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Received 22 May 2014 | Accepted 8 Sep 2014 | Published 7 Nov 2014

DOI: 10.1038/ncomms6176

# NAA10 controls osteoblast differentiation and bone formation as a feedback regulator of Runx2

Haejin Yoon[1,2], Hye-Lim Kim[2], Yang-Sook Chun[1,3,4], Dong Hoon Shin[2,4], Kyoung-Hwa Lee[3,4], Chan Soo Shin[5], Dong Yeon Lee[6], Hong-Hee Kim[7], Zang Hee Lee[7], Hyun-Mo Ryoo[8], Mi-Ni Lee[9], Goo Taeg Oh[9] & Jong-Wan Park[1,2,4]

Runt-related transcription factor 2 (Runx2) transactivates many genes required for osteoblast differentiation. The role of *N*-α-acetyltransferase 10 (NAA10, arrest-defective-1), originally identified in yeast, remains poorly understood in mammals. Here we report a new NAA10 function in Runx2-mediated osteogenesis. Runx2 stabilizes NAA10 in osteoblasts during BMP-2-induced differentiation, and NAA10 in turn controls this differentiation by inhibiting Runx2. NAA10 delays bone healing in a rat calvarial defect model and bone development in neonatal mice. Mechanistically, NAA10 acetylates Runx2 at Lys225, and this acetylation inhibits Runx2-driven transcription by interfering with CBFβ binding to Runx2. Our study suggests that NAA10 acts as a guard ensuring balanced osteogenesis by fine-tuning Runx2 signalling in a feedback manner. NAA10 inhibition could be considered a potential strategy for facilitating bone formation.

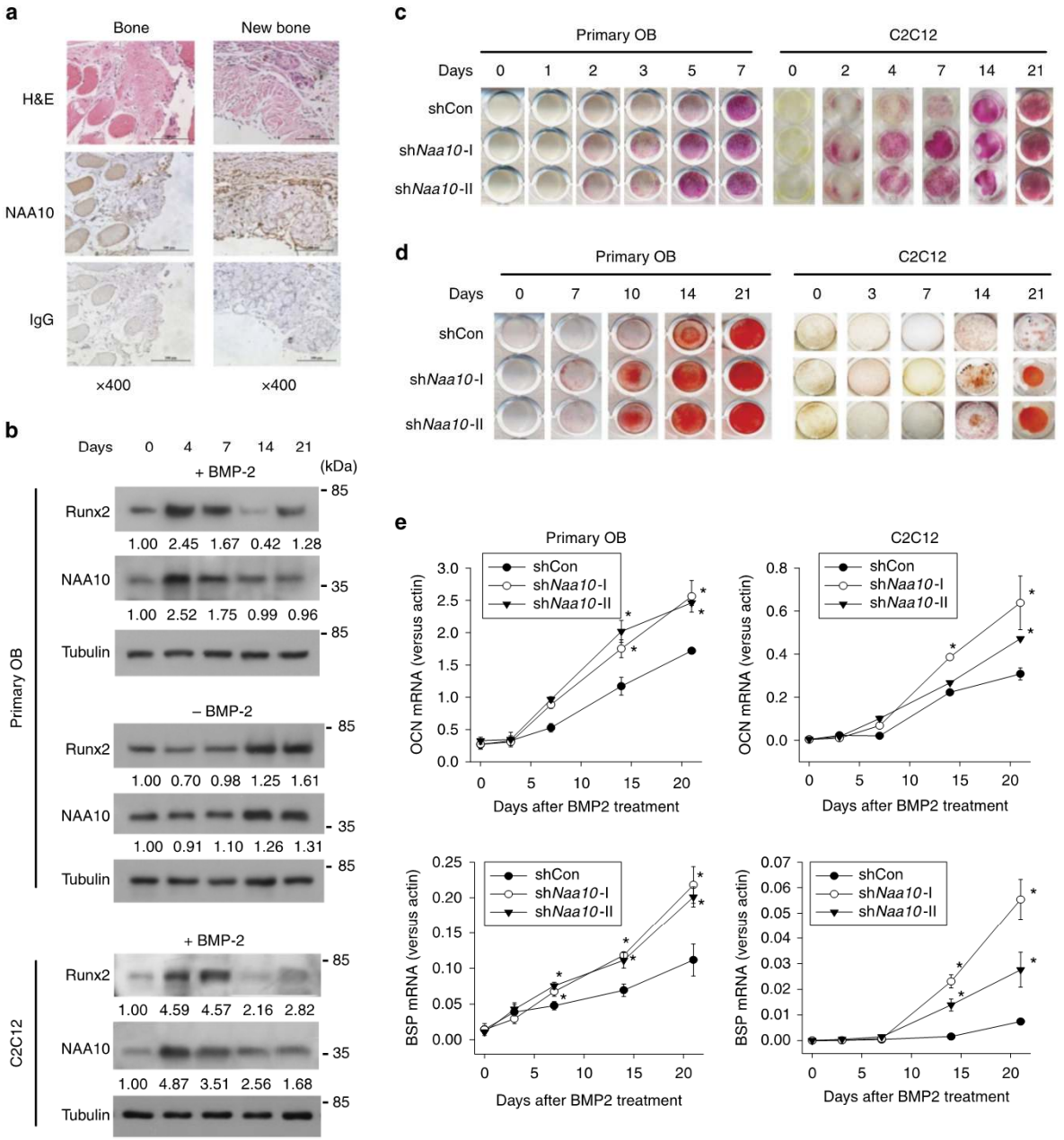



**Figure 1 | NAA10 negatively regulates osteoblast differentiation.** (**a**) A rat calvarial bone was punched out and covered with a collagen sponge containing 1 mg of BMP-2. After 2 months, the bone was removed, fixed, decalcified, paraffin embedded and cut into 5-μm sections. The sections including the margin of defect were stained with H&E or immunostained with anti-NAA10 (scale bar, 100 μm). Arrows indicate NAA10-positive osteoblasts. (**b**) Primary osteoblasts and $C_2C_{12}$ cells were incubated with PBS or BMP-2 (100 ng ml$^{-1}$) for the indicated times. Protein levels in lysates were analysed by western blotting using the indicated antibodies, and quantified using ImageJ. Runx2 and NAA10 values were divided by tubulin values and the ratios are expressed with respect to those at zero time. (**c**) Osteoblasts, which had been infected with non-targeting (shCon) or two different *Naa10*-targeting (shNaa10) shRNA viruses, were differentiated with BMP-2. ALP was stained with naphthol AS-MX phosphate. (**d**) Mineralization in differentiating osteoblasts was stained with Alizarin Red S. The results are the representative pictures of three independent experiments. (**e**) OCN and BSP mRNA levels were quantified in differentiating osteoblasts by RT-qPCR. Results are expressed as the means ± s.d. ($n = 4$) and compared statistically using Student's *t*-test (*$P < 0.05$ versus the shCon group).

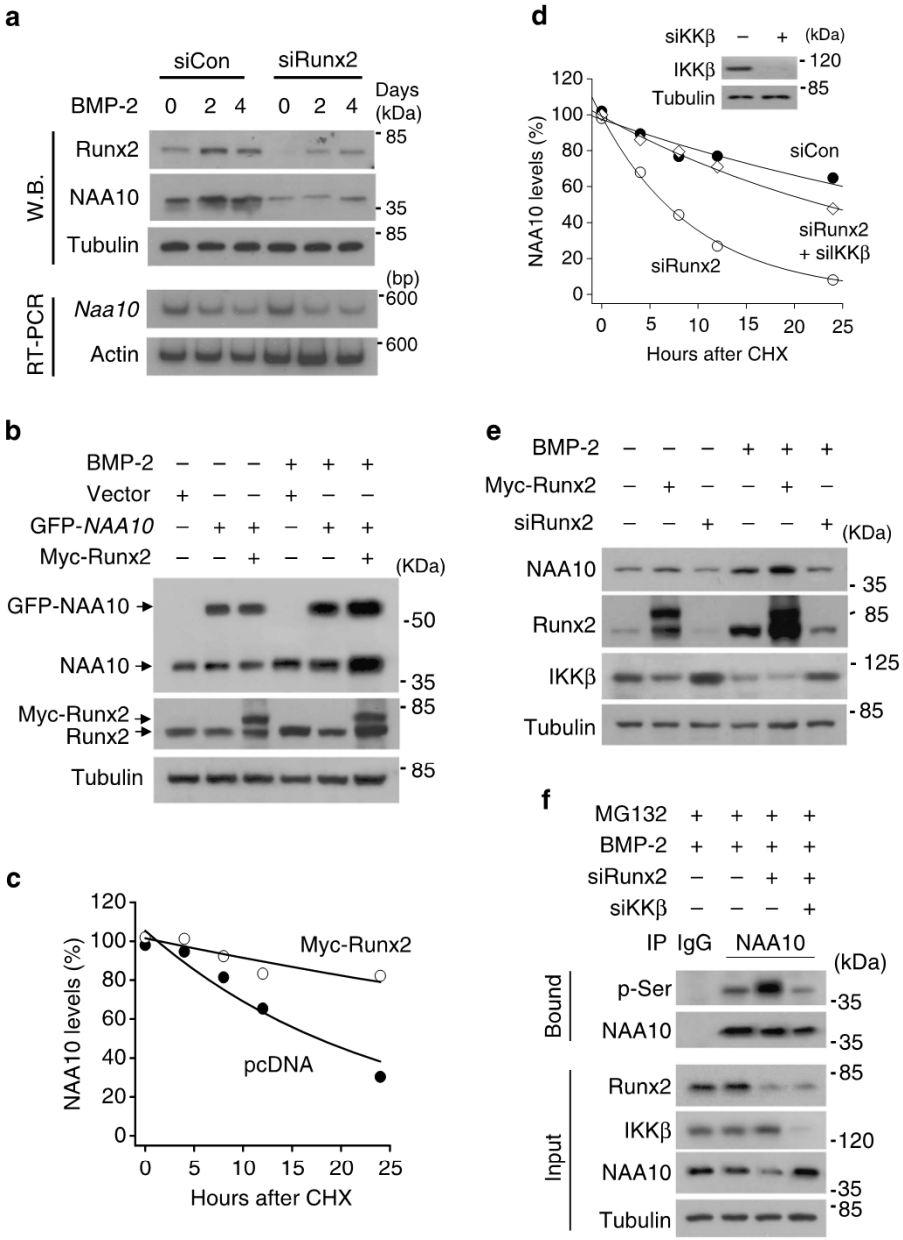



**Figure 2 | NAA10 is stabilized Runx2 dependently during osteoblastogenesis.** (**a**) $C_2C_{12}$ cells, which had been transfected with 80 nM siRNA-targeting *Runx2*, were treated with BMP-2 for 2 or 4 days. Runx2 and NAA10 expressions were analysed by western blotting or semi-quatitative RT-PCR. (**b**) Primary osteoblasts were cotransfected with the plasmids of green fluorescent protein (GFP)-*NAA10* (2 μg) and Myc-Runx2 (1 μg) and treated with BMP-2 for 2 days. Protein expressions were analysed by western blotting. (**c**,**d**) $C_2C_{12}$ cells, which had been transfected with Runx2 siRNA (80 nM) or plasmid (1 μg), were treated with 100 nM cycloheximide for the indicated times. NAA10 and tubulin levels were analysed by western blotting and quantified using ImageJ software. NAA10 values were divided by tubulin values and NAA10/tubulin ratios are expressed with respect to those at zero time. (**e**) Primary osteoblasts were transfected with the plasmids of Myc-Runx2 or Runx2 siRNA and treated with BMP-2 for 2 days. Expressed proteins were immunoblotted. (**f**) $C_2C_{12}$ cells, which were transfected with Runx2 and/or IKKβ siRNAs, were incubated with BMP-2 for 24 h and further treated with 20 μM MG132 for 8 h. Cell lysates were immunoprecipitated with anti-NAA10, and the precipitates were immunoblotted with phosphoserine (p-Ser) antibody. The input levels of NAA10, Runx2 and IKKβ were verified by western blotting.

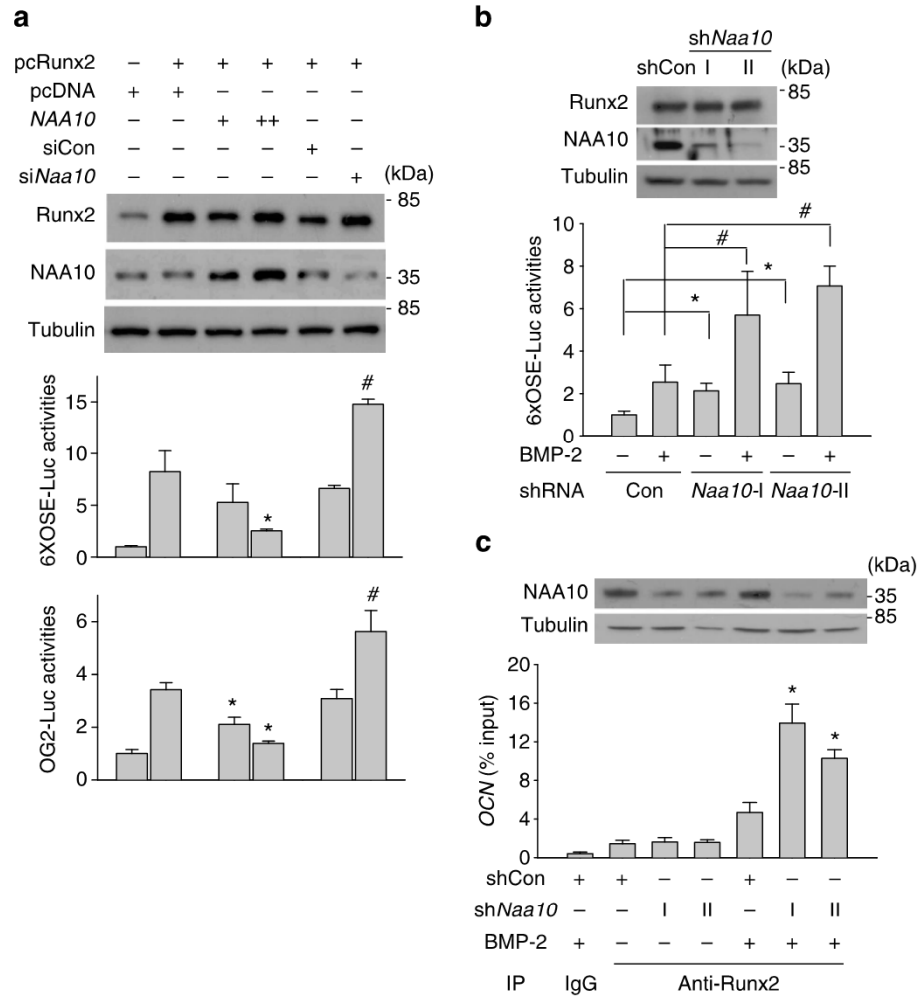



**Figure 3 | NAA10 represses the transcriptional activity of Runx2.** (**a**) $C_2C_{12}$ cells were cotransfected with 1 μg of 6XOSE-luciferase plasmid or OG2-luciferase plasmid and β-gal plasmid and with 1 or 2 μg of Runx2 plasmid or 80 nM of Runx2 siRNA. After being allowed to stabilize for 24 h, cells were lysed for western blotting (top) or reporter assays (middle and bottom). Luciferase activities (means + s.d., $n = 4$) were normalized versus β-gal activity, and are presented as relative values with respect to controls. * and #$P < 0.05$ versus the pcDNA control and versus the siRNA control, respectively. (**b**) $C_2C_{12}$ cell lines infected with sh*Naa10* were cotransfected with 6XOSE-luciferase and β-gal plasmids, and treated with BMP-2 for 24 h. Cells were lysed for western blotting (top) and reporter (bottom) analyses. Luciferase/β-gal values (means + s.d., $n = 4$) are presented as relative values versus BMP-2 ( − ) control shRNA. * and #$P < 0.05$ versus BMP-2 ( − ) control shRNA and versus BMP-2 ( + ) control shRNA, respectively. (**c**) $C_2C_{12}$ cell lines infected with sh*Naa10* or shControl virus were treated with BMP-2 for 48 h and NAA10 levels were checked by western blotting (top). Cells were then fixed with formalin and cross-linked chromatins were immunoprecipitated with non-immunized serum (IgG) or anti-Runx2. DNAs were eluted from precipitates and real-time PCR was performed to amplify the mouse OCN promoter region. Each bar represents the mean + s.d. ($n = 4$) and *$P < 0.05$ versus the BMP-2 ( + ) shControl group. Data are statistically analyzed using Student's *t*-test.

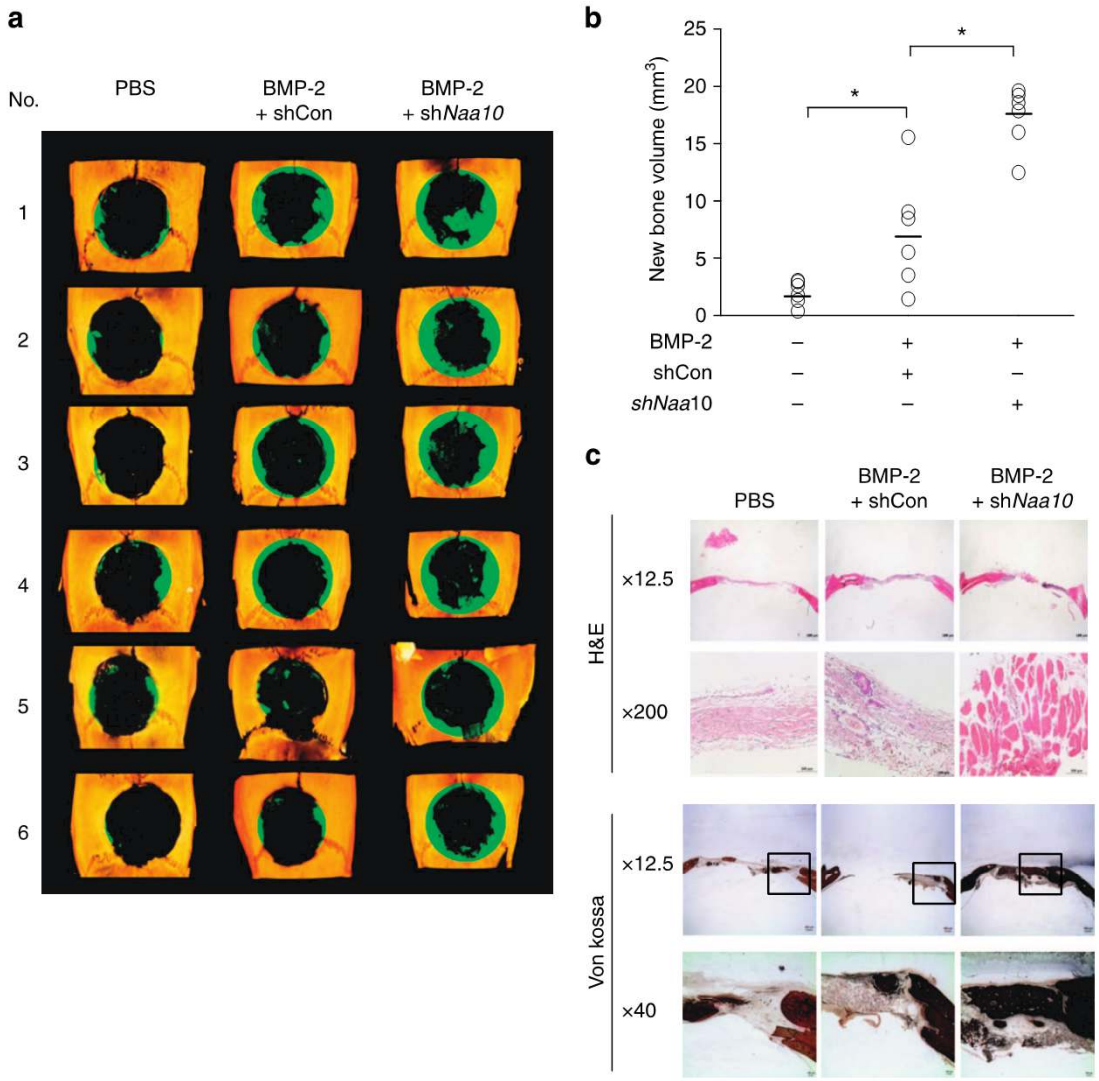



**Figure 4 | NAA10 knock down augments the healing of rat calvarial defects.** (**a**) Six rat calvarial bones per group were punched out and covered with collagen sponge containing BMP-2 (or PBS) and sh*Naa10* virus (or shControl virus). Calvarias were removed on day 42 after surgery and subjected to micro-CT analysis to evaluate defect healing. Micro-CT images were reconstructed three dimensionally; bone ingrowth from defect margins are marked with green. (**b**) Volumes of ingrown bones were calculated using three-dimensional CT images. Short horizontal bars represent group mean values and $^{*}P<0.05$ (Student's *t*-test, $n=6$) between two groups. (**c**) Calvarial tissues were fixed, decalcified, embedded and cut into 5-μm sections. The sections were stained with H&E. Calvarial tissues were fixed, embedded and cut into 50-μm sections. The sections were stained with von Kossa/Nuclear Fast Red. The images were captured under a microscope (scale bar, 100 μm).

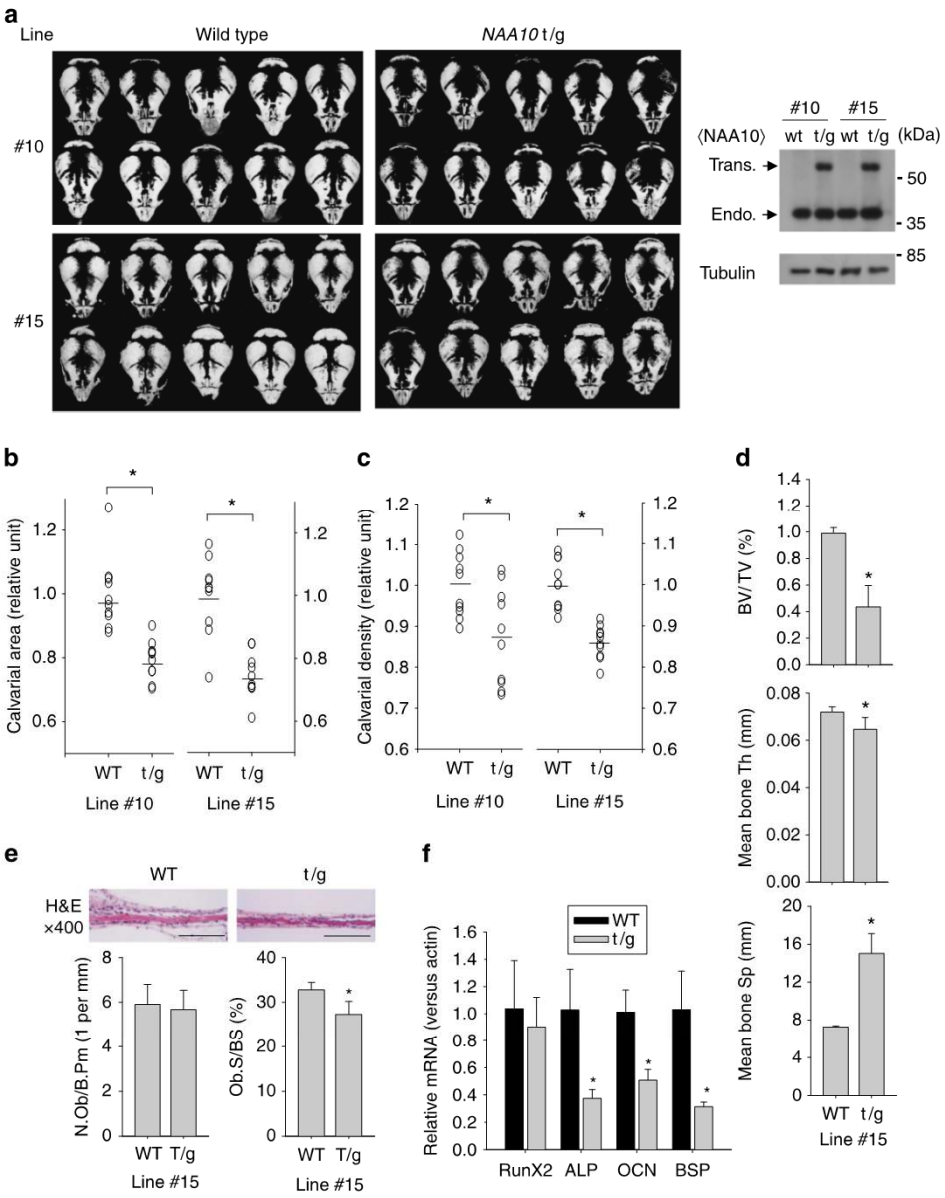



**Figure 5 | Calvarial bone development is delayed in *NAA10* transgenic mice.** (**a**) Micro-CT images of calvarias were taken from two lines (#10 and #15) of *NAA10* transgenic mice and their littermate mice at postnatal 3 day. In each line, 10 transgenic mice and 10 littermate mice were examined. All images were captured at the same magnification, and the representative images are shown. Expression of endogenous and ectopic *NAA10* in calvarias of *NAA10* transgenic mouse line #10 and #15 and their littermates. The lysates were isolated from calvaria and analysed using immunoblotting with anti-NAA10 and β-tubulin (right panel). (**b**,**c**) Calvaria area and bone density were analysed in wild-type and *NAA10* transgenic mice using ImageJ densitometry programme. Short horizontal bars represent group mean values ($n=10$). (**d**) Calvarial bone parameters, such as bone volume per tissue volume (BV/TV), mean bone thickness (Th) and mean bone separation (Sp), were analysed from reconstructed three-dimensional images on micro-CT. Results are presented as the means + s.d. ($n=5$). (**e**) Calvarias were removed from *NAA10* (line #15) transgenic mice and their wild-type littermates on postnatal day 3. Bone tissues were decalcified with EDTA and embedded in paraffin. The calvarial sections (5 μm thickness) were stained with H&E. Representative findings are presented in the top panel (scale bar, 100 μm). Bone formation parameters, such as number of osteoblasts per bone perimeter (N.Ob/B.Pm) and osteoblast surface per bone surface (Ob.S/BS), were analysed using the OsteoMeasure software system (OsteoMetrics, Decatur, GA) and presented as the means + s.d. ($n=5$). (**f**) RNAs were extracted from mouse calvarias, and Runx2, ALP, OCN and BSP mRNA levels were quantified by RT-qPCR. Each bar represents the mean + s.d. from six independent samples. $^*P<0.05$ (Student's *t*-test) versus the corresponding value in the WT group.

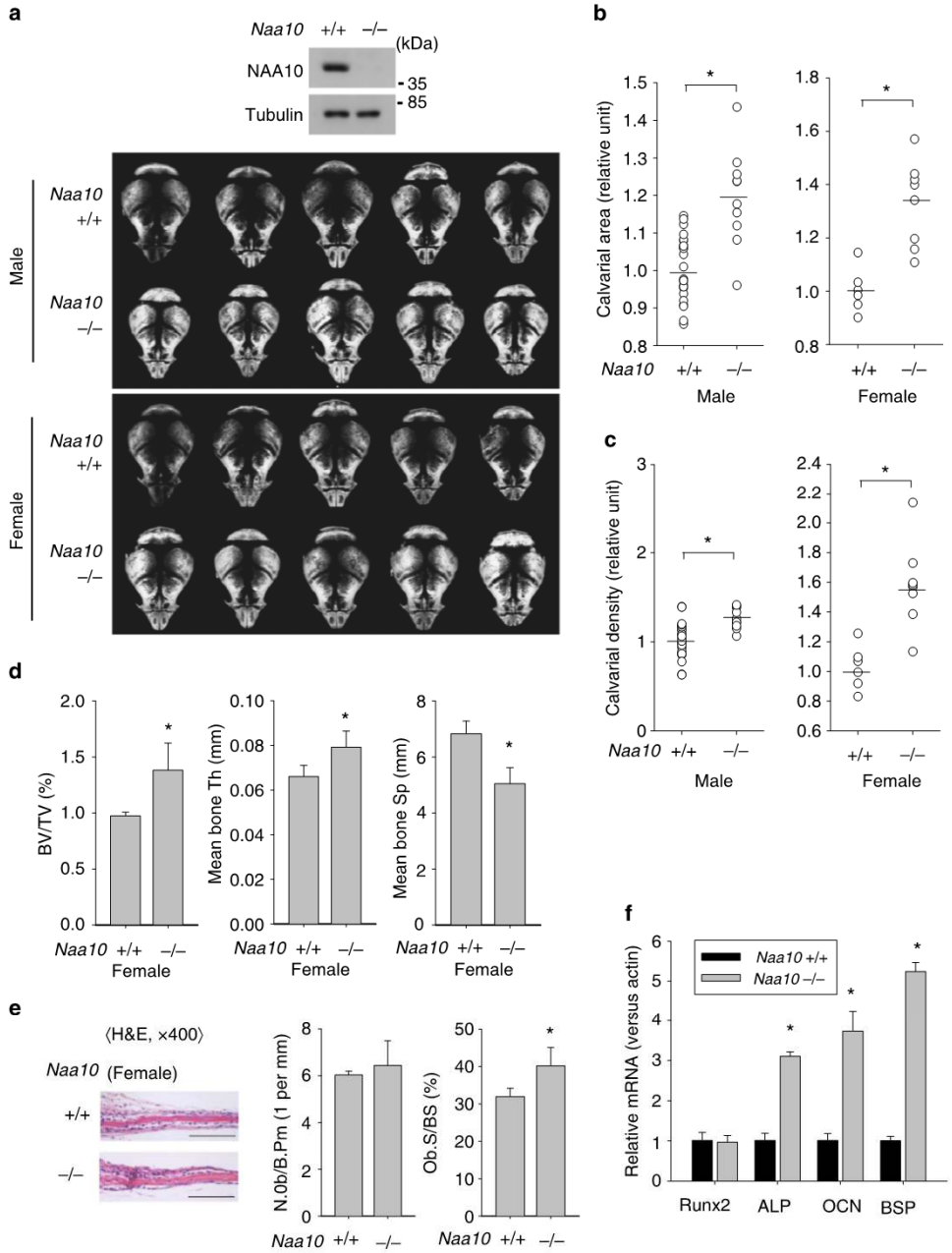



**Figure 6 | Calvarial bone development is facilitated in *Naa10*$^{-/-}$-knockout mice.** (**a**) Micro-CT images of calvarias were taken from *Naa10*$^{+/+}$ (wt) and *Naa10*$^{-/-}$ mouse on postnatal day 3. All images were captured at the same magnification, and the representative images are shown. Male and female groups were separately analysed. NAA10 and β-tubulin levels were analysed in the calvarias of *Naa10*$^{+/+}$ and *Naa10*$^{-/-}$ mice by western blotting (right panel). (**b**,**c**) Calvarial area and bone density in *Naa10*$^{+/+}$ and *Naa10*$^{-/-}$ mice were analysed using ImageJ densitometry programme. Short horizontal bars represent group mean values and $^{*}P<0.05$ between two groups. Experimental numbers are 24 for *Naa10*$^{+/+}$ males, 10 for *Naa10*$^{-/-}$ males, 6 for *Naa10*$^{+/+}$ females and 8 for *Naa10*$^{-/-}$ females. (**d**) Bone parameters, such as BV/TV, mean bone Th and mean bone Sp, were analysed from three-dimensional micro-CT images of calvarias from *Naa10*$^{+/+}$ and *Naa10*$^{-/-}$ female mice (means + s.d., $n=5$). (**e**) Calvarial sections were prepared from *Naa10*$^{+/+}$ and *Naa10*$^{-/-}$ female mice on postnatal day 3 and stained with H&E. Representative findings are presented in the left panel (scale bar, 100 μm). The bone parameters N.Ob/B.Pm and Ob.S/BS were analysed using the OsteoMeasure software and presented as the means + s.d. ($n=5$). (**f**) Runx2, ALP, OCN and BSP mRNA levels (means + s.d., $n=6$) in mouse calvarias were quantified by RT-qPCR. $^{*}P<0.05$ versus the corresponding value in the WT group. Data are statistically analysed using Student's *t*-test.

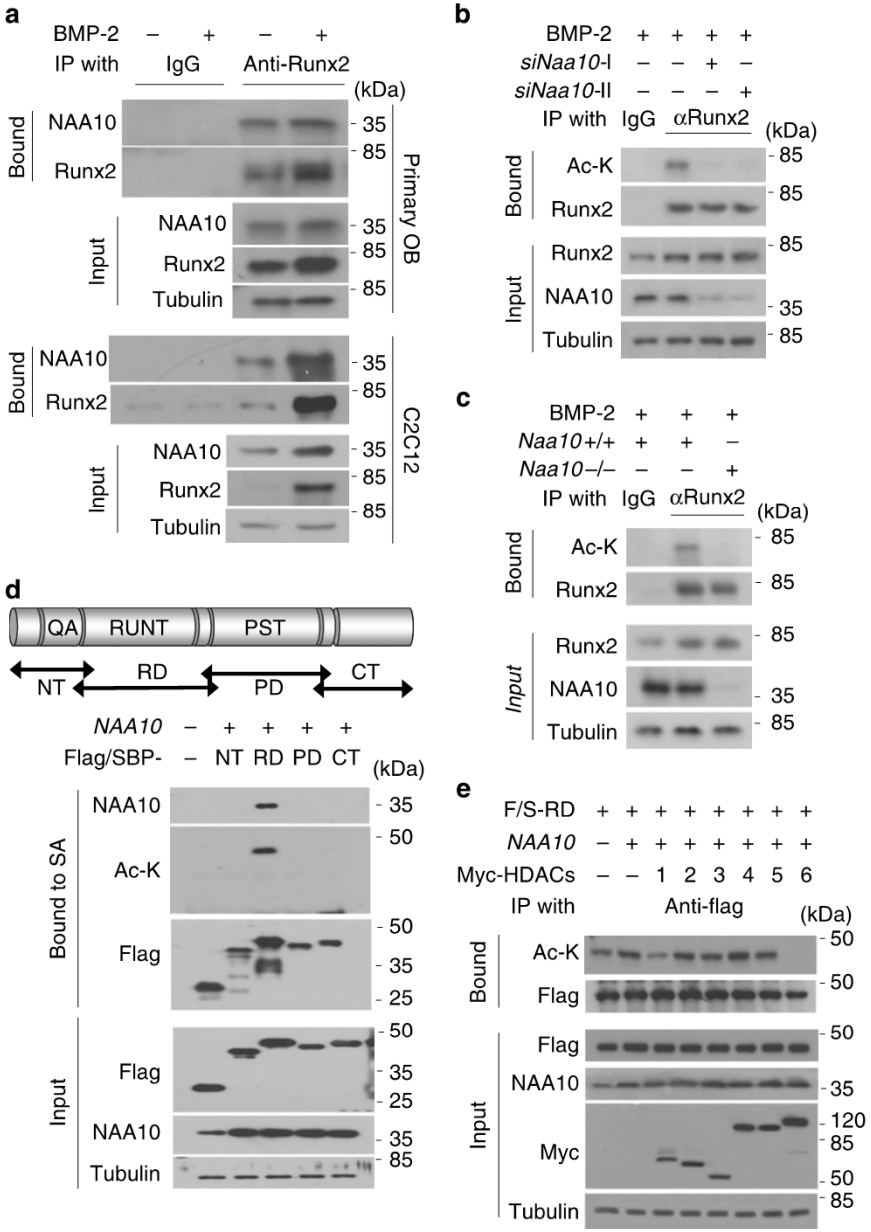



**Figure 7 | NAA10 binds and acetylates the RUNT domain of Runx2.** (**a**) Primary osteoblast cells and $C_2C_{12}$ cells were treated with BMP-2 at 100 ng ml$^{-1}$ for 4 days. Cell extracts were then immunoprecipitated with anti-Runx2, and precipitated Runx2 and NAA10 were immunoblotted with specific antibodies. (**b**) Primary osteoblast cells were transfected with *Naa10*-targeted siRNAs and treated with BMP-2 at 100 ng ml$^{-1}$ for 4 days. Later, the cells were incubated with 1 μM Trichostatin A (a deacetylase inhibitor) for 6 h. Cell extracts were then immunoprecipitated with anti-Runx2, and its acetylation was analysed by western blotting using an anti-acetyl lysine antibody. (**c**) Primary osteoblasts from wild-type and *Naa10*-knockout mice were treated with BMP-2 for 4 days. After being incubated with 1 μM Trichostatin A for 6 h, cells were subjected to immunoprecipitation with anti-Runx2, and Runx2 acetylation was analysed using anti-acetyl lysine antibody. (**d**) Flag/SBP-tagged Runx2 constructs are illustrated in the top panel. HEK293T cells were cotransfected with plasmids of a Runx2 peptide and *NAA10*, and treated with Trichostatin A for 6 h. Flag/SBP-peptides were pulled down with streptoavidin-affinity beads and NAA10 coprecipitation and lysyl acetylation were analysed by western blotting. (**e**) HEK293T cells were cotransfected with plasmids of Flag/SBP-RD, *NAA10* and Myc-HDACs, and then treated with Trichostatin A for 6 h. Flag/SBP-RD peptide was pulled down using Flag-affinity beads, and the lysyl acetylation of RD peptide was analysed by western blotting.

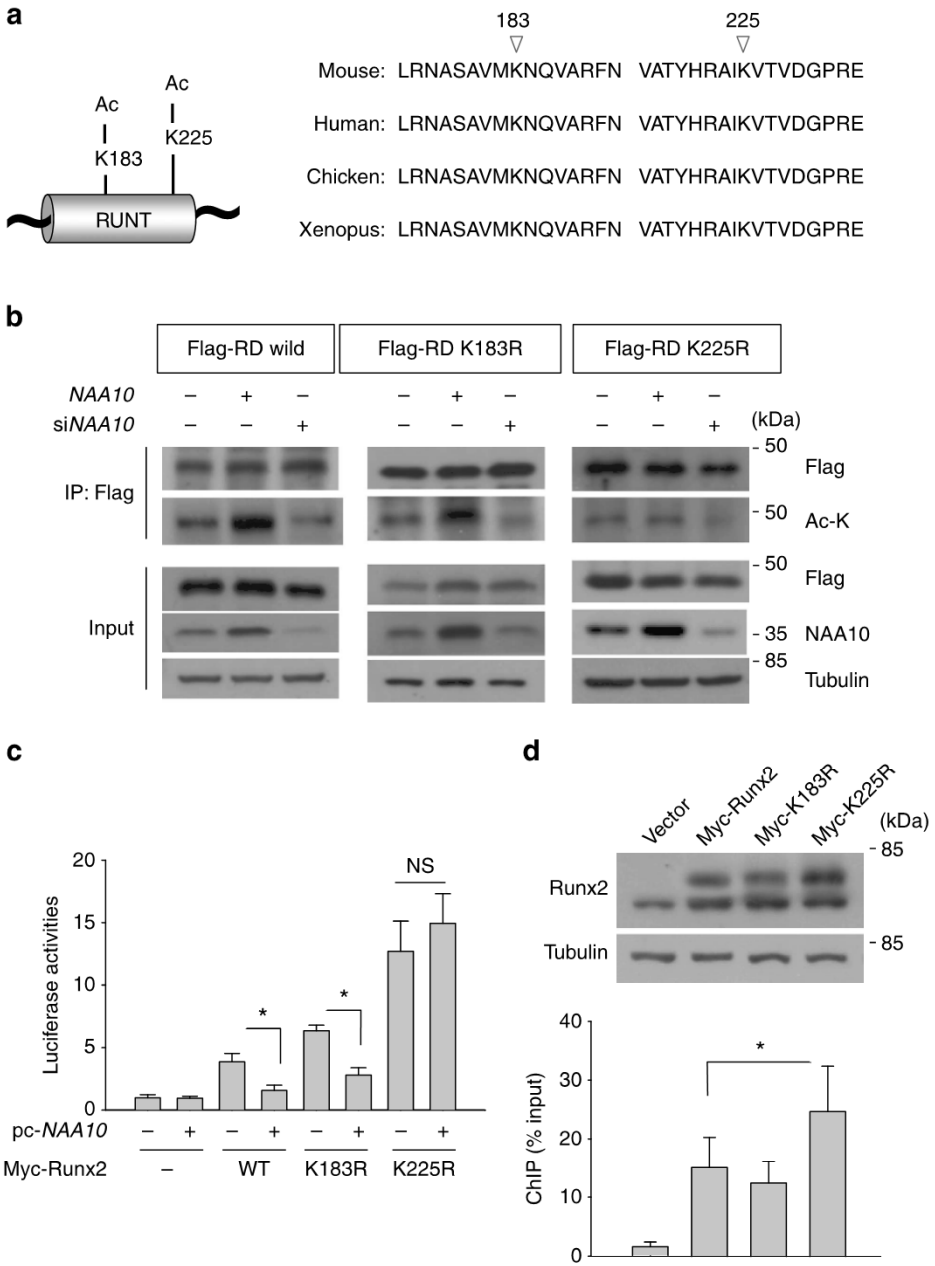



**Figure 8 | NAA10 inhibits the transcriptional activity of Runx2 by acetylating it at K225.** (**a**) Lysine residues 183 and 225 are well conserved among different species. (**b**) Flag/SBP-RD, Flag/SBP-RD K183R or Flag/SBP-RD K225R plasmids were cotransfected with *NAA10* plasmid or siRNA into HEK293T cells. After treated with 1 μM Trichostatin A, cells were lysed and incubated with anti-Flag and Flag/SBP-RD peptides and their lysyl acetylations were analysed by western blotting. (**c**) $C_2C_{12}$ cells were cotransfected with OG2-luciferase, β-gal, a Myc-Runx2 (full-length), Myc-Runx2 K183R and Myc-Runx2 K225R plasmids. Luciferase activities (means + s.d., $n = 4$) were normalized versus β-gal activities and are presented relative to NAA10( − ) and Runx2( − ) values. (**d**) $C_2C_{12}$ cells, which had been transfected with one of the indicated plasmids, were treated with BMP-2 for 48 h and Runx2 levels were checked by western blotting (top). Cells were fixed with formalin and cross-linked chromatins were immunoprecipitated with anti-Runx2. DNAs were eluted and real-time PCR was performed to amplify the mouse OCN promoter region. *$P < 0.05$ (Student's *t*-test) between two groups.

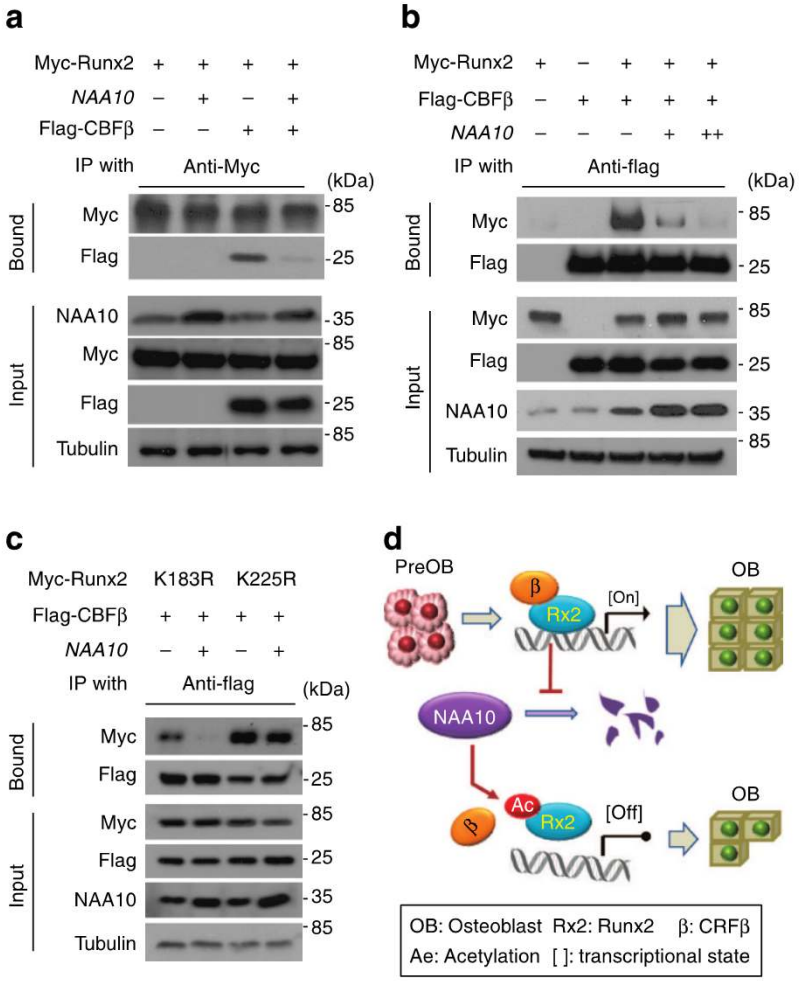



**Figure 9 | NAA10 blocks the Runx2-CBFβ interaction by acetylating Runx2 at K225.** (**a**) HEK293T cells were cotransfected with Myc-Runx2, NAA10 and Flag-CBFβ plasmids. Myc-Runx2 was precipitated with anti-Myc, and coprecipitated Flag-CBFβwas analysed using anti-Flag. (**b**) In HEK293 cells transfected with 2 or 4 μg of *NAA10*, Flag-CBFβ precipitated with anti-Flag and coprecipitated Myc-Runx2 was analysed using anti-Myc. (**c**) In HEK293 cells transfected with Myc-Runx2 K183R or Myc-Runx2 K225R plasmid, Flag-CBFβ was precipitated with anti-Flag and coprecipitated Myc-Runx2 mutants were analysed using anti-Myc. (**d**) Proposed mechanism for the role of NAA10 in Runx2-mediated osteoblast differentiation.

## Methods

**Reagents and antibodies.** Culture media were purchased from Invitrogen (Carlsbad, CA), and fetal calf serum from Sigma-Aldrich (St Louis, MO. Recombinant human BMP-2 (bone morphogenic protein 2) was purchased from Perprotech (Rocky Hill, NJ). A polyclonal antibody against NAA10 was raised in rats against full-length human NAA10 (ref. 17). Mouse NAA10 and β-tubulin primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Runx2 from MBL (Nagoya, Japan); Acetylated-Lysine antibody from Cell Signaling Technologies (Billerical, MA); anti-α-Myc-tag and Flag-tag antibody from Sigma-Aldrich.

**Preparation and transfection of plasmids or siRNAs.** Myc-tagged mouse Runx2 plasmid and 6xOSE/OG2 reporter plasmids were provided by Dr Hyun-Mo Ryoo (Seoul National University School of Dentistry). Human CBFβ cDNA was cloned using reverse transcriptase-PCR (RT-PCR) and inserted into the Myc-tagged pcDNA. The plasmids for Flag/SBP-tagged Runx2 fragments were re-cloned from the mouse Runx2 plasmid using PCR and blunt-end ligation. Site-specific mutations of Runx2 were performed using PCR-based mutagenesis (Stratagene; Cedar Creek, TX). The *NAA10* plasmid was constructed as previously described[17]. For transient transfection with plasmids or siRNAs, cells at ~40% density were transfected with plasmids or siRNAs using calcium phosphate or Lipofectamine reagent (Invitrogen). The transfected cells were allowed to be stabilized for 48 h before being used in experiments. The shRNAs containing a hairpin loop were synthesized and inserted into pLKO.1-puro vector. The viral vectors were cotransfected into HEK293T cells with pRSV-Rev, pMD2-VSVG and pMDLg/pRRE plasmids to prepare viral particles. On the third day after transfection, Lenti viruses were collected from the supernatant of HEK293T cells. $C_2C_{12}$ cells were infected with viruses (100 μl ml$^{-1}$) and selected using puromycin (2 μg ml$^{-1}$) to establish stable cell lines. The sequences of siRNAs used are 5′-CCAACACCCTC AACTTTCAGATCAG-3′ for silencing mouse *Naa10* and 5′-AUGAACGUGA AUUGCUCAATT-3′ for non-targeting control. The sequences of *Naa10* shRNAs are 5′-CCGGCCGGAGAACTACCAGATGAAGCTCGAGCTTCATCTGG TAGTTCTCCGGTTTTT-3′ for mouse sh *Naa10*-I, 5′-CCGGACACCCTCAACTT TCAGATCACTCGAGTGATCTGAAAGTTGAGGGTGTTTTTT-3′ for mouse sh *Naa10*-II and rat shNAA10, and 5′-CCGGTACAACAGCCACAACGTCTAT CTCGAGATAGACGTTGTGGCTGTTGTATTTTT-3′ for non-targeting shRNA.

**Isolation and culture of mouse primary osteoblasts.** Calvarias of newborn mice were washed in HBSS (Life Technologies, NY) containing 3% penicillin and streptomycin. Isolated calvarias were sequentially digested in α-MEM containing 0.1% collagenase (Sigma-Aldrich) and 0.2% Dispase II (Roche, Switzerland) at 37 °C. Digestion was carried out five times (for 30 min per each digestion), and the fractions were pooled. Osteoblasts were collected by centrifugation and resuspended in ascorbic acid-free α-MEM. Osteoblasts were plated onto 100-mm culture dishes and the media were replaced 24 h later. To induce differentiation, cells were treated with ascorbic acid (50 μg ml$^{-1}$), β-glycerophosphate (2 mM) and BMP-2 (100 ng ml$^{-1}$). This procedure was approved by the Institutional Animal Care and Use Committee of Seoul National University (Approve no. SNU-120313-10-1).

**Cell culture.** $C_2C_{12}$ (mouse mesenchymal precursor), MC3T3-E1 (mouse pre-osteoblast) and HEK293T (human embryonic kidney) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured at 5% $CO_2$ in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum. $C_2C_{12}$ differentiation towards osteoblast was induced by BMP-2 (100 ng ml$^{-1}$) or Wnt3a (50 ng ml$^{-1}$). ATDC5 cell line (mouse chondrocyte), which was a kind gift from Dr Dae-Won Kim (Yonsei University, Korea), was cultured at 5% $CO_2$ in 1:1 mixture of DMEM and Ham's F-12 medium containing 10% FBS. ATDC5 differentiation was induced by Insulin-Transferrin-Selenium Supplement (Life Technologies) and BMP-2 (10 ng ml$^{-1}$).

**Critical-size calvarial defects in rats.** Male Spraque–Dawley rats (10 weeks old) were purchased from Orient Bio Inc (Gyeonggi-do, Korea) and kept in specific pathogen-free rooms. General anaesthesia was induced using Zoletil and Rumpun. After a midline incision of the scalp, an 8-mm critically sized calvarial defect was created using a trephine bur (GEBR; Brasseler, Germany) under sterile saline irrigation. The calvaria was covered with a 1% collagen matrix (Bioland; Cheongwon, Korea) containing BMP-2 (1 mg) and shCon or shNAA10 ($5.0 \times 10^9$ ml$^{-1}$), and skin flaps were sutured. Six rats per each group were killed 6 weeks after surgery. The calvarias were decalcified with 10% EDTA solution for 2 weeks and dehydrated through a series of ethanol solutions of increasing concentration and embedded in paraffin. The calvarial slices (5 μm thickness) were sectioned coronally at the centre of defect, stained with H&E, and subjected to immunohistochemistry. The procedures used and the care of animals were approved by Institiue of Laboratory Animal Resources Seoul National University (approve no. SNU-111111-3) and adhered to the Seoul National University Laboratory Animal Maintenance Manual.

***NAA10* transgenic mice.** Transgenic mice overexpressing m*NAA10*[235] (mouse orthologue of human *NAA10*) were produced by using modified pCAGGS expression vector containing cytomegalovirus enhancer fused to the ubiquitously expressed chicken β-actin promoter. Purified transgenic construct DNA was microinjected into the fertilized eggs collected from the superovulated C57BL/6 females, as previously described[42]. Genotyping was performed by PCR and Southern blot analysis of genomic DNAs obtained from the tails of founder mice at 2 weeks of age. Two different founders (#10 and #15) were bred in the hemizygous state, and transgenic and non-transgenic littermates were assigned into pair-matched groups for all experiments. Mice were fed a chow diet and water *ad libitum* in the Ewha Laboratory Animal Genomics Centre under specific pathogen-free conditions. Wild-type and *NAA10* transgenic mice were killed on postnatal day 3 to examine the extent of bone development. After the skulls were carefully isolated from skin and brain, they were washed with PBS and fixed in 10% formalin for 24 h. All experiments were approved by the Institutional Animal Care and Use Committees of Ewha Women's University, and adhered to the National Research Council Guidelines (approve no. 2010-24-2).

***NAA10*-knockout mice.** *Naa10*-knockout mice were generated based on a standard gene-targeting in E14 embryonic stem (ES) cells (129/Sv). The targeting vector was constructed to delete exons 1–4 in the *Naa10* gene. ES cells were electroporated with the targeting vector that was linearized with Not I, and selected by neomycin and gancyclovir. Selected clones were screened for homologous recombination using Southern analysis. Correctly targeted ES clones were used for blastocyst microinjection and generation of chimeric mice. Chimeric mice were mated with C57BL/6 mice, and germ-line transmission of targeted alleles was detected by PCR and Southern blot analysis. *Naa10*-knockout mice were backcrossed more than 10 times into the C57BL/6J background. Genotyping was performed by PCR and Southern blot analyses of genomic DNAs obtained from the tails. All experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committees (IACUC) of Ewha Women's University.

**Histological procedure and histomorphometric analysis.** Calvaria, tibia and vertebra tissues were decalcified with 10% EDTA solution for 2 weeks and dehydrated through a series of ethanol solutions of increasing concentration and embedded in paraffin. The calvarial slices (5 μm thickness) sectioned coronally were stained with H&E. Digital images of H&E-stained calvaria sections were taken using OLYMPUS microscope and camera (Tokyo, Japan) and the following histomorphometric paramenters were analysed by OsteoMeasure version 1.01 software (Osteometrics, Decatur, GA). Each parameter was analysed in quadruplicate from four different images per H&E-stained section at 400-fold magnification and its mean value was calculated from five independent experiments. The number of osteoblasts per bone perimeter (N.Ob/B.Pm, 1 $mm^{-1}$) and the percentage of osteoblast surface to bone surface (Ob.S/BS, %) were determined according to standardized protocols using the OsteoMeasure programme. Histomorphometric data are presented as recommended by the American Society for Bone and Mineral Research.

**Micro-CT-based analysis of bone structure.** Calvarial bones were removed and fixed in 10% formalin, and micro-CT was taken using NFR Polaris-G90 manufactured by Nanofocusray (Jeonju, Korea). The scanner uses an X-ray source and has a camera that rotates around a bed holding the samples. The samples were scanned at a tube potential of 65 kVp and a tube current of 120 μA for 15 min. Data were acquired from the 1,024 images of reconstruction with an isotropic voxel spacing of $0.034 \times 0.034 \times 0.027\ mm^3$. The volume of bone ingrown in a defect site was analysed by the three-dimensional analysis using a software Amira version 5.4.1 (San Diego, CA). The bone range was reflected with CT analyzer manual, which ranged more than 1,000 CT number, and the CT number of air (1,000 HU) and water (0 HU) was used to calibrate the image values in Hounsfield units. To distinguish bone tissue from soft tissue, the CT number was given a range of brain and cortical-bone equivalent hydroxyapatite material. The mean value was determined by the CT number. The bone parameters bone volume/tissue volume (BV/TV, %), bone (for calvaria) or trabecular (for femur) thickness (Tb.Th, mm), bone or trabecular number (Tb.N, 1/mm) and trabecular separation (Tb.Sp, mm) were calculated using the analysis software CTAn v1.9 and the three-dimensional model visualization software μCTVol v2.0. The scanner was set at a voltage of 40 kVp, a current of 200 μA and a resolution of 11.55 μm pixels. The femur sample area selected for scanning was a 1.5-mm length of the secondary spongiosa, originating 1.0 mm below the epiphyseal growth plate.

**Alcian blue/Alizarin red co-staining of skeletons.** After the skin and internal organs were removed, mice were fixed in ethanol for 5 days. Mice were stained with 0.015% Alcian Blue 8GX and 0.005% Alizarin Red S in ethanol/acetic acid (9:1. v/v) at 37 °C for 48 h. After being rinsed with water, skeletons were kept in 1% KOH solution with 20% glycerol until they became clearly visible.

**Alkaline phosphatase, alizalin red S and von Kossa stainings.** $C_2C_{12}$ cells were cultured on 6-well plates at a density of $1x10^5$ cells per well, and ALP was visualized using naphthol AS-MX phosphate (Sigma-Aldrich) as a substrate. After being differentiated by BMP-2, cells were fixed with 10% formaldehyde for 5 min, briefly rinsed by water and permeabilized by 0.1% Triton X-100. Cells were incubated at 37 °C for 30 min in a mixture containing sodium nitrite, FRV-Alkaline solution (Sigma-Aldrich) and Naphthol-AS-BL alkaline solution (Sigma-Aldrich). After being rinsed by water, wells were photographed. The calcification of osteoblast was analysed by Alizarin Red staining. After being fixed in 10% formaldehyde and washed with distilled water, cells were stained with 1% Alizarin Red S at room temperature for 20 min. Wells were rinsed with water and photographed. Mineralization of bone tissues was evaluated by von Kossa staining. Rat calvarias were fixed with 3.7% paraformaldehyde and embedded in epoxy resin. Calvarial sections were incubated with 1% silver nitrate and exposed to bright light for 30 min. After silver was removed by 5% sodium thiosulfate, sections were counterstained with Nuclear Fast Red.

**ALP activity.** The cultures were rinsed twice with ice-cold PBS, solubilized in a lysis buffer (pH = 10.5, 50 mM Tris, 100 mM glycine and 0.1% Triton X-100) and sonicated twice on ice for 15 s. The supernatant was collected and used to determine total protein and ALP levels. Total protein levels were calculated using a BCA protein assay kit (Bio-Rad; Hercules, CA). A 100-μl aliquot of freshly prepared *p*-nitrophenyl-phosphate tablet solution was added to 200 μl of the supernatant samples and incubated at 37 °C for 30 min. The optical density of *p*-nitrophenol was measured at 405 nm, and ALP activity was standardized as nmoles of *p*-nitrophenol per mg of protein per minute.

**RT-PCR.** Total RNAs were extracted using TRIZOL (Invitrogen), and reverse transcribed in a reaction mixture containing M-MLV Reverse Transcriptase (Promega; Madison, WI), RNase inhibitor, dNTP and random primers at 46 °C for 30 min. For sensitive semi-quantitative RT-PCR, the cDNAs were amplified over 17 PCR cycles with 5 μCi [α-$^{32}$P]dCTP. The PCR products were electrophoresed on a 4% polyacrylamide gel, and the dried gel was autoradiographed. PCR primer sequences are 5′-AACCTCAAAGGCTTCTTCTT-3′ and 5′-ACATGGGGAATG TAGTTCTG-3′ for mouse ALP and 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-ATCTCTTGCTCGAAGTCCAG-3′ for mouse β-actin. Quantitative real-time PCR was performed in the Evagreen qPCR Mastermix, and fluorescence emitting from dye-DNA complex was monitored in CFX Connect Real-Time Cycler (BIO-RAD). The levels of mRNAs were normalized by 18S RNA levels and represented as relative values. The primer sequences are 5′-GCAATAAGGTAGT GAACAGACTCC-3′ and 5′-GTTTGTAGGCGGTCTTCAAGC-3′ for mouse OCN; 5′-AAGCAGCACCGTTGAGTATGG-3′ and 5′-CCTTGTAGTAGCTGT ATTCGTCCTC-3′ for mouse BSP; 5′-AACCTCAAAGGCTTCTTCTT-3′ and 5′-ACATGGGGAATGTAGTTCTG-3′ for mouse ALP; 5′-AACTTCTGTGGGA GCGACAA-3′ and 5′-GGGAGGGAAAACAGAGAACGA-3′ for mouse sex determining region Y-box 9 (Sox9).

**Reporter assays.** Cells were cotransfected with 6xOSE-luciferase or OG2-luciferase and CMV-β-gal using calcium phosphate or Lipofectamine reagent. The final DNA or siRNA concentration was adjusted by adding pcDNA or control siRNA, respectively. After 16 h of stabilization, transfected cells were seeded on four dishes at a cell density of $5 \times 10^4$ cells cm$^{-2}$ and further cultured for 24 h. The cells were incubated under normoxic or BMP-2 treated conditions for 48 h, and then lysed to determine luciferase. β-gal activities were measured to normalize transfection efficiency.

**Preparation of cytosolic and nuclear extracts.** Cells were centrifuged at 1,000 *g* for 5 min, and resuspended with a lysis buffer containing 10 mM Tris/HCl (pH 7.4), 10 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP40, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate and 400 μM phenylmethylsulphonyl fluoride. The cell lysates were separated into pellet (for nuclear fraction) and supernatant (for cytosolic faction) using centrifugation at 1,000 *g* for 5 min. One packed volume of a nuclear extraction buffer (20 mM Tris/HCl, pH 7.4, 420 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and 400 μM phenylmethylsulphonyl fluoride) was added to the pellet, and vortexed intermittently at low speed on ice for 30 min. The nuclear extracts were cleaned at 20,000 *g* for 10 min, and stored at − 70 °C.

**Immunoblotting and immunoprecipitation.** Cell lysates were separated on SDS-polyacrylamide (8–12%) gels, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with a Tris/saline solution containing 5% skim milk and 0.1% Tween-20 for 1 h and then incubated overnight at 4 °C with a primary antibody diluted 1:1,000 in the blocking solution. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at RT, and stained using the ECL-plus kit (Amersham Biosciences, Piscataway, NJ). To analyze protein interactions, cell lysates were incubated with anti-Runx2, anti-Myc or anti-Flag antibody (2 μg ml$^{-1}$) overnight at 4 °C, and the immune complexes were pulled down by 10 μl of protein A/G beads (Santa Cruz, CA). The bound proteins were eluted in a denaturing SDS sample buffer or by Myc/Flag peptides, loaded on SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti-Runx2, anti-NAA10, anti-Myc or anti-Flag antibody.

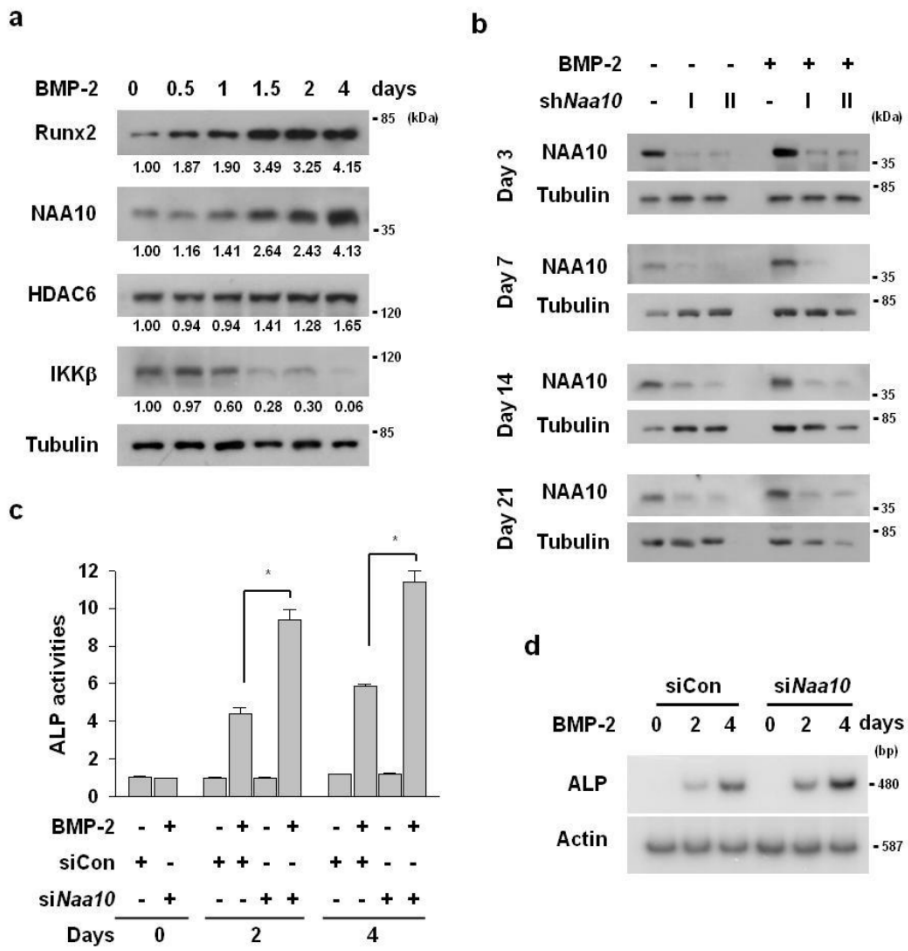
**Chromatin immunoprecipitation.** Cells were fixed with 1% formaldehyde for 10 min to crosslink chromatin complexes, and treated with 150 mM glycine to stop the crosslinking reaction. After cells were lysed and sonicated, soluble chromatin complexes were precipitated with anti-Runx2 antibody (10 μg ml$^{-1}$) overnight at 4 °C. DNAs were isolated from the precipitates and the precipitated DNA segment of the OCN promoter was quantified by real-time PCR using the Evagreen qPCR Mastermix (Applied Biological Materials, Inc., Richmond, Canada) in the CFX Connect Real-Time System (BIO-RAD). The chromatin immunoprecipitation-DNAs were amplified over 40 PCR cycles (95–53–70 °C, 20 s at each temperature) and the DNA amplification was continuously monitored. The primer sequences used for qPCR amplifying the OCN gene are 5′-TGCATAGGGTTCTTGTC TCT-3′ and 5′-CTCCACCACTCCTACTGTGT-3′.

***In vitro* acetylation assay.** GST-tagged Runt domain, its K225R mutant or His-tagged NAA10 peptide was expressed in *E. coli* using isopropyl-β-D-thiogalactoside, and then purified using GSH- or nickel-affinity beads. Recombinant His-NAA10 (0.5 μg) and GST-Runt (0.5 μg) peptides were incubated in a reaction mixture (50 mM Tris/HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 10 mM sodium butyrate, 20 mM acetyl-CoA and 10% glycerol) at 37 °C for 4 h. Acetylated Runt peptides were identified by western blotting using anti-acetyl lysine antibody. To verify the presence of peptides, the mixture was run on a SDS-polyacrylamide gel and stained with 0.1% Coomassie Brilliant Blue R-250, followed by being destained with 50% methanol and 10% glacial acetic acid.

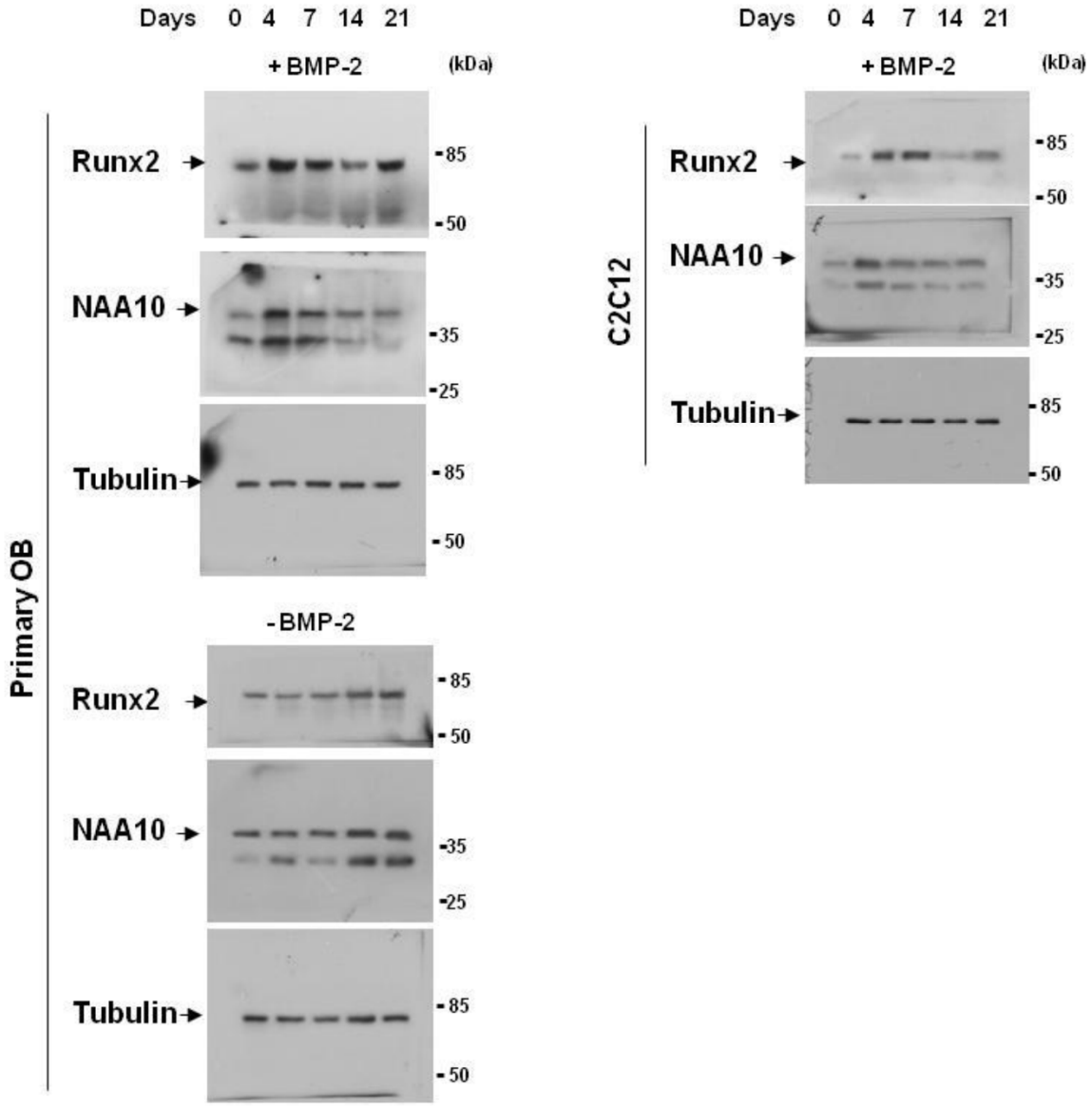
**In-gel digestion and mass spectrometric analysis.** After F/S-Runt peptide was coexpressed with *NAA10* in $C_2C_{12}$, the peptide was separated by a SDS–PAGE, digested in the gel slice with trypsin and subjected to a nanoflow ultra-performance liquid chromatography/ESI/MS/MS with a mass spectrometer (Q-tof Ultra global), comprising a three-pumping Waters nano-liquid chromatography system, a stream selection module and MassLynx 4.0 controller (Waters, UK). Five microlitres of mixed peptides was dissolved in buffer C (water/acrylonitrile/formic acid; 95:5:0.2, V:V:V), injected on a column and eluted by a linear gradient of 5–80% buffer B (water/acrylonitrile/formic acid, 5:95:0.2) over 120 min. MS/MS spectra were processed and analysed using ProteinLynx Global Server (PLGS) 2.1 software (Micromass, UK). Lysyl acetylation was identified by the additional mass of 42 Da on lysine residue.

**Antibody generation.** Antibody against acetylated K225 of Runx2 was raised from rabbits (New Zealand white). Rabbits were immunized with Keyhole limpet haemocyanin-conjugated synthetic peptide (Supplementary Fig. 22a), which contain acetylated K225 of Runt domain in Runx2. The specific, polyclonal antibody was purified by affinity chromatography using the acetylated K225 peptide.

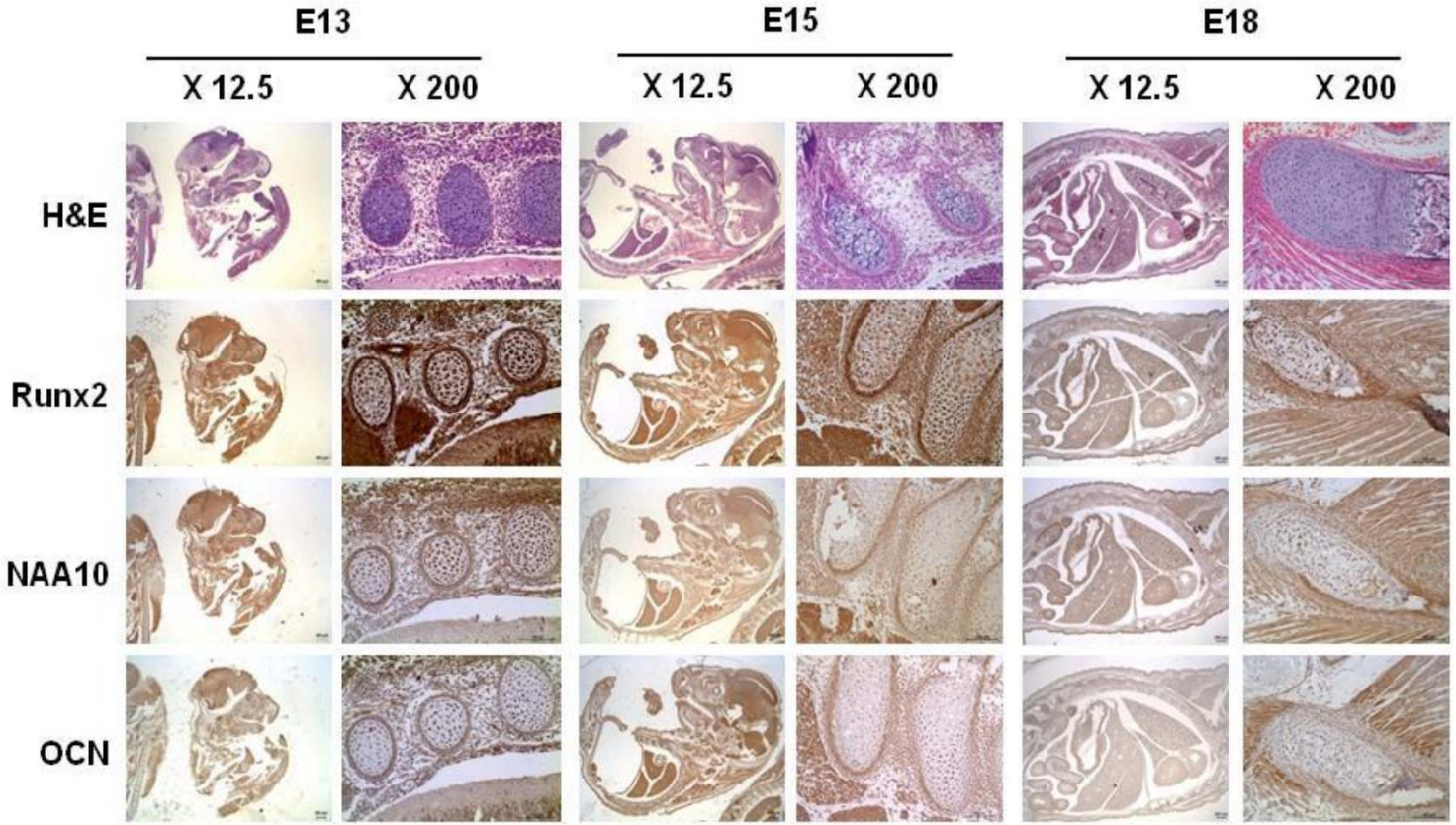
**Statistics.** All data were analysed using Microsoft Excel 2007 software, and results are expressed as means and s.d. We used the unpaired, two-sided Student's *t*-test to compare reporter activities and morphometric data from micro-CT analyses. Statistical significances were considered when *P* values were less than 0.05.



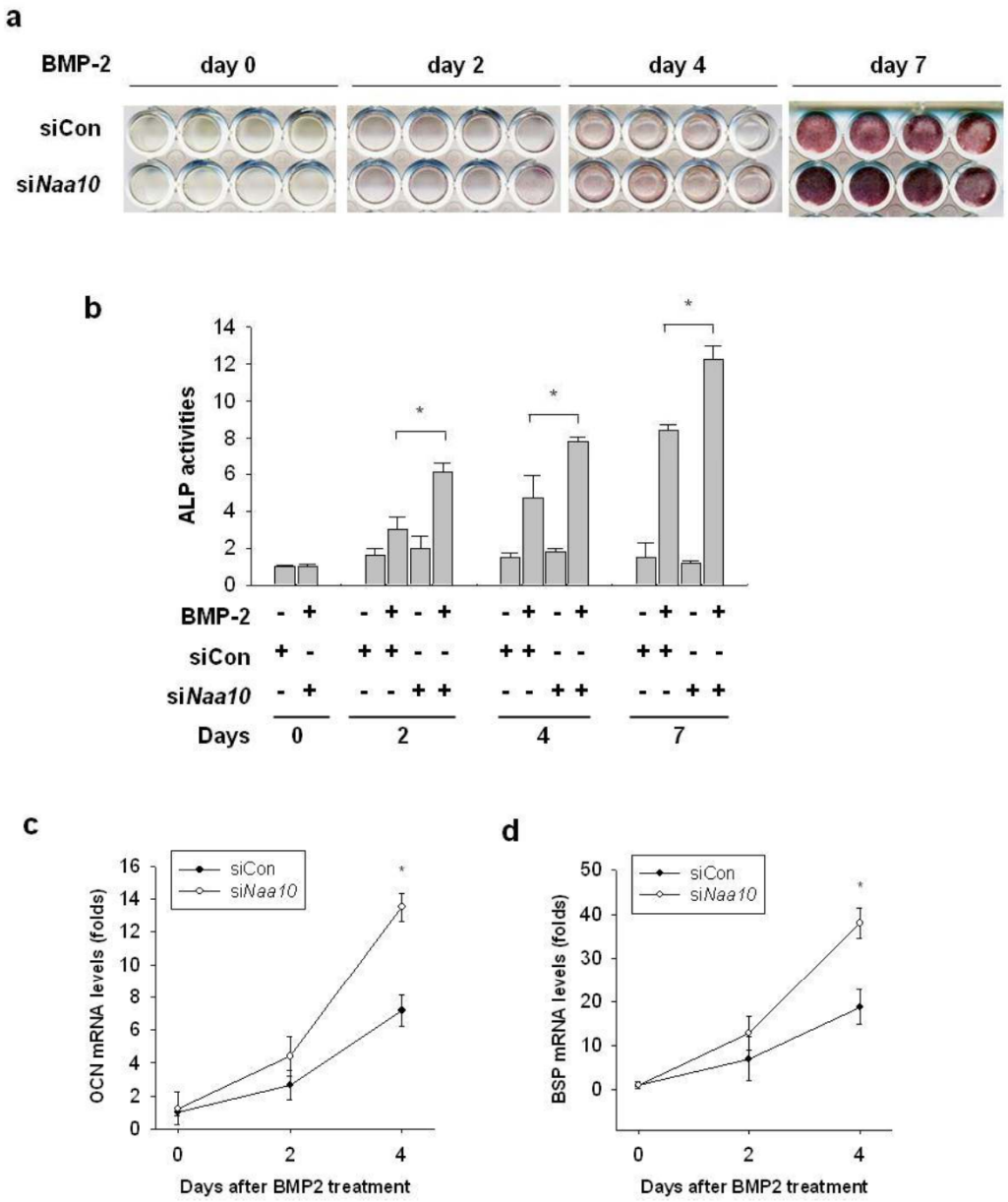

**Supplementary Figure 1. NAA10 and ALP expressions during osteoblast differentiation.** (**a**) In primary osteoblasts treated with BMP-2 (100 ng/ml) for the indicated times, Runx2, NAA10, HDAC6, IKKβ and β-tubulin protein levels were analyzed by Western blotting. The blots were quantified (intensity x area) using ImageJ, and the results were divided by corresponding tubulin values. The protein levels were relatively compared with those at zero time. (**b**) $C_2C_{12}$ cells, which had been infected with *Naa10*-targeting shRNA viruses, were differentiated with BMP-2. On the indicated dates, NAA10 levels were analyzed in cell lysates by Western blotting. (**c**) $C_2C_{12}$ cells, which had been transfected with the indicated siRNAs, were treated with PBS or BMP-2 for 4 days. ALP activities (means + s.d., n = 4) were normalized versus total protein levels and are presented as relative values versus controls. * denotes $P < 0.05$ (Student's t-test). (**d**) RNAs were extracted from transfected $C_2C_{12}$ cells and reverse-transcribed. ALP and actin cDNAs were amplified using PCR over 17 to 22 cycles with [$\alpha$-$^{32}$P]dCTP, and the cDNAs obtained were electrophoresed on 4% polyacrylamide gels and autoradiographed.



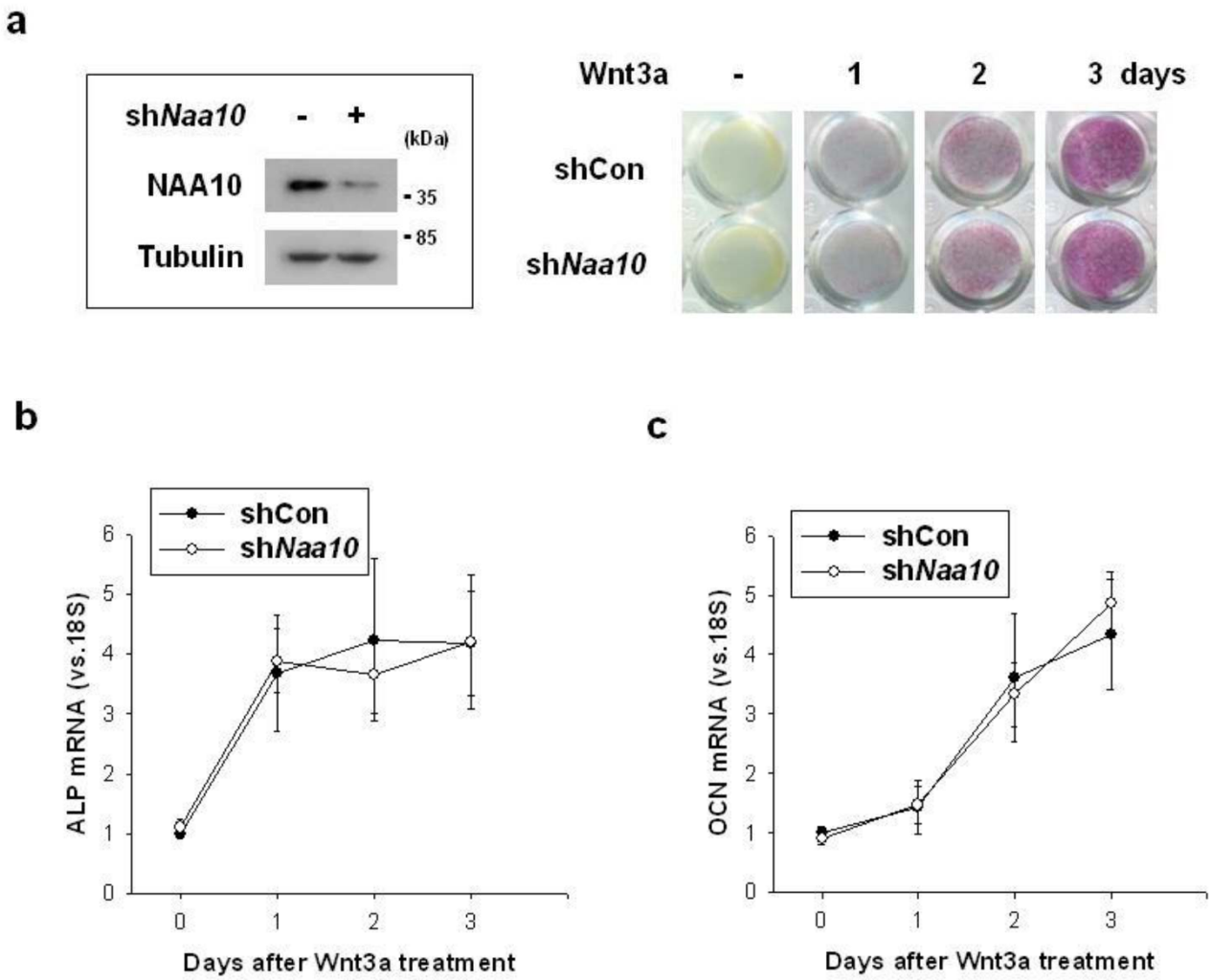

**Supplementary Figure 2. Runx2 and NAA10 expressions in differentiating osteoblasts − uncropped scans of Figure 1b.**



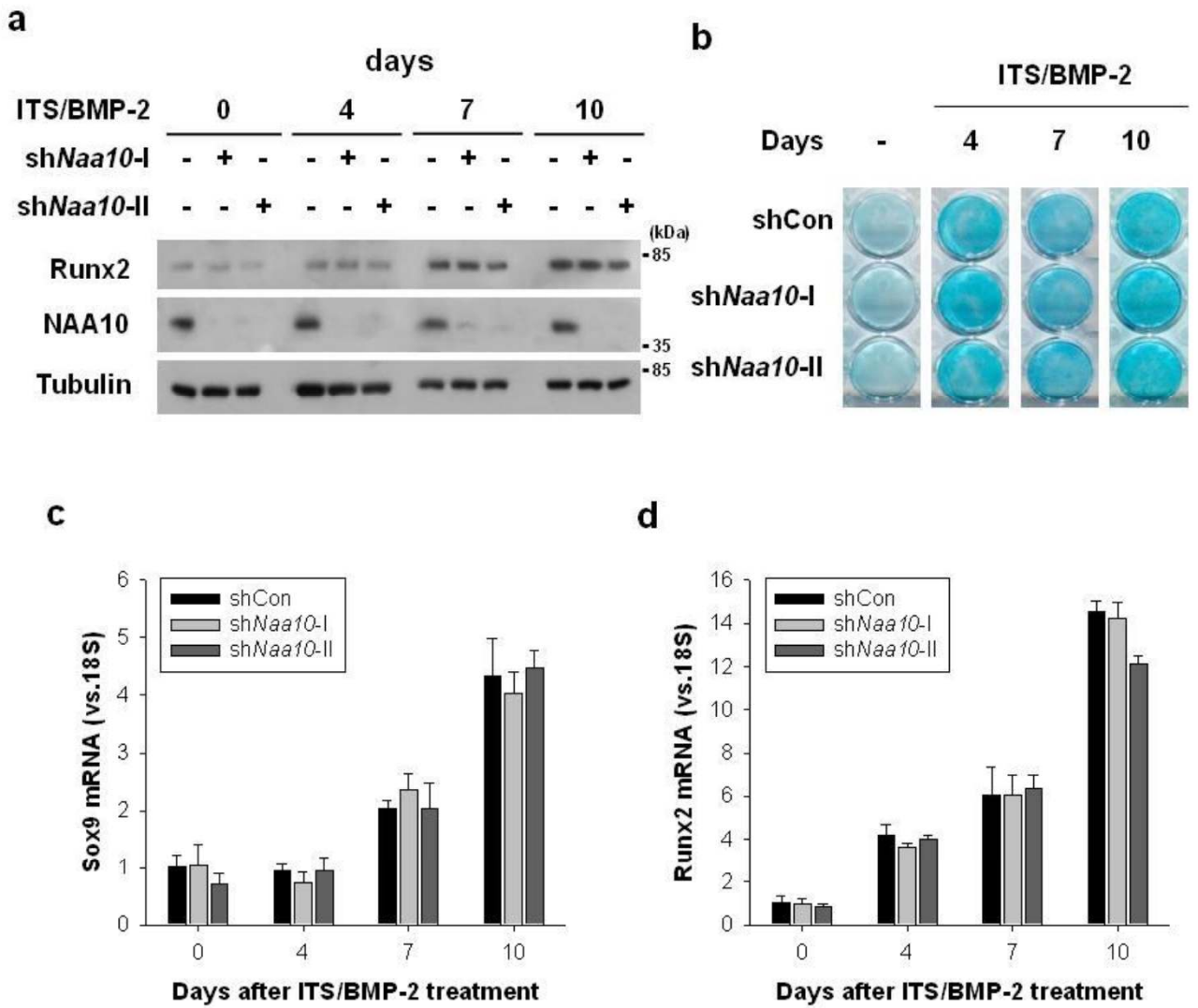

**Supplementary Figure 3. Runx2 and NAA10 expressions in embryonic stage.** Paraffin sections of mice at E13, E15, and E18 were stained with H&E and subjected to immunohistochemical analyses using anti-Runx2, anti-NAA10, or anti-OCN antibody. Scale bar = 500 μm.



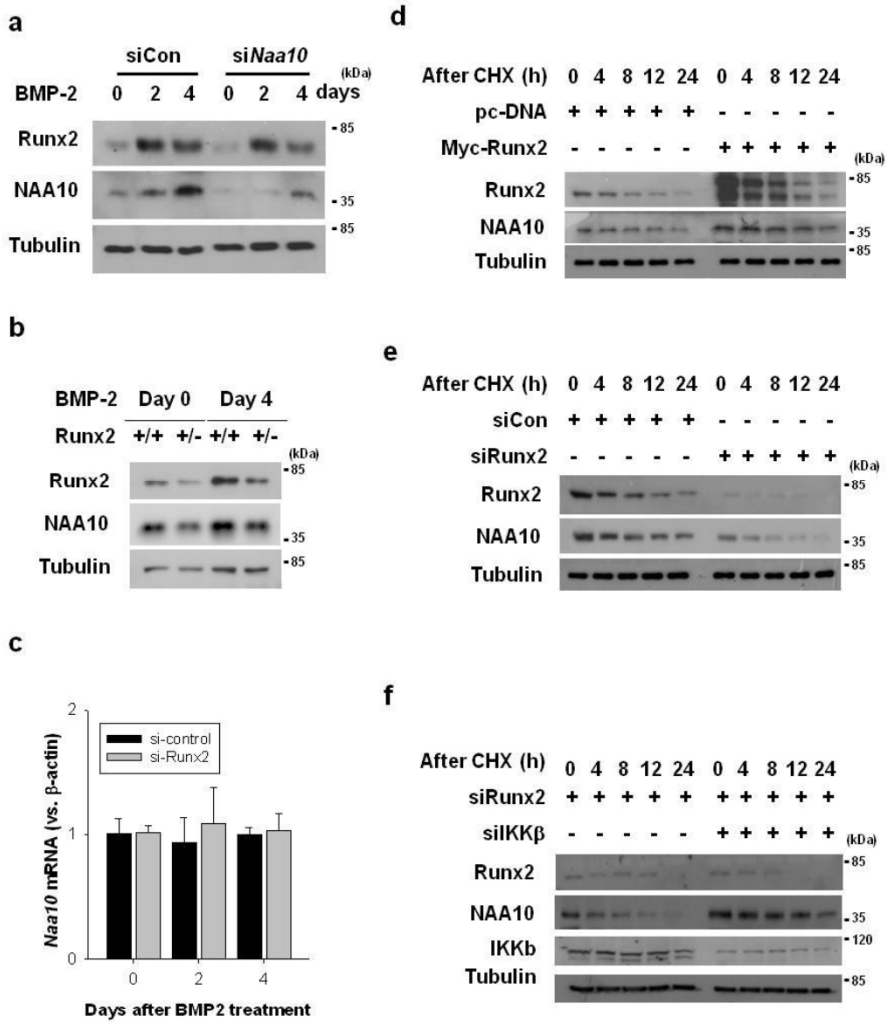

**Supplementary Figure 4. NAA10 knock-down promotes osteoblast differentiation in MC3T3-E1 cells.** *Naa10* or control siRNAs were transfected into MC3T3-E1 cells, and treated with BMP-2 (100 ng/ml) for the indicated times. Cells were stained with p-nitrophenyl phosphate (**a**) or lysed for enzymatic ALP assays (**b**). ALP activities (means + s.d., n = 4) were normalized to protein amounts, and presented as relative values versus the mock and PBS control. RNAs were extracted from transfected MC3T3-E1 cells, and the osteocalcin (**c**) and BSP (**d**) mRNA levels (means ± s.d., n = 4) were quantified by RT-qPCR. * denotes $P < 0.05$ (Student's t-test).



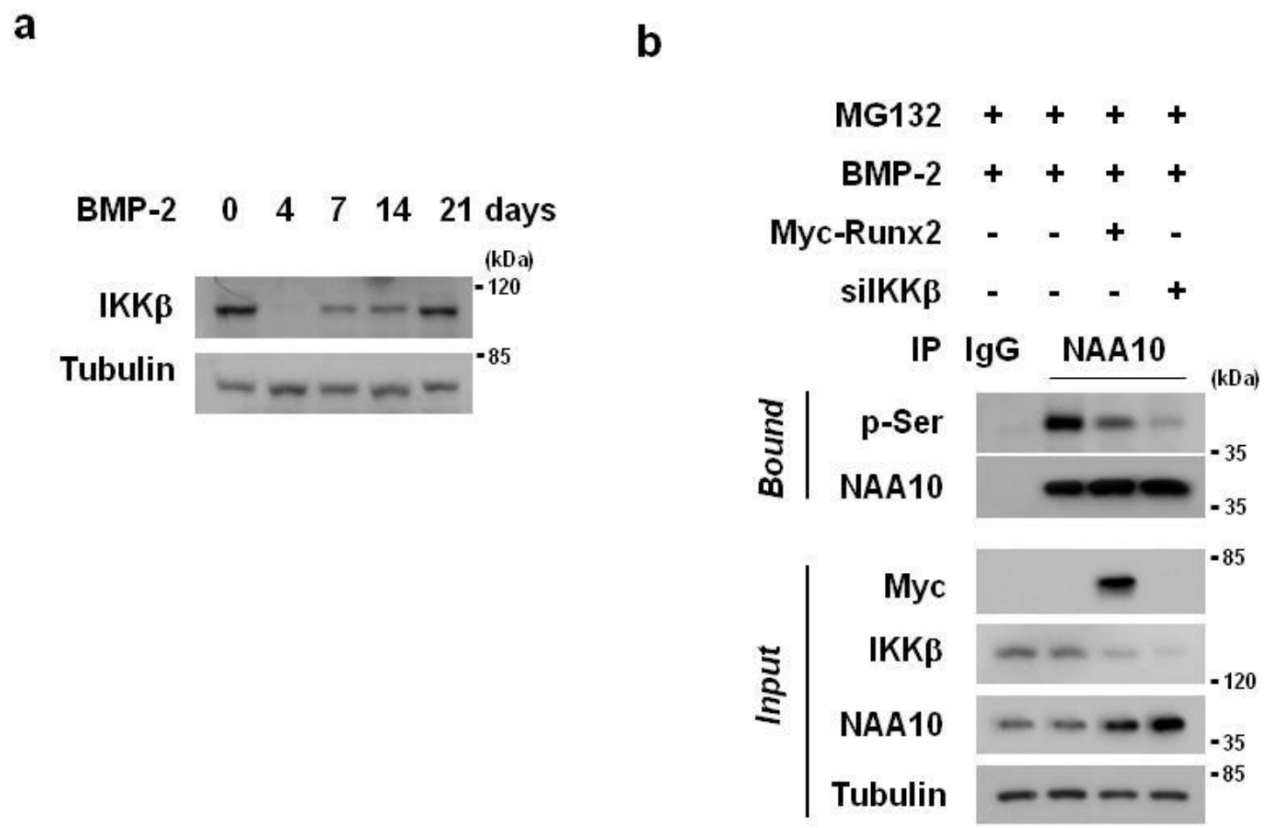

**Supplementary Figure 5. NAA10 is not involved in Wnt3a-induced osteoblast differentiation.** (**a**) $C_2C_{12}$ cells, which had been infected with *Naa10*-targeting shRNA viruses, were differentiated with Wnt3a (50 ng/ml) for the indicated days. NAA10 levels were analyzed Western blotting (left panel). Cells were stained with naphthol AS-MX phosphate at 37°C for 1 hour (right panel). (**b**, **c**) The mRNA levels of ALP and OCN were quantified by RT-qPCR. Results are expressed as the means ± s.d. (n = 4).



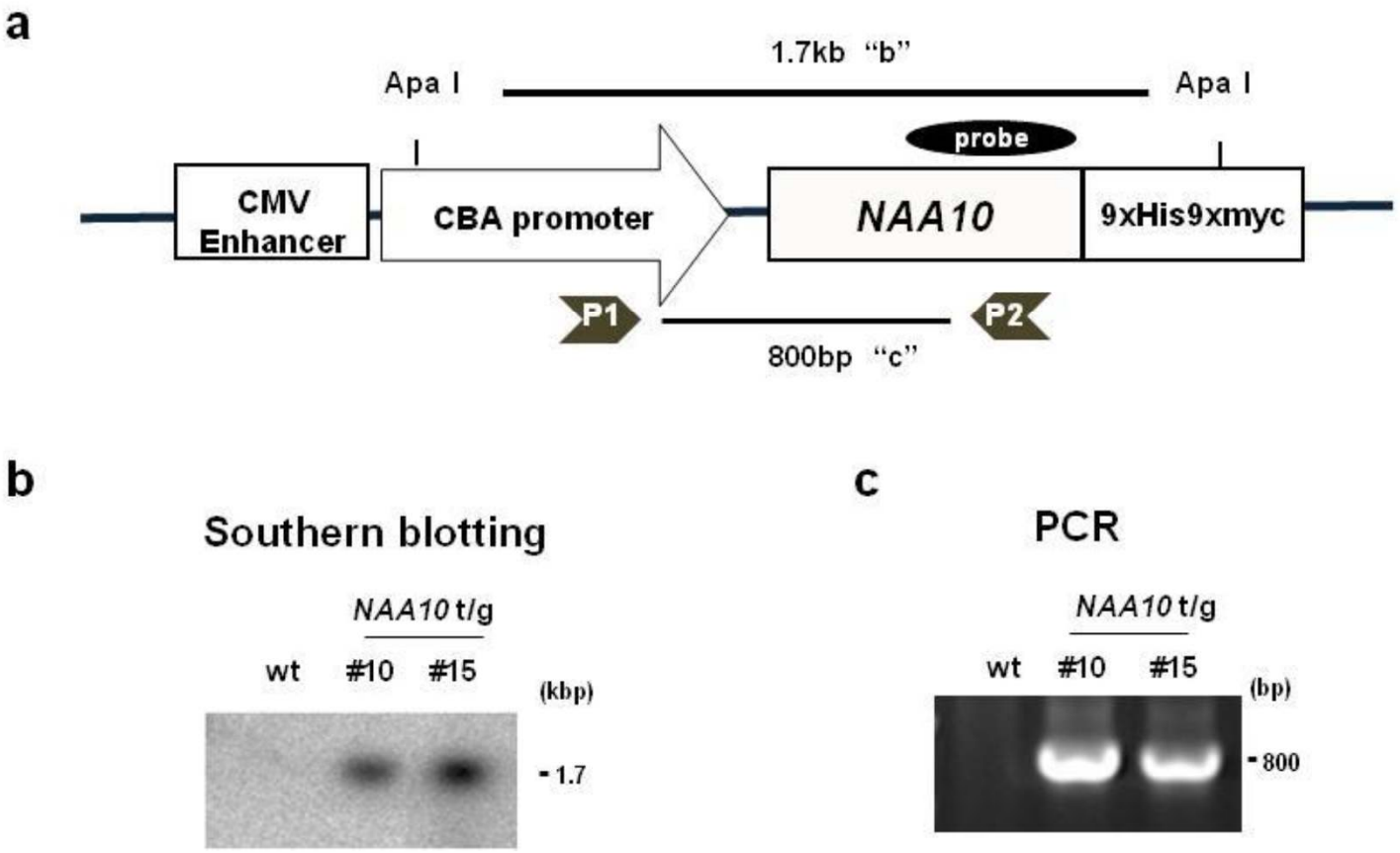

**Supplementary Figure 6. NAA10 is not involved in chondrocyte differentiation.** Mouse Chondrogenic ATDC5 cells, which had been infected with *Naa10*-targeting shRNA viruses, were treated with ITS and BMP-2 (10 ng/ml) for the indicated days. (**a**) Runx2 and NAA10 levels were analyzed by Western blotting. (**b**) ATDC5 cells were stained with Alcian blue to evaluate chondrocyte differentiation. (**c**, **d**) Sox9 and Runx2 mRNA levels were quantified by RT-qPCR. Results are expressed as the means + s.d. (n = 4).



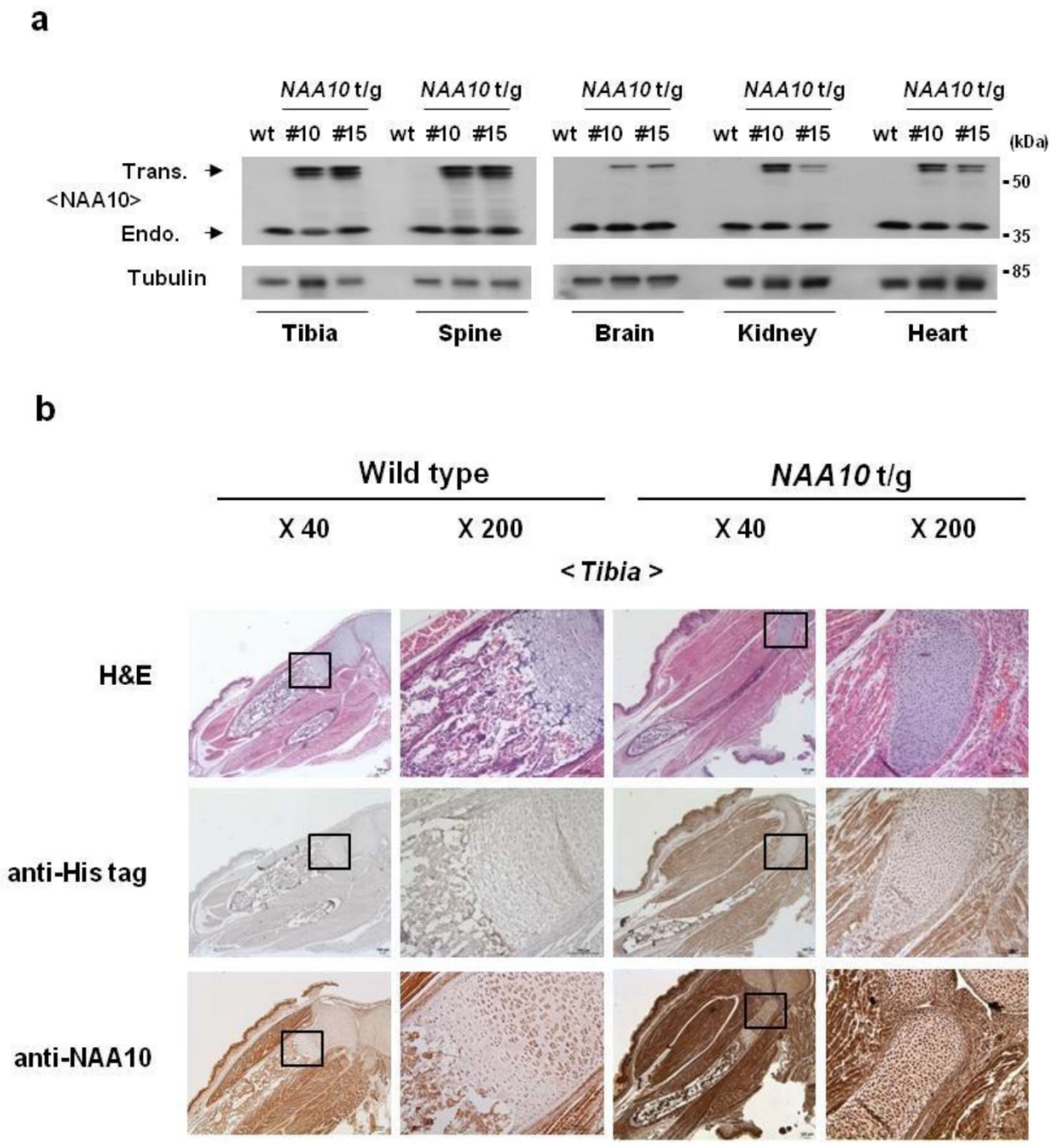

**Supplementary Figure 7. Runx2 blocks the NAA10 degradation induced by IKKβ.** (**a**) $C_2C_{12}$ cells, which had been transfected with 80 nM siRNA targeting *Naa10*, were treated with BMP-2 for 2 or 4 days. Runx2 and NAA10 expressions were analyzed by Western blotting. (**b**) Primary osteoblasts were isolated from wild type and Runx2$^{+/-}$ mice, both of which were provided by Dr. Hyun-Mo Ryoo (Seoul National University School of Dentistry, Korea). Cells were incubated with BMP-2 for 4 days, and Runx2 and NAA10 levels were analyzed by Western blotting. The heterozygous Runx knock-out (**c**) *Naa10* mRNA levels were quantified by RT-qPCR in BMP-2 treated $C_2C_{12}$ cells which had been transfected with the indicated siRNAs. Results are expressed as the means + s.d. (n = 4). (**d**) $C_2C_{12}$ cells, which had been transfected with Myc-Runx2 plasmid or pcDNA, were treated with 100 nM cycloheximide for the indicated times. (**e**) $C_2C_{12}$ cells were transfected with 80 nM Runx2 or control siRNAs, and treated with cycloheximide. (**f**) $C_2C_{12}$ cells were transfected with 80 nM Runx2 or/and IKKβ siRNAs, and treated with cycloheximide. Runx2 and NAA10 levels were analyzed by Western blotting.




**Supplementary Figure 8. Runx2 inhibits IKKβ-dependent serine-phosphorylation of NAA10.** (**a**) $C_2C_{12}$ cells were treated with BMP-2 for the indicated times, and the IKKβ levels were analyzed by Western blotting. (**b**) $C_2C_{12}$ cells, which were transfected with Myc-Runx2 plasmid and/or IKKβ siRNA, were incubated with BMP-2 for 24 hours, and further treated with 20 μM MG132 for 8 hours. Cell lysates were immunoprecipitated with anti-NAA10, and the precipitates were immunoblotted with phosphoserine (p-Ser) antibody. The input levels of NAA10, Runx2 and IKKβ were verified by Western blotting.




**Supplementary Figure 9. Establishment of *NAA*10 transgenic mouse.** (**a**) The structure of the *NAA10* expression vector introduced into transgenic mice. Gene expression was driven under the control of a chicken beta-actin promoter. (**b**, **c**) The presence of the *NAA10* vector in two t/g lines (#10 and #15) was verified using Southern blotting and PCR.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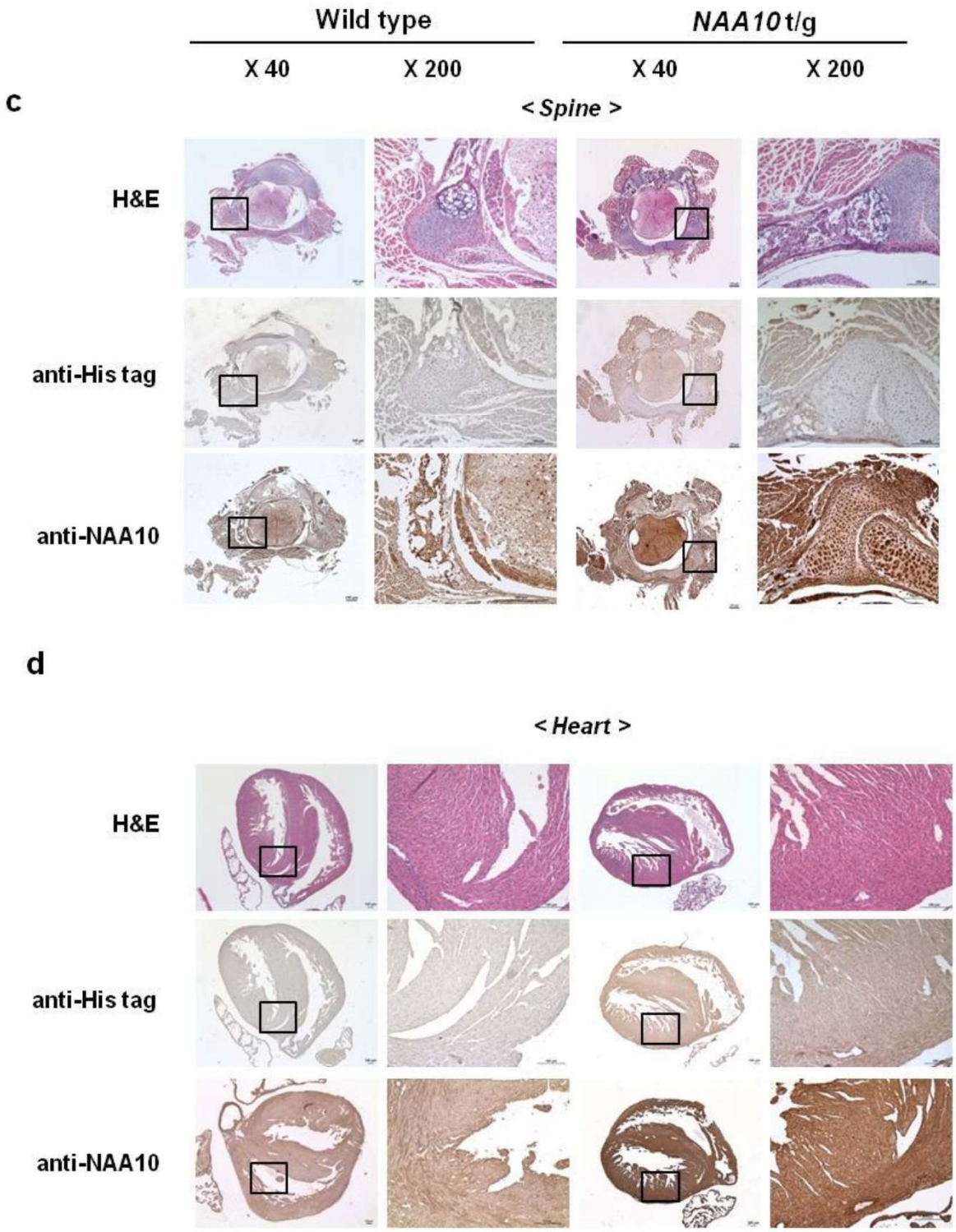
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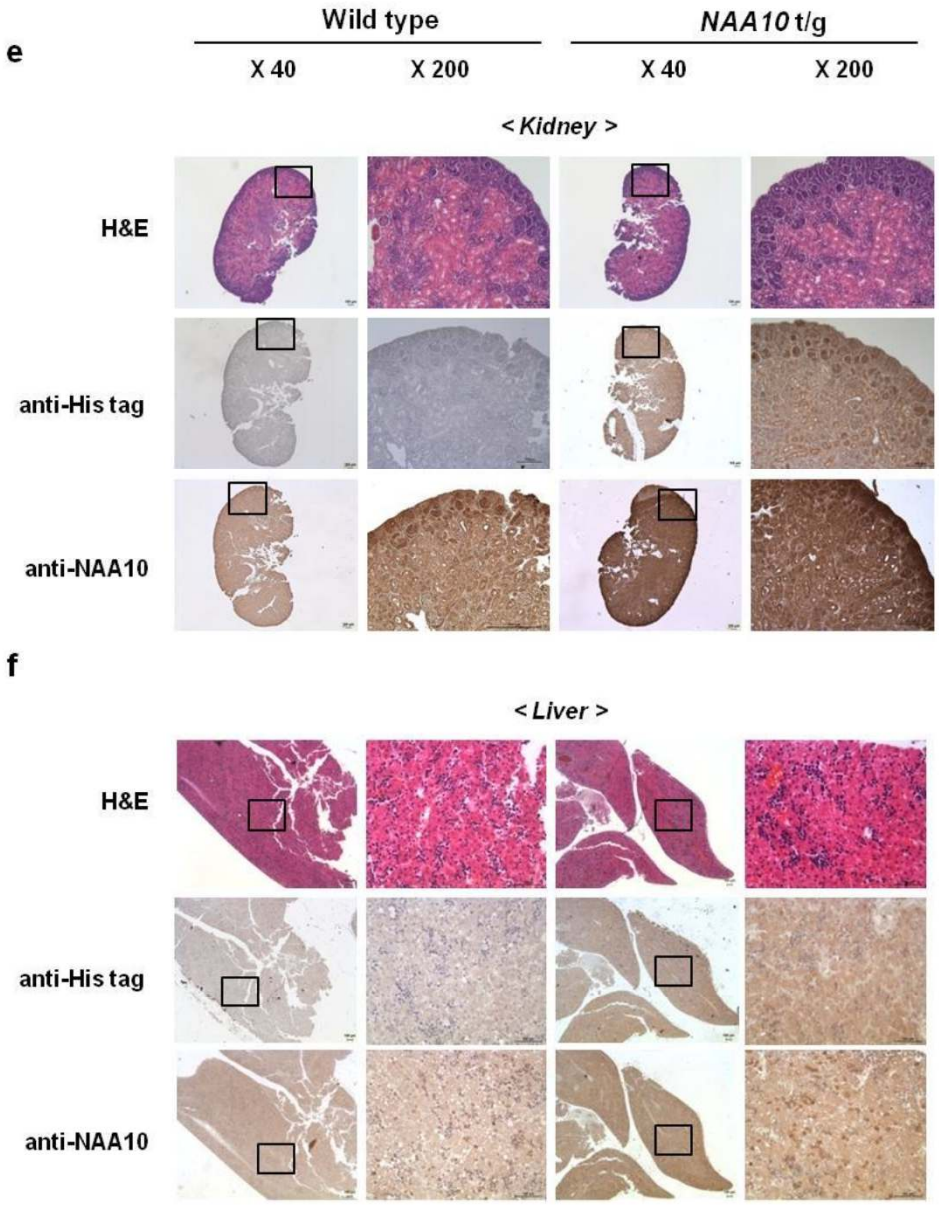
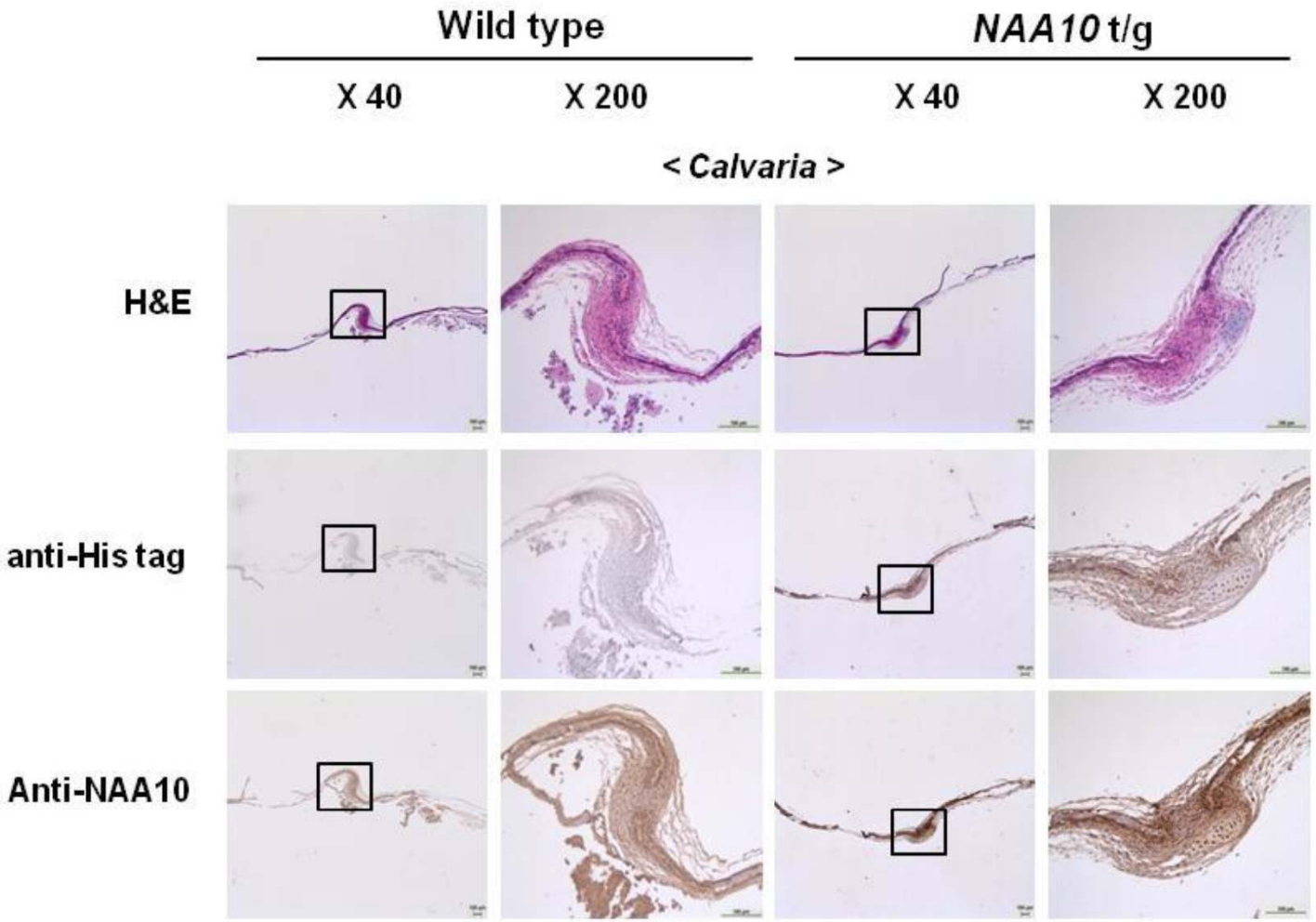
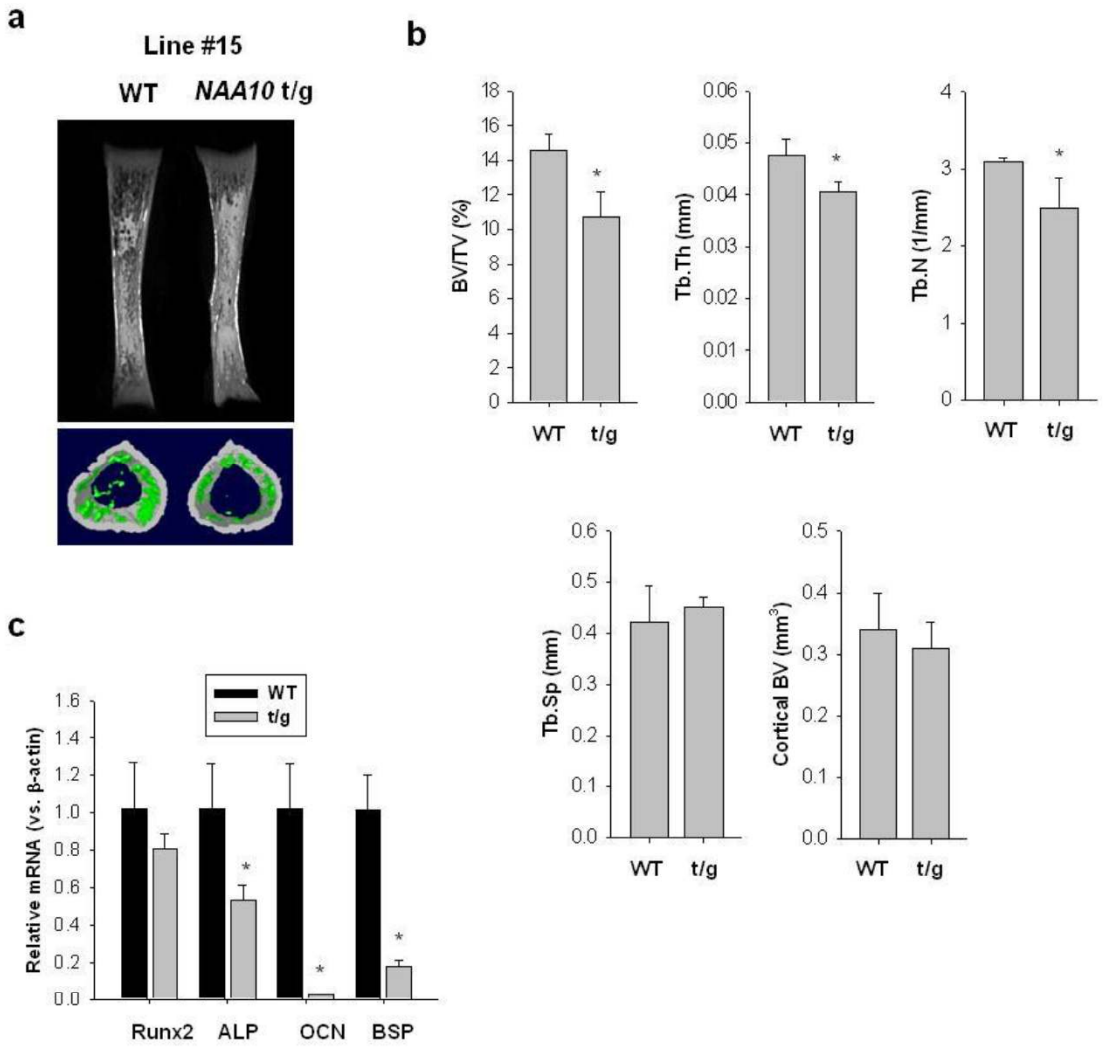


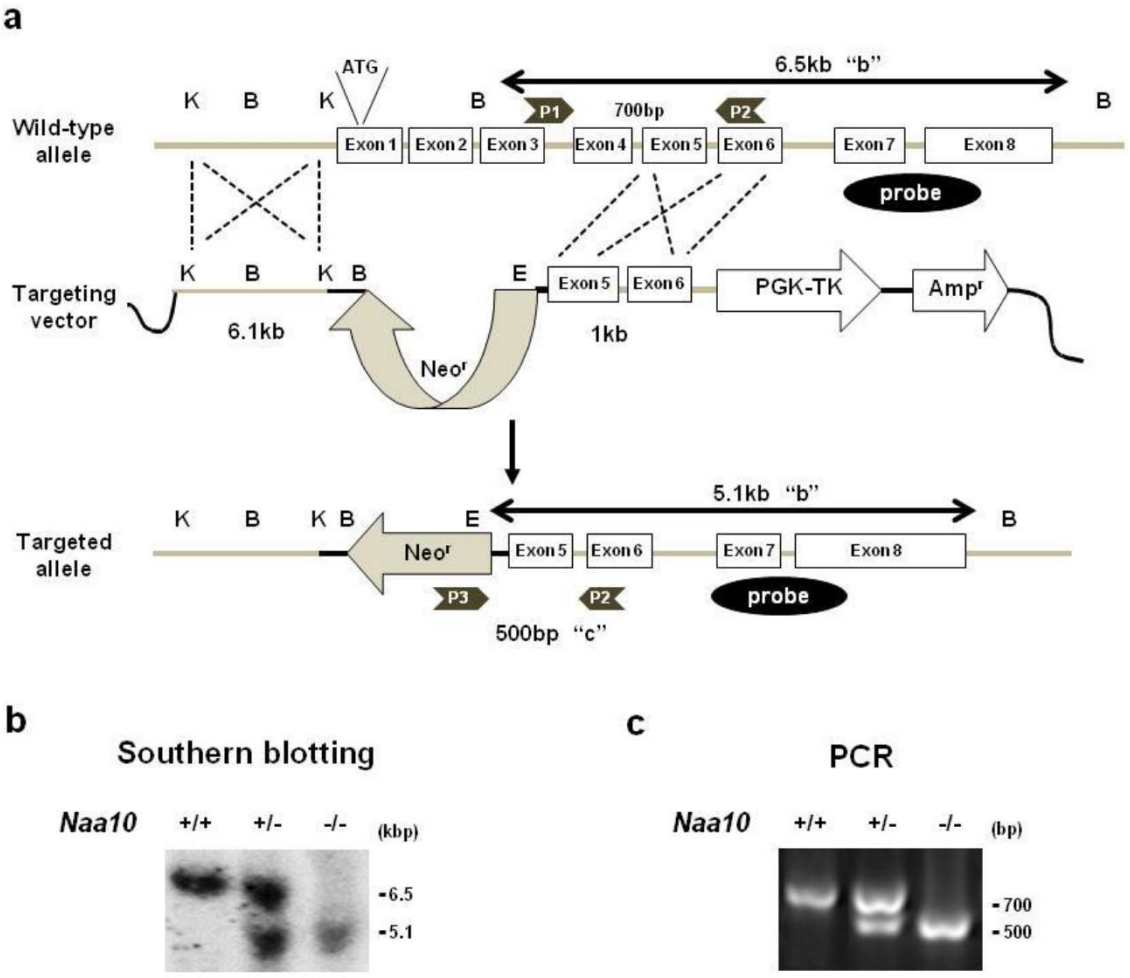

**Supplementary Figure 10. NAA10 expressions in *NAA10* transgenic mouse.** (**a**) Expression of endogenous NAA10 (Endo.) and transgenic Myc/His-tagged NAA10 (Trans.) NAA10 in wild type and two *NAA10* transgenic lines #10 and #15. Tissue homogenates were prepared from tibia, spine, brain, kidney, and heart, and analyzed by Western blotting with anti-NAA10 antibody. (**b-f**) Immunohistochemical analyses of NAA10 in wild type and *NAA10* transgenic mice (#10). Paraffin tissue sections of mice at a postnatal 3 day were subjected to H&E staining (upper panel) and immunohistochemical analyses. Scale bar = 100 μm.



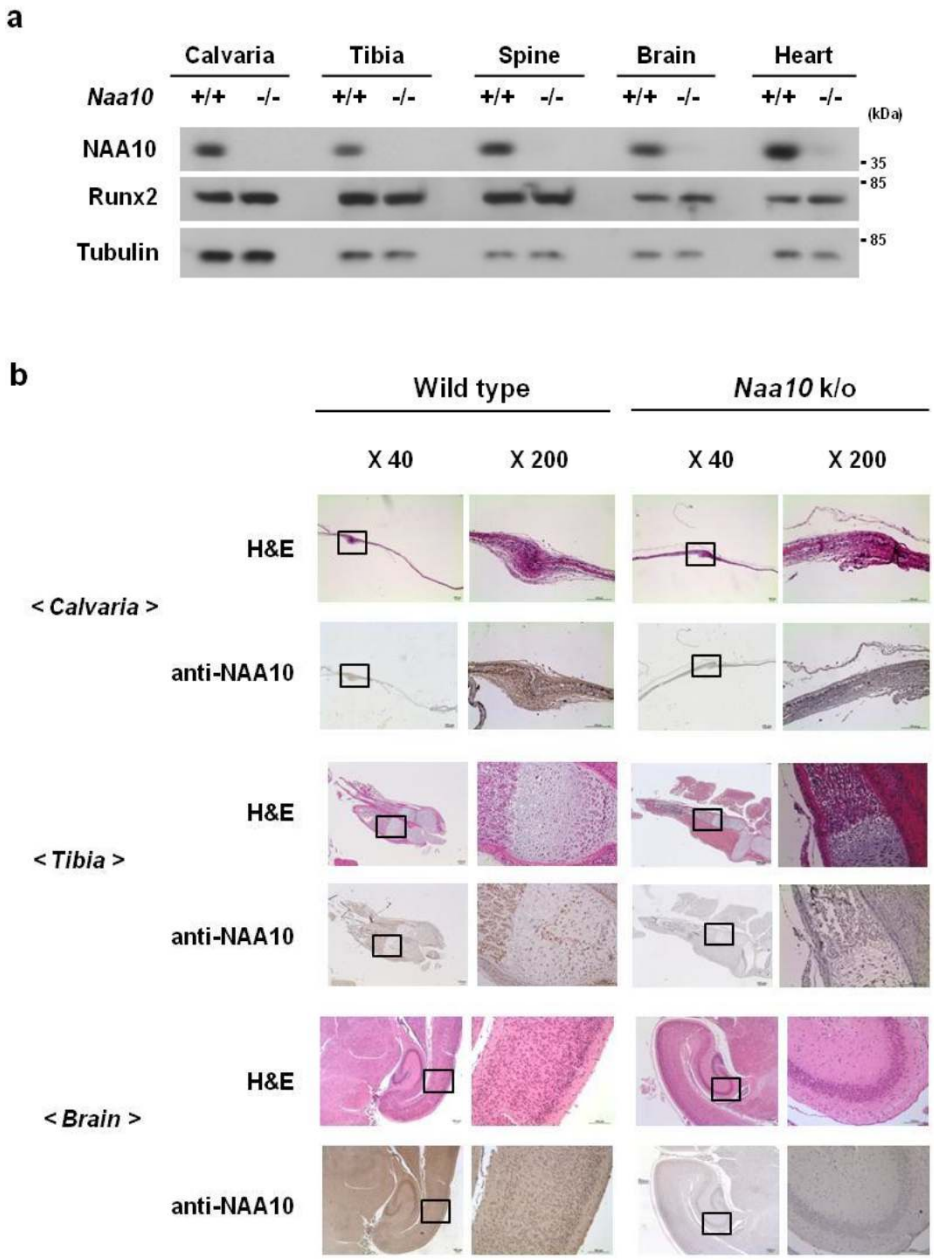

**Supplementary Figure 11. NAA10 expression in calvarias of *NAA10* transgenic mice.** Paraffin sections of calvarias from wild type and *Myc/His-tagged NAA10* t/g mice were stained with H&E (top panel), and subjected to immunohistochemical analyses with anti-His (middle panel) or anti-NAA10 antibody (bottom panel). Scale bar = 100 μm.



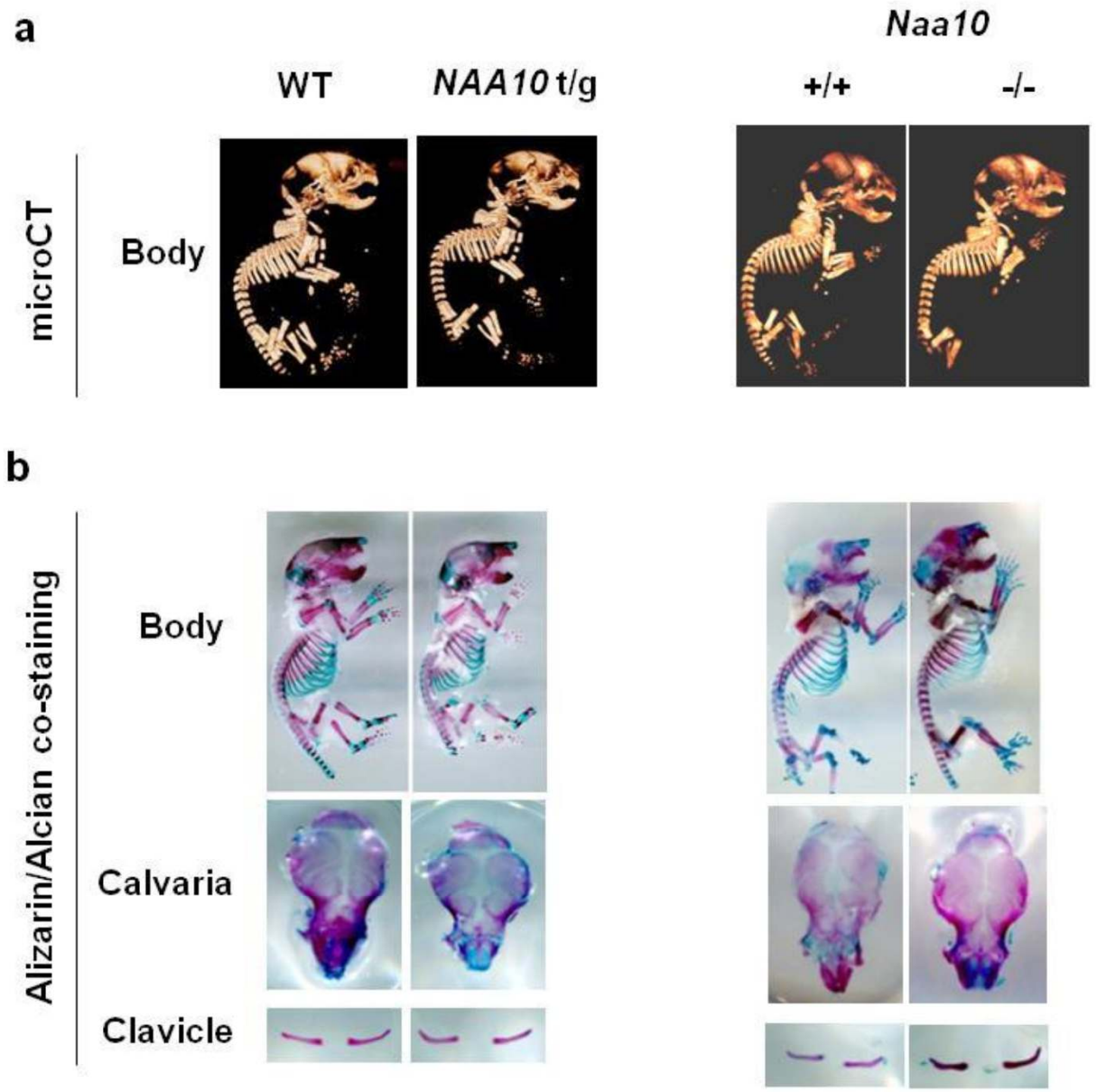

**Supplementary Figure 12. Femur bone development in *NAA10* transgenic mice.** (**a**) Representative microCT images of femurs prepared from line #15 *NAA10* transgenic (t/g) mice or their wild type littermates (WT) on the postnatal day 3. (**b**) Quantitative microCT analysis of the secondary spongiosa of proximal femur. Bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and cortical bone volume (Cortical BV) were analyzed from reconstructed 3D images. Results are expressed as the means + s.d. (n = 5). (**c**) Runx2, ALP, OCN, and BSP mRNA levels were quantified in mouse femurs by RT-qPCR and results are expressed as the means + s.d. (n = 6). * denotes $P < 0.05$ (Student′s t-test) versus the WT value.



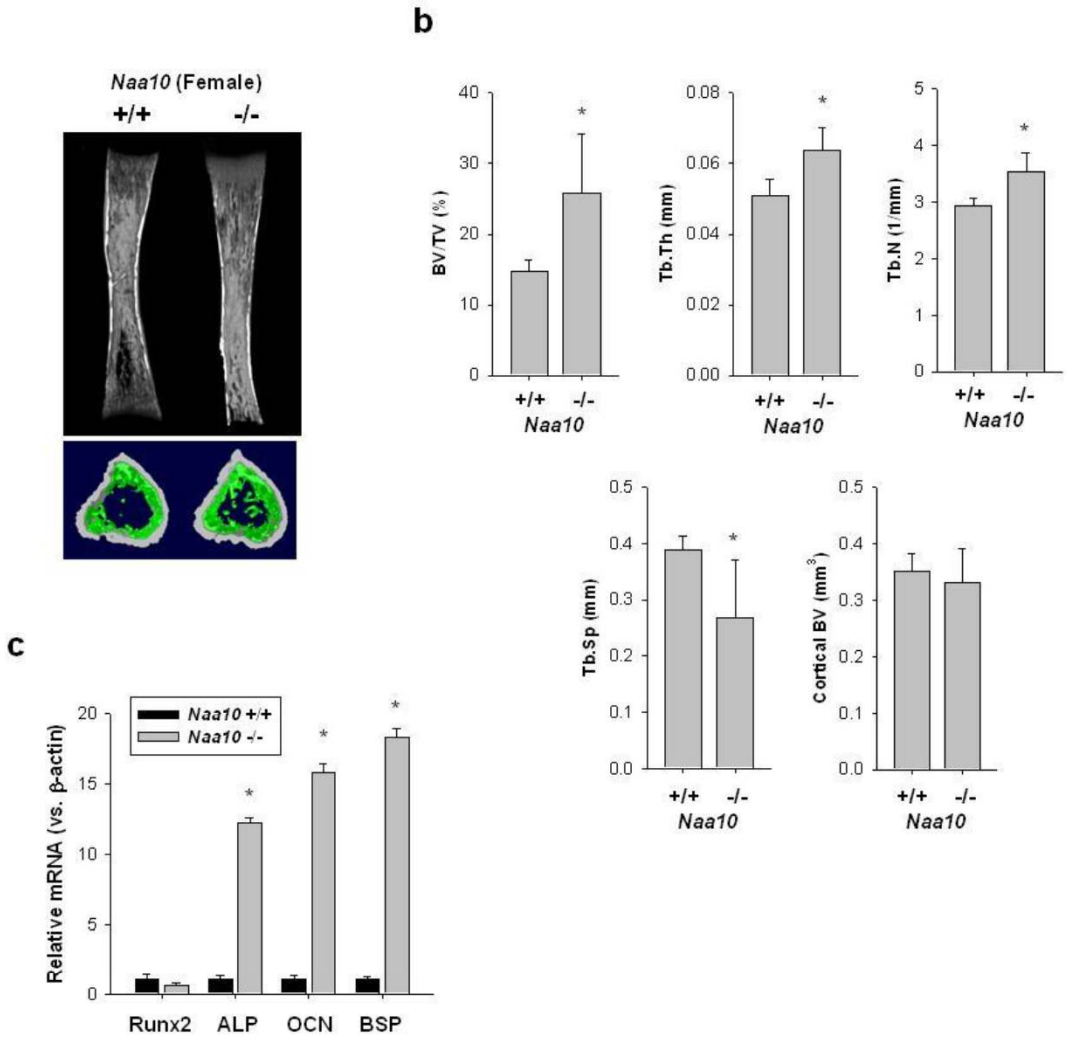

**Supplementary Figure 13. Establishment of *Naa10* knock-out mouse.** (**a**) Targeting vector construct was designed to knockout *Naa* exon1 to 4 by homologous recombination. E14 ES cells (129Sv background) were electroporated and selected for gaining of resistance to G418. Recombinant was detected in 16 of 700 clones screened by Southern blotting. *Neo*$^{r}$; neomycin-resistance cassette (for positive selection); PGK-TK ; thymidine kinase-encoding gene cassette driven by the promoter of the gene encoding phosphoglycerate kinase (for negative selection); K ; KpnI, B ; BamHI, E ; EcoRI. The location of the 3′ external probes used for Southern hybridization and PCR primers P1, P2, and P3 used for genotyping are indicated. (**b**, **c**) The partial deletion of the *Naa10* gene was verified using Southern blotting and PCR.



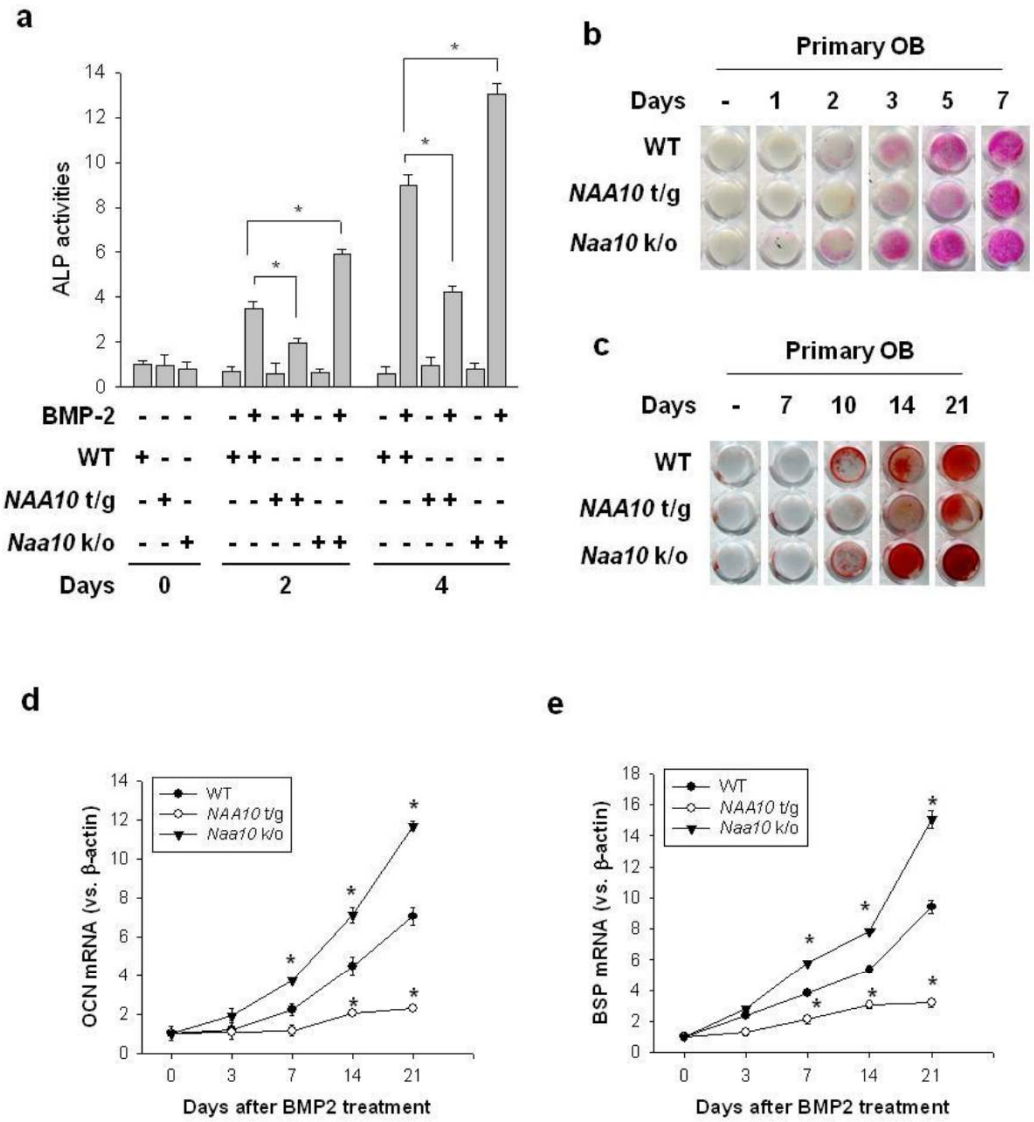

**Supplementary Figure 14. NAA10 expressions in wild type and *Naa10* knock-out mice.** (**a**) NAA10 and Runx2 levels were analyzed in various tissues form wild type ($Naa10^{+/+}$) and *Naa10* knock-out ($Naa10^{-/-}$) mice by Western blotting. (**b**) Immunohistochemical analyses of NAA10 in $Naa10^{+/+}$ and $Naa10^{-/-}$ mouse. Paraffin tissue sections of mice at a postnatal 3 day were subjected to H&E staining and immunohistochemical analysis using anti-NAA10 antibody. Scale bar = 100 μm.



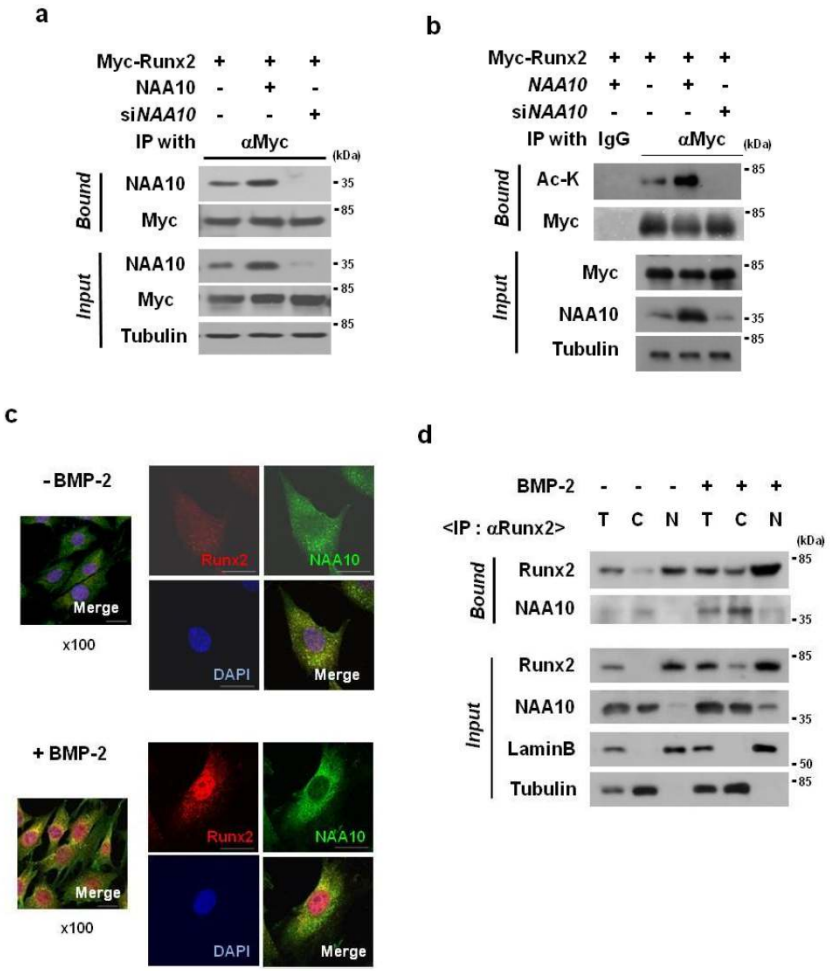

**Supplementary Figure 15. Skeletal structures of wild type, *NAA10* transgenic mice, and *Naa10* knock-out mice.** (**a**) Representative microCT images of mouse whole bodies. (**b**) Mice at a postnatal 3 day were stained with Alcian blue and Alizarin red. Calvarias and clavicles were isolated and pictured separately.



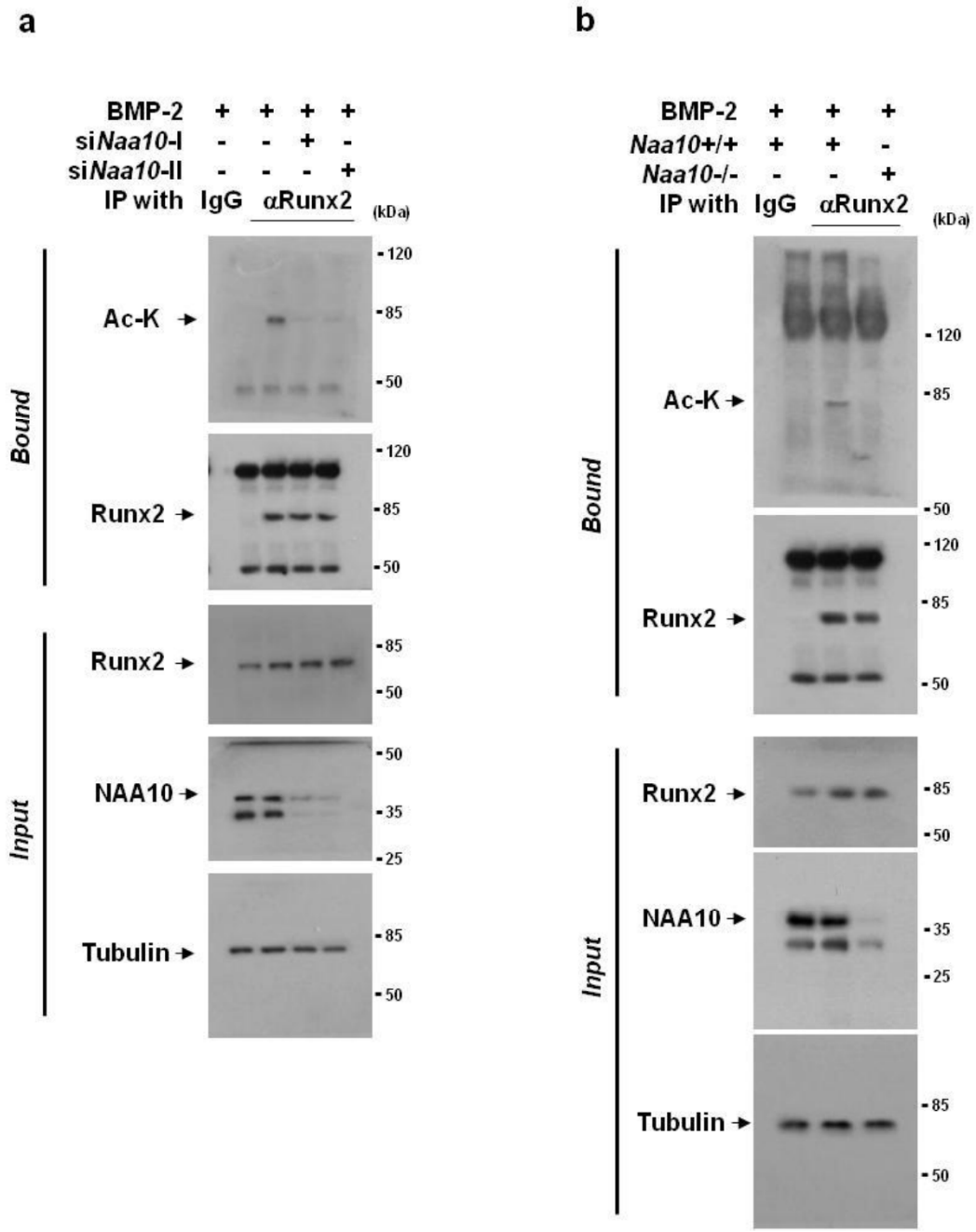

**Supplementary Figure 16. Femur bone development in *Naa10* knock-out mice.** (**a**) Representative microCT images of femurs prepared from *Naa10* knock-out (-/-) female mice or wild type female (+/+) mice at the postnatal day 3. (**b**) Quantitative microCT analysis of the secondary spongiosa of proximal femur. Bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and cortical bone volume (Cortical BV) were analyzed from reconstructed 3D images. Results are expressed as the means + s.d. (n = 5). (**c**) Runx2, ALP, OCN, and BSP mRNA levels were quantified in mouse femurs by RT-qPCR and results are expressed as the means + s.d. (n = 6). * denotes $P < 0.05$ (Student′s t-test) versus the WT value.



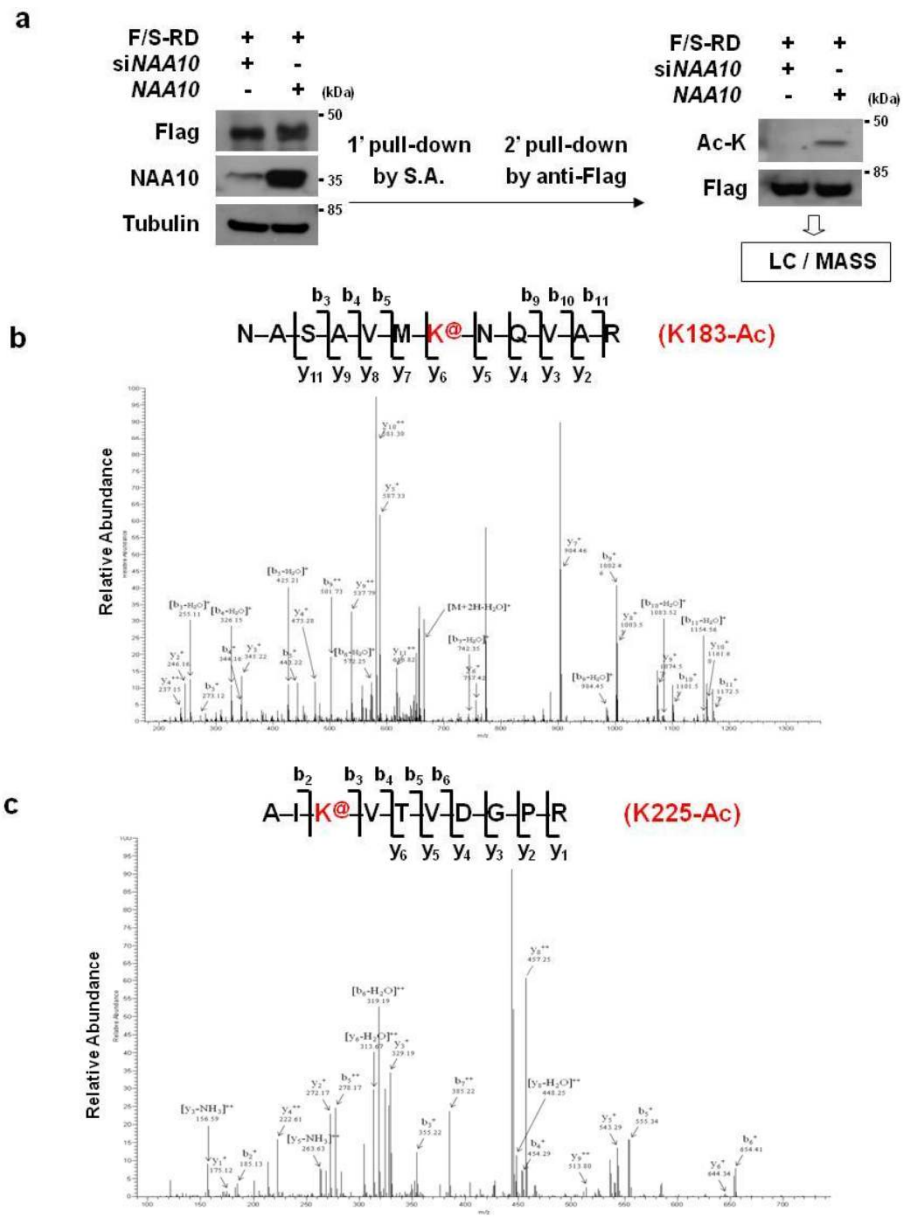

**Supplementary Figure 17. NAA10 inhibits osteoblast differentiation in primary osteoblasts from wild type, *NAA10* transgenic mice and *Naa10* knock-out mice.** (**a**) Primary osteoblasts from the indicated mice were incubated under PBS or BMP-2 for 2 or 4 days. ALP activities (means + s.d., n = 4) were presented as relative values versus those of the PBS-treated WT mice. Primary osteoblasts were stained with ALP (**b**) and Alizarin Red S (**c**). The mRNA levels of OCN (**d**) and BSP (**e**) were quantified in primary osteoblasts by RT-qPCR. Results (means ± s.d., n = 4) are expressed as relative values versus the zero time control. * denotes $P < 0.05$ (Student′s t-test) versus the WT control.



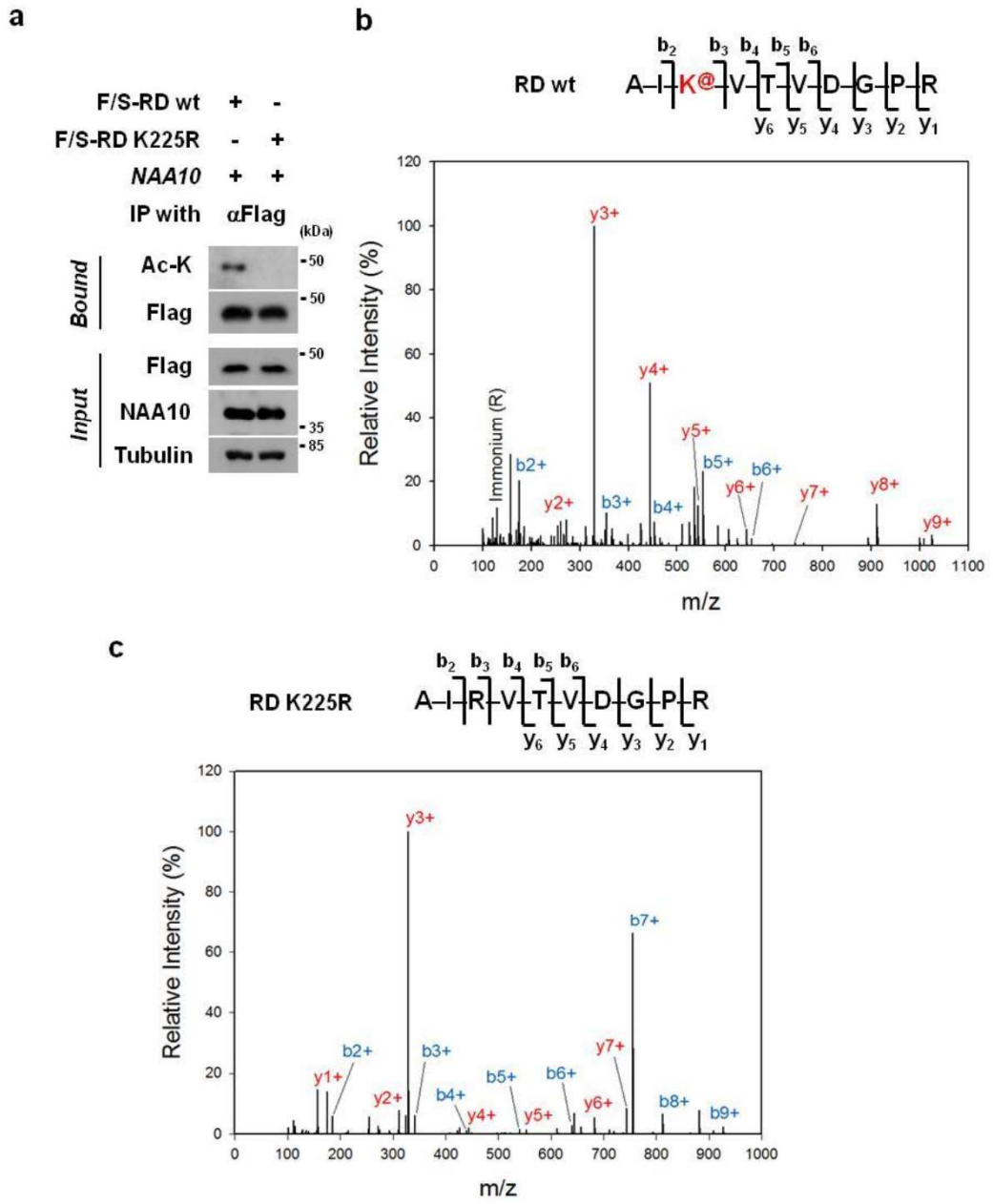

**Supplementary Figure 18. NAA10 binds and acetylates Runx2 in primary osteoblasts.** (**a**) Myc-Runx2 plasmid was co-transfected with *NAA10* plasmid or its siRNA into HEK293T cells. The interaction between Myc-Runx2 and NAA10 was identified by immunoprecipitation using anti-Myc and by immunoblotting with anti-NAA10. (**b**) Myc-Runx2 plasmid was co-transfected with *NAA10* plasmid or siRNA into HEK293T cells. Cells were treated with 1 μM Trichostatin A (a deacetylase inhibitor) for 6 hours and then lysed. Myc-Runx2 was precipitated using anti-Myc and its acetylation was analyzed by Western blotting using anti-acetyl lysine antibody. (**c**) Primary osteoblasts were treated with PBS or BMP-2, and subjected to immunocytochemical analyses with anti-Runx2 or anti-NAA10 antibody. The sections were visualized at 568 nm for Runx2 or at 488 nm for NAA10 under OLYMPUS fluorescence microscope. Scale bar, 20 μm. (**d**) Primary osteoblasts were lysed and fractionated into total (T), cytosolic (C) and nuclear (N) components. The cellular fractions were immunoprecipitated with anti-Runx2, and the precipitates were analyzed by Western blotting using indicated antibodies.



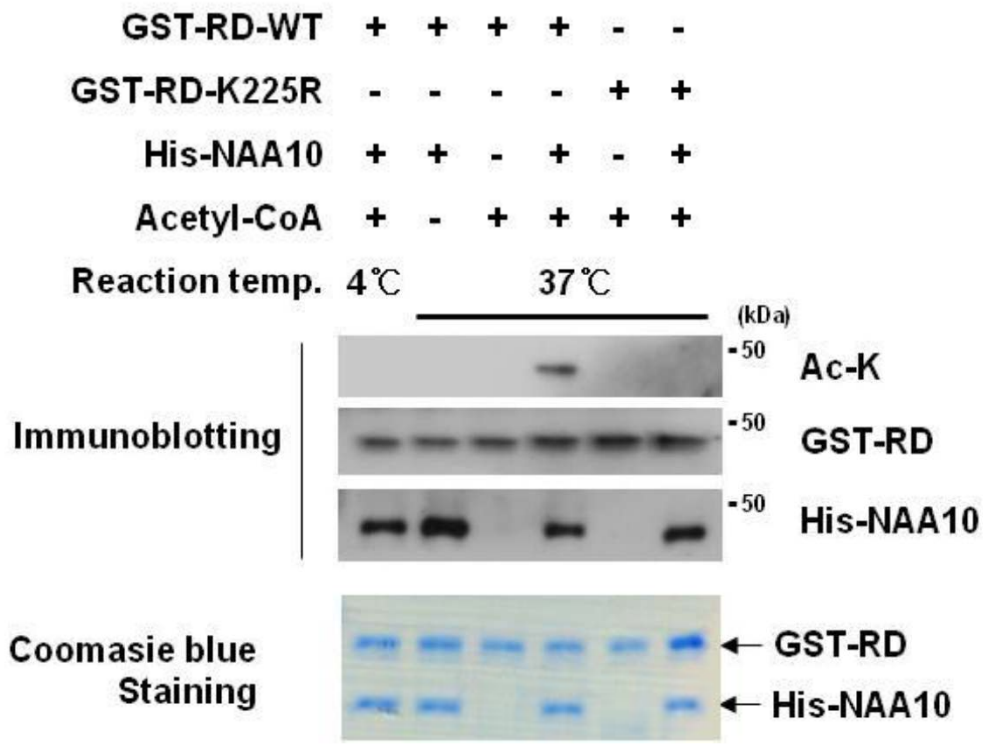

**Supplementary Figure 19. NAA10-dependent acetylation of Runx2 - uncropped scans of Figures 7b (a) and 7c (b).**



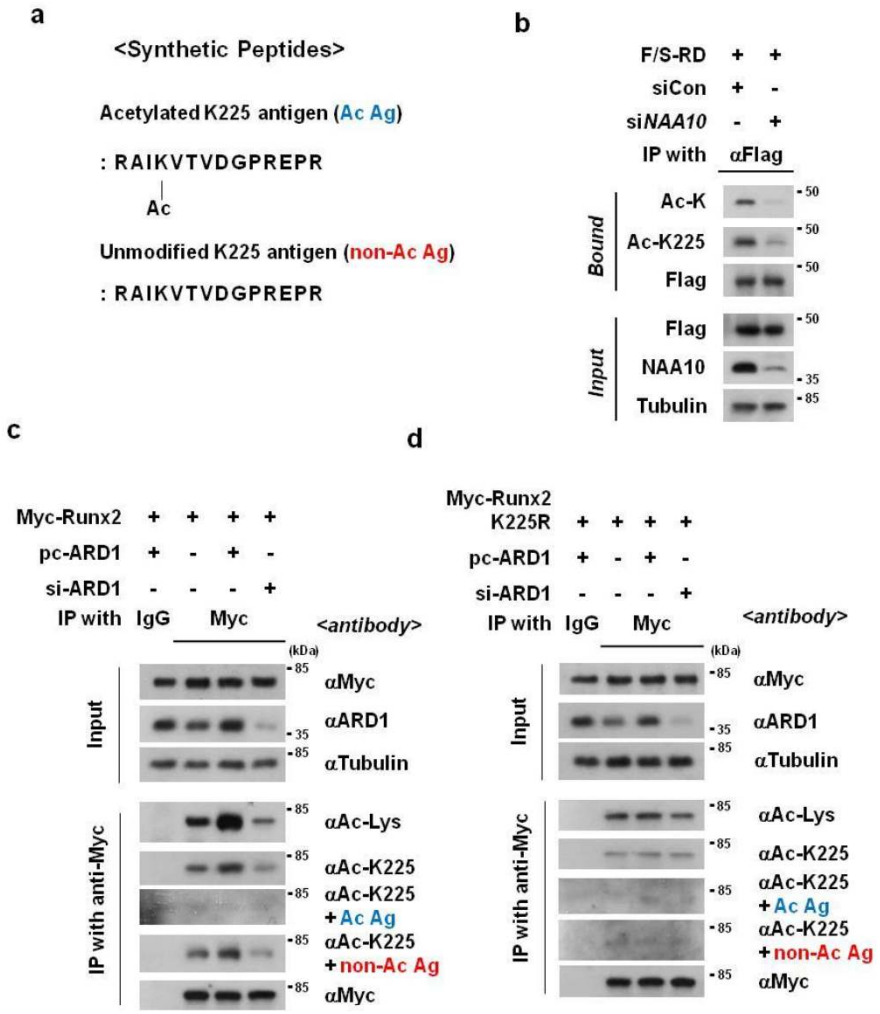

**Supplementary Figure 20. Runx2 is identified to be acetylated at K183 and K225.** HEK293T cells were co-transfected with Flag/SBP-RD and *NAA10* plasmid. Cells were lysed and Flag/SBP-RD peptide was isolated through two-step affinity chromatography using SA and Flag affinity beads. Isolated Flag/SBP-RD peptide was run on SDS-PAGE, and subjected to an in-gel trypsin digestion (**a**). The LC/MS spectra detecting K183 acetylation and K225 are shown in (**b**) and (**c**) panels, respectively. Acetylation is indicated by an additional mass of 42 Daltons.



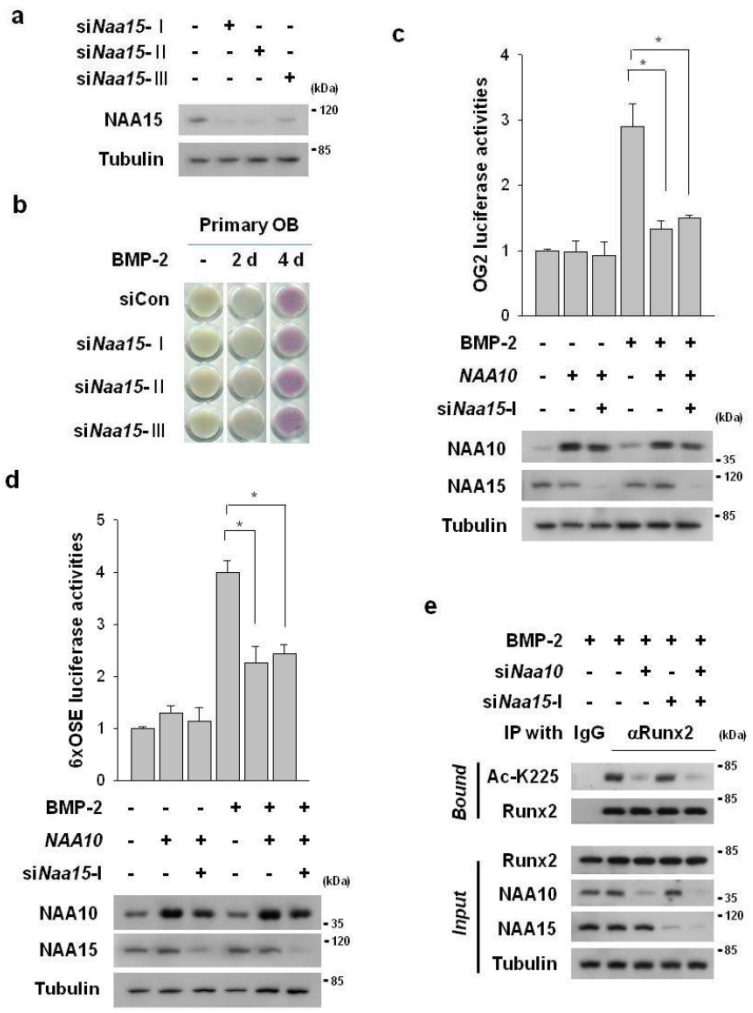

**Supplementary Figure 21. NAA10 acetylates mouse Runx2 at K225.** HEK293T cells were co-transfected with *NAA10* plasmid, Flag/SBP-RD or Flag/SBP-RD K225R. Flag/SBP-tagged peptides were purified using anti-Flag affinity beads, run on SDS-PAGE, and analyzed acetylation (**a**). The LC/MS spectra of WT and K225R-mutated peptides are shown in (**b**) and (**c**) panels, respectively.




**Supplementary Figure 22. NAA10 acetylates Runx2 at Lys 225 *in vitro*.** Recombinant His-NAA10 and GST-Runt (or its K225R mutant) peptides were incubated in the acetyl-CoA-containing reaction mixture for protein acetylation at 4$^{o}$C or 37$^{o}$C for 4 hours. The acetylation of GST-Runt peptides was identified using anti-acetyl-lysine antibody. The peptides on a gel were stained with Coommasie blue (bottom panel).




**Supplementary Figure 23. Establishment of an antiserum recognizing the acetylation of Runx2 at K225.** (**a**) The structures of synthetic peptides used as antigens to generate anti-Ac K225 antibody or as blocking peptides to verify antibody specificity. (**b**) The Flag/SBP-RUNT plasmid (F/S-RD) was co-transfected with control or *NAA10* siRNA into HEK293T cells. After treated with 1 μM trichostatin A for 6 h, cells were lysed and F/S-RD was pulled down using anti-Flag affinity beads. The lysyl-acetylation of F/S-RD was detected with anti-Ac-K antibody and the acetylation of F/S-RD at K225 was with anti-Ac-K225 antibody. HEK293T cells were transfected with Myc-Runx2 (**c**) or Myc-Runx2_K225R (**d**) plasmid, NAA10, or/and siRNAs. After cells were treated with trichostatin A, proteins in cell lysates were precipitated using anti-Myc affinity beads. Whole total and precipitated proteins were electrophoresed and immunoblotted with the indicated antibodies. To verify the specificity of the antibody against acetylated K225, acetylated or non-acetylated K225 peptide was pre-incubated with the antiserum for 1 h before immunoblotting.




**Supplementary Figure 24. NAA15 is not required for the NAA10 inhibition of Runx2 during BMP-2-induced osteoblasts differentiation.** Primary osteoblasts, which had been transfected with *Naa*15-targeting siRNAs, were treated with BMP-2 for 2 or 4 days. NAA15 knock-down and osteoblast differentiation were evaluated using Western blotting (**a**) and ALP staining (**b**), respectively. OG2-luciferase plasmid (**c**) or 6XOSE-luciferase plasmid (**d**) was co-transfected with β-gal plasmid, NAA10 plasmid, and/or Naa15 siRNA into primary osteoblasts. After being stabilized for 48 hours, cells were treated with PBS or BMP-2 for 24 hours. Cells were lysed for reporter assays (top panel) or for Western blotting (bottom panel). Luciferase activities (means + s.d., n = 4) were normalized versus β-gal activity, and are presented as relative values with respect to the vector controls. * denotes $P < 0.05$ (Student′s-t-test). (**e**) $C_2C_{12}$ cells were co-transfected with si*Naa10* or si*Naa15*, and treated with BMP-2 for 48 hours. Proteins in cell lysates were subjected to Western blotting using anti-Runx2 antibody or anti-Ac K225 antibody recognizing the acetylation of Runx2 at K225.