Supplementary Information

Non-canonical Wnt4 prevents skeletal aging and inflammation by inhibiting NF-κB

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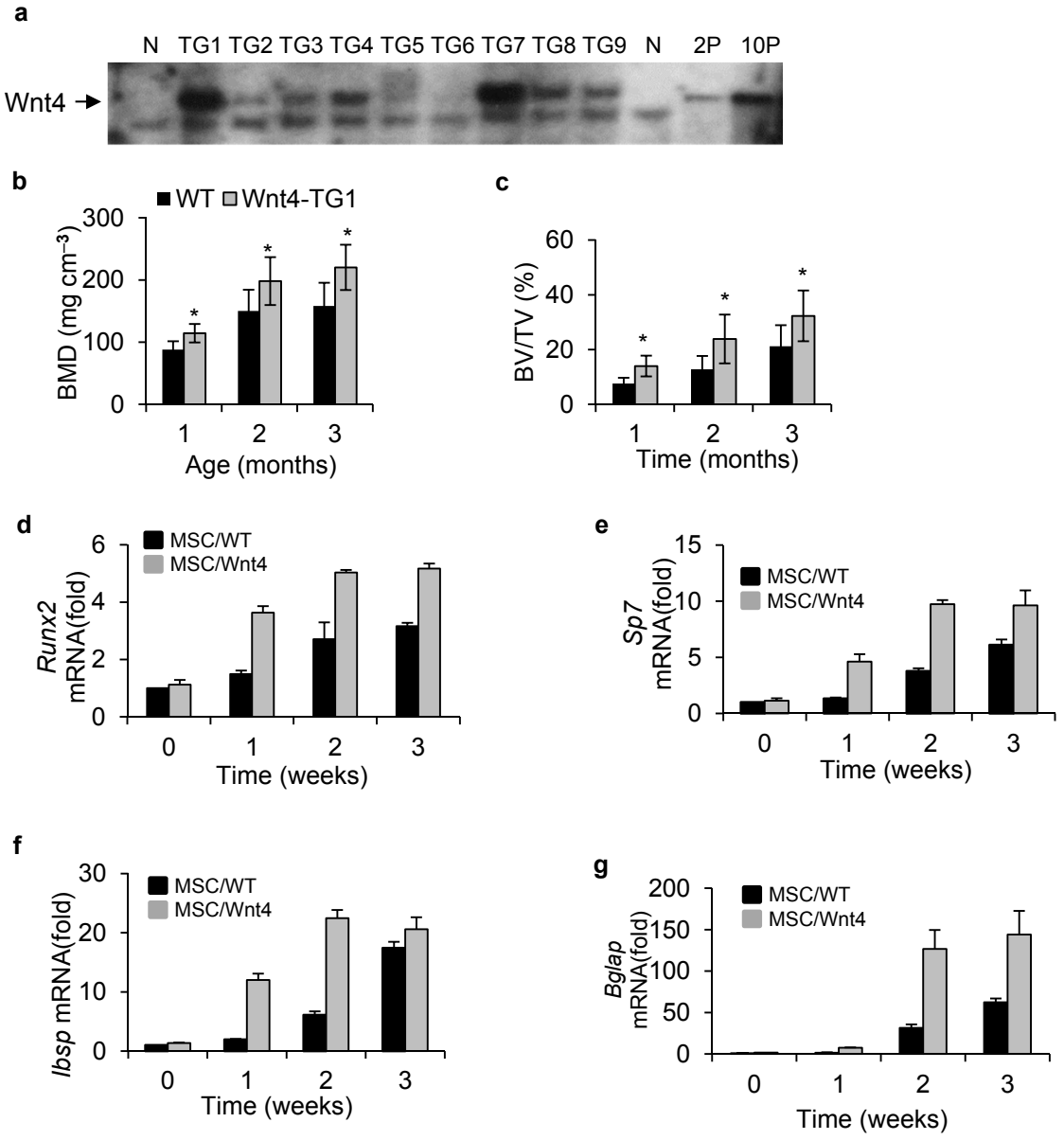
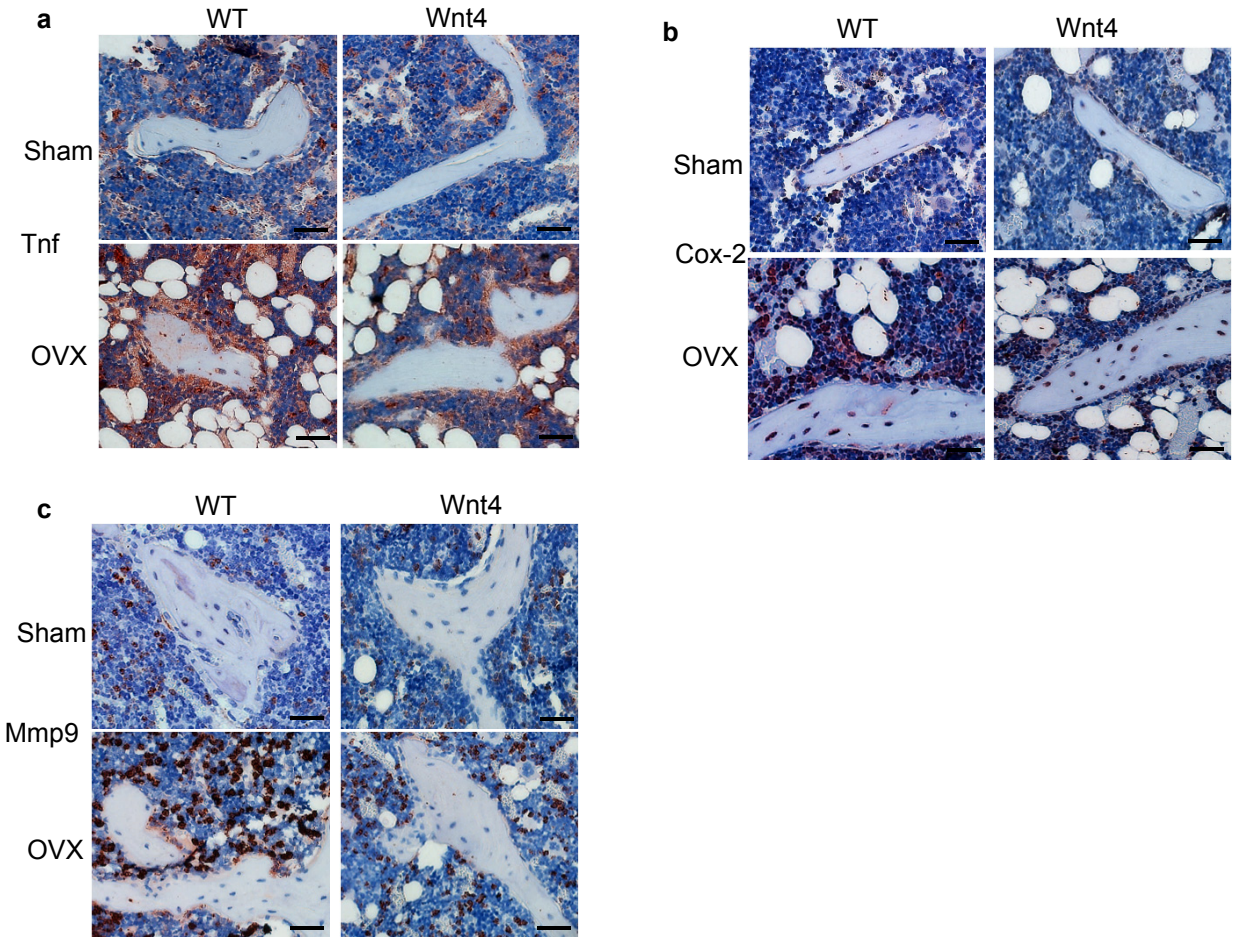
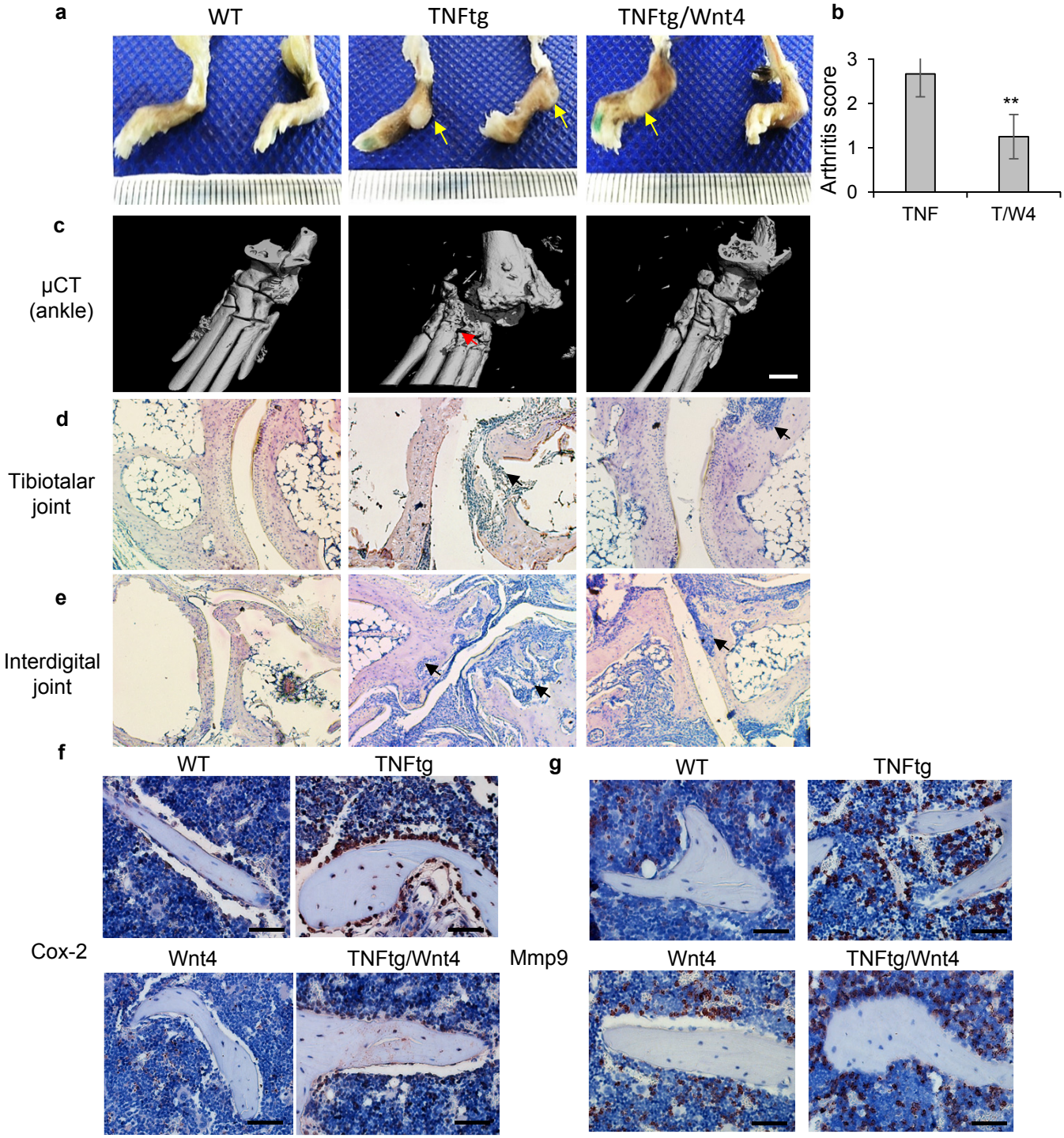
Supplementary Figures 1–7



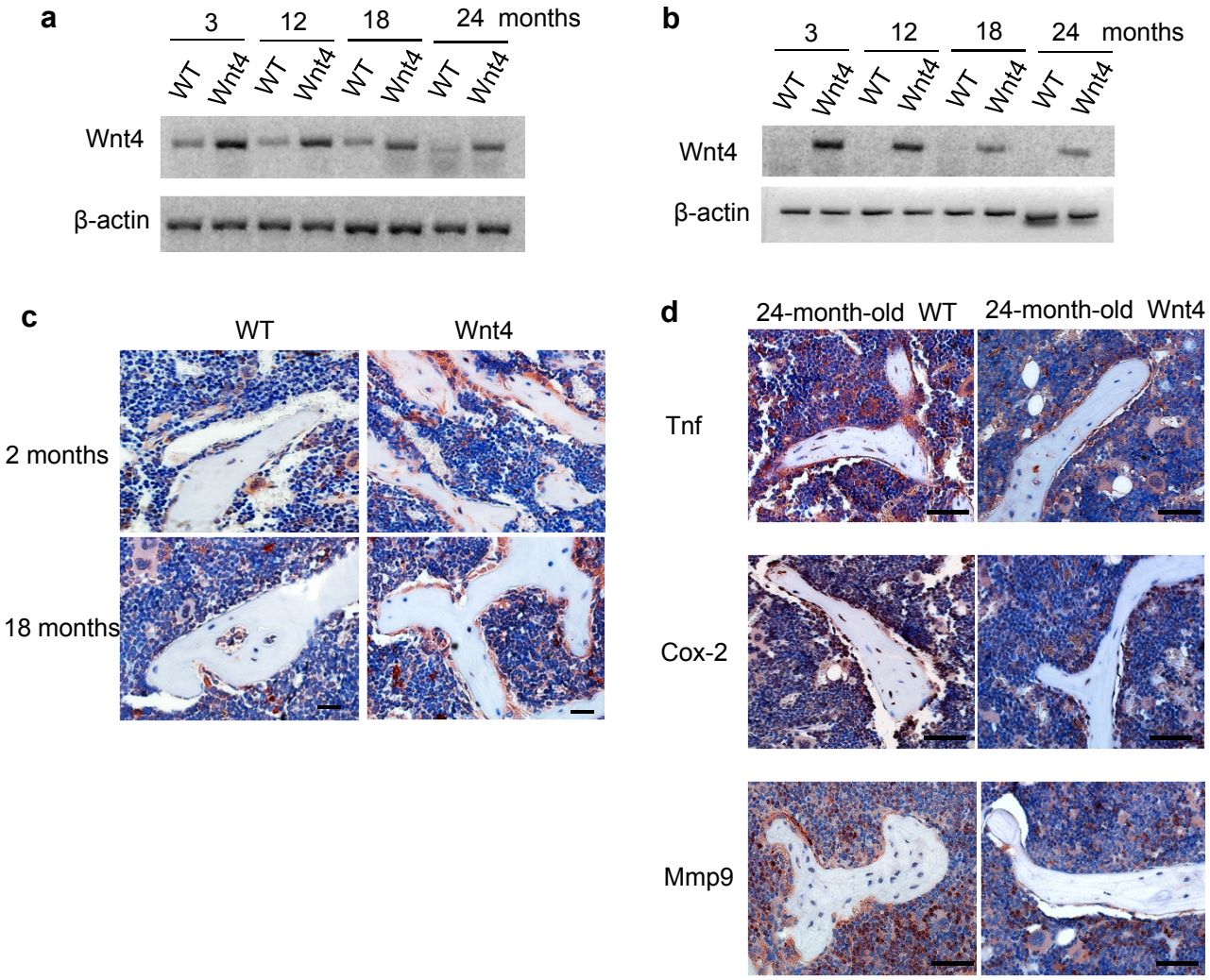
Supplementary Figure 1 Wnt4 promotes postnatal bone formation *in vivo*. (**a**) Southern blot of Wnt4 transgene expression in 10 founder mouse lines. (**b–c**) μCT analysis of BMD (**b**) and BV/TV (**c**) of 1-, 2- and 3-month-old WT and Wnt4 mice (TG-1). n = 10 mice per group. * $P < 0.05$. (**d–g**) Real time RT-PCR analysis of osteogenic marker genes including *Runx2* (**d**), *Sp7* (**e**), *Ibsp* (**f**) and *Bglap* (**g**) mRNA expression in primary bone marrow MSCs isolated from femurs of 3-month-old WT and Wnt4 mice, after osteogenic induction treatment for indicated times.



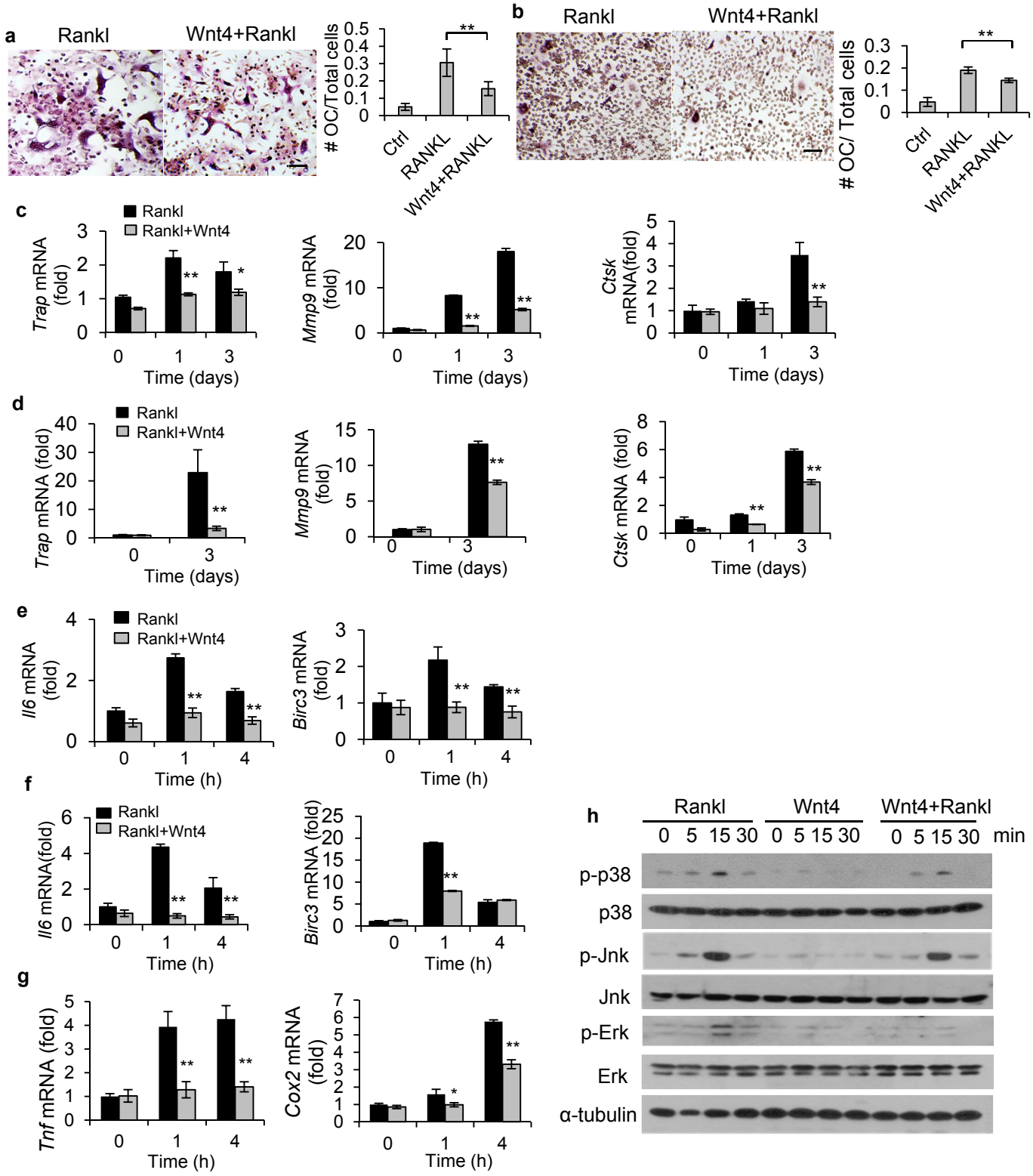
Supplementary Figure 2 Wnt4 attenuates the expression of NF-κB-regulated molecules *in vivo* induced by OVX. (**a–c**) Immunostaining of NF-κB-dependent Tnf (**a**), Cox-2 (**b**), and Mmp9 (**c**) surrounding trabecular bones in the distal metaphysis of WT and Wnt4 mice two months after OVX or sham operation. Scale bars, 60 μm.



Supplementary Figure 3 Wnt4 alleviates arthritis induced by TNF. (**a–c**) Photographs of hindpaws and ankle joints (**a**) showing swelling (yellow arrow) as well as μCT reconstruction of ankle and tibiotalar joints (**c**) showing bony erosions (red arrow) from 12-month-old WT, TNFtg and TNFtg/Wnt4 mice. Average arthritis scores (**b**) were given based on the degree of swelling and joint deviation. $n = 8$ hindpaws for WT and TNFtg/Wnt4 groups; $n = 4$ hindpaws for TNFtg group. ** $P < 0.01$. (**d–e**) H&E staining of tibiotalar (**d**) and interdigital (**e**) joints showing joint cartilage destruction and bone erosions due to invasion of inflammatory cells (black arrows). (**f,g**) Immunostaining of NF-κB-dependent Cox-2 (**f**) and Mmp9 (**g**) in distal femoral metaphysis of 12-month-old WT, Wnt4, TNFtg and TNFtg/Wnt4 mice. Scale bar, 1 mm (**c**); 200 μm (**d–e**); 40 μm (**f–g**).



Supplementary Figure 4 Wnt4 attenuated the expression of NF-κB-regulated molecules in aging mice. (**a**) RT-PCR of endogenous and transgenic Wnt4 mRNA expression in Wnt4 mice and WT mice of various ages. (**b**) RT-PCR of Wnt4 transgene mRNA expression in Wnt4 mice and WT mice of various ages. (**c**) Immunostaining of Wnt4 proteins in young (2-month-old) and aged (18-month-old) WT and Wnt4 mice. (**d**) Immunostaining of NF-κB-dependent Tnf, Cox-2 and Mmp9 in distal metaphysis of 24-month-old WT and Wnt4 mice. Scale bars, 40μm (**c**–**d**).



Supplementary Figure 5. Wnt4 directly inhibits osteoclast differentiation induced by Rankl. (**a–b**) TRAP staining showing osteoclast formation from bone marrow macrophages (**a**) and RAW264.7 cells (**b**) induced by Rankl or Rankl with Wnt4. (**c,d**) Real time RT-PCR of *Trap*, *Mmp9* and *Ctsk* mRNA in bone marrow macrophages (**c**) and RAW264.7 cells (**d**). (**e,f**) Real time RT-PCR of *Il6* and *Birc3* in bone marrow macrophages and RAW264.7 cells (**f**). (**g**) Real time RT-PCR of *Tnf* and *Cox-2* in bone marrow macrophages. (**h**) Immunoblots showing the phosphorylation of p38, Jnk, and Erk of lysates from bone marrow macrophages stimulated with Rankl, Wnt4 or Rankl with Wnt4. Scale bars, 100 μm (**a–b**). * $P < 0.05$; ** $P < 0.01$.

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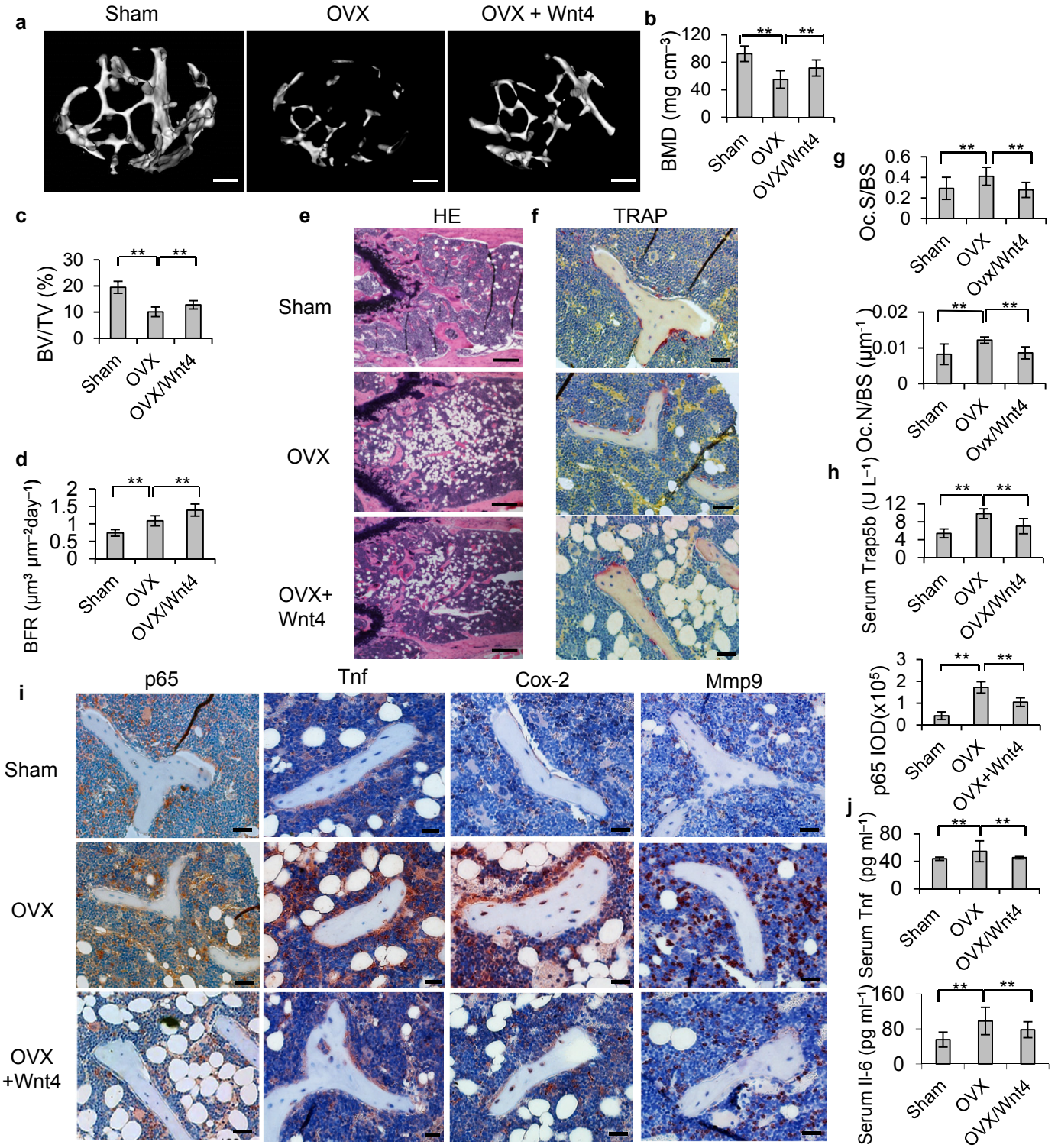
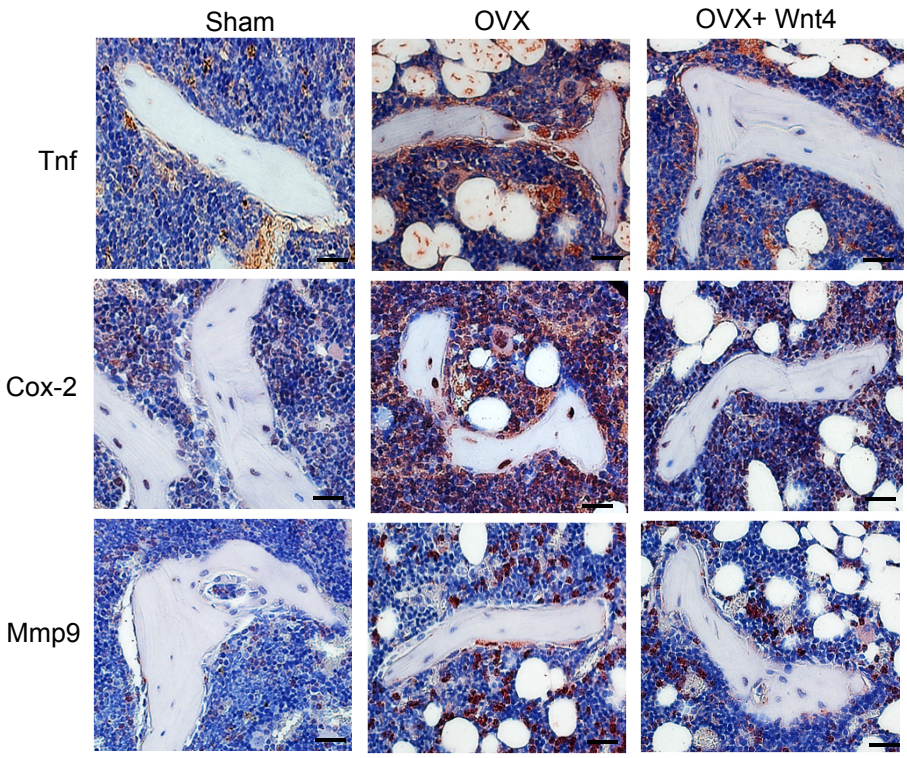


Supplementary Figure 6 rWnt4 prevents osteoporotic bone loss by inhibiting NF-κB. (**a–c**) μCT reconstruction (**a**), BMD (**b**) and BV/TV (**c**) of distal femoral metaphysis regions from mice after sham operation, OVX and OVX immediately followed by rWnt4 injection. (**d**) BFR measurement from dual calcein labeling of mice. (**e–f**) H&E staining (**e**) and TRAP staining (**f**) in distal metaphysis of mice. (**g**) Morphometric analysis of osteoclast counts in distal femoral metaphysis. (**h**) ELISA of Trap5b concentrations in serum. (**i**) Immunostaining showing active p65, Tnf, Cox-2 and Mmp9 in distal femoral metaphysis. (**j**) ELISA of serum concentrations of Tnf and Il-6. For **b**, **c**, **d,** and **g–j**, n = 8 mice for sham group; n = 12 mice per group for mice receiving OVX and OVX with preventive rWnt4 injection. *$P < 0.05$, ** $P < 0.01$, unpaired two-tailed t-test. Scale bars, 200 μm (**a**); 300 μm (**e**); 25 μm (**f**) and (**i**).



Supplementary Figure 7 rWnt4 proteins attenuate activation of NF-κB-dependent molecules induced by OVX. Immunostaining of NF-κB-dependent Tnf, Cox-2 and Mmp9 in distal metaphysis of mice. Scale bars, 40 μm.