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Supplementary Materials for

The Extracellular Calcium-Sensing Receptor (CaSR) Is a Critical Modulator of Skeletal Development

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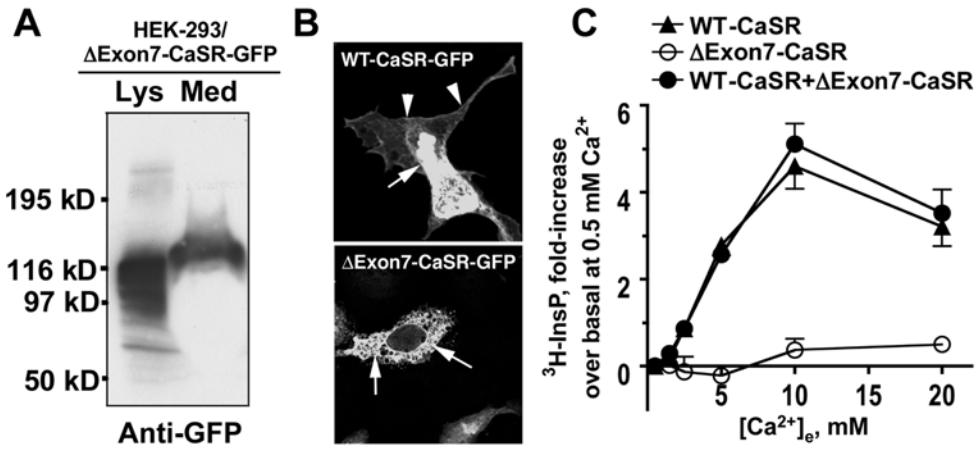

Fig. S1. Δ Exon7-CaSR encoded by exons 1-6 does not insert into the cell membrane and activate signal transduction. (**A**) Immunoblotting of cell lysates and the media from cultured HEK-293 cells expressing cDNA encoding a fusion protein (ΔExon7-CaSR-GFP) of the ΔExon7-CaSR and green fluorescent protein (GFP) with anti-GFP antibody. The ΔExon7-CaSR-GFP (≈120 kD) was found in the culture media (Med) as well as in the cell lysates (Lys). (**B**) Fluorescence confocal microscopy indicated that the ΔExon7-CaSR-GFP was localized only to intracellular compartments (arrows) while the fusion protein (WT-CaSR-GFP) of WT CaSR and GFP was detected in both intracellular organelles (arrows) and cell membranes (arrowheads). (**C**) Production of total ^{3}H-InsPs in response to raising $[Ca^{2+}]_e$ from 0.5 to 20 mM in HEK-293 cells expressing WT-CaSR or ΔExon7-CaSR individually or in co-cultures of these 2 cell populations begun 24 hrs after transfections were done and continued for 48 hrs (N= 3-4 cell transfections). Raising $[Ca^{2+}]_e$ from 0.5 to 20 mM dose-dependently increased the production of total ^{3}H-InsPs -- an index of PLC activation -- in cells expressing WT-CaSR[34]. Signaling responses were absent in cells expressing ΔExon7-CaSR. Furthermore, co-culturing cells expressing WT-CaSR with cells expressing ΔExon7-CaSR did not alter the signaling responses by the former cells. These data indicate that the ΔExon7-CaSR, encoded by exons 1-6, does not mediate PLC activation alone nor does it interfere with the signaling function of WT-CaSRs.

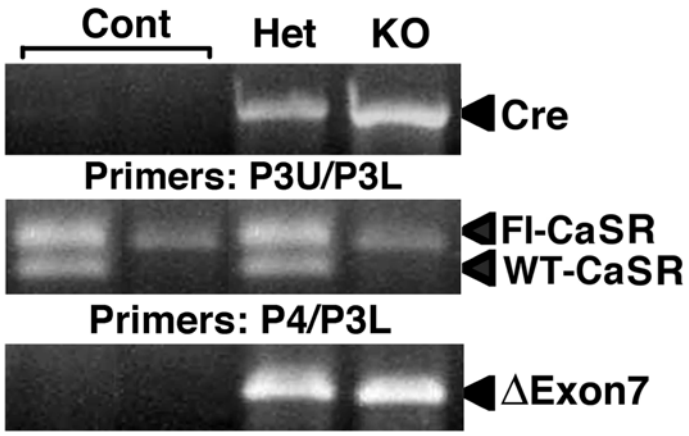

Fig. S2. PCR analyses of genomic DNAs from humeri of Cont, Het ($^{Col\text{-}Bone}CaSR^{WT/\Delta flox}$), and KO ($^{Col\text{-}Bone}CaSR^{\Delta flox/\Delta flox}$) mice for the expression of 2.3Col(I)-Cre transgene, floxed CaSR alleles, and sequences lacking exon 7 (ΔExon7).

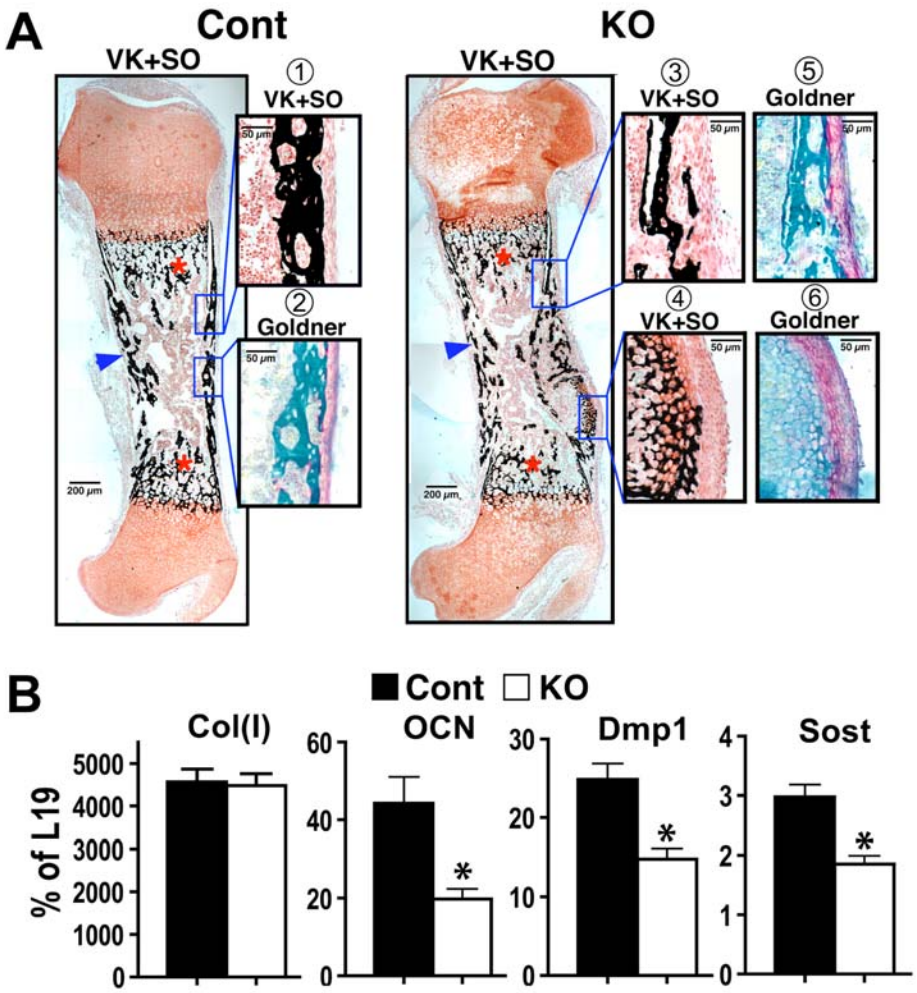

Fig. S3. Knockout of the CaSR in OBs driven by 2.3Col(I)-Cre blocks skeletal development evident in bone from newborn mice. (**A**) Histology of femurs from KO mice showed reduced mineralization (black by VK and green by Goldner staining) in Tb (red asterisks) and Ct (blue arrowheads) bone areas and thinned disorganized cortices as well as increased osteoid accumulation (pink in Goldner staining). VK+SO and Goldner staining was performed on adjacent plastic-embedded sections from newborn Cont and KO femurs and visualized (5x). Boxes in the right panels are enlarged regions of interest (Boxes 1 and 2 for Cont and boxes 3, 4, 5 and 6 for KO mice) (20x). Scale bars: 200 μm (5x); 50 μm (20x). As shown in Box 4, we consistently observed mineralized cartilage nodules stained strongly by SO in the presumed Ct bone area in KO but not in Cont bones, suggesting a delay in bone modeling and in making the transition from cartilage to bone. (**B**) Gene expression of OB markers further indicates delayed OB differentiation in bones from KO vs. Cont mice. qPCR was performed on RNA isolated from humeral cortices (no marrow) from newborn mice with primers for Col(I), DMP1, OCN, and SOST. Results were normalized to L19 (*$p<0.05$; N=4-6 mice per group).

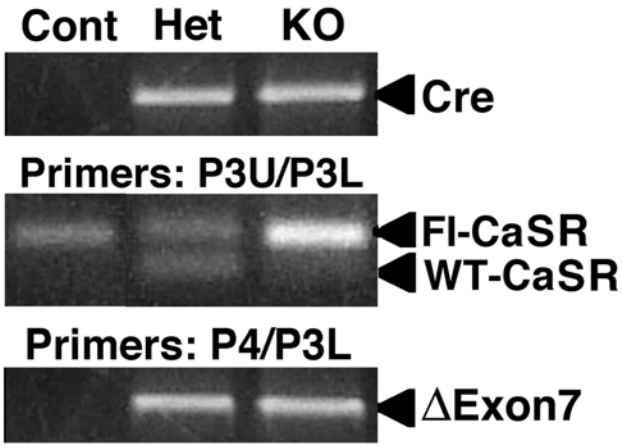

Fig. S4. PCR analyses of tail genomic DNAs from E12.5 Cont, Het ($^{Cart}CaSR^{WT/\Delta flox}$), and KO ($^{Cart}CaSR^{\Delta flox/\Delta flox}$) embryos for the expression of ^{Cart}Cre transgene, floxed CaSR alleles, and sequences lacking exon 7 (ΔExon7).

Table S1. Sequences of primers used for genotyping mice.

	5` primer		3` primer
Cre1	GCAAAACAGGCTCTAGCGTTCG	**Cre2**	CTGTTTCACTATCCAGGTTACGG
P1U	CAGGGATCCGGAAGGGCATCATTG	**P1L**	CTGACTAGGGGAGGAGTAGAAGG
P2U	TCTTGATTCCCACTTTGTGGTTCTA	**P2L**	GATGAATTCGTCTTCCAGCTCGTG
P3U	TGTGACGGAAAACATACTGC	**P3L**	CGAGTACAGGCTTTGATGC
P4	CCTCGAACATGAACAACTTAATTCGG		

Table S2. Sequences of primers and probes used in qPCR assays.

Gene	5` primer	3` Primer	Probe*	GeneBank #
CaSR-E2/3	**CTTTCCTATCCATTTTGGAGTAGCA**	**CTATGGCAAAGATCATGGCTTGT**	**TGGAGTGCATCAGGTATAACTTCCGTGG**	AF110178
OSX	**CCCTTCTGAAGCACCAATGG**	**AGGGTGGGTAGTCATTTGCATAG**	**CAGGCAGTCCTCCGGCCCCG**	NM_130458
Col(I)	**GCGAAGGCAACAGTCGCT**	**CTTGGTGGTTTTGTAATTCGATGAC**	**ACCTACAGCAGGGTTGTGGACGGC**	U08020
ALP	**TCCTGACCAAAAACCTCAAAGG**	**TGCTTCATGCAGAGCCTGC**	**CTGGTGGAAGGAGGCAGGATTGACC**	NM_007431
OCN	**CTCACAGATGCCAAGCCCA**	**GCGCCGGAGTCTGTTCACTA**	**CCCTGAGTCTGACAAAGGTTCTCCACAGC**	NM_007541
IGF-1	**ACAGGCTATGGCTCCAGCA**	**GCACAGTACATCTCCAGTCTCCTC**	**CACCTCAGACAGGCATTGTGGATGAGTG**	NM_010512
IGF-1R	**CACGACGATGAGTGCATGC**	**GAGCAGAAGTCACCGAATCGA**	**ATCCCCTGCGAAGGCCCCTG**	AF056187
AGG	**AAACAACCATGTCCCTGACAGAT**	**GTCCACCCCTCCTCACATTG**	**AGACGGAGTCAACCGTTGCAGACCAG**	NM_007424
Col(II)	**ACTGGTGGAGCAGCAAGAGC**	**GACGTTAGCGGTGTTGGGAG**	**ACGGTGGCTTCCACTTCAGCTATGGC**	NM_031163
Col(X)	**TTATGCTGAACGGTACCAAACG**	**GTTCTCCTCTTACTGGAATCCCTTA**	**CCAAGACACAATACTTCATCCCATACGCC**	NM_009925
OPN	**TGGTGCCTGACCCATCTCA**	**TGCTTGGAAGAGTTTCTTGCTTAA**	**AATCTCCTTGCGCCACAGAATGCTG**	AF515708
RUNX2	**GGCTCTGGCGTTTAAATGGTT**	**GTGCCCTCTGTTGTAAATACTGCTT**	**CCACCGAGACCAACCGAGTCATTTAAGG**	AF010284
L19	**CCAAGAAGATTGACCGCCATA**	**GTCAGCCAGGAGCTTCTTGC**	**CATCCTCATGGAGCACATCCACAA**	NM_009078

*Probes were conjugated with 6-FAM^{TM} and BHQ^{TM} at their 5' and 3' ends, respectively.