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A human importin-β-related disorder: Syndromic thoracic aortic aneurysm caused by bi-allelic loss-of-function variants in *IPO8*

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Abstract

Importin 8, encoded by *IPO8*, is an ubiquitously expressed member of the importin-β protein family that translocates cargo molecules such as proteins, RNAs and ribonucleoprotein complexes into the nucleus in a RanGTP-dependent manner. Current knowledge of the cargoes of importin 8 is limited, but TGF-β signaling components such as SMAD1-4 have been suggested to be amongst them. Here, we report that bi-allelic loss-of-function variants in *IPO8* cause a syndromic form of thoracic aortic aneurysm (TAA) with clinical overlap with Loeys-Dietz and Shprintzen-Goldberg syndrome. Seven individuals from six unrelated families showed a consistent phenotype with early-onset TAA, motor developmental delay, connective tissue findings and craniofacial dysmorphic features. A C57Bl/6N *Ipo8* knock-out mouse model recapitulates TAA development from 8-12 weeks onwards in both sexes, but most prominently shows ascending aorta dilatation with a propensity for dissection in males. Compliance assays suggest augmented passive stiffness of the ascending aorta in male *Ipo8*$^{-/-}$ mice throughout life. Immunohistological investigation of mutant aortic walls reveals elastic fiber disorganization and fragmentation along with a signature of increased TGF-β signaling, as evidenced by nuclear pSmad2 accumulation. RT-qPCR assays of the aortic wall in male *Ipo8*$^{-/-}$ mice demonstrate decreased *Smad6/7* and increased *Mmp2* and *Ccn2 (Ctgf)* expression, reinforcing a role for dysregulation of the TGF-β signaling pathway in TAA development. As importin 8 is the most downstream TGF-β-related effector implicated in TAA pathogenesis so far, it offers opportunities for future mechanistic studies and represents a candidate drug target for TAA.

Thoracic aortic aneurysm (TAA) refers to a pathological and progressive dilatation of the aorta which, if left untreated, imposes a risk for life-threatening aortic dissection or rupture. TAA presents either as an isolated condition (non-syndromic TAA) or as part of a multi-systemic connective tissue disorder (syndromic TAA). Most typically, the inheritance pattern is autosomal dominant, but rare X-linked or autosomal recessive families have also been reported. As pathogenic variants in the more than 30 known TAA genes explain less than 30% of probands with a positive family history[1], additional TAA genes remain to be identified.

Important mechanistic insights into syndromic TAA formation have largely emanated from elucidation of the etiology of two clinically overlapping autosomal dominant TAA syndromes: Marfan syndrome (MFS [MIM: 154700]) and Loeys-Dietz syndrome (LDS [MIM: 609192, MIM: 610168, MIM: 613795, MIM: 614816 & MIM: 615582])[2]. Besides TAA, MFS is characterized by ocular (e.g. ectopia lentis), skeletal (e.g. overgrowth, pectus deformity) and cutaneous (e.g. striae, hernia) manifestations. LDS can be distinguished from MFS by the unique presence of hypertelorism, cleft palate or bifid uvula and prominent arterial tortuosity, as well as by a more widespread and severe aneurysm phenotype. Whereas MFS is caused by dominant-negative or haplo-insufficient variants in the extracellular matrix (ECM) component fibrillin 1[3] (*FBN1* [MIM: 134797]), LDS results from loss-of-function variants in six key components of the canonical transforming growth factor β (TGF-β) signaling pathway (i.e. TGFBR1/2 [MIM: 190181 & MIM: 190182], SMAD2/3 [MIM: 601366 & MIM: 603109], TGFB2/3 [MIM: 190220 & MIM: 190230]) (Figure S1)[4-10]. In both conditions, analysis of the aortic wall in mouse models and affected individuals shows a clear tissue signature for enhanced TGF-β signaling, including activation of signaling intermediates and increased output of TGF-β target genes[11]. Interestingly, a third condition with extensive phenotypic overlap with MFS and LDS but less severe cardiovascular involvement and the unique presence of neurodevelopmental delay (Shprintzen-Goldberg syndrome (SGS) [MIM: 182212]) is caused

by heterozygous missense variants located in the R-SMAD-binding domain of a negative regulator of the TGF-β transcriptional response called SKI (*SKI* [MIM: 164780]) (Figure S1)[12; 13].

Using exome or genome sequencing in six unrelated probands presenting with an LDS/SGS-like phenotype (for details see supplemental materials and methods), we identified bi-allelic loss-of-function variants in *IPO8* [MIM: 605600; GenBank: NM_006390.3], encoding the nuclear import protein importin 8 (Figure 1A, Figure S2). None of the probands carried a likely pathogenic variant in any of the known TAA genes. Except for p.(Leu866Profs*12) (c.2597_2601delTTTTC) (1/250920 alleles), all identified variants are absent from the Genome Aggregation Database (gnomAD v2.1.1). Causality is further supported by segregation analysis, which demonstrated heterozygosity in the unaffected parents and siblings (Figure 1A) as well as homozygosity in one additional affected brother (individual 4-II:3; Figure 1A). Subsequent Sanger sequencing of the coding regions of *IPO*8 in 50 other genetically unsolved MFS-, LDS- or SGS-like probands did not reveal additional individuals with homozygous or compound heterozygous variants.

Recurrent phenotypic manifestations in our series of cases with bi-allelic *IPO8* variants include facial dysmorphism with dolichocephaly (5/7), frontal bossing (6/7), hypertelorism (6/7), eyelid ptosis (4/7), retrognathia (6/7) and a high arched (6/7) or cleft palate/bifid uvula (3/7); skeletal findings with arachnodactyly (6/7), joint hypermobility (7/7), pectus excavatum (7/7), foot deformity (5/7) and scoliosis (3/7); neuromuscular features including hypotonia (7/7) and developmental delay (7/7); cardiovascular abnormalities with aortic root and/or ascending aortic aneurysm (6/7), structural heart disease (atrial or ventricular septal defect (ASD, VSD) and patent ductus arteriosus (PDA), 7/7); and finally, umbilical and/or inguinal hernia (5/7) (Figure 1B, Table 1). No disproportionate body growth was observed (Figure S3). Of note, despite the severe aneurysm phenotype, none of the affected individuals experienced an arterial

or aortic dissection, but this may be due to their young age. Additionally, marked arterial tortuosity, a typical LDS feature, was reported in two cases (2-II:1 and 6-II:1), but might have been overlooked in the others as they have not yet undergone head-to-pelvis arterial imaging. Overall, the phenotype fits in the spectrum of LDS/SGS-like disorders (Table 2).

Six out of eight *IPO8* variants are predicted to result in a premature termination codon and, as a result, to induce nonsense-mediated mRNA decay (NMD). Indeed, in fibroblast cDNA of individual 3-II:3 c.2597_2601delTTTTC was only observed upon puromycin treatment (Figure S4A). In blood-derived cDNA of the same child, c.1428+5G>A was found to result in exon 13 skipping (Figure S5A-B). *In silico* protein modeling of its predicted resultant in-frame deletion p.(Lys447_Arg476del) (c.1428+5G>A) suggests abnormal folding due to removal of a single helix (Figure S5C). In fibroblast cDNA of case 1-II:3, the variant allele was seen even in the absence of inhibition of NMD with puromycin, revealing surprising escape from NMD (Figure S4B). Western blotting on fibroblast lysates of individuals 1-II:3 and 3-II:3 using an N-terminal importin 8 antibody did not show protein expression, in keeping with a loss-of-function mechanism (Figure S4C). In proband 1-II:3, the lack of importin 8 protein is possibly attributed to translational repression, which previously has been described in other conditions[14], or significant protein instability. For individual 6-II:1 fibroblasts are not available, but *in silico* modeling of the predicted resultant deletion-insertion p.(Thr967_Glu1006delinsLys) (c.2900-1G>A) suggests removal of the last structured part of the protein (Figure S6), which, based on this region's role in controlling the protein conformation in some other β-importins, may significantly affect protein stability[15-17].

Murine importin 8 is 92% identical and 95% similar to its human orthologue, rendering mouse a suitable animal model to pursue supportive *in vivo* evidence for a causal relationship between *IPO8* deficiency and TAA. We used a C57Bl/6N *Ipo8*$^{-/-}$ model that was previously only known to present with reduced grip strength and diminished vertical activity, suggesting muscle

weakness as well as decreased locomotor exploration, respectively[18], and thus corroborating with the observed hypotonia and (possibly associated) motor delay in individuals with *IPO8* bi-allelic variants. Serial transthoracic echocardiography (age 4-32 weeks) of the aortic root at the level of the sinuses of Valsalva and distal ascending aorta in *Ipo8*$^{-/-}$ mice and their wild type (WT) littermates (N=17/group) revealed statistically significant progressive dilatation in mutant mice at both anatomical locations, with aneurysms of the distal ascending aorta already becoming visible at the age of 8-12 weeks (p_{root}=1.3E-3 (Figure 2A); p_{asc}=8.4E-9 (Figure 2B)). Intriguingly, sex-stratified analyses demonstrated aortic root enlargement in both mutant females (7 *Ipo8*$^{-/-}$ vs 8 WT; p_{root_f}=2.3E-3 (Figure S7A)) and males (10 *Ipo8*$^{-/-}$ vs 9 WT; p_{root_m}=2.3E-2 (Figure S7B)), whereas the ascending aortic aneurysm phenotype is very pronounced and only statistically significant in the male *Ipo8*$^{-/-}$ animals (p_{asc_f}=6.5E-2 (Figure S7C) vs p_{asc_m}=8.4E-10 (Figure 2C)). After the last echo at 32 weeks, 14 *Ipo8*$^{-/-}$ and 17 WT animals were kept alive until the age of 48 weeks. Of these, three homozygous mutant males (3/9, 33.3%) died from an aortic rupture at the age of 32, 36 and 46 weeks, respectively, while no aortic rupture-related mortality was seen in the homozygous females (0/5, 0%) or WT animals (0/17, 0%). Sex differences in syndromic TAA penetrance and severity have been reported before, both in mice and humans[19; 20]. Generally, males are more severely affected, exhibiting larger aortas and experiencing dissection and/or rupture more frequently[21; 22]. Several studies in TAA mouse models have attempted to define the basis for the observed sex differences, revealing a context-dependent role for female and male hormone signaling, hypertension and/or exacerbated ERK activation, but no predominant mechanism has been identified[20]. The C57Bl/6N *Ipo8*$^{-/-}$ mouse model represents a promising tool to further investigate the TAA sexual dimorphism. Of note, during our echocardiography studies we did not observe severe structural outflow tract defects. Evaluation of lateral and dorsoventral total body X-rays, which are publicly available through the International Mouse Phenotyping

Consortium (IMPC) portal, did not show evidence for scoliosis (visual inspection) or increased kyphosis (quantitative evaluation; p=2.8E-1) in *Ipo8*$^{-/-}$ mice as compared to wild type animals. Given the fact that the aneurysmal phenotype is most pronounced in males at the level of the distal ascending aorta, we performed further experiments in male mice only. To study the biomechanical properties of distal ascending aortic rings, the 'rodent oscillatory tension set-up to study arterial compliance' (ROTSAC) assay was used[23]. More precisely, *ex vivo* aortic stiffness was assessed at 12 (5 *Ipo8*$^{-/-}$ vs 4 WT), 24 (4 *Ipo8*$^{-/-}$ vs 4 WT) and 52 (4 *Ipo8*$^{-/-}$ vs 2 WT) weeks of age. Different experimental conditions were used to evaluate the involvement of vascular smooth muscle cells (VSMCs) and/or endothelial cells. The Peterson modulus (Ep) was first determined in Krebs-Ringer solution at a distention pressure of 80-120 mmHg and 120-160 mmHg, revealing a trend towards higher Ep values and, thus, stiffer ascending aortas at 120-160 mmHg in 12, 24 and 52 week old *Ipo8*$^{-/-}$ male animals as compared to controls (Figure 3, Figure S8). As complete VSMC relaxation by diethylamine NONOate (DEANO) addition or VSMC stimulation with phenylephrine (PE), even upon nitric oxide synthase (NOS) inhibition through N(Ω)-nitro-L-arginine methyl ester (L-NAME) addition, did not considerably alter the Ep increase in *Ipo8* null males (Figure 3), increased basal tone nor sustained VSMC contraction seem to contribute to the increased aortic stiffness. Our data rather point towards an increased passive stiffness of the ascending aorta in male *Ipo8*$^{-/-}$ mice throughout life. Increased arterial stiffness, an important marker for cardiovascular disease, has previously been observed in genetic TAA mouse models[24] and affected individuals[25]. In an established MFS mouse model, i.e. *Fbn1*$^{mgR/mgR}$, stiffness was augmented in mutant non-aneurysmal (circa 3-fold) and aneurysmal (circa 4-fold) ascending aortas, which upon histological analysis was shown to correlate with a diffuse loss in elastic fiber integrity[24]. Compared to age-matched controls, TAA cases exhibit a stiffer mechanical response with aortic biomechanical properties resembling those of a significantly older ('aged') non-aneurysmal

cohort[26]. Given the observed trend towards stiffer ascending aortas in *Ipo8*$^{-/-}$ mice (Figure 3) and recurrent prior associations between aortic ECM deterioration and TAA[2], we evaluated the structural ECM integrity using histological elastin and collagen staining in ascending aortic sections of 12- (3 *Ipo8*$^{-/-}$ vs 3 WT), 24- (3 *Ipo8*$^{-/-}$ vs 3 WT) and 52-week (3 *Ipo8*$^{-/-}$ vs 2 WT) old mice. Whereas the collagen content did not differ noticeably (Figure S9A), the elastic fibers were more disorganized and fragmented in mutant males of all age groups as compared to their WT counterparts ($p_{age\text{-}combined}$=5.2E-4) (Figure 4A-B, Figure S9B).

Importin 8 is a nuclear transport receptor belonging to the importin-β protein family, which has not been linked to human diseases before. It is ubiquitously expressed and becomes upregulated upon TGF-β1 stimulation[27]. β-importins translocate cargo molecules such as proteins, RNAs and ribonucleoprotein complexes to the nucleus in a RanGTP-dependent manner. While a specific cargo can be shuttled by multiple β-importins, superior affinity to one of them is often observed. The most established cargoes for human importin 8 are phosphorylated SMADs 1-4 (pSMAD1-4)[28], AGO2[29], mature miRNAs[30], EIF4E[31] and SRP19[32]. Apart from being a nuclear transport receptor, importin 8 has been implicated in miRNA-guided gene silencing[29]. Given that individuals with bi-allelic *IPO8* variants phenotypically resemble individuals with TGF-β-related aortopathy syndromes such as LDS and SGS and key effectors of the canonical TGF-β pathway (i.e. pSMAD2-4) have been reported to be shuttled by importin 8[28], a plausible hypothesis is that dysregulated TGF-β signaling is involved in the pathogenesis of *IPO8*-related disease (Figure S1). We determined the levels of nuclear pSmad2, an effector of canonical TGF-β signaling, in ascending aortic sections of 12- (3 *Ipo8*$^{-/-}$ vs 3 WT), 24- (3 *Ipo8*$^{-/-}$ vs 3 WT) and 52-week (3 *Ipo8*$^{-/-}$ vs 2 WT) old mice. A larger fraction of nuclei stained positive for pSmad2 in *Ipo8*$^{-/-}$ mice as compared to WT animals ($p_{age\text{-}combined}$=3.4E-2), suggesting a role for dysregulated TGF-β signaling in the pathogenesis of *IPO8*-related TAA (Figure 4A-B, Figure S9C). Subsequent RT-qPCR analysis for nine TGF-β superfamily-related genes (i.e. *Tgfb1*,

Tgfb2, *Smad4*, *Smad6*, *Smad7*, *Mmp2, Ccn2 (Ctgf), Eln and Serpine1 (Pai1)*) in ascending aortic samples of 16-week old *Ipo8*$^{-/-}$ and WT males (N=12/group) revealed significantly reduced *Smad6* (p=6.0E-3) and *Smad7* (p=3.6E-2) mRNA expression in the mutant animals, along with a significant increase in *Mmp2* (p=4.2E-3) and *Ccn2 (Ctgf)* (p=7.8E-3) (Figure 5). SMAD6 and 7 inhibit SMAD-dependent and -independent TGF-β family signaling through various mechanisms[33]. Whereas SMAD6 preferentially inhibits bone morphogenetic protein (BMP)-related signaling[34], SMAD7 impedes both TGF-β- and BMP-induced signaling[35]. In the absence of SMAD7, TGF-β receptor activation is augmented, resulting in excessive SMAD2/3 phosphorylation. The detected decrease in *Smad7* mRNA levels in the *Ipo8*$^{-/-}$ aortic walls might thus be directly linked to the observed increase in nuclear pSmad2 levels. SMAD6, on the other hand, has mostly been linked to BMP signaling, which is less well studied in the context of TAA development. Nonetheless, our group identified loss-of-function *SMAD6* variants as a cause of bicuspid aortic valve-related TAA[36; 37], demonstrating a mechanistic link between SMAD6 deficiency and TAA development. *MMP2* and *CCN2 (CTGF)* are prototypical downstream transcriptional targets of the TGF-β signaling pathway[38]. *MMP2* belongs to the family of matrix metalloproteinases, which mediate the physiological turnover of the aortic ECM by degrading structural ECM proteins, including collagen and elastin[39]. In TAA cases and mouse models, MMP2 levels and/or activity are strongly increased[40-42]. Moreover, *Mmp2* deletion in *Fbn1*$^{mgR/mgR}$ mice inhibited TGF-β activation and subsequent Smad2 and Erk1/2 phosphorylation[43], which significantly prolonged the lifespan of the MFS *Fbn1*$^{mgR/mgR}$ mice[43]. As such, increased *Mmp2* expression might connect increased TGF-β signaling and impaired elastic fiber integrity in our *Ipo8*$^{-/-}$ mouse model. CCN2 (CTGF) is a multifunctional protein that is involved in ECM remodeling[38]. Overexpression of CCN2 (CTGF) has been proven to be associated with TAA development[44] and was previously been shown to be upregulated in the aortic walls of individuals with LDS [4;7]. Interestingly, elastic fiber fragmentation but normal

collagen content, as well as reduced *Smad6* and *Smad7* mRNA expression levels and higher Mmp activity were also described in aneurysmal aortic tissue specimens and/or VSMCs of *Smad3*$^{-/-}$ mice, an established LDS model that presents with TAA already at the age of 6 weeks[45]. Together, our histological, immunohistochemistry and RT-qPCR findings suggest a link between *IPO8* deficiency and dysregulated TGF-β signaling. Moreover, they recapitulate prior observations in an established LDS mouse model, further relating *IPO8*-related TAA to the LDS disease spectrum.

In conclusion, we describe a syndrome caused by bi-allelic loss-of-function variants in *IPO8*. The human and mouse phenotypes caused by importin 8 loss-of-function are characterized by severe early-onset TAA development. Our immunohistochemistry and RT-qPCR studies of murine *Ipo8*-deficient aortic tissue reveal pathophysiological mechanisms that have previously been described in clinically overlapping TGF-β-related signalopathies. Further research is warranted to obtain more in-depth insight into the disease's clinical course and mechanisms. First, identification of additional individuals with bi-allelic *IPO8* variants will shed better light on the variability with respect to disease expressivity and penetrance. Moreover, longitudinal follow-up of affected individuals will provide information on aortic/arterial dissection or rupture risk. Interestingly, our clinical findings are corroborated by the observations of Ziegler et al in this issue of AJHG who describe aortic dilatation in 11 out 12 individuals with bi-allelic *IPO8* variants. Second, it remains to be determined if and how abnormal cytosol-to-nucleus shuttling elicits *IPO8*-related disease and dysregulated TGF-β signaling in aneurysmal aortic walls. Finally, as we predominantly focused on the TAA phenotype, it would be interesting to have a closer look at the mechanisms involved in the other affected organ systems, especially the neuromuscular system in order to explain the motor developmental delay that was observed in individuals with *IPO8* bi-allelic variants.

Supplemental data

The supplemental data file contains details on the materials and methods, nine figures and supplemental case reports.

Consortia

Genomics England Research Consortium

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Declaration of interests

The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing conducted at Baylor Genetics Laboratories. AB is an employee of GeneDx, Inc.

Web resources

Genome Aggregation Database (gnomAD), gnomad.broadinstitute.org

International Mouse Phenotyping Consortium, www.mousephenotype.org

Online Mendelian Inheritance in Man, omim.org

Protein Databank (PDB), www.rcsb.org

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343 **Data and Code Availability**

344 The *IPO8* variants were submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)

345 (GenBank: NM_006390.3, accession numbers SCV001547250 - SCV001547257). WES

346 datasets have not been deposited in a public repository because of privacy and ethical

347 restrictions but are available from the corresponding author on request.

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Figure legends

Figure 1. Familial screening and clinical characterization of individuals with bi-allelic *IPO8* variants. A) Pedigrees of the families with their respective pathogenic variants. Squares represent males while circles represent females, filled symbols denote affected individuals, a double line connecting spouses symbolizes consanguinity and a - or + sign denotes presence or absence of the respective *IPO8* variant. Variants are annotated against NM_006390.3. B) Clinical phenotyping. Proband 1-II:3 showing prominent forehead, hypertelorism, mild ptosis left eye, retrognathia, pectus excavatum, umbilical hernia, joint hypermobility with thumb abduction and camptodactyly of the second toe. CT angiography of proband 2-II:1 demonstrating dilatation of the common carotid arteries along with marked tortuosity of the common carotid and internal carotid artery, mild tortuosity of the vertebral arteries, enlargement of the anterior and middle cerebral arteries bilaterally. Proband 3-II:3 presenting with frontal bossing with bitemporal flattening, retrognathia, downturned corners of the mouth and flat feet. Proband 5-II:2 showing prominent forehead, significant hypertelorism with flat nasal bridge, mild ptosis of left eye and retrognathia. Proband 6-II:1 demonstrating dolichocephaly, retrognathia, malar flattening, downslanting palpebral fissures and hypertelorism. MRA revealing tortuous intracranial and extracranial arterial vessels, most prominently involving the superior cervical internal carotid arteries with dilation of the left internal carotid artery at the carotid bifurcation. CT-scan (pre-surgical) showing os odontoideum with cervical spinal canal stenosis (arrows).

Figure 2. Progressive TAA development in *Ipo8*$^{-/-}$ mice. A) Log of weight-corrected aortic root diameters in male and female mice combined (N=17/group). B) Log of weight-corrected ascending aortic diameters in male and female mice combined (N=17/group). C) Log of weight-corrected ascending aortic diameters in male mice only (10 *Ipo8*$^{-/-}$ vs 9 WT). The error bars

show the standard error of the mean (SEM). P-values were calculated using mixed model analysis, which represent the interaction term between genotype and age. WT: wild type.

Figure 3. Trend towards increased ascending aortic passive stiffness in *Ipo8*$^{-/-}$ mice at a distention pressure of 120-160 mmHg. Age- and genotype-dependency of the Peterson modulus (Ep) of ascending aortic segments of male *Ipo8*$^{-/-}$ and wild type mice under control (Krebs-Ringer), maximally relaxed (DEANO) and contracted (PE or PE + L-NAME) conditions at 12 (5 *Ipo8*$^{-/-}$ vs 4 WT), 24 (4 *Ipo8*$^{-/-}$ vs 4 WT) and 52 (4 *Ipo8*$^{-/-}$ vs 2 WT) weeks of age. The error bars show the SEM. Two-way ANOVA p-values are shown (*$p < 0.05$). Sidak post-hoc testing did not reveal statistically significant genotype-based differences in Ep. PE: Phenylephrine, DEANO: diethylamine NONOate, L-NAME: N(Ω)-nitro-L-arginine methyl ester, Ep: Peterson modulus, WT: wild type, NS: non-significant.

Figure 4. Elastic fiber deterioration and nuclear pSmad2 accumulation in the ascending aorta of *Ipo8*$^{-/-}$ mice. A) Histological and immunohistochemistry images demonstrating marked elastin disorganization and fragmentation as well as prominent nuclear pSmad2 accumulation in *Ipo8*$^{-/-}$ mice. Scale bar = 50μm. B) Elastic fiber integrity scores and nuclear pSmad2 grades of the ascending aorta of all ages combined (12- (3 *Ipo8*$^{-/-}$ vs 3 WT), 24- (3 *Ipo8*$^{-/-}$ vs 3 WT) and 52-weeks (3 *Ipo8*$^{-/-}$ vs 2 WT)). Elastin grades can range from 1 to 4, with grade 1 sections presenting with continuous and well-organized elastic bundles and grade 4 sections displaying vastly disorganized fibers, marked fiber fragmentation and a thickened aortic wall. For pSmad2, grade 1, 2, 3 and 4 denote sections in which respectively <25%, 25-50%, 50-75% and 75-100% of nuclei stained positive. Averaged age-combined scores of blinded observations of three independent researchers are shown. The error bars depict the SEM. P-values were calculated using two-way ANOVA statistics (*$p < 0.05$, ***$p < 0.001$). WT: wild type

Figure 5. mRNA expression analysis of TGF-β-related genes reveals decreased *Smad6* and *Smad7* levels as well as increased *Mmp2* and *Ccn2 (Ctgf)* levels in the ascending aorta of *Ipo8*$^{-/-}$ mice. Ascending aortic samples of 16-weeks old *Ipo8*$^{-/-}$ and WT males were used (N=12/group). The error bars depict the SEM. P-values were calculated using mixed model statistics (*p < 0.05, **p < 0.01). WT: wild type, NS: non-significant

Table legends

Table 1. Detailed overview of the clinical characteristics of individuals with bi-allelic *IPO8* variants.

ND: not done, ?: unknown, L: left, R: right, +: present, -: absent, Z: z-score (calculated according to *Lopez et al.*)[46], P: percentile, Com: common, int: internal, ASD: atrial septal defect, VSD: ventricular septal defect, PDA: patent ductus arteriosus, homz: homozygous, yrs: years, mo: months, bilat: bilateral, umb: umbilical, membr: membraneous, OFC: occipitofrontal circumference. § Proband 6:II-1 also has a chromosomal duplication (1.779 Mb gain of 19q13.41) and learning disability is also present in proband's mother and maternal half-brother.

Table 2. Comparative overview of the clinical characteristics of *IPO8*-related aortopathy and phenotypically overlapping TAA syndromes.

-: absent, +: occasional, ++: common, +++: typical clinical feature, MFS: Marfan syndrome, LDS: Loeys-Dietz syndrome, SGS, Shprintzen-Goldberg syndrome, TAA: thoracic aortic aneurysm; AD: autosomal dominant, AR: autosomal recessive, BAV: bicuspid aortic valve, ASD: atrial septal defect, VSD: ventricular septal defect, PDA: patent ductus arteriosus.

Table 1. Detailed overview of the clinical characteristics of individuals with bi-allelic *IPO8* variants

	Family 1 **1-II:3**	**Family 2** **2-II:1**	**Family 3** **3-II:3**	**Family 4** **4-II:4**	**Family 4** **4-II:3**	**Family 5** **5-II:2**	**Family 6** **6-II:1**
Variant c. annotation	c.1420C>T homz	c.770_777delTATGGTGG; c.1000dupG	c.1428+5G>A; c.2597_2601delTTTTC	c.776G>A homz	c.776G>A homz	c. 2347_2369del homz	c.2900-1G>A homz
Variant p. annotation	p.(Arg474*) homz	p.(Val257Glufs*3); p.(Val334Glyfs*19)	p.(Lys447_Arg476del); p.(Leu866Profs*12)	p.(Trp259*) homz	p.(Trp259*) homz	p.(Leu783Valfs*5) homz	p.(Thr967_Glu1006 delinsLys) homz
Sex	M	M	F	M	M	F	M
Current age	10 yrs	8 yrs	8 yrs	6 yrs	10 yrs	3 yrs 9m	19 yrs
Growth Height Weight OFC	(7yrs 11mo) 124 cm (P10-25) 21 kg (P3-5) 55 cm (P97)	(8 yrs) 127 cm (P25) 19.9 kg (P1)	(7yrs 4mo) 118.7 cm (P10-25) 22 kg (P25-50) 53.5 cm (P50-75)	(6yrs) 121 cm (P75) 18.3 kg (P25-50)	(9 yrs) 126 cm (P10-25) 17.6 kg (P0.3)	(3yrs9m) 92 cm (P3) 11 kg (P0.5) 47 cm (P10)	(19 years) 175 cm (P25-50) 63 kg (P25)
Facial features							
Dolichocephaly	+	+	- (prominent sutures)	+	+	-	+
Frontal bossing	+	+	+	+	+	+	-
Hypertelorism	+	+	-	+	+	+	+
Ptosis	+ (L>R)	+ (L>R)	-	-	-	+ (L>R)	+
Retrognathia	+	+	+	-	-	+	+
Submucous cleft palate	-	+ & broad uvula	-	-	-	+ (bifid uvula)	+ (bifid uvula)
High arched palate	+	+	+	+	+	-	+
Skeletal findings							
Arachnodactyly	+	+	-	+	+	+	+
Joint hypermobility	+	+	+	+	+	+	+
Pectus excavatum	+	+	+	+	+	+	+
Pes planum	+	+	+	+	+	-	-
Cervical spine anomalies	ND	+	-	ND	-	-	+
Scoliosis	-	+	-	-	+	-	+
Other	2nd toes camptodactyly	Kyphosis	Recurrent hip, ankle dislocation	Talipes equinovarus (L) Vertical talus (R)	Sagittal clefts of midthoracic vertebrae Talipes equino varus (R)		Long toes
Neurological findings							
Hypotonia	+	+	+	+	+	+	+
Developmental delay	+ (mild)	+	+ (motor)	+ (motor)	+ (motor)	+ (motor)	+
Intellectual disability	-	-	-	-	-	mild	+[§]
Cardiovascular findings	(10yrs 8mo)	(8yrs)	(7yrs 5mo)	(1yr 8mo)	(9 yrs)	(42 months)	(19 years)
ASD	+	+	+	-	-	+	+ (aneurysmal)
VSD	-	-	+ (membr & muscular)	+ (membraneous)	+	+ (membraneous)	-

PDA	-	+	+ (surgical repair)	+	-	-	-
Aortic root	26 mm (Z=3.5)	35 mm (Z=10)	25 mm (Z=3.58)	25 mm (Z=5.7)	38 mm (Z=6.0)	15 mm (Z=0,5)	41 mm (Z=6.9)
Ascending aorta	28 mm (Z=5.7)	28 mm (Z=8.7)	21 mm (Z=2.68)	17 mm (Z=3.9)	23 mm (Z=2.7)		31 mm (Z=3.8)
Sinotubular junction		25 mm (Z=5.4)	23 mm (Z=4.99)		25mm (Z=3.8)	12 mm (Z=0.18)	23 mm (Z=1.2)
Other aneurysms	ND	Com/int carotid, cerebral arteries	ND	ND	ND	pulmonary artery, coronary sinus	ND
Arterial/aortic tortuosity		+					+
Other findings							
Hernia	Umbilical	Umb/bilat inguinal	-	-	Umbilical	Umbilical	Umb/inguinal
Easy bruising	+	+	-	-	-	-	-

571 **Table 2. Comparison of Marfan, Loeys-Dietz, Sphrintzen-Goldberg and IPO8**

572 **phenotypical characteristics**

	MFS	**LDS**	**SGS**	**IPO8**
Gene	*FBN1*	*TGFBR1/2* *SMAD2/3* *TGFB2/3*	*SKI*	*IPO8*
Inheritance	AD	AD	AD – *de novo*	AR
Ectopia lentis	+++	-	-	-
Cleft palate/bifid uvula	-	++	+	+
Hypertelorism	-	++	++	++
Proptosis	-	+	++	++
Craniosynostosis	-	+	+++	-
Arachnodactyly	+++	++	++	++
Tall stature	+++	+	++	-
Pectus deformity	++	++	++	++
Club foot	-	++	+	+
Joint hypermobility	+	++	++	+++
Cervical spine instability	-	++	+	+
Osteo-arthritis	+	++	+	?
Hernia (umbilical, inguinal,…)	+	+	+	+
Aortic root aneurysm	+++	+++	+	+++
Ascending aneurysm	+	++	+	++
Arterial aneurysm	-/+	+++	+	+
Arterial tortuosity	-	+++	+	+
Early aortic dissection	+	++	-	-
BAV/ASD/VSD/PDA	-	+	-	++
Motor developmental delay	-	-	++	++
Intellectual disability	-	-	++	-

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