

Metabolic, Endocrine and Genitourinary Pathobiology

Failure of Pelvic Organ Support in Mice Deficient In Fibulin-3

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Fibulin-5 is crucial for normal elastic fiber synthesis in the vaginal wall; more than 90% of fibulin-5-knockout mice develop pelvic organ prolapse by 20 weeks of age. In contrast, fibulin-1 and -2 deficiencies do not result in similar pathologies, and fibulin-4-knockout mice die shortly after birth. EFEMP1 encodes fibulin-3, an extracellular matrix protein important in the maintenance of abdominal fascia. Herein, we evaluated the role of fibulin-3 in pelvic organ support. Pelvic organ support was impaired significantly in female *Efemp1* knockout mice (*Fbln3*^{-[supil/-]}), and overt vaginal, perineal, and rectal prolapse occurred in 26.9% of animals. Prolapse severity increased with age but not parity. Fibulin-5 was up-regulated in vaginal tissues from *Fbln3*^{-[supil/-]} mice regardless of prolapse. Despite increased expression of fibulin-5 in the vaginal wall, pelvic organ support failure occurred in *Fbln3*^{-[supil/-]} animals, suggesting that factors related to aging led to prolapse. Elastic fiber abnormalities in vaginal tissues from young *Fbln3*^{-[supil/-]} mice progressed to severe elastic fiber disruption with age, and vaginal matrix metalloprotease activity was increased significantly in *Fbln3*^{-[supil/-]} animals with prolapse compared with *Fbln3*^{-[supil/-]} mice without prolapse. Overall, these results indicate that both fibulin-3 and -5 are important in maintaining pelvic organ support in mice. We suggest that increased vaginal protease activity and abnormal elastic fibers in the vaginal wall are important components in the pathogenesis of pelvic organ prolapse. (*Am J Pathol* 2009, 174:206–215; DOI: 10.2353/ajpath.2009.080212)

Pelvic organ prolapse is a common pelvic floor disorder with substantial financial, social, and psychological implications.^{1–3} More than 11% of women will ultimately require surgical correction of prolapse or incontinence in their lifetimes.⁴ Despite the prevalence of prolapse, the true pathophysiology of the disease is not well understood although epidemiological studies indicate that aging and vaginal parity are major risk factors.^{1,5} Connective tissues of the pelvic floor are important for support of the pelvic viscera. Elastic fibers, which are abundant in connective tissues of the vaginal wall, confer resilience to stretching and expansive forces.^{6–8} The phenotype of pelvic organ prolapse in animals with defects in elastic fiber assembly and synthesis suggests that elastic fiber homeostasis pathways are involved in maintaining pelvic organ support.

Elastic fiber synthesis and assembly is a complex process that requires tropoelastin monomers to be cross-linked in the extracellular matrix to form a growing elastic fiber. Microfibrils are the scaffold on which tropoelastin is deposited before it is cross-linked by one or more of the copper-requiring lysyl oxidases.⁹ Fibulin-5 is a protein thought to participate in this elastic fiber building process by binding lysyl oxidase-like 1 (LOXL1) and possibly targeting it to fiber assembly sites in the extracellular matrix.^{10–12}

Mice with null mutations in genes encoding either fibulin-5 (*Fbln5*^{-/-})^{10,12} or LOXL1 (*Loxl1*^{-/-})¹¹ develop elastinopathies including emphysematous lungs; defective elastic laminae of the great vessels; and loose, stretchy skin. Both mouse models also develop pelvic organ prolapse remarkably similar to that seen in primates, ie, descent of the vagina, cervix, and bladder

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herniating through the pelvic floor musculature. Previously, we reported that aging alone, regardless of parity, results in pelvic organ prolapse in more than 90% of *Fbln5*^{-/-} mice.¹³ Liu and colleagues¹⁴ reported that all parous *Loxl1*^{-/-} mice developed prolapse after their first or second litters and that ~50% of nulliparous animals ultimately progressed to prolapse after more than 1 year of age.

The phenotype of these mouse models has led us to predict that other matrix proteins involved in elastic fiber assembly or degradation may be involved in the pathophysiology of pelvic organ prolapse. The fibulin family of proteins has seven known members characterized by tandem repeats of calcium binding-epidermal growth factor-like motifs and a C-terminal fibulin module. Repeating calcium binding-epidermal growth factor motifs are thought to be involved in protein-protein interactions, and biochemical analyses have revealed many interacting binding partners for fibulin-1 and fibulin-2 including fibronectin, proteoglycans, and basement membrane proteins.¹⁵ Fibulins are divided into two subgroups by similar size and binding preferences: fibulins-1, -2, -6 and fibulins-3, -4, -5, and -7.^{15,16} Most have been shown to bind tropoelastin and are believed to contribute to elastic fiber assembly. There may be some redundancy in their roles, however, because absence of fibulin-1 or -2 does not appear to have effects on elastic fiber homeostasis *in vivo*.¹⁷ To date, only fibulin-4 and -5 have been found to be essential in elastic fiber assembly. *Fbln4*^{-/-} mice die during or immediately after birth because of rupture of aortic aneurysms.^{10,12,18} *Fbln5*^{-/-} mice are born with elastic fiber defects in the lungs, skin, and aorta, but do not develop prolapse until 12 weeks of age.¹³ The role of fibulin-3 (also known as EFEMP1, MBP1, H411, or UPH1) in elastic fiber assembly or pelvic organ prolapse is unknown. Among the seven known fibulins, fibulin-3 shares highest homology with fibulin-4 and -5.¹⁹⁻²² Recently, McLaughlin and colleagues²³ reported that fibulin-3 has a specific effect on the integrity of elastic fibers in fascial connective tissues. *Fbln3*^{-/-} mice develop early aging and herniation of the abdominal wall that increases progressively with age.²³ In this study, we report the potential role of fibulin-3 in pelvic organ support by characterizing the gross and ultrastructural changes in the vaginal muscularis of mice deficient in fibulin-3 (*Fbln3*^{-/-}). Further, studies were conducted to lend insight into potential mechanisms by which the lack of fibulin-3 led to pelvic organ prolapse.

Materials and Methods

Mice

All mice were studied and euthanized in accordance with the standards of humane animal care described by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, using protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Animals were housed under a 12-hour dark/light cycle at 22°C.

Fbln3^{-/-} mice and wild-type (WT, *n* = 81) mice were the same strain (C57BL/6x129Sv/J). At sacrifice, after disarticulation of the pubic symphysis, uterine horns together with the bladder, cervix, and vagina were dissected down to the perineal skin. The vaginal dissection included the entire vaginal muscularis and the connective tissue suspending the vaginal wall to the pubocaudalis. Using microinstruments and a dissection microscope, the uterus was removed at the utero-cervico junction. Perineal skin was removed and the bladder and urethra dissected from the anterior vaginal wall. Wet weight of the vagina was determined by subtracting the weight of the dissected cervix from that of the cervicovaginal complex. The intact vaginal wall (muscularis and epithelium) was snap-frozen in liquid N₂ and stored at -80°C.

Prolapse Quantification

A validated mouse pelvic organ quantification (MOPQ) examination was used to serially measure the degree of vaginal, perineal, and rectal prolapse in 227 *Fbln3*^{-/-} and 81 WT mice weekly for up to 75 weeks of age. Animals less than 11 weeks (*n* = 197) were not measured because prolapse was not observed before this age. The MOPQ was conducted with one investigator holding the animal by the scruff of the neck, which resulted in a prolonged, reflex valsalva (evidenced by defecation), whereas the other investigator performed the measurements using a caliper with the precision of 0.01 mm. Six assessments were performed in each animal: i) stage of perineal bulge; ii) magnitude of perineal bulge; iii) cervical descent; iv) anal prolapse; v) perineal body length, and vi) vaginal diameter.¹³ Perineal bulge (0, none; 1, detectable but small; 2, moderate size bulge; 3, huge; 4, vagina coming out), cervical descent (0, not visible; 1, visible on straining inside the introitus; 2, at the level of the introitus; 3, outside the introitus), and anal prolapse (0, none; 1, present but mild; 2, severe) were measured on an ordinal scale. Magnitude of perineal bulge was measured in mm from the point of insertion of inner thigh to maximal edge of perineum. Perineal body was measured in mm from the posterior fourchette to mid-anus. Vaginal diameter was measured in mm from the anterior to posterior vaginal walls at the level of the introitus.

Histology and Ultrastructural Morphology

WT, *Fbln3*^{-/-}, and *Fbln5*^{-/-} mice were anesthetized and perfused with 10% neutral buffered formalin (pH 7.4). Thereafter, the female urogenital tract was dissected *en bloc*. Serial transverse sections (5 μm) were obtained in 100-μm increments throughout the specimen, stained with hematoxylin and eosin or Hart's stain, and analyzed with an Eclipse E1000N microscope (Nikon, Tokyo, Japan). For transmission electron microscopy, anesthetized animals were perfused first with ice-cold phosphate-buffered saline and then with 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). Full thickness rings of vaginal wall from WT and *Fbln3*^{-/-} mice were then dissected and fixed in fresh fixative overnight. Samples

were then sequentially treated with 1% osmium tetroxide, 2% tannic acid, and 2% uranyl acetate before dehydration and Epon embedding. Thin sections (60 nm) were placed on formvar-coated grids and counterstained with 7% methanolic uranyl acetate followed by lead citrate. Sections were viewed using a Tecnai 12 (FEI Co., Hillsboro, OR) transmission electron microscope at 120 kV and images were digitally captured.

Immunohistochemistry and Antibody Production

Rabbit polyclonal antibody BSYN5128 was raised against mouse fibulin-3 polypeptide (⁷⁸IVNNEHPQQTQPAEASS⁹⁵; Biosynthesis Inc., Lewisville, TX). Purified immunoglobulin- γ (IgG) and affinity-purified antibodies were prepared. Formalin-fixed, paraffin-embedded tissues from WT and *Fbln3*^{-/-} animals were sectioned at 5 μ m. After drying, slides were deparaffinized in xylene and rehydrated in graded alcohols to distilled water. Endogenous peroxidase activity was quenched for 10 minutes at room temperature, using 0.3% H₂O₂ with 0.1% sodium azide. Slides were subjected to steam-heat epitope retrieval in ethylenediaminetetraacetic acid buffer (1 mmol/L, pH 8.0) for 30 minutes. After rinsing in phosphate-buffered saline (PBS), slides were incubated in primary antibody (BSYN5128) for 30 minutes at 25°C using gentle orbital rotation. Negative control specimens were processed simultaneously in an identical manner, with the exception that PBS was used in place of primary antibody. After another rinse in PBS, slides were incubated with appropriate horseradish peroxidase-conjugated polymer (PowerVision reagent; ImmunoVision Technologies Co., Daly City, CA) for 30 minutes at 25°C. Finally, the slides were immersed for 5 minutes in 25°C diaminobenzidine (Invitrogen, Carlsbad, CA), enhanced with 0.5% copper sulfate in PBS for 5 minutes at 25°C, counterstained in hematoxylin, dehydrated in graded alcohols, cleared in xylene, and covered with a coverslip.

Gelatin Zymography

Vaginal tissues were thawed on ice, minced, washed in PBS, and homogenized in MMP2 assay buffer (EnzoLyte 520 MMP-2 assay kit; Anaspec, San Jose, CA) containing 0.1% Triton-X 100 (95 \times volume:tissue wet weight). Thereafter, homogenates were centrifuged at 10,000 \times g for 15 minutes at 4°C and supernatants used for determination of protease activity. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples (5 μ g per lane) were applied to gelatin polyacrylamide minigels (Invitrogen) (10%) in standard sodium dodecyl sulfate loading buffer containing 0.1% sodium dodecyl sulfate with no β -mercaptoethanol, and the samples were not boiled before loading. Gels were run at room temperature at 125 V. After electrophoresis, gels were soaked in renaturing buffer (2.7% Triton X-100 in distilled water) in a shaker for 30 minutes with one change after 30 minutes to remove sodium dodecyl sulfate. Next, gels were soaked in assay buffer (50 mmol/L Tris, 200 mmol/L NaCl, 10 mmol/L CaCl₂,

0.05% Brij 35, pH 7.5) for 4 to 16 hours at 37°C and then stained with Coomassie Brilliant Blue-R 250 in 50% methanol and 10% acetic acid followed by destaining with 25% methanol and 7% acetic acid. Clear zones of lysis against a dark background indicated enzyme activity, and purified enzymes were used as positive controls (Anaspec). Conditions of zymography and analysis were quantitative because enzyme activity was linear with time of incubation and protein loading, and samples for each experiment were applied to the same gel to avoid intergel variation. Areas of lysis were quantified using a Fuji LAS 3000 image analysis system (Fujifilm Life Science, Stamford, CT). Side-by-side immunoblots for α -tubulin protein were conducted to ensure equal protein loading of the gel. MMP activity was expressed as relative units per unit α -tubulin.

Immunoblot Analysis

Relative amounts of fibulin-5 and tropoelastin in the vaginal wall from five virginal WT mice, six *Fbln3*^{-/-} mice without prolapse, and five *Fbln3*^{-/-} mice with prolapse were determined using immunoblot analysis as described previously using 10 μ g of urea-extracted protein per lane.²⁴ All mice were matched for age and estrus cycle (metestrus), except in some mice with advanced prolapse that were not cycling. Protein extracts from *Fbln5*^{-/-} mice served as negative controls for fibulin-5.

Real-Time Polymerase Chain Reaction (PCR)

Quantitative PCR was used to determine the relative levels of *Timp1*, *Timp2*, and *Timp3* in vaginal tissues as described previously.²⁵ Primer sequences for amplifications were chosen using published cDNA sequences and chosen such that the resulting amplicons would cross an exon junction thereby eliminating the potential of false-positive signals from genomic DNA contamination. Primer sets for *Timp3* (BC014713) were 5'-CACGGAA-GCCTCTGAAAGTCTT-3'(⁵¹³⁻⁵³⁴) and 5'-CATACACGC-GCCCTGTCA-3'(⁵⁹²⁻⁵⁷⁵). SYBR Green was used for amplicon detection, and gene expression was normalized to that of the housekeeping gene β 2-microglobulin (*B2M*). All assays included positive, negative, and no template controls.

Quantification of Desmosine

Elastic fiber content was assessed by radioimmunoassay for desmosine, an amino acid crosslink found only in elastin. After urea extraction, pelleted tissue extracts were hydrolyzed in 6 N HCl at 100°C for 24 hours. An aliquot was evaporated again to dryness and redissolved in 100 μ l of H₂O, vortexed, microfuged, and assayed for desmosine as previously described.²⁶

Statistical Analysis

Differences in continuous variables of MOPQ scores, protease activities, and amounts of fibulin-5 and tro-

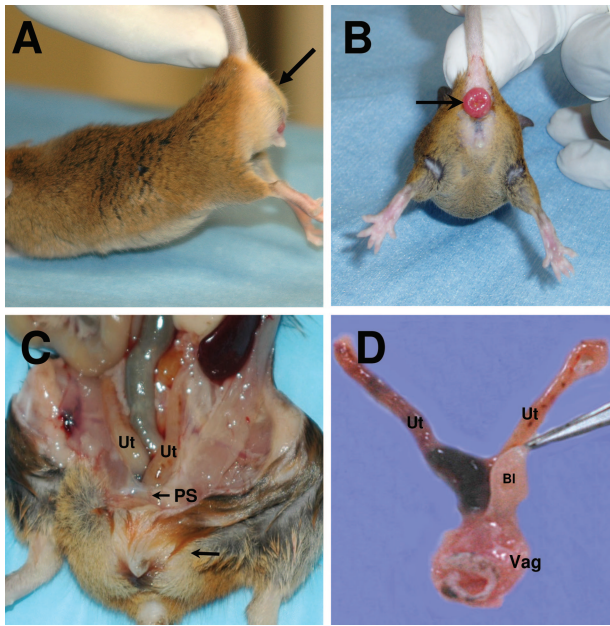


Figure 1. Pelvic anatomy of *Fbln3*^{-/-} mice with pelvic organ prolapse. Perineal bulge (stage 3; **A**, arrow) and rectal prolapse (**B**) are observed in *Fbln3*^{-/-} females. **C:** Uterine horns (Ut) descend beneath the pubic symphysis (PS). The bulge (arrow) is comprised of enlarged bladder and vagina. **D:** Dissected female reproductive tract of *Fbln3*^{-/-} mice with prolapse. Note patulous, enlarged vaginal wall (Vag). Ut, uterine horns; Bl, bladder.

poelastin for *Fbln3*^{-/-} mice with and without prolapse versus WT mice were assessed using analysis of variance with Student-Newman-Keuls method for pair-wise multiple comparisons. To determine whether age or parity were independent predictors of prolapse, a multiple logistic regression analysis was used with pelvic organ prolapse as the dependent variable and age and parity as independent variables. Vaginal weight comparisons between knockout and WT groups were made with the Student's *t*-test. Statistical software used was SigmaStat version 2.03 (Jandel Scientific, San Rafael, CA). *P* values ≤0.05 were considered statistically significant.

Results

Pelvic Organ Support in *Fbln3*^{-/-} Mice

During the course of this investigation, overt pelvic organ prolapse was noted in a significant number of *Fbln3*^{-/-} female mice (Figure 1A). Grossly, the phenotype of prolapse in *Fbln3*^{-/-} knockout animals resembled that of *Fbln5*^{-/-} and *Lox11*^{-/-} animals.^{14,24} Specifically, the perineal bulge was comprised of the bladder, vaginal vault, and descended uterine horns caudal to the pubic ligament (Figure 1C). Rectal prolapse and hernias were observed in the flank and inguinal areas (Figure 1B). Vaginal weight was increased in knockout animals compared with WT (mean ± SD): 77.6 ± 18.3 mg versus 44.2 ± 7.3 mg, respectively (*P* < 0.001). Prolapsed *Fbln3*^{-/-} vaginas were thin and patulous (Figure 1D). Like *Fbln5*^{-/-} mice, the connective tissue paravaginal and apical attachments (eg, the uterosacral ligaments) were either absent or attenuated in *Fbln3*^{-/-} mice with prolapse.

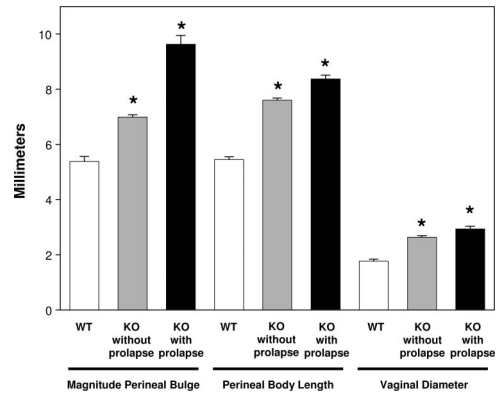


Figure 2. Pelvic organ support in *Fbln3*^{-/-} with or without pelvic organ prolapse. Magnitude of perineal bulge, perineal body length, and vaginal diameter were measured in WT and *Fbln3*^{-/-} mice without (KO without prolapse) and with (KO with prolapse) prolapse as part of the MOPQ scoring system. Prolapse was defined as the presence of ≥stage 1 perineal bulge (visually evident bulge). **P* ≤ 0.05 compared with WT.

Rectal prolapse was a common finding among *Fbln3*^{-/-} animals with concomitant perineal prolapse (18 of 53 or 34%) (Figure 1B). In contrast, rectal prolapse occurred in only 4 of 74 (5.4%) *Fbln5*^{-/-} mice.

To study the effect of fibulin-3 on subtle increases (and waxing and waning) in perineal bulge, rectal prolapse, and cervical descent, a longitudinal observation of pelvic organ support was conducted in WT and *Fbln3*^{-/-} mice. Knockout (*n* = 227) and C57BL/6 WT (*n* = 81) mice were examined weekly with MOPQ scores from birth; the oldest of which were greater than 1 year of age. The effects of pregnancy, parturition, and aging were noted. Continuous variable measurements of the MOPQ (magnitude of perineal bulge, perineal body length, and vaginal diameter) of WT and knockout animals with and without gross evidence of pelvic organ prolapse were compared and validated by examiners blinded to genotype (Figure 2). All parameters were increased significantly in knockout relative to WT animals (all *P* < 0.001, Figure 2), suggesting that pelvic organ support is abnormal in *Fbln3*^{-/-} mice even in the absence of obvious prolapse.

In agreement with our previous study involving WT mice of C3BL/6 strain,^{13,25} WT C57BL/6x129Sv mice did not develop perineal prolapse. In contrast, 53 of 197 knockout animals ≥11 weeks of age (26.9%) developed at least stage 1 perineal bulge. For these animals with prolapse, the average age at time of diagnosis was 37 weeks (range, 11.3 to 70.9 weeks). The majority of knockout animals (89%) developed prolapse after 20 weeks of age (*n* = 47, Figure 3). Of these older knockout mice with prolapse, 18 (34%) were parous and 35 (66%) were virginal. Using multivariate logistic regression analysis including age and parity as independent variables, prolapse was significantly related to age (*P* < 0.001; Figure 3, A–C) but not parity.

As described by McLaughlin and colleagues,²³ *Fbln3*^{-/-} mice developed hernias in either one or both flanks or inguinal area. These hernias were distinct from the perineal bulge of pelvic organ prolapse. Herein, we found that of 129 animals older than 40 weeks of age, 53 (53.5%) developed hernias, and of the mice with pelvic

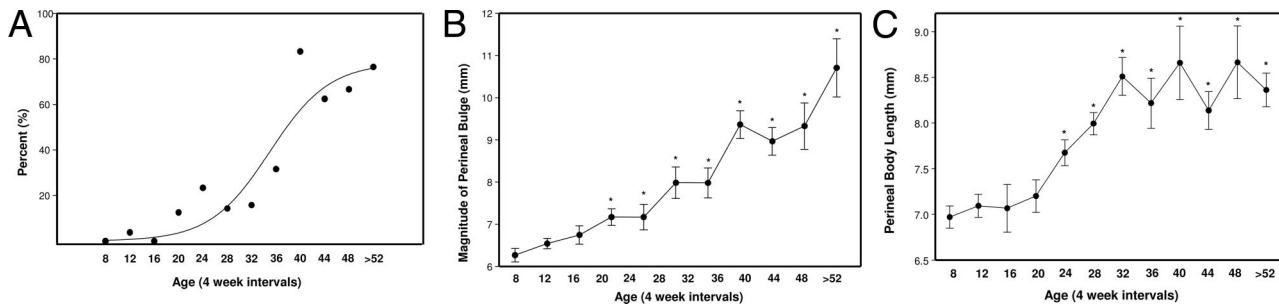


Figure 3. Rate and severity of pelvic organ prolapse in *Fbln3*^{-/-} mice. **A:** Percentage of *Fbln3*^{-/-} mice with ≥stage 1 pelvic organ prolapse as a function of age. Magnitude of perineal bulge (**B**) and perineal body length (**C**) are increased significantly in *Fbln3*^{-/-} mice with age. Measurements were derived from 197 animals >11 weeks of age. **P* < 0.05 compared with 12 weeks.

prolapse, 32% had concomitant hernias. Using multivariate logistic regression analysis, hernias were associated with mouse age (*P* < 0.001) but not parity. Interestingly, the background strain altered the prevalence of hernias in *Fbln3*^{-/-} mice.²³ To determine whether strain may alter the prevalence of prolapse, *Fbln3*^{-/-} mice on a BALB/c background were examined for 18 months. Of 36 animals younger than 1 year of age, none developed prolapse. Older BALB/c *Fbln3*^{-/-} mice (>1 year of age), however, developed severe stage 3 pelvic organ prolapse involving the rectum, uterus, and cervix (3 of 12 animals, data not shown). Thus, the background strain appears to modify the time of onset of prolapse, but pelvic organ prolapse is a phenotype of both C57BL/6 and BALB/c *Fbln3*^{-/-} mice.

Histology and Ultrastructural Morphology in *Fbln3*^{-/-} Mice

To examine the morphological changes in elastic fibers related to the loss of fibulin-3, vaginal tissues from *Fbln3*^{-/-} mice with and without perineal prolapse were fixed in formalin and stained for elastic fibers using Hart's stain. Transverse sections from the mid-vagina were analyzed. To control for potential changes induced by the estrus cycle, animals were sacrificed in metestrus, except in cases of stage 3 prolapse in which most animals were not cycling. In vaginal tissues from WT animals, elastic fibers were long and slender, with numerous fibers extending from the muscularis through the lamina propria branching just beneath the vaginal epithelium (Figure 4A). In vaginal tissues from *Fbln3*^{-/-} mice (Figure 4, B and C), elastic fibers were abnormal and somewhat similar to those observed in vaginal tissues from *Fbln5*^{-/-} mice (Figure 4D). Specifically, elastic fibers were blunt, thick, and fewer in number in the muscularis. Although normal in some areas near the subepithelium, elastic fibers were virtually always abnormal in the vaginal muscularis and adventitial layer in both *Fbln5*^{-/-} (Figure 4D) and *Fbln3*^{-/-} animals (Figure 4, B and C). The adventitial connective tissue is adjacent to the pubocaudalis muscle. In WT animals, this tissue is characterized by loose areolar tissue, neurovascular bundles, and long strands of intact elastic fibers that circumscribe the vaginal wall (Figure 4E). In *Fbln3*^{-/-} animals, elastic fibers of the

adventitial layer were grossly more abundant, fragmented, tortuous, and tangled (Figure 4F).

To gain further insight into the role of fibulin-3 and fibulin-5 in vaginal elastic fibers, vaginal tissues from 8-month-old WT, *Fbln3*^{-/-} with and without prolapse, and

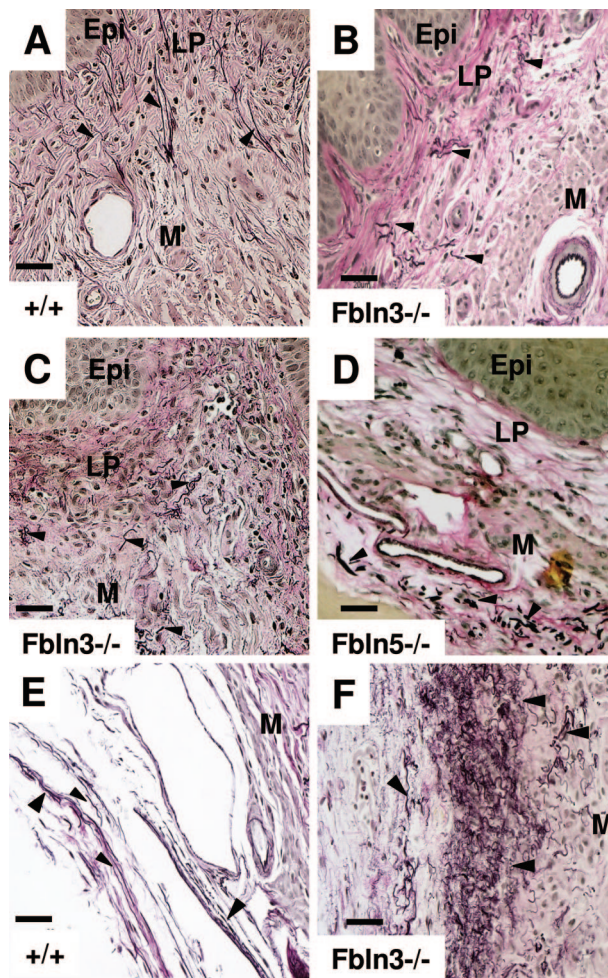


Figure 4. Elastic fiber morphology in *Fbln3*^{-/-} and *Fbln5*^{-/-} mice. Transverse sections of mid-vagina were stained with Hart's stain in WT mice (**A**, **E**), *Fbln3*^{-/-} mice without prolapse (**B**), *Fbln3*^{-/-} mice with prolapse (**C**, **F**), and *Fbln5*^{-/-} mice with prolapse (**D**). **Arrowheads** indicate elastic fibers. **A–D** represent epithelium (Epi), lamina propria (LP), and muscularis (M). **E** and **F** represent deep muscularis and adventitial layers of the vaginal wall adjacent to the pubocaudalis. Scale bars = 20 μ m.

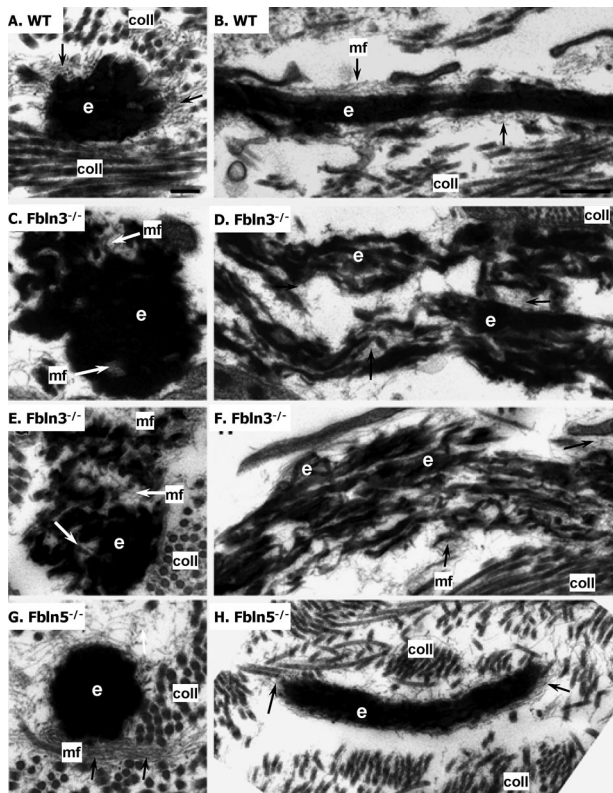


Figure 5. Ultrastructural morphology of elastic fibers in vaginal tissues from WT, *Fbln3*^{-/-}, and *Fbln5*^{-/-} mice at 6 to 8 months of age. **A** and **B**: Elastic fibers in vaginal wall from WT animals are comprised of a central elongated core of elastin (e) surrounded by numerous fine microfibrils (arrows, mf), and collagen fibers (coll) in the extracellular matrix. **C** and **D**: Elastic fibers in vaginal tissues from *Fbln3*^{-/-} mice without prolapse. Elastic fibers are disrupted resulting in evident microfibrils within the elastin core. Longitudinal section reveals significant disruption of the elastic fiber, which is porous and frayed. **E** and **F**: Elastic fibers in vaginal tissues from *Fbln3*^{-/-} mice with prolapse. Disrupted elastin core permits visualization of microfibrils throughout the elastin core. Severely disrupted and frayed elastic fibers are noted on longitudinal section. **G** and **H**: Elastic fibers in vaginal tissues from *Fbln5*^{-/-} mice. Large amorphous elastin aggregates and displaced clusters of microfibrils. Scale bars: 0.2 μm (**A**, **C**, **E**, **G**); 0.5 μm (**B**, **D**, **F**, **H**).

Fbln5^{-/-} mice with prolapse were analyzed by electron microscopy (Figure 5). In WT mice, vaginal elastic fibers were comprised of a solid core of elastin surrounded by microfibrils (Figure 5, A and B). Longitudinal sections of the fibers revealed that the fibers were long and slender with few disruptions in the elastin core region. In vaginal tissues from *Fbln3*^{-/-} animals without prolapse, elastic fibers were disrupted and tortuous (Figure 5, C and D). The elastic core was not uniform in density and small clumps of globular elastin were surrounded by microfibrils (Figure 5, C and D). Longitudinal views of the fiber revealed microfibrils located in the regions of the disrupted elastin core (Figure 5D). Disruption of elastic fibers was more prominent in vaginal tissues from *Fbln3*^{-/-} mice with prolapse with large areas of tangled microfibrils seen within the disrupted, tortuous, and frayed elastin core (Figure 5, E and F). Interestingly, although elastic fibers in the vaginal wall of *Fbln5*^{-/-} mice were similar to those of *Fbln3*^{-/-} by light microscopy, ultrastructural morphology of elastic fibers was unique between the two genotypes. In *Fbln5*^{-/-} mice, vaginal elastic fibers consisted of large globules of solid elastin

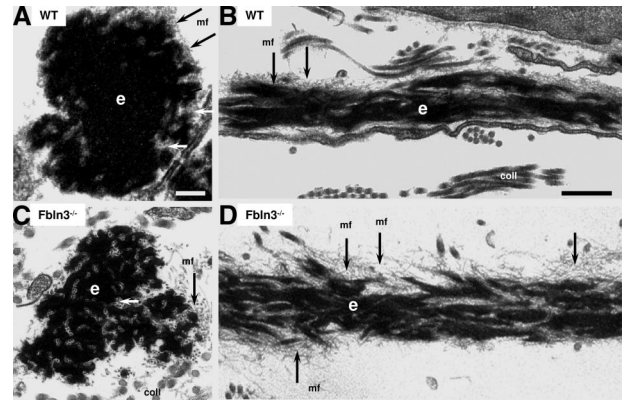


Figure 6. Ultrastructural morphology of elastic fibers in vaginal tissues from young WT and *Fbln3*^{-/-} mice. Elastic fibers from WT (**A**, **B**) and *Fbln3*^{-/-} (**C**, **D**) mice at 5 weeks of age. The elastin core (e) is more fragmented in vaginal tissues from young *Fbln3*^{-/-} females compared with WT. Microfibrils (mf) are noted with arrows. coll, collagen. Scale bars: 0.2 μm (**A**, **C**); 0.5 μm (**B**, **D**).

with displaced, more peripherally located microfibrils (Figure 5, G and H). Longitudinal sections of vaginal *Fbln5*^{-/-} elastic fibers revealed that the fibers were shorter and thicker compared with WT, but that the fibers were not disrupted to the same degree as those in the vaginal wall of *Fbln3*^{-/-} animals (Figure 5F).

The abnormal morphology of elastic fibers in older (8 months) *Fbln3*^{-/-} mice led us to investigate whether this morphology evolved with age, or whether abnormal vaginal elastic fibers were present in young knockout mice (Figure 6). Elastic fibers in the vaginal wall of young WT mice (5 weeks) were similar to those in older vaginal animals (Figure 6, A and B, compared with Figure 5, A and B). Although disruption of vaginal elastic fibers was not as severe as that in the vagina of older *Fbln3*^{-/-} mice, disruption of the elastin core was obvious even in young 5-week-old *Fbln3*^{-/-} females several months before the onset of prolapse (Figure 6, C and D).

Localization of Fibulin-3 in the Vaginal Wall

Using a polyclonal polypeptide antibody, immunohistochemistry was conducted to localize fibulin-3 in the vagina (Figure 7). Immunostaining was observed in cells juxtaposed to the basement membrane of epithelial cells, in the extracellular space lining subepithelial elastic fi-

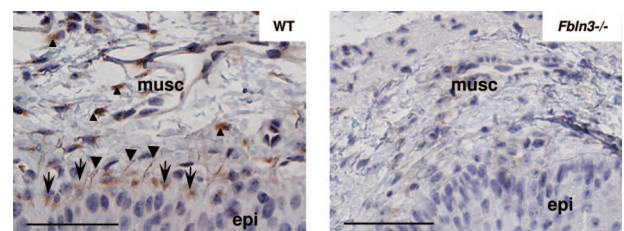


Figure 7. Localization of fibulin-3 in the vaginal wall. Transverse sections of mid vagina from WT (**left**) and *Fbln3*^{-/-} mice (**right**) at 6 months of age were immunostained with a polypeptide polyclonal antibody to fibulin-3. Immunostaining was visualized in subepithelial cells and basement membrane (arrows), subepithelial elastic fibers (arrowheads), and stromal cells of the vaginal muscularis (small arrowheads). Epi, epithelium; musc, muscularis. Scale bars = 20 μm .

bers, and in stromal cells of the vaginal muscularis (Figure 7, left). Although immunostaining was completely absent in negative control sections (not shown), faint immunostaining could be detected in the vaginal wall of *Fbln3*^{-/-} mice (Figure 7, right), suggesting that the antibody may recognize an epitope of another member of the fibulin family with much lower affinity because there is no evidence of fibulin-3 or fibulin-3 protein fragments in *Fbln3*^{-/-} mice.²³ Nevertheless, the results indicate that fibulin-3 protein is expressed in subepithelial cells of the vaginal wall and in the vaginal muscularis.

Matrix Degradation in *Fbln3*^{-/-} Mice

MMP2 and MMP9 enzyme activities were determined in vaginal tissues from *Fbln3*^{-/-} mice with and without prolapse and virginal age- and estrus phase-matched WT controls using gelatin zymography (Figure 8). Pro-MMP2, active MMP2, and active MMP9 were increased significantly in vaginal tissues from *Fbln3*^{-/-} mice with prolapse compared with WT (pro-MMP2, 1.9-fold, *P* = 0.013; MMP2, 2.0-fold, *P* = 0.039; total MMP9, 2.8-fold, *P* = 0.042) (Figure 8). MMP activity in *Fbln3*^{-/-} without prolapse did not differ significantly from WT.

Tissue inhibitors of matrix metalloproteases (TIMPs) are extracellular matrix proteins that bind to and inhibit MMP activity. In addition to its role in elastogenesis, fibulin-3 has been shown to be a strong binding partner of TIMP3.²⁷ Thus, a possible mechanism by which *Fbln3*^{-/-} mice develop pelvic organ prolapse may be through increases in MMP activity mediated through changes in regulation or activity of TIMP3, TIMP2, or TIMP1. Expression of *Timp1* mRNA was up-regulated in the prolapsed vagina from *Fbln3*^{-/-} animals; *Timp2* was not altered; and, *Timp3* was increased in *Fbln3*^{-/-} animals with or without prolapse (Figure 9). Thus, it

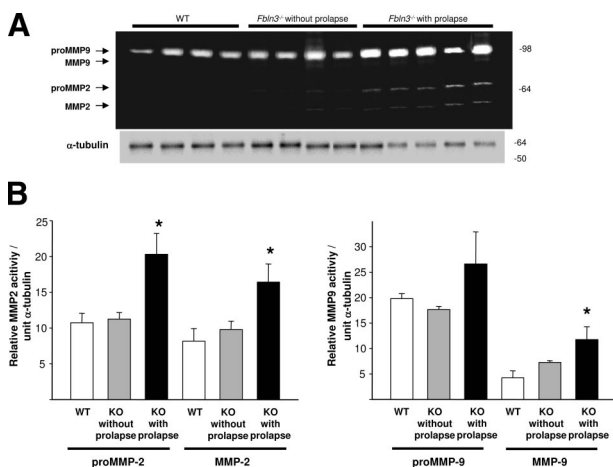


Figure 8. MMP2 and MMP9 activity in vaginal tissues from *Fbln3*^{-/-} mice. **A:** Gelatin zymogram indicating pro- and active MMP9 and MMP2 in vaginal tissue extracts from WT mice, *Fbln3*^{-/-} mice without prolapse, and *Fbln3*^{-/-} mice with prolapse. Immunoblot for α -tubulin is placed beneath the zymogram. **B:** Quantification of MMP2 and MMP9 in vaginal tissues from WT (*n* = 4) and *Fbln3*^{-/-} (KO) mice without (*n* = 4) and with prolapse (*n* = 5). Enzyme activity is expressed in relative units and normalized to α -tubulin. **P* \leq 0.05 compared with WT.

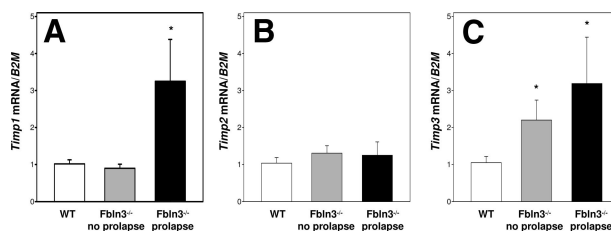


Figure 9. Expression of TIMPs in the vaginal wall. Relative levels of *Timp1* (A), *Timp2* (B), and *Timp3* (C) mRNA were quantified in vaginal tissues from WT (*n* = 5) and *Fbln3*^{-/-} mice without (*n* = 5) and with prolapse (*n* = 6) and expressed relative to that of β 2-microglobulin (*B2M*). **P* \leq 0.05 compared with WT.

does not appear that loss of tissue inhibitors (at least at the mRNA levels) is involved in up-regulation of MMP2 and MMP9 in vaginal tissues from *Fbln3*^{-/-} mice.

Elastic Fiber Synthesis in Vaginal Walls of *Fbln3*^{-/-} Mice

Fibulin-5 and tropoelastin proteins are both essential for assembly of mature elastic fibers, and both proteins are increased significantly in the vaginal wall postpartum.²⁴ To determine whether increased MMP activity in knockout mice is counterbalanced by changes in elastic fiber synthesis, fibulin-5 and tropoelastin protein levels were quantified in vaginal tissues from virginal age- and estrus phase-matched WT (*n* = 5), *Fbln3*^{-/-} mice without prolapse (*n* = 6), and *Fbln3*^{-/-} mice with prolapse (*n* = 5). In agreement with previous studies,²⁴ fibulin-5 and tropoelastin were highly expressed in urea extracts of vaginal tissues from nonpregnant WT mice (Figure 10). Fibulin-5 was increased significantly in vaginal tissues from *Fbln3*^{-/-} with or without prolapse (*P* \leq 0.01) (Figure 10, A and C). Tropoelastin was also increased in urea extracts from *Fbln3*^{-/-} mice with or without prolapse (Figure 10, B and C). To address the possibility that fibulin-5 and tropoelastin may be more extractable from vaginal tissues from *Fbln3*^{-/-} mice, we analyzed tropoelastin and fibulin-5 protein content in the initial salt/ethylenediaminetetraacetic acid extract. Although strong signals for each protein were present in urea extracts, tropoelastin and fibulin-5 were absent in all salt/ethylenediaminetetraacetic acid extracts (data not shown). The findings suggested that although increased expression of steady state levels of fibulin-5 may occur in response to the loss of fibulin-3 in the vaginal wall, it does not protect from failure of pelvic organ support.

To quantify cross-linked elastin in the vaginal wall, desmosine levels were determined in vaginal tissues from age-, strain-, and cycle-matched WT and *Fbln3*^{-/-} mice with or without prolapse (Figure 11). Desmosine levels were increased in vaginal tissues from knockout animals regardless of prolapse. Thus, the vaginal wall of knockout animals is characterized by increased levels of fibulin-5 and tropoelastin in the urea extract; increased desmosine content; and abundant, tortuous, and fragmented elastic fibers in the adventitial layer.

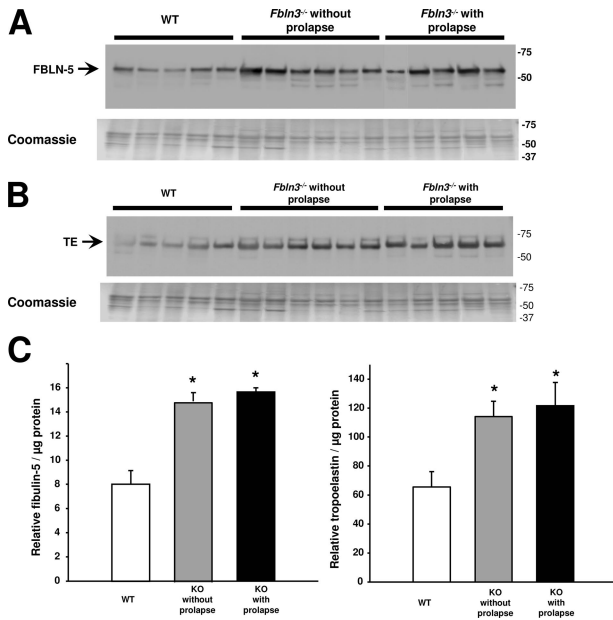


Figure 10. Expression of fibulin-5 (FBLN-5) and tropoelastin (TE) in vaginal tissues from *Fbln3*^{-/-} mice. **A:** Immunoblot of FBLN-5 was conducted in urea extract proteins from vaginal tissues of WT and *Fbln3*^{-/-} mice without and with prolapse. Coomassie staining was conducted in side-by-side gels to ensure even protein loading. **B:** Immunoblot of tropoelastin (TE) in urea extract proteins from vaginal tissues of WT and *Fbln3*^{-/-} mice without and with prolapse. Coomassie staining was conducted in side-by-side gels to ensure even protein loading. **C:** Quantification of FBLN-5 and TE in urea extract proteins from vaginal tissues of WT and *Fbln3*^{-/-} mice without and with prolapse. Data represent mean \pm SEM of five to six tissues in each group. **P* \leq 0.05 compared with WT.

Discussion

Role of Fibulin-3 in Pelvic Organ Support

Using a validated quantification tool for assessing prolapse, we found that 27% of mature *Fbln3*^{-/-} knockout animals develop overt prolapse, which was concomitant with hernia formation in 32% and rectal prolapse in 34%. Compared with WT controls, perineal bulge, perineal

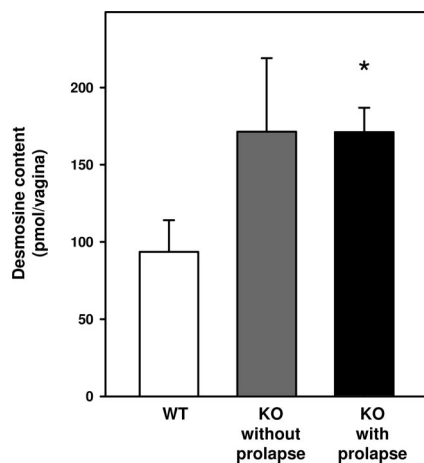


Figure 11. Desmosine content in the vaginal wall. Desmosine levels were determined in vaginal tissues from age-, cycle-, and strain-matched WT (*n* = 5) and *Fbln3*^{-/-} (KO) mice without (*n* = 5) and with prolapse (*n* = 7). Desmosine levels were expressed as the total amount of desmosine per vagina. **P* \leq 0.05 compared with WT.

body length, and vaginal diameter were increased significantly in *Fbln3*^{-/-} mice even without obvious prolapse. As in women, the frequency of prolapse increased with age. Multivariate analysis did not indicate a significant association between parity and prolapse, suggesting that the lack of fibulin-3 does not impact the ability of pelvic floor connective tissue to recover after parturition but that fibulin-3 is necessary to maintain pelvic organ support during aging. These findings suggest that although elastic fiber assembly and synthesis are important in the pathophysiology of pelvic organ prolapse, biochemical pathways to maintain or recover pelvic organ support after parturition may be different from those during aging.

Development of *Fbln5*^{-/-} and *Loxl1*^{-/-} mouse models has led to the hypothesis that elastic fiber synthesis and assembly play important roles in the maintenance of pelvic organ support. The current study underscores this concept. Fibulin-3 has been shown to be important for elastic fibers in abdominal fascia such that absence of this protein leads to age-dependent development of abdominal and inguinal hernias.²³ Herein, we show that absence of fibulin-3 also leads to age-dependent disruption of elastic fibers in connective tissue of the vaginal wall and development of pelvic organ prolapse.

Fibulin-3 Is Crucial for Normal Elastogenesis in the Vagina

Fibulin-5 and fibulin-4 have been shown to play crucial roles in elastic fiber development.^{10,12,28} Fibulin-5 preferentially binds monomeric tropoelastin²⁹ and regulates self-aggregation of elastin, a process termed coacervation.³⁰ Recent studies *in vitro* suggest that fibulin-5 promotes coacervation and alignment of tropoelastin on the microfibrillar scaffold for subsequent alignment and crosslinking.³⁰⁻³² Our electron microscopy findings are consistent with this suggestion, in that large globules of elastin with displacement of microfibrils were observed in the vaginal wall of *Fbln5*^{-/-} mice. In contrast, in the absence of fibulin-3, elastic fibers of the vaginal wall were disrupted such that microfibrils were easily visualized within the elastin core. Disruption of elastic fibers was more severe with age and prolapse.

Age-related modifications in elastic fibers (extensively described in virtually all organs and tissues) may be primarily interpreted as progressive degradation of elastin polymers produced early in life. During aging, elastic fibers become tortuous, frayed, and porous.³³ In the vaginal wall, however, elastic fiber degradation was not observed during aging. Unlike other adult organs, elastic fiber turnover appears to be continuous in the female reproductive tract.^{6,34} This unique adaptation to synthesize and assemble new elastic fibers may allow the vagina to expand during pregnancy, recover from childbirth, and cope with prolonged gravitational forces associated with aging. Unlike skin,²³ fibulin-3 deficiency may lead to progressive and severe deterioration of elastic fibers in an organ such as the vagina that undergoes

continuous remodeling with cyclic degradation and synthesis of elastic fibers.

Phenotypic Differences in *Fbln3*^{-/-} and *Fbln5*^{-/-} Females

The magnitude or severity of prolapse in *Fbln3*^{-/-} mice was similar to that of *Fbln5*^{-/-} animals. Nonetheless, a smaller percentage of *Fbln3*^{-/-} mice developed the disease, and animals were much older at the onset of prolapse. The lack of fibulin-3 was associated with progressive degradation of elastic fibers in the vaginal wall with aging. Despite increases in fibulin-5, tropoelastin, and desmosine levels, elastic fibers became disrupted with age and failure of pelvic organ support occurred in *Fbln3*^{-/-} animals, suggesting that other factors related to aging may have led to pelvic organ prolapse. One of these factors may be increased vaginal MMP activity. Because the age of prolapse was not predictable in *Fbln3*^{-/-} mice, it is unclear whether increased vaginal protease activity preceded the development of prolapse or if increased MMP activity resulted from the mechanical stress on the prolapsed vaginal tissue. Nevertheless, progressive elastin degradation in the vaginal wall may lead to accumulation of elastin degradative peptides, which activate proteases in a number of tissues.³⁵ Overall, the results indicate that development of prolapse in *Fbln3*^{-/-} mice involves both increased production and degradation of elastic fibers in the vaginal wall, a situation not unlike solar elastosis of photoaged skin in which production of matrix macromolecules by dermal fibroblasts is accompanied by significant increases in matrix-degrading enzymes.³⁶

Fbln3^{-/-} animals develop abdominal and inguinal hernias whereas *Fbln5*^{-/-} mice do not. On the other hand, *Fbln5*^{-/-} animals develop tortuosity of the great vessels whereas *Fbln3*^{-/-} mice do not. Both animals develop pelvic organ prolapse. The importance of each fibulin in different tissues is probably related not only to compensatory roles of other family members, but also to tissue-specific levels of expression. Overall, the results in this investigation indicate that both fibulin-3 and -5 are important in maintaining pelvic organ support in mice. Absence of either protein results in altered elastic fibers in the connective tissue of the vaginal wall and in failure of pelvic organ support with age. Fibulin-5 and tropoelastin are increased in the vaginal wall of *Fbln3*^{-/-} mice with or without prolapse, suggesting a redundant and compensatory mechanism for elastic fiber assembly in the vagina. MMP2 and MMP9 enzyme activities, however, are increased only in vaginal tissues of *Fbln3*^{-/-} mice with prolapse. The difference in penetrance of pelvic organ prolapse between the two mouse models may be explained by the significant up-regulation of other components of elastic fiber assembly in *Fbln3*^{-/-} mice, possible differences in the relative abundance of each fibulin in connective tissues of the pelvic floor, differences in localization throughout the pelvic floor connective tissues, and the magnitude and timing of protease activation in the vaginal wall.

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