1 **Development of migrating entheses involves replacement of progenitor populations**

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11 Keywords: Musculoskeletal development, Enthesis, Sox9, Gli1, Progenitor cell, Hedgehog signaling,

12 R26R-Confetti

13

14 **ABSTRACT**

15 Attachment sites of tendons to bones, called entheses, are essential for proper musculoskeletal function.
 16 They are formed embryonically by *Sox9*⁺ progenitors and undergo a developmental process that
 17 continues into the postnatal period and involves *Gli1* lineage cells. During bone elongation, some
 18 entheses maintain their relative positions by actively migrating along the bone shaft, while others,
 19 located at the bone's extremities, remain stationary. Despite their importance, we lack information on
 20 the developmental transition from embryonic to mature enthesis and on the relation between *Sox9*⁺
 21 progenitors and *Gli1* lineage cells. Here, by performing a series of lineage tracing experiments, we
 22 identify the onset of *Gli1* lineage contribution to different entheses during embryogenesis. We show that
 23 *Gli1* expression is regulated by SHH signaling during embryonic development, whereas postnatally it is
 24 maintained by IHH signaling. Interestingly, we found that unlike in stationary entheses, where *Sox9*⁺
 25 cells differentiate into the *Gli1* lineage, in migrating entheses the *Sox9* lineage is replaced by *Gli1*
 26 lineage and do not contribute to the mature enthesis. Moreover, we show that these *Gli1*⁺ progenitors
 27 are pre-specified embryonically to form the different cellular domains of the mature enthesis.
 28 Overall, these findings demonstrate a developmental strategy whereby one progenitor population
 29 establishes a simple, embryonic tissue, whereas another population is responsible for its maturation into
 30 a complex structure during its migration. Moreover, they suggest that different cell populations may be
 31 considered for cell-based therapy of enthesis injuries.

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33

34 INTRODUCTION

35 The proper assembly of the musculoskeletal system is essential for the function, form and stability of the
36 organism. During embryogenesis, an attachment between tendon and bone, known as enthesis, is
37 formed. Thereafter and into the postnatal period, the rudimentary entheses further develops into a more
38 complex tissue. While some knowledge on entheses formation and maturation exists, far less is known
39 about the processes that transform the simple embryonic entheses into the structure of the mature
40 entheses.

41 Traditionally, entheses are divided to either fibrous or fibrocartilaginous, according to their composition.
42 Fibrous entheses form at the attachment site of a tendon that is inserted directly into the bone shaft,
43 forming a structure that resembles a root system. This structure, which is composed of a dense
44 connective tissue (Doschak and Zernicke, 2005; Shaw and Benjamin, 2007; Wang et al., 2013), was
45 shown to be regulated by parathyroid hormone-like hormone (PTHrP, also known as PTHrP) (Wang et
46 al., 2013). Fibrocartilage entheses typically form at attachment sites of tendons to the epiphysis or to
47 bone eminences. Relative to fibrous entheses, fibrocartilage entheses are structurally more complex,
48 displaying a cellular gradient that is typically divided into four zones, namely tendon, fibrocartilage,
49 mineralized fibrocartilage and bone. The graded tissue that develops postnatally dissipates the stress that
50 forms at the attachment site, thereby providing the entheses with the mechanical strength necessary to
51 withstand compression (Benjamin et al., 2006).

52 Enthesis development is initiated by the specification of a specialized pool of progenitor cells that
53 express both SRY-box 9 (*Sox9*), a key regulator of chondrogenesis, and the tendon marker scleraxis
54 (*Scx*) (Akiyama et al., 2005; Blitz et al., 2013; Schweitzer et al., 2001; Sugimoto et al., 2013). Cell
55 lineage studies showed that *Sox9*-expressing progenitor cells contribute to the formation of the entheses
56 in neonatal mice (Akiyama et al., 2005; Soeda et al., 2010). Another marker for entheses cells is GLI-

57 Kruppel family member *GLI1* (*Gli1*), a component of the hedgehog (HH) signaling pathway. Lineage
58 studies revealed that *Gli1*-expressing cells act as progenitors that contribute to the formation of some
59 adult entheses (Dyment et al., 2015; Schwartz et al., 2015). However, the relation between progenitors
60 of the embryonic enthesis and *Gli1* lineage cells, which contribute to the mature enthesis, has not been
61 determined.

62 The relative position of an enthesis along the bone directly affects its mechanical function and,
63 subsequently, the animal's mobility (Polly, 2007; Salton and Sargis, 2009). Recently, it was shown that
64 all entheses maintain their relative position during bone elongation (Stern et al., 2015). The mechanism
65 that maintains their positions involves regulation of the relative growth rates at the two epiphyseal
66 plates. Additionally, some entheses migrate through continuous reconstruction to maintain their position,
67 a process known as bone modelling or drift (Benjamin and McGonagle, 2009; Dörfl, 1980a; Dörfl,
68 1980b). These entheses, referred to in the following as migrating entheses, face a unique developmental
69 challenge. Unlike most organs and tissues, they must develop into a complex graded tissue while
70 constantly drifting. This raises the question of whether the descendants of the embryonic enthesis
71 progenitors continue to serve as the building blocks during maturation of migrating entheses.

72 In this work, we identify the embryonic stage at which *Gli1* lineage is initiated and demonstrate its
73 contribution to the postnatal enthesis. We show that embryonic *Gli1* expression is initially under the
74 regulation of SHH. Later during postnatal development, *Gli1* expression is maintained by IHH.
75 Moreover, we show that *Sox9* lineage does not contribute to postnatal migrating entheses. Instead, *Gli1*
76 lineage cells replace the *Sox9* lineage cells and populate the enthesis. Finally, we show that embryonic
77 *Gli1* lineage cells are pre-determined to contribute to the different layers of the fibrocartilaginous
78 enthesis.

79

RESULTS

Some entheses undergo cellular and morphological changes while migrating

The development of the rudimentary embryonic attachment site into the complex structure of a mature enthesis has received little attention (Galatz et al., 2007). As mentioned, in addition to substantial cellular and morphological changes, some entheses also migrate considerably along the bone during bone growth (Benjamin and McGonagle, 2009; Dörfl, 1980a; Dörfl, 1980b; Stern et al., 2015). Therefore, to study the transition that migrating entheses undergo during maturation, we documented morphological and molecular changes as well as drifting activity in entheses from embryonic day (E) 14.5 to postnatal day (P) 14. We focused on two migrating entheses, namely the deltoid enthesis (DT), a fibrocartilaginous enthesis that forms between the deltoid tendon and the deltoid tuberosity, and the teres major enthesis (TM), a fibrous enthesis that forms between the teres major tendon and the humeral shaft. Analysis of enthesis positions during development revealed that both DT (0.778 ± 0.026 mm) and TM (1.624 ± 0.171 mm) entheses drifted considerably along the bone shaft during bone elongation (Fig. 1A). Histological sections through wild-type (WT) mouse humeri showed that both embryonic entheses displayed a simple structure of layered cells (Fig. 1B,C(a),D(a)). However, the overall shape of the enthesis dramatically changed through development, as it protruded outwards from the bone shaft and the different enthesis domains became more noticeable (Fig. 1C(a'-a'''),D(a'-a''')). Tissue complexity also increased, as a larger variety of cells, such as fibrocartilage cells and osteoblasts (Benjamin and McGonagle, 2009), were identified along with an increase in extracellular matrix (Fig. 1C(d,d',h,h'), D(d,d',h,h')). The increased complexity was also demonstrated by a change in the expression patterns of structural genes, such as bone sialoprotein (*Bsp*; also known as integrin binding sialoprotein (*Ibsp*), collagen type 2 alpha 1 (*Col2a1*), collagen type 12 alpha 1 (*Col12a1*) and tenascin C (*Tnc*), and regulatory genes such as *Gli1*. Furthermore, expression domains correlating to various structural

domains emerged, namely *Coll2a1*, *Tnc*, *Gli1* and *Colla1* in tendon and fibrocartilage (Fig. 1C(c,f-h),D(c,f-h), and *Colla1*, *Gli1* and *Bsp* in mineralized fibrocartilage and bone (Fig. 1C(c,e,h),D(c,e,h)).

***Gli1*+ cell lineage contributes to the postnatal enthesis**

Previously, lineage tracing experiments showed that *Gli1* lineage cells contribute to postnatal enthesis development (Dyment et al., 2015; Schwartz et al., 2015). Yet, the onset of this lineage during embryogenesis and its dynamics in different entheses have been missing. In order to identify the onset of *Gli1* lineage, we performed pulse-chase experiments on *Gli1-CreER^{T2}* (Ahn & Joyner, 2004) mice crossed with *R26R-tdTomato* reporter mice (Madisen et al., 2010), which allowed us to mark *Gli1* expressing cells at specific time points and follow their descendants. We analyzed three entheses representing different types, namely DT (migratory-fibrocartilaginous), TM (migratory-fibrous), and Achilles (stationary-fibrocartilaginous). Examination of neonatal (P0) TM and DT entheses following tamoxifen administration at E11.5 and E12.5 revealed only a few *Gli1* lineage cells. However, administration at E13.5 and E15.5 resulted in extensive labeling in both entheses (Fig. 2A-A''',B-B'''). In the stationary Achilles enthesis, extensive labeling was observed at P0 only after tamoxifen administration at E15.5 (Fig. 3C'''). The postnatal contribution of *Gli1* lineage to migratory entheses was further established by following E13.5 lineage induction to P14 (Fig. 2D,E).

Together, these results indicate that although *Gli1* lineage is not induced at the onset of enthesis formation (Blitz et al., 2013; Sugimoto et al., 2013), it contributes differentially to different entheses during embryonic development.

***Gli1* expression in migrating entheses is initiated by SHH and maintained by IHH**

125 The Hedgehog (HH) signaling pathway has previously been suggested to play a role in regulating the
 126 activity of *Gli1*-positive enthesis cells (Breidenbach et al., 2015; Dymment et al., 2015; Liu et al., 2013;
 127 Schwartz et al., 2015). Thus, our finding that *Gli1* expression is initiated at early stages of enthesis
 128 development (Fig. 1C(h)D(h)) raised the question of which component of the HH pathway regulates
 129 *Gli1* expression in enthesis cells and whether it also affects migratory entheses. It was suggested that
 130 Indian hedgehog (IHH), an effector of the HH signaling pathway, is a possible regulator of *Gli1* lineage
 131 cells in adult stationary entheses. In order to examine the possible role of IHH in inducing *Gli1*
 132 expression in the embryonic enthesis, we blocked *Ihh* expression in the limb by using *Prx1-Cre-Ihh*^{-/-}
 133 mice. Interestingly, at E14.5 *Gli1* was expressed in control and mutant entheses, suggesting that *Gli1*
 134 expression in embryonic enthesis progenitors is not regulated by *Ihh* (Fig. 3A,A'). We therefore
 135 examined the possible role of another regulator of HH signaling, namely sonic hedgehog (SHH), by
 136 analyzing *Gli1* expression by embryonic enthesis cells in *Shh* KO embryos (*Shh-GFPCre*^{-/-}). Results
 137 showed that *Gli1* expression dramatically decreased in the mutant entheses compared to the WT (Fig.
 138 3B,B'), suggesting that SHH is necessary for the induction of *Gli1* in embryonic enthesis cells.
 139 That result was intriguing, because we observed that *Gli1* was constantly expressed by both embryonic
 140 and postnatal enthesis cells (Fig. 1A(h-h'''),B(h-h''')), while *Shh* is expressed in the limb only during
 141 embryogenesis (Harfe et al., 2004). This raised the question of how *Gli1* expression in the enthesis is
 142 maintained after the loss of *Shh* expression. To address the possibility that *Ihh* controls *Gli1* expression
 143 in the postnatal enthesis even though it is not involved in the induction of *Gli1* expression in embryonic
 144 enthesis progenitors, we analyzed *Prx1-Cre-Ihh*^{-/-} mice at P10. Results showed that although *Gli1*
 145 expression was maintained in mutant muscle and bone, in the enthesis it was completely lost (Fig.
 146 3C,C'), indicating that *Ihh* is indeed required for the maintenance of *Gli1* expression in enthesis cells.
 147 To identify the source of *Ihh* in the enthesis, we performed immunofluorescence staining for IHH

148 protein in P6 enthesis sections. As seen in Figure 3 (D,D'), IHH was highly expressed in mineralized
149 fibrocartilage and bone regions at the enthesis center.

150 Taken together, these results suggest that *Gli1* expression by embryonic enthesis progenitors is induced
151 by SHH and later, in the maturing enthesis, maintained by IHH originating in mineralized fibrocartilage
152 and bone.

153

154 *Sox9* lineage cells of the embryonic enthesis do not contribute to postnatal migrating entheses

155 The embryonic enthesis originates from *Scx/Sox9* double-positive progenitor cells (Blitz et al., 2013;
156 Sugimoto et al., 2013). Yet, the contribution of these progenitors to the postnatal enthesis and their
157 relation to the *Gli1* lineage cells have never been studied. To fill this gap, we first examined the
158 contribution of *Sox9* lineage to the postnatal enthesis. To that end, we performed a pulse-chase cell
159 lineage experiment using mice that express Cre-ER under control of the *Sox9* promoter (*Sox9-CreER^{T2}*)
160 crossed with *R26R-tdTomato* reporter mice (Soeda et al., 2010). It was previously demonstrated that
161 tamoxifen administration at E12.5 effectively labels embryonic enthesis cells (Blitz et al., 2013; Soeda
162 et al., 2010). Indeed, examination at P0 following tamoxifen administration at E12.5 showed that the
163 DT, TM and Achilles entheses were populated by tdTomato-positive cells, suggesting that at that stage,
164 the enthesis is populated by *Sox9* lineage cells (Fig. 4A(a,b,d)). Yet, surprisingly, at P14 we observed a
165 dramatic decrease in the contribution of tdTomato-expressing cells to the two migrating entheses (Fig.
166 4A(a',b',c), although the cells in the stationary enthesis were still extensively labeled (Fig. 4A(d')).
167 These results suggest that postnatal stationary enthesis cells were descendants of the *Sox9*-positive
168 embryonic lineage, whereas in migrating entheses this lineage was lost.

169 The labeled stationary entheses could serve as a positive internal control for the effectiveness of
170 labeling. Nevertheless, to rule out the possibility that tamoxifen was administered at the wrong time

point, we repeated the experiment while administering tamoxifen at different time points from E11.5 to E17.5 (Fig. 4B). Examination at P14 showed minimal contribution of *Sox9* lineage cells to the postnatal entheses, similar to the results obtained following pulsing at E12.5. Taken together, these results indicate that the embryonic *Sox9* lineage contributes poorly to postnatal migrating entheses, suggesting that these entheses are populated by another cell lineage postnatally.

***Gli1* lineage cells replace the embryonic *Sox9* lineage during enthesis maturation**

Our finding that both embryonic *Gli1* and *Sox9* lineages contribute to the stationary postnatal enthesis suggests that these two genes mark a common cell lineage. Conversely, the finding that embryonic *Sox9* lineage contributes to embryonic but not to postnatal migrating enthesis implies that postnatally, embryonic *Gli1* lineage replaces the *Sox9* lineage to form the mature enthesis.

To study the process of lineage replacement and to follow its dynamics, we traced both lineages throughout enthesis development, from E15.5 to maturation at P14, by pulse-chase experiments. As seen in Figure 5, at E15.5 and P0 in both the DT and TM, *Sox9* lineage cells populated the embryonic enthesis. However, from P0 their number decreased dramatically and by P8, the enthesis contained only a limited number of these cells. Concurrently, the number of cells of the *Gli1* lineage gradually increased in both entheses and, by P8, *Gli1*⁺ cells inhabited most of the enthesis structure. Interestingly, the gradual population of the DT and TM entheses by *Gli1* lineage cells correlated with the temporal dynamics of enthesis migration (Fig. 1A). These results support our hypothesis that during early postnatal development of migrating entheses, a new population derived from *Gli1*-positive progenitors substitutes the *Sox9* lineage cells of the embryonic enthesis.

Cell fate of *Gli1*-positive progenitors is predetermined

194 The increasing cellular complexity of the developing enthesis and our finding that progenitors of the
 195 *Gli1* lineage that will contribute to the postnatal enthesis are present already during embryonic
 196 development led us to ask how these cells form the graded tissue of the mature enthesis. Specifically, we
 197 sought to determine whether *Gli1* lineage cells possess a multipotent capacity and, thereby, produce the
 198 different cell types of the various enthesis domains, or have predetermined cell fates already at the time
 199 of their recruitment to the embryonic enthesis. To decide between these options, we conducted pulse-
 200 chase experiments on *Gli1-CreER^{T2}* mice crossed with *R26R-Confetti* mice (Snippert et al., 2010) that,
 201 upon recombination, stochastically express GFP, RFP, CFP or YFP, allowing for the identification of
 202 different cell clones. Clones derived from multipotent progenitors would be spread among the different
 203 domains, whereas predetermined progenitors would produce clones that are confined to specific
 204 domains.

205 We focused on the two main domains of the DT enthesis, namely mineralized and non-mineralized
 206 fibrocartilage, and on the border between enthesal mesenchymal cells and bone in the TM enthesis.
 207 Examination of P14 limbs following tamoxifen administration at E13.5 revealed multiple clones in both
 208 entheses, most of which were restricted to a specific domain (Fig. 6). These results suggest that at E13.5,
 209 the cell fates of *Gli1* lineage progenitors have already been determined.

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211

212

213 **DISCUSSION**

214 The transition from the embryonic to the mature enthesis has been understudied. Here, we use genetic
 215 lineage tracing to unravel the cellular developmental sequence of fibrous and fibrocartilaginous
 216 entheses. We show that although *Gli1*⁺ progenitors contribute to entheses of both types, their

217 contribution differ greatly between stationary and migratory entheses. In stationary entheses, *Gli1*+
 218 progenitors are descendants of *Sox9*+ progenitors that have established the embryonic enthesis.
 219 However, in migrating entheses, a separate, pre-specified population of *Gli1*+ progenitors is recruited to
 220 the embryonic enthesis, co-populates it alongside *Sox9*+ progenitors and, eventually, replaces them
 221 during postnatal development.

222 As mentioned, fibrous and fibrocartilaginous entheses exhibit marked differences in structure and
 223 composition (Benjamin et al., 2002). Notwithstanding the importance of this distinction, the observed
 224 differences in cellular origin between stationary and migrating entheses calls for a revision of the
 225 traditional classification and suggests that it should be taken into account whether an enthesis is
 226 “migratory” or “stationary”.

227 Organ development can be mediated by several cellular mechanisms. In a linear mechanism, an
 228 embryonic set of progenitors forms a primordium and then continues to proliferate and differentiate to
 229 form the mature organ. Another mechanism is cell recruitment, during which cells are supplied to the
 230 forming organ after primordium establishment. A third mechanism involves template replacement, in
 231 which an initial template is formed by one type of cells to be later replaced by another cell population,
 232 which will form the mature organ. Interestingly, in the musculoskeletal system there are examples of all
 233 these modes of development. Muscles and joints develop through cell recruitment (Buckingham et al.,
 234 2003; Shwartz et al., 2016), whereas most of the skeleton develops through template replacement,
 235 namely by endochondral ossification (Kronenberg, 2003). Here, we show that stationary entheses
 236 develop linearly by embryonic *Sox9*+ progenitors that form the postnatal enthesis and later upregulate
 237 the expression of *Gli1*. However, we show that migrating entheses develop through template
 238 replacement, as *Gli1*+ progenitors replace the *Sox9*+ embryonic enthesis cells and form the mature
 239 enthesis.

240 Previous studies have shown not only that *Gli1* is a marker for the forming enthesis, but that the HH
 241 pathway plays an active role in regulating enthesis maturation and regeneration (Breidenbach et al.,
 242 2015; Dymment et al., 2015; Liu et al., 2013; Schwartz et al., 2015; Schwartz et al., 2017). *Ihh* expression
 243 was identified in proximity to *Gli1*⁺ progenitor population in stationary entheses (Dymment et al., 2015;
 244 Liu et al., 2013; Schwartz et al., 2015). Moreover, loss-of-function of *Smo*, a component of the HH
 245 pathway, in *Scx*-expressing cells resulted in reduced mineralization of the enthesis (Breidenbach et al.,
 246 2015; Dymment et al., 2015; Liu et al., 2013; Schwartz et al., 2015). Yet, the question of which ligand of
 247 the HH pathway regulates the specification of *Gli1*⁺ cells in the embryo and, specifically, in migrating
 248 entheses has remained open. Our results clearly suggest that *Gli1* expression is regulated by both SHH
 249 and IHH during enthesis development. In the embryo, we show that SHH, but not IHH, signaling is
 250 essential for *Gli1* expression in the enthesis, implicating SHH in *Gli1*⁺ progenitor specification.
 251 However, during postnatal development, *Ihh* expression is vital for maintaining *Gli1* enthesis expression
 252 (Fig. 7A).

253 During endochondral ossification, IHH regulates hypertrophic chondrocyte differentiation and, thereby,
 254 bone elongation (Vortkamp et al., 1996). It is therefore possible that by controlling both these processes,
 255 IHH coordinates enthesis migration with the concurrent bone elongation to preserve enthesis position
 256 along the shaft. However, the signals that govern enthesis positioning and migration along the bone,
 257 including the possible effect of IHH on these processes, are yet to be elucidated.

258 Another key question regards the mechanism that facilitates the replacement between *Sox9*⁺ and *Gli1*⁺
 259 cell populations. It is possible that the former cells die, while the latter cells proliferate and populate the
 260 entire enthesis. A more attractive hypothesis is that during enthesis drift, *Sox9*⁺ cells are removed
 261 together with eroded bone tissue by osteoclasts or other phagocytic cells. However, the replacement

mechanism remains an open question. Moreover, whether the replacement process is the mechanism that allows migrating entheses to maintain their relative position along the bone is yet to be determined. Finally, our finding that *Gli1*-expressing cells are pre-determined already in the embryo suggests the existence of an earlier, yet unknown player in the specification of these progenitors. The discovery of this early marker gene may enable the identification of multipotent enthesis cells, which may hold therapeutic potential.

To conclude, our findings shed light on developmental linearity in organogenesis. Using the enthesis as a model system, we identify two different strategies of development (Fig. 7). The first, found in stationary entheses, is linear development where embryonic progenitors and their descendants contribute to the entire process of enthesis development, from embryogenesis to maturity. Conversely, in migrating entheses, another developmental strategy was identified where one type of progenitor cells form an embryonic template, only to be later replaced by another cell lineage that contributes to the mature organ.

MATERIALS AND METHODS

Animals

All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute. Histology was performed on BL6 mice.

For lineage tracing experiments, *Sox9-Cre* (Akiyama et al., 2005), *Sox9-CreER* (Soeda et al., 2010) and *Gli1-CreER^{T2}* (Ahn and Joyner, 2004, Jackson laboratories) mice were crossed with *R26R-tdTomato* mice (B6;129S6-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}/J*, (Madisen et al., 2010) or with *R26R-Confetti* mice (B6.129P2-*Gt(ROSA)26Sor^{tm1(CAG-Brainbow2.1)Cle}/J*, Snippert et al., 2010).

284 To create *Shh* KO mice, mice heterozygous for a mutation in *Shh* (B6.Cg-*Shh*^{tm1(EGFP/cre)Cjt}/J; Jackson
285 Laboratory) were intercrossed; heterozygotes or WT littermates were used as a control. The generation
286 of *Prx1-Cre* (Logan et al., 2002) was previously described. To generate *Prx1-Cre-Ihh* mutant mice, *Ihh*-
287 floxed mice (B6N;129S4-*Ihh*^{tm1Blau}/J; Jackson Laboratory) (Razzaque et al., 2005) were mated with
288 *Prx1-Cre* mice. As a control, *Prx1-Cre*-negative animals were used.

289 For cell lineage experiments, *Sox9-CreER*^{T2/+} or *Gli1-CreER*^{T2/+} males were crossed with *R26R*-
290 *tdTomato* females to produce embryos carrying both the relevant *CreER*^{T2} and *R26R-tdTomato* alleles.
291 For fate mapping, *Gli1-CreER*^{T2/+} males were crossed with *R26R-Confetti* females to produce embryos
292 carrying both the *Gli1-CreER*^{T2} and *Confetti* alleles.

293 In all timed pregnancies, plug date was defined as E0.5. For harvesting of embryos, timed-pregnant
294 females were sacrificed by cervical dislocation. The gravid uterus was dissected out and suspended in a
295 bath of cold phosphate-buffered saline (PBS) and the embryos were harvested after removal of amnion
296 and placenta. Tail genomic DNA was used for genotyping.

297

298 **Histological analysis, in situ hybridization and immunofluorescence**

299 For histology and *in situ* hybridization, embryos were harvested at various ages, dissected, and fixed in
300 4% paraformaldehyde (PFA)/PBS at 4°C overnight. After fixation, tissues were dehydrated to 70%
301 EtOH and embedded in paraffin. The embedded tissues were cut to generate 7-μm-thick sections and
302 mounted onto slides. Hematoxylin and eosin (H&E) staining was performed following standard
303 protocols. Non-fluorescent and fluorescent *in situ* hybridizations were performed as previously
304 described using digoxigenin- (DIG) labeled probes (Shwartz and Zelzer, 2014). All probes are available
305 upon request.

For immunofluorescence staining, 10- μ m-thick cryosections were air-dried for 1 hour before staining. For IHH staining, sections were washed twice in PBST for 5 minutes and blocked to prevent non-specific binding with 7% goat serum and 1% BSA dissolved in PBST. Then, sections were incubated with rabbit anti-IHH antibody (Abcam, #AB39364, 1:50) at 4C° overnight. The next day, sections were washed twice with PBST and incubated for 1 hour with Cy2 conjugated fluorescent antibody (1:100, Jackson Laboratories). Slides were mounted with Immuno-mount aqueous-based mounting medium (Thermo).

For Gli1 staining, 10- μ m-thick cryosections were air dried for 1 hour and fixed in 4% PFA for 10 minutes. Then, sections were washed twice in PBST and endogenous peroxidase was quenched using 3% H₂O₂ in PBS. Next, antigen retrieval was preformed using 0.3% Triton in PBS. Non-specific binding was blocked using 7% horse serum and 1% BSA dissolved in PBST for 1 hour. Then, sections were incubated with Goat anti-GLI1 antibody (R&D systems, #AF3455, 1:100) overnight at room temperature. The next day, sections were washed twice in PBST and incubated with Biotin anti-goat (1:100, Jackson laboratories, 705065147) for 1 hour and then with streptavidin-HRP (1:200 Perkin Elmer, NEL750001EA) for 1 hour. HRP was developed using TSA amplification kit (1:100, Prekin Elmer) for 20 minutes, counterstained with DAPI and mounted with Immuno-mount aqueous- based mounting medium (Thermo).

323

324 **Physical position of entheses**

For identification of enthesis physical position, 2-4 limb samples at stages E16.5-P14 were scanned *ex vivo* using iodine contrast agent to allow visualization of the soft tissue. DT and TM positions were identified manually, and their physical position along the bone was calculated as described previously (Stern et al., 2015).

329

330 **Cell lineage analysis**

331 Tamoxifen (Sigma T-5648) was dissolved in corn oil (Sigma C-8267) at a final concentration of 5
332 mg/ml. Time-mated *R26R-tdTomato* females were administered 1 mg of tamoxifen by oral gavage
333 (FST) at designated time points as indicated.

334

335 **Cell count**

336 For each age, 2-4 limbs from different litters were harvested, embedded in OCT, and sectioned at a
337 thickness of 10 μ m. Sections were imaged using Zeiss LSM 780 microscope approximately every 80
338 μ m, capturing red and DAPI channels. Images were then processed in ImageJ as follows: Each image
339 was converted to an RGB stack and the region of interest was manually identified and cropped. Then,
340 image levels were adjusted to improve separation between nuclei. A binary threshold was set
341 automatically and conjoined nuclei were automatically separated using the binary watershed function.
342 Using a home-made Matlab script, the number of nuclei was counted. Each nucleus that was co-
343 localized with a red channel signal was counted as a tdTomato-positive cell. For each section, the
344 percentage of tdTomato-positive cells was calculated. The average percentage of these cells was
345 calculated first for each bone and then for all samples. From each line and for each age group, at least 3
346 individual bones were sampled, and 9-17 sections per bone were analyzed, depending on bone length.
347 Data are presented as mean $\pm$ SD.

348

349 **Fate mapping Confetti experiment**

350 Tamoxifen (Sigma T-5648) was dissolved in corn oil (Sigma C-8267) at a final concentration of 20
351 mg/ml. Time-mated *R26R-Confetti* females were administered 4 mg of tamoxifen by oral gavage at

E13.5. Cre-positive pups were sacrificed at P14. 1 hour prior to sacrifice, each pup was injected with Calcein blue (Sigma, #m1255; 30 mg/kg). Limbs were harvested, fixed for 30 minutes in 4% PFA at 4°C, embedded in OCT, and sectioned at a thickness of 10 µm. Sections were imaged using Zeiss LSM 780 microscope approximately every 80 µm.

Microscope settings and image analysis

At least 1024X1024 pixels, 8-bit images were acquired using the X20 lens. A Z-stack of 2-4 images was taken from each section. To detect GFP and YFP, the argon laser 488 nm was used. For RFP detection, a red diode laser emitting at 561 nm was used, and blue mCFP was excited using a laser line at 458 nm. Calcein blue staining was detected using the 405-nm laser line. GFP fluorescence was collected between ~500-598 nm, airy 1; RFP fluorescence was collected between ~606-654nm, airy 1; mCFP fluorescence was collected between ~464-500 nm, airy 1 and Calcein blue was collected between ~410-451. For each image, a corresponding bright field image was captured. The acquired images were processed using Photoshop and ImageJ.

To calculate the percentage of *Gli1*⁺ clones found in both mineralized and non-mineralized fibrocartilage, the border between the zones was identified by Calcein blue signal. Clones that crossed the mineral border were manually counted and their percentage was calculated first for each section, then for each limb and, finally, for each type of enthesis. Data are presented as mean ± SD.

Acknowledgments

We thank Nitzan Konstantin for expert editorial assistance. We thank Dr. Patrick Tschopp from the Clifford J. Tabin lab, Harvard Medical School, for his assistance in generating *Shh* KO mice. Special thanks to all members of the Zelzer laboratory for encouragement and advice.

375 This study was supported by grants from the National Institutes of Health (grant #R01 AR055580),
 376 European Research Council (ERC) (grant #310098), the Jeanne and Joseph Nissim Foundation for Life
 377 Sciences Research, the Y. Leon Benozio Institute for Molecular Medicine, Beth Rom-Rymer, the
 378 Estate of David Levinson, the Jaffe Bernard and Audrey Foundation, Georges Lustgarten Cancer
 379 Research Fund, the David and Fela Shapell Family Center for Genetic Disorders, the David and Fela
 380 Shapell Family Foundation INCPM Fund for Preclinical Studies, and the Estate of Bernard Bishin for
 381 the WIS-Clalit Program.

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470

Fig.1

Fig.2

Fig.3

Fig.4

A

B

Fig.5

Fig.6

Fig.7

A. Migratory entheses

B. Stationary entheses

471 **FIGURE LEGENDS**

472 **Figure 1. Some entheses undergo cellular and morphological changes while migrating.** (A) Graph
 473 showing the physical position of the deltoid tuberosity (DT) and teres major (TM) entheses along the
 474 bone throughout development (E16.5-P14), defined as the distance of the element (mm) from a
 475 predefined longitudinal origin roughly at the center of the shaft. Positive and negative values correspond
 476 to elements that are proximal and distal to the point of origin, respectively. As indicated by the steep
 477 slope of the curves, both DT and TM migrate considerably through development. (B) Schematic
 478 illustrations of the embryonic DT and TM entheses. (C and D) Transverse sections through the humerus
 479 showing the DT (A) and TM (B) entheses of E14.5-P14 WT mice. [C(a,b) and D(a,b)]: H&E staining.
 480 C(b) and D(b) are magnifications of the boxed areas in C(a) and D(a), respectively. [C(c-h) and D(c-
 481 h)]: *In situ* hybridization using digoxigenin-labeled, anti-sense complementary RNA probes for *Colla1*,
 482 *Col2a1*, *Bsp*, *Coll2a1*, *Tnc* and *Gli1* at E14.5 (c-h), E18.5 (c'-h'), P5 (c''-h'') and P14 (c'''-h'''). Scale
 483 bars: 50 μ m.

484 **Figure 2. *Gli1*+ cell lineage contributes to the postnatal enthesis.** Pulse-chase cell lineage
 485 experiments using *Gli1-CreER^{T2};R26R-tdTomato* mice demonstrate the contribution of *Gli1* lineage
 486 cells to various entheses. *Gli1*-positive cells were pulsed by a single tamoxifen administration at
 487 different time points (E11.5-E15.5) and their descendants were followed to P0 and P14. (A-A''') The
 488 DT enthesis was extensively marked after tamoxifen administration at E13.5 and E15.5. (B-B''')
 489 tdTomato-positive cells were identified in the TM enthesis regardless of the time of pulsing; however, a
 490 stronger signal was seen following tamoxifen administration at E13.5 or later. (C-C''') tdTomato-
 491 positive cells were identified in the Achilles tendon enthesis following tamoxifen administration at
 492 E15.5. (D-E) Both DT and TM entheses were extensively marked at P14 following tamoxifen
 493 administration at E13.5. The enthesis is demarcated by a dashed line. Scale bars: 50 μ m.

Figure 3. *Gli1* expression in migrating entheses is initiated by SHH and maintained by IHH.

(A,A') Fluorescent ISH for *Gli1* on sections through the DT of E14.5 control (A) and *Prx1-Cre-Ihh^{ff}* embryos (A') demonstrates that in the absence of IHH signaling, *Gli1* expression in the enthesis is unchanged. **(B,B')** Fluorescent ISH for *Gli1* on sections through the DT of E14.5 control (B) and *Shh^{Cre/Cre}* embryos (B') demonstrates that in the absence of SHH signaling, *Gli1* expression in the enthesis is lost. **(C,C')** Immunostaining for GLI1 protein in *Prx1-Cre-Ihh^{ff}* mutant (C') and control (C) DT sections shows that in the absence of IHH, GLI1 protein expression is lost from the DT enthesis. **(D,D')** Immunostaining for IHH protein on *Gli1-CreER^{T2};R26R-tdTomato* mice. Tamoxifen was administered at E13.5 and mice were sacrificed at E15.5 (D) or P6 (D'). IHH expression is seen in hypertrophic chondrocytes at E15.5 and in the mineralizing part of the enthesis at P6. Enthesis is demarcated by a dashed line. Scale bars: 50 μ m.

Figure 4. *Sox9* lineage cells of the embryonic enthesis do not contribute to postnatal migrating entheses.

(A) Pulse-chase cell lineage experiment using *Sox9-CreER^{T2};R26R-td Tomato* mice demonstrates that *Sox9* lineage contributes differently to migrating and stationary entheses. *Sox9*-positive cells were marked at E12.5 by a single tamoxifen administration. At P0, tdTomato-positive cells were identified in migrating DT and TM entheses (A(a,b)) as well as in stationary Achilles entheses (A(d)). At P14, only a few tdTomato-positive cells were identified in migrating entheses (A(a',b',c)), whereas extensive staining was still seen in stationary entheses (A(d')). Enthesis is demarcated by a dashed line. Scale bars: 50 μ m. **(B)** The contribution of *Sox9* lineage to the DT and TM entheses was evaluated by crossing *Sox9-Cre* or *Sox9-CreER^{T2}* mice with *R26R-tdTomato* reporter mice. (Ba,h): The total contribution of the lineage was evaluated by examination of *Sox9-Cre;R26R-tdTomato* mice at P14. (Bb-g,i-n): The relative contribution of *Sox9* lineage cells specified at different time points was

516 evaluated by pulse-chase experiments on *Sox9-CreER^{T2};R26R-tdTomato* mice, in which a single dose of
517 tamoxifen was administered at various stages from E11.5 through E17.5.

518 **Figure 5. *Gli1* lineage cells replace *Sox9* lineage cells during entheses maturation.** (A,B) *Sox9-*
519 *CreER^{T2};R26R-tdTomato* and *Gli1-CreER^{T2};R26R-tdTomato* mice were labeled by tamoxifen
520 administration at E12.5 and E13.5, respectively and sacrificed at E15.5-P14. Transverse sections
521 through the DT (A) and TM (B) show an increase in *Sox9* lineage cells at E15.5-P0. Between P0 and
522 P14, a continuous decrease in *Sox9* lineage cells is seen. Concurrently, *Gli1* lineage cell number
523 gradually increased in both DT and TM entheses. By P8, both entheses were populated by *Gli1* lineage
524 cells. Enthesis is demarcated by a dashed line. Scale bars: 50 μ m. (C,D) Graphs showing the percentage
525 of *Gli1*-tdTomato and *Sox9*-tdTomato positive cells in P0, P4, P6, and P8 DT (C) and TM (D) cells.

526 **Figure 6. Cell fate of *Gli1*+ entheses progenitors is pre-determined.** Pulse-chase cell lineage
527 experiments using *Gli1-CreER^{T2};R26R-Confetti* mice, in which *Gli1*+ progenitor cell clones were
528 labeled. *Gli1*-positive cells were pulsed by a single tamoxifen administration at E13.5 and their
529 descendants were followed to P14. One hour prior to sacrifice, mice were injected with Calcein blue.
530 (A) Schematic illustration of possible Cre recombination outcomes. (B) Multiple clones were identified
531 in the DT entheses; however, the clones were restricted to either fibrocartilage or mineral fibrocartilage
532 and did not cross the border between the layers. The clones identified in the TM entheses were restricted
533 to the fibrous part and did not penetrate the bone. (C) Graph showing the percentage of crossing vs. non-
534 crossing clones. Scale bars: 50 μ m.

535 **Figure 7. Migrating entheses develop through template replacement.** Schematic illustration of the
536 developmental sequence of migratory and stationary entheses. During embryogenesis, *Sox9* lineage cells
537 form an entheses template. (A) In migrating entheses, *Sox9* lineage cells are replaced by a second
538 population of *Gli1* lineage cells, which eventually form the mature entheses. Specification of *Gli1*

539 lineage is regulated by SHH, whereas maintenance of this population is regulated by IHH. (B) In
 540 stationary entheses, embryonic *Sox9* lineage cells proliferate and differentiate and, eventually, populate
 541 the postnatal enthesis structure. During late embryonic development, some *Sox9* lineage cells start
 542 expressing *Gli1*.

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