COLD SPRING HARBOR
Molecular Case Studies

RESEARCH REPORT

De novo variants in *EBF3* are associated with hypotonia, developmental delay, intellectual disability, and autism

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Abstract Using whole-exome sequencing, we identified seven unrelated individuals with global developmental delay, hypotonia, dysmorphic facial features, and an increased frequency of short stature, ataxia, and autism with de novo heterozygous frameshift, nonsense, splice, and missense variants in the *Early B-cell Transcription Factor Family Member 3* (*EBF3*) gene. EBF3 is a member of the collier/olfactory-1/early B-cell factor (COE) family of proteins, which are required for central nervous system (CNS) development. COE proteins are highly evolutionarily conserved and regulate neuronal specification, migration, axon guidance, and dendritogenesis during development and are essential for maintaining neuronal identity in adult neurons. Haploinsufficiency of *EBF3* may affect brain development and function, resulting in developmental delay, intellectual disability, and behavioral differences observed in individuals with a deleterious variant in *EBF3*.

[Supplemental material is available for this article.]

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Ontology terms: autism; central hypotonia; intellectual disability, mild; moderate global developmental delay; neurogenic bladder

Published by Cold Spring Harbor Laboratory Press

doi: 10.1101/mcs.a002097

INTRODUCTION

Early B-cell factor 3 (*EBF3*) is located on Chromosome 10q26.3 and is a member of the **c**ollier/olfactory-1/EBF (COE) family of transcription factors required for the development and differentiation of various cell types across species, from planarians to humans (Bu and Su 2003; Siponen et al. 2010; Cowles et al. 2014). The COE family of proteins has low sequence similarity to other protein families, but all COE proteins share a conserved amino-terminal DNA-binding domain with a unique zinc-finger binding motif, an immunogolubulin Ig-like, plexins, transcription factors (IPT/TIG) domain, and an atypical helix–loop–helix domain

with a dimerization motif (Fig. 1A; Liberg et al. 2002; Siponen et al. 2010). COE transcription factors play an important role in the nervous system and brain development, including neuronal differentiation, migration, axon guidance, and dendritogenesis, and are also necessary for the specification and maintenance of neuronal identity in adult neurons (Wang et al. 2004). Mice and humans have four COE paralogs, EBF1-4 (Daburon et al. 2008), which may have complementary and redundant roles (Dubois and Vincent 2001; Liberg et al. 2002). Among the COE paralogs, *EBF3* is highly expressed throughout the brain during development in mice and humans (Zhao et al. 2006; Tao et al. 2015) and is required for the migration of Cajal–Retzius cells during corticogenesis and the terminal differentiation and maintenance of specialized neurons in the adult brain (Dubois and Vincent 2001; Garcia-Dominguez et al. 2003; Thuret et al. 2004; Chiara et al. 2012). *EBF3* also acts as a putative tumor suppressor by activating cell cycle arrest and inducing apoptosis (Zhao et al. 2006).

Previous reports have identified approximately 100 patients with terminal 10q deletions, who demonstrate a wide range of clinical features frequently including delayed development, intellectual disability, and craniosynostosis (Faria et al. 2015). *EBF3* is within a shared deletion of Chromosome 10q26.3 in many of these patients. Four recent reports identified a total of 20 unrelated individuals and two siblings who have de novo variants in *EBF3* and a distinct neurodevelopmental syndrome (Blackburn et al. 2017; Chao et al. 2017; Harms et al. 2017; Sleven et al. 2017). Here we add further evidence for the role of *EBF3* in brain development and expand the phenotype of this syndrome caused by pathogenic variants in *EBF3*. We describe seven unrelated individuals who have heterozygous de novo variants in *EBF3* that are predicted to be deleterious and who share common features of global developmental delay, intellectual disability (ID), hypotonia, and dysmorphic features.

RESULTS

Clinical WES was performed on 8057 individuals with developmental delay and/or intellectual disabilities in a single clinical laboratory. In 11 affected individuals from 11 unrelated families, we identified de novo variants in *EBF3* as potentially causative for the neurodevelopmental phenotype. Candidate disease-causing variants in the *EBF3* gene were confirmed by Sanger sequencing to be de novo. Only seven of the 11 de novo predicted pathogenic variants we observed are reported in the manuscript based on permission of the referring physicians to report the results. Some of the patients who were diagnosed were included in other publications and were therefore not included in this series. The variants include one nonsense, two frameshift deletions, one splice, and three missense variants (Fig. 1). All seven variants identified are novel, located in highly evolutionarily conserved regions across species, and predicted to be deleterious by SIFT (http://sift.jcvi.org/), CADD (http://cadd.gs.washington.edu/), MetaSVM (https://omictools.com/meta-svm-tool), and MutationTaster (http://www.mutationtaster.org/) (Table 1; Fig. 1B). No likely gene-disrupting *EBF3* variants were detected in ExAC (http://exac.broadinstitute.org/), 1000 Genomes (http://www.internationalgenome.org/), ESP (http://evs.gs.washington.edu/EVS/), or our own internal database of 24,709 exomes of unaffected parents of children referred for testing. Additionally, the non-TCGA ExAC v0.3.1 gene tolerance scores of mis$Z$ = 4.89 and p(LI) = 1.00 indicate that the gene is highly constrained against both missense and loss-of-function (LOF) variation across humans, respectively. Using TADA (He et al. 2013) default parameters for ID/DD and the expected mutation rate of 2.331847×10^{-05} for missense variants and 8.693607×10^{-07} for LOF variants (Samocha et al. 2014), we calculate a false-discovery corrected q-value of 3.84×10^{-08} for observing six de novo LOF and five de novo missense variants in *EFB3* within our cohort.

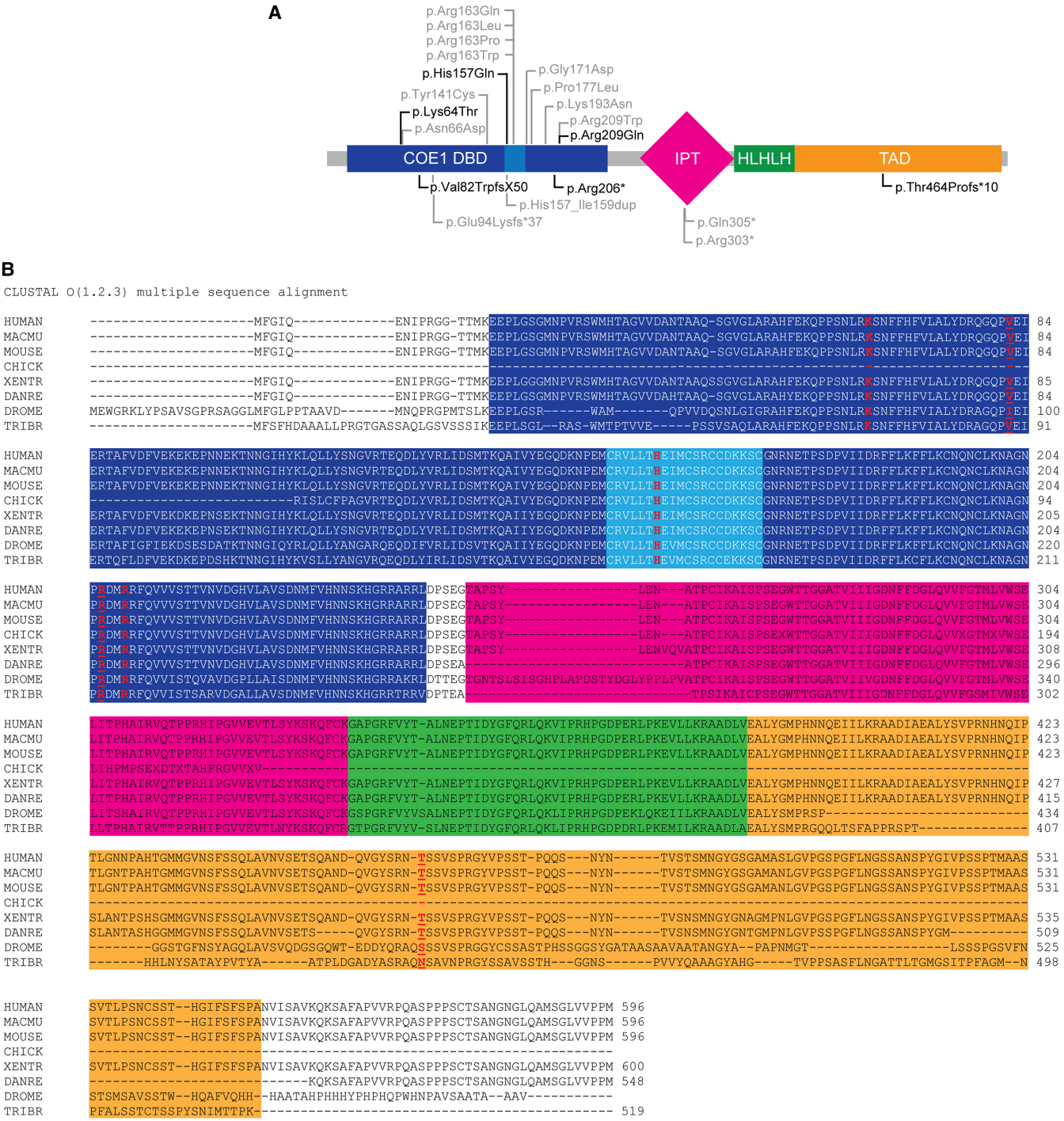

Figure 1. (*A*) De novo variants in *EBF3*. Missense variants in EBF3 (NP_001005463.1) are shown *above* the protein diagram and likely gene-disrupting mutations are *below*. The variants identified in our patients are in black and ones in individuals reported by others are in gray. COE1, collier/olfactory-1/EBF. DBD, DNA-binding domain. IPT, Ig-like, plexins, transcription factors domain. HLHLH, atypical helix–loop–helix (HLH) domain. TAD, transactivation domain. Light blue region in COE1 DBD represents unique zinc finger binding motif. (*B*) Sequence alignment of EBF3 and its homologs across species with residues mutated in our patients in red. Macmu, rhesus monkey, Xentr, *Xenopus tropicalis*, Danre, zebrafish, Drome, *Drosophila melanogaster*, Tribr, parasitic roundworm.

Table 1. Predicted pathogenicity and allele frequencies of *EBF3* variants

Patient	Chromosome 10 coordinates (GRCh37/hg19)	Nucleotide change	Amino acid change	SIFT	PROVEAN	PolyPhen2	Mutation-Taster	CADD Phred	Allele frequency in ExAC and GeneDx database of 49,418 alleles
1	131761731:T>G	c.191A>C	K64T	Damaging	Deleterious (−4.3)	Probably damaging (0.99)	Disease-causing	19.3	0
2	131761679:C>-	c.244delG	V82WfsX50	N/A	N/A	N/A	N/A	N/A	0
3	131757212:G>T	c.471C>A	H157Q	Damaging	Deleterious (−6.2)	Possibly damaging (0.9)	Disease-causing	18.3	0
4	131755591:C>T	c.486-1G>A	IVS5-1G>A	N/A	N/A	N/A	Disease-causing	32	0
5	131676052:G>A	c.616C>T	R206X	N/A	N/A	N/A	Automatic disease-causing	18.1	0
6	131676042:C>T	c.626G>A	R209Q	Damaging	Deleterious (−3.4)	Possibly damaging (0.9)	Disease-causing	20.4	0
7	131639239GTACTGCTGGGGA>-	c.1402_1414del13	T464PfsX10	N/A	N/A	N/A	N/A	N/A	0

Clinical Presentation and Family History

The seven unrelated individuals include five females and two males who range in age from 1 to 24 yr (see Table 2; Supplemental Table S1). They all share similar clinical features of global developmental delay or intellectual disabilities and of hypotonia or rarely hypertonia. Four of the five individuals over the age of three were verbal. Many of the individuals also demonstrated autism and/or attention deficit hyperactivity disorder (ADHD) and behavioral differences including poor eye contact, self-injurious behavior, or altered pain sensitivity. The only adult in our series also had tics and auditory and visual hallucinations. Notably, none had seizures nor were there consistent brain malformations, and head circumferences ranged from relatively small to relatively large. Most of the individuals have distinctive physical features including triangular face, synophrys, small ears, highly arched palate and crowded teeth, and single palmar crease (Fig. 2). All of our patients have moderate-to-severe neurodevelopmental impairment. Approximately half of the patients have autism and half of them have motor coordination problems including ataxia. Six of the seven individuals had neurological findings, including coordination issues, insensitivity to pain, muscle weakness, and ataxic gait. Abnormal brain MRIs were observed, including vermis hypoplasia, delayed myelination, or irregular frontal cerebral white matter and protuberance of optic papillae in three separate patients (Fig. 3). Additional notable features in the minority of individuals included altered pain sensitivity and sleep issues. Ophthalmologic problems were common and included strabismus, esotropia, amblyopia, and delayed visual maturation. Short stature was common and height was in the lower quartile for all but one individual. Skeletal abnormalities were less common and included pectus excavatum, severe scoliosis, pronation, and hallux valgus. Genitourinary issues included microphallus in two, urinary tract infections and hydronephrosis in one, and unilateral renal duplication, and vesicoureteral reflux in another.

Figure 2. Photographs of patients with de novo variants in EBF3. (*A*) Patient 4 at 11 yr old. Note triangular face. (*B*,*B*′) Patient 5 at 11 yr old. Note round, mildly coarse face, mild maxillary hypoplasia and prominent mandible, highly arched eyebrows and synophrys, long palpebral fissures, short philtrum and thin upper lip, and anteverted nares.

DISCUSSION

We add to the emerging evidence for a clinical syndrome characterized by global developmental delay/intellectual disability, autism, hypotonia, and dysmorphic features due to heterozygous de novo predicted pathogenic variants in *EBF3*. Although not observed in all patients, ataxia/coordination problems, strabismus, skeletal abnormalities, short stature, and urogenital anomalies are also common features. The allelic spectrum we observed includes several likely gene-disrupting variants: one nonsense, two frameshift, and one splice variant. In addition there are three de novo missense variants, (p.(Lys64Thr), p.(His157Gln), and p.(Arg209Gln), which are all in the COE1 DNA-binding domain (DBD) (Fig. 4; Siponen et al. 2010). The p.(His157Gln) mutation lies in the "EBF zinc knuckle" motif located in the COE1 DBD, which is crucial for zinc coordination and positioning of residues for DNA binding. The splice site variant, c.486-1G>A, may also cause the loss of the splice acceptor site and induce skipping of exon 6, which encodes the conserved zinc knuckle motif in the COE1 DBD. Molecular modeling of EBF3 mutations at the zinc knuckle has demonstrated decreased DNA affinity resulting in aberrant DNA binding (Blackburn et al. 2017). Altered expression or binding of *EBF3* is likely responsible for the predominance of neurological symptoms seen in our patients. Mutations in *Ebf3* in the brains of adult mice result in a modest reduction in olfactory bulb size and defective olfactory axon projections to the dorsal and lateral surfaces of the olfactory bulb (Wang et al. 2004). Jin et al. (2014) further determined

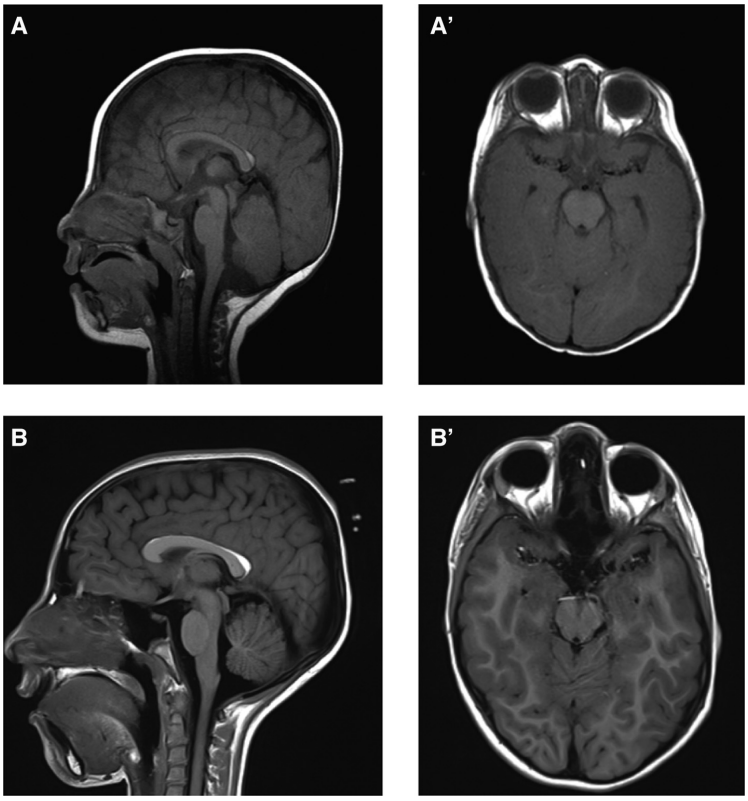

Figure 3. Brain imaging data of individual with EBF3 variant. (*A*,*B*) Sagittal and (*A*′,*B*′) axial images of Individual 4 at 9 mo (*A*,*A*′) and 9 yr (*B*,*B*′) of age show a mild hypoplasia of the cerebellar vermis, mildly dysplastic corpus callosum, and small pericallosal lipoma without evidence of significant progression.

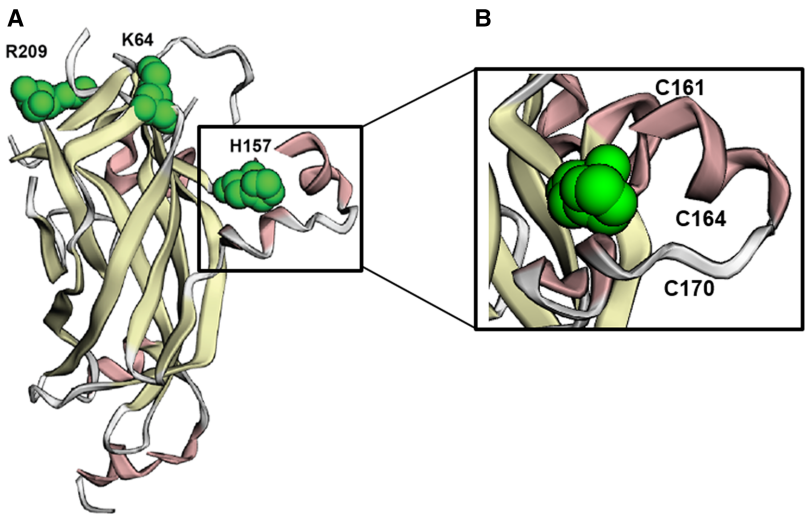

Figure 4. Deleterious de novo missense variants in the EBF DNA-binding domain (DBD). (*A*) Structural representation of the EBF DBD based on original 3D representation of EBF3 in Siponen et al. (2010) with the three residues affected by missense variants in our patients in green. The H157 residue is part of the EBF zinc knuckle (box), which coordinates EBF binding to DNA. (*B*) Top view of residues involved in zinc coordination are indicated.

that *Ebf3* is strongly expressed in the midbrain, cerebellum, and the mantle layer of the spinal cord and generated *Ebf3*-deficient mice that died from respiratory failure due to dysfunctional diaphragm relaxation within 12 h following birth (P0.5) (Jin et al. 2014). Detailed investigation of the brains of *Ebf3* mutant mice and the generation of conditional knockout mice will likely reveal additional functions for *EBF3* in regulating the genetic programs involved in developing and maintaining neuronal function. In humans, reports of deletions of a common 1-Mb region on 10q26.3 including *EBF3* have been associated with severe neurodevelopmental delay and intellectual disability (Faria et al. 2015), although additional genes in the interval including *MGMT*, *PPP2R2D*, and *BNIP3* may also contribute to the phenotype in 10q26.3 deletion patients.

Four groups independently reported a total of 22 unique individuals who have *EBF3* nonsense, missense, and splice variants with clinical features shared by our patients (Blackburn et al. 2017; Chao et al. 2017; Harms et al. 2017; Sleven et al. 2017), including one individual with a diagnosis of ADHD (Harms et al. 2017), three with autistic-like behavior (Sleven et al. 2017), and one with a family history of ADHD and sleep problems (Blackburn et al. 2017). Several of our patients exhibited behavioral differences including repetitive hair pulling, self-injurious face rubbing, and lack of eye contact or social smile. Three of the individuals with autism in our study also had abnormal brain imaging, including vermis hypoplasia, delayed myelination, and small structural defects in the right temporal lobe (Patient 5) and optic papillae (Patient 6) (Table 2; Supplemental Table S1). We provide additional evidence for the role of *EBF3* in brain development and hypothesize that decreased or aberrant binding of EBF3 to its targets lead to abnormal gene regulation during brain development and may be responsible for the global developmental delay and altered brain function consistently observed in individuals with mutations in *EBF3*. We suggest the variants we describe result in loss of function and haploinsufficiency of EBF3 and are a cause of autism and intellectual disabilities.

The missense variants identified in the individuals in this study are all amino acid substitutions that result in a change in charge, which may alter the DNA binding of EBF3 to its binding partners, including aristaless related homeobox (*ARX*) (Friocourt and Parnavelas 2011; Olivetti et al. 2014). Genes regulated by ARX include ones that are involved in or have been linked to CNS disorders with intellectual disability and autism (Fulp et al. 2008). *ARX* regulates the transcription of genes involved in cell differentiation, migration, and maturation during interneuron development in the brain and has been shown to repress *EBF3* and its putative tumor-suppressing properties, such as cell cycle arrest and apoptosis (Fulp et al. 2008; Olivetti et al. 2014). *ARX* is predominantly expressed in the fetal and adult brain, testis, skeletal muscle, and pancreas (Shoubridge et al. 2010; Uhlén et al. 2015). Patients with pathogenic variants in *ARX* exhibit heterogeneous clinical features resulting in a number of different developmental disorders including Ohtahara (OMIM ID: 308350), Partington (OMIM ID: 309510), and Proud (OMIM ID: 300004) syndromes, X-linked lissencephaly and ambiguous genitalia (XLAG; OMIM ID: 3000215), X-linked infantile spasms syndrome (ISSX; OMIM ID: 308350), and syndromic and nonsyndromic mental retardation (Shoubridge et al. 2010; Olivetti and Noebels 2012).

The phenotypes and clinical outcomes of individuals with *ARX*-related disorders resemble those with mutations in *EBF3*, and disruptions in both genes are associated with intellectual disability with various neurological symptoms and are often associated with urogenital malformations. Recent reports of individuals with mutations in *EBF3* have documented genitourinary abnormalities, including several cases of undescended testes as well as micropenis and hypospadias (Blackburn et al. 2017; Chao et al. 2017; Harms et al. 2017; Sleven et al. 2017). The male individuals in our study with missense variants, p.Lys64Thr (Patient 1) and p.Arg209Gln (Patient 6), each had a small penis and undescended testes perhaps because of aberrant binding of ARX to *EBF3*, which could result in the disruption of genitourinary

Table 2. Clinical details of patients with *EBF3* variants

Patient	Age	Sex	Variant	Age at sitting	Age at walking	Verbal skills	Muscle tone	Additional neurological findings	Vision	Seizure	Abnormal behavior	ADD/ ADHD	Brain MRI	Renal or urogenital issues	GI
1	30 mo	M	c.191A>C p. Lys64Thr;	18 mo	Cannot walk on own, able to stand without support	Nonverbal; able to communicate with sign language	Hypotonia	Coordination issues; insensitivity/ decreased sensitivity to pain; fine and gross motor difficulties	N/A	None	Moderate eye contact; did not smile until 11 mo old	N/A	N/A	Small penis	History of poor weight gain
2	24 yo	F	c.244delG, p.Val82TrpfsX50	9 mo	2 yo	Talked at 9 mo; Full sentences, normal mild articulation problems	Hypotonia	None	Strabismus, surgically repaired	None	Autistic features; tics; hallucinations (auditory and visual); emotional fragility; self-injurious behavior; hair pulling	No	Normal	Normal	Normal
3	10 yo	F	c.471C>A, p.His157Gln	1 yo	3 yo	Talked at 5 yo; normal vocabulary for age with articulation differences	Hypotonia	Fine motor difficulties; decreased pain sensitivity	Strabismus	None	Autism; tantrums and melt-downs	Hyperactivity	Normal	Vesicostomy surgery for vesicoureteral reflux and poor bladder emptying; left renal duplication; urine retention with neurogenic bladder, needs nocturnal catheter	Normal
4	11 yo	F	c.486-1G>A, IVS5-1G>A	N/A	Cannot walk on own, needs assistance or walker	Nonverbal, no words but hums tunes	Hypotonia	Muscle weakness; ataxic gait	Strabismus, surgically repaired	None	Autistic features; rubs face on hard objects to point of bruising	No	Vermis hypoplasia	Normal	Constipation; requires G-tube feeds; takes only baby foods orally

(*Continued on next page.*)

Table 2. (*Continued*)

Patient	Age	Sex	Variant	Age at sitting	Age at walking	Verbal skills	Muscle tone	Additional neurological findings	Vision	Seizure	Abnormal behavior	ADD/ ADHD	Brain MRI	Renal or urogenital issues	GI
5	11 yo	F	c.616C>T, p.Arg206X	4 mo	14 mo; Wears leg braces	Talked at 10 mo; Articulation defect	Hypotonia	Hyporeflexia or difficult to elicit DTRs; mild dysmetria and gait incoordination; inconsistent sensory exam, mild proximal muscle weakness	Refractive error	None	Autism; oppositional defiant disorder	ADHD	Small schizencephalic cleft in right temporal lobe and delayed myelination at 2 yo, later resolved	History of UTIs and hydronephrosis, resolved with growth	Excess weight gain, BMI 33.5 (≫95th percentile)
6	15 mo	M	c.626G>A p.Arg209Gln	9 mo	Cannot walk at 12 mo	Babbling at 8 mo; No clear first words at 12 mo	Hypotonia	Muscle weakness; ataxic gait	Strabismus	None	Some eye contact, but no social smile	N/A	Curvilinear tract-like signal abnormality in left frontal cerebral white matter; protuberance of right optic papillae	Small penis; testes high in scrotum	Normal
7	4 yo	F	c.1402_1414del13, p.Thr464ProfsX10	8 mo	2 yo	Talked at 2 yo; Speaking in full sentences at 4 yo	Hypertonia in hips	Muscle weakness; ataxic gait	Normal	None	Oral fixation, awakens at night	No	Normal	Recurrent urinary tract infections	Normal

See Supplemental Table S1 for additional details of clinical presentations.
yo, years old; ADD, attention deficit disorder; ADHD attention deficit hyperactivity disorder; N/A, not available.

patterning during development. It is thus possible that dysregulation of *EBF3* caused by defective binding by ARX may be part of the mechanism leading to the phenotype observed in these patients. Additional studies of *EBF3* are required to elucidate the molecular mechanisms of EBF3-associated neurodevelopmental deficiency and genitourinary abnormalities.

METHODS

Studies were approved by the Institutional Review Board of Columbia University.

Whole-Exome Sequencing (WES)

Genomic DNA was extracted from whole blood from the affected children and their parents. Exome sequencing at GeneDx was performed on exon targets captured using the Agilent SureSelect Human All Exon V4 (50 Mb) or the Clinical Research Exome kit (Agilent Technologies) according to the manufacturer's instructions. Libraries were sequenced using the Illumina HiSeq 2000 or 2500 sequencing system with 100-bp paired-end reads (Illumina). Whole-exome sequence data for all sequenced family members was analyzed using GeneDx's XomeAnalyzer (a variant annotation, filtering, and viewing interface for WES data) as described previously (Table 3; Tanaka et al. 2016). Identified sequence variants of interest were confirmed in each proband and both parents by conventional di-deoxy DNA sequence analysis using an ABI3730 (Life Technologies).

EBF3 Primer Sequences

Patient 1: Forward ACAGTGTCCAGGAACACGTGA, Reverse CAGGAGGTCTGACCAA GGGC

Patient 2: Forward CATGTTTGGGATTCAGGAGAA, Reverse CCTGCCTCCCGCTTCTA

Patient 3: Forward TAGCCCAGCTCCGAGGGTGA, Reverse CAATCGATGCCCTTCCCGGA

Patient 4: Forward ACCGCTTCATTGTCAGACGT, Reverse TTTTGTTGCTGCTGCGGTTT (This primer set has been discontinued and is not recommended.)

Patient 5: Forward TCTGATAACCCTAATAAATATCAT, Reverse GATTACTTCCTGAACA GTTGC

Patient 6: Forward TCTGATAACCCTAATAAATATCAT, Reverse GATTACTTCCTGAACA GTTGC

Patient 7: Forward GGCGCTAGAGCAGGTGGAAA, Reverse GAGATCACATCGGGCCCGTT

Table 3. Sequencing results

Patient	WES 10× cov	Mean cov	Kit	EBF3 10× cov	EBF3 mean CDS cov
1	97.32%	153	Agilent CRE	100.00%	137
2	92.88%	47	Agilent SSv4	96.77%	42
3	96.36%	142	Agilent SSv4	100.00%	118
4	91.52%	103	Agilent SSv4	100.00%	98
5	97.00%	113	Agilent CRE	100.00%	101
6	94.85%	113	Agilent CRE	100.00%	125
7	94.69%	69	Agilent SSv4	98.03%	48
Mean	94.95%	105	N/A	99.26%	95

Results from individuals identified at GeneDx. cov, coverage; CDS, coding sequence.

ADDITIONAL INFORMATION

Data Deposition and Access

Whole-exome sequencing data are not publicly available because patient consent could not be obtained. The *EBF3* variants found in this study have been deposited in ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) under accession numbers SCV000584020.1–SCV000584025.1 and SCV000570295.2.

Ethics Statement

The study was approved by the Institutional Review Board of Columbia University. Written informed consent was obtained from the probands or probands' parents for publication and accompanying images.

Acknowledgments

We thank the patients and their families for their generous participation. The Simons Foundation provided funding to W.K.C.

Author Contributions

A.J.T. and M.T.C. analyzed the data and drafted and critically reviewed the manuscript. R.W. and M.J.G.S. analyzed the data and critically reviewed the manuscript. K.R. generated and analyzed the data and critically reviewed the manuscript. Y.A.Z., K.B., V.S., K.O., A.W., G.N.W., A.B., T.B.-D., L.O., S.S., M.K.G., I.J.A., and R.E.S. provided the clinical data and critically reviewed the manuscript. W.K.C. conceived of the study, analyzed the data, and drafted and clinically reviewed the manuscript.

Competing Interest Statement

M.C., R.W., K.R., M.G.S., and R.E.S. are employees of GeneDx. W.C. is a former employee of GeneDx and a member of the Scientific Advisory Board of Regeneron Genetics Center.

Received May 2, 2017; accepted in revised form July 5, 2017.

Funding

This work was supported in part by a grant from the Simons Foundation.

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