Choline Kinase β Mutant Mice Exhibit Reduced Phosphocholine, Elevated Osteoclast Activity, and Low Bone Mass*

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Background: Bone homeostasis requires constant remodeling by bone-resorbing osteoclasts and bone-forming osteoblasts. However, the precise molecular mechanism(s) underlying the differentiation and activities of these specialized cells are still largely unknown. Here, we identify choline kinase β (CHKB), a kinase involved in the biosynthesis of phosphatidylincholine, as a novel regulator of bone homeostasis. Choline kinase β mutant mice (flp/flp) exhibit a systemic low bone mass phenotype. Consistently, osteoclast numbers and activity are elevated in flp/flp mice. Interestingly, osteoclasts derived from flp/flp mice exhibit reduced sensitivity to excessive levels of extracellular calcium, which could account for the increased bone resorption. Conversely, supplementation of cytidine 5′-diphosphocholine in vivo and in vitro, a regimen that bypasses CHKB deficiency, restores osteoclast numbers to physiological levels. Finally, we demonstrate that, in addition to modulating osteoclast formation and function, loss of CHKB corresponds with a reduction in bone formation by osteoblasts. Taken together, these data posit CHKB as a new modulator of bone homeostasis.

Results: Choline kinase β (CHKB)-deficient mice have reduced bone mass, increased osteoclast number, and impaired osteoblast function.

Conclusion: CHKB activity controls bone homeostasis via regulation of osteoclasts and osteoblasts.

Significance: This is the first report implicating a role for CHKB in bone homeostasis.

The maintenance of bone homeostasis requires tight coupling between bone-forming osteoblasts and bone-resorbing osteoclasts. However, the precise molecular mechanism(s) underlying the differentiation and activities of these specialized cells are still largely unknown. Here, we identify choline kinase β (CHKB), a kinase involved in the biosynthesis of phosphatidylincholine, as a novel regulator of bone homeostasis. Choline kinase β mutant mice (flp/flp) exhibit a systemic low bone mass phenotype. Consistently, osteoclast numbers and activity are elevated in flp/flp mice. Interestingly, osteoclasts derived from flp/flp mice exhibit reduced sensitivity to excessive levels of extracellular calcium, which could account for the increased bone resorption. Conversely, supplementation of cytidine 5′-diphosphocholine in vivo and in vitro, a regimen that bypasses CHKB deficiency, restores osteoclast numbers to physiological levels. Finally, we demonstrate that, in addition to modulating osteoclast formation and function, loss of CHKB corresponds with a reduction in bone formation by osteoblasts. Taken together, these data posit CHKB as a new modulator of bone homeostasis.

Hematopoietic precursors of the monocyte/macrophage lineage differentiate into bone-resorbing osteoclasts in response to the receptor activator of NFκB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). These essential osteoclastogenic cytokines are produced by osteoblasts/stromal cells and immune cells in the bone marrow and by osteocytes in the bone matrix. Despite its relatively static appearance, bone is a dynamic and metabolically active tissue that undergoes continuous cycles of remodeling throughout life, a process that involves the breakdown of bone by osteoclasts and the formation of new bone by osteoblasts to maintain its structural integrity. The remodeling process must be highly regulated to maintain bone homeostasis. Most adult skeletal diseases, including osteoporosis, rheumatoid arthritis, and multiple myeloma, are the result of an imbalance in bone remodeling leading to excessive osteoclast activation. These conditions cause considerable suffering and place a huge economic burden on society. Currently, little is known about the fine molecular details underlying many of these aberrant bone disorders. Therefore, deciphering the molecular mechanisms that regulate bone homeostasis holds the potential to identify novel therapeutic targets for the treatment and alleviation of bone disease.

Choline kinases represent an important, yet largely understudied, class of protein kinases that serve to catalyze the first phosphorylation reaction in the biosynthesis of phosphatidylincholine, a major membrane phospholipid. Choline kinases exist as three distinct isoforms (α1, α2, and β) encoded by two genes Chka and Chkb (8–11), which are ubiquitously expressed, although choline kinase α is highly expressed in the testes with CHKB expression predominant in the heart and liver (12, 13). Of the two choline kinase-encoding genes, Chka has been best characterized, particularly with respect to its potential involvement in malignant transformation (14). Although the precise role of choline kinases in human disease remains unclear, their physiological importance is exemplified in genetic studies in mice. Deletion of Chka is embryonically lethal in mice attesting to its fundamental role in embryonic development (15), whereas loss of Chkb results in severe rostrocaudal muscular dystrophy in skeletal muscle and bone deformity at the forelimb.
CHKB Regulates Bone Homeostasis

Recent studies have identified a subset of human muscular dystrophy patients with CHKB mutations (17–19). Using a phenomics-based approach, we screened N-ethyl-N-nitrosourea (ENU)-induced mutant mice to uncover potential modulators of bone homeostasis. We identified a mutant line that exhibited a low bone mass phenotype (osteopenia) and a forelimb deformity designated flipper (flp/flp). Sequence analysis revealed that flp/flp mice possess a mutation in the start codon of the Chkb gene. Here, we demonstrate that the low bone mass phenotype of flp/flp mice is attributed to intrinsic deficiencies in the formation and function of osteoclasts and osteoblasts, thus uncovering CHKB as an important regulator of bone homeostasis.

EXPERIMENTAL PROCEDURES

Generation of Mice—The flp/flp mice used in this study were generated by the Australian Phenomics Facility at the Australian National University in Canberra, Australia. This strain is available from the Australian Phenome Bank. The mutant mice were produced by ENU-induced mutagenesis as described previously (20). The mutant C57BL/6 mice were outcrossed to a mapping strain (NOD) to produce F1 carrier mice. The wild type and CHKB mutant mice used in this study are from the F10 to F16 progeny. Animal studies were carried out in accordance with protocols approved by the University of Western Australia animal ethics committee and the Australian National University animal ethics committee.

Radiography Assessment—Radiographic assessment of the hind limbs and tail of wild type and flp/flp mice was performed using a Senographe DS digital mammography machine at Sir Charles Gairdner Hospital, Perth, Australia. The hind limbs were imaged using an x-ray voltage of 22 kV and current of 12.5 mA. To image the tail, a 22-kV voltage was used with a current of 10 mA.

X-ray Microcomputed Tomography (Micro-CT)—Micro-CT (SkyScan 1174, Bruker) was performed on tibias of 2-month-old wild type or flp/flp male mice. Analysis of trabecular bone was performed over a 1-mm length, 0.5 mm below the growth plate. Analysis of cortical bone was performed on a region 0.6 mm in length, 3.3 mm from the growth plate. The following acquisition parameters were used: x-ray tube voltage of 50 kV; current 800 μA; isotropic voxel size 9 μm; 0.5-mm aluminum filter; angular increment 0.4° over an angular rotation of 180°. Datasets were reconstructed using cone beam reconstruction software (NRecon, Bruker) based on the Feldkamp algorithm and segmented into binary images using adaptive local thresholding. Morphometric parameters, including percentage bone volume to tissue volume, trabecular thickness, trabecular number, and trabecular separation, cortical bone volume, and cortical thickness were measured using CTAn software (Bruker) (21).

Histomorphometric Analysis—For dynamic histomorphometry measurements, calcein green (5 mg/kg) was injected into wild type and flp/flp mice. A second intraperitoneal calcein (5 mg/kg) injection followed 7 days later. The mice were sacrificed 2 days after the second injection. Undecalcified femora were embedded in methyl methacrylate. In vivo osteoclast and osteoblast numbers were generated from formalin-fixed, paraffin-embedded, and decalcified tibias stained with H&E or tartrate-resistant acid phosphatase (TRAP). Histomorphometric analysis was performed using Osteomeasurexp™ Version 1.20.0.3 software. For all analyses, a 1-mm region, 0.5 mm below the growth plate, was measured.

Forelimb Staining—Differential staining was performed on the forelimbs of mice aged 18.5 days post-coitum and 1 week old as described previously (22). The forelimbs were imaged using a stereoscopic microscope. The measurement of forelimb and hind limb length was performed with ImageJ software (version 1.47p) (23).

Osteoclast Cultures—Osteoclasts were generated from freshly isolated bone marrow cells as described previously (24). Cells were fixed at the indicated times with 4% paraformaldehyde and stained for TRAP. Flow cytometry analyses of triple negative bone marrow populations were performed as described previously (24). Apoptosis was assessed using CellEvent™ caspase 3/7 detection reagent (Invitrogen) according to the manufacturer’s instructions. Apoptotic nuclear morphology was assessed using 4’,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized on a Nikon TE2000-U microscope, and images were collected and analyzed using NIS Elements BR software (Nikon). Bone resorption assay was performed as described previously (24). The number of TRAP-positive osteoclasts was scored prior to assessment of resorptive activity. Resorption pits were visualized by scanning electron microscopy, and the area of bone resorbed was measured using ImageJ software. Reflective confocal microscopy was used to capture Z stacks of resorption pits for depth analysis, and the distance between the surface of the bone and the base of the resorption pit was measured using NIS-Elements Basic Research software (Nikon) (23). C-terminal collagen cross-links (CTX) in medium were determined using CrossLaps for Culture ELISA kit (Immunodiagnostic Systems) according to the manufacturer’s instruction.

Osteoblast Cultures—For osteoblastogenesis assays, nucleated bone marrow cells were plated into culture dishes at a cell density of 1 × 10⁶ cells/ml in complete α-minimal essential medium (α-minimal essential medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). When confluent, the osteogenic media (complete α-minimal essential medium, 10 nM dexamethasone, 2 mM β-glycerophosphate, and 50 μg/ml ascorbate) was added. After 14 days, the cells were fixed and stained with 1% alizarin red (25). ImageJ software was used to measure the mineralized area (23). Matrix vesicles were isolated from day 28 osteoblast cells cultured in the absence of β-glycerophosphate as described previously (26) and analyzed by Biomol inorganic phosphate assay (Sapphire Biosciences, Australia) according to the manufacturer’s instructions.

Detection of Choline Metabolite Levels in Bone Cells—Phosphatidylcholine levels in mature osteoclasts were measured using the phosphatidylcholine assay kit (ab83377, Abcam, Sapphire Bioscience, Australia) according to the manufacturer’s instructions. 1H NMR spectroscopy was performed to identify relative levels of choline metabolites in whole cells derived from bone marrow of mice as described previously (27). Briefly, bone marrow macrophages (BMMs) were cultured in T-75 flasks to
confluence and stimulated with M-CSF and RANKL to induce osteoclast formation. Outgrowth osteoblast cultures were prepared from long bones of adult mice as described previously (28). Cells were harvested and resuspended at 2 × 10⁶ cells in 500 μl of PBS (90% D₂O). Cells were immediately snap frozen and stored at −80 °C until analysis (27). Prior to analysis, cells were thawed and warmed to 21 °C and then transferred to an NMR tube. Samples were not spun. Gradient shimming was performed on the D₂O deuterium signal using the routine of the Bruker Avance 600 MHz spectrometer (Bruker). High resolution ¹H NMR spectra were acquired at 21 °C with water presaturation using a triple tuned ¹H-¹³C-¹⁵N inverse probe. Scan acquisition was as follows: 90° pulse, 17 μs; transverse reaction, 5.53 s; spectral width, 7716.05 Hz in 8000 complex points; 256 transients. The FIDs were exponentially multiplied with 0.2 Hz line broadening and Fourier-transformed to 64,000 complex points. Peak assignments were confirmed by addition of standard compounds. 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-d13 sodium 3-trimethylsilylpropionate was added as a chemical shift reference at 0.00 ppm. Spectra were analyzed individually off line using TopSpin and WinNMR software (Bruker). For all samples, a polynomial baseline fit of the Fourier-transformed spectrum in the extracted region 3.1–3.4 ppm was performed. Resolved peaks were fit to Lorentzian line shapes by adjusting linewidths and intensities to give a visual match between simulated and actual spectrum. Ratios of metabolite values relative to the central taurine peak were determined.

**Immunoblotting**—BMMs were cultured with M-CSF and GST-RANKL for various time periods as indicated. Western blotting was performed as described previously (24). Antibodies used were as follows: mouse anti-β-actin antibody (Developmental Studies Hybridoma Bank, University of Iowa); rabbit anti-NFATc1 antibody (Santa Cruz Biotechnology); rabbit anti-CHKB antibody (kindly donated by Dr. Chieko Aoyama); rabbit anti-1xBo antibody (Santa Cruz Biotechnology); mouse anti-pERK antibody (Santa Cruz Biotechnology); rabbit anti-ERK antibody (Promega); rabbit anti-p-p38 antibody (Cell Signaling); and rabbit anti-VATPase d2 antibody (29). Detection was done by respective peroxidase-conjugated antibodies (Sigma) and chemiluminescence reagent (PerkinElmer Life Sciences).

**Proliferation Assay**—Osteoblasts were isolated from the calvaria of 5-week-old mice as described previously (28). The resulting cell suspension was cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Proliferation assay was performed using the CellTiter 96® AQueous MTS cell proliferation assay (Promega) according to manufacturer’s guidelines. 

**PCR Analysis**—Total RNA was extracted from the hind limb or cultured cells at the indicated times, from wild type and flp/flp mice using TRIzol (Invitrogen) and phenol/chloroform incorporated with the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA was transcribed into cDNA using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega). Real time PCR was performed using SYBR Green PCR Master Mix (Qiagen). Primers used are listed in Table 1. Each sample was analyzed in triplicate and normalized to 18 S ribosomal RNA.

**Immunofluorescence**—Osteoclasts cultured on coverslips or bone discs were fixed and permeabilized with 0.1% Triton X-100. F-actin was stained with rhodamine-conjugated phalloidin 546 (Molecular Probes). Nuclei were stained with DAPI (Sigma) or PBS control, by intraperitoneal injection, three times a week for 3 weeks.

**Fluorescent Detection of Intracellular Calcium**—Intracellular calcium was monitored using the fluorescent indicator Fura-2 acetoxyethyl ester (Molecular Probes) at 1 μM in a HEPES-buffered solution containing 5.33 mM KCl, 0.41 mM MgSO₄, 139 mM NaCl, 5.63 mM Na₂HPO₄, 5 mM glucose, 20 mM HEPES, 2 mM glutamate, and 2.5 mM Ca(NO₃)₂ (adjusted to pH 7.4 with NaOH), at 37 °C as described previously (31). In some experiments, increased calcium was added as indicated. Fluorescence at 340/380 nm excitation and 510 nm emission was measured at 1-min intervals with an exposure of 50 ms on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Ratiometric 340/380 nm signals of individual osteoclasts were quantified using Metamorph 6.3 to measure signal intensity of manually traced cell regions. An equivalent region not containing cells was used for background and was subtracted.

**CDP-choline Supplementation**—To assess the effects of CDP-choline *in vivo*, wild type and flp/flp mice (female, 3 months old) were treated with CDP-choline (500 mg/kg) (Sigma) or PBS control, by intraperitoneal injection, three times a week for 3 weeks.

**Statistics**—All data presented are expressed as the means ± S.E. from at least triplicate measurements. The results presented are from a single experiment that is representative of at least three independent experiments. Where a direct comparison is made between wild type and flp/flp, Student’s t test was performed. For multiple comparisons, one-way analysis of variance statistical analysis was carried out with a post-hoc Bonfer-
**RESULTS**

ENU-induced Mutagenesis Reveals Reduced Bone Mass in flp/flp Mice—To identify novel genes regulating bone homeostasis, we employed an ENU-induced mutagenesis approach (32). Radiographic screening of several ENU-induced mutant mouse lines identified a mutant mouse line, named fliper (flp/flp), that exhibited a systemic reduction in bone density as demonstrated in the hind limb and tail compared with wild type littermate controls at 2 months of age (Fig. 1A). In addition, these mice displayed a forelimb deformity (Fig. 1B). Genetic mapping identified an A-to-T base substitution in the start codon of the Chkb gene in flp/flp mice (Fig. 1C), resulting in a loss of gene translation. Immunoblot analyses confirmed CHKB protein is present in BMM derived from wild type mice but not flp/flp mutant mice (Fig. 1D). Consistent with a loss of CHKB function (33), analysis of choline metabolite levels in bone cells (both osteoclasts and osteoblasts) derived from flp/flp mice showed significant reductions in phosphocholine, glycerophosphocholine, and phosphatidylcholine levels as compared with those derived from wild type littermates (Fig. 1, E–G).

Decreased Bone Mass of flp/flp Mice Correlates with Increased Osteoclast Numbers and Reduced Bone Formation in Vivo—Quantitative assessment of 2-month-old male mice by micro-CT showed flp/flp mice displayed a 50% reduction in trabecular bone volume compared with wild type littermate controls, with corresponding decreases in trabecular thickness and number and a concomitant increase in trabecular separation (Fig. 2, A and B). Trabecular bone mineral density was also significantly reduced in the flp/flp mice (Fig. 2B). Consistently, there was a significant reduction in cortical bone volume and cortical thickness in the flp/flp mice compared with the wild type mice. The reduction in cortical bone was accompanied by a significant reduction in bone surface (WT = 9.07 ± 0.23 mm² versus flp/flp = 7.10 ± 0.66 mm², p < 0.001) indicating that flp/flp tibias have a reduced periosteal diameter. micro-CT analysis of 3-month-old female mice revealed a similar low bone mass phenotype, which persisted into old age, as 8-month-old flp/flp mice exhibited a reduction in bone volume (data not shown).

Osteoclast and osteoblast parameters analyzed by histological examination of TRAP-stained sections and quantitative histomorphometric analysis revealed a significant increase in the number of osteoclasts in the tibias of flp/flp mice (Fig. 2C). There was no significant difference in osteoclast number in vivo (Fig. 2C). In agreement with these results, quantitative PCR analysis of osteoclast and osteoblast marker gene expression in whole bone tibias showed a significant increase in osteoclast marker gene expression with no significant change in the expression of osteoblast marker genes (Fig. 2D). However, in vivo double calcein labeling revealed a significant decrease in mineral apposition rate and bone formation rate in flp/flp mice as compared with wild type controls (Fig. 2E), indicating that osteoblast function is impaired. No significant difference was observed in mineralizing surface/bone surface. Quantitative PCR analysis of the relative levels of CHKA and CHKB in wild type bone marrow-derived osteoclasts and osteoblasts revealed that CHKB is the predominant isoform expressed in both cell types (Fig. 2F). Therefore, loss of the CHKB isoform is likely to be responsible for the differences observed in osteoclast number and osteoblastic bone formation.

flp/flp Mice Exhibit Increased Osteoclast Formation Rates—In vitro analysis of osteoclastogenesis from flp/flp and wild type M-CSF and RANKL-stimulated BMMs showed a 43 and 50% increase in the number of TRAP-positive multinucleated (nuclei >3) osteoclasts in flp/flp cultures at days 5 and 7 post-RANKL stimulation, respectively (Fig. 3A). Consistent with the results obtained from bone marrow-derived cells, osteoclast formation was also enhanced in spleen cells isolated from flp/flp mice (data not shown). It has been previously identified that the osteoclast progenitor cell population lies within the CD45R−CD3−CD11blow− bone marrow population (34). Flow cytometry showed a trend toward increases in osteoclast progenitor cells in the bone marrow of flp/flp mice (Fig. 3, B and C). Western blotting analysis of protein isolated from flp/flp BMMs revealed an increase in cathepsin K and vacuolar ATPase subunit d2 at day 5 of culture, consistent with the observed increased osteoclastogenesis in flp/flp mice (Fig. 1D). There was a clear increase in ERK and p38 phosphorylation in flp/flp BMMs stimulated with RANKL over a 1-h period (Fig. 3D). ERK and p38 have been associated with cell survival in osteoclasts, and therefore an increased activation of ERK in the CHKB mutant bone marrow cultures suggests enhanced survival of CHKB mutant osteoclasts (35, 36). However, assessment of apoptosis by fluorescent visualization of caspase 3/7 activity and nuclear morphology (DAPI) revealed no significant difference in the percentage of mononuclear or multinuclear cells undergoing apoptosis at day 7 of culture or after 6 days of RANKL stimulation followed by a 16-h cytokine (M-CSF and RANKL) withdrawal, between WT and flp/flp cultures (Fig. 3E). As ERK and p38 activity is induced by RANKL stimulation in osteoclast progenitors, this result likely indicates an increased proportion of osteoclast progenitors present in the flp/flp cultures, in agreement with the flow cytometry results. By contrast, we observed no difference in the degradation profiles of IκBα, suggesting that the NFκB pathway was largely unaffected by the loss of CHKB.

Osteoblasts from flp/flp Mice Have Impaired Mineralization Capacity and Reduced Proliferation Rates in Vitro—Consistent with the in vivo observations, osteoblasts derived from bone marrow of flp/flp mice displayed a significant reduction in mineralization activity as evidenced by a reduction in alizarin red staining in bone nodule formation assays (Fig. 3F). A similar decrease in bone nodule formation was also observed in calvaria-derived osteoblast cultures (data not shown). This reduction in mineralization capacity may be attributed, at least in part, to reduced proliferation rates of osteoblasts derived from flp/flp mice that were significantly reduced at 48 h in cell proliferation assays (Fig. 3G). Phosphocholine is a major substrate for the mineralization enzyme PHOSPHO1; consistent with the reduced levels of phosphocholine present in flp/flp osteoblasts, production of inorganic phosphate by matrix vesicles derived
from flp/flp osteoblasts was significantly reduced (Fig. 3H). The addition of phosphocholine normalized phosphate production, an effect that was abolished by the further addition of the PHOSPHO1 inhibitor lansoprazole. Taken together, these results indicate that, in addition to increased osteoclast numbers, osteoblast function is also compromised in flp/flp mice.
due to reduced proliferation and reduced production of phosphate for mineralization.

Osteoclasts Derived from flp/flp Mice Have Enhanced Bone Resorptive Activity—Mature osteoclasts derived from flp/flp mice were able to attach to the bone surface and initiate resorption when seeded onto bone slices (Fig. 4, A and B). The area of bone resorbed per osteoclast was indistinguishable between wild type and flp/flp osteoclasts (Fig. 4, C). Detailed inspection of resorptive pit parameters revealed an equivalent degree of osteoclastic polarization, as signified by the formation of a well defined F-actin ring, but with significantly deeper resorption pits formed by the flp/flp osteoclasts as compared with those formed by the wild type osteoclasts (Fig. 4, D and E) implying that osteoclasts derived from flp/flp mice exhibit enhanced resorptive activity. Confirming this observation, levels of collagen CTX released per osteoclast were significantly increased in medium from flp/flp osteoclasts cultured on bone (Fig. 4, F).

FIGURE 2. Mutation in choline kinase β results in bone loss. A, two-dimensional micro-CT images of the tibia of flp/flp mice compared with wild type. B, structural parameters of the tibial bone (n = 5 per group), including the ratio of bone volume to total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), cortical bone volume (BV), cortical thickness, trabecular bone mineral density (BMD), and cortical bone mineral density (BMD). C, histomorphometric analysis of the tibia reveals an increase in osteoclast number (N.Oc/B.Pm) but no change in osteoblast numbers (N.Ob/B.Pm) in flp/flp mice compared with wild type. D, quantitative PCR of osteoclast marker genes: TRAP, cathepsin K (CatK), and MMP9 and osteoblast marker genes as follows: alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN) in whole bone tibias. n = 3. E, calcein double labeling of flp/flp and wild type mice showing reduction in mineral apposition rate (MAR) and bone formation rate (BFR), with no change in mineralizing surface/bone surface (MS/BS). n = 5. Scale bars, 50 μm. Data are presented as mean ± S.E. *, p < 0.05; **, p < 0.01; n.s., no significance. F, quantitative PCR analysis of expression levels of CHKA and CHKB relative to 18 S RNA in osteoclasts and osteoblasts derived from wild type BMM.

Osteoclasts Derived from flp/flp Mice Have Enhanced Bone Resorptive Activity—Mature osteoclasts derived from flp/flp mice were able to attach to the bone surface and initiate resorption when seeded onto bone slices (Fig. 4, A and B). The area of bone resorbed per osteoclast was indistinguishable between wild type and flp/flp osteoclasts (Fig. 4C). Detailed inspection of resorptive pit parameters revealed an equivalent degree of osteoclastic polarization, as signified by the formation of a well defined F-actin ring, but with significantly deeper resorption pits formed by the flp/flp osteoclasts as compared with those formed by the wild type osteoclasts (Fig. 4, D and E) implying that osteoclasts derived from flp/flp mice exhibit enhanced resorptive activity. Confirming this observation, levels of collagen CTX released per osteoclast were significantly increased in medium from flp/flp osteoclasts cultured on bone (Fig. 4F).
flip/flp Osteoclasts Have Reduced Intracellular Calcium Levels in Response to Extracellular Calcium—An increase in intracellular calcium concentration, \([Ca^{2+}]_{in}\), in response to increased extracellular calcium, \([Ca^{2+}]_{ex}\), has been implicated in the retraction of the osteoclast from the resorptive lacuna, as well as in reducing the secretion of bone-degrading enzymes (37). Considering the deeper resorption pits observed from osteoclasts derived from flip/flp mice, we reasoned that flip/flp osteoclasts may have a reduced sensitivity to \([Ca^{2+}]_{ex}\), allowing them to continue resorbing bone thereby forming deeper pits.

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Wild type and flp/flp osteoclasts were subjected to increasing concentrations of \([\text{Ca}^{2+}]_{\text{Ex}}\), and resulting alterations in \([\text{Ca}^{2+}]_{\text{In}}\) were assessed by measuring changes in 340/380 nm ratiometric Fura-2 fluorescence (340/380 nm fluorescence). As shown in Fig. 5A, there was a significant increase in fluorescence in wild type but not in flp/flp osteoclasts, when exposed to increasing levels of \([\text{Ca}^{2+}]_{\text{Ex}}\), implying that either calcium uptake or retention of calcium within flp/flp osteoclasts is attenuated (Fig. 5A). Wild type osteoclasts pretreated with thapsigargin exhibited a significant increase in response to 10 and 20 mM extracellular calcium (Fig. 5B). However, flp/flp osteoclasts only exhibited a significant increase in response to 20 mM extracellular calcium (Fig. 5B). These data further suggest that calcium transport across the membrane of flp/flp osteoclasts, in response to \([\text{Ca}^{2+}]_{\text{Ex}}\), is reduced in the flp/flp osteoclasts. To complement these experiments, wild type and flp/flp osteoclasts were also treated with ionomycin, a calcium ionophore. Wild type but not flp/flp osteoclasts exhibited a significant increase in 340/380 nm fluorescence in response to ionomycin (Fig. 5C). Together, these results suggest that calcium transport across the membrane in response to high \([\text{Ca}^{2+}]_{\text{Ex}}\) is impaired in flp/flp osteoclasts.
CDP-choline Treatment in Vitro Partially Rescues the Increased Osteoclast Numbers in flp/flp Mice—To rescue the elevation in osteoclast numbers in the flp/flp BMM, we supplemented cell cultures with CDP-choline to overcome the reduction in choline metabolites associated with CHKB deficiency in flp/flp mice. This has been shown to be effective in restoring choline metabolism in CHKB-deficient animals (15). Treatment with CDP-choline at both doses (0.1 and 1 μM) attenuated the number of osteoclasts in the flp/flp-derived cultures such that their numbers returned to baseline (Fig. 6A). However, similarly treating bone marrow-derived osteoblast cultures with CDP-choline did not restore bone mineralization potential in flp/flp osteoblasts (Fig. 6B).

CDP-choline Treatment in Vivo Restores Physiological Osteoclast Numbers—The capacity of CDP-choline treatment to rescue the bone defect in vivo was assessed. Wild type and flp/flp bone marrow were subjected to increasing concentrations of extracellular calcium. Resulting alterations in intracellular calcium were assessed using the fluorescent indicator Fura-2 (340/380 nm fluorescence). n = 5. BMM-derived osteoclasts from wild type and flp/flp mice were pre-treated with thapsigargin. Alterations in intracellular calcium in response to increasing concentrations of extracellular calcium were then assessed using Fura-2. n = 10. C, wild type and flp/flp BMM-derived osteoclasts were treated with 5 μM ionomycin (calcium ionophore). Resulting alterations in intracellular calcium were assessed using Fura-2. n = 10. The curves are representative data for a single cell, and the bars are presented as mean ± S.E. of 5–10 cells. *, p < 0.05 compared with wild type basal levels; #, p < 0.05 compared with flp/flp basal levels; n.s., no significance.

The effect of CDP-choline on ex vivo osteoblast mineralization was consistent with the results in Fig. 3E, showing a significant decrease in mineralization in the control flp/flp bone marrow-derived osteoblast cultures compared with wild type. However, although treatment with CDP-choline marginally increased osteoblast mineralization in both wild type and flp/flp cultures, this regimen was not sufficient to fully rescue the decreased mineralization by flp/flp osteoblasts (Fig. 6D).

DISCUSSION

The maintenance of bone homeostasis is highly dependent upon the complementary yet opposing activities of two key cells types, namely the osteoclast and osteoblast (38); however, the precise molecular mechanisms regulating the number and activities of these two resident bone cells remain largely unclear. Using an ENU-induced mutagenesis-based approach, we showed that loss of CHKB in flp/flp mice resulted in reduced bone mass in the hind limb and tail. Furthermore, this decrease in bone mass was attributed to elevated number and/or activity...
of osteoclasts and reduced activity of osteoblasts. To our knowledge, this is the first study to implicate a direct role for CHKB in bone homeostasis.

Accumulated studies in recent years, including the generation of chka- and chkb-specific knock-out mice, have helped unveil important new roles for choline kinase in multiple cells and tissues outside of its known role in lipid homeostasis (39, 40). Loss of CHKB corresponds to a hind limb muscular dystrophy and forelimb deformity due to decreased levels of phosphatidylcholine (16, 41). A recent study has established that the forelimb deformity in CHKB-deficient mice is due to altered growth plate physiology in the embryo, implicating a role for CHKB in endochondral bone formation (42). Homozygous and compound heterozygous mutations in CHKB have been identified in patients with congenital muscular dystrophy (17–19, 43).

Our findings that flp/flp mice are osteopenic further extends the role of CHKB to the maintenance of bone mass. flp/flp mice display reduced trabecular and cortical bone mass and a notable forelimb deformity (16), a phenotype that was consistently observed in both male and female littermates. Reflecting this, flp/flp mice are anatomically smaller and have reduced total forelimb and hind limb length (data not shown), highlighting an

FIGURE 6. CDP-choline treatment partially restores the osteoclast phenotype. A, wild type and flp/flp bone marrow cells were treated with 0.1 or 1 μM CDP-choline, RANKL (100 ng/ml), and M-CSF (10 ng/ml) in vitro for 14 days. Cultures were stained with Alizarin red, and the mineralized area was calculated. C, wild type and flp/flp mice were treated with CDP-choline (500 mg/kg) or PBS control for 3 weeks. Bone marrow cells from these mice were cultured in vitro with RANKL (100 ng/ml) and M-CSF (10 ng/ml). D, area mineralized by bone marrow-derived osteoblasts from wild type and flp/flp mice treated with CDP-choline. Data are presented as mean ± S.E. **, p < 0.01; n.s., no significance.
important role for CHKB in fundamental skeletal development and growth.

At this point in time we cannot rule out the possibility that the forelimb deformity and muscular dystrophy phenotype of the flp/flp mice may contribute to mechanical unloading-induced bone loss. A reduction in muscle use has been associated with reduced bone mass in patients with Duchenne muscular dystrophy and cerebral palsy (44–46). However, we feel that the muscular dystrophy only partially accounts for the reduced bone mass of the flp/flp mice. Studies assessing the role of muscle function on bone mass, using the tail suspension model, report a 10–20% change in bone volume with a decrease in trabecular thickness but no change in trabecular number (47, 48). In the flp/flp mice, we observe at least a 50% decrease in bone mass. Furthermore, we also see a decrease in trabecular number in the flp/flp mice. The CHKB mutant mice are able to move to obtain food and water and have survived up to 18 months of age.

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Moreover, our data demonstrate that the bone loss observed in flp/flp mice is, at least in part, attributed to cell-autonomous defects in osteoclast and osteoblast numbers and/or activity. For instance, flp/flp mice exhibit significantly increased osteoclast numbers both in vitro and in vivo. Furthermore, although the total area of bone resorbed was comparable between wild type and flp/flp osteoclasts, the resorption pits formed by the flp/flp osteoclasts were morphologically deeper, implying that flp/flp osteoclasts have elevated resorptive activity compared with wild type osteoclasts.

FIGURE 7. In vivo treatment with CDP-choline does not rescue the bone phenotype in flp/flp mice. A, two-dimensional micro-CT images of the tibia of 3-month-old female flp/flp mice compared with wild type following PBS or CDP-choline treatment. B, structural parameters of the tibial bone (n = 5 per group), including the ratio of bone volume to total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), cortical bone volume (BV), cortical thickness, trabecular bone mineral density (BMD), and cortical bone mineral density (BMD). Results are presented as mean ± S.E. n = 5 mice per group. *, p < 0.05; **, p < 0.01; n.s., no significance.
CHKB Regulates Bone Homeostasis

In keeping with this position, osteoclasts derived from flp/flp mice display attenuated responses to extracellular calcium indicating a reduced capacity to transport calcium across the plasma membrane. This impairment is likely due to changes in the phospholipid composition observed in the plasma membrane of osteoclasts derived from flp/flp mice, which can alter the spatial positioning of integral membrane proteins (e.g. ion channels) (41). Osteoclasts are exposed to increasing concentrations of Ca²⁺ during bone resorption, a process that is thought to trigger a negative feedback on osteoclastic bone resorptive activity (37, 49). Therefore, it is plausible that changes in the phospholipid composition of flp/flp-derived osteoclasts alter the transport of calcium across the plasma membrane, rendering them partially insensitive to extracellular calcium and thereby prolonging their bone resorptive activity. Future comprehensive comparison of the total membrane-bound proteins present in wild type and flp/flp osteoclast membranes will therefore provide important clues as to the precise nature and identity of proteins directly contributing to the increased osteoclastic bone resorption activity in flp/flp mice.

Analysis of the mitogen-activated protein kinase (MAPK) signaling pathways, the extracellular signal-regulated kinase (ERK), and p38 pathways downstream of RANKL showed a significant increase in phosphorylated ERK and p38 levels in the flp/flp bone marrow cultures. This result suggests that there are increased numbers of progenitor cells responding to RANKL in the flp/flp cultures, in agreement with the osteoclastogenesis and flow cytometry data, and consistent with the osteopetetic phenotype of the flp/flp mice (35, 36, 50, 51).

Our findings also indicate significant reductions in osteoblast proliferation coupled with reduced bone mineralization in flp/flp osteoblasts. Phosphorylation of CHKB substrates is increased upon differentiation of osteoblasts (52). Previous studies have identified the importance of phosphatidylcholine biosynthesis in B cell proliferation (53) and the up-regulation of choline kinase in highly proliferative cancer cells (54). Furthermore, mineralization requires the activity of multiple phosphatases, including the enzyme PHOSPHO1, to generate inorganic phosphate for effective mineralization. Phosphocholine is a highly specific substrate for PHOSPHO1 (55), and loss of PHOSPHO1 activity leads to growth plate abnormalities, bowing of long bones, and hypophosphatemia (56). We demonstrated reduced phosphocholine levels in CHKB-deficient osteoblasts, resulting in reduced availability of inorganic phosphate for bone mineralization, clearly indicating that CHKB dually modulates osteoclast and osteoblast activity. This conclusion has been further verified by the recent publication by Li et al. (57) demonstrating that pharmacological inhibition of choline kinase activity in human osteoblasts resulted in defective mineralization in vitro.

Treatment with CDP-choline has been previously shown to partially rescue the muscular dystrophy phenotype of the Chkb⁻/⁻ mice, and it is in clinical use in Japan and Europe for the treatment of strokes (33, 58, 59). When we treated flp/flp bone marrow cultures with CDP-choline, we were able to suppress, albeit partially, the elevation in osteoclast numbers. However, treatment with CDP-choline had no significant effect on osteoblastic bone mineralization potential. CDP-choline is downstream of phosphocholine in the Kennedy pathway, and this result provides further evidence that the effects of CHKB loss in osteoblasts operate through reductions in phosphocho-line levels. The failure of CDP-choline supplementation to restore bone mass in vivo is likely attributable to the treatment regimen being too short, as well as the failure to rescue the osteoblast phenotype; however, our findings demonstrate that both in vitro and in vivo supplementation of CDP-choline are able to partially rescue the osteoclast-mediated effects in flp/flp mice. As CDP-choline is already approved for human use, and considering the positive effects of CDP-choline supplementation on osteoclasts, this compound warrants further study as a potential therapeutic option in treating osteoporosis.

In summary, we have shown for the first time that flp/flp mice have reduced bone mass, a forelimb deformity, and decreased limb length. Our data indicate that the bone phenotype of the flp/flp mice can be explained, at least in part, by an increase in osteoclast number and activity as well as a corresponding decrease in osteoblast-mediated bone formation. Although the precise molecular mechanism(s) through which CHKB regulates both osteoclast formation and activity and osteoblast function requires further investigation, our findings establish choline kinase B as a novel regulator of bone homeostasis and thus a potential future therapeutic target for bone metabolic disorders.

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