Genetic analysis of the contribution of LTBP-3 to thoracic aneurysm in Marfan syndrome

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Marfan syndrome (MFS) is an autosomal dominant disorder of connective tissue, caused by mutations of the microfibrillar protein fibrillin-1, that predisposes affected individuals to aortic aneurysm and rupture and is associated with increased TGFβ signaling. TGFβ is secreted from cells as a latent complex consisting of TGFβ, the TGFβ propeptide, and a molecule of latent TGFβ binding protein (LTBP). Improper extracellular localization of the latent complex can alter active TGFβ levels, and has been hypothesized as an explanation for enhanced TGFβ signaling observed in MFS. We previously reported the absence of LTBP-3 in matrices lacking fibrillin-1, suggesting that perturbed TGFβ signaling in MFS might be due to defective interaction of latent TGFβ complexes containing LTBP-3 with mutant fibrillin-1 microfibrils. To test this hypothesis, we genetically suppressed *Ltbp3* expression in a mouse model of progressively severe MFS. Here, we present evidence that MFS mice lacking LTBP-3 have improved survival, essentially no aneurysms, reduced disruption and fragmentation of medial elastic fibers, and decreased Smad2/3 and Erk1/2 activation in their aortas. These data suggest that, in MFS, improper localization of latent TGFβ complexes composed of LTBP-3 and TGFβ contributes to aortic disease progression.

extracellular matrix | transforming growth factor beta | aneurysm | LTBP

Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder caused by mutations in the gene encoding fibrillin-1 (FBN1), an extracellular matrix (ECM) glycoprotein that is the main component of microfibrils and that associates with elastin to form elastic fibers. In MFS, defects in microfibrils predispose individuals to thoracic aortic aneurysm (TAA), with ensuing vessel dissection and rupture (1, 2).

The vascular defects in MFS were initially considered a consequence of constitutive tissue weakness due to structurally abnormal fibrillin-1 microfibrils (3). However, mouse models of MFS revealed that abnormal fibrillin-1 resulted in an increase in signaling by transforming growth factor beta (TGFβ), a cytokine involved in cell proliferation, differentiation, and matrix synthesis. TGFβ signaling requires the cytokine to bind its type II cell surface receptor (TβRII), which recruits and phosphorylates the type I receptor (TβRI). TβRI phosphorylates SMAD2/3 (mothers against decapentaplegic homolog 2/3), which forms a heterodimeric complex with SMAD4 and enters the nucleus to activate the transcription of TGFβ-dependent genes. The TGFβ–TβRI–TβRII complex also can activate MAPK signaling pathways, including ERK1 and ERK2 (ERK1/2) (4). The levels of both active SMAD2/3 and ERK1/2 are heightened in the ascending aortas of MFS mouse models (5–7). Treatment of these animals with TGFβ neutralizing antibodies (TGFβ-Nab) prevents or impedes TAA progression in some studies (6, 7), while exacerbating arterial disease in others (5).

TGFβ is secreted from cells as part of a biologically inactive large latent complex (LLC), composed of LTBP-1, -3, or -4, the prodomain dimer of TGFβ, referred to as the latency associated peptide (LAP), and the mature TGFβ dimer. LAP associates noncovalently with mature TGFβ to form the small latent complex (SLC). Covalent binding of the SLC to an LTBP occurs in the secretory pathway through the formation of two disulfide bonds between LAP and the third 8-Cys domain of LTBP-1, -3, or -4. Of the four LTBPs, LTBP-1 and -3 bind efficiently to all three TGFβ (TGFβ1, -2, and -3) LAP isoforms whereas LTBP-4 binds very inefficiently and only to TGFβ1 LAP (8, 9). Moreover, LTBP-3 requires binding to TGFβ for secretion and is secreted only in the LLC form, suggesting an important role for LTBP-3 in the control of TGFβ availability (8, 9).

LTBPs regulate TGFβ activity by facilitating its secretion, by localizing the LLC to specific sites in the ECM, and by participating in latent TGFβ release from the ECM (9–12). For TGFβ to bind to its receptor, the interaction of LAP and TGFβ must be disrupted, a process known as latent TGFβ activation (13, 14). LTBP localization into the ECM is important for latent TGFβ activation. Abnormal localization is reported to alter TGFβ activity in both positive and negative ways: e.g., overexpression of a mutated form of LTBP-1 that binds TGFβ but does not interact with the ECM results in increased TGFβ activity (15) whereas mice in which the cysteines that link the propeptide of TGFβ1 to LTBP were mutated to serines, thereby blocking covalent interaction with LTBP and subsequent association to the ECM, have multiorgan inflammation resembling that observed in TGFβ1-null mice (16). In addition, cleavage of LTBP-1 by a bone morphogenetic protein 1 (BMP1)-like metalloproteinase liberates LLC from the ECM and leads to activation of TGFβ1 by MMP2 (17).

The mechanisms by which defective microfibrils perturb TGFβ signaling and cause aortic disease in MFS remain poorly understood. A current hypothesis proposes that abnormal fibrillin-1 fibers cause faulty LLC matrix incorporation, yielding increased TGFβ signaling with consequent aortic aneurysm and dissection (1, 2). However, there is no evidence demonstrating either the

Significance

Marfan syndrome (MFS) is a connective tissue disorder caused by mutations of fibrillin-1 (FBN1), the main component of extracellular matrix microfibrils. *FBN1* mutations predispose to thoracic aortic aneurysm and rupture and are associated with increased TGFβ signaling. TGFβ is secreted from cells complexed with latent TGFβ binding protein (LTBP), a protein that targets TGFβ to the ECM through interaction with fibrillin-1. One hypothesis proposes that aortic disease in MFS is due to the release of LTBP/TGFβ complexes in the aortic wall. We suppressed the expression of *Ltbp3* in an MFS mouse model and observed essentially no aortic aneurysm and rupture in these compound mice. Our data suggest a key role for LTBP-3 in MFS aortic disease and provide a potential therapeutic point for intervention.

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participation of the LLC in MFS aortic disease or which LTBP is involved. We previously reported the in vitro and in vivo absence of LTBP-3, but not LTBP-1, incorporation into matrices that lack fibrillin-1 microfibrils, implying that LTBP-3 is the functionally important LTBP affecting latent TGFβ in MFS (18). In the present study, we present data that identify LTBP-3 as an important contributor to TAA in MFS.

Results

Genetic Deletion of *Ltbp3* Prevents Premature Death of *Fbn1*$^{mgR/mgR}$ Mice. To identify a possible role of LTBP-3 in aortic disease in MFS, we generated *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice. *Fbn1*$^{mgR/mgR}$ mice are a hypomorphic MFS mouse model and represent a progressively severe model of MFS because most of the affected animals die from dissecting TAA within 3 mo after birth (19, 20). The mutant allele encodes a WT protein, but the animals produce only 10–20% of the normal amount of fibrillin-1 (19). Survival studies were performed, and the mice were followed for 3 mo (Fig. 1*A*). More than 90% of *Wt* and *Ltbp3*$^{-/-}$ mice survived for this period whereas *Fbn1*$^{mgR/mgR}$ mice displayed 70% lethality by 90 d. Of the *Fbn1*$^{mgR/mgR}$ mice that died, 75% of deaths were due to ruptured ascending aneurysms, as determined by necropsy, in agreement with published data (19). Surprisingly, survival of *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice was almost equivalent to *Wt* and *Ltbp3*$^{-/-}$ mice (Fig. 1*A*). There was no evidence of aortic rupture in any of the *Wt*, *Ltbp3*$^{-/-}$, or *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice. Together, these data suggest that the absence of LTBP-3 significantly attenuates aortic disease in *Fbn1*$^{mgR/mgR}$ mice and prolongs their survival.

Ltbp3 Deletion Prevents Elastin Degradation and Aneurysm in *Fbn1*$^{mgR/mgR}$ Mice. At 9 wk of age, obvious aneurysms of the ascending aortas on gross examination were observed in 63% (17 of 27) of killed *Fbn1*$^{mgR/mgR}$ mice compared with none observed in *Wt*, *Ltbp3*$^{-/-}$, and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice (Fig. 1*B*). We evaluated ascending aortic diameters at 9 wk of age by transthoracic echocardiography. We observed a significant dilatation of the ascending aortas of *Fbn1*$^{mgR/mgR}$ mice (2.08 ± 0.48 mm) compared with both *Wt* (1.59 ± 0.11 mm) and *Ltbp3*$^{-/-}$ (1.59 ± 0.16 mm) mice. Absence of *Ltbp3* in *Fbn1*$^{mgR/mgR}$ mice significantly attenuated the aortic enlargement (1.58 ± 0.19 mm), which was equivalent to the diameter of *Wt* or *Ltbp3*$^{-/-}$ aortas (Fig. 1*C*). Blood pressure measurements indicated no difference between *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ animals (Fig. S1).

Previous studies of *Fbn1*$^{mgR/mgR}$ mice demonstrated progressive fragmentation of aortic elastic lamellae in the ascending aorta as the animals aged (19). Histological analysis of sections from 2-mo-old *Fbn1*$^{mgR/mgR}$ ascending aortas revealed disorganized media with extensive elastic fiber fragmentation (Fig. 1 *D* and *E*). On the other hand, the aortic wall architecture and the number of elastic breaks were normalized in *Fbn1*$^{mgR/mgR}$ mice lacking LTBP-3 (Fig. 1 *D* and *E*). In line with those observations, we observed a marked increased of the metalloelastase *Mmp12* mRNA expression in *Fbn1*$^{mgR/mgR}$ compared with *Wt* (Fig. S2). Absence of LTBP-3 in *Fbn1*$^{mgR/mgR}$ significantly attenuated the expression of *Mmp12* to a level of expression equivalent to *Wt* samples (Fig. S2). Together these data indicate that LTBP-3 absence in *Fbn1*$^{mgR/mgR}$ mice preserves aortic elastic fiber integrity and protects against TAA and rupture.

Decreased Incorporation of LTBP-3 in the ECM of *Fbn1*$^{mgR/mgR}$ Aortic Smooth Muscle Cells. We determined whether hypomorphic expression of *Fbn1* has an effect on matrix targeting of LTBP-3 in aortic smooth muscle cells (ASMCs) isolated from the ascending aorta. After 14 d in culture, immunoreactivity was detected in *Wt*

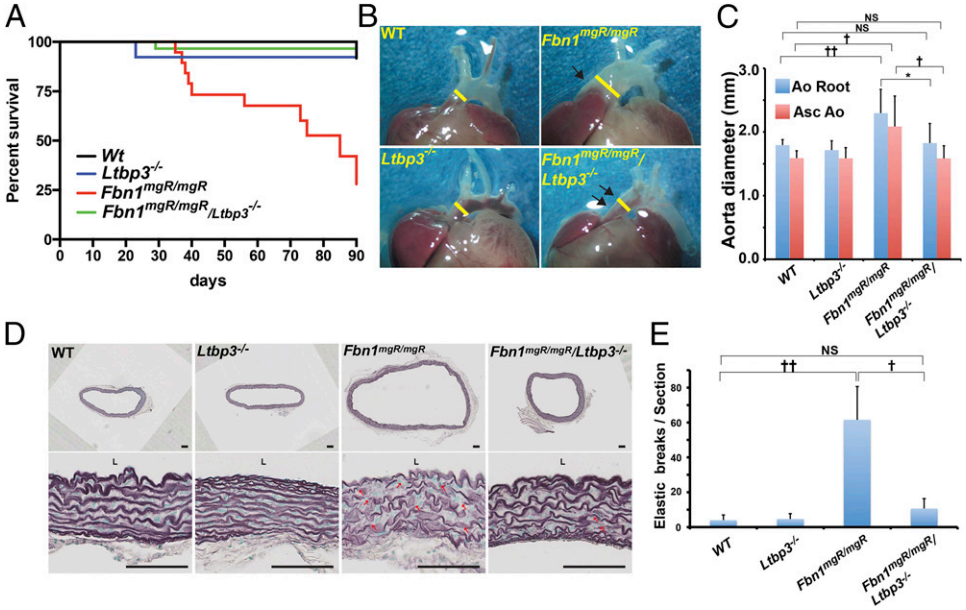

Fig. 1. Absence of *Ltbp3* prevents premature death, aneurysm of the ascending aorta, and elastic fiber breaks in *Fbn1*$^{mgR/mgR}$ mice. (*A*) Kaplan–Meier survival curves of WT ($n = 34$), *Ltbp-3*$^{-/-}$ ($n = 26$), *Fbn1*$^{mgR/mgR}$ ($n = 21$), and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ($n = 30$), demonstrating a decreased rate of death of *Fbn1*$^{mgR/mgR}$:*Ltbp-3*$^{-/-}$ mice compared with *Fbn1*$^{mgR/mgR}$ mice. Differences between *Fbn1*$^{mgR/mgR}$ and *Wt* or *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice were statistically significant ($P < 0.0001$). There was no statistically significant difference between *Wt* and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice ($P = 0.34$). (*B*) Gross morphology of the ascending aorta in 2-mo-old *Wt*, *Ltbp3*$^{-/-}$, *Fbn1*$^{mgR/mgR}$, and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ mice. *Fbn1*$^{mgR/mgR}$ mice typically develop ascending aortic aneurysms (single arrows). Ablation of LTBP-3 in *Fbn1*$^{mgR/mgR}$ mice prevented aneurysm formation (double arrows). Yellow bars highlight aortic diameter. (*C*) Average aortic root (AoR) and ascending aorta (AscAo) dimensions measured by echocardiogram of 2-mo-old *Wt* ($n = 8$), *Ltbp3*$^{-/-}$ ($n = 5$), *Fbn1*$^{mgR/mgR}$ ($n = 9$), and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ($n = 5$) mice. *Fbn1*$^{mgR/mgR}$ aortic diameter was significantly increased compared with *Wt* whereas there was no significant difference in aortic diameter between *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ and *Wt* or *Ltbp3*$^{-/-}$ mice. Data are presented as the mean ± SD. *$P < 0.05$; †$P < 0.02$; ††$P < 0.005$. ANOVA $P < 0.01$. NS, not significant. (*D*) Staining of elastic fibers in cross-sections of ascending aortas from 2-mo-old *Wt*, *Ltbp3*$^{-/-}$, *Fbn1*$^{mgR/mgR}$, and *Fbn1*$^{mgR/mgR}$/*Ltbp3*$^{-/-}$ mice. Arrows indicate elastic lamellae fragmentation. L, lumen. (Scale bars: all panels, 100 μm.) (*E*) Quantification of elastic breaks per aorta section for each genotype. Data are presented as the mean ± SD of at least three samples for each genotype. †$P < 0.01$; ††$P < 0.001$; ANOVA, $P < 0.001$. NS, not significant.

cultures with antibodies against both LTBP-3 and fibrillin-1 (Fig. 2*A*). In *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ extracellular matrix, there were fewer detectable fibrillin-1 fibers than in *Wt* cultures. This absence correlated with decreased incorporation of LTBP-3 in *Fbn1*$^{mgR/mgR}$ extracellular matrix. As expected, staining for LTBP-3 was totally absent in the ECM of *Ltbp3*$^{-/-}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ASMCs (Fig. 2*A*). On the other hand, LTBP-1 seemed to be present in the ECM of all four genotypes in roughly equivalent amounts (Fig. 2*A*). We analyzed 24 h conditioned media from the ASMCs by Western blotting for LTBP-3. We observed the absence of LTBP-3 in the *Ltbp3*$^{-/-}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ samples compared with those from *Wt* or *Fbn1*$^{mgR/mgR}$ cultures (Fig. 2*B*). There was a 60% decrease in the intensity of the LTBP-3 band in *Fbn1*$^{mgR/mgR}$ compared with *Wt* samples (Fig. 2*B*). Immunoblotting with an antibody specific for LTBP-1 revealed no differences in the amount of protein released by cells of the four genotypes (Fig. 2*B*). These experiments also revealed that, in nonreducing conditions, the molecular mass of the detected LTBP-3 band is consistent with that of the LTBP-3/LAP complex (Fig. 2*B*), implying that the changes in LTBP-3 levels would also affect latent TGFβ incorporation.

Next, we performed quantitative real-time PCR (qPCR) analyses to assess the relative levels of *Fbn1* mRNA in the ascending aortas of the different genotypes. As expected, we observed a decrease of *Fbn1* mRNA in *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ compared with *Wt* and *Ltbp3*$^{-/-}$ aortas (Fig. S3). There was no increase in *Fbn1* expression in response to the absence of LTBP-3 in either *Ltbp3*$^{-/-}$ or *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ tissues, indicating no compensation for fibrillin-1 expression in the *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ascending aortas. This result was confirmed by immunostaining against FBN1 on tissue sections of the ascending aorta. In *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ aortas, FBN1 staining in the aortic wall was greatly decreased compared with *Wt* or *Ltbp3*$^{-/-}$ (Fig. 2*C*). We observed equivalent levels of immunoreactivity against FBN1 in *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ samples, with no indication of an increase in FBN1 assembly in the absence of Ltbp3 (Fig. 2*C*). mRNA levels of LTBP-1, -3, and -4 in the ascending aortas of the four genotypes were analyzed by qPCR (Fig. S4). We observed similar levels of *Ltbp1* and *Ltbp4* transcripts in the four genotypes. Interestingly, there was a statistically significant increase in the mRNA level of *Ltbp3* in *Fbn1*$^{mgR/mgR}$ compared with *Wt* (Fig. S4). The biological significance of this increase is unclear.

In summary, these data suggest that, in *Fbn1*$^{mgR/mgR}$ ASMCs, the LLC composed of LTBP-3, LAP, and TGFβ is poorly incorporated into the matrix and abrogation of *Ltbp3* expression in *Fbn1*$^{mgR/mgR}$ mice prevents the formation of this complex and has no effect on FBN1 expression.

Absence of LTBP-3 Attenuates Activation of Smad2/3 and Erk1/2 in *Fbn1*$^{mgR/mgR}$ Aortas. Increased ERK1/2 and Smad2/3 phosphorylation has been observed in the aortic wall in human tissues and in MFS mouse models (5, 7, 21). Moreover, treatment of *Fbn1*$^{C1039G/+}$ mice, a slowly progressing and mild form of MFS, with TGFβ-neutralizing antibodies, ERK1/2 inhibitor, or AT1r antagonists ameliorates the aortic phenotype in association with attenuated activation of Smad2/3 and ERK1/2 signaling (5, 7, 21). We therefore examined the effect of *Ltbp*3 deletion in *Fbn1*$^{mgR/mgR}$ mice on ERK1/2 and Smad3 phosphorylation in the ascending aorta. We performed Western blotting analysis on ascending aorta lysates from 12-wk-old mice. Strong up-regulation of phospho-ERK1/2 (p-ERK1/2) was observed in *Fbn1*$^{mgR/mgR}$ aortas compared with *Wt* or *Ltbp3*$^{-/-}$ samples (Fig. 3*A*). The absence of *Ltbp3* in *Fbn1*$^{mgR/mgR}$ mice suppressed ERK1/2 activation to levels indistinguishable from *Wt* (Fig. 3*A*). Interestingly, we observed a small but significant decrease of ERK1/2 activation in *Ltbp3*$^{-/-}$ ascending aortas compared with *Wt* tissues (Fig. 3*A*). The significance of this change is unclear. Consistent with the Western blotting analysis, we observed, by immunostaining, increased p-ERK1/2 in the media of aortas from *Fbn1*$^{mgR/mgR}$ mice compared with *Wt* or *Ltbp3*$^{-/-}$ animals and no significant difference in *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ compared with *Wt* or *Ltbp3*$^{-/-}$ samples (Fig. 3*B*).

We also assessed TGFβ pathway signaling by measuring the levels of phosphorylated Smad3 (p-Smad3) in the ascending aortas of 11-wk-old mice. Western blotting analysis showed increased p-Smad3 in *Fbn1*$^{mgR/mgR}$ compared with *Wt* or *Ltbp3*$^{-/-}$ samples (Fig. 3*A*). Absence of LTBP-3 in *Fbn1*$^{mgR/mgR}$ mice resulted in a statistically significant reduction of Smad3 activation levels (Fig. 3*A*). There was no significant difference in p-Smad3 levels between *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ and *Wt* or *Ltbp3*$^{-/-}$ tissues (Fig. 3*A*).

To assess TGFβ signaling with a different approach, we examined the expression levels of collagen 1 alpha1 (*Col1a1*), fibronectin (*Fn*), plasminogen-activator inhibitor-1 (*Pai1*), and connective tissue growth factor (*Ctgf*), four genes whose expression is often induced by TGFβ (22). *Col1a1, Fn, Pai1, and Ctgf* expression were all

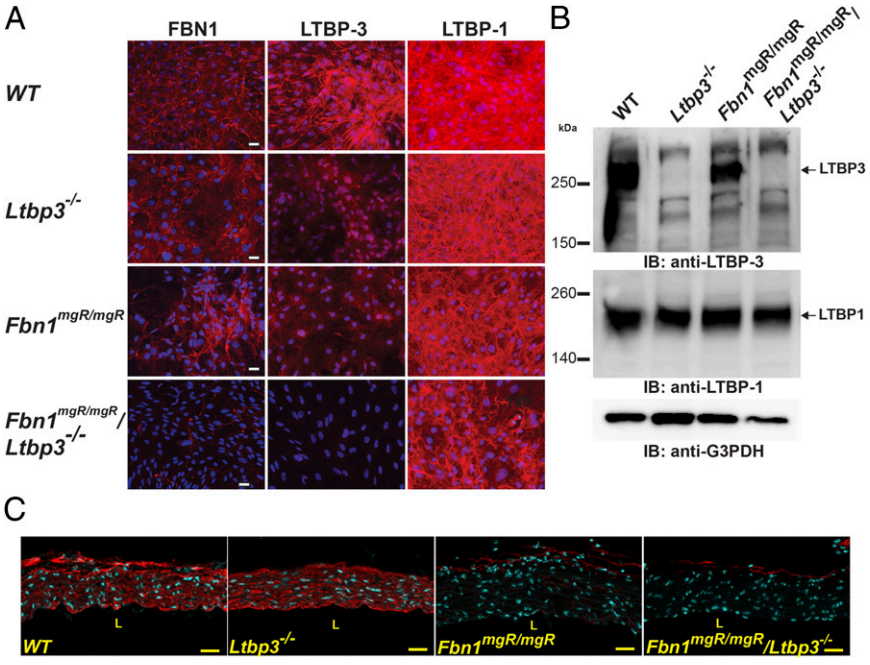

Fig. 2. Decreased incorporation of LTBP-3 in *Fbn1*$^{mgR/mgR}$ ASMCs. (*A*) Analyses by immunofluorescence of the deposition of FBN1 and LTBP-1 and -3 into the ECM of *Wt*, *Ltbp3*$^{-/-}$, *Fbn1*$^{mgR/mgR}$, and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ASMCs. Cells were cultured for 14 d on glass coverslips, fixed with ethanol, and stained with specific antibodies against LTBPs (red). Nuclei are stained with DAPI (blue). (Scale bars: all panels, 40 μm.) (*B*) Analyses of 24 h conditioned media from *Wt* and *Fbn1*$^{mgR/mgR}$ ASMCs. Conditioned media were collected and analyzed by Western blotting using antibodies specific for LTBP-3 and LTBP-1 isoforms. Housekeeping protein G3PDH is shown as a loading control. (*C*) Analysis by immunofluorescence of the distribution of FBN1 in *Wt*, *Ltbp3*$^{-/-}$, *Fbn1*$^{mgR/mgR}$, and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ascending aortas. The fibrillin-1 staining intensity was equivalent in *Wt* and *Ltbp3*$^{-/-}$ samples whereas *Fbn1*$^{mgR/mgR}$ and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ samples displayed a decrease in staining intensity compared with *Wt*. (Scale bars: all panels, 40 μm.)

significantly increased in the ascending aortas of $Fbn1^{mgR/mgR}$ mice compared with *Wt* (Fig. 3*C*). Expression of *Col1a1* and *Fn* was significantly diminished in $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ ascending aortas compared with $Fbn1^{mgR/mgR}$ samples (Fig. 3*C*). In conclusion, these data indicate that absence of LTBP-3 in $Fbn1^{mgR/mgR}$ mice reduces TGFβ signaling in the ascending aorta.

Absence of Ltbp-3 in $Fbn1^{mgR/mgR}$ Mice Normalizes Gene Expression Changes in the Aorta. We performed gene expression analysis to identify genes whose expression is affected in the ascending aortas from $Fbn1^{mgR/mgR}$ and $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ mice. Comparative gene array analysis of 2-mo-old $Fbn1^{mgR/mgR}$ versus *Wt* samples identified 786 differentially expressed genes using a threshold of 1.5 and a corrected *P* value of <0.10 (Fig. 4 and Dataset S1). However, in $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ aortas, the expression of 773 genes out of 786 genes was reversed, and, as expected, two of the genes whose expression was not reversed were *Fbn1* and *Ltbp3* (Fig. 4 and Table S1). There were no major mRNA expression changes in $Ltbp3^{-/-}$ compared with *Wt* ascending aortas, except for *Ltbp3* (>1.5-fold, corrected *P* value < 0.1) (Fig. 4 and Table S2). Overall, this result demonstrates that absence of LTBP-3 almost completely attenuates gene expression changes observed in MFS-like mice.

Upstream regulator analysis was applied to identify upstream molecular pathways that might explain the gene expression changes observed in $Fbn1^{mgR/mgR}$ aortas. The 786 genes affected in $Fbn1^{mgR/mgR}$ compared with *Wt* (>1.5-fold, $P < 0.1$) were analyzed with the Ingenuity Pathway Analysis (IPA) software package. This analysis confirmed the activation of the TGFβ pathway in $Fbn1^{mgR/mgR}$ mice (upstream analysis Z-score, 5.734; *P* value, 1.4 E−37) (Table S3). However, the TGFβ pathway was not activated in $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ ascending aortas because the expression of none of those genes regulated by TGFβ was significantly affected in these double mutants. Only *Fbn1*, a TGFβ gene target, was affected, but this result was expected because those mice are homozygous for a hypomorphic mutation in *Fbn1*. We additionally identified the activation of molecular signatures associated with inflammation, fibrosis, and immune function (Table S3), which is consistent with reports that indicated the known participation of TGFβ in these processes (22, 23).

Discussion

The purpose of the present study was to investigate the role of TGFβ/LTBP/fibrillin-1 interactions in the pathogenesis of TAA in MFS. A current view is that, in MFS, abnormal ECM sequestration of LTBP/TGFβ complexes due to defective fibrilin-1 microfibrils leads to increased TGFβ signaling and results in TAA (24). However, the validity of this hypothesis and whether LTBP and latent TGFβ sequestration are involved in MFS have yet to be determined at a molecular level.

As an initial approach to analyze the interactions of LTBPs and fibrillin-1 in MFS, we previously examined the matrix assembly of LTBPs in aortic tissue from *Fbn1*-null mice (18) and found diminished ECM incorporation of LTBP-3 and, by inference, its bound TGFβ. In the present study, we extended this observation to ASMCs isolated from *Wt* and $Fbn1^{mgR/mgR}$ mice (18, 20). $Fbn1^{mgR/mgR}$ mice represent a murine model that replicates the clinically severe and progressive form of human MFS, with death from aortic dissection and rupture during the first year of life accompanied by enhanced TGFβ signaling in the media of the thoracic aorta (19, 25). As with $Fbn1^{-/-}$ ASMCs, $Fbn1^{mgR/mgR}$ cells exhibited impaired LTBP-3 incorporation into the matrix with no significant change in LTBP-1 incorporation.

To test the role of LTBP-3 in the aortic disease observed in MFS, we generated mice containing mutations in both *Fbn1* ($Fbn1^{mgR/mgR}$) and *Ltbp3* genes. We reasoned that, if enhanced activation of latent TGFβ complexes containing LTBP-3 were causative for the vascular disease in MFS, the absence of LTBP-3 should prevent the formation of the LLC that contributes to pathological TGFβ signaling and thus attenuate aortic disease progression in $Fbn1^{mgR/mgR}$. *Ltbp3* deletion dramatically reduced aortic disease in $Fbn1^{mgR/mgR}$ mice and increased their survival to a level similar to that of Wt mice. $Fbn1^{mgR/mgR}$;$Ltbp3^{-/-}$ mice had aortas with diameters equivalent to those of normal mice, the number of discontinuities in the elastic lamellae was decreased by 80%, signaling through both Smad3 and ERK1/2 pathways was normalized, and expression of two genes regulated by TGFβ was reverted to levels approximating that observed in WT mice.

To our knowledge, this work is the first study in which ablation of a gene involved in the regulation of TGFβ action improves the

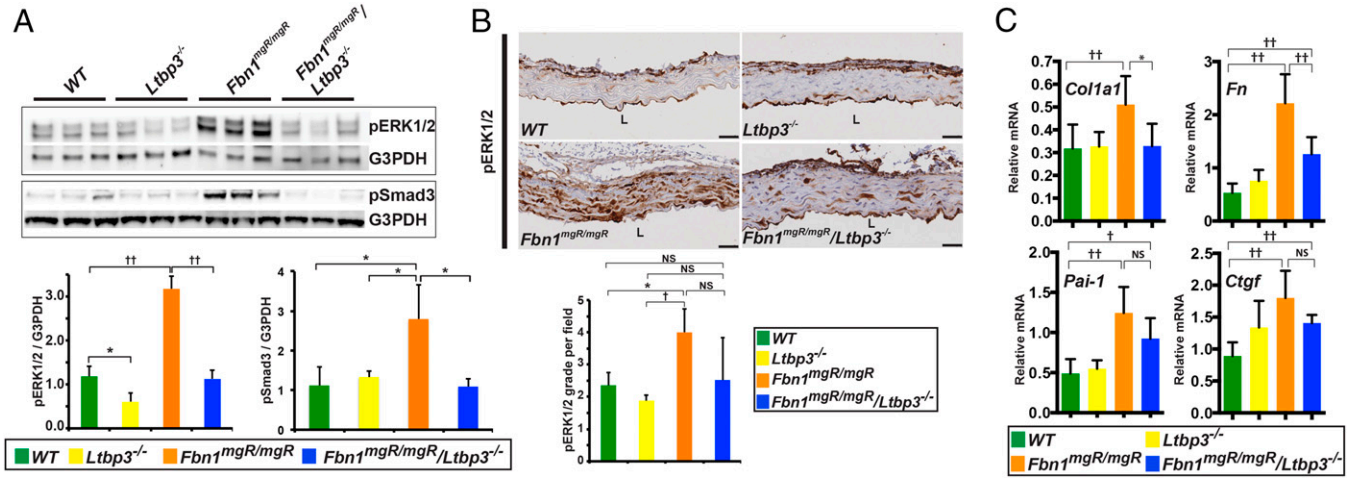

Fig. 3. Absence of LTBP-3 attenuates noncanonical (ERK1/2) and canonical (Smad2/3) TGFβ signaling in $Fbn1^{mgR/mgR}$ aortas. (*A*) Immunoblotting analysis of the ascending aorta of 12-wk-old mice. The results are expressed as densitometric levels of p-ERK1/2 and p-Smad3 intensity normalized to G3PDH. $Fbn1^{mgR/mgR}$ aortic tissue showed an increase in p-ERK1/2 compared with *Wt* or $Ltbp3^{-/-}$ tissues. Absence of LTBP-3 in $Fbn1^{mgR/mgR}$ mice substantially reduced p-ERK1/2. $Fbn1^{mgR/mgR}$ aortic tissues showed an increase in p-Smad3 compared with *Wt*, $Ltbp3^{-/-}$, and $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ tissues ($P < 0.05$). There was no significant difference in Smad3 activation between $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ and *Wt* or $Ltbp3^{-/-}$ ascending aortas. Bars represent the means ± SD of three aortas per genotype. $^{*}P < 0.05$; $^{\dagger\dagger}P < 0.01$; ANOVA $P < 0.01$. NS, not significant. (*B*) Representative p-Erk1/2 immunostaining on cross-sections of ascending aorta from 2-mo-old mice. L, lumen. (Scale bars: all panels, 40 μm.) The graph represents p-ERK1/2 positive signal quantification scored according to grade indicated in *SI Materials and Methods*. Data are presented as the mean ± SD of at least three samples for each genotype. $^{*}P < 0.05$; $^{\dagger}P < 0.02$; ANOVA, $P < 0.05$. NS, not significant. (*C*) qPCR analysis of TGFβ signaling target genes in ascending aortas from 2-mo-old *Wt*, $Ltbp3^{-/-}$, $Fbn1^{mgR/mgR}$, and $Fbn1^{mgR/mgR}$:$Ltbp3^{-/-}$ mice. mRNA expression levels were normalized to *Gapdh*. Data are presented as the mean ± SD of five to seven aortas per genotype. $^{*}P < 0.05$; $^{\dagger}P < 0.02$; $^{\dagger\dagger}P < 0.01$; ANOVA, $P < 0.02$. NS, not significant.

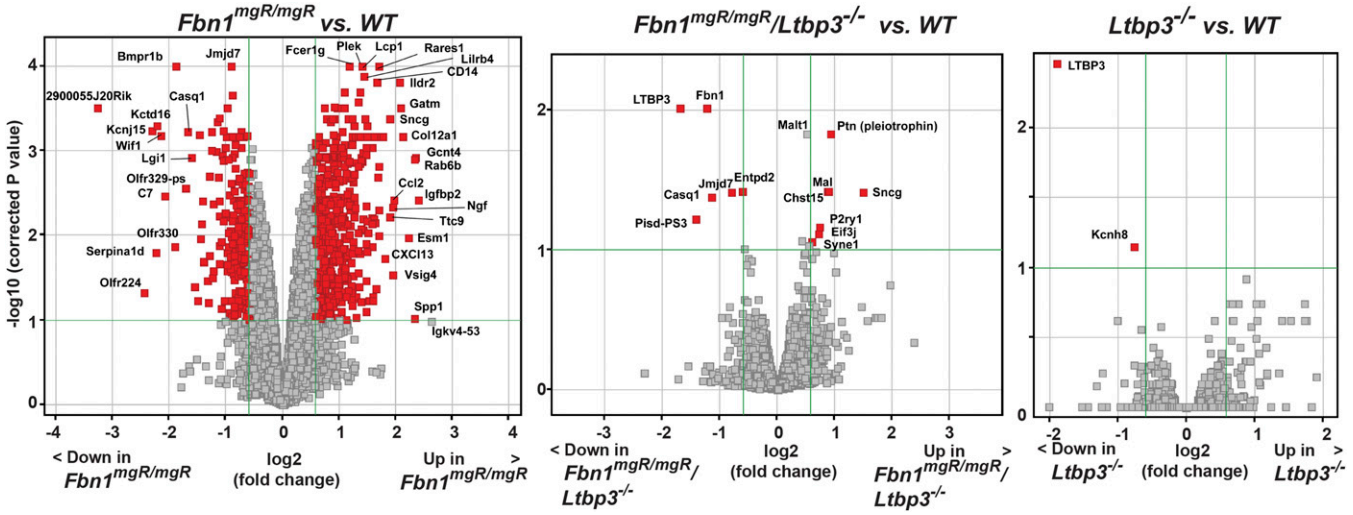

Fig. 4. Gene expression changes in the ascending aorta of 2-mo-old *Fbn1*$^{mgR/mgR}$ mice are normalized by loss of LTBP-3. Volcano plot of findings from gene array data of 2-mo-old ascending aortas. *Ltbp-3*$^{-/-}$ ($n = 3$), *Fbn1*$^{mgR/mgR}$ ($n = 4$), and *Fbn1*$^{mgR/mgR}$:*Ltbp3*$^{-/-}$ ($n = 4$) samples are compared with *Wt* ($n = 4$). Genes that met the inclusion criteria (*P* value of <0.1 and fold change of >1.5) were represented in the volcano plot. The $-\log_{10}$ of corrected *P* values (*y* axis) is plotted against the $\log_2$ of fold change between two groups. Within each of the three volcano plots, green lines delineate the cutoffs for genes significantly down-regulated (*Left*) or up-regulated (*Right*). Genes of highest statistical significance and fold change are sequestered into the upper left and upper right.

aortic disease phenotype in MFS mice. Genetic ablation of *Mmp*2 results in prolonged survival of *Fbn1*$^{mgR/mgR}$ mice, but it is uncertain whether this finding reflects a diminution of TGFβ signaling due to the absence of proteolytic activation of latent TGFβ by MMP2 or is a consequence of decreased aortic media degeneration caused by MMP2 enzymatic activity (25). Conversely, mutations in several genes that encode components of the TGFβ pathway either alone or in combination with an MFS mouse model (e.g., loss-of-function mutations in *Tgfbr1* or *Tgfbr2* genes, *Smad3*-null mutation, and *Smad4* or *Tgfb2* haploinsufficiency in *Fbn1*$^{C1039G/+}$ mice) either induce or worsen TAA (7, 26–30). Curiously, these mutations are expected to attenuate TGFβ signaling but yield paradoxical activation of TGFβ signaling in the aortas. However, deletion of *Ltbp3* in *Fbn1*$^{mgR/mgR}$ mice did not result in paradoxical increase in TGFβ signaling and prevented aortic disease in *Fbn1*$^{mgR/mgR}$ mice although LTBP-3 deletion is expected to decrease TGFβ levels and accordingly to worsen aortic disease (31).

A possible explanation for these discrepancies is that, whereas *Tgfbr1* and *-2* mutations, *Smad3* deletion, and *Smad4* haploinsufficiency affect signaling of all three TGFβ isoforms, *Ltbp3* deletion disturbs only the subset of latent TGFβ molecules bound to this specific carrier protein. It should be noted that our results also indicate that LTBP-1 present in the aorta cannot compensate for the loss of LTBP-3 even though both molecules bind all three isoforms of TGFβ. This observation implies functional specificity for these two LTBPs. It is still unclear which TGFβ isoform is the mediator of the aortic disease found in MFS because LTBP-3 interacts with TGFβ1, -β2, and -β3 isoforms with similar efficiency (9). However, it is unlikely that ablation of *Ltbp3* in *Fbn1*$^{mgR/mgR}$ mice diminished TGFβ2 signaling because mice with *Tgfb2* haploinsufficiency, when bred with *Fbn1*$^{C1039G/+}$ mice, display increased aortic dilatation and morbidity, indicating a protective role for TGFβ2 (28). Accordingly, we propose that specifically lowering either TGFβ1 or -3 in MFS mice should mitigate TAA and rupture.

To date, only pharmacological inhibition of TGFβ with either TGFβ-neutralizing antibodies (Nabs), the MEK1/2 inhibitor RDEA119, the angiotensin-II type 1 receptor-blocker losartan, or doxycycline ameliorates the aortic phenotype in MFS mice (6, 7, 25, 32). However, it is unclear whether the efficacy of losartan or doxycycline in attenuating aortic disease in MFS mice is due to direct impairment of TGFβ signaling or whether TGFβ-independent mechanisms are involved (5, 25). Pharmacological inhibition of TGFβ with neutralizing antibodies has also underscored the complex, controversial, and context-dependent roles of TGFβ in aneurysm. Whereas earlier studies of systemic TGFβ neutralization with the *Fbn1*$^{C1039G/+}$ mouse model prevented TAA formation, recent results with the *Fbn1*$^{mgR/mgR}$ mouse model have indicated that TGFβ can exert opposite effects on TAA pathology that broadly correlate with the early and late stages of TAA progression (5). Thus, early treatment (postnatal day 16; P16) with TGFβ-neutralizing antibodies enhances aneurysm formation whereas later treatment (P45) diminishes aneurysm formation. This context-dependent role of TGFβ was also underscored by a recent study in which induction of aortic dissection was dependent on the age at which *Tgfbr2* was ablated in smooth muscle cells, with enhanced incidence of the phenotype with young mice and almost no in cidence after 9 wk (33). Differential contributions of TGFβ signaling to aortic physiology early and later after birth may explain the greater incidence of aortic dissection in young mice upon targeted ablation of *Tgfbr2* in smooth muscle cells (33). Accordingly, we propose that the protective effect of LTBP-3 loss might represent the specific diminution of the pathological effects of LTBP-3/TGFβ complexes during aortic disease with no perturbation of basal physiological TGFβ signaling involved in aorta development or aortic tissue homeostasis and repair pathways (33, 34). In support of this postulate, we have not observed any evidence of vascular phenotype in this study or other studies with *Ltbp3*$^{-/-}$ mice (31, 35).

Finally, we cannot exclude the possibility that LTBP-3 has TGFβ-independent functions that participate in aortic pathology. TGFβ-independent activities have been identified for LTBP-4 in stabilizing microfibril bundles and regulating elastic fiber assembly and for LTBP-2 in the development of ciliary zonule microfibrils (36–38). Thus, LTBP-3 might directly contribute to the integrity of the matrix or the mechanical compliance of the aortic wall (39).

In summary, our work addresses the significance of the interaction between LTBP and fibrillin-1 microfibrils in the control of TGFβ action by using a genetic approach. Our results demonstrate the potential importance of the interaction between the LTBP-3/TGFβ complex and fibrillin-1 microfibrils in the control of detrimental TGFβ signaling involved in TAA pathogenesis in MFS. Blocking activation of latent TGFβ associated with LTBP-3 might represent a potentially novel and specific therapeutic approach to preventing aortic disease in MFS.

Materials and Methods

Mice. *Fbn1*$^{mgR/mgR}$ and *Ltbp3*$^{-/-}$ mice have been previously described (19, 31). Colonies of Wt and mutant mice were maintained on a mixed C57BL/6; Sv129;SW genetic background. Only age-matched males were used in all experiments. For tissue analysis, animals were euthanized by CO_2 asphyxiation. Survival of the different genotypes was calculated from weaning through death or collection at the intended time points. All procedures were performed according to the regulations of the New York University Langone Medical Center Institutional Animal Care and Use Committee.

Antibodies and Reagents. Antibodies used for Western blotting are described in *SI Materials and Methods*.

Isolation of Vascular Smooth Muscle Cells. Vascular smooth muscle cells (VSMCs) were isolated from 12-wk-old male ascending aortas of the different genotypes as described in *SI Materials and Methods*.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR Analysis. Total RNA from ascending aortas was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen) with DNase treatment included. See *SI Materials and Methods* for details. Primers used for qPCR are listed in *SI Materials and Methods*.

Immunocytochemistry. ASMCs were plated on glass coverslips and stained as described by Zilberberg et al. (18). Adjustments of brightness and contrast were applied to the whole image. Images from different genotypes underwent the same adjustments.

Conditioned Media Analysis. Cell cultures were washed with serum-free DMEM and incubated with fresh serum-free DMEM for 24 h. The conditioned media (CM) was collected, and protease inhibitors were added. CM was clarified by centrifugation and stored at –80 °C.

Echocardiography. Transthoracic echocardiography was performed in standard fashion as described in *SI Materials and Methods*.

Histology and Immunohistochemistry. Ascending aortas were harvested and fixed in 10% (vol/vol) buffered formalin, processed, and embedded in paraffin. Five-micrometer sections were used in all studies. Elastin was stained using the orcinol–new fuchsin technique (40). The number of elastic fiber breaks for the entire aortic ring was counted manually for each genotype. Immunohistochemistry was performed using rabbit anti-mouse p-ERK1/2 (clone D13.14.4E; Cell Signaling) as described in *SI Materials and Methods*.

Gene Expression Analysis. Total RNA (100 ng) from the 8-wk-old ascending aortas was analyzed as described in *SI Materials and Methods*.

Statistics. Data are presented as the mean ± SD in bar graphs. The unpaired two-tailed Student's *t* test was used to determine the significance between two groups, assuming significance at $P < 0.05$. Analyses between multiple groups used one-way ANOVA with $P < 0.05$ considered as statistically significant. Kaplan–Meier survival curves were constructed and analyzed using the log-rank (Mantel–Cox) test (GraphPad Prism software).

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