

# The CRLF2-JAK2/STAT3 Axis Promotes Bone Formation and Ameliorates OVX-Induced Osteoporosis

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## Research Article

**Keywords:** Osteoporosis, CRLF2, JAK2, STAT3, Bone Remodeling

**Posted Date:** October 27th, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-11298170/v1>

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**Additional Declarations:** No competing interests reported.

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# Abstract

## Background

Osteoporosis is a typical disorder of bone metabolism imbalance, and its occurrence and development are closely related to the immune system, which has become a core topic in the field of "bone immunology". CRLF2, a cytokine receptor-like factor, is a key molecule in immune regulation and plays an important role in lymphocyte differentiation and inflammatory factor signal transduction. There is evidence suggesting that the protein TSLPR (TSLP receptor) encoded by *Crlf2* is expressed on the precursor cells of osteoclasts (such as bone marrow-derived macrophages BMMs). TSLP can directly act on the precursor cells of osteoclasts and inhibit the osteoclast differentiation induced by RANKL. However, the specific role of CRLF2 in osteoporosis remains unknown. This study aims to explore the functional role of CRLF2 in the process of osteoporosis and further reveal its potential molecular mechanism, providing new theoretical basis for understanding the cross-regulation of immune-skeletal interactions.

## Methods

Firstly, using six public databases we evaluated the correlation between CRLF2 expression and osteoporosis, also and osteoclasts. Secondly, by constructing CRLF2 knockout (*Crlf2*-KO) mice and wild type mice with ovariectomy (OVX) to induce osteoporosis mouse models, the effects of CRLF2 on bone density, bone microstructure, and bone metabolism markers were evaluated *in vivo* through Micro-CT, HE staining, immunohistochemistry(IHC), ELISA Kit, Western Blot, and RT-qPCR methods. Finally, by transfecting the overexpressed CRLF2 plasmid into MC3T3-E1 cells and using overexpression and knockdown CRLF2 adenovirus to infect primary bone marrow cells to induce their differentiation into osteoblasts, ALP staining, co-immunoprecipitation (Co-IP), and Western blotting were conducted to investigate the effects of CRLF2 on osteogenic differentiation and the JAK2/STAT3 signaling pathway.

## Results

Bioinformatics and experimental validation have shown that CRLF2 is upregulated in bone tissue of osteoporosis patients, bone tissue of ovariectomized female mice, and osteoclasts, but downregulated in osteoblasts. *In vivo* studies have demonstrated that compared with wild-type ovariectomized female mice, the bone density, bone volume fraction, and trabecular number of CRLF2 gene knockout ovariectomized female mice significantly increased. The expression of bone formation markers PINP and key osteogenic proteins (Runx2, OPN) was upregulated, the number and area of adipocytes were decreased, and the levels of local inflammatory factors were reduced. *In vitro* experiments confirmed that overexpression of CRLF2 significantly inhibited the differentiation of MC3T3-E1 cells and primary BM cells into osteoblasts, and knockdown had the opposite effect. Mechanism studies showed that CRLF2 directly binds to JAK2, activates the JAK2/STAT3 signaling pathway, promotes Stat3

phosphorylation and nuclear translocation to regulate the expression of osteogenic-related molecules, thereby inhibiting osteogenic differentiation.

## Conclusion

This study demonstrates that Crlf2 participates in the dynamic bone balance through activating the JAK2/STAT3 signaling pathway. Knockdown of Crlf2 can effectively alleviate bone loss caused by ovarian removal (OVX). These findings suggest that the CRLF2/JAK2/STAT3 axis may be a potential new target for the treatment of osteoporosis.

## Introduction

Osteoporosis is a systemic metabolic bone disorder characterized by reduced bone mass, microstructural destruction, and increased fracture risk, and has become a major global health issue [1]. With the aging population, its incidence has risen significantly, especially in postmenopausal women and elderly men. Clinically, primary osteoporosis is divided into type I (postmenopausal) and type II (senile) [2]. Osteoporotic fractures in the hip, vertebrae, and wrist cause disability, reduced quality of life, complications, and increased mortality [3]. The pathological essence of osteoporosis is an imbalance in bone remodeling, where bone resorption exceeds bone formation. This process involves various cells, molecules, and signals, though its mechanism remains incompletely understood. Bone metabolism depends on the precise coupling between osteoclast-mediated resorption and osteoblast-mediated formation [4]. Osteoclasts differentiate from hematopoietic stem cells, while osteoblasts originate from mesenchymal stem cells [5–7]. Under healthy conditions, resorption and formation are balanced, ensuring bone integrity and mineral homeostasis [8]. However, this balance can be disrupted by aging, hormone decline, genetics, nutrition, and lifestyle, with estrogen decline making postmenopausal women particularly vulnerable. Current anti-osteoporosis drugs (e.g., bisphosphonates, denosumab, parathyroid hormone analogues) have limitations [9]. Some patients respond poorly, and long-term use may cause adverse effects or necessitate a "drug holiday." Moreover, most therapies only delay bone loss without repairing microstructure. Therefore, elucidating molecular mechanisms and developing new therapeutic targets are important research directions.

In recent years, cytokine signaling pathways in bone remodeling have gained attention. Cytokine receptor-like factor 2 (CRLF2/TSLPR) is a type I cytokine receptor superfamily member [10]. CRLF2 forms heterodimeric complexes and participates in cellular signal transduction [11, 12]. It binds thymic stromal lymphopoietin (TSLP) to activate the JAK-STAT pathway via JAK2/JAK1, promoting STAT phosphorylation and nuclear translocation to regulate target gene expression [13–15]. Under normal conditions, CRLF2 is expressed in epithelial cells, stromal cells, and mast cells of the thymus, lungs, skin, intestines, and tonsils [16]. It plays roles in dendritic cell-mediated central tolerance, T cell homeostasis, and Th2 responses. Notably, CRLF2 abnormal expression is involved in diseases such as acute

lymphoblastic leukemia, where CRLF2 rearrangement drives leukemogenesis, suggesting a core role in cell proliferation and differentiation [17].

Although direct studies of CRLF2 in bone are scarce, structurally related receptors (e.g., gp130) regulate osteoblast and osteoclast differentiation and function [18]. This provides a basis for exploring CRLF2's role in osteoporosis.

## Materials and Methods

### 1. Animals and OVX model construction

All animal experimental procedures were approved by the Animal Ethics Committee of the Institute of Innovation and Translational Medicine, Gannan Medical University (Approval No.: GIMI-K2024236). Female C57BL/6J mice aged 12 weeks (body weight 18–22 g) were selected and housed in an SPF-level animal facility under controlled environmental conditions (room temperature 22–24°C, relative humidity 40%-70%, 12-hour/12-hour light-dark cycle) with free access to food and water. For anesthesia, meloxicam (2 mg/kg) was administered subcutaneously one hour prior to surgery for preemptive analgesia, and anesthesia depth was confirmed by the absence of toe pinch reflex. The dorsal lumbar region was shaved and sequentially disinfected with 75% ethanol and povidone-iodine. A longitudinal skin incision was made along the midline of the back, centered approximately 0.5 cm above the intersection point between the line connecting the upper edges of the thighs on both sides and the midline of the back. The skin incision was pulled to the left to expose the subcutaneous white adipose pad, which was carefully dissected using microforceps. Beneath it, pink granular ovaries were visible. The distal end of the uterine horn attached to the ovary was ligated with absorbable suture, and the ovary, fallopian tube, and a small segment of the uterine horn were completely removed. The adipose pad was then repositioned, and the peritoneum was simply closed with interrupted absorbable sutures. The same procedure was repeated on the right side by pulling the skin incision to the right and removing the contralateral ovary. Finally, the skin incision was closed with interrupted absorbable sutures, and the wound was disinfected with povidone-iodine. Mice in the sham surgery group underwent identical surgical procedures except that the ovaries were exposed but not removed—instead, they were immediately repositioned and sutured; in an alternative sham protocol, only a small amount of adipose tissue beside the ovaries was removed. After surgery, mice were placed on a 37°C heating pad until fully awake and housed individually to prevent wound licking. Meloxicam (2 mg/kg) was administered subcutaneously again 24 hours post-surgery for pain relief, and penicillin was given intraperitoneally daily for three consecutive days to prevent infection. Mice were euthanized at 12 weeks post-surgery for tissue collection and subsequent analyses.

### 2. Construction of CRLF2 gene knockout (-KO) mice

2. Construction of CRLF2 gene knockout (*Crlf2*-KO) mice

A pair of trans-activating factor-like effector (TALE) nucleases targeting exon 2 of the Sestrin1 gene was designed using an online targeted design tool (<https://tale-nt.cac.cornell.edu/node/add/talen-old>). Subsequently, using the "unit assembly" method, the TALEN expression plasmid was assembled. After linearization with the PmeI endonuclease (NEB, R0560L), the mRNA was transcribed and tail-ended using the mMessage mMachine T7 Ultra Kit (Ambion, AM1345), and purified using the RNeasy Mini Kit (Qiagen, 74104) to obtain mature mRNA. Then, these purified mRNA was mixed with the injection buffer and injected into the cytoplasm of mouse single-cell stage embryos. After 19–21 days, the injected fertilized eggs were transplanted into pseudopregnant female mice. The following primers were used to identify the genotypes of the mice: Forward: 5'-CTTGCAAAATGGCGTTACTTAAGC-3'; Reverse: 5'-CTGGAGTTCCGGTGAGTGAAG-3'. The background strain of the Crlf2 knockout mice was C57BL/6J mice. Before the OVX surgery was performed to create the osteoporosis model, the mice were bred for three generations.

### **3. Micro-CT assessment of bone density and bone microstructure**

The analysis of bone tissue by Micro-CT follows a standard procedure. After the femoral samples are fixed in 10% neutral formalin, they are stored in 70% ethanol. Before scanning, the samples are fixed in dedicated sample tube and immersed in 70% ethanol to eliminate air interference. The in vitro examination was conducted using a high-resolution micro-CT system (NEMO® Micro CT NMC-200, Pingsheng Medical Technology Co., Ltd.). The tube voltage was set at 90 kV and the tube current at 50  $\mu$ A. Subsequently, the Avatar 1.6.3 software (Pingsheng Medical Technology Co., Ltd.) was used to complete the three-dimensional reconstruction. The Avatar 1.6.3 software was used to align the reconstructed images and select the areas to be analyzed, then save them. For the analysis of the cancellous bone of the femur, starting from 0.5 mm after the disappearance of the growth plate at the distal end of the femur, 1 mm was taken from the proximal end of the femur as the region for analyzing the cancellous bone. For the analysis of the cortical bone of the femur, starting from 5 mm after the disappearance of the growth plate at the distal end of the femur, 1 mm was taken from the proximal end of the femur as the region for analyzing the cortical bone. The parameter analysis referred to the guideline for evaluating the microstructure of rodent bones using Micro-CT formulated in 2010. Then we quantitatively evaluate and calculated the bone volume fraction (BV/TV), number of trabeculae (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) in the cancellous bone region, as well as the bone area fraction (Ct.Ar/Tt.Ar) and average thickness (Ct.Th) in the cortical bone region. At the same time, parameters such as bone density (BMD) and bone mineral content (BMC).

### **4. Elisa detection of PINP concentration in serum**

At the end of the experiment, blood samples were collected from each group of mice through the posterior orbital venous plexus. The whole blood samples were left at room temperature to stand for 2 hours, then centrifuged at 3000 rpm for 15 minutes to collect the supernatant serum. The serum samples were aliquoted and stored at -80°C for future use. The operation was carried out according to

the instructions of the mouse PINP ELISA kit (Jianglai Biology, China). A standard curve was plotted based on the concentration of the standard substances and the absorbance, and the serum PINP concentration of each sample was calculated.

## 5. Histology and immunohistochemistry

The femoral bone samples of mice were fixed with 4% paraformaldehyde for 24 hours, then placed in 10% EDTA decalcification solution (pH 7.4) for 2–3 weeks of decalcification. Subsequently, the tissues were dehydrated with gradient ethanol, transparented with xylene, and embedded in paraffin. The tissues were cut into consecutive coronal sections (thickness 5  $\mu$ m) using a paraffin sectioning machine and mounted on glass slides. The sections were stained with hematoxylin-eosin (HE), OCN (CST, 1:1000 dilution). All sections were observed under an upright optical microscope and images were captured. The number and area of bone marrow adipocytes in the wild-type OVX group and the CRLF2-KO OVX group were compared using image analysis software, and the positive area of OCN was statistically analyzed.

## 6. Cell culture and transfection

MC3T3-E1 cells were cultured in MEM- $\alpha$  medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO<sub>2</sub>. When the cell confluence reached 70–80%, the CRLF2 overexpression plasmid was transfected into the cells using Lipofectamine™ 3000 transfection reagent according to the instructions. The specific steps were as follows: 2  $\mu$ g of plasmid DNA was diluted in Opti-MEM® serum-free medium, mixed with an equal volume of diluted Lipofectamine™ 3000 reagent, incubated at room temperature to form a DNA-liposome complex, and then added to the cell culture plate. After 6 hours of transfection, the medium was replaced with fresh complete medium. To achieve continuous and efficient overexpression, the transfection operation was repeated every 3 days starting from the first transfection.

Also, primary bone marrow cells were obtained by flushing from the femurs and tibias of 8 week old C57BL/6 mice and were cultured and passaged up to the generation 2–4. At the same time, recombinant plasmids for Crlf2 overexpression (pcDNA3.1-Crlf2) and knockdown (pLKO.1-shCrlf2) were constructed, and corresponding recombinant adenoviruses (Ad Crlf2-OE and Ad Crlf2-Sh) were prepared using the adenovirus packaging system. The P2 bone marrow cells were infected with the above adenoviruses at an MOI of 50–100 (setting the empty vector and scramble control group as the negative control). After 48 hours, the cells were switched to osteogenic induction medium and osteoclastic induction medium.

## 7. Osteogenic differentiation

After 24 hours of the first transfection, the medium was changed to an osteogenic induction differentiation medium (containing 50  $\mu$ g/mL ascorbic acid, 10 mM  $\beta$ -glycerophosphate sodium) to

induce osteogenic differentiation. Subsequently, each time the medium was changed, fresh osteogenic induction medium was used, and the induction process lasted for 7 days and 14 days.

## 8. Osteoclast induction

RAW264.7 cells and primary bone marrow cells were seeded at a density of  $5 \times 10^5$  cells per well in a 6-well plate and cultured in DMEM medium containing 1% penicillin-streptomycin. After the cells fully adhered, the medium was changed to the osteoclast induction medium, which was composed of DMEM supplemented with 50 ng/mL RANKL and 25 ng/mL M-CSF. The cells were cultured in a 37°C, 5% CO<sub>2</sub> incubator and the induction medium was changed every 2 days. The induction process lasted for 7 days.

## 9. Western Blot analysis

Total protein samples extracted from bone tissue or cells were mixed with 4× loading buffer at a ratio of 3:1 and heated at 95°C for 10 minutes to denature. Equal amounts of protein (50 µg from bone tissue and 40 µg from cells) were separated by 10% SDS-PAGE, and then the protein was transferred to a PVDF membrane by wet transfer. The membrane was incubated in a 5% skim milk TBST solution at room temperature for 60 minutes, followed by incubation with primary antibodies overnight at 4°C: rabbit anti-CRLF2(CST, 1:1000 dilution), rabbit anti-RUNX2(CST, 1:1000 dilution), mouse anti-OPN(CST, 1:1000 dilution), rabbit anti-cathepsin K(CST, 1:1000 dilution), and mouse anti-MMP9 polyclonal antibodies (CST, 1:1000 dilution), JAK2 Rabbit Monoclonal Antibody(CST, 1:1000 dilution), p-JAK2 Rabbit Monoclonal Antibody (CST, 1:1000 dilution), Stat3 Mouse Monoclonal Antibody(CST, 1:1000 dilution), p-Stat3 Mouse Monoclonal Antibody(CST, 1:2000 dilution), Stat5a Mouse Monoclonal Antibody (CST, 1:1000 dilution), p-Stat5a Rabbit Monoclonal Antibody (Proteintech, 1:2000 dilution) and a mouse anti-β-Actin monoclonal antibody(CST, 1:1000 dilution) was used as a reference. The membrane was washed with TBST 3 times, each for 10 minutes, and then incubated with HRP-labeled goat anti-rabbit or mouse anti-mouse secondary antibody (1:1000) at room temperature for 60 minutes. Finally, the membrane was developed using the ECL system and the gray values of the bands were analyzed using ImageJ software. The relative expression of the target protein was expressed as the gray value ratio of the target protein to β-Actin.

## 10. Real-time quantitative polymerase chain reaction (RT-qPCR)

For bone tissue, the samples were first ground into powder in dry ice, then transferred to an EP tube containing grinding beads, and TRIzol was added and thoroughly lysed in a tissue grinder. After chloroform extraction and centrifugation, total RNA was obtained by isopropanol precipitation. 1 µg of total RNA was used, and reverse transcription was performed using PrimeScript RT pre-mix (20 µL system). The first strand of cDNA was synthesized. 1 µL of diluted cDNA was used as a template, and amplification was carried out in the ABI StepOnePlus™ real-time fluorescence quantitative PCR system using Power SYBR Green PCR pre-mix. The reaction program was: 30 seconds of pre-denaturation at

95°C; then 40 cycles (5 seconds at 95°C, 30 seconds at 60°C). All gene-specific primers were synthesized by Tsingke Biotechnology and normalized using  $\beta$ -actin as the internal reference (**Table S1**). The relative expression levels of each target gene were calculated using the  $2^{-\Delta\Delta Ct}$  method.

## 11. Bioinformatics analysis of public databases

Firstly we used six databases, including human samples, OVX mice and cell samples (QB80\_OVX\_vs\_WT\_sham, GSE35956, GSE35959, GSE152986, GSE311708, GSE311709) for differential gene screening. We sorted the differentially expressed genes that were simultaneously upregulated in the six common databases by their expression levels, and found that *Crlf2* has significant research significance in osteoporosis.

## 12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1. All quantitative results were expressed as mean  $\pm$  standard deviation (SD). The non-paired t-test was used to analyze the differences between the control group and the model group. The Sidak's multiple comparison method of two-way ANOVA was employed to analyze the differences among different groups after gene overexpression or knockdown in the control group and the model group. A P value less than 0.05 was considered to indicate a significant difference.

## Results

***Crlf2* is highly expressed in osteoporosis samples and osteoclasts, lowly expressed in osteoblasts.**

To investigate the role of CRLF2 in osteoporosis, firstly, we used six common mouse and cell databases (QB80\_OVX\_vs\_WT\_sham, GSE35956, GSE35959, GSE152986, GSE311708, GSE311709) for differential gene screening. We sorted the differentially expressed genes that were commonly upregulated in these six databases by their expression levels. It was found that *Crlf2* has significant research significance in osteoporosis (Fig. 1A-C), suggesting that CRLF2 may play a role in bone homeostasis. At the same time, the data on the changes in *Crlf2* expression levels in the OVX mouse model were detected using immunohistochemical experiments (Fig. 1D,E). The results showed that *Crlf2* was highly expressed in the bone tissue of OVX mice. Subsequently, these findings were validated in both *in vitro* and *in vivo* models. In cell-based experiments, CRLF2 expression at both mRNA and protein levels progressively declined during osteogenic differentiation of MC3T3-E1 cells (Fig. 1F, G), while it was markedly increased during osteoclast differentiation of RAW264.7 cells (Fig. 1H, I). Furthermore, in an OVX mouse model of osteoporosis, CRLF2 mRNA and protein expression in bone tissue was significantly elevated compared with wild-type mice (Fig. 1J, K). These data indicate that CRLF2, beyond being a critical molecule in B-cell acute lymphoblastic leukemia, is closely associated with osteoporosis..

# CRLF2 knockout alleviates osteoporosis caused by ovariectomy in mice

To evaluate the effect of CRLF2 on osteoporosis in vivo, we constructed a systemic CRLF2 knockout (CRLF2-KO) mouse model. Western blot analysis showed that the CRLF2 protein was not detected in the bone tissue of KO mice, confirming the successful construction of the model (Fig. 2A, B). Subsequently, the bone phenotypes were compared. Serum ELISA detection revealed that the content of the bone formation marker PINP in the KO-OVX group was significantly higher than that in the WT-OVX group (Fig. 2C). Three-dimensional structural analysis of the distal femur using Micro-CT revealed that the cancellous bone structure of KO-OVX mice was denser and more interconnected. Quantitative analysis showed that the bone mineral content (BMC), bone density (BMD), bone volume fraction (BV/TV), and trabecular number (Tb.N) of KO-OVX mice were significantly increased, while the trabecular separation (Tb.Sp) was significantly decreased (Fig. 2E). Cortical bone density and thickness also improved (Fig. 2D, E). Histological analysis further confirmed these findings. H&E staining showed that the number and immunological activity of adipocytes in the bone marrow of KO-OVX mice were significantly lower than those in WT-OVX mice (Fig. 2F-**HE Staining**). Immunohistochemistry results showed that the positive signal area and intensity of OCN in the cancellous bone of KO-OVX mice were significantly enhanced, but intensity of PPAR $\gamma$  in the cancellous bone of KO-OVX mice were significantly inhibited (Fig. 2F-**OCN IHC**), consistent with the trend of increased serum PINP. In summary, CRLF2 knockout can alleviate the pathological damage of bone tissue caused by OVX, and its mechanism may be related to bone remodeling.

## CRLF2 knockout enhanced bone formation ability and reduced bone resorption ability

To elucidate the molecular mechanism, we examined the protein and gene expressions of bone metabolism related key markers in the tibia of each group of mice. Western blot results showed that compared with the WT-Sham group, the expressions of osteogenic-related proteins Runx2 and OPN in the bone tissue of WT-OVX mice were significantly downregulated; while in the KO-OVX group, the expressions of these two proteins were restored to levels close to those of the Sham group (Fig. 3A, B). Osteoclastic-related proteins Cathepsin K and MMP9 were upregulated in the WT-OVX group, while they were downregulated in the KO-OVX group (Fig. 3C, D). Real-time fluorescent quantitative PCR results showed that the transcriptional levels of inflammatory factors *Tnf- $\alpha$* , *IL-6*, and *Mcp-1* were significantly decreased in the KO-OVX group of bone tissue (Fig. 3E); while the mRNA expressions of osteogenic-related genes *Osteopontin*, *Osteocalcin*, *Runx2*, and *Alpl2* were significantly upregulated. The mRNA levels of osteoclastic-related transcription factors *Mmp9*, *C-fos* and *Trap* had the trend of downregulated, but had not show significant differences among the groups, the mRNA levels of *Cathepsin k* downregulated in *Crlf2* OVX compared WT OVX mice (Fig. 3F, G). In summary, CRLF2 KO upregulates the expression of osteogenic-related factors to promote bone formation; on the other hand, it inhibits bone resorption by

suppressing local inflammation and osteoclastic-related molecules(*Cathepsin k*), thereby jointly promoting the positive balance of bone remodeling and alleviating the process of osteoporosis.

### **CRLF2 inhibits osteoblast differentiation and promote osteoclast differentiation of primary bone marrow in vitro**

To verify the function of CRLF2 in bone formation in vitro, we constructed a CRLF2 overexpression plasmid and transfected it into MC3T3-E1 cells. Also, by overexpressing and knocking down *Crlf2* and using adenovirus to infect primary bone marrow cells, osteogenic and osteoclastogenic differentiation was achieved. Western blot results showed that compared with the empty vector control group, the expression of CRLF2 protein was successfully upregulated(Fig. 4A). The verification results of *Crlf2* knockdown efficiency showed that the recombinant plasmid had a certain knockdown efficiency(Fig. 4B). Subsequently, we overexpressed CRLF2 in osteogenic precursor cells MC3T3-E1, and performed osteogenic induction 24 hours after transfection. To maintain the overexpression effect, we repeated the transfection on the 3rd day of induction and analyzed on the 7th day. Alkaline phosphatase (ALP) staining showed that under osteogenic induction conditions, overexpression of CRLF2 significantly reduced ALP staining area in MC3T3-E1 cell (Fig. 4C). After overexpression and knockdown of *Crlf2* in primary bone marrow cells and subsequent adenovirus infection for 7 days, the results showed that after *Crlf2* overexpression, the osteogenic differentiation ability of primary bone marrow cells was weakened, while knockdown enhanced it(Fig. 4D). Western blot and qPCR results further demonstrated that overexpression of CRLF2 significantly inhibited the expression of the key osteogenic protein osteocalcin in MC3T3-E1 cell (Fig. 4E, F), but had no effect on *Runx2* expression. In addition, CRLF2 overexpression suppressed the mRNA levels of osteogenic marker genes, including *Osteocalcin*, *Alpl2*, and *Col1a1* in MC3T3-E1 cell (Fig. 4G). These results consistently indicate that overexpression of CRLF2 inhibits osteoblast differentiation.

### **Crlf2 targets JAK2 to promote the phosphorylation of Stat3, revealing its involvement in osteogenic differentiation**

To explore its molecular mechanism, we predicted through the STRING and BioGRID databases that CRLF2 might interact with the tyrosine kinase JAK2 (Fig. 5A, B). Therefore, we designed an immunoprecipitation experiment to confirm the specific binding of CRLF2 to JAK2 (Fig. 5C, D). Given that JAK2 is the upstream kinase of STAT3 and STAT3/5 are closely related to the regulation of osteogenic differentiation, we further detected the activity of the JAK2-STAT3/5 pathway. The results showed that in CRLF2-overexpressing cells, the total protein levels of JAK2, STAT3, and STAT5a remained unchanged, but their phosphorylation levels (p-JAK2, p-STAT3) significantly increased (Fig. 5E, F), while the expression of p-STAT5a did not show any significant change. This suggests that in the osteogenic induction context, CRLF2 overexpression further enhanced the phosphorylation of JAK2 and STAT3. These results indicate that CRLF2 binds and activates JAK2, thereby activating the STAT3 signaling pathway.

## Discussion

Current research suggests that CRLF2 may be involved in osteoporosis by influencing estrogen and osteocyte differentiation, but the specific mechanism is still unclear. This study suggests that CRLF2 may play a crucial role in osteoporosis. Wu et al. found that TSLP from trophoblast cells promotes invasion and proliferation, implying CRLF2 may relate to estrogen and endocrine homeostasis, thereby affecting osteoporosis [21]. Ohno T et al. showed that TSLPR (encoded by *Cr1f2*) is expressed on osteoclast precursors and inhibits RANKL-induced osteoclast differentiation, indicating *Cr1f2* is involved in bone cell differentiation [22]. Bone cells secrete cytokines like RANKL, OPG, IL-6, and TNF- $\alpha$ , which regulate bone remodeling and immune interactions [23, 24]. In OVX mice, CRLF2 expression was upregulated in bone tissue and osteoclasts but decreased in osteoblasts, verified by *in vivo* and *in vitro* experiments. CRLF2 (type I cytokine receptor superfamily) is known for its role in hematological malignancies [25–27], but its function in bone metabolism, especially osteoporosis, remains unclear. We speculate CRLF2 may regulate osteoporosis by influencing bone resorption or formation.

This experiment has confirmed that the absence of CRLF2 can alleviate bone loss caused by estrogen deficiency by promoting osteogenesis and inhibiting osteoclast activity. Our results confirm *Cr1f2* is highly expressed in OVX mice. CRLF2 knockout mice showed higher bone density and better microstructure after ovariectomy, along with elevated PINP and OCN levels, indicating enhanced osteogenesis and reduced bone degeneration. HE staining showed less marrow adipogenesis. Western blotting revealed that CRLF2 knockout promoted osteopontin and Runx2, inhibited Cathepsin K and MMP9, and reduced IL-6, TNF- $\alpha$ , and MCP-1 mRNA. *In vitro*, CRLF2 overexpression inhibited osteogenic differentiation and related genes (Col1a1, Alpl, Osteocalcin), while promoting osteoclast differentiation; knockdown had opposite effects. Thus, CRLF2 mediates bone remodeling via osteoblasts and osteoclasts. Notably, *Cr1f2* deletion increased CTSK expression in Sham group, possibly due to negative feedback from reduced bone resorption or non-osteoclast sources (e.g., osteoblasts, chondrocytes, immune cells), or through immune microenvironment changes upregulating CTSK independently of RANKL. These speculations require further verification.

To explore the mechanism by which *Cr1f2* affects bone homeostasis, this study focused on the osteogenic differentiation pathway and found that CRLF2 regulates osteogenic differentiation bidirectionally through the JAK2-STAT3 signaling axis. In order to explore the mechanism by which *Cr1f2* affects bone homeostasis, we focused on osteogenic differentiation. CRLF2 can form a heterodimer with IL-7R $\alpha$  as the TSLP receptor [29], participating in Th2 inflammation and hematopoiesis [30, 31]. In acute lymphoblastic leukemia, CRLF2 transmembrane mutations cause TSLP-independent dimerization and continuous activation [32, 33], often accompanied by JAK2 activation [38] and STAT5 activation [34]. In BCP-ALL, abnormal CRLF2 expression accounts for ~ 15% of high-risk and up to 50% of Down syndrome-related cases [35–37], with JAK2 and STAT5 phosphorylation [39, 40]. We confirmed CRLF2-JAK2 interaction via co-immunoprecipitation, consistent with Mullighan et al. [39]. The STAT family (discovered in 1993) includes STAT3, which—along with JAKs—is expressed in bone [41]. In osteoporosis, STAT3, STAT5a/b directly participate in bone development, while STAT1 deficiency affects

Runx2 [42]. STAT3 promotes osteogenic development under physiological conditions; its knockout causes skeletal deformities [43]. However, in our osteogenic induction experiments with CRLF2 transfection, p-JAK2 and p-STAT3 activation were enhanced, yet osteogenic differentiation was inhibited. Levy et al. showed that inhibiting STAT3 enhanced BMP2/4-induced osteogenic differentiation of human mesenchymal stem cells [44], indicating that STAT3 activation has a bidirectional role in osteogenesis.

Our study indicates that in osteoporosis caused by estrogen deficiency, elevated levels of IL-6 and TNF- $\alpha$  inhibit osteogenic differentiation via a positive feedback loop involving JAK-STAT3. In the pathogenesis of osteoporosis caused by estrogen deficiency, pro-inflammatory cytokines IL-6 and TNF- $\alpha$  increase, which is common in postmenopausal women and ovariectomized rats [45, 46]. In chronic inflammation, continuous STAT3 activation promotes IL-6 and TNF- $\alpha$  release, exacerbating inflammation. JAK proteins respond to IL-6 and TNF- $\alpha$ , undergoing autophosphorylation and further phosphorylating downstream STAT proteins, forming a positive feedback loop. STAT3 activation may promote IL-6 and TNF- $\alpha$  expression, thereby activating NF- $\kappa$ B to inhibit Runx2 transcriptional activity, or interfering with Wnt/ $\beta$ -catenin and BMP/Smad pathways, thus inhibiting osteoblast development. However, in this article, we did not conduct more in-depth research.

Based on this study and the existing literature, CRLF2 may be involved in osteoporosis through the CRLF2-JAK2-STAT3 axis. However, its negative regulatory role in chronic inflammation still requires further exploration. Based on this study and related literature, CRLF2 may act as a key regulatory factor and participate in the occurrence and development of osteoporosis through the CRLF2-JAK2-STAT3 axis. Although STAT3 activation typically promotes osteogenic differentiation under physiological conditions, this study found that in the context of CRLF2 overexpression, JAK2-STAT3 pathway activation actually inhibits osteogenic differentiation. This contradiction suggests the complexity of STAT3's function in bone biology and may reflect its negative regulatory role in chronic inflammatory states such as osteoporosis.

In conclusion, our experimental results indicate that Crlf2 inhibits osteogenic differentiation and accelerates bone loss in OVX mice, and the JAK2/Stat signaling pathway plays an important role in this process (Fig. 6). Although we have preliminarily confirmed the existence of the CRLF2-JAK2-STAT3 axis, it is still unclear whether it directly interferes with osteogenic differentiation or indirectly exerts its effect by reshaping the inflammatory microenvironment. Therefore, further exploration of the specific mechanism of CRLF2 signaling in the skeletal system, especially in inflammatory bone diseases, is an important direction for future research.

## Declarations

## Conflicts of interest

The authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

# Funding

This work was supported by Jiangxi Province Natural Science Foundation(No.20252BAC200551), the Early-Career Young Scientists and Technologists Project of Jiangxi Province(No.20252BEJ730273) and the Ganzhou Science and Technology Project (No. 2023CZY17876).

# Author Contribution

Jie Song: Conceptualization, Methodology Wenyi Bai: Conceptualization, Software, Writing- Reviewing and Editing Ping Xie: Data curation, Software, Writing- Original draft preparation Qi Rao: Methodology, Software Jianping Wang: Software Meiting Xu: Validation Juan Yang: Supervision Chuhua Huang: Conceptualization, Supervision. All authors have read and agreed to the published version of the manuscript.

# Data Availability

Data will be made available on request.

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## Figures

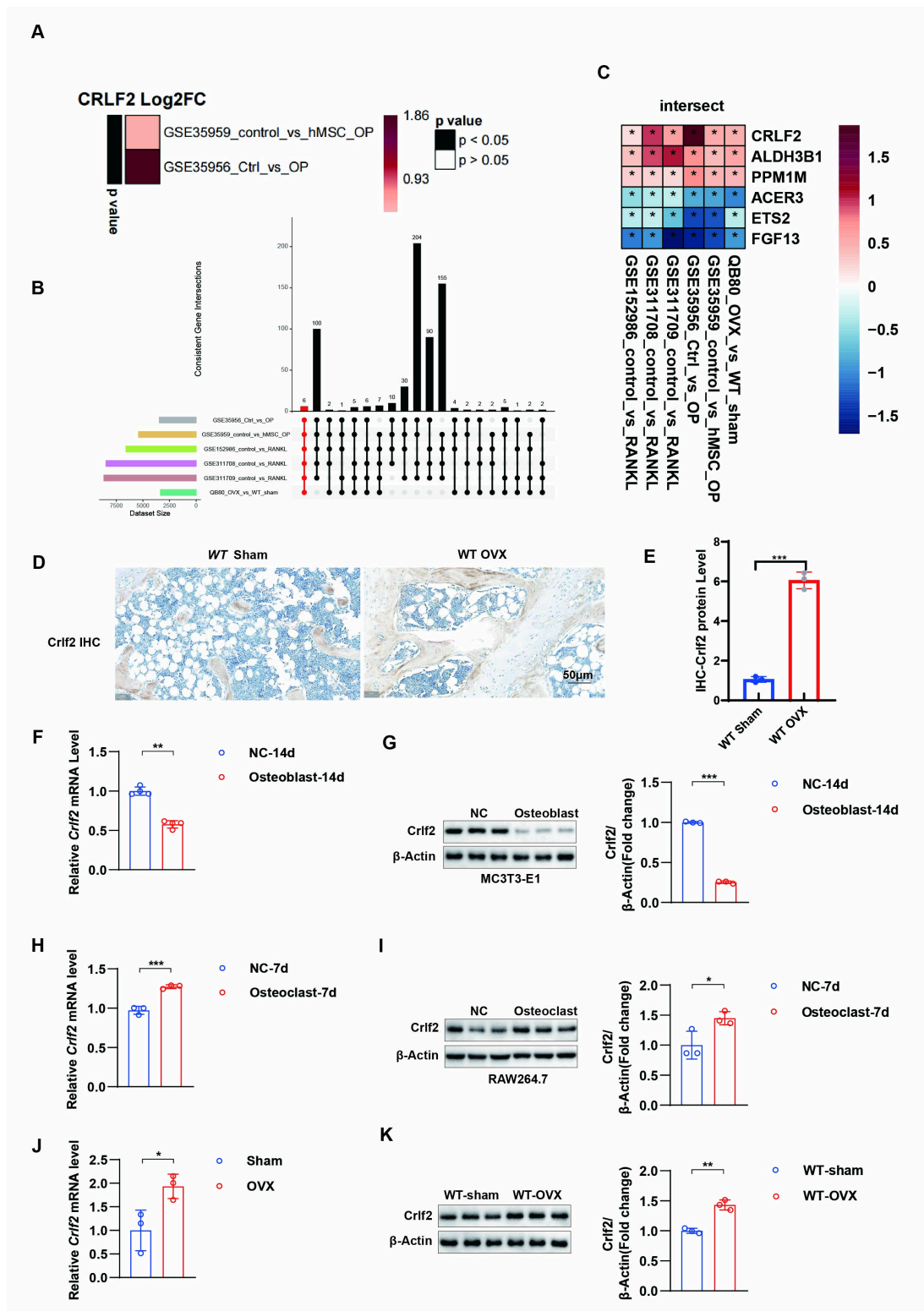


**Figure 1**

**Crlf2 is highly expressed in osteoporosis samples and osteoclasts, and is lowly expressed in osteoblasts.** (A-C) Bioinformatics analysis; (D,E) Immunohistochemical staining and quantitative analysis of Crlf2 in the bone tissue of OVX mice; (F, G) 14 days of OB-induced MC3T3-E1, protein expression levels and mRNA expression levels of CRLF2 ; (H, I) 7 days of OC-induced Raw264.7, protein expression levels and mRNA expression levels of CRLF2 ; (J, K) Protein expression levels and mRNA

**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**CRLF2 knockout alleviated osteoporosis in mice caused by ovariectomy.** (A) Construction of CRLF2 knockout mice; (B) Identification of CRLF2 knockout mice at the protein level; (C) Detection of serum PINP; (D) Micro-CT analysis of the 2D phenotypes of cortical and cancellous bone; (E) Micro-CT statistical analysis; (F) Hematoxylin and eosin staining,IHC of OCN; (G,H) Quantitative statistics of the number and area of adipocytes in HE staining; (I) Quantitative statistical analysis of OCN protein expression in F. \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ ; ns, not significant.

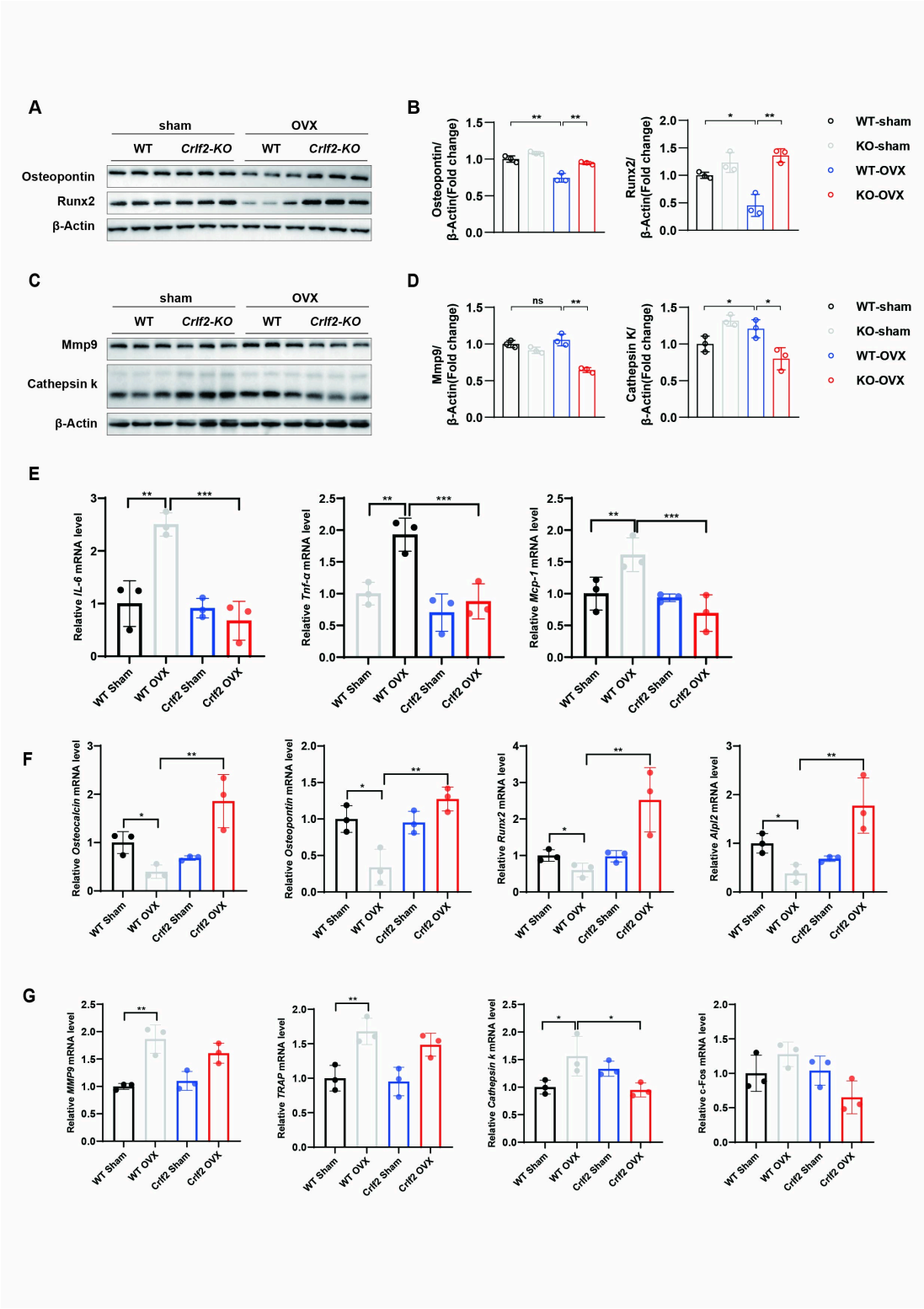
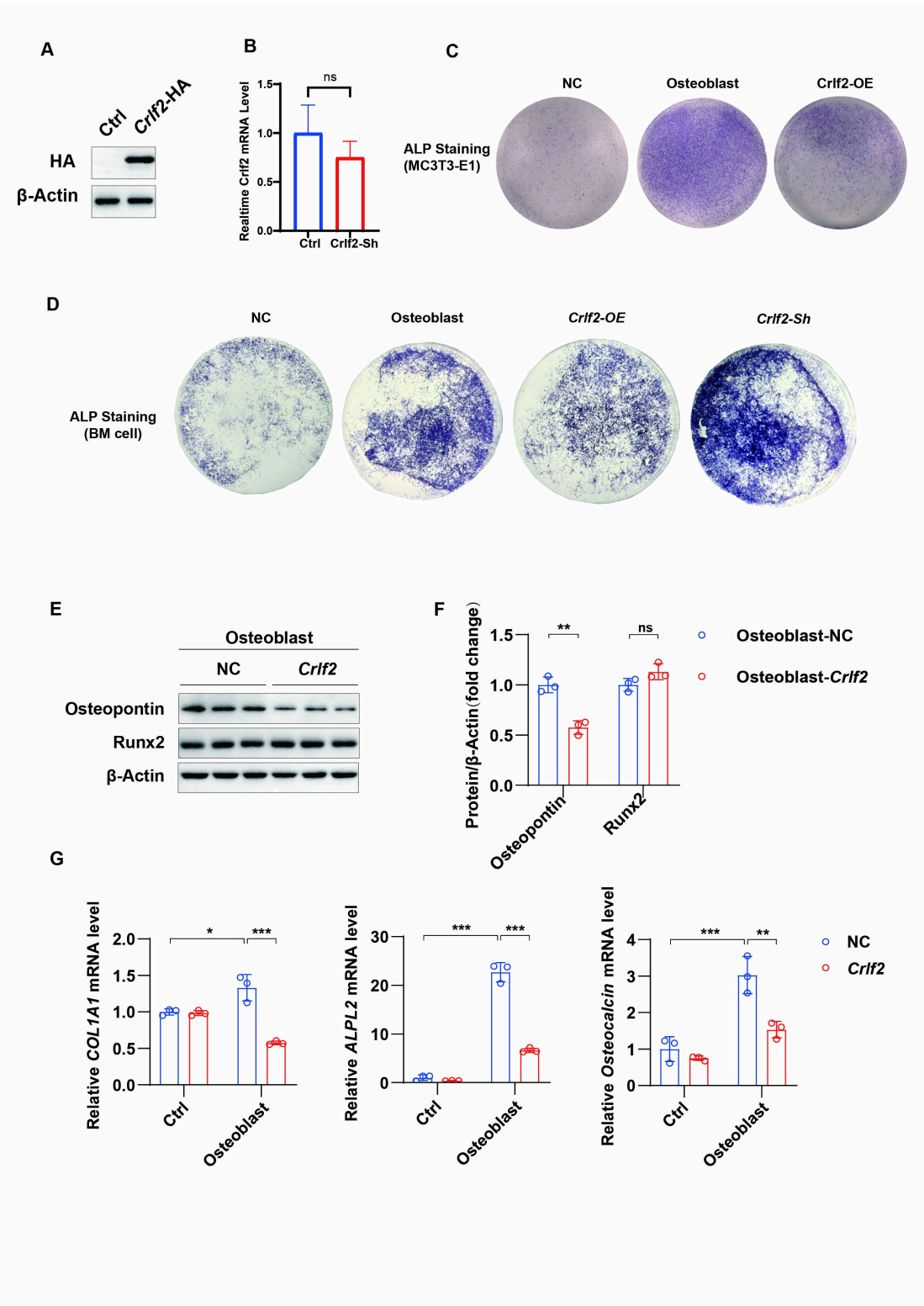


Figure 3

**CRLF2 knockout enhanced bone formation ability and reducing bone resorption ability of ovariectomized mice.** (A, B) Protein expression and visualization of Runx2 and Osteopontin; (C, D) Protein expression and visualization of Cathepsin K and Mmp9; (E) mRNA expression of inflammatory-related factors; (F) mRNA expression of osteogenesis-related factors ; (G) mRNA expression of osteoclastogenesis-related factors. n = 3/group. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, not significant.



## Figure 4

**CRLF2 Regulates Osteoblast and Osteoclast Differentiation *in vitro*.** (A, B) Effect of overexpress and reduced of CRLF2 was detected; (C) ALP staining in MC3T3-E1 cells; (D) ALP staining in primary bone marrow differentiate into osteoblasts; (E) Protein expression levels of Runx2 and Osteopontin; (F) mRNA levels of osteogenic marker genes *Runx2* and *Osteopontin*; (G) mRNA expression levels of osteogenic-related factors *Col1a1*, *Osteocalcin* and *Alpl2*. n = 3/group. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, not significant.

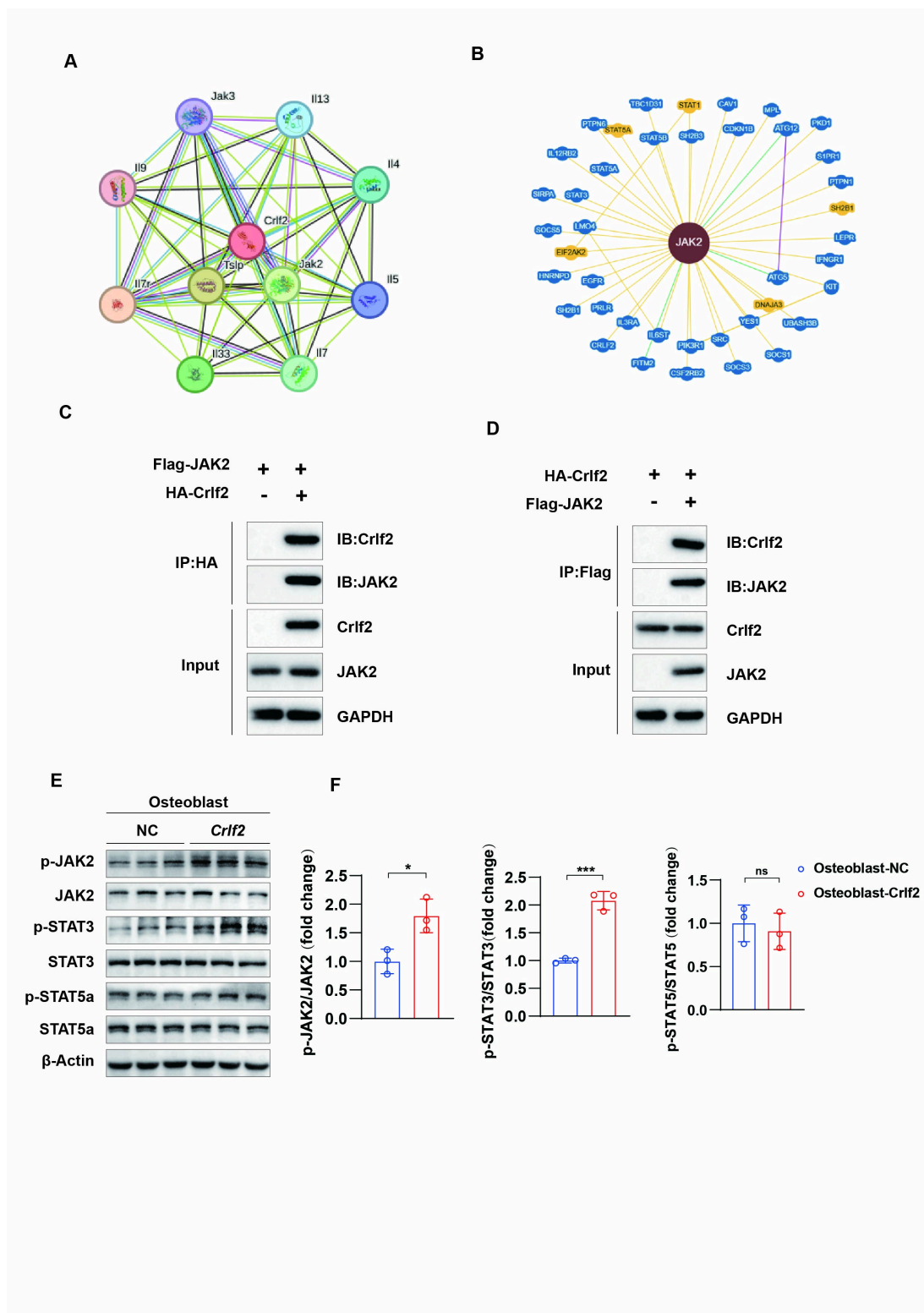


**Figure 5**

**Crlf2 targets the JAK2 phosphorylated Stat3 signaling pathway and is involved in osteogenic differentiation.** (A) STRING and BioGRID (B) databases predict the interaction between CRLF2 and tyrosine kinase JAK2; (C, D) co-immunoprecipitation detects the protein interaction between CRLF2 and JAK2; (E, F) expression levels of total proteins of JAK2, STAT3, STAT5a and their phosphorylated proteins, and their quantification.  $n = 3/\text{group}$ .  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ ; ns, not significant.

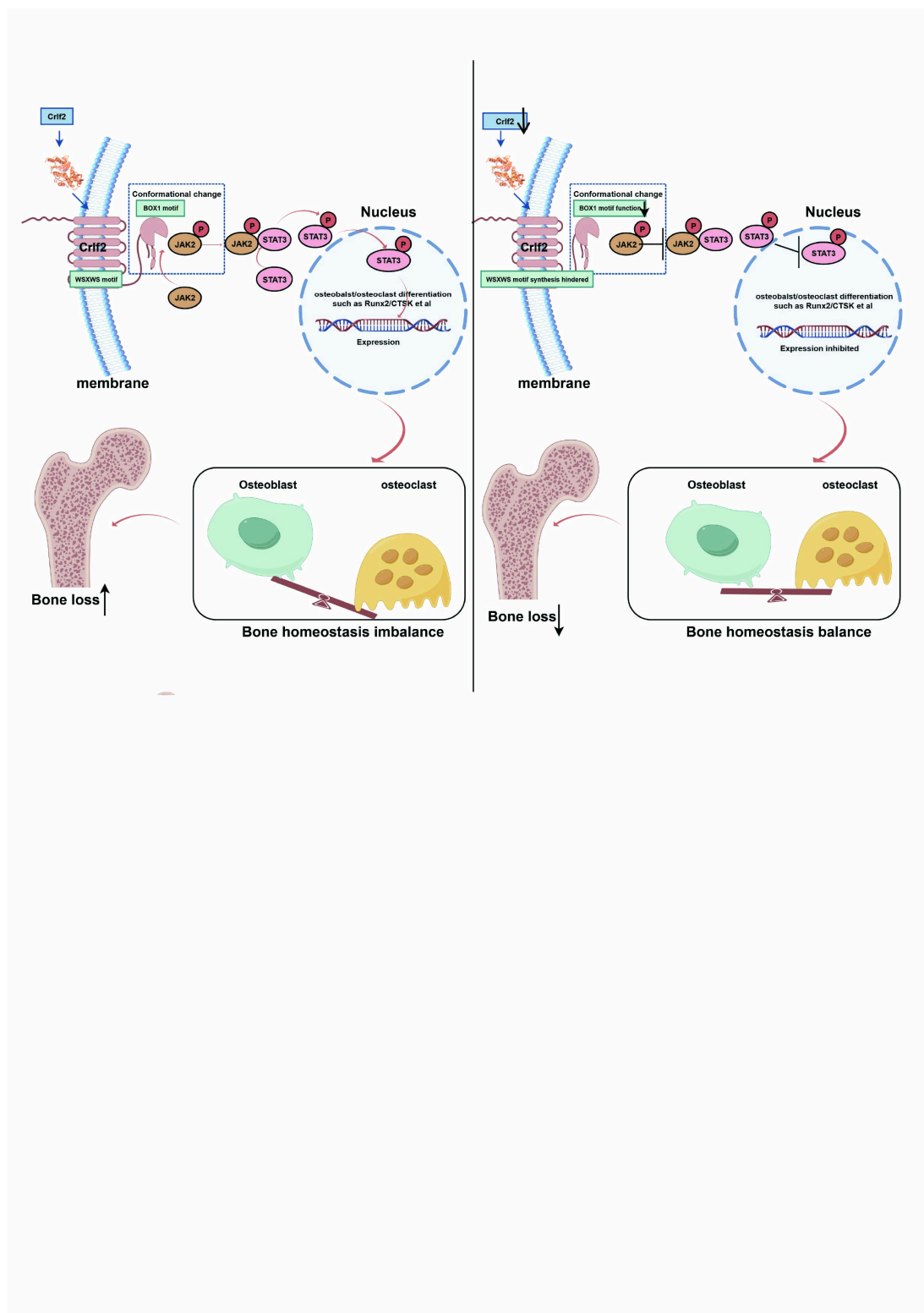


Figure 6

The abstract schematic diagram of *crlf2* promoting osteogenic differentiation through the Jak2/Stat3 pathway and affecting the bone quality of OVX mice

## Supplementary Files

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