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## ***FBN1*: The Disease-Causing Gene for Marfan Syndrome and Other Genetic Disorders**

Lynn Y. Sakai<sup>1,\*</sup>, Douglas R. Keene<sup>2</sup>, Marjolijn Renard<sup>3</sup>, and Julie De Backer<sup>3</sup>

<sup>1</sup>Departments of Molecular & Medical Genetics and Biochemistry & Molecular Biology

<sup>2</sup>Biomedical Engineering, Oregon Health & Science University and Shriners Hospital for Children, Portland, OR 97239

<sup>3</sup>Center for Medical Genetics, University of Ghent, Ghent, Belgium

### **Abstract**

*FBN1* encodes the gene for fibrillin-1, a structural macromolecule that polymerizes into microfibrils. Fibrillin microfibrils are morphologically distinctive fibrils, present in all connective tissues and assembled into tissue-specific architectural frameworks. *FBN1* is the causative gene for Marfan syndrome, an inherited disorder of connective tissue whose major features include tall stature and arachnodactyly, ectopia lentis, and thoracic aortic aneurysm and dissection. More than one thousand individual mutations in *FBN1* are associated with Marfan syndrome, making genotype-phenotype correlations difficult. Moreover, mutations in specific regions of *FBN1* can result in the opposite features of short stature and brachydactyly characteristic of Weill-Marchesani syndrome and other acromelic dysplasias. How can mutations in one molecule result in disparate clinical syndromes? Current concepts of the fibrillinopathies require an appreciation of tissue-specific fibrillin microfibril microenvironments and the collaborative relationship between the structures of fibrillin microfibril networks and biological functions such as regulation of growth factor signaling.

## **1. Introduction**

### **1.1 Fibrillin Microfibrils**

*FBN1* encodes the gene for fibrillin-1. In humans, there are three different genes (*FBN1*, *FBN2*, and *FBN3*) encoding fibrillins. Fibrillins are large (~350,000 MW) structural macromolecules that contribute to the integrity and function of all connective tissues. They are considered to be “structural macromolecules” because, like the collagens, the fibrillins form fibers that are visible in transmission electron micrographs. Unlike the collagens, fibrillins form “microfibrils” with uniform diameters (10–12 nm) that are not periodically cross-striated or “banded”. Fibrillin microfibrils display a characteristic morphology

\*To Whom Correspondence Should be Addressed: Lynn Y. Sakai, Ph.D., Shriners Hospital for Children, 3101 SW Sam Jackson Park Rd., Portland, OR 97239, lys@shcc.org.

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consisting of light and dark or hollow areas that give the appearance of railroad tracks. Fibrillin microfibrils exist as large bundles of microfibrils, as short individual microfibrils (usually in close proximity to basement membranes, for example on the endothelial cell side of the glomerular basement membrane), or as the peripheral microfibril mantle around elastin in all elastic fibers. Typical morphological features of fibrillin microfibrils are shown in Figure 1. In the various types of connective tissue, fibrillin microfibrils are organized to best suit the functional integrity of the tissue: for example, in skin, elastic fibers form a loose network of interconnecting highways; in the dermis, these highways run parallel to the epidermis with turn-offs coursing perpendicularly up from the deeper elastic fibers to the basement membrane at the dermal-epidermal junction, where bundles of microfibrils intersect the lamina densa; in tendons and perichondrium/periosteum, elastic fibers run parallel to the long axis; in muscular arteries, elastic fibers encircle the lumen.

Although “10 nm microfibrils” had been described as ultrastructural entities, the molecular components of these microfibrils were not known until 1986. Protocols to extract microfibrillar molecules used harsh denaturing conditions as well as disulfide bond reducing agents (1). Reductive guanidine extractions of fetal bovine nuchal ligament, an elastic fiber rich tissue, yielded a 31,000 MW glycoprotein, which was named MAGP (“microfibril associated glycoprotein”) (2). MAGP antiserum localized to elastin-associated microfibrils (2). Today, a number of additional molecules are known to be associated with microfibrils. These molecules have been both immunolocalized to microfibrils and shown to bind directly to fibrillin. These include the fibulins (3,4), the LTBPs (Latent TGF $\beta$  Binding Proteins) (5–7), and members of the Adamslike (8–10) and Adams (11) family of proteins.

Initially, it was thought that the main function of microfibrils is to serve as the scaffold for elastic fiber formation. This function was based on morphological studies of developing elastic tissues, which documented that microfibrils appeared first in the embryo, followed by the deposition of amorphous elastin onto the microfibril scaffold (12). Biochemical investigations as well as genetic evidence from both humans and mice have uncovered many more functions of fibrillin microfibrils. Today we know that fibrillin microfibrils perform important tissue-specific architectural functions, beyond serving as scaffolds for elastin deposition. For example, fibrillin microfibrils are specifically required for the structural integrity of both the aortic wall (which contains elastin) and the suspensory ligament of the lens (which does not contain elastin). In addition, over the last decade, a novel and highly significant function of fibrillin microfibrils has emerged: fibrillin microfibrils target and sequester members of the TGF $\beta$  superfamily of growth factors. Because this superfamily of growth factors includes more than 30 different members, this function diversifies the biological roles performed by fibrillin microfibrils, even though the microfibrils themselves are ubiquitous elements of all connective tissues. Using tissue-specific architectures, fibrillin microfibrils pattern the targeting and sequestration of a variety of growth factors and contribute to organ formation and repair. In this manner, the structures of fibrillin microfibrils collaborate with biological functions to shape and maintain connective tissues, and mutations in fibrillins exert powerful, even opposing, forces on tissue growth and homeostasis.

## 1.2 The Discovery of Fibrillin, the Major Structural Component of Microfibrils

In the early 1980's, the era of protein discovery was in full swing. Monoclonal antibody technology, developed in 1975 by Köhler and Milstein (who shared the 1984 Nobel Prize), was employed to generate specific antibodies that yielded novel immunofluorescence staining patterns on human fetal membrane (amnion and chorion) and skin sections. In a large screen of antibody clones, two yielded patterns suggestive of the microfibrillar component of elastic fibers. These were selected and used to immunoprecipitate a large intra-chain disulfide bonded molecule from the medium of cultured human fibroblasts. The molecule was named "fibrillin" because electron microscopic immunolocalization experiments demonstrated periodic labeling along the lengths of 10 nm diameter microfibrils (13). Monomeric fibrillin molecules, purified from fibroblast medium, were shown to be flexible extended strings with lengths of 148 nm and diameters of 2.2 nm (Figure 2) (14).

After rotary shadowing and electron microscopy, microfibrils extracted from tissues were visualized as "beaded strings" (Figure 2) (15). These images suggested that monomeric fibrillin molecules likely contribute to the many strings present in the beaded string structure. Based on epitope mapping studies, it was proposed that single molecules of fibrillin-1 are organized in a parallel, head-to-tail fashion within individual microfibrils (16). Eight fibrillin molecules were estimated in each individual microfibril (17).

Since monomeric fibrillin molecules assembled very quickly into disulfide-bonded aggregates (18), it is possible that fibrillin also contributes to the globular bead structure. However, other proteins, particularly small globular molecules like MAGP, might contribute to the bead. Immunolocalization of MAGP was shown to be periodic along the length of individual microfibrils, suggesting that it might also be a structural component of the microfibril (19). Extraction of connective tissue microfibrils with collagenase resulted in extended periods between beads (17). These extended lengths were sometimes greater than the length of single fibrillin molecules. Therefore, molecules in addition to fibrillins may be required to form the backbone structure of microfibrils. Alternatively, a model of staggered fibrillin molecules folding back on themselves was proposed to fit the ultrastructural images (17). However, collagenase cleaved fibrillin molecules at specific sites, and extraction of tissue microfibrils with guanidine yielded microfibrils with much shorter periods (20), suggesting that the long periods seen between beads in collagenase digested microfibrils were due to cleavage events rather than to unfolding or an unwinding of the microfibril structure. Together with additional antibody epitope mapping, a working model with fibrillin-1 molecules arranged head-to-tail and staggered in the beaded string microfibril was proposed (20). Figure 3A depicts this model.

## 1.3 The Fibrillin Gene Family

Fibrillin was first cloned from human placental cDNA libraries using a mixed pool of oligonucleotides representing all coding possibilities for a fibrillin peptide sequence (CEDIDEC) (21). Fibrillin peptide sequences were determined by amino acid sequencing of pepsin-resistant peptides extracted from human tissues and identified using fibrillin monoclonal antibodies (22). The complete deduced amino acid sequence revealed a modular domain structure consisting primarily of Epidermal Growth Factor (EGF) -like domains

(with 6 cysteines per domain) and novel domains containing 8-cysteines (21,23). Each fibrillin molecule is composed of 47 EGF-like domains, 43 of which are predicted to bind calcium (cbEGF), 7 8-cysteine containing domains (8-cys), 2 “hybrid” domains that share features of both the 8-cysteine domain and the EGF-like domain, a proline-rich domain, and amino- and carboxyl- terminal domains (Figure 4).

Cloning methods led to the discovery of a second fibrillin (24). It turned out that the monoclonal antibodies that were used to first characterize and clone fibrillin had identified fibrillin-1 (21,22). Expression of the gene for fibrillin-2 was largely limited to fetal development (25). Both fibrillin-1 and fibrillin-2 molecules were present within individual fetal microfibrils, indicating that fibrillin microfibrils are heteropolymers (26). Fibrillin-2 molecules were found in postnatal tissues but epitopes were masked by fibrillin-1 (27), suggesting that mature microfibril structure involves the accretion of fibrillin-1 molecules around a core of fibrillin-2 molecules that were assembled during fetal development. Mutations in *FBN2* were discovered to cause congenital contractural arachnodactyly (now called Distal Arthrogyrosis, Type 9). These mutations clustered between exons 23 and 34 (28).

A third fibrillin, fibrillin-3, was also found by cloning methods (29,30). Expression of *FBN3* was also limited to fetal development, and fibrillin-3 was immunolocalized to fetal microfibrils (30). All three fibrillins share the same overall organization of modular domains (Figure 4). However, in place of the proline-rich domain in fibrillin-1, there is a glycine-rich domain in fibrillin-2 and a proline- and glycine-rich domain in fibrillin-3. Specific functions for these distinctive domains are not known. The second calcium-binding EGF-like domain is missing in fibrillin-3. In mouse, the gene for fibrillin-3 was inactivated during evolution.

Figure 4 depicts the molecular structures of the three fibrillins and the LTBP, molecules structurally and functionally related to fibrillins. There are 4 LTBP, and all four are composed of domain modules found in fibrillins. The 8-cysteine module is present only in the LTBP and in the fibrillins. Interestingly, the 8-cysteine module in LTBP is used to form covalent bonds with the small latent complex of TGF $\beta$  (31,32). Unlike the fibrillins, which are restricted to a very similar size, the LTBP vary in size and are smaller than fibrillin. LTBP have been immunolocalized to microfibrils (5,6), and LTBP-1, -2 and -4 bind directly to fibrillin (6,7,33). Periodic localization of LTBP along the lengths of microfibrils has not been reported. Furthermore, because the LTBP are smaller than fibrillins and variable in size, it seems unlikely that these molecules contribute in the same way as fibrillins to the backbone beaded string structure of microfibrils.

## 2. The Marfan Syndrome

The Marfan syndrome is a relatively common (1 case in every 3–5,000 people) dominantly inherited disorder of connective tissue with variable clinical features in the musculoskeletal, cardiovascular and ocular systems (34). Individuals with the Marfan syndrome are usually very tall with long limbs, long face, and long fingers and toes, hypomusculature, and chest, spine, hip, and foot deformities. A cardinal and potentially life-threatening aspect of Marfan syndrome is aortic root aneurysm with subsequent dissection and rupture. Other important

clinical features include mitral valve prolapse, pneumothorax, dural ectasia, and myopia. Tissues affected in the Marfan syndrome (the aorta, musculoskeletal tissues, the dura, lung, and cornea) are fibrillin-rich.

Ectopia lentis is another cardinal characteristic of the Marfan syndrome, present in about 60% of patients. It is caused by a weakness of the ciliary zonule (the suspensory ligament of the lens). Immunolocalization of fibrillin to the ciliary zonule (13) prompted interest in fibrillin as the candidate gene for Marfan syndrome. Screening of skin biopsies and cultured fibroblasts demonstrated that fibrillin immunostaining patterns could distinguish Marfan samples from control samples (35), supporting the candidacy of fibrillin as the Marfan gene. At the same time, genetic mapping of the Marfan gene locus identified a region on chromosome 15 (36). Very quickly thereafter, once the gene for fibrillin-1 had been cloned (21), it was mapped to chromosome 15,q21.1 (37), and the first mutation in a patient with Marfan syndrome was identified (38). Today, identification of a mutation in *FBNI* is not mandatory for the diagnosis of Marfan syndrome, but a mutation can be very useful in cases of incomplete phenotypes and for subsequent screening of family members.

## 2.1 *FBNI* Mutations in Marfan syndrome

Since the identification of *FBNI* as the causal gene for Marfan syndrome, 1847 different mutations in 3044 DNA samples have been reported, according to the currently most extensive *FBNI* mutation database (<http://www.umd.be/FBNI/>- last updated 8/28/2014). Mutations are found throughout the entire length of the gene, and only 12% of all reported *FBNI* mutations are recurrent (39). It is likely that the numbers of known mutations are actually much larger, since not all *FBNI* mutations that have been identified worldwide have been reported or entered into this database. Penetrance of *FBNI* mutations is extremely high, but age-dependent, and no example of nonpenetrance has been documented in hundreds of pedigrees (40). About 25% of cases are caused by new or spontaneous mutations arising during zygote formation. Gonadal mosaicism has been reported rarely in Marfan syndrome (41,42). Paternal age is advanced (36 versus 29 years) at the time of conception of isolated cases, consistent with the knowledge that a new mutation in a spermatogonium is a frequent cause of sporadic Marfan syndrome (43).

Mutations in the *FBNI* gene include many different types (44). Missense mutations are most frequently encountered (about 2/3 of cases). These commonly substitute cysteine residues that form disulfide bonds within one of the cbEGF or 8-Cys domains, but missense mutations creating novel cysteine residues in these modules are also common. About one quarter of missense mutations affect modules other than cbEGF domains. Small insertions, deletions, or duplications represent around 10–15% of all reported mutations, the majority of which will create a premature termination codon (PTC). Another 10–15% of reported mutations consist of various classes of splicing errors, most commonly affecting canonical splice sequences at exon/intron boundaries. Many *FBNI* splice site mutations result in in-frame exon skipping, such that the mutant fibrillin-1 lacks an entire cbEGF domain. Some exon-skipping mutations in *FBNI* result in a frameshift and reduced mutant RNA levels caused by nonsense-mediated decay of the mutant transcript (45,46). Larger rearrangements, including both deletions and insertions, have been reported in a minority of Marfan patients

(47–50); entire gene deletions are rare (51). In view of this large array of types of mutations found in *FBNI*, mutation screening of the *FBNI* gene should not only include regular techniques for the detection of exonic mutations, but should also be supplemented by techniques for the detection of large deletions/insertions (for example, multiplex ligation-dependent probe amplification, MLPA) (52) and even for deep-intronic mutations (cDNA analysis, whole-genome sequencing) (53).

## 2.2 Genotype-Phenotype Correlations in Marfan Syndrome

Genotype-phenotype correlations have been explored, in order to investigate whether certain types of mutations in *FBNI* are associated with specific clinical features of Marfan syndrome. These efforts have been made difficult by the known clinical variability in Marfan syndrome. Both inter-familial and intra-familial variability is well appreciated in Marfan syndrome, such that the established marked phenotypic variability among individuals carrying the same mutation tends to blur distinctions between different mutations (54). Other factors to explain the high clinical variability in Marfan syndrome include the level of expression of the normal *FBNI* allele (55,56) and the role of hyperhomocysteinemia (57). It is likely that genetic modifiers, or epigenetic and environmental factors also play important roles in clinical variability in Marfan syndrome.

Table 1 provides an overview of the main genotype-phenotype correlations that have been reported in studies of large numbers of subjects. Case reports are not included in this list.

Ectopia lentis (EL) has been reported more frequently in cysteine missense mutations (58,61). In patients diagnosed by age 25, EL was present in 59% for those with a missense mutation involving a cysteine, compared with 32% for those with other missense mutations (61). In contrast, EL in patients diagnosed by age 25 was 23% for those with a mutation leading to a PTC, compared with 50% for those with inframe mutations (61). Isolated EL has been associated with mutations located in the first 15 exons of the gene (60,63). This latter association is however only significant if young patients are included, so it cannot be excluded that some of the young EL patients might develop additional features of Marfan syndrome in their adult life, necessitating reclassification out of the EL group. A recent review of all published cases over the last 20 years indicated that 57/123 (46.3%) should now be classified as Marfan, according to the revised Ghent nosology, since 37/96 mutations (38.5%) reported to cause isolated EL have also been found in patients with aortic dilation/dissection (65). Based on these findings, it was suggested that EL caused by mutations in *FBNI* is part of a spectrum of Marfan syndrome, and the term “Isolated Ectopia Lentis” should be avoided in these cases (65).

A similar distribution, but at the 3' end of the *Fbn1* gene, was observed in patients presenting an “incomplete Marfan” phenotype, with a statistically significant clustering of their mutations in exons 59–65, whether or not patients under 18 were included (63). The “incomplete” Marfan group did not fulfill the criteria for a diagnosis of classic Marfan syndrome, suggesting that this group might represent a milder phenotype. However, it should be noted that the association of milder cardiovascular features with exons 59–65 was present, but not significant (63). Previously, it was suggested that mutations in this region

was associated with mild phenotypes lacking significant aortic pathology (66), but numbers in this earlier study were small.

The most consistent genotype-phenotype correlation that has emerged is the association of mutations in the middle region of the gene (exons 24–32) with all cases of “neonatal” Marfan syndrome and with other severe forms of Marfan syndrome (58,61,62). Even if the mutation does not result in lethal neonatal Marfan syndrome, the presence of a mutation occurring in this region of the gene appears to be the best indicator of early onset aortic risk (61).

Recently, truncating and splicing mutations were found to occur more frequently in Marfan patients who have had aortic events (64). A higher frequency of truncating or splicing mutations (79%) were found in patients who had had aortic events than in those without an aortic event (39%), and those who had an aortic event before age 30 (100%) or before age 40 (95%) had truncating or splicing mutations (64). A trend toward higher frequency of truncating or splicing mutations was found in those who had dissections compared to those who had prophylactic surgery. The latter observation was also previously reported (59). Similarly, it was previously suggested that splicing mutations resulting in exon skipping of a cbEGF domain can be associated with particularly severe phenotypes, including lethal neonatal Marfan syndrome (4 of 11 cases) or with aortic involvement early in life (67).

Nonsense, frameshift, and splicing mutations were considerably underrepresented (27.8%) in exons 24–32, compared to all other exons (44.4%), and nonsense mutations were never reported in cases of neonatal Marfan syndrome (61). Therefore, the collective data indicate that a higher risk for an early aortic event is associated with mutations in exons 24–32 (61) and with truncating and splicing mutations (64). Nevertheless, in spite of these genotype-phenotype correlations, it is fair to say that we are not able to predict the phenotype for any given *FBNI* mutation.

### 3. *FBNI* Mutations in Other Disorders

#### 3.1 Marfan-related Disorders

In addition to the interfamilial, as well as intrafamilial, clinical variability known in Marfan syndrome, there is extensive clinical variability in individuals harboring mutations in the *FBNI* gene that do not give rise to Marfan syndrome. For the Marfan syndrome, the clinical spectrum ranges from mild (“incomplete” Marfan syndrome) to severe disease (lethal neonatal Marfan syndrome). For other disorders, mutations in *FBNI* have been reported in patients with mild or isolated Marfan-related phenotypes including MASS phenotype (myopia, mitral valve prolapse, mild aortic dilatation, skeletal features, skin striae) (68), familial ectopia lentis, familial kyphoscoliosis (69), adolescent idiopathic scoliosis (70), and in familial thoracic aortic aneurysms (71–73).

Familial thoracic aortic aneurysms or heritable thoracic aortic disorders (H-TAD) can present with syndromic or isolated (nonsyndromic) clinical manifestations. The prototype for syndromic disease is the Marfan syndrome. Other forms of syndromic H-TAD include Loeys-Dietz syndrome (caused by mutations in the receptors for TGF $\beta$ , *TGFBR1* and

*TGFBR2*) and aneurysm-osteoarthritis syndrome (caused by mutations in *SMAD3*, an intracellular component of the TGF $\beta$  signaling pathway). Clinical diagnosis of these syndromes has been difficult due to overlapping manifestations. Gene panel screening of individuals with H-TAD demonstrates clinical overlap in patients diagnosed with Marfan syndrome: in a study of 175 patient samples, 19 patients clinically diagnosed with Marfan syndrome yielded 10 pathogenic mutations in *FBNI* and 1 in *TGFBR1* and variants of unknown significance in *FBN2*, *MYH11*, *MLK*, *SMAD3*, and *COL3A1*; 3 patients clinically diagnosed with Loeys-Dietz syndrome yielded 1 pathogenic mutation in *FBNI* and variants of unknown significance in *FBNI* and *SMAD3* (73). Gene panel screening of 233 TAD patient samples, in which 19 patients were clinically diagnosed with Marfan syndrome but had no mutation in *FBNI*, *SMAD3* mutations were revealed in 6 patients and 1 in *TGFB2* (72).

These emerging data demonstrate the difficult clinical diagnosis of syndromic forms of H-TAD. Gene screening for nonsyndromic forms of TAD also demonstrate difficulties in clinical diagnosis. *FBNI* mutations were revealed in 12 patients who were not clinically diagnosed with Marfan syndrome, but who had some Marfanoid features; some Marfanoid features were also present in TAD patients in whom *SMAD3* and *TGFB2* mutations were detected (72). Recently, the frequency of *FBNI* mutations causing H-TAD was estimated to be about 3% (5/183) (74). None of these affected individuals fulfilled the clinical criteria of Marfan syndrome, however, two of the *FBNI* mutations identified in these H-TAD patients had been previously reported in patients with Marfan syndrome.

### 3.2 Acromelic Dysplasias

Since clinical manifestations of Marfan syndrome are pleiotropic, it may not be surprising that mutations in *FBNI* were found in nonsyndromic Marfan-related disorders. It was surprising, however, when mutations in *FBNI* were found to cause acromelic dysplasias, which manifest short stature, short hands and feet, stiff joints, and a hypermuscular build. Acromelic dysplasias include Weill-Marchesani syndrome, geleophysic dysplasia, acromicric dysplasia, and Myhre syndrome (75). McKusick described Weill-Marchesani syndrome as the “opposite” of Marfan syndrome (76). Although ectopia lentis is common to both Marfan syndrome and Weill-Marchesani syndrome, individuals with Marfan syndrome normally present with tall stature, arachnodactyly, hypermobile joints and a thin hypomuscular build. Mutations in *FBNI* were first discovered in autosomal dominant Weill-Marchesani (77,78) and then in geleophysic and acromicric dysplasias (79).

Mutations in *FBNI* that underlie the acromelic dysplasias are predominantly limited to a hot spot in fibrillin-1. An in-frame deletion of 24 nucleotides in exon 41 causes Weill-Marchesani syndrome (77), and missense mutations in exons 41 and 42 cause geleophysic dysplasia and acromicric dysplasia (79). Exons 41 and 42 encode the 5<sup>th</sup> 8-cysteine domain in fibrillin-1. A second mutation causing Weill-Marchesani syndrome was found to be an in-frame deletion of exons 9–11, encoding the first 8-cysteine domain, the proline-rich region and the fourth EGF-like domain (78). While this mutation is distinct from the other acromelic dysplasia mutations which are all in the 5<sup>th</sup> 8-cysteine domain, it was proposed that the three-dimensional structure of fibrillin molecules within a microfibril may create a



microenvironment consisting of these domains in close proximity (78). Figure 3B maps the proximity of domains in fibrillin-1 that cause acromelic dysplasias.

### 3.3 “Stiff Skin Syndrome”

Mutations in *FBNI* causing “stiff skin syndrome” occur in the 4<sup>th</sup> 8-cysteine domain (80). This domain contains the single Arginine-Glycine-Aspartic Acid (RGD) sequence present in fibrillin-1. Loss of integrin binding to this site is implicated as the cause of stiff skin syndrome, since a mouse in which the RGD site is mutated to RGE develops stiff skin (81). The 4<sup>th</sup> 8-cysteine domain is predicted to be in close proximity to the microenvironment formed by the domains which contain the known mutations for the acromelic dysplasias (see Figure 3B). Interestingly, one of the clinical features of Weill-Marchesani syndrome and geleophysic dysplasia is thick skin. Therefore, it is possible that any of these mutations within this microenvironment may perturb integrin binding to fibrillin-1, leading to thick or very stiff skin.

### 3.4 Progeroid Fibrillinopathies

Mutations in *FBNI* causing progeroid fibrillinopathies cluster in a region encoding the C-terminus of fibrillin-1 (82). Clinical manifestations resembled those in Marfan syndrome, but in addition, patients presented with a neonatal progeroid appearance. Five mutations involving nucleotide deletions or insertions and one missense mutation were described.

## 4. Mouse Models

There are multiple mouse models in which mutations have been generated in mouse *Fbn1* by homologous recombination. These mouse models include a complete *Fbn1* null, a homozygous hypomorph (mgR/mgR), several deletions (mg /+; GT-8/+; WM /+; H1 /+), and several missense mutations (C1039G/+; W1572C/+; D1545E/+). Table 2 summarizes the phenotypes of these mouse models.

It was known in 1991 that the Marfan gene is *FBNI*. Therefore, initial experiments were directed toward generating a mouse model of Marfan syndrome. The first attempt resulted in two hypomorphic mouse models, in which phenotypes were only revealed in homozygosity (mg /mg and mgR/mgR) (83,84). These mice were phenotypically normal in heterozygosity (mg /+ and mgR/+). The deletion of exons 19–24, originally called mg (83), was re-engineered in order to remove the *neo* gene which had caused decreased expression of *Fbn1* in the original mg and mgR mice. The re-engineered mg loxPneo mouse, in heterozygosity, displayed phenotypes of Marfan syndrome (85). Heterozygous C1039G/+ (86) and GT-8/+ (87) also demonstrated features of Marfan syndrome.

Homozygous C1039G and homozygous GT-8, like homozygous null mice (88), die in the early postnatal period. In humans, only three cases of compound heterozygosity at the *FBNI* locus have been reported. In one case of compound heterozygosity (W217G/G2627R), the affected child had a severe phenotype leading to early death (89). Compound heterozygous probands (R2726W/C1928S in one family and R240H/c.3861delC in a second family) consistently presented with more severe phenotypes compared to their heterozygous family members (90). In these two families, the mutations R2726W and R240H are not considered

pathogenic by themselves, but in combination with a second pathogenic mutation, these mutations were not neutral, suggesting the possibility that certain trans-located *FBNI* mutations might act as intrafamilial modifiers of phenotype (90). A single allele of *FBNI* with two mutations (I1071S and E1073D) was reported in an infant with neonatal Marfan syndrome (91). The homozygous mutations in mouse *Fbn1* leading to early postnatal death are not equivalent to these compound mutations in humans. Although early death of homozygous *Fbn1* mouse mutants have been suggested to model neonatal Marfan syndrome, clearly these homozygous mutants are not equivalent to the heterozygous *FBNI* mutations leading to neonatal Marfan syndrome and early lethality in humans.

In contrast to the first *Fbn1* mutant mouse models, which showed signs of Marfan syndrome and died in homozygosity, homozygous H1 (87) and homozygous WM (78) lived long lives. This was somewhat surprising at the time. However, it is now clear in both mouse and human that not all fibrillin-1 mutations result in Marfan syndrome and not all homozygous mouse mutations lead to early death. The WM *Fbn1* mouse was generated in order to test whether the mutation found in a human family with Weill-Marchesani syndrome would display features of Weill-Marchesani syndrome or Marfan syndrome (78). The WM *Fbn1* mice, in both heterozygosity and homozygosity, replicated features (thick skin and short bones) of Weill-Marchesani syndrome and did not show any signs of aortic disease (78). Similarly, heterozygous W1570C/+ mice phenocopied stiff skin syndrome, and homozygous W1507C mice showed accelerated skin fibrosis but remained viable (81).

Heterozygous RGE mice (D1545E/+) also phenocopied stiff skin syndrome, but homozygous RGE mice were embryonic lethal before E10.5 (81). This finding suggests that, in homozygosity, RGE fibrillin-1 acts differently than W1570C fibrillin-1. RGE fibrillin-1 must significantly perturb important interactions with molecules in addition to integrins. Since the complete *Fbn1* null mouse dies around P14 (88), loss of fibrillin-1 binding to integrins is compatible with life until that time. Mechanisms to explain the early embryonic lethality (a “gain of function” phenotype) of homozygous RGE mice are unknown. It is interesting that one allele of *tight skin (Tsk) Fbn1* results in a scleroderma-like phenotype, but homozygous *tsk* mice also die during early embryonic development (92). In *Tsk* fibrillin-1, the RGD site is duplicated. Functional differences between W1570C, RGE, and *tsk* fibrillin-1 clearly exist, but the molecular bases for these differences are not understood.

## 5. Fibrillin, Growth Factors, and Genetic Pathways

The fibrillins and the LTBP are composed of very similar domain modules. The 8-cysteine domain, which is used by LTBP to bind covalently to the propeptide of TGFβs (31,32), is present in only 7 proteins in humans: three fibrillins and 4 LTBP. Fibrillins do not bind to TGFβ propeptides (93). However, fibrillins do bind to propeptides of BMPs and GDFs. So far, biochemical studies have demonstrated interactions between fibrillins and the propeptides of BMP-2, -4, -5, -7 and GDF-5, but not the propeptide of GDF-8, which binds to a glycosaminoglycan side chain present on perlecan (94–96).

Extracellular regulation of TGFβ super-family members involves interactions between propeptides of growth factor complexes and fibrillin microfibril networks. Figure 3C shows

binding sites in fibrillins for pro-BMP complexes, for LTBP, and for perlecan/GDF-8. The functions of these interactions include targeting of growth factors to appropriate locations in specific tissues and sequestration of latent growth factor complexes. In addition, for TGF $\beta$  complexes, LTBP facilitates the correct folding of TGF $\beta$  (functioning as intracellular chaperones) and perform roles in the activation of latent TGF $\beta$  (97). Less is known about the function of interactions between fibrillins and propeptides of BMPs and GDFs. Biochemical investigations of pro-BMP-7 (98) and comparison of the structures of pro-TGF $\beta$  and pro-BMP-9 (99) demonstrate that, unlike pro-TGF $\beta$ , pro-BMP-7 and -9 are not latent. Therefore, the functions of interactions between fibrillins and pro-BMPs may be somewhat different from the functions of LTBP interactions with pro-TGF $\beta$ . Recently, abnormal activation of BMP signaling was found in limb skeletal muscle in *Fbn2* null mice, and loss of inhibition of BMP signaling normally conferred by interaction of fibrillin with pro-BMP was suggested as a potential mechanism for this abnormal activation (100). However, these and other studies of fibrillin or LTBP function in mice are complicated because of the overlapping functions of fibrillin-1 and -2 and of the LTBP *in vivo*. Moreover, teasing out regulation of growth factor signaling from the structural functions of these microfibrillar proteins is not an easy task.

LTBP binds directly to fibrillins, presumably targeting large latent TGF $\beta$  complexes to fibrillin microfibrils (6,7). Therefore, when TGF $\beta$  signaling was found to be abnormally activated in the lungs of *Fbn1* mg /mg mice (101), it was hypothesized that loss of fibrillin-1 in this severely hypomorphic mouse model might result in a specific failure to target and sequester large latent TGF $\beta$  complexes, which would then lead to abnormally activated TGF $\beta$  signaling. Abnormal activation of TGF $\beta$  signaling was also observed in *Fbn1* C1039G/+ aorta (102) and skeletal muscle (103). At the same time, mutations in a gene encoding a receptor for TGF $\beta$  (*TGFBR2*) were identified, and *TGFBR2* was suggested to be a second locus for Marfan syndrome (104). Mutations in *TGFBR1* and *TGFBR2* were subsequently reclassified as a new syndrome, which was named Loeys-Dietz syndrome (105). Originally, *TGFBR2* mutation constructs were shown to result in reduced TGF $\beta$  signaling in luciferase reporter assays (104). However, examination of cells and tissues from individuals with mutations in TGF $\beta$  receptors showed evidence of increased, rather than decreased, TGF $\beta$  signaling (105). All together, these results led to the concept that abnormally activated TGF $\beta$  signaling is the main driver of pathogenesis in Marfan syndrome and related disorders (106). Clinical trials of losartan, an angiotensin II type I receptor blocker, which also inhibited TGF $\beta$  signaling and rescued aortic disease in *Fbn1* C1039G mice (102), were carried out in children (107) and adults (108) with Marfan syndrome. Results from multiple other trials are currently being reported.

Currently, the concept that abnormally activated TGF $\beta$  signaling causes Marfan syndrome and related disorders like Loeys-Dietz syndrome is not so clear. It is clear, however, that *FBN1* and components of the TGF $\beta$  signaling pathway (*TGFBR1* and *TGFBR2* (104,105), *SMAD3* (109), *TGFB2* (110,111), and *TGFB3* (112)) share similar genetic pathways. Thoracic aortic aneurysm and dissection can be the common result from a mutation in any of these genes. However, recent findings that mutations in the ligands, TGF $\beta$ -2 and TGF $\beta$ -3, are likely to be loss-of-function mutations, together with the original proposal that mutations in *TGFBR2* were loss-of-function mutations (104), now suggest that the role of TGF $\beta$

signaling in H-TAD is “paradoxical” (111,112). The “paradox” is that loss-of-function mutations co-exist with evidence for abnormal activation of TGF $\beta$  signaling. This evidence consists primarily of immunocytochemical staining for phosphorylated Smad2/3 (102,105,111,112), a reporter for intracellular signaling initiated by TGF $\beta$ .

Proof, however, that abnormal activation of TGF $\beta$  signaling is the main driver of pathogenesis in Marfan syndrome did not come simply from reporters showing activation of intracellular TGF $\beta$  signaling pathways. Proof came from the rescue of aortic disease in Marfan mice after administration of TGF $\beta$  neutralizing antibodies (102). More recent studies in mice demonstrated that inhibiting TGF $\beta$  signaling by either TGF $\beta$  neutralizing antibodies (113) or by genetic ablation of *Tgfr2* (114) can cause severe aortic disease, consistent with mutations in TGF $\beta$  receptors and ligands causing H-TAD through loss-of-function mechanisms.

Instead of performing a primary role in pathogenesis, activated TGF $\beta$  signaling in Marfan syndrome is likely a compensatory response by aortic wall cells to the altered fibrillin extracellular matrix. TGF $\beta$  is a context-dependent “switch” that provides mechanisms whereby a cell can respond appropriately to its environment (115). As a switch, TGF $\beta$  can be either activating or inhibiting; stimulating or repressing; promoting or preventing, depending on the context. Biochemical and genetic studies establish a role for fibrillin in determining the extracellular context for TGF $\beta$  signaling.

Genetic studies implicate the structure of fibrillin microfibrils as an important determinant in clinical phenotypes. Fibrillin-1 and fibrillin-2 perform roles in genetic pathways that control musculoskeletal phenotypes such as joint mobility, arachnodactyly, and muscularity. Mutations in *FBN1* and or *FBN2* result in arachnodactyly and hypomuscularity, features of Marfan syndrome and congenital contractural arachnodactyly. However, mutations in *FBN1* usually cause joint hypermobility, while mutations in *FBN2* are associated with contractures of the small and large joints. *FBN1* mutations that cause Weill-Marchesani syndrome result in stiff joints. In Weill-Marchesani syndrome, unusual aggregates of fibrillin microfibrils were associated with thick skin (78). Similarly, aggregated masses of fibrillin microfibrils were found in skin samples from patients with stiff skin syndrome (80). These unusual structures of fibrillin microfibrils contrast with apparently normal microfibrils which undergo gradual degradation in skin from human or mouse Marfan syndrome (35,87). Proteolysis of fibrillin microfibrils in patients with thoracic aortic aneurysm and dissection is supported by the presence of circulating fibrillin-1 fragments in these individuals compared with controls (116). The effects of mutations in fibrillin-2 on the fine structure of fibrillin microfibrils are currently unknown.

An emerging genetic pathway involves *FBN1* and genes that encode members of the Adams and Adamslike family of proteins. In addition to dominant Weill-Marchesani syndrome, caused by mutations in *FBN1*, recessive Weill-Marchesani syndrome is caused by mutations in *ADAMTS10* (117). Clinical features of both the dominant and recessive forms are similar. Related acromelic dysplasias are associated with mutations in *FBN1* (acromelic dysplasia and geleophysic dysplasia) (79) and in *ADAMTS2* (geleophysic dysplasia) (118). A Weill-Marchesani related syndrome is caused by mutations in *ADAMTS17* (119), and mutations

in *ADAMTSL4* have been reported in autosomal recessive isolated ectopia lentis (120), a clinical feature common to Marfan syndrome and Weill-Marchesani syndrome.

Adamtslike proteins and Adamts enzymes have been shown to bind to fibrillin and to promote fibrillin fibril formation *in vitro* (8–11). Therefore, it might have been expected that mutations in the genes encoding these molecules would result in pathologies similar to the fibrillinopathies. However, this is a large family of molecules, and the fibrillinopathies are diverse. Genetic pathways provide invaluable information. In the cases known to date, mutations in *ADAMTSL* and *ADAMTS* genes result in phenotypes related primarily to the fibrillinopathy, Weill-Marchesani syndrome, rather than to Marfan syndrome. Mutations in specific members of the *ADAMTSL* and *ADAMTS* family and the resulting clinical features may be the combined results of effects on the fine structure of fibrillin microfibrils as well as tissue-specific gene expression. The fine structure of fibrillin microfibrils, as seen in Weill-Marchesani syndrome (78), may be common to the Weill-Marchesani-related disorders and may be the result of defects in Adamtslike-Adamts modifications on fibrillin fibril formation.

It is very interesting that Myhre syndrome, a syndrome related to the acromelic dysplasias, is caused by heterozygous mutations in *SMAD4* (121). Smad-4 partners with intracellular signaling molecules (Smad-2, -3 or Smad-1, -5, -8) to transduce signals initiated by TGF $\beta$ s or by BMPs. Therefore, the finding that mutations in *SMAD4* cause a syndrome related to the acromelic dysplasias implicates growth factor signaling in these related disorders. However, it should also be noted that heterozygous mutations in *SMAD4* cause juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome. Differences between these syndromes may be due to gain-of-function mutations associated with Myhre syndrome and loss-of-function mutations associated with juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome.

## 6. Future Directions: Cellular Interactions with Fibrillin-1

Biochemical and genetic evidence demonstrate important roles for fibrillin-1. In this review, we have underscored collaborative interactions between the architectural and biological functions of fibrillin-1. From this perspective of collaborative interactions, roles for the cell have not received sufficient attention. It is the cell that assembles fibrillin microfibrils during growth and development and that senses and responds to defects in the fibrillin microenvironment. Moreover, following a fundamental concept elucidated in work by Elizabeth D. Hay (122), the cell's extracellular environment controls cell shape and gene expression. This concept has been recently used to provide a framework unifying the finding that mutations in both intracellular cytoskeletal molecules and extracellular molecules cause thoracic aortic aneurysm and dissection (123,124). The cellular mechanosensor, coordinating the extracellular environment with the intracellular cytoskeleton, and with TGF $\beta$  signaling, in the thoracic aorta is currently unknown. Whether mechanosensing is the job of one receptor or multiple receptors is also unknown. Here genetic evidence would be very informative. Presumably, when mutated, such mechanosensors would cause thoracic aortic disease.

An important role for integrin sensing of fibrillin-1 has been established in skin (80,81). Integrin sensing of fibrillin-1 has also been implicated in the heart, since dilated cardiomyopathy is the sole clinical feature resulting from combined haploinsufficiency of fibrillin-1 and  $\beta 1$  integrins (125). From these data, it seems likely that cellular sensors for fibrillin will vary depending on the tissue and that the cellular sensor for fibrillin-1 in the thoracic aorta may not be an integrin.

As suggested by Jurgen Engel (126), the fibrillin microfibril network may work like a machine to control growth factor signaling. In addition to the microfibrillar molecules, growth factors, inhibitors, and activators that are components of the microfibril network, cellular receptors may perform critical roles in the supramolecular assemblies that are required to make the machine work properly. In order to capture the dynamics of how the fibrillin microfibril machine works, new technological approaches to unraveling microfibril fine structure and microenvironments will be required.

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## List of Abbreviations

<b><i>FBN</i></b>	fibrillin gene
<b>MAGP</b>	microfibril associated glycoprotein
<b>LTBP</b>	latent TGF $\beta$ binding protein
<b>TGF<math>\beta</math></b>	transforming growth factor $\beta$
<b><i>TGFBR</i></b>	TGF $\beta$ receptor gene
<b>EGF</b>	epidermal growth factor
<b>cbEGF</b>	calcium-binding EGF
<b>PTC</b>	premature termination codon
<b>EL</b>	ectopia lentis
<b>H-TAD</b>	heritable thoracic aortic disorders
<b>BMP</b>	bone morphogenetic protein
<b>GDF</b>	growth and differentiation factor

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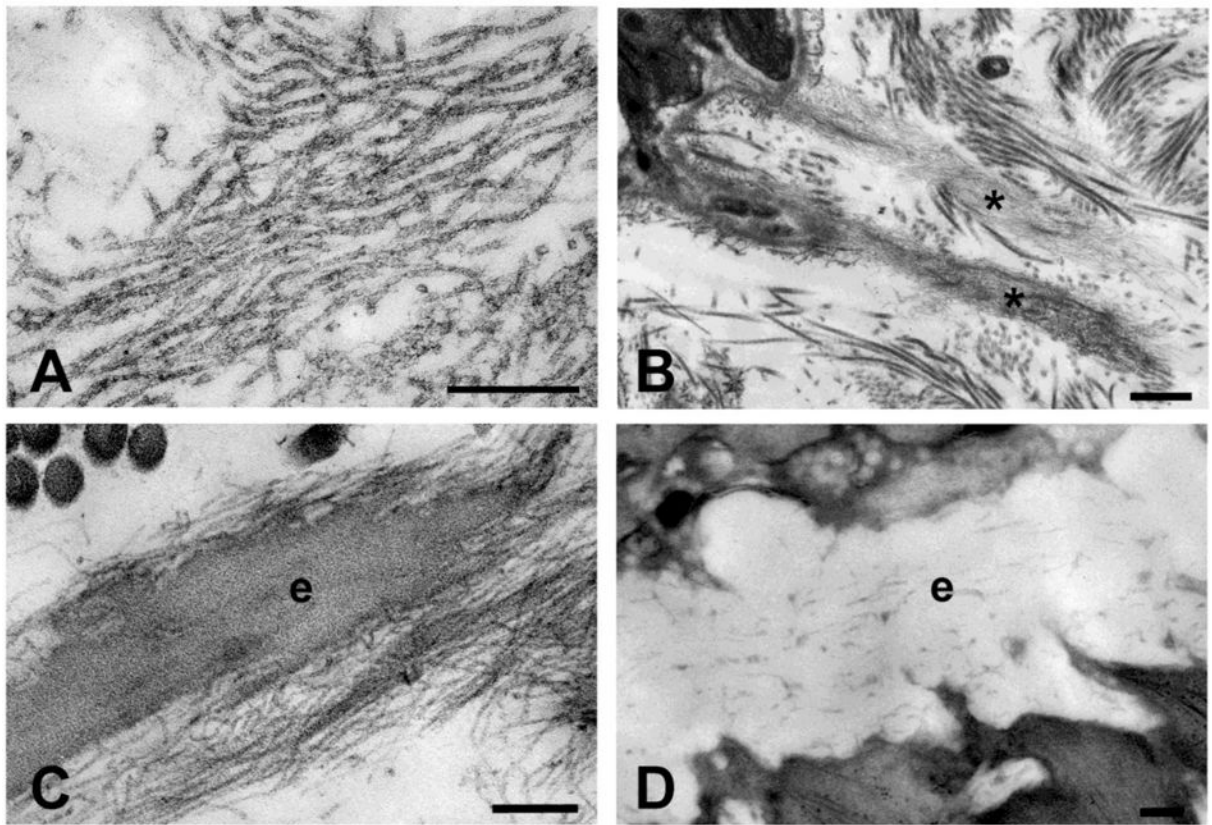
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### Highlights

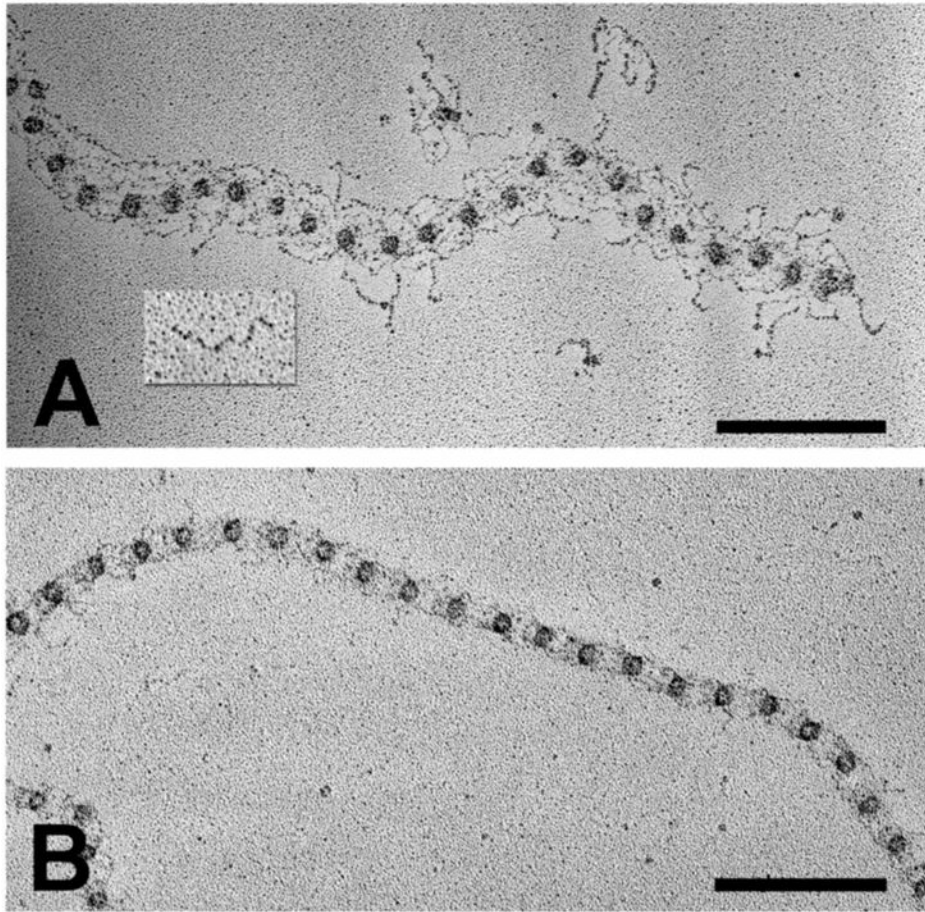
- *FBNI* encodes fibrillin-1, a structural macromolecule for extracellular microfibrils.
- Mutations in *FBNI* cause the Marfan syndrome and related disorders.
- Mutations in *FBNI* also cause acromelic dysplasias and stiff skin syndrome.
- Abnormal growth factor signaling is implicated in these fibrillinopathies.



**Figure 1. Ultrastructure of Fibrillin Microfibrils**

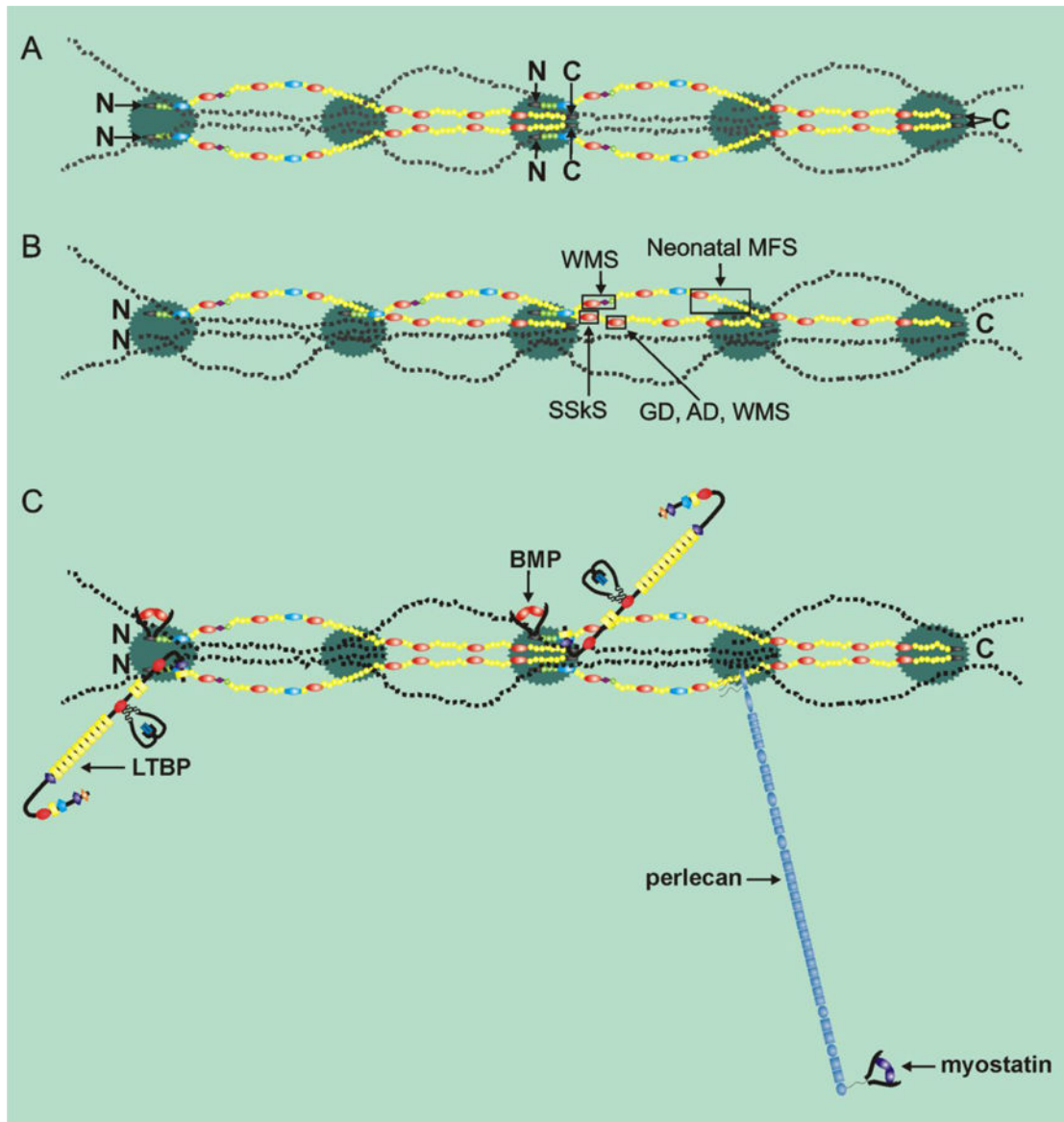
**A.** High magnification images of fibrillin microfibrils in human amnion show fibrils of uniform diameter with alternating hollow and filled (light and dark) regions. **B.** Fibrillin microfibrils exist in bundles (\*), especially in close proximity to basement membranes. Here are two bundles of microfibrils intersecting the lamina densa at the dermal-epidermal junction in human skin. **C.** Fibrillin microfibrils surround amorphous elastin (e) in all elastic fibers. Shown here is an elastic fiber in human skin. **D.** In the aorta, elastic fibers are organized circumferentially in lamellae around the lumen of the vessel. Using high pressure freezing techniques, cell processes are seen directly adjacent to and even within the elastic fiber. In the mouse aorta, microfibrils are barely visible around the amorphous elastin. Scale bars = 200 nm (A,C,D); 500 nm (B).





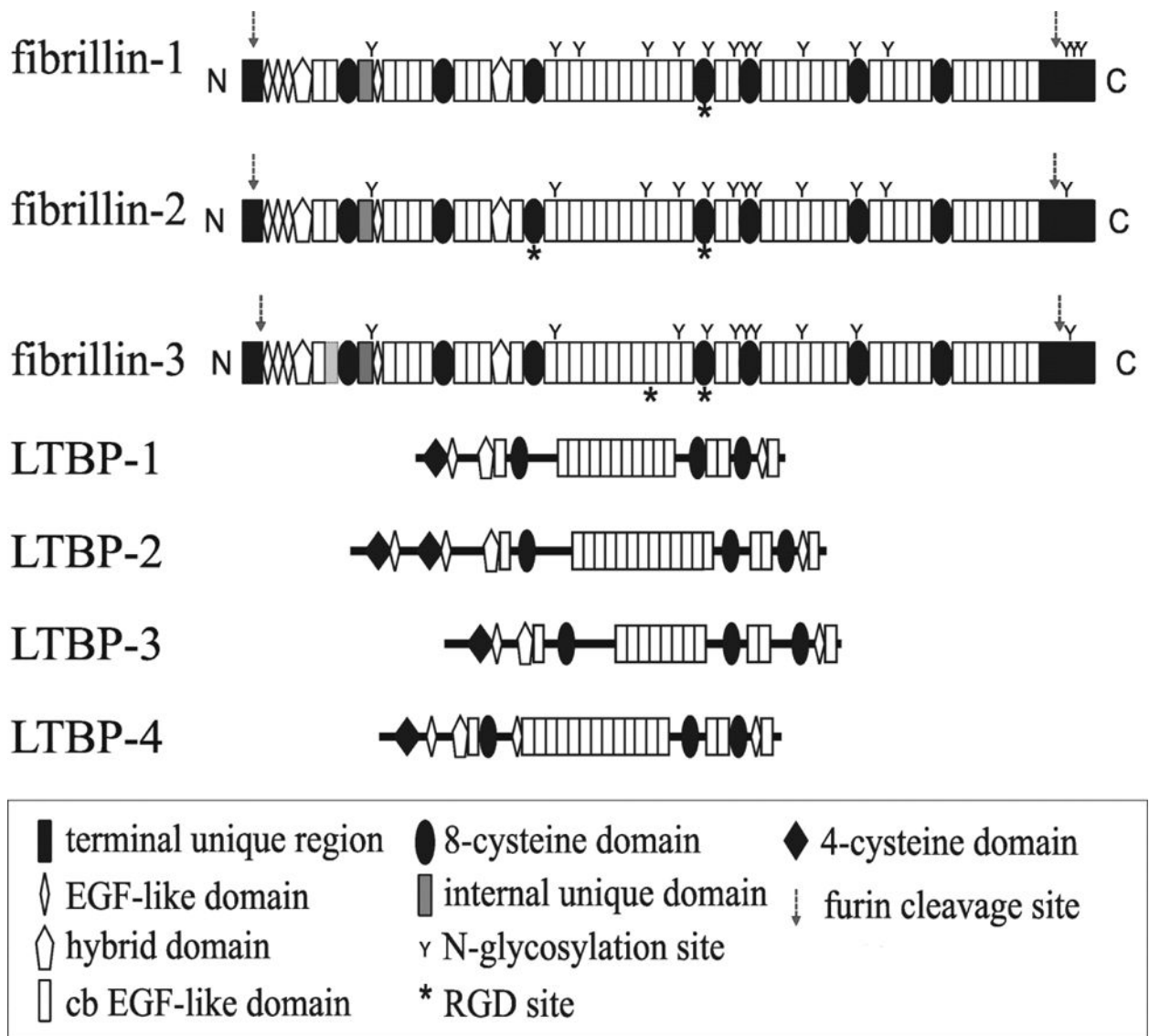
**Figure 2. Rotary-shadowed Images of Fibrillin and Extracted Microfibrils**

Fibrillin microfibrils were extracted from human fetal membranes with guanidine (**A**) or with collagenase (**B**). A fibrillin monomer isolated from the medium of fibroblast cultures is shown as an inset (**A**). Scale bars = 200 nm.



**Figure 3. Model of Fibrillin Molecules Within the Microfibril**

**A.** In this model (taken from reference 20), single fibrillin molecules span two bead lengths and are staggered. The N- and C- termini are marked for 4 molecules with colored domains (yellow=calcium-binding EGF-like domains; red=8-cysteine domains; blue=hybrid domains; green=generic EGF-like domains; purple=proline-rich domain). The dark green ovals represent the beads seen in beaded string microfibrils (Figure 2). **B.** Domains in which mutations cause Weill-Marchesani syndrome (WMS), geophysic dysplasia (GD), acromicric dysplasia (AD), and Stiff Skin Syndrome (SSkS) are boxed. These domains are predicted to be close together within the microfibril and may form a special microenvironment. The region containing mutations leading to neonatal Marfan syndrome is also boxed. **C.** Pro-BMP complexes (BMP), large latent TGFβ complexes (LTBP), and perlecan with an associated pro-GDF-8 complex (myostatin) are shown bound to fibrillin.



**Figure 4. Domain Structures of the Fibrillin-LTBP Family of Proteins**

Fibrillins and LTBPs are composed of the same types of domains. The 8-cysteine domain is present only in this family of proteins. The fibrillins are the same size, whereas the LTBPs vary in size.

**Table 1**

## Genotype-phenotype Correlations in Marfan syndrome

Type of Mutation	Phenotype	Comments	Reference
Cysteine substitutions in EGF-like domains	High incidence of ectopia lentis; severe early onset in exons 26–32	24 distinct mutations; 44 subjects	(58)
Premature termination codons (PTC)	Low incidence of ectopia lentis; high incidence of large joint hypermobility; high incidence of skin striae; higher incidence of aortic dissection?	33 distinct mutations; 60 subjects	(59)
First 15 exons; arginine to cysteine mutations?	Predominant ectopia lentis	11 subjects; younger EL subjects may develop further symptoms of Marfan syndrome	(60)
Full spectrum of mutation types	Cysteine mutations correlate strongly with ectopia lentis; PTC mutations are associated with severe skeletal and skin phenotypes; mutations in exons 24–32 are associated with severe disease	803 mutations in 1,013 probands	(61)
75% of mutations were in- frame; 25% resulted in PTCs; 33% of mutations occurred in exons 24–32; incidence of PTCs was smaller than in adult cohort	Lethal neonatal Marfan syndrome is a genuine clinical entity; clinical manifestations increase with age	320 subjects <18 years old; 15% were diagnosed with neonatal Marfan syndrome	(62)
Full range of mutation types	“Incomplete” or mild Marfan syndrome was associated with mutations in exons 59–65; mutations at the ends (in exons 1–15 and 59–65) may be milder than mutations in between	193 mutations in 503 subjects	(63)
Full range of mutation types	Truncating and splicing mutations were associated with aortic events	179 probands with FBN1 mutations	(64)

Table 2

*Fbn1* Mutant Mouse Models

Type of Mutation	Phenotypes	Comments	Reference <sup>1</sup>
<i>Fbn1</i> mg	Homozygous mg die in the early postnatal period; heterozygous mice are similar to mgR/+ and mgN/+	In-frame deletion of exons 19–24; presence of <i>Neo</i> represses expression of the deletion	(83)
<i>Fbn1</i> mg loxPneo	Heterozygous mice show Marfan phenotypes	By removing <i>Neo</i> , the deletion of exons 19–24 is fully expressed	(85)
<i>Fbn1</i> mgR	Homozygous mgR die from aortic rupture during early adulthood; heterozygous mice appear normal	Presence of <i>Neo</i> represses expression of wildtype fibrillin-1 to around 20% of normal; homozygous mgR model severe aortic disease	(84)
<i>Fbn1</i> mgN	Homozygous mgN die in the early postnatal period; heterozygous mgN appear normal	The complete null establishes early postnatal death around P14, phenocopied by homozygous mg <sup>-</sup> , C1039G, and GT-8; establishes haploinsufficient phenotypes	(88)
<i>Fbn1</i> C1039G	Homozygous C1039G mice die in the early postnatal period; heterozygous mice show aortic dilatation but have normal life spans	A heterozygous missense mutation causes Marfan phenotypes in mice	(86)
<i>Fbn1</i> GT-8	Homozygous GT-8 mice die in the early postnatal period; heterozygous mice show aortic dilatation but have normal life spans	Cre-inversion of eGFP truncates fibrillin-1 after cbEGF18 (exon 32), tagging fibrillin-1 with eGFP (preserving the “neonatal region”); presence of GFP tag shows assembly of mutant fibrillin-1, followed by proteolysis of microfibrils	(87)
<i>Fbn1</i> H1	Homozygous and heterozygous H1 mice live long lives with no signs of aortic disease and no structural defects in microfibrils	In-frame deletion of exon 7, encoding the first hybrid domain	(87)
<i>Fbn1</i> WM	Homozygous and heterozygous WM mice live long lives with no signs of aortic disease; thick skin and reduced bone growth; microfibrils are abnormally aggregated	In-frame deletion of exons 9–11, phenocopying a human family with autosomal dominant Weill-Marchesani syndrome	(78)
<i>Fbn1</i> W1507C	Heterozygous W1507C mice show dermal fibrosis and loss of subcutaneous fat; homozygous W1507C show accelerated skin fibrosis but remain viable	This missense mutation phenocopies a human mutation causing Stiff Skin Syndrome	(81)
<i>Fbn1</i> D1545E	Like heterozygous W1507C, heterozygous D1545E develop dermal fibrosis and loss of subcutaneous fat; homozygous D1545E are embryonic lethal	This missense mutation alters the RGD integrin binding site to RGE	(81)
<i>Tight skin (Tsk)</i>	Heterozygous <i>tsk</i> mice are a model for sclerodema; hets also display emphysema and myocardial hypertrophy; homozygous <i>tsk</i> die at E7–8	The <i>tsk</i> mutation results in a large tandem duplication of fibrillin-1 (the region encoding cbEGF7- cbEGF24 is duplicated after cbEGF24)	(92)

<sup>1</sup> Only the original reference is cited.