

Ablation of integrin-mediated cell-collagen communication alleviates fibrosis

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Running title Triple knockout of integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 11\beta 1$

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Abbreviations: α SMA, alpha smooth muscle actin
CCN, cellular communication network
Col, collagen
DDR, discoidin domain receptor
ECM, extracellular matrix
FA, focal adhesion
FACIT, fibril-associated collagen with interrupted triple helix
FAK, focal adhesion kinase
SRC, SRC proto-oncogene, non-receptor tyrosine kinase
TGF β , transforming growth factor beta
tKO, triple knockout of *Itga1* + *Itga2* + *Itga11*
WT, wild type
YAP, Yes-associated protein

Abstract

Objectives

Activation of fibroblasts is a hallmark of fibrotic processes. Besides cytokines and growth factors, fibroblasts are regulated by the extracellular matrix environment through receptors such as integrins, which transduce biochemical and mechanical signals enabling cells to mount appropriate responses according to biological demands. The aim of this work was to investigate the *in vivo* role of collagen-fibroblast interactions for regulating fibroblast functions and fibrosis.

Methods

Triple knockout (tKO) mice with a combined ablation of integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ were created to address the significance of integrin-mediated cell to collagen communication. Properties of primary dermal fibroblasts lacking collagen-binding integrins were delineated *in vitro*. Response of the tKO mice skin to bleomycin induced fibrotic challenge was assessed.

Results

Triple integrin-deficient mice develop normally, are transiently smaller and reveal mild alterations in mechanoresilience of the skin. Fibroblasts from these mice in culture show defects in cytoskeletal architecture, traction stress generation, matrix production and organization. Ablation of the three integrins leads to increased levels of DDR2, an alternative receptor recognizing collagens *in vivo* and *in vitro*. However, this overexpression fails to compensate adhesion and spreading defects on collagen substrates *in vitro*. Mice lacking collagen-binding integrins showed a severely attenuated fibrotic response with impaired mechanotransduction, reduced collagen production and matrix organization.

Conclusions

The data provide evidence for a crucial role of collagen-binding integrins in fibroblast force generation and differentiation *in vitro* and for matrix deposition and tissue remodeling *in vivo*. Targeting fibroblast-collagen interactions might represent a promising therapeutic approach to regulate connective tissue deposition in fibrotic diseases.

KEY MESSAGES

What is already known about this topic

Activation of fibroblasts is crucial for the development of all fibrotic processes, in particular in systemic sclerosis. Fibrogenic cytokines and growth factors play an important role in this process; also the integrin-mediated interaction of fibroblasts with the surrounding extracellular matrix environment is a crucial activator. However, the exact molecular mechanisms involved in this activation are still unknown.

What this study adds

Abrogating the interaction of fibroblasts with collagen, the main structural component in the extracellular environment, induces a major defect in force generation, matrix organization and YAP activation, thereby compromising fibroblast activation, myofibroblast formation and function. Integrin-mediated collagen interaction is required for challenging situation such as fibrotic processes but are dispensable for (murine) development.

How this study might affect research, practice or policy

The study provides novel insight into the regulation of fibroblast activation and indicates that integrin-mediated interaction of fibroblasts with collagen can be a targeted in novel therapeutic approaches for fibrosis.

Introduction

Excessive deposition of extracellular matrix (ECM) constituents and their aberrant macromolecular organization is the hallmark of fibrotic diseases. Activated fibroblasts (myofibroblasts) play a key role in this process by producing and remodeling ECM. They are regulated by a variety of cytokines and growth factors released from different cell types but also by their interaction with the ECM (1, 2). The extracellular matrix (ECM) is a complex network of multidomain proteins interacting to form large macromolecular complexes that determine the biomechanical properties of tissues. It is composed of proteins belonging to several families in which collagens constitute the most abundant ECM component. (3, 4). Besides providing structural support, the collagens and other ECM constituents have critical roles in modulating numerous cellular activities (4-6). They function as reservoir for growth factors, but also directly interact with most cell types through specific cell surface receptors (7). These interactions provide vital cues impacting cellular behavior and disease processes (8).

Integrins form a large group of receptors for ECM constituents. They are transmembrane heterodimers composed of a ligand-specific α subunit in non-covalent association with a β subunit. Integrins lack enzymatic activity but execute bidirectional signaling between the extra- and the intracellular compartments as their intracellular domains are linked to the actomyosin cytoskeleton, whereas their extracellular domains can bind ECM ligands (7). Integrin signaling thereby integrates information from the ECM and cytoskeleton and influences virtually all cellular functions (9). Through their ability to couple to the contractile actomyosin cytoskeleton, integrin-based adhesions are a major site for sensing, generating and transducing mechanical tension to downstream effectors such as YAP/TAZ (10, 11), thereby regulating fibroblast activities (12). Tension-induced signaling from ligand-bound integrins clustered in adhesion sites involves FAK, SRC-family kinases, PI3K (phosphoinositide-3 kinase), JNK pathways and RHO family GTPases leading to stress fiber formation and actin reorganization to YAP/TAZ activation and MRTF-SRF (myocardin-related transcription factors, serum response factor)-dependent transcriptional changes (10, 12-14).

The major integrins facilitating fibroblast-collagen interactions are $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ (15, 16). Their distribution across cell types and tissues and ligand preference varies. The collagen-binding $\alpha10\beta1$ integrin is restricted to cartilage (17).

Much work defining the molecular interactions of integrins with collagen or other ECM components has used cultured cells attaching to extracted ECM, ECM fragments or synthetic peptides. In vivo the situation may be more complex, since the cells encounter the surrounding ECM in a supramolecular organization. It remains an open question which binding sites are exposed and accessible or masked depending on biological context (18-21).

Single and double knockout mouse models were instrumental in delineating regulatory functions of individual collagen-binding integrins; for review see (22). $\alpha1\beta1$, mainly expressed by mesenchymal cells, supports cell proliferation, stimulates tumor angiogenesis and impacts T cell and innate immunity, while $\alpha2\beta1$, more widely expressed, regulates platelet and mast cell functions, suppresses tumor angiogenesis and impacts immune responses differently from $\alpha1\beta1$ (16, 23-25). These knockout models have also revealed various regulatory functions of collagen-binding integrins in granulation tissue formation and collagen deposition in in vivo models (26, 27). Loss of integrin subunit $\alpha11$, expressed in a restricted fashion mainly by fibroblasts, decreased collagen abundance due to impaired myofibroblast formation (28). Integrin $\alpha11\beta1$ was shown to largely contribute to collagen remodeling through TGF β mediated JNK signaling (26) whereas integrins $\alpha1\beta1$ and $\alpha2\beta1$ have minor roles in this process (29).

Besides integrins, cell-collagen interaction is facilitated by the discoidin domain receptors (DDR) -1 and -2 (30). The DDRs are receptor tyrosine kinases that undergo autophosphorylation upon collagen binding, triggering downstream signaling pathways that regulate ECM remodeling, proliferation, migration and survival. In addition, cross-talk of DDRs with integrins may either positively (31) or negatively (32) affect integrin function. DDR2 but not DDR1 is expressed by mesenchymal cells, and binds to fibrillar collagens (30).

The studies using mice with either single or double deletion of the $\alpha1\beta1$, the $\alpha2\beta1$ or the $\alpha11\beta1$ integrin revealed only slight phenotypic alterations, which could be due to functional compensation by remaining integrins. To eliminate compensation and to address the question, which role integrin-mediated contact of cells with collagen has in regulating fibroblast activities, we here generated and analyzed a mouse model lacking all three integrins that were shown to bind collagens in in vitro systems. We also evaluated the significance of fibroblast-collagen interactions in a challenging

situation, an induced fibrotic process that is characterized by excessive production and remodeling of collagen. We focused our analysis on the skin and on fibroblasts obtained from this tissue that is characterized by a collagen-rich ECM in its dermal compartment.

We observed that tKO mice are viable but smaller than WT. They show attenuated dermal fibrosis and upregulation of DDR2 receptors. tKO fibroblasts in culture have aberrant cytoskeletal and contractile properties, which disable the generation of a strong mechanical response. Our findings reveal that together, the integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ are essential for regulating cellular force balance required for fibroblast activation and the development of fibrosis. They provide the basis for a potential therapeutic approach to modulate fibroblast functions in fibrosing diseases.

Results

Mice with global ablation of integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 11\beta 1$ are viable, with reduced mechanoresilience and increased DDR2 levels in their skin

tKO mice with a full-body depletion of the collagen-binding integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 11\beta 1$ were generated by breeding mice lacking $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (25) with $\alpha 11\beta 1$ -deficient mice (33) (**Fig. 1A and S1**). They are born at expected Mendelian ratio. In the first 3 months of postnatal life, tKO mice are visibly smaller than WT littermates (**Fig. 1B**), but indistinguishable from WT mice in later life. Body weight, in contrast, is reduced and does not normalize within 3 months (**Fig. 1C**). Histological analysis of the skin of young adult tKO mice showed no major alterations in the different compartments including the dermis (**Fig. 1D**). The collagenous ECM, inspected by transmission electron microscopy, failed to show gross differences in ultrastructural organization of collagen fibrils (**Fig. 1E**). In contrast to the unaltered morphology, tensile strength tests revealed that tKO skin ruptures at lower force, is less deformable and stiff (**Fig. 1F-J**). However, maximum stress, toughness and elastic (E-) modulus of tKO back skin are slightly but not significantly reduced compared to the skin from sex- and age-matched WT mice (**Fig. 1K-M**).

Given the mild alterations in unchallenged skin, we asked if the activity of the missing integrins is compensated by other receptors. Contact with collagen in dermal fibroblasts is not restricted to the collagen-binding integrins but can also be mediated by non-integrin DDR2 (30). Indeed, increased DDR2 staining in the upper and lower dermis was detected in the skin of tKO mice (**Fig. 1N-P**).

Increased levels of active DDR2 on tKO fibroblasts do not mediate spreading on collagen

To assess if ablation of the three integrins affects levels of other relevant cell surface receptors, we investigated the levels of the major fibronectin receptor, integrin $\alpha 5\beta 1$ and of integrin $\alpha 10\beta 1$, a collagen receptor normally expressed by chondrocytes. Flow cytometry did not reveal significant differences in surface levels of integrin subunits $\alpha 5$ (ITA5) (**Fig. 2A, B**) and $\beta 1$ (ITB1) (**Fig. 2C, D**) in tKO compared to WT fibroblasts seeded on fibronectin coats for 6 hours, indicating that surface levels of the major fibronectin receptor, integrin $\alpha 5\beta 1$, are not significantly altered in tKO fibroblasts. No compensatory de novo expression of integrin $\alpha 10$, normally not present in fibroblasts,

was detected in tKO dermal fibroblasts (**Fig. S2**). However, and in accordance with increased DDR2 signals in tKO skin, increased DDR2 levels were also seen by immunoblotting of proteins from cultured primary dermal tKO fibroblasts grown on coated collagen I substrates (**Fig. 2E-H and Fig. S3**). The increase was not only detected in fibroblasts cultured for 6 hours on collagen I coats, but also in fibroblasts released from tissue culture plastic by trypsin treatment (**Fig. 2E**; 0h), i.e. prior to contact with collagen, indicating that its expression is not exclusively induced by fibroblast-collagen contact. Exposure of fibroblasts grown on tissue culture plastic to solubilized collagen (monomers) triggered a significant increase in phosphorylated DDR2 after 6 hours, which was >3 fold higher in tKO than in WT fibroblasts (**Fig. 2I-L**). Levels of total DDR2 (**Fig. 2I, K**) and the ratio of phosphorylated to total DDR2 (**Fig. 2I, L**) tended to be higher in tKO fibroblasts but did not differ significantly from those in WT fibroblasts.

To test if increased DDR2 surface levels contribute to adhesion and spreading of tKO fibroblasts, we compared the spreading behavior and morphology of WT and tKO fibroblasts after 6 hours on collagen I and fibronectin (**Fig. 3**). Clearly, less than 5% of tKO fibroblasts had spread on collagen I despite high levels of phosphorylated DDR2 detected at this time point, whereas more than 40% of WT fibroblasts were spread. In contrast, both, WT and tKO fibroblasts appeared to be spread to comparable extent on fibronectin (**Fig. 3A, B**). A closer inspection of stained cells revealed, however, distinct differences in actin cytoskeleton architecture and cell size also on fibronectin (**Fig. 3C-F**). Whereas WT fibroblasts formed distinct actin microfilaments and stress fibers as well as clearly defined paxillin-positive focal adhesions (FAs) on collagen I, no such organization was detected in tKO fibroblasts (**Fig. 3C**). These results clearly showed that the collagen receptor DDR2, although present at elevated levels in tKO fibroblasts, does not rescue their failure to adhere to and spread on collagen I. On fibronectin, both WT and tKO fibroblasts spread to larger area than on collagen I. Stress fibers and peripherally as well as centrally located adhesions were formed by WT fibroblasts, whereas size, abundance of stress fibers and matrix adhesions were reduced in tKO cells (**Fig. 3D-F**, see also Fig. 5). Clearly, the modified characteristics of the tKO fibroblasts on fibronectin were not on account of reduced levels of integrin $\alpha 5$ chain (**Fig. 2A-D**) but due to other cellular changes.

tKO fibroblasts produce and arrange an abnormal ECM

We asked if the spreading defect of tKO fibroblasts might be caused by an abnormal matrix deposited by these cells and can be rescued by a WT matrix, and vice versa, if the tKO matrix can induce spreading defects in WT fibroblasts. WT and tKO fibroblasts were therefore cultured for 10 days to deposit matrices. These were decellularized and then seeded with either WT or tKO cells to create autologous or heterologous systems, respectively. As expected, tKO fibroblasts on tKO matrix were significantly less spread than WT fibroblasts on WT matrix (**Fig. 4A top and B**). In contrast, tKO fibroblasts tended to spread to larger size on heterologous WT matrix, and WT fibroblasts were slightly, albeit not statistically significantly, smaller on tKO matrix (**Fig. 4A bottom and B**). However, differences in ECM assembled by WT and tKO fibroblasts were detected, which revealed distinct differences in collagen I network organization. Both tKO and WT fibroblasts deposited an extensive extracellular collagen I network, but in contrast to WT, tKO networks featured only few thick collagen fiber bundles, whereas fine fibers were more abundant (**Fig. 4C**) and prevailed in particular in tKO matrix after decellularization (**Fig. 4D**).

To obtain a comprehensive overview of the secretome of tKO fibroblasts, we applied SILAC to label the proteins released by tKO and WT fibroblasts into culture media over a 24 hour period. The list of significantly regulated proteins was surprisingly short and included 8 downregulated (in tKO versus WT) and 2 upregulated candidate proteins (**Fig. 4E, F**).

Several of these downregulated proteins are involved in collagen fibrillogenesis such as thrombospondin 2 (TSP2) (34) periostin (POSTN) (35), procollagen C-endopeptidase enhancer 1 (PCOC1) (36) and fibulin 4 (37). Insulin-like growth factor binding protein 5 (IBP5) (38) and fibulin 4 are known to regulate production of various ECM components including collagen and fibronectin. Additionally, fibulin 4 affects the activation of TGF β signaling and α SMA production (37). IBP5 is upregulated in the fibrotic skin lesions of SSc patients (39) and fibrotic intestine in Crohn's disease (40). Chemokine ligand 2 (CCL2) recruits macrophages critical in dermal ECM turnover upon tissue injury (41). STRING analysis (Search Tool for the Retrieval of Interacting Genes/proteins) indicated an interacting network of ECM proteins including periostin, matrix metalloproteinase-2 (MMP2) and chemokine (C-C motif) ligand 2 (CCL2) with further connections to PCOC1 and TSP2 (**Fig. 4G**). Gene ontology (GO) term

analysis used to identify the biological processes affected by the ablation of the three integrins revealed that these candidates fall predominantly in the category 'extracellular structure organization' (**Fig. 4H**). Together, these results show that the lack of the three integrins on cultured fibroblasts induces quantitative and qualitative changes in the ECM deposited by these cells, which can impact cell spreading and cytoskeletal organization.

Impaired mechanotransduction in tKO fibroblasts in vitro

To investigate the mechanisms of the cell-intrinsic differences in cell spreading (**Fig. 3**), we analyzed the ability of the cells to generate traction stresses at FAs, which is required for this process. Traction force microscopy of WT and tKO fibroblasts seeded on polyacrylamide gels coated with collagen I revealed that tKO fibroblasts produced about 50% less traction stress than WT fibroblasts (**Fig. 5A and B**). To further substantiate the defect in actomyosin contractility, we quantified the capacity of WT and tKO fibroblasts to contract and remodel collagen I fibrils in floating collagen lattices, a function that relies on cell traction (42). As expected, lattices with tKO fibroblasts remained significantly larger, reflecting severely reduced mechanotransduction needed to remodel collagen I networks (**Fig. 5C and D**).

We next analyzed potential downstream consequences of the reduced contractility of tKO fibroblasts by analyzing YAP signaling, a key mechanosensitive transcriptional coactivator of several pro-survival, cytoskeletal and ECM genes (**Fig. S4**) (14). Adhesion to collagen I for 6 hours induced robust activation of YAP in WT fibroblasts, which was reduced by more than 50% in tKO fibroblasts (**Fig. 5E, F**), while levels of total YAP did not differ between WT and tKO fibroblasts (**Fig. 5E, G**). On fibronectin, a slight, but insignificant reduction in active YAP was seen in tKO fibroblasts (**Fig. 5I-L**). In addition, remodeling of the actin cytoskeleton was impaired in tKO fibroblasts grown on collagen I. The ratio of monomeric G-actin to filamentous F-actin was significantly elevated in tKO, indicating insufficient actin remodeling in comparison to WT fibroblasts (**Fig. 5M, N**). A similar tendency was observed in tKO cells on fibronectin, but this difference was also not significant (**Fig. 5O, P**). In addition to determining YAP levels by immunoblotting, we assessed its subcellular distribution. Staining using an antibody detecting unphosphorylated (active) nuclear YAP clearly revealed decreased nuclear YAP in the tKO fibroblasts on collagen I, in comparison

to WT (**Fig. 5Q**) but active, nuclear YAP signals were completely absent in tKO fibroblasts (**Fig. 5R**). In contrast, tKO fibroblasts grown on fibronectin showed active YAP in the nuclei (**Fig. 5S, T**), indicating that tKO fibroblasts are capable of perceiving and transducing stress upon stimulation of $\alpha 5\beta 1$ integrin receptors. This finding agrees with unaltered levels of active to total YAP detected by immunoblotting (**Fig. 5I-L**).

The finding of altered G- to F-actin levels led us to study the actin cytoskeleton in more detail. As noted previously (**Fig. 3**), the reduced spreading of tKO fibroblasts seeded on collagen I was confirmed by phalloidin staining (**Fig. 5Q, R**). In addition, stress fibers were virtually absent in tKO cells, and paxillin-positive FAs were scarce in comparison to WT cells. In contrast, organization of F-actin stress fibers as well as formation of FAs, clearly visible in WT fibroblasts on fibronectin, were also reduced in tKO fibroblasts on fibronectin (**Fig. 5S, T**). In particular, numbers of FAs per tKO fibroblast, area occupied by and average size of FAs were significantly reduced (**Fig. 5U, V, W**). pFAK and pSRC signals (43) were reduced in tKO fibroblasts on collagen but normal on fibronectin (**Fig. S5**) suggesting impairment in adhesion assembly in tKO fibroblasts on collagen but not fibronectin and impaired maturation or disturbance of further downstream signaling on fibronectin.

Absence of collagen-binding integrins leads to a reduced fibrotic response in mice

To delineate the biological relevance of integrin-mediated cell-collagen interactions *in vivo* under conditions of induced collagen synthesis and remodeling, we subjected tKO and WT mice to intradermal bleomycin injections that result in skin fibrosis (44). Histological inspection of bleomycin lesions clearly showed attenuated fibrosis in tKO skin, reflected by significantly reduced dermal thickness (**Fig. 6A-D**) and a strong reduction of collagen content (**Fig. 6H**) in comparison to WT controls. These changes in the dermal connective tissue were confirmed by second harmonic generation analysis, showing a significant increase in collagen fluorescence intensity in WT compared to tKO skin lesions (**Fig. 6E-G**), together indicating that tKO fibroblasts deposit reduced amounts of collagen into fibrotic lesions. Ultrastructural analysis of the collagen network by transmission electron microscopy revealed that induction of fibrosis resulted in a change in collagen fibril organization in the upper dermis in WT

skin, which displayed a significantly denser packing of collagen fibrils than tKO lesions (**Fig. 6I and J**). This difference in collagen fibrillar organization between tKO and WT skin was not observed upon saline injection, serving as solvent control treatment. Histological analysis also did not reveal any obvious changes in the inflammatory infiltrate and vascularization between tKO and WT in the bleomycin lesions. However, significantly reduced numbers of α SMA-positive myofibroblasts (**Fig. 7D-F**) and of pSMAD2-positive nuclei (**Fig. 7A-C**) were detected in fibrotic lesions of tKO than WT mice, reflecting attenuated TGF β signaling and explaining the reduced collagen deposition in tKO fibrotic lesions (**Fig. 6**).

tKO dermal fibrotic lesions show diminished mechanoreponse

To test if the attenuated dermal fibrosis in tKO mice correlates with impaired force transduction *in vivo*, we stained sections of bleomycin-induced fibrotic skin lesions for active YAP (**Fig. 8A**). Indeed, levels of active YAP and numbers of YAP-positive nuclei were significantly lower in fibrotic lesions of tKO mice (**Fig. 8A-C and Fig. S6**). Interestingly, double staining for α SMA and active YAP showed that in WT lesions, not all α SMA-positive myofibroblasts displayed nuclear YAP signals, indicating that active YAP marks a subpopulation of myofibroblasts (**Fig. 8A**). Comparative proteomics analysis of WT and tKO fibrotic lesions revealed the potentially altered biological processes, showing 'metabolic process' and 'multiorganism process' as top hits (**Fig. 8D**). Indeed, a number of proteins known to be regulated in fibrotic reactions were identified as regulated in tKO fibrotic skin lesions. These are depicted in the volcano plot (**Fig. 8E**) and in the list in **Fig. 8F**. In accordance with a reduced fibrotic response in tKO mice, CCN1, a prominent YAP target, was identified as the most significantly downregulated protein. CCN1 is a matricellular protein associated with fibroblast proliferation and apoptosis, matrix organization as well as synthesis and stability of collagen. It belongs to the CCN family of matricellular proteins that also include CTGF (connective tissue growth factor, CCN2) and have functions in fibrosis and wound repair (45, 46). Reduced CCN1 signals throughout the dermis in tKO fibrotic skin lesions were confirmed by immunofluorescence (**Fig. 8G-I**), in accordance with the proteomic analysis.

In summary, ablating the integrin-mediated contact of fibroblasts with collagen does not lead to major alterations in skin mechanics in unchallenged tKO mice. *In vitro*,

adhesion of tKO fibroblasts to collagen is abrogated and is not compensated by upregulated levels of DDR2. tKO fibroblasts in contact with collagen display severe defects in actin cytoskeleton organization and integrin-based matrix adhesions, which result in strongly impaired force transduction and collagen organization. Of note, cell size and morphology are abnormal in tKO fibroblasts seeded on collagen I, but also on fibronectin or WT fibroblast-derived ECM, clearly reflecting cell-intrinsic alterations in cultured fibroblasts. The challenge by a fibrotic stimulus that requires synthesis and remodeling of new ECM to mice with combined absence of the integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ induces impaired mechanotransduction and myofibroblast differentiation and ultimately reduced fibrosis.

Discussion

Activation of fibroblasts and their conversion to myofibroblasts is crucial for the development of fibrosis. It is regulated by several cytokines and growth factors and also by the contact of the cells to ECM structures (1, 2, 47). Collagen is the most abundant extracellular protein in connective tissues of vertebrates. The family of collagen proteins comprises different structural assemblies ranging from long rigid fibrils (type I/III/V heteropolymers) to flat network-forming meshes found in basement membranes (type IV). In parallel, three different collagen-binding integrin receptors, $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha11\beta1$ have evolved that were thought to enable fibroblasts to interact with the diverse collagen structures in their microenvironment and regulate their functions (15, 22). Recently, the concept of these integrins 'binding to collagens' has been revised. While many in vitro studies showed that the integrins recognize and directly bind to collagen monomers, the in vivo situation might be more complex and binding to higher-order structures, e.g. fibrils, can be indirect and involve the macromolecular organization (20) and interaction with non-collagen components decorating the fibril surface (18, 19, 21).

These findings raised the question how relevant the interaction of collagen-binding integrins with collagens is in vivo, and prompted us to generate tKO mice with a global and combined absence of the integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$, thereby avoiding compensation. This combined deletion affects primarily fibroblasts being the only cell type expressing all three integrins. No compensatory changes in levels of integrin $\alpha10\beta1$ (mostly restricted to cartilage) (17) were noted at the surface of fibroblasts, but the non-integrin collagen receptor, DDR2, was strongly upregulated by fibroblasts in vitro and in vivo. We here did not address a potential role of mannose receptor uPARAP/Endo 180 for remodeling mediated by its activity in collagen internalization (48).

tKO mice showed reduced size up to about 3 months, when body size becomes almost normal. This pattern is reminiscent of the smaller size of mice lacking either $\alpha11\beta1$ or combined $\alpha2\beta1$ plus $\alpha11\beta1$ (49). In contrast to size, body weight failed to normalize with age. Given that ablation of DDR2 causes dwarfism in mice due to impaired proliferation of chondrocytes in the growth plate (50), it is unlikely that the overabundance of DDR2 in tKO mice is responsible for reduced size. Elevated DDR2 expression did not rescue the failure of tKO fibroblasts to adhere to collagen in vitro, clearly indicating that DDR2 is not an adhesion receptor, although it does respond to

soluble collagen molecules and may trigger other signals potentially including survival signals.

Ablation of the three integrins caused severe impairment in mechanotransduction. ECM-mediated integrin activation involving in particular the $\beta 1$ family has been associated with inhibition of the Hippo pathway (**Fig. S4**), thus promoting YAP activation (13, 51). Accordingly, tKO fibroblasts displayed severely impaired force transduction as shown directly by traction force microscopy, by virtual lack of active YAP in vitro and in vivo and by several downstream changes that can be attributed to impaired mechanotransduction. The lower traction forces in tKO dermal fibroblasts are thought to result from a decrease in the number of mechanical links between the ECM-bound integrins and actomyosin, called molecular clutches (52). The absence of molecular clutches between the collagen-bound integrins and actin reduces the force generated by polymerizing actin on the resistant plasma membrane, consequently decreasing the cellular traction force on the ECM (13). This agrees with our finding of increased globular relative to filamentous actin, which results in perturbed cytoskeletal architecture, and is in agreement with YAP/TAZ-mediated gene transcription regulating F-actin polymerization (14). It is known that absence of a mechanically competent F-actin network further impairs YAP/TAZ activation (53). Conversely, integrin-mediated mechanical stress on the nucleus causes nuclear F-actin polymerization favoring nuclear localization of myocardin related transcription factor (MRTF), which leads to serum response factor (SRF)-mediated transcription of genes required for YAP/TAZ activation (54). These mechanisms are likely severely disturbed in tKO fibroblasts in which mechanotransduction is perturbed by the absence of key force-transmitting structures. Analysis of WNT signaling may provide further insights into disturbed mechanosignaling in tKO fibroblasts, as canonical as well as non-canonical Wnt signaling can promote YAP activation (55).

Ligand-occupied integrins assemble to form integrin adhesion complexes that are sites at which the cell surface is in closest contact with ligand (9). Collectively termed the 'integrin adhesome' (56), their biochemical composition is highly dynamic and changes within very short time intervals in response to environmental impact and cellular activity (57). Mechanical force acting on these structures strongly promotes their maturation and, in addition, increases integrin affinity for their ligands (58). Moreover, integrin-dependent RhoA signaling maintains cell tension and rigidity essential for YAP/TAZ activation and controls actin stress fiber formation (13, 14). In

tKO fibroblasts, fewer structures are available for the assembly of matrix adhesions to be exposed to tensile stress, initiating signaling and regulating actin dynamics. This can explain the smaller size of tKO fibroblasts when grown in contact with collagen I *in vitro*. Reduced spreading was also noted when tKO fibroblasts were in contact with fibronectin that is recognized by integrin $\alpha 5 \beta 1$ and not by collagen-binding receptors, despite comparable surface levels of $\alpha 5$ and $\beta 1$ integrin chains and pSRC and pFAK levels in tKO and WT fibroblasts. These findings indicate that lack of the integrins $\alpha 1 \beta 1$, $\alpha 2 \beta 1$ and $\alpha 11 \beta 1$ in fibroblasts not only impacts collagen binding activity but affects cell adhesion in a broader way. It is conceivable that absence of the three integrins might negatively affect overall integrin clustering to assemble focal adhesions by disabling the lateral spacing of integrins of 58 nm or less required for efficient focal adhesion formation (59). Reduced numbers of integrins at the surface of tKO fibroblasts may thereby result in larger gaps that preclude proper focal adhesion function.

We also found that ablation of the three integrins severely changes the secretome of fibroblasts, reducing a number of structural ECM proteins including collagen that is deposited by tKO fibroblasts as a network of abnormally thin fibrils, and proteins affecting collagen fibrillogenesis.

Impaired mechanotransduction and altered ECM production in skin fibroblasts *in vitro* raised the question about the relevance for an *in vivo* situation and especially whether in a challenging situation, characterized by increased fibrogenic mediators, induced mechanosignaling, excessive collagen production, crosslinking and remodeling to generate a stiff ECM, the response would be attenuated in tKO mice. Bleomycin-induced fibrosis is a strong challenging condition and the lesions in tKO mice showed reduced YAP activation in the lower dermis while inflammatory cell infiltrates and abundance of vascular structures remained unaltered. YAP/TAZ activation stimulates fibrosis by translating matrix stiffness into critical fibrogenic events and promotes fibroblast to myofibroblast conversion and TGF β signaling (60). We observed decreased numbers of α SMA-positive cells in the tKO fibrotic lesions suggesting lower myofibroblast numbers, as previously seen in integrin $\alpha 11 \beta 1$ knockout mice (26, 27). We also showed that the WT fibrotic lesions contain a higher amount of active YAP/ α SMA double positive cells suggesting that active YAP is contributed by a subset of myofibroblasts, specifically in the deep dermis. The decrease of YAP activated myofibroblasts in the deep dermis of tKO fibrotic lesions

raised the question if the loss of the three integrins alters the formation of myofibroblasts from fibroblasts of the reticular (deep) dermis or of fascia underlying the deep dermis (61). The reticular fibroblast population (62) is a major contributor of a plethora of fibrogenic factors such as TGF β , α SMA and of new ECM (63). These fibroblasts deposit stiffer matrix than the papillary fibroblasts (64), thus providing sufficient mechanical tension for YAP activation. Additionally, myofibroblasts originating from reticular fibroblasts are highly contractile and capable of cooperative matrix contraction by generating collective forces through their stress fibers (47, 65, 66). The absence of the integrins α 1 β 1, α 2 β 1 and α 11 β 1 in the reticular or fascia fibroblasts seems to interfere with the process of myofibroblast generation in the deep dermis due to disrupted force transmission. Interestingly, this is also in accordance with reduced CCN1 in the deep dermis of fibrotic lesions in tKO mice compared to WT. CCN1 is a downstream YAP target, and it is associated with fibrosis where its anti- or profibrotic role depends on the specific context (46, 67). In addition, recent experiments demonstrated that reduced YAP activity is associated with cellular aging (68). Lack of these integrins may therefore also promote (myo)fibroblast senescence.

In conclusion, we have documented that the integrins α 1 β 1, α 2 β 1 and α 11 β 1 are required for activation and force generation in fibroblasts in vitro. Mice lacking them reveal only mild alterations but show a severely attenuated fibrotic reaction following bleomycin injection, indicating a role of these integrins in remodeling situations in vivo. Targeting these integrins by monoclonal antibodies or small molecules (69-71) could be implemented to ameliorate the fibrotic response. Actually, integrin inhibitors have already been used in several pathological processes (71). Some have led to clinical trials, for example, the dual α v β 1- α v β 6 integrin inhibitor PLN74809 is currently being tested in a clinical trial for the treatment of interstitial pulmonary fibrosis (69, 72). Even an inhibitor of the integrins α 1 β 1, α 2 β 1 and α 11 β 1 has been described (73). Our study provides the detailed analysis of the cellular mechanisms underlying these approaches and indicates that lack of the activity of these integrins is most crucial in challenging situations such as fibrotic reactions.

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Author contributions

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Figure legends

Fig. 1: Characteristic features of adult mice with constitutive ablation of integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 11\beta 1$. (A) Representative image of WT (left) and tKO mice at 6 weeks. (B) Body length and (C) weight of tKO mice are reduced in comparison to WT littermates. N=5. (D) Histological analysis (Hematoxylin and eosin staining) of the dorsal skin of 3 months old mice shows no major alterations. (E) Representative electron micrographs of dorsal skin from 3 months old mice shows no alterations in the collagen fibrillar arrangement. (F-M) Graphs from skin tensile strength tests of back skin from 10 weeks old mice reveal significant reduction in (F) maximum force, (G) maximum deformation, (H) stiffness, (I) energy and (J) maximum strain of tKO skin, while (K) maximum stress, (L) elastic modulus and (M) toughness do not differ from WT littermates; N=11 WT / 8 tKO. (N) Representative confocal images of back skin of 3 months old mice show significantly increased DDR2 staining in tKO (O) upper and (P) lower dermis. N=4 per genotype. Quantification in (N) was restricted to dermal cells excluding hair follicles, arrector pili muscle and blood vessels (shown in magnified insets). Red arrows in insets depict DDR2 positive fibroblasts. Each data point represents a separate mouse. P values were calculated using two-way ANOVA with Sidak's multiple comparisons test for (B and C), Student's t test for (F-M) and Mann Whitney U test for (O and P). Data are presented as mean $\pm$ SD. **** P $\leq$ 0.0001, *** P $\leq$ 0.001, ** P $\leq$ 0.01 and * P $\leq$ 0.05. Scale bars indicate 200 μ m in (D), 500 nm in (E) and 30 μ m in (N).

Fig. 2: Primary dermal fibroblasts derived from tKO mice display unaltered expression of fibronectin binding integrin $\alpha 5\beta 1$ but increased expression of non-integrin collagen receptor DDR2. (A-D) Flow cytometric analysis of WT and tKO fibroblasts isolated from mice skin seeded on fibronectin for 6 h reveals unaltered surface levels of (A and B) integrin subunit $\alpha 5$ (ITA5) and (C and D) integrin subunit $\beta 1$ (ITB1) in tKO fibroblasts. Representative western blots of primary dermal fibroblasts before (0 h, trypsinized cells) and after seeding for 6 h on (E) collagen I coating. Significantly increased levels of (F) phosphorylated DDR2 and (G) total DDR2 are detected in tKO fibroblasts seeded for 6 h on collagen I, while (H) the ratio of phosphorylated to total DDR2 is comparable. (I) Stimulation of fibroblasts adherent to tissue culture plastic with acid-solubilized collagen I (10 μ g/ml in 0.1% acetic acid) for 1 h results in a (J) significant increase in phosphorylated DDR2 and (K) a slight but not significant increase in total DDR2 and (L) in the ratio of phosphorylated to total DDR2 in tKO fibroblasts. N = 4-5 per genotype. Each data point represents fibroblasts from a separate mouse. Data are presented as mean $\pm$ SD. P values were calculated using Mann Whitney U test. * P $\leq$ 0.05.

Fig. 3: tKO fibroblasts spread to significantly smaller size than WT on collagen and fibronectin. (A) Phase contrast images of WT and tKO fibroblasts seeded for 6 h on collagen I and fibronectin. (B) Whereas virtually no tKO fibroblasts adhere and spread on collagen I, adhesion to fibronectin is comparable to WT. (C and E) Representative confocal images of fibroblasts seeded for 6 h on (C) collagen I and (E) fibronectin. (D and F) Quantification of cell size shows significant reduction in tKO

spreading on both substrates. Each data point represents cells from a separate mouse. For (B) N = 6 mice per genotype and for (D and F) N = 4 mice per genotype (≥ 22 cells per mouse) were analyzed. Data are presented as mean $\pm$ SD. P values were calculated using Kruskal-Wallis test followed by Dunn's test of multiple comparison test for (B) and Mann Whitney U test for (D). * $P \leq 0.05$. Scale bars indicate 100 μm in (A) and 30 μm in (C and E).

Fig. 4: Differences in deposited ECM and secretome between tKO and WT fibroblasts. (A) Representative confocal images showing staining for collagen I (red) and phalloidin (blue). Fibroblasts were seeded for 2 h either on autologous or heterologous (bottom) ECM deposited by either WT or tKO primary dermal fibroblasts for 10 days followed by decellularization. (B) On autologous matrix, tKO fibroblasts spread to significantly smaller size than WT. This spreading defect is partially rescued by seeding tKO fibroblasts on WT matrix. WT fibroblasts show a minor decrease in cell spreading when seeded on tKO matrix. Each data point represents fibroblasts from a separate mouse, N = 4 mice per genotype, N ≥ 25 fibroblasts per mouse. (C and D) Representative confocal images of ECM deposited by WT and tKO fibroblasts for 10 days, stained for collagen I (white) and DAPI (blue), show decreased amounts of thick collagen fibers (C) before and more clearly (D) after decellularization. (E) Volcano plot and (F) list of significantly down- (blue) and upregulated (pink) proteins in tKO fibroblast supernatants as determined by SILAC-based secretome analysis. N = 4. (G) STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis and (H) Gene ontology (GO) term analysis representing biological processes potentially affected due to the significantly altered proteins in the tKO secretome. (B) Data are presented as mean $\pm$ SD. P values were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison test. Scale bars indicate 30 μm in (A, C and D)

Fig. 5: tKO fibroblasts show significantly altered properties essential for efficient mechanotransduction and collagen remodeling. (A,B) Traction stress is significantly reduced in tKO fibroblasts seeded onto collagen I-coated polyacrylamide gels with a nominal stiffness of 8.44 kPa. N=3 mice per genotype, N > 12 cells per mouse per experiment. (C) Representative images of floating collagen lattices seeded with fibroblasts for the indicated time reveal (D) significantly reduced capacity of tKO fibroblasts to remodel and contract the lattices. N = fibroblasts from 4 mice per genotype. (E) Western blot of fibroblasts seeded on collagen I for 6 h showing (F) significantly reduced levels of active YAP, (G) unaltered amount of total YAP and (H) decreased ratio of active YAP : total YAP in tKO fibroblasts. (I) Western blot of fibroblasts seeded on fibronectin for 6 h showing slightly but not significantly reduced levels of (J) active YAP, (K) total YAP and (L) ratio of active YAP : total YAP in tKO fibroblasts. N = fibroblasts from 5 separate mice per genotype. Western blots of fibroblasts seeded for 6 h on collagen I (M) or fibronectin (O) showing (N) significantly elevated ratio of G actin : F actin in tKO fibroblasts on collagen but (P) no significant alteration on fibronectin. N = fibroblasts from 4 separate mice per genotype. (Q-T) Representative confocal images showing fibroblasts seeded for 6 h

on **(Q and R)** collagen I or on **(S and T)** fibronectin. tKO fibroblasts on collagen show **(Q)** highly reduced nuclear localization of YAP and **(R)** negligible active YAP, accompanied by **(Q and R)** drastically reduced cell spreading and absence of actin microfilaments (Phalloidin staining) and FAs (Paxillin staining). **(S and T)** tKO fibroblasts seeded on fibronectin do not show altered **(S)** nuclear YAP signals and **(T)** active YAP signals, however, **(S and T)** altered architecture of the actin cytoskeleton (Phalloidin) and of FAs (Paxillin). **(U-W)** FA analysis of tKO fibroblasts seeded on collagen I for 6 h revealed a significant decrease in **(U)** numbers, **(V)** percentage of cell area occupied by, and **(W)** size of FAs. N = fibroblasts from 4 separate mice per genotype. Data are presented as mean $\pm$ SD. P values were calculated using Mann Whitney U test. **** $P \leq 0.0001$, ** $P \leq 0.01$ and * $P \leq 0.05$. Scale bars indicate 10 μ m in (A) and 30 μ m in (Q-T).

Fig. 6: Reduced fibrotic response to bleomycin in tKO mice. (A) Representative hematoxylin and eosin staining of skin of WT and tKO mice following repeated injections with NaCl (used as solvent control) shows (B) comparable dermal thickness (indicated by black arrow), whereas (C) bleomycin treatment results in a significant reduction in dermal thickness of tKO lesions as also seen by analyzing the (D) relative dermal thickness when normalized to NaCl treated skin and (H) collagen content in tKO lesions. Representative images of skin sections (E) acquired using second harmonic generation microscopy showing collagen in green and nuclei (DAPI) in blue. Quantification shows (F) comparable collagen signals in NaCl treated skin of WT and tKO mice, however, (G) significantly lower collagen fluorescence intensity in bleomycin-injected tKO skin. N = 4-5 per genotype, each data point represents skin or fibroblasts from a separate mouse. (I) Representative transmission electron micrographs showing more densely packed collagen fibrils in the upper dermis in bleomycin-injected lesions of WT than in tKO lesions. In contrast, collagen fibril arrangement is comparable in WT and tKO skin upon NaCl injection. (J) Quantification of area occupied by collagen fibrils (percent), indicating that the interfibrillar space is larger in tKO than in WT lesions. The red dotted line marks the epidermal-dermal basement membrane. Data are presented as mean $\pm$ SD. P values were calculated using Mann Whitney U test for (B-D and F-H). ** $P \leq 0.01$ and * $P \leq 0.05$. Scale bars indicate 500 μ m in (A), 100 μ m in (E) and 500 nm in (I).

Fig. 7: Reduced TGF β signaling and myofibroblast numbers in tKO fibrotic lesions. (A) Representative immunohistochemistry images showing (B) comparable numbers of cells with nuclear pSMAD2 signals upon NaCl treatment, (C) but significantly higher numbers of cells with active TGF β signaling in WT than in tKO skin upon bleomycin treatment. (D) Representative confocal images of skin showing (E) comparable numbers of α SMA-positive myofibroblasts in NaCl-injected skin, (F) but significantly higher myofibroblast numbers in WT than tKO upon bleomycin treatment (white arrow heads in magnified areas). (A-F) Quantification was restricted to dermal fibroblasts excluding hair follicles, arrector pili muscle and blood vessels. N = 3-5 per genotype, each data point represents a separate mouse. Data are presented as mean $\pm$ SD. P values were calculated using Mann Whitney U test. ** $P \leq 0.01$

≤ 0.01 and $* P \leq 0.05$. Scale bars indicate 100 μm in (A), 50 μm in magnified inset in (A) and 50 μm in (D) and magnified inset in (D).

Fig. 8: Bleomycin-induced dermal fibrotic lesions in tKO mice show reduced YAP activation and decreased expression of downstream YAP target, CCN1.

(A) Representative confocal images of bleomycin-induced fibrotic lesions show lower abundance of active YAP-positive dermal fibroblasts (red arrows) in tKO mice as confirmed by quantification of (B) active YAP fluorescence intensity per μm^2 and (C) number of active YAP-positive nuclei per field. (A) Counterstaining with αSMA reveals that the active YAP-positive nuclei identify a subset of myofibroblasts. Quantification was restricted to dermal fibroblasts, excluding hair follicles, arrector pili muscle and blood vessels. (D-F) Results of Tandem Mass Tag (TMT) labeling-based comparative proteomics of tKO and WT fibrotic skin lesions. (D) Gene ontology (GO) term analysis showing the biological processes that may be affected by the overall significantly altered proteins in tKO lesions and known to be linked to fibrosis. (E) Volcano plot (F) List depicting the significantly downregulated (blue) and upregulated (pink) proteins related to fibrosis and (G) Representative confocal images showing decreased CCN1 expression in (H) the upper as well as (I) lower dermis of tKO lesions compared to WT. N = 4 separate mice per genotype. Data are presented as mean $\pm$ SD. (A-C) and (G-I) each data point represents a separate mouse. P values were calculated using Mann Whitney U test. $* P \leq 0.05$. Scale bars indicate 30 μm in (A) and (G).

Figure S1: Absence of integrin subunits $\alpha 1$, $\alpha 2$ and $\alpha 11$ in tKO mice. (A) PCR of genomic DNA confirming presence of targeted *Itga1*, *Itga2* and *Itga11* alleles and the absence of *Itga2* and *Itga11* alleles in tKO mice. (B) RT-PCR showing the absence of *Itga1*, *Itga2* and *Itga11* transcripts in various tissues. (C) and (D) Flow cytometric analysis showing the absence of surface-localized ITGA1 in tKO fibroblasts after seeding for 6 h on solubilized and coated collagen I (coating of collagen in 0.1% acetic acid and in PBS, respectively). Each data point represents cells from a separate mouse, N = 4. Data are presented as mean $\pm$ SD. P values were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison test. $* P \leq 0.05$. (E) Western blot confirming the absence of ITGA2 and ITGA11 proteins in tKO fibroblasts seeded for 6 h on either solubilized or coated collagen I or fibronectin.

Fig. S2: tKO fibroblasts have unaltered levels of integrin $\alpha 10$. (A and B) Flow cytometric analysis showing that $\alpha 10$ is not detected, however $\alpha 11$ integrin is observed in WT dermal fibroblasts (A), whereas both $\alpha 10$ and $\alpha 11$ could not be detected in the tKO fibroblasts (B). (C) Positive control showing that both $\alpha 10$ and $\alpha 11$ could be detected in murine rib cage chondrocytes confirming that antibodies are functional and specific.

Fig. S3: Time-dependent increase in levels of DDR2 and phospho-DDR2 in tKO fibroblasts. Western blots showing higher amounts of DDR2 and phosphorylated DDR2 in tKO fibroblasts already following trypsin-mediated detachment from tissue culture plastic (0 h) and at 2, 4 and 6 h after seeding onto collagen I coats in comparison to WT fibroblasts.

Fig. S4: Scheme depicting HIPPO/YAP signaling in fibroblasts. HIPPO signaling is turned on by interaction of fibroblasts with a soft ECM or in the absence of proper force transduction machinery, such as in tKO fibroblasts (left). Cytoplasmic MST1/2 and LATS1/2 are phosphorylated and phosphorylate YAP, which will result in cytoplasmic retention. In contrast upon interaction of fibroblasts with an ECM in which tensile forces are transduced by integrins, focal adhesions and F-actin, YAP is not phosphorylated and thereby translocates to the nucleus and enables transcription of mechanosensitive genes including collagen I and other ECM. Their production and/or activity promote fibrosis.

Fig. S5: Reduced pFAK and pSRC in tKO fibroblasts on collagen but not on fibronectin. (A) Representative western blots of primary dermal fibroblasts before (0 h, trypsinized cells) and after seeding for 6 h on either collagen I or fibronectin coating. Although minimal phospho-FAK signals were detected in fibroblasts seeded on collagen I, tKO fibroblasts expressed lower phospho-FAK than WT. Strong reduction in phospho-SRC signals were observed in tKO fibroblasts seeded on collagen I as compared to WT. Phospho-FAK and phospho-SRC levels were unaltered in tKO fibroblasts seeded on fibronectin. Graphs showing that tKO fibroblasts seeded for 6 h on collagen I have significantly decreased levels of (B) phosphorylated SRC compared to WT. (C) Total SRC did not differ from WT and (D) the ratio of phosphorylated to total SRC is lower in tKO than WT. N = 2 for FAK and N = 4 for SRC. Data are presented as mean $\pm$ SD. Each data point in (B-D) represents a separate mouse. P values were calculated using Mann Whitney U test. * $P \leq 0.05$.

Fig. S6: Intradermal NaCl injection does not lead to fibrotic lesions with active YAP expression. (A) Immunofluorescence images of NaCl injected WT and tKO skin do not show the presence of either active YAP or α SMA expression. (B) Volcano plot, (C) list of upregulated (pink) and downregulated (blue) proteins, and D. GO term analysis of TMT labeling based comparative proteomics analysis of NaCl injected WT and tKO back skin. N = 4 for each genotype. Scale bars indicate 30 μ m in (A).

Fig. 1

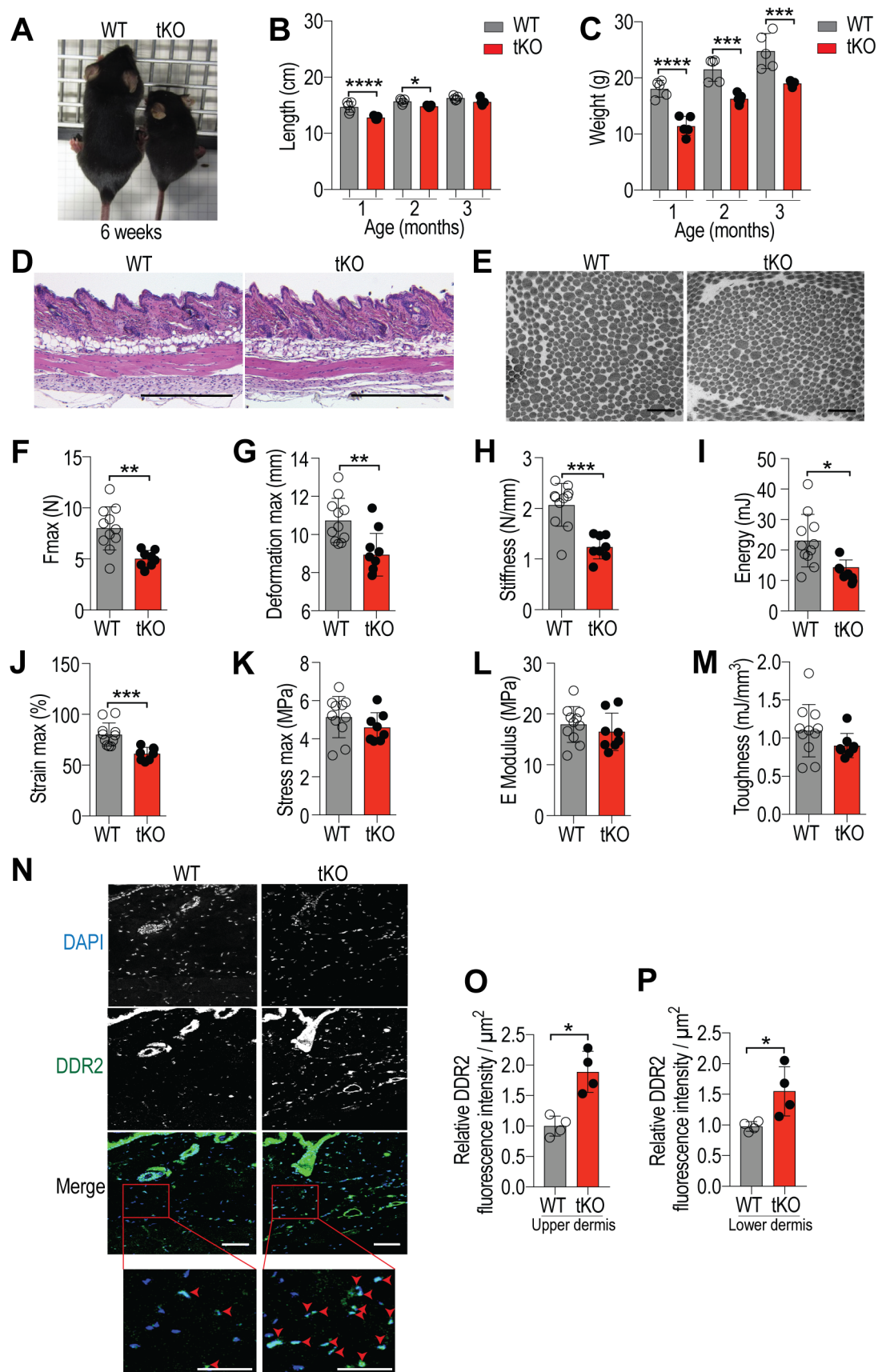


Fig. 2

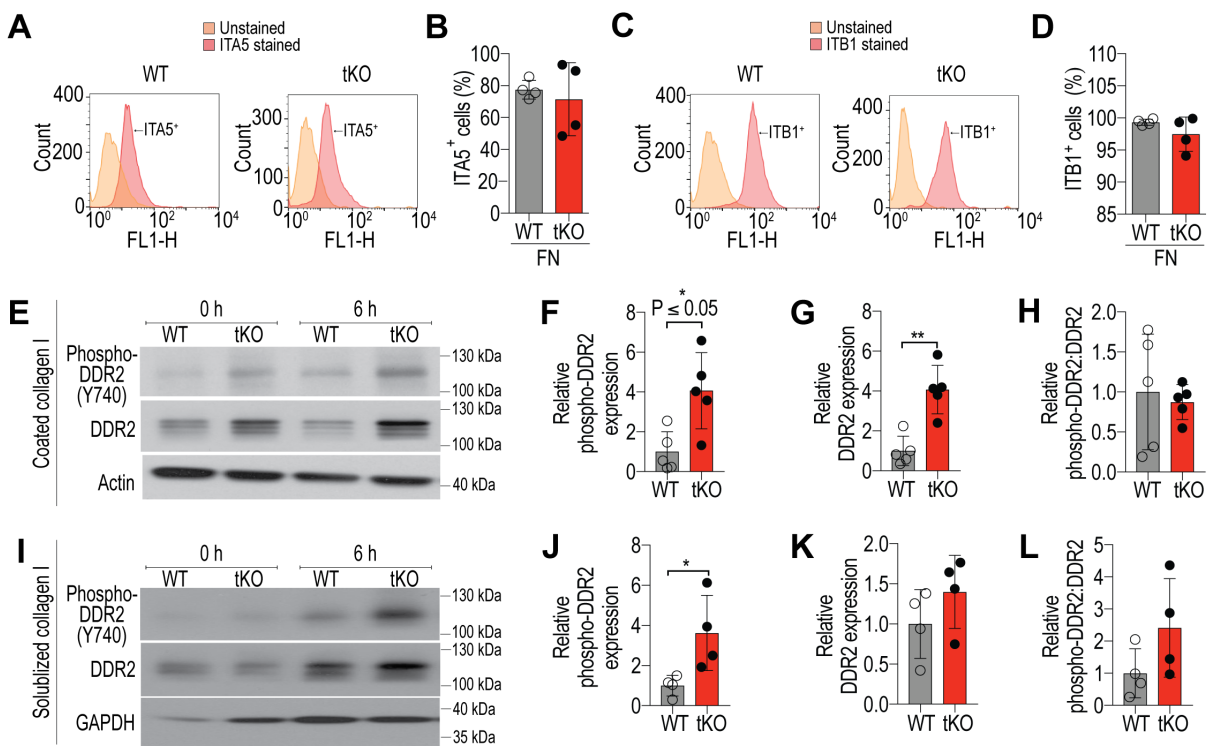


Fig. 3

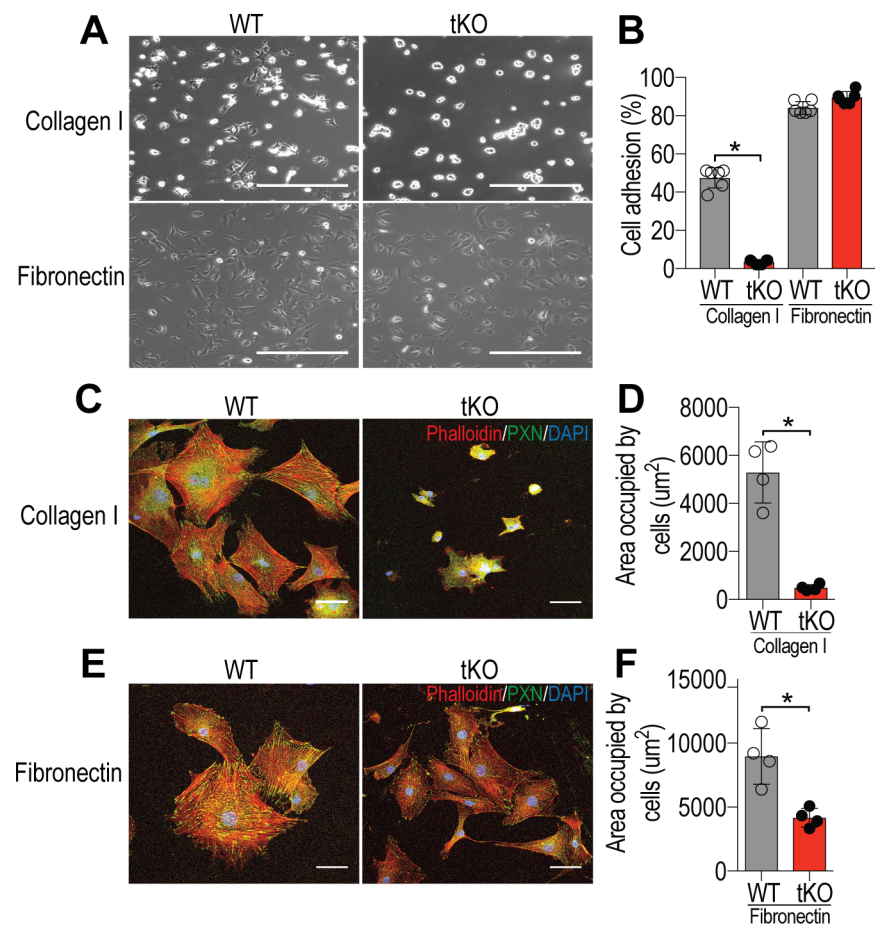


Fig. 4

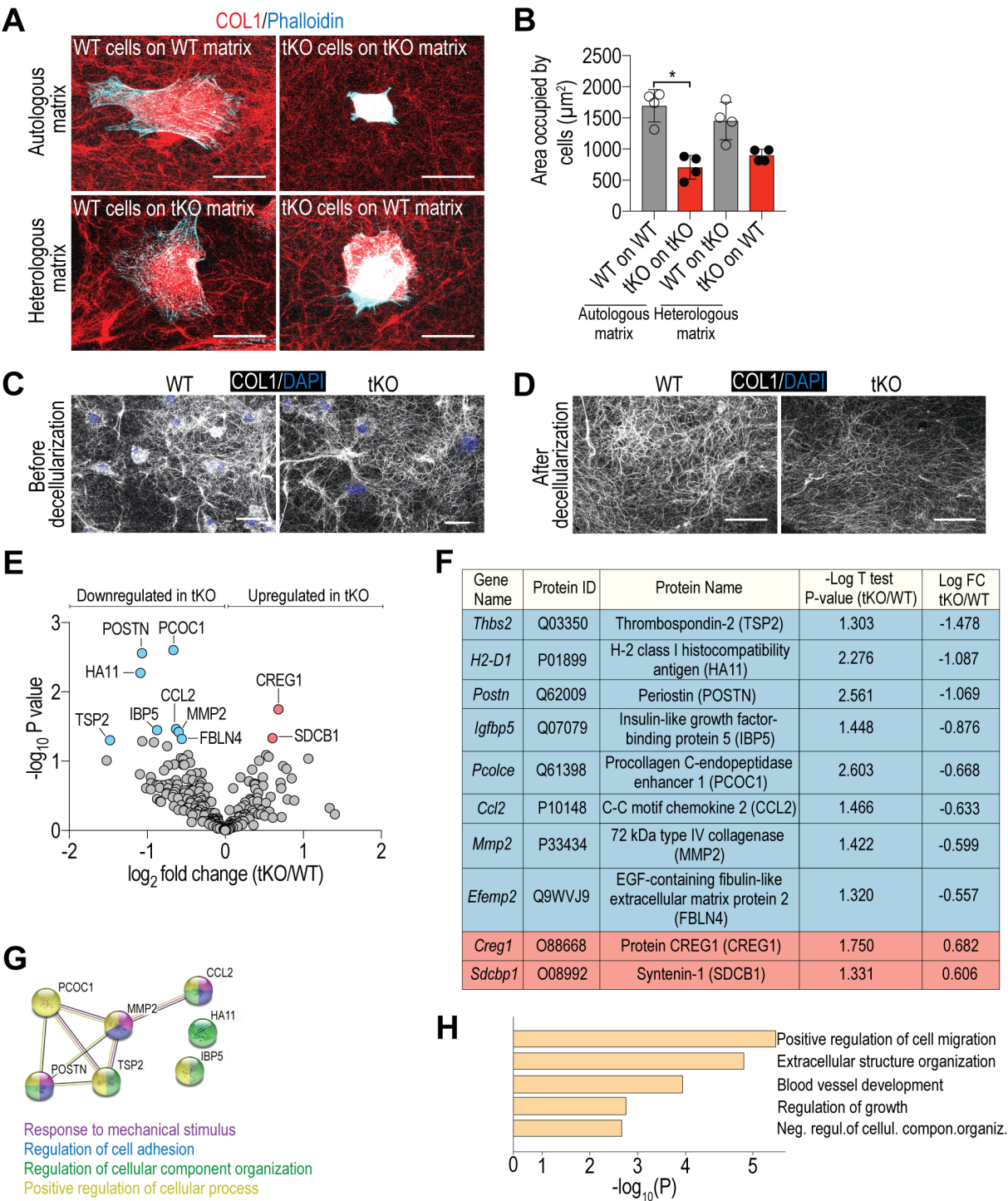


Fig. 5

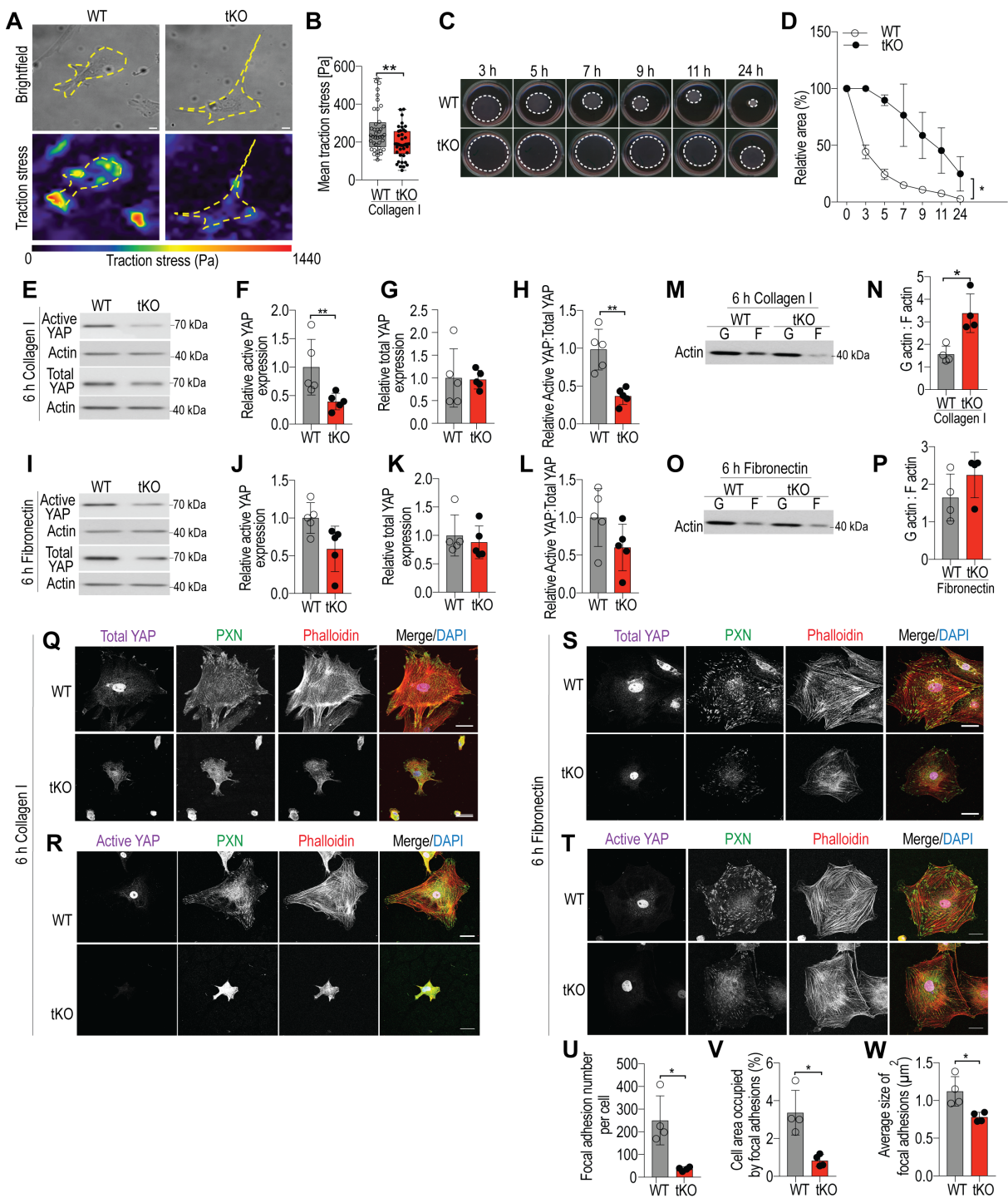


Fig. 6

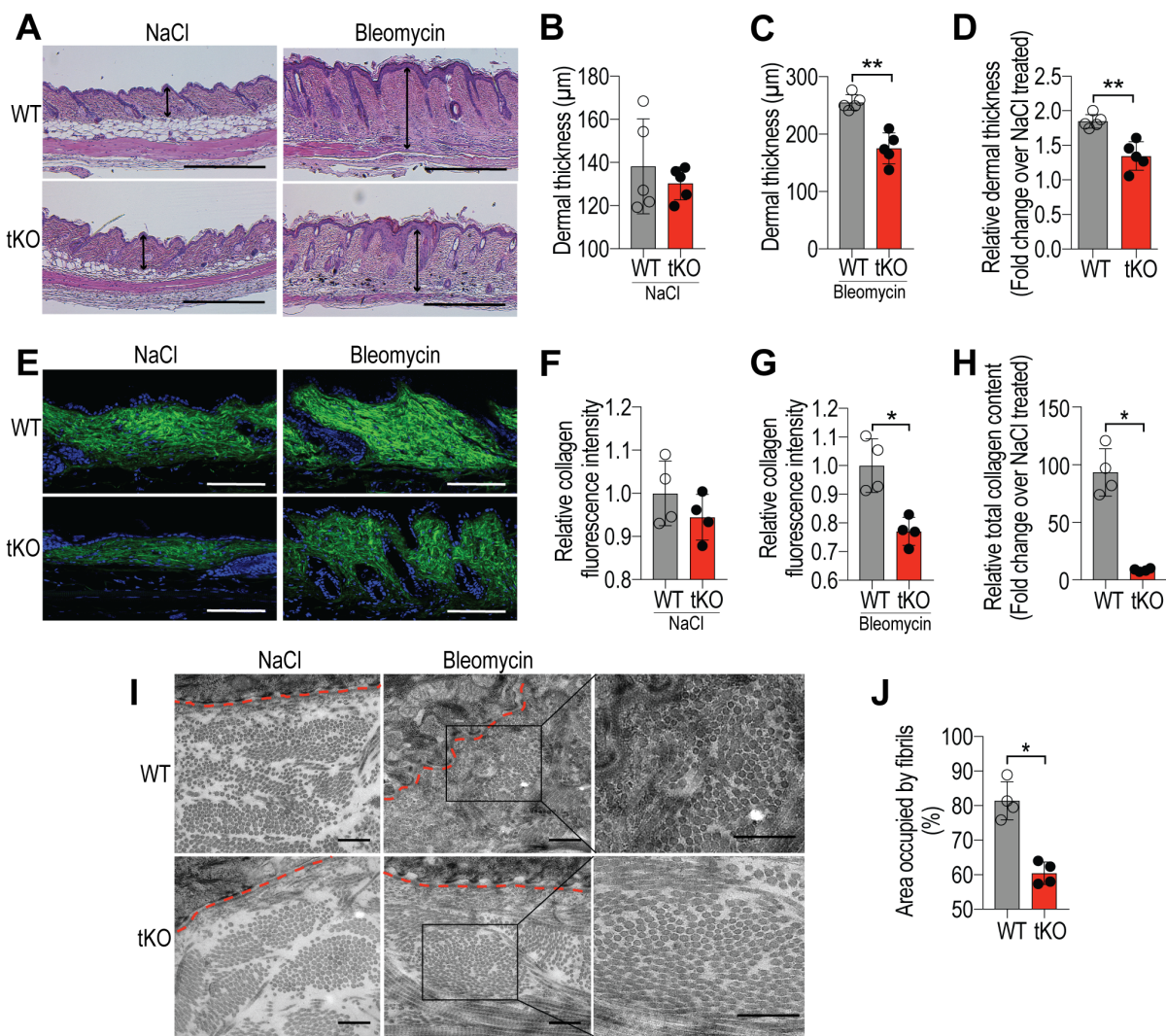


Fig. 7

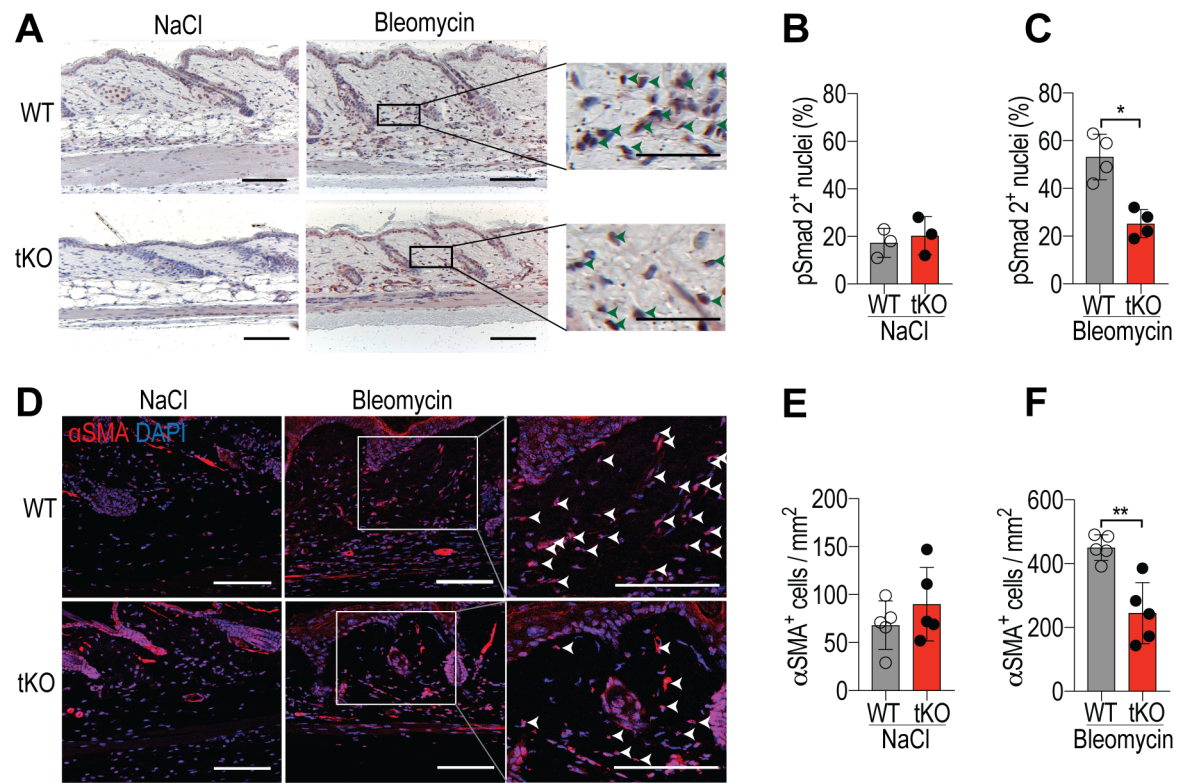


Fig. 8

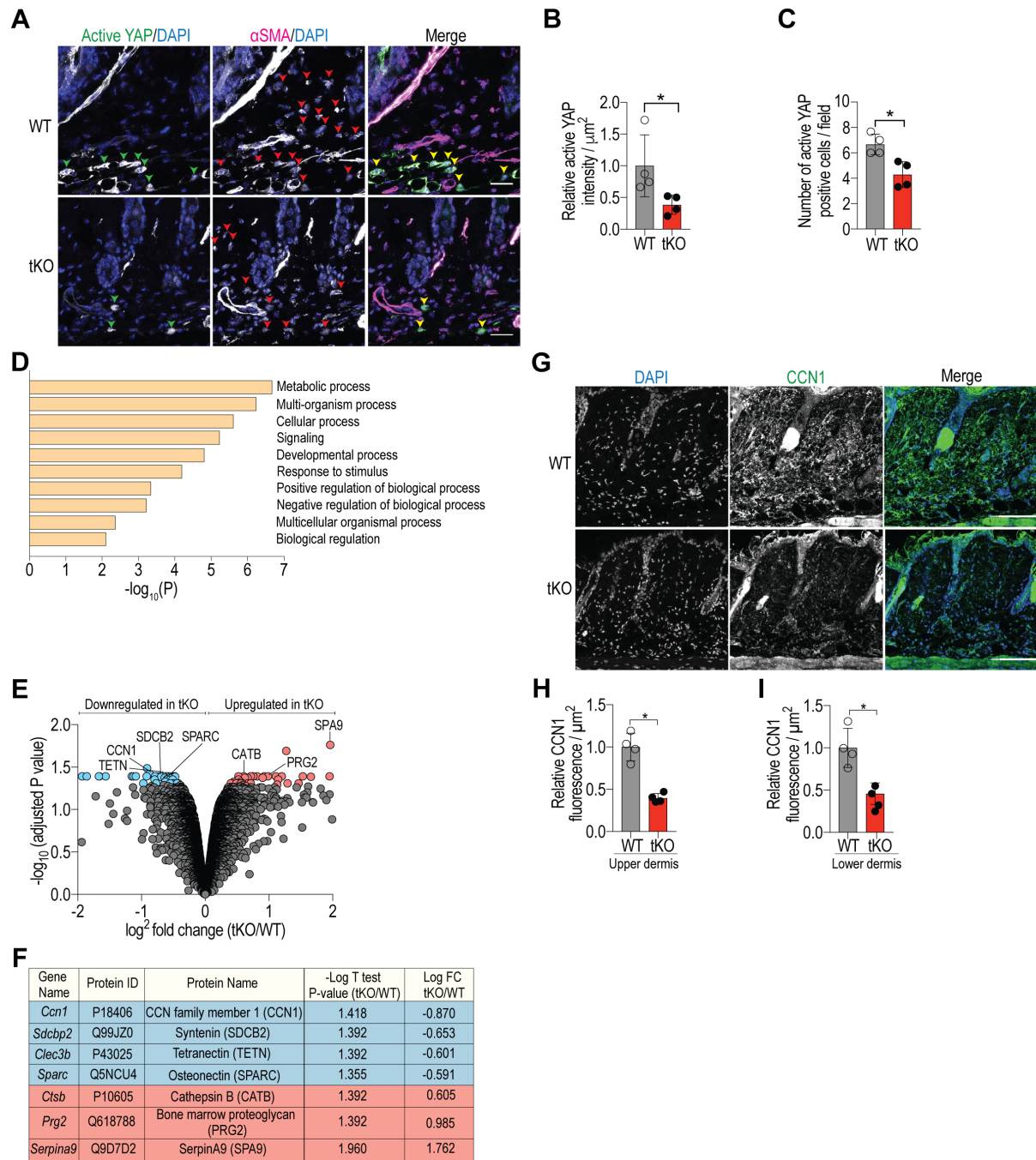


Fig. S1

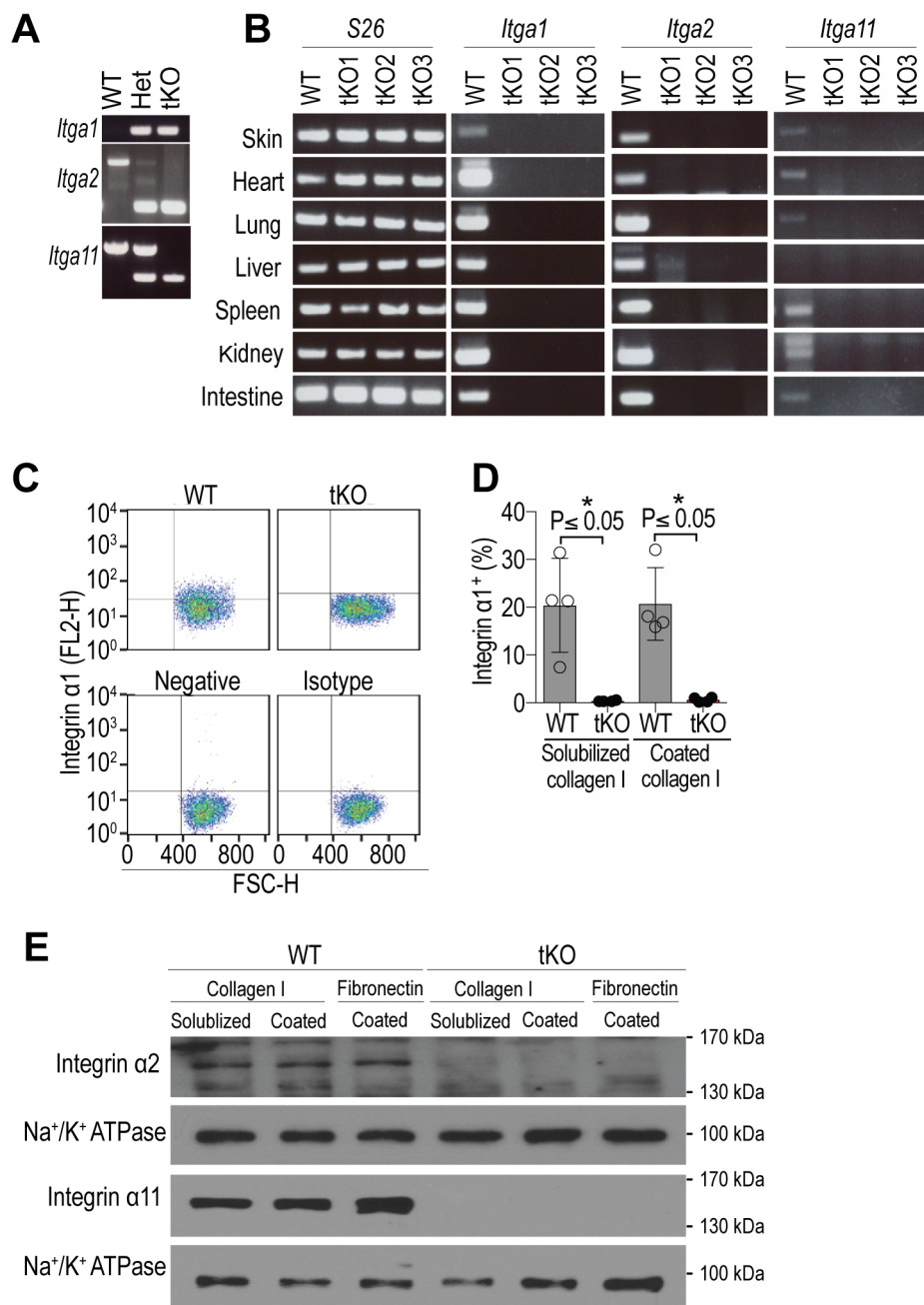


Fig. S2

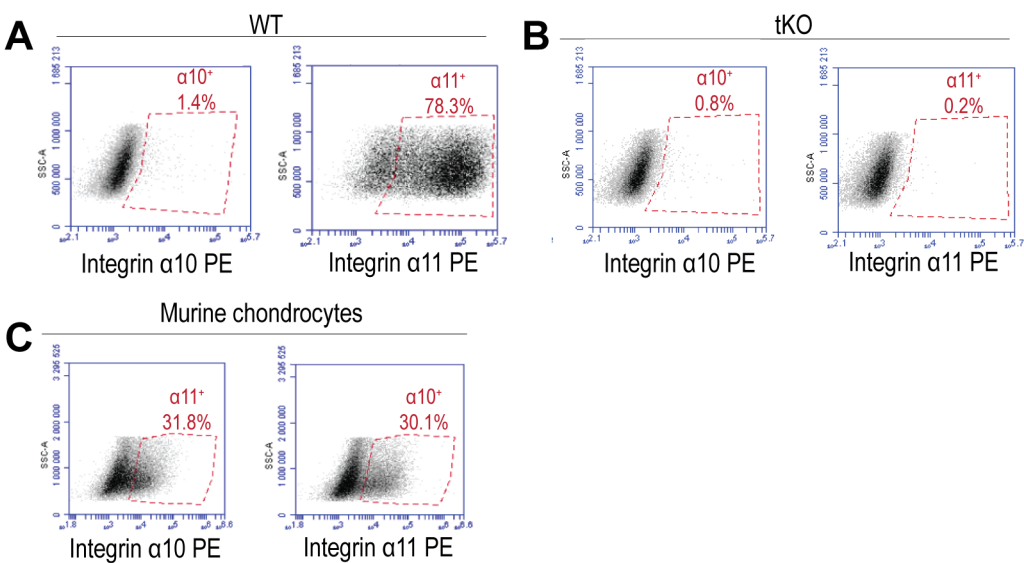


Fig. S3

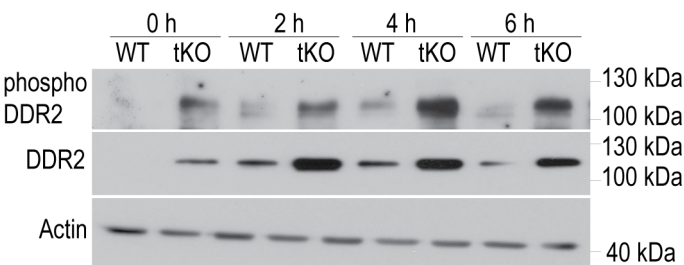


Fig. S4

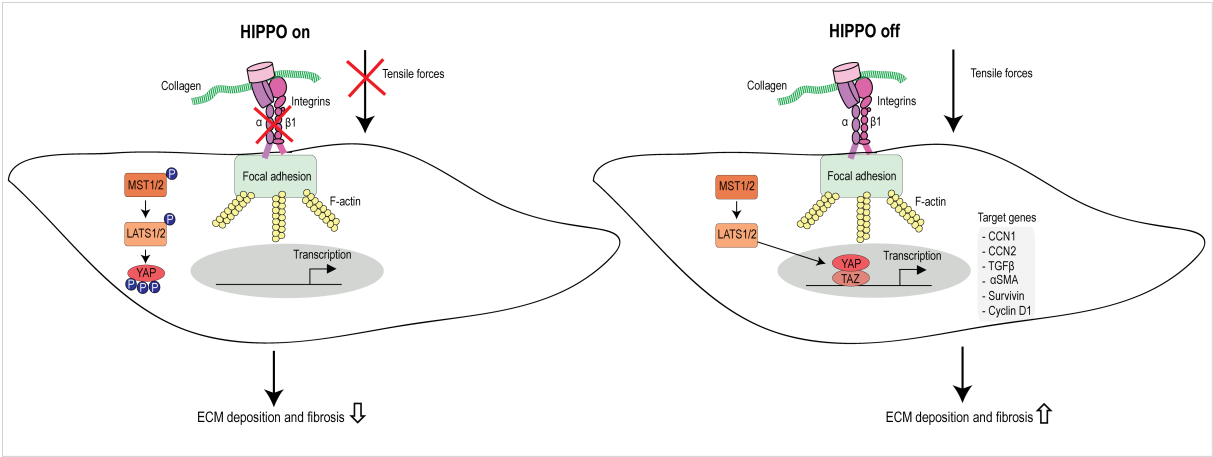


Fig. S5

