

Full Length Article

Annexin A3 accelerates osteoclast differentiation by promoting the level of RANK and TRAF6

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ABSTRACT

Annexin A3 (ANXA3), a member of Annexin family, is reported to mediate membrane transport and cancer development. However, the effect of ANXA3 on osteoclast formation and bone metabolism is still unclear. In this study, we found that knockdown of ANXA3 can significantly inhibit receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast formation through NF- κ B signaling. ANXA3 downregulation abrogated the expression of osteoclast-specific genes, including *Acp5*, *Mmp9* and *Ctsk* in osteoclast precursors. Moreover, lentiviral of shRNA against ANXA3 reversed the bone loss in osteoporosis using ovariectomized mice model. Mechanistically, we found that ANXA3 directly bound to RANK and TRAF6 to accelerate osteoclast differentiation by promoting their transcription and limiting degradation. In conclusion, we propose a fundamentally novel RANK-ANXA3-TRAF6 complex to effectively modulate the formation and differentiation of osteoclast to manipulate bone metabolism. The ANXA3-targeted therapeutic strategy may provide new insight for bone degrading-related diseases prevention and treatment.

1. Introduction

Osteoclasts, which are derived from hematopoietic stem cells, are bone-resorbing multinucleated giant cells. It controls the homeostasis of the skeleton together with osteoblasts [1,2]. Perturbation of this balance causes various skeletal disorders, such as osteoporosis and osteopetrosis [3]. Osteoclastogenesis is initiated by the binding of receptor activator of nuclear factor- κ B ligand (RANKL), which is expressed by osteoblasts and stromal cells within the marrow, to its receptor RANK in osteoclast precursors [4]. Subsequently, TNF receptor-associated factors (TRAFs), especially TRAF6, can be directly recruited into RANK cytoplasmic domains, and activates downstream NF- κ B signaling [5,6]. Activated signals trigger the expression of osteoclast-specific genes comprising tartrate-resistant acid phosphatase (*Acp5*), matrix metalloproteinase 9 (*Mmp9*) and Cathepsin K (*Ctsk*), which affect the fusion of osteoclast

precursors and the resorption of bone matrix [7,8].

Annexin A3 (ANXA3) is considered to belong to Annexin family that is expressed almost exclusively in myeloid cell lineage [9]. The protein contains four conserved domains of about 70 amino acids and plays a crucial role in membrane transport and calmodulin-dependent activities. Notably, ANXA3 has a wide cytoplasmic distribution, while translocate to the membranes in activated neutrophils and monocytes [10]. The protein is well known to lengthen cytoplasmic calcium oscillation by extracellular calcium through calcium and/or magnesium channels in the HL-60 macrophage-like cells [11]. Accumulated evidence implicate ANXA3 regulates multiple types of tumor cell migration, proliferation, invasion and angiogenesis [12–14]. Moreover, ANXA3 is also suggested to be involved in early blood vessel formation, adipocyte differentiation and myocardial cell repair [15,16]. Those findings indicate that ANXA3 has a wide range of physiological and

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pathological functions.

Our previous study revealed that mechanical force regulated the periodontal ligament stem cell derived exosome protein profiles, including the ANXA3 protein, which were capable of inducing and promoting osteoclastogenesis [17]. Because exosomes are multi-subunit protein complexes, the role and mechanism of the ANXA3 protein in osteoclast differentiation need to be further assessed. Herein we found that ANXA3 positively controlled osteoclast differentiation by activating NF- κ B signaling pathway. Moreover, ANXA3 shRNA could attenuate the bone loss in osteoporosis. In addition, we provided the first conclusive evidence that ANXA3 was associated with RANK and TRAF6 in osteoclast precursors. Inhibition of ANXA3 substantially suppressed RANK and TRAF6 expression and dampened the RANK-ANXA3-TRAF6 association. Thus, our data demonstrated a unique perspective on ANXA3 in bone homeostasis and shed light new therapeutic target for osteoporosis.

2. Materials and methods

2.1. Reagents

Anti-TRAF6 (A16991), anti-Myc-Tag (AE070), anti-RANK (A13382), anti-ANXA3 (A8763) and anti-PYK2 (A16045) antibodies were purchased from Abclonal (Wuhan, China). Anti-phospho-P65 (3033), anti-P65 (8242), anti-TRAF6 (8028), anti-GAPDH (5174), Goat anti-Mouse IgG-HRP (7076), Goat anti-Rabbit IgG-HRP (7074) antibodies were purchased from Cell Signal Technology (Beverly, MA, USA). Anti-ANXA2 (sc-28385), anti-ANXA3 (sc-390502) and anti-ANXA5 (sc-74438) antibody were purchased from Santa Cruz (Dallas, Texas, USA). Anti-RANK (ab13918) and anti-GST (ab19256) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-Flag and anti-HA antibodies were purchased from MBL (Beijing, China). RANKL (315–11) and M-CSF (315–02) were purchased from PeproTech (Rocky Hill, NJ, USA). Cycloheximide (CHX), MG132, Bafilomycin A1 (BAF) and PKH26 were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRITC Phalloidin was purchased from YEASEN (Shanghai, China).

2.2. Plasmids

pCMV6-ANXA3-Flag-Myc (MR216256) plasmid was purchased from OriGene (Rockville, MD, USA). pCMV3-TRAF6 (MG57197-UT) and pCMV3-RANK-HA (MG50490-CY) plasmids were purchased from Sino Biological (Beijing, China). The pCMV6-ANXA3-Myc, pCMV3-TRAF6-Flag and all truncations were constructed using Gibson assembly technology [18]. The truncations of ANXA3 were listed as follows: M1 (amino acids 1–161), M2 (amino acids 162–323).

2.3. Lentiviral particle production

The lentivirus-based plasmid that expressing shRNA to stably knockdown the mouse *Anxa3* gene was designed by our lab. The lentiviral construct plasmid (shANXA3), envelope plasmid (pMD2.G) and packaging plasmid (psPAX2) were co-transfected into 293 T cells to get lentiviral particles. Negative control (shNC) was transfected with pMD2.G and psPAX2. The lentiviral particles were concentrated for animal experiment. The effectively gRNA sequence of ANXA3 was listed as follow:

CCGGCCGAGCTGAACTAACATTTCTCGA-GAAATGTTAGTTTCAGCTGCGGTTTTTG.

2.4. Animals

Female C57BL/6J mice at age 10–12 weeks were purchased from laboratory animal resource center of Chinese Institute for Brain Research, Beijing. The mice were raised in a specific pathogen-free conditions with temperature, humidity, a 12 h light / 12 h dark cycle. The mice were randomly distributed into four groups of five animals in

each group, as follows: SHAM group, ovariectomized (OVX) group, OVX-shNC group, OVX-shANXA3 group. For OVX group, mice were anesthetized with 2.5 % avertin and the oviducts were ligated and the ovaries were removed. In the SHAM group, muscles between ovaries were separated without ovaries isolated. To prevent infection, penicillin was injected for 3 days after operation. After the operation, mice were raised for six weeks. The shRNA groups were injected with 5 μ L (1×10^8 TU/mL) lentivirus at the fifth lumbar vertebra, while the SHAM and the OVX group were injected with an equal volume of sterile saline. Mice were sacrificed after four weeks for further experiments. The study was authorized by the Laboratory Animal Welfare and Ethics Committee of Chinese Institute for Brain Research, Beijing (CIBR-IACUC-051) and the Animal Care and Use Committee of the Health Science Center, Peking University (No. LA2021488).

2.5. Cell culture

RAW264.7 cells were purchased from the National Infrastructure of Cell Line Resource (Beijing, China) and cultured in DMEM medium containing 10 % FBS and 1 % antibiotics under 5 % carbon dioxide. Bone marrow macrophages (BMMs) were extracted from male C57BL/6 mice about 10–12 weeks of age. Briefly, the bone marrow were irrigated using serum-free DMEM medium. Single bone marrow cells after lysing erythrocytes were sifted using 100 μ m strainer and cultured overnight in complete culture medium. Then the suspension of un-adherent cells was absorbed and added to M-CSF (30 ng/mL) for 3 days to obtain BMMs for further experiments.

2.6. Gene silencing

BMMs (4×10^5) and RAW264.7 (3×10^5) cells were seeded in 6-well plates. The next day, after the cells were attached, the corresponding siRNA was transfected using the Ribo FECTTM CP Transfections Kit (RIBOBIC, Guangzhou, China) according to the instructions. Mouse *Anxa3* siRNA and negative control siRNA were generated by RIBOBIC. The effectively siRNA mix contains two target sequences were listed as follows: *Anxa3* siRNA.

2# ACAGAACCGTGCTCTTGAA,

3# GCTGGAACAGATGAATTCA.

2.7. TRAP staining

For cells, BMMs (3.5×10^4) and RAW264.7 (3×10^4) cells were seeded in 24-well plates. The next day, after cell adherence, *Anxa3* gene was silencing or overexpression. After 6 h, 50 ng/mL RANKL were added into the medium. TRAP staining was then performed after 5 days using TRAP staining Kit (WAKO, Osaka, Japan). The number of TRAP-positive multinucleated cells (more than three nuclei) were counted as osteoclasts.

2.8. F-actin staining

RAW264.7 cells (1.5×10^4) or BMMs cells (2.5×10^4) under logarithmic growth were inoculated in circle microscope cover glass which placed at the bottom of the 24-well plate. The next day, after cell adherence, *Anxa3* gene was silencing. After 6 h, 50 ng/mL RANKL were added into the medium. After 7 days of cultivation, the cells were fixed and stained with rhodamine-conjugated phalloidin as previously described [19]. The staining of F-actin belts and nucleus were observed by OLYMPUS IX73 inverted microscope (Tokyo, Japan).

2.9. Quantitative real-time PCR (qPCR)

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNAs (cDNAs) were synthesized using the PrimeScript RT reagent Kit (Takara, Shiga, Japan). The

expression of indicated genes were detected by using a Real-Time System (ABI Quant Studio 3). The primers were listed in Supplementary table 1.

2.10. Western blot and co-immunoprecipitation

The cell lysates were harvested and subjected to western blot analysis using primary antibodies as described earlier [19]. For co-immunoprecipitation (Co-IP) assay, Cells were collected and lysed in cell lysis buffer (Cell Signal Technology, 9803). The indicated antibodies were added to cell lysates overnight at 4 °C with gentle agitation, followed by the incubation of protein A/G plus Agarose (Santa Cruz Biotechnology, sc-2003) for 2 h at 4 °C. The immunoprecipitates were collected by centrifugation at 3000g for 5 min. After washed with wash buffer for 3 times, SDS loading buffer was added and then heated at 100 °C for 10 min. The proteins were then subjected to western blot.

2.11. GST-pulldown

GST-pull-down Kit (IK-2004) was purchased from Bio-Linkedin (Shanghai, China). RANK-GST Fusion Protein, TRAF6-GST Fusion Protein and GST protein were purchased from ProteinTech (Wuhan, China). Recombinant ANXA3-His protein was purchased from Solarbio (Beijing, China). The general process was as follows: 5 µg TRAF6/RANK-GST Fusion Protein and ANXA3-His protein were mixed with pre-washed GST-labeled protein agar-beads (GST Protein was used in control group) and supplemented with GST-pull-down buffer to total volume of 500 µL. PMSF was added proportioned at the same time. The mixture was rotated and incubated overnight at 4 °C. The magnetic rack was used to collect magnetic beads, then added 500 µL GST-pull-down buffer to wash beads 3 times. Magnetic beads were collected by magnetic rack and then added 30 µL SDS loading buffer, heated at 100 °C for 10 min. The proteins were then subjected to western blot.

2.12. Immunofluorescence staining

The RAW264.7 cells (2×10^4) were seeded in glass bottom cell culture dishes. Cells cultured overnight and were fixed with 4 % paraformaldehyde for 20 min at room temperature. Then 0.1 % Triton X-100 was added to infiltrated the cells at room temperature for 5 min. After washing with PBS for two times, cells were blocked with 5 % goat serum and incubated overnight in indicated diluted primary antibodies at 4 °C. Subsequently, cells were treated with fluorescence-labeled secondary antibodies for 1 h at room temperature. Finally, the cells were mounted with anti-fade reagent containing DAPI for 5 min. The localization of proteins was visualized by Zeiss LSM880 Confocal Microscope (Oberkochen, Germany).

2.13. Hematoxylin and eosin (HE) and Alkaline phosphatase (ALP) staining

HE staining was conducted according to routine protocols. Briefly, 10 µm sections were stained with hematoxylin solution for 3min and rinsed in deion water. Then the sections were stained with eosin solution for 40s. ALP staining was performed using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) in accordance with the manufacturer's instructions. Briefly, development buffer was mixed with BCIP and NBT solution and then the sections stained for 1 h.

2.14. Micro-computed tomography (micro-CT)

Micro-CT scanning sites comprised the fifth lumbar vertebra of 5 mice per group using a high-resolution Micro-CT (NMC-200, PINGSENG Healthcare (Kunshan) Inc., China). The scanner was set at a voltage of 90kV and a resolution of 15µm per pixel. The 3D images were reconstructed and analyzed using Avatar software (PINGSENG). The region of

interest (ROI) contained 0.5 mm thick vertebra body close to vertebral foramen excluding the cortical bone. All trabecular measurements were made by manually drawing contours every 5 slices. The main parameters of cancellous bone analysis included bone volume (BV)/tissue volume (TV), trabecular number (Tb.N), bone surface (BS)/bone volume (BV) and trabecular separation (Tb.Sp).

2.15. Isolation, labeling and internalization of exosomes

The periodontal ligament stem cells were transfected with ANXA3 overexpression plasmid and cultured in α -MEM with exosome-free serum (centrifuged at 100,000 g for 10 h) for 48 h. The exosomes were isolated from culture supernatant through ultracentrifugation as previously described [20]. Exosome labeling assay was performed using PKH26 according to the manufacturer's instructions. ANXA3-overexpressed exosomes (50 µg/mL) were added in the medium for further experiments.

2.16. Statistical analysis

All experiments were repeated three times independently and the data were analyzed using SPSS 17.0 (IBM Corporation, Somers, NY, USA) and presented as means $\pm$ SD. Student's *t*-test was used for the comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among more than two groups followed by the LSD test (assuming equal variances) or Tamhane's *T2 post hoc* test (without the assumption of equal variances). *P* < 0.05 was identified as statistically significant.

3. Results

3.1. ANXA3 is a positive regulator of RANKL-induced osteoclast differentiation

Our previous study showed that ANXA3 in exosomes took participated in the osteoclast differentiation [17]. In order to analyze the regulatory role of ANXA3 in osteoclast formation, we knockdown ANXA3 using specific small interfering RNA (siRNA) in osteoclast precursors (Fig. S1A-C), and the results showed that osteoclast formation induced by RANKL was significantly decreased after Anxa3 mix siRNA treatment (Fig. 1A, B), assessed by TRAP staining. Moreover, the number of osteoclasts was significantly increased after ANXA3 overexpression (Fig. 1C, D, S1D). Next, we detected whether ANXA3 could influence F-actin belts formation, which is essential for bone resorption and the results showed that RANKL treatment could induced notable F-actin belts formation in osteoclast precursors and the number of F-actin belts were significantly reduced after ANXA3 knockdown by siRNA (Fig. 1E, F). Consistently, the mRNA levels of key marker genes involved in osteoclast differentiation and matrix dissolution [7,8], including *Acp5*, *Ctsk* and *Mmp9*, were significantly decreased after ANXA3 knockdown both in RAW264.7 and BMMs (Fig. 1G-L). Therefore, these findings indicate that ANXA3 positively regulates osteoclast differentiation.

3.2. ANXA3 silencing reverses the bone loss in OVX mice

To investigate the role of ANXA3 in osteoporosis, lentiviral of shRNA against ANXA3 was used to treat OVX mice due to the paucity of ANXA3-specific inhibitors. Ablation of ANXA3 expression was confirmed after using ANXA3 shRNA (Fig. 2A). Four weeks after injection, we examined the fifth lumbar vertebra through micro-CT, and microarchitecture parameters demonstrated that BV/TV and Tb.N were lower, while BS/BV and Tb.Sp were higher in the OVX group compare to the SHAM group (Fig. 2B, C). There is no significantly differences between the OVX and OVX-shNC (shNC) group. On the contrast, BV/TV and Tb.N were enhanced by 27 % and 10 %, respectively, whereas, BS/BV and Tb.Sp

were reduced by 10 % and 15 %, respectively, in the OVX-shANXA3 (shANXA3) group compared with the shNC group (Fig. 2B, C). Supportively, TRAP-positive osteoclasts were increased in the OVX and shNC group and decreased in the shANXA3 group (Fig. 2D). The OVX and shNC group caused apparently bone mass altered and loosened structure in the trabecular bone compared with the SHAM group, while shANXA3 could rescue the trabecular structure (Fig. 2E). Meanwhile, the number

of ALP-positive cells was no significant difference after ANXA3 knock-down (Fig. S2A). These data confirm osteoporotic phenotypes *in vivo* were significantly relieved by ANXA3 knockdown.

3.3. ANXA3 promotes the expression of RANK and TRAF6

The NF- κ B signal pathway is shown to actively regulate osteoclast

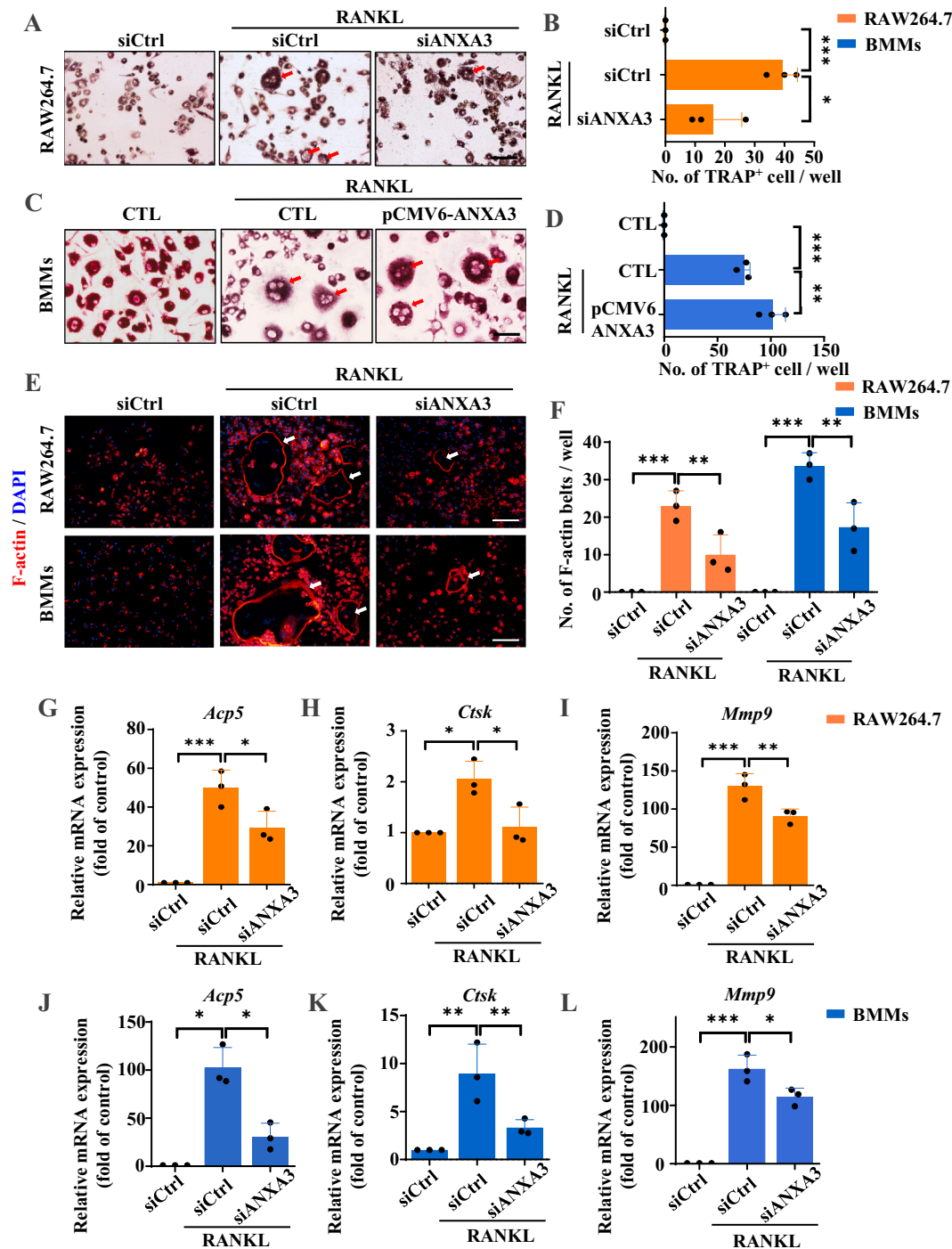


Fig. 1. Effect of ANXA3 on osteoclast formation and function.

A, B The osteoclasts formation in siControl (siCtrl) and siANXA3 groups with or without RANKL treatment for 5 days analyzed by TRAP staining in RAW264.7 cells. Arrows indicated TRAP-positive cells. Scale bar: 100 μ m. C, D TRAP staining in Control Vector (CTL) and pCMV6-ANXA3 treatment groups with or without RANKL stimulation for 5 days in BMMs. Arrows indicated TRAP-positive cells. Scale bar: 50 μ m. E, F The F-actin belts formation in siCtrl and siANXA3 groups with or without RANKL treatment for 5 days analyzed by rhodamine-conjugated phalloidin staining in RAW264.7 cells and BMMs. Arrows indicated F-actin belts. Scale bar: 50 μ m. G-L The expression levels of osteoclast-related genes (*Acp5*, *Ctsk*, *Mmp9*) in siCtrl and siANXA3 with or without RANKL treatment groups for 5 days assessed by qPCR in RAW264.7 cells (G-I) and BMMs (J-L). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

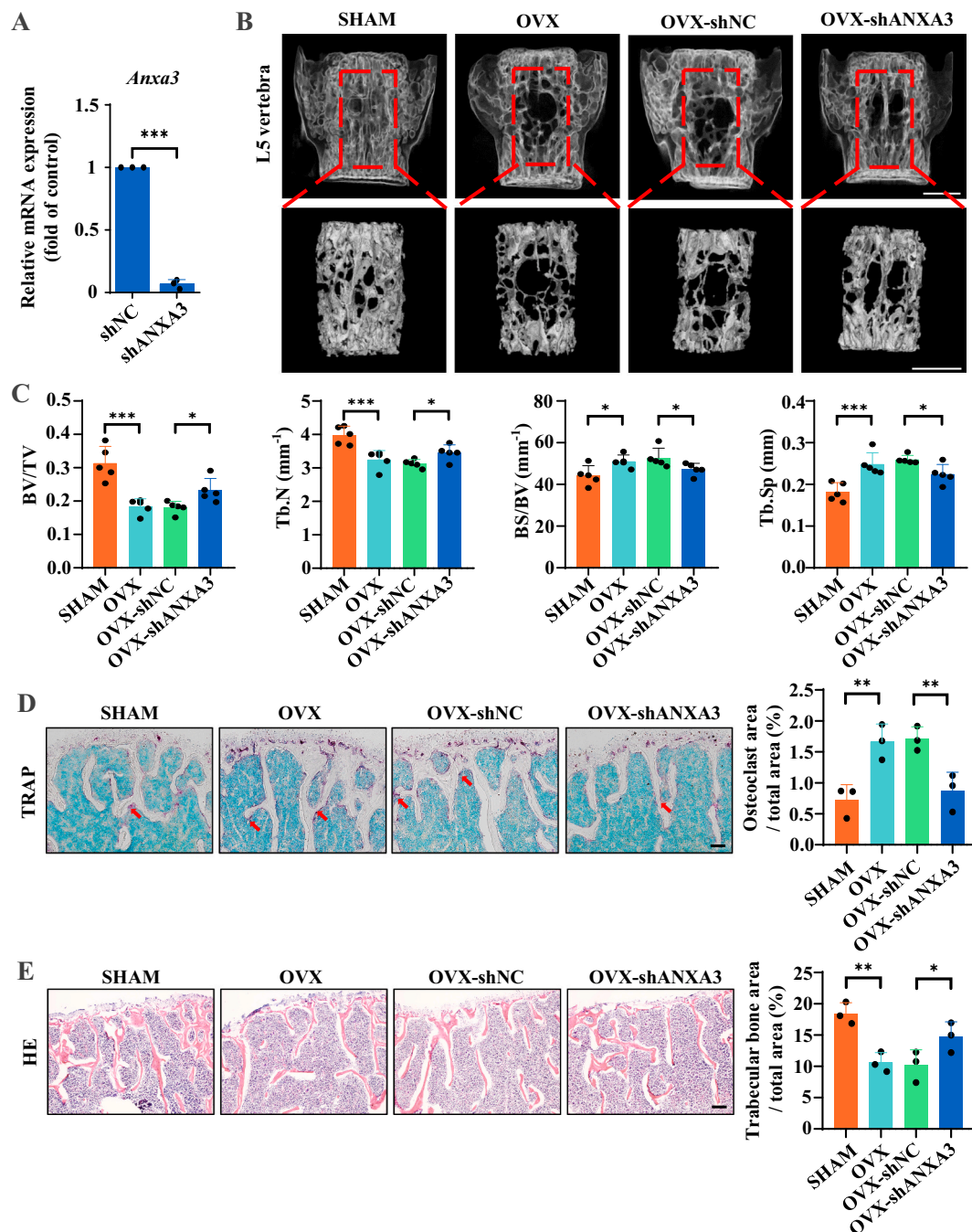


Fig. 2. ANXA3 silencing reverses the bone loss in ovariectomized mice.

A The efficacy of shANXA3 was assessed by qPCR in RAW264.7 cells. B The morphology of trabecular bone in the fifth lumbar vertebra in SHAM, OVX, OVX-shNC and OVX-shANXA3 groups were shown by micro-CT analysis. Scale bar: 1 mm. C Quantitative analysis of BV/TV, Tb.N, BS/BV and Tb.Sp in the ROI of the fifth lumbar vertebra determined by micro-CT measurements. D The TRAP positive cells in SHAM, OVX, OVX-shNC and OVX-shANXA3 groups were shown by TRAP staining. Arrows indicated TRAP-positive staining areas. Scale bar: 100 μ m. The quantification is shown on the right side. E The trabecular bone characteristics in SHAM, OVX, OVX-shNC and OVX-shANXA3 groups were shown by HE staining. Scale bar: 100 μ m. The quantification is shown on the right side. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

differentiation upon RANKL exposure [21]. To check whether this signal is related to the regulation of osteoclast differentiation by ANXA3, we found that the phosphorylation of NF- κ B subunit p65 was significantly increased after RANKL treatment, while ANXA3 knockdown inhibited the activated NF- κ B signal within 60 min (Fig. 3A). In addition, the phosphorylation of NF- κ B subunit p65 was ascended caused by ANXA3 overexpression (Fig. 3B). To verify the role of ANXA3 on osteoclast differentiation, we analyzed the genes which affected osteoclast differentiation through NF- κ B signaling. The results showed that the

transcription level of *Anxa3*, *Rank*, *Traf6*, *Stac2* and *Tbkl* were dramatically decreased after ANXA3 knockdown (Fig. 3C). It is known that RANK and TRAF6 are critical proteins in the initiation of osteoclast-related signals [4], and participate in the regulation of STAC2 and TBK1 during osteoclast differentiation [19,22]. We found that the expression of RANK and TRAF6 were inhibited after ANXA3 knockdown and promoted by ANXA3 overexpression (Fig. 3D). Furthermore, ANXA3 was detected in both osteoclast precursors (RANKL-induced 0 day) and osteoclasts (RANKL-induced 4 days) (Fig. 3E, S3A). During RANKL-

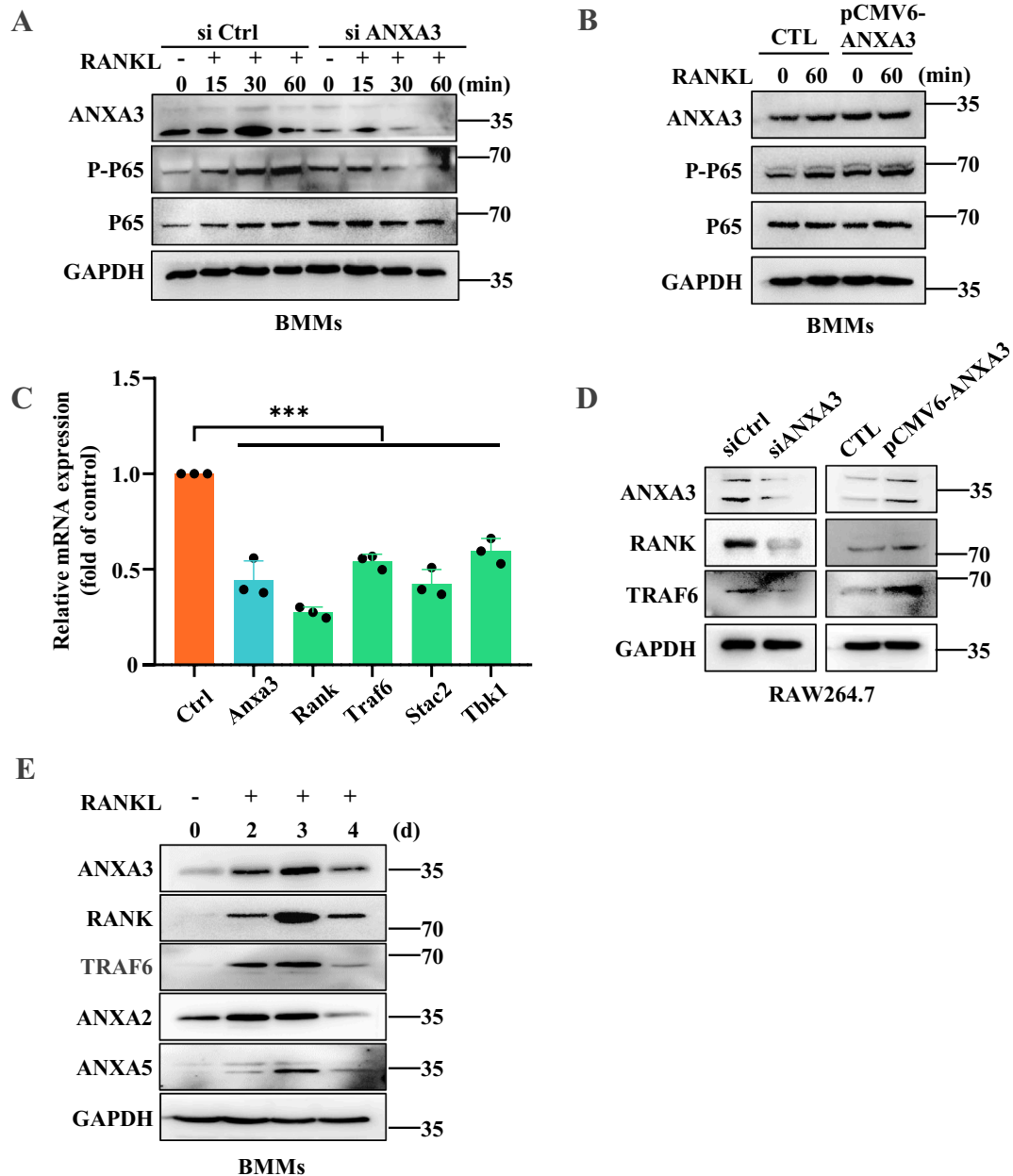


Fig. 3. The downstream target genes of ANXA3 are RANK and TRAF6.

A The protein expression of ANXA3, phospho-P65 and P65 in control and ANXA3 siRNA treatment groups treatment with or without RANKL stimulation in BMMs monitored by western blot. B The expression of ANXA3, phospho-P65 and P65 in CTL and pCMV6-ANXA3 plasmid treatment groups. C The mRNA level of genes (*Anxa3*, *Rank*, *Traf6*, *Stac2* and *Tbk1*) were examined by qPCR after RAW264.7 cells transfection with siCtrl or siANXA3. D The protein expression of ANXA3, RANK and TRAF6 in siCtrl and siANXA3 treatment groups (left panel) or CTL and pCMV6-ANXA3 plasmid treatment groups (right panel) in RAW264.7 cells. E The protein expression of ANXA3, RANK, TRAF6, ANXA2 and ANXA5 were analyzed by western blot at indicated time points in RANKL-stimulating BMMs cells. *** $P < 0.001$.

induced osteoclast differentiation, the protein level of ANXA3 was gradually increased in a time-dependent manner within 3 days and then tended to decline and the RANK, TRAF6 protein levels showed high parallelism with ANXA3 (Fig. 3E, S3A). In addition, we also tested other members of annexin family, which has been reported to be associated with bone metabolism [23,24]. A slight increase was found in the protein levels of ANXA2 and ANXA5 within RANKL-induced 3 days (Fig. 3E). These observations suggest that ANXA3 promotes the level of RANK and TRAF6.

3.4. ANXA3 enhances osteoclast formation by regulating degradation of RANK and TRAF6

To study the molecular mechanism of ANXA3 regulating RANK and TRAF6, we analyzed the degradation of RANK and TRAF6. RANK and TRAF6 have been shown previously to be essential factors for osteoclastogenesis *in vivo* [25,26]. We utilized cycloheximide (CHX) to inhibit protein biosynthesis, and investigated the effect of ANXA3 on RANK and TRAF6 degradation. ANXA3 knockdown was notably shortened the half-life of RANK and TRAF6 protein (Fig. 4A). As we know, the two major systems that eukaryotic cells used for protein degradation are the ubiquitin-proteasome system and autophagy-lysosome pathways [27]. Furthermore, we used proteasome inhibitor MG132 and lysosomal

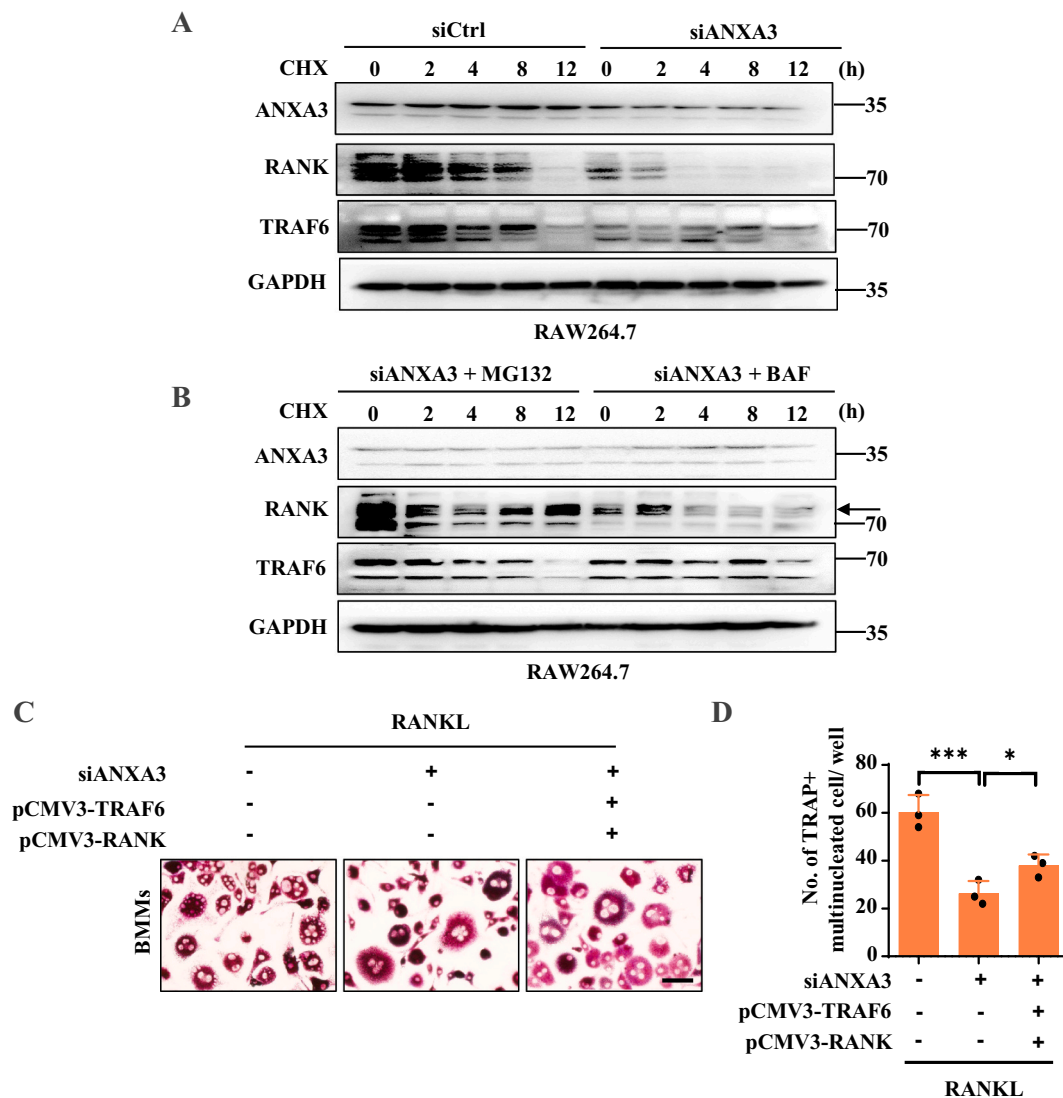


Fig. 4. ANXA3 inhibits degradation of RANK and TRAF6.

A The protein levels of ANXA3, RANK and TRAF6 in control and ANXA3 siRNA groups treatment with CHX-treated (20 μ g/mL) for different times in RAW264.7 cells. **B** The protein levels of ANXA3, RANK and TRAF6 in ANXA3 siRNA treatment with MG132-treated (10 μ mol/L) or BAF-treated (200 nmol/L) groups with or without CHX stimulation. **C, D** The osteoclasts formation in siANXA3, pCMV3-TRAF6, and pCMV6-RANK groups with or without RANKL treatment for 5 days analyzed by TRAP staining in BMMs. Arrows indicated TRAP-positive cells. Scale bar: 50 μ m. * P < 0.05, *** P < 0.001.

inhibitor BAF to determine the pathways by which ANXA3 degraded RANK and TRAF6 protein. The shortened half-life of TRAF6, which was caused by ANXA3 knockdown, was rescued by MG132 and BAF (Fig. 4B). However, RANK half-life was not significantly altered with BAF treatment but rescued by MG132 treatment (Fig. 4B). Thus, ANXA3 knockdown promoted TRAF6 degradation through the ubiquitin-proteasome and autophagy-lysosome pathways as well as accelerated RANK degradation through the ubiquitin-proteasome pathway. In addition, we analyzed whether RANK and TRAF6 are involved in the regulation of osteoclast differentiation by ANXA3, and found that overexpression of RANK and TRAF6 could reverse the reduction of osteoclast numbers due to ANXA3 downregulation (Fig. 4C, D). Collectively, ANXA3 knockdown inhibits osteoclast formation via abrogating RANK and TRAF6 expression.

3.5. ANXA3 interacts with RANK and TRAF6 in osteoclast precursors

Recruitment of TRAF6 by RANK is the initial step in osteoclast differentiation upon RANKL exposure [4]. Given that ANXA3 could

regulate RANK and TRAF6 expression, we hypothesized that ANXA3 had the ability to bind RANK and TRAF6 to regulate osteoclast differentiation. The endogenous Co-IP assay was performed using anti-TRAF6 and anti-ANXA3 antibodies, respectively. It indicated that there was a strongly binding among ANXA3, RANK and TRAF6 in osteoclast precursors (Fig. 5A). Furthermore, PYK2 localizes to cytoskeletal fractions and plays an important role in osteoclast actin belt dynamics [28]. We found that there was no endogenous interaction between ANXA3 and PYK2 (Fig. S3B). Moreover, there was an apparent interaction between ANXA3 and TRAF6, assessed by exogenous Co-IP assay (Fig. 5B). To test whether the ANXA3-RANK and ANXA3-TRAF6 interaction were direct, GST-pull-down assays suggested that there was a direct binding in ANXA3-TRAF6 as well as ANXA3-RANK (Fig. 5C, D). Meanwhile, ANXA3 colocalized with TRAF6 and RANK, respectively, assessed by confocal microscopy (Fig. 5E, F). To verify our previous results, we examined whether exosomal protein ANXA3 works inside osteoclast precursors and binds to RANK. Exosomes can be phagocytosed or incorporated to acceptor cells [29]. We found that ANXA3-overexpressed exosomes which were labeled by PKH26 could be

internalized to the cytoplasm of RAW264.7 cells (Fig. S4A). The protein level of ANXA3 was increased as well as more interaction was detected between ANXA3 and RANK after ANXA3-overexpressed exosomes treatment (Fig. S4B). These results imply that ANXA3 protein can play important roles in osteoclast precursors both directly and indirectly.

3.6. ANXA3 interacts with RANK and TRAF6 via different domains

To ascertain whether ANXA3 could affect the combination of RANK and TRAF6, we revealed that ANXA3 significantly promoted the binding of RANK to TRAF6, while more interaction was found upon RANKL exposure (Fig. 6A). Moreover, to search for the potential binding domain of ANXA3, we constructed two truncated mutants, ANXA3 1-161 aa and ANXA3 162-323 aa, based on the full length of ANXA3 (Fig. 6B). Result showed that TRAF6 bound to ANXA3 162-323 aa, while RANK bound to ANXA3 1-161aa (Fig. 6C, D).

4. Discussion

The roles for ANXA3 in the regulation of membrane transport and cancer development have been proposed, while the aspect of bone homeostasis remains poorly understood. Our previous research found that

ANXA3 in exosomes accelerated the osteoclast differentiation [17]. The study provides clues for the important role of ANXA3 on osteoclast differentiation. However, the underlying mechanism that how ANXA3 take participated in osteoclast differentiation need to be studied. In this study, we identified that ANXA3 directly controls osteoclast formation and function via NF- κ B pathway to regulate bone homeostasis. Functionally, ANXA3 shRNA relieved bone loss in OVX mice. Mechanistically, ANXA3 promoted the expression RANK and TRAF6 with inhibiting the degradation of RANK and TRAF6 protein to regulate osteoclast formation. Meanwhile, ANXA3, transported by exosomes, could be internalized to the cytoplasm of osteoclast precursors (Fig. 7). Combined with our previous reports, these results indicate that ANXA3 protein could promote osteoclast differentiation both directly and indirectly. Targeting ANXA3 may provide a novel therapeutic strategy to overcome the imbalance of bone homeostasis.

The binding of TRAFs to specific cytoplasmic domains of RANK is a crucial step for osteoclast activation [6,30,31]. To date, in the osteoclast regulation field, most studies of RANK have focused on genes competitively binding to RANK protein [22]. But few genes have reported to regulate RANK itself [32]. In our study, ANXA3 directly bound to RANK, promoted its transcription, inhibited its degradation through the ubiquitin-proteasome pathway and thus stabilized the RANK protein.

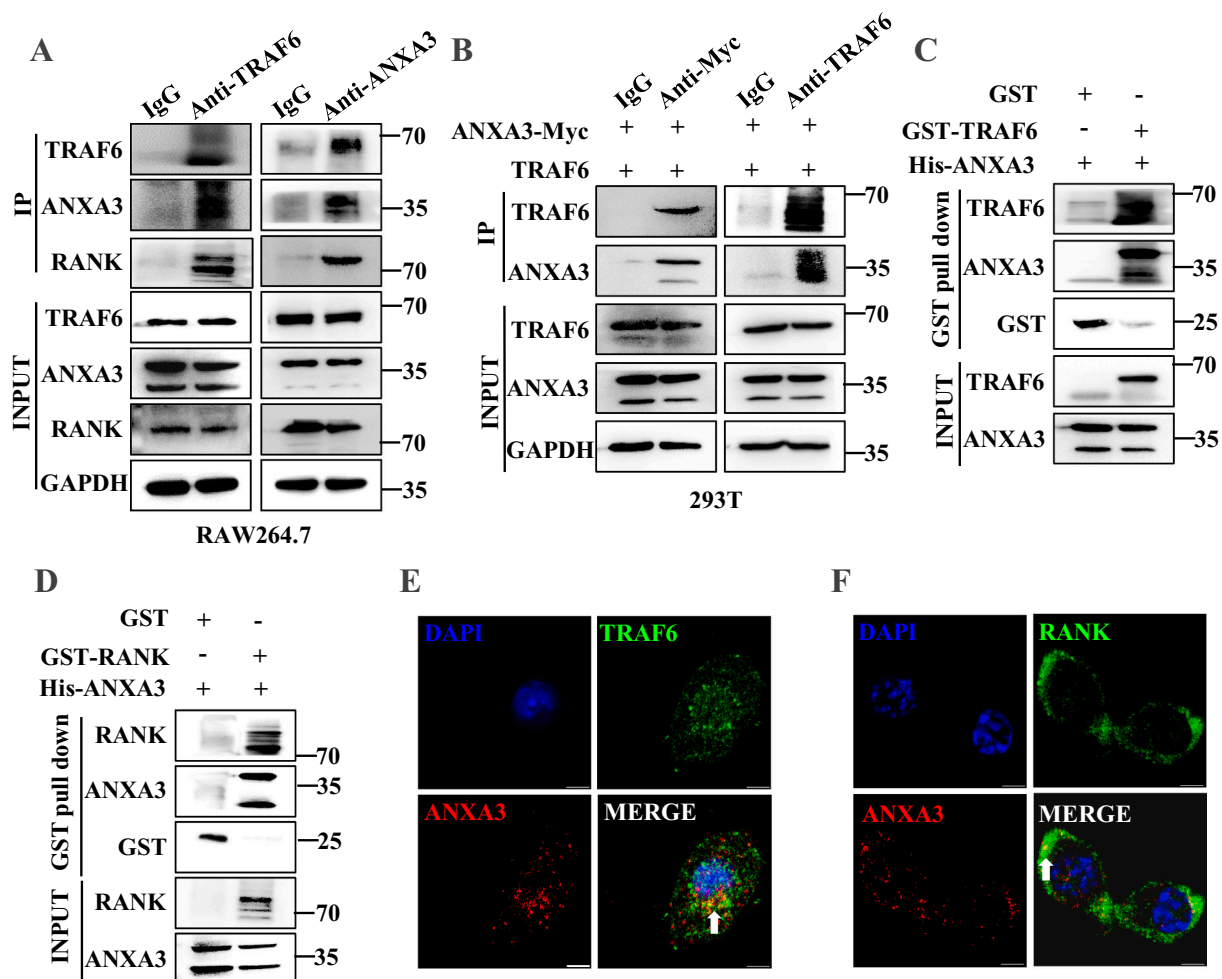


Fig. 5. ANXA3 binds with RANK and TRAF6 in osteoclast precursors.

A The endogenous interaction between TRAF6, ANXA3 and RANK assessed by Co-IP using Anti-IgG, Anti-TRAF6 and Anti-ANXA3 antibodies in RAW264.7 cells. B The exogenous interaction between TRAF6 and ANXA3 monitored by Co-IP in 293 T cells. C, D The direct interaction between TRAF6-ANXA3 (C) and RANK-ANXA3 (D) analyzed by GST-pull down assay. E Immunofluorescence co-localization analysis of ANXA3 (red) and TRAF6 (green) proteins using ANXA3 and TRAF6 antibodies in RAW264.7 cells. Scale bar: 5 μ m. F Immunofluorescence co-localization analysis of ANXA3 (red) and RANK (green) proteins using ANXA3 and RANK antibodies in RAW264.7 cells. Scale bar: 5 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

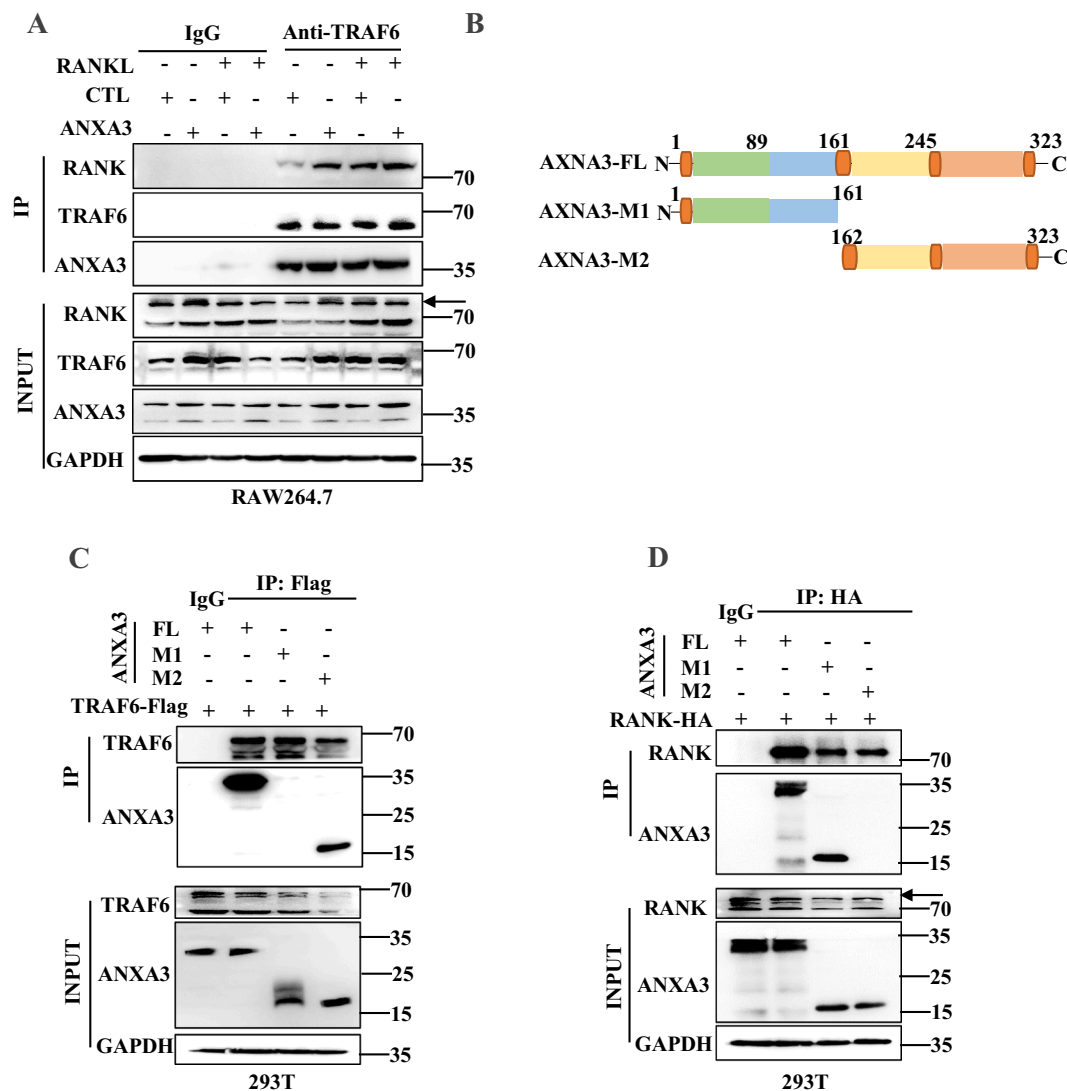


Fig. 6. The binding domains analysis of RANK and TRAF6 to ANXA3.

A The endogenous interaction between RANK and TRAF6 in Anti-IgG and Anti-TRAF6 groups with or without treatment with pCMV6-ANXA3 and RANKL for 3 days detected by Co-IP in RAW264.7 cells. **B** Schematic diagram of full-length ANXA3 and truncation mutants. **C** The binding domain analysis between TRAF6 and ANXA3 truncations assessed by Co-IP using anti-Flag (TRAF6) and anti-Myc (ANXA3 truncates) antibodies in 293 T cells. **D** The binding domain analysis between RANK and ANXA3 truncations monitored by Co-IP in 293 T cells.

Here, we showed that ANXA3 also directly bound to TRAF6, increased its transcription levels, and inhibited its degradation through ubiquitin-proteasome and autophagy-lysosomal pathways to stabilize the TRAF6 protein. Supportively, the finding of the TRAF6 degradation pathways was consistent with previous studies [33,34]. However, additional studies are required to investigate which enzyme or enzymes regulated by ANXA3 to degrade RANK and TRAF6 protein in the proteasome and/or lysosome. PYK2 is essential to the osteoclast actin ring formation [28]. However, no endogenous binding of ANXA3 with PYK2 was detected in this study. It implies ANXA3 may play a role in osteoclast in a PYK2-independent manner. Therefore, we reported a fundamentally new RANK-ANXA3-TRAF6 complex and revealed that ANXA3 promoted RANKL-induced binding of RANK and TRAF6. Meanwhile, we interpreted that ANXA3 bound to RANK and TRAF6 via different domains. To our knowledge, the binding domains of RANK and TRAF6 upon RANKL exposure has been confirmed in previous studies [35,36]. However, the binding domains which ANXA3 bound to RANK and TRAF6 need to be further explored.

ANXA3, bind to calcium ions and cell membranes [37], could extend the C5aR-mediated cytoplasmic calcium oscillation by extracellular

calcium [11]. It is generally known that cytoplasmic calcium oscillation is closely related to osteoclast differentiation [38]. Whether cytoplasmic calcium oscillation is also a regulatory mechanism of ANXA3 on osteoclast differentiation deserves further study. In view of the close relationship between inflammation and osteoclast differentiation, we speculate that ANXA3 may also regulate inflammatory bone diseases, such as osteoarthritis and rheumatoid arthritis. In terms of screening of ANXA3 downstream targets, we also observed the transcription levels of *Stac2*. It is a negative controller of osteoclast formation [22] and decreases expression after ANXA3 knockdown. The opposite trend of osteoclast regulation suggests that the regulatory effects of ANXA3 on bone remodeling are complicated, and may be signal and context dependent. The protein levels of ANXA2 and ANXA5 were slightly increased in the early phase of osteoclast differentiation. ANXA2 has been shown to promote osteoclast differentiation [23] and the role of ANXA5 in osteoclast differentiation requires further investigation. Furthermore, it has been reported that osteoblast also expressed ANXA3 [39]. However, no difference was evident in the osteoblast numbers after ANXA3 silencing. Our results indicated ANXA3 acts mostly via regulating bone resorption.

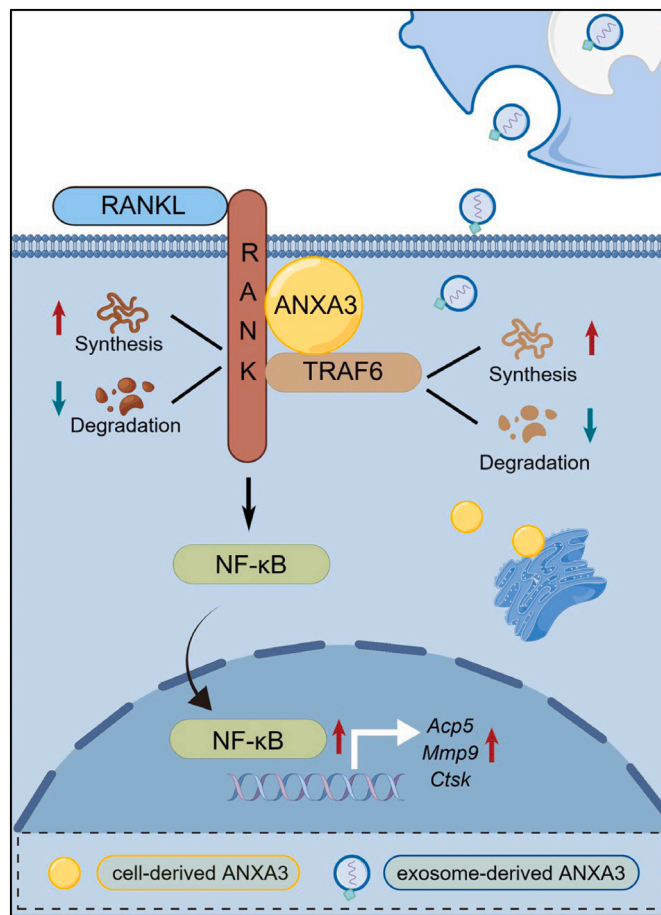


Fig. 7. Schematic diagram.

ANXA3 directly controls osteoclast formation and function via NF-κB pathway to regulate bone homeostasis. ANXA3 in exosomes also acts inside osteoclast precursors. Mechanistically, ANXA3 promotes the expression RANK and TRAF6 with inhibiting the degradation of RANK and TRAF6 protein to regulate osteoclast formation. Figure created by Figdraw.

TRAF6 protein is a key intermediate to assemble signaling proteins that activate downstream signaling cascades involved in osteoclast differentiation. The closest signal is NF-κB. The activation of NF-κB promotes the nuclear translocation of NF-κB complexes, mainly the P50/P65 heterodimer, to initiate downstream genes transcription. In our study, we found that ANXA3 knockdown significantly inhibited phosphorylation of NF-κB subunit P65 after 30 min, which is consistently with the previous reports [13]. It has been reported that mitogen-activated protein kinases (MAPKs) and protein kinase B (Akt) signals can also be activated by TRAF6 [40,41]. Whether these pathways are regulated by ANXA3 merits further study.

In summary, our data demonstrated that ANXA3 positively regulated osteoclast differentiation to regulate bone metabolism. ANXA3 promoted level of RANK and TRAF6 by directly binding to RANK and TRAF6, thus facilitated osteoclast differentiation. These findings provided novel understanding of bone homeostasis, and new promising target for treatment of bone degrading-related diseases.

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CRediT authorship contribution statement

S, L: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing. **Mz, L and Yk, Z:** Validation, Formal analysis, Investigation, Visualization. **Lj, C; Ym, W; and Zm, Z:**

Formal analysis, Investigation. **RL, Y and H, Z:** Resources, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors have no conflicts of interest.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper. Further inquiries can be directed to the corresponding authors.

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