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Supplemental information

Loss of C2orf69 defines a fatal autoinflammatory syndrome in humans and zebrafish that evokes a glycogen-storage-associated mitochondriopathy

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Supplemental Data

Supplemental Note: Case Reports

Family 1 from Turkey

The proband is a 1-year-old male who was presented to the pediatric neurology division due to fussiness, constant crying, low tone and unable to hold his head up. He was born at 41 gestational weeks via normal spontaneous vaginal delivery. Pregnancy was uneventful but he was stained with meconium at birth. He cried soon after delivery. Birth weight was 3750 g (65%ile). His development in the first 3 months were unremarkable, smiled at 2 months and had head control at 3 months. He started progressive deterioration in developmental steps starting at 3 months. His hair color became lighter, his belly became more prominent since the last 4-5 months. He was having frequent hospitalizations due to lung infection and periodic fevers.

In addition to lung infections he had a history of tibia, wrist and hip osteomyelitis requiting hospitalizations and long treatments, He had generalized tonic clonic seizures which were under control with phenobarbital and levetiracetam. He died at the age of 18 months due to aspiration pneumonia while being hospitalized for lung infection.

Anthropometric parameters at 1-year-old revealed weight 4.9 kg (-5.3 SD), height: 68 cm (-2.7 SD) and head circumference: 39 cm (-5.8 SD). Physical examination showed severely delayed with no head control. He had dysmorphic facial features including frontal bossing, thin and long philtrum, broad nasal bridge, large tongue, blonde kinky hair. Abdomen was protuberant and the liver was palpable at 4-5 cm below the lower costal ridge. Light microscopic evaluation of the hair showed brittle, hypopigmented hair with varying diameters. Brain MRI revealed severe global atrophy, diffuse white matter hypomyelination, dystrophic and thin appearing corpus callosum and Dandy-Walker malformation. Ophthalmic evaluation showed optic atrophy.

The proband had an 18 months younger brother born at 36 gestational week via normal spontaneous vaginal delivery, with a birth weight of 2.3 kg (12%ile), height of 45 cm (18%ile). Pregnancy was complicated by polyhydramnios. He was loose since birth and had frequent lung infections and fever episodes. He started having seizures at 2 months of life which was under control with low dose levetiracetam. He has a history of cardiac arrest at 3 months of life. In one of his admissions, his hemoglobin was 7 mg/dl requiring erythrocyte transfusion. He was evaluated at 6 months during one of his hospital admissions due to infection. He was hospitalized most of his life. Growth parameters showed weight 4.3 kg (-4.3 SD), height: 58 cm (-3.7 SD) and head circumference: 39.5 cm (-3.1 SD). He was intubated. Physical evaluated revealed hypotonia, decreased spontaneous movements. Had facial dysmorphia similar to brother but exam was limited due to intubation (frontal bossing and hair type was similar). Hair was sparse, thin, brittle. He had malnutrition, decreased subcutaneous fat, hepatomegaly of 4 cm. Eye examination showed optic atrophy.

Brain CT showed diffuse atrophy and thin corpus callosum. He has multiple increased blood levels of C-reactive protein and lactate.

Informed consent was provided according to the Baylor-Hopkins Center for Mendelian Genomics Research Protocol (IRB number: H-29697). Trio exome (I.1, I.2 and II.1) sequencing was performed as previously described [17]. Validation and segregation of the identified variant in all family members were performed by Sanger sequencing using primers flanking the mutation (Forward: 5'-GCTGCTTGATGGGAACCTAC-3'; Reverse 5'-GCTTGTTTTCTCCCAAAAATG-3') .Primary skin cells were obtained from punch skin biopsies performed on I:1 and II:2.

Family 2 from Tunisia

The index was a 1-year-old female, born to first degree consanguineous Tunisian healthy parents. There is no family history of a similar disease. Pregnancy and delivery were uneventful. The index was born by c-section at term for circular cord. Her birth weight was of 3.1 kg, her height birth height was of 50 cm and her head circumference at birth was of 35 cm. She presented weak sucking at birth. She had global and severe developmental delay with hold up of the head at the age of 18 months. At the age of 8 months, she developed focal seizures with chewing, eye lid and hemicorporeal clonies. The EEG showed a slow background with generalized discharges. She was initially treated by valproate until 40 mg/kg/day without improvement (3 times a week). The phenobarbuital was associated with worsening of seizure hence it was stopped and replaced by levetiracetam until 50 mg/kg/day with decrease of seizures but recurrence when infectious episodes.

Neurological examination at the age of 18 months showed a poor contact, no ocular pursuit, language delay, microcephaly (37.5cm; -6SD), axial hypotonia and spastic tetraparesis. General examination noted failure to thrive (weight was of 6.5kg (-3SD) and height was of 85 cm) abnormal facial shape, narrow forehead, hypertelorism and stereotypy. Fundus examination showed neither optic atrophy nor corneal deposits. Brain MRI concluded to cortical and subcortical atrophy. Spectroscopy was normal. CT scan showed no calcifications. Laboratory tests were normal and CSF analysis were normal. Visual evoked potential, electroretinogram and electro-neuromyogram were normal. Metabolic screening (lactate, chromatographies of amino acids and organic acids) were normal. She passed away at the age of 32 months due to status epilepticus.

Informed consents were provided, including consent for scientific publication. The form contains a section for consent for genetic testing related to the disease(s) of the individual, and consent for research (related to the main concern, but implicating genes not yet associated with human diseases). The informed consent form is available upon request with Centogene. Exome and genome sequencing were performed as previously described [18,19].

Family 3 from Saudi Arabia

The index is a 6-month-old female born to consanguineous parents from Saudi Arabia. Family history is positive, a sibling died at 18 months of age with a history of seizure, hypotonia, ventilator dependent and optic atrophy. The index and her twin sister were born by c-section at 36 weeks of gestation. Her Apgar scores were 6 and 8, and birth weight of 2.1 kg. She required active resuscitation and was admitted to the NICU for two weeks. She was noted to have hypotonia and club foot and presented focal seizures. On physical examination (6 month old), all growth parameters were below the 5th percentile for age and gender (height: 53 cm, weight, 4.6 kg, HC:36 cm). She presented hypotonia, and required mechanical ventilation (under sedation). She had fair, brittle hair and facial dysmorphism: narrow forehead, hypoplastic supraorbital ridge, upturned nostril, micrognathia, and depressed nasal bridge. Her twin sister did not present any abnormality. Her brother is similarly affected and presented neurodevelopmental delay, seizures and hypoplastic corpus callosum and cerebellum. Her male cousin was also affected, he had neurodevelopmental delay and intractable seizures. All affected subjects passed away.

Informed consents were provided, including consent for scientific publication. The form contains a section for consent for genetic testing related to the disease(s) of the individual, and consent for research (related to the main concern, but implicating genes not yet associated with human diseases). The informed consent form is available upon request with Centogene. Exome and genome sequencing were performed as previously described [18,19].

Family 4 from Iran

Chief complaint/ First clinical manifestation:
Fever, Recurrent septic arthritis, sepsis

Present illness and past medical history:
He was a two-year-old boy, from a consanguineous marriage, with no complications during the prenatal and delivery process. He was referred to our center following fever, recurrent septic arthritis, neuromuscular disorders (CP), developmental delay, weaknesses, and sepsis. He was repeatedly hospitalized with FUO (fever of unknown origin) which had a poor response to antibiotic treatment. At 19 months old His mother noticed the limited range of motion in babies' left hip and inflammation in his left elbow. The neuromuscular examinations revealed: a limited range of motion in the left elbow and hips, muscular Spasticity in four limbs, and positive bilateral Babinski in favor of Quadriparetic Cerebral palsy (CP). He also had experienced several episodes of absence seizures. (The brain MRI and EEG report are not available). The Pelvic radiography demonstrated left hip dislocation without dysplasia, suggestive of being secondary to trauma or osteomyelitis. The left hip was reduced with surgical technique and a Pavlik harness was implemented. The left elbow was tapped but had no fluid. Tissue smear and culture were negative for common pathogenic bacteria. The laboratory examinations rolled out HIV, TB, EBV, and brucellosis infection. Regarding the recent antibiotic administration, he was diagnosed with septic arthritis, treated with antibiotics for three weeks at the hospital, and discharged with oral antibiotics (Cefixime, Rifampin, and Clindamycin). He was repeatedly hospitalized with elbows and hips septic arthritis from 18 to 22 months old and underwent several surgical interventions (including elbow abscess drainage, hip fistulectomy, and debridements) as well as antibiotic treatment. Regarding recurrent infections, the patient was investigated for suspected immunodeficiency. He had completed vaccination with no adverse effect, no lymphadenopathy, no organomegaly, no leukopenia, no neutropenia, normal NBT, no complement deficiency, normal flow cytometry, a decreased IgA level (3 months-old and 21 months old), the normal response of specific antibody for tetanus-diphtheria. Unfortunately, the patient had passed away at two years and five-month-old due to sepsis following pneumonia. Family history: The parents are first cousins and the patient's sibling had passed away at 11 months old. He had similar symptoms including recurrent infections, arthritis, neuromuscular disorders (CP), and development retardation, and finally died following severe pneumonia. The parents did not recall a similar case in other relatives. Presently they have no other children and the mother had no history of abortion or miscarriage. Unfortunately, no documented report is available for his brother.Validation and segregation of the identified variant in all family members were performed by Sanger sequencing using primers flanking the mutation (Forward: 5’-GCTGCTTGATGGGAACCTAC-3’; Reverse 5’-GCTTGTTTTCTCCCAAAAATG-3’)

Drug History:

Antibiotics: Clindamycin, Rifampin, Cefotaxime, Fluconazole, mupirocin ointment, cotrimoxazole, ceftriaxone. IVIG therapy (the patient received IVIG at birth and 21

months old, for unknown reason despite having normal IgG level). Negative allergy history

Laboratory results	Date 1	Date 2	Date 3	Date 4	Date 5	Date 6
	28/1/2009	30/7/2010	25/9/2010	3/10/2010	9/10/2010	31/10/2010
WBC		15080	7300		18490	13120
RBC		3.69 * 10^6				
Hb		8.9	10.9		8.5	8
MCV		73.2				
Plt		799000	457000		1064000	551000
Neut		58/2%	49.2%		73%	79.3%

Lymph		23.9%	39.5%		17%	18%
Mono		11.7%				
NBT		100%				
CRP						
CD2	25					
CD3	66					
CD4	50					
CD8	15					
CD4/CD8	3.3					
CD19	23					
CD16						
CD56						
BMA						No pathologic finding
PBS		PBS: anisocitosis, hypochroic, microcytosis, target cell				No pathologic finding
IgG	IgG:453,	IgG:1358, IgG1:622 IgG2:312 IgG4:128				IgG1:4478 IgG2:1105 IgG3:934 IgG4:195
IgM	45	100				
IgA	5	128				
IgE	5	23				
ESR		74	82			65
CRP		48				>48
CPK						310
Aldolaze						18
C3	105	145				
C4	30	36				
CH50	95	170				
BUN:		9	10			
Cr		0.3	0.3			
PT		12	12.5			
PTT		33 INR=1	39			
Blood Suger		112	115			

Urine analyse		normal	normal			
Urine culture		Candida.	negative			
Stool exam						
Stool culture						
Blood culture		negative				
Calsium		8.5				
Na		133				
K		4.4				
Other		Wright, Coomns Write And 2me Widal =negative , HIV = negative ANA:0.7 negative Anti DS DNA : 12 negative G6pd: normal		Normal production of IL-6, No respounce to TNF-alpha		PHA:2.1 Candida: 1.75

Para-clinical Reports:

Pelvic radiography report
(19 months old) **Pelvic radiography report:** Disseminated osteopenia in bones, Left hip dislocation with no dysplasia
(19 months old) **Pelvic radiography report 2:** Thick Effusion with debris in the left hip with skin fistula. The left hip is dislocated.
(20 months old) **Pelvic radiography report 3:** Both Femurs are in the acetabulum but the left femur's head is deformed

(2 years old) **Pelvic radiography report 4:** There is Fluid effusion in both hips with acetabulum irregularity.The medial side of the Synovial sac in both hips had debris and eco (size:25 mm). There is a collection or pus in the left iliac fossa (20* 13 mm) with calcifications around the lesion, changes in the left hip in favor of osteomyelitis and dislocation.

Elbow radiography report
(19 months old) **Left elbow radiography report:** Inflammation in the deep soft tissue surrounding the elbow joint decreased bone density and periosteal reaction in distal Humerus bone
(19 months old) **Left elbow radiography report 2:** Thick Effusion with debris around the left elbow .
(20 months old) **Left elbow radiography report 3:** Left elbow-joint effusion with echogenic points.
(20 months old) **Left elbow radiography report 4:** Left Elbow had periosteal reactions due to septic arthritis and osteomyelitis, soft tissue inflammation around the elbow joint
(21 months old) **Right elbow sonography :** Hypoeco regions 9*27 mm and 5* 13 mm in lateral arm muscles in ant compartment s in favor of necrosis, abscess, or hematoma, Thick echogenic fluid in the right elbow with dissemination to the anterior capsule of the elbow joint, in favor of puss in the elbow joint.

(20 months old)**Chest and skull x-ray** Disseminated Low bone density ,Osteomyelitis in the left clavicle ,Normal lung and normal skull
(19 months old) **Inguinal ulcer biopsy result:** The microscopic examination demonstrated: congested fibromuscular and adipose tissue, foamy histocytes, and a few giant cell formation. PAS staining is negative for fungal infections. Acid-fast staining was negative for acid-fast bacillus. These finding were in favor of chronic inflammatory process.
(19 months old) **Abdominopelvic sonography:** No pathologic finding, mass, etc. The liver and spleen had normal eco and size. No gallbladder or kidney stone. Kidney, Pancrase, and bladder were normal. Testis had normal size and eco located at the inguinal canal The right hip was normal left hip was dislocated.
(21 months old) **Ecocardiography** Normal cardiac function and anatomy , no vegetation, no PE

Family 5 from Egypt

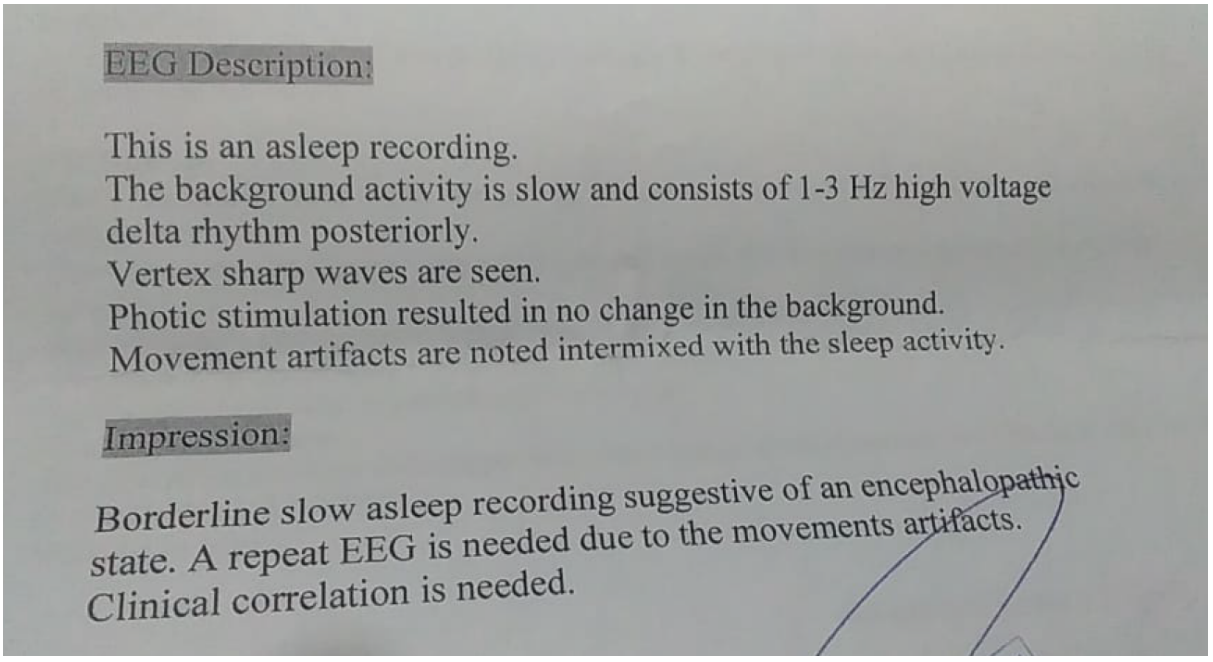
The proband was a female patient aged 6ms old from Upper Egypt. She was the first offspring of healthy first cousin parents (26 years old father and 24 years old mother). No family history of similar condition was recorded. The pregnancy history was uneventful and she was delivered vaginally with Apgar score 8, 10. Anthropometric measurements at birth were all within normal range; weight was 3 kg (-0.8 SD), length 49 cm (-0.3 SD) and head circumference 33 cm (-0.6 SD). Hypoactivity was noted in the neonatal period and at the age of 2 months the first attack of myoclonic epilepsy appeared. Myoclonic fits were several times per day reached 10-15 times as clusters and were uncontrolled with medications as valproate, levetiracetam and clonazepam. The patient was evaluated at the age of 6 months. No milestones of development were acquired she failed to support the head or reacted to surrounding. Recurrent chest infection was recorded and required hospitalization twice with combinations of antibiotics, antiviral and steroids. Hypoactivity, encephalopathy and failure to thrive were distinguished. The weight was 4.7 kg (-3.25 SD), length 59 cm (-3.4 SD) and head circumference was 37 cm (-3.5 SD). Generally, the girl wasn't reactive and didn't follow objects, she was fairly responded to sounds. She had high forehead, light tangled hair, upturned nose, retruded mandible and low set ears. Cardiac and abdominal examinations were insignificant, however, irregular wheezes and crepitations were present on chest auscultation. Neurologically, there is axial hypotonia, muscle wasting, hypotonia of extremities with some infrequent rigidity and preserved reflexes. Investigations showed normal karyotyping, extended metabolic screening, acylcarnitine profile and organic acid in urine. Blood lactate was mildly elevated (24 mg/dl) and ammonia was within normal range. Echocardiography and ABR were normal while fundus examination showed pale optic disc. Recurrent evaluation of infection profile showed elevation of CRP, microcytic hypochromic anemia and leucocytosis with neutrophilia and sometimes lymphocytosis. Neuroimaging identified mild cortical and central atrophic changes, small vermis and thin corpus callosum.

Family 6 from Syria:

This is a refugee Syrian family in which the parents are unrelated but from the same region in north Syria, with 3 unaffected and 3 affected children. Limited information is available on the first and second kids who died many years ago in Syria:

The first daughter was full term and born normal. She had feeding difficulties, irritability and poor feeding, hospitalised on and off for around 2 weeks, lost weight significantly, cachectic and suddenly died at 4 months of age, less than 2 kg at the time of death.

The second boy was full term and born normal (4 kg at birth), one week in hospital, started losing weight, irritable, encephalopathy and brain atrophy and died at 1.5 months. Proband, full term and born normal, 13 days ICU, poor feeding difficulty, NG feeding, fever first and seizures (at 4 months age), cardiac tumour, spasticity, poor vision and cachectic died 11 months, less than 2 kg at the time of death.



EEG Description:

This is an asleep recording.
The background activity is slow and consists of 1-3 Hz high voltage delta rhythm posteriorly.
Vertex sharp waves are seen.
Photic stimulation resulted in no change in the background.
Movement artifacts are noted intermixed with the sleep activity.

Impression:

Borderline slow asleep recording suggestive of an encephalopathic state. A repeat EEG is needed due to the movements artifacts.
Clinical correlation is needed.

Collection Date	02/12/2019 18:32:08		Report Date	20/12/2019 11:11
Test	**Result**		**Unit**	**Reference**
Order Number	**4020120**			
Test Date	1 03/12/2019 18:31:55			
	Chemistry			
Phosphorous	4.13		mg/dl	2.5 - 4.5
Magnesium	2.16		mg/dl	1.6 - 2.4
	Serology			
CRP	106.4	H	mg/L	<5
Order Number	**4021010**			
Test Date	1 03/12/2019 16:33:17			
	Chemistry			
Ammonia, P	47		ug/dl	27.2 - 102
Order Number	**4021445**			
Test Date	1 04/12/2019 10:38:13			
	Body Fluid			
Protein in CSF	11	L	mg/dl	15 - 45
Specimen type	CSF			
Specimen type	CSF			
Volume	1		ml	
Volume	1		ml	
Appearance	Clear			
Appearance	Clear			
Fibrin Clot	Absent			
Fibrin Clot	Absent			
Viscosity	Normal			
Viscosity	Normal			
Glucose, BF	72		mg/dl	
	Hematology			
CBCD				
WBC	12.2	H	$10^{\wedge}9/L$	4 - 1

Family 7 from Iraq:

The index is a 7-month-old female, born to consanguineous parents from Iraq. Family history is positive. Five older siblings are similarly affected (3 females and 2 males). Four of them died during early infancy (surviving up to 3 years), the living affected female sibling is in critical condition. The index patient presented feeding difficulties soon after birth. She has neurodevelopmental delay, failure to thrive and brain atrophy. Her older affected sister is currently 4-year-old, she has feeding difficulties, neurodevelopmental delay, joint contractures, and brain atrophy.

An expanded new-born screening performed at 23-days-old had normal results. No evidence of Hypothyroidism, Galactosemia, Cystic Fibrosis, Congenital Adrenal Hyperplasia, GGPD and Biotinidase Deficiency. There is no indication of amino acid disorders such as: Hyperphenylalaninemia (PKU), Maple Syrup Urine Disease (MSUD), Tyrosinemia type-I, II and 111, Argininosuccinic Aciduria, Citrullinemia, Homocystinuria, Argininemia and Non-Ketotic Hyperglycinemia.

In addition, the acylcarnitines do not show any indication of metabolic diseases. There is no evidence of the following organic acid disorders: 3-Hydroxy-3-Methylglutaric Aciduria (HMG), Glutaric Acidemia (GA) Type I and II, Isovaleric Acidemia (IVA), 3-Methylcrotonyl-CoA Carboxylase Deficiency (3-MCC), Methylmalonic Acidemia (MUT), Malonic Aciduria, B-Ketothiolase Deficiency (BKT), Propionic Acidemia (PROP), and Multiple Carboxylase Deficiency (MCD). And also, no evidence of the following fatty accid oxidation disorders: Carnitine Uptake Defect (CUD), Long Chain L-3 hydroxyl-CoA Dehydrogenase Deficiency (LCHAD), Median Chain Acyl-CoA Dehydrogenase Deficiency (MCAD), Trifunctional Protein Deficiency (TFP), Very Long Chain Acyl-CoA Dehydrogenase Deficiency (VLCAD), and Carnitine Palmitoyltransferase (CPT) I and II.

No evidence of adenosine deaminase severe combined immunodeficiency (ADA-SCIO), which is caused by a deficiency of the enzyme ADA and is the second most common SCIO. There is no evidence, also, of X-Linked Adrenoleukodystrophy (X-ALD).

Additionally, thorax X-ray was normal, Brain CT showed brain atrophy, EEG was abnormal (focal discharges), auditory evoked potentials (4-month-old) showed normal hearing but immature brain stem waves, with recommendation to follow up.

Informed consents were provided, including consent for scientific publication. The form contains a section for consent for genetic testing related to the disease(s) of the individual, and consent for research (related to the main concern, but implicating genes not yet associated with human diseases). The informed consent form is available upon request with Centogene. Exome and genome sequencing were performed as previously described [18,19]. Validation and segregation of the identified variant in all family members were performed by Sanger sequencing using primers flanking the mutation (Forward: 5'-GCTGCTTGATGGGAACCTAC-3'; Reverse 5'-GCTTGTTTTCTCCCAAAAATG-3')

Family 8 from Turkey:

The male proband (II:3) is the third born child of healthy consanguineous Turkish parents, born at 38 weeks of gestation following an uncomplicated pregnancy and vaginal delivery. He had two healthy sisters and family history was unremarkable. Anthropometric measurements at birth were within normal limits [Birth weight: 3100g (50-75p, -0.74 SD), Birth length: 49cm (50-75p, -0.44 SD), Head circumference: 35cm (75-90p, -0.4 SD)]. Soon after birth he developed signs of respiratory distress, which required intervention by ventilatory support in the intensive care unit (ICU). Due to paracardiac infiltrates in the chest X-ray, he was treated with broad-spectrum antibiotics. He received phototherapy for neonatal jaundice and anti-E minor blood group incompatibility was detected.

Complete blood count (CBC) revealed neonatal anemia (hemoglobin: 10 g/dl) with MCV of 111fl and reticulocyte ratio of 4.48%. Absolute neutrophil count and absolute lymphocyte counts were normal for age and he never became lymphopenic or neutropenic throughout his life. LDH dramatically was elevated (3217 U/l) and peripheral blood smear showed presence of normoblasts, spherocytes and schistocytes. In order to identify the underlying cause of congenital anemia, bone marrow aspiration was performed which showed intermediate cellularity, micromegakaryocytes, and erythroid hypoplasia with unremarkable changes in myeloid lineage. Hemoglobin electrophoresis and osmotic fragility tests were normal. He required regular transfusions throughout his life due to persistent anemia.

Throughout his life, the proband was hypotonic with fair hair and skin, but had no apparent dysmorphic or skeletal abnormalities. Laboratory work-up revealed significantly elevated liver enzymes (ALT: 248U/l, AST: 1291U/l) and hyperbilirubinemia (total bilirubin: 17.25mg/dl, direct bilirubin 1.07mg/dl) and was treated by ursodeoxycholic acid. He was given amlodipine as a result of continuous high blood pressure. He had poor feeding, persistent vomiting and mild respiratory distress. Size of liver and spleen were normal by abdominal ultrasonography in the first month of life. Renal artery and vein doppler ultrasound was normal and the echocardiogram showed atrial septal defect (ostium secundum), which was not apparent on follow-up. During the course, he continued to have repeated pulmonary infections, accompanied by elevation in C-reactive protein (CRP) levels and received broad-spectrum antibiotics. Under antibiotic treatment CRP levels decreased but anemia was persistent (Hb: 8.3g/dl at 2 months). Unexpectedly, he developed swollen left knee with inflammatory signs at 6 months, which lasted about 2 weeks and accompanied by mild thickening of synovial wall and increased bursal fluid on ultrasound. Due to his fair hair and skin accompanied by recurrent pulmonary infections, primary immune deficiency syndromes with pigmentation abnormalities, such as Chediak-Higashi syndrome or Griscelli syndrome were considered, but normal immunological assays and microscopic examination of proband's hair ruled out these diagnoses.

Starting from his second month of life, he developed drug-resistant focal epilepsy; received multiple antiepileptic medications, including clobazam, topiramate, carbamazepine, levetiracetam and pyridoxine, but without any improvement. EEG at

2 months revealed asynchronous multifocal epileptiform discharges initially, and left temporal dysfunction with focal epileptic discharges. at 6 months. Metabolic screening tests, including tandem mass spectroscopy and urinary organic acids, were normal. As the proband's multisystemic findings were highly suggestive of a metabolic/mitochondrial disorder, muscle biopsy was performed at the age of 5 months, which showed subtle mitochondrial proliferation, mild cytochrome-C-oxidase deficiency and presence of partially diastase-resistant Periodic Acid-Schiff (PAS) stained granules. These findings were suggestive for mitochondrial dysfunction and glycogen storage disease. Brain MRI at seven-months showed dilatation of all ventricles, cortical thinning and a decrease in cerebral white matter discordant with age. The concurrent MR spectroscopy was normal.

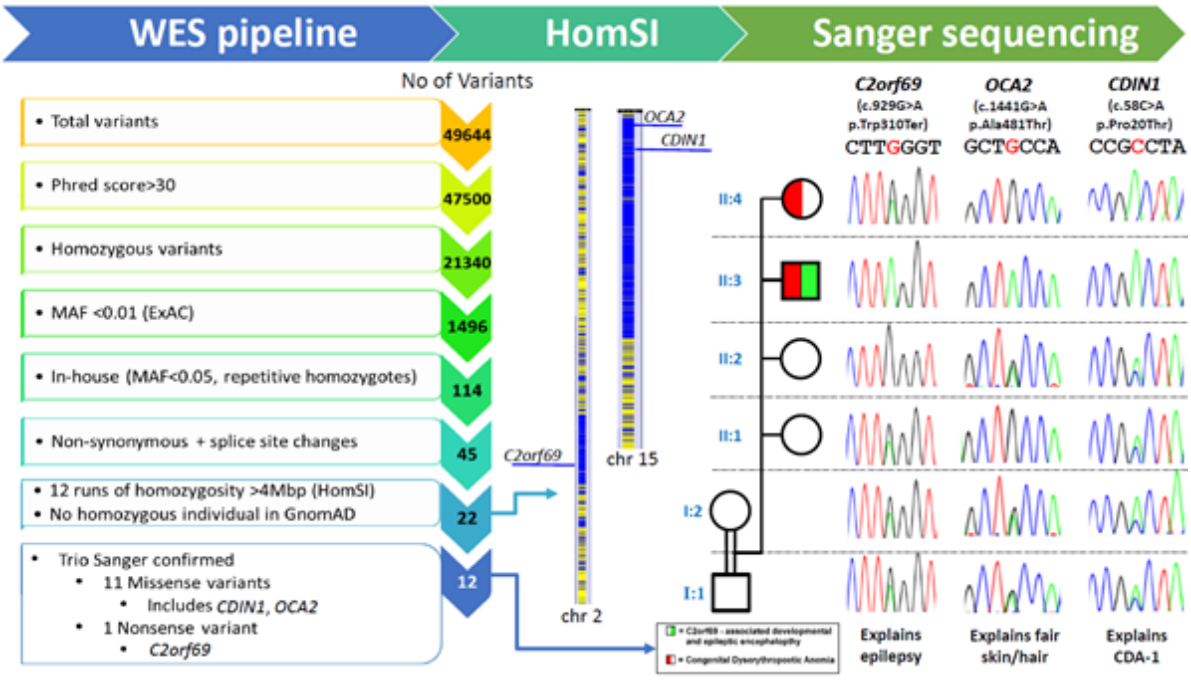
He was admitted to the hospital several times throughout his life mainly for transfusion dependent anemia, recurrent pulmonary infections and intractable seizures. He was hypotonic, microcephalic and undernourished with weight, length and head circumference below 3rd percentile after 6 months. He never gained any developmental milestones and passed away at the age of 1 due to respiratory failure.

After the male proband's death, his sister (II:4), the fourth child of the family, was born at 38th gestational week via uncomplicated vaginal delivery. Prenatal history was not remarkable. She also developed neonatal jaundice and received phototherapy. Her hemoglobin at birth was low (9 g/dl) like her affected brother. CBC was repeated at 2 months due to remarkable pallor and fatigue, which revealed marked anemia with a hemoglobin of 7.8 g/dl and high reticulocyte ratio of 3.19%. Her blood smear revealed polychromasia, hypochromia, anisocytosis and basophilic stippling. After receiving folic acid treatment her hemoglobin levels returned to normal and symptoms improved. Imaging studies included an abdominal ultrasonography showing mild renal pelvis dilation. Abdominal ultrasonography showed mild renal pelvis dilation. She did not have fair hair unlike her brother. Her anemia was under control with no additional symptoms and she did not require blood transfusions. Currently she is 13 months old and has reached developmental milestones timely and did not develop epilepsy.

The marked discordance of phenotypic expression between the 2 affected siblings prompted us to look for a blended phenotype in the proband caused by at least 2 independently segregating Mendelian phenotypes. We performed whole exome sequencing (WES) in the proband and due to apparent autosomal recessive inheritance, we focused on homozygosity (see the figure below). Twelve >4 Mbp runs of homozygosity (uninterrupted blue stretches in the middle portion of figure) were identified in the WES data using HomSI [1]. In these regions, 12 candidate variants were present. One of these variants explained the congenital anemia phenotype in both siblings through a novel homozygous missense mutation in *CDIN1* (aka. *C15orf41*) (c.58C>A, p.Pro20Thr), a newly described gene leading to congenital dyserythropoietic anemia type 1 [MIM 615631]. The proband also had a hypofunctional homozygous c.1441G>A (p.Ala481Thr) variation in *OCA2* on chromosome 15, responsible for regulating skin, hair and iris pigmentation [2]. This variant was absent in the sister, indicating that it is associated with the fair hair in the

proband, which is uncommon in his family. Based on the presence of two rare homozygous variants within the same ~60 Mbp homozygous stretch on chromosome 15, the possibility of uniparental disomy or large deletion was explored. MLPA which covers the critical region for Prader-Willi Syndrome within this genomic stretch on chromosome 15 ruled out both possibilities. The most probable candidate for explaining the divergent neurological findings found in the proband was the only highly damaging nonsense mutation that segregated in the family—a novel nonsense mutation in C2orf69 (c.929G>A, p.Trp310Ter) [MIM 619219]. The clinical and genetic findings for family 8 have also been presented in ESHG 2020 [3].

Informed consent forms for genome-wide high-throughput sequencing were obtained from each individual, which was in accordance with the ethical standards of the Declaration of Helsinki. The DNA from family members was archived in the inherited bone marrow failure syndrome registry and Genome-wide studies were performed in Hacettepe University Medical Genetics Exome Facility. Briefly, DNA libraries from peripheral blood leukocytes for WES were prepared by Ion AmpliSeq Exome RDY kit (ThermoFisher Scientific) and subsequently sequenced by Ion Proton semiconductor sequencer (ThermoFisher Scientific). IonReporter software was used for alignment, variant calling and annotation. Rare and likely damaging variants in homozygosity regions were verified and available family members were genotyped by Sanger sequencing.



Supplemental Figures

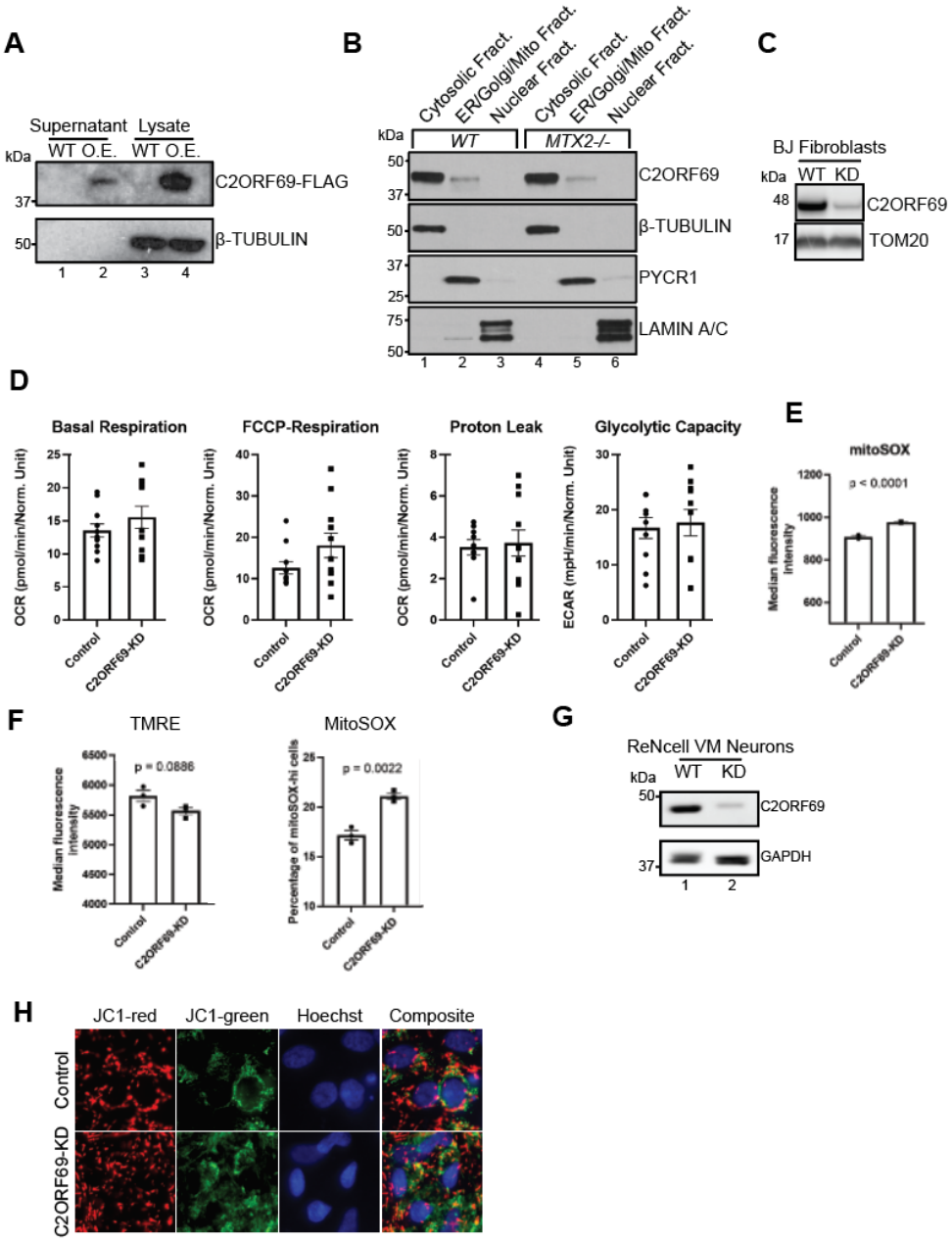


Figure S1: C2ORF69 knockdown efficiency in human cultured cells

(A) Overexpressed Flag-tagged C2orf69 can be found in the supernatant of BJ-TERT cells. The majority is seen in the cell lysate.

(B) Endogenous C2orf69 is mainly found in the cytoplasmic fraction of wt and MTX2 knockout human fibroblasts. A smaller fraction is seen with the mitochondrial extract.

(C) siRNA-mediated knockdown efficiency of endogenous C2orf69 measured by western blotting on BJ Fibroblasts.

(D) Agilent Seahorse mito-stress (3 left panels) and GlycoStress (rightmost panel) test on control and C2orf69 knockdown (KD) fibroblasts.

(E) Mitochondrial ROS sensitive dye MitoSOX (5 uM) measurement of mito-ROS production in control and C2orf69 knockdown (KD) fibroblasts. Each dot represents one well of cultured cells. Data are mean +/- SEM, p-value = unpaired student's t-test.

(F) Mitochondrial membrane potential in control and C2orf69 knockdown (KD) fibroblast measured by TMRE dye (100 nM) by flow cytometry. The same samples were stained with mitochondrial ROS sensitive dye MitoSOX (5 μM). Each dot represents values derived from one well of cells. Data are mean +/- SEM, p-value is derived from unpaired t-test.

(G) siRNA-mediated knockdown efficiency of endogenous C2orf69 measured by western blotting on ReNcell VM Neurons.

(H) Representative images of ReNcell VM neurons stained with ratiometric JC-1 dye.

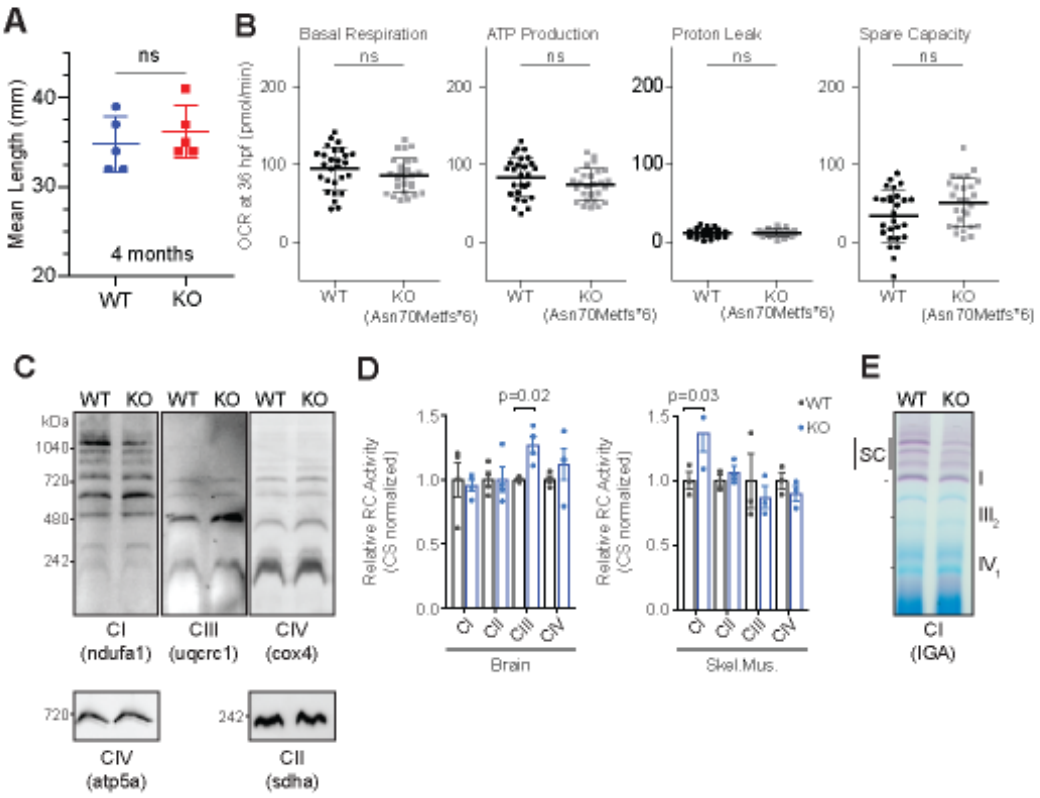


Figure S2
HH. Wong *et al.*, (2021)

Figure S2: C2ORF69 knockout fish have mild respiration defects.

(A) C2orf69 knockout fish are born at Mendelian ratios and at 4 months of age have the same length as their wiltdype siblings.

(B) C2orf69 knockout embryos at 36 hpf show no oxidative respiration defects as measured by a Seahorse mito-stress assay.

(C) Blue native PAGE of purified skeletal mitochondria and western blot for ETC complexes I-IV with the indicated proteins. SC=supercomplexes. Results are representative of 3 independent experiments.

(D) Respiratory chain enzymatic assays of CI - CIV of WT and KO skeletal muscle (left) and brain (right) homogenates. Data are represented as mean +/- SEM, p = p-value from unpaired student's t-test. n=4 biological replicates. Each dot is an average of 3 technical replicates of each biological replicate. Data are mean +/- SEM, p value = unpaired student's t-test.

(E) In-gel assay for CI activity following clear native PAGE of purified skeletal mitochondria. Results are representative of 2 independent experiments.

		Family 1		Family 2	Family 3			Family 4		Family 5	Family 6	Family 7	Family 8
	HPO	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11	Case 12
Origin		Turkey	Turkey	Tunisia	Saudi Arabia			Iran		Egypt	Syria	Iraq	Turkey
Sex		Male	Male	Female	Male	Male	Female	Male	Male	Female	Male	Female	Male
Recessive inheritance	HP:0000007	+	+	+	+	+	+	+	+	+	+	+	+
Gene (MIM619219)		*C2orf69*		*C2orf69*	*C2orf69*			*C2orf69*		*c2orf69*	*C2orf69*	*C2orf69*	*C2orf69*
C2orf69 variant (NM_153689.5)		c.298delC		c.280delG	c.588_592delTTTAA			c.298delC		c.311_313del	c.909_925del	c.298delC	c.929G>A
Predicted protein change (Q8N8R5)		p.(Q100Sfs*18)		p.(E94Sfs*24)	p.(N196Kfs*4)			p.(Q100Sfs*18)		p.(L104_Y105delinsH)	p.(S304Lfs*29)	p.(Q100Sfs*18)	p.(W310*)
Observed protein change (Q8N8R5)		p.0		n.t.	n.t.			p.0		n.t.	n.t.	p.0	n.t.
Clinical synopsis													
Disease onset		3 months	3 months	Congenital	n.a.	n.a.	Congenital	3 months	4 months	2 months	4 months	neonatal	neonatal
Antenatal findings/pregnancy		normal, 41 week ,	normal, 36 weeks,	normal, 41 week , NVD	n.a.	n.a.	preterm, needed	41 weeks, CS	41 weeks, CS	41 week, NVD	41 weeks, CS	n.a.	38 weeks, NVD
Head Circumference at birth (cm)		normal	n.a.	35	n.a.	n.a.	n.a.	normal	normal	33 (-0.6 SD)	normal	n.a.	35 (-0.4 SD)
Birth Weight (kg)		3.75	2.3	3.1	n.a.	n.a.	2.12	3.5	3.7	3.0 (-0.8 SD)	3.5	3.5	3.1 (-0.74 SD)
Birth Length (cm)		normal	45	50	n.a.	n.a.	n.a.	52	50	49 (-0.3 SD)	normal	n.a.	49 (-0.44 SD)
Age at last exam		12 months	6 months	18 months	n.a.	n.a.	6 months	24 months	n.a.	6 months	6 months	6 months	7 months
Head circumference (cm)		39 (-5 SD)	39 (-3.1 SD)	39.5 (-6 SD)	n.a.	n.a.	36 (-6 SD)	n.a.	n.a.	37 (-3.5 SD)	n.a.	n.a.	37 (-5.5 SD)
Failure to thrive, (weight at last exam, kg)	HP:0001508	+, (4.9, -5.3 SD)	+, (4.3, -4.3 SD)	+, (6.5, -3 SD)	n.a.	n.a.	+, (4.6)	+	n.a.	+, 4.7 (-3.25 SD)	+, 2.5 (-5 SD)	+	5.8 (-3 SD)
Post-natal short stature, (length at last exam, cm)	HP:0004322	+, (68, -2.7 SD)	+, (58, -3.7 SD)	+, (85)	n.a.	n.a.	+, (53)	n.a.	n.a.	+, 59 (-3.4 SD)	n.a.	+	60 (-3.5 SD)
Deceased, (age of death)		+, (18 months)	alive (in critical	+, (32 months)	+, (n.a.)	+, (18 months)	+, (24 months)	+, (29 months)	+, (11 months)	+, (9 months)	+, (9 months)	alive (in critical	+, (12 months)
Cause of death		pneumonia	-	status epilepticus	n.a.	n.a.	n.a.	pneumonia	pneumonia	pneumonia	cardiac arrest	n.a.	pneumonia
Brain anomalies													
Developmental delay	HP:0001263	+	+	+	+	+	+	+	+	+	+	+	+
Secondary microcephaly	HP:0005484	+	+	+	+	+	+	n.a.	n.a.	+	-	+	+
Cerebellar atrophy/Dandy Walker variant	HP:0001272	+	+	-	n.t.	+	+	-	-	+	-	+	-
Dysgenesis of corpus callosum	HP:0006989	+	+	-	n.a.	n.a.	n.a.	+	+	+	+	+	-
CNS hypomyelination	HP:0003429	+	+	-	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	+	+
Cerebral atrophy	HP:0002059	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	+	+
Basilar impression	HP:0005758	+	+	-	n.a.	n.a.	n.a.	n.a.	n.a.	+	-	n.a.	-
Cerebellar vermis atrophy	HP:0002335	+	+	-	n.a.	n.a.	n.a.			+	-	-	-
Cerebral cortical atrophy	HP:0006855	+	+	-	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	+	+
Seizures	HP:0001250	+	+	+ (focal, pharmacoresistant)	+ (intractable)	+	+ (focal)	+ (absent seizures, several episodes)	n.a.	+ (Myoclonic, several times a day)	+ (tonic)	+ (Focal and Myoclonic)	+ (intractable, focal)
Severely reduced visual acuity	HP:0001141	+	+	+, (abnormal pursuit)	n.a.	n.a.	n.a.	-	-	+	+	+	n.a.
Optic nerve hypoplasia	HP:0000609	+	+	-	n.t.	+	n.t.	-	-	n.t.	n.t.	n.t.	-
Immune anomalies													
Recurrent fever	HP:0001954	+	+	+	n.a.	n.a.	n.a.	+	+	+	-	+	+
Inflammatory arthritis	HP:0001369	-	-	-	n.a.	n.a.	n.a.	+	+	-	-	-	+ (knee, once)
Septic arthritis	HP:0003095	-	-	-	n.a.	n.a.	n.a.	+	+	-	-	-	-
Aseptic osteomyelitis	HP:0002754	+ (tibia, elbow, hip)	-	-	n.a.	n.a.	n.a.	+ (elbows, hip, clavicle)	n.a.	-	-		-
Elevated C-reactive protein level	HP:0011227	+	+	-	n.a.	n.a.	n.a.	+	n.a.	+	-	-	+
Pneumonia	HP:0002090	+	+	+	n.a.	n.a.	n.a.	+	+	+	+		+
Hypochromic microcytic anemia	HP:0004840	+	+	n.t.	n.a.	n.a.	n.a.	+	n.t.	+	n.a.	n.a.	Anemia due to Congenital Dyserythropoietic Anemia
Response to steroids		n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+	n.a.	n.a.	n.t.
Other phenotypes													
Muscular hypotonia	HP:0001252	+	+	+	n.a.	+	+	-	-	+	+	+	+
General muscle wasting	HP:0009055	+	+	+	n.a.	n.a.	n.a.	+	n.a.	+	+	+	+
Abdominal distention	HP:0003270	+	+	-	n.a.	n.a.	n.a.	+	+	n.a.	-	-	-
Muscular spasticity	HP:0001257	-	-	+	n.a.	n.a.	n.a.	+	+	n.a.	+	n.a.	-
Hepatomegaly	HP:0002240	+	+	-	n.a.	n.a.	n.a.	-	-	-	-	n.a.	-
Osteopenia	HP:0000938	+	n.t.	-	n.a.	n.a.	n.a.	+	+	n.a.	n.a.	n.a.	n.t.
Brittle hair	HP:0002299	+	+	-	n.a.	n.a.	+	-	-	+	+ (sparce)	-	-
Fair hair	HP:0002286	+	+	-	n.a.	n.a.	+	-	-	+	-	-	+
Dry hair	HP:0011359	+	+	-	n.a.	n.a.	+	-	-	+	-	-	-
	-: negative	+: affirmative	n.t.: not tested	n.a.: not available	CS: Caesarean	NVD: Normal							

Unabridged Supplemental Table1

Supplemental Methods

List of antibodies used

Primary antibodies used were anti-C2orf69 (rabbit, 1:500 in 5% milk in TBST, Abcam #188870), anti-Laminin A/C (rabbit, 1:1000 in 5% BSA in TBST, Cell Signalling #2032), anti-PYCR1 (rabbit, 1:2000 in 5% milk in TBST, Proteintech #13108-1-AP), anti-β-Tubulin (mouse, 1:500 in 5% milk in TBST, Merck #MAB3408), anti-MTCOI, 1:3000 (Abcam, ab14705), anti-TOMM20, 1:500 (Proteintech, 11802-1-AP), anti-TIM23, 1:5000 (Proteintech, 11123-1-AP) and anti-CS, 1:2000 (Santa Cruz, SC-390693).

List of primers used

Two guide RNAs against exon 1 of *c2orf69* was used with the following targeting sequences, 5'-AGAGCAGAACATCATTGACG-3'; 5'-GGAAAATGACCGCTGCAACG-3'.

Quantitative real time PCR

Primers used to determine gene expression in zebrafish:

Gene	Forward	Reverse
TNFα	GCTGGATCTTCAAAGTCGGGTG TA	TGAGTCTCAGCACACTTCCATC
TGFβ	CCTTGCTTGCTGGACAGTTT	AATCCGCTTCTTCCTCACCA
TGFβ1a	CCTGCACCTACATCTGGAATG	TGAGAAATCGAGCCATGAACC

TGFβ1b	ACAATGAAGGAGAAGCAGGAG	TTCTAACACAGCAACCCTCAG
IL1β	GGACTTCGCAGCACAAAATGAA	TTCACTTCACGCTCTTGGATGA
Scn1a	GAGCGGTTTGACCCCAATG	GGCAATGCGTAATGGAGGAT
Scn8aa	TGGCTGGATTTCATGGTCATC	GAATGTGCGCAGAGCTGACA
Scn8ab	GCCGTGGCTCTCTCTTCGT	AGCCAGCGGGTTAATTCGA
β-actin	AGAAAATCTGGCACCACACC	AGAGGCGTACAGGGATAGCA

Seahorse analysis on zebrafish larvae

Zebrafish Seahorse XFe24 Mito Stress experiments were performed with minor amendments to Stackeley et al. 2011[1]. Ten single dechorionated 26hpf embryos per line were trapped under meshes in a 24 well islet capture plate. Embryos were sequentially exposed to 9.4 µM Oligomycin A (Sigma 75351), 2.5 µM FCCP (Sigma C2920) and finally 2 µM of both Rotenone and Antimycin A (Sigma R8875/A8674). For basal respiration 8 measurements were taken (Mix: 2 min, Wait: 1min, Measure: 2 min), followed by 16, 8 and 20 for the drug-modified respiration stages. Three replicate runs were performed. Calculations were performed according to manufacturer's instruction.

Constructs and site directed mutagenesis

The wild type C-terminus Flag-tagged C2orf69 (C2orf69-Flag) ORF construct (was purchased from Genscript (#OHu26166). To obtain ORF encoding for patient's LY104_Y105delinH, deletions of 3 nucleotides from the wild type C2orf69 was introduced by PCR, using the Q5 Site-Directed Mutagenesis kit (New Englands Biolabs) with the following primers: Fwd: ATTTCCCTGGGGATGTGCAG and Rvs: GGACGTGATGCTGGGGCG. PCRs were run for 24 cycles of 10 s at 98°C, 30 s at 57°C and 30 s at 72°C. The resulting mutant plasmids were verified by DNA sequencing. The Flag-tagged C2orf69 construct depleted of the first 24 amino acid encoding for the MTS (Δ-MTS-C2orf69-Flag) were generated with Genscript via gene synthesis.

Blue and Clear Native PAGE

Mitochondria were further isolated by centrifuging the homogenates described above at 600 × *g* for 5 min to clear intact myofibrils and heavy cell debris and then spun at 7000 × *g* for 15 min to isolate the desired mitochondrial fraction. 50 µg of purified mitochondria were extracted with 8 g/g digitonin and resolved on 3-12% Native PAGE gels (Thermo Fisher) for Western blotting (blue native) or Complex I in-gel assay (clear native) according to [2].

Statistical analysis

Continuous variables are presented as means with standard deviations. Comparisons were performed with parametric tests for normally distributed data or nonparametric tests when not satisfying this criterion. For multiple comparisons, adjusted P values and confidence intervals were calculated with Šídák correction in one-way ANOVA tests or Bonferroni correction in Fisher's Exact tests. Detailed statistical methods used for individual assays are described in the figure legends.

Supplemental References

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