Nmp4/CIZ Closes the Parathyroid Hormone Anabolic Window

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Abstract

Chronic degenerative diseases are increasing with the aging U.S. population. One consequence of this phenomenon is the need for long-term osteoporosis therapies. Parathyroid hormone (PTH), the only FDA-approved treatment that adds bone to the aged skeleton, loses its potency within two years of initial treatment but the mechanism regulating its limited "anabolic window" is unknown. We have discovered that disabling the nucleocytoplasmic shuttling transcription factor nuclear matrix protein 4/cas interacting zinc finger protein (*Nmp4/CIZ*) in mice extends the PTH bone-forming capacity. Nmp4 was discovered during our search for nuclear matrix transcription factors that couple this hormone's impact on osteoblast cytoskeletal and nuclear organization with its anabolic capacity. CIZ was independently discovered as a protein that associates with the focal adhesion-associated mechanosensor p130Cas. The *Nmp4/CIZ*-knockout (KO) skeletal phenotype exhibits a modestly enhanced bone mineral density but manifests an exaggerated response to both PTH and to BMP2 and is resistant to disuse-induced bone loss. The cellular basis of the global *Nmp4/CIZ*-KO skeletal phenotype remains to be elucidated but may involve an expansion of the bone marrow osteoprogenitor population along with modestly enhanced osteoblast and osteoclast activities supporting anabolic bone turnover. As a shuttling Cys_2His_2 zinc finger protein, *Nmp4/CIZ* acts as a repressive transcription factor perhaps associated with epigenetic remodeling complexes, but the functional significance of its interaction with p130Cas is not known. Despite numerous remaining questions, *Nmp4/CIZ* provides insights into how the anabolic window is regulated, and itself may provide an adjuvant therapy target for the treatment of osteoporosis by extending PTH anabolic efficacy.

Keywords

BMP2; osteoblasts; osteoclast; osteoporosis; osteoprogenitors; p130Cas

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I. THE PARATHYROID HORMONE ANABOLIC WINDOW

As the U.S. population ages[1] and life expectancy continues to increase,[2] more people experience chronic degenerative diseases[3] as observed with the burgeoning osteoporosis epidemic.[4,5] This necessitates improvements in strategies for long-term osteoporosis therapy.

Bisphosphonates, which are antiresorptive drugs, are the most prescribed medication for the management of osteoporosis and are highly effective at reducing the risk of osteoporotic fractures,[6] but recent concerns have emerged that their suppression of bone turnover or remodeling may actually weaken the skeleton after years of treatment.[7–9] Additionally, bisphosphonates are only marginally effective in subjects that present with significant loss of bone mineral density.[10]

Ideally, the optimum strategy for long-term osteoporosis therapy is to increase skeletal turnover or remodeling with a positive bone balance (anabolic remodeling), thus improving biomechanical properties while adding more bone.[11,12] Bone remodeling occurs in the basic multicellular unit (BMU) and is the process by which osteoblasts synthesize new bone within pits that have been excavated by osteoclasts.[12] This bone resorption and formation process is temporally and spatially linked, occurs throughout the life of the skeleton, contributes to maintaining the biomechanical integrity of the bone, and maintains calcium and phosphorous exchange between the skeleton and extracellular fluid. Bone modeling differs from remodeling in that bone formation and resorption are uncoupled in both time and space; this process mediates changes in bone size and shape during development.[12]

Parathyroid hormone (PTH) is the sole FDA-approved anabolic therapy for osteoporosis and indeed enhances anabolic modeling and remodeling; however, its bone-forming activity is intrinsically limited. Specifically, treatment with PTH induces a rapid increase in bone-formation markers, followed by increases in bone-resorption markers, opening an "anabolic window," defined as the time period when this drug is maximally anabolic.[13–16] Intermittent doses enhance anabolic remodeling and modeling and thus significantly increase bone formation resulting in reduced risk of fractures.[12,17] Within this window, PTH may initially induce modeling by activating the bone lining cells followed by increases in remodeling activity, which comprises the most significant aspect of the anabolic effect in humans.[18–20]

A number of clinical strategies have been evaluated for keeping the anabolic window open for extended periods of time with limited success. Briefly, coadministration of PTH with antiresorptives (e.g., bisphosphonates or raloxifene) failed to enhance the anabolic effect.[21–23] The cyclical administration of PTH (alternating three months on/off) versus daily treatment did not extend the anabolic window.[16] Furthermore, even a one-year PTH "drug holiday" did not improve the attenuated response to this hormone.[24]

The mechanisms regulating the extent of the PTH anabolic window are unknown, but we recently demonstrated that disabling the nucleocytoplasmic shuttling transcription factor *Nmp4/CIZ* in mice extends and enhances PTH anabolic action.[25,26] Therefore, targeting the *Nmp4/CIZ*-pathway is a potential adjuvant treatment for enhancing anabolic remodeling-based osteoporosis therapy.[27] This, however, first requires an understanding of how this novel restraining arm of bone anabolism works. Here, we review the present knowledge on *Nmp4/CIZ* within the context of a historical perspective and describe current areas of investigation.

II. HISTORICAL PERSPECTIVE: THE SEARCH FOR MECHANISMS THAT COUPLE CHANGES IN CELL MORPHOLOGY/ADHESION WITH GENE EXPRESSION

Two research groups independently discovered *Nmp4/CIZ* by asking distinct but ultimately related questions concerning the more mechanical aspects of signal transduction and gene expression. The late Hisamaru Hirai and colleagues first cloned *CIZ* from a screen for binding partners of the focal adhesion-associated adaptor protein p130Cas.[28] As an adaptor protein, p130Cas controls several signaling events by undergoing changes in phosphorylation and associating with numerous effector proteins in multimolecular complexes.[29,30] It is responsive to integrin stimulation and participates in the remodeling of actin stress fibers.[31] As a primary mechanical sensor, the mechanical extension or unfolding of p130Cas during cell stretching favors its phosphorylation by c-Src, which in turn triggers downstream mechanotransduction signaling cascades.[32] The p130Cas-signaling hub governs proliferation, cell survival, and adhesion/migration by regulating many signaling pathways including PI3K-Akt, GSK-3beta, Erk, and the nongenomic effects of ERalpha.[29,30,33] This adaptor protein also plays a significant role in the fusion of osteoclast precursors and osteoclast adhesion.[34–36] The Hirai study showed that *CIZ* shuttles between the cytoplasm and the nucleus and as a transcription factor binds to an AT-rich consensus sequence found in the gene regulatory regions of numerous matrix metalloproteinases.[28]

PTH induces changes in osteoblast shape,[37,38] cytoskeletal organization,[37,39] nuclear organization, [40,41] and the activity of focal adhesions.[42] Thus, we inquired whether this shift in cell shape mechanically alters gene expression critical to its anabolic effect.[43] Earlier work by Maniotis et al.,[44] Dhawan et al.,[45] and Thomas et al.[46] had demonstrated physical and functional connections between integrin receptors, the cytoskeleton, nuclear shape, DNA, and gene expression. We proposed that as part of the substructure of the cell, nuclear matrix architectural transcription factors convert alterations in cell shape/adhesion into bends and twists of target genes, which in turn could alter protein-protein interactions between transacting factors that regulate activity.[43] Consistent with this hypothesis, we characterized a PTH-responsive, nuclear matrix protein DNA-binding activity along the AT-rich regulatory region of the rat type I collagen gene that we designated as *Nmp4*.[47] Binding to the minor groove of AT-rich DNA is the preferred consensus sequence for some architectural transcription factors,[48] and we demonstrated that *Nmp4* indeed binds to DNA in this manner and is capable of bending the type I collagen gene.[47]

Combining these two different pictures of *Nmp4/CIZ* led us to propose the concept of the *mechanosome*, the functional interaction between a mechanosensor associated with an adhesion complex and a shuttling architectural transcription factor.[49,50] In this hypothetical scheme, the mechanosome converts the unfolding of p130Cas in the cytoplasm to *Nmp4/ CIZ*-mediated DNA bending in the nucleus. This idea was derived in part from earlier studies on the role of beta-catenin/LEF-1 in mediating osteoblast response to mechanical load.[51,52] LEF-1 is also an architectural transcription factor,[53] and beta-catenin is part of a complex of proteins that constitute adherens junctions; LEF-1/beta-catenin is an integral component of the Wnt signaling pathway, which is a central regulator of skeletal modeling and remodeling.[54]

Although ubiquitously expressed,[55] the underlying assumption was that the impact of *Nmp4/CIZ* on bone phenotype, and thus on the mechanosome, was specific to its control of the osteoblast.[49,56] However, a recent study from our laboratory suggests that this protein regulates osteoclasts as well, and thus governs both the formation and resorption arms of

bone remodeling.[25] This suggests a need to expand the mechanosome hypothesis, which we take up again at the end of this review.

III. *Nmp4/CIZ* GLOBAL KNOCKOUT PHENOTYPE: NO GROSS ABNORMALITIES, NORMAL LIFE SPAN

A global "*CIZ*-deficient" mouse[57] and a global *Nmp4*-knockout (KO) mouse[26] were constructed independently by different homologous recombination strategies. The *CIZ*-deficient mouse was prepared by introducing β-galactosidase fused to the *CIZ*-N terminus and neo resistance gene into the second exon of the CIZ gene.[57] The *Nmp4*-KO mouse was prepared by replacing exons 4–7 with the neo gene cassette.[26] The description of the baseline phenotype of the *Nmp4*-KO mouse was determined from two colony populations: (i) the KO and wild-type (WT) F2 progeny of the mating of the first heterozygotes, which were a hybrid of 129SvEv and C57BL/6J, and (ii) these F2 mice that had been backcrossed six generations onto a C57BL/6J background (these N6 animals were compared to WT C57BL/6J mice).[26] The baseline phenotype of the *CIZ*-deficient mouse was described by comparing WT and null mice on a DBA (25%) × C57BL/6 F2 background that had been derived from the original heterozygous cross.[56,57]

Nmp4/CIZ is ubiquitously expressed, yet the global *Nmp4*-KO and *CIZ*-deficient mice are healthy, have a normal lifespan, and are free of any gross morphological defects.[26,56,57] Both the *Nmp4*-KO F2 and *CIZ*-deficient male and female mice show a modest (4–10%), but significant, decrease in body weight compared to their WT littermates, and this phenotype appears to be retained throughout adulthood.[26,57] We have not observed this decreased body weight in the N6 *Nmp4*-KO mice.[26] The male *CIZ*-deficient mice exhibit variable degrees of spermatogenic cell degeneration within the seminiferous tubules, and these animals suffer from sporadic infertility.[57] Our anecdotal observations on the *Nmp4*-KO males are consistent with this aspect of the phenotype. Additionally, we have reported that the *Nmp4*-KO litters are modestly, but significantly, smaller than WT litters.[26] Finally, the Mendelian frequencies expected from matings between *Nmp4* heterozygous mice show a significantly diminished yield of KO pups, suggesting that some of the null embryos die *in utero*.[26]

IV. *Nmp4/CIZ* GLOBAL KNOCKOUT SKELETAL PHENOTYPE: MODESTLY ENHANCED BASELINE BONE MINERAL DENSITY, YET EVIDENCE FOR AN OVERACTIVE OSTEOCLAST

The baseline skeletal phenotypes of the *Nmp4*-KO and *CIZ*-deficient mice at eight weeks of age are similar with some important distinctions, which may be due to the differences in strain backgrounds.[26,56] Both the *Nmp4*-KO and *CIZ*-deficient mice exhibited modest but significantly enhanced bone mineral density and bone mineral content throughout the skeleton. Additionally, we observed no significant difference in *Nmp4*-KO and WT femur lengths.[26] Although significant differences in trabecular bone were not detected in the *Nmp4*-KO mice, the *CIZ*-deficient animals exhibited a 40% increase in femoral BV/TV and 15% increase in vertebral BV/TV. Furthermore, there was a modest, but significant, increase in matrix apposition rate and bone formation rate in the femoral cross sections evaluated in the *CIZ*-deficient mice compared to the WT animals.[56]

On the other hand, the *Nmp4*-KO mice exhibited an osteoclast phenotype,[25] which was absent in the *CIZ*-deficient animals.[56] Specifically, the *Nmp4*-KO mice showed elevated serum C-terminal telopeptides (CTXs), a marker for resorption over a seven-week time-course experiment (10–17 weeks of age).[25] Additionally, the null marrow from eight-week-

old mice yielded twofold more osteoclasts than did WT marrow, and the isolated *Nmp4*-KO osteoclasts exhibited an elevated resorption activity compared to the WT osteoclasts.[25] Histomorphometric analysis revealed an elevated baseline number of tartrate-resistant acid phosphatase positive-staining cells (TRAP+ n/BS) in 17-week-old *Nmp4*-KO mice, which provides an estimate of the number of osteoclast precursors and mature osteoclasts normalized to the bone surface.[25] This is consistent with our recent evidence showing that the null mice exhibited a modest but significant increase in CFU-GM cells (osteoclast precursors) compared to their WT littermates.[58,59]

V. *Nmp4/CIZ* GLOBAL KNOCKOUT SKELETAL PHENOTYPE: AN EXTENDED PTH ANABOLIC WINDOW, ENHANCED RESPONSE TO BMP2, AND RESISTANCE TO DISUSE-INDUCED BONE LOSS

The response of the *Nmp4*-KO mouse to intermittent doses of PTH demonstrates that the length and amplitude of the anabolic window has been expanded in these animals.[25,26] In a typical experiment, female WT and null mice were sorted into four treatment groups and administered human PTH(1–34), 30μg/kg/day, or vehicle control for seven weeks (10–17 weeks of age). Serum analysis revealed that osteocalcin levels, a marker for bone formation and osteoblast number, in the WT animals significantly increased with PTH, peaked at three weeks of treatment, and declined to baseline levels by seven weeks of hormone challenge.[25] In contrast, the PTH-induced increase in serum osteocalcin in the *Nmp4*-KO mice continued to rise throughout the duration of the seven-week regimen.[25] Evaluation of both the raw and the percent change osteocalcin data using a two-way ANOVA indicated a strong interaction between the independent variables of genotype and treatment.[25] As described above, the null mice showed elevated serum CTX compared to the WT mice and PTH stimulated a near-significant increase in both genotypes, but evaluation of both the raw data and the percent change data showed no interaction between the independent variables of genotype and treatment.[25] At the end of seven weeks of treatment, the KO mice manifested a strikingly enhanced PTH-induced increase in trabecular bone while maintaining robust increases in cortical bone as compared to WT animals. For example, the null mice gained approximately 2.6-fold more femoral trabecular bone (BV/TV), 1.4-fold more L5 vertebral bone, and 1.1-fold higher tibial trabecular bone, all showing significant genotype x treatment interactions.[25,26]

Interestingly, the WT and *Nmp4*-KO mice exhibited equivalent PTH-induced gains in trabecular bone during the first two weeks of treatment, but at three weeks the null mice showed significantly more hormone-stimulated addition of bone than did the WT animals, which coincided with the serum osteocalcin profiles.[25,59] The femoral bone histomorphometry measurements at the end of the seven-week treatment period showed no difference in bone formation rate (BFR) between the genotypes,[25] but these experiments did not analyze vertebral or tibial histomorphmetry at this time point to determine the basis of the continued increase in serum osteocalcin. Further histomorphometric and bone microarchitecture analyses at seven weeks treatment and beyond are required to fully characterize this extended anabolic window.

The WT and KO femoral mRNA profiles harvested at two weeks and three weeks of treatment (12–13 weeks of age) represented a composite of multiple marrow and bone cell types, but provided clues as to the early events leading toward the development of the enhanced PTH-stimulated bone formation response in the null mice.[25] Consistent with the two-week μCT data showing equivalent amounts of new bone added in this early phase of treatment, the WT and KO mRNA profiles showed similar PTH-induced increases in the expression of genes that support bone formation, e.g., *Runx2, Osterix, Col1a1, Alpl*

(alkaline phosphatase), and *Pthr1*.[25] However, there was a transient enhanced response of the AP-1 transcription factors *c-fos* and *Fra-2* to PTH in the *Nmp4*-KO mice, and there was an elevated baseline expression of *Nfatc1* at two weeks but not three weeks into the treatment regimen.[25] These transcription factors have numerous important functional roles in bone physiology, but are particularly significant to the development of the osteoblast, osteoclast, and chondrocyte.[60–62] The significance of the transient nature of this increase in gene expression remains to be determined, but may involve the enhanced hormone mobilization of both osteoprogenitors and osteoclast progenitors at the beginning of the anabolic window. Additionally, the null mice exhibited a significant decrease in the baseline expression of the proapoptotic gene *Bax*, irrespective of treatment, perhaps indicative of longer-lived osteoblasts supporting the enhanced bone formation. Finally, the *Rankl/Opg* ratio was diminished in the *Nmp4*-KO animals compared to WT mice at two and three weeks of treatment, which was significant at the later time point. This is consistent with a null bone microenvironment permissive for bone formation.[25]

In addition to its role extending the PTH anabolic window, *Nmp4/CIZ* also plays a role in BMP2-induced increase in bone formation.[56] Briefly, eight-week-old female *CIZ*-deficient mice received rhBMP2 (5 μg) injections onto the parietal bones every other day for 10 days. Soft X-ray analysis demonstrated that the *CIZ*-deficient mice formed twofold more new bone than their WT counterparts during the same 10-day treatment period.[56] In a related experiment from the same study, *CIZ* deficiency also significantly enhanced the formation of new bone during the recovery from surgical marrow ablation.[56]

Another remarkable aspect the *CIZ*-deficient skeletal phenotype is its resistance to disuse-induced bone loss.[63] In these experiments, 10-week-old male *CIZ*-deficient and WT mice were subjected to hind limb unloading by tail suspension for two weeks. Unloading induced significant trabecular and cortical bone loss in the WT mice, but not in the null mice. Histomorphometric analysis showed that the unloaded WT limb exhibited decreases in mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS), and BFR in both the cancellous and cortical compartments, but these parameters were unaffected in the unloaded *CIZ*-deficient femur. Interestingly, bone marrow stromal cells (BMSCs) from unloaded WT mice showed a significantly decreased mineralization in culture as compared to cells from the loaded controls; however, this disparity was not observed in cultures from null mice.[63] Finally, osteoclastic bone resorption parameters were similar in the genotypes irrespective of treatment. Quantification of osteoclast surface per bone surface (Oc.S/BS) and osteoclast number per bone surface (Oc.N/BS) in the cancellous bones of the tibia were not affected by unloading in either genotype. Also, the WT and *CIZ*-deficient bone marrow yielded equivalent numbers of osteoclasts in cultures generated from unloaded and loaded bones.[63]

VI. CELLULAR BASIS OF THE *Nmp4/CIZ*-KNOCKOUT SKELETAL PHENOTYPE: MORE BONE CELLS AND HEIGHTENED RESPONSIVENESS TO ANABOLIC STIMULI KEEPS THE ANABOLIC WINDOW OPEN

Recent data from our laboratory demonstrated that bone marrow from female *Nmp4*-KO mice yielded approximately fourfold more osteoprogenitor cells, as assessed by the CFU-F[AlkPhos+] colony assay, than did the marrow from their WT littermates.[58,59] Additionally, the null bone marrow contained twofold more CD8+ T cells than did the WT bone marrow, although there was no difference in the number of CD8+ T cells in the peripheral blood between the genotypes.[58,59] Interestingly, the CD8+ T cells have been shown to support the PTH-induced anabolic response. Specifically, these cells express the PTH receptor (PTHR1)

and secrete Wnt10b in response to PTH, which in turn enhances osteoblast proliferation, differentiation, and life span.[64]

The impact of *Nmp4/CIZ* on the baseline phenotype of osteoblasts and its expression during bone cell development has not been fully elucidated. BMSC-derived osteoblasts from the *CIZ*-deficient mice exhibited an enhanced alkaline phosphatase activity at 10 days in culture and formed more mineralized nodules than did WT cells.[56] On the other hand, the calvarial-derived osteoblast-like cell line MC3T3-E1 also formed fewer mineralized nodules in culture when overexpressing *CIZ*.[65] Consistent with these *in vitro* observations, mRNA expression of genes that support the osteoblast phenotype, including *Col1a1*, *Osterix*, and *Opn* (osteopontin), were modestly, yet significantly, elevated in the humeri of untreated *CIZ*-deficient mice, compared to their WT counterparts.[56]

Of note, we have been unable to reproducibly demonstrate a significant impact of *Nmp4/CIZ* on the baseline mature osteoblast phenotype using cells derived from the *Nmp4*-KO mice. We have not observed striking differences in population doubling times, alkaline phosphatase expression, or mineralization in primary cultures of BMSC-derived osteoblasts obtained from WT and *Nmp4*-null animals.[26] Specifically, BMSC-derived WT and *Nmp4*-KO osteoblasts cultured under standard osteogenic conditions for 10–14 days[66] showed no difference in basal mRNA expression of *Runx2*, O*sterix*, *Alpl*, *Igf1*, *Mmp13*, *Rankl*, *Opg*, *osteocalcin (Oc)*, or *Opn*.[26] Nor did we observe an enhanced expression of these genes in the femurs of untreated eight-week-old *Nmp4*-null animals save for a modest, near-significant increase in *Col1a1*.[26] Finally, we did not discern any differences in these parameters between WT and null immortalized clonal cell lines.[67]

That said, osteoblasts derived from both the *CIZ*-deficient and *Nmp4*-KO mice showed a modestly heightened response to a variety of anabolic stimuli, and this characteristic likely contributes to the extended PTH anabolic window and underlies the augmented BMP2-stimulated bone formation. [65,67] As previously indicated, PTH-induced *c-fos* mRNA expression was transiently enhanced in *Nmp4*-KO femoral bone, compared to WT bone, one hour after the last hormone injection at two weeks of PTH treatment.[25] Consistent with the *in vivo* observation, primary *Nmp4*-KO BMSC-derived osteoblasts and an *Nmp4*-null clonal cell line both showed a modest concentration-dependent enhancement in PTH-stimulated increases in *c-fos* mRNA expression as compared to that observed in WT bone cells.[67]

With regard to BMP2 responsiveness, *CIZ*-deficient BMSC-derived osteoblasts maintained in 100 ng/ml BMP2 for 10 days exhibited an enhanced alkaline phosphatase activity as compared to WT cells maintained under the same conditions.[56] Additionally, overexpression of *CIZ* in MC3T3-E1 cells suppressed BMP2-enhanced mRNA expression of *Alpl*, *Oc*, and *Col1a1*.[65] Similarly, we demonstrated enhanced responsiveness of *Nmp4*-KO osteoblasts to BMP2, but this sensitivity was dependent on the number of days the cells were in culture and the number of days in the presence of this growth factor.[67] Specifically, the primary BMSC-derived *Nmp4*-null osteoblasts exhibited an enhanced BMP2-induced increase in mRNA *Alpl* expression after eight days in culture of which the last three days included treatment with this potent anabolic agonist. However, WT and *Nmp4*-KO BMSC-derived osteoblasts displayed equal increases in mRNA *Alpl* expression when evaluated after 10 days exposure to BMP2 and 15 days in culture.[67]

Nmp4/CIZ regulation of osteoblast responsiveness to anabolic stimuli does not appear to be limited to gene expression but also impacts cell signaling as well.[68] As an example, primary *Nmp4*-KO calvarial-derived osteoblasts exhibited an enhanced oscillatory shear stress (OSS)–induced activation of the Wnt signaling pathway.[68] OSS is the cellular analogue to mechanical loading, a strong anabolic agonist to bone.[69] OSS induced an enhanced

phosphorylation of ERK, Akt, and GSK-3beta in the *Nmp4*-KO osteoblasts compared to WT cells within minutes of challenge.[68] Consistent with these observations, the null cells exhibited an increased OSS-stimulated beta-catenin nuclear translocation.[68] Finally, in a potentially telling observation, we observed that OSS stimulated a more extensive reorganization of stress fibers and the formation of focal adhesions in the *Nmp4*-null cells than in the WT osteoblasts.[68] This is consistent with *Nmp4/CIZ* binding to the focal adhesion-associated adaptor protein p130Cas.

Recent data from our laboratory indicate that *Nmp4/CIZ*-KO osteoblasts are not the only cell type with a heightened response to agonists that regulate bone remodeling.[25] As detailed earlier, we reported that the *Nmp4*-KO mature osteoclasts exhibited an enhanced *in vitro* resorption activity in response to the cytokines RANKL/M-CSF compared to WT cells.[25] This raises the question as to whether there is a common *Nmp4/CIZ*-driven pathway in both osteoblasts and osteoclasts, and other cell types, involved in cell response. For example, this may involve *Nmp4/CIZ* association with p130Cas as a central hub for regulating multiple signal transduction pathways and *Nmp4/CIZ* association with epigenetic remodeling proteins for regulating multiple gene targets; this universal and wide-ranging molecular mechanism could act to suppress a variety of cell type–specific responses to multiple agonists (see below for further discussion).

We propose that *Nmp4* restricts the PTH anabolic window by repressing osteoprogenitor pool expansion and dampening cell sensitivity to anabolic stimuli. PTH recruits osteoprogenitors into the osteoblast differentiation pathway and enhances their survival,[70–72] Thus, the observed three-week lag period before the *Nmp4*-KO mice exhibit enhanced hormone-stimulated bone formation and show a surge in serum osteocalcin compared to the WT animals[25,59] may be due to the differential depletion of the osteoprogenitors between the genotypes as they are recruited into the anabolic window. The increased presence of the BM CD8+ T cells may bolster the expansion of the null osteoprogenitors as a consequence of enhanced secretion of Wnt10b (see Fig. 1), which, in turn, may surpass the modest elevation in bone resorption observed in the *Nmp4*-KO mice. Finally, the subtle increased sensitivity of the null osteoprogenitors/osteoblasts to anabolic stimuli (PTH, BMP2, load) may further augment and extend the PTH anabolic window. As mentioned previously, the discrepancies in cell phenotype (osteoblasts and osteoclasts) noted between the *CIZ*-deficient and *Nmp4*-KO mice may be due to strain differences between the mouse models. Alternatively, perhaps this marginally augmented bone-forming activity is difficult to reproduce *in vitro* outside the confines of the bone marrow microenvironment. The observation that the time in culture is significant to the sensitivity of the null cells to BMP2 suggests that *Nmp4/CIZ* impact on osteoblast phenotype may be specific to the stage of differentiation. If the disrupted association of *Nmp4/CIZ* and the mechanosensor p130Cas plays a significant role in the expression of the null osteoblast phenotype, then it is conceivable that subtle changes in cell culture density and adhesion could significantly impact the behavior of the cells. Clearly, more study is required on *Nmp4/CIZ* control of osteoblast and osteoclast phenotypes.

VII. *Nmp4/CIZ* MOLECULAR PROFILE: FUNCTIONAL DOMAINS FOR REGULATING CELL SIGNALING AND TRANSCRIPTION

Nmp4/CIZ is a nucleocytoplasmic shuttling protein, appears to be expressed in most if not all tissues, and is highly conserved between rodents and humans (~96%).[55] This single-copy gene is located on mouse chromosome 6 band F1.[73] The human ortholog (*ZNF384*) is located on chromosome 12p12 and was first identified as a partial cDNA, *TNRC1* (*CAGH1*) from a human brain expression library.[74] The rat gene gives rise to at least seven isoforms

with molecular weights ranging from approximately 40 to 63 kDa, but it is unknown whether these proteins have distinct cellular functions.[28,55]

All *Nmp4/CIZ* isoforms have from five to eight Cys2His2 zinc fingers, e.g., the *Nmp4*-21H/*CIZ*6-2 isoform has six fingers (Fig. 2). The zinc fingers #2, #3, and #6 of this isoform bind to the minor groove of the unusual homopolymeric (dAdT) consensus site.[75] The zinc finger domain mediates nuclear localization and association with the nuclear matrix[76] (Fig. 2). The AT-hook domain of the *Nmp4/CIZ* protein weakly associates with the DNA major groove and may provide this protein with its capacity for bending DNA.[47] The AT-hook is a common functional domain for certain architectural transcription factors such as HMGA1, proteins that regulate gene expression by bending or looping the DNA, thus altering interactions between other trans-acting proteins.[48] Architectural transcription factors typically do not have a transacting domain, but numerous conventional transcription factors alter local DNA structure as part of their regulatory function. *Nmp4/CIZ* has context-dependent transactivation domains, i.e., their activity is dependent on the organization of the regulatory elements surrounding the *Nmp4/CIZ* consensus sequence[75] (Fig. 2).

As a transcription factor, *Nmp4/CIZ* often acts as a suppressor consistent with its blunting of bone response to PTH and BMP2.[55,65,77] We have demonstrated that *Nmp4/CIZ* represses PTH-stimulated *Mmp13* transcription induction.[77] Mmp13 is significant to PTH-induced remodeling, and the human, rat, and mouse genes have a conserved PTH-response regulatory region. In the rat gene, this region spans nucleotides (nt) −148 to −38 and mediates the binding of multiple trans-acting proteins including Runx2, Ets-1, and cFos/c-Jun[77] (Fig. 3). These proteins support hormone transcription induction but the mechanistic details with respect to their interactions have not been fully elucidated. *Nmp4/CIZ* binds to this PTH response region adjacent to the distal Runx2 site (Fig. 3), and to address the significance of this interaction to PTH-stimulated induction we made a series of null-binding mutations within this PTH response region, including mutating individually the *Nmp4/CIZ* consensus site and the Runx2 consensus site, as well as mutating both the *Nmp4/CIZ* and Runx2 sites in combination.[77] These mutations were introduced into an *Mmp13* promoter-reporter construct containing the first 1329nt of the 5′-regulatory region and transfected into UMR 106-01 osteoblast-like cells. Exposure of the cells to PTH induced a twofold increase in activity in the WT *Mmp13* promoter-reporter, a fourfold increase in the construct containing the mutated *Nmp4/CIZ* site, a near twofold response in the construct containing the distal Runx2 binding site mutation, and an 11-fold increase in the *Mmp13* promoter-reporter construct harboring both the *Nmp4/CIZ* and Runx2 binding site mutations[77] (Fig. 3).

Partridge and colleagues have recently showed that histone deacetylase 4 (HDAC4) interacts with Runx2, binds the *Mmp13* promoter near the distal Runx2 consensus sequence adjacent to the *Nmp4* binding site, and suppresses *Mmp13* gene transcription in osteoblasts.[78] PTH induced the rapid cAMP-dependent protein kinase-dependent release of HDAC4 from the promoter and subsequent transcription of *Mmp13*. Knockout of HDAC4 led to an increase in *Mmp13* expression, and overexpression of HDAC4 decreased the PTH induction of *Mmp13*.[78] Additionally, PTH stimulated HDAC4 gene expression and enzymatic activity at times corresponding to the reassociation of HDAC4 with the *Mmp13* promoter and a decline in its transcription. These data, in conjunction with our previous study, raise the question as to whether *Nmp4* plays a significant role in regulating epigenetic remodeling along this site (Fig. 3).

Nmp4/CIZ may also act as a signaling molecule. As previously described, *Nmp4*-KO cells exhibit an enhanced phosphorylation of ERK, Akt, and GSK-3beta within minutes of exposure[68] suggesting that this nucleocytoplasmic shuttling protein has significant

cytoplasmic activities. The adaptor protein p130Cas regulates these signaling pathways[29,30,33] consistent with the *Nmp4*-p130Cas association.[28] The sequence APPKPPR from the 186th amino acid residue in the rat *Nmp4/CIZ* ortholog corresponds to the p130Cas SH3-binding consensus sequence XXPXKPX, and it also resembles the p130Cas SH3-binding site of FAK (APPKPSR)[28] (see Fig. 2). *Nmp4/CIZ* binding activity can distinguish between the SH3 domains of p130Cas, Abl, Ash/Grb2, Crk, Fyn, and Src as determined by far-Western screening.[28] The human ortholog ZNF384 does not bind to p130Cas but instead binds to zyxin, which in turn binds to p130Cas.[79] Although the rat *Nmp4/CIZ* and human ZNF384 are highly conserved, one proline residue in the p130Cas SH3-binding consensus sequence is absent in the latter, which prevents the human protein from binding to p130Cas directly, but instead mediates its association with zyxin and in turn zyxin binds to p130Cas in the human cell.[79] Zyxin is also an adaptor protein that associates with focal adhesions.[80] Interestingly, this SH3-binding functional domain overlaps with the AT-hook motif (Fig. 2). The functional significance of this juxtaposition, if any, remains to be elucidated but raises the question as to whether it mediates dual and distinct functions in the cytoplasm and nucleus.

VIII. SUMMARY: IS *Nmp4/CIZ* AN ADJUVANT THERAPY TARGET FOR KEEPING THE PTH ANABOLIC WINDOW OPEN?

The *Nmp4/CIZ*-KO mouse skeletal phenotype has potentially profound clinical significance in that it offers the first clues as to the nature of the regulation of the PTH anabolic window. Disabling this protein augments and extends PTH anabolic activity and thus might ultimately provide a novel target as the basis of an adjuvant therapy for improving this hormone's clinical efficacy.[27] An additional attractive aspect of the null skeletal phenotype is its resistance to disuse-induced bone loss since compromised mobility, common in the elderly population, often accompanies and exacerbates osteoporosis.[81] Finally, disabling *Nmp4* in a patient may not present problems with collateral damage since the global KO mice are healthy and long lived and appear not to suffer from the loss of this protein with the exception of sporadic male infertility.

The cellular basis of *Nmp4*'s control of the PTH anabolic window appears to involve its suppression of the size of the osteoprogenitor pool from which the hormone recruits osteoblasts and the repression of bone cell response to PTH and other anabolic stimuli. The modest elevation of bone resorption observed in the *Nmp4*-KO mice may contribute to anabolic bone turnover. Finally, the baseline increase in T cells from the knockout marrow may enhance bone gain through production of Wnt10b, a PTH response mediator.

Fully elucidating the molecular mechanisms of *Nmp4/CIZ* action will be of considerable importance for pursuing this molecule as a therapeutic target. Whether *Nmp4/CIZ* is significant to epigenetic remodeling is currently under study in our laboratory. Drugs targeting epigenetic processes, or "epidrugs," are at the forefront of drug discovery. [82] The impact of *Nmp4/CIZ* on osteoblast cell signaling is intriguing, but whether its interaction with p130Cas is functionally significant to this phenomenon and the regulation of the PTH anabolic window is unclear. Finally, this question remains: Is there a single novel *Nmp4/ CIZ* signaling-transcriptional pathway operative in both osteoblasts and osteoclasts and can this be used as the basis of a drug strategy for controlling the bone formation and bone resorption arms of the remodeling process?

Our original goal of understanding the connection between PTH-induced changes in osteoblast shape and the hormone's anabolic effect on bone remains elusive. We have shown that *Nmp4/CIZ* has a significant repressive action on PTH-stimulated bone formation and that this protein has the capacity for bending DNA as an architectural transcription

factor while localizing to the osteoblast nuclear matrix. As described above, others have demonstrated that *Nmp4/CIZ* associates with the focal adhesion-associated proteins p130Cas and zyxin; therefore, the function, location, and protein-protein associations of *Nmp4/CIZ* are all consistent with our original hypothesized function of carrying PTH-generated mechanical information from the membrane to the nucleus resulting in a change in bone formation. However, we still lack key evidence demonstrating that *Nmp4/CIZ* translates changes in cell morphology/adhesion into alterations in gene expression. In its current form, the mechanosome hypothesis is osteoblast-centric, and if the Nmp4 regulation of the PTH anabolic window involves multiple cell types, then the mechanosome hypothesis must be reevaluated in this broader context. Does *Nmp4/CIZ* play a similar role in many different kinds of cells within the bone marrow microenvironment and if so what is this novel function and how does it culminate in closing the PTH anabolic window?

Acknowledgments

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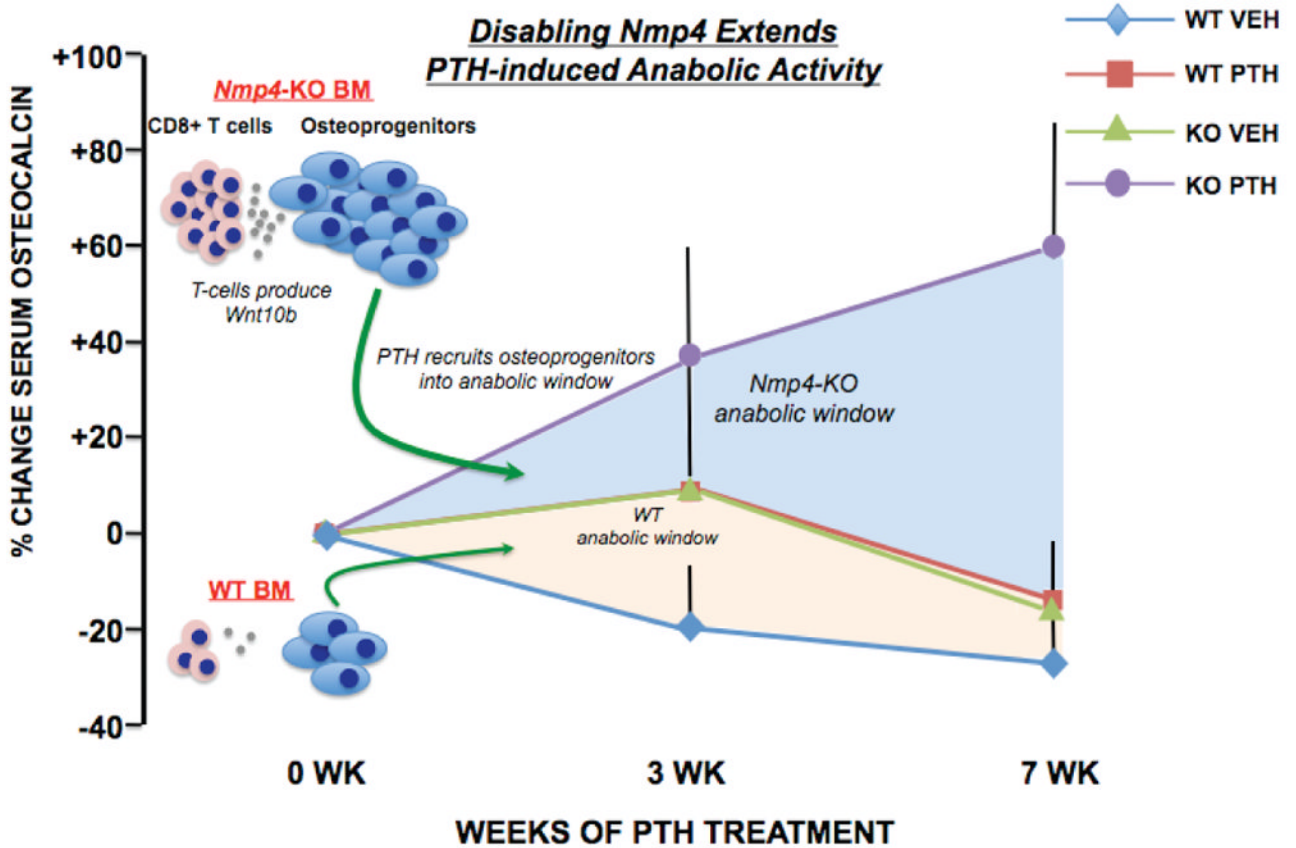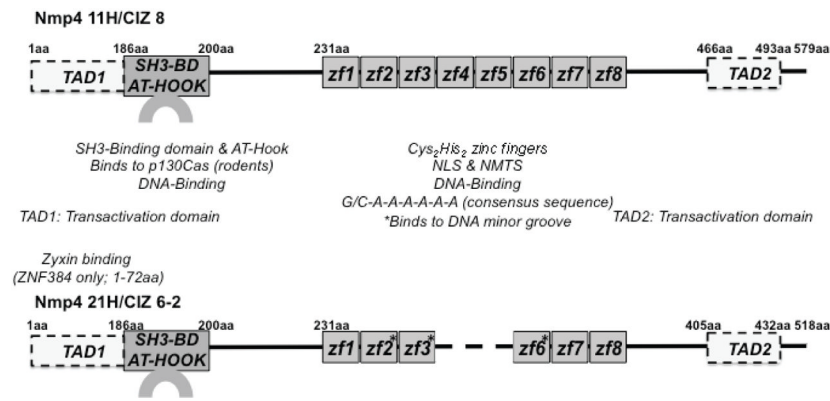
FIGURE 1.
Disabing Nmp4 in mice extends the PTH anabolic window. The percent change of serum osteocalcin (a marker for bone formation and osteoblast number) in WT and *Nmp4*-KO mice treated with intermittent PTH or vehicle (VEH) for seven weeks (see Ref. 25 for details). Null mice treated with PTH showed an elevated and extended bone-forming period compared to WT-treated mice. We propose an expanded osteoprogenitor pool bolstered by CD8+ T cells that express Wnt10b supports this extended anabolic window.[58,59]

FIGURE 2.

Nmp4/CIZ has the functional domains of a transcription factor and signaling molecule. Rat isoforms Nmp4 11H [CIZ 8] and Nmp4 21H [CIZ 6-2] are shown.[28,55] The amino acid positions are indicated by numerals but are not positioned to scale. The Cys_2His_2 zinc fingers act as a nuclear localization signal (NLS) and a nuclear matrix-targeting signal (NMTS); for Nmp4 11H, the zinc fingers #4–#8 are minimally required for the NLS and NMTS.[76] The Nmp4/CIZ SH3-binding domain (SH3-BD) associates with p130Cas in rodent cells; however, the absence of a single proline residue in the human ortholog ZNF384 obviates p130Cas association, but instead ZNF384 binds to zyxin.[28,79] The transactivation and DNA-binding domains were mapped using Nmp4 21H.[75] DNA binding to the minor groove of DNA is mediated by the zinc fingers #2, #3, and #6 (asterisks), whereas the AT-hook motif, which overlaps with the p130Cas SH3-binding sequence, weakly associates with the DNA major groove. There are context-dependent transactivation domains within the first 186aa (TAD1) of the protein and within a polyglutamine/alanine repeat (TAD2) (see Ref. 75 for full discussion).
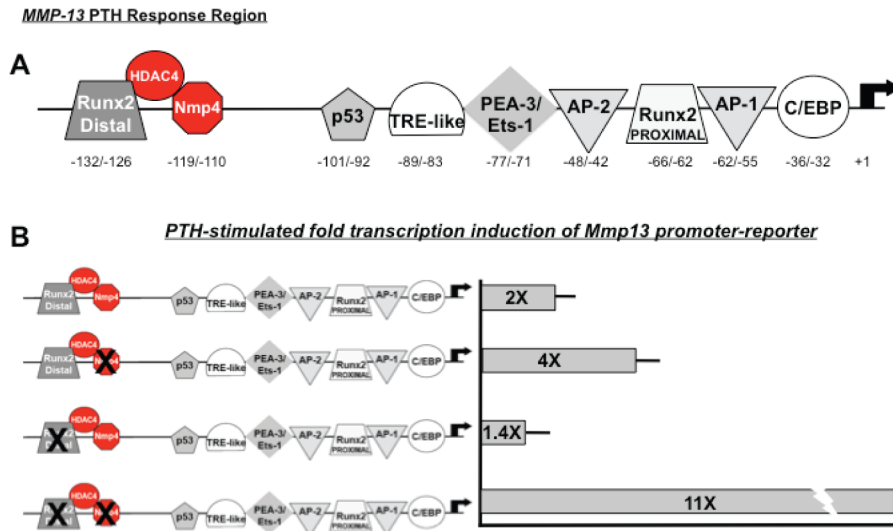
FIGURE 3.

Nmp4/CIZ is a repressive transcription factor that binds near an epigenetic remodeling site in the PTH response region of the *Mmp13* promoter. (a) The region in the *Mmp13* promoter that regulates response to PTH is conserved in mice, rats, and humans. Nmp4/CIZ binds to this region in a sequence-specific fashion between (–119/–110bp).[77] The distal Runx2 site binds to HDAC4 and mediates suppression of *Mmp13* transcription. Whether Nmp4 binds to Runx2 or HDAC4 is not yet known. PTH removes HDAC4 as part of the transcription induction mechanism. [78] (b) Introducing null-binding mutations in the Nmp4/CIZ-binding site within the *Mmp13* promoter-reporter construct containing the first 1329nt of the 5′-regulatory region and transfected into UMR 106-01 osteoblast-like cells enhances PTH-stimulated transcription induction. Mutating the distal Runx2 site does not significantly alter PTH response, but mutating both the Runx2 and Nmp4 sites dramatically increases hormone-induced transcription (see Ref. 77).