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A human importin- $\beta$ -related disorder : syndromic thoracic aortic aneurysm caused by bi-allelic loss-of-function variants in IPO8

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1 **A human importin- $\beta$ -related disorder:**

2 **Syndromic thoracic aortic aneurysm**

3 **caused by bi-allelic loss-of-function variants in *IPO8***

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71 **Abstract**

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

91

92 Thoracic aortic aneurysm (TAA) refers to a pathological and progressive dilatation of the aorta  
93 which, if left untreated, imposes a risk for life-threatening aortic dissection or rupture. TAA  
94 presents either as an isolated condition (non-syndromic TAA) or as part of a multi-systemic  
95 connective tissue disorder (syndromic TAA). Most typically, the inheritance pattern is  
96 autosomal dominant, but rare X-linked or autosomal recessive families have also been reported.  
97 As pathogenic variants in the more than 30 known TAA genes explain less than 30% of  
98 probands with a positive family history<sup>1</sup>, additional TAA genes remain to be identified.  
99 Important mechanistic insights into syndromic TAA formation have largely emanated from  
100 elucidation of the etiology of two clinically overlapping autosomal dominant TAA syndromes:  
101 Marfan syndrome (MFS [MIM: 154700]) and Loeys-Dietz syndrome (LDS [MIM: 609192,  
102 MIM: 610168, MIM: 613795, MIM: 614816 & MIM: 615582])<sup>2</sup>. Besides TAA, MFS is  
103 characterized by ocular (e.g. ectopia lentis), skeletal (e.g. overgrowth, pectus deformity) and  
104 cutaneous (e.g. striae, hernia) manifestations. LDS can be distinguished from MFS by the  
105 unique presence of hypertelorism, cleft palate or bifid uvula and prominent arterial tortuosity,  
106 as well as by a more widespread and severe aneurysm phenotype. Whereas MFS is caused by  
107 dominant-negative or haplo-insufficient variants in the extracellular matrix (ECM) component  
108 fibrillin 1<sup>3</sup> (*FBNI* [MIM: 134797]), LDS results from loss-of-function variants in six key  
109 components of the canonical transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling pathway (i.e.  
110 *TGFBR1/2* [MIM: 190181 & MIM: 190182], *SMAD2/3* [MIM: 601366 & MIM: 603109],  
111 *TGFB2/3* [MIM: 190220 & MIM: 190230]) (Figure S1)<sup>4-10</sup>. In both conditions, analysis of the  
112 aortic wall in mouse models and affected individuals shows a clear tissue signature for enhanced  
113 TGF- $\beta$  signaling, including activation of signaling intermediates and increased output of TGF-  
114  $\beta$  target genes<sup>11</sup>. Interestingly, a third condition with extensive phenotypic overlap with MFS  
115 and LDS but less severe cardiovascular involvement and the unique presence of  
116 neurodevelopmental delay (Shprintzen-Goldberg syndrome (SGS) [MIM: 182212]) is caused

117 by heterozygous missense variants located in the R-SMAD-binding domain of a negative  
118 regulator of the TGF- $\beta$  transcriptional response called SKI (*SKI* [MIM: 164780]) (Figure S1)<sup>12;</sup>  
119 <sup>13</sup>.

120 Using exome or genome sequencing in six unrelated probands presenting with an LDS/SGS-  
121 like phenotype (for details see supplemental materials and methods), we identified bi-allelic  
122 loss-of-function variants in *IPO8* [MIM: 605600; GenBank: NM\_006390.3], encoding the  
123 nuclear import protein importin 8 (Figure 1A, Figure S2). None of the probands carried a likely  
124 pathogenic variant in any of the known TAA genes. Except for p.(Leu866Profs\*12)  
125 (c.2597\_2601delTTTTC) (1/250920 alleles), all identified variants are absent from the Genome  
126 Aggregation Database (gnomAD v2.1.1). Causality is further supported by segregation  
127 analysis, which demonstrated heterozygosity in the unaffected parents and siblings (Figure 1A)  
128 as well as homozygosity in one additional affected brother (individual 4-II:3; Figure 1A).  
129 Subsequent Sanger sequencing of the coding regions of *IPO8* in 50 other genetically unsolved  
130 MFS-, LDS- or SGS-like probands did not reveal additional individuals with homozygous or  
131 compound heterozygous variants.

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

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

146 Six out of eight *IPO8* variants are predicted to result in a premature termination codon and, as  
147 a result, to induce nonsense-mediated mRNA decay (NMD). Indeed, in fibroblast cDNA of  
148 individual 3-II:3 c.2597\_2601delTTTTC was only observed upon puromycin treatment (Figure  
149 S4A). In blood-derived cDNA of the same child, c.1428+5G>A was found to result in exon 13  
150 skipping (Figure S5A-B). *In silico* protein modeling of its predicted resultant in-frame deletion  
151 p.(Lys447\_Arg476del) (c.1428+5G>A) suggests abnormal folding due to removal of a single  
152 helix (Figure S5C). In fibroblast cDNA of case 1-II:3, the variant allele was seen even in the  
153 absence of inhibition of NMD with puromycin, revealing surprising escape from NMD (Figure  
154 S4B). Western blotting on fibroblast lysates of individuals 1-II:3 and 3-II:3 using an N-terminal  
155 importin 8 antibody did not show protein expression, in keeping with a loss-of-function  
156 mechanism (Figure S4C). In proband 1-II:3, the lack of importin 8 protein is possibly attributed  
157 to translational repression, which previously has been described in other conditions<sup>14</sup>, or  
158 significant protein instability. For individual 6-II:1 fibroblasts are not available, but *in silico*  
159 modeling of the predicted resultant deletion-insertion p.(Thr967\_Glu1006delinsLys) (c.2900-  
160 1G>A) suggests removal of the last structured part of the protein (Figure S6), which, based on  
161 this region's role in controlling the protein conformation in some other  $\beta$ -importins, may  
162 significantly affect protein stability<sup>15-17</sup>.

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



167 weakness as well as decreased locomotor exploration, respectively<sup>18</sup>, and thus corroborating  
168 with the observed hypotonia and (possibly associated) motor delay in individuals with *IPO8* bi-  
169 allelic variants. Serial transthoracic echocardiography (age 4-32 weeks) of the aortic root at the  
170 level of the sinuses of Valsalva and distal ascending aorta in *Ipo8*<sup>-/-</sup> mice and their wild type  
171 (WT) littermates (N=17/group) revealed statistically significant progressive dilatation in mutant  
172 mice at both anatomical locations, with aneurysms of the distal ascending aorta already  
173 becoming visible at the age of 8-12 weeks ( $p_{\text{root}}=1.3\text{E-}3$  (Figure 2A);  $p_{\text{asc}}=8.4\text{E-}9$  (Figure 2B)).  
174 Intriguingly, sex-stratified analyses demonstrated aortic root enlargement in both mutant  
175 females (7 *Ipo8*<sup>-/-</sup> vs 8 WT;  $p_{\text{root}_f}=2.3\text{E-}3$  (Figure S7A)) and males (10 *Ipo8*<sup>-/-</sup> vs 9 WT;  
176  $p_{\text{root}_m}=2.3\text{E-}2$  (Figure S7B)), whereas the ascending aortic aneurysm phenotype is very  
177 pronounced and only statistically significant in the male *Ipo8*<sup>-/-</sup> animals ( $p_{\text{asc}_f}=6.5\text{E-}2$  (Figure  
178 S7C) vs  $p_{\text{asc}_m}=8.4\text{E-}10$  (Figure 2C)). After the last echo at 32 weeks, 14 *Ipo8*<sup>-/-</sup> and 17 WT  
179 animals were kept alive until the age of 48 weeks. Of these, three homozygous mutant males  
180 (3/9, 33.3%) died from an aortic rupture at the age of 32, 36 and 46 weeks, respectively, while  
181 no aortic rupture-related mortality was seen in the homozygous females (0/5, 0%) or WT  
182 animals (0/17, 0%). Sex differences in syndromic TAA penetrance and severity have been  
183 reported before, both in mice and humans<sup>19; 20</sup>. Generally, males are more severely affected,  
184 exhibiting larger aortas and experiencing dissection and/or rupture more frequently<sup>21; 22</sup>. Several  
185 studies in TAA mouse models have attempted to define the basis for the observed sex  
186 differences, revealing a context-dependent role for female and male hormone signaling,  
187 hypertension and/or exacerbated ERK activation, but no predominant mechanism has been  
188 identified<sup>20</sup>. The C57Bl/6N *Ipo8*<sup>-/-</sup> mouse model represents a promising tool to further  
189 investigate the TAA sexual dimorphism. Of note, during our echocardiography studies we did  
190 not observe severe structural outflow tract defects. Evaluation of lateral and dorsoventral total  
191 body X-rays, which are publicly available through the International Mouse Phenotyping

192 Consortium (IMPC) portal, did not show evidence for scoliosis (visual inspection) or increased  
193 kyphosis (quantitative evaluation;  $p=2.8E-1$ ) in *Ipo8*<sup>-/-</sup> mice as compared to wild type animals.  
194 Given the fact that the aneurysmal phenotype is most pronounced in males at the level of the  
195 distal ascending aorta, we performed further experiments in male mice only. To study the  
196 biomechanical properties of distal ascending aortic rings, the ‘rodent oscillatory tension set-up  
197 to study arterial compliance’ (ROTSAC) assay was used<sup>23</sup>. More precisely, *ex vivo* aortic  
198 stiffness was assessed at 12 (5 *Ipo8*<sup>-/-</sup> vs 4 WT), 24 (4 *Ipo8*<sup>-/-</sup> vs 4 WT) and 52 (4 *Ipo8*<sup>-/-</sup> vs 2  
199 WT) weeks of age. Different experimental conditions were used to evaluate the involvement of  
200 vascular smooth muscle cells (VSMCs) and/or endothelial cells. The Peterson modulus ( $E_p$ )  
201 was first determined in Krebs-Ringer solution at a distention pressure of 80-120 mmHg and  
202 120-160 mmHg, revealing a trend towards higher  $E_p$  values and, thus, stiffer ascending aortas  
203 at 120-160 mmHg in 12, 24 and 52 week old *Ipo8*<sup>-/-</sup> male animals as compared to controls  
204 (Figure 3, Figure S8). As complete VSMC relaxation by diethylamine NONOate (DEANO)  
205 addition or VSMC stimulation with phenylephrine (PE), even upon nitric oxide synthase (NOS)  
206 inhibition through N( $\Omega$ )-nitro-L-arginine methyl ester (L-NAME) addition, did not  
207 considerably alter the  $E_p$  increase in *Ipo8* null males (Figure 3), increased basal tone nor  
208 sustained VSMC contraction seem to contribute to the increased aortic stiffness. Our data rather  
209 point towards an increased passive stiffness of the ascending aorta in male *Ipo8*<sup>-/-</sup> mice  
210 throughout life. Increased arterial stiffness, an important marker for cardiovascular disease, has  
211 previously been observed in genetic TAA mouse models<sup>24</sup> and affected individuals<sup>25</sup>. In an  
212 established MFS mouse model, i.e. *Fbn1*<sup>mgR/mgR</sup>, stiffness was augmented in mutant non-  
213 aneurysmal (circa 3-fold) and aneurysmal (circa 4-fold) ascending aortas, which upon  
214 histological analysis was shown to correlate with a diffuse loss in elastic fiber integrity<sup>24</sup>.  
215 Compared to age-matched controls, TAA cases exhibit a stiffer mechanical response with aortic  
216 biomechanical properties resembling those of a significantly older (‘aged’) non-aneurysmal

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

224 Importin 8 is a nuclear transport receptor belonging to the importin- $\beta$  protein family, which has  
225 not been linked to human diseases before. It is ubiquitously expressed and becomes upregulated  
226 upon TGF- $\beta$ 1 stimulation<sup>27</sup>.  $\beta$ -importins translocate cargo molecules such as proteins, RNAs  
227 and ribonucleoprotein complexes to the nucleus in a RanGTP-dependent manner. While a  
228 specific cargo can be shuttled by multiple  $\beta$ -importins, superior affinity to one of them is often  
229 observed. The most established cargoes for human importin 8 are phosphorylated SMADs 1-4  
230 (pSMAD1-4)<sup>28</sup>, AGO2<sup>29</sup>, mature miRNAs<sup>30</sup>, EIF4E<sup>31</sup> and SRP19<sup>32</sup>. Apart from being a nuclear  
231 transport receptor, importin 8 has been implicated in miRNA-guided gene silencing<sup>29</sup>. Given  
232 that individuals with bi-allelic *IPO8* variants phenotypically resemble individuals with TGF- $\beta$ -  
233 related aortopathy syndromes such as LDS and SGS and key effectors of the canonical TGF- $\beta$   
234 pathway (i.e. pSMAD2-4) have been reported to be shuttled by importin 8<sup>28</sup>, a plausible  
235 hypothesis is that dysregulated TGF- $\beta$  signaling is involved in the pathogenesis of *IPO8*-related  
236 disease (Figure S1). We determined the levels of nuclear pSmad2, an effector of canonical TGF-  
237  $\beta$  signaling, in ascending aortic sections of 12- (3 *Ipo8*<sup>-/-</sup> vs 3 WT), 24- (3 *Ipo8*<sup>-/-</sup> vs 3 WT) and  
238 52-week (3 *Ipo8*<sup>-/-</sup> vs 2 WT) old mice. A larger fraction of nuclei stained positive for pSmad2  
239 in *Ipo8*<sup>-/-</sup> mice as compared to WT animals ( $p_{\text{age-combined}}=3.4\text{E-}2$ ), suggesting a role for  
240 dysregulated TGF- $\beta$  signaling in the pathogenesis of *IPO8*-related TAA (Figure 4A-B, Figure  
241 S9C). Subsequent RT-qPCR analysis for nine TGF- $\beta$  superfamily-related genes (i.e. *Tgfb1*,

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

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

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

291

292 **Supplemental data**

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

295

296 **Consortia**

297 *Genomics England Research Consortium*

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299 Caulfield, M. J., Chan, G. C., Fowler, T., Giess A., Hamblin, A., Henderson, S., Hubbard, T. J.  
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301 Leigh, S. E. A., Leong, I. U. S., Lopez, F. J., Maleady-Crowe, F., Moutsianas, L., Mueller, M.,  
302 Murugaesu, N., Need, A. C., O'Donovan P., Odhams, C. A., Patch, C., Perez-Gil, D., Pereira,  
303 M.B., Pullinger, J., Rahim, T., Rendon, A., Rogers, T., Savage, K., Sawant, K., Scott, R. H.,  
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331

### 332 **Declaration of interests**

333 The Department of Molecular and Human Genetics at Baylor College of Medicine receives  
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335 employee of GeneDx, Inc.

336

### 337 **Web resources**

338 Genome Aggregation Database (gnomAD), [gnomad.broadinstitute.org](http://gnomad.broadinstitute.org)

339 International Mouse Phenotyping Consortium, [www.mousephenotype.org](http://www.mousephenotype.org)

340 Online Mendelian Inheritance in Man, [omim.org](http://omim.org)

341 Protein Databank (PDB), [www.rcsb.org](http://www.rcsb.org)

342

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.

348

349

350



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499 **Figure legends**

500 **Figure 1. Familial screening and clinical characterization of individuals with bi-allelic**  
 501 ***IPO8* variants.** A) Pedigrees of the families with their respective pathogenic variants. Squares  
 502 represent males while circles represent females, filled symbols denote affected individuals, a  
 503 double line connecting spouses symbolizes consanguinity and a - or + sign denotes presence or  
 504 absence of the respective *IPO8* variant. Variants are annotated against NM\_006390.3. B)  
 505 Clinical phenotyping. Proband 1-II:3 showing prominent forehead, hypertelorism, mild ptosis  
 506 left eye, retrognathia, pectus excavatum, umbilical hernia, joint hypermobility with thumb  
 507 abduction and camptodactyly of the second toe. CT angiography of proband 2-II:1  
 508 demonstrating dilatation of the common carotid arteries along with marked tortuosity of the  
 509 common carotid and internal carotid artery, mild tortuosity of the vertebral arteries, enlargement  
 510 of the anterior and middle cerebral arteries bilaterally. Proband 3-II:3 presenting with frontal  
 511 bossing with bitemporal flattening, retrognathia, downturned corners of the mouth and flat feet.  
 512 Proband 5-II:2 showing prominent forehead, significant hypertelorism with flat nasal bridge,  
 513 mild ptosis of left eye and retrognathia. Proband 6-II:1 demonstrating dolichocephaly,  
 514 retrognathia, malar flattening, downslanting palpebral fissures and hypertelorism. MRA  
 515 revealing tortuous intracranial and extracranial arterial vessels, most prominently involving the  
 516 superior cervical internal carotid arteries with dilation of the left internal carotid artery at the  
 517 carotid bifurcation. CT-scan (pre-surgical) showing os odontoideum with cervical spinal canal  
 518 stenosis (arrows).

519 **Figure 2. Progressive TAA development in *Ipo8*<sup>-/-</sup> mice.** A) Log of weight-corrected aortic  
 520 root diameters in male and female mice combined (N=17/group). B) Log of weight-corrected  
 521 ascending aortic diameters in male and female mice combined (N=17/group). C) Log of weight-  
 522 corrected ascending aortic diameters in male mice only (10 *Ipo8*<sup>-/-</sup> vs 9 WT). The error bars

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

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

534 **Figure 4. Elastic fiber deterioration and nuclear pSmad2 accumulation in the ascending**  
535 **aorta of *Ipo8<sup>-/-</sup>* mice.** A) Histological and immunohistochemistry images demonstrating  
536 marked elastin disorganization and fragmentation as well as prominent nuclear pSmad2  
537 accumulation in *Ipo8<sup>-/-</sup>* mice. Scale bar = 50 $\mu$ m. B) Elastic fiber integrity scores and nuclear  
538 pSmad2 grades of the ascending aorta of all ages combined (12- (3 *Ipo8<sup>-/-</sup>* vs 3 WT), 24- (3  
539 *Ipo8<sup>-/-</sup>* vs 3 WT) and 52-weeks (3 *Ipo8<sup>-/-</sup>* vs 2 WT)). Elastin grades can range from 1 to 4, with  
540 grade 1 sections presenting with continuous and well-organized elastic bundles and grade 4  
541 sections displaying vastly disorganized fibers, marked fiber fragmentation and a thickened  
542 aortic wall. For pSmad2, grade 1, 2, 3 and 4 denote sections in which respectively <25%, 25-  
543 50%, 50-75% and 75-100% of nuclei stained positive. Averaged age-combined scores of  
544 blinded observations of three independent researchers are shown. The error bars depict the  
545 SEM. P-values were calculated using two-way ANOVA statistics (\* $p < 0.05$ , \*\*\* $p < 0.001$ ).  
546 WT: wild type

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

552

553 **Table legends**

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

556 ND: not done, ?: unknown, L: left, R: right, +: present, -: absent, Z: z-score (calculated  
 557 according to *Lopez et al.*)<sup>46</sup>, P: percentile, Com: common, int: internal, ASD: atrial septal defect,  
 558 VSD: ventricular septal defect, PDA: patent ductus arteriosus, homz: homozygous, yrs: years,  
 559 mo: months, bilat: bilateral, umb: umbilical, membr: membranous, OFC: occipitofrontal  
 560 circumference. § Proband 6:II-1 also has a chromosomal duplication (1.779 Mb gain of  
 561 19q13.41) and learning disability is also present in proband's mother and maternal half-brother.

562

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

565 -: absent, +: occasional, ++: common, +++: typical clinical feature, MFS: Marfan syndrome,  
 566 LDS: Loeys-Dietz syndrome, SGS, Shprintzen-Goldberg syndrome, TAA: thoracic aortic  
 567 aneurysm; AD: autosomal dominant, AR: autosomal recessive, BAV: bicuspid aortic valve,  
 568 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**

	<b>Family 1 1-II:3</b>	<b>Family 2 2-II:1</b>	<b>Family 3 3-II:3</b>	<b>Family 4 4-II:4</b>	<b>Family 4 4-II:3</b>	<b>Family 5 5-II:2</b>	<b>Family 6 6-II:1</b>
Variant c. annotation	c.1420C>T homz	c.770_777delTATGGTGG; c.1000dupG	c.1428+5G>A; c.2597_2601delTTTTTC	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
<b>Growth</b>	(7yrs 11mo)	(8 yrs)	(7yrs 4mo)	(6yrs)	(9 yrs)	(3yrs9m)	(19 years)
Height	124 cm (P10-25)	127 cm (P25)	118.7 cm (P10-25)	121 cm (P75)	126 cm (P10-25)	92 cm (P3)	175 cm (P25-50)
Weight	21 kg (P3-5)	19.9 kg (P1)	22 kg (P25-50)	18.3 kg (P25-50)	17.6 kg (P0.3)	11 kg (P0.5)	63 kg (P25)
OFC	55 cm (P97)		53.5 cm (P50-75)			47 cm (P10)	
<b>Facial features</b>							
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	+	+	+	+	+	-	+
<b>Skeletal findings</b>							
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
<b>Neurological findings</b>							
Hypotonia	+	+	+	+	+	+	+
Developmental delay	+ (mild)	+	+ (motor)	+ (motor)	+ (motor)	+ (motor)	+
Intellectual disability	-	-	-	-	-	mild	+ <sup>§</sup>
<b>Cardiovascular findings</b>	(10yrs 8mo)	(8yrs)	(7yrs 5mo)	(1yr 8mo)	(9 yrs)	(42 months)	(19 years)
ASD	+	+	+	-	-	+	+ (aneurysmal)
VSD	-	-	+ (membr & muscular)	+ (membraneous)	+	+ (membraneous)	-

PDA	-	+	+	+	-	-	-
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		+					+
<b>Other findings</b>							
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**

	<b>MFS</b>	<b>LDS</b>	<b>SGS</b>	<b>IPO8</b>
Gene	<i>FBN1</i>	<i>TGFBR1/2</i> <i>SMAD2/3</i> <i>TGFB2/3</i>	<i>SKI</i>	<i>IPO8</i>
Inheritance	AD	AD	AD – <i>de novo</i>	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	-	-	++	-

573