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Supplemental information

Bi-allelic variants in *IPO8* cause a connective tissue disorder associated with cardiovascular defects, skeletal abnormalities, and immune dysregulation

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Supplemental Case reports

Cohort

Affected individuals were clinically evaluated in different centers and gathered via GeneMatcher[1]. Each individual or legal guardians gave informed consent for participation in the study. This study was approved by the ethics committees and the institutional review boards at each center.

Family 1- Individual 1, a male, was born to consanguineous parents of Ashkenazi origin. He was referred at age 59 with a putative diagnosis of connective tissue disorder. He had a past history of congenital umbilical hernia, recurrent spontaneous pneumothorax, rectal prolapse and scoliosis. He was disabled by joint hypermobility and had recurrent dislocations of the knees and shoulders. He did not have skin hyperextensibility but he reported bruising easily. Palate, chest and arm span to body height ratio (1.03) were normal. At age 35, his high myopia was complicated by bilateral retinal detachment and at age 45 he developed a bilateral cataract in the absence of lens subluxation. Vascular screening performed at age 59 evidenced a dilatation of both ascending (42mm, Z score +6.79) and abdominal aorta requiring beta-blocker therapy. A mitral valve prolapse as well as dilated and calcified femoral arteries were evidenced. A large cortical cyst (11 cm) of the left kidney was incidentally discovered on CT-scan. He died at age 67 of an unknown cause. **Individual 2**, the youngest sister of individual 1, was referred at age 53 with the putative diagnosis of Marfan syndrome. Like her brother, her medical history was significant for recurrent spontaneous pneumothorax (at age 27 and 31), joint hypermobility with joint dislocation (mainly the elbows), high myopia complicated by retinal detachment at age 17 and bilateral cataract. She also had severe scoliosis, umbilical and spigelian hernias which occurred after abdominal surgery. At age 53, the diameter of the ascending aorta was normal but she had dilatation of sinus of Valsalva (39 mm, Z score +5.23) requiring beta-blocker therapy as well as an abdominal aortic aneurysm. Multiple aneurysms affecting left internal femoral and left popliteal arteries were also present. These aneurysms were associated with peripheral artery disease in lower limbs. At age 53, an ischemic nephropathy consecutive to a thrombosis of the right renal artery and stenosis of the left renal was detected. Palate, chest and arm span to body height ratio were normal. She died at age 65 after surgery for her abdominal aortic aneurysm. Both individuals 1 and 2 were normally intelligent and had no dysmorphic features.

Family 2- Individual 3, a male child was born to consanguineous parents of Indian origin. At 33 weeks of gestation, ultrasonography showed bilateral pelvicalyceal dilatation. He was born at term but was small for gestational age and required neonatal resuscitation for respiratory distress. Birth weight was 2165g (–2SD), length was 50cm (0 SD) and OFC was 35 cm (0 SD). Neonatal echocardiography showed dilatation of heart chambers and ventricular septal defect (6 mm) as well as severe pulmonary arterial hypertension. Bilateral hydroureteronephrosis was present and testes were undescended. He underwent surgery for congenital diaphragmatic hernia and left inguinal hernia at 6 and 15 months respectively. Clinical assessment at 20 months of age evidenced a developmental delay associated with microcephaly (44cm; - 4.4 SD). Dysmorphic features included low set and posteriorly rotated ears, ptosis, down-slanted palpebral fissures, micro-retrognathia, narrow mouth with downturned corners, cleft uvula, curly and hypopigmented hair. In addition, he

had a pectus carinatum, mild arachnodactyly, bilateral eversion of feet, umbilical hernia and hip dysplasia. At echocardiography, the aortic root diameter was measured at 17 mm (Z score +2.19). In addition, he had a perimembranous ventricular septal defect (4 mm), an atrial septal defect (2 mm), as well as a left to right shunt with mild dilatation of left atrium and ventricle. **Individual 4,** his younger brother presented similar clinical features. He had bilateral equinovarus talipes that was evidenced during pregnancy at 35-36 weeks' gestation. Clinical assessment at 24 months of age evidenced motor development delay with normal growth parameters. Echocardiography was normal. Dysmorphic features included micrognathia, small mouth with downturned corners, curly and hypopigmented hair. In addition, he had a pectus carinatum, mild arachnodactyly and umbilical hernia.

Family 3 - Individuals 5 and 6 were born to consanguineous parents of Algerian origin. Both were born at term and presented severe hypotonia and swallowing difficulties from neonatal period. They had both global developmental delay and facial dysmorphic features including bilateral parietal bossing, enlarged nose, micrognathia and hypertelorism. They also had joint hyperlaxity, umbilical hernia and pectus excavatum. In both of them, at age 12 and 14, respectively, brain MRI evidenced bilateral tortuous internal carotids and helicoidal arteriography combined with CT Scan showed enlarged diameters of Valsalva sinus at 32 mm (+2.4SD) and 34mm (+3SD), respectively, as well as pulmonary emphysema and bronchiectasis. **Individual 5**, a girl, also displayed congenital septal defect, severe gastroesophageal reflux (both corrected by early surgery) and pyelo-ureteral duplication. At 4 years, she developed colitis revealed by rectal bleeding and stunted growth. Colonic endoscopy showed pancolitis with mild eosinophilic infiltration. She was treated with corticosteroids and azathioprine; now at 16 years, she remains treated with methotrexate. Her weight and height are currently -2 SD. She also has severe scoliosis treated with physiotherapy and corset. **Individual 6**, a boy, was diagnosed with celiac disease at the age of 3 because of stunted growth, total duodenal villous atrophy, intraepithelial lymphocyte infiltrate, HLA-DQ2 haplotype as well as high titers of serum anti-transglutaminase and anti-endomysium IgG antibodies. He also had nodular gastritis. His growth improved with gluten free diet. At age 12, brain MRI evidenced mild bilateral ventricular dilatation and pituitary hypoplasia. At age 15, his weight and height are -2SD and -1SD of growth standards median respectively. In addition, both siblings have allergic manifestations including asthma and eczema and as well as recurrent pulmonary infections and bronchiectasis. Immunological work-up showed normal lymphocyte counts and phenotype but revealed very low serum IgA (< 0.04g/L, ref:0,45-3.5g/L) contrasting with very high serum IgE (> 1000 kIU/L, ref: <30 IU/L) and IgG (18-28 g/L, ref: <10g/L). Individual 6 has also developed vitiligo.

Family 4- Individual 7, a boy, was born at 36 weeks' gestation after a normal pregnancy to consanguineous parents originating from the United Arab Emirates. He was referred at 12 years of age with the diagnosis of connective tissue disorder. He underwent several surgeries for birth defects including palatal cleft, inguinal hernia and umbilical hernia. Since the neonatal period, he presented with hypotonia, delayed motor development (independent walking was acquired at the age of 9), severe language delay (first words spoken at the age of 6) caused by moderate to severe bilateral conductive hearing loss. He also displayed scoliosis, joint hyperlaxity with multiple dislocations/luxations of large and small joints, skin hyperlaxity associated with mammary

hypoplasia, nail hypoplasia as well as facial dysmorphic features including micrognathia, hypertelorism and proptosis. He had asthma since age 2 and was hospitalized repeatedly for pulmonary infections. Brain MRI and cardiac echocardiography performed at age 7 were considered normal.

Family 5- Individual 8, a male child was born to consanguineous parents of Moroccan origin at 40 weeks of gestation after a normal pregnancy. He had congenital umbilical hernia, bilateral hydroureteronephrosis revealed at 12 months by acute pyelonephritis and developmental delay affecting both motor and language acquisitions. At age 3, he presented digestive symptoms including diarrhea, rectal bleeding and anal fissure associated with stunted growth (-2SD). Endoscopy revealed pancolitis, mild esophageal ulcerations, inflammatory gastritis and duodenitis. Immunological work-up showed transient deficit in IgA (0.07g/L, ref:0,45-3.5g/L), which normalized two years later, high serum IgG (> 15g/L, ref: <10g/L) and IgE (279 kIU/L, ref: <30 IU/L) and hypereosinophilia, that persisted over time. He simultaneously developed allergic symptoms with atopic dermatitis in infancy followed by allergic rhinoconjunctivitis. Now at age 14, he still has high fecal calprotectin (1433 μg/g, ref: <50 μg/g) with Crohn-like inflammation (including microgranulomas) of the colon and ileum and nodular gastritis despite azathioprine and mesalazine and was therefore recently switched to anti-TNF treatment. Severe joint laxity was observed since the age of 3, causing walking difficulties, recurrent pain in the knees and he suffered several spontaneous fractures of the tibia. Beighton score was 8/9 at 9 years when cutaneous laxity with ecchymoses and blue sclera were also noted. At 6 years, echocardiography evidenced moderate dilation of the aortic root at 24 mm (N< 21mm) that was confirmed at the age of 9 (26mm, Z-score +2.63). Pituitary hypoplasia with growth delay (-2.5 SD) and very low serum GH and IGF1 was diagnosed at age 9 requiring a treatment with growth hormone. His weight and height are currently at -2SD of the growth standards median and he has developed mild scoliosis and severe myopia.

Family 6- Individual 9, a woman, was born to consanguineous parents of Arab-Muslim origin. She was referred at age 22 because of dilated aorta and clinical suspicion of a connective tissue disorder. In infancy, she presented with low muscle tone and delayed motor developmental milestones contrasting with normal intellectual, speech and language development. An atrial septal defect was surgically repaired at the age of 1 month. At age 12, an aortic dilatation affecting the aortic root and ascending aorta was diagnosed and treated with beta-blockers. She had recurrent patella dislocation, early osteopenia and hip fracture as well as high myopia without lens dislocation. At age 17, she underwent surgical repair of severe scoliosis. On physical examination, she had marfanoid habitus, her height was 157cm and weight was 36 kg. She had dysmorphic features including exophthalmos, micrognathia, high arched and narrow palate, arachnodactyly, and pectus carinatum. Her skin was normal without abnormal scarring, hyper-extensibility or stretch marks. There was joint laxity especially in the interphalangeal joints, low muscle mass and low fat mass, flat feet and hind foot valgus. Radiological and echographic examination showed lumbosacral dural ectasia, severe scoliosis, aortic root dilatation at sinus of Valsalva (35mm; Z-score +3.4) as well as bilateral tortuosity of the carotid arteries.

Family 7- Individual 10, a girl born to unrelated parents of Australian origin was persistently hypotonic following a Caesarian delivery at term. Her birth weight was 2500g (10th centile). She failed to thrive and needed a fundoplication for reflux with recurrent pneumonia at 9 months of age and surgical patch repair of ventricular and auricular septal defects at 11 months of age. She was assessed in genetics at 17 months of age for failure to thrive (weight and height below the third centile), bilateral positional talipes and delayed motor development. At age 13, she had aortic root dilatation treated with a beta blocker and two forearm fractures following trauma. She had scoliosis with a reduced upper segment to lower segment ratio (0.81), a normal span to height ratio (1.02) and associated short stature (3rd centile) in the setting of delayed pubertal development (Tanner stage 3). She had joint hypermobility (Beighton score 7/9), long fingers with positive thumb and wrist signs, long 2nd and 3rd toes, a high arched palate with a normal uvula, dysplastic teeth, and a flat midface with prominent eyes. Her hypotonia had resolved, she attended a mainstream school and had minor cognitive and fine motor delays. There was no evidence of a generalized bone pathology and her bone density was appropriate for her pubertal stage. At age 14, she had an elbow dislocation and needed a wheelchair for severe hip pain. She required bilateral hip joint replacement for severe hip dysplasia with progressive protrusio acetabulae. She required spinal fixation with anterior lumbar fusion at age 16 of for progressive scoliosis with rib-cage distortion. Her aortic root dilatation had progressed on beta-blocker therapy. Her cardiac anatomy was normal. She was seen more regularly between the age of 24 and her current age of 33 years and clinical diagnoses of Ehlers-Danlos syndrome (EDS) VI or Loeys-Dietz (LDS) syndrome were considered. Her height, weight, head circumference are 150 cm, 57 kg and 55 cm respectively. Her skin feels soft and smooth and is hyperextensible but is not loose and demonstrates normal scarring. She has generalized reduced muscle strength (3-4/5) and experiences chronic joint related pain, in the absence of inflammatory joint changes, and chronic generalized fatigue. She has prominent eyes, shallow orbits, midface retrusion, relative prognathism, dental crowding, gingivitis/ gingival retrusion, a high arched palate and a normal uvula. She has had orthopedic surgery on different occasions for dislocations of one ankle, one knee, one toe and one wrist caused by minor trauma. Painful recurrent subluxations of one shoulder was not improved by surgery. She has also dislocated both thumbs and her temporomandibular joint. She has had recurrent fractures of the metacarpals of one foot and the opposite ankle with minor but proportional trauma. She required abdominal surgery on different occasions for a large anterior abdominal wall hernia that required mesh insertion, for an impacted paraumbilical hernia, and for cholecystitis. She was investigated for gastroparesis and constipation/ decreased colonic transit time. She has always been normotensive and in sinus rhythm. She has stable asymmetrical aortic root dilatation (43mm, Z score +5.29) with aneurysmal dilatation of the right coronary cusp, borderline normal sinotubular diameters, a normal aortic arch, and normal pulmonary arteries with normal pulmonary pressures. She has not had aneurysmal dilatation of her coronary arteries or any medium sized arteries. She has exertional dyspnea and unexplained mild exertional hypoxia with no evidence of cardiac failure nor of significant right to left shunt. She has mild restrictive lung disease and mild asthma. She has moderate myopia requiring glasses, and non-specific retinal changes with no evidence of retinal detachment.

Family 8- Individual 11, a boy born to consanguineous parents of Iranian origin was referred at age 7. He had several congenital defects including umbilical hernia, club feet, bifid uvula and pectus carinatum. He had gradually developed a C-shaped scoliosis and showed mild arachnodactyly but arm spam to height ratio was normal (1.01). His motor development was more delayed (independent walking acquired at 29 months of age) than language acquisition (he spoke his first words at 17 months of age). High myopia without lens subluxation was diagnosed at the age of 3. Due to recurrent abdominal pain, he underwent esophagogastroduodenoscopy at age 3 which showed mild chronic gastritis and duodenitis. Serum immunoglobulins (G, A, M and E) were normal and there was no evidence of hypereosinophilia. Abdominal ultrasound showed a duplicated collecting system of the left kidney with mild hydronephrosis. Vascular screening performed at age 7 evidenced a dilatation of the aortic root (aorta sinuses = 31 mm Z scores+5.15) and floppy mitral valves.

Family 9- Individual 12, a woman, was born to consanguineous parents of Tunisian origin. She was referred at age 24 with the putative diagnosis of EDS. She was a full-term eutrophic baby but had an axial hypotonia that persisted until she was 18 months, bilateral hip dislocation, pes planus and umbilical hernia, that was repaired surgically. Complete hair loss occurred at age 11. She was also diagnosed with celiac disease and presented drug allergies. Her myopia and bilateral cataract required surgery at age 23. Physical assessment at age 24 found facial features evocative of vascular EDS including micrognathia, narrow nose, prominent eyes, and blue sclera. She also had skin fragility with easy bruising, abnormal scarring and skin hyperextensibility. In addition, she had varicose veins of the lower limbs that had appeared at 6 years, joint hypermobility (Beighton score 9/9) without scoliosis as well as complete absence of the permanent dentition. She was normally intelligent. Vascular screening showed carotid artery tortuosity with ectasia of the carotid bulb (15mm). Diameter of the aortic sinus was 41mm (z scores: 4.8).

Supplemental Figures

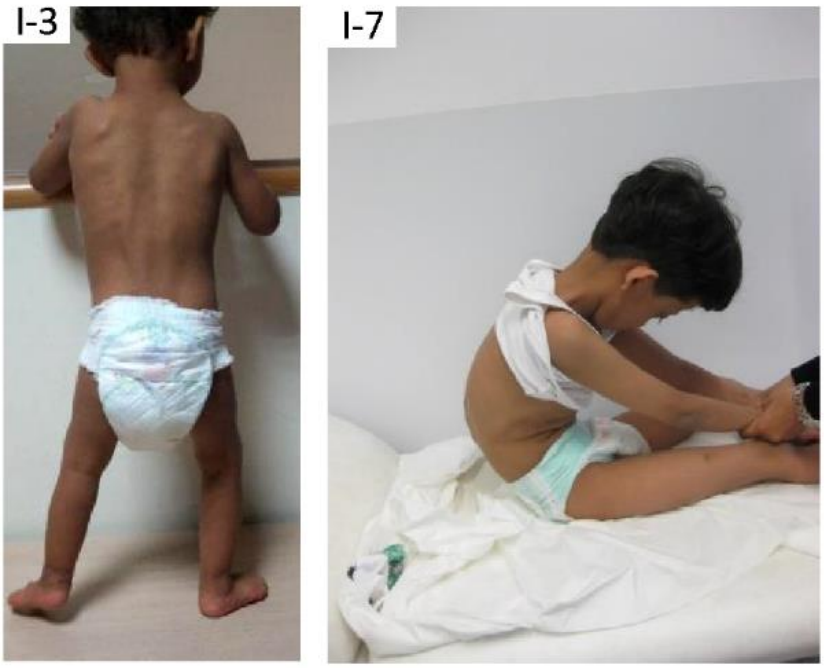

Figure S1. Additional clinical features of affected individuals.

Whole body pictures of I-3 and I-7 illustrating phenotypic variability in dolichostenomelia.

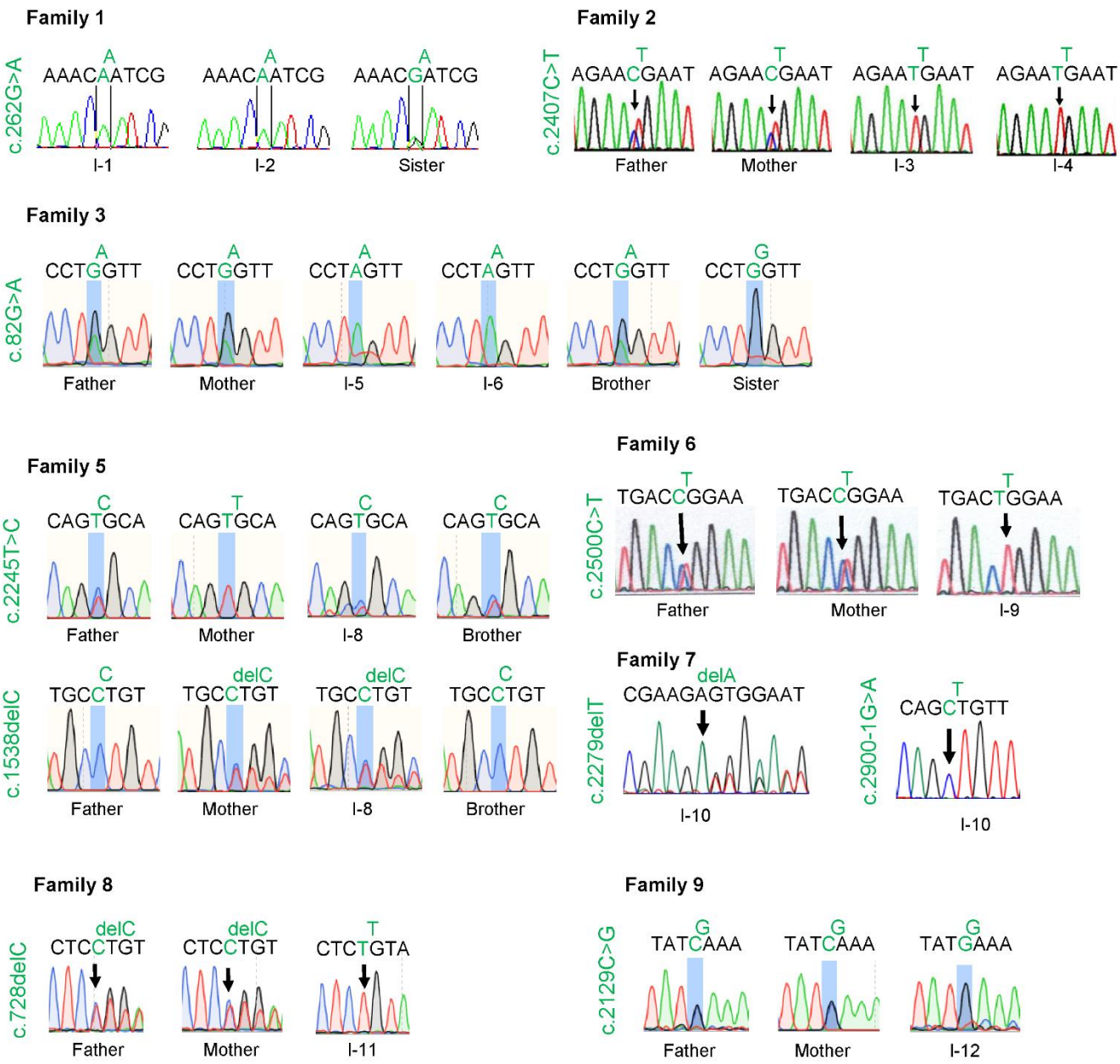

Figure S2. Confirmation of *IPO8* variants by Sanger sequencing.

Sanger tracings in families 1-2-3-5-6-7-8-9.

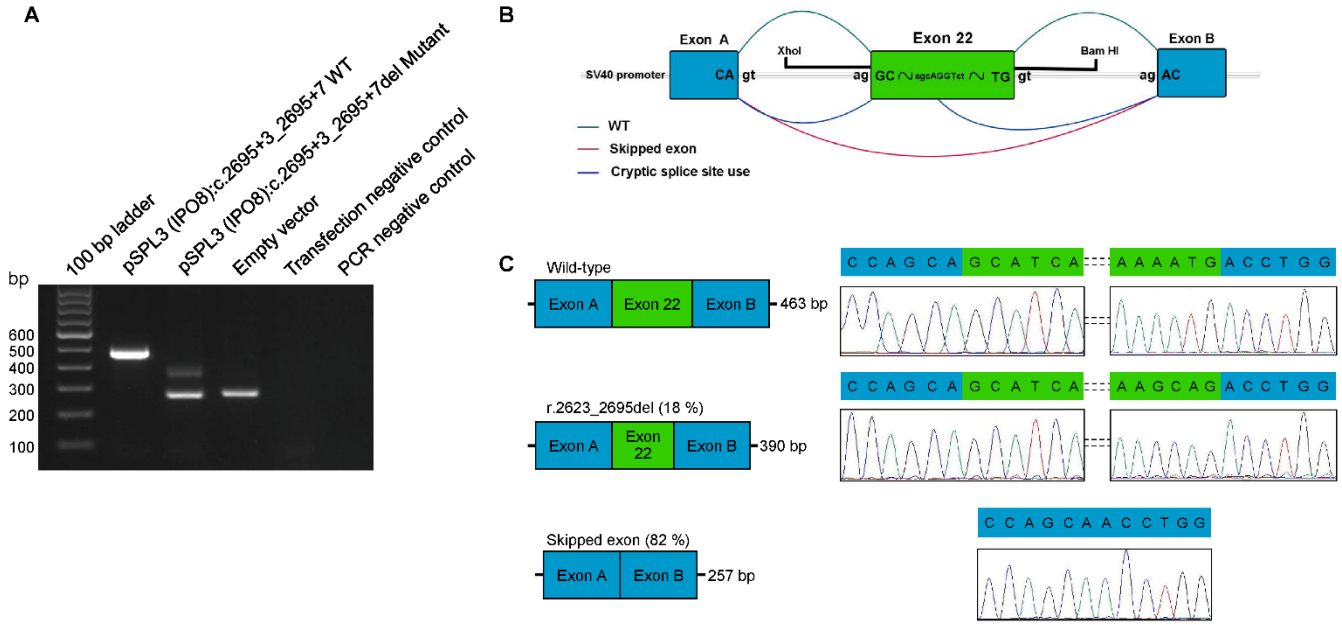

Figure S3. *In vitro* splicing assay of the *IPO8* c.2695+3_2695+7del variant.

(A) cDNA RT-PCR products amplified from constructs following transfection. Wild-type splicing yields a 463 bp product. The mutant amplicon shows two bands, one of which shows skipping of exon 22 (257 bp) while the second weaker band indicates activation of a cryptic donor site (390 bp). Transfection negative and PCR negative controls performed as expected.

(B) Schematic of the pSPL3 exon trapping vector with cloned IPO8 exon 22 (green) with splicing outcomes. Exons A and B (blue) originate from the vector. Wild-type splicing is shown in teal (top). Mutant splicing is depicted in blue (cryptic splice site activation) and red (skipping of exon 22) (bottom).

(C) Electropherograms of the splice junction sites for the wild-type (upper panel), activated cryptic splice site (middle panel, 18% of amplicon pool) and skipped exon 22 (bottom panel, 82% of amplicon pool).

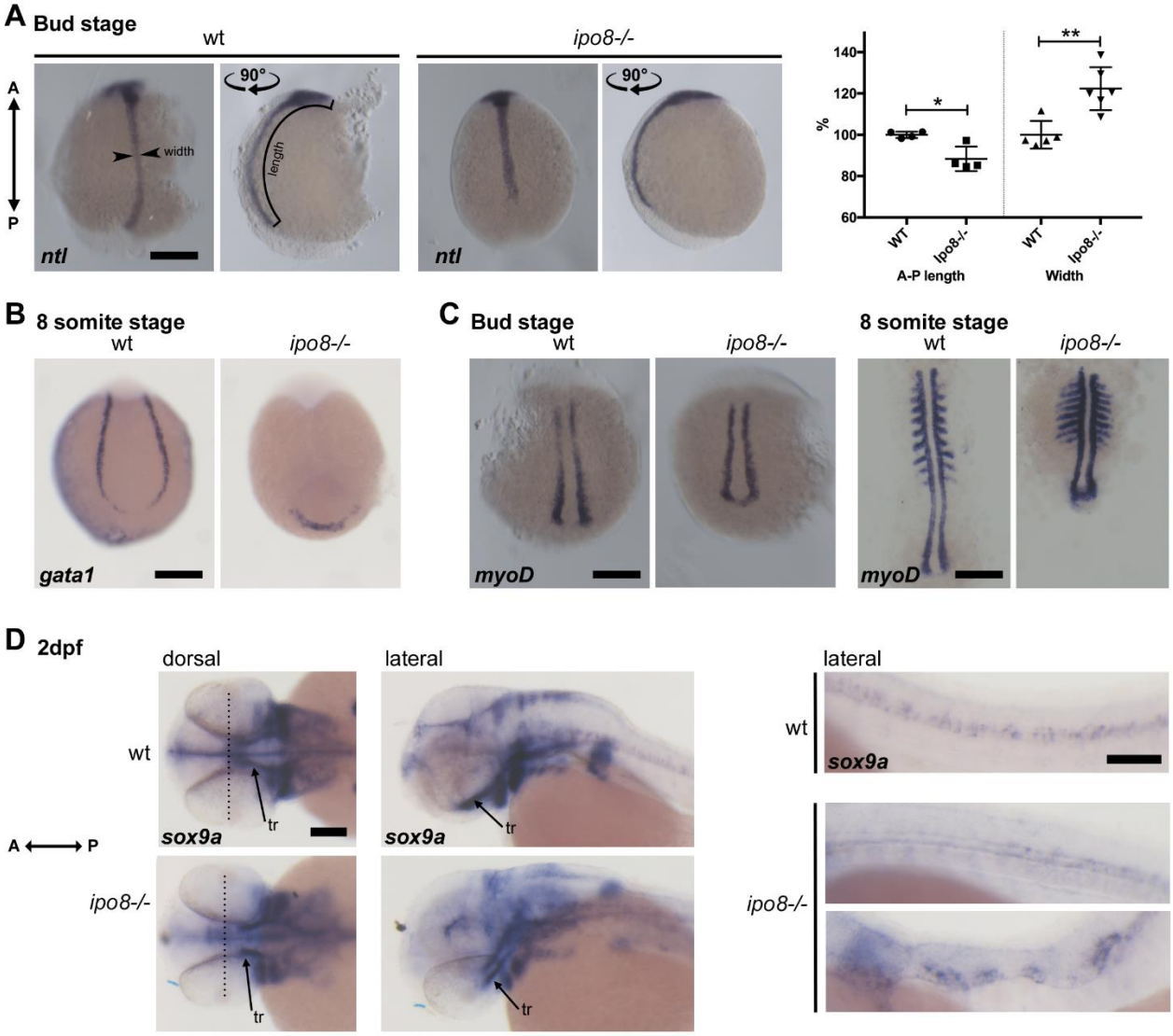

Figure S4: *Ipo8* deficiency causes dorso-ventral patterning defects and skeletal defects in zebrafish.

In situ hybridization pictures of wt and *ipo8*$^{-/-}$ embryos at the indicated stages for the early patterning genes *ntl* (A), *gata1* (B), *myoD* (C) and the chondrogenic precursor marker *sox9a* (D). Penetrance of the dorsalization phenotype is variable: only *ipo8*$^{-/-}$ embryos with strong phenotype are shown. In A, box plots show quantifications of the antero-posterior length and of the width of the ntl stripe. Medians and IQR are shown. P-values are calculated by Mann Whitney test *p<0.05, **p<0,01). A-P, antero-posterior, tr trabecula. In A-C scale bars= 200µm, in D scale bars= 50µm.

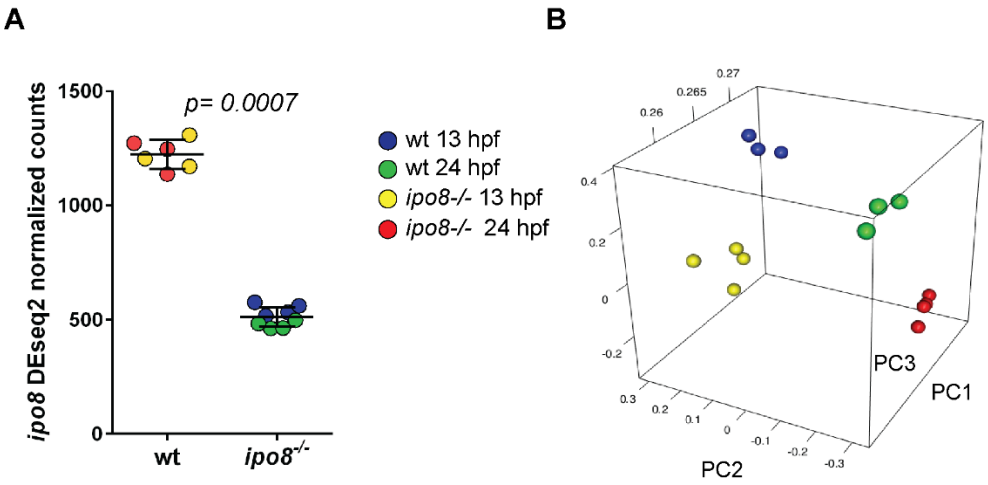

Figure S5: Global analysis of the zebrafish RNA-seq data.

(A) *Ipo8* expression (DESeq2 normalized counts).

(B) Principal component analyses (PCA) of *ipo8*$^{-/-}$ and wt samples at 13 and 24 hpf showing the distribution of the gene profile of each sample. PC1=83.1%, PC2=14.6%, PC3=1.4% on the common modulated genes ($p<0.05$, fold 1.2) between all compared conditions.

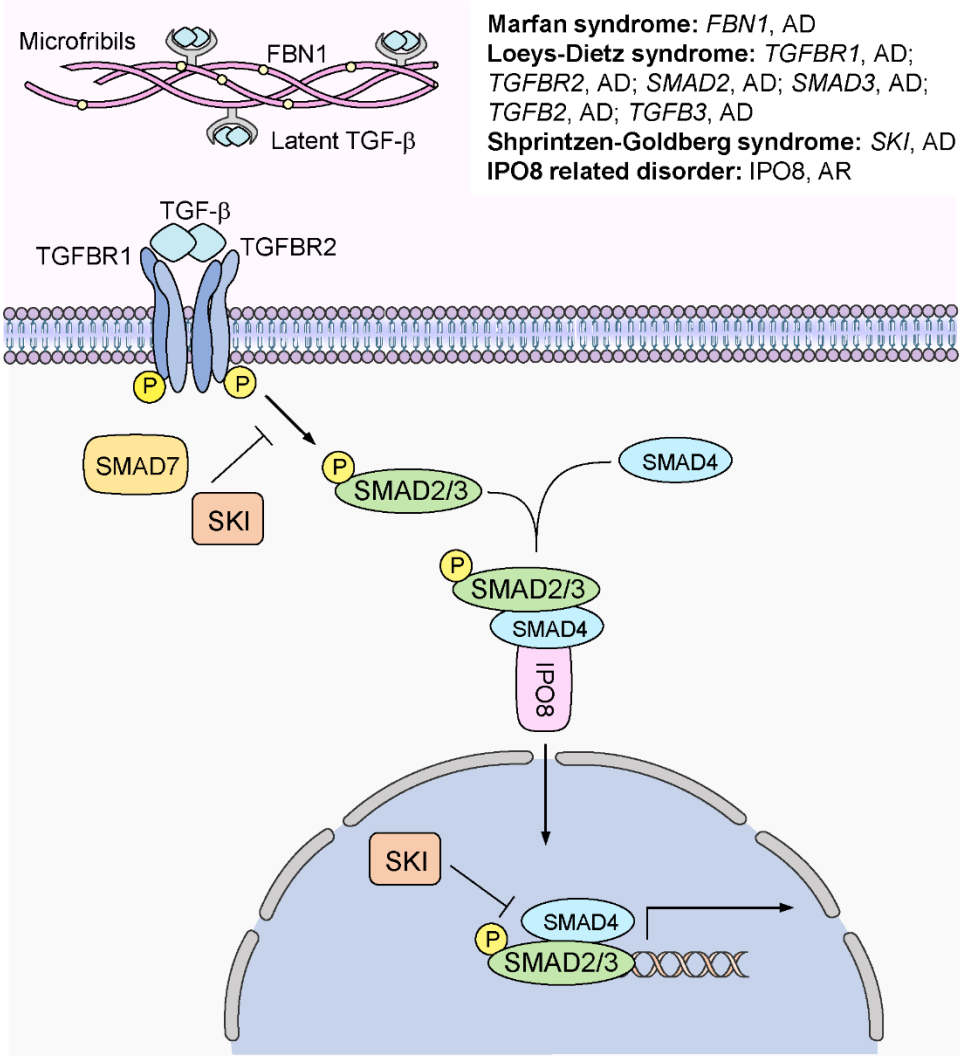

Figure S6: TGF-β signaling via canonical pathway.

In MFS individuals, AD variants in FBN-1, the gene coding for the main component of the extracellular matrix, fibrillin-1, increase the bioavailability of TGF-β ligands. In LDS individuals, AD variants have been reported in TGF-β ligands, TGF-β receptors or R-Smad effectors. In SGS individuals, AD variants have been reported in SKI, which prevents the translocation of R-Smads effectors to the nucleus or their binding to DNA. In IPO8-related disorder, AR variants have been identified in IPO8, the karyopherin which mediates the nuclear transport of the Smad complexes. AD, autosomal dominant, AR, autosomal recessive.

Table S1: Clinical features in MFS, LDS, SGS and IPO8 patients.

Clinical Features		**Marfan**	**Loeys-Dietz**	**Shprintzen-Goldberg**	***IPO8*-related disorders**
Gene(s)		*FBN1*	*TGFBR1/2* *SMAD2/3* *TGFB2/3*	*SKI*	*IPO8*
Mode of inheritance		AD	AD	AD	AR
Heart and blood vessels	Aortic root aneurysm	+++	+++	+	++
	Arterial tortuosity	-	++	+	+
	Early arterial dissection	+	+++	-	-
	Atrial or Ventricular septal defect	-	+	-	++
Skeleton	Craniosynostosis	-	++	+++	-
	Pectus	++	++	++	++
	Scoliosis	++	++	++	++
	Arachnodactyly	+++	++	++	+
	Feet malposition	-	++	++	+
Joint	Hyperlaxity	+	++	++	+++
Facial features	Hypertelorism	-	+	++	+
	Ocular proptosis	-	+	++	++
Ocular	Severe Myopia	++	+	++	++
	Ectopia lentis	+++	-	-	-
Cleft palate/ bifid uvula		-	++	+	+
Hernia		+	+	++	+++
Immune dysregulation		-	+	-	++
Developmental delay		-	-	++ (cognitive and motor delay)	+ (mostly motor delay)
Ureterohydronephrosis		-	-	-	+

+, feature is rare; ++, feature is more commonly present, +++, feature is most commonly present.
−, feature is absent.
AD, autosomal dominant; AR, autosomal recessive.

Supplemental methods

DNA Sequencing

Next generation sequencing was performed on DNA extracted from peripheral blood for all the individuals. Whole Exome sequencing (WES) was performed in all individuals except individuals 4 and 8, for whom targeted next generation sequencing was performed. Sequence validation and segregation analysis of *IPO8* variants was performed on PBMC-derived DNA from individuals 1, 2, 3, 5, 6, 7, 8, 9, 10, 11 and12 by Sanger sequencing. Target enrichment kits, sequencing platforms, family members tested and coverage are detailed below.

Individual	Capture Kit	Sequencing Platform	Family members tested	Average Coverage	Reference
1-2	Twist Human Core Exome Kit (Twist Bioscience, San Francisco, USA)	HiSeq4000 system (Illumina, San Diego, USA)	I-1 and I-2	77x (99.15% > 25x) and 70x (99.5% > 25x) for Individual 1 and 2 respectively	[2]
3-4	Twist Human Core Exome Kit (Twist Bioscience)	NovaSeq 6000 (Illumina)	Trio	178x (99.81x>40x) for Individual 3	[3]
5-6	Agilent Sure Select All Exon V5 (Agilent, Les Ulis, France)	HiSeq2500 HT system (Illumina)	Trio + two healthy siblings	150.8x (97.4% > 30x) and 155.8x (97.7% > 30x) for Individual 5 and 6 respectively	[4]
7	SureSelect Human All Exon V5 kit (Agilent Technologies)	HiSeq 2000 (Illumina)	I-7	113x	[5]
8	Capture Agilent sureselect custom made (Agilent Technologies)	NovaSeq 6000 (Illumina)	I-8	299.6x (99.4% >30x)	[4]
9	TruSeq Exome Enrichment Kit (Illumina)	HiSeq2500 HT system (Illumina)	Trio + one healthy sibling	90x-110x (85% > 20x)	[6]
10	Nextera exome capture kit (Illumina)	HiSeq_X_10 (Illumina)	Trio	80% >20x	[7]
11	Nextera Rapid Capture Enrichment library preparation kit (Illumina)	NextSeq 500 (Illumina)	Trio	27.1x (67.8% >10x)	[8]
12	Xgen-exome-research-panel (Integrated DNA technologies, Leuven, Belgium)	NextSeq 550 (Illumina)	Trio	115x (97.5% >30x)	[9]

Cell Culture

Primary fibroblasts derived from skin biopsy from individuals 2, 5, 9 and four healthy individuals (controls) were cultured in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific, Villebon sur Yvette, France) supplemented with 10% fetal bovine serum (Gibco) and penicillin-streptomycin (Gibco). EBV-B cells derived from individuals 5, 6 and 8 were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin-streptomycin (Gibco).

Western blot

Total proteins were lysed in RIPA buffer (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 1X proteinase inhibitor cocktail mix (Roche, Sigma-Aldrich). Protein concentration was measured by Bradford protein assay (Biorad, Marnes-la-Coquette, France). Equal amounts of proteins in Laemmli buffer (Biorad) were separated by SDS–PAGE, transferred to a polyvinylidene difluoride membrane (Biorad), blocked with 5% milk protein in TBST (0.5% Tween, Biorad) and probed with primary antibodies. The membranes were washed with TBST and incubated with appropriate secondary antibodies. Primary antibodies directed against IPO8 (#398854, 1:100, Santa Cruz, Dallas, USA), GAPDH (#14C10, 1:1000, Cell Signaling Technology, Leiden, The Netherlands). Secondary peroxidase–conjugated anti-rabbit and anti-mouse (1:1000, Cell Signaling Technology) were used. Specific protein bands were visualized using an ECL advanced Western blotting detection kit (GE Healthcare, Amersham, UK). The immunoblot was repeated at least twice for each patient.

Zebrafish (*Danio rerio*) husbandry and mutant generation

Wild-type Tupfel long fin zebrafish strains were used and raised according to standard protocols. All animal procedures were performed in accordance with French and European Union animal welfare guidelines with protocols approved by Sorbonne Université and Institut Curie (APAFIS#21323-2019062416186982 and APAFIS#6031-2016070822342309). To generate the *ipo8* mutant, the CRISPR/Cas9 technology was used. The crRNA (with *ipo8* exon4 specific sequences: sg5 AGTCGCAGAACAGTGGCAGC and sg6 TAAAGCATGATTTTCCTGGC) and tracrRNA sequences were ordered at IDT (Leuven, Belgium). Oligonucleotides were annealed according to the manufacturer's instructions at 45µM each in 20mM Hepes-NaOH pH 7.5, 150mM KCl. 1µL of the annealed sgRNA (45µM) was mixed with 1µL of Cas9 protein (30µM) and injected in one cell stage embryos [10]. Injected embryos were grown to adulthood and crossed with WT fish to identify founders. Pools of 20 embryos per clutch were lysed in NaOH 50mM at 95°C for at least 30min. PCR was performed on lysates to amplify the genomic region targeted by the sgRNA with primers forward 5'-GTCCATAAGGCAAGTCACAATGTTT-3' and reverse 5'-AAGAGAGTCAAAAGTGTCGTGTTTC- 3' using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The amplicons were run on a 3% agarose gel to identify deletions in the targeted region and to select the corresponding founder fish. Deletions were confirmed by sequencing (GATC biotech) and sequences were analyzed using Geneious [11] to select mutations inducing early stop codon. After selection of the founder, genotyping of the line was performed by PCR on fin clips with the same primers.

Zebrafish imaging and quantifications

For live-imaging, embryos were anaesthetized in 0.02% MS-222 and immobilized in 1% low melting point agarose. To visualize endothelial cells *in vivo,* the *ipo8* mutant line was crossed with Tg*(kdrl:Hsa.HRAS-mCherry)s916*. Live imaging was performed on a Zeiss LSM 780 confocal.

In situ hybridization

In situ hybridizations (ISH) were performed on embryos fixed in freshly made 4% paraformaldehyde (PFA) overnight at 4°C and stored in 100% methanol at -20°C. After rehydration, embryos were treated with proteinase K (20 µg/ml, Roche) at RT depending on their stage and fixed again in 4% PFA at RT for 20min. Digoxigenin-labelled antisense and sense RNA probes were synthesized by *in vitro* transcription using DIG-labelled UTP according to the manufacturer's instructions (DIG RNA labelling kit, Roche). Anti-DIG antibody conjugated to alkaline phosphatase allowed detection of hybridized riboprobes according to the manufacturer's instructions (Roche). DNA for probe synthesis was amplified from zebrafish genomic DNA using the following primers (*ntl,* Fwd: ATCATCTCCTTAGCGCCGTG, Rev: ATTGAACTGAGGAGGGCTGC; *myoD,* Fwd: CCCTTGCTTCAACACCAACG, Rev: GACAGATCCCTCATGCGGAG; *gata1,* Fwd: CAGGCTCCAGGAAGTCCTG, Rev: CACTAGTGTGGGCATCATGCC; *sox9a,* Fwd: CCAGCAAAAACAAGCCGCAC, Rev: CTGCCGTTTTGGGGTTTGGT) and transcribed *in vitro* according to the manufacturer's instructions (DIG RNA labelling kit, Roche). Imaging was performed on a Zeiss Discovery V20 stereomicroscope using the ZEN software.

Immunohistochemistry

80% epiboly embryos were fixed overnight at 4°C in 4% PFA and incubated in blocking buffer for at least 1h (10%FCS, 1%DMSO, 0.1%Tween in PBS). Embryos were incubated for 6h at RT with the anti-Phospho-Smad1(Ser463/465)/Smad5(Ser463/465)/Smad9(Ser465/467) Rabbit mAb (clone D5B10, #13820, Cell Signaling Technology) at a dilution of 1:100 in 1%FCS, 0.1%DMSO, 0.1%Tween in PBS, followed by overnight incubation at 4°C with Goat anti-rabbit Alexa Fluor488 antibody (1:400 in previous buffer, A27034, Thermo Fisher Scientific), phalloidin-Alexa Fluor 568 probe (1:40, A12380, Thermo Fisher Scientific) and Dapi (D1306, Thermo Fisher Scientific). After mounting in 1% low melting point agarose, images of the ventral sides were acquired on a Zeiss LSM 780 confocal. For quantifications of the ratio of cytoplasmic over nuclear signal, image segmentation was performed on the Dapi channel to create a binary mask used to quantify pSmad1/5/9 nuclear signal, using ImageJ. The binary image of segmented nuclei was subtracted from the pSmad1/5/9 image to quantify cytoplasmic signal.

Zebrafish RNA extraction and sequencing

Triplicates of 60 embryos were lysed in 1mL trizol (Invitrogen, Thermo Fisher Scientific) and mixed with a pestle (5min at RT) and RNA was extracted according the manufacturer. RNA pellet was dissolved in 20uL RNAse free water, treated with DNAse (TURBO DNA-free Kit, Thermo Fisher Scientific) for 30 min at 37°C, cleaned up (Qiagen RNA clean up kit, Qiagen, Venlo, The Netherlands) and resuspended in 30uL. RNA quality was assessed using RNA Screen Tape 6000 Pico LabChips with the Tape Station (Agilent Technologies) and the RNA concentration was measured by spectrophotometry using the Xpose (Trinean, Ghent, Belgium). RNAseq libraries were prepared

starting from 1 µg of total RNA using the Universal Plus mRNA-Seq kit (NuGen Technologies Inc. San Carlos, USA) as recommended by the manufacturer. The oriented cDNA produced from the poly-A+ fraction was sequenced on a HiSeq2500 from Illumina (Paired-End reads 130 bases + 130 bases). A total of ~70 millions of passing filter paired-end reads were produced per library. FASTQ files were mapped to the ENSEMBL Zebrafish (GRCz11) reference using Hisat2 and counted by *featureCounts* from the Subread R package. Read count normalizations and groups comparisons were performed by three independent and complementary statistical methods: Deseq2, edgeR, LimmaVoom. Flags were computed from counts normalized to the mean coverage. All normalized counts <20 were considered as background (flag 0) and >=20 as signal (flag=1). P50 lists used for the statistical analysis regroup the genes showing flag=1 for at least half of the compared samples. The results of the three methods were filtered at pvalue<=0.05 and folds 1.2/1.5/2 compared and grouped by Venn diagram. Cluster analysis was performed by hierarchical clustering using the Spearman correlation similarity measure and ward linkage algorithm. Functional analyses were carried out using Ingenuity Pathway Analysis (IPA, Qiagen). Heat maps were made with the R package ctc: Cluster and Tree Conversion and imaged by Java Treeview software.

Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). P value ≤ .05 was considered significant. The test used in each set of experiment is described in the corresponding figure legends.

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