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Supplemental information

BMAL1-TTK-H2Bub1 loop deficiency contributes to impaired BM-MSC-mediated bone formation in senile osteoporosis

Li Jinteng, Xu Peitao, Yu Wenhui, Ye Guiwen, Ye Feng, Xu Xiaojun, Su Zepeng, Lin Jiajie, Che Yunshu, Zhang Zhaoqiang, Zeng Yipeng, Li Zhikun, Feng Pei, Cao Qian, Li Dateng, Xie Zhongyu, Wu Yanfeng, and Shen Huiyong

Supplemental Materials:

Table S1 Sequences for Sh-RNA, qPCR primer and CUT&Tag-qPCR primer.

Lentiviruses encoding short hairpin RNA	**Sequences for Sh-RNA(5' - 3')**	
NC	TTCTCCGAACGTGTCACGTTTC	
BMAL1	GCCGAATGATTGCTGAGGAAA	
CLOCK	GCCAAGATTCTGGGTCAGATA	
TTK	CCAGTTGTAAAGAATGACTTT	
RNF40	GCAGCTTAACTCTGGCTACTA	
WAC	CTCAAACTAACACAGTCCCTA	
MDM2	GATTCCAGAGAGTCATGTGTT	
qPCR primer		
BMAL1	Forward Primer	AAGGGAAGCTCACAGTCAGAT
	Reverse Primer	GGACATTGCGTTGCATGTTGG
CLOCK	Forward Primer	TGCGAGGAACAATAGACCCAA
	Reverse Primer	ATGGCCTATGTGTGCGTTGTA
RUNX2	Forward Primer	TGGTTACTGTCATGGCGGGTA
	Reverse Primer	TCTCAGATCGTTGAACCTTGCTA
OSX	Forward Primer	CCTCTGCGGGACTCAACAAC
	Reverse Primer	AGCCCATTAGTGCTTGTAAAGG
OPN	Forward Primer	CTCCATTGACTCGAACGACTC
	Reverse Primer	CAGGTCTGCGAAACTTCTTAGAT
OCN	Forward Primer	CACTCCTCGCCCTATTGGC
	Reverse Primer	CCCTCCTGCTTGGACACAAAG
CUT&Tag-qPCR primer		
TTK Ebox1	Forward Primer	GGTTCTAAGGATAGCTTTGGAGTT
	Reverse Primer	TAGGACGGGTTGGTTTTGTGT
TTK Ebox2	Forward Primer	CCCGCGAAGATTATTACTAGGG
	Reverse Primer	TCGAAGCCTGCCCCACTTA
TTK control	Forward Primer	TTAGGGTGAGGTCATCCAGCA
	Reverse Primer	TTGATCTGTTTGCGGCCTGAT
RUNX2	Forward Primer	GGCTGTCTCTACTCACGAGC
	Reverse Primer	GGCTGCTGCGTCATCTTTTT
OSX	Forward Primer	TCCCCGTGTGGGCTTTAATC
	Reverse Primer	GCACTGGCTCACATCTTCCT
BMAL1 A	Forward Primer	GGAGTCAGGAACTGCTGCTT
	Reverse Primer	CTGCTACTTTCCTGCCACCA
BMAL1 B	Forward Primer	GGTCTGTTTGTGCAGCCAAG

	Reverse Primer	TCAAGCCCAACACAACCTGT
BMAL1 C	Forward Primer	CTTCCTTCTTCCTCCGCTGT
	Reverse Primer	AGACAAAAAGAGGCAGAGAGGTT
BMAL1 D	Forward Primer	CACCCCAGCAGTGCTTATCA
	Reverse Primer	TCCGTGGAGTAGGACTGGAG
BMAL1 E	Forward Primer	CCACCCCACTCTTCAATCCC
	Reverse Primer	ATGTGCTTAGTGGCAGGGTC
BMAL1 F	Forward Primer	GCGGATTTCCCCATGAATGC
	Reverse Primer	CCACTAGCCAACACAGGAGG

Table S2 Characteristics of the study subjects

	Young patients with traffic injuries	Patients with senile osteoporosis
Number	3	3
Sex	Male	Male
Age, years	30.54±7.30	80.21±8.67*
Height (cm)	172.77±6.23	168.23±5.05
Weight (kg)	65.23±9.41	60.34±8.31
Lumbar spine BMD (g/cm^2)	1.27±0.14	0.69±0.13*
Lumbar spine *T* score	0.35±1.81	-2.90±1.22*
Total hip BMD (g/cm^2)	1.10±0.17	0.72±0.16*
Total hip *T* score	0.33±1.69	-1.78±0.93*

Data are shown in the form of the mean ± SD, n=3 in each group. P values for all variables are the result of independent t tests between the control and osteoporosis groups, * indicates $P < 0.05$ compared with the control group. BMD: bone mineral density.

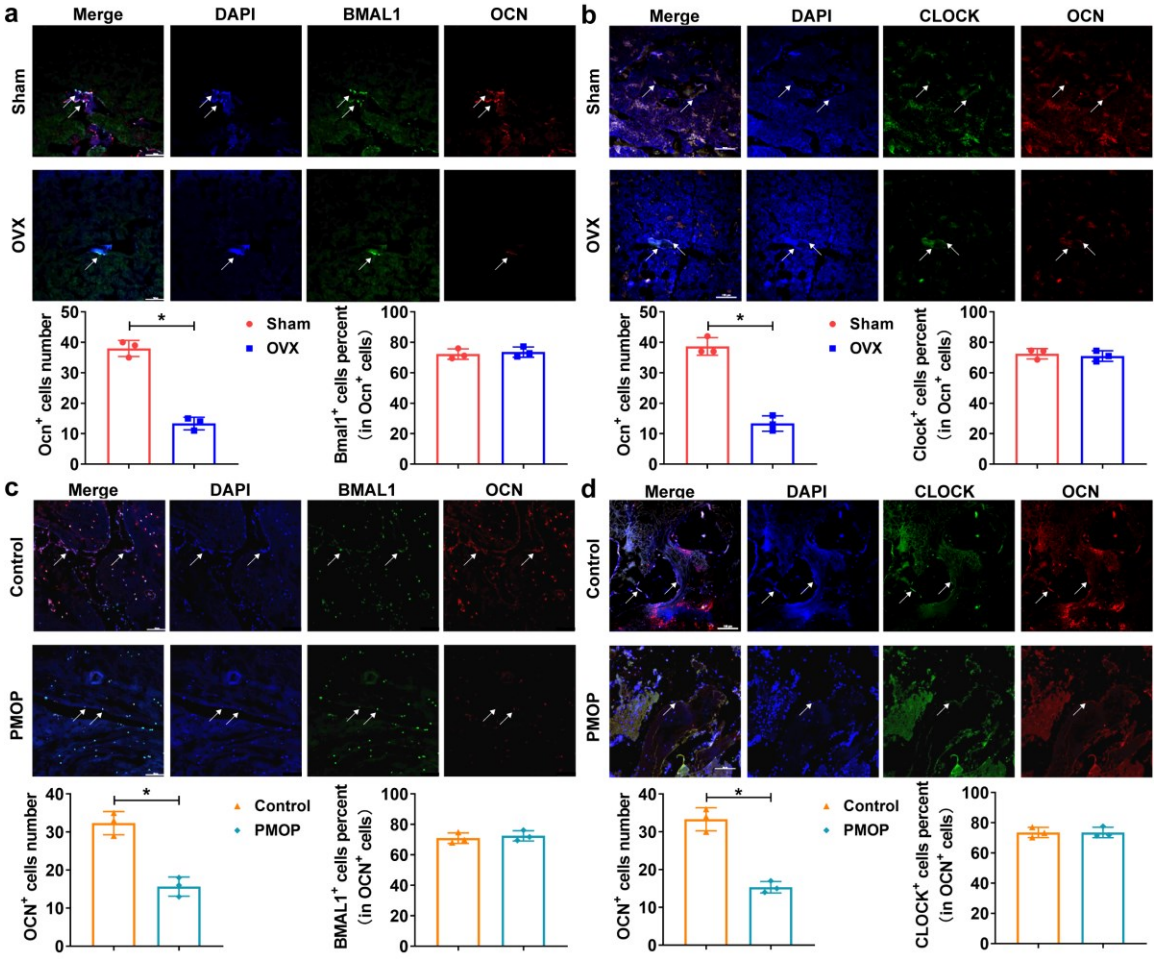

Fig. S1 BMAL1 expression in the skeletal system in postmenopausal osteoporosis. (a-b) Immunofluorescence staining (scale bar =100 μm) showed Bmal1 and Clock expression in the Ocn+ osteoblast lineage in the sham-operated and OVX mice (white arrows). (c-d) Immunofluorescence staining (scale bar =100 μm) showed BMAL1 and CLOCK expression in the OCN+ osteoblast lineage in age-matched controls and patients with postmenopausal osteoporosis (white arrows).

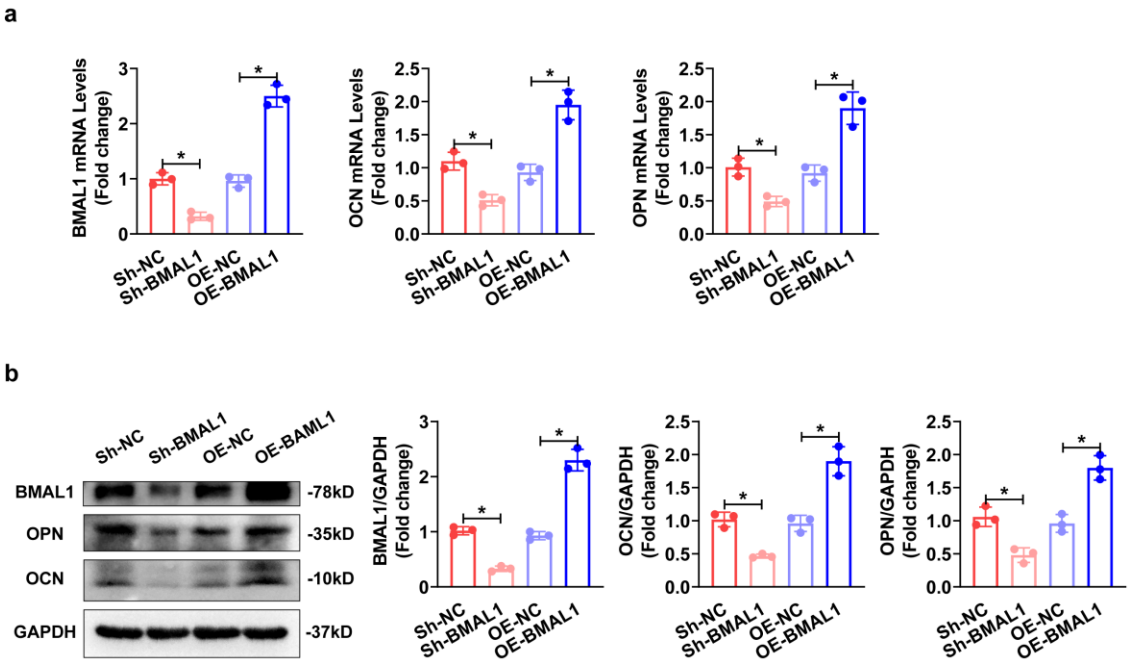

Fig. S2 BMAL1 positively regulated the osteogenic differentiation of MSCs. (a, b) Relative mRNA (a) and protein (b) expression of BMAL1 and the osteogenesis-associated markers OPN and OCN in the MSCs infected with Sh-NC, Sh-BMAL1, OE-NC, or OE-BMAL1 lentiviruses on the 10th day of osteogenic differentiation. The data are presented as the means ± SDs, n = 3, *P < 0.05.

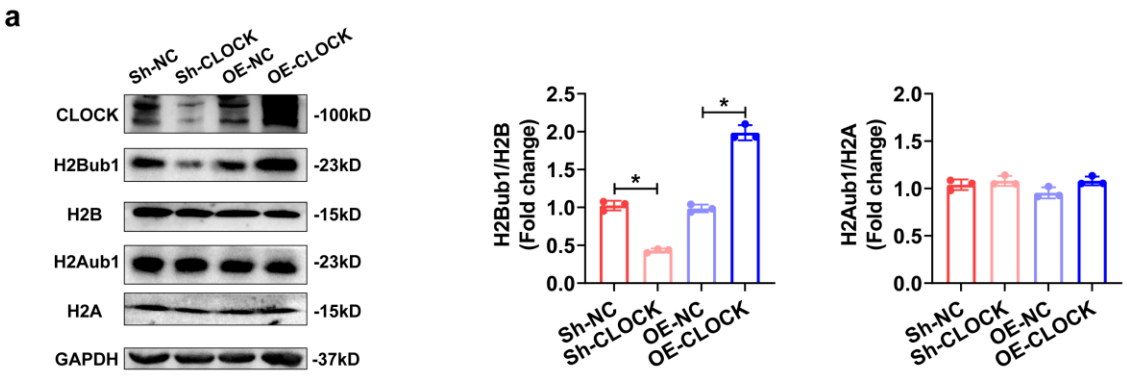

Fig. S3 The core circadian component CLOCK regulated histone H2B monoubiquitination levels. (a) H2Bub1 and H2Aub1 levels in the MSCs infected with Sh-NC, Sh-BMAL1, or Sh-CLOCK lentiviruses on the 10th day of osteogenic differentiation. H2B and H2A served as the internal controls. Bar graphs showing the relative levels. The data are presented as the means ± SDs, n = 3, *P < 0.05.

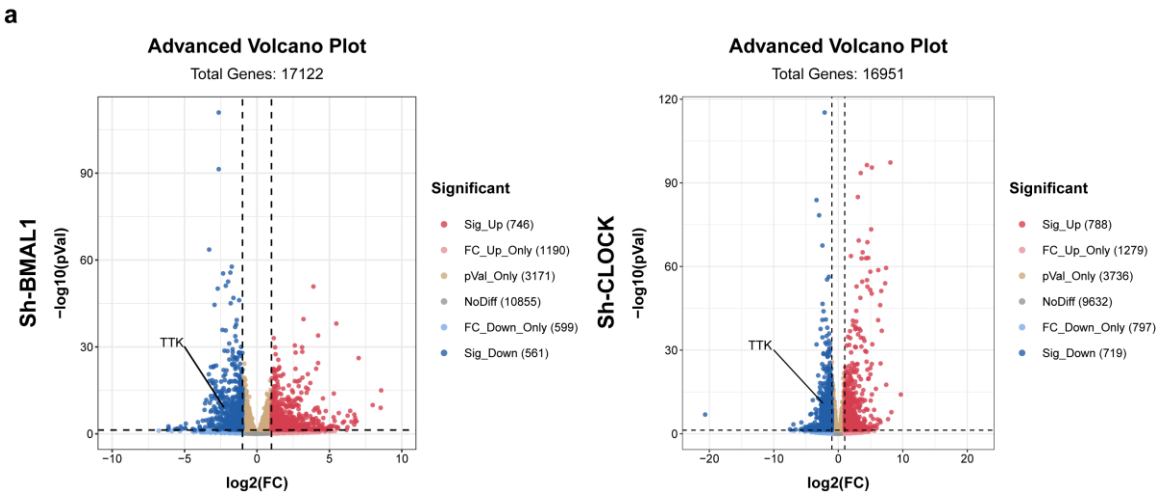

Fig. S4 The core circadian components BMAL1 and CLOCK regulated TTK expression. (a) Volcano plot showing the significantly differentially expressed gene, and TTK, the regulator of histone H2B monoubiquitination, is marked.

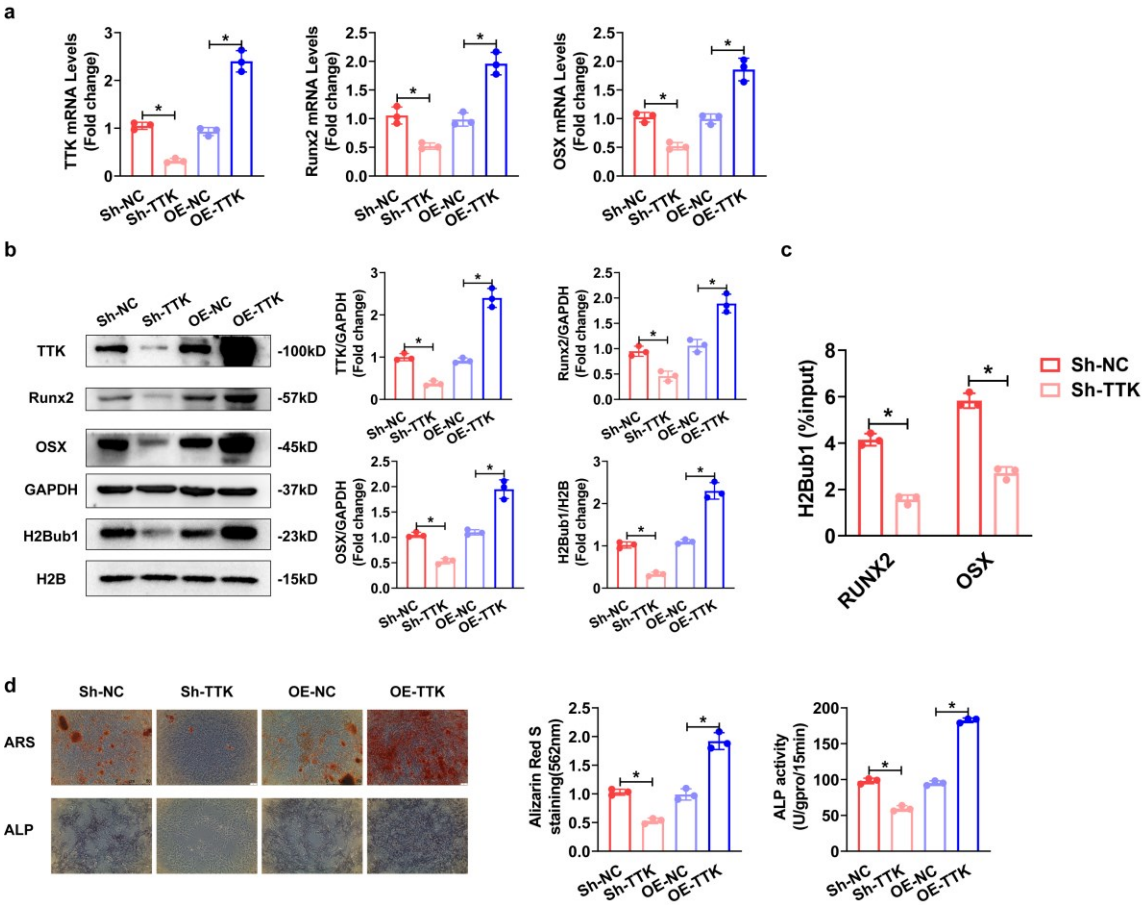

Fig. S5 TTK positively regulated the osteogenic differentiation of MSCs. (a, b) Relative mRNA (a) and protein (b) expression of TTK and the osteogenesis-associated markers RUNX2 and OSX in the MSCs infected with Sh-NC, Sh-TTK, OE-NC, or OE-TTK lentiviruses on the 10th day of osteogenic differentiation. (c) CUT&Tag-qPCR analysis showing that H2Bub1 occupancy on RUNX2 and OSX decreased in the MSCs while TTK expression was reduced. (d) ARS and ALP staining of the MSCs infected with Sh-NC, Sh-TTK, OE-NC, or OE-TTK lentiviruses on the 14th day of osteogenic differentiation. The data are presented as the means ± SDs, n = 3, *P < 0.05.

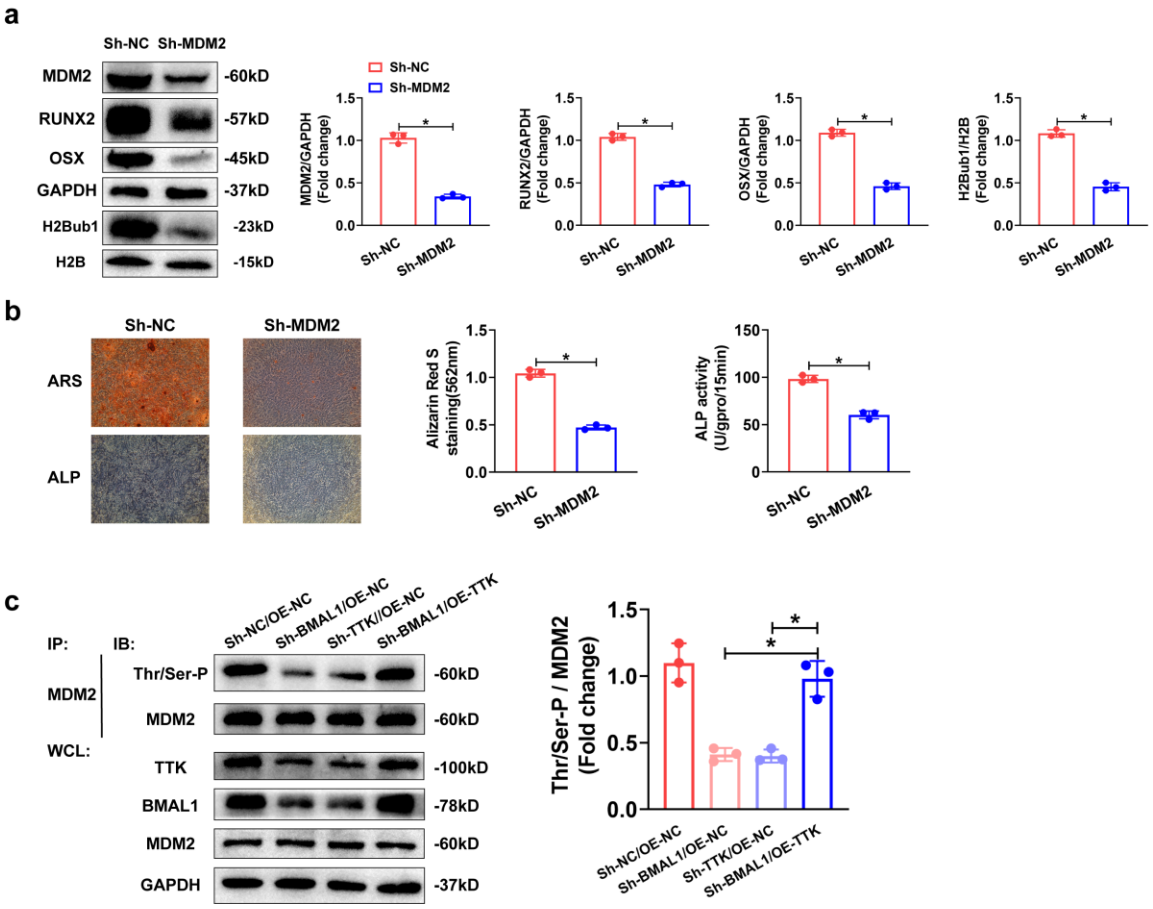

Fig. S6 MDM2 is part of the BMAL1-TTK-H2Bub1 axis, which regulates the osteogenic differentiation of MSCs. (a) Expression of MDM2 and the osteogenesis-associated markers RUNX2 and OSX in the MSCs infected with Sh-NC or Sh-MDM2 lentiviruses on the 10th day of osteogenic differentiation. (b) ARS and ALP staining of the MSCs infected with Sh-NC or Sh-MDM2 lentiviruses on the 14th day of osteogenic differentiation. (c) Co-IP/western blot assays showing that BAML1 regulates TTK expression to modulate MDM2 phosphorylation and ultimately regulate H2Bub1 level. All data are presented as the means ± SDs, n = 3, *P < 0.05.

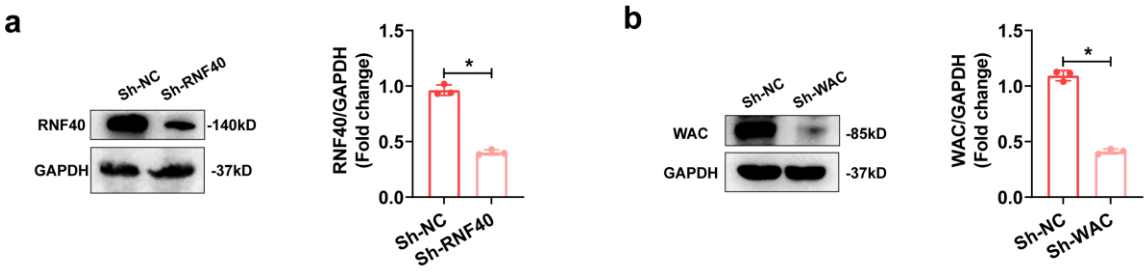

Fig. S7 Effect of Sh-RNF40 or Sh-WAC. (a, b) Protein expression of RNF40 and WAC in the MSCs infected with Sh-NC, Sh-RNF40 or Sh-WAC lentiviruses.

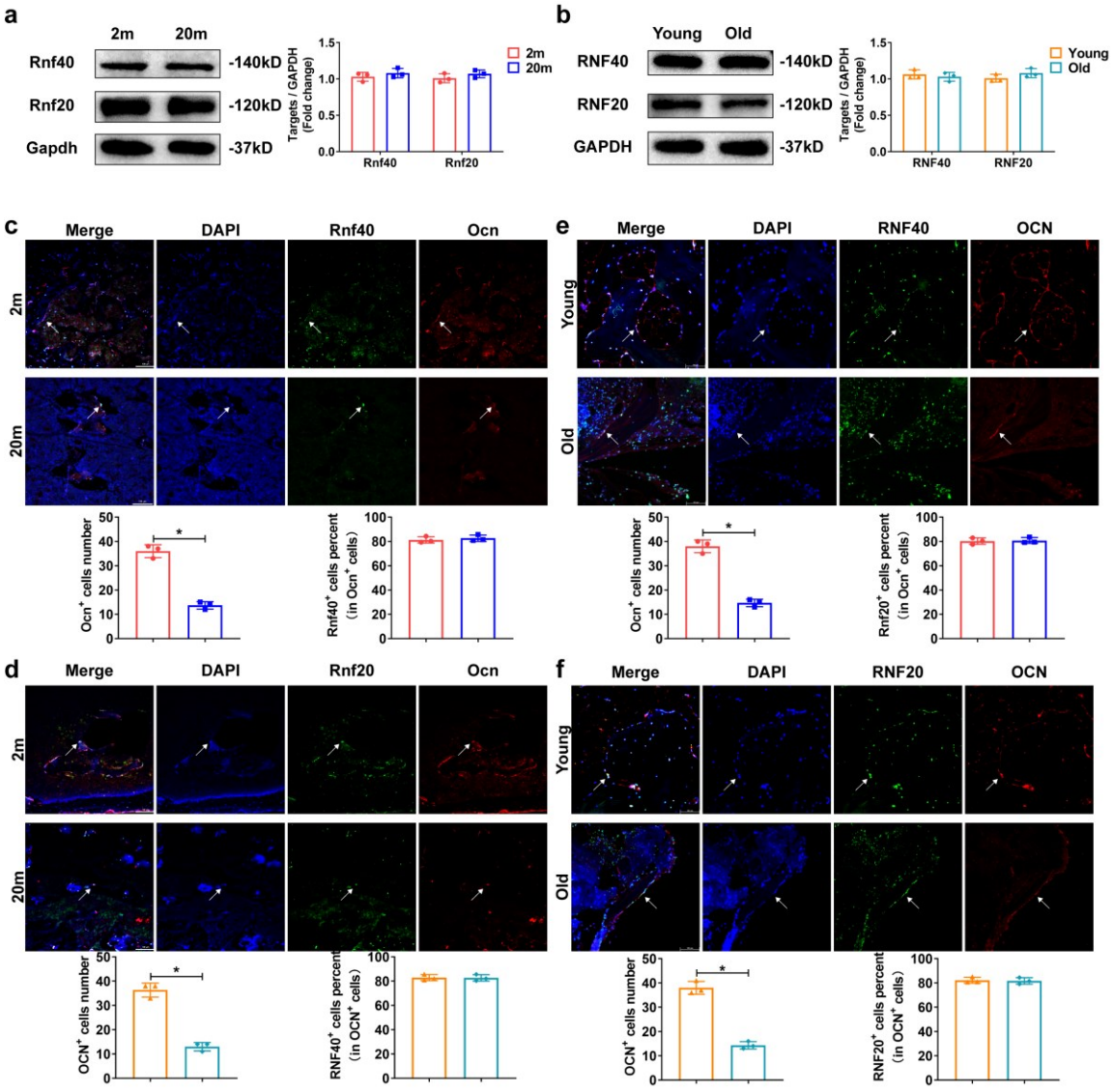

Fig. S8 RNF20/40 expression in BM-MSCs did not significantly differ between young stages and senile osteoporosis. (a-b) Western blot analysis of the expression of RNF20 and RNF40 in BM-MSCs from 2-month-old and 20-month-old mice, patients with traffic injuries and patients with senile osteoporosis. (c-d) Immunofluorescence staining (scale bar =100 μm) showing Rnf20 and Rnf40 expression in the Ocn+ osteoblast lineage in 2-month-old and 20-month-old mice (white arrows). (e-f) Immunofluorescence staining (scale bar =100 μm) showing RNF20 and RNF40 expression in the OCN+ osteoblast

lineage in young patients with traffic injuries and patients with senile osteoporosis (white arrows). All data are presented as the means ± SDs, n = 3, *P < 0.05.

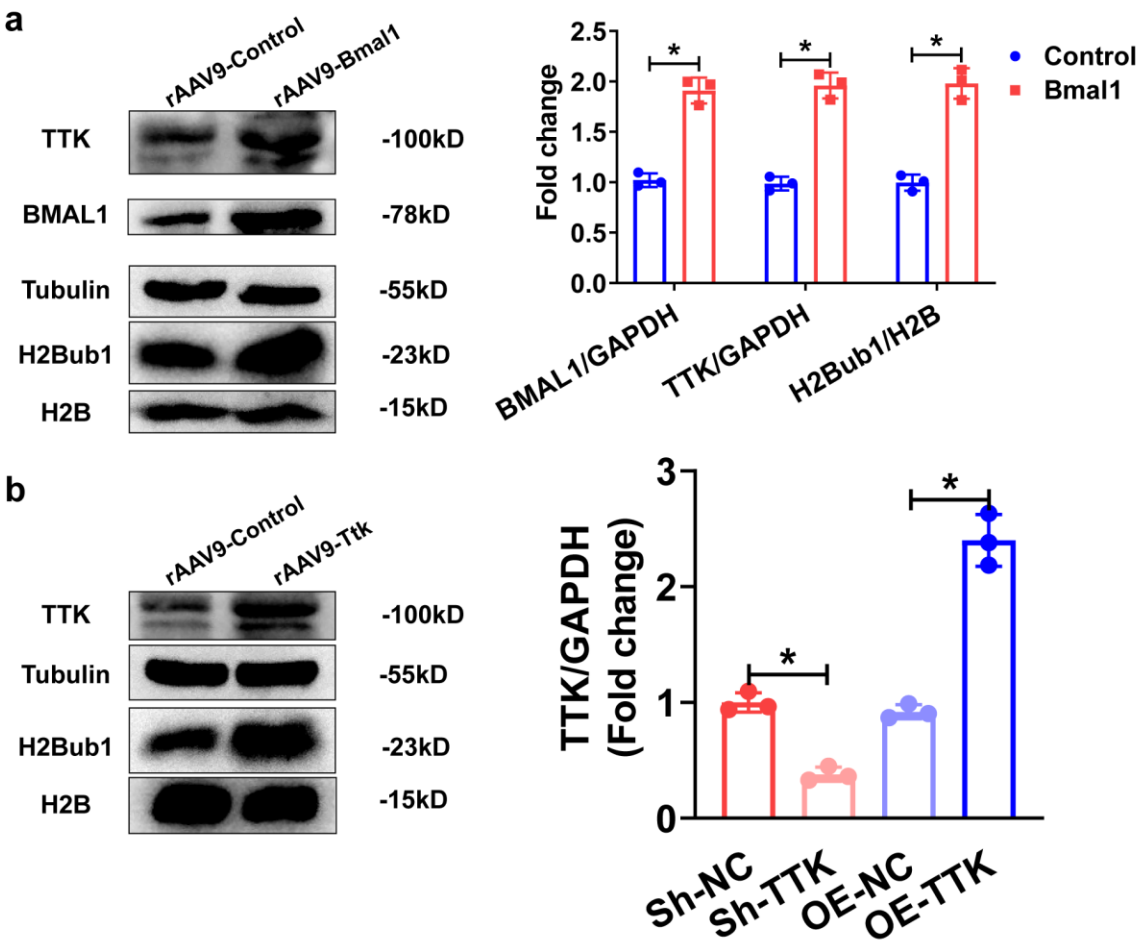

Fig. S9 Bone-targeted Bmal1 or Ttk rescue increased H2bub1 levels. (a) Immunoblot analysis showing Bmal1 and Ttk expression and H2Bub1 levels in the femoral tissue from the mice injected with rAAV9-control or rAAV9-Bmal1. (b) Immunoblot analysis showing Ttk expression and H2Bub1 levels in the femoral tissue from the mice injected with rAAV9-control or rAAV9-Ttk. The data are presented as the means ± SDs, n = 3, *P < 0.05.