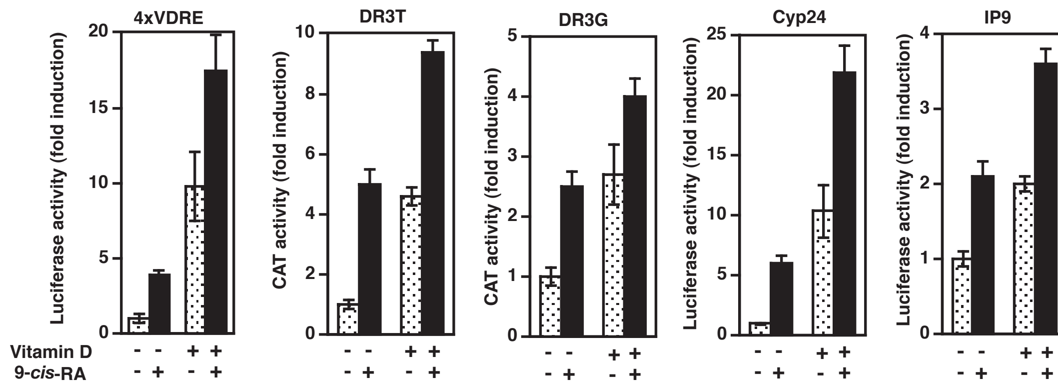
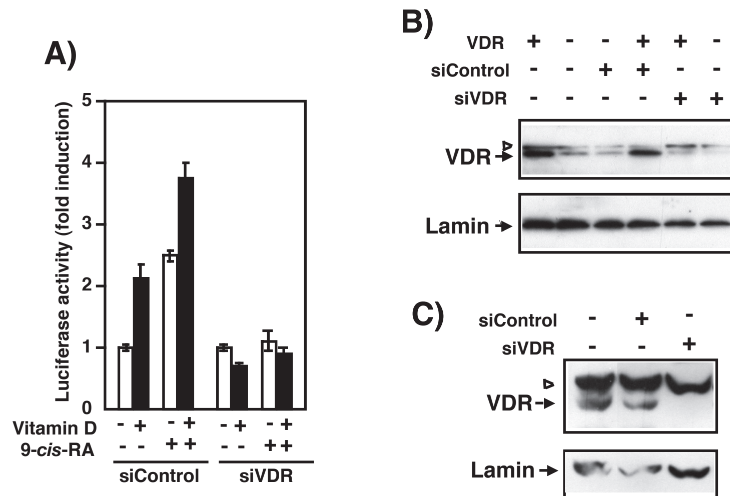
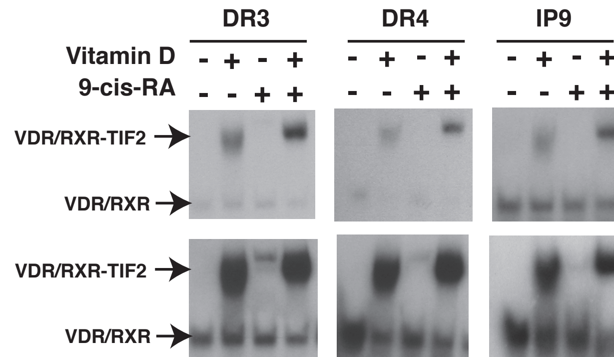


Cooperation of vitamin D and 9-*cis*-RA on different VDREs. The influence of the VDR and RXR ligands was analyzed in 293-T cells transiently transfected with VDR/RXR and various VDRE-containing reporter constructs. 4xVDRE is the luciferase reporter plasmid containing 4 copies of the DR3 (AGGTCAtgaAGGACA) present in the rat atrial natriuretic factor (ANF) promoter used in Fig.1. DR3T harbors the single response element GGTTCAcgaAGTT-CA upstream of the thymidine kinase promoter driving expression of the chloramphenicol acetyltransferase (CAT) gene. DR3G is similar, but contains the element AGGTCAaggAGGTCA. In the Cyp24 reporter, the -367 to +1 fragment of the human *cyp*24 gene is fused to luciferase. This promoter region contains two well-characterized VDREs in positions -293/-273 and -172/-143. IP9 is a luciferase reporter plasmid that harbors the sequence TGACCCtggaaaccgGGTCCA, a single palindromic element spaced by 9 oligonucleotides present in the mouse c-*fos* promoter. Regulation was similar in all cases: incubation with 9-*cis*-RA alone increased reporter activity and transactivation by vitamin D was further induced in the presence of the RXR ligand.

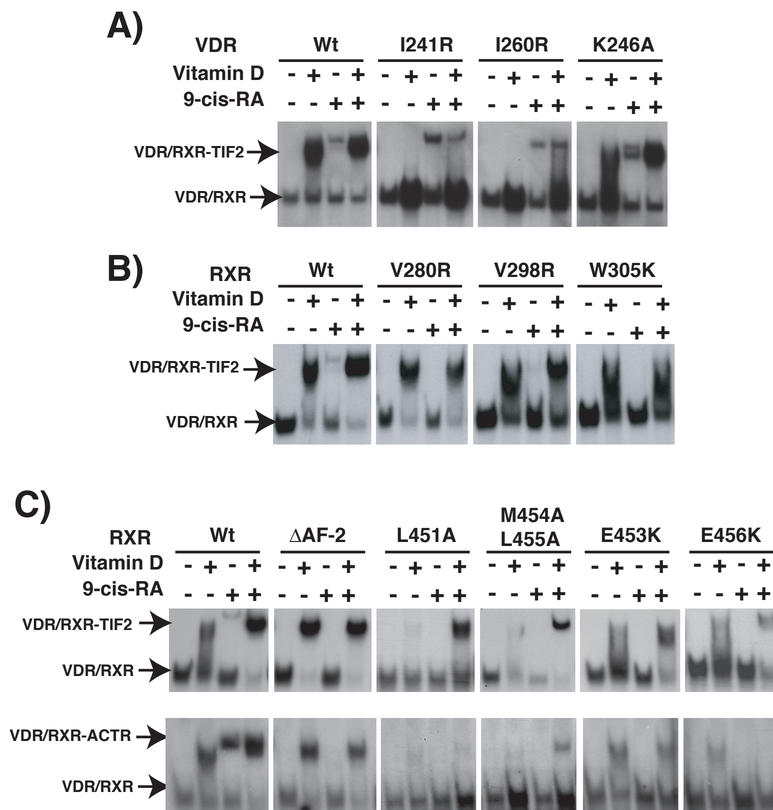


siRNA knock-down of VDR. In **panel A)** luciferase activity was determined in 293-T cells transfected with the *cyp*24 reporter plasmid described in Supplementary Fig.1 and the siRNA control (Dharmacon siCONTROL non-targeting siRNA #1) or the siRNA against VDR (Dharmacon siSMRT pool M-0034-48-00-005). Transfected cells were incubated for 48 h before treatment with vitamin D and or 9-*cis*-RA. Knock-down of endogenous VDR abolished *cyp*24 promoter stimulation by both ligands. In **Panel B**) VDR levels were analyzed by western blot in 293-T cells after 48 h of transfection with VDR or an empty vector and the siRNAs, as indicated. The empty arrowhead shows the position of a non-specific band. The VDR antibody (Chemicon) was used at a 1:1000 dilution. In the lower panel the blot was reprobed with an antibody against Lamin used as a loading control. **Panel C)** shows endogenous VDR and Lamin levels in MCF-7 cells transfected for 48 h with the control siRNA and the VDR siRNA.



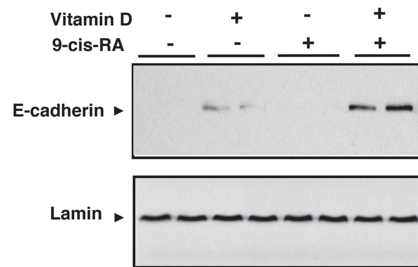
Gel retardation assays with various VDREs. In vitro translated VDR and RXR and the p160 coactivator TIF-2 fused to GST were used for band-shift assays with oligonucleotides conforming the consensus DR3-type VDRE agctcAGGTCAaggAGGTCAg, the DR4-type VDRE agcttAGTTCAtgagAGTTCAg identified in the rat Pit-1 gene, and the IP9 VDRE agctTTGCCTgggtgaatgAGGACAg of the rat osteocalcin promoter. Vitamin D and 9-*cis*-RA were present in the assays as indicated. The upper panels show low film exposures to illustrate cooperation of both ligands for coactivator recruitment. The lower panels show higher exposures suitable for observing recruitment by 9-*cis*-RA.

Supplementary Figure 4



Recruitment of coactivators by point mutants of RXR and VDR in the coactivators binding surface. Gel retardation assays were performed with the DR3 oligonucleotide agctcAGGTCAaggAGGTCAg and p160 coactivators as indicated. In **panel A)** recruitment of TIF-2 in response to vitamin D and or 9-*cis*-RA was analyzed with wild type RXR, wild type VDR (Wt) and the VDR mutants I241R (helix 3), I260R (helix 4) and K246A (helix 3). The mutations I241R and I260R had a stronger effect than the K246A mutation and totally abolished recruitment by vitamin D and the synergism with 9-*cis*-RA, although recruitment by the retinoid was not affected. In **panel B)** was analyzed the effect of equivalent RXR point mutations V280R (helix 3) and V289R (helix 4), as well as mutation W305K (helix 5) on binding of TIF-2 to VDR/RXR. These mutations inhibited coactivator recruitment by 9-*cis*-RA and and in the case of the helix 4 and helix 5 mutants also reduced the response to vitamin D. **Panel C** shows the effects of mutations in helix 12 of RXR. RXR lacking helix 12 (ΔAF-2), the point mutants L451A, E453K and E456K, and the double mutant M454A/L455A were used to study association with ACTR (upper panel) and TIF-2 (lower panel). All mutations abolished recruitment of coactivators by 9-*cis*-RA, although synergism with vitamin D was observed in some cases. Additionally, whereas deletion of RXR helix 12 did not reduce recruitment of coactivators by vitamin D, association of ACTR or TIF-2 with the heterodimer in response to the VDR ligand was markedly reduced in the case of the point mutants. These results confirm the important role allosteric communication between VDR and RXR on binding of coactivators to the VDR/RXR heterodimer.

Supplementary Fig.5



Vitamin D cooperates with a low concentration of 9-*cis*-RA to increase expression of E-cadherin in colon cancer cells. E-cadherin levels were determined by western blot in SW480-ADH cells treated with 3 nM vitamin D in the presence and absence of 10 nM 9-*cis*-RA during 48 h (upper panel). The membrane was reprobed with anti-lamin antibody (lower panel).