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SUMOylated αNAC Potentiates Transcriptional Repression by FIAT

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ABSTRACT

The transcriptional coregulator αNAC (Nascent polypeptide associated complex And Coregulator alpha) and the transcriptional repressor FIAT (Factor Inhibiting ATF4-mediated Transcription) interact but the biological relevance of this interaction remains unclear. The activity of αNAC is extensively modulated by post-translational modifications (PTMs). We identified a novel αNAC PTM through covalent attachment of the Small Ubiquitin-like MOdifier (SUMO1). Recombinant αNAC was a SUMO1 target in in vitro SUMOylation assays and we confirmed that αNAC is conjugated to SUMO1 in cultured osteoblasts and in calvarial tissue. The amino acid sequence of αNAC contains one copy of the composite "phospho-sumoyl switch" motif that couples sequential phosphorylation and SUMOylation. We found that αNAC is selectively SUMOylated at lysine residue 127 within the motif and that SUMOylation is enhanced when a phosphomimetic mutation is introduced at the nearby serine residue 132. SUMOylation did not alter the DNA-binding capacity of αNAC. The S132D, hyper-SUMOylated αNAC mutant specifically interacted with histone deacetylase-2 (HDAC2) and enhanced the inhibitory activity of FIAT on ATF4-mediated transcription from the *Osteocalcin* gene promoter. This effect required binding of SUMOylated αNAC to the target promoter. We propose that maximal transcriptional repression by FIAT requires its interaction with SUMOylated, HDAC2-interacting αNAC. J. Cell. Biochem. 115: 866–873, 2014.

KEY WORDS: αNAC; SUMOylation; FIAT; TRANSCRIPTIONAL REGULATION; OSTEOBLASTS

FIAT (Factor Inhibiting ATF4-mediated Transcription, also named γ-taxilin) is a leucine-zipper protein devoid of DNA-binding activity but capable of heterodimerizing with ATF4 to form inactive dimers and inhibit ATF4 transcriptional activity [Yu et al., 2005; St-Arnaud and Mandic, 2010]. The exhaustive phenotype analysis of FIAT transgenic mice [Yu et al., 2005] combined with a number of in vitro experiments [Yu et al., 2005, 2008, 2009] support the interpretation that FIAT interacts with ATF4 to repress its transcriptional activity, thus regulating bone mass. FIAT was initially cloned using a yeast two-hybrid screen for proteins interacting with αNAC (Nascent polypeptide associated complex And Coregulator alpha), a transcriptional coregulator of gene expression in bone cells [Akhouayri et al., 2005; Yu et al., 2005, 2006; Meury et al., 2010]. This interaction was independently confirmed [Yoshida et al., 2005] but its biological relevance has remained elusive. We reasoned that post-translational modifications of one or both of the proteins might modulate the functional outcome of the interaction.

The αNAC protein shuttles to the nucleus where it can positively or negatively regulate gene transcription during mesenchymal cell differentiation through differential interaction with histone deacetylase (HDAC) corepressor molecules [Akhouayri et al., 2005; Jafarov et al., 2012]. We have shown that αNAC is extensively post-translationally modified by phosphorylation events that regulate its half-life, subcellular localization, and activity [Quelo et al., 2004a,b, 2005]. Ongoing structure–function analysis of the αNAC protein has identified a "phospho-sumoyl switch" motif that couples sequential phosphorylation and SUMOylation [Yang and Gregoire, 2006]. Modification by the small ubiquitin-related modifier (SUMO) family is a dynamic and reversible modification whereby a SUMO moiety is covalently added to target lysine

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residues, in a three step enzymatic process similar to ubiquitination [Gill, 2004].

SUMOylation often leads to transcriptional inhibition by providing a novel protein–protein interface, allowing interaction of the SUMOylated substrate with transcriptional repressors such as HDACs, which have been shown to be both effectors, substrates or regulators of SUMOylation [Yang and Sharrocks, 2004; Gill, 2005]. There is growing evidence indicating preferential recruitment of HDAC2 by different SUMO-bound transcription factors to repress transcription of target genes [Yang and Sharrocks, 2004; Kuo et al., 2005].

We report that αNAC is selectively modified by SUMO1 at lysine residue 127 within the "phospho-sumoyl switch" motif and that this post-translational modification appears regulated by differential phosphorylation of nearby residue serine 132. A phosphomimetic S132D, hyper-SUMOylated αNAC mutant specifically interacts with HDAC2 to enhance the inhibitory activity of FIAT on ATF4-mediated transcription from the *Osteocalcin* (*Ocn*) gene promoter. These results are the first demonstration of a functional interaction between FIAT and αNAC.

MATERIALS AND METHODS

REAGENTS

The SUMOlink kit was purchased from Active Motif (Carlsbad, CA). Anti-SUMO1 (catalog No. sc-40120), anti-SUMO2/3 (sc-40220), and anti-HDAC2 (sc-7899) antibodies were from SantaCruz Biotechnologies (SantaCruz, CA), as was naïve IgG (sc-66931). The anti-T7 tag and anti-GAPDH were procured through AbCam (Toronto, ON; ab8245). The anti-FLAG antibody (F3165) and anti-FLAG M2 affinity gel (F2426) were purchased from Sigma. Other antibodies have been previously described and are referenced accordingly. Sepharose beads and ECL Western blotting detection reagents were obtained from GE Healthcare Bio-Sciences (Baie d'Urfé, QC). Lipofectamine transfection reagent was purchased from Life Technologies-Invitrogen (Carlsbad, CA) while the Bright-Glo™ luciferase assay reagents were from Promega (Madison, WI). Protease inhibitors cocktail was obtained from Roche Molecular Biochemicals (Laval, QC). Dimethyl 3,3′-dithiobis-propionimidate-2HCl (DTBP) was bought from ThermoFisher Scientific (Burlington, ON); other chemicals were obtained from Sigma.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Pellets from cultured cells or homogenized mouse calvaria were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris, pH 8, 0.1% SDS) containing protease inhibitors, in the presence or absence of 20 mM *N*-EthylMaleimide (NEM) for 30 min on ice. Lysates were centrifuged for 10 min at 4°C and then supernatants were incubated with primary antibody overnight at 4°C. Immuno complexes were collected with addition of 25 μl of protein G-agarose at 4°C with rotation. The beads were then washed three to five times with 1 ml of low IPB (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) or RIPA buffer. Immunoprecipitated proteins were resuspended in 40 μl of 2× SDS loading buffer, boiled for 5 min, and resolved electrophoretically by SDS–PAGE. Separated proteins were transferred to a nitrocellulose membrane using standard techniques. Membranes were blocked with 5% nonfat milk for 1 h at room temperature with agitation. The blocked membranes were then incubated with primary antibody overnight at 4°C with agitation followed by washing in TBS-T (150 mM NaCl, 20 mM Tris, pH 8, 0.1% Tween 20). Membranes were incubated with secondary antibody for 1 h at room temperature with agitation, then washed in TBS-T. Blots were developed using the ECL Western blotting detection reagent according to the instructions from the manufacturer.

PLASMIDS

The pSI-NAC-Flag expression vector [Quelo et al., 2004b] was used as backbone to generate all site-specific mutants using PCR-based methods [Cormack, 1997]. The SD/ΔDBD vector was constructed by removing a 520 bp AccI-MfeI fragment containing the S132D mutation (from the S132D vector) and inserting it into the corresponding position within the pSI-αNACΔ69-80-Flag expression vector [Akhouayri et al., 2005]. All mutations were confirmed by sequencing of the final plasmids; details and maps are available upon request. Other expression vectors have been previously described and are referenced accordingly. Recombinant wild-type αNAC protein was produced in *E. coli* from the $pTYB_2$-NAC plasmid [Quelo et al., 2004b] and purified using the IMPACT™ kit following the manufacturer's procedure (NEB, Mississauga, ON).

IN VITRO SUMOYLATION ASSAY

Recombinant wild-type αNAC (0.5 μg) was incubated with wild-type or mutant SUMO1 proteins, E1 activating enzyme, and E2 conjugating enzyme supplied in the SUMOlink kit, following the instruction of the manufacturer (Active Motif, Carlsbad, CA). SUMOylation was analyzed by immunoblotting with anti-SUMO1 antibodies.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Complementary oligonucleotides corresponding to the αNAC binding site within the murine *Ocn* proximal promoter region [Akhouayri et al., 2005] were synthesized with an overhang, annealed, and labeled with [^{32}P]-labeled dNTPs by Klenow fill-in using standard protocols [Ausubel et al., 1993]. Nuclear extracts from untransfected 3T3-L1 cells or 3T3-L1 cells transiently transfected with expression vectors for wild-type or site-specific mutant αNAC proteins were prepared following the technique of Dignam et al. [1983]. Recombinant proteins or nuclear extracts were incubated for 30 min at 4°C in 20 μl of binding buffer (100 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 500 mM NaCl, 2% NP-40, 10 mM DTT, 10 mM EDTA, 100 ng of polydI-dC, 30% glycerol). Labeled probe (5,000 dpm) was added to the binding reaction mixture. The bound mixtures were size-fractionated on a non-denaturing 6% polyacrylamide gel at 140 V for 4 h in 0.5× TBE (Tris-Borate-EDTA) buffer. The gels were subsequently dried and autoradiographed.

SEQUENTIAL CHROMATIN IMMUNOPRECIPITATION (CHIP-RECHIP)

MC3T3-E1 osteoblastic cells [Sudo et al., 1983] stably transfected with pSIαNAC-WT-Flag or pSIαNAC-S132D-Flag were plated on 100-mm-diameter dishes to grow until confluence. Cells were cross-linked with DTBP in conjugation with formaldehyde. Chromatin immunoprecipitation was performed as previously described [Akhouayri et al., 2005]. Immunoprecipitations were performed with anti-FLAG, anti-FIAT, or anti-GAPDH antibodies. Five microliter of purified DNA was used as template for PCR with the following primer

sequences which amplified the αNAC binding site in the promoter region of the *Osteocalcin* gene: Forward, 5′-TCGTCCACTCCCA-GACCTTGC-3′; Reverse, 5′-CTGCACCCTCCAGCATCCAG-3′. ChIP-reChIP was performed as described by Furlan-Magaril et al. [2009].

TRANSIENT TRANSFECTION ASSAYS

MC3T3-E1 cells were plated at 1.0–1.5 × 10^5 cells/well in a 6-well plate. Cells were transfected with 100 ng of p6OSE1-luc reporter construct [Ducy and Karsenty, 1995] and various concentrations of the expression vectors for ATF4, FIAT, and wild-type or mutant αNAC (for ATF4: pcDNA3.1/V5-His-ATF4, 500 ng – ref [Yang et al., 2004]; for FIAT: pcDNA3.1/V5-HisTOPO-FIAT, 300 ng – ref [Yu et al., 2005]; for WT αNAC: pSIαNAC-WT-Flag, 1,000 ng; for S132D: pSIαNAC-S132D-Flag, 100–1,000 ng; for KR/SD: pSIαNAC-K127R/S132D-Flag, 1,000 ng; for SD/ΔDBD: pSIαNAC-S132D/ΔDBD-Flag, 1,000 ng) using the Lipofectamine reagent according to manufacturer's instructions. The total amount of transfected DNA was adjusted to 2 μg in each well using pBlueScript (Stratagene, La Jolla, CA). At 24 h post-transfection, cells were lysed and 20 μl of cell lysate was used to measure luciferase activity following the manufacturer's procedure (Promega) and analyzed with a Sirius single tube luminometer (Berthold Technologies, Oakville, TN). Expression of the transfected proteins was ascertained by immunoblotting (data not shown). Each transfection was repeated three times with triplicate samples, and the data shown represent the mean and the standard error of the mean (SEM) of a representative experiment.

RESULTS

INTERACTION OF ENDOGENOUS FIAT AND αNAC PROTEINS

The interaction between FIAT and αNAC has been observed in yeast two-hybrid screens [Yoshida et al., 2005; Yu et al., 2006] or in co-immunoprecipitation assays using overexpressed, epitope-tagged proteins [Yoshida et al., 2005]. We first set out to confirm that endogenous FIAT and αNAC proteins interact in mammalian cells. Endogenous FIAT was immunoprecipitated from confluent MC3T3-E1 osteoblastic cells [Sudo et al., 1983] using the anti-FIAT peptide antibody [Yu et al., 2005]. The 66 kDa FIAT protein was specifically precipitated from the cell extract (Fig. 1, left panel). Probing of the immunoprecipitates with the anti-αNAC antibody [Yotov and St-Arnaud, 1996] showed that αNAC was co-precipitated with FIAT (Fig. 1, right panel). This data confirms that endogenous αNAC and endogenous FIAT proteins interact in osteoblastic cells cultured under steady-state conditions.

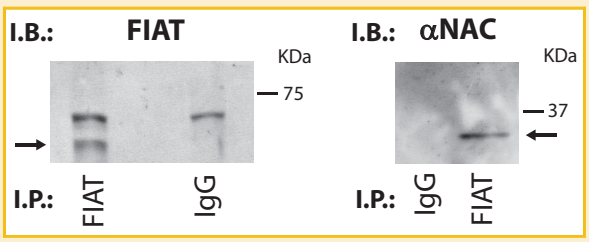

Fig. 1. Endogenous FIAT interacts with endogenous αNAC in osteoblastic cells. Endogenous FIAT was immunoprecipitated (I.P.) from MC3T3-E1 osteoblastic cells treated with 50 μM forskolin for 6 h, using the anti-FIAT antibody. The immunoprecipitates were separated by SDS–PAGE and immunoblotted (I.B.) with the anti-FIAT (left panel) or anti-αNAC (right panel) antibodies. Naïve IgG served as negative control. Specific signals are indicated by arrows.

Attempts to demonstrate the physiological relevance of this interaction using transient transfection assays with *Ocn* promoter reporter constructs and wild-type expression vectors for FIAT, αNAC, and ATF4 were not successful (Fig. 5B and data not shown). Considering the documented extensive post-translational modification of the αNAC protein, we hypothesized that modification(s) of αNAC might influence the functional outcome of the FIAT/αNAC interaction. We focused on a novel post-translational modification identified in the course of ongoing structure–function analysis of the αNAC sequence.

SPECIFIC CONJUGATION OF αNAC WITH SUMO1

In silico analysis of the αNAC primary sequence with the SUMOplot™ program (www.abgent.com/sumoplot) identified residue lysine 127 (K127) as a putative SUMOylation site with a high confidence probability. We thus tested whether recombinant αNAC could be SUMOylated in vitro using purified SUMO-activating enzyme (E1), UBE2I conjugation enzyme (E2), and WT or mutated SUMO1. Immunoblotting with an anti-SUMO1 antibody detected a ∼50 kDa band in reactions that included recombinant αNAC, activating and conjugating enzymes, and wild-type SUMO1 (Fig. 2A). No signal was detected in reactions that included recombinant αNAC alone or when mutant SUMO1 was used in the assay (Fig. 2A). The αNAC protein has a 23 kDa molecular size but runs aberrantly at around 37 kDa in SDS–PAGE [Yotov and St-Arnaud, 1996], and thus covalent modification with the 11 kDa SUMO1 moiety yields a molecular size of ∼48 kDa. These results show that αNAC can be SUMOylated by SUMO1 in vitro.

To determine the specificity of αNAC SUMOylation in cultured cells, cell extracts were prepared from UMR106 osteosarcoma cells [Bringhurst et al., 1989] in the presence or absence of the SUMO isopeptidase inhibitor NEM, which prevents de-SUMOylation. Immunoblotting analysis of whole cell extracts or immunoprecipitated αNAC using the anti-αNAC antibody showed that αNAC was readily detected at an apparent molecular mass of 37 kDa in extracts and immunoprecipitates (Fig. 2B, lanes 1–4). The 48 kDa slower migrating band was faintly detected in extracts from NEM-treated cells (lane 2) and appeared as a strong signal in immunoprecipitated fractions, both the presence and absence of NEM (lanes 3 and 4). This band was not detected in control immunoprecipitates (lane 5), ruling out that the signal could be due to immunoglobulin heavy chains. The non-specific signal observed at the αNAC migration location in control immunoprecipitates was not observed in subsequent experiments (data not shown). Stripping and re-probing the membrane with anti-SUMO1 antibody detected the 48 kDa band in immunoprecipitated fractions (lanes 8 and 9), while no signal was observed when the membrane was re-probed with an antibody against SUMO2/3 (lanes 11–15). These results demonstrate that endogenous αNAC is specifically modified by SUMO1 in UMR106 cells.

We next tested whether endogenous αNAC can be SUMOylated in vivo. Protein extracts from newborn mice calvaria were immunoprecipitated using anti-αNAC or anti-T7 epitope (negative control) antibodies. Treatment of the extracts with sepharose beads alone

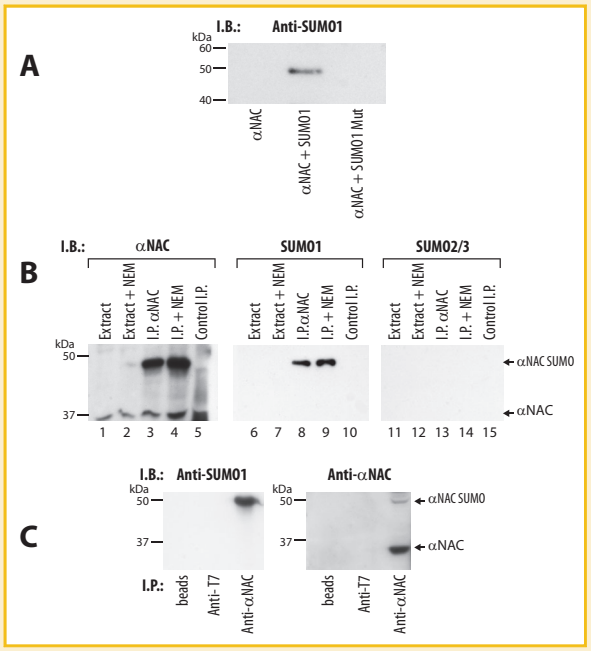

Fig. 2. αNAC SUMOylation in vitro and in vivo. A: Recombinant wild-type αNAC (0.5 μg) was incubated with wild-type or mutant (Mut) SUMO1 proteins, E1 activating enzyme, and E2 conjugating enzyme in an in vitro SUMOylation assay. SUMOylation was analyzed by immunoblotting (I.B.) with anti-SUMO1 antibodies. B: MC3T3-E1 C3T3-E1 osteoblastic cells were cultured in the presence or absence of the SUMO isopeptidase inhibitor NEM and cell extracts or immunoprecipitates (I.P.) with the anti-αNAC antibody were prepared at confluence. Samples were then immunoblotted (I.B.) with antibodies against αNAC (lanes 1–5), SUMO1 (lanes 6–10) or SUMO2/3 (lanes 11–15). Control I.P. used naïve IgG; the non-specific signal observed at the αNAC migration location in control immunoprecipitates (lane 5) was not observed in subsequent experiments. C: Protein extracts from newborn mice calvaria were immunoprecipitated using anti-αNAC or anti-T7 epitope (negative control) antibodies. Treatment of the extracts with sepharose beads alone served as an additional negative control. Immunoprecipitates were migrated on SDS–PAGE and immunoblotted with anti- αNAC (right panel) antibodies, then stripped and re-probed with anti-SUMO1 (left panel).

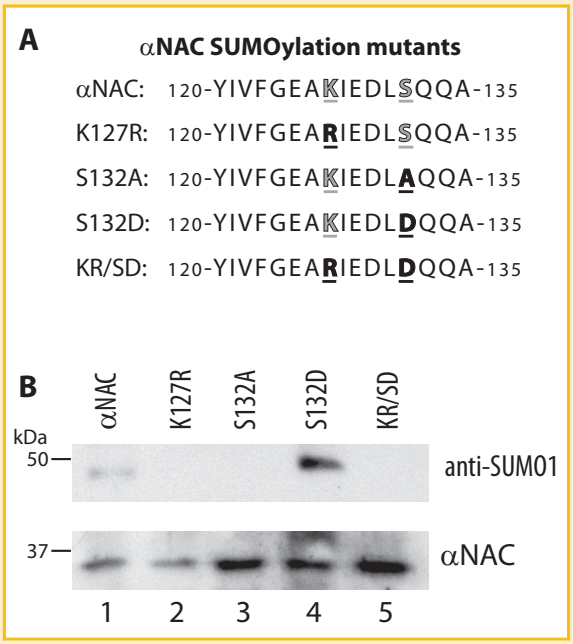

Fig. 3. Structure–function analysis of αNAC SUMOylation. A: Partial amino acid sequence (residues 120–135) of wild-type αNAC and site-specific mutants. The SUMOylation and phosphorylation site within the putative phosphorylation-dependent SUMOylation motif are shown in bold gray and underlined; mutations are shown in bold black and underlined. B: UMR106 osteosarcoma cells were transfected with expression vectors for the epitope-tagged wild-type or mutated αNAC and immunoprecipitated with anti-FLAG epitope antibodies. The immunoprecipitates were migrated on SDS–PAGE and immunoblotted with anti- αNAC antibodies. The membrane was stripped and re-probed with antibodies against SUMO1.

served as an additional control for specificity. Western blot analysis of the immunoprecipitated samples using anti-αNAC antibodies detected both the 37 kDa αNAC protein and the 48 kDa αNAC SUMO variant in the αNAC-precipitated samples but not in the negative control immunoprecipitates (Fig. 2C, right panel). Re-probing the membrane with anti-SUMO1 antibodies confirmed that the 48 kDa band has SUMO1 immunoreactivity (Fig. 2C, left panel). Together, these results confirm that αNAC can be SUMOylated by SUMO1 in osteoblast cells both in vitro and in vivo.

αNAC IS SUMOYLATED AT LYSINE 127

Further inspection of the αNAC SUMOylation sequence revealed the site to correspond to a putative phospho-sumoyl switch (or phosphorylation-dependent SUMOylation motif, PDSM) [Hietakangas et al., 2006; Yang and Gregoire, 2006], with the following alignment: ΨKxExxS (consensus); AKIEDLS (αNAC sequence; Fig. 3A). To facilitate the structure–function analysis of αNAC SUMOylation, we generated FLAG epitope-tagged site-specific mutant forms of αNAC at residues K127 or S132, alone or in combination (Fig. 3A). UMR106 osteosarcoma cells were transfected with wild-type or site-specific mutant forms of αNAC and immunoprecipitated with anti-FLAG epitope antibodies. Immunoblotting with the anti-αNAC antibody detected the αNAC 37 kDa band in all samples (Fig. 3B, lanes 1–5 of lower panel). Re-probing of the membrane with anti-SUMO1 antibodies showed that mutation K127R inhibited SUMOylation, demonstrating that residue K127 is the primary SUMO conjugation site (lane 2). Interestingly, mutation S132A also inhibited αNAC SUMOylation (lane 3), while the phosphomimetic S132D mutation increased it (lane 4), supporting the notion that the SUMOylation of αNAC is regulated through differential phosphorylation of serine 132. The compound K127R/S132D (KR/SD) mutant was not SUMOylated (Fig. 3B, lane 5), showing that a phosphomimetic residue at position 132 does not induce SUMOylation at a different lysine residue within the αNAC sequence. We interpret these results to mean that residue S132 is required for efficient SUMOylation of αNAC and that the phospho-sumoyl switch within the αNAC sequence is active.

αNAC AND SUMOYLATED αNAC INTERACT WITH FIAT AT THE *OCN* PROXIMAL PROMOTER

We have previously shown that αNAC binds the *Ocn* gene promoter through its DNA binding domain (DBD) mapping to residues 69–80 [Akhouayri et al., 2005]. To determine the impact of SUMOylation on

the DNA-binding function of αNAC, we used WT and SUMOylation mutants of αNAC in electrophoretic mobility shift assays (EMSA) and Chromatin Immunoprecipitation (ChIP) assays. The WT and site-specific αNAC mutants were transiently expressed in 3T3-L1 adipogenic cells [Green and Kehinde, 1975] that do not express detectable levels of endogenous αNAC (Fig. 4, lane 3: 3T3-L1 NE). Nuclear extracts were prepared from the transfected cells and used in EMSA with a labeled oligonucleotide probe corresponding to the αNAC binding sequence within the *Ocn* promoter [Akhouayri et al., 2005]. Recombinant WT αNAC protein interacted with the probe to yield a complex of altered electrophoretic mobility, while the use of nuclear extracts from untransfected 3T3-L1 cells in the EMSA did not result in altered migration of the labeled probe (Fig. 4). A complex with an altered migration identical to that observed with recombinant αNAC was detected when nuclear extracts from cells transfected with WT or mutated αNAC were incubated with the probe (Fig. 4). These data show that mutations at residues 127 or 132, alone or in combination, do not alter the DNA-binding activity of αNAC.

Next, we stably transfected MC3T3-E1 osteoblastic cells with expression vectors for the epitope-tagged WT or S132D (SD) αNAC proteins. Conventional ChIP with antibodies against the FLAG epitope tag confirmed that WT and hyper-SUMOylated, S132D αNAC bind to their cognate element on the *Ocn* proximal promoter in the stable transfectants (Fig. 5A, lanes 2 and 5). Antibodies against GAPDH served as negative control in these assays (lanes 3 and 6). We next performed sequential ChIP (ChIP-reChIP) to determine whether αNAC or SUMOylated αNAC and FIAT cohabit at the *Ocn* promoter. To enhance the probability of success of the procedure a modification of the ChIP assay using double cross-linking with DTBP (dimethyl-3,3-dithiobispropionimidate-2HCl) was necessary, since FIAT does not directly associate with the chromatin but could be recruited to the site through interaction with the DNA-bound αNAC.

In the first ChIP assay, the specific antibodies were either: anti-FLAG to immunoprecipitate epitope-tagged αNAC, or anti-FIAT. The negative control was the anti-GAPDH antibody. In the second ChIP round (reChIP), the eluted DNA-protein complex was immunoprecipitated using anti-FIAT or anti-FLAG. With either WT αNAC or the S132D mutant, enrichment of the *Ocn* promoter fragment was only achieved when anti-FLAG followed by anti-FIAT antibodies were used sequentially (Fig. 5A, lanes 8 and 13). No significant enrichment was detected when FIAT was immunoprecipitated first or when the first immunoprecipitation reaction involved the negative control anti-GAPDH antibody (lanes 9–11 and 14–16). Our preferred interpretation of these data is that WT αNAC and mutant, S132D αNAC bind the *Ocn* promoter and recruit FIAT to the site.

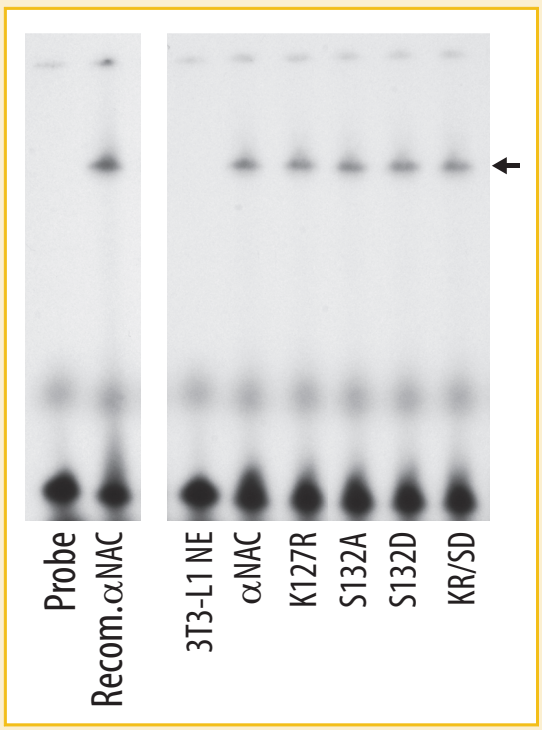

Fig. 4. Site-specific mutation of residues within the phosphorylation-dependent SUMOylation motif of αNAC does not affect its DNA binding activity. EMSAs using an oligonucleotide probe corresponding to the αNAC binding site within the murine *Ocn* proximal promoter region and purified recombinant (recom.) αNAC (left panel) or nuclear extracts (NE) from untransfected 3T3-L1 cells or 3T3-L1 cells transiently transfected with expression vectors for wild-type or site-specific mutant αNAC proteins (right panel, mutations indicated below each lane; KR/SD: K127R/S132D compound mutant). The specific complex is indicated by the arrow. Note that no complex was detected in nuclear extracts from untransfected 3T3-L1 cells that do not express endogenous αNAC (3T3-L1 NE, right panel).

SUMOYLATED αNAC AND FIAT MAXIMALLY REPRESS ATF4-MEDIATED *OCN* TRANSCRIPTION

We used transient transfection assays to examine the potential functional impact of αNAC SUMOylation on the FIAT-αNAC interaction. The reporter construct contained the luciferase reporter under the control of six copies of the OSE-1 element from the *Ocn* gene promoter, which contains both the αNAC and ATF4 binding sites [Ducy and Karsenty, 1995; Akhouayri et al., 2005]. This was co-transfected in MC3T3-E1 cells with expression vectors for ATF4, FIAT, or WT or mutant αNAC, alone or in combination. FIAT or αNAC alone had no effect on reporter gene expression (Fig. 5B and D). As previously reported [Yang et al., 2004; Yu et al., 2005], ATF4 induced transcription from the multimeric OSE1 element promoter and stimulated luciferase expression, and this effect was suppressed by FIAT (Fig. 5B). Increasing amounts of wild-type αNAC had no effect on FIAT-mediated inhibition of ATF4-dependent transcription (Fig. 5B). Interestingly, increasing amounts of hyper-SUMOylated S132D (SD) αNAC mutant further enhanced the inhibitory activity of FIAT on ATF4-mediated transcription (Fig. 5C). Mutating the lysine 127 SUMOylation site prevented the potentiation of the FIAT repressing activity observed with the S132D mutant (Fig. 5C, KR/SD bars). As suggested by the ChIP-reChIP results, maximal transcriptional repression by S132D αNAC and FIAT required DNA binding by the αNAC partner as the SD/ΔDBD αNAC mutant could not enhance the inhibitory activity of FIAT (Fig. 5C). Maximal transcriptional repression required both the FIAT and S132D αNAC partners, as co-transfection of ATF4 with WT or site-specific mutants of αNAC, in the absence of FIAT, did not lead to statistically significant repression (Fig. 5D). These results suggest that DNA-bound SUMOylated αNAC interacts with FIAT to maximally repress ATF4-mediated transcription.

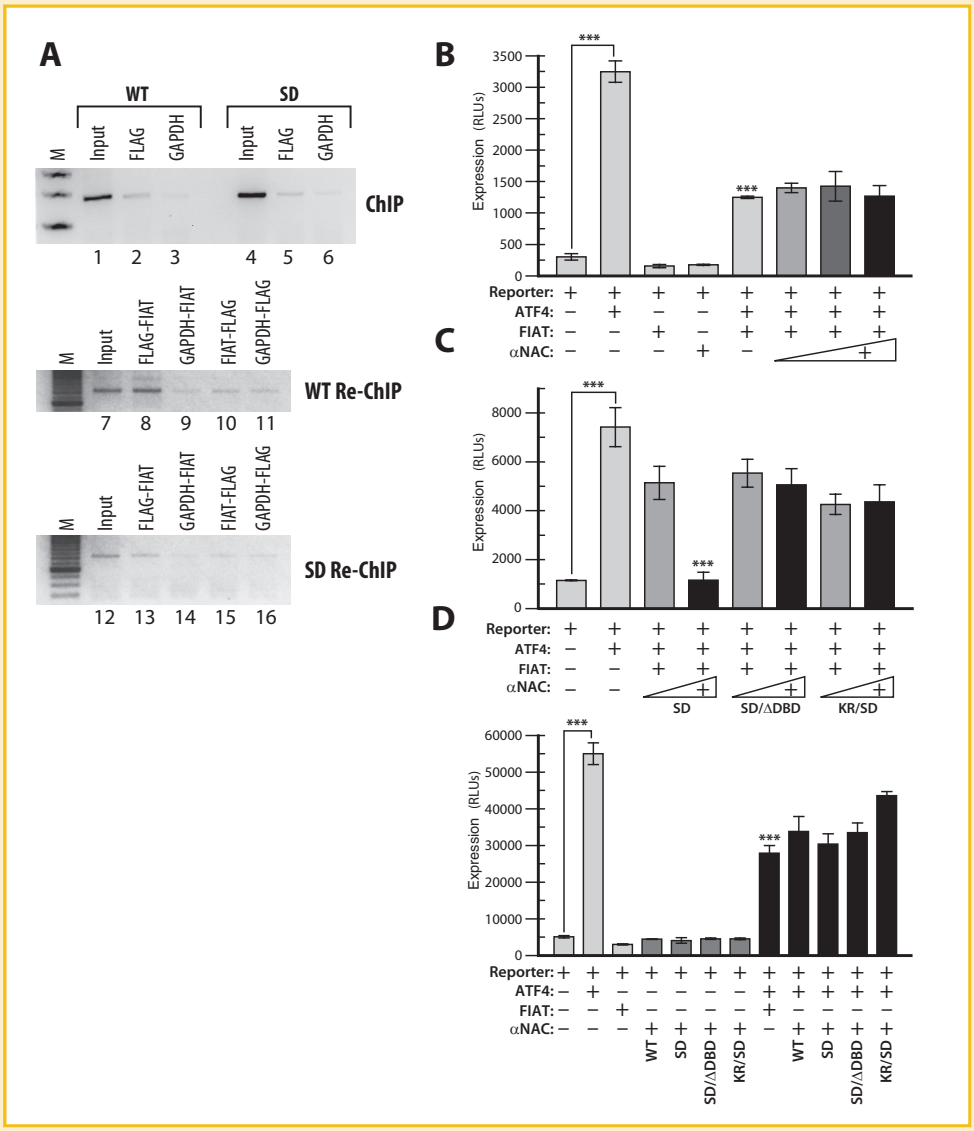

Fig. 5. Hyper-SUMOylated αNAC binds the *Ocn* promoter and interacts with FIAT to maximally repress ATF4-mediated *Ocn* transcription. A: Conventional (lanes 1–6) or sequential (lanes 7–16) ChIP assays of the *Ocn* proximal promoter. MC3T3-E1 cells were stably transfected with expression vectors for epitope-tagged wild-type (WT) or hyper-SUMOylated (SD) αNAC. Immunoprecipitation was performed with formaldehyde- and dimethyl-3,3-dithiobispropionimidate-2HCl-crosslinked chromatin and antibodies against the FLAG epitope or FIAT. Anti-GAPDH antibodies served as negative control. For sequential (ChIP-reChIP) assays, the sequence of immunoprecipitation is indicated above each lane. Ethidium bromide-stained agarose gels of PCR products obtained with primers flanking the αNAC binding site within the mouse *Ocn* gene promoter are shown. Input, amplification of DNA prior to immunoprecipitation; M, molecular size markers. B–D: Transient transfection assays in MC3T3-E1 osteoblastic cells. The reporter construct contained the luciferase gene under the control of six copies of the OSE-1 element from the *Ocn* gene promoter and was co-transfected with expression vectors for ATF4, FIAT, or WT or mutant αNAC, alone or in combination, as indicated below each bar. Each transfection was repeated three times with triplicate samples, and the data shown represent the mean and the standard error of the mean (SEM) of a representative experiment. RLUs, relative light units.

S132D αNAC SPECIFICALLY INTERACTS WITH HDAC2

The additional protein–protein interface created by SUMOylation often allows interaction of the SUMOylated substrate with transcriptional repressors. Since αNAC was shown to interact with HDAC1 or HDAC3 corepressors to inhibit gene expression in a cell- and promoter-specific context [Jafarov et al., 2012], we tested whether SUMOylated αNAC differentially interacted with specific HDAC molecules. UMR106 cells were transfected with an empty vector or expression vectors for the FLAG epitope-tagged non-SUMOylated K127R (K/R) αNAC mutant, the hyper-SUMOylated, S132D (S/D) molecule, or the non-SUMOylated compound K127R/S132D (KR/SD) mutant. Naïve or transfected UMR106 cells were lysed and the protein extracts were either used directly for immunoblotting or first immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting. Expression of the transfected mutant αNAC proteins was readily detected in the immunoprecipitates (Fig. 6C). The corepressor HDAC2 was expressed at a detectable level in cellular extracts from naïve cells, cells transfected with the empty vector, and cells transfected with the mutant αNAC expression plasmids (Fig. 6A). Interestingly, HDAC2 was co-immunoprecipitated with the hyper-

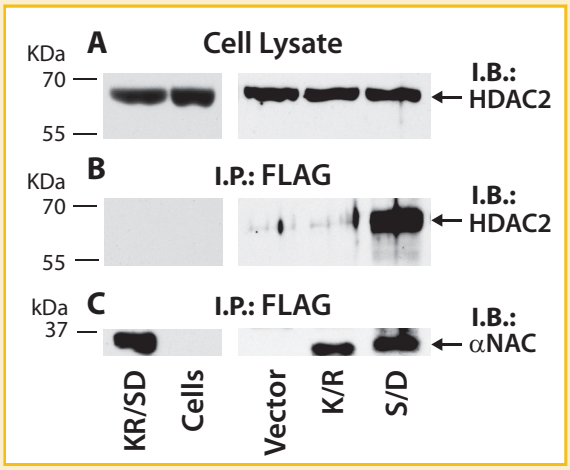

Fig. 6. Hyper-SUMOylated αNAC interacts with HDAC2. UMR106 cells were transfected with an empty vector or expression vectors for the FLAG epitope-tagged non-SUMOylated K127R (K/R) or K127R/S132D (KR/SD) αNAC mutants or the hyper-SUMOylated, S132D (S/D) molecule. The transfected cells were lysed and the protein extracts were either used directly for immunoblotting (A) or first immunoprecipitated (I.P.) with an anti-FLAG antibody, followed by immunoblotting (I.B.) with anti-HDAC2 (A and B) or anti-αNAC (C) antibodies. Cells, untransfected UMR106 cells.

SUMOylated S132D αNAC mutant, but did not interact with the non-SUMOylated, K127R (K/R) or K127R/S132D (KR/SD) mutated αNAC proteins (Fig. 6B). No differential interaction of the K/R or S/D αNAC mutants with either HDAC1, HDAC3, HDAC4, N-CoR, or Sin3a corepressors were detected (data not shown). Our data support a mechanism through which DNA-bound, SUMOylated αNAC specifically recruits HDAC2 and interacts with FIAT to repress *Ocn* gene transcription.

DISCUSSION

This is the first report of a functional outcome for the interaction of endogenous FIAT and αNAC proteins, which had previously been observed to interact in yeast two-hybrid screens or over-expression of epitope-tagged proteins [Yoshida et al., 2005; Yu et al., 2005, 2006]. Our study has focused on bone cells where we have previously described a physiological role for the αNAC transcriptional coregulator [Meury et al., 2010]. It remains to be determined if the FIAT/αNAC interaction is functional in brain tissue where FIAT (also named γ-taxilin) is expressed [Nogami et al., 2004; Yoshida et al., 2005].

Our data show that αNAC is conjugated to SUMO1 in cultured osteoblasts and in calvarial tissue. It is not clear why immunoprecipitation enriched the SUMO-conjugated form (Fig. 2B, lanes 3 and 4). We can only speculate that SUMOylation could have exposed high-affinity epitopes recognized by a fraction of the immunoglobulin molecules within the anti-αNAC polyclonal antibodies. The αNAC SUMOylation site is not an ideal match to the consensus ψKXE, where ψ is usually a large hydrophobic residue and not a small alanine [Gill, 2004]. However, in vitro SUMOylation assays combined with site-specific mutagenesis have confirmed that the αNAC site, -AKIE-, is functional. Mutagenesis of lysine 127 completely abolished modification of αNAC by SUMO1 in cultured cells, thus indicating that this lysine is the only SUMOylation site within the αNAC sequence. Moreover, the mutation of nearby serine 132 into a charged, phosphomimetic residue leads to hyper-SUMOylation, providing strong supporting evidence for an active phosphorylation-dependent SUMOylation motif with the sequence -AKIEDLS- within αNAC. Studies to identify the relevant kinase that phosphorylates serine 132 to stimulate SUMOylation are ongoing in our laboratory.

Conjugation to SUMO has been shown to affect several distinct properties of the target substrate. We have confirmed that SUMOylation of αNAC does not affect its half-life, subcellular localization, or DNA-binding activity (Fig. 4 and data not shown). We then examined if SUMOylation of αNAC affected its transcriptional coregulator function for the best characterized target gene, *Ocn* [Akhouayri et al., 2005]. Expressed alone or in combination with ATF4, site-specific mutants of αNAC that cannot be SUMOylated (K127R, S132A, and compound KR/SD) or that are hyper-SUMOylated (S132D) had no effect on transcription from a synthetic *Ocn* proximal promoter sporting six copies of the ATF4 and αNAC binding sites (Fig. 5D). *Ocn* promoter ChIP-reChIP analysis suggested the importance of stoichiometry between αNAC and FIAT for promoter occupancy and regulation of transcription. Indeed, co-expression of FIAT and the hyper-SUMOylated S132D αNAC mutant led to maximal repression of ATF4-mediated transcription at the *Ocn* promoter. This response was abolished when the αNAC SUMOylation site (K127) or the αNAC DNA binding domain were mutated (Fig. 5C), demonstrating that it is the SUMOylated, DNA-bound αNAC protein that functionally interacts with FIAT.

Histone deacetylases have been shown to preferentially interact with SUMO-modified substrates [Girdwood et al., 2003; Yang and Sharrocks, 2004]. Since we had previously demonstrated contacts between αNAC and HDACs [Jafarov et al., 2012], we tested whether SUMOylated αNAC could interact with this class of corepressors. We did not anticipate a preferential interaction of SUMOylated αNAC with HDAC1 or HDAC3 since we have mapped the HDAC1/3 contact sequence to residues 12–69 within αNAC [Jafarov et al., 2012], while SUMOylation affects amino acid K127. Co-immunoprecipitation assays demonstrated that hyper-SUMOylated αNAC bound HDAC2, and this interaction was lost upon mutation of the SUMO acceptor site, K127, suggesting that SUMOylation at lysine 127 creates a novel interaction surface that allows αNAC to recruit HDAC2. Notably, HDAC2 is an integral component of large co-repressor complexes, such as NuRD and CoREST [Lakowski et al., 2006; McDonel et al., 2009]. A role for the HDAC complexes in SUMO-mediated repression has been identified [Ouyang et al., 2009] and it has also been suggested that at least one other factor associated with HDACs, MEP-1, contributes to SUMO-dependent repression [Leight et al., 2005]. Assays more sophisticated than co-immunoprecipitation may be required to determine whether SUMOylated αNAC is part of large multimeric complexes.

Our data support a model in which phosphorylation of residue S132 promotes SUMOylation of αNAC at position K127. The SUMOylated αNAC protein binds DNA and interacts with FIAT and HDAC2 to maximally repress ATF4-mediated transcription from the

Ocn proximal promoter. The physiological relevance of this proposed mechanism will be tested using site-directed mutagenesis of αNAC at K127 or S132 in knock-in mice models.

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