European Journal of Human Genetics (2015) 23, 781–789

www.nature.com/ejhg

ARTICLE

MBD5 haploinsufficiency is associated with sleep disturbance and disrupts circadian pathways common to Smith–Magenis and fragile X syndromes

Sureni V Mullegama[1], Loren Pugliesi[2], Brooke Burns[2], Zalak Shah[2], Raiha Tahir[2], Yanghong Gu[1], David L Nelson[1] and Sarah H Elsea*[,1,2]

Individuals with autism spectrum disorders (ASD) who have an identifiable single-gene neurodevelopmental disorder (NDD), such as fragile X syndrome (FXS, *FMR1*), Smith–Magenis syndrome (SMS, *RAI1*), or 2q23.1 deletion syndrome (del 2q23.1, *MBD5*) share phenotypic features, including a high prevalence of sleep disturbance. We describe the circadian deficits in del 2q23.1 through caregiver surveys in which we identify several frequent sleep anomalies, including night/early awakenings, coughing/snoring loudly, and difficulty falling asleep. We couple these findings with studies on the molecular analysis of the circadian deficits associated with haploinsufficiency of *MBD5* in which circadian gene mRNA levels of *NR1D2*, *PER1*, *PER2*, and *PER3* were altered in del 2q23.1 lymphoblastoid cell lines (LCLs), signifying that haploinsufficiency of *MBD5* can result in dysregulation of circadian rhythm gene expression. These findings were further supported by expression microarrays of *MBD5* siRNA knockdown cells that showed significantly altered expression of additional circadian rhythm signaling pathway genes. Based on the common sleep phenotypes observed in del 2q23.1, SMS, and FXS patients, we explored the possibility that MBD5, RAI1, and FMR1 function in overlapping circadian rhythm pathways. Bioinformatic analysis identified conserved putative E boxes in *MBD5* and *RAI1*, and expression levels of *NR1D2* and *CRY2* were significantly reduced in patient LCLs. Circadian and mTOR signaling pathways, both associated with sleep disturbance, were altered in both *MBD5* and *RAI1* knockdown microarray data, overlapping with findings associated with FMR1. These data support phenotypic and molecular overlaps across these syndromes that may be exploited to provide therapeutic intervention for multiple disorders.
European Journal of Human Genetics (2015) **23,** 781–789; doi:10.1038/ejhg.2014.200; published online 1 October 2014

INTRODUCTION

Autism spectrum disorder (ASD; MIM 209850) is a neurodevelopmental disorder (NDD) that affects all areas of a child's development, including social interaction, communication, and behavior. Approximately 10% of children with ASD have an identifiable genetic condition and may present with deficits in language, cognition, motor function, speech impairment, intellectual disability (ID), or seizures.[1] In addition, behavioral phenotypes such as repetitive, stereotypical, or problematic behaviors are often present.

Significant sleep problems are highly prevalent in individuals with ASD.[2] Parents of children with ASD report 50–80% prevalence of sleep problems compared with a 9–50% prevalence rate reported by parents of age-matched typically developing children.[2] Common sleep etiological factors that are present in children with ASD are rapid eye movement sleep abnormalities, dysregulation of melatonin synthesis, difficulty in settling to sleep, night waking, irregular sleep patterns, short-duration sleep, and daytime sleepiness.[2,3] Persistent sleep problems are thought to adversely affect cognitive, physical, and social function, learning, mood, and behavior in these individuals and, in addition, cause significant stress for families and caretakers.[3] Better understanding of the sleep difficulties experienced by children with ASD may reduce the adverse effects and decrease family stress, possibly with targeted therapies.

About 10–15% of single-gene NDDs, such as fragile X syndrome (FXS; MIM 300624; *FMR1*, MIM 309550), Rett syndrome (RTT; MIM 312750; *MECP2*, MIM 300005), and Smith–Magenis syndrome (SMS, MIM 182290; *RAI1*, MIM 607642), are associated with a high prevalence of autism.[4] Further, these NDDs share many overlapping features, including a high prevalence of sleep disturbance. Understanding the molecular alterations in NDDs individually and collectively and the subsequent overlapping pathways affected that lead to the sleep disturbance could possibly lead to behavioral and pharmaceutical therapies.

The 2q23.1 deletion syndrome (del 2q23.1) (MIM 156200) is a NDD/ASD that results from haploinsufficiency of a common gene in the del 2q23.1 region called *methyl-CpG-binding domain 5* (*MBD5*) (MIM 611472).[4] Genomic disruptions of *MBD5* lead to the primary clinical phenotypes present in the disorder, including ID, epilepsy, speech impairment, unusual behaviors, ASD, and broad-based ataxic gate.[4] Sleep disturbance have been previously reported in a few cases.[4–12] Some of the sleep disturbances mentioned in these cases are waking 6–8 times per night, apparent night terrors in the early part of sleep, and waking in the early hours of the morning.[4–12] Although it is clear a sleep disturbance exists in del 2q23.1, no systematic characterization of the extent of sleep problems in this population has been published.

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA; [2]Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, VA, USA
*Correspondence: Dr SH Elsea, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, NAB2015, Houston, TX 77030, USA. Tel: +1 713 798 5484; Fax: +1 832 825 1269; E-mail: elsea@bcm.edu
Received 12 March 2014; revised 23 July 2014; accepted 26 August 2014; published online 1 October 2014

We report clinical characterization of the circadian deficits in the del 2q23.1 syndrome and couple this analysis with identification of the molecular deficits associated with haploinsufficiency of *MBD5*. We investigated circadian gene expression in del 2q23.1 in comparison to that present in SMS and FXS, illustrating that circadian gene dysregulation is a significant etiology in these disorders. Furthermore, we examined whether circadian rhythm pathways and mammalian target of rapamycin (mTOR) pathways, which are thought to be responsible for circadian deficits, are altered in these disorders.[13]

MATERIALS AND METHODS

Participants for sleep survey

Potential participants were contacted via the 2q23.1 Facebook support group page and the online support group, www.2q23.org, where online surveys were posted. Emails with a brief description of the survey and the link to the online survey were sent to clinical geneticists and genetic counselors to spread awareness of the study. Surveys were advertised and open for collection from mid-November through December 2012.

Participants of the sleep study consisted of 19 children between 9 months and 11 years with a molecular diagnosis of del 2q23.1. Parents served as participants in completing the surveys. School-age children as described by the National Sleep Foundation (5–12 years) comprised 58% of the study population (mean = 5.5 years). The majority of the children were from middle socioeconomic backgrounds and were predominantly white. Males (43.8%) and females (56.2%) were represented. The mean age of diagnosis was 3.9 years (SD = 2.136 years). Caregivers/parents reported that their child's diagnosis of 2q23.1 deletion syndrome was confirmed using chromosomal microarray (~53%), FISH (~21%), karyotype (~16%), and *MBD5* mutation analysis (~6%). The majority of the survey participants were mothers (84.2%), had a college education or greater (68.4%), and either worked part-time or did not work outside the home (63.1%). Families did not receive any incentive for taking part in the study. All information was collected after informed consent was obtained and in accordance with local institutional review board protocols from Virginia Commonwealth University (VCU).

Demographic and sleep questionnaires

Demographic and sleep questionnaires were created and administered using SurveyMonkey (https://www.surveymonkey.com/; Palo Alto, CA, USA). Demographic information consisted of nine questions, including participant's relationship to child, gender, highest level of education, employment status, and occupation, and child's age at diagnosis, diagnosis, current age, and gender. The sleep questionnaire was designed to assess questions regarding child sleep patterns, behaviors, and sleep troubles experienced and was comprised of select questions from the Pittsburgh Sleep Quality Index (PSQI), the Epworth Sleepiness Scale (ESS), and additional questions regarding specific sleep concerns and behaviors. The PSQI is a self-rated questionnaire designed to assess the sleep disturbance and overall sleep quality over a 1-month time interval.[14] The ESS is a standard measure that assesses the level of daytime sleepiness and thus the likelihood of dozing off during common sedentary daytime activities.[15] Based on response, a total score can range from 0 to 24. A score of ≥ 10 represents 'excessive daytime sleepiness'.[15] The additional questions surveyed specific sleep and nighttime behaviors of the child, including wandering, food seeking, sleepwalking, bedtime, and waking difficulties, naps, and sleepiness at school.

Cell culture

Lymphoblastoid cell lines (LCLs) were created from blood samples collected after informed consent was obtained and in accordance with the local institutional review board-approved protocols from VCU or Baylor College of Medicine (BCM). At least three LCLs from del 2q23.1, SMS, FXS, and controls were cultured as previously described.[8] Furthermore, previously published LCL controls were used.[8] LCLs utilized in this study included the following: SMS367[8] (SCV000172263) with a single-copy loss of 204 kb at chr(2)(q23.1), chr2.hg18:g.(148,447,295_148,651,456)del; SMS185[8] (SCV000172261) with a single-copy loss of 930 kb at chr(2)(q23.1), chr2.hg18:g.(148,447,496_149,377,297)del; SMS361[4] (SCV000172262) with a single-copy loss of 3.51 Mb at chr(2)(q22.3-q23.3), chr2.hg18:g.(146,798,229_150,310,317)del; SMS129[16,17] (RCV000003082.1) with a 29-basepair deletion in the coding region of *RAI1*, NM_030665.3:c.2773_2801del; SMS175[18] (rs104894634, RCV000003084.1) with an *RAI1* mutation, NM_030665.3:c.4685A>G; SMS123 (SCV000172264),[19] with a typical SMS 17p11.2 deletion, chr17:g.(AC027266_AL353996)_(AC015726_AC087393)del; SMS105 (SCV000172265),[19] with a typical SMS 17p11.2 deletion, chr17:g.(AC027266_AL353996)_(AC015726_AC087393)del; FXS1 (SCV000172267),[20] with a fully expanded CGG repeat in *FMR1* (GM09237, Coriell Cell Repositories, Camden, NJ, USA), (NM_002024.5)c.-158GGC(950); FXS2 (SCV000172266)[21] with an expanded CGG repeat in *FMR1* (NM_002024.5)c.-158GGC(500); and FXS3 (SCV000172267)[22] with a deletion of *FMR1* exons 1–7, chrX.hg18:g.(146,703,942_146,820,448)del. All variant information is available in either dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) or ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/). Neuroblastoma cell line, SH-SY5Y, was cultured according to the standard protocols (ATCC CRL-2266, Manassas, VA, USA). Human embryonic kidney cell line, HEK293T, was cultured according to the standard protocols (ATCC CRL-11268).

Quantitative real-time PCR

Total RNA was isolated from SH-SY5Y cells and 2q23.1 deletion syndrome, SMS, FXS, and control LCLs, via Trizol (Invitrogen, Carlsbad, CA, USA) according to the standard protocols. RNA was quantified using the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). First-strand cDNA synthesis was performed using the qScript cDNA SuperMix (Quanta BioScience, Gaithersburg, MD, USA) according to the manufacturer's instructions. For quantitative real-time PCR, predesigned Taqman MGB probes from Assays-on-Demand Gene Expression Products (ABI, Applied Biosystems, Life Technologies Inc., Carlsbad, CA, USA) were used for all genes in the study (Supplementary Table S1). All samples were run and analyzed according to previously published methods using either ABI Prism 7900 HT Sequence Detection System (Life Technologies Inc.) or BioRad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).[23] *GAPDH* (MIM 138400, Hs9999905_m1) mRNA served as an endogenous control.

siRNA knockdown of *MBD5* and *RAI1* in HEK293T and SH-SY5Y cells

Methodology for knockdowns of *MBD5* and *RAI1* were performed in HEK293T cells as previously described.[24] Knockdown of *MBD5* or *RAI1* was performed according to the standard protocol in SH-SY5Y using *Silencer* Select predesigned short interfering RNAs, which give the highest knockdown and lowest off-target effects, specific to *MBD5* (siRNA ID no. s31485) or *RAI1* (siRNA ID no. s21107) were designed to target constitutive exons found in human *MBD5* and *RAI1* transcripts. A scrambled siRNA (siRNA ID no. 4390849) served as the negative control and GAPDH siRNA (siRNA ID no. 4390849) was used in control experiments to test the optimization of the siRNA transfection protocol and were performing according to the manufacturer's instructions (Ambion, Austin, TX, USA) (Cat. no. 4392420 and Cat. no. 4392420). Each transfection was performed in quadruplicate, and three biological replicates were used for array studies and gene expression analysis, while the remaining sample was used for further expression studies.

Microarray

Three biological replicates from the following treatment groups from SH-SY5Y cells (*MBD5* knockdown (KD), *RAI1* KD, scrambled siRNA) were sent for microarray analysis. Microarray hybridizations were performed at the VCU Massey Cancer Center Nucleic Acids Research Facility, DNA Microarray Core, using an Illumina HumanWG-12 v4.0 expression beadchip (Illumina, San Diego, CA, USA) (containing >47 000 probes) and were read and analyzed according to previously published methods.[25] Briefly, array was read using an Illumina BeadScan confocal scanner and analyzed by the Illumina's BeadStudio software. The data were normalized by quantile method using the Illumina BeadStudio Software. *P*-values < 0.05 were used to determine which probes were detected. One-way ANOVA was subsequently performed comparing control *versus MBD5* KD or *RAI1* KD groups to identify differentially expressed genes. The criterion for a gene to be differentially expressed was set at $P \leq 0.05$ and then at $P \leq 0.01$.

Ingenuity Pathway Analysis (IPA)

IPA was used to identify gene networks and pathways and likely upstream regulators that were significantly altered in knockdown cells than in controls. Significant interactions were determined using the Ingenuity Pathway Knowledge Base and a Fisher's exact test to calculate a *P*-value determining the probability that each function network or pathway assigned to that data set is due to chance alone. Molecules from the data set that met the $P<0.05$ cutoff and that were associated with biological functions in the Ingenuity knowledge Base were considered for the analysis.

E box location in *MBD5*, *RAI1* and *FMR1* and E box conservation analysis

A Perl program was written to look for the nucleotide sequences that represent the E box, 'CANNTG', where 'NN' can be any nucleotide. This E box sequence was searched for starting 2500 bases upstream of the ATG for *FMR1*, *RAI1*, or *MBD5*. Each E box sequence identified in each gene was then aligned to the mouse counterpart to determine evolutionary conservation using BLAST from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to validate this approach, we also investigated several non-circadian genes, including *BMP7*, *GATA4*, *TBX5*, *HAND2*, and *NOG*, which are involved in bone growth and development, and looked for E box sequences upstream 2500 bases from the ATG. We found that these genes have either 0 or 1 E boxes compared with *MBD5* and *RAI1*, which have a larger count of E boxes as discussed in the Results. We also looked at known biologically confirmed circadian rhythm-dependent genes such as *SNAI1*, *SMAD3*, *WEE1*, *OTX2*, and *TSHB* and found they also have a greater count of E boxes (>3), similar to *RAI1* and *MBD5*.

Statistical analyses

Statistical analysis for gene expression data was performed with Prism 4 version 4.0b (GraphPad Software, San Diego, CA, USA), as previously described.[4,25]

RESULTS

Frequency and nature of sleep problems in children with 2q23.1 deletion syndrome

Surveys of parents and caregivers of children with del 2q23.1 demonstrated a variety of sleep anomalies affecting these individuals (Table 1). Among the school-age children (age 5–12 years), 45% reported having 'fairly bad' sleep. Among these school-age children, parents considered the sleep problems present to be associated with behaviors they observed (Table 2). Supporting these concerns, parents reported that 33% of children aged 5–12 years were napping, a rate higher than expected for school-age children. As parents reported that many of their children were not sleeping well throughout the night, an ESS score was calculated to assess each child's daytime sleepiness (see Methods). Total scores ranged from 4 to 17 (mean = 9.4, SD 3.99), which was 0.6 points below the threshold of 10 for 'excessive daytime sleepiness', as defined by the ESS among school-age children. Of note, 43.7% of the children surveyed held a score of ≥ 10, indicating that a substantial portion of the children with del 2q23.1 have excessive daytime sleepiness likely due to poor and inconsistent sleep at night. Finally, many parents reported using various methods to ensure proper sleep for their children. The most commonly used interventions were regular naps for their children (62.50%) and use of containment (enclosed beds, locked doors) to prevent their child from wandering at night (66.7%). However, many parents reported using daily pharmacological (melatonin, clonidine, and trazadone) interventions to cope with the sleep disturbances (55.6%), indicating a significant impact on the family.

Dysregulation of circadian genes in del 2q23.1 syndrome LCLs

Given that individuals with del 2q23.1 have a sleep phenotype, we sought to determine whether circadian gene expression was affected, perhaps pointing to an etiology for the sleep disturbance. We tested the expression of *MBD5* to confirm *MBD5* haploinsufficiency in the del 2q23.1 cell lines, which was consistent with previous studies (Figure 1).[26] Based on literature indicating altered expression of circadian genes in SMS LCLs, we tested the expression of four circadian genes, *NR1D2*, *PER1*, *PER2*, and *PER3*, in 2q23.1 deletion syndrome LCLs that were grown at the same time and under the same conditions.[23] The transcript levels of the above four circadian rhythm genes had significantly reduced mRNA expression in del 2q23.1 LCLs (Figure 1). Other methyl CpG-binding domain (MBD) genes *(MBD2*, *MBD3*, and *MBD4*) were tested as non-affected gene controls, and no differences were found in the mRNA levels of these genes (Figure 1).

Circadian rhythm gene expression altered by haploinsufficiency of *MBD5*

Given the association of sleep disturbance and altered circadian gene expression in del 2q23.1 patients, we hypothesized that *MBD5* can regulate additional circadian genes and that haploinsufficiency of *MBD5* causes dysregulation of the circadian rhythm pathway. Thus, through microarray technology, we investigated the effect of knocking down *MBD5* for 24 and 32 h with siRNA to levels that mimic expression seen in del 2q23.1 patients (~50–60% of normal expression) on circadian gene expression in SH-SY5Y cell lines.[4,26] We first knocked down *MBD5* in HEK293T cells to test the efficacy in knocking down *MBD5* and then proceeded to SH-SY5Y lines, which are less robust and harder to transfect. Real-time qRT-PCR showed ~60% knockdown of *MBD5* was achieved in HEK293T and SH-SY5Y cells (Supplementary Figure S1). In the microarray data on KD of

Table 1 Parent-reported sleep concerns in 2q23.1 deletion syndrome

	%			
Sleep concern	*Three or more times a week*	*Once or twice per past month*	*Less than once a month*	*Not during the past month*
Cannot get to sleep within 30 min	25	42.8	6.3	25
Wake up in the middle of the night or early morning	73.3	20	0	6.7
Cannot breathe comfortably	12.5	25	6.3	56.3
Cough or snore loudly	43.8	12.5	0	43.8
Feel too cold	12.5	18.8	6.3	62.5
Feel too hot	18.8	6.3	0	75
Had bad dreams	6.3	18.8	18.8	56.3
Other[a]	46.2	0	0	53.8

[a]Includes bed wetting, toileting, agitation, and nightmares.

Table 2 Sleep behaviors of school-age children with 2q23.1 deletion syndrome

Behavior	*%*
Wanders at night	22.2
Seeks food	11.1
Sleep walks	25.0
Difficulty putting to bed	44.4
Difficult to wake	22.2
Child naps on their own	44.4

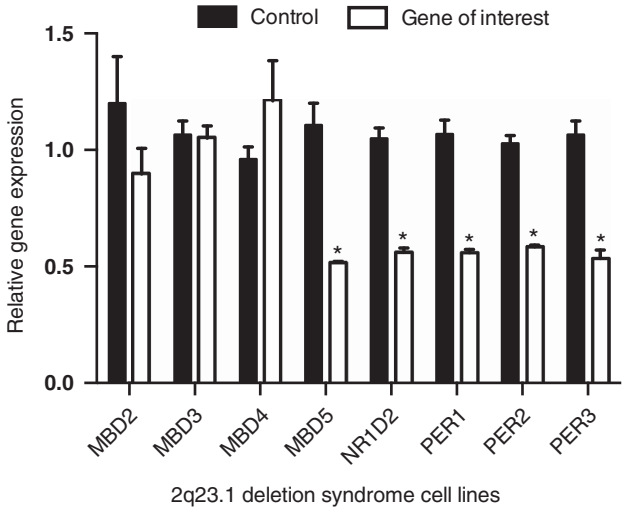

Figure 1 Altered expression of circadian genes in 2q23.1 deletion syndrome. Quantitative real-time PCR was performed on LCLs from normal controls and from individuals with 2q23.1 deletion syndrome, as described in the Methods.[4] Expression levels of key circadian genes (*NR1D2*, *PER1*, *PER2*, *PER3*) were evaluated. Data are an average of three independent experiments performed in triplicate. Haploinsufficiency of *MBD5* was confirmed in these patient samples. Expression levels of *MBD2*, *MBD3*, and *MBD4* served as non-affected gene controls, illustrating normal levels of expression in these LCLs. Quantification of the test gene was compared with that of the housekeeping gene *GAPDH*. All expression values were calculated relative to control levels set at 1.0. Error bars represent the SEM. A *P*-value <0.005 was determined by one-sample *t*-tests. Asterisks indicate significant differences in gene expression.

MBD5 in SH-SY5Y, 1903 genes were significantly upregulated ($P<0.05$); using a higher stringency ($P<0.01$), 619 genes were significantly upregulated. Furthermore, in *MBD5* knockdown array data, 1880 genes were significantly downregulated ($P<0.05$); using a high stringency ($P<0.01$) 1110 genes were significantly downregulated. The microarray revealed significant changes of mRNA abundance for 63 genes implicated in control of circadian rhythm ($P<0.05$), including *CLOCK*, *PER3*, and *RAI1* (Table 3). The top 16 significantly dysregulated circadian rhythm genes can be seen in Table 3 ($P\leq0.001$). Using real-time qRT-PCR, we confirmed that mRNA expression levels of several of these genes were significantly altered in SH-SY5Y knockdown cells, validating the microarray data (Figure 2a). Importantly, the expression of *CLOCK*, a key component of the mammalian circadian oscillator that transcriptionally regulates many key circadian genes was significantly reduced to $\leq50\%$ of control (Figure 2a). Other essential components of the circadian feedback loop *PER1*, *PER3*, *CRY1*, *CRY2*, *NR1D1* and *NR1D2* were significantly downregulated in *MBD5* knockdown cells (Figure 2a). Other *CLOCK*-related genes, *ATF2* and *ATF4*, were significantly overexpressed compared with the control (Figure 2a). IPA confirmed the observation that the circadian rhythm pathway was significantly dysregulated due to *MBD5* knockdown ($P=0.00474$; Figure 2b).

Effect of *MBD5*, *RAI1*, and *FMR1* on circadian rhythm gene expression

Based on the sleep phenotype observed in del 2q23.1, SMS, and FXS, we hypothesized that MBD5, RAI1, and FMR1 function in common or overlapping pathways involved in circadian rhythm, which when disrupted lead to the common sleep disturbances present in the above disorders. We analyzed the mRNA expression of key circadian genes *MBD5*, *RAI1*, and *FMR1* in the three syndromes in three patient-derived LCLs for each syndrome compared with three normal controls (Figure 3a–h). FXS cell lines showed normal expression for all *PER* genes; however, the mRNA levels of *PER1*, *PER2*, and *PER3* were significantly reduced ($<60\%$) in the del 2q23.1 and SMS cell lines (Figure 3a–c). mRNA levels of *NR1D2* and *CRY2* were also significantly reduced ($<60\%$) in cell lines from all three syndromes (Figure 3d–e). *CLOCK* and *CRY1* were evaluated, but the expression was too low to effectively assay in LCLs. Overall, these data alert us to a possible molecular basis for the similar phenotypes observed across these three disorders.

Due to similar mRNA expression patterns of circadian rhythm genes in LCLs from del 2q23.1, SMS, and FXS, we wanted to assess the relationships between mRNA expression of *MBD5*, R*AI1*, and *FMR1* in del 2q23.1, SMS, and FXS LCLs. As expected, each disease gene had reduced expression in its respective syndrome (Figure 3f–h). Interestingly, *MBD5* expression was significantly increased in FXS cell lines ($P<0.01$), suggesting that *FMR1* is critical for proper dosage of *MBD5* (Figure 3f). *RAI1* expression was significantly reduced in cells from del 2q23.1 subjects ($P<0.0001$), which suggests that RAI1 may function downstream of MBD5 and could be either directly or indirectly regulated by MBD5 (Figure 3f). Further, expression of *FMR1* was increased in del 2q23.1 LCLs ($P<0.02$) (Figure 3f). Based on these findings, we propose that MBD5, RAI1, and FMR1 function in an overlapping circadian network, with *NR1D2* and *CRY2* having important roles in linking these disorders together and contributing to overlapping sleep phenotypes. Overall, data demonstrate the possibility that these genes are involved in common circadian pathways.

Identification of potential E boxes in *MBD5* and *RAI1*

E boxes, which are found in the promoter or enhancer regions, are thought to have an important role in the regulation of the mammalian circadian clock and clock genes.[27] Nine closely connected circadian rhythm genes have E boxes, including *CLOCK*, *NPAS2*, *ANTL*, *CRY1*, *BHLHB2*, *PER1*, *PER2*, and *PER3*. As MBD5, RAI1, and FMR1 participate in circadian regulation, we sought to determine whether E boxes were also present in the promoter or enhancer regions, which would suggest that these genes are circadianly regulated through their E boxes. Assessment of ~2500 nucleotides upstream of each ATG translation start site revealed the presence of putative E boxes in each gene (Table 4). We then investigated whether these E boxes were evolutionarily conserved in non-coding regions of *Rai1*, *Mbd5*, and *Fmr1* in mice (Supplementary Figure S2), as these genes are conserved between human and mice. None of the E boxes of human *FMR1* were identified in *Fmr1* promoter or enhancer region. Only one E box in human *RAI1* was found in mouse *Rai1*, while all of the E boxes found in *MBD5* were found in *Mbd5* (Supplementary Figure S2).

Table 3 Circadian rhythm genes dysregulated due to *MBD5* haploinsufficiency in SH-SY5Y cells

Gene symbol	*Fold change*	*Name*	*Cellular function*
Upregulated			
ATF2	2.0396	Activating transcription factor 2	Transcription factor, member of the leucine zipper family of DNA-binding proteins
MAPK1	1.540	Mitogen-activated protein kinase 1	Integration point for multiple biochemical signals that are involved in a wide variety of cellular processes, such as proliferation, differentiation, transcription regulation, and development
CSNK1E	1.371	Casein kinase 1, epsilon	Role in regulating the phosphorylation and abundance of per proteins in animals
ATF4	1.2486	Activating transcription factor 4	Transcription factor, member of the leucine zipper family of DNA-binding proteins
PRNP	1.121	Prion protein	Membrane glycosylphosphatidylinositol-anchored glycoprotein
Downregulated			
RORB	−0.0357	RAR-related orphan receptor beta	Transcription factor possibly related to the circadian pacemaking system
CRY1	−1.0362	Cryptochrome 1	Key component of the circadian core oscillator complex
CLOCK	−1.1599	Circadian locomotor output cycles kaput	Encodes a basic helix-loop-helix (bHLH)-PAS transcription factor that is essential for circadian rhythm
RAI1	−1.227	Retinoic acid induced 1	Positive transcriptional regulator of *CLOCK* and possible role in circadian oscillator
CRY2	−1.3004	Cryptochrome 2	Central to the core autoregulatory loop of the mammalian circadian clock
NR1D1	−1.3401	Nuclear receptor subfamily 1, group D, member 1/ nuclear receptor Rev-ErbA-alpha	Major regulator of cyclic BMAL1 transcription
JUNB	−1.377	Jun B proto-oncogene	Role in regulation of light-induced gene expression in the suprachiasmatic nucleus
PER3	−1.4271	Period circadian protein homolog 3	Maintenance of circadian rhythm in cells
NOS2	−1.490	Nitric oxide synthase 2, inducible	Biologic mediator in several processes, including neurotransmission and antimicrobial and antitumor activities
NR1D2	−1.544	Nuclear receptor subfamily 1, group D, member 2/ nuclear receptor Rev-ErbA-beta	Functions as a transcriptional repressor and may have a role in circadian rhythms and carbohydrate and lipid metabolism
PER1	−2.3391	Period circadian protein homolog 1	Maintenance of circadian rhythm in cells

MBD5 and *RAI1* are involved in molecular pathways related to sleep

Based on the more common sleep phenotype and our previous mRNA expression studies, we have shown that there is a relationship between MBD5 and RAI1. We further investigated the hypothesis that *MBD5* and *RAI1* have shared molecular pathways that when altered cause some aspects of the sleep phenotype present in del 2q23.1 and SMS patients.

To test this hypothesis, we used our microarray knockdown data for *MBD5* and *RAI1* with IPA. We knocked down *MBD5* or *RAI1* using siRNA technology in SH-SY5Y cell lines (Supplementary Figure S3) and determined genome-wide levels of mRNAs using microarrays. In the microarray data on KD of *RAI1* in SH-SY5Y, 1358 genes were significantly upregulated ($P<0.05$); using a higher stringency ($P<0.01$), 836 genes were significantly upregulated. Furthermore, in the *RAI1* knockdown array data, 1459 genes were significantly downregulated ($P<0.05$); using a high stringency ($P<0.01$) 349 genes were significantly downregulated. IPA demonstrated that mRNAs for genes in two pathways related to sleep were altered in both knockdown studies. The first pathway is circadian rhythm signaling ($P=0.00329$), where 63 mRNAs showed significantly altered abundance in both *MBD5* and *RAI1* knockdowns ($P<0.05$). The mTOR signaling pathway also showed significant changes in both knockdowns ($P=0.0304$) (Figure 4a). Four mRNAs in the mTOR pathway were increased (*PLD* (MIM 602382), *ULK1* (MIM 603168), *eIF4G* (MIM 600495), *eIF3* (MIM 602039)), and three showed reduced levels (*REDD1* (MIM 607729), *REHB* (MIM 601293), and *VEGF* (MIM 192240)). Analysis of the mRNA expression level of *mTOR* in del 2q23.1 and SMS LCLs showed a significant increase in expression compared with normal controls (Figure 4b), which corroborated our microarray studies. These data support a functional role for MBD5 and RAI1 in the same pathways contributing to the sleep phenotype present in the associated disorders.

DISCUSSION

Proper circadian rhythms are vital to human physiological processes, including body temperature, feeding behavior, drug and xenobotic metabolism, glucose homeostasis, cell cycle progression, learning, memory, and development.[28] The mammalian circadian clock is composed of a core set of genes that are involved in cell autonomous transcription–translation feedback loops, including *CLOCK*, *NPAS2*, *BMAl1*, *PER* genes (*1–3*), *CRY* genes (*1–2*), *NR1D* genes (*1–2*), *ROR* genes (*A–C*), and a number of candidate clock components whose roles remain to be more clearly defined. The disruption of these genes is now thought to influence a range of disease-related pathways.[28] Defects in circadian rhythm may also affect the daily timing of neurobehavioral and endocrine functions.[29] Circadian rhythm defects seen in ASD are thought to contribute not only to the sleep disturbances but also to behavior, learning, and other phenotypes seen in these patients.[29]

2q23.1 deletion syndrome, *MBD5*, and sleep

In this study, we show that individuals with del 2q23.1 syndrome have sleep disturbances. These sleep studies further support previous reports regarding del 2q23.1 patients' sleep habits.[4–12] Although this survey may reflect a subjective measure of the sleep phenotype present in 2q23.1 deletion syndrome patients as parents/guardians answered the survey, parents are in a unique position to describe their child's regular sleep behaviors. Importantly, ~55.6% of parents reported that they use medication to improve the poor sleep patterns in their children, which is a significant indicator of the difficulties faced by the child and the family because of the sleep problems. As the majority of children were taking sleep medication during the survey, this may

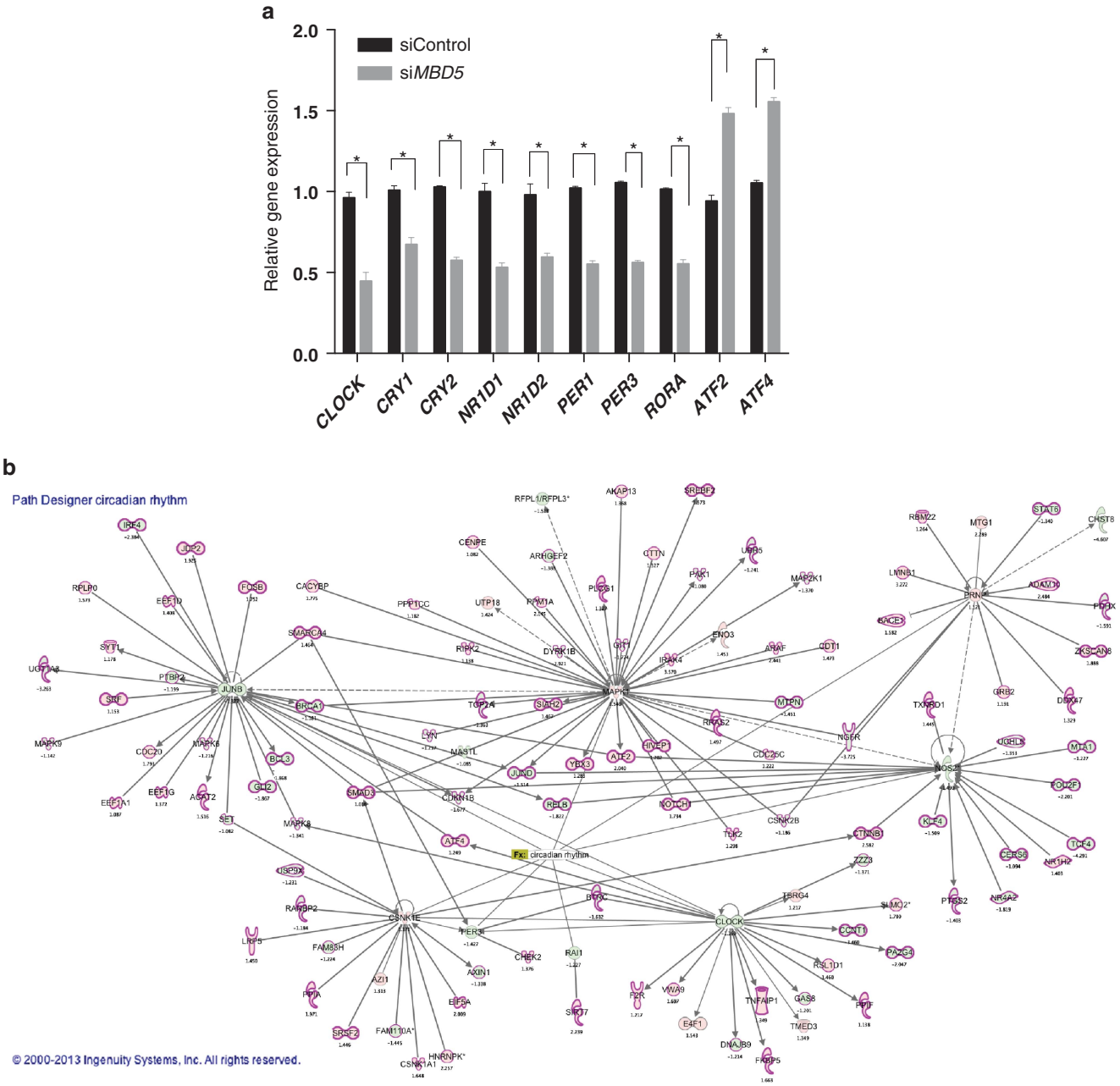

Figure 2 Knockdown of *MBD5* is associated with dysregulation of key circadian rhythm genes and altered circadian signaling pathway. (**a**) Relative expression levels obtained from quantitative real-time PCR of circadian rhythm genes found dysregulated by microarray analysis of *MBD5* siRNA knockdown cells are shown. Data are an average of three independent experiments performed in triplicate. Control siRNA is shown in black, and *MBD5* siRNA is shown in gray. Bars represent SE. Quantification of the test gene was compared with that of the housekeeping gene *GAPDH*. Circadian genes that were altered in *MBD5* knockdown microarray have altered expression in *MBD5* siRNA knockdown SH-SY5Y cells. Asterisks indicate statistical significance ($P<0.001$) as calculated with the Student's *t*-test and the Holm–Šídák test. (**b**) The pathway map of for circadian signaling ($P=0.00474$) drawn using IPA was one of the top significantly enriched canonical pathways in *MBD5* knockdown SH-SY5Y cell lines. Genes highlighted in red were upregulated, and genes in green were downregulated. The darker the color, the higher the fold change in gene expression. Values under each gene are the fold change observed in the study.

confound adequate assessment of the sleep problems in this syndrome, as medications may mask the reporting of specific sleep difficulties, limiting the extent of the data collected. More severe sleep issues may be present than were reported. Thus additional studies are required to more fully understand the specific problems in del 2q23.1 syndrome, including assessment of individuals across lifespan. Also, the molecular data presented here corroborate the sleep phenotypes present in del 2q23.1 syndrome and show that MBD5 has a role in circadian gene regulation.

2q23.1 deletion syndrome, SMS, and FXS shared sleep pathway

Disease phenotypes are complex, resulting from the interaction of many biological pathways across development. Even the phenotypes that result from single-gene disorders are likely the result of the

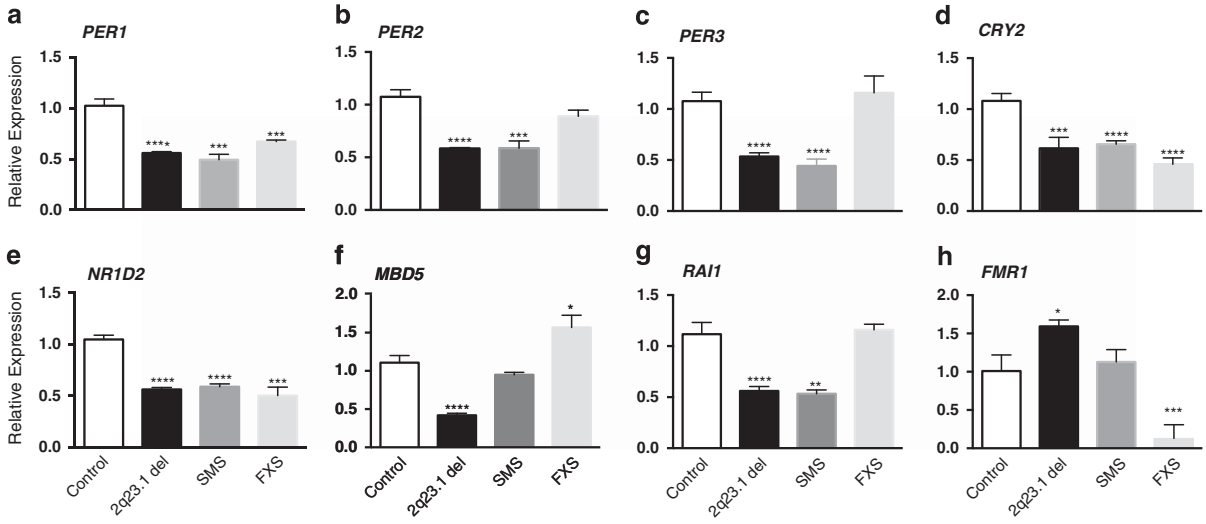

Figure 3 Gene dysregulation in del 2q23.1, Smith–Magenis, and fragile X syndrome cell lines suggests common molecular pathways. Quantitative real-time PCR of mRNA from LCLs from patients with SMS, FXS, or del 2q23.1 was performed. Data shown are calculated relative to mRNA expression levels from control subjects to assess expression of *MBD5*, *RAI1*, *FMR1*, *PER1*, *PER2*, *PER3*, *NR1D2*, and *CRY1* across each syndrome. Data are an average of three independent experiments performed in triplicate. All expression values are calculated relative to control levels set at 1.0. Asterisks indicate significant differences between samples from patients *versus* control subjects (****$P<0.0001$, ***$P<0.001$, and *$P<0.01$). *P*-values were determined by unpaired *t*-test. Error bars represent the SD from the mean. (**a**) Although *MBD5* expression is reduced in 2q23.1 deletion syndrome, overexpression is observed in *FXS* cell lines. (**b**) *RAI1* expression is reduced in 2q23.1 deletion syndrome and SMS cell lines but not in FXS. (**c**) *FMR1* expression is very low in FXS lines but overexpressed in 2q23.1 deletion syndrome cell lines, with normal expression in SMS lines. (**d**–**f**) Expression of *PER1*, *PER2*, and *PER3* is impaired in 2q23.1 deletion and SMS lines but at normal levels in FXS cell lines. (**g**) *NR1D2* expression is reduced in 2q23.1 deletion, SMS, and FXS lines. (**h**) *CRY1* expression is reduced in 2q23.1 deletion, SMS, and FXS lines.

Table 4 Putative E box sites for *MBD5*, *RAI1*, and *FMR1*[a]

	Sequence (5′–3′)	Genome coordinates (hg19)	Location upstream of ATG
MBD5			
1	CACGTG	chr2.hg19:g.148778363_148778368	−226
2	CAATTG	chr2.hg19:g.148777876_148777881	−713
3	CAGCTG	chr2.hg19:g.148777454_148777459	−1135
4	CAATTG	chr2.hg19:g.148776675_148776680	−1914
5	CAATTG	chr2.hg19:g.148776650_148776655	−1939
6	CAGCTG	chr2.hg19:g.148776217_148776222	−2372
RAI1			
1	CAGCTG	chr17.hg19:g.17582772_17582777	−2015
2	CAGCTG	chr17.hg19:g.17582365_17582370	−2422
FMR1			
1	CAGCTG	chrX.hg19:g.147008609_147008614	−579
2	CAGCTG	chrX.hg19:g.147008301_147008306	−887
3	CAGCTG	chrX.hg19:g.147007263_147007268	−1925
4	CAGCTG	chrX.hg19:g.147007055_147007060	−2133

[a] ~2500 base pairs upstream from ATG of *MBD5*, *RAI1*, or *FMR1*.

interactions of multiple pathobiological processes that interact in a complex network.[30] The disorders in this study, del 2q23.1, SMS and FXS, all clearly share common sleep phenotypes, such as difficulty falling asleep, shorter sleep duration, or night-waking episodes.[31,32] Although RAI1 and FMR1 have been shown to regulate or affect expression of circadian rhythm genes,[23,28] published sleep data suggest that SMS has a more severe sleep phenotype than FXS.[32,33] In addition, from this study, SMS may have more similar sleep phenotype to del 2q23.1 than FXS because of the more frequent concerns for daytime sleepiness and napping.[32,33]

Our bioinformatics analyses identified potential E boxes that suggest that RAI1 and MBD5 could be circadianly regulated by components of the molecular clock through their E boxes. Further studies involving whether these E boxes actually function like typical E boxes are necessary to elucidate the role of the regulation of MBD5, RAI1, and FMR1 in circadian rhythm.

The expression data presented here demonstrate that RAI1, MBD5, and FMR1 likely function in overlapping pathways that may explain etiologies for sleep disturbance and other phenotypes in each disorder. Data suggest that RAI1 and FMR1 may be more molecularly connected to MBD5 than to one another. Supporting our above hypothesis is a recent study on specific molecular pathways and circuits in autism, where ASD and ID risk genes were mapped onto co-expression networks of developmental trajectories and transcriptional profiles in the fetal and adult cortical laminae.[34] These studies showed bioinformatically that FMRP interacts with a series of ASD genes, one of them being MBD5.[34]

Finally, we show that the expression levels of *NR1D2* and *CRY2* were significantly reduced in 2q23.1 deletion syndrome, SMS, and FXS LCLs. Interestingly, loss of *CRY2* has been linked to high anxiety, and NR1D2 was recently shown to protect normal metabolic function.[35,36] Anxiety levels and abnormal metabolic function resulting in weight problems are two phenotypes present in these three disorders. The findings of dysregulation of circadian mRNA expression in connection with other phenotypes suggest that circadian rhythm defects not only contribute to sleep disturbances but also to other phenotypes as well. Overall, data suggest a potentially complex relationship for gene regulation across *MBD5*, *RAI1*, and *FMR1*, with key roles for each in the regulation of circadian gene expression.

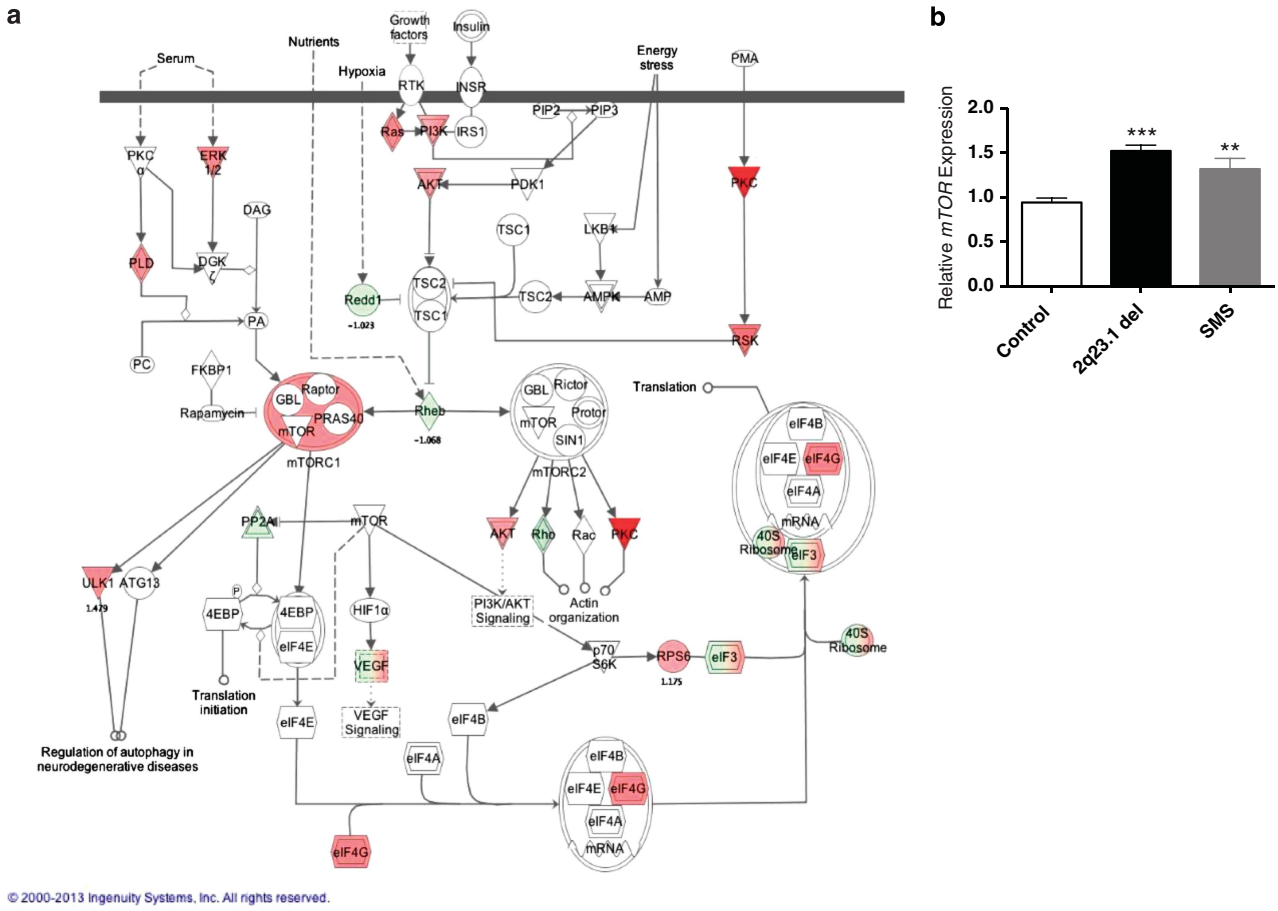

Figure 4 Dysregulation of mTOR signaling pathway and *mTOR* in sleep phenotype of del 2q23.1 and SMS. (**a**) Significantly altered mTOR signaling pathway due to knockdown of *MBD5* or *RAI1*. Illustration of a key pathway being altered by altered expression due to knockdown of *MBD5* or *RAI1* in SH-SY5Y cells (Ingenuity Pathway Analysis). The top altered pathway, mTOR signaling ($P=0.00329$), has a role in proper sleep. Genes highlighted in green were downregulated in *MBD5* or *RAI1* in knockdown SH-SY5Y cells, and genes in red were upregulated in *MBD5* or *RAI1* in knockdown SH-SY5Y cells. The darker the color, the higher the fold change in gene expression. Values under each gene represent the fold change. (**b**) Significantly increased mRNA expression of *mTOR* in del 2q23.1 and SMS LCLs. Quantitative real-time PCR of mRNA from LCLs from patients with SMS and del 2q23.1 was performed. Data shown are calculated relative to mRNA expression levels from control subjects to assess the expression of *mTOR* across each syndrome. Data are an average of three independent experiments performed in triplicate. All expression values are calculated relative to control levels set at 1.0. Asterisks indicate significant differences between samples from patients *versus* control subjects (***$P<0.005$, and **$P<0.05$). *P*-values were determined by unpaired *t*-test. Error bars represent the SD from the mean.

MBD5, RAI1, FMR1, mTOR signaling pathway, and sleep disturbance

Recently, the mTOR signaling pathway was associated with circadian rhythm. In humans, the master circadian clock is localized in the suprachiasmatic nucleus (SCN) of the hypothalamus. The circadian clock is thought to be reset by rapid induction of gene expression and, in particular, the expression of the circadian clock genes *PER1* and *PER2*.[37] In recent studies, mTOR-evoked mRNA translation alters the capacity of light to couple to the core clock timing mechanism, suggesting that the mTOR signaling pathway modulates photic entrainment of the SCN circadian clock.[37] In addition, dysregulation of mTOR signaling is found in mouse and *Drosophila* models of FXS, which show that loss of FMRP leads to altered circadian rhythm behaviors and sleep-dependent synaptic renormalization.[38] Also, in human studies, FXS patients show altered mTOR signaling.[39] Moreover, these findings suggest that *MBD5*, *RAI1*, and *FMR1* dysregulation could disrupt the mTOR signaling pathway, disrupting mammalian period (*PER*(*1–2*)) genes, which have key roles in photic entrainment of the circadian clock to light pulses.[39] Recent studies in *Fmr1*/*Fxr2* double KO mice show that absence of Fmrp/Fxr2p results in altered cyclical patterns of abundance of several core clock component mRNAs in the liver, including *Per* (*1–2*).[28] Furthermore, FMRP and FXR2P together increase PER(1–2) mediated BMAL1-Neuronal PAS2 (NPAS2) transcriptional activity, which is an essential component of circadian clock.[28] Data shown here demonstrate dysregulation of some or all of the *PER* genes in 2q23.1 deletion syndrome, SMS, and FXS cases. Further study of the mTOR pathway in relation to sleep could be a key to identifying possible drug therapies to alleviate the sleep disturbances in these disorders. Finally, understanding the pathways and mechanisms in which genes disrupt neuronal development and brain function will allow us to understand the pathogenesis of phenotypes, such as sleep.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to PRISMS and to the 2q23.1 Facebook group and all of the study participants and their families for their cooperation in this study. We thank the Fondation Jérôme Lejeune and the Smith–Magenis Syndrome

Research Foundation for funding portions of this study. This work was supported, in part, by the resources from Virginia Commonwealth University, Baylor College of Medicine, and the Jan and Dan Duncan Neurological Research Institute at the Texas Children's Hospital.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)