Reduced bone mass is a common complication in chronic inflammatory diseases, although the mechanisms are not completely understood. The PHEX gene encodes a Zn-endopeptidase expressed in osteoblasts and contributes to bone mineralization. The aim of this study was to determine the molecular mechanism involved in TNF-mediated downregulation of Phex gene transcription. We demonstrate downregulation of Phex gene in two models of colitis: naïve T-cell transfer and in gnotobiotic IL-10−/− mice. In vitro, TNF decreased expression of Phex in UMR106 cells and did not require de novo synthesis of a transrepressor. Transfecting UMR-106 cells with a series of deletion constructs of the proximal Phex promoter identified a region located within -74nt containing NF-κB and AP-1 binding sites. After TNF treatment, RelA/p50 NF-κB complex interacted with two cis-elements at positions -70/-66 and -29/-25nt in the proximal Phex promoter. Inhibition of NF-κB signaling increased the basal level of Phex transcription and abrogated the effects of TNF, while overexpression of RelA mimicked the effect of TNF. We identified PARP-1 binding immediately upstream of the NF-κB sites and showed that TNF induced poly-ADP-rybosylation of RelA when bound to Phex promoter. TNF-mediated Phex downregulation was completely abrogated in vitro by PARP-1 inhibitor and by overexpression of poly(ADP-ribose) glucohydrolase (PARG) and in vivo in PARP-1−/− mice. Our results suggest that NF-κB signaling and PARP-1 enzymatic activity cooperatively contribute to the constitutive and inducible suppression of Phex. The described phenomenon likely contributes to the loss of bone mass density in chronic inflammatory diseases, such as IBD.
genetic disorders can skew this balance leading to low bone mineralization. In vitamin D-resistant, X-linked hypophosphatemic rickets (XLH), an inactivating mutation of PHEX (Phosphate-regulating gene with Homologies to Endopeptidases on the X Chromosome) leads to hypophosphatemia, aberrant vitamin D levels, high serum alkaline phosphatase, and osteomalacia (2,3). While the Phex gene encodes a membrane-bound, Zn-metallopeptidase expressed only in OBs and odontoblasts, the effects of its mutation lead to phosphate wasting in the kidney by decreasing the expression and activity of the Na+/Pi cotransporter, NaPi-IIa (NPT2; SLC24A1) in proximal convoluted tubules (4,5). Phex inactivation was postulated to indirectly affect the kidney through bone-released, phosphaturic factors known as phosphatonins. Fibroblast growth factor 23 (FGF23) was one of the leading phosphatonin candidates due to its potent negative effects on renal phosphate reabsorption and its highly elevated expression in XLH patients (6). Although initial studies seemed to confirm this mechanism (7), it was later shown that Phex mutations lead to increased FGF23 expression rather than processing (8), and that FGF23 is cleaved by subtilisin-like proprotein convertases (SPC) and not by PHEX (9).

In addition to the renal abnormalities in XLH, PHEX inactivation also leads to osteoblast mineralization deficits. This is exemplified by the inability of immortalized osteoblasts from Phex-deficient Hyp mice to mineralize in vitro (10). While the target(s) of PHEX proteolytic activity remain uncertain, PHEX protein may influence bone metabolism by binding and stabilizing matrix extracellular phosphoglycoprotein (MEPE), dentine matrix protein 1 (DMP1) (11), and osteopontin (12). Specifically, when PHEX binds to these substrates, it prevents their cleavage and release of a small, acidic protease-resistant ASARM peptide (acidic-serine-aspartate-rich-MEPE-associated motif). These ASARM peptides have been shown to inhibit mineralization in vivo and in vitro, and most likely function by directly binding to hydroxyapatite crystals and by decreasing the expression of Phex (13).

PHEX itself is regulated by several hormones and cytokines important for skeletal homeostasis. Phex is upregulated after treatment with insulin-like growth factor 1, growth hormone (14), and glucocorticoids (15). Alternatively, Phex was found to be downregulated by parathyroid hormone (16), parathyroid hormone-related peptide (17), and vitamin D (18).

We have shown that the proinflammatory cytokine TNF decreases Phex expression in vivo and in vitro (19), an observation with significant pathophysiological implications. During chronic inflammation such as in Inflammatory Bowel Diseases (IBD), circulating and/or infiltrating lymphocytes and other mononuclear cells produce cytokines that can influence bone metabolism by altering the balance of bone mineral deposition and resorption. Decreased bone mineral density is a common outcome of Inflammatory Bowel Disease (IBD). Indeed, 31 to 59% of adult IBD patients are classified as osteopenic, whereas 5 to 41% are actually diagnosed with osteoporosis, although rates of up to 70% of adult and pediatric IBD patients with low bone mineral density (BMD) have been reported (20). In our earlier study (19), we showed that TNF treatment and chemically-induced colitis decrease Phex mRNA expression via a transcriptional mechanism, and that polyadenine (polyA) region located -116 to -110 bp upstream of the transcriptional start site was necessary for the TNF-mediated inhibition. This decrease in Phex expression correlated with decreased mineral deposition in osteoblast-like UMR-106 cells.

In the present work, we extend our previous observations to demonstrate the downregulation of Phex in two more representative models of human IBD: microflora-induced colitis in gnotobiotic IL-10−/− mice and adoptive CD4+CD45RBhigh T-cell transfer into Rag2−/− recipients. Phex gene promoter analysis showed that the polyadenine (polyA) region in the murine Phex promoter consitutively binds PARP-1, while TNF induces binding of the NF-κB complex proximally to this element. RelA is then PARylated to increase its activity as a transrepressor. PARP-1 activity was indispensable for the effects of TNF as demonstrated by a blunted response to the cytokine in the presence of a PARP-1 inhibitor or overexpressed poly (ADP-ribose) glycohydrolase (PARG), and by a complete abrogation of the response to TNF in PARP-1 knockout mice.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents - 10X TBE, 20X SSC, 100 mmol/L sodium pyruvate, 100X Antibiotic-Antimyotic, restriction enzymes, TRIzol reagent, DMEM high glucose medium, gels and T4 DNA ligase were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Mediatech, Inc and Irvine Scientific (Santa Ana, CA), γ-[32P] deoxyadenosine triphosphate was from Perkin Elmer (Boston, MA), ToxiLight BioAssay Kit was purchased from Lonza (Rockland, ME), TransIT-LT1 reagent from Mirus Bio LLC (Madison, WI), Galacto-Star β-galactosidase Reporter Gene Assay System and TaqMan primer sets for real-time RT-PCR were purchased from Applied Biosystems (Foster City, CA). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). qScript cDNA Synthesis Kit and PerfeCTa qPCR SuperMix were from Quanta Biosciences Inc. (Gaithersburg, MD). Recombinant murine (mTNF) and human (hTNF) TNF were purchased from PeproTech (Rocky Hill, NJ). Human NF-κB expression plasmids pFlag-CMV2-RelA and pFlag-CMV2-p50 were kindly provided by Dr. Christian Jobin (University of North Carolina, Chapel Hill, NC). Human poly (ADP-ribose) polymerase 1 (pCMV6-PARP-1), human poly(ADP-ribose) glucohydrolase (pCMV6-PARG) expression plasmids and control vector pCMV6-null were from OriGene Technologies Inc. (Rockville, MD). Control expression vector pTarget, Caspase-Glo 3/7 Assay Systems and Streptavidin Magna-Sphere® Paramagnetic Particles (SA-PMPsm) were from Promega (Madison, WI). All other reagents, unless otherwise indicated, were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma (St Louis, MO).

Experimental animals - Specific pathogen-free (SPF) wild-type (WT) 129/SvEv mice and germ-free IL-10−/− mice were purchased from the National Gnotobiotic Rodent Resource Center at the University of North Carolina, Chapel Hill. Germ-free IL-10−/− mice were transferred to the SPF facility and kept in sterile cages 2 days prior to being colonized with SPF fecal bacteria. 14 days after bacterial colonization allowed for development of mild to moderate colitis in SPF-associated previously germ-free IL-10−/− mice (21). Adoptive T-cell transfer colitis was induced by i.p. injection of 0.5x10^6 naive, flow-sorted CD4+CD45RB^high lymphocytes (98% purity) into Rag-2−/− host (both C57BL/6) (22). Control (PBS-injected) and colitic mice were sacrificed 8 weeks after transfer. Additionally, 4-5 week-old male WT and PARP-1 KO mice purchased from Jackson Labs (129s1/SvImJ, 129SParp1/J respectively) were injected i.p. once with murine recombinant TNF (PeproTech; Rocky Hill, NJ) at a dose of 150 μg/kg B.W. (22) or with an equal volume of vehicle (PBS). Mice were sacrificed 24 hours after injection. All mice used for experiments described above were supplied with food and water ad libitum. At respective time points mice were sacrificed by CO2 narcosis followed by cervical dislocation. Femurs and calvarias were removed, flash frozen in liquid nitrogen and stored at -80°C before RNA isolation. Colitis was evaluated histologically, as well as by cytokine analysis in colonic explant culture and lymphocytes isolated from the mesenteric lymph nodes and stimulated in vitro with CD3/CD28 or with cecal antigen extract as described earlier (21,22). Animals maintained at the University of Arizona Health Sciences Center were routinely monitored and determined as free from common murine pathogens (MHV, MPV, MVM, TMEV, Mycoplasma pulmonis, Sendai, EDIM, MNV, ecto- and endoparasites). All described animal use protocols were approved by the University of Arizona or the University of North Carolina at Chapel Hill Animal Care and Use Committee.

Cell cultures and sample collection - Rat osteogenic sarcoma cells (UMR-106; ATCC #CRL-1661) were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM high glucose media containing 10% fetal bovine serum, 1mmol/L sodium pyruvate, and 1X Antibiotic-Antimyotic at 37°C with 5% CO2. For analysis of endogenous rat Phex gene expression (rPhex), UMR-106 cells were seeded on 6-well plates at 0.5x10^5 cells per well they were treated with hTNF (10 ng/mL), cycloheximide (10μM), combination of both or vehicle (depending on experiment). In some experiments, cells were pretreated for 30 minutes...
with proteasome inhibitor clasto-lactacystin-β-lactone (10 μM, clβL). Apoptosis and cytotoxicity were evaluated with Caspase-Glo 3/7 Assay System (Promega), and ToxiLight BioAssay kit (adenylate kinase release; Lonza Rockland, Inc., Rockland, ME), respectively, according to the manufacturer’s protocols.

Real-time RT-PCR analysis –200 ng of total RNA isolated with TRIzol were reverse-transcribed using the qScript cDNA Synthesis Kit, and 2 μL of each reverse-transcribed reaction (10% of total volume of real-time reaction mixture) were used for real-time PCR analysis using TaqMan technology and commercially available primers (Rn01455648_m1 and Mm00446973_m1 for Tbp, Rn00448130_m1 and Mm 00448123_m1 for Phex; Rn01415175_m1for Parp-1, Rn00580158_m1 for Parg) using PerfeCTa qPCR SuperMix and the iCycler optical PCR cycler (BioRad, Hercules, CA). Resulting data were analyzed by the comparative cycle threshold (Ct) method as means of relative quantitation of gene expression, normalized to an endogenous reference (TATA-box binding protein [TBP]) and relative to a calibrator (normalized Ct value obtained from control mice), and expressed as 2^{-ΔΔCT} (Applied Biosystems User Bulletin No. 2: Rev B “Relative Quantitation of Gene Expression”).

Reporter Gene Constructs and transient Transfections - Progressive 5'-promoter deletions were generated between -522 and -74nt of the mPhex promoter by PCR and cloned into pβGal-Basic reporter vector (Clontech, Palo Alto, CA) as previously described (18). All promoter/reporter gene constructs were sequenced to confirm fidelity. UMR-106 cells were seeded in 6- or 24-well plates (0.5x10^6 or 0.5x10^5 cells/well, respectively), and was transfected with mPhex reporter vector DNA (0.5-1 μg/mL medium) by using TransIT-LT1 transfection reagent. In some experiments mPhex reporter vector DNA was co-transfected with the following expression vectors: pCMV6-null, pFlag-CMV2-RelA, pFlag-CMV2-p50, pCMV6-PARP-1 or pCMV6-PARG. 20-24 hours after transfection, cells were treated as described in the Results section. Promoter activity was expressed as β-galactosidase activity (β-Gal, relative light units) per microgram of protein. Cells from 6 well plates were used for total RNA isolation.

Electrophoretic Mobility Shift Assay (EMSA) - Nuclear protein (NP) for EMSA was prepared from UMR-106 cells as previously described (23). Double-stranded, synthetic oligonucleotides were end-labeled with [γ-32P]dATP. For each reaction, 30,000 cpm of the probe were incubated at room temperature for 20 minutes with 1-2.5μg of NP, 4μl of 5x binding buffer (Promega), 1μg of poly(d(I-C)) (Sigma) and H2O to a final volume of 20μl. For competition studies, 100x excess of unlabeled probe was added to the reaction. Antibodies for supershift assays ( p50, p52, RelA, cRel, RelB and PARP-1) were purchased from Santa Cruz Biotechnology (sc-114, sc-84, sc-372, sc-272, sc-226, sc-74469, respectively). Binding reactions were loaded on a 6% DNA retardation gel (Invitrogen) and separated at 250 volts in 0.5x TBE. Gels were dried and then exposed to an X-ray film. Oligonucleotides used for EMSA are depicted in Results section.

Southwestern blot analysis - NP was prepared as described above for EMSA, separated on 8% Tris-glycine SDS-polyacrylamide gels (Invitrogen), and transferred to nitrocellulose membranes. Immobilized proteins were then re-natured and blocked overnight at room temperature in 1x EMSA buffer containing 5% nonfat dried milk. Blots were then incubated with 1x 10^5 cpm of 32P end-labeled DNA probe overnight at room temperature. Following the hybridization, blots were washed three times for 15min in 1xEMSA buffer and then exposed to X-ray film (18).

DNA affinity precipitation assays (DAPA) – A double-stranded (ds) probe corresponding to -133 to +1bp of the PHEX promoter region was generated by annealing a 5′ biotin-labeled -133/+1 GS oligonucleotide (see results section) with an unlabeled complementary oligonucleotide. 37pMol biotinylated probe were mixed with: 75-100 μg of UMR-106 NP, 50 μL 5xEMSA buffer (Promega), 1mM DTT, phosphatase inhibitor (Sigma), protease inhibitor (Pierce), 1μg of poly(d(I-C)) (Sigma) and incubated 2h at 4°C with end to end rotation (final volume 250μL). For competition studies, 100x excess of unlabeled probe
(competitor) was added to the reaction 30 minutes prior to addition of the biotinylated probe. 100μL of Streptavidin Magna-Sphere® (Promega, Madison, WI) paramagnetic particles (SA-PMPs) suspended in complete 1xEMSA buffer were added 2 hours later followed by a 30 minute incubation at 4°C with rotation. SA-PMPs were separated from the supernatants in a magnetic field and washed 3 times with 1xEMSA buffer. SA-PMPs were resuspended in 70μl of Laemmli sample buffer with 5% α-mercaptoethanol, boiled for 5min and the supernatant was separated on 8% Tris-glycine SDS-polyacrylamide gels and immunoblotted, silver stained, or used with the Experion Automated Electrophoresis System. In some studies, samples from DAPA were used for proteomic identification of the polyA element binding protein, as described below and in the Supplement.

Proteomic identification of the polyA-element binding protein – DAPA was performed as described above. Biotin label was incorporated at 5'-end of either the lead-strand (containing polyA sequence) or 5'-end of the reverse-complement strand (containing poly-T sequence) to avoid bias. Affinity-precipitated nuclear protein from UMR-106 cells were separated on SDS-PAGE gel, silver-stained and band(s) which could be competed with 100x excess of unlabeled oligo were excised, digested with trypsin, and analyzed by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS). To independently verify the findings, affinity-precipitated nuclear protein with or without excess of unlabeled probe was used without gel separation with tandem mass spectrometry coupled to tandem liquid chromatography (LC-LC-MS/MS). The experimental details are provided in the Supplement. DNA interaction of the protein consistently indentified with all approaches (poly (ADP-ribose) polymerase 1; PARP-1) was further confirmed by EMSA (supershift) and chromatin immunoprecipitation assay (ChIP) as described below.

Chromatin Immunoprecipitation Assay (ChIP) – rPhex CHIP was performed with UMR-106 cells utilizing enzymatic ChIP-IT Express Express Chromatin Immunoprecipitation Kit according to the manufacturer’s protocol (Active Motif; Carlsbad, CA) with the following antibodies: RelA, PARP-1, RNA polymerase II (RNAP II) and the respective IgG (Santa Cruz and Active Motif). PCR reaction were performed primers specific for the proximal rat Phex gene promoter or 500, 1300, 3000, 4100 bp downstream from the transcription start site (RNAP II walking), or with control primers for rat β-actin. Sequences of primers and the conditions of PCR reactions are depicted in the results section.

Immunoprecipitation – TNF-treated or control UMR-106 cells were lysed with PBS with 1% NP-40 (IP buffer) and 500μg of protein extract was rotated with 2μg of RelA (Santa Cruz; sc-372x) or control IgG (Santa Cruz; sc-2027) for 2h at 4°C in a total volume of 0.5ml. 75μl of Protein A (Sigma) was then added to the samples and rotated overnight at 4°C. Samples were centrifuged (3,000xg for 2 min.); beads were washed 3 times with 750μl of the IP buffer, and resuspended and boiled in 75μl 5x Laemmlli buffer with β-mercaptoethanol. The eluted protein was used for Western blotting with antibodies against RelA, PolyADP-ribose chain (Calbiochem/EMD Chemicals, Gibbstown, NJ), or PARP-1 (Santa Cruz).

Statistical Analysis—Statistical significance was determined by the Student's t-test or ANOVA followed by followed by Fisher's protected least significant difference (PLSD) test, using the Statview software package version 4.53 (SAS Institute; Cary, NC). Data are expressed as means ± S.E

RESULTS

Chronic T-cell mediated colitis suppresses Phex expression in mouse osteoblasts in vivo - Adoptive transfer of CD4+CD45RB<sup>high</sup> T-cells (naïve T-cells) from healthy wild-type mice into syngeneic Rag2<sup>−/−</sup> recipients that lack T- and B-cells induces pancolitis and small bowel inflammation within 5–8 wk with symptoms resembling human Crohn’s disease. The CD45RB<sup>high</sup> T-cell transfer led to 80% reduction in the bone Phex mRNA compared to PBS-injected mice (Fig. 1A), coinciding with moderate-to-severe chronic colitis, characterized by
increased mucosal thickness and transmural infiltration of lymphocytes, monocytes, macrophages, and granulocytes, affecting both proximal and distal colon as we described earlier (22). Production of selected cytokines (IFN-γ, TNF, IL-1β, and IL-17) was significantly elevated in the supernatants obtained from unstimulated colonic explants cultures as well as from CD3/CD28-stimulated MLN lymphocyte culture (ref. (22) and data not shown). A similar decrease in Phex expression was observed in germ-free IL-10−/−mice 14 days following colonization with specific pathogen-free microflora (Fig. 1B). In this model, milder colitis (as determined by histology and cytokine production; ref. (21) and data not shown) was associated with comparatively smaller (50%) reduction in Phex mRNA expression compared to the more severe reaction in T-cell transfer studies.

**Inhibition of Phex gene expression by TNF may not require de novo synthesis of a transrepressor and is not related to pro-apoptotic or cytotoxic effects of the cytokine** – Our previous studies demonstrated that neutralizing anti-TNF antibodies reversed the effects of colitis on Phex expression and that TNF inhibits Phex gene transcription and osteoblast mineralization in vitro (19). To determine whether the TNF-induced repression of the Phex gene requires de novo synthesis of a transrepressor, we tested the effects of TNF on Phex mRNA expression in cycloheximide (CHX)-treated UMR-106 cells. TNF time-dependently inhibited Phex expression (Suppl. Fig. 1A). CHX alone also significantly diminished Phex transcript, suggesting the need for sustained translation of transactivator(s) and/or related signaling proteins (Suppl. Fig. 1A). However, in the presence of CHX, TNF failed to further downregulate Phex expression or to accelerate the decline of the steady-state Phex transcript levels (Suppl. Fig. 1A). We observed a transient increase in apoptosis in UMR-106 cells treated with TNF reaching statistical significance after 8 hours of treatment (Suppl. Fig. 1B). At later time points (14 and 24 hours), coinciding with the greatest inhibition of Phex mRNA expression, there was no significant change in caspase 3/7 activation (Suppl. Fig. 1B). We documented a small (~20%), albeit significant increase in TNF-induced cytotoxicity as verified by adenylate kinase release into the medium (Suppl. Fig. 1C). To test if under these experimental conditions UMR-106 were still capable of positively respond to TNF, we analyzed the expression of IkBα (NFKBAI; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), IL-6 and ICAM-1 mRNA 8 and 24 hours into the treatment. All three genes (exemplified by IkBα shown in Suppl. Fig. 1D) were significantly induced by TNF, CHX, and to a higher degree with the TNF/CHX combination. These data confirms the viability of the cells, and a strong positive response in the absence of protein-synthesis dependent negative feedback (TNF+CHX). Collectively, these findings suggest that under the selected experimental settings, TNF inhibits Phex expression independent of de novo protein synthesis. It is also possible, however, that in CHX-treated cells, TNF is unable to further inhibit PHEX transcription rate. In either case, the observed effects are not likely to be secondary to the minor changes in cell viability.

**Proximal Phex promoter region is involved in TNF–mediated inhibition of Phex transcription** – The proximal regulatory regions of the mouse and rat Phex genes are highly homologous (Fig. 2A). rPhex polyA region is shorter than in the mPhex gene, although prediction analysis of putative cis-elements by Match® software (24), including relatively weak NF-κB and AP-1 binding sites, are very similar. To characterize the mechanism of the transcriptional response of the murine Phex gene (mPhex) to TNF, we tested a series of 5’ progressive deletions of its promoter from -542 to -74 for TNF responsiveness in transiently transfected UMR-106 cells (Fig. 2B). TNF treatment reduced the activity of constructs spanning -522/+104, and -133/+104nt by ~50%. A construct truncated to remove the polyA region (-111/+104nt) was inhibited to a lesser degree (35%), whereas the shortest construct (mPhex -74/+104) containing a putative AP-1 and two NFκB binding sites was not significantly inhibited by TNF. These observations indicated that cis-element(s) required for TNF-mediated repression of Phex promoter activity are located within -111/-74 nt, while the immediately upstream polyA region is necessary for the maximal inhibition.
TNF inhibits Phex transcript elongation, but not RNA polymerase II (RNAP II) recruitment – To address the possible effect of TNF on the RNAP II recruitment to the Phex gene transcriptional start site (TSS), we performed RNAP II walking using ChIP with an RNAP II antibody followed by PCR with primers specific for the TSS vicinity, and primers designed for downstream regions of the gene (+500, +1300, +3000, +4100 bp). While TNF had no influence on the interaction of RNAP II with the Phex TSS region, significantly less PCR product was associated with RNAP II in downstream regions of the gene (Fig. 2C). These results were confirmed in three independent experiments and suggest that TNF-induced changes within the proximal Phex promoter result in impaired promoter clearance by RNAP II rather than its altered recruitment to the TSS.

Identification of polyA-element binding protein – We have previously described the polyA element in the proximal Phex promoter and postulated a transcriptional modulator of an approximate molecular weight of 110kDa, tentatively termed PAP110 (PHEX-activating protein 110) as important elements in basal as well as 1,25-(OH)2 vitamin D-mediated transcriptional control of the Phex gene, (18). DNA affinity enrichment using biotinylated ds probe spanning the polyA region (5'-CTAAAAAAAAAAAAAAAAAGT-3') followed by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) identified poly (ADP-ribose) polymerase (PARP-1) as a likely candidate with a matching molecular weight (Suppl. Table 1 and Suppl. Fig. 2). Another protein fulfilling the search criteria was SP120. The latter rat protein has been deposited to GenBank by others (BAA031336.1), but not published as a peer-reviewed report. SP120 is a putative rat nuclear scaffold protein binding to the matrix attachment regions of DNA, with high homology to the human scaffold attachment factor A. The presence of PARP-1, but not SP120, in the precipitated DNA-protein complex was independently confirmed without gel separation with MudPIT (Multidimensional Protein Identification Technology) using tandem mass spectrometry coupled to tandem liquid chromatography (LC-LC-MS/MS) and double stranded probes spanning the polyA region of the Phex promoter, with a biotin label either on polyA or polyT strands (data not shown).

DAPA assays using the sequence depicted in Fig. 2A and followed by microfluidics-based automated protein electrophoresis (Experion Automated Electrophoresis System; Bio-Rad) identified a strong and constitutive (not altered by TNF treatment) association of a protein of approximate size of 110kDa (consistent with PARP-1), and a TNF-inducible protein of approx. 65 kDa. Both were competed by excess of unlabeled probe (Fig. 3A). Western blot analysis of the same samples confirmed PARP-1 and RelA (p65) as two proteins binding to the proximal Phex promoter (Fig. 3B). ChIP assay, EMSA with PARP-1-specific antibodies, as well as southwestern blotting further confirmed the ~110 kDa protein as PARP-1 (Fig. 3 C-E).

Recruitment of NF-κB, but not AP-1 complex to the proximal Phex promoter is induced in TNF-treated UMR-106 cells – Prediction analyses indicated the presence of putative AP-1 and NF-κB binding sites between the polyA element and the transcription start site (Fig. 2B). EMSA with consensus AP-1 oligonucleotide as a probe showed no significant induction of this transcription factor complex (Fig. 4A). AP-1 binding to this consensus probe could be competed with a probe spanning the putative AP-1 site in the Phex promoter (-52/-24nt; Competitor 1, Fig. 4A), but not but the probe with a mutated AP-1 cis-element (competitor 2; Fig. 4A). This indicated that AP-1 complex can bind to the proximal Phex promoter, despite the lack of detectable induction by TNF. Indeed, in EMSA analysis with the -52/-24nt fragment used as the labeled probe, strong, but not TNF-inducible binding was observed (Fig. 4B). On the other hand, TNF activated NF-κB, as demonstrated in EMSA with a NF-κB consensus probe (Fig. 4C). Moreover, this binding could be competed with a probe spanning -133/+1nt of the Phex gene, thus confirming the observations from DAPA assays (Fig. 3A-B) and suggesting TNF-inducible binding of the NF-κB complex to the proximal Phex promoter.

A subsequent WB study showed that in UMR-106 cells, TNF induces translocation of RelA (p65)
and p50 to the cell nucleus (Fig. 5A). While TNF induced p100 processing and increased total p52 levels, western blotting with nuclear proteins did not indicate increased nuclear translocation of this subunit (Fig. 5A). EMSA with supershifting or blocking antibodies verified inducible DNA binding of RelA (p65) and p50 in TNF-treated UMR-106 cells (Fig. 5B). DAPA with biotinylated NF-κB consensus probe also demonstrated inducible binding of RelA which could be competed with the excess of both the unlabeled consensus probe or with unlabeled -133/+1nt fragment of the Phex promoter (Fig. 6A). When the -133/+1nt fragment was used as a probe, competitor spanning -90/-70nt of the promoter was not effective, while both the -76/-57nt and the -35/-16nt probes containing putative NF-κB binding sites competed for RelA binding (Fig. 6B). This was further verified in EMSA with wild type or mutated -76/-57nt and -35/-16nt probes (Fig. 6C). Collectively, obtained data indicated the involvement of two relatively weak NF-κB binding sites located in the TSS proximity in TNF-mediated Phex inhibition.

Inhibition of NF-κB signaling reverses whereas overexpression of RelA mimics TNF-mediated inhibition of Phex expression – To better define the role of NF-κB signaling in the regulation of Phex gene transcription, we used decoy peptides (IMG-2000, IMG-2001, IMG-2002), pyrrolidine dithiocarbamate (PDTC), BAY 11-7082, proteasome inhibitor clasto-lactacystin-β-lactone, as well as RelA siRNA. While the decoy peptides, PDTC, BAY and p65 siRNA were able to decrease level of RelA in the nuclei of UMR-106 cells, their combination with TNF treatment was associated with severe cytotoxicity, therefore yielding data difficult to interpret. Pretreatment with proteasome inhibitor clasto-lactacystin-β-lactone (cLβL), at a concentration resulting in no discernable cytotoxicity (10 μM), partially (but significantly) reversed the inhibitory effects of TNF on rPhex mRNA level (Fig. 7A). It also fully reversed the negative effects of TNF on the mPhex promoter activity (Fig. 7B). Overexpression of RelA in UMR-106 cells transiently transfected with pFlag-CMV2-RelA, mimicked the effects of TNF on both the expression of endogenous rPhex mRNA (Fig. 7C) and gene promoter activity (Fig. 7D).

Overexpression of p50 alone or in combination of RelA did not result in a further decrease of Phex expression or promoter activity (Fig. 7 C, D).

Inhibition of polyADP-ribosylation (parylation) increases mPhex promoter activity and reverses the effects of TNF in UMR-106 osteoblasts – The proximity of PARP-1 binding sites to the confirmed NF-κB cis-elements in the mPhex promoter, and the reported potential interaction of these two transcriptional modulators, prompted us to investigate the effects of PARP-1 inhibition on mPhex promoter activity under basal conditions and in TNF-treated UMR-106 cells. UMR106 cells were transiently transfected with a reporter gene construct containing mPhex-133/104 promoter region and treated for 24h with or without TNF in the presence of polyADP-ribosylation inhibitor 3-amino benzamide (3-AB), or in cells overexpressing poly (ADP-ribose) glycohydrolase (PARG). The latter enzyme catalyzes the removal of paryl moiety from the target proteins. 3-AB and PARG overexpression caused a significant increase in Phex promoter activity over baseline in cells not exposed to TNF, and resulted in a full reversal of the inhibitory effects of TNF on Phex gene transcription (Fig. 8A).

PARP-1 catalyzes parylation of RelA – We next aimed to determine whether RelA is a target of PARP-1 activity. In Western blot analysis with whole-cell extract of control or TNF-treated UMR-106 cells, an anti-polyADP-ribose chain (PARyl) antibody detected TNF-inducible parylation of a protein with a molecular weight corresponding with that of RelA (Fig. 8B). Similar inducible binding of a parylated protein with a molecular weight consistent with RelA was observed in the DNA affinity precipitation assay (DAPA) with a biotinylated mPhex -133/1 probe, followed by a Western blot with an anti-PARyl antibody (Fig. 8C). When RelA was immunoprecipitated from whole-cell extracts obtained from control or TNF-treated UMR-106 cells, we observed TNF-inducible parylation of this NF-κB subunit (Fig. 8D).

TNF-mediated downregulation of rPhex is completely abrogated in PARP-1 KO mice – To confirm the role of PARP-1 in TNF-driven and NF-
κB-dependent repression of Phex gene transcription in vivo, we treated wild-type or PARP-1 null knockout mice with recombinant mouse TNF. TNF dose previously reported as sufficient to inhibit Phex expression in mice (150 mg/kg), reduced mPhex expression in the bone of wild-type mice by 50%, consistent with previously reported results (19). However, in PARP-1 KO mice, TNF treatment was without effect (Fig. 8E). This in vivo observation provides a strong evidence for the involvement of PARP-1 as a modulator NF-κB activity in osteoblasts exposed to elevated TNF during inflammatory conditions.

DISCUSSION

The skeletal and immune systems share numerous key players and regulatory mechanisms, an overlap that gave rise to the emerging field of osteoimmunology. A detailed understanding of the pathogenesis of bone destruction as a result of the interaction of immune cells and inflammatory mediators with bone cells is critical in designing novel strategies for the treatment of several disorders. These include rheumatoid arthritis, periodontal disease, Paget’s disease, osteoarthritis, multiple myeloma, metastatic bone tumors, and chronic inflammation-associated loss of bone mineral density. The latter is increasingly more recognized by the gastroenterology community due to a significant association of chronic IBD with osteopenia and osteoporosis (25). Bone loss in chronic inflammation is believed to be at least in part mediated by proinflammatory cytokines such as TNF, IL-1β, IL-6, or IFN-γ. Although TNF has been believed to affect bone primarily by causing osteoclast-driven bone erosion, newer data points to a direct effect of inflammatory mediators on the osteoblast functions as well. Defective bone formation has been reported not only in animal models of IBD (26) but also in pediatric Crohn’s disease patients (27). Serum from children with CD decreases osteoblast function including bone nodule formation in vitro (28).

The Phex gene encodes a M13 family Zn-metalloendopeptidase expressed primarily in osteoblasts and odontoblasts. Phenotypically, inborn inactivating mutations in the PHEX gene result in vitamin D resistant, X-linked hypophosphatemic rickets (XLH), while in vitro, the expression of Phex is a prerequisite for bone matrix deposition (6). We described earlier that chemically induced colitis results in a TNF-mediated decrease in bone Phex expression, and that in vitro exposure of osteoblasts to TNF results in a corresponding decrease of Phex mRNA and protein and a mineralizing defect(19). In this report we confirm these observations in more relevant IBD models offered by gnotobiotic IL-10−/− mice and by the naïve T-cell transfer, and we identify the molecular mechanism underlying this phenomenon, which likely contributes to the dysfunction of osteoblast activity and bone formation in patients with chronic IBD. According to this model, TNF treatment results in the recruitment of the p65/p50 NF-κB complex to two relatively weak cis-elements located at nt -76/-57 and -35/-16 between the Phex transcription start site and a poly-A element located upstream of the NF-κB sites. Through proteomic and molecular approaches we identified the poly-A binding protein as PARP-1, an enzyme participating in TNF-inducible parylation of the p65 (RelA) subunit, likely increasing the affinity of the NF-κB complex to the proximal Phex promoter, resulting in increased retention and inhibited clearance of the RNA polymerase II complex.

The obtained results highlight the importance of Phex in the osteoblast function during chronic inflammatory conditions, but also the significance of NF-κB activity and the modulating role of PARP-1 in the osteoblast function. It also identifies PARP-1 as a potential target in mitigating IBD-associated bone loss. Recent studies using transgenic mice bearing IKK-γ dominant negative (IKK-DN) targeted to mature osteoblasts by the bone γ-carboxylutamate protein-2 (Bglap2) promoter have demonstrated the anti-mineralizing effects of NF-κB (29). These mice manifested enhanced bone formation with elevated expression of bone matrix genes such as α-1 type 1 collagen, osteocalcin, secreted phosphoprotein-1, and bone sialoprotein. Studies with these transgenic mice also demonstrated that NF-κB is constitutively active, albeit to a lesser extent, under basal conditions (29). This is consistent with our results that examined mouse promoter activity in UMR-106 cells in the presence of the proteasome inhibitor cLβL. These experiments showed that
cLB-L alone significantly increases Phex mRNA expression. More importantly, the results from our studies and the IKK-DN transgenic mouse study strongly suggest that targeting NF-κB in the treatment of osteoporosis and inflammatory bone loss will not only result in suppression of bone resorption but also in promotion of bone formation, thus facilitating the rebuilding of bone mass.

It is important to note, however, that the role of NF-κB in IBD is very complex and its pleiotropic roles in cellular proliferation, differentiation and survival, inflammation, and carcinogenesis do not make it an easy target for systemic approach (30). Identifying PARP-1 as a crucial modulator of NF-κB activity in the osteoblasts opens an alternative possibility of targeting PARP-1. Several lines of evidence suggests that PARP-1 could be a suitable pharmacological target, particularly in IBD. (A) PARP-1 has been shown to participate in trigerring the NF-κB pathway, affecting both the classical pathway and the nuclear-to-cytoplasmic DNA damage-induced NF-κB pathway. In the classical pathway, PARP-1 was shown to participate in LPS-induced monocyte chemotactic protein-1 expression (31), whereas in vascular smooth muscle cells PARP-1 was critical for TNF-induced expression of ICAM-1 (but not VCAM-1) and shown to physically interact with p65 (RelA) (32). In the nuclear-to-cytoplasmic DNA damage-induced NF-κB pathway PARP-1 contributes to the physical assembly of the nuclear signalosome including IKK7, PIASy (nuclear matrix-associated SUMO E3 ligase), and ATM (protein kinase ataxia telangietasia mutated) (33). (B) Modulating PARP-1 expression or enzymatic activity has been shown in numerous studies to ameliorate the symptoms of experimental colitis (34-39).

Although PARP-1 is an abundant and ubiquitous nuclear enzyme originally identified as a key factor in the DNA repair pathway, it has now been shown to positively and negatively affect gene transcription and chromatin structure under both basal and signal-activated conditions (40). Studies examining gene expression profiles in PARP-1 deficient embryonic stem cells and liver cells from PARP-1 KO mice showed that 3.5% of the transcriptome were regulated by PARP-1, 70% of which were positively regulated (41). More notably, PARP-1 has been described as one of the major molecules involved in the propagation of inflammatory stimuli and has been proposed as a target for anti-inflammatory treatment (42). PARP-1 has been shown to affect gene transcription in several ways: as an enhancer-binding factor similar to classical sequence specific DNA-binding activators or repressors, as a transcriptional co-regulator, or as a modifying enzyme which catalyzes the NAD⁺-dependent addition of ADP-ribose polymers (PARylation) to several nuclear proteins. We demonstrate here that in TNF-treated osteoblasts, RelA is translocated to the nucleus, where it is PARylated by PARP-1. This chemical modification is critical for TNF-induced inhibition of Phex expression since this response does not occur in PARP-1-deficient mice and is reversed by the PARP-1 inhibitor 3-AB or by an overexpression of PARG. While it is technically challenging to determine whether PARP-1 and RelA physically interact with each other in the context of the Phex gene promoter, the proximity of their binding sites and inducible recruitment of RelA suggest such possibility.

Interestingly, we initially described the Phex polyA promoter region as a positive cis-element whose affinity for a 110kD, then unidentified binding protein was decreased in response to dihydroxy-vitamin D treatment (29). While the exact mechanism of the effects of vitamin D on PARP-1 in osteoblasts requires further work, our findings suggest that PARP-1 may play a pleiotropic role in the regulation of Phex gene transcription, depending on the physiological or pathophysiological context.

In conclusion, our results describe a new mechanism of TNF-mediated gene regulation in osteoblasts involving NF-κB and PARP-1. Cooperatively, NF-κB signaling and PARP-1 enzymatic activity constitutively and inducibly suppress Phex gene expression.

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FIGURE LEGENDS

Figure 1. *Chronic colonic inflammation suppresses bone mPhex mRNA expression.* Real-time PCR analysis of mPhex mRNA expression in (A) femurs harvested from control Rag2−/− mice or Rag2−/− mice adoptively transferred with naïve CD4CD45RBHi T-cells, and (B) from calvarias of wild-type (WT) mice and germ-free IL-10−/− mice collected 14 days after colonization with SPF microflora. Values are means and ±SE of 4-6 mice per group; * p<0.05, *** p<0.001 (Student t-test).

Figure 2. *Functional analysis of the murine Phex gene promoter and its response to TNF.* (A) Alignment and schematic representation of -133/+1 Phex promoter region of mouse (mPhex) and rat (rPhex) genes with depicted poly-adenine region (PolyA) and predicted NF-κB (rectangles) and AP-1 cis-elements (line). (B) Effects of TNF (10 ng/mL, 24 hours) on mPhex promoter activity in UMR-106 cells transiently transfected with reported constructs containing progressive promoter deletions. Promoter activity is expressed as fold increase over β-galactosidase activity in cells transfected with promoter-less vector βGal basic. PolyA element, and NF-κB and AP-1 binding sites are depicted by black or gray rectangles. Values are means and ±SE from 3 independent experiments; * p<0.05, ** p<0.01, *** p<0.001 (Student t-test). (C) Chromatin immunoprecipitation analysis (ChiP) in control (CTRL) and TNF-treated (10 ng/mL, 24 hours) UMR-106 cells using RNA polymerase II antibody (RNAP II walking) and primers specific for the mPhex TSS vicinity, and primers designed for downstream regions of the gene (+500, +1300, +3000, +4100 bp; see table below). Primers specific for the rat β-actin were used as a control.

Figure 3. *PARP-1 and RelA interact with -133/+1 nt of the mPhex promoter.* (A) DAPA followed by automated protein electrophoresis identified a constitutively associating 110 kDa protein and inducible binding of a 65kDa protein in TNF-treated UMR-106 cells. These DNA-protein complexes were competed with 100-fold excess of the non-biotinylated probe. (B) DAPA was followed by SDS-PAGE separation and Western blot analysis of the complexes formed with -133/+1 nt of the mPhex gene and confirmed the bands to represent PARP-1 and RelA. (C) ChiP analysis of PARP-1 association with the proximal mPhex promoter: Chromatin was immunoprecipitated with anti-PARP-1 or RNAP II and IgG (as a positive and negative control, respectively) and PCR amplified with primers specific for mPhex TSS (see Fig. 3 for sequences). (D) Neutralization of PARP-1 binding to the polyA element of the mPhex gene in EMSA analysis in untreated UMR-106 cells. (E) Southwestern blot analysis of nuclear proteins isolated from control (Ctrl) or TNF-treated UMR-106 cells with a radiolabeled probe spanning -133/+1 nt of the mPhex gene.

Figure 4. *NF-κB and AP-1 complexes interact with the proximal mPhex promoter.* (A) EMSA analysis with nuclear protein isolated from control and TNF-treated UMR-106 cells with a radiolabeled consensus AP-1 probe. No detectable increase in AP-1 DNA binding was reported in response to TNF, although binding could be competed with an excess of the unlabeled mPhex -52/-24nt probe spanning the AP-1 consensus element but not its mutated form. (B) Consistently, only constitutive AP-1 binding was observed with -52/-24nt sequence used as a probe. (C) TNF treatment increases NF-κB binding to a consensus NF-κB element. This interaction can be effectively competed with an excess of the unlabeled -133/+1 mPhex probe. (D) Probe sequences used in EMSA analysis. An excess of the free probe is not demonstrated due to necessary complex separation and for figure clarity, but was confirmed in an independent assay (not shown).

Figure 5. *Nuclear translocation of the NF-κB complex components and their DNA association in TNF-treated UMR-106 osteoblasts.* (A) Representative Western blot analysis of NF-κB proteins in whole cell
lysate or in the nuclear fraction of control and TNF-treated UMR-106 cells (10 ng/mL, 24 hours). Only RelA (p65) and p50 demonstrated detectable and inducible nuclear translocation in response to TNF. (B) Supershift/blocking EMSA analysis of NF-κB/DNA complexes with nuclear protein from TNF-treated UMR-106 cells confirming the predominant involvement of the p50/p65 heterodimer.

Figure 6. RelA (p65) protein binds to the proximal region of the mPhex gene promoter. DAPA was performed with nuclear proteins isolated from control or TNF-treated UMR-106 cells with: (A) biotinylated NF-κB consensus DNA probe, or (B) biotinylated dsDNA probe corresponding to -133/+1 region of mPhex promoter. Western blot was performed with antibodies specific to RelA. TNF-inducible binding could be competed with an excess unlabeled NF-κB consensus probe, a probe spanning -133/+1 nt and the two putative NF-κB elements located at -76/-57 nt and -35/-16 nt of the mPhex promoter, but not another putative NF-κB site located at -90/-70 nt. (C) EMSA analysis with radiolabeled consensus NF-κB probe or probes spanning -76/-57nt (probe 1) or -35/-16nt of the mPhex promoter. Competing probes were designed to have intact or mutated core elements of the putative NF-κB sites (underlined nucleotides). All binding reactions were performed with nuclear extracts from UMR-106 cells treated with TNF. An excess of the free probe is not demonstrated due to necessary complex separation and for figure clarity, but was confirmed in an independent assay (not shown).

Figure 7. Inhibition of NF-κB signaling reverses TNF-mediated inhibition of the Phex gene expression and promoter activity while overexpression of RelA (p65) mimics the TNF effect. Proteasome inhibitor clasto-lactacycin-β-lactone (cLβL) reduces the negative effects of TNF on the endogenous Phex mRNA expression and mPhex promoter (-103/+104nt) activity in transiently transfected UMR-106 osteoblasts (A & B). Overexpression of p65 alone is sufficient to inhibit the endogenous Phex mRNA expression and mPhex promoter activity in transiently transfected UMR-106 cells. All values expressed relative to untreated/mock-transfected cells. Values are means ± SE; ** p<0.01, *** p<0.001 (Student t-test; n=4).

Figure 8. (A) Inhibition of polyADP-ribosylation reverses TNF-mediated suppression of mPhex gene promoter activity. UMR-106 cells were transiently co-transfected with a reporter construct carrying -133/+104nt fragment of mPhex gene with an “empty” or PARG expression vector. Cells were then pretreated with or without PARG expression vector. Cells were then pretreated with or without PARP-1 inhibitor 3-amino benzamide (3-AB; 1 mM) prior to 24 hour treatment with TNF (10 ng/mL). Both 3-AB and overexpression of PARG reversed the effects of TNF. Data are expressed relative to the control β-galactosidase activity in vehicle-treated cells transfected with the -133/+104 mPhex reporter construct. * p<0.05, ** p<0.01, *** p<0.001 (ANOVA and Fisher PLSD test; n=4). (B-D) TNF treatment results in inducible poly (ADP-ribosyl) ation (PARylation) of p65 in UMR-106 osteoblasts. (B) Western blot (WB) analysis of whole protein extract with antibodies specific to polyADP-ribose and RelA showed indicible PARylation of a 65kDa protein with a migration pattern corresponding to that of p65. (C) DAPA followed by Western blot detection of polyADP-ribose and p65 resulted in similar findings. (D) RelA was immunoprecipitated from control and TNF-treated UMR-106 cell lysates and probed for polyADP-ribose to confirm poly(ADP-ribosyl)ation of this NF-κB subunit. (E) TNF–mediated downregulation of mPhex gene expression in vivo is completely abrogated in PARP-1-deficient mice. Real-time PCR analysis of mPhex mRNA expression in femurs harvested from wild-type (WT) or PARP-1 KO mice (PARP-1 KO) 24h after a single i.p. injection of recombinant murine TNF (150 mg/kg). Values are means ±SE ( *** p<0.001, Student t-test; n=4).
Figure 1.
Figure 2.
Figure 3.
A. 
Probe: **AP-1 consensus**

| Nuclear proteins: | - | + | + | + | + | + | + | + |
|-------------------|---|---|---|---|---|---|---|---|
| Competitor:       | - | - | - | - | - | - | 1 | 2 |

B. 
Probe: **mPhex -52/-24**

| Nuclear proteins: | - | + | + | + | + | + | + | + |
|-------------------|---|---|---|---|---|---|---|---|
| Competitor:       | - | - | - | - | - | - | 1 | 2 |

C. 
Probe: **NF-κB consensus**

| Nuclear proteins: | - | + | + | + | + | + | + | + |
|-------------------|---|---|---|---|---|---|---|---|
| Competitor:       | - | - | - | - | - | - | 3 | 3 |

D. 

|            | 5’-CGCTTGATGAGTCAGCCGAA-3’ |
|------------|----------------------------|
| AP-1 consensus | 5’- AGTTGAGGGGACTTTCCCAGGC-3’ |
| NF-κB consensus | 5’- TGCAATGGACTATGACTGATTTTTGGCAC-3’ |
| 1- mPhex -52/-24nt | 5’-TGCAATGGACTGACTGGATTTTTGGC-3’ |
| 2- mut mPhex -52/-24nt | as depicted in Fig. 3A |
| 3- mPhex -133/+1nt |

**Figure 4.**
Figure 5.
Figure 6.
Figure 7.
Figure 8.
COOPERATIVE ROLE OF NF-κB AND POLY(ADP-RIBOSE) POLYMERASE 1 (PARP-1) IN THE TNF-INDUCED INHIBITION OF PHEX EXPRESSION IN OSTEOBLASTS
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