Loss of Nmp4 optimizes osteogenic metabolism and secretion to enhance bone quality

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ABSTRACT:

A goal of osteoporosis therapy is to restore lost bone with structurally sound tissue. Mice lacking the transcription factor Nuclear Matrix Protein 4 (Nmp4, Zfp384, Ciz, ZNF384) respond to several classes of osteoporosis drugs with enhanced bone formation compared to wild type (WT) animals. Nmp4−/− mesenchymal stem/progenitor cells (MSPCs) exhibit an accelerated and enhanced mineralization during osteoblast differentiation. To address the mechanisms underlying this hyper-anabolic phenotype, we carried out RNA-sequencing and molecular and cellular analyses of WT and Nmp4−/− MSPCs during osteogenesis to define pathways and mechanisms associated with elevated matrix production. We determined that Nmp4 has a broad impact on the transcriptome during osteogenic differentiation, contributing to the expression of over 5,000 genes. Phenotypic anchoring of transcriptional data was performed for the hypothesis-testing arm through analysis of cell metabolism, protein synthesis and secretion, and bone material properties. Mechanistic studies confirmed that Nmp4−/− MSPCs exhibited an enhanced capacity for glycolytic conversion- a key step in bone anabolism. Nmp4−/− cells showed elevated collagen translation and secretion. Expression of matrix genes that contribute to bone material-level mechanical properties were elevated in Nmp4−/− cells, an observation that was supported by biomechanical testing of bone samples from Nmp4−/− and WT mice. We conclude that loss of Nmp4 increases the magnitude of glycolysis upon the metabolic switch, which fuels the conversion of the osteoblast into a super-secretor of matrix resulting in more bone with improvements in intrinsic quality.
INTRODUCTION:

Osteoporosis is a disease of attenuated bone mass and strength that significantly increases the risk of fragility fractures (92). Teriparatide (PTH) and abaloparatide (PTHrP) are currently the only FDA-approved osteoanabolic therapies for this disease (52, 61). These drugs add new bone to the osteoporotic skeleton whereas the primary effect of anti-catabolic drugs is a reduction in the pathologically elevated bone resorption (30). The benefits of PTH treatment include an increase in bone mass through a combination of new bone modeling and the sustained bone remodeling with a positive balance as well as improved bone material properties (13, 18, 29, 32, 59). However, the potency of PTH precipitously declines and there is an FDA-mandated two-year limit on treatment (18), emphasizing the need for new strategies that improve the efficacy of the drug, such as by combining hormone treatment with an anti-catabolic drug or targeting PTH directly to bone (26, 83). Neutralizing intrinsic pathways that temper PTH-induced osteoblast secretion of bone matrix might improve drug efficacy. Indeed, a similar strategy of “inhibiting the inhibitor” (46) has led to the development of the osteoanabolic romosozumab, a monoclonal antibody that neutralizes the action of the osteoinhibitory protein sclerostin, currently under consideration by the FDA for clinical approval (3, 94).

We reported that the transcription factor Nuclear Matrix Protein 4 (Nmp4, Zfp384, Ciz, ZNF384) suppresses the action of osteoanabolics (15, 16, 41, 70, 90, 95) and thus elucidation of the upstream and downstream effectors in the Nmp4 pathway may provide a map of the innate barriers to PTH-induced bone formation. Indeed, as a trans-acting protein Nmp4 is well positioned to control multiple aspects of bone formation. Genome-wide Chromatin Immunoprecipitation followed by high-throughput sequencing (ChIP-seq) analysis in MC3T3-E1 cells suggested that Nmp4 has wide ranging effects on the transcriptome, with over 15,000 Nmp4 binding sites in the osteoblast genome. Of importance, nearly 70% of these sites are within -5 and +2 kb from a transcription start site (TSS) or within introns, both DNA regions that often harbor regulatory regions (16).
Nmp4<sup>−/−</sup> mice exhibit more bone marrow osteoprogenitors than their WT littermates (16, 41, 95). Expanded cultures of Nmp4<sup>−/−</sup> mesenchymal stem/progenitor cells (MSPCs) induced with osteogenic medium exhibit elevated mRNA expression of the bone matrix proteins type I collagen (Col1a1), osteocalcin (Bglap2), and osteopontin (Spp1). Additionally, the anabolic process of ribosome biogenesis is elevated in these cells, as is the expression of Gadd34 (PPP1r15a), which helps maintain translation and ultimately contributes to the continued trafficking of secretory protein through the endoplasmic reticulum (ER) despite increased protein loads (16, 20, 114).

To address the cellular pathways by which Nmp4 suppresses osteoblast-mediated bone formation we performed high-throughput RNA sequencing (RNA-seq) of WT and Nmp4<sup>−/−</sup> expanded MSPCs during osteogenesis. Network analyses of the RNA-seq output were used for driving hypothesis testing, i.e. select pathways that were significantly altered in the transcriptome were evaluated experimentally. The results phenotypically anchored bioinformatic predictions to changes in metabolic and biochemical properties of the Nmp4<sup>−/−</sup> osteogenic cells. Based on the RNA-seq data we hypothesized that Nmp4<sup>−/−</sup> osteoblasts elaborate a matrix that improves bone material and structural characteristics. Therefore we examined these bone properties from experimental WT and Nmp4<sup>−/−</sup> mice that had undergone various osteoporosis therapies. These data reveal new aspects of how loss of Nmp4 alters bone matrix secretion as well as the impact of this single gene on bone quality.

**MATERIALS AND METHODS**

**Cell culture:** MSPCs were derived from individual mice as previously described (16, 109). Briefly, long bone marrow (BM) was harvested from euthanized mice 6–8 weeks of age, and a Ficoll gradient was used to isolate the mononuclear cells. These cells were seeded in Mesencult Media™ + Mesencult Stimulatory Supplement™ (StemCell Technologies, Vancouver Canada) and sustained for 3–4 weeks without passage while fed every 5–7 days by removing 50% of the
old media and adding 50% fresh media, so as not to disturb the cells. Upon reaching 80% confluence, the cells were passaged at 1:3 dilution for 2 additional passages before use in experiments or were frozen for stock vials. Cells were used for study between passages 5–10. To assess the mineralization phenotype of each MSPC preparation, cells were seeded in α-MEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL amphotericin, 2 mM L-glutamine (Gibco BRL, Life technologies; Grand Island, NY, USA) and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). At 48 hours post-seeding the medium was refreshed and further supplemented with ascorbic acid (5 µg/mL; Sigma-Aldrich), dexamethasone (10 nM; Sigma-Aldrich), and 10 mM glycerol 2-phosphate disodium salt hydrate (BGP) (Sigma-Aldrich). To visualize the mineralization in culture, cells were stained with alizarin red as previously described (16).

**RNA-seq analysis:** To compare transcriptome profiles of non-differentiating and osteogenic-differentiating WT and *Nmp4*-/- MSPCs, cells were seeded into 12-well plates at either 10,000 cells/well (25 cells/mm²) or 25,000 cells/well (62 cells/mm²). The cells seeded at the lower density were maintained in Mesencult Media™ + Mesencult Stimulatory Supplement™ (non-differentiating medium) for 3 days post-seeding and then harvested for total RNA. Cells plated at the higher density were maintained in α-MEM complete medium throughout the experiment. At 48 hours post-seeding the medium was refreshed with the ascorbic acid, dexamethasone, and BGP supplement. These cells were harvested at 7 days post-seeding as early osteogenic cells.

Total RNA was harvested using RNeasy (Qiagen, Valencia, CA) and measured for quality using the Agilent 2100 Bioanalyzer, and Qubit 2.0 Fluorometer. High RNA integrity is critical for evaluating the transcriptome. The RNA integrity number (RIN) is an algorithm for assigning integrity values to RNA measurements and assigns an electropherogram a value of 1 to 10, with 10 being the least degraded. All RIN numbers for our samples ranged between 8.2-
A conservative cut-off value in the context of RNA degradation lies between 6.4 and 7.9 (31), well below our values. Four technical replicates were harvested for each time point and genotype. Total RNA samples were submitted to the Beijing Genomics Institute (BGI) for transcriptome sequencing. In brief, magnetic beads with Oligo (dT) were used to isolate mRNA. The mRNA was fragmented and then constructed into HiSeq 2000 strand-specific libraries. The 2 × 100-nt paired-end reads were generated by Illumina HiSeq™ 2000. Clean reads filtered from raw sequence reads were returned from BGI. Raw reads were filtered into clean reads by employing the following rules: (i) remove reads in which the percentage of bases with quality <10 was >50%; (ii) remove reads in which unknown bases were more than 10%; (iii) remove reads with adapters; (iv) map the clean reads to Mus musculus reference mm10 using STAR (version 2.4.2a) (23); (v) gene-based expression levels were quantified with featureCounts (58); (vi) differential expression of genes across different treatments was determined with edgeR (88) [GEO accession number GSE112694]

RNA-seq determines the relative amount of each gene in each RNA sample but does not provide any measure of the total RNA output on a per-cell basis. This can be important when some genes are very highly expressed in one sample but not another (89), which is the case for our Nmp4−/− phenotype. We have previously shown that the Nmp4−/− MSPCs express upwards to 2-fold more RNA/cell than WT cells (114). Therefore we used GusB as a scaling factor for the present RNA-seq data since our previous work identified GusB as an appropriate normalizer for microarray data (16). Pathway enrichment analysis was performed using the Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Inc., Redwood City, CA, USA) to distinguish significant canonical pathways in which the Differentially Expressed Genes (DEGs) identified in the WT and Nmp4−/− samples were enriched. Fisher’s exact test was used to compute a p-value that denotes the probability of the DEGs in the pathway being found together due to random chance.
We also applied the Benjamini-Hochberg false discovery rate (FDR) (q < 0.05) correction to account for multiple comparisons in the IPA.

We define a candidate Nmp4 direct target gene as a gene whose expression is altered with the loss of Nmp4 and also supports Nmp4 occupancy. To identify candidate genes we performed Venn diagram analysis with the gene lists from the present RNA-seq dataset and lists derived from our previous study of the Nmp4 genome-wide occupancy by ChIP-Seq in MC3T3-E1 preosteoblasts (16). This cell line is an established in vitro model for osteoblastogenesis. Genes that were identified as supporting Nmp4 occupancy exhibited ChIP-seq peaks within -5 to +2 kb from a transcription start site (TSS) and/or within the range defined by the TSS and the transcription end site, and not within the promoter range of the same gene (Table S1 https://figshare.com/s/aef3382cdc7c02151e6f, GEO accession number GSE112693 for complete ChIP-Seq dataset) (16). Additionally, we further refined this definition by using only genes contained in both the ChIP-seq and RNA-seq lists.

Seahorse assay: Four independent MSPC cell preparations were used in the metabolic stress tests. The MSPCs 1957RWT and 1957NKO were derived from male littermates obtained from an Nmp4+/x Nmp4+/x cross. The 1584LWT and 1515RRKO MSPCs were derived from mice obtained from different litters and different parents. Cells were seeded into an XFe24 well plate and grown for ~24hrs in culture. MSPCs were then subjected to mitochondrial stress tests using oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), rotenone, and antimycin A per the manufacturer's instructions (Seahorse Biosciences, Lexington, MA). Glycolysis stress tests were performed using oligomycin and 2-deoxy-D-glucose (Seahorse Biosciences). After each analysis, total cell number was quantified and normalized to O2 consumption rate (OCR) or extracellular acidification rate (ECAR), respectively. Glycolytic and mitochondrial stress tests were repeated 4-5 times each (biological replicates). We pooled all
data (each well) obtained from the glycolytic or mitochondrial tests as technical replicates for statistical analysis.

Collagen secretion analysis: All six independent MSPC cell preparations were used in the collagen secretion assays including 1957R<sup>WT</sup>, 1584L<sup>WT</sup>, 2001RL<sup>WT</sup>, 1957N<sup>KO</sup>, 1515RR<sup>KO</sup>, and 1986R<sup>KO</sup>. Collagen levels were determined using the Sircol assay (Biocolor Ltd, Carrickfergus, Northern Ireland) (1, 62). Non-differentiating WT and Nmp4<sup>−/−</sup> MSPCs, cells were seeded into 12-well plates at 10,000-20,000 cells/well (25-50 cells/mm<sup>2</sup>). These cells were maintained in Mesencult Media™ + Mesencult Stimulatory Supplement™ (non-differentiating medium) for 4 days post-seeding. To harvest the acid soluble fraction, cultures were washed twice with ice-cold PBS and then scraped into PBS containing 0.5M acetic acid and digested overnight at 4-8°C. The samples were then snap frozen. Collagen was concentrated from these acid-soluble fractions and then analyzed according to the manufacturer’s instructions. The collagen amount was normalized to cell number or presented as collagen/well vs. cell number/well. All experiments were repeated at least twice. All the data shown in the assays are an average of at least 4-5 different wells per group.

Col1a1 polysome analysis: Preparations from four independent MSPC cell preparations, designated 1957R<sup>WT</sup>, 1957N<sup>KO</sup>, 1584L<sup>WT</sup>, and 1515RR<sup>KO</sup>, were used to measure collagen mRNA in polysomes. Equal amounts of WT and Nmp4<sup>−/−</sup> MSPCs were cultured into 10cm culture plates and maintained in Mesencult Media™ + Mesencult Stimulatory Supplement™ for 4 days. On Day 4, cycloheximide was added to each culture dish for 10min prior to harvesting. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) solution containing 50 μg/ml cycloheximide and then lysed with 500μl of cold lysis buffer containing 10mM Tris-HCl (pH 7.4), 300mM KCl, 10mM MgCl<sub>2</sub>, 1mM DTT and 50μg/ml cycloheximide, followed by centrifugation at 13000 rpm for 10min at 4°C. Cell lysates were then applied to the top of 10-50% sucrose
gradients and subjected to ultracentrifugation in a Beckman SW41Ti rotor at 40,000 rpm for 2 h
at 4°C. Using a piston gradient fractionator, polysome profiles of each sample was recorded at
254 nM by a UV monitor with Data Quest software as described previously (103). TRIzol LS
reagent (Life Technologies, Inc) was used to purify RNA present in each of the sucrose gradient
fractions. To insure that there was uniform RNA preparation between fractions, equal amount of
firefly luciferase mRNA was added to each fraction. RNA prepared from equal volumes of each
fraction was then used as a template for cDNA synthesis utilizing the TaqMan RT kit (Life
Technologies, Inc.). The qPCR analyses of firefly luciferase and Col1a1 transcripts were
measured as described previously (2). Equal amounts of firefly luciferase mRNA was measured
in each of the fractions. Primer sequences for both transcripts were Col1a1 F: 5'-
ACGTCTGGTGAAGTTGGTC-3', R: 5'-CAGGGAAGCCTCTTTCTCCT-3'; firefly luciferase F:
5'-CCAGGGATTTCAGTCGATGT-3', R: 5'-AATCTCACGCAGGCAGTTCT-3'. Experiments
were carried out two independent times with similar results.

Mice: WT and Nmp4-/− mice were generated as previously described and maintained at Indiana
University Bioresearch Facility School of Dentistry (90). Briefly the strategy for preparing the
global Nmp4-/- mice involved removing the region of this gene containing coding exons 4 – 7 via
homologous recombination (90). The correctly targeted embryonic stem (ES) cell lines from
129SvEv ES clones were microinjected into C57BL/6J blastocysts and the chimeric mice were
crossbred with the C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) to generate
germline transmission. These mice were backcrossed for seven generations on the C57BL/6J
background. Their WT littermates were used as the control mice for these experiments. The
mice were housed, 2-4 mice/cage, under a 12hr light/12 hour dark regimen and Labdiet Rodent
5001 diet was provided ad libitum. The Indiana University Institutional Animal Care and Use
Committee approved all experimental procedures described in the present study.
Therapies: At 10 weeks of age virgin female mice were randomly sorted into eight treatment groups by weight and genotype. Each mouse received two sequential 100µl injections/day containing the drugs or vehicle(s) 7 days/week for 7 weeks. Mice in select groups were injected subcutaneously with synthetic human PTH (hPTH) 1–34 acetate salt (Bachem Americas, Inc. Torrance, CA) at 30 µg/kg/d, daily, a dose often used in rodents to evaluate PTH bone anabolic action in vivo (37, 63). The dose of the anti-catabolic agent raloxifene (RAL, Sigma-Aldrich) is based on human clinical doses. RAL is normally administered as a 60mg daily dose, therefore based on a 60kg patient the quantity would be 1 mg/kg/day. The assumption is 100% absorption therefore the full dose was administered as a subcutaneous injection (95). Our euthanasia protocol involves using carbon dioxide inhalation at 20%V/min followed by bilateral pneumothorax or cervical dislocation in compliance with the guidelines of our Animal Care and Use Committee. This is an approved method by the Panel on Euthanasia of the American Veterinary Medical Association.

Micro-computed tomography (μCT): Femurs and L5 vertebra were dissected from the 17 week-old mice. The femurs were soaked in 0.9% saline, wrapped with gauze and stored at –20°C. The L5 vertebra were transferred to 10% formalin for 2 days and then stored in 70% ethanol. Left femurs were thawed to room temperature and scanned while hydrated with a 8.5 µm voxel size using a Skyscan 1172 μCT system (176 mA, 0.5 mm Aluminum filter). Scans were reconstructed with voxel attenuation coefficients ranging from 0-0.11, a beam hardening correction of 40%, and a ring artifact correction of 5. Mineral density was calculated using daily scans of manufacturer supplied hydroxyapatite (HA) phantoms of 0.25 g/cm³ and 0.75 g/cm³. L5 vertebrae were scanned with a 6 µm voxel size using the Skyscan 1172 μCT system (176 mA, 0.5 mm Aluminum filter). Scans were reconstructed with voxel attenuation coefficients ranging from 0-0.08, a beam hardening correction of 20%, and a ring artifact correction of 10. Three-dimensional reconstructions using Skyscan software provided femur and L5 vertebra trabecular
bone volume per total volume (BV/TV, %). Parameters obtained for femoral cortical bone included total cross-sectional area (CSA, mm²), marrow area (mm²), cortical thickness (mm), periosteal bone surface (BS, mm), endocortical BS (mm), anterior-posterior width (AP, mm), medial-lateral width (ML, mm), AP/ML, moment of inertia about the AP axis (I_{ap}, mm⁴), moment of inertia about the ML axis (I_{ml}, mm⁴), maximum moment of inertia (I_{max}, mm⁴), minimum moment of inertia (I_{min}, mm⁴), medial extreme (mm), and tissue mineral density (TMD, g/cm³ HA).

Mechanical testing: Left femurs from each animal were thawed to room temperature and monotonically tested to failure in three-point bending at a displacement rate of 0.025 mm/sec using a support span of 9 mm (4). The bones were oriented in the anterior-posterior direction with the anterior side in tension. The moment of inertia about the medial-lateral axis and the extreme fiber in the anterior direction were obtained from the μCT images using a seven slice region centered on the failure site, and were utilized to map load-displacement to stress-strain, employing standard beam bending equations. Structural-level mechanical and tissue-level material properties were then obtained from the load-displacement and stress-strain curves.

Statistical analysis: We used the statistical package JMP version 7.0.1 (SAS Institute, Cary, NC) to evaluate osteoporosis treatment response in our experimental mice. We tested three experimental therapies and a vehicle control using two genotypes of mice, yielding a total of eight treatment groups. Outliers in the datasets were identified using the interquartile range (IQR) method to assess statistical dispersion (68). The remaining data were analyzed with a 2-way ANOVA for effects of genotype and treatment followed by a Tukey-Kramer post hoc test for comparison of more than two groups or Student t post hoc test for comparing WT and Nmp4⁻/⁻ parameters as two groups. Experimental data were sorted by either treatment or genotype to determine whether either or both influenced the value of the endpoint parameter and whether genotype affected the response to treatment (genotype x treatment interaction). To assess if the
combination treatment provided a synergistic effect over the mono-therapies we performed 2-way ANOVA tests using PTH and RAL as the independent variables on both WT and Nmp4+/− datasets. Statistical significance was set at p≤0.05. To evaluate the metabolic profiles of the MSPCs, we used the Statistical Analysis System version 9.4 (SAS Institute) and JMP to perform student t-tests in comparing specific metabolic parameters. Finally, ggplot2 was used to create all the heatmaps, volcano plots, and boxplots (107).

RESULTS:

*Nmp4 regulates a large portion of the osteogenic transcriptome*

We previously showed that expanded cultures of Nmp4+/− bone marrow MSPCs exhibited a precocious and enhanced mineralization compared to WT cells (16). For the present study, three independently derived WT MPSCs from individual isogenic mice, along with three Nmp4+/− preparations confirmed that the null cells exhibited mineralization typically within 1 week of exposure to osteogenic medium compared to 2-3 weeks for the WT cells (Figure 1A).

To address the mechanism for the hyper-anabolic phenotype elicited by loss of Nmp4, we performed transcriptome analysis on osteogenic MSPCs as a guide for hypothesis testing. Given that there were some variations in time to mineralization between the individual Nmp4+/+ and Nmp4+/− MSPC preparations, we elected to carry out RNA-seq on the MSPCs 1584L^WT vs. 1515RR^Nmp4+/− under two distinct culture conditions. These cells exhibited a striking difference in the time to mineralization onset. We then carried out the critical phenotypic anchoring experiments with the other MSPC preparations as well as the WT and Nmp4+/− mice to show that our findings are broadly applicable.

To perform RNA-seq analysis, RNA was harvested from cells at Day 3 post-seeding that were maintained in non-differentiation medium and at Day 7 in culture in which the cells had been transferred to osteogenic medium 48hrs post-seeding. All data obtained from these studies including the differences observed in mRNA expression between the WT and Nmp4+/−
MPSCs at different time points following exposure to osteogenic medium are provided in Table S2. A volcano plot shows that Nmp4-deleted cells cultured in non-differentiating medium for 3 days displayed significant ≥ 2-fold change in the expression of 5032 genes compared to WT. Of these, there was an increase in the expression of 3468 genes and a decrease in expression of 1564 genes (Figure 1B). Following this criterion, the expression profiles of 8438 genes were not significantly affected by Nmp4 status (Figure 1B).

Loss of Nmp4 had a similar impact on the transcriptome of MSPCs maintained in the osteogenic differentiating medium and harvested at Day 7, which coincided with the initiation of mineralization. At the 7-day time point, the expression profiles of 5313 genes were significantly altered by ≥ 2-fold, with 3925 genes presenting an elevation in expression compared to WT cells and 1388 genes showing a decrease (Figure 1B). Nmp4 status did not impact the expression of 8151 genes in cells maintained in the osteogenic medium (Figure 1B).

We recently reported a genome-wide ChIP-seq analysis of Nmp4 binding in MC3T3-E1 pre-osteoblasts that identified over 15,000 binding sites for this transcription factor (16). This cell line is an established in vitro model for early osteoblastogenesis that is similar to our primary MSPCs. To identify genes that are direct targets of Nmp4, we determined the overlap of the gene lists derived from the present MSPC RNA-seq datasets and those lists derived from our previous analysis of Nmp4 genome-wide ChIP-Seq analysis (16). The gene list used from the ChIP-seq dataset contained genes that had 1 or more peaks associated with the transcription start site (TSS) within -5 to +2 kb from a TSS and/or within the range defined by the TSS and the transcription end site (TES), and not within the promoter range of the same gene (Table S1) (16). Additionally, we limited the compilation to 4786 and 4787 genes expressed by our MSPCs for days 3 and 7 in culture respectively. The Venn diagrams revealed that about 28% of the genes occupied by Nmp4 exhibited a significant increase in expression upon loss of this
transcription factor after 3 and 7 days in culture, indicating gene repression by Nmp4. By contrast, ~9% showed a decrease in expression upon loss of Nmp4 suggesting that Nmp4 functions to directly activate these gene targets (Figures 1D & 1E). Expression of ~63% of the genes that supported Nmp4 with significant occupancy were not strongly impacted by loss of Nmp4, suggesting that Nmp4 status alone is not sufficient to alter the expression of these genes (Figures 1D & 1E). We conclude that in this osteogenic context Nmp4 has an extensive influence on the MSPC and osteogenic transcriptomes consistent with its widespread occupancy in their genomic landscapes.

**Loss of Nmp4 alters pathways that exhibit the dual functions of driving osteogenesis and glycolysis**

To identify cellular pathways sensitive to Nmp4 status, we performed IPA (Ingenuity pathway analysis)-based network analyses on the 5032 genes that exhibited a significant change in expression between the Nmp4\(^{-/-}\) and WT cells at Day 3 (non-differentiating medium) and on the 5313 genes that exhibited a change in expression at Day 7 (osteogenic medium) in culture. Tables S3 and S4 list the 252 significant canonical pathways derived from transcriptome analysis of Day 3 and the 201 significant canonical pathways derived from analysis of Day 7 cells, respectively [https://figshare.com/s/aef3382cdc7c02151e6f](https://figshare.com/s/aef3382cdc7c02151e6f). The large number of affected pathways is consistent with the substantial number of genes whose expression is influenced by Nmp4 status.

Many of the canonical pathways listed in Tables S3 & S4 were also identified in previous studies characterizing MSC transcriptomic changes during osteogenic differentiation (11, 72, 80), thus supporting our experimental approach. For example, transforming growth factor-β (TGF-β) signaling, IGF1, Wnt/β-catenin signaling, and bone morphogenic protein (BMP) signaling all appear to support human adipose-derived stem cells (hASC) and bone marrow
stromal cell (BMSC) osteogenesis. Additionally, many pathways related to the triggering of cell cycle, growth, differentiation, and migration, such as axonal guidance signaling, platelet-derived growth factor signaling (PDGF signaling), integrin signaling, and actin cytoskeleton signaling, have previously been distinguished in these MSPC preparations (11, 72, 80) and were identified here.

In our hypothesis-generating screen of the IPA outcomes we identified several pathways predicted to be sensitive to Nmp4 status and drive both osteogenesis and metabolic reprogramming necessary for fueling the development of the professional secretory osteoblast (Tables S3 and S4) (56, 79, 81, 96). Several pathways were common to cells harvested on either Day 3 or Day 7 and we present some of these data in graphical form for Day 7 (Figures 2A & 2B). The bar graphs in Figure 2A are color-coded to reflect the z-score calculated by the IPA algorithm, which predicts the direction of change for the pathway upon loss of Nmp4. An absolute z-score of 2 or more is considered significant. The activation state of the pathway is predicted to be increased if the z-score is ≥ 2 and these bars are color coded with an orange hue. Conversely, bar graphs with a blue hue indicate a z-score ≤ −2 representing canonical pathways with a decreased activity. Those pathways represented with a grey bar (z = NaN) indicate that the z-score algorithm cannot predict whether the pathway activity is increased or decreased in the Nmp4−/− cells.

The bar graphs in Figure 2B are color coded to reflect the percentage of genes in a particular pathway whose expressions are upregulated (red) or downregulated (green). For example, the Wnt/Ca2+ signaling pathway z scores were +3.00\textsuperscript{Day3}/+3.77\textsuperscript{Day7}, (Figure 2A; Tables S3 and S4) indicating that loss of Nmp4 enhances the activity of this pathway. Additionally a high percentage of the genes in this pathway exhibited a significant increase in expression in the Nmp4−/− MSPCs (Figure 2B). This is significant to the Nmp4−/− osteoblast phenotype since Wnt signaling is a major driver of bone anabolism and advances osteogenesis in part through its stimulation of glycolysis (27). The IPA's Molecule Activity Predictor (MAP) algorithm allowed
simulating the effects of disabling *Nmp4* on the Wnt signaling pathway, which predicted elevated beta catenin activity, a key driver of osteogenesis (49), and the attenuated activity of Nemo-like kinase (NLK), a suppressor of beta-catenin transcriptional activity and osteogenesis (9, 44, 74) (Figure 3). The accompanying Wnt pathway heatmap (Figure 3) suggests this predicted increase in Wnt signaling activity is based, in part, on the diminished expression of numerous Wnt inhibitors including *Wif1*, *Sfrp1*, *Sfrp2*, and *Apc2* (7, 101).

Of interest, loss of *Nmp4* significantly enhanced the expression of *Dkk2* mRNA (see heatmap Figure 3). Depending on the cellular context *Dkk2* can stimulate or inhibit Wnt signaling (55, 64). For example, *Dkk2* is essential for osteoblast terminal differentiation, mineralization and may be a novel mediator of the PTH-induced anabolic response in bone (57, 111). The activities of the *Igf1* (*z* = +4.13<sup>Day3</sup>) and the *Nrf2* signaling pathways (*z* = +4.33<sup>Day3</sup>; +4.23<sup>Day7</sup>) were predicted to be upregulated in *Nmp4*<sup>−/−</sup> cells and although loss of *Nmp4* was projected to alter the Hif1α signaling pathway the direction of activity could not be ascertained (*z* = NaN<sup>Day3</sup>; *z* = NaN<sup>Day7</sup>) (Figure 2; Tables S3 and S4). Nevertheless all pathways regulate osteogenesis as well as govern cellular metabolic reprogramming (28, 39, 82, 85). Furthermore, the PTEN network was significantly sensitive to *Nmp4* status and assigned *z* scores -2.50<sup>Day3</sup> and -1.76<sup>Day7</sup> (Figure 2; Tables S3 and S4) suggesting that the activity of this pathway is attenuated with loss of *Nmp4*. Indeed, depletion of PTEN signaling was reported to enhance osteoprogenitor expansion and glycolytic conversion (35, 110).

Of interest, loss of *Nmp4* did not significantly alter the expression of *Runx2* and *Sp7* (*Osterix*), master regulators of osteogenesis, but elevated expression of the transcription factors *Tcf4*, *Atf4*, and *Ddit3* (*Chop*, *Gadd153*), which all function downstream of *Runx2* and *Sp7* (Figure 4). Additionally *Nmp4*<sup>−/−</sup> cells exhibited decreased mRNA expression of transcription factors that drive adipogenesis or chondrogenesis suggesting that loss of *Nmp4* facilitates MSCP differentiation towards osteogenesis and that this predisposition is reinforced by shifts in
transcriptional networks regulating the activities of the aforementioned osteogenic/metabolic pathways (Figure 2, Figure 4 Tables S3 and S4).

Phenotype anchoring of our transcriptional data confirmed Nmp4−/− MSPCs exhibited an enhanced capacity for glycolytic conversion

The glycolytic pathway is predicted to be altered in the Nmp4−/− cells at both Day 3 and Day 7 in culture (Figure 2; Tables S3 and S4). A heatmap of several genes that comprise the glycolytic pathway showed that loss of Nmp4 greatly elevated the expression of the glucose transporter Slc2a1 (a.k.a Glut1) and increased the transcript levels of both Slc2a3 and Slc2a4 (Glut3, Glut4, Figure 5A). The lactate transporter Slc16a3 (a.k.a Mct4) was highly expressed in the Nmp4−/− MPSCs at both Day 3 and Day 7 in culture (Figure 5A). A primary function of Slc16a3 is the secretion of lactate and protons from highly glycolytic cells (22) and a recent study determined that increased levels of Slc16a3 is necessary for sustaining high glycolysis in macrophages (102). Several genes mediating the conversion of glucose to pyruvate displayed significantly elevated expression in Nmp4−/− cells (Figure 5A). Genes responsible for regulating the switch between aerobic glycolysis and oxidative phosphorylation including Hk2, Pkm, Pdk1, and LDHA showed significantly higher mRNA levels in the Nmp4−/− cells. Additionally, our ChIP-seq analysis in MC3T3-E1 cells showed that Nmp4 binds to both Pdk1 and Pkm genes (Figure 5B) indicating that this trans-acting protein directly targets key genes that regulate the glycolytic switch.

We linked our transcriptome/ChIP-seq analyses to functional data via the glycolytic stress tests (Figure 6). WT vs. Nmp4−/− cells derived from the male littermates (1957WT/1957KO) and the WT vs. Nmp4−/− cells derived from the males obtained from random litters (1584LWT/1515RRKO) were cultured in non-differentiating medium using the Seahorse analyzer. Cells were seeded directly into an analyzer well plate and grown for 24hrs in culture. Subsequently cells were incubated in medium devoid of glucose or pyruvate and the analyzer
measured the extracellular acidification rate (ECAR) before and after a saturating amount of glucose was injected. These experiments quantified glycolytic activity (glycolysis), which was significantly elevated in *Nmp4*−/− cells (Figures 6A-6C). The ECAR value was then obtained after injection of oligomycin, which inhibited oxidative phosphorylation driving the cell to use glycolysis to its maximum capacity (glycolytic capacity). Again the *Nmp4*−/− cells exhibited a significantly elevated level for this parameter (Figures 6A-6C). The final injection of 2-deoxy-glucose (2-DG), a glucose analog that inhibited glycolysis through competitive binding to glucose hexokinase, decreased ECAR confirming that the lowered medium pH was the result of increased glycolysis (Figures 6A and 6B). The glycolytic reserve, defined as the difference between glycolytic capacity and glycolysis rate was elevated with the loss of *Nmp4* (Figures 6A-6C). We conclude that loss of *Nmp4* results in the metabolic reprogramming of the MSPCs enhancing their capacity for glycolysis.

*Nmp4*−/− MSPCs exhibited an increased mitochondrial respiratory capacity

Next the mitochondrial respiratory capacity was compared in the WT and *Nmp4*−/− cells. For the mitochondrial stress test the Seahorse analyzer was used to measure basal respiration reported as oxygen consumption rate (OCR) and then the cells were sequentially exposed to various compounds to assess mitochondrial electron transport chain function (Figures 7A-7C). Our results showed that loss of *Nmp4* elevated basal respiration, maximal respiration, and ATP production in MSPCs (Figures 7A-7C). Spare respiratory capacity and non-mitochondrial respiration were also significantly elevated (data not shown). We conclude that metabolic reprogramming occurs in MSPCs as a consequence of *Nmp4* loss, enhancing the capacity of these cells for oxidative phosphorylation.

*Nmp4*−/− osteoprogenitors exhibit enhanced protein production and secretion
IPA analysis predicted that loss of Nmp4 elevates the activity of several cellular pathways driving protein production and delivery. Specifically, the activities of the eIF-2 ($z = +3.28^{\text{Day3}} + 2.72^{\text{Day7}}$), mTOR ($z = +2.949^{\text{Day3}} + 2.71^{\text{Day7}}$), and the eIF4 and p70SK6 ($z = +3.16^{\text{Day3}} + 2.56^{\text{Day7}}$) signaling pathways were predicted to be upregulated in Nmp4-- cells (Figure 2, Tables S3 and S4). This suggests that loss of Nmp4 stimulates anabolic processes including protein synthesis, translation initiation, and the regulation of energy production in mitochondria (71, 105). Loss of Nmp4 was projected to alter the tRNA signaling pathway but the direction of change could not be predicted ($z = \text{NaN}^{\text{Day7}}$) (Figure 2; Table S4). Nevertheless, several genes of this pathway were significantly upregulated (Figures 2B & 8). Indeed the expression of numerous genes comprising the pathways of amino acid transport, amino acid biosynthesis, ribosome biogenesis, and translation initiation were significantly elevated (Figure 8). Elevated protein production in Nmp4-- MSPCs is also supported by our earlier report that enhanced ribosome biogenesis was sustained during induction of the unfolded protein response (UPR) which serves to expand the processing capacity of the ER for nascent secretory proteins (25, 114). This Nmp4-directed transcriptome program may allow a large protein client load to be processed through the endoplasmic reticulum without halting global osteoblast translation or inducing apoptosis (114). Indeed, the RNA-seq analysis confirmed that Nmp4-- MSPCs exhibited elevated expression of several genes UPR pathway (Figure 9) and IPA/MAP analysis predicted that protein-folding activity is elevated and UPR-induced apoptosis is attenuated with loss of Nmp4 (Figure 9).

We validated the transcriptome data experimentally by measuring bone matrix production and delivery in WT and Nmp4-- osteoprogenitors by comparing the levels of Col1a1-mRNA associated with polyribosomes and the levels of collagen protein secretion. WT vs. Nmp4-- cells derived from the littermates (1957WT/1957KO) and the WT vs. Nmp4-- cells derived from mice obtained from random litters (1584LWT/1515RRKO) were cultured in non-differentiating medium for four days. We observed elevated levels of 40S and 60S ribosomal subunits and 80S
monosomes, and increased polysomes in Nmp4−/− MSPCs compared to WT (Figure 10A). The RNA-seq data revealed that total Col1a1 mRNA expression was elevated in the Nmp4−/− cells (Figure 10B). To address whether Col1a1 mRNA translation accompanied the enhanced global translation, qRT-PCR analysis was performed to quantify the amount of Col1a1 mRNA present in the polysome fractions prepared from the WT and Nmp4−/− cells (Figure 10C). Col1a1 mRNA was present in heavy polysomes in both WT and Nmp4−/− MSPCs, suggesting efficient translation. However, there was a reproducible increase in Col1a1 mRNA in the largest fraction 7 in Nmp4−/− cells, suggesting more robust Col1a1 translation in the Nmp4-depleted cells (Figure 10C). Thus the combination of more Col1a1 mRNA available for translation, along with increased amounts of ribosomes and more efficient Col1a1 mRNA translation, would culminate in elevated synthesis of Col1A1 protein in the Nmp4−/− cells.

Collagen deposition is coupled to osteogenic proliferation (78) and Nmp4−/− MSPCs frequently exhibit a modest but significant increase in proliferative activity compared to WT (16). To evaluate changes in collagen production induced by Nmp4 deletion, independent of the confounding effects of proliferation differences, we first measured collagen production in the 1515RRKO and 1584LWT preparations that normally do not exhibit a difference in proliferation. \1515RRKO cells produced approximately 3-4-fold more collagen/cell than the 1584LWT (Figure 10D). Next we evaluated the amount of collagen recovered/well as a function of the number of cells/well for all six MSPC preparations (Figure 10E). All three Nmp4−/− preparations produced more collagen compared to WT cells regardless of cell number during this proliferative period in culture (Figure 10E). Moreover, preliminary experiments with shRNA knockdown of Nmp4 in MC3T3-E1 cells yielded a similar Collagen/well vs. Cells/well profile (data not shown). This is consistent with our previous in vivo data showing that the Nmp4−/− mice harbor more bone marrow osteoprogenitors than WT, which in turn produce more bone when stimulated (16, 41, 95). We conclude that loss of Nmp4 converts osteoprogenitors/osteoblasts into super-secretors of bone matrix while moderately enhancing their proliferative activity.
Nmp4−/− osteoblasts produce a bone matrix with improved material properties

Several genes representing multiple protein classes comprising the bone matrix (6, 14, 17, 51, 69) were identified as upregulated in our RNA-seq dataset suggesting enhanced matrix material properties in the null animal. The mRNA expression of this collection of genes is represented by a heatmap that displays changes between the Nmp4−/− and WT MSPCs/osteogenic cells (Figure 11). Loss of Nmp4 significantly increased or decreased the expressions and relative ratio of several extracellular matrix (ECM) genes including those that support bone mechanical properties e.g. Col1a1, Col1a2, Bglap2 (osteocalcin), and Spp1 (osteopontin) (Figure 11). Also the expression of key genes that control mineralization were altered in the Nmp4−/− cells consistent with the phenotype observed in culture. For example, the genes phosphoethanolamine/phosphocholine phosphatase (Phospho1) and alkaline phosphatase, tissue-nonspecific isozyme (Alpl), encoding phosphatases responsible for initiating mineralization (5, 43, 67, 112, 113), were highly induced in the Nmp4−/− cells as was the gene Slc20a1 a sodium-phosphate symporter also involved in the initiation of skeletal mineralization (112) (Figure 11). Finally, the expression of several small leucine-rich proteoglycans (SLRPs) such as lumican (Lum) and decorin (Dec) were highly elevated in the Nmp4−/− cells (Figure 13). SLRPs play significant structural roles within the ECM and regulate collagen fibril growth, organization and ECM assembly (12, 47, 76).

To test the biological ramifications of the transcriptional changes associated with the bone matrix genes, we evaluated skeletal tissue obtained from healthy virgin mice that had been treated with the osteoporosis therapeutics RAL, PTH, PTH+RAL and vehicle-control for 7 weeks as described in the Materials and Methods. Ovariectomized mice were not used in this experiment because ovariectomy does not change the enhanced response to anabolic drugs in the Nmp4−/− animals (16). Furthermore, all the MSPCs used in this study were derived from healthy, virgin mice. Briefly, µCT analysis showed that the PTH+RAL therapy produced more
bone compared to both the PTH and RAL mono-therapies at the distal femur and L5 vertebra (Figures 12A & 12B, Tables 1 and 2). There was a synergistic (greater than additive) interaction between PTH and RAL in both the WT and \( \text{Nmp4}^{-/-} \) mice for BV/TV of the distal femur and the L5 vertebra (Table 2). However, loss of \( \text{Nmp4} \) significantly improved the femoral bone gain and the L5 bone gain in the PTH and PTH+RAL treatments (Figures 12A & 12B Table 1). \( \text{Nmp4} \) status had no impact on bone response to RAL mono-therapy (Figures 12A & 12B, Table 1). Finally, there was no significant difference between the genotypes under the VEH control treatment with respect to femoral and L5 BV/TV (Figures 12A & 12B, Table 1). However loss of \( \text{Nmp4} \) did significantly impact some aspects of femoral cortical geometry, such as cortical thickness, marrow area as well as other related parameters (Table 3). Altogether, these results are similar to the data we reported in older ovariectomized mice (95).

A key component of these functional investigations required the measurement of material and structural mechanical properties of the bone. Therefore the left femurs from each animal were monotonically tested to failure. The \( \text{Nmp4}^{-/-} \) bones exhibited a significantly higher ultimate stress, which is the stress necessary to fracture the bone at the material-level, normalized for the bone geometry (Figure 12C, Table 4). Yield stress, the stress applied to the bone after which there is permanent damage, normalized for geometry, was also significantly higher in the \( \text{Nmp4}^{-/-} \) femurs (Figure 12D, Table 4). Additionally, the higher value for the elastic modulus, a measure of the material’s stiffness, in \( \text{Nmp4}^{-/-} \) bones approached significance (genotype \( p<0.06 \), Table 4). Interestingly, numerous material properties were sensitive to the osteoporosis therapies. PTH+RAL led to significantly higher ultimate stress over RAL and PTH mono-therapies in both genotypes (treatment \( p<0.0001 \) Figure 12C and Table 4). PTH treatment led to a modest but significantly lower yield stress than VEH control and RAL cohorts, which were equivalent. The lower yield stress in the PTH cohorts is likely due to the increased amount of new and less mineralized bone. This would make the tissue less stiff, which is consistent with the modulus trending lowest in the PTH-treated mice (Table 4).
Finally, total strain, elastic modulus, and resilience were all differentially responsive to the various therapies (treatment p<0.05 Table 4).

Loss of Nmp4 also altered the structural properties of the femur. Yield force was significantly increased in the null bone (genotype p=0.004 Table 5) and the increase in ultimate force neared significance (genotype p=0.07). Total displacement, the total amount of deformation the bone undergoes before failure, was significantly lowered in the Nmp4-/− femurs (genotype p=0.04 Table 5) and the decrease in post yield displacement, the amount of deformation that occurs after the yield point, approached significance (p=0.06, Table 5). Finally, work-to-yield, the energy that goes in to deforming the sample was significantly higher in the Nmp4-/− bone (genotype p=0.03 Table 5). These results show that the Nmp4-/− osteoblast produces more matrix than WT cells and that the composition of the secretome results in improved bone material and structural properties.

**DISCUSSION:**

We investigated the mechanisms underlying the hyper-anabolic phenotype of Nmp4-/− MSPCs during osteogenesis. Transcriptomic data predicted that Nmp4-/− osteogenic cells have (i) a significantly increased capacity for metabolic conversion to glycolysis, (ii) increased Col1a1 mRNA translation, (iii) elevated collagen secretion, and (iv) elaborate a matrix that improves bone material and structural mechanical properties. In each case, we were able to anchor the predicted phenotype with experimental results. Moreover the derived mechanistic insights on Nmp4 control of bone cell phenotype were remarkably consistent between the multiple model systems used in this investigation including MSPCs, MC3T3-E1 osteoblast-like cells, and the in vivo bone studies. Nevertheless, additional model systems are required to explore Nmp4 control of bone phenotype, e.g. conditional knockout mice and CRISPR cell lines, and we are currently developing these reagents.
The Nmp4−/− MSPCs have a significantly increased capacity for metabolic conversion to glycolysis, which is a key driver of osteoblast anabolism. Our ChIP-seq and RNA-seq data show that Nmp4 directly targets and regulates key genes that direct the cell towards aerobic glycolysis including Pdk1 and Pkm. Glucose is a key nutrient for osteoblasts and aerobic glycolysis is the dominant mode of glucose utilization in these cells (49, 53). Thus, while it is a less efficient source of energy, glycolysis can generate both anabolic growth intermediates and ATP very rapidly owing to the much higher speed of glycolysis reactions (97). In vivo, PTH-induced bone anabolism is driven in part by the hormone mobilizing osteoblast autocrine IGF1 signaling. This activates mTORC2, which suppresses glucose entry into the TCA cycle and shunts it into the aerobic glycolysis pathway (28). Concomitantly, PTH downregulates Sost/sclerostin expression in osteocytes, unleashing the anabolic WNT signaling pathway (98), driving osteogenesis in part by further stimulating glycolysis via the rapid increase in GLUT1 and HK2 protein expression and escalation in LDHA and PDK1 activities thus increasing glucose consumption and driving lactate over acetyl-CoA production from pyruvate (27).

Our transcriptome data identify the HIF1α and PTEN canonical pathways as significantly sensitive to the status of Nmp4, which is consistent with the glycolytic phenotype of the null cells. Like the IGF1 and WNT pathways the HIF1α and PTEN link osteogenesis and metabolic reprogramming. Stabilization of the transcription factor HIF1α in Sp7-positive cells in postnatal mice significantly stimulated trabecular bone formation via an increase in the number of osteoblasts and also promoted bone glycolysis via the mRNA upregulation of key glycolytic enzymes including Pdk1, Ldha, and Hk2 (85). Mice expressing the stable-oxygen form of HIF1α in osteoprogenitors exhibited an expanded pool of these cells and elevated trabecular BV/TV, very reminiscent of the Nmp4−/− skeletal response to PTH (16, 41, 95). Our pathway analysis predicted that PTEN signaling is attenuated in the Nmp4−/− cells. Interestingly, PTEN signaling antagonizes the Pi3k-Akt-mTORC2-p70s6k pathway and thus decreases the glycolytic rate and favors oxidative phosphorylation (77). Specifically, PTEN decreases the levels of two key
enzymes involved in the Warburg effect, PKM2 and PFKFB3 (33). Therefore, conditional loss of
Pten in osteoprogenitors led to increased numbers of osteoblasts and expanded bone matrix
(35), whereas conditionally disabling Pten in mature osteoblasts enhanced mTOR activity and
increased bone mineral density (60). Again, this Pten-deficient bone phenotype is similar to the
Nmp4+/− skeleton under PTH challenge.

The Nmp4+/− MSPCs also exhibited an enhanced capacity for oxidative phosphorylation.
This is consistent with a recent study showing that non-differentiated MC3T3-E1 osteoblast-like
cells exhibited both spare glycolytic and oxidative capacities (34). Additionally, this study
reported that differentiated MC3T3-E1 cells met ATP demand primarily by aerobic glycolysis,
whereas non-differentiated cells generated ATP through oxidative phosphorylation (34). Further
studies with our MSPCs are required to elucidate the impact of Nmp4 on metabolic
reprogramming during differentiation.

The present study revealed that Nmp4+/− MSPCs exhibited increased Col1a1 mRNA
translation attendant with elevated collagen secretion revealing part of the mechanism by which
these cells are converted into super-secretors. Collagen comprises over 90% of the bone
protein matrix and our results suggest that a large percent of Col1a1 mRNA transcripts are
present in the heavy polysomes in Nmp4+/− MSPCs implying a high translation of Col1a1
transcript. This is consistent with our observed increase in collagen protein secretion in these
cells. Osteoprogenitor loss of Nmp4 not only redirects metabolic programming toward cellular
anabolism, but also elevates gene expression for multiple pathways involved in protein
synthesis and delivery, a key step in bone formation (27, 48). Our transcriptomic analysis
showed a striking increase in the mRNA expression of several genes that promote protein
anabolism during osteoblast differentiation including numerous amino acid transporters, the
amino acid synthase Asns and several other genes involved in amino acid synthesis, many
tRNA-charging enzymes, and multiple genes driving protein translation initiation as part of the
eif4 and eif2 pathways. The present data are consistent with our previous study showing that
659  *Nmp4*−/− MSPCs exhibited significantly elevated ribosome biogenesis, the primary determinant of
660  translational capacity and a key driver of cell growth (114).
661
662  Another key finding of the present work is that *Nmp4*−/− osteoblasts elaborate a matrix
663  that improves bone material and structural mechanical properties. The expression of matrix
664  genes that contribute to material and structural mechanical properties, e.g. collagen, osteocalcin,
665  and SLRPs were elevated in *Nmp4*−/− cells and mechanical analysis of femurs from mice treated
666  with osteoporosis therapies confirmed enhanced improvement in several of these key properties
667  in the *Nmp4*−/− bone. Enhanced and accelerated mineralization *in vitro* often does not correlate
668  with positive effects on the skeleton. For example, osteoblasts deficient in the expression of
669  *Naca* (66), *Sox8* (93), or *Foxc1* (42) showed an *ex vivo* accelerated mineralization phenotype;
670  however animals harboring deficits in any one of these genes exhibited significant defects in
671  bone development, formation, or mineralization (42, 66, 93). Sodium fluoride (NaF) is an
672  osteoanabolic that increases bone mass but the newly formed bone lacks normal structure and
673  strength (10, 86, 99). Mechanical load is a bone anabolic signal and the magnitude of the
674  applied strain determines whether the response is adaptive, forming primarily lamellar bone, or
675  injury, producing woven bone (65). Although woven bone forms faster than lamellar bone, it has
676  inferior mechanical and material properties (8, 19). These findings are in stark contrast to our
677  present model in which the precocious and enhanced mineralization of the *Nmp4*−/− osteoblast
678  directly translates into an improved skeletal response to osteoanabolics (15, 16, 41, 95).
679
680  The molecular mechanisms underlying the improved *Nmp4*−/− bone quality remains to be
681  determined but the enhanced expression of osteocalcin by pharmacologically induced *Nmp4*−/−
682  osteogenic cells (15, 16, 95) may improve the quality of the produced bone. Earlier we proposed
683  that non-collagenous proteins act as “glue” at the collagen-mineral interface to resist the
684  separation of the mineralized fibrils and therefore enhance bone toughness (69, 75, 84, 100).
685  Anabolic therapies that induce the formation of osteocalcin/osteopontin-enriched bone may
686  further enhance energy absorption capacity of bone tissue. Therefore loss of *Nmp4* may
modestly alter the ratio of collagen to non-collagenous protein matrix composition, which would be enough to improve bone quality. Additionally, the present data suggest that Nmp4\(^{-/-}\) matrix is enriched in SLRPs, which govern ECM assembly via regulation of linear/lateral fibril growth by binding to the collagen fibril surface (12, 47, 76). Thus the collagen maturation may be accelerated in Nmp4\(^{-/-}\) bone.

The present data demonstrate that Nmp4 acts cell autonomously as a barrier to bone matrix production and mineralization. As an apex regulator of bone cell anabolic output Nmp4 directly and indirectly regulates gene programs that control key stages of matrix production and secretion and the metabolic reprogramming necessary to fuel it (Figure 13). The Nmp4 transcriptional modus operandi is reminiscent of another apex regulator c-Myc, which like Nmp4 controls the expression of large sets of genes involved in ribosome biogenesis, metabolism, and protein synthesis, representing a cell-type-independent genetic program involved in biomass accumulation (38, 45). Furthermore, whereas c-Myc acts a general amplifier of gene expression (50, 73, 108), Nmp4 appears to act as a general attenuator and suppressor of biomass accrual (16, 114). Both the MSPC RNA-seq and Nmp4 MC3T3-E1 ChIP-seq pathway analyses (16) are consistent with this mechanistic profile. Like c-Myc, Nmp4 may directly govern the expression of master transcriptional regulators of these key networks in addition to broadly engaging some of their downstream target genes. Whether there is a functional relationship between c-Myc and Nmp4 remains to be determined. Nmp4 control of protein translation and movement through the bone cell ER is particularly intriguing since this is a potential promising area for drug target discovery. Several therapeutic strategies and multiple drugs are currently being developed to enhance the adaptive capability of the ER in the service of secretion for numerous diseases including diabetes, cancer, Alzheimer’s, and osteoporosis (24, 36, 40, 54, 87, 91, 104). Nmp4 control of metabolic reprogramming may also present therapeutic targets for regulating bone anabolism (48, 49, 106).
There is a critical medical need for understanding the intrinsic barriers to pharmacologically inducing bone formation in the osteoporotic skeleton (21). Interrogating these pathways may alleviate the current limits to osteoanabolic therapy.

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FIGURES AND FIGURE LEGENDS:

Figure 1: Loss of Nmp4 accelerates and enhances MSPC mineralization and has a broad impact on the transcriptome. [A] Six independently expanded MSPC preparations from individual WT and Nmp4<sup>-/-</sup> mice were established as described in Materials and Methods. Cultures were stained with alizarin red when mineralization was first observed. The cells derived from the Nmp4<sup>-/-</sup> mice consistently exhibited mineral days to weeks before this was observed in the WT cultures. The cell preparations 1957N<sup>KO</sup> and 1957R<sup>WT</sup> were derived from male littermates. The remaining lines were derived from male or female mice selected from random litters. [B] Volcano plots of RNA-seq data from MSPCs maintained in non-differentiating culture medium for 3 days and osteogenic differentiating culture medium for 7 days. The X-axis represents the logarithmic transformation to the base 2 of the mean fold-change of mRNA expression in Nmp4<sup>-/-</sup> cells versus control cells and the Y-axis represents the negative logarithm to the base 10 of the FDR value. Changes in gene expression were considered significant if the fold-change of KO/WT ≥+2 (red circles) and FDR ≤0.05 or KO/WT≤ -2 (green circles) and FDR ≤0.05. The black circles represent genes that did not meet either criteria. The dotted line demarcates FDR=0.05. [C] Venn diagrams showing gene overlap between ChIP-seq and RNA-seq data. The former was derived from MC3T3-E1 cells (16). Genes that supported Nmp4 occupancy were required to exhibit peaks (height ≥ 10) within-5 to+2 kb from a transcription start site (TSS) and/or located within the range defined by the TSS and the transcription end site.

Figure 2: Ingenuity Pathway Analysis of the RNA-seq data identified over 200 pathways significantly altered in the Nmp4<sup>-/-</sup> cells maintained in differentiation culture medium for 7 days (see Table S4). Here we show select canonical pathways that are sensitive to Nmp4 status and relevant to the metabolic reprogramming, protein synthesis and secretion of the bone cells. [A] The bar graphs are color-coded to reflect the z-score calculated by the IPA algorithm, which predicts the direction of change for the pathway upon loss of Nmp4. An absolute z-score of 2 or
more is considered significant. The activation state of the pathway is predicted to be increased if the z-score is $\geq 2$ and these bars are color coded with an orange hue. Conversely, bar graphs with a blue hue indicate a z-score $\leq -2$ representing canonical pathways with a decreased activity. Those pathways represented with a grey bar ($z = \text{NaN}$) indicate that the z-score algorithm cannot predict whether the pathway activity is increased or decreased in the $Nmp4^{-/-}$ cells. The orange line gives the ratio of the number of genes listed in the Nmp4 dataset over the total number of genes in the IPA annotated pathway. \([B]\) The bar graphs are color coded to reflect the percentage of genes in a particular pathway whose expressions are significantly upregulated with the loss of $Nmp4$ (red) and those genes whose expressions are attenuated in the null cells (green). The total numbers of genes comprising the canonical pathways are also indicated. The orange line gives the $-\log_{10}(p\text{-value})$ and significance was defined by $p$ value $\leq 0.05$ [or $1.30 = -\log_{10}(p\text{-value})$].

Figure 3: The IPA Molecule Activity Predictor (MAP) algorithm indicated that loss of $Nmp4$ elevates Wnt/$\beta$-catenin activity, a major driver of bone anabolism and suppressor of adipogenesis. Molecules in pink-red are found in the dataset and are upregulated. Molecules that are green are found in the dataset and are downregulated. Molecules that are grey are found in the dataset but did not pass any of the filter parameters originally established for the analysis. White molecules are not in the dataset but part of the pathway. Orange molecules and arrows predict activation whereas blue molecules and arrows predict inhibition. On the left-hand side of this pathway is a heatmap of genes comprising the Wnt/$\beta$-catenin pathway derived from the RNA-seq data of WT and $Nmp4^{-/-}$ MPSCs at Day 3 (uncommitted) and Day 7 (early osteogenesis) in culture. Red boxes indicate increased expression in the $Nmp4^{-/-}$ cells compared to the WT, with greater color saturation indicating higher expression, and green indicate reduced expression. The star $*$ indicates Nmp4 binds proximal to the transcription start.
site or within the intron of the gene as determined by ChIP-seq analysis (Childress et al., 2015).

Abbreviations for the IPA/MAP: Adenomatous polyposis coli protein (APC); B-cell lymphoma 9 (BCL9); the histone acetyl transferase (CBP); Casein kinase I (CKI); Dickkopf (Dkk); disheveled (Dsh); Glycogen synthase kinase 3β (GSK3β); and GSK3 binding protein (GBP); mitogen-activated protein kinase kinase kinase kinase 1 (Hpk1 a.k.a. Map4k1); NEMO-like kinase (NLK); retinoic acid receptor (RAR); nuclear receptor subfamily 2, group C, member 2 (Tak1 a.k.a. Nr2c2); T cell activation factor (TCF).

Figure 4: Loss of Nmp4 biases the MSPC transcriptome toward the osteogenic lineage. Heatmap of RNA-seq data from WT and Nmp4−/− MPSCs at Day 3 (uncommitted) and Day 7 (early osteogenesis) in culture. Red boxes indicate increased expression in the Nmp4−/− cells compared to the WT, with greater color saturation indicating higher expression, and green indicate reduced expression. The star * indicates Nmp4 binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (16). Also shown, IPA canonical pathways and z scores that support osteogenesis. Orange ovals indicate pathways that are predicted to be activated and whereas blue ovals predict that the pathways are inhibited.

Figure 5: Loss of Nmp4 perturbs the MSPC glycolytic pathway. [A] Nmp4−/− osteoprogenitors/osteoblasts exhibit significant elevated expression of several genes that drive glycolysis. Schematic of glycolysis/oxidative phosphorylation [OXPHOS] pathways with overlay of heatmap derived from RNA-seq data generated from WT and Nmp4−/− MPSCs harvested at Day 3 (uncommitted cells) and Day 7 (early osteogenesis). Red boxes indicate increased expression in the Nmp4−/− cells compared to the WT cells, with greater color saturation indicating higher expression, and green color indicates reduced expression. The star * indicates Nmp4 binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (16). [B] ChIP-seq reveals Nmp4 binding profiles at specific gene loci in
mouse MC3T3-E1 cells (Childress et al., 2015, GEO accession number GSE112693 for complete ChIP-Seq dataset). The Burrows-Wheeler algorithm was used to align sequences (50-nt reads, single end) to the mouse genome (mm10). Alignments were extended in silico at their 3'-ends to a length of 150bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The MACS algorithm (v1.4.2) with a cutoff of P = 1e-7 was used to determine Nmp4 (Znf384) peak locations. The genomic loci including the chromosome number and nucleotide interval are indicated. The y-axis indicates the read scales. Arrows indicate the transcriptional start sites and direction of transcription; vertical boxes within the gene indicate exons. The Nmp4 ChIP-seq gene profiles include Pdk1 Pkm. The input DNA profiles were devoid of peaks.

**Figure 6:** Loss of Nmp4 enhances glycolytic capacity. The line graphs show a comparison of WT vs. Nmp4−/− MSPC extracellular acidification rate (ECAR) profiles that have undergone the Glycolytic Stress test. [A] The MSPCs 1957RWT and 1957NKO were derived from male littermates. [B] The MSPCs 1584LWT and 1515RRKO were derived from a random pair of male WT and Nmp4−/− mice. These graphs are representative of 4 individual tissue culture experiments (biological replicates). [C] These graphs represent data from 5 separate experiments with cells from 5 different platings. In each experiment, 10 technical replicates with each cell preparation have been performed. The data are mean ± SD. Statistical significance was set at p<0.05. Glycolysis is the increase in ECAR measured after the glucose injection. This is the rate of glycolysis under basal conditions. Glycolytic capacity is the increase in ECAR after oligomycin injection. Glycolytic reserve is determined after 2-deoxy-glucose (2-DG) injection, which inhibits glycolysis. The difference between Glycolytic Capacity and Glycolysis rates defines Glycolytic Reserve.
**Figure 7:** Loss of *Nmp4* enhances mitochondrial respiratory capacity. The line graphs show a comparison of WT vs. *Nmp4*−/− MSPC oxygen consumption rate (OCR) profiles that have undergone the Mitochondrial Stress test. **[A]** The MSPCs 1957RWT and 1957NKO were derived from male littermates. **[B]** The MSPCs 1584LWT and 1515RRKO were derived from a random pair of male WT and *Nmp4*−/− mice. These graphs are representative of 5 individual tissue culture experiments (biological replicates). **[C]** These graphs represent data from 5 separate experiments with cells from 5 different platings. In each experiment, 10 technical replicates with each cell preparation have been performed. The data are mean ± SD. Statistical significance was set at p<0.05. Basal respiration (BASAL RESP) was first measured and then the cells were sequentially exposed to various inhibitors of the mitochondrial electron transport chain. ATP production (ATP PROD) was based on the comparison between the basal OCR and the oligomycin-induced drop in OCR. The subsequent injection of carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone (FCCP) uncoupled the electron transport chain increasing OCR and permitting the calculation of the maximal respiration rate (MAX RESP). Non-mitochondrial respiration was determined from the final injection of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This parameter was significantly higher in the *Nmp4*−/− cells (data not shown). Spare respiratory capacity was also significantly elevated (data not shown).

**Figure 8:** Genes involved in various aspects of protein synthesis are shown in the heatmaps positioned along this cellular process. These heatmaps were derived from RNA-seq data generated from WT and *Nmp4*−/− MPSCs harvested at Day 3 (uncommitted cells) and Day 7 (early osteogenesis). Red boxes indicate increased expression in the *Nmp4*−/− cells compared to the WT cells, with greater color saturation indicating higher expression, and green color indicates reduced expression. The star ★ indicates Nmp4 binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (16).
**Figure 9:** The IPA Molecule Activity Predictor (MAP) algorithm indicated that loss of *Nmp4* elevates protein folding and attenuates endoplasmic reticulum stress-induced apoptosis. Molecules in pink-red are found in the dataset and are upregulated. Molecules that are green are found in the dataset and are downregulated. Molecules that are grey are found in the dataset but did not pass any of the filter parameters originally established for the analysis. White molecules are not in the dataset but part of the pathway. On the right-hand side of this pathway is a heatmap of genes comprising the unfolded protein response pathway (UPR) derived from the RNA-seq data of WT and *Nmp4⁻/⁻* MPSCs at Day 3 (uncommitted) and Day 7 (early osteogenesis) in culture. Red boxes indicate increased expression in the *Nmp4⁻/⁻* cells compared to the WT, with greater color saturation indicating higher expression, and green indicate reduced expression. The star * indicates Nmp4 binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis. Abbreviations for the IPA/MAP: Autocrine motility factor receptor (AMFR); ER-degradation-enhancing-α-mannidose-like protein (EDEM); ER-associated protein degradation (ERAD); Membrane bound transcription factor peptidase (MBTPS); protein disulfide isomerase (PDI); SREBF chaperone (SCAP); Valosin-containing protein (VCP).

**Figure:** Loss of *Nmp4* enhances collagen expression and secretion. Data were derived from MSPC preparations 1584L<sup>WT</sup>, 1515RR<sup>KO</sup>, 1957<sup>WT</sup>, 1957<sup>KO</sup> [A] Polysome profiles of lysates prepared from WT and *Nmp4⁻/⁻* MSPCs at 4 days in culture. Representative profiles from 3 biological replicates [B] *Col1a1* mRNA expression as determine by RNA-seq in MSPCs maintained in non-differentiation medium for 3 days in culture and 7 days in culture (5 days in osteogenic medium). [C] Following polysome profiling, fractions 1-7 were collected, and the percentage of *Col1a1* mRNA present in each sucrose gradient fraction were quantified by qRT-PCR and presented as a histogram. Data is representative of 2 biological replicates and 3
technical replicates each. Statistical analyses were performed using 1W ANOVA tests and asterisks\(***\) was equivalent to \(p<0.0001\). [D] Secretion of collagen protein was measured in the acid-soluble cell-matrix layer of 1584L\(^{WT}\) and 1515RR\(^{KO}\) by using the Sircol Assay as described in Materials and Methods. Loss of \(Nmp4\) significantly enhanced the amount of collagen protein secreted/cell, * \(p<0.0001\). Data represents 3 biological replicates and 5-6 technical replicates each. [E] Secretion of collagen protein was measured in the acid-soluble cell-matrix layer by using the Sircol Assay at Day 4 post-seeding from all MSPC preparations 1584L\(^{WT}\), 1957R\(^{WT}\), 2001RL\(^{WT}\), 1515RR\(^{KO}\), 1957N\(^{KO}\), and 1986R\(^{KO}\) and presented as Collagen/well [\(\mu\)g] vs. cell number/well. All six preparations were tested independently at least twice (Experiments 1 & 2) and experiments comprised 4-6 wells/preparation. All \(Nmp4^{-/-}\) [KO] preparations produced more collagen during the first four days of culture, regardless of cell number. Data represents average ± SD, \(n=4-6\) wells/group.

**Figure 11:** \(Nmp4^{-/-}\) osteoprogenitors/osteoblasts exhibit significant elevated expression of several genes that encode proteins of the bone matrix. The schematic shows family of proteins that comprise the bone matrix. Also the expressions of key genes that control mineralization were altered in the \(Nmp4^{-/-}\) cells consistent with the observed phenotype observed in culture. The manually annotated heatmap was derived from RNA-seq data generated from WT and \(Nmp4^{-/-}\) MPSCs harvested at Day 3 (uncommitted cells) and Day 7 (early osteogenesis). Red boxes indicate increased expression in the \(Nmp4^{-/-}\) cells compared to the WT cells, with greater color saturation indicating higher expression, and green color indicates reduced expression. The star \(\ast\) indicates \(Nmp4\) binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (16).

**Figure 12:** Loss of \(Nmp4\) improves enhances therapeutically induced bone formation and femoral material properties. [A] Femoral and [B] L5 vertebral BV/TV for all the experimental
cohorts (age 17wks) comparing WT and Nmp4−/− mice. We compared the therapies RAL, PTH, and PTH+RAL to each other and to VEH. Statistical analyses were performed using 2W ANOVA tests setting genotype and treatment as the independent variables. Statistical significance was set at p≤0.05. There were a strong genotype effect and loss of Nmp4 enhanced femoral and L5 vertebral BV/TV over the cohorts. There was a strong treatment effect and PTH+RAL was the most efficacious osteoanabolic therapy for both femoral and L5 vertebral BV/TV. The analysis revealed a genotype x treatment interaction (G x T denoted by an asterisk in the dot plot showing improved response in the PTH mono-therapy and PTH+RAL combination therapy with loss of Nmp4. Results of 3pt-bending analysis for [C] Ultimate stress [D] Yield stress. There were strong genotype and treatment effects for both ultimate stress and yield stress. Data represents average ± SD, n=8-15 mice/group.

**Figure 13:** Hypothesis—Nmp4 is an apex regulator of bone cell anabolic output. This transcription factor directly and indirectly regulates gene programs that control key stages of matrix production and delivery. It may accomplish this by regulating both the expression of master transcriptional regulators of these pathways in addition to broadly engaging several of their downstream target genes.
### TABLE 1: FEMORAL AND L5 TRABECULAR ARCHITECTURE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Femur BV/TV [%]</th>
<th>Femur Tb N (mm⁻³)</th>
<th>Femur Tb Th (mm)</th>
<th>Femur Tb Sp (mm)</th>
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</thead>
<tbody>
<tr>
<td>WT VEH</td>
<td>4.57±0.83</td>
<td>0.970±0.163</td>
<td>0.047±0.003</td>
<td>0.282±0.013</td>
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<tr>
<td>Nmp4⁻ VEH</td>
<td>6.53±0.93</td>
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<td>WT RAL</td>
<td>11.13±1.03</td>
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<tr>
<td>Nmp4⁻ RAL</td>
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<tr>
<td>WT PTH</td>
<td>13.60±3.39</td>
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<td>0.230±0.020</td>
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<tr>
<td>Nmp4⁻ PTH</td>
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<td>0.076±0.008</td>
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<tr>
<td>WT PTH+RAL</td>
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<tr>
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#### 2W ANOVA

**GENOTYPE**

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<td>P:</td>
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**2W ANOVA**

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**GROUP**

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<th>L5 Tb Th (mm)</th>
<th>L5 Tb Sp (mm)</th>
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<tr>
<td>WT VEH</td>
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<td>0.056±0.002</td>
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<tr>
<td>Nmp4⁻ VEH</td>
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<tr>
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<tr>
<td>Nmp4⁻ RAL</td>
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<td>WT PTH+RAL</td>
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<tr>
<td>Nmp4⁻ PTH+RAL</td>
<td>55.76±3.02</td>
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**2W ANOVA**

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<td>Nmp4⁻ V:</td>
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**GROUP**

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**2W ANOVA**

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<td>Nmp4⁻ P+R:</td>
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<td>Nmp4⁻ V:</td>
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TABLE 1: Femoral and L5 trabecular architecture from WT and Nmp4<sup>−/−</sup> mice treated with vehicle (V), raloxifene (R), parathyroid hormone (P), and parathyroid hormone + raloxifene (P+R). Statistical analyses were performed using 2W ANOVA tests setting genotype (G) and treatment (T) as the independent variables. Statistical significance was set at p≤0.05. The statistical results list the cohorts by genotype, treatment, and genotype x treatment. Cohorts not connected by the same letter are statistically different. The average value of the specific parameter follows the letter. The data represents average ± SD, n=8-15 mice/group. See text for explanation of results.
<table>
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**TABLE 2**: Identification of synergy between PTH and the anti-catabolic SERM RAL using a series of 2 way ANOVA tests comparing the efficacy of the PTH mono-therapy, RAL mono-therapy and the combination of the two drugs. Statistical significance was set at p≤0.05.
## TABLE 3: Femoral Cortical Parameters

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<th>GROUP</th>
<th>Marrow Area (mm²)</th>
<th>Cortical Area (mm²)</th>
<th>Cortical Thickness (mm)</th>
<th>Periosteal BS (mm)</th>
<th>Endocortical BS (mm)</th>
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<tbody>
<tr>
<td>WT VEH</td>
<td>0.940±0.049</td>
<td>0.829±0.052</td>
<td>0.204±0.008</td>
<td>5.393±0.139</td>
<td>4.140±0.114</td>
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<tr>
<td>Nmp4− VEH</td>
<td>0.913±0.049</td>
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<td>5.353±0.090</td>
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<td>WT RAL</td>
<td>0.860±0.022</td>
<td>0.867±0.037</td>
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<td>Nmp4− RAL</td>
<td>0.879±0.039</td>
<td>0.865±0.047</td>
<td>0.218±0.010</td>
<td>5.332±0.111</td>
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<td>WT PTH</td>
<td>0.969±0.048</td>
<td>0.949±0.078</td>
<td>0.221±0.005</td>
<td>5.617±0.196</td>
<td>4.213±0.108</td>
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<td>Nmp4− PTH</td>
<td>0.931±0.064</td>
<td>0.951±0.062</td>
<td>0.226±0.007</td>
<td>5.564±0.087</td>
<td>4.142±0.116</td>
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<td>5.552±0.152</td>
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2W ANOVA

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<tr>
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2W ANOVA

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<th>G x T: p=0.8513</th>
<th>G x T: p=0.4973</th>
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## TABLE 3: Femoral cortical architecture from WT and Nmp4−/− mice treated with vehicle (V), raloxifene (R), parathyroid hormone (P), and parathyroid hormone + raloxifene (P+R). Statistical analyses were performed using 2W ANOVA tests setting genotype (G) and treatment (T) as the independent variables. Statistical significance was set at p≤0.05. The statistical results list the cohorts by genotype, treatment, and genotype x treatment. Cohorts not connected by the same letter are statistically different. The average value of the specific parameter follows the letter. The data represents average ± SD, n=7-15 mice/group. See text for explanation of results. ABBREVIATIONS: HA hydroxyapatite; Iap moment of inertia about the femoral anterior–posterior length axis; Imax maximum moment of inertia; Imin minimum moment of inertia; TMD tissue mineral density.
### TABLE 4: Estimated femoral material properties from WT and Nmp4⁻/⁻ mice treated with vehicle (V), raloxifene (R), parathyroid hormone (P), and parathyroid hormone + raloxifene (P+R). Statistical analyses were performed using 2W ANOVA tests setting genotype (G) and treatment (T) as the independent variables. Statistical significance was set at p≤0.05. The statistical results list the cohorts by genotype, treatment, and genotype x treatment. Cohorts not connected by the same letter are statistically different. The average value of the specific parameter follows the letter. The data represents average ± SD, n=7-14 mice/group.

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<th>GROUP</th>
<th>Ultimate Stress (MPa)</th>
<th>Yield Stress (MPa)</th>
<th>Strain to Yield (µε)</th>
<th>Total Strain (µε)</th>
<th>Modulus (GPa)</th>
<th>Resilience (MPa)</th>
<th>Toughness (MPa)</th>
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<tr>
<td>WT VEH</td>
<td>141.12±6.97</td>
<td>105.73±12.89</td>
<td>16012±1124</td>
<td>99029±30556</td>
<td>7.87±0.30</td>
<td>0.95±0.18</td>
<td>9.19±1.93</td>
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<tr>
<td>Nmp4⁻ VEH</td>
<td>150.08±8.71</td>
<td>121.23±21.47</td>
<td>16982±2858</td>
<td>82376±26470</td>
<td>8.55±0.54</td>
<td>1.08±0.31</td>
<td>8.86±2.59</td>
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<tr>
<td>WT RAL</td>
<td>155.49±5.00</td>
<td>117.92±5.13</td>
<td>16552±371</td>
<td>83863±19659</td>
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<td>1.06±0.12</td>
<td>9.15±1.86</td>
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<tr>
<td>Nmp4⁻ RAL</td>
<td>159.48±13.63</td>
<td>127.00±22.37</td>
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<td>75610±19393</td>
<td>8.24±1.13</td>
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<td>8.95±2.10</td>
</tr>
<tr>
<td>WT PTH</td>
<td>152.07±4.20</td>
<td>97.09±15.56</td>
<td>16881±3008</td>
<td>90210±29366</td>
<td>7.57±1.23</td>
<td>0.85±0.28</td>
<td>9.46±2.63</td>
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<tr>
<td>Nmp4⁻ PTH</td>
<td>157.94±5.37</td>
<td>97.77±9.56</td>
<td>15021±1932</td>
<td>91519±26401</td>
<td>7.93±0.30</td>
<td>0.82±0.23</td>
<td>10.31±2.28</td>
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<tr>
<td>WT PTH+RAL</td>
<td>166.39±3.78</td>
<td>102.76±18.16</td>
<td>15452±34324</td>
<td>69998±19460</td>
<td>8.44±0.38</td>
<td>0.89±0.32</td>
<td>8.16±1.90</td>
</tr>
<tr>
<td>Nmp4⁻ PTH+RAL</td>
<td>173.39±10.84</td>
<td>114.52±13.66</td>
<td>15321±708</td>
<td>63635±16321</td>
<td>8.64±1.06</td>
<td>0.99±0.20</td>
<td>8.09±1.91</td>
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**2W ANOVA**

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| G: p=0.0012 | G: p=0.0109 | G: p=0.0559 | G: p=0.1304 | G: p=0.8913 |
| Nmp4⁻: A 160.22 | Nmp4⁻: A 115.13 | WT: B 105.87 | WT: B 105.87 | |

**TREATMENT**

| T: p<0.0001 | T: p<0.0001 | T: p<0.0001 | T: p<0.0001 | T: p<0.0001 |
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| R: B 157.48 | R: B 157.48 | V: AB 90704 | V: AB 90704 | V: AB 90704 |
| P: B 157.48 | P: B 157.48 | P+R: BC 108.64 | P+R: BC 108.64 | P+R: BC 108.64 |
| V: C 145.60 | V: C 145.60 | P: C 97.43 | P: C 97.43 | P: C 97.43 |

**G x T**

| G x T: p=0.8141 | G x T: p=0.5067 | G x T: p=0.6425 | G x T: p=0.7598 | G x T: p=0.6545 | G x T: p=0.7901 |
| G x T: p=0.0001 | G x T: p=0.0001 | G x T: p=0.0001 | G x T: p=0.0001 | G x T: p=0.0001 | G x T: p=0.0001 |
### TABLE 5: STRUCTURAL MECHANICAL PROPERTIES

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<th>Displacement to yield (µm)</th>
<th>Post yield displacement (µm)</th>
<th>Total displacement (µm)</th>
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<tr>
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<td>10.08±0.99</td>
<td>13.52±1.16</td>
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<td>11.65±2.44</td>
<td>14.27±0.92</td>
<td>172.64±26.99</td>
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<td>WT RAL</td>
<td>11.19±0.20</td>
<td>14.67±0.41</td>
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<td>15.22±1.20</td>
<td>15.22±1.20</td>
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<td>11.24±1.79</td>
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#### 2W ANOVA

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<td>G: p=0.0137</td>
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<tr>
<td>R:</td>
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<th>Total Work (mJ)</th>
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<td>8.64±2.63</td>
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<tr>
<td>WT RAL</td>
<td>106.07±12.65</td>
<td>1.05±0.08</td>
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<td>9.15±1.90</td>
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<td>Nmp4&lt;sup&gt;−&lt;/sup&gt; RAL</td>
<td>105.42±16.10</td>
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<tr>
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<td>130.49±10.66</td>
<td>0.92±0.29</td>
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<tr>
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#### 2W ANOVA

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**GROUP**

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<th>Work to Yield (mJ)</th>
<th>Post Yield Work (mJ)</th>
<th>Total Work (mJ)</th>
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<td>104.39±10.17</td>
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<td>8.64±2.63</td>
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<td>WT RAL</td>
<td>106.07±12.65</td>
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<td>Nmp4&lt;sup&gt;−&lt;/sup&gt; RAL</td>
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<td>1.19±0.34</td>
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<td>WT PTH</td>
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<td>10.38±2.76</td>
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<td>121.91±16.51</td>
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<td>10.24±2.81</td>
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<td>1.25±0.45</td>
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<td>8.94±2.45</td>
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</table>

**TABLE 5:** Estimated femoral structural mechanical properties from WT and Nmp4<sup>−</sup> mice treated with vehicle (V), raloxifene (R), parathyroid hormone (P), and parathyroid hormone + raloxifene (P+R). Statistical analyses were performed using 2W ANOVA tests setting genotype (G) and treatment (T) as the independent variables. Statistical significance was set at p≤0.05. The statistical results list the cohorts by genotype, treatment, and genotype x treatment. Cohorts not connected by the same letter are statistically different. The average value of the specific parameter follows the letter. The data represents average ± SD, n=7-14 mice/group.
REFERENCES:


44. Ishitani T, Ninomiya-Tsuji J, and Matsumoto K. Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-


**Loss of Nmp4 optimizes osteogenic metabolism and secretion to enhance bone quality**

Yu Shao¹, Emily Wichern², Paul J. Childress³, Michele Adaway², Jagannath Misra⁴, Angela Klunk², David B. Burr², Ronald C. Wek⁴, Amber L. Mosley⁴, Yunlong Liu¹, Alexander G. Robling², Nickolay Brustovetsky⁵, James Hamilton⁵, Kylie Jacobs⁶, Deepak Vashishth⁷, Keith R. Stayrook⁸, Matthew R. Allen², ⁹, Joseph M. Wallace³, ¹⁰¶ Joseph P Bidwell¹, ²¶

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⁴. Department of Biochemistry & Molecular Biology, IUSM
⁵. Department of Pharmacology & Toxicology, IUSM
⁶. Department of Microbiology & Immunology, IUSM
⁷. Center for Biotechnology & Interdisciplinary Studies (Rm 2213) and Department of Biomedical Engineering, Rensselaer Polytechnic Institute, 110 8th Street, Troy, NY 12180, USA
⁸. Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46202
⁹. Roudebush Veterans Administration Medical Center, Indianapolis, IN
¹⁰. Department of Biomedical Engineering, Indiana University-Purdue University at Indianapolis, IN, 46202

**List of Materials Included:** [https://figshare.com/s/aef3382cdc7c02151e6f](https://figshare.com/s/aef3382cdc7c02151e6f)

**Supplemental Table S1:** ChIP-seq data, located in separate xlsx file
**Supplemental Table S2:** RNA-seq data, located in separate xlsx file
**Supplemental Table S3:** Day 3 IPA canonical pathways, located in separate xls file
**Supplemental Table S4:** Day 7 IPA canonical pathways, located in separate xls file

NOTE: The following are the legends/description for the Supplemental Tables S1-S4.
Supplemental Table S1: ChIP-seq data, located in separate xlsx file GEO accession number GSE112693 for complete ChIP-Seq dataset

Nmp4 occupancy sites in MC3T3-E1 osteoblast-like cells as determined by ChIP-seq analysis (Childress et al., 2015). Peaks are mapped to mouse genome build mm10. Column A: gene IDs from Ensembl genes, UCSC genes, etc; Column B: gene symbol; Column C: strand; Column D: chromosome; Column E: location of transcription start site (TSS); Column F: location of transcription end site (TES); Column G: Location of Nmp4 in Zones 1-4. A peak was assigned to a promoter region if it was within -5 to +2 kb from a transcription start site (TSS, Zone 1). The Nmp4 peak was assigned to Zone 2, the intragenic region, if it was located within the range defined by the TSS and the transcription end site, and not within the promoter range of the same gene. To assign a peak to Zone 3, the intergenic region, it had to be -10 000 kb from the TSS and +10 000 kb from the transcription end site, and not within the promoter range of the same gene. Peaks that did not fit any of these definitions were assigned to the classification “other” (Zone 4). A peak could be assigned to multiple functional regions in an area of the genome harboring multiple genes. Note for Nmp4 gene occupancy we used genes identified in Zones 1 & 2 and listed in Supplemental Table 2. Column H: Peak_start, the recorded peak start position; Column I: Peak_end: the recorded peak end position; Column J: Peak_position, the middle point position of a peak; Column K: Peak_value, the peak score. This parameter is the measurement of overall (usually average) enrichment for the region.
Supplemental Table S2: RNA-seq data, located in separate xlsx file [GEO accession number GSE112694] Expression of all genes was normalized based on the expression of Gusb (see Materials and Methods). The columns are defined as follows (also see file)

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<td>log2 fold change</td>
</tr>
<tr>
<td>WT.day7_vs_WT.day3_PValue</td>
<td>p value</td>
</tr>
<tr>
<td>WT.day7_vs_WT.day3_FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>KO.day7_vs_KO.day3_logFC</td>
<td>log2 fold change</td>
</tr>
<tr>
<td>KO.day7_vs_KO.day3_PValue</td>
<td>p value</td>
</tr>
<tr>
<td>KO.day7_vs_KO.day3_FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>KO-day7/WT-day7_vs_KO-day3/WT-day3_logFC</td>
<td>log2 fold change</td>
</tr>
<tr>
<td>KO-day7/WT-day7_vs_KO-day3/WT-day3_PValue</td>
<td>p value</td>
</tr>
<tr>
<td>KO-day7/WT-day7_vs_KO-day3/WT-day3_FDR</td>
<td>false discovery rate</td>
</tr>
</tbody>
</table>

*KO/day7_versus WT/day7

- KO.day3_versus WT.day3
  - FDR: false discovery rate
  - PV: p value
  - logFC: log2 fold change
- KO.day7_versus WT.day3
  - FDR: false discovery rate
- KO.day3_versus WT.day3
  - FDR: false discovery rate
- KO.day3_versus WT.day7
  - FDR: false discovery rate

**Materials and Methods.** The columns are defined as follows (also see file):

- Chr: chromosome
- Start: start position of exons
- End: end position of exons
- Strand: strand
- Length: gene length
- KO.day3_vs_WT.day3_logFC: log2 fold change
- KO.day3_vs_WT.day3_PValue: p value
- KO.day3_vs_WT.day3_FDR: false discovery rate
- KO.day7_vs_WT.day7_logFC: log2 fold change
- KO.day7_vs_WT.day7_PValue: p value
- KO.day7_vs_WT.day7_FDR: false discovery rate
- WT.day7_vs_WT.day3_logFC: log2 fold change
- WT.day7_vs_WT.day3_PValue: p value
- WT.day7_vs_WT.day3_FDR: false discovery rate
- KO.day7_vs_KO.day3_logFC: log2 fold change
- KO.day7_vs_KO.day3_PValue: p value
- KO.day7_vs_KO.day3_FDR: false discovery rate
- KO.day7/WT-day7_vs_KO-day3/WT-day3_logFC: log2 fold change
- KO-day7/WT-day7_vs_KO-day3/WT-day3_PValue: p value
- KO-day7/WT-day7_vs_KO-day3/WT-day3_FDR: false discovery rate

*KO/day7_versus WT/day7

- KO.day3_versus WT.day3
  - FDR: false discovery rate
  - PV: p value
  - logFC: log2 fold change
- KO.day7_versus WT.day3
  - FDR: false discovery rate
- KO.day3_versus WT.day7
  - FDR: false discovery rate
- KO.day3_versus WT.day7
  - FDR: false discovery rate

**Expression of all genes was normalized based on the expression of Gusb (see Supplemental Table S2: RNA-seq data, located in separate xlsx file [GEO accession number GSE112694]).**
**Supplemental Table S3:** Day 3 IPA canonical pathways, located in separate xls file

IPA Canonical pathways perturbed by loss of *Nmp4* in MPSCs harvested at Day 3 in culture. Pathways were identified as significantly sensitive to *Nmp4* status that achieved a value of – \log(p-value) ≥ 1.30.

- Column A: identity of the canonical pathway;
- Column B: –\log(p-value);
- Column C: Ratio, the number of genes listed in the dataset over the total number of genes in the pathway.
- Column D z-score of pathway. The activation state of the pathway is predicted to be increased if the z-score is ≥ 2 and attenuated if the z-score ≤ –2. Those pathways listed as #NUM indicate that the z-score algorithm cannot predict whether the pathway activity is increased or decreased in the *Nmp4*-/− cells.
- Column E: molecules in the dataset belonging to pathway.

**Supplemental Table S4:** Day 7 IPA canonical pathways, located in separate xls file

IPA Canonical pathways perturbed by loss of *Nmp4* in MPSCs harvested at Day 7 in culture. Pathways were identified as significantly sensitive to *Nmp4* status that achieved a value of – \log(p-value) ≥ 1.30. See **Supplemental Table S3** legend for identity of table columns.
A. Initial mineralization (post-seeding of WT & Nmp4-/- MSPCs)

B. Volcano plots
Day 3 & Day 7 in culture (RNA-seq)

C. Venn diagrams
Day 3 & Day 7 ChIP-seq vs RNA-seq
Extracellular space

Cytoplasm

Nucleus

Heatmap Legend

LogFC

+10

+5

0

-5

Day 3 Day 7

Ratio of Nmp4-/WT mRNA Expression (RNA-seq)

Gene

Day 3 Day 7 in culture

Direct Nmp4 gene target (ChIP-seq)
IPA canonical signaling pathways

Cell fate/differentiation
Transcription factors

Day 3    Day 7

★Tcf4
Sox4
★Ddit3
★Atf4
★Runx2
★Satb2
Sp7
Msx2
★Sox6
★Sox9
Zfp219
★Sox5
Cebpβ
Med1
Add1
Srebf1
★Ebf1
★Ppary
Cebpa
★Zfp423

Chondrogenesis
Adipogenesis

IPA Canonical Pathways Supporting Osteogenesis

MSPC
Osteogenic Commitment
Proliferation Early Differentiation
Differentiation

NaN=no prediction

IPA z score ≤ 2 → activity

Log FC
Ratio of Nmp4^WT mRNA Expression
Gene
Day 3     Day 7
in culture
Direct Nmp4 gene target (ChIP-seq)
A. RNA-seq heatmap/schematic of glycolytic pathway

B. Nmp4 ChIP-seq profiles
A Glycolytic stress test for $1957N^{\text{KO}}$ vs. $1957R^{\text{WT}}$

B Glycolytic stress test for $1515R^{\text{KO}}$ vs. $1584L^{\text{WT}}$

C Statistical analysis of combined data

**GLYCOLYSIS**

**GLYCOLYTIC CAPACITY**

**GLYCOLYTIC RESERVE**
A Mito stress test for $1957N^{KO}$ vs. $1957R^{WT}$

B Mito stress test for $1515RR^{KO}$ vs. $1584L^{WT}$

C Statistical analysis of combined data
Extracellular space

Day 3
- Extracellular space
- CYTOPLASM
- ENDOPLASMIC RETICULUM

Day 7
- Extracellular space
- CYTOPLASM
- ENDOPLASMIC RETICULUM

Gene
- Direct Nmp4 gene target (ChIP-seq)

Ratio of Nmp4−/−:WT mRNA Expression (RNA-seq)

Heatmap Legend

Day 3 Day 7 in culture
- LogFC
  +5 0

Heatmap

Predicted relationships:
- Green: increased expression
- Red: decreased expression
- Orange: decreased expression with shift of downstream marker
- Gray: not predicted
A. Total polyribosome profiling of MSPCs

B. Col1a1 mRNA expression

C. Col1a1 mRNA association with MSPC polyribosome [Day 4]

D. MSPC collagen protein secretion

E. MSPC collagen secretion and proliferation activity
1. Loss of Nmp4 alters the expression of multiple ECM genes which may change matrix composition.

2. Loss of Nmp4 alters the expression of multiple genes that regulate mineralization.

**Promote Mineralization**
- Slc20a1
- Clcn3
- Iftm5

**Inhibit Mineralization**
- Igfbp4
- Ecm1

**Heatmap Legend**
- LogFC
- Ratio of Nmp4⁺⁺:WT mRNA Expression (RNA-seq)
- Day 3 vs Day 7 in culture

**Gene Helper**
- Direct Nmp4 gene target (ChIP-seq)

**ECM Genes**
- Col1a1
- Col1a2
- Bgn
- Sparc
- Spp1
- Fbn1

**ECM Proteins Supporting Bone Mechanical Properties**
- Col1a1
- Col1a2
- Bgn
- Sparc
- Fbn1

**Glia-containing proteins**
- Mgp
- Tnni3

**Other RGD-containing glycoproteins**
- Thbs1
- Thbs2
- Thbs3
-Fn1
- Fbn1

**Collagenous proteins**
- Col2a1
- Col3a1
- Col5a1
- Col5a2
- Col6a1

**Glycosylated proteins**
- Vcan
- Aspn
- Omd
- Hspg2
- Deu
- Fmod
- Ltm

**Proteoglycans**
- Tnc
- Bgal2
- Procn
- Bgn
- Spp1
- Sparc
- Fbn2

**Cytosol**

**Nucleus**

**ECM**

**Bone Matrix**
KEY PATHWAYS OF THE Nmp4 ANTI-ANABOLIC BONE AXIS

- Transporters for Glutamine, Glucose, & Amino Acids
- Bioenergetics
  - Biosynthesis
  - Fuels
  - Proliferation
  - Secretome Synthesis
- PTH Responsiveness
- Redox Maintenance
- Secretome Delivery
  - Expanding ER Capacity (UPR)

- mTORC1
- mTOR
- Raptor
- mSRE1
- tRNA charging
- Ribosome biogenesis
- Protein Synthesis
- Amino acid Biosynthesis
- Aerobic Glycolysis
- TCA cycle

Nmp4 Apex Regulator
Key Nmp4 Targets
- c-Myc, Gadd34, Atf4, Ddit3, Pdk1, Hk2, Hif1α, Asns
- Metabolic reprogramming program
- Transporter program
- Ribosome biogenesis program
- UPR program
- Amino acid synthesis program