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Vitamin D receptor(s): In the nucleus but also at membranes?

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Abstract

The genomic actions of the vitamin D are mediated *via* its biologically most potent metabolite 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and the transcription factor vitamin D receptor (VDR). Activation of VDR by 1,25$(OH)_2D_3$ leads to change in the expression of more 1000 genes in various human tissues. Based on (epi)genome, transcriptome and crystal structure data the molecular details of this nuclear vitamin D signalling pathway are well understood. Vitamin D is known for its role on calcium homeostasis and bone formation, but it also modulates energy metabolism, innate and adaptive immunity as well as cellular growth, differentiation and apoptosis. The observation of rapid, non-genomic effects of 1,25$(OH)_2D_3$ at cellular membranes and in the cytosol initiated the question, whether there are alternative vitamin D-binding proteins in these cellular compartments. So far, the best candidate is the enzyme PDIA3 (protein disulphide isomerase family A member 3), which is found at various subcellular locations. Furthermore, also VDR seems to play a role in membrane-based responses to vitamin D. In this viewpoint, we will dispute whether these rapid, non-genomic pathways are a meaningful addition to the genome-wide effects of vitamin D.

KEYWORDS
genomic signaling, non-genomic signaling, vitamin D, vitamin D receptor

1 | INTRODUCTION

Secosteroids are steroid compounds with an open B-ring commonly found in fungi (vitamin D_2), plants, invertebrates and vertebrates (vitamin D_3). Evolutionary, vitamin D_3 is a very old molecule,[1] which is created in a non-enzymatic reaction when the direct cholesterol precursor 7-dehydrocholesterol is exposed to UV-B (290-320 nm),[2,3] *that is*, cholesterol synthesizing species use(d) vitamin D_3 for UV-B scavenging[4] (Figure 1). This implies that also humans can synthesize vitamin D_3 in their skin. Through the action of a variety of cytochrome P450 (CYP) enzymes numerous vitamin D metabolites are created,[5] of which 25-hydroxyvitamin D_3 (25$(OH)D_3$) is the most stable (serum half-life some 3 weeks[6]) and abundant (a vitamin D sufficient person should have 25$(OH)D_3$ serum concentration of at least 75 nM[7]). The starting point of what is called today vitamin D endocrinology was some 550 million years ago, when in a boneless fish a receptor evolved that bound with high affinity the metabolite 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$).[8] This high affinity

Abbreviations: 1, 25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1, 25D_3-MARRS, membrane-associated rapid response to steroid; 25$(OH)D_3$, 25-hydroxyvitamin D_3; AR, androgen receptor; CALR, calreticulin; CAMK2G, calcium/calmodulin-dependent protein kinase II gamma; CANX, calnexin; CAV1, caveolin 1; CUBN, cubilin; CYP, cytochrome P450; DBP, vitamin D-binding protein; ER, estrogen receptor; GPCR , G-coupled membrane receptor; GR, glucocorticoid receptor; HSPA, heat shock protein family A (Hsp70); LBD, ligand-binding domain; LBP, ligand-binding pocket; LRP2, LDL receptor-related protein 2; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; PDIA3, protein disulphide isomerase family A member 3; PKC, protein kinase C; PLA2, phospholipase A2; PLAA, phospholipase A2 activating protein; PLC, phospholipase C; SHH, sonic hedgehog signalling molecule; SLCO, solute carrier organic anion transporter family; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; VDR, vitamin D receptor; WNT5A, Wnt family member 5A.

is necessary, since the serum concentrations of $1,25(OH)_2D_3$ are in average 1000 times lower than that of $25(OH)D_3$.[9,10]

The vitamin D receptor (VDR) is a member of the transcription factor superfamily of nuclear receptors, thus, vitamin D found *via* $1,25(OH)_2D_3$ a direct way to regulate genes[11-13] (Figure 1). These genomic actions of vitamin D take a few hours before physiological effects can be observed, since RNA and proteins need to be synthesized. In contrast, since more than 30 years so-called non-genomic actions of vitamin D have been described, which are rapid (seconds to minutes) responses to vitamin D_3 and its metabolites that are claimed not to involve the VDR protein and do not require the activation of genes[14] (Figure 2). By analogy to common signal transduction pathways of hydrophilic compounds that cannot pass the cell membrane, membrane receptors for vitamin D were postulated.[15] In this discussion paper, one of us (C. Carlberg) will provide evidence that VDR is the unique natural target for $1,25(OH)_2D_3$, while the other (M.A. Zmijewski) will explore alternative pathways and receptors triggered by vitamin D_3 and its metabolites.

2 | VDR IS THE CENTRAL PROTEIN FOR VITAMIN D SIGNALLING

In humans, the nuclear receptor superfamily comprises 48 members, 12 of which are classical endocrine receptors, since they bind their ligands in the nanomolar range.[16] VDR is one of them and enjoys the entourage of the receptors for the steroids cortisol (GR (glucocorticoid receptor)), aldosterone (MR (mineralocorticoid receptor)), estrogen (ERα and ERβ), progesterone (PR) and testosterone (AR (androgen receptor)) as well as for thyroid hormone (THRα and THRβ) and retinoic acid (RARα, RARβ and RARγ).[17] All nuclear receptors carry a structurally conserved ligand-binding domain (LBD) being a 3-layer sandwich formed by 11-13 α-helices.[18] Within the lower part of the LBD of endocrine nuclear receptors, a cavity, referred to as ligand-binding pocket (LBP), has evolved. With a volume of 300-700 Å,[3] the LBP perfectly fits to the shape of the specific ligand[19] (Figure 1).

There is an interesting co-evolution between nuclear receptors, their ligands and other proteins binding these ligands,[20,21] where the genes encoding for specific metabolic enzymes and transporters control the concentration of metabolites, which act as specific ligands for nuclear receptors regulating these genes. In case of vitamin D, the gene *CYP27B1* encoding for the 1α-hydroxylase converting $25(OH)D_3$ into $1,25(OH)_2D_3$ is down-regulated in kidney cells by $1,25(OH)_2D_3$-activated VDR,[22] while the *CYP24A1* gene, which is encoding for the 24-hydroxylase inactivating $25(OH)D_3$ and $1,25(OH)_2D_3$, is up-regulated by VDR.[23] Another important enzyme is CYP2R1, which in the liver mediates the hydroxylation of vitamin D_3 to $25(OH)D_3$.[24,25] The vitamin D-binding protein (DBP), which is the major transport vehicle for vitamin D_3 and $25(OH)D_3$ in serum,[26] completes the list of key proteins of vitamin D endocrinology.

A general principle of biochemistry indicates that the affinity of a metabolite-binding protein, such as an enzyme, transporter or receptor, is in the order of the physiological concentrations of its ligand.[27] Accordingly, the binding affinity of the enzymes CYP2R1, CYP27B1 and CYP24A1 as well as of DBP for the abundant compounds vitamin

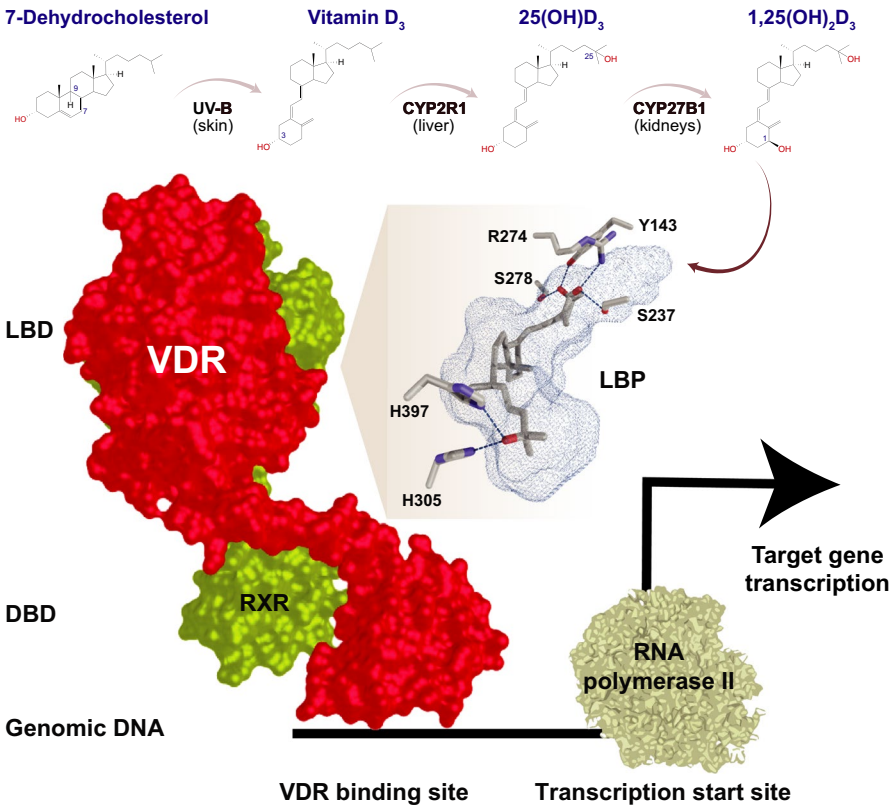

FIGURE 1 Nuclear vitamin D signalling. Vitamin D_3 is produced in the skin from UV-B radiated 7-dehydrocholesterol, in the liver converted by CYP2R1 to $25(OH)D_3$ and further in the kidneys (and other cell types) by CYP27B1 to $1,25(OH)_2D_3$ (top). The LBP is located in the lower part of VDR's LBD, in which the three indicated amino acid pairs fix the hydroxyl groups of $1,25(OH)_2D_3$ with high specificity and affinity (centre). VDR binds with its DBD (DNA-binding domain) and support by its partner receptor retinoid X receptor (RXR) to accessible sites on genomic DNA and modulates the activity of RNA polymerase II on transcription start sites of hundreds of target genes per cell type (bottom)

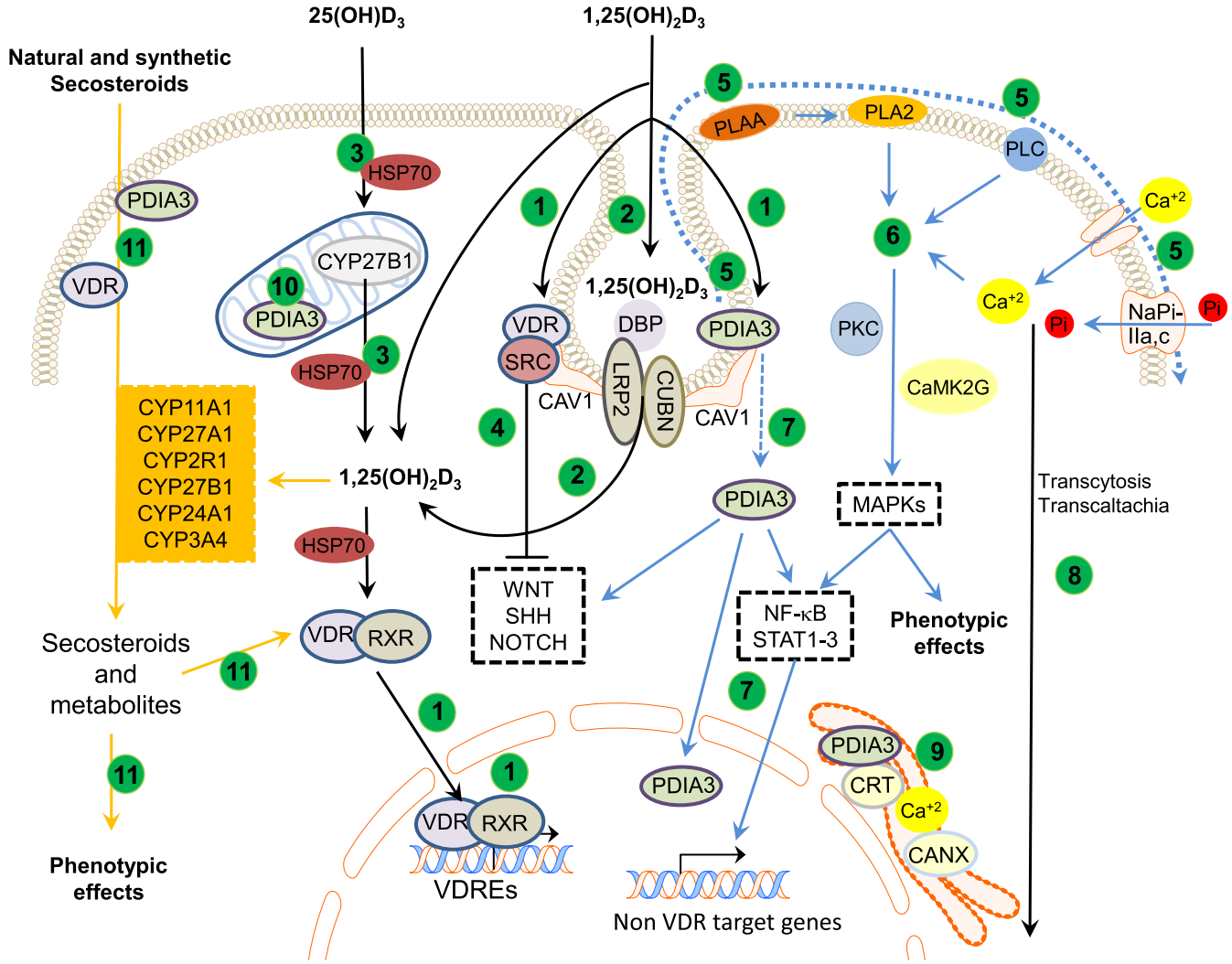

FIGURE 2 Vitamin D pathways. In the classic vitamin D pathway (black arrows) $1,25(OH)_2D_3$ penetrates the cell membrane as a free molecule (1) or, bound to DBP, it is transported *via* endocytosis mediated by the LRP2-CUBN complex (2). The VDR-RXR complex binds the ligand already in the cytosol or in the nucleus (1) resulting in the modulation of target gene expression (Figure 1). Many cell types, including keratinocytes, express CYP27B1 in mitochondria and are able to produce $1,25(OH)_2D_3$ from $25(OH)D_3$ (3) and HSP70 is believed to be intracellular a carrier for both. In alternative vitamin D pathways (blue arrows) $1,25(OH)_2D_3$ interacts also with membrane-bound proteins (4 and 5). Membrane-associated VDR interacts with CAV1 and SRC in caveolae (4) and may down-regulate WNT, SSH and NOTCH signalling. PDIA3 is the best-studied protein associated with rapid, non-genomic response of vitamin D. PDIA3 mediates $1,25(OH)_2D_3$-dependent membrane signalling cascades (5) including the activation of PLAA, PLA2, PLC and opening of Ca^+ [2] and Pi (NaPi II a,c) channels. This results in the rapid accumulation of secondary messengers (6) including DAG, IP3, cAMP and Ca^{+2} followed by PKC or CAMK2G activation and the alteration of several downstream targets including MAPK pathways. PDIA3 was postulate to associate with both sides of the plasma membrane, circulates in the cytoplasm, is targeted to the endoplasmic reticulum or even translocates to the nucleus. PDIA3 was found to be stimulated by $1,25(OH)_2D_3$ alone or together with the transcription factors NF-κB and STAT3 (7). $1,25(OH)_2D_3$ may also directly (not shown) or indirectly *via* PDIA3 (5) activate Ca^{+2} and Pi channels and stimulate intestinal or renal Ca^{+2} and Pi transcellular transport (8). At the endoplasmic reticulum PDIA3 together with CRT and CANX is involved in protein folding (9). In mitochondria, PDIA3 is believed to regulate apoptosis (10), but the involvement of $1,25(OH)_2D_3$ in this function of PDIA is unclear (11). In alternative ligand pathway (orange arrows) hundreds of natural or synthetic secosteroids were shown to be biologically active and metabolized by classic (CYP27A1, CYP2R1, CYP27B1 or CYP24A1) or non-classic (CYP11A1, CYP3A4) cytochromes (10). Some of these compounds were shown to bind VDR and may activate genomic vitamin D signalling

D_3 and $25(OH)D_3$ are in the order of 10-100 nmol/L, while the K_D-value of VDR for $1,25(OH)_2D_3$ has to be about 1000 times lower. The binding site for $25(OH)D_3$ in DBP is a cleft with partial contact to the solvent.[28] The same applies for the binding of $25(OH)D_3$ to the substrate-binding site of CYP24A1.[29] In contrast, in the VDR protein $1,25(OH)_2D_3$ is fully covered inside of the LBD.[30] The LBP within VDR's LBD is formed by 40 hydrophobic amino acids that are well-adapted to the shape of $1,25(OH)_2D_3$[30,31] as well as of three

pairs of polar amino acids that are positioned within the LBP so that they specifically contact via hydrogen bonds the hydroxyl groups of $1,25(OH)_2D_3$ at carbons 1, 3 and 25[32] (Figure 1). This leads to the even for endocrine nuclear receptors very high ligand-binding affinity of VDR of 0.1 nmol/L.[12]

From an evolutionary perspective, the first function of $1,25(OH)_2D_3$-activated VDR was the regulation of energy metabolism.[1] The immune system requires substantial amounts of energy,[33] so that *via* the control of immunometabolism vitamin D became a modulator of immunity.[34] Immune cells are very rapidly growing and vitamin D expanded its functions to the control of their differentiation, growth and death by apoptosis.[35] Some 400-million years ago some bony fish species moved from the calcium-rich ocean to the calcium-poor land and needed to control the homeostasis of this ion.[36] At that time, vitamin D endocrinology extended on the regulation of calcium homeostasis and obtained for this task central importance.[37] Accordingly, the bone malformation is the prime phenotype of vitamin D deficiency.[38] However, vitamin D still contributes to its "older" tasks in the control of immunity, growth and metabolism, as the *VDR* gene is expressed in more than half of the 400 human tissues and cell types.[39] Moreover, taking all tissues together more than 1000 genes are regulated by vitamin D and its receptor.[40]

It should be noted that the physiology of vitamin D and its metabolites is directed towards homeostasis.[41] In contrast to other nuclear hormones, such as cortisol and estrogen, ideally there should be no fluctuations in the circulating levels of vitamin D compounds. However, most insight on the mechanisms of vitamin D signalling had been obtained from cell lines that had been treated with supra-physiological concentrations of $1,25(OH)_2D_3$ in the range of 10-100 nmol/L.[42]

In summary, VDR is the only protein that evolved a sufficiently high affinity for the low natural levels of the nuclear hormone $1,25(OH)_2D_3$. The unique interaction of VDR with its ligand is structurally and mechanistically very well understood. This provides confidence that nature has not designed an alternative protein for the task of regulating genes by $1,25(OH)_2D_3$.

3 | EVIDENCE FOR MEMBRANE SIGNALLING OF VITAMIN D

The idea of a membrane receptor for vitamin D has been intriguing scientists for years.[43,44] Membrane vitamin D signalling was used as a model to explain particular effects of $1,25(OH)_2D_3$, which could not be fully attributed to the activities of VDR in the nucleus (Figure 2,1-3), but requires interactions of $1,25(OH)_2D_3$ with potential membrane-bound or intracellular targets, in order to provide rapid and efficient response to the stimulus (Figure 2,4-8). In a classic paper from 1990, rapid activation of calcium influx was observed in osteogenic sarcoma cell line ROS 17/2.8 treated with $1,25(OH)_2D_3$.[45] Transcaltachia is a rapid (seconds to minutes) transport of calcium by enterocytes and serves as another example of a rapid response to $1,25(OH)_2D_3$[46] (Figure 2,9). $1,25(OH)_2D_3$ was also found to induce a rapid (1-10 min) increase in tissue calcium uptake in primary-cultured myocytes isolated from chicken embryonic heart, an effect which was mediated by the second messenger cAMP.[44] Furthermore, rapid (15-300 seconds) activation of phospholipase C (PLC) and calcium influx triggered by $1,25(OH)_2D_3$ was found in osteoblastic cell line ROS 24/1 lacking VDR expression.[47] Most importantly, the activation of rapid response was observed under picomolar concentrations of $1,25(OH)_2D_3$ (0.13 nmol/L[48] or 0.3 nmol/L[49]). Existence of rapid non-genomic activity of vitamin D was also confirmed in vivo in chicken[50] and in cultured chondrocytes derived from VDR knockout mice.[51] This suggested the involvement of a G-coupled membrane receptor (GPCR) in connection with DBP, which also was detected in membranous cell extracts.[44,47] However, within the past 30 years no receptor of the GPCR or receptor tyrosine kinase family dedicated to vitamin D or its metabolites could be identified.

Vitamin D is a hydrophobic molecule that following the free hormone hypothesis[52] can penetrate biological membranes, thus, in non-renal cells a membrane receptor or a transmembrane transport protein seemed not to be required. However, inside these cells $25(OH)D_3$ binds to HSPA (heat shock protein family A (Hsp70)) proteins for shuttling to mitochondria and the nucleus[53] (Figure 2,3). Some 20 years ago the membrane proteins LRP2 (LDL receptor-related protein 2, also called megalin) and cubilin (CUBN) were found to be involved in the endocytosis of vitamin D compounds bound to DBP[54-56] (Figure 2,2). Thus, $25(OH)D_3$ is transported bound to DBP into proximal tubules of the kidneys, which is the main organ where it is hydroxylated by CYP27B1 into $1,25(OH)_2D_3$.[55,57-59] Interestingly, the multidrug resistance proteins SLCO (solute carrier organic anion transporter family) 1B1 and SLCO1B3 were described to bind sulfated and glucuronated forms of $25(OH)D_3$.[60] This intriguing observation requires further investigations, in order to provide more mechanistic details and physiological significance for these potential vitamin D transporters.

The best described membrane-associated protein that binds vitamin D compounds is the enzyme PDIA3 (protein disulphide isomerase family A member 3, also known as ERp57 or $1,25D_3$-MARRS (membrane-associated rapid response to steroid)), which plays a crucial role in rapid non-genomic response to vitamin D[61-68] (Figure 2,5-7,9,10). Initially, the protein was purified from basal-lateral membranes of chick intestinal epithelium on a base of its binding capability to radiolabeled $1,25(OH)_2D_3$ (K_D-value 0.72 nmol/L).[69] Further studies identified $1,25D_3$-MARRS as PDIA3.[50,70] So far, there is only partial crystal structure of PDIA3[71] and no binding site for $1,25(OH)_2D_3$ could be confirmed. However, it was postulated that $1,25(OH)_2D_3$ binds to PDIA3 with an estimated K_D of 1 nmol/L via its a' domain[72] and amino acids K214 and R282 (calreticulin (CALR) interaction site) and C406 (catalytic site) are essential for the rapid response to $1,25(OH)_2D_3$.[73] Primarily, PDIA3 is an endoplasmic reticulum chaperone for CALR and calnexin (CANX) (Figure 2,9), and its presence was also confirmed at the cell membrane, cytoplasm,

mitochondria and nucleus[74] (Figure 2,5,7,9,10). PDIA3 is involved in the proper function of immune[14,75] and musculoskeletal systems[62,76] as well as mammary gland growth and development[77] Most importantly, PDIA3 takes part in an intestinal uptake of calcium and phosphate, and thus, it is involved in one of the classic functions of $1,25(OH)_2D_3$.[49,50,65,67,69] The activation of the rapid response to vitamin D requires the interaction of membrane-associated PDIA3 with caveolin 1 (CAV1)[76,78,79] (Figure 2,5), the main protein of small invaginations of the plasma membrane called of caveolae.[80] The interaction of vitamin D with membrane-associated PDIA3 has important physiological implications. For example, PDIA3 but not VDR is essential for the activation of protein kinase C (PKC) signalling pathway[81] or the protection of cells against UV-induced DNA damage, in particular thymine dimer formation.[82] Moreover, $1,25(OH)_2D_3$-triggered PDIA3 induces extracellular Ca^+ [2] influx through L-type Ca^{+2} channels in human aortic smooth muscle cells resulting in rapid down-regulation of tumor necrosis factor receptor signalling.[83] In these studies, the non-genomic activity of vitamin D was attributed to PDIA3 but not to VDR.[81-83] However, the photo-protective effects of vitamin D are linked to VDR's genomic[84] and to non-genomic[82] activities. Therefore, it was postulated that VDR itself may be associated with plasma membranes, including caveolae[85,86] (Figure 2,4), mitochondria membranes[87-89] or even lipid droplets.[90] Interestingly, PDIA3 and VDR were found to co-localize with CAV1 on the cell membrane of the osteoblastic cell line MC3T3-E1 [85]. Moreover, a second LBP of VDR supporting the involvement of VDR in membrane signalling was postulated.[86,91]

Stimulation of cells with $1,25(OH)_2D_3$ results in rapid activation of phospholipase A2 (PLA2) triggered by interaction of PDIA3 with PLA2 activating protein (PLAA) (Figure 2,5), whereas VDR activation activates the SRC (SRC proto-oncogene, non-receptor tyrosine kinase) pathway (Figure 2,4). Furthermore, $1,25(OH)_2D_3$ binding to PDIA3 initiates a rapid signalling cascade *via* CAMK2G (calcium/calmodulin-dependent protein kinase II gamma), PLA2, PLC, PKC and ultimately results in the activation of MAPK (mitogen-activated protein kinase) 1 and MAPK276,78. Rapid activation of WNT5A (Wnt family member 5A) by $1,25(OH)_2D_3$ was also shown to be dependent on the PDIA3 membrane complex.[61] In contrast, VDR is implicated in vitamin D-triggered downstream regulation of transcriptional activity of WNT,[92-95] sonic hedgehog signalling molecule (SHH)[96-101] and NOTCH[102-104] signalling pathways[95] (Figure 2,4). A detailed dissection of the genomic and non-genomic effects of $1,25(OH)_2D_3$ requires further investigations and other potential vitamin D-binding proteins may have to be taken into consideration.

Another import argument that PDIA3 is involved in vitamin D signalling came from animal knockout studies. Although an ubiquitous *Pdia3* deletion in mice is embryonically lethal,[68,75] heterozygous $Pdia3^{+/-}$ knockout mice showed abnormalities in skeletal tissues[68] indicating effects on calcium homeostasis. In fact, targeted disruption of the *Pdia3* gene in chicken intestinal epithelial cells resulted in the attenuation of rapid non-genomic responses to $1,25(OH)_2D_3$ including rapid calcium uptake and down-regulation of PKA signalling.[67] Moreover, disrupting the *PDIA3* gene results in bone abnormalities and attenuation of the $1,25(OH)_2D_3$-induced rapid activation of PKC.[68] Interestingly, VDR-PDIA3 interference was observed in $1,25(OH)_2D_3$-mediated proliferation control of rat growth plate chondrocytes. *VDR* knockdown chondrocytes expressing Pdia3 showed a rapid escalation in PKC levels in response to $1,25(OH)_2D_3$.[51] This may suggest that PDIA3 has other functions in addition to acting as a $1,25(OH)_2D_3$-binding protein. Furthermore, after $1,25(OH)_2D_3$ treatment rapid translocation of PDIA3 to the nucleus was observed in the cell lines IEC-6[70] and HepG2.[105] Vitamin D stimulates also the nuclear translocation of PDIA3-STAT3[106] and PDIA3-NF-κB[107] complexes (Figure 2,7) although the significance of this PDIA3 localization is not fully understood.[108] Interestingly, VDR and NF-κB share binding sites that may explain the influence of vitamin D on immune response.[109] Interestingly, it was shown that alternative metabolites of vitamin D generated by CYP450 enzymes, such as $20(OH)D_3$ and $20,23(OH)_2D_3$, also are able to inhibit NF-κB[110,111] or stimulate NRF2.[112,113] It seems that new biologically active metabolites of vitamin D bring additional complexity to the vitamin D world, with possible involvement of alternative pathways including regulation of biological activity of other transcription factors, such as RORα and RORγ[114,115] or AhR.[116] Taken together, a non-genomic vitamin D-triggered pathway *via* PDIA3 and VDR has significant impact on the regulation of musculoskeletal biology.[61,62,73,76,78,79,85,117,118]

It should be taken into account that the majority of the in vitro studies investigating rapid, non-genomic effects of $1,25(OH)_2D_3$ use potentially non-physiological concentrations (1-100 nmol/L). However, it is not fully understood how much of free $1,25(OH)_2D_3$ in the medium is getting into cells. Moreover, many organs and cell types, such as skin and immune cells, express the full enzymatic machinery for the local production of $1,25(OH)_2D_3$,[119,120] thus, its cellular or tissue concentrations might be of several fold higher than in the serum. On the other hand, in proliferation assays, the IC_{50} for $1,25(OH)_2D_3$ was found to be in the low nanomolar or even picomolar range depending on melanoma cellular model.[121,122] In addition, the expression of *CYP24A1* was found to be stimulated by $1,25(OH)_2D_3$ with an EC_{50} of 0.2 nmol/L,[123] although mostly concentration of 10 nmol/L is used.[23,121] Furthermore, the presence of foetal bovine serum in medium decreases the efficiency of $1,25(OH)_2D_3$ in stimulating the *CYP24A1* promoter more than 1000 times.[124] In general, similar concentration ranges are used for the study of genomic and non-genomic pathways, while PDIA3-mediated activation of PKC was observed at 0.3 nM $1,25(OH)_2D_3$.[49] Finally, even if the activation of non-genomic pathways may require higher concentrations of $1,25(OH)_2D_3$ than the stimulation of VDR, the concentration of $1,25(OH)_2D_3$ may serve as a switch between genomic and non-genomic responses. It may also explain why the physiological effects of vitamin D are concentration dependent. For example, the serum level of $25(OH)D_3$ of 50 nmol/L is sufficient for rickets or

osteomalacia eradication, while other beneficial effects of vitamin D (anti-osteoporotic, anti-preeclampsia) require higher levels (>75 nmol/L).[125-128] Potential anti-cancer effects of vitamin D are also strongly concentration dependent and required even higher serum levels of 25(OH)D_3.[129-131]

4 | SOLVING THE DISPUTE?

There is no doubt that VDR is the only protein in the human body that binds with high affinity the biologically most active vitamin D metabolite 1,25(OH)$_2D_3$ (Figure 1). Millions of years of evolution have optimized the LBP within VDR's LBD, in order to create an endocrine system that regulates not only calcium homeostasis, but also immunity, cell growth and differentiation as well as energy metabolism. However, there are a number of metabolic and transport processes involved, in order to obtain from a molecule produced in UV-B irradiated keratinocytes of the skin a nuclear hormone binding to a transcription factor in the nucleus of hundreds of human tissues and cell types. Each of these steps allows a fine-regulation of this endocrine process, such as the efficiency of hydroxylation *via* CYP enzymes or the transport through membranes *via* the LRP2-CUBN endocytosis complex in kidney cells. In this context, vitamin D_3 and its metabolites are not only bound by VDR but also by transport proteins, such as DBP in serum and HSPA proteins inside cells, and enzymes, such as CYP2R1, CYP27B1 and CYP24A1. However, all these proteins do not carry any binding pocket with an affinity for 1,25(OH)$_2D_3$ comparable to that of VDR.

The enzyme PDIA3 is the best-studied candidate for an alternative binding protein for 1,25(OH)$_2D_3$. However, no crystal structure has confirmed direct contact of 1,25(OH)$_2D_3$ with PDIA3, thus, mechanistically the observed non-genomic effects of vitamin D and its metabolites are not fully understood. Nevertheless, in vivo studies on VDR or PDIA3 mice knockouts strongly indicate the involvement of PDIA3 in intestinal calcium absorption and skeletal development.[51,63,65,67,77] Moreover, there is a more severe phenotype of *Cyp27b1* knockout mice (including infertility)[132] in comparison with *Vdr* knockout mice[133] suggesting VDR-independent functions of 1,25(OH)$_2D_3$. It seems that PDIA3 activity is essential for 1,25(OH)$_2D_3$ membrane signalling, so, even if PDIA3 may not directly bind the hormone, the protein may serve as a molecular chaperone[73] for VDR or DBP or presently unknown proteins. Non-genomic effects have been described also for the steroids estrogen,[134,135] testosterone,[136] aldosterone[137] and cortisol.[138] The respective nuclear receptors, ER, AR, MR and GR, have an evolutionary history comparable to VDR. Each of them evolved a high-affinity LBP within their LBD, *thus*, the nuclear receptors are indisputably the prime targets of the respective steroid hormones. For none of these steroids has a membrane receptor been identified, which was designed by nature to bind exclusively them. Thus, membrane receptors and a rapid response to steroids may represent a further evolutionary adaptation of intracellular signalling to growing complexity of organisms.

The complexity of vitamin D signalling includes genomic and non-genomic pathways with presumable implication of membrane-bound and nuclear VDR. Thus, it is hard to identify diseases that are exclusively related a deficiency in rapid, non-genomic actions. Moreover, in the endocrine system of vitamin D, which is primarily designed for homeostasis,[41] rapid actions are not required under in vivo conditions. On the other hand, some processes, such as intestinal calcium absorption or skin response to UV, may benefit from rapid membrane responses. Nevertheless, even if the rapid, non-genomic actions of 1,25(OH)$_2D_3$ take place under natural conditions in vivo, they seem to be restricted to a few limited scenarios, such as fine-tuning of VDR-driven responses.

Although the focus of vitamin D endocrinology is on the actions of 1,25(OH)$_2D_3$, there are reports on hormonal effects of other vitamin D metabolites, such as 25(OH)D_3 as direct VDR ligand in the context of prostate cancer.[139,140] 20(OH)D_3 and 20,23(OH)$_2D_3$ as agonists of the transcription factor AhR[116] and reverse agonist of the nuclear receptors RORα and RORγ[114,115] in the skin and 24R,25(OH)$_2D_3$ in bone *via* the membrane protein FAM57B2.[141] Finally, there is growing evidence that CYP11A1 is responsible for alternative metabolism of vitamin D and its precursor 7-dehydrocholesterol[142,143] with generation of new hydroxy derivatives of vitamin D[144-148] or lumisterol[149] with hydroxyl group at carbons 17, 20, 22 or 23. Their physiological impact in relation to the actions of 1,25(OH)$_2D_3$ needs to be further investigated.

5 | CONCLUSION

Vitamin D is an interesting compound with a biological profile that clearly exceeds musculoskeletal effects.[150] There is no doubt that VDR plays the central role in 1,25(OH)$_2D_3$ signalling. Membrane response to vitamin D, including PDIA3 and membrane-associated VDR, may modulate its activity and broaden the spectrum of intracellular targets, with a special emphasis to rapid and non-genomic responses. The presence of alternative pathways may help to explain the activity of low or non-calcemic analogs of vitamin D.[151-153] However, further in vivo studies are required to confirm physiological and clinical significance of membrane receptors for vitamin D.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

M.A.Z. wrote the section concerning membrane vitamin D signalling and prepared Figure 2. C.C. wrote the section concerning nuclear vitamin D and prepared Figure 1. Other parts of the manuscript were written jointly. Both authors read and approved the final manuscript.

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