β-catenin Activity in Late Hypertrophic Chondrocytes locally orchestrates Osteoblastogenesis and Osteoclastogenesis

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Summary Statement

Conditional modulation of β-catenin activity in late hypertrophic chondrocytes locally regulates osteoclast differentiation and affects transdifferentiation of chondrocytes into osteoblasts.

Abstract

Trabecular bone formation is the last step in endochondral ossification. This remodeling process of cartilage into bone involves blood vessel invasion and removal of hypertrophic chondrocytes (HTCs) by chondroclasts and osteoclasts. Periosteal- and chondrocyte-derived osteoprogenitors utilize the leftover mineralized HTC matrix as a scaffold for primary spongiosa formation. Here we show genetically, that β-catenin (encoded by the Ctnnb1 gene), a key component of the canonical Wnt pathway, orchestrates this remodeling process at multiple levels. Conditional inactivation or stabilization of β-catenin in HTCs by a Col10a1-Cre line locally modulated osteoclastogenesis by altering the Rankl:Opg ratio in HTCs. Lack of β-catenin resulted in a severe decrease of trabecular bone in the embryonic long bones. Gain-of β-catenin activity interfered with removal of late HTCs and bone marrow formation leading to the presence of a continuous mineralized hypertrophic core in the embryo, and resulted in an osteopetrotic-like phenotype in adult mice. Furthermore, β-catenin activity in late HTCs is required for chondrocyte-derived osteoblastogenesis at the chondro-osseous junction. The latter contributes to the severe trabecular bone phenotype in mutants lacking β-catenin activity in HTCs.
Introduction

Severe loss of trabecular bone leads to osteopenia / osteoporosis, while a gain in trabecular bone mass is referred to as osteopetrosis. Trabecular bone formation is the final step in endochondral bone formation during fetal development occurring at the chondro-osseous front in conjunction with the formation of a bone marrow cavity. For this, terminally differentiated HTCs need to be turned over. They undergo apoptosis and are actively removed by osteoclasts and chondroclasts (Bianco et al., 1998; Shapiro et al., 2005), or alternatively transdifferentiate into osteoblasts (Park et al., 2015; Yang et al., 2014a; Yang et al., 2014b; Zhou et al., 2014).

HTC differentiation is accompanied by extracellular matrix changes, such as type X collagen (Col10a1) production, matrix mineralization, as well as matrix remodeling due to the up-regulation of matrix metalloproteinase 13 (MMP13) (Gack et al., 1995; Mattot et al., 1995). Mature HTCs also produce vascular endothelial growth factor (VEGF). VEGF induces blood vessel invasion into the transition zone between the two hypertrophic domains facilitating bone marrow cavity formation and attracts blood vessels to the chondro-osseous front (Gerber and Ferrara, 2000; Gerber et al., 1999; Maes et al., 2002; Zelzer et al., 2004; Zelzer et al., 2002). Along with the blood vessels, precursors of osteoclasts and perichondrial-derived osteoblasts invade. The latter utilize, together with the chondrocyte-derived osteoblast precursors, the mineralized matrix remnants of late HTCs as a template for trabecular bone formation. The HTC matrix is degraded by matrix metalloproteases such as MMP13 produced by late HTCs and MT1-MMP and MMP9 produced by osteoclasts and chondroclasts (Zhou et al., 2000; Vu et al., 1998; Inada et al., 2004; Stickens et al., 2004). Besides VEGF, which stimulates osteoclast recruitment, HTCs produce factors, such as osteopontin (Opn), Rankl and its antagonist Osteoprotegerin (Opg),
regulating osteoclastogenesis at the chondro-osseous front (Kishimoto et al., 2006; Nakashima et al., 2011; Silvestrini et al., 2005; Usui et al., 2008; Xiong et al., 2011). On the other hand, osteoclast- and in particular MMP9 activity has been proposed to facilitate the release of matrix-bound bioactive VEGF (Bergers et al., 2000; Colnot et al., 2003; Engsig et al., 2000; Ortega et al., 2010).

The Wnt/β-catenin pathway regulates skeletogenesis at many levels; influencing cell lineage decisions (Day et al., 2005; Hill et al., 2005; Spater et al., 2006), cell differentiation within the perichondrium-derived osteoblast lineage (Rodda and McMahon, 2006) or the chondrocyte lineage (Tamamura et al., 2005). The cytosolic levels of β-catenin determine pathway activity. Activation by a Wnt-ligand results in increased cytosolic β-catenin levels and its subsequent translocation into the nucleus, where it acts as a transcriptional coactivator (MacDonald et al., 2009). Continuous Wnt/β-catenin signaling in immature chondrocytes leads to their dedifferentiation and blocks hypertrophy (Guo et al., 2004; Miclea et al., 2009; Tamamura et al., 2005). Inducible β-catenin activation in chondrocytes in vivo and β-catenin activation or Wnt9a overexpression in more mature chondrocytes in vitro stimulated hypertrophy and expression of the late hypertrophic markers, Mmp13 and Vegf, while slightly decreasing Col10a1 levels (Dao et al., 2012; Tamamura et al., 2005). Moreover, in perichondrium-derived osteoblasts the pathway postnatally influences bone homeostasis (Glass et al., 2005; Holmen et al., 2005; Kramer et al., 2010; Sato et al., 2009; Spencer et al., 2006). A recent study associated β-catenin signaling in Col2a1-expressing chondrocytes with the regulation of postnatal bone development (Wang et al., 2014).

Previous experimental settings may have obscured the requirement for β-catenin in differentiated HTCs. Hence, we specifically modulated β-catenin function
in HTCs using a \textit{Col10a1-Cre} line (Gebhard et al., 2008) and either conditionally deleted the \textit{Ctnnb1} gene or stabilized β-catenin at the protein level. Recently, \textit{Ctnnb1} deletion from HTCs has been shown to result postnatally in decreased trabecular bone density due to a local increase in osteoclast number through upregulation of \textit{Rankl} (Golovchenko et al., 2013). Here we show that this phenotype is already present embryonically and that the augmented osteoclastogenesis by increased \textit{Rankl} does not fully account for the phenotype, as decreasing \textit{Rankl} expression specifically in HTCs only partially reverts the phenotype. Our analysis uncovered an additional need for β-catenin in the differentiation process of HTC-derived osteoblasts. Stabilization of β-catenin in HTCs, in contrast, interfered with late HTC turnover affecting bone embryonic and postnatal marrow formation. The embryonic phenotype was associated with a reduction in osteoclast number due to reduced \textit{Rankl} expression and could be reverted in part by additional removal of \textit{Opg}. In the long bones of adult mice the HTC-derived osteoblast differentiation was promoted at the more active growth plates.

\textbf{Results}

\textit{Loss of β-catenin activity from HTCs results in reduced trabecular bone formation}

Consistent with a previous report, conditional \textit{Ctnnb1} inactivation from HTCs using \textit{Col10a1-Cre} resulted in viable mice (\textit{Ctnnb1^{LacZ/0};Col10a1-Cre^+} = \textit{Ctnnb1^{LOFHTC}}) with an osteopenic phenotype (Golovchenko et al., 2013). The embryonic phenotype was analyzed from stage E16.5 onwards focusing primarily on the humerus. At this stage, the primary spongiosa begins to form in the wild-type. Alcian blue and von Kossa staining revealed a severe reduction of calcified structures in the bone marrow
cavity of $Ctnnb1^{\text{LOFHTC}}$ mutants (Fig. 1A). Based on histology and $Col10a1$ in situ staining, the hypertrophic domains were not altered in size by the loss of $Ctnnb1$ (Fig. 1A,B). In situ hybridization for the osteoclast markers cathepsin K ($Ctsk$) and tartrate-resistant acid phosphatase ($Trap/Acp5$) revealed an accumulation of $Ctsk$ and $Trap$ positive cells at the chondro-osseous front (Fig. 1C,D). Staining for the metalloproteinase $Mmp13$ and osteopontin ($Opn$), which are both expressed in terminal mature HTCs and osteoblasts, appeared slightly increased at the chondro-osseous front in $Ctnnb1^{\text{LOFHTC}}$ embryos (Figs 1E, S1H). In contrast, staining for the osteoblastic marker $Coll1$ was restricted to the central region of the maturation zone aside from its strong periosteal expression (Fig. 1F). Histology and marker expression in E18.5 and P0 humeri was similar to that at E16.5, showing reduced trabeculation, reduction in $Coll1$ positive structures and an increase in cells positive for osteoclast markers at the chondro-osseous front (Figs 1G-I, S1A-F). $Mmp9$ positive osteoclasts/chondroclasts were also increased in numbers at the chondro-osseous front (Fig. S1G). The staining for the prehypertrophic marker Indian hedgehog ($Ihh$) appeared to be slightly more intense (Fig. S1D). CD31 staining for endothelial cells revealed no apparent differences in vascularization close to the chondro-osseous front, but hypervascularity was noticed in the diaphysis (Fig. 1J). $Vegf$ expression in mature HTCs appeared to be similar to control at E18.5 (Fig. S1C). Quantitative PCR analysis at P0 (Fig. 1K) and E16.5 (Fig. 1L) revealed that $Rankl$ transcript levels were increased in $Ctnnb1^{\text{LOFHTC}}$ mutants compared to controls at both time points, as were $Mmp13$ and $Opn$ transcript levels (Fig. 1K,L). Surprisingly, $Vegf$ and $Opg$ levels were both increased at E16.5, but not significantly altered at P0 (Fig. 1K,L). $Ihh$ expression was slightly, but not statistically significant increased at P0 (Fig. 1K). In summary, inactivation of $\beta$-catenin from HTCs results in an increase in osteoclast number, loss
of mineralized structures and increased expression of \textit{Mmp13}, \textit{Opn} and \textit{Rankl} - factors that promote osteoclastogenesis.

\textit{Stabilization of β-catenin in HTCs results in an expansion of the mineralized hypertrophic zone}

Conditional stabilization of β-catenin in HTCs using the \textit{Col10a1-Cre} (\textit{Ctnnb1}^{\text{ex3f/+}},\textit{Col10a1-Cre}^{+} = \textit{Ctnnb1} \text{GOFHTC}) revealed a massive expansion of the two mineralized HTC zones in the mutant E16.5 humeri, which remained connected (Fig. 2A). In the mutant, high levels of β-catenin were be detected in all HTCs throughout the expanded zone (Fig. 2B). At the molecular level, the two \textit{Col10a1} expression domains were expanded in the mutant, but did not connect (Fig. 2C). In the central most region where the cells still appeared to be of chondrogenic nature based on their roundish morphology and their alcian blue positive matrix only a few cells expressed \textit{Col10a1} (Fig. 2C). These roundish, chondrocyte-like cells expressed \textit{Col1a1}, which is normally not expressed by HTCs, but rather by osteoblast precursors and mature osteoblasts as seen in the control (Fig. 2D). The expression of \textit{Col10a1} and \textit{Col1a1} in the expanded HTC zone in the mutants was nearly mutually exclusive (Fig. S2A,C). These \textit{Col1a1} expressing chondrocyte-like cells also expressed the osteoblast marker \textit{parathyroid hormone receptor 1 (Ppr1)} (Fig. S2B), but not the more mature osteoblast marker \textit{osteocalcin} (data not shown). Sox9 protein persisted in the expanded hypertrophic zone of the mutants (Fig. 2E). In the \textit{Ctnnb1} \text{GOFHTC} mutants, osteoclasts positive for \textit{Ctsk} and \textit{Mmp9} were restricted to the bone collar region and reduced in number (Fig. 2G, and data not shown). Accordingly, blood vessel invasion visualized by CD31 staining was compromised in \textit{Ctnnb1} \text{GOFHTC} mutants (Fig. 2H). Cells in the central region stained intensively for Opg (Fig. 2I).
Analysis of histology and molecular markers in E18.5 mutant humeri was very similar to E16.5 (Fig. 2J-Q, and data not shown). The Col10a1 and Ihh domains were expanded (Fig. 2K,L). Interestingly, late HTC markers such as Opn and Mmp13 were not expressed in the expanded hypertrophic zone (Fig. 2M,N). Staining for the aggrecan neoepitope DIPEN (Fosang et al., 1996), which was absent in the expanded region, together with a concurrent expansion of the collagen type II positive region (Fig. 2O,P) confirmed the lack of Mmp13 in the central core. DIPEN positive cells were only present at the edges of the expanded HTC zone and the two surrounding rows of cells, which by morphology appeared chondrocyte-like (Fig. 2O). Blood vessel invasion had proceeded but was still different compared to the control (Fig. 2Q).

Quantitative PCR using mRNA from P0 (Fig. 2R) skeletal elements and E16.5 (Fig. 2S) sorted chondrocytes revealed that upon stabilization of β-catenin, Mmp13, Opn and Rankl levels were decreased, Ihh and Sox9 levels were slightly increased, as were Opg levels but to our surprise not all cells that produce stabilized β-catenin produced Opg (Fig. 2I). Although the overall phenotype of Ctnnb1GOFHTC mutants resembled that of transgenic animals expressing Sox9 under the control of the Col10a1 promoter (Hattori et al., 2010), we did not observe down-regulation of Vegf, instead the levels of anti-angiogenic factors, such as chondromodulin-I (Lect-1/ChM-I) and tissue inhibitors of metalloproteinases, Timp1-3, were increased (Fig. 2R). In addition to their function as metalloproteinase inhibitors, Timp2 and Timp3 can directly inhibit Vegf-signaling (Qi et al., 2003; Seo et al., 2003). The upregulation of these anti-angiogenic factors might contribute to the fact that blood vessels failed to invade the hypertrophic region.
We next asked whether the mineralized HTCs would be removed after birth. In long bones of three-day-old mutant pups (P3) the mineralized hypertrophic zones of the proximal and distal growth plates were still expanded and connected (Fig. 3A). The mutant long bones were slightly shorter. This shortening was more pronounced in 28 day-old mice (P28), which overall were smaller than their littermate controls (Ctnnb1<sup>ex3fl/+;Col10a1-Cre<sup>-</sup>) (Fig. S3A,B). MicroCT-analysis and histological examination of four-week-old humeri and femora revealed a locally restricted osteopetrotic phenotype in Ctnnb1<sup>GOFHTC</sup> compared to littermate controls (Figs 3B,C, S3C). Closer examination uncovered differences between distal and proximal regions of the skeletal elements: in the humerus, bone marrow space was present proximally, while it was almost completely replaced by abnormally mineralized tissue distally (Fig. 3B,C). In the femur this pattern was reversed (Fig. S3C). Interestingly, bone remodeling and formation of bone marrow space was always associated with the side of the growth plate that has been described in humans to be the more active – e.g. the proximal humeral and the distal femoral growth plate (Figs 3B,C, S3C, and data not shown) (Pritchett, 1991; Pritchett, 1992). The mineralized HTC zone was increased in the proximal growth plates of humeri and femora and the distal femoral growth plate (Figs 3C, S3C). Furthermore, in the proximal humeri and distal femora, trabecular bone volume with respect to total volume (BV/TV) and trabecular number were increased, accordingly trabecular spacing was decreased, while trabecular thickness was unaltered (Figs 3D, S3D).

In essence, stabilization of β-catenin in HTCs, with the exception of the active growth plates in juveniles, interferes with the removal of mineralized hypertrophic chondrocytes and blood vessel invasion. This is associated with the down-regulation
of pro-osteoclastic factors, a reduced number of osteoclasts and up-regulation of anti-
angiogenic factors.

**Genetic alteration of osteoclastogenesis partially restored phenotypic changes caused by loss- or stabilization of β-catenin in late HTCs**

Conditional loss or stabilization of β-catenin in late HTCs led to an alteration in expression of osteoclastogenesis modulating factors, such as *Opg* and *Rankl*, and as a result altered the location and/or number of osteoclasts. Hence, using a genetic approach, we examined whether local downregulation or increased activation of osteoclastogenesis can restore the phenotypic changes occurring upon conditional loss or stabilization of β-catenin. Examination of E19.5 *Ctnnb1*\textsuperscript{GOFHTC};*Opg*\textsuperscript{-/-} double-mutant humeri (*Ctnnb1\textsuperscript{x3fl/+};Col10a1-Cre\textsuperscript{+};*Opg*\textsuperscript{-/-}) revealed a partially restored phenotype in all examined specimens (5/5) (Fig. 4). Bone marrow space was restored but trabecular bone formation was still abnormal and the mineralized HTC zones were still enlarged (Fig. 4A,B). *Col1a1* and *Mmp13* staining revealed the presence of osteoblasts and *Ctsk* staining the presence of osteoclasts in the bone marrow of *Ctnnb1*\textsuperscript{GOFHTC};*Opg*\textsuperscript{-/-} humeri (Fig. 4C-E). Unlike in the *Ctnnb1*\textsuperscript{LOFHTC} mutants, no pronounced reduction of trabeculae was observed in *Opg* mutants at P0 (Fig. S4A). According to the *Col10a1* and *Ctsk* staining the hypertrophic zone appeared normal and osteoclast numbers appeared not to be substantially increased (Fig. S4A).

Given our hypothesis that β-catenin activity represses *Rankl* expression in HTCs, we decided to down-regulate *Rankl* specifically in HTCs in the *Ctnnb1* mutant background. Control, *Ctnnb1*\textsuperscript{LOFHTC}, and conditional *Ctnnb1* and *Rankl* double mutants (*Ctnnb1\textsuperscript{LacZ/fl};*Rankl*\textsuperscript{fl/fl};Col10a1-Cre\textsuperscript{+} = *Ctnnb1*\textsuperscript{LOFHTC};*Rankl*\textsuperscript{AHTC}) were examined by microCT (see Fig. S5A). BV/TV measurements revealed that trabecular
bone formation was restored to a variable degree in the Ctnnb1<sup>LOFHTC</sup>;Rankl<sup>AHTC</sup> specimens and that this restoration was statistically significant (Fig. 5A). Based on the microCT data, the eight Ctnnb1<sup>LOFHTC</sup>;Rankl<sup>AHTC</sup> specimens fell into two groups: four with an almost ‘complete’ phenotypic restoration and four with a very local ‘rescue-effect’ restricted to the chondro-osseous front (Figs 5A, S5A). The average BV/TV ratio for the four ‘complete’ restored specimens increased to 39.56% (± 2.40) as compared to about 18% in the Ctnnb1<sup>LOFHTC</sup> mutants, but was still at the low-end range of controls with a BV/TV ratio of 45.90% (± 4.51) (Fig. S5B). Histological and osteoblast marker analysis confirmed the variability of the restoration effect (Fig. 5B-F, and data not shown). In ‘complete’ Ctnnb1<sup>LOFHTC</sup>;Rankl<sup>AHTC</sup> mutants increased trabeculae numbers, and an increase in Col1a1 positive cells and trabeculae-associated Osterix positive (Osx<sup>+</sup>) cells were observed (Fig. 5B-D). Local Rankl modulation levels did not alter the vascular pattern (Fig. 5E). However, local loss of Rankl in the Ctnnb1<sup>LOFHTC</sup>;Rankl<sup>AHTC</sup> specimens decreased overall the osteoclasts in number compared to Ctnnb1<sup>LOFHTC</sup> littermates (Fig. S5C). The BV/TV ratio in Rankl<sup>AHTC</sup> (Rankl<sup>fl/fl</sup>;Col10a1-Cre<sup>+</sup>) newborns was increased to 61.16% (± 5.89) (Figs 5A, S5A,B), but no obvious decrease in Ctsk stained cell numbers was observed (Fig. S5C). In contrast to previous reports in adult mice (Nakashima et al., 2011; Xiong et al., 2011) the width of the hypertrophic zone was not altered upon deletion of Rankl in HTCs (Figs 5F, S5C). In mutants lacking the receptor Rank no osteoclasts are present (Fig. S4B, and data not shown), while some Mmp9 positive cells can still be detected primarily at the chondro-osseous border (Fig. S4B). Here, the Col10a1 staining revealed a noticeable enlargement of the HTC zone (Fig. S4B). Nevertheless, in contrast to the Ctnnb1<sup>GOFHTC</sup> mutants, remodeling of the hypertrophic zone was only
slightly affected and bone marrow cavity formation was normal (compare Figs S4B and 2J,K).

Taken together, genetic modulation of osteoclastogenesis is able to partially revert the β-catenin loss- and gain-of-function phenotypes, demonstrating that the alterations in osteoclast numbers are in part responsible for the phenotypic changes.

**β-catenin activity in late HTCs is required for differentiation of chondrocyte-derived osteoblasts**

Given that the phenotypic restoration achieved by deleting Rankl in HTCs in the Ctnnb1LOFHTC mutant background was not 100%, and that β-catenin plays a role in periosteal osteoblast differentiation (Day et al., 2005; Hill et al., 2005), as well as in the light of the recent publications demonstrating that HTC descendants can differentiate into osteoblasts (Yang et al., 2014a; Yang et al., 2014b; Zhou et al., 2014), we addressed the question whether chondrocyte-derived osteoblastogenesis was altered in Ctnnb1LOFHTC mutants. For this, we examined Ctnnb1LOFHTC;RosaYFP/+ humeri and determined the percentage of all osteoblasts (Osx+(total)), all chondrocyte-derived cells (YFP+(total)), of chondrocyte-derived (Osx+;YFP+) and perichondrial-derived (Osx−;YFP+) osteoblasts, as well as the osteogenic (YFP+;Osx+) and non-osteogenic (YFP+;Osx−) subpopulations of YFP+ cells in the subchondral growth plate region compared to littermate controls (Ctnnb1∆ColX+/+;RosaYFP+/−) (Fig. 6). We noted that the overall cellularity (DAPI+ cells) was increased in the Ctnnb1LOFHTC mutant bone marrow (Fig. 6A). The percentage of Osx+(total) and YFP+(total) populations were both reduced by about 50% compared to controls (Fig. 6B). The perichondrial-derived Osx+;YFP population decreased by 18%, and the chondrocyte-derived Osx+;YFP+ population dropped from 34% in the controls to 2% in Ctnnb1LOFHTC mutants (Fig.
In contrast, the non-osteoblastic YFP\(^+\);Osx\(^-\) population increased by 18%, while only 4% of all YFP\(^+\) cells differentiated towards the osteoblastic lineage (YFP\(^+\);Osx\(^+\)) in the mutant (Fig. 6D). These findings were corroborated by the analysis of Runx2/Cbfa1, a second osteoblastic marker, which acts upstream of Osx in osteoblastogenesis (Nakashima et al., 2002). Compared to the control the Runx2\(^+\) (total) population dropped by 44% (Fig. 6E), the chondrocyte-derived Runx2\(^+\);YFP\(^+\) population from 25% to 6%, and the perichondrial-derived Runx2\(^+\);YFP\(^+\) population by 24% in the \(Ctnnb1^{LOFHTC}\) mutants (Fig. 6F). Interestingly, unlike the non-osteogenic YFP\(^+\);Osx\(^-\) population, the YFP\(^+\);Runx2\(^-\) population did not increase and the osteogenic YFP\(^+\);Runx2\(^+\) subpopulation did not decrease to the same extent as the YFP\(^+\);Osx\(^+\) population (Figs 6D,G). The fact that osteoblastic chondrocyte-derived bone marrow cell numbers decreased dramatically in the long bones lacking β-catenin activity in late HTCs, suggests that β-catenin is required for the differentiation of chondrocyte-derived osteoblasts and that the remaining osteoblasts are essentially all periosteal-derived.

Next we asked whether this decrease in chondrocyte-derived osteoblast number is due to the fact that the precursor cells upregulated Sox9, similar to what is observed in perichondrial-derived osteoblasts that lack β-catenin (Hill et al., 2005). Sox9 protein expression and levels were similar to the control (Fig. S6). Furthermore, no increase in TUNEL or cleaved caspase 3 positive cell numbers was observed in the growth plate or at the chondro-osseous front in \(Ctnnb1^{LOFHTC}\) mutants (Fig. S6, and data not shown). According to the Ki67 staining proliferation in the growth plate or of YFP\(^+\) cells was not altered (Fig. S6). Next we investigated whether the increased number of non-osteoblastic YFP\(^+\);Osx\(^-\) cells may be due to a cell-lineage shift into adipocytes. Adipocytes were increased in number in four-month-old \(Ctnnb1^{LOFHTC}\)
mutant tibiae (Fig. S7A). Similarly, the overall number of fatty acid binding protein 4 (FABP4) positive cells was increased in newborn Ctnnb1\textsuperscript{LOFHTC} mutant limbs compared to controls (Fig. S7B). However, none of the FABP4\textsuperscript{+} cells, located in close proximity to blood vessels, stained positive for YFP (Fig. S7B-D). Thus, we conclude that the increase in adipocyte number is not caused by a lineage-shift of chondrocyte-derived osteoblast precursor.

**Increased \(\beta\)-catenin activity promotes transdifferentiation of chondrocyte-derived osteoblasts**

Finally, we analyzed whether increased \(\beta\)-catenin activity in late HTCs affects the number of chondrocyte-derived osteoblasts. Counting the YFP\textsuperscript{+} as well as the YFP\textsuperscript{+};Osx\textsuperscript{+} cells in an area spanning 250 \(\mu\)m below the chondro-osseous border (Fig. 7A) at the proximal growth plates of P28 humeri revealed a significant increase in the total number of YFP\textsuperscript{+} cells and in the number of YFP\textsuperscript{+};Osx\textsuperscript{+} cells by about 70% each compared to the controls (Fig. 7C). At the growth plates undergoing active remodeling in humeri and femora, TRAP\textsuperscript{+} osteoclasts were detected but compared to the control the number of osteoclasts lining the cartilage erosion zone was decreased by about 35% (Fig. 7B,D). Thus, the combination of increased chondrocyte-derived osteoblast precursors formation and decreased osteoclastogenesis observed at the growth plate undergoing remodeling is probably responsible for the increased BV/TV in the P28 Ctnnb1\textsuperscript{GOFHTC} limbs (see Figs 3D, S3D).

**Discussion**

During the late phase of fetal development, remarkable remodeling processes occur in long bones – resorption of hypertrophic calcified cartilage, bone marrow cavity, and
trabecular bone formation. Our data show that the severe reduction of trabecular bone in mutants lacking β-catenin activity in late HTCs is the result of a combinatorial mechanism: decreased chondrocyte-derived osteoblast differentiation and increased recruitment of osteoclasts to the chondro-osseous front. The latter is due to local deregulation of the Rankl:Opg ratio, and probably further supported by Opn and Mmp13 upregulation, molecules potentiating osteoclast function (Franzen et al., 2008; Pivetta et al., 2011; Ross et al., 1993). Our results on Rankl regulation are consistent with previous results (Golovchenko et al., 2013; Wang et al., 2014) and resemble the situation in osteoblasts (Holmen et al., 2005; Kramer et al., 2010; Sato et al., 2009; Spencer et al., 2006). Yet, the molecular mechanisms may differ between the cell-types; being direct mechanism in osteoblasts (Spencer et al., 2006), and potentially indirect in chondrocytes via interference with a glucocorticoid receptor (GR)-dependent mechanism (Wang et al., 2014). Yet, the latter is questioned by the fact that the cartilage-specific GR knockout has no apparent growth plate or bone phenotype (Tu et al., 2014). Wang and colleagues used the Col2a1-CreER in their study to inactivate β-catenin. This Cre-line is also active in mesenchymal precursors of perichondrium-derived osteoblasts (Ono et al., 2014), which also require β-catenin. Hence, some of the phenotypic changes may be due to altered β-catenin activity in perichondrial-derived osteoblasts (Wang et al., 2014). In contrast to Wang and colleagues, we did not observe a decrease in height upon Ctnnb1 loss or cellular disorganization upon β-catenin stabilization, suggesting that these changes correlate with β-catenin functions in Col2a1 expressing chondrocytes (Wang et al., 2014). Our hypothesis that increased Rankl expression in HTCs upon Ctnnb1 deletion contributes substantially to the Ctnnb1LOFHTC phenotype is supported by the reversal of the phenotype upon conditional removal of Rankl from HTCs in 50% of the specimens.
The incomplete penetrance can likely be explained by the fact that two \textit{Rankl} alleles need to be recombined and Cre recombination efficiency at the cellular level varies between specimens (Nagy, 2000). Even in the four ‘complete’ \textit{Ctnnb1}^{\text{GOFHTC};\text{Rankl}^{\text{AHTC}}} specimens the average BV/TV was at the lower end of the control. This indicates that \textit{Rankl} deregulation is not the sole cause of the phenotype.

The 17 fold decrease in the number of osteoblasts derived by chondrocyte-transdifferentiation together with the decrease in perichondrial-derived Osx$^+$;YFP$^+$ precursor number by 18% in \textit{Ctnnb1}^{\text{LOFHTC}} mutants likely contributes to the slightly lower BV/TV ratio of the ‘complete’ \textit{Ctnnb1}^{\text{GOFHTC};\text{Rankl}^{\text{AHTC}}} specimens. Perichondrial-derived osteoblast precursors migrate along blood vessels into the bone marrow cavity (Maes et al., 2010). In the \textit{Ctnnb1}^{\text{LOFHTC}} mutants blood vessels reach the chondro-osseous border and there is even hypervascularity noticeable primarily in the diaphysis. The latter may be due to the transient increase in Vegf observed at E16.5, or increased liberation of matrix-bound VEGF due to increased osteoclastic activity at the chondro-osseous front (Bergers et al., 2000; Huang et al., 2002). Thus, perichondrial-derived osteoblast precursors should be able to migrate in normally, but either due to the lack of mineralized matrix, the remains of late HTCs, or to other environmental changes, not all of them may find the right environment to survive or to differentiate along the osteoblastic lineage. While both osteoblastic populations decreased, the number of non-osteoblastic, chondrocyte-derived bone marrow cells increased by about 18% in the \textit{Ctnnb1}^{\text{LOFHTC}} mutants. The exact cellular nature of the YFP$^+$;Osx$^-$ population observed here and in previous lineage-tracing transdifferentiation studies has not yet been clarified (Park et al., 2015; Yang et al., 2014a; Yang et al., 2014b; Zhou et al., 2014). A part of the YFP$^+$;Osx$^-$ cells in the \textit{Ctnnb1}^{\text{LOFHTC}} mutants apparently remained in a more undifferentiated state being
positive for Runx2. Given that Wnt/β-catenin signaling plays an important role in the osteoblastogenesis versus adipogenesis lineage decision process of bone marrow cells (Bennett et al., 2005; Gambardella et al., 2011; Krishnan et al., 2006; Song et al., 2012) it was an intriguing possibility that some YFP⁺;Osx⁻ cells differentiated to adipocytes. However, the observed increase in adipocyte and adipocyte progenitor numbers in Ctnnb1LOFHTC mutants was not due to a lineage-switch of chondrocyte-derived osteoprogenitors. Instead our data suggests that the FABP4⁺ progenitors originate from perichondrium-derived osteoprogenitors explaining in part their reduction. Osteoblast precursors in the perichondrium that lack β-catenin activity differentiate into chondrocytes and express Sox9 (Hill et al., 2005; Rodda and McMahon, 2006). Yet, the mechanism that blocks Ctnnb1-deficient cells from undergoing transdifferentiation appears to be distinct as no alteration in Sox9 was observed. Altered apoptosis or proliferation appears also not to be causal for the decrease in chondrocyte-derived osteoblastogenesis. Thus, the mechanism by which β-catenin controls this process is currently unclear.

In osteoblasts, Opg has been reported to be a direct, positively regulated β-catenin/Tcf target (Glass et al., 2005; Sato et al., 2009). However, this seems not to be the case in HTCs. In Ctnnb1GOFHTC specimens, Opg transcript levels were not increased and only the most centrally located cells, which also expressed osteoblast markers, stained positive for Opg. Hence, β-catenin may only be able to regulated Opg in osteoblastic cells. Nevertheless, genetic loss of Opg reverted the Ctnnb1GOFHTC phenotype to a certain extent, but only very late in embryonic development. Increased systemic Rankl levels in Opg mutants (Bennett et al., 2006) are likely responsible for the increase in osteoclast numbers. Yet, trabecular bone formation was abnormal and the mineralized HTC domains were still enlarged. The reason for the latter is probably
multifactorial: despite Opg loss, Rankl expression by HTCs is probably still decreased and hence local osteoclastogenesis may not occur at the same rate as in wild-type. This may contribute to the persistence of the enlarged mineralized hypertrophic zone in the Ctnnb1^{LOFHTC};Opg^{−/−} animals, as the hypertrophic zone is also increased in mutants with compromised osteoclastogenesis. In addition, Opn and Mmp13 expression in late HTCs was still altered. As Mmp13 mutants also display a widening of the HTC zone, the lack of its expression in late HTCs possibly contributes to the shortcomings of the phenotypic reversal (Inada et al., 2004).

Chondrocyte remodeling was absent in Ctnnb1^{GOFHTC} mice and late hypertrophic markers were not expressed, while the domains of immature markers such as Ihh and Sox9 were expanded. These data indicate that stabilization of β-catenin in HTCs amongst others delays terminal HTC differentiation. On the other hand, a dramatic expansion of mineralized HTCs was observed. Here, the central most chondrocytes started to express osteoblastic markers such as Colla1 and Pprl and produced Opg. We interpret this as an attempt of the cells to undergo osteoblastic transdifferentiation. Yet, these cells did not express osteocalcin a marker for mature osteoblasts. Similar to the situation in periosteal osteoblast precursors, their attempt to mature might be stalled at an intermediate stage due to the high levels of stabilized β-catenin (Rodda and McMahon, 2006).

While no true bone marrow cavity was formed in the Ctnnb1^{GOFHTC} skeletal elements up to postnatal age P3, partial bone marrow cavity formation had occurred in four-week-old mice. Interestingly, its occurrence was always associated with the more active growth plate (Farnum, 1994; Pritchett, 1991; Pritchett, 1992). In all postnatal growth plates the number of new chondrocytes produced was equal to the HTCs lost but in the more active growth plates these numbers were 2-3 fold higher
(Wilsman et al., 1996). The factors responsible for this phenomenon are not yet known. Nevertheless, given that more chondrocytes are produced and turned over in the same amount of time in active growth plates, this means that chondrocytes spend less time in the hypertrophic zone (Wilsman et al., 1996). Consequently, Col10a1-Cre efficacy may decrease as the activity window becomes narrower and as such fewer cells recombine the Ctnnb1 exon 3 floxed allele. As a result, Rankl would not be repressed in all HTCs and could support local osteoclastogenesis. Hence, bone marrow formation can reoccur and due to increased chondrocyte transdifferentiation, trabecular bone formation is enhanced at these growth plates.

In conclusion, we show that in late HTCs, β-catenin has dual functions regarding trabecular bone formation: firstly, it locally regulates osteoclastogenesis, by repressing the expression of the pro-osteoclastic factor Rankl and secondly, it is involved in the newly discovered transdifferentiation process of HTCs giving rise to osteoblast precursors (see Fig. 8). As such β-catenin is a key-player in early trabecular bone formation during embryonic skeletal development.

Material and Methods

Mouse husbandry

The following strains were used: Ctnnb1 floxed (Huelsken et al., 2001), Ctnnb1 LacZ knock-in null (Huelsken et al., 2000), Ctnnb1 ex3 floxed (Harada et al., 1999), BAC-Col10a1-Cre (Col10a1-Cre, #1465 and #1421 (Gebhard et al., 2008), Opg/Tnfrsf11b knock-out (Mizuno et al., 1998), Rankl floxed (Xiong et al., 2011), Rank/Tnfrsf11aΔ/Δ (Hanada et al., 2009), and Rosa26-flox-Stop-flox-EYFP (RosaYFP) (Srinivas et al., 2001). For breeding details see Supplementary material and methods.
For timed pregnancies, the plug date was considered to be embryonic day (E) 0.5. Genotyping was performed using previously published PCR protocols. Animals were sacrificed by cervical dislocation, embryos and newborns by decapitation. All mouse experiments were performed in accordance with local, institutional and national regulations and licenses (84-02.05.20.12.261).

**Microcomputed tomography (microCT) and histomorphometric analysis**

Long bones were fixed for 24 h in 4% paraformaldehyde followed by two PBS and one wash in 70% ethanol for 24 h each. Prior to the scan, tissue was stored in 70% ethanol at 4°C and wrapped in parafilm for the scan. Scans were performed using a SkyScan 1176 scanner (SkyScan, Kontich, Belgium). For scanning details see Supplementary material and methods.

**Processing of specimens**

For a detailed description see Supplementary material and methods.

**Histology**

Histological stainings such as alcian blue, alcian blue / von Kossa, H&E, TRAP were performed on 5 μm paraffin sections. For details see Supplementary material and methods.

**Immunohistochemistry and Immunofluorescence**

Staining for CD31, Opg, Osx, Sox9, β-catenin, Col II, DIPEN, Runx2, YFP, Ki67, FABP4, endomucin, cleaved caspase 3, TUNEL was performed on deparaffinized and
rehydrated 5 µm sections. For more detailed information see Supplementary material and methods.

**Alkaline phosphatase based and double fluorescent in situ hybridization**

In situ hybridization using digoxygenin (DIG) labeled RNA probes was performed according to (Murtaugh et al., 1999). Double-fluorescent in situ hybridization on paraffin sections were performed using antisense biotin-labeled *Col1α1* probe and DIG-labeled *Col10α1* or *PPR1* probes according to (Taschner et al., 2008). All probes have been previously published and are available on request.

**RNA isolation from FACS sorted primary HTCs and total skeletal elements**

RNA was isolated from dissociated whole P0 skeletal elements or from YFP positive cells isolated from E16.5 skeletal elements using the RNeasy Kits (Qiagen) according to the manufacturer’s instructions, including a DNase I treatment. RNA concentration was determined by OD measurement. For more detailed information see Supplementary material and methods.

**cDNA synthesis**

For first-strand cDNA synthesis, 0.5 - 1 µg of total RNA was reverse transcribed using oligo dT primers. For qRT-PCR analysis cDNA was diluted 1 in 10. For further details see Supplementary material and methods.

**Real-time PCR analysis**

2-3 µl of 1 in 10 diluted 1st strand cDNA were mixed with either Fast Start SBG master mix (Roche, #04673484001) or SYBR Premix Ex Taq II (TaKaRa,
Gene expression was monitored using a BioRad CFX96 cycler. For more details see Supplementary material and methods and Table S1 for primer sequences.

**Image acquisition**

Histological images were acquired using either the Zeiss AxioPlan2 equipped with a Leica DFC320 – 3.45 µm pixel colour camera or the Zeiss AxioImager.M2 equipped with an AxioCam MRC 6.45 µm colour camera (Zeiss, Jena). Immunofluorescent images were acquired using a Zeiss AxioImager.M2 equipped with an ApoTome.2 and an AxioCam MRm 6.45 µm monochromatic camera (Zeiss, Jena) using the Zen software (Zeiss, Jena).

**Statistical analysis**

Statistical analysis was performed by two-tailed, unpaired Student’s t-test using the GraphPad Prism software 6.0. Data are displayed as mean values ± standard deviation (s.d.).

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**Competing interest**

Authors declare no financial or non-financial competing interests.
Author contribution

A.H. and D. K-P. are responsible for the majority of data acquisition, analyses and data interpretation, M.W. and J.G. were involved in data generation and acquisition, S.T. helped with the microCT and histomorphometric analyses, K.v.d.M. provided reagents and contributed conceptionally, C.H. is responsible for the conception, data interpretation and wrote the manuscript.

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Figures

**Fig. 1 Phenotypic analysis of Ctnnb1<sup>LOFHTC</sup> mutants.**

(A–F) Representative images of alternating sections through humeri of E16.5 control and Ctnnb1<sup>LOFHTC</sup> mutant littermates. (A) Alcian blue / von Kossa staining. (B) Hypertrophic zones visualized by Col10a1 in situ hybridization. (C) Ctsk in situ hybridization. (D) Trap positive osteoclasts visualized by in situ hybridization. (E) Mmp13 in situ hybridization. (F) Coll1a1 in situ hybridization. (G–J) Representative alternating sections through humeri of P0 control and Ctnnb1<sup>LOFHTC</sup> mutant littermates. (G) Alcian blue / von Kossa. (H) Coll1a1 in situ hybridization. (I) Ctsk in situ hybridization. (J) Visualization of the vascular network by CD31 (PECAM-1) immunostaining. Arrowheads point to the chondro-osseous border. The dashed line indicates the beginning of the diaphysis (DP). (K) qPCR analysis of P0 Ctnnb1<sup>LOFHTC</sup> relative to control long bone material (n=3) ± s.d. *P-value <0.05; **P-value <0.01. Genotype of control in A–K: Ctnnb1<sup>fl/+</sup>; Col10a1-Cre<sup>+</sup>. (L) qRT-PCR analysis using E16.5 YFP sorted material from Ctnnb1<sup>LOFHTC</sup>; Rosa26<sup>YFP/+</sup> long bones relative to material isolated from E16.5 Coll10a1-Cre<sup>+</sup>; Rosa26<sup>YFP/+</sup> limbs (n=2).
Fig. 2 Phenotypic analysis of E16.5 and E18.5 Ctnnb1\textsuperscript{GOFHTC} mutants.

(A-I) Representative images of alternating sections through humeri from E16.5 Ctnnb1\textsuperscript{GOFHTC} and control (Ctnnb1\textsuperscript{ex3fl/+};Col10a1-Cre\textsuperscript{-}) littermates. (A) Alcian blue / von Kossa staining. (B) β-catenin immunohistochemical staining. (C) Col10a1 in situ hybridization. (D) Coll1 in situ hybridization showing the presence of chondrocyte-like, Coll1 positive cells (red asterisk). (E) Sox9 immunohistochemical staining. (G) Ctsk in situ hybridization for osteoclasts. (H) CD31 immunohistochemical staining for blood vessels. (I) Opg immunohistochemical staining. (J-Q) Representative images of E18.5 humerus sections of Ctnnb1\textsuperscript{GOFHTC} and control (Ctnnb1\textsuperscript{ex3fl/+};Col10a1-Cre\textsuperscript{-}) littermates. (J) Histological changes visualized by alcian blue / von Kossa staining. (K) Coll10a1 in situ hybridization. (L) Ihh in situ hybridization. (M) Osteopontin (Opn) in situ hybridization. (N) Mmp13 in situ hybridization. (O) Immunohistochemical staining for the aggrecan neoepitope DIPEN, counterstained with methyl green. (P) Immunohistochemical staining for type II collagen (Col II). (Q) Immunohistochemical staining for CD31. (R) qRT-PCR analysis of P0 Ctnnb1\textsuperscript{GOFHTC} relative to control long bone material (n=3) ± s.d. *P-value <0.05; **P-value <0.01. (S) qRT-PCR analysis using E16.5 YFP sorted material from Ctnnb1\textsuperscript{GOFHTC};Rosa26\textsuperscript{YFP/+} long bones relative to material isolated from E16.5 Coll10a1-Cre\textsuperscript{+};Rosa26\textsuperscript{YFP/+} limbs (n=2).
Fig. 3  Postnatal phenotypic analysis of $Ctnnb1^{GOFHTC}$ mutants.

(A) Representative images of humeri from 3-day-old (P3) control and $Ctnnb1^{GOFHTC}$ littermates stained with alcian blue / von Kossa or alcian blue. (B) MicroCT images of control and $Ctnnb1^{GOFHTC}$ humeri (P28, frontal and lateral view). Note: the lateral view reveals anterior-posterior differences in the trabecular density underneath the proximal growth plate in the mutant humerus. (C) Toluidine-blue / von Kossa stained sections of control and $Ctnnb1^{GOFHTC}$ P28 humeri. Note: orientation is proximal up and distal down. (D) Histomorphometric quantification of the bone volume (BV) to total volume (TV), trabecular thickness (Tb. Th), trabecular number (Tb. N) and trabecular spacing (Tb. Sp) of P28 proximal humeri comparing $Ctnnb1^{GOFHTC}$ (n=8) and littermate controls (n=9). **P-value < 0.01, ***P-value < 0.001, ± s.d. Genotype of control = $Ctnnb1^{en3H/+};Col10a1-Cre^-$. 
Fig. 4 Partial restoration of the $Ctnnb1^{GOFHTC}$ phenotype by the loss of $Opg$.

(A-E) Representative images of sections through E19.5 humeri of control ($Ctnnb1^{ex3fl/+};Col10a1-Cre}$), $Ctnnb1^{GOFHTC}$ and $Ctnnb1^{GOFHTC};Opg^{-/-}$ specimens. (A) Histological changes visualized by alcian blue / von Kossa staining showing remodeling of the extended zone of mineralized HTCs in the $Ctnnb1^{GOFHTC}$ upon loss of $Opg$. (B) $Col10a1$ in situ hybridization. (C) $Colla1$ in situ hybridization. (D) $Mmp13$ in situ hybridization. (E) $Ctsk$ in situ hybridization.
Fig. 5 Partial reversal of the $Ctnnb1^{\text{LOFHTC}}$ phenotype by conditional deletion of $Rankl$ in HTCs.

(A) Scatter plot showing the distribution of the bone volume (BV) to total volume (TV) in % of the different controls (n=17), $Ctnnb1^{\text{LOFHTC}}$ (n=6), $Ctnnb1^{\text{LOFHTC};Rankl^{\text{AHTC}}}$ ($Ctnnb1^{\text{LacZ/LacZ}};Rankl^{\text{fl/fl};Col10a1-Cre^{+}}$) (n=8), and $Rankl^{\text{AHTC}}$ ($Rankl^{\text{fl/fl};Col10a1-Cre^{+}}$) (n=8) specimens. * P-value <0.05, ** P-value <0.01, *** P-value <0.001. (B-F) Representative images of sections through control, $Ctnnb1^{\text{LOFHTC}}$, and ‘complete’ $Ctnnb1^{\text{LOFHTC};Rankl^{\text{AHTC}}}$ humeri. (B) Alcian blue / von Kossa staining. (C) $Colla1$ in situ hybridization. (D) Osx immunohistochemical staining. (E) CD31 immunohisto-chemical staining. (F) $Colla1$ in situ hybridization.
Fig. 6 Chondrocyte-derived osteoblastogenesis is affected by the loss of Ctnnb1 from HTCs.

(A) Representative images of control and Ctnnb1LOFHTC P0 specimens taken from the region below the chondro-osseous front. Immunofluorescent staining for Osx (red), YFP (green), and DAPI for nuclei (blue). The dashed line indicates the position of the chondro-osseous border. Note: the increase in DAPI+ cells in the Ctnnb1LOFHTC mutant reflects an increase in cellularity. Higher magnification images of the boxed regions with only the Osx (red) and YFP (green) signals are provided on the right, double positive cells are marked by arrows. (B) Bar graph showing the mean value of the percentage of Osx+ (total) and YFP+ (total) cells relative to control. (C) Pie diagrams showing the distribution of Osx++;YFP− (perichondrial-derived) and Osx++;YFP+ (chondrocyte-derived) cells normalized with respect to the Osx+ (total) population (100%) of the control. (D) Pie diagrams showing the distribution of YFP++;Osx+ (osteoblastic) and YFP++;Osx− (non-osteoblastic) cells normalized with respect to the YFP+ (total) population (100%) of the control. (E) Bar graph showing the mean value of the percentage of Osx+ (total) and Runx2+ (total) cells relative to control. (F) Pie diagrams showing the distribution of Runx2++;YFP− (perichondrial-derived) and Runx2++;YFP+ (chondrocyte-derived) cells normalized with respect to the Runx2+ (total) population (100%) of the control. (G) Pie diagrams showing the distribution of YFP++; Runx2+ (osteoblastic) and YFP++; Runx2− (non-osteoblastic) cells normalized with respect to the YFP+ (total) population (100%) of the control. Note: Cells for B-D were counted within the square indicated by the thin white line in (A) and for E-G in a square of the same size on 3-4 sections per specimen and genotype (control = Ctnnb1fl/fl;Col10a1-Cre+;Rosa26YFP/+, Ctnnb1LOFHTC = Ctnnb1LacZ/fl;Col10a1-Cre+;Rosa26YFP/+). (B, E) **P-value <0.01; *** P-value < 0.001, ± s.d.; the n of specimens analyzed is indicated by the numbers in the bars.
Fig. 7 Alteration in chondrocyte-derived osteoblast and osteoclast numbers in P28 Ctnnb1\textsuperscript{GOFHTC} specimens.

(A) Representative images of control and Ctnnb1\textsuperscript{GOFHTC} specimens below the chondro-osseous front at the proximal humeral growth plate. Immunofluorescent staining for Osx (red), YFP (green), and DAPI for nuclei (blue). The dashed line indicates the position of the chondro-osseous border. (B) TRAP staining on control and Ctnnb1\textsuperscript{GOFHTC} littermates. Asterisks mark the osteoclasts at the chondro-osseous border. (C) Bar graph showing the mean value of the percentage of YFP\textsuperscript{+ (total)} (= progeny of Col10a1-Cre expressing HTCs) and YFP\textsuperscript{+;Osx\textsuperscript{+}} (chondrocyte-derived osteogenic population) cells determined within the area 250 $\mu$m below the chondro-osseus front. (D) Bar graph showing the mean number of osteoclasts lining the chondro-osseous front (marked by asterisks in B) in humerus and femur of control and Ctnnb1\textsuperscript{GOFHTC} specimens. (C,D) The n of the samples analyzed is indicated by the numbers in the bars; ± s.d. Genotypes: control = Col10a1-Cre\textsuperscript{+;Rosa26\textsuperscript{YFP/+}}, Ctnnb1\textsuperscript{GOFHTC} = Ctnnb1\textsuperscript{ex3β;Col10a1-Cre\textsuperscript{+;Rosa26\textsuperscript{YFP/+}}.}
Fig. 8 Summary model.

Schematic summary of the involvement of β-catenin in late HTCs: here it locally regulates the Rankl:Opg ratio controlling osteoclastogenesis and negatively influences the expression of Mmp13 and Opn. Inactivation of Ctnnb1 from late HTCs leads to an increased Rankl:Opg ratio, increased expression of Mmp13 and Opn and increased osteoclast numbers but simultaneously affects the differentiation of osteoblasts derived from HTCs. Thus, this leads to a severe defect in trabecular bone formation.

Stabilization of β-catenin results on the one hand in a decrease in the Rankl:Opg ratio and a subsequent reduction in osteoclast number and, on the other hand, affects terminal differentiation of HTCs reflected in the absence of Mmp13 and Opn and their overall reduced expression while anti-angiogenic factors are increased in their expression. Thus, endochondral bone formation is severely impaired upon stabilization of β-catenin in HTCs, due to impaired cartilage resorption and bone marrow formation.
Supplementary Material and Methods

Mouse husbandry

Conditional Ctnnb1 loss-of-function mutant mice (Ctnnb1\textsuperscript{LacZ/flo},Col10a1-Cre\textsuperscript{+} = Ctnnb1\textsuperscript{LOFHTC}) were generated by intercrossing Ctnnb1\textsuperscript{flo/flo} females with males double heterozygous for Ctnnb1 LacZ knock-in null (Huelsken et al., 2000) and BAC-Col10a1-Cre (Col10a1-Cre, #1465 and #1421) transgenic alleles (Gebhard et al., 2008). Conditional Ctnnb1 gain-of-function mutant mice (Ctnnb1\textsuperscript{ex3flo/+};Col10a1-Cre\textsuperscript{+} = Ctnnb1\textsuperscript{GOFHTC}) were generated by intercrossing females homozygous for the Ctnnb1 exon3 floxed allele (Harada et al., 1999) with Col10a1-Cre (#1465 and #1421) heterozygous males.

For genetic restoration experiments, either females double-homozygous for Opg/Tnfrsf11b knock-out (Mizuno et al., 1998) and Ctnnb1 exon3 floxed alleles were crossed with males double-heterozygous for the Opg knock-out and Col10-Cre transgenic alleles, or males double-homozygous for the floxed Rankl/Tnfsf11 (Xiong et al., 2011) and Ctnnb1 alleles were crossed to Rankl\textsuperscript{flo/+};Ctnnb1\textsuperscript{LacZ/+};Col10a1-Cre\textsuperscript{+} females. Homozygous Opg (Mizuno et al., 1998) and Rank/Tnfrsf11a\textsuperscript{Δ/Δ} (Hanada et al., 2009) mutants were generated by the intercrossing of heterozygous animals, conditional Rankl mutants by the intercrossing of Rankl\textsuperscript{flo/flo} males with Rankl\textsuperscript{flo/+};Col10a1-Cre\textsuperscript{+} females.

For quantitative gene expression analysis in sorted HTCs and for the transdifferentiation assays, the following crosses were set up: for conditional Ctnnb1\textsuperscript{LOFHTC} embryos, males double heterozygous for Ctnnb1 LacZ-null and Col10a1-Cre alleles were crossed with females double-homozygous for Rosa26-EYFP (Srinivas et al., 2001) and Ctnnb1 floxed alleles. For controls and
Ctnnb1GOFHTC embryos, males heterozygous for the Col10a1-Cre transgenic allele were crossed with females homozygous for the Rosa26-EYFP transgene or with females double homozygous for the Rosa26-EYFP transgene and the Ctnnb1 exon3 floxed allele, respectively.

Microcomputed tomography (microCT) and histomorphometric analysis
Scanning was carried out at 40 kV, 600 µA, w/o filter, 0.5° rotation steps, an image pixel size of 8.52 µm and an exposure set to 525 ms. Reconstruction of sections was carried out with software associated with the scanner (Nrecon v1.6.9.8) with beam hardening correction set to 40%. Analysis of trabeculae in P28 specimens was carried out using the CT Analyzer v1.13.11.0 software, at a region from 852.35 µm to 1707.7 µm from the growth plate.

Processing of specimens
For histology, immunohistochemistry, and immunofluorescent stainings, the limbs of embryos, newborn (P0) and three-day-old (P3) specimens were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated and embedded in paraffin. Bones obtained from four-week-old mice (P28) were fixed for 24 h in 4% paraformaldehyde at 4°C and embedded in paraffin or methyl methacrylate (MMA) (Technovit 9100, Heraeus Kulzer GmbH, Germany). For paraffin embedding, bones were decalcified for one week in 0.5 M EDTA pH 8.0 and dehydrated into xylene. For MMA embedding, fixed bones were washed overnight in PBS containing 10% sucrose, dehydrated (2 days 70% ethanol, 2 days 95% ethanol, twice for 1 day 2-propanol, twice for 2 days xylene) and embedded in methyl methacrylate (MMA).
Histology

For histological stainings sections were deparaffinized and rehydrated into H2O. For alcian blue / von Kossa staining mineralized tissue was stained by incubation of sections in 2% silver-nitrate solution under light exposure (60W bulb) for 1 h, washed with 1% acetic acid and subsequently stained for 15 min in alcian blue staining solution (20 mg alcian blue in 70 ml EtOH, 30 ml acetic acid), tissue was counterstained with eosin, dehydrated and mounted with DPX (Sigma). Alcian blue / eosin staining was performed in principle as described above, omitting the incubation step in silver-nitrate solution. For hematoxylin and eosin (H&E) staining sections were incubated in Mayer’s Haemalaun (Waldeck GmbH & Co. KG, Divison Chroma) for 5 min, washed in running tap water, counterstained with an alcoholic solution of eosin Y (Sigma) for 45 sec, dehydrated and mounted with DPX. For von Kossa / toluidine blue staining on 5 µm MMA sections, sections were deplasticized (two times 20 min xylene, 20 min 2-methoxyethyl acetate, 10 min acetone), rehydrated into H2O and processed as following: sections were stained in 2% silver-nitrate solution under light exposure for 1 h, washed with distilled water, incubated in toluidine blue solution (0.1% toluidine blue O and 0.1% sodium tetraborate in H2O) for 30 sec, dehydrated and mounted with DPX (Sigma). For tartrate-resistant acid phosphatase (TRAP) staining, rehydrated sections were incubated for 1 h at 37°C in freshly prepared TRAP staining solution: buffer (40 mM sodium acetate and 10 mM sodium tartrate, pH 5.0) containing 0.1 mg/ml naphtol AS-MX phosphate disodium salt (Sigma) and 0.6 mg/ml fast red violet LB salt (Sigma). Sections were washed with distilled water, counterstained for 1 min with Mayer’s Haemalaun, washed in running tap water and mounted with Faramount (Dako).
**Immunohistochemistry and Immunofluorescence**

For immunohistochemical stainings deparaffinized and rehydrated sections were treated with 3% H$_2$O$_2$ for 30 min and blocked with appropriate 10% blocking serum for 30 min. Antigen retrieval was achieved by incubating sections in 10 mM sodium citrate (pH 6.0) at 95°C for 20 min for β-catenin, by proteinase K treatment 40 µg/ml for 15 min at 37°C, followed by 10 min at RT for CD31/PECAM-1, and by using chondroitinase ABC (Sigma, #C3667, 2 mU/ml in PBS (pH 8.0)) at 37°C for 2 h for the aggrecan neoepitope DIPEN. No antigen retrieval was required for Opg, Osterix and Sox9. Incubation with the respective primary antibodies was performed either for 1 h at room temperature or overnight at 4°C: mouse anti-β-catenin (1:200, BD Transduction Laboratories, #610154), rat anti-mouse CD31 (1:25, BD Pharmingen, #550274), rabbit anti-mouse DIPEN (1:1000, clone BC4, a gift from Professor Amanda Fosang, University of Melbourne, Australia), biotinylated goat anti-mouse OPG/TNFRSF11B (15 µg/ml, R&D systems, #BAF459), rabbit anti-mouse Sp7/Osterix (1:500, Abcam, #ab22552) and rabbit anti-mouse Sox9 (1:1000, Millipore, #AB5535). Sections were subsequently incubated with the appropriate biotinylated secondary antibody (Vector Laboratories) for 1 hour at room temperature followed by color development with the Vectastain Elite ABC Kit (Vector Laboratories) and DAB substrate (Sigma). For Osterix and Sox9, HRP-conjugated secondary antibodies were used (Promega). Immunostaining for Collagen type II was performed with a mouse anti-chicken Col II antibody (1:25, Chemicon, #MAB8887) using the Dako ARK Peroxidase kit for Mouse Primary Antibodies (Dako, #K3954) according to the manufacturer’s instructions. Antigen retrieval for Col II was achieved by incubating sections with 5 µg/ml proteinase type XXIV (Sigma, #P8038) for 10 min at 37°C followed by 10 µg/ml hyaluronidase type 1-S (Sigma, #H3506) for
30 min at 37°C. For double immunofluorescence, sections were incubated with rabbit anti-mouse Sp7/Osterix (1:250, Abcam, #ab22552), rabbit anti-mouse Runx2 (1:50, Abcam, #ab23981), rabbit anti-mouse Sox9 (1:1000, Millipore, #AB5535), rabbit anti-human Ki67 (1:1000, Novocastra Laboratories, #NCL-Ki67p), rabbit anti-mouse Cleaved Caspase-3 (Asp175) (1:20, Cell Signaling, #9661) or rabbit anti-mouse FABP4 (1:500, Abcam, #ab13979). Previous heat-mediated antigen retrieval was achieved by incubation in 10 mM sodium citrate (pH 6.0) at 95°C for 20 min for Runx2, Ki67 and FABP4, and by Tris/EDTA-Buffer (pH 9.0) at 95°C for 15 min for cleaved Caspase-3. To detect TUNEL-positive cells sections were pretreated for 15 min at 37°C with 20 µg/ml proteinase K in 10 mM Tris/HCl, pH 7.5. TUNEL assay was performed using the In situ Cell Death Detection Kit from Roche (Roche, #11684795910) according to the manufacturer’s instructions. After visualization of the first primary antibody with Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes), sections were incubated with chicken anti-mouse GFP (1:500, Abcam, #ab13970) or rat anti-mouse Endomucin (1:50, Santa Cruz, #sc-65495) followed by detection with Alexa Fluor 488 goat anti-chicken IgG or Alexa Fluor 488 goat anti-rat IgG (Molecular Probes). Nuclei were counterstained with DAPI. Antibody specificity was confirmed using the respective IgG or IgY isotype control antibodies.

**RNA isolation from FACS sorted primary HTCs and total skeletal elements**

E16.5 or P0 limbs were dissected in PBS and isolated skeletal elements were sequentially digested with 0.3% Trypsin (Invitrogen, GIBCO, #15090-046) for 30 min at 37°C, washed with culture medium (DMEM/F12, 10% FCS), followed by digestion with 0.1% collagenase type 1-S (Sigma, # C1639) for 3 h at 37°C in a bacterial dish in a tissue culture incubator, while separating the cells every hour by
pipetting. For RNA-isolation from whole P0 skeletal elements, cells were collected, washed twice with PBS, filtered through a cell strainer and cell pellets were stored at -80°C until RNA isolation. For cell sorting of YFP positive cells from E16.5 skeletal elements, fresh culture medium was added and the cells were incubated overnight. Prior to cell sorting, cells were washed with PBS/2% FCS, debris was removed using a cell strainer and the cell suspension was subjected to FACS sorting for transgenic, endogenous YFP fluorescence by the staff of the BioOptics unit of the IMP (Vienna) using a FACS Aria III (BD Biosciences, Franklin Lakes, New Jersey, USA). FACS sorted E16.5 hypertrophic cells were washed twice with PBS and either frozen or directly processed. Total RNA was extracted from the respective cell pellets using the RNeasy Micro Kit (Qiagen, #74004) or RNeasy Mini Kit (Qiagen, #74106) according to the manufacturer’s instructions, including a DNase I treatment. RNA concentration was determined by OD measurement on a nano-drop 2000 (Thermo Scientific, Waltham, Massachusetts, USA) or a BioPhotometer plus (Eppendorf AG, Hamburg, Germany).

cDNA synthesis

For first-strand cDNA synthesis, 1 µg of total RNA from FACS sorted primary material was reverse transcribed by using Oligo(dT)₁₂-₁₈ and SuperScript II Reverse Transcriptase (Invitrogen, #18064-022). Reverse transcriptase was inactivated at 70°C for 15 min and RNA in RNA:DNA duplex was digested by incubation with RNaseH (Roche, #10786357001) for 20 min at 37°C. A total of 500 ng RNA from whole P0 skeletal elements was reverse transcribed using PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa, #RR037A) with oligo dT primer. For quantitative RT-PCR, cDNA was diluted 1 in 10 with distilled water.
Real-time PCR analysis

2 µl of 1 in 10 diluted 1st strand cDNA of the RNA from E16.5 sorted chondrocytes were mixed with Fast Start SBG master mix (Roche, #04673484001) in a total reaction volume of 25 µl and quantitative PCR was performed using the Bio-Rad CFX96 cycler. Reaction conditions were 95°C 10 min, 45x (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), 72°C 5 min, plate read at 80°C, melting curve: from 55°C to 99°C, in 0.5°C increments for 5 sec + plate read. For the RNA from whole P0 skeletal elements SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, #RR820Q) and 3 µl of 1 in 10 diluted cDNA in a final volume of 25 µl were used. Gene expression was monitored using a BioRad CFX96 cycler and following protocol: 95°C 30 sec, 45x (95°C 15 sec, 60°C 30 sec, 72°C 20 sec + plate read), 95°C 10 sec, melting curve: 55°C to 99°C, in 0.5°C increments for 5 sec + plate read. For quantitative real-time PCR, each reaction was performed in at least in duplicate and repeated using independent sample sets. Products were analyzed by agarose gel electrophoresis and melting curves. Values were calculated using the comparative ΔC(t) method and normalized to the expression levels of housekeeping genes (Hprt for sorted HTCs and Hprt, Actb for whole skeletal elements). For primer sequences, product size, and Genbank accession number see Table S1.
| Gene | Sequence | Product size (bp) | GenBank Access No. |
|------|----------|------------------|-------------------|
| Mmp13 | 5’-TTCTGGTCTTTCTGGCACACGCTTT-3’ 5’-CCAAAGTCTATGGCAGCAAGAAATA-3’ | 132 | NM_008607 |
| Vegf | 5’-AAGGAGACAGAAAGATCCATGA-3’ 5’-CTGTAATTGAGGCATGACTGCT-3’ | 74 | NM_009505 |
| Sox9 | 5’-AACAGCCACACGTCGACG-3’ 5’-AAGGCTCTCTCTCTCCCTC-3’ | 161 | NM_011448 |
| Ihh | 5’-AGCGCTCTCAAGAGGCAGCCTC-3’ 5’-CATCTCTCATCCGCCATCTCTC-3’ | 184 | NM_010544 |
| Opg | 5’-GAAGCCACGCAAAATGTTG-3’ 5’-CTACACTCTCGGCATCTTTG-3’ | 145 | NM_008764 |
| Opg* | 5’-CCAAAGACCCAGTCTTTCT-3’ 5’-CAAAGACCCAGCTTTGTAAT-3’ | 115 | NM_008764 |
| Rankl | 5’-GGCCACAGCCGCTCTCAG-3’ 5’-GAGTGACTTTATGGGAACCCGAT-3’ | 144 | NM_011613 |
| Rankl* | 5’-CCAAAGATCTCTCTCTTGACG-3’ 5’-CAGGAACTTTATGGGAACCCGAT-3’ | 139 | NM_011613 |
| Opn | 5’-CCCATCTCAGAAGAGAGCTC-3’ 5’-TTCCATCTCAGAAGACCTC-3’ | 187 | NM_009263 |
| Opn* | 5’-GATGATGATGAGCAATGGAG-3’ 5’-CGACTCTAGAGATGCAATGGAG-3’ | 147 | NM_009263 |
| ChM-I | 5’-TCCTTGAACTCTCTGCTGGCCTA-3’ 5’-GAGGCAGTTCTCTGCTGGCCTA-3’ | 99 | NM_010701 |
| Timp-1 | 5’-CTATAGTGCTGCTGTGATGGA-3’ 5’-TTCCATCTCAGAAGACCTC-3’ | 150 | NM_011593 |
| Timp-2 | 5’-CCAGAGAGAGAGCTCGTGGCCTA-3’ 5’-GTCCATCTCAGAAGACCTC-3’ | 112 | NM_011594 |
| Timp-3 | 5’-GGCCCTGCTCTTGCGTGGACCTC-3’ 5’-CTGATAGGCCAGCTTGACCTC-3’ | 135 | NM_011595 |
| Hprt | 5’-AGCTACTGTAATATGATGATCAACG-3’ 5’-AGAGGCTCTCTCTCCACGCA-3’ | 198 | NM_013556 |
| Actb | 5’-TTGCTGACAGTTGACGAGGAG-3’ 5’-ACTCTCTGCTGCTGTGAGGAG-3’ | 159 | NM_007393 |

**Supplementary Table 1**

Primer sequences for real-time PCR. All primers span exon-intron boundaries with the exception of primers marked with an asterisk used for amplification on YFP-sorted E16.5 material.
Supplementary Figures

Fig. S1 Phenotypic analysis of Ctnnb1\textsuperscript{LOFHTC} mutants.

(A-F) Representative images of alternating sections through E18.5 humeri of control (Ctnnb1\textsuperscript{fl/+};Col10a1-Cre\textsuperscript{−/−}) and Ctnnb1\textsuperscript{LOFHTC} (Ctnnb1\textsuperscript{LacZ/fl};Col10a1-Cre\textsuperscript{−/−}) littermates. (A) In situ hybridization for Mmp13 showing increased Mmp13 signal at the chondro-osseous front and less staining in the region of the primary spongiosa in the mutant compared to the littermate control. (B) In situ hybridization for Opn showing a slight increase in Opn staining at the chondro-osseous front and less staining in the region of the primary spongiosa in the mutant compared to the littermate control. (C) In situ hybridization for Vegf showing no obvious difference between mutant and littermate control. (D) In situ hybridization for Ihh showing a slightly more intense signal in the mutant compared to the littermate control. (E) In situ hybridization for Col10a1 showing no obvious difference between mutant and littermate control. (F) In situ hybridization for Ctsk reflecting an increase in osteoclasts at the chondro-osseous border in the mutant compared to the littermate control. (G) In situ hybridization for Mmp9 on sections through P0 humeri showing a slight increase in Mmp9 positive cells at the chondro-osseous front in the mutant compared to the littermate control. (H) In situ hybridization for Opn on sections through E16.5 humeri showing a slight increase in the signal at the chondro-osseous front in the mutant compared to the littermate control. The arrowhead marks the position of the chondro-osseous front.
**Fig. S2 Extended phenotypic analysis of Ctnnb1<sup>GOFHTC</sup> mutants**

(A-C) Double fluorescent in situ hybridization for *Col10a1*, *Col1a1*, and *Ppr1* on E16.5 (A,B) and P0 (C) humeri of control (*Ctnnb1<sup>ex3fl/+;Col10a1-Cre</sup>*) and *Ctnnb1<sup>GOFHTC</sup> (<em>Ctnnb1<sup>ex3fl/+;Col10a1-Cre</sup></em>) specimens. Single channels and merged images are shown. **(A)** The signals for *Col10a1* (green) and *Col1a1* (red) are restricted to the HTC zone and the bone collar / trabecular bone, respectively in the control, while *Col1a1* positive cells are also found in the central most chondrogenic region of the *Ctnnb1<sup>GOFHTC</sup> humerus. Note: the *Col10a1* and the *Col1a1* signals in the chondrogenic regions hardly overlap - only a few yellow cells can be seen (white arrow heads). **(B)** The signals for *Col1a1* (red) and *Ppr1* (green) overlap in the control in the bone collar and trabecular bone. In the *Ctnnb1<sup>GOFHTC</sup> mutant, the signals also overlap in the central most chondrogenic region (asterisk). *Ppr1* is also expressed in prehypertrophic chondrocytes (PH). **(C)** Double fluorescent in situ hybridization for *Col10a1* and *Col1a1* on P0 humeri, confirming the minimal coexpression of the two markers at the transition from *Col10a1* to *Col1a1* positive cells in the central chondrogenic region in the *Ctnnb1<sup>GOFHTC</sup> specimens.
**Fig. S3 Phenotypic characterization of \textit{Ctnnb1}^{GOFHTC} at postnatal stage P28.**

(A) \textit{Ctnnb1}^{GOFHTC} mutant mice are smaller than their control (\textit{Ctnnb1}^{ex3fl/+};\textit{Col10a1}-Cre) littermates. (B) Image of a humerus showing a reduction in the overall length and partial loss of hematopoiesis in the mutant compared to the littermate control. (C) Composite toluidine blue / von Kossa stained images showing sections through P28 femora of \textit{Ctnnb1}^{GOFHTC} and control littermates. In the mutant, there is an abnormal dense mineralized structure visible in the proximal region. (a-b') show higher magnification of the boxed proximal and distal growth plate regions revealing an expansion of the mineralized hypertrophic region in the mutant (a', b') compared to the littermate control (a, b). (D) Histomorphometric quantification of the bone volume (BV) to total volume (TV), trabecular thickness (Tb. Th), trabecular number (Tb. N) and trabecular spacing (Tb. Sp) of P28 distal femora regions comparing \textit{Ctnnb1}^{GOFHTC} (n=8) and littermate controls (n=9). **P-value < 0.01, ***P-value < 0.001, ± s.d.
Fig. S4 Characterization of the Opg and Rank-deficient specimens.

(A) Histological and marker analysis on sections through P0 humeri of Opg mutants and littermate controls (Opg+/+): showing a slight reduction in trabecular bone formation compared to the wild type control visualized by von Kossa staining, no difference in the width of the hypertrophic zone visualized by the Col10a1 in situ hybridization, and no obvious increase in osteoclasts visualized by Ctsk in situ hybridization. (B) Histological and marker analysis on sections through P0 humeri of germ line deleted Rank mutants and littermate controls (Rank−/−): showing an increase in mineralized structures particularly in the diaphysis compared to the wild type control visualized by the von Kossa staining. Col10a1 in situ hybridization revealed an increased width of the hypertrophic zone in the mutant compared to wild type littermate. Osteoclasts were completely absent based on the Ctsk in situ hybridization while Mmp9 positive cells are still present and primarily localized at the chondro-osseous junction.
Fig. S5 Characterization of Ctnnb1\textsuperscript{LOFHTC};Rankl\textsuperscript{HTC} specimens.

(A) Representative microCT images of humeri from control, Ctnnb1\textsuperscript{LOFHTC} (Ctnnb1\textsuperscript{LacZ/fl};Col10a1-Cre\textsuperscript{+}), Ctnnb1\textsuperscript{LOFHTC};Rankl\textsuperscript{HTC} (‘complete’ and ‘locally’ restored) (Ctnnb1\textsuperscript{LacZ/fl};Rankl\textsuperscript{fl/fl};Col10a1-Cre\textsuperscript{+}), and Rankl\textsuperscript{HTC} (Rankl\textsuperscript{fl/fl};Col10a1-Cre\textsuperscript{+}) specimens. The red line shown in the control humerus outlines the quantified area in an exemplary manner. Note: the ‘locally restored’ Ctnnb1\textsuperscript{LOFHTC};Rankl\textsuperscript{HTC} specimen show only a local increase in the width of the dense area right around the chondro-osseous junction compared to the Ctnnb1\textsuperscript{LOFHTC} specimen (indicated by the double arrow in the insets).

(B) Table showing the mean values of the BV/TV in % of control, Ctnnb1\textsuperscript{LOFHTC}, Ctnnb1\textsuperscript{LOFHTC};Rankl\textsuperscript{HTC} (‘complete’ and ‘local’), and Rankl\textsuperscript{HTC} specimens (± s.d.), on which the dot plot shown in Fig. 5A is based.

(C) Representative images of sections through humeri of control, Ctnnb1\textsuperscript{LOFHTC}, Ctnnb1\textsuperscript{LOFHTC};Rankl\textsuperscript{HTC} (‘complete’ and ‘local’), and Rankl\textsuperscript{HTC} specimens, hybridized with Ctsk and Col10a1 probes showing that the increase in Ctsk positive cells in the Ctnnb1\textsuperscript{LOFHTC} mutant is corrected by the additional loss of Rankl in HTCs in the Rescue\textsuperscript{Rankl\textsuperscript{HTC} mutants. Note: no obvious differences in the number of Ctsk positive cells are visible comparing the Rankl\textsuperscript{HTC} mutants with control. The width of the HTC zone is not significantly altered in any of the mutants compared to the control (Ctnnb1\textsuperscript{fl/+};Col10a1-Cre\textsuperscript{+}).
Fig. S6 Effects of the loss of Ctnnb1 in HTCs on chondrocyte-derived osteoblastogenesis.

Representative fluorescent images of control and Ctnnb1/LOFHTC P0 proximal growth plates triple-stained for Sox9/YFP/DAPI on the left, TUNEL/YFP/DAPI in the middle, and Ki67/YFP/DAPI on the right. PH: prehypertrophic zone, H: hypertrophic zone. The dashed lines in the middle and right images indicate the position of the chondro-osseous front. Genotypes: control = Ctnnb1fl/fl; Col10a1-Cre+; Rosa26YFP/+, Ctnnb1/LOFHTC = Ctnnb1LacZ/fl; Col10a1-Cre+; Rosa26YFP/+.

Fig. S7 Increased adipogenesis in Ctnnb1/LOFHTC mutants

(A) Images of H&E stained sections of four-month-old control (Ctnnb1fl/fl; Col10a1-Cre+) and Ctnnb1/LOFHTC femora showing an increase in adipocytes in the bone marrow of the mutant (whitish, round cells). (B) Representative composites of three images each of control (Ctnnb1fl/fl; Col10a1-Cre+; Rosa26YFP+) and Ctnnb1/LOFHTC (Ctnnb1LacZ/fl; Col10a1-Cre+; Rosa26YFP+) P0 specimens taken from the region below the chondro-osseous front. Immunofluorescent staining for FABP4 (red), YFP (green), and DAPI for nuclei (blue), showing an increase in cells that are positive for FABP4 but negative for YFP in the Ctnnb1/LOFHTC primary spongiosa. The dashed line indicates the position of the chondro-
osseous border. (C) Higher magnification images of the boxed regions in (B) with only the FABP4 (red) and YFP (green) signals shown. Note: There are no yellow FABP4⁺;YFP⁺ cells visible. (D) Immunofluorescent staining for FABP4 (red), endomucin (green), and DAPI (blue) showing that the FABP4-positive cells are closely associated with endomucin-positive blood vessels.