ATF4 mediation of *NF1* functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae

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Summary

The transcription factor ATF4 enhances bone formation by favoring amino acid import and collagen synthesis in osteoblasts, a function requiring its phosphorylation by RSK2, the kinase inactivated in Coffin-Lowry Syndrome. Here, we show that in contrast, RSK2 activity, ATF4-dependent collagen synthesis, and bone formation are increased in mice lacking neurofibromin in osteoblasts ($Nf1_{ob}^{-/-}$ mice). Independently of RSK2, ATF4 phosphorylation by PKA is enhanced in $Nf1_{ob}^{-/-}$ mice, thereby increasing *Rankl* expression, osteoclast differentiation, and bone resorption. In agreement with ATF4 function in amino acid transport, a low-protein diet decreased bone protein synthesis and normalized bone formation and bone mass in $Nf1_{ob}^{-/-}$ mice without affecting other organ weight, while a high-protein diet overcame $Atf4^{-/-}$ and $Rsk2^{-/-}$ mice developmental defects, perinatal lethality, and low bone mass. By showing that ATF4-dependent skeletal dysplasiae are treatable by dietary manipulations, this study reveals a molecular connection between nutrition and skeletal development.

Introduction

The molecular elucidation of human skeletal dysplasiae has greatly contributed to our current knowledge of skeletogenesis (Olsen et al., 2000). Indeed, many of the transcription factors implicated in this process such as Msx1, Sox9, Runx2, Twist-1 and Atf4 are mutated or their activity is affected in several dysplasiae (Bi et al., 2001; Bialek et al., 2004; Ducy et al., 1997; Satokata and Maas, 1994; Wagner et al., 1994; Yang et al., 2004). Conversely, the identification through molecular means and/or mouse genetics of transcription factors regulating chondrocyte and osteoblast differentiation led, in few cases, to a better molecular understanding of the pathogenesis of human skeletal dysplasiae. A potential implication of these advances is that one could propose and test, at least in mice, adapted therapies for some of these diseases based on the knowledge of the molecular mode of action of these transcription factors.

In the vast majority of cases, however, molecular elucidation of skeletal dysplasiae has not led yet to a better understanding of the disease or to a more rational treatment. Neurofibromatosis type I (NF1), a disease caused by loss of function mutations in *NF1*, a gene encoding the Ras GAP (GTPase Activating Protein) neurofibromin (Dasgupta and Gutmann, 2003; Klose et al., 1998; Parada, 2000) belongs to this latter category. Indeed, besides well-described tumors of the nervous system, NF1 patients often display well-documented skeletal abnormalities whose cellular and molecular pathogenesis is not fully elucidated, in part because no animal model has been available to study them (Alwan et al., 2005; Jacquemin et al., 2003; Kuorilehto et al., 2004, 2005; Kwok et al., 2002; Ruggieri et al., 1999; Sigillo et al., 2002; Simsek et al., 2003; Stevenson et al., 1999). As a result of the paucity of molecular knowledge of how neurofibromin affects bone biology the only available treatment for these often debilitating manifestations remains surgery.

ATF4, an osteoblast-enriched member of the CREB family of transcription factors, is necessary for the latest phases of osteoblast differentiation, for extracellular matrix (ECM) synthesis by osteoblasts, i.e., bone formation, and for osteoclast differentiation (Elefteriou et al., 2005; Yang et al., 2004). These different functions require phosphorylation of ATF4 by distinct kinases. RSK2, the kinase mutated in Coffin-Lowry syndrome (CLS), regulates the first two activities of ATF4 while PKA regulates its third function. Remarkably, ATF4 favors bone formation through two distinct mechanisms, both of which depend on its phosphorylation by RSK2. On the one hand, it positively regulates osteoblast-specific genes at the transcriptional level, and on the other hand, it promotes synthesis of Type I collagen, the main component of the bone ECM, by favoring amino acid import (Yang et al., 2004). In that respect the fact that the skeletal manifestations of CLS worsen overtime (Hunter, 2002; Touraine et al., 2002) raises the hypothesis that it may be due, primarily, to a lack of collagen synthesis during skeletal growth. This hypothesis is testable using mouse models. In any case the central role played by ATF4 in regulating osteoblasts main functions suggests that it may be implicated in the development of additional bone diseases beside CLS.

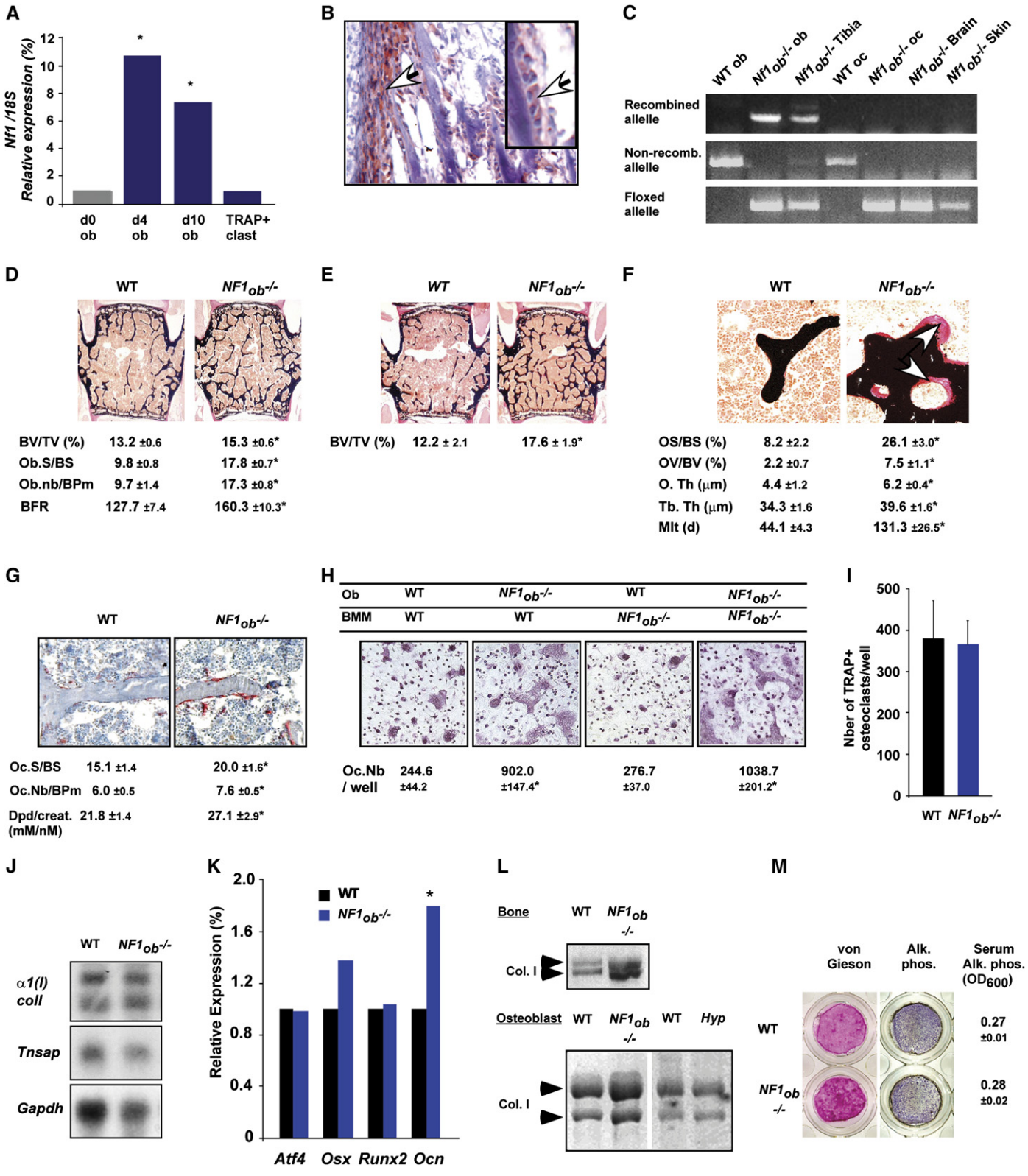

Figure 1. Increased bone formation, osteoid thickness, and bone resorption in $Nf1_{ob}^{-/-}$ mice

A) *Nf1 mRNA* is expressed at higher level in differentiated osteoblasts (d4 to d10 of culture) than in $TRAP^+$ multinucleated osteoclasts (qPCR).

B) Neurofibromin immunoreactivity (brown staining) in tibia periosteal and trabecular osteoblasts.

C) *Nf1 cre* recombination occurred in $Nf1^{-/-}$ osteoblasts (ob) and bones but not in osteoclasts (oc) or other cell types where *Nf1* is expressed.

D and E) Increased bone volume/tissue volume (BV/TV), osteoblast surface/bone surface (ObS/BS), osteoblast number/bone perimeter (Ob.nb/BPm), and bone formation rate (BFR, $\mu m^3/\mu m^2$/year) in 3- (**D**) and 6-month-old (**E**) $Nf1_{ob}^{-/-}$ mice (n = 6–9, $p < 0.05$).

In the present study, we generated mice lacking *Nf1* only in osteoblasts ($Nf1_{ob}^{-/-}$ mice) with the primary goal to elucidate the transcriptional events accounting for NF1 skeletal manifestations. This conditional deletion bypasses the embryonic lethality of a somatic deletion of the gene allowing us to analyze the function of *Nf1* in bone (Zhu et al., 2001). Through molecular and genetic analyses of this mouse model we demonstrate here that ATF4 plays a crucial role in mediating neurofibromin signaling in osteoblasts. Surprisingly, further use of this mouse model and of $Atf4^{-/-}$ and $Rsk2^{-/-}$ mice led to the conclusion that, at least in mice, one can reverse and even prevent skeletal manifestations that are secondary to abnormal phosphorylation of ATF4 by RSK2 through simple dietary manipulations. Thus, these results not only provide a molecular basis for NF skeletal manifestations but also identify a hitherto unanticipated connection between amino acid intake and skeletal development.

Results

Multiple bone phenotypes in $Nf1_{ob}^{-/-}$ mice

To identify molecular events accounting for NF skeletal manifestations we studied neurofibromin functions in osteoblast, the bone cell type where *Nf1* is expressed at the highest level (Figures 1A and 1B). Because *Nf1*-deficient embryos die before skeletogenesis is initiated (Jacks et al., 1994) and $Nf1^{+/-}$ mice do not display any appreciable bone abnormalities (Yu et al., 2005), we generated a mutant mouse strain lacking *Nf1* only in osteoblasts ($Nf1_{ob}^{-/-}$ mice) by crossing mice harboring a floxed allele of *Nf1* (Zhu et al., 2001) with mice expressing *Cre* recombinase specifically in osteoblasts beyond E14.5 (*α1(I) Collagen Cre* transgenic mice) (Dacquin et al., 2002; Zhu et al., 2001). Excision at the *Nf1* locus exceeded 90% in primary osteoblast culture originating from 4-day-old $Nf1_{ob}^{-/-}$ mice while no excision could be detected in any other cell type tested including osteoclasts, the bone-resorbing cells (Figure 1C).

$Nf1_{ob}^{-/-}$ mice were born at the expected Mendelian ratio, did not display any skeletal patterning abnormalities and had a normal life span (Figure S1 in the Supplemental Data available with this article online, and data not shown). Histological and 3D microtomography analyses performed on vertebrae and long bones of male and female $Nf1_{ob}^{-/-}$ mice at 2, 3, and 6 months of age showed a progressive increase in bone volume, i.e., a high bone mass phenotype (Figures 1D and 1E, S1C, and data not shown). Static and dynamic histomorphometric analyses revealed a marked increase in osteoblast surface and number and in the bone formation rate (Figure 1D) indicating that this high bone mass phenotype was secondary to an increase in bone formation.

This histomorphometric analysis also uncovered two other and unanticipated abnormalities in $Nf1_{ob}^{-/-}$ mice. The first one was, in all mutant bones analyzed, a significant increase in osteoid volume, i.e., in the amount of bone ECM that is not mineralized (Figure 1F). Second, $Nf1_{ob}^{-/-}$ mice displayed an increased bone resorption characterized by an increase in the number of tartrate resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts, and in urinary elimination of deoxypiridinoline (Dpd), a biomarker of osteoclasts activity (Figure 1G). A similar increase in Dpd urinary elimination has been reported in NF1 patients, underscoring the relevance of the $Nf1_{ob}^{-/-}$ mice as a model of NF1 bone disease (Stevenson et al., 2005).

Consistent with the osteoblast-specific gene inactivation performed, coculture of $Nf1^{-/-}$ osteoblasts with bone marrow macrophages (BMMs), originated from either WT or $Nf1_{ob}^{-/-}$ mice yielded a significantly higher number of multinucleated differentiated osteoclasts than identical cocultures of BMMs (of either genotypes) with WT osteoblasts (Figure 1H). Despite the role of *Nf1* signaling in osteoblast-dependent osteoclast differentiation, vitamin D was still required for osteoclast differentiation in this ex vivo assay. In addition, spleen cells isolated from WT or $Nf1_{ob}^{-/-}$ mice differentiated equally well into multinucleated osteoclasts in the presence of RANKL and m-CSF excluding an autonomous defect in osteoclasts from $Nf1_{ob}^{-/-}$ mice (Figure 1I). Thus *Nf1* inactivation in osteoblasts affects osteoclast differentiation in addition to bone formation.

Molecular bases of the increased osteoid volume in $Nf1_{ob}^{-/-}$ mice

To elucidate the molecular mechanisms whereby neurofibromin deletion in osteoblasts affects altogether bone formation, bone resorption and ECM mineralization, we used this latter phenotype as an entry point. *Type I Collagen* and *tissue nonspecific alkaline phosphatase (Tnap)*, which are together necessary and sufficient to induce bone ECM mineralization in mice, were normally expressed in $Nf1_{ob}^{-/-}$ mice as were *Runx2*, *Osterix* and *Atf4 mRNA*, the three main transcription factors governing osteoblast differentiation (Stein et al., 1996; Murshed et al., 2005; Wagner and Karsenty, 2001; Yang et al., 2004) (Figures 1J and 1K). The only molecular abnormality observed in $Nf1^{-/-}$ osteoblasts was an increase in the expression of *Osteocalcin*, a gene whose inactivation or overexpression in osteoblasts does not affect ECM mineralization (Ducy et al., 1996; Murshed et al., 2004). Furthermore, there was no overt abnormality of calcium and phosphate metabolism that could explain this defect in ECM mineralization (Figure S2). Taken together, these data ruled out that the increase in osteoid thickness in $Nf1_{ob}^{-/-}$ mice was due to an abnormal expression of genes involved in osteoblast differentiation or ECM mineralization.

We also asked whether the ECM mineralization abnormality observed in $Nf1_{ob}^{-/-}$ mice could be secondary to posttranscriptional events. To that end we measured Type I collagen production and TNAP activity in WT and $Nf1_{ob}^{-/-}$ bones and osteoblasts. Bone collagen content, as assessed by total trabecular thickness and by Western blot was increased in $Nf1_{ob}^{-/-}$ bones and osteoblasts compared to WT controls (Figures 1F and 1L).

F and G) Increased osteoid surface/bone surface (OS/BS), osteoid thickness (O. Th), trabecular thickness (Tb. Th), mineralization lag time (Mlt) (**F**), osteoclast surface/bone surface (Oc.S/BS), osteoclast number/bone perimeter (Oc.Nb/BPm), and urinary elimination of deoxypiridinoline (Dpd/creat.) (**G**) in 3-month-old mice $Nf1_{ob}^{-/-}$ mice ($n = 6–9$, $p < 0.05$).

H and I) Number of TRAP-positive multinucleated osteoclasts (Oc. Nb, red staining) in osteoblasts (Ob)/osteoclasts (BMM) cocultures (**H**) and M-CSF and RANKL differentiated BMM cultures ($n = 3$, $p < 0.05$) (**I**).

J and K) *α1(I) collagen* (*α1(I) coll*, *Tnsap* (j, Northern), *Atf4*, *Osterix* (*Osx*), *Runx2*, and *Osteocalcin (Ocn)* ([**K**] qPCR) expression in WT and $Nf1^{-/-}$ osteoblasts ($n > 3$, $p < 0.05$).

L) Type 1(I) collagen content in WT, $Nf1^{-/-}$, and *Hyp* bones and osteoblasts.

M) Collagen deposition (Van Gieson), alkaline phosphatase (Alk. Phos.) activity, and level in WT and $Nf1^{-/-}$ osteoblasts in culture ($n = 3$) and serum ($n = 16$, $p < 0.05$). The data represent the mean ± the SEM.

In contrast, TNAP activity was normal in *Nf1*$^{-/-}$ osteoblasts as was TNAP serum levels in *Nf1*$_{ob}$$^{-/-}$ mice (Figure 1M). This increase in collagen secretion was not observed in osteoblasts isolated from another mouse model characterized by an increased osteoid thickness such as the *Hyp* mice (Eicher et al., 1976) indicating that it was a specific consequence of *Nf1* deletion in osteoblasts (Figure 1L). Lastly, mineralization lag time, i.e., the time period between collagen synthesis and the initiation of mineralization, was increased in *Nf1*$_{ob}$$^{-/-}$ mice, suggesting the existence of an additional function of *Nf1* in bone mineralization (Figure 1F). Taken together, these observations indicated that a posttranscriptional increase in collagen synthesis as well as an increase in osteoblast number along with a delay in mineralization contributed to the increase in osteoid amount in *Nf1*$_{ob}$$^{-/-}$ mice.

ATF4 mediates neurofibromin signaling in osteoblasts

The increase in collagen content in the face of a normal expression of *Type I collagen* along with the increase in *Osteocalcin* expression is the mirror image of what is seen in mice lacking *Atf4*. ATF4 is an osteoblast-enriched member of the CREB family of transcription factors regulating *Osteocalcin* expression, osteoclast differentiation and Type I collagen synthesis via its ability to favor amino-acid import (Elefteriou et al., 2005; Harding et al., 2000, 2003; Yang et al., 2004). This observation led us to test whether an increase in ATF4 activity contributed to the skeletal abnormalities in *Nf1*$_{ob}$$^{-/-}$ mice.

To regulate Collagen synthesis and *Osteocalcin* expression ATF4 must be phosphorylated at serine 251 by RSK2, a substrate of the Ras/MEK/ERK signaling pathway (Dalby et al., 1998; Fisher and Blenis, 1996). Accordingly, RSK2 and ERK1/2 phosphorylation were increased in *Nf1*$^{-/-}$ osteoblasts and Ras activity was higher in *Nf1*$^{-/-}$ than in WT osteoblasts (Figure 2A). These alterations were specific of the ERK pathway as AKT and p38 phosphorylation was not affected in *Nf1*$^{-/-}$ osteoblasts. As expected the increase in RSK2 activation enhanced ATF4 phosphorylation at serine 251, conversely treatment of *Nf1*$^{-/-}$ osteoblasts with the MEK inhibitor U0126 reduced ^{251}S-ATF4 phosphorylation, leading to a reduction of *Osteocalcin* expression (Figures 2A–2C). Likewise, treatment of *Nf1*$_{ob}$$^{-/-}$ mice with a MEK inhibitor for 5 weeks reduced collagen synthesis and osteoid thickness without altering *Type I collagen* mRNA levels and bone volume (Figures 2D and 2E and data not shown). Taken together, these results established that, in osteoblasts, neurofibromin is a negative regulator of the RSK2-mediated phosphorylation of ATF4, thereby a negative regulator of *Osteocalcin* expression and of Type I collagen synthesis, the most abundant protein component of the bone ECM.

On the other hand, to favor *Rankl* expression and osteoclast differentiation, ATF4 must be phosphorylated by PKA on serine 254, which is a RSK2-independent event (Elefteriou et al., 2005). PKA activity was increased in *Nf1*$^{-/-}$ compared to WT osteoblasts (Figure 2F), consequently ATF4 was more highly phosphorylated at serine 254 and *Rankl* expression was higher in *Nf1*$^{-/-}$ than in WT osteoblasts, while expression of *Opg*, a decoy receptor for RANKL, was unaffected (Figures 2G–2I). The PKA inhibitor H89 blunted the increase in *Rankl* expression in *Nf1*$^{-/-}$ osteoblasts further supporting the notion that neurofibromin-dependent PKA phosphorylation of ATF4 contributes to the osteoblast-mediated increase in bone resorption observed in *Nf1*$_{ob}$$^{-/-}$ mice (Figure 2I).

Genetic evidence of ATF4 involvement in *Nf1*$_{ob}$$^{-/-}$ mice bone phenotypes

To provide further support to the notion that ATF4 mediates neurofibromin signaling in osteoblasts we next used mouse models. We first constructed and analyzed transgenic mice overexpressing *Atf4* specifically in osteoblasts (*α1(I) Collagen-Atf4* mice) (Figures 3A and S3). *α1(I) Collagen-Atf4* mice displayed the same three bone phenotypes observed in *Nf1*$_{ob}$$^{-/-}$ mice: an increase in bone formation and bone mass, in bone resorption and in osteoid thickness (Figures 3B and 3D). At the molecular level the similarities between the two models were also striking and included an increase in *Ocn* and *Rankl* expression, two target genes of ATF4, and an increase in collagen content (but not its mRNA expression) in *α1(I) Collagen-Atf4* mice (Figures 3E and 3F). The increase in *Rankl* expression explains the increase in bone resorption observed in these transgenic mice while the increase in collagen content together with an increase in osteoblast number provides an explanation for their bone formation and osteoid thickness abnormalities. This is consistent with the known role of *Atf4* as a regulator of osteoblast differentiation and collagen synthesis (Yang et al., 2004).

Next we generated and analyzed *Nf1*$_{ob}$$^{-/-}$ mice lacking one allele of *Atf4 or* of *Runx2* or *Osterix*, two other osteoblast-specific transcription factors. Six-month-old *Nf1*$_{ob}$$^{-/-}$;*Atf4*$^{+/-}$ mice displayed a significant reduction in collagen synthesis that was not observed in *Nf1*$_{ob}$$^{-/-}$ mice lacking one allele of either *Runx2* or *Osterix* (Figure 3G). Accordingly, osteoid thickness was decreased in *Nf1*$_{ob}$$^{-/-}$;*Atf4*$^{+/-}$ mice but not in *Nf1*$_{ob}$$^{-/-}$;*Runx2*$^{+/-}$ or *Nf1*$_{ob}$$^{-/-}$;*Osx*$^{+/-}$ mice (Figure 3H and data not shown). Taken together, these results provide genetic support to the notion that ATF4 mediates neurofibromin signaling in osteoblasts.

Low-protein diet corrects *Nf1*$_{ob}$$^{-/-}$ mice bone formation abnormalities

What could be the therapeutic implications of these molecular findings? It is known that ATF4 promotes amino acid import into cells by activating genes encoding membranes transporters (Harding et al., 2003). Consequently, *Atf4*$^{-/-}$ osteoblasts are subject to amino acid depletion, a phenotype corrected by addition of nonessential amino acid to their culture medium (Yang et al., 2004). Thus we asked whether the ATF4-mediated increase in intracellular amino acid availability plays a role in the development of bone abnormalities in *Nf1*$_{ob}$$^{-/-}$ mice.

WT and *Nf1*$_{ob}$$^{-/-}$ mice were fed with either a normal diet (ND) or a LPD from birth to 4 months of age. To determine if the LPD affected amino acid pools in bone cells, we measured the activity of GCN2, a kinase that is activated by uncharged transfer RNAs and that phosphorylates serine 51 on the α subunit of the translation initiation factor 2 (eIF2α) resulting in reduced translation initiation and reduced protein synthesis (Dong et al., 2000; Harding et al., 2000). Levels of activated GCN2 and phosphorylated eIF2α were both increased in bones of LPD-fed WT mice but not in bones of LPD-fed *Nf1*$_{ob}$$^{-/-}$ mice (Figure 4A). These observations suggested that dietary manipulation can affect amino acid pools in bone cells and that, as predicted these pools are greater in *Nf1*$_{ob}$$^{-/-}$ mice. Accordingly, the amount of Type I collagen detectable by Western blot was markedly decreased in bones of *Nf1*$_{ob}$$^{-/-}$ mice fed a LPD (Figure 4B). Despite the fact that GCN2 remained inactive in mutant mice, histomorphometric analyses showed that the high bone

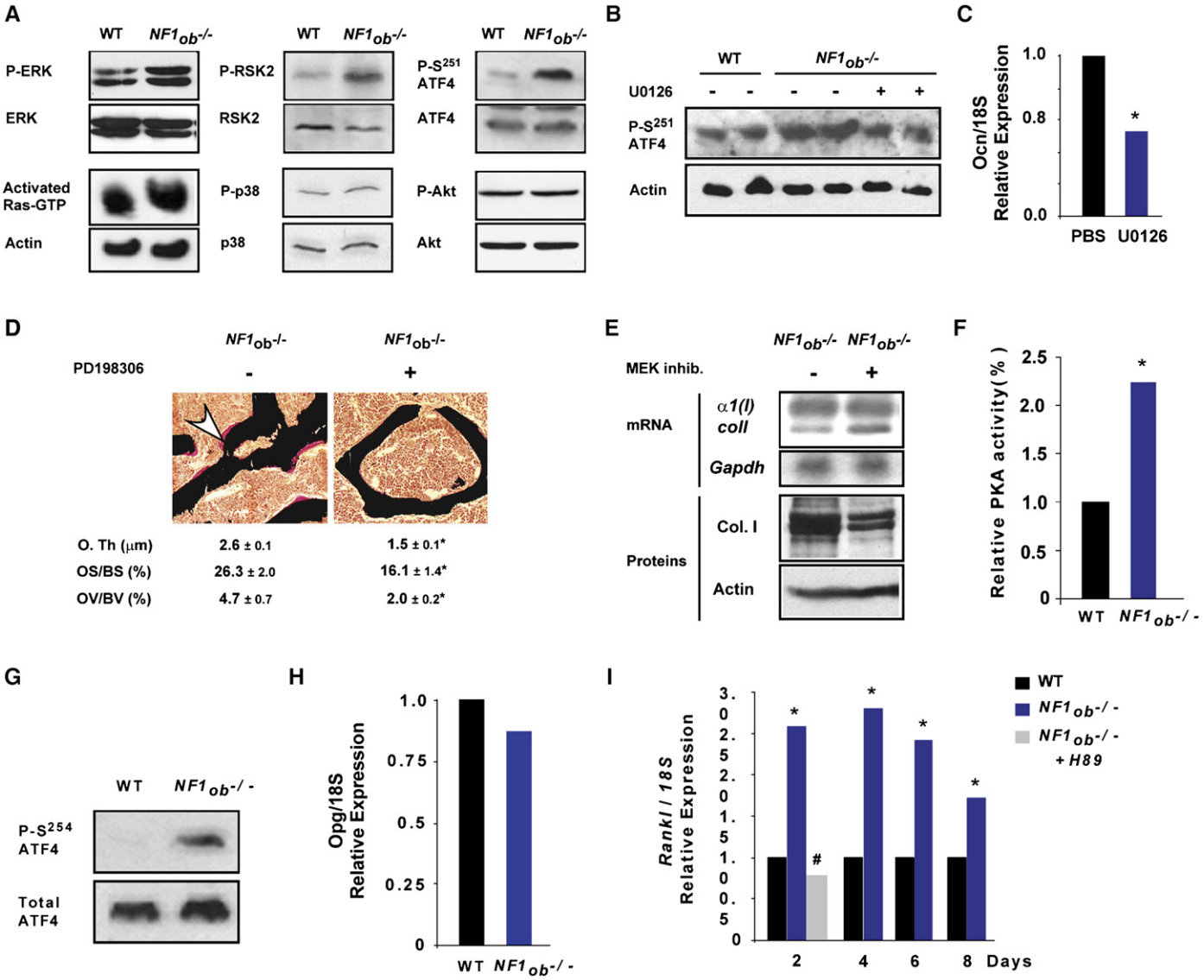

Figure 2. *Nf1* deficiency in osteoblasts activates ATF4 via the MAPK pathway

A) Western blots. Phosphorylation of ERK1/2, RSK2, S^{251}-ATF4, p38, AKT, and Ras activation in osteoblasts (n = 3).

B and C) MEK inhibition by U0126 decreases S^{251}-ATF4 phosphorylation ([**B**] Western blots) and *Ocn* expression ([**C**] qPCR) in cultured osteoblasts (n = 3, $p < 0.05$).

D and E) MEK inhibition by PD198306 decreases osteoid parameters (OS/BS and OV/BV) (**D**) and collagen content ([**E**] Western blots) in $Nf1_{ob}^{-/-}$ bones and mice (n = 7, $p < 0.05$).

F) PKA activity in osteoblasts (n = 4, $p < 0.05$).

G) Western blots. Phosphorylation of S^{254}-ATF4 in osteoblasts (n = 3).

H and I) qPCR. *Osteoprotegerin* (*Opg*) expression in osteoblasts ([**H**] n = 3, $p < 0.05$) and *Rankl* expression in immature (d2) to mature (d8) untreated or H89-treated osteoblasts ([**I**] n = 3, *: $Nf1^{-/-}$ versus wt, #: $Nf1^{-/-}$ versus $Nf1^{-/-}$ +H89, $p < 0.05$).

The data represent the mean ± the SEM.

mass, increase in bone formation parameters and in osteoid thickness characteristic of $Nf1_{ob}^{-/-}$ mice fed a normal diet were all corrected by the LPD (Figure 4C). Importantly, the LPD did not affect any of these parameters in WT mice nor the overall weight of mice or of their internal organs regardless of their genotypes (Figure S4). In addition, LPD did not influence bone resorption parameters in $Nf1_{ob}^{-/-}$ mice indicating that the increase in PKA activity observed in *Nf1*-deficient osteoblasts was not secondary to the increase in RSK2 activity (Figure 4C). The LPD also normalized bone mass, osteoid thickness and bone formation parameters in 2-month-old *α1(I) Collagen-Atf4* mice further demonstrating the involvement of ATF4 as a mediator of neurofibromin signaling in osteoblasts (Figure 4D).

High-protein diet corrects $Rsk2^{-/-}$ and $Atf4^{-/-}$ bone phenotypes

The remarkable efficacy of a LPD in rescuing skeletal manifestations of a disease caused by an increase in ATF4 activity suggested that, conversely, a high-protein diet (HPD) could rescue the skeletal manifestations observed in *Rsk2*-deficient mice, a mouse model of Coffin-Lowry syndrome (CLS) characterized by a decrease in ATF4 function.

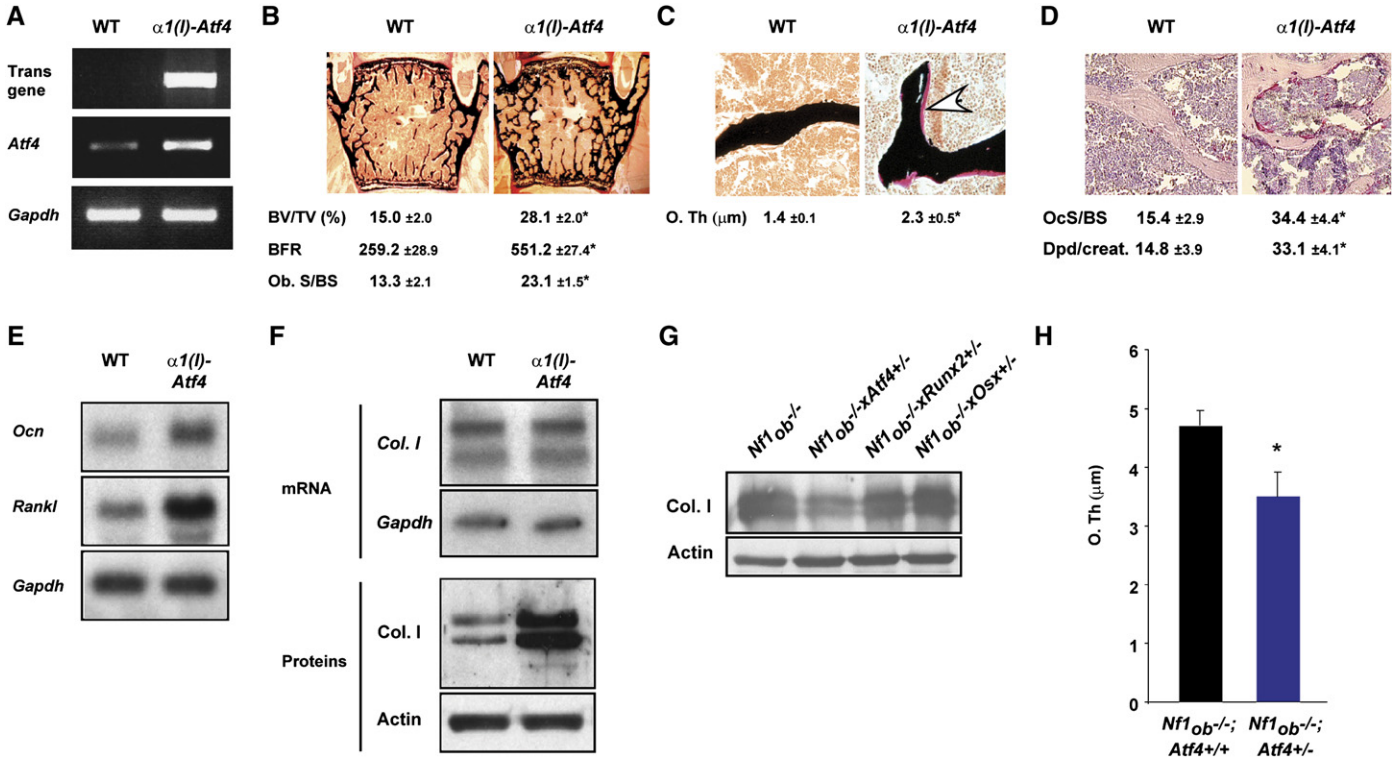

Figure 3. In vivo evidence of ATF4 involvement in $Nf1_{ob}^{-/-}$ bone phenotype

A) Transgenic and endogenous *Atf4* mRNA expression in WT and *α1(I) collagen-Atf4* bones.
B–D) BV/TV, BFR, Ob.S/BS (**B**), O.Th (**C**), Oc.S/BS and Dpd/creat. (**D**) in 2-month-old *α1(I) collagen-Atf4* mice (n = 4, $p < 0.05$).
E and F) Increased *Ocn* and *Rankl* expression ([**E**] n = 3, Northern blot) and bone α1(I) Collagen content ([**F**] n = 3, Western blot) in *α1(I) collagen-Atf4* bones.
G and H) Bone α1(I) collagen content ([**G**] n = 3, Western blot), O.Th ([**H**] n = 4, $p < 0.05$) in mice lacking one copy of *Atf4*, of *Runx2* or of *Osx*.
The data represent the mean ± the SEM.

To test this hypothesis, $Atf4^{+/-}$ and $Rsk2^{+/-}$ mothers were fed from the first day of gestation with a ND, a high-fat diet (HFD) or a HPD. After weaning, pups of each genotype were kept on this same diet up to 28 days of age. As previously reported (Tanaka et al., 1998), when $Atf4^{+/-}$ mothers and $Atf4^{-/-}$ pups received a ND only 10% of the pups recovered at 1 month of age were $Atf4^{-/-}$. This perinatal lethality was not corrected by a HFD (Figure 5A), in contrast, the proportion of 1-month-old $Atf4^{-/-}$ mice alive doubled when $Atf4^{+/-}$ mothers and $Atf4^{-/-}$ pups were fed a HPD. This rescue was specific of the *Atf4* mutation since HPD did not affect the perinatal lethality of mice lacking *Runx2* or *Osterix*, the two other osteoblast-specific transcription factors whose deletion prevents osteoblast differentiation (Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997). That phosphorylation of GCN2 as well as eIF2α was decreased in HPD-fed *Atf4*- and *Rsk2*-deficient bones suggested a state of amino acid sufficiency in mutant osteoblasts (Figure 5B). In agreement with this assumption, histomorphometric analyses showed that HPD, but not HFD, fully rescued the low bone formation and low bone mass otherwise observed in 1-month-old $Atf4^{-/-}$ and $Rsk2^{-/-}$ mice (Figures 5C and S5A) and normalized Type I collagen synthesis in $Atf4^{-/-}$ and $Rsk2^{-/-}$ but not in WT bones without affecting *Type I collagen* expression (Figure 5E). Return to normal diet following 1 month of HPD reversed the HPD-induced bone mass rescue observed in $Atf4^{-/-}$ mice (Figure 5C), indicating that nutritional manipulations of ATF4's function are effective postnatally as well.

A surprising feature of *Atf4*- and *Rsk2*-deficient mice fed a HPD was the normalization of their osteoblasts number (Figure 5C). This suggested that this diet may have also rescued the delay in osteoblast differentiation characterizing these mutant embryos. To address this possibility, we analyzed $Atf4^{-/-}$ and $Rsk2^{-/-}$ embryos whose mothers were fed either a ND or a HPD at the time osteoblast differentiation occurs. Molecular analysis of E15.5 and E18.5 mutant embryos showed that feeding pregnant mothers with HPD but not HFD sufficed to correct the lack of bone trabeculae otherwise observed in $Atf4^{-/-}$ and $Rsk2^{-/-}$ embryos (Figure 5D and S5B). This rescue was specific of the RSK2-ATF4 pathway as the HPD did not correct the osteoblast differentiation defect observed in embryos lacking *Runx2* or *Osterix*, two transcription factors not phosphorylated by RSK2 (Yang et al., 2004) (Figure 5D). The restoration of the expression of *Bone sialoprotein* (*Bsp*) and *Osteocalcin*, two target genes of ATF4, in E15.5 and E18.5 $Atf4^{-/-}$ embryos to levels seen in WT embryos verified that osteoblast differentiation has been rescued by the HPD (Figure 5F). Taken together, these results demonstrate that ATF4 regulation of osteoblast differentiation and bone formation during development and postnatally occurs mainly through its ability to favor amino acid import and thereby protein synthesis.

Discussion

This study identifies ATF4 as a crucial transcriptional mediator of *NF1* signaling in osteoblasts, the bone cells in which *Nf1* is

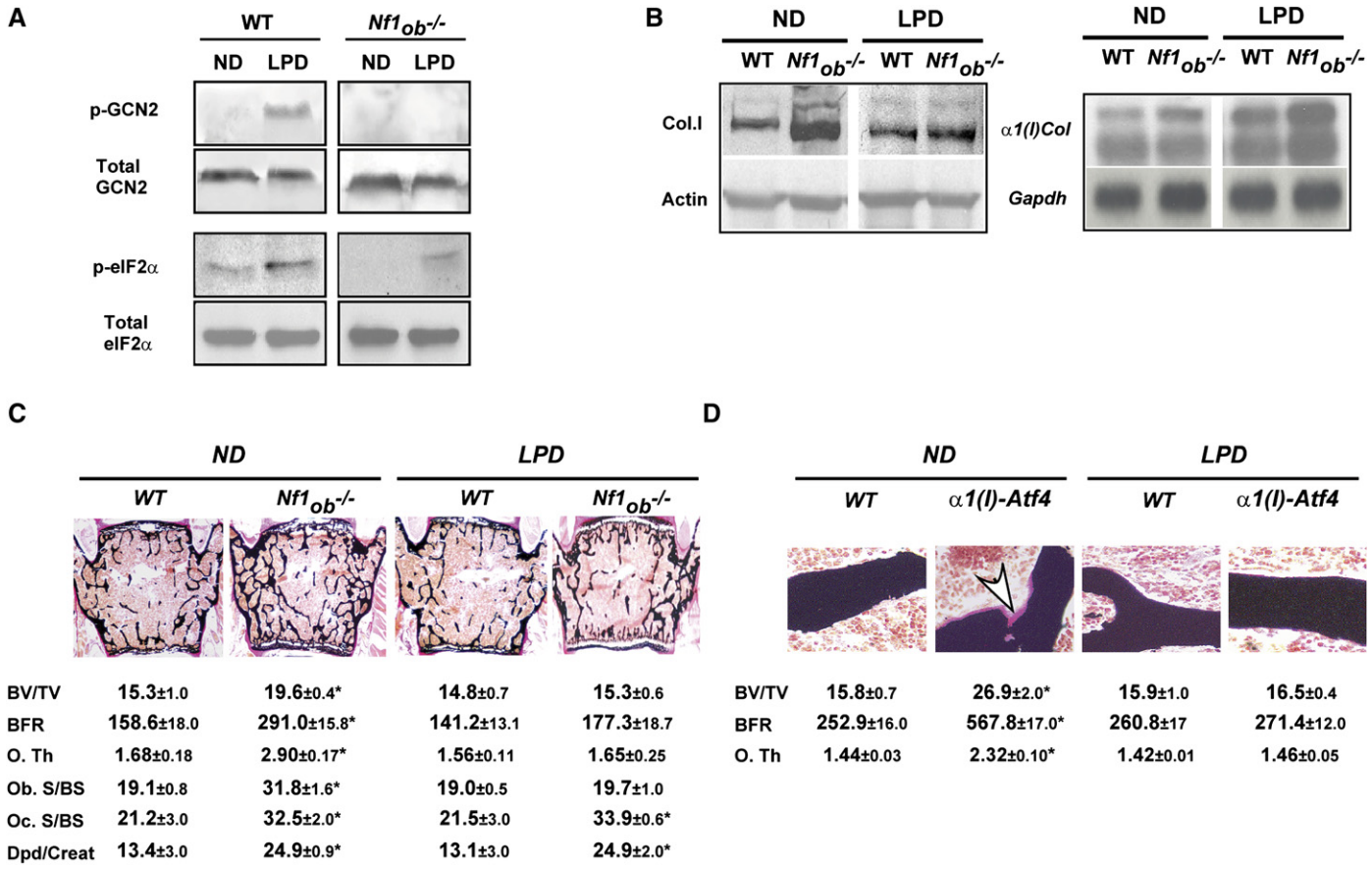

Figure 4. Low-protein diet corrects RSK2-dependent bone abnormalities in $Nf1_{ob}^{-/-}$ mice

A and B) GCN2 and eIF2 phosphorylation (**A**) and bone α1(I) Collagen content (**B**) in WT and $Nf1_{ob}^{-/-}$ bones under ND or LPD (n = 3, Western blots).
C and D) BV/TV, BFR, Ob.S/BS, Oc.S/BS and Dpd/Creat and O.Th in WT and $Nf1_{ob}^{-/-}$ bones under ND or LPD (n = 6, $p < 0.05$).
The data represent the mean ± the SEM.

expressed at the highest level and identifies neurofibromin as a negative regulator of bone remodeling acting via distinct pathways on bone formation and bone resorption (Figure 6). Moreover, further use of $Nf1_{ob}^{-/-}$ mice elicited by the knowledge surrounding ATF4 molecular mode of action showed that development of ATF4-dependent skeletal dysplasiae can be corrected by simple diet manipulations thus uncovering a novel mechanism regulating skeletal development and suggesting a simple treatment for a relatively frequent skeletal dysplasia.

A mouse model of NF1 skeletal manifestations

Pleiotropic skeletal manifestations ranging from bowing of long bones to pseudoarthrosis are observed in a significant portion of NF1 patients. Remarkably, these manifestations often worsen with age (Ruggieri et al., 1999; Hermanns-Sachweh et al., 2005; Kuorilehto et al., 2004; Lammert et al., 2005). Little was known about the cellular and molecular mechanisms accounting for NF1 skeletal manifestations since no animal model has been available until now. Indeed, $Nf1^{-/-}$ mice die in utero before skeletogenesis is initiated and $Nf_1^{+/-}$ mice, unlike human patients, do not have any overt skeletal manifestations (Yu et al., 2005). Thus, to elucidate the histological, cellular, and molecular bases of NF1 skeletal manifestations, we generated mice lacking *NF1* only in the bone-specific cell in which *Nf1* expression is the highest, namely the osteoblast. We show here that in mice, through expression in osteoblasts, *Nf1* affects both bone formation and bone resorption. In addition, it also affects the overall degree of mineralization of bone ECM. These results define an animal model of NF1 skeletal manifestations that may contribute to elucidate the pleiotropic nature of NF1 skeletal manifestations in humans. They also uncovered a molecular mechanism of action for neurofibromin in osteoblasts.

ATF4, a trancriptional mediator of neurofibromin signaling in osteoblasts

The only molecular abnormalities observed in $Nf1_{ob}^{-/-}$ mice and bones were an increase in collagen synthesis and in *Osteocalcin* expression. Those features were the mirror image of what was seen in mice lacking one of the major osteoblast-enriched transcription factor, ATF4 (Yang et al., 2004). Several lines of evidence of molecular, genetic or pharmacological nature establish that ATF4 is a mediator of neurofibromin signaling in osteoblasts. First, RSK2 phosphorylation is increased as is ATF4 phosphorylation on serine 251 in $Nf1^{-/-}$ osteoblasts, thus explaining the increase in collagen synthesis and in *Osteocalcin* expression; second, PKA activity is increased as is ATF4 phosphorylation on serine 254 and as a result, *Rankl* expression, osteoclast differentiation, and bone resorption in $Nf1_{ob}^{-/-}$ mice. That neurofibromin can affect PKA–dependent signaling events has been shown in other cell types such as schwann cells (Kim

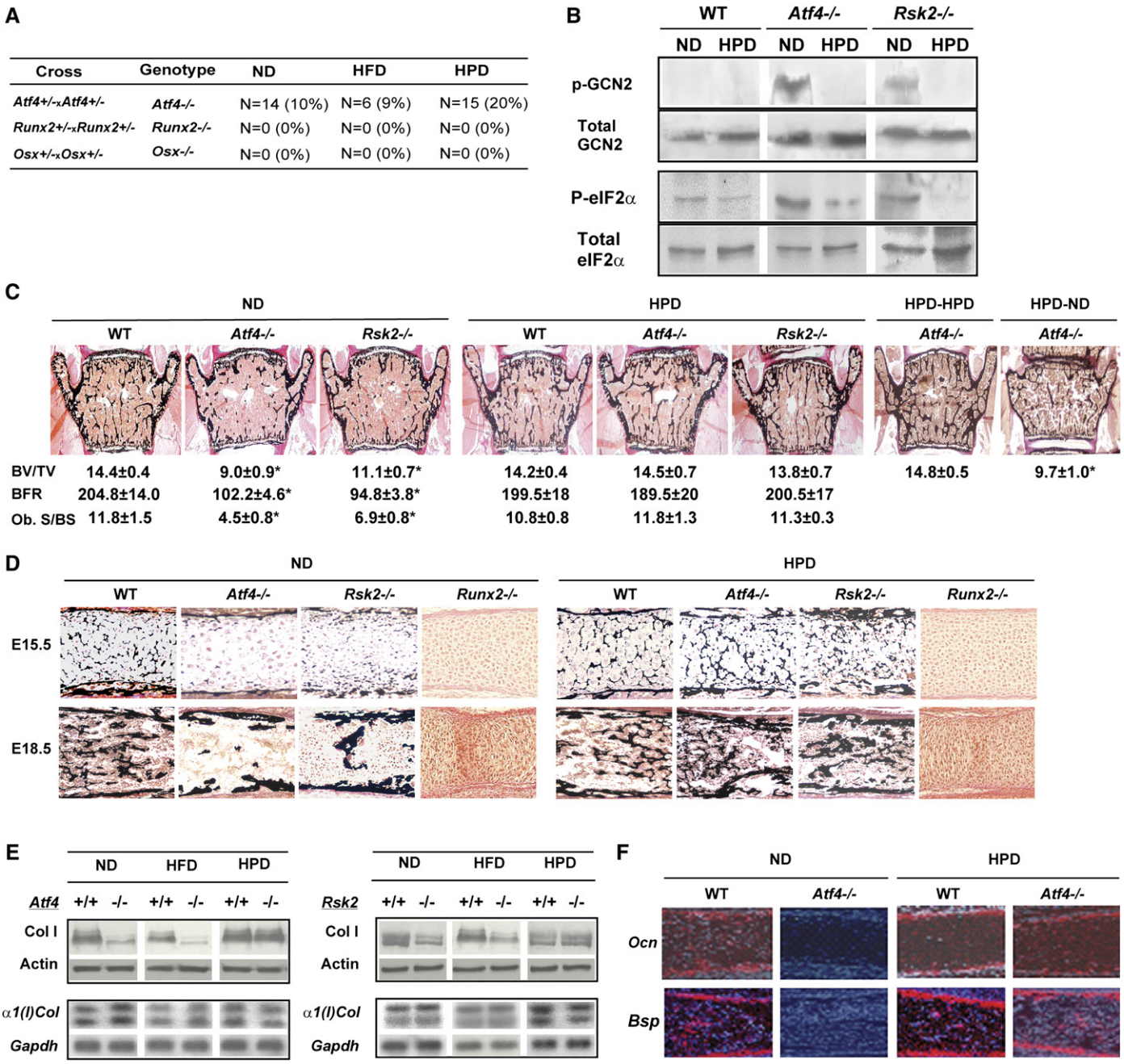

Figure 5. High-protein diet corrects the bone abnormalities in *Atf4*$^{-/-}$ and *Rsk2*$^{-/-}$ mice

A) Percentages of *Atf4*$^{-/-}$, *Runx2*$^{-/-}$, and *Osx*$^{-/-}$ pups reaching 1 month of age when mothers and pups were fed ND, HFD, or HPD (*Atf4:*272, *Runx2:* 66, *Osx*: 60 pups counted).

B and C) Bone GCN2 and eIF2a phosphorylation ([**B**] n = 3), BV/TV, BFR and Ob.S/BS in 1-month-old mice for each diet (except HPD-HPD/HPD-ND, 2-month-old) ([**C**] n = 6, $p < 0.05$).

D) Trabeculae Von Kossa staining in long bones of E15.5 and E18.5 embryos for each diet (n = 4-8 per group, $p < 0.05$).

E) Type I Collagen content and mRNA expression (*α1(I) Col*) in 1-month-old mice for each diet.

F) In situ hybridization for *Bsp* and *Ocn* expression in long bone of E15.5 or E18.5 embryos for each diet.

The data represent the mean ± the SEM.

et al., 2001; Xu et al., 2002). Third, transgenic mice overexpressing *Atf4* in osteoblasts have a phenotype similar to the one of *Nf1*$_{ob}$$^{-/-}$ mice; fourth and more importantly, removing one allele of *Atf4* sufficed to correct, at least partially, *Nf1*$_{ob}$$^{-/-}$ mice bone phenotypes while removing one allele of either *Runx2* or *Osterix* could not; fifth, a low-protein diet corrected all RSK2-dependent abnormalities, i.e., the increase in bone formation and the defect in ECM mineralization in both *Nf1*$_{ob}$$^{-/-}$ and *α1(I) Collagen-Atf4* mice.

These findings, however, do not exclude that other transcription factors whose activity can be modulated by RSK2 and/or PKA may also contribute to *Nf1*$_{ob}$$^{-/-}$ mice skeletal phenotypes.

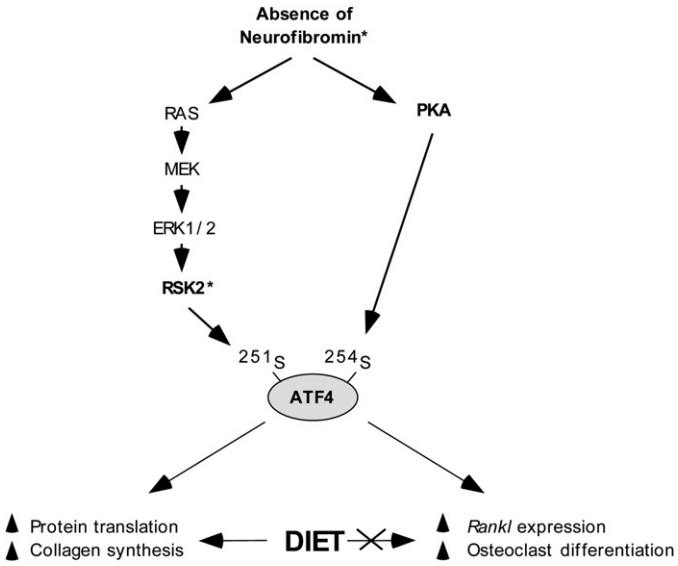

Figure 6. Roles of neurofibromin in the regulation of bone remodeling

In osteoblasts, neurofibromin reduces Ras, MAPK and RSK2 signaling, leading to a decrease in ATF4 transcriptional activity via reduced phosphorylation of ^{251}Serine. This leads to a reduction in protein translation and collagen synthesis. Neurofibromin also decreases PKA activity leading to a reduction of ATF4 phosphorylation at Serine 254. As a result, *Rankl* expression and osteoclast differentiation are normally decreased by neurofibromin. The asterisk denotes proteins whose loss causes skeletal dysplasiae treatable by dietary manipulations. Modulation of amino acid supply can correct the defects of protein translation and collagen synthesis mediated by *Nf1* or RSK2 mutations.

In that respect it is worthwhile to note that CREB phosphorylation was increased in *Nf1*-deficient osteoblasts (data not shown). The possible involvement of CREB (or of other transcription factors) in this signaling cascade will have to await the generation of mutant mice lacking CREB only in osteoblasts.

Rescue of ATF4-dependent skeletal dysplasia through diets in mice

ATF4 is a peculiar player in the field of transcriptional control of bone formation as it has at least two mechanisms of action. It regulates osteoblast differentiation and the expression of osteoblast-specific genes such as *Osteocalcin*, however, *Osteocalcin* deletion does not delay bone formation (Ducy et al., 1996; Yang et al., 2004). At the same time, it promotes amino acid import in osteoblasts by activating genes encoding membrane transporters (Harding et al., 2003). The observation that NF1 skeletal manifestations worsen overtime and reappear after surgical treatment in patients suggested to us that the former mode of action of ATF4 may explain this feature of NF skeletal manifestations. Consistent with this hypothesis, reducing protein intake normalized all skeletal manifestations related to the ability of ATF4 to favor amino acid import in cells of $Nf1_{ob}{}^{-/-}$ mice. Conversely, increasing protein intake in a mouse model of Coffin-Lowry Syndrome rescued the skeletal manifestations observed in adult mice. Remarkably, these latter manipulations also rescued all skeletal developmental abnormalities in both $Atf4^{-/-}$ and $Rsk2^{-/-}$ embryos, thus revealing that food intake affects skeletal development or at least ATF4-dependent events in this process.

These findings raise several questions beyond NF1 skeletal manifestations per se; first, are other manifestations in NF1 secondary, at least in part, to an increase in amino acid import in other cell types? That *Rsk2* and other members of this family of kinases as well as other members of the CREB family of transcription factors are expressed in neurons give some credence to this hypothesis that can easily be tested. Second, one can wonder whether development of other skeletal dysplasiae may not be affected by amino acid intake. Although this is a valid hypothesis, we should stress here that this notion applies only to ATF4-related diseases. More generally, our results illustrate how the precise knowledge of the molecular mechanism of action of a given gene involved in the pathogenesis of human genetic disease can potentially translate into therapeutic interventions.

Experimental procedures

Generation of mutant mice and animal treatments

The generation of *2.3kb α1 (I) collagen cre* mice, *Nf1* flox, $Atf4^{-/-}$, $Rsk2^{-/-}$, $Runx2^{-/-}$, and $Osx^{-/-}$ mice have been described (Dacquin et al., 2002; Nakashima et al., 2002; Otto et al., 1997; Yang et al., 2004; Zhu et al., 2001). WT are cre(−); $Nf1_{flox}/Nf1_{flox}$, $Nf1_{ob}{}^{-/-}$ are cre(+); $Nf1_{flox}/Nf1_{flox}$. *Alpha(I) collagen-Atf4* construct was generated by cloning the *Atf4* genomic sequence 3′ of the 2.3 *a1(I) Collagen* promoter and 5′ of a mp1 polyA signal in the pJ251 vector. All mice used in this study were backcrossed on a C57BL6 background. Four week-old $Nf1_{ob}{}^{-/-}$ mice were administered PD198306 daily (20 mg/kg/day) for 5 weeks by oral gavage. Normal diet (Picolab-Roden 20% #5053, TEKLAD, Madison, WI) contained 23.6% protein, 64.5% carbohydrate and 11.9% fat. High-protein diet (TD94266, TEKLAD) contained 53.3% protein, 33.6% carbohydrate and 13.1% fat. High-fat diet (DI12331, Research Diets, New Brunswick, NJ) contained 16% protein, 26% carbohydrate, and 58% fat. Low-protein diet (TD92208, TEKLAD) contained 10.8% protein, 76.0% carbohydrate, and 13.2% fat. All animal protocols were approved by the Animal Care Committees of Baylor College of Medicine.

Morphometric measurements

Static and dynamic histomorphometry measurements were performed as previously described in accordance with standard nomenclature, using the Osteomeasure Analysis System (Osteometrics, Inc) (Ducy et al., 2000; Parfitt et al., 1987; Takeda et al., 2002). 3D microtomographic measurements were performed at 12microns resolution under the growth plate (Scanco Medical, Bassersdorf, Switzerland). Four to 9 animals were assigned per group.

Cell cultures

Calvaria osteoblasts were extracted by triple collagenase/trypsine digestion from 4 day-old CD1 pups and differentiated with ascorbic acid as previously described (Ducy et al., 2000). Cre-mediated recombination efficiency was performed with differentiated calvarial osteoblasts. PCR was performed as originally described {Zhu, 2001 #1650}. Osteoclast differentiation assays were performed using Percoll gradient-purified BMM isolated from WT and $Nf1_{ob}{}^{-/-}$ bones, in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 4 days. Osteoblast/osteoclasts cocultures were performed in triplicate in 48-well plates using WT and $Nf1^{-/-}$ calvaria osteoblasts and BMM in the presence of 1,25(OH)$_2$ vitamin D$_3$ (10^{-8}M, Calbiochem) for 4 days (Takahashi et al., 1988). In vitro TRAP staining was performed at day 4 using the Acid Phosphatase Leukocyte kit (Sigma). Only TRAP-positive multinucleated (n > 3 nuclei) were counted. Van Gieson and alkaline phosphatase staining was performed using established protocols using buffered 10% formalin-fixed osteoblasts.

Gene expression studies

Osteoblasts were treated in serum-free medium with vehicle, U0126 (30 μM, Promega) or H89 (10 μM, Sigma) for 24 hr. Total RNA were extracted with Trizol (Invitrogen). cDNA were generated using the ABI Reverse transcriptase system and random hexanucleotide primers. Real-time PCR was performed using Taqman ABI probes/primers on a 9700 ABI cycler and 18S rRNA expression as endogenous control. Northern blot analyses were performed using 15 μg of total RNA and ^{32}P-labeled probes. PCR primers used for transgenic and endogenous *Atf4* expression are available upon request. In situ

hybridization analyses were performed using 4% PFA-fixed embryos embedded in paraffin, according to standard procedures (Ducy et al., 1997).

Biochemical studies

Osteoblasts were treated in serum-free medium with vehicle or U0126 (30 μM) for 6 hr. Lysates from primary osteoblasts or crushed frozen bones were prepared in RIPA buffer in the presence of protease and phosphatase inhibitors. Twenty to 60ug of proteins were separated by SDS-PAGE in reducing conditions and transferred on nitrocellulose membrane using standard protocols. Membranes were incubated with primary antibodies including total or anti-Phospho ATF4 (Elefteriou et al., 2005; Yang et al., 2004), total and anti-Phospho GCN2 (Harding et al., 2003), eIF2α (Cell Signaling), RSK2 (Dumont et al., 2005), CREB (Upstate), AKT, p38 (Santa Cruz) antibodies. Type I collagen from crushed frozen bones (20 mg) or calvaria primary osteoblast was extracted twice with pepsin (1 mg/ml) in 0.5 M acetic acid for 72 hr at 4°C. Collagen was precipitated by NaCl (0.7% final concentration) overnight and collected by centrifugation. Collagen pellets were resolubilized in 0.5M Tris-HCl (pH 7.8) and separated on a 7.5% SDS-PAGE in presence of 2 M urea, followed by Western blot analysis using anti-collagen type I antibody (Rockland, Gilbertsville, PA) (Bashey et al., 1989). Anti-actin antibody (Santa Cruz Biotechnology, Inc.) was used for loading. Protein kinase A assay (Stressgen) was performed in the presence of 10% FBS. Ras assay (Cell Signaling) was performed using differentiated WT and *Nf1*$^{-/-}$ osteoblasts following overnight starvation and 2 min stimulation with 10% FBS and according to manufacturer's recommendations. In short, cells were washed and lysed in the presence of protease and phosphatase inhibitors, supernatants were assessed for protein concentration and kinase reaction was performed in the presence of ATP. PKA activity values were corrected for protein concentration. All biochemical assays were performed using WT and *Nf1*$^{-/-}$ osteoblasts grown and treated simultaneously. Serum measurements for PTH (Immunotopics), calcium, phosphate, and alkaline phosphatase (Sigma) were performed according to the manufacturer recommendations. Deoxypyridinoline cross-links and Creatinine were measured using the Pyrilinks-D and creatinine kits (Metra Biosystems).

Statistical analyses

Data are expressed as mean ± SEM. Statistical significance was assessed by Student's test. Values were considered statistically significant when $p < 0.05$.

Supplemental data

Supplemental Data include five figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/6/441/DC1/.

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