Cell Reports

Supplemental Information

A Vitamin D Receptor Selectively Activated by Gemini Analogs Reveals Ligand Dependent and Independent Effects

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Supplemental Experimental Procedures.

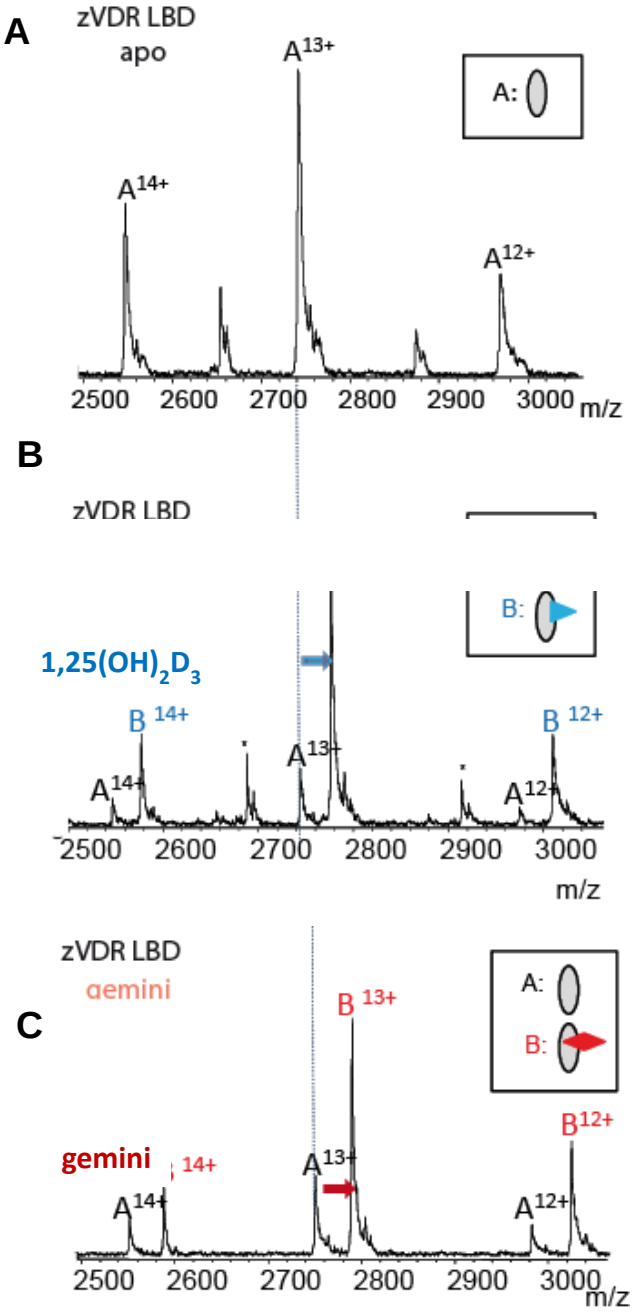
Fluorescence anisotropy measurements. Steady-state fluorescence anisotropy measurements were performed with a T-format SLM 8000 spectrofluorometer, thermostated at 20 °C. A home-built device ensured the automatic rotation of the excitation polarizer. Anisotropy titrations were carried out by adding increasing concentrations of $zVDR_{gem}$ LBD-complexes saturated with $1,25(OH)_2D_3$ or gemini, to a fixed concentration of tetramethylrhodamine (TAMRA) – SRC-1 (RHKILHRLLQEGSPS) peptide (F-SRC-P) in 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM DTT buffer. The binding stoichiometry was determined at peptide concentrations between 1 and 10 μM, while the binding constants were determined at peptide concentrations between 0.5 and 1 μM. The excitation wavelength was 530 nm and the emitted light was monitored through high-pass filters (550 nm) (Kodak). Titration curves were fitted with the Microcal Origin 6.1 software based on the nonlinear, least-squares method and the Levenberg–Marquardt algorithm as described (Sato et al., 2010).

Mammalian two-hybrid assay. Cells were transfected with 100 ng of pVPVDR or of $pVPVDR_{gem}$, 100 ng of pM-DRIP205 or pSG424-SRC-1, 100 ng of pG5-LUC reporter plasmid, 3 ng of pRL plasmid (Promega) containing the Renilla luciferase gene (transfection and cell viability control), and 698 ng of the carrier plasmid pBluescript (Stratagene). Transfections were performed with jetPEI (Polyplus transfection) according to the manufacturer's instructions. Six hours after transfection, tested compounds were added at 10 nM concentration. Cells were harvested after eighteen hours of incubation with ligands. The amount of reporter gene product (firefly luciferase) and constitutively expressed Renilla luciferase produced in cells were measured using the Dual-Luciferase® Reporter Assay System (Promega) on a luminometer plate reader (LB96P; BertholdThechnologies). Luminescence of firefly luciferase value was

normalized to the Renilla luciferase activity. Luciferase activities are expressed as relative units of light intensity. Data points represent the mean of assays performed in triplicate for at least three independent experiments.

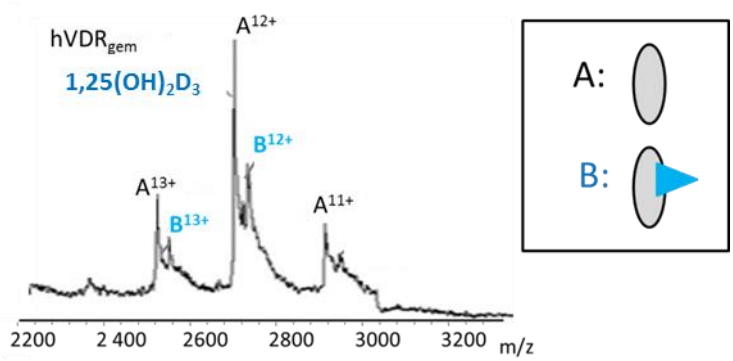
Supplemental References

Sato, Y., Ramalanjaona, N., Huet, T., Potier, N., Osz, J., Antony, P., Peluso-Iltis, C., Poussin-Courmontagne, P., Ennifar, E., Mely, Y., *et al.* (2010). The "Phantom Effect" of the Rexinoid LG100754: structural and functional insights. PLoS One *5*, e15119.



Figue lS1. Non-denaturing electrospay ionization (ESI-MS) analysis of ligand binding to zVDR, related to Figure 1. Enlarged view of the 12+, 13+ and 14+ ions of the ESI-MS spectrum of zVDR LBD in the absence of ligand (**A**) and in presence of fivefold molar excess of $1,25(OH)_2D_3$ (blue; **B**) or gemini (red; **C**). Arrows indicate the shift of the peak upon ligand binding.

A



B

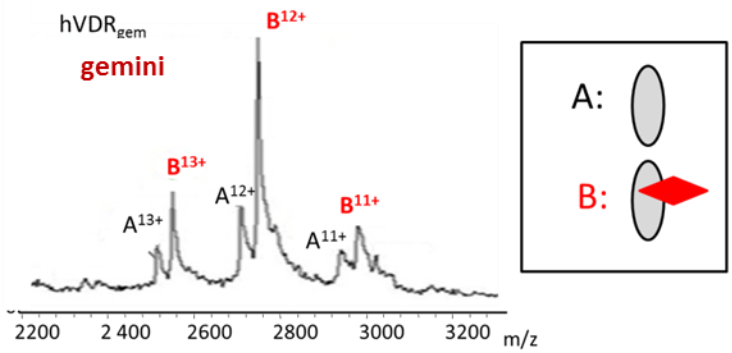


Figure S2. Non-denaturing electrospay ionization (ESI-MS) analysis of ligand binding to hVDR, related to Figure 1. Enlarged view of the 11+, 12+ and 13+ ions of the ESI-MS spectrum of hVDR$_{gem}$ LBD in the presence of fivefold molar excess of 1,25(OH)$_2$D$_3$ (bue; **A**) or gemini (red; **B**).

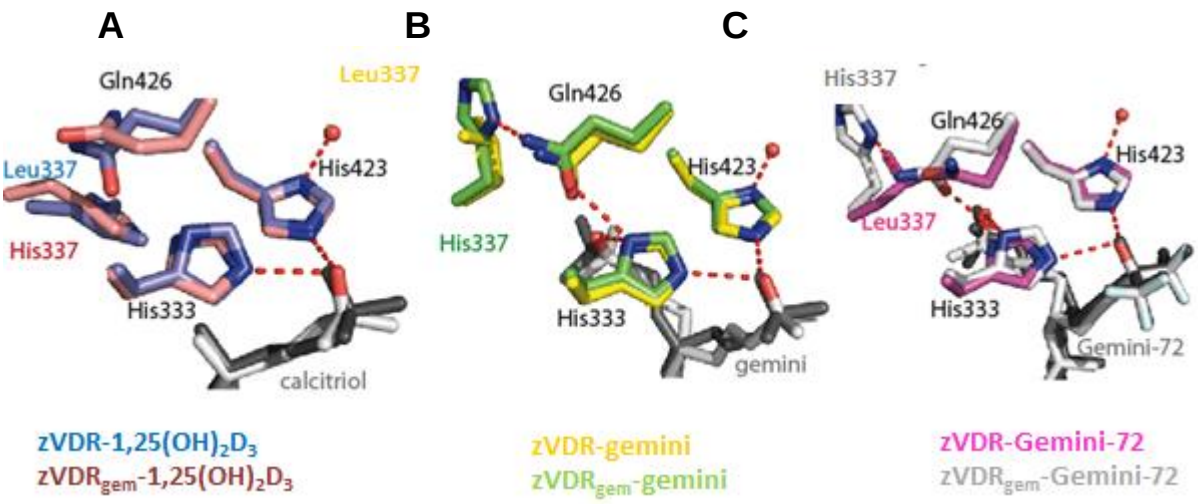


Figure S3. Detailed view of the VDR LBP in the superimposed VDR and VDR_{gem} structures in complexes with $1,25(OH)_2D_3$ (A), gemini (B) and Gemini-72 (**C**), **related to Figure 1**. zLeu337 and zHis337 side chains point towards the LBP in both zVDR-$1,25(OH)_2D_3$ (cyan) and $zVDR_{gem}$-$1,25(OH)_2D_3$ (pink) complexes. In VDR_{gem}, zHis337 perturbs Gln426 conformation and prevents its interaction with zHis333. In complex with gemini, Leu337 and His337 rotate out of the pocket in the zVDR-gemini (yellow) and $zVDR_{gem}$-gemini (green) complexes making similar interactions with His333. His337 further stabilizes Gln426 and stabilizes the hydrogen bonds network around the 25-hydroxyl group of $zVDR_{gem}$-Gemini-72 complex (grey). Red sphere is a conserved water molecule.

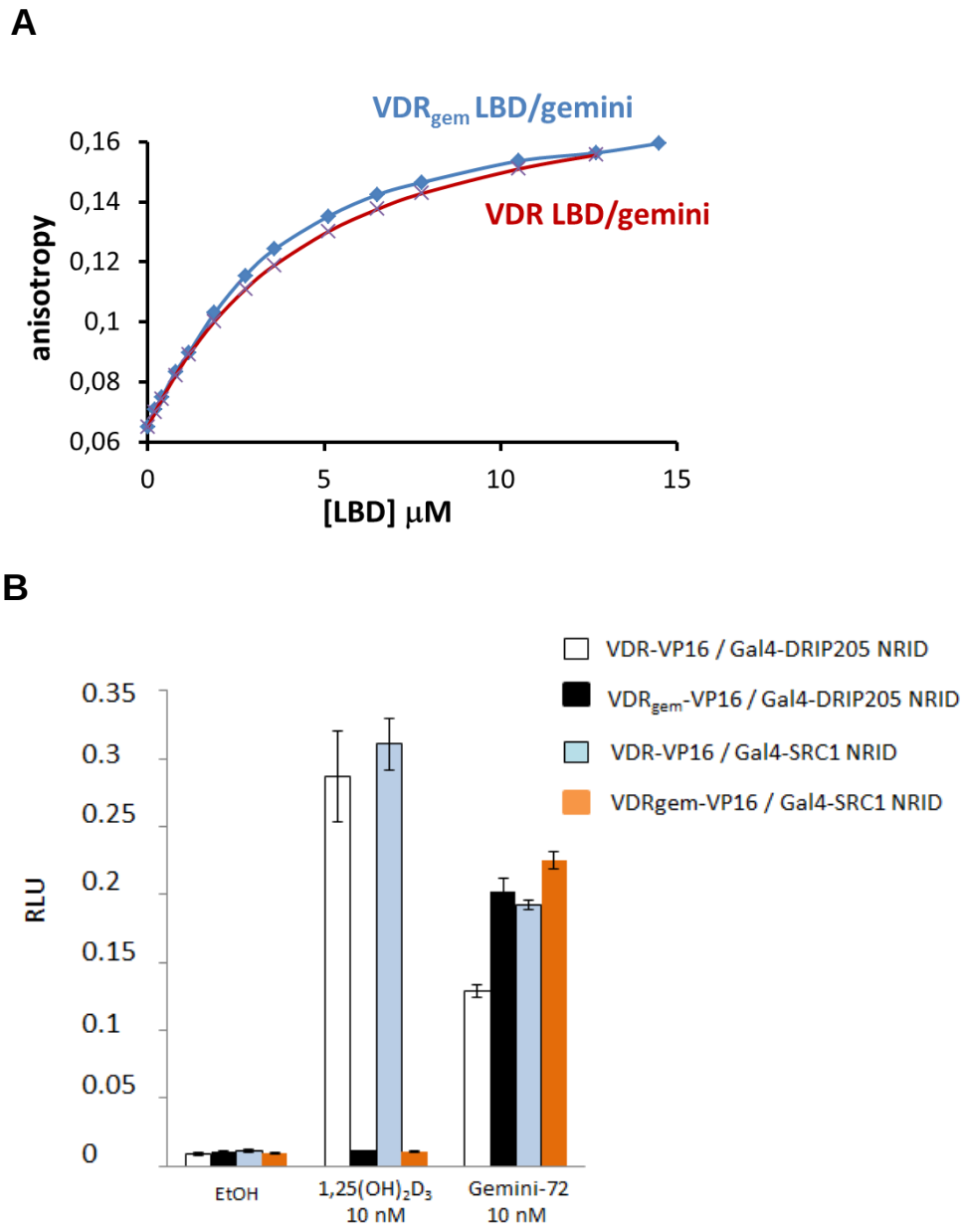


Figure S4. SRC-1 and Drip205 coactivator recruitment by VDR_{gem} related to the experimental procedures. (A) Gemini-bound VDR_{gem} LBD efficiently recruits SRC-1 coactivator peptide. Titration of the F- SRC-1 peptide with gemini bound $zVDR_{gem}$ LBD (in blue) or zVDR LBD (in red). **(B)** Mammalian two-hybrid assay showing that SRC-1 and Drip205 coactivator recruitment by VDR_{gem} is more efficient in the presence of gemini than of $1,25(OH)_2D_3$. HEK293 EBNA cells were transfected with pVPVDR or pVPVDRgem, pM-DRIP205 NRID or pSG424-SRC-1 NRID and pG5-LUC reporter plasmid, and treated with

1,25(OH)2D3, Gemini-72 or vehicle. Luciferase activities are expressed as relative units of light intensity (RLU). Results shown are the mean ± SEM of at least three independent experiments performed in triplicate.

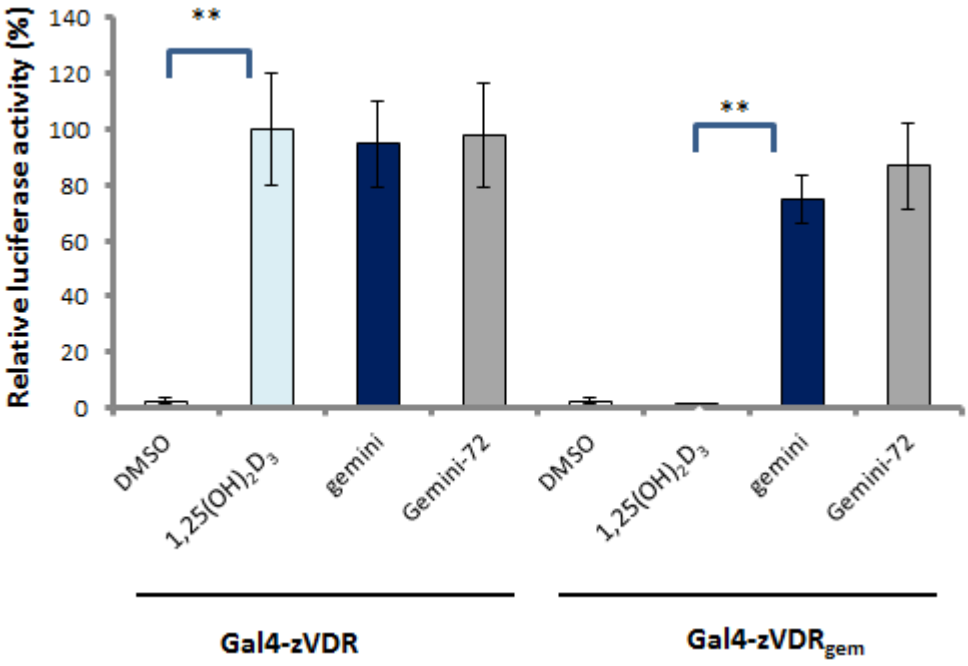


Figure S5. Transactivation efficiencies of zVDR and zVDR$_{gem}$ in response to 100 nM of 1,25(OH)$_2$D$_3$, gemini or Gemini-72, related to Figure 2. Transient transfection assays performed in HEK293 EBNA cells using Gal4-zVDR and Gal4-zVDR$_{gem}$ fusion proteins and the reporter plasmid 5x17m-TATA-luciferase. Bars represent the mean ± SEM (n=3). ** P <0.01.

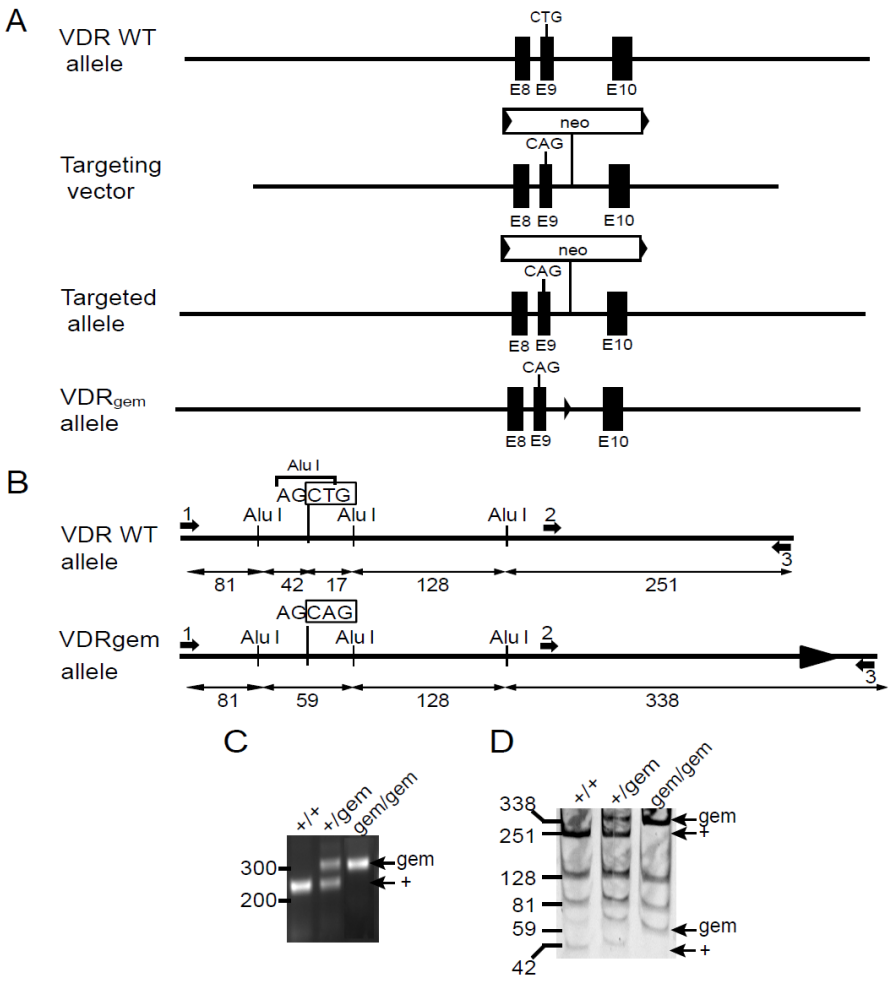


Figure S6. Generation of VDR_{gem} mice, related to the generation and characterization of mice expressing VDR_{gem}., related to Figure 3 (A) Schematic representation of the wild-type (WT) VDR allele, the targeting vector, the targeted allele in ES cell and the VDR_{gem} allele generated after Cre-mediated excision of the LoxP-flanked neomycin (neo) resistance gene. VDR exon (E) 8 -10 are indicated by black boxes. The codon CTG and CAG encoding VDR Leu304 and His304, respectively, are indicated. The open box (neo) represents the neomycin resistance gene. LoxP sites are depicted by arrowheads. **(B)** Schematic representation of the genomic region encompassing the codon encoding the VDR Leu304 and His304. Alu 1 restriction sites and the length of the DNA segments amplified by PCR with the primers indicated by arrowheads are indicated. **(C)** Visualisation of the PCR products amplified from tail genomic DNA isolated from littermates bearing one WT (+) and one VDR_{gem} allele (gem), two VDR_{gem} alleles (gem/gem) or two WT alleles (+/+) with primer 2 and 3, resolved by agarose

gel electrophoresis. **(D)** Visualisation of the PCR products amplified from tail genomic DNA isolated from littermates bearing one WT (+) and one VDR_{gem} allele (+/gem), two VDR_{gem} alleles (gem/gem) or two WT alleles (+/+) with primer 1 and 3, restricted by Alu 1, and resolved by acrylamide gel electrophoresis.

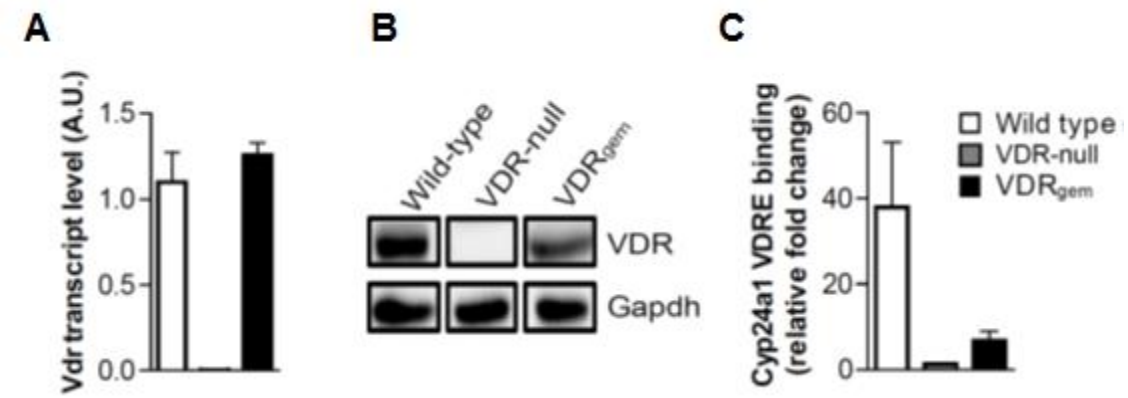


Figure S7. Duodenal VDR and VDR_{gem} transcript and protein levels and VDR and VDR_{gem} binding to Cyp24a1 VDRE, related to Figure 6. Transcript **(A)** and protein **(B)** levels of VDR and VDR_{gem} in duodenum of 16 week-old wild type, VDR-null mice and VDR_{gem}. Gapdh is used as internal control. **(C)** Binding of VDR and VDR_{gem} to the 3' region of Cyp24a1 encompassing a VDRE analysed by immunoprecipitation of chromatin from duodenum of 10 week old wild type, VDR-null and VDR_{gem} mice, with an antibody directed against VDR. Data are normalized against Ig G to account for non-specific binding. Bars represent the mean +/- SEM (n=3).

Protein	VC50	
	1,25(OH)$_2$D$_3$	**gemini**
zVDR	149 V	153 V
zVDR$_{gem}$	-	152 V
hVDR	51 V	60 V
hVDR$_{gem}$	-	60 V

Table S1. Ligands and peptide dissociation constants: Ligand dissociation experiments monitored by mass spectrometry, related to Figure 1. Accelerating voltage of the ions (VC) needed to dissociate 50% of the non-covalent complex initially present (VC50). Experiments on hVDR and zVDR were done on two distinct mass-spectrometers with different settings.

	$zVDR_{gem}$LBD-1,25$(OH)_2D_3$	$zVDR_{gem}$LBD-gemini	$zVDR_{gem}$LBD-Gemini-72
Data Processing			
Resolution (Å)	50.0-2.35 (2.43-2.35)	50.0-2.80 (2.9-2.8)	50.0-2.75 (2.85-2.75)
Crystal space group	$P6_522$	$P6_522$	$P6_522$
Cell parameters (Å)	a=b=65.85 ; c=264.37	a=b=66.09; c=265.24	a=b=65.75; c=268.85
Unique reflections	13825	9101	9462
Mean redundancy	6.2	6.2	3.8
Rsym (%)	7.5 (32.5)	8.2 (39.3)	8.8 (40.7)
Completeness (%)	91.2	98.5	98.3
Mean I/σ (%)	21.9	18.2	18.7
Refinement			
R.m.s.d. bond length (Å)	0.003	0.002	0.002
R.m.s.d. bond angles (°)	0.742	0.7	0.648
Rcryst (%)	20	20.4	20.2
Rfree (%)	25.5	25.4	24.9

Table S2. Data collection and refinement statistics, related to Figure 1.

Values in parentheses correspond to the highest resolution shell.

$R_{sym}(I) = \Sigma_{hkl} \Sigma_i | I_{hkl,i} - < I_{hkl} > | / \Sigma_{hkl} \Sigma_i | I_{hkl,i} |$ with $< I_{hkl} >$ the mean intensity of the multiple $I_{hkl,i}$ observations for symmetry-related reflections.

Root-mean-square deviation (R.m.s.d.) values are given from ideal ones.

$R_{cryst} = \Sigma_{hkl} | F_{obs} - F_{calc} | / \Sigma_{hkl} | F_{obs} |$, where F_{obs} and F_{calc} are the observed and calculated structure amplitudes, respectively. R_{free} is the same as R_{cryst}, but calculated on the 10% of data excluded from refinement.

Complexes	Kd (μM)
zVDR apo	-
zVDR-1,25$(OH)_2D_3$	1.6 ± 0.1
zVDR-gemini	3.1 ± 0.2
$zVDR_{gem}$-1,25$(OH)_2D_3$	9.0 ± 1.0
$zVDR_{gem}$-gemini	3.1 ± 0.2

Table S3. Dissociation constants of the F-SRC-1 peptide from zVDR and $zVDR_{gem}$ ligand binding domain in absence and presence of 1,25$(OH)_2D_3$ or gemini ligand, related to the SRC-1 and Drip205 coactivator recruitment by VDR_{gem}.