Dephosphorylation of the transcriptional cofactor NACA by the PP1A phosphatase enhances cJUN transcriptional activity and osteoblast differentiation

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ABSTRACT

The transcriptional cofactor nascent polypeptide-associated complex and co-regulator alpha (NACA) regulates osteoblast maturation and activity. NACA functions, at least in part, by binding to Jun proto-oncogene, AP-1 transcription factor subunit (cJUN) and potentiating the transactivation of AP-1 targets such as osteocalcin (Bglap) and matrix metallopeptidase 9 (Mmp9). NACA activity is modulated by phosphorylation carried out by several kinases, but a phosphatase regulating NACA’s activity remains to be identified. Here, we used affinity purification with MS in HEK293T cells to isolate NACA complexes and identified protein phosphatase 1 catalytic subunit α (PP1A) as a NACA-associated Ser/Thr phosphatase. NACA interacted with multiple components of the PP1A holoenzyme complex: the PPP1CA catalytic subunit and the regulatory subunits PPP1R9B, PPP1R12A and PPP1R18. MS analysis revealed that NACA co-expression with PPP1CA causes dephosphorylation of NACA at Thr-89, Ser-151 and Thr-174. NACA Ser/Thr-to-Alanine variants displayed increased nuclear localization, and NACA dephosphorylation was associated with specific recruitment of novel NACA interactants, such as basic transcription factor 3 (BTF3) and its homolog BTF3L4. NACA and PP1A cooperatively potentiated cJUN transcriptional activity of the AP-1-responsive Mmp9-luciferase reporter, which was abolished when Thr-89, Ser-151 or Thr-174 were substituted with phosphomimetic aspartate residues. We confirmed the NACA-PP1A interaction in MC3T3-E1 osteoblastic cells and observed that NACA phosphorylation status at PP1A-sensitive sites is important for the regulation of AP-1 pathway genes and for osteogenic differentiation and matrix mineralization. These results suggest that PP1A dephosphorylates NACA at specific residues, impacting cJUN transcriptional activity and osteoblast differentiation and function.

AP-1 (Activator Protein-1) is a dimeric transcription factor involved in the regulation of multiple cellular processes such as proliferation, differentiation and apoptosis (1-4). The AP-1 complex consists of members of the JUN (e.g. cJUN – gene symbol: JUN, JUND) and FOS (e.g.
FOSB, FRA-1) family of basic leucine zipper proteins, in several interfamilial combinations of JUN/JUN homodimers or JUN/FOS heterodimers (1-4). AP-1 characteristically mediates immediate early transcriptional responses to extracellular stimuli such as growth factors and cytokines by binding to sequence-specific cis-acting response elements in gene promoters (1-4). AP-1 factors canonically bind to the TPA (12-O-tetradecanoylphorbol-13-acetate) response element (TRE; TGA G/C TCA) leading to the activation of transcription (5,6). The activity of AP-1 factors is enhanced by synergistic cooperation with general transcription factors and transcriptional coactivators (7,8).

NACA (nascent polypeptide associated complex and co-regulator alpha, aNAC) is a 215 amino acids transcriptional co-factor involved in the regulation of AP-1 transcription (9-11). NACA was originally cloned as the alpha chain of the nascent-polypeptide-associated complex (NAC), responsible for the sorting and translocation of polypeptides from the ribosome (12). NACA contains a DNA binding domain, a calcium-binding EF-hand motif, and a NAC domain (13-15). The NAC domain, a region sharing homology with basic transcription factor 3 (BTF3), contains 3 alpha helices and 6 beta sheets, which are critical for nuclear localization and DNA binding (13,14). NACA can bind to cJUN and stabilize the interaction of cJUN homodimers with chromatin thereby enhancing AP-1 transcriptional activity (9,10,16). In addition to cJUN, NACA can also coactivate the activity of JUND (17). Physiologically, in osteoblast cells, we have previously shown that coactivation of AP-1 dependent transcription by NACA is important for the regulation of Type I Collagen (Collla1), Mmp9, Osteocalcin (Bglap) and Lrp6 gene expression during differentiation (9,11,16-19). Furthermore, NACA also associates with TATA-binding protein (TBP) and is thus hypothesized to act as a bridge between cJUN homodimers and the basal transcriptional machinery on target promoters (11).

An important mechanism for the regulation of protein function is the post-translational modification of threonine, serine, and tyrosine residues by phosphorylation (20,21). Phosphorylation events are fast and reversible, thus allowing for temporal modulation of protein activity. Phosphorylation of a single residue can alter protein localization, target proteins for degradation, enhance or inhibit protein-protein interactions, modify protein conformations or stimulate or repress enzymatic activity (21-24). Despite there being more than 500 kinases and over 100 phosphatases in the proteome, protein phosphorylation events are often highly specific (22-24). We, as well as others, have shown that the activity of NACA is extensively regulated by post-translational modifications including phosphorylation. NACA is phosphorylated by casein kinase 2 (CK2) and integrin linked kinase (ILK) at an N terminus cluster of phosphoacceptor sites (25,26). CK2 phosphorylation of NACA at Ser-25, Thr-27, Ser-29 and Ser-34 leads to CRM1-mediated nuclear export of NACA (26). In osteoblasts, cell adhesion to fibronectin triggers ILK phosphorylation of NACA on Ser-43 leading to nuclear import of NACA (25). Nuclear import of NACA is also induced by protein kinase A phosphorylation of Ser-99 downstream of a parathyroid hormone signaling cascade in osteoblast cells (27). Additionally, NACA phosphorylation by glycogen synthase kinase 3β (GSK3β) on Thr-159 triggers the degradation of NACA by the 26S proteasome complex (28,29). Nuclear accumulation of NACA as a result of ILK or PKA phosphorylation results in an increase of NACA coactivation of AP-1 transcriptional activity (17,25,27). Moreover, mutation of the CK2 export or the GSK3β degradation phosphoacceptor sites on NACA enhance its coactivation potency (25,26,29). To demonstrate the physiological relevance of NACA phosphorylation in vivo, we previously generated knock-in mice harboring a non-phosphorylatable serine-to-alanine mutation at NACA Ser-43 (19). NACA S43A mice were deficient in nuclear NACA and exhibited a

PP1A dephosphorylates NACA
PP1A dephosphorylates NACA

reduction in bone formation and bone mass. Taken together, these data indicate that a balance between potentiating and inhibitory phosphorylation events of NACA alters the activation of AP-1 signaling pathways. Given the transient nature of phosphorylation events, NACA activity is likely to be fine-tuned by the dynamic removal of phosphate modifications. Despite our extensive understanding of NACA kinases, identification and characterization of a NACA phosphatase has remained elusive.

In the present study we used an unbiased proteomics approach to identify a NACA phosphatase. We report that NACA interacts with a major Ser/Thr phosphatase, PP1A (enzyme complex abbreviated as protein phosphatase 1 alpha; catalytic unit gene symbol PPP1CA). PP1A counteracts NACA phosphorylation on Thr-89, Ser-151 and Thr-174. Additionally, PP1A enhances NACA nuclear localization and coactivation activity.

RESULTS

PP1A associates with NACA

To identify potential NACA phosphatases, we utilized an unbiased affinity purification and proteomics approach (Fig. 1A). FLAG-tagged NACA protein complexes were immunopurified from HEK293T cells (Fig. 1B) and NACA-associated proteins identified by tandem mass spectrometry analysis. Several components of the Protein Phosphatase 1 alpha holoenzyme complex (PP1A), including the regulatory subunits PPP1R9B, PPP1R12A, PPP1R18 and the catalytic subunit PPP1CA were identified (Fig. 1C). The catalytic subunit, PPP1CA was detected at a level lower than the standard threshold of 2 peptides. This low abundance most likely reflects the short-lived and transient nature of the catalytic unit/substrate reaction. PP1A components were the only phosphatase proteins found in the NACA complex. Co-immunoprecipitation confirmed the association of PPP1R9B, PPP1R12A, PPP1R18 and PPP1CA with NACA (Fig. 1D). Amongst these PP1A components, interaction with PPP1CA and PPP1R18 are likely to be direct, because a full-length glutathione-S-transferase NACA fusion protein (GST-NACA) purified from E. Coli bacteria (Fig. 1E, left panel), bound to in vitro translated MYC-tagged PPP1CA and PPP1R18 protein (Fig. 1E, right panel).

PP1A mediates the dephosphorylation of NACA

Increased mobility in SDS-PAGE is characteristic of hypo-phosphorylated protein forms. To determine whether PP1A could specifically dephosphorylate NACA, we treated NACA with recombinant serine/threonine phosphatases including PP1A, PP2 and PPM. As shown in Figure 2A, PP1A activity increased the mobility of NACA, whereas incubation of NACA with PP2 or PPM did not result in a faster migrating hypo-phosphorylated form of NACA. The highly promiscuous Lambda phosphatase served as a positive control of NACA dephosphorylation. To determine whether PP1A could modify the phosphorylation status of NACA in vivo, we next overexpressed NACA together with or without the PP1A catalytic unit in HEK293T cells and examined NACA migration by western blotting. Co-expression of NACA with PPP1CA resulted in a faster migrating hypo-phosphorylated NACA band indicative of NACA dephosphorylation in the presence of PPP1CA (Fig. 2B).

PP1A mediates dephosphorylation of NACA at Thr-89, Ser-151 and Thr-174

To identify NACA phosphorylation sites modified by PP1A, we performed a phosphoproteomic characterization of NACA from HEK293T cells expressing NACA alone or NACA together with PPP1CA (Fig. 3A). In cells expressing NACA alone, we detected four NACA phosphorylation sites at Thr-89, Ser-151, Ser-166 and Thr-174 (Fig. 3B & 3C). Interestingly, PPP1CA co-expression led to a loss of phosphorylation at Thr-89, Ser-151 and Thr-174. Ser-166, however, was not regulated by PPP1CA overexpression (Fig. 3B).
NACA dephosphorylation leads to recruitment of novel interactants

Protein phosphorylation is often linked to alterations in protein interactions. To better understand the molecular consequences of altered NACA phosphorylation status, we compared the NACA interactomes following affinity purification in the presence or absence of PPP1CA overexpression (Fig. 4A). Network analysis of the NACA interactomes revealed that in addition to the aforementioned PP1A complex, NACA is associated with 5 major functionally related protein clusters. Consistent with the diverse and multifunctional nature of NACA, we uncovered clustered enriched for components of the intermediate filaments, Cajal bodies, cytoskeleton, mRNA processing and transcriptional regulation. From the 40 high-confidence NACA-interacting proteins in both datasets, we found a total of 9 proteins that uniquely interacted with NACA following PPP1CA-mediated dephosphorylation (Fig. 4B). Of note was the induction of NACA interaction with BTF3 and BTF3L4 following PPP1CA co-expression. BTF3 is a general transcription factor shown to initiate transcription through interactions with TATA-binding protein and RNA polymerase II at proximal promoter regions (13,14,30-32). Furthermore, BTF3 has also previously been shown to form heterodimeric complexes with NACA (12,33). BTF3-NACA complexes have been implicated in DNA binding as well as the sorting and targeting of cytosolic proteins from the ribosome. We further validated the PPP1CA-regulated association of NACA and BTF3 by immunoprecipitation and western blotting (Fig. 4C). As shown in Fig. 4C, overexpression of PPP1CA significantly enhanced the interaction of BTF3 with NACA.

NACA cooperates with PPP1CA and BTF3 to coactivate cJUN transcriptional activity

To determine the functional consequences of NACA dephosphorylation by PP1A, we examined the effect of co-expression of PPP1CA and BTF3 on cJUN transcriptional activity. Luciferase reporter gene assays with the cJUN-responsive MMP9-promoter luciferase showed that NACA and PPP1CA cooperatively enhanced cJUN transcriptional activity (Fig. 5A). Similarly, BTF3 and NACA cooperatively stimulated transcriptional activity in the cJUN-mediated activation of an AP-1 luciferase reporter assay (Fig 5B). Taken together, these data indicate that NACA can functionally interact with PPP1CA and BTF3 in the regulation of AP-1 transcriptional activity. Interestingly, PPP1CA was able to coactivate cJUN on its own. This may be a result of PPP1CA dephosphorylating endogenous NACA or another unknown AP-1 regulator. We next examined whether dephosphorylation of NACA was required for coactivation of transcription with PPP1CA. As shown in Figure 5C, coactivation of NACA and PPP1CA was abolished when Thr-89 (T89) or Ser-151 (S151) were mutated into phosphatase-resistant phosphomimetic aspartate residues. This suggests that dephosphorylation of NACA on T89 and S151 contributes to coactivation of cJUN transcription.

Regulation of NACA localization by PP1A-sensitive NACA residues

Changes in NACA phosphorylation are strongly linked to alterations in its subcellular localization. Since dephosphorylation of NACA by PPP1CA increased NACA coactivation of transcriptional activity, we examined the possibility that NACA phosphorylation at T89, S151 and Thr-174 (T174) may alter NACA subcellular localization. As shown in Figure 6A and quantified in Figure 6B, nuclear localization of NACA was increased in a mutant harboring phosphorylation-resistant threonine-serine-to-alanine mutations [T89A; S151A; T174A] compared to the phosphomimetic mutant [T89D; S151D; T174D]. Furthermore, AP-1 luciferase reporter assays showed that phosphorylation-resistant NACA mutant had increased AP-1 coactivation activity relative to the phosphomimetic mutant (Figure 6C). In conclusion, these findings suggest that dephosphorylation of NACA at multiple residues
by PPP1CA enhances nuclear targeting of NACA thereby leading to an increase in cJUN coactivation.

**NACA cooperates with PPP1CA to coactivate cJUN activity in osteoblast cells**

We have previously shown that in osteoblast cells, NACA is required for the potentiation of cJUN-mediated transcriptional activity during osteoblast differentiation (18). We next sought to determine whether NACA functionally interacts with PPP1CA in the context of osteoblast cell biology.

MC3T3-E1 cells are a calvaria-derived murine osteoblast cell line commonly used as a model for the differentiation and mineralization properties of osteoblast cells. To assess whether NACA interacts with PPP1CA in bone cells, MC3T3-E1 cells were co-transfected with constructs expressing NACA-FLAG and MYC-PPP1CA. Cell lysates were then immunoprecipitated with anti-MYC antibody and immunoblotted for NACA. As shown in Figure 7A, a NACA-specific band was observed in immunoprecipitates from co-transfected cells but not from singly-transfected cells indicating that NACA interacts specifically with PPP1CA in osteoblast cells. To further verify the interaction of endogenous PPP1CA with endogenous NACA, we used immunofluorescence imaging to co-localize PPP1A and NACA in untransfected MC3T3-E1 cells. We observed that a fraction of endogenous perinuclear NACA foci also contain PPP1CA (Fig. 7B). To investigate the role of NACA and PPP1CA in regulating cJUN function in osteoblast cells, we co-expressed NACA, cJUN and PPP1CA together with the AP-1 luciferase reporter. Co-expression of NACA and PPP1CA led to a more significant activation of cJUN transcriptional activity compared to expression of either NACA or PPP1CA alone (Fig. 7C). These data indicate that NACA and PPP1CA physically interact and potentiate cJUN activity in osteoblast cells.

We next sought to determine whether phosphorylation of NACA at the PP1A-sensitive residues modulates AP-1 activity. To this end we stably expressed wildtype NACA, the phosphorylation-resistant mutant [T89A; S151A; T174A] and the phosphomimetic mutant [T89D; S151D; T174D] in MC3T3-E1 cells (Fig. 7D) and examined the expression levels of four direct AP-1/cJUN targets. Overexpression of NACA [T89A; S151A; T174A] led to a significantly enhanced expression of the AP-1 targets, Mmp9, Jun, Fos and Ccnd1 compared to NACA [T89D; S151D; T174D]. This suggests that dephosphorylation of NACA at residues T89, S151 and T174 is indeed important for regulation of native AP-1 target promoters in osteoblast cells. Osteocalcin (Bglap), a marker of terminal osteoblast differentiation, is also a well-characterized target of NACA/cJUN activity in osteoblasts (18). We examined whether the increased AP-1 activity induced by NACA mutant [T89A; S151A; T174A] also affects Bglap expression and/or osteoblast differentiation. MC3T3-E1 cells stably expressing NACA or phosphorylation mutants were differentiated in osteogenic media for 12 days after which gene expression and mineralization were assessed. RT-qPCR analysis of Bglap and Runx2 expression revealed that overexpression of NACA mutant [T89A; S151A; T174A] led to increased expression of these osteoblast differentiation markers compared to phosphomimetic mutant [T89D; S151D; T174D] or wildtype NACA. Interestingly, cells expressing wildtype NACA had lower levels of Bglap and Runx2 expression than control cells and behaved similar to the phosphomimetic mutant [T89D; S151D; T174D]. This suggests that in terminally differentiated osteoblasts, NACA may be highly phosphorylated under basal conditions. In addition to regulating Bglap expression and osteoblast differentiation via cJUN activity, NACA also influences osteoblast matrix mineralization by a separate distinct and as yet unclear mechanism (19). Examination of matrix mineralization by von Kossa staining revealed that cells expressing NACA mutant [T89A; S151A; T174A] deposited more mineral than cells expressing wildtype or mutant [T89D;
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S151D; T174D] (Fig 7G). However, it must be noted that all forms of NACA displayed less mineral than control cells, perhaps indicative of the predictable involvement of other phosphorylation sites in the regulation of matrix mineralization by NACA. Nonetheless, taken together, these data indicate that the phosphorylation state of NACA at PPP1CA-sensitive residues is important for the regulation of AP-1-dependent transcription in a way that influences osteoblast differentiation and activity.

DISCUSSION

Nuclear NACA functions as an important transcriptional coactivator of cJUN and AP-1 signaling (11,16-19,25,27). Phosphorylation of NACA is known to alter its subcellular localization, transcriptional functions and its proteolytic degradation (25,27,29). Although several kinases are known to phosphorylate NACA, the process by which these modifications are reversed or negated has remained elusive. Here in this study, we have identified PP1A as the major serine/threonine phosphatase responsible for NACA dephosphorylation. We report that NACA associates with multiple PP1A holoenzyme constituents and that dephosphorylation of NACA is linked to functional changes in the localization and AP-1 coactivation potential of NACA.

The reversible phosphorylation of proteins is achieved by a balance between kinases and phosphatases. The human genome encodes for 428 known serine/threonine kinases but only about 30 serine/threonine phosphatases (23,24). Serine/threonine phosphatases can be further divided into three major groups: phosphoprotein phosphatases (PPP), metal-dependent phosphatases (PPM) and the aspartate-based phosphatases. In our study, we found that NACA interacted with one major phosphatase: PP1, a PPP family member. PP1 is a ubiquitously expressed serine/threonine phosphatase with a diverse array of cellular functions (34,35). In humans PP1 is encoded by 3 closely related genes (PP1α, PP1β, PP1γ). These PP1 holoenzyme isoforms are combinatorial complexes of a catalytic subunit interacting with >90 distinct regulatory proteins that define enzyme localization, substrate specificity and activity (34,35). We demonstrated that NACA binds directly to the PP1A α isoform catalytic subunit as well as the PPP1R18 regulatory subunit. Amongst the PP1 isoforms which can all be also found in the cytosol, the α isoform is unique in its localization to the nuclear matrix (23,36,37). It is easy to speculate that such close proximity to nuclear factors makes PP1A an important regulator of transcriptional events. Indeed, dephosphorylation of transcription factors such as CREB (38) and RUNX2 (39) by PP1A is a major mechanism for the attenuation of their transcriptional activity. Here, we likewise show that dephosphorylation of NACA by PP1A has implications for AP-1 transcription. Our results suggest a model wherein PP1A controls the phosphorylation state of NACA thereby altering the relative distribution of nuclear and cytoplasmic pools of NACA.

In addition to the catalytic subunit of PP1A, we also observed that NACA was associated with the regulatory subunits PPP1R9B, PPP1R18 and PPP1R12. This suggests that there are multiple PP1A holoenzymes interacting with NACA or that each of the specific residues are targeted by a particular PP1A complex. Regulatory subunits often target catalytic units to specific subcellular compartments (23,40). Indeed, given that there are multiple pools of NACA it is understandable that specific regulatory subunits would be required to target the PPP1CA catalytic subunit to each compartment. Thus, it follows that regulatory subunits may also competitively inhibit each other. Further work would be required to dissect the function of these regulatory subunits in relation to NACA function. Withstanding some exceptions, PP1A usually interacts with regulatory subunits or substrates through two well-defined docking motifs: the RVXF motif ([KR][X]{0,1}[VI][P][FW]) and the SILK motif ([GS][IL][RK]) (41-43). Consistent with our findings that NACA associates with
PP1A, NACA does indeed contain a putative RVXF motif at amino acids 100-104 (KNILF).

Several prior studies using in vitro kinase assays have demonstrated phosphorylation of NACA at multiple residues. We have used a mass spectrometry-based proteomics approach to dynamically map NACA phosphorylation in the presence and absence of PP1A. We showed that NACA is indeed phosphorylated at Thr-89, Ser-151, Ser-166 and Thr-174. Amongst these sites, Thr-89, Ser-151 and Thr-174 are completely novel and have not previously been described. Given the varied expression of kinases and phosphatases across cell types it is highly likely that NACA phosphorylation sites and phosphatases could be cell-type specific. Phosphorylation of Ser-166 has previously been described in embryonic stem cells as an induced response to bone morphogenetic protein during differentiation (44). In our study Ser-166 phosphorylation was resistant to PP1A, whereas Thr-89, Ser-151 and Thr-174 were dephosphorylated by PP1A. Thus, PP1A dephosphorylation of NACA is site specific and perhaps other phosphatases, in various cell types, might be responsible for dephosphorylation of Ser-166 and the other important NACA phosphorylation sites that we have previously described.

To our knowledge, this is the first comprehensive NACA interactome analysis performed in mammalian cells. NACA has previously been purified in yeast cells with a focus on its interaction with ribosomal proteins (45,46). Consistent with this aspect of NACA function, we did observe, as part of the 31 NACA interactants that were not PPP1CA-dependent (Fig. 4A), several ribosome-associated proteins interacting with NACA including ribosomal protein L38 (RPL38). More importantly, amongst the previously known transcriptional partners of NACA, we observed interaction with TXLNG (FIAT) and TXLNA (47-52). Taken together these observations are supportive of the fidelity of our purification method. Our study focused on NACA associations that were differentially regulated by PPP1CA – namely BTF3. NACA has long been known to dimerize with BTF3 (also known as βNAC) in the formation of the nascent polypeptide-associated complex, a multifunctional protein complex involved in both protein translation and nuclear DNA binding (12-14,51,53). In the absence of the binding partner, NACA and BTF3 can also homodimerize and function independently. Until now there has been no study describing how hetero- and homodimerization of NACA and BTF3 are regulated. Here we show that phosphorylation of NACA is a possible mechanism regulating NACA/BTF3 dimerization. Crystal structures of NACA/BTF3 heterodimers have shown that the β sheets between NACA amino acids 70 and 135 are important for dimerization (13,14). Interestingly, Thr-89, which we have shown to be sensitive to PPP1CA, was a residue shown to be specifically involved in heterodimerization by the formation of intermolecular hydrogen bonds. The phosphorylation status of Thr-89 will therefore be important for NACA interaction with BTF3. BTF3 is one of several general transcription factors involved in TATA-box mediated proximal promoter transcription via interactions with RNA polymerase II (32,54). Our own previous data demonstrated that NACA interacts with TATA-binding protein, and stabilizes cJUN heterodimers (9,11). Here we add to this model, by showing that NACA and BTF3 can cooperatively enhance cJUN activity. BTF3 could therefore be the bridge between NACA, cJUN, TBP and RNA Pol II during cJUN-activated transcription.

Interestingly, our earlier attempts to understand the role of NACA/BTF3 dimers on transcription had suggested that BTF3 could be inhibitory to NACA transcriptional activity (11). In this system, NACA coactivated the transcriptional activity of a hybrid transcriptional activator (yeast GAL4 transcription factor fused to the activator domain of the herpes simplex virus VP16 protein) and this activity was inhibited by BTF3b (11). BTF3b is an alternative splice isoform of BTF3, lacking the first 44 amino
acids (32). Indeed, Zheng et al demonstrated that BTF3b is transcriptionally inactive despite being able to interact with RNA Pol II (32). Thus, it is possible that BTF3b acts as a dominant negative inhibitor of NACA as we previously observed whereas full-length BTF3 co-activates NACA activity as demonstrated in this study. We also cannot exclude the possibility that NACA/BTF3 activity is promoter- and activator-dependent. In summary, we have identified PP1A as a major NACA phosphatase. We propose that dephosphorylation of NACA by PP1A is a mechanism for regulating AP-1 transcriptional activity. These findings extend to osteoblast biology where NACA and PP1A function cooperatively in the regulation of gene expression and osteoblast activity. Further studies into the cellular outcomes of PP1A and NACA interactions will permit us to understand the role of NACA in other signaling pathways.

EXPERIMENTAL PROCEDURES

Cell culture

HEK293T cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Life Technologies). MC3T3-E1 cells (a generous gift from Dr. Hiroko Sudo, Tohoku Dental University, Japan) (55) were maintained in alpha modified Eagle’s minimum essential medium (αMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were transfected with X-tremeGENE HP (Roche) according to manufacturer’s instructions. Stable cells lines were selected with 5 µg/ml Puromycin (Life) for 3 days and thereafter maintained in complete medium with 2.5 µg/ml Puromycin. Osteoblast differentiation was induced in MC3T3-E1 cells, by culturing cells in αMEM supplemented with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid for 12 days. von Kossa staining for mineral was performed using 5% silver nitrate.

Plasmids

To generate pCMV6-Naca-FLAG, the mouse Naca coding sequence was PCR amplified from pLVX-Naca-FLAG (27) and inserted into EcoRI-linearized pCMV6-Kan/Neo (Origene) by DNA assembly (In-Fusion; Takara). The Ppp1ca (pCMV6-MYC-Ppp1ca) and Btf3 (pCMV6-MYC-Btf3) expression vectors were constructed by PCR amplification from mouse MC3T3-E1 cell cDNA with MYC-containing primers and inserted into the EcoRI-linearized pCMV6-Kan/Neo. The MMP9 promoter reporter plasmid (MMP9-Luc), containing the 670 bp proximal promoter fragment in the pGL3 luciferase backbone was a kind gift from Dr. Shoukat Dedhar (University of British Columbia, Canada). The AP-1 reporter plasmid (pAP1(PMA)-TA-Luc) containing 6 tandem repeats of the AP-1 response element was obtained from Clontech. The cJUN expression vector as well as the GST-fusion constructs, pGEX-4T-3 and pGEX-4T-3-Naca have previously been described (11). pCMV6-Ppp1r9b, pCMV6-Ppp1r12a, pCMV6-Ppp1r18 were obtained from Origene. Site-directed mutagenesis to obtain pCMV6-Naca[T89A; S151A; T174A]-FLAG and pCMV6-Naca[T89D; S151D; T174D]-FLAG was performed with the Q5 Site-directed mutagenesis kit (NEB). All high-fidelity PCR amplifications were performed with CloneAMP HiFi Taq polymerase (Takara). All expression vectors were verified by sequencing. Primer sequences for cloning and mutagenesis are available upon request.

Antibodies

Rabbit Anti-FLAG, Mouse Anti-FLAG, Rabbit Anti-MYC, Anti-Rabbit HRP, Anti-Rabbit (Conformation specific)-HRP, Anti-Mouse (Light chain specific)-HRP, Anti-Mouse HRP, Anti-Rabbit Alexa Fluor 594, Anti-Rabbit Alexa Fluor 488, Anti-Mouse Alexa Fluor 594, AntiMouse Alexa Fluor 488, Anti-PP1A, Anti-PPP1R9B, Anti-PPP1R12A and Anti-GAPDH-HRP antibodies were from Cell Signaling. Anti-BTF3 and Anti-PPP1CA antibody was obtained from Abcam. Anti-PPP1R18 was obtained from
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Santa Cruz Biotechnology. Anti-NACA antibody has previously been described (27).

Affinity purification, co-immunoprecipitation and mass spectrometry

HEK293T cells stably expressing NACA-FLAG, empty vector, or NACA-FLAG and MYC-PPP1CA were washed twice in cold PBS and scraped into PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation, cells were resuspended in lysis buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% NP40 and 10% glycerol), lysed with a Dounce homogenizer and cell debris pelleted by centrifugation. To purify NACA-FLAG complexes, 15 µl of Anti-FLAG M2 magnetic beads (Sigma) were added to clarified cell lysates (3 mg of protein) and incubated with gentle rotation for 3 hours. Beads were then washed 4 times with a high-salt buffer (25 mM Hepes pH 7.4, 900 mM NaCl, 1% Triton X, 0.5% NP40 and 10% glycerol), twice with regular lysis buffer and then twice more with a detergent-free buffer (25 mM Hepes pH 7.4, 150 mM NaCl and 10% glycerol). All buffers were supplemented with 1 mM DTT, 0.25 mM PMSF and protease/phosphatase inhibitor cocktail (Cell Signaling). All steps were carried out in a 4°C cold room. Beads were then subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or immunoprecipitated proteins eluted with Laemmli buffer and separated by SDS-PAGE for silver staining (SilverQuest Silver Staining Kit; Thermo Fisher) and western blotting analysis. LC-MS/MS was performed at the Proteomics Core Facility of the Research Institute of the McGill University Health Centre using a Dionex Ultimate 3000 uHPLC and a Thermo Orbitrap Fusion mass spectrometer. Peptides were identified and analyzed using Mascot 2.3 and Scaffold Q+ Scaffold 4.7.5. Protein identifications with >99.9% probability by the Protein Prophet algorithm were considered valid. Common background proteins and contaminants such as keratin were removed (56,57). Network visualization and ontology enrichment was performed with GeNets (58) (Broad Institute of MIT and Harvard) and Enrichr (59).

For co-immunoprecipitation assays, cells were lysed with HKMG lysis buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5mM MgCl2, 10% glycerol, 1 mM DTT, and 0.5% NP-40) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling). After syringe homogenization with a 27-gauge needle, cell lysates were clarified by centrifugation and protein concentrations determined with the Bio-Rad Protein Assay reagent (Bio-Rad). One mg of protein was then incubated with 15 µl of Anti-FLAG M2 or Anti-MYC (Cell Signaling) magnetic beads for 3 hours at 4°C. Beads were washed three times in HKMG lysis buffer, twice in high salt buffer and twice more in HKMG lysis buffer. Bound proteins were eluted with 2X SDS sample buffer and analyzed by western blotting.

GST pull-down assay

Recombinant GST-NACA fusion protein and GST control protein were expressed and purified from T7 Express Escherichia coli (E. coli) strain (NEB) using anti-GST magnetic beads (Cell Signaling) according to standard manufacturer’s protocols. Prey proteins were in vitro transcribed and expressed in rabbit reticulocytes with the TNT Quick Coupled Transcription/Translation system (Promega). GST-fusion-protein bound beads were incubated with prey protein in a GST pull-down buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP40, 10% glycerol with protease/phosphatase inhibitor cocktail) for 90 minutes at 4°C. After washing 5 times with pull-down buffer, associated proteins were eluted in 2X SDS sample buffer and analyzed by SDS-PAGE and western blotting.

In vitro phosphatase assays

Recombinant FLAG-tagged NACA was expressed and purified from HEK293T cells. Forty-eight hours after transfection with Naca-FLAG cDNA plasmid, cells were lysed in a harsh lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2% SDS, 1% Triton) supplemented with
protease inhibitor cocktail (Sigma). Following sonication and clarification by centrifugation, lysates were incubated with anti-FLAG M2 magnetic beads (Sigma) for 3 hours at 4°C. Beads were then washed 5 times with a wash buffer containing 10 mM Tris-HCl pH 8.0, 1M NaCl, 1% NP-40. NACA-FLAG fusion protein was then eluted with 200 μg/ml FLAG peptide (Sigma). In vitro phosphatase assays were carried out for 30 minutes at 30°C in 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 1 mM MnCl₂ for PP1A (Millipore), PPM (Millipore) and Lambda Phosphatase (NEB). For PP2 (Millipore), buffers were further supplemented with 1 mM MgCl₂.

**Luciferase reporter assays**

Luciferase reporter plasmids together with expression plasmids were transfected into HEK293T or MC3T3-E1 cells using X-tremeGENE HP (Roche). Luciferase activity was measured 24 hours after transfection with the Dual-Glo Assay System (Promega). Renilla luciferase (pRL-TK or pRL-SV40) was used as an internal control for transfection efficiency. For responses to PMA (phorbol 12-myristate 13-acetate, also known as 12-O-tetradecanoylphorbol-13-acetate; TPA) (Cell Signaling), cells were maintained in 0.5% FBS and treated with 200 nM PMA or vehicle 16 hours prior to assay measurement.

**Immunofluorescence and confocal microscopy**

Cells cultured on coverslips or chamber slides were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X in PBS. Cells were then blocked with 3% BSA for 30 minutes and then incubated with primary antibody in PBS with 1% BSA overnight at 4°C. Cells were incubated with Alexa Fluor labelled secondary antibodies for 1 hour at room temperature and then mounted with Prolong Gold Antifade containing DAPI (Life Technologies). Images were acquired on a Zeiss LSM880 Laser Scanning Confocal or a Zeiss Spinning Disk Confocal Microscope at the Molecular Imaging Platform of the Research Institute of the McGill University Health Centre (Fig. 6) or a Leica DMR fluorescence microscope (Leica Microsystems) connected to a digital DP70 camera (Olympus) (Fig. 7). Image analysis and fluorescence intensity quantification was performed with ImageJ (NIH) and the Zen Software Suite (Carl Zeiss Microscopy).

**Statistical analysis**

Data are presented as means ± one standard deviation (SD). Comparisons were made by analysis of variance (ANOVA) with Fisher’s LSD post hoc test. Statistical significance relative to the specified control is represented as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001. Experiments were performed in triplicate.

**Author contributions:** W.N.A. generated data. W.N.A. and R.St-A. participated in data analysis and interpretation. M.P. provided materials and technical support. R.St-A. obtained the funding. W.N.A. and R.St-A. participated in the conception and design of the study, and wrote the manuscript.

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**Conflict of interest:** None.

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Figure 1. PP1A associates with NACA. (A) Schematic overview of the proteomics approach used to identify NACA-interacting proteins. (B) Silver-stained SDS-PAGE gel showing expression and purification of NACA protein. (C) Mass spectrometry (MS/MS) analysis identified numerous NACA-associated proteins, including several components of the PP1A holoenzyme complex. (D) Validation of interaction of NACA-FLAG with endogenous PP1A complex proteins. HEK293T cells expressing NACA-FLAG or FLAG empty vector were lysed and immunoprecipitated (IP) with anti-FLAG antibody beads and blotted with anti-FLAG antibody or the indicated PP1A-complex antibodies. (E) GST-pulldown analysis showing direct interaction between NACA and PPP1CA as well as PPP1R18. GST-NACA fusion protein or GST alone expressed in bacteria (left panel) were incubated with in vitro translated PPP1R9B, PPP1R12A, PPP1R18 and PPP1CA, and then subjected to SDS–PAGE and western blot analysis (right panel).
Figure 2. PP1A dephosphorylates NACA in vitro and in vivo. (A) Recombinant NACA (arrow) was treated with the indicated recombinant enzymes for 90 minutes at 30°C and then analyzed by western blotting (WB). (B) HEK293T cells were transfected with plasmid vectors expressing NACA or the PP1A catalytic unit (PPP1CA). Forty-eight hours after transfection, cell lysates were separated by SDS-PAGE and analyzed by western blotting. In vitro dephosphorylation of NACA (arrow) with recombinant phosphatase enzymes (A) or in vivo co-expression of NACA (B) with PP1A resulted in a faster migrating hypo-phosphorylated form of NACA (arrowhead).
Figure 3. PP1A mediates dephosphorylation of NACA at specific residues. (A) NACA was immunopurified from HEK293T cells expressing NACA with or without the PP1A catalytic unit (PPP1CA). Post-translational modifications were identified by liquid chromatography-tandem mass spectrometry. (B) Heat maps indicating the differential abundance of phosphorylation sites detected on NACA in the presence or absence of PPP1CA. Black indicates zero spectral counts and increasing quantities of spectral counts are represented in yellow. (C) A representative mass spectra of a NACA phosphopeptide modified at Serine 151.
Figure 4. NACA dephosphorylation leads to recruitment of novel interactants. (A) Venn diagram showing overlap and comparison between the sets of NACA-associated proteins identified by mass spectrometry. Network analysis and visualization of the identified NACA interactants based on curated protein-protein interaction databases (GeNets). Interactants unique to PP1A overexpression are delineated within the red rectangle. Closely connected protein clusters are color coded (green, intermediate filaments; purple, cajal bodies; orange, cytoskeleton; blue, mRNA processing; red, transcriptional regulation; interactants that are not part of defined clusters are shown in grey). (B) Table of the PP1A-dependent NACA interactome. NACA-associated proteins identified by mass spectrometry in the presence or absence of PPP1CA. (C) Validation of NACA interaction with BTF3 in a PP1A-dependent manner. HEK293T cells expressing NACA-FLAG and/or MYC-PPP1CA were lysed and immunoprecipitated (IP) with anti-FLAG antibody beads and probed for endogenous BTF3. Overexpression of MYC-PPP1CA induces interaction of NACA and BTF3.
Figure 5. PPP1CA and BTF3 functionally cooperate with NACA to regulate cJUN transcription. (A) Transcriptional activity of a cJUN-responsive MMP9-promoter luciferase (MMP9-Luc) reporter construct in the presence of the indicated expression vectors. (B) Luciferase activity of a cJUN responsive construct containing 6 tandem repeats of the AP-1 binding element (AP1-Luc) in cells transfected with the indicated expression vectors. (C) Effect of NACA serine/threonine-to-aspartate mutations on cJUN activity on the MMP9-Luc reporter. HEK293T cells were transiently transfected with reporter vectors together with cJUN, NACA, BTF3 and/or PPP1CA expression vectors alone or in combination and relative luciferase activity measured 24 hours after transfection. Data are presented as means ± SD; n ≥ 3. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ANOVA with post-hoc test.
Figure 6. Regulation of NACA localization by PP1A-sensitive NACA residues. (A) HEK293T cells were transiently transfected with wildtype NACA or NACA mutant constructs and immunostained for NACA with anti-FLAG antibody. (B) Quantification of NACA distribution in cells treated as in (A) by fluorescence intensity analysis with ImageJ software. (C) Effect of NACA serine/threonine-to-aspartate-or-alanine mutations on AP-1 activity using the AP1-Luc reporter. HEK293T cells were transiently transfected with reporter vectors together with wildtype NACA or the indicated mutants. Relative luciferase activity was measured 16 hours after treatment with 200 nM PMA. Data are presented as means ± SD; n ≥ 3. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ANOVA with post-hoc test. Magnification bars, 50 µm.
Figure 7

(A) Interaction of NACA with PPP1CA. MC3T3-E1 cells expressing NACA-FLAG and/or MYC-PPP1CA were lysed and immunoprecipitated (IP) with anti-MYC antibody beads and blotted with the indicated antibodies. (B) Localization of NACA and PPP1CA in MC3T3-E1 cells was examined by immunofluorescence using antibodies against endogenous NACA (red) and endogenous PPP1CA (green). Areas of colocalization (yellow) were detected in the merged image. (C) Transcriptional activity of a cJUN-responsive luciferase reporter construct (AP1-Luc) in the presence of the indicated expression vectors in MC3T3-E1 cells. (D) Immunoblots of whole-cell lysates from MC3T3-E1 cells stably expressing wildtype NACA or NACA serine/threonine-to-aspartate or -alanine mutations. (E) RT-qPCR analysis of AP-1 target gene expression in MC3T3-E1 cells stably expressing NACA or the indicated mutants. (F) RT-qPCR analysis of osteoblast differentiation markers after 12 days of differentiation. (G) MC3T3-E1 cells differentiated as in (F) were stained for mineral by von Kossa staining. Data are presented as means ± SD; n ≥ 3. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ANOVA with post-hoc test. Magnification bars, 50 µm except inset, 9 µm.
Dephosphorylation of the transcriptional cofactor NACA by the PP1A phosphatase enhances cJUN transcriptional activity and osteoblast differentiation
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