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Permalink

<https://escholarship.org/uc/item/7683d0mf>

Journal

Genetics in Medicine, 22(5)

ISSN

1098-3600

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Publication Date

2020-05-01

DOI

10.1038/s41436-019-0747-z

Peer reviewed



Published in final edited form as:

Genet Med. 2020 May ; 22(5): 878–888. doi:10.1038/s41436-019-0747-z.

GATAD2B-associated neurodevelopmental disorder (GAND): clinical and molecular insights into a NuRD-related disorder

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Abstract

Purpose: Determination of genotypic/phenotypic features of *GATAD2B*-associated neurodevelopmental disorder (GAND).

Methods: Fifty GAND subjects were evaluated to determine consistent genotypic/phenotypic features. Immunoprecipitation assays utilizing *in vitro* transcription-translation products were used to evaluate *GATAD2B* missense variants' ability to interact with binding partners within the Nucleosome Remodeling and Deacetylase (NuRD) complex.

Results: Subjects had clinical findings that included macrocephaly, hypotonia, intellectual disability, neonatal feeding issues, polyhydramnios, apraxia of speech, epilepsy, and bicuspid aortic valves. Forty-one novel *GATAD2B* variants were identified with multiple variant types (nonsense, truncating frameshift, splice-site variants, deletions and missense). Seven subjects were identified with missense variants that localized within two Conserved Region domains (CR1 or CR2) of the *GATAD2B* protein. Immunoprecipitation assays revealed several of these missense variants disrupted *GATAD2B* interactions with its NuRD complex binding partners.

Conclusions: A consistent GAND phenotype was caused by a range of genetic variants in *GATAD2B* that include loss-of-function and missense subtypes. Missense variants were present in conserved region domains that disrupted assembly of NuRD complex proteins. GAND's clinical phenotype had substantial clinical overlap with other disorders associated with the NuRD complex that involve *CHD3* and *CHD4*, with clinical features of hypotonia, intellectual disability, cardiac defects, childhood apraxia of speech, and macrocephaly.

Keywords

GATAD2B; NuRD complex; Apraxia of speech; Chromatin remodeling; Macrocephaly

INTRODUCTION

The Nucleosome Remodeling and Deacetylase (NuRD) Complex is involved in the management of genomic integrity, stem cell differentiation, and neurodevelopment.^{1,2} This important multi-enzyme complex regulates transcription by linking two independent

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SUPPLEMENTARY INFORMATION

Please see Supplementary Information Section

Disclosures: The authors declare no conflict of interest.

chromatin-regulating activities: histone deacetylase and ATP-dependent nucleosome remodeling activity.^{1,3} Several neurodevelopmental disorders have been associated with variants in NuRD subunit proteins that include *CHD3*, *CHD4*, and *GATAD2B*.^{4–8} Previously identified clinical features shared by each of these dominant disorders include intellectual disability, motor delays and distinct facies.^{4–8} *De novo CHD4* variants were also associated with hearing loss, bony fusions, palatal abnormalities, hypogonadotrophic hypogonadism, and heart defects (MIM# 617159; *CHD4*-related syndrome (CHD4RS) or Sifrim-Hitz-Weiss syndrome (SIHIWES));^{5–7} while *de novo CHD3* variants were associated with neonatal feeding issues, childhood apraxia of speech, joint laxity, undescended testes, and high forehead with frontal bossing (MIM#618205; *CHD3*-related syndrome (CHD3RS) or Snijders Blok-Campeau syndrome (SNIBCPS)).⁴ Both disorders were also associated with enlarged CSF spaces and macrocephaly.^{4–6} To date, previous reports of *GATAD2B*-associated neurodevelopmental disorder (GAND; MIM 615074) had identified a total of ten subjects that possessed deletion or truncating variants in *GATAD2B* and presented with intellectual disability, impaired language development, strabismus, and characteristic facies.^{8–12}

In this report, we have characterized the phenotypic data of fifty subjects with multiple types of *GATAD2B* variants with the purpose of defining the common genetic and clinical features of GAND. Pathogenic variants included deletions (3), nonsense (17), truncating frameshift (16), splice-site (7), and missense (7) changes. Missense variants have not been previously associated with GAND and all of them were located within two highly conserved domains, Conserved Region-1 (CR1) and Conserved Region-2 (CR2) of the *GATAD2B* protein (also known as p66 β) (Fig. 1, Table S1). Several of these missense variants were shown to disrupt interactions between the *GATAD2B* protein and its NuRD binding partners. All subjects had phenotypes that included intellectual disability, impaired language development, strabismus, and characteristic facies. Several additional phenotypic features were identified that included polyhydramnios, neonatal feeding difficulties, anisocoria, aortic valve defects, epilepsy, abnormal brain MRIs, and macrocephaly. This expanded GAND phenotype closely overlaps other NuRD-associated neurodevelopmental disorders indicating that these associated proteins (*CHD3*, *CHD4*, and *GATAD2B*) may have converging molecular functions and result in clinically-related syndromes.

MATERIALS AND METHODS

Standard Protocol Approvals, Registrations and Patient Consents

Research protocols were approved through an institutional review board (CSMC IRB protocol Pro00037131). The families of fifty subjects identified with *GATAD2B* variants gave informed consent and were enrolled in our retrospective study. Subjects GAND21, GAND24, GAND26, GAND27, GAND29, GAND30, GAND32–35 were identified and referred to our study through the Deciphering Developmental Disorders (DDD) study (UK Research Ethics Committee approval: 10/H0305/83, granted by the Cambridge South REC).¹³ Authorization for disclosure of recognizable subjects in photographs and information was obtained by parents.

Clinical Genetic Evaluations

Genomic DNA was extracted from saliva or blood and genetic testing was performed and analyzed per previous protocols for clinically-available trio-based exome sequencing^{14,15} (N=44; the DDD study; the Undiagnosed Diseases Network; GeneDx, Gaithersburg, MD; Centogene, Rostock, Germany; Baylor College of Medicine, Houston, TX), intellectual disability panels (N=4; GeneDx, Gaithersburg, MD; University of Chicago, Chicago, IL; Genome Diagnostics Nijmegen, Nijmegen, NL), and chromosomal SNP microarrays (N=1; GeneDx, Gaithersburg, MD).

Genome sequencing and analysis

Subject GAND50 was diagnosed via genome sequencing analysis. Genomic DNA was extracted from the subject and her unaffected parents. Genome sequencing was performed at Human Longevity Inc (San Diego, CA) on the Illumina Hiseq/novaseq platform to an average depth of 35–40X per sample. Approximately 450M 150bp-paired-end reads (132Gb of sequences) were generated across the genome for each sample using Illumina HiSeq X system with median insert size of ~340bp. Data analysis was performed on the DNAnexus platform using a UCLA custom built pipeline that incorporates BWA-mem v0.7.5 for alignment;¹⁶ Picard v2.0.1 (<http://broadinstitute.github.io/picard/>) for PCR and optical duplicate marking; GATK v3.4^{17–19} for depth of coverage analysis, indel realignment, base quality score recalibration, haplotype calling, joint genotyping, and variant recalibration; and PLINK v1.07²⁰ for determining regions of excess homozygosity. GoldenHelix VarSeq™ v1.4.5 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com) was used for variant annotation, filtering and interpretation. Manta v1.4.0,²⁰ ERDS v1.1,²¹ and CNVnator v0.3.3²² were used for structural variant (SV) detection. Annotation of SV calls for genic content was performed using in-house scripts and validated by manual inspection and Sanger confirmation at a CAP/CLIA accredited laboratory (UCLA Orphan Disease Testing Center). No clinically significant de novo, homozygous, hemizygous or compound heterozygous SNVs or small indels were identified. However, a 1.5Kb de novo deletion, NC_000001.10: g.153788220_153789753del, was observed in the proband that removes a single exon in *GATAD2B* (exon 7 in transcript NM_020699.3), resulting in a predicted frameshift. No common SVs with similar breakpoints are observed in the Database of Genomic Variants²³ or among 2,504 individuals called for SV by the 1000 Genomes Project.²⁴

Clinical Data

Retrospective clinical, diagnostic, and neuro-diagnostic information was collected, analyzed and reported from medical records and family interviews. Physical exams were performed whenever possible. Please note that the average values of the reported parameters are based on available data with raw data supplied in parentheses wherever pertinent.

Plasmids

Full-length genes for mouse methyl-binding domain protein-3 (MBD3; UniProt ID:Q9Z2D8), and human methyl-binding domain 2-alpha (MBD2-alpha; UniProt ID:Q9UBB5), *GATAD2B* (UniProt ID:Q8WXI9), and partial gene sequences for the C-

terminal domains (CTD) of Chromodomain-helicase DNA binding protein-3 (CHD3; UniProt ID:Q12873), Chromodomain-helicase DNA binding protein-4 (CHD4; UniProt ID:Q14839) and Chromodomain-helicase DNA binding protein-5 (CHD5; UniProt ID:Q8TDI0) were cloned into pcDNA3.1 expression vectors to generate HA-tagged GATAD2B, and FLAG-tagged MBD2, MBD3, and the CTD of CHD3 (residues 1246–1944), CHD4 (residues 1230–1912) and CHD5 (residues 1218–1954). Five missense variants (L180P, G406R, R414Q, C420R, and C420S) were introduced into the HA-GATAD2B plasmid using site-directed mutagenesis.

In vitro Rabbit Reticulocyte Lysate Protein Expression and Immunoprecipitation Assays

Protein expression and immunoprecipitation experiments were performed as previously described.^{25,26} *In vitro* transcription-translation in rabbit reticulocyte lysates (IVT) and pulldown studies were used to generate interaction data for: i) FLAG-tagged MBD2 or MBD3 co-expressed with versions of HA-GATAD2B (Wild Type (WT) or L180P) and ii) FLAG-tagged C1-C2 region²⁶ of CHD3, CHD4, and CHD5 co-expressed with HA-GATAD2B (WT, G406R, R414Q, C420R, or C420S). In all experiments, FLAG-fusion proteins were immobilized on α -FLAG affinity beads and used as baits to pull down the co-expressed HA-GATAD2B proteins. Wild-type HA-GATAD2B was expressed individually as a negative control. Lysates to which no plasmids were added were also used as negative controls. In each case, 10% of inputs and 50% of elutions were loaded on an SDS-PAGE gel and PVDF membranes were probed with α -HA-HRP (#2999S, Cell Signalling Technology, Danver, MA, USA) in 1:20,000 dilution and α -FLAG-HRP (#A8592, Sigma Aldrich, USA) in 1:80,000 dilution.

RESULTS

GATAD2B subjects and demographics

Fifty subjects were enrolled in our study. Most subjects were unrelated, although two sets of identical twins and one set of non-twin siblings were identified. Subjects were located in the United States, Canada, United Kingdom, Australia, Portugal, Spain, United Arab Emirates, and Brazil. Fifty-eight percent of subjects were female (29/50). Parents were primarily of Caucasian background (95.5%); a minority of parents were of other backgrounds (Asian (1.5%), Arabic (2%), Hispanic (2%) and African descent (1%)). The average age at diagnosis was 6.8 years (SD 4.6). Average age at enrollment in our study was 7.0 years (SD 4.5; range: 20 months to 21 years).

GATAD2B pathogenic variants

Forty-three different *GATAD2B* pathogenic variants were identified in the fifty enrolled subjects, forty-one of these were novel. Almost all subjects (96%) had *de novo* pathogenic variants, with two subjects inheriting variants via parental mosaicism. Five of the novel variants were found in multiple subjects (p.R116X (2 unrelated subjects); p.Q188Efs36X (2 subjects, siblings via paternal mosaicism); p.R414Q (2 unrelated subjects); p.C420R (2 unrelated subjects); p.E476X (2 subjects, monozygotic twins); p.R478X (3 subjects, monozygotic twins and one unrelated child). Two pathogenic variants in our cohort had been previously reported (c.1217–2 A>G splice-site; p.Q190Afs34X)^{8,9} (Fig. 1 and Table S1).

Multiple types of variants were identified in our subjects that included: nonsense (17; 34%), truncating frameshift (16; 32%), splice-site (7; 14%), deletions (3; 6%), and missense (7; 14%) changes. One deletion (~1.5kb) involved exon 7 and another deletion (~3.5kb) involved exons 8–11. A third deletion (~175kb) extended from the non-coding exon 1 of *GATAD2B* upstream to include seven other genes of unknown function terminating within the *NUP210L* gene. Interestingly, the missense variants were all located within the Conserved Region-1 (CR1; p.L180P) or Conserved Region-2 (CR2; p.G406R, p.R414Q, p.C420R, C420S) domains of the GATAD2B protein. These domains have important interactions with MDB and CHD proteins within the NuRD complex (Fig. 1).^{25–28}

Facial features

In addition to macrocephaly, the majority of GAND subjects also had distinct facial features. Dysmorphisms were evaluated in thirty-seven children whose families provided adequate photos. Findings included a high wide forehead/frontal bossing (100%), prominent supraorbital ridges (62.2%), posteriorly angulated ears (59%), ocular hypertelorism (78.4%), down-slanting palpebral fissures (45.6%), epicanthal folds (29.7%), prominent or bulbous nasal tip (83.8%), wide nasal base (35.1%), elongated wide nose (35.1%), short philtrum (51.3%), small recessed jaw (24.3%), and a pointed chin (91.9%)(Fig. 2, Table S2). Face2Gene image analysis (FDNA Inc., USA) was used on photographs of 19 GAND subjects to produce a composite model of the facial features associated with GAND as compared to age-, ethnicity-, and gender-matched controls. The facial features of the GAND cohort was significantly different from healthy controls (N=19; area under the curve [AUC] value of 0.933 of receiver operating characteristic [ROC] curve, *p*-value: 0.004).

Birth and Development

Average parental age at birth was in the early thirties. The average gestational age at birth was 38.0 weeks (SD 1.8). Polyhydramnios (45%) was the most common complication during pregnancy. The average birthweight and birth length were within normal limits. Macrocephaly was seen in 60.4% of subjects at birth, with an average birth head circumference of 36.7 cm (SD 1.7). The average percentile rank of the birth head circumference was ~87 (SD 23.5). Macrocephaly at older timepoints was present in 91.8% of subjects, with average head circumference percentile score of 96.0 (SD 6.4) (Tables 1 and S3).

All developmental milestones were abnormal from early infancy, including feeding, language, motor ability, and intellectual ability. Infantile hypotonia was present in all subjects (100%), with most being described as “floppy” in infancy. Children sat up on average at 14.7 months (SD 5.2) and most children were ambulatory by an average age of 33.1 months (SD 12.6). Five subjects were non-ambulatory (ages: 1.3, 2, 2.5, 5, and 6 years) (Table S4). Most ambulatory children had a persistent unsteady gait (91%). Infantile feeding issues and gastro-esophageal reflux disease were reported in 82% of subjects. Two subjects required gastrostomy-tube placement (4%). Reflux disease often resolved with age, but most children had persistent oro-mechanical issues and excessive drooling. (Tables 1, S3, S4, and S5)

All subjects had intellectual disability associated with expressive and receptive language issues. The average age of first spoken words was 3.9 years (SD 2.0), although none of the subjects attained an extensive vocabulary. Eleven subjects were non-verbal (ages: 1.3, 2, 3, 3, 3, 5, 5, 6, 6, 11, 14, and 21 years) (Tables S4, S5). All children over 7 years could follow a one-step command (Table S5), although 20% of subjects 0–3 years and 10.5% in the 4–7 years age group could not. A substantial number of subjects never attained complete toilet-training (Table S5). Almost all subjects exhibited normal eye contact and social reciprocity (98% each).

Neurological, neuroimaging, and ophthalmological findings

Epilepsy was present in 24% of subjects. Interestingly, 58% of all subjects underwent electroencephalograms (EEGs) for suspected seizure events, with most EEGs being normal (79.3%). Primary generalized and focal features were noted in several subjects (13.7% and 6.9%; respectively). Most epileptic subjects were well-controlled with anti-seizure medication (75%; 8/12). MRI of the brain was performed on 88% of subjects, with abnormalities being present in 60% of all neuroimaging studies (Tables 1, S6). Predominant findings were enlarged extra-axial spaces/ventriculomegaly (35.6%), white matter signal abnormalities (22.2%), thin corpus callosum (8.9%), and hypomyelination (13.3%) (Fig. 3). Ophthalmological findings included strabismus (88%), astigmatism (41.3%), hypermetropia (30.4%), myopia (21.7%), anisocoria (13.6%) and optic nerve hypoplasia (9.1%) (Table 1).

Cardiac findings

Many subjects underwent cardiology consultations and echocardiograms (34%). One subject had left pulmonary artery stenosis, while four other subjects had a bicuspid aortic valve (8%). Two of these subjects underwent balloon valvulotomy. One of these two children had neonatal critical aortic stenosis and subsequently developed aortic regurgitation that required a Ross cardiac procedure for correction.

Evaluation of cohorts with specific variant types

All of the above evaluations were subdivided into cohorts consisting of nonsense, truncating frameshift, deletions, splice-site, and missense variants. There were no obvious or dramatic differences between these variant groups with regards to the above-mentioned parameters, with the exception of the frequency of epilepsy in subjects with missense variants (57%), compared to the total (24%) (Tables S2, S4, and S6).

Disruption of NuRD interactions by GATAD2B missense variants

GAND-associated missense variants were all located in conserved regions of GATAD2B: namely CR1 (L180P) and CR2 (G406R, R414Q, C420R, C420S). The CR1 motif is known to bind MBD2/3 NuRD subunits through the formation of a coiled coil.³⁷ The CR2 region has been shown to interact with the C-terminal region of CHD4.^{26,36} In order to understand the effect of the GATAD2B missense variants in the context of NuRD assembly, we performed pairwise interaction experiments to assess the binding of GATAD2B to MBD or CHD proteins. Full-length HA-tagged GATAD2B (wildtype or missense variants) was co-expressed with either FLAG-tagged MBD2/3 or the FLAG-tagged C-terminal region of

CHD proteins (the C1-C2 region that follows the helicase domain) and their interaction examined in pulldown experiments.²⁶ The L180P variant abrogated the interaction of GATAD2B with both MBD2 and MBD3 (Fig. 4a). The L180 residue lies at the interface of the GATAD2-MBD coiled-coil, and the leucine to proline substitution is likely to disrupt both the interaction interface and the intrinsic helical propensity of the CR1 region. As compared to wild type GATAD2B, the CR2-region variants variable effects on the interactions between GATAD2B and the CHD paralogues (Fig. 4b). The C420R and C420S variants substantially inhibited these interactions with the CHD paralogues as compared to wild-type controls, whereas R414Q variant caused a mild reduction in these interactions. The G406R variant showed no clear effect. The C420 residue is one of the zinc-binding ligands in the GATAD2B zinc-finger domain found in CR2 and substitution of this cysteine is likely to severely disrupt the structure of the CR2 domain. In contrast, G406 and R414 are upstream of the zinc finger domain and are predicted to be in a region that is unstructured – consistent with their smaller (R414Q) or negligible (G406R) effect on the GATAD2B-CHD4 interaction. As compared to controls, the effect of these variants on interactions between GATAD2B and the CHD paralogues was consistent with the close sequence similarity between the CHD paralogues in the C2 region of these proteins (Fig. 4b).²⁶

DISCUSSION

We report the genetic and clinical features of 50 individuals with *GATAD2B*-associated neurodevelopmental disorder (GAND). Prior to our work, a handful of GAND patients had been reported with distinct facies, infantile hypotonia, intellectual disability, limited language ability, and strabismus.^{8–12} Our large cohort has allowed us to identify an expanded range of new phenotypic and genotypic features associated with GAND. These new features included macrocephaly, frontal bossing, polyhydramnios, infantile feeding difficulties, cardiac defects, anisocoria, astigmatism, epilepsy (focal and/or primary generalized), and abnormal neuroimaging, as well as a variety of new variant types including missense variants.

The clinical phenotype of GAND subjects was relatively consistent across our cohort. Pregnancies were mostly without complications, with the exception of polyhydramnios (45%). Several children were noted to have macrocephaly on prenatal ultrasound and macrocephaly was frequently present at birth (69%), and became more common with age (91.8%). All subjects had global developmental delay and intellectual disability. All subjects had infantile hypotonia and delayed motor milestones. Most children learned to ambulate. A majority of subjects were noted to have infantile feeding difficulties and GERD (82%). These oromechanical issues were also associated with delayed and limited expressive language development in all subjects that was reminiscent of childhood apraxia of speech.⁴ Receptive language was not as severely affected, with most children being able to follow multi-step commands at older ages. Epilepsy was present in a minority of subjects (24%), and could be of focal or primary generalized onset. Most children responded well to anti-epileptic treatment. Neuroimaging was abnormal in the majority of subjects (60%), with common features including ventriculomegaly/enlarged CSF spaces, white matter signal abnormalities, hypomyelination, and thin corpora callosi. Almost all subjects made good eye contact and exhibited social reciprocity. Toilet training was not attained in the majority of

subjects. Most subjects had ophthalmological issues that included strabismus (88%), which was sometimes associated with astigmatism (41.3%), anisocoria (13.6%) and/or hypoplastic optic nerves (9%). Lastly, a minority of subjects were born with bicuspid aortic valves (10%, as compared to 2% in the general population),²⁹ with two subjects requiring surgical intervention. Of note, the two pairs of monozygotic twins in our cohort (GAND33/GAND34; GAND40/GAND41) had similar overall phenotypes with some differences. GAND33 developed epilepsy in her teens and continues to require seizure medication, while GAND34 does not have epilepsy. GAND33 has also plateaued developmentally, while GAND34 continues to progress with school. The other set of monozygotic twins shared very similar phenotypes, although GAND40 has had an easier time with school performance and GAND41 has been more adept at potty training. Of course, with only two sets of twins it is difficult to make any definitive comments on phenotypic trends of monozygotic siblings. As the NuRD complex plays an active epigenetic role in corticogenesis,³⁰ the identification of more monozygotic twin sets will be valuable to determine variable aspects of the GAND phenotype, especially with regards to cortical function.

Almost all GAND variants were *de novo*, with a diverse range in variant types that included nonsense (34%), truncating frameshift (32%), splice-site variants (14%), and deletions (6%). Seven subjects had missense variants (14%), which had not been previously reported in GAND. All of the missense variants were located within the two conserved region domains (CR1 and CR2), of the GATAD2B protein. Aside from an increased incidence of epilepsy in subjects with missense variants as compared to subjects with other variants (57 to 19%, respectively), there were no dramatic phenotypic differences between subjects with missense versus loss-of-function (deletion, splice-site, nonsense, and truncating frameshift) variants (Table S4). Of course, in the context of these relatively low numbers of subjects (N = 7 vs. N = 43, respectively) that were across a range of ages, it is difficult to make any firm conclusions about specific variant types. If, with larger numbers, subjects with missense variants continue to be similar to subjects with loss-of-function variants, this similarity may indicate that haploinsufficiency of the GATAD2B protein may likely be the primary pathological mechanism in most GAND subjects.⁸

The GATAD2B protein functions as a subunit within the NuRD complex, which is an important regulator of gene expression during neurodevelopment. NuRD is a multi-protein holoenzyme possessing both histone deacetylase and chromatin remodeling activity. The complex can consist of an assortment of paralogous subunits, which in addition to GATAD2B (and its paralogue, GATAD2A) includes several scaffold proteins: i) Retinoblastoma binding proteins (RBBP4/RBBP7), ii) metastasis associated proteins (MTA1/MTA2/MTA3), and iii) methyl-domain binding proteins (MBD2/MBD3), along with enzymatically active subunits that include: iv) histone deacetylases (HDAC1/HDAC2), and v) chromatin-helicase DNA-binding proteins (CHD3/CHD4/CHD5).^{1,2} CDK2AP1 is also present in specific cell types.²⁸ The MBD, GATAD2, and CHD subunits exist as interacting monomers;^{28,30–33} therefore, in principle, many configurations can exist with unique combinations of these paralogues that may have specific regulatory functions.²⁰ For example, recent work suggested that MBD2-NuRD converts open chromatin into compacted chromatin, while MBD3-NuRD was associated with more active promoter regions.³¹ Other reports indicate that the CHD proteins are developmentally regulated during corticogenesis,

which influences the generation of neural progenitors and neurons, as well as their subsequent migration and laminar identity.³⁰ Of note, the GATA zinc-finger proteins (GATAD2B and its paralogue, GATAD2A) may have distinct effects on NuRD activity. For example, GATAD2A plays a specific role in DNA repair,³³ as well as having a role in repression of pluripotency factors during iPSC reprogramming,³⁴ however, when GATAD2A expression was reduced, GATAD2B was incapable of rescuing the lost GATAD2A activity. To date, no specific roles for GATAD2B have been identified, although it has been shown to be the predominant GATAD2 gene expressed in the brain.³⁵

One hypothesis regarding the general function of GATAD2 proteins is that they provide a “bridge” that links MBD proteins (via CR1) and the CHD chromatin remodeling proteins (via CR2) in the NuRD complex.^{2,25,26} GATAD2B-CR1’s interaction with MBD proteins occurs through coiled-coil domains within each protein that links GATAD2B with the core proteins of NuRD through MBD.²⁶ In turn, the GATAD2-CR2 domain extends this bridge by its direct interactions with the carboxy-terminal C2 region of CHD proteins, thereby linking CHD proteins to the NuRD core.³⁶ The validity of this bridge model is corroborated by the fact that all of our cohort’s pathogenic missense variants were located within these two regions. Furthermore, our immunoprecipitation assays indicated that several of these missense variants disrupted GATAD2B interactions as predicted by the model. For example, our L180P variant (analogous to L159 of GATAD2A) is located within the coiled-coil domain of CR1 (CR1-CC) that is a key contact point for MBD binding (Fig. S1).³⁷ Our immunoprecipitation assays showed this variant prevented GATAD2B from interacting with MBD2 or MBD3 (Fig. 4a). This result is in line with the prediction that the substituted proline is likely to destabilize the CR1-CC α -helix that forms a coiled-coil with MBD. It had been shown previously that a GATAD2A CR1-CC missense change (K149R) abolished the interaction of GATAD2A with MBD proteins, as well as MBD-mediated transcriptional repression.²⁷ Our other missense variants were clustered within the GATAD2B-CR2 domain. The C420S and C420R variants affect the first of four zinc-binding cysteines of the GATA zinc-finger domain and so would be predicted to disrupt the proper folding and the known interaction of this domain with the CHD carboxy-terminal C2 region.³⁶ Our immunoprecipitation assays confirmed this to be the case. It is also important to note that several subjects with CHD3-related syndrome and CHD4-related syndrome have been reported with carboxy-terminal variants (CHD3: p.R1881L, p.F1935Efs108X; CHD4: p.R1870X) that lie within the CHD carboxy-terminal C2 region.^{4,7} These variants could also disrupt the GATAD2B-CHD bridge and lead to the exclusion of CHD activity from NuRD complexes causing their associated neurodevelopmental disorders.^{4,7,8} The R414Q variant was present in two subjects and resulted in a milder disruption of *in vitro* GATAD2B-CHD interactions. It may be that, in the context of expression within a complete NuRD complex, this substitution may be more disruptive or alter interactions with other proteins associated with NuRD activity. In contrast to the effects of these other missense variants, the G406R substitution did not alter the *in vitro* interactions between GATAD2B and CHD proteins. This variant involves highly-conserved GATAD2B-CR2 residue (Fig. S1) that is located in a region that has not been characterized structurally or functionally and that lies just N-terminal to the GATA zinc finger (Fig. S1). Further work to determine the mechanism by which this variant affects GATAD2B function is required; however, the nucleotide

substitution (c.1216 G>C) associated with G406R involves the terminal nucleotide of exon 7 and so might interrupt proper splicing of intron 8. Whether these missense variants produce proteins capable of antagonizing CHD-MBD-NuRD interactions in a dominant negative manner or work via a dosage effect similar to haploinsufficiency with a similar resulting phenotype remains to be determined; nonetheless, their observed phenotypes are not dramatically altered from subjects with loss-of-function variants. Identification of more missense subjects and additional work are required to answer these questions.

Currently, no other germline disorders have been associated with *GATAD2A* or most of the other NuRD proteins. Nevertheless, numerous mouse models have indicated the importance of each of these subunits in development.² Besides *GATAD2B*, the only other NuRD complex genes that are associated with human disease are *CHD3* and *CHD4*, which have a substantial phenotypic overlap with *GAND* (although an HDAC1 missense variant was reported in a large exomic screen associated with epilepsy without a detailed report of the phenotype).^{4-8,38} *CHD4RS* (or *SIHIWES*) has been recently reported in 32 subjects, while *CHD3RS* (or *SNIBCPS*) has been reported in thirty-five subjects.⁴⁻⁷ Our expanded *GAND* phenotype indicates these three neurodevelopmental disorders share features that include macrocephaly, developmental delay, intellectual disability, infantile hypotonia, and ventriculomegaly. Interestingly, each of these disorders share some facial features that are associated with macrocephaly that include a high broad forehead, frontal bossing, ocular hypertelorism, and a wide nasal bridge (Fig. 2).⁴⁻⁸ Many *GAND* and *CHD3RS* subjects also had a prominent nose/nasal tip and pointed chins, while *CHD4RS* subjects had a small nose and ears with a square chin.⁴⁻⁸ Besides these facial characteristics, other variable features across these disorders include male genital abnormalities in both *CHD3RS* and *CHD4RS*, inguinal hernia in *CHD3RS*, and deafness in *CHD4RS*. Alternatively, *GAND* and *CHD4RS* are associated with congenital heart defects,^{5-7,29} whereas *GAND* and *CHD3RS* subjects presented with oromechanical dysfunction that was absent in *CHD4RS* that included neonatal feeding issues (and perhaps polyhydramnios) and language deficits reminiscent of childhood apraxia of speech.^{4,38}

Childhood Apraxia of Speech (CAS) is a rare neurodevelopmental disorder associated with oromotor incoordination that limits fluent speech.³⁹ Pathogenic variants in several members of the forkhead transcription factor family (*FOXP1*, MIM 613670; *FOXP2*, MIM 602081) have been associated with oromotor incoordination and language issues classified as CAS.³⁹⁻⁴¹ Similar to *CHD3*,⁴ *GATAD2B* is one of a few documented proteins that interact with *FOXP1/FOXP2* (via its CR2 domain and the zinc-finger/leucine-zipper repressor domain of *FOXP*),^{4,42} with *GAND*, *CHD3RS*, and *FOXP1* subjects sharing several phenotypic features that include high forehead, frontal bossing, intellectual disability, and delayed language milestones. These data may indicate that these proteins may undergo important interactions during neurodevelopment and corticogenesis that have essential roles in language development. Further work on the specific interactions of *CHD3*, *GATAD2B*, and the *FOXP* proteins is warranted and may provide further clarification of the molecular events required for corticogenesis and language development.

In summary, our *GAND* cohort has provided a more established picture of the genotypic and phenotypic features of this disorder. At this time, this diagnosis has been associated with

several developmental, cardiac, ophthalmological, and neurological issues. Therefore, we recommend any child who is genetically diagnosed with GAND undergo consultations with specialists in these fields. Additional evaluations should include speech, physical, and occupational therapy consultations. Notably, the identification of macrocephaly as a feature of GAND has aligned this phenotype with other NuRD-associated disorders that include CHD4RS and CHD3RS. One hypothesis for this could be that GATAD2B dosage effects cause associated deficiencies in CHD3 and/or CHD4 chromatin remodeling activity in GAND children by the decreased availability of haploinsufficient GATAD2B levels to bring CHD-associated chromatin remodeling activity to NuRD complexes. Therefore, it may be prudent to evaluate GAND subjects for additional clinical features seen in CHD3RS and CHD4RS (e.g. bony lesions, hearing loss, hernias, or genital abnormalities), as these findings may also be present in GAND, albeit at lower frequency (e.g. one male subject had an undescended atrophic testis, which is more frequent in CHD3RS).⁴

GAND-associated missense variants were another important finding within our cohort. These variants were only present within GATAD2B's CR1 or CR2 domains, which are known to interact with other NuRD proteins. Whether these variants act via a dominant negative mechanism by sequestering NuRD components or by blocking their interactions with other effector proteins (e.g. CHD4, CHD3, FOXP) and/or altering its DNA binding will require further evaluations with affected samples. It is interesting to note that subjects possessing missense variants and loss-of-function variants had similar phenotypes for the most part, which may indicate that they act through similar dosage-dependent mechanisms. More work will be required to unravel these interesting questions with regards to GATAD2B, the NuRD complex, and their phenotypic overlap with other neurodevelopmental disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ACKNOWLEDGEMENTS:

We are grateful and thank the subjects and their families for their help, for their providing medical information, and providing consent to post photographs in this article. We also would like to extend special thanks to the Helping Hands for GAND Foundation for their assistance and continued cooperation. We are grateful to Dr. Charles Simmons and Clive Svendsen for mentorship and Kelli DeJohn, Roxana Ramirez, Joanne Baez, and Carmela Brito for clinical and technical assistance. We would like to acknowledge Jaemin Kim and Yogesh Kushwaha for their contribution to the work. We would like to thank Barrington Burnett for their critical review of the manuscript. Research reported in this manuscript was supported by the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director under Award Number(s) [U01HG007672]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. GAND50's seq+analysis was supported by NIH National Center for Advancing Translational Science (NCATS) UCLA CTSI Grant Number UL1TR001881. JPM received funding from the National Health and Medical Research Council (APP1012161, APP1063301, APP1126357, APP1058916). TMP and this research was supported by the Cedars-Sinai institutional funding program and the Cedars-Sinai Diana and Steve Marienhoff Fashion Industries Guild Endowed Fellowship in Pediatric Neuromuscular and the Fashion Industries Guild Endowed Fellowship for the Undiagnosed Diseases Program. TMP is especially grateful for the wonderful and continued support from the Cedars-Sinai Fashion Industries Guild.

GLOSSARY

GAND	<i>GATAD2B</i> -associated neurodevelopmental disorder
GATAD2A/B	GATA zinc finger domain containing 2A or 2B
CR1	Conserved Region 1
CR2	Conserved Region 2
NuRD	nucleosome remodeling and deacetylase complex
CHD	Chromodomain-helicase-DNA-binding protein
MBD	methyl-CpG binding domain protein
MTA	metastasis-associated protein
RBBP	retinoblastoma associated protein
HDAC	histone deacetyltransferase

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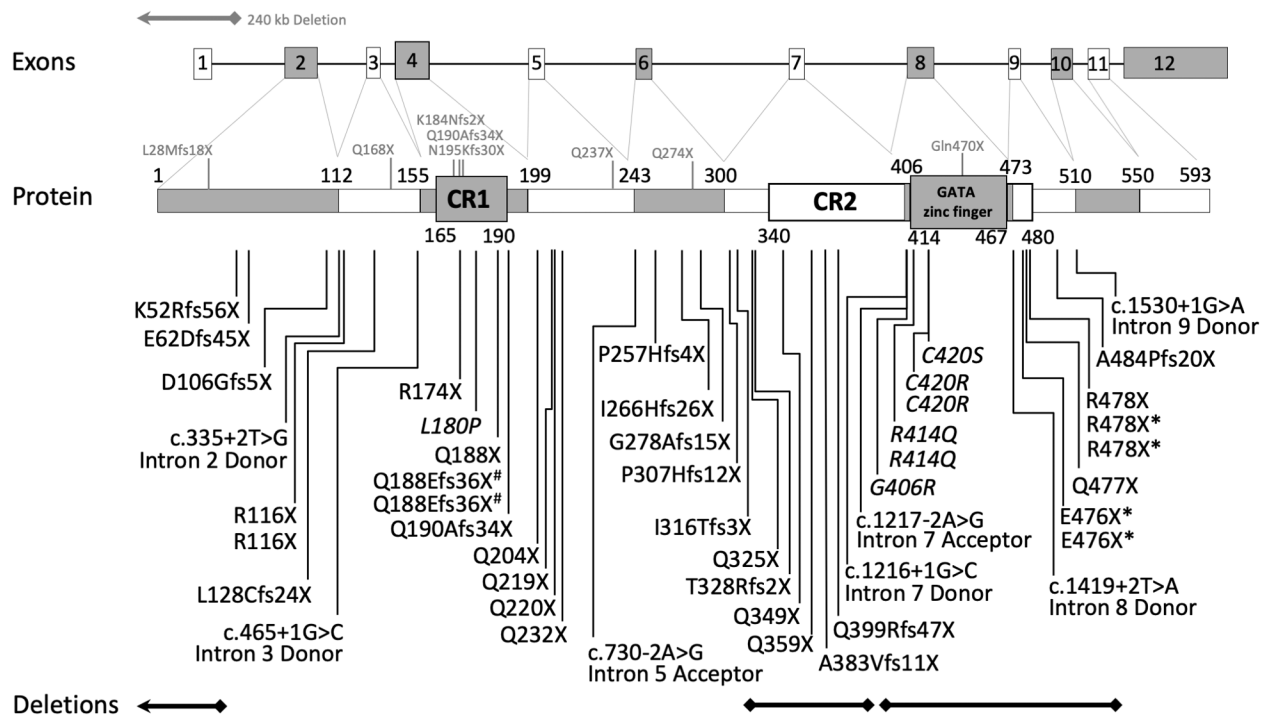


Fig. 1. Genomic and Protein Schematic Diagram with GAND variants.

All reported pathogenic *GATAD2B* variants from our study are represented as deletions, splice-site or protein changes below the protein diagram. Genomic deletions are represented as bars below the figure. Previously reported variants and deletions are represented with grey letters and bars above the diagram. * = monozygotic twins; # = somatic mosaic family

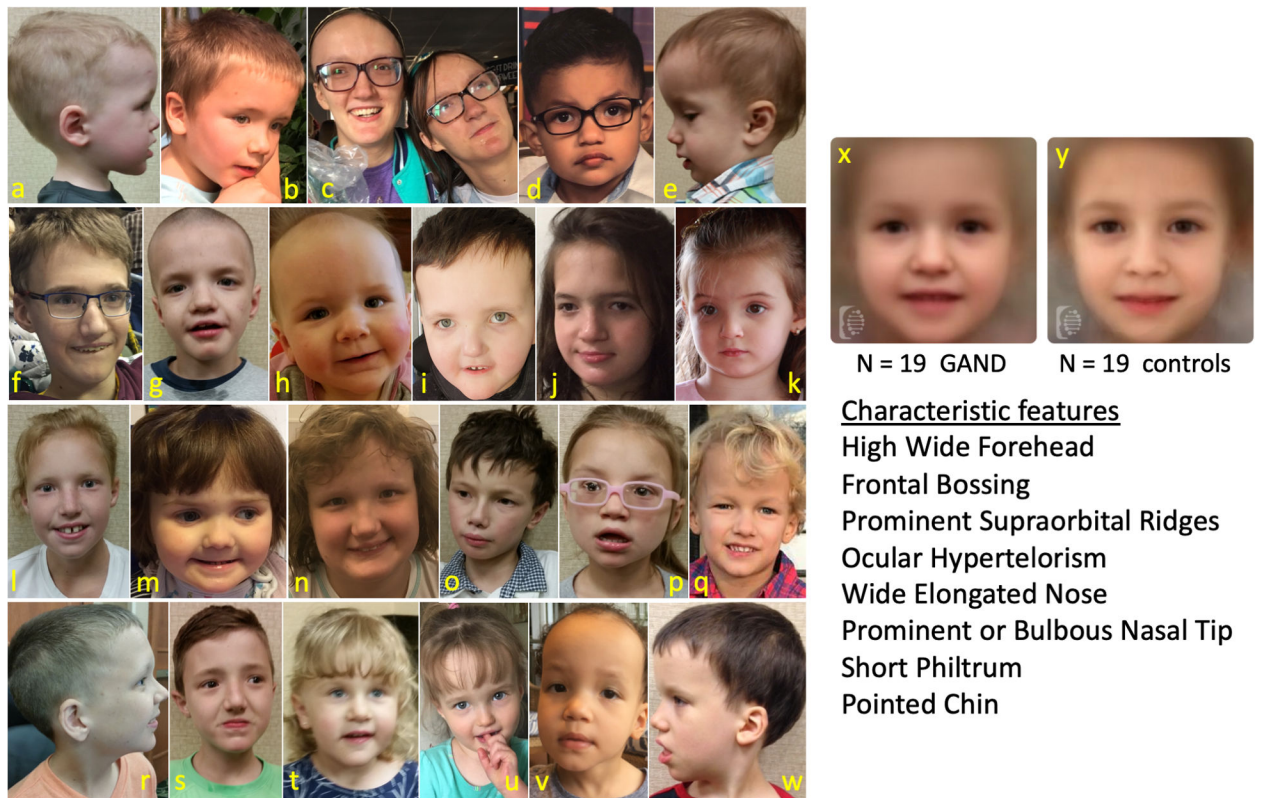


Fig. 2. Photographs of affected individuals with associated with variant types. a-e: Nonsense or truncating frameshift variants: **a.** GAND19 **b.** GAND18 **c.** GAND33 & GAND34 **d.** GAND53 **e.** GAND20 **f-k:** Missense variants: **f.** GAND28 **g.** GAND15 **h.** GAND52 **i.** GAND32 **j.** GAND55 **k.** GAND49 **l-q:** Splice-site variants: **l.** GAND17 **m.** GAND27 **n.** GAND42 **o.** GAND21 **p.** GAND6 **q-v:** Nonsense or truncating frameshift variants: **q.** GAND40 **r.** GAND12 **s.** GAND3 **t.** GAND13 **u.** GAND8 **v.** GAND36 **w.** GAND2 **x.** composite of 19 photographs of GAND subjects and **y.** 19 healthy controls (FDNA Inc. USA).

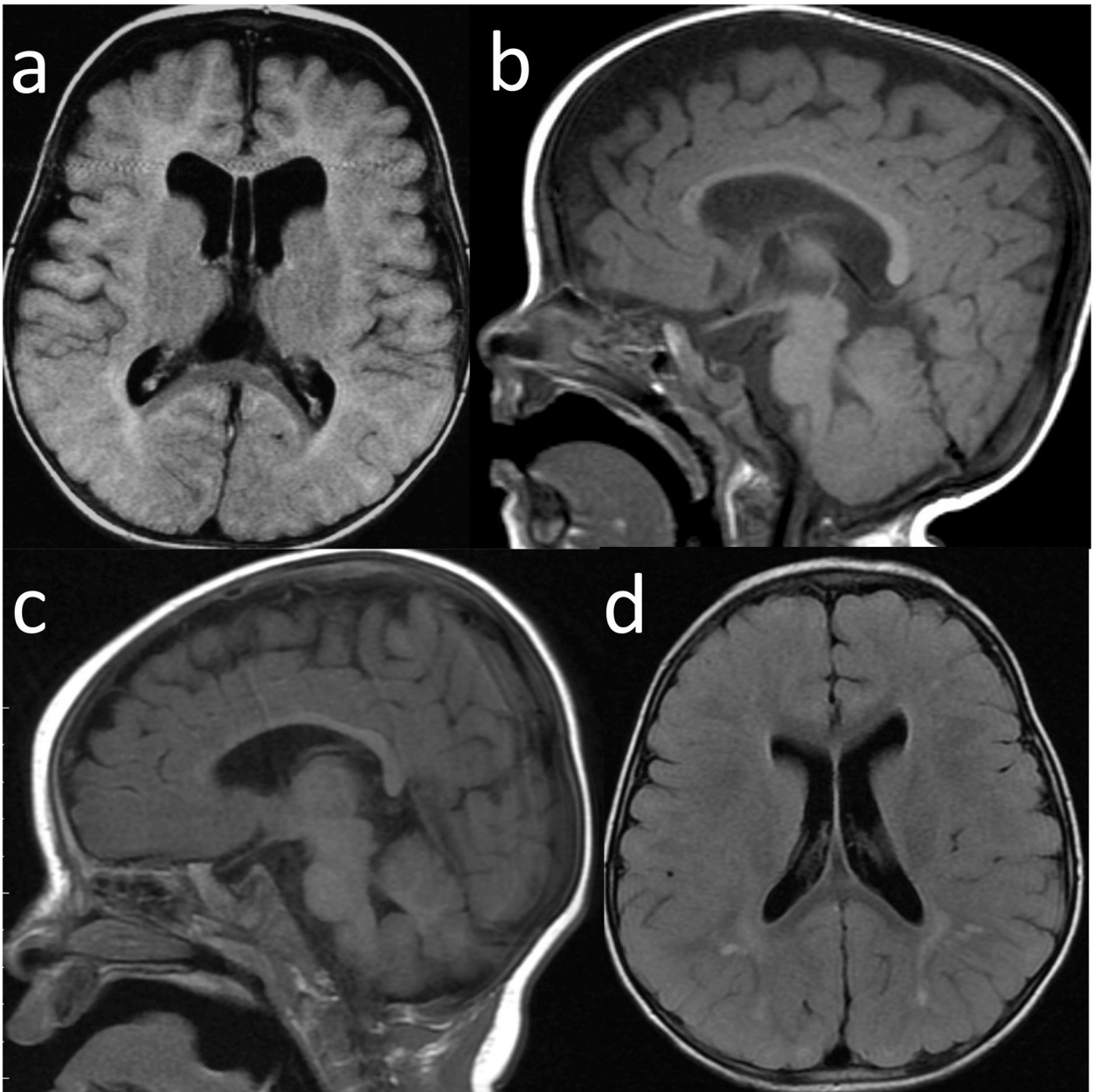
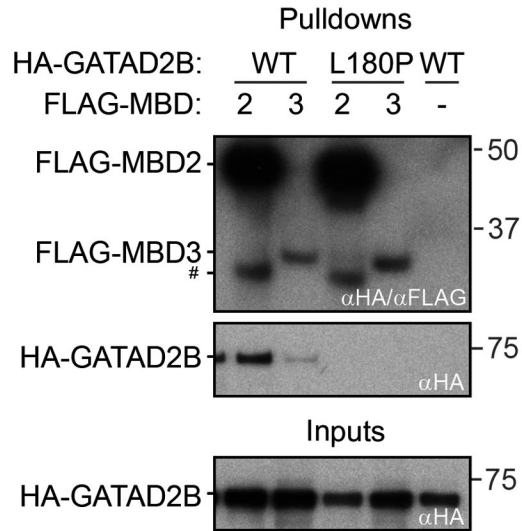


Fig. 3. Neuroimaging of GAND8 and GAND53.

a-b: GAND8 **a.** Axial FLAIR image showed enlarged subarachnoid spaces and nonobstructive ventriculomegaly of the lateral ventricles (cavum vergae was also present) **b.** Sagittal T1 showed macrocephaly, enlarged extra-axial fluid, and a thin corpus callosum **c-d: GAND53** **c.** Sagittal T1 with normal corpus callosum and structures (prominence of the extraaxial CSF spaces previously seen on earlier imaging had resolved) **d.** Axial FLAIR image showing multiple punctate and patchy foci of nonspecific signal hyperintensity scattered in the bilateral cerebral white matter, particularly involving the parietal lobes.

a



b

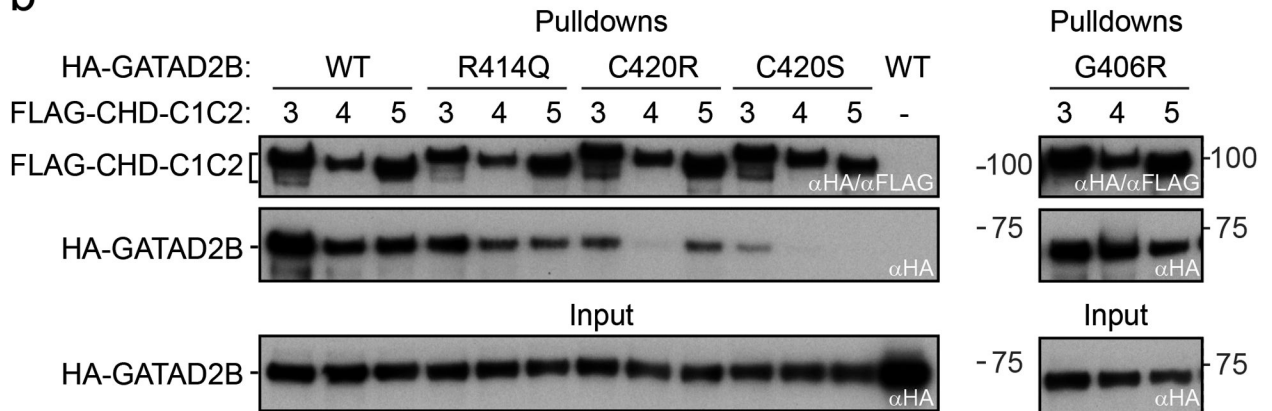


Fig. 4. GAND-associated missense variants L180P, R414Q, C420S and C420R disrupt binding to NuRD components CHD3/4/5 or MBD2/3.

a) FLAG-tagged MBD2 or MBD3 were co-expressed with HA-GATAD2B (wild type (WT) or L180P) by *in vitro* transcription-translation (IVT) in a rabbit reticulocyte lysate. b) The FLAG-tagged C1-C2 domains of CHD3, CHD4 or CHD5 was co-expressed with HA-GATAD2B (WT, R414Q, C420R, C420S, and G406R) in IVTs. In all experiments, FLAG-fusion proteins were immobilized on α FLAG affinity beads and used as baits to pull down the co-expressed HA-GATAD2B. As a negative control, wild-type (WT) HA-GATAD2B was added to beads to which no FLAG fusion protein had been immobilized. In each case, 10% of inputs and 50% of elutions were loaded on an SDS-PAGE and proteins were detected by Western blot, using α HA and/or α FLAG antibodies.

= degradation product of FLAG-MBD2.

Table 1.

Phenotypic Findings

Phenotypic Findings	Affected/Total (%)
Neonatal	
Polyhydramnios	19/45 (42.2)
Neonatal Feeding Issues	41/50 (82.0)
Infantile Hypotonia	50/50 (100)
Neurology	
Intellectual Disability/DD	50/50 (100)
Macrocephaly-Birth	26/43 (60.4)
Macrocephaly-Current	45/49 (91.8)
Seizures/Epilepsy	12/50 (24)
Abnormal MRI	27/45 (60)
Non-Verbal (<4 years)	5/15 (33.3) *
Non-Verbal (>4 years)	7/35 (20) *
Non-Ambulatory (<3 years)	3/9 (33.3) *
Non-Ambulatory (>3 years)	2/41 (4.9) *
Wide-based Gait	41/45 (91.1)
Orthotics	25/41 (61.0)
Deafness	1/50 (2)
Behavioral	
Good Eye Contact	49/50 (98)
Social Reciprocity	49/50 (98)
Not Toilet Trained (<4 years)	15/15 (100)
Not Toilet Trained (>4 years)	22/35 (62.9)
Cardiac	
Bicuspid Aortic Valve	5/50 (10)
Cardiac intervention	2/50 (4)
Ophthalmologic	
Strabismus	44/50 (88)
Anisocoria	7/46 (13.6)
Astigmatism	19/46 (41.3)
Hyperopia	14/46 (30.4)
Myopia	10/46 (21.7)
Optic Nerve Hypoplasia	4/44 (9.1)
Deafness	1/50 (2.0)
Dysmorphology	
Prominent Forehead	37/37 (100)
Hypertelorism	29/37 (78.4)

Phenotypic Findings	Affected/Total (%)
Prominent/Bulbous Nasal Tip	31/37 (83.8)
Pointed Chin	34/37 (91.9)

* specific ages of non-verbal and non-ambulatory subjects are noted in Table S4.

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