2007]. Moreover, ACF1 was recently shown to contribute to the susceptibility to depression, and in regulating stress related behaviors [Sun et al., 2015]. Here, we found that the identified variant in *BAZ1A* affects the function of ACF1 as a transcription regulator, causing differential expression in genes important in vitamin D metabolism, the Wnt signaling pathway and in the development of proper synaptic function. We further investigate the general function of *Baz1a* by RNA-seq in knockout mice.

## Materials and Methods

### DNA Extraction

Genomic DNA was extracted from peripheral blood lymphocytes according to standard procedures. Patient samples were studied with the approval of the local Research Ethics Committee, Faculty of Medicine, Uppsala University, Sweden.

### Copy Number Variation Analysis

Chromosomal microarray analysis (CMA) was performed using Affymetrix 250K Nsp 1 array according to the standard Affymetrix GeneChip protocol (Affymetrix Inc., Santa Clara, CA). Data analysis was carried out using Genotyping Console 4.0.

### Exome Capture

Exome enrichments for the trio family were prepared from 3.0  $\mu$ g genomic DNA from the trio. The DNA samples were sheared with

Covaris instrument (Covaris, Inc., Woburn, MA). Libraries were created from the sheared DNA using AB Library Builder System and captured using the Agilent SureSelect 50 Mb exome enrichment kit, according to the manufacturer's protocols. Exome capture was conducted by hybridizing the DNA libraries with biotinylated RNA baits for 24 hr followed by extraction using streptavidin-coated magnetic beads. Captured fragments were amplified and emulsion PCR was conducted using Bead System and sequenced using SOLiD4 producing 68–80 million reads per sample.

### Exome Capture Data Analysis

Exome sequence data was aligned using BioScope (Applied Biosystems, Foster City, CA). SNP calls were made using DiBAYES with default settings and resulted in 16,723 nonsynonymous single-nucleotide variants (SNVs) with a sequence read coverage  $>3$  in the patient. Filtering was performed using ANNOVAR [Wang et al., 2010] and dbSNP 132 [Sherry et al., 2001]. Further filtering was done using the parental samples and our in-house database of previously analyzed exomes. Manual inspection of each SNP remaining was then performed resulting in three putative de novo coding variant. Sanger sequencing only validated a single de novo mutation in the *BAZ1A* gene (NM\_182648.2:c.4043T>G, g.35231163A>C). The other two variants were found to be false positives. The DNA mutation numbering system is based on genomic DNA. The de novo variant in *BAZ1A* identified in the patient is submitted to LOVD 3 ([www.lovd.nl](http://www.lovd.nl)).

### PCR and Sanger Sequencing

The de novo mutation in *BAZ1A* was validated using standard PCR and Sanger sequencing. Briefly, PCR was carried out with initial denaturation at 95°C for 3 min and 35 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min. The reaction contained 10x DreamTaq buffer (Fermentas, Waltham, MA), 200  $\mu$ M of each dNTP, 1.25 U of DreamTaq DNA polymerase (Fermentas), primers harboring the mutation site (Supp. Table S1), and 12.5 ng DNA. PCR products were visualized on 1.5% agarose and purified using PCR purification kit (QiaGen, Hilden, Germany). The mutation was then validated in the patient by Sanger sequencing, whereas the parents confirmed not to carry the mutation.

### In Silico Prediction Methods

#### *The combined annotation-dependent depletion*

This score was assigned using the v1.0. Precalculated combined annotation-dependent depletion (CADD) scores were found at <http://cadd.gs.washington.edu/>.

#### *MutationTaster (build; NCBI 37/Ensembl 69)*

This test was used to predict the disease-causing potential of the variant (<http://www.mutationtaster.org>). *BAZ1A* transcript (ID ENST00000382422) was used in the analysis.

#### *MUpro (MUpro 1.0)*

This test was used to evaluate if the de novo variant compromises protein stability (<http://mupro.proteomics.ics.uci.edu>).

## RNA Extraction and cDNA Synthesis

Total RNA was extracted from transfected cells or from trio member's whole blood using Ribopure kit (Ambion, Waltham, MA). RNA samples were then treated with TURBO DNase (Ambion). Single-stranded cDNA was synthesized from 1  $\mu$ g RNA using Maxima<sup>®</sup> first-strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, Waltham, MA).

## Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to measure the relative expression of differentially expressed genes (DEGs) between the patient and the parents and between the wt-ACF1 or mut-ACF1-transfected cells. The qPCR was carried out using Stratagene Mx3000P in 96 well plates. The reactions were conducted with initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. The cycles were followed by a melting curve step. The reaction contained 10 ng single-stranded cDNA, 0.4  $\mu$ M of each primer (Supp. Table S1), and 12.5  $\mu$ l Maxima SYBR green/ROX master mix (Fermentas) in a total reaction volume of 25  $\mu$ l. The relative expression levels in the samples were determined using  $\Delta\Delta$ CT method normalized to the levels of  $\beta$ -actin. All samples were run in triplicates and the average values were used to calculate the expression levels for each target. Raw data were analyzed using MxPro software (Stratagene, Santa Clara, CA).

## Vitamin D Test

A test for vitamin-D (25-(OH)D) levels in serum revealed an insufficiency (70 nmol/l, normal range is 125–200 nmol/l). Calcium level was normal (2.34 nmol/l).

## Cloning and Mutagenesis

Wild-type (wt) and mutant (mut) hACF1 transcripts were cloned in pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). The original acf1 clone in pEGFP-N1 expression vector (CLONTECH) was a generous gift from Prof. PD Varga-Weisz. Briefly, the hACF1 transcript was amplified from the original transcript using full-length primers (Supp. Table S1). The hACF1 transcript was then cloned into pcDNA3.1/V5-his-TOPO vector by TOPO Cloning reaction, using pcDNA3.1 Directional TOPO expression kit (Invitrogen). The mutant hACF1 construct was produced by PCR-based site-directed mutagenesis using PFU Turbo (Stratagene). Positive hACF1 constructs were further validated by Sanger sequencing.

## Transient Transfection

Saos-2 cells were maintained in DMEM medium (Sigma, Saint Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 2 mM of L-glutamin and 1 $\times$  penicillin/streptomycin (Sigma). All transfection experiments were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer recommendations. Briefly, 1 $\times$ 10<sup>6</sup> cells were transfected in 6-wells plates using 4  $\mu$ g of wt or mut hACF1pcDNA3.1 plasmid. All transfections were performed in duplicate. Cells were collected for analysis 48 hr post-transfection.

## RNA Sequencing and Mapping for the Trio Family

RNA sequencing of the trio family was performed using a SOLiD4 instrument. Each parent was sequenced once and two replicates were

sequenced for the child. Sequenced reads were mapped to the HG19 version of the human reference genome using the BioScope software (Applied Biosystems). The number of reads mapping to each RefSeq gene (based on the refFlat table from the UCSC genome browser) was then counted using htseq-count (<http://wwwhuber.embl.de/users/anders/HTSeq/doc/count.html>). Differential expression was calculated using the DESeq2 [Love et al., 2014] and EdgeR [Robinson et al., 2010], comparing the replicated patient samples to the parents, generating a lists of DEGs. Candidate genes were then chosen using an adjusted *P* value of 0.0001 as cutoff and required that each gene had a *P* value below the cut-off in both analysis programs. Gene ontology (GO) analysis was performed using the R-package GOseq, and the REVIGO Webserver was used to identify nonredundant GO categories [Supek et al., 2011]. The list of Wnt target genes used for enrichment analysis was compiled from the Nousse laboratory ([http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target\\_genes](http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes)), for genes found in both humans and other organisms (if a human orthologue could be identified)

## BAZ1A siRNA Knockdown

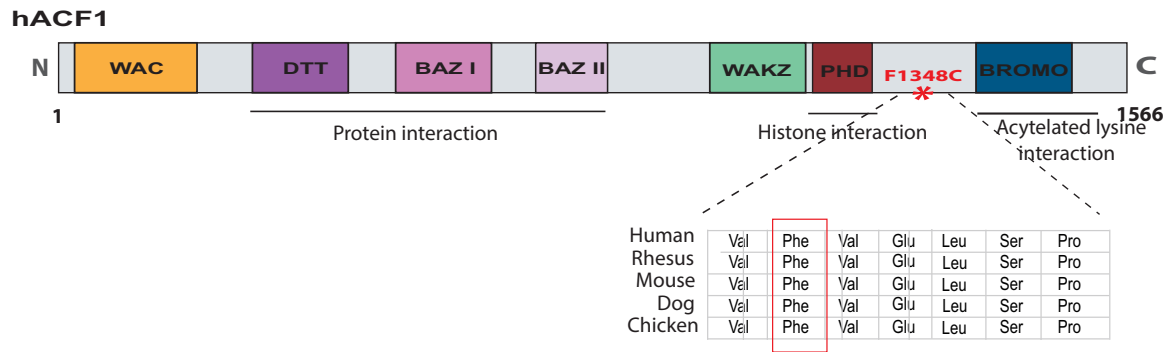
siRNA-mediated knockdown of BAZ1A expression was performed in Saos-2 cells using ON-TARGETplus BAZ1A siRNA SMARTpool (Cat#: L-006941; GE Dharmacon, Lafayette, CO) according to the manufacturer recommendations. ON-TARGETplus nontargeting pool (Cat#: D-001810-10-05; GE Dharmacon) was used as negative control for the siRNA experiment. Briefly, 5 $\times$ 10<sup>4</sup> cells were seeded in 24-wells plate 24 hr before transfection with complete growth media (DMEM; Cat#: D 5546; Sigma-Aldrich). Cells were then transfected with either BAZ1A siRNA SMARTpool or nontargeting pool to a final concentration of 25 nm and 0.65  $\mu$ l DharmaFECT transfection reagent 1 were added to the cells to a final concentration of 25 nM. The media containing the siRNA and the transfection reagent was replaced with complete growth media 24 hr after transfection. The cells were harvested at 48 hr post-transfection. All transfections were performed in triplicates.

## SiRNA Sequencing

Poly(A) selected RNA extracted from the siRNA-treated Saos-2 cells and controls was sequenced on an Illumina HiSEQ instrument. Sequenced reads were aligned using the program STAR and reads for each gene was counted using the Ensembl human gene list (version 70). Differential expression was detected using the DESeq2 R package, using an adjusted *P* value of 0.05. GO enrichment analysis was performed as described for the trio family. All further analysis was made using in-house R scripts.

## Mouse Transcriptome Sequencing

*Baz1a* knockout mice (C57BL/6N, *Baz1a*<sup>tm1b(EUCOMM)Hmgu</sup>) were generated by the International Knockout Mouse Consortium [Skarnes et al., 2011; Bradley et al., 2012]. Mouse tissue was obtained from The Canadian Mouse Mutant Repository at The Hospital for Sick Children (Toronto, Canada). Four knockout mice (two males, two females) and four littermate controls (two males, two females) were euthanized at 6 weeks of age. Heart and brain tissue was removed and each brain was sliced into four pieces (corresponding to frontal cortex, striatum/hippocampus, cerebellum, and brain stem). Tissue pieces were immediately snap frozen in liquid nitrogen. Total RNA was extracted from tissues using Ribopure kit



**Figure 1.** A schematic representation of the human ACF1 protein indicating known interaction domains. ACF1 domains include a WAC motif necessary for the binding of ACF1 to the DNA and involved in ACF-mediated chromatin assembly [Fyodorov and Kadonaga, 2002], and a DTT motif (DNA binding homeobox and transcription factors). It further contains three conserved motifs, two BAZ and a WAKZ (responsible for the binding of ACF1 to ISWI, the other subunit of the ACF complex) [Jones et al., 2000; Eberharter and Becker, 2004], a PHD finger (plant homeodomain) and bromodomain (Brds) motifs. The mutation (p.Phe1348Cys) is located in the linker region between the PHD and Brd domains. The evolutionary conservation is shown for the mutation and the surrounding amino acids.

(Ambion). *Baz1a* knockout was validated in RNA sequencing data and using qrtPCR (Supp. Fig. S1). RNA was sequenced using Illumina HiSeq2500, generating 100-bp paired-end strand-specific sequence from polyA<sup>+</sup> RNA. Standard protocols were used for library preparation and sequencing and an average of 97 million reads per sample was produced. Reads were aligned using the same method as described for the siRNA sequencing but using the GRCh38 version of the mouse reference genome. Reads were counted using the Gencode GRCh38 mouse gene list. Differential expression analysis was performed using the R package DESeq2, and DEGs were identified using an adjusted *P* value of 0.05 as cutoff. GO analysis was performed using the R-package Goseq, all further analysis of the data was made using in-house R scripts. In order to compare overlaps between DEGs in knockout mice and previously published ChIP-seq data, DEGs (*P* value < 0.05) in the knockout mice brain (*n* = 2,160) and knockout mice heart (*n* = 1,586) were compared with the list of regions indicated in Sun et al. (2015). All sequencing data are deposited in the EMBL-EBI Sequence Read Archive (European Nucleotide Archive) under accession number PRJNA319519.

## Results

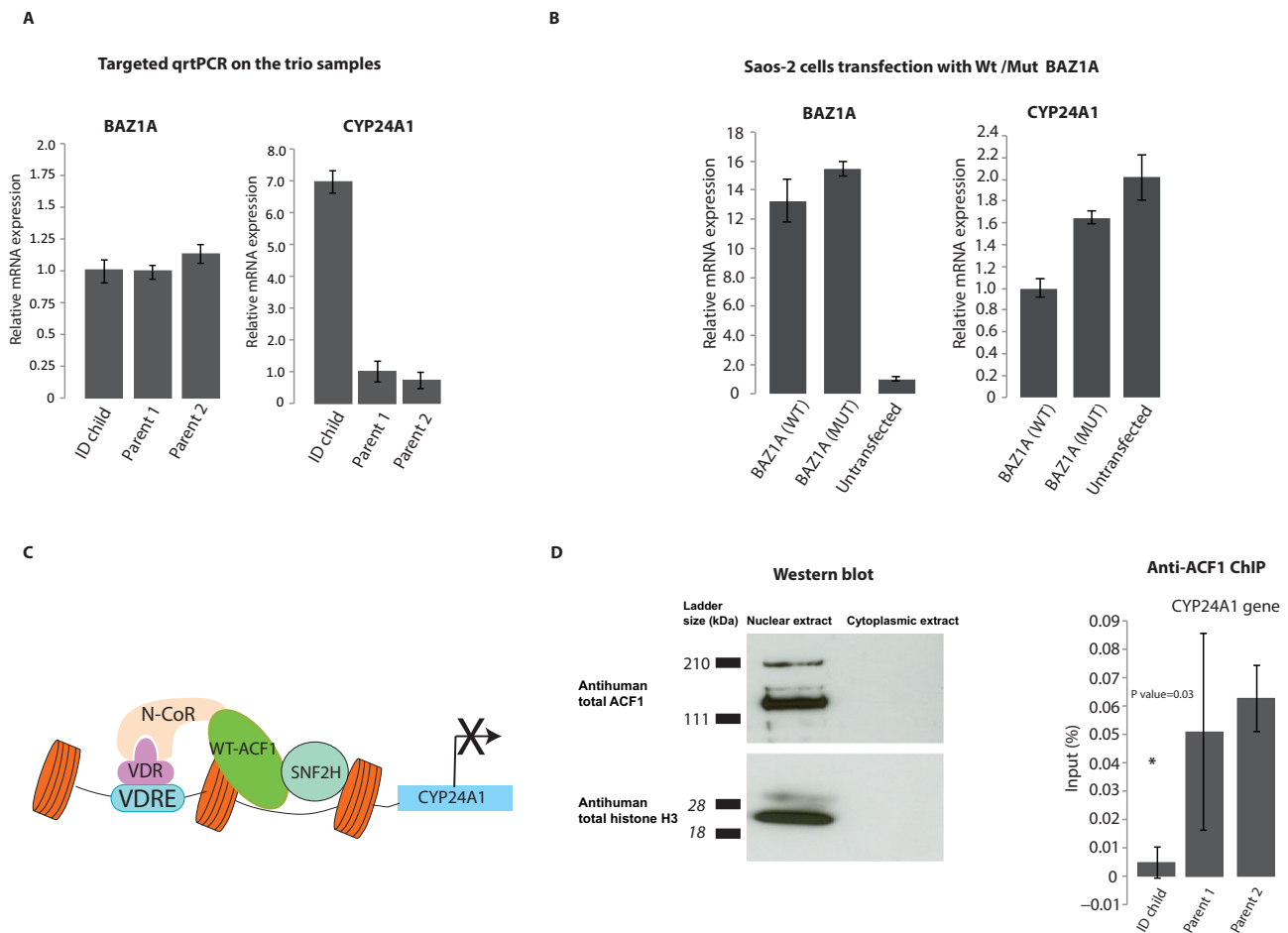
### Exome Sequencing

Aiming to identify the disease-causing mutation in the patient, we first performed CMA using Affymetrix 250K Nsp 1 array and found no evidence of any chromosomal aberrations. We then performed target enrichment of exome sequences on DNA from the patient and his parents using the Agilent SureSelect 50 Mb kit. Captured targets were then sequenced using SOLiD4. Analysis of variants was performed to identify potential recessive-causative variants as well as de novo mutations in the patient. We found no potentially causative homozygous or compound heterozygous recessive variants in ID candidate genes. After filtering the exome sequencing data followed by Sanger sequencing of candidate de novo mutations, one single de novo variant could be validated in the child, a mutation in the chromatin-remodeling gene *BAZ1A* (NM\_182648). The de novo variant is a T > G substitution in the gene *BAZ1A* (NM\_182648.2:c.4043T > G, NC\_000014.8:g.35231163A > C), and it leads to a phenylalanine (Phe) to cysteine (Cys) change at amino acid 1,348 (p.Phe1348Cys) in ACF1, the protein product of *BAZ1A*. The

substitution thus changes a nonpolar hydrophobic residue (Phe) to a polar uncharged residue (Cys). The variant is located in an evolutionarily conserved region in the oligopeptide linker motif that connects the PHD domain and the bromodomain (Brd) (Fig. 1). We evaluated the potentially damaging effect of the variant using CADD [Kircher et al., 2014], which yielded a CADD-score of 16.71, indicating that it is among the 10% most damaging substitutions in the genome. We also used MutationTaster [Schwarz et al., 2010] to predict the disease-causing potential of the variant and it was scored as likely pathogenic. To test whether the variant compromises protein stability, we used the prediction of protein stability changes for single-site mutation from sequences software (MUpro) [Cheng et al., 2006]. The analysis predicted a decreased stability with a confidence score -0.799. The confidence score of MUpro ranges between -1 and 1. A score less than zero is indication of decreased protein stability, and a score of more than zero is an indication that the mutation increases protein stability. This observation further suggests that the variant has a negative effect on the function of the protein.

### Effect of BAZ1A De Novo Variant on Vitamin D-Regulated Genes

ACF1 has been previously reported to interact with the nuclear receptor corepressor (N-CoR) and act as a suppressor of the VDR-regulated genes in the absence of VD3 [Ewing et al., 2007]. The PHD and brd domains in ACF1 are critical for the repression process through binding to histone structures of VDR-regulated genes. To test the impact of the variant on the expression of VDR-regulated genes, we performed targeted qrtPCR on RNA extracted from blood samples from the trio and measured the expression of *RANKL*, *VDR*, and *CYP24A1*. Interestingly, *CYP24A1* [OMIM 126065], which has been implicated in the metabolism of VD3 levels [Jones et al., 2012], was significantly upregulated (sevenfold, *P* value = 0.005) in the patient compared with both parents (Fig. 2A). Meanwhile, *BAZ1A* itself showed no difference in expression between the patient and the parents. The patient showed equal biallelic expression of the reference allele and the mutation, as determined by Sanger sequencing of the qrtPCR product, indicating no effect of the mutation on the expression level of *BAZ1A* itself. No significant difference was detected in *VDR* and *RANKL* expression.

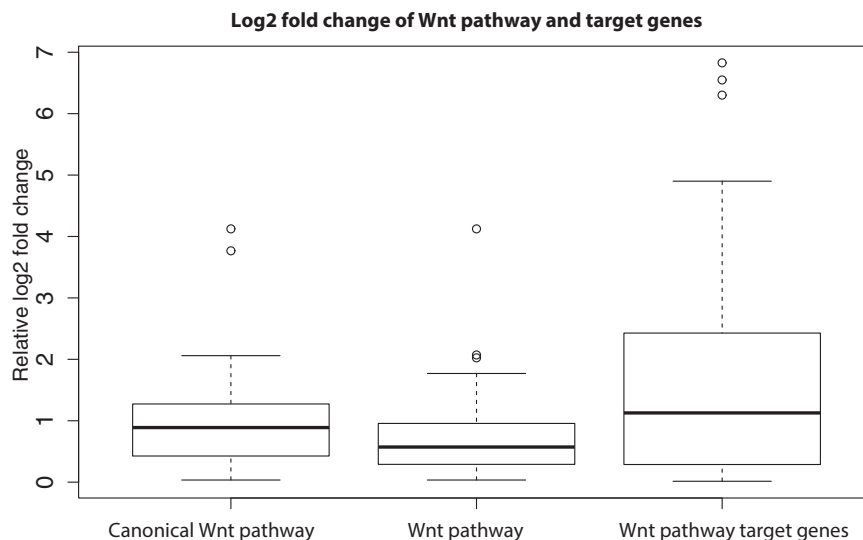


**Figure 2.** Differential expression of *CYP24A1*. **A:** The left graph shows levels of *BAZ1A* expression in the trio. To the right are the results of targeted qPCR validation of the *CYP24A1* expression in the affected child and the healthy parents. **B:** The left graph shows overexpression of the *BAZ1A* in the transfected Saos-2 cells compared with untransfected cells. The right graph shows the qPCR validation of the *CYP24A1* expression in Saos-2 cells transfected with either wt-*BAZ1A* or mut-*BAZ1A*. Saos-2 cells transfected with the wt-*BAZ1A* is considered as control. All expression values were normalized to the level of B-actin in each respective sample. Expression levels represent mean values of the biological and technical replicates and error bars are  $\pm$ SD. **C:** Simplified schematic model showing the role of ACF1 in the regulation of expression of *CYP24A1*. In the absence of vitamin D3, ACF1 and SNF2H (the other member of the ACF complex) interact with N-CoR and suppress the expression of *CYP24A1* through binding to the VDR. **D:** ACF-1 protein is localized to the nucleus of Saos-2 cells. To the left, protein expression analysis was performed by western blot of nuclear versus cytoplasmic protein lysates using specific antibodies against ACF-1 and total histone H3. Histone H3 was used as a positive control for the nuclear fraction purification. To the right, ChIP analysis of anti-ACF1 precipitated DNA measured by qPCR from fresh blood monocytes of the trio family. Chromatin precipitated from each sample is reported as a percentage of input. Error bars are  $\pm$ SD of the biological and technical replicates. Asterisks indicate significant statistical difference ( $P \leq 0.05$ ) of *CYP24A1* enrichment in anti-ACF1-precipitated DNA between the ID patient and the parents. Student's *t*-test was used to calculate *P* values.

To test whether the elevated level of *CYP24A1* in the patient also affected the levels of VD3, we performed a clinical test to measure the levels of VD3 in patient blood. The test showed that the patient suffers from moderate VD3 insufficiency (70 nmol/l). To determine whether the de novo variant is the cause of *CYP24A1* upregulation, and to exclude the possibility that the upregulation is caused by other variants in the patient, we performed transient transfection of wild-type and mutant ACF1 transcripts (wt-ACF1, mut-ACF1) in Saos-2 cells. The mut-ACF1 harbors the same mutation identified in the patient. Saos-2 cells were chosen due to their well-characterized VD3 pathway. qPCR results showed that *CYP24A1* is indeed upregulated in the cells transfected with mut-ACF1 (Fig. 2B), highlighting the role of the de novo variant on the expression levels of *CYP24A1*.

ACF1 is known to directly interact with a sequence motif upstream of the *CYP24A1* gene through VDR, causing repression of

*CYP24A1* transcription (Fig. 2C) [Ewing et al., 2007; Luo et al., 2010]. As further validation on the effect of the variant, we sought to evaluate whether the de novo mutation decreases the association of ACF1 to the *CYP24A1* promoter. To test this, we conducted chromatin-immunoprecipitation (ChIP) using human ACF1 antibodies on freshly purified blood monocytes from the trio (Supp. Methods). We first validated the specificity of the human ACF1 (hACF1) antibody in Saos-2 cells using western blot (Fig. 2D). We then performed qPCR on the immunoprecipitated DNA to determine the occupancy of ACF1 on *CYP24A1* promoter. The results demonstrated that the patient's monocytes have significantly less ACF1 occupancy on the *CYP24A1* promoter as compared with the parents ( $P$  value = 0.03) (Fig. 2D). These results indicate that the mutated ACF1 has a decreased ability to bind and repress *CYP24A1*, leading to an increase in *CYP24A1* expression.



**Figure 3.** The log<sub>2</sub> fold change of the genes in the GO categories Wnt pathway ( $n = 123$ ), canonical Wnt pathway ( $n = 45$ ), and Wnt target genes ( $n = 44$ ). Genes having no reads aligned in the RNA sequencing experiments were not counted.

### RNA Sequencing of the Trio

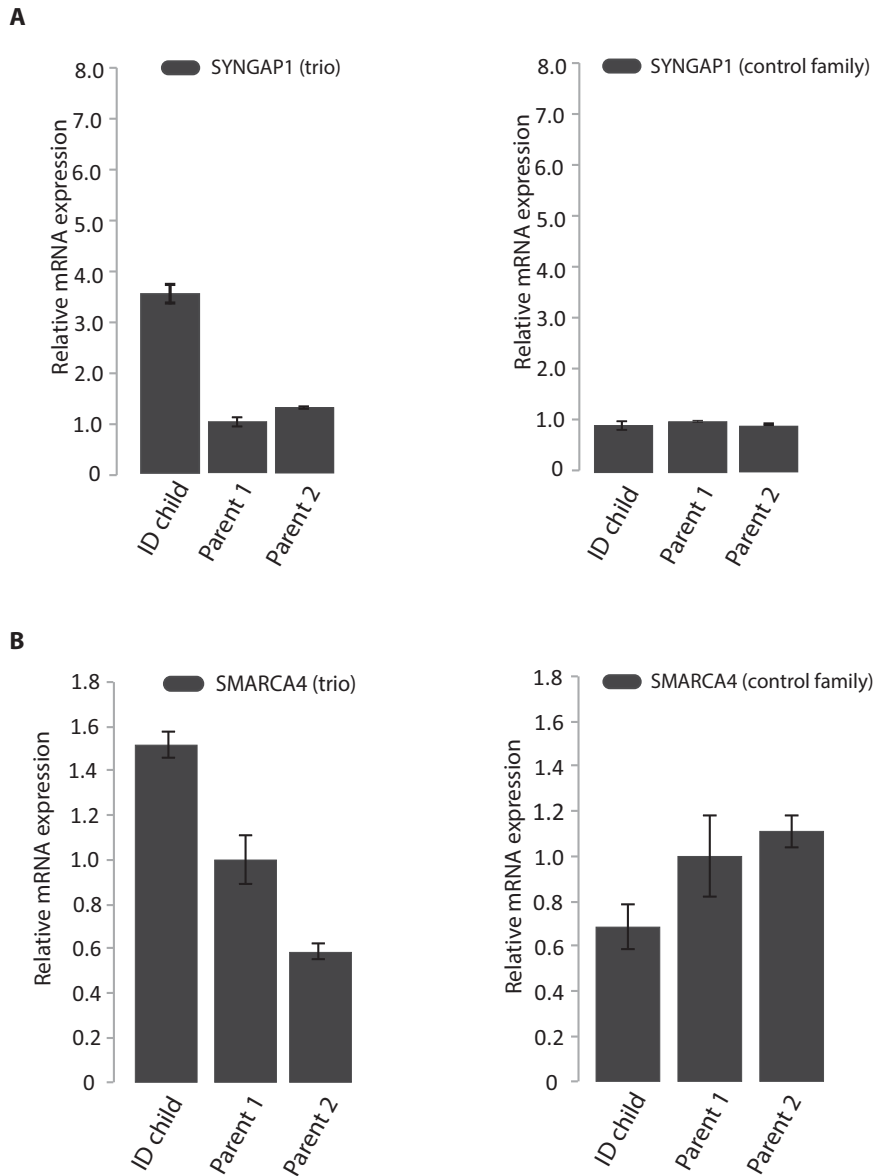
To investigate whether the de novo variant has an impact on genes involved in other pathways, we performed global RNA-seq on polyA<sup>+</sup> RNA extracted from blood samples from the trio family, producing 60–90 million mapped sequence reads per sample. In order to identify DEGs in the patient compared with the parents, and to minimize false-positive findings, the two R programs DESeq [Anders and Huber, 2010] and EdgeR [Robinson et al., 2010] were used and only genes identified as differentially expressed by both programs were further used in our analysis. Using a stringent  $P$  value of  $<0.0001$ , 823 genes were determined to be differentially expressed using this strategy (Supp. Table S2). The RNA-seq results also confirmed the previously identified upregulation of *CYP24A1* in the patient (fold change 2.38).

A GO analysis of the DEGs resulted in 108 significantly enriched nonredundant GO categories (Supp. Table S3). In line with the role of ACF1 as a suppressor of the VD3 pathway, we note that the GO category “response to vitamin D” is significantly enriched in the analysis (adjusted  $P$  value = 0.018). We then compared the genes differentially expressed in our analysis with a more detailed list of genes known to be regulated by vitamin D [Ramagopalan et al., 2010]. Again, a significant over-representation of vitamin D-regulated genes was observed among the DEGs ( $P$  value = 0.0016, Fisher’s exact test). In a previous study, four genes in the vitamin D pathway have been experimentally shown to be regulated by ACF1 [Ewing et al., 2007]. Of these four, we found two to be differentially expressed in the patient (*CYP24A1* and *IGFBP3*), whereas the other two genes were expressed at very low levels in blood in the trio samples (*TNFSF11* and *IGF1*).

The GO analysis of DEGs also yielded significant enrichment for both the canonical and noncanonical Wnt signaling pathways ( $P$  value =  $9.0E-5$  and  $9.7E-5$ , respectively). Consistent with these findings, a previous study in *Drosophila melanogaster* revealed that ACF1 is involved in the basal repression of Wnt target genes in the absence of active Wnt signaling [Liu et al., 2008]. To further pursue the enrichment of the Wnt signaling pathway genes, we created a list of genes previously identified as targets of the Wnt pathway ( $n = 76$ , [http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target\\_genes](http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes))

and compared these genes with the list of DEGs resulting from our RNA-seq analysis. Of the 76 genes, 14 genes showed differential expression in the patient (Supp. Table S4) yielding a significant over-representation of Wnt target genes among the DEGs identified ( $P$  value =  $3.63E-7$ , Fisher’s exact test). To investigate the possibility that this is an effect of the age difference between the patient and his parents, the number of DEGs overlapping with the Wnt target genes was counted for a second unrelated trio family with healthy parents and a child with mild mental retardation. This test gave a nonsignificant result, indicating that the differential expression in Wnt target genes in the patient is not an influence of age difference. To further investigate the impact on Wnt pathway target genes, the expression fold change was measured for the genes in the Wnt pathway GO categories as well as the Wnt target genes. The results showed a stronger response in fold change of the Wnt target genes as compared with the Wnt pathway genes (see Fig. 3).

Considering the phenotype of the patient, we note that additional significantly enriched GO categories include axon guidance and nervous system development. To find specific genes relevant to the phenotype of the patient, we searched our list of DEGs for genes known to be involved in ID [Veltman and Brunner, 2012] and found that 25 of the DEGs have previously been shown to cause ID. The most significant differential expression among these ID genes was found for *SYNGAP1* [OMIM 603384] and *SMARCA4* [OMIM 603254]. *SYNGAP1* is known to cause both epilepsy and ID [Berryer et al., 2013; Carvill et al., 2013]. We identified *SYNGAP1* to be overexpressed in the patient compared with his parents. Overexpression of *SYNGAP1* has been previously shown to significantly affect neuronal signaling and synapse function [Rumbaugh et al., 2006]. *SMARCA4*, a member of SWI/SNF chromatin-remodeling complex previously linked to Coffin-Siris syndrome, was also overexpressed in the patient. To experimentally validate the differential expression of *SYNGAP1* and *SMARCA4*, we performed qrtPCR on the trio samples, confirming overexpression of both genes in the patient compared with the parents (fold change 3 and 1.5, respectively) (Fig. 4A and B). To exclude the possibility that the differential expression is age related, we also measured relative expression of the two genes in an independent control trio family with two healthy



**Figure 4.** The relative expression of *SYNGAP1* and *SMARCA4* in the trio. **A:** qrtPCR validation of the differential expression of *SYNGAP1* between the patient and the parents (left). To the right are the expression levels of *SYNGAP1* in a control trio family, indicating no age-related differential expression. **B:** qrtPCR validation of the differential expression of *SMARCA4* between the patient and the parents (left). To the right are the expression levels of *SMARCA4* in a control trio family showing no age-related differential expression. All qrtPCR results were normalized to the level of B-actin in each respective sample. Expression levels represent mean values of the biological and technical replicates and error bars are  $\pm$ SD.

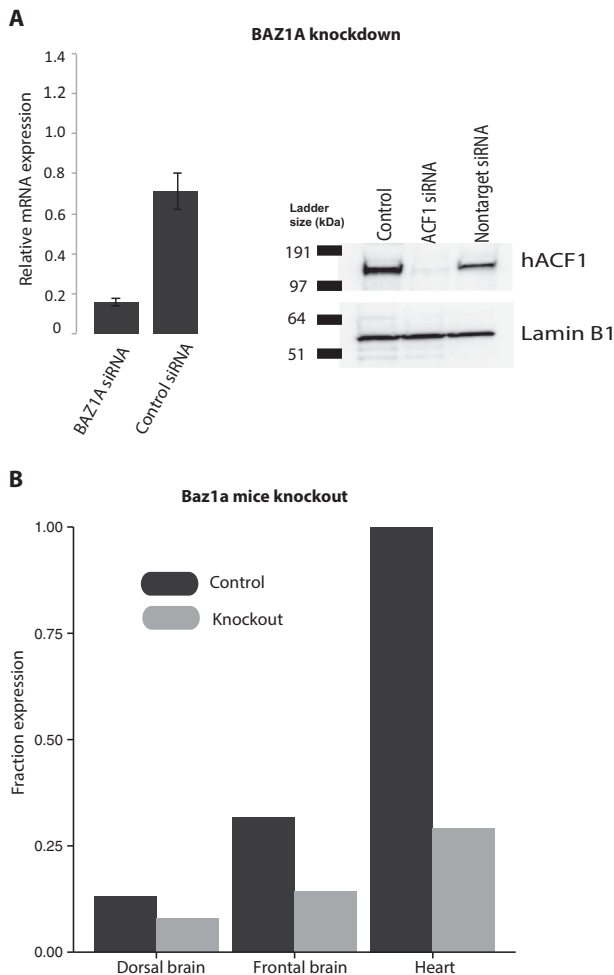
parents and a child of similar age suffering from mental retardation and epilepsy. The results showed no differential expression between the child and the parents in the unaffected trio (Fig. 4A and B).

### The Role of ACF1 in Regulation of Gene Expression

To date, there is limited knowledge about the general function of *BAZ1A*. Therefore, we next sought to investigate the role of ACF1 in gene expression regulation. We performed siRNA-mediated knockdown of *BAZ1A* in Saos-2 cells (Fig. 5A) followed by polyA<sup>+</sup> RNA-seq on Saos-2 cells with *BAZ1A* knockdown and cells transfected with control siRNA. RNA-seq analysis indicated a large number of genes to be differentially expressed upon knockdown ( $n = 1,256$ , data not shown). GO analysis of the DEGs showed enrichment of GO

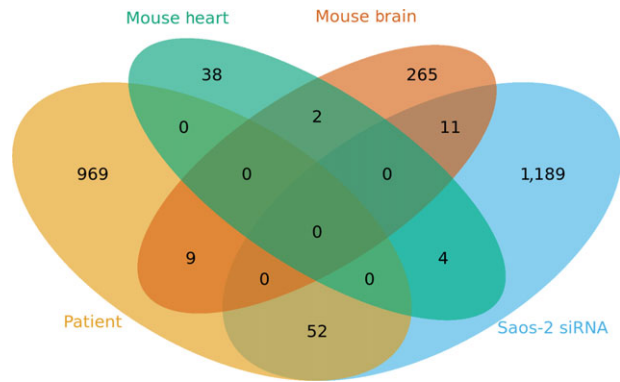
categories related to several biological pathways including cell signaling and system development. The top 10 GO categories are shown in Supp. Table S5. Consistent with previously published *BAZ1A* loss-of-function data [Ewing et al., 2007], our RNA-seq data showed no differential expression of *CYP24A1* in Saos-2 cells with *BAZ1A* knockdown. However, in the same study, Ewing et al. (2007) demonstrated that overexpression of ACF1 lacking the C terminus, where the de novo variant is located, in cells resulted in increased levels *CYP24A1*. These data suggest that the de novo variant in the patient does not cause loss-of-function of *BAZ1A*. It may rather reflect a dominant negative gain-of-function, at least for the transcriptional regulation of *CYP24A1*.

We also investigated the role of ACF1 on gene expression in vivo using polyA<sup>+</sup> RNA-seq on frontal brain, dorsal brain, and heart



**Figure 5.** Validation of *BAZ1A* knockdown in Saos-2 cells and *Baz1a* knockout in mouse. **A:** Left, expression levels of *BAZ1A* in Saos-2 cells transfected with *BAZ1A* siRNA and Saos-2 with control siRNA. All expression values were normalized to the level of B-actin in each respective sample. Expression levels represent mean values of the biological and technical replicates and error bars are  $\pm$ SD. Right, protein expression of ACF1 in Saos-2 cells transfected with *BAZ1A* siRNA, Saos-2 with control siRNA and untransfected cells. **B:** Bar plot showing the fraction of *Baz1a* expression in each mouse tissue as compared with the tissue with the highest *Baz1a* expression (heart).

tissues from four *Baz1a* knockout mice and four wild-type litter mate controls (two males and two females for each condition). Even though the highest expression of *Baz1a* was observed in the heart compared with frontal and dorsal brain (Fig. 5B), we found that the frontal brain harbored the highest number of DEGs between knockout and control mice (DEG = 287) compared with heart (DEG = 44). No DEGs were found in the dorsal brain, where expression of *Baz1a* was very low. These results indicate a larger influence of ACF1 on gene expression regulation in the frontal brain compared with the dorsal brain and heart. The results also suggest that ACF1 carries out distinctive functions in different tissues. GO analysis of the DEGs of the knockout mice revealed enrichment for several GO categories including synaptic transmission ( $P$  value =  $4.6E-10$ ), neurological system process ( $P$  value =  $3.6E-7$ ), and ensheathment of neurons ( $P$  value =  $5.5E-6$ ) in the frontal brain (most significant GO terms are listed in Supp. Table S6). These results indicate an important role of ACF1 in regulation of gene expression in the nervous system.



**Figure 6.** Venn diagram showing the number of DEGs in mouse heart (green), mouse frontal brain (brown), patient blood (yellow) and Saos-2 cells (blue), and the overlap of the DEGs between the different tissues.

On the other hand, the heart showed enrichment for categories associated with cardiovascular system development and extracellular matrix. Most significant GO categories enriched in the heart are listed in Supp. Table S7. It is obvious in our data that DEGs and GO categories show weak overlap between the different tissues, highlighting that ACF1 may be involved in different regulatory networks in different tissues. Nevertheless, it is interesting to note that ACF1 influences the expression of genes related to development in mouse frontal brain and heart, and in Saos-2 cells, indicating a role for ACF1 in development across several tissues. The weak overlap between the different tissues is also evident from the low number of DEGs that overlap between the different RNA-seq analyses (Fig. 6). We do note that the *SYNGAP1* gene, which was found as differentially expressed in the patient blood (compared with parents), and has previously been linked to ID and epilepsy, was also identified as a DEG in the frontal cortex of knockout mice.

A recent study reported the genomic locations of ACF1 binding in the nucleus accumbens in the brain of control and depression mouse models using ChIP-seencing (ChIP-seq) [Sun et al., 2015]. We investigated whether the genes showing differential expression ( $P$  value < 0.05) in the knockout mice overlap with these genomic-binding locations. As expected, the genomic locations of ACF1 bindings overlapped more with the DEGs in the brain of the knockout mice ( $n = 14$ ) compared with the heart ( $n = 3$ ) ( $P$  value = 0.048, Fisher's exact test). The overlapping genes are shown in Supp. Tables S8 and S9. We also note that the ChIP-seq experiment revealed ACF1 binding on the promoter region of *SMARCA4*, which was found as differentially expressed in the patient blood. These results further support a role of ACF1 in the regulation of gene expression in the brain.

Although ACF1 has been mainly reported as a transcriptional repressor, we observed several downregulated genes in the RNA-seq data from the *Baz1a* null mice. Similarly, we found downregulated genes in Saos-2 cells with *BAZ1A* knockdown and in the patient among the DEGs using a  $P$  value cut-off of 0.0001. The number of upregulated and downregulated genes in RNA-seq data from all samples is shown in Supp. Table S10. These results indicate that ACF1 is also involved in functions that could indirectly lead to transcriptional activation.

## Discussion

ACF1 is a multidomain protein, with 1,556 amino acids, containing several conserved motifs for interaction with DNA, histones,



and the other member of the ACF complex (Fig. 1). In this study, we identified a de novo p.Phe1348Cys variant in *BAZ1A* in a child with ID from a trio family. Since the general function of *BAZ1A* is not well characterized, we further investigated the role of *BAZ1A* in a cell line, and in mice brain and heart. The variant we identified in the patient is located in a conserved region in the linker motif between the PHD finger and the Brd domains. The PHD finger interacts with the central domain of the core histone and is known to be involved in chromatin-mediated transcriptional regulation [Aasland et al., 1995; Eberharter et al., 2004]. Brd domains bind to acetylated histone H4 and H3 N-termini to promote the interaction of chromatin-remodeling factors to the DNA and facilitate the function of chromatin-remodeling machinery [Jenuwein and Allis, 2001; Marmorstein and Berger, 2001]. Moreover, in agreement with our results, recent evidence implicated a role for Brd-containing proteins in the initiation and development of ID and other neurodevelopmental diseases [Li et al., 2013]. In addition, cooperation of Brds together with PHD fingers has been implicated in chromatin-mediated transcriptional regulation [Aasland et al., 1995; Schultz et al., 2001; Zeng et al., 2008]. Linker regions in multidomain proteins have been suggested to bridge flanking domains and support interdomain communication. Their conformation and structure is critical for the function of the flanking domains [Bhaskara et al., 2013]. The exchange of the phenylalanine (nonpolar, hydrophobic) to cysteine (polar, uncharged) in a highly conserved motif will likely impact the folding of the linker region and affect the flanking domains, that is, binding of the PHD and Brd to the histones, especially in the presence of other cysteines upstream the mutation. In fact, it has been shown that cysteine-rich domains create disulfide-bonded cysteine loops that influence the function of a protein [Roseman, 1988; Koticha et al., 2002]. Furthermore, analysis of post-translational modification previously reported in ACF1 revealed large numbers of phosphorylation sites in the vicinity of the mutation [Hornbeck et al., 2012], which further supports the potential impact of the mutation on protein function. In line with this, a previous study has reported that deletion of the C-terminus of ACF1, spanning the PHD and the bromodomain, influenced the function of ACF1 in vitamin D pathway and relieved repression of the VDR-regulated genes [Ewing et al., 2007]. Depletion of hACF1 in human cells causes delay in cell cycle progression [Collins et al., 2002]. In *Drosophila*, disruption of *BAZ1A* compromises fly viability [Chioda et al., 2010]. To date, there is only one publication on *Baz1a* knockout mice, showing that male mice suffer from reduced fertility due to global misregulation of gene expression in sperm cells [Dowdle et al., 2013]. In addition to male infertility, a number of more modest phenotypes are described by the International Mouse Phenotyping Consortium (measures with  $P$  value < 0.005) and these include hypoactivity, decreased body mass and length, decreased body fat, increased mean platelet volume, and increased blood urea nitrogen levels. Interestingly, the mice also display abnormal bone mineralization, potentially linked to the established role of ACF1 in VD3 metabolism. *BAZ1B*, a *BAZ1A* homolog, has been previously implicated in Williams syndrome, a well-known neurodevelopmental disorder [Lu et al., 1998]. *Baz1b*-deficient mice die at the neonatal stage [Yoshimura et al., 2009].

The specific variant we identified is not present in >60,000 unrelated individuals accessible through the Exome Aggregation Consortium database [Exome Aggregation Consortium (ExAC), 2015]. Deletions encompassing *BAZ1A* have been described in the literature, but no clear phenotypic associations have been made to date. In a comprehensive study of copy-number variants identified in patients with developmental delay and healthy controls [Coe et al., 2014], heterozygous deletions spanning *BAZ1A* have been

identified in six patients and no controls ( $P$  value = 0.045), whereas duplications have been identified in four patients and five controls. We further note that *BAZ1A*, along with several other chromatin remodelers in the SWI/SNF complex, have been implicated in carcinomas [Jones et al., 2014]. Interestingly, the mutations detected in *BAZ1A* in patients with carcinomas accumulate in the same part of the protein (linker region and flanking domains) where the de novo mutation in the present study is located.

A large number of chromatin-remodeling genes have been implicated in ID syndromes [Iwase et al., 2011; Harakalova et al., 2012; Kosho et al., 2013]. The majority of these genes are components of the SWI/SNF chromatin-remodeling complex. In all cases, mutations in these genes give rise to a range of symptoms in addition to ID. The patient in the present study shares several clinical manifestations with patients carrying mutations in components of the SWI/SNF complex [Kosho et al., 2013]. Shared symptoms include severe ID, autism, profound speech impairment (in agreement with patients with mutations in any component of the SWI/SNF complex), seizures (mutations in *SMARCA2* and *SMARCB1*), hypotonia (mutations in *SMARCB1*, *SMARCA2*, and *SMARCA4*), hypoplastic finger nails (*SMARCA4* mutations), and distal phalanges (*SMARCB1*, *SMARCA4*, and *ARID1B* mutations). The most striking clinical overlap is found with patients with Coffin–Siris syndrome, caused by mutations in *SMARCA4*. The fact that we also find significant changes in the expression of *SMARCA4* in our patient raises the possibility that there is an interaction between the ISWI and SWI/SNF complexes.

Since we are not aware of any other patients with similar phenotype with mutation in *BAZ1A*, we prioritized functional analysis of the mutation, and of *BAZ1A* in general. We initially focused on the known involvement of *BAZ1A* in the VD3 pathway in Saos-2 cells. Targeted qRT-PCR revealed overexpression of the *CYP24A1* in the patient compared with the parents, which was also confirmed in Saos-2 cells transfected with the mutated ACF1 (mut-ACF1) transcript, verifying the effect of the mutation on the expression of *CYP24A1*. The ChIP-qPCR analysis revealed this effect was caused by the reduced efficiency of the mut-ACF1 to bind and repress the expression of *CYP24A1*. *CYP24A1* was previously shown to control vitamin D levels in the cell in a negative feedback process. In the absence of VD3, *CYP24A1* expression is suppressed by ACF1 and the N-CoR protein [Vaisanen et al., 2005; Ewing et al., 2007]. The overexpression of *CYP24A1* could therefore explain the vitamin D insufficiency detected in the patient. On the other hand, individuals with ID and epilepsy commonly suffer from vitamin D deficiency [Knutzson et al., 1987], and the deficiency may be further exacerbated by anticonvulsant drug treatment [Silver et al., 1974; Harijan et al., 2013]. However, to our knowledge, no direct link to changes in expression of factors in vitamin D metabolism, as we find for *CYP24A1*, has previously been established in this patient group. VD3 deficiency has been linked to a range of clinical symptoms, including effects on the developing brain. Mice with VD3 deficiency suffer from profound brain structural alterations [Eyles et al., 2003]. Our results show that the role of *BAZ1A* in VD3 regulation is similar to that of *BAZ1B* [Fujiki et al., 2005], a homolog to *BAZ1A* known to be involved in the causation of Williams syndrome [Lu et al., 1998].

Consistent with the function of ACF1 as a chromatin-remodeling factor, our RNA-seq data revealed many genes in the patient to be differentially expressed, most notably, genes involved in the Wnt signaling pathway. The link between chromatin-remodeling factors and Wnt/ $\beta$ -catenin signaling pathway has been highlighted in several studies [Barker et al., 2001; Griffin et al., 2011; Holik et al., 2014]. Wnt signaling is a highly conserved cascade known to play

key roles in many developmental processes including the central nervous system [Salinas, 2012]. Mutations in genes encoding members of the Wnt signaling pathway have previously been implicated in human cognitive disorders such as ID and autism [de Ligt et al., 2012; O’Roak et al., 2012]. Our observation is supported by previous studies of the role of ACF1 in the basal repression of Wnt target genes in *Drosophila* [Liu et al., 2008]. In the absence of Wnt signaling, ACF1 binds to target genes and antagonizes the acetylation of histone H4 causing transcriptional repression. Consistent with the role of the C terminus of ACF1 in binding histones H3 and H4, the mutation in the patient could therefore explain the misregulation of Wnt target genes observed in the RNA-seq data. Similarly, ARID1B, a chromatin-remodeling factor, was recently shown to repress Wnt/ $\beta$ -catenin signaling. Moreover, ID patients with loss-of-function mutations in *ARID1B*, show upregulation Wnt/ $\beta$ -catenin target genes [Vasileiou et al., 2015]. The influence of ACF1 on Wnt signaling further adds to the growing evidence of a link between chromatin-remodeling factors and cancer. Additionally, studies in cultured cells and animal models revealed a crosstalk between VDR and Wnt signaling where VD3 antagonizes the activity of Wnt signaling [Larriba et al., 2013]. Therefore, the reduced levels of VD3 in the patient could further contribute to the differential expression of Wnt targets in the patient.

Our analysis also revealed DEGs involved in synaptic function, including overexpression of *SYNGAP1* encoding synaptic GTPase-activating protein. *SYNGAP1* is a major member of the postsynaptic density [Chen et al., 1998] and is essential for postsynaptic signaling [Komiyama et al., 2002]. Mutations in this gene have previously been associated with ID [Hamdan et al., 2009] and epilepsy [Carvill et al., 2013]. Moreover, *SYNGAP1* overexpression was reported to hinder axon outgrowth in granule cells [Tomoda et al., 2004]. *SMARCA4*, a member of SWI/SNF chromatin-remodeling complex, was also overexpressed in the patient. As most chromatin remodelers, *SMARCA4* is known to have broad range of functions including roles in the development of the heart and the nervous system in a dosage-dependent manner [Lessard et al., 2007; Wu et al., 2007; Takeuchi et al., 2011; Smith-Roe and Bultman, 2013]. Mice lacking *SMARCA4* die in early development [Bultman et al., 2000]. Targeted depletion of *SMARCA4* in neural stem/progenitor cells result in reduced numbers of both neurons and glia, and reduced brain size and the absence of the cerebellum in mouse embryos [Lessard et al., 2007]. In line with this, mutations in *SMARCA4* have been linked to Coffin–Siris syndrome [Tsurusaki et al., 2012; Kosho et al., 2013]. It is also interesting to note that both *SYNGAP1* and *SMARCA4* are interconnected with the Wnt signaling pathway [Griffin et al., 2011; Krumm et al., 2014].

We also investigated the role of ACF1 on regulating gene expression using RNA-seq. Upon *BAZ1A* knockdown in Saos-2 cells, we found large numbers of gene to be differentially expressed including genes important for cell signaling and development. In agreement with our results, ACF1 was reported to be involved in chromatin remodeling during development and in several signaling processes in human and *drosophila*, especially in hormone responsiveness and the signaling necessary for DNA damage repair [Ewing et al., 2007; Sanchez-Molina et al., 2011]. In *Baz1a* knockout mice, we demonstrate that the frontal brain harbors the largest number of DEGs as compared with heart and dorsal brain. Interestingly, we observed that many DEGs in the brain are important for neurological processes and synaptic function, highlighting a previously unknown function of ACF1 in nervous system. We also found that ACF1 function is tissue specific, obvious from the weak overlap between the DEGs in different tissues. Similarly, a recent study demonstrated that overexpression of *Baz1a* specifically in the nucleus accumu-

lates contributes to susceptibility to stress-induced depressive-like behaviors [Sun et al., 2015]. Nevertheless, we observed that DEGs in the different tissues are enriched in pathways important for development, accentuating a general role for ACF1 in tissue development. Although ACF1 is generally recognized as a transcription repressor, we show several genes to be upregulated upon *BAZ1A* depletion, highlighting new role of ACF1 in transcription regulation.

In this study, we report, for the first time, a de novo mutation in *BAZ1A*, in a patient with a syndromic form of ID. Consistent with the function of ACF1 as a chromatin-remodeling factor, the de novo mutation affects the expression of genes involved in several biological pathways. Our data support a role for *BAZ1A* on three distinct pathways: vitamin D regulation, Wnt signaling, and postsynaptic signaling. The diverse roles of *BAZ1A* could explain several syndromic clinical phenotype of our patient, similar to the symptoms identified in other patients with mutations in chromatin-remodeling factors. We particularly demonstrate that the differential expression of *CYP24A1*, *SYNGAP1*, and *SMARCA4* correlates with clinical findings in the patient, raising the possibility that the de novo mutation in *BAZ1A* may contribute to the phenotypes of the patient. However, further functional studies are needed to prove unambiguously the pathogenicity of this de novo variant in ID. We also report new roles of *BAZ1A* in nervous system and neurological process. Our findings add to the growing evidence for a role of chromatin-remodeling factors in cognition and in syndrome related to neurodevelopment.

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