Supporting Information

Khan et al. 10.1073/pnas.1714313114

SI Materials and Methods

Generation of *Gnas^{R201H}* Conditional "Knockin" Mice. The BAC clone RPCI23-312C1 containing the *Gnas* gene was purchased from Invitrogen. As shown in Fig. S1, a DNA fragment containing exons 4 to 12 of the *Gnas* gene was retrieved from the BAC clone and inserted into the targeting vector using a recombineering method. Next, we cloned a minigene cassette containing a 250-bp upstream sequence of exon 7, a partial cDNA sequence containing exons 7 to 12, and three consecutive polyA sequences. The minigene cassette was inserted into intron 6 of the *Gnas* gene and flanked by LoxP sequences. The mutation in exon 8 corresponding to human R201H was generated by changing a single nucleotide (CGT to CAT). The targeting vector containing the wild-type minigene cassette and the FD mutation was used in mouse ES cells along with Cas9 and a guide RNA to increase targeting efficiency. Correctly targeted ES clones were identified by both 5′ and 3′ long-range PCR, and sequencing and mouse lines were generated by blastocyst injection of the targeted ES cells according to standard protocols. One of the correctly targeted ES clones was used to generate the *Gnas$^{f(R201H)}$* mice, and two independent founder lines gave rise to the same phenotypes in subsequent experiments.

***Gnas$^{f(R201H)}$* Genotyping PCR.** PCR amplification of the conditional allele and wild-type allele was done in the same reaction mixture using the following primers: forward-1, ATGCGGCGCGCCACTTTTGCCTTTTGCAGGACTGAG; forward-2, CTTTGGGGATCACACCACTT; and reverse, AGCTTGGCGTGAAGTTCCTATTCTC. PCR amplification conditions used were as follows: step 1, 94 °C for 3 min; step 2, 94 °C for 30 s; step 3, 58 °C for 30 s; step 4, 72 °C for 1 min; and step 5, cycle 35 times to step 2.

μCT Scanning. μCT scanning of long bones was performed using a SCANCO μCT 35.

Tamoxifen Injection. Tamoxifen (1 mg) was injected into the lactating mother in our *Sox9CreER; Rosa26Tdtomato; Gnas$^{f(R201H)}$* mating at postnatal day 5 (P5), and the pups were collected at P21 for analysis.

Skeletal Preparation: Alizarin Red and Alcian Blue Staining. Embryos were harvested and skinned carefully. Internal organs were then eviscerated, and the embryos were placed in 100% ethanol overnight. Staining was performed according to previously described protocol (18).

von Kossa Staining. Paraffin sections were deparaffinized in xylene and hydrated in distilled water. Then a 5% silver nitrate solution was added to the slides and placed under a 60-W lamp for 1 h. Slides were rinsed three times in distilled water. Sodium thiosulfate (5%) was added to the slides for 5 min. Slides were rinsed three times in distilled water and counterstained with nuclear fast red for 5 min. Slides were rinsed three times in distilled water and then dehydrated and cleared in xylene before mounting with Permount.

Alkaline Phosphatase and Alizarin Red Staining. Cells were rinsed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 to 15 min. After washing with PBS, cells were stained with NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) (purchased from Thermo Fisher Scientific; 34042) for alkaline phosphatase activities or with 1% alizarin red (pH 4.2) in water for 20 min followed by PBS washing (three times). Quantification of alizarin red staining was performed as described before (71).

Antibodies Used for Immunohistochemistry. Osx (ab22552; Abcam), Opn (AF808; R&D), Col10 (X-AC9; DSHB), αSMA (A5228; Sigma), CD31 (550274; BD Biosciences), p-Creb (06-519; Millipore), and β-catenin (610154; BD Biosciences).

Antibodies Used for Western Blotting. $G\alpha_s$ (sc-55546; Santa Cruz Biotechnology), Creb (sc-25785; Santa Cruz), p-Creb (06-519; Millipore), β-catenin (610154; BD Biosciences), αSMA (A5228; Sigma), Gapdh (G8795; Sigma), and α/β-tubulin (2148; Cell Signaling Technology) according to the manufacturers' recommendations.

Quantitative Real-Time PCR. qRT-PCR was performed with SYBR Green Real-Time PCR Master Mix purchased from Thermo Fisher Scientific. Primer pairs used in this study are listed in Table S1.

Fig. S1. Generation of the *Gnas*$^{f(R201H)}$ conditional knockin allele. (*A*) Maps of the genomic locus of mouse *Gnas* gene, targeting vector, and correctly targeted and mutant alleles. Relative position of exons 4 to 12 is indicated by black boxes. The map of the targeting vector shows insertion of a minigene cassette between exons 6 and 7. The R-to-H FD mutation is CGT→CAT in exon 8, which was recreated in the targeting vector. When there is no Cre activity, the targeted *Gnas* allele expresses a wild-type *Gnas* gene containing the minigene cassette. After Cre-mediated recombination, the minigene cassette is deleted and the *Gnas*^{R201H} mutant is expressed instead. (*B*) Representative chromatogram of DNA sequencing of long PCR-positive ES cell clones showing a single-nucleotide base change from G to A. (*C*) PCR analysis of genomic DNA obtained from mouse ES cells or tails. f, *Gnas*$^{f(R201H)}$.

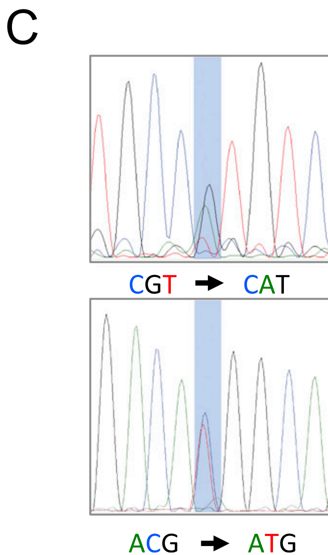
A

```
                 2650      2660      2670      2680      2690      2700      2710      2720
Mouse GNAS mRNA  TTCCCACCTGAATTCTATGAGCATGCCAAGGCTCTGTGGGAGGATGAGGGAGTGCGTGCCTGCTACGAGCGCTCCAATGA  2720
hGNAS mRNA       TTCCCTCCCGAATTCTATGAGCATGCCAAGGCTCTGTGGGAGGATGAAGGAGTGCGTGCCTGCTACGAACGCTCCAACGA  503

Majority         GTACCAGCTGATTGACTGTGCCCAGTACTTCCTGGACAAGATXGAXGTGATCAAGCAGGCXGACTAXGTGCCXAGXGAXC
                 2730      2740      2750      2760      2770      2780      2790      2800
Mouse GNAS mRNA  GTACCAGCTGATTGACTGTGCCCAGTACTTCCTGGACAAGATTGATGTGATCAAGCAGGCCGACTACGTGCCAAGTGACC  2800
hGNAS mRNA       GTACCAGCTGATTGACTGTGCCCAGTACTTCCTGGACAAGATCGACGTGATCAAGCAGGCTGACTATGTGCCGAGCGATC  583

Majority         AGGACCTGCTTCGCTGCCGTGTCCTGACXTCTGGAATCTTTGAGACCAAGTTCCAGGTGGACAAAGTCAACTTCCACATG
                 2810      2820      2830      2840      2850      2860      2870      2880
Mouse GNAS mRNA  AGGACCTGCTTCGCTGCCGTGTCCTGACCTCTGGAATCTTTGAGACCAAGTTCCAGGTGGACAAAGTCAACTTCCACATG  2880
hGNAS mRNA       AGGACCTGCTTCGCTGCCGTGTCCTGACTTCTGGAATCTTTGAGACCAAGTTCCAGGTGGACAAAGTCAACTTCCACATG  663

Majority         TTXGAXGTGGGXGGCCAGCGCGATGAXCGCCGCAAGTGGATCCAGTGCTTCAAXGATGTGACTGCCATCATCTTCGTGGT
                 2890      2900      2910      2920      2930      2940      2950      2960
Mouse GNAS mRNA  TTCGATGTGGGCGGCCAGCGCGATGAGCGCCGCAAGTGGATCCAGTGCTTCAATGATGTGACTGCCATCATCTTCGTGGT  2960
hGNAS mRNA       TTTGACGTGGGTGGCCAGCGCGATGAACGCCGCAAGTGGATCCAGTGCTTCAACGATGTGACTGCCATCATCTTCGTGGT  743

Majority         GGCCAGCAGCAGCTACAACATGGTCATXCGGGAGGACAACCAGACXAACCGCCTGCAGGAGGCTCTGAACCTCTTCAAGA
                 2970      2980      2990      3000      3010      3020      3030      3040
Mouse GNAS mRNA  GGCCAGCAGCAGCTACAACATGGTCATTCGGGAGGACAACCAGACTAACCGCCTGCAGGAGGCTCTGAACCTCTTCAAGA  3040
hGNAS mRNA       GGCCAGCAGCAGCTACAACATGGTCATCCGGGAGGACAACCAGACCAACCGCCTGCAGGAGGCTCTGAACCTCTTCAAGA  823

Majority         GCATCTGGAACAACAGATGGCTGCGCACCATCTCTGTGATXCTXTTCCTCAACAAGCAAGAXCTGCTXGCTGAGAAAGTC
                 3050      3060      3070      3080      3090      3100      3110      3120
Mouse GNAS mRNA  GCATCTGGAACAACAGATGGCTGCGCACCATCTCTGTGATTCTCTTCCTCAACAAGCAAGACCTGCTTGCTGAGAAAGTC  3120
hGNAS mRNA       GCATCTGGAACAACAGATGGCTGCGCACCATCTCTGTGATCCTGTTCCTCAACAAGCAAGATCTGCTCGCTGAGAAAGTC  903
```

B

```
                    IDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETKFQVDKVNFHMFDVGGQRDERRKWIQCFNDV
                           920       930       940       950       960       970       980
Human  GNAS  mR     IDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETKFQVDKVNFHMFDVGGQRDERRKWIQCFNDV 241
Mouse  GNAS  mR     IDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETKFQVDKVNFHMFDVGGQRDERRKWIQCFNDV 2938

                    TAIIFVVASSSYNMVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLAGKSKIEDY
                           990      1000      1010      1020      1030      1040      1050
Human  GNAS  mR     TAIIFVVASSSYNMVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLAGKSKIEDY 311
Mouse  GNAS  mR     TAIIFVVASSSYNMVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVLAGKSKIEDY 3148
```

C



D

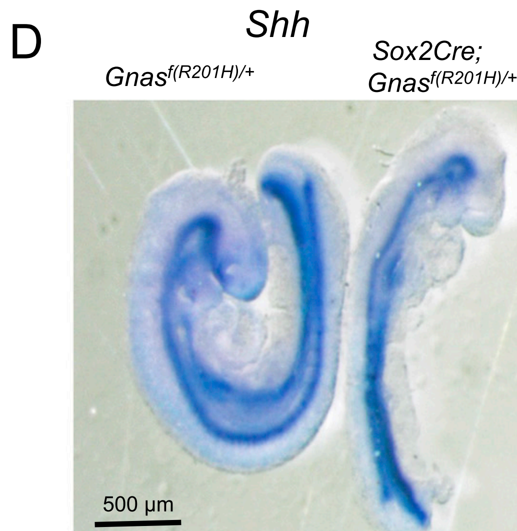


Fig. S2. (Continued)

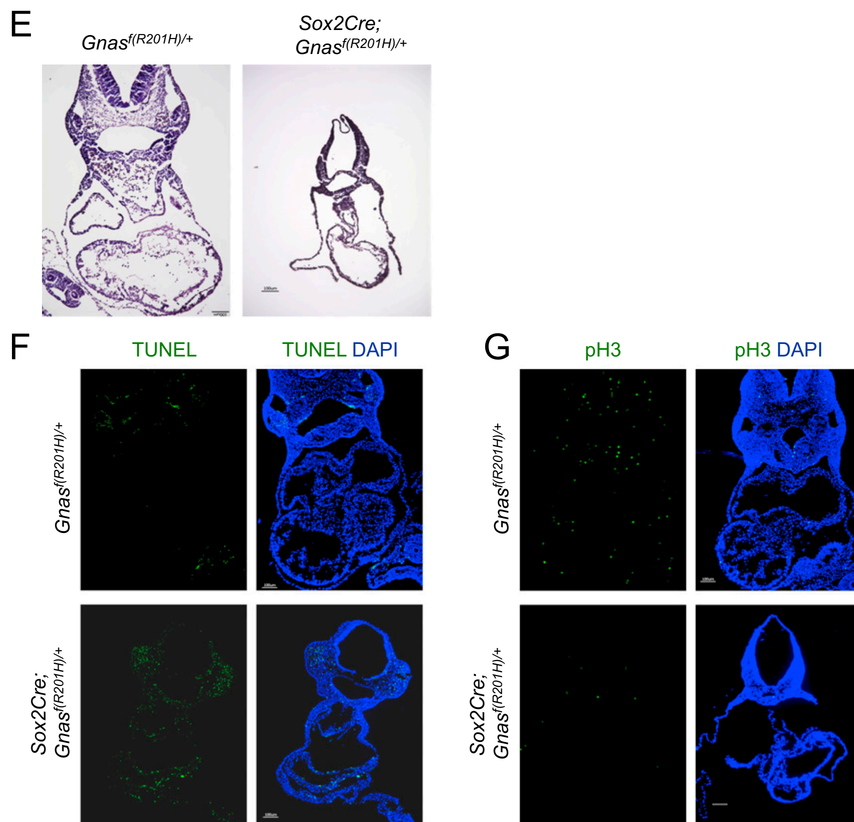


Fig. S2. Sequence identity between mouse and human Gnas. (*A*) Nucleotide sequence identity between mouse and human *Gnas* coding sequences. The yellow highlighted region indicates exon 8 nucleotide sequence, and the nucleotide highlighted in blue is the mutation site. (*B*) Sequence identity at the amino acid level in exons 7 (highlighted in yellow on left), 8 (not highlighted region; amino acid highlighted in blue is the site of mutation), and 9 (highlighted in yellow on right) between mouse and human GNAS protein. (*C*) Representative chromatogram of DNA sequencing of cDNA, prepared from total RNA isolated from an E10.5 *Sox2Cre; Gnas*$^{f(R201H)}$ embryo, showing a single-nucleotide base change. (*D*) Whole-mount mRNA in situ hybridization of *Shh* in E9.5 *Gnas*$^{(R201H)/+}$ littermate control and mutant embryos. (Scale bar, 500 μm.) (*E*) Representative H&E staining of embryo sections at E10.5 showing retarded heart development of the mutant embryo. (*F*) Representative images of TUNEL staining in control and mutant embryo sections at E10.5. (*G*) Representative images of the proliferation marker pH3 in control and mutant embryos at E10.5. (Scale bars, 100 μm.)

Fig. S3. Histomorphometric analysis of long bones from P21 mice. (*A*) Dorsal view of 3- and 8-wk-old littermate control [*Gnas*$^{f(R201H)/+}$] and mutant [*Prrx1-Cre; Gnas*$^{f(R201H)/+}$] mice. While the limbs were shortened significantly, the body length was not altered in the mutant mice. (*B*) X-ray images of forelimbs and hind limbs of 3- and 8-wk-old littermate control and mutant mice. White arrows indicate translucent X-ray areas of cartilage tissue. (*C*) Whole-mount skeletal preparation of trunk bones. (Magnification: 5×.) (*D*) μCT scans of humerus from 2- and 4-mo-old littermate control [*Gnas*$^{f(R201H)/+}$] and mutant [*Prrx1-Cre; Gnas*$^{f(R201H)/+}$] mice. (*E* and *F*) Toluidine blue staining and quantification by histomorphometric analysis of bone parameters. (*G* and *H*) H&E staining of the growth plate of littermate control and mutant humerus. BV, bone volume; Sp, space; Tb, trabecular; Th, thickness; TV, trabecular volume. (*I*) Quantification of TRAP^{+} cell. (*J*) qRT-PCR analysis of *Rankl* expression. *$P < 0.05$, **$P < 0.001$; data are presented as mean ± SD.

Fig. S4. *Gnas*^{R201H} expression in early limb mesenchyme leads to delayed bone formation at postnatal stages. (*Top*) Whole-mount limb skeleton preparation. (*Middle*) H&E staining of humerus sections (*A*–*C*) and tibia sections (*D*). (*Bottom*) von Kossa staining of the similar sections shown (*Middle*). Arrows indicate enchondroma-like lesions. (Magnification: *A*–*D, Top*, 5×.)

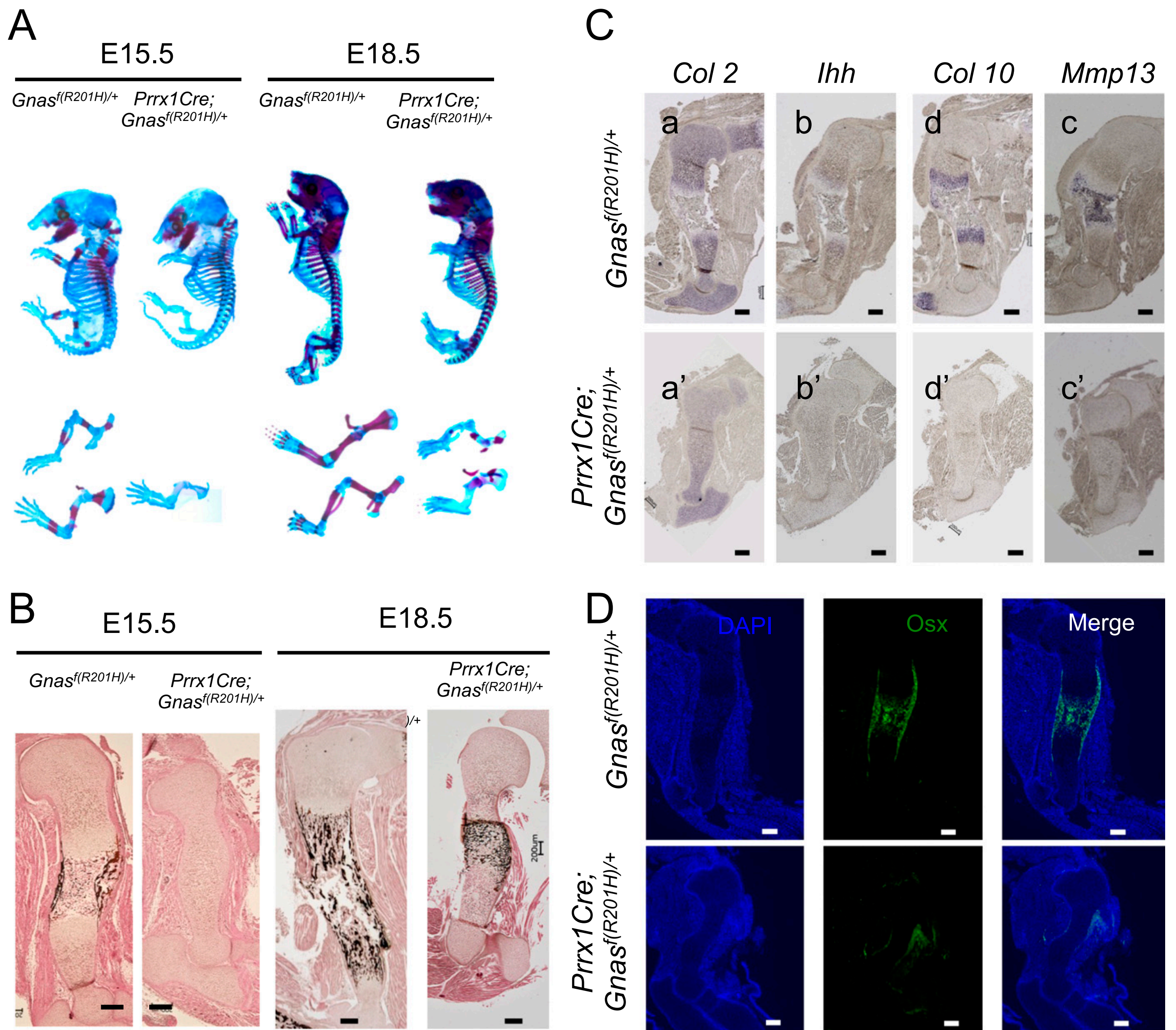


Fig. S5. *Prrx1-Cre; Gnas*$^{f(R201H)/+}$ mutant embryos recapitulate PTHrP/PTH receptor activation phenotypes. (*A*) Alcian blue and alizarin red staining of E15.5 and E18.5 whole embryonic skeleton. Mineralization is delayed in *Prrx1-Cre; Gnas*$^{f(R201H)/+}$ embryos compared with the *Gnas*$^{f(R201H)/+}$ littermate control. (Magnification: 5×.) (*B*) von Kossa staining showing mineralization in E15.5 and E18.5 humerus sections. (*C*) In situ hybridization performed at E15.5 and E18.5 on humerus sections for *Collagen type 2 (Col2)*, *Ihh*, *Collagen type 10* (*Col10*), and *Mmp13* from control (*a*–*c*) and mutant (*a′*–*c′*) embryos. (*D*) Immunofluorescence staining of Osx on E18.5 humerus sections. (Scale bars, 200 μm.)

Fig. S6. (Continued)

Fig. S6. Expression of the *Gnas*^{R201H} allele in Sox9 lineages postnatally leads to bone fibrosis. TM injection was performed at P5 and the mice were analyzed at P21. (*A*) μCT scans of littermate control (*Sox9CreER; RosaTdTomato*) and mutant [*Sox9CreER; RosaTdTomato; Gnas*$^{f(R201H)}$] long bones and quantification of bone parameters. (*B*) H&E staining for littermate control and mutant long bones at P21. TM injection was performed at P5. Boxed areas are magnified (*Bottom*). (Scale bars, 200 μm.) (*C*) Immunofluorescence staining of pCREB in TdTomato$^+$ cells; TdTomato marks *Gnas*$^{(R201H)}$-expressing cells. (*C′*) Quantification of the ratio of pCREB$^+$; TdTomato$^+$ double-positive cells versus TdTomato$^+$ cells. (Scale bars, 50 μm.) (*D*) Western blot analysis of αSMA from BMSCs (7 d after osteogenic differentiation) transduced with Ad-GFP or Ad-Cre. (*E*) Coimmunofluorescence staining of αSMA and β-catenin in littermate control and mutant long bones from P21 mice with the indicated genotypes. (Scale bars, 100 μm.) (*F*) Coimmunofluorescence staining of αSMA and CD31 in littermate control and mutant long bones from P21 mice. (Scale bars, 50 μm.) (*G*) Quantification of the ratio of β-catenin$^+$; TdTomato$^+$ double-positive cells versus TdTomato$^+$ cells, and αSMA$^+$ cells that are not associated with CD31$^+$ cells (αSMA$^+$/CD31$^-$) versus all αSMA$^+$ in littermate control and mutant long bone sections from P21 mice.

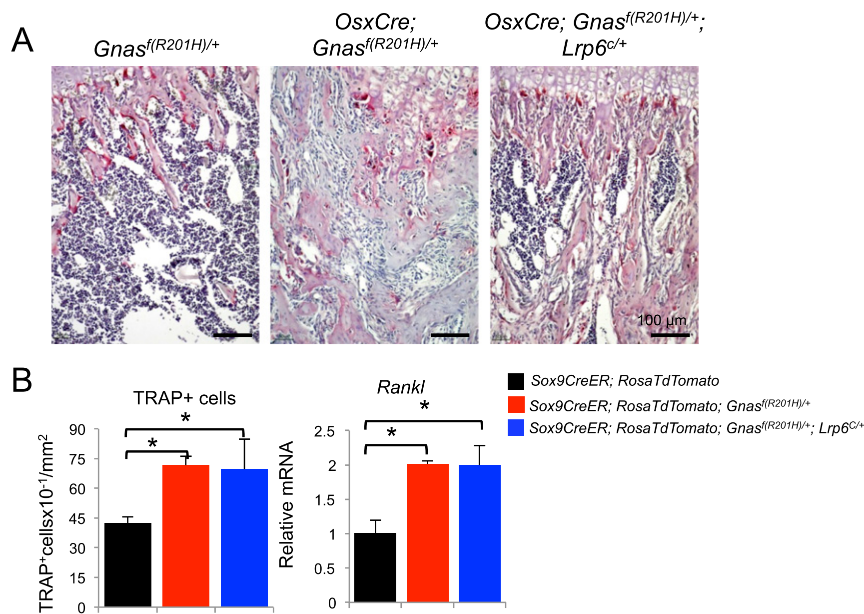


Fig. S7. Osteoclast differentiation was not rescued by *Lrp6* removal. (*A*) TRAP staining in the bone sections of littermate control [$Gnas^{f(R201H)}$] and *OsxCre*-$Gnas^{f(R201H)}$ and *OsxCre*-$Gnas^{f(R201H)}$; $Lrp6^{C/+}$ mutant mice. (Scale bar, 100 μm.) (*B*) Quantification of $TRAP^+$ cells and qRT PCR analysis of *Rankl* from the RNA isolated from bone littermate control and mutant mice ($n = 3$). $^*P < 0.05$; data are presented as mean ± SD.

Table S1. Sequences of primers used in this study

No.	Gene name	Forward primer, 5′–3′	Reverse primer, 5′–3′
1	*β-Actin*	TGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT
2	*Axin2*	GAAGCTGGAGTTGGAGAGCCGCC	TCTCTCTTCATCCTCTCGGATCTGC
3	*Tcf1*	ACATGAAGGAGATGAGAGCCA	CTTCTTCTTTCCGTAGTTATC
4	*Ptch1*	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
5	*Gli1*	GAA AGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
6	*Lef1*	TCTCAAGGACAGCAAAGCTC	CACTTGAGGCTTCATGCACAT
7	*Hip1*	GGGAAAAACAGGTCATCAGC	ATCCACCAACCAAAGGGC
8	*Osx*	CCCACTGGCTCCTCGGTTCTCTCC	GCTGAAAGGTCAGCGTATGGCTTC
9	*ALP*	CACGCGATGCAACACCACTCAGG	GCATGTCCCCGGGCTCAAAGA
10	*Opn*	TACCGGCCACGCTACTTTCTTTAT	GACCGCCAGCTCGTTTTCATCC
11	*Ocn*	ACCCTGGCTGCGCTCTGTCTCT	GATGCGTTTGTAGGCGGTCTTCA