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Supplemental Data

A Syndromic Neurodevelopmental Disorder Caused by De Novo Variants in *EBF3*

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SUPPLEMENTAL NOTE

Proband #1 NIH Undiagnosed Diseases Program - *de novo EBF3* chr10: 131755588C>T (hg19): (NM_001005463.2: c.488G>A): p.Arg163Gln (R163Q)

Proband 1 is a seven-year old Pacific Islander male of Chinese and Japanese descent with expressive speech delay, mild dysmorphic facial features, hypotonia, global developmental delay, and genital hypoplasia.

His prenatal history was significant for maternal age of 33-years old and paternal age of 41-years old. Pregnancy was complicated by borderline hypertension and gestational diabetes, but responded to diet management. Mother reported that fetal movement was decreased compared to her previous pregnancy. There were no maternal exposures to drugs, alcohol, or trauma. Birth history was significant for delivery at 38-weeks gestation by repeat Caesarean section that was complicated by a loose nuchal cord wrapped once around the neck and a fractured clavicle. Birth weight: 7 lbs, 9 ozs; birth length: 20.5 inches; OFC: 36.2, Apgar scores: 7, 8.

Newborn exam was significant for micropenis and bilateral undescended testes. He was discharged in on day three of life. However, during the first 24-48 hours of life, he was reported to have episodes of choking, back arching, and a dusky appearance during breastfeeding that was followed by a period of decreased alertness. MRI brain was unremarkable for age per report. Sepsis evaluation was unrevealing. These episodes of choking and back arching during breastfeeding were attributed to gastro-esophageal reflux. His first-week of life was significant for reportedly absent crying and generalized hypotonia.

His first-year of life was notable for dysphagia, hypotonia, strabismus, and developmental delay. At nine-months of age he was only able to roll over and sit with support.

Therapies were initiated. By one-year of age, he achieved head control and dysphagia improved. An endocrine evaluation at this time confirmed testicular failure and he underwent bilateral orchiopexy. Repeat MRI of the brain at one-year of age was significant for mild prominence of the ventricles and sulci per report.

Genetics evaluation at 2-years of age was significant for dysmorphic facial features including myopathic facies with oval-shaped face, over-folding of the superior helices, bilateral epicanthal folds, short anterverted nostrils, and downturned corners of the mouth. He also had noticeable expressive language delay, generalized hypotonia, strabismus, and microphallus. Targeted biochemical and genetic testing was negative for Allan-Herndon-Dudley, Mowat-Wilson, Prader-Willi, and Rett syndromes. A complete metabolic evaluation was unremarkable.

At 7-years of age notable physical exam findings included astigmatism and hyperopia without retinal or optic nerve abnormalities, isolated right peroneal neuropathy of uncertain significance, and wide-based ataxic gait. Neuropyschiatric testing was significant for moderate fine motor delay and significant expressive, greater than receptive, language delays. His overall speech was limited verbally to approximately two words and approximately 100 signs. However, non-verbal receptive and comprehensive skills tested close to normal. Repeat MRI of the brain revealed bilateral small inferior posterior cerebellar lobes and posterior vermian hypoplasia with mildly abnormal ventricles and sulci.

Proband #2 Texas Children's Hospital - *de novo EBF3* chr10: 131755588C>T (hg19): (NM_001005463.2: c.488G>A): p.Arg163Gln (R163Q)

Proband 2 is a five-year old African-American female with expressive speech delay, mild dysmorphic facial features, hypotonia, and global developmental delay.

Her prenatal history was significant for maternal age of 43-years old and paternal age of 47-years old. Pregnancy was uncomplicated, but mother reports reduced fetal movements compared to her previous pregnancy. There were no maternal exposures to drugs, alcohol, or trauma. An amniocentesis was performed due to advanced maternal age and the results were normal. At 40-weeks gestation, oligohydramnios was noted on ultrasound but was absent on prior ultrasounds. Birth history was significant for delivery at 40-weeks gestation by induced vaginal delivery that was uncomplicated. Birth weight: 7 lbs, 6 ozs; birth length: 20 inches; OFC: 33.5 cm, Apgar scores: 8/8.

Newborn exam was unremarkable except for some noted compression of the left ear and left toes consistent with oligohydramnios. She was discharged in the first week of life.

Her first-year of life was notable for hypotonia, dysphagia, strabismus, and developmental delay. Hypotonia was noted within the first three months of life due to delayed head control and she continued to have poor head control at 9-10 months of age. She also had significant difficulty with control of oral secretions and was unable to swallow solid foods at 12-months of age. Therapies were initiated at 9-10 months of age.

Gross motor delays included the inability to sit without support until 12-months old, inability to roll over until 10-11 months old, and did not crawl independently until 18-months old. She achieved independent ambulation at 30-months old but continued to have a wide-based ataxic gait with frequent falls. Fine motor delays included the acquisition of raking grasp at 11-months old, pincer grasp at 30 months old, at 4-years of age she could assist with dressing

herself, and at 5-years of age she remains ambidextrous. Language delays included babbling at 11-months of age, first word at 11-months of age, and 2-3 word sentences at 4-years of age. Now at 5-years of age she is able to speak in short sentences with apraxic speech. Additionally, at 5-years of age she continues to have difficulty controlling her oral secretions and achieved bowel-control but not bladder-control. Her social behavior is noteworthy at 5-years of age for a short attention span, very curious and interactive, overly sociable with behavioral perseveration, and decreased awareness of boundaries or consequences.

Interestingly, she is reported to exhibit decreased pain sensitivity based absent signs of discomfort with vaccinations or falls until around 2-years of age and at 5-years of age is still described by mother as "tough to pain". She is also noted to have significantly decreased spontaneous facial expressions since birth that was most notably described as the inability to smile with emotion or on command. Physical exam at 5-years of age was notable for mild dysmorphic features including prominent forehead, triangular facies, facial hypotonia, overfolding of superior helices, epicanthus inversus, and abnormal palmar creases. Mild hypoplasia of the labia majora was noted.

Brain MRI was significant for a cleft in the superior cerebellar vermis, and reduced size of the middle cerebellar vermis. Cerebellar hemispheres were unremarkable.

Proband #3 NYU Langone Medical Center- *de novo EBF3* chr10: 131755588C>A (hg19): (NM_001005463.2: c.488G>T): p.Arg163Leu (R163L)

Proband 3 is a 3-year old Caucasian female of English, Irish, German, and Polish descent withexpressive speech delay, mild dysmorphic facial features, hypotonia, and global developmental delay.

Her prenatal history was significant for maternal age of 32-years old and paternal age of 31-years old. Pregnancy was uncomplicated and no invasive prenatal tests were performed. There were no reports of decreased fetal movements. There were no maternal exposures to drugs, alcohol, or trauma. Birth history was significant for delivery at 39-weeks gestation by Caesarean section due to breech position. Birth weight: 2.7 kg; Apgar scores: 7-9. Newborn exam was normal. The proband was discharged home within the first week of life.

Her first-year of life was notable for dysphagia, hypotonia, strabismus, and developmental delay. Occupational therapy was started at 3-weeks of age due to deficient latching and torticollis. At 3-months old she was diagnosed with gastroesophageal reflux (GERD) by upper gastrointestinal imaging series following an apparent life-threatening event and was treated with omeprazole. Developmental evaluation at 5-months of age was notable for significant hypotonia and global developmental delays. Gross motor delays include rolling over from prone to supine at 3-months of age but unable to roll over from supine to prone until 10-months of age. She was able to sit without support at 11-months of age and was able to put herself into a seated position at 13-months of age. By 12-months of age, she was commando crawling but did not pull to stand or cruise. Now at 30-months of age, she is ambulating independently with a wide-based waddling ataxic gait and occasionally drags her feet when fatigued. Language delays included babbling with consonant sounds but no words at 13-months of age. She has near normal receptive language with minimal expressive language (only has one or two words). She occasionally has flat affect with decreased facial expressions. Notably, parents report that she appears to exhibit increased tolerance to pain.

Clinical examination at 13-months old revealed head circumference at 20^{th}-%ile for length, 10^{th}-%ile for weight, difficulty controlling oral secretions, and torticollis. Heart murmur

was not heard. Spleen was not palpable, but liver was palpable. Mild dysmorphic features include a prominent occiput, triangular facies, metopic ridge, low set ears, down-slanting palpebral fissures, hypertelorism, beaked nose, facial hypotonia, small umbilical hernia, inverted nipples, small hemangioma on the back, tapered fingers, and small feet. She had stereotypic motor movements including bilateral writhing or piano tapping finger movements as well as simultaneous asynchronous knee and hip flexion with kicking movements of both feet. The stereotypies decreased in frequency at severity by 30-months of age.

At 2-years of age she had placement of myringotomy tubes due to conductive hearing loss after chronic otitis media with effusion. She also had recurrent urinary tract infections (UTI) and was diagnosed with urinary retention associated with incomplete bladder emptying and vesicoureteral reflux grade I. She was treated with Macrodantin for one year and had no further UTIs. At 30-months of age the GERD has resolved and torticollis is minimal.

Due to the motor stereotypies MRI of the brain and 24-hour ambulatory EEG studies were obtained at 13-months of age, both studies were unremarkable and appropriate for age. Chromosomal microarray, urine organic acids, plasma amino acids, carnitine levels, creatine kinase, acylcarnitine profile, Angelman/Prader Willi DNA methylation test and Pompe disease testing were unrevealing. Pelvic X-rays were normal. Follow-up MRI of the brain was unremarkable.

Table S1: Clinical Features of *EBF3* Probands

	Proband 1	**Proband 2**	**Proband 3**
Variant	Chr10: 131755588C>T (hg19): NM_001005463.2 c.488G>A p.Arg163Gln *de novo*	Chr10: 131755588C>T (hg19): NM_001005463.2 c.488G>A p.Arg163Gln *de novo*	Chr10: 131755588C>A (hg19) NM_001005463.2 c.488G>T p.Arg163Leu *de novo*
Age (evaluation)	7 year 4 month	5 years	3 year
Gender	Male	Female	Female
Ethnicity	Chinese and Japanese	African-American	English, Irish, German, and Polish
Prenatal findings	Reduced fetal movements	Oligohydramnios, reduced fetal movements	None
Birth growth parameters	Weight 7lbs 9oz Length 20.5 inches OFC 36.2	Weight 7lbs 9oz Length 20 inches OFC 33.5 cm	Weight 2.7 kg
Growth parameters at last exam	HT 109.7 cm (25-%ile) WT19.5 kg (25-%ile) OFC 52.5 (50-75-%ile)	WT 17.5 kg (41-%ile) HT 114.8 cm (91-%ile) OFC 51 cm (85-%ile)	WT 10-%ile HT 10-%ile OFC 20-%ile
Hypotonia	Resolved to proximal weakness about age 4 ½ years	Present since at least 3 months	Present since at least 3 months
Coordination	Ataxic wide based gait	Ataxic wide based gait, dysmetria	Ataxic wide based gait
Development	Expressive language and Fine motor delays (testing= 5 years, 10 months)	Global developmental delay	Global developmental delay
Speech delay	Severe expressive, 2-3 single words	Expressive delay, apraxic speech with short sentences at 5-years of age	Severe expressive, 1 word
Seizures	None	None	None
Ophthalmologic	Strabismus	Strabismus, surgical correction of extraocular movement dysfunction	Strabismus, surgical correction of extraocular movement dysfunction
Facial features	Oval shaped face, overfolding of superior helices, short anterverted nostrils, downturned corners of the mouth, hockey stick palmar creases	triangular shaped face, facial hypotonia, overfolding of superior helices, epicanthus inversus, abnormal palmar creases	Prominent occiput, triangular facies, metopic ridge, low set ears, down-slanting palpebral fissures, hypertelorism, beaked nose, facial hypotonia, small umbilical hernia, inverted nipples, small hemangioma on back, tapered fingers and small feet.
Facial Weakness	Present	Present	Present
Behavior	Stereotypic behaviors reported in the past. Full criteria were not formally evaluated.	Atypical social interactions – persistent eye gaze, perseveration, occasional flat affect	Motor stereotypies, occasional flat affect
Pain Insensitivity	None	Present	Present
MRI	Mild prominence of ventricles and sulci, decreased cerebellar hemisphere volume, vermian hypoplasia	Vermian hypoplasia	Normal
GU	Micropenis, testicular failure	Mild underdevelopment of the labia majora	Grade 1 reflux

Table S2: *EBF3* Variant Prediction Scores

	Proband 1+2	**Proband 3**
Variant	Chr10: 131755588C>T (hg19): NM_001005463.2 c.488G>A p.Arg163Gln *de novo*	Chr10: 131755588C>A (hg19) NM_001005463.2 c.488G>T p.Arg163Leu *de novo*
SIFT (Score)	0.01	0
SIFT (prediction)	Deleterious	Deleterious
PolyPhen-2 (Score)	0.994	0.993
PolyPhen(Prediction)	Probably Damaging	Probably damaging
Mutation Taster (Prediction)	Disease Causing	Disease Causing

Table S3: Sequencing Methods

	Proband 1	Proband 2	Proband 3
Sequencing laboratory	NIH Intramural Sequencing Center (NISC)	Baylor Genetics Laboratory	Baylor Genetics Laboratory
Sequencing type	Quad exome sequencing (Sanger sequencing of *EBF3* in proband, parents, and unaffected sibling)	Proband exome sequencing (Sanger sequencing of *EBF3* in proband and parents) (https://www.bcm.edu/research/medical-genetics-labs/index.cfm?PMID=21319)[1]	Proband exome sequencing (Sanger sequencing of *EBF3* in proband and parents) (https://www.bcm.edu/research/medical-genetics-labs/index.cfm?PMID=21319)[1]
Capture and library construction	Illumina TrueSeq capture kit	Biotin-labeled VCRome 2.1 in-solution Exome probes	Biotin-labeled VCRome 2.1 in-solution Exome probes
Sequencing platform	Illumina HiSeq2000	Illumina HiSeq2000	Illumina HiSeq2000
Sequence data aligned to human reference genome (hg19)	Novoaling (Novocraft Technologies, Selangor, Malaysia)	Human Genome Sequencing Center Mercury analysis pipeline (http://www.tinyurl.com/HGSC-Mercury)	Human Genome Sequencing Center Mercury analysis pipeline (http://www.tinyurl.com/HGSC-Mercury)
Analysis, sorting, and filtering of variants	Variants were analyzed, sorted and filtered with VarSifter and a graphical java tool to view, sort, and filter variants[2]. Variants were filtered based on allele frequencies in the NIH-UDP cohort[3-5] (<0.06) and variants were prioritized using CADD[6] and Exomiser[7]. Primers GAAACCAAGCAAGGCAAAAC and AATTCTCCAAACTGCCTTGG were used for the amplification of the region of genomic DNA around the mutation in *EBF3* (NM_001005463 c.488G>A; CADD 28.4). Sanger dideoxy sequencing of the PCR products was performed by Macrogen (Rockville, MD, USA). The sequences were aligned and analyzed using Sequencher v.5.0.1 (Gene Codes, Ann Arbor, MI, USA). Mutation interpretation analysis was conducted using Alamut 2.0 (Interactive Biosoftware, San Diego, CA, USA).	Variants were determined and called using the Atlas2 suite to produce a variant call file[8]. For the population comparisons we utilized data from the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [November 2015] and Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [November 2015].	Variants were determined and called using the Atlas2 suite to produce a variant call file[8]. For the population comparisons we utilized data from the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [November 2015] and Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [November 2015].

Table S4: Fly Genotypes Used in Study and Maintained at 21°C

Genotype	Allele	Source	Reference
w; kn^{col-1}/CyO*	Amorphic	Drosophila Genomics and Genetic Resources	http://www.ncbi.nlm.nih.gov/pubmed/10477305
kn^{1}/SM6a	Hypomorphic	Drosophila Genomics and Genetic Resources	http://www.ncbi.nlm.nih.gov/pubmed/10375526
w^{1118}; Df(2R)BSC429/CyO	Genomic deficiency allele including *knot* locus	Bloomington Drosophila Stock Center	http://www.ncbi.nlm.nih.gov/pubmed/22445104
M[UAS-kn.ORF.3xHA.GW]ZH-86Fb	Wildtype fly allele	FlyORF	http://www.ncbi.nlm.nih.gov/pubmed/24922270
y[1] w*; Mi{MIC}knot[MI15480]/SM6a	MiMIC	Bloomington Drosophila Stock Center	http://www.ncbi.nlm.nih.gov/pubmed/21985007
y[1] w; Mi{Trojan-GAL4.2}kn[MI15480-TG4.2]/SM6a*	T2A-GAL4	Bellen Lab	Line MI15480 was converted to *kn-T2A-GAL4* via recombinase mediated cassette exchange as previously described[9, 10].
y[1] w; M[UAS-EBF3.ORF.3xHA.GW]ZH-86Fb/TM3, Sb Ser*	UAS	Bellen Lab	Transgenic flies were generated by ϕC31-mediated transgenesis with the pUASattB vector and integrated into the same *86Fb* (chromosome 3) docking sites to minimize position effects on transgene expression[11-13].
y[1] w; M[UAS-EBF3-p.Arg163Gln.ORF.3xHA.GW]ZH-86Fb/TM3, Sb Ser*	UAS	Bellen Lab	Transgenic flies were generated by ϕC31-mediated transgenesis with the pUASattB vector and integrated into the same *86Fb* (chromosome 3) docking sites to minimize position effects on transgene expression[11-13].
y[1] w; M[UAS-EBF3-p.Arg163Leu.ORF.3xHA.GW]ZH-86Fb/TM3, Sb Ser*	UAS	Bellen Lab	Transgenic flies were generated by ϕC31-mediated transgenesis and with the pUASattB vector integrated into the same *86Fb* (chromosome 3) docking sites to minimize position effects on transgene expression[11-13].

Table S5: Antibodies Used in Study

Antibody	Dilution	Source	Reference
rat anti-Elav-7E8A10	1:50	Developmental Studies Hybridoma Bank	http://www.ncbi.nlm.nih.gov/pubmed/8033205
Mouse anti-HA	1:200	Covance (Catalog #MMS-101P)	n/a
DAPI	1:100	Thermo-Fisher (Catalog #D1306)	n/a
Donkey anti-rat Alexa 647	1:300	Jackson ImmunoResearch (Catalog #712-605-153)	n/a
Donkey anti-mouse Cy3	1:300	Jackson ImmunoResearch (Catalog #715-165-150)	n/a

Table S6: Oligonucleotide Pairs Used in Study

Purpose	Forward or Reverse	Sequence
Site directed mutagenesis for EBF3 p.Arg163Gln	Forward	5'-CACGAGATCATGTGCAGTCAATGCTGTGACAAGAAAAGTTG-3'
Site directed mutagenesis for EBF3 p.Arg163Gln	Reverse	5'-AACTTTTCTTGTCACAGCATTGACTGCACATGATCTCGTGG-3'
Site directed mutagenesis for EBF3 p.Arg163Leu	Forward	5'-ATGTGCAGCCTGTGCTGTGAC-3'
Site directed mutagenesis for EBF3 p.Arg163Leu	Reverse	5'-GATCTCGTGGGTCAGCAG-3'
Concatamerized COE binding sequence	Forward	5'-CTAGCTCTCAGGATTCCCCAGGGAGGGGACACTCTCAGGATTCCCCA GGGAGGGGACACTCTCAGGATTCCCCAGGGAGGGGACACCTAGGC-3'
Concatamerized COE binding sequence	Reverse	5'-TCGAGCCTAGGTGTCCCCTCCCTGGGGAATCCTGAGAGTGTCCCC TCCCTGGGGAATCCTGAGAGTGTCCCCTCCCTGGGGAATCCTGAGAG-3'
Site directed mutagenesis for EBF3 deletion of the Zn^{2+} finger COE motif (ΔCOE)	Forward	5'-GGCAATAGAAACGAAACGCCC-3'
Site directed mutagenesis for EBF3 deletion of the Zn^{2+} finger COE motif (ΔCOE)	Reverse	5'-GGTCAGCAGCACACGGCA-3'

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