

Supplementary Information

Bone-targeting AAV-mediated silencing of Schnurri-3 prevents bone loss in osteoporosis

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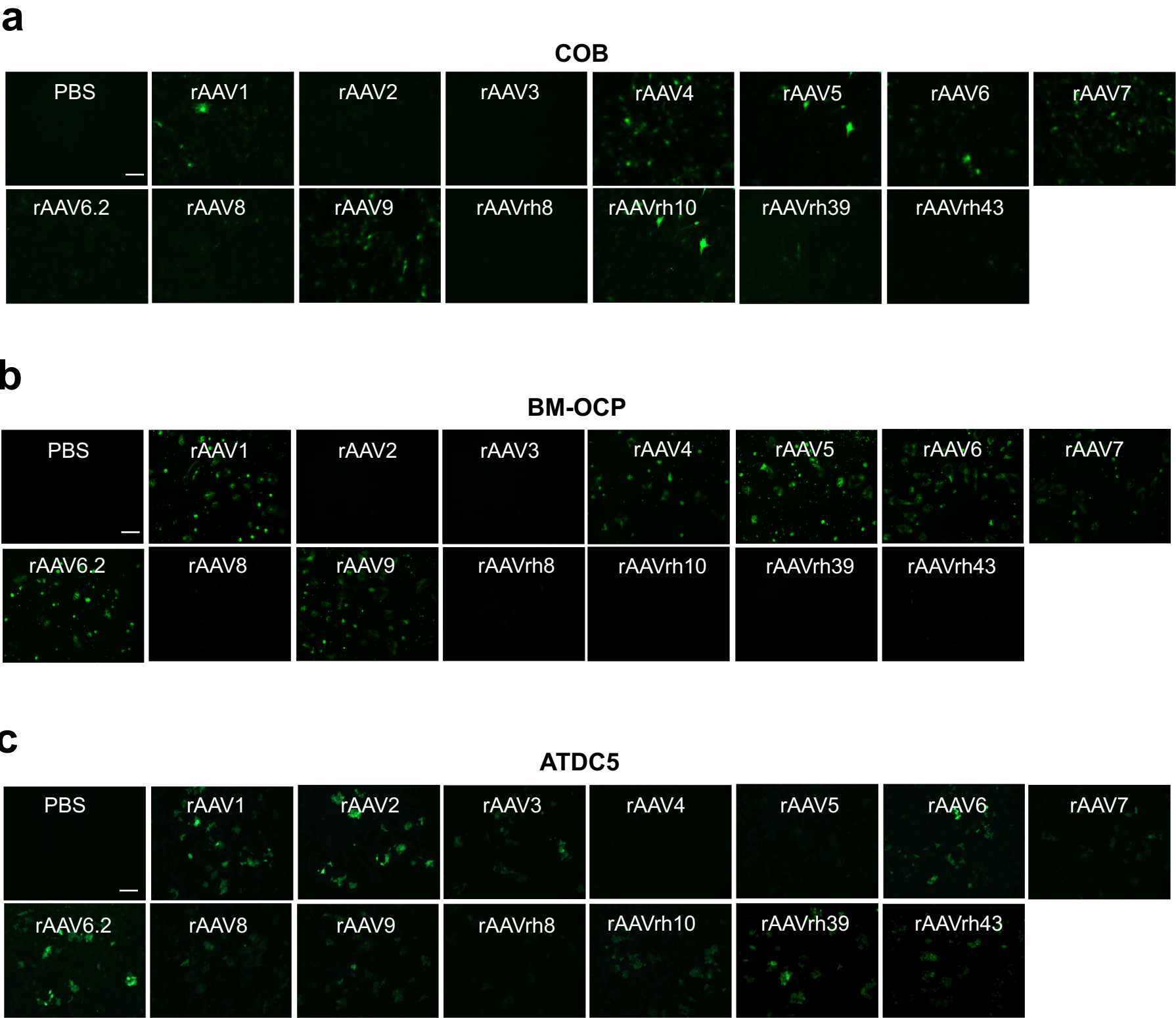
Jae-Hyuck Shim and Guangping Gao

18 **Supplementary Table 1: Sequences of primers, probes and gblocks**

Gene	Forward	Reverse
Mouse <i>shn3</i>	AGAGGCCATTTCAGACGAGTGT	CTGCGGAAGCTGAGAGATGT
Mouse <i>Alp</i>	CACAATATCAAGGATATCGACGTGA	ACATCAGTTCTGTTCTTCGGGTACA
Mouse <i>Runx2</i>	TACAAACCATACCCAGTCCCTGTTT	AGTGCTCTAACCACAGTCCATGCA
Mouse <i>Bsp</i>	CAGGGAGGCAGTGA CTCTTC	AGTGTGGAAAGTGTGGCGTT
Mouse <i>Osx</i>	ATGGCGTCCTCTCTGCTTGA	GAAGGGTGGGTAGTCATTTG
Mouse <i>Ocn</i>	GCAGCACAGGTCCTAAATAG	GGGCAATAAGGTAGTGAACAG
Mouse <i>Col1a1</i>	ACTGTCCCAACCCCAAG	ACGTATTCTTCCGGGCAGAA
Mouse <i>Hprt</i>	CTGGTGAAAAGGACCTCTCGAAG	CCAGTTTCACTAATGACACAAACG
EGFP	AGCAAAGACCCCAACGAGAA	GGCGGCGGTCACGAA
EGFP-probe	6FAM-CGCGATCACATGGTCCTGCTGG-TAMRA	
(AspSerSer) ₆	GATTCATCAGATTCTTCTGATTCATCCGACTCTTCTGACAGTTCAGACAGCTCT	
amiR-33-ctrl (<i>amiR-ctrl</i>)	TTTGTCTTTTATTTTCAGGTCCCAGATCTAGGGCTCTGCGTTTGCTCCAGGTAG TCCGCTGCTCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGCC GGCTGCACACCTCCTGGCGGGCAGCTGTGTACAACTACTTGAGAGCAGGT GTTCTGGCAATACCTGCCTGCTCTGTAATAGTTTGTACACGGAGGCCTGCCC TGACTGCCCACGGTGCCGTGGCCAAAGAGGATCTAAGGGCACCGCTGAGGG CCTACCTAACCATCGTGGGGAATAAGGACAGTGTCACCCCTGCAGGGGATCC GGTGGTGGTGCAAATCA	
amiR-33-SHN3 (<i>amiR-shn3</i>)	TTTGTCTTTTATTTTCAGGTCCCAGATCTAGGGCTCTGCGTTTGCTCCAGGTAG TCCGCTGCTCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGCC GGCTGCACACCTCCTGGCGGGCAGCTGTGTACAACTACTTGAGAGCAGGT GTTCTGGCAATACCTGCCTGCTCTGTAATAGTTTGTACACGGAGGCCTGCCC TGACTGCCCACGGTGCCGTGGCCAAAGAGGATCTAAGGGCACCGCTGAGGG CCTACCTAACCATCGTGGGGAATAAGGACAGTGTCACCCCTGCAGGGGATCC GGTGGTGGTGCAAATCA	

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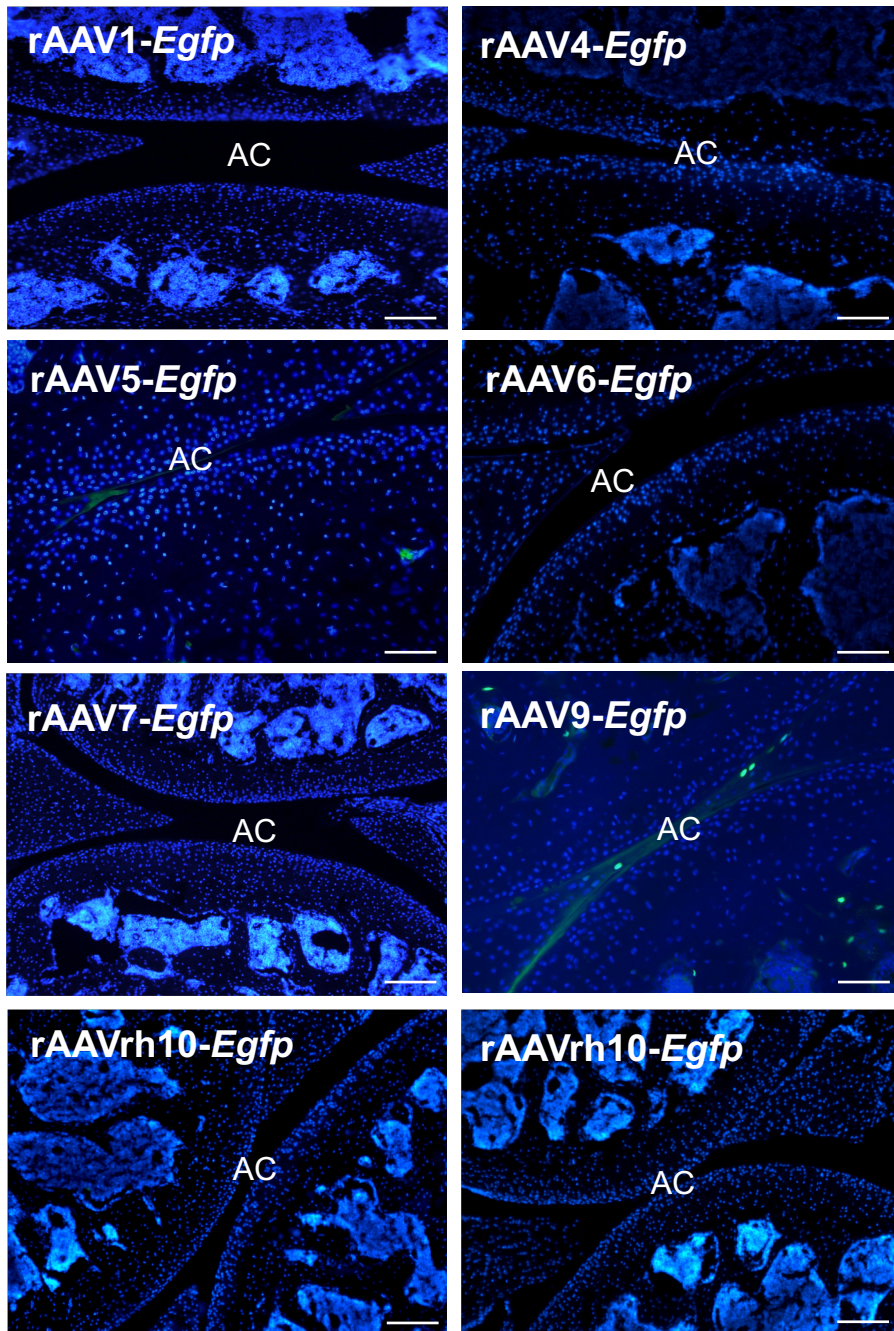
Supplementary Figure. 1



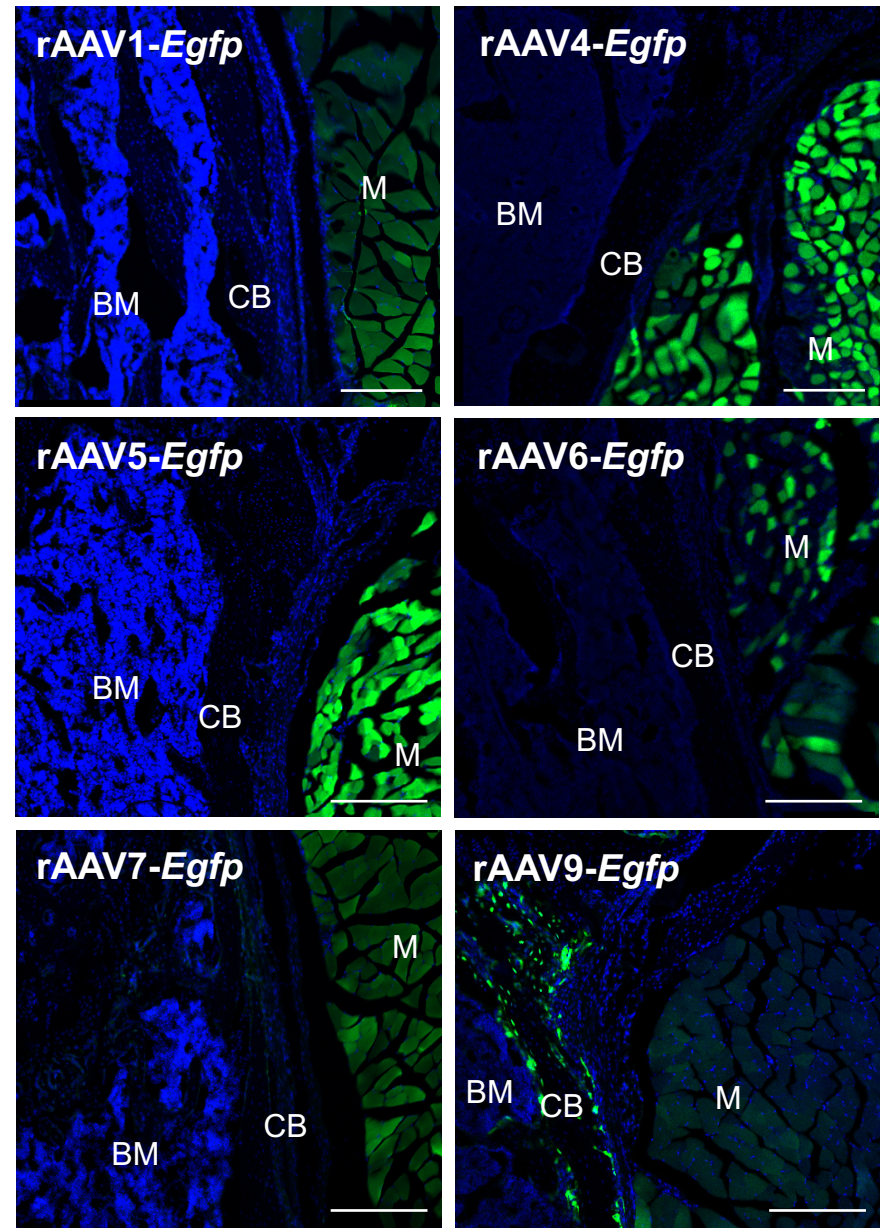
Supplementary Fig. 1: *In vitro* transduction assay of rAAV serotypes.
Calvarial osteoblasts (COB, **a**), bone marrow-derived osteoclast precursors (BM-OCP, **b**), or a chondrogenic cell line (ATDC5, **c**) were treated with PBS or 14 different AAV serotypes. Two days later, EGFP expression was monitored by fluorescence microscopy. Scale bar: 100 μ m.

Supplementary Figure. 2

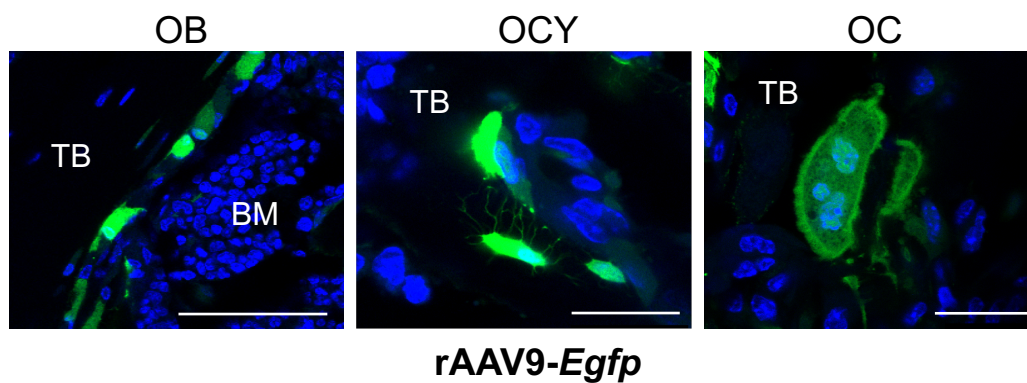
a



b



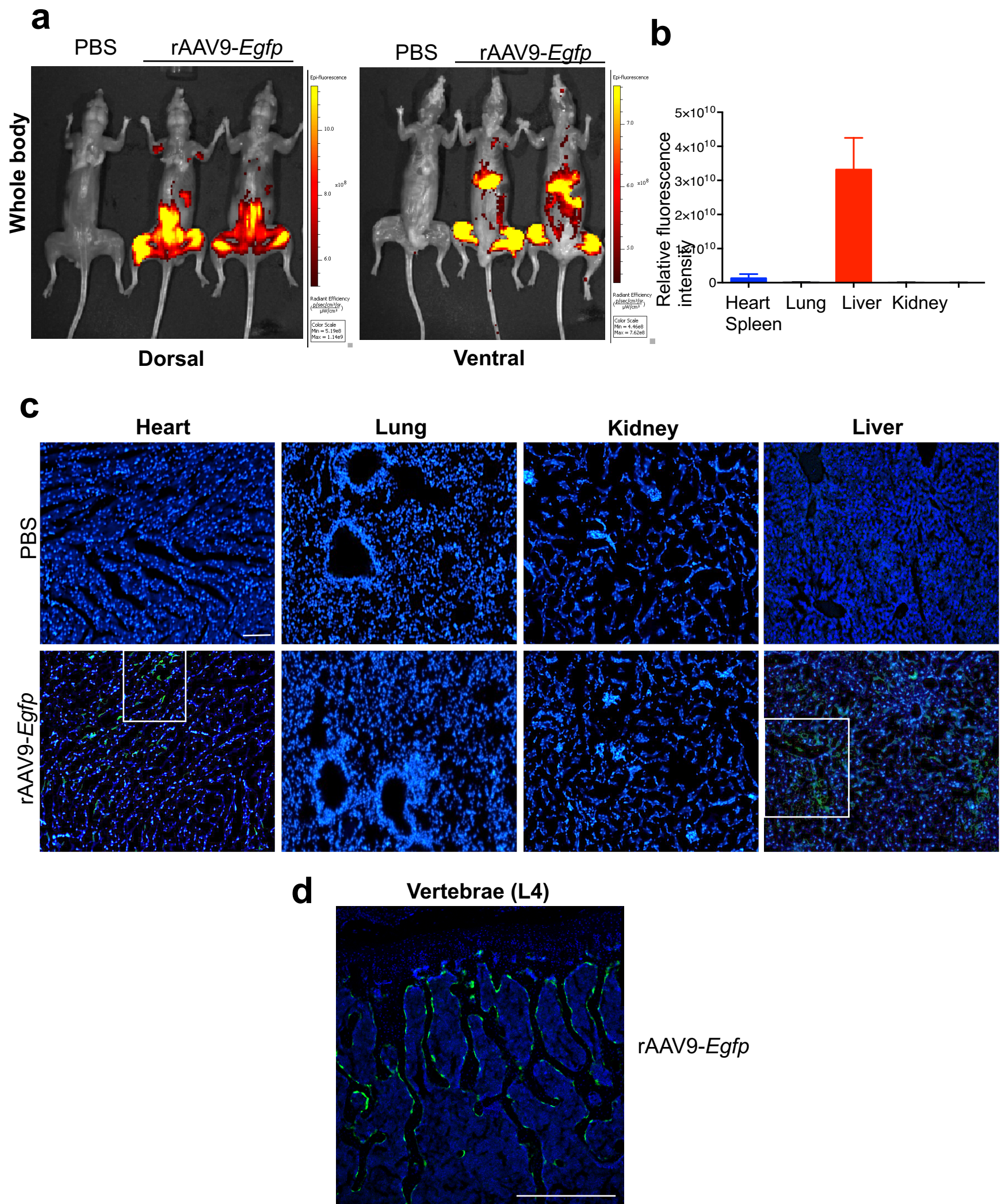
c



Supplementary Fig. 2: Identification of rAAV serotypes that transduce bone and cartilage.

A single dose of PBS or 1×10^{11} genome copies of rAAV9-Egfp was intraarticularly (i.a.) injected into knee joints of two-month-old male mice. Knee joints (**a**) and femurs (**b**) were cryo-sectioned to identify EGFP-expressing cells. DAPI was used to stain nuclei. **Panel c** represents high-magnification images of EGFP-expressing osteoblasts (OB), osteocytes (OCY), and mature osteoclasts (OC). AC, articular cartilage; M, muscle; CB, cortical bone; BM, bone marrow. Scale bars: 100 μ m, **panel a**; 250 μ m, **panel b**; 75 μ m (**left**) and 25 μ m (**middle, right**), **panel c**.

Supplementary Figure. 3

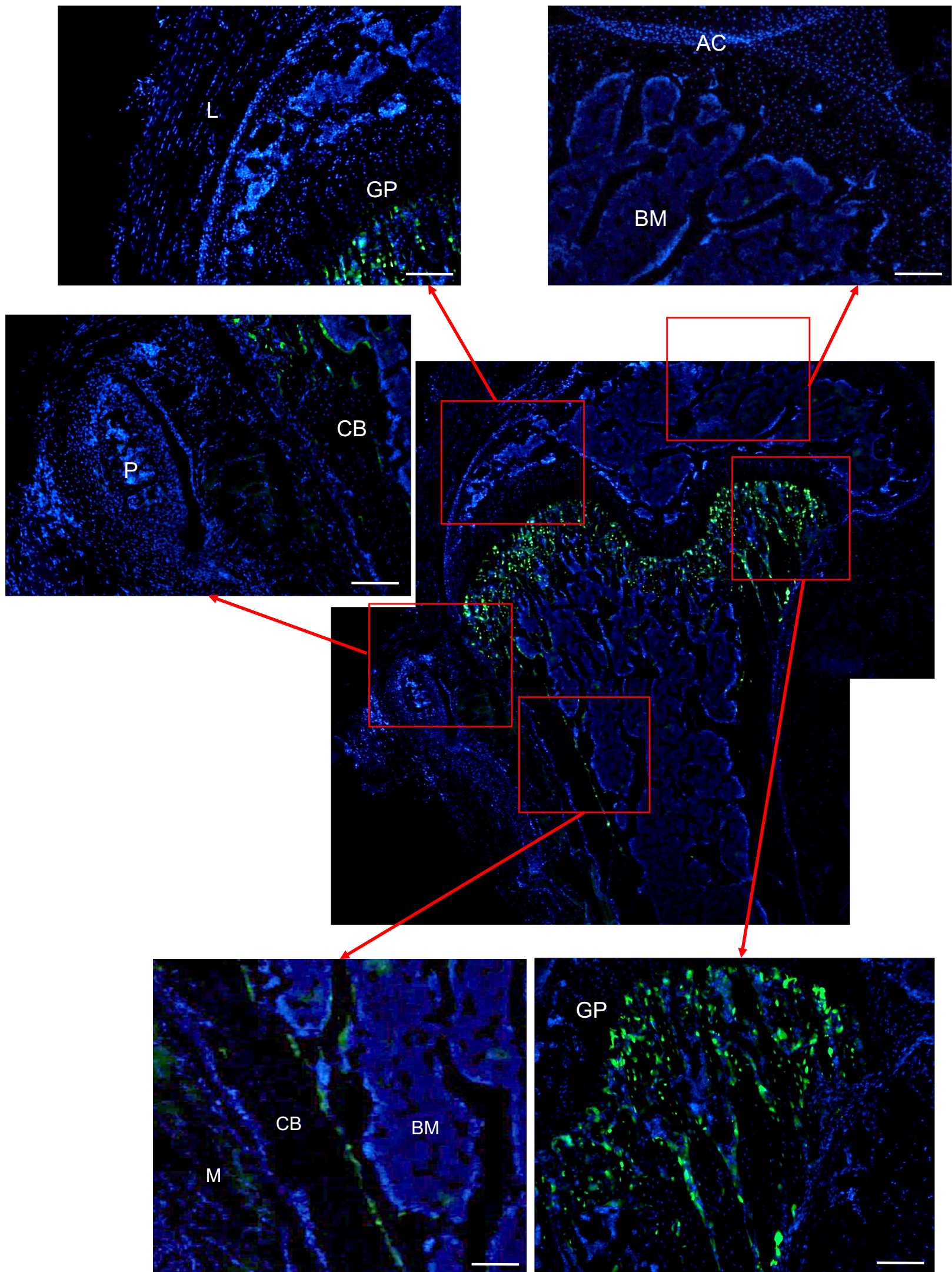


Supplementary Fig. 3: Tissue distribution of systemically-delivered rAAV9 in mice.

(a, b) A single dose of PBS or 4×10^{11} genome copies of *rAAV9-Egfp* was intravenously (i.v.) injected into two-month-old male mice and EGFP expression was monitored by IVIS-100 optical imaging two weeks post-injection. EGFP expression in whole body (a) and the quantification of EGFP expression in the dissected tissues shown in Fig. 2a are displayed (b). PBS-injection was used as a negative control.

(c, d) Tissues dissected from PBS- or *rAAV9-Egfp*-injected mice were cryo-sectioned to locate EGFP-expressing cells. Scale bars: 100 μ m, panel c; 500 μ m, panel d.

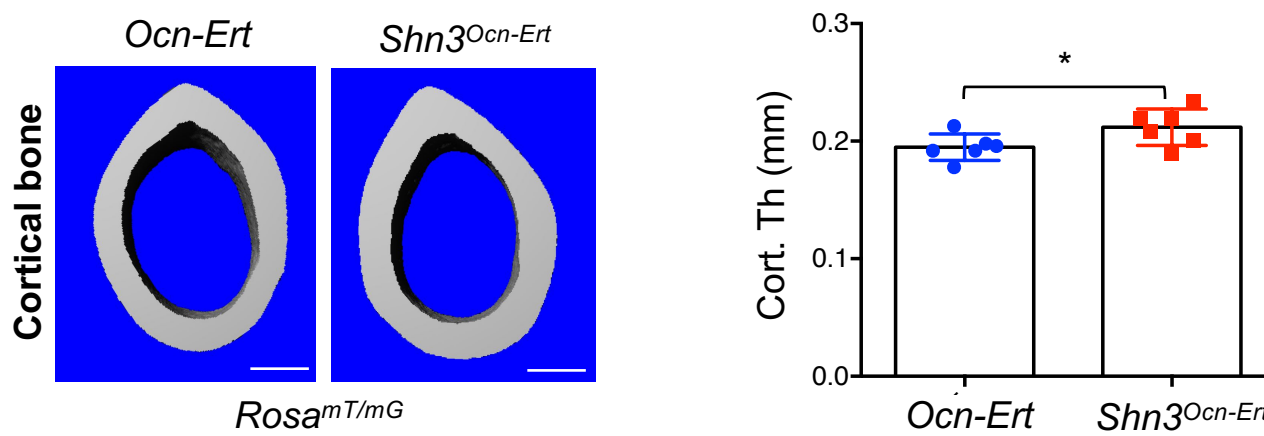
Supplementary Figure. 4



Supplementary Fig. 4: Distribution of systemically-delivered rAAV9 in the femur.

A single dose of 4×10^{11} genome copies of rAAV9-*Egfp* was i.v. injected into two-month-old male mice and EGFP expression was assessed in the cryo-sectioned femurs two weeks post-injection. L, Ligament; GP, growth plate; AC, articular cartilage; BM, bone marrow; CB, cortical bone; P, patella; M, muscle. Scale bars: 100 μ m.

Supplementary Figure. 5

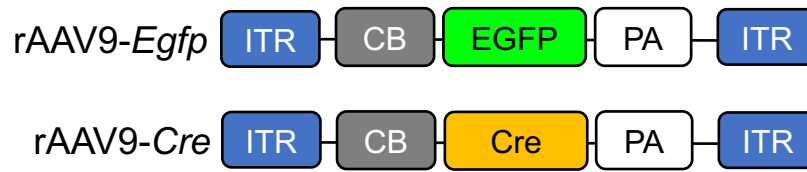


Supplementary Fig. 5: Inducible deletion of *Shn3* increases bone accrual in adult mice.

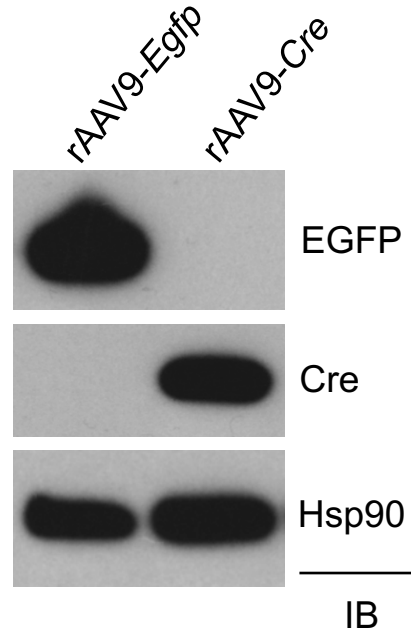
Two-month-old female *Ocn-Ert;Rosa^{mT/mG}* and *Shn3^{Ocn-Ert};Rosa^{mT/mG}* mice were treated with 100 mg/kg of tamoxifen for five consecutive days; two months later, femoral cortical bone mass was assessed by microCT. Representative 3D-reconstruction (**left**) and relative quantification (**right**) are displayed. Cortical Thickness (Cort.Th) (n = 6/group). Values represent mean $\pm$ SD: *, P < 0.05 by an unpaired two-tailed Student's t-test. Scale bars: 1 mm.

Supplementary Figure. 6

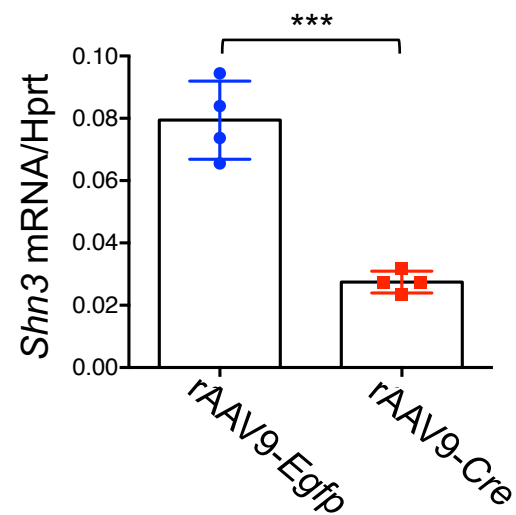
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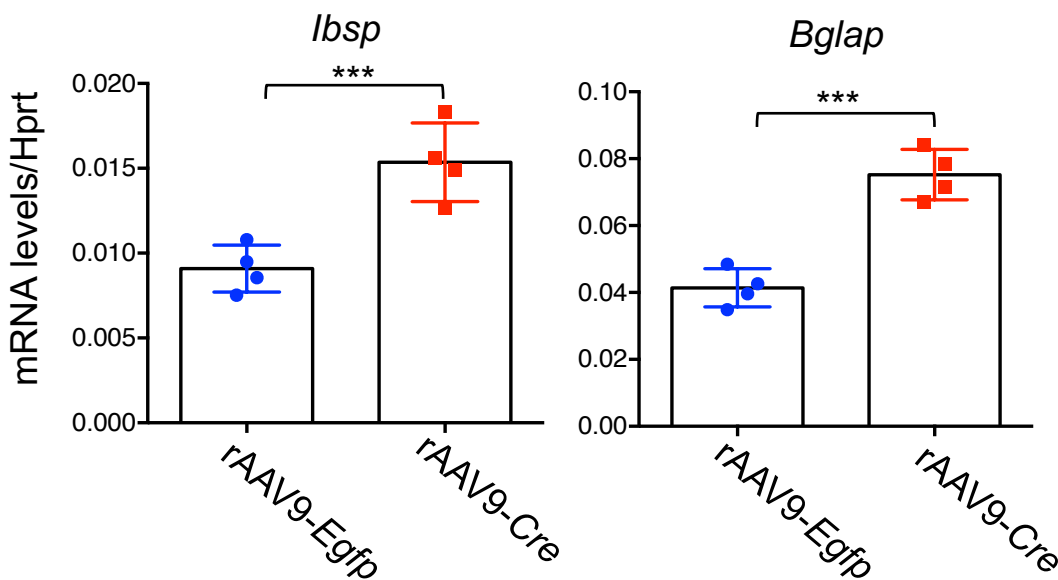
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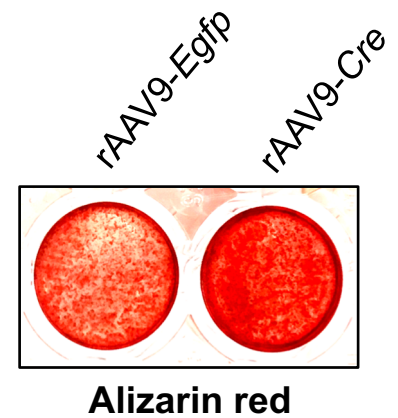
c



d



e



Supplementary Fig. 6: *In vitro* characterization of rAAV9-Cre in *Shn3^{fl/fl}* osteoblasts.

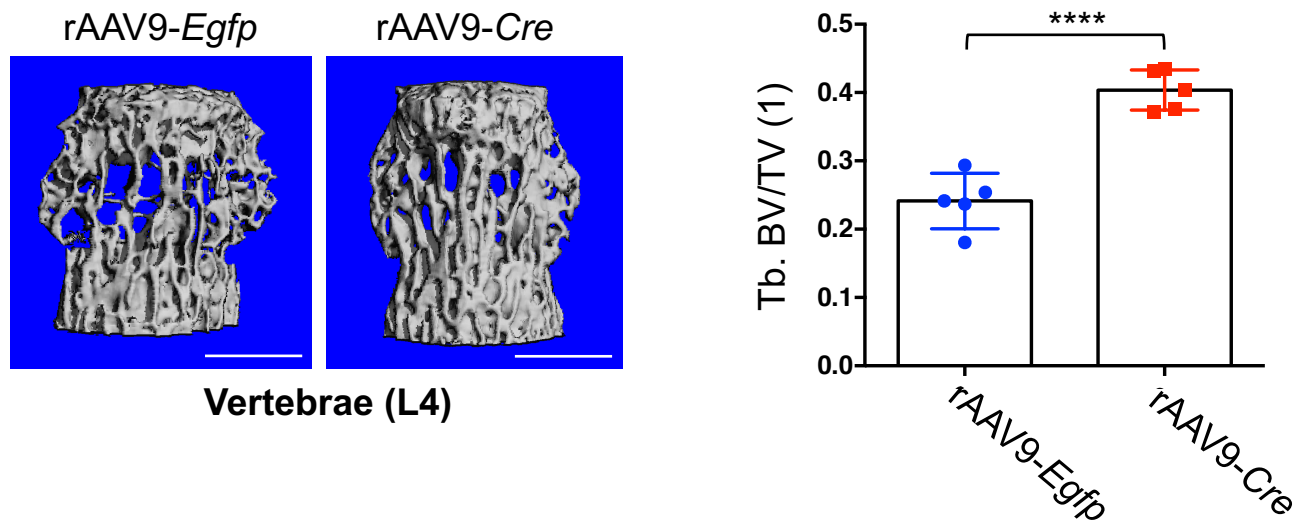
(a) Diagram of rAAV9 constructs containing the CMV enhancer/chicken β -actin promoter (CB), an *Egfp* reporter gene (EGFP), or Cre recombinase (Cre), β -globin polyA sequence (PA), and inverted terminal repeat (ITR).

(b) COBs were infected with rAAV9-*Egfp* or rAAV9-*Cre* for two days and cells were lysed and immunoblotted with the indicated antibodies.

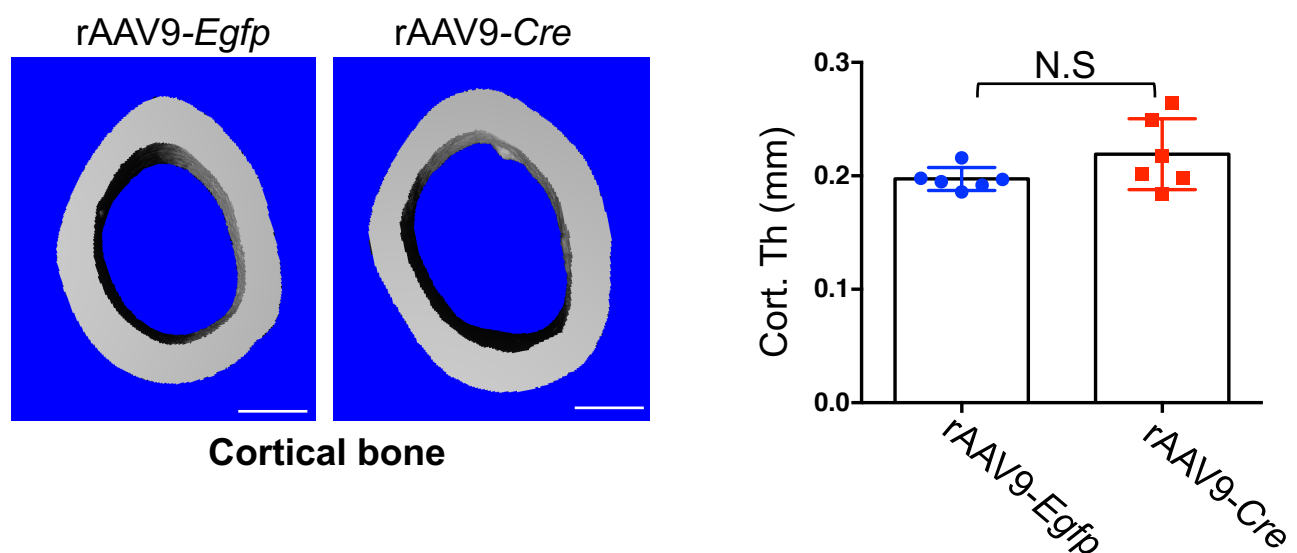
(c-e) Two days after treatment with rAAV9-*Egfp* or rAAV9-*Cre*, COBs were cultured under osteogenic conditions for six days and mRNA levels of *Shn3* (c) and osteogenic genes (d) were measured by RT-PCR. After 21 days of the culture, mineralization was assessed by alizarin red staining (e). Values represent mean $\pm$ SD: ***, P < 0.001 by an unpaired two-tailed Student's t-test (c, d).

Supplementary Figure. 7

a



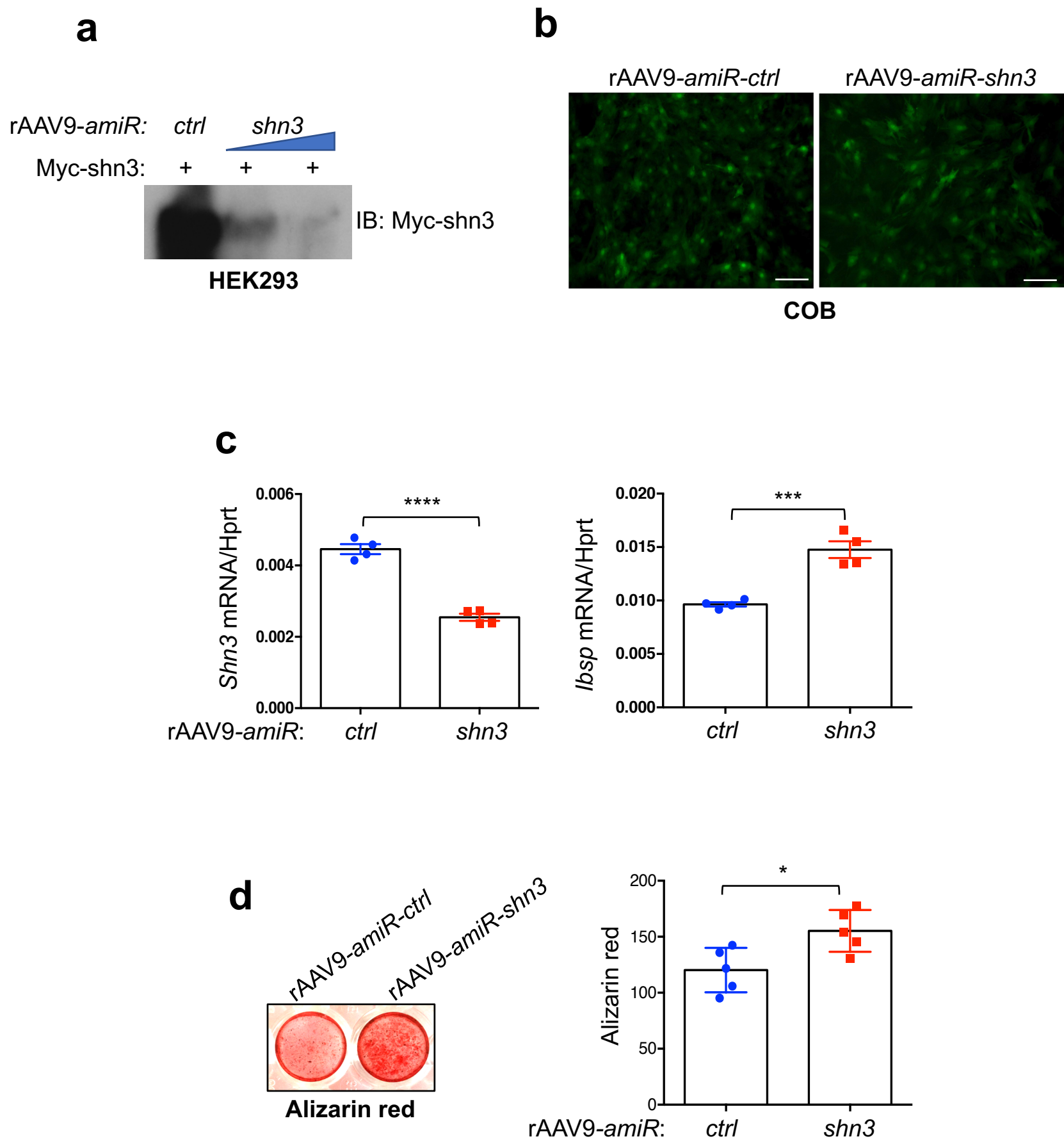
b



Supplementary Fig. 7: *In vivo* characterization of rAAV9-Cre in *Shn3^{fl/fl}* mice.

A single dose of 4×10^{11} genome copies of rAAV9-Egfp or rAAV9-Cre was i.v. injected into three-month-old male *Shn3^{fl/fl};Rosa^{mTmG}* mice. Two months later, trabecular bone mass in the lumbar vertebrae (**a**) and cortical bone thickness in the femur (**b**) were assessed by microCT. Representative 3D-reconstruction (**left**) and relative quantification (**right**) are displayed. Scale bars: 1 mm.

Supplementary Figure. 8

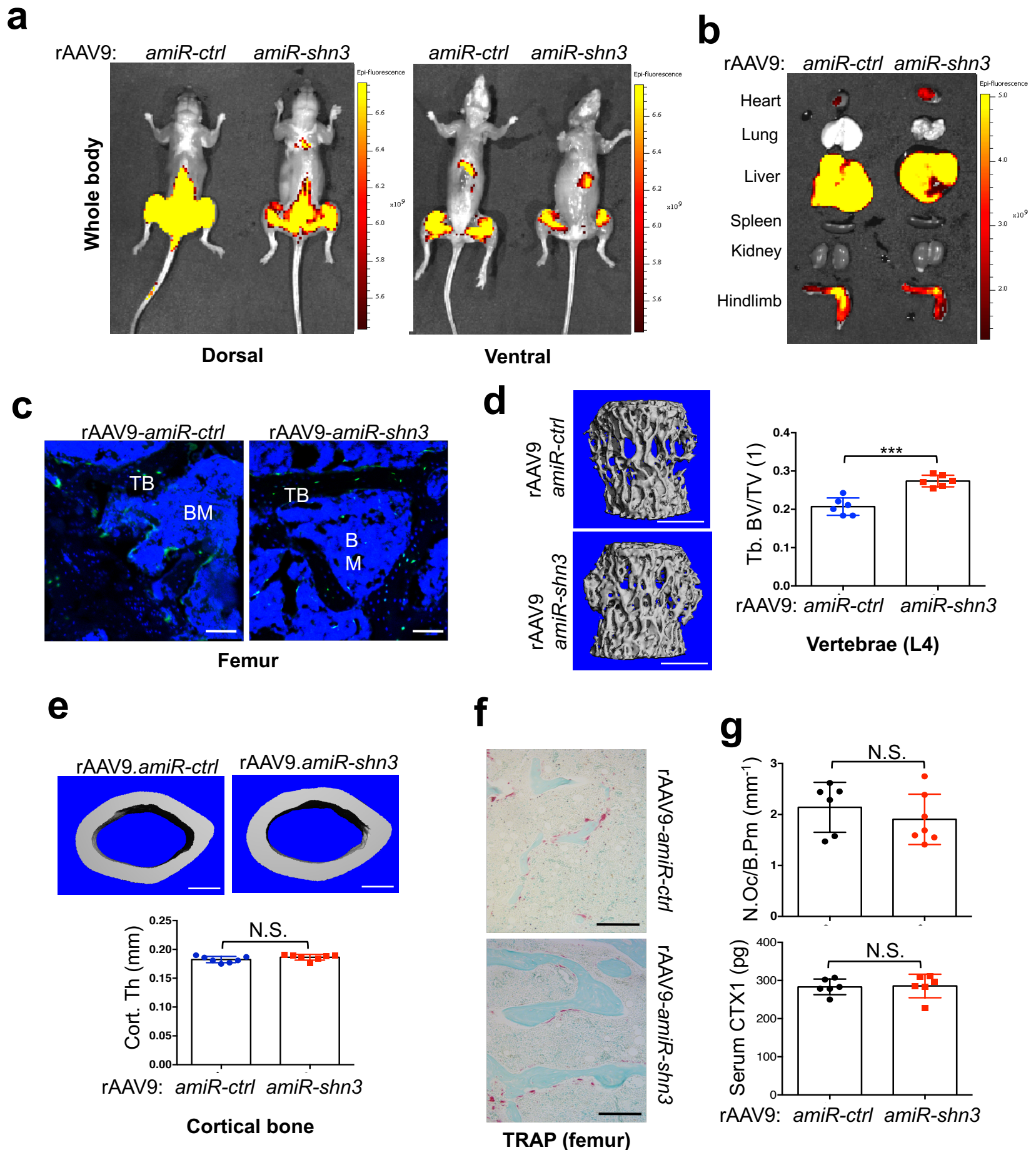


Supplementary Fig. 8: *In vitro* characterization of rAA9-amiR-*shn3* in osteoblasts.

(a) HEK293 cells were transiently transfected with Myc-tagged mouse *shn3* along with plasmids encoding *amiR-shn3* or *amiR-ctrl*. Two days later, cell lysates were immunoblotted with anti-Myc antibody.

(b-d) Two days after treatment with rAAV9 carrying *amiR-ctrl* or *amiR-shn3*, COBs were cultured under osteogenic conditions. EGFP expression **(b)**, mRNA levels of *Shn3* and osteogenic gene **(c)**, and mineralization **(d)**, were assessed by fluorescence microscopy, RT-PCR, and alizarin red staining, respectively. Scale bar: 100 μ m, **panel b**. Values represent mean $\pm$ SD: *, $P < 0.05$; ***, $P < 0.001$; and ****, $P < 0.0001$ by an unpaired two-tailed Student's t-test **(c, d)**.

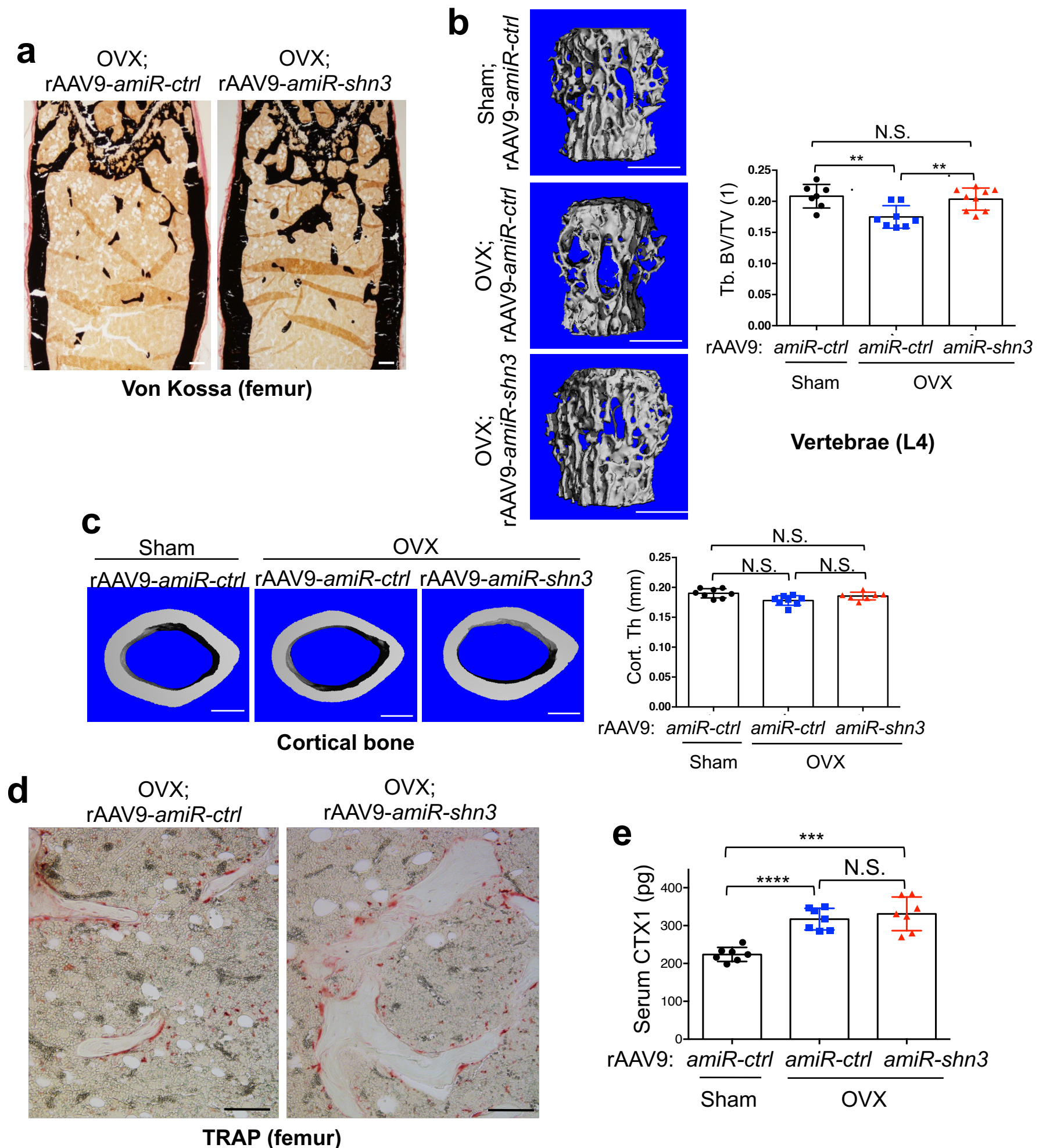
Supplementary Figure. 9



Supplementary Fig. 9: Characterize mice with systemically delivered rAAV9-*amiR-shn3*.

A single dose of 4×10^{11} genome copies of rAAV9 carrying *amiR-ctrl* or *amiR-shn3* was i.v. injected into three-month-old female mice. Two months following treatment, mice were labeled with calcein and alizarin red for dynamic histomorphometry. Non-labeled mice were used to monitor EGFP expression using IVIS-100 optical imaging. EGFP expression in the whole body (**a**) and the dissected tissues (**b**) are displayed. Femurs were cryo-sectioned to identify EGFP-expressing osteoblast lineage cells (**c**). Trabecular bone mass in the lumbar vertebrae (**d**) and cortical bone thickness in the femur (**e**) were assessed by microCT. TRAP-stained longitudinal sections (**f**) and histomorphometric analysis (**top, g**) of femurs from five-month-old female mice treated with rAAV9 carrying *amiR-ctrl* or *amiR-shn3* ($n = 6\sim7$). The number of osteoclasts per bone perimeter (N.Oc/B.Pm). Serum CTX levels were assessed by ELISA ($n = 6$) (**bottom, g**). Scale bars: 100 μm , **panel c**; 1 mm, **panel d and e**; 50 μm , **panel f**. Values represent mean $\pm$ SD; N.S., not significant; ***, $P < 0.001$ by an unpaired two-tailed Student's *t*-test (**d, e, g**).

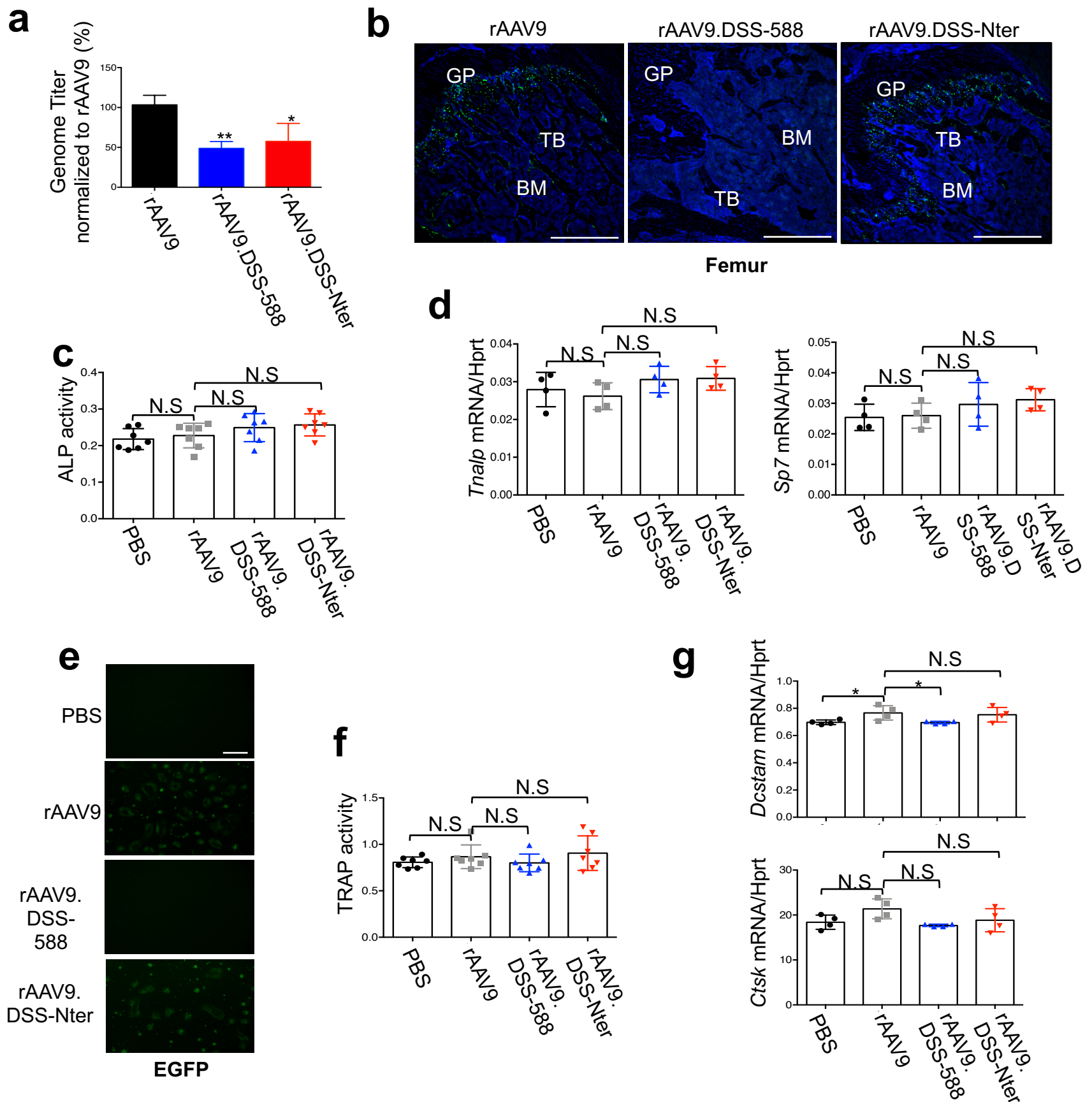
Supplementary Figure. 10



Supplementary Fig. 10: Effects of rAAV9-mediated silencing of *shn3* in osteoporosis.

Sham or OVX surgery was performed on three-month-old female mice and six weeks later, a single dose of 4×10^{11} genome copies of rAAV9 carrying *amiR-ctrl* or *amiR-shn3* was i.v. injected. Seven weeks after injection, mice were labeled with calcein and alizarin red for dynamic histomorphometry. Staining by Von Kossa (**a**) or TRAP (**d**) was performed in longitudinal sections of femurs from five-month-old female OVX mice treated with rAAV9 carrying *amiR-ctrl* or *amiR-shn3*. Trabecular bone mass in the lumbar vertebrae (**b**) and cortical bone thickness in the femur (**c**) were assessed by microCT. Serum CTX levels were assessed by ELISA ($n = 7$) (**e**). Scale bar: 1 mm, **panels a-c**; 50 μ m, **panel d**. Values represent mean $\pm$ SD; NS, not significant, **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$ by an unpaired two-tailed Student's *t*-test (**c**).

Supplementary Figure. 11



Supplementary Fig. 11: Characterization of rAAV9.DSS vectors *in vitro* and *in vivo*.

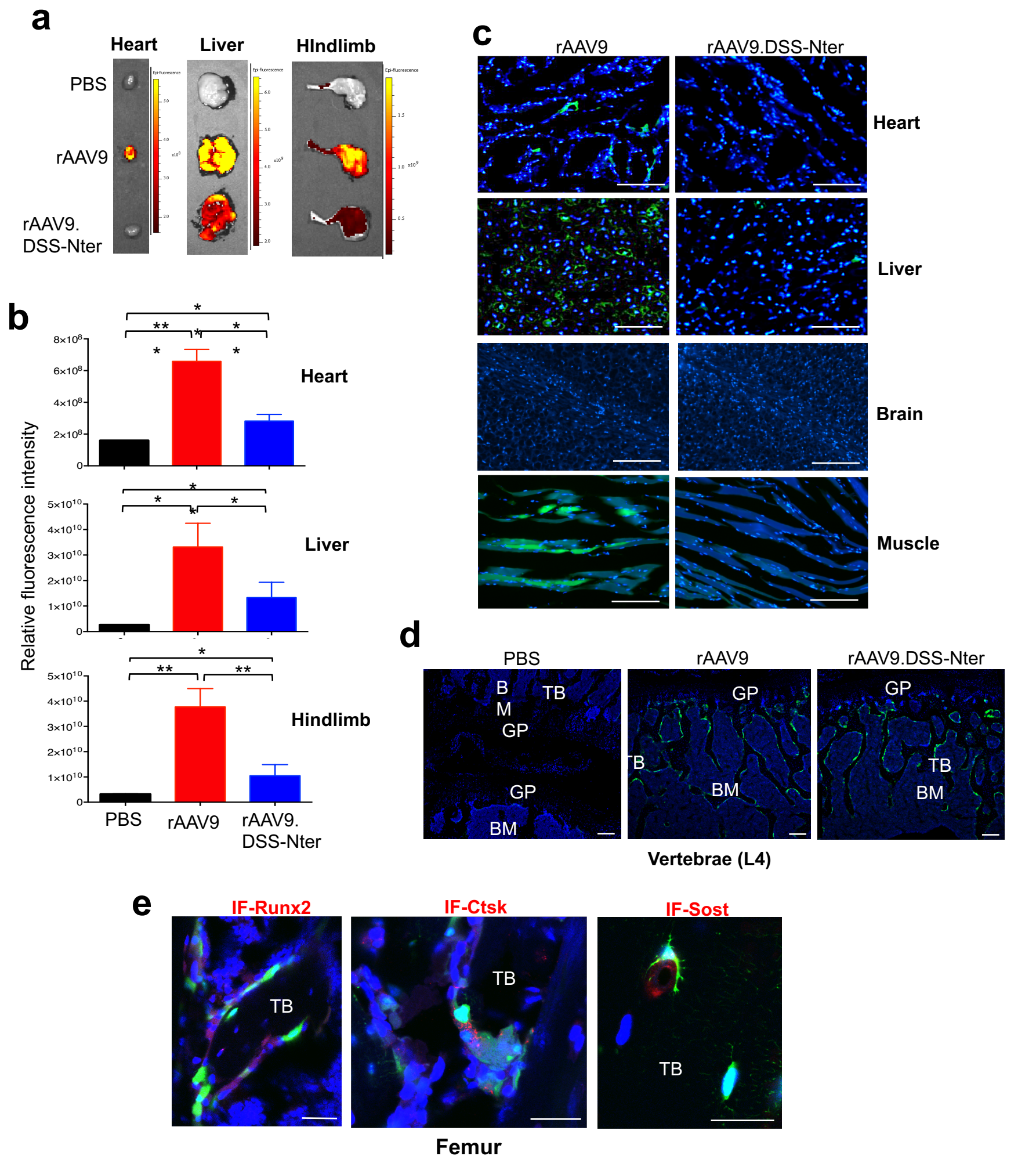
(a) The genome titers of rAAV9.DSS-588 and rAAV9.DSS-Nter were measured by ddPCR and normalized to that of rAAV9.

(b) A single dose of 1×10^{11} genome copies of rAAV9-*Egfp*, rAAV9.DSS-588-*Egfp* or rAAV9.DSS-Nter-*Egfp* was i.a. injected into knee joints of two-month-old male mice and femurs were cryo-sectioned to identify EGFP-expressing cells two weeks post-injection. GP, growth plate; CB, cortical bone; BM, bone marrow. Scale bar: 500 μ m, **panel b**.

(c, d) Two days after infection with rAAV9-*Egfp*, rAAV9.DSS-588-*Egfp* or rAAV9.DSS-Nter-*Egfp*, COBs were cultured under osteogenic conditions for six days. Osteoblast differentiation was assessed by ALP activity (**c**) and osteogenic gene expression (**d**).

(e-g) Two days after treatment with M-CSF (20 ng/ml) and Rank ligand (10 ng/ml), bone marrow-derived monocytes were incubated with rAAV9-*Egfp*, rAAV9.DSS-588-*Egfp* or rAAV9.DSS-Nter-*Egfp* and EGFP expression was monitored by fluorescence microscopy two days post-infection (**e**). Osteoclast differentiation was assessed by TRAP activity (**f**) and osteoclast gene expression (**g**). Values represent mean $\pm$ SD: N.S, non-significant; *, $P < 0.05$ by one-way ANOVA test. Scale bars: 500 μ m, **panel b**; 1 mm, **panel e**.

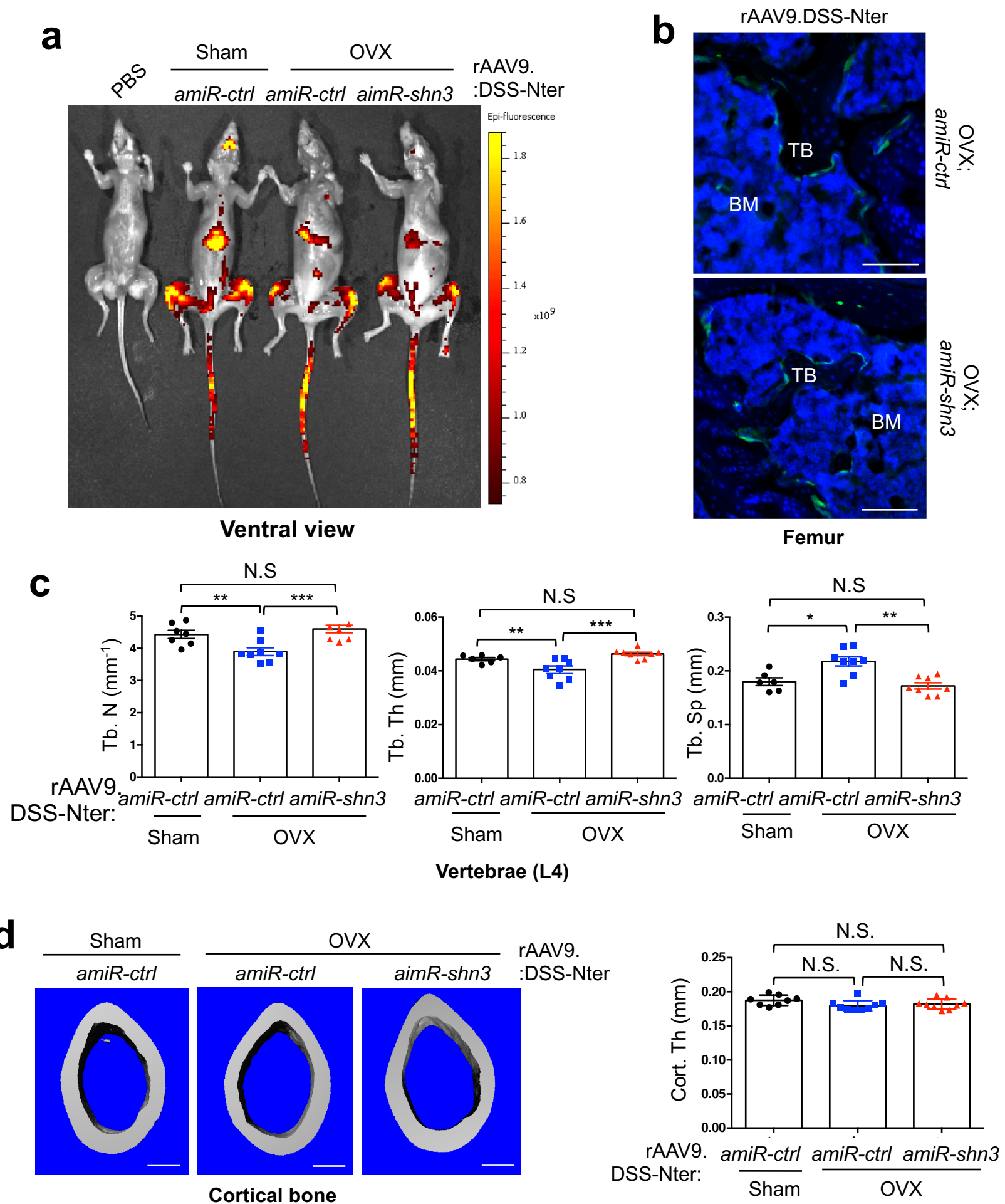
Supplementary Figure. 12



Supplementary Fig. 12: Tissue distribution of systemically delivered rAAV9.DSS-Nter.

A single dose of PBS or 4×10^{11} genome copies of rAAV9, rAAV9.DSS-588, or rAAV9.DSS-Nter was i.v. injected into two-month-old male mice and EGFP expression was monitored using IVIS-100 optical imaging two weeks post-injection. EGFP expression in the heart, liver, and hindlimb (**a**) and quantification of EGFP expression in dissected tissues (**b**) are displayed. Heart, liver, brain, muscle (**c**) and lumbar vertebrae (**d**) were cryo-sectioned to identify EGFP-expressing cells. Cryo-sectioned femurs were also immunostained for Runx2, Ctsk, and Sost to identify osteoblasts, mature osteoclasts, and osteocytes, respectively (**e**). GP, growth plate; BM, bone marrow; TB, trabecular bone. Scale bar: 100 μ m, **panels c and d**; 25 μ m, **panel e**. Values represent mean $\pm$ SD: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ by one-way ANOVA test (**b**).

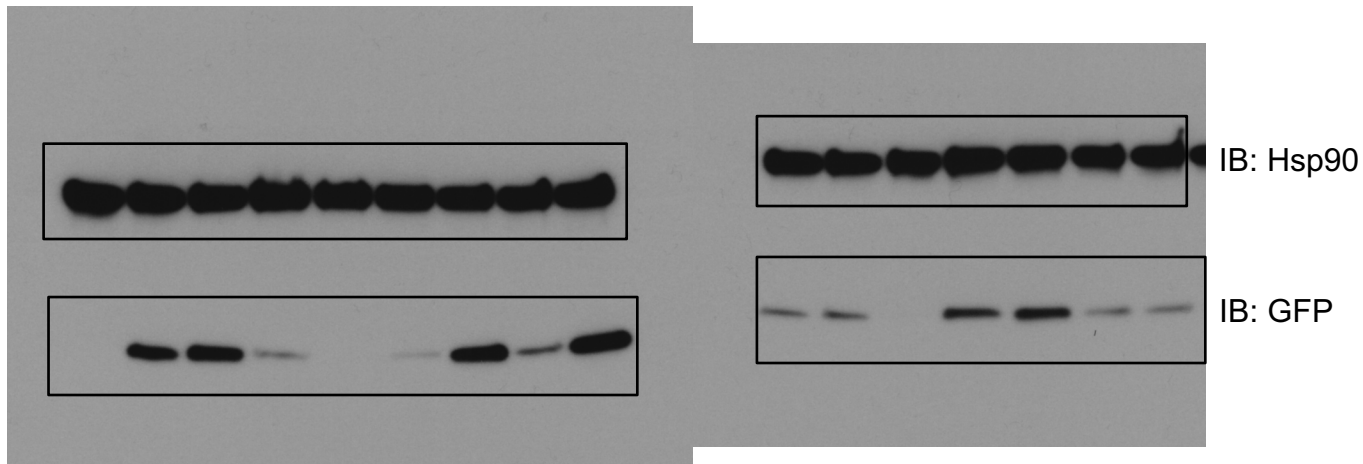
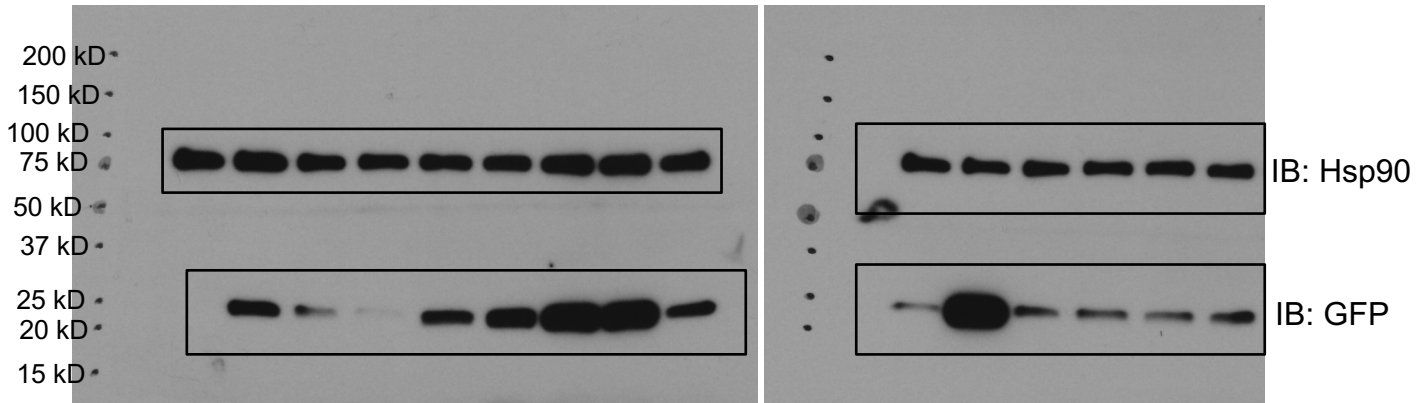
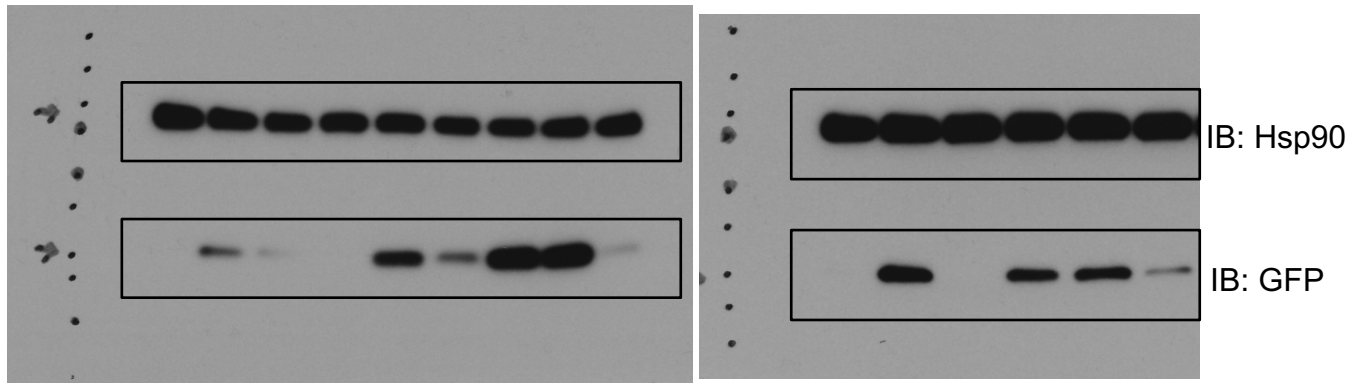
Supplementary Figure. 13



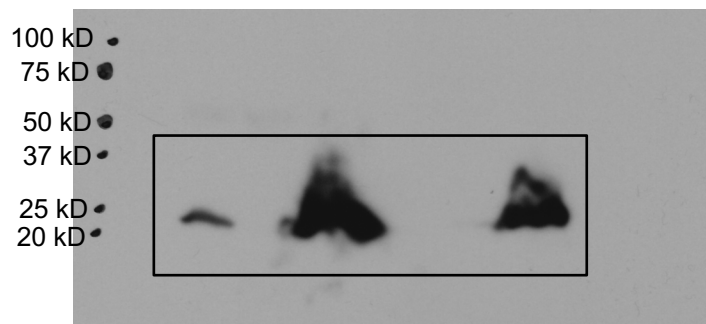
Supplementary Fig. 13: Effects of rAAV9.DSS-Nter carrying *amiR-shn3* in osteoporosis.

Sham or OVX surgery was performed on three-month-old female mice and six weeks later, a single dose of PBS or 4×10^{11} genome copies of rAAV9.DSS-Nter carrying *amiR-ctrl* or *amiR-shn3* were i.v. injected. Seven weeks after injection, EGFP expression was monitored by IVIS-100 optical imaging (**a**). EGFP-expressing cells in the cryo-sectioned femurs were identified by fluorescence microscopy (**b**). Trabecular bone mass in lumbar vertebrae (**c**) and cortical bone thickness in the femur (**d**) was assessed by microCT ($n = 7\sim 8/\text{group}$). Trabecular number per cubic millimeter (Tb.N), trabecular thickness (Tb.Th), and trabecular space (Tb. Sp). TB, trabecular bone; BM, bone marrow. Scale bar: $100\ \mu\text{m}$, **panel b**. Values represent mean $\pm$ SD: N.S, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA test (**c**).

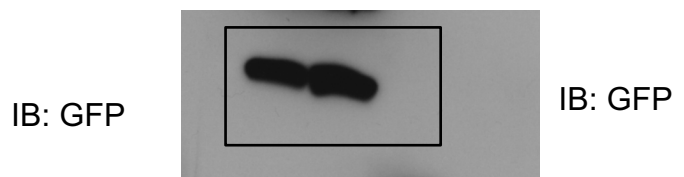
Full uncut gel for Figure 1a



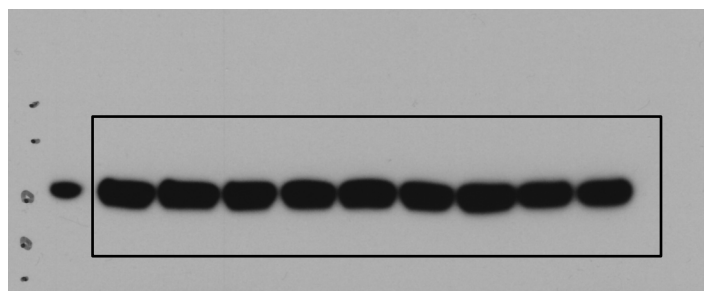
Full uncut gel for Figure 2d



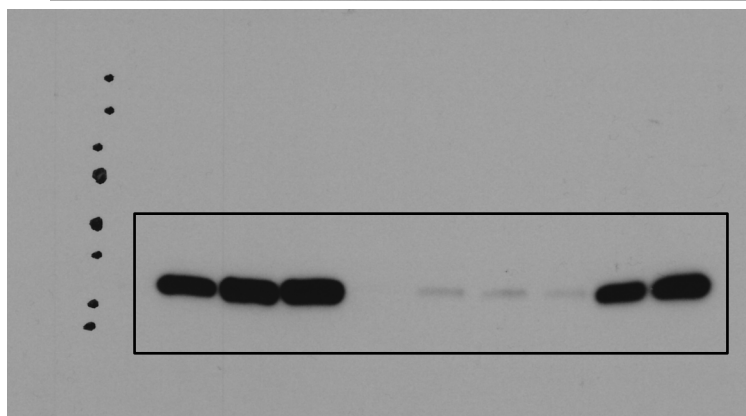
Full uncut gel for Figure 3d



Full uncut gel for Figure 5b

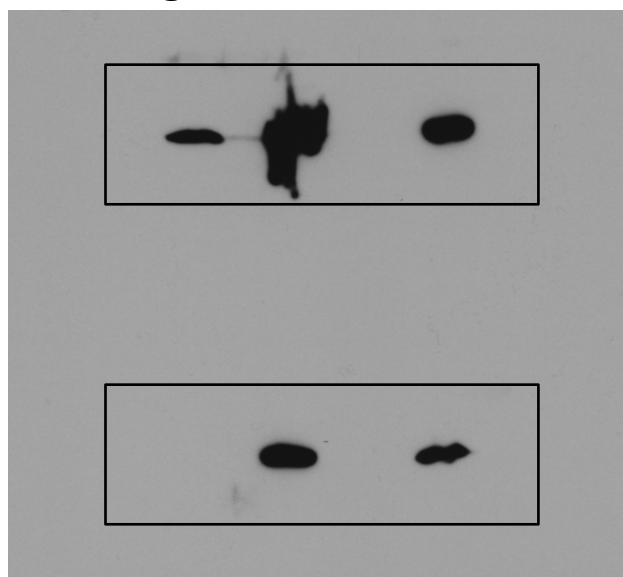
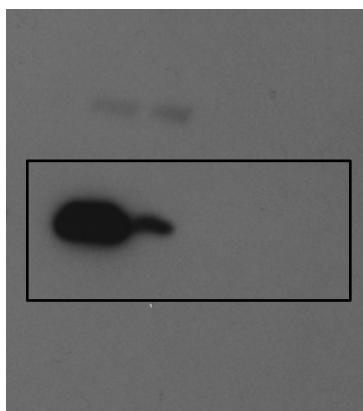


IB: Hsp90



IB: GFP

Full uncut gel for Figure 5e



IB: GFP

IB: GFP

18 **Supplementary Table 1: Sequences of primers, probes and gblocks**

Gene	Forward	Reverse
Mouse <i>shn3</i>	AGAGGCCATTTCAGACGAGTGT	CTGCGGAAGCTGAGAGATGT
Mouse <i>Alp</i>	CACAATATCAAGGATATCGACGTGA	ACATCAGTTCTGTTCTTCGGGTACA
Mouse <i>Runx2</i>	TACAAACCATACCCAGTCCCTGTTT	AGTGCTCTAACCACAGTCCATGCA
Mouse <i>Bsp</i>	CAGGGAGGCAGTGA CTCTTC	AGTGTGGAAAGTGTGGCGTT
Mouse <i>Osx</i>	ATGGCGTCCTCTCTGCTTGA	GAAGGGTGGGTAGTCATTTG
Mouse <i>Ocn</i>	GCAGCACAGGTCCTAAATAG	GGGCAATAAGGTAGTGAACAG
Mouse <i>Col1a1</i>	ACTGTCCCAACCCCAAG	ACGTATTCTTCCGGGCAGAA
Mouse <i>Hprt</i>	CTGGTGAAAAGGACCTCTCGAAG	CCAGTTTCACTAATGACACAAACG
EGFP	AGCAAAGACCCCAACGAGAA	GGCGGCGGTCACGAA
EGFP-probe	6FAM-CGCGATCACATGGTCCTGCTGG-TAMRA	
(AspSerSer) ₆	GATTCATCAGATTCTTCTGATTCATCCGACTCTTCTGACAGTTCAGACAGCTCT	
amiR-33-ctrl (<i>amiR-ctrl</i>)	TTTGTCTTTTATTTTCAGGTCCCAGATCTAGGGCTCTGCGTTTGCTCCAGGTAG TCCGCTGCTCCCTTGGGCCTGGGCCCACTGACAGCCCTGGTGCCTCTGGCC GGCTGCACACCTCCTGGCGGGCAGCTGTGTACAACTACTTGAGAGCAGGT GTTCTGGCAATACCTGCCTGCTCTGTAATAGTTTGTACACGGAGGCCTGCCC TGACTGCCCACGGTGCCGTGGCCAAAGAGGATCTAAGGGCACCGCTGAGGG CCTACCTAACCATCGTGGGGAATAAGGACAGTGTACCCCTGCAGGGGATCC GGTGGTGGTGCAAATCA	
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