SHARPIN regulates collagen architecture and ductal outgrowth in the developing mouse mammary gland

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Abstract

SHARPIN is a widely expressed multifunctional protein implicated in cancer, inflammation, linear ubiquitination and integrin activity inhibition; however, its contribution to epithelial homeostasis remains poorly understood. Here, we examined the role of SHARPIN in mammary gland development, a process strongly regulated by epithelial–stromal interactions. Mice lacking SHARPIN expression in all cells (*Sharpin*^{cpdm}), and mice with a stromal (*S100a4-Cre*) deletion of *Sharpin*, have reduced mammary ductal outgrowth during puberty. In contrast, *Sharpin*^{cpdm} mammary epithelial cells transplanted *in vivo* into wild-type stroma, fully repopulate the mammary gland fat pad, undergo unperturbed ductal outgrowth and terminal differentiation. Thus, SHARPIN is required in mammary gland stroma during development. Accordingly, stroma adjacent to invading mammary ducts of *Sharpin*^{cpdm} mice displayed reduced collagen arrangement and extracellular matrix (ECM) stiffness. Moreover, *Sharpin*^{cpdm} mammary gland stromal fibroblasts demonstrated defects in collagen fibre assembly, collagen contraction and degradation *in vitro*. Together, these data imply that SHARPIN regulates the normal invasive mammary gland branching morphogenesis in an epithelial cell extrinsic manner by controlling the organisation of the stromal ECM.

Keywords collagen; ductal morphogenesis; fibroblast; mammary gland; SHARPIN

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Development & Differentiation

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Introduction

The mammary gland develops post-natally in response to growth and steroid hormones, and local growth factors. During mammary ductal elongation and branching morphogenesis, the pubertal mammary epithelium invades into the fat pad stroma to form the gland that later evolves further during the menstrual cycle, and terminally differentiates/dedifferentiates during pregnancy, lactation and involution. Mammary ductal outgrowth takes place at the tips of the ducts, in the terminal end buds (TEBs). Here, in areas of active cell division, hollow ducts are formed through luminal apoptosis, and cells undergo differentiation into luminal and basal mammary epithelial layers (Hinck & Silberstein, 2005; Ewald *et al*, 2008). Complex signalling between the epithelium and the stroma orchestrates the mammary ductal outgrowth and branching through the adipose tissue (Sternlicht *et al*, 2006; Howard & Lu, 2014). This process involves significant regulation of the surrounding extracellular matrix (ECM) (Zhu *et al*, 2014; Gomes *et al*, 2015). Epithelial cell adhesion to the surrounding ECM via integrins, heterodimeric transmembrane adhesion receptors, plays an important role in mammary ductal outgrowth (Klinowska *et al*, 1999), in preserving the regenerative capacity of the mammary epithelium (Taddei *et al*, 2008), and during breast cancer invasion

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and metastasis (Levental *et al*, 2009). However, the signalling pathways that regulate mammary gland stromal cell adhesion and collagen architecture, and thereby ductal outgrowth, are largely unknown.

SHARPIN (Shank-associated RH domain-interacting protein, also known as SIPL1) binds to intracellular integrin alpha tails of inactive integrins and inhibits recruitment of talin and kindlin to the beta tail, thereby functioning as an integrin inhibitor (Rantala *et al*, 2011; Pouwels *et al*, 2013; De Franceschi *et al*, 2015). SHARPIN is also an essential component of the linear ubiquitination assembly complex (LUBAC) (Gerlach *et al*, 2011; Ikeda *et al*, 2011; Tokunaga *et al*, 2011) that regulates canonical nuclear factor- κ B (NF- κ B) signalling in response to cytokines, bacteria and genotoxic stress through linear ubiquitination (Gerlach *et al*, 2011; Tokunaga *et al*, 2011; Fujita *et al*, 2014). Furthermore, SHARPIN is involved in negative regulation of T-cell receptor signalling (Park *et al*, 2016), priming of the NLRP3 inflammasome complex in macrophages (Rodgers *et al*, 2014; Gurung *et al*, 2015), and it has been reported to bind and regulate key signalling proteins such as eyes absent homolog 1 (EYA1) (Landgraf *et al*, 2010), SH3 and multiple ankyrin repeat domains protein (SHANK) (Lim *et al*, 2001), and phosphatase and tensin homolog (PTEN) (He *et al*, 2010). SHARPIN-deficient (*Sharpin*^{cpdm}) mice display defective secondary lymphoid organ development (Hogen-Esch *et al*, 1993; Seymour *et al*, 2013) and suffer from progressive multiorgan inflammation with chronic eosinophilic hyperproliferative dermatitis due to increased tumour necrosis factor receptor (TNFR)-mediated keratinocyte apoptosis (Seymour *et al*, 2007; Gerlach *et al*, 2011; Rickard *et al*, 2014). Increased SHARPIN expression has previously been linked to prostate tumorigenesis (He *et al*, 2010; Li *et al*, 2015), elevated breast cancer risk (De Melo & Tang, 2015) and breast cancer metastasis (Bii *et al*, 2015), suggesting a role for SHARPIN in regulating epithelial homeostasis. Many of the molecular features driving invasive breast carcinoma are also essential during normal mammary ductal outgrowth, including collective cell migration, ECM remodelling and epithelial–stromal communication (Polyak & Kalluri, 2010). The potential involvement of SHARPIN in processes related to breast cancer invasion and metastasis prompted us to investigate post-natal mammary gland development in *Sharpin*^{cpdm} mice. Here, we report that *Sharpin*^{cpdm} mice have defective mammary ductal outgrowth during puberty and demonstrate an epithelial cell extrinsic requirement for SHARPIN in regulating normal stromal collagen architecture and stiffness. Accordingly, *Sharpin*^{cpdm} fibroblasts demonstrate an inability to generate traction forces on collagen and to assemble, contract and degrade collagen fibres.

Results

SHARPIN is expressed in the mammary gland

To examine the expression of SHARPIN in the mammary gland, paraffin-embedded human tissue sections were stained by immunohistochemistry (IHC) (Fig 1A). SHARPIN expression was detected in the luminal epithelial cell layer and in the scattered stromal cells, but not in the basal epithelial cells directly adhering to the basal

lamina (Fig 1A). Co-staining of SHARPIN with vimentin confirmed that the majority of the SHARPIN-positive stromal cells were spindle-shaped and vimentin expressing fibroblasts (Fig EV1A). For further characterisation, mouse mammary gland epithelial cells (MECs) and mammary gland stromal fibroblasts (MSFs) were isolated, and the expression of SHARPIN was analysed by Western blotting (Fig 1B). SHARPIN was expressed at the protein level in both mammary gland primary cell populations although more prominently in the epithelial portion (Fig 1B). The specific expression of CDH1 (also called E-cadherin), detected as a double band (upper band represents the unprocessed receptor form) (Fujita *et al*, 2002), in the epithelial cells and vimentin in the stromal cells confirmed the purity of the two populations (Fig 1B). MECs and MSFs were further sorted by flow cytometry to basal epithelial, luminal epithelial (progenitors/mature) and stromal populations based on CD24 and ICAM1 surface expressions (Di-Cicco *et al*, 2015; Fig 1C). Lineage-specific marker mRNA expression [basal, keratin 5 (*Krt5*); luminal, keratin 18 (*Krt18*); stromal, *Pdgfra*] was controlled by quantitative PCR (qPCR) (Fig EV1B). *Sharpin* mRNA expression was low in basal epithelial cells (Lin^{neg}CD24^{int}ICAM1^{hi}), higher in luminal progenitor (Lin^{neg}CD24^{hi} ICAM1^{int}) and mature luminal epithelial cells (Lin^{neg}CD24^{hi} ICAM1^{neg}) and highest in stromal fibroblasts (Lin^{neg}CD24^{neg}) when measured by qPCR (Fig 1D). Taken together, our results show that SHARPIN mRNA and protein are expressed both in the epithelial and in the stromal cells of the mouse mammary gland.

SHARPIN-deficient mammary glands exhibit reduced ductal outgrowth during puberty

Mammary ductal morphogenesis in pubertal female mice has been previously investigated to identify mechanisms potentially hijacked by breast cancer cells for adhesion, invasion and metastasis (Lanigan *et al*, 2007; Polyak & Kalluri, 2010). As SHARPIN is expressed in the mammary gland (Fig 1) and is known to regulate both cell adhesion (Rantala *et al*, 2011) and breast cancer metastasis (Bii *et al*, 2015; De Melo & Tang, 2015), we hypothesised that SHARPIN could play an important role during mammary ductal outgrowth. To examine this, the 4th mammary glands were isolated from 3- to 7-week-old female wt and SHARPIN-deficient (*Sharpin*^{cpdm}) mice (Figs 2A and EV2A), and ductal outgrowth and branching were quantified from carmine alum-stained whole mounts (Fig 2B and C). While the ductal trees of the pre-pubertal 3- to 4-week-old mice were equal in size, a clear reduction in ductal outgrowth area was observed in the *Sharpin*^{cpdm} mammary glands at puberty (5–7 weeks old; Fig 2A and B), indicating impaired pubertal (allometric) mammary growth. Additionally, the number of ductal branches per gland was significantly lower in pubertal *Sharpin*^{cpdm} mice (Fig 2C). These differences were not attributed to disturbances in the onset of puberty in the *Sharpin*^{cpdm} mice, as it occurred normally close to 5 weeks of age similarly to their wt female littermate controls, as judged based on the evaluation of vaginal opening (Fig EV2B). Furthermore, oestrogen receptor and progesterone receptor expressions were similar in both wt and *Sharpin*^{cpdm} mammary glands indicative of normal systemic steroid hormone production at puberty (Fig EV2C). The polarity of the mammary ductal cell layers was also similar in wt and *Sharpin*^{cpdm} mice as examined by hematoxylin-eosin (HE) and IHC labelling of luminal

Figure 1. SHARPIN is expressed in the stromal and luminal epithelial cells of the mammary gland.

- A** Immunohistochemical analysis of SHARPIN expression in the human mammary gland. Cross section of a mammary duct (upper panel) and magnification of the marked area (lower panel). SHARPIN-positive luminal (grey arrow) and stromal cells (red arrow), and the approximate position of the basal lamina (dashed red line) are indicated. Scale bar represents 50 μ m.
- B** Western blot analysis of SHARPIN protein expression in isolated primary mammary epithelial cells (MECs) and mammary stromal fibroblasts (MSFs). CDH1 and vimentin were used as markers of epithelial and stromal cell lineages, respectively. GAPDH served as a control for protein loading.
- C** FACS-based isolation of mouse mammary gland basal epithelial cells (Lin^{neg}CD24^{int}ICAM-1^{hi}), mature luminal epithelial cells (Lin^{neg}CD24^{hi}ICAM-1^{neg}), luminal progenitor cells (Lin^{neg}CD24^{hi}ICAM-1^{int}) and stromal cells (Lin^{neg}CD24^{neg}).
- D** Quantitative PCR analysis of *Sharpin* mRNA expression in cell populations isolated in (C) (mean $\pm$ SEM, $n = 3-5$).

(CDH1) and basal (integrin alpha 6; ITGA6) epithelial cells from histological sections of 7-week-old mouse mammary glands (Fig 2D).

As TEBs of the mammary ductal epithelium are known to be responsible for ductal growth and invasion through the mammary fat pad (Hinck & Silberstein, 2005), the number of TEBs in wt and *Sharpin*^{cpdm} mouse mammary gland whole mounts was analysed (Fig 2E). The number of *Sharpin*^{cpdm} TEBs was reduced (Fig 2F), likely reflecting the reduced ductal growth and branching. In histological sections, the overall cellular organisation of the *Sharpin*^{cpdm} TEBs was normal (Fig 2G), and similar numbers of proliferating (BrdU-positive) and apoptotic (cleaved caspase-3-positive) cells were observed in wt and *Sharpin*^{cpdm} mammary gland TEBs (Fig EV2D). Together, these data show that the *Sharpin*^{cpdm} mammary gland has reduced ductal outgrowth *in vivo* suggesting a

previously unknown role for SHARPIN in the regulation of mammary branching morphogenesis.

Epithelial SHARPIN is dispensable for mammary gland morphogenesis

Since SHARPIN expression was detected in the mature luminal and luminal progenitor cells in the mouse mammary gland, and only at low level in basal epithelial cells (Fig 1D), we postulated that SHARPIN deficiency could affect mammary epithelial regeneration and differentiation. To investigate this, MECs were isolated from pubertal wt and *Sharpin*^{cpdm} mice and surface labelled to detect CD24 expression in combination with CD29 (integrin beta 1) or CD49f (integrin alpha 6) and quantified basal epithelial (Lin^{neg}CD24^{int}CD29⁺ or Lin^{neg}CD24^{int}CD49f⁺) and luminal

Figure 2. Mammary ductal outgrowth during puberty is impaired in SHARPIN-deficient (*Sharpin^{cpdm}*) mice.

Mammary ductal outgrowth in 3- to 7-week-old wt or *Sharpin^{cpdm}* female mice.

- A** Representative carmine alum-stained mammary gland whole mounts. Arrow indicates the inguinal lymph node. Scale bars represent 2 mm.
- B** Quantification of mammary ductal outgrowth area ($n = 4-15$ glands).
- C** Quantification of the number of ductal branch points per mammary gland ($n = 7-9$ glands, 7-week-old mice).
- D** Cryosections from 7-week-old wt or *Sharpin^{cpdm}* mouse mammary glands stained with hematoxylin-eosin (HE) (upper panel) or immunolabelled with the indicated antibodies. Scale bars represent 20 μ m.
- E, F** (E) Representative carmine alum-stained images highlighting terminal end buds (TEBs) in 7-week-old wt and *Sharpin^{cpdm}* mouse mammary glands and (F) quantification of the number of TEBs per gland ($n = 9-10$ glands).
- G** TEBs in paraffin sections from wt or *Sharpin^{cpdm}* mouse mammary glands stained with HE (upper panel) or immunolabelled with the indicated antibodies (lower panel). Scale bars represent 50 μ m.

Data information: (B, C, F) Mean $\pm$ SEM. (B) Unpaired Student's *t*-test or Mann-Whitney test, (C) Mann-Whitney test. (F) Unpaired Student's *t*-test.

epithelial (Lin^{neg}CD24^{hi}CD29^{neg} or Lin^{neg}CD24^{hi}CD49f^{neg}) cell populations (Taddei *et al*, 2008) (Fig EV2E). Interestingly, both labelling strategies indicated that 50–60% of the MEC population expressed luminal and 30–40% basal markers in both wt and *Sharpin*^{cpdm} samples (Fig EV2E), demonstrating that the *Sharpin*^{cpdm} mammary epithelium has normal proportions of basal and luminal epithelial cells.

Next, to conclusively evaluate whether *Sharpin*^{cpdm} mammary epithelium retained normal regenerative capacity, indicative of normal stem cell population, small pieces of wt or *Sharpin*^{cpdm} mammary glands were transplanted into virgin wt recipients' cleared (epithelium-free) mammary fat pads (Fig 3A). Ductal outgrowth from transplants was evaluated from mammary gland whole mounts 7–11 weeks after transplantation. The *Sharpin*^{cpdm} (donor) epithelium was able to fully regenerate the mammary gland in the wt (recipient) stroma (Fig 3A and B) and invade into the fat pad to a similar extent as the wt donor epithelium (Fig 3C). To evaluate the role of SHARPIN in terminal differentiation of the mammary gland, virgin wt mice were transplanted with wt or *Sharpin*^{cpdm} mammary epithelium and mated 10 weeks post-transplantation. Mammary gland whole mounts were prepared 15 days after conception (P15; Fig 3D). In five out of six transplants of both genotypes, mammary epithelium was able to regenerate and undergo tertiary branching and alveologenesis during pregnancy, when grown in wt mammary stroma (Figs 3D and EV2F), demonstrating that epithelial SHARPIN is dispensable for terminal differentiation of the mammary gland. Due to the reduced lifespan of the *Sharpin*^{cpdm} mice (Potter *et al*, 2014), the reciprocal transplantation of wt epithelium into *Sharpin*^{cpdm} recipients was not feasible. Together, our data demonstrate that wt fat pad stroma is able to support mammary ductal growth and branching, as well as the terminal differentiation of the *Sharpin*^{cpdm} epithelium, suggesting that SHARPIN deficiency is predominantly affecting the mammary stromal compartment.

SHARPIN in stromal cells regulates ductal outgrowth in mammary glands

The stroma of the developing mammary gland is composed of several different cell types (including fibroblasts, endothelial cells and leukocytes), which undergo complex dialog with the invading mammary epithelium (Reed & Schwertfeger, 2010; Howard & Lu, 2014). Macrophages, eosinophils, mast cells and lymphocytes have previously been shown to regulate mammary branching morphogenesis (Gouon-Evans *et al*, 2000; Lilla & Werb, 2010; Plaks *et al*, 2015). Significantly more leukocytes (CD45⁺), neutrophils (CD11b⁺Ly6G^{hi}), eosinophils (CD11b⁺Siglec F^{hi}) and macrophages (CD11b⁺F4/80⁺) were detected in the pubertal mammary gland of *Sharpin*^{cpdm} mice compared to wt animals (Fig EV3A and B), which is consistent with the *Sharpin*^{cpdm} multiorgan inflammation phenotype (HogenEsch *et al*, 1993; Seymour *et al*, 2007). While the increase in neutrophils (+65%) and macrophages (+51%) was similar to the increase in total pool of mammary gland leukocytes (+35%) in *Sharpin*^{cpdm} mice, the proportion of eosinophils (+331%) was clearly higher in the *Sharpin*^{cpdm} mouse mammary gland (Fig EV3A and B). However, angiogenesis, measured by PECAM1 IHC, was similar in wt and *Sharpin*^{cpdm} mammary gland stroma (Fig EV3C).

To evaluate further the role of stromal SHARPIN in the regulation of mammary ductal outgrowth, a conditional *Sharpin* knockout in stromal cells was generated using the S100 Calcium Binding Protein A4 (*S100a4*), commonly known as the fibroblast-specific protein-1 (*Fsp1*), promoter for *Cre* expression. The *S100a4* promoter has also previously been used for conditional knockout and selective gene expression in the mammary gland stroma (Cheng *et al*, 2005; Trimboli *et al*, 2009; O'Connell *et al*, 2011; Pickup *et al*, 2015; Koledova *et al*, 2016). *S100a4* expression was detected in lysates of isolated wt and *Sharpin*^{cpdm} mammary gland fibroblasts by Western blotting (Fig EV4A) and by IHC labelling of the mammary gland stroma (Fig EV4C). No *S100a4* expression was detected in the mammary epithelium (Fig EV4C). Importantly, expression of *Sharpin* mRNA was specifically reduced in the *S100a4-Cre; Sharpin*^{fl/fl} mammary gland stromal cells surrounding the mammary ducts when compared to *S100a4-Cre; Sharpin*^{fl/+} control (Fig EV4B). Consistent with our transplantation results, mouse mammary ductal outgrowth and number of TEBs were reduced when *Sharpin* was deleted specifically in stromal cells (Fig 3E–G). Comparable numbers of F4/80-positive cells (macrophages and eosinophils) were detected around invading TEBs in *S100a4-Cre; Sharpin*^{fl/fl} and control mice, while elevated density of F4/80 expressing cells was observed in *Sharpin*^{cpdm} samples (Fig EV4D). Our findings demonstrate that SHARPIN in stromal fibroblasts rather than in epithelial cells is an important regulator of mammary gland ductal outgrowth.

SHARPIN regulates ECM in the mammary gland stroma

In addition to the many secreted signals originating from the stromal cells (Sternlicht *et al*, 2006), the pattern of extracellular collagen fibre deposition has been suggested to function as an important guide for mammary ductal outgrowth and branching morphogenesis that are known to occur under strict spatial regulation (Hovey *et al*, 2002; Ingman *et al*, 2006; Brownfield *et al*, 2013). Multiphoton laser scanning microscopy and second harmonic generation (SHG) imaging enable label-free visualisation and analysis of the collagen-rich ECM in tissues *in vivo* and *in vitro*. This technique was utilised to view the stromal collagen fibres proximal to the invading mammary epithelium in wt and *Sharpin*^{cpdm} pubertal mouse mammary gland whole mounts. SHG imaging revealed that in the *Sharpin*^{cpdm} stroma individual collagen fibres around TEBs appeared less organised compared to corresponding areas in wt samples (Fig 4A). Quantification of the SHG signal by scoring maximum intensity projection images for the presence of clear collagen bundles (as opposed to mesh-like or “curly” collagen fibres) demonstrated that distinct collagen bundles were significantly less abundant around the *Sharpin*^{cpdm} TEBs (Fig 4B). Furthermore, fibre extraction image analysis (Fig EV4E) revealed that *Sharpin*^{cpdm} collagen fibres had a tendency to be shorter and thicker than wt fibres, particularly when the longest collagen fibres (> 75.8 µm) were examined (although the results were not statistically significant; Fig EV4F and G). Other measured parameters, such as the overall alignment or straightness of the collagen fibres, did not show any similar trend in *Sharpin*^{cpdm} mammary gland stroma (data not shown). These data suggest that stromal collagen patterning is altered in

Figure 3. Stromal SHARPIN regulates mammary gland ductal outgrowth during puberty.

A–C Monitoring of mammary gland development following transplantation of wt or *Sharpin*^{cpdm} mouse mammary epithelium in the mammary fat pads (epithelium-free) of virgin wt mice. (A) Representative carmine alum-stained images of mouse mammary glands generated from wt or *Sharpin*^{cpdm} epithelial tissue transplants. (B) Quantification of transplant growth take-on-rate ($n = 17$). (C) Quantification of fat pad filling rate of grown transplants ($n = 7–10$) in wt hosts.

D Monitoring of mammary gland differentiation during pregnancy following transplantation of wt or *Sharpin*^{cpdm} mouse mammary epithelium into wt hosts. Host animals were mated and mammary glands isolated at P15. Representative carmine alum-stained mammary gland whole mounts generated from wt and *Sharpin*^{cpdm} mammary epithelial transplants (upper panel), and magnifications of the branched ductal epithelium (lower panel) are shown ($n = 5$ mice). Scale bars represent 1 mm.

E Representative images of carmine alum-stained mouse mammary gland whole mounts generated from 7-week-old *S100a4-Cre; Sharpin*^{fl/fl} conditional knockout mice and their littermate controls (*S100a4-Cre; Sharpin*^{fl/+}).

F Quantification of the normalised *Sharpin*^{cpdm} and *S100a4-Cre; Sharpin*^{fl/fl} mammary ductal outgrowth area relative to littermate control mice (wt and *Sharpin*^{cpdm} $n = 10$ glands; *S100a4-Cre; Sharpin*^{fl/+} and *S100a4-Cre; Sharpin*^{fl/fl} $n = 8$ glands).

G Quantification of the number of TEBs per gland ($n = 8–9$ glands).

Data information: (F, G) Mean $\pm$ SEM; Mann–Whitney test.

the absence of SHARPIN, which may impair the ECM cues for mammary epithelial outgrowth.

Atomic-force microscopy (AFM) analyses revealed a tissue stiffness in the range of 0.1–1 kPa (Fig 4C) for the wt mammary gland stroma, comparable to previously published data (Lopez et al, 2011; Plodinec et al, 2012). Interestingly, the *Sharpin*^{cpdm} mammary fat pad stroma at corresponding fat pad regions was clearly softer compared to wt samples ($P = 8.82 \times 10^{-8}$; Fig 4C) potentially reflecting the altered collagen fibre architecture

observed in *Sharpin*^{cpdm} mice. However, we cannot formally exclude that the difference in stromal stiffness is secondary to the mammary ductal outgrowth defect in *Sharpin*^{cpdm} mice. Such differences were not detected in pre-pubertal wt and *Sharpin*^{cpdm} mammary fat pad stroma at the age of 4 weeks when ductal outgrowth is still comparable in both genotypes ($P = 0.0963$; Fig EV4H). Our results provide evidence that SHARPIN positively regulates mammary gland stromal tissue stiffness and affects collagen arrangement *in vivo*.

Figure 4. SHARPIN deficiency causes aberrant collagen fibre organisation and reduced tissue stiffness in the mouse mammary gland stroma.

A Second harmonic generation (SHG) and unfiltered multiphoton (MP) imaging of collagen fibres surrounding TEBs (dashed line) in 6- to 7-week-old wt and *Sharpin^{cpdm}* mouse mammary glands ($n = 6-9$ mice, 5–13 TEBs analysed per mouse; scale bars represent 100 μm). Magnified images of collagen fibres are also shown (scale bars represent 50 μm).

B Frequency of clear collagen bundles around TEBs per mouse (mean $\pm$ SEM, $n = 6-9$ mice, Mann–Whitney test).

C AFM indentation analysis of stromal stiffness in 6- to 7-week-old wt and *Sharpin^{cpdm}* mouse mammary gland tissue sections ($n = 3$ mice; 3–6 sample sections per mouse and 192 indentation curves per sample, paired Student's *t*-test).

SHARPIN-deficient mammary gland stromal fibroblasts have reduced capacity for collagen degradation

Mammary gland stromal fibroblasts were studied further *in vitro* due to their established role in ECM remodelling. First, the global mRNA expression patterns in isolated and *in vitro* cultured wt and *Sharpin^{cpdm}* primary MSFs (passage 0) were compared (Figs 5A and EV5A and B, and Appendix Table S1) with Ingenuity Pathway Analysis. Significant alterations in canonical pathways related to leukocyte adhesion and transmigration, and tissue fibrosis ($P < 0.05$) were detected in *Sharpin^{cpdm}* MSFs, indicating an important role for SHARPIN in processes associated with immune cell chemotaxis and ECM remodelling (Appendix Table S1). Among the differentially expressed genes in this category were several previously identified target genes of the TNF signalling pathway (*Agt*, *Agtr1*, *Ccl22*, *Cdh5*, *Col4a3*, *Cxcl10*, *Cxcl3*, *Cxcr4*, *Cxcr5*, *Ednrb*, *Figf*, *Icam2*, *Igfbp4*, *Il1b*, *Il1r2*, *Il1rl1*, *Lbp*, *Mmp13*, *Mmp2*, *Mmp3*, *Mmp8*, *Mmp9*, *Pecam1* and *Selp*), likely related to the well-documented role of SHARPIN in the LUBAC complex (Gerlach *et al*, 2011; Ikeda *et al*, 2011; Tokunaga *et al*, 2011), several collagen genes (upregulated: *Col10a1*; downregulated: *Col23a1*, *Col4a3*, *Col6a6*, *Col24a1*, *Col13a1* and *Col25a1*) and multiple collagenases (upregulated: *Mmp9*; downregulated: *Mmp2*, *Mmp3*, *Mmp8* and *Mmp13*) (Fig 5A). As the proteolytic degradation of fibrillar collagen by stromal matrix metalloproteases

(MMPs) is an important step in mammary ductal outgrowth and branching (Wiseman *et al*, 2003), we investigated whether collagen degradation was compromised in *Sharpin^{cpdm}* MSFs with reduced MMP expression. MSFs, isolated from wt and *Sharpin^{cpdm}* mice, were plated on fluorogenic fibrillar DQ™ collagen-coated crossbow-shaped micropatterns, to constrain cell shape and allow cell polarisation (Thery *et al*, 2006), and collagen degradation was monitored. Indeed, *Sharpin^{cpdm}* MSFs displayed diminished collagenolytic activity as indicated by a lower fluorescence signal (Fig 5B). These data demonstrate that SHARPIN influences the expression of both collagens and collagen-degrading enzymes, most likely reflecting the altered stromal environment observed *in vivo*.

SHARPIN deficiency reduces traction forces applied on collagen

In connective tissue, fibroblasts generate traction forces and reorganise collagen fibres in response to mechanical and chemical cues (Tschumperlin, 2013). To investigate how SHARPIN deficiency affects the capacity of cells to contract collagen *in vitro*, MSFs were seeded inside a floating 3D collagen plug (Fig 5C). Indeed, *Sharpin^{cpdm}* MSFs were significantly less capable of contracting collagen, compared to wt MSFs (Fig 5C and D). Similar results were obtained with the partial silencing of SHARPIN achievable in these primary cells, as *Sharpin* siRNA also significantly reduced

Figure 5.

collagen contraction of wt cells (Fig 5D and E). As mechanical forces are required for large-scale collagen contraction, we also analysed how SHARPIN deficiency influenced the traction forces exerted by MSFs on the microenvironment. To this end, the cells

were plated on collagen-coated hydrogels (1.1 kPa) and imaged by traction force microscopy (Fig 5F and G). As expected, wt cells created significantly higher traction stresses than *Sharpin^{cpdm}* cells (Fig 5F) indicating a difference in the ability of the cells to apply

Figure 5. SHARPIN regulates collagen remodelling and traction force generation.

- A Ingenuity Pathway Analysis of differential gene expression [fold-change (FC) > 1.5, $P < 0.05$] in wt and *Sharpin*^{cpdm} MSFs (passage 0). The differentially expressed genes implicated in the most significantly altered canonical pathways (leukocyte adhesion and transmigration, and tissue fibrosis) are shown. Three independent cell isolation replicates were compared pairwise. FC colour scale represents a range from 0 to 4.
- B Analysis of collagen degradation on crossbow-shaped micropatterns coated with the DQ™ collagen substrate. Mean intensity projection images of the DQ™ collagen fluorescence (top panel; cell area is outlined by dashed line; original integer range 0–255) and single confocal images of the fibrinogen-AlexaFluor647-marked micropatterns (bottom panel) are shown ($n \geq 53$ from four independent experiments). Scale bars represent 10 μ m.
- C, D Collagen plug contraction assay using wt and *Sharpin*^{cpdm} MSFs or wt MSFs with SHARPIN siRNA silencing. (C) Representative light microscopy images of the collagen plugs 3 days post-cell culture. (D) Quantification of the average collagen plug area 1–3 days after seeding ($n = 3–6$). Scale bars represent 2 mm.
- E SHARPIN protein level after silencing with negative control siRNA or SHARPIN siRNA in wt MSFs. Tubulin was used as loading control.
- F–I Traction force microscopy analysis of wt and *Sharpin*^{cpdm} MSFs on collagen-coated matrices. (F) Representative images of traction force maps observed with wt and *Sharpin*^{cpdm} cells (colour scale represents the magnitude of traction stresses in Pa; cell boundaries are indicated by dashed lines). (G) Quantification of strain energy per cell ($n = 8–11$ cells from two independent experiments). (H) Representative images of the traction force maps and mCherry fluorescence of mCherry-Ctrl-transfected wt cells and mCherry-Ctrl or mCherry-SHARPIN-transfected *Sharpin*^{cpdm} cells (colour scale represents the magnitude of traction stresses in Pa; cell boundaries are indicated by dashed lines). Scale bars represent 20 μ m. (I) Quantification of strain energy per cell (wt mCherry-Ctrl $n = 26$; *Sharpin*^{cpdm} mCherry-Ctrl $n = 21$; *Sharpin*^{cpdm} mCherry-SHARPIN $n = 24$ cells from five independent experiments).
- J, K Focal adhesion dynamics. (J) Visualisation of GFP-paxillin dynamics in a wt and a *Sharpin*^{cpdm} cell over time. Colour scale represents a range from early to late occurring focal adhesions. (K) Quantification of focal adhesion average size, assembly rate and disassembly rate (wt $n = 17,527, 5,250, 5,311$ FAs; and *Sharpin*^{cpdm} $n = 9,052, 2,790, 3,264$ FAs, respectively from two independent experiments).

Data information: (D, G, I, K) Mean $\pm$ SEM. (D) Wilcoxon test, (G, I, K) Mann–Whitney test.

mechanical force (strain energy, Fig 5G) on the substrate in the absence of SHARPIN. Importantly, traction force generation (on 1.8 kPa gels) was rescued to wt level by ectopic expression of SHARPIN-mCherry in *Sharpin*^{cpdm} mammary gland fibroblasts (Fig 5H and I).

The relative activity of integrin beta 1 heterodimers that bind cells to collagen and mediate cell traction was elevated in *Sharpin*^{cpdm} MSFs compared to wt cells when measured by an active integrin beta 1 conformation-specific antibody and flow cytometry (Fig EV5C), suggesting that the negative regulation of integrin activity by SHARPIN might play a role in traction force generation (Rantala *et al*, 2011). *Sharpin*^{cpdm} mammary gland fibroblasts also activated focal adhesion-induced signalling [phosphorylation of protein tyrosine kinase 2 (PTK2), also called focal adhesion kinase (FAK)] more rapidly than wt cells (Fig EV5D and E) indicative of faster formation and maturation of focal adhesions. Furthermore, our analysis of focal adhesion dynamics by live cell total internal reflection (TIRF) imaging of GFP-paxillin expressing cells on collagen revealed that *Sharpin*^{cpdm} cells form on average larger focal adhesions and have faster focal adhesion assembly and disassembly rates (Fig 5J and K), suggesting that SHARPIN deficiency leads to abnormal cell adhesion dynamics and compromised traction force generation.

Taken together, reduced collagen contraction and traction force in *Sharpin*^{cpdm} MSFs suggest that SHARPIN is required for efficient collagen fibre arrangement, a function important for ECM regulation during mammary branching morphogenesis.

SHARPIN regulates collagen fibre assembly in cell-derived matrices

To indirectly analyse the capability of *Sharpin*^{cpdm} MSFs to convert newly synthesised collagen into insoluble fibrillar collagen, the amount of acid-soluble collagen in wt and *Sharpin*^{cpdm} MSF cultures was measured by Sirius red assay (Marotta & Martino, 1985; Tullberg-Reinert & Jundt, 1999). Similar assays have previously demonstrated that during the first 2 days, fibroblasts produce an increasing amount of soluble collagen in the culture medium and starting from day 4, soluble collagen levels decline, and deposition of fibrillar collagen increases (Chen *et al*, 2013). Despite initially lower levels of soluble

collagen production in *Sharpin*^{cpdm} MSFs when compared and normalised to wt values (Fig 6A), the *Sharpin*^{cpdm} culture media contained significantly more soluble collagen than wt media on day 7 post-plating (Fig 6A). These data indirectly suggest a defect in fibrillar collagen deposition in the absence of SHARPIN (Fig 6A). Fibroblasts, *in vitro*, are capable of producing a cell-derived matrix (CDM) composed of fibrillar collagen, fibronectin and laminin, closely resembling the *in vivo* ECM (Cukierman *et al*, 2001). Immunofluorescence (IF) labelling of collagen I revealed that distinct collagen bundles were the predominant form of collagen in the CDM produced by wt MSFs (Fig 6B and C), whereas cells isolated from *Sharpin*^{cpdm} mice more frequently produced CDM consisting only of a collagen fibre mesh (Fig 6B and C). These *in vitro* data recapitulate the less organised stromal phenotype observed *in vivo* by SHG imaging of the collagen ECM in *Sharpin*^{cpdm} mouse mammary glands (Fig 4A and B). Our data suggest that in addition to affecting traction force generation, SHARPIN supports ECM remodelling by regulating fibroblast-mediated fibrillar collagen assembly and degradation in the mammary gland.

Discussion

Breast cancer-associated changes in ECM structure and stiffness promote invasion and metastasis (Schedin & Keely, 2011; Acerbi *et al*, 2015; Robertson, 2015). However, it is unclear how changes in stromal cell activity translate into alterations in the ECM structure, and which molecules are involved in this process. Mammary gland branching morphogenesis has been used as a developmental model system to decipher the mechanisms of collective cell invasion and epithelial–stromal crosstalk (Howard & Lu, 2014; Zhu *et al*, 2014). Here, we show that *Sharpin*^{cpdm} mice lacking SHARPIN, a protein involved in the regulation of NF- κ B signalling and integrin activity regulation among other functions, have impaired mammary ductal outgrowth during puberty. The *Sharpin*^{cpdm} mouse phenotype stems from an abnormal stromal environment where collagen distribution is altered and tissue stiffness is reduced. We find that SHARPIN is required for cell traction force generation on collagen matrices and for collagen fibre assembly, contraction and degradation by MSFs. The importance of SHARPIN in these ECM-related

Figure 6. SHARPIN regulates the assembly of cell-derived ECM *in vitro*.

A Quantification of soluble collagen levels in wt and *Sharpin*^{cpdm} MSF cultures on days 2, 5 and 7 post-serum removal ($n = 6$ from two independent experiments). **B, C** Analysis of the collagen network in cell-derived matrices (CDMs) produced by wt and *Sharpin*^{cpdm} MSFs using COL1A1 immunolabelling. **(B)** Maximum intensity projections of confocal images are shown. Scale bars represent 50 μm . **(C)** Quantification of distinct collagen fibre bundles in wt and *Sharpin*^{cpdm} CDM samples ($n = 5$).

Data information: (A, C) Mean $\pm$ SEM; Mann–Whitney test.

remodelling processes is reflected *in vivo* in *Sharpin*^{cpdm} mice that demonstrate reduced stromal stiffness during mouse mammary ductal outgrowth.

In mouse mammary epithelium that lacks the inhibitor of kappaB alpha gene, increased NF- κ B signalling triggers excessive cell proliferation and ductal branching when transplanted into wt fat pads (Brantley *et al*, 2001). The data presented here demonstrate that *Sharpin*^{cpdm} mammary epithelium, with a potential reduction in NF- κ B signalling due to impaired LUBAC function (Gerlach *et al*, 2011; Tokunaga *et al*, 2011; Fujita *et al*, 2014), develops normally in the wt fat pad. Furthermore, deletion of *Sharpin* specifically from stromal cells using the *S100a4* promoter impairs mammary ductal outgrowth, suggesting that epithelial SHARPIN is not required for normal mammary gland branching morphogenesis. Instead, SHARPIN might play a role in TNF-induced NF- κ B signalling in the mammary gland stromal cells. For example, TNF has been shown to increase the expression of pro-tumorigenic chemokines via NF- κ B in breast cancer-associated fibroblasts (Katanov *et al*, 2015).

We also observed increased accumulation of innate leukocytes in the mammary glands of *Sharpin*^{cpdm} mice. Since the lack of macrophages in CSF1-deficient mice and the lack of eosinophils in eotaxin-deficient mice lead to defective mammary branching morphogenesis (Gouon-Evans *et al*, 2000), we find it unlikely that the enhanced leukocyte infiltration in the *Sharpin*^{cpdm} mice would critically contribute to the defective ductal invasion observed in these mice. However, increase in eosinophils may also affect branching morphogenesis, since interleukin-5 (IL5) transgenic mice (Tg(CD2-IL5) 5C2Ldt) with a massive 265-fold increase in blood eosinophils (Dent *et al*, 1990) show mammary gland eosinophilia and delayed TEB formation, ductal branching and ductal elongation in puberty (Sferruzzi-Perri *et al*, 2003). Nevertheless, we observed impaired mammary fat pad invasion also in the presence of normal numbers of eosinophils and macrophages in *S100a4-Cre; Sharpin*^{fl/fl} mice. Fibroblast-specific transgene expression *in vivo* is complicated by the lack of naturally occurring lineage-specific marker genes, and in this study, we chose to use one of the most specific and commonly used promoters, the *S100a4* promoter, for *Cre* expression (Bhowmick *et al*, 2004).

In addition to being expressed in fibroblasts of the mammary gland (Cheng *et al*, 2005; Trimboli *et al*, 2009; Pickup *et al*, 2015; Koledova *et al*, 2016), kidney (Strutz *et al*, 1995), lung (Lawson *et al*, 2005) and skin (Osterreicher *et al*, 2011), the *S100a4* promoter is also expressed in liver macrophages (Osterreicher *et al*, 2011), and kidney interstitial leukocytes (Le Hir *et al*, 2005). Thus, although our data collectively suggest that the defects in stromal fibroblasts may be the primary contributor to the *Sharpin*^{cpdm} mammary gland phenotype, the specific contributions of SHARPIN in mammary gland fibroblasts and the stromal immune cells during mammary gland ductal invasion *in vivo* remain to be formally studied.

The mammary gland ECM is subject to change during development, pregnancy and lactation, and upon formation of breast tumours (Schedin & Keely, 2011). Numerous genes expressed in the mouse mammary epithelium have been identified as important regulators of mammary gland morphogenesis, but only relatively few studies have analysed the contribution of stromal-dependent ECM regulation. In pubertal mice, stromal macrophages promote mammary ductal outgrowth (Gouon-Evans *et al*, 2000) and regulate the shape of TEBs as well as the surrounding collagen fibrillogenesis (Ingman *et al*, 2006). Furthermore, the patterning of collagen fibres in the mammary gland stroma was found to regulate the orientation of TEBs during branching morphogenesis (Brownfield *et al*, 2013). Our data show that SHARPIN is involved in regulating ECM stiffness and collagen arrangement in the developing mammary gland stroma *in vivo*. In addition, *Sharpin*^{cpdm} MSFs have reduced capacity to contract and organise collagen *in vitro*. Thus, SHARPIN plays an important role in regulating collagen ECM in the mammary gland.

Mammary stromal MMP-2 expression facilitates TEB invasion and represses precocious lateral branching, while MMP-3 regulates secondary branching during mid-puberty (Wiseman *et al*, 2003). Furthermore, lack of stromal MMP-11 leads to decreased periductal collagen content and reduced ductal outgrowth (Tan *et al*, 2014). These studies highlight the importance of stromal collagen proteolysis for pubertal mouse mammary gland development. Reduced expression of several collagenases and impaired collagenase activity was observed in primary MSFs as a result of SHARPIN deficiency

indicating that reduced mammary ductal branching and outgrowth in *Sharpin^{cpdm}* mice could be due to diminished stromal MMP activity. Some of the differentially expressed genes have also been linked to angiogenesis. However, there was no apparent difference in blood vessel density between wt and *Sharpin^{cpdm}* mammary glands, suggesting that the developmental defects were not secondary to impaired angiogenesis.

The data presented here also demonstrate that SHARPIN deficiency triggers altered focal adhesion turnover and reduced traction force generation in MSFs plated on collagen. As integrins in focal adhesions couple the ECM to the actin cytoskeleton and mediate cellular force transmission (Schwarz & Gardel, 2012), the ability of SHARPIN to inhibit integrin activity (Rantala *et al*, 2011) could represent an important regulatory step in focal adhesion dynamics and mechanotransduction. Recent data suggest that dynamic integrin regulation is required for effective matrix contraction as expression of constitutively active integrin beta 1 reduces cell traction force (Elloumi-Hannachi *et al*, 2015). In line with this, elevated integrin beta 1 heterodimer activity was observed in primary *Sharpin^{cpdm}* MSFs. Whether SHARPIN affects fibroblast traction forces directly via integrin regulation, or by other mechanisms regulated by this multifunctional protein, remains to be investigated.

Pregnancy-induced changes in the stromal collagen content and stiffness protects rats from breast tumorigenesis (Maller *et al*, 2013). On the other hand, radial collagen alignment at the mammary tumour-stromal boundary is associated with increased local invasion in mice (Provenzano *et al*, 2006), and increased collagen fibre straightness, alignment and length in tumour-associated ECM correlate with poor prognosis in cancer patients (Conklin *et al*, 2011; Hanley *et al*, 2015). SHARPIN expression has been shown to correlate with disease grade, poor patient survival and metastasis in breast cancer (Bii *et al*, 2015; De Melo & Tang, 2015). Reduced stiffness, altered collagen organisation and impaired epithelial invasion were observed in the *Sharpin^{cpdm}* mouse mammary gland suggesting that altered SHARPIN expression in cancer cells or cancer-associated stromal cells could also contribute to the previously reported changes in the breast tumour ECM (Conklin *et al*, 2011; Hanley *et al*, 2015). While our results show that SHARPIN regulates ECM in the mouse mammary gland stroma, the possible implications in cancer stroma remain to be studied.

Taken together, our study identifies SHARPIN as a stromal regulator of mammary gland ductal outgrowth. SHARPIN loss correlates with reduced amount of collagen bundles *in vivo* and *in vitro*, and this is most likely linked to the inability of *Sharpin^{cpdm}* MSFs to contract, pull and degrade collagen ECM. Our findings indicate that SHARPIN regulates stromal collagen architecture and mammary ductal outgrowth during development and thereby provide new insight on the stromal mechanisms that may also regulate breast cancer cell invasion.

Materials and Methods

Animals

The inbred C57BL/KaLawRij-*Sharpin^{cpdm}*/RijSunJ mouse strain (Stock No: 007599) with a spontaneous mutation leading to the complete loss of SHARPIN protein (HogenEsch *et al*, 1993; Seymour

et al, 2007) was acquired from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/KaLawRij-+/+*Sharpin^{cpdm}* were crossed to generate C57BL/KaLawRij-*Sharpin^{cpdm}*/*Sharpin^{cpdm}*, C57BL/KaLawRij-+/+*Sharpin^{cpdm}* and C57BL/KaLawRij-+/+ mice. The colony was maintained in heterozygote breeding and genotyped for the *Sharpin^{cpdm}* mutation. DNA was extracted with KAPA Mouse Genotyping Kit (KK7302) and the *Sharpin^{cpdm}* mutation detected using 40× genotyping assay mix (TaqMan SNP Genotyping Assays, 5793982, Applied Biosystems) and TaqMan Universal PCR Master Mix. For timed mating, mice were examined for mating plug appearance and sacrificed on P15. Female *Sharpin^{cpdm}* mice (homozygous for the *cpdm* allele) with the indicated age were used in the experiments with wild-type littermate controls (negative for the *cpdm* allele). The *Sharpin^{cpdm}* and wt mice had not established an oestrus cycle by the time samples were collected (maximum 49 days old) and were therefore not synchronised for oestrus.

For cell sorting and qPCR experiments, mammary glands were collected from adult virgin wild-type BALB/c mice. BALB/c-Tg (S100a4-cre)1Egn/YunkJ (Stock number 012641, The Jackson Laboratory) X B6(Cg)-*Tyr^{cre-2J}* *Sharpin^{tm1Sun}*/Sun (repository number 029265) conditional SHARPIN knockout strain was created at The Jackson Laboratory. Detailed characterisation of the mice will be reported elsewhere (C.S. Potter, C.H. Pratt, K.A. Silva, V.E. Kennedy, T.M. Stearns, L. Godwin, H. HogenEsch, and J.P. Sundberg, manuscript under preparation). Mice were housed in standard conditions (12-h light/dark cycle) with food and water available *ad libitum*. All animal experiments were ethically assessed and authorised by the National Animal Experiment Board and in accordance with The Finnish Act on Animal Experimentation (Animal licence number 7522/04.10.03/2012) and The Jackson Laboratory Animal Care and Use Committee (approval number 07005).

Antibodies and plasmids

The following antibodies were used in the study: ITGA6 (integrin alpha 6; clone GoH3, Serotec), CDH1 (E-cadherin, 24E10, Cell Signaling), ACTA2 (alpha smooth muscle actin; clone 1A4, Sigma), KRT8 (keratin 8, clone Troma-I, Hybridoma Bank), SHARPIN (14626-1-AP, Proteintech), COL1A1 (collagen I alpha 1, NB600-408, Novus), GAPDH (5G4 Mab 6C5, Hytest), S100a4 (ab27957, Abcam), tubulin (12G10, Hybridoma Bank) and vimentin (VIM, D21H3, 5741, Cell Signalling). AlexaFluor-conjugated (Life Technologies) and HRP-linked (GE Healthcare) secondary antibodies against rat, rabbit and mouse IgG were used in immunolabelling and Western blotting, respectively. For flow cytometry, CD45-APC (PTPRC, clone 30-F11), CD54-PE (ICAM1, clone YN1/1.7.4), PECAM1-APC (PECAM1, clone MEC13.3), CD24-Brilliant Violet 421, CD49f-PEcy7 (ITGA6) (all from BioLegend) and CD24-FITC (clone M1/69; BD Biosciences) antibodies were used.

For construction of mCherry-SHARPIN, the GFP in GFP-C1 (Clontech) was replaced with mCherry using primers introducing NheI and EcoRI restriction sites, resulting in pmCherry-C1. Subsequently, the human *Sharpin* coding sequence (with four silent mutations that render the mRNA insensitive to siRNA1) was amplified from the GFP-Sharpin expression vector (Rantala *et al*, 2011) using primers introducing EcoRI and BamHI restriction sites and cloned into pmCherry-C1, resulting in mCherry-Sharpin. Full-length paxillin (PXN) in pEGFPC2 vector was a gift from Vic Small (IMBA, Vienna, Austria).

Whole-mount staining and quantification

The fourth mammary gland was placed on an object glass, left to adhere briefly, and submerged in Carnoy's medium (60% EtOH, 30% chloroform, 10% glacial acetic acid). Tissue was fixed overnight at +4°C, rehydrated in decreasing ethanol series and stained with carmine alum (0.2% carmine, 0.5% aluminium potassium sulphate dodecahydrate) overnight at room temperature. Samples were dehydrated and subsequently bleached in xylenes for 2–3 days. After mounting in DPX Mountant for histology (Sigma), the samples were imaged with Zeiss SteREO Lumar V12 stereomicroscope (NeoLumar 0.8× objective, Zeiss AxioCam ICc3 colour camera). Several images were automatically combined into a mosaic picture with Photoshop.

Ductal outgrowth was quantified by measurement of the area (mm²) covered by the ductal tree in mosaic images of the mammary gland whole mounts in ImageJ. Branch number was measured from whole-mount images where the ductal tree was traced manually (virgin mice) and analysed with “skeletonise” and “analyse skeleton” plug-ins in ImageJ. The number of TEBs was measured individually from each whole-mount image in ImageJ.

Isolation and culture of mouse mammary gland cells

Primary MSFs were isolated from the mammary glands of 6- to 7-week-old virgin mice. Briefly, the 2nd, 3rd, 4th and 5th mammary glands from 2 to 4 wt and *Sharpin*^{cpdm} mice were removed aseptically without lymph nodes, minced with surgical blades and incubated in a shaker for 2–3 h at 37°C in 25–30 ml of digestion media [DMEM (Dulbecco's modified Eagle's medium)/F12, 5% foetal calf serum (FCS), 5 µg/ml insulin, 50 µg/ml gentamicin] containing 2 mg/ml collagenase type XI (Sigma). Then, the cell suspensions were centrifuged 10 min at 400 g to eliminate floating fat cells. Cell pellets were resuspended in isolation media (DMEM/F12, 50 µg/ml gentamicin, pen/strep) with 20 U/ml DNase I (Roche) and incubated for 3 min at room temperature with occasional shaking. Cells were pelleted and disaggregated by pipetting up and down 10 times in 10 ml of isolation media. After each round of pulse centrifugation at 400 g, the supernatant containing single cells was collected and pooled. After four rounds of pulse centrifugation, the pellet containing mammary epithelial ducts was collected. Mouse MECs were trypsinised and pushed through a cell strainer (70 µm, 352350 BD Biosciences) to obtain single cells. The supernatants containing MSFs were also pelleted and resuspended in fibroblast growth media (DMEM/F12, 5% FCS, L-glutamine, pen/strep). Medium was replaced the following day to remove dead cells. Passages 1–4 were used in the experiments if not otherwise indicated, and at least two independent isolates of MSFs per genotype were used in each experiment. The MSF cell cultures were all adherent and homogeneous in appearance, expressed vimentin and did not express epithelial (CDH1) or endothelial (PECAM1) cell markers (data not shown).

Western blotting

Snap-frozen mouse mammary glands were homogenised in TX lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 5% glycerol, 1% SDS, Complete protease inhibitor, PhosSTOP (Roche)] using MagNA Lyser instrument (Roche) with MagNA Lyser

green beads (Roche). *In vitro* cultured cells were also lysed in TX lysis buffer. Samples were sonicated with BioRuptor, protein concentration was measured by Bio-Rad, and equal amounts of protein were loaded on 4–20% Mini-PROTEAN[®] TGX[™] Gel SDS-PAGE gradient gels (Bio-Rad). Proteins were transferred with Trans-Blot Turbo Transfer Pack (Bio-Rad). In immunoblotting, the following primary antibodies were used (diluted 1:1,000 in 5% milk, 0.1% Tween-20 in TBS): GAPDH (5G4 Mab 6C5, Hytest), tubulin (12G10, Hybridoma Bank, 1:5,000), vimentin (D21H3, 5741, Cell Signalling), CDH1 (E-cadherin, 24E10, Cell Signalling), S100a4 (ab27957, Abcam) and SHARPIN (14626-1-AP Proteintech). Amersham ECL Plus[™] and Odyssey[®] CFX (LICOR) Western blotting reagents were used for signal detection.

Mouse tissue samples

Formalin-fixed or Carnoy's medium-fixed and paraffin-embedded mouse mammary gland tissue sections were deparaffinised, rehydrated and stained with conventional HE. For IHC, epitope unmasking was performed in citrate buffer using 2100 Antigen Retriever (Aptum, UK). Samples were blocked with 0.5% FCS in PBS for 45 min and incubated overnight with primary antibodies in blocking buffer. Samples were washed with PBS and incubated with fluorochrome-conjugated secondary antibodies for 2 h at RT. For cryosections, mammary gland tissue was embedded in Tissue-Tek[®] O.C.T. compound (Sakura) and snap-frozen, and 6-µm sections were cut for HE and staining. For IF, cryosections were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min RT, and permeabilised and blocked in 2% bovine serum albumin (BSA) 0.1% Triton X-100 for 20 min at RT. Sections were labelled with primary antibodies in 2% BSA/PBS for 1 h at RT, followed by fluorochrome-conjugated secondary antibodies for 1 h at RT. All samples were washed, stained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Life Technologies) and mounted in Mowiol containing DABCO[®] (Sigma) anti-fading reagent. Samples were imaged with Zeiss Axiovert 200M inverted wide-field microscope (HE) and Zeiss Axiovert 200M with Yokogawa CSU22 spinning disc confocal microscope unit with Hamamatsu Orca ER CCD camera (Hamamatsu Photonics K.K.) (IF).

Human tissue samples

Formalin-fixed paraffin-embedded human mammary gland tissues were collected from the archives of the Department of Pathology, Helsinki University Central Hospital, Helsinki, Finland. An Institutional Review Board of the Helsinki University Central Hospital approved the study. Samples were deparaffinised and rehydrated. Endogenous peroxidase activity was removed with 3% hydrogen peroxide, and heat-mediated epitope unmasking was performed in 10 mM sodium-citrate buffer (pH 6.0). Samples were incubated with SHARPIN antibody (1:75 in PowerVision blocking solution overnight at 4°C) followed by Histofine[®] Simple Stain anti-rabbit secondary antibody (Nichirei Biosciences, 30 min at RT). Antibody complexes were detected with 3,3'-diaminobenzidine (DAB) solution (brown precipitate, ImmPact), and nuclei were counterstained with haematoxylin. After dehydration, samples were mounted and viewed with Olympus BX43 microscope and Lumenera Lt425 camera using a 20× objective (200× magnification).

Cleared fat pad transplantation

A mammary gland piece (approx. 1 mm³) from 7-week-old wt or *Sharpin*^{cpdm} female donor mice was transplanted under isoflurane anaesthesia and analgesia (Temgesic, Rimadyl) to the fourth fat pad of 3-week-old wt hosts (wt and *Sharpin*^{cpdm} transplants on each side of the host) after clearing the fat pad up to, and including, the lymph node. The removed part of the fat pad was fixed and stained to confirm complete removal of the recipient mouse mammary epithelium. Growth of transplants was analysed after 7–11 weeks in virgin mice ($n = 9$) or after 13 weeks on day 15 of the first pregnancy (P15; $n = 8$) by carmine alum staining of the 4th mammary gland whole mounts. Growth take-on-rate was calculated from both virgin and P15 transplant samples ($n = 17$), and fat pad filling rate was evaluated from the virgin mouse transplants that had initially begun to grow ($n = 7$ –10). Terminal differentiation of the 2nd–3rd mammary gland (P15) was monitored as a control sample.

Cell preparation, sorting and qPCR

Single cells were prepared from inguinal mammary glands taken from virgin adult, P15 BALB/cByJ females according to a detailed protocol described previously (Di-Cicco *et al*, 2015). Freshly isolated cells were incubated at 4°C for 20 min with the conjugated antibodies. Labelled cells were sorted on a FACS Vantage flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analysed using FlowJo software. CD45⁺ immune cells and CD31⁺ endothelial cells were excluded during the cell sorting procedure. Mammary gland lineage-specific gene expression (*Krt5*, *Krt18* and *Pdgfr*) in the purified cell populations was checked by qPCR as reported (Di-Cicco *et al*, 2015). RNA was reverse-transcribed with MMLV H(–) Point reverse transcriptase (Promega, Madison, WI, USA), and qPCR was performed by monitoring, in real time, the increase in fluorescence of the SYBR Green dye in a LightCycler[®] 480 Real-Time PCR System (Roche Applied Science, Basel, Switzerland). The primers used for qPCR analysis (*Sharpin*, *Krt5*, *Krt18*, *Pdgfr* and *Gapdh*) were purchased from SABiosciences/Qiagen (Hilden, Germany).

Collagen contraction

The SHARPIN siRNA (5'-GCUAGUAAUUAAGACACAd(TT)-3') and the scramble Allstars negative control siRNA were ordered from QIAGEN. Transient transfection was performed with Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to manufacturer's instructions. Transfection efficiency was determined by Western blotting of total cell lysates. Cells were seeded in collagen contraction assays as in (Vuoriluoto *et al*, 2011). Briefly, 1×10^5 MSFs in 30 µl medium were mixed per 170 µl collagen (PureCol[®] EZ Gel Bovine Collagen Solution, type I in DMEM/F-12 medium; Advanced Biomatrix, San Diego, CA, USA) and plated on a 24-well ($V = 500$ µl) or 48-well ($V = 170$ µl) plate. After polymerisation at 37°C for 1–2 h, fibroblast growth media was added and the collagen plug was gently detached from the well edges. After 1 day (48-well) or 3 days (24-well), in culture the collagen plug area was imaged with Bio-Rad ChemiDoc MP gel analysis instrument and quantified by ImageJ software. Average plug areas in each experiment were normalised to wt or ctrl siRNA.

Traction force microscopy

Traction force microscopy was performed as described in Elkhathib *et al* (2014), with minor modifications. Glass-bottom dishes (World Precision Instrument Inc.) were silanised with 3-aminopropyl-trimethoxysilane (Sigma-Aldrich, St. Louis, MO, USA). The dishes were washed extensively with water, and the glass surface was treated for 30 min with 0.5% glutaraldehyde followed by a final wash. 40% acrylamide (47 µl) and 2% bis-acrylamide (7.5 µl or 25 µl) (Bio-Rad Laboratories, Richmond, CA, USA) were mixed in PBS solution to a final volume of 250 µl to achieve a Young's modulus of approximately 1,100 and 1,750 Pa, respectively, as experimentally measured by AFM indentation. For traction force measurements, FluoSphere bead solution (0.2 µm, 505–515 nm; Invitrogen) was added at 8% volume and the mixture was briefly sonicated. Polymerisation was initiated by addition of 2.5 µl freshly prepared ammonium persulphate (10% w/v solution) and 0.5 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED). Immediately after initiation, the polyacrylamide solution (12 µl) was pipetted onto the glass-bottom dish, and a coverslip was quickly placed on top of the gel droplet and gently pressed down. After polymerisation, the gel was immersed in PBS for 10 min, and then, the top coverslips were gently removed under PBS, followed by three washes with PBS for 10 min. Then, the gel surface was activated to allow for collagen coating by applying a solution containing 50 mM Hepes pH 7.5, 10 mg/ml of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Thermo Scientific) and 1 mg/ml of Sulfo-SANPAH (Pierce) for 30 min at RT. After the incubation, the cross-linker Sulfo-SANPAH was photo-activated by UV light for 10 min. After several washes with PBS, the gels were coated with 20 µg/ml of collagen for 1 h at RT. Before seeding the cells, the gels were incubated in the cell culture media for a minimum of 30 min at 37°C. The non-transfected, mCherry-Ctrl-transfected or mCherry-SHARPIN-transfected cells were plated at least 12 h before imaging to allow for proper spreading. For time-lapse imaging, Roper/Nikon wide confocal spinning disc microscope and for single-point imaging a Zeiss LSM780 microscope was used. In both cases, a fluorescence image of beads and a phase contrast image of the cells were recorded, cells were detached by adding 10% Triton (Euromedex) or 10× trypsin, and a reference image without cells was recorded. To ensure good quality imaging of fluorescent beads, Z stacks of 15–30 images with a distance of 1 µm were performed and the best focus chosen (MetaMorph and ImageJ softwares). For data analysis, a previously described correlation algorithm was used to extract the bead displacement fields (Betz *et al*, 2011). Traction forces were determined using the Fourier transform traction force algorithm as introduced by Butler *et al* (2002). To quantify the contractile potential per cell, the strain energy was measured, which corresponds to the mechanical energy the cells expend to deform the substrate, and is as such a measure for the absolute tensile force generation per cell.

Atomic-force microscopy indentation

Tissues were prepared for AFM as in Lopez *et al* (2011) with slight modifications. The fourth mammary glands from wt and *Sharpin*^{cpdm} female mice were snap-frozen in liquid nitrogen, stored at –80°C. O.C.T.-embedded tissues in TissueTEK[®] standard plastic moulds, cut with Leica 819 low profile microtome blades into 30-µm

sections directly on 22-mm coverslips with Cryostat Leica CM 1950 and refrozen.

All AFM indentations were performed using a JPK NanoWizard II AFM with its CellHesion module (JPK Instruments), mounted on a Carl Zeiss confocal microscope Zeiss LSM510 (Carl Zeiss AG). Triangular silicon nitride cantilevers with a spring constant of 0.06 Nm^{-1} were custom fitted with borosilicate glass spheres $5 \mu\text{m}$ in diameter (Novascan Tech) and calibrated using the thermal noise method prior to each experiment (Hutter & Bechhoefer, 1993). The deflection sensitivity was determined in fluid using glass substrates as an infinitely stiff reference material. Tissue was indented in DMEM supplemented with 10% FCS and at 37°C . A calibrated force of 5 nN was applied, and the Hertz model of impact (Hertz, 1881) was used to determine the elastic properties of the tissue. The Young's elastic modulus was calculated using the JPK data processing software (JPK DP version 4.2) and an input Poisson's ratio of 0.5. Indentation was performed in tissue coming from three independent mice per condition and using 3–6 sections of $20\text{-}\mu\text{m}$ thickness per mouse. For each slice, a total of 192 indentation curves distributed in three regions were performed with an 8×8 point grid ($100 \times 100 \mu\text{m}^2$) in each region. A total of 21 mammary gland slices coming from six mice were analysed. All individual stiffness values for a sample were summarised in Igor Pro 8.37 (WaveMetrics) to obtain the distribution of stiffness values. The bin width was set to 25 Pa, and counts were normalised to the total amount of measured stiffness value per sample. For data fitting, peaks were located and fitted with the Multi-Peak Fit analysis option of Igor Pro. The statistical difference in mean values was assessed with the paired Student's *t*-test in R: Language and Environment for Statistical Computing (R Core Team).

Micropatterns

Crossbow micropatterns were produced on glass coverslips as described by Azioune *et al* (2009). Cells were plated on the micropatterns coated with type I collagen from rat tail ($20 \mu\text{g/ml}$; 08-115, Millipore) or DQTM collagen type I fluorescein conjugate ($100 \mu\text{g/ml}$; D12060, Life Technologies) and AlexaFluor 647-conjugated human plasma fibrinogen ($5 \mu\text{g/ml}$; Molecular Probes) for 4 h in fibroblast growth medium and analysed by IF labelling. The samples were fixed for 10 min in 4% PFA in PBS and stained with AlexaFluor 546 phalloidin (Life Technologies) and DAPI. Washed samples were mounted in Mowiol containing DABCO and imaged with Zeiss LSM780 laser scanning confocal microscope.

Micropattern average intensity maps were obtained as in Thery *et al* (2006) and Vonaesch *et al* (2013). Briefly, the signal of single-cell 2D images from one condition was assembled into one stack and aligned using the corresponding fluorescent micropattern for each cell. Then, the average intensity of each pixel over the assembled stack of aligned cells was calculated by an Average Intensity Projection. A heat map (16 colours Lookup Table) was applied to the Z-projected image to facilitate examination and interpretation of the experimental results.

Second harmonic imaging

Second harmonic generation (SHG) microscopy was used to investigate collagen fibres of mouse mammary gland tissue samples.

SHG images were acquired from carmine alum-stained mammary gland whole-mount samples from 6- to 7-week-old wt and *Sharpin*^{cpdm} female mice by using a Leica SP5 MP multiphoton microscope system on DM6000 CFS upright microscope, with a $20\times 1.0 \text{ W}$ objective, and two photon excitation set to 890 nm wavelength (Leica Microsystems, Mannheim, Germany). Non-filtered emission signal was used to observe a common tissue structure, and the SHG signal was separated with a 440/20 emission filter. Individual TEBs were imaged over a whole-tissue volume with a z-axis step size of $5.0 \mu\text{m}$.

Maximum intensity projection images were made from selected four representative continuous slices for all terminal end bud (TEB) image stacks per mouse (wt $n = 9$ mice, 5–13 TEBs per mouse; *Sharpin*^{cpdm} $n = 6$, 5–12 TEBs per mouse). In some images, the artificial fibres such as from blood vessels or membrane structures were excluded by only considering the fibres in the manually defined regions of interest. Quantification of the collagen fibres from the SHG image was done by blindly scoring randomised maximum intensity projection images by two people independent for presence of clear collagen bundles or only mesh-like, curly collagen fibres. The percentage of images with clear collagen bundles for each mouse was calculated and analysed.

Cell-derived matrix production

Cell-derived matrices were prepared as described in Cukierman *et al* (2001). Shortly, coverslips were coated with 0.2% gelatin (Sigma G1393 in PBS) for 60 min at 37°C . Gelatin cross-linking was done with 1% glutaraldehyde for 30 min at room temperature followed by 20 min incubation with 1 M glycine at RT. 5×10^4 wild-type or *Sharpin*^{cpdm} low-passage mammary gland fibroblasts were seeded on the gelatin-coated coverslips. To promote collagen synthesis, ascorbic acid treatment ($50 \mu\text{g/ml}$) was started when the cell layer was fully confluent. Ascorbic acid-containing medium was changed every day over 10 days. Cells were extracted using extraction buffer (0.5% Triton X and 20 mM NH_4OH in PBS), and the remaining DNA was cleared by $10 \mu\text{M}$ DNase (11284932001, Roche) treatment for 1 h at 37°C , to produce the acellular CDM prepares.

Immunofluorescence

Cell-derived matrices were fixed with 4% PFA for 10 min at RT followed by blocking with 30% horse serum (HRS, 16050-122, Gibco) in PBS for 10 min at RT. Collagen I antibody (NB600-408, Novus) was diluted in 30% HRS (1:100 dilution) and incubated for 1 h at RT. CDMs were wash three times with PBS and incubated with secondary antibody (Alexa Fluor 568 or 488 Goat Anti-Rabbit IgG) for 1 h at RT followed by PBS washes and Mowiol mounting. Randomly chosen sample areas were imaged with Zeiss Axiovert 200M with Yokogawa CSU22 spinning disc confocal microscope unit with Hamamatsu Orca ER CCD camera (Hamamatsu Photonics K.K.), 3i CSU-W1 spinning disc confocal microscope (Intelligent Imaging Innovations) with Hamamatsu CMOS Orca Flash 4 or with Carl Zeiss LSM780 laser scanning confocal microscope using $40\times/1.2 \text{ W}$ objective. Maximum intensity projection images were evaluated for presence of collagen bundles, and all results were normalised to wt samples.

TIRF imaging

Transient transfections with GFP-paxillin were performed with Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to manufacturer's instructions, and cells were plated overnight on collagen-coated (20 µg/ml) 35-mm glass-bottom dishes (Mattek). GFP-paxillin dynamics were recorded on a TIRF microscope (Zeiss Laser-TIRF 3 Imaging System, Carl Zeiss) using a 63× (NA 1.46 Oil, alpha Plan-Apochromat, DIC) objective and an extra 1.6× magnification was provided by an internal Optovar. Cells were imaged every 1 min, at 37°C in presence of 5% CO₂, using multiposition capabilities. Images were recorded using an EMCCD camera (Hamamatsu ImageEM C9100-13; chip size 512 × 512; Hamamatsu Photonics K.K., Hamamatsu City, Japan) controlled by the Zen software (Zen 2012 Blue Edition Systems; Carl Zeiss). After acquisition, videos were opened and processed in ImageJ. In particular, contrast was adjusted, and images smoothened and aligned using the rigid body algorithm. Focal adhesion (FA) dynamics were then analysed using the Focal Adhesion Analysis Server (Berginski & Gomez, 2013). Only FA with lifetime minimum of 10 frames was analysed, and FA that was assembled and disassembled during the course of imaging was used for measurement of FA kinetics.

Soluble collagen assay

MSFs were plated on a 24-well plate and grown to confluency. Culture medium was replaced by serum-free medium, and the conditioned medium was collected from parallel samples after 2, 5 and 7 days in culture. Cell debris was removed by centrifugation, and the supernatant was stored at −20°C until analysis. Immediately after medium collection, the relative cell number in each well was quantified with Cell Proliferation Reagent WST-1 (Roche). Briefly, the tetrazolium salt WST-1 was diluted 1:10 in serum-free medium and 200 µl was added per well. After 10 min, 100 µl of the WST-1 medium was collected from each well to 96-well plate and analysed for absorbance at 450 nm with Multiscan Ascent plate reader (Thermo Scientific).

Soluble collagen was quantified by precipitation with Sirius red (Direct Red 80, 365548, Sigma) in acidic solution using protocol adapted from Marotta and Martino (1985) and Tullberg-Reinert and Jundt (1999). One millilitre of 50 µM Sirius red in 0.1 M acetic acid was added to each triplicate medium sample (100 µl) or collagen standard sample (0–12 µg/ml collagen type I, rat tail, Millipore). Samples were incubated at room temperature for 30 min with mixing every 5 min by inverting the tubes. The precipitated soluble collagen was pelleted by centrifugation (16,000 g 10 min), and pellets were washed ones with acetic acid. The drained collagen pellets were resuspended in 0.1 M KOH (250 µl) by vortexing, and the absorbance of the solution was analysed on a 96-well plate at 570 nm with Multiscan Ascent plate reader. Soluble collagen concentration in each sample was determined using the standard curve and normalised to the cell amount. Data were further normalised to the soluble collagen content of the wt samples.

RNA sequencing and bioinformatics

Freshly isolated adherent mammary gland stromal cells (without passaging) were cultured until confluency, and the cells were

lysed in RNA extraction lysis buffer. RNA was isolated and DNase-treated using NucleoSpin[®] RNA kit 740955.10 (Macherey-Nagel). RNA quality was controlled with Bioanalyzer 2100 (Agilent). RNA sequencing libraries were performed using TruSeq RNA Library Preparation Kit v2 (Illumina) and sequenced with HiSeq2500 (Illumina) using TruSeq v3 sequencing chemistry. Single-read sequencing with 1 × 50 bp read length was used, followed by 6 bp index run. Twelve samples were run on one lane. Technical quality of the sequencing run was good, and the cluster amount was as expected. Greater than 80% of all bases were above Q30. The base calling was performed using standard bcl2fastq software (Illumina).

The raw sequencing reads were aligned with Tophat (v. 2.0.1) to mm10 reference genome derived from UCSC database and downloaded from Illumina's iGenomes website (https://support.illumina.com/sequencing/sequencing_software/igenome.html). Gene-wise read counting was carried out using HTSeq (v 0.5.4p3) based on RefSeq gene annotations. Downstream data analysis was done with R (v. 3.1) and Bioconductor (v. 2.14). Raw count values were normalised using TMM method of the edgeR package. The normalised expression values were also summarised to the reads per kilobase of exon per million reads mapped (RPKM) although these values were not used for statistical testing. Statistical testing was carried out applying voom transformation and limma package taking the pairing of the samples into account, and the differentially expressed genes were filtered in Ingenuity Pathway Analysis software with (P -value < 0.05 and fold-change > 1.5). A heat map was generated for the differentially expressed genes with RNA expression above 0.2 in the most significantly altered canonical pathways by GENE-E matrix visualisation and analysis platform (<http://www.broadinstitute.org/cancer/software/GENE-E/index.html>).

Accession numbers

The RNA sequencing data set has been added to Gene Expression Omnibus (GEO) database repository (GSE83795).

Statistical analysis

Sample size for the studies was chosen according to previous studies in the same area of research. A minimum of three mice was analysed for each genotype for comparison, except for Fig EV4B and F, where samples from one mouse for each genotype were analysed. Mice with less than three imaged TEBs were excluded from SHG image analysis. The exclusion criterion was pre-established. No randomisation procedure was used for animal studies. Blinding was used for visual analysis of SHG images (Fig 4B) and RNAscope *in situ* hybridisation samples (Fig EV4B). GraphPad program was used for all statistical analyses. Student's *t*-test (unpaired, two-tailed) was used when normality could be confirmed by D'Agostino & Pearson omnibus normality test. Nonparametric Mann–Whitney *U*-test was used when two non-normally distributed groups were compared or when normality could not be tested [due to a too small data set ($n < 8$)]. Wilcoxon matched-pairs signed rank test was used if samples with unequal variance were compared. Data are presented in column graphs with mean ± standard error of mean (SEM) and *P*-values.

Individual data points per condition are shown when $n \leq 15$, and n -numbers are indicated in figure legends.

Expanded View for this article is available online.

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Author contributions

EP and JI contributed to the conception and design of the study. EP, RK, ML, NDF and MG conducted and analysed *in vitro* experiments. MSa and GJ performed microscopy. M. Saari, YL and KWE conducted the computational collagen fibre analysis. ML, YA and JI performed the TFM experiments, and CG analysed the data with help from TB. CG performed and analysed the AFM studies. PR and M. Salmi designed and performed the immune cell flow cytometry. EP, EM, AW, RV and KAS performed the *in vivo* studies. EP and JI wrote the manuscript, and AW, MG, GJ, JPS, MSa, MAD and KWE edited the manuscript. JPS, M. Salmi, MAD, KWE and JI supervised the research.

Conflict of interest

Dr. JP Sundberg and KA Silva have a sponsored research project with BIOCON LLC that is unrelated to this research. All other authors declare that they have no conflict of interest.

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