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Discovery of novel vitamin D receptor interacting proteins that modulate 1,25-dihydroxyvitamin D₃ signaling

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Abstract

The nuclear vitamin D receptor (VDR) modulates gene transcription in 1,25-dihydroxyvitamin D₃ (1,25D) target tissues such as kidney, intestine, and bone. VDR is also expressed in heart, and 1,25D deficiency may play a role in the acceleration of cardiovascular disease. Employing a yeast two-hybrid system and a human heart library, using both a 1,25D-independent and 1,25D-dependent screen, we discovered six candidate VDR interacting proteins (VIPs). These novel VIPs include CXXC5, FASTK, NR4A1, TPM2, MYL3 and XIRP1. Mammalian two-hybrid assays as well as GST pull-downs were used to confirm VIP-VDR interaction, and the combination of these two assays reveals that CXXC5, XIRP1, FASTK and NR4A1 interactions with VDR may be modulated by 1,25D. The functional effects of these VIPs on 1,25D-mediated gene expression were explored in transcriptional assays employing three separate and distinct 1,25D-responsive element (VDRE)-driven luciferase reporter genes in transfected Caco-2 and HEK-293 cells, and in a C2C12 myoblast line. FASTK and TPM2 activated expression in all cell line and promoter contexts, while CXXC5 and XIRP1 exhibited differing effects depending on the cell line and promoter employed, suggesting promoter and cell-specific effects of these unique VIPs on VDR signaling. Further evaluation of the interaction between CXXC5 and VDR revealed that CXXC5 acts in a dose-dependent manner to stimulate VDR-mediated transcription on select VDREs. Identification of novel heart VIPs and their influence on VDR activity may increase our understanding of how vitamin D impacts cardiac physiology and may facilitate development of VDR/VIP drug analogs to combat heart disease.

Keywords

vitamin D; vitamin D receptor; vitamin D responsive elements; transcription; co-modulators; CXXC5

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1. Introduction

The active hormonal metabolite of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25D), has a broad spectrum of biological actions, which have been extensively reviewed [1,2]. 1,25D stimulates intestinal calcium and phosphate absorption, bone calcium and phosphate resorption, and renal calcium and phosphate reabsorption. These bioactions of 1,25D affect calcium and phosphate homeostasis and ensure proper remodeling of the mineralized skeleton. An obligate mediator of 1,25D action is a transcription factor, the vitamin D receptor (VDR), a member of the nuclear and steroid receptor superfamily. Binding of 1,25D elicits significant conformation changes in VDR that lead to recruitment of its co-receptor, the retinoid X receptor (RXR). A liganded VDR-RXR heterocomplex then binds to short sequences of DNA, termed vitamin D responsive elements (VDREs), which are typically in the vicinity of 1,25D-regulated genes. Once bound to a VDRE, the VDR-RXR duplex induces transcription by recruiting coactivators with histone acetyl transferase activity, such as steroid receptor coactivator-1 (SRC-1), as well as recruiting other components of the RNA polymerase promoter complex.

Transcriptional control mediated by VDR as well as other transcription factors is a highly sensitive and adaptive cellular process modulated by multiple layers of regulation. Transcription is directed by a plethora of transcription factors, receptors, bound ligands, and DNA response elements that work in concert to fine-tune gene expression to the needs of each individual cell and tissue. In order to tailor vitamin D-mediated transcription, VDR is modulated by additional proteins, collectively termed vitamin D receptor interacting proteins (VIPs). VIPs identified thus far range in function from traditional modulators of transcription to other proteins that do not participate directly in nuclear transcription but that possess non-genomic functions. Steroid receptor co-activator (SRC-1), DRIP205/mediator, glucocorticoid receptor interacting protein 1 (GRIP1 or SRC-2), and the activator of thyroid hormone and retinoic acid receptor (ACTR or SRC-3) are recognized regulators of transcription of the p160 class that interact with ligand-bound VDR and modulate VDRE transcription (reviewed in [3]). Additional proteins that interact with VDR and modulate transcription include WSTF [4], Runx2, [5], NCoA-62 [6,7], and RIP140 [8,9]. TRIP/SUG1, a subunit of the proteasome, interacts with VDR to target ligand-bound VDR for proteasome mediated degradation, perhaps to downregulate signaling [10]. Ligand-bound VDR also interacts with a component of the Wnt signaling pathway, β -catenin, to increase VDRE-dependent transcription and decrease TCF/LEF-mediated transcription [11–13]. LEF1 binds directly to the first zinc finger of unliganded VDR [14], perhaps to regulate the Wnt and Hedgehog pathways in the hair cycling pathway. Some VIPs like IGFBP-5 and IGFBP-6 block VDR transcription and 1,25D-VDR mediated differentiation in osteoblasts. IGFBP-5 binds to VDR and blocks VDR:RXR heterodimerization and seems to impair vitamin D induced VDR-mediated transcription and cell cycle arrest [15]. IGFBP-6 binds nuclear VDR, may inhibit the VDR:RXR interaction and inhibits 1,25D induced VDR-mediated transcription [16].

Recently VDR has also been shown to interact with proteins not directly associated with DNA binding and gene regulation. Vitamin D treated cells expressing VDR activate outwardly rectifying chloride channel currents [17,18] and mutational analysis of the VDR indicates that the ligand binding domain is required for this 1,25D effect, suggesting that vitamin D-bound VDR can modulate plasma membrane events [18]. VDR also binds to caveolin-3 and the sarcoplasmic reticulum Ca²⁺-ATPase, Serca-2, in rat cardiomyocytes and when these cells are treated with vitamin D, contraction is less pronounced than cells treated with vehicle [19,20].

The well-established role of the nuclear vitamin D receptor is to modulate gene transcription in its traditional target tissues of kidney, intestine, and bone, but the receptor is also known to be expressed in a variety of tissues including heart [21]. Before the receptor was discovered in heart tissue, the 1,25D ligand was shown to normalize impaired heart contractility that accompanied hypovitaminosis D₃ [22]. These two discoveries suggest a receptor-mediated mechanism of action for vitamin D in the heart. Other studies have documented a role for 1,25D in cardiac physiology. In a clinical observational study, low serum levels of the hormone and its subsequent metabolites were frequent occurrences in patients who had severe congestive heart failure [23]. In hemodialysis patients who were experiencing myocardial hypertrophy, a reduction in left ventricular posterior wall thickness and left ventricle mass index were observed after treatment with intravenous calcitriol [24]. More recent studies have demonstrated that vitamin D concentrations inversely correlate with levels of heart failure, low levels of vitamin D correlate with high levels of myocardial infarction, myofibrils shrink in size after prolonged vitamin D deficiency, and VDR knockout mice have cardiac hypertrophy (reviewed in [25,26]) [27]. Moreover, it has become more evident that there is an increased mortality risk among chronic kidney disease (CKD) patients [28], or in patients that are vitamin D deficient [29]. There is also an increasing appreciation that vitamin D metabolism may likely play a vital role in myocardial and cardiovascular physiology [29]. Treatment with vitamin D or its analogs have resulted in documented improvement in cardiovascular function, especially in CKD and dialysis patients [30]. The bioeffects exerted by vitamin D and its analogs in the cardiovascular system are consistent with the tissue distribution of the vitamin D receptor. The VDR has been reported in both arterial smooth muscle cells, in cardiac myocytes and in cells of the immune system [28]. Vitamin D receptor-knockout as well as CYP27B1 (the 1-hydroxylase enzyme responsible for the synthesis of active 1,25D) KO mice develop heart failure even after calcium levels are normalized [31]. Thus, vitamin D and VDR appear to play a crucial role in normal cardiac physiology, and there is ample circumstantial evidence to implicate VDR and its natural ligand vitamin D in maintenance of healthy heart function.

Therefore, we were interested in probing the heart, a nonclassical vitamin D target tissue, for novel VIPs that may explain the reported effects of vitamin D on the cardiovascular system, and the goal of the current study is to identify comodulator proteins expressed in heart tissue that interact with VDR. These vitamin D interacting proteins (VIPs) could potentially interact with VDR in its traditional genomic pathway or may facilitate other yet unknown functions in a non-genomic pathway. Identification of novel heart VIPs and their impact on VDR transcriptional or cytosolic activity may prove instructive in developing VDR/VIP drug analogs to combat heart disease.

2. Materials and Methods

2.1 Yeast Two-Hybrid Assay

Yeast two-hybrid analysis was performed [32] to identify VDR-interacting proteins. The vitamin D receptor gene was cloned into the pDEST32 bait plasmid (ProQuest Two-Hybrid, Invitrogen, Carlsbad, CA). The library probed was a human heart library (26 year old male cloned into the pPC86 prey vector, Invitrogen, Carlsbad, CA). MaV203 *Saccharomyces cerevisiae* yeast were transformed with the bait plasmid (600 ng) using the EZ Yeast Transformation Kit II (Zymo Research Corporation, Irvine, CA) then subsequently transformed with the prey library (600 ng per plate to be spread) and selected on plates lacking histidine with 50 mM 3-amino-1,2,4-triazole added to reduce background interactions. For vitamin D induced interaction screening, 10⁻⁷ M 1,25D in ethanol was added to the transformation reaction for the last hour of the reaction (while the cells were incubating in solution 3, a proprietary solution of the Zymo Yeast Transformation Kit) as

well as to the selective plates. Transformants were screened for LacZ activity [33]. Prey plasmids were rescued and retransformed to confirm interactions and then sequenced.

2.2 Mammalian Two-Hybrid Assay

C2C12 myoblasts were transfected with components of the Mammalian Two-Hybrid (M2H) system from Stratagene (Santa Clara, CA), employing similar transfection reagents and procedures to those outlined for VDRE-luciferase based assays below. RXR α (positive control) or the indicated candidate VIP was cloned into pCMV-BD (bait), while human VDR was cloned into pCMV-AD (prey). Each well of a 24-well plate was seeded with 40,000 cells and was transfected with 50 ng of bait; 50 ng of prey vector; 20 ng of pRL-Null; and 250 ng of pFR-luc, a firefly luciferase reporter construct containing tandem copies of the DNA-binding site that binds to the GAL4 BD and allowed to incubate for 18–24 hours. The cells were then treated with either ethanol vehicle or 10^{-8} M 1,25D for 24 hours and assayed for luciferase activity as indicated below. Results (performed in triplicate) were expressed relative to the positive control VDR-RXR interaction in the presence of 1,25D (set to 100%). Negative controls employing "empty" AD vectors (without VDR) were also tested. Statistical analysis was performed to determine if the VDR-VIP interaction was statistically different than the empty vector control using an unpaired Student's t-test with unequal variance and setting the significance to $p < 0.05$.

Competition M2H experiments were also carried out in C2C12 cells that were seeded in 24-well plates and transfected as described. Each well received 50 ng of BD-RXR, 50 ng AD-VDR, 20 ng of pRL-Null, and 250 ng of pFR-luc plus treatment with either ethanol vehicle or 10^{-8} M 1,25D for 24 hours. In addition, some wells received the empty mammalian expression vector, pSG5, or pSG5 containing the indicated VIP. The resulting VIP overexpression in the presence of BD-RXR and AD-VDR allowed testing for VIP-mediated disruption of the well-documented RXR-VDR interaction. Experiments were performed in triplicate. Statistical analysis was performed to determine if the VDR-VIP interaction was statistically different with RXR than the empty vector control using an unpaired Student's t-test with unequal variance and setting the significance to $p < 0.05$.

2.3 GST Pulldown Assay

GST-VDR or GST, cloned into pGEX-4T (GE Healthcare, Piscataway, NJ) [13,34], was expressed in *E. coli* cells. Putative VIP genes were cloned into the pSG5 eukaryotic expression vector (Agilent Technologies, Santa Clara, CA) and expressed in a rabbit reticulocyte *in vitro* transcription/translation reaction (TNT coupled Reticulocyte Lysate Kit, Promega Corp, Madison, WI) (IVTT). 1.0 μ g of each VIP plasmid was used in the reaction with [35 S] methionine and HALT protease inhibitor (Thermo Fisher Scientific, Rockford, IL) to generate labeled VIP polypeptide. Glutathione sepharose beads containing bound GST or GST-VDR were incubated with each labeled VIP or α RXR (as a positive control for GST-VDR interaction) in TEZ buffer (10 mM Tris HCl pH 7.6, 1 mM EDTA, 0.3 mM zinc chloride, 5 mM DTT, 10% Tween 20, 140 mM KCl, BSA and HALT Protease inhibitor (Thermo Fisher Scientific, Rockford, IL)) supplemented with 1% ethanol vehicle (control) or 10^{-7} M 1,25D for 90 minutes at 4°C on a rocking platform. The beads were then washed three times by adding 1 ml of TEZ wash buffer with subsequent centrifugation and elimination of the supernatant, and separated via 12% SDS-PAGE. The gel was then fixed and dried prior to autoradiography using Kodak X-OMAT film. The "Input" lane represents 5% of the total amount of IVTT lysate that was mixed with the beads, and is a control to indicate the relative synthesis of each VIP in the reticulocyte *in vitro* transcription/translation reaction.

2.4 Transcriptional Assays

Each cell line (Caco-2, HEK-293, or C2C12) was transfected with a 20 ng Renilla luciferase construct (to control for transfection efficiency), 50 ng pSG5-hVDR expression plasmid [35] and 200 ng pSG5 expression plasmid containing each full length VIP or empty pSG5 (baseline control), and 250 ng reporter plasmid by liposome-mediated transfection (either Lipofectamine LTX and Plus Reagent, Invitrogen, Carlsbad, CA or Express-In Transfection Reagent, Thermo Scientific, Rockford, IL). Reporter plasmids contained the luciferase gene, whose expression is driven by the indicated VDRE. The 24-OHase plasmid contains a 5500 bp fragment of the promoter region from the human 24-hydroxylase (CYP24A1) gene upstream of luciferase; this plasmid contains two antisense DR3, the sequences are AGGTGAN₃AGGGCG and AGTTCAN₃GGTGTG (sense direction) [34]. The XDRE reporter plasmid contains two copies of the anti-sense distal direct repeat of the human CYP3A4 gene; this sequence is GGGTCAGcgGGTGCG [34]. The ROC reporter plasmid has four copies of the rat osteocalcin VDRE upstream of the luciferase gene; this VDRE sequence is GGGTGAatgAGGAGA [34]. Depending on the cell line, 50,000 to 90,000 cells in one ml volume were plated into a 24-well plate followed by liposome-mediated transfection. Cells were then treated (6–18 hours after transfection) with 1 ml of Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Rockford, IL) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100mg/ml streptomycin, and either 10⁻⁸ M 1,25D or ethanol vehicle (as a control). Cells were incubated for 24h at 37°C and then were assayed for luciferase using a DLR Kit (Promega, Madison, WI) following the manufacturer's instructions. The cells were lysed with 150 µl of passive lysis buffer, incubated at 37°C for 30 min with shaking at 225 rpm. Reagents of the DLR kits were added and measurements were taken in turn of firefly luciferase and Renilla activities using a Sirius tube Luminometer (Berthold, Pforzheim, Germany). We calculated the ratio of the firefly to Renilla activity to normalize for transfection activity; data were normalized to pSG5 empty vector activity (pSG5 vector = 100%); and this activity is reported in Figures 3–5. Experiments were performed in triplicate. Statistical analysis was performed to determine if the VDR-VIP was able to change the transcription of the luciferase reporter gene as compared to the empty vector control using an unpaired Student's t-test with unequal variance and setting the significance to $p < 0.05$.

2.5 Preparation of Total Cell Lysates

Human colorectal carcinoma cells (HCT-116) were cultured in DMEM-high glucose (Hyclone, Thermo Fisher Scientific, Rockford, IL), supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were either transfected with the indicated plasmids, or empty vector, and incubated with 1,25D ligand or ethanol vehicle for 24 hours. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS), and total cell lysates were prepared in ice-cold lysis buffer (20 mM HEPES-KOH pH7.5, 150 mM NaCl, 1% Triton-X 100 and protease inhibitor cocktail (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). The cells were incubated 10 minutes to initiate lysis, vortexed, and centrifuged at 12,000 g at 4°C for 30 minutes. The supernatants were harvested as total cell lysates. The protein concentration was determined using the BCA protein assay reagent kit (Thermo Fisher Scientific, Rockford, IL).

2.6 Western Blotting

Cell lysates were heated at 95°C in SDS sample buffer in the presence of 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred by electrophoresis to Amersham Hybond-P polyvinylidene difluoride transfer membranes (GE Healthcare, Wauwatosa, WI) and then treated with diluted antibodies as follows: CXXC5 (H-6) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:300), VDR (D-6) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)

(1:3,000), or VDR (9A7) [36] (1:30,000) antibodies. Horseradish peroxidase-conjugated anti-rabbit, rat or mouse IgG was utilized as the secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and signals were detected using SuperSignal (Pierce Biotechnology, Rockford, IL).

2.7 Co-Immunoprecipitation

Transfections of cell used for co-immunoprecipitation (CoIP) experiments were performed in HCT-116 cells (100 mm dishes) using Express-In transfection reagent (Thermo Fisher Scientific, Lafayette, CO). Total cell lysates were harvested (as described above) from the cells co-transfected with 50 ng each of VDR/CXXC5 or VDR/RXR and treated with ethanol or 1,25D. Proteins (500 µg) were incubated with 2 µg each of the appropriate antibody overnight at 4°C. Antibodies are as described above; RXR D-20 antibody was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitates were collected using Protein A/G-Agarose (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Protein/antibody complexes bound to the agaroses were eluted by boiling with SDS sample buffer in the presence of 2-mercaptoethanol. Proteins were then separated by SDS-PAGE. The CXXC5 or VDR proteins were subsequently detected by western blotting analysis.

2.8 Gene Knockdown Analysis

To probe the functional effects of select VIPs on VDR activity, genetic knockdown using siRNA was performed in HCT-116 cells. The cells were plated at 80,000 cells per well in 24-well plates and co-transfected with an XDR3-Luciferase reporter gene (VDRE from human CYP3A4 distal vitamin D responsive element), a pSG5-hVDR expression vector, a Renilla control plasmid, a pSG5-empty or pSG5-CXXC5 expression plasmid, and -/+CXXC5 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A liposome-mediated transfection was employed according to the manufacturer's protocol using 2 µL/well of Express-In transfection reagent (Thermo Fisher Scientific, Rockford, IL) and allowed to incubate for 24 hours. The cells were then treated with ethanol or 10^{-8} M 1,25D and incubated for 24 hours. The amount of VDR-mediated activity was measured by luciferase output utilizing a dual-luciferase reporter assay system according to the manufacturer's protocol (Promega, Madison, WI) in a Sirius FB12 luminometer (Berthold Detection Systems, Zylux Corporation, Huntsville, AL). Results are expressed as the ratio of Firefly/Renilla relative light units $\times$ 1000. Statistical significance was determined by a two-tailed Student's unpaired t-test (unequal variance) for comparison between two results or ANOVA analysis for multiple result analysis.

3. Results

3.1 Yeast Two-Hybrid Screen

We were interested in identifying novel vitamin D receptor interacting proteins (VIPs) in unorthodox target tissues. Recently heart has been shown to be a nonclassical target of vitamin D (reviewed in [26]). Thus we set out to identify VIPs through a yeast two-hybrid screening [32] using a VDR bait and a human heart prey library. Within the context of the yeast system, we identified four potential VIPs that interacted with VDR in the absence of vitamin D, TPM2, FASTK, NR4A1, and CXXC5, and two that interacted with VDR in the presence of vitamin D, MYL3 and XIRP1 (Table 1). We also isolated previously known VIPs, including RXR and NCoA-62 (data not shown), which serve to demonstrate the validity of our yeast screen.

3.2 Mammalian Two-Hybrid Analysis of Putative VIPs

Although a yeast two-hybrid screen is an excellent method for identifying potential interacting proteins, the yeast nucleus and the mammalian nucleus have distinct differences and all yeast two-hybrid interactions should be confirmed in additional systems. To determine if these putative VIPs were indeed true interactors with VDR and not spurious results, we undertook a mammalian two-hybrid assay, to determine specificity in cells other than *Saccharomyces cerevisiae* cells.

C2C12 mouse myoblast cells were transfected with a bait and a prey plasmid, as indicated in Figure 1, and luciferase activity, indicating bait and prey interaction, was assayed as described. Control plasmids (bait or prey, indicated as empty, in the figure) were employed to detect background binding and luciferase activity. The well-established interaction of VDR with RXR was used as a bona fide positive control. In the mammalian two-hybrid assay, several putative VIPs interact with VDR, as indicated by the increase in luciferase activity above that observed in the negative control assay of empty “bait” plasmid with VIP-prey plasmid (Figure 1A). FASTK interacts with VDR in a vitamin D-dependent manner, as the luciferase activity is significantly higher ($P = 0.003$, one tailed Student's t-test, heteroscedastic distribution) in the transfected cell line treated with 1,25D versus the ethanol vehicle control. NR4A1 interacts with the unliganded VDR in this assay ($P = 0.001$), and addition of 1,25D decreases the luciferase activity (Figure 1A); however, the interaction of NR4A1 with liganded VDR is still significant ($P = 0.02$). CXXC5 and XIRP also interact with VDR in a vitamin D-dependent manner in this assay ($P < 0.001$ for each), although the 1,25D-mediated increase in VDR interaction is less than that observed with FASTK.

We also employed a mammalian two-hybrid competition assay to determine interactions between putative VIPs and VDR. In this assay (Figure 1B), a known interacting pair, VDR and RXR α are expressed in a two-hybrid system. These two proteins interact and are able to stimulate luciferase activity. Then each putative VIP in turn is expressed at high levels to assess whether the indicated VIP can compete (i.e., block) the interaction between AD-VDR and BD-RXR α with a resultant decrease in luciferase activity, indicating interaction between VDR and the VIP. Although this assay has limitations since any VIP that does not interact at the interface of the VDR-RXR heterodimer (or influences its stability) will have no effect on the RXR-VDR heterocomplex, the assay can nonetheless help reveal the nature of the VIP-VDR interaction. Figure 1B illustrates the results of this “competition” assay. As a positive control, RXR was expressed (pSG5-RXR), and was indeed able to inhibit the two-hybrid interaction between AD-VDR and BD-RXR, compared to the pSG5 empty vector control without RXR (Figure 1B, compare first and second set of bars on left; $P < 0.001$, one-tailed Student's t-test, unequal variance). In this assay, FASTK was able to inhibit the interaction of VDR-RXR ($P < 0.001$), indicating that FASTK likely interacts with VDR at the VDR-RXR interface. Interestingly, CXXC5 consistently appeared to increase the interaction between AD-VDR and BD-RXR ($P = 0.01$). A negative result in this assay does not disagree with other experimental assay systems that demonstrate a VIP-VDR interaction, as the VIP interaction surfaces could most certainly be on other parts of the VDR molecule that do not affect the RXR-VDR interface.

3.3 *In vitro* Interaction Assays

To support the yeast and mammalian two-hybrid results, “GST-pulldown” assays were performed to demonstrate *in vitro* binding. GST or GST-VDR was used to probe interactions between VIPs expressed in an *in vitro* transcription translation system. Each VIP was expressed in an *in vitro* transcription translation assay incorporating [35 S] methionine for tracking (left of each panel in Figure 2). GST, as a negative control, or GST-VDR was linked to glutathione sepharose beads and then incubated with each radiolabeled

VIP either without (–1,25D) or with 1,25-dihydroxyvitamin D (+1,25D) and then the complex was analyzed using SDS-PAGE and autoradiography. We controlled for nonspecific GST interaction by utilizing GST alone to try to isolate the VIP (GST lane). As a positive control, we used a known VDR-interacting protein, RXR, to demonstrate the validity of our assay. Additionally, RXR binds to VDR more effectively in the presence of 1,25D [34], and this assay effectively demonstrates 1,25D-dependent binding, as observed in the case of RXR (Figure 2, panel A). To control for differences in transcription and translation in the assay, 5% of each *in vitro* transcription/translation assay was separated using SDS-PAGE to demonstrate protein synthesis. CXXC5, MYL3, and XIRP1 interact with VDR regardless of the presence or absence of 1,25D (panels B, F, and G), as both the –1,25D and +1,25D lanes demonstrate equivalent amounts of protein. Alternatively, FASTK (panel C) and NR4A1 (panel D) interact with VDR in a 1,25D-regulated manner. FASTK binding to VDR is enhanced in the presence of vitamin D, as the +1,25D lane demonstrates more protein bound to VDR (panel C). NR4A1 binds to VDR in the absence of vitamin D (panel D), and 1,25D apparently inhibits this association because less of the protein is observed in the +1,25D lane. TPM2 association with VDR is weak (panel E) in this system.

The results described in Figures 1A (mammalian two-hybrid) and 2 (in vitro GST assays) reveal unique patterns of interaction between VDR and each VIP, and we have summarized these in Table 2. This difference between *in vivo* and *in vitro* interactions is to be expected as the *in vitro* IVVT assay does not contain all of the cellular proteins that may be influencing the interaction of VDR and each VIP. The GST-pulldown assays serve as a potential confirmation of the yeast- and mammalian- two hybrid interactions but have to also be considered within the context of an *in vitro* system. For example in Figure 2, RXR, which is known to interact with VDR in a 1,25D-dependent manner [3], interacts with unliganded VDR to a minor extent. These observed differences in VDR-VIP interactions, outlined in Table 2, represent the diversity in the proteins and small molecules present in each system; thus the data in Figure 1 may be more representative of a more natural system than the data in Figure 2, although in many cases the two systems reinforce each other.

3.4 VIP-Mediated Transcriptional Assays

To probe further the interactions of each VIP and VDR to determine the nature (genomic or nongenomic) of the interactions, we undertook transcriptional assays in which VDR-mediated transcription was assessed in conjunction with expression of each VIP. Furthermore, in order to assess if there are tissue-specific differences in the functional activity of each VIP, we measured VDR transcriptional activity in the presence of each VIP in three different cell types, colon (Caco-2), kidney (HEK-293), and myoblasts (C2C12). Additionally, to discern any VDRE-specific differences between the VIPs, we utilized three distinct VDRE-reporter constructs. One reporter construct utilizes a VDRE in the natural context of 5500 bp of promoter region from the human cytochrome P450 24-hydroxylase gene (24-OHase). The other two VDREs utilize a classical direct repeat-type (DR3) element from the human CYP3A4 gene promoter (XDR3) and from the rat osteocalcin gene (ROC) [3].

We were interested in testing transactivation in colon (Caco-2) cells, as the intestine is a classical target tissue for vitamin D [3]. Caco-2 cells were co-transfected with VDR and either expression plasmid alone (pSG5) or expression plasmid containing the indicated VIP insert, along with a Renilla plasmid to control for transfection affinity. VDR-mediated transcription with empty pSG5 plasmid was set at 100% and each VIP assay was expressed relative to this baseline. Transcription in Caco-2 cells mediated by the 24-OHase VDRE demonstrates that TPM2 and XIRP1 are able to potentiate VDR-mediated transcription as revealed by greater transcription than VDR alone, all in a 1,25D-dependent manner (Figure 3A). TPM2 stimulated vitamin D dependent transcription about 2-fold utilizing the 24-

OHase VDRE in Caco-2 cells, the highest levels observed under these conditions. Interestingly, we obtained diverse results when we probed VIP-mediated VDR transcription on additional VDREs. Cells expressing CXXC5 were able to stimulate VDR- and XDR3-dependent transcription an additional 12-fold, and ROC dependent transcription almost 5-fold (Figure 3B and C), although CXXC5 acted as a repressor on the 24-OHase construct. XIRP1 acted as a repressor of VDR-mediated transcription on the XDR3 VDRE, while FASTK and TPM2 acted to stimulate transcription mediated via VDR utilizing the ROC construct.

We wanted to compare and contrast VIP-mediated expression in additional tissues, and we chose human embryonic kidney (HEK-293). In these cells, FASTK stimulated VDR-dependent 1,25D-stimulated transcription on the 24-OHase VDRE above empty vector, although less than two-fold (Figure 4A). Interestingly, on this promoter, the presence of CXXC5 or NR4A1 actually inhibited transcription of 1,25D/VDR to about 30% or 55% (respectively) of VDR alone. In contrast, utilizing the additional VDRE constructs, cells expressing CXXC5 were able to significantly stimulate 1,25D/VDR transcription above VDR alone on both the XDR3 and ROC-driven luciferase constructs (Figure 4B and C). In embryonic kidney cells, when assaying transcription on the XDR3 construct, both TPM2 and XIRP1 are able to stimulate VDR-mediated transcription over baseline in a vitamin D-dependent manner (Figure 4B). Using an ROC-driven construct, the presence of FASTK and TPM2 was able to stimulate VDR-dependent transcription approximately 1.75-fold and 2.5-fold, respectively. Cells expressing NR4A1 demonstrated lower activity with 1,25D treatment than VDR alone on the 24-OHase and ROC VDRE constructs in this cell line (Figure 4A and C).

To probe association and activation in muscle cells, we used the mouse myoblast cell line, C2C12. Cells expressing FASTK had less than two-fold increases in VDR-mediated vitamin D-dependent transcription with the P450 and the XDR3 promoter (Figure 5A and B) and 2-fold stimulation on the ROC VDRE (Figure 5C). Interestingly, FASTK expression also significantly increased transcription on all VDRE constructs in cells not treated with 1,25D (Figure 5A, B, and C). CXXC5 inhibits transcription on the 24-OHase VDRE and ROC VDRE (Figures 5A and C) and stimulates 1,25D/VDR-dependent transactivation on the XDR3 VDRE in the myoblast cell line (Figure 5B). NR4A1 consistently acts as a 1,25D-VDR inhibitor of transcription on all VDREs tested in the myoblast cell line (Figures 5A, B, and C). Finally, XIRP1 acted as an inhibitor of 1,25D-mediated VDR expression on the hydroxylase and XDR3 VDREs.

In conclusion, we probed three tissue types expressing luciferase driven by three different promoter configurations to determine VDRE- and tissue-specific transcriptional profiles of the novel VIPs. The effects of the VIPs on 1,25D/VDR-mediated transactivation in different cellular contexts and utilizing distinct VDREs are summarized in Figure 6. Interestingly, in all configurations probed save for the ROC construct in myoblast cells, MYL3 did not have an effect on transcription. CXXC5 acted as an activator on XDR3 and ROC in Caco-2 and HEK-293 cells, and as an inhibitor on 24-OHase in Caco-2, HEK-293, and C2C12 cells. CXXC5 acted as an inhibitor on the ROC VDRE in C2C12 cells. FASTK acted as an activator on all three VDREs tested, but fold-activation differed depending on tissue type. NR4A1 acted as a repressor on all VDREs, independent of tissue type. TPM2 acted as an activator on the 24-OHase and ROC VDREs in Caco-2 cells and on XDR3 and ROC VDREs in HEK. XIRP1 was most interesting, as it acted in an opposing fashion depending on the tissue type and VDRE tested. XIRP1 performed as an activator on the 24-OHase VDRE in Caco-2 and as a repressor in C2C12 cells on that same VDRE. On the XDR3 VDRE, XIRP1 acted as a weak activator in HEK-293 cells and as a repressor in Caco-2 and

C2C12 cells. Finally, the XIRP1 VIP did not affect the VDR-mediated transcription on the ROC VDRE.

Given the striking promoter-dependent repression or enhancement of 1,25D/VDR-mediated transcription by the CXXC5, we were especially interested in further probing the interaction of this novel VIP with VDR. To demonstrate the validity of our two hybrid and *in vitro* approach, we probed the *in vivo* interactions of VDR and CXXC5. We used co-immunoprecipitation followed by western blotting of transfected HCT-116 cells to determine if VDR and CXXC5 interact in the context of a cellular coimmunoprecipitation. We immunoprecipitated with either a CXXC5 or a VDR antibody (Figure 7A) and then probed the immunoprecipitates with the alternative antibody using western blotting, using the bona fide VDR-RXR α interaction as a positive control (Figure 7B). Using co-immunoprecipitations, the ligand-independent VDR-CXXC5 interaction we observed *in vitro* (Figure 2) was again observed, demonstrating this to be a genuine interaction. We further wished to determine if the transactivation results seen in Figures 3–5 are indeed the result of CXXC5. We performed a CXXC5 expression dose-response experiment, to determine if increasing amounts of CXXC5 plasmid during transfection lead to increased CXXC5 expression and a concomitant increase in VDR transactivation. We demonstrated increasing CXXC5 expression with higher concentrations of plasmid added during transfection (Figure 8A). This increased CXXC5 expression does in fact lead to higher levels of VDR-mediated transactivation of a reporter gene expressed from the XDR3 promoter (Figure 8B), indicating that the transactivation observed in Figures 3–5 is due to CXXC5 expression in the assay.

Finally, we were interested in assessing the CXXC5-mediated transcriptional activation after CXXC5 knockdown with siRNA. We utilized increasing amounts of siRNA (Figure 9) to assess CXXC5 expression via western blot (Figure 9A) to demonstrate effectiveness of the siRNA methodology. After determining that the siRNA treatment did decrease CXXC5 expression, we utilized several experimental protocols to assess the effect of CXXC5 knockdown on 1,25D-dependent VDR-mediated transactivation. We transfected either empty vector or CXXC5 overexpression plasmid and assessed 1,25D/VDR transcription with or without the siRNA vector (Figure 9B). In mock transfected cells, the siRNA was able to significantly decrease the VDR-mediated transcription. In CXXC5 overexpression, the siRNA decreased the transcription, but not significantly ($P=0.08$), perhaps because there was excess CXXC5 mRNA compared to the siRNA as might be expected with a potent expression vector. Importantly however, in cells not transfected with CXXC5 or mock vector (Figure 9C), when we knocked down only the endogenous CXXC5, we observed a significant result. Increasing amounts of CXXC5 siRNA vector caused a direct and significant attenuation of 1,25D/VDR-mediated transcription of the reporter gene. The loss of CXXC5 in the HCT-116 cell line decreased 1,25D-mediated transcription in a dose-dependent manner, indicating that CXXC5 is an authentic VIP, and that diminution of endogenous CXXC5 levels continues to produce a functional effect on VDR activity.

4. Discussion

There are a number of mechanisms that could account for the effects of vitamin D on the cardiovascular system. One potential mechanism involves the intricate balance of immune cells and their cytokine modulators, both of which are influenced by treatment with vitamin D. This could also result in modulation of anti-inflammatory mediators that are known to be regulated by VDR [28,37]. Ultimately, improvement in endothelial function, as well as reduced vascular calcification may be the result of optimal levels of circulating vitamin D and its metabolites. Other possibilities include the observed anti-hypertrophic actions of

vitamin D, as well as modulation in cardiac contractility and suppression of the renin-angiotensin system [29,31].

In evaluating the potential molecular mechanisms that might contribute to the cardioprotective effects of vitamin D, it is also important to consider the cellular phenotype and the network of genes that may be regulated by VDR in cardiovascular target sites. For example, cardiomyocytes cultured in the presence of 1,25D display inhibited cell proliferation, enhanced cardiomyocyte formation, diminished apoptosis, and an attenuation in the expression of genes related to the regulation of the cell cycle, such as cyclins A1, C, and E and cyclin-dependent kinases Cdk2 and Cdk4 [38]. In addition to studying genes regulated by 1,25D, another approach that has been used in other vitamin D target cells, but not in cardiac tissue, is to identify VDR interacting proteins (VIPs) that might modulate vitamin D signaling. Thus, in the current study we employed a two-hybrid screening methodology to identify putative VIPs using a human heart library. We identified six known proteins as novel VIPs: CXXC5, FASTK, NR4A1, TPM2, MYL3, and XIRP1. These vary a great deal in their cellular functions and thus add significantly to the catalog of VIPs now known. CXXC5 (also known as the retinoid-inducible nuclear factor) was first identified as a novel retinoid responsive gene that was implicated in myeloid differentiation [39] and later correlated with poor prognosis in solid tumors [40] as well as being implicated as an inhibitor of Wnt signaling [41,42] and is required for p53 signaling after DNA damage [43]. FASTK (Fas-activated serine threonine kinase) is activated in response to Fas proapoptotic signaling and phosphorylates TIA1, an RNA binding protein that regulates alternative splicing of Fas [44,45]. NR4A1 (also called nerve growth factor IB or Nur77) is a member of the Nur nuclear receptor family [46] and has been shown to mediate inflammation [47] and play a role in both pro- and anti-apoptotic pathways depending on its partners and subcellular distribution [48]. TPM2 is tropomyosin beta chain, a subunit of tropomyosin, an actin binding protein [49]. MYL3, myosin light chain 3, is a subunit of myosin, a protein that contracts muscle [50]. Finally XIRP1 (CMYA1) protects actin filaments from depolymerization [51] and mice carrying null mutants of this polypeptide demonstrate cardiomyopathy [52].

Interestingly, most of the novel VIPs we identified in this study do not function as traditional transcription factors. CXXC5 interacts significantly with VDR in our system: *in vitro* in a 1,25D-independent manner (Figure 2), in the mammalian two-hybrid assay mediated by 1,25D (Figure 1A), and ligand-independently when probed via coimmunoprecipitation (Figure 7). We also demonstrate CXXC5 dose-dependent effects on VDR transactivation in HCT-116 cells (Figure 8 and 9) employing both an overexpression system (Figure 8) and in siRNA knockdown of endogenous CXXC5 (Figure 9). CXXC5 is an inhibitor of the Wnt pathway [41,42]. VDR is also a Wnt effector that inhibits Wnt signaling by binding β -catenin and restricting its nuclear localization [11,13], especially when bound to the 1,25D ligand. The observation that both VDR and CXXC5 can independently inhibit the Wnt pathway is intriguing, especially because CXXC5 also is able to potentiate VDR-mediated transcription in some instances. This suggests that the cooperative effect of CXXC5 and VDR may lead to a synergistic inhibition of Wnt/ β -catenin signaling. Preliminary experiments (data not shown) indicate that VDR and CXXC5 appear to synergize to inhibit β -catenin. Future experiments will continue to determine the nature of this interaction. Such a mechanism for Wnt inhibition may be significant not only for suppression of colon cancer progression, but also in β -catenin mediated adaptive cardiac remodeling and homeostasis as recently reported in a transgenic mouse model system [53].

Another VIP that interacts in both the *in vitro* pulldown assays, and the mammalian two-hybrid assay is FASTK, a pro-apoptotic serine-threonine protein kinase that is stimulated in response to Fas signaling to induce apoptosis [44]. Vitamin D has been implicated in

controlling cancer by inducing VDR-mediated apoptosis in tumor tissue (for example [54,55]). Perhaps VDR is interacting directly with FASTK to induce apoptosis in these cancerous tissues. In our assays, liganded VDR interacts with the FASTK, lending support to this idea. Moreover, in all tissues tested (on a subset of the VDREs), FASTK also acts as a potentiator of VDR-mediated transcription, perhaps by phosphorylating VDR and further stimulating receptor activity.

NR4A1 is a nuclear transcription factor induced in tumor cell lines, which will potentiate tumor migration and growth, and NR4A1 expression is induced by β -catenin [56]. NR4A1 interacts with unliganded VDR (Figure 1A and Figure 2) but acts as an inhibitor of vitamin D-mediated VDR mediated transcription in our assays (Figures 3, 4, and 5). Perhaps NR4A1 is acting directly to suppress VDR in the absence of ligand to promote tumor growth, and in the presence of 1,25D is titrating away an important factor for VDR-mediated transcription. NR4A1 homodimerizes and also heterodimerizes with RXR [48] suggesting the possibility that NR4A1 competes with VDR for RXR binding, thus inhibiting VDR transcription in our assays (Figures 3, 4, and 5).

Other VIPs we identified that do not appear to function in nuclear signal transduction directly are TPM2, MYL3, and XIRP1. Recent work implicates VDR in a nongenomic manner in modulation of contraction of heart muscle [19,20], and 1,25D decreases sarcomere shortening in a dose-dependent manner. Perhaps TPM2 (tropomyosin subunit that binds actin) and XIRP1 (an actin capping protein) work together with actin and MYL3 (myosin light chain), to contract heart muscle, and 1,25D-VDR can modulate this interaction directly in the cytosol to decrease sarcomere shortening. Thus, the VIP-VDR interactions we have elucidated are suggestive of a novel nongenomic activity of VDR to modulate heart muscle contraction.

We identified an additional VIP in the yeast two-hybrid assay, ORAI3 [57], a calcium release-activated calcium modulator, but this interaction could not be confirmed due to lack of expression in the additional assays, possibly due to the fact that ORAI3 is an integral membrane protein. Nonetheless, previous work has implicated nongenomic effects of VDR as the receptor binds to ion channels to potentially modulate their activity [17,18]. Although the interaction between VDR and ORAI3 is speculative at this point, other studies have revealed that VDR interacts and regulates channels [17,18], and perhaps VDR is binding and regulating calcium channels in heart to regulate muscles contraction.

Previous investigations with VDR and VIPs in the context of cardiac function are limited. However, a few studies hint at a potential role for VDR-specific interactions with cardiac/myocyte-expressed proteins that may mediate important actions of vitamin D in cardiac physiology. For example, Zhao et al. recently reported that the VDR localizes to the t-tubule and sarcolemma in rat cardiomyocytes [20]. Moreover, VDR co-immunoprecipitates and localizes with the membrane protein caveolin-3, and 1,25D modulates the contraction of isolated cardiomyocytes and affects the interaction of caveolin-3 and the VDR. Another study [58] reported that in adult cardiomyocytes VDR is located in the t-tubular structure, and a portion of VDR translocates to the nucleus after treatment of cardiomyocytes with 1,25D. Ablation of VDR in mice resulted in chronic changes in contractile kinetics and 1,25D also had rapid effects on myocyte contraction that were absent in VDR-KO myocytes. Interestingly, several proteins that contain a Jumonji-like domain have been implicated as important mediators of cardiomyocyte development [59–61], and two of these Jumonji-family proteins, namely HR and JARID1A, have now been shown to interact physically with VDR [62], and are thus classic VIPs. Finally, another known VIP that functions as a VDR corepressor, NCoR1, has been shown to function as a physiological modulator of muscle

mass and oxidative function, thus suggesting that NCoR1 plays an adaptive role in muscle physiology [63].

As summarized in Figure 6, the differing VIPs have unique effects on the transcriptional profile of the luciferase reporter gene, differing often by tissue type or VDRE. We speculate that these differences are due to the diverse transcriptional enhancer and repressor proteins that are expressed in differing tissue types as well as the various cis-acting factors we are employing in the transcriptional assays. For example, in Figure 3, CXXC5 acts as a repressor on the 24-OHase VDRE but as an enhancer on the XDR3 and ROC DNA constructs, all in the Caco2 cell line. Thus in this case, the trans-acting factors are the same, but the cis-acting elements differ substantially, indicating that each VDRE must bind its own specific milieu of additional factors. Furthermore, CXXC5 acts as a repressor in HEK293 and C2C12 cells as well on the 24-OHase VDRE, indicating that the trans-acting factors that bind to this promoter are repressed in the presence of CXXC5. It will be interesting in the future to try and tease out the CXXC5/VDR interacting proteins that modulate these transcriptional activities that are apparently opposed depending on VDRE type, as well as the synergistic inhibition of β -catenin activity by CXXC5 and VDR.

In summary, we have discovered several novel VDR interacting proteins from a human heart library that add to the growing list of VIPs, and we demonstrated that each VIP interacts with VDR using mammalian two-hybrid and GST-pulldown technology. We also evaluated each VIP in VDR transcriptional assays to assess any potential impact on the genomic actions of VDR. Some of the VIPs appear to display a novel profile of stimulation or repression of VDR-mediated transcription, depending on the tissue type and VDRE employed, while others do not impact VDR genomic activity. Thus, future work will evaluate the functional effects of each VIP on VDR signaling under limiting and excess levels of 1,25D that would mimic hypo/hyper vitaminosis D. Dose-response experiments with increasing amounts of VIPs will also be performed to probe the role of VIP under/over expression. The molecular basis of FASTK enhancement and NR4A1 repression of 1,25D-stimulated transcription will also require further study. Although we have not yet elucidated the exact mechanism(s) of action attributed to these novel VIP interactions, it is clear from the nature of the VIPs we have identified that both genomic and nongenomic activity of VDR may play a role in regulation of heart physiology.

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Highlights

- VDR interacting proteins (VIPs) bind to VDR to yield genomic and nongenomic effects
- We identified new VIPs via a yeast two-hybrid screen of a human heart library
- These novel VIPs include CXXC5, FASTK, NR4A1, TPM2, MYL3 and XIRP1
- We demonstrated the activity and interaction of the VIPs with VDR *in vivo*
- CXXC5 is a potent and dose-dependent activator of VDR/VDRE-mediated transcription

A. **B.** **Figure 1.**

Mammalian two-hybrid assay analysis of VDR and VIPs. A. C2C12 cells were transfected with the indicated plasmid and luciferase activity was assayed in the presence (+1,25D) or absence (–1,25D) of 10^{-8} M 1,25-dihydroxyvitamin D. Asterisks indicate significance at $p < 0.05$ (unpaired Student's t test, unequal variance, compared to empty plasmid control treated with or without 1,25-D). B. C2C12 cells were transfected with the indicated VIP-expressing plasmid along with plasmids AD-VDR and BD-RXR, and luciferase activity was assayed in the presence (+1,25D) or absence (–1,25D) of 10^{-8} M 1,25-dihydroxyvitamin D. Asterisks indicate significance at $p < 0.05$ (unpaired Student's t test, unequal variance, compared to empty plasmid control treated with or without 1,25D).

Figure 2.

GST-pulldown assay of VDR and VIPs. Each VIP was expressed in an *in vitro* transcription/translation (IVTT) assay and incubated with either GST alone or GST-VDR with (+1,25D) or without (–1,25D) 10^{-7} M 1,25-dihydroxyvitamin D. Complexes were isolated on glutathione beads and resolved by SDS-PAGE. The input lane represents 5% of each IVTT reaction for comparison of polypeptide concentration.

A. Caco-2 24-OHase

B. Caco-2 XDR3

C. Caco-2 ROC

Figure 3.

Effects of VIP expression on VDR/RXR-mediated transcription using select VDREs in a luciferase-based assay system in colon cells. Human colon cancer cells (Caco-2) were cotransfected with expression vectors encoding VDR and the indicated VIP using liposome-mediated transfection. A firefly luciferase reporter vector was also introduced into the cells. The reporter construct in (A) utilizes VDREs in the natural context of 5500 bp of promoter region from the human cytochrome P450 24-hydroxylase gene (CYP24A1). The other two VDREs utilize a classical direct repeat-type (DR3) element from the human CYP3A4 gene promoter (XDR3) (B) and from the rat osteocalcin gene (ROC) (C). Cells were incubated for 24 hours in the presence of 10^{-8} M 1,25D or ethanol vehicle followed by measurement of

luciferase activity. Activity was normalized to empty vector control treated with 1,25D (which was set at 100%). Asterisks indicate significance at $p < 0.05$ (unpaired Student's *t* test, unequal variance, compared to empty plasmid control treated with or without 1,25D).

A. HEK293 24-OHase

B. HEK293 XDR3

C. HEK293 ROC

Figure 4.

VIP effects on VDR/RXR-mediated transcription in kidney cells. Human embryonic kidney cells (HEK293) were cotransfected with expression vectors encoding VDR and the indicated VIP as in Figure 3. Luciferase activity was assayed as a measure of transactivation on three VDREs: (A) human cytochrome P450 24-hydroxylase gene (CYP24A1) VDRE; (B) XDR3 VDRE; (C) ROC VDRE. Activity was normalized to empty vector control treated with 10^{-8} M 1,25D (which was set at 100%). Asterisks indicate significance at $p < 0.05$ (unpaired Student's *t* test, unequal variance, compared to empty plasmid control treated with or without 1,25D).

A. C2C12 24-OHase **B. C2C12 XDR3** **C. C2C12 ROC** **Figure 5.**

VIP effects on VDR/RXR-mediated transcription in muscle cells. Mouse myoblast cells (C2C12) were cotransfected with expression vectors encoding VDR and the indicated VIP as in Figure 3. Luciferase activity was assayed as a measure of transactivation on three VDREs: (A) human cytochrome P450 24-hydroxylase gene (CYP24A1) VDRE; (B) XDR3 VDRE; (C) ROC VDRE. Activity was normalized to empty vector control treated with 10^{-8} M 1,25D (which was set at 100%). Asterisks indicate significance at $p < 0.05$ (unpaired Student's *t* test, unequal variance, compared to empty plasmid control treated with or without 1,25D).

Figure 6.

1,25D-dependent VIP effects on VDR/RXR-mediated transcription in all cell types tested. Transcriptional assays employing the CYP24A1, XDR3 and ROC VDRE-luciferase reporter constructs are summarized with respect to empty vector control treated with 10^{-8} M 1,25D (which was set at 1).

Figure 7.

Binding of CXXC5 protein to the VDR complex as assessed by co-immunoprecipitation analysis. (A) Lysates from HCT-116 cells transfected with pSG5-CXXC5 and pSG5-hVDR were precipitated with anti-VDR (9A7), anti-CXXC5 (H-6) or control IgG. The immunoprecipitated protein complexes were analyzed by western blotting with anti-CXXC5 (H-6) or anti-VDR (9A7) antibody. The co-immunoprecipitation was performed with both anti-CXXC5 (upper panel) or anti-VDR (lower panel) antibodies. (B) Lysates from HCT-116 cells transfected with pSG5-hRXRα and pSG5-hVDR were precipitated with anti-RXRα (D-20) or control IgG. The immunoprecipitated proteins were analyzed by western blotting with anti-VDR (D-6) antibody. Anti-VDR (9A7), rat monoclonal antibody; anti-VDR (D-6), mouse monoclonal antibody; anti-CXXC5 (H-6), mouse monoclonal antibody; anti-RXR (D-20), rabbit polyclonal antibody. All lysates contained either ethanol (–1,25D) vehicle or 10^{-7} M 1,25D (+1,25D).

Figure 8.

Dose-dependent expression of CXXC5 and assessment of VDR-mediated transcription. (A) CXXC5 overexpression was assessed by western blotting utilizing HCT-116 cells. Total cell lysates (50 μ g) from transfected cells (with indicated amount of pSG5-CXXC5 plasmid) were loaded onto each lane on 10% SDS-PAGE gel followed by western blotting with anti-CXXC5 antibody. (B) Evaluation of 1,25D/VDR-mediated transcriptional activity as a function of CXXC5 overexpression. HCT-116 cells were co-transfected with XDR3-Luciferase, pSG5-hVDR, pRL-null and pSG5-CXXC5. After treatment with EtOH vehicle (-1,25D) or 1,25D (10^{-8} M) (+1,25D), the cells were lysed with 150 μ l passive lysis buffer. Firefly and Renilla activities were measured sequentially from each well. After normalization for transfection efficiency, results were expressed as relative light units (RLU) per well. VDR transactivation in the presence of 1,25D from cells without pSG5-

CXXC5 was set at 100%. Results are expressed as the mean $\pm$ SD of three independent experiments.

Figure 9.

Functional analysis of CXXC5 expression on VDR signaling via siRNA technology. HCT-116 cells were co-transfected (XDR3-Luc, pSG5-hVDR, pRL-null, pSG5-empty/pSG5-CXXC5) along with CXXC5 siRNA (CXXC5 siRNA 0 pmoles/well (-), CXXC5 siRNA 20 pmoles/well (+), CXXC5 siRNA 40 pmoles/well (++)). The cells were harvested after treatment with EtOH (-1,25D) or 1,25D (10^{-8} M) (+1,25D) for 24 hours. (A) Effect of CXXC5 protein knockdown was assessed by western blotting. Total cell lysates (7.5 μ g) from the co-transfected cells were loaded onto each lane on 10% SDS-PAGE gel. Anti-CXXC5 (H-6) was used as primary antibody. (B) Evaluation of 1,25D/VDR-mediated transcriptional activity after CXXC5 knockdown. The cells were co-transfected as described

above, including pSG5-empty or pSG5-CXXC5 as indicated, treated with EtOH (–1,25D) or 1,25D (+1,25D), and then lysed in 150 µl passive lysis buffer. (C) Evaluation of 1,25D/VDR-mediated transcriptional activity after dose-dependent knockdown of endogenous CXXC5. The cells were co-transfected (XDR3-Luc, pSG5-hVDR, pRL-null and siRNA) without additional pSG5-CXXC5, treated with EtOH (–1,25D) or 1,25D (+1,25D) and then lysed with 150 µl passive lysis buffer. Firefly and Renilla activities were measured sequentially from each well. After normalization for transfection efficiency, results were expressed as relative light units (RLU) per well. Values are means ± SD of triplicate determinations. Statistical differences between –siRNA and +siRNA were determined by a two-sided Student's *t* test. Differences among multiple groups were analyzed by ANOVA. * *p* value < 0.01.

Table 1

VIPs identified through yeast two-hybrid screening. Those colonies displaying the most vigorous growth on - trp, -leu, -his agar plates that also exhibited positive results in the LacZ colorimetric assay were scored as VIPs, and were restreaked onto “master” plates. Screen indicates with (+1,25D) or without (–1,25D) 1,25-dihydroxyvitamin D₃ included in the incubation and plating. Subsequent sequencing of the prey plasmid led to the identification of the six genes shown above. CXXC5 was previously identified in a yeast two-hybrid screen using a HeLa cDNA library indicating that our yeast screens performed with the human heart cDNA library are reproducible in that they revealed previously identified VIPs from other cDNA libraries. Also shown in the table is the known or putative function of each VIP. Not shown are known VIPs (RXR and NCoA-62) that were also isolated in our yeast system.

Official Gene Symbol	Accession Number	Protein	Screen
TPM2	NM_213674	Tropomyosin	–1,25D
FASTK	NM_006712.3	Fas-activated serine/threonine kinase	–1,25D
NR4A1	NM_002135.3	Nuclear receptor subfamily 4, group A, member 1	–1,25D
CXXC5	BC016207	CXXC finger 5	–1,25D
MYL3	NM_194293	Myosin light chain 3	+1,25D
XIRP1	NM_194293	Xin actin-binding repeat containing protein 1	+1,25D

Table 2

Summary of Figure 1A and Figure 2 comparing and contrasting *in vivo* and *in vitro* interaction data. ND = experiment was not performed. – indicates no interaction; + indicates a weak interaction; ++ indicates a modest interaction; +++ indicates an interaction comparable to the VDR/RXR interaction.

VIP	Mammalian Two Hybrid		GST Pulldown	
	– 1,25D	+ 1,25D	– 1,25D	+ 1,25D
RXR	–	+++	+	+++
CXXC5	–	+	++	++
FASTK	–	++	–	++
NRA41	+++	+	++	–
TPM2	–	–	+	+
MYL3	ND	ND	++	++
XIRP1	–	+	++	++