

Fibrillin-1 G234D mutation in the hybrid1 domain causes tight skin associated with dysregulated elastogenesis and increased collagen cross-linking in mice

ASM Sakhawat Hossain^{a,c,k}, Maria Thea Rane Dela Cruz Clarin^{b,c,d}, Kenichi Kimura^c, George Biggin^e, Yuki Taga^f, Koichiro Uto^d, Ayana Yamagishi^g, Eri Motoyama^c, Narenmandula^{a,c}, Kazunori Mizuno^f, Chikashi Nakamura^g, Keiichi Asano^c, Sumio Ohtsuki^h, Tomoyuki Nakamuraⁱ, Sachiko Kanki^j, Clair Baldock^e, Erna Raja^{c,*}, Hiromi Yanagisawa^{c,*}

^a Graduate School of Comprehensive Human Sciences, University of Tsukuba, Japan

^b School of Integrative and Global Major, University of Tsukuba, Japan

^c Tsukuba Advanced Research Alliance (TARA), Life Science Center for Survival Dynamics, University of Tsukuba, Japan

^d National Institute for Material Science, Japan

^e Division of Cell Matrix Biology and Regenerative Medicine, Faculty of Biology, Medicine and Health, Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, UK

^f Nippi Research Institute of Biomatrix, Japan

^g National Institute of Advanced Industrial Science and Technology, Japan

^h Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, Japan

ⁱ Department of Pharmacology, Kansai Medical University, Japan

^j Department of Surgery, Osaka Medical and Pharmaceutical University, Japan

^k Department of Pharmacy, Varendra University, Bangladesh

ARTICLE INFO

Keywords:

Fibrillin-1
Tight skin
Fibroblasts
Extracellular matrix
Collagen cross-linking
Elastic fibers
Fascia

ABSTRACT

Fibrillin-1, an extracellular matrix (ECM) protein encoded by the *FBN1* gene, serves as a microfibril scaffold crucial for elastic fiber formation and homeostasis in pliable tissue such as the skin. Aside from causing Marfan syndrome, some mutations in *FBN1* result in scleroderma, marked by hardened and thicker skin which limits joint mobility. Here, we describe a tight skin phenotype in the *Fbn1*^{G234D/G234D} mice carrying a corresponding variant of *FBN1* in the hybrid1 domain that was identified in a patient with familial aortic dissection. Unlike scleroderma, skin thickness and collagen fiber abundance do not change in the *Fbn1*^{G234D/G234D} mutant skin. Instead, increased collagen cross-links were observed. In addition, short elastic fibers were sparsely located underneath the panniculus muscle layer, and an abundance of thin, aberrant elastic fibers was increased within the subcutaneous fascia, which may have tightened skin attachment to the underlying skeletal muscle. Structurally, *Fbn1*^{G234D/G234D} microfibrils have a disrupted shoulder region that shares similarities with hybrid1 deletion mutant microfibrils. We then demonstrate the consequence of fibrillin-1 G234D mutation on dermal fibroblast functions. Mutant primary fibroblasts produce fewer elastic fibers, exhibit slower migration and increased cell stiffness. Moreover, secretome from mutant fibroblasts are marked by enhanced secretion of ECM, ECM-modifying enzymes, proteoglycans and cytokines, which are pro-tissue repair/fibrogenic. The transcriptome of mutant fibroblasts displays an increased expression of myogenic developmental and immune-related genes. Our study proposes that imbalanced ECM homeostasis due to a fibrillin-1 G234D mutation impacts fibroblast properties with potential ramifications on skin function.

* Corresponding authors.

E-mail addresses: rajaerna@tara.tsukuba.ac.jp (E. Raja), hkyanagisawa@tara.tsukuba.ac.jp (H. Yanagisawa).

<https://doi.org/10.1016/j.matbio.2024.11.006>

Received 4 June 2024; Received in revised form 25 November 2024; Accepted 27 November 2024

Available online 28 November 2024

0945-053X/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Fibrillin-1 is a glycoprotein encoded by the *FBN1* gene. It plays a pivotal role in the architecture of connective tissues such as blood vessels, lungs, and skin, and acts as a scaffold for elastic fibers that provide elasticity and structural integrity crucial for various organs and systems [1–4]. Among fibrillin protein isoforms, fibrillin-1 is most abundant in adult tissue, while fibrillins-2 and –3 are prominent during development [1,5–7]. Beyond their structural roles, fibrillin microfibrils regulate extracellular matrix (ECM) function by interacting with cells via integrins [8], participating in mechanosensation, and sequestering growth factors such as latent transforming growth factor- β (TGF β) through interactions with latent TGF β -binding proteins (LTBPs) [9,10] and the prodomains of several bone morphogenetic proteins (BMPs) [11].

The diverse functionality of fibrillin-1 is regulated by its distinct domains such as 43 calcium-binding epidermal growth factor (cbEGF)-like domains, 4 EGF-like domains, 2 hybrid domains and 7 TGF β -binding protein-like (TB) domains, which underscores its ability to modulate protein-protein interactions, ECM assembly, and the regulation of TGF β signaling pathways [9]. Of particular interest is the hybrid1 (H1) domain, located in the N-terminus of fibrillin-1 [12]. The H1 domain plays a crucial role in establishing intermolecular disulfide bonds that stabilize assembled fibrillin microfibrils [13]. Furthermore, H1 functions as a protein interaction site, which requires its adjacent domains (cbEGF-like and TB) to allow binding with proteins such as fibulins-2, –4 and –5, LTP1, among others [2,12,14,15].

FBN1 mutations predominantly cause Marfan syndrome (MFS), a connective tissue disorder of the cardiovascular, musculoskeletal, and pulmonary systems, with clinical presentations that include aortic root aneurysm and dissection, tall stature, arachnodactyly, and ectopia lentis [16]. Aside from MFS, *FBN1* mutations, including W1572C and D1545E mutations [17], underlie stiff skin syndrome (SSS), typified by rigid, thickened skin distributed across the body, culminating in restricted joint mobility and flexion contractures [18]. SSS pathology is associated with collagen accumulation indicative of fibrosis. In addition, a spontaneous tandem duplication in the *Fbn1* gene leads to the tight skin (Tsk/+) mouse phenotype, which exhibits myocardial, skeletal, and pulmonary abnormalities that resemble MFS and some fibrotic features similar to SSS [19,20].

In the skin, fibrillin-1 is localized at the dermal-epidermal junction, the dermis, around the hair follicles [21] and in the subcutaneous fascia region where the wound-healing and scar-promoting fibroblasts reside [22,23]. This wide distribution of fibrillin-1 in the skin allows molecular contact with multiple cell types. Thus, *FBN1* mutations are likely to influence many cellular processes, whether through altering ECM mechanical properties or via the modulation of growth factors bioavailability.

Skin fibroblasts reside in the dermis and subcutaneous fascia, which tethers the skin to the underlying muscle tissue [24]. Fibroblast functions are pivotal in ECM synthesis and remodeling [25] pertinent in the connective tissue, especially in skin physiology and pathology. Evidence suggests that *Fbn1* mutations in fibroblasts lead to loss of integrin-mediated cell adhesion, aberrant elastogenesis, collagen production, and signaling pathway activation [9,17,18,26–28]. Fibrillin-1 interactions with fibroblasts regulate intracellular signaling kinase activities and gene expression, which is translated into cellular functions [29–31]. However, there remains a limited understanding of how disease-associated fibrillin-1 mutations affect fibroblast function and response.

In this study, we investigated the mouse skin phenotype containing a missense variant (c.701 G > A, p.234 Gly>Asp, G234D) in the H1 of fibrillin-1, initially found in a Japanese patient with familial aortic dissection carrying a heterozygous mutation. Although the patient did not exhibit tight skin, the corresponding homozygous mutation in mice resulted in altered elastic fibers in the dermis and subcutaneous fascia,

with a concomitant increase in collagen cross-linking but without the characteristic fibrosis or collagen accumulation. Screening analysis of mutant dermal fibroblasts indicates changes in secreted proteins and the upregulation of immune-related and skeletal and muscle developmental-related genes.

Results

Fbn1^{G234D/G234D} mutation results in mouse skin tightness

Fbn1^{G234D/G234D} (from here on referred to as ‘mutant’) mice exhibited tight skin phenotype (Fig. 1A–D) in addition to developing aortic dissection [32]. While sharing some resemblance to previously reported mouse models of scleroderma [33], the mutant skin exhibited a decrease in the stretchable dorsal skin area (SSA) as a ratio to the total body surface area (TSA) (Fig. 1B). However, body weight did not significantly differ between the wild-type (WT) or the mutant mice (Fig. S1A, [32]). With a blinded examiner, this phenotype was observed in half of the mutant mice as early as postnatal day (PD)3 with subsequent 100% penetrance in all the mutant mice from PD7 onwards (Fig. 1C). A quantification of the SSA/TSA ratio confirmed the tight skin phenotype in all mutant mice at 3–4 weeks of age (Fig. 1D).

To evaluate whether the mutation affected the skin’s biomechanical properties, we measured its elasticity at 3 weeks of age by rheometry and tensile testing (Fig. S1B). There was no significant increase in the complex modulus of the mutant skin compared to WT skin, indicating unaltered ex-vivo skin viscoelasticity (Fig. 1E). However, when tensile testing was performed at 0–10% of the deforming strain, the mutant skin showed lower tensile strength compared to WT (Fig. 1F), indicating compromised elastic fibers [34]. Hematoxylin/eosin staining demonstrated that mutant skin histological features and thickness were unaffected in the epidermis, dermis, dermal adipose, panniculus carnosus (PC) muscle, and the subcutaneous fascia layer at 3 or 4 weeks of age (Fig. 1G–H). Hence, *Fbn1*^{G234D/G234D} in vivo skin tightness is not associated with augmented thickness in any skin compartments or increases in ex vivo skin mechanical stiffness.

Alterations in elastic fiber abundance and patterns in the dermis and subcutaneous fascia of *Fbn1*^{G234D/G234D} mice

To investigate whether ECM modifications were the underlying cause of skin tightness, we first examined the deposition of fibrillin-1 in young mice at PD28 (Fig. 2A–B) and at PD3 (S2A–B). Although previously reported *Fbn1* mutations with tight skin are associated with more pronounced fibrillin-1 microfibrils in the dermis [18,35,36], at PD3 and PD28, the G234D mutation consistently lowered fibrillin-1 protein abundance in the dermis while the levels in the subcutaneous fascia appeared comparable to WT (Fig. S2A–B, 2A–B). Similarly, elastin immunostaining illustrated its reduced presence in the mutant dermis at PD28 (Fig. 2C–D), but no significant change was detected at PD3 (Fig. S2C–D). In contrast to the dermis, elastin immunostaining in the fascia indicated an increased abundance at PD28 with fibrillin-1 G234D mutation (Fig. 2E). A similar trend was observed at PD3 although it was not statistically significant (Fig. S2C–D).

In line with decreased levels of fibrillin-1 and elastin in the mutant dermis, elastic fibers were shorter, thinner, and sparser in the mutant dermis compared to WT (Fig. 2F upper panels, red arrowheads, 2H; S2E–F upper panel). Similar elastic fiber impairment at the PC muscle-fascia interface of the mutant skin was also detected, which exhibited short, thin, and sparse fiber morphology (Fig. 2F lower panels, red arrowheads, 2I; S2E–F, fascia-lower panels, black arrows). Moreover, elastic fibers were scarcely present within the fascia compartment in WT skin, in contrast to an increased amount of thin elastic fibers scattered throughout the mutant fascia region (Fig. 2G, red dotted boxes, 2J; S2E–F, fascia-lower panels, black arrowheads), where the G234D mutant fibrillin-1 protein was maintained (Fig. 2A; S2A, fascia, lower panels).

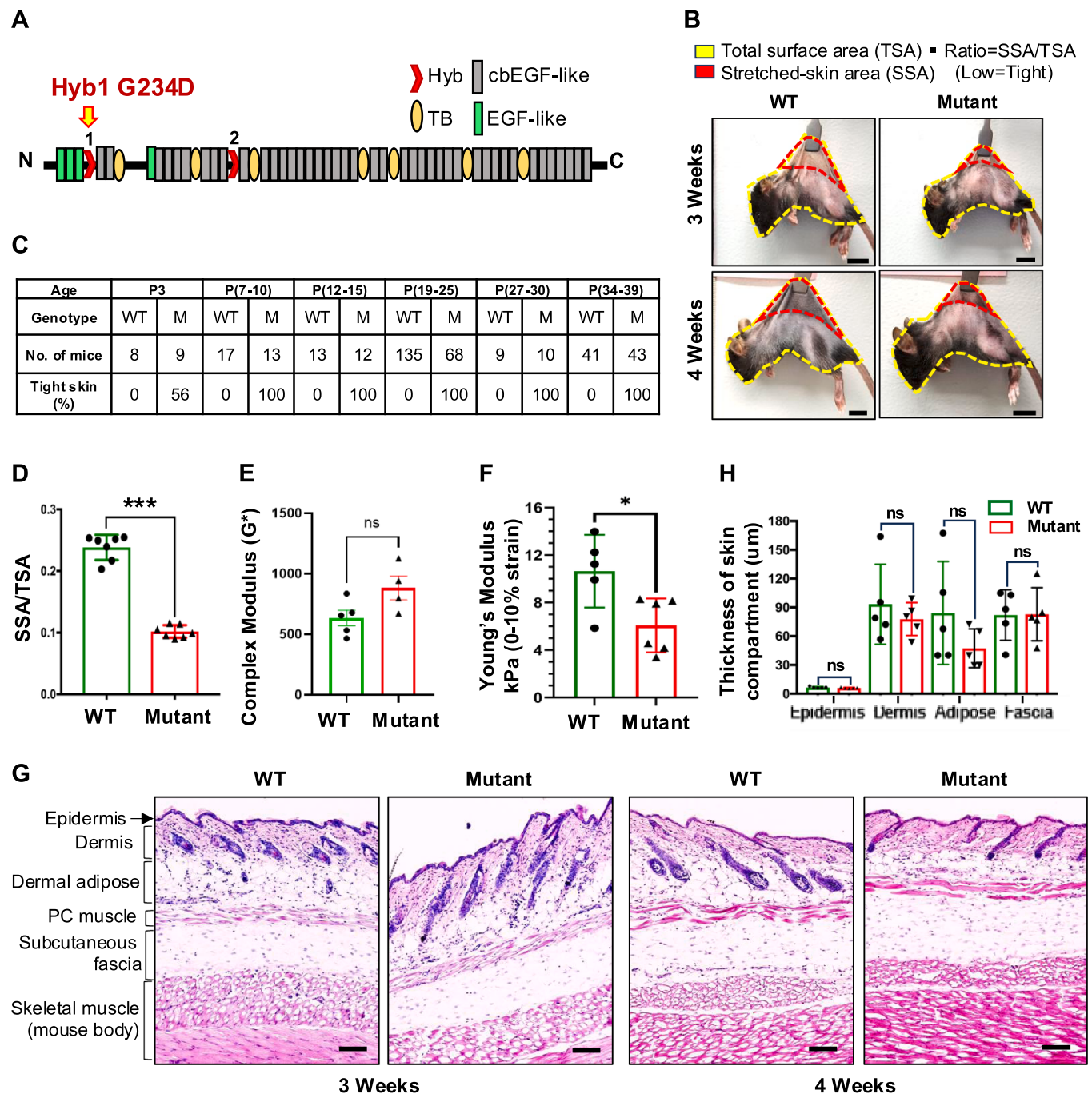


Fig. 1. The tight skin phenotype in *Fbn1*^{G234D/G234D} mice. A, Diagram of fibrillin-1 structure and the location of G234D mutation within the hybrid1 domain. Hyb, hybrid domain; TB, TGF- β -binding protein-like domain; EGF-like, epidermal growth factor-like domain; cbEGF, calcium-binding EGF-like domain. B, Representative mouse photographs showing dorsal skin extensibility and the method used to measure the ratio of stretched skin area to total surface area (SSA/TSA). Scale bar, 1 cm. Yellow dotted line shows the total surface area of the mouse skin image. Red dotted line describes the stretchable back skin area. C, Table summarizes mice of different age groups and genotypes with the observed tight skin phenotype by grabbing the dorsal skin by hand. Half of the population exhibited the tight skin phenotype on postnatal day 3 (P3), and by P7 onwards the manifestation showed 100 % penetrance. D, Bar graph shows the ratio of SSA/TSA at 3–4 weeks of age (wild type; WT, $n = 7$; Mutant, $n = 7$; *** $P < 0.001$; student t -test; mean $\pm$ S.D.). E, Viscoelasticity measurement of WT and mutant skin by rheometer at 3 weeks of age (WT, $n = 5$; Mutant, $n = 4$; ns, not significant; student t -test; mean $\pm$ S.D.). F, Skin tensile measurement at low strain (0–10 %), mutant compared to WT skin at 3 weeks of age (WT, $n = 5$; Mutant, $n = 6$; * $P < 0.05$; student t -test; mean $\pm$ S.D.). G, Representative images from Haematoxylin & Eosin (H&E) staining of WT and mutant mice at 3 and 4 weeks of age. $n = 5$ per genotype per age group. Scale bar, 100 μ m. PC, panniculus carnosus. H, Thickness measurements of skin compartments in WT and mutant mice using images from H&E staining at 3 weeks of age ($n = 5$ per genotype, ns, not significant; student t -test; mean $\pm$ S.D.).

We further investigated other elastic fiber-forming components, such as fibulin 5 (Fig. S2G) and fibronectin (Fig. S2H–J), and found no obvious changes in their abundance between WT and mutant mice.

To test whether the deposition of elastic fibers in the mutant fascia is partly due to increased fibroblast secretion of mutant fibrillin-1 within

this compartment, we compared fibrillin-1 abundance in the cell lysate and the conditioned media (CM) of WT and mutant fibroblasts from dermis and fascia, respectively (Fig. S3A–H). Our analysis revealed that CM from both mutant dermal and fascia fibroblasts contained significantly less fibrillin-1 compared to their WT counterparts (Fig. S3C, D, G,

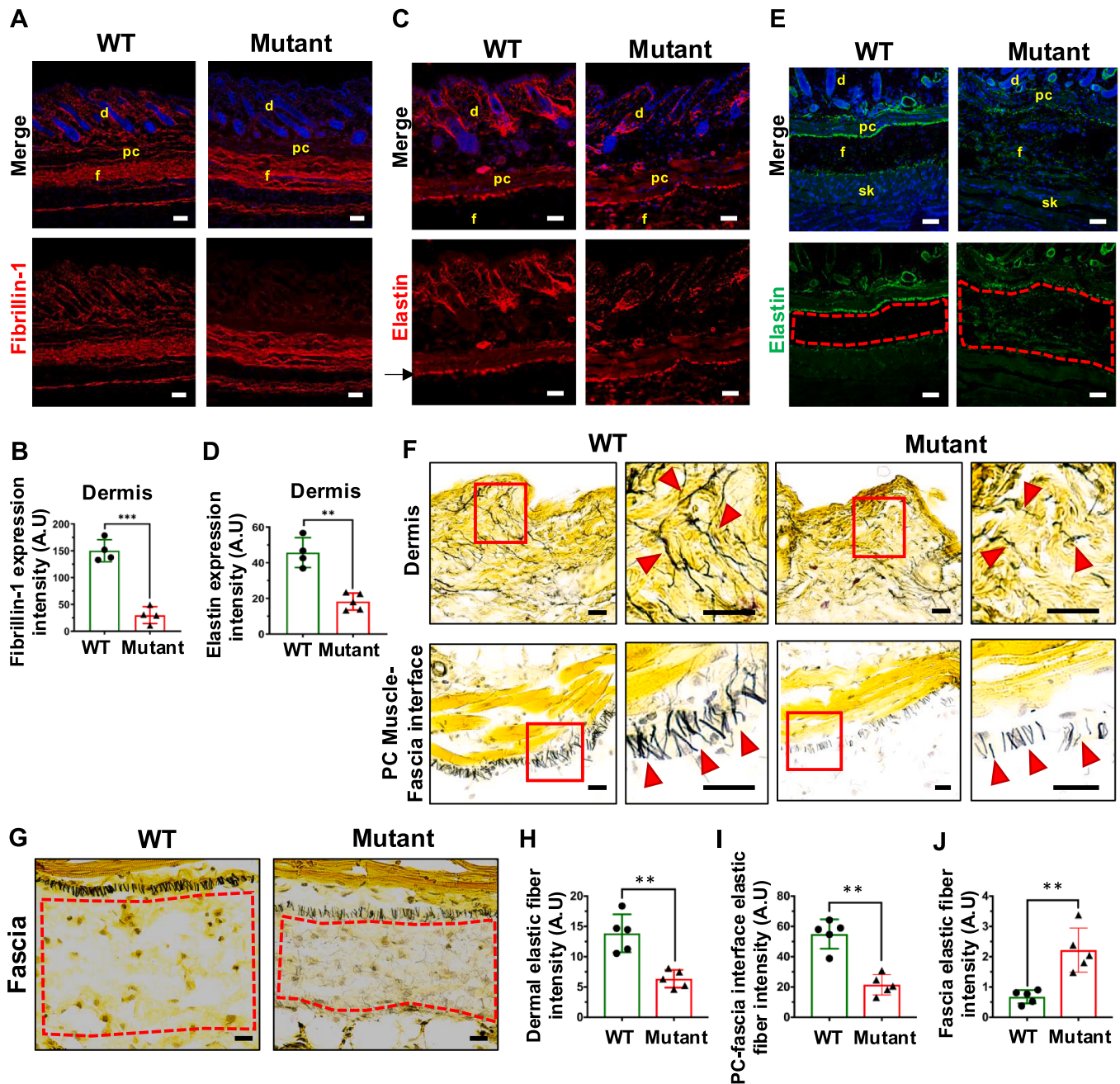


Fig. 2. Skin elastic fiber abnormalities due to G234D mutant fibrillin-1. **A**, Fluorescence microscopy images from fibrillin-1 immunostaining and **B**, quantification of fibrillin-1 dermal signal intensity from immunostaining in (A). **C**, Fluorescence microscopy images from elastin immunostaining, and **D**, quantification of dermal elastin signal intensity using immunofluorescence in (C). d, dermis; pc, panniculus carnosus muscle; f, fascia. A black arrow indicates elastin expression at the PC muscle-fascia interface. Scale bar, 50 μ m. Hoechst nuclear stain (blue fluorescence). WT, $n = 4$; *Fbn1*^{G234D/G234D} (Mutant), $n = 4$ –5; *** $P < 0.001$; ** $P < 0.01$; student t -test; mean $\pm$ S.D. A.U.; arbitrary unit. **E**, Elastin immunostaining in the subcutaneous fascia region (red boxed regions). sk, skeletal muscle. Scale bar, 50 μ m. Hoechst nuclear stain (blue fluorescence). **F**, Representative images of Weigert's iron resorcin fuchsin staining of elastic fibers in the dermis and at the interface between the PC muscle and fascia in WT (left panels) and mutant (right panels). Elastic fibers are stained black with metanil yellow connective tissue counterstain. Areas of disrupted elastic fibers in both the dermis and at the PC muscle-fascia interface are highlighted with red boxes and are enlarged. Red arrowheads indicate the elastic fibers. **G**, Micrographs of elastic fiber staining in the fascia region. Mutant fascia shows elastic fibers as fine grey fragments (red-dotted boxed area). Number of mice observed in E, F, and G; $n = 5$ per group, scale bar, 20 μ m. **H**, **I**, **J**, Quantification of elastic fiber intensity in the dermis, PC muscle-fascia interface, and fascia, respectively. $n = 5$ per genotype, ** $P < 0.01$; student t -test; mean $\pm$ S.D. A.U.; arbitrary unit. In Fig. 2 all comparisons were made between WT and mutant mice at 4 weeks of age.

H), suggesting that the unique tissue microenvironment in the mutant dermis vs fascia may influence the stability of secreted mutant fibrillin-1 (Fig. 2A, S2A-B).

Nonetheless, the deposition of thin elastic fibers within the fascia and impaired elastic fibers at the PC muscle-fascia interface may modify the mechanical properties of the connective tissue layer. Indeed, young *Tsk*/

+ dorsal skin tightness is already discernible from the WT counterparts at an early age of PD 5–7 [37,38]—before the occurrence of fibrosis and hypodermal thickening [33]. This was linked to the absence of elastic fibers in the PC muscle-fascia interface and the presence of short fibers within the fascia, which may modify skin tethering [37]. Therefore, skin tightness due to the *Fbn1* G234D mutation may, in part, be attributed to

accrued and distorted elastic fibers in the connective tissue fascia, resulting in a tighter attachment of the mutant skin to the underlying skeletal muscle.

Structural abnormality in *Fbn1*^{G234D/G234D} microfibrils

Fibrillin microfibril repeating units consist of several regions including the bead, arm, interbead, and shoulder that, overall, measure about 57 nm in length (Fig. 3A). Visualisation of WT and G234D mutant fibrillin microfibrils from 22-day-old mouse skin by negative stain transmission electron microscopy (TEM) showed no obvious differences in microfibril organization or periodicity (Fig. 3B). Periodicity measurements of 332 WT and G234D mutant microfibrils from 3 biological repeats revealed that WT and mutant microfibrils have an average repeat length of 57.30 ± 0.22 nm and 57.63 ± 0.32 nm, respectively, which was not significantly different (Fig. 3C). This aligns with a previously reported typical fibrillin microfibril period length [39]. Two-dimensional class averaging of WT and mutant microfibril repeating units was then performed and analyzed for changes in microfibril structure. Although class averages were similar between WT and mutant microfibrils, there appeared to be structural variability present around the shoulder region of the mutant microfibrils, represented by a blurred density to the left of the bead, as shown by the stain exclusion profile of the mutant compared to WT microfibrils (Fig. 3D, E, red arrows). These structural changes appear to be similar but less severe than those previously observed in H1 domain deleted microfibrils and Weill-Marchesani syndrome (WMS) mutant microfibrils, of which the latter exhibits tight skin as well [39,40]. Taken together, these data suggest structural rearrangement in the same region of the microfibril with the G234D mutant.

Enhanced collagen cross-linking without collagen accumulation in *Fbn1*^{G234D/G234D} mice

Collagen-mediated tissue stiffening is a crucial factor in multiple pathological tissue fibrosis. To investigate whether collagen homeostasis was affected by the fibrillin-1 G234D mutation, Masson trichrome staining (Fig. 4A, S4A) and immunostaining of type I collagen (Fig. 4B, S4B) and its quantitation (Fig. 4C) were performed. Both results indicated that collagen expression level and localization were unaffected by the fibrillin-1 G234D mutation. This remained true in the adult mutant mouse skin at PD49, which consistently showed a lack of dermal thickening (Fig. S4C).

Collagen orientation is another important determinant of tissue stiffness. In homeostasis, collagen bundles are oriented in a basket weave-like structure, whereas in fibrosis, they exhibit an increasingly parallel alignment [41]. Picosirius red staining with polarized light microscopy has been used to analyze collagen and its microarchitecture [42]. We used this method to evaluate the collagen arrangement between WT and mutant dermis. We found that the basket weave-like structure of collagen fibrils was maintained in both genotypes, suggesting unaltered collagen orientation and alignment (Fig. 4D, white arrows, 4E-F). TEM was further employed to scrutinize the structural arrangement of collagen fibrils; however, no obvious difference between WT or mutant skin was observed (Fig. S4D).

We last assessed collagen cross-linking, which also influences tissue stiffness by regulating fiber stability, movement, and its ability to withstand deformations [43,44]. Using liquid chromatography-mass spectrometry, we measured the amounts of collagen cross-linking, including pyridinoline (Pyr), lysinonorleucine (LNL), hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL) [45] in the full-thickness dorsal skin of WT and mutant mice (Fig. 4G). The mutant skin contained a significantly higher amount of mature collagen cross-link Pyr and immature cross-links such as LNL and DHLNL per collagen, suggesting that the fibrillin-1 G234D mutation

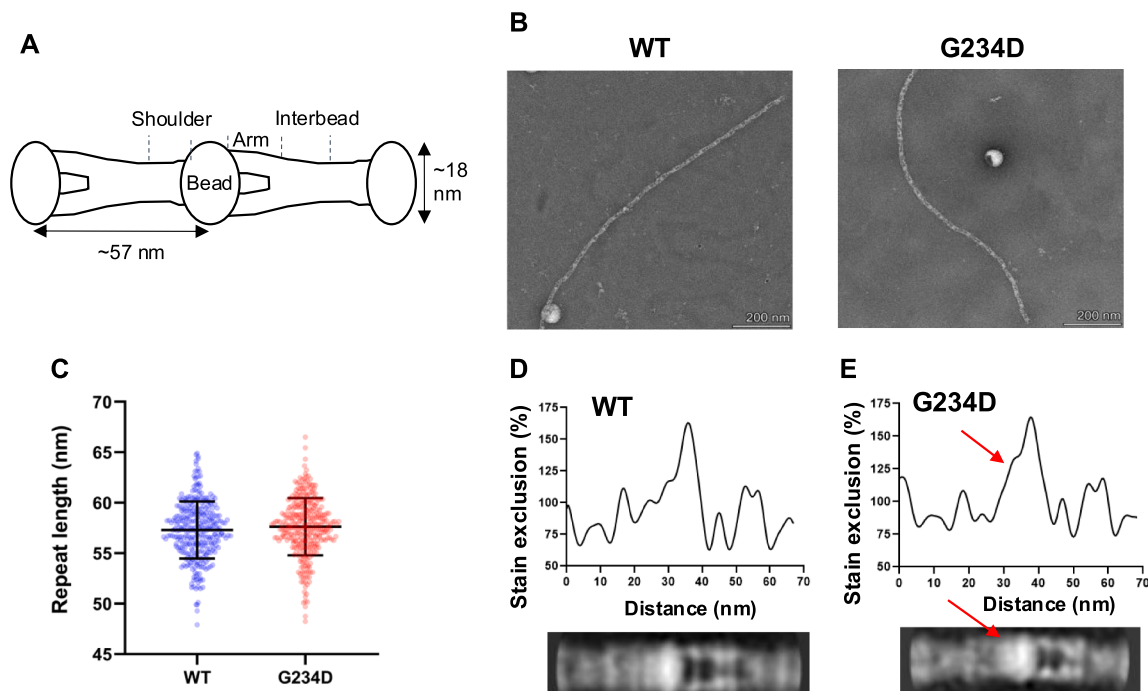


Fig. 3. Structural rearrangement in WT vs G234D mutant fibrillin microfibrils. A, Fibrillin microfibril diagram, illustrating the bead, arm, interbead and shoulder regions. B, Representative negative stain images of WT control (left panel) and G234D mutant microfibrils (right panel) from 22-day-old mouse skin. C, Scatter plot showing periodicities of the WT and G234D mutant extracted microfibrils. Graph represents mean $\pm$ SEM. 332 microfibrils were measured per group and no significant difference ($P = 0.145$) was found. Unpaired student *t*-test. D, 2D class averages of 50 microfibril periods and associated stain exclusion profiles for WT control and E, G234D mutant. Red arrow highlights a region at one side of the bead which is disrupted.

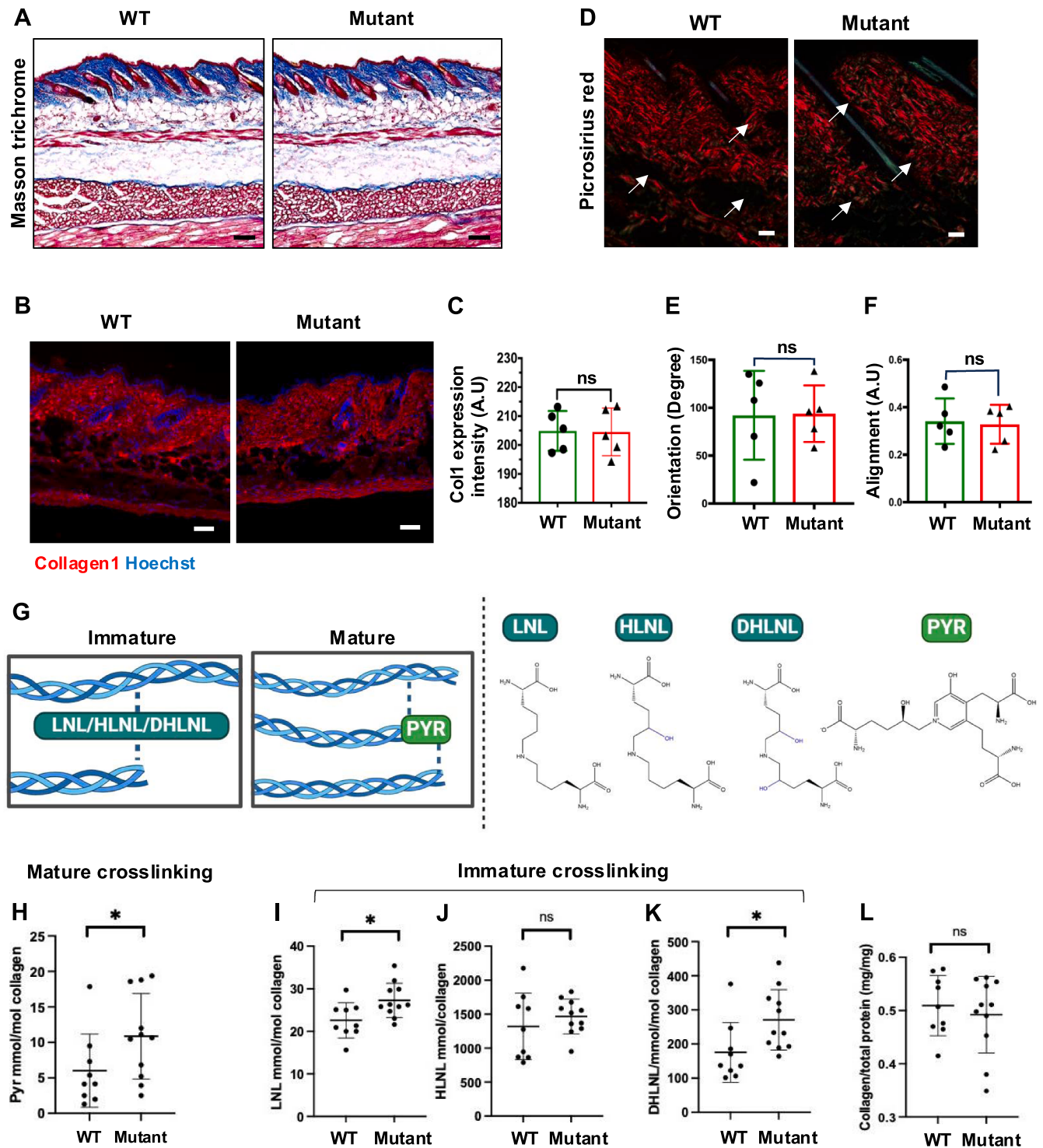


Fig. 4. Collagen associated assessment in *Fbn1*^{G234D/G234D} tight skin. **A**, Representative Masson trichrome staining micrographs show the amount of collagen in WT and mutant skin. Scale bar, 100 μ m. **B**, Type I collagen immunostaining comparing between WT and mutant skin. Scale bar, 50 μ m. Hoechst, nuclear staining. **C**, Quantification of type I collagen signal intensity from (B). **D**, Picrosirius red staining under polarized light microscopy reveals skin collagen arrangement in both WT and mutant tissue, scale bar 20 μ m. **E**, **F**, Quantification of collagen fibers orientation and alignment from (D). $n = 5$; ns, not significant, Student *t*-test, mean $\pm$ S.D). **G**, Schematic diagram describing immature and mature collagen cross-links and their corresponding chemical structures. **H** – **L**, Quantitative biochemical analysis of collagen by LC-MS and amino acid analysis using mice dorsal skin tissue. The amount of mature collagen cross-links, as measured by pyridinoline (Pyr) (**H**), and immature cross-links, as measured by lysinonorleucine (LNL) (**I**), hydroxylysinonorleucine (HLNL) (**J**) and dihydroxylysinonorleucine (DHLNL) (**K**). Collagen amount per total protein (**L**). WT, $n = 9$; Mutant, $n = 11$; * $P < 0.05$; Mann-Whitney U test (**H**, **K**); Student *t*-test (**I**, **J**, **L**); mean $\pm$ S.D). In **Fig. 4** all comparisons were made between WT and mutant mice at 3 weeks of age.

promotes a stiffer ECM microenvironment (Fig. 4H, I, K) independent of collagen quantity modulation (Fig. 4L).

Transcriptomics of neonatal $Fbn1^{G234D/G234D}$ fibroblasts denote a tendency for stiffer cells expressing muscle-related developmental genes

Although the ECM microenvironment is perturbed in mutant skin, the underlying mechanism for this is unknown. Fibroblasts are the primary cell type for ECM secretion in the skin [25]. To examine whether $Fbn1^{G234D/G234D}$ dermal fibroblasts undergo intrinsic changes that lead to deviations in ECM-regulating gene expression, RNA-sequencing (RNA-seq) was performed on primary cultures of new-born dermal fibroblasts (PD 0–2) (Fig. 5A). A volcano plot and heat map from 2-fold differentially expressed genes (DEG) show the transcriptomic differences between WT and mutant fibroblasts (Fig. 5B–C), albeit more pronounced differences were detected in 2 out of 7 mutant fibroblasts (designated as M1 and M2 in Fig. 5C). This could be due to the beginning of the tight skin development, as indicated by the lower frequency or a milder tight skin phenotype detected at the neonatal stage (Fig. 1C).

We confirmed the positive expression of fibroblast marker genes [46, 47] in our RNA-seq results, but ECM genes and some ECM-modifying genes, such as the lysyl oxidase (LOX) family, were relatively unaffected by the mutation, including *Fbn1* expression itself (Table S1). *Fbn1* mutations or loss of function that lead to decreased fibrillin-1 protein abundance have been linked to the misregulation of TGF β /BMP signaling [10,48]. Although the *Fbn1* G234D mutation resulted in less fibrillin-1 in the dermal area (Fig. 2A, S2A), our RNA-seq data indicates that TGF β /BMP signaling-related genes (ligands, receptors and target genes) remained comparable to WT in the mutant fibroblasts (Table S2). We further examined TGF β /BMP receptor signaling by measuring the phosphorylation of Smad2 and Smad1/5 [10] in the dermal fibroblasts. As shown in Fig. S5A–C, both TGF β /BMP Smads signaling were unaffected by mutant fibrillin-1.

Intriguingly, the 224 upregulated genes in the mutant fibroblasts included muscle developmental-related genes, immune response genes, and some cell-cell adhesion/cytoskeletal genes (Fig. 5B–E, S5D, Table S3). The muscle-related genes enriched in mutant fibroblasts included transcription factors (TFs) such as *Myod1* and *Myog* and contractile genes such as myosin heavy chains (*Myh* family), tropomodulin (*Lmod3*), and troponins (*Tnn* family) (Fig. 5E). Given the enhanced expression of myogenic and contractile-related genes in mutant fibroblasts (Fig. 5E) and that myoblasts are stiffer than fibroblasts [49], we tested the cell stiffness of fibroblasts by atomic force microscopy to evaluate whether muscle-related gene expression was associated with changes in cell mechanical properties. Our results indicate that mutant fibroblasts were modestly stiffer than WT (Fig. 5F). In the down-regulated gene list (48 genes, Table S3), there was an enrichment of histone genes and developmental TFs with some of the gene examples shown in a heatmap (Fig. 5G–H), suggesting a possible modification of the chromatin/transcriptional state.

$Fbn1^{G234D/G234D}$ fibroblasts secrete pro-fibrogenic proteins in ECM-inducing conditions and are defective in elastogenesis

To assess the changes in dermal fibroblasts-secreted proteins, we performed a secretome analysis between WT and mutant fibroblasts cultured in an ECM-inducing medium. Mass-spectrometry results demonstrated that 73 proteins were more abundant, and 13 proteins were less abundant in the mutant fibroblasts compared to WT (Fig. 6A, Table S4). Among the significantly upregulated proteins in the mutant fibroblasts were those involved in elastic fiber formation and collagen fibril organization, including cross-linking, modifying enzymes such as lysyl oxidase-like 1 (Loxl1) [50], which may participate in the collagen cross-linking observed in Fig. 4H–L (Fig. 6B–C, S6A). Immunoblotting of Loxl1 using mouse dorsal skin lysate further confirmed the significantly increased Loxl1 abundance in the mutant skin compared to WT (the

uncleaved form, 63kDa) (Fig. 6D–E), while immunostaining of Loxl1 uncovered its augmented presence in the mutant dermis (Fig. 6F). A higher secretion of proteoglycans such as lumican was also detected within factors regulating collagen fibrils organization (Fig. 6C). This was confirmed by immunostaining that showed enhanced intensity in the mutant dermis (areas highlighted by white rectangles, Fig. 6G). Indeed, a previous report described fragile and loose skin in mice lacking lumican expression [51].

Although fibrillin-1 secretion was compromised in the mutant fibroblasts in agreement with the reduced fibrillin-1 deposition in the mutant dermis (Fig. 2A, S2A, S3C–D), the secretion of multiple elastic fiber components was augmented in mutant cells (Fig. 6A, B, S6A). Therefore, we examined elastic fiber formation in vitro by performing an elastogenesis assay and immunostaining of elastin, fibrillin-1, and fibronectin (Fig. 6H). Mutant dermal fibroblasts deposited less fibrillin-1, and fewer elastic fibers were produced as a net result (Fig. 6H). This correlated to a slower fibroblast migration in a scratch assay (Fig. 6I), which could be associated with the reduced level of fibrillin-1 [31,52] or secretion of ECM such as collagen type VI that is known to downregulate cell motility (Fig. 6B, C) [53]. In line with the RNAseq data (Fig. 5E, S5D), the secretome data revealed the enrichment of extracellular proteins involved in skeletal system development and immune responses categorized as leukocyte chemotaxis and tumor necrosis factor (TNF) production (Fig. 6B, S6B–C). Interestingly, insulin-like growth factor-1 (IGF1) and insulin growth factor binding proteins (IGFBP3, 4, 6) were significantly augmented in mutant fibroblasts, suggesting that G234D fibrillin-1 may play further roles in regulating skin growth and regeneration (Fig. S6D). Furthermore, among the top 3 categories of secreted proteins (ECM, collagen fibril organization, and skeletal system development), 6 proteins were shared between them, including those encoded by *Col11a1* and *Col6a1*, *Fbn1*, *Fbn2*, *Ltbp3*, and *Adamts7* (Fig. S6E). This suggests that changes in the ECM due to the *Fbn1* G234D mutation are, in part, related to collagen reorganization (in line with the collagen cross-linking shown in Fig. 4H–L) and skeletal-related development (as indicated by RNA-seq data shown in Fig. 5C–E).

Discussion

Our study describes a new category of tight skin found in $Fbn1^{G234D/G234D}$ mice that highlights molecular, cellular, and phenotypic tissue changes due to a single amino acid substitution in the H1 domain. We have presented evidence indicating an altered structure and distribution of elastic fibers with sub-regional differences in mutant fibrillin-1 abundance in the skin (particularly the dermis versus fascia), possibly caused by a distinct microenvironment. The *Fbn1* G234D mutation promoted collagen cross-linking without fibrotic changes typified by collagen accumulation and deviations in fibroblast characteristics (Fig. 7).

Tight skin develops without collagen accumulation

The tight skin phenotype in $Fbn1^{G234D/G234D}$ mice is physically characterized by a decrease in the SSA (Fig. 1), similar to other mouse models such as TSK, SSS, and WMS, implicating a common dysfunction of fibrillin-1 [18,19,40]. However, unlike the previous mouse models that exhibit excessive collagen accumulation and thicker skin [17,40,54], $Fbn1^{G234D/G234D}$ mice develop tight skin independent of increases in skin thickness or collagen amount. Instead, the deposition of collagen cross-links and abnormal elastic fibers in the fascia may cause the mutant skin to be more firmly attached to the underlying skeletal muscle, thus increasing skin tightness. This is consistent with the abundance of mutant fibrillin-1 in the fascia, which coincides with the observation of accrued aberrant elastic fibers within this region (Fig. 2).

However, fibrillin-1 is diminished in the mutant dermis, and elastic fiber quality is compromised (Fig. 2), which is in line with fibrillin-1's role in maintaining elastic fiber organization and integrity—crucial for

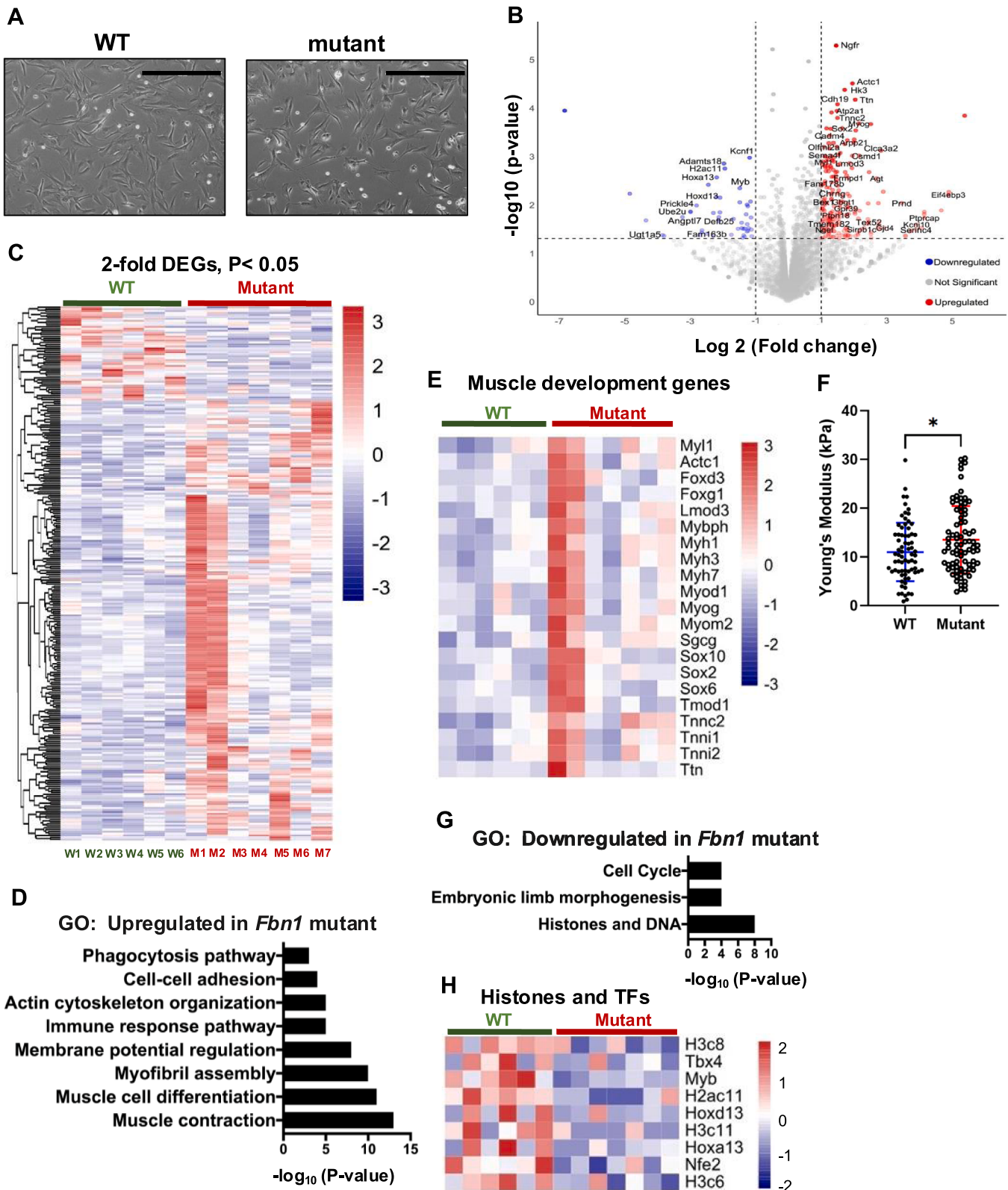
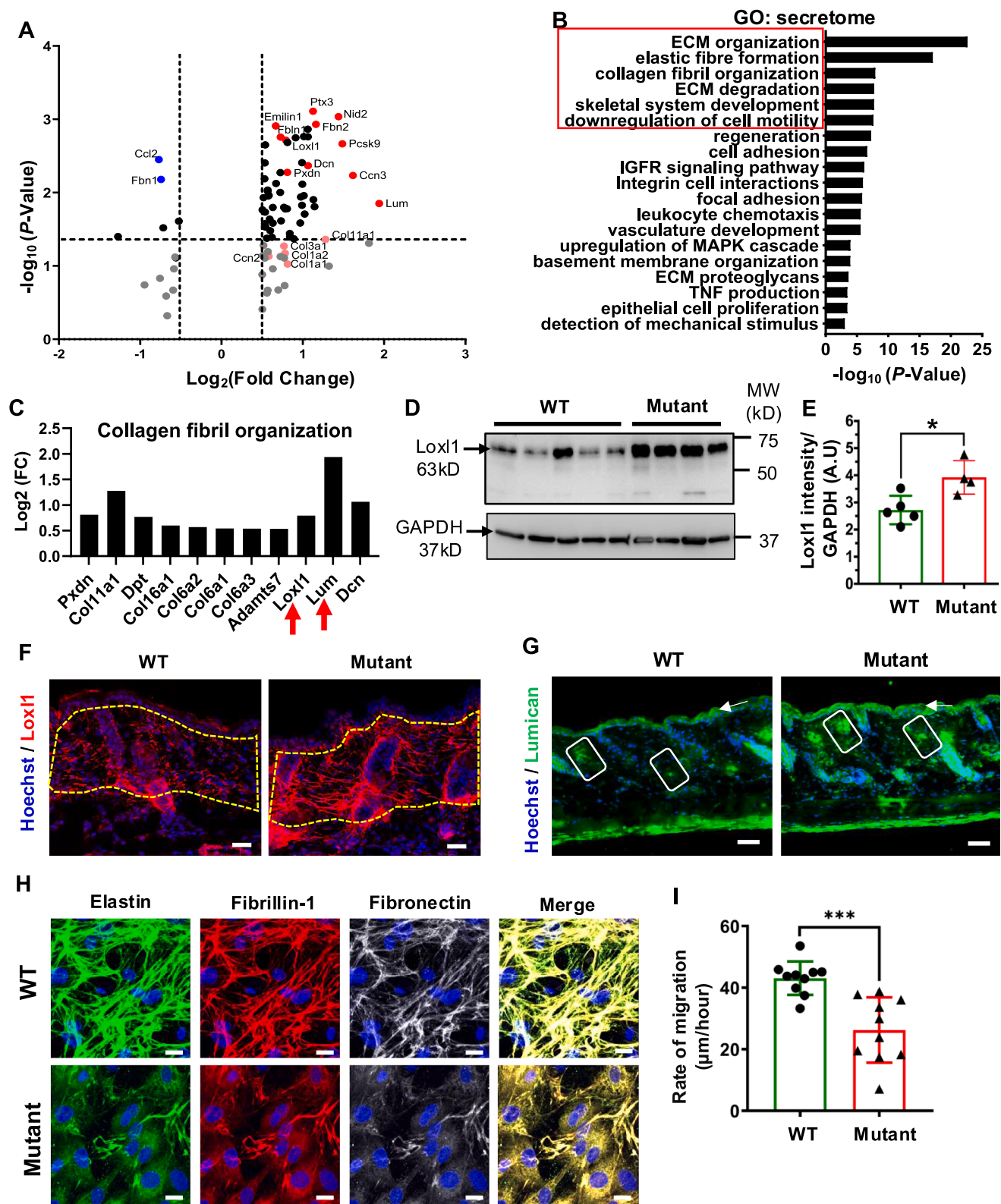


Fig. 5. Comparison of transcriptome between wild-type and *Fbn1*^{G234D/G234D} dermal fibroblasts. **A**, Phase contrast micrographs of primary neonatal dermal fibroblasts. Scale bar, 400 μ m. **B**, Volcano plot shows differentially expressed genes (DEGs) up- or down-regulated in mutant fibroblasts compared to WT. **C**, Heatmap displays DEGs that are up- and down-regulated by a two-fold difference in mutant fibroblasts compared to WT. **D**, Gene ontology (GO) analysis obtained from two-fold differentially upregulated genes in mutant fibroblasts compared to WT in (C). **E**, Heatmap highlights muscle developmental genes identified from the GO of upregulated genes, including transcription factors (*Foxd3*, *Foxg1*, *Myod1*, *Myog*, *Sox2*, *Sox6*, *Sox10*) while others are cytoskeleton-contractile genes. **F**, Cell stiffness measured by atomic force microscopy (AFM) on 70–80 cells per group (primary fibroblasts from 3 mice per group, **P* < 0.05, Mann-Whitney test; mean $\pm$ S.D.). **G**, GO analysis conducted on two-fold downregulated genes in mutant neonatal fibroblast compared to WT. **H**, Heatmap representing downregulated genes categorized as histones and developmental transcription factors (TFs) from GO analysis in (G).



(caption on next page)

skin elasticity [1]. Compromised dermal elastic fibers in the mutant mice may have mitigated dermal ECM pliability (becoming more loose skin-like [55]). This correlates with the decrease in skin tensile strength, which is dependent on elastic fibers, at a low deformation strain (Fig. 1)

[56].

The finding that a G234D mutation, H1 deletion, or WMS deletion produces fibrillin microfibrils with a shared disruption at the shoulder region points to their similar localization within the microfibril structure

Fig. 6. The secretome of *Fbn1*^{G234D/G234D} dermal fibroblasts. **A**, Volcano plot depicts secreted proteins significantly up-regulated or down-regulated in mutants compared to WT fibroblasts cultured in an ECM-inducing medium for 24 h (CnT-Prime ECM). Data obtained from mass spectrometry-based protein identification (primary fibroblasts from 3 mice were analyzed per group, x axis FC, fold change cut off 0.5 and P-value < 0.05. **B**, GO enrichment analysis of differentially secreted proteins in mutant fibroblasts vs WT. Bar graph depicts the top biological processes in up- and down-regulated proteins, sorted by P-value. Red box indicates top 6 gene categories affected by *Fbn1*^{G234D} mutation. **C**, Bar graph shows the fold change expression level of some candidate proteins known to modulate collagen fibril organization. (in their gene names: *Pxdn*, *Col11a1*, *Dpt*, *Col6a2*, *Col6a1*, *Col16a3*, *Adams7*, *Loxl1*, *Lum*, *Dcn*). **D**, Immunoblotting of Loxl1 protein abundance in dorsal skin tissue lysates from WT and mutant mice. **E**, Band intensities of Loxl1 were quantified and normalized to GAPDH. WT, *n* = 5; mutant, *n* = 4; **P* < 0.05; Student *t*-test; mean ± S.D. **F**, Loxl1 immunostaining comparing WT and mutant skin. Yellow dotted area indicates Loxl1 expression in the dermis. **G**, Micrographs of lumican immunostaining in mutant skin compared to WT. Epidermal signals (white arrow) are non-specific. White boxes show dermal expression of lumican. (D – G) Mouse samples were collected at 3 weeks of age (*n* = 5 per group, scale bar, 50 μm). Hoechst, nuclear staining. **H**, Fluorescence microscopy images from fibroblast elastogenesis assay, staining for fibronectin, fibrillin-1, and elastin secreted by mutant fibroblasts compared to WT (representative images from 3 independent experiments). Scale bar, 20 μm. Hoechst, nuclear staining. **I**, In vitro scratch assay of mutant fibroblasts migration compared to WT. 10 fields of view per genotype were assessed. *** *P* < 0.001. Student *t*-test; mean ± S.D. From 2 independent experiments using fibroblasts from 2 different mice per group.

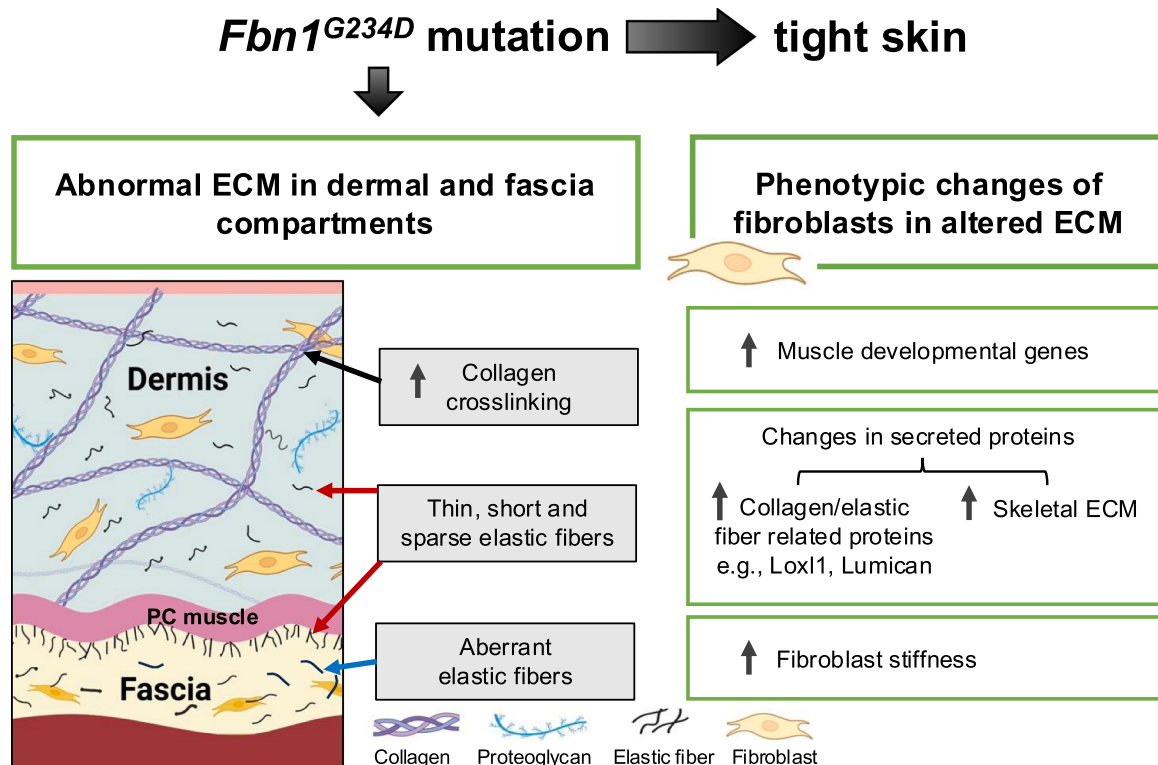


Fig. 7. Molecular and cellular changes in tight skin phenotype due to *Fbn1*^{G234D/G234D} mutation. **Left panel** depicts structural alterations in the mutant skin. These changes include collagen crosslinking (black arrow), more sparsely distributed thin and short elastic fibers in the dermis and the interface between the panniculus carnosus (PC muscle) and fascia (dark red arrows), while elastin and thin elastic fibers are accumulated in the fascia compartment (blue arrow) compared to WT skin. **Right panel** illustrates dermal fibroblast changes in the altered ECM due to the *Fbn1*^{G234D} mutation. Genes related to muscle development and contraction are upregulated. Proteins secreted by mutant fibroblasts are enriched for collagen-regulating factors, elastic fiber components, and ECM proteins related to skeletal system development. Mutant fibroblasts display increased stiffness compared to WT fibroblasts.

(Fig. 3, [39]). However, it remains unknown whether these 3 mutant forms of fibrillin-1 share a common molecular mechanism as they cause distinct biological outcomes in the skin, with the WMS deletion mutant causing tight skin with collagen deposition and skin thickening, whereas the H1 deletion mutant appears to have normal skin with a largely preserved microfibril assembly [40,57]. Indeed, the homozygous deletion of the H1 domain leads to a mild phenotype in the aorta, resulting in a normal life span, as opposed to the G234D homozygous mutation in the H1 domain [32,57,58]. This raises the question of whether the H1 domain contains site(s) that both positively and negatively regulate fibrillin-1 function.

The effect of the fibrillin-1 G234D mutation on dermal fibroblast transcriptomic characteristics

Investigating the effect of *Fbn1* mutations on fibroblasts may serve as a model to further unravel the molecular basis of connective tissue

disorders. The transcriptomic analysis of *Fbn1*^{G234D/G234D} fibroblasts revealed cellular and molecular alterations. The overall preservation of ECM and TGFβ/BMP signaling-related gene expression differed from previous observations of *Fbn1* mutations with perturbed TGFβ/BMP signaling and ECM dysregulation [9,11,17]. Nevertheless, we found the upregulation of genes related to muscle development and immune response in mutant fibroblasts, necessitating further investigation. Additionally, *Fbn1*^{G234D/G234D} fibroblasts demonstrated an increase in cell stiffness, which could result from an altered ECM on mutant fibroblasts [59]. The downregulation of histone genes and developmental transcription factors in mutant fibroblasts hints at epigenetic alterations, potentially influencing cellular functions/phenotypes [60]. Although it remains unexplored whether the acquisition of altered properties is intrinsic to the mutation or is a response to a modified ECM microenvironment, our data supports the idea that a change in the ECM has an effect on fibroblast characteristics, which may contribute to the tight skin phenotype.

The effect of fibrillin-1 G234D mutation on dermal fibroblast secretome

Although the transcriptomics analysis did not find altered ECM gene transcription, aberrant protein secretion in *Fbn1*^{G234D/G234D} fibroblasts was detected. Despite an increased secretion of elastic fiber components (possibly as a compensatory response to reduced mutant fibrillin-1 secretion), elastogenesis was compromised in vitro. This disruption aligns with prior evidence demonstrating that *FBN1* mutations impede elastic fiber assembly [1]. Importantly, the decrease in elastogenesis correlated with slower fibroblast migration, suggesting a functional association between elastic fibers, ECM structural integrity and dynamics with fibroblast motility [31,61], which may be crucial for tissue homeostasis and repair.

The augmented secretion of various ECM components, including the cross-linking enzymes Loxl1 and Pxdn, suggests another compensatory response aimed at preserving ECM homeostasis in the presence of mutant fibrillin-1 [62–65]. Moreover, mutant fibroblasts displayed a predisposition towards activated fibroblasts, as demonstrated by the heightened secretion of proteins associated with tissue repair. The overall promotion of ECM secretion, cross-linking related enzymes, proteoglycans such as lumican and decorin, matricellular proteins such as CCN3 and periostin, chemotactic factors, and pentraxin are reminiscence of a tissue injury response and fibrosis [21,34,50,66–72]. This suggests that although no skin fibrosis was detected due to the G234D mutation at homeostasis, mutant fibroblasts/skin may be more susceptible to fibrogenesis upon wounding or other skin insults.

The enrichment of proteins involved in skeletal system development and the immune response emphasizes the effect of the fibrillin-1 G234D mutation on cellular functions beyond ECM regulation. Given the intimate link between the skeletal system and connective tissue homeostasis [73], alterations in skeletal development proteins may reflect perturbations in tissue architecture and mechanical properties. Additionally, the presence of immune-related proteins suggests potential interactions between mutant fibroblasts and the immune system, further modulating tissue remodeling processes and potentially contributing to disease pathogenesis.

The upregulation of IGF1 and IGF1R in mutant fibroblasts suggests further roles for fibrillin-1 in regulating skin growth and repair. IGF1 plays a critical role in tissue growth, wound healing, and regeneration by promoting cell proliferation, survival, and ECM synthesis [74,75]. IGF1 is also one of the early cytokines identified that promote myogenesis [76,77], and it may be associated with the enhanced expression of muscle developmental genes in mutant fibroblasts transcriptome (Fig. 5). In addition, changes in secreted proteins such as CCN2, CCN3, VEGF and CCL2 (Fig. 6 and S6) have been reported to regulate myogenesis as well [77]. Although fibroblasts have some plasticity, including conversion into myogenic progenitor cells [78,79], in our context, due to the limitation of bulk-RNAseq, we could not distinguish whether muscle-related genes were expressed by mutant fibroblasts or by PC muscle satellite cells [80] that remained in skin fibroblast culture and were responding to the altered fibroblast secretome.

Unexpectedly we observed enhanced secretion of some fibroblast senescent-related markers such as IGF1R and IGF1R [81,82] and along with it some chemokines such as CCL2, CXCL12, and CX3CL1. Cellular senescence has been implicated in the secretion of the senescence-associated secretory phenotype (SASP), such as those involved in chemotaxis as indicated in our mutant fibroblasts secretome (Fig. S6C), and concomitant to increased immune-response genes (Fig. S5D). A pro-inflammatory state in some of these mutant fibroblasts may account for the tendency towards the promotion of a tissue repair/fibrogenesis-related secretome [83,84] without an actual fibrosis or skin thickening, raising the possibility for a modified wound healing response.

Conclusion and future directions

Although we have not provided direct evidence of the mechanistic link between a novel hybrid1 domain *FBN1* mutation and cellular changes, our study describes the interplay and correlations between mutant fibrillin-1, ECM dynamics, fibroblast behavior, and skin physiology. While the fibrillin-1 G234D mutant tight skin phenotype resembles scleroderma-like mouse models, the absence of characteristic fibrosis suggests a distinct mechanism involving compromised elastic fiber integrity and an altered ECM that affects fibroblast secretomes, and transcriptomic characters. Future studies should elucidate the effects of the fibrillin-1 mutation on the skin wound-healing response and its molecular mechanism. In conclusion, the characterization of the fibrillin-1 G234D mutation in a mouse model expands our understanding of diverse phenotypic consequences due to fibrillin-1 mutation and provides some insights into the underlying mechanisms that lead to skin tightness.

Experimental procedures

Mice

The C57BL/6 wild-type mice were obtained from Jackson Laboratory, and *Fbn1*^{G234D/G234D} mice were developed on a pure C57BL/6 background by the Laboratory Animal Resource Centre at the University of Tsukuba [32]. Genotyping was conducted using GoTaq green master mix (Promega, M7123) and specific primers (Forward: 5'-GACACACGGGTAGCTTCCAT-3', Reverse: 5'-ACCTTCCGGACTC CAAGAAT-3'). To detect the *Fbn1*^{G234D/G234D} mutant allele, the restriction enzyme Sac II (Takara,1079A) was used. Homozygous alleles are detected at 829 bp (uncleaved) and WT alleles at 617 bp and 212 bp (Sac II cleaved). Both male and female mice were included in the experiments. All mice were housed in a pathogen-free environment on a 12-hour light/12-hour dark cycle. All animal procedures were conducted following animal experimentation guidelines approved by the Institutional Animal Experiment Committee at the University of Tsukuba (20–262).

Skin sample collection and processing for histology

Mice were anesthetized with a combination of three compounds (Medetomidine hydrochloride, butorphanol tartrate, and midazolam) then euthanized by cervical dislocation. The tissue from the back skin was collected from both WT and *Fbn1*^{G234D/G234D} mice for various immunohistological analyses. Briefly, the back skin, including skeletal muscle, was excised, washed with phosphate-buffered saline (PBS), and fixed with 4 % paraformaldehyde (PFA; (FUJIFILM Wako,162–16065)) overnight at 4 °C. The following day, the tissue was washed with PBS for 3 h on a shaker and incubated with 20 % sucrose (FUJIFILM Wako, 190–00013) overnight at 4 °C before being embedded in an optimal cutting temperature compound (Tissue-Tek® OCT Compound, 4583) and snap-frozen by dry ice. Finally, 10 µm thin sections were prepared by cryo-sectioning (CryoStar NX70).

Tissue staining

Hematoxylin and Eosin (H&E) staining: Frozen sections were stained with Hematoxylin (FUJIFILM Wako, 131-09665) for 20 min and Eosin (FUJIFILM Wako, 058-00062) for 30 s at room temperature (RT). **Hart's staining:** To visualize elastic fibers, tissue sections were pre-treated with (100 ml 70 % Ethanol+1ml HCl) for 5 min before being incubated in Maeda's Resorcin-Fuchsin (Muto Pure Chemical, 4032) solution for 2 h, and Metanil Yellow (FUJIFILM Wako, 137–01502) solution for 1 min. All incubations at RT. **Masson trichrome Staining (MTS):** Tissue sections were fixed in Bouin's fluid (Polysciences,16045–1) for 1 hour at 60 °C and rinsed in distilled water.

Slides were then treated with Weigert's Hematoxylin (FUJIFILM Wako, 298–21741) for 10 min, Ponceau-Xylidine-Acid Fuchsin-Azophloxine (FUJIFILM Wako, 166–19765) for 5 min, Phosphomolybdic-Phosphotungstic acid solution (Sigma-Aldrich, 221856 and P6395, respectively) for 25 min, Aniline Blue (FUJIFILM Wako, 015–18045) for 30 seconds at RT. **Picrosirius Red Staining (PRS):** Tissue sections were stained with Picrosirius Red Stain Kit and protocol (ScyTek Laboratories, PSR-1). After xylene washes, all stained tissue sections were mounted using Entellan (Merck Millipore, HX73846161). H&E, Harts, and MTS images were captured using a Zeiss Axio Imager Z2 microscope and Zen 2.3 Pro software. For PRS, images were captured with polarized light microscopy (Olympus BX53). To quantify collagen fiber orientation and alignment, ImageJ converted PRS images into 8-bit images with enhanced contrast. The output images were then imported into the CurveAlign for ROI analysis [85,86]. For each image, two to five regions were selected as region of interest (ROI: 256 H x 256 W). The fiber analysis method and boundary method chosen were “CT” and “No Boundary”, respectively, with a fraction of coefficient set as 0.06.

Immunofluorescence staining: Skin section staining procedures were performed as previously described [87,88]. Briefly, frozen sections were fixed with 4 % PFA for 10 min at RT. Afterward, the sections were incubated with 0.1 % Triton X-100 in PBS and subjected to blocking in normal goat and donkey serum solution containing bovine serum albumin (BSA; Sigma-Aldrich, A3059). Sections were incubated overnight at 4 °C with primary antibodies listed below: rabbit anti-fibrillin-1 (1:400, pAb 9543, a kind gift from Lynn Sakai), goat anti-elastin (1:200, Elastin Products Company, RA75), rabbit anti-fibulin 5 (1:200, BSYN 1923), rabbit anti-fibronectin (1:200, Sigma-Aldrich, F3648), rabbit anti-collagen I (1:500, EMD Millipore, AB756), rabbit anti-Lox1 (1:200, Abcam, ab313585), rabbit anti-lumican (1:200, Abcam, ab168348). The following day, sections were washed and incubated for 1 h with secondary antibodies (1:200, Alexa 488, 546, Invitrogen) and hoechst nuclear stain (1:1000, Sigma-Aldrich, B2261) at RT before mounting. Images were captured using a Zeiss Axio Imager Z2 microscope and Zen 2.3 Pro software. Image analysis was performed using ImageJ fiji (<http://imagej.nih.gov/ij/>). ROI was drawn, and fluorescence intensity was measured.

Mechanical testing of dorsal skin

The dorsal skin of 3-week-old mutant or WT mice was harvested after hair removal by shaving and hair removal cream treatment. For tensile testing, dorsal skin was cut into dog bone-shaped specimens. After thickness measurement, uniaxial tensile tests were performed using a tensile tester (DMA 850, TA Instruments, New Castle, DE, USA) at RT with a strain rate of 5 mm min⁻¹. The slope of the stress-strain curve at 0–10 % was used to obtain the Young's Modulus [34]. For the rheological measurement, the dorsal skin was cut into 1 cm² specimens and tested using an MCR 301 rheometer (Anton Paar GmbH, Austria) with a parallel PP10/P2 plate. The samples were placed with the epidermis in contact with the plate and a specimen-dependent gap distance. Frequency sweep mode was used to obtain the viscoelastic spectra (storage modulus, G' and loss modulus, G'') by applying 1 % strain from 0.1 to 100 rad/s at 25 °C. To calculate the complex modulus (G^*), the following formula was used: $G^* = \sqrt{(G')^2 + (G'')^2}$ [89].

Collagen biochemical analysis

A full-thickness skin of 0.5 cm² was excised from the back skin of 3-week-old mice and frozen pulverized with mortar and pestle (Spectrum Bessmann, 189470). The skin samples were hydrolyzed with 6 N HCl after reduction with NaBH₄ as described [45]. An aliquot of the acid hydrolysate was subjected to amino acid analysis using an l-8900 amino acid analyzer (Hitachi). The amount of total protein and collagen was calculated from the total of amino acids and hydroxyproline (Hyp) with

collagen conversion factor 7.46 [90], respectively. Collagen cross-links were quantitated by LC-MS on a QTRAP 5500+ hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex) coupled to an ExionLC AD HPLC system (AB Sciex) with ¹⁸O-labeled internal standards as described previously [91]. For analysis of Pyr, derivatization with propionic anhydride was further done to sensitively detect the cross-link by LC-MS as reported previously [92]. Contents of the cross-links in a collagen molecule were calculated based on the value of 300 residues of Hyp per collagen molecule.

Skin microfibril structural analysis

Fibrillin microfibril extraction from WT and G234D mice: Mouse back skin samples from 22-day-old WT and mutant mice were thawed and resuspended in buffer (20 mM Tris-HCl, 400 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Skin was minced using scissors, then supplemented with protease inhibitors (NEM (5 mM), PMSF (3 mM)) and collagenase type 1A (0.1 mg/ml). Samples were incubated overnight at room temperature with rotation. Subsequently, samples were centrifuged at 800 g for 10 min at 4 °C. Supernatant was then collected and centrifuged at 5,000 g for 10 min at 4 °C. Size exclusion chromatography (SEC) was performed using a Sepharose CL-2B column and the void volume collected. **Negative stain electron microscopy:** Microfibrils eluted from the SEC column were adsorbed onto glow-discharged carbon-coated copper grids and stained with 2 % uranyl acetate. Grids were imaged on a Talos L120C TEM operating at an accelerating voltage of 120 kV and equipped with a Ceta CMOS camera. Images were recorded at a magnification of 57,000x and 2.45 Å pixel⁻¹. The microfibril repeat was manually picked in RELION-5.0 and subjected to 2D class averaging. To determine the period length of the WT and mutant microfibrils, the periodicities of 332 microfibril repeats were measured using ImageJ. Microfibrils were extracted from images and straightened using the straighten tool before measuring the bead-to-bead repeat length.

Transmission electron microscopy

Mice were euthanized at 3 weeks of age and back skin was cut into 1 mm x 1 mm x 2 mm dimensions which were then fixed with 3 % glutaraldehyde (Sigma-Aldrich, 1.12179.0025) in phosphate buffered solution (pH 7.4) prior to processing as previously described [93] (without tannic-acid staining). Ultra-thin sections (80 nm) were viewed and captured using a JEM1400 transmission electron microscopy (JEOL).

Isolation of fibroblast from mouse neonatal skin and primary cell culture

The isolation and culture of primary dermal fibroblasts from post-natal day 0–2 (PD 0–2) mouse pups are as follows. 70 % ethanol solution was used for surface sterilization of the pups before limb and tail dissection, followed by careful removal of the skin without disturbing the skeletal muscle or internal organs [94]. Tissue specimens were then subjected to overnight incubation at 4 °C with agitation in CnT-Prime (CnT-PR) medium supplemented with antibiotics (CnT-GAB-10) and dispase solution (CnT-DNP-10, all reagents from CELLnTEC) followed by a 30-min incubation at RT. Epidermal and dermal layers were separated, and the dermal tissue was finely minced and subjected to enzymatic digestion using 1 mg/ml collagenase A (Sigma-Aldrich, 10103578001) in DMEM medium (Gibco, 11965092) for 1.5 h at RT. Post-digestion, cells were resuspended in 20 % FBS/DMEM, centrifuged, and filtered through a 70 µm mesh. Cell count and viability were determined before seeding on collagen I-coated plates (Corning Biocoat, 356450) for passage 0 (P0) cell culture, after which passages were conducted on non-coated plates.

For isolation of primary fascia fibroblasts from PD 0–2 skin, mice were dissected as above and the skin lay flat on a culture dish with the fascia side facing up [95]. Fragments of fascia layers were collected with

forceps then digested with 1 mg/ml collagenase A (Sigma-Aldrich, 10103578001) containing antibiotics (CnT-GAB-10) in DMEM/F12 (Gibco, 11320033) for 1 h at RT followed by incubation at 37 °C for 15 min. Cells suspension were then filtered (70 µm mesh), centrifuged and cultured as above. P0 cells were cultured in DMEM/F12 with 10 % FBS.

Bulk RNA-sequencing

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, 74,136) from WT and mutant primary dermal fibroblasts at P2 culture (WT $N = 6$, mutant $N = 7$). RNA quality was analyzed with Agilent 2100 bioanalyzer and subsequently submitted to (Azenta, Project 60–808522843 and 60–1074343755) for sequencing. The FASTAQ data were converted and analyzed with CLC Genomics 22 software (Qiagen) for gene expression analysis. Then hierarchical clustering/heatmaps, Venn diagram and GO analysis were performed using the R packages (R version 4.4.1 with DESeq2, ggplot2, pheatmap, ggfortify, plotly and htmlwidgets packages), online tools Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>) and Metascape (<http://metascape.org>) [96], respectively. Raw data is stored at Gene Expression Omnibus (GSE268383).

Cell stiffness measurement by AFM

WT and mutant Fibroblasts (30,000 cells) from P3–4 cultures were seeded on 40mm culture plates (TPP, 93040) and individual cell stiffness was measured using AFM (Nanowizard II, JPK Instruments, Berlin, Germany) by a cantilever attached with a 10 µm-diameter glass bead with a spring constant of 0.02 Nm⁻¹ (CP-CONT-BSG, NanoAndMore, Watsonville, CA, USA). Force spectroscopy was performed by probing on the cytoplasmic cell area immediately outside the nucleus under phase contrast imaging guidance. The end-point of force measurement was set at 10 nN, and the cantilever was driven at a velocity of 10 µms⁻¹. Results were analysed by JPKSPM data processing software using Hertz model for spherical indenter

$$F = \frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R} I_2^3$$

where F , R , E , I , and ν represent force, radius of indenter (5 µm), Young's modulus, indentation depth, and Poisson's ratio (0.5 in this study), respectively.

Western blot analysis

Dorsal skin tissue was collected from 3-week-old mice. Neonatal dermal fibroblasts were cultured, and cells were collected between passages 2–4. Both tissue and cells lysate were prepared in RIPA lysis buffer (Sigma Aldrich, R0278) containing 1 % protease inhibitor (Thermo Scientific, 78430) and 1 % phosphatase inhibitor (FUJIFILM Wako, 160–24371). The lysates were mixed with 3 x SDS sample buffer with 2-mercaptoethanol (Wako, 133–14371) and boiled at 95 °C for 5 min, and then were subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore, IPVH00010) and immunoblotted with primary antibodies as follows: mouse anti-fibronectin (1:1000, BD Transduction Laboratories, 610078), rabbit anti-Lox1 (1:1000, Abcam, ab313585), rabbit anti-pSmad2 (1:500, Cell Signaling, 31084S), rabbit anti-Smad2/3 (1:3000, Cell Signaling, 8685S), rabbit anti-pSmad1/5 (1:1000, Cell Signaling, 9516S), rabbit anti-fibrillin-1 (1:1000, rf18, a kind gift from Tomoyuki Nakamura), mouse anti-collagen-1 (1:1000, Millipore, AB765P) and rabbit anti-GAPDH (1:1000, Cell Signaling, 2118). Membranes were then incubated with respective anti-rabbit or -mouse HRP-conjugated secondary antibody (1:1000, Bio-Rad) and visualized with a chemoluminescence kit (Ez WestLumiplus, Atto Corporation, WSE 7120S HRP).

Secretome analysis

250,000 of primary skin fibroblasts of P2–3 culture were seeded on a 6-well plate and cultured with 10 % FBS/DMEM ($N = 3$ per group). Next day, medium was replaced by fibroblast ECM production medium (CELLnTEC, CnT-PR-ECM) for 1-day incubation. Because of proprietary reasons, we could not obtain the information for the exact components in the CnT-PR-ECM medium. Conditioned medium from each sample was collected and column centrifugation with Sartorius Vivaspin 2 (Membrane: 3000 MWCO PES, VS0291) was performed at 8000 g to wash and finally concentrate the samples in PBS prior to proteomics analysis as previously described [97]. Raw data is accessible from (iPOST ID: JPST003131/PXD052580). GO analysis was performed using Metascape (<http://metascape.org>).

Elastogenesis assay

To visualize elastic fiber development, primary fibroblasts (P3–4) were grown to confluence on an 8-well culture plate (Ibidi, 80806), seeded at 100,000 cells per well and cultured with 10 % FBS/DMEM medium. On the 4th day of culture, the cells were fixed with –20 °C methanol for 20 min. After several washes with PBS, fixed cells were incubated with a blocking solution (5 % BSA/PBS) for 1 h. Subsequently, the cells were treated with the following primary antibodies: rabbit anti-fibrillin-1 (1:400, rf18 from Tomoyuki Nakamura), goat anti-elastin (1:400, Elastin Products Company, RA75), and mouse anti-fibronectin (1:400, BD Transduction Laboratories, 610078), and incubated overnight at 4 °C. The next day, the cells were washed with PBS and incubated for 1 hour with secondary antibodies (1:200, Alexa 488, 546, 647, Invitrogen). Mounting solution containing DAPI (Ibidi, 50011) was used for nuclear staining. The stained elastin fiber components were observed under a confocal microscope (Zeiss LSM 700) and images were captured and analysed using ZEN 2010 software.

Scratch assay

Primary fibroblast of P3–4 (200,000 cells) were seeded on a 6-well plate with 10 % FBS/DMEM. The next day a scratch was made on the confluent culture using a 1 ml pipette tip. Time-interval phase contrast images were obtained using ZEISS Cell Discoverer 7. The 12-hour interval images were analysed by ZEN 2010 software and quantitated using Image J.

Statistics

Graphs and statistical tests were constructed and analyzed using GraphPad Prism ver. 8.4.2. All data are described as mean±S.D. Data normality was assessed with Saphiro-Wilk test. Statistics were determined by unpaired t -tests. Mann-Whitney tests (non-parametric) were conducted where data did not conform with normality assumption.

Declaration of competing interest

The authors declare no conflict of interest

Acknowledgements

We thank Choi Jun Hyeok for performing preliminary experiments, the Laboratory Animal Resource Center at the University of Tsukuba for their excellent animal care, the TEM Facility at the University of Tsukuba and the EM facility University of Manchester (RRID:SCR_021147; Faculty of Biology, Medicine and Health) for support. We also thank Ryutaro Ishii for RNA-sequencing administration, Aiko Sada and Lynn Y. Sakai for discussion. Figure S1B was made with Biorender. ASM.S.H. is supported by a Ph.D. study scholarship from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT). G.B. is

supported by a PhD studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) UK (BB/T008725/1). We thank research funding from Japan Society for the Promotion of Science (23K07737) and Lydia O'leary Memorial Pias Dermatological Foundation for Elastin molecular research grant 2022 (to E.R.), Japan Agency for Medical Research and Development (grant number 23ek0109553h003 and 23ek0210183h0001), Grant-in-Aid for Scientific Research (A, grant number 23H00431), and Grant-in-Aid for Fund for the Promotion of Joint International Research (Fostering Joint International Research(B)) (grant number 21KK0151), and the Everest Grant from the Marfan Foundation (to H.Y.), and JSPS KAKENHI Grant Number 23H04937 and Japan Agency for Medical Research and Development (grant number 23ama121018) (to S.O.).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.matbio.2024.11.006](https://doi.org/10.1016/j.matbio.2024.11.006).

Data availability

Data will be made available on request.

References

- [1] J. Thomson, M. Singh, A. Eckersley, S.A. Cain, M.J. Sherratt, C. Baldock, Fibrillin microfibrils and elastic fibre proteins: functional interactions and extracellular regulation of growth factors, *Semin. Cell Dev. Biol.* 89 (2019) 109–117.
- [2] E. El-Hallous, T. Sasaki, D. Hubmacher, M. Getie, K. Tiedemann, J. Brinckmann, B. Batge, E.C. Davis, D.P. Reinhardt, Fibrillin-1 interactions with fibulins depend on the first hybrid domain and provide an adaptor function to tropoelastin, *J. Biol. Chem.* 282 (12) (2007) 8935–8946.
- [3] A.R.F. Godwin, M. Singh, M.P. Lockhart-Cairns, Y.F. Alanazi, S.A. Cain, C. Baldock, The role of fibrillin and microfibril binding proteins in elastin and elastic fibre assembly, *Matrix. Biol.* 84 (2019) 17–30.
- [4] L.Y. Sakai, D.R. Keene, E. Engvall, Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils, *J. Cell Biol.* 103 (6) (1986) 2499–2509.
- [5] H. Zhang, W. Hu, F. Ramirez, Developmental expression of fibrillin genes suggests heterogeneity of extracellular microfibrils, *J. Cell Biol.* 129 (4) (1995) 1165–1176.
- [6] F. Quondamatteo, D.P. Reinhardt, N.L. Charbonneau, G. Pophal, L.Y. Sakai, R. Herken, Fibrillin-1 and fibrillin-2 in human embryonic and early fetal development, *Matrix. Biol.* 21 (8) (2002) 637–646.
- [7] L. Sabatier, N. Miosge, D. Hubmacher, G. Lin, E.C. Davis, D.P. Reinhardt, Fibrillin-3 expression in human development, *Matrix. Biol.* 30 (1) (2011) 43–52.
- [8] M. Pfaff, D.P. Reinhardt, L.Y. Sakai, R. Timpl, Cell adhesion and integrin binding to recombinant human fibrillin-1, *FEBS Lett.* 384 (3) (1996) 247–250.
- [9] S.S. Chaudhry, S.A. Cain, A. Morgan, S.L. Dallas, C.A. Shuttleworth, C.M. Kielty, Fibrillin-1 regulates the bioavailability of TGFβ1, *J. Cell Biol.* 176 (3) (2007) 355–367.
- [10] G. Sengle, L.Y. Sakai, The fibrillin microfibril scaffold: a niche for growth factors and mechanosensation? *Matrix. Biol.* 47 (2015) 3–12.
- [11] A.P. Wohl, H. Troilo, R.F. Collins, C. Baldock, G. Sengle, Extracellular regulation of bone morphogenetic protein activity by the microfibril component fibrillin-1, *J. Biol. Chem.* 291 (24) (2016) 12732–12746.
- [12] S.A. Jensen, S. Iqbal, E.D. Lowe, C. Redfield, P.A. Handford, Structure and interdomain interactions of a hybrid domain: a disulphide-rich module of the fibrillin/LTBP superfamily of matrix proteins, *Structure* 17 (5) (2009) 759–768.
- [13] D.P. Reinhardt, J.E. Gambee, R.N. Ono, H.P. Bächinger, L.Y. Sakai, Initial steps in assembly of microfibrils, *J. Biol. Chem.* 275 (3) (2000) 2205–2210.
- [14] D.P. Reinhardt, T. Sasaki, B.J. Dzamba, D.R. Keene, M.L. Chu, W. Gohring, R. Timpl, L.Y. Sakai, Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues, *J. Biol. Chem.* 271 (32) (1996) 19489–19496.
- [15] I.B. Robertson, H.F. Dias, L.H. Osuch, E.D. Lowe, S.A. Jensen, C. Redfield, P.A. Handford, The N-Terminal Region of Fibrillin-1 Mediates a Bipartite Interaction with LTBP1, *Structure* 25 (8) (2017) 1208–1221, e5.
- [16] H.C. Dietz, G.R. Cutting, R.E. Pyeritz, C.L. Maslen, L.Y. Sakai, G.M. Corson, E. G. Puffenberger, A. Hamosh, E.J. Nanthakumar, S.M. Currstin, et al., Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene, *Nature* 352 (6333) (1991) 337–339.
- [17] E.E. Gerber, E.M. Gallo, S.C. Fontana, E.C. Davis, F.M. Wigley, D.L. Huso, H. C. Dietz, Integrin-modulating therapy prevents fibrosis and autoimmunity in mouse models of scleroderma, *Nature* 503 (7474) (2013) 126–130.
- [18] B.L. Loeys, E.E. Gerber, D. Riegert-Johnson, S. Iqbal, P. Whiteman, V. McConnell, C.R. Chillakuri, D. Macaya, P.J. Coucke, A. De Paeppe, D.P. Judge, F. Wigley, E. C. Davis, H.J. Mardon, P. Handford, D.R. Keene, L.Y. Sakai, H.C. Dietz, Mutations in fibrillin-1 cause congenital scleroderma: stiff skin syndrome, *Sci. Transl. Med.* 2 (23) (2010) 23ra20.
- [19] L.D. Siracusa, R. McGrath, Q. Ma, J.J. Moskow, J. Manne, P.J. Christner, A. M. Buchberg, S.A. Jimenez, A tandem duplication within the fibrillin 1 gene is associated with the mouse tight skin mutation, *Genome Res.* 6 (4) (1996) 300–313.
- [20] T. Barisic-Dujmovic, I. Boban, D.J. Adams, S.H. Clark, Marfan-like skeletal phenotype in the tight skin (Tsk) mouse, *Calcif. Tissue Int.* 81 (4) (2007) 305–315.
- [21] C.S. Adamo, A.V. Zuk, G. Sengle, The fibrillin microfibril/elastic fibre network: a critical extracellular supramolecular scaffold to balance skin homeostasis, *Exp. Dermatol.* 30 (1) (2021) 25–37.
- [22] D. Jiang, S. Christ, D. Correa-Gallegos, P. Ramesh, S. Kalugde Gopal, J. Wannemacher, C.H. Mayr, V. Lupperger, Q. Yu, H. Ye, M. Muck-Hausl, V. Rajendran, L. Wan, J. Liu, U. Mirastschijski, T. Volz, C. Marr, H.B. Schiller, Y. Rinkevich, Injury triggers fascia fibroblast collective cell migration to drive scar formation through N-cadherin, *Nat. Commun.* 11 (1) (2020) 5653.
- [23] D. Correa-Gallegos, D. Jiang, S. Christ, P. Ramesh, H. Ye, J. Wannemacher, S. Kalugde Gopal, Q. Yu, M. Aichler, A. Walch, U. Mirastschijski, T. Volz, Y. Rinkevich, Patch repair of deep wounds by mobilized fascia, *Nature* 576 (7786) (2019) 287–292.
- [24] A. Gatt, S. Agarwal, P.M. Zito, Anatomy, Fascia Layers, StatPearls, Treasure IslandFL, 2024.
- [25] K.Y. DeLeon-Pennell, T.H. Barker, M.L. Lindsey, Fibroblasts: the arbiters of extracellular matrix remodeling, *Matrix. Biol.* 91–92 (2020) 1–7.
- [26] C.M. Kielty, T. Rantamaki, A.H. Child, C.A. Shuttleworth, L. Pelttonen, Cysteine-to-arginine point mutation in a 'hybrid' eight-cysteine domain of FBN1: consequences for fibrillin aggregation and microfibril assembly, *J. Cell Sci.* 108 (Pt 3) (1995) 1317–1323.
- [27] C.M. Kielty, J.E. Phillips, A.H. Child, F.M. Pope, C.A. Shuttleworth, Fibrillin secretion and microfibril assembly by Marfan dermal fibroblasts, *Matrix. Biol.* 14 (2) (1994) 191–199.
- [28] J.S. Del Cid, N.I. Reed, K. Molnar, S. Liu, B. Dang, S.A. Jensen, W. DeGrado, P. A. Handford, D. Sheppard, A.B. Sundaram, A disease-associated mutation in fibrillin-1 differentially regulates integrin-mediated cell adhesion, *J. Biol. Chem.* 294 (48) (2019) 18232–18243.
- [29] R.M. Zhang, K.A. Zeyer, N. Odenthal, Y. Zhang, D.P. Reinhardt, The fibrillin-1 RGD motif posttranscriptionally regulates ERK1/2 signaling and fibroblast proliferation via miR-1208, *FASEB J.* 35 (5) (2021) e21598.
- [30] K.A. Zeyer, R.M. Zhang, H. Kumra, A. Hassan, D.P. Reinhardt, The Fibrillin-1 RGD integrin binding site regulates gene expression and cell function through microRNAs, *J. Mol. Biol.* 431 (2) (2019) 401–421.
- [31] S.E. McGowan, A.J. Holmes, R.P. Mecham, T.M. Ritty, Arg-Gly-Asp-containing domains of fibrillins-1 and -2 distinctly regulate lung fibroblast migration, *Am. J. Respir. Cell Mol. Biol.* 38 (4) (2008) 435–445.
- [32] K. Kimura, E. Motoyama, S. Kanki, K. Asano, M.A.A. Sheikh, M.T.R.D.C. Clarin, E. Raja, M. Takeda, R. Ishii, K. Murata, V. Deleuw, P. Sips, L. Muino-Mosquera, J.D. Backer, S. Mizuno, L.Y. Sakai, T. Nakamura, H. Yanagisawa, A novel genetic mouse model of fatal aortic dissection reveals massive inflammatory cell infiltration in the thoracic aorta, *bioRxiv* (2024) 2024.05.02.592287.
- [33] T.G. Osborn, N.E. Bauer, S.C. Ross, T.L. Moore, J. Zuckner, The tight-skin mouse: physical and biochemical properties of the skin, *J. Rheumatol.* 10 (5) (1983) 793–796.
- [34] M. Nakasaki, Y. Hwang, Y. Xie, S. Kataria, R. Gund, E.Y. Hajam, R. Samuel, R. George, D. Danda, M. JP, T. Nakamura, Z. Shen, S. Briggs, S. Varghese, C. Jamora, The matrix protein Fibulin-5 is at the interface of tissue stiffness and inflammation in fibrosis, *Nat. Commun.* 6 (2015) 8574.
- [35] S. Chatterjee, M.E. Mark, P.H. Wooley, W.D. Lawrence, M.D. Mayes, Increased dermal elastic fibers in the tight skin mouse, *Clin. Exp. Rheumatol.* 22 (5) (2004) 617–620.
- [36] C.M. Kielty, M. Raghunath, L.D. Siracusa, M.J. Sherratt, R. Peters, C. A. Shuttleworth, S.A. Jimenez, The Tight skin mouse: demonstration of mutant fibrillin-1 production and assembly into abnormal microfibrils, *J. Cell Biol.* 140 (5) (1998) 1159–1166.
- [37] R. Lemaire, J.H. Korn, W.P. Schiemann, R. Lafyatis, Fibulin-2 and fibulin-5 alterations in tsf mice associated with disorganized hypodermal elastic fibers and skin tethering, *J. Invest. Dermatol.* 123 (6) (2004) 1063–1069.
- [38] B. Gayraud, D.R. Keene, L.Y. Sakai, F. Ramirez, New insights into the assembly of extracellular microfibrils from the analysis of the fibrillin 1 mutation in the tight skin mouse, *J. Cell Biol.* 150 (3) (2000) 667–680.
- [39] A.R.F. Godwin, R. Dajani, X. Zhang, J. Thomson, D.F. Holmes, C.S. Adamo, G. Sengle, M.J. Sherratt, A.M. Roseman, C. Baldock, Fibrillin microfibril structure identifies long-range effects of inherited pathogenic mutations affecting a key regulatory latent TGFβ-binding site, *Nat. Struct. Mol. Biol.* 30 (5) (2023) 608–618.
- [40] G. Sengle, K. Tsutsui, D.R. Keene, S.F. Tufa, E.J. Carlson, N.L. Charbonneau, R. N. Ono, T. Sasaki, M.K. Wirtz, J.R. Samples, L.I. Fessler, J.H. Fessler, K. Sekiguchi, S.J. Hayflick, L.Y. Sakai, Microenvironmental regulation by fibrillin-1, *PLoS. Genet.* 8 (1) (2012) e1002425.
- [41] P.P. van Zuijlen, J.J. Ruurda, H.A. van Veen, J. van Marle, A.J. van Trier, F. Groenewelt, R.W. Kreis, E. Middelkoop, Collagen morphology in human skin and scar tissue: no adaptations in response to mechanical loading at joints, *Burns* 29 (5) (2003) 423–431.
- [42] L. Rittie, Method for picrosirius red-polarization detection of collagen fibers in tissue sections, *Methods Mol. Biol.* 1627 (2017) 395–407.
- [43] S. Ricard-Blum, G. Baffet, N. Theret, Molecular and tissue alterations of collagens in fibrosis, *Matrix. Biol.* 68–69 (2018) 122–149.
- [44] M.L. Tanzer, Cross-linking of collagen, *Science* (1979) 180 (4086) (1973) 561–566.
- [45] M. Yamauchi, Y. Taga, S. Hattori, M. Shiiba, M. Terajima, Analysis of collagen and elastin cross-links, *Methods Cell Biol.* 143 (2018) 115–132.

- [46] U. Lendahl, L. Muhl, C. Betsholtz, Identification, discrimination and heterogeneity of fibroblasts, *Nat. Commun.* 13 (1) (2022) 3409.
- [47] P. Chitturi, A. Leask, The role of positional information in determining dermal fibroblast diversity, *Matrix. Biol.* (2024).
- [48] E.R. Neptune, P.A. Frischmeyer, D.E. Arking, L. Myers, T.E. Bunton, B. Gayraud, F. Ramirez, L.Y. Sakai, H.C. Dietz, Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome, *Nat. Genet.* 33 (3) (2003) 407–411.
- [49] A.J. Engler, M.A. Griffin, S. Sen, C.G. Bonnemann, H.L. Sweeney, D.E. Discher, Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments, *J. Cell Biol.* 166 (6) (2004) 877–887.
- [50] A.G. Greene, S.B. Eivers, E.W.J. Dervan, C.J. O'Brien, D.M. Wallace, Lysyl Oxidase Like 1: biological roles and regulation, *Exp. Eye Res.* 193 (2020) 107975.
- [51] S. Chakravarti, T. Magnuson, J.H. Lass, K.J. Jepsen, C. LaMantia, H. Carroll, Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican, *J. Cell Biol.* 141 (5) (1998) 1277–1286.
- [52] D.V. Bax, Y. Mahalingam, S. Cain, K. Melody, L. Freeman, K. Younger, C. A. Shuttleworth, M.J. Humphries, J.R. Couchman, C.M. Kielty, Cell adhesion to fibrillin-1: identification of an Arg-Gly-Asp-dependent synergy region and a heparin-binding site that regulates focal adhesion formation, *J. Cell Sci.* 120 (8) (2007) 1383–1392.
- [53] G. Theodoridis, S. Drymoussi, A.P. Kao, A.H. Barber, D.A. Lee, K.M. Braun, J. T. Connelly, Type VI collagen regulates dermal matrix assembly and fibroblast motility, *J. Invest. Dermatol.* 136 (1) (2016) 74–83.
- [54] J. Manne, M. Markova, L.D. Siracusa, S.A. Jimenez, Collagen content in skin and internal organs of the tight skin mouse: an animal model of scleroderma, *Biochem. Res. Int.* 2013 (2013) 436053.
- [55] H. Yanagisawa, E.C. Davis, B.C. Starcher, T. Ouchi, M. Yanagisawa, J. A. Richardson, E.N. Olson, Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo, *Nature* 415 (6868) (2002) 168–171.
- [56] F.H. Silver, G.P. Seehra, J.W. Freeman, D. DeVore, Viscoelastic properties of young and old human dermis: a proposed molecular mechanism for elastic energy storage in collagen and elastin, *J. Appl. Polym. Sci.* 86 (8) (2002) 1978–1985.
- [57] N.L. Charbonneau, E.J. Carlson, S. Tufa, G. Sengle, E.C. Manalo, V.M. Carlberg, F. Ramirez, D.R. Keene, L.Y. Sakai, In vivo studies of mutant fibrillin-1 microfibrils, *J. Biol. Chem.* 285 (32) (2010) 24943–24955.
- [58] V. Deleuw, E. Carlson, M. Renard, K.D. Zientek, P.A. Wilmarth, A.P. Reddy, E. C. Manalo, S.F. Tufa, D.R. Keene, M. Olbinado, M. Stamparoni, S. Kanki, H. Yanagisawa, L.M. Mosquera, P. Sips, J. De Backer, L.Y. Sakai, Unraveling the role of TGFbeta signaling in thoracic aortic aneurysm and dissection using Fbn1 mutant mouse models, *Matrix Biol.* 123 (2023) 17–33.
- [59] J. Solon, I. Levental, K. Sengupta, P.C. Georges, P.A. Janmey, Fibroblast adaptation and stiffness matching to soft elastic substrates, *Biophys. J.* 93 (12) (2007) 4453–4461.
- [60] G. Delsante, P. Pietta, Epigenetics in autoimmune connective tissue diseases, *Acta Biomed.* 85 (2) (2014) 91–107.
- [61] K.M. Yamada, J.W. Collins, D.A. Cruz Walma, A.D. Doyle, S.G. Morales, J. Lu, K. Matsumoto, S.S. Nazari, R. Sekiguchi, Y. Shinsato, S. Wang, Extracellular matrix dynamics in cell migration, invasion and tissue morphogenesis, *Int. J. Exp. Pathol.* 100 (3) (2019) 144–152.
- [62] C.M. Kielty, Elastic fibres in health and disease, *Expert. Rev. Mol. Med.* 8 (19) (2006) 1–23.
- [63] E. Lazar, Z. Peterfi, G. Sirokmany, H.A. Kovacs, E. Klement, K.F. Medzihradsky, M. Geiszt, Structure-function analysis of peroxidase provides insight into the mechanism of collagen IV crosslinking, *Free Radic. Biol. Med.* 83 (2015) 273–282.
- [64] S.J. Chen, D.E. Birk, The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly, *Febs J.* 280 (10) (2013) 2120–2137.
- [65] B. Reinboth, E. Hanssen, E.G. Cleary, M.A. Gibson, Molecular interactions of biglycan and decorin with elastic fiber components: biglycan forms a ternary complex with tropoelastin and microfibril-associated glycoprotein 1, *J. Biol. Chem.* 277 (6) (2002) 3950–3957.
- [66] A. Hall, C. Cotoi, T.V. Luong, J. Watkins, P. Bhathal, A. Quaglia, Collagen and elastic fibres in acute and chronic liver injury, *Sci. Rep.* 11 (1) (2021) 14569.
- [67] D. Pilling, V. Vakil, N. Cox, R.H. Gomer, TNF-alpha-stimulated fibroblasts secrete lumican to promote fibrocyte differentiation, *Proc. Natl. Acad. Sci. USA* 112 (38) (2015) 11929–11934.
- [68] M. Pehrsson, J.H. Mortensen, T. Manon-Jensen, A.C. Bay-Jensen, M.A. Karsdal, M. J. Davies, Enzymatic cross-linking of collagens in organ fibrosis - resolution and assessment, *Expert. Rev. Mol. Diagn.* 21 (10) (2021) 1049–1064.
- [69] N. Chaudhari, A.D. Findlay, A.W. Stevenson, T.D. Clemons, Y. Yao, A. Joshi, S. Sayyar, G. Wallace, S. Rea, P. Toshniwal, Z. Deng, P.E. Melton, N. Hortin, K. S. Iyer, W. Jarolimek, F.M. Wood, M.W. Fear, Topical application of an irreversible small molecule inhibitor of lysyl oxidases ameliorates skin scarring and fibrosis, *Nat. Commun.* 13 (1) (2022) 5555.
- [70] H. Yin, N. Liu, X. Zhou, J. Chen, L. Duan, The advance of CCN3 in fibrosis, *J. Cell Commun. Signal.* 17 (4) (2023) 1219–1227.
- [71] Y. Yamaguchi, Perioitin in skin tissue and skin-related diseases, *Allergol. Int.* 63 (2) (2014) 161–170.
- [72] J.Y. Chi, Y.W. Hsiao, H.Y. Liang, T.H. Huang, F.W. Chen, C.Y. Chen, C.Y. Ko, C. C. Cheng, J.M. Wang, Blockade of the pentraxin 3/CD44 interaction attenuates lung injury-induced fibrosis, *Clin. Transl. Med.* 12 (11) (2022) e1099.
- [73] S. Nassari, D. Duprez, C. Fournier-Thibault, Non-myogenic contribution to muscle development and homeostasis: the role of connective tissues, *Front. Cell Dev. Biol.* 5 (2017).
- [74] S.S. Ahmad, K. Ahmad, E.J. Lee, Y.-H. Lee, I. Choi, Implications of insulin-like growth factor-1 in skeletal muscle and various diseases, *Cells* 9 (8) (2020) 1773.
- [75] Z. Garoufalia, A. Papadopetraki, E. Karatza, D. Vardakostas, A. Philippou, G. Kouraklis, D. Mantas, Insulin-like growth factor-I and wound healing, a potential answer to non-healing wounds: a systematic review of the literature and future perspectives, *Biomed. Rep.* 15 (2) (2021).
- [76] S.E. Tollefsen, R. Lajara, R.H. McCusker, D.R. Clemmons, P. Rotwein, Insulin-like growth factors (IGF) in muscle development. Expression of IGF-I, the IGF-I receptor, and an IGF binding protein during myoblast differentiation, *J. Biol. Chem.* 264 (23) (1989) 13810–13817.
- [77] R.J. Waldemer-Streyer, D. Kim, J. Chen, Muscle cell-derived cytokines in skeletal muscle regeneration, *FEBS J.* 289 (21) (2022) 6463–6483.
- [78] M.V. Plikus, X. Wang, S. Sinha, E. Forte, S.M. Thompson, E.L. Herzog, R.R. Driskell, N. Rosenthal, J. Biernaskie, V. Horsley, Fibroblasts: origins, definitions, and functions in health and disease, *Cell* 184 (15) (2021) 3852–3872.
- [79] I. Kim, A. Ghosh, N. Bundschuh, L. Hinte, E. Petrosyan, F. von Meyenn, O. Bar-Nur, Integrative molecular roadmap for direct conversion of fibroblasts into myocytes and myogenic progenitor cells, *Sci. Adv.* 8 (14) (2022) eabj4928.
- [80] N. Naldaiz-Gastesi, M. Goicoechea, S. Alonso-Martin, A. Aiausti, M. Lopez-Mayorga, P. Garcia-Belda, J. Lacalle, C. San Jose, M.J. Arauzo-Bravo, L. Trouilh, V. Anton-Leberre, D. Herrero, A. Matheu, A. Bernad, J.M. Garcia-Verdugo, J. J. Carvajal, F. Relaix, A. Lopez de Munain, P. Garcia-Parra, A. Izeta, Identification and characterization of the dermal panniculus carnosus muscle stem cells, *Stem Cell Rep.* 7 (3) (2016) 411–424.
- [81] S. Goldstein, E.J. Moerman, R.C. Baxter, Accumulation of insulin-like growth factor binding protein-3 in conditioned medium of human fibroblasts increases with chronologic age of donor and senescence in vitro, *J. Cell Physiol.* 156 (2) (1993) 294–302.
- [82] L. Micutkova, T. Diener, C. Li, A. Rogowska-Wrzesinska, C. Mueck, E. Huetter, B. Weinberger, B. Grubeck-Loebenstein, P. Roepstorff, R. Zeng, P. Jansen-Duerr, Insulin-like growth factor binding protein-6 delays replicative senescence of human fibroblasts, *Mech. Ageing Dev.* 132 (10) (2011) 468–479.
- [83] N.S. Reyes, M. Krasnikov, N.C. Allen, J.Y. Lee, B. Hyams, M. Zhou, S. Ravishanker, M. Cassandras, C. Wang, I. Khan, P. Matatia, Y. Johmura, A. Molofsky, M. Matthey, M. Nakanishi, D. Sheppard, J. Campisi, T. Peng, Sentinel p16(INK4a+) cells in the basement membrane form a reparative niche in the lung, *Science* (1979) 378 (6616) (2022) 192–201.
- [84] P. Strzyz, Senescent fibroblasts support lung repair, *Nat. Rev. Mol. Cell Biol.* 23 (12) (2022) 775.
- [85] Y. Liu, A. Keikhosravi, G.S. Mehta, C.R. Drifka, K.W. Eliceiri, Methods for quantifying fibrillar collagen alignment, *Methods Mol. Biol.* 1627 (2017) 429–451.
- [86] J.S. Bredfeldt, Y. Liu, C.A. Pehlke, M.W. Conklin, J.M. Szulzewski, D.R. Inman, P. J. Keely, R.D. Nowak, T.R. Mackie, K.W. Eliceiri, Computational segmentation of collagen fibers from second-harmonic generation images of breast cancer, *J. Biomed. Opt.* 19 (1) (2014) 16007.
- [87] A. Sada, F. Jacob, E. Leung, S. Wang, B.S. White, D. Shalloway, T. Tumber, Defining the cellular lineage hierarchy in the interfollicular epidermis of adult skin, *Nat. Cell Biol.* 18 (6) (2016) 619–631.
- [88] E. Raja, G. Changarathil, L. Oinam, J. Tsunozumi, Y.X. Ngo, R. Ishii, T. Sasaki, K. Imanaka-Yoshida, H. Yanagisawa, A. Sada, The extracellular matrix fibulin 7 maintains epidermal stem cell heterogeneity during skin aging, *EMBO Rep.* 23 (12) (2022) e55478.
- [89] K. Uto, S.S. Mano, T. Aoyagi, M. Ebara, Substrate fluidity regulates cell adhesion and morphology on poly(epsilon-caprolactone)-based materials, *ACS. Biomater. Sci. Eng.* 2 (3) (2016) 446–453.
- [90] K. Tarnutzer, D. Siva Sankar, J. Dengjel, C.Y. Ewald, Collagen constitutes about 12% in females and 17% in males of the total protein in mice, *Sci. Rep.* 13 (1) (2023) 4490.
- [91] Y. Taga, K. Tanaka, C. Hamada, M. Kusubata, K. Ogawa-Goto, S. Hattori, Hydroxyhomocitrulline Is a Collagen-Specific Carbamylation Mark that Affects Cross-link Formation, *Cell Chem. Biol.* 24 (10) (2017) 1276–1284, e3.
- [92] M. Boutin, I. Ahmad, M. Jauhainen, N. Lachapelle, C. Rondeau, J. Roy, P. Thibault, NanoLC-MS/MS analyses of urinary desmosine, hydroxyllysylpyridinoline and lysylpyridinoline as biomarkers for chronic graft-versus-host disease, *Anal. Chem.* 81 (22) (2009) 9454–9461.
- [93] Y. Yamashiro, C.L. Papke, J. Kim, L.J. Ringuette, Q.J. Zhang, Z.P. Liu, H. Mirzaei, J. E. Wagenseil, E.C. Davis, H. Yanagisawa, Abnormal mechanosensing and cofilin activation promote the progression of ascending aortic aneurysms in mice, *Sci. Signal.* 8 (399) (2015) ra105.
- [94] U. Licht, J. Anders, S.H. Yuspa, Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice, *Nat. Protoc.* 3 (5) (2008) 799–810.
- [95] D. Correa-Gallegos, H. Ye, B. Dasgupta, A. Sardogan, S. Kadri, R. Kandi, R. Dai, Y. Lin, R. Kopplin, D.S. Shenai, J. Wannemacher, R. Ichijo, D. Jiang, M. Strunz, M. Ansari, I. Angelidis, H.B. Schiller, T. Volz, H.G. Machens, Y. Rinkevich, CD201(+) fascia progenitors choreograph injury repair, *Nature* 623 (7988) (2023) 792–802.
- [96] Y. Zhou, B. Zhou, L. Pache, M. Chang, A.H. Khodabakhshi, O. Tanaseichuk, C. Benner, S.K. Chanda, Metascape provides a biologist-oriented resource for the analysis of systems-level datasets, *Nat. Commun.* 10 (1) (2019) 1523.
- [97] Y. Yamashiro, B.Q. Thang, K. Ramirez, S.J. Shin, T. Kohata, S. Ohata, T.A. V. Nguyen, S. Ohtsuki, K. Nagayama, H. Yanagisawa, Matrix mechanotransduction mediated by thrombospondin-1/integrin/YAP in the vascular remodeling, *Proc. Natl. Acad. Sci. USA* 117 (18) (2020) 9896–9905.