Supplemental Methods

Generation of Osx1-Cre, FoxO3$^{f/f}$ mice

To delete FoxO3 from Osx1-expressing cells, Osx1-Cre transgenic mice (mixed background crossed into C57BL/6 for more than 9 generations) were crossed with FoxO3$^{f/f}$ mice (FVBn), a gift from Ronald DePinho (University of Texas, MD Anderson Cancer Center, Houston, Texas) (1), to generate mice that were heterozygous for floxed FoxO3 allele with and without the Osx1-Cre transgene. These mice were intercrossed to generate the experimental wild-type, Osx1-Cre, FoxO3$^{f/f}$ and FoxO3$^{f/f}$;Osx1-Cre mice.

Micro-CT

Micro-CT analysis was done after the bones were dissected, cleaned, fixed in 10% Millonig's formalin and transferred to 100% ethanol, loaded into 10 mm diameter scanning tubes and imaged (μCT40, Scanco Medical) and the vertebral and femoral cancellous bone was analyzed as previously described (2). At medium resolution (nominal isotropic voxel size = 12 μm), 550 slices were acquired and 400 were used for analysis. Scans were integrated into 3-D voxel images (1024 x 1024 pixel matrices for each individual planar stack). A Gaussian filter (sigma = 0.8, support = 1) was applied to all analyzed scans. Key parameters were x-ray tube potential = 55 kVp, x-ray intensity = 145 μA, integration time = 200 ms, and threshold = 200 mg/cm^3. Image processing language scripts including the "cl_image" command were used to obtain the femoral endocortical and periosteal circumference. Micro-CT measurements were expressed in 3-D nomenclature. After a 1-hour warm-up period, calibration and quality control was done weekly using five density standards and spatial resolution was verified monthly using a tungsten wire rod. Beam-hardening correction was based on the calibration records. Corrections were made for 200 mg/cm^3 hydroxyapatite for all energies. Over the past 3 years, the coefficient of variation for the fifth density standard (mean five) was 0.97% (787 ± 7.6 SD mg HA/cm^3) and for rod volume was 2.18% (0.0642 ± 0.0014 SD cm^3).

Quantitative RT-PCR

Taqman quantitative PCR was performed to determine mRNA levels using the primers Mm00490672_m1 (FoxO1); Mm00490673_m1 (FoxO3); Mm00840140_g1 (FoxO4);

Mm00432359_m1 (Ccnd1); Mm00475831_m1 (ALP); Mm00501578_m1 (Runx2); Mm00801666_g1 (Col1a1); Mm00435452_m1 (OPG); Mm00470479_m1 (Sost); Mm00439105_m1 (Cnx43); Mm01210646_m1(Mmp16); Mm00487160_m1 (Col6a1); Mm00440945_m (Pparγ2); Mm01350394_m1 (β-catenin) and Mm00475528_m1 (ribosomal protein S2) manufactured by the TaqMan® Gene Expression Assays service (Applied Biosystems). Osterix, osteocalcin FABP4 and DKK1 levels were determined using custom made Taqman Assay by Design primer sets 5'ATCTGACTTTGCTCCCCTTAACC3' and 5'GGGCCCTGGTTGCAAGA3'; 5'GCTGCGCTCTGTCTCTCTGA3' and 5' TGCTTGGACATGAAGGCTTTG3'; 5'GCGTGGAATTCGATGAAATCA3' and 5'GGGCCCCGCCATCTAG3'; 5' GGGCTGTGTTGTGCAAGACA3' and 5' GGTGCACACCTGACCTTCTTTAA3', respectively.

Supplemental References

1. Paik JH et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell.* 2007;128:309-323.

2. Jilka R.L. et al. Decreased oxidative stress and greater bone anabolism in the aged, as compared to the young, murine skeleton by parathyroid hormone. *Aging Cell.* 2010;9:851-867.

Supplemental Table 1

Effects of FoxO deletion in Osx1-Cre expressing cells in growing mice. Micro-CT measurements in the vertebrae and femurs at four (n=8/group) and seven weeks of age (n=9-11/group). BV/TV, bone volume per tissue volume; Tb, trabecular. Values represent mean s.d., *p<0.05 versus respective FoxO1,3,4$^{f/f}$ by Student's t-test.

		4 weeks		7 weeks	
		FoxO1,3,4$^{f/f}$	FoxO1,3,4$^{f/f}$; Osx1-Cre	FoxO1,3,4$^{f/f}$	FoxO1,3,4$^{f/f}$; Osx1-Cre
Vertebra	BV/TV (%)	27.0 ± 4.5	29.7 ± 2.7	30.8 ± 3.5	35.1 ± 6.2
	Connectivity (/mm^3)	306.0 ± 49.8	304.9 ± 40.2	159.0 ± 15.6	173.0 ± 25.6
	Tb. Number (/mm)	5.4 ± 0.82	5.0 ± 0.23	4.4 ± 0.34	4.6 ± 0.31
	Tb. Thickness (mm)	0.05 ± 0.010	0.05 ± 0.002	0.07 ± 0.005	0.07 ± 0.008
	Tb. Spacing (mm)	0.18 ± 0.023	0.20 ± 0.007	0.23 ± 0.019	0.22 ± 0.018
Femur	BV/TV (%)	23.97 6.8	45.43 7.1*	23.85 ± 5.6	36.78 ± 8.8*
	Connectivity (/mm^3)	247.4 89.6	322.2 36.5	180.9 ± 40.1	248.1 ± 36.5*
	Tb. Number (/mm)	4.9 1.18	7.8 0.82*	4.9 ± 0.64	6.5 ± 0.88*
	Tb. Thickness (mm)	0.06 0.002	0.07 0.006*	0.07 ± 0.005	0.08 ± 0.011*
	Tb. Spacing (mm)	0.22 0.072	0.12 0.021*	0.21 ± 0.031	0.15 ± 0.024*
	Cortical Thickness (mm)	0.18 ± 0.02	0.19 0.01	0.21 ± 0.012	0.23 ± 0.013*

Supplemental Table 2

Changes in antioxidant gene expression resulting from FoxO deletion in Osx1-GFP calvaria cells by microarray

Gene symbol	Gene name	Average Intensity		Ratio (FoxO1,3,4$^{f/f}$; Osx1-Cre/ Osx1-Cre)
		Osx1-Cre	FoxO1,3,4$^{f/f}$; Osx1-Cre	
CAT	Catalase	355.92	284.71	0.80
Gsta4	Glutathione-S-transferase alpha 4	171.04	762.35	4.46*
SOD1	Superoxide dismutase 1	3665.78	2930.62	0.80
SOD2	Superoxide dismutase 2	2319.08	1653.88	0.71
SOD3	Superoxide dismutase 3	298.86	240.97	0.81
PRDX1	Peroxiredoxin 1	10932.28	8827.29	0.81
PRDX2	Peroxiredoxin 2	7928.59	7709.44	0.97
PRDX3	Peroxiredoxin 3	897.78	653.21	0.73
PRDX4	Peroxiredoxin 4	4395.18	3889.81	0.89
PRDX5	Peroxiredoxin 5	3894.06	2401.34	0.62
PRDX6	Peroxiredoxin 6	92.18	83.25	0.90
SESN1	Sestrin1	167.67	214.26	0.78
SESN2	Sestrin 2	119.30	122.33	0.98
SESN3	Sestrin 3	66.54	85.55	0.78

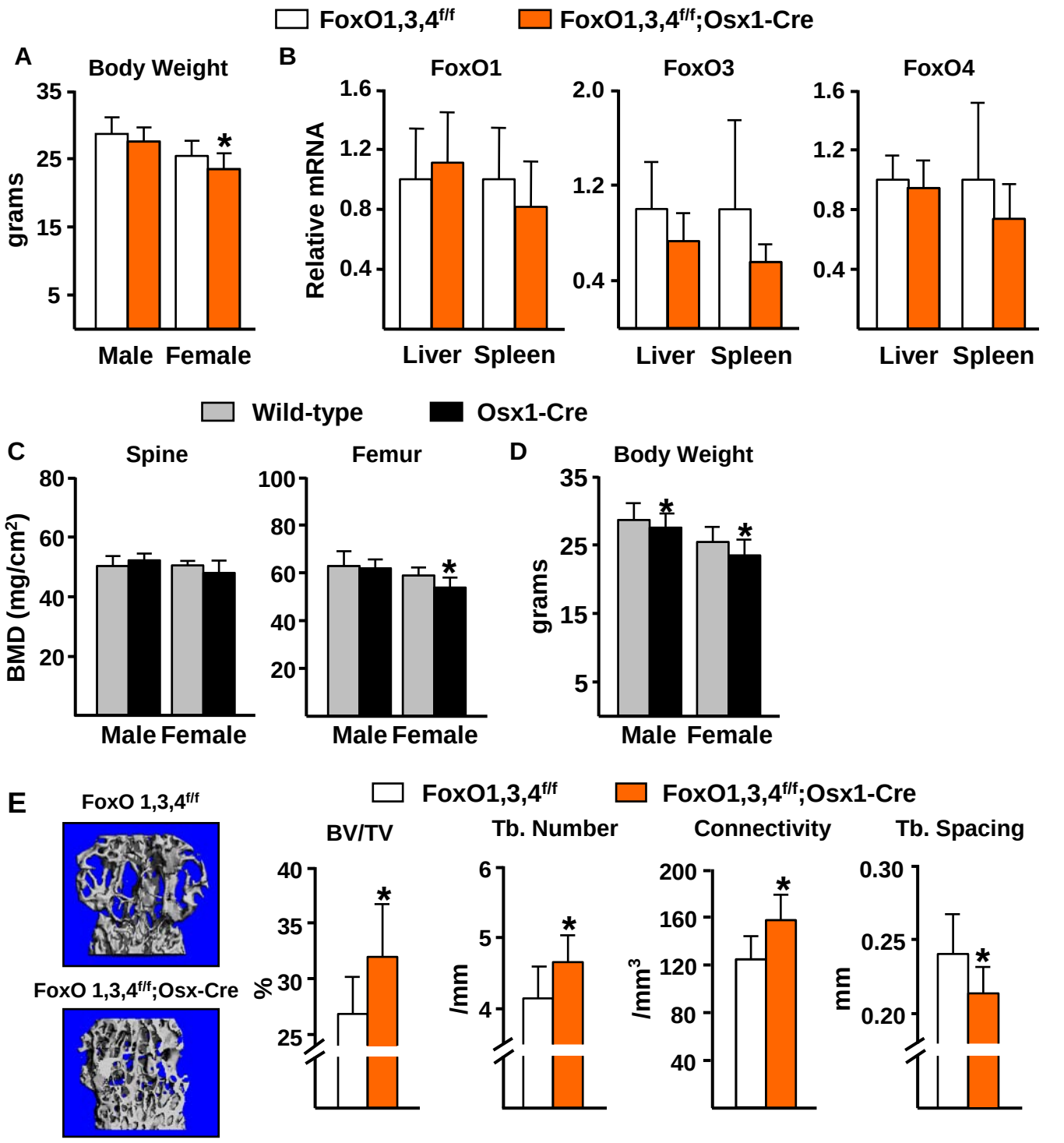
***p ≤ 0.0001; shown in red are previously described FoxO-target genes**

Supplemental Table 3

Changes in gene expression resulting from FoxO deletion in Osx1-GFP calvaria cells by microarray

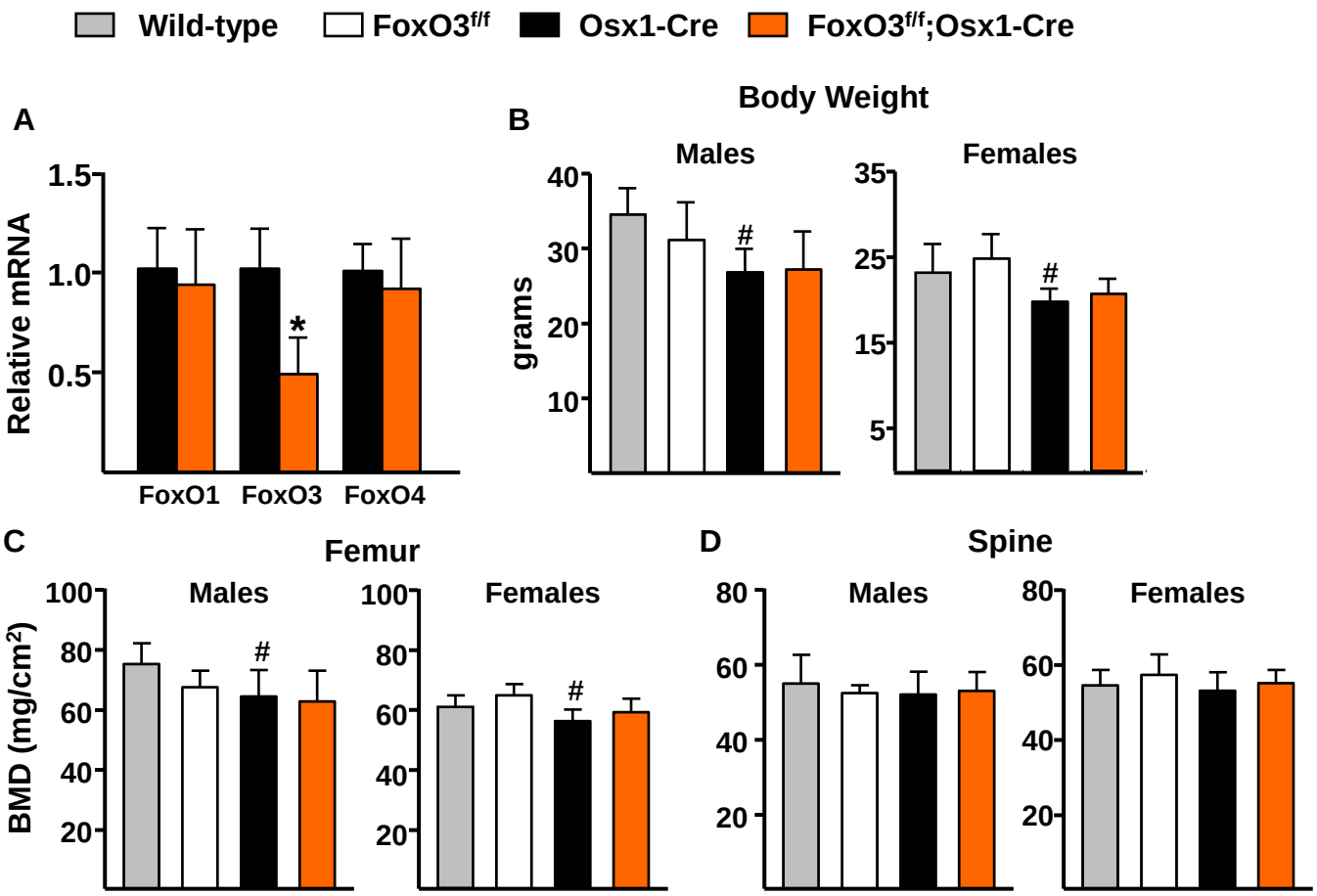
Gene symbol	Gene name	Average Intensity		Ratio (FoxO1,3,4$^{f/f}$; Osx1-Cre/ Osx1-Cre)
		Osx1-Cre	FoxO1,3,4$^{f/f}$; Osx1-Cre	
Rankl	Receptor activator of NFκB ligand	85.91	90.31	1.05
M-csf	Colony stimulating factor 1 (macrophage)	4043.10	2724.91	0.67
Alpl	Alkaline phosphatase, liver/bone/kidney	88.38	83.74	0.95
Runx2	Runt-related transcription factor 2	65.63	62.46	0.95
Osx1	Sp7 transcription factor 7	567.21	315.44	0.56
Col1a1	Collagen, type 1, alpha1	5168.77	4607.22	0.89
Dkk1	Dickkopf homolog 1 (*Xenopus laevis*)	57.83	55.81	0.96
Sost	Sclerostin	54.28	50.83	0.94
Sfrp1	Secreted frizzled related protein 1	340.87	618.44	1.81*
Sfrp2	Secreted frizzled related protein 2	76.31	87.11	1.14
WNT1	Wingless-related MMTV integration site 1	56.17	50.74	0.9
WNT2	Wingless-related MMTV integration site 2	136.65	115.74	0.85
WNT2B	Wingless-related MMTV integration site 2B	60.16	58.44	0.97
WNT3	Wingless-related MMTV integration site 3	61.72	53.56	0.87
WNT3A	Wingless-related MMTV integration site 3B	56.83	57.19	1.01
WNT4	Wingless-related MMTV integration site 4	80.48	72.58	0.9
WNT5A	Wingless-related MMTV integration site 5A	1021.69	395.54	0.39*
WNT5B	Wingless-related MMTV integration site 5B	108.44	111.77	1.03
WNT6	Wingless-related MMTV integration site 6	54.42	51.13	0.94
WNT7A	Wingless-related MMTV integration site 7A	52.33	52.22	1
WNT7B	Wingless-related MMTV integration site 7B	56.92	57.5	1.01
WNT8A	Wingless-related MMTV integration site 8A	52.84	51.74	0.98
WNT8B	Wingless-related MMTV integration site 8B	53.06	55.66	1.05
WNT9A	Wingless-related MMTV integration site 9A	63.08	62.94	1
WNT9B	Wingless-related MMTV integration site 9B	57.52	56.19	0.98
WNT10A	Wingless-related MMTV integration site 10A	69.9	68.8	0.98
WNT10B	Wingless-related MMTV integration site 10B	75.55	57.89	0.77
WNT11	Wingless-related MMTV integration site 11	59.79	57.33	0.96
WNT16	Wingless-related MMTV integration site 16	55.98	53.79	0.96

***$p \leq 0.005$**



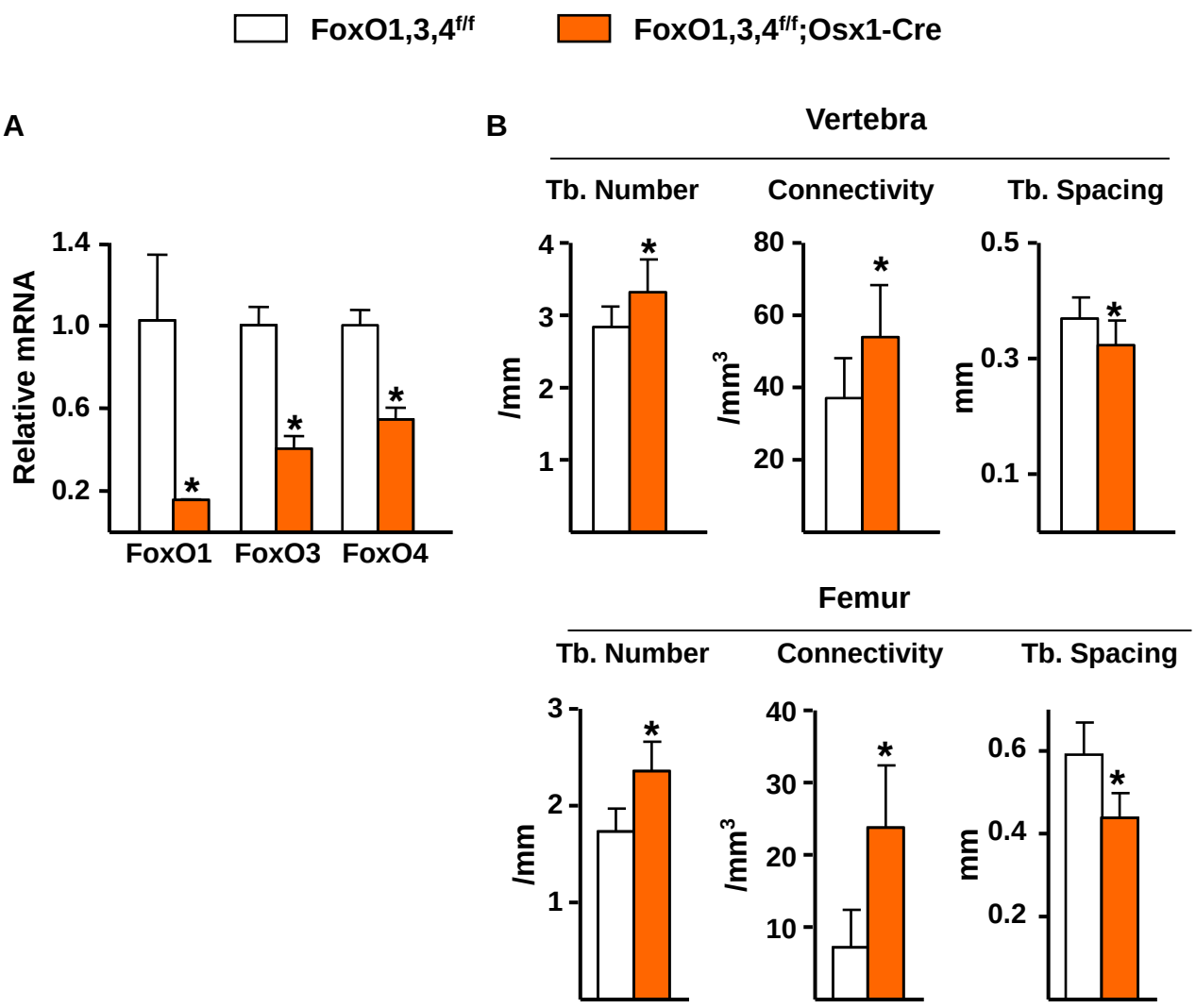
Supplemental Figure 1

Mice with FoxO1, 3 and 4 deletion in osteoprogenitors have high bone mass. (**A**) Body weights of 3 month old male (n=15-20/group) and female mice (n=23-30/group). (**B**) mRNA levels by quantitative RT-PCR in liver and spleen of 3 month old mice (n=8-10/group). (**C**) DEXA BMDs and (**D**) body weights of 3 months old mice (n=7-10/group). (**E**) Micro-CT measurements in the vertebra (L5) of 3 month old male mice. Representative images of vertebral cancellous bone are shown. BV/TV, bone volume per tissue volume; Tb, trabecular. Bars represent mean and s.d., *p<0.05 versus FoxO1,3,4$^{f/f}$ or wild-type by Student's t-test.



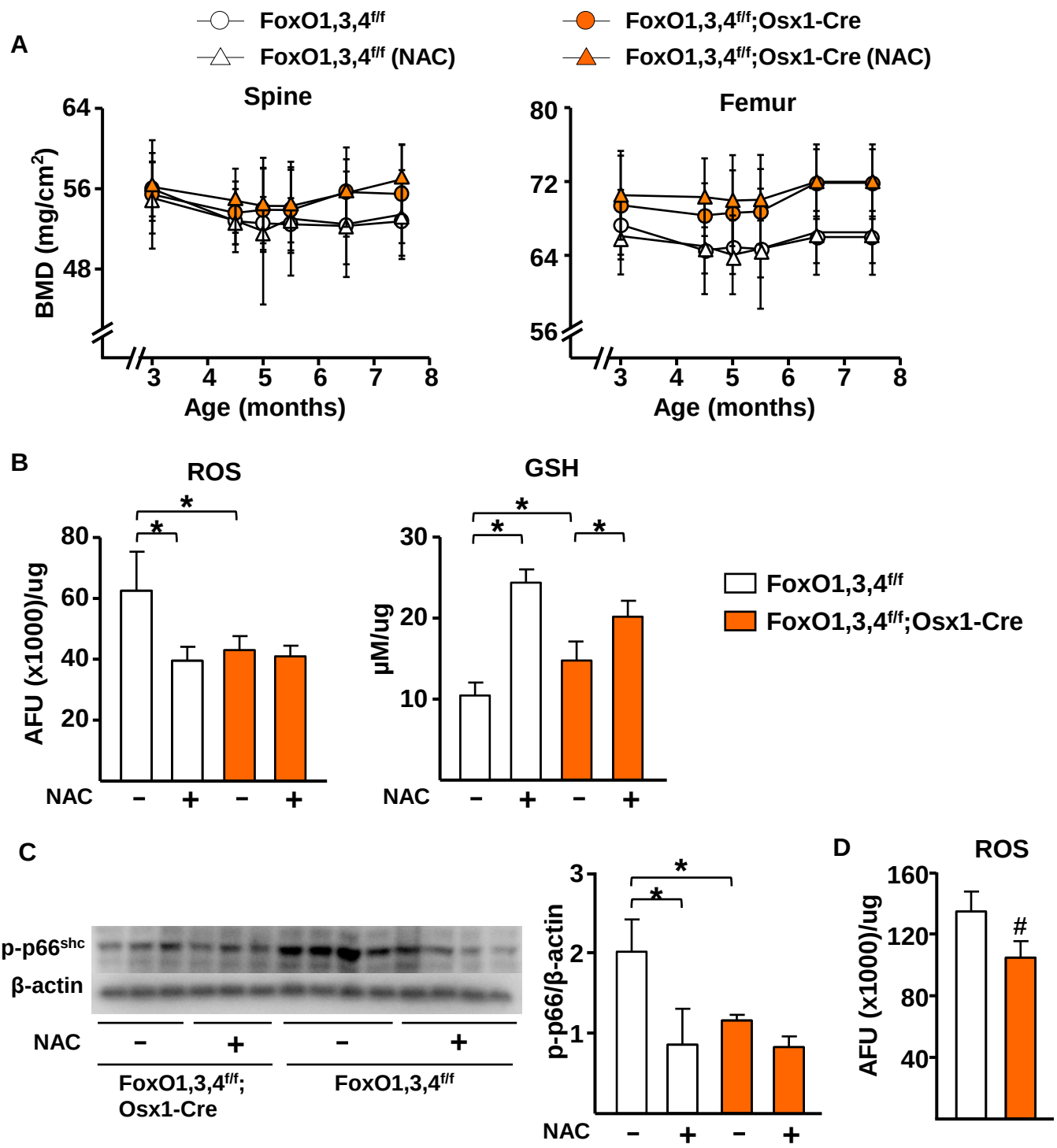
Supplemental Figure 2

FoxO3 deletion in osteoprogenitors does not affect bone mass. (**A**) mRNA levels determined by qRT-PCR in high density bone marrow stromal cells (triplicates) cultured with 1% ascorbate for 21 days. (**B**) Body weights of 3 month old male (n=7-11/group) and female (n=8-15/group) mice. (**C**) Femur and (**D**) Spine DEXA BMDs of the mice described in A. Bars represent mean and s.d., #$p<0.05$ versus wild-type or $FoxO3^{f/f}$ by two-way ANOVA, *$p<0.05$ versus Osx1-Cre by Student's t-test.



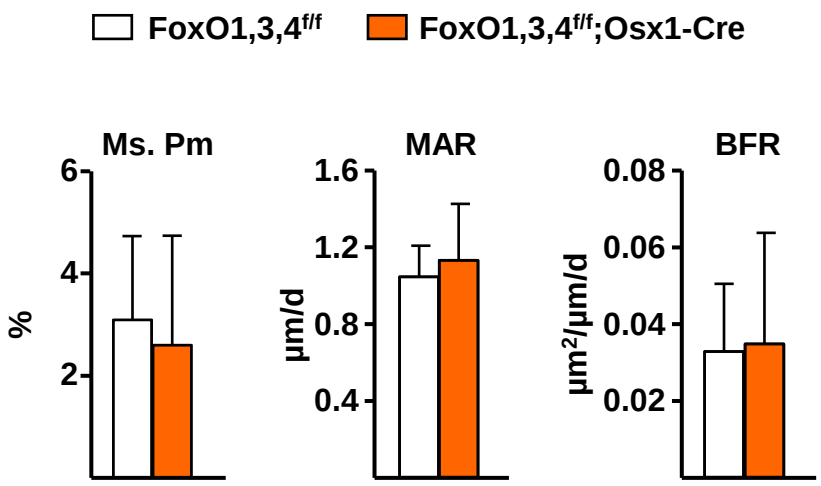
Supplemental Figure 3

FoxO deletion in osteoprogenitor cells of adult mice increases bone mass. (**A**) FoxO deletion was induced in 3 month old females by replacing the doxycycline containing diet with regular chow. FoxO mRNA levels were determined by quantitative RT-PCR in cultured bone marrow stromal cells obtained after euthanasia at 7.5 months of age (triplicates). (**B**) Micro-CT measurements in vertebra (L5) and femur of the mice described in A (n=10-11/group). BV/TV, bone volume per tissue volume, Tb, trabecular. Bars represent mean and s.d.*$p<0.05$ versus FoxO1,3,4$^{f/f}$ by Student's t-test.



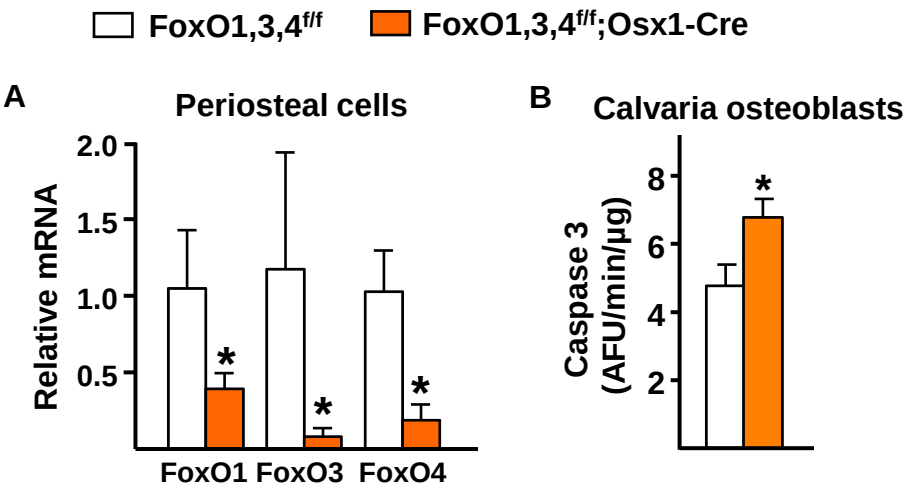
Supplemental Figure 4

Administration of the anti-oxidant NAC to FoxO1,3,4$^{f/f}$;Osx1-Cre mice had no effect on bone mass. (**A-D**) FoxO deletion was induced in 3 month old females by replacing the doxycycline containing diet with regular chow or a diet containing 100 mg kg^{-1} N-acetyl-cysteine (NAC). (**A**) Longitudinal BMD measurements determined by DXA (n=10-11/group). Data represents mean and s.d. (**B**) ROS and GSH levels in the bone marrow. (**C**) phospho-p66^{shc} by Western blot in vertebral bone lysates. Each lane represents one animal; the quantification is shown on the right. (**D**) ROS levels in the bone marrow of 24 month old females. Bars represent mean and s.d. *p<0.05 by two-way ANOVA; #p<0.05 by Students t test.



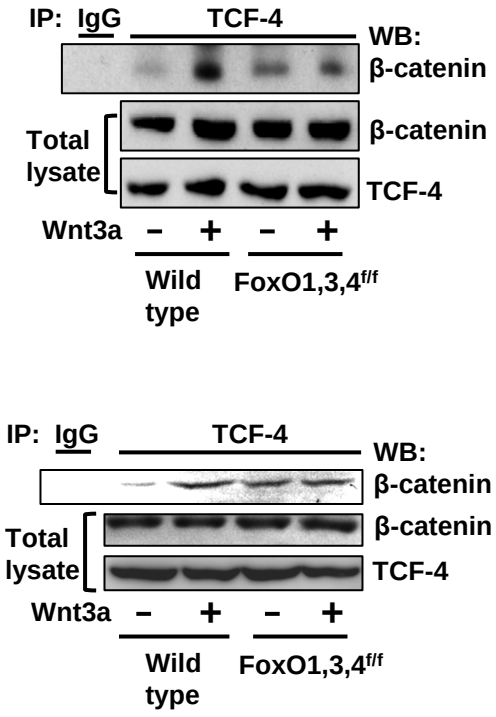
Supplemental Figure 5

Bone formation in aged FoxO1,3,4$^{f/f}$;Osx1-Cre mice. Mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR) as determined by tetracycline labels, in the cancellous bone of longitudinal undecalcified vertebral (L1-L3) sections from 24 month old female mice (n=6-7/group). Bone sections in three FoxO1,3,4$^{f/f}$ and two FoxO1,3,4$^{f/f}$;Osx1-Cre mice had less than five double labels, the minimum recommended by the ASBMR Histomorphometry Nomenclature Committee (Dempster et al. *J Bone Miner Res.* 2013, 28:1-16; Recker et al. *Bone* 2011, 49:955-964). Bars represent mean and s.d.



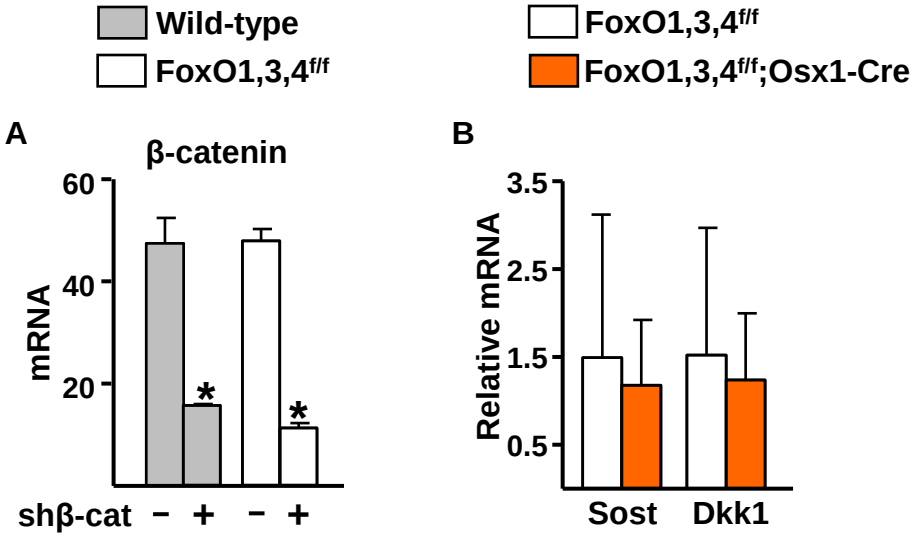
Supplemental Figure 6

Apoptosis is increased in osteoblast cultured from FoxO1,3,4$^{f/f}$;Osx1-Cre mice. (**A**) mRNA levels of FoxOs determined by qRT-PCR in cultured periosteal cells isolated from 7-week-old mice (triplicates). (**B**) Caspase 3 activity in calvaria cells from 5 month-old mice cultured for 10 days in the presence of ascorbic acid (triplicates). Bars represent mean and s.d.; *p<0.05 by Student's t-test.



Supplemental Figure 7

The binding of β-catenin to TCF4 is increased in the absence of FoxOs. Calvaria cell cultures from the indicated mice were infected with Adeno-Cre to induce FoxO deletion and treated with vehicle or 50 ng/ml Wnt3a. Total cell lysates were immunoprecipitated with an anti-TCF-4 or anti-IgG antibody and probed with an anti-β-catenin antibody. Two independent experiments are depicted.



Supplemental Figure 8

Gene expression in cells and bone from FoxO1,3,4$^{f/f}$;Osx1-Cre mice. (**A**) mRNA levels determined by quantitative(q) RT-PCR in calvaria-derived cells infected with Adeno-Cre and with empty viral particles (-) shRNA or one clone expressing shRNA directed against β-catenin (triplicates). (**B**) mRNA levels determined by qRT-PCR in bone shafts from 3 month old mice (n=8-9/group). Bars represent mean and s.d.; *$p<0.05$ versus respective cells infected with empty viral particles by Student's t-test.