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Supplemental information

Variants in *ZFX* are associated with an X-linked neurodevelopmental disorder with recurrent facial gestalt

James L. Shepherdson, Katie Hutchison, Dilan Wellalage Don, George McGillivray, Tae-Ik Choi, Carolyn A. Allan, David J. Amor, Siddharth Banka, Donald G. Basel, Laura D. Buch, Deanna Alexis Carere, Renée Carroll, Jill Clayton-Smith, Ali Crawford, Morten Dunø, Laurence Faivre, Christopher P. Gilfillan, Nina B. Gold, Karen W. Gripp, Emma Hobson, Alexander M. Holtz, A. Micheil Innes, Bertrand Isidor, Adam Jackson, Panagiotis Katsonis, Leila Amel Riazat Kesh, Genomics England Research Consortium, Sébastien Küry, François Lecoquierre, Paul Lockhart, Julien Maraval, Naomichi Matsumoto, Julie McCarrier, Josephine McCarthy, Noriko Miyake, Lip Hen Moey, Andrea H. Németh, Elsebet Østergaard, Rushina Patel, Kate Pope, Jennifer E. Posey, Rhonda E. Schnur, Marie Shaw, Elliot Stolerman, Julie P. Taylor, Erin Wadman, Emma Wakeling, Susan M. White, Lawrence C. Wong, James R. Lupski, Olivier Lichtarge, Mark A. Corbett, Jozef Gecz, Charles M. Nicolet, Peggy J. Farnham, Cheol-Hee Kim, and Marwan Shinawi

SUPPLEMENTARY CASE REPORTS

Patient 1

The proband is the second child born to non-consanguineous parents. He was born via C section secondary to severe preeclampsia at 32 4/7 weeks of gestation. Pregnancy was complicated by fetal growth restriction, short femur length and left prenatal pyeliectasis. Family history was unremarkable. His older sister is healthy.

At birth, Apgar score was 9/10/10. Growth parameters were at 5th percentile for weight (1650g) and length (41.5cm), and 10th percentile for OFC (30cm). He exhibited a transient neonatal respiratory distress and central hypotonia. Facials features at birth included bulging eyes, coarse facial traits, pointed chin, smooth philtrum, thin upper lip, deep plantar creases, squared finger tips and deep-seated nails.

His physical examination and additional workup revealed hypospadias (operated at 2 years old), bilateral inguinal hernias and horseshoe kidney. He had hepatic angiomatosis and cutaneous haemangiomas. Evaluation of the heart revealed a repolarization abnormality with normal cardiac architecture and normal heart function.

His evaluation by audiology revealed moderate hearing loss and his ophthalmological examination showed hyperopia and astigmatism. Initial MRI and skeletal X-rays were normal.

During first weeks of life, his main medical issues were nephrocalcinosis secondary to hyperoxaluria and hypoglycaemia secondary to cortisol deficiency. He also presented feeding difficulties, persistent hypotonia and joint hypermobility.

Thought the clinical follow-up, he exhibited a global developmental delay compatible with mild intellectual disability after testing. He acquired walking at 3.5 years old with persistent fine motor difficulties. First words were said after 2 years old with 2-word phrases at age 3 years. He did not acquire complete sentences, leading to communication through isolated words and sign language.

He went to specialized school and exhibited initial difficulties during social interaction and intolerance to frustration. The proband still continues receiving speech therapy, psychomotricity and physical therapy. He is depended for some daily life activities (personal hygiene, self-dressing).

Hi height gain slowed around 8 years of age without endocrinal etiology. Last height was around -1.7 SD at 11 years old and weight and OFC remained in the norms.

Several medical conditions appeared, including digestive issues (esophagitis with gastric ulcer, constipation, dysphagia), respiratory issues (asthma), recurrent otitis (ear tube placement) and cutaneous lipomas. Hyperoxaluria remained while cortisol deficiency resolved.

At 8 years of age, an MRI revealed new cerebral features with moderate cerebellum atrophy, choroid plexus cyst and enlarged frontal sub-arachnoid space.

Several non-conclusive genetic investigations were performed including an array-CGH, metabolic workup, and sequencing of HRAS and other genes in the RAS signalling pathway.

Finally, a trio exome found a missense VUS, c.2290C>T p.(Arg764Trp), in the ZFX gene. It is inherited from his asymptomatic mother and asymptomatic maternal grandmother with no other male carrier.

Patient 2

This patient was born at 42 weeks to non-consanguineous parents by emergency caesarean section for fetal distress. He weighed 2.68kg (2nd centile) with relative macrocephaly (50th centile). He was hypotonic and required intubation and ventilation.

At 5 months, he was seen in the genetics clinic. He had a large anterior fontanelle, a high prominent forehead and dysplastic, and low set, posteriorly rotated ears. He had long, down slanting, palpebral fissures, with ectropion of the temporal aspect of his lower lids. He had blocked nasolacrimal ducts and a tear duct repair. He had a bulbous nasal tip and a prominent pointed chin. His hair was fine and slow growing. He had hyper-extensibility of his finger joints, a single palmar crease and small nails. A vascular lesion on the dorsum of his little finger was noted. Over time, he developed red-brown, discrete macules and papules scantily distributed on his face, trunk and limbs. His skin was soft, pale and translucent. He had seborrheic eczema of his scalp for several years. He had delayed tooth eruption and possible extra teeth. He had bilateral undescended testes with bilateral inguinal hernias and an umbilical hernia. He was noted to have scoliosis in his teenage years.

He had delayed development. He sat late but before 1 year and was walking at 2 years, 1 month of age. He couldn't hold a pen at 5 years, 4 months. He had no sounds or words at 2 years, 10 months. His first words were at 3.5 years. At 5 years, 4 months, he had nasal speech which was difficult to understand. He had input from the speech and language therapy and physiotherapy teams at various ages. He was social at 5 years and 4 months and played with other children. At 12 years of age, he had an IQ of 48. He had full time support in school. At age 17, he has new diagnosis of autism. A re-assessment of IQ suggests this is in the normal range (report awaited). He is in college and completing foundation level 2.

He had a cranial ultrasound that showed an abnormality in his ventricles. His brain MRI revealed an unusually large choroid plexus. He has severe sensorineural deafness in his left ear. He had an abdominal ultrasound scan which was normal. He initially had a normal echocardiogram but developed aortic dilatation at 6 years of age. This has remained stable on atenolol. An array CGH in 2014 showed a - VOUS 7q21.13-7q21.13 0.42Mb loss. A buccal smear karyotype was normal – 46XY with no 12p tetrosomy. In 2014, he had normal 15 gene Rasopathy panel testing. He had normal FBN1, SKI, TGFBR1 and TGFBR2 sequencing. The DDD study and 100,000 genomes project initially found no pathogenic results.

He has the X:24229365-24229365 C>T missense variant which was picked up from the DDD study and parental testing confirmed that the variant was *de novo*.

Patient 3

The proband is the first male child to non-consanguineous parents. The pregnancy was complicated by polyhydramnios. He postnatally exhibited feeding difficulties and prolonged neonatal jaundice. He had pyloric stenosis, which required surgery at 6 months of age.

The proband had macroglossia, which required surgery, hypospadias and bilateral cryptorchidism, umbilical hernia and three cutaneous hemangiomas. He exhibited global developmental delay and learning difficulties. His independent walking was at 3 years of age. He also exhibited delays in speech and fine motor skills. He is able to read and write

but with difficulties. He also exhibited recurrent respiratory infections and had mild hypogammaglobulinemia.

At last evaluation at the age of 10yand 6m, his weight was 35kg/+1SD, height was 147 +1.5SD, and OFC was 56 cm (+1.5SD). His main dysmorphic features were large mouth, everted lower lip, thin upper lip, deep plantar creases, and deep-seated nails. Bilateral hallux valgus and supernumerary tooth were also noted.

Because of his clinical course and findings, Simpson-Golabi Behmel and Costello syndromes were clinically considered on the differential diagnosis.

Several non-conclusive genetic investigations were performed including an array-CGH, metabolic workup, and sequencing of HRAS and other genes in the RAS signaling pathway. His trio exome sequencing revealed a de novo missense, c.2312C>T p.(Thr771Met) in the ZFX gene.

Patient 4

This female proband was born at 39w5d gestational age by uncomplicated vaginal delivery. Bilateral inguinal hernias were identified and repaired in infancy. Macroglossia was noted in early childhood prompting a genetics evaluation where an exam revealed additional findings including large hands and feet, prominent lashes, and macrotia. Her workup revealed normal Beckwith-Wiedemann methylation studies, a normal chromosome microarray, and normal urine glycosaminoglycans. Other issues in childhood included recurrent otitis media requiring bilateral tympanostomy tubes and a small, narrow palate that required a palate expander. She also has atypical scoliosis with grade 2 spondylolisthesis at L5 relative to S1 and L5 bilateral spondylolysis.

This individual developed multiple endocrinopathies during adolescence including persistent hypercalcemia. Comprehensive evaluation revealed inappropriately normal PTH level and urine calcium excretion leading to a working diagnosis of familial hypocalciuric hypercalcemia. She also has a history of amenorrhea with evidence of hypogonadotropic hypogonadism. Her first menses was at the age of 15 years and she had a total of 11 cycles before developing amenorrhea. Labs have shown persistently low LH, borderline low FSH, and low estradiol levels with normal testosterone, DHEA-S, prolactin, and thyroid studies. Brain MRI did not reveal causative pituitary findings.

This individual met her developmental milestones on time and did not require any special educational services. She is an excellent student and is attending college. She does have neuropsychiatric challenges including generalized anxiety disorder, ADHD, and autism spectrum disorder. She re-established care with genetics during early adulthood due to her history of hypercalcemia. Exam at the time revealed thick eye brows, down-slanting palpebral fissures, and a prominent chin. Fragile X repeat testing was sent due to her amenorrhea and this returned normal. Trio exome sequencing was sent that was non-diagnostic, but did reveal a heterozygous, *de novo*, candidate variant of uncertain significance in the *ZFX* gene [c.2312 C>T; p.(T771M)].

Patient 5

Proband ZFX05 was born at 41 4/7 weeks gestational age to a healthy non-consanguineous couple with no known family history except for a bicuspid aortic valve in the father. Pregnancy was complicated by decreased fetal movements around the time of his due date. Respiratory effort at birth was weak and there was no heartbeat detected. He received chest compressions, was intubated, and underwent a cooling protocol. He was noted to have macroglossia, organomegaly, and hypo- then hyperglycemia. A clinical diagnosis of Beckwith-Wiedemann was made. Cardiac imaging showed a bicuspid aortic

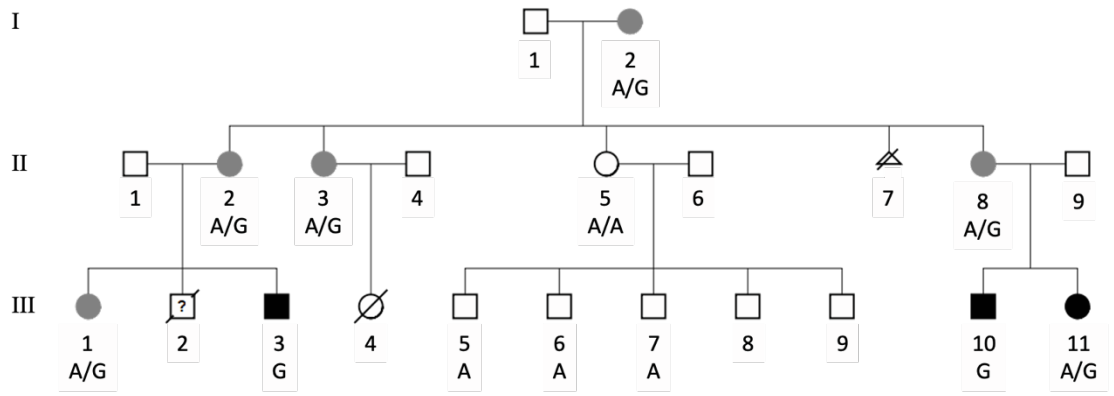
valve, coarctation of the aorta and PDA. He had repair of the PDA and coarctation of the aorta within the first 3 weeks of life and then repair of a pyloric stenosis and inguinal and umbilical hernias. He had a 9 weeks NICU stay. At 9 months of life he had a tongue reduction and then required re-repair of an inguinal hernia and hypospadias repair. He had many episodes of otitis media during childhood and required several PE tubes, an adenoidectomy, and repeat tongue reduction.

Developmental milestones were all delayed. He sat at 1 year of age, walked at 2 years of age, and had his first expressive words at 5 years of age. He was diagnosed with autism and has always been in special classes in school and continues to receive speech, physical, and occupational therapies.

Work-up included array CGH showing a paternally inherited 10q21.3 duplication of uncertain significance and normal Beckwith-Wiedemann methylation studies. Urine organic acids, plasma amino acids, a peroxismal disorders panel, and carnitine levels were normal or inconclusive.

At 10 years of age a whole exome sequencing trio was done showing a de novo ZFX variant of uncertain significance.

Family 6



Pedigree of Family ZFX-6: Dark black circles and squares- affected individuals. Light black circles- carrier females diagnosed with hyperparathyroidism (except for III-1). A is the wild type ZFX allele chrX-24229396 A>G (c.2438A>G:p.(Tyr774Cys)); G is the mutated ZFX allele at same position.

Patient 6A (III:3 in the pedigree): This is a male proband who was born at 39 weeks of gestation via Caesarean section for symptoms of pre-eclampsia. The pregnancy was otherwise uncomplicated. This was his mother's third pregnancy. Amniocentesis was performed and results of karyotype (46, XY) and alpha-fetoprotein were normal. He was macrosomic at birth with a weight of 4.125kg, a length of 53cm, and a head circumference of 37.5cm all above the 90thcentile for gestational age. He was noted after delivery to have dysmorphic and "coarse features" including thick lips, macroglossia and large hands and feet. He was normoglycemic. He had palpable liver and spleen 2cm below the costal margin. A small umbilical hernia, a large right inguinal hernia, undescended testes and hypospadias were also found. Because of the constellation of findings, Beckwith-Wiedemann syndrome was suspected clinically. At 11 years of age, a diagnosis of Simpson-Golabi-Behmel syndrome (SGBS) was made based on an X-linked inheritance pattern in the family,

diaphragmatic hernia in deceased sibling III:2, and III:3's history of neonatal overgrowth, coarse facial features with thick lips and macroglossia, hypotonia and intellectual disability. Proband III:3 had a right inguinal herniorrhaphy with orchidopexy at 2 weeks of age. At 12 months of age, he underwent a reduction surgery for his macroglossia and rerouting of his sublingual salivary ducts for excessive drooling. His hypospadias and umbilical hernia were repaired surgically at 4 years of age. He required multiple sets of tympanostomy drainage tubes for chronic serous otitis media. He also had an adenoidectomy and tonsillectomy. Ultrasound surveillance was recommended for Beckwith-Wiedemann related tumours but these were unremarkable. Developmentally, Proband III:3 had global developmental delay presenting with hypotonia and delayed motor milestones in infancy. He received physiotherapy in infancy and early intervention with physiotherapy, occupational therapy and speech therapy in early childhood. His neuropsychological evaluation at 6 years of age confirmed mild intellectual disability and his cognitive abilities were consistent with those of a three-year-old child. He attended a mainstream primary school with support from an integration aide. His social and self-help skills as well as his behaviours were judged to be satisfactory. He was noted to have mild coordination problems (not specified).

On physical examination at 6 years and 7 months, his head circumference was at the 80%ile, weight at the 50%ile, and height at the 5%ile. His build was described as "bulky", he had a prominent tongue despite a reduction surgery and an "erythematous nevus" over his eyebrows, outer cheeks and chin. He had small longitudinal pits in the skin behind his left ear lobe and helix. He had prominence of the right posterior thoracic wall but scoliosis was not confirmed. He did not have any evidence of hemihypertrophy. Subsequently, a mild thoracic scoliosis was diagnosed radiologically 8y8m with associated short stature (height < 3%ile). Scoliosis surgery was not required.

In addition to scoliosis, III:3's skeletal phenotype included flexion contractures of both elbows (20degrees) and both knees (10 degrees). He had broad hands with mild limitation of extension of the MCP and PIP joints of the fingers of both hands. His DIP joints in the fingers of both hands could hyperextend. He had bilateral hallux valgus deformity, very short 4th metatarsal, and his 4th toe crossed over his third toe bilaterally.

Proband III:3's ectodermal features included abnormal anterior and posterior head hair whorls, sparse eyebrows with lateral thinning and general hypertrichosis. As a child, he had soft skin generally, keratosis pilaris on his trunk and upper arms. His childhood facial findings included naevus simplex over his medial forehead, both upper eyelids and his chin. As a young man, he had seborrheic dermatitis of the forehead and eyebrows. Proband III:3's fingernails and toenails were small in childhood. As a young adult, his fingernails were hyperconvex and angled back with a more acute nail-bed: nail angle.

Proband III:3 had five supernumerary maxillary teeth adjacent to his central upper incisors and medial to his upper canine's preventing the normal eruption of his canines and his lateral upper incisors. These were removed surgically prior to orthodontic treatment. Additional concerns were a crossbite, thickened gums, dental caries and poor oral hygiene. The risk of caries and oral hygiene issues was exacerbated by a dry mouth following the redirection of III:3's salivary ducts.

Proband III:3 was referred for an ophthalmology assessment at 7 years for significant myopia requiring glasses. His corrected visual acuity was 6/12 on the right and 6/24 on the left. Complete absence of the sphincter papillate bilaterally was noted. Proband III:3 had bilateral retinal detachment as a teenager. He had multiple surgeries but vision could only be saved in one eye.

Proband III:3 developed epilepsy that was more difficult to control in his early adult years. His epilepsy presented with a 2-minute non-febrile generalized convulsion at 9 years of age. He was not hypoglycaemic at the time. He had additional generalized tonic colonic seizures in childhood and was treated with carbamazepine. His epilepsy was well controlled until his early 20's when he had more frequent seizures. EEG at 22 years of age for frequent partial seizures showed frequent bilateral interictal activity that was more prominent in the posterior temporal regions. A single generalized seizure was recorded from sleep. Levetiracetam, Lamotrigine and lacosamide treatments were not effective. He was reviewed by a neurologist at 23 years of age and was treated with clonazepam and carbamazepine. By 25 years of age, his seizure control had improved with infrequent dyscognitive and convulsive seizures on a combination of carbamazepine, phenytoin and clobazam.

A striking feature of III:3's phenotype was his long-standing hypothalamic hypogonadism with secondary osteopenia. An initial clinical finding was an absence of body hair persisting into adulthood. At 18 years of age, despite absent body hair, his penis and testicles were noted to be "normal". He did not have gynaecomastia. His bone age was 14 years at a chronological age of 18 years and 3 months. He suffered an ankle fracture at age 16 years following a fall. He had fracture of his left upper humerus following another fall at 21 years of age and a different fracture of his left mid-shaft of his humerus during a seizure shortly after the first humerus fracture healed. His bone density showed a T-score of -3.9 at both his lumbar spine and hip and he was commenced on Strontium ranelate by his GP. He was formally assessed by an Endocrinologist at 22 years of age. He had a "generally hypogonadal" appearance but without gynaecomastia. His left testis was 12.5 mL and his right testis 10 mL in volume. Both were soft in texture. Penile length was 5 cm. Despite his testicular volume, his androgen profile was consistent with hypogonadotropic hypogonadism with baseline serum testosterone levels of 2.2 nmol/L and 2.3 nmol/L and an LH of 2 IU/L and FSH of 4 IU/L. His SHBG was elevated (126 nmol/L, his calculated free testosterone was 16pmol/L and his oestradiol level was not detectable. Cortisol, prolactin, thyroid function, parathyroid hormone level and serum calcium, phosphate, magnesium and vitamin D levels were all normal. Screening for other secondary causes of osteopenia was negative. Mild thrombocytopaenia was thought to be caused by anti-epileptic medications and did not require treatment. Proband III:3's androgen replacement was commenced slowly, initially using testogel, subsequently Axiron transdermal testosterone solution and more recently intramuscular reandron. At 33 years of age III:3's testosterone level was 18.2 nmol/L on Testosterone undecanoate 1000mg IM every 16 weeks.

Proband III:3 had a friendly and happy predisposition as a child. His behaviour in his teenage years was repeatedly noted to be challenging and of concern to his parents. Repeat educational psychology testing placed his full-scale IQ at 60. He was enrolled in a special education secondary school for his schooling for additional support. As an adult in his mid-twenties, Proband III:3's behaviour is much less challenging and no longer a concern for his family. Currently, he attends a supported work program one day a week and music and theatre groups 2 days per week. He lives with his parents and is not in a relationship currently.

Male baby III:2's clinical status is uncertain and DNA was not available for genetic testing due to neonatal demise shortly after delivery in 1986. He presented antenatally with maternal polyhydramnios at 30 weeks' gestation. Ultrasound identified hydrops fetalis and what was thought to be an intrathoracic "growth". Antenatal banded karyotype following amniocentesis was normal (46, XY). No further genetic testing was undertaken at the time. He was delivered at 35 weeks' gestation by Caesarian section. He died 25 minutes after

birth secondary to respiratory insufficiency. A post mortem examination was performed and revealed a large right congenital diaphragmatic hernia with liver herniation. There was cardiac distortion and presumed cardiac compromise with hydrops fetalis, and pulmonary hypoplasia bilaterally, more severe on the right side. At autopsy, syndromic features were not noted nor discussed with the family and it is possible that II:2 had incidental isolated right congenital diaphragmatic hernia. However, it was partly on the basis of III:2's CDH that a possible diagnosis of Simpson-Golabi Behmel syndrome was considered for III:3 and III:10.

Female III:1 died at 38 years and 8 months of age from metastatic colorectal carcinoma that had been diagnosed at 37 years and 2 months of age. She had no symptoms or signs of hyperparathyroidism and her pertinent biochemical studies were normal. She had no other medical problems and had normal childhood development and cognitive abilities.

Female child III:4 had agenesis of the corpus callosum, a possible malformation of cortical development, global developmental delay, epilepsy and severe visual impairment. Her problems were judged not to be related to the problems in her cousins III:2 and III:3. DNA was not available for retrospective ZFX gene testing.

At 34-week gestation, a prenatal ultrasound scan identified a fetal brain anomaly with increased intracerebral fluid filled spaces thought to be dilated ventricles with fetal hydrocephalus. However, this was not confirmed on postnatal imaging. A CT scan at one week of age identified agenesis of the corpus callosum with a high-riding and dilated third ventricle and colpocephaly with dilated posterior horns of the lateral ventricles. Delayed sulcation and reduced grey-white matter differentiation raised the possibility of a malformation of cortical development. MRI was not performed. As a newborn III:4 had a relatively small head with a narrow forehead. She fed normally and was not noted have any abnormal neurological findings. She needed phototherapy for unconjugated hyperbilirubinemia. She was seen by an ophthalmologist at 10 weeks of age for reduced visual acuity. She was not reacting to light and her pupils constricted sluggishly. Her eyes were both normal in structure with the exception of optic disc pallor and her left optic disc being smaller than her right. These changes were not thought to be significant enough to diagnose optic nerve hypoplasia. She was reviewed by a clinical geneticist at the same time. She was feeding and sleeping well. Her head circumference was 39cm (25^{th} -50^{th} centile) with open fontanelles and she had good head control. She had mild dysmorphic facial features including mild hypotelorism. She had pectus carinatum with "some curving of the dorsal spine". A clinical diagnosis of holoprosencephaly was made and her parents were counselled that III:4 was at the mild end of the holoprosencephaly spectrum. III:4 was fostered at approximately 6 months of age and there are no medical records of her findings thereafter. The family reports that she subsequently struggled with epilepsy and was not able to sit independently, walk or talk at 18 months of age. The family did not have significant contact with III:4's adoptive parents after this time.

Female II:2 (Mother of Patient 6A) had regular health and development in early childhood. She was diagnosed with scoliosis at 9 years of age and managed with a Milwaukee brace for three years. The angle of her scoliosis curve exceeded 45 degrees despite bracing and she had surgery with Harrington rod fixation at age 12 years. II:2 had cognitive abilities in the normal range (not formally tested) and attended mainstream schooling. She did not complete her final year of schooling but married at a young age and had her daughter, III:1, at the age of 18. She and her partner had two more male children over 5 years, including III:2 who died shortly after birth due to CDH and the family proband III:3. II:2 stayed home to raise her children and to manage III:3's needs. She reports that back pain was a chronic issue and she had secondary problems with right hip pain as a result of her spinal curve. II:2 joined the workforce at 43 years of age as a supermarket cashier. She had increased back pain as a result and needed time off work intermittently in her 40's. Ongoing symptoms resulted in an extensive two stage anterior and posterior spinal fusion using rib grafts at 50 years of age. She experienced post-operative back pain, pain and paresthesia related to the

harvesting of her ribs, unexplained right sided abdominal, and right foot paresthesia in the absence of bladder symptoms. She experienced restricted movement of her spine but also limited tolerance for sitting and reduced effort tolerance. She was not able to work and received a disability pension. At 51 years of age, II:2 saw a gastro-enterologist for persistent abdominal pains and constipation in the setting of opioid analgesia requirements. Endoscopy and laparoscopy were negative. Blood tests at the time identified hypercalcemia and she was referred to an endocrinologist with bone aches, lethargy, short term memory loss and hyperparathyroidism. II:2 was assessed for parathyroidectomy. Pre-operative blood biochemistry showed calcium 2.64 mmol/L (RR 2.15-2.65), corrected calcium 2.70 mmol/L (RR 2.15-2.65), phosphate 0.8mmol/L (RR 0.8-1.4), ALP 149 U/L (RR 30-115), and otherwise unremarkable LFTs. PTH was 15.6pmol/L (RR 1.5-7.6). A 24hr urine collection showed a urinary calcium excretion of 12.5 mmol/24hr (RR 2.0-7.5). Pre-operative ultrasound neck imaging identified a 12mm left parathyroid lesion inferior to the left thyroid lobe. A parathyroid Sestamibi scan noted retention in left and right inferior poles of the thyroid gland. Ultrasound images of the thyroid gland revealed bulkiness of both thyroid lobes with multiple nodules in the right lobe, left lobe and isthmus, and small cysts in the left lobe totaling 16 in number. The largest two nodules were in the left isthmus and 19 and 16mm in size, respectively. Fine needle aspirate from the largest left nodule and the largest right nodule (11mm) showed thyroid tissue with benign changes.
Bilateral neck exploration and parathyroidectomy was performed.
The left inferior parathyroid was markedly enlarged (640mg), contained packed sheets of chief cells. The right superior parathyroid was moderately enlarged (290mg) with predominantly shaved chief cells. The right inferior parathyroid was mildly enlarged. Atypia or malignancy was not detected. Histology was judged to be in keeping with parathyroid gland hyperplasia. The left superior parathyroid gland was not identified. Postoperative biochemistry 3weeks after surgery showed persistent hypercalcemia (corrected calcium 2.97 mmol/L) and increased parathyroid hormone (9.8pmol/L). Repeat localization studies placed the 4th parathyroid gland at/ within the left lower pole of the thyroid gland. The gland was removed approximately one year after the initial surgery. It was moderately enlarged (252g) with predominantly chief and clear cells seen on histology. Blood biochemistry had normalized 2 weeks later. Osteopenia/ osteoporosis was diagnosed 5 months post-surgery on DEXA scan with left femoral neck BMD 0.698 g/cm2 (T score -1.4, in the osteopenic range) and left forearm BMB 0.494 g/cm2 (T score -3.3, in the osteoporotic range). She had a fractured toe with significant trauma. She was treated with combined vitamin D3 and calcium tablets and magnesium but not bisphosphonates. In addition to calcium and vitamin D supplementation, II:2's current regular medications at 57 years of age include levodopa / carbidopa for restless legs, a statin for hypercholesterolemia, Gabapentin for nerve pain, paracetamol and turmeric for musculoskeletal pain, and vitamin C, CoQ10 and zinc supplements for wellbeing. II:2 has hyperconvex and ridged finger-nails and small toes nails similar to her son III:3's. She has an interpupillary distance of 5.8cm with a head circumference of 55cm and is not considered to be dysmorphic.

Female II:3 is a 56-year-old woman who was diagnosed with hyperparathyroidism when she was 50 years old. He initial symptoms included lethargy but she had no constipation or kidney stones. She underwent parathyroidectomy of 2 parathyroid glands.
She fractured her right femur 10 years ago after a fall from standing height. Her other medical issues include osteoarthritis, depression, fibromyalgia, and osteoporosis.

Female II-8 is the mother of Probands ZFX06_B and ZFX06_C. She is 48 years old and was diagnosed with hyperparathyroidism at age 43 when she presented with lethargy. She underwent parathyroidectomy of 3 ½ glands. Her past medical history is unremarkable. She had no developmental or cognitive concerns. She currently receives no medications.

Male Patient 6B (III:10 in the pedigree) was delivered vaginally following face presentation at 39 weeks of gestation. Mild polyhydramnios was noted late in the pregnancy but routine ultrasound scans were otherwise unremarkable. He was normally grown with a birth weight of 3,315g (50%ile), a length of 51cm (50-90%ile) and a head circumference of 36.5cm (90%ile). Facial swelling settled over 24 hours but his macroglossia persisted. He was treated with triple phototherapy at 36 hours for neonatal jaundice (unconjugated bilirubin 315umol/L, conjugated 5umol/l). He and his mother were O positive and direct Coombs was negative. A left inguinal hernia was noted. He was transferred to a tertiary neonatal unit on day of life 3 with the combination of the hernia, jaundice, breathing difficulties and dysmorphic facial features. On admission, he was treated with oxygen, antibiotics and phototherapy. Echocardiogram showed a PDA, a PFO, persistent pulmonary hypertension and poor left ventricle contraction that was treated with oxygen and dobutamine for 6 days and captopril for 10 days. Capillary glucose was monitored and hypoglycemia was not recorded. Jaundice resolved on day 6. He was assessed by a clinical geneticist and given a diagnosis of Beckwith-Wiedemann syndrome. A renal ultrasound scan showed large but otherwise normal kidneys (R5.6cm/Lt5.9cm). His macroglossia caused feeding difficulties that improved with a Habermann teat feeder.

His banded karyotype was normal. He was diagnosed with pyloric stenosis at four weeks of age after he had vomiting with increasing frequency for 72 hours. He had a pyloromyotomy and bilateral inguinal herniotomies.

III:10 was evaluated in genetics at 4 years of age. He had mild global developmental delay. He had walked at 21 months of age and could run and climb stairs at 4 years. His first words were at 18 months and he was speaking in full sentences by four years of age. As a young child, III:10 attended an early childhood intervention program for speech and occupational therapy. Bilateral chronic serous otitis media had been treated with ear ventilation tubes. He had mild atopic eczema and infrequent episodes of wheezing. He attended a regular kindergarten. His height was on the 75th centile, his weight was 2 kg above the 97th centile and his head circumference was just above the 98th centile. A diagnosis of Simpson-Golabi- Behmel was considered. However, GPC3 sequencing was normal.

He was seen again at 8 years of age with dysmorphic features and mild global developmental delay. He had a number of morphological features in common with his maternal male cousin III:3 and his sister III:11. His speech was not clear as a result of his macroglossia. He could feed himself, color and paint. He struggled with scissors and his drawings were immature. He attended a mainstream primary school with an integration aide for support. Formal testing recorded his full-scale IQ as 67. His growth followed his previously noted centiles. His medical problems included persistent macroglossia, bilateral supernumerary upper jaw teeth (above the premolar/ molar tooth junction), myopia without retinal detachment, enlarged adenoids and tonsils treated surgically, scoliosis, and an admission for pneumonia.

III:10 attended a special education school for his secondary schooling. He had delayed puberty onset (Tanner stage 2 pubic hair and 5-6ml testicle volume at 14y6mo) with a growth spurt, concordant bone and chronological ages and some early blood testosterone (0.9 nmol/L) and LH (1.0 IU/L) level changes as assessed by an endocrinologist pre-scoliosis surgery. When reviewed at 15y3mo of age, puberty had progressed with 12 ml testicle volumes and Tanner stage 3 pubic hair development and he was discharged from endocrinology follow up. As age 16 years, he had a staged anterior thoracolumbar release and posterior instrumentation and fusion procedure for progressive scoliosis. Two subsequent lumbar CSF leaks were treated with blood patches. Chronic rhinosinusitis was treated surgically by endoscopic sinus washout, nasal septoplasty, inferior turbinoplasty, nasal polypectomy and partial adenoidectomy. Glaucoma was diagnosed and treated medically. As a young adult he currently has a mixed weekly program including part time work at a disability service in a supported environment two days a week, a day of performing art and dance, a day volunteering in an animal shelter and a day for therapies (physiotherapy and speech therapy) and medical appointments.

In addition to initial clinical diagnoses of familial Beckwith-Wiedemann syndrome and Simpson-Golabi-Behmel syndrome, a diagnosis of FG syndrome had been considered at 8 years of age but ruled out based on careful review of his clinical course and facial features.

Patient 6C (III:11 in the pedigree) is an 18-year-old female who completed secondary school and who works as a waitress. Fetal macrosomia and polyhydramnios were noted antenatally. She was delivered at 38 weeks of gestation by emergency Caesarian section following maternal antepartum hemorrhage due to abruptio placentae and an abnormal cardiotocograph. She required resuscitation (initial Apgar scores of 2^{1}, 5^{5} and 5^{10}) and 48 hours of ventilation for blood aspiration with right lung atelectasis. She was noted to have coarse facial features, macroglossia and macrosomia and clinically diagnosed with Simpson-Golabi-Behmel syndrome in a manifesting female with reference to the same clinical diagnosis in her older male sibling. She had neonatal jaundice that did not require treatment (peak bilirubin of 249/8 (umol/l) on day 5 of life). She had cyanotic episodes observed at 2 weeks of age that were attributed to her macroglossia causing intermittent upper airway obstruction documented on polysomnography. Her obstruction improved when sleeping prone and she did not require surgery. GERD was diagnosed in the neonatal period by pH study and managed with thickened feeds. Her auditory brainstem response was normal for both ears. She attended mainstream schooling and had a part time integration aide during primary school. She struggled with mathematics. She attended mainstream secondary school without an aide and completed her senior education certificate in a technical stream. As a young adult, she has a learner driving license and is aiming for her driver's license. She lives with her parents.

Female I:2 is a 76-year-old woman who was diagnosed with hyperparathyroidism and underwent right inferior minimally invasive parathyroidectomy when she was 73 years old. Her medical history is otherwise unremarkable.

Methods: Whole genome sequencing (Complete Genomics) was performed on a single individual from family 6 (Patient 6B). Single nucleotide and structural variants were filtered against public databases (gnomAD, minor allele frequency (MAF) < 0.0002; Complete Genomics 69 genomes MAF = 0) and based on an X-linked inheritance pattern. The coding c.2321A>G variant was segregated through gDNA extracted from blood of consented family members by Sanger sequencing.

Patient 7

This is a male patient who was born at term via spontaneous vaginal delivery to non-consanguineous parent. He has 2 healthy older siblings. His birth weight was 2.75kg. The pregnancy was complicated with gestational diabetes mellitus, requiring insulin treatment, and polyhydramnios, which was detected at 23 weeks of gestation. Labor was complicated by shoulder dystocia with low Apgar scores. He was admitted to neonatal ward as he had poor sucking, cleft palate, hypotonia, coarse and dysmorphic features. He had poor feeding since birth, requiring orogastric tube feeding, anti-reflux formula milk and anti-reflux medication. Due to feeding difficulty, a magnetic resonance imaging (MRI) of brain and spine was performed on day 6 of life, and it revealed subacute left parietal and posterior fossa subdural haemorrhage, right parieto-occipital subarachnoid haemorrhage, and bilateral choroid plexus cysts. This was treated conservatively.
He had stridor and required non-invasive ventilation since 1 week of age. Airway assessment revealed laryngotracheomalacia and mid-tracheal stenosis. He underwent aryepiglottoplasty surgery and bilateral inguinal hernia repair at 3 months of age. He was discharged home with non-invasive ventilation at 4 months old. Besides the history of

bilateral middle ear effusion, his hearing and eye assessment were reported to be normal. He stopped using non-invasive ventilation and orogastric tube feeding at one year old. He was doing well without frequent hospitalisation after that.
He had global developmental delay, for which he required speech therapy and occupational therapy since infancy. At his last assessment at age 9 years and 10 months, he has mild intellectual disability. He is attending special education classes. He can communicate in short sentences and interacts well with friends in school. He understands a lot and able to identify many objects. He is independent for his daily activities. His weight and height were at the 75th centile and head circumference was at the 50th centile. Physical examination showed prominent forehead, hypertelorism, downslanting palpebral fissures, thin upper lip, prominent jaw, low set posteriorly rotated ears, pectus carinatum, reducible umbilical hernia, lax finger joints, hallux valgus bilaterally. He has a normal parathyroid hormone level, echocardiogram, kidney scan, chromosomal microarray and metabolic workup.
Trio whole exome sequencing was performed using NovaSeq was performed at Takara Bio Inc. (Kusatsu, Japan).

Patient 8

Patient ZFX08 is a 13-year-old male with global developmental delay, dysmorphic features, hearing loss, congenital heart defects, Pierre-Robin sequence (cleft palate repaired), feeding difficulties (G tube dependent), chromic respiratory failure (tracheostomy-dependent), and hyperparathyroidism complicated by hypercalcemia requiring parathyroidectomy at age 13 years.

He was at 35 weeks of gestation via C-section with low Apgar scores and pregnancy was complicated by oligohydramnios, fetal growth restriction 2 vessel cord. His birth weight was 1.96 kg, and his birth length was 48 cm. He did not pass the hearing newborn screening. The patient was in the neonatal intensive care unit for 4 months because of respiratory distress and feeding problems. He underwent tracheostomy at 3 weeks of age. He exhibited feeding difficulties poor weight gain during the first few months of life. He was found postnatally to have aortic coarctation, which required 2 repair procedures and he remained with mildly dilated ascending aorta. His subsequent echocardiography revealed mildly redundant mitral and tricuspid valves. He required hearing aids for his conductive hearing loss. His sleep study showed obstructive events. He was diagnosed with gastroesophageal reflux and feeding difficulties, for which he had G-tube placement and Nissen fundoplication. He had 2 corrective eye surgeries for strabismus and continues exhibiting myopia and astigmatism and was diagnosed with bilateral posterior polar cataracts. He is status post cataract extraction and intraocular lens and Yag in both eyes.

Patient ZFX08 developed hypercalcemia secondary to primary hyperparathyroidism. He required 2 ½ gland parathyroidectomy at age 13 years and pathology was remarkable for one hypercellular parathyroid tissue with no adenoma. He was found to have bilateral non-obstructing renal calculi but his renal function studies are normal.

He exhibits recurrent tracheobronchitis and asthma. He failed decannulation but currently is not mechanically ventilated and doesn't require oxygen supplementation.

He underwent umbilical hernia reduction and inguinal hernia repair and is followed for chronic mild thrombocytopenia of unknown etiology. His thrombocytopenia panel was negative.

Patient ZFX08 started exhibiting significant motor and language delays since 1 year of age. He started sitting unsupported around 3 years, crawling 3 years and walking at 4 years but continues requiring some support with walking. He started using utensils at 4-1/2 years. He

is nonverbal and currently exhibits global developmental delay, autism and significant behavioral concerns, specifically aggression and agitation. He is not potty trained yet. He attends special education classes at 7th grade. He receives physical, occupation and speech therapies as well as ABA for autism

Family history is significant for a brother who died at 4 months of age due to multiple complications of extreme prematurity (24 weeks gestation) including liver failure, NEC, sepsis and respiratory failure. No genetic testing was performed for him. The mother had a history of delayed speech and needed speech therapy. She underwent 3 surgeries for parathyroid glands due to hyperparathyroidism. Histology showed parathyroid hyperplasia. She had a history of chronic anemia due to bleeding gastric ulcers. She underwent an excision of a benign tumor on the back of her knee (fibroma). The proband's father had a history of bipolar and ADHD. The parents are of Northern European ancestry.

On most recent physical examination, he was small for his age: his weight was 27.2 kg (<1 %ile; Z= -2.94), height 127 cm (<1 %ile; Z= -3.61) and OFC was 52.3 cm (12 %ile; Z= -1.15). Tracheostomy and G tube were in place. He had dysmorphic features including relative macrocephaly, broad forehead with metopic ridging, low anterior hairline, smooth and long philtrum, thin upper lip, micrognathia, thickened helices with asymmetric ears that were relatively large. Pectus excavatum was noted. He had squared finger tips, symmetric abnormal creases in feet, deep seated toenails, hallux valgus deformity. Small phallus and small scrotum sac with retractable testes. He had truncal hypotonia and stiffness in both elbows.

Results of initial exome sequencing were negative. However, exome reanalysis revealed that the patient is hemizygous for the p.R786Q (c.2357G>A) variant in the X-linked gene ZFX.

Patient 9

Proband ZFX09 is an 18-year-old biological female who is now a transgendered male and was the third-born child of non-consanguineous parents. He was born at 38-week gestation by induced vaginal delivery due to pregnancy-induced hypertension. There was a nuchal cord, but Apgars quickly returned to normal. At two months of age, he had an echocardiogram due to possible murmur and the study was essentially normal. Prominent coronary arteries without obstruction or aneurysm were noted but repeat study at 13 months of age was normal with normal coronary arteries noted.

At 3.5 months of age, the patient had herniorrhaphy for an umbilical and two epigastric hernias. He had seven hemangiomas of infancy on skin, scalp and liver. A right scapular hemangioma and a right parietooccipital scalp dermoid were concurrently excised at the time of the herniorrhaphies.

The patient has a history of longstanding eustachian tube dysfunction and was first seen in ENT at 12 months of age due to a history of recurrent episodes of otitis media and bilateral middle ear effusions, at which time he underwent bilateral tympanotomies and insertion of middle ear vent tubes. At 15 months of age, the patient was re-evaluated in ENT due to snoring at night and restless sleeping. Vent tubes were replaced and a partial selective adenoidectomy was performed at 16 months of age. The adenoids were 4+ and almost totally obstructing the posterior choanae. The patient's breathing initially improved and there was one episode of tonsillitis. The patient was seen again in ENT and the impression was dysphagia, for which speech therapy consultation was recommended.

The patient achieved developmental milestones as follows: sat alone at 6 months, crawled at 9 months, walked with support at 11 months, walked alone at 15 months. Speech/language milestones were difficult to evaluate. The patient had avoidance of meat and other difficult to chew and swallow textures due to choking, which could be severe enough to result in vomiting. He had a tonsillar voice quality which impacted speech acceptability and intelligibility, with an occasional slight wet sound to phonation. Speech was essentially unintelligible to the unfamiliar listener on first production. Speech output was reduced. ENT impression was that the patient manifested a significant feeding/swallowing problem related to oropharyngeal airway restriction secondary to significantly enlarged palatine tonsils and possibly adenoids.

A sleep study performed at 22 months of age showed evidence of sleep-disordered breathing characterized mainly by hypopneas associated with moderate hypoxemia. There was elevated pCO2 suggestive of possible obstructive hypoventilation. The patient underwent an adenotonsillectomy for upper airway obstruction as well as dysphagia associated with tonsillar hypertrophy.

At 2y3m of age, the patient was seen by dermatology for a lymphatic malformation of the left axilla. At 2y6m of age, the patient had intermittent rectal prolapse and constipation. A solitary rectal polyp was removed during colonoscopy. At 4y10m of age the patient was seen by Genetics due to history of multiple hemangiomas and lymphangiomas. Clinical exam noted long and down slanting palpebral fissures, upturned nose, high arched palate, normal uvula, small chin, and hypoplastic fifth toenails bilaterally. Chromosome analysis was 46,XX.

The patient was noted to have prolonged bleeding with cuts and nosebleeds, and testing by Haematology was negative. Concern for possible Ehlers-Danlos syndrome was raised. The patient was noted to have a history of hypermobility and low tone.

The patient underwent reduction of lingual tonsillar hypertrophy and expansion pharyngoplasty for low lying palate and had another abnormal sleep study, showing evidence of residual obstructive sleep apnea. He followed up in Genetics at age 5y10m, at which time clinical exam noted prominent nose, slightly cupped ears, pectus excavatum and flaring of ribs inferiorly, hypermobility of finger, knees, and thumbs, skin showed some hyperextensibility. The geneticist felt that the patient's clinical features were consistent with Ehlers-Danlos syndrome type 3 (hypermobility type), and the history of vascular malformation suggested the possibility Klippel-Trenaunay-Weber syndrome but insurance did not authorize testing. DNA microarray was recommended and came back normal.

At age 7y, the patient was seen in the ER for acute onset of left-sided chest pain described as sharp and constant, and chest wall tenderness on physical exam led to consideration of costochondritis. The patient was seen repeatedly in the ER for chest pain.

He had septal cautery for epistaxis as well as re-excision of recurrent lymphatic malformation from left axilla and excision of melanocytic nevi from temporal scalp, periumbilical area, and posterior scalp.

The patient underwent neuropsychology evaluation at age 10y and his general intellectual abilities were average. He demonstrated relative weaknesses in visuo-motor coordination and executive functioning skills related to planning and decision-making. The weaknesses noted were not at a level consistent with a diagnosis, and the deficits described were likely related to difficulties in learning math. Re-evaluation occurred at age 15y, and the patient was felt to be at risk for a diagnosis of a specific learning disorder in math.

The patient underwent total thyroidectomy at age 13y. Pathology showed a focus of papillary thyroid cancer classified by ATA pediatric thyroid cancer as low risk. He underwent remnant ablation and there was no cervical lymphadenopathy. At age 15y the patient had surgical removal of a neck mass and histology was consistent with a parathyroid adenoma. He required a short course of calcitriol and calcium carbonate and has since maintained eucalcemia without any medications. His calcium baseline is now normal whereas he previously had slight hypercalcemia before the parathyroid adenoma was removed.

Other medical concerns include congenital QT syndrome due to a parentally inherited *KCNH2* variant, bilateral mixed conductive and sensorineural hearing loss, cyclic vomiting syndrome, gastroparesis, gastroesophageal reflux, chronic abdominal pain, chronic headaches, migraine, idiopathic intracranial hypertension, proteinuria and microscopic hematuria, POTS, euthyroid autoimmune thyroiditis, familial hypocalciuric hypercalcemia, generalized anxiety disorder and major depressive disorder. He also had a year long history of fatigue, nausea, dizziness, shortness of breath, and rapid weight gain. A core biopsy was performed of a lesion on the left 10th rib and showed benign/reactive fibro-osseous lesion with no evidence of malignancy. He has had a longstanding history of generalized body and bone pain.

The patient underwent WGS trio analysis in 2018 was found to have a *de novo* variant in ZFX (R786Q) through research analysis of WGS raw data. A paternally inherited KCNH2 c.526C>T (p.Arg176Trp) was also identified in this patient, consistent with a family history of prolonged QT interval.

Genome sequencing for patient 8 and parental samples was conducted through Illumina (Medical Genomics Research, Illumina Inc) using the Illumina NovoSeq at an average of 40 x coverage and evaluated single nucleotide variants (SNVs), small indels, copy number variants and mitochondrial DNA SNVs.

Patient 10

Proband ZFX10 is a 10-year-old male who was the first-born child of his non-consanguineous parents. His mother was 20 years old at the time of conception. There were no teratogen exposures. During the pregnancy, nonspecific findings on ultrasound were found including a 2-vessel cord and enlarged cisterna magna. Owing to poor fetal growth, there was an induction of delivery at 35 weeks gestational age. Birth weight was 1860 grams, reflecting fetal growth restriction, and Apgar scores were 6 and 9, at 1 and 5 minutes, respectively. At birth, other features were noted including bilateral post axial polydactyly of the feet and hypospadias. No specific syndrome was apparent at the time on physical exam.

His early childhood was complicated by a vein of Galen thrombosis and bilateral subdural bleeds after a minor trauma. He was also diagnosed with congenital nystagmus in infancy. He has mesocardia and a history of a ventricular septal defect, now closed. He also has bilateral hydronephrosis. He has a history of mild to moderate developmental delay, and now probable mild intellectual disability with ADHD. However, on formal assessment at 11 years of age, he does not have evidence of autism. In follow up, his height is below the 3rd percentile with head circumference at the 55th percentile. He has a distinctive facial appearance characterized by turricephaly, a long face, eyebrows that are sparse laterally and full medially with marked synophrys. He has short and upslanting palpebral fissures with remnant epicanthal folds, a long nose, prominent columella and relatively indistinct philtrum. After birth, he had normal chromosomal microarray. Over the course of his childhood, other

diagnostic investigations included normal 7- dehydrocholesterol and a ciliopathy sequencing panel (Blueprint Genetics) both nondiagnostic. Calcium and parathyroid hormone levels are normal in the patient and his mother. The family was then enrolled for clinical trio exome sequencing (Blueprint Genetics) with the detection of a novel maternally inherited frameshift variant in *ZFX*.

Patient 11

Proband ZFX11 was born at 37 weeks' gestation by Caesarean section. He spent 30 days in the NICU following delivery with APGARs of 0 at 1 minute and 5 at 5 minutes. He had an extensive workup in the NICU with concern for sepsis, was noted to have feeding difficulties, and required a g-tube while admitted. While admitted, he was observed to have a mild/moderate central renal collecting structure prominence of questionable significance and an increasing bilateral hydronephrosis with moderate right and moderately left pyelocaliectasis. His g-tube was removed at discharge.

He was noted to have mild developmental delays early on. Fine motor weakness, gross motor delays, and history of speech delay are all noted in this patient's history. He required surgical intervention for bilateral inguinal hernias (2010) and for superior oblique palsy of the right eye (2019). He was also noted to have left sided strabismic amblyopia. He has also had ventilation tubes inserted in bilateral ears and required removal of both his tonsils and his adenoids.

Over the course of his genetics workup, he was evaluated by several different geneticists and had multiple genetic tests performed. A chromosomal microarray and a next generation sequencing analysis of 23 genes associated with the RAS pathway were completed in 2015, both of which were normal.

Physical examinations revealed multiple distinctive features, though nonspecific for a known genetic syndrome. Features noted on his physical examination included asymmetry of the face, hypertrichosis to the forehead, a prominent forehead, thick hair, a low posterior hairline, synophrys, thick eyebrows, low-set posteriorly-rotated cupped ears (left>right), leftward nasal deviation, high palate, retained baby teeth, a short and asymmetric neck with prominent right posterior musculature, a grade II-III/VI systolic murmur best heard over the left sternal border, jagged nail edges, and slightly decreased extension to his bilateral elbows.

He was evaluated through the school system in 2015 and was noted to have a level of intellectual functioning within the average range, although with varied abilities, and his academic achievement was observed to be adequate aside from math, with his adaptive functioning in the average to above average range. Following evaluation by Cardiology in 2016, he was found to have a small hemodynamically insignificant muscular ventricular septal defect. In 2018 he was evaluated through Paediatric Psychology and was noted to have a full-scale IQ of 76 in the 5th percentile and a 95% confidence interval of 71-83, denoted as in the borderline to low average range. He was worked up for a palpable lump of the right popliteal region in 2019 with bilateral superficial varicosities without evidence of thrombophlebitis found.

In 2020 he had whole exome sequencing trio performed through the Greenwood Genetic Center, which identified a de novo variant in *ZFX*. Following this, PTH and Calcium levels were tested and were normal.

Patient 12

This 8-year-old boy was born at 38+5 weeks by vaginal delivery following an unremarkable pregnancy. He was the first child to non-consanguineous parents. His mother has a clinical diagnosis of oculocutaneous albinism and Autistic Spectrum Disorder (Pedigree Figure). His father has no medical problems. He has a younger brother who is now 6 years of age and has nystagmus with abnormal visual evoked potentials but his development has been normal.

Born at 2.58kg (-1.70 SD), he struggled to feed and required nasogastric feeding support for the first week of life. He otherwise made good progress. At the age of 7 months, he was noted to have nystagmus and small nails and was hence referred to genetics.

He sat at 11 months, started commando crawling at 12 months but did not walk independently until 23 months. He babbled by 12 months but did not say single words until two and a half years. He started speaking in sentences by 4 years, although his pronunciation was poor due to his protruding tongue.

He has several autistic traits, including intolerance to change and lack of understanding of social norms, although does not have a formal diagnosis. He has sensory processing difficulties and is under dental review as he has a tendency to push his tongue against his bottom teeth, which has resulted in wearing down of the secondary roots. He attends a specialist school for the visually impaired and his English and Maths skills are currently at Key Stage 1 (5-7 years) whilst all other subjects are at Key Stage 2 level (7-11 years). He has recently developed tightness in his quadriceps, which is worse when he has been sat for long periods of time. He has regular physiotherapy but this does not limit his ability to walk or exercise.

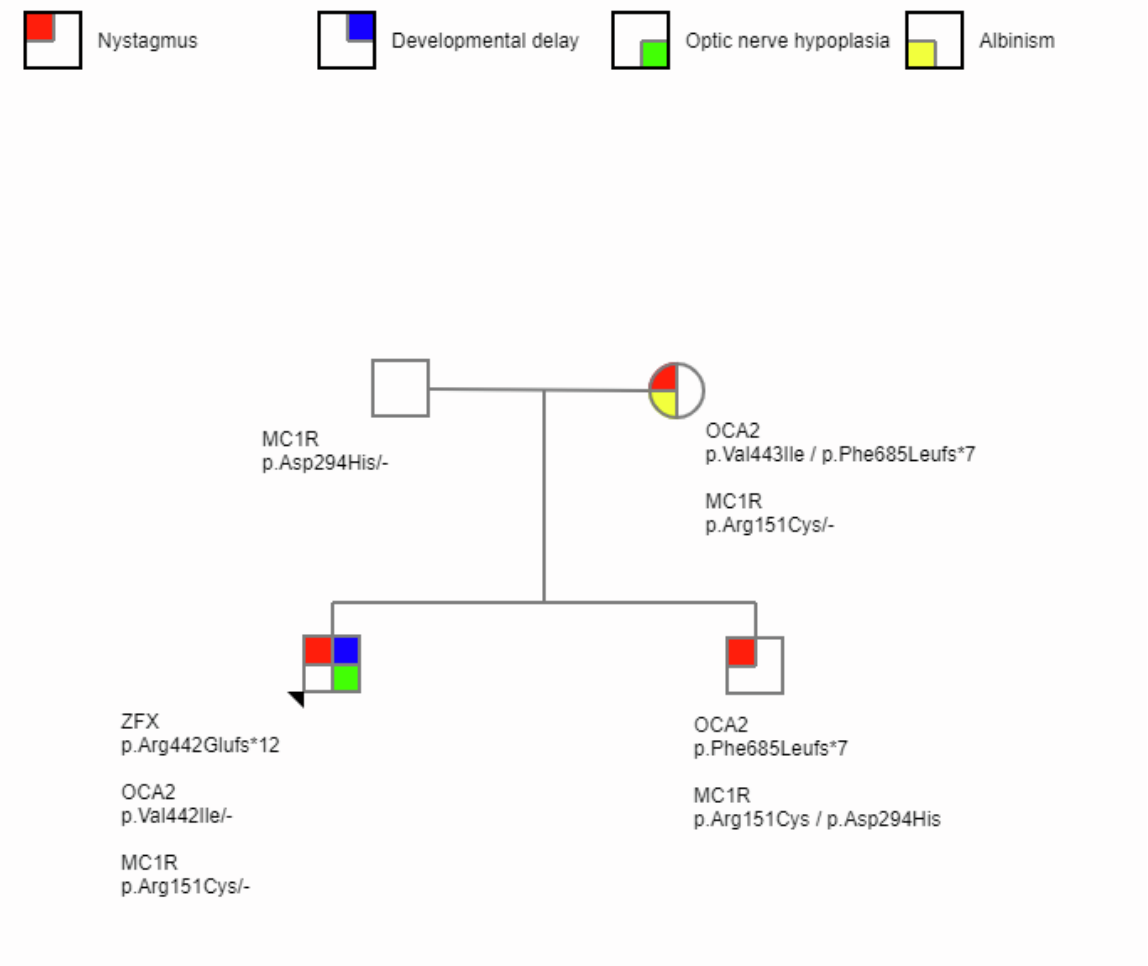
On physical examination at 7 years of age, hypotonia, small deep-set nails, tongue protrusion, long philtrum and deep palmar creases were noted. Appearance of blepharophimosis. He had normal anterior segments of both eyes and clear corneas. Well pigmented irides and well pigmented retinae but small grey optic discs, indicating optic nerve hypoplasia.

An MRI brain aged 1 year showed thin optic tracts and chiasm, a relatively small pituitary, a retrocerebellar arachnoid cyst and periventricular leukomalacia. An ultrasound of the renal tract was normal at this time also. His arginine stimulation test for growth hormone was low at age 5 years and he was commenced on growth hormone injections.

Karyotype and chromosomal microarray were unremarkable. He was recruited to the 100 000 Genomes Project where no causative variants were identified using panels: Infantile nystagmus v1.3, Ocular and oculocutaneous albinism v1.21, Optic neuropathy v2.1, Retinal disorders v2.6, IUGR and IGF abnormalities v1.30 and Intellectual disability v3.3.

Subsequent panel agnostic re-analysis identified a *de novo* frameshift variant in *ZFX* (NM_001330327.1:c.1322_1323dupGA, p.Arg442Glufs*12). This variant is absent from population databases, occurs in exon 10/10 and is likely to escape nonsense-mediated decay, although this is predicted to truncate the C2H2 zinc fingers 2-13. C2H2 fingers 11-13 have recent been shown to be essential for DNA-binding of ZFX and for its subsequent function as a transcriptional activator.

We also identified likely pathogenic and pathogenic variants in both *OCA2* and *MC1R* in the mother and brother of the proband, which explain their ocular albinism, although the proband is unaffected in this respect (see pedigree).



Patient 13

The patient is a 24-year-old man. He is the younger of two sons born to healthy, unrelated Caucasian parents. There is no significant family history of intellectual disability.

At 12-week gestation increased nuchal thickening and mild pericardial effusion was noted on ultrasound scan. CVS testing showed normal karyotype. Pericardial effusion persisted on antenatal scans at 25 and 28 weeks. At 32 weeks his mother suffered a large antepartum haemorrhage. He was born by Caesarean Section at 33 week-gestation due to maternal pre-eclampsia, with a birth weight of 2.01kg (50th centile).

He had prolonged neonatal jaundice and feeding difficulties, requiring admission to special care baby unit (SCBU) for 4 months. He underwent bilateral inguinal hernia repair at 2 days of age and repair of pyloric stenosis at 6 weeks. There was initial unconjugated hyperbilirubinaemia, followed by cholestasis. A liver biopsy showed evidence of giant cell hepatitis, which gradually resolved throughout early childhood.

At 11 years, a routine follow-up abdominal ultrasound showed a large suprarenal mass. This was found to be a benign suprarenal ganglioneuroma, for which he underwent complete surgical excision.

His early developmental milestones were all delayed, with particular delay in expressive language. He was late to sit and walked independently at 25 months. He has intellectual disability and attended Special School. At 12 years of age his verbal and non-verbal IQ were

in the 3-4 year age range. He is currently at college studying life skills and enjoys playing basketball and football.

He has required surgery for hypospadias. He had a large patent ductus arteriosus and small atrial septal defect on echocardiogram which closed spontaneously. He also suffered from recurrent respiratory infections and was found to have mild IgG and IgM deficiency.

Ophthalmological review was reported as showing partial ocular albinism and astigmatism. He also has very dry eyes for which he uses regular eye drops. He has bilateral moderate conductive hearing loss for which he had three sets of grommets in the past and has been prescribed hearing aids.

He remains under the care of the adult immunology service but no longer has recurrent chest infections. He takes Solifenacin for night-time enuresis. There is no history of seizures and he is otherwise generally healthy.

His final height is 173 cm (28^{th} centile) and head circumference 59.4 cm (93^{rd} centile), with current weight 69.3kg (60^{th} centile). He has distinctive facial features with long palpebral fissures, long eyelashes, thick medial eyebrows, up-turned ear lobes, a thin upper lip, smooth philtrum, and micrognathia. He has limited mouth opening. His teeth are crowded with a pointed incisor on the left. His speech is nasal in tone. He has mild ulnar deviation of his 3^{rd} and 4^{th} fingers in the left hand and fetal pads on his fingertips on both sides. His 4^{th} toes are proximally placed. He also has hallux valgus and thick soles. There is slight restriction of elbow extension bilaterally.

Previous genetic investigations included array CGH, *NSD1* gene sequencing and 11p15 methylation analysis, all of which were normal. His brain MRI scan at 3 years was reported as showing delayed myelination for age.

A recent spinal X-ray showed no evidence of scoliosis but several other bony changes. Both clavicles are angulated with an apparent pseudoarthrosis forming with the coracoid processes of the scapula. There are 12 pairs of ribs with angulated slender posterior elements. In the thoracic spine the vertebral body endplates appear irregular. The femoral necks appear short with irregular ossification of the ischium.

Patient 14

Proband ZFX14 was an 8-year-old girl at her last evaluation. She initially presented for a medical genetic evaluation at age 3 years for growth hormone deficiency, pituitary stalk interruption syndrome, developmental dysplasia of the hip, and sacral dysgenesis with deviation of rectum and anus. Prenatal history was remarkable for abnormal ultrasounds identifying congenital heart defect and macrocephaly. Neonatal history was remarkable for hypoglycemia and NG feeds. Postnatally, she followed with cardiology and had a VSD that closed spontaneously. Head and neck imaging revealed hypoplasia of the facial bones and temporomandibular joints, as well as cervical spine stenosis. On physical exam she was noted to have short stature, mild pectus excavatum, hypotonia, macrocephaly, dolichocephaly, prominent forehead, sparse hair, mid-face hypoplasia, epicanthal folds, low and depressed nasal bridge, wide and upturned short nasal tip, long and flattened philtrum, unilateral preauricular tag, spatulate fingers and scooped toenails. She rolled over at age 10-11 months and walked at 26 months. She had hip surgery and spica cast at 12 months. She required PT and OT. Her motor skills improved after starting growth hormone therapy at age 3 years. Her first words were at 10 months, and she was putting 2-3 words together at 13 months. At age 8 years she was doing well in school without additional support or therapies. Her height was in the 25^{th}-50^{th} centile for age. Previous genetic testing revealed 46, XX

chromosomes and a Xp11.23 de novo duplication on SNP array. She also had a Growth Hormone Receptor multigene panel that was non diagnostic. Given non diagnostic testing, trio exome sequencing was recommended which identified a paternally inherited variant of uncertain significance in *ROBO1* (c.928C>T; p. R130X) and a *de novo* variant of uncertain significance in *ZFX* (c.529dupT; p. S177Ffs*12).

Patient 15

Proband ZFX15 is a now 13-year-old male of White British origin. His older sister who is well. Pregnancy was complicated by hyperemesis and pre-eclampsia. A risk of Down syndrome was identified by maternal serum biochemical screening. He was born via emergency C section following secondary to poor fetal movements at 36 weeks. He was transferred to the NICU due to respiratory distress. He required NG feeding. MRI showed small left intraventricular haemorrhage. He had relative macrocephaly at birth and Sotos syndrome was queried but *NSD1* testing was normal. Other early features noted included bilateral inguinal hernia, seizures (later resolved), hypospadias, wide palpebral fissures, prominent forehead. He was discharged when he was 3 weeks of age. He was noted to have developmental delay; he was able to walk at 2 years, but not running until 4 years of age. Examination then revealed a high arched palate in addition to the other previously noted features noted. He was recruited into the DDD study (PMID: 25529582). However, no pathogenic variants were identified. He was then recruited to the 100,000 Genomes study (PMID: 34758253) and the genome sequencing identified the two variants: ZFX (subject of this paper and TFAP2A (Branchiooculofacial syndrome). The TFAP2A was not thought to cause patient's phenotype and it is likely not relevant.

The proband developed thoracolumbar scoliosis around 10 years of age. He is wearing a brace but the scoliosis continues progressing and a spinal fusion planned. He also exhibited vision deterioration (vision was 6/12 iat age 7 yo and now it is less than 6/60) around 10 years of age. Electrophysiology revealed no evidence of significant retinal pathway dysfunction but cortical visual evoked potentials were consistent with significant bilateral optic nerve or posterior visual pathway dysfunction. His retinal photo with optos showed macula changes affecting outer retinal layers and pigment epithelium (but not clear if that is the reason for his visual problem or if it is related to his previous diagnosis of papilledema); these changes remained stable between images in 10/2020 and optos images from 3/2022 and 9/2022. Currently he is registered as severely sight impaired and his vision is further down (6/76 RE and 6/150 left eye). Bilateral optic atrophy was also noted. He also had deterioration in hearing at age 12 when he found to have high frequency sensorineural hearing loss - recently had cochlear implants. He continues exhibiting occasional "blank spells.". He used to sleep very well but recently he started to wake in the night and needs to sleep with parents or becomes very distressed

Patient 16

Proband ZFX16 is a 15-year-old boy, the third child of healthy non-consanguineous parents. During pregnancy, right-sided hydronephrosis was found by ultrasound. The delivery was uncomplicated. He had surgery for pelviourethral obstruction at 4 years of age. His initial development regarding contact, including eye contact was described as normal. Crawling and walking were delayed (not specified), and he still has slight motor problems. His language development was also delayed and he used non-verbal language. He spoke in short sentences by age 3 years. At age 5½ years, he started receiving physiotherapy due to delayed motor skills and at age 14 he started receiving occupational therapy. He is described as having problems with social interaction, concentration and organization. He

was diagnosed with infantile autism at age 14 years. At age 15 years, he goes to normal school, but his cognitive level is below for what is expected for his age.

His physical exam at 14 years of age showed dysmorphic features including thin upper lip, long, almost flat philtrum, downslanting palpebral fissures, long and big nose, big nostrils, large ears and slightly large tongue.

SUPPLEMENTARY FIGURES

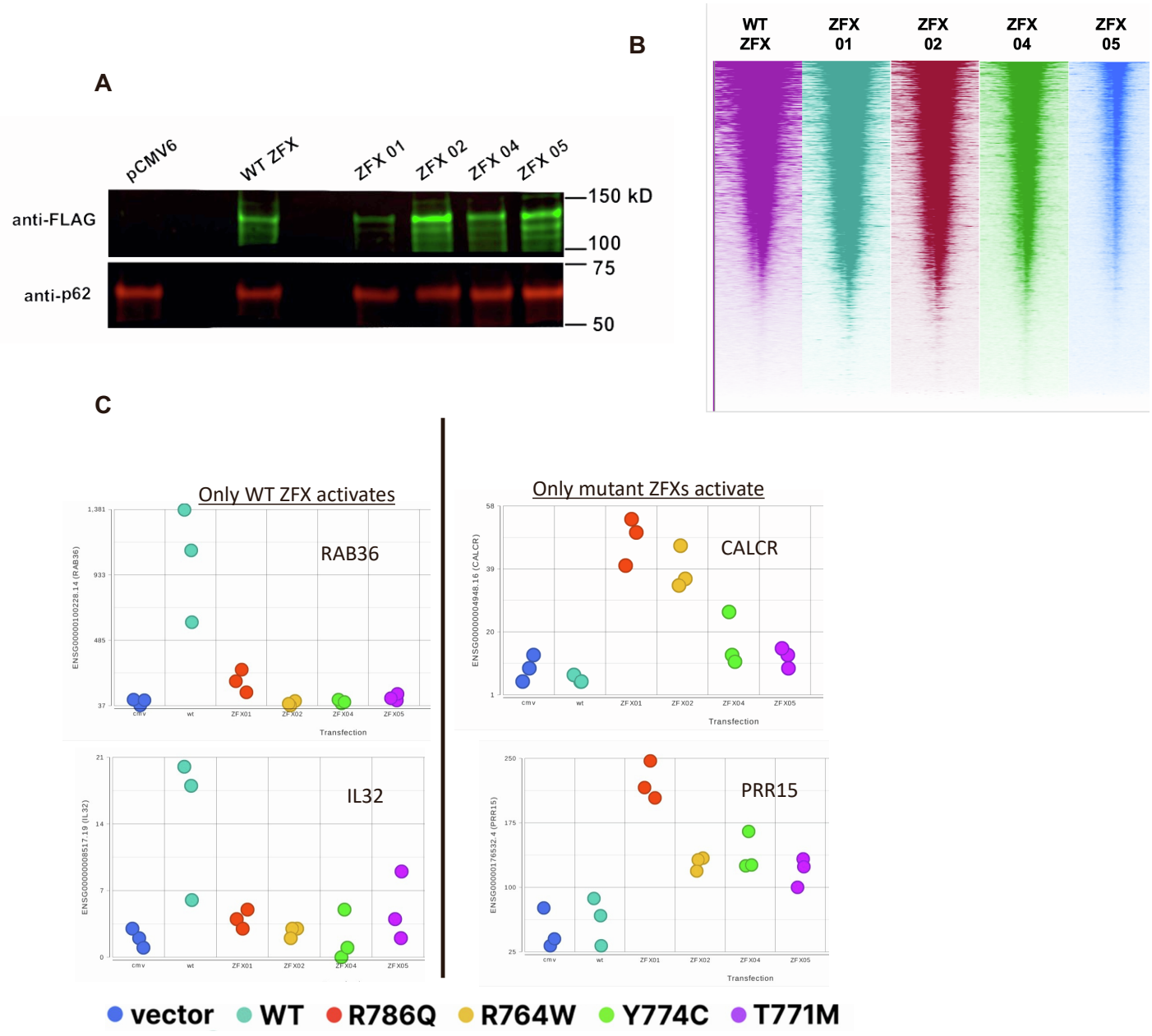
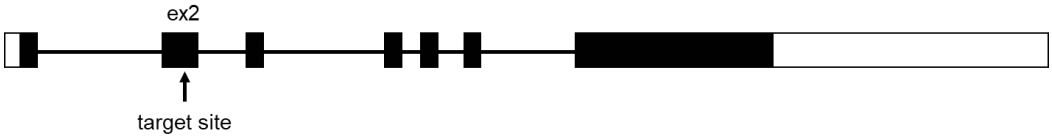


Figure S1: (A) Western blot analysis of WT and mutant ZFX proteins in DKO cells. Equal amounts of nuclear lysate (~ 40 ug) were loaded into each well of a 4-15% gel (Bio-Rad CAT#4561085) After transfer, the membrane was probed with anti-FLAG (green) and anti-p62 (red) antibodies. WT ZFX or mutant ZFX proteins are indicated with the green arrow; the nuclear-localized protein p62 loading control is indicated with the red arrow. (B) Shown are heat maps of ChIP-seq data for wt ZFX and ZFX missense mutants at promoters of all protein-coding genes; all missense ZFX datasets are ranked according to the wt ZFX-bound promoters. (C) Shown are activation levels for two direct targets of wt ZFX only (left) and 2 direct targets of the variant ZFX proteins only (right) in cells transfected with the wt and mutant ZFX proteins; values on the Y-Axis represent normalized read counts for all transcripts of the indicated gene. Key: R786Q = ZFX01; R764W= ZFX02; Y774C= ZFX04; and T771M =ZFX05.

A



B

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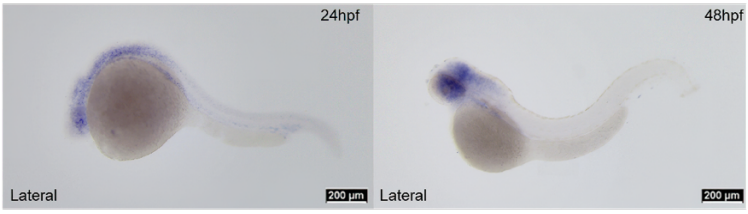
WT        5’- GCTCTGGAGGATGAAGGGCTGCAGGTGGATGTGGTGACCGACGCTCAGGTACAAGAGGAT- 3’
-25 bp        GCTCTGGAGGATGAAGG-------------------------GCTCAGGTACAAGAGGAT

WT         5’-GTGACCGACG----------------------CTCAGGTACAAGAGGATCCAGACACCTGC -3’
+23 bp        GTGACCGACGGTACAAGACGGTACTCAGGTACAAGAAGATCCAGACACCTGCGGACACCTGC
```

C



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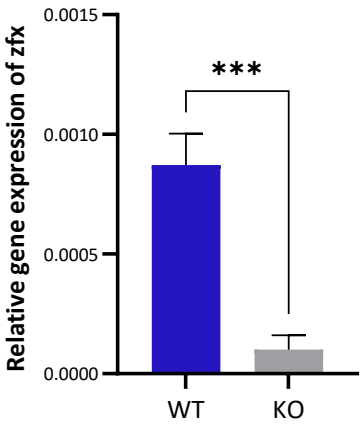

E

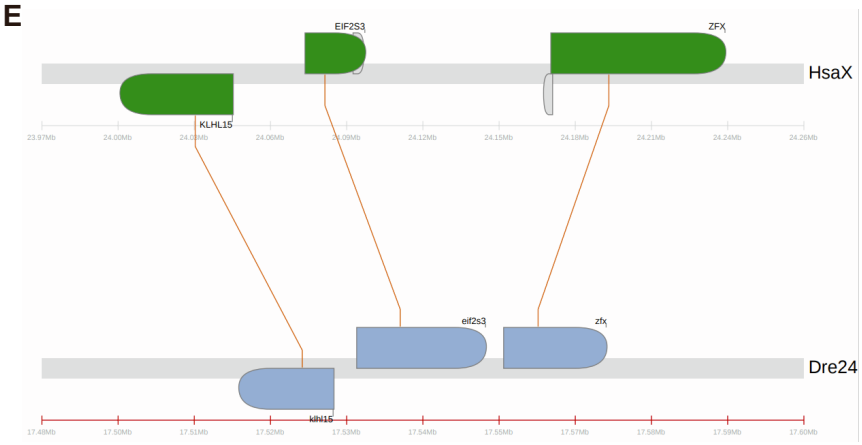

Figure S2: (A) Generation of zfx mutant zebrafish. Schematic showing the genomic structure of zebrafish zfx and CRISPR-Cas9 target site in exon 2. Black boxes, coding regions; white box, untranslated region; black lines, introns. (B) Targeted sequence for WT and 25-bp deletion and 23-bp insertion mutants. (C) Whole mount *in situ*

hybridization of *zfx* in wildtype zebrafish larvae. (D) Whole brain extracts measured by qPCR reveals *zfx* mRNA expression level was significant low in KO compered to WT (p=0.008)(E) Visualization of synteny map of ZFX surrounding region comparing human and zebrafish reference genomes obtained by Synteny Database (http://syntenydb.uoregon.edu/synteny_db/). hpf = hours post fertilization

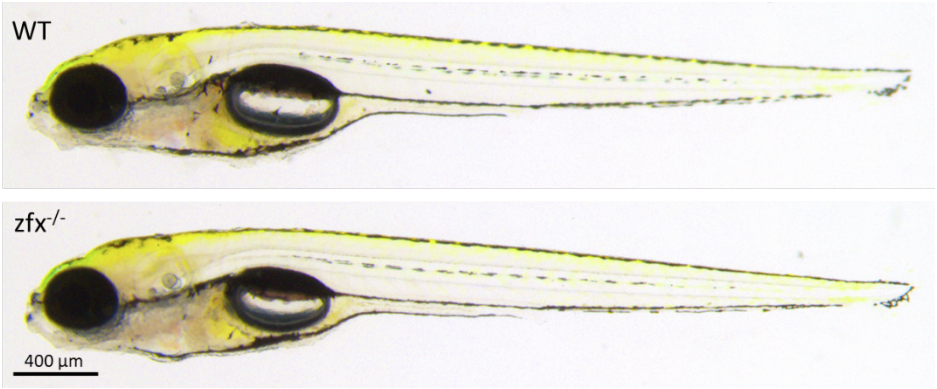

Figure S3: Representative bright field images of WT and *zfx* mutant zebrafish at 5 dpf. *zfx* homozygous mutants show relatively normal development at 5 dpf.

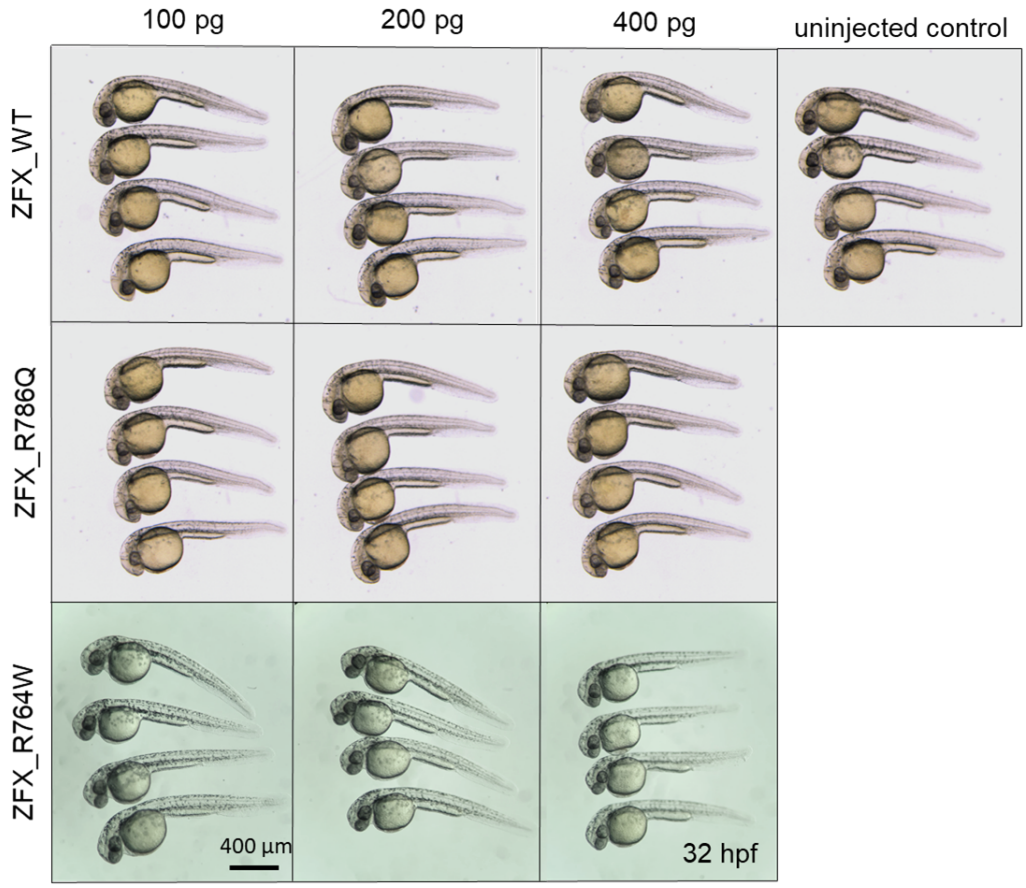

Figure S4. Functional analysis of human ZFX variants using *zfx* mutant zebrafish model. Overexpression experiments by microinjection of mRNAs. Three different concentration (100, 200 and 400 pg) of mRNAs, human wild-type or variant forms of ZFX (R764W and R786Q) were injected.

SUPPLEMENTARY TABLES

Table S1: Clinical, molecular, and demographic summaries for cohort participants.

See Supplementary_Table_S1.xlsx

Table S2: Primers used for creating mutant ZFX expression constructs. The red nucleotide indicates the site of the mutation. ZFX01: p.Arg786Gln; ZFX02: p.Arg764Trp; ZFX04: p.Tyr774Cys; ZFX05: p.Thr771M

Primer name	Primer sequence (5' to 3')
ZFX01_3	GGAAGCCTTTCTTGCAGTACTCACACCGGTGAGG
ZFX01_5	GTACTGCAAGAAAGGCTTCCAAAGACCTTCAGAAAAGAACCAG
ZFX02_3	TAAAGCCTGAGGCATCTGTAGTGCTATACTCACAGTACTCACACTG
ZFX02_5	GCACTACAGATGCCTCAGGCTTTAAACGGCACGTTATTTCCATTCACAC
ZFX05_04_3	GTGAATGGAAATAACGTGCCGTTTAAAGCCTGAGGCATCTGTAGTGC
ZFX04_5	ACGGCACGTTATTTCCATTCACACGAAAGACTGTCCTCACCGGTGTG
ZFX05_5	ACGGCACGTTATTTCCATTCACATGAAAGACTATCCTCACCGGTGTG

Table S3: RNA-seq and ChIP-seq data used in this study.

See Supplementary_Table_S3.xlsx

Table S4: ChIP peaks called for the indicated ZFX genotypes.

See Supplementary_Table_S4.xlsx

Table S5: Gene-level differential expression values computed for the indicated genotype comparisons.

See Supplementary_Table_S5.xlsx

Table S6: Identified direct target genes of the indicated ZFX mutants.

See Supplementary_Table_S6.xlsx

Table S7: Primer sequences used for generation of zfx CRISPR knockout zebrafish model.

Name	Information	Sequence	Purpose
zfx-FP	Forward	5'-GATGATGAAGGGCTTGGCAC-3'	Genotyping
zfx-RP	Reverse	5'-cgctacaatctcactcacAGG-3'	Genotyping
Oligo		5'-taatacgactcactataGGATGTGGTGACCGACGCTCgttttagagctagaa-3'	sgRNA

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