ARTICLE

UBA2 variants underlie a recognizable syndrome with variable aplasia cutis congenita and ectodactyly

Rhonda E. Schnur1,2,17✉, Sairah Youssaf3,17, James Liu3,17, Wendy K. Chung4, Lindsay Rhodes1, Michael Marble5, Regina M. Zambrano6, Nara Sobreira7, Parul Jayakar8, Mary Ella Pierpont9, Matthew J. Schultz10, Pavel N. Pichurin10, Rory J. Olson10, Gail E. Graham11, Matthew Osmond12, Gustavo A. Contreras-García13, Karina A. Campo-Neira14, Camilo A. Peñaloza-Martíll14, Mark Flage3, Srikar Kuppa3, Karina Navarro3, Maria J. Guillen Sacoto1, Ingrid M. Wentzensen1, Maria I. Scarano2, Jane Juusola1, Carlos E. Prada15,16 and Robert B. Hufnagel13✉35

PURPOSE: The human chromosome 19q13.11 deletion syndrome is associated with a variable phenotype that includes aplasia cutis congenita (ACC) and ectodactyly as specific features. UBA2 (ubiquitin-like modifier-activating enzyme 2) lies adjacent to the minimal deletion overlap region. We aimed to define the UBA2-related phenotypic spectrum in humans and zebrafish due to sequence variants and to establish the mechanism of disease.

METHODS: Exome sequencing was used to detect UBA2 sequence variants in 16 subjects in 7 unrelated families. Uba2 loss of function was modeled in zebrafish. Effects of human missense variants were assessed in zebrafish rescue experiments.

RESULTS: Seven human UBA2 loss-of-function and missense sequence variants were detected. UBA2-phenotypes included ACC, ectodactyly, neurodevelopmental abnormalities, ectodermal, skeletal, craniofacial, cardiac, renal, and genital anomalies. Uba2 was expressed in zebrafish eye, brain, and pectoral fins; uba2-null fish showed deficient growth, microcephaly, microphthalmia, mandibular hypoplasia, and abnormal fins. uba2-mRNAs with human missense variants failed to rescue nullizygous zebrafish phenotypes.

CONCLUSION: UBA2 variants cause a recognizable syndrome with a wide phenotypic spectrum. Our data suggest that loss of UBA2 function underlies the human UBA2 monogenic disorder and highlights the importance of SUMOylation in the development of affected tissues.

INTRODUCTION

Features of the chromosome 19q13.11 deletion syndrome include early growth deficiencies, developmental delay, distinctive facial features, aplasia cutis congenita (ACC), hip dysplasia, digital and limb anomalies including ectodactyly, and other malformations.1–8 Deletions range in size from 1.37–11 Mb with a minimum overlapping region (MOR) of 324 kb, without clear genotype-phenotype correlation.3,4,6 UBA2 lies adjacent to the MOR and has been proposed to underlie key aspects of the deletion phenotype including ACC and ectodactyly.1–3,5,7 Limited patient data and lack of an animal model have prevented establishing UBA2 as the causative gene.

UBA2 plays a key role in the post-translational modification of protein (SUMOylation) by the addition of SUMO1 (small ubiquitin-like modifier) protein. UBA2 forms a heterodimer with SAE1 (SUMO-Activating Enzyme Subunit 1) and binds with SUMO1 in an ATP-dependent manner.9–11 Unlike ubiquitination, SUMOylation does not only target proteins for degradation, but is also involved in cell cycle regulation, subcellular trafficking, signal transduction, stress responses, and chromatin structure dynamics. SUMOylation alters protein kinases and transcription factors to maintain transcriptional regulation of tissue-specific gene expression.12

In this study, we report 16 additional individuals from seven unrelated families with de novo and familial UBA2 sequence variants who have highly variable but overlapping clinical presentations. In silico modeling and a zebrafish uba2 nullizygous phenotype provide further functional evidence for the pathogenicity of UBA2 as the key gene underlying the chromosome 19q13.11 microdeletion syndrome.
MATERIALS AND METHODS

Subject enrollment and clinical evaluations
Each described patient was evaluated by a clinical geneticist. Written informed consent was obtained for exome sequencing either on a clinical or research basis. A written informed consent was also obtained from subjects to publish their photos. Genomic DNA was extracted from whole blood from affected probands and their biological parents for exome sequencing. See supplement for details.

Zebrafish modeling of the phenotypic effects of uba2 variants
All animal experiments were conducted in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (protocol NEI-679). Adult AB (Tubingen) and ABTL (Tubingen long fin) zebrafish strains were raised and maintained according to standard protocols as described.13

Whole-mount in situ hybridization
Wild type (WT) zebrafish embryos at different developmental stages (5 somite, 24, 35, 48, 72 hpf [hours postfertilization]), 5 and 7 dpf (days postfertilization) were fixed in preparation for performing in situ hybridization. See Supplemental methods for details.

CRISPR/Cas9 uba2 knockout line generation
CRISPR/Cas9 method was used to generate uba2 knockout zebrafish lines. See Supplemental methods for details.

mRNA rescue
To evaluate the impact of human UBA2 variants on encoded protein products, we utilized uba2-mutant fish to perform rescue studies with capped full-length human WT and missense alleles in messenger RNA (mRNA) transcribed with the T7 mMESSAGE mMACHINE kit (Ambion). Please see Supplement for other methodology details.

RESULTS

Clinical studies
The cohort was gathered through GeneDx, a clinical molecular laboratory, and GeneMatcher. Investigators independently ascertained families with related phenotypes and rare candidate variants. Table 1 and the Supplement contain additional clinical details.

Family 1: Family 1 (Figs. 1 and 2) is comprised of an affected mother and her four offspring. Two children have ACC. By report, the maternal grandmother and great grandmother also have histories of ACC. Other ectodermal changes are variable including thin scalp hair, xerosis, and dental anomalies. The index case (IV-4, Fig. 1a, b) has unilateral ectrodactyly of the hand. All of the other affected examined individuals have more subtle digital variations including camptodactyly, syndactyly, clinodactyly, and diminished distal flexion creases of the fingers. All affected individuals share a high anterior hairline and mild frontal bossing, and several, including the proband (IV-4), have slightly downslanted palpebral fissures. All have had highly variable neurodevelopmental problems, ranging from hypotonia to autism spectrum disorder in two of the brothers. Hypotonia generally persisted throughout childhood. Affected individuals had early growth deficiencies that improved with age. See Supplement for other details. All affected individuals studied are heterozygous for a UBA2 frameshift variant: c.816_817delAT, p.Trp273Alafs*13.

Family 2: This family consists of three affected brothers (Fig. 1b: II-1, II-2, II-3); neither parent is affected. Parentage was genetically confirmed prior to exome sequencing. All affected individuals have histories of hypotonia through childhood that impeded motor development and even feeding ability in early infancy, and sensory integration problems, but normal cognitive abilities. Neither ACC nor other ectodermal changes are noted, but the youngest brother (II-3) has unilateral cleft hand and polydactyly.

None of the detected UBA2 variants was found in the gnomAD database.15 Results of in silico predictor analyses for missense variants and variant classification are provided in Supplemental Tables 1 and 2. All would be classified as pathogenic or likely pathogenic using American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) guidelines (classification criteria)16 in Supplemental Table 2.
### Table 1. Clinical features in study subjects and previous publications.

| Family ID | Subject ID | Gender | OBA2 variant, transcript | Current age or age at exam (years) | Developmental delay or neurodevelopmental delays | Height percentile (most recent or at stated age) | Weight percentile (most recent or at stated age) | Head circumference percentile (most recent or stated age) | Early growth problems | Craniofacial features | Apalasia cutis congenita | Other ectodermal variations | Ectodactyly/ syndactyly | Other skeletal anomalies | Other anomalies/features: cardiac, renal, genital, ocular, miscellaneous | Other genetic/ chromosomal results |
|-----------|------------|--------|--------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-------------------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-----------------------------------------------|-----------------------------------------------|
| 1         | II-2       | F      | c.816_817del/AT; p. Thr273Alafs*13maternal NM_005499 | 37 | Normal development, but had behavior problems as child, history of seizures, mini strokes | 45 | Increased 2 | Tall forehead/high hairline, hypertelorism, broad nasal root, facial asymmetry, cleft chin, ptosis, simple lower-set ears | Hypoplastic distal flexion creases, clinodactyly, camptodactyly, hip abnormality | Xerosis, keratosis pilaris, unruly hair, atopic dermatitis, history of heat exhaustion | Yes, multiple areas | N/A | N/A | N/A | N/A | Heterozygous for FGG LPATH variant: D344N; BLM71B heterozygous U5.48T |
| 1         | II-3       | M      | c.816_817del/AT; p. Thr273Alafs*13maternal NM_005499 | 8 | Autism spectrum disorder, behavior problems, encopresis, stereotypies, mood swings, hypotonia, normal MRI | 54 | ~30 | Tall forehead/high hairline, orbital asymmetry, syndactyly, square uvala, atrophalgoglossia, cleft chin | Hypoplastic distal flexion creases, clinodactyly, camptodactyly, hip abnormality | Xerosis, keratitis,�pulmonary hypoplasia, tracheostenosis, bronchial hyperplasia, scoliosis, pectus excavatum | Yes, unilateral hand | N/A | N/A | N/A | N/A | Heterozygous for FGG LPATH variant: p.D344N; microarray, dup12q11.2 |
| 1         | II-4       | F      | c.816_817del/AT; p. Thr273Alafs*13maternal NM_005499 | 7 | Mild delays, intermittent attention deficit, brain parietal reflexes, poor balance, hypotonia, normal cognitive skills, MRI normal | 11 | ~2 | Tall forehead/high hairline, hypotelorism, keratoconus, kyphoscoliosis, diplopia, cleft palate, dental crowding | Hypoplastic distal flexion creases, brachydactyly of toes, clinodactyly, camptodactyly, hip abnormality, mild pectus excavatum and hypertelorism, wormian bones | Xerosis, mild ichthyosis, keratoconus, kyphoscoliosis, pectus excavatum, postauricular pits, hydrops, facial dysmorphism, inguinal hernia | Yes, unilateral hand | N/A | N/A | N/A | N/A | Normal SNP microarray; normal IPI gene sequencing |
| 2         | II-1       | M      | c.1336_1337del/AT; p. Thr460Aspfs*24 de novo NM_005499 | 21 | Delayed: hypotonia, sensorimotor integration problems, normal cognitive development | 10 | <3 | ~5 | Tall forehead/high hairline, hypertelorism, broad nasal root, facial asymmetry, keratoconus, kyphoscoliosis, pectus excavatum, plagiocephaly | Long thin fingers, first anomalies, clinodactyly, pectus excavatum, plagiocephaly | N/A | N/A | N/A | N/A | N/A | Cryptorchidism, hydrocele |
| 2         | II-2       | M      | c.1336_1337del/AT; p. Thr460Aspfs*24 de novo NM_005499 | 12 | Delayed motor skills; hypotonia, sensorimotor integration problems | "Low" | <5 | Yes | Downslanted palpebral fissures, broad nasal root, high arched palate, dental crowding | Dypastic malformations, low point outward, hyperplastic subungual keratosis, inguinal hernia | N/A | N/A | N/A | N/A | N/A | N/A |
| Family ID | Subject ID | Gender | UBA2 variant, transcript | Current age or age at exam (years) | Developmental delay/ neurodevelopmental details | Height percentile (most recent or at stated age) | Weight percentile (most recent or at stated age) | Head or circumference percentile (most recent or stated age) | Early growth problems | Craniosynostosis | Apalae cutis congenita | Other ectodermal variations | Other skeletal anomalies | Other anomalies/ features: cardiac, renal, genital, ocular, miscellaneous | Other genetic/ chromosomal results |
|-----------|------------|--------|--------------------------|---------------------------------|-------------------------------------------|------------------|------------------|-----------------------------|------------------|----------------|----------------|--------------------------|-------------------|-------------------------------------------------|-----------------------------|
| 2         | II-3       | M      | c.1336_1337insT: p. Thr446Pro+1 ins denovo NM_005499.2 | 6                               | Delays, unstable gait, poor fine motor skills, sensory integration problems, poor balance, hypertonia, normal cognitive skills | c.1336_1337insT: p. Thr446Pro+1 | c.1336_1337insT: p. Thr446Pro+1 | <3 <3 <3 | Yes | Arched palate, micrognathia | Not noted | No | Yes, unilateral partial central drift of hand, polydactyly of third finger | Syndactyly, camptodactyly | Cryptorchidism, hydrocele, XY and normal microarray |
| 3         | I-2        | M      | c.364C>T: p.Arg122* NM_005499 | 45                              | Delays, learning difficulties in school, depression in adulthood | C.364C>T: p.Arg122* | C.364C>T: p.Arg122* | <5 >95 ~5–10 | Tall forehead/high hairline, hypertelorism, broad nasal root, low-set ears, micrognathia | Yes, multiple areas | Supernumerary nipples | No | None reported | Recurrent urinary tract infections; asymmetric renal sizes with reduced function of smaller kidney; hypothyroidism, s/p cholesteatoma; congenital hemiarchathy; history of paralytic cerebral palsy |
| 3         | II-1       | F      | c.364C>T: p.Arg122* maternal NM_005499 | 24.5                             | Delays (walked at 17 months, first words at 22 months), special education, depression and anxiety as an adult | C.364C>T: p.Arg122* | C.364C>T: p.Arg122* | ~15 ~93 <3rd | Tall forehead/high hairline, hypertelorism, broad nasal root, thin upper lip, smooth philtrum, everted lower lip, thick, low-set, and laterally protruding ears, medial eyebrows, thin vermilion border, mild micrognathia | Yes, 3 areas | Supernumerary nipple | No | None reported | Bicuspid aortic valve, astigmatism, s/p cholesteatoma; migraines; low back pain |
| 3         | II-2       | M      | c.364C>T: p.Arg122* maternal NM_005499 | 21.5                             | Delays recognized at 16 months, learning difficulties and special education, bipolar disorder, panic attacks and social phobia as an adult | C.364C>T: p.Arg122* | C.364C>T: p.Arg122* | ~20 ~15 ~60–70 | Tall forehead/high hairline, dolichostenomelia, broad nasal root, prominent cipitum, bulbous tip of nose, micrognathia | Yes, single area | Supernumerary nipple | No | None reported | Cryptorchidism, asthma, rhinitis |
| 3         | III-1      | F      | c.364C>T: p.Arg122* maternal NM_005499 | 2.75                             | Delays (at 16 months, cognitive function was at the 8 month old level, motor skills were at the 9 month level, at 30 months still no sentences) | C.364C>T: p.Arg122* | C.364C>T: p.Arg122* | 50 75-90 3–10 | No | C.364C>T: p.Arg122* | Supernumerary nipple | No | None reported | Frequent otitis, constipation |
| 4         | II-1       | F      | c.167A>C: p.Asn56Thr de novo NM_005499 | 4.75                             | Delayed motor skills, attention deficit disorder, set independently at 12 months, walked at 22 months, first word at 18 months, sentencs after 2 years | C.167A>C: p.Asn56Thr | C.167A>C: p.Asn56Thr | <3 <3 <3 | Yes | High bilaterally, broad forehead, hypertelorism, broad nasal root, delayed bone age, kyphoscoliosis treated with bracing | Yes | This, sparse hair, coarse skin, poor sweating, skin with scars | No | Clinodactyly, overhanging toes on right foot (CL4), delayed bone age, kyphoscoliosis treated with bracing | Renal hypoplasia, chronic kidney disease, stable bilateral optic nerve hypoplasia with normal vision, postural orthostatic hypotension, hypothyroidism, growth hormone deficiency, headaches, no brain development (budding only), menarche at 14 years, pubic hair, no axillary hair | MA288: heterozygous W5.S, de novo fs c.3317insA; SO3: heterozygous variant c.2817–1C > G; 22:8:17 homozygous p.11; W5.H; heterozygous W5.S, p.G384R (maternal) |
| 5         | II-1       | F      | c.1447G>A: p.Glu483Lys de novo NM_005499 | 4.75                             | Global delay (gross motor and speech), nonverbal, refractory seizures, infantile spasms, hypertonia | C.1447G>A: p.Glu483Lys | C.1447G>A: p.Glu483Lys | ~75 ~10 ~25 | No | Epicanthal folds | Yes | Normal hair and nails | Pes planus | Hemangiomalmas (left ear, back); anteriorly placed anus | Normal microarray, normal Pode–WBB, Angelman methylation, |
Table 1 continued

| Family ID | Subject ID | Gender | U5A2 variant, transcript | Current age or age at exam (years) | Developmental delay/ neuromotor developmental details | Height percentile (most recent or stated age) | Weight percentile (most recent or stated age) | Head circumference percentile (most recent/stated age) | Early growth problems | Craniofacial features | Other ectodermal variations | Other skeletal anomalies | Other anomalies/features: cardiac, renal, genital, ocular, miscellaneous | Other genetic/ chromosomal results |
|-----------|------------|--------|-------------------------|-----------------------------------|-----------------------------------------|---------------------------------|----------------------------------|---------------------------------|-----------------|------------------|-----------------------|-----------------|-----------------------------------------------|-------------------------------|
| 6         | 8-1        | M      | c.807T>A; p.Leu270*     | 1.5                               | Normal development                      | 10–25                           | 10–25                           | 5–10                           | No              | No               | Tall forehead/ high hairline, hypertelorism, epicanthal folds, pseudohypoparathyroidism | No, bilateral ectrodactyly of the feet | Cleft palate, syndactyly of the feet | Yes, bilateral encephalocele, bilateral cleft lip and palate |
| 7         | 8-1        | M      | c.547C>G; p.Arg120Lys    | 3.0                               | Gross, fine motor and speech delays, persistent | 75–90                           | -75                             | 25                             | No              | Diffuse patches of hypopigmentation | No               | Diffuse patches of hypopigmentation | Yes, bilateral cleft palate |
| Marble    | et al. 2019 | F      | c.71G>T; p.Gly24Val     | 2.5                               | Delayed motor development, normal cognitive ability | 25–50                           | 3rd                             | 25–50                          | Yes             | Yes              | Tall forehead/ high hairline, hypertelorism, downslanted palpebral fissures, suspected hypertelorism and broad nasal root | Yes, single large area | Cleft palate, syndactyly of both feet | Normal SNP microarray |
| Yamoto    | et al. 2019 | M      | c.1324dupT; p.Tyr442Leufs*17 | 4                                | Normal development                      | 10–25 (birth)                   | <3 (birth)                      | <10 (birth)                    | Yes, two areas | Bilateral ectrodactyly, dysplasia, hands, and feet | Yes              | Bilateral ectrodactyly, dysplasia | Clitoral hypoplasia, long bone deficiency of tibia | Underamnionization of external genitalia |
| Wang      | et al. 2019 | M      | c.3216delT; p.Phe109Leufs*3 | 35                               | Normal development                      | Yes                             | No                             | None reported                  | Yes             | No               | Yes                   | No              | None reported |
| Wang      | et al. 2019 | F      | c.3216delT; p.Phe109Leufs*3 | 35                               | Normal development                      | Yes                             | No                             | None reported                  | Yes             | No               | Yes                   | No              | None reported |
| Aerden    | et al. 2020 | M      | c.637delA; p.Glu213Lys   | 8                                | Speech delay, normal motor milestones; learning difficulties, autism diagnosed at 8 years, intelligence quotient 76 | 25–50 (5.6 years)               | 25                             | 20–30 (3.6 years)              | Yes             | Retrognathia, low-set and prominent ears, fullness of upper eyelids | Yes              | Supernumerary nipple, increased hair on back, dry, sparse scalp hair | Yes, polydactyly with six metatarsals on right foot, multiple bony anomalies in feet, syndactyly of toes, normal hands, transient hip instability; normal hands | Strabismus, hypermetropia |

ASD atrial septal defect, CGH comparative genomic hybridization, ES exome sequencing, GU genitourinary, LPATH likely pathogenic, MRI magnetic resonance image, PFO patent foramen ovale, SNP single nucleotide polymorphism, VUS variant of uncertain significance.
Modeling effects of missense variants on UBA2 function

UBA2 in complex with SAE1 plays a key role in the SUMOylation pathway. Observed human UBA2 variants are distributed across the gene (Fig. 2a, b). All truncating variants are expected to undergo nonsense-mediated decay based on their position within the mRNA. Missense variants occur at residues that are strongly conserved across vertebrates (Fig. 2c). Given the similarities in phenotypes between individuals with truncating and missense alleles, we hypothesized that missense alleles also lead to loss of function.
To understand how missense alleles might disrupt UBA2 function, molecular modeling using published crystal structures\textsuperscript{17} and simulated substitutions were performed for each detected human missense variant. In the UBA2 protein, p.Gly24\textsuperscript{18} is directly involved in ATP binding; its substitution with valine results in altered protein conformation and is predicted to result in loss of ATP binding and ectopic interactions with nearby residues (Fig. 2d).\textsuperscript{17} Similarly, asparagine replacement with threonine at position 56 putatively abolishes ATP-dependent activation. The p.Arg122Gly substitution is predicted to result in loss of interaction with ATP. Human UBA2 protein interacts with a conjugating enzyme called UBC9 (amino acids 6–38) via amino acid residues 478–509, which include Glutamate 483. UBA2 forms a hydrophobic bond with Leu6, Met36, and Leu38 of UBC9; replacing Glutamate 483 with Lysine is predicted to disrupt UBA2–UBC9 binding. In summary, missense alleles observed in patients with UBA2-associated syndrome are observed to occur at functionally critical residues and potentially disrupt ATP binding, protein folding, or protein–protein interactions.

Zebrafish uba2 expression in affected tissues
By whole-mount in situ hybridization, uba2 transcript was detected on the dorsoventral axis of 5-somite stage embryos (Fig. S1a, b). At later stages, uba2 is expressed in developing brain, eye, craniofacial structures, and fins. At 24 hpf, uba2 expression was restricted to the head region, including the eye and nervous system (Fig. S1c). At 35 hpf, prominent signal was observed in pectoral fins (arrows, Fig. S1d). At all other examined stages (48 and 72 hpf, 5 and 7 dpf), uba2 mRNA signal localized to the head region, specifically brain, neural retina, and lens (Fig. S1e–h). Therefore, zebrafish uba2 is expressed in some structures that are analogous to those affected in humans harboring deleterious UBA2 variants.

Variable expressivity observed with uba2 loss of function
uba2 knockout zebrafish lines were generated by CRISPR/Cas9-targeted deletion. The phenotype of homozygous fish was notable for failure to inflate swim bladders. At 5–8 dpf, we observed severe gross morphological defects in uba2\textsuperscript{−/−} zebrafish (Fig. 3) including...
small eyes, hydrocephalus and craniofacial edema, ventrally curved body axis, and uninflated swim bladder. Faint heartbeat and severe pericardial edema were observed in 41% of embryos (Fig. 3a, b). Edema became generalized at 8 dpf when most lethality was noted. To further examine the effect of uba2 on zebrafish development, we calculated the survival rate of uba2-/− zebrafish which was significantly lower than control (WT) and heterozygous fish. uba2-/− zebrafish showed a mortality rate of approximately 50% at 8 dpf; however, 100% of mutant fish were dead by day 12 (Fig. 3d).

Nullizygous fish exhibited a wide phenotypic range. We observed a pair of normal extended pectoral fins in WT zebrafish versus uba2−/− fish, where pectoral fins were found to be short and upright-oriented (Fig. 3a) confirming uba2 function in fish extremity development. WT zebrafish had thin lines originating from base to fin tips showing normal actinotrichia. In contrast, uba2−/− fish displayed collapsed (Fig. 3b, middle image) and irregular fin fold edges (Fig. 3b, last image).

To better characterize variable expression and the relationship between the zebrafish knockout and the human disorders, we quantified craniofacial (F), brain (B), pectoral fin (PF), tail fin (TF), and swim bladder (SB) defects. Defects at later stages of development were studied in uba2−/− fish bred from the same parent at 8 dpf, when approximately half the fish survive (n = 32; Fig. 3c). Tissue-level malformations were observed in craniofacial structures (9.38%), brain size (90.6%), tail fin (25%), pectoral fin (100%), and swim bladder (93.75%) (Fig. 3c and as described below). Thus, across individual fish with similar genetic backgrounds, total uba2 function loss recapitulates some tissue-level phenotypes and the variable expression observed in human UBA2-related phenotypes.

Neuronal reduction in uba2 zebrafish
Tissue-level analysis was performed in zebrafish to elucidate abnormalities resulting from uba2 loss of function. First, we conducted immunohistochemistry studies on 8 dpf zebrafish cryosections through eye and brain. Compared to WT controls, uba2-null fish showed small heads, reduced midbrain size, low nuclei cell count with high accumulation of actin signal (orange, Fig. S2), implying a decreased proportion of gray to white matter. In addition, uba2−/− fish had smaller eyes, reduced retinal thickness, retinal laminations, and lens defects (see Supplement).

Skeletal and extremity phenotypes in the uba2 zebrafish model
To investigate the impact of uba2 on zebrafish skeletal development, we stained uba2 WT (+/+), heterozygous (+/-), and homozygous (-/-) fish with alcian blue dye at 5 dpf. In both uba2 WT (Fig. 4a) and heterozygous zebrafish (data not shown), alcian staining demonstrated a normal pattern of cartilage element development including typical ceratohyoid, Meckel’s cartilage, ceratobranchials arches, and pectoral fin cartilage.
However, complete loss of *uba2* in homozygous fish resulted in abnormal craniofacial development. In addition to jaw malformations, other craniofacial malformations included malformed and hypoplastic ventral and dorsal cartilage structures with lack of basihyal and hypohyal development. We also noted an apparently abnormal fusion of Meckel’s cartilage with the palatoquadrate, resulting in a small, narrow mandible (Fig. 4b). Moreover, Meckel’s cartilage was flattened at the midline fusion point with completely absent ceratohyal cartilage and ceratobranchials arches, the equivalent of micrognathia in these fish.
To further confirm the specificity of the uba2 knockout phenotype, we attempted phenotypic rescue of developmental fish malformations by injecting human UB2A mRNA. Injected fish were grouped into three phenotypic classes and genotyped at 5 dpf, and the uba2"−/−" subset was analyzed. Embryos were classified as class I (grossly normal body structure), class II (decreased head size, absent swim bladder), and class III (small head and body, generalized edema) (Fig. 4d). As compared to H2O-injected controls, injecting human WT UB2A mRNA grossly rescued phenotypes in a significant number of fish. The proportion of class I fish increased from 5% to 33%, and the proportion of class III fish decreased from 47% to 6% (p < 0.0001) (Fig. 4e). Even though WT UB2A mRNA injection rescued gross phenotypes, most uba2"−/−" zebrafish still did not show inflated swim bladder (data not shown), suggesting that early uba2 deficiency permanently impacts zebrafish physiology despite substitution with human mRNA.

Human mRNAs encoding p.Gly24Val, p.Arg122Gly and p.Glu483Lys all failed to rescue the uba2"−/−" phenotypes in contrast to WT mRNA. The p.Asn56Thr substitution demonstrated statistically similar rescue to control mRNA; however, there were more class III fish (23% vs. 6%) and fewer class I fish (18% vs. 33%) following p.Asn56Thr injection, indicating possible partial loss of function for this missense substitution (Fig. 4e). Because the mRNAs containing the missense variants failed to rescue uba2-null phenotypes to a similar level as did WT UB2A mRNA, we conclude that the most likely mechanism of disease is loss of function.

**DISCUSSION**

In this study, we describe a cohort of patients harboring deleterious variants in the UB2A gene. They show highly variable inter- and intrafamilial expression of dermatologic, skeletal, extremity, neurologic, cardiac, and renal features, similar to those of the chromosome 19q13.11 microdeletion syndrome.1,2 These observations further support UB2A as the critical gene in the microdeletion syndrome and suggest its essential role in early human growth and development. There are only a few other reports of intragenic UB2A variants (summarized in Table 1). Marble et al.18 reported a de novo UB2A missense variant (c.71G>T, p.Gly24Val) in a 2.5-year-old female with ACC, thin hair, tall forehead, Duane anomaly, hip dysplasia, clinodactyly, and poor weight gain. Wang et al.18 reported an inherited UB2A frameshift variant (c.327delT, p.Phe109Leufs*3) in a young boy and his mother. The mother had ACC but was otherwise healthy. The son had ACC, microcephaly, bilateral clinodactyly, low-lying conus medullaris, horseshoe kidney, and tracheoesophageal fistula. A de novo UB2A loss-of-function variant (c.1324dupT, p.Tyr442Leufs*17) was associated with four extremity split hand and foot malformation with tibial deficiency and undermasculinized external genitalia.19 Aerden et al.20 reported a male proband with ectrodactyly of the feet, autism spectrum disorder, craniofacial variations, dry sparse scalp hair, strabismus, and hydronephrosis who was heterozygous for a de novo frameshift variant in UB2A (c.612delA, p.Glu205Lysfs*63); this was considered to be responsible for the phenotype.20

The four patients previously reported with intragenic UB2A variants were added to our clinical summary table (Table 1) to compare phenotypes.18,21 We’ve estimated the percentage of key traits in UB2A subjects (Fig. 1c) based on available clinical information. The most specific aspects of the UB2A-related phenotype are ACC, seen in 61%, and ectrodactyly, which is less common (37%). Early growth deficiency and neurodevelopmental delay are reported in 61% and 80% of affected individuals, respectively. More variable digital and skeletal abnormalities are also present (56%) but are sometimes subtle and potentially overlooked (e.g., Fig. 1a, panels C, D). These include clinodactyly (62%), syndactyly (59%), camptodactyly (57%), and hip abnormality (35%). The most common craniofacial variations are tall forehead/high hairline (76%), downsloanted palpebral fissures (47%), hypertelorism (62%), broad nasal root (81%), microcephaly (37%), and micrognathia (53%). Other observed features among our subjects include other ectodermal variations (~82%), ocular abnormalities (53%), and cardiac (43%), genital (50%, in males), and renal (36%) abnormalities.
In *C. elegans*, *Ubo-2* is also noted to be a critical element of the SUMOylation pathway; its ablation leads to embryonic lethality.\(^2\)

UBA2 acute knockdown in xenograft tumors by conditional short hairpin RNA (shRNAs) causes marked growth arrest, cell proliferation defects, and increased apoptosis.\(^3\) In mice, loss of any key component of the SUMOylation pathway can lead to severe impairment of cellular functions and lethality.\(^4\)\(^5\) An in situ hybridization study conducted in mouse embryos (8.5 to 11.5 days postcoitum) revealed *Uba2* ample expression at multiple morphogenetic activity sites, e.g., neural folds, branchial arches, and limb buds,\(^6\) suggesting that *Uba2* is essential for normal cellular function/development. Recentely, SUMOylation was reported to regulate differentiation of several ocular tissues.\(^7\)\(^8\)

Phenotypic features in our human UBA2-related syndrome cohort and the uba2 knockout zebrafish are reminiscent of disorders associated with pathogenic variants in *DLX5/6* (split hand/foot malformation [SHFM1], OMIM 220600), *TP63* (e.g., ectodactyly, ectodermal dysplasia, and cleft lip/palate syndrome [SHFM1], OMIM 220600; sh (33%). Notably, three of four human missense *UBA2* sense variants, who present with highly variable related phenotypes,\(^9\) related autosomal dominant syndrome. De

In conclusion, we report clinical details in 16 individuals from seven unrelated families with inherited or de novo heterozygous UBA2 sequence variants, who present with highly variable phenotypes. Definition of the UBA2-related autosomal dominant phenotypic spectrum in humans, in silico modeling predictions, *uba2* expression, and characterization of the knockout phenotype in zebrafish support the significance of UBA2/*uba2* in development, potentially by affecting post-translational modification of SHFM-associated genes. mRNA rescue experiments in zebrafish also suggest that loss of gene function is the primary mechanism of disease. The highly variable expressivity of the human UBA2 phenotype, either via sequence alteration or contiguous gene deletion, even within the same family, remains incompletely explained; there are likely other modifiers, still to be identified. However, our studies define a human disorder associated with UBA2 sequence variants with a phenotype that overlaps key aspects of the chromosome 19q13.11 microdeletion syndrome.

Web Resources
ClinVar Database https://www.clinicalgenome.org/data-sharing/clinvar

gnomAD https://gnomad.broadinstitute.org/

GeneMatcher https://genematcher.org/

Pathogenicity predictions https://varsome.com/

OMIM http://www.omim.org/

Clustal omega https://www.ebi.ac.uk/Tools/msa/clustalo/

DATA AVAILABILITY
All data is mentioned in the main text and supplement, available to readers.

Received: 10 February 2021; Revised: 7 April 2021; Accepted: 7 April 2021;

Published online: 26 May 2021

REFERENCES
1. Abe, K. T. et al. 19q13.11 microdeletion: clinical features overlapping ectodactyly/ectodermal dysplasia-clefting syndrome phenotype. *Clin. Case Rep.* 6, 1300–1307 (2018).

2. Chowdhury, S. et al. Phenotypic and molecular characterization of 19q12q13.1 deletions: a report of five patients. *Am. J. Med. Genet. A.* 164A, 62–69 (2014).

3. Gana, S. et al. 19q13.11 cryptic deletion: description of two new cases and indication for a role of WTI haploinsufficiency in hypospadias. *Eur. J. Hum. Genet.* 20, 852–856 (2012).

4. Malan, V. et al. 19q13.11 deletion syndrome: a novel clinically recognisable genetic condition identified by array comparative genomic hybridisation. *J. Med. Genet.* 46, 635–640 (2009).

5. Melo, J. B., Estevinho, A., Seraiva, J., Ramos, L. & Carreira, I. M. Cutis aplasia as a clinical hallmark for the syndrome associated with 19q13.11 deletion: the possible role for UBA2 gene. *Mol. Cytogenet.* 8, 21 (2015).

6. Schuurs-Hoeijmakers, J. H. et al. Refining the critical region of the novel 19q13.11 microdeletion syndrome to 750 Kb. *J. Med. Genet.* 46, 421–423 (2009).

7. Urquhart, J. E. et al. Deletion of 19q13 reveals clinical overlap with Dubowitz syndrome. *J. Hum. Genet.* 60, 781–785 (2015).

8. Venegas-Vega, C. et al. 19q13.11 microdeletion concomitant with ins(2;19)(p25.3;q13.1q34) in a boy: potential role of UBA2 in the associated phenotype. *Mol. Cytogenet.* 7, 61 (2014).

9. Desterro, J. M., Rodriguez, M. S., Kemp, G. D. & Hay, R. T. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. *J. Biol. Chem.* 274, 10618–10624 (1999).

10. He, P. et al. UBA2 promotes proliferation of colorectal cancer. *Mol. Med. Rep.* 18, 5552–5562 (2018).

11. Olsen, S. K., Capilli, A. D., Lu, X., Tan, D. S. & Lima, C. D. Active site remodelling accompanies thioester bond formation in the SUMO E1. *Nature.* 463, 906–912 (2010).

12. Chang, S. C. & Ding, J. L. Ubiquitination and SUMOylation in the chronic inflammatory tumor microenvironment. *Biochim. Biophys. Acta Rev. Cancer.* 1870, 165–175 (2018).

13. Westerfield, M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish* (Danio rerio). (M. Westerfield, Eugene, OR, 2007).

14. Marble, M. & Pridjian, G. Scalp defects, polythelia, microcephaly, and developmental delay: a new syndrome with apparent autosomal dominant inheritance. *Am. J. Med. Genet.* 108, 327–332 (2002).

15. Lek, M. et al. Analysis of protein-coding genetic variation in 60,706 humans. *ClinVar Database* https://www.clinicalgenome.org/data-sharing/clinvar

16. Schuurs-Hoeijmakers, J. H. et al. Reanalysis of 19q13.11 deletions: a report of five patients. *Am. J. Med. Genet. A.* 164A, 62–69 (2014).

17. Richards, S. et al. 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 (2015).

18. Lois, L. M. & Lima, C. D. Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *EMBO J.* 24, 439–451 (2005).

19. Marble, M., Guellin Sacoto, M. J., Chikarmane, R., Gargiulo, D. & Jususola, J. Missense variant in UBA2 associated with aplasia cutis congenita, duane anomaly, hip dysplasia and other anomalies: a possible new disorder involving the SUMOylation pathway. *Am. J. Med. Genet. A.* 173, 758–761 (2017).
20. Aerden, M. et al. Genotype-phenotype correlations of UBA2 mutations in patients with ectrodactyly. *Eur. J. Med. Genet.* **63**, 104009 (2020).

21. Wang, Y., Dupuis, L., Jobling, R. & Kannu, P. Aplasia cutis congenita associated with a heterozygous loss-of-function UBA2 variant. *Br. J. Dermatol.* **182**, 792–794 (2020).

22. Jones, D., Crowe, E., Stevens, T. A. & Candido, E. P. Functional and phylogenetic analysis of the ubiquitlation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol.* **3**, RESEARCH0002 (2002).

23. Carrington, B., Varshney, G. K., Burgess, S. M. & Sood, R. CRISPR-STAT: an easy and reliable PCR-based method to evaluate target-specific sgRNA activity. *Nucleic Acids Res.* **43**, e157 (2015).

24. Costa, M. W. et al. Complex SUMO-1 regulation of cardiac transcription factor Nhox2-S. *PLoS One* **6**, e24812 (2011).

25. Zhao, J. Sumoylation regulates diverse biological processes. *Cell. Mol. Life Sci.* **64**, 3017–3033 (2007).

26. Nie, Q. et al. Analysis of the differential expression patterns of sumoylation enzymes E1, E2 and E3 in ocular cell lines. *Curr. Mol. Med.* **18**, 509–515 (2018).

27. Gong, X. et al. Localization patterns of sumoylation enzymes E1, E2 and E3 in ocular cell lines predict their functional importance. *Curr. Mol. Med.* **18**, 516–522 (2018).

28. Santos-Pereira, J. M., Gallardo-Fuentes, L., Neto, A., Acemel, R. D. & Tena, J. J. Pioneer and repressive functions of p63 during zebrafish embryonic ectoderm specification. *Nat. Commun.* **10**, 3049 (2019).

29. Lee, H. & Kimelman, D. A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev. Cell.* **2**, 607–616 (2002).

30. Liedtke, D. et al. ECM alterations in Fndc3a (Fibronectin Domain Containing Protein 3A) deficient zebrafish cause temporal fin development and regeneration defects. *Sci. Rep.* **9**, 13383 (2019).

31. Yousaf, R. et al. Modifier variant of METTL13 suppresses human GAB1-associated profound deafness. *J. Clin. Invest.* **128**, 1509–1522 (2018).

32. Yousaf, S. et al. Molecular characterization of SLC24A5 variants and evaluation of Nitisinone treatment efficacy in a zebrafish model of OCA6. *Pigment Cell Melanoma Res.* **33**, 556–565 (2020).

33. Stainier, D. Y. R. et al. Guidelines for morpholino use in zebrafish. *PLoS Genet.* **13**, e1007500 (2017).

34. Pykhzhij, S. V. & Berman, J. N. Zebrafish knock-ins swim into the mainstream. *Dis. Model Mech.* **11**, dmm037515 (2018).

ACKNOWLEDGEMENTS

We thank the individuals and families who participated in this project. We express our deepest gratitude to Mary Ella Pierpont for her valuable contribution and dedicate this report to her memory. W.K.C. received financial support from the JPB Foundation. N.S.’s work is supported by National Human Genome Research Institute (NHGRI) grant 1U54HG006542. We would like to thank Sunit Dutta (National Eye Institute, National Institutes of Health [NIH], Bethesda, MD) for assistance in establishing uba2 zebrafish knockout lines. We thank the zebrafish facility, Confocal, transmission electron microscopy, and microcomputed tomography mouse imaging facilities at NIH for their support and technical assistance. The research work carried out at NIH was supported by funds provided by National Eye Institute, NIH (Bethesda, MD).

AUTHOR CONTRIBUTIONS

R.E.S. and R.B.H. designed and organized the study. S.Y. and J.L. generated and analyzed zebrafish-related data. R.E.S. collated and composed sections describing human clinical data; S.Y. and J.L. composed the core manuscript. R.E.S. and R.B.H. supervised and validated data and reviewed and edited the manuscript. M.F. generated microcomputer tomography data. S.K. performed zebrafish genotyping and alcin staining. L.R. coordinated all clinical collaborations. R.E.S., W.K.C., M.M., R.M.Z., N.S., P.J., M.E.P., M.J.S., P.N.P., R.J.O., G.E.G., M.O., G.A.C.-G., K.A.C.-N., C.A.P.-M., K.N., M.I.S., C.E.P. all contributed clinical patient information. M.J.G.S., I.M.W., J.J. analyzed exome data and provided clinical variant interpretations.

COMPETING INTERESTS

R.E.S., I.M.W., M.J.G.S., L.R., and J.J. are employees of GeneDx, Inc., Gaithersburg, Maryland. The other authors declare no competing interests.

ETHICS DECLARATION

Study participants were enrolled in approved protocols as per the policies of the Institutional Review Board (IRB) Committees of the institutions at which patients were identified, or via GeneDx, following the tenets of the Declaration of Helsinki. The main IRB for this study is Western Institutional Review Board, study number 1175260, WIRB protocol 20171030 (GeneDx). Written informed consent for inclusion in this study was obtained as required from all subjects, including specific consent to use photographs. All zebrafish-related experiments were conducted in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, protocol NEI-679.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41436-021-01182-1.

Correspondence and requests for materials should be addressed to R.E.S. or R.B.H.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.