The Role of Fibrillin-1 in Eye Development and Disease

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The Role of Fibrillin-1 in Eye Development and Disease

by

Wendell Brooks Jones Jr.

A dissertation presented to
The Graduate School
of Washington University in
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of Doctor of Philosophy

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List of Abbreviations

ADAMTSL4; A disintegrin and metalloproteinase with a thrombospondin motif-like protein 4
AD WMS; Autosomal dominant Weill-Marchesani syndrome
BMP; Bone morphogenetic protein
Ca^{2+}; Calcium
cbEGF; Calcium binding epidermal growth factor
CCA; Congenital Contractural Arachnodactyly
DN; Dominant Negative
EL; Ectopia lentis
E; Embryonic day
ECM; Extracellular matrix
FBN-1; Fibrillin-1
FBN-2; Fibrillin-2
FUN; Fibrillin unique region
FN; Fibronectin
HI; Haploinsufficient
ECTOL1; Isolated ectopia lentis-1
ECTOL2; Isolated ectopia lentis-2
ILM; Inner limiting membrane
IOP; Intraocular pressure
LTBPs; Latent-transforming growth factor beta-binding proteins
LTBP2; Latent-transforming growth factor beta-binding protein 2
MFS; Marfan syndrome
MFAP2; Microfibrillar-associated protein 2
NPCE; Non-pigmented ciliary epithelium
OMIM; Online Mendelian Inheritance in Man
PCE; Pigmented ciliary epithelium
P; Postnatal day
SEM; Scanning electron microscopy
8-cys-TB; 8 cysteine-TGF-β binding-like
TGF-β; Transforming growth factor beta
TEM; Transmission electron microscopy
Weill-Marchesani syndrome-3; WMS3
Zonular fibers; ZFs
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Washington University in St. Louis

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Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

The role of fibrillin-1 in eye development and eye disease

Wendell Brooks Jones Jr.

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Genetics and Genomics

Washington University in St. Louis, 2018

Professor Steven Bassnett, Chair

The ciliary zonule of the human eye consists of a circumferential array of fibers that connect the ocular lens to the nonpigmented ciliary epithelium (NPCE) located at the inner wall of the eye. Zonular fibers consist of bundles of beaded filaments called microfibrils. Microfibrils are major structural elements of the extracellular matrix and are present in pure form in the ciliary zonule. Microfibrils are composed principally of fibrillin-1 (FBN-1); a large extracellular matrix glycoprotein. In humans, mutations in FBN1 underlie Marfan syndrome; a pleiotropic connective tissue disorder that profoundly affects the eye. Ocular manifestations include ectopia lentis (dislocated lenses), cataracts, glaucoma and axial myopia. The ocular phenotypes in Marfan syndrome suggest an important role for FBN-1 in eye development.

In this report, I used mice as a model system to test the role of FBN1 in eye development and disease with an emphasis on understanding the role of FBN-1 in the synthesis of the ciliary zonule. Our lab has shown that the organization and composition of the mouse ciliary zonule is similar to humans. A recent proteomic study identified FBN-1 as the main component of the ciliary zonule, accounting for 60% to 70% of total zonule protein. In order to understand the role of FBN-1 in eye development, I had to first identify the cells in the eye responsible for
expressing FBN-1 and other core components of the zonule (FBN-2, MFAP2, LTBP2 and ADAMTSL4). I used in situ hybridization on wild type mouse eye tissue to show that some zonule components (Fbn1, Fbn2, Ltbp2, Mfap2) are expressed by cells of the NPCE while others (Adamtsl4, Mfap2) are expressed by the lens. This suggests that the ciliary zonule is synthesized by both tissues. Immunofluorescence experiments on adult wild type mouse eyes using antibodies against core zonule proteins showed that the ciliary zonule is heterogeneous in composition and nonuniform along the length of fibers suggesting that the spatial distribution of proteins in the ciliary zonule could reflect the temporal expression of zonule components during eye development.

The identification of NPCE cells as the likely source of FBN-1 in the zonule allowed me to test the contribution of Fbn1 directly. I used a conditional knockout approach to delete Fbn1 in the nasal and temporal regions of the mouse NPCE. Three-dimensional reconstructions of conditional Fbn1 knockout mouse eyes (Fbn1-NPCE) demonstrated that the zonule was produced in the nominal absence of its most abundant component. However, the Fbn1-NPCE mice developed ectopia lentis; the major ocular manifestation and diagnostic criterion for MFS in humans. Given the clinical importance of the disease, I decided to investigate the structural and biomechanical changes leading to ectopia lentis in the Fbn1-NPCE mouse model. Ultrastructural studies on FBN-1 deficient mouse eyes using scanning electron microscopy revealed that Fbn1-NPCE mice had significant changes in zonular fiber density and thickness. Biomechanical testing on wild type and Fbn1-NPCE mouse zonules showed that FBN-1 deficient zonular fibers were significantly weaker (~50%) than their wild type counterparts. Conditional deletion of Fbn1 in the mouse lens (Fbn1-lens) had no discernible effect on the structure or function of the ciliary zonule, suggesting that FBN-1 protein synthesized by the lens
is not required for the stability of the ciliary zonule. Together, these data show that the ectopia lentis phenotype in Fbn1-NPCE mice is secondary to changes in ciliary zonule ultrastructure and biomechanical properties and that these effects may give some insights to the mechanism of ectopia lentis in Marfan syndrome. In addition to ectopia lentis, Fbn1-NPCE mice express other ocular phenotypes commonly associated with Marfan syndrome including cataracts, increased axial length and, in rare cases, glaucoma.

The data presented in this report provide insights into the role of FBN-1 in eye development and the biology of the ciliary zonule. In addition, the development of the Fbn1-NPCE mouse model will serve as a platform for understanding the role of FBN-1 in ocular disease associated with Marfan and related syndromes.
Chapter 1: Introduction

1.1 Eye development

The human eye functions to gather and focus light on the retina in order to produce a clear image of the outside world. In 1901, Hans Spemann proposed the first mechanism of eye development by studying the interactions of embryonic tissues using amphibians as a model organism. Over the past century, scientists have gravitated towards using higher ordered animals such as mice (*Mus musculus*) to study the mechanisms of human eye development. The anatomy and developmental processes of the mouse eye resemble those of the human eye. As a result, many studies have shown that mice are useful tools for investigating the roles of genes and proteins implicated in human eye disease due to their highly conserved and homologous genomes.

Vertebrate eye development commences during the early stages of embryogenesis. Eye morphogenesis is a collaboration between three embryonic tissues: the neural ectoderm, the mesoderm and the surface ectoderm. In mice, eye development begins on embryonic day 8.5 (E8.5) with the evagination of the neural ectoderm to form the optic vesicle (Fig. 1.1 A). Between E9.0 and E9.5, the optic vesicle makes contact with the overlying surface ectoderm, causing the ectodermal cells to thicken and form the lens placode (Fig. 1.1 B). On E10.5, coordinated invagination of the lens placode and optic vesicle results in the formation of the lens pit and optic cup, respectively (Fig. 1.1 C). At E11.5, the lips of the lens pit fuse to form the lens vesicle (Fig. 1.1 D). Subsequently, the surface ectoderm separates from the lens vesicle,
giving rise to the corneal epithelium (Fig. 1.1 E). On E12.5, growth factors secreted from the inner layer of the optic cup induce the posterior lens cells to differentiate into primary lens fiber cells (Fig. 1.1 E) ³. Between E12.5 to E13.0, the primary lens fibers cells elongate anteriorly to fill the lumen of the lens vesicle, giving rise to embryonic lens (Fig.1.1 F).

In mice, most of the ocular tissues are established by E13.0 (equivalent to gestational week 10 in humans) ⁴. Like mice, the exterior and interior embryonic structures of the human eye continue to grow and form specialized tissues that carry out specific functions necessary for vision. For example, the corneal epithelium develops into the adult cornea which contributes nearly two-thirds of the eyes focusing power of the eye (Fig. 1.2) ⁴. The anterior rim of the optic cup differentiates into the iris, which is responsible for controlling the amount of light that enters the eye, and the ciliary body (Fig. 1.2, see sections below for more detail) ⁴. The ocular lens undergoes rapid growth to become the correct size and shape to focus light on the retina (Fig. 1.2) ⁵. The inner layer of the optic cup will give rise to the neural retina, the light-sensing tissue of the eye, which contains specialized cells called rods and cones (Figure 1.1C) ⁴. Rod cells are responsible for night vision while cone cells are responsible for day vision. The outer layer of the optic cup constitutes the retinal pigment epithelium, which prevents photons of light from escaping the eye (Fig. 1.1C) ⁴. The cells of the periocular mesenchyme (Fig. 1.1) gives rise to specialized tissues necessary for eye movement and intraocular dynamics, such as the extraocular muscles, sclera, corneal stroma, corneal endothelium, iris stroma, ciliary muscle, ciliary body stroma, and trabecular meshwork ⁴.
1.1.1 Ciliary body development

The human eye can be divided into the anterior segment and the posterior segment. The anterior segment consists of the cornea, pupil, iris, lens, and ciliary body, while the posterior segment constitutes the vitreous gel, retina, and optic nerve (Fig. 1.2). The ciliary zonule, an organized system of fibers that span the gap between the lens and the inner wall of the eye, is located in the anterior segment of the eye directly behind the iris. The zonule is an extension of the ciliary body; a circumferential ring of folded tissue that extends from the ora serrata and posterior surface of the iris.

Most of what is known about ciliary body development comes from histological studies on the mouse eye. In mice, ciliary body morphogenesis begins on E14.5 with thinning of the outer rim of the optic cup. By E15.5, distinct zonal regions between the neural retina and presumptive ciliary body/iris are visible. Between E16.5 to E17.5, the cells of the presumptive ciliary body zone start to differentiate, giving rise to two distinct epithelialized cell layers. The innermost layer is the non-pigmented ciliary epithelium (NPCE), which is continuous with the neural retina. The pigmented layer nearest the wall of the eye is the pigmented ciliary epithelium (PCE) and is continuous with the retinal pigment epithelium. Collectively, the NPCE and PCE make up the ciliary epithelium; a bi-layered epithelium that covers the surface of the ciliary body. Between E18.5 and postnatal day 0 (P0), the ciliary folds (also known as the pars plicata or ciliary processes) start to form with the evagination of the PCE toward the NPCE. By P30, the ciliary folds are fully developed and radially extended. Each fold forms around a network capillaries located in the stroma of the ciliary body. At this stage, the ciliary body can be divided into three regions: the pars plana (flat portion of the ciliary body), pars plicata and ciliary muscle.
The ciliary muscle, located at the base of the ciliary body, is responsible for initiating the accommodation process (see below) \(^6,^7\).

The mechanisms that drive ciliary body development are not clear. Some studies suggest that ciliary body morphogenesis depends on interactions between the inner wall of the eye and the lens. In the mouse eye, the presumptive ciliary body comes into contact with the lens around E12.0 and subsequently separates from the lens around P1. Removal of the embryonic lens between E12.0 and E17.5 results in the absence of the ciliary epithelium \(^8\). Interestingly, removal of the lens after E19.0 does not disturb the formation of the ciliary epithelium, suggesting time-dependent interactions between the embryonic lens and the rim of the optic cup. Others have suggested that intraocular pressure (IOP) may play a role in the formation of the ciliary folds. Working on bird eyes, Bard and Ross observed a rapid increase in IOP around the time of the formation of the ciliary folds\(^9\). A complementary experiment performed by Coulombre on embryonic chicken eyes showed that reducing the IOP in the eye slows eye growth and inhibits the formation of the folds \(^10\). A more recent study suggests that the formation of the ciliary folds is caused by a surge in proliferation of NPCE and PCE cells in the ciliary epithelium between P0 and P2 \(^6\).

In addition to tissue-tissue interactions, studies show that genes such as \(Otx1\) and \(Lmx1b\) play important roles in ciliary body development. \(Otx1\), a gene involved in brain and sensory organ development, is strongly expressed in the presumptive ciliary epithelium from E12.5 onward \(^11\). Mice lacking \(Otx1\) do not develop ciliary bodies \(^11\). Likewise, mice lacking \(Lmx1b\), a gene involved in anterior segment development, display iris and ciliary hypoplasia \(^12\). The precise roles of these genes in eye development are not known, however, overexpression of
Noggin has been shown to block ciliary body development, implicating bone morphogenetic protein (BMP) signaling in ciliary body formation.

1.1.2 The ciliary epithelium

The ciliary epithelium of the eye serves as a blood-aqueous barrier separating the aqueous humor from the complex network of blood vessels located in the stroma of the ciliary body. The ciliary epithelium is composed of NPCE and PCE cells. NPCE and PCE cells are orientated such that their apical membranes are touching. NPCE and PCE cells are bordered by basement membranes called the inner limiting membrane (ILM) and external limiting membrane, respectively. The two epithelia are connected by gap junctions, suggesting that they function as a syncytium, facilitating the exchange of ions and small metabolites. Adjacent NPCE and PCE cells are linked together by tight junctions that are expressed on the apical and basolateral membranes. These prevent the leakage of large, light-scattering proteins from the capillary bed into the aqueous humor. Ultrastructural studies on the ciliary epithelium show that NPCE and PCE cells contain specialized membranes called β-cytomembranes. β-cytomembranes are folded membranes that extended into the cell body. In other cell types, such as renal tubular and salivary gland cells, they play a role in the transport of fluids and ions across blood-aqueous barriers. This suggest that NPCE and PCE cells may function to facilitate the movement of aqueous humor into the anterior chamber.
Although NPCE and PCE cells share many of the same physiological functions, there are distinct differences in the morphology and genes that are expressed between these cell types. The most obvious macroscopic difference between NPCE and PCE cells is the absence of pigmentation from the NPCE. Histological studies on the show that NPCE cells have a different appearance compared to PCE cells. NPCE cells in the pars plicata region of the ciliary body are cuboidal in shape while NPCE cells in the pars plana are columnar and face in the direction of the lens \(^6\). In contrast, PCE cells have a more flattened cellular morphology. Another key difference is the relative abundance of mitochondria in NPCE cells, suggesting that NPCE cells are more metabolically active than PCE cells \(^{16}\). This idea is supported by the observation that that Na, K-ATPases are particularly abundant in the basolateral membranes of NPCE cells. This is consistent with the notion that NPCE cells actively transport ions across their membranes thereby establishing an electrochemical gradient across the inner wall of the eye \(^{14}\). Water channels called aquaporins are also expressed in the plasma membrane of NPCE cells, suggesting a role for these cells in the secretion of aqueous humor into the anterior chamber \(^{14}\). Finally, gene expression analyses on NPCE and PCE cells demonstrates the major difference between the two transcriptomes is the expression of genes in PCE cells involved in pigmentation \(^{17}\). A few studies also suggest that NPCE cells are solely responsible for the synthesis of the ciliary zonule; a system of fibers that connect the lens to the ciliary epithelium of the eye \(^7,^{18},^{19}\) (see section 1.2).
1.2 The ciliary zonule of the eye

1.2.1 Structural organization of the human ciliary zonule

The ciliary zonule, also known as the Zonule of Zinn, is named after Johann Gottfried Zinn who first described it in his 1755 monograph, *Descriptio Anatomica Oculi Humani Iconibus Illustrata*. Zinn described the ciliary zonule as a fibrillar structure that spanned from the ora serrata (the junction between the retina and the pars plana of the ciliary body) to the anterior surface of the lens. In 1855, Muller added to Zinn’s description, stating that the zonular fibers (ZF; sometimes referred to as the suspensory ligaments or zonules) were glassy in appearance and adherent to the ciliary body. An important advancement in elucidating the microscopic anatomy of the ciliary zonule came in the mid-20th century with the invention of the electron microscope. Electron microscopy studies on human and monkey tissues revealed that the zonule consists of an elaborate system of fibers that emerge from the surface of the ciliary body and attach to the surface of the lens (Fig. 1.3). In this section, I discuss our current knowledge about the anatomy of the ciliary zonule.

In human and non-human primate eyes, ZFs emerge from the pars plana of the ciliary body (Fig. 4). Scanning electron microscopy (SEM) images of ZFs in this region show that ZFs are linear filaments, aligned in parallel, and closely associated with the surface of the pars plana. Some studies suggest that ZFs are attached to the surface of the pars plana region of the ciliary body by merging with the ILM of the ciliary body. In contrast, one study suggests
that ZFs penetrate the ILM and terminate in the intercellular spaces between the NPCE cells, although this observation has yet to be confirmed\(^1\).

Before approaching the ciliary folds, the ZFs fuse with one another to form larger fibers (Fig. 1.4)\(^2\). Ultrastructural studies suggest that ZFs are attached to the walls of the ciliary folds by smaller fibers called tension or transversal fibers\(^2,25\).

As the ZFs exit the ciliary folds, they split, forming the signature fan-like structure of the ciliary zonule called the zonular fork (Fig. 1.5). In the human eye, the zonular fork gives rise to three sets of fibers: the anterior, equatorial and posterior ZFs. The anterior and posterior ZFs vary in size between 25 µm and 60 µm in diameter\(^2\) while equatorial ZFs have been variously reported to be 10 µm to 40 µm in width\(^2\)\(^6,27\). Recent studies suggest that the zonule includes a fourth set of fibers called the vitreous zonule. The vitreous zonule consists of ZFs that are closely associated with the anterior face of the vitreous called the anterior hyaloid membrane\(^2\)\(^8\), although the function of these fibers is not understood.

As the ZFs approach the lens, the anterior, equatorial and posterior ZFs often split or fuse with one another forming larger or smaller fibers. Before making contact with the lens, ZFs splay again into “pitch-fork”-like fibers measuring 5 µm to 10µm in diameter\(^2\)\(^5,26\). These “pitchfork” like fibers merge into an unorganized meshwork of fibrils (measuring 35 nm to 55nm in diameter) on the surface of the lens capsule; a specialized basement membrane composed largely of collagen IV that envelopes the crystalline lens\(^2\)\(^6,29,30\). One study suggests that the tips of the ZFs penetrate into lens capsule approximately 600 nm\(^1\)\(^5,31,32\). The insertion of ZFs into the lens capsule of human eyes is thought to be important for the accommodation processes.
1.2.2 Composition of the ciliary zonule

In 1966, Buddecke and Wollensak were the first to describe the biochemical composition of the zonule by analyzing the amino acid and carbohydrate profiles on acetone-treated bovine ZFs. Their results showed that ZFs were composed of protein(s) rich in acidic and cysteine amino acids (38/1000 amino acids) \(^{33}\). This study also showed that ZFs were rich in monosaccharides, accounting for 5% of total zonule material. These results were later confirmed by Streeten et al. on freshly dissected bovine ZFs; however, it is important to note that the content of cysteines (83/1000 amino acids) and carbohydrates (9.25%) was significantly higher in this study \(^{34}\).

Many researchers hypothesized that the ciliary zonule was an extension of the vitreous humor (a clear gel composed of collagen proteins that fills the space between the surface of the lens and the retina) and thus must be composed of vitreous protein \(^{35}\). A comparative amino acid composition analysis on isolated human ZFs and vitreous humor collagen fibers revealed that ZFs were devoid of hydroxylysine and hydroxyproline residues; amino acids found only in collagen proteins \(^{36}\). However, a comparative analysis of ZFs and other fibrillar structures found in the human body revealed that ZFs shared a similar amino acid profile with that of elastin-associated microfibrils (Fig. 1.6; small beaded filaments found in the extracellular matrix (ECM) of connective tissues) \(^{36, 37}\).

To confirm the identity of ZFs, Streeten et al. stained sections of connective tissues and ocular tissues with antibodies raised against bovine zonule. The antibodies bound specifically to beaded filaments in connective tissues and the ciliary zonule, confirming that ZFs were composed of microfibrils \(^{38}\). In addition, Streeten et al. performed western blot analysis on
bovine ZFs and showed that zonular microfibrils were heterogeneous in composition and composed of multiple proteins ranging from 35 to 200 kDa in size 34.

To date, two proteomic studies have addressed the composition of the ciliary zonule. The first identified a protein named fibrillin-1 as the major component 39. A more comprehensive analysis of the human and bovine zonule concluded that the composition of the ciliary zonule is conserved between species and that a set of 52 proteins constitute more than 95% of zonule protein 40. To better define the assemblage of proteins present in the zonule, De Maria et al. their proteome dataset through a database called the matrisome. The matrisome is an ensemble of over 1000 genes that encode ECM components 41. Two main classes of matrisomal proteins are defined: the core matrisome and those proteins that bind or associate with the ECM (matrisome-associated proteins) 41. The core matrisome is further divided into three categories (glycoproteins, collagens and proteoglycans), while the matrisome-associated proteins include ECM affiliated, regulators, and ECM secreted factors 41. When filtered through the matrisome database, De Maria et al. showed that all six classes of matrisomal proteins are present in the ciliary zonule. Ranked in order of abundance, the ciliary zonule was found to be composed primarily of glycoproteins with the most abundant glycoproteins being remarkably similar between human and bovine. The study confirmed fibrillin-1 as the main component of the ciliary zonule, accounting for 60% to 70% of zonule protein 40. Other core components include microfibrillar-associated protein 2, latent-transforming growth factor beta binding-protein 2, fibrillin-2, and A disintegrin and metllaproteinase with a thrombospondin motifs 40. Interestingly, moderate levels of cross-linking and proteolytic enzymes and their inhibitors were detected, suggesting important roles for these proteins in the stability of this ciliary zonule 40. In this next
section, I discuss the function and disease implications of some of the most abundant proteins found in the ciliary zonule.

1.2.3 Fibrillin-1 (FBN-1)

Fibrillins are ancient molecules that emerged from a common ancestral gene approximately 600 million years ago \(^42\). At least one functional fibrillin gene is found in all organisms with the exception of *Drosophila melanogaster* and *Caenorhabditis elegans* \(^43\). The human genome contains three functional fibrillin genes (*FBN1-3*) \(^44\). The *FBN1* gene (237,000 kb) is located on the long arm of human chromosome 15 at position 15q15-21.1 \(^44\). It is predicted that transcription factors such as GATA 1/2/3 or Nkx-2.5 may play a role in regulating fibrillin gene expression \(^45\).

The *FBN1* gene encodes a 350 kDa precursor protein called profibrillin-1. Profibrillin-1 is processed by furin proteases at the N- and C- termini, converting profibrillin-1 to a functional 330 kDa protein called fibrillin-1 (FBN-1) \(^46-48\). It is unclear whether the furin mediated processing of profibrillin-1 occurs intracellularly or extracellularly \(^49,50\).

The FBN-1 protein was first isolated from cultured human skin fibroblast and characterized as a large ECM glycoprotein rich in cysteine amino acids \(^51\). Fibrillins are modular proteins consisting of 47 epidermal growth factor like domains of which 43 are calcium binding epidermal growth factor domains (cbEGF), seven 8-cysteine TGFβ binding-like domains (8-cys-TB), and two are hybrid domains (Fig. 1.7) \(^43\). Each fibrillin contains at least one (FBN-1) or two (FBN-2 and -3) integrin binding sites \(^43\), which are shown to play important roles for the binding of cell surface \(\alpha_4\beta_3, \alpha_5\beta_1\), or \(\alpha_4\beta_3\) integrins to FBN-1 proteins in microfibrils \(^52-56\).
cbEGF domains are the most common motif found in fibrillins. As there name implies, cbEGF domains have the ability to bind $\text{Ca}^{2+}$\textsuperscript{57, 58}. Approximately 80% (282 out of the 361) of cysteine residues reside within these domains\textsuperscript{59}. Each cbEGF domain is stabilized by six cysteine residues that form disulfide bonds in a 1-3, 2-4, 5-6 arrangement\textsuperscript{57}. Each cbEGF domain contains a highly conserved $\text{Ca}^{2+}$ binding consensus sequence rich in asparagine and aspartic amino acids\textsuperscript{60}. Together, these residues stabilize $\text{Ca}^{2+}$ ions in the binding pockets of cbEGF domains through covalent interactions between by six oxygen atoms extending from the hydroxyl groups of asparagine and aspartic amino acids\textsuperscript{56}. The binding of $\text{Ca}^{2+}$ to cbEGF domains plays an important role in the secondary and tertiary structure of FBN-1\textsuperscript{61}.

8-cys-TB domains are the second most common expressed motif and are found only in fibrillins and structurally-related proteins called latent-transforming growth factor $\beta$-binding proteins (LTBPs)\textsuperscript{43}. Approximately 15% (56/361) of the cysteine residues are located in 8-cys-TB domains\textsuperscript{59}. Each 8-cys-TB domain consist of eight cysteine residues involved in forming disulfide bonds. 8-cys-TB domains are stabilized by all eight cysteine residues that pair in a 1-3, 2-6, 4-7, 5-8 arrangement\textsuperscript{62}.

8-cys-TB domains are important for binding TGF-$\beta$\textsuperscript{63}. Although fibrillins contain these domains, they lack the ability to bind TGF-$\beta$ due to a two amino acid deletion in the third 8-cys-TB motif; a domain known to bind TGF-$\beta$ molecules in LTBPs\textsuperscript{64}. Although fibrillins lack the ability to bind TGF-$\beta$ molecules, BMPs (members of the TGF-$\beta$ superfamily) have been shown to bind to fibrillin proteins. Solid phase binding assays and immunohistological experiments show that BMP -2, -4, and -7 bind to the N-terminus of fibrillins and co-localize with fibrillin microfibrils in tissues\textsuperscript{65, 66}. The physiological importance of BMPs binding to fibrillins is not known.
Sequence analysis of the two hybrid domains suggests that these result from the fusion of the N-terminus of a 8-cys-TB domain with the C-terminus a cbEGF domain \(^{43}\). Cysteine residues in the hybrid domains participate in a 1-3, 2-5, 4-6, 7-8 disulfide-bonding pattern \(^{67}\). The first hybrid domain is located proximal to the N-terminus of FBN-1 while the second hybrid domain is located close to the C -terminus \(^{43}\). It is suggested that the N-terminal hybrid domain contains a free thiol that participates homotypic interactions between FBN-1 proteins and is believed to be one of the initial steps in microfibril assembly \(^{59}\).

All fibrillins share a similar modular organization but, differ by a stretch of 20 amino acids known as the fibrillin unique region (FUN) located at the N-terminus \(^{43}\). The FUN regions of FBN-1 is rich in proline while the FUN region of FBN-2 and FBN-3 is rich in glycine and proline/glycine, respectively \(^{43}\). The FUN regions of fibrillins are thought to act as a flexible hinges or springs contributing to the extensibility and packing of fibrillins in microfibrils \(^{68}\).

FBN-1 proteins have been shown to form interactions with itself and other ECM proteins. FBN-1 proteins form homodimers with the strongest homotypic interactions occurring between the N- (exons 1-17, amino acids (aa) 1-72) and C-terminal (exons 57-64, aa 2402-2871) ends in a Ca\(^{2+}\)-dependent manner, suggesting that FBN-1 proteins may be arranged head-to-tail ( N- to C- terminus) \(^{46,69,70}\). Studies using recombinant FBN-1 proteins support the hypothesis that FBN-1 proteins are arranged head-to-tail, by showing that mutations in the C-terminal end of FBN-1 (cbEGF 41-43) inhibit N- to C-terminal binding and microfibril formation \(^{71}\). In addition, retention of the furin-mediated processing of the C-terminal pro-peptide, supports the idea that FBN-1 proteins interact via N- and C-terminal ends with the prevention of FBN-1 secretion into the ECM and FBN-1 assembly (see section 1.4 for more details on fibrillin microfibril assembly) \(^{71}\).
In addition to self-interactions, fibrillins also bind other proteins in the ECM such as fibronectin (FN). FN is a large ECM glycoprotein that plays an important role in cell adhesion, migration and differentiation \(^{72}\). FBN-1 and FN networks co-localize \textit{in vitro}\(^{73-75}\) and solid phase binding assays show that the N- and C- termini of FBN-1 and the C- terminus of FBN-2 and -3 bind to FN. The strongest interactions between FBN-1 and FN proteins occur at the C-termini \(^{73,74}\). The FBN-1 binding site in FN has been localized to the gelatin-binding domain of FN \(^{73}\). Inhibition of FBN-1 binding to the gelatin-binding domain of FN significantly reduces microfibril assembly \textit{in vitro}\(^{73-75}\). Transcriptional interference of FN gene using siRNA probes in cultured human fibroblast has been shown to reduce FBN-1 networks in the ECM \(^{73,75}\). In addition, primary cultured fibroblasts from mice lacking α5β1 cell surface integrin show a significant decrease in FN and FBN-1 fibrils in the matrix, suggesting that FN and fibrillin microfibril assembly occurs close to the cell \(^{75}\). Collectively, these data suggest that FBN-1 microfibril assembly depends on a pre-existing scaffold of FN in the ECM.

Fibrillins also bind heparan sulfate proteoglycans (HSPG) \(^{76}\). HSPG are ECM proteins decorated with sugar groups called glycosaminoglycans \(^{77}\). HSPGs have important roles ranging from cell-matrix interactions to cell signaling \(^{77}\). Fibrillins have high binding affinities for heparan and heparan sulfate \(^{78}\). FBN-1 binds specifically to heparan proteins via their N-terminal (residues 1-489) and C-terminal (1528-2871) ends in a Ca\(^{2+}\)-independent manner \(^{79}\). Addition of heparan to cells in culture results in reduced cell adhesion to the ECM (presumably FBN-1 proteins) and inhibits FBN-1 microfibril assembly leading to a decrease in FBN-1 immunofluorescence in the matrix \(^{78-81}\). How heparan molecules facilitate the formation of FBN-1 microfibrils is not known.
Human mutations in FBN1 underlie Marfan syndrome (Online Mendelian Inheritance of Man [OMIM 154700]) and autosomal dominant Weill-Marchesani syndrome (AD WMS; [OMIM 608328]) \(^{44}\). Individuals with Marfan syndrome are characterized by their tall stature and life-threatening cardiovascular complications while AD WMS patients have short stature with mild cardiovascular symptoms \(^{44}\). Interestingly both diseases share ocular manifestations that profoundly affect the eye (see section 1.5). Mutations in FBN1 also give rise to a rare disease called Marfan Lipodystrophy syndrome (MFLS; [OMIM 616914]). Individuals with MFLS are characterized by their aged appearance due to reduced amounts of subcutaneous fat beneath their skin (subcutaneous lipoatrophy), failure to thrive, and resistance to insulin production, which is controlled by an increase in glucose in the blood. MFLS patients express mutations in the C-terminal furin cleavage site of profibrillin-1, resulting in the expression of a truncated FBN-1 protein and the absence of the C-terminal 140 amino acid long cleavage product named asprosin\(^{82}\). In normal individuals, asprosin is present in the blood and is correlated with increased insulin production during fasting periods \(^{82}\). In MFLS, patients, asprosin and insulin levels are significantly reduced \(^{82}\). Mice lacking asprosin develop a MFLS phenotype similar to humans \(^{83}\). Interestingly, subcutaneous injection of asprosin into MFLS-expressing mice rescues the MFLS phenotype showing increased food intake, body weight, blood glucose levels and insulin production \(^{83}\). Together, these data show that the C-terminal cleavage product of FBN-1 may serve as a hormone that regulates glucose and insulin production.

Together, these studies show that fibrillins are large modular proteins that bind ions, growth factors and other fibrillin proteins in order to form structural elements (microfibrils) of the ECM.
1.2.4 Microfibrillar-associated protein 2 (MFAP2)

The human MFAP2 gene is located on the short arm of chromosome 1 at position 1p36.3-p35. The MFAP2 gene encodes a 31 kDa glycoprotein called microfibrillar-associated protein 2 (MFAP2). MFAP-2 proteins are major constituents of microfibrils. MFAP-2 binds specifically to the beaded domains of FBN-1 microfibrils.

In humans, the role of MFAP2 proteins in the body is unknown. In the eye, MFAP-2 proteins localize to the cornea, tunica vasculosa lentis, pupillary membrane, and ciliary zonule. However, the cells responsible for synthesizing these proteins have yet to be identified. In regards to the zonule, one study suggests that NPCE cells synthesize MFAP-2 and these proteins are required to synthesize FBN-1 zonular microfibrils. To date, no diseases have been firmly associated with mutations in the MFAP2 gene. However, obesity and diabetes traits have been linked to the 1p36 locus, which includes MFAP2 gene. Mice lacking MFAP-2 show a significant increase in body weight and fat deposition compared controls. Thus, these data suggest that MFAP-2 may be involved in metabolic disease.

1.2.5 Latent-transforming growth factor beta-binding protein 2 (LTBP2)

LTBPs belong to the fibrillin/LTBP superfamily. Like fibrillins, LTBPs are characterized by their high content of cysteine residues and signature 8-cys-TB motifs. The human genome contains four LTBP genes (LTBP1-4). The LTBP2 gene is located on the long arm of chromosome 14 at position 14q24.3. LTBP2 encodes a 250 kDa cysteine rich glycoprotein (LTBP2). LTBPs are composed of the same types of modular domains as fibrillins (Fig. 7). LTBPs localize and bind to FBN-1 proteins in microfibrils. As there name implies, LTBPs have the ability to bind and sequester TGF-β in the ECM by forming strong covalent bonds.
between with cysteine residues in the third 8-cys-TB of LTBP\(_2\) and the TGF-\(\beta\) molecule. Biochemical assays show that LTBP-1, -3, and -4 bind all TGF-\(\beta\) isoforms (TGF-\(\beta\) 1-3)\(^{93}\). However, LTBP2 does not bind TGF-\(\beta\) due to a dibasic amino acid deletion in the third 8-cys-TB\(^{64,93}\).

Recessive human mutations in LTBP2 underlie Weill-Marchesani syndrome-3 (WMS3; [OMIM 614819]). Interestingly, like AD WMS, WMS3 patients also have short stature with mild cardiovascular complications and obvious ocular manifestations. Ocular manifestations include ectopia lentis, microspherophakia, glaucoma and myopia. LTBP2 has been localized to the ciliary zonule but, its precise role in eye development is not known\(^{94}\).

Proteomic analysis on the human ciliary zonule showed that LTBP2 accounts for 10\% of zonule protein\(^{40}\). A recent study using Ltbp2\(^{-/-}\) mice suggests that LTBP2 proteins are required for the synthesis of the ciliary zonule. In knockout mice, the zonular microfibrils were fragmented and ectopia lentis (EL; lens dislocation) developed\(^{94}\). However, ectopic expression of Ltbp4 in Ltbp2\(^{-/-}\) mice has been shown to restored zonular microfibril morphology\(^{95}\), suggesting a possible role for LTBP-4 in the synthesis of the ciliary zonule.

1.2.6 A disintegrin and metalloproteinase with a thrombospondin motif-like protein 4 (ADAMTSL4)

The human genome encodes 28 functional A disintegrin and metalloproteinase with a thrombospondin motif (ADAMTs) proteins, including A disintegrin and metalloproteinase domain with thrombospondin motif-like (ADAMTSL) proteins. Importantly, ADAMTSLs lack
proteolytic activity. The ADAMTSL4 gene is located on the long arm of human chromosome 1 at position 1q21.2. ADAMTSL4 encodes a 116 kDa protein that binds directly to FBN-1 and contributes to microfibril formation.

In humans, mutations in ADAMTSL4 result in isolated ectopia lentis 2 (ECTOL2; [OMIM 225100]) and ectopia lentis et pupillae. Ocular symptoms include ectopia lentis, asymmetric pupils and axial myopia. The role of ADAMTSL4 in eye development is unknown. A recent study examining the expression profile of Adamtsl4 in the mouse eye showed that Adamtsl4 transcripts are strongly expressed by lens epithelial cells at the lens equator, although ADAMTSL4 protein has yet to be localized in the eye. Proteomic analysis indicates that ADAMTSL4 proteins are expressed at low levels in the ciliary zonule compared to other components such as FBN-1. Nevertheless, the genetic evidence suggests an important role for ADAMTSL4 in eye development.

1.2.7 Fibrillin-2 (FBN-2)

The human FBN2 gene is located on the long arm of chromosome 5 at position 5q23.3. FBN2 encodes a 350 kDa ECM glycoprotein called fibrillin-2 (FBN-2). At the amino acid level, FBN-2 is ≈ 69% identical to FBN-1 and 68% identical to FBN-3. Fibrillin-2 differs from FBN-1 and -3 by its FUN located at the N-terminus of the protein.

In-situ hybridization and immunohistochemical experiments show that FBN-2 is expressed strongly during embryonic development (in contrast, to FBN-1 which is predominately expressed in adult tissues). Immunogold labeling experiments on elastic and non-elastic
tissues show that FBN-2 proteins co-localize with FBN-1 proteins in microfibrils \(^\text{100, 103, 104}\). Like FBN-1, biochemical experiments using recombinant proteins show that FBN-2 forms homodimers and heterodimers with FBN-1 \(^\text{70, 105}\). Homodimer and heterodimer formation between FBN-2 and FBN-1 a mediated via intra- and inter- molecular disulfide bonds \(^\text{70, 105}\). Like FBN-1, FBN-2 shares the ability to bind Ca\(^{2+}\) and BMPs.

To date, more than 60 different mutations in the \textit{FBN2} have been reported \(\text{http://www.umd.be/FBN2/}\) \(^\text{106}\). In humans, mutations in \textit{FBN2} mutations give rise to Congenital Contractural Arachnodactyly (CCA; \{OMIM 121050\}) also known as Beals syndrome \(^\text{107}\). CCA is an autosomal dominant connective tissue disorder characterized by severe skeletal abnormalities (joint contractures, scoliosis, kyphosis, chest deformities, and crumpled ears) \(^\text{107}\). Cardiovascular complications are rare but have been reported \(^\text{108}\). Ocular manifestations such as ectopia lentis, lens coloboma, cataracts, and blue sclera are also rare, occurring in only 20% of CCA patients \(^\text{109, 110}\).

The role of FBN-2 in eye development is unclear. A recent study shows that FBN-2-deficient mice develop mislocated pupils (corectopia), iris coloboma, and ciliary body hypoplasia \(^\text{7}\). In regards to the zonule, ZFs are still synthesized in the absence of FBN-2 but their organization is disrupted. In \textit{Fbn2} \(^\text{7}\) mice, FBN-1 expression is significantly increased in the tunica vasculosa lentis, suggesting that FBN-1 expression is upregulated to compensate for the absence of FBN-2 \(^\text{7}\). The mechanism by which FBN-1 is upregulated is not clear, but does raise the question of whether FBN-2 expression might be upregulated in eyes of patients with MFS.
1.2.8 Expression and synthesis of ciliary zonule components

The identity of cells responsible for expressing core ciliary zonule components has not been established definitively. In principle, ciliary zonule components could be expressed by the ciliary body, the lens, or both tissues. Previous in situ hybridization experiments on guinea pig and monkey eyes suggest that FBN1 expression is restricted to cells of the NPCE. Similar results were reported in another study analyzing the expression of Fbn1 and Fbn2 in the developing mouse eye. In that study, Fbn2 was identified as the dominant ciliary zonule component expressed in the embryonic eye while Fbn1 predominated in the adult eye.

Expression of other zonule components of the ciliary zonule such as LTBP2 and MFAP2 have been documented elsewhere in the body but has yet to be characterized in the eye. Although present at low levels in the zonule, recent evidence suggests a role for ADAMTSL4. One study shows that Adamtsl4 transcripts are expressed by lens epithelial cells at the lens equator, suggesting that ADAMTSL4 proteins may have anchorage roles at the tips of the ZFs near the lens surface.

The mechanisms of zonule synthesis and microfibril assembly are also not known. Studies (reviewed above) suggest that microfibril assembly requires a pre-existing scaffold of FN. In the human eye, FN is localized to the basement membranes of the cornea and retina but is nearly undetectable in the ILM of the ciliary epithelium or the lens capsule whether the ZFs attach. Therefore, in the eye, the zonule appears to be assembled in the nominal absence of FN.

In conclusion, relatively little is known about the expression and/or synthesis of ciliary zonule components in the eye. The current literature suggests that zonule proteins may be
produced by cells of the NPCE or the lens. In this report, I attempt to address these questions by determining the mRNA and protein expression profiles of zonule components in the developing mouse eye using quantitative in-situ hybridization and whole-mount immunofluorescence methods.

1.2.9 Mechanical function and properties of the ciliary zonule

In the human eye, the zonule has two functions: to center the lens on the optical axis and facilitate the changes in lens shape that take place during accommodation\(^\text{115}\). The mechanism of accommodation has been debated for many years, and several theories of accommodation have been put forward. The most widely accepted mechanism was proposed by German physicist Hermann Von Helmholtz in 1855\(^\text{116}\).

According to Helmholtz, to achieve near vision, the eye must switch from the unaccommodated to the accommodated state. In the unaccommodated state, the ciliary muscle (a circumferential sphincter-like muscle located at the base of the ciliary body) is relaxed, with the ZFs stretched and lens flattened (Fig. 1.8). During accommodation, centripetal contraction of the ciliary muscle moves the ciliary body forward in the eye, which in turn releases tension in the ZFs. The release in tension in the ZFs allows the lens to assume a spherical shape, increasing its diopteric power, and allowing the eye to focus on near objects (Fig. 8)\(^\text{116}\).

To achieve distant vision, the eye must switch back to the unaccommodated state. Centripetal relaxation of the ciliary muscle (to its original position), restores tension in the ZFs.
As a result, the lens adopts a more flattened shape, allowing the eye to come into focus on distant objects (Fig. 8)\textsuperscript{116}.

In the human eye, biomechanical studies on the ciliary zonule show that ZFs are relatively elastic and capable of stretching up to four times their initial length\textsuperscript{117}. The elastic modulus of the ciliary zonule has been variously reported to be between 0.2-0.25, 0.27-0.34, 0.35 and 1.5 MPa \textsuperscript{118-121}. Mutations in genes encoding zonular proteins often result in breakage of the ZFs and EL \textsuperscript{122}. In this report, I investigate the contributions of zonule components to the strength and integrity of ciliary zonule using mice as a model system (see data section).

### 1.3 Microfibrils

#### 1.3.1 Zonular fibers are composed of microfibrils

Electron microscopy studies on the human ciliary zounule show that ZFs are composed of small-beaded filaments called microfibrils (Fig 1.6)\textsuperscript{15}. Below, I briefly discuss microfibril structure and function.
1.3.2 Microfibril morphology

To isolate microfibrils for study, tissues are either fixed, frozen or extracted using harsh denaturing agents and/or proteolytic enzymes (collagenase, elastase, etc.) \(^{37}\). Due to their small size, microfibrils are typically visualized using various high resolution imaging modalities. Transmission electron microscopy (TEM) and rotary shadowing EM have revealed that microfibrils are 10-12 nm-wide filaments with a “beads-on-a-string” appearance (Fig 1.6). Studies show that untensioned microfibrils have an average periodicity (bead-to-bead distance) of 50 nm to 60 nm. In contrast, microfibrils under tension can reach periodicities as high as 160 nm, indicating that microfibrils are extendable structures \(^{123}\). Three dimensional reconstructions of isolated untensioned microfibrils using electron tomography show that the “beaded” domains of microfibrils are 9 nm in length and 18.7 nm wide \(^{124}\). High resolution analysis reveals that microfibrils have an average interbead length and interbead width of 41.5 nm and 10.2 nm, respectively \(^{124}\). A recent study analyzing the structural morphology of microfibrils isolated from various human tissues found no appreciable difference in microfibril morphology between tissues \(^{125}\). However, a significant difference in microfibril periodicity was found between microfibrils produced \textit{in vitro} versus microfibrils isolated from native tissues, suggesting that microfibrils produced in culture are synthesized under more tension than those microfibrils synthesized \textit{in vivo} \(^{125}\).

Immunogold labeling experiments using monoclonal antibodies against different regions of the FBN-1 revealed that the bead and interbead regions of microfibrils are composed of FBN-1 proteins \(^{51,124,125}\). Measurements on FBN-1 monomers show that FBN-1 proteins are 2.2 nm wide and 150 nm long \(^{51}\). Based on previous studies (those mentioned above), it has been proposed that eight FBN-1 monomers constitute a single microfibril \(^{124}\).
1.3.3 Arrangement of fibrillin proteins in microfibrils

The precise arrangement and/or organization of FBN-1 monomers within microfibrils has become a topic of debate for many years. Two competing models have been proposed. The first, known as the *unstaggered model*, was put forward by Sakai et al. using antibody epitope mapping to full length FBN-1 and immunogold electron microscopy. The *unstaggered* model proposes that FBN-1 molecules are aligned in parallel and arranged in a non-overlapping head-to-tail (N-terminus to C-terminus) fashion within microfibrils (Fig. 1.9). This model is supported by the finding that monoclonal antibodies recognizing the N- and C-terminal ends of FBN-1 bind to opposite sides of the beaded domains. In contrast, the second model, known as the *staggered model*, suggests that FBN-1 molecules are arranged in an anti-parallel head-to-tail arrangement spanning two or three interbead distances (Fig. 1.9). This model was supported by nuclear magnetic resonance and X-ray diffraction studies showing exposure of certain 8-cys-TB and cbEGF within microfibrils.

A recent study using the same antibodies as described previously showed that incubating microfibrils with bacterial collagenase ablated the outer filaments of microfibrils, revealing the interbead regions of microfibrils. The authors concluded that the arrangement FBN-1 proteins revealed in these studies may favor the staggered model.
1.3.4 Fibrillin microfibril formation is mediated via intermolecular binding of FBN-1 proteins

It has been proposed that the first step in microfibril assembly is homotypic interactions between FBN-1 molecules, governed via intermolecular disulfide bonding between cysteine residues of FBN-1 molecules. The third cysteine residue in the first hybrid domain of FBN-1, Cys 204, exists as a free thiol (-SH) and can facilitate dimer formation. It is unclear whether these interactions occur intracellularly or extracellularly.

The mechanisms by which fibrillin monomers polymerize to form microfibrils are not well understood. Some studies suggest that the assembly of FBN-1 microfibrils involve scaffolding and/or interactions with other microfibril proteins. For example, FBN-1 has been shown to form heterotypic interactions with FBN-2, although it remains unclear whether these interactions occur inside or outside of the cell. FBN-1 and FBN-2 co-localize in the same microfibrils, suggesting that interactions between these two isoforms may be important for microfibril synthesis. Adult tissues stained with an antibody to FBN-1 show that FBN-1 is abundant in postnatal tissues, while FBN-2 protein expression was limited, consistent with previous studies. However, collagenase treatment on postnatal tissues revealed that FBN-2 epitopes persisted in adult tissues, suggesting that postnatally, the presence of FBN-1 protein masks the presence of FBN-2. These data are consistent with the notion that FBN-2 may have a scaffolding function, facilitating the assembly of FBN-1 microfibrils.
1.3.5 The arrangement of microfibrils in ZFs

The precise manner in which zonular microfibrils are packed in ZFs is not known. Ultrastructural studies show that microfibrils are orientated in parallel and tightly packed within ZFs. Quick freeze deep-etch microscopy on the bovine ciliary zonule show that individual zonular microfibrils are connected by 4 nm-wide filaments (composition unknown). This observation was confirmed in a recent study by Godwin et al. analyzing the hierarchical organization of fibrillin microfibrils in the bovine zonule. In that study, Godwin et al. also noted that ZFs were wrapped in 5 µm wide-filaments, suggesting that these filaments are responsible for stabilizing ZFs. However, these observations have yet to be confirmed.

1.3.6 Microfibril Biomechanics

Atomic force microscopy on individual zonular microfibrils suggests that these filaments are stiff structures with a modulus of 78-96 MPa. X-ray diffraction studies on hydrated ZFs revealed that zonular microfibrils are reversibly extensible filaments with periodicities ranging from 56 nm to 80 nm. X-ray diffraction studies on untensioned zonular microfibrils show that a 50% extension of microfibrils, with a resting length (periodicity) of ~56 nm, does not result in increased periodicity and is reversible. However, a 100% or 150% extension of microfibrils the results in periodicities of 80 nm and 100 nm, respectively. Previous reporting of microfibril periodicities of 160 nm, equivalent to a strain of 270%, have been reported but are unlikely to occur under physiological conditions because of irreversible molecular deformation of FBN1 proteins and loss of tissue integrity. Further examination on the behavior fibrillin microfibrils during extension reveals a decrease in fibril diameter and
interfibrillar spacing between individual FBN-1 molecules suggest that FBN-1 proteins undergo lateral compaction molecular unfolding or when stretched extension \(^{133}\). All together, these data show that microfibrils are extendable filaments with an average periodicity of 56 nm in their resting state and a 90 nm periodicity when stretched.

The mechanism by which microfibrils extend and retract is not known but, some studies suggest that these properties may be influenced by interdomain linker regions between protein domains or by binding Ca\(^{2+}\) ions. Proline-rich linker regions between 8-cys-TB/cbEGF and hybrid/cbEGF domains are predicted to provide up to 35 nm of extensibility ((5 nm of extension per 8-cys-TB/cbEGF domain (7)) \(^{68}\). Linker regions between hybrid and cbEGF domains are estimated to contribute up to 7 nm of extensibility (3.5 nm of extensibility per hybrid/cbEGF domain (2)) \(^{68}\). Ca\(^{2+}\) binding to cbEGF domains has been shown to play an important role in the linearity and structure of fibrillin protein \(^{61}\). Biochemical experiments on FBN-1 monomers (150 nm) deprived of Ca\(^{2+}\) ions significantly shorten the length (25%) of FBN-1 monomers, suggesting that the binding of Ca\(^{2+}\) to cbEGF promotes FBN-1 extension \(^{61}\). Together, these data suggest that proline-rich linker domains and the binding or release of Ca\(^{2+}\) may play important role in the extensibility of FBN-1 proteins in microfibrils but the precise physiological contributions are not known.
1.4 Marfan syndrome

1.4.1 The history of Marfan syndrome and the fibrillin-1 gene

In 1896, the French pediatrician Antoine Marfan, was the first to describe the disease that we now know as Marfan syndrome (MFS)\textsuperscript{134}. MFS is a pleiotropic connective tissue disorder affecting the cardiovascular, skeletal, and ocular systems\textsuperscript{135}. The original description concerned a five year-old-girl who had dolichostenomelia (long limbs), arachnodactyly (long fingers) and severe scoliosis\textsuperscript{134,136}.

Histological analyses of tissues collected from MFS patients revealed abnormalities in the connective tissues. Initially, these findings led investigators to suspect that genes encoding collagens were implicated. However, collagens were soon excluded on the basis of genetic linkage analysis\textsuperscript{137}. Significantly, an immunohistological study on human skin biopsied from MFS patients showed reduced levels of fibrillin microfibrils in the dermis\textsuperscript{138}. Shortly after, another MFS study identified defects in fibrillin microfibril synthesis, secretion, and incorporation into the ECM\textsuperscript{139}. Using linkage analysis, and known genetic markers, Kainulainen \textit{et al.}, mapped the Marfan locus to human chromosome 15\textsuperscript{140}. Shortly thereafter, Dietz \textit{et al.} localized the mutation to a centromeric region on chromosome 15 at position 15q-21\textsuperscript{141}. In order to locate the \textit{FBN1} gene and determine whether the Marfan mutation mapped to the same location in genome, Lee \textit{et al.} used molecular cloning and genetic linkage analysis\textsuperscript{99}. Partial cloning of the \textit{FBN1} gene, isolated from cultured human fibroblasts, produced a 1.6 kb cDNA fragment. The open reading frame of this cDNA fragment matched the amino acid
sequence of a previously described FBN-1 peptide, thus confirming that the isolated cDNA fragment was cloned from the FBN1 gene. In situ hybridization experiments using fibrillin cDNA probes, mapped the gene to the same centromeric region on chromosome 15 as described previously. Lee et al. performed genetic linkage analysis on three separate multi-generational families with MFS and linked the mutations to chromosome 15 at position 15q-21 thus confirming fibrillin as the defective gene in MFS. These results were later confirmed by Dietz et al. showing tight linkage of two de novo missense mutations (codon 239, R239P) to chromosome 15q15-21.

1.4.2 MFS is a pleiotropic connective tissue disorder

MFS is a pleiotropic connective tissue disease that affects the cardiovascular, skeletal and ocular tissues of the body. MFS patients live relatively normal lives but can be shortened due to cardiovascular complications (thoracic aortic aneurysms). In addition, individuals with MFS develop severe skeletal abnormalities, which include dolichostenomelia, scoliosis, arachnodactyly, and pectus excavatum (sunken chest). Furthermore, mutations in FBN1 profoundly affects the eye. The major ocular manifestation is EL. Other ocular complications include cataract, microspherophakia, axial myopia, and glaucoma (see section 1.5).

1.4.3 Diagnosis of MFS

According to the revised Ghent Nosology (Fig. 10), an accurate diagnosis for MFS is as follows: In the absence of a family history of the disease, a combination of aortic aneurysm with
EL and/or a systemic score ≥7 and/or a bonafide *FBN1* mutation previously reported to be associated with aortic aneurysm is a positive diagnosis for MFS (Fig. 10). In the presence of a family history, the development of an aortic aneurysm or EL, or a systemic score ≥ 7 constitutes a positive diagnosis for MFS (Fig. 1.10)\(^{144}\). Since the revision to the Ghent Nosology, more than 15% of patients have been classified as having MFS or some other connective tissue disease\(^{145}\).

Epidemiological studies suggest that the prevalence of MFS is 1 in 5,000 to 10,000 births\(^{146}\). There is no ethnic, racial, or gender bias in MFS\(^{147}\). The average age of diagnosis is between 19-20 years of age with roughly 10% to 25% of cases being diagnosed before the age of seven\(^{148}\).

### 1.4.4 Genomic mutations in MFS

The predicted mutation rate in MFS is five per million genes per generation\(^{149}\). Approximately 75% of mutations in MFS are inherited, while 25% of mutations occur sporadically (de novo mutations)\(^{149}\). Human mutations in MFS are completely penetrant with variable expressivity between individuals with the same mutation and within families, making genotype-phenotype correlations challenging.

To date, more than 3,000 mutations have been reported in the *FBN1* gene and linked to MFS (http://www.umd.be/FBN1/). At the chromosome level, nearly all (97%) *FBN1* mutations are small chromosomal rearrangements involving only a few base pairs while large chromosomal rearrangements affecting 1 ≥ exons (3%) are rare. At the gene level, most of the *FBN1* mutations occur in exons, while relatively few are reported in the intronic and untranslated regions of the gene (www.umd.be/FBN1). Exome sequencing has identified an
overrepresentation of mutations in exon 13, 26, 27, 28, 43, 45, and 57, while exons 45 and 57 are significantly underrepresented.

Mutations in FBN1 give rise to different modes of the disease ranging from classical to severe MFS. Approximately 53% of exonic mutations in FBN1 give rise to classical MFS, while 35% of exonic mutations give rise to the most severe form of MFS known as neonatal MFS (nMFS). nMFS is associated with mutations in exons 24-32 and is characterized by early onset thoracic aortic aneurysms. A mouse model expressing an in frame internal deletion of exons from 19-24 recapitulates some of the cardiovascular complications associated with MFS. The remaining 12% of exonic mutations give rise to fibrillinopathies.

Mutations in non-coding intronic and untranslated regions of the FBN1 gene have occasionally been reported. Such mutations are predicted to affect splice donor and splice acceptor sites. An overrepresentation of splicing mutations have been reported in exon/intron junctions between exon/intron 18/19 and 50/51 affecting a cbEGF and 8-cys-TB domain, respectively (www.umd.be/FBN1).

1.4.5 Types of FBN1 mutations

In MFS, mutations are scattered throughout the entire FBN1 gene. According to a recent study characterizing the different types of FBN1 mutations in 1013 probands with MFS, missense mutations (56%) are the most common type of mutation while frameshift mutations (17%), nonsense (14%), splicing (11%), and in frame deletions/insertions (2%) occur at lower frequencies.
1.4.6 Mutations in FBN-1 protein domains

At the protein level, 80% of FBN1 mutations affect cbEGF domains. This is unsurprising because of the fact that cbEGF motifs comprise 60% of the FBN-1 protein. Of the cbEGF domain mutations, approximately 60% are missense mutations while 15% are non-sense mutations. Missense mutations in cbEGFs domains either create (~8%) or substitute (~50%) cysteine residues implicated in disulfide bonding. Mutations in cysteine residues are likely to promote protein misfolding and proteolytic degradation of FBN-1 proteins. The second motif most commonly affected in MFS is the 8-cys-TB domain. Thirteen percent of FBN1 mutations occur in 8-cys-TB domains. Missense mutations dominate in 8-cys-TB-domains (63%), although appreciable amounts of frameshift mutations (24%) have also been reported (www.umd.be/FBN1). In addition to MFS, FBN1 mutations in 8-cys-TB domains can also give rise to rare diseases that mimic MFS known as fibrillinopathies, which include Stiff Skin Syndrome, Acromelic dysplasia and WMS.

1.4.7 Genotype-phenotype correlations

Genotype-phenotype correlations are difficult to make in MFS because of the range in expressivity of the disease. However, some tentative conclusions can be drawn. For example, patients with missense mutations involving a cysteine residue, either creating or substituting a cysteine, have a higher probability of developing EL compared to other types of FBN1 mutations (this is also true for patients that express in-frame mutations in FBN1 compared to patients that express premature stop mutations). In contrast, patients expressing premature stop mutations have an increased risk of developing cardiovascular and skeletal complications (aortic
dilation/dissection) compared to other types of FBN1 mutations. Interestingly, individuals with mutations in the neonatal region of the FBN1 gene, exons 24-32, have a higher probability of developing aortic root dilation/dissection, ectopia lentis, and scoliosis compared to mutations in other exons. More specifically, patients with mutations in exon 25 (cbEGF #11) have a lower rate of survival and higher probability of developing aortic dilation/dissection and mitral valve regurgitation compared to those with mutations in other exons of the neonatal region (exons 24-32).

1.4.8 Is MFS a Dominant negative or Haploinsufficient disease?

Over the years, there has been considerable debate in the field whether MFS functions as a dominant negative (DN) or haploinsufficient (HI) disease. DN mutations constitute inframe mutations, which include missense mutations, inframe deletions/insertions and inframe splicing mutations. HI mutations comprise out-of-frame mutations, which include premature stop mutations, non-sense mutations, frame-shift mutations, and out-of-frame deletions and splicing mutations. At the protein level, heterozygous DN mutations are predicted to produce an abnormal protein that would interfere with the normal FBN-1 protein function. HI mutations are predicted to be more deleterious and are likely to be loss of function mutations resulting in depleted FBN-1 and a reduced complements of microfibrils in the ECM. Recent evidence suggest that ≥ 30% of MFS patients have HI mutations and that those patients are at increased risk of cardiovascular complications and mortality compared to patients with DN mutations. The majority of mutations in MFS are missense mutations that occur in cbEGF domains. Therefore, one might predict that MFS is a DN disorder, leading to the production of
antimorphic FBN-1 proteins that are incorporated into the ECM. However, in the case of nMFS, mutations in the neonatal region of the FBN1 (exons 24-32) are dominated by missense mutations. These are predicted to give rise to a less severe form of MFS compared to HI mutations but, in fact, nMFS is very severe thus contradicting the null hypothesis that DN mutations lead to a less severe form of the disease. As a result, the mode of DN and HI mutations in MFS is unclear and continues to complicate the predicted clinical outcomes of the disease.

1.4.9 Disease pathology of MFS

Over the years, there has been much debate in the field as to the underlying molecular mechanism of MFS. The cause of premature death in MFS is the development of thoracic aortic aneurysms\textsuperscript{144}. Studies investigating the pathogenesis of cardiovascular complications in MFS have shown that MFS patients with aortic aneurysms have elevated levels of TGF-\(\beta\) signaling in connective tissues.

To test the role of FBN-1 in cardiovascular disease, mouse models expressing different types of Fbn1 mutations have been generated\textsuperscript{162}. As with humans, FBN-1 mutant mice develop aortic aneurysms with increased TGF-\(\beta\) signaling\textsuperscript{152,163}. To determine whether TGF-\(\beta\) signaling played a role in the pathogenesis of MFS, mutant mice were treated with drugs targeting TGF-\(\beta\) or its receptors. In one study, FBN-1 mutant mice were treated with Losartan; an angiotensin II type 1 receptor antagonist that binds to TGF-\(\beta\) receptors and inhibits TGF-\(\beta\) signaling. Treatment of mutant mice with Losartan rescued the aortic aneurysm phenotype in FBN-1 mutant mice and improved cardiovascular tissue morphology\textsuperscript{164}. These data suggest that increased TGF-\(\beta\) signaling causes MFS in mice. Based on such studies, a model has emerged in which
dysregulated TGF-β signaling plays a central role in the etiology of the disease \(^{165}\).

Accordingly, several clinical trials were initiated to test the efficacy of Losartan in treatment of MFS in humans. \(^{166}\). Unfortunately, the results of the trials have been largely disappointing. In most studies, MFS patients treated with Losartan showed delayed aortic root dilation but this did not prevent the development of aortic aneurysms. In a few studies, Losartan treatment showed significant regression of the aortic root \(^{167-169}\). These studies suggest that the mechanism of MFS may differ from that in humans and that the current disease model in MFS (which places defective TGF-β signaling in a central role) may be incorrect.

### 1.5 Marfan syndrome and the eye

#### 1.5.1 Ectopia lentis: The major ocular manifestation in MFS

Ectopia lentis (EL) is defined as the displacement of the crystalline lens in the eye (Fig. 1.11). In humans, two types of EL are recognized: lens subluxation and lens luxation. A *subluxated* lens is partially dislocated \(^{170}\) while a *luxated* lens is completely dislocated \(^{122}\).

In MFS, EL is the major ocular manifestation and one of two main diagnostic criteria, the other being aortic aneurysm, used to diagnose the disease \(^{144}\). EL occurs in \(\approx 60\%\) of MFS patients \(^{136}\). Symptoms of EL include headaches, dizziness, blurred vision, diplopia (double vision), and eye pain \(^{171}\). In most cases, MFS patients develop EL between the ages of 10 to 20, although the lens can dislocate at any time in life \(^{136}\). Lens dislocation is typically bilateral and
usually occurs in the superior direction (Fig. 1.11), although the lens can dislocate in any direction\textsuperscript{136}. A large study analyzing the eyes of 160 MFS patients showed that eyes without EL had an average visual acuity of 20/20 while eyes with EL have a visual acuity ranging from 20/25 to 20/80. Blindness due to a dislocated lens is rare but, can occur if the eye is left untreated.

In cases where the lens is only slightly subluxated, ophthalmologists may suggest using a noninvasive optical correction approach such as specialized glasses or contact lenses\textsuperscript{171}. However, in cases where the lens is luxated, it must be removed immediately in a procedure known as lensectomy. Lensectomy is required if the malpositioned lens bisects the pupillary space, is displaced into the vitreous cavity or becomes cataractous\textsuperscript{171}.

Ocular surgery is complicated in MFS patients due to fragility of the eye and risk of secondary complications such as corneal edema, iris prolapse, or retinal detachment.\textsuperscript{171} Nevertheless, if surgical intervention is necessary, ophthalmologists perform a procedure called vitreolensectomy; removal of the lens and partial removal of the vitreous gel. After the lens is removed, the eye may be left aphakic (lensless) or an artificial intraocular lens (IOL) may be implanted. Many ophthalmologist favor leaving the eye aphakic to reduce the risk of retinal detachment and glaucoma\textsuperscript{171}. This option is particularly favored in young children where the eye has not yet fully developed. In adults, an IOL is usually placed in the eye. Typically, during cataract procedures the IOL is placed in the capsular bag. However, because the lens capsule is no longer supported by ZFs in MFS patients (or, if present, the ZFs are weak) the IOL must be sutured to the sclera in the posterior chamber of the eye\textsuperscript{171}. 

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Studies have shown that patients with EL have a pre-operative visual acuity ranging from 20/25 to 20/80 while post-operatively visual acuities can be restored to 20/20-20/25, although in some patients, the visual acuity is never improved. Slit lamp examinations on MFS patients suggest that EL is due to breakage of the ZFs. However, it is unclear where the breakage occurs or why the mutant ZFs are prone to breakage. Studies on isolated ectopic lenses from MFS patients suggest that the fibers are abnormal structure and/or that the number of ZFs attached to the lens is decreased.

There is little information about the mechanism of EL in MFS and other connective tissue disorders. Animal models have been generated to investigate the role of FBN1 mutations in the cardiovascular and skeletal manifestations associated with MFS, but in none of the existing models do the ocular symptoms that characterize MFS manifest. Therefore, a primary goal of my thesis was to develop a suitable model to gain insights into the role of FBN-1 in eye development and disease.

1.5.2 Genetics of ectopia lentis

In humans, EL occurs either in isolation or in association with a systemic disorder (such as MFS). Isolated EL has two modes of inheritance: an autosomal dominant and autosomal recessive form. Autosomal dominant EL is caused by missense mutations in FBN1. Mutations in FBN1 can give rise to isolated ectopia lentis-1 (ECOL1; [OMIM 129600]). ECOL1 is a difficult disease to diagnosis due to tight genetic linkage of FBN1 mutations with MFS alongside mild cardiovascular and skeletal abnormalities. According to the revised Ghent nosology, a patient is to be diagnosed with ECOL1 only in the presence of a dislocated lens(es).
and a *FBN1* mutation that has not been previously reported or known to cause thoracic aortic dilation/dissection (Fig. 10) 144.

Isolated EL can also result from recessive mutations in the *ADAMTSL4* gene; isolated ectopia lentis 2 (ECTOL2; [OMIM 225100]). 176. The most common type of mutation in ECTOL2 patients are 20 base pair deletions in exons 6 through 19 of the *ADAMTSL4* gene, resulting in premature stop codons and truncation of the ADAMTSL4 protein, although point mutations have also been reported 179,180. The genetic evidence thus suggests an important role for *ADAMTSL4* in the structure and stability of the ciliary zonule but its precise contribution is unknown.

EL is also a feature of systemic disorders such as MFS, AD WMS, and metabolic diseases such as Homocystinuria 122,176. Homocystinuria is caused by mutations in the *CBS* gene that encodes the enzyme cystathionine beta-synthase. Previously, homocystinuria was sometimes mistaken for MFS due to the high incidence of EL (90%) in homocystinuria patients 181. Consequently, a differential diagnosis and testing criteria have been put in place to differentiate between the two diseases. Interestingly, however, subcutaneous injection of the enzyme cystathionine beta-synthase into *Cbs*- mice partially restores the structural organization of the zonule, suggesting a role for the enzyme in the synthesis of the ciliary zonule 182.
1.5.3 Minor ocular manifestations of MFS

Ocular manifestations of MFS, other than EL, are not weighted heavily in the nosology. This is because of the relatively high incidence of these conditions in the general population.

Below is a short summary of some of the “minor” ocular manifestations seen in MFS.

1.5.4 Cornea

In MFS, the cornea is often too big and flat; a condition in humans known as megalocornea 136. The horizontal corneal diameters of MFS patients are significantly larger than the corneal diameters of non-MFS patients (13 mm and 11mm, respectively) 136. It is not known why the cornea should be enlarged but, it has been hypothesized that flattened corneas may be secondary to the increased axial length of the eye globe seen in many MFS patients.

1.5.5 Cataract and Microspherophakia

Cataracts are common in MFS and occurs earlier in life (30’s to 40’s) than in the general population (60’s) 147. MFS is associated with the development of posterior subcapsular and cortical cataracts 171. Cataracts are often seen in association with EL, suggesting that lens clarity may be dependent on its position in the eye 171.

Microspherophakia (small spherical lens) has been reported in MFS but is more commonly associated with AD WMS and WMS3 183. It has been hypothesized that decreased
tensile force exerted on the lens surface by weakened fibers results in a smaller and more spherical lens.

1.5.6 Glaucoma

Glaucoma is an optic neuropathy characterized by loss of optic nerve axons and retinal ganglion cells. The most common form of glaucoma in MFS is primary open angle glaucoma (POAG). POAG is often but not always associated with an increase in intraocular pressure (IOP). An increase in IOP in the eye can cause severe eye pain and, if left untreated, can result in irreversible vision loss. Some studies have reported that MFS patients have elevated IOPs (25 mmHg to 30 mmHg), however, most MFS patients have IOPs that fall within the normal range (12 mmHg to 22 mmHg). The glaucomatous phenotype in MFS may reflect structural changes in the trabecular meshwork of the eye (the tissue responsible for the drainage of aqueous humor) or the lamina cribrosa (the structure through which the ganglion cell axons leave the eye).

Other forms of glaucoma in MFS include pupillary block glaucoma and phacolytic glaucoma. Pupillary block glaucoma is perhaps the second most common form of glaucoma in MFS. Pupillary glaucoma is caused by sealing of the ectopic lens against the pupil, resulting in increased resistance of aqueous humor outflow through the pupillary space. This form of glaucoma is treatable by removal of the ectopic lens. Phacolytic glaucoma is caused by blockage of the outflow pathways of the eye by leaking lens proteins from cataractous lenses. Phacolytic glaucoma is treatable by removal of the opaque lens. If left untreated, however, the
lens particulate material can cause an immune response inside the eye leading to inflammation, obstruction of the trabecular meshwork, corneal edema, etc 171.

1.5.7 Myopia

Axial myopia is the second most common ocular manifestation in MFS 136. Myopia is present in 34% to 44% of MFS patients. MFS patients can have as many 15 diopters of myopia, although most patients fall within the range of -1 to -3 diopters 136, 189. The eyes of MFS patients are significantly longer than usual. Interestingly, the increase in axial length of the eye globe is correlated with EL 136. Studies show that eyes of MFS patients with EL have significantly longer axial lengths (25.96 mm) compared to eyes without EL (23.29 mm) 136. The cause of axial elongation in MFS is unknown but conceivably mutations in FBN1 could alter the biomechanical properties of the sclera making the wall of the eye compliant and facilitating axial elongation 7, 171.

Other eye globe abnormalities in MFS include strabismus (eye globe misalignment), amblyopia (abnormal vision in one eye), and blue (thin) sclera 171. Strabismus is relatively common in MFS and seen in 19% to 39% of cases 190. The most common form of strabismus in MFS is exotropia (outward eye misalignment). The cause of strabismus in MFS is not known but it has been speculated that expression of mutant FBN-1 in the extraocular muscles may alter the mechanical properties of the tissues causing slippage of the eye globe. 190.
1.5.8 Retinal detachment

Retinal detachment, the primary cause of blindness in MFS, occurs in 5% to 25% of patients\textsuperscript{171}. Retinal detachment is typically bilateral\textsuperscript{171}. Several studies have shown that retinal detachment is correlated with EL and increased axial myopia. In one study, \(\approx10\%\) of MFS patients with EL developed retinal detachment\textsuperscript{181}. Another showed that retinal detachment was common in myopic eyes with axial lengths approaching 28 mm, while eyes without retinal detachment had an average axial length of 24 mm\textsuperscript{136}. It is has been speculated that the increase in axial length of the eye globe causes the retina to tear or detach from wall of the eye.
1.6 Figures

Figure 1.1 Embryonic mouse eye development. On embryonic day 8.5 (E8.5) the optic vesicle evaginates towards the overlying surface ectoderm (A). Cells of the periocular mesenchyme are enclosed between the optic vesicle and surface ectoderm. Around E9.0 and E9.5, contact between the optic vesicle and surface ectoderm induces cells of the ectodermal cells to thicken forming the lens placode (B). On E10.5, the invagination of the optic vesicle and lens placode forms the optic cup and the lens pit, respectively (C). The inner layer of the optic cup will give rise to the neural retina, while the outer layer will give rise to the presumptive retinal pigment epithelium (C). On E11.5, the anterior lips of the lens pit fuse and detach from the overlying surface ectoderm giving rise to the corneal epithelium and the lens vesicle (D, E). At E12.5, the primary lens fibers cells (1°LFs) elongate anteriorly to fill the lumen of the lens vesicle (E). By E13.0, the lumen of the lens vesicle is filled and lens polarity is established consisting of the anterior lens epithelium facing the front of the eye and the lens fiber cells facing the back of the eye (F). Diagram is adapted from Cvekl et al. 3 OV, optic vesicle; SE, surface ectoderm, POM, periocular mesenchyme; LP, lens placode; LP, lens pit; NR, neural retina; RPE, retinal
Figure 1.2 Diagram of the adult human eye. The eye can be divided into the anterior segment and the posterior segment. Anterior segment constitutes the cornea, iris, pupil, lens, and ciliary body. The posterior segment consist of the vitreous gel, retina, and optic nerve. Adapted from the National Eye Institute (nei.nih.gov). Note: This figure does not include the ciliary zonule (fibers that bridge the gap between the lens and the ciliary body located directly behind the iris).
Figure 1.3 The ciliary zonule of the adult monkey eye. Anterior view of the monkey ciliary zonule. The circumferential array of MFAP2-positive ZFs (green) extend radially from the surface of the lens near its equator. Image ciliary body and attach to the surface of the equator of the lens. This image was adapted from Parel JM et al. 19
Figure 1.4 Zonular fibers in the pars plana region of the ciliary body. As the zonular fibers (Z) approach the ciliary folds (labeled as “CP” in this image), fibers merge with one another (arrow) to form larger fibers that run a lateral course along the surface of the ciliary folds. Magnification (200X). Adapted from Rohen et al. 22
**Figure 1.5** The zonular fork of the monkey ciliary zonule. MFAP2-positive ZFs (green) originate from the pars plana region of the ciliary body and fuse together to form larger fibers before entering the valleys of the ciliary folds. As the ZFs exit between the ciliary folds, fibers split into a “fan-like” structure called the zonular fork. ZFs span the narrow gap between the ciliary body and lens before attaching to the equator of the lens. Scale bar: 10 µm. This image was adapted from De Maria *et al.*^40^
**Figure 1.6** Rotary shadowing electron microscopy on a guanidine-extracted microfibril isolated from human fetal tissue in the presence of calcium. Chemically extracted microfibrils are composed principally of FBN-1 proteins and have a characteristic “beads on a string” appearance. Scale bar: 100 nm. Figure adapted from Kuo *et al.* 126
Figure 1.7 Fibrillin protein structure. The human genome encodes three fibrillin proteins (FBN 1-3). Fibrillins are modular proteins, consisting of epidermal growth factor domains (4/47 (EGF)), calcium binding epidermal growth factor binding domains (43/47 (cbEGF)), seven 8-cysteine-TGF-β binding like motifs (TB), and two hybrid domains (hybrid). Each fibrillin contains at least one (FBN-1) or two (FBN-2 and -3) integrin binding sites. Fibrillins also contain N-glycosylation sites dispersed throughout the protein. This image was adapted from Gossack AP et al. 43
**Figure 1.8** Accommodation of the human eye. ZFs of the human eye emerge from the surface of the ciliary epithelium and attach to the anterior and posterior surface of the lens. In the unaccommodated eye (*top image*), the ciliary muscle (CM) is relaxed creating tension (T) in the ZFs. This conformation of the eye flattens the lens, allowing the eye to focus on distant objects. In the accommodated eye (*bottom image*), the CM moves forward (dashed lines) and centripetally in the eye. Forward movement of the CM loosens the T on the fibers, causing the lens to assume a more spherical shape (dashed lines). This conformation of the eye is suitable for viewing objects up close. Anterior zonule; AZ, ciliary muscle; CM, Posterior zonule; PZ, Tension, T. This image was adapted from Charman WN *et al.* 115
**unstaggered parallel arrangements**

N- and C-termini close to the beads

![Unstaggered Parallel Arrangements](image)

**staggered parallel arrangements**

N- and C-termini close to the beads

![Staggered Parallel Arrangements](image)

**Figure 1.9** Fibrillin arrangement in microfibrils. In the *unstaggered model*, microfibrils are fully extended with the N- and C-termini of FBN-1 proteins aligned in parallel. The beaded regions of microfibrils (circles) represent clustering of the N and C-terminal ends of fibrillin proteins while the interbead regions (lines) correspond to the central regions of fibrillin proteins. In the *staggered model*, microfibrils are fully extended, with FBN-1 proteins arranged in an antiparallel fashion with the N-terminus of one fibrillin protein overlapping the C-terminus of another fibrillin protein. The beaded domains in this model are thought to represent the overlapping N and C-termini of fibrillin proteins. This image was adapted from Reinhardt et al. 47
Figure 1.10 The revised Ghent nosology (2010). The diagnosis of MFS is contingent on the presence of two phenotypes: increased aortic root diameter (Ao) and/or ectopia lentis (EL). This nosology also provides a differential diagnosis for disorders that resemble MFS. This table was adapted from Loeys et al. 144
Figure 1.11 Ectopia lentis in MFS. Split lamp examination through the dilated eye of a 27-year-old MFS patient with ectopia lentis (EL) shows superior dislocation of the crystalline lens. ZFs appear as “glassy” filaments when visualized by slit lamp and appear stretched in the direction of the dislocated lens. Large gaps (arrows) are present between ZFs, suggesting the deletion or breakage of ZFs in the eye. This image was adapted from Lally DR. 170
1.7 References

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Chapter 2: Targeted deletion of fibrillin-1 in the mouse eye results in ectopia lentis and other ocular phenotypes associated with Marfan syndrome

Preface

The ciliary zonule consists of a system of extracellular fibers that connect the lens to the wall of the eye. ZFs are composed of small filaments called microfibrils. Microfibrils are major structural elements of the extracellular matrix. Proteomic analysis on the human and bovine zonule show that the ZFs are composed principally of FBN-1. Human mutations in FBN1 underlie MFS and AD WMS; pleotropic connective tissue disorders that affect the eye. Several models of MFS have been generated to investigate the role of FBN-1 in the cardiovascular and skeletal systems of the body. None, however, manifests the ocular symptoms associated with the disease. The purpose of this study was to identify the cells responsible for synthesizing the ciliary zonule and investigate the role of FBN-1 in eye development and disease using mice as a model system.

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2.1 ABSTRACT

Fibrillin is an evolutionarily ancient protein that confers elasticity and resiliency on a variety of tissues. In humans, mutations in fibrillin-1 cause Marfan and related syndromes, conditions in which the eye is often severely affected. To gain insights into the ocular sequelae of Marfan syndrome, we targeted Fbn1 in mouse lens or non-pigmented ciliary epithelium (NPCE).

Conditional knockout of Fbn1 in NPCE, but not lens, profoundly affected the ciliary zonule, the system of fibrillin-rich fibers that centers the lens in the eye. The tensile strength of the fibrillin-depleted zonule was reduced substantially, due to a shift toward production of smaller caliber fibers. By three months, zonular fibers invariably ruptured and mice developed ectopia lentis, a hallmark of Marfan syndrome. At later stages, untethered lenses lost their polarity and developed cataracts, and the length and volume of mutant eyes increased. This model thus captures key aspects of Marfan-related syndromes, providing insights into the role of fibrillin-1 in eye development and disease.
2.2 INTRODUCTION

Fibrillin-1 is a large, cysteine-rich glycoprotein of the extracellular matrix. Mutations in \textit{FBN1} underlie Marfan Syndrome (MFS; Mendelian Inheritance in Man [MIM]: 154700) and the autosomal dominant form of Weill-Marchesani Syndrome (AD WMS; [MIM]: 608328), \textsuperscript{1,2} conditions that severely affect the eye. Ocular manifestations include lens dislocation (ectopia lentis; EL), microspherophakia, cataract, lens thickening, glaucoma, iris trans-illumination defects, flattened cornea, and axial myopia.\textsuperscript{3-6} EL is almost ubiquitous in WMS patients and sufficiently common (>60\% \textsuperscript{3,7}) in MFS patients to be included as one of two cardinal features in the disease nosology (the other being aortic root dilation).\textsuperscript{8}

To investigate the roles of \textit{FBN1} mutations in the pathophysiology of MFS and related conditions, the \textit{Fbn1} locus has been targeted extensively in mice. Numerous models have been generated, including hypomorphs, nulls, and missense mutations\textsuperscript{9}. Collectively, these capture key features of human MFS, including kyphoscoliosis, rib overgrowth,\textsuperscript{10} and aneurysms of the ascending aorta.\textsuperscript{11} None, however, manifests the spectrum of ocular symptoms that characterize MFS and WMS in humans. As a result, we know relatively little about the role of fibrillin-1 in normal eye development or the pathophysiological impact of \textit{FBN1} mutations in MFS or WMS.

EL, the most common ocular symptom of MFS and WMS, results from instability or rupture of the ciliary zonule, a network of fibrillin-rich extracellular fibers that suspends and centers the lens in the eye. The zonular fibers span the narrow gap between the non-pigmented ciliary epithelium (NPCE; located at the inner wall of the eye) and the lens equator. In species that accommodate, the zonule transmits the forces that flatten the lens, bringing distant objects into focus. Each zonular fiber is composed of hundreds or thousands of microfibrils,\textsuperscript{12} long
filamentous structures $\approx$10-12 nm in diameter, with a characteristic “beads-on-a-string” appearance when examined by electron microscopy.\textsuperscript{13} Partial breakage of zonular fibers manifests as iridodonesis, or tremulousness of the iris.\textsuperscript{14} More profound rupture leads to the complete untethering of the lens (EL).

Proteomic studies indicate that zonular fibers are composed of several dozen proteins, of which fibrillin-1 is by far the most abundant\textsuperscript{15-17}. Other important components include microfibrillar-associated protein-2 (MFAP-2), and latent-transforming growth factor β-binding protein 2 (LTBP2), each comprising about 10% of the proteome by mass. Fibrillin-2, the predominant fibrillin isoform during embryonic development, persists in the adult zonule.\textsuperscript{18} The zonule is also enriched in ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs)-like proteins (notably ADAMTSL-6 and ADAMTSL-4), which promote microfibril formation in vitro\textsuperscript{19} and cross-linking enzymes such as transglutaminase-2 and lysyl oxidase-like 1, which may stabilize the fibrillar structure.

In the current study, we identified the cells responsible for synthesizing zonular proteins. We then used Cre-Lox technology to conditionally disrupt the $Fbn1$ locus in selected tissues and examine how fibrillin-1 depletion affected the structural and mechanical properties of the zonular fibers. Critically, conditional $Fbn1$ knockout mice developed EL and other sequelae of MFS in humans. As such, the mice constitute a powerful model for testing clinical strategies to treat MFS in the eye.
2.3 RESULTS

2.3.1 Zonular proteins are produced by NPCE and lens cells.

The processes leading to synthesis and assembly of the ciliary zonule are unclear. One possibility is that ocular tissues collaborate in its production. Alternatively, a single tissue could generate the fifty-or-so proteins that constitute the core “zonulome”\textsuperscript{16}. We used multiplexed fluorescence \textit{in situ} hybridization to monitor the expression of transcripts encoding some of the major zonule components (\textit{Fbn1}, \textit{Fbn2}, \textit{Mfap2}, \textit{Ltbp2}, and \textit{Adamtsl4})\textsuperscript{16} in tissues of the anterior mouse eye (Fig. 1). Of the genes examined here, \textit{Fbn2} was expressed most strongly in the eyes of young mice. At post-natal day 1 (P1), \textit{Fbn2} transcripts were abundant and restricted largely to the NPCE (Fig. 1A), consistent with previous reports\textsuperscript{20}. \textit{Mfap2}, by contrast, was expressed at comparable levels in the NPCE and lens epithelium (Fig. 1A). At later stages, expression of zonular transcripts in the NPCE became concentrated in the pars plana region, the narrow band of cells interposed between the folded pars plicata region and the retina (Fig. 1B). Both \textit{Fbn1}, encoding the most abundant zonular protein in the adult, and \textit{Ltbp2} were expressed strongly in this region of the NPCE at P30. We quantified expression of the various transcripts in the lens and NPCE from P1 to 1 year of age (Fig. 1C). This analysis showed that \textit{Fbn1}, \textit{Fbn2}, and \textit{Ltbp2} were expressed predominantly in the NPCE throughout development, with comparatively low expression levels in the adjacent lens epithelium. \textit{Adamtsl4} was the only transcript to show preferred expression in the lens at all time points. Collectively, these data indicated that while some zonular proteins (fibrillin-1, fibrillin-2, LTBP2) were derived largely from NPCE, others
(ADAMTS1-4) were produced solely by the lens, and some (MFAP-2) were synthesized by both lens and NCPE.

2.3.2 Conditional deletion of Fbn1 in NPCE cells (but not lens) depletes the zonule of fibrillin-1.

Mice homozygous for a Fbn1 germline deletion die shortly after birth,\(^1\) precluding their use in studies of zonule synthesis or stability. Therefore, we used the Cre-Lox conditional knockout strategy to target the Fbn1 locus in lens or NPCE, the two tissues flanking the zonule. We first obtained transgenic mice that expressed Cre in NPCE or lens. In Pax6aCre transgenic mice, Cre is expressed in the nasal/temporal segments of the NPCE/retina\(^2\). Similarly, the MLR10Cre transgene directs Cre expression to the mouse lens.\(^3\) We verified the published tissue-specific Cre expression patterns using the mTmG reporter line (Supplemental Fig. 1) and then crossed the mice with animals carrying a floxed Fbn1 allele.\(^4\) Animals derived from the crosses were indistinguishable in appearance and body weight from wild types (Supplemental Fig. 2), suggesting that expression of the Cre transgenes and/or the presence of the floxed Fbn1 allele did not have obvious untoward effects.

The zonule was not noticeably affected in Fbn1\(^{Lox/Lox}\); MLR10Cre\(^{Tg/-}\); mTmG\(^{Tg/-}\) (hereafter, referred to as Fbn1-lens) mice (Fig. 2). Radially-oriented zonular fibers projected from the folded surface of the NPCE, attaching to the lens at its equator. Fibrillin-1 immunofluorescence was uniform along the length of zonular fibers and in fibers located at different points around the circumference. The gap between the tips of the ciliary processes and the lens equator (approximately 250 \(\mu\)m) was consistent at all radial locations and did not differ from age-matched controls.
In Fbn1+/+;Pax6aCreTg/;mTmGTg/ mice (hereafter, called controls), all fibers were labeled strongly with antibodies against fibrillin-1 (Fig. 3A). In contrast, in Fbn1Lox/+;Pax6aCreTg/;mTmGTg/ mice (hereafter, referred to as Fbn1-NPCE mice), fibrillin-1 immunofluorescence was largely abolished in fibers extending from the Cre-positive nasal/temporal segments of NPCE (Fig. 3B) but preserved in fibers projecting from the Cre-negative superior/inferior segments. Immunolabeling with antibodies against LTBP2, revealed that despite the nominal absence of fibrillin-1, zonular fibers were present in the Cre-positive nasal/temporal quadrants, and at densities approaching those of the superior/inferior quadrants (Fig. 3C). It has been shown previously that upregulation of one fibrillin isoform in the eye may compensate for loss of another.18, 25 Hence, we examined the distribution of fibrillin-2 protein in fibrillin-1-depleted regions. Fibrillin-2 immunofluorescence was increased in fibers projecting from the Cre-positive nasal/temporal regions of the NPCE (Fig. 3D), consistent with the hypothesis that upregulated Fbn2 expression may compensate for the absence of fibrillin-1. Quantitative multiplexed in situ hybridization was used to assess the recombination efficiency in Cre-positive regions of the NPCE and determine whether the observed increase in fibrillin-2 immunofluorescence in the nasal/temporal quadrants (Fig. 3D) signified an increase in Fbn2 transcription in those regions. Compared to age-matched wild types, controls, or Cre-negative regions of Fbn1-NPCE mice, Fbn1 expression levels in Cre-positive regions were reduced significantly (by >80% (Fig.4)). With regard to Fbn2 expression, there was no significant difference between Cre-positive and Cre-negative regions of Fbn1-NPCE mice (Fig. 4B). Together, these data indicate that Fbn1 expression was reduced substantially in Cre-positive regions and that increased fibrillin-2 fluorescence in the fibrillin-1-depleted zonule was not associated with increased Fbn2 transcription.
Close examination of fibers projecting from Cre-positive regions of the NPCE revealed that they were not entirely devoid of fibrillin-1 (Fig. 3B and Fig. 5). At higher magnification, faint fibrillin-1 immunofluorescence was visible in those segments of the zonular fibers proximal to the lens surface. Due to mild mosaicism in transgene expression, small islands of Cre-negative cells were sometimes present in the nasal/temporal quadrants (see supplemental Fig. 1). In contrast to the surrounding fibers, zonular fibers projecting from the Cre-negative islands were labeled throughout their lengths by the fibrillin-1 antibody (Fig. 5B).

2.3.3 EL develops in Fbn1-NPCE mice but not in Fbn1-lens mice or controls.

The position of the lens in Fbn1-NPCE, Fbn1-lens, or control mice was assessed non-invasively by optical coherence tomography (OCT; Fig. 6). In control animals, the iris had a convex configuration, due to the support of the underlying lens. The central region of the anterior lens surface was faintly visible through the pupil, allowing the anterior chamber depth (ACD; the distance between the inner surface of the cornea and the front surface of the lens) to be measured. Until 6 weeks of age, lens position was indistinguishable between the various genotypes (Fig. 6A). However, between 6 weeks and 8 weeks of age, approximately 70% of Fbn1-NPCE mice developed unilateral or bilateral EL. By 3 months, 100% of the mice had developed bilateral EL. Mice were followed for periods of >1 year. Over this period, EL did not develop in control, Fbn1-lens animals, or mice heterozygous for the floxed Fbn1 allele. The change in ACD was quantified for the various genotypes (Fig. 6B). Mean ACD increased sharply (from ≈0.3 to ≈0.8 mm) between 6 and 12 weeks of age in Fbn1-NPCE mice only. The imaging depth of the OCT system was 1.6 mm. As a result, the final disposition of ectopic lenses within the eye could not be verified. We overcame this limitation by examining fixed eyes in three dimensions by X-ray microscopy (Fig. 7). The X-ray images of control eyes revealed the
relative positions of the lens, ciliary body, and iris (Fig. 7A). In 3-month-old Fbn1-NPCE mice, the ectopic lenses were located immediately adjacent to the retina (Fig. 7B). In the absence of the supporting lens, the iris adopted a flattened configuration and the ciliary processes, which are normally angled forward in the eye were, instead, oriented perpendicular to the eye wall. ACD measurements suggested that EL began to develop between 6- and 8-weeks-of-age. Focusing on this period, we processed Fbn1-NPCE eyes for confocal microscopy, to determine the pattern of breakage of the zonular fibers. In most cases, we found zonular fibers to be either completely broken or entirely intact. However, in two eyes (out of 12 examined) we identified eyes in which a subset of zonular fibers was broken (Fig. 8). In those cases, the stretch of broken fibers was located in the fibrillin-1-depleted nasal region. The fact that only rarely did we observe the zonule in the process of breaking suggests that the partially ruptured zonule is mechanically unstable and breakage of the remaining fibers occurs quite quickly. Close observation indicated that the zonular fibers break in mid-span (Fig. 8B) rather than, for example, detaching from anchorage points on the lens or surface of the ciliary processes.

2.3.4 The tensile strength of the Fbn1-depleted zonule is reduced but breaking displacement is not.

To test whether fibrillin-1 depletion affected the tensile strength of the zonule, we measured the force and vertical displacement required to break the zonular fibers of 1-month-old mice (Fig. 9). At that age, the appearance of the fibrillin-1-depleted fibers at the level of resolution of the light microscope, was indistinguishable from controls (Fig. 3A-C). In wild type (C57/Bl6) mice, fibers broke after approximately ≈10 mN of tensile force were applied (Fig. 9A). Ultimate
tensile strength increased substantially (by about 30%) in older (1- or 2-year-old) wild type animals. The breaking displacement (the vertical distance the lens could be raised from the immobilized eye cup before the zonule ruptured) was also substantially greater in older eyes than younger eyes (0.55 mm and 0.85 mm, respectively; Fig. 9B). In 1-month-old Fbn1-NPCE mice, the breaking force was reduced by ≈ 55% compared to age-matched controls (Fig. 9C), a significant decrease (p=0.002). The reduction in breaking force was not paralleled by changes in displacement distance (Fig. 9D).

In 2-month-old mice, the breaking force for the Fbn1-NPCE zonule was reduced by >90% compared to age-matched controls (Fig. 9E), while the breaking displacement was comparable between genotypes (Fig. 9F). The tensile strength of the zonule in Fbn1-lens mice showed no such reduction (Supplemental Fig.3A) and the displacement distance was not significantly different from controls (Supplemental Fig. 3B). Thus, after disruption of Fbn1 expression in the NPCE (and not lens) the structural integrity of the zonule was severely compromised.

In Fbn1-NPCE mice, fibrillin-1-rich zonular fibers coexist with fibrillin-depleted fibers (Fig. 3B,C). The pull-up technique measured the properties of the two types of fibers in parallel. To better distinguish the properties of wild type (fibrillin-1-rich) and fibrillin-1-depleted fibers, the superior/inferior and nasal/temporal sets of fibers were cut selectively in control or Fbn1-NPCE eyes (Fig. 10). In control eyes, cutting the fibers in the nasal/temporal quadrants or superior/inferior quadrants had comparable effects, in both cases reducing the overall breaking force by 50-60% (Fig. 10A). However, in Fbn1-NPCE mice, selectively cutting the superior/inferior fibers caused a larger proportional reduction in tensile strength than cutting the nasal/temporal fibers (70% vs 39%, respectively). We conclude that the residual tensile strength of the mutant zonule is largely attributable to the presence of fibrillin-1-rich zonular fibers in the
superior and inferior quadrants. The vertical displacement values were comparable in the two
genotypes, whether subpopulations of fibers were severed or not (Fig. 10B).

2.3.5 Loss of Fbn1 is associated with reduced fiber density and fewer microfibrils per
bundle.

Loss of fibrillin-1 from zonular fibers was associated with a substantial reduction in tensile
strength (Figs. 9 and 10). To identify structural correlates, we examined the zonule in 1-month-
old controls and the Cre- positive and Cre-negative regions of Fbn1-NPCE mice, by high
resolution SEM (Figs. 11).

The wild type zonule consisted of a fine web of radially-oriented fibers (Fig. 11A). The fibers
emerged from a fibrous cladding on the surface of the ciliary processes and spanned the 200-300
μm gap separating the NPCE from the lens equator. Converging towards the lens, fibers
commonly coalesced into larger bundles. These were notably absent from fibrillin-1-deficient
regions of Fbn1-NPCE eyes (Fig. 11B), where, instead, individual fibers projected from the
NPCE directly to the lens. Imaged at their midpoints, fibers in control eyes or the superior (Cre-
negative) regions of Fbn1-NPCE eyes (Fig. 11C) were substantially thicker than fibrillin-1-
depleted fibers (Fig. 11D), although a wide range of fiber diameters was present in all cases.
Fibrillin-rich and fibrillin-depleted fibers were both composed of microfibrils, visible at high
magnification. The diameter of the component microfibrils did not differ significantly between
the Cre-positive and Cre-negative regions of Fbn1-NPCE eyes (diameter = 10.8±1.8 nm (mean ±
SD; n=33) for Fbn1-depleted fibers, 10.5 ± 2.3 nm (n=30) for Fbn1-rich fibers, and 10.65 ± 1.9
nm (n=141) for controls).
In addition to influencing fiber diameter, depletion of fibrillin-1 in the NPCE had a marked effect on the appearance of the ciliary processes (Fig. 12). In control mice, the surface of the processes was obscured by the presence of a loose basket weave of orthogonally oriented microfibril bundles (Fig. 12A,B). The microfibril cladding was largely absent from Cre-positive regions of the ciliary epithelium in Fbn1-NPCE mice (Fig. 12C,D).

To quantify the effect of fibrillin depletion on zonular fiber structure we used SEM to measure the diameter of several hundred randomly selected fibers from control mice, and the Cre-positive (nasal/temporal) and Cre-negative (superior) regions of Fbn1-NPCE mice (Fig. 13). For each genotype, a broad distribution of fiber diameters was recorded, ranging from 0.1-4.0 μm (Fig. 13A). This range of fiber widths corresponds to <100 microfibrils per fiber to > 100,000 microfibrils per fiber (under the assumption that fibers are circular in cross-section, microfibrils are 11 nm in diameter, and microfibril packing is efficient (i.e. a hexagonal lattice)). The fiber size distributions were positively skewed in all cases, with a high proportion of small diameter fibers and relatively few large (i.e., >1.0 μm) fibers. The size distribution was similar in control eyes or fibers emanating from the superior regions of Fbn1-NPCE eyes. In contrast, compared to control or Cre-negative regions, the size distribution of fibers emanating from Cre-positive nasal/temporal regions was skewed toward smaller diameter fibers. For example, ~30% of fibers from the nasal/temporal regions had diameters <0.2 μm, whereas only 10% of control or Cre-negative fibers fell in this range. Similarly, large diameter fibers were comparatively rare in the fibrillin-1-depleted nasal/temporal regions. Thus, fibers with diameters >1 μm accounted for 20% (174/868) and 14 % (43/297) of control and Cre-negative Fbn1-NPCE fibers, respectively, but only 4% (25/603) of Cre-positive NPCE fibers. The size distributions of fibers emanating from various quadrants of control and Fbn1-NPCE eyes were compared using the Kolmogorov-
Smirnov test (Fig. 13B-D). There was no significant difference in the diameter of fibers projecting from the superior and nasal/temporal regions of the NPCE in control eyes (Fig. 13B). Similarly, there was no significant difference in fiber diameter between the superior region of wild type eyes and the superior (i.e., Cre-negative region) of Fbn1-NPCE eyes (Fig. 13C). Finally, we compared the diameters of fibers emanating from the superior (Cre-negative) region of Fbn1-NPCE eyes with those projecting from the nasal/temporal (Cre-positive) region of the same eyes (Fig. 13D). Fibers from the Cre-negative region had a mean diameter of 0.61 μm and a median diameter of 0.48 μm (n=123). In comparison, fibrillin-1-depleted fibers emanating from the Cre-positive region had a mean diameter of 0.35 μm and a median diameter of 0.27 μm (n=267). The Kolmogorov-Smirnov statistic, D, was 0.35 and the null hypothesis (that fibers from the Cre-positive and Cre-negative regions share a common probability distribution) was rejected (p<0.001). Thus, fibrillin-1 depletion in the nasal/temporal quadrants of Fbn1-NPCE mice was associated with significant thinning of the zonular fibers emanating from those regions. We also noted that fiber density (i.e. the number of fibers observed per microscopic field) was also markedly reduced (by approximately 50%), although we did not attempt quantify that effect. In summary, morphometric analysis suggests that the number of zonular fibers per unit length of NPCE is reduced in fibrillin-1-depleted areas compared to fibrillin-1-rich areas and that the fibers are significantly thinner.
2.3.6 Aging Fbn1-NPCE mice develop additional Marfan-like ocular symptoms, including cataract and axial elongation.

In wild type mice, lenses generally remain transparent, even in aged animals (Fig. 14A). Ectopia lentis developed in 100% of Fbn1-NPCE eyes by 3 months of age (Fig. 11B). Initially, dislocated lenses retained their transparency but, as the animals aged, the ectopic lenses usually became opaque (Fig. 14B-D). Faint cataracts were first noted at 6 months. Thereafter, prevalence increased such that, by one year, 86% of eyes from Fbn1-NPCE mice had dense cataracts. In some cases, the cataractous lenses disintegrated, leading to the expulsion of the lens core, which was expressed through the pupil and visible in the anterior chamber (Fig. 14C).

Histological analysis of the ectopic lenses (Fig.15) showed a range of pathophysiological changes, including liquefaction of the cortical layers and, in extreme cases, rupture of the lens capsule. In all cases, the cataractous lenses were smaller than age-matched controls. An early aspect of the lens phenotype was the overgrowth of the anterior lens epithelium, which often expanded to envelop the entire lens (Supplemental Fig.4). Proliferating lens cells, which in wild type lenses are restricted to the germinative zone (a region of the equatorial epithelium immediately anterior to the lens equator), were instead distributed across the anterior and posterior surface of Fbn1-NPCE lenses. These data suggest that tissue polarity was disturbed in the ectopic lenses. Gross observation suggested that the volume of the eye globe was often increased in Fbn1-NPCE mice compared to controls (Fig. 16A), a finding confirmed subsequently by biometry. Beginning at about 8 months of age, eyes of Fbn1-NPCE mice had a significantly larger volume (Fig. 16B). This was attributable to an increase (of about 14%) in the axial length of the globe (rather than an increase in equatorial diameter). At later stages, the cornea became hazy or vascularized in the buphthalmic eyes. In humans, mutations in FBN1 can
result in glaucoma and pressure-induced buphthalmia.\textsuperscript{26} To test whether increases in ocular volume were the result of elevated intraocular pressure (IOP) within the eye, we measured IOP in Fbn1-NPCE and age-matched controls. In both genotypes, there was a modest decline in IOP from \(\approx 18 \text{ mm/Hg}\) in young animals to \(\approx 12 \text{ mm/Hg}\) in aged animals but pressure differences between control type and mutant eyes did not reach statistical significance. On two occasions, a sharply elevated IOP (>50 mm/Hg) was measured in eyes of aged Fbn1-NPCE mice. In both cases, the cataractous lens had ruptured and particulate material was visible by OCT in the anterior chamber (Supplemental Fig. 5). This condition may be analogous to phacolytic glaucoma in humans, which is similarly associated with release of lens material from hypermature cataracts. The pressure readings from those two eyes were not included in the aggregated data shown in Fig. 16.

\section*{2.4 DISCUSSION}

The mouse eye may serve as a useful model for understanding the ophthalmic sequelae of Marfan and related syndromes. Here, we developed two conditional knockouts and used them to gain insights into the role of fibrillin-1 in eye development and disease.

\subsection*{2.4.1 Zonule synthesis.}

The source of the proteins that make up the zonule is a long-standing question in eye development. Our in situ hybridization data suggest that the major structural proteins (fibrillin-1, fibrillin-2, LTBP2) are most likely derived from the pars plana portion of the NPCE (although
transcripts for MFAP-2, another key zonule protein, were equally abundant in lens and NPCE). Mutations in ADAMTSL4 cause autosomal-recessive isolated EL ([MIM]: 225100)\textsuperscript{27} and ectopia lentis et pupillae ([MIM]:225200)\textsuperscript{28}, implying that ADAMTSL-4 has a role in zonule stability. Our in situ hybridization experiments on Adamtsl4 confirmed earlier observations that the gene is expressed strongly in equatorial lens with very low levels in the NPCE.\textsuperscript{29} Thus, although the NPCE is the probable source of the major structural components of the zonule, production of the full proteome likely requires the coordinated synthetic activity of the NPCE and lens. With regard to the most abundant zonular protein, fibrillin-1, we generated mice in which the Fbn1 locus was disrupted in either NPCE or lens. It was only in the former case that fibrillin-1 immunofluorescence was markedly depleted in the zonule. Similarly, tensile strength and zonular fiber diameter were affected only when Fbn1 was targeted in NPCE cells, and EL developed in Fbn1-NPCE mice only. These observations lend further credence to the notion that the NPCE is the major site of fibrillin-1 synthesis. We noted that, in Fbn1-NPCE mice, residual fibrillin-1 immunofluorescence was detected in segments of the zonular fibers proximal to the lens. Fibrillin-1 incorporated at this location may originate in the lens (where in situ hybridization analysis identified weak Fbn1 expression early in development). The generation of double-conditional knockout (lens plus NPCE) mice would be necessary to test this hypothesis.

The relative expression levels of the various zonule genes fluctuated over time. For example, Fbn2 was expressed strongly in young eyes, whereas Ltbp2 transcripts were undetectable at P1 but abundant by one month of age. This implies an age-dependence to the molecular composition of zonular fibers. Presumably, fibers synthesized early in life will be enriched in fibrillin-2, whereas those produced later will contain a higher proportion of LTBP2.
The impact (if any) of such compositional fluctuations on zonule biomechanics remain to be determined. The observation that *Fbn1* transcripts were abundant in the NPCE of aged mice allows the possibility that microfibril components turnover with time. Our data are thus consistent with early in situ hybridization and radiolabeling experiments, which suggested that zonule synthesis continues, albeit at reduced levels, in the aged eye\textsuperscript{30, 31}. Pulse-chase experiments would be required to test this hypothesis. Such studies are of particular interest because they speak to whether the repair of zonular fibers is possible in MFS patients with compromised zonules.

Although fibrillin-1 is the major structural component of microfibrils, conditional knockout of *Fbn1* in the NPCE did not completely eliminate the zonular fibers. There are several explanations for this somewhat surprising finding. First, as a result of the segmental expression pattern of the *Pax6aCre* transgene, *Fbn1* expression in the superior and inferior regions of the knockout eye was unaffected. Thus, even in knockout eyes, a substantial contingent of wild type fibers remained. Fibrillin-1 produced in the *Cre*-negative superior and inferior regions of the NPCE may have been incorporated into zonular fibers in the *Cre*-positive nasal and temporal regions. It is also likely that *Fbn1* transcription was not eliminated completely in the *Cre*-positive regions. There was a small degree of mosaicism in *Cre* transgene expression and *Cre*-mediated recombination may not have been 100% efficient, as suggested by the in situ hybridization experiments. Finally, it is clear for this and earlier studies that fibrillin-2 is expressed in the zonule. Although we did not find evidence of a compensatory increase in *Fbn2* transcription, extant fibrillin-2 (in combination with residual fibrillin-1 expression), may have been sufficient to produce a zonule that was initially serviceable but failed at later time points.
2.4.2 Structural and Mechanical Properties.

We used a novel pull-up technique to measure the ultimate tensile strength of the mouse zonule. The force required to break the Fbn1-NPCE zonule in 1-month-old mice (about 5 mN) was less than half that required to break the zonule of age-matched control eyes. This value fell further, to about 10%, in 2-month-old animals. Due to the presence of a contingent of wild type fibers (in the superficial and inferior quadrants), mechanical measurements on the entire zonule likely overestimated the tensile strength of the fibrillin-1-depleted fibers. The displacement values (about 0.6 mm at one month of age) did not differ significantly between genotypes, consistent with the notion that there were fewer fibers in the mutant eyes rather than fibers of a qualitatively different type. Scanning electron microscopy provided support for this view. Fibrillin-1-rich and fibrillin-depleted fibers had a similar morphology but the size distribution of the fibers was shifted significantly toward smaller diameters in the Fbn1-NPCE mice. The number of microfibrils per fiber is a function of the cross sectional area of the fiber, so relatively modest differences in diameter have large effects on the total number of microfibrils present. We also noted decreased fiber density in the Fbn1-NPCE eyes. Together the shift in fiber diameter and reduced fiber density provides a satisfying explanation for the measured reduction in tensile strength of the mutant zonule.

The displacement distance was approximately 0.6 mm in most cases. The gap between the lens equator and the NPCE is about 0.2 mm. Simple geometry suggests that equatorial fibers stretch by >3 fold before finally rupturing. Thus, mouse zonular fibers are apparently similar in extensibility to human zonular fibers. In larger species, where such measurements are practicable, the elastic modulus of the zonule has been calculated. In pig, for example, the modulus is 200-250 kPa, while in humans it is about 300 kPa. In the mouse, the small size of
the eye and the broad distribution of fiber diameters make a determination of the elastic modulus problematic. Interestingly, the modulus of the component microfibrils is believed to be orders of magnitude greater than that of the zonule as a whole. Thus, the elastic behavior of zonular fibers probably derives from interactions between microfibrils rather than the elastic properties of the microfibrils themselves.

2.4.3 Mutational mechanisms

More than 3,000 mutations in \textit{FBN1} have been identified in human MFS (http://www.umd.be/FBN1/). Because of the dominant mode of inheritance and the observation that fibrillin-1 forms the core of multimeric structures (microfibrils), it was long assumed that \textit{FBN1} mutations function as dominant negatives (DN). Recently, however, MFS patients have been identified with unambiguous deletions of an \textit{FBN1} allele.\textsuperscript{36, 37} Significantly, these patients exhibit the full spectrum of symptoms associated with MFS, including EL. Such instances imply that classical MFS can also arise via haploinsufficiency (HI). Indeed, some studies estimate that >30\% of adult MFS patients carry HI mutations and there is accumulating evidence that HI mutations may be associated with a more aggressive disease course.\textsuperscript{38} The risk of cardiovascular death, for example, is elevated by 2.5 fold in patients with HI relative to DM mutations.\textsuperscript{39, 40} In \textit{Fbn1}-NPCE mice, \textit{Cre} expression was directed to the nasal/temporal segments of the NPCE and fibrillin-1 incorporation into the adjacent ciliary zonule was significantly reduced. Thus, genetically, our mice most closely resemble human MFS patients harboring HI \textit{FBN1} mutations. Mice heterozygous for a germline \textit{Fbn1} knockout do not have an ocular phenotype.\textsuperscript{21} Similarly, in our study, mice heterozygous for the floxed allele did not develop EL. This suggests that in mice, fibrillin-1 expression in the NPCE must be reduced substantially (i.e., by >50\%) in order to generate an ocular phenotype.
EL was first observed in 8-week-old mice and the EL phenotype was 100% penetrant by 3 months of age. This time course generally parallels that of human MFS patients, where the lens is supported initially by the zonule and EL often manifests during childhood or early adolescence.\(^4^1\) In the mice, it is possible that lens dislocation is triggered simply by increased physical activity of the young animals, which would place increasing mechanical demands on the mutant zonular fibers. Alternatively, fibrillin-1-depleted fibers may be more prone to proteolysis during aging. Microfibrils have been shown to serve as substrates for matrix metalloproteases, several of which are expressed in the eye.\(^4^2\)

### 2.4.5 Ophthalmic sequelae

In human MFS patients, ectopic lenses are normally removed surgically. In the Fbn1-NPCE mice, luxated lenses remained in place, becoming gradually cataractous and in some cases disintegrating completely. Even in transparent dislocated lenses, the fundamental organization of the lens tissue was found to be severely disturbed. The epithelial layer, which is normally restricted to the anterior surface, expanded to envelop the entire lens. In healthy wild type lenses, S-phase cells are confined to the germinative zone, a swath of the equatorial epithelium.\(^4^3\) The germinative zone was unrecognizable in the dislocated lenses, with S-phase cells distributed throughout the epithelium. The eye contains diffusible signals that serve to specify the polarity of the lens.\(^4^4\) One such molecule is fibroblast growth factor (FGF), which is produced by the retina and consequently found at higher concentration in the posterior segment of the eye than the anterior. The FGF gradient triggers fiber cell differentiation specifically at the lens posterior and thus has a critical role in lens polarity. Presumably, morphogen gradients are scrambled for a
free-floating lens, leading to dysregulated patterns of lens cell proliferation and differentiation and, ultimately, opacification.

There was a significant increase in ocular volume at later stages in Fbn1-NPCE mice, attributable largely to an increase in axial length. In emmetropic human eyes mean axial length is about 23.5 mm\(^{45}\) compared to 24-25 mm for MFS patients.\(^{5, 6, 46}\) As a result, MFS patients are often highly myopic. The cause of axial elongation in the Fbn1-NPCE mice is unclear. Clinically, a buphthalmic (enlarged) eye can result from increased intraocular pressure, sometimes secondary to lens distintegration (so-called phacolytic glaucoma). We excluded increased IOP as the cause of axial elongation in all but two animals. In most cases, IOP was indistinguishable between control and Fbn1-NPCE animals (with or without cataract). Another possibility is that elongation is a response to blurring of the retinal images following the onset of EL. Form-deprivation myopia is a well-known but poorly understood phenomenon in many vertebrate species, including mice\(^{47}\) and describes a process whereby low contrast retinal images stimulate scleral growth and axial elongation. The mispositioning of the lens in the Fbn1-NPCE mice would undoubtedly blur the retinal image but it is not known whether this evokes a scleral growth response, especially in adult mice. Finally, it is possible that the enlarged globes observed in aged Fbn1-NPCE mice were the result of edema secondary to an inflammatory reaction triggered by the release of protein from hypermature cataracts. Lens-induced uveitis is a well-recognized clinical entity, resulting from an immune response to antigenic material released from ruptured lenses.

In conclusion, we have shown that in mice, disrupting Fbn1 expression in the temporal/nasal regions of the NPCE is sufficient to produce EL, cataract and buphthalmia. Because the onset of EL (between 6 and 8 weeks of age) is predictable, and changes in the mechanical properties of
the zonule readily quantifiable, these mice represent a useful model for testing clinical interventions aimed at strengthening or preserving the zonular fibers.

2.5 MATERIALS AND METHODS

2.5.1 Animals

Procedures were approved by the Washington University Animal Studies Committee.

2.5.2 In situ hybridization

Mice (C57BL/6J; Jackson Laboratory, Bar Harbor, ME) were killed by CO₂ inhalation. Tissues were prepared from 1-day-old to 1-year-old mice. Enucleated eyes were fixed for 24 h in 4% RNase-free paraformaldehyde/PBS, embedded in paraffin, and sectioned in the midsagittal plane at 4 µm. Transcripts encoding zonular components were localized by multiplexed fluorescence in situ hybridization (RNAscope Multiplex Fluorescence Assay; Advanced Cell Diagnostics, Hayward, CA). The following probe sets were utilized: Fbn1 (NM_007993.2; bases 2290-3183), Fbn2 (NM_010181.2; bases 2933-3893), Ltbp2 (NM_013589.3; bases 5453-6485), Mfap2 (NM_008546.3; bases 36-1064), and Adamtsl4 (NM_144899.2; 2624-3725). In situ hybridization was also used to visualize Cre (KC845567.1; bases 1058-2032) and GFP (U55763.1; bases 628-1352) expression in transgenic animals. Each probe consisted of 20 sets of oligonucleotides designed to hybridize to a contiguous ≈1kb region of target mRNA. Following hybridization and labeling, sections were counterstained with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI). As a negative control, adjacent sections were hybridized with DapB, a
bacterial gene encoding the enzyme dihydrodipicolinate. Pre-mixed PolR2A (DNA-directed RNA polymerase II polypeptide A), Ppib (Peptidylprolyl Isomerase B), and Ubc (Ubiquitin C) probes were used as positive controls. The specificity of target probes was verified using samples from Fbn1Lox/Lox;Pax6αCre+/−, Ltbp2−/−, Mfap2−/−, and Fbn2−/− mice.

The hybridization/labeling procedure generated a punctate pattern of fluorescence on the tissue sections. Each fluorescent punctum corresponds to a single mRNA transcript. To gauge the relative abundance of transcripts over time in tissues flanking the zonule, the number of puncta over the NPCE and equatorial lens epithelium was determined using ImageJ analysis software.

2.5.3 Generation of Conditional Fbn1 KO mice

Targeted deletion of Fbn1 in the NPCE and retina was achieved by crossing Fbn1Lox mice24 with Pax6αCre mice (Jackson Lab, Bar Harbor, ME).22 In Pax6αCre animals, Cre recombinase is expressed from E10.5 in the inner layer of the optic cup (corresponding, in adults, to NPCE and retina). To visualize the distribution of Cre-expressing cells, Fbn1Lox/Lox;Pax6αCre+/− mice were crossed with the Cre reporter strain, mTmG (Jackson Laboratory, Bar Harbor, ME).49 On the mTmG background, Cre activity causes a shift in expression of fluorescent membrane-targeted proteins, from tandem dimer Tomato (tdTomato) to green fluorescent protein (GFP). Conditional deletion of Fbn1 in lens was achieved by crossing Fbn1Lox/Lox mice with MLR10Cre mice, which express Cre in lens epithelial and fiber cells from E10.5 onward.23
2.5.4 Immunofluorescence

Mice were anesthetized with a mixture of ketamine (80 mg/kg body weight) and xylazine (6 mg/kg) and perfused transcardially with 4% paraformaldehyde/PBS (pH 7.4). Perfusion fixation preserved the delicate structure of the zonular fibers, while simultaneously stabilizing the wall of the eye. Following fixation, eyes were enucleated and a small hole was made in the posterior wall of the globe to facilitate the penetration of fixative. Isolated globes were then immersed in 4% paraformaldehyde/PBS for a further 24 hours. Fixed eyes were washed thoroughly in PBS and the posterior portion of the globe was dissected to the level of the pars plana and discarded. The remaining tissue was incubated in 8% bovine serum albumin (BSA)/PBS blocking solution to minimize non-specific antibody binding. Samples were incubated overnight with primary antibodies in 4% BSA/PBS. After several washes in PBS, samples were incubated with appropriate fluorescently-labeled secondary antibodies (1:200) for 2 h at room temperature. Samples were washed in PBS, placed in a glass bottom dish, and imaged by confocal microscopy. Nuclei were counterstained with Draq5 (Cell Signaling Technology, Danvers, MA, USA). Eyes from three or more animals were examined. To verify the specificity of antibody labeling, antibodies were tested on germline Fbn1-null and Fbn2-null mouse eye tissues (data not shown).

2.5.5 Antibodies

Goat-polyclonal anti-MAGP1 was obtained commercially (R&D Systems, Minneapolis, MN) and used at a dilution of 1:100. Anti-mouse fibrillin-1 (pAb 9543; 1:200) and anti-mouse fibrillin-2 (pAb 0868; 1:100) were provided by Dr. Lynn Sakai (Oregon Health and Science
University, Portland, OR) and anti-Ltbp2 was obtained from Tomoyuki Nakamura (Kansai Medical University, Osaka, Japan).

2.5.6 Ocular Phenotyping

**Gross analysis**

Mice were examined under a dissecting microscope for the presence of dislocated lenses, cataracts, or buphthalmos (enlarged eyes).

**Intraocular pressure (IOP)**

Intraocular pressure measurements were made using a rebound tonometer (iCare TonoLab tonometer, Vantaa, Finland). To avoid injury to the animal or misfiring of the probe, the tonometer was fixed to a stage and anesthetized mice were positioned such that the center of the cornea was aligned with the center of the probe. Three or more measurements were made per eye.

**Optical Coherence Tomography (OCT)**

The anterior segments of anesthetized mice were visualized non-invasively by OCT (Bioptogen Envisu R2210) using a 10 mm telecentric bore lens with an imaging depth of 1.6 mm and an
optical resolution of 2.4 µm. Measurements were made on both eyes and completed within five minutes. Two hundred B-scans (each composed of 1000 A-scans) were averaged to generate the final images. Anterior chamber depth (ACD) was measured along the optical axis from the inner surface of the corneal endothelium to the anterior surface of the lens capsule using software supplied with the instrument.

**Biometry**

The outer dimensions of the globe were determined from digital photos of enucleated eyes. Axial length was measured from the corneal surface to the posterior sclera adjacent to the optic nerve. Equatorial dimensions were measured perpendicular to the axial axis at the widest point. Ocular volume was calculated assuming the globe to be an oblate spheroid.

**2.5.7 Lens optical analysis**

To gauge their transparency and refractive properties, lenses were dissected from euthanized mice, placed into pre-warmed tissue culture medium, and photographed against a grid pattern. Dark field illumination was used to assess light scattering within the tissue.
2.5.8 Confocal Microscopy

Images were collected using an LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) or FV1000 confocal microscope (Olympus). Stacks of optical sections were visualized as maximum intensity projections.

2.5.9 Scanning Electron Microscopy

Eyes from euthanized 1-month-old Fbn1+/+;Pax6aCRE and Fbn1Lox/Lox;Pax6aCRE mice were carefully enucleated, fixed for 5 min in 4% paraformaldehyde and dissected down to the level of the pars plana. The dissected eyes were rinsed with PBS to remove any vitreous humor that might adhere to and thereby obscure the zonular fibers. Eyes were then fixed further in 2.5% paraformaldehyde/2.5% glutaraldehyde. Fixed tissue was critical point dried, sputter coated with gold and imaged using a Merlin–FE scanning electron microscope (Zeiss). The diameters of zonular fibers were determined from the SEM images at 1000 X magnification. A small incision in the sclera served as a fiduciary mark, allowing the eye to be oriented in the microscope and the superior, inferior, nasal and temporal quadrants to be identified unambiguously. Differences in the diameters of zonular fibers between wild type and Fbn1-depleted regions were assessed using the two sample Kolmogorov-Smirnov test.
2.5.10 X-ray Microscopy.

To visualize the disposition of the lens within the eye, enucleated globes were fixed using a freeze substitution method\textsuperscript{51} and then incubated in 1:4 dilution of Lugol’s iodine solution for 48 h, embedded in 2.0 % agarose, and imaged using a Zeiss Xradia Versa 520 microscope.

2.5.11 Zonule Biomechanics

The biomechanical properties of the mouse zonule were measured on fixed (24 h in 4% paraformaldehyde/PBS) tissue (Fig. 17). The corneal surface of the enucleated eye was glued to the base of a custom-fabricated chamber positioned on a sensitive balance (Fig. 17A). The back of the eye was removed by dissection, exposing the posterior surface of the lens, zonule, and the ciliary body. The tip of a small glass probe was brought into contact with

\begin{figure}[h]
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\caption{Pull-up procedure for measuring the tensile strength and breaking displacement of zonular fibers. (A) The hemisected eye is immobilized on a balance. A probe is affixed to the posterior surface of the lens and raised in 50 μm increments, while the reduction in weight (at equilibrium, equal to the lifting force) is recorded. (B) Representative data from the eye of a 1-month-old wild type mouse. The weight decreases until position 3 (corresponding to a lifting force of approximately 1.1 g (11 mN) and a vertical displacement of about 0.6 mm), at which point the zonular fibers begin to break. By position 4, the fibers have broken, and the weight has returned to baseline. (C) Photographs of the lens as it is lifted from the hemisected eye (the numbers correspond to those shown in B).}
\end{figure}
the posterior pole of the lens and attached using a drop of Lazer bond™ adhesive. The chamber was then filled with PBS. The probe, which was mounted on a motorized micromanipulator, was raised in 50 µm steps (2 µm/s² acceleration/deceleration) each lasting 10 s. At each step, the associated reduction in sample weight was recorded over a period of 1 minute (Fig. 17B). Due to the slow acceleration, we assumed the eye to be in equilibrium and that the measured decrease in weight was equal to the lifting force. In this fashion, the lens was raised steadily from the eye, the zonular fibers becoming increasingly stretched. At a certain point (between position 3 and 4 in Fig. 17B and 17C), the zonular fibers ruptured and the weight measurements returned to baseline values. Using this apparatus, we measured the tensile strength of the zonular fibers and generated force: displacement curves for each genotype. We also measured the breaking displacement (the distance the lens could be raised from the eye before the zonular fibers failed). Measurements were made on at least three eyes in each case. The person performing the procedure was unaware of the genotype of the mice when making the measurements.
2.6 Figures

Figure 2.1 Expression of genes encoding zonular proteins in ocular tissues during development and aging. (A) An example of multiplexed fluorescence in situ hybridization in the eye of a 1-day-old (P1) wild type mouse. *Fbn2* transcripts (red puncta) are restricted largely to the non-pigmented ciliary epithelium (NPCE). In contrast, *Mfap2* (green puncta) is expressed in the NPCE and the lens epithelium (LE) at comparable levels. (B) Representative image from a P30 eye probed for *Fbn1* and *Ltbp2* expression. Note that for both transcripts, the signal is stronger in the pars plana region of the ciliary epithelium than the pars plicata region. (C) Expression of zonule genes shows marked spatial and temporal fluctuations. *Fbn2*, *Fbn1* and *Ltbp2* are expressed predominantly in the NPCE, *Mfap2* has approximately equal expression levels in NPCE and lens, and *Adams4* is expressed predominantly in the lens epithelium. Non-specific binding, assessed using a probe set to a bacterial gene *DapB*, was negligible (data not shown). Data represent mean ± SD. NPCE = non-pigmented ciliary epithelium; PCE = pigmented ciliary epithelium; LE = lens epithelium. Scale bar (A) = 50 µm.
Figure 2.2. Conditional deletion of \textit{Fbn1} in lens does not prevent fibrillin-1 incorporation into zonular fibers. In this and other images, the interior of the eye is viewed from the posterior aspect (the retina and posterior sclera having been removed). A network of extracellular fibers, the ciliary zonule, projects from the folded surface of the non-pigmented ciliary epithelium (NPCE) to the lens equator. \textit{Cre} expression in the lens is marked by a shift from red to green fluorescence (see also Supplemental Fig. 1). Note that \textit{Cre} is not expressed in the NPCE. Despite conditional deletion of \textit{Fbn1} in the lens, the zonular fibers are intact and strongly immunofluorescent for fibrillin-1 (light blue).
Figure 2.3 Conditional deletion of \(Fbn1\) in the nasal/temporal NPCE causes a reduction in fibrillin-1 immunofluorescence in the adjacent zonule and a corresponding increase in fibrillin-2 immunofluorescence. (A) In 1-month-old control mice, the large, centrally located lens (L) is suspended from the non-pigmented ciliary epithelium (NPCE) by the ciliary zonule (light blue and arrow). \(Cre\) expression (green) is restricted to the nasal (N) and temporal (T) segments of the NPCE. \(Cre\)-negative regions (red) are located superiorly (S) and inferiorly (I). Fibrillin-1 immunofluorescence is equally strong in zonular fibers projecting from the \(Cre\)-positive and \(Cre\)-negative regions of the NPCE. Fibers attach at the lens equator and some elements run a short distance along the posterior lens surface before terminating (arrowhead). (B) In Fbn1-NPCE mice, fibrillin-1 immunofluorescence is reduced to near background levels in fibers projecting from the \(Cre\)-positive nasal/temporal segments of the NPCE, although residual immunofluorescence is present near the lens surface (arrow). Note that fibrillin-1 immunofluorescence persists in fibers projecting from \(Cre\)-negative regions located superiorly and inferiorly. (C) Despite the nominal absence of fibrillin-1, fibers projecting from the nasal and temporal regions have a relatively normal appearance, as judged by immunofluorescence labeling using an antibody to \(LTBP2\), another abundant zonular protein. Note that the posterior extensions of zonular fibers on the lens surface are eliminated in \(Cre\)-positive regions but persist in \(Cre\)-negative regions. (D) Fibrillin-2 immunofluorescence exhibits a reciprocal pattern to fibrillin-1 (compare (D) with (B)) with relatively strong labeling in \(Cre\)-positive regions and weak labeling in \(Cre\)-negative regions.
Figure 2.4 Fibrillin mRNA expression in the NPCE of wild type or in the Cre-positive and Cre-negative regions of Fbn1-NPCE mice at 1 month of age. Fbn1 transcript levels are reduced by >80% in the Cre-positive region compared to Cre-negative regions in wild type or transgenic mice. There was no compensatory increase in Fbn2 expression in Cre-positive nasal/temporal regions. Counts were n ≥3 histological sections from each of three or more mice. Data represent mean ± SD.
Figure 2.5 Conditional deletion of Fbn1 in the NPCE does not eliminate fibrillin-1 immunofluorescence in that portion of the zonular fibers proximal to the lens. (A) In Fbn1-NPCE eyes, MFAP-2 immunofluorescence (red) labels the entire length of zonular fibers, from the surface of the nasal/temporal NPCE to the lens equator. (B) By contrast, fibrillin-1 immunofluorescence (red) is restricted to that segment of the zonular fibers closest to the lens. An exception is a region of the NPCE containing a small island of Cre-negative cells (red, arrowed). Fibers emanating from this region are labeled for their entire length. Scale bar = 100 µm.
Figure 2.6. Onset of EL in Fbn1-NPCE mice. (A) Anterior segment is visualized by optical coherence tomography (OCT) in living mice. The cornea (C), iris (I), and lens (L) are visible. The distance between the inner surface of the cornea and the anterior surface of the lens is the anterior chamber depth (ACD; arrowed). The position of the lens is stable in control mice or Fbn1-lens mice. In Fbn1-NPCE mice, the lens is initially in the correct position. However, between 6- and 8-weeks-of-age the lens dislocates backward in the eye (lower right panel). As a result, the ACD increases sharply and the iris, which is normally convex because of the support of the underlying lens, flattens. (B) In Fbn1-NPCE mice, there is a sharp increase in ACD beginning at 6-weeks-of-age. This reflects the onset of EL, a condition that becomes 100% penetrant by 3 months. Note that EL did not develop in control, heterozygous Fbn1-NPCE mice, or conditional lens knockouts. N>10 eyes at all ages. Data represent mean ± SD.
Figure 2.7 X-ray imaging of ectopic lenses. (A) In eyes from 3-month-old control mice, the central position of the lens (green) is evident. The iris has a convex configuration and the ciliary processes project forward. (B) In Fbn1-NPCE mice, EL is accompanied by an increase in anterior chamber depth due to the posterior displacement of the lens (which now is in contact with the retinal surface). In the absence of the supporting lens, the iris and ciliary processes are angled perpendicular to the eye wall.
Figure 2.8 Breakage of zonular fibers in Cre-positive (green) regions precedes lens dislocation. (A) Eye from an 8-week-old Fbn1-NPCE mouse. The zonular fibers (light blue) are visualized using MAGP1 immunofluorescence. Note the breakage of a stretch of zonular fibers (between the arrows) projecting from the nasal (N) region of the NPCE. (B) The fibers break near their midpoints, with fiber remnants visible on the surface of lens and NPCE. N, nasal; T, temporal; S, superior; I, inferior.
Figure 2.9 Biomechanical properties of the ciliary zonule in wild type and Fbn1-NPCE mice. Tensile strength and breaking displacement were determined using a pull-up assay (see text for details). (A) The tensile strength of the wild type zonule increases with age. (B) The breaking displacement distance of the zonule in wild type mice increases with age. (C) Compared to the zonule in 1-month-old wild type mice, the breaking force of age-matched Fbn1-NPCE zonules is significantly decreased ($p=0.002$). (D) Zonule breaking displacement does not differ significantly between wild type and Fbn1-NPCE mice. (E) The tensile strength of the Fbn1-NPCE zonule in 2-month-old mice is reduced by >90% compared to wild type but the displacement distance is unaffected (F). N≥3 or more eyes per age and genotype. Data represent mean ± SD.
Figure 2.10 Effect of selectively cutting subsets of zonular fibers on tensile strength and breaking displacement. Experiments are performed on eyes from 1-month-old control (gray) or Fbn1-NPCE mice (orange). (A) In control mice, the tensile strength is reduced by ≈55% if the nasal/temporal (N/T) or superior/inferior (S/I) fibers were cut. The tensile strength of the intact Fbn1-NPCE zonule was ≈40% of the control zonule. Cutting the S/I fibers in Fbn1-NPCE caused a larger reduction in breakage force than cutting the N/T fibers, suggesting that the S/I fibers make a greater contribution to the tensile strength of the mutant zonule. (B) The vertical displacement required to break the zonular fibers was approximately 0.6 mm in all cases. N ≥ 3 eyes per condition. Data represent mean ± SEM.
Figure 2.11 Ultrastructure of control (A,C) and Fbn1-depleted (B,D) zonular fibers, imaged at midspan. The images are oriented such that the ciliary epithelium is at the top and lens at the bottom (although neither tissue is visible in these high magnification images). (A) As they course toward the lens, control fibers coalesce to form thicker bundles (arrowed). (B) In contrast, fibrillin-1-depleted fibers are generally thinner and do not converge to form bundles. (C) At higher magnification, the individual microfibrils that comprise the fibers are visible. Each microfibril is 10-12 nm in diameter. (D) Although thinner on average, zonular fibers in Fbn1-NPCE mice are otherwise indistinguishable from control fibers.
Figure 2.12 Microfibril cladding covering the surface of the ciliary processes is absent in Fbn1-NPCE mice. (A) In control animals, occasional gaps between the zonular fibers allow the surface of the ciliary processes to be visualized (boxed region, shown at high magnification in B). (B) A layer of microfibrils, arranged in a loose basket weave pattern, covers the epithelial surface. (C) The surface of the ciliary processes in Cre-positive regions of Fbn1-NPCE mice is visible, due to the reduced density of zonular fibers. (D) At high magnification, there is no evidence of the basket weave cladding found in controls.
Figure 2.7.13 Zonular fiber diameter is reduced in Fbn1-depleted regions. Fiber diameter was measured by SEM in randomly selected fibers from the superior, or nasal/temporal quadrants of control and Fbn1-NPCE mice. The resulting size distributions were evaluated using the two-sample Kolmogorov-Smirnov test. (A) Histogram of fiber size distributions in the three samples. Note that all distributions are positively skewed and that a higher proportion of small diameter fibers is observed in the nasal/temporal (Cre-positive) region of Fbn1-NPCE eyes. (B) Cumulative frequency plots of fiber diameters in the superior and nasal/temporal regions of control eyes. There is no significant difference in fiber diameters between the two regions. (C) There is no significant difference in diameter between superficial fibers in control eyes and Fbn1-NPCE eyes. (D) Fibers in the nasal/temporal region of Fbn1-NPCE eyes are significantly thinner than fibers in the superior region of the same eyes. S, superior; N, nasal; T, temporal; D, Kolmogorov-Smirnov statistic (i.e., maximum difference between the cumulative distributions).
Figure 2.14  Ectopic lenses become cataractous. (A) In wild type mice, lenses retain their clarity, even in aged animals (see also lower right panel in D). (B) In aged Fbn1-NPCE animals, ectopic lenses lose their transparency. (C) In some cases, the lens cortex disintegrates and the lens nucleus is expressed through the pupil into the anterior chamber. (D) Gallery showing the effect of age on the prevalence of cataract in Fbn1-NPCE mice. Cataracts are rare in young animals but increasingly common in aged Fbn1-NPCE mice. M=month.
Figure 2.15 Mid-sagittal sections of ectopic lenses from 1-1.5 year-old Fbn1-NPCE mice. A wild type lens (A) is provided for comparison. In all cases, the anterior surface of the lens, identified by virtue of its thickened lens capsule, is oriented to the right. To various degrees, the lenses show gross distortions of shape, loss of eosinophilic material, liquefaction of cortical layers, shrinkage and frank disintegration. Scale bar = 1 mm.
Figure 2.16 Eyes from aged Fbn1-NPCE mice have a larger volume than age-matched wild types. (A) Representative examples of eyes from 10-month-old Fbn1-NPCE or control mice showing increased axial length and corneal hazing in the knockout animals. (B) Aggregated data show a difference in ocular volume that is evident at 8 months and statistically significant (*, \( p < 0.05 \)) by 10 months (data represent mean±S.D.; \( n \geq 6 \) eyes at each time point). (C) For both types of mice, IOP declines with age but differences between genotypes are not significant (\( n \geq 6 \) eyes at each time point).
**Supplemental Figure 2.1** Verifying Cre expression patterns in Pax6αCre (A-C) and MLR10Cre (D) mice. On the mTmG reporter background, Cre-mediated recombination causes a shift in expression from membrane-targeted TdTomato (red) to membrane targeted GFP (green). (A) The Pax6αCre transgene is expressed in the eye from E11 onwards, as indicated by GFP fluorescence in the nasal and temporal segments of the inner layer of the optic cup. (B) The segmental labeling pattern persists in eyes of 1-month-old mice. The circumferential arc of Cre-negative cells in the superior (S) region of the NPCE is larger than the inferior arc. (C) A pixel histogram of mTdT (red) and mGFP fluorescence (green) in the NPCE (measured in the direction indicated by the arrow in (B)), confirms that the expression pattern is consistent between eyes and that the nasal and temporal Cre-positive arcs are longer than the Cre-negative superior arc. The inferior arc is the shortest (n=11). (D) In MLR10Cre transgenic animals, Cre activity is restricted to the lens.
**Supplemental Figure 2.2** Body weights of control and conditional *Fbn1* knockouts are indistinguishable.
Supplemental Figure 2.3 Mechanical properties of the zonule in Fbn1-lens mice. Eyes in which Fbn1 is conditionally deleted in the lens do not show decreased tensile strength (A) or altered displacement distance (B) compared to aged-matched (1-month-old) wild type mice.
Supplemental Figure 2.4 Flocculent material in the anterior chamber of Fbn1-NPCE mouse imaged by OCT.  
(A) Eye of an 18-month-old control mouse.  
(B) Eye of 21-month-old Fbn1-NPCE mouse. Note the presence of light-scattering material in the anterior chamber and the folded surface of the partially disintegrated ectopic lens protruding through the pupil (arrow).  
Scale bar = 0.5 mm.
**Supplemental Figure 2.5** Loss of tissue polarity in ectopic lenses. In control lenses, the epithelial layer covers the anterior surface only. In this ectopic lens from a 14-month-old Fbn1-NPCE mouse, the epithelium has enveloped the entire lens, making the original orientation difficult to discern. Cells cover the “equator” (A), the “anterior” hemisphere (B), and the “posterior” hemisphere (C). Acellular regions are visible throughout the epithelium (arrowed in B). S-phase cells (green; identified by EdU nuclear labeling) are generally located near the edge of the acellular regions. Nuclei (purple) are stained with Draq5.
2.7 References

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Chapter 3: Enzyme Replacement Therapy Ameliorates Multiple Symptoms of Murine Homocystinuria

Preface

Homocystinuria is a systemic metabolic disorder caused by recessive human mutations in the CBS gene. The CBS gene encodes the enzyme cystathionine beta synthase. Cystathionine beta synthase is involved in the metabolism of methionine by converting homocysteine to cystathionine. Lack of the enzyme results in high levels of homocysteine and low levels of methionine in the blood. This chemical imbalance has profound effects on the cardiovascular and neurological systems in homocystinuria patients. The eye is also severely affected. Ocular manifestations include EL (the cardinal ocular feature of the disease), myopia and retinal detachment. The relationship between abnormal levels of homocysteine and disturbed eye development is not known. During the course of my training, I had collaborated with Tomas Majtan (University of Colorado School of Medicine) who previously developed and described a CBS-deficient mouse model that expresses many of the metabolic dysfunctions seen in homocystinuria. My role in the project was to characterize and phenotype the eyes of CBS/$^-$ and enzyme-treated Cbs/$^-$ mice. In Cbs/$^-$ eyes, I showed that the stability of the ciliary zonule was compromised resulting in fewer and broken fibers. However, in Cbs/$^-$ mice treated with CBS via subcutaneous injection, the structural organization of the ciliary zonule was partially rescued.

This work was done in collaboration with the following authors: Tomas Majtan (T.M), Jakub Krjit (J.K.), Insun Park (I.P.), Warren D. Kruger (W.D.K.), Viktor Kozich (V.K.), Steven
Bassnett (S.B.), Erez M. Bubil (E.M.B.), and Jan P. Kraus (J.P.K). T.M. designed and performed animal studies, prepared and analyzed PEG-CBS conjugates, analyzed data, prepared figures, wrote the initial draft, and coordinated the collaborations. W.J. and S.B. performed analyses of the eyes and interpreted the results. J.K. and V.K. determined sulfur amino acid metabolites in tissues and matching plasmas. I.P. took care of the animal colony and executed animal studies. W.D.K. generously provided the I278T mice. E.M.B. co-designed the studies and reviewed the data. J.P.K. conceived the idea, reviewed the data, and coordinated the project. All authors reviewed the draft, contributed to its revisions, and approved the final form of the manuscript.

Chapter 4: Conclusions and Future Directions

4.1 Conclusions and Future Directions

In this report, I investigated the role of FBN-1 in eye development and disease, focusing on the role of FBN-1 in the synthesis of the ciliary zonule. The bulk of this work involved the identification of the cells that express zonule components and the generation of a mouse model to test the role of Fbn1 in eye development. Additional experiments not discussed in Chapter 2, are included in this section of the thesis. Here, I will discuss conclusions, alternative experiments/methods and future directions of the work.

4.2 The ciliary zonule is synthesized by cells of the NPCE and the lens

In Chapter 2 of the thesis, I identified the cells in the eye that express core zonule components. In this chapter, I will discuss in greater detail the expression behavior of these genes during mouse eye development.

To visualize the expression of zonule components, I utilized multiplexed fluorescence in situ hybridization. Before starting those experiments, I first tested the specificity of probes targeting zonule transcripts (Fbn1, Mfap2, Fbn1, Ltbp2, on germline knockout mouse eye tissues
(i.e. Fbn2 probes tested were tested on Fbn 2⁻/⁻ eye tissue). These data showed that the probes were specific and able to differentiate homologous proteins (i.e. FBN-1 and FBN-2). To simplify, I will first describe the expression of zonule components in the NPCE followed by the expression of these components in the lens.

On P1, Fbn2 and Mfap2 transcripts were localized to cells of the NPCE. In the NPCE, Fbn2 transcripts were concentrated towards the apical pole of the cells while Mfap2 transcripts were localized to the basal side of (Appendix fig. 1.1, 1.3). This localized expression of Fbn2 and Mfap2 in NPCE cells might suggest a role for establishing the anchorage points of ZFs to the ciliary epithelium. Fbn1 and Ltbp2 were expressed at low levels in the NPCE at this stage of eye development (Appendix fig. 1.5, 1.7). Adamts14 transcripts were also expressed at low levels in the NPCE and at subsequent stages of eye development (Appendix fig. 1.9). By P7, Fbn2 expression started to decline in the NPCE while Mfap2 expression remained constant (Appendix fig. 1.1, 1.3). At this stage, Fbn1 and Ltbp2 transcripts were significantly increased (Appendix fig. 1.5, 1.7). By P30, a dramatic shift in expression of zonule components was observed. Fbn2 and Mfap2, the dominant zonule components in embryonic and early postnatal eye, were now the lowest expressed genes in the NPCE (Appendix fig. 1.1, 1.3). In contrast, Fbn1 and Ltbp2, genes that were expressed weakly in the embryonic and early postnatal eye, were strongly expressed in the NPCE (Appendix fig. 1.5, 1.7). Interestingly, Fbn1 and Ltbp2 expression was restricted to NPCE cells in the pars plana region of the ciliary body and posterior folds of the ciliary epithelium suggesting that these cells are responsible for synthesizing ZFs in the eye (Appendix fig. 1.5, 1.7). By P360, Fbn1 and Ltbp2 were the only genes expressed in the aged NPCE.
In the lens, *Mfap2* and *Adams14* were expressed at comparable levels in the lens on P1 (Appendix fig. 1.4, 1.10). *Mfap2* transcripts were expressed by lens epithelial cells in the peripheral lens epithelium, the lens equator and at low levels in differentiating lens fiber cells. *Adams14* expression was restricted to the lens epithelium just above the lens equator (Appendix fig. 1.4, 1.10). *Fbn2*, *Fbn1*, and *Ltbp2* transcripts were detected at low levels in the lens at this stage of eye development and declined to background levels in the ageing eye (Appendix fig. 1.1, 1.2, 1.5, 1.6, 1.7, and 1.8). At P30, *Mfap2* and *Adams14* expression dropped by ~50% in the lens compared to earlier stages (Appendix fig. 1.4, 1.10). In aged (P360) eyes, *Mfap2* and *Adams14* were expressed at near background levels in the lens (Appendix fig. 1.4, 1.10).

These results in addition to those discussed in Chapter 2 suggest that synthesis of the ciliary zonule is likely a collaboration between NPCE and the lens. Of the transcripts examined here, some zonule components are expressed predominately by cells of the NPCE (*Fbn1*, *Fbn2*, *Ltbp2*), while other are preferentially expressed by lens epithelial cells (*Adams14*). *Mfap2* is expressed by both cell types. The expression patterns of *Fbn1*, *Fbn2*, and *Adams14* described in this report is consistent with the literature 1, 2.

There are some caveats to these studies. The first is that expression of zonule components in the human eye may not follow the same patterns as in the mouse eye. Importantly, unlike humans, mice do not accommodate. Perhaps for this reason, the zonular structure is more elaborate in the human eye and this may be reflected in different patterns of gene expression in the NPCE. In our studies, we also assumed that the presence of an mRNA transcript is indicative of active protein synthesis. However, these two cellular processes are not necessarily tightly correlated.
To address these concerns and expand our understanding of zonule protein synthesis, a more sensitive and quantitative approach must be taken. Future experiments will include a comparative gene expression profile analysis of zonule components in the developing mouse and human eye. RNA sequencing analysis on NPCE and lens tissue could be performed using lazer microdissected tissue. This would provide an improved quantification of zonule components expressed during eye development. To monitor protein expression directly, a pulse chase protocol would be required using radiolabeled amino acids or fluorescently-labeled or His-tagged protein. In principle, these experiments could be attempted through a transgenic approach but would be challenging for the fibrillins due to the very large size of the genes involved.

4.3 Zonule proteins are expressed early in eye development and are unevenly distributed in ZFs

The in-situ hybridization data presented in this report indicate that the NPCE and the lens synthesize the ciliary zonule. To track the expression of zonule proteins and the synthesis/maturation of the ciliary zonule during eye development, I stained sections of paraffin-embedded wild type mouse eye tissues (P5- P30) using antibodies targeting zonule proteins. On P5, microfibrils were localized to a narrow cleft between the NPCE and retina (Appendix fig. 1.11 and 1.12). By P7, a small gap between NPCE and the lens is formed with microfibrils adherent on the surface on the ciliary epithelium. Microfibrils lining the surface of the ciliary epithelium connect with microfibrils coating the surface of blood vessels associated with the tunica vasculosa lentis (Appendix fig. 1.11 and 1.12). By P14, individual microfibrils bundle together to form ZFs (Appendix fig. 1.11 and 1.12). The ZFs form a fan-like structure similar to
the zonular fork in the human eye \(^4\). By P30, the ciliary zonule is fully erect and mature (Appendix fig. 1.11-1.12).

To analyze the distribution of zonule components in the zonule, eyes from adult wild type (P30) were fixed, dissected down to the level of the ciliary epithelium and stained with antibodies targeting FBN-1, LTBP-2, and MFAP2 proteins (Appendix fig. 1.13). Three-dimensional confocal microscopy shows that ZFs of the mouse eye emerge between the folds of the ciliary body and splay into small “pith-fork” like fibers before making contact with the lens equator similar to humans (Appendix figures 1.14A and 1.15A) \(^5\). The attachment of the posterior zonule forms a dense mat of radially oriented fibers known as the fibrillar girdle (Appendix figures 1.14B and 1.15B) \(^1\). Fibers of the fibrillar girdle continue to travel across the posterior surface of the lens capsule where they terminate (Appendix figures 1.14A and 1.15A).

Immunostaining results show that FBN-1 and MFAP2 these proteins co-localize in fibers emerging from the ciliary epithelium, at their midpoints, at the lens equator, and in fibers on the posterior surface of the lens capsule (Appendix fig. 1.14A-E). Eyes stained for the presence of LTBP2 and MFAP2 show that these proteins co-localize in fibers projecting between the ciliary epithelium (Appendix fig. 1.15A and C). Interestingly, however, LTBP2 protein expression terminates in fibers just before they splay and attach to the lens (Appendix fig. 1.15D). LTBP2 proteins were also absent in fibers on the back of the lens capsule (Appendix fig. 1.15E).

Together, these data show that the ciliary zonule of the mouse eye is synthesized between P7 and P14 of eye development and is fully mature by P30. The structural organization and arrangement of the mouse ciliary zonule is similar to that of humans \(^4\-6\). In addition, these data show for the first time that distribution of zonule proteins in the fibers is not uniform.
The gene and protein expression profiles of zonule components in the developing mouse eye suggest that the composition of the ciliary zonule changes with age and may reflect temporal expression of zonule components in the mature ciliary zonule. This would imply that as the zonule is being synthesized, developing ZFs are rich in FBN-2 and MFAP2 while mature ZFs are composed principally of FBN-1 and LTBP2.

To determine whether the composition of the ciliary zonule differs with age, future experiments include performing western blot and/or proteomic analysis on isolated ZFs from the developing wild type mouse eye (P7, P14, P30, P360). These experiments would be technically challenging because mouse ZFs are delicate structures (0.2 to 4.0 µm in diameter) and are difficult to manipulate. If isolating ZFs from the mouse eye poses a challenge, ZFs isolated from young and adult human, non-human primate or cow eyes will suffice. I predict that the composition of the zonule will differ significantly with age and that the young zonule will be enrich in FBN-2 and MFAP2 proteins while the adult ciliary zonule will be composed mostly of FBN-1 and LTBP2 proteins.

An additional experiment is to visualize the distribution of zonular proteins in cross sectioned ZFs. ZFs contain thousands of microfibrils packed tightly together (see SEM images Chapter 2). If one were to picture a ZF cut cross section as an onion, the core of the onion might consist of zonule proteins expressed during early eye development (FBN-2 and MFAP2) while the outer layers would be enriched in proteins synthesized at later times (FBN-1 and LTBP2). If this were the case, it would support the notion that ZFs are synthesized by adding microfibrils to the sides of pre-existing fibers. To test this idea, mouse or human ZFs will be cut in cross section, stained for various zonule proteins, and visualized edge-on using super resolution light microscopy. If this hypothesis is correct, I would predict that ZFs will be composed of a core
enriched in FBN-2 or MFAP2 proteins with an outer cortex composed mostly FBN-1 or LTBP2 proteins.

Lastly, whole mount immunofluorescence on wild type mouse eyes show that LTBP2 is absent from fibers near the lens equator and in fibers located on the posterior surface of the lens capsule. In humans, mutations \textit{LTBP2} gene give rise to WMS-3\textsuperscript{7}. In WMS-3, EL is the dominant ocular manifestation and yet the cause mechanism disease is not known\textsuperscript{7}. Preliminary OCT data on \textit{Ltbp2}\textsuperscript{−/−} mouse eyes show that lenses dislocate between one and four months of age. Preliminary immunofluorescence data on wild type mouse eyes presented in this report show that LTBP2 is absent from the portions of ZFs closest to the lens. Thus, it would be of interest to know whether the detachment event in \textit{Ltbp2}\textsuperscript{−/−} null mice occurs near the lens surface where LTBP2 proteins are absent.

\textbf{4.4 The attachment of zonular fibers to the ciliary epithelium and lens}

The mechanism by which the zonule attaches to the lens and ciliary epithelium has been a topic of debate for many years\textsuperscript{1}. Some argue that ZFs merge with the ILM of the ciliary epithelium or terminate between the NPCE cells\textsuperscript{4,8}. To determine how ZFs attach to the surface of the ciliary epithelium and the lens, sections of wild type mouse eyes (P5-P30) were stained with antibodies targeting MFAP2 and laminin proteins to visualize the interactions between microfibrils and basement membranes, respectively.
Immunohistochemistry experiments show that mouse ZFs merge with laminin proteins in the ILM of the ciliary epithelium (Appendix fig. 1.16) consistent with human studies on the ciliary zonule \(^9\). Based on these data, it appears that ZFs of the mammalian eye are connected to the ciliary epithelium by coalescing with the ILM. Although these observations are consistent with most human studies studying the attachment of the ciliary zonule to tissues in the eye, these data do not provide an actual mechanism for ZF attachment to the ciliary body. It would be interesting to know whether a particular protein expressed in the fibers or the ILM is responsible for attaching these tissues.

The attachment mechanism of the ciliary zonule to the lens remains unclear. In the human eye, some studies suggest that ZFs penetrates the fibrous layer on the surface of the lens and terminate \(\approx 600\) nm into the lens capsule \(^10\). The penetration of ZFs into the lens capsule of the human eye is thought to aid in the accommodation process. Confocal microscopy images on mouse eyes show that ZFs adhere to rather than penetrate the lens capsule (Appendix fig 1.16). If penetration of the ZFs in the human eye is required for accommodation, then the adherence ZFs to the surface of the lens capsule may reflect the fact that the mouse eye does not accommodate.

Together, these data suggest that the zonule is attached to the ciliary body by merging with the ciliary epithelium and that the ZFs, at least in the mouse eye, are anchored to the lens by merging with a meshwork of fibrils on the surface of the lens capsule.

4.5 Deletion of \textit{Fbn1} in the NPCE does not prevent the production of zonular fibers or microfibrils
It is not known whether FBN-1, the main component by mass of the ciliary zonule, is required for ZF synthesis. In situ hybridization experiments suggest that FBN-1 is synthesized by cells of the NPCE. To test the role of FBN-1 in the synthesis of the ciliary zonule, I used a conditional knockout approach by segmentally deleting Fbn1 in the mouse NPCE. To target Fbn1 in the NPCE, I utilized the Pax6aCRE mouse strain. Pax6aCRE transgenic mice, express Cre recombinase from E10.5 onward in the nasal and temporal regions of the inner layer of the optic cup (corresponding to the NPCE and retina in the adult eye). CRE expression was visualized by crossing Pax6aCRE mice with the mTmG mouse strain. On the mTmG background, Cre activity causes a shift in expression of fluorescent membrane targeted proteins, from tandem dimer Tomato protein (tdTomato; orange) to the Green Fluorescence Protein (GFP; green). Before targeting Fbn1 expression in the NPCE, I verified that the spatial expression of CRE was consistent in the ciliary epithelium throughout eye development. Three-dimensional reconstructions of P1, P7, and P30 Pax6aCRE +/−; mTmG Tg/− mouse eye cups imaged from the posterior aspect using confocal microscopy show that CRE expression is restricted to the nasal/temporal regions of the NPCE throughout eye development (Appendix fig. 1.17-1.19). Wild type expressing cells are present in the superior and inferior regions of the NPCE. Images taken from the anterior aspect shows that CRE is also expressed by the anterior part of the folds (Appendix fig.1.20). A vibratome section through the nasal/temporal regions of the NPCE show that CRE-positive regions of the NPCE contain small islands of wild type cells (Appendix fig. 1.20). These data were quantified in Chapter 2.

After the spatial expression of CRE was confirmed, conditional Fbn1 KO mice (Fbn1-NPCE) were generated by crossing Pax6aCRE +/−; mTmG Tg/− mice with Fbn-1 Lox/Lox mice. Before, analyzing the ciliary zonule of Fbn1-NPCE mouse eyes, I tested the specificity of the
mouse FBN-1 and FBN-2 antibodies (provided by Dr. Lynn Sakai (OHSU)) on P14 Fbn1−/− and control mouse eye tissues (provided by Dr. Francesco Ramirez, Icahn of Medicine at Mount Saini)13,14. To visualize microfibrils, mouse eye tissues, FBN-1 or FBN-2 stained eye tissues were co-stained with an antibody to MFAP2. In control eye tissues, FBN-1 proteins were enriched in microfibrils of the regressing tunica vasculosa lentis (TVL) while FBN-2 proteins were barely detectable in microfibrils (Appendix fig. 1.21 A and B). However, in Fbn1−/− null mouse eyes, FBN-1 protein expression was significantly reduced in microfibrils of TVL (despite the presence of MFAP2- positive microfibrils) whereas FBN-2 immunofluorescence was increased (Appendix fig. 1.21 C and D). These data showed that the FBN-1 and FBN-2 antibodies used in the experiments performed in this report were able to differentiate between the two homologous proteins.

Three-dimensional reconstructions on P30 control eyes show ZFs mouse emerging from the surface of the ciliary epithelium, attaching to the lens equator and terminating on the back of lens capsule identical to controls (Appendix fig. 1.22 and 1.23). In Fbn1-NPCE eyes, fibers projected from both CRE-negative (superior/inferior) and CRE-positive (nasal/temporal) regions of the eye (Appendix fig. 1.24; 1.25 A-C). ZFs projecting from the CRE- negative regions of the ciliary epithelium were brightly stained for FBN-1 and looked similar to fibers in control eyes (Appendix fig. 1.24). In contrast, deletion of Fbn1 in CRE-positive regions of the ciliary epithelium led to decreased FBN-1 immunofluorescence and the absence of the terminal ends of ZFs on the posterior surface of the lens capsule (Appendix fig. 1.25 D-F). Fbn1-NPCE eyes stained for MFAP2 (Appendix fig. 1.26 and 1.27) and LTBP2 proteins (Appendix fig. 1.28 and 1.29) confirmed the presence of ZFs in CRE-positive regions of the eye suggesting that ZFs can be synthesized in the absence of FBN-1 proteins.

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The presence of ZFs in CRE-positive regions of the ciliary epithelium was perplexing. We hypothesized that although CRE was effective in driving GFP expression in NPCE cells, it was unable to repress the *Fbn1* locus. To determine whether the *Fbn1* locus was inactivated in Fbn1-NPCE mouse eyes, I performed multiplex in situ hybridization on sections of P30 control and Fbn1-NPCE mouse eye tissues using molecular probes targeting *Fbn1* transcripts. Ideally, probes targeting *Cre* transcripts would be sufficient for detecting *Cre* activity in NPCE cells. However, in situ data on adult *Pax6aCRE*<sup>+</sup>; *mTmG*<sup>Tg</sup> eye tissues showed that *Cre* expression levels in the nasal/temporal regions of the eye were significantly weaker in the NPCE compared to the expression of *GFP* transcripts (Appendix fig. 1.30). Therefore, probes targeting *GFP* transcripts were used a marker to identify CRE-expressing cells in the ciliary epithelium.

Multiplex in-situ hybridization experiments on sections of control tissues showed that *Fbn1* and *Fbn2* expression levels were comparable between CRE-positive and CRE-negative regions of the ciliary epithelium. This suggested that expression of CRE does not alter *Fbn1* or *Fbn2* gene expression (Appendix fig. 1.31-1.34). In Fbn1-NPCE eyes, *Fbn1* and *Fbn2* expression levels in CRE-negative regions were comparable to wild type (Appendix fig. 1.36 and 1.38). However, in CRE-positive regions of the eye, *Fbn1* expression was significantly reduced compared to *Fbn1* expression in CRE-positive regions in control eyes (Appendix fig. 1.35). *Fbn2* expression levels remained consistent in CRE-positive regions in the NPCE in the absence of *Fbn1* (Appendix fig. 1.37). Therefore, these data suggest that CRE recombinase was successful in silencing the *Fbn1* locus. The residual low level *Fbn1* expression in the nasal/temporal regions of the ciliary epithelium in Fbn1-NPCE KO mice was most likely attributable to the presence of occasional wild type cells. Alternatively, recombination efficiency may not have been 100% in CRE-positive cells.
The data discussed above suggest that CRE recombinase was largely effective in silencing the \textit{Fbn1} locus. This being said, why did ZFs persist in CRE-positive regions of the eye? One explanation is that the fibers are synthesized by wild type cells dispersed in the nasal/temporal regions of the NPCE. Confocal images of CRE-positive regions of the ciliary epithelium in Fbn1-NPCE eyes show areas of FBN-1 rich ZFs projecting from small patches of wild type cells amongst a sea of CRE-positive cells (Appendix fig 1.25). Another possible explanation for the presence of ZFs in FBN-1 deficient zones of the eye is that FBN-1 proteins may diffuse some distance (potentially hundreds of micrometers) from a cell and become incorporated into a ZF. The current theory of microfibril assembly suggests that microfibrils are synthesized close to the cell surface on a scaffold of FN. However, 3-D confocal images of Fbn1-NPCE eyes show that FBN-1 immunofluorescence in CRE-negative regions of the eye extends \( \approx 500 \) \( \mu \)m into CRE positive regions (Appendix fig. 1.24). This is consist with the hypothesis that FBN-1 incorporation can occur a considerable distance from the expressing cell. To test this hypothesis, future experiments would involve isolating and culturing CRE-positive and CRE-negative NPCE cells from the NPCE of Fbn1-NPCE mice. CRE-positive and CRE-negative cells will be cultured for several days in a two-chambered dish with CRE-positive cells on one side of the dish and CRE-negative on the other. To determine whether FBN-1 can travel from wild type expressing cells and become incorporated into FBN-1 deficient microfibrils, cultures will be stained at different time points with an antibody to FBN-1 proteins. If, for example, the staining intensity of FBN-1 is increased microfibrils produced by CRE-positive cells, then these data would support the hypothesis that microfibrils can be assembled either locally or across long distances.
Lastly, immunostaining on Fbn1-NPCE mouse eyes using an antibody to FBN-2 showed an increase in FBN-2 immunofluorescence in CRE-positive regions of the eye. This observation supports the hypothesis that the production of fibers in the nasal/temporal regions of the eye is the result of a compensatory increase in Fbn2 expression. This hypothesis is reasonable, given the fact that in germline Fbn2<sup>−/−</sup> mice, FBN-1 protein expression is increased to compensate for the loss of FBN-2<sup>15</sup>. The in situ data showed no significant increase in Fbn2 expression in adult Fbn1-NPCE mouse eyes. However, it is possible that Fbn2 expression was increased in the eye earlier time points and that the resultant FBN-2 immunofluorescence and reflects increased FBN-2 protein synthesis during early eye development. To test this hypothesis, additional in situ hybridization experiments would need to be performed on early postnatal Fbn1-NPCE mouse eyes (P1-P30). It is also possible, that Fbn2 expression was not upregulated in the eye and that the apparent increase in FBN-1 immunofluorescence reflects epitope unmasking in the absence of FBN-1<sup>16</sup>.

4.6 A role for metallproteinases?

In this report, I propose that the EL-like phenotype in this new Marfan mouse model, and perhaps human MFS, is caused by changes in the ultrastructure and biomechanical properties. My data show, for example, that ZFs only fail after a period of two to three months. Confocal images of Fbn1-NPCE mouse eyes with partial lens dislocation show that ZF breakage first occurs in the nasal/temporal regions of the eye and at their midpoints. Broken ZFs retract and adhere to the surface of the ciliary folds and the lens. Curiously, the broken fibers do not persist. Examination of the surface of dislocated lens or the CRE-positive regions of the eye show that
the ruptured fibers disappear over time leaving the surface of the lens and ciliary body fiber-free. We hypothesize that proteolytic enzymes inside the eye, may participate in fiber removal.

A few studies investigating the cardiovascular aspects of MFS identified an increase in metalloproteinase expression in the cardiovascular tissues of MFS patients with ascending thoracic aortic aneurysms compared to controls. Metalloproteinases (MMPs) are zinc dependent proteolytic enzymes involved in ECM remodeling and other processes. Multiple tissues of the eye express MMPs. In fact, the NPCE and lens, tissues that flank the ciliary zonule, express a variety of MMPs, which can be detected in the aqueous humor in their active form where most of the ZFs reside. In vitro studies and biochemistry experiments have shown that FBN-1 microfibrils are excellent substrates for several MMPs.

Proteomic analysis on the ciliary zonule shows that MMPs are present at low levels in ZFs. However, the fibers are enriched in endogenous protease inhibitors such as tissue inhibitors of metalloproteinases (TIMPs), Serpins, alpha-1 microglobulin/bikini precursor proteins (AMBPs). TIMPs, serpins, and AMBPs are protease inhibitors that regulate MMP, serine protease, and trypsin proteolytic activity, respectively. In the human eye, TIMP-3 is the most abundant protease inhibitor in the ciliary zonule and accounts for ~ 2% of total zonule protein.

Under normal conditions, MMPs and other proteolytic enzymes are expressed at moderate to low levels and are only activated or upregulated in response to some sort of stimulus. Thus, any of the enzymes expressed in the eye (MMPs, Serpins, etc) could act on the zonule in Fbn1-NPCE mice. Due to the high levels of TIMP-3 inhibitors present in the zonule, MMPs are candidate enzymes. To determine whether the EL phenotype in Fbn1-NPCE mice is
caused by proteolytic degradation of fibrillin-rich ZFs by endogenous proteases, future experiments include isolating RNA from the ciliary bodies and lenses of Fbn1-NPCE and control mice. Once the RNA is collected, I will perform RNA seq. A comparative analysis between control and Fbn1-NPCE mouse eye tissues will allow me to identify genes that are up- or down-regulated in the eye in the absence of Fbn1. Currently the results of such experiments are difficult to predict. However, if one were to assume that the breakage of ZFs is caused by increased proteolytic activity, then I predict that MMPs will be upregulated in either the NPCE, lens, or both. To verify these data, I will perform in situ hybridization on sections cut through CRE-positive and CRE-negative regions of Fbn1-NPCE mice using probes targeting MMP transcript(s). If MMPs expression is upregulated in the eye, additional experiments would include rescuing the EL phenotype by administering protease inhibitors to the eye. Studies have shown doxycycline, a tetracycline chelator that binds to metal ions, inhibits MMP activity in culture and rescues ocular phenotypes in animal models. 

4.7 Additional ocular abnormalities manifest in Fbn1-NPCE mice

In Chapter 2, we observed that Fbn1-NPCE mice expressed additional ocular phenotypes similar to MFS. By three months, 100% of Fbn1-NPCE mouse eyes developed dislocated lenses. At 6 months, ectopic lenses developed opacities (30%). In aged mice (≥1 year-old), the incidence of cataracts was increased to 65% which were clearly visible through the dilated pupil (Appendix fig. 1.43). Cataracts are common in MFS but it is not known why dislocated lenses become opaque the mechanism is unknown. Studies show that cataract formation is highly
correlated with EL. Interestingly, cataracts did not form in aged Fbn1-NPCE mouse eyes where lenses were sealed against the pupil (Appendix fig. 1.43). Thus, these data suggest that cataractogenesis in Fbn1-NPCE mouse eyes, and perhaps MFS, is in partial caused by malpositioning of the lens in the eye. Note that young and aged control mice did not develop cataracts (Appendix fig. 1.39).

To analyze the refractive properties of Fbn1-NPCE mouse eyes, freshly dissected lenses of control and Fbn1-NPCE mouse lenses were placed on grids. Control lenses showed no obvious changes in refractive properties (Appendix 1.42). In contrast, young intact Fbn1-NPCE mouse lenses demonstrated slight distortions in the center of the lens (Appendix 1.45, P30). These distortions in the lens were exacerbated in ectopic lenses and spread towards the outer periphery of the lens. These data suggest that the refractive properties of Fbn1-NPCE mouse lenses is lost once dislocated (Appendix fig. 1.45, P60-P360).

In addition to cataracts, aged Fbn1-NPCE mice exhibited changes in ocular dimensions of the eye globe as early as 8 months of age. Eye measurements on Fbn1-NPCE mice show that knockout mouse eyes are significantly larger in volume compared to age matched controls (see Chapter 2). I observed that the eyes of Fbn1-NPCE mice developed hazy and vascularized corneas (Appendix fig. 1.44) compared to corneas of control mouse eyes, which were transparent (Appendix fig. 1.40). In MFS, the eye is often too big and too long; a condition called axial myopia. Studies have shown that increased axial myopia in MFS is highly correlated with EL, suggesting that the increase in eye growth may be caused by a lens induced effect. In contrast, some studies suggest that the increase in eye size of the eye is a mechanical defect caused by thinning or an stretching of the sclera.
To determine whether the increase in axial length in Fbn1-NPCE mice is secondary to lens dislocation, future experiments will include performing lensectomy on Fbn1-NPCE mice. Ectopic lenses of 6 month-old Fbn1-NPCE mice will be surgically removed leaving the eye aphakic. Treated Fbn1 NPCE mice, Fbn1-NPCE sham, control shame and control animals will be allowed to age to one year or older. Starting at 6 months of age, the eyes of Fbn1-NPCE mice will be carefully removed from the orbit and measured (axial length and axial width). The results of these experiment are difficult to predict. However, if one were to assume that the increase in axial length is caused by the ectopic lens, then I predict that the eyes of Fbn1-NPCE sham mice (ectopic lens not removed) will be significantly larger than Fbn1-NPCE treated (ectopic lens removed) and control mouse eyes. In other words, aged Fbn1-NPCE with the ectopic lenses removed will not experience an increase in eye size. If this hypothesis is correct, it would suggest that the increase in eye size in Fbn1-NPCE mice is a lens induced effect.

Other studies have proposed the idea that the development of large eyes in MFS is caused by increased scleral elasticity. This cannot be the mechanism in our experiments because the Fbn1 locus was targeted in the NPCE and not the sclera. It is possible, however, that disruption of Fbn1 expression in the NPCE could effect the mechanical properties of the sclera. If one were to think of the ciliary zonule as a network of internal springs, then springs may function to stabilize the walls of the eye. If the springs break, this would release tension on the walls of the eye, perhaps allowing the eye globe to expand.

To determine whether the increase in eye growth is due to an increase in elasticity of the sclera (caused indirectly by breakage of the ZFs), or not, future experiments include harvesting Fbn1-NPCE and control mouse eyes (P30-P360) and carefully removing the scleras. Once the scleras have been removed, I will perform stress/strain mechanical assays on dissected scleral
tissues. The results from such experiments are difficult to predict. It is possible that the presence of an intact zonule or the compromised zonule of Fbn1-NPCE mice have no effect on the elastic properties of mutant eye scleras. This would suggest that the increased in dimensions of the knockout eyes occurs via some other mechanism. However, if a difference in the elastic properties of the scleras is detected between Fbn1-NPCE and control mouse eyes, then one might predict that the mutant sclera may initially have the same elastic properties as the control mouse sclera but, overtime, steadily increases the after the lenses has dislocated in the eye. If this prediction is correct, it would suggest that the increase eye size is caused by scleral growth secondary to the EL phenotype.

It is also possible that the increase in size of the eye is caused by increased cell proliferation in the sclera. The sclera is composed of two cell types: chondrocytes and fibroblast. Scleral cell division is thought to be regulated by retinal pigment epithelium but the mechanism is unclear.31 Eye measurements on control mouse eyes show that the eye gets larger with age (see Chapter 2) suggesting steady growth of sclera tissue. To determine whether the increase in axial length is caused by an increased cell proliferation in the scleral, future experiments would involve injecting young and aged control Fbn1-NPCE mice (P30-P360) with 5- ethynyl-2’-deoxyuridine (EdU; Invitrogen, Carlsbad,CA). EdU is a thymidine analog that is incorporated into DNA in S-phase cells. One hour after EdU injection, eyes will be enucleated and fixed. Scleras will be removed, flat mounted, stained for EdU, and counter stained. The S-phase labeling index of mutant and control scleras will be determined. The results of this experiment are difficult to predict. It is possible that there is no increase in the number dividing cells in the sclera between Fbn1-NPCE control and KO mouse eye tissues. However, if there is a change in the labeling index in Fbn1-NPCE scleral tissues compared to control, then I would predict that
the rate of cell division in the scleras of one-month-old Fbn1-NPCE when the lens is still suspended in the eye is comparable to control. In contrast, scleras from eyes with ectopic lenses will be a significant increase in the number of dividing cells in Fbn1-NPCE scleras compared to control scleras.

Initially, we hypothesized that the increase in size of the eye was caused by an increase in IOP; a common feature of glaucoma in humans. IOP measurements on young and aged Fbn1-NPCE mice showed no significant difference in IOP compared to aged control eyes. However, in a few cases, OCT analysis on Fbn1-NPCE mice >1 year of age, showed particulate material floating in the anterior chamber of the eye. The presence of the particulate material was often associated with a slight increase in IOP ≥ 20mm HG. OCT images suggest that this particulate material is most likely lens protein that has leaked from the ruptured cataractous lenses into the anterior chamber of the eye. Based on these observations, it is possible that the increase in eye volume is a result of an inflammatory immune response to lens proteins expelled from the hyper mature cataractous lens resulting in swelling or edema of the eye; a condition in humans called phacolytic glaucoma. It has been reported that patients with phacolytic glaucoma experience corneal edema and an increased in lymphocytes. To determine whether the enlarged eye is caused by an immune response to the free-floating lens proteins in the eye, future experiments include collecting aqueous humor samples from several Fbn1-NPCE mice. Aqueous humor samples will be screened for lymphocytes. If the samples contain large numbers of immune cells, then this would indicate that the presence of a hypermature cataractous lens eye has triggered an inflammatory response in the eye.

To verify that the immune response is caused by leakage of lens proteins into the eye, eyes with particulate material, verified by OCT, will be enucleated, paraffin embedded, and
sectioned. Eye sections will be co-stained using antibodies to a lens crystallin protein (i.e. \( \alpha \)-crystallin) and lymphocytes (CD14) and then counterstained for nuclei. If there is co-localization or association between immune cells and free-floating lens proteins in the eye than this would suggest that the enlarged eye phenotype in Fbn1-NPCE mice is caused by an immune response to the hyper mature cataractous lens resulting in of the swelling eye.

4.8 Is there a relationship between the ciliary zonule and the proliferative region of the lens?

In MFS, the lens is sometimes too small and too spherical; a condition in humans called microspherophakia (MSP). The cause of MSP in MFS and other diseases is not known. The small lens phenotype could be caused by the inability of the weakly synthesized ciliary zonule to effectively pull and stretch the lens into the correct shape and size. However, one study noted that attachment points of the anterior and posterior ZFs of the mouse eye span the proliferative region of the lens epithelium (called the germinative zone). Cell division in the lens is restricted to the lens epithelium. In mice, the germinative zone is a 300 \( \mu \)m wide region of the lens epithelium, located ~150um above the lens equator, where more than ~70% of lens epithelial cell division occurs. Both proposed theories are possible, especially in the accommodating eye where forces exerted by the ZFs are responsible for stretching the lens.

Biomechanical and OCT data presented in this report on P30 Fbn1-NPCE mouse eyes show that the weak FBN-1 deficient zonule, is capable of suspending the lens within the eye up to six weeks of age. Given the fact that the fibers are weaker (see Chapter 2) one could speculate that there could be reduced tension exerted on the lens equator that could result in a small lens.
To test this hypothesis, I dissected Fbn1-NPCE mouse lenses and measured their equatorial diameters. These data showed that there was no difference in the equatorial diameters of P30 Fbn1-NPCE and control lenses, although a significant decrease in equatorial diameter was seen in deteriorating cataractous lenses of aged mice (>1 yr of age; data not shown). Anterior and posterior poles of the lenses were not measured. Together, these data suggest that there is no apparent difference in the size of Fbn1-NPCE and control mouse lenses.

To determine whether the attachment of the FBN-1 deficient zonule has an effect on lens cell behavior, two-month-old Fbn1-NPCE and control mice were injected once with EdU, as described. After one hour of EdU, mouse eyes were enucleated and stained using an MFAP2 antibody to label the ZFs, and imaged using confocal microscopy. In two-month-old control mice (n=2), the anterior and posterior ZFs spanned the germinative zone. The pattern of lens cell division CRE-positive and CRE-negative regions of the NPCE were comparable.

In Fbn1-NPCE mice, cell division in the CRE-negative regions of the eye was similar to control mice (data not shown). In some cases, ZFs were still intact in the CRE-positive regions of the eye. The EdU labeling index in those regions was similar to cell proliferation patterns in the superior regions of the NPCE in Fbn1-NPCE. Interestingly, images of CRE-positive regions where the ZFs were broken show a marked reduction in the number of dividing cells (n=2 eyes; data not shown).

The reason for decreased cell division in regions of the Fbn1-NPCE lens subjacent to broken ZFs not known. It is possible that the reduction is due to a mechanical defect. One could hypothesize that lens epithelial cells sense their physical environment and that the pull on the lens by the zonule stimulates cell division in the germinative zone. To test the idea that zonular
tension stimulates lens cell division, eyes of larger animals such as a human, cow, or pig, will be enucleated and dissected to remove the cornea, retina and sclera. This will produce a ring of tissue compromising of the ciliary epithelium, the lens, and the zonule. Ring of tissues will be placed in culture dish and allowed to grow in culture for several days. In control non-tensioned experiments, tissues will be incubated with EdU and the number of dividing cells in the lens epithelium will be calculated as a reference. In tensioned experiments, the ZFs will be stretched prior to EdU labeling. The number of EdU labeled lens epithelial cells in the stretched lens will be calculated and compared to EdU labeling in non-stretched tissues.

The results of these experiments are difficult to predict. The pattern of cell division in the lens is stochastic. Thus, the images shown in this report of regions containing few Edu-positive cells might just be coincidental. However, if the labeling index of EdU positive cells in stretched lens tissue is increased compared to non-stretched lens tissues, then this would strongly suggest that lens epithelial cell division is influenced, in part, by the mechanical forces of the ciliary zonule.
4.1 References


Appendix
Figure 1.1 *Fbn2* expression in the developing wild type mouse (C57BL/6J) ciliary body. Representative images are triplicate experiments of *Fbn2* expression in aging (P1, P7, P30; columns) mouse eye. Paraffin sections were probed using complementary *Fbn2* mRNA oligonucleotides that span a ≈1-kb contiguous region of the mRNA transcript. Sections were counterstained with 4',6-diamidino-2 phenylindole (DAPI) to visualize cell nuclei (cyan). *Fbn2* mRNA transcripts (green puncta) were detected using confocal microscopy. *Fbn2* transcripts are strongly expressed by NPCE cells. *Fbn2* expression is strongest between Postnatal day 1 (P1; the earliest time point examined) and P7. In adult (P30) and aged (P360) mice, *Fbn2* expression in the NPCE is reduced to near-background levels. L; lens, I; iris, NPCE; non-pigmented ciliary epithelium; RET, retina; S; sclera. Scale bar: 100 µm.
Figure 1.1 Continued. Representative images are triplicate experiments of Fbn2 expression in aging (P360; rows) mouse eye. *Scale bar: 100 µm.*
Figure 1.2 Fbn2 expression in the developing wild type mouse lens. Representative images are triplicate experiments of Fbn2 expression in aging (P7, P30, P360; columns) mouse eye. Fbn2 (green puncta) is expressed at low levels by lens cells. LE; lens epithelium, LQ; lens equator, LF; lens fiber cells. Scale bar: 100 µm.
Figure 1.3 *Mfap2* expression in the developing wild type mouse ciliary body. Representative images are triplicate experiments of *Mfap2* expression in aging (P1, P7, P30; columns) mouse eye. Paraffin sections were probed using complementary *Mfap2* mRNA oligonucleotides that span a ≈1-kb continuous region of the mRNA transcript. Sections were counterstained with DAPI to visualize cell nuclei (cyan). *Mfap2* mRNA transcripts (green puncta) were detected using fluorescence light microscopy. *Mfap2* transcripts are strongly expressed by NPCE cells. *Mfap2* expression is strongest between P1 and P7. In adult (P30) and aged (P360) mice, *Mfap2* expression is reduced to near-background levels. L; lens, I; iris, NPCE; non-pigmented ciliary epithelium; RET, retina; S; sclera. Scale bar: 100 µm.
Figure 1.3 Continued. Representative images are triplicate experiments of *Mfap2* expression in aged (P360; row) mouse eye. Scale bar: 100 µm.
Figure 1.4 Mfap2 expression in the developing wild type mouse lens. Representative images are triplicate experiments of Mfap2 expression in ageing (P7, P30, P360; columns) mouse eye. Mfap2 mRNA transcripts (green puncta) is strongly expressed by lens epithelial cells in the peripheral lens epithelium and lens equator. Mfap2 is also expressed by newly differentiated lens fiber cells. Mfap2 expression is strongest between P1 (see Figure 3) and P7. In adult (P30) and aged mouse eyes (P360), Mfap2 expression in the lens is reduced to near-background levels. LE; lens epithelium, LQ; lens equator, LF; lens fiber cells. Scale bar: 100 µm.
Figure 1.5 Fbn1 expression in the developing wild type mouse ciliary body. Representative images are triplicate experiments of Fbn1 expression in ageing (P1, P7, P30; columns) mouse eye. Paraffin sections were probed using complementary Fbn1 mRNA oligonucleotides spanning a ≈1-kb continuous region of the mRNA transcript. Sections were counterstained with DAPI to visualize cell nuclei (cyan). Fbn1 mRNA transcripts (green puncta) were detected using confocal microscopy. Fbn1 expression is low at P1 but steadily increases and is restricted to the NPCE at P7. Fbn1 expression peaks at P30. Fbn1 transcripts are strongly expressed by NPCE cells in the pars plana region of the ciliary epithelium. In aged eyes (P360), Fbn1 is still expressed by NPCE cells, although levels have fallen compared to P30. L; lens, I; iris, NPCE; non-pigmented ciliary epithelium; RET, retina; S; sclera. Scale bar: 100 µm.
Figure 1.5 continued. Representative images are triplicate experiments of Fbn1 expression in ageing (P360; row) mouse eye. Scale bar: 100 µm.
Figure 1.6 Fbn1 expression in the developing wild type mouse lens. Representative images are triplicate experiments of Fbn1 expression in ageing (P7, P30, P360; columns) mouse eye. Fbn1 mRNA transcripts (green puncta) are expressed at low levels by lens cells at all ages. LE; lens epithelium, LQ; lens equator, LF; lens fiber cells. Scale bar: 100 μm.
Figure 1.7  *Ltbp2* expression in the developing wild type mouse ciliary body. Representative images are triplicate experiments of *Ltbp2* expression in aging (P1, P7, P30; columns) mouse eye. Paraffin embedded sections were probed using complementary *Ltbp2* mRNA oligonucleotides that span a ≈1-kb continuous region of the mRNA transcript. Sections were counterstained with DAPI to visualize cell nuclei (cyan). *Ltbp2* mRNA transcripts (green puncta) were detected by confocal microscopy. *Ltbp2* is expressed at low levels in P1 mouse eyes but is gradually restricted to the NPCE by P7. *Ltbp2* expression peaks at P30. *Ltbp2* is strongly expressed by NPCE cells in the pars plana region of the ciliary epithelium. In aged eyes, *Ltbp2* is still expressed in by NPCE cells despite being markedly reduced in the NPCE. L; lens, I; iris, NPCE; non-pigmented ciliary epithelium; RET, retina; S; sclera. *Scale bar*: 100 µm.
Figure 1.7 Continued. Representative images are triplicate experiments of *Ltbp2* expression in ageing (P360; row) mouse eye. *Scale bar*: 100 µm.
Figure 1.8 Ltbp2 expression in the developing wild type mouse lens. Representative images are triplicate experiments of Ltbp2 expression in ageing (P7, P30, P360; columns) mouse eye. Ltbp2 mRNA transcripts (green puncta) is expressed at low levels by lens cells. LE; lens epithelium, LQ; lens equator, LF; lens fiber cells. Scale bar: 100 µm.
Figure 1.9 *Adamts14* expression in the developing wil-type mouse ciliary body. Representative images are triplicate experiments of *Adamts14* expression in ageing (P1, P7, P30; columns) mouse eye. Paraffin-embedded sections were probed using complementary *Adamts14* mRNA oligonucleotides that span a ≈1-kb continuous region of the mRNA transcript. Sections were counterstained with DAPI to visualize cell nuclei (cyan). *Adamts14* mRNA transcripts (green puncta) were detected using fluorescence light microscopy. *Adamts14* transcripts were expressed at very low levels in the ciliary body throughout development (P1-P360). L; lens, I; iris, NPCE; non-pigmented ciliary epithelium; RET, retina; S; sclera. Scale bar: 100 µm.
Figure 1.9 Continued. Representative images are triplicate experiments of *Adams14* expression in ageing (P360; columns) mouse eye. *Scale bar*: 100 µm.
Figure 1.10. *Adamsl4* expression in the developing wild type mouse lens. Representative images are triplicate experiments of *Adamsl4* expression in aging (P7, P30, P360; columns) *Adamsl4* transcripts (green puncta) were strongly expressed by lens epithelial cells just above the lens equator. *Adamsl4* expression was strongest between P1 (see figure 10) and P7. *Adamsl4* was moderately expressed in the adult lens epithelium. In aged eyes (P360), *Adamsl4* expression in the lens was reduced to near-background levels. LE; lens epithelium, LQ; lens equator, LF; lens fiber cells. Scale
Figure 1.11. The emergence of the ciliary zonule in the developing wild type mouse eye. Enucleated eyes were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned in the mid-sagittal plane. Microfibrils were stained using anti-mouse FBN-2 (red) and anti-mouse MFAP2 (green) antibodies. Sections were counterstained with methyl green to visualize cell nuclei (blue). At P5, FBN-2 and MFAP2 proteins were concentrated in the narrow cleft between the lens, the adjacent NPCE and retina (A). By P7, a small gap between the lens and the NPCE is formed (B). Within this narrow space, microfibrils lining the surface of the ciliary epithelium connect with microfibrils associated tunica vasculosa lentis (a network of blood vessels that nourishes the developing lens) on the surface of the lens (C). By P14, the signature fan-like structure of the ciliary zonule has formed (D). By P30, the ciliary zonule is fully developed and the enriched in FBN-2 and MFAP2 proteins. Fibers emerge from the pars plana of the ciliary body and attach to the surface of the lens. L; lens; NPCE; non-pigmented ciliary epithelium, RET; retina, TVL; tunica vasculosa lentis. Scale bar: 50µm.
Figure 1.11 The emergence of the ciliary zonule in the developing wild type mouse eye. Repeated experiment. The results from this experiment are comparable to figure 1.11. L; lens; NPCE; non-pigmented ciliary epithelium, RET; retina, TVL; tunica vasculosa lentis. *Scale bar: 50 µm*
Figure 1.12 The emergence of the ciliary zonule in the developing wild type mouse eye. Microfibrils were stained using anti-mouse LTBP2 (red) and anti-mouse MFAP2 (green) antibodies. Sections were counterstained with methyl green to visualize cell nuclei (blue). At P5, MFAP2-positive microfibrils are sandwiched between the NPCE and the lens (A). By P7, a small gap between the lens and the NPCE is formed (B). Within this space, microfibrils lining surface of the ciliary epithelium connect with microfibrils associated with the tunica vasculosa lentis (a network of blood vessels that nourishes the lens) on the surface of the lens (B). At this stage of eye development, LTBP2 proteins are expressed in microfibrils but only in filaments lining the posterior surface of the ciliary epithelium (*white asterisk*). On P14, an organized network of LTBP2 and MFAP2 rich ZFs can be seen bridging the gap between the lens and the NPCE (C). By P30, the ciliary zonule is fully developed. Fibers emerge from the pars plana of the ciliary body and attach to the surface of the lens. L; lens; NPCE; non-pigmented ciliary epithelium, RET; retina, TVL; tunica vasculosa lentis. *Scale bar: 50 µm*
Figure 1.13 Alternative method for preserving and visualizing the mouse ciliary zonule. P30 C57/BL 6 mouse eyes were placed either directly into 4% paraformaldehyde (A) or fixed via transcardial perfusion (C). After fixation, mouse eyes were dissected down to the level of the ciliary epithelium by removing the most of the retina and posterior sclera. Stereomicroscope images of eyes placed directly into fixative revealed heterogeneous fixation of ocular tissues. In some areas, the retina, sclera, and ciliary body were stabilized while in other areas, ocular tissues collapsed inward toward the lens (black arrow). In contrast, eyes fixed via transcardial perfusion showed homogenous fixation of ocular tissues (C). It is important to note that the ciliary zonule cannot be visualized using standard light microscopy due its natural transparent properties (black asterisk). To determine whether the structural organization of the ciliary zonule was preserved, ZFs (red) were labeled using an anti-mouse MFAP2 antibody. Tissues were counter-stained with methylgreen to label cell nuclei (magenta). Mouse eye tissues were placed in a glass bottom dish with posterior surface of the lens in contact with the surface of the dish and the eye centered with the cornea facing up. The ciliary zonule was visualized using confocal microscopy and imaged from the posterior aspect. Three-dimensional (3-D) reconstructions of mouse eyes show circumferential expression of MFAP2-positive ZFs throughout the inner circumference of the eye (B, D). The structural integrity of the zonule in eyes placed directly into fixative was not preserved (B). ZFs were often stretched and broken (B, white asterisks). In contrast, the structural organization of the zonule in mouse eyes that were fixed via transcardial perfusion was well preserved (D). ZFs were radially organized and bridged the gap between the ciliary body and the lens (white asterisk). To prevent damaging the ciliary zonule (white triangle) during dissection tissues, it is best to leave a thin layer of overlying retinal-tissue. CB; ciliary body, L; lens, RET; retina, S; sclera. **Scale bar:** (B, D) = 200 µm
Figure 1.14 Expression of FBN-1 and MFAP2 in a P30 wild type mouse ciliary zonule. Mouse eyes were processed for whole mount immunostaining. ZFs are visualized with anti-mouse FBN-1 (green) and MFAP2 (red) antibodies. Tissues were counterstained with methyl green to label cell nuclei (blue). To visualize the attachment of the ciliary zonule to the lens, eyes were tilted at 45° imaged at their equator by confocal microscopy. The ciliary zonule is visualized using confocal microscopy. A) The ciliary zonule connects the lens to the ciliary epithelium. B) Higher magnification images show that the fixed posterior ZFs show that ZFs are rich in FBN-1 and MFAP2. Fibers of the posterior zonule attach to the lens equator and form a dense mat of radially oriented fibers on the surface of the lens capsule known as the fibrillar girdle. C) Near the surface of the ciliary epithelium (40X), FBN-1 and MFAP2 proteins co-localize in fibers emerging between the ciliary folds (arrow). D) Approximately 50 µm above the surface of the lens, fibers splay into pitchfork-like filaments (arrow) 0.4 µm to 0.5µm in width. E) Terminal ends of the posterior ZFs on the back surface of the lens capsule are rich in FBN-1 and MFAP2. CE, ciliary epithelium; CF, ciliary folds; CZ, ciliary zonule; FG; fibrillar girdle, PZ; posterior zonule. Scale bar: (A) = 200 µm; (B) = 100 µm; (C, D, E) = 20 µm
Figure 1.14 Expression of FBN-1 and MFAP2 in a P30 wild type mouse ciliary zonule. The results from this experiment are comparable to figure 1.14. CE, ciliary epithelium; CF, ciliary folds; CZ, ciliary zonule; FG; fibrillar girdle; PZ; posterior zonule. Scale bar: (A) = 200 µm; (B) = 100 µm; (C, D, E) = 20 µm
Figure 1.15 Expression of LTBP2 and MFAP2 proteins in a P30 wild type mouse ciliary zonule. ZFs are visualized with anti-mouse LTBP2 (green) and MFAP2 (red) antibodies. Eye tissue was counterstained with methyl green to label cell nuclei (blue). Mouse eyes were angled at 45° and imaged by confocal microscopy. A) The ciliary zonule bridges the gap between the ciliary epithelium and to the ocular lens. B) Images fixed on the posterior zonule, show that the posterior ZFs attach to the lens equator and form a dense mat of radially oriented fibers on the surface of the lens capsule known as the fibrillar girdle. C) Near the surface of the NPCE, LTBP2 and MFAP2 co-localize in ZFs emerging between the ciliary folds. LTBP2 proteins show high affinity to bundles of microfibrils that wrap around the folds of the ciliary body (figure B, asterisk). D) Approximately 50 µm above the surface of the lens, fibers splay into pitchfork-like filaments (arrow) measuring 0.4 µm to 0.5µm in width. LTBP2 protein expression stops just before the fibers splay and attach to the lens. E) Terminal ends of the posterior ZFs on the back surface of the lens capsule are rich in MFAP2 but devoid of LTBP2 proteins. CE, ciliary epithelium; CF, ciliary folds; CZ, ciliary zonule; FG; fibrillar girdle, PZ; posterior zonule. Scale bar: (A) = 200 µm; (B) = 100 µm; (C, D, E) = 20 µm.
Fig 1.15 Expression of LTBP2 and MFAP2 in a P30 wild type mouse ciliary zonule. The results from this experiment are comparable to figure 16. CE, ciliary epithelium; CF, ciliary folds; CZ, ciliary zonule; FG, fibrillar girdle, PZ; posterior zonule. Scale bar: (A) = 200 µm; (B) = 100 µm; (C, D, E) = 20 µm
Figure 1.16 The relationship between microfibrils and the basement membranes of the ciliary epithelium and lens. Basement membranes (red) were labeled using an anti-mouse laminin. Microfibrils (green) were labeled with anti-mouse MFAP2 antibody. Sections were counterstained using methyl green to label cell nuclei (blue). A) In young mouse eyes (P5), laminin and microfibrils are highly concentrated in the narrow cleft between the retina, NPCE, and lens. B) As the gap forms between the lens and the NPCE (P7), microfibrils connect with the inner limiting membrane and blood vessels of the hyaloid vasculature. C) As the blood vessels of the hyaloid vasculature regress (P14), microfibrils adhere to the surface of the ciliary epithelium by merging or coalescing with the ILM. D) In the adult eye (P30), ZFs adhere to the surface of the lens capsule. The attachment of the ZFs to the lens forms a thin layer of microfibril material on the surface of the lens capsule. The ZFs do not appear to penetrate the lens capsule. BV; blood vessel, L; lens; LC; lens capsule, M; microfibrils, NPCE; non-pigmented ciliary epithelium, RET; retina, TVL; tunica vasculosa lentis. Scale bar: 20 µm
Figure 1.17 Visualizing CRE expression in P1 Pax6αCRE^+/--; mTmG^tg/- mouse eyes. Mouse eyes were enucleated, placed into agar and cut in the mid-sagittal plane using a vibratome. Sections were placed on a glass side and CRE expression was visualized using confocal microscopy. A) In mTmG^tg/- control mice, membrane targeted tandem-dimer tomato proteins (tdTomato; red) are ubiquitously expressed in all tissues of the eye (sclera, cornea, lens, retina, hyaloid vasculature). B) In Pax6αCRE^+/--; mTmG^tg/-, CRE activity (green) causes a shift in expression of the fluorescent from tdTomato to the green fluorescent protein (GFP). CRE is expressed in the neural retina and ciliary epithelium. C) A vibratome section cut through the nasal/temporal region of the mouse eye cup (inset) shows that CRE is restricted to the outermost layer of the ciliary epithelium (corresponding to the NPCE of the adult eye) and the neural retina. CRE is not expressed in the sclera, iris, pigmented ciliary epithelium, or ciliary body stroma. Scale bar: (A, B) = 200 µm; (C) = 20 µm
Figure 1.18 Visualizing CRE expression in P7 Pax6aCRE<sup>+/−</sup>; mTmG<sup>tg/−</sup> mouse eyes. Left and right eye cups of the same animal were processed as described, dissected down to the level of the ciliary epithelium and the lenses removed from the eye cup. Eye cups were imaged from posterior aspect using confocal microscopy. On P7, the developing ciliary folds of the ciliary body bulge inward towards the interior of the eye cup. 3-D reconstructions of P7 mouse eye cups show that CRE (green) is expressed by NPCE cells in the nasal/temporal regions of ciliary epithelium while the superior/inferior regions are populated by cells that do not express CRE (red). S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200µm.
Figure 1.19 Visualizing CRE expression in P30 Pax6αCRE<sup>+/−</sup>; mTmG<sup>+/−</sup> mouse eyes. By P30, the ciliary body is fully mature. The ciliary folds are radially extended facing inward towards the interior of the eyecup. 3-D reconstructed images of left and right PaxαCRE<sup>+/−</sup>; mTmG<sup>+/−</sup> mouse eye cups show that CRE (green) is expressed by NPCE cells nasal/temporal regions of the ciliary epithelium while the superior/inferior regions are populated by NPCE cells that do not express CRE (red). It is important to note that the pattern of CRE expression is preserved throughout eye development and consistent between animals and between eyes of the same animal. S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200 µm
*Pax6αCRE<sup>+/−</sup>; mTmG<sup>τα/−</sup>*

A: Posterior Aspect

B: Anterior Aspect

C

D

E

F

Scale bars indicate...
Figure 1.20 CRE expression in the posterior ciliary epithelium is continuous with expression in the anterior ciliary epithelium in Pax6αCRE\textsuperscript{+/−}; mTmG\textsuperscript{+/−} mouse eyes. CRE expression in the posterior ciliary epithelium (A, C, E) and the anterior ciliary epithelium (B, D, F) was visualized using confocal microscopy. 3-D reconstructions of the posterior ciliary epithelium show that CRE (green) is expressed by NPCE cells in the nasal/temporal regions of the ciliary epithelium while the superior and inferior regions are populated by cells that do not express CRE (red) (A). High magnification images of the ciliary epithelium in the nasal/temporal regions of the eye show that these regions contain small islands CRE-negative expressing cells (C, asterisk) while superior/inferior regions consist primarily of CRE-negative cells (E). These data are quantified in Chapter 2. To visualize CRE expression in the anterior ciliary epithelium, the cornea and iris of the same eye were carefully removed. At low magnifications, CRE expression in the anterior ciliary epithelium mirrors the expression of CRE in the posterior ciliary epithelium (B). At high magnification, CRE expression in the nasal/temporal region of the anterior ciliary epithelium is continuous with the posterior ciliary epithelium (D). Additionally, the expression of CRE-negative cells in the superior/inferior regions of the eye is continuous with posterior ciliary epithelium (F). A and B were imaged at 10X whereas C, D, E, and F were imaged at 40X. S; superior, T; temporal, I; inferior, N; nasal. Scale bar: (A, B) = 200 µm; (C, D, E, F) = 50 µm
Figure 1.21 Fibrillin expression in P10 germline Fbn1−/− mouse eyes. Mouse eyes were processed for whole mount immunostaining. Microfibrils were visualized with anti-mouse FBN-1 (A and C, green), FBN-2 (B and D, green) and anti-MFAP2 (A-D). Tissues were counterstained using methyl-green to label cell nuclei (magenta). In Fbn1+/+ eyes, overlay of MFAP2 immunofluorescence with either anti-FBN-1 or anti-FBN-2 revealed that FBN-1 was present in microfibrils associated with the tunica vasculosa lentis (A) whereas FBN-2 proteins were barely detectable (B). In Fbn1−/− mouse eyes, FBN-1 expression was eliminated in microfibrils while MFAP2 expression was unchanged (C). Conversely, FBN-2 immunofluorescence was enriched in microfibrils in the absence of FBN-1 (D). TVL, tunica vasculosa lentis. Scale bar: 50 µm.
Figure 1.22 3-D reconstructions of P30 Fbn1-NPCE control mouse ciliary zonules. Eyes were processed for whole mount imaging. ZFs (magenta) were visualized using anti-mouse FBN-1. Representative images of the control ciliary zonules show that CRE (green) is expressed by NPCE cells in the nasal/temporal regions of the ciliary epithelium where as CRE-negative cells are present in the superior/inferior regions. In control eyes, ZFs span the gap between the pars plana region of the ciliary epithelium and the lens. At the surface of the lens, ZFs run a few hundred microns across the back of the lens where they terminate. S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200 µm
Figure 1.23 Structural arrangement of FBN-1 rich ZFs ciliary in control ciliary zonules. Eyes were processed for whole mount immunostaining (figure 14) and imaged in the same orientation as described in figure 14 using confocal microscopy. ZFs (red) were visualized using an anti-mouse FBN-1 antibody. Tissues were counterstained using Draq5. A representative images of CRE-negative (A) and CRE-positive regions (D-E) of ciliary epithelium in control eyes show that ZFs emerge from the surface of the ciliary epithelium and attach to the lens equator. At the surface of the lens, ZFs run a few hundred microns across the back of the lens where they terminate (white arrows). CE; ciliary epithelium, CZ; ciliary zonule, L; lens. Scale bar: 200 µm
Figure 1.24. 3-D reconstructions of P30 mouse ciliary zonules. ZFs (magenta) were visualized using anti-mouse FBN-1. Representative images of the Fbn1-NPCE ciliary zonules show that CRE (green) is expressed by NPCE cells in the nasal/temporal regions of the ciliary epithelium whereas as CRE-negative cells (red) are present in the superior/inferior regions. In Fbn1-NPCE mice, FBN-1 immunofluorescence is greatly reduced in fibers projecting from CRE-positive regions on the nasal and temporal sides of the eye (asterisks). FBN-1 staining in fibers projecting from superior and inferior regions is comparable to wild-type control Fbn1-NPCE mice (triangles). S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200 µm
Figure 1.25 Structural arrangement of FBN-1 rich ZFs in Fbn1- NPCE mouse ciliary zonules. Eyes were processed for whole mount immunostaining. ZFs (red) were visualized using an anti-mouse FBN-1 antibody. Tissues were counterstained using Draq5. Representative images of CRE-negative regions of the eye (A-C) show that ZFs bridge the gap between the ciliary epithelium and the lens. The structural organization of the ciliary zonule in CRE-negative regions is similar to the superior/inferior regions in Fbn1-NPCE control mice. In CRE-positive regions of the ciliary epithelium (D-F), FBN-1 immunofluorescence is greatly diminished and restricted mostly to distal ends of the fibers closest to the lens (D, white arrow). Occasionally, FBN-1 rich ZFs can be seen emerging from islands of CRE-positive cells in the nasal/temporal regions of the eye. Most strikingly, deletion of Fbn1 in the NPCE leads to the ablation of the terminal ends of ZFs on the posterior surface of the lens capsule (white triangle). CE; ciliary epithelium, CZ; ciliary zonule, L; lens. Scale bar: 200 µm
Figure 1.26 3-D reconstructions of P30 Fbn1-NPCE mouse ciliary zonules. Eyes were processed for whole mount immunofluorescence. ZFs (magenta) were visualized using an anti-mouse MFAP2. In control mouse eyes (left image), MFAP2 rich ZFs are present throughout the entire inner circumference of the eye. ZFs emerge from the surface of the ciliary epithelium and attach to the lens equator. In Fbn1-NPCE mouse eyes (right image), MFAP2 positive ZFs are present in the nasal/temporal and superior/inferior regions of the eye. The intensity of the MFAP2 immunofluorescence is comparable between CRE-positive (triangles) and CRE-negative (asterisk) regions of the ciliary epithelium. S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200 µm
Figure 1.27 ZFs persist in Fbn1-NPCE mouse ciliary zonules. Eyes were processed for whole mount immunostaining. ZFs (red) were visualized using an anti-mouse MFAP2. In control mouse eyes (A and B), MFAP2 rich ZFs bridge the gap between the ciliary epithelium and the lens. ZFs emerge from the surface of the ciliary epithelium and attach to the lens equator. After the fibers attach to the lens, they travel several hundred microns across the posterior surface of the lens capsule before terminating (white arrow). Fbn1-NPCE mouse eyes stained with anti-MFAP2 (B and D), reveal the presence of ZFs in CRE-expressing regions of the eye. ZFs extend from the surface of the ciliary epithelium and attach to the lens similar to control. However, deletion of Fbn1 in the NPCE leads to the ablation of the terminal ends of ZFs ((triangles; (C, D)). CE; ciliary epithelium, CZ; ciliary zonule, L; lens. Scale bar: 200 µm
Figure 1.28 Fbn1-NPCE mouse ciliary zonules are rich in LTBP2. Eyes were processed for whole mount immunostaining. ZFs were visualized using an anti-mouse LTBP2 (green). 3-D representative confocal images of a Fbn1-NPCE ciliary zonule shows circumferential expression of LTBP2-rich ZFs emerging from the nasal/temporal and superior/inferior regions of the ciliary epithelium. LTBP2 immunofluorescence is comparable between CRE-positive and CRE-negative regions of the eye. S; superior, T; temporal, I; inferior, N; nasal. Scale bar: 200 µm
Figure 1.29 Structural arrangement of LTBP2 rich zonular fibers in Fbn1-NPCE mouse eyes. Eyes were processed for whole mount immunostaining. ZFs were visualized using anti-mouse LTBP2 (green). 3-D representative confocal images of the superior region of the ciliary zonule show ZFs emerging from the ciliary epithelium and attach to the lens equator (A). Reconstructed image of the mosaic inferior region of the eye (B) show that LTBP2 rich fibers expressed by CRE-positive cells (asterisk), are identical in appearance to ZFs synthesized by CRE-negative cells (triangle). Note, distal ends of ZFs on the posterior surface of the lens capsule are negative for LTBP2 proteins similar to wild-type control studies (figure 17 and 18). CE; ciliary epithelium, CZ; ciliary zonule, L; lens. Scale bar: 200 µm
Figure 1.30 Cre versus GFP expression in CRE-expressing regions of the ciliary epithelium in P30 control mouse eyes. Tissue sections were co-labeled using probes using targeting Cre (red puncta) and GFP (green puncta) mRNA transcripts. Confocal images on Fbn1-NPCE control mouse eyes show that Cre transcripts are at near-background levels in the NPCE (arrow) whereas GFP transcripts are strongly expressed by NPCE cells in the pars plana (arrow) and ciliary folds (arrow) as well as cells of the retina. These data show that probes targeting GFP expression are restricted to the inner wall of the eye and is good indicator for identifying CRE-expressing cells in the eye. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. Scale Bar: 100 µm
Figure 1.31 *Fbn1* and *GFP* expression in CRE-expressing regions of the ciliary epithelium in P30 control mouse eyes. Tissue sections were co-labeled using probes targeting *Fbn1* (red puncta) and *GFP* (green puncta) mRNA transcripts. Confocal images on Fbn1-NPCE control mouse eyes show that *Fbn1* transcripts are strongly expressed by NPCE cells in the pars plana (arrow) and posterior ciliary folds (arrow) of the NPCE. CRE-expressing cells were identified using probes targeting *GFP* mRNA transcripts. The data show that *GFP* is strongly expressed throughout the entire NPCE layer as well as the retina. *GFP* transcripts were not expressed in the PCE, iris or sclera. These data show that CRE is strongly expressed in the nasal/temporal regions of the NPCE, is active in NPCE cells, and does not alter *Fbn1* expression in control eyes. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. *Scale Bar: 100 µm*
Figure 1.32 *Fbn1* and *GFP* expression in CRE-negative regions of the ciliary epithelium in P30 control mouse eyes. Sections were co-labeled using probes targeting *Fbn1* (red puncta) and *GFP* (green puncta) mRNA transcripts. *Fbn1* transcripts are strongly expressed by NPCE cells in the pars plana of the ciliary body (arrow) and posterior ciliary folds (arrow). *Fbn1* expression in the superior region of the NPCE is comparable to *Fbn1* expression in CRE-expressing regions of the eye (see figure 45). CRE-expressing cells were identified indirectly using probes targeting *GFP* mRNA transcripts. In contrast to *Gfp* expression in the nasal/temporal regions of the ciliary epithelium, *GFP* transcripts are not expressed at high levels by NPCE cells in the superior regions of the eye. These data show that CRE activity is low in the superior region of the NPCE of Fbn1-NPCE wild-type control eyes. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. *Scale Bar:* 100 µm
Figure 1.33 *Fbn2* and *Gfp* expression in CRE-expressing regions of the ciliary epithelium in P30 control mouse eyes. Eyes were co-labeled using probes targeting *Fbn2* (red puncta) and *GFP* (green puncta) mRNA transcripts. *Fbn2* is expressed at near-background levels by NPCE cells in the pars plana of the ciliary body (arrow) and posterior ciliary folds (arrow) of the NPCE. CRE-expressing cells were identified indirectly, using probes targeting *GFP* mRNA transcripts. The data show that *GFP* is strongly expressed throughout the entire NPCE layer and the retina. *GFP* transcripts were not expressed by cells of the PCE, iris or sclera. These data show that CRE is expressed in the nasal/temporal regions of the NPCE, is active in NPCE cells, and does not alter *Fbn2* expression in wild-type control eyes. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. *Scale Bar:* 100 µm
Figure 1.34 *Fbn2* and *GFP* expression in CRE-negative regions of the ciliary epithelium in P30 control mouse eyes. Sections were co-labeled using probes targeting *Fbn2* (red puncta) and *GFP* (green puncta) mRNA transcripts. *Fbn2* expression in the nasal/temporal regions of the eye is comparable to *Fbn2* expression in the superior/inferior regions. *GFP* transcripts are not expressed at high levels by NPCE cells in the superior region. These data show that CRE activity is low in the superior region of the NPCE of *Fbn1*-NPCE control mouse eyes. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. Scale Bar: 100 µm
Figure 1.35 *Fbn1* and *GFP* expression in CRE-expressing regions of the ciliary epithelium in P30 Fbn1-NPCE mouse eyes. Sections paraffin-embedded tissues were cut in the sagittal plane through the nasal and temporal regions of the eye and co-labeled using probes using targeting *Fbn1* (red puncta) and *GFP* (green puncta) mRNA transcripts. *Fbn1* expression is markedly reduced in the nasal/temporal regions of the NPCE compared to *Fbn1* expression in control tissues (compare to figure 31). CRE-expressing cells were identified indirectly using probes targeting *GFP*. The data show that *GFP* is expressed throughout the NPCE and the retina. *GFP* transcripts were not expressed by cells of the PCE, iris or sclera. CRE is expressed in the nasal/temporal regions of the NPCE where it is largely effective in silencing the *Fbn1* locus. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. *Scale Bar:* 100 µm
Figure 1.36 Fbn1 and GFP expression in CRE-negative regions of the ciliary epithelium in P30 Fbn1-NPCE mouse eyes. Sections were co-labeled using probes targeting Fbn1 (red puncta) and GFP (green puncta) mRNA transcripts. Fbn1 expression in the superior regions of the NPCE is similar to controls (compare to fig.32). CRE-expressing cells were identified indirectly using GFP probes. GFP transcripts are not expressed at high levels in the superior NPCE. These data suggest that CRE activity is low in the superior regions and does not have a significant effect on Fbn1 expression. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. Scale Bar: 100 µm
Figure 1.37 Fbn2 and GFP expression in CRE-expressing regions of the ciliary epithelium in P30 Fbn1-NPCE mouse eyes. Sections were co-labeled using probes targeting Fbn2 (red puncta) and GFP (green puncta) mRNA transcripts. Fbn2 expression in the nasal/temporal regions of the NPCE is comparable to Fbn2 expression in control tissues (compare with fig.33). CRE-expressing cells were identified indirectly, using probes targeting GFP. GFP is strongly expressed throughout the NPCE and retina. GFP was not expressed by cells of the PCE, iris or sclera. Therefore, these data suggest that CRE is expressed in the nasal/temporal regions of the NPCE and that knockdown of Fbn1 does not result in increased Fbn2 expression. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. Scale Bar: 100 μm
Figure 1.38. *Fbn2* and *GFP* expression in CRE-negative expressing regions of the ciliary epithelium in P30 *Fbn1*-NPCE mouse eyes. Sections co-labeled using probes using targeting *Fbn2* (red puncta) and *GFP* (green puncta) mRNA. *Fbn2* expression in the superior regions of the NPCE is comparable with *Fbn2* expression in the nasal/temporal regions of the NPCE in knockout eyes and control eye tissues (compare with fig. 34). CRE-expressing cells were identified indirectly, using probes targeting *GFP*. *GFP* was not expressed by cells of the PCE, iris or sclera. These data show that *GFP* is not expressed at high levels in the superior and inferior regions of the NPCE compared to the nasal and temporal regions of the eye. These results suggest that CRE activity is low in the superior regions of the NPCE in knockout mouse eyes and that knockdown of *Fbn1* does not result in the increase of *Fbn2* transcripts. Images are from three wild-type control littermates. I; iris, RET; retina, S; Sclera. *Scale Bar*: 100 µm
Figure 1.39. Live images of young and aged control mice (P30-P420). Headshots of mice were photographed using a stereomicroscope. CRE expression in control mice does not interfere with craniofacial development. Eyes of Fbn1-NPCE control mice develop normally and are transparent.
Fig 1.39. Continued.
Figure 1.40 Eyes of young and aged control mice are normal in appearance. Mouse eyes were enucleated, positioned on their equators and imaged photographed using a stereomicroscope. Corneas of control eyes are normal in shape and transparent. The lens and iris are in the correct position. The sclera and optic nerve are normal in appearance. Eye size measurements are described in Chapter 2 of the thesis.
Figure 1.41 Young and aged control mouse lenses are transparent. Lenses of control mice were freshly dissected and placed in a dish containing prewarmed tissue culture medium. To gauge their transparency, lenses were photographed on a dark background using a stereo microscope. All lenses were transparent and did not develop lens opacities.
Figure 1.42 Optical properties of control mouse lenses. To gauge their refractive properties, freshly dissected lenses of P30 to P300 mice were photographed on 0.22 mm electron microscope grid. Control lenses are completely transparent and display normal refractive properties.
Figure 1.42 Continued.
Figure 1.43 Live images of young and aged Fbn1-NPCE mice (P30-P630). Headshots of mice were photographed using a stereomicroscope. CRE expression in Fbn1-NPCE mice does not disturb craniofacial development. In young Fbn1-NPCE mice (P30-P180), eye globes were initially transparent. At later stages (P240-P360), Fbn1-NPCE mice developed hazy corneas, corneal cysts, cataracts, and buphthalmos. Note that not all eyes of aged Fbn1-NPCE mice developed cataracts.
Figure 1.43 Continued.
Figure 1.43. Continued.
Figure 1.44 Fbn1-NPCE develop enlarged eyes. Eyes of Fbn1-NPCE mice (P30-P360) were enucleated, positioned on their equators and imaged using a stereomicroscope. Eyes of Fbn1-NPCE mice become larger in volume and axial length with age (see Chapter 2 for eye size data). Corneas of Fbn1-NPCE mice are initially normal in shape and transparent (P30-P180). At later stages, corneas become enlarged, elongated, and vascularized (P240-P630). In young Fbn1-NPCE mice, the iris and the lens are initially in the correct position similar to eyes of wild-type control animals (see Chapter 2). In aged mice, the lens dislocates back ward in the eye. As a result, the iris, which is normally supported in the eye by the suspended lens, flattens (see Chapter 2). Scleras of aged Fbn1-NPCE mice develop a dark ring around the equator of the eye globe (≥P300). In some cases, scleral tissues started to engulf the cornea suggesting late onset scleral growth (images with black arrows).
Figure 1.44 Continued.
Figure 1.44 Continued.
Figure 1.45 Optical properties of young and aged Fbn1-NPCE mouse lenses. To gauge their refractive properties, freshly dissected lenses and were photographed on a 0.22 mm electron microscope grid. Only non-cataractous Fbn1-NPCE lenses were examined. In young Fbn1-NPCE mice (P30), 3 out of 5 non-dislocated lenses showed slight distortions in the grid. Distortions in the pattern of the grid were exacerbated in aged Fbn1-NPCE mice with ectopic lenses (P30-P630). The refractive properties of the inner core and periphery of ectopic lenses are greatly disturbed. It is important to note that not all Fbn1-NPCE lenses showed changes in refractive properties.
Figure 1.45 Continued.
Figure 1.45. Continued.
Figure 1.46 Late onset of EL in aged Mfap2−/− mice. The anterior segment is visualized by optical coherence tomography (OCT). In most aged Mfap2−/− mouse eyes, the position of the lens is stable similar to wild-type control mice (A-H). Only 1 out of 7 Mfap2−/− mice (or 2/12 mouse eyes, I and J) examined in this study developed lens dislocation. The dislocated lens falls backward in the eye toward the retina (not shown). As a result, the iris, which is normally convex because of the support of the underlying lens, flattens. C; cornea, I; Iris, L; lens.
Figure 1.47 The ciliary zonule persists in germline Mfap2−/− mouse eyes. P30 mouse eyes were processed for whole mount immunostaining and imaged as described in figure 15. ZFs (red) were stained using an anti-mouse FBN-1 antibody. Eyes were counterstained using Draq5 to label cell nuclei. FBN-1 rich ZFs are expressed around the entire inner circumference of the Mfap2−/− mouse eye similar to wild-type (figure 14). ZFs in Mfap2−/− null mice emerge from the ciliary folds and attach to the lens equator. Some fibers extend over large distances across the back of the lens before terminating (green triangle). A few FBN-1 ZFs appear to be broken in the MFAP2 deficient zonule. CZ; ciliary zonule, RET; retina, S; sclera. Scale bar: 200 um
Figure 1.48 Mfap2 -/- null mouse ciliary zonules show subtle changes in zonule structure. The eye was processed for whole immunostaining using an anti-mouse FBN-1 and anti-mouse MFAP2. In MFAP2 deficient mouse eyes, FBN-1 rich ZFs bridge the gap between the ciliary epithelium and lens (A) similar to wild-type (see figure 15). The posterior ZFs emerge from the ciliary epithelium and attach to the lens equator forming the fibrillar girdle (B). However, the FBN-1 immunofluorescence in the fibrillar girdle region of the lens in Mfap2 -/- mouse eyes is greatly reduced compared to wild-type. At higher magnifications, ZFs appear thinner as they emerge from the ciliary folds (C). An unusual “dusting” of FBN-1 rich microfibrill material is seen coating the surface of the ciliary folds (asterisk, B and C). Near the lens equator, ZFs are similar to wild-type fibers and splay before making contact with the lens (D, note the reduced FBN-1 immunofluorescence). Fibers present on the posterior surface of the lens capsule are rich in FBN-1 proteins but have a frail appearance. MFAP2 staining on negative Mfap2 -/- mouse eyes was negative in the ciliary zonule confirming the specificity of the antibody and genotype of the animals. CE, ciliary epithelium; CF, ciliary folds; CZ, ciliary zonule; FG; fibrillar girdle, PZ; posterior zonule. Scale bar: (A) = 200 µm; (B) = 100 µm; (C, D, E) = 20 µm