1 Y.B.A. Wan^{1,17}, M.A. Simpson^{2,17}, J.A. Aragon-Martin¹, D.P.S. Osborn³, E.
2 Regalado⁴, D.C. Guo⁴, C. Boileau^{5,6,7,8}, G. Jondeau^{5,6,7,9}, L. Benarroch⁵, Y.
3 Isekame¹, J. Bharj³, J. Sneddon¹⁰, E. Fisher¹¹, J. Dean¹², M.T. Tome
4 Esteban^{1,13}, A. Sagar¹⁴, D. Milewicz⁴, M. Jahangiri^{13,15}, E. R. Behr^{1,13}, A.
5 Smith^{16,18} & A. H. Child^{1,13,18}.

6
7 1) Molecular and Clinical Sciences Research Institute, St. George's,
8 University of London, United Kingdom;
9 2) Division of Genetics and Molecular Medicine, Kings College London,
10 United Kingdom;
11 3) Cell Biology and Genetics Research Centre, St. George's, University of
12 London, United Kingdom;
13 4) Department of Internal Medicine, University of Texas, USA;
14 5) Laboratory of Vascular Translational Science, INSERM, Paris, France;
15 6) Centre de référence pour le syndrome de Marfan et apparentés, APHP
16 hopital Bichat, Paris, France;
17 7) Université Paris Diderot, Paris France;
18 8) Department de Genetique, APHP, Hopital Bichat Paris, France;
19 9) Service de Cardiologie, APHP, Hopital Bichat, Paris, France;
20 10) East Surrey Hospital, Redhill, United Kingdom;
21 11) The Park Surgery, Albion Way, Horsham, United Kingdom;
22 12) Clinical Genetics, NHS Grampian, Forester hill, Aberdeen, United
23 Kingdom;
24 13) Cardiology Academic Group, Cardiology Department, St George's
25 Hospital, United Kingdom;
26 14) Clinical Genetics Unit, St George's, University of London, United
27 Kingdom;
28 15) Department of Cardiothoracic Surgery, St. George's Healthcare NHS
29 Trust, London, United Kingdom;
30 16) Academic Department of Vascular Surgery, Cardiovascular Division, BHF
31 Centre of Research Excellence, Kings College London, United Kingdom;
32 17) These authors contributed equally to this work.
33 18) These authors contributed equally to this work. Correspondence should
34 be addressed to A Child (achild@sgul.ac.uk).
35

36 37 38 **A mutation in the *LMOD1* actin-binding domain** 39 **segregating with disease in a large British family with** 40 **thoracic aortic aneurysms and dissections.**

41 42 **Abstract**

43 We describe a mutation in *LMOD1*, which predisposes individuals to thoracic
44 aortic aneurysms and dissections in a large multi-generation British family.
45 Exome variant profiles for the proband and two distantly related affected

46 relatives were generated and a rare protein-altering, heterozygous variant
 47 was identified, present in all the exome-sequenced affected individuals. The
 48 allele c.1784T>C, p.(V595A) in *LMOD1* is located in a known actin-binding
 49 WH2 domain and is carried by all living affected individuals in the family.
 50 *LMOD1* was further assessed in a consecutive series of 98 UK TAAD patients
 51 and one further mutation was found, yielding an incidence of ~2% in our study
 52 group. Assessment of *LMOD1* in international TAAD cohorts discovered nine
 53 other missense variants of which three were classed as likely pathogenic.

54 Validation of *LMOD1* was undertaken using a zebrafish animal model. Knock-
 55 down of both *lmod1a* and *lmod1b* paralogs using morpholino oligonucleotides
 56 showed a reproducible abnormal phenotype involving the aortic arches under
 57 off-target controls. Injection of the human *LMOD1* c.1784T>C, p.(V595A)
 58 mutation demonstrated a likely dominant negative effect and illustrated a loss
 59 of function cause.

60 Mutations found in the WH2 actin-binding domain of *LMOD1* may delay actin
 61 polymerization and therefore compromise actin length, dynamics and
 62 interaction with myosin in the smooth muscle contraction pathway.

63

64

65 **Introduction**

66

67 TAAD (thoracic aortic aneurysm and dissection) is a severe disease
 68 accounting for approximately 15,000 deaths per year in the USA¹. TAAD can
 69 be present in genetic syndromes such as Marfan and Loeys-Dietz syndromes
 70 and the vascular form of Ehlers Danlos syndrome. However, most TAAD

71 patients do not satisfy the characteristic syndromes². Approximately a quarter
 72 of non-syndromic forms of TAAD are familial³. Within affected kindred the
 73 disorder shows variability in penetrance and severity, notably between males
 74 and females³⁻⁵. The underlying molecular genetics of this disorder is
 75 heterogeneous, with 36 genes reported to date as sites of pathogenic
 76 mutation in syndromic and non-syndromic TAAD (OMIM). Mutations in
 77 reported genes contribute to the genetic aetiology of approximately 20-30% of
 78 TAAD families⁶, with the most mutations, in approximately 14% of families,
 79 found in *ACTA2*. Investigations into the biological roles of this group of 36
 80 genes⁷⁻³⁷ have revealed some common pathways, including those relating to
 81 TGF- β signaling³⁸ as well as smooth muscle contraction⁷.

82

83 To extend the understanding of the genetic basis of TAAD, a whole exome
 84 sequencing approach was used in a large multigenerational family of British
 85 ancestry (M441) with autosomal dominant inheritance of TAAD. The proband
 86 initially consulted a cardiologist as he had lost both his father and half-brother
 87 to type A dissection involving the ascending aorta. Doppler transthoracic
 88 echocardiography (TTE) measurements revealed he had a mildly dilated
 89 aortic root (4.1cm). Clinical investigation of other family members revealed
 90 five living relatives who had previously undergone surgery for TAAD or who
 91 had a dilated aortic root (figure 1). Physical examination of these subjects
 92 revealed that they shared some phenotypic features. Family history revealed
 93 aortic dissection as the cause of death for four males (mean age of death
 94 52.25 years, range 39-62 years).

95

96

97 **Subjects and Methods**

98

99 Subjects

100 All individuals in this study were ascertained through the Aortopathy clinic at
101 St George's University Hospitals Foundation NHS Trust.

102 Ethical consent has been obtained from the National Research Ethics Service
103 Committee London- Bloomsbury (reference; 14/LO/1770, project ID; 99752).

104 Letter of invitation to participants, participant information sheets, DNA analysis
105 consent forms and skin biopsy consent forms have all been approved by the
106 ethical committee. License has been granted for the storage of samples under
107 section 16 (2) (e) (ii) of the Human Tissue Act 2004 (No. 12335). Patient and
108 family participants have read and signed the relevant consent forms.

109

110 Sequencing

111 We undertook a whole exome sequencing (WES) approach to identify the
112 causative mutation in this family, generating exome variant profiles for the
113 proband and two distantly affected relatives (figure 1). It was performed at the
114 KCL-GSTT BRC Genomics facility at Guys Hospital, London as a
115 collaborative research service. Exome libraries were generated using Agilent
116 SureSelect kits on an Agilent Bravo Liquid handling robot and the exome
117 enriched libraries were sequenced by paired-end reads on an Illumina
118 HiSeq2000 platform and interpreted using their own in-house pipeline.

119

120

121 Variant Interpretation

122 In-silico prediction tools Polyphen-2³⁹, GVG⁴⁰, UMD Predictor⁴¹, SIFT⁴², and
 123 PROVEAN⁴³ were used along with the genomic database The Genome
 124 Aggregation Database (gnomAD)⁴⁴ to determine pathogenicity of alleles found
 125 upon exome analysis. Functional annotation tools Combined Annotation-
 126 Dependent Depletion (CADD)⁴⁵ and PhastCons 100 way⁴⁶ were used to
 127 predict if variants were amongst the top 1% of deleterious variants and
 128 estimate the probability that the nucleotide belongs to a conserved element
 129 based on multiple alignment. The Universal Protein Resource (UniProt)⁴⁷ and
 130 The Genotype Tissue Expression (GTEx)⁴⁸ were used to assess domain
 131 architecture and tissue specific gene expression. Sanger sequencing was
 132 performed to determine cosegregation of candidate genes in the study family
 133 as well as detect further mutations in our consecutive 98 TAAD cohort.

134

135 TAAD gene analysis

136 Exome profiles were screened for disease-causing mutations in all 36 TAAD
 137 associated genes. These genes included *ACTA2* (OMIM: *102620), *BGN*
 138 (OMIM: *301870), *COL1A1* (OMIM: *120150), *COL1A2* (OMIM: *120160),
 139 *COL3A1* (OMIM: *120180), *COL4A5* (OMIM: *303630), *COL5A1* (OMIM:
 140 *120215), *COL5A2* (OMIM: *120190), *EFEMP2* (OMIM: *604633), *ELN*
 141 (OMIM: *130160), *FBN1* (OMIM: *134797), *FBN2* (OMIM: *612570), *FLNA*
 142 (OMIM: *300017), *FOXE3* (OMIM: *601094), *SLC2A10* (OMIM: *606145),
 143 *JAG1* (OMIM: *601920), *LRP1* (OMIM: *107770), *MAT2A* (OMIM: *601468),
 144 *MFAP5* (OMIM: *601103), *MYH11* (OMIM: *160745), *MYLK* (OMIM:
 145 *600922), *NOTCH1* (OMIM: *190198), *PKD1* (OMIM: *601313), *PKD2* (OMIM:

146 *173910), *PLOD1* (OMIM: *153454), *PRKG1* (OMIM: *176894), *SKI* (OMIM:
147 *164780), *SMAD2* (OMIM: *601366), *SMAD3* (OMIM: *603109), *SMAD4*
148 (OMIM: *600993), *TGFB2* (OMIM: *190220), *TGFB3* (OMIM: *190230),
149 *TGFBR1* (OMIM: *190181), *TGFBR2* (OMIM: *190182), *TGFBR3* (OMIM:
150 *600742) and *ULK4* (OMIM: *617010).

151

152

153 Animal studies

154 Ethics Statement Animal maintenance, husbandry, and procedures are defined
155 and controlled by the Animals (Scientific Procedures) Act 1986. All animal
156 experimentation has been carried out under licenses granted by the Home
157 Secretary (PIL No. 70/10999) in compliance with Biological Services
158 Management Group and the Biological Services Ethical Committee, UCL, London,
159 UK.

160 Wild type (AB x Tup LF) and Tg(kdrl:GFP) zebrafish were maintained and staged
161 as previously described by Westerfield et al⁴⁹. Tg(kdrl:GFP) fish were used to
162 study the development of the aortic arches⁵⁰.

163 Zebrafish (*Danio rerio*) were used to assess the involvement of *LMOD1* as a
164 potential TAAD-causing gene as this species have proven to be a versatile
165 vertebrate model to study human genetic disease and have already been
166 used to demonstrate pathogenicity of human TAAD genes^{20; 34}. *LMOD1* in
167 zebrafish identifies two paralogs, *lmod1a* and *lmod1b*. Gene duplication is a
168 common feature in zebrafish and these paralogs are the result of a genome
169 duplication that occurred in teleost fish some 300 million years ago⁵¹. Whole
170 mount in-situ hybridization was performed to determine expression of

171 candidate genes. Morpholino oligonucleotides (MO) for splice blocking
172 *lmod1a* exon1/intron1 (5'-TATCATCATCATCACCTCTGCGCTC-3') and
173 intron1/exon2 (5'-CCTCCCTACAAACAGACACATTATT-3') and splice
174 blocking *lmod1b* (5'- ATTTCCATTTCCTACCTTTTTGAGC-3') were injected
175 into 1-2 cell embryos to knock down candidate genes and evaluate the
176 phenotype of morphants compared to un-injected wild-type control fish using
177 confocal microscopy at 3-4 days post fertilization (dpf). Fluorescent
178 microscopy was employed to investigate MO injected fish processed with an
179 actin-filament phalloidin stain and Dextran dyes, highlighting the vasculature.
180 To obtain an enhanced resolution at confocal microscopy, *Tg(kdrl:GFP)*⁵⁰ fish
181 were used to highlight the vasculature, in particular the aortic arches of MO
182 injected fish. A standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-
183 3') targeting a splice site of human beta-globin⁵² was used to identify toxicity
184 concentrations. A zebrafish control MO targeting *p53* (5'-
185 GCGCCATTGCTTTGCAAGAATTG-3') was injected singularly (6ng/ul) as
186 well as co-injected with *lmod1a/lmod1b* MOs (6ng/ul total, 2ng/ul + 2ng/ul
187 +2ng/ul) as MOs have been reported to cause off-target activation of p53 and
188 therefore causing non-specific phenotype in injected fish⁵³.
189 Injection of full length human *LMOD1* mRNA and zebrafish *lmod1a* and
190 *lmod1b* at 1-cell stage were performed to rescue the phenotype caused by
191 MO knockdown. Injection of the c.1784T>C mRNA from the study family
192 mutation at 1-cell stage was performed to assess the phenotype of the
193 embryos.
194
195

196 Results

197 Initial analysis of exome variant call format (VCF) files for all 36 reported
 198 TAAD genes showed no pathogenic mutation present in all three affected
 199 individuals. Further analysis revealed three rare ($MAF < 0.0001$), protein-
 200 altering, heterozygous variants present in the profiles of each of the
 201 sequenced individuals. The three candidate variants located in *LMOD1*,
 202 *SPHKAP* and *ZNF483* were each evaluated for cosegregation with the trait in
 203 the pedigree by Sanger sequencing. Neither the variant in *SPHKAP* nor the
 204 variant in *ZNF483* cosegregated with the disease. However, the c.1784T>C,
 205 p.(V595A) (NM_012134) allele in *LMOD1* was carried by each of the five
 206 affected living individuals (figure 1) and was also present in the DNA sample
 207 obtained from a deceased affected half-brother of the proband. A further 10
 208 heterozygous individuals were identified in the pedigree who had an
 209 ambiguous phenotype, meaning that none of them had detectable aortic
 210 dilation by transthoracic echo but they presented with two or more minor
 211 features associated with a marfanoid phenotype⁵⁴. Seven of these 10
 212 individuals shared phenotypic features found in the family members who had
 213 previously undergone surgery for TAAD or who had a dilated aortic root. One
 214 heterozygous clinically unaffected individual was found who did not present
 215 with a detectable aneurysm, but this individual, IV:18, aged 28 at the time of
 216 examination, was documented as having only a high arched palate consistent
 217 with the phenotypic presentation in the proband and 10 other heterozygous
 218 individuals. A parametric linkage analysis of the observed segregation of the
 219 *LMOD1* c.1784T>C allele and the affection status, generated a LOD score in
 220 excess of 2.3, $p=0.0005$. (LOD score 6.7 from an affected-only analysis,

221 dominant model, disease frequency 0.0001, penetrance 0.9). These data
222 suggested that the allele located in *LMOD1* was likely to be disease-causing
223 in this family and should therefore be investigated further.

224 The c.1784T>C *LMOD1* variant is located in the third and terminal exon of the
225 encoded 1839bp transcript (figure 2). The predicted p.(V595A) substitution in
226 the mature protein product replaces a valine residue that has been highly
227 conserved throughout evolution. This residue is located in the C-terminal WH2
228 domain known to be an actin-binding motif. The c.1784T>C allele was not
229 observed gnomAD and was not seen in ~5,500 exomes sequenced and
230 processed using the same laboratory protocols and data processing pipeline
231 as used in the current study. The rarity of this allele suggests that it must have
232 a role in the pathogenesis of aortic aneurysm in a population of TAAD
233 patients.

234

235 The possibility that further rare *LMOD1* alleles may explain additional cases of
236 TAAD was explored using Sanger sequencing of the three *LMOD1* exons and
237 their associated splice sites in a cohort of 98 (36/98 familial) unrelated British
238 individuals with TAAD. Sanger sequencing of the three amplicons identified
239 three of the 98 individuals carrying heterozygous missense alleles in *LMOD1*
240 and one heterozygous silent allele (table 1 showing only rare missense alleles
241 and figure 2 showing all variants found). The observation of three missense
242 alleles in this gene in a total of 196 alleles does not deviate from the expected
243 rate based on allele counts in the ExAC (Chi Square $p = 0.49$). However, it is
244 noteworthy that one proband, individual M508.1, carries a heterozygous
245 c.1631C>A, p.(P544H) (MAF = 0.0004342, rs202184893) allele, which was

246 also predicted to be damaging by all *in-silico* prediction tools used (table 1).
 247 This residue substitution is highly conserved across species and appears
 248 within the proline-rich region found upstream of the WH2 domain of the
 249 protein. This affected female (M508.1) at age 32 had a type A dissection at 36
 250 weeks of pregnancy necessitating emergency caesarean section and aortic
 251 root and valve replacement. Aortic histology revealed cystic medial necrosis
 252 found typically but not specifically in Marfan syndrome patients. Physical
 253 examination revealed individual M508.1 to have a high palate, overcrowded
 254 teeth, wrinkled forehead, and small joint hypermobility similar to features seen
 255 in the proband and affected members of our initial proband's family.
 256 Individual M508.1, a proband from the consecutive TAAD series reported her
 257 maternal grandmother died at age 40 of ruptured dissecting aortic aneurysm,
 258 verified by the death certificate. DNA was obtained from the proband's mother
 259 and Sanger sequencing confirmed the c.1631C>A allele was present,
 260 although at age 55, after two pregnancies, her echocardiogram was reported
 261 to be within normal range. In this family, the proband's mother appears to be
 262 unaffected by aortic disease at present based on echocardiogram
 263 measurements, however is physically inactive due to an unrelated illness
 264 causing an inflammatory neurological condition. This may be a contributing
 265 factor to the variable expressivity seen in this family. The proband's clinically
 266 unaffected sister, whose cardiac evaluation including echocardiogram is
 267 normal, does not carry the mutation. Clinical evaluation suggests similarity in
 268 phenotypic presentation of affected individuals in both UK families.
 269 The phenotypic presentation seen in affected individuals in both families
 270 M441 and M508 are generally 'soft signs' also seen in normal control

271 populations. Arm span greater than height, high arched palate, blue sclerae,
272 down-slanting palpebral fissures, wrinkled forehead, small joint hypermobility
273 and hyperextensible skin were recorded and compared with the same findings
274 in all our TAAD probands. Only the presence of small joint hypermobility was
275 found to be significantly higher in family M441 and proband M508.1 compared
276 to our TAAD proband cohort ($p=0.0000003$).

277

278 Comparison of our TAAD study family with our cohort of 98 consecutive TAAD
279 probands (table 2), reveals that only blue sclerae and small joint hypermobility
280 appear significantly more often in the M441 family. These clinical features
281 were not assessed in international cohorts as they were not recorded upon
282 physical examination.

283 The incidence of mutations in *LMOD1* was also evaluated in international
284 familial TAAD cohorts; GenTac Research Consortium (800 TAAD families)
285 and Paris Marfan Syndrome Study Group (225 families and 178 isolated
286 cases), which revealed further variants in this gene (Table 1). Nine rare
287 missense variants were detected in 12 familial probands of the GenTac
288 Research Consortium and two rare missense variants were identified in the
289 Paris Marfan Study Group. All heterozygous alleles were found spanning
290 across all three exons of the gene. Variants from all three cohorts were
291 evaluated as likely pathogenic if they were scored in the top 1% of most
292 deleterious substitutions, high conservation and damaging in 2 or more in-
293 silico tools (table 1, highlighted in grey).

294 Further investigations through the use of *in-vivo* models were performed to
295 show that a non-functional *LMOD1* is associated with TAAD in the zebrafish

296 animal model. Conservation of the human LMOD1 (P29536) WH2 domain at
297 residues 574-600 results in 62.5% shared identity to the predicted zebrafish
298 *lmod1a* (F1Q5S5) and 84.6% to zebrafish *lmod1b* (E7F062) and conservation
299 of the proline-rich region at residues 509-544 results in 51.2% and 56.8%
300 shared identity with *lmod1a* and *lmod1b*, respectively.

301 These data suggest that zebrafish *lmod1b* is more closely related to human
302 LMOD1 and thus offers the better gene to study, however both paralogs are
303 likely to be expressed and have overlapping functions, therefore functional
304 redundancy is likely to exist.

305 *In-situ* hybridization revealed strong expression of both *lmod1a* and *lmod1b* in
306 the heart, fin buds and as well as expression from the telencephalon to the
307 tegmentum of the zebrafish. To determine the functional involvement of these
308 genes during development, antisense Morpholino oligonucleotides (MO) were
309 employed that block gene-specific translation or mRNA processing. Gene
310 knockdown was assessed by co-injection of two non-overlapping splice-
311 directed MOs in *lmod1a*, and one splice-directed MO in *lmod1b*. The zebrafish
312 embryos were classified as normal if the phenotype was indistinguishable
313 from wild-type un-injected embryos, moderately affected if they had
314 pericardial oedema and tail curvature, and severely affected if widespread
315 oedema was seen with severe tail curvature (figure 3A). This classification
316 was confirmed independently by two trained observers. Additional phenotypic
317 features observed in the injected morphants included smaller eyes and body
318 size, abnormal distribution of melanocytes compared to wild type un-injected
319 controls (figure 3A).

Initially, injection of one splice-directed *lmod1a* MO at a 4ng concentration generated a 67% combined moderate/severe affected rate and this increased to 93% when only *lmod1b* MO was injected at the same concentration. Two splice-directed MO (*lmod1a* and *lmod1b*) were co-injected at 4ng (2ng *lmod1a* and 2ng *lmod1b*) as a final concentration giving higher percentage of affected embryos (97%), as well as a higher survival rate (figure 4). This may suggest that as both *lmod1a* and *lmod1b* are expressed in zebrafish, when one is knocked-down, the other may have a compensatory effect. Therefore, knocking-down both paralogs more closely resemble the human deficient *LMOD1*. A human beta-globin standard control was used and injected at a 4ng concentration and the resulting fish revealed no increase in abnormal phenotype associated with toxicity compared with un-injected controls. *p53* MO was injected as an apoptosis management control at 6ng concentration and the fish did not present with any abnormal phenotype. A co-injection of *lmod1a/lmod1b/p53* MO at a final concentration of 6ng revealed no reduction in severity of the phenotype observed compared to *lmod1a/lmo1b* only co-injection. Therefore, the data presented suggests that the phenotype observed was unlikely due to off-target effects.

Fifty out of sixty-six *lmod1a/lmod1b* MO injected embryos analysed (76%) demonstrated pooling of blood in the yolk sac by 4 days post fertilization (dpf) and as a consequence developed significant oedema in their yolk sac by 5dpf.

Injection of the full-length human wild-type *LMOD1* mRNA at 60ng/ul into 1-cell embryos did not give an overexpression phenotype and demonstrated a similar effect (7% affected) as in the standard control MO and un-injected

344 control experiments indication no toxic effects at that concentration were
345 causing a phenotype (figure 4).

346 Only the full-length mRNA of the human c.1784T>C mutation (60ng/ul
347 concentration) was injected into the 1-cell embryos, which resulted in
348 embryonic death in 141/174 (81%) of cases, with the remaining 32/33
349 zebrafish demonstrating oedema, tail curvature, abnormal distribution of
350 melanocytes, smaller eyes and body size as seen in the MO injected
351 moderate to severe phenotype.

352 Next a full length wild-type human *LMOD1* mRNA was injected at 1-cell stage
353 followed by *lmod1a/lmod1b* MO (4ng/ul) and the resulting phenotype from MO
354 knock down could not be rescued at 60ng/ul dosage or even with an
355 increased dosage up to 200ng/ul, as most were still affected (figure 4). Wild-
356 type zebrafish *lmod1a* and *lmod1b* were also injected singularly and
357 combined with MO but also did not show significant rescue of the phenotype.
358 This suggested that *LMOD1* mRNA may be required at specific time point
359 which is not possible with zebrafish injections because these are injected at
360 the 1-cell stage.

361 An F-actin (Phalloidin) marker was used to determine if knockdown of
362 *lmod1a/lmod1b* in zebrafish had any effect on the actin filament production in
363 the vasculature as well as the aortic arches at 4dpf (figure 3B). Crossing-over
364 of muscle fibres through adjacent somites was also identified and
365 disturbance/loss of the ventral pharyngeal arches was observed in the
366 morphant fish compared to controls suggesting that *lmod1a* may be required
367 for aortic arch development or F-actin stabilisation.

368 Next a high molecular weight (500kDa) fluorescein-conjugated Dextran dye
 369 was used to highlight the vasculature of the *lmod1a/lmod1b* injected
 370 morphants to assess for perturbations as observed in the study family.
 371 Fluorescent microscopy showed abnormal branching of the aortic arches and
 372 intermittent flow through the vessels and absent flow towards the caudal half
 373 of the trunk, including fin vasculature (figure 3C). This suggested that *lmod1a*
 374 might play a role in vascular development and maintenance.

375 Transgenic fish were used to further describe the abnormalities observed in
 376 the aortic arches by injection of *lmod1a/lmod1b* MO into *Tg(kdrl:GFP)* fish and
 377 found that MO injected fish showed minimal branching of the aortic arches
 378 compared to that of the un-injected control (figure 3D). These arches are
 379 evolutionary important because in fish, they later become the vasculature of
 380 the gills, but in humans they elaborate more extensively to carry blood to the
 381 lungs and form the carotid arteries, subclavian arteries, aorta and other
 382 associated structures in the upper mediastinum and proximal upper limb and
 383 neck. Importantly, the resulting phenotype seen in all affected fish from
 384 *lmod1a* and *lmod1b* MO as well as the mRNA of the human c.1784T>C
 385 mutation injections were seen consistently through repeat injections and
 386 toxicity controls indicate that the phenotype recorded was the result of a non-
 387 functioning protein rather than off-target toxic effects.

388

389 Discussion

390 Leiomodlin-1 (LMOD1) is a 64-kDa actin-binding protein and a homolog of the
 391 only known tropomyosin-coated pointed-end capping tropomodulin protein
 392 (TMOD) family⁵⁵. Vertebrate Lmods consist of 3 isoforms; *LMOD1* (smooth
 393 muscle), *LMOD2* (cardiac muscle) and *LMOD3* (skeletal muscle). LMOD1, is
 394 expressed predominantly in human arteries (tibial, aorta and coronary),
 395 oesophagus, colon, bladder and uterus (GTEx Analysis Release V6 (dbGaP
 396 Accession phs000424.v6.p1)⁵⁶.

397 No mutations in *LMOD2* have been reported yet but the Lmod2 knockout
 398 mouse exhibits shortened cardiac actin filaments and dilated
 399 cardiomyopathy⁵⁷. Mutations in the human *LMOD3* are reported to cause thin
 400 filament disorganization and nemaline myopathy⁵⁸ associated with the rod-like
 401 nemaline bodies reported to cause muscle weakness. The Lmod3-null mouse
 402 showed skeletal muscle weakness associated with nemaline bodies^{59; 60}.

403 An autosomal recessive mutation causing a premature terminating codon in
 404 exon 2 of *LMOD1* was recently described as causing a fatal rare condition,
 405 megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS) in a
 406 newborn child⁶¹. Knock-down of human LMOD1 in intestinal smooth muscle
 407 cells showed decrease in actin filament formation and contractility.

408 Phalloidin staining showed consistently a lower concentration of actin
 409 filaments in the Lmod1-null bladder tissue and electron microscopy revealed a
 410 presence of rod-like structures in the smooth muscle, similarly found in the
 411 Lmod3-null mouse. The authors also reported attenuation in the transitional
 412 epithelium of the bladder and stomach in the Lmod1-null mouse but not in the
 413 intestine and suggested that it may be due to decreased mechanical stress.

414 The data demonstrated that *LMOD1* has significant importance in regulating
 415 smooth muscle cytoskeletal-contraction coupling. In contrast to TMODs,
 416 leiomodins also harbor a C-terminal extension and in all three isoforms
 417 this consists of a proline-rich region followed by a WH2 domain⁶². Recent
 418 studies have shown that constructs of *Lmod1* lacking this C-terminal,
 419 containing proline-rich region and WH2 domain, caused a delay in actin
 420 polymerization and increased dependence of *LMOD1* concentration relative to
 421 the polymerization rate⁶³.

422 Many of the proteins known to regulate actin dynamics contain a WH2
 423 domain, such as the β -thymosin⁶⁴ family of proteins, and WASP proteins bind
 424 the actin nucleating protein complex Arp2/3⁶⁵.

425 These data above suggest that the mutations found in the C-terminal
 426 extension containing the actin-binding domain (WH2) of *LMOD1* may delay
 427 actin polymerization and therefore compromise the length and dynamics of
 428 actin filaments.

429 The non-synonymous *LMOD1* variant [c.1784T>C/p.(V595A)] found in family
 430 M441 changes the amino acid at the WH2 domain at residues 575-600⁶³ of
 431 the protein. The mutant residue is smaller than the wild-type residue, which
 432 may lead to loss of interactions with other molecules⁶⁶. Changes in the
 433 structure of the WH2 domain may be interfering with binding and assembly of
 434 actin filaments and therefore disrupting the actin dynamics and interaction
 435 with myosin in the smooth muscle cell contraction pathway. Delayed actin
 436 polymerization by abnormal *LMOD1* seen in other studies⁶³ may lead to

437 earlier capping of the pointed actin filaments by tropomodulin, resulting in
438 shorter actin filaments.

439 The *LMOD1* c.1631C>A, p.(P544H) allele found in the proline-rich region is
440 predicted to bind profilin, which is thought to be involved in actin dynamics,
441 namely actin polymerization⁶⁷. The mutant residue is larger than the wild-type,
442 which may lead to disturbances in multimeric interactions⁶⁶ and the
443 hydrophobic interactions in the core or surface of the protein may become
444 lost. Protein structure stability prediction tools forecast a decreased stability of
445 the protein structure in both c.1631C>A [p.(P544H)] and c.1784T>C
446 [p.(V595A)] alleles⁶⁸.

447 Therefore as proline-rich regions have been reported to be contributing to
448 actin binding alongside the WH2 domain⁶⁹, mutations in this polyproline
449 domain may account for a TAAD phenotype as seen in the second proband,
450 individual M508.1.

451 The incidence of *LMOD1* disease-related mutations in our UK study is ~2%
452 (2/99) and the phenotypic presentation of this gene may be of late onset, as
453 observed by the mean age of dissection in males (50.4 years, range 39-58
454 years) and variable expression of this dominant gene between carrier males
455 and females in our study family. Of 18 adults over the age of 18 (7M:11F)
456 carrying the c.1784T>C allele, those six with demonstrated aneurysm were
457 predominantly male (5M:1F). Three further variants in our international
458 cohorts (two in USA cohort and one in French cohort) were classed as likely
459 pathogenic and further segregation studies and animal modelling are needed
460 to confirm these.

461 Injection of the human mutant mRNA from the proband of the study family into
462 zebrafish embryos may demonstrate either a dominant negative effect or
463 haploinsufficient state. The human mutation illustrates a 'loss of function' as
464 the resulting phenotype is similar to that observed in *lmod1a/lmod1b* 'knock
465 down' in zebrafish.

466 The likely dominant negative phenotype suggested by the animal studies
467 indicates a loss of function mutation carried by our study family. The mutation
468 is likely to cause a delay in the nucleation of actin monomers to actin
469 filaments because of a non-functioning WH2 domain, resulting in decreased
470 actin filaments⁶¹. Therefore, the crosslinking assembly of actin filaments may
471 be impaired as abnormal distributions and lower concentrations of phalloidin
472 staining have been observed by us and *Halim et al*⁶¹, which may
473 consequently be leading to aortic cystic medial necrosis and permitting
474 dilatation and dissection in the affected individuals of our study family.

475 The abnormal branching of the aortic arches observed in the *Tg(kdrl:GFP)*
476 zebrafish may become an aneurysm specific gene phenotype as it has now
477 been reported in a third TAAD gene following the *MAT2A*²⁰ and *FOXE3*³⁴
478 reports.

479

480 **Conclusion**

481 In summary, we have shown that mutations in the C-terminal end of *LMOD1*
482 may underlie TAAD with an incidence of ~2% in our cohort. Additional
483 mammalian *in-vivo* knock-down studies may help to further confirm a role of
484 *LMOD1* in actin dynamics of the aorta. Histopathology will be required to
485 explore the pathological effects (such as demonstration of cystic medial

486 necrosis) of these mutations on the vascular smooth muscle wall in TAAD. An
 487 associated phenotype may be established, when more families and
 488 individuals with pathogenic alleles have been found. This could include blue
 489 sclerae and small joint hypermobility, features that were seen significantly
 490 more frequently in the c.1784T>C carriers than in the 98 other TAAD
 491 probands.

492 *LMOD1* should be investigated in TAAD exome profiles already available
 493 internationally and it may be useful to add this small three-exon gene to future
 494 TAAD panels.

495 Sequencing of other TAAD cohorts will determine a worldwide incidence. In
 496 addition, screening other genes in this important actin-myosin pathway may
 497 highlight other TAAD candidate genes. This should lead to more specific
 498 collaborative therapeutic trials once enough affected *LMOD1* deficient
 499 patients have been identified internationally.

Figure 1. Family M441 pedigree showing affected individuals in black, ambiguous phenotype in grey and unaffected in white. The proband is indicated with a P with an arrow. Affected individuals, who have undergone exome sequencing, circled in red. Individuals carrying c.1784T>C allele (-/+); reference allele (-/-). ND- No DNA; NE- No echo; --y (e.g. 20y)- age when echo was taken (e.g. 20 years old); d.-- (e.g. d.39) age at death (e.g. death at age 39).

Figure 2. Diagram representing domain architecture of *LMOD1*. TMBS1- Tropomyosin binding site 1 (blue area). ABS1 and ABS2- Actin binding site 1 and 2 (green areas). PR- Proline rich region (yellow area) and WH2- Wiskott-Aldrich syndrome homology region 2 (red area). Adapted from Boczkowska et al, 2015⁶³. Upward lines indicate alleles found in three cohorts (red variants from the UK cohort, black from the USA and green from French cohort). Downward lines indicate variants present on gnomAD, and the length indicates the number of times it has been reported from their database. Downward lines truncated from 20X.

Cohort	Mutation HGVS	Consequence HGVS	CADD score	phastCons 100 score	<i>In-silico</i> predictions	gnomAD allele frequency	Cosegregation tested
UK	c.1631C>A	p.(P544H)	25.8	1	+	0.0003	Yes
UK	c.1784T>C	p.(V595A)	23.6	0.913	+	0	Yes
USA	c.137C>T	p.(P46L)	23.4	1	+	0.0003	N/A
USA	c.350G>A	p.(R117Q)	18.26	0	-	0.00002	N/A
USA	c.404C>G	p.(S135C)	24.4	0.003	+	0.00001	N/A
USA	c.466C>T	p.(R156W)	25.9	0.01	+	0.00005	N/A
USA	c.500T>C	p.(V167A)	11.17	0.996	+	0	N/A
USA	c.607G>C	p.(D203H)	23.6	0.008	+	0.0001	N/A
USA	c.728G>A	p.(G243D)	0.102	0	+	0.0002	N/A
USA	c.757G>A	p.(E253K)	8.519	0	-	0.0001	N/A
USA	c.1723C>T	p.(R575C)	33	0.999	+	0	N/A
FRANCE	c.578G>A	p.(G193E)	0.016	0	+	0.0002	N/A
FRANCE	c.1686G>A	p.(M562I)	23.7	1	+	0.000008	N/A

Table 1. *In-silico* analysis of only the rare missense alleles detected in 98 UK, 800 USA and 403 French TAAO probands. The table includes columns for the cohort, mutation at nucleotide level, consequence on protein level (HGVS- Human Genome Variation Society nomenclature- NM_012134), Combined Annotation-Dependent Depletion (CADD) score, PhastCons score, *In-silico* predictions (Polyphen-2, GVG, UMD-Predictor, SIFT and PROVEAN (+) - Damaging in two or more in-silico tools used (-) - Damaging in one or none of the in-silico tools used.), gnomAD allele frequency, cosegregation tested (N/A- No cosegregation performed). Rows highlighted in grey are predicted to be disease-causing mutations due to high CADD scores (>20), PhastCons 100 score (>0.9) and damaging (+) upon in-silico predictions.

Study group	AS>HT	HP	BS	RE	DSPF	WF	SJH	HS
Group A (Family M441 c.1784T>C carrier >18yrs)	12/18	12/18	6/18	2/18	3/18	6/18	9/18	9/18
Group B (All TAAD probands)	51/98	48/98	3/98	11/98	11/98	18/98	24/98	45/98
Chi Square between group A (c.1784T>C carrier >18years) and group B (all TAAD probands) P=	0.25	0.17	0.00001	0.43	0.51	0.15	0.0000003	0.7

Table 2. Phenotype correlation between group A- c.1784T>C affected status (n=18) with group B- all clinically diagnosed TAAD probands (n=98). AS>HT- Arm span greater than height, HP- High palate, BS- Blue sclera, RE- Redundant eyelids, DSPF- Downward-slanting palpebral fissures, WF- Wrinkled forehead, SJH- Small joint hypermobility and HS- Hyper extensible skin.

Figure 3. Microscopy of control and *lmod1a/lmo1b* MO fish. **A-** Phenotypic representation of un-injected wild-type zebrafish compared to moderately affected and severely affected *lmod1a/lmod1b* co-injected fish. Moderate to severe tail curvature (yellow arrows), pericardial edema (blue arrows), reduced eye size (green arrows) and abnormal distribution of melanocytes (white arrows), abnormal pooling of blood (red arrow) can be seen. **B-** Fluorescent microscopy of phalloidin stained Wild Type un-injected control (WT UIC) and *lmod1a/lmo1b* morpholino (MO) injected fish. Top left- WT UIC showing aortic arches (white arrows). Top right *lmod1a/lmo1b* MO injected fish showing aortic arches (white arrows). Bottom left- WT UIC fish showing normal muscle fibers in the tail. Bottom right- *lmod1a/lmo1b* MO injected fish showing crossing over of muscle fibers. **C-** Fluorescent microscopy of dextran injected WT UIC and *lmod1a/lmo1b* MO injected fish. Top left- WT UIC showing aortic arches (white arrows). Top right *lmod1a/lmo1b* MO injected fish showing aortic arches (white arrows). Bottom left- WT UIC fish showing normal vasculature in the tail. Bottom right- *lmod1a/lmo1b* MO injected fish showing absent flow towards the caudal half of the trunk, including fin vasculature. **D-** Confocal microscopy imaging of un-injected kdrl: GFP zebrafish compared with *lmod1a/lmod1b* MO injected kdrl: GFP fish. Normal branching of the pharyngeal arches can be seen in the un-injected wild-type fish but were disturbed/missing in the *lmod1a/lmod1b* MO injected kdrl: GFP fish (white arrows). Scale bar shown in the bottom right of images (100um length). *- Marks the eye of the zebrafish.

Figure 4. Bar chart showing normal (green), moderate (yellow) and severely affected (red) embryos associated with the following injections; Un-injected control (UIC); Human beta-globulin control MO (4ng/ul); p53 toxicity control MO (6ng/ul); *lmod1a* MO (4ng/ul); *lmod1b* MO at (4ng/ul); *lmod1a/lmod1b* MOs combined (4ng/ul; 2ng/ul + 2ng/ul); *lmod1a/lmod1b/p53* MOs combined (6ng/ul; 2ng/ul + 2ng/ul + 2ng/ul); Full length *LMOD1* Wild-type human mRNA (60ng/ul); Full length *LMOD1* Wild-type human mRNA (60ng/ul) and *lmod1a/lmod1b* (4ng/ul, 2ng/ul + 2ng/ul); Human mutated *LMOD1* mRNA (c.1784T>C), * A significant number of human mRNA c.1784T>C injected fish died by 24hpf compared to UIC.

Acknowledgements

The authors would like to thank the families participating in this research study. This work was supported with grants from the British Heart Foundation to E.R.B, The Rosetrees Trust to Y.B.A.W. and A.C, The Marfan Trust, The Marfan association and The Everett Roseborough Legacy to Y.B.A.W, Peter and Sonia Field Charitable Trust to A.C, St George's Medical School and NHS Hospital Trust to A.C. and Guy's and St Thomas's charity to A.S. The zebrafish study was conducted in collaboration with Zebsolutions.

1. Heron, M., Hoyert, D.L., Murphy, S.L., Xu, J., Kochanek, K.D., and Tejada-Vera, B. (2009). Deaths: final data for 2006. *Natl Vital Stat Rep* 57, 1-134.
2. Milewicz, D.M., Carlson, A.A., and Regalado, E.S. (2010). Genetic testing in aortic aneurysm disease: PRO. *Cardiol Clin* 28, 191-197.
3. Albornoz, G., Coady, M.A., Roberts, M., Davies, R.R., Tranquilli, M., Rizzo, J.A., and Elefteriades, J.A. (2006). Familial thoracic aortic aneurysms and dissections--incidence, modes of inheritance, and phenotypic patterns. *Ann Thorac Surg* 82, 1400-1405.
4. Biddinger, A., Rocklin, M., Coselli, J., and Milewicz, D.M. (1997). Familial thoracic aortic dilatations and dissections: a case control study. *J Vasc Surg* 25, 506-511.
5. Coady, M.A., Davies, R.R., Roberts, M., Goldstein, L.J., Rogalski, M.J., Rizzo, J.A., Hammond, G.L., Kopf, G.S., and Elefteriades, J.A. (1999). Familial patterns of thoracic aortic aneurysms. *Arch Surg* 134, 361-367.
6. Pyeritz, R.E. (2014). Heritable thoracic aortic disorders. *Curr Opin Cardiol* 29, 97-102.
7. Guo, D.C., Pannu, H., Tran-Fadulu, V., Papke, C.L., Yu, R.K., Avidan, N., Bourgeois, S., Estrera, A.L., Safi, H.J., Sparks, E., et al. (2007). Mutations in smooth muscle alpha-actin (ACTA2) lead to thoracic aortic aneurysms and dissections. *Nat Genet* 39, 1488-1493.
8. Deak, S.B., Scholz, P.M., Amenta, P.S., Constantinou, C.D., Levi-Minzi, S.A., Gonzalez-Lavin, L., and Mackenzie, J.W. (1991). The substitution of arginine for glycine 85 of the alpha 1(I) procollagen chain results in mild osteogenesis imperfecta. The mutation provides direct evidence for three discrete domains of cooperative melting of intact type I collagen. *J Biol Chem* 266, 21827-21832.
9. Sasaki, T., Arai, K., Ono, M., Yamaguchi, T., Furuta, S., and Nagai, Y. (1987). Ehlers-Danlos syndrome. A variant characterized by the deficiency of pro alpha 2 chain of type I procollagen. *Arch Dermatol* 123, 76-79.
10. Nicholls, A.C., Valler, D., Wallis, S., and Pope, F.M. (2001). Homozygosity for a splice site mutation of the COL1A2 gene yields a non-functional pro(alpha)2(I) chain and an EDS/OI clinical phenotype. *J Med Genet* 38, 132-136.

11. Kuivaniemi, H., Kontusaari, S., Tromp, G., Zhao, M.J., Sabol, C., and Prockop, D.J. (1990). Identical G+1 to A mutations in three different introns of the type III procollagen gene (COL3A1) produce different patterns of RNA splicing in three variants of Ehlers-Danlos syndrome. IV. An explanation for exon skipping some mutations and not others. *J Biol Chem* 265, 12067-12074.
12. Ziganshin, B.A., Bailey, A.E., Coons, C., Dykas, D., Charilaou, P., Tanriverdi, L.H., Liu, L., Tranquilli, M., Bale, A.E., and Elefteriades, J.A. (2015). Routine Genetic Testing for Thoracic Aortic Aneurysm and Dissection in a Clinical Setting. *Ann Thorac Surg*.
13. Renard, M., Holm, T., Veith, R., Callewaert, B.L., Adès, L.C., Baspinar, O., Pickart, A., Dasouki, M., Hoyer, J., Rauch, A., et al. (2010). Altered TGFbeta signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency. *Eur J Hum Genet* 18, 895-901.
14. Szabo, Z., Crepeau, M.W., Mitchell, A.L., Stephan, M.J., Puntel, R.A., Yin Loke, K., Kirk, R.C., and Urban, Z. (2006). Aortic aneurysmal disease and cutis laxa caused by defects in the elastin gene. *J Med Genet* 43, 255-258.
15. Dietz, H.C., Pyeritz, R.E., Hall, B.D., Cadle, R.G., Hamosh, A., Schwartz, J., Meyers, D.A., and Francomano, C.A. (1991). The Marfan syndrome locus: confirmation of assignment to chromosome 15 and identification of tightly linked markers at 15q15-q21.3. *Genomics* 9, 355-361.
16. Dietz, H.C., Cutting, G.R., Pyeritz, R.E., Maslen, C.L., Sakai, L.Y., Corson, G.M., Puffenberger, E.G., Hamosh, A., Nanthakumar, E.J., and Curristin, S.M. (1991). Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature* 352, 337-339.
17. Wang, M., Clericuzio, C.L., and Godfrey, M. (1996). Familial occurrence of typical and severe lethal congenital contractural arachnodactyly caused by missplicing of exon 34 of fibrillin-2. *Am J Hum Genet* 59, 1027-1034.
18. Sheen, V.L., Jansen, A., Chen, M.H., Parrini, E., Morgan, T., Ravenscroft, R., Ganesh, V., Underwood, T., Wiley, J., Leventer, R., et al. (2005). Filamin A mutations cause periventricular heterotopia with Ehlers-Danlos syndrome. *Neurology* 64, 254-262.
19. McElhinney, D.B., Krantz, I.D., Bason, L., Piccoli, D.A., Emerick, K.M., Spinner, N.B., and Goldmuntz, E. (2002). Analysis of cardiovascular phenotype and genotype-phenotype correlation in individuals with a JAG1 mutation and/or Alagille syndrome. *Circulation* 106, 2567-2574.
20. Guo, D.C., Gong, L., Regalado, E.S., Santos-Cortez, R.L., Zhao, R., Cai, B., Veeraraghavan, S., Prakash, S.K., Johnson, R.J., Muilenburg, A., et al. (2015). MAT2A Mutations Predispose Individuals to Thoracic Aortic Aneurysms. *Am J Hum Genet* 96, 170-177.
21. Pannu, H., Tran-Fadulu, V., Papke, C.L., Scherer, S., Liu, Y., Presley, C., Guo, D., Estrera, A.L., Safi, H.J., Brasier, A.R., et al. (2007). MYH11 mutations result in a distinct vascular pathology driven by insulin-like growth factor 1 and angiotensin II. *Hum Mol Genet* 16, 2453-2462.

22. Wang, L., Guo, D.C., Cao, J., Gong, L., Kamm, K.E., Regalado, E., Li, L., Shete, S., He, W.Q., Zhu, M.S., et al. (2010). Mutations in myosin light chain kinase cause familial aortic dissections. *Am J Hum Genet* 87, 701-707.
23. Stittrich, A.B., Lehman, A., Bodian, D.L., Ashworth, J., Zong, Z., Li, H., Lam, P., Khromykh, A., Iyer, R.K., Vockley, J.G., et al. (2014). Mutations in NOTCH1 cause Adams-Oliver syndrome. *Am J Hum Genet* 95, 275-284.
24. Guo, D.C., Regalado, E., Casteel, D.E., Santos-Cortez, R.L., Gong, L., Kim, J.J., Dyack, S., Horne, S.G., Chang, G., Jondeau, G., et al. (2013). Recurrent gain-of-function mutation in PRKG1 causes thoracic aortic aneurysms and acute aortic dissections. *Am J Hum Genet* 93, 398-404.
25. Doyle, A.J., Doyle, J.J., Bessling, S.L., Maragh, S., Lindsay, M.E., Schepers, D., Gillis, E., Mortier, G., Homfray, T., Sauls, K., et al. (2012). Mutations in the TGF- β repressor SKI cause Shprintzen-Goldberg syndrome with aortic aneurysm. *Nat Genet* 44, 1249-1254.
26. Coucke, P.J., Willaert, A., Wessels, M.W., Callewaert, B., Zoppi, N., De Backer, J., Fox, J.E., Mancini, G.M., Kambouris, M., Gardella, R., et al. (2006). Mutations in the facilitative glucose transporter GLUT10 alter angiogenesis and cause arterial tortuosity syndrome. *Nat Genet* 38, 452-457.
27. Micha, D., Guo, D.C., Hilhorst-Hofstee, Y., van Kooten, F., Atmaja, D., Overwater, E., Cayami, F.K., Regalado, E.S., van Uffelen, R., Venselaar, H., et al. (2015). SMAD2 Mutations Are Associated with Arterial Aneurysms and Dissections. *Hum Mutat*.
28. van de Laar, I.M., Oldenburg, R.A., Pals, G., Roos-Hesselink, J.W., de Graaf, B.M., Verhagen, J.M., Hoedemaekers, Y.M., Willemsen, R., Severijnen, L.A., Venselaar, H., et al. (2011). Mutations in SMAD3 cause a syndromic form of aortic aneurysms and dissections with early-onset osteoarthritis. *Nat Genet* 43, 121-126.
29. Teekakirikul, P., Milewicz, D.M., Miller, D.T., Lacro, R.V., Regalado, E.S., Rosales, A.M., Ryan, D.P., Toler, T.L., and Lin, A.E. (2013). Thoracic aortic disease in two patients with juvenile polyposis syndrome and SMAD4 mutations. *Am J Med Genet A* 161A, 185-191.
30. Boileau, C., Guo, D.C., Hanna, N., Regalado, E.S., Daint, D., Gong, L., Varret, M., Prakash, S.K., Li, A.H., d'Indy, H., et al. (2012). TGFB2 mutations cause familial thoracic aortic aneurysms and dissections associated with mild systemic features of Marfan syndrome. *Nat Genet* 44, 916-921.
31. Bertoli-Avella, A.M., Gillis, E., Morisaki, H., Verhagen, J.M., de Graaf, B.M., van de Beek, G., Gallo, E., Kruithof, B.P., Venselaar, H., Myers, L.A., et al. (2015). Mutations in a TGF- β ligand, TGFB3, cause syndromic aortic aneurysms and dissections. *J Am Coll Cardiol* 65, 1324-1336.
32. Loeys, B.L., Schwarze, U., Holm, T., Callewaert, B.L., Thomas, G.H., Pannu, H., De Backer, J.F., Oswald, G.L., Symoens, S., Manouvrier, S., et al. (2006). Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med* 355, 788-798.

33. Meester, J.A., Vandeweyer, G., Pintelon, I., Lammens, M., Van Hoorick, L., De Belder, S., Waitzman, K., Young, L., Markham, L.W., Vogt, J., et al. (2016). Loss-of-function mutations in the X-linked biglycan gene cause a severe syndromic form of thoracic aortic aneurysms and dissections. *Genet Med*.
34. Kuang, S.Q., Medina-Martinez, O., Guo, D.C., Gong, L., Regalado, E.S., Reynolds, C.L., Boileau, C., Jondeau, G., Prakash, S.K., Kwartler, C.S., et al. (2016). FOXE3 mutations predispose to thoracic aortic aneurysms and dissections. *J Clin Invest* 126, 948-961.
35. Liu, D., Wang, C.J., Judge, D.P., Halushka, M.K., Ni, J., Habashi, J.P., Moslehi, J., Bedja, D., Gabrielson, K.L., Xu, H., et al. (2014). A Pkd1-Fbn1 genetic interaction implicates TGF- β signaling in the pathogenesis of vascular complications in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 25, 81-91.
36. Wågsäter, D., Paloschi, V., Hanemaaijer, R., Hultenby, K., Bank, R.A., Franco-Cereceda, A., Lindeman, J.H., and Eriksson, P. (2013). Impaired collagen biosynthesis and cross-linking in aorta of patients with bicuspid aortic valve. *J Am Heart Assoc* 2, e000034.
37. Guo, D.C., Grove, M.L., Prakash, S.K., Eriksson, P., Hostetler, E.M., LeMaire, S.A., Body, S.C., Shalhub, S., Estrera, A.L., Safi, H.J., et al. (2016). Genetic Variants in LRP1 and ULK4 Are Associated with Acute Aortic Dissections. *Am J Hum Genet* 99, 762-769.
38. Dietz, H.C. (2010). TGF-beta in the pathogenesis and prevention of disease: a matter of aneurysmic proportions. *J Clin Invest* 120, 403-407.
39. Adzhubei, I., Jordan, D.M., and Sunyaev, S.R. (2013). Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* Chapter 7, Unit7.20.
40. Tavtigian, S.V., Deffenbaugh, A.M., Yin, L., Judkins, T., Scholl, T., Samollow, P.B., de Silva, D., Zharkikh, A., and Thomas, A. (2006). Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 43, 295-305.
41. Salgado, D., Desvignes, J.P., Rai, G., Blanchard, A., Miltgen, M., Pinard, A., Lévy, N., Collod-Bérout, G., and Bérout, C. (2016). UMD-Predictor: A High-Throughput Sequencing Compliant System for Pathogenicity Prediction of any Human cDNA Substitution. *Hum Mutat* 37, 439-446.
42. Ng, P.C., and Henikoff, S. (2001). Predicting deleterious amino acid substitutions. *Genome Res* 11, 863-874.
43. Choi, Y., Sims, G.E., Murphy, S., Miller, J.R., and Chan, A.P. (2012). Predicting the functional effect of amino acid substitutions and indels. *PLoS One* 7, e46688.
44. Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., O'Donnell-Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B., et al. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 536, 285-291.
45. Kircher, M., Witten, D.M., Jain, P., O'Roak, B.J., Cooper, G.M., and Shendure, J. (2014). A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 46, 310-315.

46. Pollard, K.S., Hubisz, M.J., Rosenbloom, K.R., and Siepel, A. (2010). Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res* 20, 110-121.
47. Pundir, S., Martin, M.J., and O'Donovan, C. (2017). UniProt Protein Knowledgebase. *Methods Mol Biol* 1558, 41-55.
48. Consortium, G. (2015). Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science* 348, 648-660.
49. Westerfield, M. (2007). *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th Edition. (University of Oregon Press, Eugene).
50. Covassin, L.D., Siekmann, A.F., Kacergis, M.C., Laver, E., Moore, J.C., Villefranc, J.A., Weinstein, B.M., and Lawson, N.D. (2009). A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signaling during artery development. *Dev Biol* 329, 212-226.
51. Wittbrodt, J., and Meyer, A., M. (1998). **More genes in fish?** More genes in fish? In. (BioEssays.
52. Eisen, J.S., and Smith, J.C. (2008). Controlling morpholino experiments: don't stop making antisense. *Development* 135, 1735-1743.
53. Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., and Ekker, S.C. (2007). p53 activation by knockdown technologies. *PLoS Genet* 3, e78.
54. Loeys, B.L., Dietz, H.C., Braverman, A.C., Callewaert, B.L., De Backer, J., Devereux, R.B., Hilhorst-Hofstee, Y., Jondeau, G., Faivre, L., Milewicz, D.M., et al. (2010). The revised Ghent nosology for the Marfan syndrome. *J Med Genet* 47, 476-485.
55. Conley, C.A., Fritz-Six, K.L., Almenar-Queralt, A., and Fowler, V.M. (2001). Leiomodins: larger members of the tropomodulin (Tmod) gene family. *Genomics* 73, 127-139.
56. Consortium, G. (2013). The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 45, 580-585.
57. Pappas, C.T., Mayfield, R.M., Henderson, C., Jamilpour, N., Cover, C., Hernandez, Z., Hutchinson, K.R., Chu, M., Nam, K.H., Valdez, J.M., et al. (2015). Knockout of Lmod2 results in shorter thin filaments followed by dilated cardiomyopathy and juvenile lethality. *Proc Natl Acad Sci U S A* 112, 13573-13578.
58. Yuen, M., Sandaradura, S.A., Dowling, J.J., Kostyukova, A.S., Moroz, N., Quinlan, K.G., Lehtokari, V.L., Ravenscroft, G., Todd, E.J., Ceyhan-Birsoy, O., et al. (2014). Leiomodins-3 dysfunction results in thin filament disorganization and nemaline myopathy. *J Clin Invest* 124, 4693-4708.
59. Cenik, B.K., Garg, A., McAnally, J.R., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., Olson, E.N., and Liu, N. (2015). Severe myopathy in mice lacking the MEF2/SRF-dependent gene leiomodins-3. *J Clin Invest* 125, 1569-1578.
60. Tian, L., Ding, S., You, Y., Li, T.R., Liu, Y., Wu, X., Sun, L., and Xu, T. (2015). Leiomodins-3-deficient mice display nemaline myopathy with fast-myofiber atrophy. *Dis Model Mech* 8, 635-641.

61. Halim, D., Wilson, M.P., Oliver, D., Brosens, E., Verheij, J.B., Han, Y., Nanda, V., Lyu, Q., Doukas, M., Stoop, H., et al. (2017). Loss of LMOD1 impairs smooth muscle cytocontractility and causes megacystis microcolon intestinal hypoperistalsis syndrome in humans and mice. *Proc Natl Acad Sci U S A* 114, E2739-E2747.
62. Chereau, D., Boczkowska, M., Skwarek-Maruszewska, A., Fujiwara, I., Hayes, D.B., Rebowski, G., Lappalainen, P., Pollard, T.D., and Dominguez, R. (2008). Leiomodin is an actin filament nucleator in muscle cells. *Science* 320, 239-243.
63. Boczkowska, M., Rebowski, G., Kremneva, E., Lappalainen, P., and Dominguez, R. (2015). How Leiomodin and Tropomodulin use a common fold for different actin assembly functions. *Nat Commun* 6, 8314.
64. Hertzog, M., van Heijenoort, C., Didry, D., Gaudier, M., Coutant, J., Gigant, B., Didelot, G., Prat, T., Knossow, M., Guittet, E., et al. (2004). The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. *Cell* 117, 611-623.
65. Zalevsky, J., Lempert, L., Kranitz, H., and Mullins, R.D. (2001). Different WASP family proteins stimulate different Arp2/3 complex-dependent actin-nucleating activities. *Curr Biol* 11, 1903-1913.
66. Venselaar, H., Te Beek, T.A., Kuipers, R.K., Hekkelman, M.L., and Vriend, G. (2010). Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC Bioinformatics* 11, 548.
67. Lambrechts, A., Jonckheere, V., Dewitte, D., Vandekerckhove, J., and Ampe, C. (2002). Mutational analysis of human profilin I reveals a second PI(4,5)-P2 binding site neighbouring the poly(L-proline) binding site. *BMC Biochem* 3, 12.
68. Cheng, J., Randall, A., and Baldi, P. (2006). Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins* 62, 1125-1132.
69. Urbanek, A.N., Smith, A.P., Allwood, E.G., Booth, W.I., and Ayscough, K.R. (2013). A novel actin-binding motif in Las17/WASP nucleates actin filaments independently of Arp2/3. *Curr Biol* 23, 196-203.