Sex-determining region Y (SRY) attributes to gender differences in RANKL expression and incidence of osteoporosis

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Abstract
Receptor activator of nuclear factor κB ligand (RANKL) plays a crucial role in bone metabolism. RANKL gene misregulation has been implicated in several bone and cancer diseases. Here, we aimed to identify novel transcription regulators of RANK expression. We discovered that transcription factors, sex-determining region Y (SRY) and c-Myb, regulate RANKL expression. We demonstrated that c-Myb increases and male-specific SRY decreases RANKL expression through direct binding to its 5’-proximal promoter. These results are corroborated by the gene expression in human bone samples. In osteoporotic men, expression of RANKL is 17-fold higher, which correlates with the drastically reduced expression (200-fold) of Sry, suggesting that in osteoporotic men, the upregulation of RANKL is caused by a decrease of Sry. In healthy men, the expression of RANKL is 20% higher than that in healthy women. Our data suggest that gender differences in RANKL expression and bone quality could be due to the sex-specific transcription factor SRY.

Introduction
Receptor activator of nuclear factor κB ligand (RANKL) is part of the RANKL/RANK/OPG system and is one of the most important molecules in bone remodeling. RANKL was identified as a key signaling molecule for the differentiation of bone macrophages into mature osteoclasts and for the activation of these cells, which leads to bone resorption. RANKL functions by binding to the RANK receptor on osteoclasts. RANKL belongs to the tumor necrosis factor (TNF) family of cytokines and was also named TNFSF11. RANKL is found as membrane-bound and soluble forms, the latter of which is produced by alternative splicing or by proteolytic cleavage of membrane-bound RANKL. Osteoprotegerin (OPG) is a RANKL decoy receptor that can bind RANKL and prevent its function. In addition to cells involved in bone formation (e.g., osteoblasts, their precursors, and osteocytes), there are other cells that express RANKL, such as T and B lymphocytes, mammary epithelial cells, vascular endothelial cells, synovial fibroblasts, chondrocytes, keratinocytes, malignant cells, cells in periodontal tissue, and other cell types. Studies have shown that RANKL is involved in numerous physiological processes, as well as bone metabolism, such as in T cell functions and lymph node and mammary gland development. In addition, RANKL is involved in pathological processes, such as the migration of tumor cells and the development of bone metastases in diseases, such as breast, lung and prostate cancer.

While the biological functions of RANKL have been extensively studied, much less is known about the regulation of the RANKL gene at the transcription level. RANKL is regulated by transcription factors that bind to distal and proximal regulatory regions. The major expression regulator of RANKL is parathyroid hormone (PTH), which acts on the 76,000-bp-distant cAMP-response elements (CREs) through the protein kinase A–cAMP pathway, which requires CREB. CREB is...
also required for 1,25(OH)2D3 and oncostatin M (OSM)-stimulated RANKL expression19. In addition to the binding of CREB, the same DNA region also contains a RUNX2 binding site. These binding sites and their importance were confirmed with electrophoretic mobility shift assays (EMSA) and ChIP and transgenic mouse models16,19,22. The same distant enhancer also contains 1,25(OH)2D3 response elements—the VDREs19,22,23. VDREs were also found at −96, −87, −75, −25, and −20 kb upstream of the transcription start site13–25. Other known RANKL transcription regulators acting through distal regulatory regions are interleukin (IL)-6-type cytokines26,27 molecules of the Wnt/β-catenin signaling pathway28–35 and c-Fos in activated T cells26. Previous studies have indicated that Vitamin D, PTH, and other transcriptional regulators could also regulate RANKL gene expression through its proximal promoter37,38. A recent study revealed the involvement of C/EBPβ in the transcriptional regulation of RANKL through binding to a CCAAT-box in the region −56/−51 upstream of the transcription start site of the RANKL gene39.

Overall, very little is known about the factors that regulate RANKL expression at the 5′-proximal region of the gene. The aim of the present study was therefore to identify other transcription factors that bind to this 5′-proximal region and regulate the expression of RANKL and to evaluate their in vivo significance. Two novel transcription factors were identified here: SRY and c-Myb. SRY is encoded by the sex determining region Y (Sry) gene that initiates testis development. SRY is implicated in sex determination and belongs to the SRY-box (Sox) gene family. SRY is not expressed in females, and this protein is responsible for the development of the male phenotype in therian mammals40. C-Myb is a transcription factor that has previously been associated with bone formation and chondrogenesis41–43. We hypothesized that SRY-mediated RANKL gene expression might be the molecular mechanism underlying the sex differences in bone metabolism and bone diseases, such as osteoporosis.

Materials and methods

Patients

Bone tissue samples from 112 Slovenian patients were included in this study44. Fifty-eight patients (46 female, 12 male) were undergoing hip arthroplasty because of low energy femoral neck fracture (osteoporosis; OP), and 42 patients (29 female, 13 male) had primary hip osteoarthritis (osteoarthritis; OA). Twelve bone tissue samples were collected from male autopsy subjects (control; CTL). All subjects included in the study signed written informed consent prior to inclusion. The study was approved by the Republic of Slovenia National Medical Ethics Committee (consent numbers: KME 91-93/08/10, KME 106/03/06, KME 137/03/06, KME 45/10/16, KME 91/08/10, KME 45/10/16) and is summarized in Supplementary Table 1. Detailed descriptions of the patient samples have been reported previously44. Briefly, the exclusion criteria for all of these patients included a history of systemic or metabolic diseases that impact bone or mineral metabolism or taking any drugs known to impact bone or mineral metabolism in the 12 months prior to the surgery. Patients with any diseases or drug use that might influence bone metabolism were excluded from the study.

Cell experiments

Human bone osteosarcoma (HOS) cells (ATCC CRL-1543) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Massachusetts, USA), 1% L-glutamine (Gibco, Thermo Fisher Scientific, Massachusetts, USA) and 1% antibiotic/antimycotic (Gibco, Thermo Fisher Scientific, Massachusetts, USA) at 37 °C under 5% CO2. Primary human osteoblasts (Promocell, Heidelberg, Germany) were grown in Osteoblast Growth Medium (Ready-to-use; Promocell, Heidelberg, Germany) at 37 °C under 5% CO2 and were subcultured according to the manufacturer’s instructions. Briefly, after thawing, the cells were grown until they reached 70 to 90% confluency, and then the cells were subcultured using DetachKit (Promocell, Heidelberg, Germany). The osteoblast growth medium was changed every 2 days to 3 days. All experiments were performed between the third and the fifth passages. The presence of osteoblast markers was confirmed by quantitative real-time polymerase chain reaction (q-PCR) (Supplementary Fig. 1).

Plasmid construction and site-specific mutagenesis

Ensembl ENST00000398795 (Human GRCh38.p2) was used as the reference sequence for the RANKL gene. Five different fragments of the human RANKL proximal promoter region were PCR amplified from the genomic DNA with a 5′-primer harboring a BglII site and a reverse primer complementary to the sequences near the transcription start site of the RANKL gene harboring a HindIII site. The primers used for PCR amplifications of the fragments are listed in Supplementary Table 1. The resulting PCR fragments were digested with BglII and HindIII and cloned into the pGL3 basic vector (Promega, Madison, USA). With PCR amplification, five fragments of the RANKL proximal promoter were obtained, with sizes of 898 bp (pGL3-F4), 762 bp (pGL3-F3), 538 bp (pGL3-F2), 346 bp (pGL3-F1), and 1769 bp (pGL3-F5). Mutants in the putative Sex-determining region Y (Sry) binding site (−732/−726 bp upstream of the RANKL transcription start, −731 AA > CC, pGL3-F4-SRY) and putative Myb proto-oncogene C (c-Myb) binding site (691/−674 bp upstream of the RANKL transcription start,
−680 TT > GG, pGkβ-F4-c-Myb) were prepared using a Quick-Change II Site-Directed Mutagenesis kit (Stratagene, San Diego, California, USA) and the primers listed in Supplementary Table 2.

The pCIneo-hc-Myb-HA expression vector was kindly donated by Prof. Odd Stokke Gabrielsen (Department of Biosciences, University of Oslo, Oslo, Norway). The pcDNA3-hSRY expression vector was constructed from the pcDNA3-FLAG-hSRY vector, which was kindly donated by Dr. Vincent Harley (Hudson Institute of Medical Research, Melbourne, Victoria, Australia), through the removal of the FLAG tag by PCR using the primers listed in Supplementary Table 2. The pEGFP-SRY plasmid was constructed by amplifying the Sry open reading frame from plasmid pcDNA3-FLAG-hSRY, kindly donated by Dr. Vincent Harley (Prince Henry’s Institute, Australia), using the primers listed in Supplementary Table 1 to add the restriction sites for ligation into the pEGFP-N1 plasmid (Clontech, CA, USA). All constructs were verified by DNA sequencing (GATC, Konstanz, Germany).

Western blotting

Human bone osteosarcoma cells were seeded onto 10-cm² dishes and transfected with empty pcDNA3 expression vector, Sry-FLAG expression vector, or c-Myb-HA expression vector. The cells were lysed after 24 h and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes, subsequently blocked in 5% milk/TTBS, and incubated overnight with the primary antibodies against FLAG (clone M2; Sigma-Aldrich, St. Louis, Missouri, USA) or HA (H3663; Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer’s instructions. HOS and HeLa cells were used for loading controls. Whole WB membranes are shown in Supplementary Fig. 2.

Overexpression of Sry-FLAG and c-Myb-HA in human primary osteoblasts

Male and female human primary osteoblasts were plated in 24-well tissue-culture plates (5 × 10⁴ cells per well), incubated overnight, and transfected with 500 ng pcDNA3, Sry-FLAG, or c-Myb-HA using Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA), according to the manufacturer’s instructions. RANKL expression was analyzed by q-PCR after 48 h, as described below. At least three biological replicates were analyzed for each gender.

Electroporation of human primary osteoblasts

Human primary osteoblasts were trypsinized with 0.05% Trypsin/EDTA, resuspended in fresh osteoblast growth medium (Promocell) and counted. Then, 1 × 10⁶ cells per nucleofection were transferred to fresh tubes and centrifuged at 100 × g for 5 min. After removing the supernatant, the cell pellets were resuspended in 100 µL Mouse Primary Neurons Nucleofector Solution (Amaxa Biosystems) per transfection, with 2 µg pEGFP-SRY added to the cell suspensions, which were transferred to Amaxa electrode cuvettes and electroporated in Amaxa Nucleofector Device II using program T-030. Immediately thereafter, the cells were diluted in 500 µL Osteoblast Growth Medium supplemented with 20% FBS and seeded onto glass coverslips in 12-well plates. The medium was replaced 2 h after nucleofection with 750 µL osteoblast growth medium supplemented with 10% FBS.

RNA interference knock-down of gene expression

siRNAs for silencing of Sry (sc-38443), c-Myb (sc-29855) and a negative control, nc (sc-37007), were from Santa Cruz Biotechnology. Here, 30-pmol siRNA (final concentration, 300 nM) was nucleofected into 1 × 10⁶ human primary osteoblasts, as described above. After 24 h and 48 h of nucleofection, the growth medium was changed. Then, at 72 h after nucleofection, the RNA was isolated using PeqGold RNA extraction kits (VWR), reverse transcribed, and subjected to quantitative PCR for the genes of interest. Three biological replicates were analyzed.

Luciferase reporter assay

Luciferase activity was measured with the dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, 3.5 × 10⁴ cells were plated per well in 24-well tissue culture plates. After 24 h, cells were transfected with X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany), with 500 ng DNA per well. In the case of human primary osteoblasts, 2 µg of pDNA was nucleofected using the Amaxa System as described above for silencing experiments. A plasmid containing the Renilla luciferase gene was used as an internal control. Luciferase activity was measured at 24 h after transfection (BIO-TEK Synergy HT, Fisher Scientific, Pittsburgh, PA, USA). All experiments were carried out at least three times independently.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using LightShift Chemiluminescent EMSA kits (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer’s instructions. HOS and HeLa nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer’s instructions. Double-stranded
Biotinylated oligonucleotides were prepared by hybridization in a C1000 thermal cycler by stepwise 0.5°C decreases in the temperature, ranging from 95°C to 35°C, over 120 cycles, each 30 s long. The binding reactions between oligonucleotides and SRY/c-Myb were performed in 20 μL reactions containing 2 μL 10× binding buffer, 1 μL 100 mM MgCl₂, 2 μL Poly(dI-dC), 2 μL 50% glycerol, 3 μL nuclear extract, and 20 fmol hybridized oligonucleotides (c-Myb) or 4 μmol hybridized oligonucleotides (SRY). Control reactions were performed without nuclear extracts. The specificity of the DNA-protein complexes was verified by dilution of the nuclear extracts and by competitive binding with nonbiotinylated DNA or mutated nonbiotinylated DNA. A primary rabbit polyclonal antibody against c-Myb (117635, Abcam, Cambridge, UK) was used to confirm the identity of the protein. For SRY, the mouse monoclonal anti-FLAG clone M2 was used (Sigma-Aldrich, St. Louis, Missouri, USA). The biotinylated oligonucleotides listed in Supplementary Table 1 were used in the EMSA experiments.

After the binding reactions, the samples were loaded onto electrophoretic gels and run for 1 h (100 V), then transferred to nylon membranes (30 min, 100 V), and cross-linked on a transilluminator. Biotin-labeled nuclear probes were detected with Chemiluminescent Nucleic Acid Detection Module kits (Pierce, Thermo Scientific, Wilmington, DE, USA) according to the manufacturer’s instructions.

**Immunofluorescence**

Cells were fixed with 3% paraformaldehyde (Sigma Aldrich, MO, USA) in phosphate-buffered saline (PBS) (Sigma Aldrich, MO, USA) at 37°C with 0.2 μg/mL Hoechst 33342 (Sigma Aldrich, MO, USA) for 20 min. The cells were permeabilized with 0.1% Triton X-100 (Sigma Aldrich, MO, USA) in PBS for 5 min, stained with the primary antibodies anti-c-Myb (ab117635, Abcam, Cambridge, UK) for 30 min, washed 3 times with PBS, stained with the secondary antibodies anti-rabbit CF568 (20102, Biotium, Fremont, CA, USA) for 30 min, washed 3 times with PBS, and mounted on glass slides with prolong anti-fade with DAPI (Thermo Fischer Scientific, MA, USA). Images were taken with a laser-scanning confocal microscope (710 META; Carl Zeiss Ltd, Germany) at 40× magnification using ZEN software (Carl Zeiss Ltd).

**Human bone tissue immunohistochemistry and immunofluorescence**

Bone samples were collected as described previously. Briefly, trabecular bone from the intertrochanteric region of patients undergoing hemi- or total hip arthroplasty due to low-energy osteoporotic fracture or primary osteoarthritis or post-mortem donors with no musculoskeletal disorders were fixed in neutral buffered formalin for up to 24 h and decalcified with 4% EDTA for 2 to 6 weeks. Wax sections were prepared following a routine procedure at the Institute of Pathology, University of Ljubljana (Slovenia). Sections were dewaxed and rehydrated, and antigen retrieval was performed using 0.01 M sodium citrate buffer (pH 6) for 45 min in a microwave. Immunohistochemistry was performed using mouse-specific and rabbit-specific HRP/DAB detection IHC kits (ab64264; Abcam, Cambridge, UK), according to the manufacturer’s procedure, and TBS with Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for washing. The samples were incubated overnight at 4°C in a humidified chamber with primary rabbit polyclonal antibodies against c-Myb and SRY (ab117635 and ab135239, respectively; Abcam, Cambridge, UK) diluted 1:100 in TBS buffer. Control slides were prepared for each sample using TBS buffer only. The tissue sections were counterstained with hematoxylin solution (Thermo Shandon, Pittsburgh, USA) and examined under a microscope (BX50; Olympus, Hamburg, Germany). The intensity of the staining in osteoblasts, lining cells and osteocytes was compared between all of the samples by two independent blinded evaluators. Images were acquired using a digital camera (Olympus XC50) and the CellSens Dimension program 1.6.0 (Olympus, Hamburg, Germany).

Immunofluorescence for colocalization studies was performed using the following primary antibodies: SRY ab135239 (1:100) and c-Myb ab226470 (1:500) (both Abcam, Cambridge, UK); CD3 D7A6E (1:50) (Cell Signaling Technology, Danvers, Massachusetts, USA); and CD19 sc-19650 (1:50) and CD3 sc-20047 (1:50) (both Santa Cruz Biotechnology, Dallas, Texas, USA). Primary antibodies were applied overnight. The secondary antibodies of goat anti-rabbit Alexa Fluor 633 and goat anti-mouse Alexa Fluor 488 (1:500) (both Thermo Fisher Scientific Waltham, Massachusetts, USA) were applied for 1 h. The slides were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific Waltham, Massachusetts, USA), and images were taken using a laser-scanning confocal microscope (710 META; Carl Zeiss Ltd, Germany) at 40× magnification using ZEN software (Carl Zeiss Ltd).

**Quantitative PCR**

The expression of RANKL was analyzed using qPCR with RNA samples obtained in gain-of-function and loss-of-function experiments. The RNA was extracted from cells, and the complementary DNAs (cDNAs) were synthesized, with gene expression analyses performed as described below. The expression of RANKL, Sry, and c-Myb was also analyzed using qPCR assays with the bone samples of Slovenian patients. RNA was extracted from the bone samples and the complementary DNA (cDNA)
synthesized according to our previously described procedure\textsuperscript{44,45}. Pairs of oligonucleotides for RANKL, RPLP0, GAPDH, and Sry were designed using the Primer Designing Tool (NCBI) (Supplementary Table 1), while for c-Myb, previously optimized oligonucleotides were used\textsuperscript{46}. For qPCR, \textit{5x} Hot FirePol EvaGreen qPCR Mix Plus (Solis, BioDyne, Tartu, Estonia) was used, following the manufacturer recommendations, on a LightCycler 480 (Roche Diagnostics, Rotkreuz, Switzerland). All samples were quantified in triplicate. Dilution series of cDNA were prepared to create a relative standard curve, and absolute quantification of the data was performed using the second derivative maximum method (LightCycler 480, software version 1.5; Roche Diagnostics, Rotkreuz, Switzerland). All data were normalized to the internal housekeeping gene of ribosomal protein, large, P0 (RPLP0) or glycer-aldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis and bioinformatics

The TF Search program was used to predict transcription factor binding to specific DNA regions. Matrices for vertebrates and threshold scores of more than 85 were used. The results for the luciferase activities were first transformed by calculating their z-scores and then normalized from 0 to 1 using unity scaling. The normalized results were then compared using ANOVA. Gene expression results were logarithmically transformed where needed to obtain normal distributions. The results were compared using Student’s t-tests or ANOVA.

Results

The region \(-662/-798\) bp in the \textit{RANKL} proximal promoter appears to contain binding sites for the transcription factors

In this study, we aimed to characterize the proximal promoter region of the \textit{RANKL} gene. First, to analyze the contributions of particular regions of the 5’-proximal promoter of the \textit{RANKL} gene to its activation, various lengths of the proximal promoter region were cloned in front of the luciferase reporter gene (pG13-Basic), and luciferase activity was measured in the HOS cells (Fig. 1a). These data showed that the F2 region \((-438\) bp to \(+100\) bp) has 20\% lower luciferase activity \((p = 0.048)\) compared to that of the F1 region \((-246\) bp to \(+100\) bp), and the F3 region \((-662\) bp to \(+100\) bp) has 57\% lower luciferase activity \((p = 0.001)\) compared to that of the F2 region. This result suggests that the region from \(-662\) to \(-246\) contains binding sites for transcriptional repressors. Surprisingly, the luciferase activity of the F4 region \((-798\) bp to \(+100\) bp) was increased by 22\% compared to that in the F3 region \((p = 0.03)\), which suggested that the region from \(-798\) to \(-662\) of the \textit{RANKL} gene contains binding sites for transcriptional activators.

Next, to identify potential transcription factors that bind to the \textit{RANKL} proximal promoter, the F4 region of the \textit{RANKL} promoter (Fig. 1a) was analyzed using the TF SEARCH program. SRY (possibly binding to position \(-726/-732\) bp upstream of the \textit{RANKL} transcription start; sequence AAACCTAA; score 94.5) and c-Myb (possibly binding to position \(-674/-691\) bp upstream of the \textit{RANKL} transcription start; sequence TTCTCGAG TGTTGGGTG; score 85.2) were identified as probable candidates. To examine whether SRY or c-Myb putative binding sites contribute to \textit{RANKL} promoter activity, the putative binding sites in the F4 region were mutated. At the putative SRY binding site, mutation \(-732/-730\) AA was created, and at the putative c-Myb binding site, mutation \(-674/-680\) TT was created (35–37). The results of the luciferase assays showed that the mutations introduced at the putative c-Myb \((-674/-674\) or SRY \((-732/-726)\) binding sites abolished the effects of region \(-798/-622\), as the promoter activity decreased to the activity of the F3 region (Fig. 1b), indicating that both SRY and c-Myb binding sites are important regulatory regions in the \textit{RANKL} proximal promoter.

SRY represses the activity of the \textit{RANKL} proximal promoter

Next, we examined whether SRY affects the activity of the \textit{RANKL} proximal promoter by measuring the luciferase activity of the F4 region of the \textit{RANKL} promoter in the presence of the overexpressed SRY protein (pcDNA3-hSry) in HOS cells. In the presence of SRY, the activity of the F4 region of the \textit{RANKL} proximal promoter decreased compared to that of the empty vector. However, mutation of the putative SRY binding site \((-731/-730\) did not alleviate the inhibitory action of SRY (Fig. 1d). In contrast, the luciferase activity of the mutated promoter region was lower than in the presence of the F4 \textit{RANKL} promoter region, which suggested that another site is important for SRY regulation of \textit{RANKL} promoter activity. We identified another possible SRY binding site, 5-[AT]AACAA[AT]-3, at position \(-1606/-1600\) in the proximal \textit{RANKL} promoter region.

Next, we examined whether SRY affects the activity of a longer \textit{RANKL} proximal promoter region by measuring the luciferase activity of the longer proximal \textit{RANKL} promoter region (F5 region, from \(-1669\) to \(+100\) in the presence of the overexpressed SRY protein (pcDNA3-hSry) in HOS cells. In the presence of SRY, the activity of the F5 region of the \textit{RANKL} proximal promoter decreased by 30\%, which suggested that this site is important for \textit{RANKL} promoter regulation (Fig. 1e). To verify that SRY affects the \textit{RANKL} promoter in human primary cells, human POB were also employed in the luciferase assay, resulting in the same effect (Fig. 1f), which suggests that SRY decreases \textit{RANKL} promoter activity, regardless of the
Fig. 1 c-Myb increases and SRY decreases the luciferase activity of the RANKL promoter region. a Different lengths of the RANKL proximal promoter region (F1, F2, F3, F4, F5) were cloned into the luciferase reporter vector pGL3-basic. Mutations were induced in the predicted SRY binding site (−731 AA > CC) and the predicted c-Myb binding site (−680 TT > GG) in the F4 vector using site-directed mutagenesis. b HOS (human bone osteosarcoma) cells were transfected with pGL3-F1/-F2/-F3/-F4/-F4-SRY/-F4-c-Myb and pRL normalization vector. Luciferase activities were measured 24 h after transfection. c Luciferase assays of HOS cells cotransfected with the pGL3-F4 RANKL promoter region and pcDNA3 (empty) or c-Myb-HA and cotransfected with the mutated pGL3-F4 RANKL promoter regions and c-Myb-HA. d Luciferase assays of HOS cells cotransfected with the pGL3-F4 RANKL promoter region and pcDNA3 (empty) or Sry-FLAG and cotransfected with the mutated pGL3-F4 RANKL promoter regions and Sry-FLAG. e Luciferase assays of HOS cells cotransfected with the pGL3-F5 RANKL promoter region and pcDNA3 (empty) or Sry-FLAG. f Luciferase assays of human primary osteoblasts nucleofected with pGL3-F5 RANKL promoter region and pcDNA3 (empty) or Sry-FLAG or c-Myb-HA. g Western blotting of human bone osteosarcoma cells after transfection with Sry-FLAG or c-Myb-HA expression vector and of empty human bone osteosarcoma cells. All of the data are presented as the mean luciferase activities after normalization ± SEM. Asterisks indicate significant differences between luciferase activities.
host cells, implying the role of SRY in the regulation of RANKL expression. Indeed, the importance of the −1606/−1600 SRY binding site was further confirmed by EMSA (Fig. 2) and silencing experiments (Fig. 3). Western blotting was used to confirm the presence of overexpressed SRY in HOS cells (Fig. 1g).

c-Myb increases the activity of the RANKL proximal promoter

After identification of the putative c-Myb binding site in the RANKL proximal promoter region, we investigated whether c-Myb impacts the activity of the RANKL promoter. We cotransfected HOS cells with the c-Myb expressing vector (pcDNA3-c-Myb) and the pGl3-F4 reporter vector. In the presence of c-Myb, the activity of the F4 RANKL promoter increased 4-fold compared to that of the empty vector. Mutation in the putative c-Myb binding site (−680/−679 TT > CC) partially decreased the RANKL promoter activity, which indicated that c-Myb activates the RANKL promoter (Fig. 1c, f). Western blotting was used to confirm the presence of overexpressed c-Myb in HOS cells (Fig. 1g).

c-Myb and SRY bind to the RANKL proximal promoter region

Electrophoretic mobility shift assays were used to examine whether the transcription factor c-Myb binds to the site −691/−674 bp upstream of the RANKL transcription start. Figure 2a shows the appearance of the shifted biotinylated oligonucleotides in the presence of nuclear extracts from HOS cells compared to the control without nuclear extract (Fig. 2a, lane 2, 1), which indicated that a protein from the nuclear extract binds to the oligonucleotides. The specificity of the binding was confirmed using competitive oligonucleotides without biotin in 200-fold molar excess (Fig. 2a, lane 3). The identification of c-Myb as the binding protein was confirmed using an antibody against c-Myb that prevented the interaction...
between protein and DNA (Fig. 2a, lane 4). The effects of binding site mutation were tested with the mutated competitive oligonucleotides in 200-fold excess. The shifted biotinylated oligonucleotides indicated that the mutated unlabeled competitive oligonucleotides did not bind the c-Myb protein, which confirmed that the site −691/−674 bp upstream of the RANKL transcription start is responsible for c-Myb binding (Fig. 2a, lane 5).

Electrophoretic mobility shift assays were also used to determine whether the transcription factor SRY binds to the site −1606/−1600 bp upstream of the RANKL transcription start site. Figure 2b shows the appearance of the shifted biotinylated oligonucleotides in the presence of the nuclear extracts from Sry-FLAG-transfected HOS cells compared to the control without the nuclear extract (Fig. 2b, lanes 2, 1), which indicated that a protein from the transfected nuclear extract binds to the oligonucleotides. The specificity of the binding was confirmed using nontransfected nuclear extracts (Fig. 2b, lane 3) and using competitive oligonucleotides without biotin in 200-fold molar excess (Fig. 2b, lane 4). The effects of binding site mutations were tested with mutated competitive oligonucleotides in 200-fold excess. The shifted biotinylated oligonucleotides indicated that the mutated unlabeled competitive oligonucleotides did not bind the Sry-FLAG protein, which confirmed that the site −1606/−1600 bp upstream of the RANKL transcription start is responsible for the SRY binding (Fig. 2b, lane 5). The identification of Sry-FLAG as the binding protein was confirmed using an antibody against FLAG, which caused a supershift (Fig. 2b, lane 6). Western blotting was used to confirm the presence of Sry-FLAG in the nuclear extract used for EMSA (Fig. 2c).

Our results demonstrate that both c-Myb and SRY directly bind to the RANKL proximal promoter region and could consequently directly impact RANKL gene expression. Overexpression of Sry in human female osteoblasts lowers the expression of RANKL.

As c-Myb increased and SRY decreased the activity of the RANKL promoter in our luciferase assays (Fig. 1) and as both of these transcription factors bind to the RANKL...
proximal promoter (EMSA; Fig. 2), we hypothesized that these transcription factors affect the expression of the RANKL gene in primary human osteoblasts. To test this hypothesis, Sry-FLAG and c-Myb-HA were transfected into male and female human primary osteoblasts, and at 48 h after transfection, RANKL expression was measured. As shown in Fig. 3a, overexpression of Sry decreased and overexpression of c-Myb increased expression of the RANKL gene in male human osteoblasts. In female human osteoblasts (Fig. 3b), overexpression of Sry decreased expression of the RANKL gene. These results indicated that SRY and c-Myb can regulate RANKL expression in human primary osteoblasts. To further confirm this hypothesis, RNA interference assays were performed using siRNAs against c-Myb or SRY in human primary osteoblasts. Figure 3c, d show the nonsignificant differences in expression of the RANKL gene upon silencing of c-Myb (Fig. 3c) and Sry (Fig. 3d). However, there was a trend of decreased expression of the RANKL gene upon silencing of c-Myb and increased expression of the RANKL gene upon silencing of Sry, which might suggest that SRY downregulates and c-Myb upregulates the expression of RANKL in human primary osteoblasts.

SRY and c-Myb localize in human primary osteoblasts
To examine whether both transcription factors localize to the nucleus of human primary osteoblasts, which is a prerequisite for their activity on the RANKL promoter, we examined the subcellular localization of SRY and cMyb in primary osteoblasts of male origin. We designed a fusion between SRY and green fluorescent protein (GFP) and overexpressed the fusion protein in male primary osteoblasts. The SRY-GFP localized to the nucleus and did not show any other localization phenotype (Fig. 4, Supplementary Fig. 3). The localization of endogenous cMyb was also examined by staining with an antibody. Endogenous cMyb localized to the nucleus; however, the intensity was low. c-Myb is an oncogene, and therefore high levels would not be expected in primary cells. Our data suggest that both the transcription factors SRY and c-Myb localize to the nucleus in human primary osteoblasts, which are prerequisites the activation/inhibition of the RANKL promoter.

SRY and c-Myb are present in diverse human bone cells
Since SRY and c-Myb impacted RANKL promoter activity and gene expression in human primary osteoblasts and HOS cells, we investigated whether these proteins are present in physiologically relevant human bone tissues where they could actually influence the bone phenotype. To this end, we studied the localization of c-Myb and SRY in human bone tissues after an osteoporotic fracture using immunohistochemistry (Fig. 5). Intensive c-Myb staining was observed in the nuclei of lining cells and in individual bone marrow cells, but very little staining was observed in osteocytes (Fig. 5a). In dormant bone, intensive SRY staining was observed in various cells of the bone marrow (Fig. 5b, blue arrow) and with less staining of the lining cells (Fig. 5b, black arrows), while osteocytes were mostly negative for SRY (Fig. 5b, red arrow), similar to the results observed for c-Myb. During the process of active bone remodeling, SRY was observed in bone marrow cells, in active osteoblasts (Fig. 5c, black arrows), and in the osteocytes located just beneath the trabecular bone surface (Fig. 5c, red arrows), which suggested a role for SRY in bone remodeling involving RANKL. No positive SRY staining was observed in female patients (Fig. 5d), which confirmed the specificity of the SRY antibody. Double staining of SRY and c-Myb using human tissue samples from male patients with osteoporotic fractures (Fig. 5e, f) and osteoarthritis (Fig. 5g) or no such disorder (i.e., controls, Fig. 5h) showed double-positive and single-positive cells. The SRY and c-Myb double-positive cells were observed in

![Fig. 4 Localization of SRY and cMyb in primary osteoblast cells of male origin. Localization of SRY-GFP fusion protein in male primary osteoblasts. Color legend for merged image: blue, Hoechst 33342 nuclear stain; green, SRY-GFP fusion protein; red, antibody targeting endogenous cMyb. POB primary osteoblasts; GFP green fluorescent protein. Scale bar: 10 μm](image-url)
Fig. 5 Immunolocalization of cMyb and SRY in human bone tissue. 

- Bone tissue of a male patient with osteoporotic fracture. 
  - cMyb staining is observed in the nuclei of lining cells, individual bone marrow cells, and osteocytes.
  - In inactive bone, SRY staining is observed in various cells of the bone marrow (blue arrow) and in the lining cells (black arrows), while osteocytes are negative for SRY (red arrow), similar to what was observed with cMyb.
  - In bone with active processes of bone remodeling, in addition to the cells in bone marrow, SRY was observed in active osteoblasts (closed arrows) and osteocytes (open arrows) just beneath the trabecular bone surface, which suggests its role in bone remodeling via RANKL.
  - No positive SRY staining was observed in female patients with osteoporotic fracture.

- Bone tissue of a male patient with osteoarthritis and in a male donor with no musculoskeletal disease show similar patterns of localization; i.e., SRY single-positive cells (arrowheads) and SRY and cMyb double-positive cells (arrows) in bone marrow (BM).

Immunohistochemistry (a-d) and double immunofluorescence (e-h) of human bone tissue. N = 3 donors per group (fracture, osteoarthritis, and control group). Scale bars: 50 µm.
osteoblast-like cells (Fig. 5e, arrows) on the surface of trabecular bone, while single-positive SRY or c-Myb cells (Fig. 5e, f, arrowheads) were present in bone marrow, and c-Myb single-positive cells were also present on the surface of trabecular bone (Fig. 5e).

Immunolocalization studies showed both SRY and c-Myb single-positive cells and the colocalization of these proteins with the T-lymphocyte marker CD3 in bone marrow cells (Fig. 6a, c, Supplementary Fig. 4A–D). Moreover, c-Myb colocalization with the B-lymphocyte marker CD19 was shown (Fig. 6b, Supplementary Fig. 4E–H). The results of SRY and CD19 colocalization, which required different CD19 antibodies, showed nonspecific staining of CD19 (data not shown). Bone tissue from a female patient with osteoporotic fracture showed negative staining for SRY and similar patterns of c-Myb staining as for the male osteoporotic fracture tissue (Fig. 6d). The gene expression of these markers in human bone tissue is shown in Fig. 7.

Osteoporotic males show significantly decreased expression of Sry and highly increased expression of RANKL

As male-specific transcription factor SRY decreased the activity of the RANKL promoter in vitro, we hypothesized that SRY downregulates RANKL expression in vivo and that lower RANKL expression will be observed in males. To quantify the expression of RANKL, Sry, and c-Myb in human bone samples, real-time PCR was used (Fig. 7). Indeed, the expression of RANKL was 20% lower in males than in females, which supported our hypothesis ($p = 0.037$; Fig. 7a). When comparing RANKL expression between osteoporotic, osteoarthritic, and healthy individuals, RANKL was significantly increased in both male and female osteoporotic patients. Indeed, in male osteoporotic patients, RANKL expression was 17-fold higher than that in healthy controls ($p = 0.001$; Fig. 7b) and 3-fold higher than that in osteoarthritic patients ($p = 0.002$;
**Fig. 7** (See legend on next page.)
Fig. 7b). In female osteoporotic patients, RANKL expression was almost twice that in osteoarthritic patients \( (p = 0.049; \text{Fig. 7c}) \). Interestingly, the expression of RANKL in male osteoporotic patients was 40% higher than that in female osteoporotic patients. These data indicate that the expression of RANKL is upregulated in male osteoporotic patients and that the expression of RANKL increases more in male than female osteoporotic patients, which associates the expression of RANKL with osteoporosis.

As our in vitro data suggested that male-specific transcription factor SRY represses the activity of the RANKL promoter, the expression of Sry was examined in osteoporotic patients and compared with healthy and osteoarthritic individuals. Sry expression was 210-fold lower in osteoporotic men compared to osteoarthritic men \( (p = 0.001) \) and 105-fold lower compared to healthy men \( (p = 0.001; \text{Fig. 7d}) \). These data demonstrate that Sry expression is significantly downregulated in osteoporotic patients. Together with the in vitro data, our results suggest that downregulation of Sry causes upregulation of RANKL and may consequently cause the development of osteoporosis in male patients. Moreover, our data suggest that SRY is one of the regulators of RANKL expression in males and might protect men from osteoporosis.

Given that our in vitro data indicate that RANKL promoter activity is controlled by the transcription factor c-Myb, we next examined the expression of c-Myb in osteoporotic and osteoarthritic patients and healthy individuals. The expression of c-Myb appeared to be lower in male osteoporotic patients compared to that in male osteoarthritic patients and healthy males; however, these data were not significant (Fig. 7e). The expression of c-Myb was half in osteoporotic than osteoarthritic females \( (p = 0.031; \text{Fig. 7f}) \). However, no significant differences in c-Myb expression were observed between male and female bone samples \( (p = 0.641; \text{Fig. 7g}) \). These data demonstrate that c-Myb is slightly downregulated in osteoporotic patients and that both female and male patients express similar levels of c-Myb, which indicates that the expression of c-Myb is not gender dependent.

Figure 7h shows the comparison of expression of Sry and c-Myb in male patients. Again, a drastic down-regulation of Sry was observed in osteoporotic males compared to osteoarthritic and healthy individuals. Intriguingly, in osteoporotic males, the expression of c-Myb also appeared lower than that in osteoarthritic patients and healthy individuals; however, these data were not significant. In healthy and osteoarthritic male subjects, the expression of Sry was 5-fold and 12-fold higher, respectively, than the expression of c-Myb. On the other hand, in osteoporotic patients, the expression of c-Myb was 3-fold higher than the expression of Sry. Altogether, these data reveal that in osteoporotic male patients, the expression of Sry was downregulated.

**Discussion**

RANKL is a protein that is involved in bone metabolism and modulates the activation and differentiation of osteoclasts. Its activation leads to increased bone resorption. In bone diseases, such as osteoporosis, RANKL expression is highly increased; therefore, RANKL represents a great target for osteoporosis treatment. Indeed, a monoclonal antibody against RANKL (denosumab) is currently used for osteoporosis treatment and prevention of skeletal-related events in patients with bone metastases. Playing a central role in bone remodeling, RANKL represents a good drug target for the development of novel cheaper approaches for the prevention of fragility fractures and osteoporosis. Thus, deciphering the regulation of RANKL gene expression is necessary. The expression of RANKL is regulated by transcription factors that bind to its distal and proximal promoter regions. Here, we aimed to identify transcription factors that bind to the RANKL proximal promoter region and can regulate its expression. We confirmed that the −662/−798 bp region in the RANKL proximal promoter contains binding sites for transcription factors, c-Myb and SRY. c-Myb is one of the oncogenes previously shown to be involved in bone formation and chondrogenesis. SRY is a sex-specific transcription factor, expressed only in men and responsible for sex determination. We demonstrated that c-Myb increased and SRY decreased the activity of the RANKL promoter (Fig. 1). Using EMSA, we confirmed that c-Myb directly binds to the −691/−674 bp site (Fig. 2a) and showed that SRY binds to the −1606/−1600 bp region (Fig. 2b) of the RANKL promoter. When SRY was
overexpressed in female human primary osteoblasts, the expression of RANKL was significantly reduced, which indicates that SRY decreases RANKL expression in human primary osteoblasts (Fig. 3a). Gene expression in the human bone samples revealed that in women bone tissues RANKL expression is 20% higher compared to that in men bone tissues, which suggested that higher expression of RANKL accounts for lower bone mineral density in women. This finding is in agreement with observations that men have higher bone mineral density and suffer from osteoporosis less frequently than women. Importantly, in our osteoporotic patients, the expression of RANKL was highly upregulated and dependent on gender. In osteoporotic men, the expression of RANKL was significantly increased (17-fold). We demonstrated that the expression levels of RANKL were highly inversely correlated with the expression levels of Sry, which implies that the gender differences in the RANKL expression levels might be due to the sex-specific transcription factor SRY. Moreover, the drastic downregulation of Sry in male osteoporotic patients together with the upregulation of RANKL suggests that lower Sry expression might cause upregulation of RANKL and cause the development of osteoporosis in males. The mechanism behind the downregulation of Sry in men, leading to the upregulation of RANKL and osteoporosis, remains unknown. This study is the first to identify the gender-specific transcription factor SRY as a regulator of RANKL expression by directly binding to the RANKL promoter region. Our results indicate that SRY could be one of the reasons for gender differences in bone mineral density and for gender differences in the pathogenesis of osteoporosis. Previous studies have shown differences in the microarchitecture between osteoporotic and osteoarthritic bone could now be explained by SRY. Strong correlations between the reduced levels of Sry and incidence of osteoporosis in male osteoporotic patients suggest that SRY can serve as a biomarker for male osteoporosis. Moreover, our results provide a novel target for the development of new therapeutic approaches for the prevention of bone loss in men and women. Namely, by the stimulation of SRY-mediated downregulation of RANKL expression or by application of SRY analogs, the expression of RANKL could be lowered, and consequently, bone resorption prevented.

One of the major gender differences that contributes to bone phenotype and is dependent on sex is hormones. The role of hormones in bone remodeling has been well established. Indeed, the decline of estrogens in postmenopausal women is associated with a decrease in bone mineral density, which indicates that lower estrogen levels influence bone mineral density. Estrogen protection of bone mass is mediated via estrogen receptor α. However, this mechanism is not limited to females. Estrogen metabolism has also been demonstrated to influence bone density in males, which indicates that estrogen is a regulator of bone remodeling both in males and females (reviewed in). On the other hand, androgen hormones are required for the maintenance of trabecular bone in males, and their role has also been implicated in female bone remodeling. Notably, RANKL-mediated increases in bone resorption have already been associated with sex hormones. Although bone mass, size and quality differ between genders, no gender-distinctive transcriptional regulation mechanism that affects bone remodeling has been described to date. Given the important contribution of hormones to bone remodeling in our male patients, changes in estrogen or androgen levels might have contributed to the onset of osteoporosis. However, this explanation is less probable as patients with abnormal levels of hormones were excluded from the study. Here, we unveil the first gender-related transcriptional mechanism that distinctly regulates bone remodeling in males.

The role of SRY in bone biology is plausible because the Sry gene is part of the SOX gene family with an HMG box DNA binding domain involved in bone development and turnover. Mutations of Sry cause XY gonadal dysgenesis (Swyer syndrome), in which a genetically male fetus fails to develop testes, which consequently leads to the development of female genitalia with a defective reproductive system. Hormonal replacement therapy is necessary to assure a normal menstrual cycle and secondary sex characteristics and to reduce the risks of osteopenia, which suggests the importance of SRY in normal bone development and hormonal status. The tissue widespread expression of Sry in adult men has also been observed, which indicates functions of SRY other than the development of testes. Importantly, RANKL upregulation and c-Myb downregulation were also observed in osteoporotic women (Fig. 7c, f), which suggested that in females, another transcription factor might have a similar role as SRY in males. Further studies are required to determine the transcription factors that regulate RANKL expression in women. We speculate that other members of the SOX family might be involved in c-Myb-dependent regulation of RANKL in women. Altogether, this is the first study that demonstrates the importance of SRY in the regulation of RANKL expression in males.

Here, we show that c-Myb activates the RANKL promoter by direct binding and causes upregulation of RANKL expression in primary osteoblasts. Moreover, we demonstrate that c-Myb is expressed in human bone tissues by immunohistochemistry and Q-PCR, suggesting the importance of c-Myb in bone biology. Indeed, it has already been shown that c-Myb is involved in bone biology and, more specifically, in novel bone formation and chondrogenesis. In addition to the upregulation of RANKL in osteoporosis, dysregulation of RANKL has also been
associated with tumorigenesis\textsuperscript{66} and metastases\textsuperscript{67} in several cancers. The importance of RANKL in tumorigenesis has been underpinned by clinical trials where denosumab decreased the risk of metastasis development\textsuperscript{68–70}. c-Myb has recently been associated with the Wnt/β-catenin pathway as a mediator of metastasis in breast cancer\textsuperscript{71}, and has been shown to promote migration and invasion of mammmary tumor cells through regulation of cathepsin D and matrix metalloproteinase 9\textsuperscript{72}. Our data demonstrate that c-Myb overexpression activates the RANKL promoter in vitro and might be the reason for the elevated levels of RANKL in bone cancer diseases.

In conclusion, we identified two novel transcription factors, SRY and c-Myb, that regulate the expression of RANKL in human primary osteoblasts and that might influence the expression of RANKL in vivo. Importantly, we unveiled a novel gender-specific transcriptional mechanism that contributes to the regulation of RANKL in a sex-specific manner and that might contribute to gender differences in bone phenotypes.

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Author contributions

Cloning site, specific mutagenesis, plasmid preparation, sequencing, and luciferase assays: VM, KK, MNL; immunohistochemistry and immunolocalization: JZ, VM, TK; EMSA: VM, KK, MNL; sample collection and RNA extraction: JZ, RK, JM; real-time PCR: JZ, KK, VM; statistical analysis: JZ, VM, KK, NML; study design and conceiving the research: VM, MNL, JM; paper preparation: VM, KK, MNL, JZ, JM, RK; critical review: VM, KK, MNL, JZ, JM, RK

Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. NCBI TNFSF11 tumor necrosis factor superfamily member 11 (Homo sapiens (human)). Gene ID: 8600 (2016).
2. Hikita, A. et al. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. J. Biol. Chem. 281, 36846–36855 (2006).
3. Ikeda, T., Kaca, M., Ushiyama, M. & Horigawa, K. Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus. Endocrinology 142, 1419–1426 (2001).
4. Nagai, M., Kyakumoto, S. & Sato, N. Cancer cells responsible for humoral hypercalcemia express miRNA encoding a secreted form of ODF/TRANCE that induces osteoclast formation. Biochem. Biophys. Res. Commun. 269, 532–536 (2000).
5. Kong, Y.-Y. et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397, 315–319 (1999).
6. Lacey, D. L. et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93, 165–176 (1998).
7. O’Brien, C. A. Control of RANKL gene expression. Bone 46, 911–919 (2010).
8. Wong, B. R. et al. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J. Biol. Chem. 272, 25190–25194 (1997).
9. Dougall, W. C. et al. RANK is essential for osteoclast and lymph node development. Genes Dev. 13, 2412–2424 (1999).
10. Fata, J. E. et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell 103, 41–50 (2000).
11. Zhang, J. et al. Osteoprotegerin ligand inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J. Clin. Invest. 107, 1235–1244 (2001).
12. Brown, J. M. et al. Osteoprotegerin and rank ligand expression in prostate cancer. Urology 51, 611–616 (2001).
13. Huang, L., Cheng, Y. Y., Chow, L. T., Zheng, M. H. & Kamata, S. M. Tumor cells produce receptor activator of NF-kappaB ligand (RANKL) in skeletal metastases. J. Clin. Pathol. 55, 877–878 (2002).
14. Farrugia, A. N. et al. Receptor activator of nuclear factor-kappaB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. Cancer Res. 63, 5438–5445 (2003).
15. Jones, D. H. et al. Regulation of cancer cell migration and bone metastasis by RANKL. Naturo 440, 692–696 (2006).
16. Ducy, P. & Karsenty, G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol. Cell Biol. 15, 1858–1869 (1995).
17. Fan, X. et al. Regulation of RANKL promoter activity is associated with histone remodeling in murine bone stromal cells. J. Cell Biochem 93, 807–818 (2004).
18. Kabé, Y. et al. NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes. Mol. Cell Biol. 25, 512–522 (2005).
19. Fu, Q., Manolagas, S. C. & O’Brien, C. A. Parathyroid hormone controls receptor activator of NF-kappaB ligand gene expression via a distant transcriptional enhancer. Mol. Cell Biol. 26, 6453–6468 (2006).
20. Kondo, H., Guo, J. & Brighenti, F. R. Cyclic adenosine monophosphate/protein kinase A mediates parathyroid hormone/parathyroid hormone-related protein receptor regulation of osteoclastogenesis and expression of RANKL and osteoprotegerin mRNAs by marrow stromal cells. J. Bone Miner. Res. 17, 1667–1679 (2002).
21. Oral, M., St. John, H. C., Danielson, A. L. & Pike, J. W. Deletion of the Distal Tnf11 Rl20 Enhancer That Contributes to PTH-Mediated RANKL Expression in Osteoblast Lineage Cells Results in a High Bone Mass Phenotype in Mice. J. Bone Miner. Res. 31, 416–429 (2016).
22. Gall, C. et al. Targeted deletion of a distant transcriptional enhancer of the receptor activator of nuclear factor-kappaB ligand gene reduces bone remodeling and increases bone mass. Endocrinology 149, 146–153 (2008).
23. Kim, S., Yamazaki, M., Zella, L. A., Shevde, N. K. & Pike, J. W. Activation of Receptor Activator of NF-kB Ligand Gene Expression by 1,25-Dihydroxyvitamin D(3) Is Mediated through Multiple Long-Range Enhancers. Mol. Cell 26, 6469–6486 (2006).
24. Nerenz, R. D., Martowicz, M. L., Pike, J. W. An enhancer 20 kilobases upstream of the human receptor activator of nuclear factor-kappaB ligand gene mediates dominant activation by 1,25-dihydroxyvitamin D3. Mol. Endocrinol. 22, 1044–1056 (2008).
25. Martowicz, M. L., Meyer, M. B. & Pike, J. W. The mouse RANKL gene locus is defined by a broad pattern of histone H4 acetylation and regulated through distinct distal enhancers. J. Cell Biochem. 112, 2030–2045 (2011).
26. O’Brien, C. A., Gubrij, I., Lin, S. C., Sayiors, R. L. & Manolagas, S. C. STAT3 activation in stromal/osteoblastic cells is required for induction of the receptor activator of NF-kappaB ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D3 or parathyroid hormone. J. Biol. Chem. 274, 19301–19308 (1999).
