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FIAT Control of Osteoblast Activity

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ABSTRACT

The basic domain-leucine zipper transcription factor activating transcription factor 4 (ATF4) regulates most functions of the osteoblast. It is therefore not surprising that its activity should be regulated through several mechanisms. Factor inhibiting ATF4-mediated transcription (FIAT) is a leucine zipper nuclear molecule lacking a basic domain for DNA binding that interacts with ATF4 to repress its transcriptional activity. FIAT expression was monitored in parallel with ATF4 during osteoblastogenesis. The mechanism of ATF4 repression by FIAT was investigated through structure–function analysis. The physiological significance of FIAT expression in osteoblasts was studied through silencing FIAT in osteoblasts by RNA interference, as well as through characterization of two genetic mouse models: FIAT transgenic mice which overexpress FIAT in osteoblasts, and FIAT knockout mice. Studies to date show that FIAT and ATF4 are co-expressed in osteoblasts, and that FIAT inhibition of matrix mineralization requires dimerization with ATF4 through the second leucine zipper. Furthermore, transgenic mice overexpressing FIAT exhibit osteopenia. The phenotype of FIAT knockout mice is still under evaluation but the salient aspects are discussed here. Taken together, the results accumulated to date support the hypothesis that FIAT is a transcriptional repressor that modulates osteoblast function. J. Cell. Biochem. 109: 453–459, 2010.

KEY WORDS: FIAT; γ-TAXILIN; ATF4; BASIC DOMAIN-LEUCINE ZIPPER TRANSCRIPTION FACTORS; OSTEOBLASTS

The study of the transcriptional control of osteoblastogenesis has been an extremely useful and rewarding strategy to unravel key aspects of bone biology and disease. Such research has led to the identification and characterization of critical molecules like runt-related transcription factor 2 (RUNX2) and Osterix (OSX), and to the elucidation of the post-natal role of the basic domain-leucine zipper (bZIP) factors Fos-related antigen-1 (FRA-1) and ΔFOSB in the control of bone mass. Another central player in osteoblast biology is the bZIP factor activating transcription factor 4 (ATF4), which regulates most functions of the bone-forming cell. Several mechanisms have evolved to regulate the activity of ATF4, one of which is heterodimerization with factor inhibiting ATF4-mediated transcription (FIAT), leading to the formation of inactive dimers that repress the transcription of ATF4 target genes. This *Prospect* will review salient aspects of ATF4 function as they relate to osteoblast biology, before presenting current knowledge and future perspectives on the role of FIAT in the regulation of ATF4-mediated transcription and bone cell activity.

ATF4 STRUCTURE AND ACTIVITY

ATF4 is a member of the ATF/cyclic adenosine monophosphate (cAMP) responsive element-binding (CREB) family. This gene family consists of transcription factors that bind the cAMP response element (CRE) through highly related, carboxy-terminal bZIP dimerization domains [Hai and Hartman, 2001]. Over the years, several cDNA clones encoding proteins that can bind to the ATF/CRE site have been isolated. All these cDNAs encode proteins with the bZIP domain, and the proteins can be grouped into subgroups on the basis of their amino acid similarity. Proteins within each subgroup share significant similarity both inside and outside the bZIP domain. Proteins between the subgroups, however, do not share much similarity other than the bZIP motif. The ATF4 subgroup includes ATF4 (also known as CREB2, TAXREB67, mATF4, C/ATF, or mTR67) [Ameri and Harris, 2008] and ATFx (also known as ATF5) [Hai and Hartman, 2001; Persengiev et al., 2002].

ATF4 is a 381 amino acid protein containing three acidic regions, hallmark of transactivation domains [Ameri and Harris, 2008]. Its

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mRNA is ubiquitously expressed and the protein exhibits a short half-life controlled through ubiquitination [Lassot et al., 2001; Yang and Karsenty, 2004]. Interestingly, the ATF4 protein has a second leucine zipper in addition to the C-terminal bZIP moiety [Liang and Hai, 1997]. It remains unclear whether this second zipper is functionally important. ATF4 can form homodimers [Hai and Curran, 1991; Vallejo et al., 1993] but can also heterodimerize with a variety of partners, including transcription factors [Hai and Curran, 1991; Chevray and Nathans, 1992; Vallejo et al., 1993; Tominaga et al., 2008] and kinases [Kawai et al., 1998]. The dimerization partner appears to influence specificity of DNA binding [Vallejo et al., 1993] as well as transcriptional activity [Fawcett et al., 1999; Lim et al., 2000].

ATF4 interacts with transcriptional coactivators and components of the basal transcriptional machinery [Liang and Hai, 1997; Lassot et al., 2005; Yu et al., 2008a]. Although ATF4-dependent transcriptional repression has been reported [Karpinski et al., 1992], it is generally assumed that ATF4 acts as a transcriptional activator [Ameri and Harris, 2008]. In addition to *Osteocalcin*, ATF4 targets include genes involved in amino acid import, metabolism, and assimilation [Harding et al., 2003]. This role of ATF4 in regulating amino acid import appears responsible for the decrease in type I collagen synthesis measured in ATF4-deficient osteoblasts [Yang et al., 2004].

ATF4 AND BONE BIOLOGY

Results have accumulated in the last few years to establish that ATF4 plays a pivotal role in the regulation of most post-natal osteoblast functions. While expression of the *Atf4* mRNA is ubiquitous, the ATF4 protein is unstable and degraded in most cell types through ubiquitination by the $SCF^{\beta TrCP}$ ubiquitin ligase [Lassot et al., 2001], except in osteoblasts where it accumulates [Yang and Karsenty, 2004]. In the bone-forming cell, ATF4 affects several key functions such as the synthesis of type I collagen [Yang et al., 2004]. Considering that this effect is modulated through availability of non-essential amino acids [Yang et al., 2004], it is surmised that it involves ATF4 targets regulating amino acid import, metabolism, and assimilation [Harding et al., 2003]. Type I collagen is the most abundant protein of the bone matrix, and the reduction in its synthesis measured in *Atf4*-deficient mice accounts for the low bone mass and virtual absence of bone trabeculae in the mutant animals [Yang et al., 2004].

ATF4 is also involved in the transcriptional control of several major osteoblastic genes: *Osteocalcin*, *Rankl* (receptor activator of NF-κB ligand), and *Esp* (embryonic stem cell phosphatase). The potent activation of the *Osteocalcin* gene is crucial for osteoblast differentiation and involves cooperative interactions between TFIIA-γ, RUNX2, SATB2 (special AT-rich binding protein 2), and ATF4 at the proximal *Osteocalcin* promoter [Xiao et al., 2005; Dobreva et al., 2006; Yu et al., 2008a]. Transcriptional activation of *Osteocalcin* by ATF4 further requires its post-translational modification by RSK2, the ribosomal S6 kinase 2 [Yang et al., 2004]. Mutations in *RSK2* cause Coffin-Lowry syndrome, an X-linked mental retardation condition associated with skeletal abnormalities [Trivier et al., 1996]. Lack of ATF4 phosphorylation by RSK2 most probably contributes to the bone phenotype in Coffin-Lowry syndrome, linking ATF4 to the molecular etiology of skeletal dysplasias. This link is further strengthened by the observation that ATF4 mediates neurofibromin (NF1) signaling in osteoblasts, suggesting a role in the development of the skeletal abnormalities of patients with neurofibromatosis type I [Elefteriou et al., 2006; Wu et al., 2006].

Control of *Rankl* transcription by ATF4 is regulated through phosphorylation of residue serine 254 by protein kinase A (PKA) [Elefteriou et al., 2005]. RANKL expression on the surface of osteoblasts serves as a potent stimulator for osteoclast differentiation and activation [Kobayashi et al., 2009]. Thus, ATF4 controls bone formation through regulating both osteoblast function and osteoclast differentiation, with a dominating role in promoting osteoblast activity since *Atf4*-null mice have an osteoporotic phenotype [Yang et al., 2004].

More recently, ATF4 was shown to be involved in the osteoblastic control of energy metabolism [Lee et al., 2007] through transcriptional regulation of the expression of *Esp* [Yoshizawa et al., 2009]. Esp indirectly promotes the γ-carboxylation of osteocalcin [Lee et al., 2007]. The secreted, non-carboxylated form of osteocalcin favours insulin secretion by the pancreatic β cells and modulates insulin sensitivity in liver, muscle, and adipocytes [Lee et al., 2007]. This posits the osteoblast as an endocrine cell type. ATF4, but not CREB, binds to the CRE within the *Esp* gene proximal promoter and activates *Esp* transcription [Yoshizawa et al., 2009]. Global or osteoblast-specific *Atf4*-deficient mice have reduced *Esp* expression and an increased proportion of uncarboxylated osteocalcin [Yoshizawa et al., 2009]. The metabolic phenotypes of $Esp^{-/-}$, $Atf4^{-/-}$, and osteoblast-specific *Atf4*-deficient mice are strikingly similar: low blood glucose, increased insulin secretion, and enhanced insulin sensitivity [Yoshizawa et al., 2009]. These data establish a genetic and transcriptional basis for the endocrine function of osteoblasts and show that ATF4 regulates most functions of the bone-forming cell: matrix formation (through control of type I collagen synthesis), coupling to resorption (via RANKL expression), and energy metabolism.

CONTROL OF ATF4 ACTIVITY

With such a plethora of critical functions, the activity of ATF4 needs to be tightly regulated. As mentioned, a first level of control involves protein stability: the ATF4 protein is degraded via ubiquitinylation in most cell types, except in osteoblasts where it can accumulate [Lassot et al., 2001; Yang and Karsenty, 2004]. Post-translational modifications such as acetylation by CREB-binding protein (CBP) or p300, or phosphorylation by PKA or RSK2 enhances its transcriptional activity [Liang and Hai, 1997; Gachon et al., 2002; Yang et al., 2004; Elefteriou et al., 2005]. An additional level of control is provided through interaction with specific partners. For example, heterodimerization of ATF4 with C/EBP changes its binding site specificity from a CRE to a CAAT element [Vallejo et al., 1993]. Interaction of ATF4 with Runx2 has also been suggested as necessary for osteoblast-specific activity of ATF4 [Xiao et al., 2005].

Partnering can also inhibit ATF4 activity. The bZIP transcriptional repressor ICER (inducible cAMP early repressor), a CRE modulator (CREM) isoform, antagonizes the activity of ATF4, presumably through protein–protein interaction via the leucine zipper [Chandhoke et al., 2008]. The formation of inactive heterodimers was also characterized as a significant mechanism explaining the regulation of ATF4 activity by the leucine zipper containing protein FIAT [Yu et al., 2005].

FIAT STRUCTURE

FIAT was first identified in a yeast two-hybrid screen for proteins interacting with the nascent polypeptide associated complex and coactivator alpha (αNAC) transcriptional coactivator [Yu et al., 2006a]. A protein identical to FIAT (named γ-taxilin) was characterized on the basis of its extended C-terminal coiled-coil, and shown to interact with syntaxin family members and αNAC [Nogami et al., 2004; Yoshida et al., 2005]. To alleviate the text, the acronym FIAT, which defines the function of the protein mainly discussed here, will be used throughout.

The *FIAT* gene (accession number NM_018360) maps to Xp22.1 and its mRNA, of around 4.5 kb in length with an extended 3′-untranslated region, is ubiquitously expressed [Nogami et al., 2004]. The full-length *FIAT* cDNA translates into a 66 kDa protein (accession number NP_060830). In silico analysis of the primary structure of FIAT revealed three putative leucine zippers, with the last zipper contained within the extended C-terminal coiled-coil. Despite the presence of the zipper structure, the computer modeling failed to identify a putative DNA-binding basic domain, which suggested that FIAT could not act as a sequence-specific bZIP transcriptional regulator. However, it was important to determine whether FIAT could homodimerize or interact with other zipper-containing factors. Yeast two-hybrid protein interaction assays with reciprocal FIAT "bait" and "prey" vectors demonstrated that it does not homodimerize [Yu et al., 2005]. Using the FIAT "bait" to screen cDNA expression libraries from primary osteoblasts or osteoblastic cell lines identified several independent clones for ATF4. FIAT and ATF4 are co-expressed in osteoblasts, and reciprocal co-immunoprecipitation of both endogenous proteins from osteoblastic cells confirmed that they interact in mammalian osteoblasts [Yu et al., 2005].

Since FIAT does not have a readily identifiable DNA-binding domain, it was hypothesized that it could prevent ATF4 from binding to the DNA to activate transcription by forming inactive heterodimers. This was confirmed using recombinant FIAT and electrophoretic mobility shift assays (EMSAs) with nuclear extracts from ROS 17/2.8 osteoblastic cells. Recombinant FIAT protein dose-dependently inhibited the binding of ATF4 to its response element [Yu et al., 2005]. In transient transfection assays in various cell types with heterologous or natural target reporters, co-expression of FIAT with ATF4 significantly inhibited ATF4-mediated transcription, even in the presence of RSK2 [Yu et al., 2005, 2006a]. Taken together, these results show that FIAT heterodimerizes with nuclear ATF4 to prevent its binding to DNA and repress its transcriptional activity.

Mutational analysis was performed to identify the functional motifs within the FIAT sequence. Deletion or site-specific mutations that inactivated each zipper individually showed that deletion or inactivation of the second leucine zipper motif (amino acids 194–222) prevented FIAT interaction with ATF4 and its ability to inhibit ATF4 transcriptional activity [Yu et al., 2008b]. Stable expression of wild-type FIAT in osteoblastic cells inhibits mineralization, but this inhibitory activity was lost in zipper 2-inactivated FIAT mutants [Yu et al., 2008b]. Thus, this structure–function analysis revealed that FIAT interacts with ATF4 and modulates its activity through its second leucine zipper domain. One interesting finding of the structure–function study was the biological impact of mutating the third and last leucine zipper within FIAT. This mutant could interact with ATF4 to inhibit its transcriptional activity, but it was compromised in its ability to inhibit mineralization [Yu et al., 2008b]. One possible interpretation of these results is that the third leucine zipper of FIAT acts as a subordinate interaction domain that strengthens the FIAT–ATF4 complex. This is unlikely since single inactivation of the second zipper completely abolished the FIAT–ATF4 interaction. Alternatively, it remains a formal possibility that FIAT, through its C-terminal domain, interacts with additional proteins (bZIP or coiled-coil) that control osteoblast activity. The identification of all FIAT dimerization partners and the characterization of the physiological relevance of all interactions represents an active area of investigation.

FIAT EXPRESSION

The *Fiat* mRNA is detected in several tissues [Nogami et al., 2004] but the expression pattern of the FIAT transcript and protein has only been studied exhaustively in bone. *Fiat* and *Atf4* mRNA levels remain constant throughout the osteoblastic differentiation sequence [Yu et al., 2009a]. While ATF4 protein expression increased throughout differentiation, FIAT protein levels were high in early osteoblasts but became nearly undetectable in differentiated cells at longer times in culture. Both ATF4 and FIAT were detected in the cytosol and nucleus of differentiating osteoblasts. The regulation of ATF4 activity by FIAT could thus happen in both compartments. Detection of FIAT in the cytosol is in accord with the identification of FIAT as a protein that also interacts with the cytoplasmic syntaxin family members [Nogami et al., 2004]. FIAT was also shown to contact the transcriptional coactivator αNAC [Yoshida et al., 2005; Yu et al., 2006b], a protein that shuffles between the cytoplasm and the nucleus [Quelo et al., 2004a,b, 2005]. The decrease in FIAT protein levels observed in differentiating osteoblasts in culture coincided with increased binding of ATF4 to the *Osteocalcin* gene promoter, and with increased *Osteocalcin* transcription [Yu et al., 2009a]. The preferred interpretation of the expression data is that in mature osteoblasts, the stoichiometric excess of ATF4 over FIAT frees ATF4 from sequestration by FIAT, thereby allowing ATF4 to homodimerize and activate the transcription of the *Osteocalcin* gene and other targets important for bone function.

In bone tissue, the FIAT and ATF4 proteins were co-detected in osteoblasts of developing and early post-natal bone. ATF4 protein expression was detected in cuboidal osteoblasts and in lining cells,

but not in osteocytes. On the other hand, strong FIAT protein expression was observed in osteocytes as early as post-natal day 2 [Yu et al., 2009a].

The study of the expression pattern of FIAT and its dimerizing partner ATF4 during osteoblastogenesis raises several interesting questions. Since the *Fiat* mRNA levels remain relatively constant while the protein decreases in abundance, what is the mechanism involved in the post-transcriptional control of FIAT expression? It will also prove interesting to determine if the subcellular localization of FIAT depends solely on its interaction with partner targets or whether it is controlled by more elaborate post-translational mechanisms. Finally, the exclusive localization of FIAT to osteocytes suggests that FIAT interacts with other targets than ATF4 in these cells. A protein–protein interaction screen using an expression library from an osteocyte cell line might identify novel dimerization partners of FIAT.

FIAT ACTIVITY IN CULTURED OSTEOBLASTS

The interaction of FIAT with ATF4 leading to inhibition of ATF4-mediated transcriptional activity suggested that modifying the expression levels of FIAT might modulate ATF4 function. Indeed, stable overexpression of a FIAT transgene inhibits transcription from the *Osteocalcin* gene promoter and reduces mineralization, both in primary osteoblasts cultures or in an osteoblastic cell line [Yu et al., 2005, 2008b]. Conversely, siRNA-mediated knockdown of FIAT expression enhanced all ATF4 functions tested: *Osteocalcin* transcription and promoter occupancy, bone sialoprotein (*Bsp*) gene transcription, as well as type I collagen synthesis [Yu et al., 2009b]. FIAT-knockdown osteoblasts also displayed increased mineralization and an increased number of nodules [Yu et al., 2009b].

In a parallel line of investigation, it was observed that FIAT can repress the transcriptional activity of the FRA-1/cJUN heterodimer [St-Arnaud and Elchaarani, 2007]. Since FIAT does not affect cJUN function [St-Arnaud and Elchaarani, 2007], the logical conclusion is that FIAT specifically inhibits FRA-1 activity. Ectopic expression of FRA-1 leads to a progressive increase in bone mass due to enhanced osteoblast differentiation [Jochum et al., 2000], while targeted inactivation of the *Fra-1* gene produces a reciprocal osteopenic phenotype [Eferl et al., 2004]. These studies identified a second target of the FIAT transcriptional repressor activity and suggest that FIAT can modulate early osteoblast activity by interacting with ATF4, as well as regulate later osteoblast function through inhibition of FRA-1.

These conclusive in vitro studies set the stage for manipulating the expression of FIAT in genetically engineered mice models.

FIAT ACTIVITY IN VIVO

Transgenic mice in which the full-length human FIAT cDNA was expressed under the control of the osteoblast-restricted 2.3 kb α1(I) collagen promoter fragment were engineered. The bone phenotype of these mice was fully characterized. Expression of the FIAT transgene did not affect mineral homeostasis nor osteoblast proliferation or survival [Yu et al., 2005]. Bone resorption, assessed through TRAP staining of osteoclasts and quantification of osteoclast number per bone perimeter, as well as serum levels of collagen cross-links, was not changed. However, bones from FIAT transgenic animals were osteopenic with decreased bone mineral density, bone volume, mineralized volume, mineral apposition rates, and decreased trabecular thickness, trabecular number, and rigidity of long bones [Yu et al., 2005]. Since osteoblast differentiation, proliferation, or apoptosis was not modified by the transgene, the reduced mineral apposition rates suggested a decline in osteoblast activity, which was confirmed by placing osteoblasts from transgenic mice in primary cultures [Yu et al., 2005]. Transcription of the ATF4 target *Osteocalcin* was lower in transgenic bones, supporting the interpretation that FIAT repressed ATF4-mediated transcription to regulate bone mass.

It is worth noting that type I collagen synthesis was not affected in FIAT transgenic bones. It is unlikely that this was observed because FIAT does not modulate this ATF4 function since FIAT knockdown in cultured osteoblasts impacted on the synthesis of type I collagen [Yu et al., 2009b]. Most likely, the unaffected type I collagen synthesis in FIAT transgenic bones was caused by the relatively moderate overexpression of the FIAT transgene (estimated at 20% above endogenous) achieved in this model system [Yu et al., 2005].

Pursuing our efforts to unravel the physiological role of FIAT, we have engineered a floxed allele of *Fiat* and are currently characterizing the phenotype of mice in which the *Fiat* gene was inactivated either globally or specifically in osteoblasts. Surprisingly, deleting the *Fiat* gene at different stages of osteoblast differentiation by crossing the *Fiat* floxed mice with transgenic mice expressing the Cre recombinase under the control of the *Osterix* promoter or the 2.3 kb α1(I) collagen promoter fragment did not yield a detectable bone phenotype (V.W.C. Yu, V. Mandic, and R. St-Arnaud, unpublished work). We surmised that this result could be due to inefficient excision of the floxed allele and therefore bred the floxed mice to a general deleter Cre transgenic strain (CMV-Cre). Both RT-qPCR and immunoblotting analysis confirmed that expression of the *Fiat* mRNA and the FIAT protein were not detectable in the mutant progeny. Based on the demonstrated inhibition by the FIAT activity of the bone mass promoting bZIP factors ATF4 and FRA-1, we expected a high-bone mass phenotype in mice totally deficient for FIAT. However, micro-CT and bone histomorphometry did not reveal any change in bone mass between mutant and wild-type littermates of both genders at any age. Consequently, the biomechanical properties assessed by three-point bending assay were similar in both genotypes. Gene expression monitoring of calvarial mRNA did not reveal any significant changes in the expression of the ATF4 or FRA-1 targets *Osteocalcin*, *Bsp*, *Esp*, or *MGP* (matrix Gla protein).

FIAT was shown to interact in vitro with Syntaxin 4 [Nogami et al., 2003a], which regulates vesicle trafficking and is important for regulating glucose homeostasis through GLUT4. Thus, *Fiat* inactivation could potentially affect glucose homeostasis. We performed glucose tolerance tests in FIAT-deficient mice and wild-type littermates. Fasted animals were challenged with a glucose injection. The response to the glucose treatment was similar in wild-type and knockout groups, indicating that FIAT did not

affect glucose homeostasis in vivo (V. Mandic, V.W.C. Yu, and R. St-Arnaud, unpublished).

Thus, FIAT deletion appears well tolerated in vivo. Considering the convincing evidence accumulated both in tissue culture and in transgenic mice that demonstrates a role for FIAT in the regulation of osteoblast biology, it is unlikely that FIAT serves no function in living bone. Elucidating this biological role now appears like an interesting challenge to investigators.

PERSPECTIVE AND CHALLENGES

ATF4 transcriptionally controls the expression of key effectors of osteoblast activity such as *Esp*, *Rankl*, and *Osteocalcin*. Several mechanisms could be involved in mediating the FIAT-dependent inhibition of ATF4-mediated transcription (Fig. 1). First and foremost, it was shown that the interaction of FIAT with ATF4 leads to the creation of inactive dimers that cannot bind the ATF4 response element (OSE1) within the *Osteocalcin* gene promoter (Fig. 1B) [Yu et al., 2005]. As previously mentioned, the transcriptional coactivator αNAC, that binds to the osteocalcin proximal promoter element near the OSE1 site [Akhouayri et al., 2005], also interacts with FIAT [Yoshida et al., 2005; Yu et al., 2006a]. Thus, the interaction of FIAT with αNAC could lead to steric hindrance at the proximal promoter and contribute to the FIAT-mediated inhibition of *Osteocalcin* gene transcription (Fig. 1C). Additional, non-mutually exclusive, mechanisms include recruitment of co-repressors to the chromatin through αNAC, FIAT, or both (Fig. 1D,E). FIAT expression may also regulate bone formation by other mechanisms in addition to suppressing ATF4 transcriptional activity. FIAT heterodimerizes with FRA-1 [St-Arnaud and Elchaarani, 2007], which has been shown to regulate bone mass accrual [Jochum et al., 2000]. The inhibition of FRA-1 activity in bone may represent a mechanism through which FIAT could modulate osteoblastic gene transcription (Fig. 1F).

There is convincing evidence demonstrating that FIAT interacts with ATF4 to inhibit its function. A number of assays and model systems were used to accumulate data leading to this conclusion: yeast two-hybrid interaction, co-immunoprecipitation, EMSAs, overexpression in osteoblastic and in non-bone cells, siRNA-mediated knockdown, chromatin immunoprecipitation, and gene expression monitoring. The inhibitory effect of FIAT on ATF4 was also observed in vivo in FIAT transgenic mice. Yet, a true null allele of *Fiat* appears innocuous to mouse development in general, and to bone development specifically.

Evolution did not preserve genes that have no function. Thus, the failure to identify a phenotypic consequence of *Fiat* inactivation reflects either redundancy of function or points to the fact that the endpoints assayed to date were not appropriate to evaluate the physiological role of FIAT in bone. Concerning the possibility of redundancy of function, compensatory expression of the related family members α- and β-taxilin was not observed in the FIAT knockouts (V. Mandic, V.W.C. Yu, and R. St-Arnaud, unpublished). However, functional redundancy (i.e., control of ATF4 activity through formation of inactive heterodimers) could be achieved in bone through expression of ICER [Chandhoke et al., 2008], or other

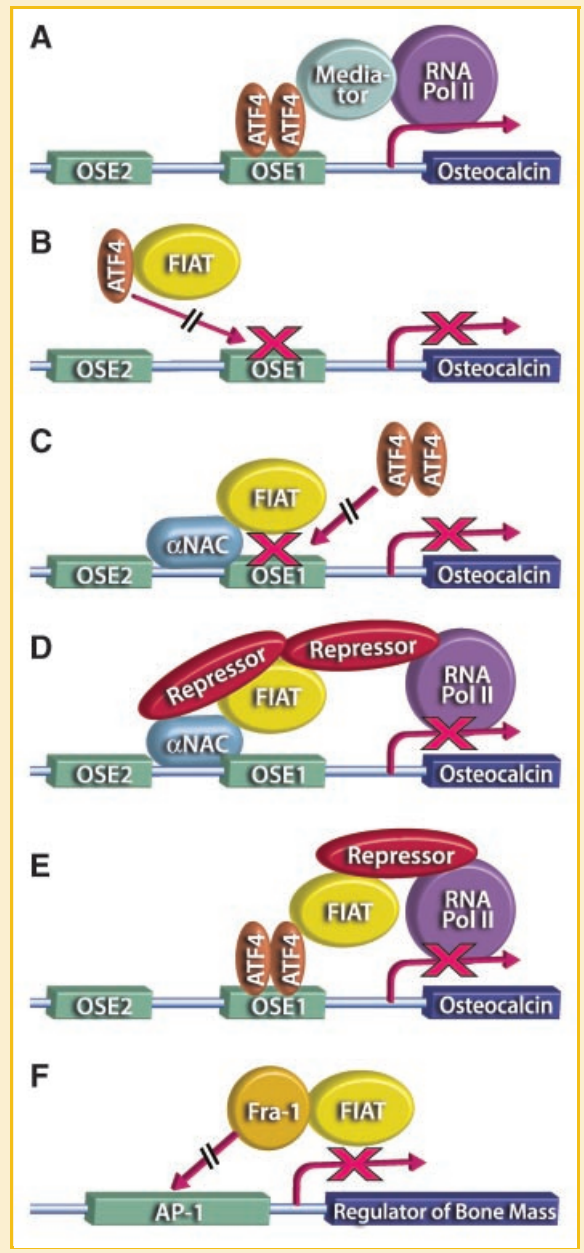

Fig. 1. Putative mechanisms of FIAT-mediated transcriptional repression. A: Schematized, partial description of *Osteocalcin* gene transcription. The binding of the ATF4 homodimer at the OSE1 (osteoblast specific element 1) site recruits accessory factors (here, globally represented as mediator) and leads to increased RNA polymerase II (RNA Pol II)-mediated transcription. OSE2 (osteoblast-specific element 2) which is the binding site for RUNX2. B: Interaction of ATF4 with FIAT forms inactive dimers that cannot bind the OSE1 site. C: FIAT also interacts with the αNAC transcriptional coactivator, which has been shown to bind the osteocalcin promoter near the OSE1 site. This could lead to steric hindrance at the proximal promoter and prevent ATF4 dimers from binding to the OSE1 element. D,E: Alternatively, αNAC or FIAT could recruit repressor molecules to the promoter. F: FIAT has also been shown to interact with the AP-1 bZIP family member FRA-1 that controls the transcription of target genes regulating bone mass. Interaction of Fra-1 with FIAT also forms inactive dimers that cannot bind the target gene promoter.

ZIP factors such as CHOP (CCAAT/enhancer-binding protein homologous protein) [Pereira et al., 2007]. This has not yet been evaluated in FIAT mutant bones. Considering the key roles played by ATF4 in osteoblasts, these cells may have evolved a sophisticated and redundant set of checkpoints to prevent aberrant ATF4 activity. It may be necessary to inactivate more than one repressor of ATF4, such as in compound FIAT/ICER knockouts, to observe a phenotypic consequence.

Concerning the parameters assayed to study the phenotypic consequences of *Fiat* inactivation in bone, more sophisticated analyzes of the bone matrix, such as ultrastructural examination of the architecture of the mineral phase and extracellular matrix composition and organization, could be performed to evaluate putative subtle effects of the *Fiat* mutation on bone mineral structure. Alternatively, a challenge to bone development or function could reveal the physiological role of FIAT in osteoblasts. Experiments along those lines include the ovariectomized (OVX)/estrogen deficiency model of post-menopausal osteoporosis, fracture repair and/or distraction osteogenesis, or skeletal unloading. Manipulation of dietary protein content was shown to modulate ATF4's role in bone [Sowa and Karsenty, 2007] and such perturbations of the diet could illuminate a biological function of FIAT. Studying primary cultures of FIAT-deficient osteoblasts might also release the cells from compensatory endocrine or paracrine regulation, and allow to unravel the activity of endogenous FIAT in bone-forming cells.

In vitro studies also hint at FIAT functions outside of bone [Nogami et al., 2003a,b]. The release of growth hormone upon stimulation with calcium in the PC12 cell line was shown to be regulated by FIAT, which raises a question as to whether growth and/or neuronal functions might be affected by the lack of FIAT in vivo. While FIAT mutant mice are of normal size, appear healthy, and do not display gross abnormal behavior, more thorough and sophisticated behavioral analysis, and study of neuronal differentiation and activity will be necessary to address these putative functions.

As previously mentioned, it was also reported that FIAT physically interacts with Syntaxin 4 [Nogami et al., 2003b], a protein involved in regulating vesicle trafficking. More specifically Syntaxin 4 is crucial for the insulin-induced translocation of GLUT4, the major glucose transporter [Spurlin et al., 2004; Yang et al., 2001]. When FIAT-deficient mice were challenged with glucose, the rate of glucose clearance in the blood was not different from what was measured in wild-type littermates. This seems to indicate that FIAT has no impact on glucose metabolism in vivo, but additional tests could be performed to more fully address this issue.

Several interesting questions remain unanswered. Does FIAT interact with the related ATF4 family member ATF5/ATFx to influence its activity? How is FIAT transcription and expression regulated? What is the physiological relevance of the interaction of FIAT with syntaxin family members or αNAC? Are the subcellular localization of FIAT, or its interaction with dimerizing targets, regulated post-translationally? Which protein(s) interact with FIAT in osteocytes? Does the C-terminal domain and third leucine zipper modulate mineralization by interacting with an as yet unidentified partner or solely through structural effects? Answers to these questions will further our knowledge of FIAT structure and function and of its physiological role in bone and other target tissues.

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