

RESEARCH ARTICLE

COMP-assisted collagen secretion – a novel intracellular function required for fibrosis

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ABSTRACT

Cartilage oligomeric matrix protein (COMP) is an abundant component in the extracellular matrix (ECM) of load-bearing tissues such as tendons and cartilage. It provides adaptor functions by bridging different ECM structures. We have previously shown that COMP is also a constitutive component of healthy human skin and is strongly induced in fibrosis. It binds directly and with high affinity to collagen I and to collagen XII that decorates the surface of collagen I fibrils. We demonstrate here that lack of COMP–collagen interaction in the extracellular space leads to changes in collagen fibril morphology and density, resulting in altered skin biomechanical properties. Surprisingly, COMP also fulfills an important intracellular function in assisting efficient secretion of collagens, which were retained in the endoplasmic reticulum of COMP-null fibroblasts. Accordingly, COMP-null mice showed severely attenuated fibrotic responses in skin. Collagen secretion was fully restored by introducing wild-type COMP. Hence, our work unravels a new, non-structural and intracellular function of the ECM protein COMP in controlling collagen secretion.

KEY WORDS: Collagen, COMP, Extracellular matrix, Secretion, Fibrosis

INTRODUCTION

The extracellular matrix (ECM) comprises various families of macromolecules that form the structural scaffold of the tissue but that also carry distinct biological activities (Eckes et al., 2010; Hynes, 2009). The most abundant proteins belong to the family of collagens. Fibrillar collagens assemble into large insoluble aggregates that are mostly combinations of different collagen types with tissue-specific composition (Bruckner, 2010). In skin, tendons and bone, collagen I is the predominant structural collagen that confers mechanical stability to the tissue. It forms fibrils that are decorated with the fibril-associated collagens with interrupted triple helices (FACIT) XII and XIV (Bruckner, 2010; Ricard-Blum, 2011).

Collagen assemblies and fibrils interact not only among themselves but extensively with non-collagen ECM components in order to form higher-order structures and networks into which resident cells are embedded. One of these non-collagenous proteins, originally discovered as an abundant component in cartilage ECM, is cartilage oligomeric matrix protein, COMP (Hedbom et al., 1992; Morgelin et al., 1992).

COMP is the fifth member of the thrombospondin family and comprises five identical subunits. The globular domains of the five C-termini are free to interact with other ECM components. COMP binds to the fibril-forming collagens I and II (Halasz et al., 2007; Rosenberg et al., 1998), the FACIT collagens IX, XII and XIV (Agarwal et al., 2012; Holden et al., 2001; Thur et al., 2001), and to distinct non-collagenous proteins such as fibronectin and matrilins (Di Cesare et al., 2002; Mann et al., 2004). Binding sites for COMP in collagens are not restricted to the triple helical domain but are also present in the propeptides of collagen I and II (Rosenberg et al., 1998).

In light of the important structural function proposed for COMP in the assembly in cartilage ECM, the lack of an overt phenotype in mice with constitutive ablation of COMP is unexpected (Svensson et al., 2002). In humans, mutations in the COMP gene lead to chondrodysplasias with short limb dwarfism, such as pseudoachondroplasia (PSACH) (Hecht et al., 1995; Posey et al., 2009) or multiple epiphyseal dysplasia (MED) (Briggs et al., 1995; Posey and Hecht, 2008). Most of the disease-causing mutations have been shown to result in misfolding of COMP, leading to retention and accumulation of the mutant protein in the secretory pathway (Dinsler et al., 2002; Hecht et al., 1998; Hou et al., 2000; Kleerekoper et al., 2002). Other mutations in COMP still allow secretion but seem to cause an inappropriate ECM assembly (Pirog et al., 2010).

We have previously described COMP as a constitutive component present in human skin that is deposited by fibroblasts into the ECM of human skin, accumulating in a characteristic linear pattern in the upper dermis (Agarwal et al., 2012). In individuals with systemic sclerosis, COMP production is markedly increased, and the protein is deposited throughout the entire dermis (Agarwal et al., 2013). Systemic sclerosis is a fibrotic condition affecting the skin and internal organs that is characterized by deposition of large amounts of collagens and other ECM components into the connective tissue, and by conversion of fibroblasts into myofibroblasts (Eckes et al., 2014; Gabrielli et al., 2009; Varga and Abraham, 2007). These are the key cells depositing the excess amounts of ECM that are characteristic of fibrosis (Hinz et al., 2012; Tomasek et al., 2002). COMP is produced by myofibroblasts in systemic sclerosis and has reached diagnostic relevance as one component of a four-gene signature that is typically seen in individuals with systemic sclerosis (Farina et al., 2010, 2009). We

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have also shown that high COMP levels are not restricted to fibrosis occurring in systemic sclerosis but that high levels also occur also in the fibrotic stroma adjacent to epidermal tumors as well as in the fibrosis underlying chronic non-healing ulcers (Agarwal et al., 2013), all characterized by overproduction of collagen.

Given that COMP binds with high affinity to collagen I both directly (Rosenberg et al., 1998) and indirectly through FACIT collagens such as collagen XII (Agarwal et al., 2012), and because elevated COMP levels are found in virtually all pathologies associated with fibrosis, we questioned which structural and functional defects might arise from COMP deficiency in skin, and whether COMP is required for maintaining the characteristic supramolecular organization of the dermal collagen network in healthy as well as fibrotic conditions.

We show here that COMP fulfills a dual role – in the extracellular space it orchestrates a functional collagen fibril network, which imparts the biomechanical properties of healthy skin, whereas, intracellularly, COMP is important for the efficient secretion of collagens into the extracellular space. Consequently, the fibrotic response of COMP-deficient mice to either bleomycin treatment or injury is attenuated. We show that the attenuated response is caused by retention of large amounts of collagens in COMP-null

fibroblasts, in which COMP is unable to exert the function as collagen carrier, whereas in normal fibroblasts, COMP forms a complex with collagens intracellularly that is a prerequisite for collagen secretion.

RESULTS

COMP-deficient skin exhibits inconspicuous histology but increased deformability

Mice with constitutive ablation of COMP develop normally as expected (Svensson et al., 2002), and their skin does not differ from that of wild-type littermates upon histological analysis (hematoxylin and eosin, H&E) (Fig. 1A). Unlike in humans (Agarwal et al., 2012), COMP is deposited abundantly throughout the dermis of wild-type mice and enriched along hair follicles (Fig. S1A), particularly during phases of hair follicle growth – e.g. at postnatal day 7 (Fig. S1B). Despite inconspicuous histology, the skin of adult mice lacking COMP showed functional alterations in its biomechanical properties. COMP-deficient skin exhibited significantly higher ultimate strain (Fig. 1B), reflecting an increased deformation in response to applied force. In addition, COMP-null skin was able to withstand higher ultimate stress before it ruptured (Fig. 1C). However, we were unable to detect significant

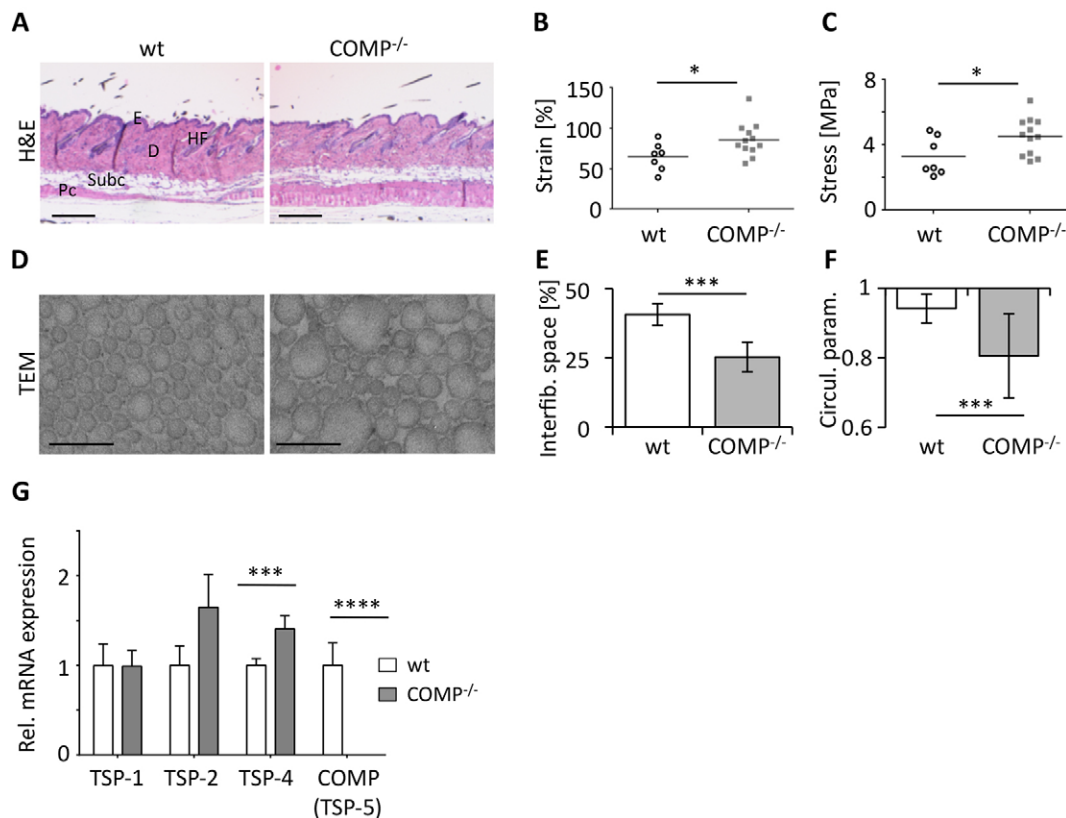


Fig. 1. Altered supramolecular arrangement of the collagen network and impaired biomechanical properties of COMP-deficient skin. (A) The skin of COMP-null mice (6 weeks), stained with H&E, showed no obvious morphological alterations in comparison to that of wild-type (wt) littermates ($n > 10$ mice). Scale bars: 100 μ m. D, dermis; E, epidermis; HF, hair follicle; Pc, panniculus carnosus muscle; Subc, subcutaneous fat. (B) Mechanical properties of wild-type and COMP-deficient skin were determined by testing tensile strength. The ultimate strain of COMP-deficient skin was significantly elevated ($*P = 0.039$). Line represents the mean. (C) Higher ultimate stress was required to rupture COMP-deficient versus wild-type skin ($*P = 0.037$). Line represents the mean. Each symbol represents one mouse. (D) Morphology and arrangement of collagen fibrils in the skin of wild-type and COMP-deficient mice at 10 weeks after birth (transmission electron micrograph). Scale bars: 1 μ m. (E) The inter-fibrillar space (percent of total space) was significantly reduced in COMP-deficient skin ($***P < 0.001$, $n = 6$ electron micrographs per genotype; mean \pm s.d.) and reflected a considerably higher fibril packing density. (F) Circularity parameter of individual fibrils was determined as described in Fig. 2. Skin of COMP-deficient mice showed a significant increase in collagen fibrils with a reduced circularity parameter ($***P < 0.001$, $n = 138$ fibrils in wild type and 109 fibrils in COMP-deficient skin; mean \pm s.d.). (G) Relative (Rel.) mRNA levels for different thrombospondin genes were determined in lysates of cultured (4 days) skin fibroblasts from wild-type and COMP-deficient mice. Expression of TSP-4 was significantly increased in COMP-null cells. $n = 3$ –5 independent fibroblast strains; non-parametric Mann–Whitney U test was used for $n = 3$ biological samples; mean \pm s.d. $***P < 0.001$ and $****P < 0.0001$.

differences in either stiffness (Fig. S1C) or elastic modulus (Fig. S1D) between COMP-null and wild-type skin.

COMP-deficiency affects collagen fibril morphology and packing density in the skin

Because tensile properties of the skin crucially depend on the ultrastructural organization of the dermal collagen network (Bailey, 2001), we performed an ultrastructural analysis of the skin of wild-type and COMP-deficient mice. Electron micrographs clearly showed fibrils of highly irregular shape and larger diameter in COMP-deficient dermis (Fig. 1D). These were more densely packed, as reflected by reduced interfibrillar space (Fig. 1E), and asymmetrically shaped (Fig. 1F) (Starborg et al., 2013). These changes were reminiscent of the larger collagen fibrils with irregular contours seen in the skin of mice deficient for thrombospondin-2 (TSP-2) (Kyriakides et al., 1998). We therefore asked whether a lack of COMP might be compensated for by upregulation of another thrombospondin protein (Fig. 1G). Expression of trimeric TSP-2 was consistently elevated in COMP-deficient versus wild-type fibroblasts, but this trend failed to reach statistical significance. Expression of thrombospondin-1 (TSP-1) in COMP-deficient cells, which has also been implicated in impacting collagen expression as well as fibril organization (Sweetwyne et al., 2010; Sweetwyne and Murphy-Ullrich, 2012) did not differ from controls. Interestingly, the other pentameric thrombospondin, thrombospondin-4 (TSP-4), which is also stimulated by mechanical load (Cingolani et al., 2011), implicated in cardiac fibrosis (Frolova et al., 2012) and in an adaptive endoplasmic reticulum (ER) stress response (Lynch et al., 2012) might be a candidate for functional compensation because its expression was significantly up-regulated in COMP-deficient fibroblasts.

Similar structural alterations of the collagen network were observed in Achilles tendons (Fig. 2A). Collagen fibrils in wild-type tendon exhibited a gain in symmetry with increasing diameter, whereas fibrils in COMP-deficient tendon (Fig. 2B–D) revealed an overabundance of fibrils with diameters of more than 180 nm (Fig. 2C) and were less circular with an increasing diameter (Fig. 2D). These results clearly demonstrate that COMP is involved in the control of the supramolecular collagen architecture by regulating collagen fibrils morphology and fibril-volume-packing (Starborg et al., 2013) in the connective tissue of skin and tendon.

COMP facilitates collagen secretion in dermal fibroblasts

The ultrastructural analysis further showed that in addition to structural abnormalities of the collagen network, COMP-deficient dermal fibroblasts embedded in such matrices displayed clear morphological differences. In contrast to wild type, they showed strongly dilated cisternae in the ER, indicating secretion defects (indicated by arrows in Fig. 3A). This result prompted us to ask whether collagen is not properly released from COMP-deficient fibroblasts. Indeed, we found that secretion of collagen I and collagen XII by COMP-deficient fibroblasts into culture medium was strongly reduced in comparison to wild-type fibroblasts (Fig. 3B). This correlated with elevated intracellular amounts of collagen I and collagen XII in corresponding lysates of the same COMP-null fibroblasts (Fig. 3C). Retention of collagens appears to be specific because secreted amounts of fibronectin, which also binds to COMP (Di Cesare et al., 2002), and of latent TGF β binding protein-1 (LTBP-1; used here as loading control), not known to bind to COMP, did not differ from controls (Fig. 3B). Interestingly, retention of collagens did not coincide with altered levels of the collagen chaperone HSP47 (Fig. 3C). In line with the ultrastructural

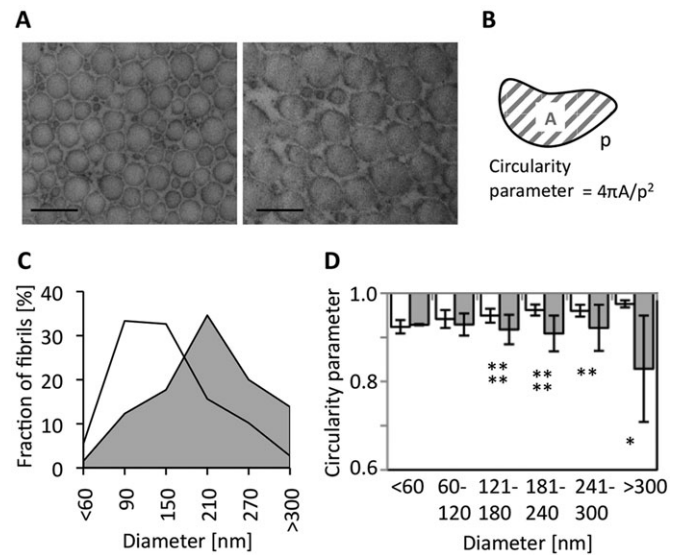


Fig. 2. Altered supramolecular morphology of the collagen network in tendons of COMP-deficient mice. (A) The arrangement of collagen fibrils in Achilles tendons was compared between wild-type and COMP-deficient mice using transmission electron microscopy at 10 weeks after birth. Scale bars: 1 μ m. (B) Symmetry of individual fibrils was determined by calculating the circularity parameter, which reaches the maximal value of 1 when fibrils are perfectly round. (C) The distribution of collagen fibril diameters in tendons of COMP-deficient mice (shaded area under curve) differed from that of wild-type tendons (open area under curve). Mutants showed fewer fibrils with small diameter (<150 nm) but a higher abundance of fibrils with diameters >180 nm. (D) The circularity parameter of collagen fibrils in wild-type tendons (clear bars) increased with larger diameters and reached nearly the maximum value of 1. By contrast, COMP-deficient tendons (shaded bars) comprised thicker collagen fibrils with significantly lower circularity parameters ($P=0.683$ for <60 nm diameter fibrils; $P=0.05$ for 60–120 nm; $****P<0.0001$ for 121–180 nm; $****P<0.0001$ for 181–240 nm; $**P=0.006$ for 241–300 nm; $*P=0.024$ for diameters >300 nm; $n=147$ wild-type and 130 COMP-null fibrils).

signs of ER stress (Fig. 3A) and intracellular collagen retention (Fig. 3C), COMP-deficient fibroblasts displayed slightly but significantly elevated expression of the ER stress marker CCAAT-enhancer-binding protein homologous protein (CHOP; also known as DDIT3) (Nishitoh, 2012), indicating that intracellular retention of collagens causes ER stress (Fig. 3D). However, in the conditions applied here, cultured COMP-null fibroblasts did not exhibit increased apoptosis, as indicated by a cleaved caspase-3 assay (Fig. 3C). Of note, the mRNA levels of collagen I (*COL1A2*) and collagen XII (*COL12A1*) were unaffected by ablation of COMP (Fig. 3E).

Collagen retention in COMP-null fibroblasts was confirmed by immunofluorescence staining of collagen I (Fig. 4A) and collagen XII (Fig. 4B). Wild-type cells had deposited an extensive extracellular network containing collagen I and collagen XII. Co-staining for the ER marker protein disulphide isomerase (PDI) revealed that only small amounts of both collagens were retained in the ER of wild-type fibroblasts. By contrast, strong signals for both collagens were detected in the ER of COMP-deficient fibroblasts, and extracellular networks had been less-well developed by those cells. Quantification of colocalized PDI and collagen I as well as of PDI and collagen XII (Fig. 4C,D) reflected the presence of low amounts of both collagens in the ER of wild type but also of large amounts in the ER of COMP-null fibroblasts.

As these findings indicated that COMP is directly involved in efficient collagen secretion, we tested whether we could rescue collagen secretion by restoring COMP expression in COMP-

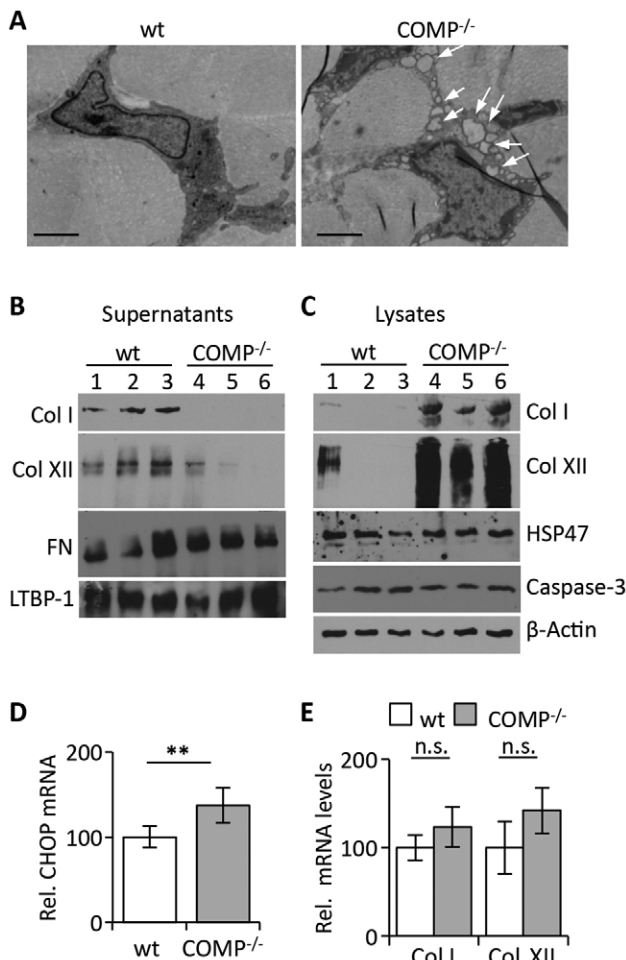


Fig. 3. ER stress caused by collagen retention in COMP-deficient fibroblasts. (A) Electron micrographs of wild-type and COMP-deficient mouse skin (10 weeks). COMP-deficient but not wild-type (wt) fibroblasts showed markedly dilated ER cisternae (arrows), suggesting disturbed secretion. Scale bars: 500 nm. (B,C) Wild-type and COMP-deficient fibroblasts were cultured for 4 days, and corresponding supernatants and lysates were analyzed. (B) Equal volumes of supernatants were collected to determine the amounts of secreted collagen I (col I) and collagen XII (col XII) by western blotting. Levels of both collagen types were markedly reduced in the supernatants of COMP-deficient fibroblasts, whereas levels of fibronectin (FN) and LTBP-1 were comparable to those in controls. (C) Lysates of the corresponding fibroblasts were probed for intracellular levels of collagen I and collagen XII. Levels of both collagens were strongly augmented intracellularly in COMP-deficient fibroblasts, whereas amounts of HSP47 and activated caspase-3 did not differ from controls. Signals for β -actin indicate comparable loading. (D) COMP-null fibroblasts expressed significantly elevated mRNA levels of the ER stress marker CHOP (** $P=0.004$; $n>5$ per genotype) relative (Rel.) to those in wild-type mice. (E) Primary fibroblasts were isolated from the dermis of wild-type and COMP-deficient newborn mice and cultured for 96 h. Levels of mRNA for collagen I and collagen XII were quantified by using RT-qPCR analysis, showing comparable levels in both genotypes ($P=0.203$ for collagen I; $P=0.138$ for collagen XII; $n=6$ for each genotype). Ns, not significant. Error bars are mean \pm s.d.

deficient fibroblasts. Using a Myc-tagged construct encoding rat COMP (Schmitz et al., 2006), we confirmed a direct role for COMP in efficient collagen secretion because collagen XII was retained in non-transfected cells but efficiently secreted by transfected COMP-expressing fibroblasts (Fig. 5A). The mechanism whereby COMP assists in collagen secretion involves formation of intracellular complexes between COMP and collagen, as demonstrated by

co-immunoprecipitation of collagen I and collagen XII with Myc-COMP that had been transfected into human Wi26/SV-40 fibroblasts (Fig. 5B), as well as by co-precipitating collagen XII (Fig. 5C) and collagen I (Fig. 5D) with endogenous COMP.

Thus, in addition to its well-characterized extracellular structural functions in the ECM (Halasz et al., 2007; Heinegård, 2009; Heinegård and Saxne, 2011), COMP exerts a crucial intracellular function in the ER to ensure efficient secretion of collagens through an intracellular interaction.

Defects in collagen secretion in COMP-deficient mice result in attenuated fibrosis

We have recently reported that highly elevated levels of COMP are deposited into the skin of individuals that show fibrosis of varying etiology – including scleroderma and chronic non-healing wounds, and in the stroma of tumors – owing to sustained activation of fibroblasts that secrete large amounts of collagen (Agarwal et al., 2013; Farina et al., 2009). To determine the *in vivo* relevance of COMP for collagen secretion, COMP-null and control mice were subjected to repeated intradermal injections of bleomycin (Yamamoto et al., 1999) or to excisional wounding (Blumbach et al., 2010; Gurtner et al., 2008; Schulz et al., 2015; Zweers et al., 2007).

The skin of control mice that had been injected with solvent (saline) for 4 weeks showed normal histology (Fig. S2A) and dermal thickness (Fig. S2B), and only basal levels of COMP and of α smooth muscle actin (α SMA). One week of bleomycin treatment was sufficient to augment COMP levels, which increased further with prolonged treatment (Fig. S2C). A similar increase was seen in α SMA levels, reflecting the number of biosynthetically active myofibroblasts. By contrast, the fibrotic response elicited by bleomycin injection in COMP-null mice was clearly attenuated (Fig. 6A). Histological analysis of COMP-deficient skin revealed significantly reduced dermal thickness compared to bleomycin-treated control skin (Fig. 6B). In agreement with reduced dermal thickness, the levels of total collagen were also significantly diminished in COMP-deficient fibrotic lesions (Fig. 6C). A similar attenuated response was observed upon excisional wounding; thus, the area of newly formed granulation tissue (Fig. 7A; marked by a white line), which is rich in collagen produced by wound myofibroblasts, was significantly smaller in COMP-deficient mice than in wild-type controls (Fig. 7B).

Ultrastructural analysis of fibrotic lesions confirmed high secretion activity in wild-type fibroblasts, illustrated by dilated ER cisternae (arrows) (Fig. 8A). By contrast, intact fibroblasts were hardly detectable in fibrotic lesions of COMP-deficient animals, whereas large amounts of cell debris were visible. Immunohistochemical detection of α SMA revealed a significant reduction in the number of myofibroblasts (Fig. 8B,D). This finding corresponded with significantly elevated numbers of cleaved-caspase-3-positive cells, which were already evident after 2 weeks of bleomycin treatment (Fig. 8C,E), whereas numbers of such cells were comparable in unchallenged skin (Fig. S1F).

DISCUSSION

In this study, we characterized in detail the skin of mice with a constitutive ablation of COMP. Ultrastructural analysis revealed alterations in dermal collagen fibril morphology and arrangement, and functional tests demonstrated changes in the biomechanical properties of COMP-deficient skin. This was not reported in the original paper describing the phenotype of COMP-deficient mice (Svensson et al., 2002). However, at that time, COMP was

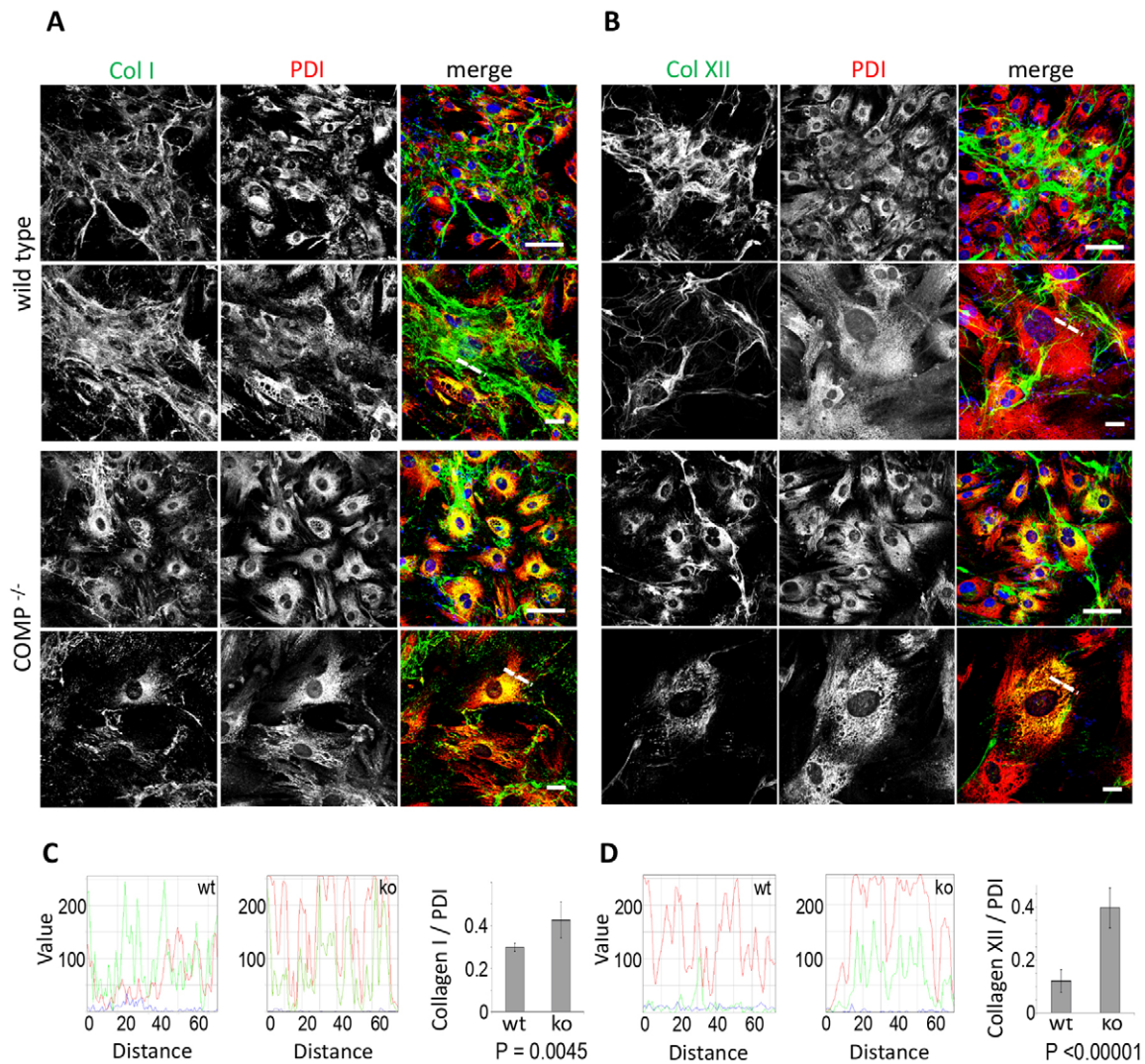


Fig. 4. Collagen secretion is impaired in COMP-null fibroblasts. Primary fibroblasts from the dermis of wild-type (wt) or COMP-deficient mice were stained with antibodies against collagen I (A, col I) or collagen XII (B, col XII), and protein disulfide isomerase (PDI), an integral ER membrane component. Top rows for each genotype show an overview (scale bars: 50 μ m), the row below displays cells at higher magnification (scale bars: 10 μ m). Well-developed fibrillar collagen networks were detected in the extracellular space surrounding wild-type fibroblasts. These networks were less well developed in COMP-null fibroblasts. Conversely, COMP-deficient, but not wild-type, fibroblasts exhibited strong signals to indicate that both collagens colocalized intracellularly with PDI, indicating retention of collagen I and collagen XII within the ER. Intensity profiles were acquired along the indicated lines in 'merge' images at higher magnification. Representative line scans of collagen I (green line in C) and collagen XII (green line in D) and PDI (red lines in both) were used to determine Pearson's correlation coefficient, which reflects the extent of colocalization (collagen I / PDI and collagen XII / PDI) used here to indicate the presence of these collagens in the ER. This analysis confirmed extensive colocalization in mutant fibroblasts but significantly less colocalization in wild-type fibroblasts ($P=0.0045$ for collagen I and PDI; $P<0.00001$ for collagen XII and PDI; $n=30$ cells per genotype). Error bars are mean \pm s.d. KO, knockout.

thought to be mainly expressed in cartilage, which was therefore the focus of that study. The alterations in the macromolecular arrangement of collagen fibrils described here are thought to contribute to the observed changes in the biomechanical properties of the dermal connective tissue of COMP-deficient skin. Similar structural alterations were also identified in collagen-I-rich tendons, and they are probably due to the well-documented structural functions of COMP, which interacts directly and indirectly with fibril-forming collagens. Absence of COMP affects the fibril packing volume and probably the fibril fraction volume, which is the fraction of the ECM occupied by collagen fibrils and which is affected by fibril number and volume (Starborg et al., 2013). These changes could be the result of reduced levels of collagen XII, which associates with fibrils, in the ECM of COMP-null mice.

Unexpectedly, our study uncovered an abundance of collagens in the ER of dermal COMP-deficient fibroblasts. Introducing wild-type COMP into COMP-null fibroblasts efficiently rescued collagen secretion, clearly demonstrating the crucial importance of COMP in this process. The mechanism whereby COMP assists in the export of collagens from wild-type fibroblasts, shown here for mouse and human fibroblasts, involves the intracellular formation of COMP-collagen complexes, suggesting a function in collagen export. An alternative explanation could be that an imbalanced ECM, one that is assembled in the absence of COMP (Halasz et al., 2007), might induce changes in collagen secretion. This explanation appears unlikely in view of the very efficient restoration of collagen secretion upon expression of COMP, which was detected within 12 to 24 h – i.e. at a time when no COMP is detectable in the surrounding matrix.

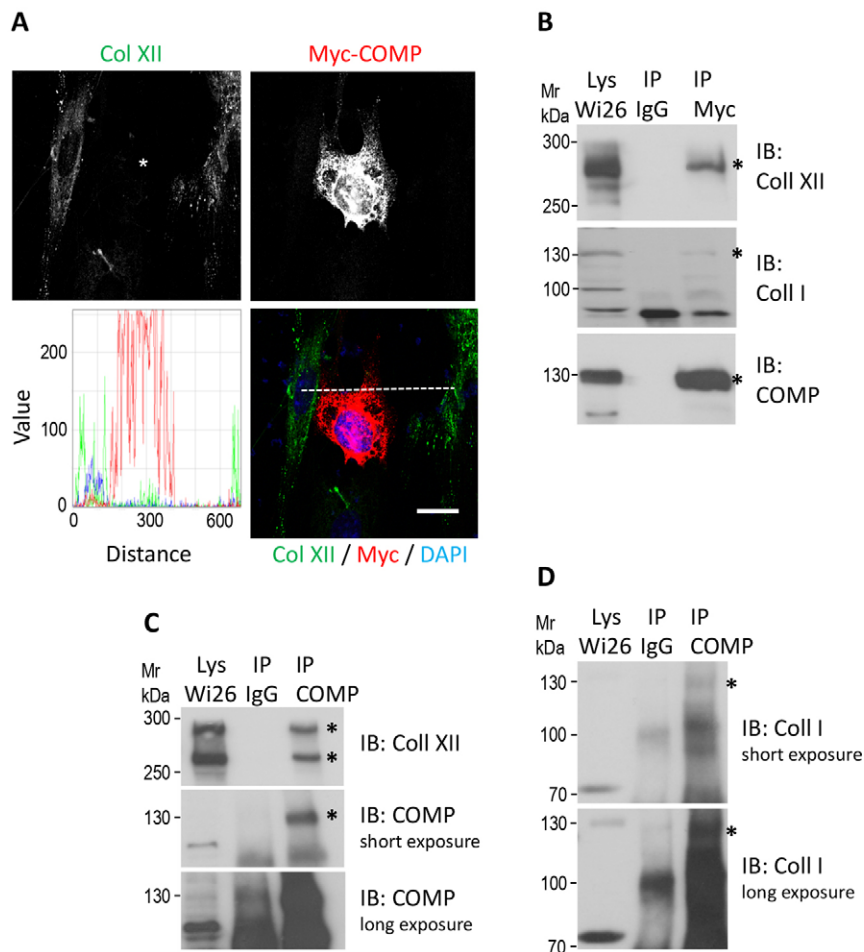


Fig. 5. COMP is important for collagen secretion through formation of intracellular complexes with collagen. (A) COMP-deficient dermal fibroblasts were transfected with a Myc-tagged wild-type COMP construct and stained 24 h later. Intracellular accumulation of collagen XII (green, col XII) was detected only in untransfected Myc-negative cells, whereas Myc-positive COMP-expressing fibroblasts (red and marked by an asterisk in A, top left panel) showed no intracellular collagen XII signals, indicating efficient secretion. Intensity profiles were acquired along the indicated line. Scale bar: 10 μ m. (B) The intracellular interaction of Myc-COMP with collagen XII (coll XII) or collagen I (coll I) was analyzed by immunoblotting (IB) of Myc-immunoprecipitates from Myc-COMP-transfected Wi26/SV-40 fibroblasts with antibodies against the indicated proteins. Why fibroblasts overexpressing COMP preferentially produce the smaller collagen XII variant (compare C) is not understood. IP, immunoprecipitation; Lys, lysate. (C) Representative immunoblot of endogenous COMP immunoprecipitates from Wi26/SV-40 fibroblasts probed with antibodies against collagen XII and COMP. (D) Representative immunoblot of endogenous COMP immunoprecipitates from Wi26/SV-40 fibroblasts probed with an antibody against collagen I. Specific signals are indicated by asterisks in blots.

Thus, our studies reveal that COMP has two different functions – first, an intracellular activity that leads to efficient export of collagen from the ER, which depends on the intracellular formation of a COMP–collagen complex; second, COMP exerts an extracellular activity that ensures the correct spatial arrangement of collagen fibrils in the ECM.

Fibrillar collagens are synthesized as single pro-collagen chains as they enter the ER lumen, where C-terminal pro-peptides mediate assembly of three α -chains, giving rise to triple helical pro-collagen molecules (Bulleid et al., 1997). Correct collagen folding is assisted, for example, by the chaperone HSP47, which is a pre-requisite for secretion; accordingly, deficiency in HSP47 in mice is lethal owing to retention of misfolded collagens in the ER (Nagai et al., 2000; Widmer et al., 2012). Expression of HSP47 is normal in COMP-null fibroblasts, suggesting that this mechanism contributes to the basal level of collagen secretion that must be in place for mice to develop and thrive in unchallenged conditions. Further mechanisms involved in collagen secretion have been postulated that include the formation of fibripositors (Canty et al., 2004) and mega-carriers that are large enough to accommodate very long cargo molecules (Jin et al., 2012; Saito et al., 2011). These models postulate that protein complexes assemble intracellularly before secretion. The ability of COMP to bind to pro-collagen I and pro-collagen II (Halasz et al., 2007; Rosenberg et al., 1998), its intracellular presence in complexes containing collagen in fibroblasts and the finding that collagen secretion is efficiently rescued by restoring COMP expression in COMP-null fibroblasts strongly support the concept that complexes containing

COMP and collagens need to be assembled intracellularly before secretion.

Evidence for such intracellular protein complexes also comes from cartilage biopsies of individuals with chondrodysplasia caused by mutations in COMP (Maddox et al., 2000; Merritt et al., 2007). In these individuals and in cell culture models expressing selected mutant variants, the retention of misfolded COMP leads to co-retention of other ECM components, including collagens (types II, IX and XI) (Hecht et al., 2005; Maddox et al., 1997; Vranka et al., 2001). Most of these mutations have been shown to induce improperly folded COMP that, along with its bound interaction partners, is not secreted (Cousty et al., 2012; Posey et al., 2009; Schmitz et al., 2006, 2008). It remains to be determined for each mutation individually whether the extent of co-retention is caused by different binding affinities to collagens or by a complete absence of functional COMP. The latter would reflect the situation in COMP-deficient mice and would be expected to result in a milder phenotype than that resulting from the retention of misfolded COMP together with its ligands.

The new non-structural function of COMP in collagen secretion postulated here for skin and tendon might reflect a more general feature in connective tissue homeostasis. We have preliminary evidence that chondrocytes of COMP-deficient mice also accumulate collagens in the ER, indicating that similar molecular defects could occur in cartilage. Furthermore, like COMP, matrilin-1 is a multimeric ECM protein that is abundantly present in cartilage (but not in skin) (J.-N. Schulz and B. Eckes, unpublished data) and is known to bind to fibrillar collagen II (Klatt et al., 2011). Recently,

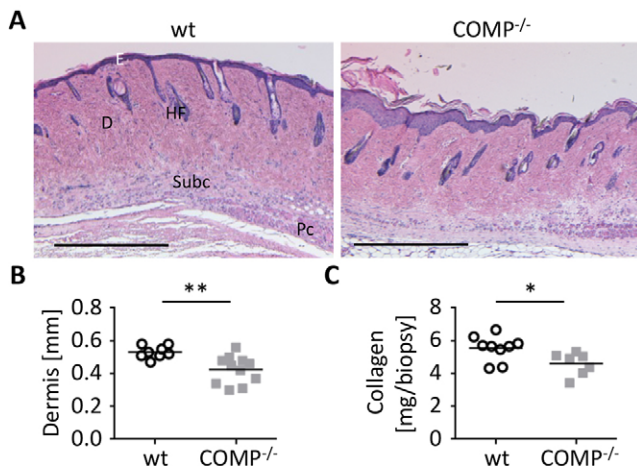


Fig. 6. Attenuated development of skin fibrosis in COMP-null mice.

(A) Histology (H&E staining) of wild-type (wt) and COMP-deficient skin fibrotic lesions produced by bleomycin injections revealed thickening of the dermis (D) and replacement of subcutaneous fat (Subc) and panniculus carnosus muscle (Pc) by connective tissue. E, epidermis; HF, hair follicle. Scale bars: 500 μ m. (B) Dermal thickness, used as read-out for the extent of the fibrotic response, was significantly reduced in fibrotic lesions of COMP-deficient animals in comparison to that of wt (** $P=0.007$). Lines in B and C represent means. (C) Hydroxyproline content reflecting collagen content was significantly reduced in fibrotic lesions of COMP-deficient versus wild-type mice (* $P=0.023$). Each symbol represents one mouse.

morpholino-mediated knockdown of matrilin-1 has revealed marked retention of collagen II in zebrafish chondrocytes, leading to overall growth defects (Neacsu et al., 2014).

Intracellular formation of complexes comprising collagen and other ECM components, and subsequent ECM-assisted collagen secretion might vary with tissue-specific demands. The crucial role of ECM-assisted collagen secretion in cartilage, tendons and fibrotic skin seems to indicate that this mechanism is particularly important in tissues that are exposed to high mechanical loads. In agreement with this notion, a mechano-responsive region in the COMP promoter has been identified (Amanatullah et al., 2012). Also, expression of collagen I and collagen XII is upregulated in response to mechanical load (Kessler et al., 2001; Trächslin et al., 1999).

Recently, mutations in *B3GALT6*, encoding galactosyltransferase II, have been associated with a spectrum of connective tissue disorders, including spondyloepimetaphyseal dysplasia with joint laxity type 1 (SEMDJL1) and Ehlers–Danlos-like syndrome. A microarray-based transcriptome analysis of skin fibroblasts from two individuals with mutations in this gene has revealed the pronounced downregulation of COMP, which is accompanied by alterations in the secretion and matrix assembly of collagen I and collagen III, eventually leading to hyperextensible skin (Ritelli et al., 2015). These changes in the biomechanical properties of the

skin of such individuals are reminiscent of the softer skin that we observed in COMP-deficient mice.

Despite the clear features arising from ablation of COMP, knockout mice survive birth and are viable, and the total amount of collagen in unchallenged skin was normal (Fig. S1E). This could be due to normal expression of HSP47 and to potential compensation by TSP-4 and, possibly, by TSP-2. Future studies will dissect the mechanisms of compensation in situations of COMP loss or malfunction.

Intracellular retention of collagen did not grossly impair fibroblast survival, and we did not observe changes in the apoptotic cell numbers in the skin of COMP-null mice, which might be expected to result from the absence of the described anti-apoptotic function of COMP (Gagarina et al., 2008). However, upon induction of excessive collagen production, apoptosis increased significantly. This property might be exploited for novel therapeutic approaches, which would target COMP preferentially in fibroblasts that are engaged in high levels of collagen production during the development of fibrosis. These cells would be expected to undergo apoptosis owing to ER overload, whereas moderately active cells would deal with the challenge – e.g. through proteasomal degradation.

MATERIALS AND METHODS

Mice

Mice with a constitutive disruption of the COMP gene were kindly provided by Dr Åke Oldberg (Lund University) (Svensson et al., 2002) and compared to wild-type littermates for experimental procedures. Mice were bred to the C57BL/6N background for more than six generations and housed in a specific pathogen-free facility. Animals were killed through cervical dislocation except those carrying wounds or fibrotic lesions, which were exposed to CO₂. All animal protocols were approved by the veterinary agency of North-Rhine Westphalia (LANUV NRW, Germany).

Determination of skin tensile strength

The tensile strength of skin was analyzed using a materials testing machine (Z2.5/TN1S; Zwick, Ulm, Germany), which recorded force–deformation curves. Two strips of back skin were harvested from male animals aged 6 weeks using a punch (hourglass-shaped, width: 5 mm in the middle and 10 mm at the ends, length: 25 mm). Tissue strips were fixed between two riffled clamps and preloaded (0.05 N, 0.1 mm/s) to assess the initial length to calculate strain (%). Then the skin was stretched until failure with a crosshead speed of 15 mm/min. Values for ultimate load (N), deformation (mm) and energy (mJ) were obtained from the load–deformation curves. To calculate ultimate stress (MPa), skin thickness was assessed in histological sections. Stiffness (N/mm) and elastic modulus (MPa) were determined from the slope of the linear portion of the load–deformation and stress–strain curves, respectively.

Wound healing and fibrosis

Wounds were created and harvested according to standard protocols (Blumbach et al., 2010; Schulz et al., 2015; Zweers et al., 2007). In brief, female mice at the age of 10 weeks were anesthetized by intraperitoneal

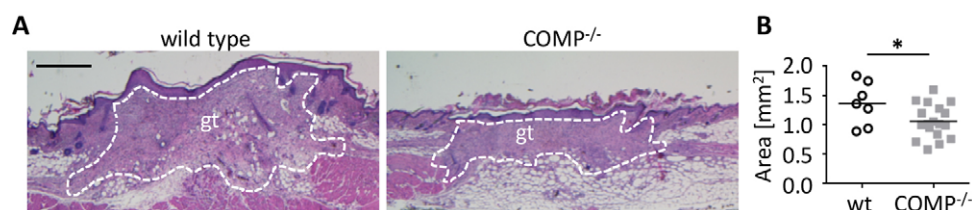


Fig. 7. COMP-deficiency affects the formation of granulation tissue during wound healing. (A) Histology (H&E staining) of excisional wounds at 7 days after injury. (B) The area of newly formed granulation tissue (gt; encircled by white lines in A) was significantly reduced in wounds of COMP-deficient versus wild-type animals (* $P=0.037$; each symbol represents one wound). Scale bar: 500 μ m.

injection of ketamine (10 g/l) and xylazine (8 g/l) solution (10 µl/g body weight) (Park Davis, Karlsruhe, Germany and Erlangen, Germany, respectively). Two full-thickness excisional wounds were created next to the spine at the scapular region using biopsy punches (6 mm; Stiefel, Offenbach, Germany). Wounds were left uncovered and harvested 7 days after injury.

Skin fibrosis was induced in female mice aged 6 weeks by daily intradermal injections of bleomycin (100 µl; 1 mg/ml in 0.9% NaCl; Medac, Wedel, Germany) (Yamamoto et al., 1999) for 4 weeks. Skin that had been injected with 0.9% NaCl served as control.

Wounds and fibrotic lesions were bisected for histological analyses. One half was embedded in optimal cutting temperature (O.C.T.) compound (Sakura, Staufen, Germany), frozen, sectioned (8 µm) and processed for immunofluorescence staining. The other half was fixed [2 h in 4% paraformaldehyde (PFA)] and processed for paraffin embedding.

Hydroxyproline determination analysis

The amount of collagen in skin was assessed by quantification of hydroxyproline content, using a total collagen assay according to the manufacturer's protocol (QuickZyme Biosciences, Leiden, The Netherlands). In brief, skin biopsies (6 mm diameter), including entire

fibrotic lesions, were hydrolyzed in 6 M HCl for 20 h at 95°C. After a colorimetric reaction based on the chloramine-T method (Prockop and Udenfriend, 1960), hydroxyproline residues were quantified by measuring the extinction at 570 nm.

Transmission electron microscopy

Biopsies of healthy skin were taken at 6 weeks after birth; Achilles tendons were dissected at 10 weeks. All specimens were immersion-fixed for 8 h (2% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, room temperature), rinsed (0.1 M cacodylate buffer) and stored in PBS. After washing (0.1 M cacodylate buffer, pH 7.2, 40°C), samples were fixed with OsO₄ (2%, 2 h) and stained in 1% uranyl acetate. Dehydrated biopsies were embedded in araldite resin. Ultra-thin sections (30–60 nm; Reichert ultramicrotome) were placed on copper grids. Transmission electron microscopy was performed using a Zeiss 902A electron microscope (Zeiss, Oberkochen, Germany). Collagen fibril parameters were determined according to Starborg et al. (2013). In brief, the deviation of collagen fibril shape from a circle was estimated by calculating the circularity parameter (circularity parameter = $4\pi A/p^2$, where p is the perimeter and A is the transverse area of the fibril). This parameter reaches its maximum value of 1 in circular objects.

Cell culture and transfection

Primary fibroblasts were isolated from murine skin at postnatal day 3 and floated overnight on 0.1% trypsin, 0.02% EDTA in PBS at 4°C to separate epidermis from dermis. Fibroblasts were liberated from the dermis by incubation with 400 U/ml of collagenase I (Worthington, Troisdorf, Germany) for 1 h at 37°C and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% FCS (GE Healthcare, Freiburg, Germany), 50 µg/ml Na-ascorbate, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Seromed-Biochrom, Berlin, Germany) in 5% CO₂ (Zhang et al., 2006). For transfections, primary murine dermal fibroblasts and Wi26/SV-40 human lung fibroblasts were seeded onto glass coverslips that had been placed in 24-well plates and transfected with 1 µg plasmid DNA per well using XtremeGENE HP (Roche, Mannheim, Germany) according to the manufacturer's protocol. As described previously (Thur et al., 2001), rat COMP was cloned in-frame with the sequence of the BM-40 signal peptide into a modified episomal expression vector, pCEP-Pu (Kohfeldt et al., 1997; Smyth et al., 2009), carrying an N-terminal Myc tag (Schmitz et al., 2006). After 24 h, cells were fixed with 4% PFA in PBS for 10 min at room temperature.

Antibodies

Antibodies against murine COMP (Agarwal et al., 2012) and collagen XII (Veit et al., 2006), or rabbit anti-bovine COMP serum (kindly provided by Dr D. Heinegard, Dept. of Clinical Sciences, Lund, Sweden) were used as described previously. Antibodies against α-SMA (western blotting 1:500, catalog number A5228) immunohistochemistry 1:250, catalog number F3777, clone 4A1, Sigma-Aldrich), β-actin (western blotting 1:1000; catalog number sc-47778, Santa Cruz), cleaved caspase-3 (immunofluorescence 1:400, western blotting 1:200; catalog number 9661, Cell Signaling), collagen I (immunofluorescence 1:100, catalog number 600-401-103, Rockland, Limerick, Ireland; western blotting 1:500, catalog number 2031503505, Quartett, Berlin, Germany), fibronectin (western blotting 1:500; catalog number 23750, Abcam); HSP47 (western blotting 1:200; catalog number sc-8352, Santa Cruz), LTBP-1 (western blotting 1:1000; catalog number sc-98275, Santa Cruz) and Myc (immunofluorescence 1:100; clone 9E10; catalog number sc-40, Santa Cruz) and PDI (immunofluorescence 1:200; catalog number ADI-SPA-891, Stressgen, San Diego) were used according to the manufacturers' protocols.

Histology, immunofluorescence and immunohistochemistry

Paraffin sections (6 µm) were de-waxed in xylol, rehydrated and stained with H&E according to standard procedures and photographed with a DM4000B light microscope (Leica, Wetzlar, Germany). Dermal thickness was determined by measuring the distance between the epidermal–dermal junction and the dermal–subcutaneous-fat junction in the center of injected

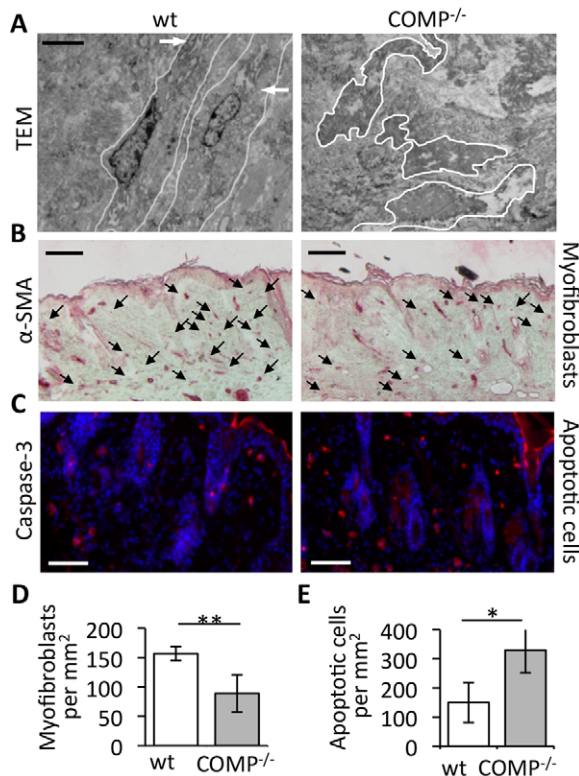


Fig. 8. Increased apoptosis of (myo)fibroblasts in fibrotic lesions of COMP-null mice. (A) Ultrastructural analysis (transmission electron microscopy) revealed highly active fibroblasts in bleomycin-induced fibrotic lesions of wild-type (wt) mice, as indicated by dilated ER (white arrows). Only a few intact fibroblasts were detected in fibrotic lesions of COMP-deficient animals, which displayed abundant amounts of cell debris. Putative cell margins are indicated by white lines. Scale bar: 5 µm. (B) Immunohistochemical staining of bleomycin-induced fibrotic skin lesions identified α-SMA-expressing cells, including vasculature. Myofibroblasts were identified by their spindle-shaped morphology and are marked by arrows. Scale bars: 100 µm. (C) Staining for cleaved caspase-3 identified apoptotic cells in fibrotic lesions. Red, cleaved caspase-3; blue, DAPI. Scale bars: 60 µm. (D) Myofibroblast numbers were significantly decreased (***P*=0.005; *n*≥6 samples for each genotype) and (E) numbers of apoptotic cells were strongly increased (**P*=0.013; *n*≥4 mice for each genotype) in fibrotic lesions of COMP-deficient versus wild-type mice.

skin areas, using ImageJ software (<http://rsbweb.nih.gov/ij>). Paraffin sections were also used for the detection of myofibroblasts, which were visualized by immunohistochemical staining of α -SMA (Tomasek et al., 2005). After incubation in 10% normal goat serum for 30 min, endogenous biotin was blocked using the Biotin Blocking System (Dako). Sections were incubated with a primary fluorescein-conjugated antibody against α -SMA (1:250 in 1% BSA in PBS, clone 1A4; catalog number F3777, Sigma-Aldrich) for 1 h, rinsed in PBS and incubated for 1 h with an anti-fluorescein antibody (1:750, catalog number A889, Molecular Probes). After washing in PBS, a biotinylated goat anti-rabbit IgG (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was applied for 1 h. The biotinylated complex was detected using ABC-alkaline-phosphatase complex, and the color was developed with Vector red alkaline phosphatase substrate (both Vector Laboratories).

Cryosections were postfixed in 2–4% PFA for 10 min and rinsed in PBS, and cells were permeabilized for 10 min in 0.2% Triton X-100 in PBS. Unspecific binding sites were blocked for 1 h with 5% BSA in PBS. Primary antibodies were applied in 1% BSA in PBS for 1 or 24 h. Fluorescence-conjugated secondary antibodies (Alexa-Fluor-488 or Alexa-Fluor-568; Molecular Probes) were applied for 1 h (1:500 in 1% BSA in PBS). Nuclei were stained with DAPI (1:1000; Life Technologies), slides were rinsed (PBS) and mounted (Dako). Photomicrographs were acquired using a Nikon Eclipse 800E fluorescence microscope equipped with a digital camera (DS-Q1Mc, Nikon) or with a BIOREVO BZ-9000 fluorescence microscope (Keyence, Neu-Isenburg, Germany).

For indirect immunofluorescence of cultured fibroblasts, cells were grown on glass coverslips for 7 days in medium containing 50 μ g/ml Na-ascorbate. Cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.5% NP-40 in PBS for 10 min and blocked with 1% FCS in PBS for 30 min. After primary antibody incubation (diluted in 1% FCS in PBS) for 1 h, cells were washed several times and incubated with appropriate secondary antibodies for an additional 45 min. After four washing steps in PBS, cells were mounted using DAPI-containing mounting medium (Dako). Images were acquired using a Leica TCS SP5 microscope controlled with LAS AF Software (Leica). Image analysis was performed with ImageJ. Intensity profiles along indicated lines were analyzed with the RGB-profiler plugin for ImageJ. For calculation of Pearson's correlation coefficients, single cells were marked and analyzed with the JACoP plugin for ImageJ.

Protein extraction and immunoblotting

Skin punch biopsies (6 mm diameter) were mechanically disrupted by homogenization using a Mixer Mill (Retsch, Haan, Germany) for 2 min at 30 Hz in RIPA buffer (150 mM NaCl, 50 mM Tris base, 0.1% sodium dodecyl sulfate, 12 mM Na-deoxycholate, 1% Nonidet P-40, pH 8), supplemented with protease inhibitor cocktail (Sigma-Aldrich). COMP was extracted from skin biopsies by using sequential extraction, as described previously (Di Cesare et al., 1994), by disrupting the skin in RIPA buffer without EDTA (Mixer Mill), then re-extracting the pellet after centrifugation with RIPA buffer containing 10 mM EDTA overnight at 4°C. Debris was removed by centrifugation. Protein concentration was determined by BCA assay (Pierce). Equal volumes of extracts were loaded to detect COMP in skin lysates.

Cultured fibroblasts were detached by incubation with 0.1% trypsin, 0.02% EDTA in PBS for 5 min at 37°C, lysed in RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich) and sonicated. Protein concentration in cell lysates was determined by BCA assay (Pierce). Equal volumes of supernatants were mixed with loading buffer directly or concentrated by using trichloroacetic acid (TCA) precipitation (to analyze LTBP-1). For western blot analysis under reducing conditions (LTBP-1 and fibronectin), samples were incubated with Laemmli buffer containing 2-mercaptoethanol for 5 min at 95°C. Proteins were resolved in 6% or 7.5% SDS-PAGE gels and transferred to nitrocellulose (GE Healthcare) or PVDF (Millipore) membranes. Membranes were stained with Ponceau Red (Sigma-Aldrich) and blocked with 5% milk powder in TBS-T (150 mM NaCl, 10 mM Tris-HCl, 0.1% Tween-20, pH 7.6) for 1 h at room temperature, probed with primary antibodies diluted in 5% milk powder in TBS-T, washed (TBS-T) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000 in 5% milk powder in TBS-T, 45 min; Dako). Signals

were detected by chemiluminescence using SuperSignal West Pico or Femto Substrate (ThermoScientific).

Co-immunoprecipitation of collagens

Human Wi26/SV-40 fibroblasts were transfected with Myc-COMP, as described above, and used 24 h after transfection. Cells were detached by incubation with 0.1% trypsin, 0.02% EDTA in PBS for 5 min at 37°C, lysed in 20 mM Hepes in KOH pH 7.4, 150 mM NaCl, 1.7 mM CaCl₂ and 0.5% NP-40 supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Roche, Berlin, Germany) with 20 strokes in a glass Dounce homogenizer and sonication. Equal amounts of lysate were incubated with either unspecific rabbit serum (IgG), an antibody against Myc or COMP-specific antiserum (rabbit anti-bovine COMP) and 40 μ l of Protein G agarose beads (Roche) overnight at 4°C with rotation. Beads were washed five times with lysis buffer, and bound proteins were eluted with Laemmli buffer containing 2-mercaptoethanol by heating for 5 min at 95°C. Eluted proteins were resolved by reduced SDS-PAGE (6%) and analyzed by immunoblotting using antibodies directed to collagen I, collagen XII and COMP. All incubations with primary antibodies were performed overnight at 4°C.

RT-qPCR

For real-time quantitative PCR (RT-qPCR) analyses, 1 μ g of total RNA (RNeasy, Qiagen) was used for cDNA synthesis with oligo(dT) primers (revertAid™ first strand cDNA synthesis kit, Fermentas, Schwerte, Germany). Gene fragments of interest were amplified using PowerSYBR Green PCR Master Mix and a 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany). Relative expression levels were calculated by the comparative $\Delta\Delta$ Ct-method after normalization to GAPDH levels. The following primers were used: CHOP (forward 5'-TGTTGAAGATGAGCGGGTGG-3'; reverse 5'-CGTGGACCAGGTTCTGCTT-3'); collagen I (forward 5'-GATGAGGAGACGGGCAGCTTG-3'; reverse 5'-GAGCAGCCATCGACTAGGAC-3'); collagen XII (forward 5'-AGACATTGTGTTGCTGGTGA-3'; reverse 5'-GAGAAATGAAGCTTCGCACAGT-3'); COMP (forward 5'-GCGCCAGTGTGCAAGGACAA-3'; reverse 5'-TGGGTTTTCGAACCAGCGGGC-3'); GAPDH (forward 5'-CATGTTTGTGATGGGTGTGA-3'; reverse 5'-AATGCCA-AAGTTGTCATGGA-3'); TSP-1 (forward 5'-TATGTGCTAATGCCA-ACCA-3'; reverse 5'-GCCATCACCATCAGATGCTC-3'); TSP-2 (forward 5'-TGTCTCGTCCATTGAAAACA-3'; reverse 5'-TCTTCAATCCCGTCAATTA-3'); TSP-4 (forward 5'-TCCTCGCTACCTGAAGAATGATGG-3'; reverse 5'-TTCAATGGACTCTGGGTTCTGGGTG-3').

Statistical analysis

Results are presented as means \pm s.d. Statistical analysis was performed using unpaired Student's *t*-test unless stated differently. **P*<0.05 was considered statistically significant; ***P*<0.01, ****P*<0.001 and *****P*<0.0001. Gaussian distribution was verified by Kolmogorov–Smirnov test. Calculations were performed using GraphPad Prism (GraphPad Software).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.-N.S. performed most experiments; A.N. performed tensile strength testing; J.N., K.S. and M. Plomann performed co-immunoprecipitation experiments; J.N. and M.K. performed collagen retention and rescue analyses; W.B. performed all electron microscopy work; J.B. performed hydroxyproline determination analysis; S.H. determined COMP expression in skin; J.-N.S., J.N., M. Paulsson, M. Plomann, T.K.,

F.Z. and B.E. designed the study and wrote the paper. All authors analyzed data and edited the manuscript.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.180216/-/DC1>

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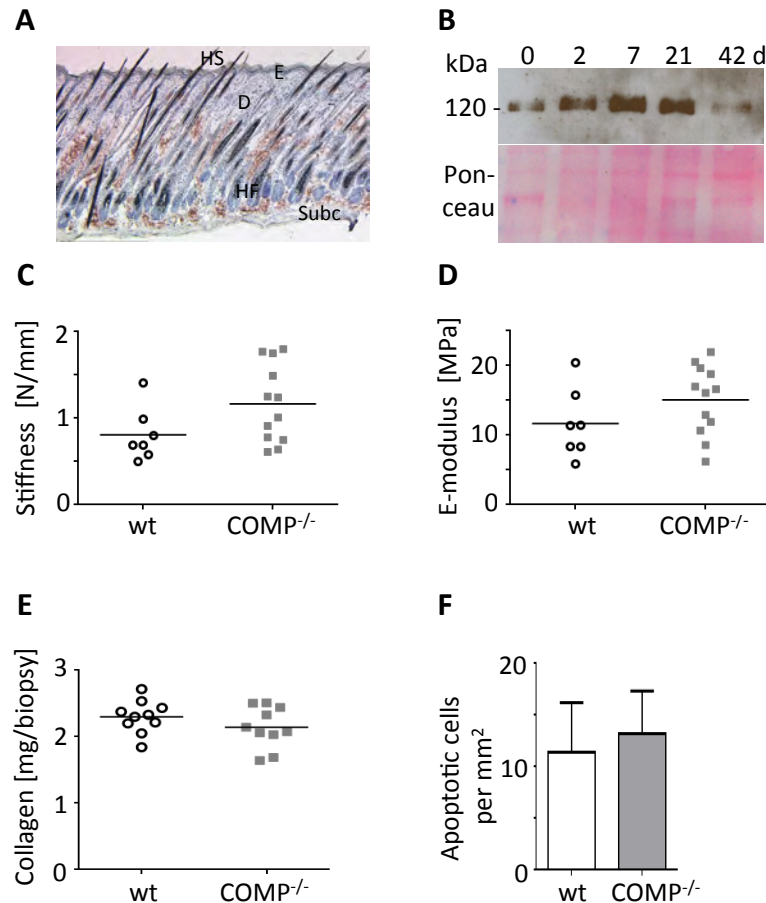


Figure S1: Properties in unchallenged skin that are not affected by absence of COMP. (A) Distribution of COMP in wild type murine skin at P7. Unlike in human skin, COMP is deposited throughout the dermis (D) of murine skin and concentrated around hair follicles (HF) especially during follicle growth. E: epidermis; HS: hair shaft; Subc: subcutaneous fat. Bar represents 500 μm . (B) Western blot showing levels of COMP in murine skin at the indicated time points. (C) Stiffness [N/mm] and (D) elastic modulus [MPa] were determined from the slope of the linear portion of the load-deformation and stress-strain curves, respectively. No significant differences were detected in stiffness ($P = 0.0807$) or elastic modulus ($P = 0.1681$). Each symbol represents the skin of one mouse. (E) The content of total collagen was assessed in biopsies of unchallenged skin from wild type and COMP-deficient mice (6 weeks) by determining hydroxyproline amount. The amount of collagen (mg/biopsy) was comparable in the skin of wild type and COMP-null mice. Each symbol represents one mouse. (F) Apoptotic cells were identified in healthy skin of mice at 3 months by staining cleaved caspase-3 ($n = 3$ per genotype). No significant difference was detected ($P > 0.05$, $n = 6$ for each genotype).

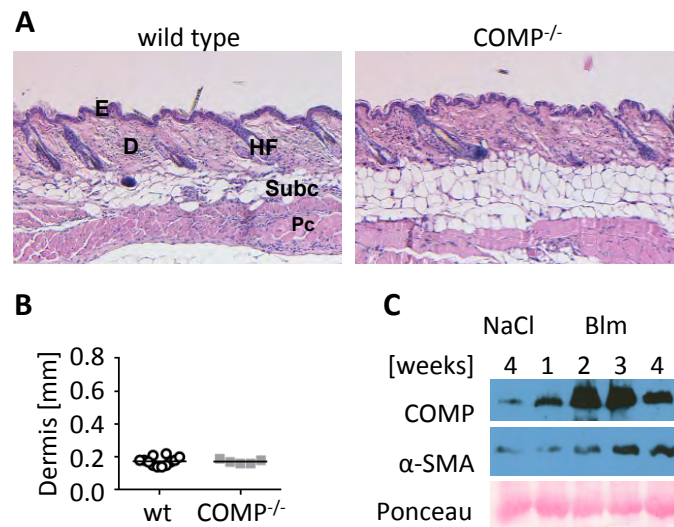


Figure S2: Induction of fibrosis results in elevated levels of COMP and α -SMA in wild type murine skin. (A) Comparable histology of wild type and COMP-deficient skin after 4 weeks of injection with 0.9% NaCl (solvent control for bleomycin injection). D: dermis; E: epidermis; HF: hair follicle; Pc: panniculus carnosus muscle; Subc: subcutaneous fat. Bar represents 200 μ m. (B) Thickness of the dermis was comparable ($P = 0.866$). Each symbol represents one lesion. (C) Levels of COMP and α -SMA were determined by immunoblot analysis in skin lysates of wild type animals after repeated injection with bleomycin (Blm) or 0.9% NaCl for the indicated number of weeks. COMP was highly elevated after treatment with bleomycin while only basal levels were detected in the skin of solvent-treated animals. The amount of α -SMA correlated with the duration of bleomycin treatment and confirmed progression of the fibrotic process.