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COMBINATORIAL CONTROL OF ATF4-DEPENDENT GENE TRANSCRIPTION IN OSTEOBLASTS

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Abstract

Osteoblast-specific gene transcription requires interaction between bone cell-specific transcription factors and more widely expressed transcriptional regulators. This is particularly evident for the basic domain-leucine zipper factor Activating Transcription Factor 4 (ATF4), whose activity can be enhanced or inhibited through interaction with other leucine zipper proteins, intermediate filament proteins, components of the basic transcriptional machinery, nuclear matrix attachment molecules, or ubiquitously expressed transcription factors. We discuss the results supporting the relevance of these interactions and present the first evidence of a functional interaction between ATF4, FIAT (Factor Inhibiting ATF4-mediated Transcription) and αNAC (Nascent polypeptide associated complex And Coactivator alpha), three proteins that have been previously shown to associate using various protein-protein interaction assays.

Keywords

Activating Transcription Factor 4 (ATF4); Factor Inhibiting ATF4-mediated Transcription (FIAT); Nascent polypeptide associated complex And Coactivator alpha (αNAC); osteoblast; transcription

Our understanding of tissue-specific gene transcription has increased considerably since the purification and cloning of the first sequence-specific DNA binding transcription factor.[1, 2] From the naïve notion that gene expression in each tissue would be controlled by factors uniquely expressed in that tissue, the knowledge has evolved to include a plethora of coregulatory proteins acting as scaffolds to recruit multiunit complexes bridging the DNA-binding factors to the general transcriptional machinery. Many of these cofactors exhibit enzymatic activity that impacts chromatin structure.[3]

In addition to the assembly of these macro-molecular complexes that are crucial to differential gene transcription, nature has developed other means to restrict gene transcription in specific cell types. The interaction of distinct transcription factor families to

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regulate gene expression is an established concept, and this interaction may require promoter DNA binding by each partner or not. These combinatorial interactions have been shown to involve not only tissue-restricted proteins but also ubiquitously expressed transcription factors.

Initially, the interaction between distinct families of DNA-binding transcription factors was observed both in the context of transcriptional activation or repression, and this notion remains valid nowadays. Early examples include the interaction between AP-1 family members and nuclear hormone receptors leading to inhibition of nuclear receptor-mediated transcription by cFOS/cJUN.[4-7] Cooperative activation of gene transcription through protein-protein combination of heterologous classes of DNA-binding factors was first clearly demonstrated by the interaction between MEF2 and myogenic bHLH proteins in muscle gene expression. In this context, the cooperativity required direct protein-protein interaction, but only one of the factors needed to be bound to DNA.[8] Many combinatorial interactions regulating bone-cell specific transcription have been described involving the osteoblast-specific factor RUNX2. Discussing every characterized interaction is beyond the scope of this perspective and the reader is referred to other reviews.[9-11] Here, we will restrict the topic to interactions involving the recent arrival on the scene of osteoblast biology, the basic domain-leucine zipper (bZIP) transcription factor ATF4.

ATF4 is a member of the activating transcription factor (ATF)/cyclic adenosine monophosphate responsive element-binding (CREB) family. This gene family consists of transcription factors that bind the CRE (cAMP response element) through highly related bZIP dimerization domains.[12] There is extensive sequence similarity between the different family members within the basic DNA binding domain and the leucine zipper dimerization interface, but members of the family that are not part of the same subgroup do not share much similarity other than the bZIP motif. ATF4 plays a pivotal role in the regulation of osteoblast function. While expression of the *Atf4* mRNA is ubiquitous, the ATF4 protein is unstable and degraded in most cell types through ubiquitination by the SCF^{bTrCP} ubiquitin ligase,[13] except in osteoblasts where it accumulates.[14] In the bone-forming cell, ATF4 affects several key functions such as the synthesis of type I collagen[15] and the transcriptional control of several major osteoblastic genes: *Osteocalcin, Rankl* (Receptor activator of NF-kB ligand), and *Esp* (Embryonic stem cell phosphatase).[15-17] In addition to *Osteocalcin*, ATF4 targets include genes involved in amino acid import, metabolism, and assimilation.[18] This role of ATF4 in regulating amino acid import appears responsible for the decrease in type I collagen synthesis measured in ATF4-deficient osteoblasts.[15]

Combinatorial interactions activating ATF4

a) Dimerization partners

The ATF4 leucine zipper dimerization interface consists of heptad repeats of leucine residues which align along one face of an alpha helix. When aligned in parallel, the hydrophobic faces of two complementary helices form a coiled coil.[19] Leucine zipper dimerization serves to juxtapose adjacent regions of each of the dimer's partners that are rich in basic amino acid residues and that serve as the DNA binding domain of the dimer.[20, 21] ATF4 can form homodimers[22, 23] but can also heterodimerize with a variety of partners.

Indeed, the first ATF4 cDNAs (identified under several different names such as CREB2, TAXREB67, C/ATF, or mTR67) were cloned through the interaction of the ATF4 bZIP domain with the leucine zipper of other transcription factors.[22, 24] The dimerization partner appears to influence specificity of DNA binding, with a preference for the CRE binding site: cJUN/ATF4 and cFOS/ATF4 dimers bind the CRE site, while the FRA-1/ATF4 heterodimer can interact with both CRE and AP-1 binding sites.[23] Interaction of ATF4 with members of the CCAAT/Enhancer-binding protein (C/EBP) family diverts C/EBP factors from CCAAT-box binding sites to CRE motifs.[22]

The dimerization of ATF4 with C/EBP family members is significant in bone biology. A C/EBPβ/ATF4 dimer binds a response element within the proximal promoter of the collagen receptor, discoidin domain receptor tyrosine kinase (DDR2).[25] This dimer enhances *Ddr2* transcription in cultured cells.[25] More importantly, mice deficient for C/EBPβ exhibit delayed bone formation and one of the mechanisms characterized also involves heterodimerization of C/EBPβ with ATF4.[26] A number of sophisticated assays confirmed the interaction of the two proteins and their association with the proximal *Osteocalcin* gene promoter at the ATF4 binding site (osteocalcin-specific element 1 or OSE1) to activate *Osteocalcin* transcription.[26] Interestingly, C/EBPβ allowed ATF4 to form a complex and synergize with RUNX2 to increase *Osteocalcin* expression (Fig 1A, panel i).[26] The osteoblast appears to use several accessory molecules such as C/EBPβ to promote the formation of complexes containing both ATF4 and RUNX2 to activate *Osteocalcin* gene transcription, as will be further detailed below.

b) Cooperative interactions

Soon after the initial description of the role of ATF4 in osteoblast-specific gene expression[15], it was observed that ATF4 activates transcription from the proximal *Osteocalcin* gene promoter via cooperative interaction with RUNX2.[27] The co-stimulation required an intact RUNX2 DNA binding site, and while it was maximal when both the ATF4 and RUNX2 elements were present, cooperativity was still observed in the absence of the ATF4 binding site (OSE1), suggesting that some of the effects of ATF4 occur in the absence of DNA binding.[27] RUNX2 and ATF4 could be co-immunoprecipitated from osteoblastic nuclear extracts, but assays using purified recombinant proteins failed to show a direct interaction between the two factors. This data was interpreted to mean that accessory factors are involved in stabilizing the interaction between the two molecules.[27] A follow-up study by the same investigators identified the smallest subunit of the general transcription factor IIA, TFIIAγ, as a protein bridge between the two factors.[28] Protein-protein interactions were demonstrated between RUNX2 and TFIIAγ, as well as between ATF4 and TFIIAγ, and the same region of RUNX2 was necessary for interaction with TFIIAγ or for association with ATF4. All three proteins associated with the same region of the *Osteocalcin* gene promoter, and co-transfection of all three factors synergistically enhanced transcription of the endogenous *Osteocalcin* gene or of reporter constructs under the control of the proximal *Osteocalcin* promoter.[28] Interestingly, one of the mechanisms through which TFIIAγ increases *Osteocalcin* expression involves stabilization of the ATF4 protein.[28] Thus the general transcription factor TFIIAγ, just like C/EBPβ, maximizes *Osteocalcin* gene transcription by promoting the formation of multimeric complexes containing ATF4 and

RUNX2 (Fig 1A, panel ii). The association of components of the general transcriptional machinery with osteoblast-relevant sequence-specific DNA binding transcription factors has been described before, such as the interaction of the vitamin D receptor or OSTERIX with TFIIB.[29, 30] However, the mechanism through which TFIIAγ increases *Osteocalcin* expression, i.e. stabilization of the half-life of the ATF4 protein,[28] is a first.

The regulation of gene transcription occurs in a specific subcellular compartment, the nucleus, and the role of the nuclear matrix in controlling transcription is now clearly recognized.[31] RUNX2 associates with the nuclear matrix, which impacts on its subnuclear localization and function.[32] While its interaction with the nuclear matrix may be direct, RUNX2 could be targeted to subnuclear regions through its association with SATB2, a member of the special AT-rich binding protein family that binds to nuclear matrix-attachment regions.[33, 34] *Satb2*-deficient mice have compromised osteoblast differentiation and function, with decreased expression of osteoblast-specific genes, including, amongst others, the RUNX2 and ATF4 targets *Osteocalcin* and *Bone Sialoprotein* (*Bsp*).[35] Chromatin immunoprecipitation assays confirmed that SATB2 is recruited to the *Osteocalcin* and *Bsp* promoters. While SATB2 directly binds the *Bsp* gene 5'-flanking region, its association with the *Osteocalcin* promoter was shown to be indirect. Direct protein-protein interactions between SATB2 and RUNX2, as well as between SATB2 and ATF4, were demonstrated using pull-downs with recombinant molecules, and their association in cells was confirmed by co-immunoprecipitation. SATB2 appears to stabilize the interaction of RUNX2 and ATF4 with their cognate DNA binding sites within the *Osteocalcin* promoter, leading to enhanced transcription. The model proposed, once again, involves the formation of complexes associating SATB2, RUNX2, and ATF4 (Fig 1A, panel iii).[35]

As previously mentioned, ATF4 controls the transcription of genes involved in amino acid import, metabolism, and assimilation[18] in addition to *Osteocalcin* and *Bsp*. A link between oxidative stress, amino acid import, and osteoblast biology was uncovered by the study of the phenotype of mice with osteoblast-specific inactivation of the transcription factor FoxO1.[36] The ubiquitously expressed FoxO family of transcription factors is involved in the response of cells to reactive oxygen species and oxidative stress.[37, 38] Inactivating the *FoxO1* gene specifically in osteoblasts reduces their number and leads to a decrease in bone volume and bone formation rates.[36] These effects can be rescued by treating the mice with the antioxidant N-acetyl L-cysteine, demonstrating that the phenotype results from oxidative stress. Antioxidant administration had no effect on the reduced levels of glutathione measured in FoxO1-deficient bones, however. Since ATF4 is part of the pathway controlling amino acid import, leading to glutathione synthesis, these observations suggested a link between FoxO1 and ATF4. Such a link was further supported by the findings that in FoxO1-deficient osteoblasts, just like in ATF4-deficient bone cells,[15] type I collagen protein synthesis was reduced while the level of α_1*(I) collagen* mRNA was not affected.[36] Similarly, a high protein diet corrected the phenotype of FoxO1 osteoblast-deficient mice,[36] as has been described in mutants that affect ATF4 function.[39, 40] In studying the mechanisms involved, it was found that ATF4 and FoxO1 co-localize in osteoblasts and that both proteins co-immunoprecipitated from nuclear extracts of primary osteoblasts or bone tissue. Transcriptional cooperativity was observed between FoxO1 and ATF4 in the expression of reporter constructs that were either FoxO targets or controlled by the *Osteocalcin* proximal

promoter region (Fig 1A, panel iv).[36] These results show that FoxO1 affects osteoblast proliferation and function by controlling redox balance, and by regulating amino acid import through its interaction with ATF4. Moreover, they implicate a ubiquitously expressed transcription factor in the regulation of osteoblast function, in addition to the better-studied osteoblast-specific transcriptional regulators.

Combinatorial interactions inhibiting ATF4

Partnering can also inhibit ATF4 function. In a search for functional partners of ATF4 that utilized His-ATF4 affinity chromatography with ROS17/2.8 osteosarcoma cells[41] nuclear extracts followed by mass spectrometry of the purified partners, the intermediate filament protein, vimentin, was identified.[42] It was confirmed that vimentin can localize to the nucleus and interacts with ATF4 through a putative leucine zipper comprised of amino acid residues 124 to 138. The interaction of vimentin with ATF4 prevents the binding of ATF4 to its cognate response element and inhibits osteoblast differentiation and the transcription of the ATF4 targets, *Bsp* and *Osteocalcin*.[42] Inhibition of vimentin expression using siRNA-mediated knockdown induces endogenous *Osteocalcin* expression in preosteoblasts. These results suggest a novel mechanism through which a cytoskeletal protein re-localizes to the nucleus to interact with ATF4 and act as an inhibitor of osteoblastogenesis.[42]

Dimerization with nuclear transcriptional regulators have also been shown to inhibit ATF4 activity. Co-transfection of ATF4 with ICER (inducible cAMP early repressor) specifically represses ATF4-dependent transcription.[43] The ICER proteins are differentially-spliced products of the cAMP responsive element modulator (*Crem*) gene that act as transcriptional repressors.[44] All four ICER isoforms are induced in osteoblasts following PTH treatment[45, 46] and the measured inhibition of ATF4 transcriptional activity by ICER was invoked as a mechanism to explain the reduced bone mass and impaired osteoblast differentiation observed in ICER transgenic mice (Fig. 1B, panel i).[43]

Our laboratory has cloned and characterized FIAT (Factor Inhibiting ATF4-mediated Transcription, also named γ-taxilin),[47, 48] a leucine-zipper protein devoid of DNA-binding activity but capable of heterodimerizing with ATF4 to form inactive dimers and inhibit ATF4 transcriptional activity (Fig. 1B, panel ii).[48] Stable overexpression of a *Fiat* transgene was shown to inhibit transcription from the *Osteocalcin* gene promoter and to reduce mineralization, both in cultures of primary osteoblasts or in established osteoblastic cells.[48, 49] Conversely, siRNA-mediated inhibition of FIAT expression enhanced all ATF4 functions tested: *Osteocalcin* transcription and promoter occupancy, *Bsp* gene transcription, as well as type I collagen synthesis.[50] FIAT-depleted osteoblasts also displayed increased mineralization and an increased number of nodules.[50] Bones from FIAT transgenic animals were osteopenic with decreased bone mineral density, bone volume, mineralized volume, mineral apposition rates, and reduced trabecular thickness, trabecular number, and rigidity of long bones.[48] The exhaustive phenotype analysis of the FIAT transgenic mice[48] combined with a number of *in vitro* experiments[48-50] support the interpretation that FIAT interacts with ATF4 to repress its transcriptional activity, thus regulating bone mass.

We cloned FIAT in a yeast two-hybrid screen for proteins interacting with the αNAC (Nascent polypeptide associated complex And Coactivator alpha) transcriptional coregulator.[48, 51] This interaction was independently confirmed[52] but its biological relevance has remained elusive. In the course of our exhaustive structure-function analysis of the αNAC protein,[53-60] we have identified a mutation affecting a putative phosphoacceptor site that define a functional interaction between ATF4, FIAT, and αNAC. The αNAC protein is extensively post-translationally modified by phosphorylation events that modulate its half-life, subcellular localization, and activity.[56-58] Our recent mutational analysis involved the replacement of a putative phosphoacceptor site, residue serine132, by a charged aspartic acid (D) moiety, thus mimicking a permanent phosphorylated state for the mutant S132D protein. When co-transfected with ATF4 and FIAT, the S132D αNAC mutant potentiated the FIAT-mediated repression of ATF4 activity, leading to complete suppression of transcription from a synthetic promoter containing six copies of the OSE1 binding site for ATF4 (Fig. 2). Transfected alone, FIAT and the αNAC mutant had no effect on reporter gene expression (Fig. 2), and the wild-type αNAC protein did not affect FIAT activity in this assay (not shown). These results are the first demonstration of a functional interaction between ATF4, FIAT, and αNAC, proteins that had been previously shown to associate using various protein-protein interaction assays.[48, 51, 52] We have previously shown that αNAC binds the *Osteocalcin* proximal promoter.[53] Thus the interaction of FIAT with αNAC could lead to steric hindrance at the proximal promoter and contribute to the FIAT-mediated inhibition of ATF4-dependent *Osteocalcin* gene transcription (Fig. 1B, panel iii).

Summary and perspectives

Considering its critical roles in osteoblast biology, the activity of ATF4 needs to be tightly regulated. Cells have evolved several mechanisms to this effect, including ubiquitinylation,[13, 14] post-translational modifications,[15, 16] and interaction with specific partners.[25, 26, 28, 35, 42, 48] It is likely that the list of accessory factors that modulate ATF4 function is not complete yet.

While the studies discussed herein all involved sophisticated experiments that support the relevance of the observed interactions with ATF4, incontrovertible genetic evidence of the physiological relevance of the interaction was only provided for SATB2. The observation that compound *Satb2*$^{+/-}$;*Atf4*$^{+/-}$ as well as *Satb2*$^{+/-}$;*Runx2*$^{+/-}$ heterozygous animals exhibit a reduced bone formation phenotype provided genetic proof of the synergy between SATB2, ATF4, and RUNX2.[35] Such breeding experiments remain to be performed to confirm that all the identified ATF4-interacting molecules form part of a common genetic pathway, and these might require tissue-specific inactivation in the case of ubiquitously expressed proteins such as vimentin, TFIIAγ or FoxO1.

Other unanswered questions concern the intricacies of the reported protein-protein interactions. What mechanisms control the nuclear re-localization of vimentin? Are the interactions direct or indirect? Some of the assays used to demonstrate ATF4-partner interactions do not allow to unequivocally conclude that the two proteins directly interact. Moreover, it is never evident to determine if a given partner interacts with a monomeric ATF4 protein or an ATF4 dimer. This question could be addressed using single-chain ATF4

dimers in which monomers are joined via a flexible polypeptide tether to force pairing.[61] Finally, considering the relevance of post-translational modifications for ATF4 activity, it will prove interesting to determine whether the observed associations are modulated through phosphorylation or other means. This is particularly relevant for the functional interaction between ATF4, FIAT, and αNAC, which was only unraveled through a mutational mimic of phosphorylation. The characterization of the kinase regulating this interaction could identify novel signal transduction pathways involved in the control of osteoblastic gene transcription. Attractive, testable mechanisms include the recruitment of corepressors to the chromatin by differentially phosphorylated αNAC (Fig. 1B, panel iv).

At any rate, it has become clear that combinations of transcriptional regulators are the norm rather than the exception and that they contribute significantly to achieve the exquisite fine control of gene expression observed during osteoblastic differentiation and in the mature bone-forming cell.

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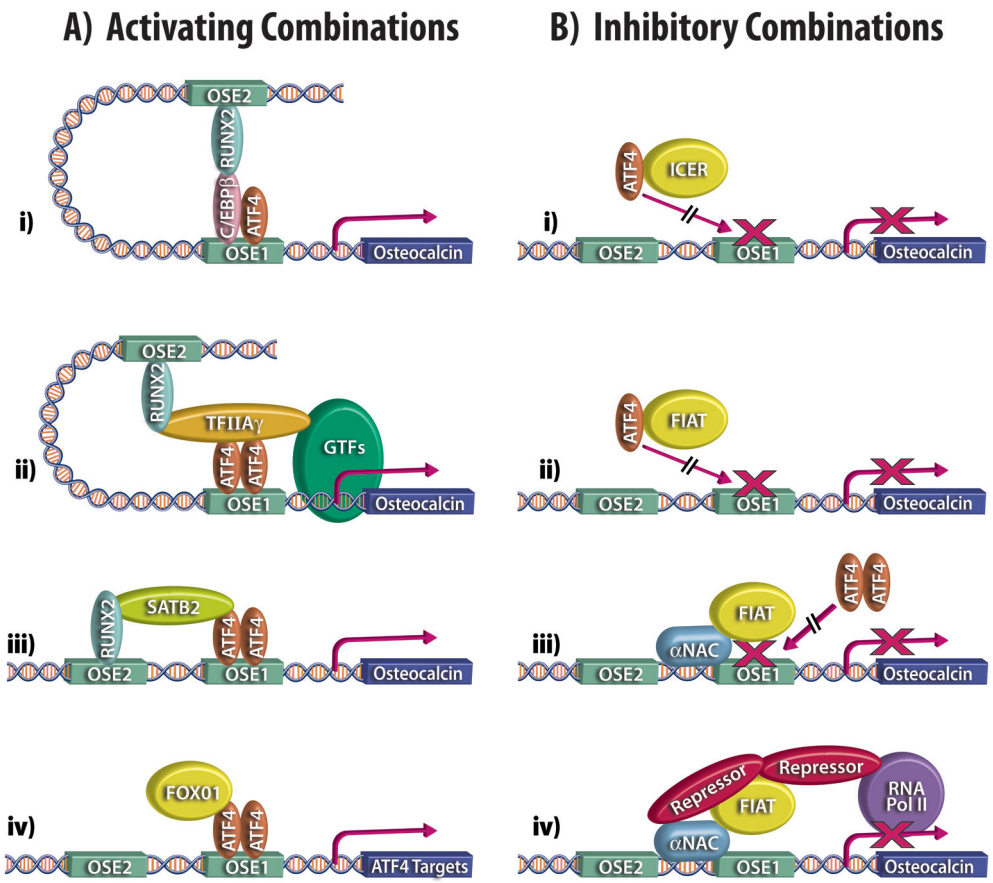

Figure 1:

Combinatorial control of ATF4 activity. Schematized, partial depiction of the proximal promoter fragment of the *Osteocalcin* gene: the RUNX2 binding site, OSE2 (Osteoblast Specific Element 2) and the ATF4 binding site, OSE1 (Osteoblast Specific Element 1) are shown. A. activating combinations: i) dimerization of ATF4 with C/EBPβ allows formation of a complex with RUNX2 to increase *Osteocalcin* expression; ii) TFIIAγ acts as a protein bridge between ATF4, RUNX2, and general transcription factors (GTFs); iii) SATB2 stabilizes the interaction of RUNX2 and ATF4 with DNA, leading to enhanced transcription; iv) transcriptional cooperativity between FoxO1 and ATF4. B. inhibitory combinations: i, ii) interaction of ATF4 with ICER or FIAT forms inactive dimers that cannot bind the OSE1 site; iii) steric hindrance at the proximal promoter caused by the binding of αNAC prevents ATF4 dimers from binding to the OSE1 element; iv) αNAC or FIAT could recruit repressor molecules to the promoter to block ATF4 activity.

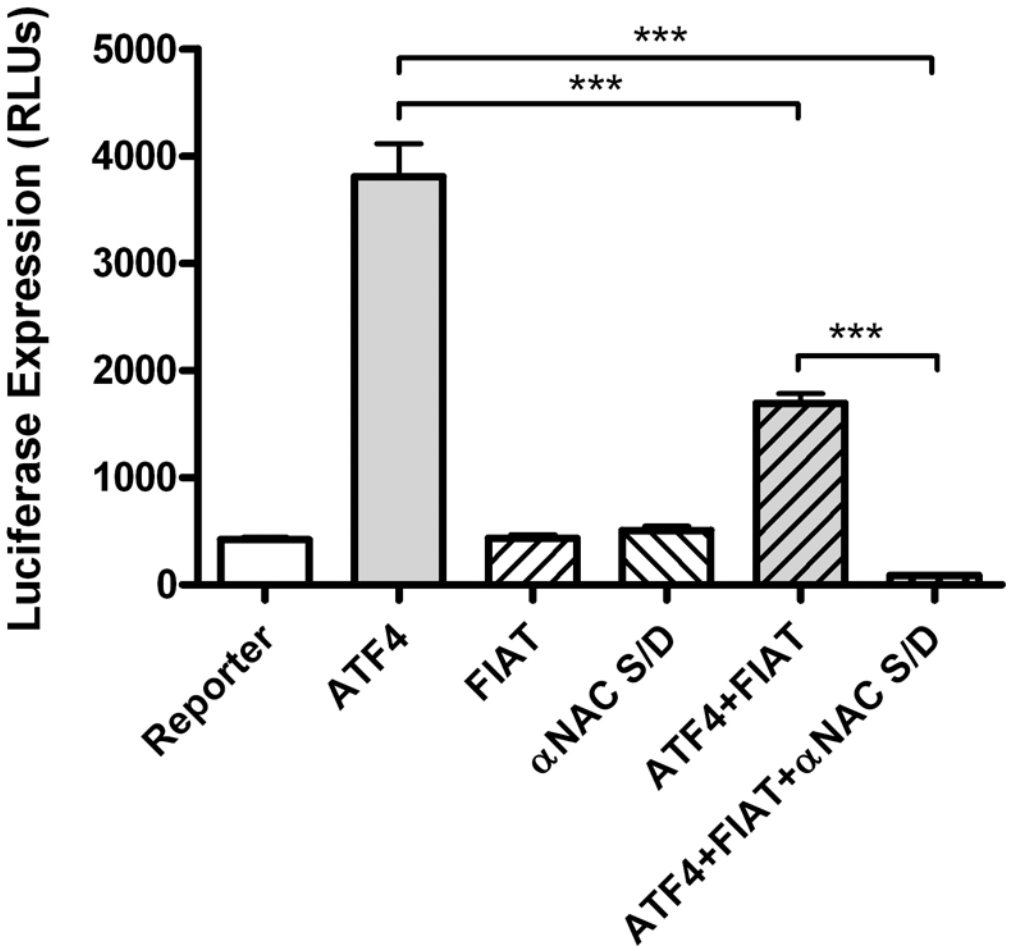

Figure 2:

Functional interaction between FIAT and αNAC completely inhibits ATF4-dependent transcription. Osteoblastic cells were transfected with a reporter construct under the control of multiple copies of the ATF4 binding site, OSE1 (Osteoblast Specific Element 1), together with expression vectors for ATF4, FIAT, and a site-directed mutant (S/D) of αNAC, alone or in combination. FIAT inhibited ATF4 transcriptional activity, and this effect was further enhanced by addition of the αNAC S/D mutant. RLUs, relative light units; ***, $p<0.001$.