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Vitamin D receptor is a novel transcriptional regulator for *Axin1*

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Abstract

Background—Axin1 is a scaffold protein in the β -catenin destruction complex, which, if disrupted, contributes to pathogenesis of various human diseases, including colorectal carcinogenesis and inflammatory bowel diseases (IBD). We have previously demonstrated that *Salmonella* infection promotes the degradation and plasma sequestration of Axin1, leading to bacterial invasiveness and inflammatory responses. Vitamin D and the vitamin D receptor (VDR) appear to be important regulators of IBD and colon cancer. Although VDR and Axin1 are all involved in intestinal inflammation, it remains unclear whether these processes are related or function independently. In the current study, we hypothesize that VDR is an important regulator for the maintenance of physiological level of Axin1.

Methods—Using the intestinal epithelial conditional VDR knockout mouse model (VDR^{IEC}) and cultured cell lines, influences of VDR status on the expression of *Axin1* was evaluated by Western blots and real-time PCR. Loss- and gain-of-function assays were used to investigate the regulation of VDR on *Axin1* at the transcriptional and translational levels. Cells were treated with cycloheximide or actinomycin for molecular mechanistic studies. Candidate genomic VDR binding sites for *Axin1* were tested by chromatin immunoprecipitation (ChIP) assay. Physical interactions among VDR, Axin1, and β -catenin were tested by immunoprecipitation. Cellular localization of Axin1 with different VDR status was determined by fractionation and immunohistochemistry.

Results—We found that VDR deletion led to lower protein and mRNA levels of *Axin1*, whereas knockdown of *Axin1* did not change the expression level of VDR protein. Immunoprecipitation data did not support physical interaction between VDR and Axin1. The VDR regulation of *Axin1* was through a VDR genomic binding site for *Axin1* gene on the regulatory region. Fractionation data showed that cytosolic Axin1 was significantly reduced due to VDR deletion, leaving the

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Conflicts of Interest

The authors have no conflicts of Interest.

nuclear fraction unchanged. In ileum, Axin1 was distributed in the cytosol of apical epithelium and crypts.

Conclusion—VDR is important for the maintenance of physiological level of Axin1. The discovery of *Axin1* as a VDR target gene provides novel and fundamental insights into the interactions between the VDR and β -catenin signaling pathways.

Keywords

Axin1; beta-catenin; vitamin D; VDR; VDR genomic binding site; IBD; intestine; inflammation; transcriptional factor; Wnt

Introduction

β -catenin, an adhesion molecule and a component of Wnt signaling, is closely related to development, proliferation, differentiation and carcinogenesis [1–6]. Axin1 is a scaffold protein in the β -catenin destruction complex. The sequence is located on chromosome 17 qA3.3. Its protein consists of an RGS domain in the N terminus, which binds with APC, and a DIX domain in the C terminus important for the homodimerization of the protein [7]. It is supposed to be rate-limiting in the regulation of β -catenin, and restricts the Wnt signaling to basal level, and is expressed constitutively [8]. Deletion of *Axin1* leads to lethality *in utero*, and its mutations have been associated with a wide variety of cancer types including colorectal cancer [8]. Our recent study has demonstrated that *Salmonella* exposure promotes the degradation and plasma sequestration of Axin1, leading to bacterial invasiveness and inflammatory responses [9]. Furthermore, Axin1 also intermediates TGF- β signaling, SAPK signaling, *etc.* [8]. Therefore, *Axin1* plays a vital role in gene expression and pathway activation, which potentially cause pathogenesis of various diseases upon dysregulation.

VDR plays a vital role in innate/adaptive immunity, and is widely expressed in a variety of organs and tissues. Vitamin D exerts its regulatory effects mainly via VDR, a nuclear receptor, which forms a heterodimer with retinoid-X receptor (RXR), and then binds to genomic VDR binding sites on regulated regions of target genes, triggering expression of target genes. It is known that VDR transcriptionally regulates the expression of cathelicidin antimicrobial peptides (CAMPs), β -defensins autophagy regulator ATG16L1, and tight junction protein Claudin-2 [10–13]. For example, 1,25(OH) $_2$ D $_3$ leads to upregulation of CAMPs and the killing of intracellular *Mycobacterium tuberculosis* in human monocytes [14]. The absence of VDR is associated with shifts in the bacterial load and profile [12, 15]. In our recent study, both taxonomic assignment and functions of the intestinal microbiota are altered due to VDR status [16]. In the experimental models, VDR knockout increases the risks of infection, inflammation and risks to intestinal disorders. VDR negatively regulates bacteria-induced inflammation through NF- κ B pathway by affecting I κ B α [17, 18].

VDR activation represses signaling and oncogenic effects of β -catenin, which, in reverse, has the potential to enhance 1,25(OH) $_2$ D $_3$ initiated transcription [19]. Vitamin D/VDR inhibits β -catenin signaling by competitively replacing it from the LEF/TCF complex [20]. However, the relationship between VDR and Axin1 remains largely unknown. Although

VDR and Axin1 are both involved in intestinal inflammation and cancer, it remains unclear whether these processes are related or function independently.

In the current study, we hypothesize that VDR is an important regulator for the maintenance of physiological level of Axin1. The regulation was mainly on transcriptional level. Moreover, we showed that intestinal epithelial conditional VDR knockout in mice altered the expression of Axin1 at the protein and mRNA levels and changed their distribution in intestine. Our study provides novel insights into the regulatory role of VDR, supplementing a novel link in the delicate Wnt/ β -catenin signaling network.

Methods and Materials

Statement of Ethics

All animal work was approved by the Rush University Medical Center Committee on Animal Resources and the Animal Care Committee (ACC) at the University of Illinois at Chicago.

Animal models

Conditional VDR knockout (VDR^{IEC}) mice [12] were obtained by crossing the VDR^{flox/flox} mice [21] with villin-cre mice (Jackson Laboratory, 004586). Tail snips were collected 4 weeks after the mice were born. Six- to eight-week-old littermates for each group were chosen and co-housed till experiments.

Cell culture

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos bred from VDR^{+/-} $\times$ VDR^{+/-} as was described previously [22]. Genomic DNA extracted from the cells was analyzed by PCR genotyping. After 15 passages, when the VDR^{-/-} and VDR^{+/-} MEFs were immortalized, they were used in subsequent experiments. HCT116 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) (Hyclone, SH30243.01) supplemented with 10% (v/v) fetal bovine serum (GEMINI, 900-108), 50 μ g/mL streptomycin and 50 U/mL penicillin (Mediatech, Inc., 30-002CI) as previously described [12].

Immunohistochemistry and quantification of images

Ileum tissues were freshly isolated, fixed with 10% neutral buffered formalin and paraffin embedded. Immunohistochemistry (IHC) was performed using 4- μ m paraffin sections as previously described [23]. The samples were permeabilized for 1 h with 0.2% Triton X-100 and rinsed with Hanks. The coverslips were incubated for 1 h in block buffer (5% BSA +0.1% Tween 20 in Hanks), primary antibody (1:50 dilution for Axin1 (Cell Signal, Beverly, MA, USA) in block buffer) at 4°C overnight, washed three times with 0.1% Tween in PBS, and then incubated with biotin-conjugated secondary antibody at room temperature for 1 h, washed, incubated with ABC reagent at RT for 1 h (Vector lab PK-6100 standard), washed, visualized with DAB kit (Vector lab SK-4100) and counterstained with hematoxylin. The areas of positive staining were quantified using Image J2x (2.1.4.7 version, Wayne Rasband, National Institutes of Health, USA).

Western blotting

Intestinal tissues from mice were isolated and sonicated in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA pH 8.0, 0.2 mmol/L sodium ortho-vanadate, and protease inhibitor cocktail), as previously described [12]. MEFs, HCT116, and HT29 cells rinsed twice with ice-cold PBS, were lysed and sonicated in protein loading buffer (50 mmol/L Tris, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Primary antibodies to mouse VDR, β -actin (Sigma-Aldrich, Milwaukee, WI, USA), and Axin1 (Cell Signal, Beverly, MA, USA) were used, and were visualized by ECL. Relative abundance of protein was determined using Quantity One software (Bio-Rad, Hercules, CA).

Fractionation

The adherent cells were harvested with trypsin-EDTA and then centrifuged at 500×g for 5 min. The cells were washed by PBS and pelleted at 500×g for 2–3 min. Nuclear and cytoplasmic fractions of the cells were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, USA) according to the manufacturer's instructions.

1, 25(OH)₂D₃ treatment

The HCT116 cells were treated with 20 nM 1,25(OH)₂D₃ (Sigma-Aldrich, Milwaukee, WI, USA) and then harvested at 0 h, 1 h, 3 h, 6 h, and 9 h for further assays.

Cycloheximide (CHX) treatment

The VDR^{+/-} and VDR^{-/-} MEFs were treated with 1 μ g/mL CHX, an effective inhibitor of protein synthesis. The cells were harvested at 4 h, 6 h, and 8 h post treatment for further assays.

Actinomycin treatment

The VDR^{+/-} and VDR^{-/-} MEFs were treated with 1 μ g/mL actinomycin D, the RNA II polymerase inhibitor of eukaryotes, for 2 h. With the medium being refreshed, the MEFs were further cultured for 22 h at 37 °C with 5% CO₂ before sample collection for Western blots.

Real-time PCR

Total RNA was extracted from isolated MEFs and mouse tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA products, prepared via reverse transcription, were subjected to quantitative real-time PCR with MiQ single-color real-time PCR detection system (Bio-Rad) and iQ SYBR green supermix (Bio-Rad) according to the manufacturer's instructions. Expression levels were normalized to the levels of β -actin, the internal control. Percentage expression was calculated as the ratio of the normalized value of each sample to that of corresponding untreated controls. The experiments were performed in triplicate. Primer sequences were listed in Supplementary Table S1.

Transient transfection

Transient transfection was performed with Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Protein was extracted with immunoprecipitation (IP) buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate) containing protease inhibitor cocktail at 24 h after transfection for immunoblotting.

Coimmunoprecipitation

Cells were rinsed twice in ice-cold PBS, and lysed in cold IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate) containing protease inhibitor cocktail. Cell lysates were precleared with protein A-agarose, and then incubated with 2 µg of primary antibodies for 1 hour at 4 °C. Protein A-agarose was added to the lysate and incubated for 30 min with agitation at 4 °C, and washed with precooled IP buffer by incubation with agitation at 4 °C. Washing was repeated 3 times. With the supernatant being removed, the samples were diluted with concentrated (2×) electrophoresis sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 200 mM β-mercaptoethanol), boiled for 5 minutes, assayed with SDS-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. Blots were probed with primary and secondary antibodies and visualized by ECL.

Chromatin immunoprecipitation (ChIP) assay

Interactions between VDR and the –10kb to 5kb regions of the *Axin1* gene in mouse ileum was tested by ChIP assay as previously described [12]. The epithelium of mouse ileum was fixed with 1% formaldehyde for 10 min at 37°C, and then washed twice in ice-cold phosphate buffered saline containing protease inhibitor cocktail (Roche). The cells were pelleted and lysed in SDS lysis Buffer. The lysate was sonicated to shear DNA into fragments of 200–1000 bp (two cycles of 30 s sonication, 30 s pausing, Branson Sonifier 250, USA). The chromatin samples were precleared with salmon sperm DNA–bovine serum albumin-sepharose beads, and then incubated overnight at 4°C with VDR antibody (Santa Cruz Biotechnology). Immune complexes were precipitated with salmon sperm DNA–bovine serum albumin-sepharose beads. DNA samples were digested with proteinase K, and extracted with phenol and chloroform, and then purified by ethanol precipitation. Primers targeting candidate VDR genomic binding sites were listed in supplementary Table S2. *Claudin-2* [13] and *IκBa* were used as the positive controls for the ChIP assay [17].

Statistical analysis

Data were expressed as mean ± SD. Differences between two samples were analyzed by Student's t test. P-values of 0.05 or less were considered statistically significant. Differences among three or more groups were analyzed using ANOVA (SAS 9.2 version, SAS Institute Inc., Cary, NC).

Results

VDR deletion led to lower protein level of Axin1, whereas knockout of *Axin1* did not change the expression of VDR

We reasoned if there were some cross-talks between Axin1-mediated Wnt signaling and VDR, VDR status might alter the expression of *Axin1*. *In vivo*, we found that VDR knockout significantly decreased Axin1 protein level in ileum (Fig. 1A). We used VDR^{-/-} and VDR^{+/-} mouse embryonic fibroblasts (MEFs) cells to test whether one allele of the VDR gene was sufficient to enhance the *Axin1* expression. The results of Western blots showed that VDR^{+/-}MEFs had significantly higher expression levels of Axin1, whereas complete lack of VDR led to significantly lower level of Axin1 protein in VDR^{-/-}MEFs (Fig. 1B). In reverse, however, knockout of *Axin1* with siRNA did not interfere with the expression of VDR protein (Fig. 1C). These data indicate that VDR is the upstream regulator of *Axin1*, whereas the level of Axin1 expression has no effect on VDR.

Increased VDR led to enhanced protein level of Axin1

We then manipulated the VDR expression and investigated the effects of VDR level on *Axin1* expression. In the human epithelial HCT116 cells, 1,25(OH)₂D₃ treatment increased VDR expression to as high as 6 fold (Fig. 2A), and also the Axin1 expression by about 3 fold. Upon exposure to cycloheximide (CHX), an inhibitor of protein synthesis, the protein level of Axin1 was significantly decreased, concomitant with a dramatic reduction of VDR level in VDR^{+/-} MEFs (Fig. 2B). Therefore, there was a positive correlation between VDR level and Axin1 level. We further tested the correlation between VDR and Axin1 in human epithelial HT29 cells (Fig. 2C). There was also an increase of VDR and a concomitant significant upregulation of Axin1 after 1,25(OH)₂D₃ treatment. It indicated the response of VDR regulation on Axin1 is universal in both intestinal epithelial and fibroblast cells.

The mechanism underlying the influences of VDR on Axin1 may involve physical binding of the two proteins. To test the VDR-Axin1 interactions at the protein level, immunoprecipitation (IP) assay was performed between VDR, Axin1 and related proteins. While VDR and β -catenin mutually pulled down each other, no physical interactions of VDR and Axin1 were detected (Fig. 2D). Input is to show the corresponding total extract for comparison. As expected, there were interactions between β -catenin and GSK3 β (Fig. 2D). We also confirmed the interactions between β -catenin and Axin1 (Fig. S1).

VDR deletion led to significantly decreased *Axin1* at the mRNA level

At the transcriptional level, we examined the mRNA level of *Axin1* by using real-time PCR. VDR knockout significantly reduced mRNA of *Axin1* both in ileum (Fig. 3A) and in MEFs (Fig. 3B). The VDR^{+/-} and VDR^{-/-} MEFs were treated with actinomycin to inhibit the synthesis of mRNA. The expression of *Axin1* mRNA was significantly reduced in both VDR^{+/-} and VDR^{-/-} MEFs ($P < 0.05$), to the basal level (Fig. 3C). These results indicate that transcriptional regulation contributes to differential expression of *Axin1* with different VDR status.

VDR regulates *Axin1* expression through a VDR genomic binding site on the promoter region

The regulation of VDR on *Axin1* at transcriptional level is likely through the activation of VDR genomic binding sites in *Axin1* gene. We mapped candidate VDR genomic binding sites on the –10kb to 5kb region of *Axin1* gene (Fig. 4A). ChIP assay was then performed, followed by PCR to identify candidate VDR genomic binding sites. Epithelium of ileum was harvested from VDR^{flx/flx} mice. Primers targeting candidate VDR genomic binding sites were listed in supplementary Table S2. One genomic VDR binding site was found in the coding region of *Axin1* gene (Fig. 4B). The primers with positive PCR band target 4303 to 4317 region (*Axin1*-P12 in Table S2). The colon was harvested for the CHIP assay using the *Axin1*-P12 primer (Fig. 4C). VDR antibody pull down showed one band after PCR detection, whereas IgG pull down showed no signal. IgG was added to get the negative control for pull-down by VDR. The PCR result using primers targeting *Axin1*-P5 site had no specific band, shown here as a negative control (Fig.4B and 4C). Based on our previous publications [13, 17], Claudin-2 and IκBα were used as the positive controls for the ChIP assay (in Fig.4B and 4C). All primers targeting candidate VDR genomic binding sites were listed in Table S2. We have tested all these primers for the potential VDR genomic binding sites by CHIP assays (data not shown). Taken together, we found substantial and specific interactions between VDR and the target region on *Axin1* gene.

VDR is important for the maintenance of physiological levels of Axin1 in intestine

Still unknown are the influences of VDR on the cellular localization of Axin1. Cell fractionation revealed Axin1 was predominantly distributed in cytosol in both VDR^{-/-} and VDR^{+/-} MEFs. In VDR^{+/-} MEFs cytosolic fraction of Axin1 was significantly ($P<0.05$) higher than that in VDR^{-/-} MEFs (Fig. 5A), whereas the nuclear fraction of Axin1 was unchanged with altered VDR status. By immunohistochemistry, it was observed that intestinal Axin1 was mainly located in cytosol of the apical epithelium of ileum (Fig. 5B), regardless of the VDR status. Consistent with the WB assay, the expression of Axin1 was reduced in the epithelium of ileum, especially in the cytosol. There was a plasma sequestration of Axin1 with conditional VDR knockout, which presumably renders it nonfunctional. In Fig. 5C, further image analysis showed that the Axin1 positive staining is significantly decreased in the epithelium of ileum of VDR^{IEC} mice, compared to the VDR^{lox} control mice.

Discussion

In the current study, we reported that VDR deletion led to lower protein and mRNA levels of *Axin1*. The VDR transcriptional regulation of *Axin1* was through a VDR genomic binding sites on the promoter region. Fractionation data showed that cytosolic Axin1 was significantly reduced due to VDR deletion, leaving the nuclear fraction unchanged. In ileum, Axin1 was distributed in the cytosol of apical epithelium and crypts. Thus, VDR is important for maintaining physiological level of Axin1.

The discovery of *Axin1* as a VDR target gene provides novel and fundamental insights into the interactions between the VDR and β-catenin signaling pathways. Insufficient vitamin D

has been associated with over 17 types of cancer, osteoarthritis, diabetes mellitus, multiple sclerosis, stroke, hypertension, autoimmune diseases, periodontal disease, *etc.* [24–31] Dwindling vitamin D/VDR signaling has been associated with susceptibility to IBD [32] and colorectal cancer [33], aberrant immune responses [24, 25], and pre-inflammatory states [31, 34]. Stimulation of vitamin D/VDR signaling enhances mucosal barrier in gut [35]. Accumulating evidence is correlating VDR and Wnt signaling [36–39]. The regulators or intermediates may involve Snail1 or LRP5 [40, 41]. In the current study, we reported that the VDR status is important for the maintenance of normal level of *Axin1*.

With VDR knockout, Axin1 was reduced at protein level in ileum and in MEFs. VDR increases with elevated environmental vitamin D [42]. Induced overexpression of VDR was accompanied by an increase of Axin1 in HCT116 cells. Treatment with CHX resulted in dramatic decrease of the Axin1 and VDR proteins. However, IP data did not support physical interaction between VDR and Axin1, so there was no evidence for protein level regulation.

Previous observations revealed that Axin1 is located on plasma membrane and cytoplasmic puncta [9, 54]. Consistent with it, we found that Axin1 was located in cytosol of MEFs and ileum. The nuclear signal was quite weak. In ileum, it is more strongly expressed at the apical side of the villi. VDR status, however, did not alter the cellular localization of Axin1 in MEFs and in the epithelium of ileum.

VDR can either recruit co-repressors or co-activators: which end it favors depends on the ligands that bind to it. VDR-mediated repression of gene expression can be through VDR interacting repressor (VDIR) [43]. Axin1 plays its role in Wnt signaling by forming GSK3 β /Axin-1/ β -catenin complex, is closely related to a variety of cancer types if disrupted or stabilized, and functions to prevent microbial invasion and inflammation in intestinal cells [9, 44–47]. VDR genomic binding sites have a consensus, which is direct or everted repeats of two PuG(G/T)TCA motifs separated by 3 bp (DR3) or 6 bp (ER6) [48]. VDR genomic binding sites regulate the expression of antimicrobial peptide β -defensin, *ATG16L1*, *I κ B α* , *CYP24A1*, *etc.* [10–12, 17, 49]. While most of VDR genomic binding site regulated genes have only one VDR genomic binding site, in the promoter region of *CYP24A1*, are two VDR genomic binding sites, indicating the complexity of the regulation [49]. In this research, *Axin1* was consistently downregulated at transcriptional level both *in vitro* and *in vivo* with conditional VDR knockout. Treatment with actinomycin, an inhibitor of RNA synthesis, significantly reduced *Axin1* mRNA to the basal level in both VDR^{+/-} and VDR^{-/-} MEFs. We found one VDR genomic binding site for *Axin1* in the –10kb to 5kb region.

Molecular methods, such as VDR ChIP-seq, can locate the likely VDR genomic binding sites in the range of from –100kb to 100kb, even at –185kb [50, 51]. These methods may be able to research more distant regions with high efficiency, as all high throughput assays may have in common, however, the reliability could be compromised. Currently, most of the VDR ChIP-seq experiments use 1,25(OH)₂D₃ treatment *in vitro*, like lymphoblastoid cell lines [50] and THP-1 cells [52, 53], which may be different from the VDR knockout and conditional knockout models and the *in vivo* models, compromising their value as accurate references. Therefore, VDR ChIP-seq data do provide us important indication, however,

these data need to be further supported by gene-specific ChIP assays and other physiologically related studies. In our *in vivo* models, further study of the influences of bacteria, e.g. *Salmonella*, on Axin1 expression with different VDR status would extend the understanding on the importance of VDR in intestinal homeostasis and diseases.

The VDR regulation of Axin1 at protein level was not thoroughly investigated in the current study. Although the IP data did not support physical interaction between VDR and Axin1, other mechanisms may underlie the regulation of VDR on Axin1 at protein level. For example, it is possible that β -catenin serves as an adapter for VDR and Axin1. The potential interactions of VDR/ β -catenin/Axin1 complex may stabilize Axin1 and prevent it from degradation. However, direct evidence is still missing.

In summary, our research identified *Axin1* as a downstream target of VDR and demonstrated a crosstalk between VDR-mediated signaling and β -catenin pathway through Axin1. The discovery of a VDR genomic binding site in the regulatory region of *Axin1* gene provides a molecular basis for the VDR regulation. For the future plan, we will explore the potential of *Axin1* as a novel candidate for therapeutic intervention against abnormal activation of β -catenin in intestinal inflammation and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlight

1. VDR deletion leads to lower protein and mRNA levels of Axin1.
2. VDR regulation of *Axin1* is through a VDR genomic binding site on the promoter region of *Axin1*.
3. VDR is important for the maintenance of physiological levels of Axin1 in intestine.
4. The discovery of *Axin1* as a VDR target gene provides novel and fundamental insights into the interactions between the VDR and β -catenin signaling pathways.

Fig 1. VDR regulates Axin1 expression at protein level both *in vitro* and *in vivo*

The expression of Axin1 in (A) epithelium of ileum from $VDR^{flox/flox}$ and $VDR^{\Delta IEC}$ mice; and in (B) $VDR^{-/-}$ and $VDR^{+/-}$ MEFs; and (C) the expression of VDR in HCT116 cells with Axin1 knockout by siRNA. VDR knockout reduced the protein level of Axin1 both *in vitro* and *in vivo*, whereas Axin1 knockout did not interfere with VDR expression. Data were expressed as mean $\pm$ SD. * P <0.05, and ** P <0.01.

Fig 2. Protein level regulation of VDR on Axin1 expression

(A) Time course of Axin1 expression in HCT116 cells upon induced overexpression of VDR by 1,25(OH)₂D₃ treatment. Overexpression of VDR significantly ($P < 0.05$) increased Axin1 expression. (B) protein levels of Axin1 in MEFs with different VDR status treated with CHX, an inhibitor of protein synthesis. CHX treatment inhibited the expression of VDR and Axin1 as expected in VDR^{+/-} cells, whereas there was not observable regulation in VDR^{-/-} MEFs upon exposure, indicating an indispensable role of VDR in modulating Axin1. (C) Time course of Axin1 expression in HT29 cells upon induced overexpression of VDR by 1,

25(OH)₂D₃ treatment. Upregulated VDR significantly ($P<0.05$) increased the Axin1 expression in HT29 cells. (D) Physical interactions among VDR, Axin1 and β -catenin. Immunoprecipitation (IP) and immunoblot (IB) detection after pull-down by antibodies against VDR and β -catenin in HCT116 cells. Input is to show the corresponding total extract for comparison. There are physical interactions between β -catenin and VDR, but not between VDR and Axin1. Data were expressed as mean $\pm$ SD. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. # $P<0.05$ in comparison to VDR^{-/-} MEFs at 0 h. n =3.

Fig 3. *Axin1* both *in vitro* and *in vivo*

The mRNA levels of *Axin1* in (A) ileal epithelium from VDR^{flox/flox} and VDR^{IEC} mice; in (B) VDR^{+/+} and VDR^{-/-} MEFs, and in (C) VDR^{+/+} and VDR^{-/-} MEFs treated with actinomycin, an inhibitor of mRNA synthesis. Actinomycin treatment inhibited the transcription of *Axin1* to basal levels in both VDR^{+/+} and VDR^{-/-} MEFs. Data were expressed as mean±SD. **P*<0.05, and ****P*<0.001. n =3.

Fig 4. The mechanism underlying the transcriptional regulation of VDR on *Axin1*
 (A) mapping of candidate VDR genomic binding sites on the -10kb to 5kb region of *Axin1* gene. (B) and (C) the VDR genomic binding site found for *Axin1* gene. ChIP assay was performed, followed by PCR to identify VDR genomic binding sites in both ileum (B) and colon (C). Epithelium of ileum and colon was harvested from VDR^{flx/flx} mice. IgG was added to get the negative control. PCR analysis was performed on the Axin1 promoter regions as indicated, showing Axin1-P12 as a new VDR genomic binding site and Axin1-P5 as a negative control. Claudin-2 and IκBα served as positive controls. Axin1-P12 sequence: 5'-GGATCAGCCTGTTCA-3' Axin1-P5 sequence: 5'-GATTGATGGGGGTGA-3'

Fig 5. Cellular localization of Axin1 with different VDR status

(A) Fractionation of VDR^{+/-} and VDR^{-/-} MEFs. Axin1 was mainly distributed in cytosolic fraction. VDR knockout didn't influence the localization of Axin1, but reduced the cytosolic expression. Data were expressed as mean±SD. ***P*<0.01. (B) Immunohistochemistry of Axin1 in ileum of VDR^{flox/flox} and VDR^{ΔIEC} mice. Axin1 was distributed in the cytosol of epithelium and crypts in ileum. (C) Axin1 expression was quantified based on the areas of positive staining. ****P*<0.001, n=5.