Supplemental Materials

Moonlighting Cytosolic Function of ACAD9: Suppression of TRAF6-Mediated Osteoclastogenesis and Protection Against Osteoporosis

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Methods

Cell culture and NAC treatments

For in vitro osteoclastogenesis, seeded cells in 12-well plates and the medium supplemented with 100ng/ml RANKL for differentiation under cells reached confluence, and the differentiation medium was changed every 2 days. For the N-acetyl-L-cysteine (NAC) intervention experiment, during the osteoclastogenesis, pretreated cells with NAC(5μM) or not and conducted other detection experiments.

Functional enrichment analysis

The pathway terms categorized by the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database and Gene Ontology (GO) Process were collected. Osteoporosis-related biological terms were selected, and the matching genes were counted.

Supplemental Table 1

gene	forward	reverse
Cathepsin K	5'- ccgaataaatctagcacccttagt-3'	5'- gaaacttgaacacccacatcc-3'
c-Src	5'-tgagccaggatctgaacca-3'	5'-Tcctgctccgtgtcccta-3'
RANK	5'-caggacagggctgatgagag-3'	5'-ttactgtttccagtcacgttcc-3'
Traf6	5'-ttgcacattcagtgtttttgg-3'	5'-tgcaagtgtcgtgccaag-3'
Nfatc1	5'-tccaaagtcattttcgtgga-3'	5'-ctttgcttccatctcccaga-3'
c-Fos	5'-gggacagcctttcctactacc-3'	5'-agatctgcgcaaaagtcctg-3'
Fra-1	5'-cccagtacagtccccctca-3'	5'-tcctcctctgggctgatct-3'
Trap	5'-gagtcagactaatgtcatctgtggtt-3'	5'-accccgaaaatggtgatg-3'
β-actin	5'-cgctgtcaaccccaagtt-3'	5'-ggcacgttcttgtctactcgt-3'
Acad9	5'-cgcagccttctgcctaac3'	5'-cgtagctctggtctggatgg-3'

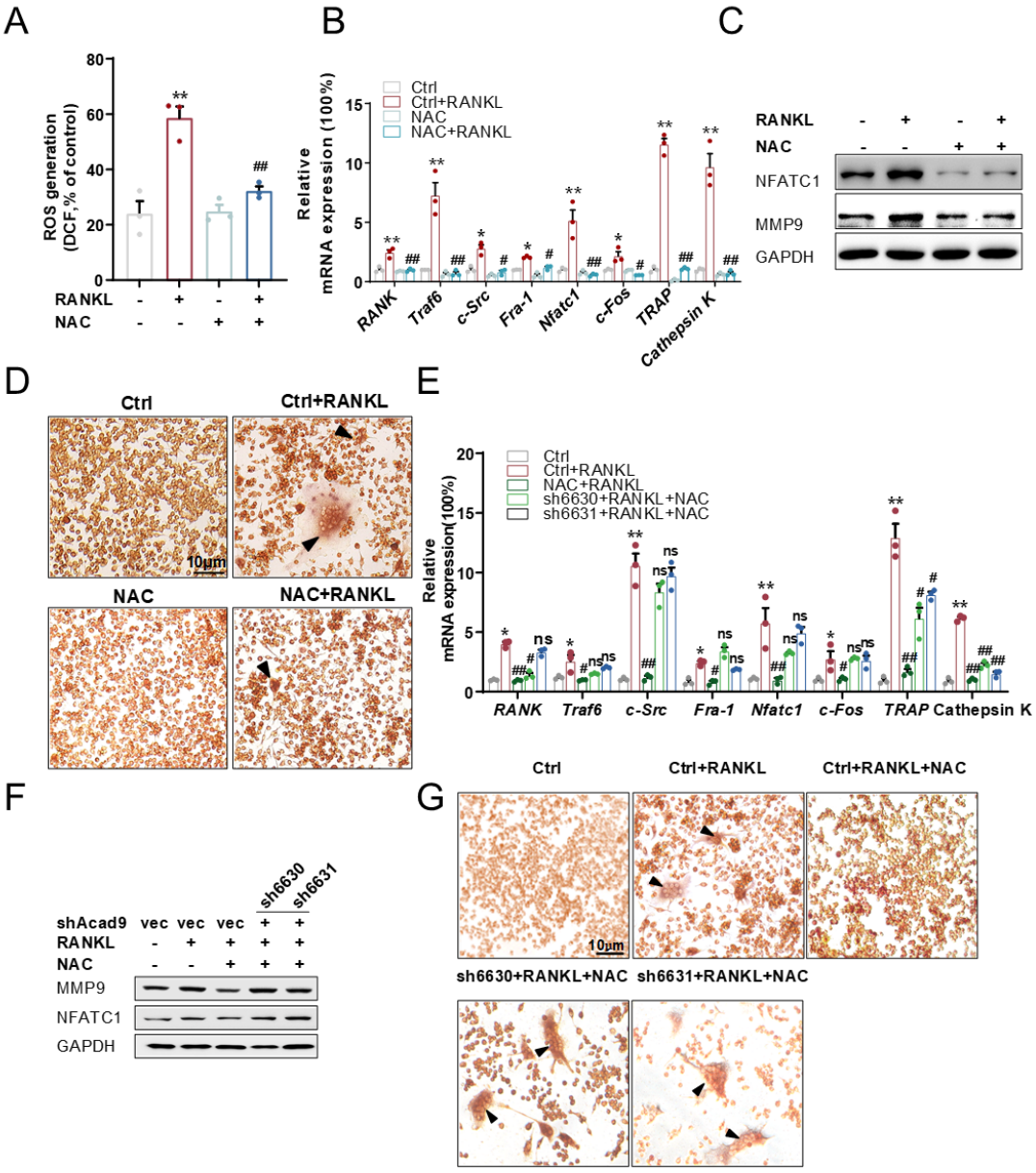

Fig. S1: Clearing ROS alone does not completely inhibit osteoclast differentiation and maturation due to loss of ACAD9 function. (A) ROS levels were detected using DCFH2-DA, (B) the relative mRNA levels of RANKL, Traf6, c-src, Fra-1, NFATc1, c-fos, TRAP, and cathepsin K were tested by qRT-PCR, (C) the levels of relative osteoclast differentiation marker protein NFATc1 and MMP9 were detected by western blot and (D) TRAP staining was performed from cells pre-treated NAC or not after

induced 4 days.(E) the relative mRNA levels of RANKL, Traf6, c-src, Fra-1, NFATc1, c-fos, TRAP, and cathepsin K were tested by qRT-PCR, (F) the levels of relative osteoclast differentiation marker protein NFATc1 and MMP9 were detected by western blot and (G) TRAP staining was performed from cells that stably knockdown ACAD9 pre-treated with NAC or not after induced 4 days.

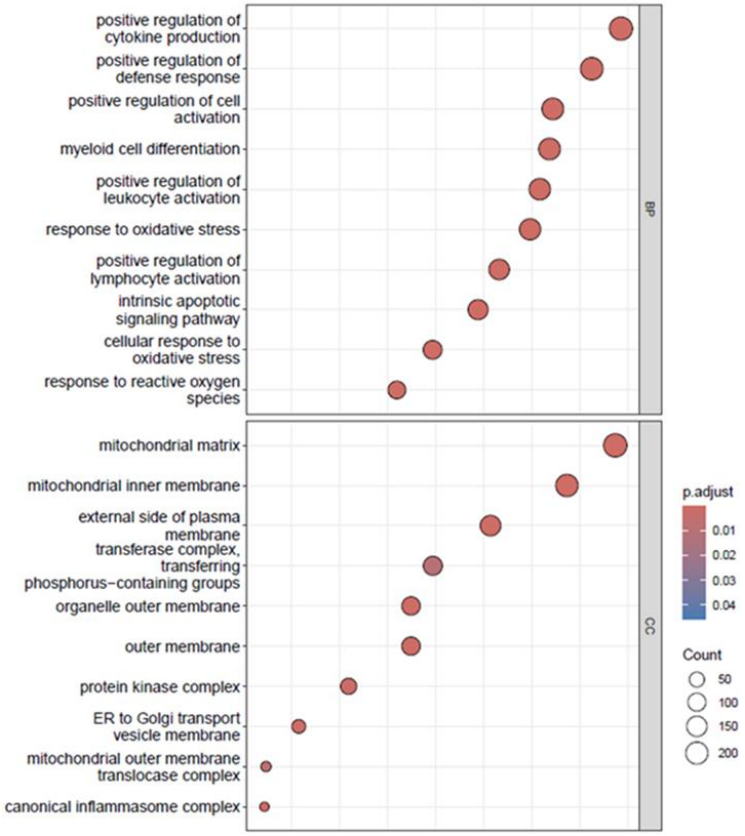

Fig. S2: ACAD9 is related to the composition of the mitochondrial inner membrane. (A) Gene Ontology (GO) enrichment analyses and functional annotations.

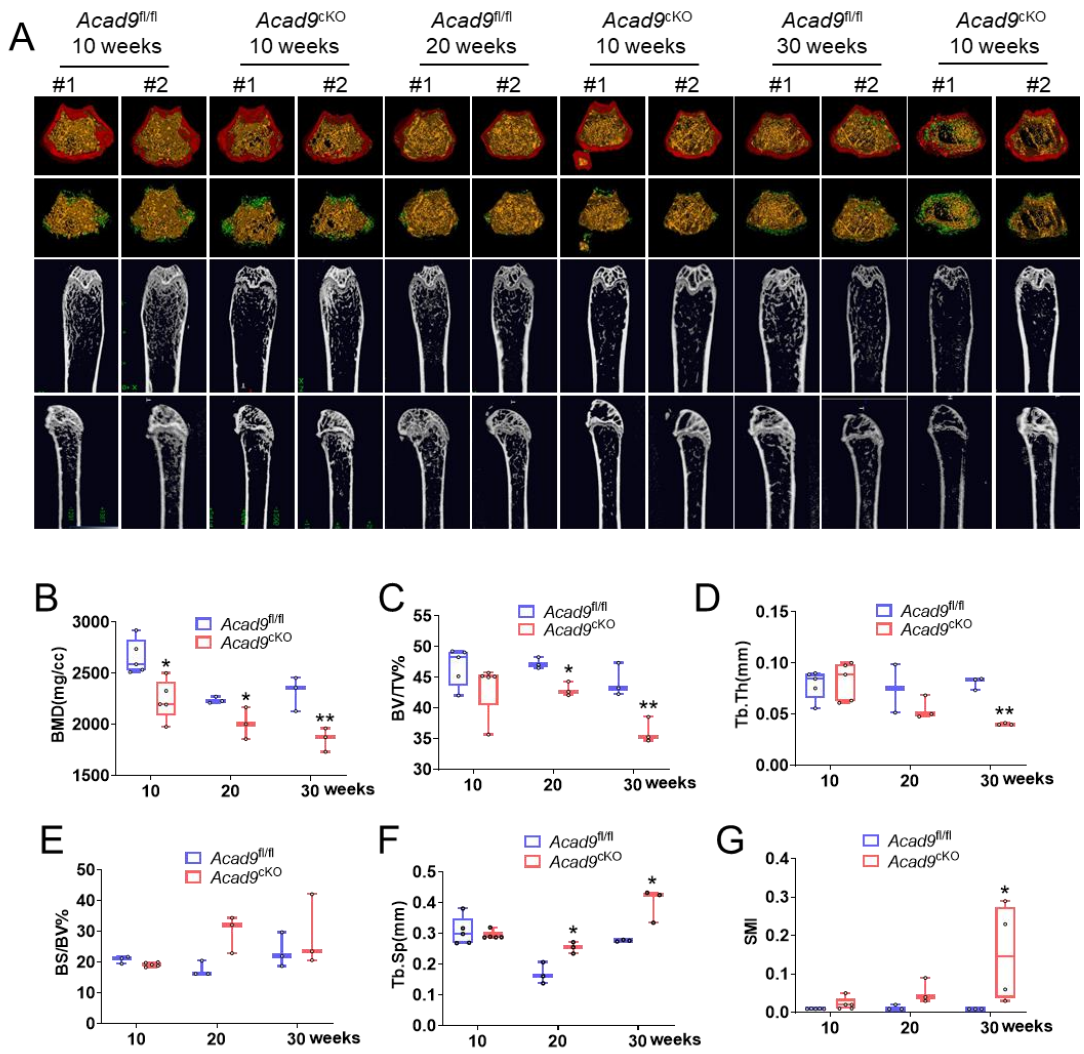

Fig. S3: Osteoclast precursors loss of *Acad9* provoke osteoporosis in male mice. Micro-CT images of the femurs of *Acad9*$^{fl/fl}$and *Acad9*^{cKO} male mice over time (A). The trabecular bone and architecture analyzed by MicroView v2.1.1 Software: (B) bone mineral density (BMD), (C) bone volume per tissue volume (BV/TV); (D) trabecular thickness (Tb. Th); (E) bone surface to bone volume (BS/BV); (F) trabecular spacing (Tb. Sp); and (G) structure model index (SMI), $*p<0.05$, $**$ $p<0.01$ vs *Acad9*$^{fl/fl}$ mice.

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