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TITLE: MAXIMIZING PTH ANABOLIC OSTEOPOROSIS THERAPY

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14. ABSTRACT The purpose of this study is to test the efficacy of parathyroid hormone (PTH) mono-therapy and PTH+ anti-catabolic combination therapies on <i>Nmp4</i>^{-/-} and wild type (WT) mice. The scope of the research comprises the following specific aims: (i & ii) to determine the impact of <i>Nmp4</i> on the efficacy of PTH mono- and combination therapies with various anti-catabolics in ovariectomized (ovx) mice; (iii) to determine the cell type-specific contributions to the enhanced response of the <i>Nmp4</i>^{-/-} mouse to osteoporosis therapies. The most significant findings during the entire study include the following ➤ Loss of <i>Nmp4</i> improved bone response to PTH combination therapies including PTH+RAL and PTH+ZOL ➤ Loss of <i>Nmp4</i> improved bone response to the anti-catabolic drug RAL ➤ Estrogen was not required for the enhanced PTH-induced bone formation in <i>Nmp4</i>^{-/-} mice but these animals were not protected from ovx-induced bone loss without therapeutic intervention ➤ Disabling <i>Nmp4</i> enhanced bone quality ➤ Loss of <i>Nmp4</i> enhanced bone formation but did not impact resorption under osteoporosis therapies. ➤ The osteoprogenitor/osteoblast appears to be a key driver of the <i>Nmp4</i>^{-/-} hyper-anabolic bone phenotype. ➤ Disabling <i>Nmp4</i> converts the osteoblast into a super-secretor by metabolically reprogramming the cell to support the concomitant elevated matrix production and expansion of the cell's delivery capacity.					
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INTRODUCTION: The **subject of this research** is the need for improved osteoporosis therapies. The **study purpose** is to test the efficacy of parathyroid hormone (PTH) mono-therapy and PTH+anti-catabolic combination therapies on ovariectomized *Nmp4*^{-/-} and wild type (WT) mice. We previously determined that *Nmp4* represses bone response to osteoanabolics¹⁻³. The **research scope** comprises the following goals: **Specific Aims #1 & #2**, determine *Nmp4* impact on the efficacy of PTH mono- and combination therapies with bisphosphonates and raloxifene; **Specific Aim #3**, to determine the cell type-specific contributions to the enhanced response of the *Nmp4*^{-/-} mouse to these osteoporosis therapies.

This **revised final report** briefly summarizes the experiments, rationale, & results for the tasks of the Statement of Work (SOW). *This final report will be organized by the Specific Aims and indicate the SOW tasks covered by that part of the project.*

BODY:

SPECIFIC AIM #1:

- **EXPERIMENTS:** The primary goal of Specific Aim #1 was to determine the impact of *Nmp4* on the duration/magnitude of the PTH anabolic window during osteoporosis PTH combination therapy with bisphosphonates. Ovariectomized (ovx) WT and *Nmp4*-KO^{global} animals were treated with the following therapies:
 - Daily administration of PTH in ovx WT and *Nmp4*^{-/-} mice
 - Daily co-administration of PTH and alendronate (ALN)
 - A single dose of zoledronate (ZOL) followed by daily PTH.
 - Sham-operated mice were used for baseline phenotype analysis. Therapy started at 4wks post-op ovx and bone restoration was evaluated for 8wks by standard parameters.
- **RATIONALE:** The following key questions underlie this Specific Aim rationale:
 - Is estrogen required for the PTH-induced *Nmp4*^{-/-} hyper-anabolic bone phenotype¹⁻³?
 - Does loss of *Nmp4* protect mice from ovx-induced bone loss?
 - Do *Nmp4*^{-/-} mice exhibit an enhanced response to ALN or ZOL mono-therapies?
 - Are PTH+bisphosphonate combination therapies more efficacious in *Nmp4*^{-/-} mice?
- **SUMMARY OF KEY RESULTS:**
 - ***Nmp4*^{-/-} mice were not protected from ovx-induced bone loss**
 - **SOW Subtask 2.1:** Both WT and *Nmp4*^{-/-} mice exhibited significant bone loss 4wks after ovx surgery as measured in the trabecular bone compartment of the distal femur and the L5 vertebra [see Table 1, Childress et al.,⁴].
 - **SOW Subtask 2.1:** The *Nmp4*^{-/-} mice exhibited a trend towards enhanced loss of bone that neared significance in the distal femur and reached significance in the L5 vertebra Table 1, Childress et al.,⁴). However, *Nmp4*^{-/-} animals maintained more trabecular bone compared to WT mice during the first 4wks after ovariectomy.
 - **Key Conclusion:** *Nmp4* is not a primary regulator of bone resorption.
 - **Ovx did not diminish the enhanced PTH-induced bone formation in *Nmp4*^{-/-} mice.**
 - **SOW Subtasks 2.1-2.2:** Disabling *Nmp4* enhanced PTH restorative therapy in the distal femur of ovx *Nmp4*^{-/-} mice as measured by BV/TV [Figures 2A & 2B, Table 2, Childress et al., 2015⁴; Figures 2A-2C, Figure 3C, Table 2, Shao et al., 2017⁵]
 - **SOW Subtasks 2.1-2.2:** The exaggerated response to anabolic PTH persisted in the L5 vertebra of ovx *Nmp4*^{-/-} mice [Figures 3A & 3B, Childress et al., 2015⁴]. However, in a second experiment there was trend to enhanced L5 response in the *Nmp4*^{-/-} mice but it did not achieve significance [Figures 2D-2F, Shao et al., 2017⁵]. This may have been the result of the differences in the number of cohorts being compared involving multiple statistical comparisons employed in this latter study.
 - **Key Conclusion:** Estrogen is not required for the enhanced PTH-induced bone formation in *Nmp4*^{-/-} mice.
 - **Loss of *Nmp4* did not enhance the response to ALN or ZOL mono-therapies**
 - **SOW Subtask 2.1-2.2:** Disabling *Nmp4* did not enhance ALN or ZOL restorative therapies in the distal femur or the L5 vertebra [Figures 2A-2F, Tables 5 & 7 Shao et al., 2017⁵].
 - **Key Conclusion:** *Nmp4* does not regulate the skeletal response to the anti-catabolic bisphosphonate mono-therapies

- **Loss of *Nmp4* significantly improved the restorative response of ovx mice to the combination treatments of PTH+ZOL but not PTH+ALN**
 - *SOW Task 1 & 2; Subtask 2.1 & 2.2:* ovx *Nmp4*^{-/-} mice showed an improved response to PTH+ZOL but not PTH+ALN in the distal femur [Figures 2A-2C, Table 2, Shao et al., 2017⁵].
 - *SOW Task 1 & 2; Subtask 2.1 & 2.2:* The PTH+ZOL therapy showed a significant improved efficacy in L5 bone gain compared to the PTH mono-therapy in both WT and *Nmp4*^{-/-} mice [Figures 2D-2F, Table 2, Shao et al., 2017⁵].
 - **Key Conclusion:** Loss of *Nmp4* distinguishes the interactions between PTH and the bisphosphonates ZOL and ALN

SPECIFIC AIM #2:

- **EXPERIMENTS:** The primary goal of Specific Aim #2 was to determine the impact of *Nmp4* on the PTH anabolic window during osteoporosis combination therapy with the milder and distinctive acting anti-catabolic selective estrogen receptor modulator (SERM) raloxifene (RAL).
 - OvX WT and *Nmp4*^{-/-} mice were treated daily with co-administration of PTH and RAL using the same treatment regimen outlined in Specific Aim #1.
- **RATIONALE:** The following key questions underlie this Specific Aim rationale:
 - The anti-catabolic activity of RAL has a mechanism distinct from bisphosphonates⁶, and RAL has tonic effects on osteogenic cells^{7,8}. Do *Nmp4*^{-/-} mice exhibit a response to RAL distinct from bisphosphonates?
- **SUMMARY OF KEY RESULTS:**
 - **Loss of *Nmp4* improved bone response to RAL mono-therapy**
 - *SOW Subtask 3.1-3.5:* Disabling *Nmp4* enhanced PTH restorative therapy in the distal femur of ovx *Nmp4*^{-/-} mice [Figures 2A-2C, Figure 3, Table 5, Shao et al., 2017⁵].
 - *SOW Subtask 3.1-3.5:* Disabling *Nmp4* did not enhance RAL restorative therapy in the L5 vertebra [Figures 2D-2F, Figure 4, Table 7, Shao et al., 2017⁵].
 - **Key Conclusion:** This was an unexpected but welcome result inasmuch as these data suggest that *Nmp4* regulates the skeletal response to the SERMs.
 - **Loss of *Nmp4* significantly improved the restorative response of ovx mice to the combination treatments of PTH+RAL**
 - *SOW Subtask 3.1-3.5, 4.2, 4.2.a:* ovx *Nmp4*^{-/-} mice showed an improved response to PTH+RAL in the distal femur [Figures 2A-2C, Figure 3, Table 2, Shao et al., 2017⁵].
 - *SOW Subtask 3.1-3.5, 4.2, 4.2.a:* The PTH+RAL therapy showed a significant improved efficacy in L5 bone gain compared to the PTH mono-therapy in both WT and *Nmp4*^{-/-} mice [Figures 2D-2F, Table 2, Shao et al., 2017⁵].
 - **Key Conclusion:** Loss of *Nmp4* improves bone response to several classes of osteoporosis drugs.
 - ***Nmp4* status did not influence serum profile response to any treatment**
 - *SOW Subtasks 2.1.g.1, 2.2.e, 3.5.a:* Loss of *Nmp4* elevated levels of the serum bone formation marker osteocalcin in mice under the anabolic therapies; however, there was no genotype x treatment interaction [Figures 8A and 8B, Shao et al., 2017⁵].
 - *SOW Subtasks 2.1.g.1, 2.2.e, 3.5.a:* Disabling *Nmp4* had no impact on serum CTX and serum RANKL/OPG [Figures 8C-8G, Shao et al., 2017⁵].
 - **Key Conclusion:** The bone marker serum profiles suggest that the loss of *Nmp4* enhances the bone formation response to osteoporosis therapies but has little impact on bone resorption.
 - **Loss of *Nmp4* improved both material and structural mechanical properties of bone**
 - *SOW Subtasks 2.1.g.6, 2.2.e.4, 2.2.e.6, 3.5.f:* To evaluate material and structural mechanical properties, the left femurs from each animal were monotonically tested to failure. The *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 11A; Shao et al., submitted to Journal of Biological Chemistry, Appendix].
 - *SOW Subtasks 2.1.g.6, 2.2.e.4, 2.2.e.6, 3.5.f:* Yield stress, the stress applied to the bone after which there is permanent damage, normalized for geometry, was also significantly

higher in the *Nmp4*^{-/-} femurs [Figure 11B; Shao et al., submitted to Journal of Biological Chemistry, Appendix].

- *SOW Subtasks 2.1.g.6, 2.2.e.6, 3.5.f*: The work-to-yield, the energy that goes in to deforming the sample was significantly higher in the *Nmp4*^{-/-} bone [Supplemental Table 6; Shao et al., submitted to Journal of Biological Chemistry, Appendix].
- *SOW Subtasks 2.1.g.6, 2.2.e.6, 3.5.f*: Loss of *Nmp4* also altered the structural properties of the femur. Yield force was significantly increased in the *null* bone and the increase in ultimate force neared significance [Supplemental Table 7; Shao et al., submitted to Journal of Biological Chemistry, Appendix]. Total displacement, the total amount of deformation the bone undergoes before failure, was significantly lowered in the *Nmp4*^{-/-} femurs [Supplemental Table 7 Shao et al., submitted to Journal of Biological Chemistry, Appendix].
 - **Key Conclusion:** Disabling *Nmp4* enhances bone quality

SPECIFIC AIM #3:

- **EXPERIMENTS:** The primary goal of Specific Aim #3 was to determine the cell type-specific contributions to the extended null PTH anabolic window.
 - A delay in successfully engineering the *Nmp4*-floxed mouse forced us to rely more heavily on our other experimental approaches for identifying the key cell type driving the *Nmp4*^{-/-} bone phenotype. This included a combination of immunohistochemical, fluorescence-activated cell sorting (FACS), and biochemical analyses to define the key cell types.
- **RATIONALE:** The following key questions underlie the Specific Aim rationale:
 - Does loss of *Nmp4* influence the impact of osteoporosis therapies on the size of specific cell populations in the bone marrow including mesenchymal stem/progenitors (MSPCs), osteoclasts, CD8+ T cells or adipocytes?
 - Does loss of *Nmp4* alter the anabolic activity of bone cells?
- **SUMMARY OF KEY RESULTS:**
 - **The combination of PTH, RAL, and loss of *Nmp4* significantly increased BM osteoprogenitor number, but did not affect adipogenesis or osteoclastogenesis**
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b, 5.5.b, 6.2*: Ovx did not abrogate the expanded population of osteoprogenitors and CD8+ T cells in *Nmp4*^{-/-} mice. The FACS analysis of ovx WT and *Nmp4*^{-/-} BM and PBL samples showed that the *null* mice exhibited an increase in the number of BM osteoprogenitors and CD8+ T cells compared to WT mice [Figure 4A-4F, Childress et al., 2015⁴].
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: RAL mono-therapy but not ZOL mono-therapy, increased bone marrow osteoprogenitors in both genotypes [Figures 6C and 6F, Shao et al., 2017⁵].
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: The *Nmp4*^{-/-} mice under the PTH+RAL treatment showed the most BM osteoprogenitors as measured by immunohistochemical analysis as well as by FACS analysis [Figure 6, Shao et al., 2017⁵].
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: Loss of *Nmp4* did not influence therapy-induced changes in adipogenesis and osteoclastogenesis [Figure 7, Shao et al., 2017⁵].
 - **Key Conclusion:** These data suggest that the populations of osteoprogenitors undergo the most significant expansion in the BM of *Nmp4*^{-/-} mice under select osteoporosis therapies.
 - ***Nmp4*^{-/-} osteoprogenitors exhibit a hyper-anabolic phenotype**
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: Isolated *Nmp4*^{-/-} MSPCs exhibited an accelerated and enhanced mineralization [Figure 5, Childress et al., 2015⁴; Figure 1 Shao et al., submitted to Journal of Biological Chemistry, Appendix].
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: *Nmp4* was observed to be a key regulator of ribosome biogenesis and the unfolded protein response (UPR), which together contribute to the *Nmp4*^{-/-} MPSCs super-secretor phenotype [Figures 2, 3, and 4 Young et al., 2016⁹].
 - *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b*: *Nmp4*^{-/-} MSPCs exhibited an elevated level of collagen protein secretion [Figure 9, Shao et al., submitted to Journal of Biological Chemistry, Appendix].

- *SOW: Subtask 2.1.g.2, 2.2.e.2, 3.5.b:* The *Nmp4*^{-/-} MSCs have a significantly increased capacity for metabolic conversion to glycolysis, which is a key driver of osteoblast anabolism [Figure 6, Shao et al., submitted to Journal of Biological Chemistry, Appendix]
 - **Key Conclusion:** Disabling *Nmp4* converts the osteoblast into a super-secretor by metabolically reprogramming the cell to support the concomitant elevated matrix production and expansion of the cell's delivery capacity. Therefore the central cell that appears to be driving the *Nmp4*^{-/-} hyper-anabolic bone phenotype is the MSC/osteoprogenitor.

KEY RESEARCH ACCOMPLISHMENTS/FINDINGS FOR ENTIRE PROJECT

- Loss of *Nmp4* improved bone response to PTH combination therapies including PTH+RAL and PTH+ZOL
- Loss of *Nmp4* improved bone response to the anti-catabolic drug RAL
- Estrogen was not required for the enhanced PTH-induced bone formation in *Nmp4*^{-/-} mice but these animals were not protected from ovx-induced bone loss without therapeutic intervention
- Disabling *Nmp4* enhanced bone quality
- Loss of *Nmp4* enhanced bone formation but did not impact resorption under osteoporosis therapies.
- The osteoprogenitor/osteoblast appears to be a key driver of the *Nmp4*^{-/-} hyper-anabolic bone phenotype.
- Disabling *Nmp4* converts the osteoblast into a super-secretor by metabolically reprogramming the cell to support the concomitant elevated matrix production and expansion of the cell's delivery capacity.

REPORTABLE OUTCOMES FOR ENTIRE PROJECT including published papers, proceeding abstracts (posters), and invited seminar presentations in which DOD support was acknowledged:

- **INVITED SEMINAR PRESENTATION:** Childress P., Bidwell JP *Developing Nmp4 as a drug target to enhance parathyroid hormone therapy for osteoporosis* 5th annual National Clinical and Translational Science Predoctoral Programs Meeting, April 9-11, 2014, Washington, DC
- **INVITED SEMINAR PRESENTATION:** Bidwell JP *Boosting Bone Anabolism: Engineering a Super Secretory Osteoblast* November 18, 2015, Richard L Roudebush VA Medical Center, Indianapolis, IN
- **INVITED SEMINAR PRESENTATION:** Bidwell JP *Boosting Bone Anabolism: Engineering a Super Secretory Osteoblast* December 11, 2015 IUPUI Department of Biology, Indianapolis, IN
- **INVITED SEMINAR PRESENTATION:** Bidwell JP *Engineering a Super Secretory Osteoblast* January 14, 2016 Indiana University School of Medicine, Indianapolis, IN
- **POSTER:** Childress P, Shao Y, Hernandez-Buquer S, et al. Increasing endogenous osteoprogenitors and improving parathyroid hormone (PTH) therapy in an osteoporotic mouse model. American Society of Bone and Mineral Research Annual Meeting; September 12-15, 2014; Houston, TX.
- **POSTER:** Shao Y, Hernandez-Buquer S, Childress P, et al. Improving PTH/Raloxifene Combination Osteoporosis Therapy In a Preclinical Model American Society of Bone and Mineral Research Annual Meeting; October 9-12, 2015; Seattle, WA.
- **PUBLISHED PAPER:** Childress P, Stayrook KR, Alvarez MB, et al. Genome-Wide Mapping and Interrogation of the *Nmp4* Antianabolic Bone Axis. *Molecular endocrinology* 2015;29:1269-85.
- **PLENARY POSTER:** Young SK, Shao Y, Bidwell JP, Wek R. Engineering A Hyper-anabolic, Super-secreting Osteoblast. American Society for Bone and Mineral Research Annual Meeting; September 16-19, 2016; Atlanta, GA.
- **PUBLISHED PAPER:** Young SK, Shao Y, Bidwell JP, Wek RC. 2016 Nuclear Matrix Protein 4 Is a Novel Regulator of Ribosome Biogenesis and Controls the Unfolded Protein Response via Repression of Gadd34 Expression. *J Biol Chem.* 291(26):13780-8.
- **PUBLISHED PAPER:** Shao Y, Hernandez-Buquer S, Childress P, et al. Improving Combination Osteoporosis Therapy In a Preclinical Model of Heightened Osteoanabolism. *Endocrinology* 2017;158:2722-40.
- **PLENARY POSTER:** Shao Y, Jacobs K, Hamilton J, et al. Identifying a Novel Regulator of Anabolic Bone Metabolism American Society For Bone and Mineral Research Annual Meeting 2017. Denver, CO.

- **SUBMITTED MANUSCRIPT:** Shao Y, Wichern E, Childress JP et al., 2018 Engineering a super-secreting osteoblast Journal of Biological Chemistry (Appendix)

Funding applied for based on work supported by this award:

Agency: NIH
 Program: NIAMS
 Title: Engineering a super-secreting osteoblast
 Date submitted: 02/05/2018
 Annual direct costs: \$305,000
 In review

Agency: NIH
 Program: NIAMS
 Title: The Nmp4 Link Between Bone & Beta cells
 Date submitted: 10/05/2017
 Annual direct costs: \$125,000
 48 Percentile.

Agency: NIH
 Program: NIAMS
 Title: The Nmp4 Anti-Anabolic Bone Axis
 Date submitted: 10/05/2015
 Annual direct costs: \$308,277
 Score: 31 PERCENTILE.

Submission of revised proposal (07/05/2017) scored a 15 PERCENTILE. Funding decision is in administrative review

Agency: NIH
 Program: MGB
 Title: The Nmp4-UPR control of proteostasis
 Date submitted: 06/05/2016
 Annual direct costs: \$250,000
 Score: Not scored

CONCLUSION: *Key experimental discovery for entire DOD study*

Our finding that disabling the single gene *Nmp4* improves the potency of several classes of osteoporosis treatments in a preclinical model for osteoporosis including PTH mono-therapy, PTH+anti-catabolic combination therapies, and RAL mono-therapy is medically significant. Moreover, the discovery that loss of this gene maximizes the response of osteogenic cells to any anabolic component of a particular therapy is exciting. Our mechanistic model based on the data gathered in this study breaks new ground in proposing that disabling *Nmp4* increases the number of osteoprogenitors and enhances the secretory capacity of their osteoblast progeny leading to bone with improved material and mechanical properties. Also novel is the new data that supports our proposed mechanism by which disabling *Nmp4* conjointly alters protein synthesis and delivery thus converting osteoblasts into hyper-responsive, super-secretory cells. This premise builds on an idea advocated by Jilka that inhibiting a repressor of anabolism is a promising therapeutic strategy for osteoporosis¹⁰.

“So what?”

Women comprise the fastest growing group of the US veterans contributing to the looming osteoporosis epidemic within the veteran population. The Veterans Affairs (VA) health care system will be in high demand by female veterans of Operation Enduring Freedom and Operation Iraqi Freedom. PTH and PTHrp are the only FDA-approved anabolic osteoporosis therapies and add significant amounts of bone to the osteoporotic skeleton. Therefore, these drugs have the potential to restore the bone lost in a variety of VA clinical settings. However, a drawback to these medications is that potency declines within 2 years and thus it is not suitable as a long-term therapy, which is problematic in treating a chronic degenerative disease. Clinicians have attempted to improve PTH therapy by adding an anti-catabolic (e.g. raloxifene or a bisphosphonate) to the treatment.

This has not met with success. The present discovery supports our contention that disabling *Nmp4* or some component of its pathway will unlock the block on PTH combination therapies and enhance/extend regeneration of osteoporotic bone in post-menopausal female veterans. We have made significant advances in understanding the molecular mechanisms that support the *Nmp4*^{-/-} phenotype. This research will lead to identifying new pharmacological targets for boosting bone anabolism in the VA patient population.

Personnel receiving pay from the research effort on W81XWH-13-1-0129 (PR120563)

- Michele Adaway
- Matthew Allen
- Joseph Bidwell
- Yongzheng He
- Selene Hernandez-Buquer
- Yu Shao
- Stuart Warden

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APPENDIX FOR REVISED FINAL REPORT W81XWH-13-1-0129 PR120563:

The original final report provided all the outcomes (publications and abstracts) that we had generated at the time of submission. However, we have recently submitted a manuscript to the *Journal of Biological Chemistry*, describing work that was partially supported by this grant. This manuscript and the supporting material are included in this appendix. The large Supplemental Tables (S1-S4) containing RNA-seq data and ChIP-seq data and not included in this report but are presently being uploaded into GEO Database.

Engineering a super-secreting osteoblast

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ABSTRACT:

A goal of osteoporosis therapy is to restore lost bone with structurally sound tissue. Mice lacking the transcription factor *Nmp4* respond to several classes of osteoporosis drugs with enhanced bone formation compared to wild type (WT) animals. To address how loss of *Nmp4* maximizes anabolic output we used RNA-seq and network analyses, along with biochemical and bone mechanical measurements to define this anti-anabolic axis. Several independent preparations of expanded mesenchymal stem/progenitor cells (MSPCs) were derived from individual *Nmp4*^{-/-} and WT mice. The *Nmp4*^{-/-} cells exhibited accelerated and enhanced mineralization. Loss of *Nmp4* significantly altered the expression of over 5,000 genes. While *Nmp4* status did not alter the mRNA expression of *Runx2* and *Sp7*, key regulators of osteoblast differentiation, *Nmp4*-depletion enhanced expression of genes that drive osteogenesis and conversion to aerobic glycolysis, a key step in bone anabolism. Functional studies confirmed that *Nmp4*^{-/-} MSPCs exhibited an enhanced capacity for glycolytic conversion. Several canonical pathways sensitive to *Nmp4* status controlled protein production and delivery and as predicted the *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. Mechanical analysis of femurs from *Nmp4*^{-/-} mice treated with osteoporosis therapies resulted in enhanced bone material properties compared to WT mice. In conclusion, disabling *Nmp4* converts the osteoblast into a super-secretor by metabolically reprogramming the cell to support the concomitant elevated matrix production and expansion of the cell's delivery capacity. Resolving this pathway will guide advances in osteoporosis therapy.

INTRODUCTION:

Osteoporosis is a disease of attenuated bone mass and strength that significantly increases the risk of fragility fractures (1). Teriparatide (PTH) and abaloparatide (PTHrP) are currently the only FDA-approved osteoanabolic therapies for this disease (2,3). 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 (4). 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 (5-9). However, the potency of PTH precipitously declines and there is an FDA-mandated two-year limit on treatment (8). The short treatment lifespan of PTH therapy has prompted further investigation into strategies that improve the drug's efficacy such as combining hormone treatment with an anti-catabolic drug or targeting PTH directly to bone (10,11). Neutralizing intrinsic pathways that temper PTH-induced bone matrix production and delivery might improve drug efficacy by boosting osteoblast secretion. Indeed, a similar strategy of "inhibiting the inhibitor" (12) has led to the development of the osteoanabolic romosozumab, a monoclonal antibody that neutralizes the action of the osteoinhibitory protein sclerostin, currently in clinical trials (13,14).

We reported that the transcription factor Nuclear Matrix Protein 4 (*Nmp4* or *Ciz*, *Zfp384*) suppresses the action of osteoanabolics (15-20) 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 of MC3T3-E1 cells revealed that *Nmp4* occupies over 15,000 sites within the osteoblast genome. 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^{-/-} mice harbor more bone marrow osteoprogenitors than their WT littermates (15,17). Expanded cultures of *Nmp4*^{-/-} 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 the increased protein load (16,21,22).

To address the mechanisms by which *Nmp4* suppresses osteoblast-mediated bone formation we sought to (i) identify the cellular pathways that are significantly altered upon loss of this transcription factor and (ii) determine how these perturbations in cellular processes alter osteoblast phenotype and ultimately bone material properties. We performed high-throughput RNA sequencing (RNA-seq) and network analyses of WT and *Nmp4*^{-/-} expanded MSPCs maintained in non-differentiating or osteogenic differentiating media. Our results suggest that loss of *Nmp4* leads to cellular metabolic reprogramming, a key step in bone anabolism (23,24). Consistent with these ideas loss of *Nmp4* promoted aerobic glycolysis in these cultured cells. Furthermore, we showed an increase in mRNA translation in the *Nmp4*^{-/-} cells leading to elevated collagen production and secretion. Finally, mRNA expression profile for extracellular matrix genes suggested that the *Nmp4*^{-/-} bone matrix is enriched with proteins that mediate improved material and mechanical properties, which ex vivo testing confirmed. We conclude that *Nmp4* suppresses several pathway networks that serve collectively to enhance osteoblast secretion of bone matrix in response to osteoanabolics.

RESULTS:

Loss of Nmp4 accelerates and enhances osteogenic mineralization in both male- and female-derived cells

We previously showed that expanded cultures of *Nmp4*^{-/-} bone marrow MSCs derived from male mice exhibited a precocious and enhanced mineralization compared to WT cells(16). This finding raised the question as to whether this phenotype is gender-specific. We compared the mineralization of male- and female-derived expanded WT and *Nmp4*^{-/-} MSC cultures. Three independently derived WT MSCs, along with three *Nmp4*^{-/-} preparations showed that the *null* cells exhibited mineralization within 1 week of exposure to osteogenic medium compared to 2-3 weeks for the WT cells, regardless of donor gender (**Figure 1**). Additionally, two of MSC preparations were derived from WT and *Nmp4*^{-/-} littermates while the others were derived from individuals obtained from random litters. These results suggest that *Nmp4* repression of osteogenic mineralization and matrix production is gender-neutral and is not influenced by kinship.

Nmp4 regulates a large portion of the osteogenic transcriptome

Next we addressed the transcriptome changes underlying the hyper-anabolic phenotype of the *Nmp4*^{-/-} MSCs. RNA-seq analysis was carried out on the MSCs 1584L^{WT} vs. 1515RR^{KO}. These cells exhibited a striking difference in the time to mineralization onset and as we discuss further below, key findings were confirmed using the other MSCs. 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*^{-/-} MSCs at different time points following exposure to osteogenic medium are provided in **Supplemental Table 2**. 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 2A**). Following this criterion, the expression profiles of 8438 genes were not significantly affected by *Nmp4* status (**Figure**

2A). Loss of *Nmp4* had a similar impact on the transcriptome of MSCs maintained in the osteogenic differentiating medium for 7 days, 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 2B**). *Nmp4* status did not impact the expression of 8151 genes in cells maintained in the osteogenic medium (**Figure 2B**).

We recently reported the results of a genome-wide ChIP-seq analysis of *Nmp4* binding in MC3T3-E1 pre-osteoblasts, which identified over 15,000 sites supporting occupancy of this transcription factor (16). This cell line is an established *in vitro* model for early osteoblastogenesis that is similar to our primary MSCs. To identify genes that are direct targets of *Nmp4*, we determined the overlap of the gene lists derived from the present MSC RNA-seq datasets and those lists derived from our previous analysis of *Nmp4* genome-wide ChIP-Seq analysis (16). The gene list we 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 (see **Supplemental Table 1 and Supplemental Figure 1**) (16). Additionally, we limited the compilation to 4786 and 4787 genes expressed by our MSCs 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 2C & 2D**). The expression of ~63% of these genes that supported *Nmp4* with significant occupancy were not strongly impacted by loss of *Nmp4* (**Figures 2C & 2D**). We conclude that in this osteogenic context *Nmp4* has an extensive influence on the MSC and osteogenic transcriptomes consistent with its widespread occupancy in their genomic landscapes.

Loss of Nmp4 alters several 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 genes that exhibited a significant change in expression between the *Nmp4*^{-/-} and WT cells at Day 3 (non-differentiating medium) and at Day 7 (osteogenic medium) in culture. **Supplemental Tables 3 and 4** 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. 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 **Supplemental Tables 3 & 4** were also identified in previous studies characterizing MSC transcriptomic changes during osteogenic differentiation (25-27). 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 MSC preparations (25-27) and were identified here.

From these comprehensive lists we display 17 IPA canonical pathways that are most likely to be integral to the precocious and enhanced mineralization phenotype of the *Nmp4*^{-/-} cells (**Figures 3 & 4**). The bar graphs in **Figures 3A and 4A** 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*. The bar graphs in **Figures 3B and 4B** are color coded to reflect the percentage of genes in a particular pathway whose expressions were significantly enhanced with the loss of *Nmp4* (red) and those genes whose expressions were attenuated in the *null*

cells (green). The total numbers of genes comprising the canonical pathways are also indicated (**Figures 3B and 4B**).

The IPA outcomes identified several pathways, which are predicted to be sensitive to *Nmp4* status and drive both osteogenesis and metabolic reprogramming necessary for fueling the development of the professional secretory osteoblast (**Figures 3 & 4**) (28-31). Both Igf1 and Wnt signaling advance osteogenesis in part through their stimulation of glycolysis (32,33). These two pathways featured several genes that exhibited a significant increase or decrease in expression in the *Nmp4*^{-/-} cells and forecast significant activation of these pathways (Igf1 signaling $z = +4.131^{\text{Day3}}$; Wnt/Ca⁺² signaling $z = +3.00^{\text{Day3}}/+3.77^{\text{Day7}}$, **Figures 3 & 4**). 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 (**Supplemental Figure 2**). The Hif1 α ($z = \text{NaN}^{\text{Day3}}$; $z = \text{NaN}^{\text{Day7}}$) and Nrf2 ($z = +4.33^{\text{Day3}}$; $+4.23^{\text{Day7}}$) signaling pathways were altered by *Nmp4* loss. Both pathways regulate osteogenesis as well as govern cellular metabolic reprogramming (34-36). Furthermore, the PTEN network was significantly sensitive to *Nmp4* status and assigned z scores -2.50^{Day3} and -1.76^{Day7} (**Figures 3 & 4**) 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 (37,38).

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*), which all function downstream of *Runx2* and *Sp7* (**Supplemental Figure 3**). Additionally *Nmp4*^{-/-} cells exhibited decreased mRNA expression of transcription factors that drive adipogenesis or chondrogenesis suggesting that loss of *Nmp4* facilitates MSPC differentiation towards osteogenesis and that this predisposition is reinforced by shifts in transcriptional networks regulating the activities of the aforementioned osteogenic/metabolic pathways (**Figures 3 & 4 and Supplemental Figure 3**).

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 (**Figures 3 & 4**). 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 5**). 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 5**). A primary function of *Slc16a3* is the secretion of lactate and protons from highly glycolytic cells (39) and a recent study determined that increased levels of *Slc16a3* is necessary for sustaining high glycolysis in macrophages (40). Several genes mediating the conversion of glucose to pyruvate displayed significantly elevated expression in *Nmp4^{-/-}* cells (**Figure 5**). 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. Interestingly, the expression of several genes linking glycolysis to the TCA cycle, e.g. *Pdha1*, *Pdhb*, *Dlat*, and *Dld* were not significantly increased in the *Nmp4^{-/-}* cells (**Figure 5**). These findings suggest that loss of *Nmp4* may have a more modest impact on oxidative phosphorylation than on glycolysis.

To establish the validity of the IPA canonical pathway forecasts, we evaluated the impact of *Nmp4* status on the glycolytic pathway (**Figure 6**). WT vs. *Nmp4^{-/-}* cells derived from the male littermates (1957^{WT}/1957^{KO}) and the WT vs. *Nmp4^{-/-}* cells derived from the males obtained from random litters (1584L^{WT}/1515RR^{KO}) were cultured in non-differentiating medium using the Seahorse Xfe24 Bioanalyzer. Cells were seeded directly into an XFe24 well plate and grown for 24hrs in culture. Subsequently cells were incubated in medium devoid of glucose or pyruvate and the extracellular acidification rate (ECAR) was measured by the bioanalyzer 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 and 6B**). 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 and 6B**). 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 and 6B**). The ECAR prior to glucose injection is the non-glycolytic acidification originating from processes other than glycolysis, and this too was significantly higher in the *Nmp4* cells (**Figures 6A and 6B**). 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 bioanalyzer was used to measure basal respiration reported as oxygen consumption rate (OCR) and then the cells were sequentially exposed to various inhibitors of the mitochondrial electron transport chain (**Figures 7A and 7B**). The first injection of oligomycin inhibited ATP synthase (complex V) allowing for the estimation of mitochondrial respiration associated with ATP production 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 permitting the calculation of the maximal respiration rate. The FCCP-stimulated OCR was then used to calculate spare respiratory capacity, which is a measure of the ability of the cells to respond to increased energy demand and is defined as the difference between maximal respiration and basal respiration. Non-mitochondrial respiration was determined from the final injection of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor,

which shuts down mitochondrial respiration oxidation. Thus the oxygen consumption that persisted is due to other metabolic processes, such as glycolysis or fatty acid oxidation. Our results showed that loss of *Nmp4* elevated ATP production, maximal respiration, spare respiratory capacity and non-mitochondrial respiration (**Figures 7A and 7B**). We note that there were significant differences in the basal respiration between the 1957^{WT}/1957^{KO} MSPCs, whereas there was only a trend between the 1584L^{WT}/1515RR^{KO} cells. 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

Loss of *Nmp4* perturbed several cellular pathways controlling protein synthesis and delivery including mTOR, tRNA charging, translation initiation, and the unfolded protein response (UPR), which serves to expand the processing capacity of the endoplasmic reticulum for nascent secretory proteins (41) (**Figures 3 & 4**). Expression of numerous genes comprising these pathways including genes that drive amino acid transport and amino acid biosynthesis were significantly elevated in the *Nmp4^{-/-}* cells (**Figure 8**). Support for elevated protein production in *Nmp4^{-/-}* MSPCs also includes our earlier report that enhanced ribosome biogenesis was sustained during induction of the unfolded protein response (UPR) through a mechanism suggested to involve GADD34-mediated dephosphorylation of eIF2 (21). 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 (21). Indeed the RNA-seq analysis confirmed that *Nmp4^{-/-}* MSPCs exhibited elevated expression of several genes in the ribosome biogenesis and UPR pathways (**Supplemental Figure 4**) and IPA/MAP analysis predicted that protein-folding activity is elevated and UPR-induced apoptosis is attenuated with loss of *Nmp4* (**Supplemental Figure 4**).

To further address whether bone matrix production and delivery were enhanced in *Nmp4*^{-/-} osteoprogenitors, we measured the levels of *Col1a1*-mRNA associated with polyribosomes and the levels of collagen protein secretion. We observed elevated levels of 40S and 60S ribosomal subunits and 80S monosomes, and increased polysomes in *Nmp4*^{-/-} MSPCs compared to WT (**Figure 9A**). The RNA-seq data revealed that total *Col1a1* mRNA expression was elevated in the *Nmp4*^{-/-} cells (**Figure 9B**). 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 9C**). *Col1a1* mRNA was present in heavy polysomes in both WT and *Nmp4*^{-/-} MSPCs, suggesting efficient translation. However, there was a reproducible increase in *Col1a1* in the largest fraction 7 in *Nmp4*^{-/-} cells, suggesting more robust translation in the *Nmp4*-depleted cells. Thus the combination of more *Col1a1* mRNA available for translation, along with more increased amounts of ribosomes and more efficient *Col1a1* mRNA translation, would culminate in elevated synthesis of *Col1A1* protein in the *Nmp4*^{-/-} cells. Consistent with these results, analysis of total acid-soluble collagen protein levels in the cell-matrix layer, normalized for cell number revealed that *Nmp4*^{-/-} cells produced approximately 4-fold more collagen than WT cells (**Figure 9D**). We conclude that loss of *Nmp4* converts bone cells into super-secretors of matrix.

Nmp4^{-/-} osteoblasts produce a bone matrix with improved material properties

Next we addressed whether loss of *Nmp4* altered the expression of bone matrix genes and if so, whether *Nmp4* depletion influenced the structural and material properties of the skeleton. Several genes representing multiple protein classes comprising the bone matrix (42-46) were identified in our RNA-seq database and 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 10**). 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 10**). Also the expression of key genes that control mineralization were altered in the *Nmp4*^{-/-} cells consistent with the observed phenotype observed in culture (**Figure 10**). For example, the gene *Phospho1*, encoding a soluble phosphatase responsible for initiating HA crystal formation inside matrix vesicles (47-50), was highly induced in the *Nmp4*^{-/-} cells as was the gene *Slc20a1* a sodium-phosphate symporter also involved in the initiation of skeletal mineralization(50) (**Figure 10**). 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 10). SLRPs play significant structural roles within the ECM and regulate collagen fibril growth, organization and ECM assembly (51-53).

The transcriptome analysis predicted that the *Nmp4*^{-/-} osteoblasts deliver elevated amounts of matrix that serve to improve bone material properties. Therefore 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. We did not use ovariectomized mice because ovariectomy does not change the enhanced response to anabolic drugs in the *Nmp4*^{-/-} animals (16) and all the MSCs used in this study were derived from healthy, virgin mice. Briefly, μ CT 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 (**Supplemental Figures 5 and 6**). There was a synergistic (greater than additive) interaction between PTH and RAL in the WT and *Nmp4*^{-/-} for BV/TV of the distal femur and the L5 vertebra (Table 1). Loss of *Nmp4* significantly improved the femoral bone gain and the L5 bone gain in the PTH and PTH+RAL treatments (**Supplemental Figures 5 & 6**). *Nmp4* status had no impact on bone response to RAL mono-therapy (**Supplemental Figures 5-7**) nor did it alter the effect of any treatment on cortical area (**Supplemental Figure 7**). Finally, there was no significant difference between the genotypes under the VEH control

treatment with respect to femoral and L5 BV/TV or cortical area (**Supplemental Figures 5-7**). However loss of *Nmp4* did significantly impact some aspects of femoral cortical geometry, such as cortical thickness, marrow area as well as other related parameters (**Supplemental Table 5**). Altogether, these results are similar to the data we reported in older ovariectomized mice (15).

To measure material and structural mechanical properties of the bone, the left femurs from each animal were monotonically tested to failure. The *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 11A**, genotype $p < 0.0001$). Yield stress, the stress applied to the bone after which there is permanent damage, normalized for geometry, was also significantly higher in the *Nmp4*^{-/-} femurs (**Figure 11B**, genotype $p = 0.0023$). Additionally, the higher value for the elastic modulus, a measure of the material's stiffness, in *Nmp4*^{-/-} bones approached significance (genotype $p < 0.06$, **Supplemental Table 6**). 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 11A**). However, RAL mono-therapy resulted in a significantly higher yield stress than PTH treatment in WT and *Nmp4*^{-/-} mice (treatment $p = 0.0048$ **Figure 11B**). Finally, total strain, elastic modulus, and resilience were all differentially responsive to the various therapies (treatment $p < 0.05$ **Supplemental Table 6**).

Loss of *Nmp4* also altered the structural properties of the femur. Yield force was significantly increased in the *null* bone (genotype $p = 0.004$ **Supplemental Table 7**) 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 $p = 0.04$ **Supplemental Table 7**) and the decrease in post yield displacement, the amount of deformation that occurs after the yield point, approached significance ($p = 0.06$, **Supplemental Table 7**). Finally, work-to-yield, the energy that goes in to deforming the sample was significantly higher in the *Nmp4*^{-/-} bone (genotype $p = 0.03$

Supplemental Table 6). Together these results show that the *Nmp4*^{-/-} osteoblast produces more matrix than the WT cells and that the composition of the secretome results in improved bone material and structural properties.

DISCUSSION:

There is a critical medical need for understanding the intrinsic barriers to pharmacologically inducing bone formation in the osteoporotic skeleton (54). This information will ultimately guide advances in the development of osteoanabolic therapies (55). The present data demonstrate that *Nmp4* acts cell autonomously as a barrier to bone matrix production and mineralization in both male and female mice. *Nmp4*^{-/-} MSPCs in culture exhibited a precocious and enhanced mineralization compared to WT cells. Enhanced mineralization in vitro often does not correlate with positive effects on the skeleton. For example, osteoblasts deficient in the expression of *Naca* (56), *Sox8* (57), or *Foxc1* (58) showed an *ex vivo* accelerated mineralization phenotype; however animals harboring deficits in any one of these genes exhibited significant defects in bone development, formation, or mineralization (56-58). These findings are in stark contrast to our present model in which the precocious and enhanced mineralization of the *Nmp4*^{-/-} osteoblast directly translates into an improved skeletal response to osteoanabolics (15-18).

Loss of *Nmp4* had a profound and wide-ranging impact on the MSPC transcriptome significantly altering the mRNA expression of approximately 5000 genes. The mRNA levels of the osteogenic master regulators *Runx2* and *Sp7* were not influenced by *Nmp4* status but several other osteogenic transcription factors were elevated in expression and IPA identified several downstream osteogenic pathways as significantly activated. Additionally the *Nmp4*^{-/-} cells exhibited decreased levels of transcription factors that drive adipogenesis or chondrogenesis suggesting that *Nmp4*^{-/-} MSPCs are biased toward bone. These results are

consistent with our previous genome-wide ChIP-seq analysis of MC3T3-E1 cells showing that *Nmp4* occupied over 15,000 DNA sites equivalent to over 6000 genes (16). We conclude that *Nmp4* is an apex regulator of several transcriptional networks in the osteogenic genome.

The *Nmp4*^{-/-} MSPCs have a significantly increased capacity for metabolic conversion to glycolysis, which is a key driver of osteoblast anabolism. Glucose is a key nutrient for osteoblasts and aerobic glycolysis is the dominant mode of glucose utilization in these cells (23,59). 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 (60). Aerobic glycolysis was the principal mode of glucose utilization in primary calvarial osteoblasts although oxidative phosphorylation increased as the cells began to mineralize (61). Indeed, loss of *Nmp4* modestly but significantly increased the respiratory capacity of the MSPCs. 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 (32). Concomitantly, PTH downregulates Sost/sclerostin expression in osteocytes, unleashing the anabolic Wnt signaling pathway (62), 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 (33).

In addition to the Igf1 and Wnt signaling pathways *Nmp4* appears to target Hif1 α , Nrf2, and the Pten canonical pathways, which also influence osteogenesis and metabolic reprogramming. Stabilization of the transcription factor HIF1 α 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* (34). Mice expressing the stable-oxygen form of Hif1 α in osteoprogenitors exhibited an expanded pool of osteoprogenitors and elevated trabecular

BV/TV, very reminiscent of the *Nmp4*^{-/-} skeletal response to PTH (15-17). The impact of Nrf2 on osteogenesis is complex and its influence on skeletal homeostasis depends on multiple factors, e.g. age, gender, genetic background, and physiological versus pathological conditions (35).

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 (63). Specifically, Pten decreases the levels of two key enzymes involved in the Warburg effect, Pkm2 and Pfkfb3 (64). Therefore, conditional loss of *Pten* in osteoprogenitors led to increased numbers of osteoblasts and expanded bone matrix (38), whereas conditionally disabling *Pten* in mature osteoblasts enhanced mTOR activity and increased bone mineral density (65). Again, this Pten-deficient bone phenotype is similar to the *Nmp4*^{-/-} skeleton under PTH challenge.

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 (24,33). 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 *Nmp4*^{-/-} MSPCs exhibited significantly elevated ribosome biogenesis, the primary determinant of translational capacity and a key driver of cell growth (21). Indeed here we showed that loss of *Nmp4* culminates in increased *Col1a1* transcription and translation. 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. Clearly, loss of *Nmp4* converts bone cells into super-secretors of matrix.

The expression of matrix genes that contribute to material and structural mechanical properties, e.g. collagen, osteocalcin, and SLRPs were elevated in *Nmp4*^{-/-} cells and mechanical analysis of femurs from mice treated with osteoporosis therapies confirmed enhanced improvement in several of these key properties in the *Nmp4*^{-/-} bone. This is significant since pharmacological or mechanical induction of accelerated and enhanced bone formation does not necessarily produce high quality mineralized tissue. Sodium fluoride (NaF) is an osteoanabolic that increases bone mass but the newly formed bone lacks normal structure and strength (66-68). Mechanical load is a bone anabolic signal and the magnitude of the applied strain determines whether the response is adaptive, forming primarily lamellar bone, or injury, producing woven bone (69). Although woven bone forms faster than lamellar bone, it has inferior mechanical and material properties (70,71).

The molecular mechanisms underlying the improved *Nmp4*^{-/-} bone quality remains to be determined but the enhanced expression of osteocalcin by pharmacologically induced *Nmp4*^{-/-} osteogenic cells (15,16,18) may improve the quality of the produced bone. Earlier we proposed that non-collagenous proteins act as “glue” at the collagen-mineral interface to resist the separation of the mineralized fibrils and therefore enhance bone toughness (43,72-74). Anabolic therapies that induce the formation of osteocalcin/osteopontin-enriched bone may 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 (51-53). Thus the collagen maturation may be accelerated in *Nmp4*^{-/-} bone.

As an apex regulator of bone cell anabolic output *Nmp4* directly and indirectly regulates gene programs that control key stages of matrix production and delivery and the metabolic reprogramming necessary to fuel it (**Figure 12**). 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 (75,76). However, whereas c-Myc acts a general amplifier of gene expression (77-79), Nmp4 appears to act as a general attenuator and suppressor of biomass accrual (16,21). 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 (80-86). Furthermore, Nmp4 control of metabolic reprogramming may also present therapeutic targets for controlling bone anabolism (23,24,87). Interrogating these pathways within the context of pharmacologically-induced bone formation will provide a detailed map of the barriers that currently limit osteoanabolic therapy.

MATERIALS AND METHODS

Cell culture: MSPCs were derived from individual mice as previously described (16,88). 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 μ g/mL streptomycin, 25 μ g/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 μ g/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. All RNA integrity numbers (RIN) ranged between 8.2-9.7. The miRNA was harvested using miRNeasy (QIAGEN). Four technical replicates were harvested for each time point and genotype.

The 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 $\times$ 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) (89); (v) gene-based expression levels were quantified with featureCounts (90); (vi) differential expression of genes across different treatments was determined with edgeR (91).

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 (92), 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 (21). 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 these 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 (**Supplemental Table 1**) (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 1957R^{WT} and 1957N^{KO} were derived from male littermates obtained from an *Nmp4*^{+/-} x *Nmp4*^{+/-} cross. The 1584L^{WT} and 1515RR^{KO} 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, trifluorocarbonylcyanide phenylhydrazone, rotenone, and antimycin A per the manufacturer's instructions (Seahorse Biosciences). Glycolysis stress tests were performed using oligomycin and 2-deoxy-D-glucose (Seahorse Biosciences, Lexington, MA). After each analysis, total cell number was quantified and normalized to O₂ consumption rate (OCR) or extracellular acidification rate (ECAR), respectively. All Seahorse experiments were repeated at least twice. All the data shown in the assays are an average of at least 3 different wells per group.

Collagen secretion analysis: Collagen levels were determined using the Sircol assay (Biocolor Ltd, Carrickfergus, Northern Ireland) (93,94). Non-differentiating WT and *Nmp4*^{+/-} MSPCs, cells were seeded into 12-well plates at 10,000 cells/well (25 cells/mm²). These cells were maintained in Mesencult MediaTM + Mesencult Stimulatory SupplementTM (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. All experiments were repeated at least twice. All the data shown in the assays are an average of at least 5 different wells per group.

Col1a1 polysome analysis: Equal amounts of WT and *Nmp4*^{-/-} 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 MgCl₂, 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 (95). 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 (96). Equal amounts of firefly luciferase mRNA was measured in each of the fractions. Primer sequences for both transcripts were *Col1a1* F: 5'-ACGTCCTGGTGAAGTTGGTC-3', R: 5'-CAGGGAAGCCTCTTTCTCCT-3'; *firefly luciferase* F: 5'-CCAGGGATTTTCAGTCGATGT-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 (19). 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 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 (97,98). 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 (15).

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 (99). 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 μ CT 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 for bone architecture and mechanical data: 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 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 (100). 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 $p \leq 0.05$. To evaluate the metabolic profiles of the MSPCs, we used the Statistical Analysis System version 9.4 (SAS Institute) to perform student t-tests in comparing specific metabolic parameters. Finally, ggplot2 was used to create all the heatmaps, volcano plots, and boxplots (101).

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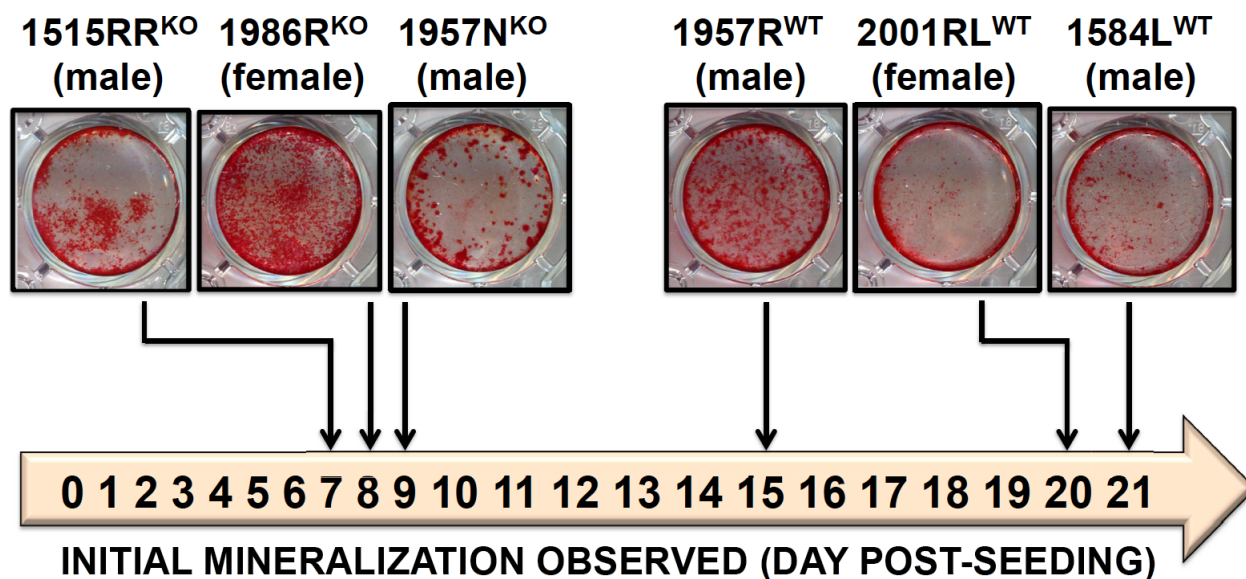


Figure 1: *Nmp4*^{-/-} osteogenic cells exhibit a precocious and enhanced mineralization regardless of donor gender or kinship. Six independently expanded MSC preparations from individual WT and *Nmp4*^{-/-} mice were established as described in Materials and Methods. Cultures were stained with alizarin red when mineralization was first observed. The cells derived from the *Nmp4*^{-/-} mice consistently exhibited mineral days to weeks before this was observed in the WT cultures. The cell preparations 1957N^{KO} and 1957R^{WT} were derived from male littermates. The remaining lines were derived from male or female mice selected from random litters.

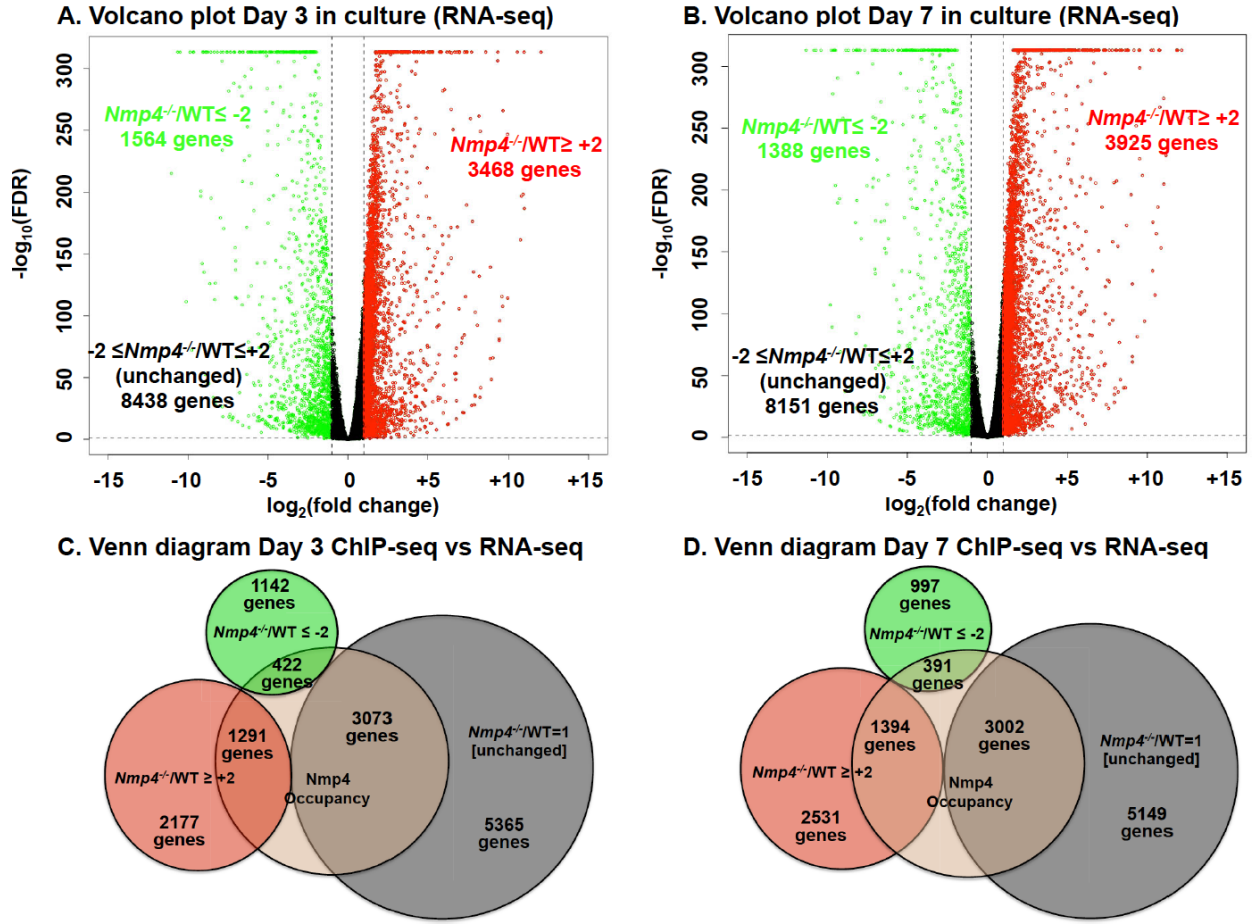


Figure 2: *Nmp4* has a broad impact on the genomic and transcriptomic landscape of MSCs. **[A & B]** Volcano plots of RNA-seq data from MSCs maintained in **[A]** non-differentiating culture medium for 3 days and **[B]** osteogenic differentiating culture medium for 7 days. The X-axis represents the logarithmic transformation to the base 2 of the mean fold-change of mRNA expression in *Nmp4*^{-/-} 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 $\geq +2$ (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 both criteria. The dotted line demarcates FDR=0.05. **[C & D]** 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.

A. DAY 3: Significant pathways (p-value)/Predicted activity (z-value) B. Percent genes upregulated/downregulated

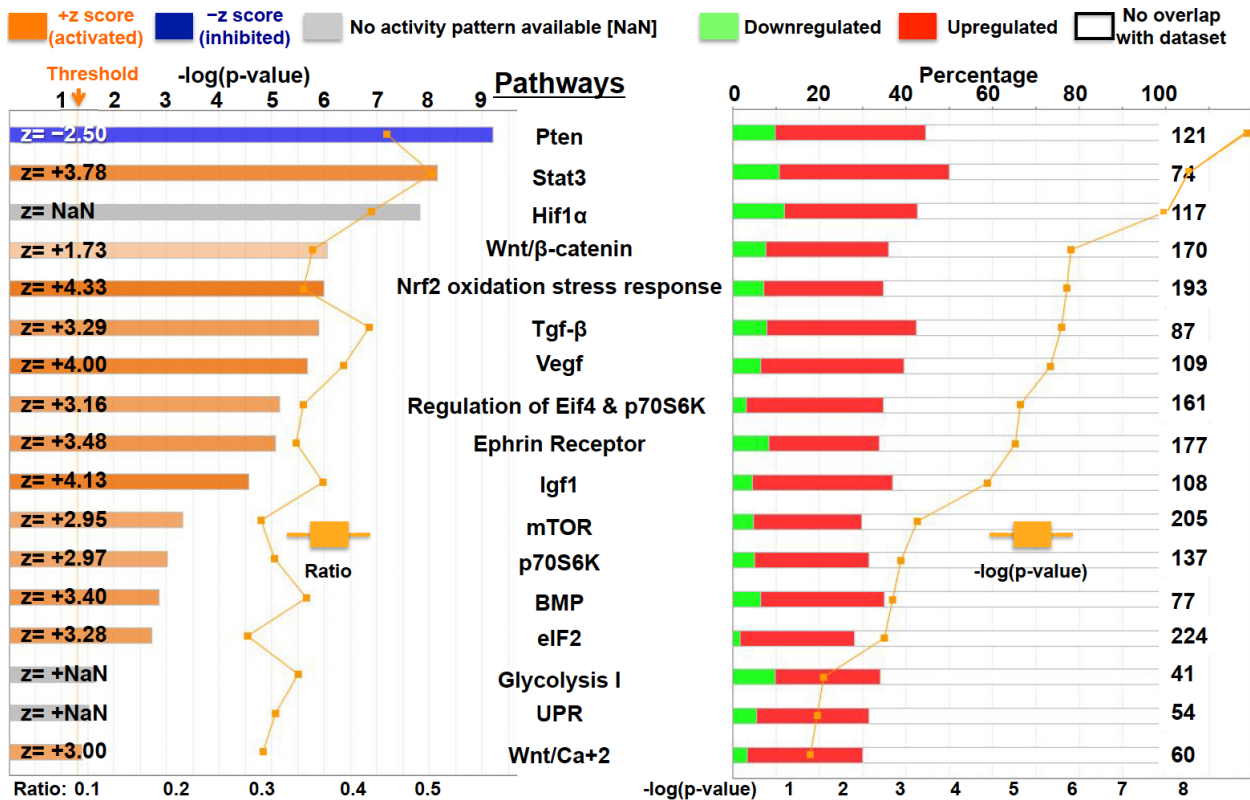


Figure 3: Loss of *Nmp4* alters pathways that drive metabolic reprogramming. Ingenuity Pathway Analysis of the RNA-seq data identified over 200 pathways significantly altered in the *Nmp4*^{-/-} cells maintained in non-differentiation culture medium for 3 days (see Supplemental Table 3 for full list). Here we show select canonical pathways that are sensitive to *Nmp4* status and relevant to the metabolic reprogramming 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 ≥ 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 orange line gives the ratio of the number 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 $-\log_{10}(\text{p-value})$ and significance was defined by p value ≤ 0.05 [or $1.30 = -\log_{10}(\text{p-value})$].

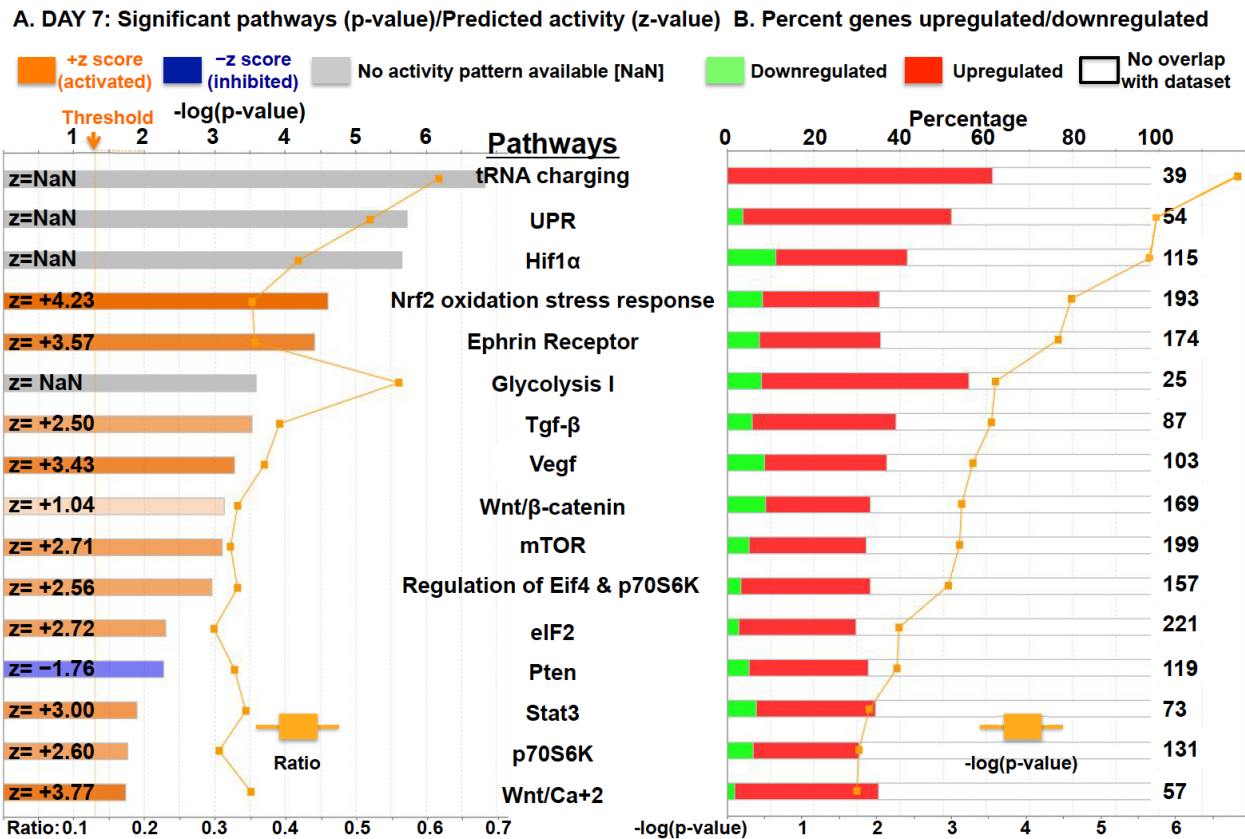


Figure 4: Select IPA canonical pathways from Supplemental Table 4 that were identified in WT vs. *Nmp4*^{-/-} MPSCs at Day 7 in culture, maintained in osteogenic medium. **[A]** The bar graphs are color-coded to reflect the z-score calculated by the IPA algorithm (see Supplemental Table 4 and Figure 3A for full explanation). **[B]** The bar graphs are color coded to reflect the percentage of genes in a particular pathway whose expressions are upregulated or downregulated (see Figure 3B for full explanation).

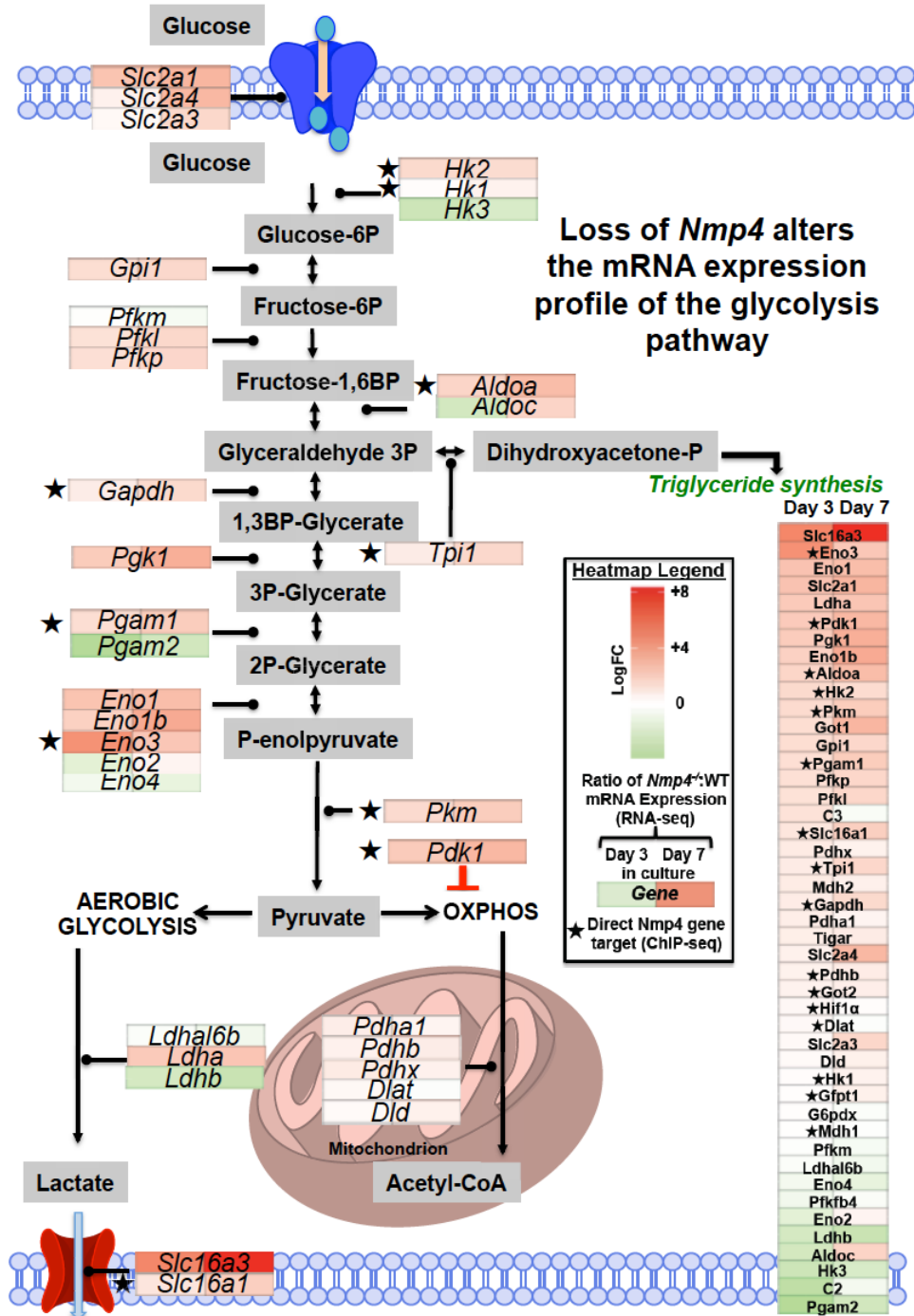


Figure 5: *Nmp4*^{-/-} osteoprogenitors/osteoblasts exhibit significant elevated expression of several genes that drive glycolysis. Schematic of glycolysis/oxidative phosphorylation [OXPHOS] pathways with overlay of heatmap derived from RNA-seq data generated from WT and *Nmp4*^{-/-} MPSCs harvested 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).

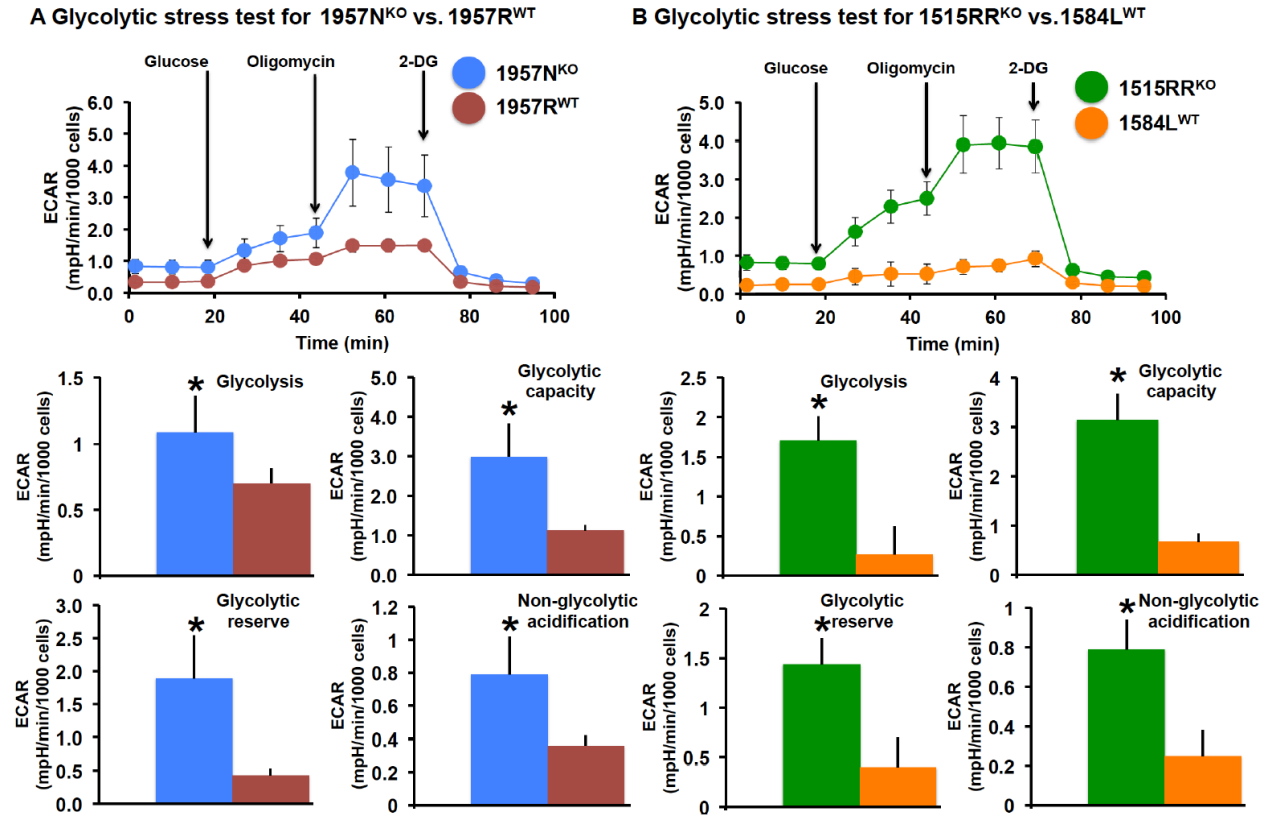


Figure 6: Loss of *Nmp4* enhances glycolysis. The line graphs show a comparison of WT vs. *Nmp4*^{-/-} MSPC extracellular acidification rate (ECAR) profiles that have undergone the Glycolytic Stress test. **[A]** The MSPCs 1957R^{WT} and 1957N^{KO} were derived from male littermates. **[B]** The MSPCs 1584L^{WT} and 1515RR^{KO} were derived from a random pair of male WT and *Nmp4*^{-/-} mice. These graphs are representative of 2-3 individual tissue culture experiments. 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. Non-glycolytic acidification is the ECAR, prior to glucose injection and is caused by processes in the cell other than glycolysis. Endpoint parameters were compared by t-tests. Statistical significance was set at $p < 0.05$.

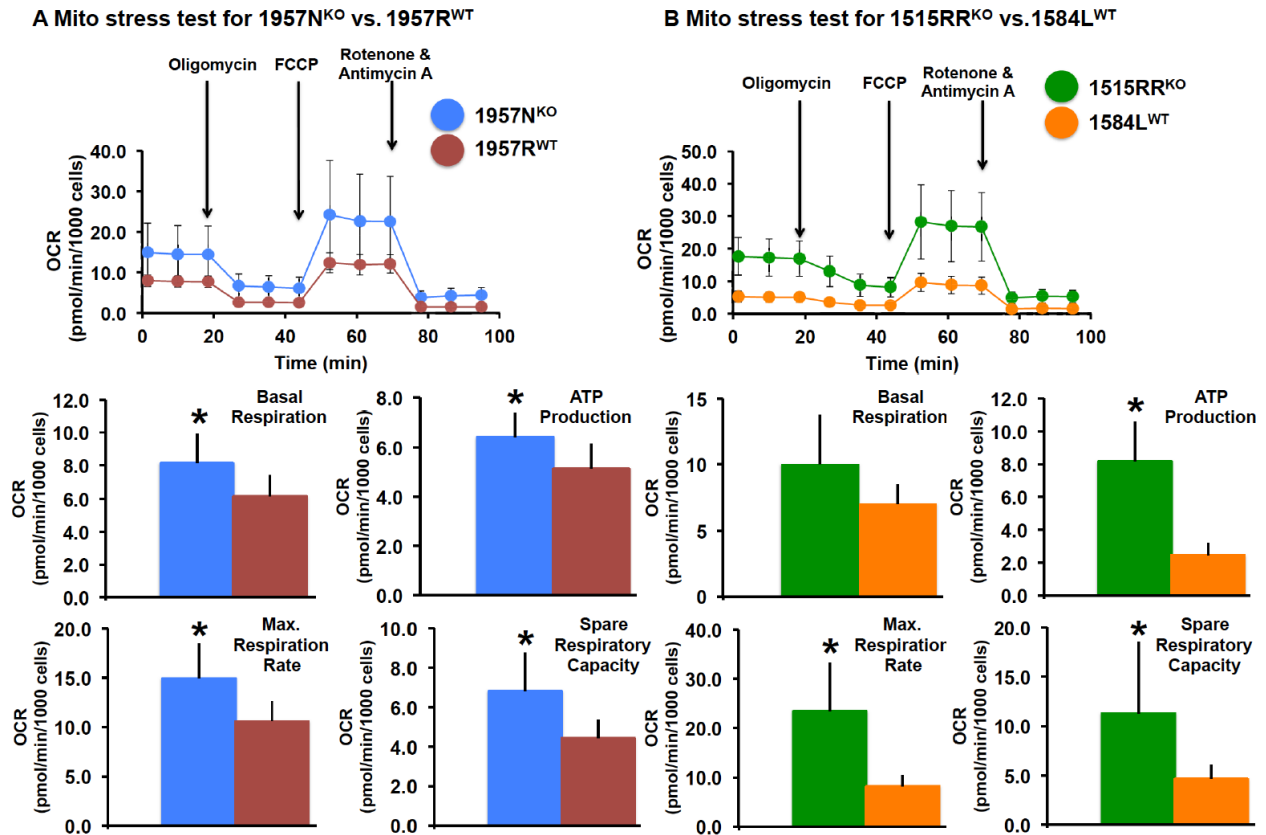


Figure 7: Loss of *Nmp4* enhances oxidative phosphorylation. The line graphs show a comparison of WT vs. *Nmp4*^{-/-} MSPC oxygen consumption rate (OCR) profiles that have undergone the Mitochondrial Stress test. **[A]** The MSPCs 1957R^{WT} and 1957N^{KO} were derived from male littermates. **[B]** The MSPCs 1584L^{WT} and 1515RR^{KO} were derived from a random pair of male WT and *Nmp4*^{-/-} mice. These graphs are representative of 2-3 individual tissue culture experiments. For the mitochondrial stress test the bioanalyzer first measured basal respiration and then sequentially exposed the cells to various inhibitors of the mitochondrial electron transport chain. The ATP production 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. The FCCP-stimulated OCR was used to calculate spare respiratory capacity. 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 consistently significantly higher in the *Nmp4*^{-/-} cells (data not shown).

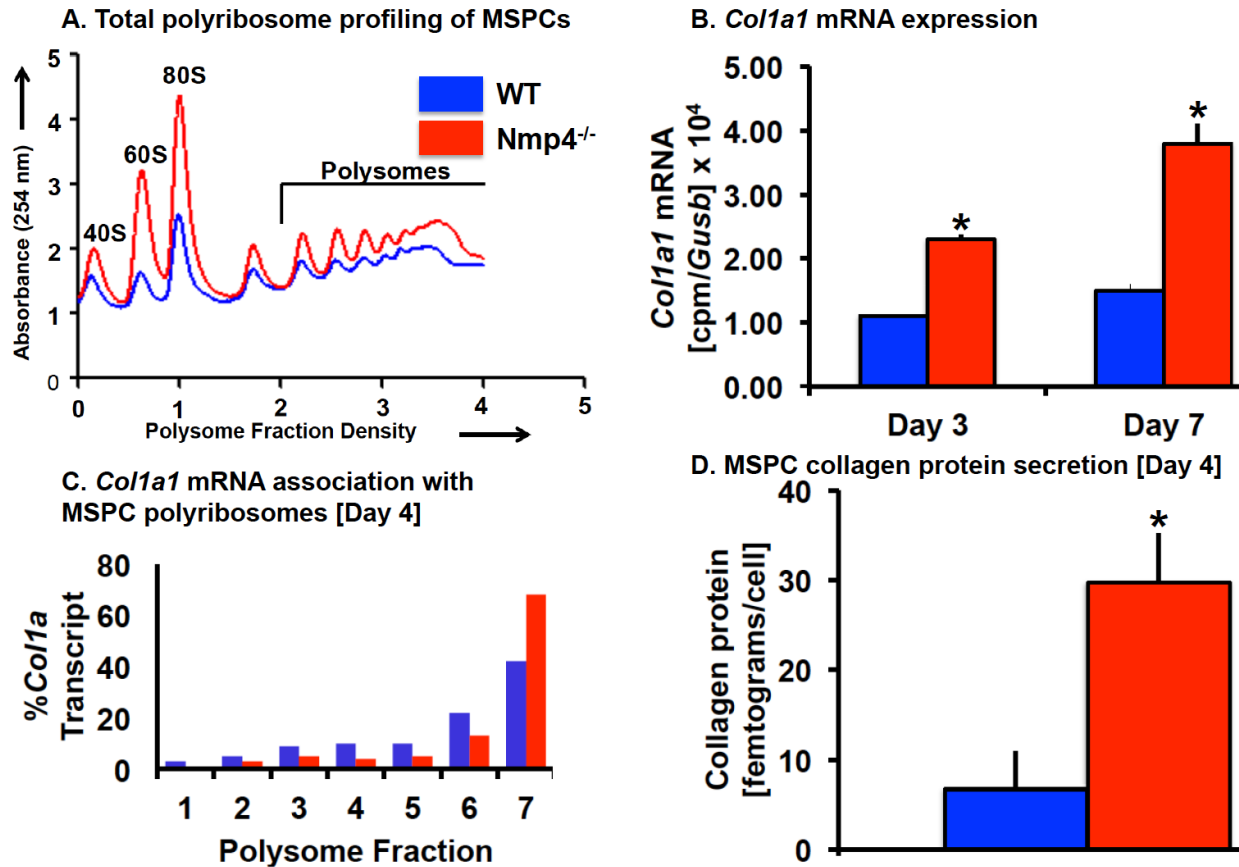


Figure 9: Loss of *Nmp4* enhances collagen expression and secretion. **[A]** Polysome profiles of lysates prepared from WT and *Nmp4*^{-/-} MSCs at 4 days in culture. Representative profiles from 3 biological replicates **[B]** *Col1a1* mRNA expression as determined by RNA-seq in MSCs maintained in non-differentiation 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. **[D]** Secretion of collagen protein was measured in the acid-soluble cell-matrix layer 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.

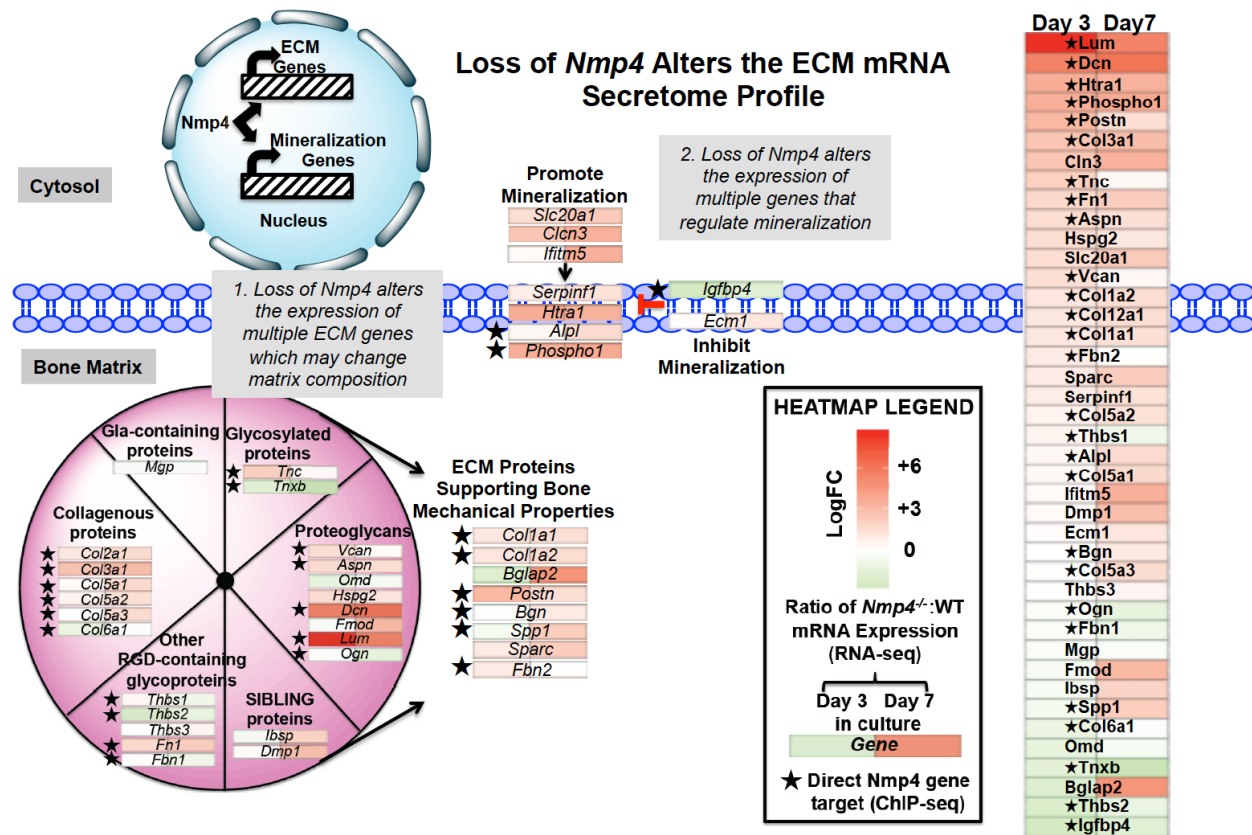


Figure 10: *Nmp4*^{-/-} osteoprogenitors/osteoblasts exhibit significant elevated expression of several genes that encode proteins of the bone matrix. The schematic shows family of proteins that comprise the bone matrix. Also the expressions of key genes that control mineralization were altered in the *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 ★ indicates *Nmp4* binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (16).

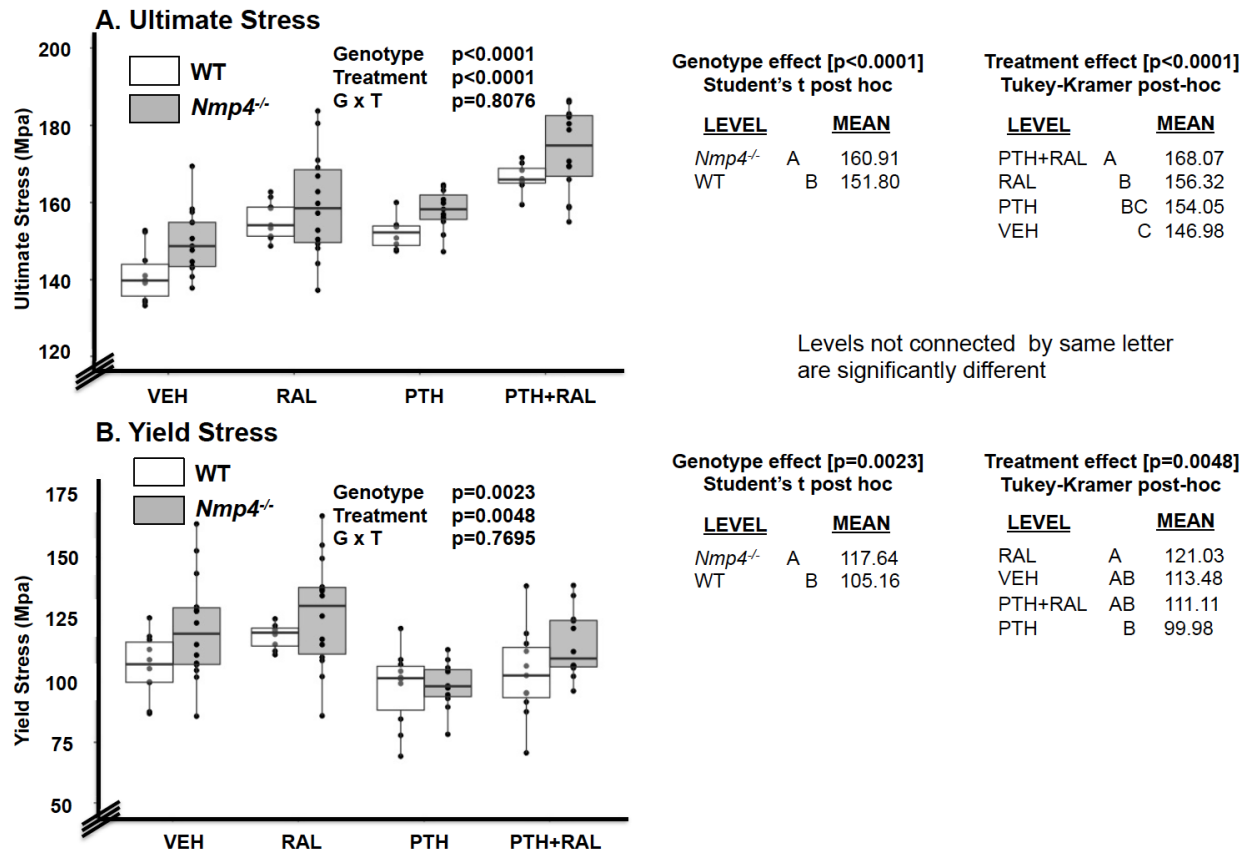


Figure 11: Loss of *Nmp4* improves femoral material properties. Results of 3pt-bending analysis. **[A]** Ultimate stress **[B]** Yield stress. Healthy female WT and *Nmp4*^{-/-} mice (10wks) were treated with RAL, PTH, PTH+RAL or vehicle for 7wks as described in the Materials and Methods. A 2W ANOVA was used to compare response ($p \leq 0.05$). Genotype and treatment were the independent variables. There were strong genotype and treatment effects for both ultimate stress and yield stress. Data represents average $\pm$ SD, $n=8-15$ mice/group.

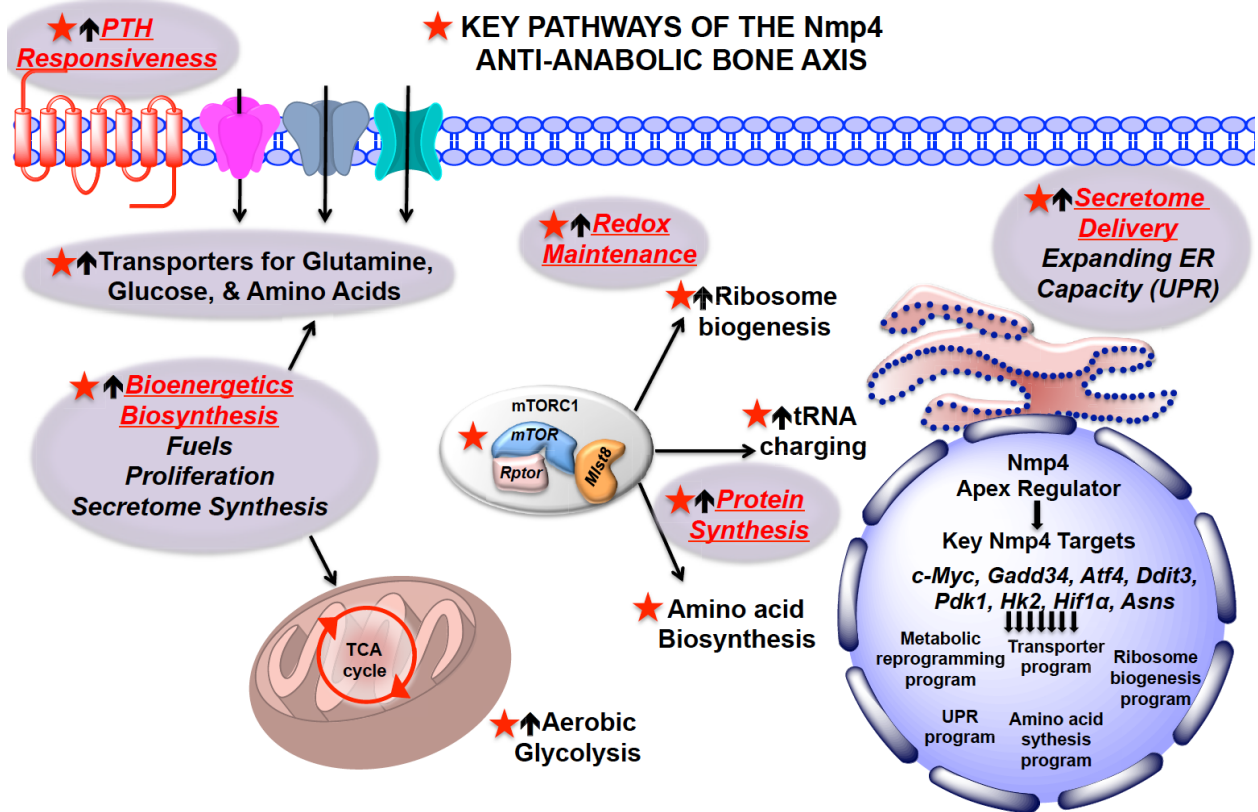


Figure 12: Hypothesis—Nmp4 is an apex regulator of bone cell anabolic output. This transcription factor directly and indirectly regulates gene programs that control key stages of matrix production and delivery. It may accomplish this by regulating both the expression of master transcriptional regulators of these pathways in addition to broadly engaging several of their downstream target genes.

TABLES:

THERAPY	p-value PTH Treatment	p-value RAL Treatment	p-value PTH x RAL interaction
Table 1A: FEMUR BV/TV			
PTH+RAL [WT]	p<0.0001	p<0.0001	p<0.0001
PTH+RAL [<i>Nmp4^{-/-}</i>]	p<0.0001	p<0.0001	0.001
Table 1B: L5 BV/TV			
PTH+RAL [WT]	p<0.0001	p<0.0001	0.0008
PTH+RAL [<i>Nmp4^{-/-}</i>]	p<0.0001	p<0.0001	p<0.0001
Table 1C: CORTICAL BONE AREA			
PTH+RAL [WT]	<0.0001	0.0591	0.8056
PTH+RAL [<i>Nmp4^{-/-}</i>]	<0.0001	0.0166	0.5938

Table 1: 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 $p \leq 0.05$

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Engineering a super-secreting osteoblast

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List of Materials Included:

Supplemental Table S1:	ChIP-seq data, located in separate xlsx file
Supplemental Table S2:	RNA-seq data, located in separate xlsx file
Supplemental Figure S1:	Nmp4 ChIP-seq profiles
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
Supplemental Figure S2:	IPA Molecule Activity Predictor of Wnt/ β -catenin pathway
Supplemental Figure S3:	Heatmap of MSC lineage commitment transcription factors
Supplemental Figure S4:	IPA Molecule Activity Predictor of ER-mediated protein folding
Supplemental Figure S5:	Femoral BV/TV for osteoporosis treatment groups
Supplemental Figure S6:	L5 BV/TV for osteoporosis treatment groups
Supplemental Figure S7:	Femoral cortical area for osteoporosis treatment groups
Supplemental Table S5:	Femoral cortical parameters
Supplemental Table S6:	Estimated material properties
Supplemental Table S7:	Structural mechanical properties

Supplemental Table S1: ChIP-seq data, **located in separate xlsx file**

Nmp4 occupancy studies 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.

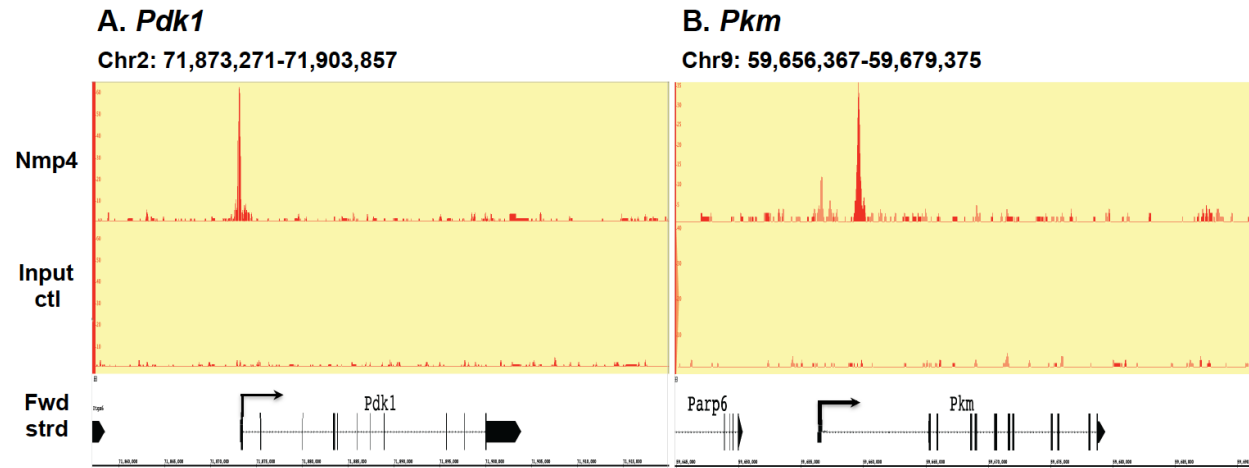
Supplemental Table S2: RNA-seq data, **located in separate xlsx file**

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)

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
*KOD3_5	cpm (counts per million reads)
KOD3_6	cpm (counts per million reads)
KOD3_7	cpm (counts per million reads)
KOD3_8	cpm (counts per million reads)
KOD7_13	cpm (counts per million reads)
KOD7_14	cpm (counts per million reads)
KOD7_15	cpm (counts per million reads)
KOD7_16	cpm (counts per million reads)
WTD3_1b	cpm (counts per million reads)
WTD3_1	cpm (counts per million reads)
WTD3_3	cpm (counts per million reads)
WTD3_4	cpm (counts per million reads)
WTD7_10b	cpm (counts per million reads)
WTD7_10	cpm (counts per million reads)
WTD7_11	cpm (counts per million reads)
WTD7_9	cpm (counts per million reads)
KOD3_5	raw read count
KOD3_6	raw read count
KOD3_7	raw read count
KOD3_8	raw read count
KOD7_13	raw read count
KOD7_14	raw read count
KOD7_15	raw read count
KOD7_16	raw read count
WTD3_1b	raw read count
WTD3_1	raw read count
WTD3_3	raw read count
WTD3_4	raw read count
WTD7_10b	raw read count
WTD7_10	raw read count
WTD7_11	raw read count
WTD7_9	raw read count

*KOD3_5, KOD3_6, KOD3_7; KOD3_8:
 KOD7_13, KOD7_14, KOD7_15, KOD7_16:
 WTD3_1b, WTD3_1, WTD3_3, WTD3_4:
 WTD7_10b, WTD7_10, WTD7_11, WTD7_9:

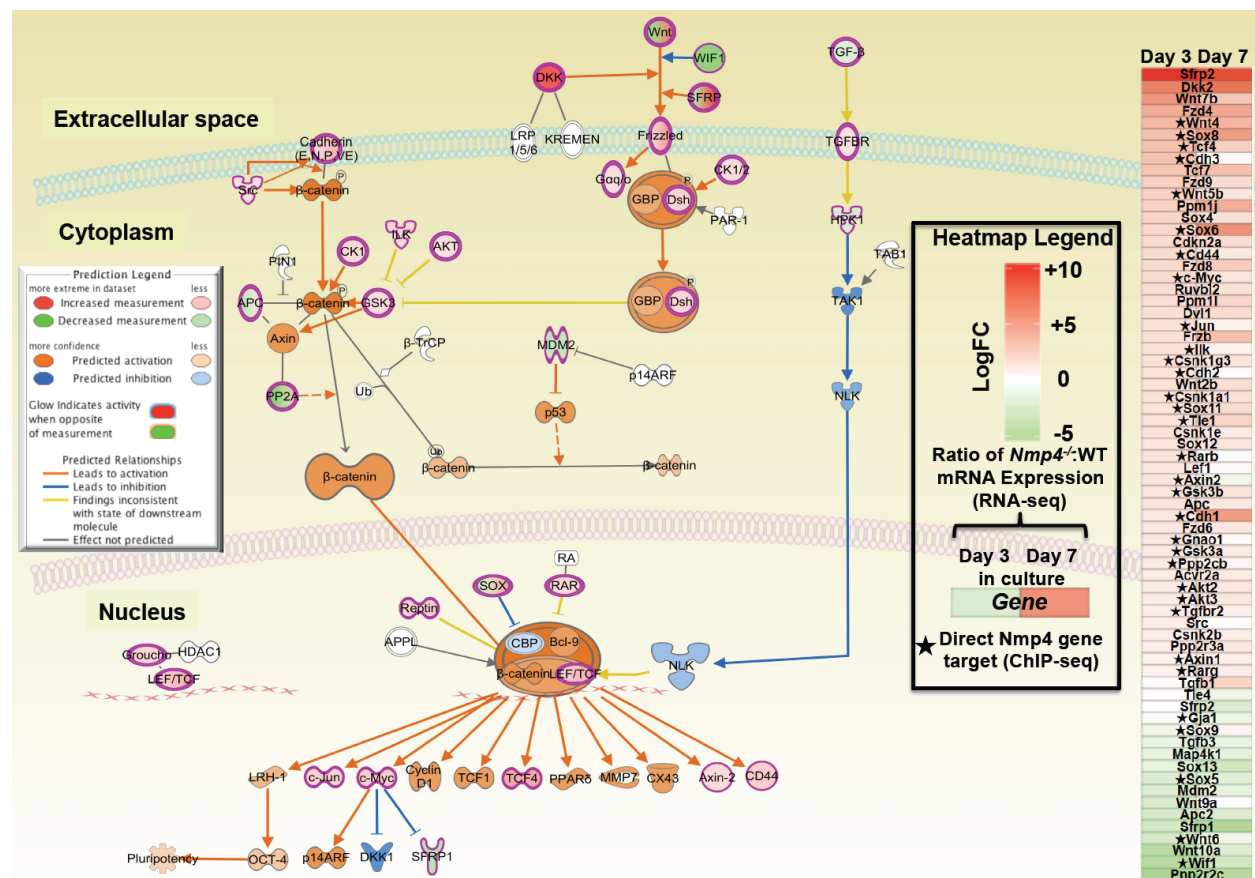
4 technical replicates for Knockout (*Nmp4^{-/-}*) Day 3 in culture
 4 technical replicates for Knockout (*Nmp4^{-/-}*) Day 7 in culture
 4 technical replicates for wild type (WT) Day 3 in culture
 4 technical replicates for wild type (WT) Day 7 in culture



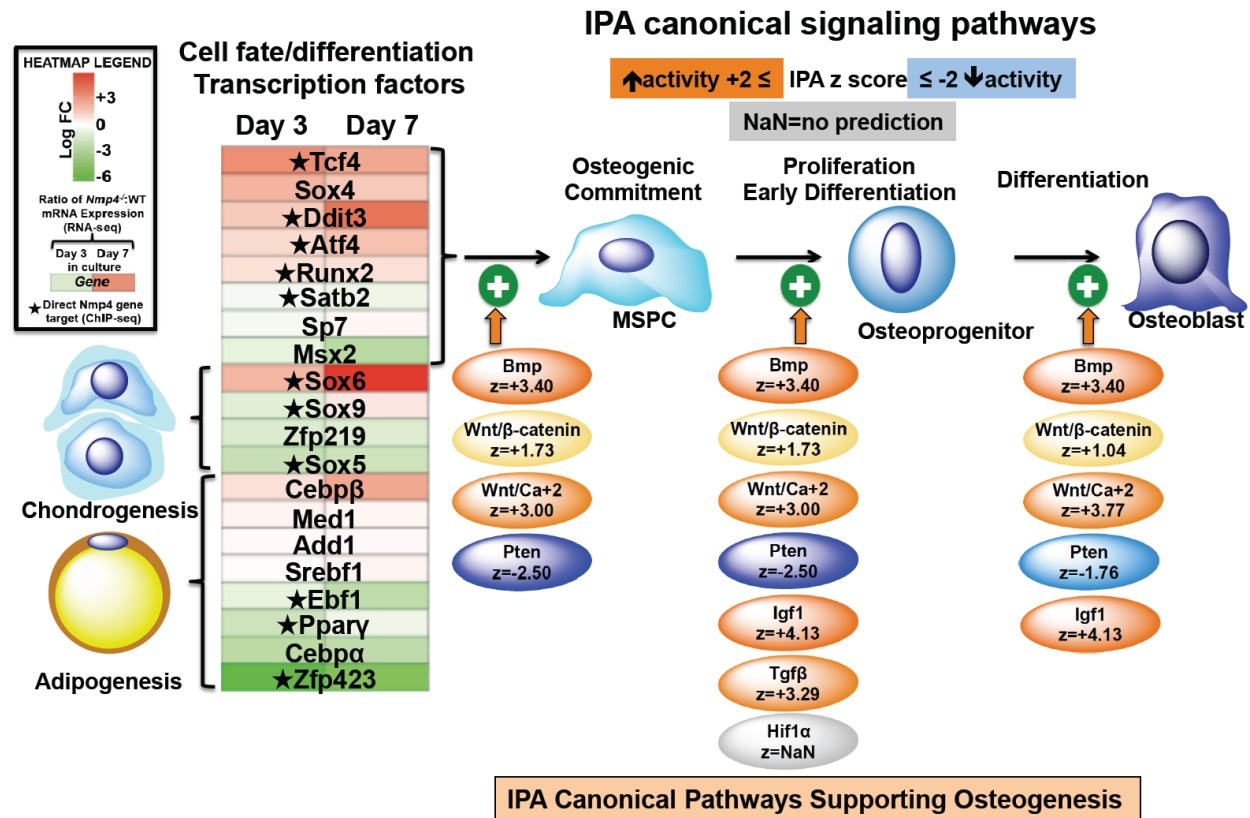
Supplemental Figure S1: ChIP-seq reveals Nmp4 binding profiles at specific gene loci in mouse MC3T3-E1 cells (Childress et al., 2015). The Burrows-Wheeler algorithm was used to align sequences (50-nt reads, single end) to the mouse genome (mm10). Alignments were extended in silico at their 3'-ends to 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 (A) *Pdk1* (B) *Pkm*. The input DNA profiles were devoid of peaks.

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 $-\log(\text{p-value}) \geq 1.30$. Column A: identity of the canonical pathway; Column B: $-\log(\text{p-value})$; 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.

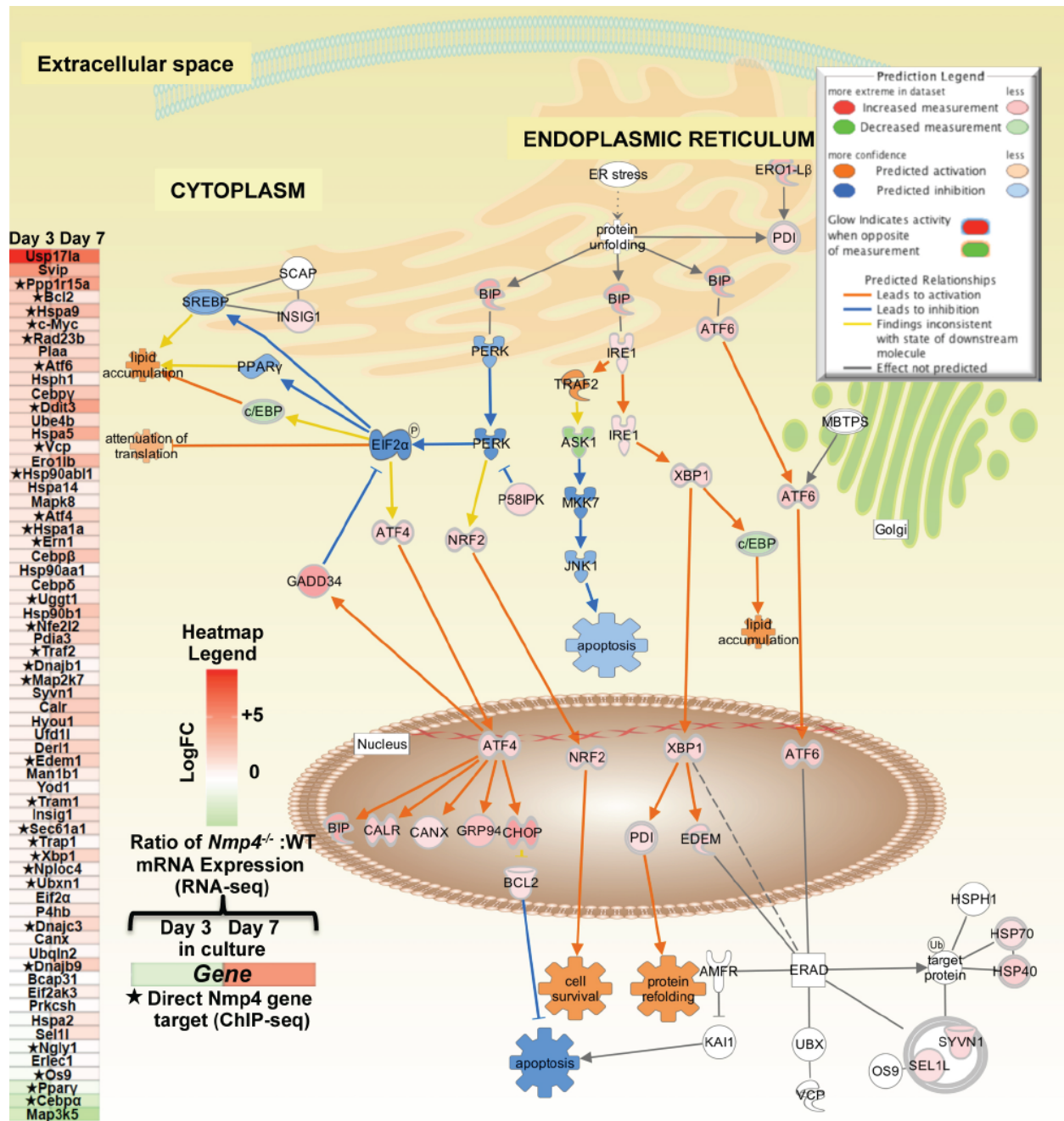
Supplemental Table S4: Day 7 IPA canonical pathways, [located in separate xls file](#)
IPA Canonical pathways perturbed by loss of *Nmp4* in MPSCs harvested at Day 7 in culture. Pathways were identified as significantly sensitive to *Nmp4* status that achieved a value of $-\log(\text{p-value}) \geq 1.30$. See Supplemental Table 3 legend for identity of table columns.



Supplemental Figure 2: The IPA Molecule Activity Predictor (MAP) algorithm indicated that loss of *Nmp4* elevates Wnt/β-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. On the left-hand side of this pathway is a heatmap of genes comprising the Wnt/β-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 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).

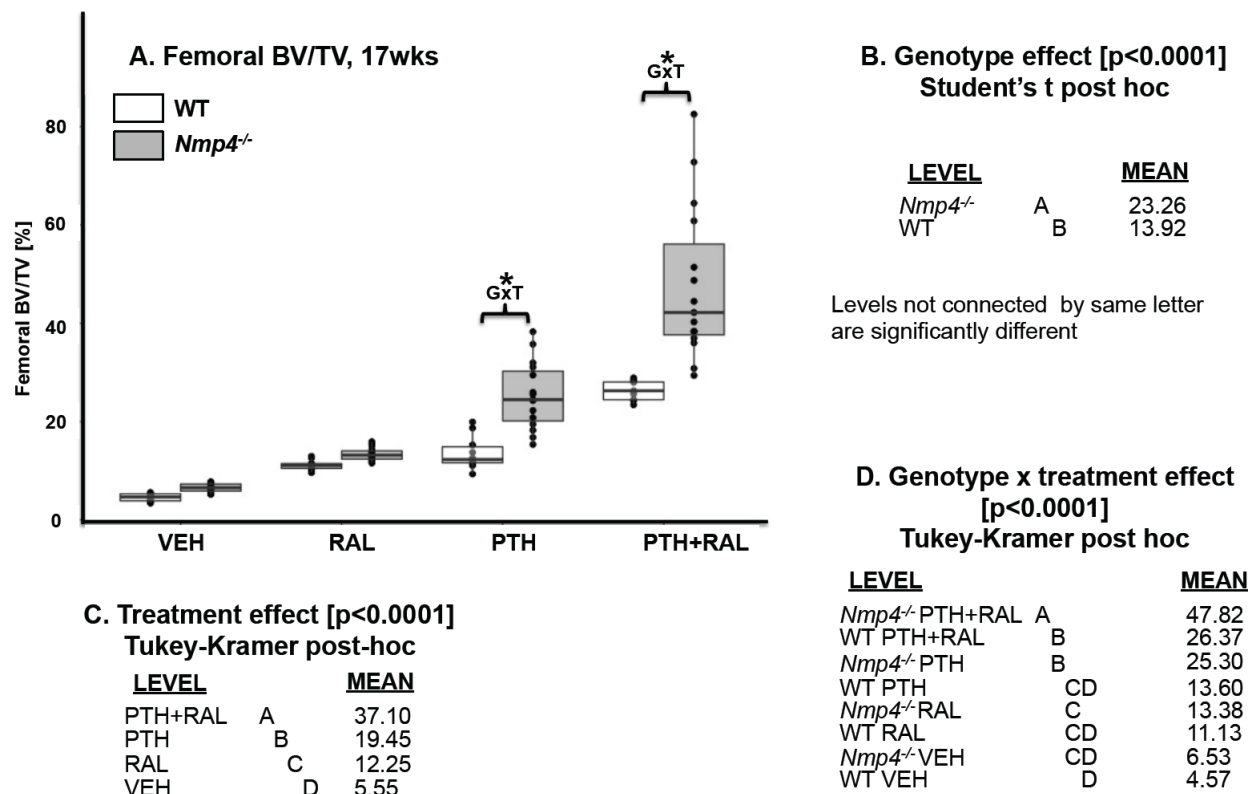


Supplemental Figure 3: Loss of *Nmp4* biases the MSC transcriptome toward the osteogenic lineage. Heatmap of RNA-seq data from WT and *Nmp4*^{-/-} MPSCs at Day 3 (uncommitted) and Day 7 (early osteogenesis) in culture. Red boxes indicate increased expression in the *Nmp4*^{-/-} cells compared to the 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.

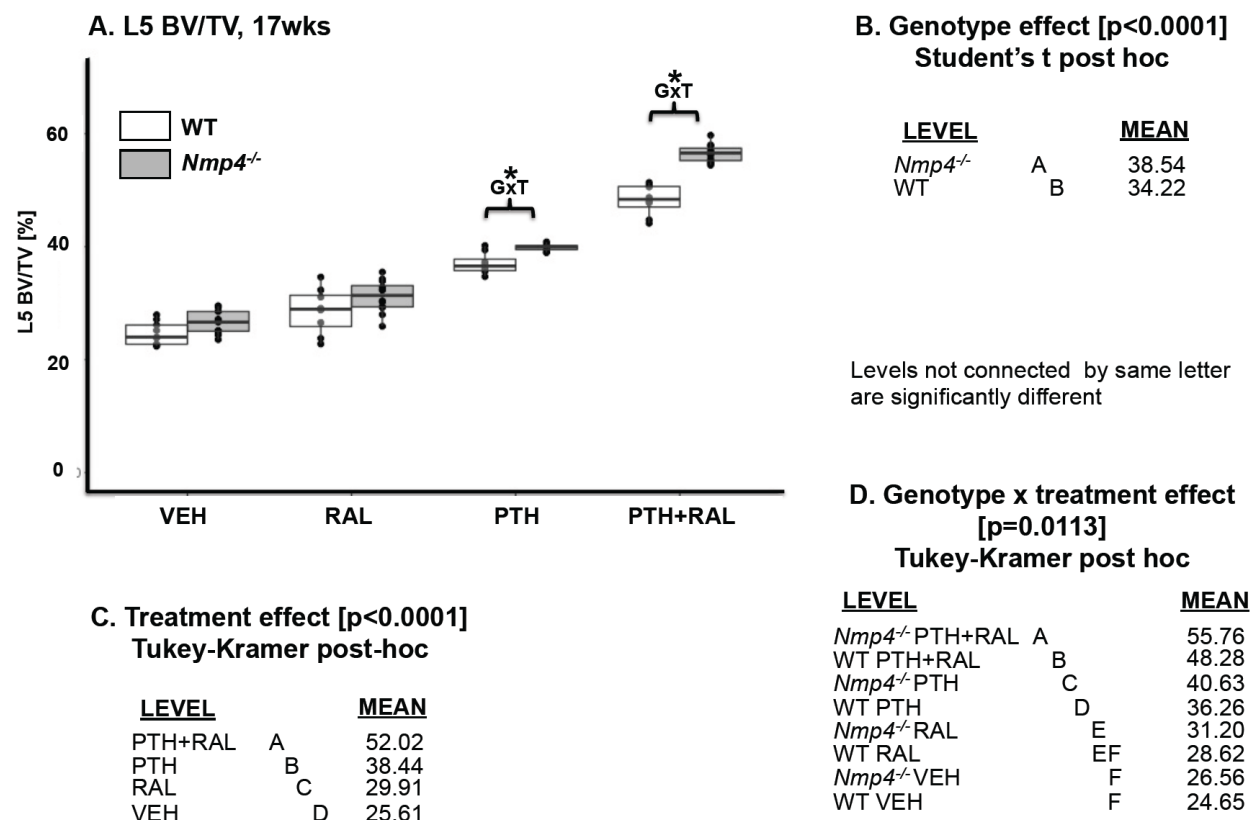


Supplemental Figure 4: The IPA Molecule Activity Predictor (MAP) algorithm indicated that loss of *Nmp4* elevates protein folding and attenuates endoplasmic reticulum stress-induced apoptosis. Molecules in pink-red are found in the dataset and are upregulated. Molecules that are green are found in the 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

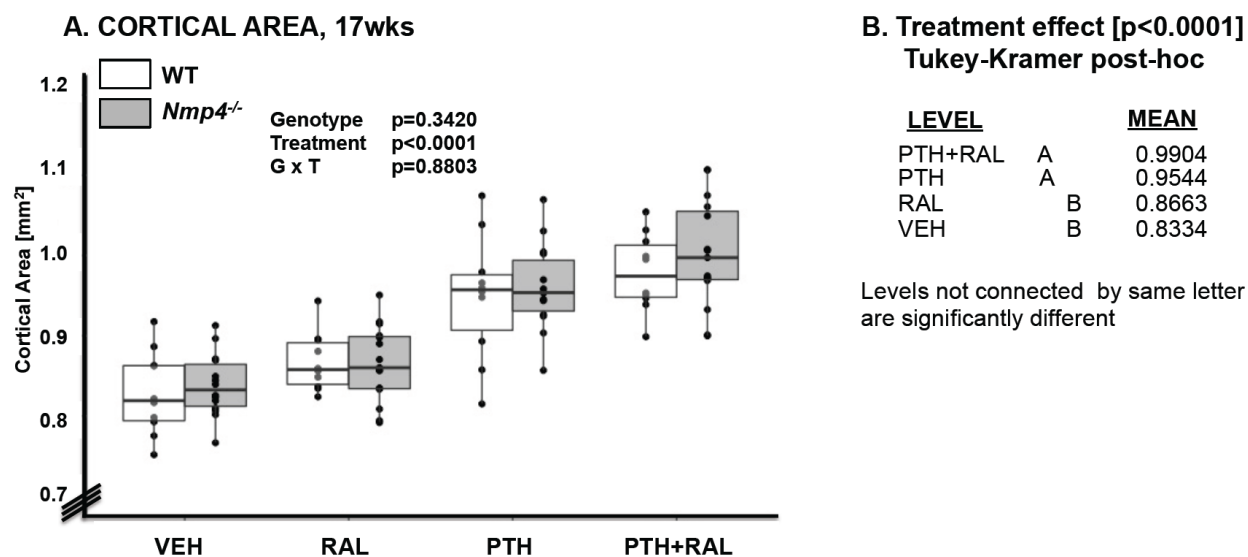
compared to the WT, with greater color saturation indicating higher expression, and green indicate reduced expression. The star ★ indicates Nmp4 binds proximal to the transcription start site or within the intron of the gene as determined by ChIP-seq analysis (Childress et al., 2015). Abbreviations for 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).



Supplemental Figure 5: **[A]** Femoral 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 $p \leq 0.05$. **[B]** There were a strong genotype effect and loss of Nmp4 enhanced femoral BV/TV over the cohorts **[C]** There was a strong treatment effect and PTH+RAL was the most efficacious osteoanabolic therapy **[D]** The analysis revealed a genotype x treatment interaction (denoted by an asterisk in the dot plot **[A]** showing improved response in the PTH mono-therapy and PTH+RAL combination therapy with loss of Nmp4. The data represents average $\pm$ SD, n=9-15 mice/group. See text for explanation of results.



Supplemental Figure 6: [A] L5 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 $p \leq 0.05$. **[B]** There were a strong genotype effect and loss of *Nmp4* enhanced femoral BV/TV over the cohorts **[C]** There was a strong treatment effect and PTH+RAL was the most efficacious osteoanabolic therapy **[D]** The analysis revealed a genotype x treatment interaction (denoted by an asterisk in the dot plot [A] showing improved response in the PTH mono-therapy and PTH+RAL combination therapy with loss of *Nmp4*. The data represents average $\pm$ SD, n=8-13 mice/group. See text for explanation of results.



Supplemental Figure 7: [A] Femoral cortical area (mid-shaft) 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. Note that the Y-axis is expanded for clarity. Statistical analyses were performed using 2W ANOVA tests setting genotype and treatment as the independent variables. Statistical significance was set at $p \leq 0.05$. There was no genotype effect or genotype x treatment interaction. **[B]** There was a strong treatment effect and PTH+RAL and PTH mono-therapy were more efficacious than the RAL mono-therapy. The data represents average $\pm$ SD, $n=10-15$ mice/group. See text for explanation of results.

SUPPLEMENTAL TABLE S5: FEMORAL CORTICAL PARAMETERS

GROUP	Total CSA (mm ²)	Marrow Area (mm ²)	Cortical Thickness (mm)	Periosteal BS (mm)	Endocortical BS (mm)	AP Width (mm)	ML Width (mm)
WT VEH	1.77±0.10	0.940±0.049	0.204±0.008	5.393±0.139	4.140±0.114	1.33±0.03	1.74±0.06
<i>Nmp4</i> ^{-/-} VEH	1.75±0.06	0.913±0.049	0.209±0.009	5.353±0.090	4.078±0.106	1.32±0.03	1.73±0.06
WT RAL	1.73±0.05	0.860±0.022	0.216±0.006	5.344±0.090	4.019±0.095	1.31±0.02	1.74±0.04
<i>Nmp4</i> ^{-/-} RAL	1.75±0.07	0.879±0.039	0.218±0.010	5.332±0.111	4.004±0.103	1.32±0.04	1.72±0.04
WT PTH	1.94±0.13	0.969±0.048	0.221±0.005	5.617±0.196	4.213±0.108	1.39±0.04	1.82±0.09
<i>Nmp4</i> ^{-/-} PTH	1.88±0.03	0.931±0.064	0.226±0.007	5.564±0.087	4.142±0.116	1.38±0.03	1.78±0.07
WT PTH+RAL	1.89±0.10	0.892±0.043	0.239±0.007	5.552±0.152	4.084±0.106	1.37±0.03	1.82±0.08
<i>Nmp4</i> ^{-/-} PTH+RAL	1.85±0.06	0.852±0.029	0.249±0.013	5.478±0.081	3.978±0.038	1.38±0.02	1.75±0.05
2W ANOVA <i>Levels not connected by same letter are significantly different</i>	G: p=0.20 T: p<0.0001 PTH: A P+R: A VEH: B RAL: B GXT: p=0.52	G: p=0.03 T: p<0.0001 PTH: A VEH: A P+R: B RAL: B GXT: p=0.15	G: p=0.008 T: p<0.0001 P+R: A PTH: B RAL: B VEH: C GXT: p=0.47	G: p=0.08 T: p<0.0001 PTH: A P+R: A VEH: B RAL: B GXT: p=0.85	G: p=0.003 T: p<0.0001 PTH: A VEH: A P+R: B RAL: B GXT: p=0.49	G: p=0.33 T: p<0.0001 PTH: A P+R: A VEH: B RAL: B GXT: p=0.37	G: p=0.01 T: p=0.0002 PTH: A P+R: A VEH: B RAL: B GXT: p=0.41
GROUP	AP/ML	I _{ap} (mm ⁴)	I _{ml} (mm ⁴)	I _{max} (mm ⁴)	I _{min} (mm ⁴)	Medial Extreme (mm)	TMD (g/cm ³ HA)
WT VEH	0.765±0.008	0.228±0.029	0.144±0.015	0.237±0.029	0.135±0.015	0.906±0.035	1.29±0.03
<i>Nmp4</i> ^{-/-} VEH	0.769±0.041	0.219±0.008	0.143±0.012	0.224±0.013	0.137±0.012	0.666±0.019	0.89±0.01
WT RAL	0.758±0.011	0.231±0.016	0.144±0.012	0.237±0.016	0.138±0.010	0.905±0.021	1.27±0.01
<i>Nmp4</i> ^{-/-} RAL	0.763±0.026	0.228±0.021	0.142±0.012	0.235±0.022	0.138±0.014	0.674±0.024	0.89±0.01
WT PTH	0.765±0.031	0.285±0.048	0.179±0.022	0.297±0.053	0.167±0.019	0.946±0.050	1.28±0.03
<i>Nmp4</i> ^{-/-} PTH	0.782±0.027	0.279±0.025	0.173±0.007	0.281±0.022	0.169±0.010	0.704±0.023	0.89±0.01
WT PTH+RAL	0.754±0.029	0.291±0.044	0.173±0.016	0.302±0.043	0.162±0.016	0.923±0.047	1.28±0.02
<i>Nmp4</i> ^{-/-} PTH+RAL	0.786±0.020	0.270±0.030	0.174±0.019	0.276±0.031	0.167±0.017	0.701±0.008	0.88±0.01
2W ANOVA <i>Levels not connected by same letter are significantly different</i>	G: p=0.01 T: p=0.46 GXT: p=0.26	G: p=0.13 T: p<0.0001 PTH: A P+R: A RAL: B VEH: B GXT: p=0.73	G: p=0.52 T: p<0.0001 PTH: A P+R: A RAL: B VEH: B GXT: p=0.90	G: p=0.03 T: p<0.0001 P+R: A PTH: A RAL: B VEH: B GXT: p=0.62	G: p=0.46 T: p<0.0001 PTH: A P+R: A RAL: B VEH: B GXT: p=0.93	G: p<0.0001 T: p=0.0005 PTH: A P+R: AB RAL: B VEH: B GXT: p=0.90	G: p<0.0001 T: p=0.23 GXT: p=0.36

ABBREVIATIONS: AP femoral anterior–posterior length; BS bone surface; CSA cross-sectional area; G genotype; GXT genotype x treatment interaction; I_{ap} moment of inertia about the AP axis; I_{max} maximum moment of inertia; I_{min} minimum moment of inertia; I_{ml} moment of inertia about the ML axis; ML the resected femoral medial–lateral; P+R PTH+RAL; RAL raloxifene; TMD tissue mineral density

SUPPLEMENTAL TABLE S6: ESTIMATED MATERIAL PROPERTIES

GROUP	Strain to Yield ($\mu\epsilon$)	Total Strain ($\mu\epsilon$)	Modulus (GPa)	Resilience (MPa)	Toughness (MPa)
WT VEH	16012 $\pm$ 1124	99029 $\pm$ 30556	7.87 $\pm$ 0.30	0.95 $\pm$ 0.18	9.19 $\pm$ 1.93
<i>Nmp4</i> ^{-/-} VEH	16982 $\pm$ 2858	82378 $\pm$ 26470	8.55 $\pm$ 0.54	1.08 $\pm$ 0.31	8.86 $\pm$ 2.59
WT RAL	16552 $\pm$ 371	83863 $\pm$ 19659	8.06 $\pm$ 0.75	1.06 $\pm$ 0.12	9.15 $\pm$ 1.86
<i>Nmp4</i> ^{-/-} RAL	16999 $\pm$ 2556	75610 $\pm$ 19939	8.24 $\pm$ 1.13	1.20 $\pm$ 0.35	8.95 $\pm$ 2.10
WT PTH	16881 $\pm$ 3008	90210 $\pm$ 29366	7.57 $\pm$ 1.23	0.85 $\pm$ 0.28	9.46 $\pm$ 2.63
<i>Nmp4</i> ^{-/-} PTH	15021 $\pm$ 1932	91519 $\pm$ 26401	7.93 $\pm$ 0.30	0.82 $\pm$ 0.23	10.31 $\pm$ 2.28
WT PTH+RAL	15452 $\pm$ 34324	69998 $\pm$ 19460	8.44 $\pm$ 0.38	0.89 $\pm$ 0.32	8.16 $\pm$ 1.90
<i>Nmp4</i> ^{-/-} PTH+RAL	15321 $\pm$ 708	63635 $\pm$ 16321	8.64 $\pm$ 1.06	0.99 $\pm$ 0.20	8.09 $\pm$ 1.91
2W ANOVA Levels not connected by same letter are significantly different	G: p=0.07 T: p=0.27 GXT: p=0.22	G: p=0.13 T: p=0.002 PTH: A VEH: A RAL: AB P+R: B GXT: p=0.64	G: p=0.06 T: p=0.03 P+R: A VEH: AB RAL: AB PTH: B GXT: p=0.75	G: p=0.13 T: p=0.003 RAL: A VEH: AB P+R: AB PTH: B GXT: p=0.65	G: p=0.89 T: p=0.06 GXT: p=0.79

ABBREVIATIONS: G genotype; P+R PTH+RAL; RAL raloxifene; T treatment

SUPPLEMENTAL TABLE S7: STRUCTURAL MECHANICAL PROPERTIES

GROUP	Yield force (N)	Ultimate force (N)	Displacement to yield (μm)	Post yield displacement (μm)	Total displacement (μm)	Stiffness (N/mm)	Work to Yield (mJ)	Post Yield Work (mJ)	Total Work (mJ)
WT VEH	10.08±0.99	13.52±1.16	162.51±12.49	843.05±320.03	1005.56±312.72	95.73±6.19	0.92±0.16	8.01±2.16	8.93±2.05
<i>Nmp4</i> ^{-/-} VEH	11.65±2.44	14.27±0.92	172.64±26.99	619.27±224.97	792.43±217.81	104.39±10.17	1.05±0.31	7.53±2.63	8.64±2.63
WT RAL	11.19±0.20	14.67±0.41	168.22±12.96	689.72±208.95	861.14±199.79	106.07±12.65	1.05±0.08	8.10±1.91	9.15±1.90
<i>Nmp4</i> ^{-/-} RAL	15.22±1.20	15.22±1.20	173.53±15.73	594.32±202.51	769.68±193.22	105.42±16.10	1.19±0.34	7.63±2.14	8.91±2.17
WT PTH	11.04±2.06	16.94±1.67	163.51±28.73	711.57±297.33	875.08±288.88	120.02±22.68	0.94±0.32	9.44±2.92	10.38±2.76
<i>Nmp4</i> ^{-/-} PTH	11.24±1.79	17.06±1.38	146.49±18.81	742±255.98	894.10±252.89	121.91±16.51	0.89±0.23	10.24±2.81	11.18±2.74
WT PTH+RAL	11.50±1.18	18.38±2.31	152.56±34.07	540.96±205.28	693.53±202.12	130.49±10.66	0.92±0.29	7.98±1.97	8.97±1.85
<i>Nmp4</i> ^{-/-} PTH+RAL	13.66±2.51	19.43±2.18	152.09±8.08	458.30±166.36	578.77±109.62	132.83±17.91	1.25±0.45	7.69±2.61	8.94±2.45
2W ANOVA Levels not connected by same letter are significantly different	G: p=0.004 T: p=0.02 P+R: A RAL: AB PTH: AB VEH: B GXT: p=0.41	G: p=0.07 T: p<0.0001 P+R: A PTH: B RAL: C VEH: C GXT: p=0.78	G: p=0.91 T: p=0.01 RAL: A VEH: AB PTH: AB P+R: B GXT: p=0.18	G: p=0.06 T: p=0.003 VEH: A PTH: A RAL: AB P+R: B GXT: p=0.35	G: p=0.04 T: p=0.0005 VEH: A PTH: A RAL: A P+R: B GXT: p=0.39	G: p=0.33 T: p<0.0001 P+R: A PTH: A RAL: B VEH: B GXT: p=0.76	G: p=0.03 T: p=0.10 GXT: p=0.22	G: p=0.83 T: p=0.01 PTH: A RAL: B P+R: B VEH: B GXT: p=0.78	G: p=0.89 T: p=0.02 PTH: A RAL: AB P+R: B VEH: B GXT: p=0.85

ABBREVIATIONS: G genotype; P+R PTH+RAL; RAL raloxifene; T treatment

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