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YAP1 is essential for osteoclastogenesis through a TEADs-dependent mechanism

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ABSTRACT

Yes-associated protein 1 (YAP1), the core effector of the Hippo signaling pathway, has been identified as a key regulator of tissue homeostasis and organ development by controlling cell proliferation and differentiation. Previous studies have shown that YAP1 regulates multiple steps during skeletal development and bone remodeling, including the self-renewal and differentiation of mesenchymal stem cells (MSCs). However, its role in osteoclastogenesis remains largely unknown. Here, we report that YAP1 is an essential regulator for osteoclast differentiation and activity. Both mRNA and protein levels of YAP1 were downregulated during RANKL-induced osteoclastogenesis. Short hairpin RNA-mediated knockdown of YAP1 in bone marrow-derived macrophages (BMM) prevented the formation and function of multinucleated osteoclasts, and markedly abrogated the expression of osteoclast marker genes. Furthermore, the suppression of osteoclastogenesis and bone resorption activity were also observed in the BMM treated with verteporfin, a small molecule that inhibits the association of YAP1 with the transcriptional enhancer-associated domain (TEAD) family of transcription factors, the major partner of YAP1. Mechanistically, the interaction of YAP1/TEADs with AP-1 and cooperation on downstream gene transcription were confirmed, and RANKL-induced NF-κB signaling was also impaired in the YAP1-inhibited condition. Our results revealed the essential role of YAP1 and the YAP1-TEADs complex in regulating osteoclastogenesis and related gene expression.

1. Introduction

Bone undergoes persistent remodeling by balancing bone-forming osteoblasts and bone-resorbing osteoclasts. Excessive osteoclast activity is associated with diseases, such as osteoporosis, arthritis, and cancer bone metastasis [1]. Osteoclasts are derived from the monocyte/macrophage cell lineage, and their activities are intricately regulated by various hormones and cytokines, among which macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (RANK) ligand (RANKL) are essential and sufficient. M-CSF acts as a crucial survival and proliferation factor for osteoclast precursor cells, while RANKL provides the crucial signal to drive osteoclast development and activate mature osteoclasts [2]. Upon binding of RANKL to its receptor RANK, tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are recruited, and as a result, mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling are activated, followed by the upregulation of the transcription factors NF-κB and activator protein 1 (AP-1). The osteoclastic master transcription factor nuclear factor of activated T-cells c1 (NFATc1) is consequently induced by the coordination of AP-1, NF-κB, and calcium signaling [3,4].

Yes-associated protein 1 (YAP1), the core effector of the Hippo pathway, plays a central role in tissue homeostasis and tumorigenesis by regulating cell proliferation and differentiation. In the Hippo pathway, LATS1/2, which are activated by MST1/2, directly phosphorylate and inhibit YAP1. As a transcriptional co-activator, YAP1 cannot bind DNA directly as it must interact with DNA-binding transcription factors to regulate target gene expression [5,6]. The transcriptional enhancer-associated domain (TEAD) family of transcription factors is believed to be the major partners of YAP1 in transcriptional regulation [7]. It has recently been shown that YAP1 is also able to interact with many other transcription factors through its WW domain that binds to Pro-Pro-X-Tyr motifs, suggesting that YAP1 serves as an important regulator during various biological processes [8–10].

Previous studies have indicated that Hippo signaling probably plays a role in osteoclastogenesis [11,12]. Upstream kinase MST2, which can restrain the activity of YAP1, has also been shown to be involved in bone homeostasis, functioning as a reciprocal regulator of osteoclasts and osteoblasts differentiation through the NF-kB pathway [12]. However, it is still not known if MST2 exerts its function by restraining the

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activity of YAP1. Transcription factor AP-1 has been shown to associate with YAP1/TEADs to coordinate target gene transcription, thereby directing cell migration and invasion [13–15]. Furthermore, AP-1-promoted tumorigenesis has been prevented in YAP1 conditional knockout mice [13]. These results suggest that YAP1 may play an essential role in osteoclastogenesis, as the two main signaling pathways are regulated by YAP1.

Here, we investigated the role of YAP1 in osteoclasts and provide insights into the cooperation of YAP1 with other osteoclastic transcription factors and canonical signaling pathways in regulating osteoclast differentiation and resorption activity.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies against YAP1, p-YAP1, NFATc1, c-JUN, c-FOS, IKKβ, p-IKKα/β, IκBα, p-IκBα, NF-κB (p65), p-NF-κB (p-p65), JNK, p-JNK, ERK, p-ERK, P38, p-P38, and DDK-Tag (binds to the same epitope as Sigma-Aldrich's anti-FLAG M2 Antibody) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against CTSK and TRAP were obtained from Proteintech Group (Wuhan, Hubei, China). Antibodies against β-Actin and the MTT cell proliferation and the cytotoxicity assay kit were bought from Boster Biological Technology (Wuhan, Hubei, China). Recombinant soluble murine M-CSF and RANKL were purchased from PeproTech (Rocky Hill, NJ, USA). The secondary antibodies were acquired from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The TRAP staining kit, verteporfin (VP), and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The basal culture mediums were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Osteoclast differentiation and function

Bone marrow-derived macrophages (BMM) were isolated from the tibias and femurs of 8-week-old male C57BL/6 mice as previously described [16]. In brief, the femurs and tibias were separated from the mice that had been killed, and soft tissue was removed; then, the marrow cavities were flushed with culture medium into a 10-cm dish. The cells were cultured in α-MEM medium containing 10% fetal bovine serum and 30 ng/mL M-CSF. On the following day, the floating cells were collected and plated in another dish. After 3 days, the adherent cells were used as osteoclast precursors and further cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 5–7 days, then multinucleated osteoclasts were fixed and stained using the TRAP kit according to the manufacturer's protocol. Mature osteoclasts were identified as multinucleated (≥3 nuclei) TRAP+ cells. RAW264.7 cells, from a murine monocytic cell line, were cultured in DMEM medium containing 10% fetal bovine serum and 75 ng/mL RANKL for 3–5 days, and subsequent fixing and staining were the same as with the BMM.

Actin ring and pit formation assays were conducted as previously described to analyze the function of the osteoclasts [16]. BMM were cultured on a 0.2% collagen-gel coated 6-well plate with M-CSF and RANKL for 5 days. Osteoclasts were digested with type I collagenase (Sigma-Aldrich) and seeded in Corning osteo assay strip wells, and then treated with different concentrations of VP for 3 days in the presence of M-CSF and RANKL. Then, the cells were incubated with 0.1% Triton-X for permeabilization, followed by staining with actin-tracker green for 1 h at 25 °C. After washing 5 times with PBS, the nuclei were counterstained with DAPI for 5 min. Actin rings were visualized fluorescently and the total number per well was counted. No distinction was made between large and small actin ring [17]. Then the cells were washed with a bleach solution and pit formation was quantitatively measured through the resorption area.

Animals were supplied by the University Laboratory Animal Center. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology.

2.3. Adenovirus and plasmids

Adenovirus carrying shRNA-targeting murine YAP1 and control viruses were cloned and packaged by Vigene Biosciences (Rockville, MD, USA). Four shRNAs were designed in one vector targeting different regions of YAP1. The sequences for each shRNA were as follows: 5′-GAAGCGCTGAGTTCCGAAATCTTCAAGAGAGATTTCGGAACTCAGCGCTTC TTTTTT-3′ for shRNA1, 5′-GTGAGAACAA TGACAACCAATATTCAAG AGATATTGGTTGTCATTGTTCTCACTTTTTT-3′ for shRNA2, 5′-GCCAGTACTGATGCAGGTACTTTCAAGAGAAGTACCTGCATCAGTACTGGC TTTTTT-3′ for shRNA3, and 5′-GGCAGGCAATACGGAATATCATTC AAGAGATGATATTCCGTATTGCCTGCCTTTTTT-3′ for shRNA4. DDK-tagged TEAD4, DDK-tagged YAP1, and the control vector were purchased from OriGene Technologies (Rockville, MD, USA).

2.4. Quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), and real-time PCR was performed using KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosystems, Hallandale, FL, USA). The following primer sequences were used: YAP1, 5′-AGACACCATCAGCCAAAGC-3′ (sense) and 5′-CACAGACTCCACGTCCAAG-3′ (antisense); NFATc1, 5′-TCTTCCGAGTT CACATCCC-3′ (sense) and 5′-GACAGCACCATCTTCTTCC-3′ (antisense); c-FOS, 5′GGTGAAGACCGTGTCAGGAG-3′ (sense) and 5′-TATTCCGTT CCCTTCGGATT-3′ (antisense); TRAP, 5′-GATGCCAGCGACAAGAGGTT-3′ (sense) and 5′-CATACCAGGGGATGTTGCGAA-3′ (antisense); CTSK, 5′-GAAGA AGACTCACCAGAAGCAG-3′ (sense) and 5′-TCCAGGTTATGGG CAGAGATT-3′ (antisense); GAPDH, 5′-CTCCCACTCTTCCACCTTCG-3′ (sense) and 5′-TTGCTGTAGCCGTATTCATT-3′ (antisense). The relative mRNA levels of target genes were calculated by the comparative C_T method (also known as the $2^{-\Delta\Delta CT}$ method) using GAPDH as an internal control and normalized to the control [18].

2.5. Co-immunoprecipitation and Western blotting

The interactions of YAP1 with AP-1 proteins were detected both in transfected NIH3T3 cells and un-transfected BMM. Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton, 1 mM EDTA, and 10% glycerol) containing protease and phosphatase inhibitor. Cell lysates were pre-cleared by incubating with the control IgG and protein A/G beads for 1 h to soak up any proteins that non-specifically bound to the immunoprecipitation components. For immunoprecipitation, the pre-cleared cell lysates were incubated with the indicated antibodies and protein A/G beads overnight. The immunoprecipitates were washed 5 times with lysis buffer, boiled in the SDS sample buffer, and then subjected to Western blotting. Western blotting was performed following the protocol from Cell Signaling Technology using the antibodies listed above. All western blot bands were quantitated (Gel-Pro Analyzer software) and analyzed based on at least three samples.

2.6. Electrophoretic mobility shift assay

The DNA-binding activity of NF-κB and AP-1 was detected using a LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) Kit (Thermo Fisher Scientific, Shanghai, China) as described previously [19]. Nuclear extracts of BMM in a 10 cm dish were prepared using a nuclear and cytoplasmic protein extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China) and quantified. An equal amount of nuclear extract

was incubated with the probe in the reaction buffer for 20 min. Reactants were loaded onto a 6% native polyacrylamide gel, transferred onto a positively charged nylon membrane, and then the DNA was cross-linked by a UV cross-linker. The biotin end-labeled DNA was detected using a Streptavidin-HRP conjugate and a chemiluminescent substrate. The AP-1 and NF-κB probes (Beyotime Institute of Biotechnology, Jiangsu, China) used for EMSA, containing the recognition sites for AP-1 and NF-κB, were as follows: AP-1, 5-TACATAATGACTT CAGCATGC-3 (NFATc1 site containing) and NF-κ B, 5′-AGTTGA GGGGACTTTCCCAGGC-3′.

2.7. Statistical analysis

All experiments were performed at least 3 times independently and the results are presented as the mean ± standard deviation (SD). Non-parametric tests were used for analysis of quantitative PCR results. In Fig. 1A and 3D, Kruskal–Wallis test was used for comparisons among more than two groups followed by Dunn-Bonferroni pairwise comparisons for post hoc comparisons. In Fig. 2D, four experiment groups were divided into two group sets (with or without RANKL treatment), so Mann–Whitney *U* test was used for comparisons between the two groups within each group sets [20]. In dealing with other data (including osteoclast cell counting, western blot protein quantification, bone resorption area), a two-tailed Student's *t*-test was used for comparisons between 2 groups, and a One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used in the cases of comparison involving more than 2 groups. Statistical significance was considered as $P < 0.05$. *P < 0.05, **P < 0.01, and ***P < 0.001, versus the control.

3. Results

3.1. Expression and activity of YAP1 are regulated during osteoclast differentiation

In the Hippo pathway, YAP1 is directly phosphorylated and inhibited by LATS1/2, and LATS1/2 are phosphorylated and activated by MST1/2. Phosphorylation of YAP1 on serine 127 generates a 14-3-3 binding site, and binding with 14-3-3 sequesters YAP1 in the cytoplasm [6].

We measured the level and activity of YAP1 during osteoclast differentiation to determine whether YAP1 is involved in osteoclastogenesis. The mRNA and protein levels of osteoclast-related genes, including c-FOS, NFATc1, CTSK (cathepsin K), and TRAP (tartrate-resistant acid phosphatase) gradually increased during osteoclast differentiation (Fig. 1A and B). However, the mRNA level of YAP1 was significantly decreased from day 1 (Fig. 1A), and the protein level of YAP1 had a significant reduction from day 3 (Fig. 1B). Moreover, RANKL stimulation

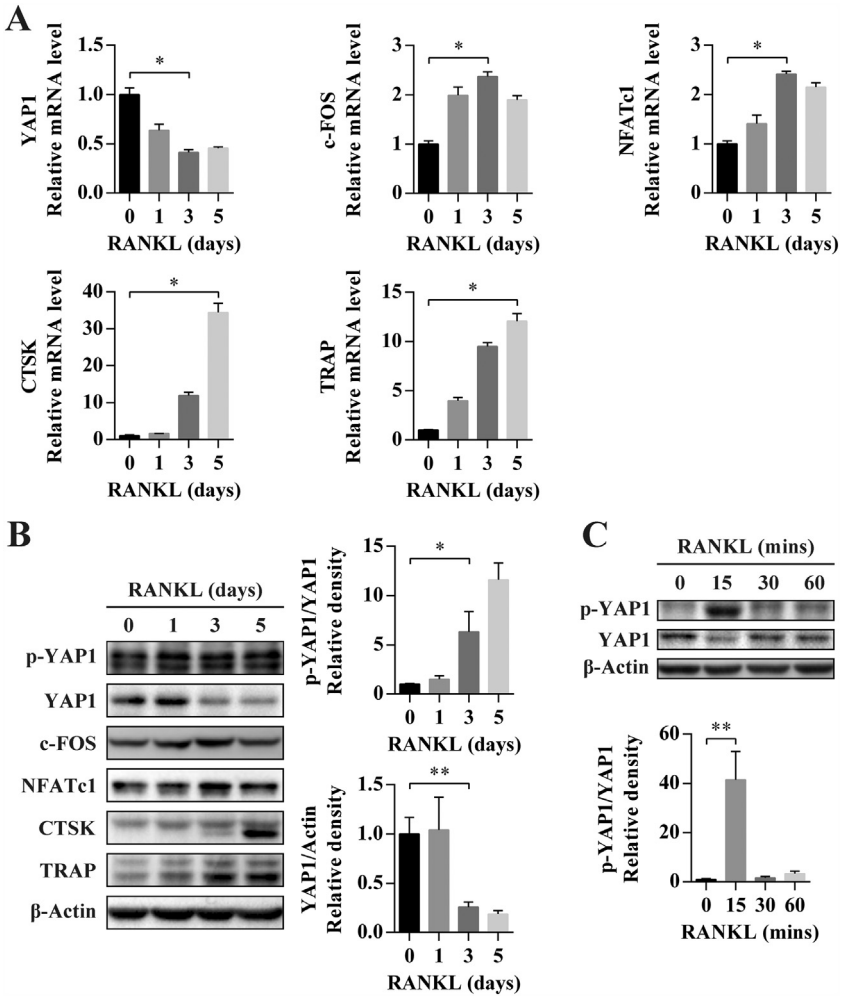

Fig. 1. Expression and activity of YAP1 are regulated during osteoclast differentiation. BMM were cultured with M-CSF and RANKL for the indicated time periods. (A) The mRNA levels of YAP1 and osteoclast-related genes were assessed by qPCR. (B) The protein levels of YAP1 and osteoclast-related genes were analyzed by Western blotting. (C) The total and phosphorylated protein levels of YAP1 were analyzed by Western blotting. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, versus day 0.

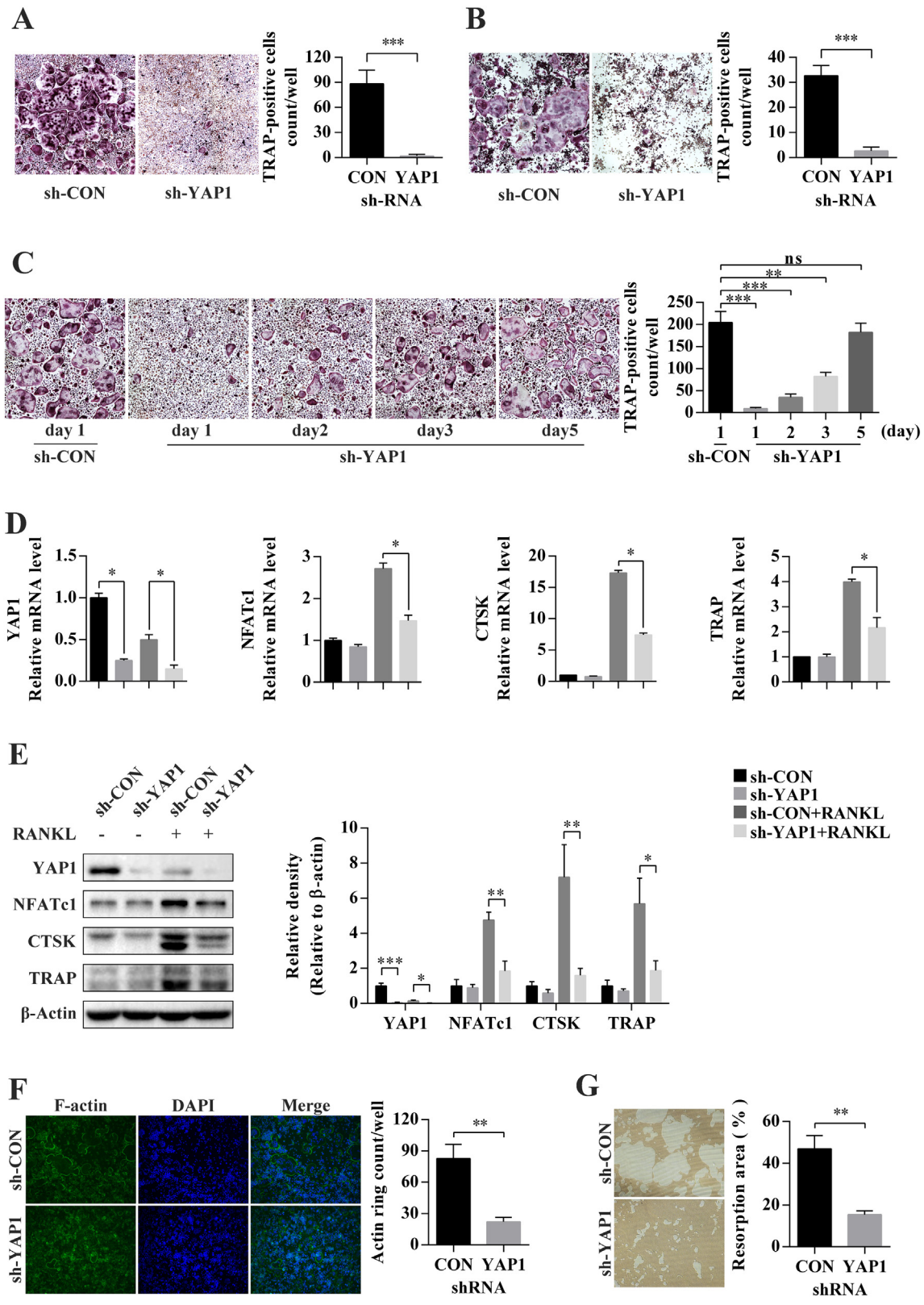

Fig. 2. Genetic knockdown of YAP1 severely impairs osteoclast differentiation and related gene expression. (A, B) BMM (A) or RAW264.7 cells (B) were infected with an adenovirus carrying YAP1-specific shRNA or a control adenovirus and then cultured in the presence of M-CSF (for BMM only) and RANKL for 7 (BMM) or 4 (RAW264.7) days. TRAP staining was performed, and TRAP-positive cells with three or more nuclei were counted. (C) BMM were cultured in the presence of M-CSF and RANKL, and were infected with YAP1-specific shRNA at indicated time of osteoclastogenesis. TRAP staining was performed when control wells formatted osteoclast mostly. (D, E) BMM were treated as described in (A), and the mRNA (D) and protein (E) levels were measured by qPCR or Western blotting, the relative density of all proteins of interest is expressed relative to β-actin. (F, G) Mature osteoclasts from BMM were seeded in Corning osteo assay strip wells and infected with the adenovirus carrying YAP1-specific shRNA or control adenovirus, then cultured for 3 days in the presence of M-CSF and RANKL. F-actin staining (F) or pit formation assays (G) were performed. The actin rings and resorption area were quantified in the right column. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, versus the control adenovirus.

induced a rapid and late phase phosphorylation of YAP1 (Fig. 1B and C), indicating that the activity of YAP1 was also regulated. Taken together, the levels and activity of YAP1 were simultaneously regulated during osteoclastogenesis.

3.2. Knockdown of YAP1 severely impairs osteoclast differentiation and related gene expression

BMM or RAW264.7 cells were infected with an adenovirus carrying YAP1-specific shRNA or the control adenovirus to examine the role of YAP1 in osteoclast differentiation. The mRNA and protein levels of YAP1 were significantly reduced by an adenovirus carrying YAP1 shRNA, compared with that in the control adenovirus (Fig. 2D and E). Knockdown of YAP1 significantly attenuated RANKL-induced osteoclast differentiation, as revealed by the decreased TRAP-positive multinuclear osteoclast formation (Fig. 2A for BMM and 2B for RAW264.7). However, the early phase knockdown of YAP1 had a more effective inhibition effects on osteoclast formation (Fig. 1C). The expression of osteoclast marker genes such as NFATc1, TRAP, and CTSK were significantly impaired in YAP1 deficient BMM (Fig. 2D), accompanied by down regulation of protein levels (Fig. 2E).

Bone resorption is initiated by the formation of an actin-rich sealing zone that isolates the resorptive microenvironment from the general extracellular space [21], we examined the effect of YAP1 knockdown on osteoclast function by performing actin ring formation and bone resorption pit formation assays. Mature osteoclasts were seeded on an osteo assay strip well and infected with the adenovirus carrying YAP1-specific shRNA or the control adenovirus, and subsequently cultured for 3 more days in the presence of M-CSF and RANKL to measure the resorption activity of the osteoclasts. The F-actin staining results revealed that actin ring formation was noticeably inhibited by the YAP1 deficiency (Fig. 2F). Consequently, the quantitative results of the resorption

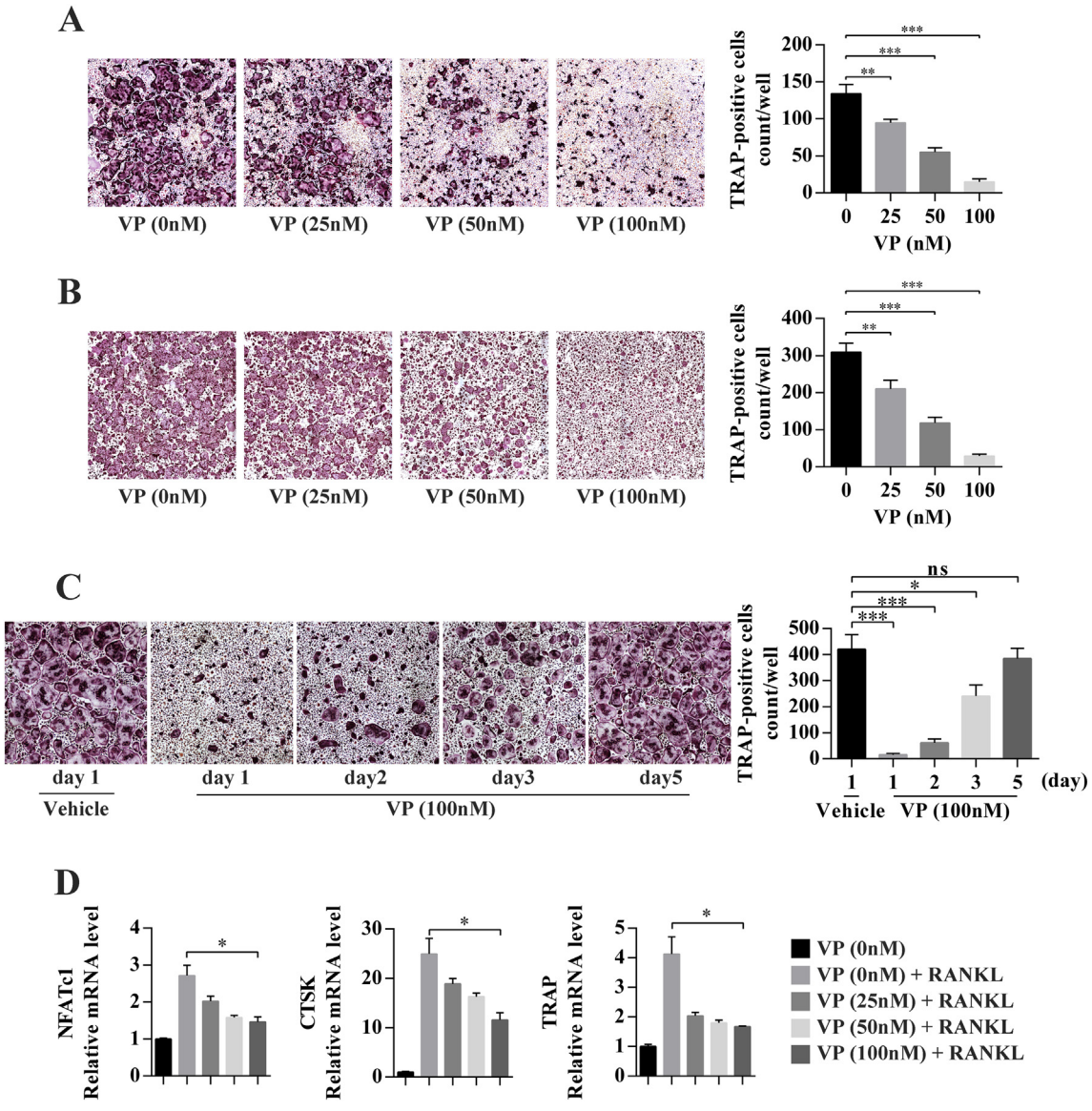

Fig. 3. Verteporfin suppresses osteoclast formation and related gene expression. (A, B) BMM (A) or RAW264.7 cells (B) were seeded in 96-wells plates overnight and then treated with different concentrations of verteporfin in the presence of M-CSF (for BMM only) and RANKL for 7 (BMM) or 4 (RAW264.7) days. TRAP staining was performed, and the TRAP-positive cells with three or more nuclei are counted in the right column. (C) BMM were cultured in the presence of M-CSF and RANKL, and were treated with verteporfin at indicated time of osteoclastogenesis. TRAP staining was performed when control wells formatted osteoclast mostly. (D) BMM were treated with different concentrations of verteporfin in the presence of M-CSF and RANKL for 3 days. The mRNA levels of osteoclast-related genes were assessed by qPCR. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, versus the vehicle.

pits showed that the YAP1 deficiency had strong inhibitory effects on osteoclasts resorption activity (Fig. 2G). These results revealed that YAP1 is essential for osteoclast differentiation and function.

3.3. Pharmacological disruption of the YAP1-TEADs association suppresses osteoclast formation and activity

Since the TEAD family of transcription factors is believed to be the major partners of YAP1 in transcriptional regulation [22], we checked the role of the YAP1-TEADs association in YAP1-regulated osteoclastogenesis. Verteporfin has recently been identified as a small molecule that inhibits the YAP1-TEADs association and YAP1-induced gene expression [23,24]. We treated the BMM and RAW264.7 cells with verteporfin to validate the important role of YAP1 in osteoclastogenesis and determine whether the effect is TEAD-dependent. TRAP staining results showed that verteporfin inhibited the formation of osteoclasts in a dose-dependent manner at 25–100 nM both in BMM (Fig. 3A) and RAW264.7 (Fig. 3B), without obvious inhibition of cell proliferation detected by MTT assay (data not shown). Verteporfin inhibited the formation of osteoclasts mainly in early phase (Fig. 3C). At the same time, the mRNA levels of the osteoclast master transcription factor NFATc1 as well as osteoclast marker genes CTSK and TRAP were downregulated in the presence of verteporfin (Fig. 3D).

The effects of verteporfin on osteoclast function were also examined by performing actin ring formation and bone resorption pit formation assays. The F-actin staining results revealed that actin ring formation was significantly inhibited by verteporfin treatment, even at a very low dose (Fig. 4A). Quantitative results of the resorption pits showed that verteporfin had strong concentration-dependent inhibitory effects on osteoclast resorption activity (Fig. 4B).

Taken together, these results indicate that verteporfin is a potent inhibitor of osteoclast differentiation and activity, which is in agreement with the knockdown of YAP1. In other words, the transcriptional coactivator YAP1 certainly plays an essential role in osteoclastogenesis, and this effect is particularly dependent on the YAP1-TEADs association.

3.4. YAP1/TEADs interact with AP1 and are required for AP-1 transcriptional activity

Previous studies showed that YAP1/TEADs and AP-1 proteins (TEAD4 with JUND, TEAD1 with FOSL1, and c-JUN and JUND) can form a complex and synergistically activate target gene transcription [13–15]. During osteoclastogenesis, the most important AP-1 dimers are formed by c-FOS and c-JUN proteins [25,26]. Here, we detected the interaction of YAP1/TEADs and c-JUN and c-FOS through co-immunoprecipitation.

Our co-immunoprecipitation assays detected DDK-tagged TEAD4 binding to endogenous YAP1 and c-JUN in the NIH3T3 cells (Fig. 5A). However, the direct interactions between DDK-tagged YAP1 and c-JUN were not detected (Fig. 5B). Further, we confirmed the endogenous interactions in the BMM cells (Fig. 5C). The transcriptional activity of AP-1 was partially represented by its DNA-binding capacity, which can be measured by EMSA. The basal and RANKL-induced DNA-binding capacity of AP-1 were both impaired by verteporfin (Fig. 5D), indicating that the YAP1/TEADs association plays a role in AP-1 transcriptional activity. Since the reduced DNA-binding capacity can be caused by intranuclear AP-1 protein level change or DNA-binding activity change, we examined the MAPK signaling activity, which is the main regulator of the intranuclear protein level of AP-1 [27]. As shown in Fig. 5E and F, verteporfin had little effect on the RANKL-induced phosphorylation of ERK and P38, and it even enhanced the phosphorylation of JNK; therefore, it can be concluded that the reduced DNA-binding capacity was caused by the reduced DNA-binding activity in the presence of verteporfin. These results indicated that YAP1 and the YAP1/TEADs association were both required for AP-1 transcriptional activity.

3.5. RANKL-induced NF-κB signaling is impaired in the YAP1-inhibited condition

To further explore the molecular mechanism through which NFATc1 was downregulated in the YAP1-inhibited condition, the canonical NF-

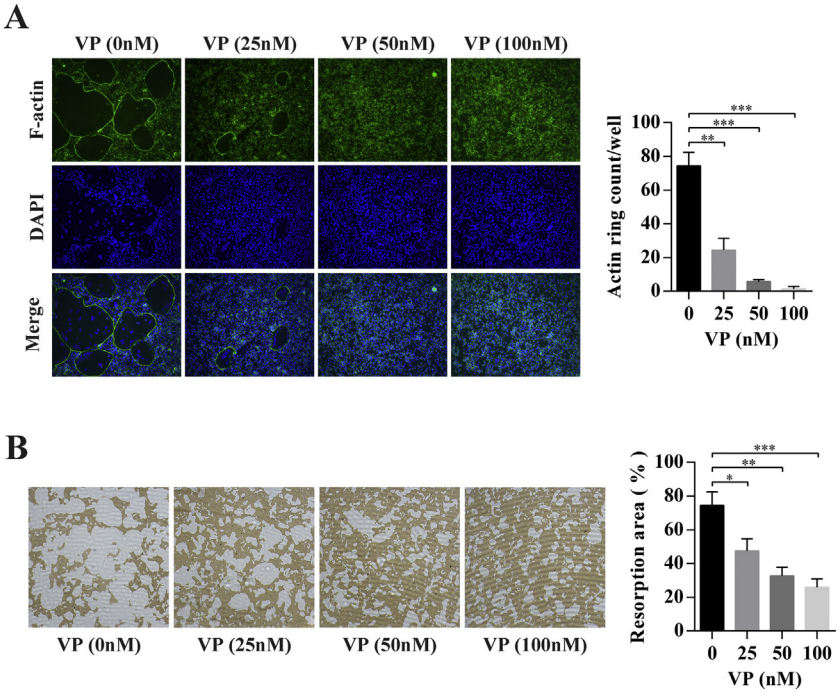

Fig. 4. Verteporfin inhibits the bone resorption activity of osteoclasts. Mature osteoclasts from BMM were seeded in Corning osteo assay strip wells, and they were treated with different concentrations of verteporfin for 3 days in the presence of M-CSF and RANKL. (A) F-actin staining was performed. The actin rings are quantified in the right column. (B) Pit formation assays were performed. The resorption areas are quantified in the right column. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, versus the vehicle.

κB signaling pathway, which is also responsible for NFATc1 induction, was examined by Western blotting and EMSA [4]. The Western blotting results showed that the phosphorylation of all three of the NF-κB signaling cascades was inhibited by verteporfin, especially at 15 min (Fig. 6A and C). In other words, the activity of NF-κB signaling was repressed by verteporfin. The EMSA results revealed the DNA-binding capacity of transcription factor NF-κB induced by RANKL was also repressed by verteporfin (Fig. 6B). These results indicated that impaired NF-κB signaling was also involved in YAP1-inhibited osteoclastogenesis.

4. Discussion

In the present study, we investigated the role of YAP1 in osteoclasts. We found that YAP1 was essential for RANKL-induced osteoclast

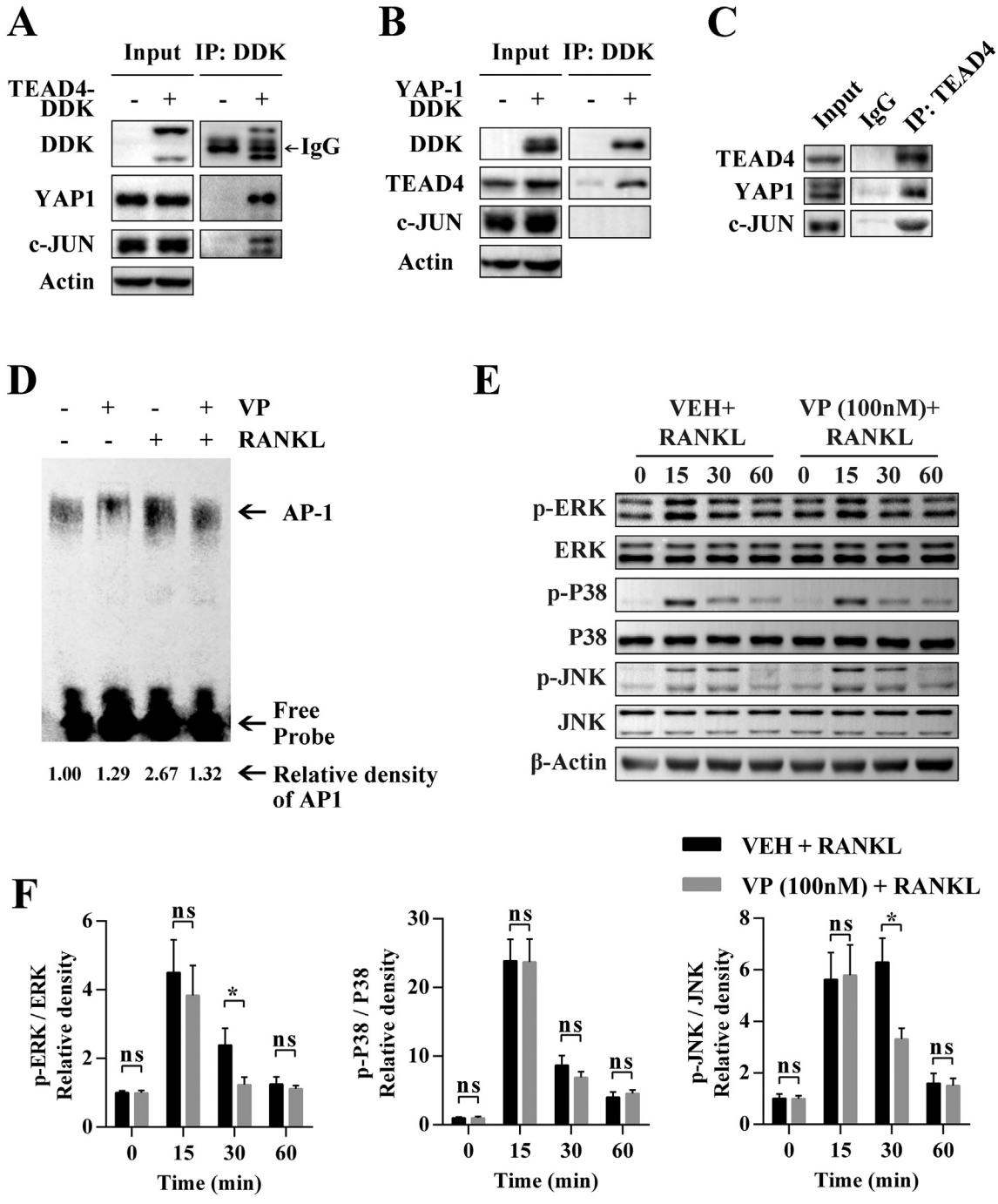

Fig. 5. YAP1/TEADs interact with AP1 and are required for AP-1 transcriptional activity. (A) NIH3T3 cells were transiently transfected with TEAD4-DDK or the control vectors, and TEAD4 was immunoprecipitated with the anti-DDK antibody. Immunoblot analysis showed that interactions between TEAD4 with c-JUN and YAP1 were detected by the indicated antibody. (B) NIH3T3 cells were transiently transfected with YAP1-DDK or the control vectors, and YAP1 was immunoprecipitated with the anti-DDK antibody. Immunoblot analysis showed that interactions between YAP1 and c-JUN were not detected by the indicated antibody. (C) Cell lysates were collected from BMM, and TEAD4 was immunoprecipitated with the anti-TEAD4 antibody. Immunoblot analysis showed that the interactions between TEAD4 with c-JUN and YAP1 were detected by the indicated antibody. (D) BMM were treated with verteporfin (100 nM) or the vehicle and stimulated with or without RANKL (100 ng/mL) for 24 h. Then, the nuclear protein was prepared and subjected to EMSA. The position of probe-AP-1 complexes was confirmed by competitive probe. (E, F) BMM were cultured with α-MEM in the absence of FBS for 16 h, and then pretreated with verteporfin (100 nM) or the vehicle for 2 h. Finally, BMM were stimulated with or without RANKL (100 ng/mL) for the indicated times. Total and phosphorylated protein levels of MAPK signaling components were analyzed by Western blotting. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, versus the vehicle.

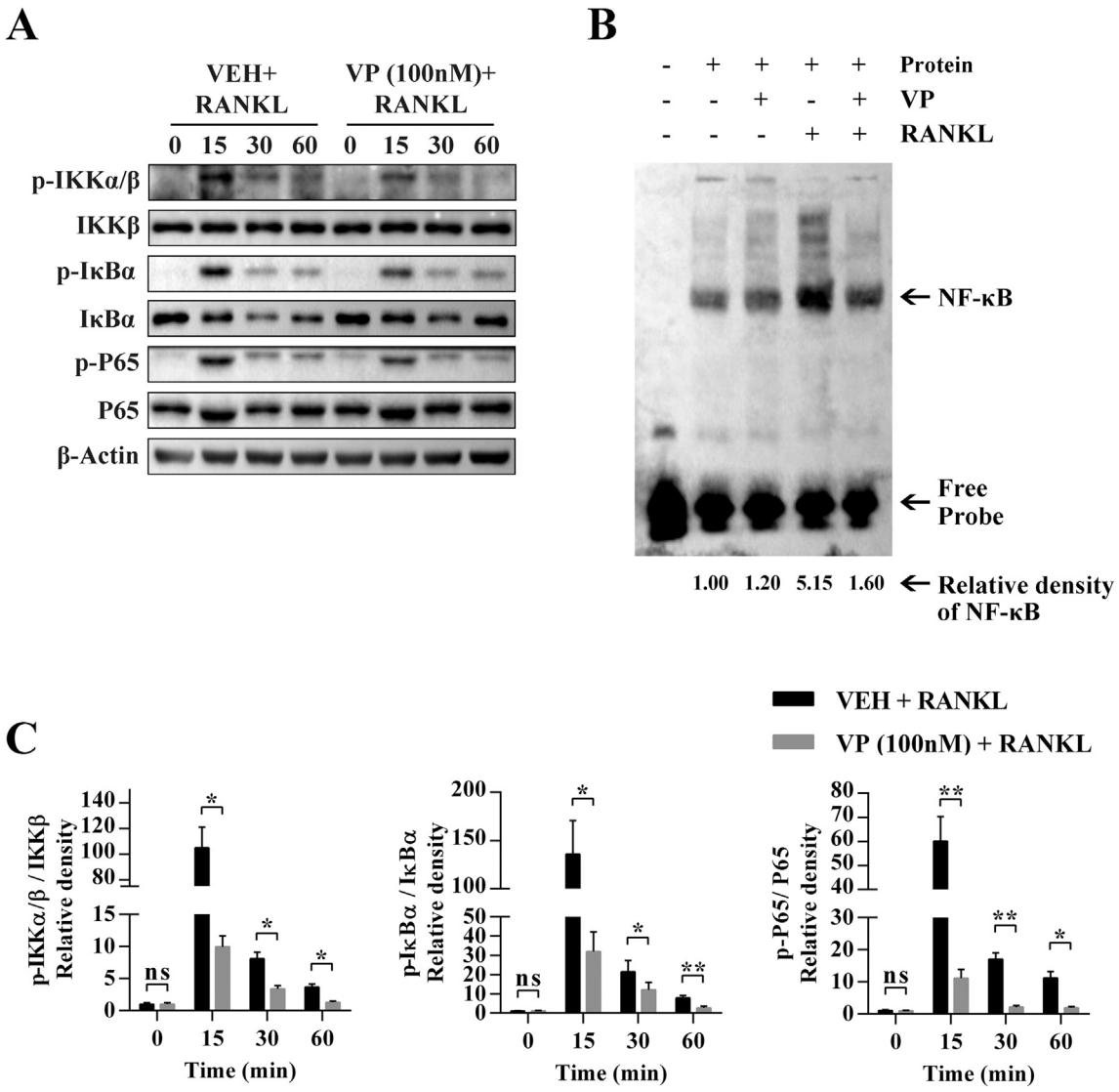

Fig. 6. RANKL-induced NF-κB signaling is impaired in the YAP1-inhibited condition. (A, C) BMM were cultured with α-MEM in the absence of FBS for 16 h, and then pretreated with verteporfin (100 nM) or the vehicle for 2 h. Finally, BMM were stimulated with or without RANKL (100 ng/mL) for the indicated times. Total and phosphorylated protein levels of NF-κB signaling components were analyzed by Western blotting. (B) BMM were pretreated with verteporfin (100 nM) or vehicle for 2 h and then stimulated with or without RANKL (100 ng/mL) for 30 min. Then, the nuclear protein was prepared and subjected to EMSA. The position of the probe-NF-κB complexes was confirmed by competitive probe. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, versus the vehicle.

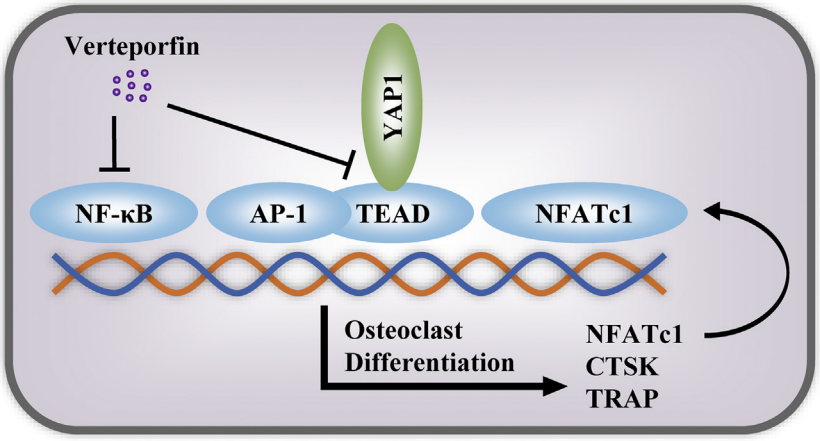

Fig. 7. Graphical summary of the regulatory mechanism of YAP1 in osteoclasts. YAP1/TEAD4 interact with AP-1, and synergistically activate the transcription of NFATc1 and osteoclast marker genes. Verteporfin inhibits formation and bone resorption activity by disturbing the association of the YAP1-TEAD4-AP-1 complex and repressing the activity of NF-κB signaling.

differentiation and bone resorption activity. The association of YAP1 with its major partners, TEAD family transcription factors, was indispensable. Multiple aspects of osteoclastogenesis were regulated by YAP1/TEADs, in which transcription factor AP-1 was confirmed to interact with YAP1/TEADs to potentiate downstream gene transcription, and canonical NF-κB signaling was also impaired when YAP1/TEADs association was disturbed (summarized in Fig. 7).

The critical role of the Hippo pathway in development and regeneration has been well established. The Hippo pathway components are extensively involved in regulating bone-forming osteoblasts during bone development and remodeling [28–30]; however, their role in regulating bone-resorbing osteoclasts was insufficiently investigated. A few studies focusing on MST1/2, the upstream inhibitory kinase of YAP1, have provided insights into this field. In an earlier study, MST1 was confirmed to be a mediator of bisphosphonate-induced potent inhibition of bone resorption through the apoptotic pathway [11]. A recent study using knockout mice showed that MST2 was a negative regulator of osteoclast differentiation through the NF-κB pathway [12]. These two studies both confirmed that MST1/2 were negative regulators in osteoclastogenesis, but these investigations have not provided insight into the role of the Hippo pathway core intranuclear effector YAP1 in osteoclasts.

Our results in the present study first revealed the role of YAP1 in osteoclasts. The protein and mRNA levels of YAP1 were downregulated during osteoclast differentiation, indicating that YAP1 was possibly involved in regulating osteoclastogenesis. Subsequently, we knocked down YAP1 through adenovirus-mediated shRNA, and the results of TRAP staining, F-actin ring, and pit formation showed a considerable repression on osteoclast formation and function. These results proved the essential role of YAP1 in osteoclastogenesis, which is in agreement with the negative regulation effects of MST1/2 on osteoclasts. Taken together, these studies suggested that the Hippo signaling pathway plays an important role in osteoclastogenesis. However, further evidence is needed to confirm that MST1/2 exhibits its function by regulating the activity of YAP1. The overall view of how the Hippo pathway regulates bone development and remodeling is still obscure due to limited investigations.

The TEAD family of transcription factors has been proven to be the major partners of YAP1 and they mediate YAP1's abundant functions [22,31]. We examined the role of the YAP1/TEADs association in osteoclastogenesis using verteporfin, a small molecule that inhibits YAP1-TEADs interactions [23,24]. Our results showed that verteporfin is a potent inhibitor of both osteoclast differentiation and function, indicating that TEADs mediate the effect of YAP1 in osteoclasts, at least partially. Whether TEADs are sufficient for YAP1-regulated osteoclastogenesis has not been investigated here, as various signaling cascades are involved in osteoclastogenesis.

Osteoclast differentiation is regulated by complex signaling cascades that are triggered by RANKL. The activation of transcription factors, such as PU.1, microphthalmia-associated transcription factor (MITF), NF-κB, AP-1, and NFATc1, is required for sufficient osteoclast differentiation [32,33]. In particular, NFATc1 plays the role of a master regulator of osteoclast differentiation, and regulates a number of osteoclast-specific genes, such as TRAP and CTSK [34,35]. Our results showed that NFATc1 and its target genes were significantly downregulated in both YAP1-deficient and verteporfin-treated conditions. Several transcription factors, including AP-1 and NF-κB and calcium signaling, have been confirmed to regulate NFATc1 expression during osteoclastogenesis [4]. In this study, we focused on AP-1 and NF-κB. Several AP-1 proteins have been shown to interact with YAP1/TEADs in previous studies, including TEAD4 with JUND, TEAD1 with FOSL1, and c-JUN and JUND [13,14]. Through co-immunoprecipitation (using exogenously and endogenously proteins), we confirmed that TEAD4 mediated the interaction between YAP1 and c-JUN. The interaction of YAP1/TEADs with AP-1 promoted the DNA-binding activity and transcriptional activity of AP-1, which is shown by EMSA. The NF-κB signaling was also impaired by verteporfin treatment. These results indicated that both AP-1 and NF-κB mediated the effects of YAP1/TEADs in regulating osteoclastogenesis.

In conclusion, we have established the essential role of YAP1 as well as the YAP1/TEADs association in RANKL-induced osteoclast differentiation. We have also shown that the interaction between AP-1 and YAP1/TEADs was crucial for AP-1 transcriptional activity and the expression of NFATc1, which is crucial for osteoclastogenesis. These findings provide new insights into the molecular mechanism of osteoclastogenesis, and provide a potential therapeutic strategy for osteoclast-related disorders through the inhibition of YAP1 or the YAP1/TEAD association.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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