

1   **Chondrocytes in the resting zone of the growth plate are maintained in a Wnt-inhibitory**  
2   **environment**

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15

16 **Abstract**

17

18 Chondrocytes in the resting zone of the postnatal growth plate are characterized by slow cell  
19 cycle progression, and encompass a population of parathyroid hormone-related protein (PTHrP)-  
20 expressing skeletal stem cells that contribute to the formation of columnar chondrocytes.  
21 However, how these chondrocytes are maintained in the resting zone remains undefined. We  
22 undertook a genetic pulse-chase approach to isolate slow cycling, label-retaining chondrocytes  
23 (LRCs) from the growth plate using a chondrocyte-specific doxycycline-controllable Tet-Off  
24 system regulating expression of histone 2B-linked GFP. Comparative RNA-seq analysis  
25 identified significant enrichment of inhibitors and activators for Wnt/β-catenin signaling in  
26 LRCs and non-LRCs, respectively. Activation of Wnt/β-catenin signaling in PTHrP<sup>+</sup> resting  
27 chondrocytes using *Pthrp-creER* and *Apc*-floxed allele impaired their ability to form columnar  
28 chondrocytes. Therefore, slow-cycling chondrocytes are maintained in a canonical Wnt-  
29 inhibitory environment within the resting zone, unraveling a novel mechanism regulating  
30 maintenance and differentiation of PTHrP<sup>+</sup> skeletal stem cells of the postnatal growth plate.

31 **Introduction**

32

33 The epiphyseal growth plate, a disk of cartilaginous tissues with characteristic columns of  
34 chondrocytes formed between the primary and secondary ossification centers, is an innovation of  
35 amniotes (reptiles, birds and mammals) that facilitates explosive endochondral bone growth  
36 (Wuellung & Vortkamp, 2019). The postnatal growth plate is composed of three morphologically  
37 distinct layers of resting, proliferating and hypertrophic zones, in which chondrocytes continue to  
38 proliferate well into adulthood, especially in mice, therefore functioning as the engine for  
39 endochondral bone growth (Hallett et al., 2019; Kronenberg, 2003). By adulthood, a large  
40 majority of hypertrophic chondrocytes undergo apoptosis or transdifferentiate into osteoblasts,  
41 marking the completion of the longitudinal growth phase and skeletal maturation (Roach et al.,  
42 1995; Yang et al., 2014; Zhou et al., 2014).

43

44 Of the three layers, the resting zone has two important functions in maintaining the growth  
45 plate. First, early studies postulated that resting chondrocytes feed their daughter cells into the  
46 adjacent proliferating zone and contribute to longitudinal growth of postnatal endochondral  
47 bones (Hunziker, 1994). More recently, the resting zone has been established as a niche for  
48 skeletal stem cells, initially demonstrated by surgical auto-transplantation experiments in rabbits  
49 (Abad et al., 2002), and subsequently by lineage-tracing experiments in mice (Mizuhashi et al.,  
50 2018; Newton et al., 2019). Second, chondrocytes in the resting zone express parathyroid  
51 hormone-related protein (PTHrP) that maintains proliferation of chondrocytes in a cell non-  
52 autonomous manner and delays their hypertrophic differentiation, thus sustaining longitudinal  
53 growth (E. et al., 1997). The proliferating zone is concertedly maintained by PTHrP released  
54 from the resting zone and Indian hedgehog (Ihh) synthesized by pre-hypertrophic chondrocytes;  
55 the proliferating zone in turn provides instructive cues to regulate cell fates of PTHrP<sup>+</sup>  
56 chondrocytes (Mizuhashi et al., 2018). Therefore, the resting zone functions as a critical  
57 constituent of the tight feedback system (the PTHrP–Ihh feedback loop) that maintains growth  
58 plate structures and longitudinal bone growth. However, mechanisms regulating self-renewal and  
59 differentiation capabilities of resting zone chondrocytes remain largely unknown.

60

61 In this study, we set out to undertake an unbiased approach to define the molecular  
62 mechanism regulating maintenance and differentiation of chondrocytes in the resting zone  
63 ('slow-cycling chondrocytes'). To achieve this goal, we developed a chondrocyte-specific  
64 genetic label-retention strategy to isolate slow-cycling chondrocytes from the postnatal growth  
65 plate. Our comparative transcriptomic analysis revealed unique molecular signatures defining the  
66 characteristics of slow-cycling chondrocytes, with particular enrichment for inhibitors of the  
67 canonical Wnt signaling pathway. Subsequent functional validation based on a cell-lineage  
68 analysis identified that, when Wnt/β-catenin signaling was activated, PTHrP<sup>+</sup> resting  
69 chondrocytes were decreased in number during initial formation and established columnar  
70 chondrocytes less effectively in the subsequent stages. These data lead to a new concept that  
71 PTHrP<sup>+</sup> skeletal stem cells may be maintained in a canonical Wnt inhibitory environment within  
72 the resting zone niche of the postnatal growth plate.

73 **Results**

74

75 **1.1. A genetic label-retention strategy to identify slow-cycling chondrocytes**

76

77 Chondrocytes in the resting zone of the postnatal growth plate ('resting' or 'reserve'  
78 chondrocytes) are characterized by their slow cell cycle progression that is much slower than that  
79 of chondrocytes in the proliferating zone. As a result, these slow-cycling chondrocytes retain  
80 nuclear labels much longer than their more rapidly dividing progeny in the proliferating zone,  
81 which are therefore termed as label-retaining chondrocytes (LRCs) (Walker & Kember, 1972).  
82 First, we undertook a genetic approach to fluorescently isolate LRCs from the growth plate based  
83 on a chondrocyte-specific pulse-chase protocol. To this end, we first generated transgenic mice  
84 expressing a tetracycline-controlled transactivator under a *Col2a1* promoter (hereafter, Col2a1-  
85 tTA), and combined this line with a *Col1a1* locus harboring a Tet-responsive element (TRE)-  
86 histone 2B-bound EGFP (H2B-EGFP) cassette (hereafter, TRE-H2B-EGFP) (Fig.1a, left panel).  
87 In this Tet-Off system, tTA binds to TRE in the absence of doxycycline and activates H2B-  
88 EGFP transcription (pulse), whereas tTA dissociates from TRE in the presence of doxycycline,  
89 shutting off H2B-EGFP transcription (chase) (Fig.S1a). In Col2a1-tTA; TRE-H2B-EGFP mice,  
90 *Col2a1*<sup>+</sup> chondrocytes accumulate H2B-EGFP in the nucleus without doxycycline, and upon  
91 initiation of doxycycline feeding, *de novo* transcription of *H2B-EGFP* mRNA becomes  
92 suppressed. After a long chase period, H2B-EGFP is preferentially diluted in highly proliferating  
93 cells and their progeny, whereas slow-cycling cells retain a high level of the label, marking them  
94 as LRCs.

95

96 In order to evaluate the labeling efficiency of the system, we first analyzed double  
97 heterozygous Col2a1-tTA/+; TRE-H2B-EGFP/+ mice at postnatal day (P) 7 and P21 without  
98 doxycycline. While most of chondrocytes in the growth plate were marked by a high level of  
99 H2B-EGFP at P7 (Fig.S1b), fewer than half of columnar chondrocytes in the growth plate were  
100 marked by H2B-EGFP at P21 (Fig.S1c), demonstrating the inefficiency of the Tet system in  
101 postnatal growth plate chondrocytes. To circumvent this problem, we further generated double  
102 homozygous Col2a1-tTA/Col2a1-tTA; TRE-H2B-EGFP/TRE-H2B-EGFP mice and analyzed  
103 these mice at P21 without doxycycline. A great fraction of columnar chondrocytes was marked

104 by H2B-EGFP (Fig.S1d), indicating that the labeling efficiency can be improved in a transgene  
105 dosage-dependent manner in this system.

106

107 Subsequently, we tested the effectiveness of this chondrocyte-specific Tet-Off system by  
108 pulse-chase experiments. We fed double heterozygous *Col2a1-tTA*/+; TRE-H2B-EGFP/+ mice  
109 with doxycycline for 5 weeks starting from P21 to shut off de novo H2B-EGFP expression. We  
110 started the chase at P21 because the secondary ossification center was fully developed within the  
111 epiphysis by this time. After the chase, the H2B-EGFP signal was largely abrogated in the  
112 growth plate region, with only a small fraction of cells in the resting zone near the top of the  
113 growth plate retaining H2B-EGFP (Fig.S1e, arrowheads) expression. However, we also noticed  
114 that a low level of H2B-EGFP signal persisted in adjacent osteoblasts and osteocytes in the  
115 epiphysis even after the chase (Fig.S1e, arrows), making it difficult to distinguish LRCs from  
116 these cells. Analysis of TRE-H2B-EGFP/+ mice without a *Col2a1-tTA* transgene at P28 revealed  
117 that osteoblasts and osteocytes expressed a low level of H2B-EGFP (Fig.S1f, arrows). These  
118 findings indicate that LRCs can be identified within the top of the growth plate by a  
119 chondrocyte-specific Tet-Off system regulating H2B-EGFP expression, although these cells  
120 cannot be easily distinguished from adjacent osteoblasts and osteocytes solely based on  
121 fluorescent intensity in histological sections.

122

## 123 **1.2. Col2-Q system: A double-color quadruple transgenic strategy to identify LRCs in the 124 growth plate**

125

126 To circumvent the technical issues hampering isolation of LRCs from the growth plate  
127 resting zone, we further included a *Col2a1-creER* transgene that activates an *R26R-tdTomato*  
128 reporter in a tamoxifen-dependent manner, as a means to specifically mark growth plate  
129 chondrocytes (M. Chen et al., 2007). We generated quadruple homozygous transgenic mice –  
130 “Col2-Q” mice: *Col2a1-tTA*; TRE-H2B-EGFP; *Col2a1-creER*; *R26R-tdTomato* (Fig.1a), and  
131 treated these mice with tamoxifen (4 mg) twice shortly before analysis (3 and 2 days before  
132 analysis, “short protocol”) to obtain *Col2a1-creER-tdTomato*<sup>+</sup> cells (hereafter, *Col2<sup>CE</sup>-tdT*<sup>+</sup>  
133 cells). After the pulse-chase protocol with doxycycline, LRCs are expected to be identified as  
134 cells with green nuclei and red cytoplasm, which are localized in the resting zone of the growth

135 plate (Fig.1b,c). First, we analyzed Col2-Q mice at P21 without doxycycline (“No Chase”). A  
136 great majority of cells in the epiphysis, including those in the growth plate and the secondary  
137 ossification center, but not on the articular surface, were H2B-EGFP<sup>high</sup> (Fig.1d, cells with green  
138 nuclei). This short protocol of tamoxifen injection marked a great number of chondrocytes in the  
139 growth plate, but not in the articular cartilage (Fig.1d), indicating that this double-color strategy  
140 can effectively identify H2B-EGFP<sup>high</sup> growth plate chondrocytes at this stage. Second, Col2-Q  
141 mice were fed with doxycycline from P21 to shut off new H2B-EGFP synthesis for 4 weeks  
142 (chase) and were then treated with the short protocol of tamoxifen injection. After the chase,  
143 LRCs were identified at a specific location near the top of the growth plate in the resting zone,  
144 retaining a higher level of H2B-EGFP signal (Fig.1e). In addition, most of these H2B-EGFP<sup>high</sup>  
145 cells in the growth plate were simultaneously marked as Col2<sup>CE</sup>-tdT<sup>+</sup> (Fig.1e, right panel,  
146 arrowhead), while more rapidly dividing and morphologically distinct columnar chondrocytes  
147 were not marked by H2B-EGFP signal. Therefore, our Col2-Q quadruple transgenic strategy can  
148 effectively mark LRCs primarily in the resting zone of the postnatal growth plate.

149

### 150 1.3. A flow cytometry-based identification and isolation of LRCs from Col2-Q mice

151

152 We next established a protocol to harvest chondrocytes from the postnatal growth plate. We  
153 manually removed epiphyses from four long bones (bilateral distal femurs and proximal tibias  
154 [Fig.1f, left panel, shown is a dissected epiphysis from a tibia]). With this protocol, the growth  
155 plate was sheared at the hypertrophic layer with the remainder attached to the epiphysis. We  
156 further digested dissected epiphyses serially with collagenase to release these cells into single-  
157 cell suspension. Five rounds of digestion completely liberated cells from the growth plate, while  
158 cells on the articular surface were almost undigested (Fig.1f, right panel). Subsequently, we used  
159 a flow cytometric approach to analyze single cells dissociated from the Col2-Q postnatal growth  
160 plate at sequential time points before and after the chase, particularly in a CD45-negative non-  
161 hematopoietic fraction. Col2a1<sup>CE</sup>-tdT<sup>+</sup> cells were clearly distinguishable from unlabeled cells at  
162 all time points investigated (Fig.1g). Before the chase started at P21 (therefore without  
163 doxycycline feeding), 86.4±5.0% of Col2a1<sup>CE</sup>-tdT<sup>+</sup> cells retained >10<sup>4</sup> units of H2B-EGFP  
164 (Fig.1g, leftmost panel). The fraction of a label-retaining population (GFP<sup>high</sup>, retaining >10<sup>4</sup> unit  
165 of H2B-EGFP signal) within a Col2<sup>CE</sup>-tdT<sup>+</sup> population gradually decreased as the chase period

166 extended (Fig.1g). These plots fit into a non-linear decay curve (Y0:  $86.5\pm1.3\%$ ; Plateau:  
167  $2.6\pm0.9\%$ ;  $T^{1/2}=0.99\sim1.18$  week) (Fig.1i). Virtually no GFP<sup>+</sup> cells were observed in the absence  
168 of a Col2a1-tTA transgene (Fig.1h, magenta line), while levels of GFP<sup>+</sup> cells were maintained  
169 from five to ten weeks of chase (Fig.1h, orange and teal lines). Therefore, these findings  
170 demonstrate that LRCs can be effectively identified and isolated from postnatal Col2-Q growth  
171 plates by combined microdissection, enzymatic digestion and flow cytometry-based approaches.  
172

## 173 **2. A comparative RNA-seq analysis reveals a unique molecular signature of LRCs**

174

175 Subsequently, we isolated slow-cycling chondrocytes using fluorescence-activated cell  
176 sorting (FACS) at a 4 week-chase time point, when the GFP<sup>high</sup> label-retaining fraction ( $>10^4$   
177 unit) was sufficiently enriched (Fig.1i). In this experiment, LRCs were defined as GFP<sup>high</sup> cells  
178 retaining H2B-EGFP signal at the top 10% brightness ( $x > 10^4$  unit), whereas non-LRCs were  
179 defined as other GFP<sup>mid-low</sup> cells ( $10^3 < x < 10^4$  unit) (Fig.2a). Cells were collected from multiple  
180 littermate mice for each of three independent experiments. To assess RNA quality, we conducted  
181 an RNA Integrity Number (RIN) assay (Schroeder et al., 2006) from total RNAs isolated from  
182 LRCs and non-LRCs. Cellular RNA levels from each population had sufficient quality for  
183 downstream application (RIN $>8.0$ , Fig.2b), which were further subjected to amplification and  
184 deep sequencing. An unsupervised clustering analysis demonstrated that LRCs and non-LRCs  
185 biological triplicate samples each clustered together (Fig.2c), indicating that slow-cycling  
186 chondrocytes in the postnatal growth plate possess a biologically unique pattern of  
187 transcriptomes compared to more rapidly diving non-LRCs. Analyses of differentially expressed  
188 genes (DEGs) revealed that 799 genes were differentially expressed between the two groups  
189 (fold change  $\geq \pm 2$ ), of which 427 and 372 genes were upregulated in LRCs and non-LRCs,  
190 respectively (Fig.2d). Representative genes upregulated in LRCs included known markers for  
191 resting chondrocytes, such as *Pthlh* (also known as *Pthrp*, x2.6) (X. Chen et al., 2006) and *Sfrp5*  
192 (x2.4); (Chau et al., 2014; Lui et al., 2010) in addition to novel markers, such as *Gas1* (x12),  
193 *Spon1* (x10) and *Wif1* (x3.8). Similarly, representative genes upregulated in non-LRCs included  
194 both known and novel markers for proliferating and pre-hypertrophic chondrocytes, such as *Ihh*  
195 (St-Jacques et al., 1999) (x54), *Col10a1* (Gu et al., 2014) (x11), *Mef2c* (Arnold et al., 2007)  
196 (x5.1), *Pthlr* (Hirai et al., 2011) (x3.0), *Sp7* (Nakashima et al., 2002) (x2.4) and *Dlx5* (Robledo

197 et al., 2002) (x2.2) as well as *Clec11a* (Yue et al., 2016) (x2.9) and *Cd200* (Etich et al., 2015)  
198 (x2.1). Therefore, these identified enriched genes support the precision and accuracy of  
199 comparative RNA-seq analysis of LRCs and non-LRCs isolated by cell sorting from the growth  
200 plate.

201

202 We further set out to validate the LRC markers using several independent approaches. First,  
203 to test if PTHrP<sup>+</sup> cells overlap with LRCs, we performed an EdU pulse-chase experiment by  
204 serially pulsing *PTHrP-LacZ* knock-in mice (X. Chen et al., 2006) (Fig.S2a). Shortly after the  
205 pulse at P7, PTHrP<sup>+</sup> cells were preferentially localized in an EdU-low zone, wherein 17.9±2.7%  
206 of EdU<sup>+</sup> cells were PTHrP<sup>+</sup> (Fig.S2a, left panel, and Fig.S2b). After 22 days of chase at P28, a  
207 great majority of EdU-retaining cells were PTHrP<sup>+</sup>, wherein 77.6±9.6% of EdU<sup>+</sup> cells were  
208 PTHrP<sup>+</sup> (Fig.2e, Fig.S2a, right panel, and Fig.S2b). Therefore, LRCs become increasingly  
209 enriched among PTHrP<sup>+</sup> chondrocytes in the postnatal growth plate. Second, we validated  
210 expression of a novel LRC marker, *Gas1*. Analysis of *Gas1-LacZ* knock-in mice (Martinelli &  
211 Fan, 2007) at P14 revealed that *Gas1*<sup>+</sup> cells were exclusively found at the top of the growth plate  
212 corresponding to the resting zone (Fig.2f). Therefore, *in vivo* expression patterns of two  
213 representative LRC markers – *PTHrP* and *Gas1* – using knock-in reporter lines further support  
214 the validity of the gene expression profile of LRCs that accurately reflects that of the resting  
215 zone of the growth plate.

216

217 Pathway analysis of DEGs revealed significant enrichment of four KEGG terms ( $p<0.05$ ,  
218 FDR), including *Oxidative phosphorylation* (KEGG:00190), *Wnt signaling pathway*  
219 (KEGG:04310), *Endocrine and other factor-regulated calcium reabsorption* (KEGG:04961) and  
220 *Pathways in cancer* (KEGG:05200) (Fig.2g). Notably, all DEGs annotated under the *Oxidative*  
221 *phosphorylation* KEGG term were upregulated in non-LRCs, highlighting a biochemically  
222 unique feature of non-LRCs undergoing active processes such as cell division and  
223 differentiation. Out of 21 DEGs annotated in *Wnt signaling pathway*, 16 genes were relevant to  
224 the canonical Wnt/β-catenin signaling pathway (Komiya & Habas, 2008) (Fig.2h). Interestingly,  
225 LRCs were enriched for genes encoding canonical Wnt inhibitors such as *Sfrp1*, *Sfrp5*, *Dkk2*,  
226 *Wif1*, *Notum* and *Fzd6*, as well as genes encoding Wnt receptors such as *Fzd1*, *Fzd3* and *Fzd8*.  
227 Conversely, non-LRCs were enriched for genes encoding canonical Wnt activators such as

228 *Rspo3*, *Rspo4*, *Wnt7b* and *Wnt10b* (Fig.2h). Therefore, these RNA-seq analyses demonstrate that  
229 LRCs reside in a microenvironment in which inhibitors for canonical Wnt signaling are  
230 abundantly present in the milieu (Fig.2i).

231

### 232 **3. Activation of canonical Wnt signaling impairs formation, expansion and differentiation 233 of PTHrP<sup>+</sup> resting chondrocytes**

234

235 We next set out to define how canonical Wnt signaling regulates slow-cycling chondrocytes  
236 of the postnatal growth plate. For this purpose, we activated Wnt/β-catenin signaling in PTHrP<sup>+</sup>  
237 resting chondrocytes by conditionally inducing haploinsufficiency of *adenomatous polyposis coli*  
238 (*Apc*), which is a critical component of the β-catenin degradation complex, using a *Pthrp-creER*  
239 (Mizuhashi et al., 2018) line and *Apc*-floxed allele (Cheung et al., 2010), and simultaneously  
240 traced the fates of these Wnt-activated PTHrP<sup>+</sup> cells using an *R26R-tdTomato* reporter allele  
241 (Fig.3a,b). Littermate triple transgenic mice with two corresponding genotypes – *PTHrP-creER*;  
242 *Apc*<sup>+/+</sup>; *R26R-tdTomato* (Control, PTHrP<sup>CE</sup>APC<sup>++</sup> cells) and *PTHrP-creER*; *Apc*<sup>fl/+</sup>; *R26R-tdTomato*  
243 (APC cHet, PTHrP<sup>CE</sup>Apc<sup>Het</sup> cells) mice – were pulsed with tamoxifen (250 µg) at P6 and  
244 analyzed at five consecutive time points after the chase, i.e. P9, P12, P21 P36 and P96 (Fig.3c).  
245 Immunohistochemical analysis revealed that the β-catenin protein was upregulated in the resting  
246 zone of APC cHet growth plates and PTHrP<sup>CE</sup>tdTomato<sup>+</sup> cells therein (Fig.3d, leftmost panels,  
247 arrows), indicating that *Apc* haploinsufficiency indeed slowed β-catenin degradation and  
248 activated canonical Wnt signaling specifically in the resting zone of the growth plate.

249

250 Subsequently, we quantified the numbers of lineage-marked tdTomato<sup>+</sup> cells in the resting  
251 zone, as well as short (composed of  $\leq$  tdTomato<sup>+</sup> 10 cells) and long (composed of  $>10$  tdTomato<sup>+</sup>  
252 cells) columns of tdTomato<sup>+</sup> chondrocytes based on serial sections of femur growth plates  
253 (Fig.3d, right panels). Consistent with our prior report (Mizuhashi et al., 2018), PTHrP<sup>CE</sup>APC<sup>++</sup>  
254 Control chondrocytes transiently increased in the resting zone during the first week of chase, and  
255 decreased thereafter due to the formation of columnar chondrocytes (P9: 718.7±132.7, P12:  
256 910.3±209.9, P21: 655.4±125.0, P36: 200.3±187.2; P96: 116.1±48.5 cells, Fig.3e, blue line,  
257  $n=3-4$  mice). In contrast, PTHrP<sup>CE</sup>APC<sup>Het</sup> resting chondrocytes did not increase in number  
258 during the initial stage of chase, the numbers of which were significantly lower than those of

259 Control at the initial three time points (P9:  $474.8 \pm 134.8$  [ $p=0.04$ ], P12:  $558.4 \pm 64.3$  [ $p=0.03$ ],  
260 P21:  $443.4 \pm 79.2$  [ $p=0.03$ ] cells, [Fig.3e, red line](#),  $n=4-5$  mice), and fell to levels that were similar  
261 to those in the Control at the latter two time points ([Fig.3d, rightmost panel](#), [Fig.3e](#)). These data  
262 indicate that the formation and the expansion of PTHrP<sup>+</sup> cells in the resting zone are impaired  
263 when canonical Wnt signaling is activated in these cells.

264

265 As expected, PTHrP<sup>CE</sup>APC<sup>++</sup> resting chondrocytes established short columns (fewer than 10  
266 cells/column) of tdTomato<sup>+</sup> chondrocytes across the growth plate that peaked at P21 (P12:  
267  $20.0 \pm 7.1$ , P21:  $67.4 \pm 10.1$ , P36:  $27.5 \pm 19.4$ , P96:  $44.3 \pm 11.1$  tdTomato<sup>+</sup> columns, [Fig.3f, blue line](#),  
268  $n=4$  mice). The number of tdTomato<sup>+</sup> short columns in APC cHet growth plates was reduced at  
269 P21 (P21:  $45.9 \pm 7.7$  tdTomato<sup>+</sup> columns, [Fig.3f, red line](#),  $n=4$  mice [ $p=0.03$ ]), indicating that the  
270 formation of short columnar chondrocytes in the proliferating zone is inhibited upon canonical  
271 Wnt signaling activation. We suspect that this result reflects the reduction of PTHrP-creER<sup>+</sup> cells  
272 in the resting zone in the preceding stages, though we cannot rule out direct effects of APC  
273 haploinsufficiency in the proliferating zone as well.

274

275 PTHrP<sup>+</sup> resting chondrocytes continue to generate long columns (more than 10 cells/column)  
276 of tdTomato<sup>+</sup> chondrocytes in the long term, the number of which gradually decreases until six  
277 months and reaches a plateau thereafter (Mizuhashi et al., 2018). Accordingly, PTHrP<sup>CE</sup>APC<sup>++</sup>  
278 cells generated gradually decreasing but still substantial numbers of tdTomato<sup>+</sup> long columns  
279 after 3 months of chase at P96 (P21:  $44.4 \pm 23.2$ , P36:  $36.1 \pm 34.1$ , P96:  $26.5 \pm 12.4$  tdTomato<sup>+</sup>  
280 columns, [Fig.3g, blue line](#),  $n=4$  mice). In contrast, the number of tdTomato<sup>+</sup> long columns in  
281 APC cHet growth plates was significantly decreased at P96 (P96:  $7.3 \pm 2.5$  tdTomato<sup>+</sup> columns,  
282 [Fig.3g, red line](#),  $n=4$  mice [ $p=0.03$ ]). Therefore, the ability for PTHrP<sup>+</sup> resting chondrocytes to  
283 clonally establish columnar chondrocytes is impaired in response to activation of canonical Wnt  
284 signaling in the resting zone.

285

286 Taken together, these findings indicate that activation of canonical Wnt signaling impairs  
287 formation, expansion and differentiation of PTHrP<sup>+</sup> chondrocytes in the resting zone ([Fig.3h](#)).  
288 Thus, PTHrP<sup>+</sup> resting chondrocytes are required to be maintained in a Wnt-inhibitory  
289 environment to maintain themselves and their column-forming capabilities.

290 **Discussion**

291

292 In this study, we investigated the molecular mechanisms regulating the maintenance and the  
293 differentiation of slow-cycling chondrocytes localized in the resting zone of the postnatal growth  
294 plate. To date, our understanding of the molecular regulators of this special subclass of  
295 chondrocytes is largely grounded in histological and immunohistochemical observations and  
296 extrapolations from conditional gene ablation studies (Hallett et al., 2019). To address this gap in  
297 knowledge, we established a quadruple transgenic murine reporter model, “Col2-Q” system, to  
298 genetically label slow-cycling chondrocytes in an unbiased manner using a pulse-chase approach  
299 based on a chondrocyte-specific doxycycline-controllable Tet-Off system regulating expression  
300 of histone 2B-linked GFP. We successfully isolated label-retaining chondrocytes (LRCs) and  
301 their proliferating counterparts (non-LRCs) to profile the transcriptome of these cells. As the  
302 resting zone of the growth plate is considered to represent a resident stem-cell niche (Abad et al.,  
303 2002; Mizuhashi et al., 2018; Newton et al., 2019), our experiments serve as an approach to  
304 interrogate the fundamental characteristics of one of the stem-like cells residing in the postnatal  
305 growth plate.

306

307 It is unclear how slow-cycling chondrocytes in the resting zone maintain low mitotic  
308 capabilities while differentiating into columnar chondrocytes in the proliferating zone. Using a  
309 comparative bulk RNA-seq transcriptomic analysis, we discovered that LRCs are enriched for a  
310 unique set of genes associated with hallmark (e.g. *Pthrp* and *Sfrp5*) and novel (e.g. *Gas1*, *Spon1*  
311 and *Wif1*) markers for resting chondrocytes, in addition to Wnt inhibitory molecules (e.g. *Sfrp1*,  
312 *Dkk2*, *Notum* and *Fzd6*). Conversely, non-LRCs were enriched for markers of pre-hypertrophic  
313 (e.g. *Ihh*) and hypertrophic (e.g. *Col10a1*) chondrocytes, and represent differentially expressed  
314 genes commonly associated with metabolically active cellular processes, such as oxidative  
315 phosphorylation. We further validated the expression of *Pthlh*, which is a hallmark marker for  
316 resting chondrocytes, and *Gas1*, a novel marker, using *PTHrP-LacZ* and *Gas1-LacZ* knock-in  
317 reporter alleles, respectively. We found that PTHrP<sup>+</sup> chondrocytes in the resting zone maintain  
318 low levels of mitotic activity, indicated by EdU labeling and pulse-chase experiments. Thus, the  
319 genes identified by our comparative transcriptomic analysis appear to represent accurate  
320 transcriptomic features of distinct populations of slow-cycling versus differentiating

321 chondrocytes in the postnatal growth plate. Future investigations aimed at assessing the roles of  
322 novel marker genes may lead to the identification of novel skeletal stem cell populations that are  
323 important for the postnatal growth plate.

324

325 Wnt/β-catenin signaling is essential for endochondral ossification (Regard et al., 2012), and  
326 is shown to regulate initiation of chondrocyte hypertrophy by inhibiting PTHrP signaling  
327 activities (Guo et al., 2009). Moreover, Wnt/β-catenin signaling is essential during skeletal  
328 development for regulating mesenchymal progenitor differentiation in favor of osteoblasts (Day  
329 et al., 2005), or for preventing transdifferentiation of osteoblast precursors into chondrocytes  
330 (Hill et al., 2005). However, no previous report ties Wnt signaling to the maintenance of putative  
331 skeletal stem cell populations in the resting zone of the growth plate. In order to determine the  
332 functional contribution of Wnt signaling to PTHrP<sup>+</sup> resting chondrocyte skeletal stem cells and  
333 their differentiation, one copy of *adenomatous polyposis coli* (*Apc*), a critical signaling  
334 component of the β-catenin degradation complex, was selectively ablated using a resting  
335 chondrocyte-specific *Pthrp-creER* line. In the resting zone, *Apc* haploinsufficiency led to  
336 increased β-catenin protein expression specifically in the resting zone including in PTHrP<sup>+</sup>  
337 chondrocytes, decreased formation and expansion of PTHrP<sup>+</sup> chondrocytes, and reduced  
338 differentiation capabilities of these cells into columnar chondrocytes in the proliferating zone.  
339 Therefore, canonical Wnt signaling plays an important role in modulating PTHrP<sup>+</sup> chondrocytes  
340 in the resting zone and regulating their differentiation.

341

342 Taken together, our data support a novel paradigm that slow-cycling PTHrP<sup>+</sup> chondrocytes  
343 are maintained in a canonical Wnt-inhibitory environment within the resting zone of the growth  
344 plate, and that this relationship is critical to regulating the formation, the expansion and the  
345 differentiation of chondrocytes of the resting zone.

346

347 **Materials and Methods**

348

349 **Generation of *Col2a1-tTA* transgenic mice.**

350 *Col2a1-tTA* transgenic mice were generated by pronuclear injection of a NotI-digested 8.4kb  
351 gene construct containing a 3kb mouse *Col2a1* promoter and a 3kb fragment of intron 1 ligated  
352 to a splice acceptor sequence followed by an internal ribosome-entry site (IRES) (Ovchinnikov  
353 et al., 2000), tetracycline-controlled transactivator (tTA) and the SV40 large T antigen  
354 polyadenylation signal (Takara Bio, Mountain View, CA), into B6SJLF1 fertilized eggs. The G0  
355 founder mice were backcrossed with C57/BL6 mice at least for three generations. Of the two  
356 lines established, the high expresser line (Line H) was used for subsequent studies. The insertion  
357 site of the *Col2a1-tTA* transgene was determined based on the Genome Walker Universal system  
358 (Takara Bio). The *Col2a1-tTA* transgene was inserted 16kbp downstream of *Pellino2* on  
359 Chromosome 14. *Col2a1-tTA* mice were genotyped using PCR primers discriminating  
360 heterozygosity and homozygosity of the transgene (85: SV40pA\_End\_Fw:  
361 ACGGGAAGTATCAGCTCGAC, 86: Mm14\_5WT\_Fw: TTGAGAGTCTCCCAGGCAAT, 87:  
362 Mm14\_3WT\_Rv: CTCCTGATCTCCTGGCAAAG, ~600bp for wild-type, ~300bp for *Col2a1-*  
363 tTA allele).

364

365 **Mice.**

366 *TRE-H2B-EGFP* (Foudi et al., 2009) knock-in, *Col2a1-creER* transgenic (Nakamura et al.,  
367 *PThrP-LacZ/null* knock-in (X. Chen et al., 2006), *Gas1-LacZ/null* knock-in (Martinelli &  
368 Fan, 2007), *PThrP-creER* transgenic (Mizuhashi et al., 2018) mice have been described  
369 elsewhere. *Rosa26-CAG-loxP-stop-loxP-tdTomato* (Ai14: *R26R-tdTomato*, JAX007914), *Apc-*  
370 floxed (JAX009045) mice (Cheung et al., 2010) were acquired from the Jackson Laboratory. All  
371 procedures were conducted in compliance with the Guidelines for the Care and Use of  
372 Laboratory Animals approved by the University Michigan's Institutional Animal Care and Use  
373 Committee (IACUC), protocol 7681 and 9496. All mice were housed in a specific pathogen-free  
374 condition, and analyzed in a mixed background. Mice were identified by micro-tattooing or ear  
375 tags. Tail biopsies of mice were lysed by a HotShot protocol (incubating the tail sample at 95°C  
376 for 30 min in an alkaline lysis reagent followed by neutralization) and used for GoTaq Green  
377 Master Mix PCR-based genotyping (Promega, and Nexus X2, Madison, WI). Mice were

378 euthanized by over-dosage of carbon dioxide or decapitation under inhalation anesthesia in a  
379 drop jar (Fluriso, Isoflurane USP, VetOne, Boise, ID).

380

381 **Doxycycline.**

382 Mice were weaned at postnatal day (P) 21 and fed with a standard diet containing 2mg/g  
383 doxycycline (Bio-Serv F3893, Flemington, NJ) for up to 9 weeks.

384

385 **Tamoxifen.**

386 Tamoxifen (Sigma T5648, St. Louis, MO) was mixed with 100% ethanol until completely  
387 dissolved. Subsequently, a proper volume of sunflower seed oil (Sigma S5007) was added to the  
388 tamoxifen-ethanol mixture and rigorously mixed. The tamoxifen-ethanol-oil mixture was  
389 incubated at 60°C in a chemical hood until the ethanol evaporated completely. The tamoxifen-oil  
390 mixture was stored at room temperature until use. Mice with 21 days of age or older received  
391 two doses of 2mg of tamoxifen intraperitoneally at 3 and 2 days prior to analysis, or mice with 6  
392 days of age received a single dose of 0.25mg tamoxifen intraperitoneally for lineage-tracing  
393 analysis.

394

395 **Cell proliferation and EdU label-retention assay.**

396 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen A10044, Carlsbad, CA) dissolved in PBS was  
397 administered to mice at indicated postnatal days. Click-iT Imaging Kit with Alexa Flour 488-  
398 azide (Invitrogen C10337) was used to detect EdU in cryosections. For EdU label-retention  
399 assay, *PThrP-LacZ* mice received serial doses of EdU (50µg each) between P4 and P6, and  
400 chased for 3 weeks.

401

402 **X-Gal staining of dissected femur epiphyses.**

403 Distal epiphyses of femurs were manually dislodged, and attached soft tissues were carefully  
404 removed to ensure the maximum penetration of the substrate. Dissected epiphyses were fixed in  
405 2% paraformaldehyde for 30 min. at 4C°, followed by overnight X-gal staining at 37°C. Stained  
406 samples were further postfixed in 4% paraformaldehyde, overnight at 4°C, then decalcified in  
407 15% EDTA for 7 days. Decalcified samples were cryoprotected in 30% sucrose/PBS followed by  
408 30% sucrose/PBS:OCT (1:1) solution, each overnight at 4°C.

409

410 **Histology.**

411 Bilateral femurs were dissected under a stereomicroscope (Nikon SMZ-800, Minato City,  
412 Japan) to remove soft tissues, and fixed in 4% paraformaldehyde for a proper period, typically  
413 ranging from 3 hours to overnight at 4°C, then decalcified in 15% EDTA for a proper period,  
414 typically ranging from 0 hours to 14 days. Decalcified samples were cryoprotected in 30%  
415 sucrose/PBS solutions and then in 30% sucrose/PBS:OCT (1:1) solutions, each at least overnight  
416 at 4°C. Samples were embedded in an OCT compound (Tissue-Tek, Sakura, Torrance, CA)  
417 under a stereomicroscope and transferred on a sheet of dry ice to solidify the compound.  
418 Embedded samples were cryosectioned at 14–50µm using a cryostat (Leica CM1850, Wetzlar,  
419 Germany) and adhered to positively charged glass slides (Fisherbrand ColorFrost Plus).  
420 Cryosections were stored at –20°C (quantification) or –80°C (immunofluorescence) in freezers  
421 until use. Sections were postfixed in 4% paraformaldehyde for 15 min at room temperature. For  
422 functional conditional knockout experiments, 50µm serial sections were collected through the  
423 entire growth plate. For immunofluorescence experiments, epiphyses were popped out of  
424 bilateral femurs, processed for 24 hours in 4% paraformaldehyde and sectioned at 14µm.  
425 Sections were incubated with anti-β-catenin primary antibody (Abcam ab16051, Cambridge,  
426 UK) overnight at 4°C and further stained with 1:200 Alexa Fluor 633 Goat anti-Rabbit IgG  
427 (H+L) Secondary Antibody (Invitrogen A21071) at a 20°C for 3 hours. Sections were further  
428 incubated with DAPI (4',6-diamidino-2-phenylindole, 5µg/ml, Invitrogen D1306) to stain nuclei  
429 prior to imaging. For EdU assay, sections were incubated with Alexa Fluor 488-azide (Invitrogen  
430 A10266) for 30 min at 43°C using Click-iT Imaging Kit (Invitrogen C10337). Sections were  
431 further incubated with DAPI to stain nuclei prior to imaging. Stained samples were mounted in  
432 TBS with No.1.5 coverslips (Fisher, Waltham, MA).

433

434 **Imaging and cell quantification.**

435 Images were captured by a fluorescence microscope (Nikon Eclipse E800) with prefigured  
436 triple-band filter settings for DAPI/FITC/TRITC, and merged with Spot Advanced Software  
437 (Spot Imaging, Sterling Heights, MI), or an automated inverted fluorescence microscope with a  
438 structured illumination system (Zeiss Axio Observer Z1 with ApoTome.2 system) and Zen 2  
439 (blue edition) software. Confocal images were acquired using LSM510 and Zen2009 software

440 (Zeiss, Oberkochen, Germany) with lasers and corresponding band-pass filters for DAPI  
441 (Ex.405nm, BP420-480), GFP (Ex.488nm, BP505-530) and tdTomato (Ex.543nm, BP565-595).  
442 LSM Image Viewer and Adobe Photoshop software were used to capture and align images. Cells  
443 were counted by two individuals using single blinded methods to ensure unbiased data  
444 interpretation.

445

#### 446 **Growth plate cell preparation.**

447 Distal epiphyses of femurs and proximal epiphyses of tibias were manually dislodged using  
448 dull scissors, and attached soft tissues and woven bones were carefully removed using a cuticle  
449 nipper. Cells were dissociated from dissected epiphyses using five serial rounds of collagenase  
450 digestion, incubating with 2 Wunsch units of Liberase TM (Roche, Basel, Switzerland) in 2ml  
451 Ca<sup>2+</sup>, Mg<sup>2+</sup>-free Hank's Balanced Salt Solution (HBSS, Sigma H6648) at 37°C for 30 min. each  
452 time on a shaking incubator (ThermomixerR, Eppendorf, Hamburg, Germany). Single cell  
453 suspension was generated using an 18-gauge needle and a 1ml Luer-Lok syringe (BD), and  
454 filtered through a 70µm cell strainer (BD) into a 50ml tube on ice.

455

#### 456 **Flow cytometry.**

457 Dissociated cells were stained by standard protocols with allophycocyanin (APC)-conjugated  
458 anti-mouse CD45 (30F-11) antibodies (1:500, eBioscience, San Diego, CA). Flow cytometry  
459 analysis was performed using a four-laser BD LSR II (Ex. 355/407/488/633 nm) and FACSDiva  
460 software. Acquired raw data were further analyzed on FlowJo software (TreeStar).  
461 Representative plots of at least three independent biological samples are shown in the figures.

462

#### 463 **Fluorescence-activated cell sorting (FACS) and RNA isolation.**

464 Cell sorting was performed using a five-laser BD FACS Aria II  
465 (Ex.355/407/488/532/633nm) and FACSDiva. CD45<sup>neg</sup>GFP<sup>high</sup> cells at the top 10 percentile of  
466 GFP brightness (LRCs) and CD45<sup>neg</sup>GFP<sup>mid-low</sup> cells with 10~70 percentile of GFP brightness  
467 (non-LRCs) were directly sorted into TRIzol LS Reagent (ThermoFisher 10296010, Waltham,  
468 MA). Total RNA was isolated using NucleoSpin RNA XS (Macherey-Nagel, 740902). RNA  
469 Integrity Number (RIN) was assessed by Agilent 2100 Bioanalyzer RNA 6000 Pico Kit. Samples  
470 with RIN>8.0 were used for subsequent analyses.

471

472 **RNA amplification and deep sequencing.**

473 Complementary DNAs were prepared by SMART-Seq v4 Ultra Low Input RNA Kit for  
474 Sequencing (Takara 634888) using 150~800pg of total RNA. Post-amplification quality control  
475 was performed by Agilent TapeStation DNA High Sensitivity D1000 Screen Tape system. DNA  
476 libraries were prepared by Nextera XT DNA Library Preparation Kit (Illumina) and submitted  
477 for deep sequencing (Illumina HiSeq 2500).

478

479 **RNA-seq analysis.**

480 cDNA libraries were sequenced using following conditions; six samples per lane, 50 cycle  
481 single end read. Reads files were downloaded and concatenated into a single .fastq file for each  
482 sample. The quality of the raw reads data for each sample was checked using FastQC to identify  
483 quality problems. Tuxedo Suite software package was subsequently used for alignment (using  
484 TopHat and Bowtie2), differential expression analysis, and post-analysis diagnostics. FastQC  
485 was used for a second round of quality control (post-alignment). HTSeq/DESeq2 was run using  
486 UCSC mm10.fa as the reference genome sequence. Expression quantitation was performed with  
487 HTSeq, to count non-ambiguously mapped reads only. HTSeq counts per gene were then used in  
488 a custom DESeq2 paired analysis. Normalization and differential expression were performed  
489 with DESeq2, using a negative binomial generalized linear model, including a term for mouse of  
490 origin for a paired analysis. Plots were generated using variations or alternative representations  
491 of native DESeq2 plotting functions, ggplot2, plotly, and other packages within the R  
492 environment. Heatmaps were generated with updated rlog normalized count values for each  
493 sample for all plus top sets (500) of differentially expressed genes with the gplots package (v  
494 3.0.1). Two types of clustering were used: 1) averaging across rows with Pearson correlation  
495 distance with average linkage and 2) Ward's squared dissimilarity criterion. Top differentially  
496 expressed genes were determined after ranking genes by standard deviation across all samples.  
497 Independent of iPathway, GO term enrichment was performed on DE results, with a logFC  
498 threshold of 2 and adjusted *p*-value < 0.05 with the GOseq package (v 1.36) with probability  
499 weighting function and GO enrichment specified with mm10 as genome and gene symbol  
500 specified as gene ID format. Results were plotted for the top ten of selected terms related to the  
501 Wnt pathways, ranked by overrepresented *p*-value using ggplot2 (v 3.2.1). KEGG results with

502 FDR correction and gene tables for Wnt signaling pathway were downloaded from iPathway  
503 (report ID: 41865). KEGG gene tables for each pathway were used to subset the DE results  
504 before restricting results to genes for which both log fold change and adjusted *p*-value statistics  
505 were available.

506

### 507 **Replicates.**

508 All experiments were performed in biological replicates. For all data presented in the  
509 manuscript, we examined at least three independent biological samples (three different mice) to  
510 ensure the reproducibility. Biological replicates were defined as multiple experimental samples  
511 sharing common genotypes and genetic backgrounds. For each series of the experiments, all  
512 attempts at biological replication were successful. Technical replicates were generated from a  
513 single experimental sample. For example, serial sections of the femur growth plate from a single  
514 mouse were considered technical replicates. Outliers were uncommon in our datasets and did not  
515 impact the trend and the significance of our quantitated results. As a result, all quantitative data  
516 were included to ensure transparency in our data interpretation.

517

### 518 **Statistical analysis.**

519 Results are presented as mean values  $\pm$  S.D. Statistical evaluation was conducted based on  
520 Mann-Whitney's *U*-test. A *p* value  $<0.05$  was considered significant. No statistical method was  
521 used to predetermine sample size. Sample size was determined on the basis of previous literature  
522 and our previous experience to give sufficient standard deviations of the mean so as not to miss a  
523 biologically important difference between groups. The experiments were not randomized. All of  
524 the available mice of the desired genotypes were used for experiments. The investigators were  
525 not blinded during experiments and outcome assessment. One femur from each mouse was  
526 arbitrarily chosen for histological analysis. Genotypes were not particularly highlighted during  
527 quantification.

528

### 529 **Data availability**

530 The bulk RNA-seq datasets presented herein have been deposited in the National Center for  
531 Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO), and are accessible  
532 through GEO Series accession numbers GSE160364

533 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160364>]. The source data underlying  
534 all Figures and Supplementary Figures are provided as a Source Data file. All the raw images  
535 and flow cytometry files supporting the conclusion of this study will be deposited in Dryad  
536 Digital Repository during the revision.

537

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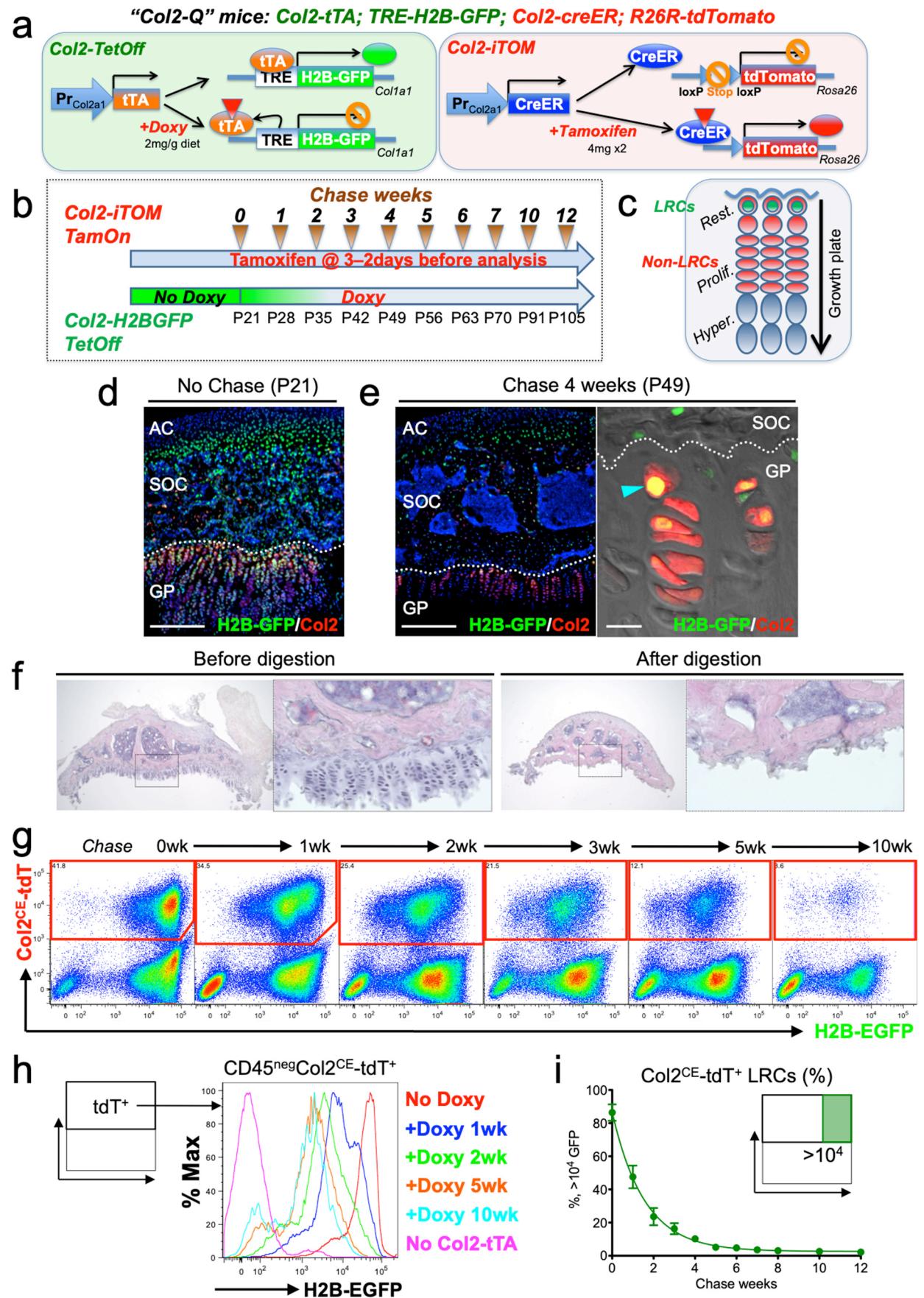
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663

# Figure 1



**Figure 1. A double-color genetic label-retaining strategy to identify and isolate slow-cycling chondrocytes of the growth plate.**

**(a)** “Col2-Q” quadruple transgenic system composed of two chondrocyte-specific bigenic Col2-Tet-Off (*Col2a1-tTA*; *TRE-H2B-EGFP*) and Col2-iTOM (*Col2a1-creER*; *R26R-tdTomato*) systems. H2B-EGFP expression can be shut off by doxycycline diet (2mg/g diet), while tdTomato expression can be induced by two doses of tamoxifen (4mg) administered shortly prior to analysis (3 and 2 days before).

**(b)** Experimental design to identify label-retaining chondrocytes (LRCs) in the growth plate. Col2-Q mice are fed with doxycycline (Doxy) starting from postnatal day (P) 21 (Chase). The mice are analyzed after the indicated number of weeks; at each time point, two doses of tamoxifen are administered shortly before analysis to induce tdTomato expression.

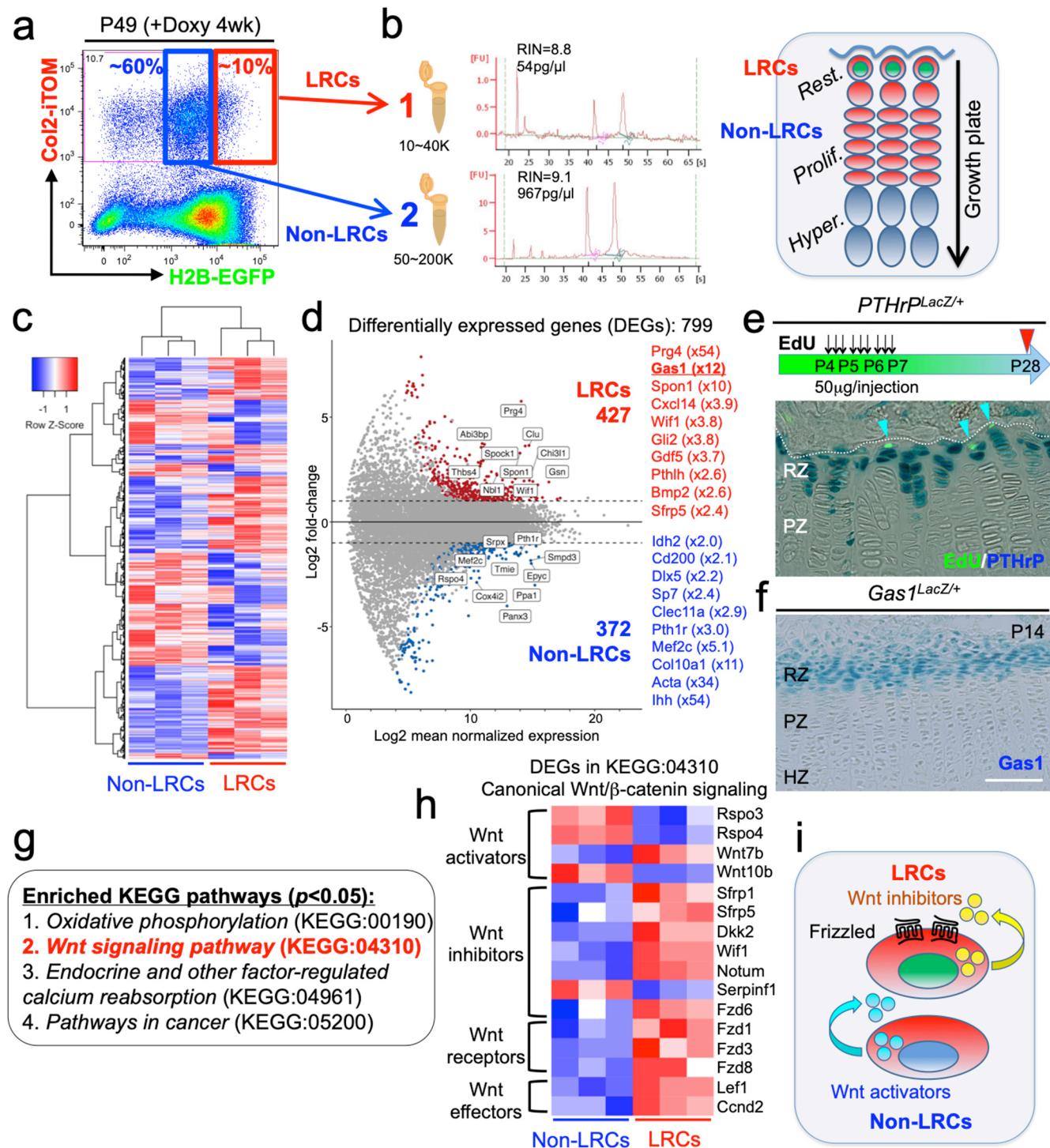
**(c)** Diagram for predicted outcomes. LRCs are expected to retain green nuclei with red cytoplasm, and located at the resting zone. Rest.: resting zone, Prolif.: proliferating zone, Hyper.: hypertrophic zone.

**(d,e)** Col2-Q distal femur growth plates with tamoxifen injection shortly before analysis. (d): No chase, without Doxy at P21. (e): After 4 weeks of chase, on Doxy for 4 weeks at P49, right panel: high-power confocal image. Arrowhead: label-retaining chondrocytes. AC: articular cartilage, SOC: secondary ossification center, GP: growth plate. Dotted line: border between growth plate and secondary ossification center. Blue: DAPI, grey: DIC. Scale bars: 500 $\mu$ m, 20 $\mu$ m (confocal in e).  $n=3$  mice at each time point.

**(f)** Epiphysis of proximal tibia, before and after serial collagenase digestions. Right panels: magnified views of the dotted areas showing growth plate.  $n=3$  mice at each step.

**(g-i)** Flow cytometry analysis of dissociated Col2-Q growth plate cells. (g): Pseudo-color plots of CD45<sup>neg</sup> cells at the indicated number of weeks in chase. Red gates: *Col2a1-creER/tdTomato*<sup>+</sup> (*Col2<sup>CE</sup>-tdT*<sup>+</sup>) cells. (h): Histogram of CD45<sup>neg</sup>*Col2<sup>CE</sup>-tdT*<sup>+</sup> cells showing the distribution of H2B-EGFP<sup>+</sup> cells as the percentage of the maximum count. Red line: P21 (No Doxy), blue line: P28 (+Doxy 1wk), green line: P35 (+Doxy 2wk), orange line: P56 (+Doxy 5wk), light blue line: P91 (+Doxy 10wk), pink line: No *Col2-tTA* control at P21. (i): Percentage of  $>10^4$  H2B-EGFP<sup>+</sup> LRCs among total *Col2<sup>CE</sup>-tdT*<sup>+</sup> cells.  $x$  axis: weeks in chase,  $y$  axis: % of cells  $> 10^4$  unit of GFP.  $n=9$  mice (0 week, 1 week),  $n=7$  mice (2 weeks, 5 weeks),  $n=6$  mice (3 weeks, 4 weeks),  $n=5$  mice (6 weeks) and  $n=3$  mice (7 weeks, 8 weeks, 10 weeks, 12 weeks). Data are presented as mean  $\pm$  s.d.

## Figure 2



**Figure 2. The unique molecular signature of label-retaining chondrocytes (LRCs) in the growth plate.**

**(a)** Cell sorting strategy to isolate LRCs (1: red box) and non-LRCs (2: blue box) after the chase, at P49 (+Doxo 4wk).

**(b)** RNA integrity number (RIN) enumerated from bioanalyzer traces (28S/18S) of LRCs (top) and non-LRCs (bottom). Cartoon representation of  $GFP^+$ / $tdTomato^+$  LRCs populating resting zone and  $GFP^-$ / $tdTomato^+$  non-LRCs populating proliferating zone of growth plate (right).

**(c)** Heatmap of top 500 differentially expressed genes (DEGs) with hierarchical clustering, between isolated non-LRCs and LRCs.  $n=3$  biological replicates (i.e. three independent littermates of mice).

**(d)** MA plot (Log2 fold change) of differentially expressed genes (DEGs) between isolated non-LRCs (372 total) and LRCs (427 total) with representative upregulated genes in each cell population.

**(e)**  $PTHrP^{LacZ^+}$  distal femur growth plates with EdU administration, serially pulsed 9 times between P4 and P6 and analyzed after 22 days of chase at P28. Arrowheads: EdU label-retaining  $LacZ^+$  cells. RZ: resting zone, PZ: proliferating zone.  $n=6$  mice.

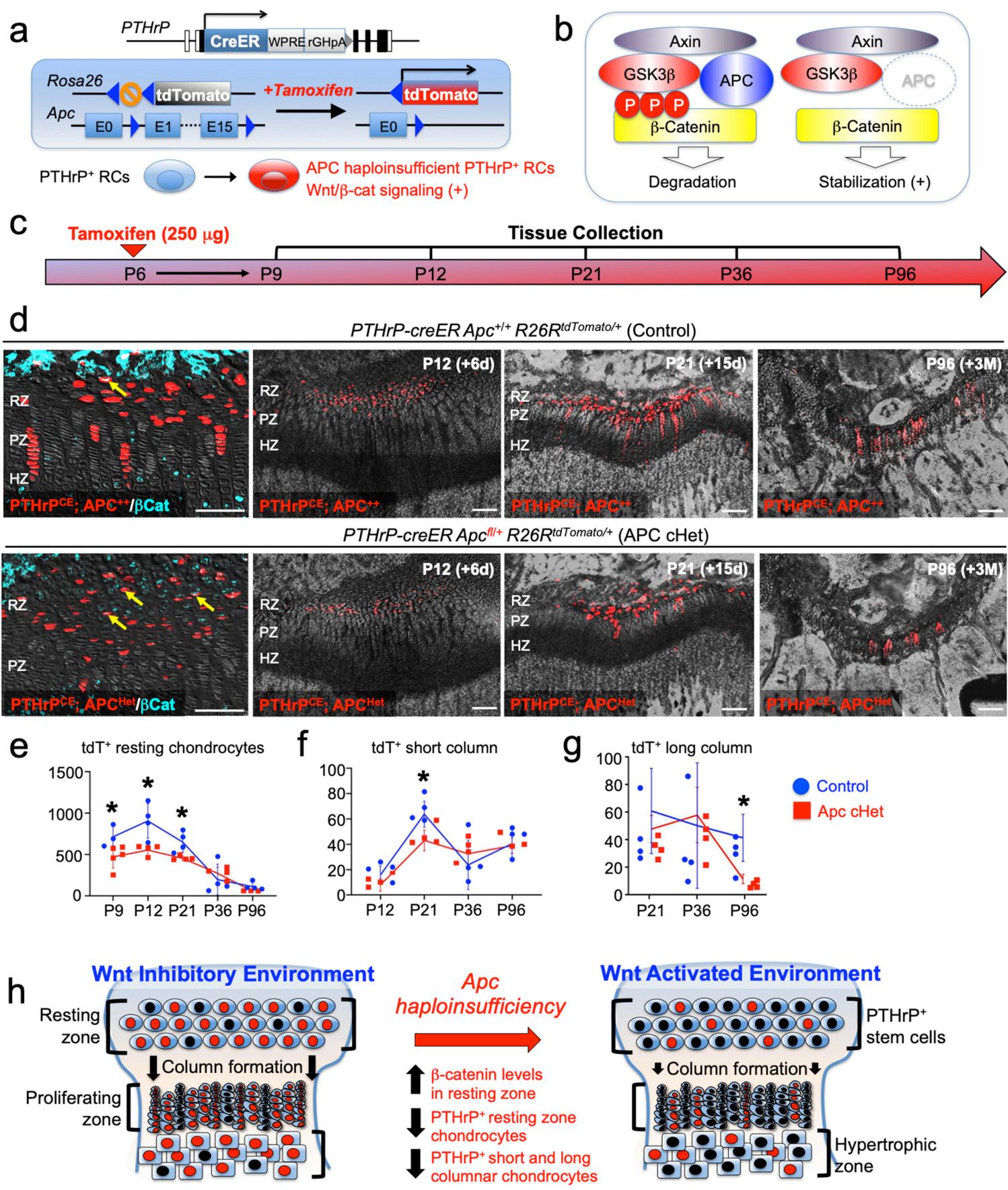
**(f)**  $Gas1^{LacZ^+}$  distal femur growth plates at P14. RZ: resting zone, PZ: proliferating zone, HZ: hypertrophic zone. Scale bar: 100 $\mu$ m.  $n=2$  mice.

**(g)** Enriched KEGG pathway terms ( $p<0.05$ ) based on 799 differentially expressed genes (DEGs).

**(h)** Heatmap of differentially expressed genes (DEGs) related to KEGG:04310 (canonical Wnt/ $\beta$ -Catenin signaling pathway). The DEGs were further classified by their functions in Wnt/ $\beta$ -Catenin signaling (e.g. Wnt activators, Wnt inhibitors, Wnt receptors and Wnt effectors).  $n=3$  biological replicates (i.e. three independent littermates of mice).

**(i)** Schematic diagram of Wnt activation and inhibition in non-LRCs and LRCs, respectively.

# Figure 3



**Figure 3. Activation of canonical Wnt/β-catenin signaling causes failure of formation and differentiation of PTHrP<sup>+</sup> chondrocytes.**

**(a)** *PTHrP-creER; R26R<sup>tdTomato</sup>* lineage-tracing model crossed with an *adenomatous polyposis coli (Apc)* floxed allele (flanking exons 1 and 15). Single intraperitoneal injection of tamoxifen (0.25 mg) at P6 induces *cre* recombination, leading to activation of canonical Wnt/β-catenin signaling in PTHrP<sup>+</sup> chondrocytes via *Apc* haploinsufficiency (*PTHrP-creER; APC<sup>f/+</sup>; R26R-tdTomato*).

**(b)** Schematic diagram of β-catenin degradation complex. Phosphorylation of β-catenin protein leads to degradation (left). *Apc* haploinsufficiency leads to β-catenin stabilization by impairing the degradation complex (right).

**(c)** Timeline for pulse-chase experiment. Tamoxifen injection (0.25 mg) at P6 and chase to P9, P12, P21, P36 and P96.

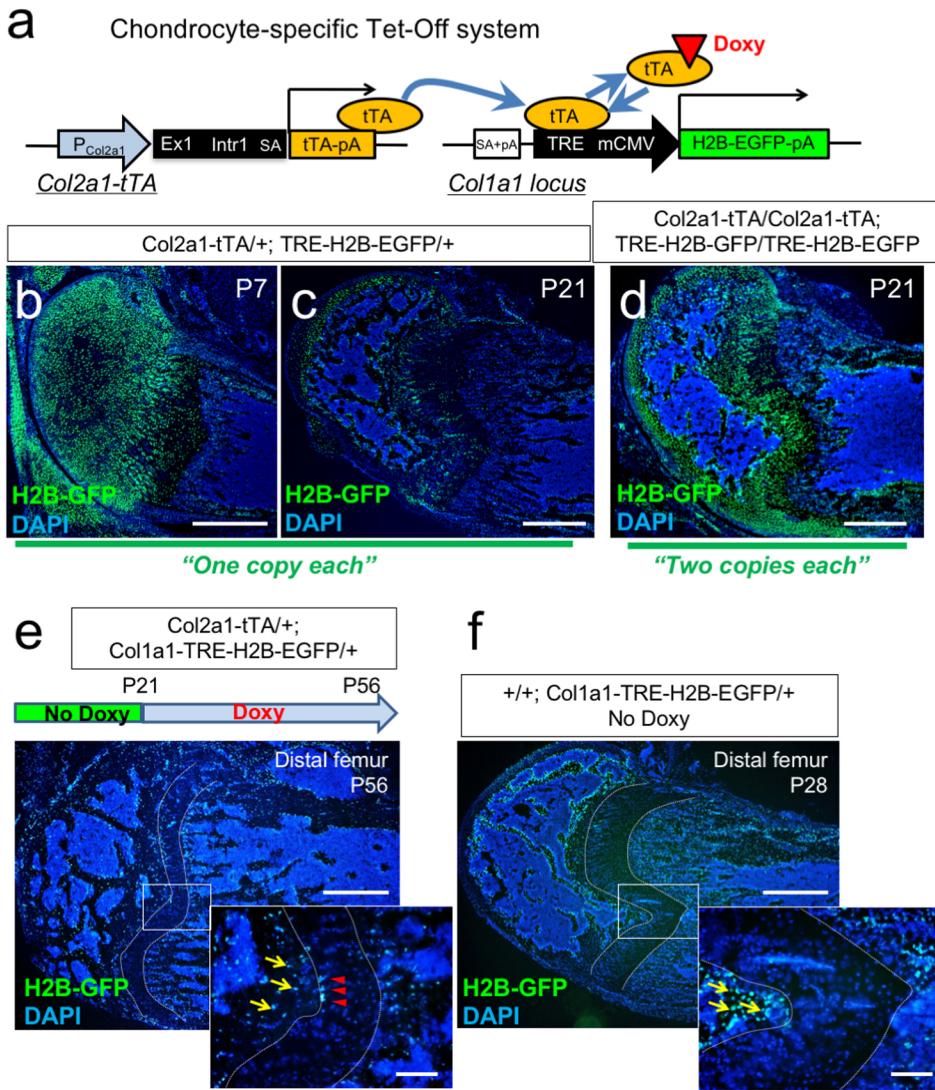
**(d)** (Leftmost panel): β-catenin staining in *PTHrP-creER; Apc<sup>+/+</sup>; R26R<sup>tdTomato</sup>* (Control) and *PTHrP-creER; Apc<sup>f/+</sup>; R26R<sup>tdTomato</sup>* (APC cHet) distal femur growth plates at P15. Arrows: β-catenin<sup>+</sup>tdTomato<sup>+</sup> cells in RZ. (2<sup>nd</sup>-4<sup>th</sup> panels): Distal femur growth plates of *PTHrP-creER; Apc<sup>+/+</sup>; R26R<sup>tdTomato</sup>* (Control) and *PTHrP-creER; Apc<sup>f/+</sup>; R26R<sup>tdTomato</sup>* (APC cHet) at P12, P21 and P96. RZ: resting zone, PZ: proliferating zone, HZ: hypertrophic zone. Blue: β-catenin-Alexa633, red: tdTomato, gray: DAPI and DIC. Scale bars: 100 μm. *n*=4 mice per genotype per time point.

**(e-g)** Compiled quantification data of total numbers of (e) resting chondrocytes, (f) short columnar chondrocytes ( $\leq 10$  tdTomato<sup>+</sup> cells) and (g) long columnar chondrocytes ( $> 10$  tdTomato<sup>+</sup> cells) (P9: *n*=3 mice for Control, *n*=5 mice for Apc cHet, P12–P36: *n*=4 mice per genotype, P96: *n*=4 mice for Control, *n*=3 mice for Apc cHet), collected from serial sections of femur growth plates (2 femurs/mouse) at all time points. Asterisks represent significant differences between control and mutant groups based on *p*<0.05 using a Mann-Whitney's *U*-test. Data are presented as mean  $\pm$  s.d.

Control versus Apc cHet, resting chondrocytes; P9: *p*=0.036, mean difference = 243.9 $\pm$ 97.4, 95% confidence interval (4.2, 483.5); P12: *p*=0.029, mean difference = 351.9 $\pm$ 109.8, 95% confidence interval (83.3, 620.5); P21: *p*=0.029, mean difference = 198.5 $\pm$ 63.9, 95% confidence interval (42.1, 355.0); P36: *p*=0.343, mean difference = -76.3 $\pm$ 100.3, 95% confidence interval (-321.8, 169.3); P96: *p*=0.057, mean difference = 55.3 $\pm$ 28.7, 95% confidence interval (-18.5, 129.1). Control versus Apc cHet, short columns; P12: *p*=0.020, mean difference = 7.9 $\pm$ 4.3, 95% confidence interval (-2.7, 18.5); P21: *p*=0.029, mean difference = 20.8 $\pm$ 6.5, 95% confidence interval (5.0, 36.5); P36: *p*=0.343, mean difference = -8.9 $\pm$ 10.7, 95% confidence interval (-35.0, 17.3); P96: *p*=0.343, mean difference = 1.3 $\pm$ 7.2, 95% confidence interval (-17.2, 19.7). Control versus Apc cHet, long columns; P21: *p*=0.886, mean difference = 10.0 $\pm$ 12.1, 95% confidence interval (-19.6, 39.6); P36: *p*=0.686, mean difference = -5.9 $\pm$ 18.6, 95% confidence interval (-51.3, 39.5); P96: *p*=0.029, mean difference = 22.3 $\pm$ 6.5, 95% confidence interval (6.2, 38.3).

**(h)** PTHrP<sup>+</sup> chondrocytes are maintained in a Wnt inhibitory environment within the resting zone. *Apc* haploinsufficiency increases β-catenin level in the resting zone, and decrease formation of PTHrP<sup>+</sup> chondrocytes and their differentiation to columnar chondrocytes.

# Figure S1



**Figure S1. A genetic label-retention strategy to identify slow-cycling chondrocytes.**

**(a)** Chondrocyte-specific Tet-Off system by *Col2a1-tTA* and *TRE-H2B-EGFP* transgenes. During development, *Col2a1*<sup>+</sup> cells accumulate H2B-EGFP in the nucleus. Binding of tetracycline-controlled transactivator (tTA) to Tet-responsive element (TRE) is prevented in the presence of doxycycline. As a result of this chase, slow-cycling cells retain a high level of H2B-EGFP, while proliferating cells dilute H2B-EGFP signal as they continue to divide.

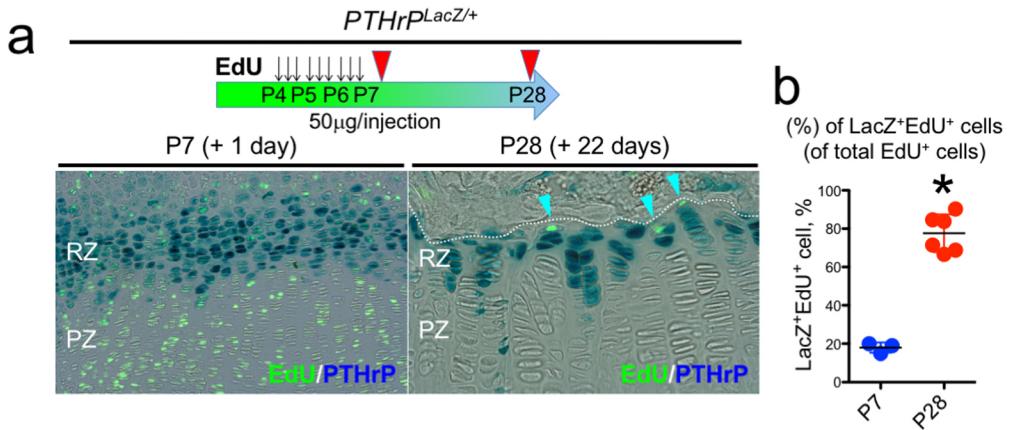
**(b,c)** Distal femur growth plates of *Col2a1-tTA/+; TRE-H2B-EGFP/+* double heterozygous mice at P7 (b) and P21 (c). Note that only a small fraction of growth plates marked by GFP in (c). Scale bars: 500μm. *n*=3 mice.

**(d)** Distal femur growth plates of *Col2a1-tTA/Col2a1-tTA; TRE-H2B-EGFP/TRE-H2B-EGFP* double homozygous mice at P21. Note that a greater number of growth plate cells are marked by GFP than in (c). Scale bars: 500μm. *n*=3 mice.

**(e)** Distal femur growth plates of *Col2a1-tTA/+; Col1a1-TRE-H2B-EGFP/+* mice, after 5 weeks of chase at P56. Arrowheads: GFP<sup>high</sup> label-retaining chondrocytes, arrows: GFP<sup>+</sup> osteoblasts/cytes. Scale bars: 500μm, 200μm (inset). *n*=3 mice.

**(f)** Distal femur growth plates of *+/+; Col1a1-TRE-H2B-EGFP/+* mice at P28. Arrows: GFP<sup>+</sup> osteoblasts/cytes. Scale bars: 500μm, 200μm (inset). *n*=3 mice.

## Figure S2



**Figure S2. Label-retaining chondrocytes (LRCs) are enriched among PTHrP<sup>+</sup> chondrocytes.**

**(a,b)** *PTHrP*<sup>LacZ/+</sup> distal femur growth plates with EdU administration, serially pulsed 9 times between P4 and P6. (a, left panel): Immediately after the pulse at P7. (a, right panel): After 22 days of chase at P28. Arrowheads: EdU label-retaining LacZ<sup>+</sup> cells. RZ: resting zone, PZ: proliferating zone. Scale bars: 100μm. (b): The percentage of LacZ<sup>+</sup>EdU<sup>+</sup> cells among total EdU<sup>+</sup> cells, at P7 ( $n=3$  mice) and P28 ( $n=6$  mice). \* $p<0.05$ , Mann-Whitney's *U*-test. Data are presented as mean  $\pm$  s.d. P7 versus P28:  $p=0.024$ , mean difference =  $-59.7 \pm 6.0$ , 95% confidence interval ( $-73.8$ ,  $-45.5$ ).