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Stimulation of Sirt1-Regulated FoxO Protein Function by the Ligand-Bound Vitamin D Receptor▽

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Hormonal vitamin D, 1,25-dihydroxyvitamin D (1,25D), signals through the nuclear vitamin D receptor (VDR). 1,25D regulates cell proliferation and differentiation and has been identified as a cancer chemopreventive agent. FoxO proteins are transcription factors that control cell proliferation and survival. They function as tumor suppressors and are associated with longevity in several organisms. Accumulating data have revealed that 1,25D and FoxO proteins regulate similarly common target genes. We show here that the ligand-bound VDR regulates the posttranslational modification and function of FoxO proteins. 1,25D treatment enhances binding of FoxO3a and FoxO4 within 4 h to promoters of FoxO target genes and blocks mitogen-induced FoxO protein nuclear export. The VDR associates directly with FoxO proteins and regulators, the sirtuin 1 (Sirt1) class III histone deacetylase (HDAC), and protein phosphatase 1. In addition, phosphatase activity and trichostatin A-resistant HDAC activity coimmunoprecipitate with the VDR. 1,25D treatment rapidly (in <4 h) induces FoxO deacetylation and dephosphorylation, consistent with activation. In contrast, ablation of VDR expression enhances FoxO3a phosphorylation, as does knockdown of Sirt1, consistent with the coupling of FoxO acetylation and phosphorylation. 1,25D regulation of common VDR/FoxO target genes is attenuated by blockade of phosphatase activity or by small interfering RNA (siRNA)-mediated knockdown of Sirt1 or FoxO protein expression. Finally, 1,25D-dependent cell cycle arrest is blocked in FoxO3a-deficient cells, indicating that FoxO proteins are key downstream mediators of the antiproliferative actions of 1,25D. These studies link 1,25D signaling through the VDR directly to Sirt1 and FoxO function and provide a molecular basis for the cancer chemopreventive actions of 1,25D.

Vitamin D is obtained from limited dietary sources and UVB-stimulated photoconversion of 7-dehydrocholesterol in skin (36). Hepatic hydroxylation catalyzed by CYP27A1, CYP2R1, and possibly other enzymes generates the major circulating metabolite 25-hydroxyvitamin D (25D). 25D is a relatively long-lived metabolite and is a marker of vitamin D status. 25D is 1α hydroxylated in kidney and peripheral tissues to produce hormonal 1,25-dihydroxyvitamin D (1,25D). While renal 1α hydroxylation generates much of the circulating 1,25D, extrarenal 1α hydroxylation is a critical source of 1,25D *in situ* in a number of tissues (61). Moreover, while renal CYP27B1 expression/activity is regulated by calcium homeostatic signals (e.g., parathyroid hormone), extrarenal 1α hydroxylation is regulated by distinct physiological inputs.

1,25D binds the nuclear vitamin D receptor (VDR), which heterodimerizes with related retinoid X receptors (RXRs) to recognize vitamin D response elements (VDREs) in target genes (36). Although initially identified as a regulator of calcium homeostasis, 1,25D is now known to have a broad spectrum of actions. For example, it acts as a chemopreventive agent in several animal models of cancer and induces cell cycle arrest and nonmalignant and malignant cell differentiation (14, 24, 27, 34, 35, 37, 46, 49). Moreover, epidemiological data provide associations between lack of UVB exposure, vitamin D insufficiency, and the prevalence of certain cancers (16). Notably, a large prospective study associated 25D sufficiency with reduced total cancer incidence and mortality, particularly in digestive cancers (head and neck squamous cell carcinoma [HNSCC] and esophageal, pancreatic, stomach, and colorectal cancers) and leukemias (23). *VDR* gene polymorphisms also correlate with protection against different malignancies, including HNSCC (16, 39). The above is noteworthy, as numerous studies have shown that vitamin D insufficiency or deficiency is widespread in temperate populations (26, 61).

FoxO1, FoxO3a, FoxO4, and FoxO6 transcription factors regulate cell proliferation, differentiation, and metabolism and control longevity (1, 10, 21, 25, 52). Serial ablation in mice of genes encoding FoxO proteins revealed that these proteins are bona fide tumor suppressors (7, 17, 28). FoxO function is inhibited by mitogen-activated PI3 kinase, which stimulates Akt-dependent phosphorylation, nuclear export (1, 10, 25), and proteasomal degradation (17, 28). FoxOs are also regulated by acetylation, which can be reversed by the NAD-dependent sirtuin 1 (Sirt1) class III lysine deacetylase (15, 30). Acetylation reduces DNA binding and enhances phosphorylation and inactivation (43). Notably, FoxO and c-MYC target genes partially overlap, and FoxO factors repress a subset of c-MYC-induced genes, including *CCND2*, which encodes cyclin D2 (18, 52).

We have been interested in understanding the mechanisms regulating the anticancer properties of vitamin D, in particular the molecular genetic events underlying its control of cell proliferation. We noted that there was an overlap in the target genes regulated by the hormone-bound VDR and FoxO pro-

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teins. For example, similar to FoxO proteins, 1,25D represses *CCND2* expression and induces transcription of *CCNG2*, which encodes the inhibitory cyclin G2 (42, 54, 59). Here, we show that the VDR interacts with FoxO proteins and their regulator Sirt1 and that 1,25D rapidly induces Sirt1- and phosphatase-dependent dephosphorylation and activation of FoxO protein function. Ablation of Sirt1 or FoxO protein expression attenuates regulation by 1,25D of common VDR/FoxO target genes, and loss of FoxO3a eliminates 1,25D-induced cell cycle arrest in human HNSCC cells, providing evidence for a hormonally regulated VDR-Sirt1-FoxO protein partnership and a molecular basis for the chemopreventive actions of 1,25D.

MATERIALS AND METHODS

Immunoprecipitation and Western blot analysis. Protein extracts from SCC25 cells were prepared in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40) and preincubated with protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4C and exposed to anti-VDR (αVDR) (D-6; Santa Cruz), αFoxO3a (H-144; Santa Cruz), or αIgG (Santa Cruz) antibodies overnight. Immunoprecipitates (IPs) were then washed, eluted in 2× SDS loading buffer, and processed for Western blotting, performed with standard protocols. Antibodies directed against FoxO3a (N15), VDR (C-20), RXR (C-20), PP2A (C-20), PP1 (FL-18), Sirt1 (H-300), and Ccnd2 (C-17) were from Santa Cruz Biotechnology, Inc., and antibodies against FoxO4 (9472) and p53 acetylated on Lys382 (2525s) were purchased from Cell Signaling. Anti-Ph-Th32 FoxO3a (Upstate 07-695) was also used. Antiactin antibody (C-2; Santa Cruz) was used for loading controls.

GST pulldown and Western blot analysis. The VDR ligand binding domain was cloned into the vector pGEX-4T2 to create a glutathione *S*-transferase (GST)-VDR fusion. The Sirt1-Flag expression vector was a kind gift from X. J. Yang (McGill University), and the Foxo3a expression vector was obtained from Addgene, Inc. (Cambridge, MA). GST pulldowns were performed using the MagneGST pulldown system (Promega) per the manufacturer's instructions. Pulldown eluates were subsequently analyzed by Western blotting using standard protocols. Antibodies directed against FoxO3a (N15) and Sirt1 (H-300) were purchased from Santa Cruz Biotechnology, Inc.

HDAC, phosphatase, and proliferation assays. Histone deacetylase (HDAC) assays were performed essentially as described previously (60). Total cell lysates were immunoprecipitated with αVDR (C-20; Santa Cruz) or mouse αIgG (Santa Cruz). IPs were then preincubated with trichostatin A (TSA; 300 nM) with NAD^+ (0.1 mM) for 5 min before Boc-Lys(acetyl[Ac])-AMC (4-amino-7-methylcoumarin) (a gift from Jim Gleason, Chemistry, McGill University) substrate addition. Deacetylation of the substrate renders it susceptible to trypsin cleavage, liberating AMC, which is a fluorescent compound (see reference 60 for assay details). Phosphatase and proliferation assays were performed by using an immunoprecipitation phosphatase assay kit (Upstate) and a Click-iT EdU Alexa Fluor 647 high-throughput imaging assay kit (Invitrogen) in accordance with the supplier's protocol. SCC25 cells were transfected with FoxO3a, FoxO4, or control small interfering RNAs (siRNAs) and, after 24 h of transfection, treated with 1,25D for 24 h. Cells were then incubated with Alexa-EdU for 2 h, fixed, and assayed. Images were captured using ImageXpressMicro (Molecular Devices) and analyzed with MetaXpress (Molecular Devices).

ChIP assays and RT-PCR. Chromatin immunoprecipitation (ChIP) assays and reverse transcription-PCRs (RT-PCRs) were performed essentially as described previously (59), and primers are shown in Fig. 1 (also data not shown). SCC25 cells were treated with 1,25D (100 nM) or vehicle as indicated in the figures. Immunoprecipitations were performed by using normal rabbit IgG, anti-VDR (D-6; Santa Cruz Biotechnology), anti-FoxO3a (H-144; Santa Cruz Biotechnology), anti-Flag (F1804; Sigma), or anti-FoxO4 (Cell Signaling). The forkhead response elements (FHREs) targeted are indicated in Fig. 2.

RNA interference. The sequences of siRNAs are as follows: for *FOXO3A*, 5′-CTGAATGATGGGCTGACTGAA-3′; for *FOXO4*, 5′-TCCGTCCACGAAGCAGTTCAA-3′; for *VDR*, 5′-TCAGACTCCATTTGTATTATA-3′; and for *SIRT1*, 5′-CACGGATAGGTCCATATACTT-3′. SCC25 cells were transfected with *FOXO3A*, *FOXO4*, *SIRT1*, *VDR* or control siRNAs for 24 h and treated with 1,25D as indicated. *FOXO3A*, *FOXO4*, *SIRT1*, *VDR*, and Allstars negative-control siRNAs were purchased from Qiagen.

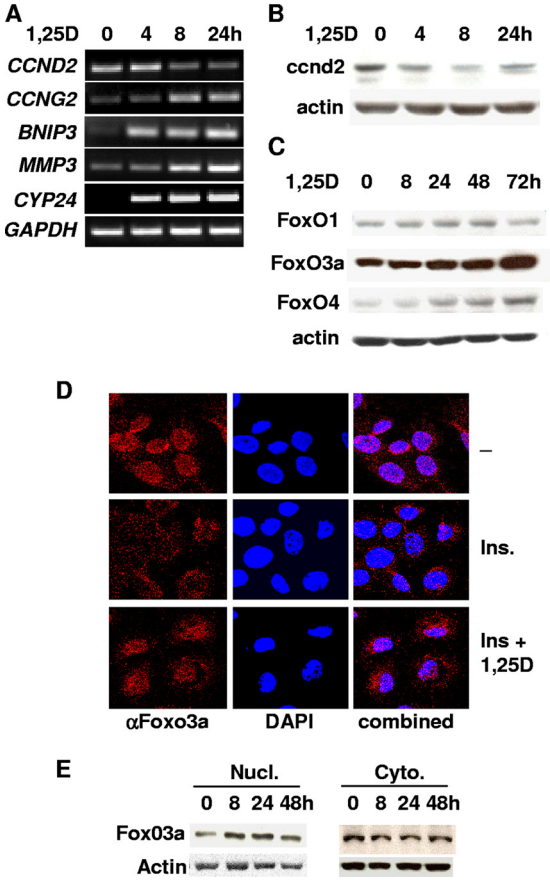

FIG. 1. The VDR and FoxO proteins regulate common target genes. (A) RT-PCR analysis of regulation by 1,25D (100 nM) of *CCND2*, *CCNG2*, *BNIP3*, and *MMP3* in SCC25 cells. *CYP24* and *GAPDH* are included as positive and negative controls, respectively, for 1,25D-regulated gene expression. (B) Treatment of SCC25 cells with 1,25D reduces cyclin D2 levels. SCC25 cells were incubated with 1,25D (100 nM) as indicated prior to isolation of protein for Western blotting. (C) Western blotting of the expression of FoxO1, FoxO3a, and FoxO4 in SCC25 cells treated with 100 nM 1,25D over a 72-h period. (D) SCC25 cells were incubated with vehicle (−), insulin (Ins), or insulin and 1,25D as indicated for 1 h. Subcellular localization of FoxO3a was determined by immunocytochemistry. Nuclei were stained with DAPI. See Materials and Methods for details. (E) SCC25 cells were treated with 1,25D as indicated prior to harvesting and isolation of nuclear and cytoplasmic fractions. Western blots of FoxO3a and actin controls are presented.

VDRE-luciferase reporter gene assay. Luciferase assays for the VDRE3/tk-luc expression vector were performed as described previously (4). SCC25 cells were transfected with the VDRE3/tk-luc expression vector (59) with or without siRNAs. After 24 h of incubation, the cells were treated with 1,25D, and the extracts were assayed for *luciferase* activity. β-Galactosidase activity was used to normalize transfection efficiencies, with commercially available reagents (Promega Corp.).

Immunocytochemistry. Immunocytochemistry on SCC25 cells was performed by using αFoxO3a (Cell Signaling) followed by goat anti-rabbit IgG–Texas Red (TR) secondary antibody (Santa Cruz Biotechnology). The cells were serum starved for 16 h and preincubated with insulin (100 nM) for 1 h before 1,25D treatment. The slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen), and confocal microscopy was carried out by using a Zeiss LSM 510 microscope.

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed with a kit (SYBR green on MiniOpticon; Bio-Rad). The qRT-PCR primer sequences are listed in Fig. 2 (also data not shown). PCRs include 2 μl of DNA template,

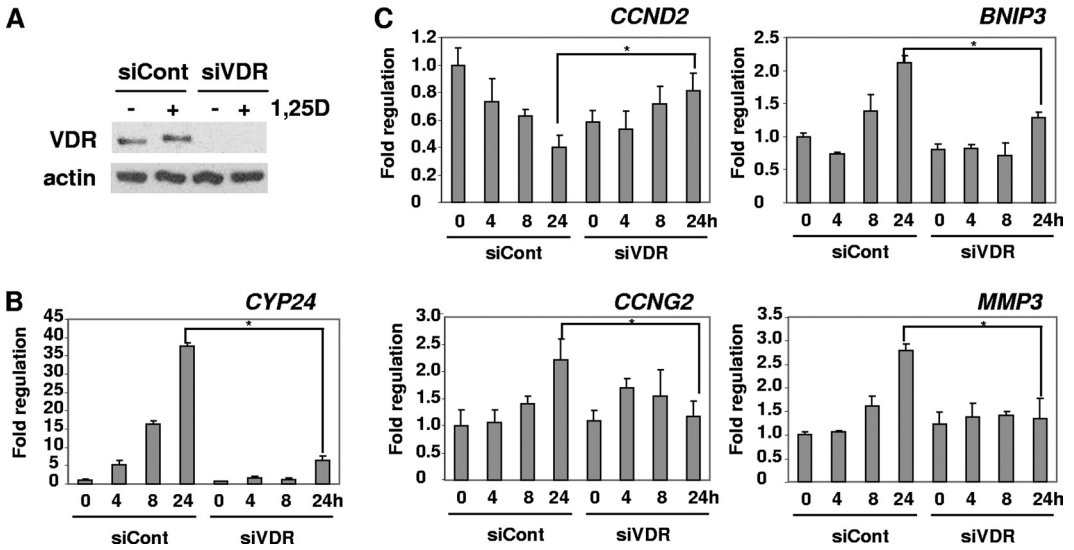

FIG. 2. Ablation of VDR expression blocks regulation by 1,25D of FoxO target genes. (A) siRNA-mediated knockdown of VDR expression. SCC25 cells were transfected with siRNAs recognizing the *VDR* gene or scrambled siRNA controls, and expression of the VDR was assessed by Western blotting. (B) Effect of VDR knockdown on regulation by 1,25D of VDR target gene *CYP24*. (C) Effects of VDR knockdown on regulation by 1,25D of FoxO target genes *CCND2*, *BNIP3*, *CCNG2*, and *MMP3*. Error bars represent SD. *, $P < 0.05$ for control (siCont) versus VDR-specific (siVDR) siRNAs at 24 h.

300 nM each primer, 10 μl of master mix (iQ Sybr green supermix; Bio-Rad), and RNase-free water to bring the reaction mixture volume to 20 μl. Each target gene was run concurrently with the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, a control gene. The qRT-PCR was performed with hot-start denaturation step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 58°C for 20 s. All reactions were run in triplicate.

Data analysis. All the experimental data are shown as the means ± standard deviations (SD) of results from three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) using the computer software program PRISM (GraphPad Software, Inc., San Diego, CA). Data were considered significantly different from each other at P values of <0.05.

RESULTS

1,25D induces VDR and FoxO protein binding to target gene promoters. Epidemiological and genetic studies have revealed that vitamin D sufficiency reduces incidence and mortality of head and neck squamous cancers (3, 16, 23, 34–36, 39, 51, 59). Our previous experiments have shown that 1,25D inhibits HNSCC cell proliferation and tumor growth (34, 59). SCC25 cells are a well-differentiated, 1,25D-sensitive HNSCC line derived from a floor of the mouth/base of the tongue tumor and are arrested in G_0/G_1 upon 1,25D treatment (3). As expected, 1,25D strongly induces expression in SCC25 cells of the *CYP24* gene (Fig. 1A), which encodes the enzyme that initiates 1,25D catabolism (36). Treatment also inhibits the expression of the G_1 cyclin D2 (*CCND2*) transcripts and protein (Fig. 1A and B). Conversely, 1,25D induces expression of the gene encoding inhibitory cyclin G2 (*CCNG2*) (Fig. 1A). Note that all experiments presented in this study are typical of multiple biological replicates.

CCND2 and *CCNG2* are well-characterized FoxO protein target genes that are repressed and induced, respectively, upon FoxO activation (43, 54). 1,25D also induced expression (Fig. 1A) of *MMP3*, and *BNIP3*, both of which are also stimulated by FoxO proteins (21, 32, 41, 56). FoxO1, FoxO3a, and FoxO4 are all present in SCC25 cells (FoxO6 is mostly neuronal [29] and was not studied here). Long-term 1,25D treatment gradually but substantially enhanced expression of FoxO3a and FoxO4 and more weakly affected FoxO1 (Fig. 1C). The slow increases in FoxO protein levels observed are consistent with previous studies showing that 1,25D signaling inhibits expression of the $p45^{SKP2}$ E3 ubiquitin ligase required for FoxO protein proteasomal degradation (33). We probed FoxO protein function further by analyzing the effect of 1,25D on the nuclear and cytoplasmic distribution of FoxO3a. Treatment of SCC25 cells with insulin induced export of nuclear FoxO3a, which was largely blocked by simultaneous treatment with 1,25D (Fig. 1D). In similar experiments, 1,25D treatment enhanced nuclear localization of FoxO3a and partially depleted cytoplasmic protein (Fig. 1E), suggesting that 1,25D influences FoxO3a function as well as expression.

We decided to investigate possible functional interactions between the VDR and FoxO proteins. Expression of the VDR in SCC25 cells was ablated by siRNA-mediated knockdown (Fig. 2A), which, as expected, blocked 1,25D-induced *CYP24* expression (Fig. 2B). Ablation of the VDR blocked induction by 1,25D of *BNIP3*, *MMP3*, and *CCNG2* expression (Fig. 2C). Repression of *CCND2* expression by 1,25D was also blocked in cells lacking the VDR (Fig. 2C). However, we consistently observed reductions in basal *CCND2* levels in untreated cells lacking the VDR in several independent experiments, suggesting that the unliganded VDR indirectly or directly controls expression of *CCND2* to some degree. As a further control for VDR function, we incubated cells with a recently developed VDR antagonist (M. Lamblin, R. Spingarn, T.-T. Wang, M. C. Burger, B. Dabbas, N. Moitessier, J. H. White, and J. L. Gleason, submitted for publication; also data not shown), which inhibited induction by 1,25D of *CCNG2*, *MMP3*, and *BNIP3* and inhibited repression of *CCND2* expression. Taken together, these experiments show that regulation by 1,25D of the FoxO target genes under study requires the VDR.

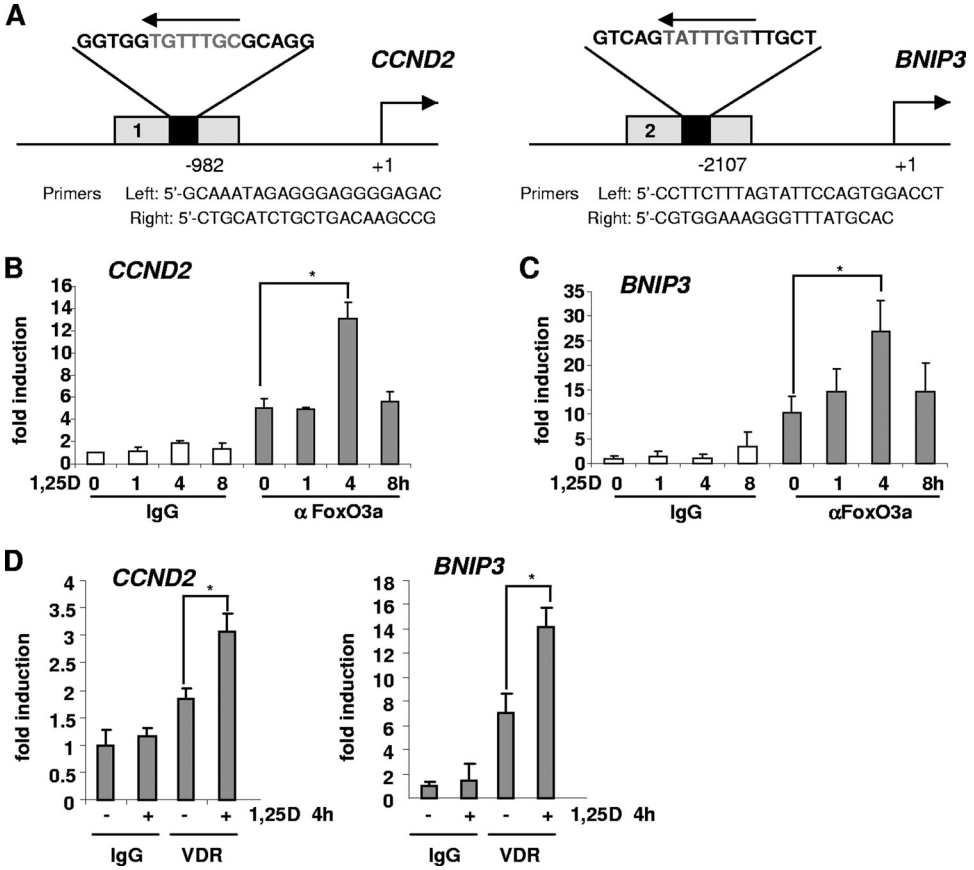

FIG. 3. Short-term 1,25D treatment induces binding of FoxO3a to target gene promoters. (A) FHREs in the promoter-proximal regions of the *CCND2* and *BNIP3* genes. (B and C) ChIP analysis of 1,25D-dependent binding of FoxO3a to the *CCND2* (B) and *BNIP3* (C) promoters in SCC25 cells as assessed by qRT-PCR. (D) ChIP analysis of 1,25D-dependent association of the VDR with the FoxO binding regions of the *CCND2* and *BNIP3* promoters. Error bars represent SD. *, $P < 0.05$.

Given that 1,25D increases the ratio of nuclear/cytoplasmic FoxO3a and that FoxO proteins and 1,25D share common target genes, we investigated the effects of 1,25D treatment on binding of FoxO proteins to the *CCND2* and *BNIP3* promoters. We focused on FoxO3a and FoxO4, as their expression was most strongly regulated by 1,25D (Fig. 1). FoxO proteins bind to the negative regulatory region of the *CCND2* promoter (50). A forkhead response element (FHRE) in the murine *BNIP3* gene has been defined (41, 42). The promoters of the human and murine *BNIP3* genes are well conserved, and we identified a conserved FHRE in the human *BNIP3* promoter (Fig. 3A). Remarkably, ChIP assays revealed that 1,25D enhanced binding of FoxO3A to both promoters, which peaked after 4 h of treatment (Fig. 3B and C). Association of the VDR with the same regions of the *CCND2* and *BNIP3* promoters was also enhanced by 4 h of treatment with 1,25D (Fig. 3D). Weak 1,25D-dependent binding of FoxO4 to the *CCND2* promoter was also detected (data not shown). Taken together, these results suggest that 1,25D signaling is inducing relatively rapid regulation of FoxO function through the VDR.

The VDR interacts with FoxO proteins and FoxO regulators Sirt1 and PP1c. FoxO protein DNA binding and subcellular localization are strongly influenced by their states of phosphorylation and acetylation. Moreover, the relatively rapid induction of FoxO DNA binding could not be accounted for by the relatively slow increase in total FoxO3a and FoxO4 seen in 1,25D-treated cells (Fig. 1), suggesting that 1,25D rapidly affected FoxO protein posttranslational modification. Changes in FoxO3a and FoxO4 phosphorylation with 1,25D treatment were investigated with phosphoprotein-specific antibodies. 1,25D induced transient dephosphorylation of FoxO3a and FoxO4 within 4 to 8 h (Fig. 4A and B), which paralleled the kinetics of 1,25D-induced FoxO DNA binding seen in Fig. 3. The relatively rapid dephosphorylation of FoxO3a and FoxO4 in the presence of 1,25D is intriguing given a previous report that the VDR forms ligand-independent complexes with the catalytic subunits of PP1 and PP2A and stimulates their phosphatase activities in the presence of 1,25D (9). Our results suggest that the VDR associated with FoxO proteins as well. Indeed, under conditions where, as expected, the nuclear receptor p160 coactivator AIB1 was recruited to the VDR in a ligand-dependent manner, coimmunoprecipitation of FoxO3a and FoxO4 was ligand independent (Fig. 4C). Both PP1c and PP2Ac are abundant in SCC25 cells, and their expression is not altered by 1,25D treatment over 24 h (data not shown). We confirmed the ligand-independent association of the VDR with PP1c by coimmunoprecipitation (Fig. 4D). However, any as-

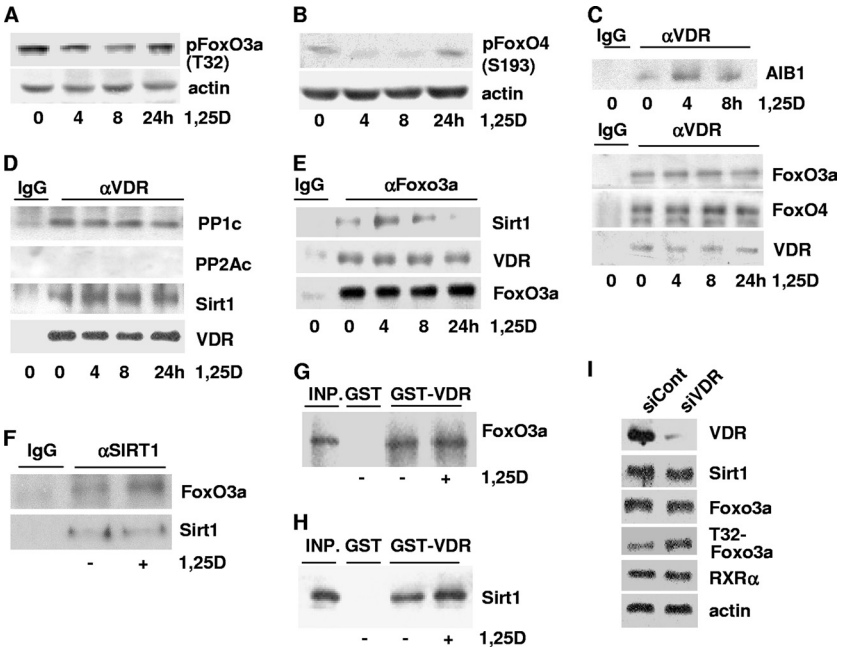

FIG. 4. Interactions of the VDR with FoxO proteins and their regulators Sirt1 and PP1. (A and B) 1,25D induces dephosphorylation of FoxO3a (A) and FoxO4 (B). FoxO3a and FoxO4 phosphorylation on specific residues were assessed by Western blotting using specific antibodies recognizing FoxO3a phosphorylated on Thr32 or FoxO4 phosphorylated on Ser193. (C) FoxO3a and FoxO4 associate with the VDR, as determined by immunoprecipitation of the VDR from SCC25 cells treated with 1,25D as indicated, followed by Western blotting for FoxO3a, FoxO4, or the VDR. Coimmunoprecipitation of p160 coactivator AIB1 with the VDR was used as a control for 1,25D-dependent association of the VDR with a cofactor. (D) Association of the VDR with PPI and Sirt1. The VDR was immunoprecipitated from SCC25 cells, and immunoprecipitates were probed for the presence of the catalytic subunits of PP1, PP2a, Sirt1, or the VDR. (E) 1,25D augments the association of Sirt1 with FoxO3a. FoxO3a was immunoprecipitated from SCC25 cells, and immunoprecipitates were probed for the presence of Sirt1, the VDR, or FoxO3a. (F) Sirt1 was immunoprecipitated from SCC25 cells treated with vehicle or 1,25D, followed by Western blotting for associated FoxO3a. (G) Analysis by a GST pulldown assay of the binding of *in vitro*-translated FoxO3a to the VDR ligand binding domain in the absence and presence of 1,25D. (H) Analysis of 1,25D-dependent binding of *in vitro*-translated SIRT1 to the VDR ligand binding domain by a GST pulldown assay. (I) Effect of siRNA-mediated VDR ablation on expression levels of Sirt1, total FoxO3a, and RXRα and an analysis of the effect of VDR ablation on the level of Th32 FoxO3a phosphorylation.

sociation of the VDR with PP2A in SCC25 cells was either weak or absent (Fig. 4D).

Given that deacetylation enhances FoxO protein dephosphorylation and that Sirt1 is a FoxO protein deacetylase (30, 43), we probed for Sirt1 association with the VDR and found that VDR coimmunoprecipitated in a partially ligand-dependent manner (Fig. 4D). Moreover, 1,25D treatment enhanced the association of Sirt1 with FoxO3a, as assessed by immunoprecipitations performed with either αFoxO3A (Fig. 4E) or αSirt1 (Fig. 4F) antibodies. Direct interactions of FoxO3a or Sirt1 with the VDR were tested in GST pulldown assays, which revealed ligand-independent interaction of FoxO and partially ligand-dependent binding of Sirt1 to GST-VDR (Fig. 4G and H), consistent with the results of coimmunoprecipitation experiments. Knockdown of VDR expression had no effect on expression of Sirt1, FoxO3a, or the VDR cofactor RXRα. Notably, however, loss of VDR expression enhanced FoxO3a phosphorylation, consistent with a role for FoxO3a-associated VDR in regulating the state of posttranslational modification of FoxO3a (Fig. 4I). Taken together, these data indicate that the VDR associates with FoxO proteins and two key regulators of their posttranslational modification and function, Sirt1 and PP1, and that interactions with FoxO proteins and PP1 are ligand independent but that binding of Sirt1 is partially ligand dependent.

Inhibition of phosphatase or Sirt1 activity blocks regulation by 1,25D of common VDR/FoxO target genes. The functional consequences of the association of the VDR with PP1c were assessed by inhibition of phosphatase activity with okadaic acid, which blocked 1,25D-induced FoxO3a dephosphorylation (Fig. 5A). In addition, short-term (10 to 30 min) 1,25D treatment of extracts of SCC25 cells increased phosphatase activity (Fig. 5B). Phosphatase activity also coimmunoprecipitated with the VDR in the absence of 1,25D, consistent with the constitutive interaction between the VDR and PP1c observed by coimmunoprecipitation (Fig. 5C). Moreover, 4 h of incubation with 1,25D augmented VDR-associated phosphatase activity (Fig. 5C). Pharmacological blockade of phosphatases with okadaic acid attenuated or inhibited regulation by 1,25D of FoxO target genes *CCNG2*, *BNIP3*, and *MMP3* (Fig. 5D) and eliminated suppression by 1,25D of cyclin D2 protein expression (Fig. 5E). It also attenuated regulation by 1,25D of the *CYP24* target gene. However, as argued below, this is likely to be independent of any effects on 1,25D-regulated FoxO protein function.

The consequences of the 1,25D-dependent association of

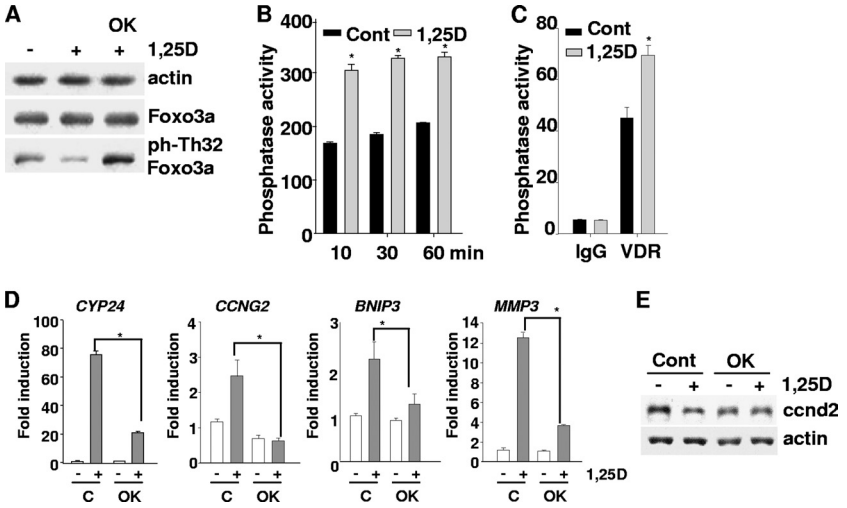

FIG. 5. Phosphatase inhibition blocks 1,25D-induced FoxO3a dephosphorylation and activation. (A) SCC25 cells were treated with 1,25D for 8 h alone or in the presence of the phosphatase inhibitor okadaic acid (Ok; 1 nM). Expression of total and phospho-FoxO3a was assessed by Western blotting. (B) 1,25D treatment enhances total phosphatase activity in SCC25 cells. Extracts of SCC25 cells were incubated with 1,25D, as indicated, and total phosphatase activity was determined. (C) Association of phosphatase activity with the VDR. SCC25 cells were treated for 4 h with vehicle or 1,25D, and extracts were immunoprecipitated with an αVDR antibody. Immunoprecipitates were assayed for phosphatase activity. (D) Inhibition of phosphatase activity blocks or attenuates regulation by 1,25D of FoxO target genes. SCC25 cells were treated with 1,25D or vehicle for 24 h in the absence of inhibitors (Cont.) or in the presence of the phosphatase inhibitor okadaic acid (Ok; 1 nM) prior to isolation of RNA for qRT-PCR analysis. (E) Okadaic acid (1 nM) blocks suppression by 1,25D of cyclin D2 expression, as analyzed by Western blotting. Error bars represent SD. *, $P < 0.05$.

Sirt1 with FoxO proteins were also tested. TSA-resistant HDAC activity immunoprecipitated with the VDR and was enhanced by short-term treatment with 1,25D (Fig. 6A). Consistent with these findings, immunoprecipitation of total FoxO3a and probing with an anti-acetyllysine antibody revealed that 1,25D treatment reduced the levels of FoxO3a acetylation (Fig. 6B). In contrast, acetylation of p53, another Sirt1 substrate, was unaffected or modestly enhanced by 1,25D treatment (Fig. 6B), suggesting that the effect of 1,25D on Sirt1 is substrate specific.

The role of Sirt1 in regulation by 1,25D of FoxO3a function was further investigated by siRNA-mediated knockdown (Fig. 6C). Although Sirt1 has been shown to contribute to FoxO protein regulation (43), it was not clear how critical its role was, as loss of Sirt1 deacetylase activity could be compensated for by other sirtuins or class I or II HDACs. Importantly, while ablation of Sirt1 had no effect on total FoxO3a expression, it markedly enhanced FoxO3a phosphorylation, consistent with the idea that Sirt1 is a key regulator of FoxO function in SCC25 cells (Fig. 6C).

We further probed the role of Sirt1 in regulation by 1,25D of FoxO target genes. In a control experiment, ablation of neither Sirt1 expression nor FoxO3a altered the levels of the VDR or RXRα in SCC25 cells (Fig. 6D). Similarly, knockdown of Sirt1 had no substantial effect on regulation of VDR target gene *CYP24* (Fig. 6E). However, loss of Sirt1 attenuated inhibition by 1,25D of *CCND2* expression and diminished induction of *BNIP3*, *CCNG2*, and *MMP3* expression (Fig. 6F). We note here that, as resveratrol has been described as an activator of Sirt1, we performed several experiments to determine whether resveratrol would enhance (1,25D-regulated) FoxO function, consistent with stimulation of Sirt1 activity. However, we failed to observe effects of resveratrol consistent with Sirt1 activation. For example, while inhibitor nicotinamide altered regulation by 1,25D of FoxO target genes in a manner consistent with blockade of Sirt1, the effects of resveratrol were inconsistent with Sirt1 activation (data not shown). In this regard, recent publications have suggested that effects of resveratrol on Sirt1 function are indirect (8, 47).

A role for Sirt1 in 1,25D-regulated FoxO target gene expression was further supported by the results of ChIP assays revealing an association of Sirt1 with the *CCND2* and *BNIP3* promoters that was modestly enhanced by 1,25D (Fig. 6G). Moreover, knockdown of Sirt1 expression blocked 1,25D-dependent binding of the VDR to the *CCND2* and *BNIP3* promoters (Fig. 6H). Taken together, the results of the ChIP assays provide evidence that 1,25D stimulates the formation of a complex containing FoxO proteins, the VDR, and Sirt1 on FoxO target promoters.

Ablation of FoxO protein expression abrogates hormonal regulation of common FoxO/1,25D target genes and 1,25D-induced cell cycle arrest. Selective siRNA knockdown blocked expression of either FoxO3a or FoxO4 mRNA and protein in SCC25 cells (Fig. 7A and data not shown). Note that knockdown of FoxO3a did not alter FoxO4 protein levels and vice versa (not shown). Loss of FoxO3a or FoxO4 had no significant effects on basal or 1,25D-inducible expression of a VDRE-containing *luciferase* promoter-reporter vector or of VDR target gene *CYP24* (Fig. 7B and C). Conversely, regulation by 1,25D of *CCND2*, *BNIP3*, *CCNG2*, or *MMP3* was either markedly attenuated or abolished in cells lacking FoxO3a (Fig. 7D), whereas ablation of FoxO4 largely abolished regulation of *BNIP3* and *CCNG2* but had little or no effect on regulation of *CCND2* or *MMP3*. This indicates that the functions of FoxO3a and FoxO4 are nonredundant and at least FoxO4 is acting gene specifically.

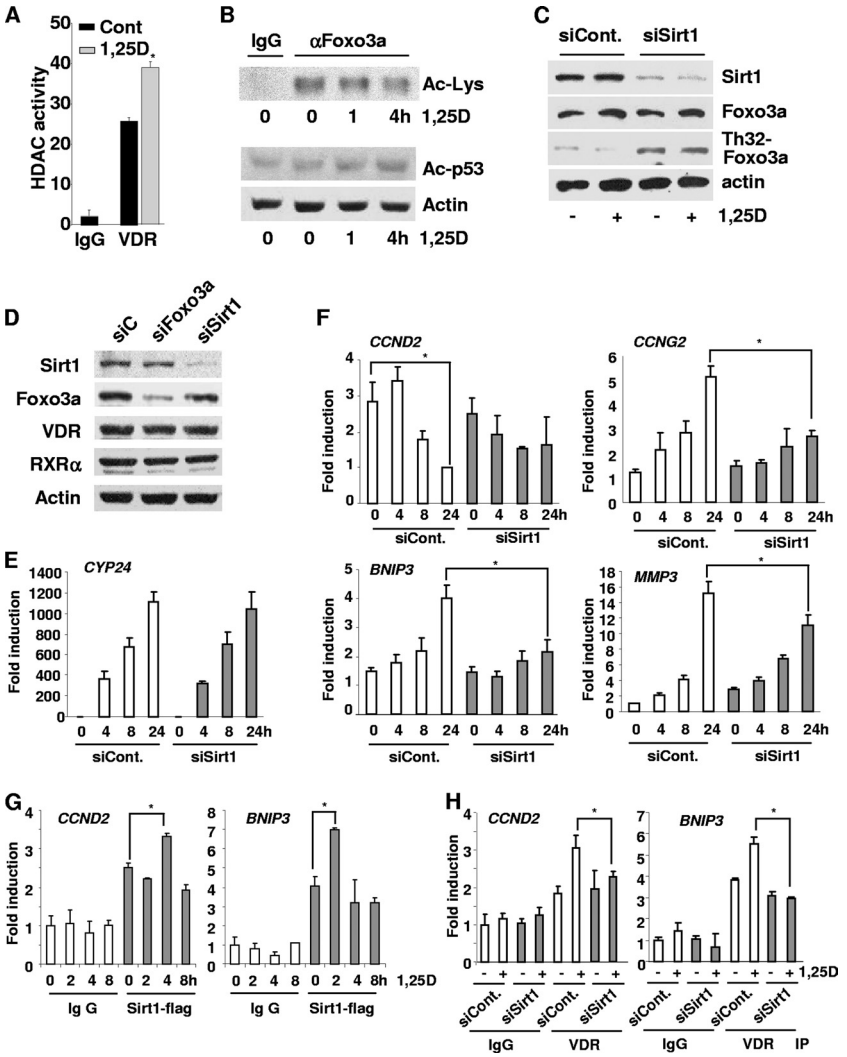

FIG. 6. Association of the VDR with TSA-resistant lysine deacetylase activity and the effects of Sirt1 on FoxO3a phosphorylation and target gene regulation. (A) Association of the VDR with lysine deacetylase activity. SCC25 cells were treated with vehicle or 1,25D for 4 h, and extracts were immunoprecipitated with an αVDR antibody or IgG control and assayed for lysine deacetylase activity in the presence of NAD and the class I and II HDAC inhibitor TSA. (B) 1,25D treatment induces deacetylation of FoxO3a. SCC25 cells were treated with 1,25D for 0, 1, or 4 h, and extracts were immunoprecipitated with an anti-FoxO3a antibody, followed by Western blotting for acetyllysine. As a control for specificity of 1,25D-induced deacetylation, SCC25 cells were treated with 1,25D for 0, 1, or 4 h and extracts analyzed by Western blotting with an anti-acetylated-p53 antibody. Note that the treatment at the zero time point was performed in duplicate in this experiment. (C) Ablation of Sirt1 expression enhances phosphorylation of FoxO3a. SCC25 cells were transfected with siRNAs recognizing Sirt1 or scrambled siRNA controls, and expression of Sirt1 was assessed by Western blotting. Expression of total and Th32-phosphorylated FoxO3a in extracts of cells transfected with control or *SIRT1*-specific siRNA was analyzed. (D) Western blotting of the effects of siRNA-mediated ablation of Sirt1 or FoxO3a expression on levels of the VDR and RXRα. (E) Effect of Sirt1 ablation on regulation of 1,25D target gene *CYP24*. SCC25 cells transfected with control or *SIRT1*-specific siRNAs were incubated with 1,25D as indicated, and regulation of *CYP24* was analyzed by qRT-PCR. (F) Effect of Sirt1 ablation on regulation by1,25D of FoxO target genes. SCC25 cells transfected with control or *SIRT1*-specific siRNA were incubated with 1,25D as indicated, and regulation of FoxO target genes was assessed by qRT-PCR. (G) ChIP analysis of association of transiently expressed Flag-Sirt1 with the FoxO binding regions of the *CCND2* and *BNIP3* promoters. (H) Analysis of the effects of Sirt1 ablation on 1,25D-dependent binding of the VDR to the FoxO binding region of the *BNIP3* promoter. Error bars represent SD. *, $P < 0.05$.

Consistent with the effects of FoxO3a and FoxO4 on regulation by 1,25D of *CCND2* mRNA, knockdown of FoxO3a abolished the reduction in cyclin D2 protein levels seen in 1,25D-treated cells, whereas loss of FoxO4 had little effect (Fig. 7E), suggesting that FoxO3a is more important contributor to 1,25D-induced cell cycle arrest. Treatment of SCC25 cells with 1,25D induces a dose- and time-dependent decrease in cell proliferation (Fig. 8A and B), characterized by reduced incorporation of nucleoside analogue EdU. Knockdown of FoxO3a expression completely abolished inhibition by 1,25D of cell proliferation as measured by EdU incorporation (Fig. 8C), whereas loss of FoxO4 had no significant effect, in agreement with the relative roles of FoxO3a and FoxO4 in regulating *CCND2* expression. Taken together, these results show that the hormone-bound VDR in association with Sirt1 is a regulator of FoxO protein posttranslational modification and func-

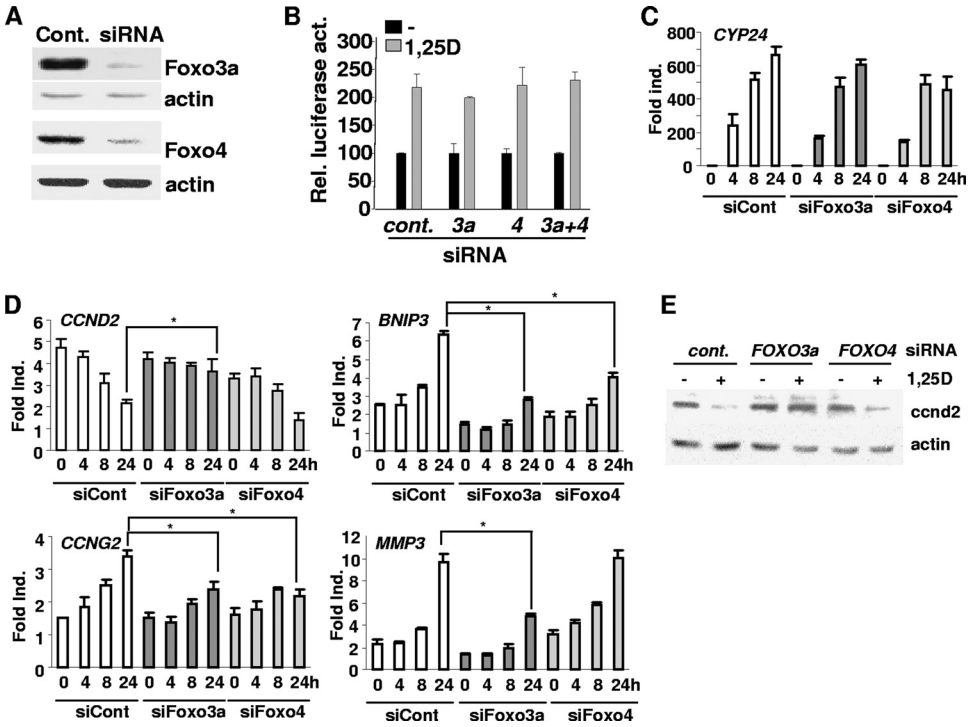

FIG. 7. Ablation of FoxO expression attenuates or blocks inhibition of SCC25 cell proliferation by 1,25D. (A) siRNA-mediated knockdown of FoxO3a or FoxO4. SCC25 cells were transfected with siRNAs recognizing *FOXO3A*, *FOXO4*, or control siRNA, and expression of FoxO proteins was assessed by Western blotting. (B) Ablation of FoxO expression does not affect regulation by 1,25D of a VDRE-containing promoter. SCC25 cells were transfected with the VDRE3/tk-luc expression vector along with control siRNAs or siRNAs directed against *FOXO3A* and/or *FOXO4*, as indicated. Cells were treated with vehicle or 1,25D, and extracts were assayed for *luciferase* activity. (C) Effects of ablation of FoxO expression on regulation by 1,25D of target gene *CYP24*. SCC25 cells were transfected with control or FoxO-specific siRNAs, and transfected cells were treated with 1,25D as indicated. Expression of *CYP24* was analyzed by qRT-PCR. (D) Effects of ablation of FoxO expression on regulation by 1,25D of FoxO target genes. SCC25 cells were transfected with control or FoxO-specific siRNA, and transfected cells were treated with 1,25D as indicated. Expression of FoxO target genes was analyzed by qRT-PCR. (E) Effects of FoxO knockdown on regulation by 1,25D of cyclin D2 expression. SCC25 cells were transfected with control or FoxO-specific siRNAs, and transfected cells were treated with 1,25D for 8 h. Cyclin D2 expression and actin control were assessed by Western blotting. Error bars represent SD. *, $P < 0.05$.

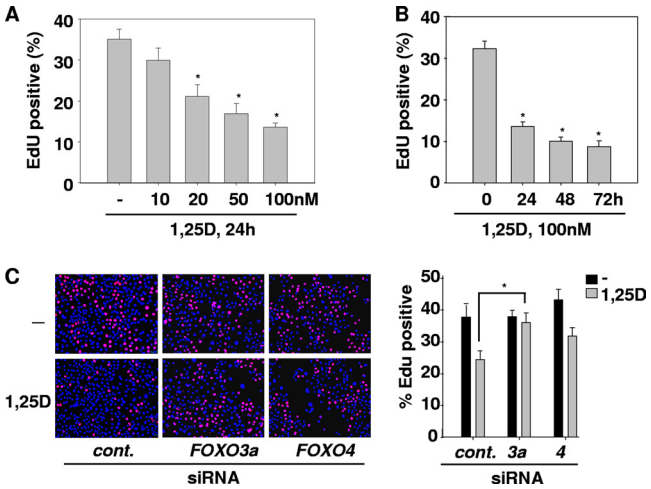

FIG. 8. Knockdown of FoxO3a expression blocks the antiproliferative activity of 1,25D. Dose (A)- and time (B)-dependent effect of 1,25D on SCC25 cell proliferation as assessed by an EdU incorporation assay. (C) SCC25 cells were transfected with control or *FOXO*-specific siRNAs and then treated with vehicle or 1,25D for 24 h. Cell proliferation was analyzed by determining EdU incorporation. Quantification of results and analysis by the *t* test are presented on the right. Error bars represent SD. *, $P < 0.05$.

tion and reveal an important role for FoxO3a as a mediator of the antiproliferative actions of 1,25D.

DISCUSSION

Our results provide direct links between 1,25D signaling through the VDR and regulation of Sirt1 and FoxO protein function and reveal that 1,25D regulates the expression of a subset of its target genes through control of FoxO protein function. The VDR coimmunoprecipitated with FoxO3a and FoxO4 and bound to FoxO3a in GST pulldown assays in a hormone-independent manner. We also found that the VDR associated constitutively with the catalytic subunit of protein phosphatase 1, consistent with previous studies showing hormone-independent interaction of the VDR and PP1 in CaCo2 colon carcinoma cells (9). The VDR associated directly with Sirt1 in a partially hormone-dependent manner, as assessed by a GST pulldown assay and coimmunoprecipitation. In addition, 1,25D enhanced the recruitment of Sirt1 to FoxO3a, as judged by the coimmunoprecipitation of Sirt1 with an anti-FoxO3a antibody. In addition, 1,25D enhanced the amount of TSA-resistant HDAC activity that coimmunoprecipitated with the VDR.

Importantly, we also found that knockdown of Sirt1 expres-

sion substantially enhanced FoxO3a phosphorylation, consistent with the previously established link between Sirt1-catalyzed FoxO deacetylation and dephosphorylation (43) and confirming that Sirt1 is a key regulator of FoxO function in SCC25 cells. Notably, FoxO3a phosphorylation was also elevated in cells lacking the VDR. This experiment was performed in the absence of 1,25D, suggesting that the hormone-free VDR associated with FoxO proteins, along with the basal association of Sirt1, leads to partial activation of FoxO protein function in the absence of hormone. It is not clear, however, whether the VDR acts merely as a scaffold under these conditions or whether it regulates the enzymatic activity of associated Sirt1 and PP1. PP1 activity may indeed be hormone regulated, as previous work revealing the ligand-independent association of PP1 with the VDR in CaCo2 cells also showed that 1,25D enhanced associated phosphatase activity (9). While ligand-dependent recruitment of Sirt1 may contribute to the observed 1,25D-stimulated binding of FoxO3a and the VDR to target promoters, we cannot rule out the possibility that association with the VDR may regulate Sirt1 enzymatic activity and substrate specificity.

The functional significance of these interactions was borne out by gene ablation studies. Loss of Sirt1 expression attenuated regulation by 1,25D of FoxO target genes *CCNG2* and *BNIP3* but had little or no effect on regulation of *MMP3*. Knockdown of FoxO3a attenuated regulation by 1,25D of all of the genes under study, whereas the loss of FoxO4 had a more gene-specific effect. In contrast, knockdown of Sirt1, FoxO3a, or FoxO4 had little or no effect on 1,25D-induced *CYP24* expression, and FoxO knockdown had no effect on induction by 1,25D of *luciferase* expression driven from a VDRE-containing minimal promoter, consistent with the idea that the hormone-bound VDR regulates a subset of its target gene via association with FoxO proteins. It will be of interest to compare the (1,25D-regulated) binding patterns of FoxO proteins and the VDR in genome-wide studies to determine the extent of the overlap.

Although long-term treatment with 1,25D gradually enhanced FoxO protein expression, consistent with 1,25D-dependent suppression of the FoxO-modifying E3 ubiquitin ligase $p45^{SKP2}$ (35), the effects on FoxO posttranslational modification and DNA binding observed here occurred with similar kinetics and were relatively rapid (<4 h) and thus largely independent of effects on total FoxO protein levels. The ramifications of these findings are severalfold. First, the induction of FoxO function provides a molecular basis for the chemopreventive properties of vitamin D, as FoxO proteins are well established as tumor suppressors (5, 48, 55). FoxO3a knockdown blocked inhibition of cyclin D2 mRNA and protein expression and cell proliferation by 1,25D, substantiating a role for stimulation of FoxO function in 1,25D-induced cell cycle arrest and consistent with the idea that FoxO proteins mediate regulation by 1,25D of the *CCND2* and *CCNG2* genes.

The control by 1,25D of Sirt1 function is intriguing, as other work has suggested that Sirt1 controls FoxO function selectively, enhancing the regulation of genes implicated in cell cycle arrest but suppressing regulation of proapoptotic genes (12, 15, 44, 57). Our data also suggest that the VDR is a selective regulator of Sirt1 function. P53 is a well characterized substrate of Sirt1, and deacetylation inhibits p53-dependent transactivation (11). However, we found no evidence for 1,25D-induced deacetylation of p53. It will be of interest to investigate further the extent of the regulation of Sirt1 function by the hormone-bound VDR. For example, a recent study showed that Sirt1 activity suppresses intestinal tumorigenesis in a β-catenin-driven model of colon cancer in mice and that Sirt1 deacetylated β-catenin and promoted its nucleocytoplasmic shuttling (19). 1,25D-driven VDR function has been strongly implicated in colon cancer chemoprevention, and the VDR interacts directly with β-catenin and suppresses its activity (45). This raises the question of whether 1,25D stimulates Sirt1-induced β-catenin deacetylation. Similarly, Sirt1 and 1,25D signaling through the VDR inhibit NF-κB function (2, 31), suggesting that 1,25D may enhance NF-κB deacetylation though its interaction with Sirt1.

The breadth of regulation of Sirt1 by the VDR is also of interest to determine because Sirt1 has turned out be a double-edged sword in terms of its relationship with cancer prevention and tumorigenesis (11, 38, 40). For example, Sirt1-null embryos display impaired DNA damage responses and genomic instability and $Sirt^{+/-}$ $p53^{+/-}$ mice spontaneously develop multiple tumors (58). In addition, the above-mentioned study implicated Sirt1 function in suppression of β-catenin-driven colon tumorigenesis in mice (19). However, Sirt1 is overexpressed in a number of tumors and can block cellular senescence and differentiation, and Sirt1 inhibitors have anticancer properties in animal models (38).

The 1,25D-dependent regulation of FoxO proteins also links VDR function to longevity. FoxO protein function has been associated with longevity in a wide variety of organisms, from *Caenorhabditis elegans* to mammals (13, 53). Moreover, recent genome-wide association studies have linked variants in the human FoxO3a gene to extreme longevity in humans of Japanese and German ancestry (20, 62). While the VDR is not considered a longevity factor *per se*, numerous studies have documented a role for vitamin D sufficiency in cancer chemoprevention (16). Moreover, a meta-analysis of randomized controlled trials taking into account death from all causes revealed an association between vitamin D supplementation and longevity in humans (6, 22). 1,25D-dependent control of FoxO proteins may thus contribute to human longevity.

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REFERENCES

1. **Accili, D., and K. C. Arden.** 2004. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell **117:**421–426.
2. **Adorini, L.** 2005. Intervention in autoimmunity: the potential of vitamin D receptor agonists. Cell. Immunol. **233:**115–124.
3. **Akutsu, N., R. Lin, Y. Bastien, A. Bestawros, D. J. Enepekides, M. J. Black, and J. H. White.** 2001. Regulation of gene expression by 1{{alpha}},25-dihydroxyvitamin D3 and its analog EB1089 under growth-inhibitory conditions in squamous carcinoma cells. Mol. Endocrinol. **15:**1127–1139.
4. **An, B.-S., D. M. Selva, G. L. Hammond, A. Rivero-Muller, N. Rahman, and P. C. K. Leung.** 2006. Steroid receptor coactivator-3 is required for progesterone receptor trans-activation of target genes in response to gonadotropin-releasing hormone treatment of pituitary cells. J. Biol. Chem. **281:**20817–20824.

5. **Arden, K. C.** 2007. FoxOs in tumor suppression and stem cell maintenance. Cell **128:**235–237.
6. **Autier, P., and S. Gandini.** 2007. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. Arch. Intern. Med. **167:**1730–1737.
7. **Barreyro, F. J., S. Kobayashi, S. F. Bronk, N. W. Werneburg, H. Malhi, and G. J. Gores.** 2007. Transcriptional regulation of Bim by FoxO3A mediates hepatocyte lipoapoptosis. J. Biol. Chem. **282:**27141–27154.
8. **Beher, D., J. Wu, S. Cumine, K. W. Kim, S. C. Lu, L. Atangan, and M. Wang.** 2009. Resveratrol is not a direct activator of SIRT1 enzyme activity. Chem. Biol. Drug Des. **74:**619–624.
9. **Bettoun, D. J., D. W. Buck II, J. Lu, B. Khalifa, W. W. Chin, and S. Nagpal.** 2002. A vitamin D receptor-Ser/Thr phosphatase-p70 S6 kinase complex and modulation of its enzymatic activities by the ligand. J. Biol. Chem. **277:** 24847–24850.
10. **Bouchard, C., J. Marquardt, A. Bras, R. H. Medema, and M. Eilers.** 2004. Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. EMBO J. **23:**2830–2840.
11. **Brooks, C. L., and W. Gu.** 2009. How does SIRT1 affect metabolism, senescence and cancer? Nat. Rev. Cancer **9:**123–128.
12. **Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell **96:**857–868.
13. **Calnan, D. R., and A. Brunet.** 2008. The FoxO code. Oncogene **27:**2276–2288.
14. **Dace, A., C. Martin-el Yazidi, J. Bonne, R. Planells, and J. Torresani.** 1997. Calcitriol is a positive effector of adipose differentiation in the OB 17 cell line: relationship with the adipogenic action of triiodothyronine. Biochem. Biophys. Res. Commun. **232:**771–776.
15. **Daitoku, H., M. Hatta, H. Matsuzaki, S. Aratani, T. Ohshima, M. Miyagishi, T. Nakajima, and A. Fukamizu.** 2004. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc. Natl. Acad. Sci. U. S. A. **101:**10042–10047.
16. **Deeb, K. K., D. L. Trump, and C. S. Johnson.** 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat. Rev. Cancer **7:**684–700.
17. **Dehan, E., and M. Pagano.** 2005. Skp2, the FoxO1 hunter. Cancer Cell **7:**209–210.
18. **Delpuech, O., B. Griffiths, P. East, A. Essafi, E. W. Lam, B. Burgering, J. Downward, and A. Schulze.** 2007. Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol. Cell. Biol. **27:**4917–4930.
19. **Firestein, R., G. Blander, S. Michan, P. Oberdoerffer, S. Ogino, J. Campbell, A. Bhimavarapu, S. Luikenhuis, R. de Cabo, C. Fuchs, W. C. Hahn, L. P. Guarente, and D. A. Sinclair.** 2008. The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth. PLoS One **3:**e2020.
20. **Flachsbart, F., A. Caliebe, R. Kleindorp, H. Blanche, H. von Eller-Eberstein, S. Nikolaus, S. Schreiber, and A. Nebel.** 2009. Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc. Natl. Acad. Sci. U. S. A. **106:**2700–2705.
21. **Fu, Z., and D. J. Tindall.** 2008. FOXOs, cancer and regulation of apoptosis. Oncogene **27:**2312–2319.
22. **Giovannucci, E.** 2007. Can. vitamin D reduce total mortality? Arch. Intern. Med. **167:**1709–1710.
23. **Giovannucci, E., Y. Liu, E. B. Rimm, B. W. Hollis, C. S. Fuchs, M. J. Stampfer, and W. C. Willett.** 2006. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. J. Natl. Cancer Inst. **98:**451–459.
24. **Gurlek, A., M. R. Pittelkow, and R. Kumar.** 2002. Modulation of growth factor/cytokine synthesis and signaling by 1alpha,25-dihydroxyvitamin D(3): implications in cell growth and differentiation. Endocr. Rev. **23:**763–786.
25. **Ho, K. K., S. S. Myatt, and E. W. Lam.** 2008. Many forks in the path: cycling with FoxO. Oncogene **27:**2300–2311.
26. **Holick, M. F.** 2007. Vitamin D deficiency. N. Engl. J. Med. **357:**266–281.
27. **Hosomi, J., J. Hosoi, E. Abe, T. Suda, and T. Kuroki.** 1983. Regulation of terminal differentiation of cultured mouse epidermal cells by 1 alpha,25-dihydroxyvitamin D3. Endocrinology **113:**1950–1957.
28. **Huang, H., K. M. Regan, F. Wang, D. Wang, D. I. Smith, J. M. van Deursen, and D. J. Tindall.** 2005. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl. Acad. Sci. U. S. A. **102:**1649–1654.
29. **Jacobs, F. M., L. P. van der Heide, P. J. Wijchers, J. P. Burbach, M. F. Hoekman, and M. P. Smidt.** 2003. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J. Biol. Chem. **278:** 35959–35967.
30. **Kim, M. J., K. Ahn, S. H. Park, H. J. Kang, B. G. Jang, S. J. Oh, S. M. Oh, Y. J. Jeong, J. I. Heo, J. G. Suh, S. S. Lim, Y. J. Ko, S. O. Huh, S. C. Kim, J. B. Park, J. Kim, J. I. Kim, S. A. Jo, and J. Y. Lee.** 2009. SIRT1 regulates tyrosine hydroxylase expression and differentiation of neuroblastoma cells via FOXO3a. FEBS Lett. **583:**1183–1188.
31. **Lavu, S., O. Boss, P. J. Elliott, and P. D. Lambert.** 2008. Sirtuins—novel therapeutic targets to treat age-associated diseases. Nat. Rev. Drug Discov. **7:**841–853.
32. **Lee, H. Y., H. J. You, J. Y. Won, S. W. Youn, H. J. Cho, K. W. Park, W. Y. Park, J. S. Seo, Y. B. Park, K. Walsh, B. H. Oh, and H. S. Kim.** 2008. Forkhead factor, FOXO3a, induces apoptosis of endothelial cells through activation of matrix metalloproteinases. Arterioscler. Thromb. Vasc. Biol. **28:**302–308.
33. **Li, P., C. Li, X. Zhao, X. Zhang, S. V. Nicosia, and W. Bai.** 2004. p27Kip1 Stabilization and G1 arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-cullin-F-box protein/Skp2 ubiquitin ligase. J. Biol. Chem. **279:** 25260–25267.
34. **Lin, R., Y. Nagai, R. Sladek, Y. Bastien, J. Ho, K. Petrecca, G. Sotiropoulou, E. P. Diamandis, T. J. Hudson, and J. H. White.** 2002. Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. Mol. Endocrinol. **16:**1243–1256.
35. **Lin, R., T. T. Wang, W. H. Miller, Jr., and J. H. White.** 2003. Inhibition of F-Box protein p45(SKP2) expression and stabilization of cyclin-dependent kinase inhibitor p27(KIP1) in vitamin D analog-treated cancer cells. Endocrinology **144:**749–753.
36. **Lin, R., and J. H. White.** 2004. The pleiotropic actions of vitamin D. Bioessays **26:**21–28.
37. **Liu, M., M. H. Lee, M. Cohen, M. Bommakanti, and L. P. Freedman.** 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev. **10:**142–153.
38. **Liu, T., P. Y. Liu, and G. M. Marshall.** 2009. The critical role of the class III histone deacetylase SIRT1 in cancer. Cancer Res. **69:**1702–1705.
39. **Liu, Z., J. I. Calderon, Z. Zhang, E. M. Sturgis, M. R. Spitz, and Q. Wei.** 2005. Polymorphisms of vitamin D receptor gene protect against the risk of head and neck cancer. Pharmacogenet. Genomics **15:**159–165.
40. **Longo, V. D., and B. K. Kennedy.** 2006. Sirtuins in aging and age-related disease. Cell **126:**257–268.
41. **Mammucari, C., G. Milan, V. Romanello, E. Masiero, R. Rudolf, P. Del Piccolo, S. J. Burden, R. Di Lisi, C. Sandri, J. Zhao, A. L. Goldberg, S. Schiaffino, and M. Sandri.** 2007. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab. **6:**458–471.
42. **Martinez-Gac, L., M. Marques, Z. Garcia, M. R. Campanero, and A. C. Carrera.** 2004. Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. Mol. Cell. Biol. **24:**2181–2189.
43. **Matsuzaki, H., H. Daitoku, M. Hatta, H. Aoyama, K. Yoshimochi, and A. Fukamizu.** 2005. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc. Natl. Acad. Sci. U. S. A. **102:**11278–11283.
44. **Motta, M. C., N. Divecha, M. Lemieux, C. Kamel, D. Chen, W. Gu, Y. Bultsma, M. McBurney, and L. Guarente.** 2004. Mammalian SIRT1 represses forkhead transcription factors. Cell **116:**551–563.
45. **Mulholland, D. J., S. Dedhar, G. A. Coetzee, and C. C. Nelson.** 2005. Interaction of nuclear receptors with the Wnt/{beta}-catenin/Tcf signaling axis: Wnt you like to know? Endocr. Rev. **26:**898–915.
46. **Munker, R., T. Kobayashi, E. Elstner, A. W. Norman, M. Uskokovic, W. Zhang, M. Andreeff, and H. P. Koeffler.** 1996. A new series of vitamin D analogs is highly active for clonal inhibition, differentiation, and induction of WAF1 in myeloid leukemia. Blood **88:**2201–2209.
47. **Pacholec, M., B. A. Chrunyk, D. Cunningham, D. Flynn, D. A. Griffith, M. Griffor, P. Loulakis, B. Pabst, X. Qiu, B. Stockman, V. Thanabal, A. Varghese, J. Ward, J. Withka, and K. Ahn.** 2010. SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J. Biol. Chem. **285:**8340–8351.
48. **Paik, J. H., R. Kollipara, G. Chu, H. Ji, Y. Xiao, Z. Ding, L. Miao, Z. Tothova, J. W. Horner, D. R. Carrasco, S. Jiang, D. G. Gilliland, L. Chin, W. H. Wong, D. H. Castrillon, and R. A. DePinho.** 2007. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell **128:**309–323.
49. **Palmer, H. G., M. Sanchez-Carbayo, P. Ordonez-Moran, M. J. Larriba, C. Cordon-Cardo, and A. Munoz.** 2003. Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells. Cancer Res. **63:**7799–7806.
50. **Park, Y., E. T. Maizels, Z. J. Feiger, H. Alam, C. A. Peters, T. K. Woodruff, T. G. Unterman, E. J. Lee, J. L. Jameson, and M. Hunzicker-Dunn.** 2005. Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. J. Biol. Chem. **280:**9135–9148.
51. **Prudencio, J., N. Akutsu, N. Benlimame, T. Wang, Y. Bastien, R. Lin, M. J. Black, M. A. Alaoui-Jamali, and J. H. White.** 2001. Action of low calcemic 1alpha,25-dihydroxyvitamin D3 analogue EB1089 in head and neck squamous cell carcinoma. J. Natl. Cancer Inst. **93:**745–753.
52. **Ramaswamy, S., N. Nakamura, I. Sansal, L. Bergeron, and W. R. Sellers.** 2002. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell **2:**81–91.

53. **Salminen, A., and K. Kaarniranta.** 2009. Regulation of the aging process by autophagy. Trends Mol. Med. **15:**217–224.
54. **Schmidt, M., S. Fernandez de Mattos, A. van der Horst, R. Klompmaker, G. J. Kops, E. W. Lam, B. M. Burgering, and R. H. Medema.** 2002. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol. Cell. Biol. **22:**7842–7852.
55. **Tothova, Z., R. Kollipara, B. J. Huntly, B. H. Lee, D. H. Castrillon, D. E. Cullen, E. P. McDowell, S. Lazo-Kallanian, I. R. Williams, C. Sears, S. A. Armstrong, E. Passegue, R. A. DePinho, and D. G. Gilliland.** 2007. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell **128:**325–339.
56. **Tran, H., A. Brunet, J. M. Grenier, S. R. Datta, A. J. Fornace, Jr., P. S. DiStefano, L. W. Chiang, and M. E. Greenberg.** 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science **296:**530–534.
57. **van der Horst, A., L. G. Tertoolen, L. M. de Vries-Smits, R. A. Frye, R. H. Medema, and B. M. Burgering.** 2004. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J. Biol. Chem. **279:**28873–28879.
58. **Wang, R. H., K. Sengupta, C. Li, H. S. Kim, L. Cao, C. Xiao, S. Kim, X. Xu, Y. Zheng, B. Chilton, R. Jia, Z. M. Zheng, E. Appella, X. W. Wang, T. Ried, and C. X. Deng.** 2008. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell **14:**312–323.
59. **Wang, T. T., L. E. Tavera-Mendoza, D. Laperriere, E. Libby, N. B. MacLeod, Y. Nagai, V. Bourdeau, A. Konstorum, B. Lallemant, R. Zhang, S. Mader, and J. H. White.** 2005. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol. Endocrinol. **19:**2685–2695.
60. **Wegener, D., F. Wirsching, D. Riester, and A. Schwienhorst.** 2003. A fluorogenic histone deacetylase assay well suited for high-throughput activity screening. Chem. Biol. **10:**61–68.
61. **White, J. H.** 2008. Vitamin D signaling, infectious diseases, and regulation of innate immunity. Infect. Immun. **76:**3837–3843.
62. **Willcox, B. J., T. A. Donlon, Q. He, R. Chen, J. S. Grove, K. Yano, K. H. Masaki, D. C. Willcox, B. Rodriguez, and J. D. Curb.** 2008. FOXO3A genotype is strongly associated with human longevity. Proc. Natl. Acad. Sci. U. S. A. **105:**13987–13992.