Supplementary data

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

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Materials and Methods

Institutional Review Boards (IRB) approval

The main IRB for this study is the Western Institutional Review Board, Study Number 1175206, WIRB protocol # 20171030 (GeneDx). The following IRB committees approved the current study:

Family 1: Human Research Protections Program, Cooper University Health Care

Family 2: Columbia University IRB-AAAJ8651

Family 3: Louisiana State University Health Science Center

Family 4: Western Institutional Review Board, Study Number 1175206, WIRB protocol # 20171030

Family 5: Mayo Clinic Institutional Research Board IRB-12.00934600

Family 6: Fundacion Cardiovascular de Colombia protocol # 2012-1017

Family 7: The Research Ethics Board for the Children's Hospital of Eastern Ontario Research Institute, IRB # 11/04E

Exome sequencing

Trio exome analysis was performed in most cases, depending on family structure, and multiple subjects also underwent chromosome microarray analysis. Clinical exome sequencing for Family 1 was performed at Ambry Genetics using the SeqCap EZ VCRome 2.0 (Roche NimbleGen) or the IDT xGen Exome Research Panel V1.0. Families 2, 4 and 5 were analyzed at GeneDx. For these samples, exons were

captured using the SureSelect Human All Exon V4 (50 Mb) or Clinical Research Exome probes, or the IDT xGen Exome Research Panel probes. Sequencing was done on an Illumina HiSeq with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19 and analyzed for sequence variants using a custom-developed analysis tool as previously described[1].

For family 3, exome sequencing was performed for the proband (II-2, Fig. 1) and his affected mother (I-2, Fig. 1) as part of the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) project utilizing genomic DNA extracted from saliva (chemagic™ MSM 1 instrument, PerkinElmer). Libraries were prepared with the Agilent SureSelect HumanAllExonV4_51MbKit_S03723314 and sequenced using the TruSeq Rapid PE Cluster Kit-HS with 100 bp pair-end runs on the Illumina HiSeq2500. Alignment to the reference genome 1000 genomes phase 2 (GRCh37) was performed using BWA mem 0.7.8[2] and local realignment utilized GATK 3.1-1 joint genotype calling with HaplotypeCaller[3]. Variant filtering was done using the Variant Quality Score Recalibration (VQSR) method[4]. Variants that passed VQSR filtering were annotated using Annovar (version 2013_09_11) against a variety of data sources. Variants were prioritized by using the PhenoDB analysis tool[5] to identify coding (not synonymous), and rare variants (minor allele frequency <1% in the public databases ExAC, gnomAD, 1000 Genomes, or Exome Variant Server) that were present in both the proband and his mother based on autosomal dominant and recessive and X-linked recessive modes of inheritance. Individuals II-1 and III-1 were tested for the previously detected familial variant at a clinical lab.

For Family 6, Exome capture platform Nimble Gen's Seq Cap EZ Exome Library (Roche Diagnostics, San Francisco, CA, USA); subsequently, sequencing was performed on the Illumina HiSeq200 platform (Illumina, San Diego, CA, USA). The VCF file was annotated by the Ingenuity Variant Analysis (IVA) webtool (Qiagen, USA) and applied standard filters to find the underlying genetic factor.

For Family 7, exome sequencing was performed using DNA extracted from peripheral blood samples, with exons being captured using an Agilent SureSelect Clinical Research Exome kit and captured regions being sequenced with Illumina NextSeq technology. The raw sequencing data was then run through a custom bioinformatics pipeline and analyzed as previously described[6].

Clustal alignment and 3D protein structures

Clustal omega multiple sequence alignment was used to evaluate the protein evolutionary sequence conservation. PDB (Protein Data Bank) templates, 1Y8R and 4W5v protein 3D structures were used to predict the functional impact of *UBA2* identified variants on the encoded protein. Adobe Illustrator and Photoshop were used to generate illustrations of the *UBA2* gene and protein structure.

Whole mount in situ hybridization

For in situ hybridization, a cDNA fragment of *uba2* was PCR amplified from full-length zebrafish cDNA clone (Dharmacon, 5604607) and inserted into pCS2+ vector. As the next step, pCS2+ vector with *uba2* insert was digested with ApaI and BamHI, digoxigenin (Roche, Indianapolis, IN), and labeled sense and antisense RNA probes

were synthesized by SP6 and T3 RNA polymerase (Invitrogen) respectively. Wild type zebrafish embryos at different developmental stages (5 somite, 24, 35, 48, 72 hpf, 5 and 7 dpf) were fixed in 4% PFA (in PBS) at 4°C overnight with slow agitation. All incubation steps of in situ hybridization were carried out as described[7] at 65°C using *uba2* probes. Hybridized embryos were then mounted in 3% methylcellulose and imaged by Leica stereo microscope (M205 FA).

Zebrafish modeling of the effects of *UBA2* variants on the phenotype

Briefly, zebrafish were kept in water with pH (7-7.5), conductivity 500-530 Ω/cm and temperature 28°C under constant light cycles:10 hours of dark and 14 hours of light. A CRISPR/Cas9 *uba2* knock out zebrafish line was generated with a 14 bp deletion/5 bp insertion in exon 1. Healthy and knockout embryos were then studied for survival and morphology at 8 dpf (days post fertilization) using a Leica Stereomicroscope (M205FA) and then fish were genotyped and imaged using immunohistochemistry and whole mount antibody staining.

CRISPR/Cas9 uba2 knock out line generation

CRISPR guides were designed using CHOPCHOP [8], using reference sequence query #NM_213363 (zebrafish assembly GRCz10/danRer10). sgRNAs were amplified, synthesized via in vitro transcription (NEB), and purified (Zymo). One-two cell ABTL embryos were injected with 50pg of sgRNA and Cas9 protein (NEB, cat# M0386M) in a solution of 0.1M KCl and phenol red. sgRNA activity level was measured via somatic tissue activity test (STAT) to genotype the zebrafish as previously described[9]. In F0 founders, we obtained 5 different independent mutant lines harboring predicted

frameshift alleles, of which we choose two lines (with a 14 bp deletion and another with a 5 bp insertion) that result in premature truncation of the *uba2* encoded protein. All fish were genotyped for experiments.

Genotyping the F0 population of zebrafish, a 14bp deletion/5 bp insertion in exon 1 [p.(Gly28Alafs*9) and p.(Gly28Alafs*6) respectively] was discovered and subsequently outcrossed to generate a stable line.

Generated zebrafish knock out survival assay and gross morphology

To determine the impact of *uba2* knock out on fish survival, we set up *uba2* heterozygous fish (14bp del) breeding and collected embryos. Embryos were kept at 28°C in regular water tank system till 21 days (first 7 days 28°C incubator in petri dish) and were collected dead/and or sick (hydrocephaly, enlarged swim bladder, abnormal swimming pattern) fish on a daily basis. We also kept sick fish in separate tanks from healthy ones to prevent competition for food. After 21 days, all fish were euthanized under an animal husbandry protocol, DNA was isolated for genotyping and the survival curve was plotted using GraphPad prism 8. For gross morphological studies, we imaged healthy and sick fish at 8 dpf using a Leica stereo microscope (M205FA) and then the fish were genotyped. Images and measurements were then processed with image j and Photoshop to carry out further analysis.

Restriction fragment length polymorphism assay

After generating stable *uba2* knock out zebrafish lines, genotyping was done using a restriction fragment length polymorphism assay. To extract genomic DNA, 5-8 dpf zebrafish tail fin clips or heads were incubated in 20 ul of DNA extraction buffer (10

mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.45 % NP40 and 0.45 % Tween-20, pH 8.5, add 20 mg/ml Proteinase fresh before use) for 3 hours at 56°C. Samples were then heated at 95°C for 10 min to heat inactivate the Proteinase K. The extracted DNA was then used as template to PCR amplify the sgRNA target site with the following set of primers Fw: 5'-CAACAGAGGTCGCCAGTTAAA-3'; Rv: 5'-CATTCCAAGTCTGTGGTTTTCA-3'. A PCR product of 378 nucleotides was then digested with SfaNI (Biolabs) overnight at 37°C. This digestion results in two fragments (220 and 158 bp) in wild type fish. However, the SfaNI restriction site is lost in zebrafish carrying the 14 bp deletion/5 bp addition, facilitating genotyping of larvae.

Alcian staining

Alcian blue pyridine variant dye (Sigma) was used to stain zebrafish cartilage at 5 dpf after euthanization and fixation of *uba2* fish in 4% paraformaldehyde at 4°C overnight. Then fish were incubated in 0.02% alcian blue solution containing MgCl2 (80mM) and ethanol (70%) overnight at room temperature with gentle shaking. On the following day, stained larvae were serially rehydrated in ethanol containing MgCl2 (10 mM) and Tris-HCl (100 mM) pH 7.5 for 5-10 min each and then placed into 3% hydrogen peroxide (H_2O_2) in 1% potassium hydroxide (KOH) for 2 hours without shaking to bleach the pigment of the larvae (kept tubes open to prevent gas accumulation by H_2O_2). After 2 hours of H_2O_2 incubation, larvae were washed with 25% glycerol solution containing 0.1% KOH for 30 minutes with gentle shaking. 1 mg/ml of trypsin (prepared in 35 % sodium borate solution) was then used to digest soft tissues for 24 hours (or till larvae looked clear) at room temperature. Afterwards, larvae were fixed and stained in 95% ethanol (in series: 25, 50, 70 and 95%) for 10 min each and

stored in 100% glycerol containing 0.1% KOH at room temperature (used glycerol series in 0.1% KOH as following: 25, 50, 70 and 100% for 2-3 hours each). A Zeiss STEMI SV-11 microscope (Brightfield) set up was used to image the stained fish (n=10 each) at 4X magnification.

Immunohistochemistry

For immunohistochemistry, *uba2* zebrafish at 8 dpf were euthanized and fixed in 4% PFA overnight. On the following day, they were washed with PBS briefly and sequentially cryoprotected in 30% sucrose (in PBS) and embedded in OCT (Tissue-Tek) for cryo-sectioning. Cryosections were produced with a thickness of 10 microns and collected on glass slides. Zebrafish cryosections were rehydrated with 0.2% PBST (1X PBS in 0.2% Triton X-100) and incubated in blocking solution (10% goat serum in 0.2% PBST) for 1-2 hours at room temperature. Photoreceptor cells were labeled with arrestin-3 primary antibody (Abcam, ab174435) at 1:500 dilution and incubated at 4°C overnight and counterstained with Alex Fluor 488 Goat anti-mouse IgG (Invitrogen) secondary antibody (1:1000), Phalloidin (1:1000) to label F-actin, and Hoechst 33342 (Invitrogen, H3570) (1:2000) to label cell nuclei for 1 hour at room temperature. The Zeiss LSM 800 was used to make the confocal images. n=8 each.

Whole mount antibody staining in zebrafish

For whole mount antibody staining in zebrafish tails, fish were euthanized, and heads were clipped to extract DNA for genotyping, however, tails were fixed in 4% PFA overnight in 96 well plates at 4°C. Fixed tails (n=15 each) were then washed two times with PBS and water (once) for 5 min each. Then treated with chilled acetone for 7 min (-

20°C) to permeabilize larvae tails, followed by two washes with water and PBS (5 min each). Afterwards, tails were incubated in blocking buffer (3% bovine serum albumin in PBST (0.1% Tween-20)) on slow shaking for one hour at room temperature. They were then incubated with primary monoclonal antibody for type II collagen (II-II6B3) in 1 % BSA prepared in PBST (1:10 dilution) overnight at 4°C with slow agitation. On the following day, tails were extensively washed with PBST for at least 4 times (15 min each) with gentle shaking. After that tails were then incubated with anti-mouse secondary antibody (A32723, Invitrogen), rhodamine-phalloidin and Dapi in 1% BSA prepared in PBST for 2 hours at room temperature with shaking. After secondary labels staining, larvae tails were washed with PBST several times and mounted on slides by using Prolong Gold antifade reagent (Invitrogen) mounting media. Z-stacks of the labelled tails were generated by LSM 800.

Transmission Electron Microscopy (TEM)

For TEM, zebrafish were fixed in fixative containing 2.5 % PBS-buffered glutaraldehyde (pH 7.4) and osmium tetroxide at 5 dpf. Fixed fish (n=3 each) were then embedded in epoxy resin and 90 nm thin longitudinal sections were collected using 200-mesh copper grids. Sections were allowed to dry for 24 hours and then double stained with uranyl acetate and lead citrate. A JEOL JM-1010 electron microscope was used to image obtained sections.

Animal Micro-Computed Tomography

Euthanized zebrafish (n=3 each) were imaged in the National Institutes of Health Mouse Imaging Facility by micro-Computed Tomography. Adult fish were fixed in 4%

PFA and mounted in sealed plastic tube sample holder. Bones were scanned using Skyscan1172 (Bruker-microCT, Kontich, Belgium) at a nominal resolution of 15 microns for whole body fish and 5 microns for head and tail fins. Projections were acquired every 0.4 degrees for whole body scans and every 0.3 degrees for high-resolution fins/heads for a 180-degree rotation utilizing five frame averages. Modified Feldkamp[ii] algorithm was used to carry out reconstruction by using SkyScan™ NRecon software accelerated by GPU[ii].

mRNA rescue

A total of 250 pg of each mRNA was injected into 1-4 cell stage embryos generated through *uba2*$^{+/-}$ to *uba2*$^{+/-}$crosses. The resulting impact on the larvae phenotype was documented at 5 dpf (days post fertilization), followed by genotyping of each of the larvae. Differences between phenotype classes were assessed by the Chi Square test using GraphPad prism. p-values considered to be significant were less than 0.05.

Results

Additional Clinical Details

Family 1: In addition to other features described, the proband has an intermittent tremor and problems with coordination and balance, possibly worsening over time. There is an apparently coincidental history of bleeding dysfunction and hypofibrinogenemia in the mother (III-2), and three of the children (IV-1, IV-3, IV-4); these individuals are each

heterozygous for a likely pathogenic missense variant in *FGG* (p.Asp344Asn). Individual IV-1 also has a diagnosis of chromosome 22q11.2 duplication syndrome. The proband (IV-4) and her mother (III-2) are also both heterozygous for an additional VUS in *WNT10B* (p.Ile285Thr) which was not assessed in the other siblings.

Family 4: The proband (II-1) sweats poorly and has underdeveloped breasts, with sparse axillary hair, but normal pubic hair; she underwent menarche at age 14. She has mildly distinctive facial features, including a broad forehead, high anterior hairline and broad nasal root. She also has a history of kyphoscoliosis requiring bracing, as well as renal hypoplasia with stage 3 kidney disease. She is reported to have bilateral optic nerve hypoplasia but has normal vision. She has a broad forehead, high anterior hairline, and broad nasal root. She also has a history of hypothyroidism and was treated with growth hormone for her short stature. The same proband was also found to harbor several other DNA sequence variations that might contribute to her phenotype (Table 1) including a *de novo* frameshift variant in the *BAZ1B* gene, a candidate gene for growth and neurodevelopmental defects.

Neuronal reduction in *uba2* zebrafish

Arrestin-3 was used to stain photoreceptors and the pineal gland. Photoreceptor density and length were both decreased (Fig. S2). We also observed a decrease in size, and in some cases, absence of the pineal gland in *uba2*$^{-/-}$ fish. Additionally, there was a decrease in overall neuronal number in eye, brain, and pineal gland in the *uba2* loss-of-function model.

Micro-computed tomography data

Inter-fish variability limited our ability to conclude with certainty that skeletal malformations are universally present in adult fish. Abnormally-developed pectoral fins at 5 dpf in our alcian stained *uba2*$^{-/-}$ fish further confirmed *UBA2*'s important role in human limb development. Common but nonspecific limb defects in our affected human cohort included syndactyly, clinodactyly and camptodactyly. Possibly analogous malformations in zebrafish were malformed shoulder girdle and shorter and upright oriented pectoral fins with reduced blades. The developmental mechanism of paired fins in zebrafish and fore/hind limbs in tetrapod's share similarities in molecular pathways during development[10, 11, 12].

Supplementary Figures

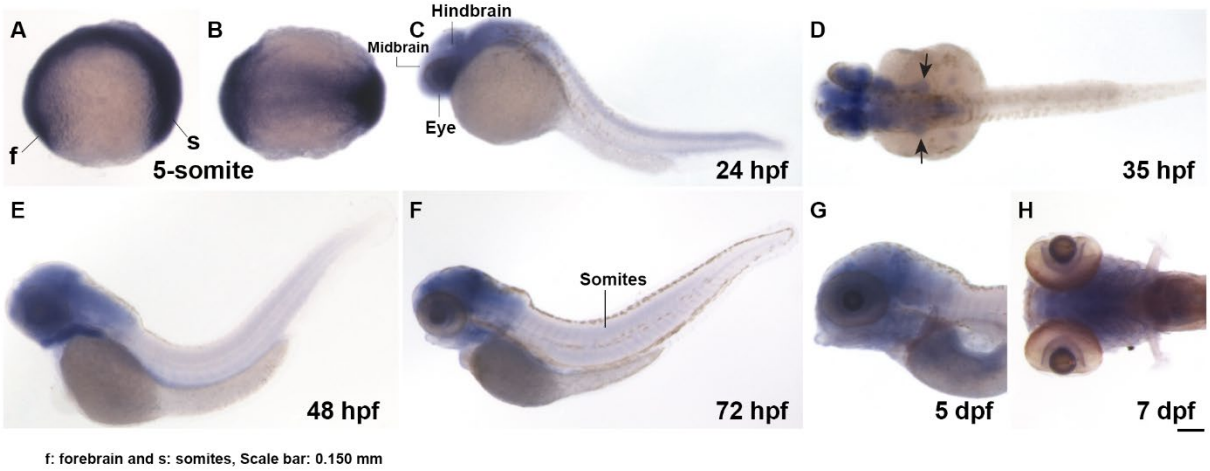

Figure S1: *uba2* expression during zebrafish development. A-B. Lateral and dorsal views of 5-somite zebrafish embryos. Expression of *uba2* is most prominent in the developing brain region from the 5-somite stage onward. C. Lateral view of a wild type embryo showing *uba2* expression concentrated in the eye and subsections of brain such as mid- and hindbrain. D. Dorsal view of a 35 hpf embryo showing signal in pectoral fins. E-G. Lateral views of embryos at 48, 72 hpf and 5 dpf with *uba2* expression in eyes and head. H. Dorsal view of head of hybridized embryo at 7 dpf. f (forebrain), s (somite). Scale bar is 0.150 mm.

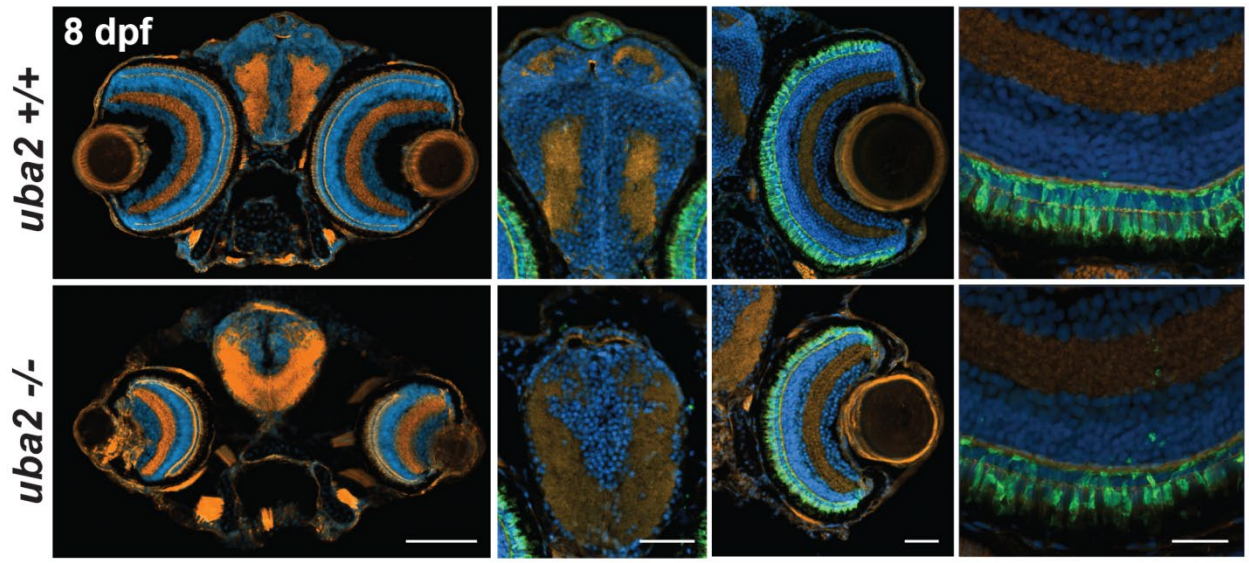

Figure S2: Brain and eye histology of *uba2*$^{-/-}$ zebrafish at 8 dpf. Top panel shows WT and bottom panel shows *uba2*$^{-/-}$ eye sections. Arrestin-3 (Green), Phalloidin (Red) and Hoechst (blue). Scale bar: 100, 40 and 20 µm.

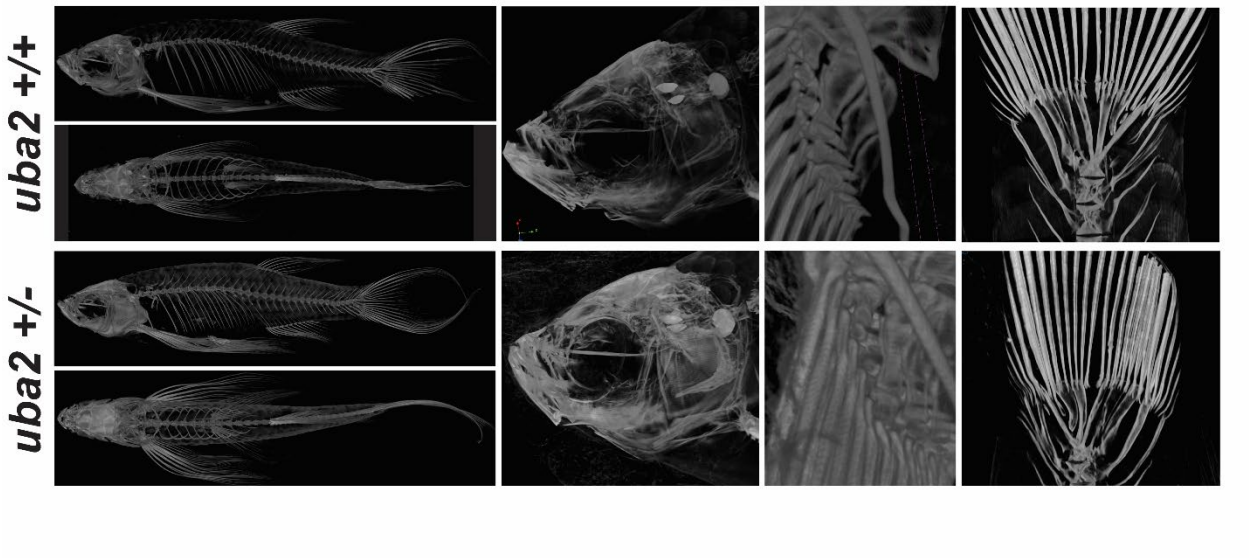

Figure S3: Adult *uba2* zebrafish skeletal survey. Micro-CT of adult wild type (+/+) and heterozygous (+/-) zebrafish revealed grossly normal structures. Rib anomalies (dorsal view, left panel), craniofacial differences (second panel), and pelvic girdle malformations (third panel) were noted to variable degrees in heterozygous fish. Tail fin anatomy appeared similar between fish (right panel).

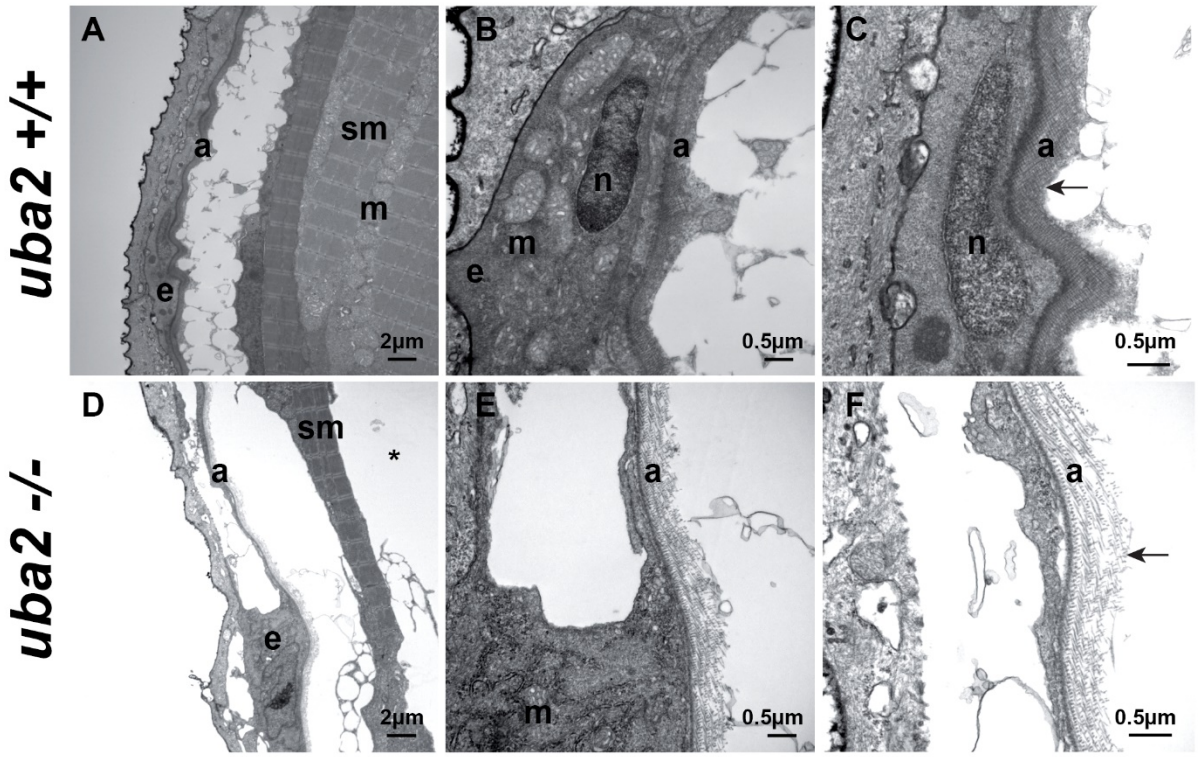

Figure S4: Cellular defects in *uba2* zebrafish fin development. Transmission electron microscopy photographs of *uba2* zebrafish. A-C. TEM images of *uba2* WT (+/+) zebrafish body at 5 dpf. D-F. TEM images of *uba2* mutant ($^{-/-}$) fish at 5 dpf. Longitudinal sections in both panels. There were dense and tightly packed actinotrichia observed in WT fish versus mutant fish. Similarly, in WT zebrafish, we observed normal and well-defined mitochondria, skeletal muscles, and nuclei however, *uba2*$^{-/-}$ fish showed an

abnormal skeletal muscle pattern as well as disorganized mitochondria.

a:(actinotrichia), sm (skeletal muscles), e (epidermis), m (mitochondria) and n (nucleus).

Supplement Table 1: Pathogenicity predictions for *UBA2* missense variants found in our cohort.

Family ID	Family 4	Family 5	Family 7
cDNA change	c.167A>C	c.1447G>A	c.364C>G
Amino acid change	p.Asn56Thr	p.Glu483Lys	p.Arg122Gly
GnomAD	Not found	Not found	Not found
DANN	0.99	0.99	0.99
EIGEN PC	Pathogenic	Pathogenic	Pathogenic
FATHMM-MKL	Damaging	Damaging	Damaging
LRT	Deleterious	Deleterious	Deleterious
MetaSVM	Damaging	Tolerated	Damaging
MutationTaster	Disease causing	Disease causing	Disease causing
MutationAssessor	High	Medium	High
Provean	Damaging	Benign	Damaging
SIFT	Damaging	Tolerated	Damaging
GERP	5.75	5.3	5.5

DANN: pathogenicity score based on deep neural networks. The value range is 0 to 1, with 1 given to the variants predicted to be the most damaging.

EIGEN PC: spectral approach for functional annotation of coding and noncoding region gene variants.

FATHMM-MKL: predicts functional impact of coding and noncoding single nucleotide variants.

LRT: Likelihood Ratio Test, tests significant amino acid conservation in human proteome.

MetaSVM: integrates results of nine prediction tools, MutationTaster, SIFT, Polyphen-2, MutationAssessor, GERP, LRT, PhyloP, SiPhy and 1KG database allele frequency.

MutationTaster: pathogenicity prediction based on evolutionary conservation.

MutationAssessor: functional impact predictor of a variant based upon protein homologue sequence conservation.

Provean: Protein Variation Effect Analyzer predicts effect of variant on protein biological function.

SIFT: *in silico* prediction of impact of nonsynonymous variants based upon sequence homology of closely related sequence.

GERP: Genomic Evolutionary Rate Profiling is a conservation score calculated by quantifying substitution deficits across multiple alignments of orthologues using the genomes of 35 mammals. It ranges from -12.3 to 6.17, with 6.17 being the most conserved.

Supplement Table 2: ACMG/AMP variant classification for *UBA2* variants found in our cohort.

Family ID	***UBA2* variant**	**Inheritance**	**ACMG/AMP classification**
Family 1	p.Trp273Alafs*13	Maternal	Pathogenic: PVS1, PM2, PP1
Family 2	p.Thr460Aspfs*24	*Unknown**	Pathogenic: PVS1, PS2, PM2
Family 3	p.Arg122*	Maternal	Pathogenic: PVS1, PM2, PP1
Family 4	p.Asn56Thr	*de novo*	Likely pathogenic: PS2, PM2, PP3
Family 5	p.Glu483Lys	*de novo*	Likely pathogenic: PS2, PM2
Family 6	p.Leu267*	*de novo*	Pathogenic: PVS1, PS2, PM2
Family 7	p.Arg122Gly	*de novo*	Likely pathogenic: PS2, PM2, PP3

*Presumed occult mosaicism in one parent.

PVS1: Null variant (nonsense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease.

PS2: De novo (BOTH maternity AND paternity confirmed) in a patient with the disease and no family history.

PM2: Absent from controls (or at extremely low frequency if recessive) in genome Aggregation Database (gnomAD).

PP1: Co-segregation with disease in at least 3 affected family members in a gene known to cause the disease.

PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product.

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