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Mutation goals in the vitamin D receptor predicted by computational methods

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ABSTRACT

The mechanism through which nuclear receptors respond differentially to structurally distinct agonists is a poorly understood process. We present a computational method that identifies nuclear receptor amino acids that are likely involved in biological responses triggered by ligand binding. The method involves tracing how structural changes spread from the ligand binding pocket to the sites on the receptor surface, which makes it a good tool for studying allosteric effects. We employ the method to the vitamin D receptor and verify that the identified amino acids are biologically relevant using a broad range of experimental data and a genome browser. We infer that surface vitamin D receptor residues K141, R252, I260, T280, T287 and L417 are likely involved in cell differentiation and antiproliferation, whereas P122, D149, K321, E353 and Q385 are linked to carcinogenesis.

1. Introduction

The vitamin D receptor (VDR) belongs to a family of nuclear receptor transcription factors that affect nearly every aspect of human physiology. Research has revealed that the VDR hormone 1α,25-$(OH)_2D_3$ (1,25D) performs several biological functions apart from its basic role in calcium-phosphorous homeostasis. For example, 1,25D and its analogs regulate cell differentiation and proliferation of tumor cells [1,2] and affect immunological responses [3,4]. Pathways through which the VDR regulates gene expression are remarkably complex [5,6]. The liganded VDR forms a complex with a retinoid X receptor (RXR) [7] that binds to DNA and recruits coregulator proteins that boost transcription, alter biological responses and change the genome-wide VDR binding profile [2]. Up to 80% of VDR binding sites lack the DR3 motif, implying that many of the receptor's functions result from *cis* and *trans* genomic interactions [8–10]. The biological activity of VDR ligands is likely mediated by changes in the receptor surface [11,12,6].

The aim of this study is finding amino acids on the VDR surface that exhibit structural variation upon binding 1,25D analogs. Identifying these residues is of considerable interest, as they are likely to influence VDR functions through their impact on VDR genomic targeting. We identify subtle structure changes due to ligand-protein interactions in VDR crystals using two programs: CCOMP [13] and MSITE [14]. CCOMP identifies residues with reoriented side chains while MSITE lists the nearest neighbors of specified amino acids (for example neighbors of residues which are in contact with the ligand). Paired and iteratively applied these two programs allow for identification of VDR structure changes caused by the substitution of a vitamin ligand. Combining this information with a measure of residue solvent exposure from the DSSP program [15] completes the process.

To validate our method we proceed to verify the biological relevance of these structurally active amino acids by looking at mutation studies [16–19], HDX-MS (hydrogen deuterium exchange mass spectrometry) [20], SANS (small-angle neutron scattering) and SAXS (small-angle X-ray scattering) [7], as well as associated genetic variants listed in the Ensembl genome database [21]. Given that biological experiments involving the VDR are not systematically conducted (Table 1), this study contributes an efficient methodology for finding VDR amino acids that could serve as targets for biological experiments, including mutations and interaction with comodulators.

We conducted pairwise comparisons of twelve holoVDR complexes with different biological activities (Table 1), holding as ligands analogs of 1,25D modified at carbons 2, 3, 20 and 23 (Fig. 1). To isolate functional VDR responses to ligand modifications, we compared complexes involving ligands that only differ by a single structural change in

Abbreviations: VD, vitamin D; VDR, vitamin D receptor; 1,25D, 1α,25-$(OH)_2D_3$; LBP, ligand binding pocket; RXR, retinoid X receptor; VDRRE, vitamin D receptor response element (DNA-DR3); VDRR, vitamin D-resistant rickets; SRC, steroid receptor coactivator; DRIP, vitamin D receptor interacting protein; TIF, transcriptional intermediary factor; TAF, a specific TATA binding protein associated factor; SFR, structure function relationship; HDX-MS, hydrogen deuterium exchange - mass spectrometry; SKIP, SKI oncoprotein interacting with VDR

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Table 1
Differences in biological activity of ligands in compared complexes.[a]

Compared complexes	VDR binding	Transcription	Cell differentation	Ant[i]proliferation	Calcic potency[b]		Sensitivity to p160 CoA	References for biol. data
					BCM	ICA		
1DB1-2HB8	↑	↑	↑	↑	↑	↑↑		[22,23,24,25,26]
2HB8-2HAM	↓↓		↓↓		≈			[22,23,27]
2HAM-2HB7	↑↑		↑↑		↑↑			[22,23,27]
2HAM-2HAS	↑		↑					[22]
2HB7-2HAR	≈		↓					[22,28,29]
2HAS-2HAR	↑		↓					[22]
3CS4-3A3Z	↑	↑	↑	↑			≈	[30,31]
3CS6-3A40		≈	≈	≈			↓	[30,31]
1DB1-3A78	↓↓	≈ or ↓	≈ or↓	≈				[32,33]
1DB1-1IE9	↑	↑↑↑	↑↑↑	↑↑↑	↑			[34,35,17]
3CS4-3CS6	≈	↓↓	↓	↓	↓		≈	[31,30]
3A3Z-3A40		↓	↓	↓↓	↓		↓	[30]

[a] The first ligand in every pair of complexes serves as the reference (*e.g.*, for 2HB8-2HAM the reference complex is 2HB8). The ↑ or ↓ arrows denote higher or lower activity of the second ligand *versus* the reference one. Small differences in biological functions (1.1 up to 1.9) times are denoted by ≈. Two arrows represent differences in the 10-fold to 100-fold range. Differences larger than 100-fold are indicated by three arrows.

[b] Calcemic activity is described by elevation of a serum calcium level (BCM) and intestinal calcium transport (ICA).

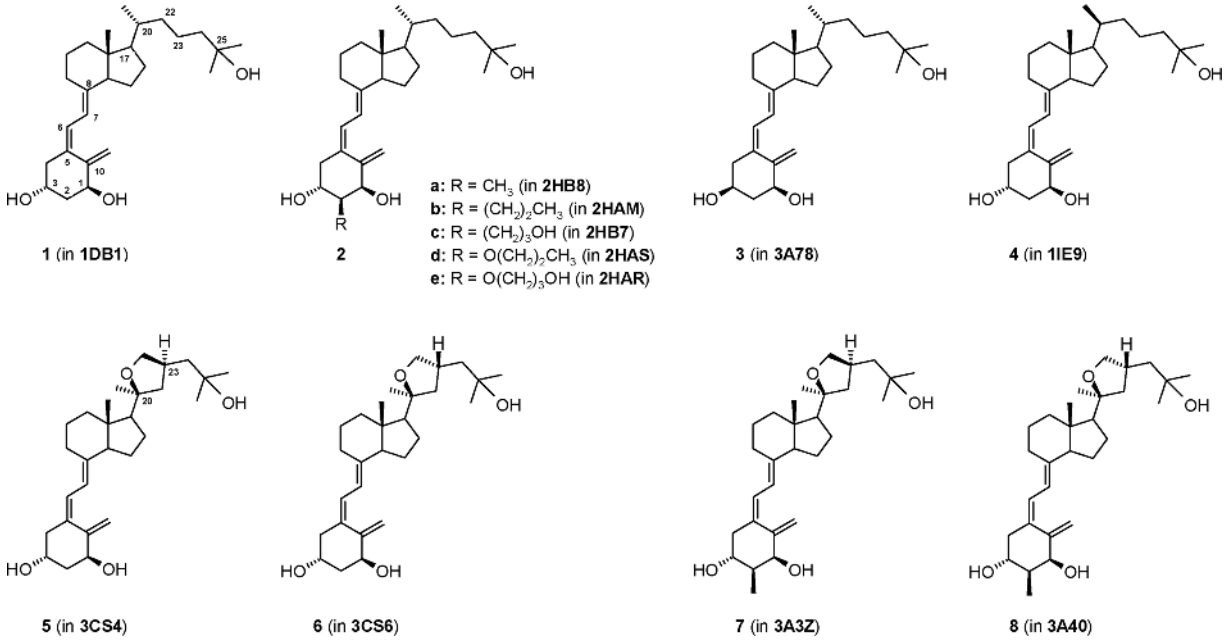

Fig. 1. Chemical structure of vitamin D ligands in studied complexes.

the A ring or side chain. The selected complexes have a high resolution (1.5–2.0 Å) and belong to the same space group (Table 2). In such high-quality crystals, one can reasonably expect that changes in the inner and surface VDR structure were induced by ligands rather than measurement error. However, to minimize the risk of finding false positives, we only consider VDR amino acids with B-factors below 40 for each of their atoms for the computational analysis (Table S1). A detailed validation of crystal complexes we published earlier [11].

2. Experimental

2.1. CCOMP

CCOMP [13] detects ligand-induced conformational changes of the side chains of VDR amino acids. This program automates the process of aligning and superimposing protein structures and calculates conformational differences between individual amino acids. By default, all amino acids with $\Delta\chi$ angle differences exceeding 10° are output by CCOMP. Considering the quality of the studied complexes (as quantified by B and R crystallographic factors), we consider differences exceeding 40° to be significant, and only amino acids exhibiting such differences are discussed in this paper (Fig. 3). The main chain backbones of complexes compared in this study are superimposed with an average value of C_α atoms (RMSD on 250 atoms) of approximately 0.2 Å.

2.2. MSITE

MSITE [14] is a simple command-line program that outputs the nearest neighbors of selected amino acids in an arbitrary number of compared complexes. MSITE takes the names of protein structures (in PDB format) as arguments and generates a list of residues grouped by the input structures. It considers amino acids to be in the neighborhood of residues specified in the input list if the distance between heavy atoms or protons does not exceed 3.5 Å.

In this study, the initial input of MSITE consisted of amino acids in contact with the ligand (Table S2) and amino acids identified by CCOMP as reoriented (Fig. 3) upon ligand binding. When an amino acid

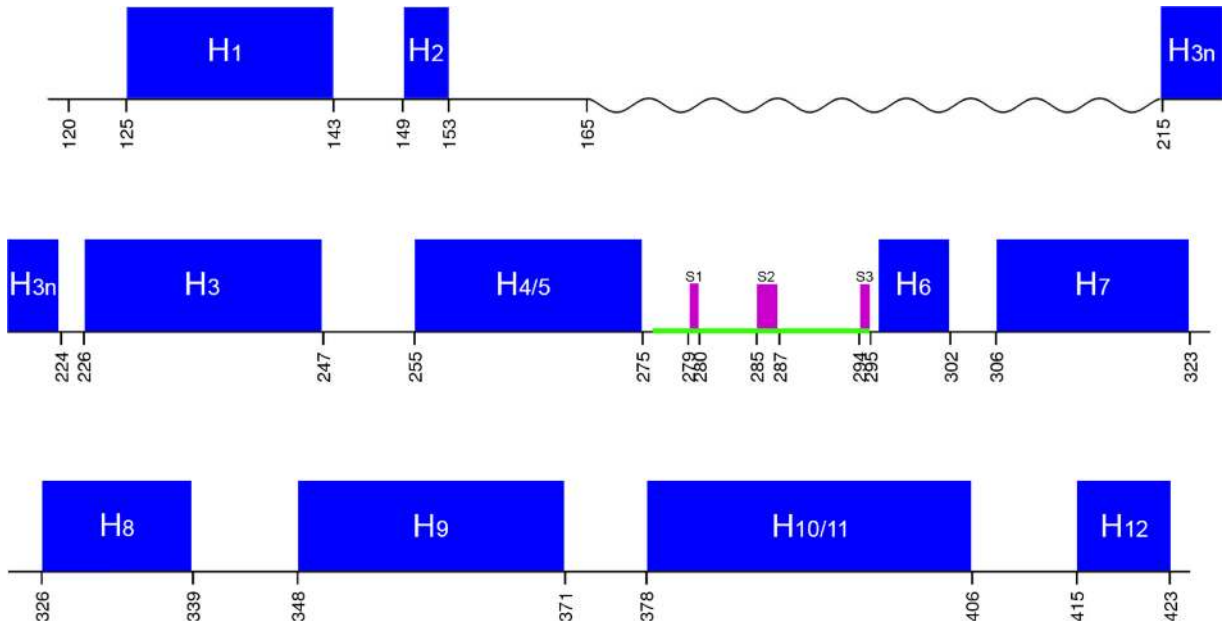

Fig. 2. Scheme of the secondary structure of the vitamin D receptor.
The figure is based on the structure of the 1DB1 complex, VDR construct 118–425, Δ(165–215). Helices are marked by solid blue rectangles. The ß hairpin loop (N276-Y295) containing three sheets is marked with a bold green line. The sheets S_1 (F279-T280), S_2 (285-T287), S_3 (K294-Y295) are denoted by solid purple rectangles. Loops are represented by thin black lines and the deleted dynamic loop (165–215) is marked with a black wavy line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 2
PDB codes of crystal VDR complexes.

PDB code	Resolution in Å	Ligand	Reference
1DB1	1.80	**1**	[36]
2HB8	2.00	**2a**	[22]
2HAM	1.90	**2b**	[22]
2HB7	1.80	**2c**	[22]
2HAS	1.96	**2d**	[22]
2HAR	1.90	**2e**	[22]
3A78	1.90	**3**	[33]
1IE9	1.40	**4**	[31]
3CS4	2.00	**5**	[31]
3CS6	1.80	**6**	[31]
3A3Z	1.72	**7**	[30]
3A40	1.45	**8**	[30]

was not part of both neighborhoods, we considered it affected by ligand binding and appended it to the next MSITE input list. This process was repeated until no new residues were identified. The results of computational analysis by MSITE are summarized in Figs. 4–6. It should be noted that our analysis was limited to amino acids detected in all twelve holoVDR complexes, specifically L120-G423, Δ [165–215].

2.3. SASA (solvent accessible surface area)

Since the transcription process is managed by co-activators, knowledge of whether the reoriented residues are exposed to solvents and capable of attracting CoA is of significant interest [6]. In this study, values for the solvent accessible surface area (SASA) were calculated using the DSSP program [15] and then normalized to the average amino acid surface. The average surface value per amino acid is defined as the surface of the central residue in a reference AXA tripeptide [56]. Table S3 contains the normalized SASA values of hVDR residues for all analyzed complexes.

2.4. Genetic variants database

Genome browsers catalog and make vast amounts of information about known variations in human DNA easily available. For this study, we used the Ensembl browser [49] to examine whether any genetic variants are present in structurally active amino acids singled out by our computational analysis, and if so, we assessed that variant's effect on protein function. Ensembl provides predictions from SIFT [57] and PolyPhen-2 [58] algorithms for variants resulting in different amino acid sequences. Additionally, FATTHM [59] predictions are available for all variants that appear in the Catalogue of Somatic Mutations in Cancer [60] (COSMIC).

SIFT, which is the oldest of the three models, builds its predictions based on the degree of conservation of amino acid residues in related proteins. PolyPhen-2 builds predictions based on sequence, phylogenetic and structural information about the substitution. FATTHM uses a machine learning approach that integrates sequence conservation measures and genomic annotations. Unlike the previously discussed algorithms, FATTHM provides predictions for both coding and non-coding sequence variants.

There are 469 genetic variations on the DNA fragment coding the VDR protein listed in the Ensembl genome database [49]. The entries relevant for this study appear in Table S4.

3. Discussion

This study compares VDR complexes liganded with analogs of 1α,25-$(OH)_2D_3$ that differed by a single structural change: carbon epimerization at positions 3-, 20-, and 23- or substitution at C-2 (Fig. 1). All of the analyzed ligands show agonistic activity, and all lactones have an *S* configuration at carbon 20. The most abundant biological data exist for ligand binding with the VDR and cell differentiation. Unfortunately, vitamin binding is not a precise predictor of the VDR-RXR complex activity. For example, a *des*-C,D vitamin D analog (compound 7 in [61]) with binding potency to the full-length recombinant rat VDR 500 times lower than the natural hormone can still induce HL-60 differentiation with potency only 100 times lower and *Cyp24a1*

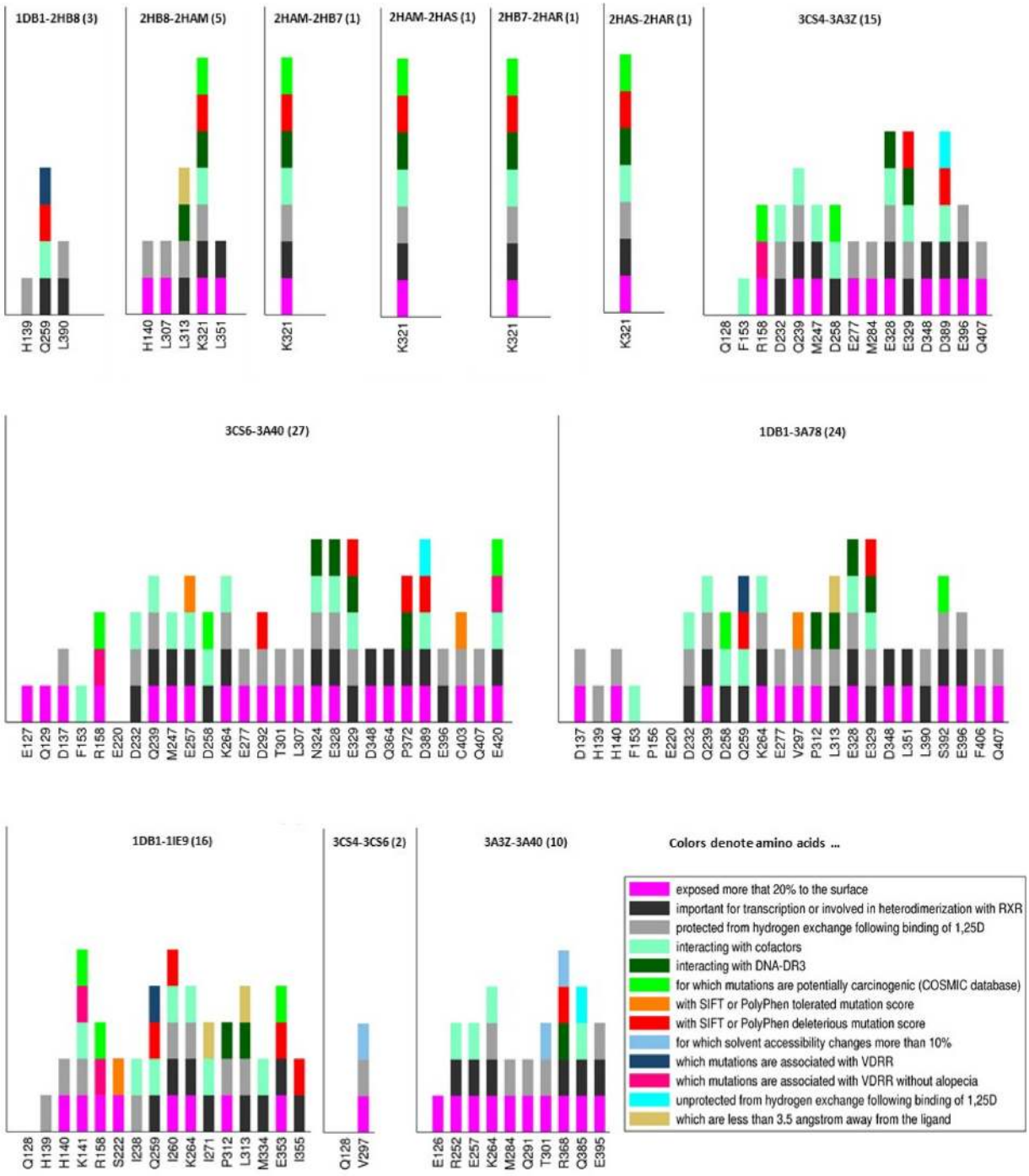

Fig. 3. VDR residues significantly ($\Delta\chi > 40^{\circ}$) changing their side chain orientation in compared complexes.
Reoriented VDR residues were identified by CCOMP. All amino acids are numbered according to the hVDR sequence. Number of reoriented residues in compared complexes is given in parenthesis next to the name of the complex pair. The location of amino acids in the VDR structure can be found in Fig. 2. The functions of residues depicted in the figure are based on the following references: for **D137**, **H139** and **H140** [20], **K141** [18], **F153** [37], **D232** [20,37], **I238** [38], **Q239** [37–41,20], **M247**, **R252**, **E257** and **D258** [39,38], **Q259** [39,42,40,43], **I260** [38–40,20], **K264** [38–40,44,20], **I271** [38,16], **E277**, **M284**, **Q291**, **D292**, **V297**, **T301** and **L307** [20], **P312** [20,45], **L313** [16,20,45], **K321** [39,20,45], **N324**, **E328** and **E329** [39,38,45,20], **M334** [39,38], **D348** [39], **L351**, **E353**, **I355** and **Q364** [39,46], **R368** [39,46,45], **P372** [39,45], **Q385** [39,47,46], **D389** [39,47,20,46], **L390**, **S392** and **E395** [39,46], **E396** [46], **C403**, **F406** and **Q407** [20], **E420** [48,40]. SIFT and PolyPhen mutation score as well as presence in the COSMIC database are sourced from [49]. The distinction between VDRR and VDRR without alopecia is based on [19,18].

transcriptions 250 times lower than 1,25D. It is worth noting that cell differentiation is directly linked to the anticancer functions of D vitamins, which makes the COSMIC variants appearing in Figs. 3–6 particularly interesting.

We started the comparison of VDR complexes using CCOMP (Fig. 3). The conformational changes of side chains are subtle and occur on approximately 11% of all protein residues. Only two reoriented amino acids (I271, L313) are part of the ligand binding pocket (LBP). Most of the residues with altered side chain conformations occupy the protein surface and have significant biological relevance, as demonstrated by the *in vivo* and *in vitro* experiments as well as the analytical and genetic data (Fig. 3 and Table S4). Since these residues are distributed

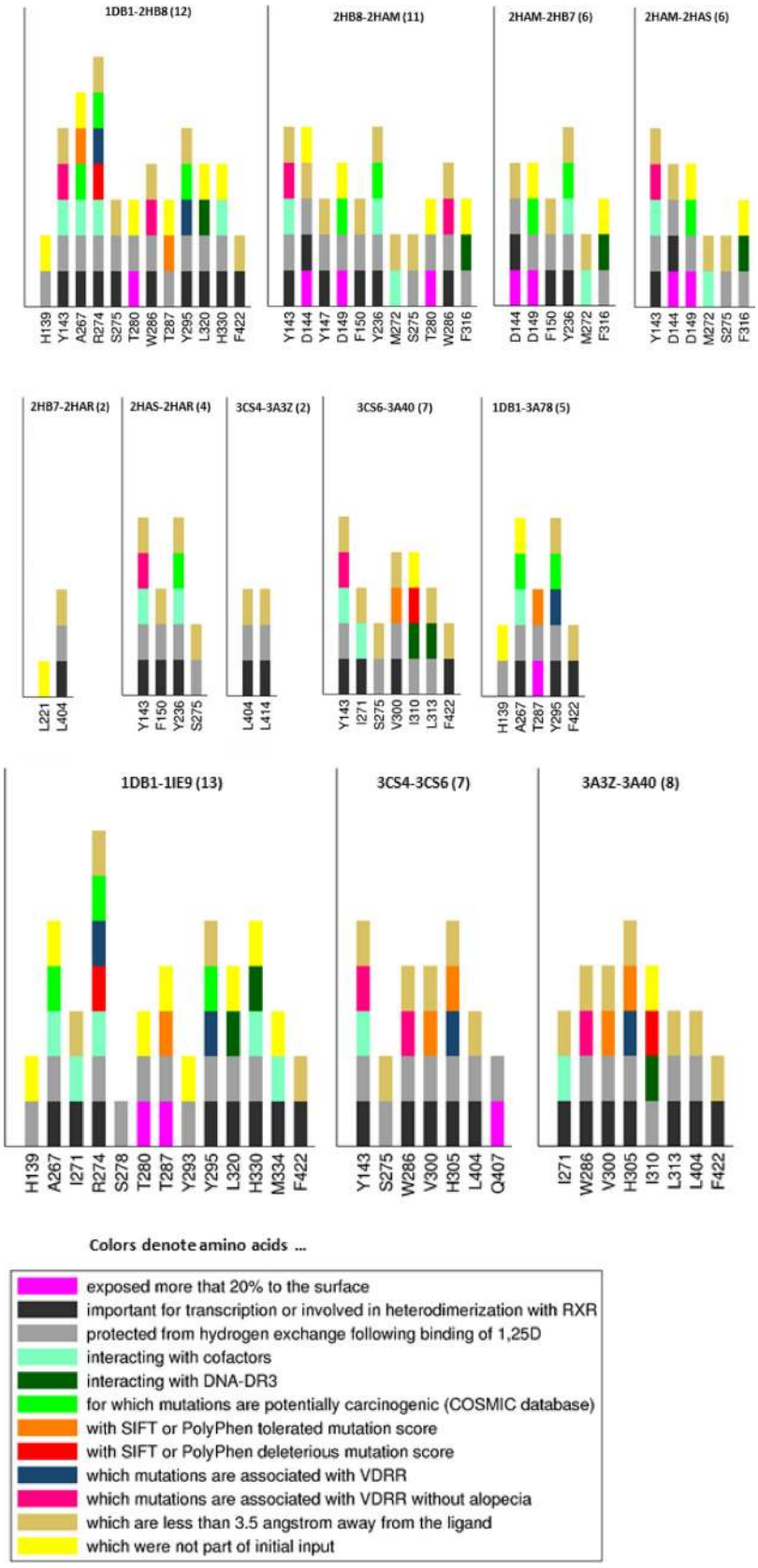

Fig. 4. VDR residues appearing as neighbors in MSITE analysis. Amino acids lining the VDR binding pocket and in contact with the vitamin ligand (distance ≤ 3.5 Å) were used as initial input for MSITE. All residues are numbered according to the human VDR sequence. The location of amino acids in the VDR structure can be found in Fig. 2. Number of residues with different neighbors in compared complexes is given in parenthesis next to the name of the complex pair. Residue functions shown in the figure are based on the following references: transcription [39,50–52,18,16], heterodimerization with RXR [39,50,18] interactions with cofactors (TAFII55 [50], DRIP205 [18], SRC1 and SRC2 [38]), interactions with DNA-DR3 from [20]. Residues protected/unprotected from hydrogen exchange following binding of 1,25D_3 to unliganded VDR-LBD are listed in [20]. SIFT and PolyPhen mutation score as well as presence in the COSMIC datasbase are sourced from [49]. The distinction between VDRR and VDRR without alopecia is based on [19,18].

throughout the LBD surface, identifying them without the help of computer tools would be impossible. In addition, these amino acids are predominantly hydrophilic and are therefore likely to be involved in electrostatic interactions and recognition of protein coactivators [62].

Our CCOMP results agree with a previous study of seventy two X-ray structures of proteins from the serine protease family [63], which used graph theory to show how small variations at the level of side-chain interactions as a result of ligand binding spread to distal sites in the

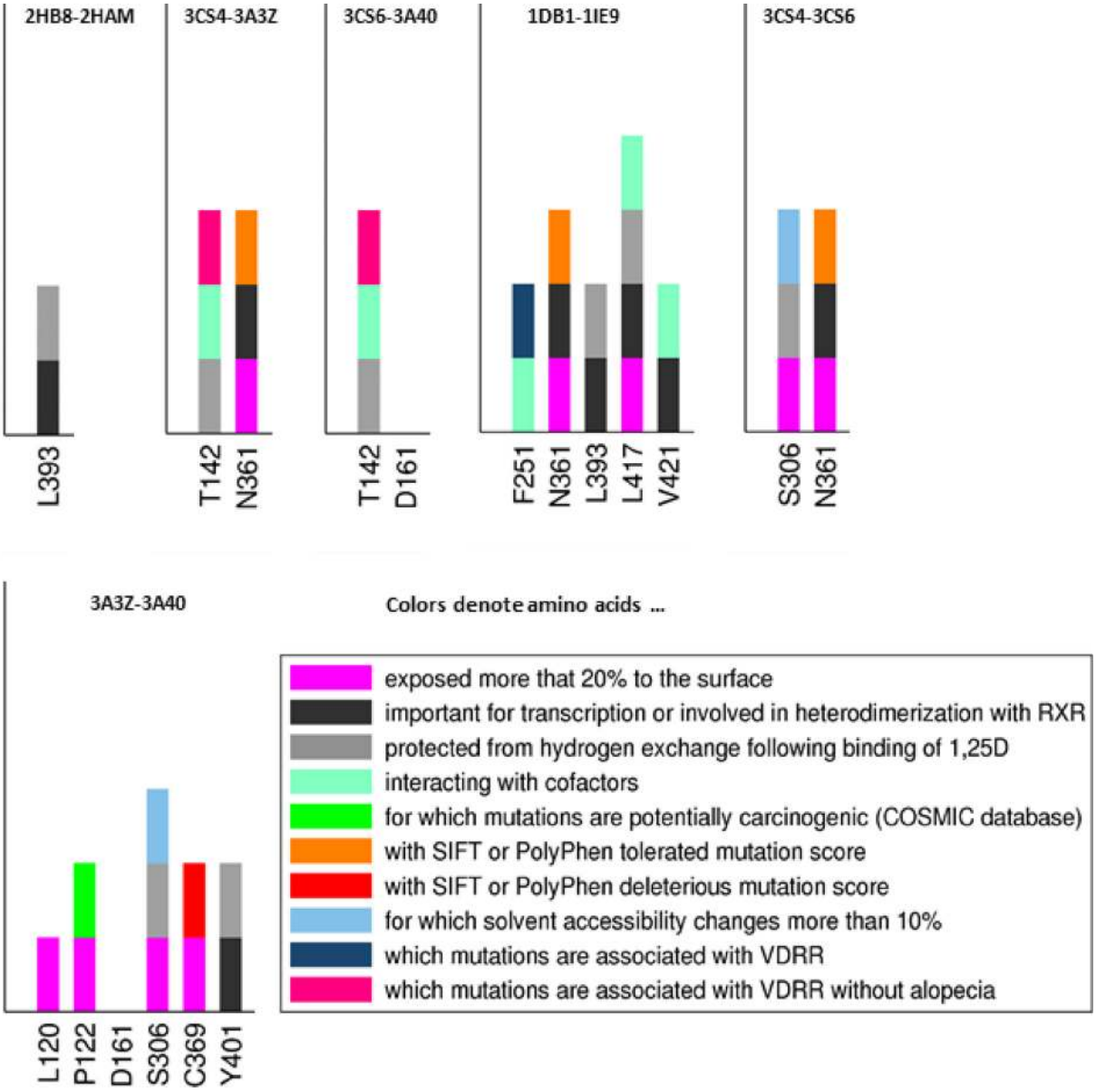

Fig. 5. VDR residues appearing as new neighbors in sequential CCOMP-MSITE analysis.
This table contains only amino acids absent in Figs. 3 and 4 in the same complex pair. Amino acids identified by CCOMP as reoriented constituted the initial MSITE input. All residues are numbered according to the human VDR sequence. The location of amino acids in VDR structure can be found in Fig. 2. Residue functions shown in the figure are based on the following references: transcription and heterodimerization with RXR [39,53–55,16,46], interactions with cofactors [40,38], interactions with DNA-DR3 [45]. Residues protected/unprotected from hydrogen exchange following binding of $1,25D_3$ to unliganded VDR-LBD are listed in [20]. SIFT and PolyPhen mutation score as well as presence in the COSMIC database are sourced from [49]. The distinction between VDRR and VDRR without alopecia is based on [19,18].

protein.

Changes in surface amino acids in complexes with significantly different biological activity are particularly interesting. In Fig. 3, two pairs of complexes, (1DB1-1IE9) and (3A3Z-3A40), fulfill these conditions (*i.e.*, they contain ligands with inverted configurations at side chain carbon 20 or 23). In the 1DB1-1IE9 pair, four amino acids exposed to the surface (K141, S222, I260, I353) were present in the CCOMP output only once (from this point on, we will refer to the amino acids that appear in a table only once as unique). It is worth noting that K141 interacts with DRIP [18] and I260 with SRC1 (PDB code: 2HC4 in [43]) coactivators, which affect cell arrest and differentiation [64,65]. Additionally, mutation of lysine 141 leads to hereditary vitamin D-resistant rickets and is listed in COSMIC, so K141 is most likely involved in the Wnt/β-catenin signaling pathway [44] and carcinogenesis. For the two remaining unique amino acids, S222 and E353, Ensembl is the only source of information (Table S4). Variants of E353 are damaging based on predictions from all three models and are linked to cancer, while the mutation for S222 is tolerated (Table S4).

In the 3A3Z-3A40 pair, all unique amino acids are exposed to the surface. The change in configuration from C-23*S* in compound **7** to C-23*R* in compound **8** reduces cell differentiation up to 10 times and antiproliferative activity almost 100 times [30]. The unique amino acids in 3A3Z-3A40 (E126, R252, Q291, R368, Q385, E395) do not appear in the COSMIC database, so their mutations are unlikely to be cancerous. However, R252 and Q385 both interact with transcription factors: arginine 252 with TAFII55 [38], and glutamine 385 with TFIIB [47]. Moreover R252 is known to come in contact with SRC1 in the 2HC4 complex ($1,25D\text{-}zVDR\text{-}SRC1_{motif}$) [43]. The R368 mutation might

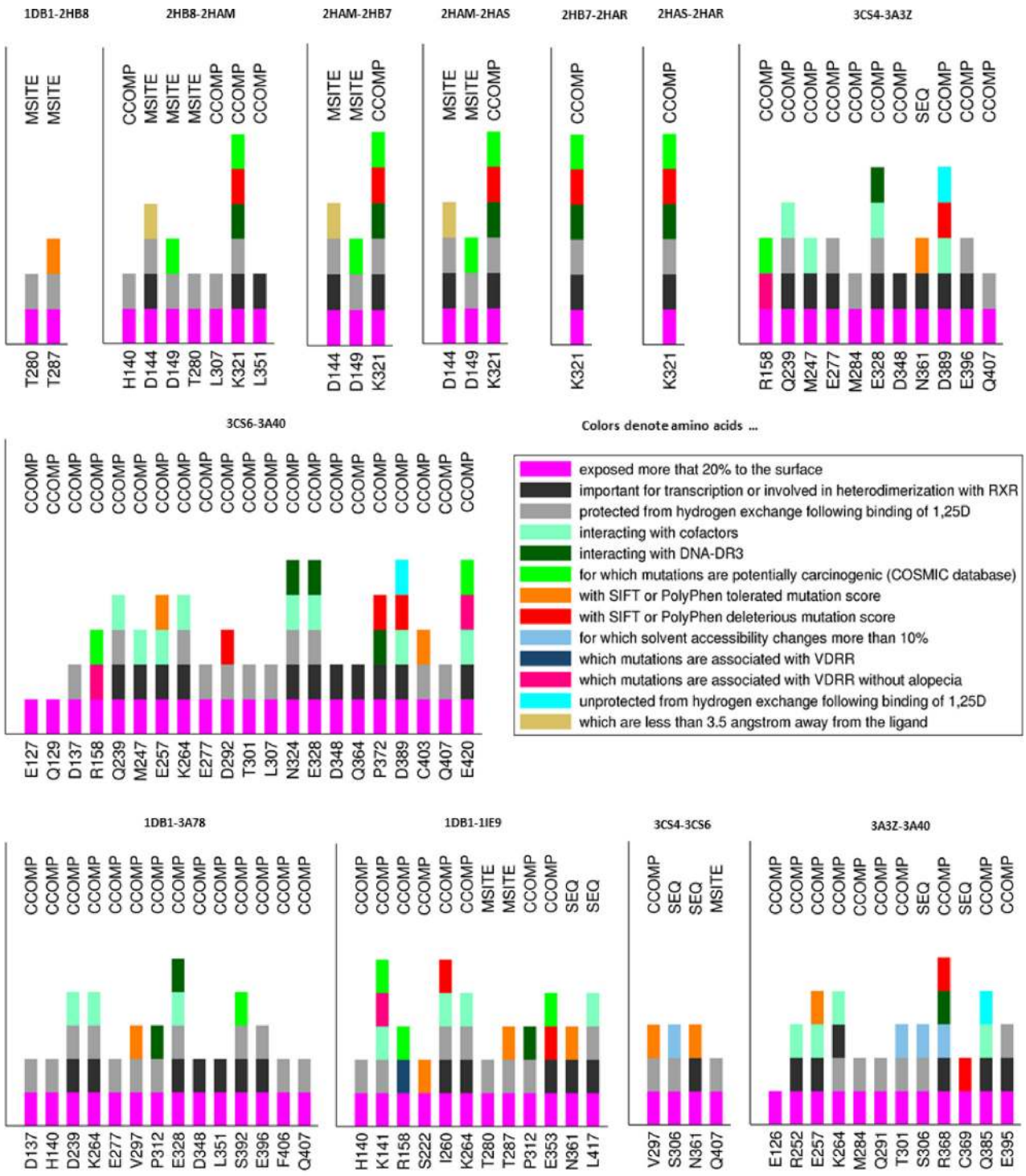

Fig. 6. Surface amino acids exhibiting changes in compared complexes.
Only amino acids with normalized solvent accessible surface area ≥ 20% are listed in this figure. Biological functions of amino acids listed here are based on a manual review of literature related to VDR complexes. All residues are numbered according to the human VDR sequence. The location of amino acids in VDR can be found in Fig. 2. Residue functions shown in the figure are based on the following references: transcription and heterodimerization with RXR [39,41,55,16,46], interactions with cofactors [38–41,47,12], interactions with DNA-DR3 [45]. Residues protected/unprotected from hydrogen exchange following binding of $1,25D_3$ to unliganded VDR-LBD are listed in [20]. SIFT and PolyPhen mutation score as well as presence in the COSMIC database are sourced from [49]. The distinction between VDRR and VDRR without alopecia is based on [19,18].

affect other protein functions and is predicted to be deleterious in the SIFT and PolyPhen models. Additionally, R368 becomes stabilized upon VDR-RXR binding to DNA [45]. We also know that Q385 binds with SKIP [47], which is an oncoprotein, and in turn, SKIP selectively interacts with holoVDR-RXR SRCs complexes to augment vitamin D receptor-activated transcription. Arginine 252 also interacts with a cofactor (SRC1) involved in cell differentiation and arrest. Only E126 has no known biological functions. Taken together, these results suggest that these amino acids constitute essential elements of the mechanism driving the biological activity of 3A3Z and 3A40 complexes.

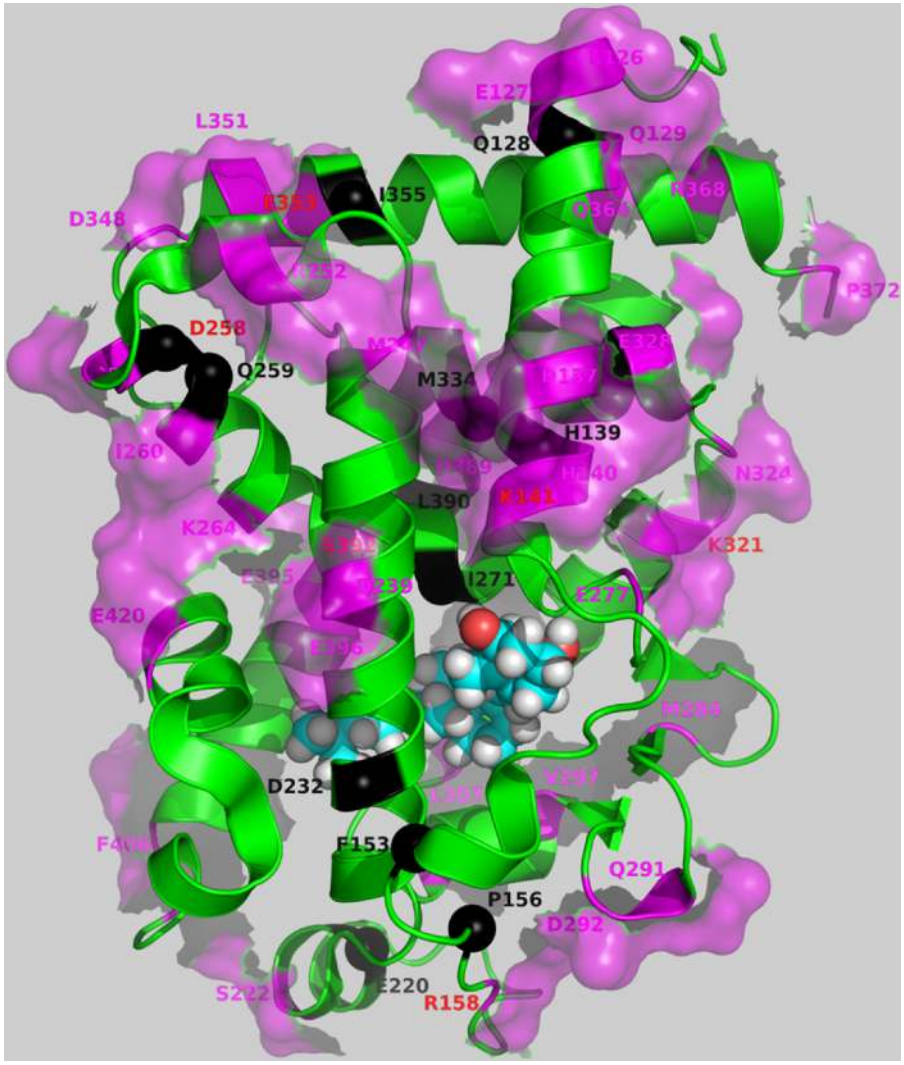

Fig. 7. VDR residues significantly ($\Delta\chi > 40^{\circ}$) changing their side chain conformations in compared complexes. Amino acids in magenta (E126, E127, Q129, D137, H140, K141, R158, S222, Q239, M247, R252, E257, I260, K264, E277, M284, Q291, D292, V297, T301, L307, P312, K321, N324, E328, D348, L351, E353, Q364, R368, P372, Q385, D389, S392, E395, E396, C403, F406, Q407, E420) are exposed to the receptor's surface and presented as spheres. They can interact with cofactors regulating biological responses of vitamin D ligands. Black solid circles denote burried residues (Q128, H139, F153, P156, E220, D232, I238, D258, Q259, I271, L313, E329, M334, D348, I355, L390). The name and number of amino acids linked to cancer are in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

In complex pairs involving 1,25D analogs with 2α-substituents none of the reoriented amino acids are unique. Lysine 321 is of particular interest because it is the only reoriented residue in four of the compared complex pairs. K321, similar to its neighbor N324, is exposed to the surface and is in the area of the VDR that interacts with the basal transcription factor-TAFII55 [38]. Extreme SIFT and PolyPhen scores indicate that substitution of this residue is expected to affect VDR function, while the fact that a K321 variant appears in the COSMIC database points to a link with cancer (Table S4).

Recent years observed the publication of many articles that suggest that not only helix 12 but also helices H6 and H7 [66] are critical for stabilizing the receptor for efficient interactions with coactivators. Our results are consistent with this finding. In all but two pairs of compared complexes (3CS4-3A3Z and 1DB1-3A78) we find reoriented amino acids from helix 6 (T301, V297) and helix 7 (L307, P312, L313, K321). Altogether, we believe that CCOMP proves to be a suitable tool to zero in on functionally important amino acids in the vitamin D receptor (Fig. 7).

Aside from conformational change, the switching of ligands also affects the neighborhood composition of amino acids lining the receptor binding pocket. The analysis of vitamin D-VDR complexes by MSITE traces transmission pathways of these neighborhood changes and identifies where they would be reflected on the protein's surface. Most changes (84%) seen in Fig. 4 occur on amino acids from the receptor's interior. In this study, we focus on changes to surface amino acids because these residues interact directly with comodulators involved in transcription and specific biological responses. Surface amino acids found by MSITE that are not present in the initial input are of particular interest ($H139_{H1}$, $D149_{H2}$, $T280_{\beta1}$, $T287_{\beta2}$, $Y293_{\Omega5\text{-}6}$, I_{310}). Three of them, specifically T280, T287, and Y293, encircle the amide bond of T286, which is the central amino acid in the VDR pocket. It is known from previous studies [67] that this β-hairpin region (F279-Y295) is important for the growth-inhibitory properties of $1,25D_3$, so these residues could be responsible for cell arrest. The variant on I310 has a deleterious SIFT score, although it is not confirmed by PolyPhen. In contrast to β-hairpins, isoleucine 310 surrounds the end of the vitamin's side chain.

The rate of amide hydrogen exchange with deuterium is highly dependent on local fluctuations in protein structure. Slower proton exchange is indicative of local stiffening of the molecule. Recent HDX-MS studies [20,45,68] revealed which parts of the VDR are stiffened by ligands. In Fig. 4, these amino acids (gray background) are very heavily represented, which confirms the validity of our method for tracking the transfer of structure changes from the pocket of the receptor to its surface.

Sequential analysis of CCOMP → MSITE found 32 distinct amino acids, 44% of which are exposed to the surface, namely $L129_{Hinge}$, $P122_{Hinge\text{-}H1}$, $D137_{H1}$, $S222_{H3n}$, $M247_{H3}$, $E277_{\Omega:H5\text{-}\beta1}$, $V297_{H6}$, $T301_{H6}$, $S306_{H7}$, $N361_{H9}$, $R368_{H9}$, $C369_{H9}$, $Q407_{\Omega:H11\text{-}H12}$, and $L417_{H12}$. They are distributed throughout the VDR surface and could not have been identified without computational analysis. Fig. 5 lists amino acids that were not found by CCOMP and MSITE alone. Of those six amino acids,

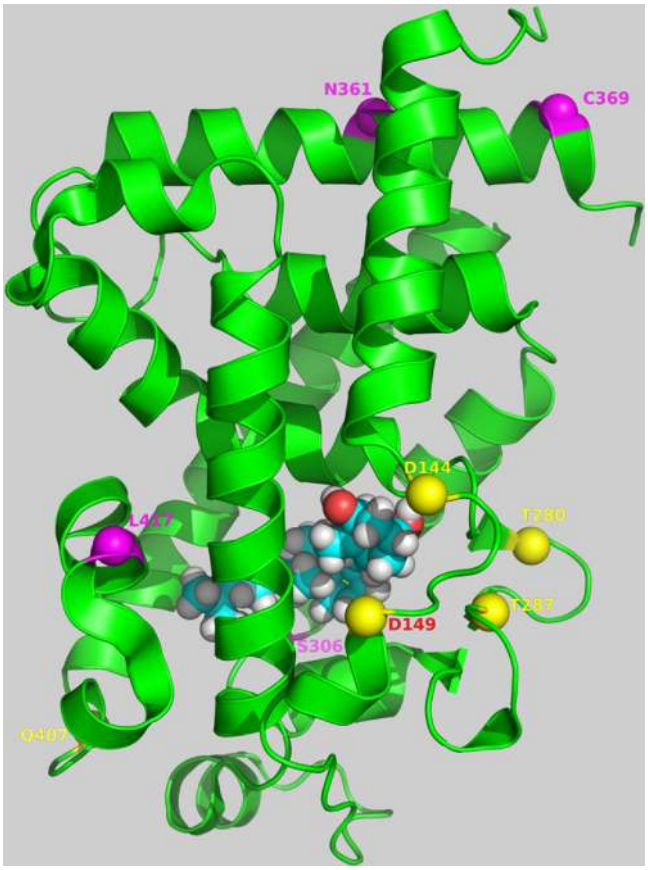

Fig. 8. VDR surface residues found by MSITE (yellow circles): D144, D149, T280, T287, Q407 and sequential CCOMP-MSITE analysis (magenta circles): S306, N361, C369, L417. The name and number of D149 linked to cancer is in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

$L120_{Hinge}$, $P122_{Hinge\text{-}H1}$, $S306_{H7}$, $N361_{H9}$, $C369_{H9}$, and $L417_{H12}$ are exposed to the surface. We limited our focus to amino acids involved in cell differentiation and carcinogenic activity in human cells. Leucine L417 interacts with DRIP and takes part in Wnt/β-catenin signaling because its mutation to serine impairs VDR-β-catenin interaction [44]. Proline 122 appears in COSMIC and has a pathogenic FATHMM score, whereas C369 is deleterious based on the SIFT model.

This result indicates that the rotation of side chains induced by changing ligands also affects the neighborhoods of amino acids, which are not on the "direct" transmission path from the binding pocket to the outer parts of the VDR and expand the possibilities for ligands to influence the VDR surface as well as the type of coactivators that bind with it (Fig. 8).

In Fig. 6, we collect amino acids from Figs. 3–5, filtering out amino acids that do not have at least 20% of their surface accessible to solvents. In this manner, we focus attention on amino acids that may interact with cofactors and modulate the biological responses of the receptor. The results from Fig. 6 are depicted in Figs. 7 and 8.

4. Summary

Uncovering the network of amino acids that are responsible for the dissemination of ligand-induced changes in the vitamin D receptor is a major challenge. A comparison of our twelve chosen crystallographic structures shows that a ligand exchange in the VDR cavity primarily leads to the reorientation of amino acid side chains (Fig. 3 *vs.* Fig. 4).

Most residues found by CCOMP and MSITE are involved in the processes leading to transcription of genes controlled by the vitamin D receptor. We found (Fig. 3) that vitamin ligands induce reorientation of side chains in less than 11% of the receptor residues. Only two of these amino acids (I271, L313) are part of the ligand binding pocket. Most of them occupy the protein's surface and have significant biological relevance, as demonstrated by *in vivo/in vitro* experiments, genetic variant data and HDX-MS analytical data. Almost 5% of the VDR amino acids experience changes in neighborhood composition when the ligands are switched (Fig. 4). These residues are mostly buried and are positioned mainly on helices $H_{4/5}$, H_1 and H_9; they probably lie on the pathway between LBP and the biologically active outer surface residues. Sequential analysis by CCOMP and MSITE revealed an additional eighteen residues, which indicates that rotation of side chains induced by ligand replacement also disturbs amino acid vicinities away from the LBP and the direct pathway to the VDR surface (Fig. 5). The results presented in Figs. 6–8 show that amino acids sensitive to ligand exchange are found all over the receptor surface and could not be spotted by visual inspection of crystal structures.

We believe that this study convincingly establishes links between ligand binding and the topology of the protein surface. We hope that our three-step method for finding active centers in holoVDR crystalline complexes can provide meaningful insight into global structural reorganization upon ligand/cofactor binding. In particular, this work can inform the selection of mutation candidates. Richer data on the effects of mutations on coactivator binding could help establish the sequence in which coactivators interact with the VDR and, consequently, determine the sources of selective activity of vitamin D analogs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2018.06.016.

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