

1 Genetic and molecular mechanism for distinct clinical 2 phenotypes conveyed by allelic truncating mutations 3 implicated in *FBNI*

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56

57 **Abstract**

58 The molecular and genetic mechanisms by which different single nucleotide
59 variant (SNV) alleles in specific genes, or at the same genetic locus, bring about
60 distinct disease phenotypes often remain unclear. Allelic truncating mutations of
61 *fibrillin-1(FBN1)* cause either classical Marfan syndrome (MFS) or a more severe
62 phenotype associated with Marfanoid-progeroid-lipodystrophy syndrome (MPLS). A
63 total of three Marfan syndrome/Marfanoid patients (2 singletons and 1
64 parent-offspring trio) were recruited. Targeted next-generation sequencing was
65 performed on all the participants. We analyzed the molecular diagnosis, patient
66 clinical features, and the potential molecular mechanism involved in the MPLS
67 subject in our cohort. We investigated a small cohort, consisting of two classical MFS
68 and one MPLS patient from China, whose clinical presentation included scoliosis
69 potentially requiring surgical intervention. We provide evidence that most nonsense
70 and frameshift mutations lead to *FBN1* null alleles due to mutant mRNA transcript
71 degradation. In contrast, the more severe disease phenotype, MPLS, is caused by
72 mutant mRNAs that are predicted to escape the nonsense mediated decay (NMD)
73 surveillance pathway, making a mutant protein that exerts a dominant negative

74 interference effect to *FBNI* thus generating a gain-of-function (GoF) rather than a
 75 loss-of-function (LoF) allele as in MFS. Overall, we provide direct evidence that a
 76 dominant negative interaction of *FBNI* potentially explains the distinct clinical
 77 phenotype in MPLS patients through genetic and functional analysis of the first
 78 Chinese patient with MPLS. Moreover, our study expands the mutation spectrum of
 79 *FBNI* and highlights the potential molecular mechanism for MPLS patients.

80 **Keywords**

81 *FBNI*, Marfan syndrome (MFS), Marfanoid-progeroid-lipodystrophy syndrome
 82 (MPLS), Targeted next-generation sequencing, Dominant negative mechanism

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86 Marfan syndrome (MFS; MIM: #154700) refers to a heritable autosomal dominant
 87 disease trait of fibrous connective tissue due to heterozygous mutations in the
 88 fibrillin-1 gene (*FBNI*; 134797) on chromosome 15q21. The cardinal phenotypic
 89 features allowing for clinical diagnosis primarily occur in the skeletal, ocular, and
 90 cardiovascular systems (DIETZ 2015). MFS is manifest by clinical features/findings
 91 involving the skeletal (tall stature, disproportionately long limbs and digits
 92 [arachnodactyly], anterior chest wall deformity, mild to moderate joint laxity and
 93 frequent spinal deformity [especially scoliosis], cardiovascular (increased risk for
 94 aortic root dilation and/or dissection), and ocular (*ectopia lentis*) systems (LOEYS *et al.*
 95 2010). Marfanoid-progeroid-lipodystrophy syndrome (MPLS; MIM: #616914) is a
 96 more recently-clarified fibrillinopathy, and also a complex disease characterized by
 97 accelerated aging and postnatal lipodystrophy, poor postnatal weight gain and
 98 characteristic dysmorphic facial features that have very rarely been clinically
 99 recognized and reported (GOLDBLATT *et al.* 2011; PASSARGE *et al.* 2016). Recent
 100 studies have implicated a potential hormone, named asprosin, encoded by the *FBNI*
 101 locus as a mediator of the lipodystrophy phenotype (ROMERE *et al.* 2016;
 102 DUERRSCHMID *et al.* 2017). All previously reported MPLS individuals consistently
 103 harbor heterozygous truncating mutations in exon 64, which leads to premature stop

104 codons in the C-terminal domain of *FBNI* (GOLDBLATT *et al.* 2011; SONG *et al.* 2012;
105 GARG AND XING 2014; JACQUINET *et al.* 2014; PASSARGE *et al.* 2016; ROMERE *et al.*
106 2016).

107 In this study, we present clinical and genetic, genomic and molecular data from
108 three unrelated subjects with Marfan/Marfanoid syndrome, including the first case of
109 clinically recognized MPLS in the Chinese population. The potential effects of
110 truncating mutations of *FBNI* which are implicated in MPLS subjects conveying
111 distinct dominant negative alleles and clinically distinguishable autosomal dominant
112 (AD) disease traits were ascertained with functional assays.

113 **Materials and methods**

114 **Participant recruitment and Sample preparation**

115 A total of three Marfan syndrome/Marfanoid patients (2 singletons and 1
116 parent-offspring trio) were consecutively recruited through the Deciphering Disorders
117 Involving Scoliosis & Comorbidities (DISCO) project (www.discostudy.org) at
118 Peking Union Medical College Hospital (PUMCH). Written informed consent was
119 provided by patients or their parents. Peripheral blood samples were extracted from
120 affected probands and corresponding unaffected parents if available. Genomic DNA

121 was extracted with the QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according
122 to the manufacturer's instructions.

123 **Targeted next-generation sequencing, Variant processing, filtering and** 124 **annotation**

125 To establish an NGS panel maximally covering known genes implicated in the
126 cause of Mendelian diseases with vertebral malformation phenotypes, we performed a
127 systematic literature and database search for congenital scoliosis (CS) related disease.
128 After manual review, a total of 344 genes were found, corresponding to 457 CS related
129 monogenic phenotypes. Also included was an additional set of 220 genes whose
130 protein products are involved in the pathways related to vertebral development or in
131 which pathogenic variant alleles have been reported in association with CS
132 phenotypes in animal models, but not yet linked to human diseases with CS
133 phenotypes. The target panel set comprises 564 genes, 6458 exons and flanking 30bp
134 regions, and 2.97 Mb in total genomic size. DNA Probes for target capture were
135 designed and purchased from NimbleGen (Roche, Switzerland)
136 (<https://design.nimblegen.com/nimbledesign/app>). The final set of DNA probes was
137 expected to cover 99.7% of the targeted regions. In addition to the previously
138 published *TBX6* gene featuring a compound heterozygous inheritance model (WU *et*

139 *al.* 2015), the *FBNI* gene was also targeted in the panel. A targeted sequence
140 enrichment library was prepared following a previously described protocol (ASAN *et*
141 *al.* 2011). For each sample, 1 µg of genomic DNA was used as starting material.
142 Genomic DNA was fragmented to a size of ~200-250 bp. The fragmented DNA was
143 end-repaired, capped with A-tailing and subject to ligation of indexed adaptors. After
144 5 cycles of PCR amplification, each indexed product was pooled and hybridized with
145 DNA probes for targeted capture in one solution capture reaction. The product of
146 targeted enrichment DNA was further subject to 14 cycles of PCR amplification,
147 followed by final library yield validation by Bioanalyzer analysis (Agilent, USA) and
148 qPCR quantification. Sequencing was performed with the PE100 mode on an Illumina
149 HiSeq2500 sequencer (Illumina, CA, USA).

150 After generating raw sequence data, a perl script was used to remove low-quality
151 and adaptor-contaminated reads. The remaining reads were mapped onto human
152 reference genome assembly hg19 (GRCh37, <http://genome.ucsc.edu/>) with the BWA
153 mapper. Additionally, single nucleotide variants (SNVs) and small insertions and
154 deletions (indels) were called with the GATK (v2.2-3) bioinformatics package.
155 Variants were annotated using an in-house developed annotation pipeline. We filtered
156 out variants with common and high frequencies (Minor allele frequency >1%) in the

157 public databases (the 1000 Genomes Project, the Exome Sequencing Project 6500
158 (<http://evs.gs.washington.edu/EVS/>), ExAC (<http://exac.broadinstitute.org>) or an
159 in-house WES control dataset) that were also not functionally relevant (deep intronic
160 (>30bp), untranslated regions, or synonymous SNVs, or noncoding indels). We also
161 annotated the detected variants using a customized database based on the Human
162 Gene Mutation Database (HGMD) and Online Mendelian Inheritance in Man (OMIM)
163 (<https://omim.org/>).

164 **Sanger sequencing**

165 Genomic DNA from all individuals was subjected to Sanger sequencing for
166 orthogonal confirmation of all identified variants.

167 **Prediction of probability of NMD events**

168 For allelic truncating frameshift variants identified in our cohort and previously
169 reported, *in-silico* predictions of NMD events were implemented using the online
170 NMDEscPredictor (COBAN-AKDEMIR *et al.* 2018).

171 **Plasmid construction and mutagenesis**

172 A cDNA fragment containing full-length *FBNI* (GenBank ID: NM_000138.4)
173 cDNA derived from human muscle and having suitable restriction sites was
174 PCR-amplified using KOD-Plus-Neo (Toyobo, Japan). The PCR amplicons were

175 cloned into the *NheI* and *SacII* sites of the pEGFP-N1 expression vector (Clontech,
176 Takara Bio, Japan). Mutant EGFP-FBN1 plasmids were generated by site-directed
177 mutagenesis and their construction confirmed by direct Sanger dideoxy sequencing.
178 G2003R which was previously reported in an adolescent idiopathic scoliosis (AIS)
179 case was used as a positive control (BUCHAN *et al.* 2014). Mutations of
180 Tyr2596Thrfs*86, Glu2759Cysfs*9 and G2003R were introduced into a wild-type
181 (WT) pEGFP-FBN1 QuikChange Lightning Site-directed Mutagenesis Kit (Agilent
182 Technologies, CA, USA) according to the manufacturer's instructions. The resulting
183 three mutant plasmids pEGFP-FBN1-Tyr2596Thrfs*86,
184 pEGFP-FBN1-Glu2759Cysfs*9 and pEGFP-FBN1-Gly2003Arg were used for
185 functional studies.

186 **Cell culture and transfection**

187 HEK293T cells were cultured in DMEM (Gibco, Waltham, MA, USA) + 10%
188 FCS (Biological Industries, Cromwell, USA) + 1% penicillin/streptomycin
189 (Gibco-Life Technologies, USA) at 37°C with 5% CO₂. Cells were
190 transfected/co-transfected with a mutant and/or a WT pEGFP-FBN1 using
191 Lipofectamine3000 (Invitrogen, CA, USA) according to the manufacturers'
192 instructions. Six hours after transfection, the medium was replaced with fresh

193 complete DMEM culture medium, and the cells were further incubated for 48 h. Total
194 proteins were extracted and analyzed by Western blot and SDD-AGE, respectively.

195 **Western blot**

196 Cells were lysed with modified RIPA (50 mM Tris-HCL, 1% NP40, 0.25%
197 Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA; Complete™ Protease Inhibitor
198 Cocktail [Roche]), and protein concentrations were determined with the BCA-Kit
199 (Pierce). A total amount of 5 mg protein was size separated on an 8% SDS
200 polyacrylamide gel, and proteins were electrophoresed and transferred to
201 nitrocellulose membranes. Membranes were blocked in powdered milk for 30 min at
202 room temperature (RT, 25-degrees Celsius), and primary antibodies (Phospho-Smad2
203 (Ser245/250/255) Antibody, Cell Signaling Technology; anti-GAPDH, Millipore)
204 were incubated overnight at 4°C. After washing, the corresponding
205 horseradish-peroxidase-coupled goat anti-rabbit secondary antibodies (KPL) were
206 incubated for 1h at RT. Bands were visualized with the WesternBright ECL
207 chemiluminescence system (Advansta, CA, USA). This experiment was performed
208 three times with different cell lysates. Chemiluminescent signals were quantified
209 using ImageJ (SCHNEIDER *et al.* 2012).

210 **SDD-AGE**

211 For Semi-Denaturing Detergent-Agarose Gel Electrophoresis (SDD-AGE)
 212 (BERCHOWITZ *et al.* 2015), HEK293T cells were co-transfected with wildtype
 213 (pEGFP-FBN1) and mutants (pEGFP-FBN1-Tyr2596Thrfs*86;
 214 pEGFP-FBN1-Glu2759Cysfs*9) for 6 h, and then incubated for 48 h. After 48 h
 215 incubation at 37°C with 5% CO₂ in the thermotank, cells were harvested by
 216 centrifugation at 3000 rcf for 2 min, resuspension in 200 µL water, and subsequent
 217 centrifugation. Approximately 100 µl of acid-washed cells were then added to each
 218 well followed by 120 µL lysis buffer (100 mM Tris pH 8, 1% Triton X-100, 50 mM
 219 β-mercaptoethanol, 3% HALT protease inhibitor cocktail, 30 mM N-ethylmaleimide,
 220 and 12.5 U/mL Benzonase nuclease). Blocks were then sealed with a rubber mat
 221 (Nunc 276002) and shaken at max speed two times for 3 min on a Qiagen Tissuelyzer
 222 2. To each well was then added 35 µL 4X sample buffer (2X TAE, 20% glycerol, 8%
 223 SDS, 0.01% bromophenol blue). The blocks were then vortexed briefly and allowed to
 224 incubate at RT for three minutes, followed by centrifugation for 2 min at 3000 rcf to
 225 remove cell debris. Electrophoresis and capillary blotting to Hybond ECL
 226 nitrocellulose were performed as described. Proteins were transferred to a
 227 polyvinylidene difluoride membrane and probed with well-characterized monoclonal
 228 antibody of anti-Fibrillin 1 (Abcam ab124334, Cambridge, UK).

229 **Statistical analysis**

230 SPSS software, version 17.0 (IBM Corporation; USA), was used to conduct
231 student's two-tailed t-test comparing values of test and control samples. *P*-values of
232 less than 0.05 were considered statistically significant.

233 **Data availability statement**

234 All reagents and plasmids are available upon request. Table S1 and S2 contain a
235 list and descriptions of summary statistics of targeted genes for CS panel and targeted
236 sequencing of 564 genes, respectively. Supplemental files have been submitted to
237 figshare. All mutations identified in this study have been submitted to the Clinvar
238 database (Accession ID: VCV000617941, VCV000617942, VCV000617943).

239

240 **Results**

241 **Clinical characterization and novel allelic variants of individuals with** 242 **MFS/Marfanoid disease**

243 **Subject XH253.** The proband was a 10-year-old Chinese girl (**Fig.1A-D**) who
244 presented with kyphoscoliosis confirmed by radiologic imaging. She was referred to
245 our orthopedic spine specialist for further evaluation and management. Whole-spine
246 X ray examination revealed scoliosis with three curves. Cobb angle of the major

247 curvature was 43 degrees, and she displayed a flat back and thoracolumbar kyphosis.
 248 Transthoracic echocardiographic evaluation revealed moderate mitral valve
 249 insufficiency and an aortic root measurement of 3.6 cm (Z-score=4.5 when
 250 standardized to age and body surface area). Skeletal system abnormalities such as long
 251 and thin limbs, and arachnodactyly were observed in the proband.
 252 Diminution of vision (myopia) occurred when she was 5 years old. The proband
 253 fulfilled the clinical criteria for classical MFS according to Ghent classification
 254 (**Table.1**), with a total of 7 points for the score in the revised Ghent Criteria (LOEYS *et*
 255 *al.* 2010). A heterozygous frameshift deletion c.7785delC (p.Tyr2596Thrfs*86) was
 256 identified in *FBNI* (RefSeq transcript number: NM_000138.4) in the proband via
 257 targeted NGS, and subsequently confirmed by further validation using an orthogonal
 258 experimental approach of Sanger sequencing (**Fig.1E**).

259 **Subject XH474.** The proband was a 16-year-old Chinese girl (**Fig.1F, G**) who
 260 presented with *pectus excavatum* and scoliosis confirmed by radiologic imaging.
 261 Transthoracic echocardiographic evaluation revealed anterior mitral leaflet prolapse
 262 and moderate mitral valve insufficiency and an aortic root diameter measurement at
 263 the level of the sinuses of Valsalva of 2.6 cm (Z-score=1.3 when standardized to age
 264 and body surface area). Skeletal system abnormalities were present, such as tall stature

265 with a height of 171 cm (P₉₇; +3SD), slender body habitus with a weight of 48 kg (P₂₅,
266 -1SD), and very thin and long upper and lower limbs. Both of her hands indicated an
267 appearance of arachnodactyly; wrist sign and thumb sign were positive. According to
268 the revised Ghent nosology in 2010, an *FBNI* mutation is identified in this sporadic
269 case but aortic root measurements are still below Z score=3 (Z=1.3), the term
270 'potential MFS' should be proposed to use until the aorta reaches threshold. Herein,
271 the second patient here should be categorized as 'potential MFS' (**Table.1**), with a
272 total of 8 points for the systemic score according to the revised Ghent Criteria (LOEYS
273 *et al.* 2010). Heterozygous *FBNI* missense variants c.4890_4891delGTinsTG
274 (p.Gln1630_Cys1631delinsHisGly) were identified in the proband via targeted NGS
275 and validated by Sanger sequencing (**Fig.1H**). The missense variants were confirmed
276 to be *in cis* and comprising a complex allele resulting from a dinucleotide variant by
277 visualizing read alignment views from the Integrative Genomics Viewer (IGV)
278 (**Fig.1I**). Notably, the missense variant of c.4891T>G (p.Cys1631Gly) has been
279 previously reported as a disease-causing mutation in a classical MFS individual,
280 whose clinical course and phenotype was characterized by aortic root dissection,
281 aortic root dilation, mitral valve regurgitation, tricuspid valve prolapse, *ectopia lentis*
282 (ARBUSTINI *et al.* 2005). Conservation analysis of amino acid residues of Gln1630

283 and Cys1631in *FBNI* demonstrate that they are highly conserved throughout
284 evolution and across many selected species, suggesting they are required for the
285 normal function of the protein. Thus, the monoallelic missense variants were most
286 likely damaging and putatively deleterious and possibly accounted for 'potential MFS'
287 in this proband (**Fig.1J**).

288 **Subject XH601.** The proband was a 9-year-old Chinese girl (**Fig.1K, L**). When
289 she was 4 years old at the time of her first evaluation, she presented with a height of
290 101 cm (P₂₅; -1SD), an extremely thin body habitus with a weight of 12 kg (P₃, -3SD).
291 Physical examination revealed bilateral arachnodactyly (**Fig.1M, N**), uneven
292 asymmetric back whilst bending and congenital dislocation of the hip joint.
293 Whole-spine X ray examination displayed severe right thoracic scoliosis with a main
294 Cobb angle of 117 degrees (**Fig.1O, P**). Transthoracic echocardiogram demonstrated
295 anterior and posterior mitral leaflet prolapse and moderate mitral valve insufficiency
296 without apparent aortic diameter enlargement at the sinuses of Valsalva or aortic root
297 dissection. Ocular system abnormalities indicated bilateral down-slanting palpebral
298 fissures, epicanthus and astigmatism. However, there were no signs or symptoms of
299 ectopia lentis. The predominant features in this patient were an extreme congenital
300 lack of subcutaneous fat, consistent with a lipodystrophy by physical exam, and a

301 subsequent bodily progeroid appearance. However, this proband did not meet revised
302 Ghent Criteria for classical MFS. Consecutive follow-ups and assessment of the
303 proband were conducted. Upon genotype driven reverse phenotyping of the proband
304 when she was 9, the proportion of upper and lower segment lengths was basically
305 normal with a ratio of 0.87. The proband had a height of 136 cm (P₅₀, 0). For
306 comparison, her father's height measurement was 170 cm and her mother's was 160
307 cm. The body mass index (BMI) of the proband was extremely low (11.1 kg/m²)
308 potentially due to disproportionate weight gain by age. More specifically, the patient
309 had an extremely thin habitus with a weight of 20.5 Kg (P₃, -3SD). Furthermore, hand
310 and foot radiographs to potentially assess 'bone age' suggested bilateral
311 metacarpophalangeal joint dislocation, interosseous atrophy and dolichostenomelia
312 (**Fig.1Q, R**). The proband was eventually diagnosed with
313 Marfanoid-progeroid-lipodystrophy syndrome (**Table.2**). Through targeted NGS, a
314 heterozygous variant of c.8275_8291delGAGAAGACAGCCATCTT
315 (c.8275_8291del; p.Glu2759Cysfs*9) in *FBN1* was identified in the proband. We
316 consequently confirmed this variant in subject XH601 and it was indeed *de novo*
317 through parental Sanger sequencing and trio analysis (**Fig.1S**). In addition to Sanger

318 sequencing validation, we also performed cloning sequencing on Subject XH601 to
319 further verify that this indel is subject to frame-shift variation (data not shown).

320 Moreover, these two allelic truncating variants of p.Tyr2596Thrfs*86 and
321 p.Glu2759Cysfs*9 were not present in ExAC, 1000 Genome, ESP6500 database,
322 universal mutation database (UMD) (COLLOD-BEROU *et al.* 2003) and our in-house
323 database (with 2000+ Chinese exomes). Clinical and genetic characteristics of subject
324 XH253 with classical MFS and XH474 with 'potential MFS' based on the revised
325 Ghent Criteria (LOEYS *et al.* 2010) are presented in Table 1.

326

327 **NMD-degradation prediction**

328 The variant of Y2596Tfs*86 identified in XH253 was a single nucleotide
329 frameshift deletion leading to a premature termination codon (PTC) in Exon 64, while
330 E2759Cfs*9 identified in XH601 was a frameshift deletion leading to a PTC in Exon
331 66, the final exon of *FBNI* (**Fig. 2A**). Although both variants are very close in linear
332 space, they may lead to distinct alterations to the *FBNI* transcript. To investigate this
333 hypothesis, we performed NMD predictions of the two truncating variants on
334 NMDescPredictor (COBAN-AKDEMIR *et al.* 2018). The result showed that
335 Y2596Tfs*86 is predicted to be degraded by NMD, i.e. NMD⁺, while E2759Cfs*9 is

336 predicted to escape NMD, i.e. NMD⁻ (**Fig. 2B**). Using the same analytical tools, we
 337 further tested whether previously reported protein-truncating *FBN1* frameshift
 338 variants implicated in MPLS subjects (GRAUL-NEUMANN *et al.* 2010; GOLDBLATT *et*
 339 *al.* 2011; TAKENOUCI *et al.* 2013; GARG AND XING 2014) uniformly shared the
 340 mechanism of escaping NMD. Our analysis shows that all of the truncating *FBN1*
 341 frameshift variants within the C-terminal gene region may escape NMD, supporting
 342 the contention that the underlying disease mechanism in MPLS is the same and
 343 distinct from MFS (**Fig. 2B**). These findings implicate a mechanism distinct from
 344 loss-of-function (LoF) alleles, and potentially gain-of-function (GoF) variant alleles
 345 (COBAN-AKDEMIR *et al.* 2018), at the *FBN1* locus causing MPLS.

346

Perturbation of native aggregation process by MPLS-causative truncating mutation

349 To further determine if the mutant FBN1 protein in MPLS is able to lead to
 350 dominant negative or GoF effects, plasmids expressing mutants
 351 (pEGFP-FBN1-Tyr2596Thrfs*86; pEGFP-FBN1-Glu2759Cysfs*9) and WT plasmid
 352 (pEGFP-FBN1) were co-transfected into HEK293T cells at a 1:1 ratio. After 48h of
 353 expression, lysates from cells expressing EGFP-FBN1 fusions were analyzed by
 354 SDD-AGE. Detection of SDS-resistant aggregates by SDD-AGE in cell lysates of

HEK293T transiently expressing EGFP-FBN1 fusions were investigated with SDD-AGE and Western blot. Protein expression was induced for 48 h and detected with a monoclonal FBN1-specific antibody. Remarkably, WT plasmid (EGFP-FBN1 fusions) had the SDS-resistance properties of an amyloidogenic protein structure. Since amyloid formation is time- and concentration-dependent, we set the time of transient expression to 48 h. WT plasmid (EGFP-FBN1 fusions) were aggregated after 48 h expression, whereas co-transfection of pEGFP-FBN1-Glu2759Cysfs*9 and WT plasmid resulted in the presence of a small fraction of monomer protein. This suggests that pEGFP-FBN1-Glu2759Cysfs*9 apparently prohibited the native aggregation process of WT plasmid, possibly revealing an intracellular dominant-negative mechanism. In addition, we observed that co-transfection of pEGFP-FBN1-Tyr2596Thrfs*86 and WT plasmid presented a small fraction of aggregated protein. As this fraction was less than that of the WT plasmid, it can be suggested that Tyr2596Thrfs*86 may undergo an NMD mechanism thus leading to *FBN1* haploinsufficiency (**Fig.3A**). Moreover, these variations in the range of particle sizes were observed, and again were reproducible for individual EGFP-FBN1 fusions validated by another two independent replication experiments.

373 **Disruption of downstream TGF- β signaling**

374 Previous studies have demonstrated that deleterious *FBNI* mutations causing
 375 Marfan syndrome result in upregulated endogenous transforming growth factor β
 376 (TGF- β) receptor signaling (ANDELFINGER *et al.* 2016). Such signaling can be
 377 measured in plasma or indirectly measured through aberrant activation of downstream
 378 targets, including excessive phosphorylation of SMAD2 (pSMAD2) (VERSTRAETEN
 379 *et al.* 2016). To determine the functional consequences of the novel truncating *FBNI*
 380 variant of E2759Cfs*9 in the MLPS patient, plasmids expressing mutant EGFP-FBN1
 381 cDNAs were transfected into HEK293T cells. Transfected cells with truncating *FBNI*
 382 variants of Y2596Tfs*86 (P=0.007), E2759Cfs*9 (P=0.017) and Gly2003Arg
 383 (P=0.006) showed significantly elevated phosphorylation of SMAD2 (pSMAD2) in
 384 Western blots compared to the WT plasmid (**Fig.3B, C**). This observation is consistent
 385 with the contention that the TGF- β signaling pathway is perturbed in a
 386 SMAD-dependent manner in all 3 subjects.

387

388 **Genotypic and phenotypic features of reported MPLS patients**

389 Intriguingly, the mechanism underlying *FBNI*-related diseases involves either
 390 loss-of-function (LoF) or NMD surveillance pathway escape as conveyed by allelic
 391 truncating mutations. Such distinct mechanisms could contribute to distinct disease

phenotypes of varying severity. According to a previous report, *FBNI* LOF mutations led to classical MFS (PARK *et al.* 2017), while predicted NMD surveillance pathway escape in *FBNI* can cause a MPLS phenotype (ROMERE *et al.* 2016), Patient XH601 in our cohort harbored a heterozygous *de novo* variant of E2759Cfs*9 in *FBNI*, which is located in the final exon. Notably, our MPLS patients presented with bilateral down-slanting palpebral fissures, epicanthus and eye astigmatism. The main clinical features of this complicated disorder, clustered in Table 2, include accelerated aging and postnatal lipodystrophy, poor weight gain since birth, premature birth, hyperextensible digits and generalized subcutaneous fat reduction leading to a progeroid appearance of the body in all patients. Mental and motor development remain mostly normal. Overlapping clinical features of MPLS with MFS are phenotypically diverse. Ocular system involvement like myopia have been observed in all individuals, although hyperextensible joints, arachnodactyly, and other significant signs of classical MFS are not always present, i.e. mitral valve prolapse in 4/8, lumbosacral dural ectasia in 2/3 (5 data points unavailable), *pectus excavatum* in 3/8, and ectopia lentis in 3/8. Scoliosis was reported in two patients aged 23 and 17 years, and kyphosis was reported in a patient aged 27 years, but not described in the others (**Table.2**). Remarkably, the major Cobb angles of Subject XH253 with classical

410 MFS and Subject XH474 with MASS were 43 degrees and 85 degrees, respectively.
411 Our MPLS patient presented a thoracic scoliosis with a major Cobb angle magnitude
412 of 117 degrees—a condition much more severe compared to the MFS patients,
413 counterparts.

414 Discussion

415 In the present report, we provide direct evidence for potential dominant negative
416 alleles in MPLS patients caused by truncating *FBNI* variants escaping NMD. These
417 genetic and functional investigations also include description of the first patient with
418 MPLS of Chinese ancestry.

419 Several case reports previously identified an association between monogenic
420 *FBNI* mutations and reduction of subcutaneous fat and/or progeroid features
421 (GRAUL-NEUMANN *et al.* 2010; GOLDBLATT *et al.* 2011; TAKENOUCI *et al.* 2013;
422 JACQUINET *et al.* 2014; PASSARGE *et al.* 2016). Here, we have provided further
423 evidence for the existence of a distinct genetic disease, MPLS, caused by *FBNI*
424 mutations. The MPLS patient in our study is characterized by accelerated aging and
425 postnatal lipodystrophy, disproportionate weight gain since birth, severe scoliosis,
426 downslanting palpebral fissures, bilateral metacarpophalangeal joint dislocation,

mitral valve prolapse, bilateral interosseous atrophy, and congenital dislocation of the hip. Intriguingly, this patient was previously diagnosed with Marfanoid disease (**Table.1**) due to an unclear molecular diagnosis. We consequently performed targeted NGS on the affected proband and her unaffected parents. As a result, we identified c.8275_8291del(p.Glu2759Cysfs*9) in *FBN1* as the responsible variant for the proband. Sanger sequencing was then conducted on genomic DNA from both parents to confirm the *de novo* occurrence and segregation with phenotypes. Subsequently, we proceeded with a comprehensive phenotypic analysis of all family members through intensive clinical follow-up. By combining genetic data with reverse phenotyping, we eventually diagnosed the proband with MPLS. Notably, the severity of spinal deformity in the MPLS individual was significantly more severe than that in Subject XH253 and XH474 (with classical MFS and potential MFS, respectively). We consider that MPLS is a distinct fibrillinopathy because it can be clinically distinguished from other fibrillinopathies, including classical MFS. To our knowledge, there are no other syndromes that simultaneously comprise the clinical phenotypes of marfanoid features, accelerated aging, postnatal lipodystrophy, poor weight gain since birth, and progeroid appearance. Particularly, the involvement of both reduced subcutaneous fat tissue and progeroid appearance is a prominent characteristic of

445 MPLS. These comprehensive findings predominate in determining the severity of
 446 defects in individuals and thus significantly influence the prognosis: lipodystrophic
 447 disorders are frequently associated with metabolic disturbance, such as insulin
 448 resistance and life-threatening hypertriglyceridemia (VANTYGHEM *et al.* 2012). Such
 449 potential metabolic disturbances are particularly important to note prior to surgical
 450 intervention as has been noted for the ocular-scoliotic form of Ehlers Danlos
 451 syndrome due to lysyl hydroxylase deficiency (YEOWELL *et al.* 1995). Secondly,
 452 elucidation of the phenotype of MPLS has been limited due to the paucity of prior
 453 clinical reports, with only seven relevant patients previously described. Up to now, it
 454 is known only to be caused by a single gene with a monogenic AD inheritance pattern
 455 for the disease trait. Finally, we have shown that, in terms of pathogenic mechanisms,
 456 the truncating *FBNI* mutations that cause MPLS cluster in 3' gene regions encoding
 457 the extreme C-terminal domains and these variant alleles represent a subset that differ
 458 from those that cause classical MFS. Most of such mutations will be anticipated to
 459 escape NMD and we propose these to function via a potential mechanism between
 460 GoF and dominant negative and not a LoF mechanism.

461 Fibrillin-1 acts as the precursor to a recently-described glucogenic hormone:
 462 aprosin (DAVIS *et al.* 2016a). Specifically, Exon 65 of *FBNI* encodes 11 amino acids,

463 while Exon 66 of *FBNI* encodes 129 amino acids of asprosin (ROMERE *et al.* 2016).
 464 Recently, Chen et.al. have successfully constructed a gene-edited rabbit model with a
 465 truncated C-terminus of fibrillin-1 involving the ultimate two exons which could
 466 recapitulate the histopathological alterations and functional defects associated with
 467 MPLS (CHEN *et al.* 2018). Since individuals with low fibrillin-1 level may fail to
 468 differentiate adipocytes and/or to accumulate adipocyte lipids, truncating variants
 469 located adjacent to the two exons of *FBNI* are prone to cause lipodystrophic
 470 phenotypes (DAVIS *et al.* 2016b).

471 PTCs located in the final coding exon are specifically prone to escape NMD and so
 472 are processed distinctly from those in internal exons in terms of transcript degradation,
 473 leading to the stable translation of truncated proteins (BAYRAM *et al.* 2017). Excessive
 474 amounts of mutated protein are therefore produced (INOUE *et al.* 2004), leading to a
 475 dominant negative molecular mechanism and the severe MPLS disease phenotype.
 476 Consistent with this interpretation, all truncating mutations associated with MPLS are
 477 located adjacent to the penultimate exon (Exon 65) and are predicted to escape NMD
 478 (**Figure.4**). From this perspective, it is perhaps most parsimonious to postulate that the
 479 incorporation of relatively few mutant monomers would be sufficient to impair the
 480 systemic processes of microfibrillar assembly and function.

481 Aberrant activation of the TGF- β pathway has been observed in MFS and may
 482 account for some of the musculoskeletal deformities, such as scoliosis (BUCHAN *et al.*
 483 2014). The MPLS patient with a truncating *FBNI* variant presented with severe
 484 scoliosis, which suggests that the protein change also alters TGF- β signaling. Our
 485 results showed upregulation of the TGF- β pathway in plasmids expressing mutant
 486 EGFP-FBN1 cDNAs, confirming that the variant of E2759Cfs*9 identified in the
 487 MPLS patient has functional effects in a SMAD-dependent manner in all 3 subjects.

488 Our study indicated that the two phenotypes associated with *FBNI* mutations,
 489 MPLS and MFS are caused by two distinct molecular mechanisms. The more severe
 490 phenotype of MPLS, which had its first report in the Chinese population, is caused by
 491 nonsense mutations that produce truncated *FBNI* mutant proteins. These proteins
 492 disrupt the process of native amyloid-like structure conformation and exert potent
 493 intracellular dominant-negative activity. In this view, abnormal protein derived from
 494 the mutant allele interacts and interferes with protein derived from the normal allele,
 495 consequently resulting in substantial loss of function. Supportive evidence includes (a)
 496 *de novo* monogenic AD inheritance pattern, and (b) aggregation of fibrillin-1 in which
 497 the mutated monomer derived from the mutant allele impairs the fundamental
 498 structure of its wildtype protein derived from the normal allele counterpart. This

499 deficiency could be supported by SDD-AGE analysis, suggesting that dominant
 500 negative interference is not restricted to enhanced proteolytic clearance of mutant
 501 microfibrils over time (BRENN *et al.* 1996), but rather could also occur at the level of
 502 intracellular aggregate formation processes. The more moderate phenotype, classical
 503 MFS, is caused by nonsense mutations that activate the NMD RNA surveillance
 504 pathway, thereby degrading mutant transcripts and resulting in *FBNI*
 505 haploinsufficiency. The data support the concept of distinct pathogenetic mechanisms,
 506 GoF *versus* LoF (BAYRAM *et al.* 2017; COBAN-AKDEMIR *et al.* 2018; POLI *et al.* 2018),
 507 for each well-established subgroup of fibrillinopathy, which mostly hinges on the
 508 intrinsic features of fibrillin-1 mutations. It is of crucial significance to pinpoint and
 509 appreciate the locus heterogeneity, clinical relevance and overlapping clinical features
 510 with other syndromes/disorders in a phenotype-oriented approach that characterizes
 511 much of clinical medicine practice (WHITE *et al.* 2018).

512 In conclusion, we provide direct evidence of dominant negative effects of
 513 truncating *FBNI* variants predicted to escape NMD in MPLS patients. Our study
 514 expands the mutational spectrum of *FBNI* and highlights the potential molecular
 515 mechanism for MPLS patients, which facilitates our understanding of
 516 genotype-phenotype correlations in terms of *FBNI* to provide effective genetic

517 counseling, implementation and timing of therapy (e.g. mitigation of TGF- β
518 hypersignaling, surgical intervention for cardiovascular complications or for
519 scoliosis), or early intervention.

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536

537 **Conflict of interest**

538 J.R.L has stock ownership in 23andMe, is a paid consultant for Regeneron
539 Pharmaceuticals, and is a co-inventor on multiple the United States and European
540 patents related to molecular diagnostics for inherited neuropathies, eye diseases and
541 bacterial genomic fingerprinting. The Department of Molecular and Human Genetics
542 at Baylor College of Medicine derives revenue from the chromosomal microarray
543 analysis and clinical exome sequencing offered in the Baylor Genetics Laboratory
544 (<http://bmgl.com>).

545

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646

647 **Figure Legends**

648 **Figure 1** Clinical and genetic manifestations of the research subjects

649 (A, B) Pictures of patient XH253 indicated bilateral arachnodactyly of hands and
650 feet.

651 (C, D) The anterior and lateral view of Patient XH253 demonstrated scoliosis on
652 whole-spine X-ray images.(E) Sanger sequencing of XH253 verified a novel
653 heterozygous frameshift variant of p.Tyr2596Thrfs*86 in *FBNI*. (F, G) The anterior
654 and lateral view of subject XH474 demonstrated scoliosis on whole-spine X-ray
655 images. (H) Sanger sequencing verified monoallelic missense mutations of
656 p.Gln1630His and p.Cys1631Gly in *FBNI*. (I) Integrative Genomics Viewer (IGV)
657 displays varying level of alignment reads detail depending on the zoom level and
658 uses red box and transparency to highlight monoallelic missense variants in the
659 exome data from XH474.

660 (J) Conservation analysis of amino acid residues of *FBNI* among vertebrates.
661 Lines indicate homologous amino acid sequences in selected vertebrates. Note the
662 strong conservation of Cys1631 among vertebrates highlighted in red and Gln1630
663 highlighted in green, respectively. (K, L, M, N) Photographic images of subject
664 XH601 who presented with scoliosis, short stature and subcutaneous fat reduction

665 and arachnodactyly anomalies of hands and feet.(O, P) The anterior and lateral view
666 demonstrated severe scoliosis on whole-spine X-ray images.
667 (Q, R) Hand and foot radiographs showed bilateral metacarpophalangeal dislocation,
668 interosseous atrophy, claw hands and dolichostenomelia. (S) Sanger sequencing of
669 XH601 parent-offspring trio verified a novel de novo heterozygous frameshift
670 mutation of p.Glu2759Cysfs*9 in *FBNI*. The RefSeq transcript number of *FBNI* is
671 NM_000138.4.

672

673 **Figure 2** Schematic representations and NMD predictions of the p.Y2596Tfs*86 and
674 p.E2759Cfs*9 mutations other MPLS-affected frameshift mutations in *FBNI*

675 (A) Schematic diagrams of full-length WT and mutant EGFP-*FBNI* plasmids
676 transiently expressed in HEK293T. The numbers indicate the amino acid positions in
677 *FBNI*. PTC location is marked with red circle. The resulting frameshift and
678 reduction in the deduced amino acid sequence caused by the p.Y2596Tfs*86 and
679 p.E2759Cfs*9 mutations are indicated by the yellow region, respectively.

680 (B) Prediction of frameshift variants that are potentially subject to
681 nonsense-mediated mRNA decay (NMD)-escape or NMD-degradation. The
682 mutation of p.Y2596Tfs*86 in *FBNI* gene is predicted to be subject to degradation

683 by triggering NMD. The p.E2759Cfs*9 mutation in *FBN1* gene is predicted to
684 escape NMD. All of other MPLS-affected frameshift mutations in *FBN1* are
685 predicted to escape NMD.

686

687 **Figure 3** Functional effects of the Y2596Tfs*86 and E2759Cfs*9 mutations on
688 *FBN1* protein expression

689 (A) Detection of SDS-resistant aggregates by SDD-AGE in cell lysates of HEK293T
690 transiently expressing EGFP-*FBN1* fusions were investigated by SDD-AGE and
691 Western blot. Expression of the proteins was induced for 48 h and detected with a
692 monoclonal *FBN1*-specific antibody.

693 (B) Elevated phosphorylation of SMAD2 (pSMAD2) in individuals with truncating
694 *FBN1* variants, respectively. G2003R is used as a positive control. GAPDH is used
695 as a loading control. pSMAD2/GAPDH ratio is shown normalized to the unaffected
696 control (WT).

697 (C) Gray-scale analysis results show significantly upregulated (pSMAD2) in
698 constructs encoding truncating *FBN1* variants. EV denotes empty vector (pEGFP).

699 RT: room temperature. Data are represented as mean \pm SD of three independent
700 experiments. * denotes P value <0.05 and ** denotes P value <0.01 .

701

702 **Figure 4** The partial gene structure of the C-terminus of *FBNI* and overview of
703 previously reported truncating variants in and around Exon 65 in patients presenting
704 with MPLS

705 Blue, purple and red boxes denote specific domain. Light green, orange and black
706 boxes denote exons and the gray area between the two boxes denote introns. All
707 truncating variants were annotated by small green circles except that c.8226+1G>T
708 previously reported twice was annotated by a big circle. COOH denotes COOH
709 unique region. Deep blue box denotes purported asprosin encoded by the exons of
710 *FBNI*.

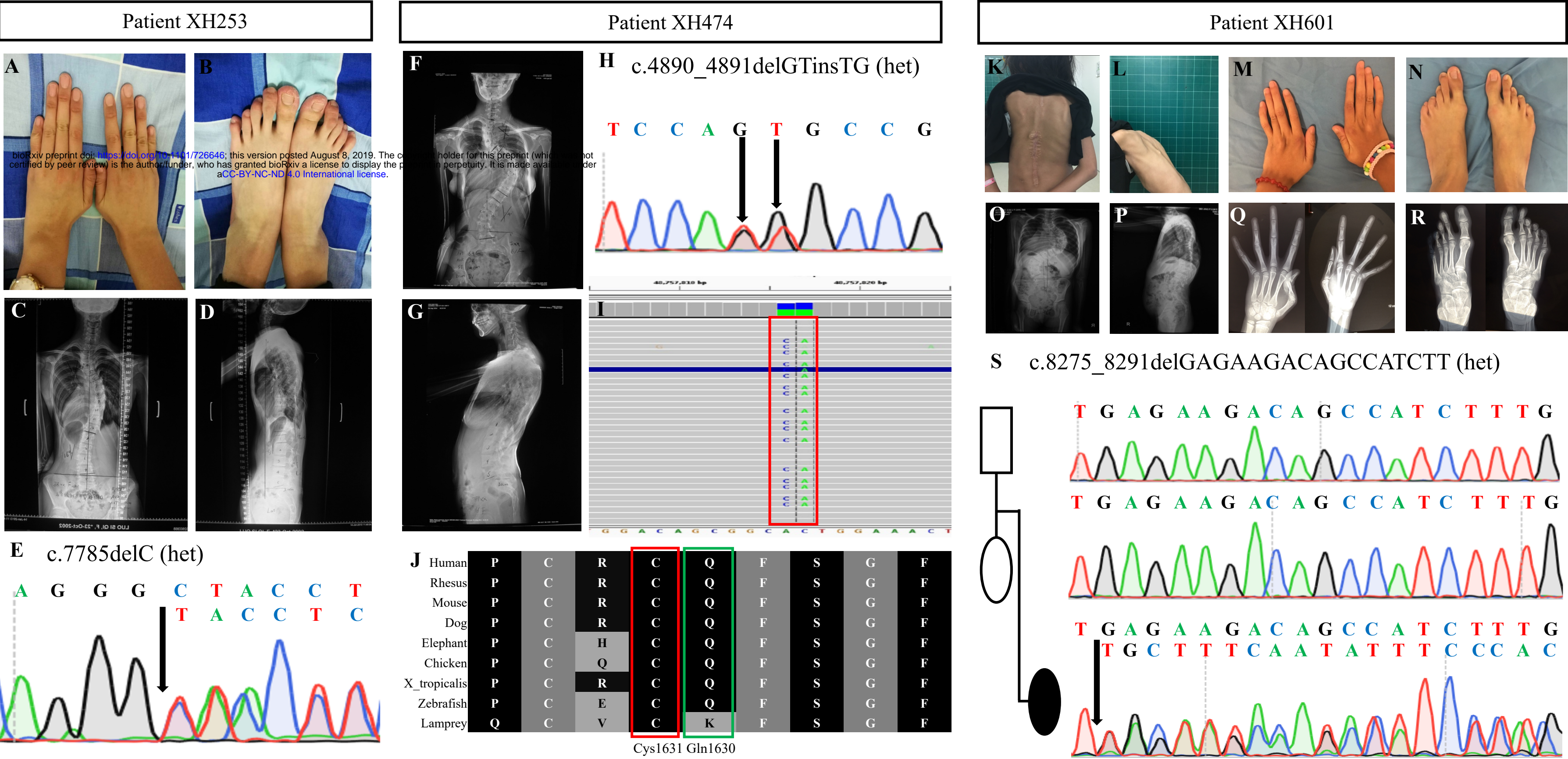
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Figure 1



Patient XH601

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c.8275_8291delGAGAAGACAGCCATCTT (het)

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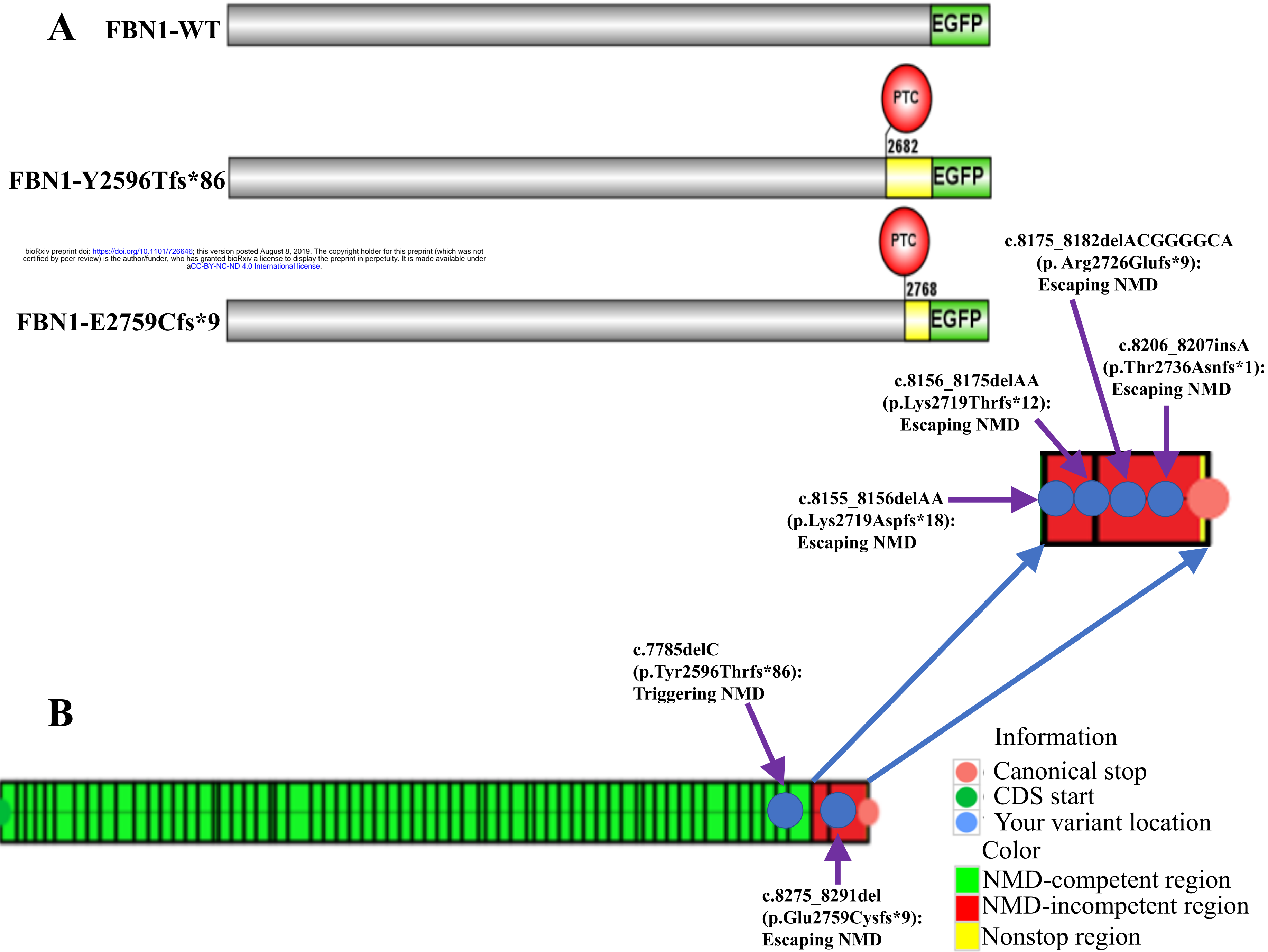
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Figure 2



A

RT

Anti-FBN1

P-SMAD2/GAPDH

Monomer

+

- + -

- - +

B

GAPDH

WT

p.Y2596Tfs*86

p.E2759Cfs*9

P.G2003R

1.0

1.8

1.8

1.4

C

P-SMAD2/GAPDH

WT

WT

p.Y2596Tfs*86

p.E2759Cfs*9

P.G2003R

Figure 4

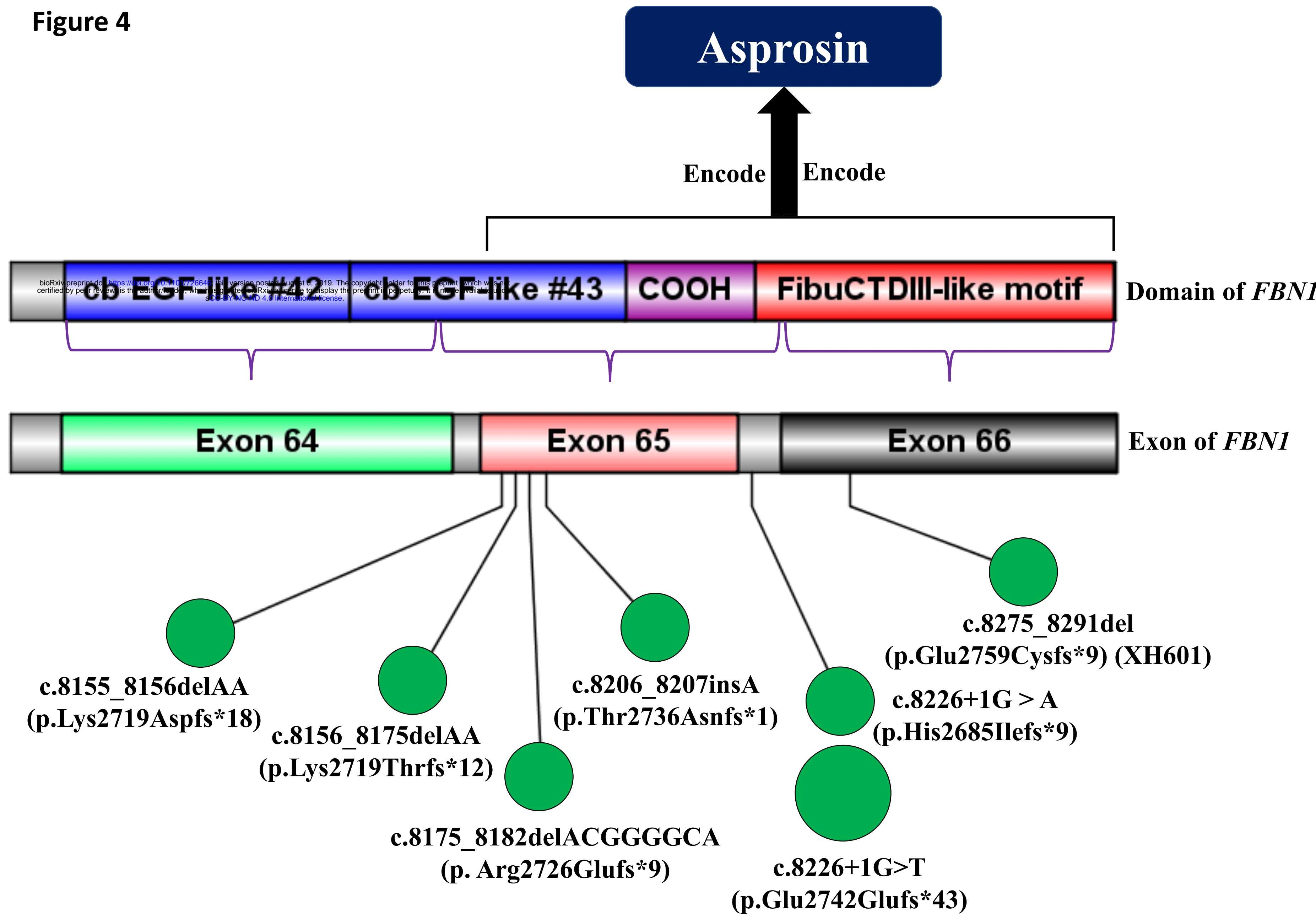


Table 1 Clinical features of Marfan syndrome patients re-evaluated for according to the revised Ghent nosology

Subject		XH253	XH474	XH601
	General patient information			
	Gender	F	F	F
	Age (years)	10	16	9
	Height (m)	1.72	1.71	1.36
	Weight (kg)	48	48	20.5
	Major Cobb angle (degrees)	43	85	117
	Major Curve type	R-T	R-T	R-T
	Curve number	3	3	2
	Curve details	UTC (38), MTC (43), LC (16)	UTC (60), MTC (85), LC (40)	MTC (117), LC (89)
	Treatment	Surgery	Surgery	Surgery
Causal <i>FBNI</i> variant	Nucleotide alteration	c.7785delC	c.4890_4891delGTinsTG	c.8275A8291delGAGAAGACAGCCATCTT
	Protein change	p.Tyr2596Thrfs*86	p.Gln1630_Cys1631delinsHisGly	p. Glu2759Cysfs*9
	Variant reported in MFS	None	p. Cys1631Gly	None
MFS evaluation	Diagnosis	MFS	MASS	Marfanoid
	Aortic root dilation (z-score)	4.5	1.3	1.6
	Ectopia lentis	A	A	A
	Systemic features score	7/20	8/20	7/20
	Wrist/thumb sign	P	P	A
	Pectus carinatum/ excavatum	A	A	A
	Hindfoot deformity	A	A	A
	Pneumothorax	A	A	A
	Dural ectasia	A	A	A
	Protrusio acetabuli	A	A	A
	UPer/lower segment	0.92	0.94	0.86
	Scoliosis or thoracolumbar kyphosis	P	P	P
	Reduced elbow extension	NA	NA	NA
	Facial features (3/5)	P	P	P
	Skin striae	A	A	A
	Myopia	-0.7 dioptries	NA	-0.4 dioptries
	Mitral valve prolapses (all types)	P	P	P

MFS denotes Marfan syndrome; MASS denotes myopia, mitral valve prolapses, borderline ($Z < 2$) aortic root dilatation, striae, skeletal findings phenotype NA denotes not available; F denotes female; R-T denotes right thoracic spine; P denotes present; A denotes absent. UTC denotes upper thoracic curve; MTC denotes major thoracic curve; LC denotes lumbar curve.

Table 2 Clinical manifestations of previously reported and our patient XH601 with Marfanoid-progeroid-lipodystrophy (MPL) syndrome with allelic truncating mutations in *FBN1*

	Graul-Neumann et al. [2010]	Goldblatt et al. [2011]	Horn and Robinson et al. [2011]	Takenouchi et al. [2013]	Jacquinet et al. [2014]	Garg and Xing et al. [2014]		XH601 (this study)
						Patient 1	Patient 2	
Clinical characteristics								
Age (year)	27	20	3.5	10	16	23	17	9
Gender	Female	Male	Female	Female	Female	Female	Female	Female
Ancestry	Caucasian	Caucasian	Caucasian	Asian (Japanese)	Caucasian	Hispanic	Caucasian	Asian (Chinese)
Height (cm; [centile])	170	50-75	108	149	177	157.5	176	136
Weight (Kg; [centile])	39	<3	14.5	21.7	41.8	26.4	41.9	20.5
Causal <i>FBN1</i> mutation								
cDNA analysis	c.8155_8156delAA	c.8156_8175del	c.8226+1G>T, splice mutation, exon 65 skipping	c.8175_8182del8bp	c.8226+1G>A, splice mutation, exon 65 skipping and subsequent frameshift	c.8206_8027insA	c.8226+1G>T, splice mutation, exon 65 skipping	c.8275_8291del GAGAAGACAGCCATCTT
protein change	p.(Lys2719Aspfs*18)	p.(Lys2719Thrfs*12)	p.(Glu2742Glufs*43)	p.(Arg2726Glufs*9)	p.(His2685Ilefs*9)	p.(Thr2736Asnfs*1)	p.(Glu2742Glufs*43)	p.(Glu2759Cysfs*9)
Adjacent to Exon 65 in <i>FBN1</i>	Y	Y	Y	Y	Y	Y	Y	Y
Inheritance pattern	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>
BMI (kg/m2)	13.3	NA	12.4	9.8	13.3	10.6	13.5	11.1
Body fat (%)	20.5	NA	NA	NA	NA	28.4	27.7	NA
Premature birth	Y	Y	Y	Y	Y	Y	Y	Y
Birth weight (Kg)	1.78	1.04 (<3)	1	1.427	1.72	1.19	1.17	NA
Birth length (cm)	41.5	NA	40	40	45	40	40.75	NA
Progeroid appearance	Y	Y	Y	Y	Y	Y	Y	Y
Subcutaneous fat reduction	Y	Y	Y	Y	Y	Y	Y	Y
Arm span/height	0.99	NA	NA	NA	NA	0.94	0.98	NA
Upper/lower segment	0.95	NA	NA	NA	NA	0.99	0.8	0.87
Proptosis	Y	Y	Y	Y	Y	Y	Y	Y
DPF	NA	NA	NA	NA	NA	NA	NA	Y
Myopia	Y	Y	N	Y	Severe	Y	Y	Y
Ectopia lentis	Bilateral	Bilateral	N	N	N	Left eye	N	N
High-arched palate	Y	Y	Y	NA	NA	Y	N	NA
Pectus excavatum	NA	Y	NA	Y	N	N	Y	N
scoliosis / kyphosis	Kyphosis	NA	NA	NA	N	Scoliosis	Scoliosis	Severe scoliosis
Cobb angle (degrees)	NA	NA	NA	NA	NA	NA	NA	117
Wrist sign	Y	Y	NA	NA	NA	Y	Y	Y
Thumb sign	Y	N	NA	NA	NA	Y	Y	Y
BMJD	NA	NA	NA	NA	NA	NA	NA	Y
Interosseous atrophy	NA	NA	NA	NA	NA	NA	NA	Y
Arachnodactyly	NA	NA	NA	Y	Y	NA	NA	Y
Hyperextensible digits	NA	Y	NA	Y	Y	Y	Y	Y
Pes planus /valgus	NA	Pes planus	NA	NA	Pes valgus	NA	Pes planus	NA
Easy bruisability	Y	Y	NA	NA	NA	N	N	Y
MVPS	Y	N	N	N	N	NA	Y	Y
Arrested hydrocephalus	NA	Y	Y	Y	NA	NA	NA	NA
RGC	NA	NA	NA	Hydronephrosis	N	NA	NA	N
Dural ectasia	Lumbosacral	NA	NA	Y	Y	NA	NA	N
Hypertension	NA	NA	NA	Y	NA	NA	NA	N
CHD	NA	NA	NA	NA	NA	NA	NA	Y

Abbreviations: Y, yes; N, no; NA, not available. DPF, downslanting palpebral fissures; BMJD: bilateral metacarpophalangeal joint dislocation; MVPS, mitral valve prolapse syndrome; RGC, renal/genitourinary complications. CHD, congenital hip dislocation