De Novo Truncating Variants in *ASXL2* Are Associated with a Unique and Recognizable Clinical Phenotype

Vandana Shashi,[1,37,*] Loren D.M. Pena,[1,37] Katherine Kim,[2] Barbara Burton,[2] Maja Hempel,[3] Kelly Schoch,[1] Magdalena Walkiewicz,[4] Heather M. McLaughlin,[5] Megan Cho,[5] Nicholas Stong,[6] Scott E. Hickey,[7] Christine M. Shuss,[7] Undiagnosed Diseases Network,[8] Michael S. Freemark,[9] Jane S. Bellet,[10] Martha Ann Keels,[11] Melanie J. Bonner,[12] Maysantoine El-Dairi,[13] Megan Butler,[14] Peter G. Kranz,[15] Constance T.R.M. Stumpel,[16] Sylvia Klinkenberg,[17] Karin Oberndorff,[18] Malik Alawi,[19,20,21] Rene Santer,[22] Slavé Petrovski,[6,23] Outi Kuismin,[24,25,26] Satu Korpi-Heikkilä,[27] Olli Pietilainen,[28,29] Palotie Aarno,[26,28,30,31,32,33] Mitja I. Kurki,[31,32,34] Alexander Hoischen,[35] Anna C. Need,[36] David B. Goldstein,[6] and Fanny Kortüm[3]

The *ASXL* genes (*ASXL1*, *ASXL2*, and *ASXL3*) participate in body patterning during embryogenesis and encode proteins involved in epigenetic regulation and assembly of transcription factors to specific genomic loci. Germline de novo truncating variants in *ASXL1* and *ASXL3* have been respectively implicated in causing Bohring-Opitz and Bainbridge-Ropers syndromes, which result in overlapping features of severe intellectual disability and dysmorphic features. *ASXL2* has not yet been associated with a human Mendelian disorder. In this study, we performed whole-exome sequencing in six unrelated probands with developmental delay, macrocephaly, and dysmorphic features. All six had de novo truncating variants in *ASXL2*. A careful review enabled the recognition of a specific phenotype consisting of macrocephaly, prominent eyes, arched eyebrows, hypertelorism, a glabellar nevus flammeus, neonatal feeding difficulties, hypotonia, and developmental disabilities. Although overlapping features with Bohring-Opitz and Bainbridge-Ropers syndromes exist, features that distinguish the *ASXL2*-associated condition from *ASXL1*- and *ASXL3*-related disorders are macrocephaly, absence of growth retardation, and more variability in the degree of intellectual disabilities. We were also able to demonstrate with mRNA studies that these variants are likely to exert a dominant-negative effect, given that both alleles are expressed in blood and the mutated *ASXL2* transcripts escape nonsense-mediated decay. In conclusion, de novo truncating variants in *ASXL2* underlie a neurodevelopmental syndrome with a clinically recognizable phenotype. This report expands the germline disorders that are linked to the *ASXL* genes.

The three additional sex-combs-like genes (*ASXL1* [MIM: 612990], *ASXL2* [MIM: 612991], and *ASXL3* [MIM: 615115]) code for polycomb proteins that act as histone methyltransferases,[1] serve as epigenetic scaffolding proteins, and are involved in body patterning.[2] Somatic mutations in all three *ASXL* genes occur across a range of malignancies.[3] *Drosophila Asxl* genes are involved in homeotic gene activation and silencing.[4,5] In mice, *Asxl2* has been reported to regulate skeletal, lipid, and glucose homeostasis and cardiac development.[6,7] Additionally, *Asxl1* and *Asxl2* fine-tune adipogenesis in mice, whereby *Asxl2* promotes adipogenesis and *Asxl1* inhibits it.[8,9] Mice with homozygous *Asxl2* knockout demonstrate premature death, growth retardation, impaired cardiac

[1]Division of Medical Genetics, Department of Pediatrics, Duke Health, Durham, NC 27710, USA; [2]Department of Pediatrics, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL 60611, USA; [3]Institute of Human Genetics, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; [4]Baylor College of Medicine, Houston, TX 77030, USA; [5]GeneDx, Gaithersburg, MD 20877, USA; [6]Institute for Genomic Medicine, Columbia University, New York, NY 10032, USA; [7]Division of Molecular and Human Genetics, Nationwide Children's Hospital, Columbus, OH 43082, USA; [8]NIH Common Fund, Bethesda, MD 20892, USA; [9]Division of Endocrinology and Diabetes, Department of Pediatrics, Duke Health, Durham, NC 27710, USA; [10]Departments of Pediatrics and Dermatology, Duke Health, Durham, NC 27710, USA; [11]Departments of Pediatrics and Surgery, Duke Health, Durham, NC 27710, USA; [12]Psychiatry and Behavioral Sciences, Duke Health, Durham, NC 27710, USA; [13]Duke Eye Center, Duke Health, Durham, NC 27710, USA; [14]Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, Duke Health, Durham, NC 27710, USA; [15]Division of Neuroradiology, Department of Radiology, Duke Health, Durham, NC 27710, USA; [16]Department of Clinical Genetics and School for Oncology & Developmental Biology, Maastricht University Medical Center, 6202 AZ Maastricht, the Netherlands; [17]Department of Neurology, Maastricht University Medical Center, 6202 AZ Maastricht, the Netherlands; [18]Department of Pediatrics, Zuyderland Medical Center, 6162 BG Sittard, the Netherlands; [19]Bioinformatics Service Facility, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; [20]Center for Bioinformatics, University of Hamburg, 20246 Hamburg, Germany; [21]Heinrich-Pette-Institute, Leibniz-Institute for Experimental Virology, Virus Genomics, 20246 Hamburg, Germany; [22]Department of Paediatrics, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; [23]Department of Medicine, Austin Health and Royal Melbourne Hospital, University of Melbourne, Melbourne, VIC 3050, Australia; [24]PEDEGO Research Unit, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, 90014 Oulu, Finland; [25]Department of Clinical Genetics, Oulu University Hospital, 90029 Oulu, Finland; [26]Institute for Molecular Medicine Finland, University of Helsinki, 00290 Helsinki, Finland; [27]Northern Ostrobothnia Hospital District, Center for Intellectual Disability Care, 90220 Oulu, Finland; [28]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA; [29]Harvard Stem Cell Institute, Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA; [30]Analytic and Translational Genetics Unit, Department of Medicine, Massachusetts General Hospital, Boston, MA 02114, USA; [31]Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA; [32]Psychiatric and Neurodevelopmental Genetics Unit, Department of Psychiatry, Massachusetts General Hospital, Boston, MA 02114, USA; [33]Department of Neurology, Massachusetts General Hospital, Boston, MA USA, 02114; [34]Genetic Analysis Platform, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA; [35]Department of Human Genetics, Donders Centre for Neuroscience, Radboud University Medical Center, 6500 HB Nijmegen, the Netherlands; [36]Division of Brain Sciences, Department of Medicine, Imperial College London, London W12 0NN, UK
[37]These authors contributed equally to this work
*Correspondence: vandana.shashi@duke.edu
http://dx.doi.org/10.1016/j.ajhg.2016.08.017.

function, and vertebral abnormalities, indicating that this gene is required for embryonic and postnatal development.[5]

Germline mutations in *ASXL1* and *ASXL3* have been associated with specific genetic syndromes.[10] Truncating variants in *ASXL1* cause Bohring-Opitz syndrome (MIM: 605039), a severe disorder with growth retardation, microcephaly, profound intellectual disability, nevus flammeus of the face, flexion of the elbows and wrists, and ulnar deviation of the hands.[11,12] *ASXL3* germline truncating variants are associated with Bainbridge-Ropers syndrome (MIM: 615485), characterized by severe intellectual disability, growth retardation, and clinical features overlapping those of Bohring-Opitz syndrome.[13] Variants in *ASXL3* have also been reported in autism spectrum disorder.[14] In contrast, *ASXL2* (MIM: 612991) has thus far not been implicated in human Mendelian disease. One individual with a t(2;9) translocation resulting in a fused transcript of *ASXL2* and *KIAA1803* had a complex phenotype of agenesis of the corpus callosum, ocular colobomas, and periventricular heterotopias,[15] but the relative contributions of the two genes to this individual's phenotype are unclear. Similarly, DECIPHER lists five individuals with cytogenetic deletions encompassing *ASXL2* and other genes (n = 35–141) and phenotypes including developmental delays or intellectual disabilities, among other manifestations (Table S1). Because of the contiguous deletion of several other genes, the specific contribution of the *ASXL2* deletion to these phenotypes cannot be determined. Also included is an individual with a single de novo base-pair deletion causing head, neck, nervous, skeletal, skin, and respiratory system abnormalities, and potential overlapping manifestations are detailed in our individuals.

In this study, we present six unrelated individuals ranging from 11 months to 31 years of age with de novo heterozygous truncating *ASXL2* variants detected by whole-exome sequencing (WES). All individuals share overlapping clinical features including developmental or intellectual impairments, macrocephaly, distinct facial dysmorphisms, facial nevus flammeus, feeding difficulties in the newborn period, and hypotonia (Table 1 and Figure 1). Detailed clinical summaries are available in the Supplemental Note. Five of the individuals (individuals 1–3, 5, and 6) and their biological parents underwent trio WES, whereas individual 4 was sequenced alone, followed by Sanger sequencing of *ASXL2* on the child and the biological parents. WES was performed after written informed consent was obtained through approval by institutional review boards and ethics committees. Experienced pediatricians and geneticists clinically assessed the individuals.

For all six individuals, DNA was extracted from maternal, paternal, and proband blood samples. The exome was captured with biotin-labeled VCRome 2.1 in-solution exome probes (individuals 1 and 4), the Agilent Clinical Research Exome Kit (individual 2), the Nextera Rapid Capture Exome Kit (individuals 3 and 6), or Agilent SureSelect v.4 (individual 5), and the exomes were sequenced on an Illumina HiSeq 2000 (individuals 2 and 5) or 2500 (individuals 1, 3, 4, and 6). Two paired-end 100 bp reads were used for the exome-capture sequencing. For individual 3, Trimmomatic[16] was employed to remove adapters and low-quality (Phred quality score < 5) bases from the 3′ ends of sequence reads. Reads shorter than 36 bp were subsequently removed. The sequencing methodology, further processing, and variant-interpretation protocols have been described previously.[17–19,20,21] Functional annotation and alteration filtering in all cases were performed against public databases (the NHLBI Exome Sequencing Project [ESP] Exome Variant Server, dbSNP138, 1000 Genomes, and Exome Aggregation Consortium [ExAC] Browser; see Web Resources). Quality metrics for all WES results are provided in Table S2. GeneMatcher, a web-based tool for researchers and clinicians working on identical genes, connected the investigators from the different institutions.[22]

Processing of the whole-exome data on the six unrelated individuals with overlapping clinical features (Figure 1 and Table 1) and the healthy parents of five of them took into account X-linked, autosomal-recessive, and autosomal-dominant inheritance models to identify genes with functionally relevant variants (new, clinically associated, or of low or unknown frequency) (Table S3). In the five trios, we identified one to two putatively de novo variants not present in any variant database (Table S3). De novo variants (frameshift and stop gain) in *ASXL2* were detected among all individuals (Table 1): c.2424delC (p.Thr809Profs*32) in individual 1, c.2081dupG (p.Gly696Argfs*11) in individual 2, c.1225_1228delCCAA (p.Pro409Asnfs*13) in individual 3, c.2472delC (p.Ser825Valfs*16) in individual 4, c.2971_2974delGGAG (p.Gly991Argfs*3) in individual 5, and c.1288G>T (p.Glu430*) in individual 6 (mutation designations refer to transcript GenBank: NM_018263.4, NCBI Genome build GRCh37 [individuals 1, 4, and 6], and UCSC Genome Browser build hg19 [individuals 2, 3, and 5]). All *ASXL2* variants were validated by Sanger sequencing, and the de novo origin was confirmed by parental-segregation studies (Figure S2A). Interestingly, all identified variants locate to the penultimate or last exon of *ASXL2* (Figure 2).

Examination of the ExAC Browser (release 0.3) for loss-of-function (LoF) variants that passed the browser's variant-quality thresholds revealed two distinct frameshift, two distinct nonsense, and two distinct canonical splice variants, all with only a single carrier (c.2002A>T [p.Lys668*], c.1973dupC [p.Ala658Glyfs*49], c.1895dupA [p.His632Serfs*17], c.1400C>A [p.Ser467*], c.505−1G>C, and c.940−2A>G). Except for c.505−1G>C and c.940−2A>G, these variants are located in a region between the variants found in individuals 2 and 6, upstream of the variants in individuals 1, 4, and 5, and downstream of the variant found in individual 3 (Figure 2). The annotation of these LoF variants in the ExAC Browser prompted us to calculate the probability of finding *ASXL2* variants by chance in exomes for neurodevelopmental

Table 1. Clinical Features of Six Individuals with *ASXL2* Variants in Comparison to Those of *ASXL1*- and *ASXL3*-Associated Disorders

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Bohring-Opitz Syndrome	Bainbridge-Ropers Syndrome
Gene	*ASXL2*	*ASXL2*	*ASXL2*	*ASXL2*	*ASXL2*	*ASXL2*	*ASXL1*	*ASXL3*
Mutation[a]	c.2424delC (p.Thr809Profs*32)	c.2081dupG (p.Gly696Argfs*11)	c.1225_1228delCCAA (p.Pro409Asnfs*13)	c.2472delC (p.Ser825Valfs*16)	c.2971_2974delGGAG (p.Gly991Argfs*3)	c.1288G>T (p.Glu430*)	truncating	truncating
Prenatal findings	none	none	none	none	left renal agenesis, cerebral ventriculomegaly, intrauterine growth restriction	none	IUGR, polyhydramnios	IUGR
Growth parameters at birth	height and weight > 97th percentile, OFC = 92nd percentile	height, weight, and OFC > 97th percentile	height = 39th percentile, length > 97th percentile, OFC = 91st percentile	weight = 35th percentile (length and OFC not available)	weight = 2nd percentile, length = 23rd percentile, (OFC not available)	weight = 30th percentile, length = 60th percentile, OFC = 70th percentile	SGA	SGA
Growth parameters at last examination	at 8 years: height, weight, and OFC = 100th percentile (macrocephaly)	at 10 months: normal height and weight, OFC = 96th percentile (macrocephaly)	at 5 years: normal height and weight, OFC > 97th percentile (macrocephaly)	at 4 years: normal height and weight, OFC > 97th percentile (macrocephaly)	at 7 years 10 months: height = 99th percentile, weight = 92nd percentile, OFC = 99th percentile (macrocephaly)	at 12.8 years: weight = 88th percentile, height = 1st percentile, OFC = 80th percentile (relative macrocephaly)	severe growth retardation, microcephaly	severe growth retardation, microcephaly
Feeding difficulties	present only shortly after birth	present	present only in neonatal period	present only shortly after birth	present for several weeks after birth	present only shortly after birth	present (persistent)	present (severe)
Hypotonia	present	present (persistent)	present, but hypertonia in limbs	present	present	present	present	present
DD and/or ID	low average cognition	moderate	severe	severe	borderline	moderate	severe	severe
Seizures	febrile	none	febrile and non-febrile	febrile	suspected	present	present	NA
Facial features	hypertelorism, arched eyebrows, long face, prominent eyes, ptosis of eyelids, epicanthal folds, broad nasal tip	hypertelorism, arched eyebrows, proptosis, epicanthal folds, long eyelashes, synophrys, broad nasal tip	hypertelorism, arched eyebrows, prominent eyes, ptosis of eyelids, epicanthal folds, prominent glabella, synophrys, small upper vermilion, broad nasal tip	hypertelorism, arched eyebrows, long face, proptosis, small mouth	hypertelorism, arched eyebrows, prominent eyes, long eyelashes, small upper vermilion, broad nasal tip	hypertelorism, malar hypoplasia, low nasal bridge, short philtrum, ptosis, broad nasal tip, high narrow palate	trigonocephaly, hypertelorism, prominent forehead, long face, micrognathia, prominent eyes, upslanting palpebral fissures	high and broad forehead, anteverted nares, hypertelorism
Ears	low-set, cupped, overfolded	posteriorly rotated	posteriorly rotated	posteriorly rotated	low-set, posteriorly rotated	thick ear lobes	low-set, posteriorly rotated	low-set, posteriorly rotated

(*Continued on next page*)

Table 1. ***Continued***

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Bohring-Opitz Syndrome	Bainbridge-Ropers Syndrome
Skin	glabellar nevus flammeus; capillary malformations on trunk, neck, and behind ears; deep palmar creases	glabellar nevus flammeus, deep palmar creases	glabellar nevus flammeus, capillary malformations on back and neck, deep palmar creases, hypertrichosis	glabellar nevus flammeus, normal palmar creases	glabellar nevus flammeus, fetal fingertip pads	glabellar nevus flammeus, hypertrichosis, deep palmar and plantar creases	glabellar nevus flammeus, capillary malformations on philtrum and neck, deep palmar creases, hypertrichosis	deep palmar creases, hypertrichosis
Episodes of hypoglycemia	none known	has required continuous feeding since neonatal period	episodic, starting at 2.5 years of age	none known	none known	present during neonatal period	NA	NA
Congenital heart disease	ASD, PDA	ASD, left ventricular dysfunction	ASD	none known	none known	thickened pulmonary valve	ASD, VSD	PDA, pulmonary artery stenosis
Skeletal and/or extremity manifestations	increased density of alveolar bone, advanced bone age	kyphosis	scoliosis	multiple bilateral fractures, overlapping second and fourth toes	none known	advanced bone age, thick calvarium, fusion of second and third cervical vertebrae, short metacarpals and distal phalanges	scoliosis, ulnar deviation of hands, elbow and wrist flexion, scoliosis	ulnar deviation of hands, clenched hands
Brain MRI	white-matter volume loss	increased extra-axial cerebral space, choroid plexus papilloma	increased extra-axial cerebral space	increased extra-axial cerebral space	white-matter volume loss, ventriculomegaly	normal	agenesis of corpus callosum, Dandy-Walker malformation	white-matter volume loss, Dandy-Walker malformation

Abbreviations are as follows: ASD, atrial septal defect; DD, developmental delay; ID, intellectual disability; IUGR, intrauterine growth retardation; NA, not available; OFC, occipitofrontal head circumference; PDA, patent ductus arteriosus; SGA, small for gestational age; and VSD, ventricular septal defect.
[a]Mutation designations refer to transcript GenBank: NM_018263.4, NCBI Genome build GRCh37 (individuals 1, 4, and 6), and UCSC Genome Browser build hg19 (individuals 2, 3, and 5).

Figure 1. Clinical Photographs of Four Individuals with De Novo *ASXL2* Mutations
(A–C) Individual 1 at ages 3 years (A) and 8.5 years (B and C). Epicanthal folds with a wide nasal bridge, arched eyebrows, ptosis of the eyelids, prominent eyes, hypertelorism, a broad nasal tip, and a V-shaped glabellar nevus flammeus are evident, along with a capillary malformation on the neck and shoulder (C).
(D–F) Individual 2 at ages 6 weeks (D), 5 months (E), and 10 months (F). Note the large glabellar nevus flammeus, thick and arched eyebrows with synophrys, proptosis of the eyes, hypertelorism, epicanthal folds, broad nasal tip, and retrognathia.
(G–I) Individual 3 presented at the ages of 10 months (G) and 4 years (H [frontal view] and I [lateral view]) with a flat face, broad forehead, prominent glabella, glabellar nevus flammeus, hypertelorism, synophrys, arched eyebrows, ptosis, downslanting palpebral fissures, broad nasal tip, long philtrum, small upper vermilion, and small mouth.
(J–L) Individual 4 after birth (J) and at ages 20 months (K) and 3 years (L) presented with proptosis, a small mouth, arched eyebrows, and a glabellar nevus flammeus.
(M–O) Individual 5 at ages 3 months (M) and 7 years 10 months (N and O). Note the glabellar nevus flammeus, prominent eyes, hypertelorism, arched eyebrows, and broad nasal tip.
(P–R) Individual 6 at ages 6 years (P) and 16 years (Q and R). Note the hypertelorism, likely macrocephaly, a broad nasal tip, and a glabellar nevus flammeus.

phenotypes: the six *ASXL2* truncating variants in our individuals occur in a collection of 12,030 subjects ascertained for neurological and neurodevelopmental disorders (GeneDx = 3,677; University Medical Center Hamburg = 123; Baylor = 6,198; Radboud University Medical Center = 1,866; and Broad Institute = 166). We used the CRAN function denovolyzeR[23] package with the parameters set for six truncating variants in *ASXL2* and estimated that the probability of the chance occurrence of the six de novo *ASXL2* mutations among 12,030 individuals is 1.47e−10. Correcting for the 18,668 protein-coding genes present in the consensus coding sequence (CCDS release 14) shows that this observation is significant genome-wide (2.744196e−6). Conversely, for the probability of finding six *ASXL2* truncating variants in the ExAC Browser (60,000 samples), we found a p value of 1.79e−6, which translates to 0.03341572 when corrected for 18,668 genes. These data indicate that there are fewer than expected truncating *ASXL2* variants within the control database as well. *ASXL2* is also highly intolerant of LoF variants (probability of 0.99) according to the ExAC Browser pLI calculation.[24] The ExAC Browser endeavors not to include subjects with severe pediatric disease but is enriched with adults with heart and metabolic diseases, cancer, schizophrenia, and Tourette syndrome.[24] Interestingly, truncating variants in ExAC Browser control individuals have been reported for both *ASXL1* and *ASXL3*; these overlap the disease-associated variants occurring within the last two exons of both genes.[13,25] Similarly, other genes involved in intellectual disability, such as *ARID1B*, have also been found to have truncating variants in ExAC Browser control individuals.[25] The exact explanation for these ExAC Browser variants that overlap the disease-associated variants for the three *ASXL* genes remains unclear, but possibilities include somatic mosaicism, variable expressivity depending on the location of the variants in the gene, or reduced penetrance. The finding that clonal hematopoiesis in healthy subjects results in acquired somatic variants in genes (including *ASXL1*) that are also mutated in myeloid cancers supports the possibility that

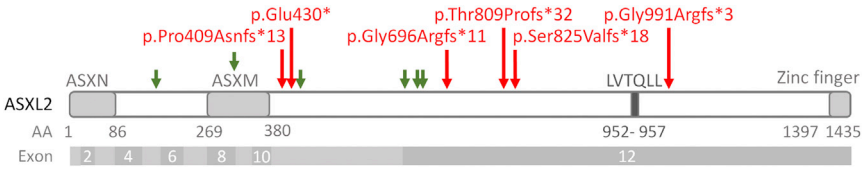

Figure 2. Schematic Structure of ASXL2
The ASXN, ASXM, and PHD-type zinc-finger domains, which are conserved throughout the ASXL family, are represented as light-gray boxes, and the LVTQLL motif, a nuclear receptor binding motif, is indicated in dark gray. The amino acid (AA) positions of all domains and motifs, as well as a schematic representation of the exons encoding the above depicted part of the protein, are given underneath. The positions of the de novo mutations identified in this study are marked with vertical red arrows. Small green arrows point to positions of known heterozygous variants (stop gain, frameshift, and splice site) found in apparently healthy individuals (from the ExAC Browser).

the variants in the ExAC Browser could be somatic rather than germline.[24] Options such as examining read counts to determine whether a variant could be postzygotic are subject to errors due to sequencing factors such as variability in capture, and so at this time, definite inferences cannot be made about the origin of these variants. Non-penetrance would be a less likely explanation, given the severity of the germline *ASXL* disorders, but a recent report has confirmed that incomplete penetrance of Mendelian diseases is likely to be more common than realized.[26] Future studies on all three *ASXL* genes are necessary for better understanding the overlap between truncating variants in control and affected individuals.

As for *ASXL1* and *ASXL3*, we initially postulated that haploinsufficiency would be responsible for the *ASXL2* phenotype; alternatively, a dominant-negative mechanism could cause disease. When truncating variants preferentially accumulate toward the end of a gene, the resulting transcript could escape nonsense-mediated decay (NMD) and interfere with the wild-type protein, causing an abnormal phenotype. The truncating variants in our six affected individuals are within the last two exons of *ASXL2*, similar to the distribution of disease-causing variants in *ASXL1* and *ASXL3*.[11,13,25] To get an idea of whether haploinsufficiency or a dominant-negative effect could be the underlying mechanism of disease, we performed cDNA studies. Fresh venous blood samples of individuals 1, 3, and 4 were deposited in PAXgene Blood RNA Tubes (PreAnalytiX, QIAGEN), and total RNA was extracted with the PAXgene Blood RNA Kit (QIAGEN) in accordance with the manufacturer's instructions. 1 μg of RNA from each individual was reverse transcribed into cDNA (Superscript III, Invitrogen) with the use of random hexanucleotides (Invitrogen) according to the manufacturer's protocol. PCR amplification for allele expression analysis of *ASXL2* was carried out with the Advantage cDNA PCR Kit (Clontech, Takara Bio) and primers spanning exon-exon boundaries of *ASXL2*. The primers used are as follows: ASXL2_RT_11for (5′-CGACAAGAGATTGAGAAG GAG-3′) in exon 11 and ASXL2_RT_12rev (5′-CCAT CAGCTGCACAATGAA-3′) in exon 12 for individuals 1 and 4 and ASXL2_RT_10for (5′-TGGAAAGAACAATTCTTT GAAAG-3′) in exon 10 and ASXL2_RT_11rev (5′-CACTCT GACTGGGAGACTACTG-3′) in exon 11 for individual 3. Three temperature "touchdown" PCRs were performed with an initial annealing temperature of 60°C, which was decreased twice by 2°C after three cycles each and then maintained at 56°C for 29 more cycles. Each cycle consisted of a denaturation step for 30 s at 94°C, an annealing step for 30 s, and a 1 min, 68°C elongation. ASXL2_ RT_12seq (5′-TCATAGTGTCTCTGGA-3′) was used for sequencing the PCR products of individuals 1 and 4.

We found that both wild-type and mutant transcripts were detectable at equal levels in all investigated individuals (Figure S2B). This suggests that at least for the three analyzed *ASXL2* mutations, the mRNA containing the respective mutations is unlikely to undergo NMD, thus providing support for a dominant-negative rather than haploinsufficient mechanism of disease. Similarly, the disease mechanism with *ASXL1* and *ASXL3* needs further clarification. Bainbridge et al. demonstrated that in a cell line of an individual with an *ASXL3* truncating variant, both alleles were expressed, suggesting that the truncating variant escaped NMD.[13] In contrast, others have demonstrated that transcripts from truncating variants in *ASXL3* were indeed subject to NMD, making a dominant-negative effect less likely. Nonetheless, because NMD is a variable process depending on the tissue and time in development, this remains a possibility in *ASXL*-related disorders.[27] Additional evidence for a dominant-negative or gain-of-function effect of truncating *ASXL1* variants in cancers[28] emphasizes the importance of further functional studies across all *ASXL* genes for elucidating mechanisms of disease.

The recognizable pattern of features and the corresponding Human Phenotype Ontology (HPO) terms seen in our individuals include macrocephaly (HP: 0000256), a typical facial appearance consisting of glabellar nevus flammeus (HP: 0001052), hypertelorism (HP: 0000316), arched eyebrows (HP: 0002553), prominent eyes, a broad nasal tip (HP: 0000455), and minor ear abnormalities. Minor congenital heart disease (HP: 0030680), neonatal feeding difficulties (HP: 0011968), hypotonia (HP: 0001252), seizures (HP: 0001250), and brain MRI findings suggestive of cerebral atrophy (Figure S1), as well as developmental delays (HP: 0001263) and intellectual disabilities (HP: 0001249) ranging from low average cognition to severe impairments, are also consistently seen. Less consistent features include multiple capillary malformations and skeletal findings. Persistent hypoglycemia occurred in individuals 2 and 3. The cause of hypoglycemia in both cases is not clear, but the observation of macrosomia at birth and variably in later life, the inappropriately low free fatty acids and ketones, and the high insulin concentration in

individual 3 on one occasion all suggest that an impaired regulation of insulin production or a disturbed peripheral effect on adipose tissue cells could play a role.

Germline damaging variants in *ASXL1* and *ASXL3* cause Bohring-Opitz and Bainbridge-Ropers syndromes, respectively.[11,13] Overlapping features between our individuals and those with these disorders include developmental delays, arched eyebrows, feeding difficulties, and prominent eyes. A glabellar nevus flammeus is a striking feature in our six individuals and is seen in the majority of individuals with Bohring-Opitz syndrome, but not in Bainbridge-Ropers syndrome. Features that distinguish our individuals from those with these disorders include macrocephaly, normal height and weight, the absence of a characteristic positioning of the hands, and more variability in the degree of cognitive impairments. The overlap between the manifestations in the three different disorders could be due to the similarities in function between the members of the *ASXL* family of genes. Conversely, the distinctive features of the *ASXL2*-associated disorder could be attributed to the differences between the function of *ASXL2* and those of the other two *ASXL* genes.[8] Overall, the presence of a glabellar nevus flammeus with developmental disabilities should prompt consideration of an *ASXL*-related disorder.

ASXL proteins function as tumor suppressors or oncogenes, and truncating somatic variants of all three *ASXL* genes are found in many malignancies.[3,29] Interestingly, Wilms tumor and nephroblastomatosis have been described in individuals with Bohring-Opitz syndrome.[30,31] Thus, surveillance for Wilms tumor has been recommended for individuals with Bohring-Opitz syndrome.[30] It is unknown at this time whether germline damaging variants in *ASXL2* would confer a risk of cancer; although none of our individuals has had a malignancy, follow-up clinical surveillance would be important.

In *Asxl2*$^{-/-}$ mice, fetuses have reduced body weight, skeletal anomalies, and cardiac dysfunction with enlarged hearts and premature death.[5] *ASXL2* is also ubiquitously expressed in the brain of mouse embryos[5] and is reported to regulate bone mineral density and be responsible for the genesis of osteoclasts, as well as for bone resorption.[6,32] *Asxl2*$^{-/-}$ mice have been variably reported as having decreased bone density or osteopetrotic bone. Interestingly, individual 1 in our series has been noted to have increased alveolar bone density, a highly unusual dental finding. Individual 4 has decreased bone density with fractures, and individual 6 has mild osteoporosis. It is unclear at this point whether mineralization could be abnormal in other individuals with this *ASXL2*-related disorder; more clarity on this should become available with more reports of individuals with *ASXL2* truncating variants.

ASXL2 plays an important role in heart morphogenesis and the transition from fetal to postnatal circulation.[33] *Asxl2*$^{-/-}$ mice display congenital heart malformations including a thickened compact myocardium in the left ventricle, membranous ventricular septal defects, and dilated cardiomyopathy.[5] Four of the six individuals in our report have minor congenital heart disease, suggesting that cardiac malformations could be common in this condition.

In conclusion, we have identified a syndrome characterized by developmental disabilities, macrocephaly, and dysmorphic features attributable to germline truncating variants in *ASXL2*. Further clinical reports of individuals with damaging *ASXL2* variants and related clinical features, as well as functional investigations, will reveal the full phenotype of this syndrome.

Supplemental Data

Supplemental Data include a Supplemental Note, two figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.08.017.

Consortia

The members of the Undiagnosed Diseases Network are Carlos A. Bacino, Brendan H. Lee, Ashok Balasubramanyam, Lindsay C. Burrage, Gary D. Clark, William J. Craigen, Shweta U. Dhar, Lisa T. Emrick, Brett H. Graham, Mahim Jain, Seema R. Lalani, Richard A. Lewis, Paolo M. Moretti, Sarah K. Nicholas, Jordan S. Orange, Jennifer E. Posey, Lorraine Potocki, Jill A. Rosenfeld, Daryl A. Scott, Neil A. Hanchard, Tran A. Alyssa, Alejandro E. Mercedes, Azamian S. Mashid, Hugo J. Bellen, Shinya Yamamoto, Michael F. Wangler, Monte Westerfield, John H. Postlethwait, Christine M. Eng, Yaping Yang, Donna M. Muzny, Patricia A. Ward, Rachel B. Ramoni, Alexa T. McCray, Issac S. Kohane, Ingrid A. Holm, Matthew Might, Paul Mazur, Kimberly Splinter, Cecilia Esteves, Vandana Shashi, Yonghui Jiang, Loren D.M. Pena, Allyn McConkie-Rosell, Kelly Schoch, Rebecca C. Spillmann, Jennifer A. Sullivan, Nicole M. Walley, David B. Goldstein, Nicholas Stong, Alan H. Beggs, Joseph Loscalzo, Calum A. MacRae, Edwin K. Silverman, Joan M. Stoler, David A. Sweetser, Richard L. Maas, Joel B. Krier, Lance H. Rodan, Chris A. Walsh, Cynthia M. Cooper, Juan C. Pallais, Laurel A. Donnell-Fink, Elizabeth L. Krieg, Sharyn A. Lincoln, Lauren C. Briere, Howard J. Jacob, Elizabeth A. Worthey, Joe Lazar, Kim A. Strong, Lori H. Handley, J. Scott Newberry, David P. Bick, Molly C. Schroeder, Donna M. Brown, Camille L. Birch, Shawn E. Levy, Braden E. Boone, Dan C. Dorset, Angela L. Jones, Teri A. Manolio, John J. Mulvihill, Anastasia L. Wise, Jyoti G. Dayal, David J. Eckstein, Donna M. Krasnewich, Carson R. Loomis, Laura A. Mamounas, Brenda Iglesias, Casey Martin, David M. Koeller, Thomas O. Metz, Euan A. Ashley, Paul G. Fisher, Jonathan A. Bernstein, Matt T. Wheeler, Patricia A. Zornio, Daryl M. Waggott, Annika M. Dries, Jennefer N. Kohler, Katrina M. Dipple, Stan F. Nelson, Christina G.S. Palmer, Eric Vilain, Patrick Allard, Esteban C. Dell Angelica, Hane Lee, Janet S. Sinsheimer, Jeanette C. Papp, Naghmeh Dorrani, Matthew R. Herzog, Hayk Barseghyan, David R. Adams, Christopher J. Adams, Elizabeth A. Burke, Katherine R. Chao, Mariska Davids, David D. Draper, Tyra Estwick, Trevor S. Frisby, Kate Frost, William A. Gahl, Valerie Gartner, Rena A. Godfrey, Mitchell Goheen, Gretchen A. Golas, Mary G. Gordon, Catherine A. Groden, Andrea L. Gropman, Mary E. Hackbarth, Isabel Hardee, Jean M. Johnston, Alanna E. Koehler, Lea Latham, Yvonne L. Latour, Chyau Yueh C. Lau, Paul R. Lee, Denise J. Levy, Adam P. Liebendorder, Ellen F. Macnamara, Valerie V. Maduro, May V. Malicdan, Thomas C. Markello, Alexandra J. McCarty, Jennifer L. Murphy, Michele E. Nehrebecky, Donna Novacic, Barbara N. Pusey, Sarah Sadozai, Katherine E. Schaffer, Prashant Sharma, Ariane

G. Soldatos, Sara P. Thomas, Cynthia J. Tifft, Nathanial J. Tolman, Camilo Toro, Zaheer M. Valivullah, Colleen E. Wahl, Mike Warburton, Alec A. Weech, Lynne A. Wolfe, Guoyun Yu, Rizwan Hamid, John H. Newman, John A. Phillips, and Joy D. Cogan.

Acknowledgments

We are grateful to the inidividuals and their families who contributed to this study and allowed us to publish their information and pictures. We thank I. Jantke for skillful technical assistance. This work was supported by the Undiagnosed Diseases Network (1U01HG007672-01 to V.S.) and the Deutsche Forschungsgemeinschaft (KO 4576/1-1 to F.K.). The authors disclose the following: the Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from molecular testing offered at the Baylor Genetics Laboratories (M.W.); D.B.G. has received consultancy fees and has equity ownership in Pairnomix LLC; H.M.M. and M.C. are employees of GeneDx; B.B. has received honoraria, consulting fees, or funding for clinical trials from Sanofi- Genzyme, Shire, Biomarin, Ultragenyx, Alexion, Armagen, and ReGenX Bio; and E.A.A. from the Undiagnosed Diseases Network is a co-founder of Personalis Inc.

Received: May 2, 2016
Accepted: August 24, 2016
Published: September 29, 2016; corrected online: December 15, 2016

Web Resources

1000 Genomes Project, http://www.1000genomes.org/
Combined Annotation Dependent Depletion (CADD), http://cadd.gs.washington.edu/score
CRAN packages, https://www.r-project.org/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
DECIPHER, https://decipher.sanger.ac.uk
Ensembl Variant Effect Predictor release 83, http://ensembl.org/info/docs/tools/vep/index.html
Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org/
Human Phenotype Ontology (HPO) Browser, http://www.human-phenotype-ontology.org/hpoweb?id=HP:0000118
LOFTEE, https://github.com/konradjk/loftee/
MutationTaster, http://www.mutationtaster.org/
NCBI Genome, http://www.ncbi.nlm.nih.gov/genome/guide/human/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RefSeq, http://www.ncbi.nlm.nih.gov/refseq/
SIFT, http://sift.jcvi.org/www/SIFT_enst_submit.html
Trimmomatic, http://www.usadellab.org/cms/?page=trimmomatic
UCSC Genome Browser, http://genome.ucsc.edu

References

1. Fisher, C.L., Berger, J., Randazzo, F., and Brock, H.W. (2003). A human homolog of Additional sex combs, ADDITIONAL SEX COMBS-LIKE 1, maps to chromosome 20q11. Gene *306*, 115–126.
2. Katoh, M. (2015). Functional proteomics of the epigenetic regulators ASXL1, ASXL2 and ASXL3: a convergence of proteomics and epigenetics for translational medicine. Expert Rev. Proteomics *12*, 317–328.
3. Katoh, M. (2013). Functional and cancer genomics of ASXL family members. Br. J. Cancer *109*, 299–306.
4. Katoh, M., and Katoh, M. (2004). Identification and characterization of ASXL3 gene in silico. Int. J. Oncol. *24*, 1617–1622.
5. Baskind, H.A., Na, L., Ma, Q., Patel, M.P., Geenen, D.L., and Wang, Q.T. (2009). Functional conservation of Asxl2, a murine homolog for the Drosophila enhancer of trithorax and polycomb group gene Asx. PLoS ONE *4*, e4750.
6. Izawa, T., Rohatgi, N., Fukunaga, T., Wang, Q.T., Silva, M.J., Gardner, M.J., McDaniel, M.L., Abumrad, N.A., Semenkovich, C.F., Teitelbaum, S.L., and Zou, W. (2015). ASXL2 Regulates Glucose, Lipid, and Skeletal Homeostasis. Cell Rep. *11*, 1625–1637.
7. Khan, F.F., Li, Y., Balyan, A., and Wang, Q.T. (2014). WTIP interacts with ASXL2 and blocks ASXL2-mediated activation of retinoic acid signaling. Biochem. Biophys. Res. Commun. *451*, 101–106.
8. Park, U.H., Seong, M.R., Kim, E.J., Hur, W., Kim, S.W., Yoon, S.K., and Um, S.J. (2014). Reciprocal regulation of LXRα activity by ASXL1 and ASXL2 in lipogenesis. Biochem. Biophys. Res. Commun. *443*, 489–494.
9. Park, U.H., Yoon, S.K., Park, T., Kim, E.J., and Um, S.J. (2011). Additional sex comb-like (ASXL) proteins 1 and 2 play opposite roles in adipogenesis via reciprocal regulation of peroxisome proliferator-activated receptor gamma. J. Biol. Chem. *286*, 1354–1363.
10. Russell, B., and Graham, J.M., Jr. (2013). Expanding our knowledge of conditions associated with the ASXL gene family. Genome Med. *5*, 16.
11. Hoischen, A., van Bon, B.W., Rodríguez-Santiago, B., Gilissen, C., Vissers, L.E., de Vries, P., Janssen, I., van Lier, B., Hastings, R., Smithson, S.F., et al. (2011). De novo nonsense mutations in ASXL1 cause Bohring-Opitz syndrome. Nat. Genet. *43*, 729–731.
12. Bohring, A., Silengo, M., Lerone, M., Superneau, D.W., Spaich, C., Braddock, S.R., Poss, A., and Opitz, J.M. (1999). Severe end of Opitz trigonocephaly (C) syndrome or new syndrome? Am. J. Med. Genet. *85*, 438–446.
13. Bainbridge, M.N., Hu, H., Muzny, D.M., Musante, L., Lupski, J.R., Graham, B.H., Chen, W., Gripp, K.W., Jenny, K., Wienker, T.F., et al. (2013). De novo truncating mutations in ASXL3 are associated with a novel clinical phenotype with similarities to Bohring-Opitz syndrome. Genome Med. *5*, 11.
14. De Rubeis, S., He, X., Goldberg, A.P., Poultney, C.S., Samocha, K., Cicek, A.E., Kou, Y., Liu, L., Fromer, M., Walker, S., et al.; DDD Study; Homozygosity Mapping Collaborative for Autism; UK10K Consortium (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. Nature *515*, 209–215.
15. Ramocki, M.B., Dowling, J., Grinberg, I., Kimonis, V.E., Cardoso, C., Gross, A., Chung, J., Martin, C.L., Ledbetter, D.H., Dobyns, W.B., and Millen, K.J. (2003). Reciprocal fusion transcripts of two novel Zn-finger genes in a female with absence of the corpus callosum, ocular colobomas and a balanced translocation between chromosomes 2p24 and 9q32. Eur. J. Hum. Genet. *11*, 527–534.
16. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114–2120.

17. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., et al.; ACMG Laboratory Quality Assurance Committee (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Med. *17*, 405–424.
18. Yang, Y., Muzny, D.M., Xia, F., Niu, Z., Person, R., Ding, Y., Ward, P., Braxton, A., Wang, M., Buhay, C., et al. (2014). Molecular findings among patients referred for clinical whole-exome sequencing. JAMA *312*, 1870–1879.
19. Tanaka, A.J., Cho, M.T., Millan, F., Juusola, J., Retterer, K., Joshi, C., Niyazov, D., Garnica, A., Gratz, E., Deardorff, M., et al. (2015). Mutations in SPATA5 Are Associated with Microcephaly, Intellectual Disability, Seizures, and Hearing Loss. Am. J. Hum. Genet. *97*, 457–464.
20. de Ligt, J., Willemsen, M.H., van Bon, B.W., Kleefstra, T., Yntema, H.G., Kroes, T., Vulto-van Silfhout, A.T., Koolen, D.A., de Vries, P., Gilissen, C., et al. (2012). Diagnostic exome sequencing in persons with severe intellectual disability. N. Engl. J. Med. *367*, 1921–1929.
21. Lelieveld, S.H., Reijnders, M.R., Pfundt, R., Yntema, H.G., Kamsteeg, E.J., de Vries, P., de Vries, B.B., Willemsen, M.H., Kleefstra, T., Löhner, K., et al. (2016). Meta-analysis of 2,104 trios provides support for 10 new genes for intellectual disability. Nat. Neurosci. *19*, 1194–1196.
22. Sobreira, N., Schiettecatte, F., Valle, D., and Hamosh, A. (2015). GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. *36*, 928–930.
23. Ware, J.S., Samocha, K.E., Homsy, J., and Daly, M.J. (2015). Interpreting de novo Variation in Human Disease Using denovolyzeR. Curr. Protoc. Hum. Genet. *87*, 1–15.
24. Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., O'Donnell-Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B., et al.; Exome Aggregation Consortium (2016). Analysis of protein-coding genetic variation in 60,706 humans. Nature *536*, 285–291.
25. Ropers, H.H., and Wienker, T. (2015). Penetrance of pathogenic mutations in haploinsufficient genes for intellectual disability and related disorders. Eur. J. Med. Genet. *58*, 715–718.
26. Chen, R., Shi, L., Hakenberg, J., Naughton, B., Sklar, P., Zhang, J., Zhou, H., Tian, L., Prakash, O., Lemire, M., et al. (2016). Analysis of 589,306 genomes identifies individuals resilient to severe Mendelian childhood diseases. Nat. Biotechnol. *34*, 531–538, advance online publication.
27. MacArthur, D.G., Balasubramanian, S., Frankish, A., Huang, N., Morris, J., Walter, K., Jostins, L., Habegger, L., Pickrell, J.K., Montgomery, S.B., et al.; 1000 Genomes Project Consortium (2012). A systematic survey of loss-of-function variants in human protein-coding genes. Science *335*, 823–828.
28. Balasubramani, A., Larjo, A., Bassein, J.A., Chang, X., Hastie, R.B., Togher, S.M., Lähdesmäki, H., and Rao, A. (2015). Cancer-associated ASXL1 mutations may act as gain-of-function mutations of the ASXL1-BAP1 complex. Nat. Commun. *6*, 7307.
29. Metzeler, K.H. (2014). ASXL genes and RUNX1: an intimate connection? Blood *124*, 1382–1383.
30. Russell, B., Johnston, J.J., Biesecker, L.G., Kramer, N., Pickart, A., Rhead, W., Tan, W.H., Brownstein, C.A., Kate Clarkson, L., Dobson, A., et al. (2015). Clinical management of patients with ASXL1 mutations and Bohring-Opitz syndrome, emphasizing the need for Wilms tumor surveillance. Am. J. Med. Genet. A. *167A*, 2122–2131.
31. Brunner, H.G., van Tintelen, J.P., and de Boer, R.J. (2000). Bohring syndrome. Am. J. Med. Genet. *92*, 366–368.
32. Farber, C.R., Bennett, B.J., Orozco, L., Zou, W., Lira, A., Kostem, E., Kang, H.M., Furlotte, N., Berberyan, A., Ghazalpour, A., et al. (2011). Mouse genome-wide association and systems genetics identify Asxl2 as a regulator of bone mineral density and osteoclastogenesis. PLoS Genet. *7*, e1002038.
33. Lai, H.L., Grachoff, M., McGinley, A.L., Khan, F.F., Warren, C.M., Chowdhury, S.A., Wolska, B.M., Solaro, R.J., Geenen, D.L., and Wang, Q.T. (2012). Maintenance of adult cardiac function requires the chromatin factor Asxl2. J. Mol. Cell. Cardiol. *53*, 734–741.