CHIP regulates skeletal development and postnatal bone growth

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Abstract
C terminus of Hsc70-interacting protein (CHIP) is a chaperone-dependent and U-box containing E3 ubiquitin ligase. In previous studies, we found that CHIP regulates the stability of multiple tumor necrosis factor receptor-associated factor proteins in bone cells. In Chip global knockout (KO) mice, nuclear factor-κB signaling is activated, osteoclast formation is increased, osteoblast differentiation is inhibited, and bone mass is decreased in postnatal Chip KO mice. To determine the role of Chip in different cell types at different developmental stages, we created Chipflox/flox mice. We then generated Chip conditional KO mice ChipCMV and ChipOSXER and demonstrated defects in skeletal development and postnatal bone growth in Chip conditional KO mice. Our findings indicate that Chip conditional KO mice could serve as a critical reagent for further investigations of functions of CHIP in bone cells and in other cell types.

Keywords
bone formation, CHIP, conditional knockout, NF-κB signaling, skeletal development

1 | INTRODUCTION

Skeletal development and endochondral bone formation is a complicated process involving chondrocyte proliferation, differentiation and hypertrophy, cartilage calcification, resorption of calcified cartilage, vascular invasion, and osteoblast differentiation. All of these steps are regulated precisely by multiple growth factors and signaling molecules. Nuclear factor-κB (NF-κB) signaling controls osteoclast formation (Boyce, Yao, & Xing, 2010) and is involved in the process of removal of calcified cartilage. In NF-κB p50/p65 double knockout (KO) mice, osteoclast formation is severely impaired leading to defects in the resorption of calcified cartilage and largely expanded hypertrophic zone at the postnatal stage (Xing, Chen, & Boyce, 2013).

NF-κB signaling plays an important role in bone biology. C terminus of Hsc70-interacting protein (CHIP) is a chaperone-dependent and U-box containing E3 ubiquitin ligase. It targets the degradation of proteins critical for multiple cellular functions and signaling pathways. In previous studies, we found that CHIP controls the degradation of multiple tumor necrosis factor receptor-associated factor proteins and regulates NF-κB signaling in both osteoclasts and osteoblasts and NF-κB signaling is activated in Chip KO mice (Li et al., 2014; Wang et al., 2018). It is known that activation of NF-κB signaling leads to stimulation of osteoclast formation and inhibition of osteoblast differentiation (Abu-Amer, 2013; Boyce et al., 2010; Jimi et al., 2016; Otero, Chen, Zhang, & Abu-Amer, 2012; Park et al., 2007; Swarnkar, Zhang, Mbalaviele, Long, & Abu-Amer, 2014; Yao et al., 2014). However, the role of NF-κB signaling in skeletal development remains unclear. In the present studies, we investigated changes in skeletal development in Chip conditional KO mice.

To study the role of CHIP in bone remodeling and in aging in adult mice and to determine the tissue-specific effects of Chip in bone and cartilage and other organs in the body, we have generated...
**Chipflox/flox** mice. To demonstrate that **Chipflox/flox** mice have been properly developed, we then created **ChipSV** and **ChipOxER** conditional KO mice. We analyzed bone phenotype of **Chip** conditional KO mice and demonstrated that **Chipflox/flox** mice could be used as an important tool to investigate functions of **Chip** in multiple tissues at different developmental stages.

2 | MATERIALS AND METHODS

2.1 | Chip KO Mice

**Chipflox/flox** mice were generated in Nanjing Biomedical Research Institute (Nanjing University, Nanjing, China). In these mice the **Chip** gene was floxed at the flanking sites of exon 1 and exon 3. The primer sequences for genotyping **Chipflox/flox** mice were: P1 (forward primer): 5′-CATATCTCACCAGGCTCAT-3′, P2 (reverse primer): 5′-ACACACAATGACCCACAT-3′ and P3 (reverse primer): 5′-GGCCCTACCCGCTTCCATTGCTC-3′. The genotyping result was presented in Figure 1. CMV-Cre and Osx-CreER transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, Maine).

2.2 | Whole embryo alizarin red/alcian blue staining

Embryos at E14.5 and E18.5 were collected and the skin, viscera, and adipose tissues were carefully removed. Whole skeletons were fixed in 95% ethanol for 2 days followed by fixation in acetone for an additional day, and stained with 0.015% alcian blue and 0.005% alizarin red for 3 days. Images of the skeletons were taken when most of the soft tissue was digested in 1% potassium chloride.

2.3 | Microcomputed tomography analysis

We used a Scanco microcomputed tomography 35 scanner (Scanco Medical, Brüttisellen, Switzerland) with 55 kVp source and 145 μA current for formalin-fixed mouse legs with a resolution of 10 μm. The scanned images from each group were evaluated at the same thresholds to allow three-dimensional structural rendering of each sample.

2.4 | Histology

Tibiae were harvested and fixed in 10% neutral-buffered formalin for 3 days and decalcified for 14 days in 14% EDTA and then paraffin-embedded. Three-micrometer sections were cut and alcian blue/H&E orange G staining and tartrate-resistant acid phosphatase (TRAP) staining were performed (Shu et al., 2013; B. Wang et al., 2014; Wang et al., 2017).

2.5 | Statistical analysis

Data are presented as the mean ± standard deviation. For experiments comparing two groups of data, unpaired Student’s t test was performed. A value of p < .05 was considered to be significant.
Skeletal development was delayed in Chip KO embryos. (a–e) Chip<sup>flx/flx</sup> mice were bred with CMV-Cre mice and E18.5 Chip<sup>CMV</sup> KO embryos and Cre-negative littermate embryos were analyzed by whole embryo alizarin red/alcian blue staining. (a) The size of Chip<sup>CMV</sup> KO embryo was smaller. The mineralization of the supraoccipital bone (a, blue arrowheads) and the digits (d, black arrowheads) was delayed in Chip<sup>CMV</sup> KO embryos. (f–i) Chip<sup>flx/flx</sup> mice were bred with CMV-Cre mice and E14.5 and E18.5 Chip<sup>CMV</sup> KO embryos and Cre-negative littermate embryos were analyzed by histology. (f) Analysis of E14.5 embryos showed that the size and width (red line) of the skeletal were significantly reduced in Chip<sup>CMV</sup> KO embryos. (g and h) Analysis of E18.5 embryos showed that the width (red lines) of the skeletal and the length of hypertrophic zone (yellow lines) were reduced in Chip<sup>CMV</sup> KO embryos. (i) The size of primary ossification center was smaller in E18.5 Chip<sup>CMV</sup> KO embryos. (j) Results of IHC staining showed that ColX expression (red arrowheads) was decreased in E18.5 Chip<sup>CMV</sup> KO embryos. IHC, immunohistochemistry; KO, knockout.
Bone mass was decreased in Chip<sup>CMV</sup> conditional KO mice. Chip<sup>flox/flox</sup> mice were bred with CMV-Cre mice and Chip<sup>CMV</sup> conditional KO mice and Cre-negative control mice were killed at 4 weeks of age. (a–c) The size, body weight, and body length of Chip<sup>CMV</sup> KO mice were significantly smaller than Cre-negative controls. (d–i) Changes in bone mass were analyzed by µCT. (d) Bone mass, (e) bone volume, (f) bone mineral density (BMD), (g) trabecular number (Tb.N.), and (h) In contrast, trabecular separation (Tb.Sp.) was increased (did not reach the statistical significant) in Chip<sup>CMV</sup> KO mice. (i) connectivity density (Conn.D.) was significantly reduced in Chip<sup>CMV</sup> KO mice. (j–m) Changes in bone morphology were also analyzed by histology. (j) Bone mass was significantly decreased in Chip<sup>CMV</sup> KO mice (trabecular bone was indicated by yellow arrowheads). (k) No significant changes in the length of growth plate cartilage in Chip<sup>CMV</sup> KO mice. (l and m) In addition, histological analysis showed that the loss of articular chondrocytes and reduced alcian blue staining (blue arrowheads) was observed in Chip<sup>CMV</sup> KO mice. 

BV/TV, bone volume/total volume; KO, knockout; µCT, microcomputed tomography; WT, wild-type.
3 | RESULTS

3.1 | Skeletal development was delayed in Chip<sup>CMV</sup> conditional KO embryos

In previous studies, we investigated the role of CHIP in postnatal bone growth. To determine the role of CHIP in skeletal development, in the present studies, we generated Chip<sup>flox/flox</sup> mice and Chip<sup>CMV</sup> conditional KO mice and analyzed changes in skeletal development in E14.5 and E18.5 Chip<sup>CMV</sup> KO embryos and Cre-negative littermate embryos. Results of whole embryo alizarin red/alcian blue staining showed that sizes of Chip<sup>CMV</sup> KO embryos were smaller (Figure 2a). We also found that the mineralization in the supraoccipital bone and the distal phalanx
of the limb were delayed in Chip<sup>CMV</sup> KO embryos (Figure 2b–d). Axial bone seems developed normally in Chip<sup>CMV</sup> KO mice (Figure 2e). Results of histological analysis showed that the width of limb of E14.5 embryos was reduced in Chip<sup>CMV</sup> KO embryos (Figure 2f) and the width and the length of the hypertrophic zones were also decreased in Chip<sup>CMV</sup> KO embryos (Figure 2g,h). The size of primary ossification center in the tibiae was smaller in Chip<sup>CMV</sup> KO embryos (Figure 2i). In addition, we also found that CoX expression was reduced in the hypertrophic zone of E18.5 Chip<sup>CMV</sup> KO embryos (Figure 2j).

### 3.2 Bone mass was decreased in Chip<sup>CMV</sup> conditional KO mice

Our long-term goal is to understand the specific roles of CHIP in bone remodeling and in aging in adult mice. To achieve this goal, we generated Chip<sup>flox/flox</sup> mice. We have bred Chip<sup>flox/flox</sup> mice with CMV-Cre transgenic mice to determine if Chip<sup>CMV</sup> conditional KO mice mimic bone loss phenotype that we have observed in Chip<sup>−/−</sup> global KO mice. We analyzed 4-week-old Chip<sup>CMV</sup> KO mice and found that body weight and body length of Chip<sup>CMV</sup> KO mice were significantly reduced compared to the Cre-negative littermates (Figure 3a–c). The μCT analysis showed that bone volume (bone volume/total volume, %) and bone mineral density (BMD) were significantly decreased in Chip<sup>CMV</sup> KO mouse compared to the Cre-negative littermates (Figure 3d–f). Consistent with the findings of bone mass reduction, the trabecular number was decreased and trabecular separation was increased in Chip<sup>CMV</sup> KO mice (Figure 3g,h). In contrast, the connectivity density was significantly decreased in Chip<sup>CMV</sup> KO mice (Figure 3i), suggesting that the bone quality were also reduced in Chip<sup>CMV</sup> KO mice. We then performed histological analysis and observed significant bone loss phenotype in Chip<sup>CMV</sup> KO mice (Figure 3j). In contrast, no significant changes in growth plate cartilage were found in Chip<sup>CMV</sup> KO mice (Figure 3k). In addition to the changes in bone mass, we also found defects in articular cartilage in Chip<sup>CMV</sup> KO mice, including loss of articular chondrocytes and reduced Alcian blue staining (Figure 3l,m), reflecting the loss of proteoglycan in articular cartilage in postnatal Chip<sup>CMV</sup> KO mice.

#### 3.3 Bone mass was decreased in Chip<sup>OsxER</sup> conditional KO mice

In previous studies, we found bone loss phenotype in Chip global KO mice. However, it is not known if CHIP exerts its effect directly through bone cells or CHIP acts on bone through an indirect mechanism. To determine the specific effect of Chip on postnatal bone growth, we generated Chip<sup>OsxER</sup> conditional KO mice by breeding Chip<sup>OsxER</sup> mice with Osx-CreER transgenic mice, which target osteoblast precursor cells (Maes et al., 2010). Tamoxifen was administered to 2-week-old Chip<sup>OsxER</sup> mice and Cre-negative littermates (Chip<sup>Δ<sub>lox/lox</sub></sup>) mice. The results of a significant decrease in body weight and slightly reduced body size were found in Chip<sup>OsxER</sup> KO mice (Figure 4a–c). Results of μCT analysis showed that bone volume, BMD, trabecular numbers, and connectivity density were significantly reduced and trabecular separation was significantly increased in Chip<sup>OsxER</sup> KO mice (Figure 4d–i). We also analyzed bone morphological changes with histological method in 4-week-old Chip<sup>OsxER</sup> KO mice. Trabecular bone loss phenotype was found in Chip<sup>OsxER</sup> KO mice (Figure 4j). These findings suggest that bone mass and bone quality were reduced in Chip<sup>OsxER</sup> KO mice and indicate that local produced CHIP could directly regulate bone mass. In contrast, we did not observe significant changes in growth plate cartilage (Figure 4k). To further determine changes in bone formation in Chip<sup>OsxER</sup> KO mice, we performed calcein/calcein double labeling assay. We found that mineral apposition rates and bone formation rates were significantly decreased in Chip<sup>OsxER</sup> KO mice (Figure 4l–n). Consistent with these findings, results of von Kossa staining showed that mineralized trabecular bone volume was significantly reduced in Chip<sup>OsxER</sup> KO mice (Figure 4o).

In previous studies, we demonstrated that NF-κB signaling is activated in Chip global KO mice (Wang et al., 2018). In this study, we performed TRAP staining in subchondral bone areas and in trabecular bone areas underneath the growth plate. We found that osteoclast formation was significantly increased in Chip<sup>OsxER</sup> KO mice (Figure 5a–c), suggesting that osteoclast formation was also affected by Chip<sup>OsxER</sup> KO mice.

### 4 DISCUSSION

In previous studies, we demonstrated that CHIP controls NF-κB signaling and regulates postnatal bone growth. In Chip global KO mice, osteoclast
formation and bone resorption were increased and osteoblast differentiation was decreased (Li et al., 2014; Wang et al., 2018). In the present studies, we generated Chip^{flox/flox} mice and then created Chip^{CMV} and Chip^{OsxER} conditional KO mice. We analyzed changes in skeletal development in Chip^{CMV} KO embryos and we also analyzed changes in postnatal bone growth and bone mass in 1-month-old Chip^{CMV} and Chip^{OsxER} conditional KO mice. Our findings suggest that CHIP plays an important role in skeletal development and postnatal bone growth and regulates bone mass. Our findings indicate that we have successfully generated Chip conditional KO mice and confirmed that Chip is expressed in Osx-expressing osteoblast precursor cells and plays a specific role in the regulation of bone mass.

Our previous study demonstrated that CHIP regulates NF-κB signaling through inducing the degradation of multiple TRAF proteins, including TRAF2, TRAF5, and TRAF6 (Li et al., 2014; Wang et al., 2018). It is known that NF-κB controls growth plate cartilage development. In NF-κB p50/p65 double KO mice, osteoclast formation is severely impaired leading to defects in the resorption of calcified cartilage and largely expanded hypertrophic cartilage zone at the postnatal stage (Xing et al., 2013). In this study, we found that growth plate cartilage development relatively normal in postnatal Chip^{CMV} and Chip^{OsxER} KO mice. These findings suggest that the activation of NF-κB signaling may not be able to significantly affect postnatal growth plate cartilage development although osteoclast formation is increased in Chip KO mice.

Bone is an endocrine organ and bone remodeling is a dynamic process that is active throughout the entire life. To determine the specific role of CHIP at different cell populations and at the different developmental stages of life, it requires the generation of inducible Chip conditional KO mice. As a long-term goal of this project, we will determine the roles of Chip in osteoclast and osteoblast lineage cells at different developmental stages. We have recently generated Chip^{flox/flox} mice and Chip conditional KO mice which allow us to perform tissue-specific and longitudinal studies to investigate the role of CHIP in bone remodeling.

CHIP may play an important role in aging. Sirtuin 6 (SirT6) is a stress-responsive protein deacetylase and mono-ADP ribosyltransferase enzyme encoded by the SirT6 gene (Min et al., 2008). Studies in mice have revealed that SirT6 is essential for postnatal development and survival. SirT6 functions in multiple molecular pathways related to aging, including DNA repair, telomere maintenance, glycolysis, and inflammation (Frye, 2000). SirT6 promotes resistance to DNA damage and oxidative stress, the defects closely related to age-associated diseases (Beauharnois, Bolivar, & Welch, 2013). CHIP prevents proteasome-dependent degradation of SirT6 and SirT6 stability is increased in Chip-deficient cells. These results suggest that CHIP protects proteasomal degradation of SirT6. To study the roles of CHIP in SirT6 and other proteins associated with aging, we need to create Chip^{flox/flox} mice and Chip conditional KO mice since Chip global KO mice died at postnatal or early adult stages. We have recently

![Figure 5](image)

**FIGURE 5** TRAP-positive osteoclast formation was increased in Chip^{OsxER} conditional KO mice. Chip^{flox/flox} mice were bred with Osx-CreER transgenic mice and resultant Chip^{OsxER} KO mice were administered with tamoxifen in 2-week-old Chip^{flox/flox} (Cre-negative control) mice and Chip^{OsxER} mice and the mice were killed at 4 weeks of age. TRAP staining was performed. Significant increases in the numbers of TRAP-positive cells were detected in subchondral bone areas (a) and in trabecular bone areas underneath the growth plate (b and c) in Chip^{OsxER} KO mice. KO, knockout; TRAP, tartrate-resistant acid phosphatase
generated Chip\textsuperscript{CMV} and Chip\textsuperscript{OsxER} conditional KO mice, which showed bone loss phenotype as we observed in Chip global KO mice (Li et al., 2014; Wang et al., 2018), suggesting that Chip\textsuperscript{flox/flox} mice could be used for long-term and longitudinal studies.

The roles of CHIP in physiological functions and disease initiation and progression have been extensively investigated in recent years. For example, CHIP plays a critical role in neurodegenerative diseases, inflammation, and cardiovascular diseases. Generation and making the availability of Chip\textsuperscript{flox/flox} mice to other fields will make studies of CHIP functions in other organ systems easier.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

W. W., J. L., F. C. K., X. Z., Y. Q., and R. S. L. carried out experiments. T. W. and D. C. prepared the manuscript, contributed to the experimental design, data interpretation, and finalized the manuscript. D. R. S. revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal protocol of this study has been approved by the IACUC of the Rush University Medical Center and all experimental methods and procedures were carried out in accordance with the approved guidelines.

REFERENCES

Abu-Amer, Y. (2013). NF-\textsuperscript{x}B signaling and bone resorption. Osteoporosis International, 24(9), 2377–2386.

Beauharnois, J. M., Bolivar, B. E., & Welch, J. T. (2013). SirTuin 6: A review of biological effects and potential therapeutic properties. Molecular BioSystems, 9(7), 1789–1806.

Boyce, B. F., Yao, Z., & Xing, L. (2010). Functions of NF-\textsuperscript{x}B in bone. Annals of the New York Academy of Sciences, 1192, 367–375.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochemical and Biophysical Research Communications, 273(2), 793–798.

Jimi, E., Kokabu, S., Matsubara, T., Nakamoto, C., Matsuoka, K., & Watanabe, S. (2016). NF-\textsuperscript{x}B acts as a multifunctional modulator in bone invasion by oral squamous cell carcinoma. Oral Science International, 13, 1–6.

Li, S., Shu, B., Zhang, Y., Li, J., Wang, Y., Ren, F., ... Chen, D. (2014). CHIP/STUB1 regulates osteoclast formation through degradation of TRAF6. Arthritis Rheumatol, 66(7), 1854–1863.

Maes, C., Kobayashi, T., Selig, M. K., Torrekins, S., Roth, S. I., Mackem, S., ... Kronenberg, H. M. (2010). Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Developmental Cell, 19, 329–344.

Min, J. N., Whaley, R. A., Sharpless, N. E., Lockyer, P., Portbury, A. L., & Patterson, C. (2008). CHIP deficiency decreases longevity, with accelerated aging phenotypes accompanied by altered protein quality control. Molecular and Cellular Biology, 28, 4018–4025.

Otero, J. E., Chen, T., Zhang, K., & Abu-Amer, Y. (2012). Constitutively active canonical NF-\textsuperscript{x}B pathway induces severe bone loss in mice. PLoS One, 7(6), e38694.

Park, B. K., Zhang, H., Zeng, Q., Dai, J., Keller, E. T., Giordano, T., ... Wang, C.-Y. (2007). NF-\textsuperscript{x}B in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nature Medicine, 13(1), 62–69.

Shu, B., Li, T. F., Li, X. F., Tang, D. Z., Zhang, Y., Shi, Q., ... Chen, D. (2013). Chondrocyte-specific inhibition of \( \beta \)-catenin signaling leads to dysplasia of the caudal vertebrae in mice. Spine, 38(24), 2079–2084.

Swarnkar, G., Zhang, K., Mbalaviele, G., Long, F., & Abu-Amer, Y. (2014). Constitutive activation of IKK2/NF-\textsuperscript{x}B impairs osteogenesis and skeletal development. PLoS One, 9(3), e91421.

Wang, B., Jin, H., Zhu, M., Li, J., Zhao, L., Zhang, Y., ... Chen, D. (2014). Chondrocyte \( \beta \)-catenin signaling regulates postnatal bone remodeling through modulation of osteoclast formation in a murine model. Arthritis & Rheumatology, 66(1), 107–120.

Wang, T., Li, S., Yi, D., Zhou, G.-Q., Ma, P., Chang, Z., ... Chen, D. (2018). CHIP regulates bone mass by targeting multiple TRAF family members in bone marrow stromal cells. Bone Research, 6(1), 10.

Wang, T., Li, J., Zhou, G., Ma, P., Zhao, Y., Wang, B., & Chen, D. (2017). Specific deletion of \( \beta \)-catenin in Col2-expressing cells leads to defects in epiphyseal bone. International Journal of Biological Sciences, 13(12), 1540–1546.

Xing, L., Chen, D., & Boyce, B. F. (2013). Mice deficient in NF-\textsuperscript{x}B p50 and p52 or RANK have defective growth plate formation and postnatal dwarfism. Bone Research, 1(4), 336–345.

Yao, Z., Li, Y., Yin, X., Dong, Y., Xing, L., & Boyce, B. F. (2014). NF-\textsuperscript{x}B RelB negatively regulates osteoblast differentiation and bone formation. Journal of Bone and Mineral Research, 29(4), 866–877.

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