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1 # TGF-β signaling is critical for maintenance of the tendon cell fate

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10

11 **Abstract**

12 Studies of cell fate focus on specification, but little is known about maintenance of the
13 differentiated state. We find that TGFβ signaling plays an essential role in maintenance of the
14 tendon cell fate. To examine the role TGFβ signaling in tenocytes TGFβ type II receptor was
15 targeted in the Scleraxis cell lineage. Tendon development was not disrupted in mutant
16 embryos, but shortly after birth tenocytes lost differentiation markers and reverted to a more
17 stem/progenitor state. Targeting of Tgfbr2 using other Cre drivers did not cause tenocyte
18 dedifferentiation suggesting a critical significance for the spatio-temporal activity of ScxCre.
19 Viral reintroduction of Tgfbr2 to mutants was sufficient to prevent and even rescue mutant
20 tenocytes suggesting a continuous and cell-autonomous role for TGFβ signaling in cell fate
21 maintenance. These results uncover the critical importance of molecular pathways that
22 maintain the differentiated cell fate and a key role for TGFβ signaling in these processes.

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30 **Introduction**

31 Studies of cell fate determination are in most cases focused on the signaling pathways and
32 transcription factors that direct naïve cells to assume a specific cell fate (Li et al., 2012; James,
33 2013; Huang et al., 2015). It is commonly accepted that once fully differentiated the cells enter
34 a stable cellular phenotype, but relatively little is known about the molecular mechanisms that
35 reinforce and maintain this differentiated state. Maintenance of the differentiated state is
36 however essential for tissue function and identifying the molecular pathways involved in these
37 processes may be of great importance for understanding tissue homeostasis and pathology.

38 Tendons are connective tissues that transmit forces from muscle to bone to generate
39 movement (Kannus, 2000). Despite their importance to overall musculoskeletal function and
40 their slow and limited healing capabilities, relatively little is known about tendon development,
41 the tendon cell fate, maturation and pathology. Elucidating the key molecular regulators of
42 these processes is thus essential for improvements in the management of tendon healing, the
43 treatment of tendinopathy and for bioengineering efforts for this tissue.

44 A limited number of transcription factors were so far identified as key regulators of the
45 tendon cell fate including most notably, Scleraxis (Scx), a bHLH transcription factor expressed in
46 tendon cells from progenitor stages and through development (Schweitzer et al., 2001) and
47 Mohawk (Mkx), an atypical homeobox protein with essential roles in the development of the
48 collagen matrix in tendons (Ito et al., 2010). Prototypic markers for the tendon cell fate also
49 include the transmembrane protein tenomodulin (Tnmd) and collagen type I (Kannus, 2000;
50 Huang et al., 2015), the major building blocks of the tendon fibrillar extracellular matrix that
51 mediates the transmission of force by tendons.

52 Previous studies have also established a central role for the transforming growth factor-β
53 (TGFβ) signaling pathway in early events of tendon development (Pryce et al., 2009; Havis et al.,
54 2016). Notably, TGFβ is a potent inducer of Scleraxis (Scx) both *in vivo* and in cultured cells and
55 disruption of TGFβ signaling in mouse limb bud mesenchyme resulted in complete failure of
56 tendon formation (Pryce et al., 2009). This phenotype manifested at the onset of embryonic
57 tendon development but robust expression of TGFβ ligands and associated molecules in later

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58 stages of tendon development suggested possible additional roles for TGFβ signaling in tendon
59 development (Kuo et al., 2008; Pryce et al., 2009). Moreover, subcutaneous application of
60 growth and differentiation factors (GDFs), members of the TGFβ superfamily, can induce
61 ectopic neo-tendon formation in rats (Wolfman et al., 1997). The goal of this study was
62 therefore to ask if TGFβ signaling plays essential roles at later stages of tendon development.

63 The TGFβ superfamily comprises secreted polypeptides that regulate diverse developmental
64 processes ranging from cellular growth, differentiation and migration to tissue patterning and
65 morphogenesis (Santibanez et al., 2011; Sakaki-Yumoto et al., 2013). These ligands act by
66 binding to transmembrane type II receptors, which in turn recruit and activate a type I receptor.
67 The activated receptor complex subsequently phosphorylates and activates receptor-regulated
68 transcription factors called Smads (Smad2/3 for TGFβ signaling) that subsequently complex
69 with the common-mediator Smad4 and translocate into the nucleus where they promote or
70 repress responsive target genes (Vander Ark et al., 2018). The TGFβ proper ligands (TGFβ1-3) all
71 bind to a single type II receptor. Consequently, disrupting this one receptor is sufficient to
72 abrogate all TGFβ signaling. To test for additional roles of TGFβ signaling in tendon
73 development and biology we wanted to bypass the early essential function in tendon formation,
74 and decided to target TGFβ type II receptor (Tgfbr2) explicitly in tendon cells. We therefore
75 targeted the receptor using ScxCre (Blitz et al., 2013), a tendon specific Cre driver, so that TGFβ
76 signaling will be disrupted specifically in tendon cells and only after the initial events of tendon
77 formation.

78 We find that tendon differentiation function and growth during embryonic development
79 was not disrupted following targeted deletion of TGFβ signaling in tenocytes, but shortly after
80 birth the cells lost tendon cell differentiation markers and reverted to a more progenitor-like
81 state. Moreover, viral reintroduction of Tgfbr2 to mutant cells was sufficient to prevent
82 dedifferentiation and even to rescue the tendon cell fate in a cell-autonomous manner,
83 highlighting a continuous and essential role of TGFβ signaling in maintenance of the tendon cell
84 fate.

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86 **Results**

87 **Targeting TGFβ type II receptor in Scx-expressing cells resulted in tendon disruption and limb**
88 **abduction**

89 Our previous studies showed that disruption of TGFβ signaling in limb mesenchyme resulted in
90 the complete failure of tendon formation (Pryce et al., 2009). To examine later roles of TGFβ
91 signaling in tendon development we targeted Tgfbr2 with ScxCre thereby bypassing the early
92 role of TGFβ signaling for tendon development. Tgfbr2;ScxCre mutant embryos indeed
93 developed a complete network of tendons by E14.5, indicating they have bypassed the early
94 requirement for TGFβ signaling in tendon development (Fig. 1A).

95 Mutant tendon development was not perturbed through embryogenesis and mutant pups
96 appeared normal at birth (Fig. 1C). However, by day 3 after birth (P3), mutant pups showed
97 physical abnormalities that manifested in abducted paws, splayed limbs (Fig. 1C, black
98 arrowhead) and severe movement limitations. Examination of forelimb tendons of P7 mutant
99 pups using the tendon reporter ScxGFP revealed severe tendon disruptions. A few lateral
100 tendons, e.g. the extensor carpi radialis longus tendon underwent fragmentation and
101 disintegrated (Fig. 1B, yellow arrowhead and Fig. S1) whereas the majority of other tendons,
102 notably the extensor digitorium communis tendons, retained structural integrity with a
103 substantial loss of ScxGFP signal (Fig. 1B, white arrowhead). The substantial loss of ScxGFP
104 signal was also detected in all tendons and related tissues, including hindlimb- and tail tendons,
105 as well as ligaments and annulus fibrosus of the intervertebral disc (Fig. 1D,E). Movement
106 limitations were exacerbated as mutant pups became older and all mutants died at or before
107 P14. This phenotypic progression was observed in most mutant pups but intriguingly, in rare
108 cases (~2%) the mutant pups showed physical abnormalities and severe tendon phenotypes
109 already at birth.

110 A closer examination of the mutant embryos identified the first indication of a tendon
111 phenotype already at E16.5. The flexor carpi radialis tendons of mutant embryos were
112 consistently torn by E16.5 (Fig. S2). Interestingly, this phenotype was highly reproducible while
113 the patterning and development of other tendons in mutant embryos was not perturbed

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114 through embryogenesis. Moreover, expression of the prototypic tenocyte markers Scx, Tnmd
115 and collagen I (Fig. 2A-D) and the development of the collagen matrix were not disrupted in any
116 tendon of mutant embryos (Fig. 2E,F), including the flexor carpi radialis tendon before it
117 snapped. A direct cause for the specific tear of the flexor carpi radialis tendon in mutant
118 embryos was not identified to date.

119 Tendons are rich in collagen fibers that provide structural integrity to the tendons and
120 transmit the forces generated by muscle contraction (Kannus, 2000). Since young mutant pups
121 exhibited movement difficulties we first examined possible structural effects in the collagen
122 matrix. The ultrastructure of mutant tendons that remained intact was therefore analyzed by
123 transmission electron microscopy (TEM). Surprisingly, despite the functional defects starting
124 around P3, collagen fibers in mutant tendons appeared organized and indistinguishable from
125 those of wild-type (WT) littermates at this stage (Fig. 3). Apparent collagen degradation was
126 observed only in older mutant pups (≥ P7) (Fig. 3), suggesting the disruption to the matrix of
127 these tendons may be a secondary consequence of the cellular changes in these mutants
128 and/or of their movement difficulties. Furthermore, epitenon, a monolayer of cells that engulf
129 and define the boundary of the tendon (Kannus, 2000) (Fig. 3, black arrowhead), was gradually
130 disrupted and in some cases was almost undetectable in older mutant pups (Fig. 3, white
131 arrowhead), suggesting that loss of the tendon boundary is an additional feature of the
132 phenotype in these mutants.

133

134 **Loss of the tendon cell fate in mutant tenocytes**

135 As mentioned earlier, the ScxGFP signal in mutant tendons appeared patchy contrary to the
136 smooth appearance of WT tendons (Fig. 1B), suggesting a disruption at the cellular level. To
137 examine this phenotype at the cellular level we analyzed cross sections through the extensor
138 communis tendons of P7 WT and mutant pups. In P7 WT pups, all tendon cells were positive for
139 ScxGFP, *Tnmd* and *Col1a1* (Fig. 4A,C). Conversely, most cells in mutant tendons lost the ScxGFP
140 signal and tendon marker gene expression (Fig. 4B, white arrowhead and 4C). Interestingly,
141 some cells in mutant tendons retained ScxGFP signal and appeared rounded and enlarged from
142 P3 onwards (Fig. 4B, yellow arrowhead). Further investigation suggests that these cells are

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143 newly recruited tendon cells. Analysis of this aspect of the mutant phenotype will be published
144 in a separate manuscript (Tan et al., manuscript in preparation).

145 The fact that most cells in the mutant tendons do not express tendon markers is surprising,
146 since the cells in these tendons were functional tenocytes at embryonic stages as evidenced by
147 tendon marker gene expression and by the development of a functional collagen matrix (Fig. 2).
148 We next sought to determine if the ScxGFP-negative cells were indeed tendon cells that lost
149 tendon gene expression or if the mutant tendons were simply repopulated by non-tenogenic
150 cells. Using TUNEL assay we did not detect cell death in mutant tendons and the rate of
151 tenocyte proliferation as examined by EdU assay was also not altered in these tendons (Fig.
152 S3A,B), suggesting the cell population of mutant tendons was not altered. To directly determine
153 if the cells in mutant tendons were tenocytes whose cell fate was altered, we took advantage of
154 the fate mapping feature of the Rosa26-tdTomato (RosaT) Cre reporter system (Madisen et al.,
155 2010). When the reporter is activated by ScxCre, expression of the RosaT reporter is restricted
156 to the Scx-expressing cells and their progeny. We found that all ScxGFP-negative cells within
157 mutant tendons were positive for the RosaT reporter (Fig. 4D, white arrowhead), proving that
158 the cells in the mutant tendons were indeed derived from tenocytes. This result thus
159 highlighted an unexpected reversibility for the tendon cell fate where it was possible for
160 committed and functional tenocytes to lose their differentiation status.

161 To test if a continuous requirement for TGFβ signaling is essential for maintenance of the
162 tendon cell fate, we next targeted Tgfbr2 either selectively in Scx-expressing cells or in all cells
163 using the tamoxifen-inducible ScxCreERT2 (Howell et al., 2017) and RosaCreERT2 (Madisen et
164 al., 2010) drivers respectively during different developmental stages (E13 and P0). Interestingly
165 the cell fate of targeted cells was not disrupted in these mutants as evidenced by retention of
166 differentiation marker expression (data not shown). This result suggests that tenocyte
167 dedifferentiation in the Tgfbr2;ScxCre mutant tendons is dependent not simply on the loss of
168 TGFβ signaling in these cells but also on additional features in these mutants associated with
169 the specific spatial and temporal features of ScxCre activity.
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172 **Mutant tenocytes acquired stem/progenitor features**

173 Loss of cell differentiation marker can be the outcome of a few cellular processes, including
174 most notably cell death, change of cell fate (transdifferentiation) or reversion to a less
175 differentiated state (dedifferentiation) (Cai et al., 2007; Talchai et al., 2012; Tata et al., 2013).
176 As aforementioned, we found no apparent cell death in mutant tendons (Fig. S3A). Using
177 histological staining for the prototypic markers of osteocytes, adipocytes and chondrocytes we
178 found that loss of tendon gene expression in the cells of mutant tendons was also not due to
179 transdifferentiation (Fig. S3C), suggesting that the changes in mutant tendons may reflect a
180 process of cellular dedifferentiation.

181 One hallmark of cellular dedifferentiation is the loss of differentiation markers, which is
182 what we observed in mutant tendon cells. When cells dedifferentiate they also assume
183 stemness features e.g. colony forming potential, and in most cases these cells also acquire
184 expression of stem/progenitor cell markers (Sun et al., 2011; Tata et al., 2013; Nusse et al.,
185 2018). To date, very little is known about the specific gene expression and cellular behavior of
186 embryonic tendon progenitors. The only defined feature of these cells is the expression of the
187 Scx tendon progenitor marker (Schweitzer et al., 2001), which was evidently lost in the mutant
188 tendon cells. We therefore next directed our attention to similarities with stem/progenitor cells
189 isolated from tendons (tendon-derived stem/progenitor cells) (Bi et al., 2007; Rui et al., 2010;
190 Zhang and Wang, 2010; Mienaltowski et al., 2013) and with stem/progenitor cell markers
191 reported in other studies (Blitz et al., 2013; Dyment et al., 2013; Tan et al., 2013; Runesson et
192 al., 2015; Yin et al., 2016).

193 To test the colony-forming capacity of the mutant tendon cells, P7 mutant tendons were
194 dissociated and FACS-sorted to collect ScxGFP-negative and RosaT-positive cells, which were
195 then seeded at one cell per well in 96-well plates. As shown in Fig. 5A, about 1-2% of cells
196 (ScxGFP-positive and RosaT-positive) isolated from tendons of P7 WT and heterozygous
197 controls formed colonies in culture, similar to the frequency of colony forming cells reported in
198 other studies (Bi et al., 2007; Rui et al., 2010). On the other hand, we found a significant 8-fold
199 increase (p<0.01) in the frequency of colony forming cells in mutant tendons (Fig. 5A).

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200 We next screened the mutant tendons for expression of stem/progenitor cell markers. We
201 found that while Tgfbr2;ScxCre mutant tendon cells exhibited weak or negative expression of
202 some progenitor markers e.g. CD90.2, Oct-3/4, Sox2, nucleostemin, alpha-SMA, nestin and Sox9
203 (data not shown), the mutant tendon cells gradually acquired expression of stem cell antigen-1
204 (Sca-1) and CD44 in postnatal stages (Fig. 5B). Notably, expression of Sca-1 was undetectable
205 and CD44 was detected only in very few WT tendon cells, but surprisingly robust expression of
206 these markers was detected in the epitenon (Fig. 5C, white arrowheads), a possible source of
207 progenitor cells (Mendias et al., 2012; Dyment et al., 2013; Mienaltowski et al., 2013). The
208 similarity of marker expression between the mutant tenocytes and epitenon cells therefore
209 reinforces the notion that the mutant tenocytes acquired progenitor features.

210 Dedifferentiation is frequently associated with reversion to an earlier progenitor cell fate
211 (Cai et al., 2007). We therefore next examined the expression of these markers during
212 embryonic tendon development. At E12.5, when tendon progenitors are first detected (Pryce et
213 al., 2009), expression of Sca-1 and CD44 could not be detected in ScxGFP-positive tendon
214 progenitors (data not shown). At E14.5, at the onset of tendon cell differentiation, we found
215 low or no expression of both markers in the differentiating tendon cells. Robust positive
216 staining for both markers was however detected in the cells that surround the tendon at this
217 stage, likely the precursors of the epitenon/paratenon (Fig. S4). Similar expression patterns
218 were also found in mutant embryos (data not shown). These findings suggest that Sca-1 and
219 CD44 are not markers for tendon progenitor *in vivo*, and possibly simply reflect a generic
220 stemness state of the dedifferentiated mutant tendon cells.

221 Taken together, our findings show that mutant tendon cells acquired some stem/progenitor
222 properties while losing their cell fate. It should be noted however that although these
223 dedifferentiated tendon cells demonstrate some stem/progenitor properties, absence of TGFβ
224 signaling in these cells might prevent them from acquiring the full spectrum of stemness or
225 plasticity.
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229 **Molecular profile of the dedifferentiated mutant tenocytes**

230 We next performed single cell RNA sequencing analysis (scRNASeq) to establish a
231 comprehensive profile of the cellular state and molecular changes in mutant tenocytes. A
232 targeted retention of 2300-2600 cells from P7 WT- and mutant tendon was obtained, and the
233 transcriptomes were analyzed using the 10X Genomics platform. Using unsupervised
234 hierarchical clustering analysis, we identified two major clusters corresponding to WT tenocytes
235 and mutant (dedifferentiated) cells in the respective samples. Expression of close to 1000 genes
236 (mean UMI count ≥ 0.5, adjusted *p*-value ≤ 0.05) was identified in each of these clusters.

237 Pairwise comparison of the gene set between the P7 WT tenocyte and mutant cell clusters
238 was next performed to determine changes in gene expression associated with tenocyte
239 dedifferentiation. In total, expression of 186 genes was significantly different between the two
240 cell populations ($\geq$ 2-fold change and adjusted *p*-value ≤ 0.05), in which expression of 89 genes
241 was upregulated and 97 genes was downregulated in the mutant tendon cells (Table S2).
242 Almost 30% of the downregulated genes (29 genes) were identified in transcriptome analyses
243 as tendon distinctive genes [(Havis et al., 2014) and our unpublished data]. Notably, the genes
244 *Scx, Fmod, Tnmd, Pdgfrl, Col1a1, Col1a2, Col11a1 and Col11a2* were among the top 25 down-
245 regulated genes in Tgfbr2;ScxCre mutant tendon cells (Table 1), further confirming the loss-of-
246 cell fate phenotype in these cells. On the other hand, expression of the *Ly6a* gene (encoding
247 Sca-1) was greatly enriched in P7 mutant cells, corroborating the IHC findings presented above
248 (Table 2 and Fig. 5B). Moreover, we also found a significant increase in the expression of the
249 *CD34* gene, another common marker for diverse progenitor cells. This observation was further
250 confirmed at protein level, where positive immunostaining for CD34 was detected in mutant
251 cells but not in normal tendon cells (Fig. 6A). Interestingly, the genes upregulated in the mutant
252 cells included several genes (*Dpt*, *Anxa1*, *CD34*, *CD44*, *Mgp* and *Mfap5*) whose expression was
253 previously reported to be enriched during embryonic tendon development (Havis et al., 2014).
254 These findings thus do not only lend support to our notion that the mutant cells lost their
255 differentiation state, but also suggest the possibility of induction of some developmental
256 programs in these cells, a general feature in cellular dedifferentiation (Tata et al., 2013; Stocum,
257 2017; Nusse et al., 2018).

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258 To gain insights into biological functions activated in the P7 mutant cells, differentially
259 expressed genes (DEGs) in these cells (Table S2) were further analyzed via GO enrichment tools
260 clusterProfiler (Yu et al., 2012) and PANTHER Classification System (http://pantherdb.org/).
261 Intriguingly, GO enrichment analysis revealed that one of the prominent biological changes
262 observed in P7 mutant cells was upregulation of gene sets associated with wound healing (Fig.
263 6B and Table S3). These genes include protease inhibitors (*Serpine2, Serping1*), inflammatory
264 mediator *Anxa1* and extracellular matrix (*Col3a1* and *Col5a1*). This finding suggests a possible
265 role for tendon cells in the responses to pathological conditions, in line with findings reported
266 by others (Dakin et al., 2015; Stolk et al., 2017; Schoenenberger et al., 2018). On the other hand,
267 many biological processes downregulated in P7 mutant cells involved collagen synthesis and
268 organization (Fig. 6C and Table S3). Since tendon biology is not annotated in most databases,
269 changes in the collagen matrix, the most prominent structural component in tendons is the
270 best indicator for the disruption of the tendon cell fate. Disruption of the collagen matrix in
271 tendons was also detected in older mutant pups by ultrastructural analysis using TEM (Fig. 3).

272 Using PANTHER, we also investigated which protein classes were significantly altered in P7
273 mutant cells relative to WT tenocytes. Genes found to be most downregulated in mutant cells
274 encode for receptors, signaling molecules, membrane traffic proteins and ECM (Table 3A). On
275 the other hand, the upregulated genes in the mutant cells encode most prominently for
276 proteins involved in nucleic acid binding, enzyme modulators, cytoskeletal protein, signaling
277 molecules and transcription factors (Table 3B). Notably, expression of the activating protein 1
278 (AP-1) transcriptional complex, associated with numerous cellular processes including cell fate
279 regulation (Hess et al., 2004), was significantly induced in mutant cells. Expression of both AP-1
280 components, i.e. the *Fos* and *Junb* genes was induced more than two fold, and the *Jun* gene was
281 induced only slightly less than 2 fold. Moreover, the *Id3* gene encoding for a general bHLH
282 transcription factor inhibitor was also induced. Due to its broad selection of targets, *Id3* was
283 also implicated in numerous cellular processes including the regulation of cellular
284 differentiation (Norton, 2000). A possible role for these transcriptional activities in tenocyte
285 dedifferentiation will be addressed in future studies.

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286 We next conducted PANTHER Pathway Analysis using different values of the filter
287 parameter (mean UMI count and fold change) for enriching DEGs in P7 mutant cells. In general,
288 we found that pathways that stood out as relevant for this study included integrin signaling,
289 insulin/IGF, Wnt and inflammation mediated by chemokine and cytokine signaling pathways
290 (Table 4). Insulin/IGF- and Wnt signaling are often implicated in cell proliferation and cell fate
291 specification (Stewart and Rotwein, 1996; Sadagurski et al., 2006; Goessling et al., 2009; Salazar
292 et al., 2016). It is interesting to note that their activation has also been associated with cellular
293 dedifferentiation in skin, gut and neuron (Weber et al., 2003; Zhang et al., 2012; Perekatt et al.,
294 2018). Further investigation is required to determine the specific roles of these signaling
295 pathways in tenocyte dedifferentiation.

296

297 **Tenocyte dedifferentiation is dependent on cell autonomous loss of TGFβ signaling**

298 Lastly we wanted to ask if tenocyte dedifferentiation in these mutants reflected a cell
299 autonomous requirement for TGFβ signaling in tenocytes, or if it was the result of global
300 changes that occurred in mutant tendons. To address this question we reactivated TGFβ
301 signaling in isolated mutant tendon cells and determined the effects on tenocyte
302 dedifferentiation. We previously found that transuterine injection of AAV viruses into
303 embryonic limbs resulted in sporadic infection of limb tendons [unpublished data and (Huang et
304 al., 2013)]. We therefore decided to address this question by injection of a Cre-activatable virus
305 encoding an epitope tagged version of the receptor, AAV1-FLEX-Tgfbr2-V5 (Fig. 7A). Injection of
306 this virus into embryonic mutant limbs would result in expression of Tgfbr2-V5 only in infected
307 tendon cells due to the tendon-restricted activity of ScxCre in mutant embryos.

308 AAV1-FLEX-Tgfbr2-V5 was injected into mutant limbs at two stages during embryonic
309 tendon development: (a) E12.5 at the onset of ScxCre activity, ensuring that Tgfbr2-V5
310 expression will be activated in infected cells concurrent with the loss of the endogenous Tgfbr2,
311 resulting in isolated Tgfbr2 expressing cells surrounded by mutant cells. (b) E16.5, before the
312 onset of tenocyte dedifferentiation in mutant embryos. Interestingly, targeted re-expression of
313 Tgfbr2-V5 in individual mutant tendon cell at both developmental stages was able to prevent

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314 the loss of tendon markers as observed in postnatal pups (Fig. 7B-D), suggesting a cell
315 autonomous role for TGFβ signaling in maintenance of the tendon cell fate.

316 Recognizing that cell autonomous activity of Tgfbr2-V5 was sufficient to prevent
317 dedifferentiation of mutant tenocytes, we next wanted to test if reactivation of TGFβ signaling
318 in a dedifferentiating tenocyte could also reverse the process and rescue a tenocyte from
319 dedifferentiation. Activity of ScxCre may be lost in the dedifferentiating tenocytes due to the
320 loss of Scx expression and therefore of Scx enhancer driven expression of Cre in ScxCre mice.
321 We therefore used in this case a virus encoding constitutive expression of Tgfbr2 in which the
322 virus was tagged with a FLAG Tag (AAV1-Tgfbr2-FLAG). The virus was injected locally into P1
323 mutant limbs and the limbs were harvested at P7. We found that all infected mutant tendon
324 cells expressed the tendon markers ScxGFP and tenomodulin (Fig. 7D and Fig. S5A), suggesting
325 that reactivation of TGFβ signaling was indeed sufficient to rescue the dedifferentiated
326 tenocytes. Taken together, these findings demonstrate that TGFβ signaling is sufficient to
327 prevent and to rescue the loss-of-tendon cell fate in a cell-autonomous manner.

328 The constitutive expression of Tgfbr2-FLAG driven by the AAV1-Tgfbr2-FLAG virus ensured
329 that the neonatal infection with this virus resulted in Tgfbr2-FLAG expression both within and
330 outside of tendons. Notably, induction of tendon gene expression following activation of
331 Tgfbr2-FLAG expression was detected only in dedifferentiated tenocytes and not in cells located
332 outside of tendons (Fig. S5B). It was previously shown that TGFβ signaling is a potent inducer of
333 ScxGFP and other tendon markers (Pryce et al., 2009; Maeda et al., 2011; Sakabe et al., 2018).
334 This result reflects the fact that induction of tendon markers by TGFβ signaling is context-
335 dependent and further indicates that the tenocytes in mutant pups have dedifferentiated to a
336 state with tenogenic potential that retained the capacity to respond to TGFβ signaling.

337 Taken together these results highlight a surprising cell-autonomous role for TGFβ signaling
338 in maintenance of the tendon cell fate. In Tgfbr2;ScxCre mutants tenocyte differentiation and
339 function are normal during embryonic development but the tenocytes dedifferentiate in early
340 postnatal stages. Tenocyte dedifferentiation is directly dependent on the loss of TGFβ signaling
341 since retention or reactivation of the TGFβ receptor in isolated cells prevents or reverses the

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342 process of dedifferentiation. TGFβ signaling is thus essential for maintenance of the tendon cell
343 fate.

344

345 **Discussion**

346 In this study we find that the tendon cell fate requires continuous maintenance *in vivo* and
347 identify an essential role for TGFβ signaling in maintenance of the tendon cell fate. To examine
348 the different roles that TGFβ signaling may play in tendon development the Tgfbr2 gene was
349 targeted in Scx-expressing cells (Tgfbr2;ScxCre mutant), ensuring disruption of TGFβ signaling in
350 tendon cells. Mutant embryos appeared normal at birth and showed movement difficulties
351 from early neonatal stages. Tendon formation and maturation was not affected in mutant
352 embryos, but one flexor tendon snapped consistently at E16.5 and a few additional tendons
353 disintegrated in early postnatal stages. Surprisingly, we find that in all other tendons the
354 resident tenocytes lost tendon gene expression and dedifferentiated, assuming behavior and
355 gene expression associated with stem/progenitor cells. While a direct loss of TGFβ signaling in
356 individual tenocytes was not sufficient to cause tenocyte dedifferentiation, we found that
357 tenocyte dedifferentiation could be reversed by reactivation of TGFβ signaling in mutant cells
358 (Fig. 8). These results uncover an essential role for molecular pathways that maintain the
359 differentiated cell fate in tenocytes and a key role for TGFβ signaling in these processes.

360 Dedifferentiation has mostly been studied *in vitro* (Weinberg et al., 2007; Zhang et al., 2010;
361 Pennock et al., 2015; Mueller et al., 2016; Guo et al., 2017; Nordmann et al., 2017) and there
362 are only a handful of reported cases of dedifferentiation *in vivo* (Talchai et al., 2012; Tata et al.,
363 2013; Zhang et al., 2019). It was therefore important to establish if the tenocytes of
364 Tgfbr2;ScxCre mutants indeed dedifferentiated. Cellular dedifferentiation manifests in most
365 cases by loss of features associated with the differentiated state and reversion to an earlier
366 progenitor state within their cell lineage. In tendons of Tgfbr2;ScxCre mutants we indeed found
367 that the tenocytes lost tendon gene expression and showed enhanced clonogenic potential.
368 Moreover, the mutant tenocytes gained expression of the prototypic somatic stem/progenitor
369 markers Sca-1, CD34 and CD44 (Holmes and Stanford, 2007; Sung et al., 2008; Hittinger et al.,
370 2013; Sidney et al., 2014). Notably, of these stem/progenitor markers only Sca-1 and CD44 are

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371 also expressed at high levels in cultured tendon-derived stem/progenitor cells (Bi et al., 2007;
372 Mienaltowski et al., 2013). Neither of these markers has so far been established as markers for
373 tenocytes or for tendon progenitors. However, both expression of the CD34 and CD44 genes
374 and expression of some additional signature genes identified in the dedifferentiated tenocytes
375 by the scRNASeq analysis was previously shown to be significantly enriched in E14.5 mouse
376 limb tendon cells when compared to cells from E11.5 (Havis et al., 2014). These observations
377 suggest that some aspects of the embryonic tendon development program may be reactivated
378 in dedifferentiated mutant tendon cells. Interestingly, we found that Sca-1, CD34 and CD44 are
379 expressed in the wild-type epitenon/paratenon, thin layers of cells that surround the tendon
380 and has been implicated as a possible source of stem/progenitor cells for tendons
381 (Mienaltowski et al., 2013; Cadby et al., 2014). We further verified that mutant tendons are not
382 repopulated by epitenon/paratenon cells since there is no evidence of elimination of the
383 resident tenocytes by cell death.

384 Most studies of cellular dedifferentiation have focused on the regulation of this process *in*
385 *vitro*. There is, however, evidence demonstrating this phenomenon *in vivo* especially in the
386 context of pathological scenarios, as part of the regeneration process. One of the well-studied
387 examples is limb regeneration in amphibians. Following limb amputation, cells near to the
388 wound dedifferentiate to blastema, proliferate and eventually re-differentiate to replace all the
389 components of the lost limb (McCusker et al., 2015). In zebrafish it has also been reported that
390 following partial heart amputation, sarcomeres in mature cardiomyocytes disassembled, lost
391 their differentiation gene expression profile and switched to embryonic hyperplastic growth to
392 replace the missing tissues (Poss et al., 2002). Cellular dedifferentiation has also been observed
393 in murine mature hepatocytes (Gournay et al., 2002), pancreatic β cells (Talchai et al., 2012)
394 and skeletal muscle cells (Mu et al., 2011). More recently, Nusse and colleagues (Nusse et al.,
395 2018) have shown that disruption of the mouse intestinal barrier, via either parasitic infection
396 or cell death, led to reversion of crypt (epithelial) cells to a fetal-like stem cell state.
397 Interestingly, expression of Sca-1 was highly induced in these cells, and when cultured the Sca-1
398 positive crypt cells exhibited characteristics of fetal intestinal epithelium including re-
399 expression of fetal signature genes and loss of differentiated markers. The results presented in

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400 this study therefore suggest that a similar process may be activated in tenocytes as part of the
401 regenerative process in response to pathology. Taken together, this growing body of evidence
402 suggests that dedifferentiation may be a generalized cellular response to tissue damage that
403 warrants further investigation. Moreover, these observations may also suggest that induction
404 of Sca-1 may serve as a marker for a pathology-related dedifferentiation process. Intriguingly,
405 Sca-1 positive cells were also found in the wound window in rat patellar tendon incisional injury
406 model, but in this case it was not determined if Sca-1 expression was associated with
407 dedifferentiation (Tan et al., 2013). Sca-1 expression has been identified on putative
408 stem/progenitor cell populations in various tissues (Holmes and Stanford, 2007; Hittinger et al.,
409 2013), but little is known about its biological function. It may therefore be interesting to
410 examine whether Sca-1 functions as a stemness marker in dedifferentiated cells or if it also
411 plays additional roles in cellular responses to pathological conditions.

412 Tenocyte dedifferentiation as observed in this study reveals an unexpected flexibility in the
413 tendon cell fate where differentiated tenocytes can revert to a progenitor state under the
414 mutant conditions Significantly, reintroduction of Tgfbr2 not only prevented tenocyte
415 dedifferentiation when it was performed during embryogenesis but was also able to rescue the
416 cell fate of dedifferentiated tenocytes when the virus was introduced after birth. This result
417 suggests that TGFβ signaling may have a continuous role in protecting the differentiated
418 tenocytes from dedifferentiation, identifying TGFβ signaling as a key regulator of tendon
419 homeostasis. Moreover, these results also highlight the importance of the molecular pathways
420 involved in maintenance of the differentiated cell fate not only for tissue homeostasis and
421 function, but also for processes associated with tissue regeneration or with the onset and
422 unfolding of pathology. Previous studies have implicated TGFβ signaling in cell fate
423 maintenance in various tissues, e.g. preserving chondrocyte identity in cultures (Bauge et al.,
424 2013) and suppressing intestinal cell dedifferentiation (Cammareri et al., 2017). While TGFβ
425 signaling has been associated with different aspects of tendon biology (Pryce et al., 2009; Havis
426 et al., 2016), to the best of our knowledge this is the first report of its role in maintenance of
427 the tendon cell fate.

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428 The fact that the mutant phenotype was caused by disruption of TGFβ signaling in tenocytes
429 and the rescue of the tendon cell fate by virus mediated reintroduction of Tgfbr2 even to
430 individual mutant cells provides direct evidence for a continuous and cell autonomous role for
431 TGFβ signaling in maintenance of the tendon cell fate. However, targeting of Tgfbr2 using both
432 tendon specific and ubiquitous inducible Cre drivers at different developmental stages did not
433 result in tenocyte dedifferentiation, suggesting that tenocyte dedifferentiation in these mutants
434 was dependent on specific spatial and temporal features of the ScxCre driver. These
435 observations suggest that tenocyte dedifferentiation in these mutants may not merely be the
436 result of the loss of intrinsic TGFβ signaling in tendon cells, but rather may be caused by an
437 interplay between intrinsic loss of TGFβ signaling and additional external factors contributed by
438 the environment of the mutant tendons, e.g. cell-cell or cell-matrix interaction.

439 The tendon phenotype of Tgfbr2;ScxCre mutants highlights a likely role for tenocyte
440 dedifferentiation in regenerative processes in tendons and possibly also in the progression of
441 tendon pathology. Uncovering the molecular pathways involved in this process may therefore
442 be important for new strategies for treatments of tendon pathologies. The Tgfbr2;ScxCre
443 mutants provide a unique opportunity to analyze these pathways, and the experimental
444 approaches employed in this study may be developed into an experimental paradigm for
445 molecular dissection of this process. Briefly, transcriptional and epigenetic analyses of the
446 mutant tenocytes through the dedifferentiation process can provide a landscape of the
447 molecular changes that initiate and drive the dedifferentiation process. Promising candidates
448 can then be tested using the AAV-mediated cell fate rescue experiments to identify genes or
449 groups of genes that can protect the tenocytes from dedifferentiation to establish the
450 molecular process of cellular dedifferentiation. Of particular interest will be the early molecular
451 changes in the mutant tenocytes that drive and promote the onset and progression of tenocyte
452 dedifferentiation.

453 Our findings underscore the fact that the tendon cell fate requires continuous maintenance
454 and that it is not an irreversible state, a long-standing biological dogma that has been
455 challenged by recent research (Takahashi and Yamanaka, 2006; Ladewig et al., 2013).
456 Nevertheless, it is important to recognize that the dramatic cell fate changes in Tgfbr2;ScxCre

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457 mutant happens in the context of a genetic modification. The occurrence of such phenomenon
458 *in vivo* might not be a simple direct outcome of changes to TGFβ signaling. Most importantly,
459 while the initiating events for tenocyte dedifferentiation may vary in different scenarios, it is
460 likely that the molecular events that drive the dedifferentiation process downstream of the
461 initiation event are similar or related. Uncovering these pathways in this experimental system
462 may therefore facilitate the analysis of such processes in various other contexts.

463

464 **<u>Materials and Methods</u>**

465 **Mice**

466 All mouse work was performed in accordance to the guidelines issued by the Animal Care and
467 Use Committee at Oregon Health & Science University (OHSU). Floxed TGFβ type II receptor
468 ($Tgfbr2^{f/f}$) mice (Chytil et al., 2002) were crossed with mice carrying the tendon deletor
469 Scleraxis-Cre recombinase (ScxCre) (Blitz et al., 2013), to disrupt TGFβ signalling in tenocytes.
470 All mice in this study also carried a transgenic tendon reporter ScxGFP (Pryce et al., 2007), and a
471 Cre reporter Ai14 Rosa26-tdTomato (RosaT) (Madisen et al., 2010) for the lineage tracing of
472 Scx-expressing cells. For embryo harvest, timed mating was set up in the afternoon, and
473 identification of a mucosal plug on the next morning was considered 0.5 days of gestation
474 (E0.5). Embryonic day 14.5 to postnatal day 13 (E14.5-P13) limb tendons were used for analysis.
475 Mouse genotype was determined by PCR analysis of DNA extracted from tail snip using a lysis
476 reagent (Viagen Biotech, Cat 102-T) and proteinase K digestion (55°C, overnight).

477

478 **Transmission electron microscopy (TEM)**

479 Skinned mouse forelimbs were fixed intact for several days in 1.5% glutaraldehyde/1.5%
480 formaldehyde, rinsed, then decalcified in 0.2M EDTA with 50mM TRIS in a microwave (Ted
481 Pella, Inc.) operated at 97.5 watts for fifteen 99 min cycles. Samples were fixed again in 1.5%
482 glutaraldehyde/1.5% formaldehyde with 0.05% tannic acid overnight, then rinsed and post-
483 fixed overnight in 1% OsO_4. Samples were dehydrated and extensively infiltrated in Spurr's
484 epoxy and polymerized at 70°C (Keene and Tufa, 2018). Ultrathin sections of tendons of

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485 interest were cut at 80 nm, contrasted with uranyl acetate and lead citrate, and imaged using a
486 FEI G20 TEM operated at 120 kV with montages collected using a AMT XR-41 2 x 2K camera.
487 The acquired images were stitched using ImageJ software (https://imagej.nih.gov/ij/) (Preibisch
488 et al., 2009). Three pups per time point were harvested for TEM analysis.
489

490 **In situ hybridization and histological staining**

491 Dissected forelimbs were fixed with 4% paraformaldehyde in PBS, decalcified in 5 mM EDTA (1-
492 2 weeks at 4°C) and incubated with a 5-30% sucrose/PBS gradient. The tissues were then
493 embedded in OCT (Tissue-Tek, Inc), sectioned at 10-12 μm using a Microm HM550 cryostat
494 (Thermo Scientific, Waltham, MA) and mounted on Superfrost plus slides (Fisher). In situ
495 hybridization was performed as previously described (Murchison et al., 2007).

496 For immunofluorescence staining, sections were air-dried, rinsed thrice with PBS and
497 blocked with 2% BSA and 2% normal goat serum in PBS for 1h at RT. The sections were then
498 incubated overnight at 4°C with specific primary antibody as listed in Table S1. This was
499 followed by incubation with the matching Cy3- or Cy5-conjugated secondary antibody (Jackson
500 ImmunoResearch; diluted at 1:250 or 1:400) in PBS containing 2% normal goat serum for 1h at
501 RT. DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Life Technologies) was used to
502 counterstain cell nuclei. Immunolabelled sections were mounted in Fluorogel (Electron
503 Microscopy Sciences, PA; Cat 17985-10) and visualized using a Zeiss ApoTome microscope. A
504 washing step with PBS containing 0.1% Triton-X 100 was performed after the change of
505 antibodies. Controls included omission of primary antibodies.

506 For examination of cell death and proliferation, TUNEL and EdU assays were performed
507 using Click-iT EdU (Life Technologies) and In Situ Cell Death Detection (Roche) kits, respectively,
508 following manufacturer's instructions. For all studies, sections from 2-4 pups were examined to
509 ensure reproducibility of results.
510

511 **Isolation and culture of tendon-derived stem/progenitor cells**

512 Mice at P7 were used for tendon progenitor cell isolation using a protocol modified from that
513 in Mienaltowski et al (Mienaltowski et al., 2013). Briefly, both forelimbs and hindlimbs were

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514 harvested from euthanized mice, skinned and exposed to 0.5% collagenase type I (Gibco, Cat
515 17100-017) and 0.25% trypsin (Gibco, Cat 27250-018) in PBS for 15 min at 37°C with gentle
516 shaking. The surfaces of tendons were then scraped carefully with a pair of forceps to remove
517 epitenon/paratenon cells. The middle portion of tendons was then harvested, cut into small
518 pieces and tendon cells were released by digestion for 30 min at 37°C with gentle shaking in a
519 solution of 0.3% collagenase type I, 0.8% collagenase type II (Cat 17101-015), 0.5% trypsin and
520 0.4% dispase II (Cat 17105-041) (all from Gibco). The released cells were strained with a 70-µm
521 cell strainer (BD Falcon, Cat 352350) and collected by centrifugation for 5 min at 300 g. The cells
522 were then resuspended in PBS with 1% BSA, and fluorescence-activated cell sorting (FACS) was
523 used to separate the cells for colony-forming assay.
524

525 **Colony-forming unit (CFU) assay**

526 CFU assay was used to examine the self-renewal potential of cells (Bi et al., 2007). The
527 enzymatically-released WT and heterozygous tenocytes as well as dedifferentiated mutant
528 tendon cells (i.e. ScxGFP-negative and RosaT-positive cells) were sorted by FACS and plated at
529 one cell per well in a 96-well plate using a BD Influx^{TM} cell sorter (BD Bioscience, USA). About
530 10-12 days into the culture, the colonies were fixed in 4% paraformaldehyde (10 min, RT),
531 stained with 0.5% crystal violet for 30 min, and rinsed twice with water. Percentage of colony-
532 forming unit was calculated as: Number of wells with colonies ÷ 96 x 100. Each data point
533 represents the mean of duplicate plates from 3-5 separate experiments. Each experiment
534 represents limb tendons collected from 2-4 pups.
535

536 **Re-expression of Tgfbr2 in mutant cells using adeno-associated virus (AAV) vector**

537 FLAG or V5 epitope tag sequences were added at the C-terminus of the murine TGFbR2
538 Consensus Coding Sequence (CCDS23601). The Tgfbr2-FLAG (*Tgfbr2-FLAG*) and reverse-
539 compliment Tgfbr2-V5 (*FLEX-Tgfbr2-V5*) insert sequences were synthesized and subcloned by
540 GenScript into an AAV vector. The FLEX backbone vector (Atasoy et al., 2008) was purchased
541 from AddGene and modified. Vectors were then packaged into AAV1 capsid, purified, and
542 titered by the OHSU Molecular Virology Support Core. AAV insert expression was under the

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543 control of a chicken beta-actin (CBA) promoter and an SV40 polyadenylation sequence. All
544 experimental procedures were evaluated and approved by the institutional Animal Care and
545 Ethics Committee.

546 Re-expression of Tgfbr2 in embryos was done by delivery of AAV-FLEX-Tgfbr2-V5, a Cre-
547 dependent expression cassette, specifically to Tgfbr2$^{f/-}$;ScxCre mutant tendon cells.
548 Transuterine microinjection of the viral vector into embryos was performed according to a
549 published protocol (Jiang et al., 2013). Briefly, a laparotomy was performed on anesthetized
550 pregnant females to expose the uterus. The left wrist field of the forelimb bud of each embryo
551 was injected with ~2 μl of concentrated viral inoculum (3.8 x 10^{13} vg/ml) using a borosilicate
552 glass capillary pipette (25-30 μm outer diameter and 20 degree bevel). The abdominal and skin
553 incisions were closed with resorbable sutures. The dams were recovered overnight with
554 supplementary heating and then returned to main colony housing.

555 For postnatal re-expression of Tgfbr2, ~10 μl of AAV-Tgfbr2-FLAG inoculum (4.1 x 10^{12} vg/ml)
556 was injected subcutaneously into the left forelimb of P1 pups using an 8 mm x 31G BD Ultra-
557 Fine™ insulin syringe and needle (Becton Dickinson and Company, NJ, USA). For both
558 experiments, forelimbs from P5 to P7 mutant pups (n = 3 pups for each stage) were harvested,
559 fixed, cryosectioned and examined for expression of tendon differentiation markers in infected
560 tendon cells.

561

562 **Single-cell RNA sequencing (scRNA-seq) and data analysis**

563 Tendons were collected and pooled from both forelimbs and hindlimbs as described above
564 from two pups with the omission of tissue-scraping step. The enzymatically-released cells were
565 centrifuged, resuspended in α-MEM with 5% FBS and submitted to the OHSU Massively Parallel
566 Sequencing Shared Resource (MPSSR) Core facility. scRNA-seq analysis was then performed
567 using the 10x Genomics Chromium™ Single Cell 3' Reagent Kits and run on a Chromium™
568 Controller followed by sequencing using the Illumina NextSeq® 500 Sequencing System (Mid
569 Output), as per the manufacturer's instructions (10x Genomics Inc, CA; Illumina Inc, CA).

570 Sequencing data processing and downstream analysis were performed using Cell Ranger
571 version 2.0 (10x Genomics, CA) (Zheng et al., 2017) with the default settings. Briefly, sequencing

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572 reads were aligned to the mm10 genome and demultiplexed and filtered using total UMI count
573 per cell to generate the gene barcode matrix. Principle component analysis was performed and
574 the first ten principle components were used for the tSNE dimensional reduction and clustering
575 analysis. Cells were clustered using K-means clustering. For each cluster, genes with an average
576 UMI count ≥ 0.5, fold change ≥ 1.5 and p-value ≤ 0.05 were identified as signature genes for
577 each cluster. Gene Ontology (GO) enrichment analysis (clusterProfiler) (Yu et al., 2012) and
578 the PANTHER Classification System (http://pantherdb.org/) were used to elucidate the
579 biological process and signaling pathway associated with individual gene. Enriched canonical
580 pathways were defined as significant if adjusted p-values were < 0.05.
581

582 **Statistical analysis**

583 Unless stated otherwise, all graphs are presented as mean ± standard deviation (SD). Student's
584 t-tests were performed to determine the statistical significance of differences between groups
585 (n ≥ 3). A value of $p<0.05$ is regarded as statistically significant.
586

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594

595 **Competing interests**

596 The authors declare no competing interests.
597
598
599

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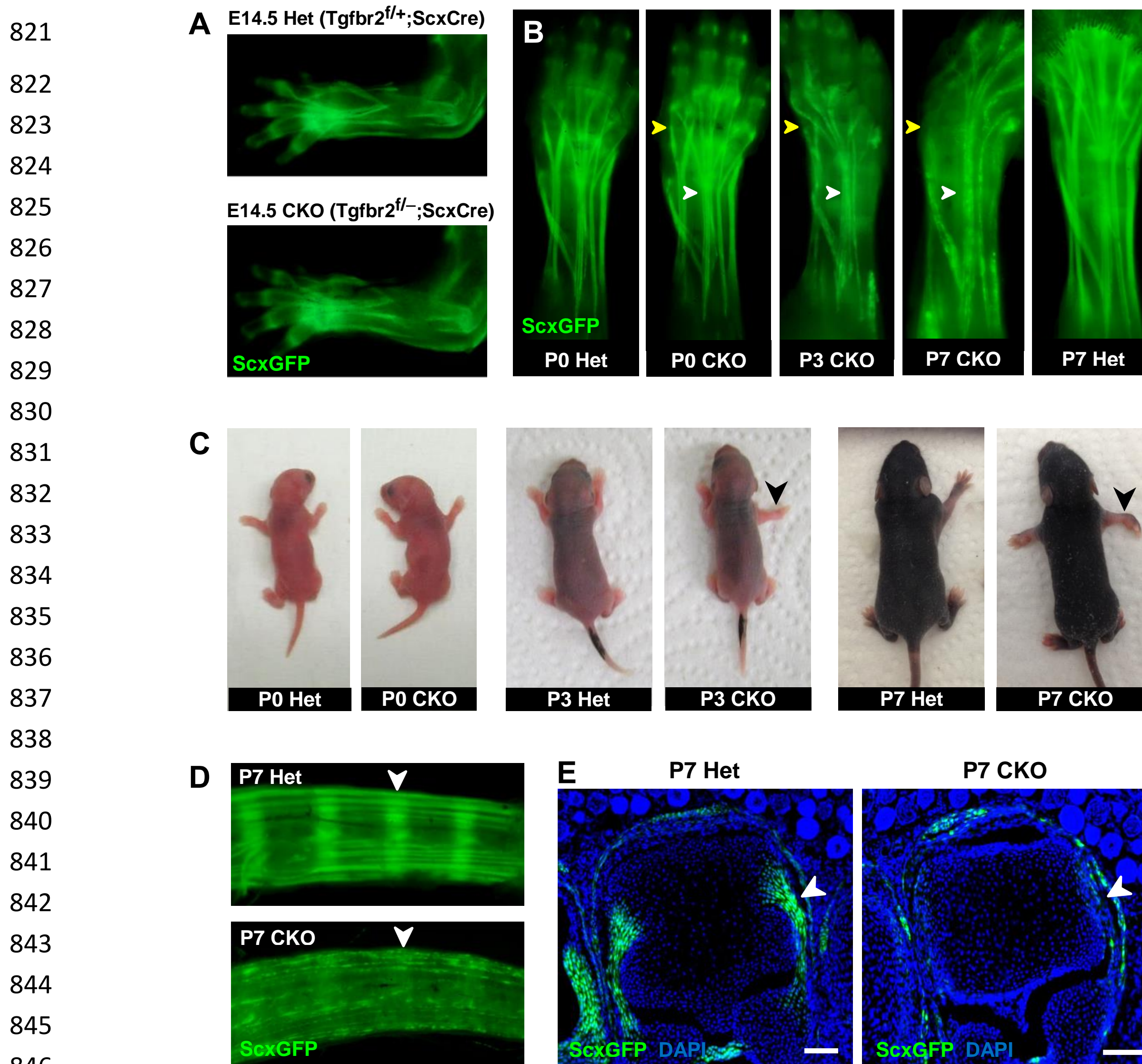

Fig 1. Tendon phenotypes manifested in Tgfbr2;ScxCre mutants. (A-D) Whole-mount imaging in fluorescent ScxGFP signal or brightfield. (A) Dorsally-viewed embryo forelimb shows the formation of a complete network of tendons in both mutant and heterozygous control by E14.5. (B) Tendons of mutant pups appeared intact at birth, but by P3 lateral tendons disintegrated and were eventually eliminated (yellow arrowheads), whereas the majority of other tendons persisted with a substantial loss of the ScxGFP signal (white arrowheads). (C) Mutant pups appeared normal at birth but showed physical abnormalities including abducted paw and splayed limb (black arrowheads) by P3. (D-E) Substantial loss of ScxGFP signal was also detected in all tendons and related tissues. (D) Tail tendons and annulus fibrosus of the intervertebral disc (white arrowheads) in P7 pups. (E) Collateral ligaments of the metacarpophalangeal joint imaged in transverse section through the joints of heterozygous control and mutant pups at P7 (white arrowhead). Scale bar, 100 µm. Mutant: CKO, Heterozygous: Het.

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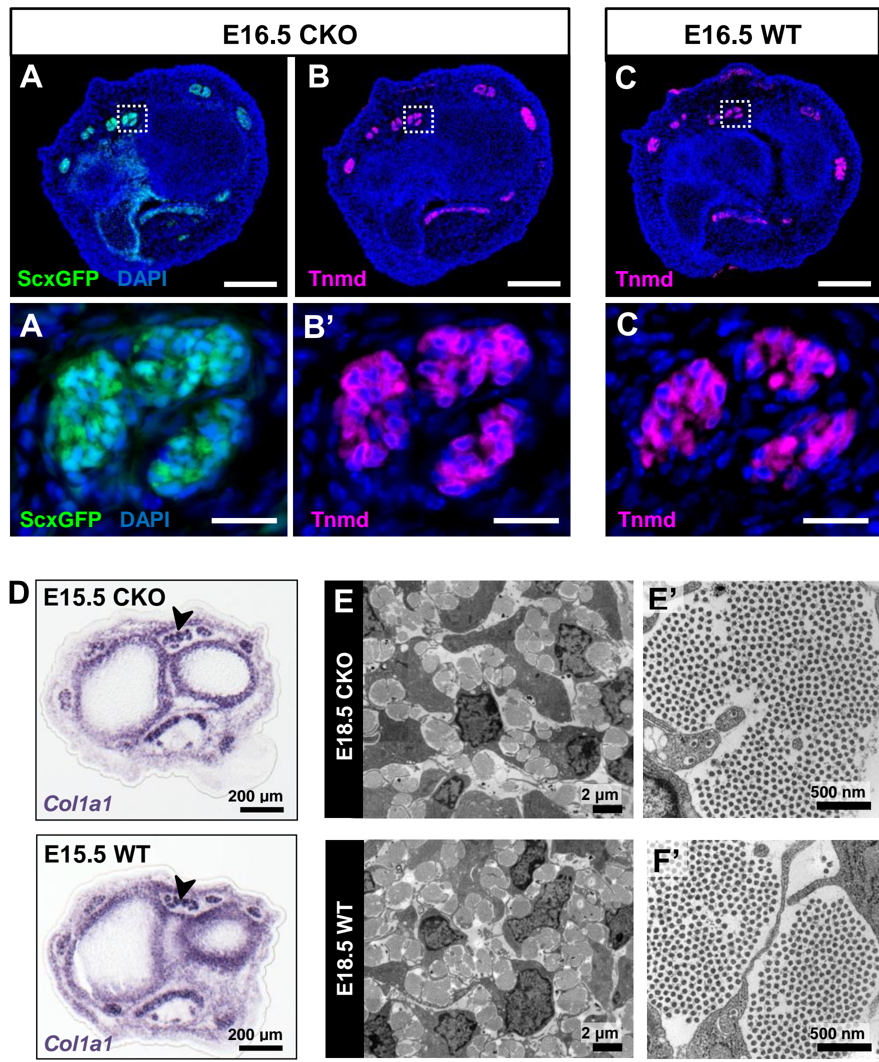

880 **Fig 2. Tendon development in Tgfbr2;ScxCre mutant embryos was not perturbed through**
881 **embryogenesis.** (A) ScxGFP signal and (B) tenomodulin (Tnmd) immunofluorescence on
882 transverse sections at wrist level of E16.5 mutant embryos demonstrate that the pattern and
883 expression of prototypic tenocyte markers was not disrupted in mutant tendons. (C) Tnmd
884 immunofluorescence in E16.5 wild-type tenocytes. (A'), (B') and (C') are higher magnifications
885 of extensor digitorium communis tendons as boxed in (A), (B) and (C). (D) In situ hybridization
886 for *Col1a1* on transverse sections of the forelimb from E15.5 mutant and wild-type littermates
887 reveals that expression of the major matrix genes was not altered in mutant embryos (black
888 arrowhead). (E,F) TEM images of tendons from forelimbs of E18.5 mutant and wild-type
889 embryos reveals that organization and accumulation of the tendon extracellular matrix was not
890 disrupted in the mutant. (E',F') Higher magnification views of (E) and (F) for direct visualization
891 of the collagen fibers. Scale bars, 200 µm (A-C) and 20 µm (A'-C'). Mutant: CKO, Wild-type: WT.
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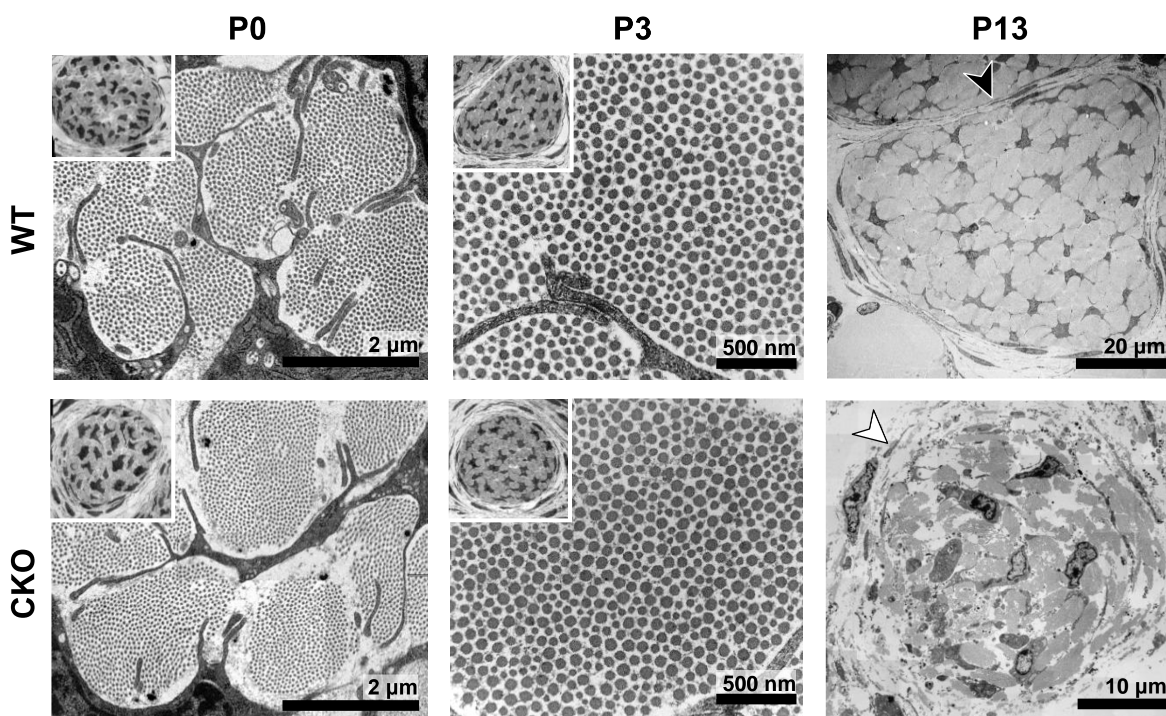

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913 **Fig 3. Tendon degeneration observed in Tgfbr2;ScxCre mutants at later postnatal stages.** TEM
914 images of tendons from forelimbs of mutant and wild-type littermates at P0, P3 and P13.
915 Images were taken at high magnification for direct examination of structural details. Insets
916 show transverse section TEM images of entire tendons at low-magnification (not to scale).
917 Despite detectable functional defects starting around P3 in mutant pups, collagen matrix
918 organization in mutant neonates was indistinguishable from that of their wild-type littermates.
919 Apparent collagen degradation and disrupted epitenon structures (white arrowhead) were
920 detected only in older mutant pups as shown here for P13 pups. Black arrowhead indicates
921 epitenon in wild-type pups. Mutant: CKO, Wild-type: WT.
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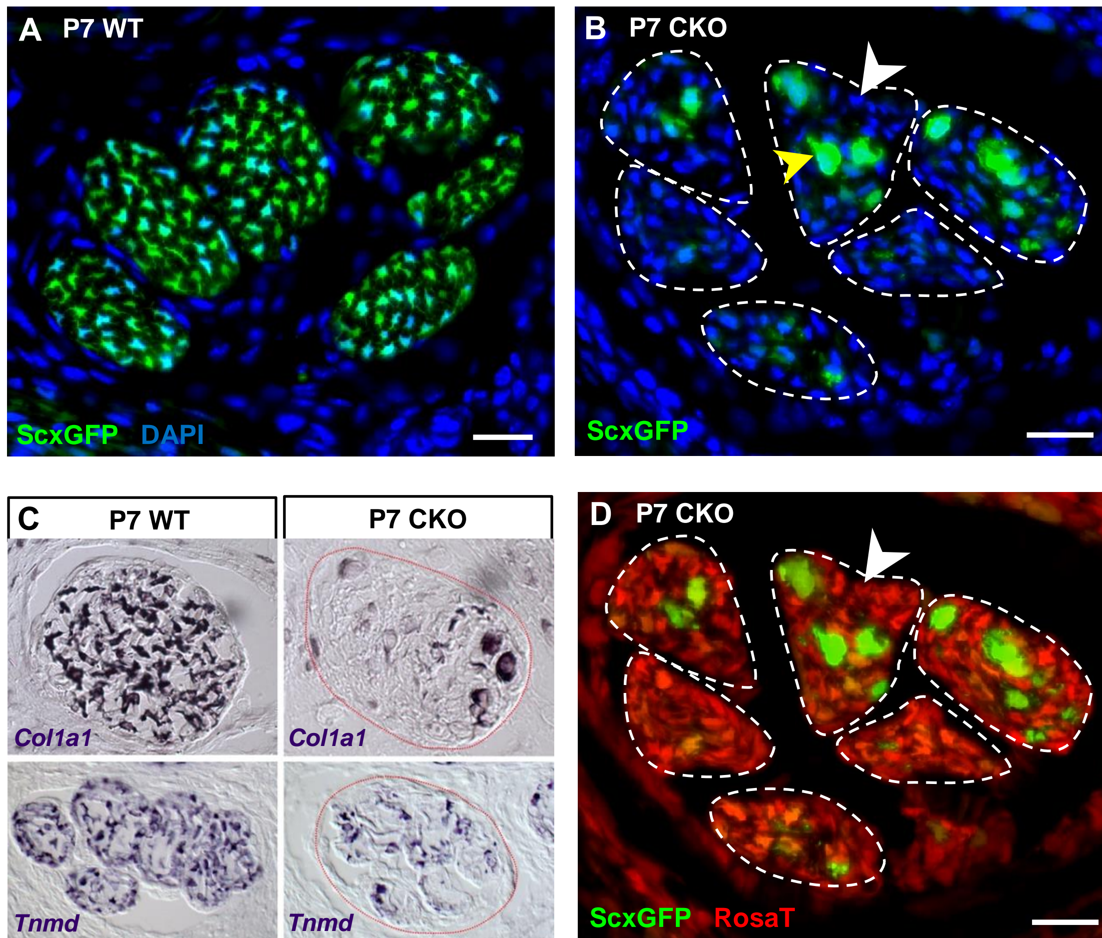

956 **Fig 4. Deletion of Tgfbr2 in Scx-expressing cells (Tgfbr2;ScxCre) results in loss of tenocyte
957 differentiation markers.** (A-D) Transverse sections of extensor digitorium communis tendons
958 of wild-type and mutant pups at wrist level. (A) In P7 wild-type pups, all tenocytes were positive
959 for tendon reporter ScxGFP signal. (B) Conversely, most cells in P7 mutant tendons lost the
960 ScxGFP signal (white arrowhead) whereas the cells positive for ScxGFP signal are newly
961 recruited cells (yellow arrowhead; Tan et al, manuscript in preparation). (C) In situ hybridization
962 shows that the mutant cells also lost gene expression of tendon markers *Col1a1* and *Tnmd*
963 (images not to scale). (D) Lineage tracing using ScxCre shows that all ScxGFP-negative cells in (B)
964 were positive for the Cre reporter Rosa26-tdTomato (RosaT; white arrowhead), proving that the
965 ScxGFP-negative cells in mutant tendons were derived from the embryonic tenocytes. Dashed
966 lines demarcate the mutant tendons. Scale bar, 20 µm. Mutant: CKO, Wild-type: WT.
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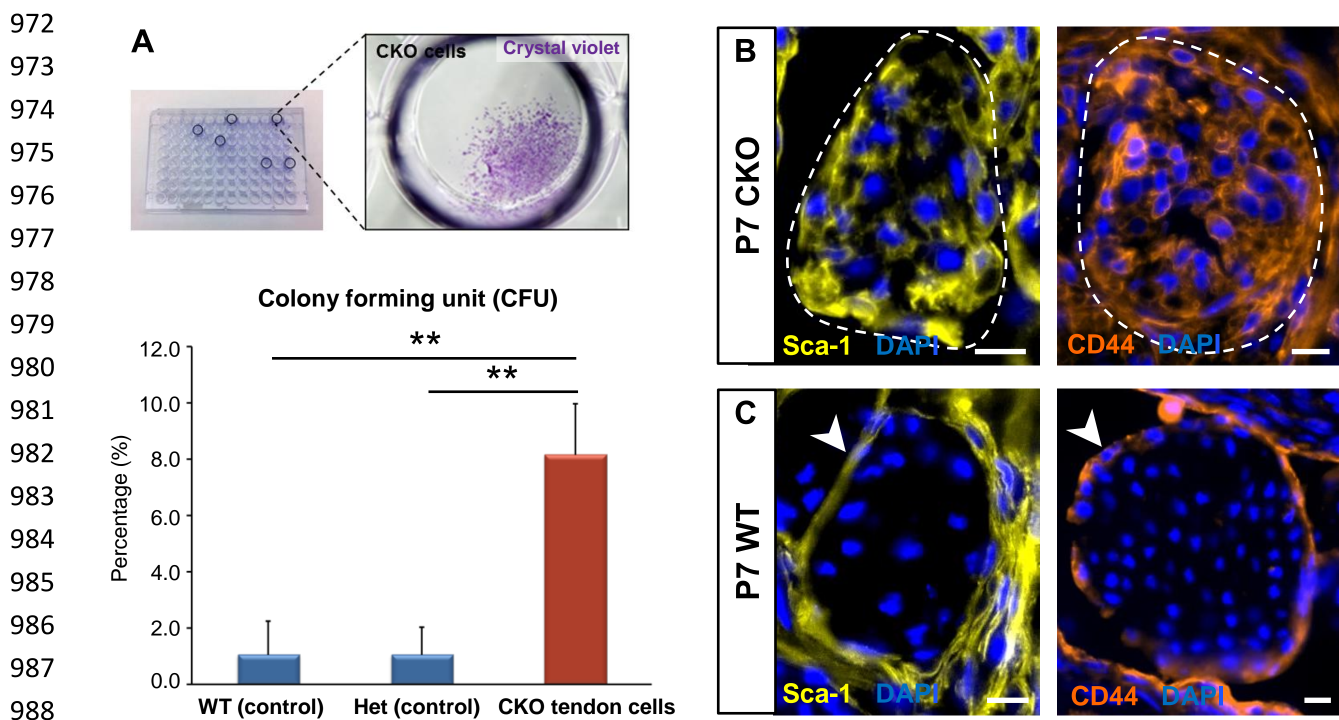

990 **Fig 5. Tgfbr2;ScxCre mutant tenocytes acquired stem/progenitor features.** (A) The colony-
991 forming efficiency of P7 wild-type and heterozygous tenocytes as well as mutant tendon cells
992 were evaluated by seeding one cell per well of the FACS-sorted cells in 96 well plates, and
993 colonies formed were visualized with crystal violet staining. Mutant tenocytes exhibited
994 significantly higher clonogenic capacity compared to wild-type and heterozygous controls. The
995 results shown are mean ± SD (n=5-6, ***p*<0.01). (B) Immunofluorescence staining for
996 stem/progenitor markers in transverse sections of mutant tendons shows that mutant tendon
997 cells acquired in postnatal stages expression of stem cell antigen-1 (Sca-1) and CD44. (C) In wild-
998 type littermate controls, expression of both markers was detected in epitenon (white
999 arrowhead), but not in tenocytes. Dashed line demarcates the mutant tendon. Scale bars, 10
1000 µm. Mutant: CKO, Wild-type: WT, Heterozygous: Het.
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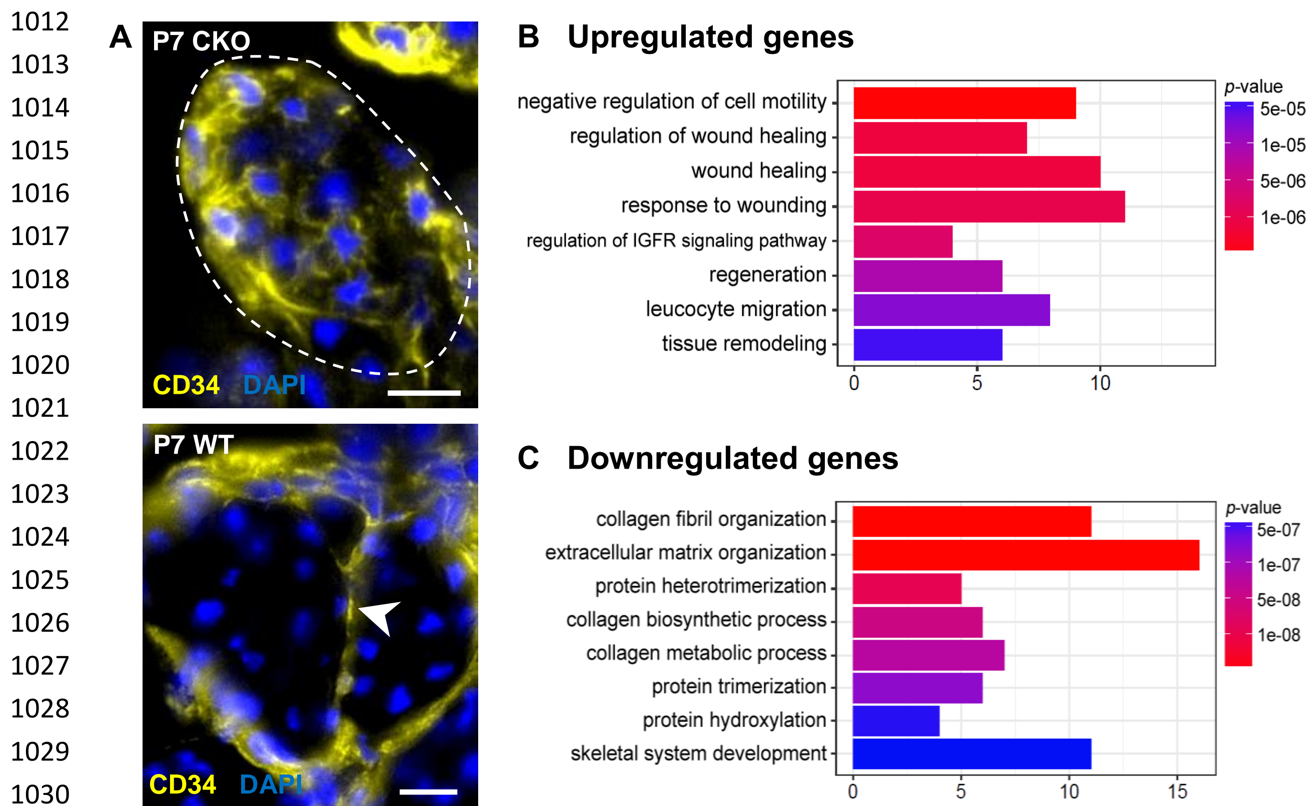

Fig 6. Molecular profile of the dedifferentiated mutant tenocytes. (A) Upregulated expression of *CD34* gene in P7 mutant tenocytes as revealed by scRNA-Seq analysis (see also Table 2) was determined using immunostaining. Transverse section of forelimb tendons shows that CD34 was indeed expressed by mutant tenocytes, while in wild-type controls CD34 was detected only in epitenon cells (white arrowhead). Dashed line demarcates the mutant tendon. (B,C) Gene ontology (GO) enrichment analysis in terms of biological processes associated with the (B) upregulated and (C) downregulated genes in P7 mutant compared with wild-type tenocytes. Selected GO terms are included in this figure, and genes annotated to the GO terms are available in Table S3. Scale bar, 10 µm. Mutant: CKO, Wild-type: WT.

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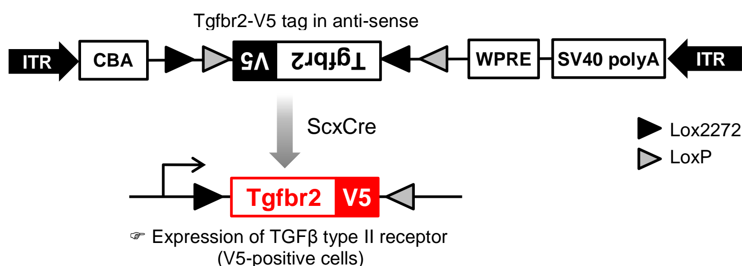

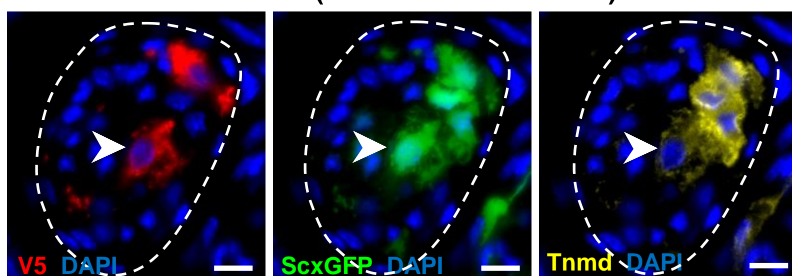

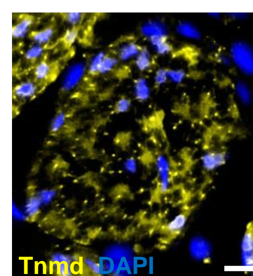

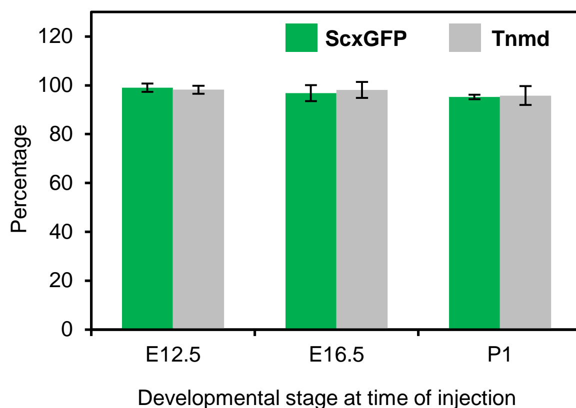

Injection stage	Total counted infected cells
E12.5	82
E16.5	40
P1	315

Fig 7. Tenocyte dedifferentiation is dependent on cell autonomous loss of TGFβ signaling. (A) AAV1-FLEX-Tgfbr2-V5 virus contains the reverse-complement sequence of Tgfbr2 with a C-terminal V5 epitope tag. Cre activity will lead to a permanent inversion of the cassette that will then express the V5-tagged TGFβ type II receptor. (B) Targeted expression of TGFβ type II receptor in E16.5 mutant tendon cells using the AAV1-FLEX-Tgfbr2-V5 prevented the loss of tendon markers in the infected tenocytes. The forelimb of E16.5 mutant embryos was infected with AAV1-FLEX-Tgfbr2-V5 virus and harvested at P6. Transverse forelimb sections were stained

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1090 with antibodies for V5 (red) to detect AAV-infected cells and tenomodulin (Tnmd; yellow), a
1091 prototypic tendon marker expressed by (C) all tenocytes in the wild-type tendon at this stage.
1092 Dashed line demarcates the mutant tendon. (D) Quantification shows that about 95-98% of the
1093 AAV-infected (V5-positive) mutant tendon cells retained or re-expressed tendon differentiation
1094 markers after viral injection at different developmental stages (n = 3 pups for each stage). Scale
1095 bar, 10 μm. Mutant: CKO, Wild-type: WT.

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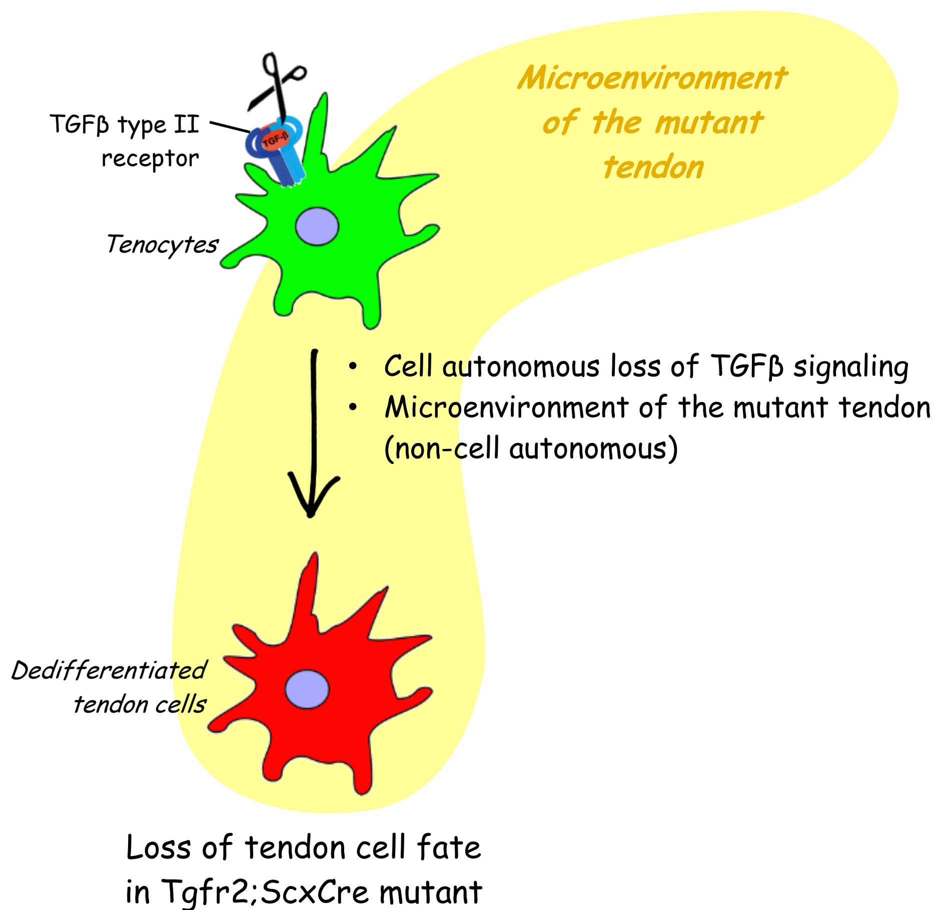

Fig 8. Proposed roles of TGFβ signaling in the maintenance of tendon cell fate. Targeted disruption of the TGFβ type II receptor by ScxCre resulted in tenocyte dedifferentiation in early postnatal stages. Tenocyte dedifferentiation was reversed by reactivation of TGFβ signaling in individual mutant cells, demonstrating a cell autonomous role for TGFβ signaling for maintenance of the cell fate. Conversely, direct loss of the TGFβ type II receptor using Cre drivers with different spatio-temporal features was not sufficient to cause tenocyte dedifferentiation, suggesting that mere loss of TGFβ signaling by tendon cells was not sufficient to cause dedifferentiation. We therefore propose that maintenance of the tendon cell fate is dependent on a combination of a cell autonomous function of TGFβ signaling and an additional, likely non cell-autonomous factor, e.g. the microenvironment of the tendon in the Tgbfbr2;ScxCre mutant.

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1171 **Table 1. Top 25 downregulated genes (2-fold change, adjusted *p*<0.05) in P7 mutant cells**
1172 **compared with P7 wild-type tenocytes.** See also Table S2 for a complete list of the
1173 downregulated genes.
1174

Gene symbol	Gene name	Fold change
Wif1	Wnt inhibitory factor 1	157.4
Col11a2[#]	Collagen, type XI, alpha 2	92.0
Scx[#]	Scleraxis	66.2
Col2a1[δ]	Collagen, type II, alpha 1	58.9
Car9	Carbonic anhydrase 9	58.1
Sema3b	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	43.9
Cgref1	Cell growth regulator with EF hand domain 1	33.2
Fmod[#]	Fibromodulin	27.9
Cilp2	Cartilage intermediate layer protein 2	24.7
Matn4	Matrilin 4	19.3
P4ha1[δ]	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	13.5
Pcolce2[δ]	Procollagen C-endopeptidase enhancer 2	11.8
Tpm1	Tropomyosin 1, alpha	10.0
Wisp1	WNT1 inducible signaling pathway protein 1	9.7
Tnmd[#]	Tenomodulin	8.5
Loxl2[δ]	Lysyl oxidase-like 2	8.3
1500015O10Rik	RIKEN cDNA 1500015O10 gene	7.1
Col11a1[#]	Collagen, type XI, alpha 1	7.1
Pdgfrl[δ]	Platelet-derived growth factor receptor-like	7.0
Mfap4	Microfibrillar-associated protein 4	6.5
Col1a1[#]	Collagen, type I, alpha 1	6.4
Ptgis	Prostaglandin I2 (prostacyclin) synthase	6.4
Col1a2[#]	Collagen, type I, alpha 2	6.2
Itgbl1	Integrin, beta-like 1	5.7
Tpm2	Tropomyosin 2, beta	5.4

1175 Note:
1176 1) #=Tendon differentiation or specific marker; δ=genes related to tendons.
1177 2) Note that the expression level detected for Scx also included that of ScxGFP, and therefore do not reflect the
1178 expression level of endogenous Scx.
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1188 **Table 2. Top 25 upregulated genes (2-fold change, adjusted *p*<0.05) in P7 mutant cells**
1189 **compared with P7 wild-type tenocytes.** See also Table S2 for a complete list of the
1190 downregulated genes.
1191

Gene symbol	Gene name	Fold change
Dlk1	Delta-like 1 homolog (Drosophila)	137.9
Serpine2	Serine (or cysteine) peptidase inhibitor, clade E, member 2	118.2
Dpt	Dermatopontin	95.7
Ly6a	Lymphocyte antigen 6 complex, locus A	54.3
H19	H19	51.1
Cd34	CD34 antigen	47.8
Lum	Lumican	36.6
Lgmn	Legumain	31.8
Cxcl12	Chemokine (C-X-C motif) ligand 12	26.1
Mfap5	Microfibrillar associated protein 5	22.5
Ly6c1	Lymphocyte antigen 6 complex, locus C1	21.7
Igf2	Insulin-like growth factor 2	21.4
Serping1	Serine (or cysteine) peptidase inhibitor, clade G, member 1	19.2
Mgst1	Microsomal glutathione S-transferase 1	18.3
Aspn	Asporin	15.9
Mt1	Metallothionein 1	15.4
Mgst3	Microsomal glutathione S-transferase 3	13.1
Col3a1$^{\delta}$	Collagen, type III, alpha 1	13.0
Postn	Periostin, osteoblast specific factor	13.0
Itm2a	Integral membrane protein 2A	12.7
Ptn	Pleiotrophin	10.3
Rps18-ps3	Ribosomal protein S18, pseudogene 3	9.7
Gsn	Gelsolin	8.3
Ifitm3	Interferon induced transmembrane protein 3	8.2
Col5a1$^{\delta}$	Collagen, type V, alpha 1	8.1

1192 Note: δ=genes related to tendons.

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Supplementary files

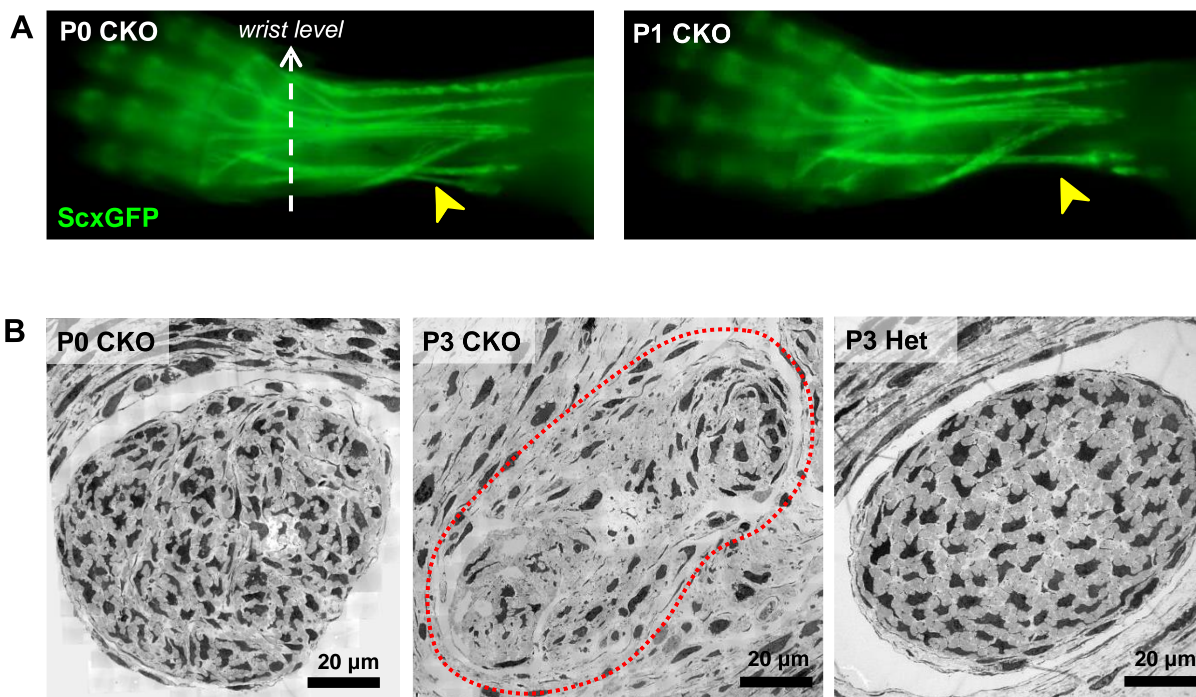

Fig S1. Fragmentation and elimination of lateral tendons in mutant neonates. (A) Rapid disruption of lateral extensor tendons in neonatal stages of mutant pups revealed by examination of forelimb tendons using the tendon reporter ScxGFP. The extensor carpi radialis longus tendon (yellow arrowheads) is present in a mutant pup at P0 but lost in a P1 mutant. (B) TEM images of the extensor carpi radialis longus tendon at wrist level. The mutant tendon shows signs of fragmentation already at P0, and by P3 the tendon appears disintegrated accompanied by complete loss of the epitenon and structural definition of the tendon circumference. The red dotted line in (B) demarcates the mutant tendon. Mutant: CKO, Heterozygous: Het.

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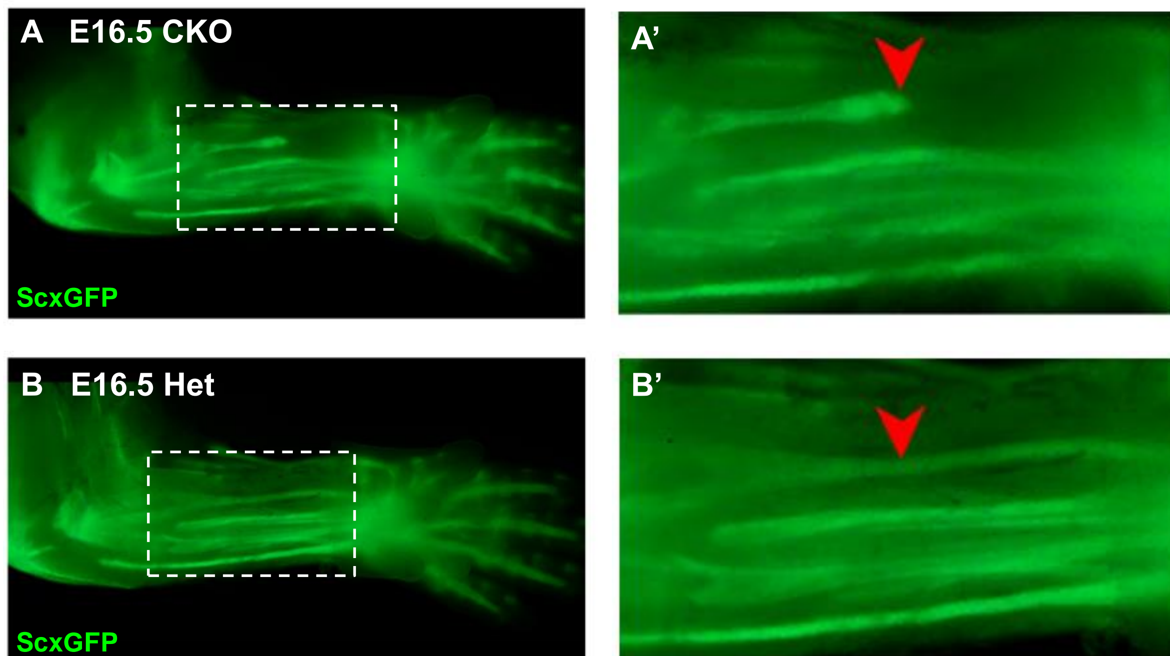

1260 **Fig S2. Disruption of the flexor carpi radialis tendon in mutant embryos.** Examination of flexor
1261 tendons in E16.5 (A) mutant and (B) heterozygous littermates using the tendon reporter ScxGFP.
1262 Boxed regions in (A) and (B) are shown enlarged in (A') and (B'). While most tendons appeared
1263 normal in mutant embryos, starting at E16.5 the flexor carpi radialis tendon (red arrowheads)
1264 was consistently torn in mutant embryo. Mutant: CKO, Heterozygous: Het. Figures not to scale.

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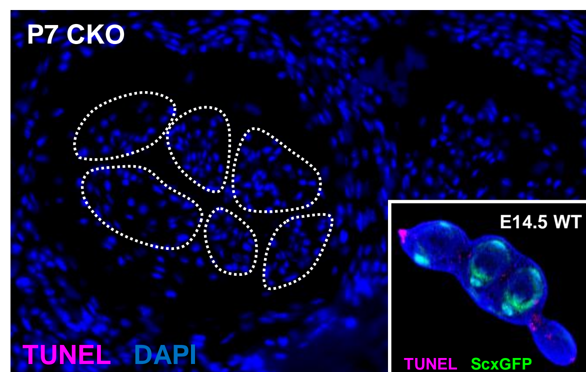

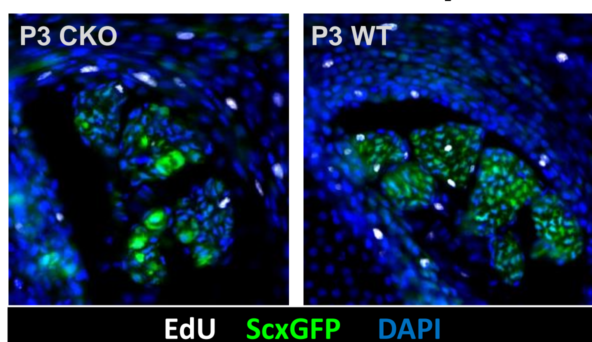

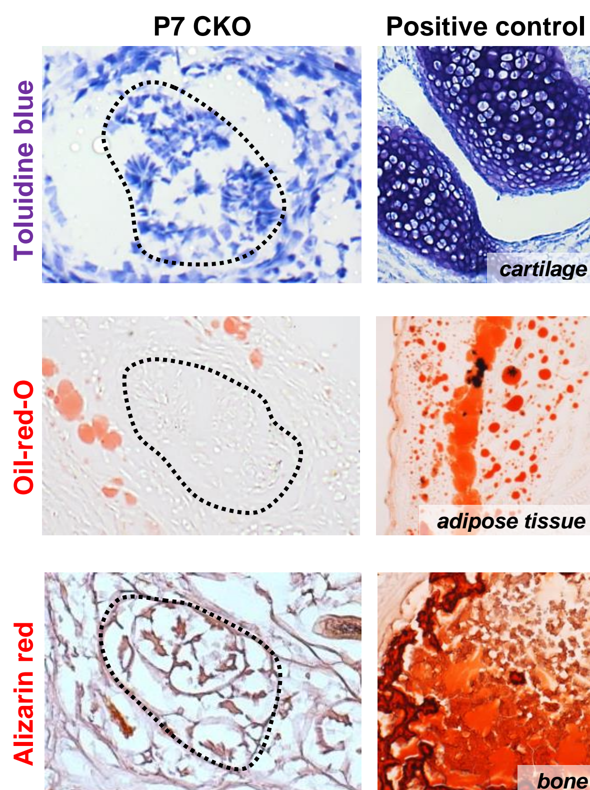

1304 **Fig S3. Evaluating cell death, proliferation and transdifferentiation in mutant tendons.**
1305 (A) TUNEL assay did not detect significant cell death in mutant tendons. The extensor
1306 digitorium communis tendons are demarcated in a transverse section of P7 mutant forelimb.
1307 Inset in (A) shows a transverse section of E14.5 forelimb paw that serves as a positive control
1308 for TUNEL staining. As expected, cell death is detected only at the distal edge of the autopod,
1309 but not in tendons (ScxGFP) at this stage. (B) EdU labeling of proliferating cells in transverse
1310 sections of the forelimb from P3 pups. The rate of proliferation was also not altered in mutant
1311 tendons compared with the wild-type littermates. The pups were injected i.p. with 100 µg of
1312 EdU in PBS and tissues were harvested two hour post injection. (C) Histological staining for the
1313 prototypic markers of chondrocytes (toluidine blue), osteocytes (alizarin red) and adipocytes
1314 (oil-red-o) showed that the loss of tendon markers in mutant tenocytes was also not due to
1315 transdifferentiation. The positive control tissues for the respective staining are cartilage,
1316 adipose tissue and bone from the same section. Dotted lines demarcate tendons. Mutant: CKO,
1317 Wild-type: WT. Figures not to scale.
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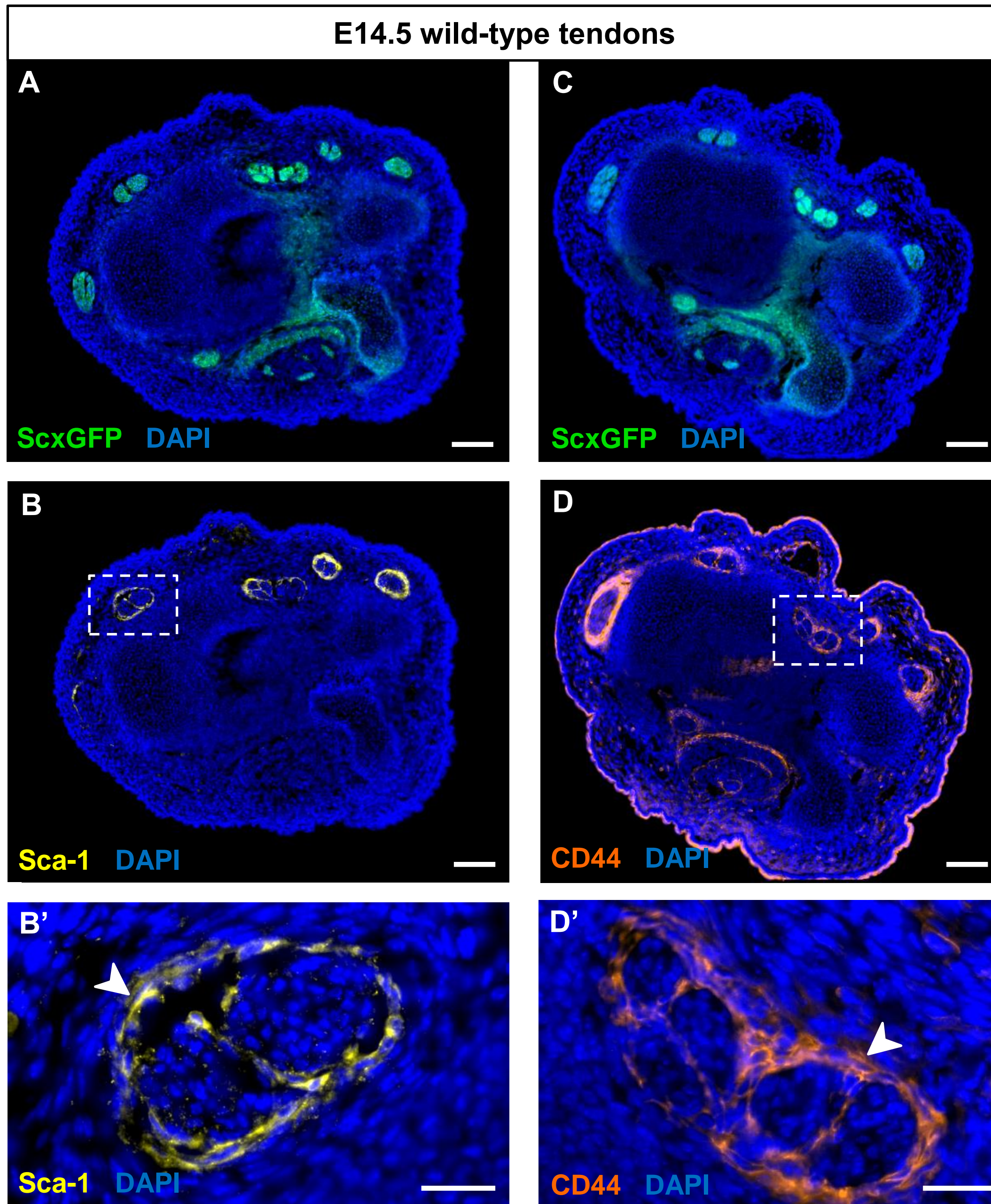

Fig S4. Expression of Sca-1 and CD44 during embryonic tendon development. (A-D) Immunofluorescence staining for Sca-1 and CD44 on wrist-level transverse sections from E14.5 ScxGFP-carrying wild-type embryos. Robust expression of (B) Sca-1 and (D) CD44 was detected in cells that surround the tendons at E14.5 (boxed areas), likely the precursors of the epitenon/paratenon. (B', D') Higher magnification views of the boxed areas in (B) and (D). The epitenon/paratenon layer is indicated by white arrowheads. Note that both markers were not expressed by the tenocytes at E14.5, the onset of tenocyte differentiation or at any other stages during embryonic tendon development (not shown). Scale bars, 100 µm (A-D) and 25 µm (B',D').

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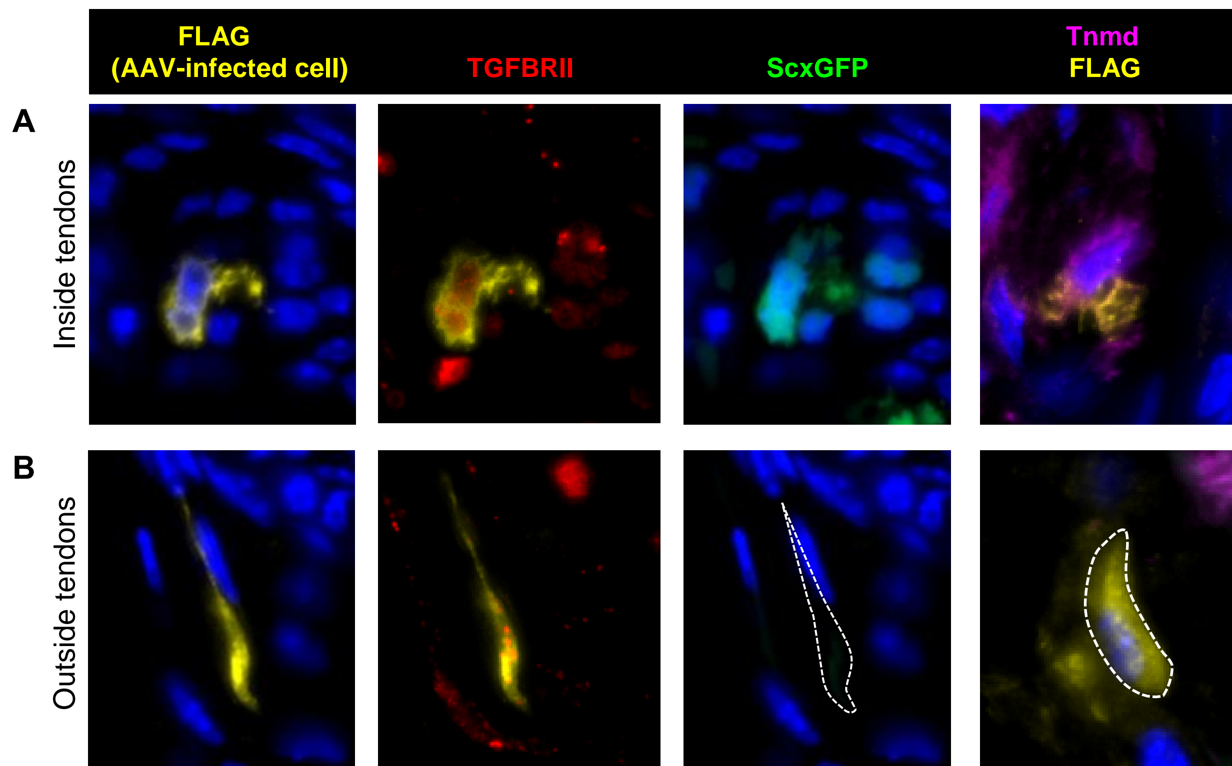

1384 **Fig S5. Induction of tendon markers by TGFβ signaling is context dependent.** AAV1-Tgfbr2-
1385 FLAG viral infection resulted in constitutive expression Tgfbr2-FLAG expression in cells both
1386 within and outside of tendons. The virus was injected locally into P1 mutant limbs and the limbs
1387 were harvested at P7. Sections from infected limbs were stained with antibodies to FLAG
1388 (yellow) to detect infected cells, and TGFβ type II receptor (TGFBRII) to confirm the re-
1389 expression of the receptor. ScxGFP signal and tenomodulin (Tnmd) antibody staining were used
1390 to identify induction of tendon markers. (A) Infected mutant tendon cells expressed the tendon
1391 markers ScxGFP and Tnmd. (B) In cells located outside of tendons (demarcated lines), the viral
1392 infection as detected by positive FLAG and TGFBRII immunofluorescence did not result in
1393 induction of the tendon markers ScxGFP and Tnmd. Figures not to scale.

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1403 **Table S1. List of primary antibodies used for immunofluorescence staining.**

Primary antibody	Host	Dilution	Company (Cat number)	Pre-treatment
Alpha-smooth muscle actin	Rabbit	1:500	Abcam (ab5694)	Permeabilization
CD34 (Clone RAM34)	Rat	1:200	BD Pharmingen (553731)	Antigen retrieval
CD44 (Clone IM7)	Rat	1:40	BD Biosciences (550538)	Cold acetone for 10 min at -20C
CD90.2 (Clone 53-2.1)	Rat	1:40	BD Biosciences (550543)	Permeabilization
FLAG (DYKDDDDK)	Rabbit	1:200	ThermoFisher (740001)	Antigen retrieval
FLAG (DYKDDDDK)	Rat	1:100	Novus Biologicals Inc (NBP1-06712SS)	Antigen retrieval
Nestin	Chicken	1:2000	Aves Labs Inc (NES)	-
Nucleostemin	Goat	1:300	Neuromics (GT-15050)	Permeabilization
Oct3/4	Rabbit	1:250	Santa Cruz (sc-9081)	Permeabilization
Sca-1/Ly6	Goat	1:80	R&D Systems (AF1226)	-
Sca-1/Ly6	Rat	1:50	R&D Systems (MAB1226)	-
Sox-2	Goat	1:250	Santa Cruz (sc-17320)	Permeabilization
Sox-9	Rabbit	1:150	Millipore (AB5535)	Permeabilization
Tenomodulin (C-20)	Goat	1:50	Santa Cruz (sc-49324)	Antigen retrieval
TGFβ type II receptor	Rabbit	1:250	Bioworld Inc (BS1360)	-
V5	Rabbit	1:500	Abcam (ab206566)	Antigen retrieval
V5	Rat	1:500	Abcam (ab206570)	Antigen retrieval

1404 Note:
1405 1) Permeabilization: Incubated with 0.3% Triton X-100 for 10 min.
1406 2) Antigen retrieval: Incubated with warm citrate buffer (10 mM sodium citrate with 0.05% Tween 20, pH 6) at
1407 550W, 50°C for 5 min using a PELCO BioWave®.
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1415
1416 **Table S2. Differentially expressed genes (2-fold change, adjusted *p*<0.05) in P7 Tgfbr2;ScxCre**
1417 **mutant tendon cells compared with P7 wild-type tenocytes.** Note that the expression level
1418 detected for Scx also included that of ScxGFP, and therefore do not reflect the expression level
1419 of endogenous Scx.
1420 **>> See the attached excel file "Table S2-DEGs in P7 mutant cells".**
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Table S3. Gene Ontology (GO) term enrichment of differentially expressed genes in P7 Tgfbr2;ScxCre mutant cells compared with P7 wild-type tenocytes. A complete list of differentially expressed genes (2-fold change, $p<0.05$) used for the analysis is available in Table S2.

(A) Upregulated genes

GO ID	GO terms (biological process)	*p*-value	Genes annotated to the term	Count
GO:2000146	negative regulation of cell motility	4.77E-07	*Cxcl12/Col3a1/Ptn/Nbl1/Apod/Sfrp2/Igfbp3/Apoe/Igfbp5*	9
GO:0061041	regulation of wound healing	1.34E-06	*Serpine2/Cd34/Serping1/Gsn/Plpp3/Apoe/Anxa1*	7
GO:0042060	wound healing	1.38E-06	*Serpine2/Cd34/Serping1/Col3a1/Gsn/Col5a1/Igf1/Apoe/Anxa1/Naca*	10
GO:0009611	response to wounding	1.61E-06	*Serpine2/Cd34/Serping1/Col3a1/Gsn/Col5a1/Igf1/Apoe/Mmp2/Anxa1/Naca*	11
GO:0043567	regulation of insulin-like growth factor receptor (IGFR) signaling pathway	2.18E-06	*Igf1/Igfbp3/Igfbp5/Igfbp4*	4
GO:0031099	regeneration	9.23E-06	*Cxcl12/Igf1/Apoe/Mmp2/Anxa1/Naca*	6
GO:0050900	leukocyte migration	2.83E-05	*Cd34/Cxcl12/Nbl1/Apod/Anxa1/Cxcl14/Mmp14/Rps19*	8
GO:0048771	tissue remodeling	7.54E-05	*Dlk1/Igf1/Igfbp5/Mmp2/Anxa1/Mmp14*	6

Table S3 (Continued). Gene Ontology (GO) term enrichment of differentially expressed genes in P7 Tgfbr2;ScxCre mutant cells compared with P7 wild-type tenocytes.

(B) Downregulated genes

GO ID	GO terms (biological process)	*p*-value	Genes annotated to the term	Count
GO:0030199	collagen fibril organization	1.86E-17	*Col11a2/Scx/Col2a1/P4ha1/Loxl2/Col11a1/Col1a1/ Col1a2/Lox/P3h4/Serpinh1*	11
GO:0030198	extracellular matrix organization	5.57E-16	*Col11a2/Scx/Col2a1/P4ha1/Loxl2/Col11a1/Mfap4/Col1a1/ Col1a2/Lox/Abi3bp/Creb3l1/Comp/P3h4/Tgfbi/Serpinh1*	16
GO:0070208	protein heterotrimerization	1.44E-08	*Col1a1/Col1a2/Col6a2/Col6a1/C1qtnf6*	5
GO:0032964	collagen biosynthetic process	4.92E-08	*Scx/Col1a1/Creb3l1/P3h4/Serpinh1/P3h3*	6
GO:0032963	collagen metabolic process	8.63E-08	*Scx/Mfap4/Col1a1/Creb3l1/P3h4/Serpinh1/P3h3*	7
GO:0070206	protein trimerization	1.60E-07	*Col1a1/Col1a2/C1qtnf3/Col6a2/Col6a1/C1qtnf6*	6
GO:0018126	protein hydroxylation	1.79E-06	*P4ha1/Crtap/P3h4/P3h3*	4
GO:0001501	skeletal system development	6.72E-06	*Col11a2/Scx/Col2a1/Loxl2/Col11a1/Col1a1/Col1a2/Sparc/ Comp/Vkorc1/Serpinh1*	11