

SCIENTIFIC REPORTS



OPEN

Tight junction *CLDN2* gene is a direct target of the vitamin D receptor

Received: 13 January 2015

Accepted: 30 April 2015

Published: 27 July 2015

Yong-guo Zhang¹, Shaoping Wu¹, Rong Lu¹, David Zhou², Jingsong Zhou³, Geert Carmeliet⁴, Elaine Petrof⁵, Erika C. Claud⁶ & Jun Sun^{1,2}

The breakdown of the intestinal barrier is a common manifestation of many diseases. Recent evidence suggests that vitamin D and its receptor VDR may regulate intestinal barrier function. Claudin-2 is a tight junction protein that mediates paracellular water transport in intestinal epithelia, rendering them “leaky”. Using whole body *VDR*^{-/-} mice, intestinal epithelial VDR conditional knockout (*VDR*^{ΔIEC}) mice, and cultured human intestinal epithelial cells, we demonstrate here that the *CLDN2* gene is a direct target of the transcription factor VDR. The Caudal-Related Homeobox (*Cdx*) protein family is a group of the transcription factor proteins which bind to DNA to regulate the expression of genes. Our data showed that VDR-enhances Claudin-2 promoter activity in a *Cdx1* binding site-dependent manner. We further identify a functional vitamin D response element (VDRE) 5′-AGATAACAAAGGTCA-3′ in the *Cdx1* site of the Claudin-2 promoter. It is a VDRE required for the regulation of Claudin-2 by vitamin D. Absence of VDR decreased Claudin-2 expression by abolishing VDR/promoter binding. *In vivo*, VDR deletion in intestinal epithelial cells led to significant decreased Claudin-2 in *VDR*^{-/-} and *VDR*^{ΔIEC} mice. The current study reveals an important and novel mechanism for VDR by regulation of epithelial barriers.

Tight junction (TJ) structural components determine epithelial polarization and intestinal barrier functions^{1–4}. Claudins, with approximately 24 members, are integral membrane proteins and components of tight junctions⁵. The complex expression pattern of claudins creates diversity in the barrier/channel property of TJs, which varies depending on the type of epithelium⁶. Claudin-2 and -10 tend to make tight monolayers leakier^{5,7–9}. Claudin-2, a “leak” protein uniquely restricted to the proliferative zone of the crypt base^{10,11}, forms a paracellular water channel that mediates paracellular water transport in epithelia and renders it more “leaky”^{11–15}. Defective epithelial barrier function has been implicated in IBD¹⁶ and similarly, elevation of Claudin-2 is associated with active IBD^{12,17}. In addition, Claudin-2 likely participates in cellular functions other than its known effects on TJ function. For example, we have demonstrated that *Salmonella* targets Claudin-2 to facilitate bacterial invasion¹⁸, and epithelial cells with Claudin-2 knockdown have significantly less internalized *Salmonella* than control cells with normal Claudin-2 expression. Claudin-2 has also been identified as a target of Wnt/ β -catenin signaling¹⁹, which is essential for intestinal development, and a recent study reported that Claudin-2 and -12 contributed to vitamin D-dependent calcium homeostasis²⁰. Because of this latter report, and because Vitamin D and its receptor (VDR) are implicated in the pathogenesis of various intestinal illness, including IBD^{21–31}, we sought to determine how Claudin-2 is regulated by VDR signaling.

¹Department of Biochemistry, Rush University, Chicago, Illinois, USA. ²Department of Pathology, University of Rochester, Rochester, New York, USA. ³Department of Physiology, Kansas City University of Medicine and Bioscience, Kansas City, Missouri, USA. ⁴Lab of Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium. ⁵Department of Medicine, GI Diseases Research Unit and Division of Infectious Diseases, Queen’s University, Ontario, Canada. ⁶Departments of Pediatrics and Medicine, The University of Chicago Medical Center, Chicago, Illinois, USA. Correspondence and requests for materials should be addressed to J.S. (email: jun_sun@rush.edu)

In the current study, we hypothesize that Claudin-2 is a direct target of the VDR. Using whole body $VDR^{-/-}$ mice, intestinal epithelial VDR conditional knockout ($VDR^{\Delta IEC}$) mice, and cultured human intestinal epithelial cells; we perform a series of molecular and biochemical experiments *in vivo* and *in vitro* to investigate VDR regulation of Claudin-2 expression in enterocytes.

Results

Intestinal VDR deficiency in epithelial cells leads to reduction of Claudin-2 at the mRNA and protein levels. In whole $VDR^{-/-}$ mice, we detected significantly decreased mRNA levels of Claudin-2 in intestine (Fig. 1A), whereas other Claudins, such as Claudin-1, -4, -7, -10, and -15, were not altered by the absence of VDR (data not shown). By immuno-blots, we further found that $VDR^{+/+}$ mice had the highest protein level of Claudin-2 in intestine. $VDR^{+/-}$ mice had intermediate levels of Claudin-2, whereas the $VDR^{-/-}$ had the lowest levels of Claudin-2 protein (Fig. 1B). Therefore, VDR expression correlates with protein levels of Claudin-2 in colonic epithelial cells *in vivo* (Fig. 1C).

We further tested the specificity of intestinal VDR on expression levels of Claudin-2, in $VDR^{\Delta IEC}$ mice. We found that the mRNA levels of Claudin-2 were significantly lower in $VDR^{\Delta IEC}$ mice, compared to the VDR-lox mice (Fig. 1D). Claudin-2 protein was also significantly decreased in $VDR^{\Delta IEC}$, where no VDR protein was detected in $VDR^{\Delta IEC}$ colon by Western blot (Fig. 1E). As expected, Claudin-3 and Claudin-7 were unchanged. These data indicate that intestinal VDR specifically regulates the expression levels of Claudin-2.

Colonic Claudin-2 expression is uniquely restricted to the proliferative zone^{10,11,18}. In Fig. 1F, Claudin-2 staining (Green) was observed at the crypt base in $VDR^{+/+}$ mice. The density of Claudin-2 fluorescence staining was weak in the $VDR^{+/-}$ and $VDR^{-/-}$ intestinal epithelial cells. Claudin-7 was very stable in these mice and showed unchanged distribution and density (Fig. 1F).

Vitamin D₃ treatment upregulates mRNA levels of Claudin-2. For molecular mechanism studies *in vitro*, we used the human colonic epithelial SKCO15 cell line, which is widely used in studying TJs^{32,33}. Vitamin D₃ is known to increase VDR expression and activate VDR signaling. Claudin-2 mRNA was significantly elevated in SKCO15 cells treated with 1, 25 vitamin D₃ (20 nM) for 24 hours, whereas Claudin-7 mRNA was not altered by vitamin D₃ treatment (Fig. 2A). Moreover, protein levels of Claudin-2 were increased by vitamin D₃ treatment in a dose-dependent manner (Fig. 2B). In contrast, the expression of Claudin-3 and 7 was unchanged in cells receiving vitamin D₃ treatment. These data suggest that the Claudin-2 gene could be a direct transcriptional target of the VDR.

VDR is generally present in the cytosol or bound to DNA in an inactive state and requires activation by binding ligand³⁴. Upon binding to vitamin D, VDR translocates to the nucleus and binds to vitamin D response elements (VDREs) in target genes and induces gene expression. A previous study showed that ongoing protein synthesis is not required for this process to occur³⁵. We treated human SKCO15 cells with vitamin D₃ (20 nM) in the presence or absence of cyclohexamide (CHX) to block protein synthesis. We chose to treat cells with vitamin D₃ at 20 nM because our dose-response data in Fig. 2B indicated that 20 nM is a suitable concentration to induce Claudin-2 expression. CHX is an inhibitor of eukaryotic protein biosynthesis and is commonly used to determine protein half-life. Therefore, in the cells treated with CHX only, the expression of Claudin-2 was significantly decreased (Fig. 2C SKCO15). CHX+vitamin D₃ treatment was able to stabilize the expression of Claudin-2 (Fig. 2C SKCO15+Vit.D3). Whereas vitamin D₃-induced Claudin-2 gene expression occurred in the absence of ongoing protein synthesis (presence of CHX), Vitamin D treatment did not induce Claudin-3 gene expression (Fig. 2C SKCO15+Vit.D3). These data further support the hypothesis that the Claudin-2 gene is a direct target of the VDR and not activated by secondary events, such as the synthesis of other transcription factors that are induced by VDR.

To study the effect of VDR overexpression on Claudin-2, we transiently transfected the human SKCO15 cells with a pCDNA-hVDR plasmid expressing human VDR. We found Claudin-2 expression increased after SKCO15 cells were transfected with pCMV-hVDR plasmids, whereas no change of Claudin-3 with VDR overexpression (Fig. 2D).

VDR binds the Claudin-2 promoter *in vitro* and *in vivo*. VDR is a nuclear receptor that acts as a transcription factor to regulate expression of its target genes^{21,36}. We reasoned that VDR may bind to DNA promoters of Claudin-2, thus changing mRNA expression of the Claudin-2 genes. VDR's effect on promoters of Claudin-2 was analyzed by CHIP assay. We designed primers to the nonrepetitive region near the transcriptional start site that specifically amplifies the Claudin-2 promoter. For negative controls, chromatin was immunoprecipitated with IgG or villin. The samples were amplified by conventional PCR. We found that VDR bound to the Claudin-2 promoter *in vitro* (SKCO15 cells, Fig. 3A) and *in vivo* (mouse colon, Fig. 3B). The expression of the other Claudin members, such as Claudin-1, was also tested. We found that VDR did not bind to the Claudin-1 promoter, either *in vitro* or *in vivo* (Figs. 3A,B). VDR is known to interact with nuclear receptor RXR in regulating gene expression. However there was no significant change in mRNA level of RXR in the $VDR^{-/-}$ intestine (Fig. 3C).

Lacking VDR decreases Claudin-2 by abolishing VDR/promoter binding. We reasoned that less Claudin-2 was generated in cells lacking VDR if VDR binds to the promoter of Claudin-2. We further

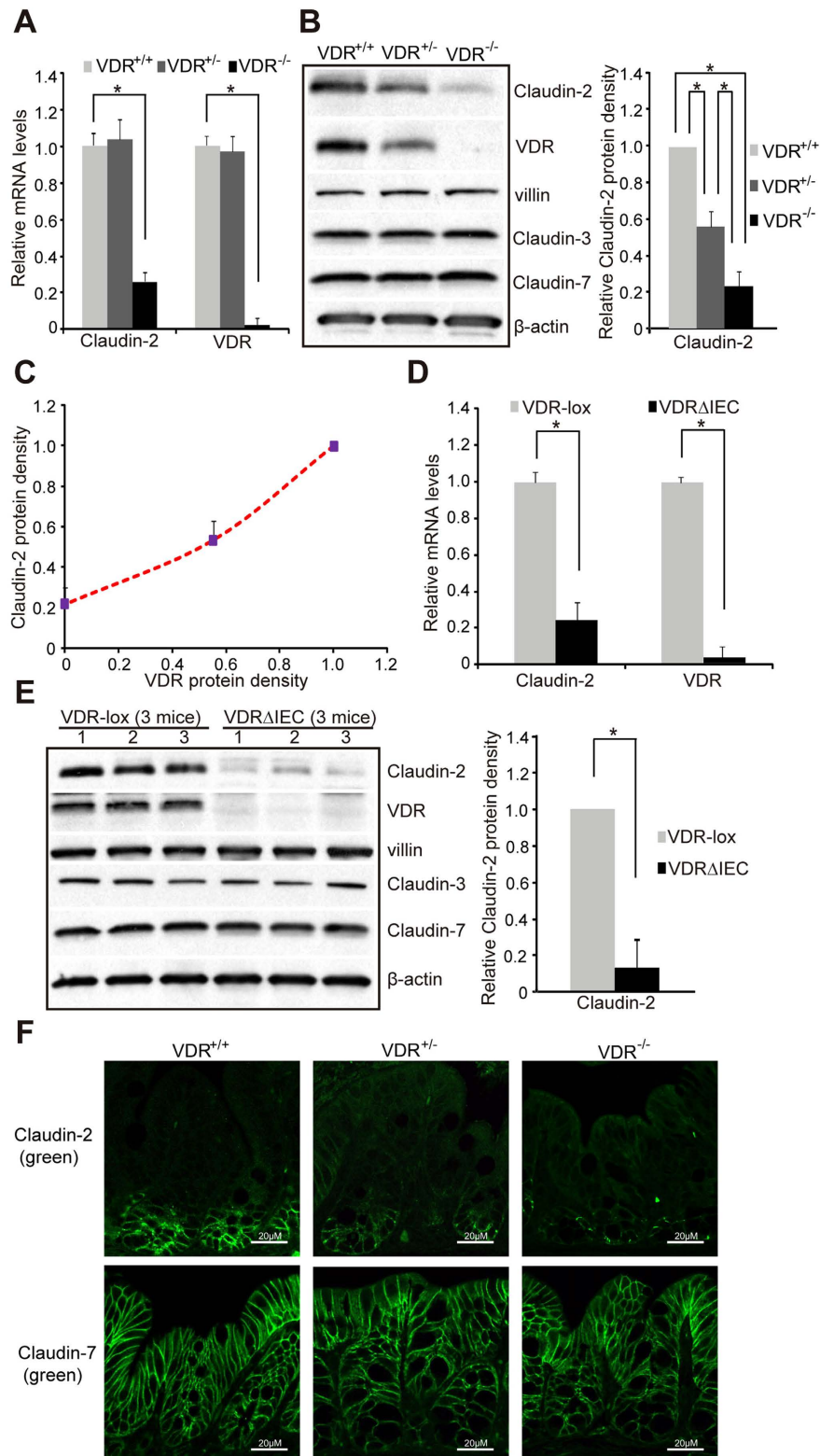


Figure 1. VDR status in intestinal epithelial cells leads to the change of Claudin-2 at both mRNA and protein levels *in vivo*. (A) Claudin-2 mRNA level and (B) Claudin-2 protein level in the intestinal epithelial cells of VDR^{+/+}, VDR^{+/-}, or VDR^{-/-} mice. (C) Claudin-2/VDR protein relative in the intestinal epithelial cells of VDR^{+/+}, VDR^{+/-}, or VDR^{-/-} mice. (D) Claudin-2 mRNA level and (E) Claudin-2 protein level in the intestinal epithelial cells of VDR KO (VDR Δ IEC) mice. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ mice/group. (F) Location and quantification of Claudin-2 protein in colons of mice *in vivo*. Images for each protein shown represent three separate experiments. $n = 3$ mice/group.

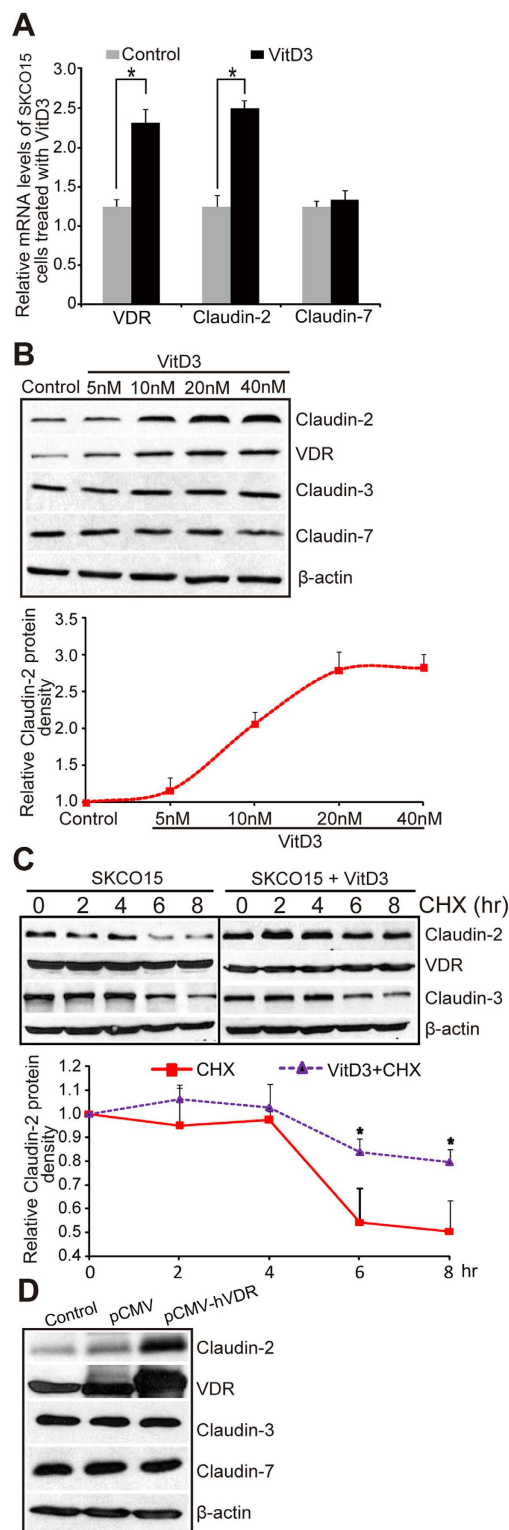
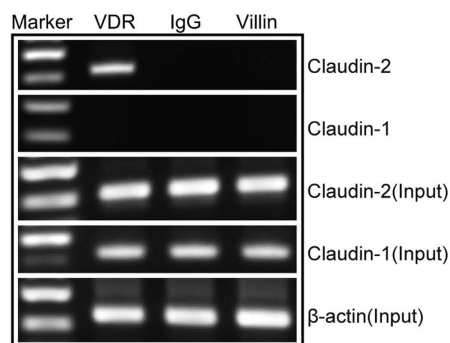
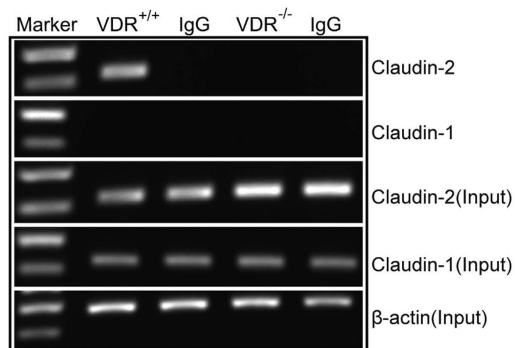


Figure 2. High levels VDR lead to increased Claudin-2 in human colonic epithelial SKCO15 cells *in vitro*. **(A)** Claudin-2 mRNA level increased post vitamin D₃ treatment. SKCO15 cells were treated with vitamin D₃ (20 nM) for 24 hours. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments. **(B)** Claudin-2 protein level and vitamin D₃ dose-dependent curve. SKCO15 cells were treated with indicated vitamin D₃ concentrations for 24 hours. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments. **(C)** Protein synthesis of Claudin-2 is high in vitamin D₃-treated SKCO15 cells. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments. **(D)** Claudin-2 expression after SKCO15 cell transfection with human VDR in a pCMV-hVDR plasmid. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments.

A SKCO15



B VDR KO mouse



C

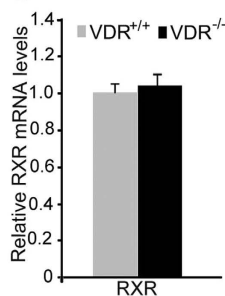


Figure 3. VDR binds to the Claudin-2 promoter. (A) CHIP-PCR amplification demonstrated binding of VDR to the promoter regions of human Claudin-2 in SKCO15 cells. PCR were performed including input-positive controls and IgG/villin-negative controls. n = 3 separate experiments. (B) ChIP-PCR assays of VDR binding to the Claudin-2 promoter in mouse colon. n = 3 separate experiments. (C) PCR analysis of RXR in VDR^{+/+} or VDR^{-/-} mice. Data are expressed as mean ± SD. *P < 0.05. n = 3 separate experiments.

tested the effects of VDR in regulating mRNA and protein levels of Claudin-2 in human colonic epithelial SKCO15 cells with VDR-siRNA. We found that Claudin-2 mRNA and protein expression were reduced when VDR was knocked down by siRNA (Fig. 4A,B). To examine the effect of one allele of VDR gene on the expression of Claudin-2, we chose VDR^{-/-} and VDR^{+/-} mouse embryonic fibroblast (MEF) cells³⁷. We found that one allele of the VDR gene in the VDR^{+/-} MEF cells was able to increase the expression of Claudin-2 protein (Fig. 4C). In contrast, Claudin-3 and -7 remained unchanged. At the transcriptional level, increased VDR mRNA was associated with elevated Claudin-2, but not Claudin-7 in VDR^{+/-} MEFs. Claudin-2 mRNA was significantly decreased in VDR^{-/-} MEF cells (Fig. 4D). This result suggests that VDR deletion affects Claudin-2 mRNA. Additionally, if we knocked down Claudin-2 by siRNA, there was no reduction of VDR at either the protein or mRNA level (Fig. 4E,F). These data suggest that Claudin-2 is downstream of VDR signaling.

VDR-enhances Claudin-2 promoter activity in a Cdx1 binding site-dependent manner. Cdx is a member of the caudal-related homeobox gene family^{38,39}. Suzuki *et al.* reports that IL-6-induced Claudin-2 promoter activity requires Cdx binding sites⁴⁰. To assess whether vitamin D3 could enhance Claudin-2 promoter activity through Cdx binding sites, we used an *in vitro* reporter Luciferase assay. A schematic drawing of transcriptional binding sites in the wild-type (WT) Claudin-2 promoter and its

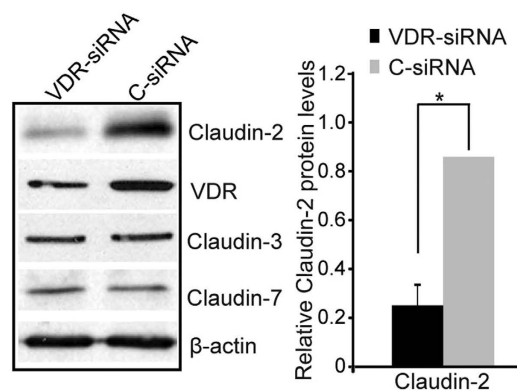
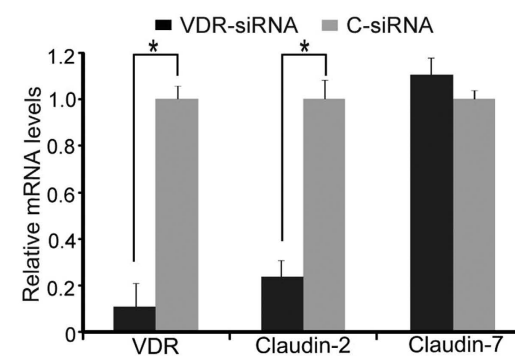
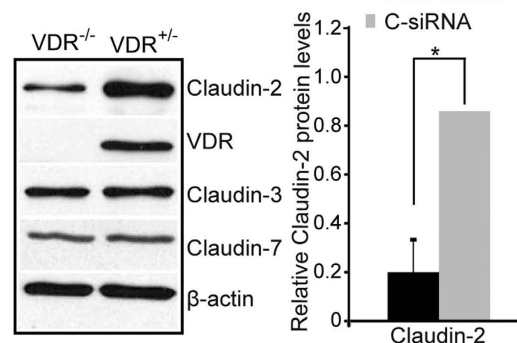
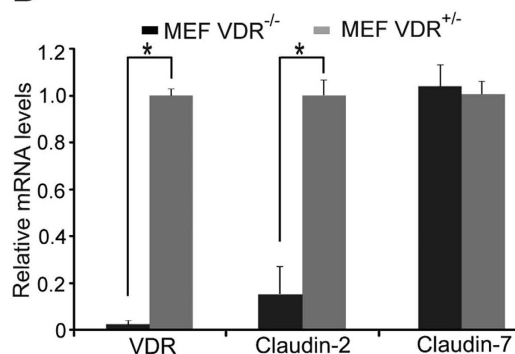
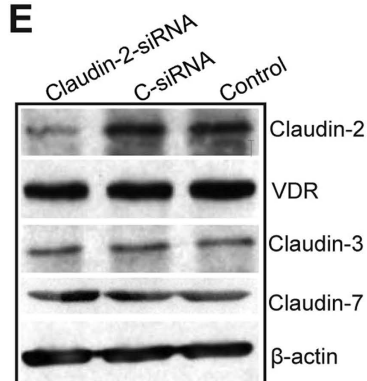
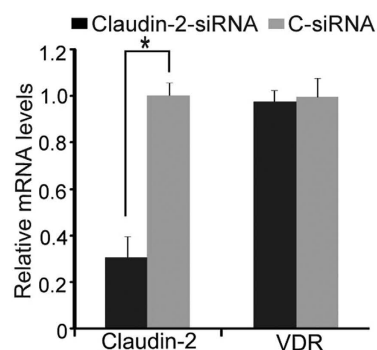
A SKCO15 cells**B****C MEF cells****D****E****F**

Figure 4. Lacking VDR decreases Claudin-2 protein and mRNA expression. (A) Claudin-2 protein and (B) mRNA were reduced by using siRNA against VDR. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments. (C) Claudin-2 protein and (D) mRNA were decreased in MEF VDR^{+/-}/VDR^{-/-} cells. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments. (E) VDR protein and (F) mRNA did not change after Claudin-2 was knocked down with Claudin-2 siRNA for 72 hours in SKCO15 cells. Data are expressed as mean \pm SD. * $P < 0.05$. $n = 3$ separate experiments.

mutants is shown in Fig. 5A. Plasmids with WT or deletions of NF κ B, STAT, or Cdx1 in the Claudin-2 promoter binding site (of Δ NF κ B, Δ STAT, or Δ Cdx) were transfected into cells, respectively, and then treated with vitamin D₃. Vitamin D₃ enhanced WT-Claudin-2 promoter activity in both HCT116 and CaCO2 cells (Fig. 5B,C). Deletions of NF κ B and STAT binding sites did not affect the Claudin-2 promoter activity. In contrast, deletions of Cdx1 binding sites clearly suppressed the promoter activity (Fig. 5B,C). These results demonstrate that vitamin D₃-induced Claudin-2 expression requires Cdx1 binding sites in the Claudin-2 promoter sequence.

Identification of a functional VDRE sequence in the Claudin-2 promoter. As Claudin-2 promoter activity is strongly elevated by exposure to 1, 25 vitamin D₃, we predicted the existence of a VDRE in the Claudin-2 promoter. Our results from the reporter assay suggested that Cdx binding sites are involved in vitamin D₃-mediated increases in Claudin-2 expression. Therefore, studies were conducted

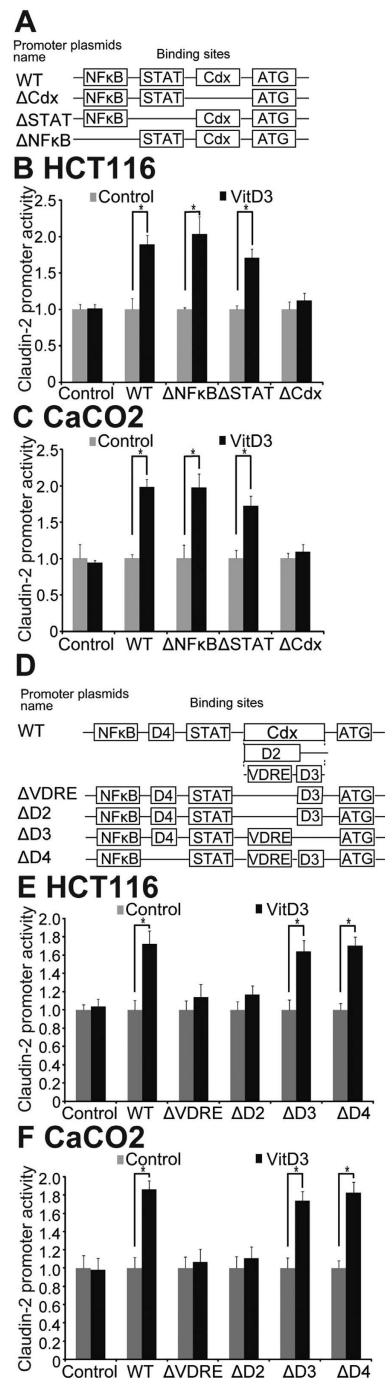


Figure 5. Identification of a functional VDRE sequence in the Claudin-2 promoter. **(A)** A schematic representation of transcriptional binding sites in the WT Claudin-2 promoter and deletion mutants. Plasmids include wild-type (WT), binding site deletions of NFκB (ΔNFκB), STAT (ΔSTAT), or Cdx1 (ΔCdx) in the Claudin-2 promoter. **(B)** WT or mutant Claudin-2 reporter plasmids were transfected in HCT116 and **(C)** CaCO2 cells. Luciferase activity was measured in the cell monolayers incubated in the absence or presence of vitamin D₃ (20 nM) for 24 hours. Dual luciferase assays were performed and firefly luciferase activity was normalized to renilla luciferase activity. Data are expressed as mean ± SD. *P < 0.05. n = 3 separate experiments. **(D)** A schematic representation of VDRE deletion construct plasmids. Putative VDR-binding sites (containing AGATAACAAAGGTCA sequence) are designated as VDRE. Deletions of all VDRE binding sites (ΔVDRE), deletion of VDRE binding sites and adjacent bases (ΔD2), and non-VDRE deletion controls (ΔD3/ΔD4). **(E)** WT Claudin-2 reporter gene plasmids and the deletion mutant plasmids were transfected to HCT116 and **(F)** CaCO2 cells. Luciferase activity was measured in the cell monolayers incubated in the absence or presence of vitamin D₃ (20 nM) for 24 hours. Data are expressed as mean ± SD. *P < 0.05. n = 3 separate experiments.

to investigate the Cdx binding site region. VDRE sequence is AGATAACAAAGGTCA⁴¹. A search of the Cdx region revealed a DR3-type, which binds preferentially to directly arrangements of two hexameric binding sites with three spacing nucleotides. PCR was used to construct deletions of all VDRE binding sites (Δ VDRE), deletion of VDRE binding sites and adjacent bases (Δ D2), and non-VDRE deletion controls (Δ D3/ Δ D4). These fragments were separately subcloned into the pGL3-basic firefly luciferase reporter plasmid. A schematic drawing of the VDRE deletion and control mutants is shown in Fig. 5D. The Claudin-2 promoter VDRE deletion constructs were transfected into cells and were subsequently treated with vitamin D₃ (20 nM). Deletions of VDRE (Δ VDRE and Δ D2) clearly lower the promoter activity of vitamin D₃ in HCT116 (Fig. 5E) and CaCO2 cells (Fig. 5F). In contrast, non-VDRE deletion controls (Δ D3 and Δ D4) did not affect the Claudin-2 promoter activity induced by vitamin D₃ (Fig. 5E&5F). Our results demonstrate that deletion of the VDRE sequence 5'-AGATAACAAAGGTCA-3' in the Claudin-2 promoter region causes loss of its responsiveness to vitamin D₃, and thus confirm that Claudin-2 is a direct target of vitamin D receptor signaling in intestinal epithelial cells.

Discussion

The experimental focus of our current study was to investigate the molecular mechanisms whereby VDR may act as a transcriptional factor to regulate the expression of Claudin-2. First, we provide molecular biological evidence that the Claudin-2 gene is a direct target of the transcription factor VDR. A transcriptional reporter study demonstrated Claudin-2 up-regulation by over-expressed VDR. CHIP-PCR data demonstrated specific binding of VDR to the Claudin-2 promoter. VDR enhanced Claudin-2 promoter activity in a Cdx1 binding site-dependent manner. Next, we identified a functional VDRE sequence within in the Claudin-2 promoter. Knockout of VDR led to lower Claudin-2 at both mRNA and protein levels. Increased VDR by vitamin D₃ pretreatment was associated with elevated Claudin-2 mRNA and protein levels. This study highlights an important and novel mechanism for VDR regulation of Claudin-2 critical to intestinal homeostasis.

Claudin-2 is a unique member of the Claudin family of transmembrane proteins as its expression is restricted to leaky epithelium *in vivo* and correlates with epithelial leakiness *in vitro*. VDR is a nuclear receptor that mediates most functions of vitamin D^{42–44}. Our data showed that activation of the *CLDN2* gene occurred via a consensus VDRE in the promoter that is bound by VDR. VDR is expressed in a wide range of tissues. Therefore, potentially, Claudin-2 can be induced in various tissues. We know that multiple factors contribute to the upregulation of Claudin-2 at the transcriptional level^{4,13,40}. TNF- α and IL-1beta contribute to elevated Claudin-2 *in vitro*^{45,46}. IL-6 enhances claudin-2 promoter activity in a Cdx binding site-dependent manner⁴⁰. VDR has multiple critical functions in regulating innate and adaptive immunity, intestinal homeostasis, host response to invasive pathogens and commensal bacteria, and tight junction structure^{35,47–54}. TJ structure plays a critical role in intestinal barrier and inflammation^{55–60}. Claudin-2 is enhanced in the inflamed gut of patients with IBD^{12,17}. The pathobiological importance of the VDR regulation of Claudin-2 could be complex. Hence, further insight into the mechanisms responsible for VDR and barrier dysfunction in mucosal inflammation is needed, especially in *in vivo* systems and disease models.

In summary, for the first time, we identify *CLDN2* gene is a direct target of VDR. Our findings reveal a novel activity of VDR in regulation of TJs in primary cell structure and intestinal homeostasis. This study fills an existing gap by characterizing the precise molecular mechanism of VDR in regulating Claudin-2 and highlights the complex role of VDR in intestinal homeostasis⁶¹. It also brings up the possibility for restoration VDR-dependent functions and prevention of the intestinal barrier breakdown in patients with intestinal disorders.

Materials and Methods

Animals. VDR^{+/+}, VDR^{+/-} and VDR^{-/-} mice on a C57BL6 background were obtained by breeding heterozygous VDR^{+/-} mice⁶². VDR flox mice were originally reported by Dr. Geert Carmeliet⁶³. VDR ^{Δ IEC} mice were obtained by crossing the VDR flox mice with villin-cre mice (Jackson Laboratory, 004586, Bar Harbor, Maine, USA), as we previously reported²¹. Experiments were performed on 2–3 months old mice. All animal work was approved by the Rush University Committee on Animal Resources. Euthanasia method was sodium pentobarbital (100 mg per kg body weight) I.P. followed by cervical dislocation.

Ethics statement. The methods in animal models were carried out accordance with the approved guidelines by the Rush University Committee on Animal Resources.

Mouse colonic epithelial cells. Mouse colonic epithelial cells were collected by scraping the tissue from the colon of the mouse, including the proximal and distal regions^{64,65}. The cells were sonicated in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, and protease inhibitor cocktail). The protein concentration was measured using the BioRad Reagent (BioRad, Hercules, CA, USA).

Cell culture. Human epithelial CaCO2 and SKCO15 cells were maintained on transwell inserts (0.33 or 4.67 cm², 0.4 mm pore. Costar, Cambridge, MA, USA) in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (Penicillin, 100 I.U./ml/Streptomycin, 100 μ g/ml), and L-glutamine

(4.5 g/L). Human colonic epithelial HCT116 cells, VDR^{+/+} and VDR^{-/-} MEF cells were cultured in DMEM medium supplemented with 10% (vol/vol) fetal bovine serum, as previously described^{62,65}.

Immunofluorescence. Colonic tissues were freshly isolated and embedded in paraffin wax after fixation with 10% neutral buffered formalin. Immunofluorescence was performed on paraffin-embedded sections (4 μm), after preparation of the slides as described previously⁶² followed by incubation for 1 hour in blocking solution (2% bovine serum albumin, 1% goat serum in HBSS) to reduce nonspecific background. The tissue samples were incubated overnight with primary antibodies at 4 °C. The following antibodies were used: anti-Claudin-2, anti-Claudin-7 (Invitrogen, Grand Island, NY, USA). Samples were then incubated with secondary antibodies (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 488, Molecular Probes, CA; 1:200) for 1 hour at room temperature. Tissues were mounted with SlowFade Antifade Kit (Life technologies, s2828, Grand Island, NY, USA), followed by a coverslip, and the edges were sealed to prevent drying. Specimens were examined with a Zeiss laser scanning microscope (LSM) 710 (Carl Zeiss Inc., Oberkochen, Germany).

Analysis of claudins distribution. Fluorescence images were analyzed using image analysis software (LSM 710 META, version 4.2; Carl Zeiss Inc., Oberkochen, Germany). Each analysis was performed in triplicate from each tissue section on a total of 10 images per mouse sample (n = 5).

Transient transfections. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) in accordance with the manufacturer's instructions. Cells were seeded on 60 mm dishes overnight before transfection with DNA and were mixed with liposome reagent at a ratio of 1:1 before addition to cells. After a 24-hour transfection period, the proteins were extracted for western-blot analysis.

Chromatin immunoprecipitation (ChIP) assays. The ChIP assays were performed essentially as described by the manufacturer (Upstate Inc., Charlottesville, VA, USA). Briefly, SKC015 cells or scraped VDR^{+/+}/VDR^{-/-} colonic epithelial cells were treated with 1% formaldehyde for 10 min at 37 °C. Cells were washed twice in ice-cold phosphate buffered saline containing protease inhibitor cocktail tablets (Roche, Nutley, NJ, USA). Cells were scraped into conical tubes, pelleted and lysed in SDS Lysis Buffer. The lysate was sonicated to shear DNA into fragments of 200–1000 bp (4 cycles of 10 s sonication, 10 s pausing, Branson Sonifier 250, Danbury, CT, USA). The chromatin samples were pre-cleared with salmon sperm DNA-bovine serum albumin-sepharose beads, then incubated overnight at 4 °C with VDR antibody (Santa Cruz Biotechnology Inc., Dallas, Texas, USA). Immune complexes were precipitated with salmon sperm DNA-bovine serum albumin-sepharose beads. DNA was prepared by treatment with proteinase K, extraction with phenol and chloroform, and ethanol precipitation, and was subjected to PCR (Primers see supplement table 1).

Western-blot analysis. Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, and protease inhibitor cocktail) and the protein concentration was measured. SKC015 and MEF cells were rinsed three times in ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and sonicated (Branson Sonifier, 250). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose (162-0112, Bio-rad, Hercules, CA, USA), and immunoblotted with primary antibodies. The following antibodies were used: anti-Claudin-2, anti-Claudin-3, anti-Claudin-7 (Invitrogen, Grand Island, NY, USA), anti-VDR, anti-Villin (Santa Cruz Biotechnology Inc., Dallas, Texas, USA), or anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) and were visualized by ECL (Thermo Scientific, Rockford, IL, USA). Membranes that were probed with more than one antibody were stripped before reprobing.

Transcriptional activation. After a 24 hour transfection period, the cells were lysed and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Firefly luciferase activity was normalized to *Renilla* luminescence activity and the activity was expressed as relative units.

Identification of functional VDRE. PCR was used to construct deletion of entire VDRE binding sites (ΔVDRE), deletion of VDRE binding site with adjacent bases (ΔD2), and control (ΔD3/ΔD4). These fragments were separately subcloned into the firefly luciferase reporter plasmid pGL3-basic (Primers see supplement table 2). Deletions of different domains of the Claudin-2 promoter cloned into the in pGL3 vector, driving luciferase expression, were transfected into HCTC116/CaCO2 cells. Luciferase activity in cell lysates was assayed by the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Real-time quantitative PCR analysis. Total RNA was extracted from mouse epithelial cells or cultured cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA). The RNA integrity was verified by electrophoresis. RNA reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The RT cDNA reaction products were

subjected to quantitative real-time PCR using CTFX 96 Real-time system (Bio-Rad, Hercules, CA, USA) and SYBR green supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. All expression levels were normalized to β -actin levels of the same sample. Percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control cells. All real-time PCR reactions were performed in triplicate. Optimal primer sequences were designed using Primer-BLAST or were obtained from Primer Bank primer pairs listed in Supplement Table 3.

Statistical Analysis. All of the data are expressed as means \pm SD. All of the statistical tests were two-sided and P values of less than 0.05 were considered to be statistically significant. Differences between two samples were analyzed using Student's t-test. The statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC).

References

- Su, L. *et al.* Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. *Gastroenterology* **136**, 551–563, doi:10.1053/j.gastro.2008.10.081 (2009).
- Al-Sadi, R. *et al.* Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. *American journal of physiology. Gastrointestinal and liver physiology* **300**, G1054–1064, doi:10.1152/ajpgi.00055.2011 (2011).
- Rahner, C., Mitic, L. L. & Anderson, J. M. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology* **120**, 411–422, doi:10.1053/gast.2001.21736 (2001).
- Sakaguchi, T. *et al.* Cloning of the human claudin-2 5'-flanking region revealed a TATA-less promoter with conserved binding sites in mouse and human for caudal-related homeodomain proteins and hepatocyte nuclear factor-1alpha. *J Biol Chem* **277**, 21361–21370, doi:10.1074/jbc.M110261200 (2002).
- Furuse, M. Molecular basis of the core structure of tight junctions. *Cold Spring Harb Perspect Biol* **2**, a002907, doi:10.1101/cshperspect.a002907 (2010).
- Anderson, J. M. & Van Itallie, C. M. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* **1**, a002584, doi:10.1101/cshperspect.a002584 (2009).
- Amasheh, S. *et al.* Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *Journal of cell science* **115**, 4969–4976, doi:10.1242/jcs.00165 (2002).
- Colegio, O. R., Van Itallie, C. M., McCrea, H. J., Rahner, C. & Anderson, J. M. Claudins create charge-selective channels in the paracellular pathway between epithelial cells. *Am J Physiol Cell Physiol* **283**, C142–147, doi:10.1152/ajpcell.00038.2002 (2002).
- Van Itallie, C. M. & Anderson, J. M. Claudins and epithelial paracellular transport. *Annual review of physiology* **68**, 403–429, doi:10.1146/annurev.physiol.68.040104.131404 (2006).
- Escaffit, F., Boudreau, F. & Beaulieu, J. F. Differential expression of claudin-2 along the human intestine: Implication of GATA-4 in the maintenance of claudin-2 in differentiating cells. *Journal of cellular physiology* **203**, 15–26, doi:10.1002/jcp.20189 (2005).
- Holmes, J. L., Van Itallie, C. M., Rasmussen, J. E. & Anderson, J. M. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. *Gene Expr Patterns* **6**, 581–588, doi:10.1016/j.modgep.2005.12.001 (2006).
- Zeissig, S. *et al.* Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* **56**, 61–72, doi:10.1136/gut.2006.094375 (2007).
- Rosenthal, R. *et al.* Claudin-2, a component of the tight junction, forms a paracellular water channel. *Journal of cell science* **123**, 1913–1921, doi:10.1242/jcs.060665 (2010).
- Schulzke, J. D. & Fromm, M. Tight junctions: molecular structure meets function. *Ann N Y Acad Sci* **1165**, 1–6, doi:10.1111/j.1749-6632.2009.04925.x (2009).
- Van Itallie, C. M., Holmes, J., Bridges, A. & Anderson, J. M. Claudin-2-dependent changes in noncharged solute flux are mediated by the extracellular domains and require attachment to the PDZ-scaffold. *Ann N Y Acad Sci* **1165**, 82–87, doi:10.1111/j.1749-6632.2009.04052.x (2009).
- Schulzke, J. D. *et al.* Epithelial tight junctions in intestinal inflammation. *Ann N Y Acad Sci* **1165**, 294–300, doi:10.1111/j.1749-6632.2009.04062.x (2009).
- Weber, C. R., Nalle, S. C., Tretiakova, M., Rubin, D. T. & Turner, J. R. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. *Lab Invest* **88**, 1110–1120, doi:10.1038/labinvest.2008.78 (2008).
- Zhang, Y. G., Wu, S., Xia, Y. & Sun, J. Salmonella infection upregulates the leaky protein claudin-2 in intestinal epithelial cells. *PLoS one* **8**, e58606, doi:10.1371/journal.pone.0058606 (2013).
- Mankertz, J. *et al.* Functional crosstalk between Wnt signaling and Cdx-related transcriptional activation in the regulation of the claudin-2 promoter activity. *Biochem Biophys Res Commun* **314**, 1001–1007, doi:10.1016/j.bbrc.2003.12.185 (2004).
- Fujita, H. *et al.* Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca²⁺ absorption between enterocytes. *Mol Biol Cell* **19**, 1912–1921, doi:10.1091/mbc.E07-09-0973 (2008).
- Wu, S. *et al.* Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. *Gut* **2014-307436**, doi:10.1136/gutjnl-2014-307436 (2014).
- Sentongo, T. A. *et al.* Vitamin D status in children, adolescents, and young adults with Crohn disease. *The American journal of clinical nutrition* **76**, 1077–1081 (2002).
- Abreu, M. T. *et al.* Measurement of vitamin D levels in inflammatory bowel disease patients reveals a subset of Crohn's disease patients with elevated 1,25-dihydroxyvitamin D and low bone mineral density. *Gut* **53**, 1129–1136, doi:10.1136/gut.2003.036657 (2004).
- Lim, W. C., Hanauer, S. B. & Li, Y. C. Mechanisms of disease: vitamin D and inflammatory bowel disease. *Nat Clin Pract Gastroenterol Hepatol* **2**, 308–315, doi:10.1038/ncpgasthep0215 (2005).
- Wang, T. T. *et al.* Direct and indirect induction by 1,25-dihydroxyvitamin D₃ of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. *J Biol Chem* **285**, 2227–2231, doi:10.1074/jbc.C109.071225 (2010).
- Rufo, P. A. & Bousvaros, A. Current therapy of inflammatory bowel disease in children. *Paediatr Drugs* **8**, 279–302, doi:10.2165/00148581-200608050-00002 (2006).
- Simmons, J. D., Mullighan, C., Welsh, K. I. & Jewell, D. P. Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. *Gut* **47**, 211–214, doi:10.1136/gut.47.2.211 (2000).
- Pei, F. H. *et al.* Vitamin D receptor gene polymorphism and ulcerative colitis susceptibility in Han Chinese. *Journal of digestive diseases* **12**, 90–98, doi:10.1111/j.1751-2980.2011.00483.x (2011).
- Naderi, N. *et al.* Association of vitamin D receptor gene polymorphisms in Iranian patients with inflammatory bowel disease. *Journal of gastroenterology and hepatology* **23**, 1816–1822, doi:10.1111/j.1440-1746.2008.05525.x (2008).

30. Kim, J. H. *et al.* Implication of intestinal VDR deficiency in inflammatory bowel disease. *Biochimica et biophysica acta* **1830**, 2118–2128, doi:10.1016/j.bbagen.2012.09.020 (2013)).
31. Hughes, D. J., McManus, R., Neary, P., O'Morain, C. & O'Sullivan, M. Common variation in the vitamin D receptor gene and risk of inflammatory bowel disease in an Irish case-control study. *European journal of gastroenterology & hepatology* **23**, 807–812, doi:10.1097/MEG.0b013e328349283e (2011).
32. Capaldo, C. T. *et al.* Tight function zonula occludens-3 regulates cyclin D1-dependent cell proliferation. *Mol Biol Cell* **22**, 1677–1685, doi:10.1091/mbc.E10-08-0677 (2011).
33. Ivanov, A. I. *et al.* Microtubules regulate disassembly of epithelial apical junctions. *BMC cell biology* **7**, 12, doi:10.1186/1471-2121-7-12 (2006).
34. Carlberg, C., Polly, P. Gene regulation by vitamin D3. *Critical reviews in eukaryotic gene expression* **8**, 19–42, doi:10.1615/CritRevEukarGeneExpr.v8.i1.20 (1998).
35. Gombart, A. F., Borregaard, N. & Koeffler, H. P. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1067–1077, doi:10.1096/fj.04-3284com (2005).
36. Sun, J. *et al.* Increased NF- κ B activity in fibroblasts lacking the vitamin D receptor. *American journal of physiology. Endocrinology and metabolism* **291**, E315–322, doi:10.1152/ajpendo.00590.2005 (2006).
37. Wu, S., Xia, Y., Liu, X. & Sun, J. Vitamin D receptor deletion leads to reduced level of IkappaBalpha protein through protein translation, protein-protein interaction, and post-translational modification. *The international journal of biochemistry & cell biology* **42**, 329–336, doi:10.1016/j.biocel.2009.11.012 (2010).
38. Charite, J. *et al.* Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development* **125**, 4349–4358 (1998).
39. Duprey, P. *et al.* A mouse gene homologous to the Drosophila gene caudal is expressed in epithelial cells from the embryonic intestine. *Genes & development* **2**, 1647–1654, doi:10.1101/gad.2.12a.1647 (1988).
40. Suzuki, T., Yoshinaga, N. & Tanabe, S. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J Biol Chem* **286**, 31263–31271, doi:10.1074/jbc.M111.238147 (2011).
41. Toell, A., Polly, P. & Carlberg, C. All natural DR3-type vitamin D response elements show a similar functionality *in vitro*. *The Biochemical journal* **352** (Pt 2), , 301–309 (2000).
42. Haussler, M. R. *et al.* The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone and Mineral Research* **13**, 325–349, doi:10.1359/jbmr.1998.13.3.32 (1998).
43. Xue, Y. & Fleet, J. C. Intestinal vitamin D receptor is required for normal calcium and bone metabolism in mice. *Gastroenterology* **136**, 1317–1327, e1311-1312, doi:10.1053/j.gastro.2008.12.051 (2009).
44. Bouillon, R. *et al.* Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev* **29**, 726–776, doi:10.1210/er.2008-0004 (2008).
45. Mankertz, J. *et al.* TNFalpha up-regulates claudin-2 expression in epithelial HT-29/B6 cells via phosphatidylinositol-3-kinase signaling. *Cell and tissue research* **336**, 67–77, doi:10.1007/s00441-009-0751-8 (2009).
46. Yamamoto, T. *et al.* IL-1beta regulates expression of Cx32, occludin, and claudin-2 of rat hepatocytes via distinct signal transduction pathways. *Experimental cell research* **299**, 427–441, doi:10.1016/j.yexcr.2004.06.011 (2004).
47. Ogura, M. *et al.* Vitamin D3 modulates the expression of bile acid regulatory genes and represses inflammation in bile duct-ligated mice. *J Pharmacol Exp Ther* **328**, 564–570, doi:10.1124/jpet.108.145987 (2009).
48. Kamen, D. L. & Tangpricha, V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. *J Mol Med* **88**, 441–450, doi:10.1007/s00109-010-0590-9 (2010).
49. Waterhouse, J. C., Perez, T. H. & Albert, P. J. Reversing bacteria-induced vitamin D receptor dysfunction is key to autoimmune disease. *Ann N Y Acad Sci* **1173**, 757–765, doi:10.1111/j.1749-6632.2009.04637.x (2009).
50. Liu, P. T., Krutzik, S. R. & Modlin, R. L. Therapeutic implications of the TLR and VDR partnership. *Trends Mol Med* **13**, 117–124, doi:10.1016/j.molmed.2007.01.006 (2007).
51. Kong, J. *et al.* Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *American journal of physiology. Gastrointestinal and liver physiology* **294**, G208–216, doi:10.1152/ajpgi.00398.2007 (2008).
52. Lagishetty, V. *et al.* Vitamin d deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* **151**, 2423–2432, doi:10.1210/en.2010-0089 (2010).
53. Adams, J. S. & Hewison, M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab* **4**, 80–90, doi:10.1038/ncpendmet0716 (2008).
54. Yu, S., Bruce, D., Froicu, M., Weaver, V. & Cantorna, M. T. Failure of T cell homing, reduced CD4/CD8alphaalpha intraepithelial lymphocytes, and inflammation in the gut of vitamin D receptor KO mice. *Proc Natl Acad Sci U S A* **105**, 20834–20839, doi:10.1073/pnas.0808700106 (2008).
55. Shen, L. & Turner, J. R. Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. *American journal of physiology. Gastrointestinal and liver physiology* **290**, G577–582, doi:10.1152/ajpgi.00439.2005 (2006).
56. Laukoetter, M. G. *et al.* JAM-A regulates permeability and inflammation in the intestine *in vivo*. *J Exp Med* **204**, 3067–3076, doi:10.1084/jem.20071416 (2007).
57. Blikslager, A. T., Moeser, A. J., Gookin, J. L., Jones, S. L. & Odle, J. Restoration of barrier function in injured intestinal mucosa. *Physiol Rev* **87**, 545–564, doi:10.1152/physrev.00012.2006 (2007).
58. Farhadi, A., Banan, A., Fields, J. & Keshavarzian, A. Intestinal barrier: an interface between health and disease. *Journal of gastroenterology and hepatology* **18**, 479–497, doi:10.1046/j.1440-1746.2003.03032.x (2003).
59. Rajapaksa, T. E., Stover-Hamer, M., Fernandez, X., Eckelhoefer, H. A. & Lo, D. D. Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery. *J Control Release* **142**, 196–205, doi:10.1016/j.jconrel.2009.10.033 (2011).
60. Ling, J., Liao, H., Clark, R., Wong, M. S. & Lo, D. D. Structural constraints for the binding of short peptides to claudin-4 revealed by surface plasmon resonance. *J Biol Chem* **283**, 30585–30595, doi:10.1074/jbc.M803548200 (2008).
61. Zhang, Y. G., Wu, S. & Sun, J. Vitamin D, Vitamin D Receptor, and Tissue Barriers. *Tissue barriers* **1**, e23118, doi:10.4161/tisb.23118 (2013).
62. Lu, R. *et al.* Chronic effects of a Salmonella type III secretion effector protein AvrA *in vivo*. *PLoS one* **5**, e10505, doi:10.1371/journal.pone.0010505 (2010).
63. Van Cromphaut, S. J. *et al.* Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci U S A* **98**, 13324–13329, doi:10.1073/pnas.231474698 (2001).
64. Duan, Y. *et al.* beta-Catenin activity negatively regulates bacteria-induced inflammation. *Lab Invest* **87**, 613–624, doi:10.1038/labinvest.3700545 (2007).
65. Sun, J. *et al.* Crosstalk between NF- κ B and beta-catenin pathways in bacterial-colonized intestinal epithelial cells. *American journal of physiology. Gastrointestinal and liver physiology* **289**, G129–137, doi:10.1152/ajpgi.00515.2004 (2005).

Acknowledgements

We want to thank Dr. Takuya Suzuki from Hiroshima University, Japan for generously providing the Claudin-2 reporter plasmids. This work was supported by Brian Piccolo Cancer Award and Swim Across America Cancer Research Award to JS.

Author Contributions

Y. Z., S. W. and R. L.: acquisition, analysis and interpretation of data; drafting of the manuscript; statistical analysis. D. Z., J. Z., G. C., E. P. and E. C. C.: technical or material support; drafting of the manuscript. J. S.: study concept and design; critical revision of the manuscript for important intellectual content; obtained funding; study supervision.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, Y.-g. *et al.* Tight junction *CLDN2* gene is a direct target of the vitamin D receptor. *Sci. Rep.* **5**, 10642; doi: 10.1038/srep10642 (2015).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>