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Dancr-BRG1 regulates Nfatc1 transcription and Pgc1β-dependent metabolic shifts in osteoclastogenesis

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Long non-coding RNA (lncRNA) serves as a vital regulator of bone metabolism, but its role in pathologically overactive osteoclast differentiation remains elusive. Here, we identify lncRNA Dancr (Differentiation Antagonizing Non-protein Coding RNA) as a critical suppressor of osteoclastogenesis and bone resorption, which is down-regulated in response to estrogen deficiency. Global or osteoclast-specific Dancr Knockout mice display significant trabecular bone deterioration and enhanced osteoclast activity, but minimal alteration of bone formation. Moreover, the bone-targeted delivery of Dancr by Adeno-associated viral remarkably attenuates ovariectomy-induced osteopenia in mice. Mechanistically, Dancr establishes a direct interaction with Brahma-related gene 1 to prevent its binding and preserve H3K27me3 enrichment at the nuclear factor of activated T cells 1 and proliferator-activated receptor gamma coactivator 1-beta promoters, thereby maintaining appropriate expression of osteoclastic genes and metabolic programs during osteoclastogenesis. These results demonstrate that Dancr is a key molecule maintaining proper osteoclast differentiation and bone homeostasis under physiological conditions, and Dancr overexpression constitutes a potential strategy for treating osteoporosis.

LncRNA | Dancr | osteoclast | bone resorption | osteoporosis

Human bone undergoes constant remodeling maintained by osteoblast-mediated bone formation and osteoclast-mediated bone resorption (1). Osteoclasts are specialized, giant and multinucleated cells responsible for the dissolution and degradation of bone tissue, which are generated from monocytes-macrophage lineage under the stimulation of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (2). Pathological circumstances, including but not limited to post-menopause, chronic inflammation, and aging, excessively provoke the differentiation and formation of osteoclasts, leading to the deterioration of bone structure and the onset of osteopenia/osteoporosis (3). In recent decades, the prevalence of osteoporosis continuously rises due to the growing aging population, with about 200 million individuals affected worldwide, thus presenting a significant healthcare challenge (4). However, the mechanisms driving uncontrolled osteoclastogenesis in pathological conditions remain elusive.

Long non-coding RNA (lncRNA) is transcripts with no more than 200 nucleotides that are not translated into proteins (5). lncRNA plays a vital role in regulating multiple cellular processes including proliferation, apoptosis, canceration, senescence, and differentiation at epigenetic, transcriptional, and translational levels (6). Several lncRNAs, such as Bmncr, lnc-ob1, and SNHG3, have been reported to participate in bone metabolism by regulating osteogenesis (7–9), while Nron, Neat1, and MALAT1 have been associated with osteoclastogenesis (10–12). Nevertheless, the lncRNAs involved in the overactivation of osteoclast functions under pathological states, especially estrogen deficiency, remain largely unknown. In addition, their therapeutic potential in vivo has yet to be determined.

In this study, we revealed that lncRNA Dancr (Differentiation Antagonizing Non-protein Coding RNA) (Mouse) and its ortholog DANCR (Human) were significantly down-regulated in response to estrogen deficiency. Global and osteoclast-specific Dancr (KO) knockout in mice led to severe trabecular bone loss and enhanced osteoclastogenesis. Mechanistically, Dancr directly interacted with Brahma-related gene 1 (BRG1) protein to inhibit its binding to the promoter regions of nuclear factor of activated T cells 1 (Nfatc1) and peroxisome proliferator-activated receptor gamma coactivator 1-beta (Pgc1β), two key regulators mediating osteoclastic gene expression and metabolic shifts respectively during osteoclast differentiation. In addition, the bone-targeting overexpression of Dancr by Adeno-associated virus (AAV) significantly reversed ovariectomy (OVX) induced bone deterioration in vivo, suggesting its prospective as pharmacological agents for treating osteoporosis.

Significance

Osteoporosis arising from dysregulation of osteoclastogenesis is a leading bone disorder. Nevertheless, the molecular mechanisms underlying osteoclast overactivation, particularly pertaining to lncRNAs (Long non-coding RNAs), remain elusive. Here, we identified Dancr (Differentiation Antagonizing Non-protein Coding RNA) as a hub lncRNA, which is down-regulated in osteoclast progenitors under ovariectomy (OVX) condition. Functionally, Dancr directly interacts with BRG1 (Brahma-related gene 1) to maintain the enrichment of H3K27me3 at the promoters of Nfatc1 (nuclear factor of activated T cells 1) and Pgc1β (proliferator-activated receptor gamma coactivator 1-beta). Deletion of Dancr leads to augmented Nfatc1 transcription and PGC1β-dependent cellular respiration, culminating in excessive bone resorption. Our findings underscore the critical significance of Dancr in bone homeostasis, thereby offering promising therapeutic targets for osteoporosis treatment.

The authors declare no competing interest.

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Results

Identification of Dancr as a Candidate Regulatory lncRNA in OVX-Induced Excessive Osteoclastogenesis. To identify the hub lncRNAs implicated in overactivated osteoclastogenesis induced by estrogen deficiency, we established an OVX model in mice. Four weeks after Sham or OVX surgery, the femurs were dissected for detection. Micro-CT showed that estrogen deficiency led to significant trabecular bone loss manifested as the reduction of bone volume/total volume (BV/TV), trabecular bone mineral density, and trabecular number (Tb.N) (*SI Appendix*, Fig. S1 *A* and *B*). In contrast, cortical thickness (Ct.Th) remained unaffected (*SI Appendix*, Fig. S1*B*). Hematoxylin and eosin (HE) staining also confirmed that OVX prompted the decrease of trabecular area (Tb.Ar) in distal femur (*SI Appendix*, Fig. S1 *C* and *E*). Additionally, osteoclasts visualized by tartrate-resistant acid phosphatase (TRAP) staining significantly increased in OVX group compared to Con group (*SI Appendix*, Fig. S1 *D* and *F*). These findings support previous studies suggesting that OVX-induced bone loss is caused by excessive osteoclastogenesis and high bone turnover.

Subsequently, we performed RNA-seq on primary bone marrow monocytes (BMMs), which are the precursors of mature osteoclasts. The top 20 lncRNAs ranked by log2 (fold change) between OVX and Con group were listed in Fig. 1*A*, in which Dancr is the only lncRNA having ortholog (DANCR) in humans (*SI Appendix*, Tables S1 and S2). Further detection of Dancr expression levels in BMMs was performed via qPCR, revealing a downregulation of Dancr expression in OVX group (Fig. 1*B*). To further investigate the correlation between DANCR and postmenopausal osteoporosis (POMP), we purified primary BMMs from human bone marrow samples for qPCR analysis, which showed DANCR expression levels were significantly lower in BMMs from POMP patients compared to the Con population (Fig. 1*C*). Moreover, we observed no significant changes in Dancr expression during osteoclast differentiation (Fig. 1*D*). These results indicate that the decline of Dancr expression may be associated with pathological osteoclastogenesis and bone resorption.

Global or Osteoclast-Specific Loss of Dancr Leads to Osteopenia due to Exorbitant Bone Resorption. In order to explore the role of Dancr in bone metabolism, we generated global Dancr knockout mice by crossing Cre-deleter mice with Dancr$^{fl/fl}$ mice (*SI Appendix*, Fig. S2 *A*–*C*). Then, 12-mo-old female Con (Dancr$^{fl/-}$ or Dancr$^{fl/fl}$) and Dancr-KO (Cre-deleter; Dancr$^{fl/fl}$) mice were killed for measurement. A remarkable trabecular bone loss characterized by the reduction in BV/TV, BMD, Tb.N, and increase of Trabecular Space (Tb.Sp) was detected in Dancr-KO mice by Micro-CT (Fig. 2 *A* and *B*). Like OVX, Ct.Th was not significantly affected (Fig. 2*C*). Quantification of Tb.Ar from HE staining also revealed a significant decline in the Dancr-KO group (Fig. 2 *D* and *E*).

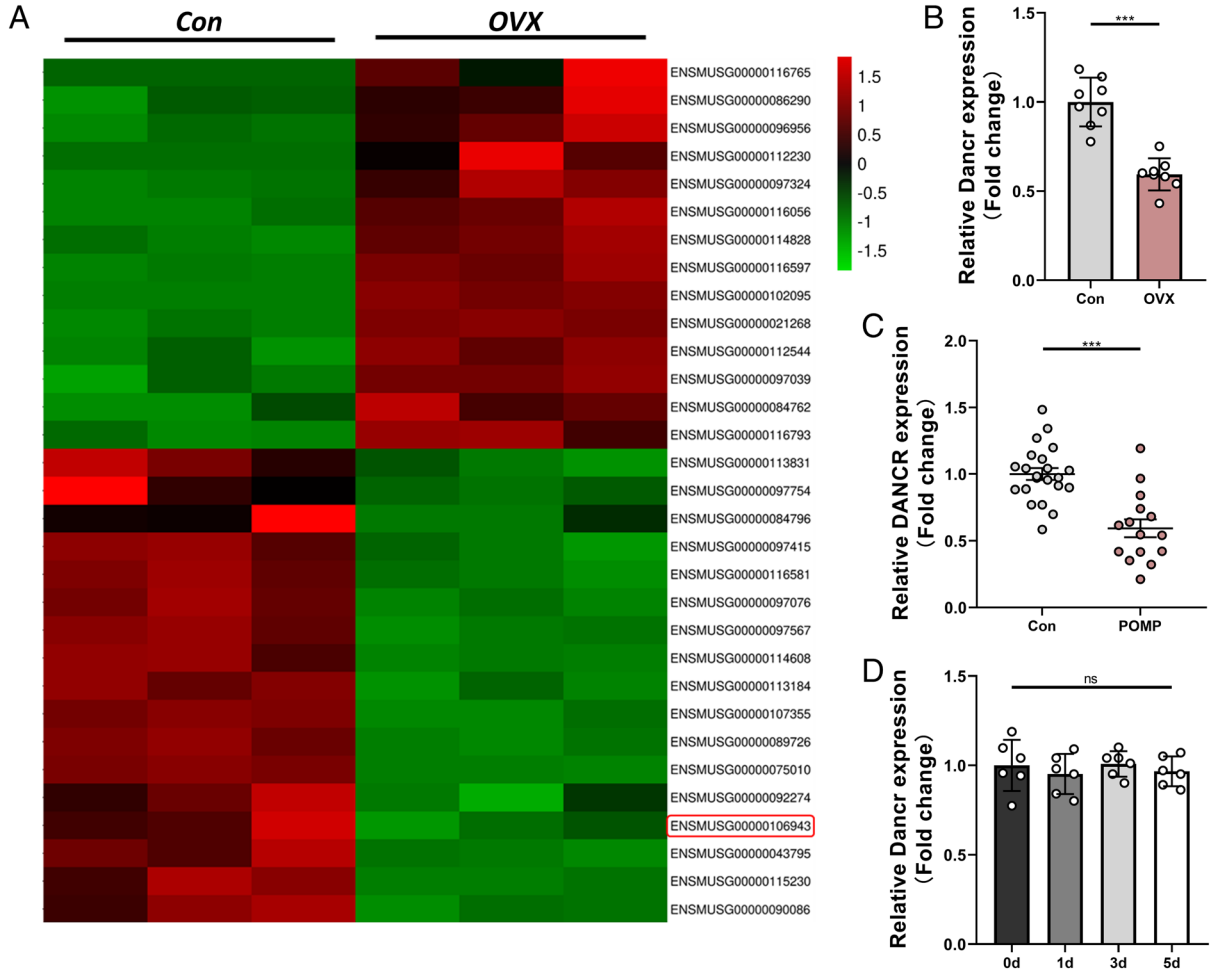

Fig. 1. Identification of Dancr as a candidate hub lncRNA in OVX-mediated osteoclast overactivation. (*A*) Heatmap of the Top 20 differential lncRNAs expression in BMMs from Con AND OVX mice. (*B*) Dancr expression in BMMs from Con AND OVX mice. (*C*) DANCR expression in BMMs from Control (Con) and POMP populations. (*D*) Dancr expression during osteoclastogenesis. Data are expressed as mean ± SEM in Fig. 1*C* and as mean ± SD in other panels. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; ns: not significant.

Furthermore, serum enzyme-linked immunosorbent assay (ELISA) analysis demonstrated a notable increase in bone resorption-related indicator Type I collagen cross-linked C-telopeptide (CTX-1) in the Dancr-KO group, while the bone formation-associated indicator procollagen type I N-terminal propeptide (PINP) was similar between the two groups (Fig. 2*F*). Therefore, we further evaluated the changes of osteoclasts and osteoblasts in femur sections. TRAP staining showed a significant enhancement of osteoclast quantity in global Dancr knockout mice (Fig. 2 *G* and *H*). In comparison, osteoblast number and bone formation rate, measured by OCN immunohistochemistry and calcein double staining, respectively, were indistinguishable between those two groups (Fig. 2 *I*–*L*).

We further isolated primary BMMs from Con and Dancr-KO mice and induced in vitro osteoclastogenesis. After 5 d of M-CSF and RANKL incubation, TRAP and F-actin staining revealed that the number of mature osteoclasts from Dancr-KO group significantly increased (Fig. 2 *M* and *N*). Pit formation assay showed an elevation in bone resorption ability of Dancr-KO osteoclasts (Fig. 2*O*). The expression levels of osteoclastic genes including Acp5, Ctsk, Dcstamp, and Mmp9 were also higher in Dancr-KO group compared to Con group (Fig.2*P*). Moreover, we performed primary bone marrow stromal cells (BMSCs) isolation and in vitro assays to examine the balance of osteogenic/adipogenic differentiation. Interestingly, Dancr-KO remarkably

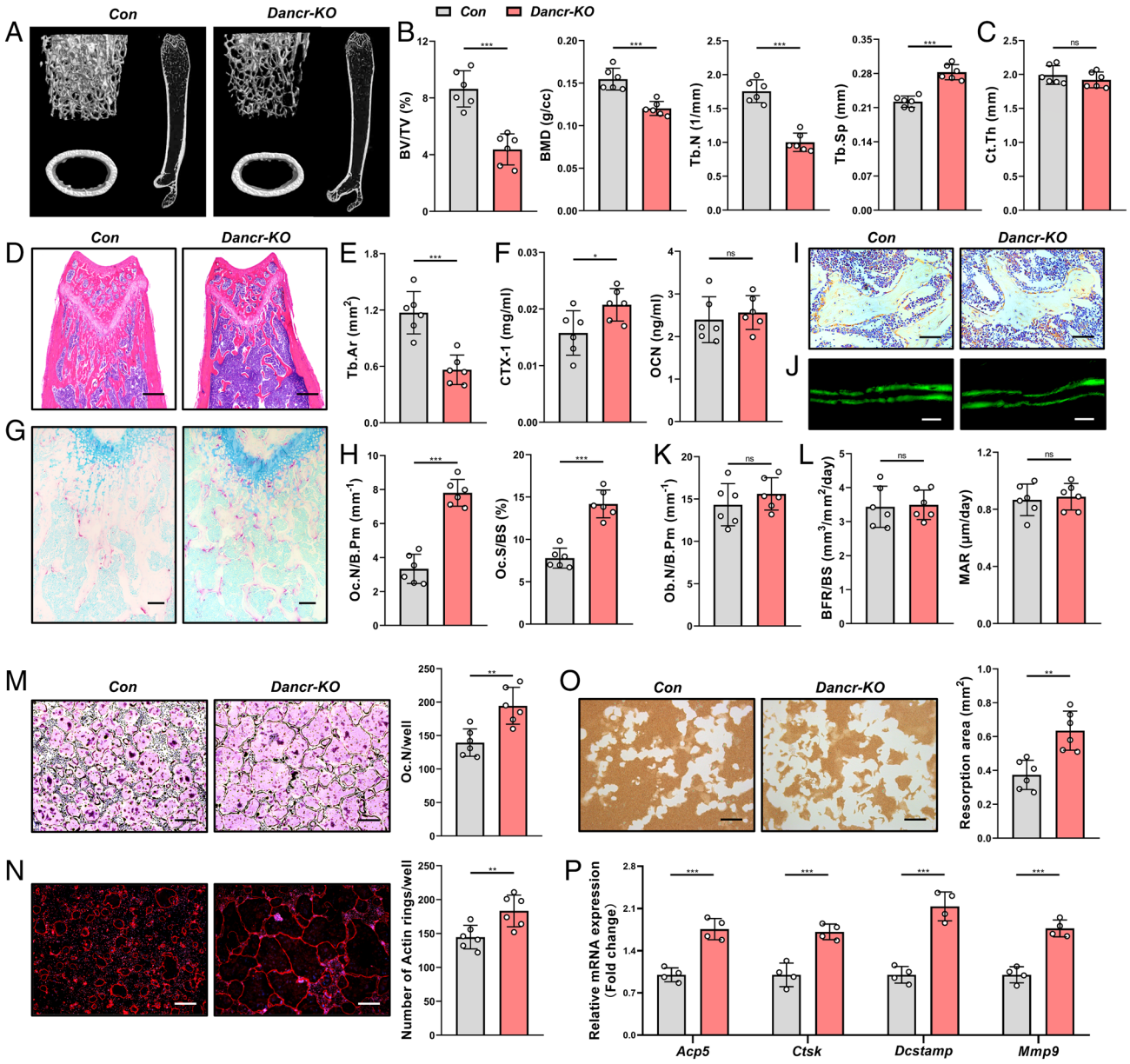

Fig. 2. Global Dancr knockout mice exhibit decreased bone mass and enhanced osteoclastogenesis. (*A*) Representative μCT analysis of the distal femur in Control (Con) and Dancr-KO mice. (*B* and *C*) Calculations of BV/TV, BMD, Tb.N, Tb.Sp (*B*) and Ct.Th (*C*). (*D*) Representative HE staining of distal femoral sections. (Scale bar: 200 μm.) (*E*) Quantification of the Tb.Ar from HE staining. (*F*) Serum levels of Type I collagen cross-linked C-telopeptide (CTX-1) and PINP. (*G*) TRAP-stained sections of the distal femur. (Scale bar: 50 μm.) (*H*) Quantification of Oc.N/B.pm and osteoclast surface/bone surface (Oc.S/BS) from TRAP staining. (*I*) Representative OCN immunohistochemical sections of distal femur. (Scale bar: 20 μm.) (*J*) Representative calcein staining of trabecula of distal femoral sections. (Scale bar: 20 μm.) (*K*) Quantification of osteoblast number per bone perimeter (Ob.N/B.pm) from OCN immunohistochemical sections. (*L*) Quantification analysis of calcein staining. Bone formation rate per unit of bone surface (BFR/BS) and mineral apposition rate (MAR). (*M*) TRAP staining and quantification of mature osteoclasts differentiated from primary BMMs of Con and Dancr-KO mice. (Scale bar: 200 μm.) (*N*) F-actin staining and its quantification. (Scale bar: 200 μm.) (*O*) Pit formation and its quantification. (Scale bar: 100 μm.) (*P*) Transcriptional expression levels of osteoclastic genes including Acp5, Ctsk, Dcstamp, and Mmp9. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; ns: not significant.

elevated osteogenesis, showed by alizarin red S and alkaline phosphatase staining, as well as osteoblastic genes' expression (*SI Appendix*, Fig. S3 *A–E*); simultaneously, Dancr-KO also promoted adipogenesis detected by oil red staining and adipogenic genes' levels (*SI Appendix*, Fig. S3 *F–H*).

To further exclude the possible effects of Dancr on bone formation, we bred osteoclast-specific Dancr knockout springs by crossing Ctsk-cre and Dancr$^{fl/fl}$ mice (*SI Appendix*, Fig. S2 *D–G*). Then, serum and femurs of 12-mo-old female Dancr$^{fl/fl}$ and Ctsk-cre; Dancr$^{fl/fl}$ mice were collected for evaluation. Similar to Dancr-KO, Ctsk-cre; Dancr$^{fl/fl}$ mice also exhibited osteopenia, immoderate osteoclastogenesis, and unaffected bone formation (*SI Appendix*, Figs. S4 and S5). Taken together, Dancr deficiency leads to trabecular bone loss by promoting osteoclast differentiation and bone resorption.

Dancr Modulates Nfatc1 Expression in Osteoclastogenesis. To explore the mechanisms underlying Dancr regulates osteoclast differentiation, RNA-seq was performed on osteoclasts differentiated in vitro from primary BMMs of Con and Dancr-KO mice. A total of 320 up-regulated and 323 down-regulated differential expressed genes (DEGs) were identified with the threshold of adjusted p (adj.p) value < 0.05 (Fig. 3*A*). KEGG (Kyoto encyclopedia of genes and genomes) enrichment was used to analyze these DEGs and the term "Osteoclast differentiation" ranked Top3 by adj.p value (Fig. 3*B*). Thus, we listed the genes under Osteoclast differentiation in a heatmap and perceived that Junb and Fos, two members of Activator protein 1 (AP1), were not significantly altered, while Nfatc1 and other osteoclastic genes including Car2, Dcstamp, and Ocstamp notably increased in the Dancr-KO group (Fig. 3*C*).

The process of osteoclastogenesis is a complex interplay of multiple pathways and cascades. Of particular importance is the binding of RANKL to RANK, which triggers the intracellular segment of RANK to stimulate AP-1, nuclear factor kappa-B (NF-κB), and PLCγ2/Ca^{2+} signaling pathway, thereby initiating Nfatc1 transcription (Fig. 3*D*). Nfatc1, in turn, triggers the expression of downstream osteoclastic genes (Fig. 3*D*). To ascertain at which point Dancr affects osteoclast differentiation, we quantified the phosphorylation of PLCγ2 (PLCγ2/Ca^{2+} signaling), p65, and IkBα (NF-κB signaling), along with c-JUN, c-FOS, and AKT (AP-1 signaling) proteins in BMMs upon RANKL stimulation within a 60-min timeframe (*SI Appendix*, Fig. S6). The activation of these three signaling pathways was observed; however, no significant disparity was detected between the Con and Dancr-KO groups (*SI Appendix*, Fig. S6). Subsequently, the expression levels of FOS and NFATC1 were assessed at 0 d, 3 d, and 5 d during osteoclastogenesis, revealing that the Dancr-KO group exhibited elevated levels of NFATC1 on 3 d and 5 d, while no discernible deviation in c-FOS expression was observed across all time points (Fig. 3 *E* and *F*). Furthermore, the downstream osteoclastic markers regulated by NFATC1, including DCSTAMP and CTSK, displayed enhanced expression in the Dancr-KO group at the 5 d time point (Fig. 3 *E* and *F*).

To confirm the role of Nfatc1 and Dancr in osteoclastogenesis, we designed three siRNAs to interfering Nfatc1 mRNA and selected si-Nfact1-b for its highest silencing efficiency (*SI Appendix*, Fig. S7*A*). TRAP staining demonstrated that si-Nfact1-b significantly inhibited the formation of mature osteoclasts in both Con and Dancr-KO groups (Fig. 3 *G* and *H*). However, Dancr-KO BMMs exhibited stronger osteoclastic ability even after siRNA transfection (Fig. 3 *G* and *H*). In addition, the transfection of si-Nfact1-b showed no influence on Dancr levels (Fig. 3*I*). Furthermore, qPCR analyses showed that the expression of Nfatc1 and two osteoclastic genes (Acp5 and Ctsk) were higher in Dancr osteoclasts with or without si-Nfact1-b interference (Fig. 3*K*). Hence, these findings suggest that Nfatc1 is a key downstream molecule mediated by Dancr in osteoclast differentiation.

Dancr Regulates Pgc1β-Dependent Metabolic Shifts in Osteoclastogenesis. Metabolic shift is an important process involved in osteoclast differentiation (13). KEGG analysis showed the tricarboxylic acid (TCA) cycle was significantly altered by Dancr-KO in osteoclasts (Fig. 3*B*). To further investigate this phenomenon, we conducted Gene Set Enrichment Analysis (GSEA) of TCA cycle and oxidative phosphorylation (OXPHOS) using RNA-seq data, which revealed that Dancr-KO dramatically promoted these two biological processes (Fig. 4 *A* and *B*). Furthermore, we observed a significant increase in mitochondrial DNA (mtDNA), which serves as a reflection of the quantity of mitochondria, in the Dancr-KO group during osteoclastogenesis (Fig. 4*C*). Mito-Tracker staining also showed an increase of mitochondria number in multinucleated preosteoclasts of Dancr-KO group (Fig. 4*D*). To assess cellular respiration activity, we used the Seahorse XFe96 Analyzer and observed a significant enhancement in mitochondrial activity, including basal respiration, adenosine triphosphate (ATP) production, respiratory capacity, and respiratory reserve, after the induction of M-CSF/RANKL (Fig. 4 *E* and *F*). Of note, mitochondrial functions were similar in BMMs between two groups without RANKL stimulation but notably boosted in Dancr-KO osteoclasts after RANKL induction (Fig. 4 *E* and *F*). Accordingly, the mRNA levels of TCA cycle-related genes including Aco1 and Idh2 and OXPHOS-related genes including Sdhd and Cyc1 were significantly enhanced during osteoclastogenesis in the Dancr-KO group compared to the Con group (*SI Appendix*, Fig. S8*A*). Taken together, these data demonstrate that Dancr knockout elevates the number and functions of mitochondria during osteoclast differentiation.

The literature reports that estrogen-related receptor alpha (ERRα) and PGC1β are two hub proteins modulating osteoclastogenesis-associated mitochondrial biogenesis and metabolic shift (Fig. 4*I*) (14). Western blot assay showed that ERRα expression was comparable between Dancr-KO and Con groups at all time points of osteoclast differentiation (Fig. 4 *G* and *H*). In contrast, PGC1β expression was significantly higher in Dancr-KO osteoclasts after 5 d of induction with M-CSF and RANKL (Fig. 4 *G* and *H*). To confirm the association of PGC1β and Dancr, three siRNAs interacting with PGC1β mRNA were synthesized and si-PGC1β-c with the highest efficiency were chosen (*SI Appendix*, Fig. S7*B*). Transfection with si-PGC1β-c led to a significant decrease in mtDNA and mitochondrial function-related genes, including Aco1, Idh2, Sdhd, and Cyc1 (*SI Appendix*, Fig. S8*B*). Additionally, the formation of osteoclasts was inhibited by si-PGC1β-c, while Dancr expression was not unaltered; and osteoclast differentiation and Pgc1β expression were still stronger in the Dancr-KO group (Fig. 4 *J–N*). However, when co-transfected with si-Nfact1-b and si-PGC1β-c, osteoclast formation of Dancr-KO group was further suppressed to the same level as the Con group (Fig. 4 *J–N*). Taken together, these findings suggest that Dancr-KO promotes Nfact1 transcription and PGC1β-dependent cellular respiration to facilitate osteoclastogenesis.

Dancr Interacts with BRG1 to Regulate Chromatin Accessibility of Nfatc1 and Pgc1β Promoter Region. Dancr has been previously identified as transcriptional factor-binding lncRNA mediating the chromatin accessibility of various genes (15). To elucidate the mechanisms by which Dancr affects the expression of Nfatc1 and Pgc1β, we performed an RNA pull-down assay using sense (Dancr+) and anti-sense Dancr (Dancr–) transcripts followed by

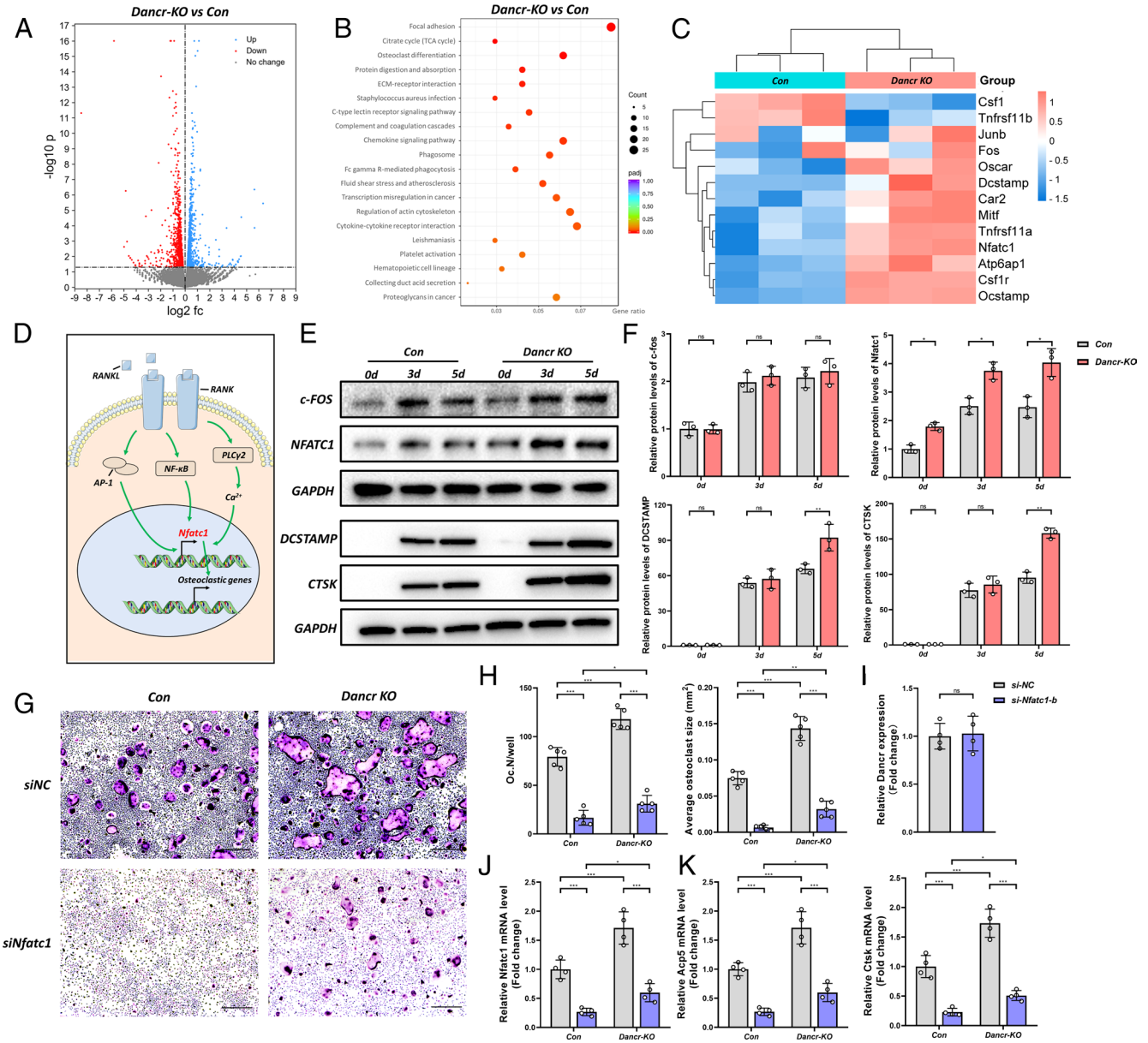

Fig. 3. Dancr regulates Nfatc1 expression in osteoclastogenesis. (*A*) Volcano plots showing the DEGs in mature osteoclasts differentiated from primary BMMs of Dancr-KO and Con mice. n = 3. (*B*) KEGG enrichment analysis of DEGs between Dancr-KO and con groups. (*C*) Heatmap showing the expression of genes under the KEGG term (Osteoclast Differentiation). (*D*) The scheme briefly depicts signaling pathways driving osteoclastogenesis. After RANKL binds to RANK, the intracellular segment of RANK activates AP-1 and NF-κB, which translocate into nucleus to stimulate Nfatc1 transcription. Then, Nfatc1 further triggers the expression of osteoclastic genes. (*E*) Western blot measuring the protein levels of c-FOS, NFATC1, DCSTAMP, and CTSK. (*F*) Quantification of Western blot. (*G*) TRAP staining of mature osteoclasts differentiated from primary BMMs of Dancr-KO and Con mice transfected with negative control siRNA (siRNA-NC) and siRNA interfering Nfatc1 (si-Nfatc1-b). (Scale bar: 200 μm.) (*H*) Quantification of osteoclast number per well (Oc.N/well) and average osteoclast size from TRAP staining. (*I*) Expression level of Dancr in Con osteoclasts after the transfection of siRNA-NC and si-Nfatc1-b. (*J*) Expression level of Nfatc1 in different groups. (*K*) Expression level of Acp5 and Ctsk in different groups. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001; ns: not significant.

mass spectrometry analysis (*SI Appendix*, Fig. S9*A*). A total of 97 proteins were identified only in Dancr+ pulled-down compounds (Fig. 5*A*). We then focused on functional proteins located in the nucleus, including BRG1, CHD6, ZFHX4, RPC5, and SOX6 (Fig. 5 *B* and *C*). BRG1 is an important ATP-dependent chromatin remodeler that decreases H3K27me3 methylation to promote gene transcription (16). Interestingly, previous studies have reported that BRG1, CHD6, and ZFHX4 are co-located in the genome and BRG1 is an RNA-binding protein (17–19). Based on these observations, we hypothesized that Dancr directly interacts with BRG1 to regulate the expression of downstream genes. The binding of Dancr with BRG1 was then confirmed by RNA immunoprecipitation (RIP) assay and RNA pull-down followed by western blot (Fig. 5 *D* and *E*). Chromatin immunoprecipitation (CHIP) was further performed to investigate whether Dancr affects BRG1-mediated H3K27me3 methylation. We found that the BRG1 enrichment was in a relatively low level at Nfatc1 and Pgc1β promoter regions in BMMs without stimulation and similar between Dancr-KO with Con group (Fig. 5 *F* and *G*). However, after 5 d of RANKL induction, the BRG1 levels remarkably increased, with higher BRG1 enrichment observed in the Dancr-KO group (Fig. 5 *F* and *G*. In contrast, H3K27me3 levels declined after RANKL activation and were lower in Dancr-KO osteoclasts (Fig. 5 *H* and *I*). These findings suggest that Dancr may interact with BRG1 to modulate H3K27me3 methylation and regulate downstream gene expression during osteoclastogenesis.

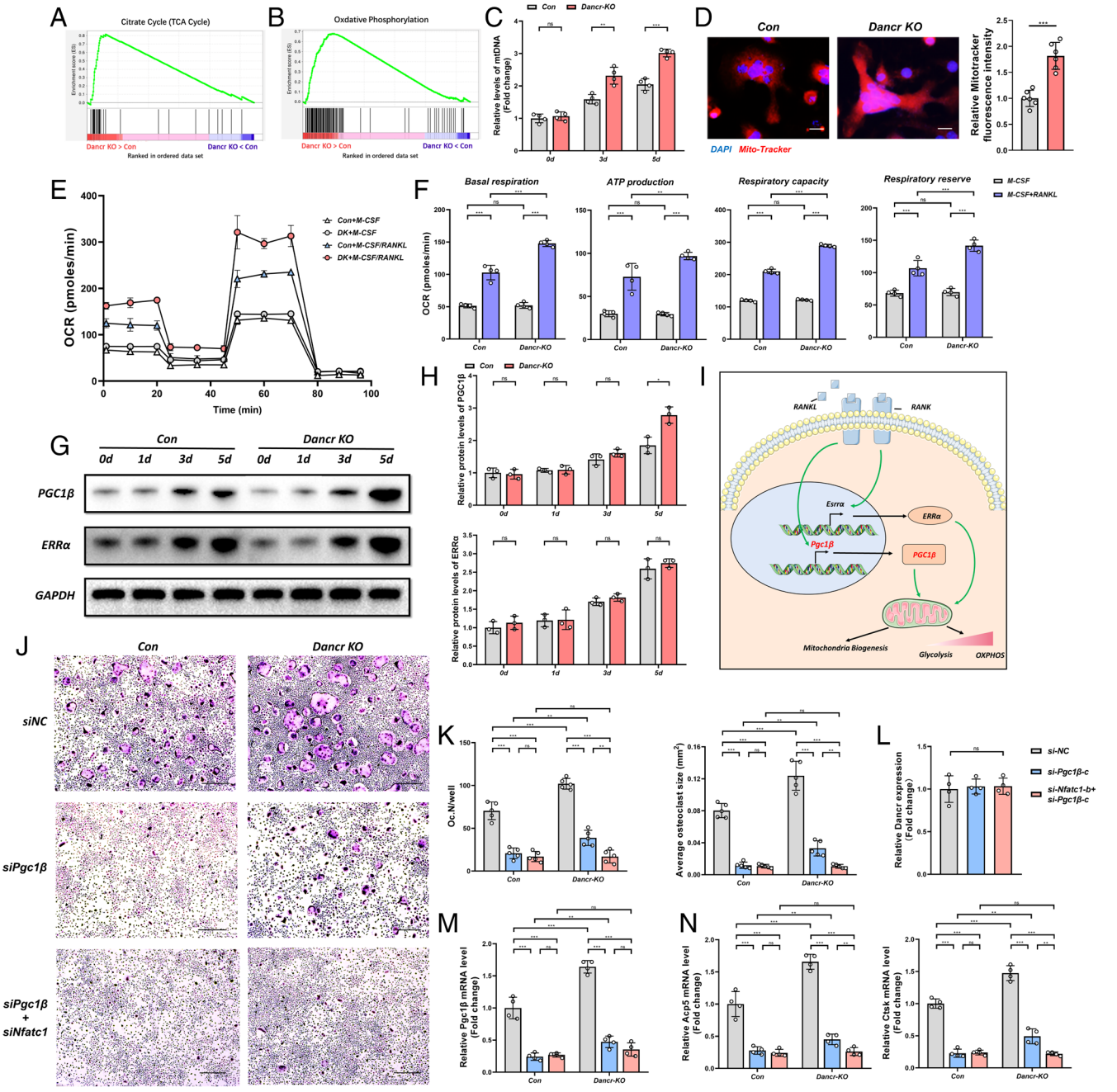

Fig. 4. Dancr regulates Pgc1β expression and mitochondrial functions in osteoclastogenesis. (*A* and *B*) GSEA analysis of Citrate Cycle (TCA Cycle) (*A*) and Oxidation Phosphorylation (*B*). (*C*) Relative levels of mtDNAs during osteoclastogenesis of Con and Dancr-KO BMMs. (*D*) Visualization and quantification of mitochondria by Mitotracker in preosteoclasts differentiated from Con and Dancr-KO BMMs. (Scale bar: 20 μm.) (*E*) Mitochondrial function was assessed by real-time OCR measurement after sequential treatment of compounds modulating mitochondrial function. The OCR was normalized to the relative amount of DNA. Dancr-KO (DK). (*F*) Assessment of mitochondrial activity including basal respiration, ATP production, respiratory capacity, and respiratory reserve calculated from real-time OCR measurement. (*G*) Western blot measuring the protein levels of PGC1β and ERRα. (*H*) Quantification of Western blot. (*I*) The scheme briefly describes mitochondrial activation during osteoclastogenesis. After RANKL binds to RANK, the intracellular segment of RANK activates the transcription of Esrrα and Pgc1β, which further promote mitochondrial biogenesis and metabolic shift from glycolysis to oxidation phosphorylation (OXPHOS) during osteoclastogenesis. (*J*) TRAP staining of mature osteoclasts differentiated from primary BMMs of Dancr-KO and Con mice transfected with negative control siRNA (siRNA-NC), siRNA interfering Pgc1β (si-Pgc1β-c), and siRNA interfering both Nfatc1 and Pgc1β (si-Nfatc1-b + si-Pgc1β-c). (Scale bar: 200 μm.) (*K*) Quantification of Oc.N/well and average osteoclast size from TRAP staining. (*L*) Expression level of Dancr in Con osteoclasts after the transfection of siRNA-NC, si-Pgc1β-c, or si-Nfatc1-b plus si-Pgc1β-c. (*M*) Expression level of Pgc1β in different groups. (*N*) Expression level of Acp5 and Ctsk in different groups. Data are expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; ns: not significant.

Subsequently, we employed a BRG1 inhibitor (compound 14, MCE) to investigate the role of BRG1 in osteoclast differentiation. To assess the cytotoxicity of BRG1 inhibitor on primary BMMs, we conducted a CCK-8 assay, which demonstrated that concentrations of 150 nM and below did not affect cell proliferation (*SI Appendix*, Fig. S9*B*). Therefore, we treated Dancr-KO and Con BMMs with 20 nM, 40 nM, and 80 nM BRG1 inhibitor during osteoclast differentiation. TRAP staining revealed that 20 nM and 40 nM of BRG1 inhibitor significantly suppressed osteoclast generation, but the Dancr-KO group still exhibited a greater number of osteoclasts than the Con group (Fig. 5 *J* and *K*). In comparison, 80 nM of BRG1 inhibitor completely prevented osteoclast formation and abolished the differentiation between Dancr-KO and Con cells (Fig. 5 *J* and *K*). Then, we selected 20 nM as the optimal

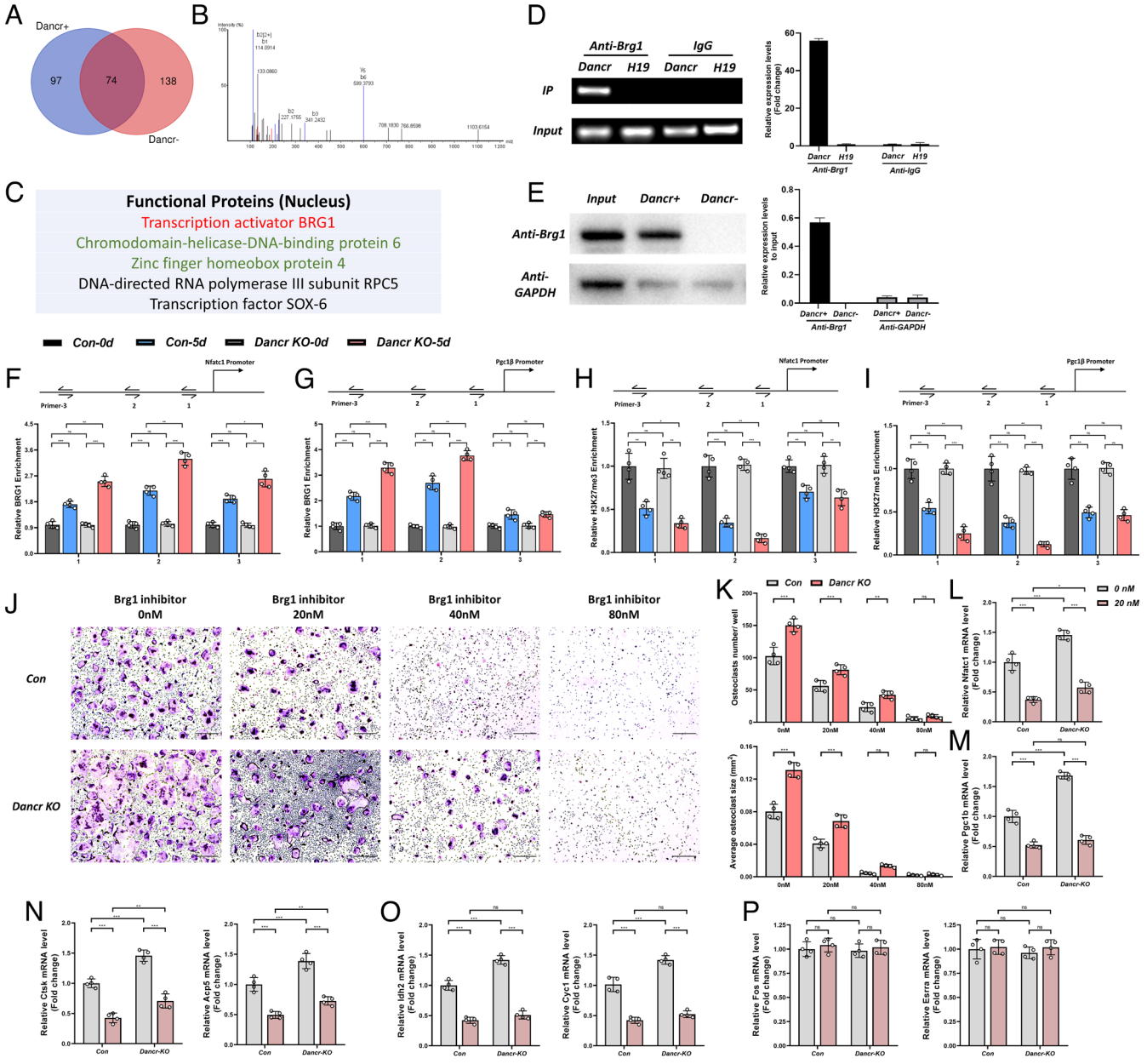

Fig. 5. Dancr directly binds to BRG1 and regulates H3K27me3 methylation in Nfatc1 and Pgc1β promoters. (*A*) Proteins interacting with Dancr+ and Dancr− identified by mass spectrum after RNA pull-down in BMMs. (*B*) BRG1 protein were identified by mass spectrum. (*C*) All functional proteins in nucleus identified by mass spectrum. (*D*) BRG1 was immunoprecipitated from cell extracts of formaldehyde-cross-linked BMMs, and the associated RNAs were evaluated by qPCR. lncRNA H19 was used as a negative control. n = 4. (*E*) Biotin-Dancr pulldown using Dancr+ and Dancr− transcripts and BMMs protein extracts showed Dancr-specific binding to BRG1. The experiment was repeated three times independently. (*F* and *G*) Nfatc1 (*F*) and Pgc1β (*G*) promoters were subjected to ChIP with an anti-BRG1 antibody in BMMs and differentiated osteoclasts from Con and Dancr-KO mice and then evaluated by qPCR. (*H* and *I*) Nfatc1 (*H*) and Pgc1β (*I*) promoters were subjected to ChIP with an anti- H3K27me3 antibody in BMMs and differentiated osteoclasts from Con and Dancr-KO mice and then evaluated by qPCR. (*J*) TRAP staining of mature osteoclasts differentiated from primary BMMs of Dancr-KO and Con mice treated with various concentrations of Brg1 inhibitor. (*K*) Quantification of Oc.N/well and average osteoclast size from TRAP staining. (*L* and *M*) Transcriptional expression levels of Nfatc1 (*L*) and Pgc1β (*M*) in osteoclasts from Dancr-KO and Con mice treated with 20 nM Brg1 inhibitor. (*N*) Transcriptional expression levels of osteoclastic genes Acp5 and Ctsk. (*O*) Transcriptional expression levels of mitochondrial function-related genes Idh2 and Cyc1. (*P*) Transcriptional expression levels of Fos and Esrrα. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; ns: not significant.

concentration for further analysis. Treatment with 20 nM of BRG1 inhibitor significantly decreased the transcriptional levels of Nfatc1, Acp5, and Ctsk, while Dancr-KO group still exhibited higher expression levels (Fig. 5 *L* and *N*). The mRNA levels of Pgc1β, Idh2, and Cyc1 were also down-regulated by 20 nM BRG1 inhibitor, with no significant difference observed between Dancr-KO and Con groups (Fig. 5 *M* and *O*). However, the mRNA levels of Fos and Esrrα were not altered by either BRG1 inhibitor or Dancr-KO (Fig. 5*P*). In conclusion, lncRNA Dancr directly interacts with BRG1 to inhibit its binding to Nfatc1 and Pgc1β promoter regions, thereby maintaining physiological osteoclastogenesis.

Bone-Targeting Delivery of Dancr Attenuates Bone Loss in OVX Mice. Finally, we evaluated the potential therapeutic effects of bone-targeted Dancr delivery on OVX-induced osteopenia (Fig. 6*A*). Negative control and Dancr overexpression AAV-8 vectors were synthesized and injected to mice through tail vein. The bone enrichment of AAV-expressed fluorescence was confirmed by in vivo imaging systems and femoral sections

(Fig. 6*B* and *SI Appendix*, Fig. S10). The qPCR analysis showed the successful overexpression of Dancr in BMMs following AAV treatment (Fig. 6*C*). Ten weeks after AAV treatment, mice were killed and femurs were collected for detection. Micro-CT and HE staining revealed that the trabecular bone parameters deteriorated by OVX, including BV/TV, BMD, Tb.N, and Tb.Sp, were significantly attenuated by Dancr overexpression (Fig. 6 *D*–*K*). Furthermore, the increased osteoclast quantity observed in the OVX group was significantly reversed by AAV-Dancr (Fig. 6 *L* and *M*). Taken together, our findings demonstrate that Dancr treatment exerts protective effects on trabecular bone in osteoporotic mice and highlight the potential translational implications of lncRNAs, such as Dancr, in the treatment of osteoporosis.

Discussion

Excessive osteoclast differentiation, fusion, and function expedite bone resorption, further prompting bone mass loss and structure damage, clinically manifested as osteopenia/osteoporosis (20). At present, anti-resorptive drugs such as bisphosphonates constitute the primary pharmacological treatments for osteoporosis (21).

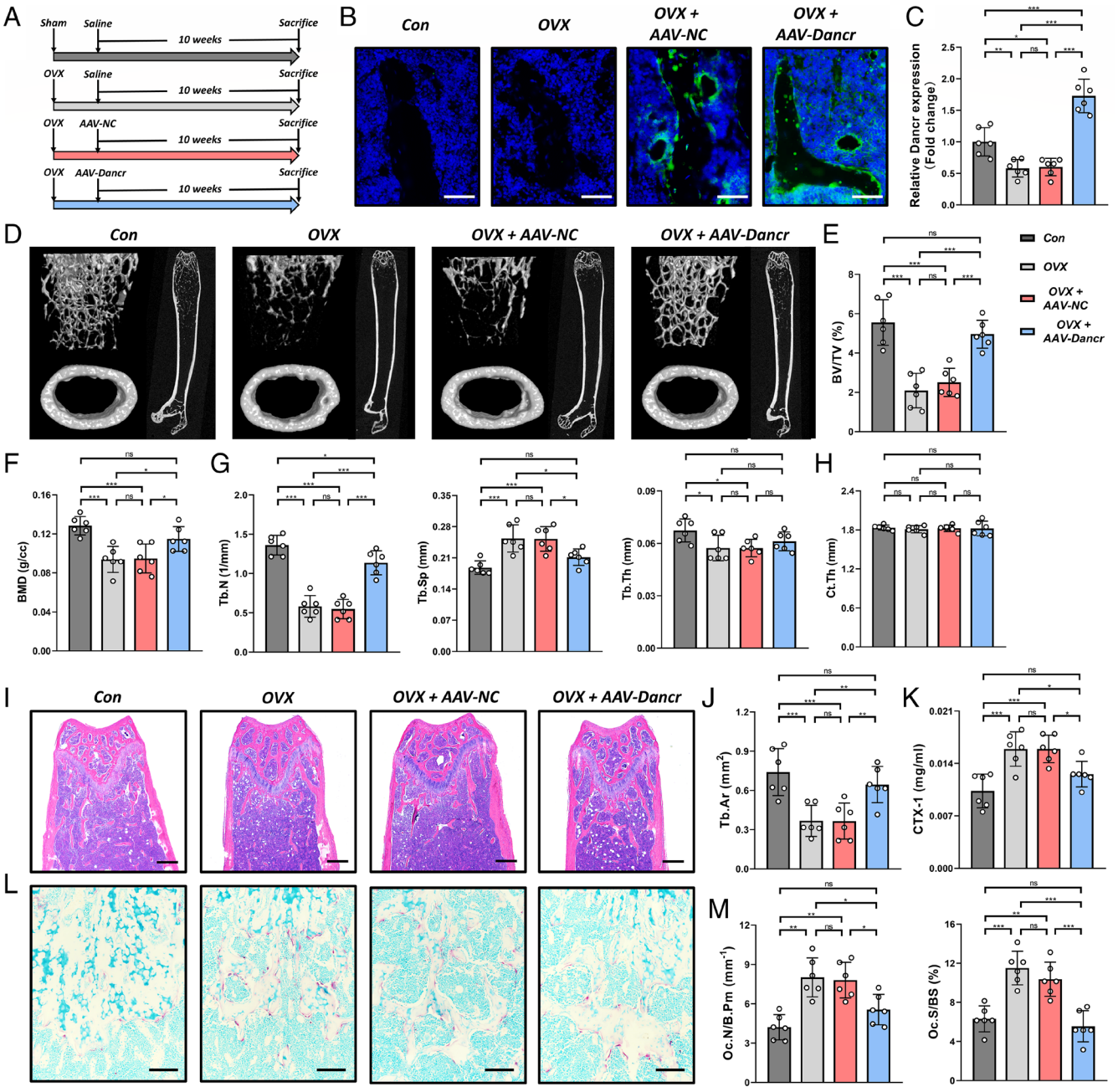

Fig. 6. Osteoclastic delivery of Dancr inhibits bone loss in OVX mice. (*A*) Mice were randomly subjected into four groups. Con: mice underwent sham surgery treated with saline; OVX: mice underwent OVX surgery treated with saline; OVX+AAV-NC: mice underwent OVX surgery treated with negative control AAV; OVX+AAV-Dancr: mice underwent OVX surgery treated with Dancr overexpression AAV. (*B*) Autofluorescence of femoral sections from different groups. (Scale bar: 5 μm.) (*C*) Transcriptional expression level of Dancr in BMMs from different groups of mice. (*D*) Representative μCT analysis of the distal femur. (*E*–*H*) Calculations of BV/TV (*E*), BMD (*F*), Tb.N, Tb.Sp, Tb.Th (*G*), and Ct.Th (*H*). (*I*) Representative HE staining of distal femoral sections. (Scale bar: 200 μm.) (*J*) Quantification of Tb.Ar from HE staining. (*K*) Serum levels of CTX-1. (*L*) TRAP-stained sections of the distal femur. (Scale bar: 50 μm.) (*M*) Quantification of Oc.N/B.pm and Oc.S/BS from TRAP staining. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; ns: not significant.

However, they are far from perfect because of several severe side effects including osteonecrosis of the jaw and increased risk of atypical fracture (22). Therefore, it is imperative to decipher the factors underlying pathological osteoclastogenesis and explore novel therapeutic targets.

Over 95% of the human transcriptome comprises ncRNAs, of which transcripts longer than 200 bases are categorized as lncRNAs (23). Moreover, the expression of most ncRNAs is cell-specific, endowing them with distinct biological functions across diverse cellular and molecular processes (24). Several lncRNAs have been implicated in the regulation of osteoclast differentiation (25). For instance, lncRNA Nron preserves ERα stability and impedes osteoclast differentiation by interacting with E3 ubiquitin ligase CUL4B (10). Meanwhile, lncRNA AK077216 boosts RANKL-induced osteoclastogenesis by enhancing the expression of NFATc1 and inhibiting NIP45 (26). Other lncRNAs, including MALAT1, Neat1, and AK131850 also modulate osteoclast differentiation and function by sponging specific miRNAs (11, 12, 27). Nevertheless, the lncRNAs responsible for hyperactive osteoclastogenesis remain elusive. In our study, we conducted high-throughput RNA-seq analysis of primary BMMs (the precursor cells of osteoclasts) derived from Con and OVX mice, which identified Dancr as a significantly changed and evolutionarily conserved lncRNA. We further confirmed that Dancr and its human ortholog DANCR were both down-regulated following estrogen deficiency, but remained unaltered during normal osteoclastogenesis. Additionally, global and osteoclast-specific Dancr knockout mice displayed significant trabecular bone loss and increased osteoclast quantity. Consistent with these findings, in vitro assays revealed that BMMs from Dancr-deficient mice exhibited enhanced osteoclastic capacity. Collectively, our results indicate that Dancr serves as a negative regulator of osteoclast differentiation to maintain bone homeostasis.

Dancr was named in accordance with its role as an anti-differentiation non-coding RNA that preserves the stemness of epidermal progenitor cells (28, 29). Additionally, DANCR/Dancr has been acknowledged as an onco-lncRNA that exerts diverse oncogenic effects in a panoply of cancer types, such as hepatocellular carcinoma, ovarian cancer, pancreatic cancer, and so on (15, 30). Conspicuously, DANCR/Dancr implicates in bone metabolism, and several studies have substantiated that its abrogation in human BMSCs augments osteogenic differentiation (31–35). Nevertheless, our research revealed that global Dancr ablation in mice manifested no discernible alterations in osteoblast quantity or bone formation capabilities in vivo. Intriguingly, BMSCs isolated from Dancr-deficient mice displayed enhanced capacities of both osteogenesis and adipogenesis in vitro, which is consistent with a previous study (28). Considering that the programs of osteogenic and adipogenic differentiation are mutually exclusive, it is plausible that Dancr knockout may engender intricate alterations in the regulatory network of BMSCs, culminating in no change in osteogenesis. In conjunction with the data demonstrating that global and osteoclast-specific knockout of Dancr resulted in similar osteopenia phenotypes, we could conclude that Dancr modulates bone homeostasis mainly by orchestrating osteoclastogenesis.

The activation of RANKL is an essential prerequisite for osteoclast differentiation, which initiates a cascade of signaling events, among which the transcription of Nfact1, activated by AP-1, NF-κB, and PLCγ2/Ca^{2+}, serves as a crucial step (36). This step necessitates epigenetic preparation (37). Prior research has indicated that the promoter region of the Nfatc1 gene in osteoclast precursors is highly enriched with H3K27me3, which can be reduced by the epigenetic regulators JMJD3 and PU.1 during osteoclastogenesis (38, 39). The increased NFATC1 protein also operates as a transcription factor, binding to the Nfatc1 promoter to form a forward loop and initiate the expression of osteoclastic genes including Acp5, Ctsk, Dcstamp, and Car2 (6). Our findings demonstrated that Dancr-KO had no impact on the activity of AP-1, NF-κB, and PLCγ2 but considerably enhances Nfatc1 expression. Furthermore, transfection with siRNA targeting Nfatc1 did not alter Dancr levels but significantly suppressed Dancr-KO induced overactive osteoclast differentiation, implying that Dancr mediates osteoclastogenesis at the Nfatc1 level.

Metabolic shift, primarily characterized by mitochondrial biogenesis and increased OXPHOS, is fundamental to osteoclast differentiation (14). Upon the stimulation with M-CSF/RANKL, activated mTOR, AMPK signaling pathways, and CREB collaborate to instigate the transcription of Pgc1β (40, 41). Simultaneously, MYC as an upstream regulator induces Essrα expression (42). PGC1β along with ERRα enhances cellular respiration and ROS production to support osteoclastogenesis (13). Our findings revealed that Dancr-KO resulted in an increase in the TCA cycle and OXPHOS in osteoclast differentiation, which was associated with PGC1β but not ERRα. The decrease of PGC1β expression by siRNA significantly inhibited osteoclast formation in both Con and Dancr-KO groups, with no effect on Dancr expression. Notably, co-transfection of si-Nfatc1 and si-Pgc1β abolished the differences of osteoclast quantity between Dancr-KO and Con groups. Collectively, our results indicated that Nfatc1 and Pgc1β are two hub downstream molecules of Dancr in osteoclastogenesis.

Reportedly, Dancr has the capacity to directly bind with nuclear proteins, thereby regulating the transcription of downstream genes (43). A plethora of studies have demonstrated that Dancr can interact with EZH2, a subunit of polycomb repressive complex 2 complex, to induce its recruitment to specific promoter regions, ultimately affecting multiple processes such as osteogenic differentiation and the development of cholangiocarcinoma (15, 31, 44). Therefore, we conducted RNA pull-down, RIP as well as mass spectrum analyses and identified BRG1 as a potential protein that interacts with Dancr during the process of osteoclast differentiation. BRG1, also recognized as SMARCA4 and a member of the switch/sucrose nonfermentable family, functions as a chromatin remodeler by eliciting structural changes in reconstituted chromatin particles in an ATP-dependent manner (45). BRG1 is a pivotal subunit of numerous protein complexes that regulate gene transcription via histone modifications (46, 47). Notably, certain lncRNAs such as Evf2 and Mhrt possess the ability to directly bind to BRG1, thereby inhibiting its enrichment on specific promoters (19, 48). In this study, we revealed that Dancr is another binding lncRNA that interacts with BRG1, consequently impeding its binding to the promoter regions of Nfatc1 and Pgc1β. This leads to the accumulation of H3K27me3, which diminishes chromatin accessibility and subsequently represses the transcription of the corresponding genes (Fig. 7).

Nucleic acid–based therapies are rapidly advancing due to their amenability of design and synthesis to target specific molecules (49). During the previous decade, a considerable endeavor has been undertaken to translate RNA-based therapeutics into clinical practice, predominantly utilizing antisense oligonucleotides and small interfering RNAs, with several approved by the US Food and Drug Administration (FDA) (50). Nevertheless, concerns regarding the low serum stability and immunogenicity of nucleic acid–based medicines persist, underscoring the importance of developing efficient delivery systems (51). Among the leading platforms for gene delivery, AAVs have been employed as vectors for vaccines and several medications, with different serotypes and surface modifications enabling tissue-specific affinity (52). In this study, we utilized AAV-9 vectors conjugated with

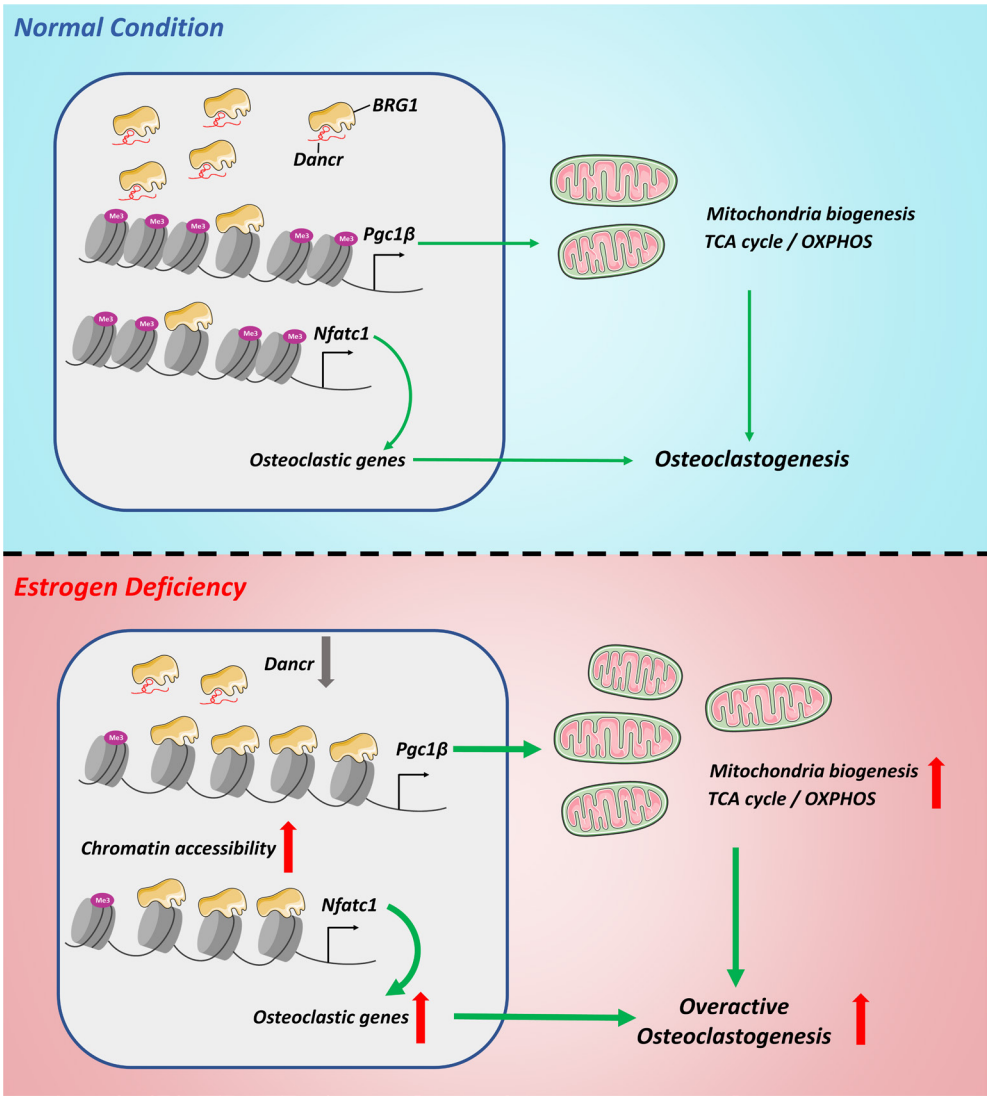

Fig. 7. Graphical abstract. In normal condition, Dancr directly interacts with BRG1 to impede its binding to Nfatc1 and Pgc1β promoters. Therefore, osteoclastogenesis activities are strictly controlled. In estrogen deficiency, Dancr expression is significantly down-regulated. Then, more BRG1 binds to the promoters of Nfatc1 and Pgc1β, which decrease the H3K27me3 levels and increase chromosome accessibility. The overexpression of Nfatc1 and Pgc1β then enhances osteoclastic genes' levels and mitochondrial activities, which jointly contribute to excessive osteoclastogenesis.

Asp8 peptide for delivering Dancr. Our data demonstrated that Dancr overexpression AAV has a significant bone-target property and remarkably alleviated OVX-induced trabecular bone loss and excessive osteoclastogenesis in vivo.

Certain limitations persist within this study. Given that the OVX surgery was conducted on mice during their comparatively juvenile stages (10 wk and 3 mo), when skeletal development is characterized by modeling rather than remodeling, it becomes evident that this model falls short of fully emulating the clinical manifestation of POMP osteoporosis. Furthermore, our assessment solely encompassed the evaluation of bone mass alterations in global or conditional knockout mice at the age of 12 wk, with no consideration for the influence of chronological age. Hence, in forthcoming investigations, we shall endeavor to explore the synergistic interplay between Dancr and other contributing factors in the pathogenesis of osteoporosis.

In summary, Dancr serves as a negative regulator of osteoclast differentiation, and its expression is reduced in response to estrogen deficiency. Mechanically, Dancr directly binds to BRG1 to preserve H3K27me3 enrichment at the Nfatc1 and Pgc1β promoters, thereby maintaining appropriate osteoclastic gene expression and metabolic shifts during osteoclastogenesis (Fig. 7). Notably, in vivo overexpression of Dancr significantly mitigated OVX-induced bone loss, indicating its potential as a therapeutic agent.

Materials and Methods

Ethical Approval. The ethical committee of the Naval Medical University granted approval for the preparation of human bone marrow samples (82172516). Informed consent was obtained from all participants. Furthermore, all mouse studies were performed in accordance with the guidelines of the Ethics Committee on Animal Experiments of the Naval Medical University. The comprehensive depiction of the gathering and arrangement of human osseous specimens was encompassed within *SI Appendix*.

In Vivo Experiments. All animals were housed in the Specific Pathogen Free laboratory room of the Animal Experimental Center at the Naval Medical University, where they were provided with an appropriate environment and fed a standard diet. The elucidation of OVX, generation of global or osteoclast-specific Dancr knockout mice, micro-CT analysis, bone histomorphometry, and osteoclast-targeting AAV-delivery in OVX mice was incorporated within *SI Appendix*.

In Vitro Experiments. Mouse primary osteoclasts were differentiated from bone marrow–derived monocytes (BMMs) through the stimulation of M-CSF and RANKL, as previously described (53). Detailed protocols of in vitro osteoclast

differentiation, pit formation assay, synthesis and cell transfection of siRNA, and mitochondrial oxygen consumption measurement were described in *SI Appendix*.

Molecular Experiments. The protocols of high-throughput RNA-seq and bioinformatic analysis, RNA extraction and qPCR analysis, western Blot, the calculation of mtDNA, RNA pulldown assay, mass spectrum, RIP, and CHIP combined with qPCR were included in *SI Appendix*.

Statistical Analysis. All statistical analyses were carried out by GraphPad prism version 8.0 (GraphPad Software, USA) and R software. Differences were considered significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The detailed description of this part was encompassed within *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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