

**Proteomic profiling of *TGFBI*-null mouse corneas reveals only minor changes in matrix composition supportive of *TGFBI* knockdown as therapy against *TGFBI*-linked corneal dystrophies**

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Abbreviations: ECM, extracellular matrix; iTRAQ, isobaric tags for relative and absolute quantitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; *POSTN*, periostin gene; SRM; selected reaction monitoring, TEM, transmission electron microscopy; *TGFBI*, transforming growth factor beta-induced protein

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## Abstract

*TGFBIp* is a constituent of the extracellular matrix in many human tissues including the cornea, where it is one of the most abundant proteins expressed. *TGFBIp* interacts with Type I, II, IV, VI and XII collagens as well as several members of the integrin family, suggesting it plays an important role in maintaining structural integrity and possibly corneal transparency as well. Significantly, more than 60 point mutations within the *TGFBI* gene have been reported to result in aberrant *TGFBIp* folding and aggregation in the cornea, resulting in severe visual impairment and blindness. Several studies have focused on targeting *TGFBIp* in the cornea as a therapeutic approach to treat *TGFBI*-linked corneal dystrophies, but the effect of this approach on corneal homeostasis and matrix integrity remained unknown. In the current study, we evaluated the histological and proteomic

profiles of corneas from *TGFBI*-deficient mice as well as potential redundant functions of the paralogous protein POSTN. The absence of TGFBIp in mouse corneas did not grossly affect the collagen scaffold, and POSTN is unable to compensate for loss of TGFBIp. Proteomic comparison of wildtype and *TGFBI*<sup>-/-</sup> mice revealed 11 proteins were differentially regulated, including Type VI and XII collagens. However, as these alterations did not manifest at the macroscopic and behavioral levels, these data support partial or complete *TGFBI* knockdown as a potential therapy against *TGFBI*-linked corneal dystrophies. Lastly, *in situ* hybridization verified *TGFBI* mRNA in the epithelial cells but not in other cell types, supportive of a therapy directed specifically at this lineage.

## Introduction

Visual impairment has a negative impact on quality of life and is estimated to affect approximately 285 million people worldwide [1]. While some visual disorders such as myopia can be treated noninvasively by corrective lenses, other diseases require surgical intervention. The latter category includes 5q31-linked transforming growth factor beta-induced (*TGFBI*) corneal dystrophies, a heterogeneous group of genetic disorders in which mutations within the *TGFBI* gene result in a progressive accumulation and aggregation of the secreted extracellular TGFBI protein (TGFBIp, UniProt entry Q15582) in the cornea [2]. Treatment of *TGFBI*-linked corneal dystrophies relies on surgical removal of the affected area using laser photoablation or ultimately replacing the affected cornea with a donor cornea. However, donor shortage and the recurrence of protein accumulation indicate the need to develop new treatment strategies.

Several studies have suggested that downregulating corneal TGFBIp expression may be a way to treat dystrophic patients, using either siRNA-based methods [3-5] or drugs targeting the molecular pathways involved in TGFBIp expression [6]. However, the practical aspects of drug delivery and the potential side effects of the partial or complete elimination of one of the most abundant proteins in the human cornea remain unclear [7]. In addition, TGFBIp is known to interact with a wide range of extracellular macromolecules, including the proteoglycans decorin and biglycan [8] and Type I, II, IV, VI and XII collagens [9-12], and is thought to link cells to the extracellular matrix (ECM) through a number of integrin binding sites [13-19]. Additionally, TGFBIp is considered a matricellular protein, meaning that TGFBIp is rapidly turned over and could play a regulatory role via sequestration and modulation of specific growth factors [20]. Thus, changing the expression level of TGFBIp in

the cornea may have unforeseen consequences on the structural integrity of the cornea and aggravate the disease condition.

In this study, we investigated the role of TGFBIp in maintaining the structural integrity of corneal cells and the collagenous matrix using a *TGFBI*-deficient mouse model (*TGFBI*<sup>-/-</sup>). A histological and ultrastructural examination demonstrated that TGFBIp deficiency has little to no effect on the gross structure of the cornea, which remained transparent, suggesting the existence of functional redundancy. Comparing the corneal proteomes of wildtype (*TGFBI*<sup>+/+</sup>) and *TGFBI*<sup>-/-</sup> mice resulted in the identification of 11 differentially expressed proteins; including Type VI and XII collagens, annexin A2, fibromodulin and fibulin-5 which are all known for their roles in maintaining corneal structural integrity. Thus, even though expression changes were observed at the molecular level, the macroscopic, microscopic and ultrastructural appearance of the *TGFBI*<sup>-/-</sup> mouse cornea was unaffected, indicating that partial or complete *TGFBI* knockdown would be a feasible treatment option for *TGFBI*-linked corneal dystrophies.

## Results

### Corneal origin of TGFBIp expression

Systemic *TGFBI*-null mice were generated as previously described [21], and these mice are fully viable, fertile and indistinguishable from wildtype littermates. The corneal transparency and mouse behavior remained unaffected throughout adulthood, suggesting that *TGFBI*<sup>-/-</sup> mice have normal vision. To verify that TGFBIp protein deposition is completely absent in adult *TGFBI*<sup>-/-</sup> mouse corneas, we examined *TGFBI* mRNA and TGFBIp protein expression using *in situ* hybridization and immunoblotting (Figs. 1, 2). *In situ* hybridization demonstrated that *TGFBI* mRNA is specifically expressed within the corneal epithelium (Fig. 1A) and not seen in the stromal embedded keratocytes nor in the endothelium. Moreover, *in situ* hybridization confirmed that *TGFBI* mRNA is absent in *TGFBI*<sup>-/-</sup> mouse eyes (negative data not shown). Immunoblotting verified that TGFBIp was undetectable in the corneal tissue of *TGFBI*<sup>-/-</sup> mice and that a single 68-kDa TGFBIp band was present in isolated wildtype adult corneas (Fig. 2C). In addition, no obvious changes in the total protein expression profile were observed between *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneal tissues subjected to SDS-PAGE followed by Coomassie brilliant blue staining (Fig. 2B), indicating that there were no major alterations in protein expression in *TGFBI*<sup>-/-</sup> mice.

### **POSTN is located at the corneal periphery**

Additionally, we sought to determine if *periostin* (*POSTN*), a closely related gene family member of *TGFBI* [22], was similarly expressed. Although *POSTN* is widely expressed in adult organs under normal and pathological conditions [23], wildtype mouse corneas do not express *POSTN* mRNA. Using both *in situ* hybridization and real-time PCR data, we found that *POSTN* mRNA is absent in adult corneas (Figs. 1A, B), and thus is not co-expressed with *TGFBI*. Likewise, immunohistochemistry revealed that *POSTN* protein is deposited at the corneal periphery, where ciliary body/scleral venous sinus are (Fig. 1C). As the same degree of *POSTN* deposition was observed for wildtype and *TGFBI*-deficient mouse corneas, this also suggests a non-compensatory role for *POSTN*. The apparent discrepancy between site of *POSTN* mRNA expression and protein deposition indicates that corneal surrounding tissues are responsible for active *POSTN* expression, which then translocates to the corneal periphery. This is supported by a previous study showing that human *POSTN* is expressed by limbal stem cells which differentiate into the corneal epithelium [24].

### **Gross structure of the *TGFBI*<sup>-/-</sup> cornea appears normal**

To assess major effects of *TGFBI* knockout in mouse corneas, tissue sections of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas were histologically compared (Fig. 2). No significant structural changes were observed in the stroma, or the Descemet's membrane. Likewise, the epithelial and endothelial cell layers appeared unaffected in the *TGFBI*<sup>-/-</sup> corneas. Therefore, we conclude from these data that the absence of TGFBIp does not affect the gross architecture of the corneal structure.

### **Proteomic analysis of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas**

The histological observations described above led us to hypothesize that either TGFBIp is not essential for maintaining the structure of the developed cornea or functional redundancy exists due to the expression of other proteins to compensate for the lack of TGFBIp. To test these alternatives, a comparative analysis of the *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> mouse cornea proteomes was performed. The iTRAQ MS analysis resulted in the quantification of 877 proteins across all datasets, of which 516 proteins were quantified in all three biological samples of both the *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> groups (Figs. 3 and Table SI). Of the 516 proteins quantified in all samples, 11 were significantly mis-regulated using a significance threshold of 0.01 and a fold change of at least 0.5 on the log<sub>2</sub> scale (Table 1, Fig. 3).

## **POSTN does not compensate for TGFBIp deficiency in the cornea**

Of note, even though POSTN was above the significance threshold of 0.01 ( $p = 0.3$ ), the protein was upregulated in *TGFBI*<sup>-/-</sup> mice, with a log<sub>2</sub> fold change of 1.9 in the iTRAQ MS analysis (Table S1). Although POSTN is a TGFBIp paralog that exhibits high sequence similarity, and the two are often co-expressed, we show that *POSTN* mRNA is absent within the wildtype cornea (Fig. 1A, B). Increased POSTN protein levels in *TGFBI*<sup>-/-</sup> mice would support the idea that one or more proteins could compensate for the functional loss of TGFBIp. To test this, we performed a targeted quantitative selected reaction monitoring (SRM) analysis of POSTN in the three biological *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> replicates in each group (Fig. 4). However, the LC-SRM-MS analysis supported the iTRAQ data and immunohistochemical staining and revealed no significant upregulation of POSTN protein in the *TGFBI*<sup>-/-</sup> mice, thus disproving the hypothesis that POSTN is upregulated to compensate for the lack of TGFBIp in the *TGFBI*<sup>-/-</sup> corneas.

## **TGFBIp absence does not affect corneal ultra-structure**

The macroscopic appearance and animal behavior of the *TGFBI*<sup>-/-</sup> mice seem normal, although the proteomic analysis indicates several changes in expression of ECM proteins known to be expressed and involved in corneal stroma collagenous matrix organization. Consequently, we analyzed potential ultrastructural differences between *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> mouse corneas using transmission electron microscopy (TEM), to quantify subepithelial collagen fibril diameters and interfibrillar spacing (Fig. 5). Collagen fibril diameters were similar for both *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas (mean: 30±3 vs. 31±3nm). However, the mean values of the center-to-center interfibrillar spacing were 58±10nm and 62±11nm in the *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas, respectively. This supports the proteomic data and indicates that the collagenous matrix organization is marginally affected in *TGFBI*<sup>-/-</sup> corneas, resulting in a less densely packed collagen fibril corneal arrangement.

## **Humans express elevated corneal TGFBIp amounts compared to mouse**

Unpublished observations suggest differential expression of TGFBIp between species. Mice appear to express substantial lower corneal levels of TGFBIp compare to human. In order to determine the degree of expression levels we performed a relative label-free MS1 quantification between corneal TGFBIp in humans and mice. This analysis showed that human corneal TGFBIp levels exceed mice by more than 10-fold (Table S2). Thus,

completely removing TGFBIp as a therapy against corneal dystrophies in humans may result in a larger effect on the protein expression profile than observed in mice mutants.

## Discussion

### TGFBIp is a major constituent of adult cornea

We have previously demonstrated that TGFBIp is one of the most abundant proteins in the human cornea [7]. We have also shown that TGFBIp and POSTN can exhibit both overlapping and mutually exclusive gene expression patterns [22], and that *TGFBI* null mice are viable and live a normal lifespan [21]. Our expression analysis (Fig. 1) revealed that only TGFBIp is robustly expressed in the adult corneal epithelium, suggesting continual secretion and deposition of TGFBIp from the epithelium into the corneal stroma. Additionally, we show that it is unlikely that the TGFBIp paralogue, POSTN, is able to compensate for the systemic loss of *TGFBI* (Fig. 1). However, future analysis of viable double *TGFBI:POSTN* knockout mice [25], will be helpful in determining whether low level corneal POSTN, as observed in both wildtype and *TGFBI*<sup>-/-</sup> corneas via iTRAQ MS analysis (Fig. 3), is sufficient to maintain normal corneal morphology and definitively conclude whether TGFBIp and POSTN indeed share any redundant and/or parallel functions in the adult cornea.

### Preservation of corneal integrity in *TGFBI*<sup>-/-</sup> mice

Studies using mouse models lacking proteins important for proper corneal collagen architecture have demonstrated changes to the cornea including stromal thickness, disorganization of collagen fibrils and loss of transparency [26-29]. TEM of corneas from *keratocan*-null mice showed less organized collagen fibers than those of wildtype mice [27]. Corneas of *lumican*-null mice exhibited major changes in expression of ECM proteins when analyzed by a similar quantitative MS approach as performed in this study [28]. Severe disruption in collagen fibril structure and organization was observed in the corneas of a *decorin* and *biglycan* double-deficient mouse model [29], whereas fibromodulin was shown to be involved in and affect collagen fibrillogenesis in the peripheral cornea in *fibromodulin*-deficient mice [26]. As TGFBIp binds several ECM proteins involved in collagen organization, it was anticipated that knocking out *TGFBI* would introduce changes in the collagenous matrix similar to that observed for *keratocan*-, *lumican*-, *decorin/biglycan*-, or *fibromodulin*-deficient mice. However, our examination of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> mice did not reveal structural changes in the corneas of *TGFBI*-null mice. This is in agreement with observations

of the corneas from another *TGFBI*-deficient mouse model, where no histological changes or differences in animal development or behavior were observed [30].

In addition to histological examination, our analysis of the ultrastructure using TEM only showed minor changes in collagen fibril diameter and interfibrillar spacing. This is in sharp contrast to the profound changes in the arrangement of collagen fibrils observed in the *keratocan*-, *lumican*-, *decorin/biglycan*- and *fibromodulin*-null mouse corneas [26-29]. However, the small changes in interfibrillar spacing seen in our TEM data support the findings in our quantitative proteomic analysis, which identified 11 proteins as being differentially expressed in the *TGFBI*-deficient mice (Table 1). Six of these proteins are known to be involved in the assembly of the collagen scaffold and cell adhesion to the ECM. The exact functional consequences of this expression profile alteration remain unclear, but it is likely that TGFBIp functions as a regulatory mediator between the ECM and the embedded corneal cell lineages. Whether TGFBIp directly interacts with these mis-expressed ECM components and if they could themselves contribute to altered TGFBIp protein aggregation will be key in the development of therapeutic agents capable of dissolving and/or inhibiting the formation of mutant TGFBIp aggregates in the cornea. The recently published high resolution crystal structure of TGFBIp [31] (PDB entry: 5NV6) will provide new insight into the functional role of TGFBIp and its interaction to the extracellular matrix and may be a help to interpret and link to the differential expressed proteins identified in this study.

The TGFBIp paralog POSTN is well characterized as a matricellular protein [32], a term that may also be applied to TGFBIp. Matricellular proteins are a diverse group of non-structural molecules present in the ECM that are vital for maintaining the integrity of the ECM in health and disease. In addition to being important components of the ECM itself, matricellular proteins also play crucial regulatory roles and can modulate cellular responses to extracellular signals induced during tissue remodeling and repair as well as in numerous disease states [33]. Interestingly, matricellular protein-deficient mice models often show mild changes in phenotypic appearance [33], which correlates well with the observations of the mouse model presented in the current study.

### **Differential expression of collagen scaffold proteins**

The cornea accounts for approximately 70% of the refractive power of the eye and together with the aqueous humor and the lens protects the retina from UV light [34, 35]. Corneal transparency is dependent on the highly ordered collagen network found in the stroma

comprising approximately 90% of the corneal thickness. The corneal collagen network consists of approximately 200 interwoven lamellae, and each lamella consists of collagen Types I and V heterotypic fibrils arranged in a hexagonal pattern [36]. Collagen Types VI, XII and XIV together with the proteoglycans decorin, lumican, keratocan, mimecan, biglycan and fibromodulin provide structural support for proper collagen fibril assembly and spacing [37]. Notably, Types VI and XII collagens were upregulated in our LC-MS/MS analysis, and both collagen types have previously been shown to covalently bind TGFBIp [9, 12]. This suggests that one of the important physiological roles of TGFBIp is to bind Type VI and XII collagen and thereby participate in matrix organization and regulation of collagen constituent composition. TGFBIp was found to be upregulated in the cartilage of a collagen *Type IX*-deficient mouse model [38]. Both Type IX and XII collagens belong to the FACIT (Fibril Associated Collagens with Interrupted Triple helices) collagen family and may therefore share similar functionalities. In addition to increased levels of TGFBIp in the cartilage of the *Type IX collagen*-deficient mouse, a change in TGFBIp degradation was observed. This is noteworthy because corneal TGFBIp is similarly proteolytically processed [39-41], implying that TGFBIp degradation is sensitive to changes in its matrix environment.

In addition to increased expression of Type VI collagen, we observed annexin A2 upregulation. Annexin A2 has been suggested to mediate secretion of Type VI collagen in epithelial cells [42], and we found that these two proteins were upregulated to a comparable extent, supporting a similar connection in our mouse mutant model.

Furthermore, our iTRAQ analysis indicated highest upregulation of fibromodulin, one of the proteoglycans involved in accurate matrix assembly. Fibromodulin is expressed in the developing cornea but is located only peripherally in the mature cornea [26]. Even though no interaction between fibromodulin and TGFBIp has been reported in the literature, the fibromodulin homologs decorin and biglycan have both been shown to bind TGFBIp in a non-covalent manner [8]. Hence, fibromodulin may possess unappreciated binding properties similar to those of decorin and biglycan. In our previous quantitative analysis of corneal proteins, decorin and biglycan are among the most abundant proteins in the corneal stroma and endothelium [7]. Moreover, TGFBIp has been shown *in vitro* to enhance the effect of biglycan and decorin on Type VI collagen aggregation, and *decorin/biglycan* double-deficient mice display major defects in collagen fibril morphology [29]. However, decorin and biglycan interactions with TGFBIp may be less pronounced in the mouse cornea, as neither protein was represented on our list of differentially regulated proteins (Table 1), and no major alterations in collagen fibril diameter or spacing was observed in the *TGFBI*-deficient mouse corneas, as indicated by our TEM analysis.

Prolargin, like lumican, decorin and biglycan, is a small leucine-rich repeat proteoglycan. Prolargin has been shown to be a major proteoglycan of both the sclera and the cornea [7, 43] and can bind Type I collagen and function as an anchor between the ECM and basement membranes. Thus, the fact that *TGFBI* knockout results in an upregulation of prolargin in the mouse cornea suggests that TGFBIp may play a role in anchoring the stromal collagen scaffold to the epithelium basement membrane.

Fibulin-5 has been shown to be important for elastic fiber organization but does not affect collagen assembly [44], and we have previously identified it as one of the more abundant proteins in the Descemet's membrane [45] and endothelial layer in the cornea [7]. It contains the RGD integrin-binding motif that is also found in TGFBIp [46]. This motif mediates binding to cells; hence, the significant fibulin-5 upregulation we observed may act to compensate for loss of TGFBIp cell adhesion. Whether fibulin-5 is able to form a complex with TGFBIp via the RGD motif and why fibulin-5 is upregulated in the absence of TGFBIp may be important for our understanding of normal cell adhesion in the cornea.

### ***TGFBI* knockdown as a therapeutic treatment in corneal dystrophies**

The genetic origins of *TGFBI*-associated corneal dystrophies are heterogeneous, with new disease-causing mutations being reported on a regular basis. Therefore, a therapeutic strategy with a broad effect is desirable. This strategy could rely on suppression of the disease-causing protein. Several groups have addressed this using *ex vivo* and *in vitro* systems. Courtney *et al.* used siRNA targeting only the mutant allele of *TGFBI* in heterozygous primary corneal epithelial cells from a patient with lattice corneal dystrophy [3]. As this allele-specific strategy only knocked down the mutant protein, the patient still expressed wildtype TGFBIp in the cornea and therefore presumably exhibited a non-affected cornea. The same group later demonstrated the use of mutant allele-specific suppression of keratin 12 using CRISPR/Cas9 in a model of Meesmann's epithelial corneal dystrophy. They concluded that a similar approach may be applicable to a substantial number of the *TGFBI* mutations reported to date [47]. However, the allele-specific approach can only be applied to heterozygous patients, whereas for homozygous patients, complete *TGFBI* knockdown will be required. Combined, our data indicate that it is possible to maintain structural integrity and to sustain protein expression following a complete removal of TGFBIp in the mouse cornea. This opens up possibilities for therapeutic strategies relying on complete elimination of TGFBIp. Yuan *et al.* and Yellore *et al.* both address this possibility using siRNA for total *TGFBI* knockdown in human corneal cell lines [4, 5], whereas Choi *et al.* used lithium treatment on corneal fibroblasts to inhibit TGFBIp expression [6]. In the latter case, lithium

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treatment affected the upstream signaling pathway involved in TGFBIp expression, which likely affects the expression of many other proteins including TGFBIp, giving rise to undesirable side-effects. Another consideration in the design of therapies, is which corneal cell to target for TGFBIp suppression or knockdown to achieve the desired effect. Our *in situ* hybridization data (Fig. 1) show that *TGFBI* mRNA is restricted to the corneal epithelium and does not appear to be transcribed by the corneal keratocytes embedded in the stroma or in the innermost endothelial layer of the cornea. This suggests that generation of a therapy against *TGFBI*-related corneal dystrophies can be achieved by targeting only the corneal epithelium, which in terms of therapeutic delivery would most likely be less complicated than targeting all corneal cell types. Of note, Allaman-Pillet *et al.* showed that corneal TGFBIp was mainly present in the corneal stroma [30], suggesting that TGFBIp is expressed and secreted by the epithelium and diffuses throughout the underlying cornea. This may also partly explain why the epicenter of TGFBIp deposition in the corneal stroma appears to be different depending on the site and type of mutation in the *TGFBI* gene [48]. Recently, Liu *et al.* using quantitative PCR showed *TGFBI* mRNA expression in human keratocytes [49], however the authors did not compare mRNA expression levels between corneal cell types and the contribution by keratocytes may be significant lower than seen for the epithelium as supported from our *in situ* hybridization data. Our unpublished observation of human corneas maintained in culture medium where the epithelial layer has detached, shows unusual low levels of TGFBIp in the stroma when analyzed by mass spectrometry than normally seen for corneas without pre-detachment of the epithelium. Hence, all together we interpret these data as that the majority of TGFBIp originate from the corneal epithelium. This assumption is also supported by *in situ* hybridization experiments on rabbit corneas only detecting *TGFBI* mRNA in the epithelium [50]. Lastly, it should be noted with care that corneal TGFBIp expression levels in humans and mice differ substantially. The relative comparison between TGFBIp expression levels shows that it is 10-fold more abundant in humans than in mice (Table S2), which may amplify the minor changes observed in ECM arrangement in our *TGFBI*-null mouse to a more severe situation in humans.

Several potential non-invasive therapeutic strategies to treat *TGFBI*-linked corneal dystrophy have been published, all relying on partial or complete knockdown of TGFBIp without considering the consequences of this on corneal homeostasis and integrity. In this study, we examined TGFBIp expression and characterized the gross structural and proteomic changes in mice deficient in TGFBIp compared to wildtype mice. Our data revealed only minor changes in the structure of the corneas in the *TGFBI*-null mice. In addition, the expression levels of only a few proteins were affected upon removal of TGFBIp. Hence, these data

suggest that total TGFBIp knockdown in corneas is a viable therapeutic strategy for the treatment of *TGFBI*-linked dystrophic patients.

## Experimental Procedures

### Transgenic mice

Mice with null genetic deletions of *POSTN* or *TGFBI* were generated and genotyped as previously described [21, 51]. After euthanization, eyes from *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> adult mice (8-10 weeks) and *POSTN*<sup>-/-</sup> adult (8-10 weeks) were harvested in cold phosphate-buffered saline (PBS) and either placed immediately in liquid nitrogen or fixed in 4% paraformaldehyde or 4% paraformaldehyde/2% glutaraldehyde. Equal ratios of male and female mice were used in all experiments, and the mice are maintained on a C57BL/6J background. The mice were housed in standard barrier rack cages supplied with Purina Rodent Chow 5001 with automatic watering dispensers. All procedures were performed with the approval of the Institutional Animal Care and Use Committee at Indiana University School of Medicine (Indianapolis, IN; protocol 10807).

### RNA Isolation and Quantitative PCR

mRNA from micro-dissected 10 week old adult wildtype corneas (n = 6 corneas from 3 mice) was isolated using RNeasy (Qiagen, Venlo, The Netherlands) kit. To compare *TGFBI* and *POSTN* mRNA expression levels within isolated cornea, we used quantitative PCR (qPCR) on cDNA synthesized using a Superscript-II kit (Invitrogen, Waltham, MA). cDNA was amplified within the linear range using primers (*TGFBI* forward 5'-ACCATGGACCGGATGTTGAC-3' and reverse 5'-GGCCACCAGCATGCTAAAAC-3'; *POSTN* forward 5'-AAGGAAAAGGGTCATACACGTACTIONTTC-3' and reverse 5'-CCTCTGCGAATGTCAGAATCC-3' from Qiagen) and normalized to *GAPDH* (PPM02946E-200), as described [52]. qPCR reactions were carried out using SyberGreenER (Invitrogen) and run on the same plate. All qPCR results are representative of three separate experiments (n = 3) and  $\Delta\Delta C_t$  based fold-changes were calculated using *GAPDH* as a housekeeping standard.

### ***In situ* hybridization and Immunohistochemistry**

Murine 8-10 week old eyes were harvested, fixed in 4% paraformaldehyde overnight at 4°C, dehydrated through a graded ethanol series, cleared in xylenes, and processed for paraffin embedding and sectioning at 10µm thickness. Sense and anti-sense [<sup>35</sup>S]UTP-radiolabeled *TGFBI* and *POSTN* cDNA probes were transcribed for *in situ* hybridization, as previously described [22]. Following de-waxing and *in situ* analysis, a specific signal pattern was observed only with the anti-sense probe and was detected in at least three consecutive wildtype eye sections (n = 3). Subsequently, following de-waxing, whole wildtype, *TGFBI*<sup>-/-</sup> and *POSTN*<sup>-/-</sup> eye sections were used to examine POSTN protein deposition. POSTN was detected using a rabbit polyclonal antibody (1:10,000 dilution) and ABC kit (Vectorstain, Burlingame, CA) with DAB and hydrogen peroxide as a chromogen, as described [53]. Serial sections probed with the POSTN antibody were examined for signal detection in consecutive sections in at least three eyes of each genotype (n = 3 per genotype).

### **SDS PAGE**

Equal amounts of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneal tissue were boiled for 10-min in SDS sample buffer under reducing condition (5mM dithiothreitol), and separated on 5-15% (w/v) gradient polyacrylamide sodium dodecyl sulfate gels, cast in-house [54].

### **Western blotting**

The SDS-PAGE gels were equilibrated with blotting buffer consisting of 10mM CAPS, pH 11, 10% methanol (VWR chemicals) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA) by electroblotting at 0.5A for 15min. The membranes were blocked using 5% milk (Milex 240) in TBS-T for an hour at 23°C followed by incubation overnight at 4°C with rabbit polyclonal anti-TGFBIp serum [12] diluted 1:10,000 in 2% skim milk TBS-T. The following day, the membrane was washed three times in TBS-T before incubation with secondary antibodies against rabbit (peroxidase conjugated anti-rabbit IgG, Sigma Aldrich, Missouri, US) diluted 1:25,000 in 2% skim milk TBS-T for two hours at 23°C. Finally, the membrane was developed using Amersham Hyperfilm and the enhanced chemiluminescence (ECL) kit (GE healthcare Life Sciences, Chalfont, UK).

### **Light microscopy**

Formaldehyde-fixed and paraffin-embedded *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneal tissue sections 7µm thick were cut perpendicular to the epithelium. The sections were deparaffinized and

stained with toluidine blue using standard histological procedures. Histological examinations (n = 6 eyes from each genotype) were performed using light microscopy.

### **Preparation of corneas for mass spectrometry**

Eyes from *TGFBI*<sup>+/+</sup> control mice and *TGFBI*<sup>-/-</sup> mice (n = 6 eyes from each genotype) were removed and immediately frozen in liquid nitrogen. The corneas were subsequently excised using a 2mm biopsy needle (Miltex, Tuttlingen, Germany) and washed three times in 200 $\mu$ L of ice cold PBS and once in 1mL of PBS. The corneas from two mice were pooled, resulting in three biological replicates within each group, and lyophilized in a Speed-Vac concentrator (Thermo Scientific, Waltham, MA) for 18h. Proteins were then chemically cleaved by adding 100 $\mu$ L of 0.66M CNBr in 70% trifluoroacetic acid and incubating at 23°C for 20h. The digest was then lyophilized and washed twice in 500 $\mu$ L of Milli-Q H<sub>2</sub>O with intermittent drying in the Speed-Vac. The washed samples were then dissolved in 100 $\mu$ L of 8M urea, 0.2M Tris-HCl, pH 8.3, and the peptide concentrations were determined using a 2-D Quant Kit (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Disulfide bridges were reduced by incubating with 10mM dithiothreitol for 1h at 23°C. The reduced cysteines were then alkylated by adding 30mM iodoacetamide and incubating for 1h in the dark at 23°C. The samples were then diluted five times in 0.1M Tris-HCl, pH 8.3, and 10 $\mu$ g was incubated overnight at 37°C with 200ng of trypsin (Promega, Madison, WI). The samples were centrifuged for 5min at 17,000 x g to remove precipitates, and the supernatants were desalted using POROS R2 C8/C18 resin (Life Technologies, Paisley, UK) packed in 200 $\mu$ L gel-loading pipet tips.

### **iTRAQ labeling and strong cation exchange (SCX) fractionation**

For each biological sample, 2.5 $\mu$ g of desalted peptide was pooled in a reference “master mix” to be labeled with iTRAQ 114, and 7.5 $\mu$ g of each sample was dissolved in 25 $\mu$ L of iTRAQ dissolution buffer (Life Technologies, Carlsbad, CA, US). The samples were labeled with 4plex iTRAQ (115, 116, or 117) reagents according to the manufacturer’s protocol, resulting in two iTRAQ sets to be analyzed by LC-MS/MS. Briefly, each iTRAQ label was diluted with 50 $\mu$ L of ethanol, mixed with the sample and incubated for 2h at 23°C. The samples were then mixed, lyophilized and desalted using POROS R2 C8/C18 resin as described above. The two iTRAQ sets were then applied to an SCX column (PolyLC Inc., Columbia, MD) equilibrated in 30% acetonitrile, 5mM KH<sub>2</sub>PO<sub>4</sub>, pH 2.7 and eluted with a

linear gradient from 0mM to 500mM KCl in 30% acetonitrile, 5mM KH<sub>2</sub>PO<sub>4</sub>, pH 2.7. Eluted peptides were collected in 12 fractions including the flow-through for each iTRAQ set. Each fraction was desalted using POROS R2 micro purification and lyophilized.

### LC-MS/MS of iTRAQ samples

Samples were dissolved in 0.1% formic acid and analyzed by nanoflow LC-MS/MS on an EASY-nLC II system (Thermo Scientific, Waltham, MA, US) coupled to a TripleTOF 5600 mass spectrometer (Sciex, Framingham, MA, US). The samples were loaded on a 0.1x21mm C18 trap column and a 0.075x100mm C18 analytical column (NanoSeparations, Nieuwkoop, Netherlands). Peptides were eluted and electrosprayed directly into the mass spectrometer using a 50min gradient from 5-40% acetonitrile in 0.1% formic acid at a flow rate of 250nL/min. The data were acquired using an ion spray voltage of 2.3kV, a curtain gas setting of 30 and an interface heater temperature of 150°C.

### Processing of iTRAQ data

All 12 wiff files generated for either iTRAQ set 1 or 2 were searched simultaneously against the SwissProt *Mus musculus* database (2016\_8) using ProteinPilot v.4.5 (Sciex, Framingham, MA) and the Paragon algorithm v. 4.5.0.0. Trypsin was specified as the digestion enzyme, allowing up to 5 missed cleavages. Iodoacetamide was entered as a fixed modification of cysteine, and the default standard workup modification set was used as the variable modifications. The instrument setting was specified as TripleTOF 5600, defining the default charge state and mass accuracy of the instrument, and the iTRAQ 4plex (peptide labeled) protocol was selected as the sample type. Quantitative data were normalized using the bias and background corrections to correct for any experimental and technical bias. Thorough ID and false discovery rate (FDR) analysis were both selected. The searches were imported to and parsed using MS Data Miner v. 1.3.0 [55], using a 1% global FDR at the protein level. To find significantly mis-regulated proteins, Student's *t*-test with a significance threshold of 0.01 was performed for proteins quantified in all 3 biological replicates of both the *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> groups. Proteins with a p-value less than 0.01 and a minimum fold change of 0.5 on the log<sub>2</sub> scale were considered up- or downregulated in *TGFBI*<sup>-/-</sup> mice. TGFBIp was excluded from the data processing, as it is not possible to obtain a ratio between *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> samples.

## Targeted MS analysis of POSTN – LC-SRM-MS

The SRM assay was developed using Skyline 1.4.0.4421 [56] and based on the optimization of 4 stable isotope-labeled POSTN analog peptides (SpikeTides, JPT Peptide Technologies GmbH, Germany) (DQGPNVCALQQILGTK (+2), NGVIHLIDEVLIPDSAK (+2), LLYPADIPVGNLQLELLNK (+2), and IIDGVPVEITEK (+2)). The final optimized assay included 4 transitions per peptide monitored, including both light and heavy peptide variants. New digests of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas were prepared as described above. The LC-SRM-MS analysis was performed on an EASY-nLC II system (Thermo Scientific, Waltham, MA, US) connected in-line to a Qtrap 6500 mass spectrometer (Sciex) equipped with a NanoSpray III source (AB Sciex) and operated under Analyst 1.6.1 control. Peptides were eluted at a flow rate of 250nL/min using a 12min gradient from 5% to 35% phase B (0.1% formic acid and 90% acetonitrile), followed by re-equilibration for 10min to the starting conditions. The Qtrap 6500 was used in positive ion mode with an ion spray voltage of 25kV, a curtain gas setting of 30, an ion source gas setting of 5 and an interface heater temperature of 150°C. The eluted peptides were measured with a scheduled (3min window) SRM method using a 20ms dwell time for all transitions (32 in total) and a target scan time of 0.8s.

## Relative comparison of human and mouse corneal TGFBIp amounts

Human tissue was used with full ethical approval and in accordance with the Declaration of Helsinki. Equal amounts of post-mortem healthy human cornea (n = 2, female, age 86; and male, 79) and wildtype mouse cornea (n = 2, 8-10 weeks) were processed as described above using a combination of CNBr chemical cleavage and trypsin digestion. The digested samples were normalized according to the OD280 value, and equal amounts were subjected to LC-MS/MS analysis using a method allowing for post-acquisition label-free quantification at the MS1 level. Wiff files were imported to Skyline 1.4.0.4421 [56], and four TGFBIp peptides (NNVSVNKK, QHGPNVCAVQK, STVISYECCPGYEK, SPYQLVLQHSR) identical in sequence between species were used for a relative MS1 quantitative comparison (Table S2).

## Transmission electron microscopy (TEM)

Corneas from *TGFBI*<sup>-/-</sup> and age-matched *TGFBI*<sup>+/+</sup> controls (n = 2 corneas from each genotype), which had all been stored at -20 °C before use, were fixed in 4%

paraformaldehyde, 2% glutaraldehyde, and 0.1M sodium cacodylate, pH 7.2 and postfixed with 1% osmium tetroxide followed by 0.5% uranyl acetate. The corneas were then dehydrated using an ethanol series and propylene oxide. The corneal specimens were infiltrated and embedded using an EMBED 812 Kit according to the manufacturer's protocol (Electron Microscopy Sciences, Hatfield, PA.). Thin sections of 40-60nm on average were cut using a Leica Ultracut UCT (Leica Mikrosysteme, Vienna, Austria) with a 45° diamond knife (Diatome, Biel, Switzerland) and post-stained with saturated aqueous uranyl and lead citrate [57]. The samples were imaged on a Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, Oregon) equipped with a TemCam F416 CCD camera (Tvips, Gauting, Germany), operating at 120kV. All micrographs used in the morphometric analysis represent the central anterior corneal stroma and were acquired at 11,000x magnification.

### **Morphometric analysis of collagen fibrils**

TEM micrographs were analyzed using ImageJ v.1.42q software (imagej.nih.gov). Collagen fibril diameter (n = 250) and center-to-center interfibrillar spacing (n = 250) were measured for *TGFBI*<sup>-/-</sup> and *TGFBI*<sup>+/+</sup> corneas (n = 2 corneas from each genotype). Each measurement relied on 5 randomly chosen TEM micrographs per cornea, all representing the central anterior stroma.

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [58] partner repository with the dataset identifier PXD006494.

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## Author contributions:

ETP, KR, SJC and JJE designed the study, analyzed the results and wrote the paper. ETP, KR, NSN and MVL performed LC-MS/MS analyses, MS data processing, SDS-PAGE, and WB experiments. PS, OS and SJC were responsible for animal work, qPCR, IHS and the ISH analyses. KT and ETP performed the TEM analysis. HV was responsible for human tissue samples and involved in the histological examination. All authors approved the final version of the manuscript.

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## Supporting Information

**Table S1:** Proteins quantified in all biological replicates of both TGFB1p wildtype and deficient mice corneas.

**Table S2:** Relative comparison of human and mouse corneal TGFB1p.

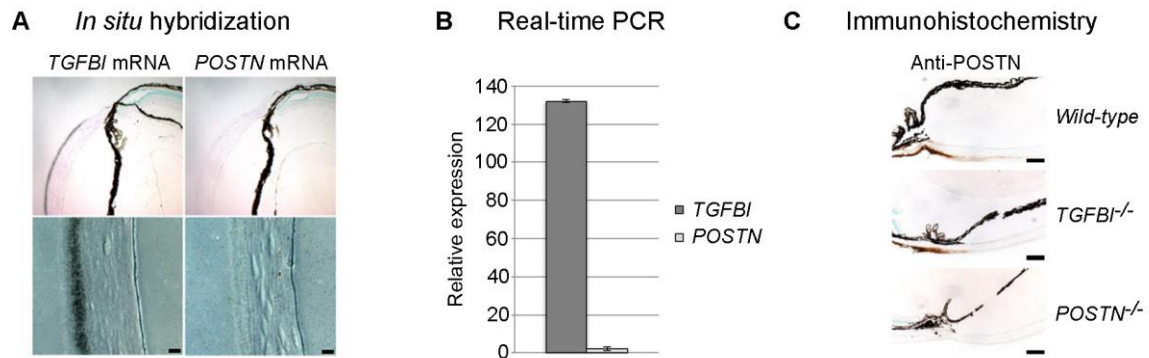
## Table 1

**Table 1. Differential expressed proteins in *TGFB1*<sup>-/-</sup> corneas**

| Accession Nr | Name                                  | Mass kDa | P - value | Log2 (-/+) |
|--------------|---------------------------------------|----------|-----------|------------|
| P40124       | Adenylyl cyclase-associated protein 1 | 51.565   | 0.003     | -0.76      |
| P07356       | Annexin A2                            | 38.676   | 0.004     | 1.00       |
| Q60847       | Collagen alpha-1(XII) chain           | 340.214  | 0.004     | 1.98       |
| Q02788       | Collagen alpha-2(VI) chain            | 110.334  | 0.005     | 0.94       |
| P50608       | Fibromodulin                          | 43.055   | 0.003     | 2.38       |
| Q9WVH9       | Fibulin-5                             | 50.193   | 0.005     | 2.34       |
| P14602       | Heat shock protein beta-1             | 23.014   | 0.002     | -1.51      |
| Q9Z331       | Keratin, type II cytoskeletal 6B      | 60.322   | 0.001     | 0.96       |
| Q9JK53       | Prolargin                             | 43.293   | 0.003     | 0.64       |
| Q9R1P1       | Proteasome subunit beta type-3        | 22.965   | 0.003     | -0.62      |
| P07724       | Serum albumin                         | 68.693   | 0.006     | -2.09      |

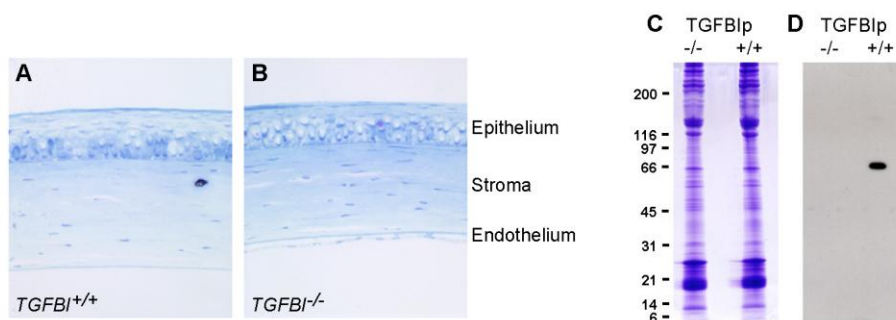
Table shows mis-regulated proteins in *TGFB1*<sup>-/-</sup> compared to *TGFB1*<sup>+/+</sup> mice corneas with a p value below 0.01 and a minimum 0.5 fold mis-regulation on the log2 scale.

## Figure legends



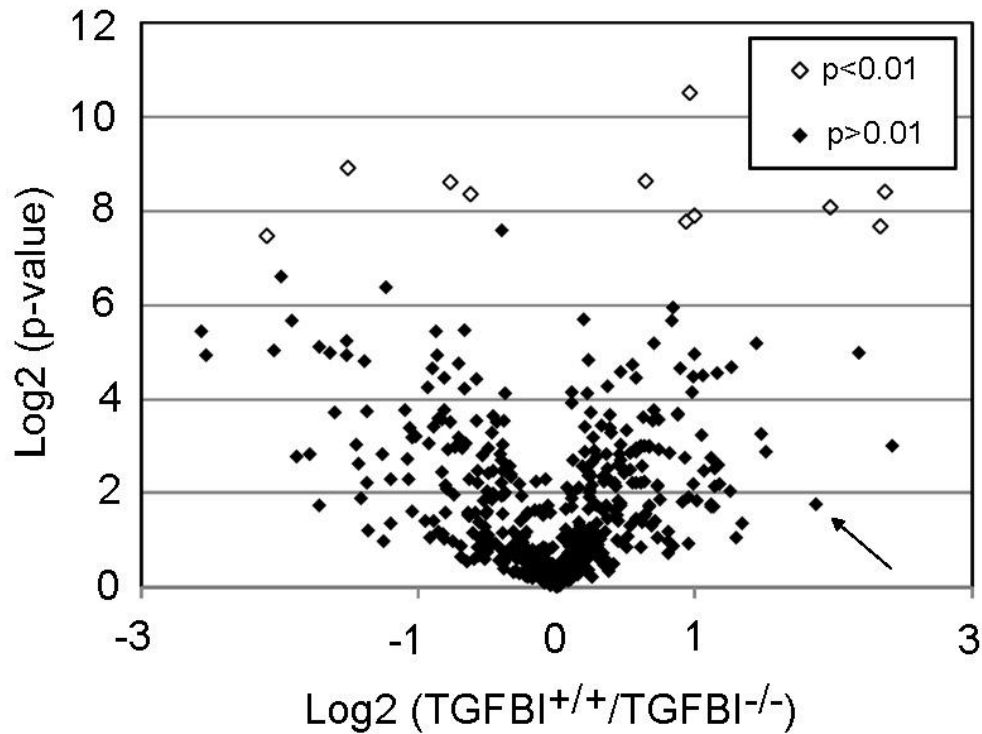
**Figure 1. TGFBIp and POSTN expression in the cornea**

(A) *In situ* hybridization analysis of *TGFBI* and *POSTN* mRNA in wildtype mouse corneas. A strong signal (indicated via silver grains) for *TGFBI* mRNA is restricted to the corneal epithelium, whereas no apparent signal is observed for *POSTN* mRNA (scale bar = 20 $\mu$ m). (B) Real-Time PCR supports the presence of *TGFBI* and the lack of *POSTN* mRNA in isolated corneal tissues (n = 3). Error bars represents the SD. (C) Immunohistochemically staining using a POSTN antibody verifies the absence of POSTN in the central cornea and reveals that POSTN protein is localized at the corneal periphery. Moreover, the extent of POSTN deposition in wildtype and *TGFBI*<sup>-/-</sup> mice corneas is unchanged, whereas no POSTN was detected in *POSTN*<sup>-/-</sup> corneas (scale bars = 100 $\mu$ m).



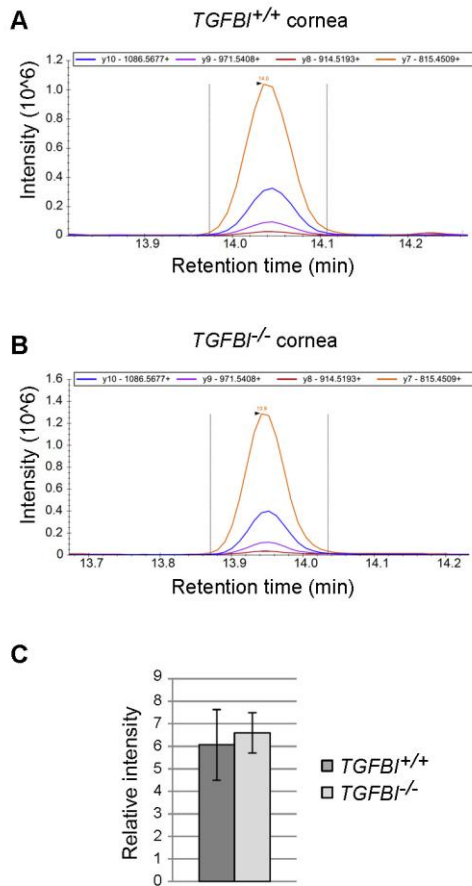
**Figure 2. *TGFBI*<sup>-/-</sup> corneas show no aberrant changes**

(A, B) No morphological changes are observed throughout the corneal in sections of (A) *TGFBI*<sup>+/+</sup> and (B) *TGFBI*<sup>-/-</sup> corneas examined by light microscopy. Tissue sections are stained with toluidine blue; a 4x objective was used. (C) SDS-PAGE of *TGFBI*<sup>-/-</sup> corneas shows no major differences in protein expression when compared to control *TGFBI*<sup>+/+</sup> corneas. (D) Western blotting using TGFBIp anti-serum confirms complete knockout in the *TGFBI*-deficient mouse eyes.



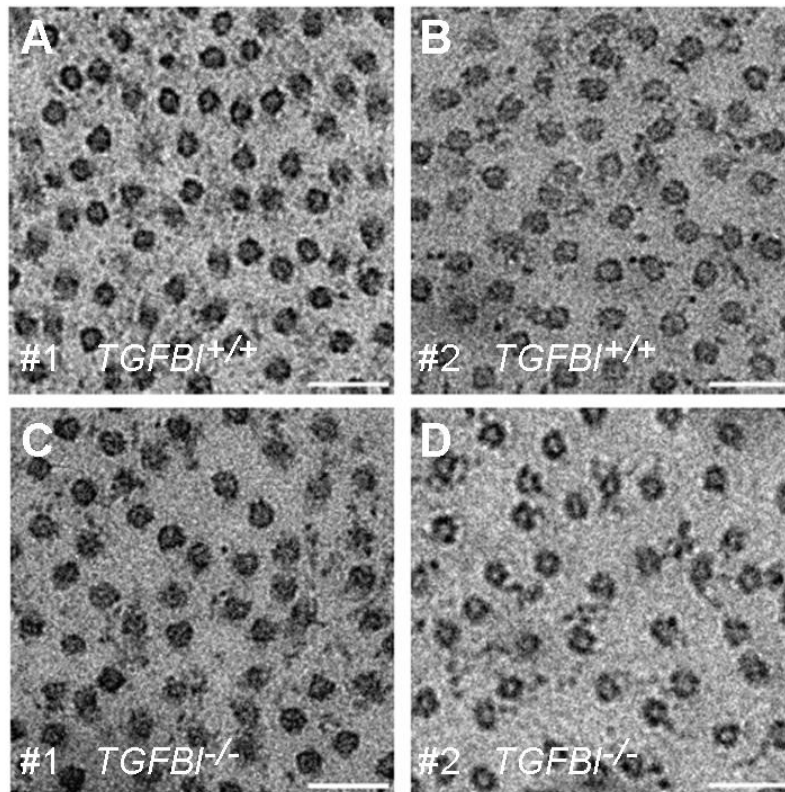
**Figure 3. The ECM of *TGFBI*-deficient corneas is affected at the protein level**

The relative quantification of *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> corneas using the iTRAQ methodology followed by LC-MS/MS resulted in 516 proteins being quantified in all three biological replicates of both the *TGFBI*<sup>+/+</sup> and *TGFBI*<sup>-/-</sup> groups. The volcano plot shows the *TGFBI*<sup>+/+</sup>/*TGFBI*<sup>-/-</sup> ratio distribution of quantified proteins using the log<sub>2</sub> scale compared to the log<sub>2</sub> of the p-values. Using a p-value cut-off of 0.01, we identified 11 proteins (Table 1) that were differentially expressed in the *TGFBI*<sup>-/-</sup> cornea (empty dots). Six proteins (Type VI collagen, Type XII collagen, annexin, fibromodulin, prolargin and fibulin-5) known for their role in ECM arrangement were among the significantly mis-regulated proteins identified, suggesting that the ECM is altered in the *TGFBI*<sup>-/-</sup> cornea. The arrow indicates POSTN, a TGFBIp paralog, that shows a 1.9-fold upregulation on the log<sub>2</sub> scale in the *TGFBI*<sup>-/-</sup> cornea; however, it was not significantly mis-regulated at p < 0.01.



**Figure 4. The TGFB1p paralog POSTN does not compensate for the lack of TGFB1p expression**

(A, B) Targeted LC-SRM-MS assay was developed to assess whether the TGFB1p paralog POSTN is upregulated in the *TGFB1*<sup>-/-</sup> cornea to compensate for loss of TGFB1p. POSTN was one of the most regulated proteins in the iTRAQ analysis; however, its change in expression was not significant at  $p < 0.01$ . The SRM assay was designed to monitor four proteotypic peptides for POSTN, including four transitions per proteotypic peptide. Each represent one of the biological replicates ( $n = 3$ ) and show the intensity of the four transitions for the tryptic POSTN peptide IIDGVPVEITEK in *TGFB1*<sup>+/+</sup> (A) and *TGFB1*<sup>-/-</sup> (B). (C) No significant difference between the *TGFB1*<sup>+/+</sup> and *TGFB1*<sup>-/-</sup> groups ( $n = 3$ ) was observed using a Student's t-test and a p-value cut-off of 0.01, thus rejecting the hypothesis that the functional loss of TGFB1p in the *TGFB1*-deficient cornea is compensated by POSTN upregulation. Error bars represents the SD.



**Figure 5. Stromal interfibrillar spacing is slightly affected in *TGFB1*<sup>-/-</sup> corneas**

(A-D) *TGFB1*p is known to interact with Type VI and XII collagens, which were both mis-regulated in the iTRAQ analysis (Table 1). To investigate the effect of *TGFB1* deficiency on the delicate collagen architecture of the cornea, TEM micrographs were taken of *TGFB1*<sup>+/+</sup> (A, B) and *TGFB1*<sup>-/-</sup> corneas (C, D). All micrographs taken represent the central anterior stroma, and the subsequent morphometric analysis relied on five randomly chosen TEM micrographs per cornea. Collagen fibril diameter (n = 250) and center-to-center interfibrillar spacing (n = 250) were measured and compared between the *TGFB1*<sup>+/+</sup> and *TGFB1*<sup>-/-</sup> groups. The morphometric analysis showed no change in collagen fibril diameter (mean: 30±3 vs. 31±3nm), whereas interfibrillar spacing increased slightly in the *TGFB1*-deficient cornea (58±10nm vs. 62±11). Scale bar = 500nm.