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Molecular Mechanisms of Vitamin D Action

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Abstract The hormonal metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), initiates biological responses via binding to the vitamin D receptor (VDR). When occupied by 1,25D, VDR interacts with the retinoid X receptor (RXR) to form a heterodimer that binds to vitamin D responsive elements in the region of genes directly controlled by 1,25D. By recruiting complexes of either coactivators or corepressors, ligand-activated VDR-RXR modulates the transcription of genes encoding proteins that promulgate the traditional functions of vitamin D, including signaling intestinal calcium and phosphate absorption to effect skeletal and calcium homeostasis. Thus, vitamin D action in a particular cell depends upon the metabolic production or delivery of sufficient concentrations of the 1,25D ligand, expression of adequate VDR and RXR coreceptor proteins, and cell-specific programming of transcriptional responses to regulate select genes that encode proteins that function in mediating the effects of vitamin D. For example, 1,25D induces RANKL, SPP1 (osteopontin), and BGP (osteocalcin) to govern bone mineral remodeling; TRPV6, $CaBP_{9k}$, and claudin 2 to promote intestinal calcium absorption; and TRPV5, klotho, and Npt2c to regulate renal calcium and phosphate reabsorption. VDR appears to function unliganded by 1,25D in keratinocytes to drive mammalian hair cycling via regulation of genes such as *CASP14*, *S100A8*, *SOSTDC1*, and others affecting Wnt signaling. Finally, alternative, low-affinity, non-vitamin D VDR ligands, e.g., lithocholic acid, docosahexaenoic acid, and curcumin, have been reported. Combined alternative VDR ligand(s) and 1,25D/VDR control of gene expression may delay chronic disorders of aging such as osteoporosis, type 2 diabetes, cardiovascular disease, and cancer.

Keywords Coactivator · Corepressor · 1α,25-Dihydroxyvitamin D_3 · Retinoid X receptor · Transcription · Vitamin D receptor · Vitamin D responsive element

Vitamin D Bioactivation and Its Endocrine/Mineral Feedback Control

The hormonal precursor vitamin D_3 can be either obtained in the diet or formed from 7-dehydrocholesterol in skin via a nonenzymatic, UV light-dependent reaction (Fig. 1). Vitamin D_3 is then transported to the liver, where it is hydroxylated at the C-25 position of the side chain to produce 25-hydroxyvitamin D_3 (25D), the major circulating form of vitamin D_3. The final step in the production of the hormonal form occurs mainly, but not exclusively, in the kidney via a tightly regulated 1α-hydroxylation reaction (Fig. 1). The cytochrome P-450-containing (CYP) enzymes that catalyze 25- and 1α-hydroxylations are microsomal CYP2R1 and mitochondrial CYP27B1, respectively. As depicted in Fig. 1, 1α,25-dihydroxyvitamin D_3 (1,25D) circulates, bound to plasma vitamin D binding protein (DBP), to various target tissues to exert its endocrine actions, which are

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mediated by the vitamin D receptor (VDR). Many of the long-recognized functions of 1,25D involve the regulation of calcium and phosphate metabolism, raising the blood levels of these ions via intestinal absorption and renal reabsorption to facilitate bone mineralization, as well as activating bone resorption as part of the skeletal remodeling cycle [1].

The parathyroid gland also expresses VDR [2], and VDR liganded with 1,25D suppresses parathyroid hormone (PTH) synthesis by a direct action on gene transcription [3]. This negative feedback loop, which curtails the stimulation of CYP27B1 by PTH under low-calcium conditions (Fig. 1), serves to limit the bone-resorbing effects of PTH in anticipation of 1,25D-mediated increases in both intestinal calcium absorption and bone resorption, thus preventing hypercalcemia. More recent understanding of the homeostatic control of phosphate has emerged, emanating originally from characterization of unsolved familial hypo- or hyperphosphatemic disorders, which we now know are caused by deranged levels of bone-derived FGF23 [4]. In short, FGF23 has emerged as a dramatic new phosphate regulator and a second phosphaturic hormone after PTH. It has been demonstrated [5] that 1,25D induces the release of FGF23 from bone, specifically from osteocytes of the osteoblastic lineage (Fig. 1), a process that is independently stimulated by high circulating phosphate levels (Fig. 1). Thus, in a striking and elegant example of biological symmetry, PTH is repressed by 1,25D and calcium, whereas FGF23 is induced by 1,25D and phosphate, protecting mammals against hypercalcemia and hyperphosphatemia, respectively, either of which can elicit ectopic calcification.

In addition to effecting bone mineral homeostasis by functioning at the small intestine, kidneys, bone, and parathyroid glands, 1,25D acts through its VDR mediator to influence a number of other cell types. These extraosseous actions of 1,25D-VDR include differentiation of certain cells in skin [6] and in the immune system (Fig. 1) [7]. Interestingly, the skin and the immune system are recognized as extrarenal sites of CYP27B1 catalysis to produce 1,25D locally, creating intracrine and paracrine systems (Fig. 1) distinct from the endocrine actions of 1,25D-VDR on the small intestine, kidneys, skeleton, and parathyroid. Apparently, higher circulating 25D levels are required for optimal intracrine actions of 1,25D (Fig. 1). This insight stems from a multitude of epidemiologic associations between low 25D levels and chronic disease, coupled with statistically significant protection against a host of pathologies by much higher circulating 25D [8]. Thus, as depicted schematically in Fig. 1, locally produced 1,25D appears to be capable of protecting the vasculature to reduce the risk of heart attack and stroke, controlling the adaptive immune system to lower the incidence of autoimmune disease while boosting the innate immune system to fight infection, effecting xenobiotic detoxification, and exerting anti-inflammatory and anticancer pressure on epithelial cells prone to fatal malignancies.

As illustrated in Fig. 1 for kidney, an important mechanism by which the 1,25D/VDR-mediated endocrine or intracrine signal is terminated in all target cells is the catalytic action of CYP24A1, an enzyme that initiates the process of 1,25D catabolism [9]. The *CYP24A1* gene is transcriptionally activated by 1,25D [10], as well as by FGF23 (Fig. 1). In addition, the 1α-hydroxylase (1α-OHase) *CYP27B1* gene is repressed by FGF23 and 1,25D, with the latter hormone acting via a short negative feedback loop to limit the production of 1,25D [11]. Therefore, the

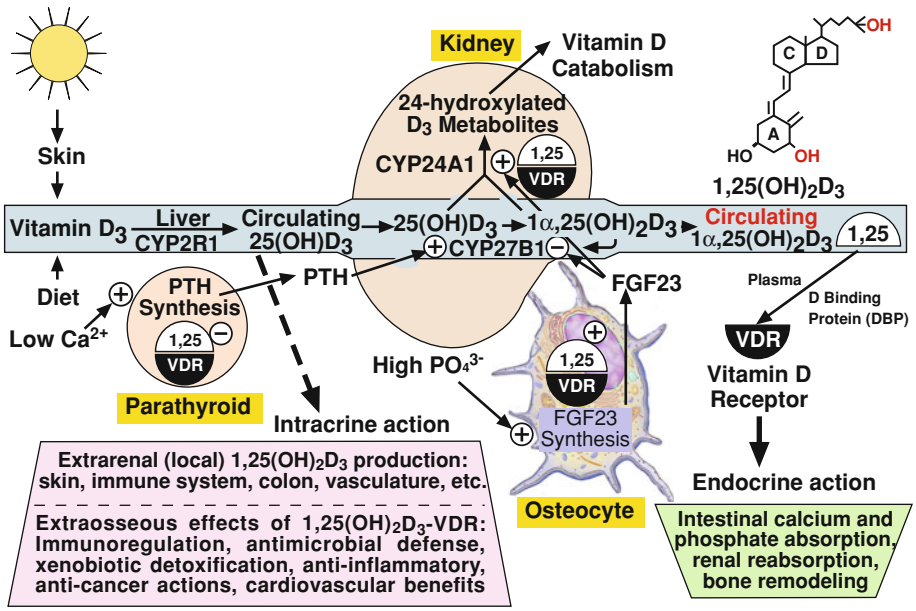

Fig. 1 Vitamin D acquisition, regulation of metabolic activation/catabolism, and receptor-mediated endocrine and intracrine actions of the 1,25D hormone in selected tissues

vitamin D endocrine system is elegantly governed by feedback controls of vitamin D bioactivation that interpret bone mineral ion status and prevent the pathologies of hypervitaminosis D via feedforward induction of 1,25D catabolism. The vitamin D intracrine system, in contrast, appears to be dependent more on the availability of ample 25D substrate to generate local 1,25D to lower the risk of chronic diseases of the epithelial (skin, colon, etc.), immune, and cardiovascular systems.

Molecular Structure and Function of VDR, a Member of the Nuclear Receptor Superfamily

The biological responses to the 1,25D hormone are mediated by VDR, originally identified as a chromatin-associated protein [12] that binds 1,25D with high affinity and specificity. The liganding of VDR triggers tight association between VDR and its heterodimeric partner, a retinoid X receptor (RXR) isoform (Fig. 2b); and this liganded VDR-RXR heterodimer is conformed to recognize vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes [13]. Table 1 provides a list of VDR-RXR target genes recognized by the combined DNA binding domain (DBD) zinc fingers of the two receptors and their C-terminal extensions (CTEs) (Fig. 2a). In general, VDREs consist of either a direct repeat of two hexanucleotide half-elements with a spacer of three nucleotides (DR3) or an everted repeat of two half-elements with a spacer of six nucleotides (ER6), with DR3s being the most common. In positive DR3 VDREs, VDR has been shown to occupy the 3′ half-element, with RXR residing on the 5′ half-site [14]. The "optimal" VDRE, which was experimentally determined via binding of randomized oligonucleotides to a VDR-RXR heterodimer [15], possesses a 3′ (VDR) half-site of PGTTCA, where P is a purine base, and a 5′ (RXR) half-site of PGGTCA. The half-sites that exist in natural DR3 elements usually contain one to three bases that do not match the optimal VDRE. The multiple sequence variations in natural VDREs (Table 1) may provide a spectrum of affinities for the VDR-RXR heterodimer. Another possibility, for which evidence is accumulating [16], is that variant VDRE sequences induce unique conformations in the VDR-RXR complex, thereby promoting association of the heterodimer with distinct subsets of comodulators to permit differential actions in diverse tissues.

Several VDREs occur as a single copy in the proximal promoter of vitamin D-regulated genes (Table 1). However, ChIP and ChIP scanning [17–20] of genomic DNA introduced the properties of multiplicity and remoteness to VDREs. Genes possessing multiple VDREs require all VDR-RXR docking sites for maximal induction by 1,25D,

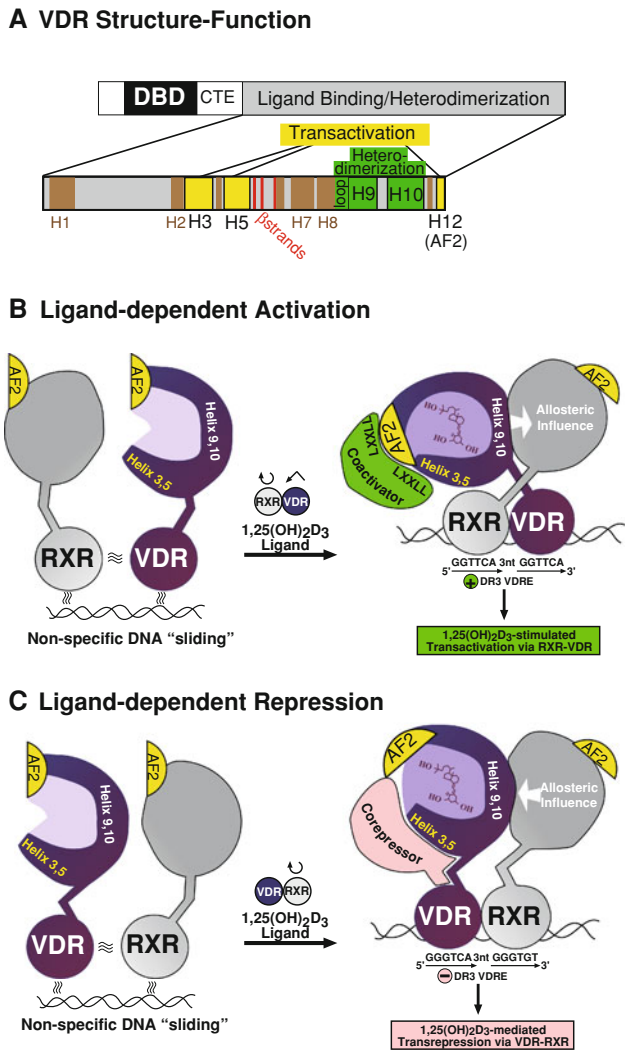

Fig. 2 Structure–function relationships and proposed mechanisms of gene induction and repression by VDR. **a** A schematic view of the functional domains in human VDR. **b** Allosteric model of RXR-VDR-mediated transactivation after binding 1,25D and recruiting coactivator/docking on a high-affinity positive VDRE (mouse osteopontin). **c** Allosteric model for VDR-RXR-mediated transrepression after binding 1,25D and attracting corepressor/docking in reverse polarity on a high-affinity negative VDRE (chicken PTH)

and the individual VDREs appear to function synergistically in attracting coactivators and basal factors for transactivation. The concept, from analyzing a number of VDR-regulated genes, is that the docking sequences for VDR-RXR consist of clearly defined DR3 and ER6 motifs, often in multiple copies dispersed up to 100 kb 5′ or 3′ of the transcription start site. The most logical model is that remote VDREs are juxtaposed with more proximal VDREs via chromatin looping, creating a single platform that supports regulation of the transcription machine by VDR-RXR and complexed comodulators. Table 1 paints a picture of the biological breadth of VDR-mediated gene control. The catalog of VDRE-containing genes can be

Table 1 Representative VDREs in genes directly modulated in their expression by 1,25D

Gene	Network	Bioeffect (s)	Type	Location	5′-Half	Spacer	3′-Half	References
rBGP	Bone	Bone metabolism	Positive	−456	GGGTGA	atg	AGGACA	[120]
mSPP1	Bone	Bone metabolism	Positive	−757	GGTTCA	cga	GGTTCA	[120]
mLRP5	Bone	Bone anabolism	Positive	+656	GGGTCA	ctg	GGGTCA	[20]
mLRP5	Bone	Bone anabolism	Positive	+19 kb	GGGTCA	tgc	AGGTTC	[18]
mRANKL	Bone	Bone resorption	Positive	−22.7 kb	TGACCT	cctttg	GGGTCA	[1]
mRANKL	Bone	Bone resorption	Positive	−76 kb	GGTTGC	ctg	AGTTCA	[28]
cPTH	Mineral	Mineral homeostasis	Negative	−60	GGGTCA	gga	GGGTGT	[121]
hTRPV6	Mineral	Intestinal Ca^{2+} transport	Positive	−2100	GGGTCA	gtg	GGTTCG	[17]
hTRPV6	Mineral	Intestinal Ca^{2+} transport	Positive	−2155	AGGTCT	tgg	GGTTCA	[17]
hFGF23	Mineral	Renal phosphate reabsorption	Positive	−32.9 kb	TGAACT	caaggg	AGGGCA	[38]
hklotho	Mineral	Renal phosphate reabsorption	Positive	−31 kb	AGTTCA	aga	AGTTCA	[63]
hCYP24A1	Detox	1,25D detoxification	Positive	−151	AGGTGA	gcg	AGGGCG	[120]
hCYP24A1	Detox	1,25D detoxification	Positive	−274	AGTTCA	ccg	GGTGTG	[120]
hCYP3A4	Detox	Xenobiotic detoxification	Positive	−169	TGAACT	caaagg	AGGTCA	[85]
hCYP3A4	Detox	Xenobiotic detoxification	Positive	−7.7 kb	GGGTCA	gca	AGTTCA	[84]
hp21	Cell life	Cell cycle control	Positive	−765	AGGGAG	att	GGTTCA	[120]
hFOXO1	Cell life	Cell cycle control	Positive	−2856	GGGTCA	cca	AGGTGA	[120]
rPTHrP	Cell life	Mammalian hair cycle	Negative	−805	AGGTTA	ctc	AGTGAA	[103]
hSOSTDC1 (*Wise*)	Cell life	Mammalian hair cycle	Negative	−6214	AGGACA	gca	GGGACA	[118]
hCAMP	Immune	Antimicrobial peptide	Positive	−615	GGTTCA	atg	GGTTCA	[120]
mCBS	Metabolism	Homocysteine clearance	Positive	+5923	GGGTTG	atg	AGTTCA	[74]

grouped into major biological networks influenced by VDR as follows: (1) bone, (2) mineral, (3) detoxification, (4) cell life (proliferation, differentiation, migration, and death), (5) immune, and (6) metabolism (amino acid, lipid, and carbohydrate). In toto, it is clear that VDR affects some of life's most fundamental processes.

Various domains of the 427-amino acid human VDR are highlighted in Fig. 2a, with the two major functional units being the N-terminal zinc finger DBD and the C-terminal ligand binding (LBD)/heterodimerization domain. DNA-binding point mutations in the zinc finger DBD of hVDR [21] confer the phenotype of hereditary hypocalcemic vitamin D-resistant rickets type II (HVDRR). The VDR LBD is a sandwich-like structure of at least 12 α-helices [22] presenting VDR surfaces for heterodimerization with RXR (predominantly helices [H] 9 and 10 and the loop between H8 and H9) as well as interaction with coactivators. Coactivator interfaces in VDR, as depicted in Fig. 2a, b, consist of portions of H3, H5, and H12 [the last constituting the activation function-2 (AF-2) domain].

Mechanisms of VDR-Mediated Regulation of Gene Expression

Very recently, the structure of intact hVDR, heterodimerized with full-length RXRα, docked on a VDRE, and occupied with 1,25D plus a single coactivator, was determined in solution via small-angle X-ray scattering and fluorescence resonance energy transfer techniques [23]. In addition, allosteric communication between interaction surfaces of the VDR-RXR complex has been elucidated by hydrogen–deuterium exchange technology [16]. These advances allow for visualization of the arrangement of the DBD and LBD relative to one another, revealing that binding to ligand, DNA, and coactivators generates a number of VDR/RXR conformations.

Induction of Gene Expression

The process of gene control by vitamin D is best understood for VDR mediation of 1,25D-stimulated transcription, for which RXR heterodimerization is an obligatory initial step. Figure 2b illustrates, in hypothetical fashion, the hormonal ligand influencing VDR to interact more efficaciously with its heterodimeric partner, with a VDRE, and with coactivators. Several steps apparently are set in motion by the ligand binding event. The presence of bound 1,25D ligand results in a dramatic conformational change in the position of H12 at the C terminus of VDR, bringing it to the "closed" position to serve the AF-2 role as part of a platform for coactivator binding [24]. The attraction of a coactivator to the H3, H5, and H12 platform of liganded VDR likely allosterically stabilizes the VDR-RXR heterodimer on the

VDRE and may even assist in triggering strong heterodimerization by inducing the VDR LBD to migrate to the 5′ side of the RXR LBD and in so doing rotate the RXR LBD 180°, employing the driving force of the ionic and hydrophobic interactions between H9 and H10 in hVDR and the corresponding helices in RXR (Fig. 2b). Therefore, ligand-intensified heterodimerization, VDRE docking, and coactivator recruitment by VDR appear to be functionally inseparable, yet experimentally dissociable, events that occur in concert to effect 1,25D-elicited gene transcription. Finally, as depicted in Fig. 2b and supported experimentally [25], the liganding of VDR conformationally influences its RXR heteropartner to cause the AF-2 region of RXR to pivot into the "closed" or active position. RXR may potentially bind an additional coactivator (not shown in Fig. 2b). In contrast, VDR is referred to as a nonpermissive primary receptor because RXR may not be able to bind its 9-*cis* retinoic acid ligand when heterodimerized to liganded VDR [25].

Repression of Gene Expression

Ligand-dependent repression of gene transcription by VDR-RXR likely shares some molecular features with induction but appears to occur via multiple routes. One theme of repression is the recruitment of a nuclear receptor corepressor(s) to alter the architecture of chromatin in the vicinity of the target gene to that of heterochromatin. This restructuring of chromatin is catalyzed by histone deacetylases and demethylases attracted to the receptor-tethered corepressor. The initial step in VDR-mediated repression, as illustrated in Fig. 2c, is hypothesized to be docking of liganded VDR-RXR on a negative VDRE, which then conforms liganded VDR such that it binds corepressor rather than coactivator. We postulate that the information driving this conformational shift in VDR is intrinsic to the negative VDRE sequence [13]. Further, because nonconsensus nucleotides in negative VDREs appear to occur in either or both half-elements, we contend that such base-pair changes may drive RXR-VDR into reverse polarity on the negative VDRE (Fig. 2c), an event that favors the recruitment of corepressor over coactivator to overlapping docking sites in H3–H6. A key question is the role of the RXR heteropartner in gene repression by VDR. One possibility is that RXR is simply a "silent" partner in VDRE binding, with negative nucleotides in the 5′ half-site allosterically conforming VDR to attract corepressor. However, because the negative cPTH VDRE (Fig. 2c) possesses nonconsensus base pairs in the terminus of the 3′ half-site only and can be converted to a positive VDRE by altering these 3′ terminal bases from GT to CA [26], we favor a role for the RXR LBD in allosterically locking VDR into a corepressor docking conformation (Fig. 2c). This concept is consistent with the data that Zhang et al. [16] obtained using hydrogen–deuterium exchange, but more direct experiments will be required to verify the hypothetical model presented in Fig. 2c. Negative regulation by VDR appears also to involve epigenetic mechanisms; for example, the *CYP27B1* gene is repressed by 1,25D through epigenetic DNA demethylation [27].

Integrated Model for the Induction of Gene Expression by 1,25D-VDR

An integrated picture of gene expression control by 1,25D shows liganded VDR-RXR serving as a "nucleus" to recruit comodulators for signal transduction and is presented in Fig. 3a in the form of a sequential recruitment hypothesis. The key tenet of this sequential model is DNA looping to facilitate contact between comodulators tethered to enhancer elements and the transcriptional start site. VDR initially heterodimerizes with RXR in response to ligand binding in order to recognize direct repeat responsive elements in the promoters of regulated genes (steps 1 and 2 in Fig. 3a). Previous research with VDR-activated genes indicates that many factors participate in transactivation [13]. These include sequential recruitment of six additional groups of factors, as detailed in Fig. 3a. Many of these factors interact with the same C-terminal AF-2 motif; thus, it is difficult to conceive of these factors all interacting with VDR to effect transactivation except in a sequential manner (Fig. 3a) or in a complex in which multiple VDR-RXR heterodimers are present (Fig. 3c).

The *RANKL* gene promoter (Fig. 3b) is a model system for studying the steps in transcriptional activation by the liganded VDR-RXR heterodimer using in silico analysis as well as ChIP, gel mobility shift, and transcription assays. Based on our studies [20] and those of Pike and associates [28], Fig. 3c depicts a postulated chromatin looping model for the mouse *RANKL* gene. Instead of separate events in which various factors sequentially bind to a single VDR-RXR heterodimer (Fig. 3a), we propose that in genes such as *RANKL*, which possess multiple VDREs, the chromatin looping model (Fig. 3c) allows for simultaneous binding of multiple factors in a supercomplex at the promoter. Indeed, direct evidence for chromosomal looping in VDR-mediated transcriptional modulation has been obtained via chromosome conformation capture technology [29]. Moreover, active VDREs are located anywhere from 76 kb upstream in the *RANKL* gene (Fig. 3b) [28] to 19–29 kb downstream in the mouse *LRP5* [18, 20] and *VDR* [30] genes and 2–4 kb upstream of the *TRPV6* gene [17, 20]. It therefore seems likely that nuclear receptors, including VDR, utilize chromosomal looping in their mechanism of transactivation, allowing for the formation of a "cloverleaf" structure (Fig. 3c) that permits the functioning of

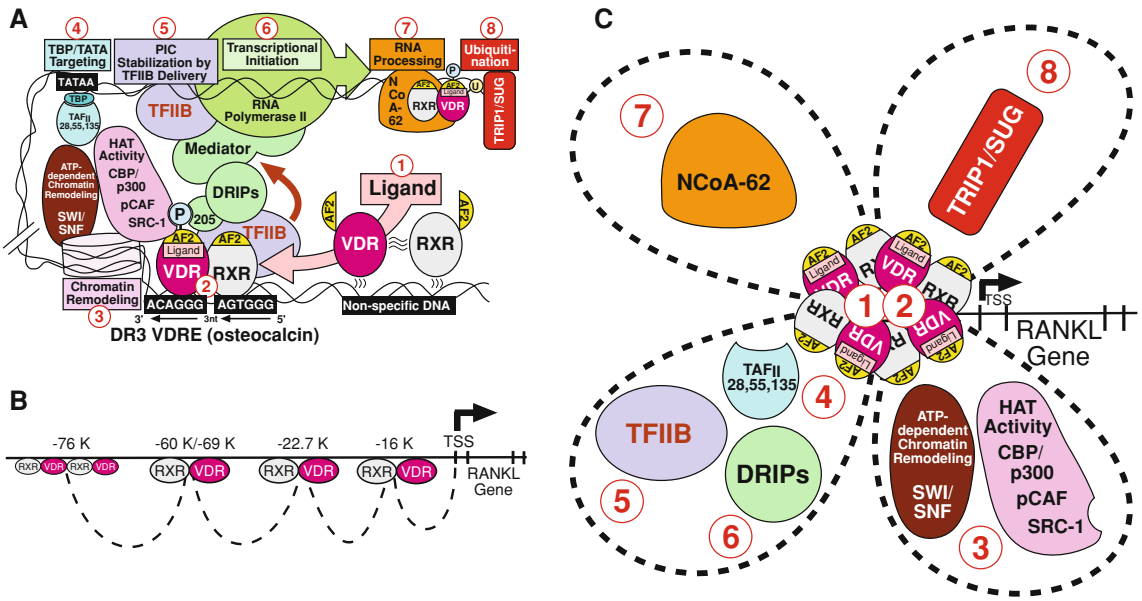

Fig. 3 Chromatin looping model of gene regulation by VDR. **a** Sequential model of gene activation by the 1,25D-bound VDR-RXR heterodimer, exemplified here on the rat osteocalcin gene (containing a single VDRE). *Numbers inside circles* refer to discrete stages in the activation of a VDR target gene, with steps 1 and 2 constituting ligand-intensified heterodimerization of VDR with RXR and recognition of the VDRE by the liganded heterodimer, respectively. Further steps include sequential recruitment of the following groups of six additional factors: step 3, histone acetyl transferases (HATs), such as SRC-1, CBP/p300, and pCAF, or factors involved in ATP-dependent chromatin remodeling, such as the mammalian homologs of SWI/SNF; step 4, TATA binding protein associated factors (TAFs, especially TAFs 28, 55, and 135); step 5, basal transcription factors such as TFIIB; step 6, D-receptor interacting proteins (DRIPs, especially DRIP205, a subunit of the mediator complex that couples transactivators to the C-terminal tail of RNA polymerase II); step 7, NCoA-62, a coactivator for VDR and related nuclear receptors that might also couple transcription to RNA splicing; and step 8, TRIP1, the mammalian homolog of the yeast SUG factor, resulting in progressive ubiquitination of VDR, ultimately leading to its recognition and degradation by the proteasome. **b** The presence of several potential VDREs in the 5′ flanking region of the mouse *RANKL* gene (Table 1) [1]. **c** Depiction of how these VDREs might cooperate in a chromatin-looping model

multiple coactivators immediately upstream of the transcriptional start site (TSS).

1,25D Action in Bone

1,25D-VDR regulates the expression of a number of genes in bone cells, many of which encode bone remodeling effectors that are either catabolic or anabolic, and secreted hormones that influence vitamin D and mineral metabolism as well as other endocrine systems such as glucose control and fertility. The skeleton serves as a sensor of phosphate levels in a manner analogous to the function of the parathyroid glands as a calcium monitor, and both tissues employ secreted hormones to effect mineral homeostasis through regulation of circulating 1,25D concentrations. Analogous to parathyroid chief cells, osteocytes and osteoblasts respond to 1,25D-VDR, with bone cells expressing and releasing FGF23 to control phosphatemia and repress CYP27B1 as well as to induce CYP24A1 for a feedback reduction in 1,25D levels. In terms of the direct action of 1,25D-VDR on the skeleton, a major question is whether the net effect of vitamin D on bone is anabolic or catabolic. As will be discussed below, 1,25D appears to facilitate bone formation at physiologically optimal concentrations, while higher levels of the hormone promote resorption and limit mineralization to sculpt bone.

Catabolic Actions of Vitamin D on Bone

It is well established that the primary effect of 1,25D-VDR is to promote bone resorption, both in experimental animals and in cultures of calvaria, leading to the conclusion that, like PTH, vitamin D is catabolic to the skeleton. With respect to gene regulation in osteoblasts, 1,25D-VDR enhances the expression of RANKL (Fig. 4; Table 1) [1] to stimulate bone resorption through osteoclastogenesis. Osteoprotegerin, the soluble decoy receptor for RANKL that tempers its activity, is repressed by 1,25D in osteoblasts [1] to amplify the bioeffect of RANKL. Moreover, 1,25D represses Runx2 expression, thereby blunting osteoblast differentiation through the bone morphogenetic protein (BMP) pathway that normally cooperates with Wnt signaling in determining cell fate. Runx2 is a known mediator of the anabolic consequences of intermittent, low-dose PTH in bone [31], indicating that 1,25D opposes PTH by

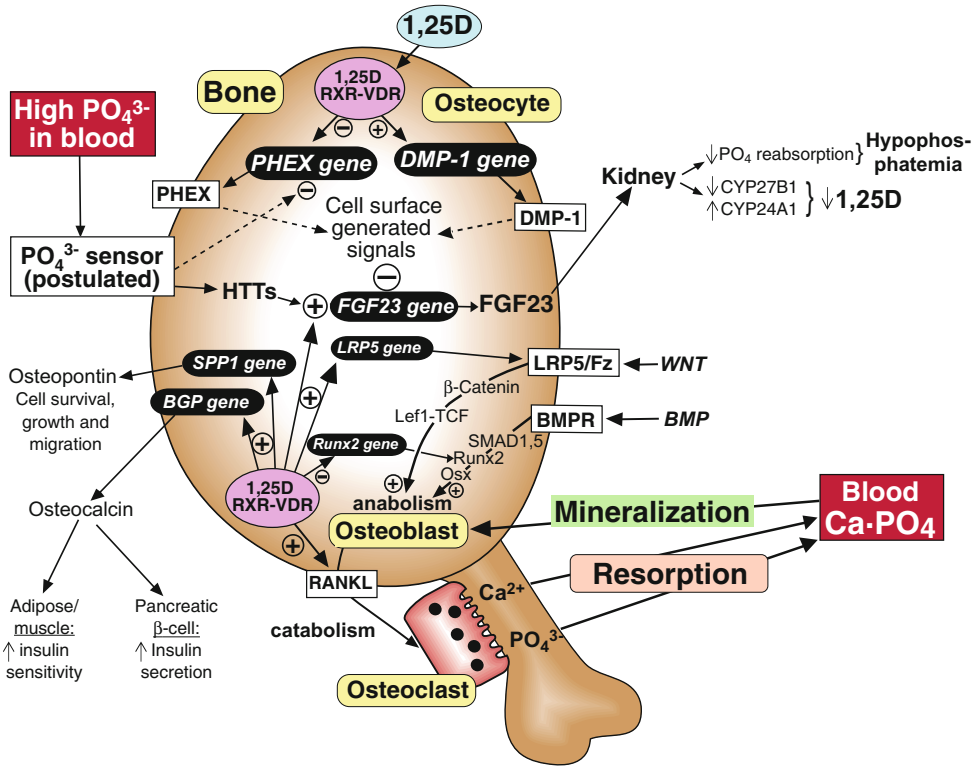

Fig. 4 1,25D-VDR action in bone cells. Vitamin D hormone signals via VDR-RXR liganding in osteoblasts and osteocytes. *BGP(BGLAP)* encodes osteocalcin, *SPP1* encodes osteopontin, Fz is the Frizzled coreceptor (together with LRP5) for Wnt ligands. HTT: hyperphosphatemia transducing transfactors. Other factors are defined and discussed in the text

attenuating osteoblastogenesis and, therefore, functions in this capacity as an antianabolic signal, thereby favoring catabolism. Supporting this concept, Tanaka and Seino [32] transplanted $VDR^{-/-}$ mouse bone into a $VDR^{+/+}$ background of normal mice and observed significantly increased mineralization of the *VDR*-null bone, suggesting that, directly in the skeleton, 1,25D-VDR action favors catabolism and/or antimineralization.

Anabolic Actions of Vitamin D on Bone

Osteopontin or *SPP1* (Fig. 4; Table 1) is induced by 1,25D in osteoblasts, increases osteoblast survival, and triggers ossification of the skeleton, while serving as an inducible inhibitor of vascular calcification and associated disease [33]. However, secreted osteopontin enhances cell survival, growth, and migration, rendering it one of the few cancer-promoting principles induced by 1,25D. 1,25D significantly increases the expression of *LRP5* (Fig. 4; Table 1) [18, 20], a gene product that stimulates osteoblast proliferation via enhanced canonical Wnt signaling and is thereby anabolic to bone [34]. Osteocalcin (bone Gla protein, or BGP) (Fig. 4; Table 1) is another classical 1,25D target in osteoblasts. Recently, utilizing *BGLAP*-null animals, it has been shown that osteocalcin expression is important for robust, fracture-resistant bones [35]. Finally, osteocalcin has been identified by Karsenty and coworkers [36] as a bone-secreted hormone that improves insulin release from pancreatic β-cells, increases insulin metabolic responsiveness, and is required for optimal fertility in male mice [37].

FGF23 is another bone-secreted hormone which, like PTH, elicits phosphaturia; and FGF23 is elaborated by bone under conditions of hyperphosphatemia (Fig. 4). *FGF23* gene expression is markedly upregulated by 1,25D [5] in osteocyte-like cells of the osteoblast lineage, and a VDRE (Table 1) has been identified in the human *FGF23* gene [38]. 1,25D also controls two osteocyte-expressed genes (*PHEX* and *DMP-1*) upstream of FGF23, rendering the modulation of FGF23 by 1,25D quite complex (Fig. 4). FGF23-null mice [39] are hyperphosphatemic and display ectopic calcification and markedly elevated 1,25D in blood, exhibiting the additional phenotypes of skin atrophy, osteoporosis, vascular disease, and emphysema. Many of these pathologies are also the consequence of hypervitaminosis D [40], and therefore 1,25D must be "detoxified" and sustained in an optimal range to maintain healthful aging. The biological effects of 1,25D are curtailed by CYP24A1-catalyzed catabolism of 1,25D in all tissues including bone, providing an "off" signal when the hormone has executed its actions. *CYP24A1* is induced by FGF23 [41] as well as by the 1,25D hormone, with potent VDREs identified in the *CYP24A1* gene (Table 1). Mice with ablation of the *CYP24A1* gene can die early, with survivors displaying defective endochondral bone formation and fracture healing [42]. Indeed, loss-of-function mutations in *CYP24A1* have been identified in patients with

idiopathic infantile hypercalcemia [43], highlighting the importance of CYP24A1-catalyzed detoxification of 1,25D. Furthermore, Gardiner et al. [44] transgenically overexpressed VDR specifically in mature osteoblasts and observed enhanced formation and decreased resorption in vivo, suggesting that the physiologic effect of 1,25D in bone is anabolic rather than catabolic.

Integration of the Catabolic and Anabolic Actions of Vitamin D on Bone

Insight can be gained into the relative significance of catabolic versus anabolic effects of vitamin D on bone if one hypothesizes that low physiologic levels of 1,25D promote bone formation via VDR signaling, perhaps acting to elicit osteoblast proliferation, with higher levels favoring catabolism/resorption or serving as an antimineralization signal. This latter concept is supported by the well-known effects of hypervitaminosis D to cause bone mineral loss and impaired mineralization [45]. These conclusions are further supported by observations both in vitro [46] and in vivo [47] that proliferation of osteoblast-like osteosarcoma cells is stimulated by physiologic levels of 1,25D (0.1 nM) but retarded by higher doses (10 nM) of the hormone. The notion that moderate to excessive vitamin D levels act to limit bone may seem counterintuitive. However, if one considers that 1,25D represses *COLIA1* gene expression in osteoblasts [48], thereby curtailing the organic matrix, and that the observed phenotype in rickets/osteomalacia resulting from vitamin D deficiency includes excess osteoid, it is clear that the mission of vitamin D is a well-mineralized, efficiently remodeled, fracture-resistant skeleton rather than simply more bone.

1,25D Action on Intestinal Enterocytes

The major action of 1,25D-VDR on the small intestine is the stimulation of calcium absorption, a process that occurs transcellularly in the duodenum and paracellularly across the length of the small intestine via active and passive mechanisms, respectively. The passive paracellular route dominates in the unweaned neonate, in aging animals, and when calcium intakes are high. Conversely, the active transcellular avenue predominates in weaned, growing animals on a limited-calcium diet. Calcium absorption is highly dependent upon VDR and its 1,25D ligand, as evidenced by greatly reduced absorption in *VDR*-ablated [49] and *CYP27B1*-null [50] mice. Both of these knockout mice are rachitic, which is rescued by a diet high in calcium, lactose, and phosphate, proving that the bone defects are entirely the consequence of eliminating the positive influence of 1,25D-VDR on intestinal calcium (and phosphate) absorption. Thus, the molecular mechanisms by which vitamin D and its receptor enhance intestinal calcium absorption are difficult to delineate [51], especially considering that this system evolved as an emergency endocrine phenomenon, allowing animals to adapt to a scarcity of calcium in their terrestrial environment.

Dietary Calcium Absorption

Enterocyte calcium uptake is mediated, in part, by 1,25D-VDR induction of *TRPV6* (Fig. 5a) [17, 20], a gene that possesses several classic VDREs (Table 1). *TRPV6* encodes a key calcium channel intrinsic to the apical membrane that transports calcium from a limited diet to build the mineralized skeleton and prevent rickets. However, although TRPV6 is depressed in concert with calcium absorption in *VDR*-null mice [49], intestinal calcium absorption is not affected in *TRPV6*-null mice receiving a normal-calcium diet [52]. Similarly, deletion of $CaBP_{9k}$, another 1,25D-induced gene [53], does not impact calcium absorption in mice fed a normal-calcium diet [52]; and only double knockout of *TRPV6* and $CaBP_{9k}$ plus feeding a low-calcium diet elicits depressed calcium absorption [52]. Thus, calcium absorption under normal dietary conditions does not absolutely require *TRPV6* and $CaBP_{9k}$, the latter of which is thought to "buffer" and/or mediate the transit of intracellular absorbed calcium. A third player in transcellular transport of calcium is a Ca-ATPase, PMCA1b, encoded by *ATP2B1* (Fig. 5a), which lacks the dramatic vitamin D dependence of *TRPV6* and $CaBP_{9k}$ expression [51] but nevertheless is critically important for the extrusion of calcium at the basolateral membrane to complete the transcellular transport of this ion. An alternative Ca-ATPase gene (*ATP2C2*) encodes $PMCA_{2c}$ (Fig. 5a), with the *ATP2C2* gene being induced by 1,25D in cultured intestinal cells (Table 2). Thus, the extrusion of calcium at the basolateral membrane is likely constitutive in part and is executed by $PMCA_{1b}$/$PMCA_{2c}$ but could be amplified via 1,25D induction of these Ca-ATPases. Further enhancement could occur through delivery of calcium by $CaBP_{9k}$ (Fig. 5a) or via alkaline phosphatase functioning as yet a second alternative Ca-ATPase [54]. However, although alkaline phosphatase I (*ALPI*) is induced in intestinal enterocytes by 1,25D at both the mRNA (Table 2) and protein/enzyme (Fig. 5b) levels, its temporal response to 1,25D lags behind that of 1,25D-initiated calcium absorption in vivo (Fig. 5c). Interestingly, $CaBP_{9k}$ appearance in response to 1,25D similarly trails calcium absorption [55], whereas *TRPV6* induction by 1,25D is more rapid and coincident with vitamin D-stimulated calcium absorption [56]. These observations suggest that TRPV6 mediates the rate-limiting step in transcellular calcium transport induced by 1,25D-VDR under low-calcium

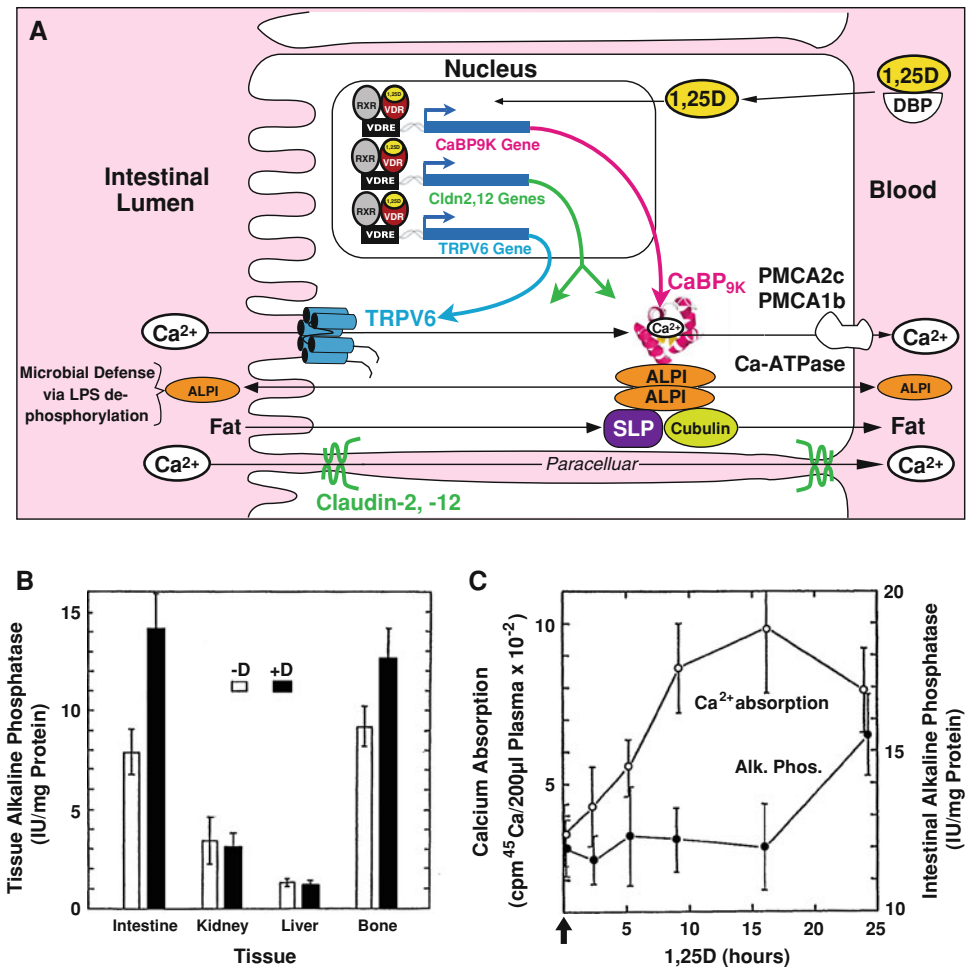

Fig. 5 1,25D-VDR action in small intestine. **a** Model for 1,25D-VDR signaling of transepithelial and paracellular calcium transport in the small intestine. *SLP* secreted lipid particle, for which exocytosis at the basolateral membrane is initiated by cubilin; *DBP* vitamin D binding protein, *ALPI* alkaline phosphatase I, *PMCA1b* plasma membrane calcium-ATPase 1b. **b** Vitamin D increases alkaline phosphatase activity selectively in intestine and bone of vitamin D-deficient chicks. Alkaline phosphatase enzyme activity was assayed as described elsewhere [54] in 20 % butanol extracts of tissue homogenates or slices (bone) 40 h after a single oral dose of 50 IU of vitamin D_3. Each result is the average of separate determinations in 10 animals (±SD). **c** Comparison of the time courses of 1,25D-mediated increases in calcium absorption and intestinal alkaline phosphatase after dosing vitamin D-deficient chickens with 390 pmoles of 1,25D at "0" time, as indicated by the *bold arrow*. Intestinal calcium absorption was measured as described previously [122]. Each value represents the average of four animals (±SD)

conditions and that $CaBP_{9k}$ and ALPI play supportive or secondary roles.

Multiple Roles of Alkaline Phosphatase

Besides a possible secondary role as a calcium ATPase in calcium translocation, *ALPI*, which encodes a membrane protein that is also secreted on both the apical and basolateral sides, may perform other functions in response to 1,25D. Knockout of *ALPI* in mice [57] accelerates fat absorption, indicating that *ALPI* governs lipid absorption, likely through attachment to a secreted lipid particle (SLP) transferring fat (Fig. 5a). By inducing *ALPI*, 1,25D-VDR could be considered "antiobesity" in animals on a high-fat diet. Moreover, ALPI associates with $CaBP_{9k}$ [58], raising the possibility of a link between calcium and fat translocation, with ALPI serving as a switching mechanism connecting these two phenomena (Fig. 5a). Finally, ALPI (tethered to the apical membrane) has been shown to function as a microbial defense barrier via the dephosphorylation of lipopolysaccharide [59]. It is instructive to compare the intestinal action of 1,25D with that in bone, the only other tissue in which we observe ALP induction (Fig. 5b). Bone, liver, and kidney express a tissue-nonspecific isozyme of ALP (TNAP). Mice with ablated *TNAP* and humans with loss-of-function mutations in this gene display hypophosphatasia, characterized by rickets and osteomalacia, epileptic seizures, and increased levels

Table 2 Key genes upregulated by 1,25D in cultured human colon cancer cells and keratinocytes via Affymetrix DNA Genechip microarray

Human colon cancer cell (Caco-2) mRNA induced by 1,25D[a]		Human keratinocyte (KERTr CCD-1106) mRNA induced by 1,25D[a]	
Class	Genes	Class	Genes
Calcium transport-related	*TRPV6*, *CLDN2*, *ATP2C2*, *ALPI*, *ALPPL2*	Epidermal differentiation, keratin-related	*LCE* (*1D*, *1F*, *2B*), *S100A* (*2*, *4*, *6*), *SPRR1B*, *KRT* (*13*, *16*, *34*, *38*, *71*), *KRTAP* (*4*, *5-1*, *5-4*, *8-1*, *10-2*, *10-4*, *10-7*, *10-9*, *12-2*)
Detoxification	*CYP* (*24A1*, *1A1*, *2S1*, *3A5/43*), *SULT* (*1A2*, *1C2*), ABC transporters (*A11*, *B1*, *D1*)	Detoxification	*CYP* (*24A1*, *2D6*)
Transcription factors	*CDX-2*, *MED9*, *JUNB*, *CEBPA*, *MX2*	Transcription factors	*JUNB*, *CREG2*, *ID1*, *SALL4*, *ZNF257*, *HNF1A*
Immunomodulation, inflammation, oxidation	*S100A4*, *NOX1*, *G6PD*, *KNG1*, *IRF8*, *ORM1*, *ORM2*, *DEFB32*, *CDC34*	Immunomodulation, inflammation, oxidation	*CAMP*, *DEFB109*, *DEFB132*, *G6PD*, *EFCAB4A/B*, *COLEC11*, *NFATC2*, *LGALS9*, *IGSF9B*, *IL25*
Development and cancer-related	*TGFB2*, *CEACAM6*, *EPHB4*, *EFNA5*, *DACT2*, *GLT8D4*, *TIMP2*, *TIMP3*	Development and cancer-related	*CASP14*, *BMP6*, *SFRP1*, *DNER*, *CST1*, *ADRB2/A1B*, *CA9*, *PNOC*, *DND1*, *MEG8*, *DUSP10*

At least a 1.2-fold upregulation

[a] [1,25D] = 10 nM for 24 h

of inorganic pyrophosphate [57]. The bone phenotype in hypophosphatasia is the result of lack of cleavage of extracellular pyrophosphate, a mineralization inhibitor, by TNAP. Thus, by inducing TNAP in bone, 1,25D functions as a promineralization hormone, yet another anabolic effect of vitamin D in the skeleton.

Dietary Phosphate Absorption and Importance of the Paracellular Route

Intestinal phosphate absorption per se is promoted by 1,25D via the induction of Npt2b in rat intestinal enterocytes [60], but because phosphate is abundant in the diet, the phosphate absorption effect of 1,25D may not be as physiologically relevant as the profound effect on calcium transport. Yet, phosphate is a fundamental biologic component of not only mineralized bone but essential biomolecules such as DNA, RNA, phospholipids, phosphoproteins, ATP, and metabolic intermediates; and under conditions of limiting dietary phosphate, the significance of 1,25D-mediated phosphate absorption likely is exposed. Despite the contribution of vitamin D-dependent translocation of calcium and phosphate across the enterocyte, it is clear that paracellular mechanisms also participate in the absorption of these bone minerals. In fact, phosphate is predominantly absorbed in this fashion, possibly because of its abundance in the diet. In the case of calcium, 1,25D induces the expression of genes encoding participants in the intercellular junction that function as paracellular cation channels, such as claudin-2 and -12 (Fig. 5a; Table 2), and influences cadherins and connexins to modulate intercellular adhesion. Therefore, a fraction of vitamin D-mediated calcium absorption is facilitated by paracellular mechanisms, which clearly cannot be ascribed to the induction of enterocyte transport proteins such as TRPV6, $CaBP_{9k}$, and $PMCA_{1b}$. Finally, a component of intestinal calcium absorption is probably mediated by nongenomic vitamin D mechanisms [61].

1,25D Action in the Kidney

The premier role of the kidney in the vitamin D endocrine system is generation of the 1,25D hormone by the catalytic action of CYP27B1 in the proximal tubule (Fig. 6). Renal CYP27B1 is induced by PTH under low-calcium conditions, whereas the enzyme is feedback-repressed by 1,25D in a short feedback loop and by FGF23 via a long loop. Bone-derived FGF23, like PTH, concomitantly signals inhibition of renal phosphate reabsorption in the proximal tubule by suppressing Npt2a (Fig. 6). Interestingly, FGF23 binds to different FGF receptors to mediate CYP27B1 repression (and CYP24A1 induction) versus Npt2a inhibition, namely, FGFR3/4 and FGFR1, respectively [62]. However, all three isoforms of FGFR require a coreceptor, klotho, a bona fide longevity gene expressed primarily in renal tubules, to bind FGF23 with high affinity. 1,25D induces klotho mRNA [63], and a VDRE (Table 1) in the human *Klotho (KL)* gene has been identified. Upregulation of klotho by 1,25D is consistent with potentiation of FGF23 signaling in the kidney and perhaps protection of other cell types (e.g., vascular) where a secreted form of klotho may exert beneficial effects (Fig. 6) [64].

The renal vitamin D hormone 1,25D acts directly on the proximal tubule via VDR to stimulate phosphate reabsorption through induction of Npt2a [65] and Npt2c [66, 67]. The physiologic impact of 1,25D on renal

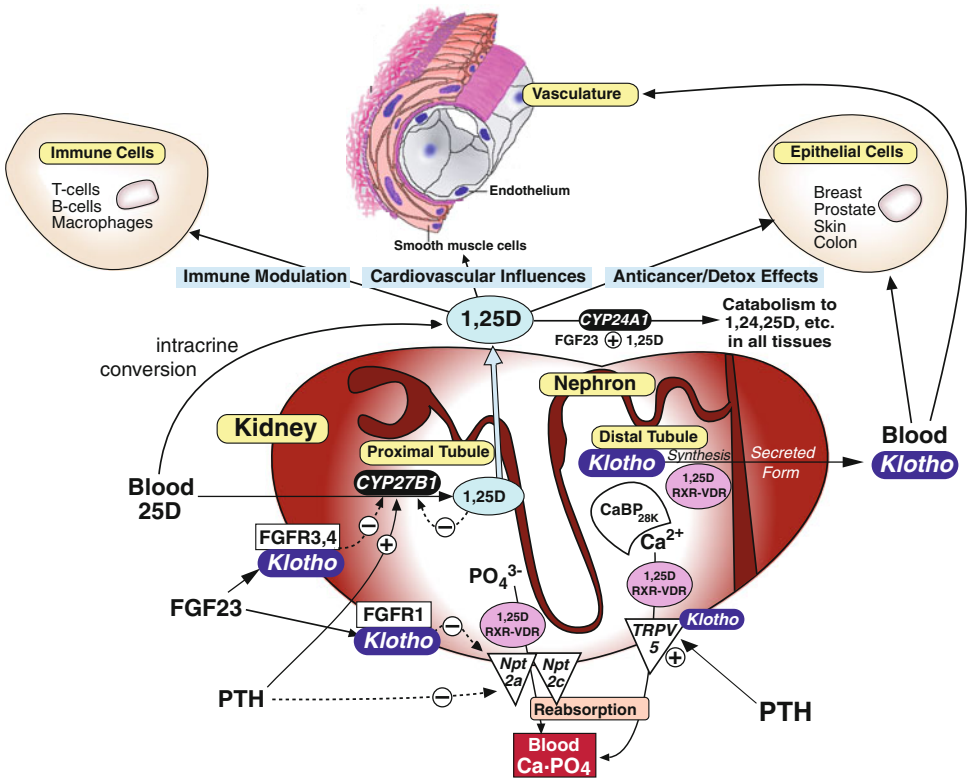

Fig. 6 The kidney responds to 1,25D, FGF23, and PTH to regulate vitamin D bioactivation and calcium/phosphate reabsorption and serves as an endocrine source of 1,25D and klotho. The effects of kidney-derived 1,25D and klotho on various tissues are discussed in the text

phosphate reabsorption is not fully understood, although patients with loss-of-function mutations in *Npt2c* display significant hypercalciuric, hypophosphatemic rickets/osteomalacia [68]. In contrast, mice with ablated *Npt2c* exhibit no phosphate imbalance but display deranged calcium and 1,25D metabolism [68], suggesting that in rodents Npt2c is more relevant to calcium homeostasis, with phosphate reabsorption carried out predominantly by Npt2a.

Calcium is reabsorbed actively in the distal renal tubules by a process analogous to 1,25D-VDR-induced duodenal calcium absorption (Fig. 5a). In the distal nephron, the rate-limiting step is the induction by 1,25D of the *TRPV5*-encoded calcium channel, which is fixed in the membrane facing the glomerular filtrate through the glycosidase enzymatic activity of klotho on extracellular carbohydrate moieties in TRPV5 [69]. TRPV5 in the distal renal tubule (Fig. 6) thus plays a role in 1,25D-induced calcium translocation akin to that of TRPV6 in the intestine. Moreover, renal klotho could be considered analogous to intestinal ALP in that both membrane enzymes are induced by 1,25D and perform secondary, supportive extracellular functions in regulating calcium and phosphate transport, as well as possibly acting systemically to promote immune function and elicit longevity. Intracellularly, calcium is buffered and transferred by $CaBP_{28k}$ in the distal nephron (Fig. 6) after induction by 1,25D in a manner similar to the induction of $CaBP_{9k}$ (*CALB3*) in the intestine. Extrusion of calcium into the bloodstream by the distal tubules is accomplished by $PMCA_{1b}$, identical to the mechanism in enterocytes, and via NCX1, a sodium/calcium exchanger [51]. In summary, as depicted in Fig. 6, renal calcium reclamation is crucial to calcium homeostasis and is accomplished by the activation of TRPV5 by PTH plus the induction of *TRPV5* by 1,25D (Fig. 6), both under conditions of low calcium. Supportive roles in calcium reabsorption are played by klotho, $CaBP_{28k}$, $PMCA_{1b}$, and NCX1, with klotho and $CaBP_{28k}$ being the most dependent on 1,25D-VDR.

Therefore, the kidney is the source of two essential hormones, 1,25D and klotho. As such, the kidney, and vitamin D actions therein, impacts virtually every cell in the body, especially those that express VDR in reasonable concentrations. Illustrated in Fig. 6 are three examples of this phenomenon. In the case of immune function (Fig. 6, upper left), 1,25D-VDR induces cathelicidin [70] to activate the innate immune system to fight infection (Table 1); and evidence is accumulating that the risk of human infections such as tuberculosis is reduced by sufficient circulating levels of 25D [71]. In addition, 1,25-VDR represses IL-17 [6] to temper the adaptive immune system and possibly lower the risk of autoimmune disorders such as type 1 diabetes mellitus, multiple sclerosis, and rheumatoid arthritis that have been linked to vitamin D deficiency in association studies. 1,25D-VDR is anti-inflammatory by blunting NFκB [72] and COX2 [73], and inflammation is considered a common denominator in

maladies such as cardiovascular disease and ischemic stroke, as well as cancer. In the realm of cardiovascular disease (Fig. 6, upper center) as well as neurodegenerative disorders of aging such as Alzheimer disease, excess circulating homocysteine is considered a negative risk factor; and 1,25D-VDR has recently been shown [74] to induce cystathionine β-synthase (*CBS*, Table 1) to catalyze the elimination of homocysteine. More directly with respect to 1,25D and heart disease, the three relevant cell types, namely, endothelial, smooth muscle, and cardiac myocyte, all express VDR; and knockout of the receptor in mice elicits left ventricular hypertrophy and fibrosis [75]. In addition, klotho likely cooperates with 1,25D to the benefit of the vasculature, with amelioration of hypertension, oxidative stress, and vascular smooth muscle proliferation/migration [64], although controlled studies are required to demonstrate that these effects of vitamin D and klotho occur in humans. Prevention of epithelial cell cancers by 1,25D and klotho (Fig. 6, upper right) represents the third extrarenal realm wherein 1,25D-VDR-regulated genes encode factors impacting cell life/cancer. VDR likely reduces the risk for many cancers by inducing the p53 and p21 (Table 1) tumor suppressors [76], as well as DNA mismatch repair enzymes in the colon [77]. *VDR* knockout mice exhibit enhanced colonic proliferation [78] plus amplified mammary gland ductal extension, end buds, and density [79], indicating that the fundamental actions of VDR to promote cell differentiation and apoptosis [80] play an important role in reducing the risk of age-related epithelial cell cancers such as those of the colon and breast. Finally, klotho has been implicated recently in the prevention of pancreatic neoplasia [81]. However, as pointed out by Manson et al. [82], evidence that vitamin D and/or klotho are preventative for human cancer is lacking. Nevertheless, through control of vital genes and cell functions, 1,25D-VDR and/or klotho are excellent candidates to allow one to age well, not only by delaying osteoporosis, fractures, and ectopic calcification via control of calcium and phosphate but also by tempering malignancy, oxidative damage, infections, autoimmunity, inflammation/pain, and cardiovascular and neurodegenerative diseases.

1,25D Action in the Colonocyte

Of the epithelial cells postulated to be protected by VDR signaling, those of the colon and skin express the highest levels of VDR, comparable to those in the calcemic tissues: intestine, kidney, and bone. One theme for liganded VDR action in colon, analogous to the role of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [83] in the liver, is the induction of CYP enzymes for xenobiotic detoxification. As illustrated in Fig. 7, a major

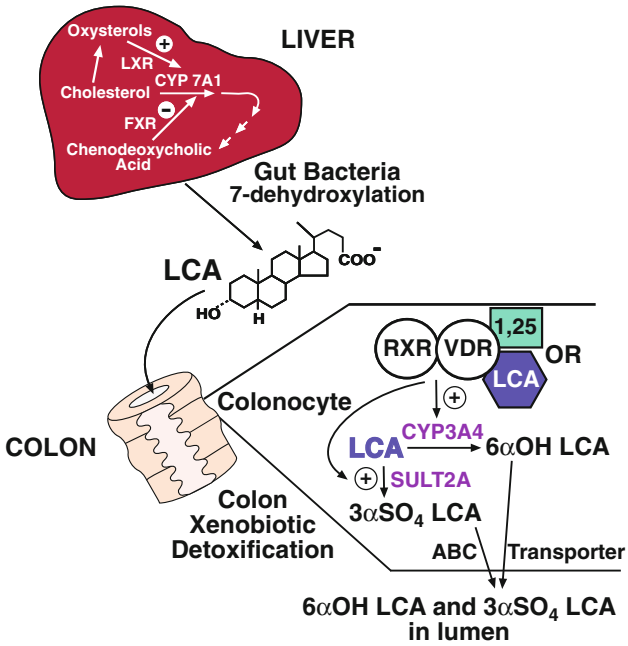

Fig. 7 Pathophysiologic roles of two VDR ligands in preventing colon cancer

target for VDR (and PXR) in humans is *CYP3A4* [84, 85], for which the detoxification substrates include lithocholic acid (LCA), a toxic secondary bile acid generated in the enteric system by bacteria. Initial studies focused on VDR liganded to 1,25D as a regulator of *CYP3A4*, but later experiments revealed that LCA is also capable of binding VDR to upregulate expression of human *CYP3A4* or its equivalent in rats (*CYP3A23*) or mice (*CYP3A11*) [84]. There is also evidence that CYP enzymes other than CYP24A1 and CYP3A4 may be VDR targets [86]. Additionally, 1,25D induces *SULT2A* (Fig. 7), an enzyme that detoxifies sterols via 3α-sulfation [87]. In fact, when we screened mRNAs induced by 1,25D in human (Caco-2) colon cancer cells (Table 2), besides mRNAs encoding calcium translocating proteins, a major group of induced mRNAs coded for detoxification agents, including CYPs, SULTs, and ABC transporters. In accordance with this observation, Meyer et al. [88], utilizing ChIP-Seq technology in the human LS180 colon cell line, demonstrated that VDR/RXR binds in the vicinity of many *CYP* and other detoxification-related genes.

A model for the pathophysiologic significance in humans of LCA detoxification as a consequence of liganded VDR signaling is depicted in Fig. 7. The precursor to LCA, chenodeoxycholic acid, is produced in the liver via a pathway that is controlled in a positive fashion by LXR and in a negative feedback loop by FXR [89] (both of these receptors form heterodimers with RXR, not shown). LCA, formed through 7-dehydroxylation by gut bacteria, is not a good substrate for the enterohepatic bile acid reuptake

system and, thus, remains in the enteric tract and passes to the colon, where it can exert carcinogenic effects [90]. VDR in the colonocyte is proposed to bind LCA (or its 3-keto derivative) and induce *CYP3A4* [84]. CYP3A4 then catalyzes the 6α-hydroxylation of LCA, thus converting it into a substrate for an ABC efflux transporter [89]. We have shown that 1,25D, which is formed in the kidney or locally in colon via the action of CYP27B1, can also induce *CYP3A4* [83]. Thus, both of these nutritionally modulated ligands for VDR possess the important potential to serve as agents for promoting detoxification of LCA and possibly other intestinal endo- or xenobiotics, with the end result likely being a reduction in colon cancer incidence.

Of course, 1,25D-VDR no doubt elicits additional effects besides detoxification in normal or malignant colonic cells, and we observed that 1,25D induces the expression of transcription factors (e.g., *CDX-2*, involved in intestinal development), immunomodulators (e.g., *DEFB32*, an antimicrobial), anti-inflammatory (e.g., *NOX1*) and antioxidant (e.g., *G6PD*) enzymes, as well as many anticancer principles (e.g., *TGFB2, TIMP2, TIMP3*) in Caco-2 cells (Table 2). Finally, 1,25D has been shown to suppress β-catenin signaling in colon cancer cells [80], and β-catenin is a proto-oncogene that participates in the growth-promoting Wnt signaling pathway that is thought to play a significant role in the etiology of colon cancer. These findings are all consistent with the proposed prodifferentiative, antioxidant, and proimmune influences of 1,25D-VDR that are thought to contribute to the anticancer potential of vitamin D.

1,25D Action in Skin

VDR is abundantly expressed in the skin. Moreover, VDR-null mice display the phenotype of alopecia and dermal cysts, which is not ameliorated with a high-calcium, -lactose, and -phosphate rescue diet. Surprisingly, however, there exists no corresponding pathologic phenotype in the skin of mice unable to synthesize 1,25D, suggesting that the action of VDR in the skin is independent of the 1,25D ligand. Along with VDR [91], β-catenin is absolutely required in keratinocytes [92] to permit mammalian hair cycling, and the ligand-independent action of VDR to mediate the hair cycle is thought to involve Wnt signaling in skin stem cells [91, 93].

As illustrated schematically in Fig. 8, the regulation of mammalian hair cycling is complex, consisting of the convergence of two signaling pathways, BMP and Wnt. Starting at the upper left of Fig. 8, Noggin from the dermal papilla initially antagonizes BMP4 signaling in bulb (or bulge) keratinocytes, allowing for the accumulation of Lef1/TCF, a transcriptional coactivator which targets genes

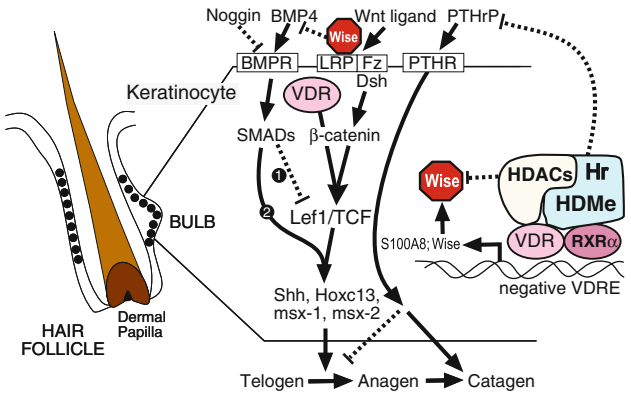

Fig. 8 Model for VDR action in keratinocytes to sustain the mammalian hair cycle. *Wnt* ortholog of *Drosophila* wingless and mouse int-1, *Lef1* lymphoid enhancer factor-1, *TCF* T cell-specific factor, *msx-1/-2* orthologs of *Drosophila* muscle-specific homeobox protein. Factors that are membrane receptors or transporters are *boxed*. *Solid arrows* indicate activation, and *dotted lines ending in a solid perpendicular line* denote inhibition

via DNA-binding partners such as β-catenin. Cessation of Noggin signaling reinstates BMP signal transduction via SMADs, provided that the Wnt modulator in surface ectoderm (Wise or SOSTDC1), which antagonizes both Wnt and BMP pathways [94], has also been repressed. Wnt ligand, e.g., Wnt 10b, signaling leads to accumulation of β-catenin, which facilitates cooperation with Lef1/TCF to induce genes encoding factors, such as sonic hedgehog (Shh), that trigger the hair cycle to transition from telogen (resting) to anagen (growth). VDR apparently promotes β-catenin-Lef1/TCF function by either serving as a coactivator of the β-catenin transcription complex or inducing a positive member of this protein complex.

Moreover, we propose that VDR also functions in keratinocytes to drive the hair cycle by controlling gene expression through negative as well as positive VDREs, based upon gene ablation studies which indicate that VDR, its RXRα [95] DNA binding heteropartner, as well as the coactivator DRIP205 [6] and the corepressor hairless (Hr) [96] all are required for normal hair cycling. The most important role of VDR in controlling the hair cycle appears to be repression of key target genes. This conclusion is based upon the observation that knockout of *Hr*, a *VDR* corepressor which colocalizes with VDR in the outer root sheath of the hair follicle [97], produces a phenocopy of the *VDR*-null mouse with respect to alopecia but does not affect bone and mineral metabolism [98]. At least one of the molecular functions of Hr is recruitment of histone deacetylases (HDACs) to promote a repressive heterochromatin architecture in the vicinity of the target gene [99]; another proposed function of Hr is to catalyze histone demethylation (HDMe) to further attenuate transcription [100].

What genes are targeted by the VDR-RXRα-Hr complex for repression? Thompson and coworkers [93] defined *SOSTDC1* (encoding Wise) as a gene overexpressed in keratinocytes from Hr-null mice, and Kato and coworkers [101] characterized *S100A8* as a gene overexpressed in VDR-null keratinocytes. In determining the effect of activated VDR on the expression of *SOSTDC1* and *S100A8* mRNA levels in human keratinocytes [1], we observed that *SOSTDC1* is strikingly repressed after 18 h of 1,25D treatment of keratinocytes. Suppression of *SOSTDC1* mRNA by 1,25D was verified utilizing cDNA microarray analysis of human cells [1]. Because Wise not only antagonizes the Wnt pathway by binding to LRP but also inhibits the BMP pathway through neutralization of BMP4 [94], repression of *SOSTDC1* by VDR could constitute a major event in initiating the mammalian hair cycle (Fig. 8). Similarly, 1,25D rapidly represses expression in human keratinocytes of *S100A8* and its obligatory *S100A9* heteropartner in calcium binding [1]. This inhibition of *S100A8/-A9* expression by 1,25D-VDR is in stark contrast to the induction of *S100A8/-A9* observed in HL-60 promyelocytic leukemia cells when differentiated by 1,25D along the macrophage lineage [102]. Thus, 1,25D regulates *S100A8/-A9* expression in a cell-selective fashion. One additional gene repressed by 1,25D-VDR, namely, *PTHrP* [103], is known to encode a suppressor of the telogen to anagen transition in the hair follicle, as well as to promote entry into catagen [104], providing yet another VDR-RXRα-Hr repressed gene target that participates in hair cycle control (Fig. 8). In conclusion, VDR is crucial for the regeneration of hair, an obvious shield that protects skin and facilitates healthful aging, via both protein–protein and protein–DNA interactions that potentiate Wnt, BMP, and calcium signaling.

Although unliganded VDR is the dominant force in driving the mammalian hair cycle, 1,25D, either produced locally or generated in the kidney, is capable of signaling keratinocyte differentiation and is used clinically as an antiproliferative agent in the treatment of psoriasis [105]. Thus, vitamin D acts in the skin to depress growth and to promote differentiation, just as it does in many cancer cell lines. In fact, the VDR-null mouse is supersensitive to dimethylbenz[*a*]anthracene-induced skin cancer [106] as well as UV light-induced skin malignancy [107], and 1,25D is a candidate for the prevention of skin cancer. Interestingly, photoirradiation of the skin produces vitamin D, and the CYPs catalyzing bioactivation to 1,25D are expressed in the skin and, therefore, able to produce local 1,25D to protect the epithelium against UV-induced photodamage and malignancy. In addition, 1,25D induces the expression of a number of genes in cultured keratinocytes, the products of which are potential prodifferentiative and structural components as well as detoxification, immunomodulation, and anti-inflammatory/antioxidation principles (Table 2). For example, 1,25D induces caspase-14 (*CASP14*) in keratinocytes (Table 2), and this nonapoptotic caspase is crucial for keratinocyte differentiation [98]. Also, genes in the epidermal differentiation complex (*LCE-1D*, *-1F*, *-2B*) are induced by 1,25D in human keratinocytes (Table 2). 1,25D induces cathelicidin (*CAMP*) and several defensins in keratinocytes (Table 2), indicating that vitamin D modulates the immune complement in skin. Finally, 1,25D increases the expression of a number of keratin-related transcripts, as well as the late cornified envelope (LCE) proteins, suggesting that vitamin D signaling supports the skin structurally and mediates barrier function development. In summary, unliganded VDR functions to drive the mammalian hair cycle in cooperation with Hr, primarily via the repression of gene expression, whereas 1,25D acts via VDR binding to signal the development and barrier function of the skin. This latter activity is apparently redundant with other signalers, such as calcium, but nevertheless is important therapeutically in the prevention and treatment of hyperproliferative skin diseases.

Ontogeny and Evolution of VDR and Its 1,25D Ligand

VDR appears to have adapted evolutionarily to become a "specialty" regulator of intestinal calcium absorption and hair growth in terrestrial animals, providing both a mineralized skeleton for locomotion in a calcium-scarce environment and physical protection against the harmful UV radiation of the sun. Yet, as discussed above specifically for the colon, VDR also has retained its ancient PXR-like ability to effect xenobiotic detoxification via CYP induction. In fact, based upon examination of the evolutionary position of VDR among the 48 human nuclear receptors, VDR is extremely closely related to PXR, both structurally and functionally [13]. The major action of PXR is in liver protection via induction of CYP enzymes that participate in xenobiotic detoxification. VDR could conceivably complement PXR by serving as a general guardian of epithelial cell integrity, especially at environmentally or xenobiotically exposed sites such as the intestine, kidney, and skin.

In this section we examine the evolutionary origin of VDR and its hormonal ligand. In other words, do VDR and 1,25D predate their modern functions of promoting intestinal calcium and phosphate absorption to create calcified tissue and of signaling keratinocyte differentiation to drive mammalian hair cycling? A second issue addressed in this section is VDR ontogenesis, which includes the temporal expression of VDR in the development of classic vitamin D target tissues such as the intestine, kidney and bone as well as in "non-vitamin D target tissues" such as liver and muscle.

With respect to VDR ontogeny, we examined VDR concentrations in the developing chicken, focusing on the small intestine, which expresses high levels of the receptor in adults (positive control), as well as skeletal muscle and liver, which express very low levels of VDR in the avian adult (negative controls). Immunoblotting (Fig. 9a, right portion of inset) confirms the high expression of avian intestinal VDR compared to the paucity of VDR in muscle and liver in the adult chicken. In striking contrast, immunoblotting (Fig. 9a, left portion of inset) reveals a complementary expression pattern of VDR in these same tissues in the 9 day-old embryo. VDR is barely expressed in embryonic intestine but moderately expressed in embryonic skeletal muscle and liver. To track the apparent

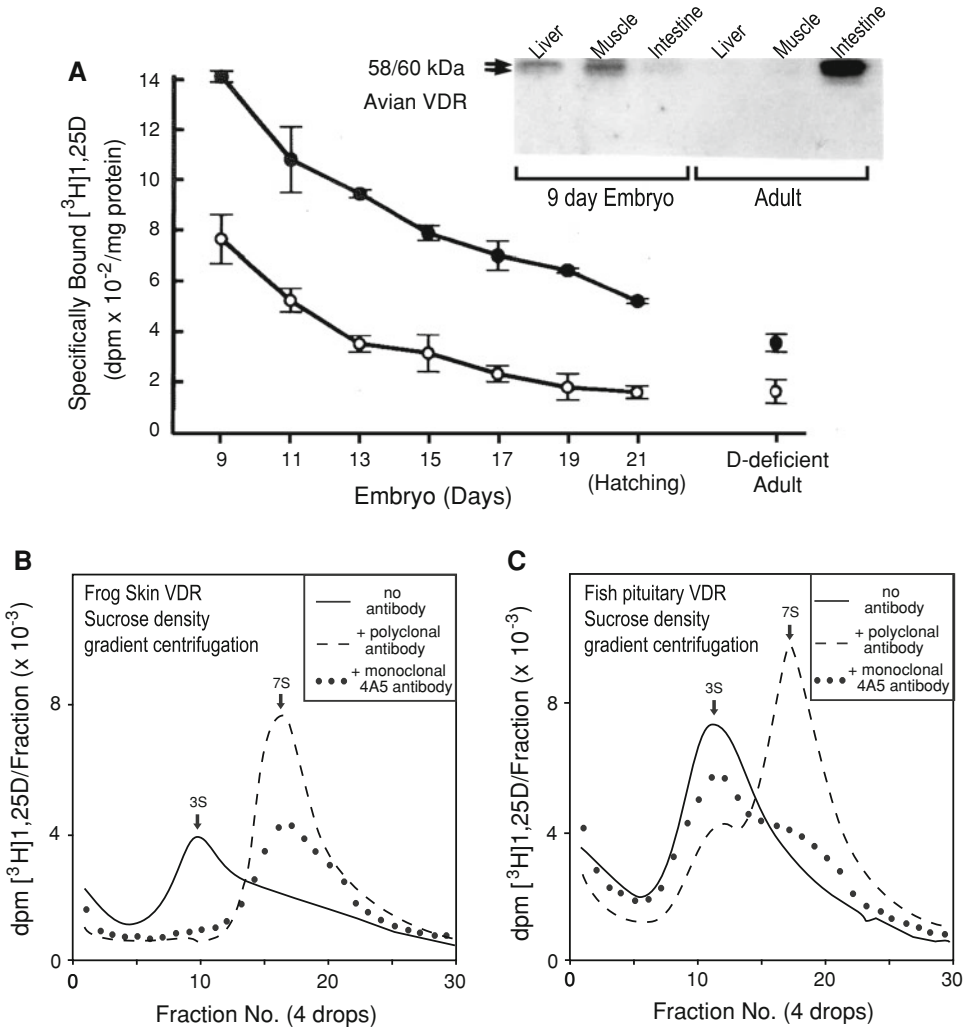

Fig. 9 Ontogenesis and evolution of VDR. **a** Profile of VDR as monitored by high-affinity/specific binding of [^{3}H]1,25D in skeletal muscle (*open circles*) and liver (*solid circles*) of the developing chicken. Nuclear extracts were incubated 16 h at 4 °C with 1 nM [^{3}H]1,25D (180 Ci/mmol) to radiolabel the receptor and a 50-fold excess of radioinert 25(OH)D_3 to eliminate any ligand binding to contaminating DBP. Radiolabeled VDR was isolated and counted by DEAE filter assay-liquid scintillation procedures [123]. Data are the average of triplicate determinations ± SD. *Inset* immunoblot detection of VDR in embryonic and adult chicken tissues. Nuclear extracts from each tissue were incubated as detailed above to radiolabel the receptor, which was then purified by DNA-cellulose chromatography, concentrated, and subjected to SDS-PAGE, followed by transfer to nitrocellulose membranes for immunological probing with 9A7 monoclonal antibody [124]. For embryo samples, total protein levels applied to each lane were as follows: liver, 1.5 mg; muscle, 5.0 mg; intestine, 1.0 mg. For adult tissue samples, total protein levels applied to each lane were as follows: liver, 1.5 mg; muscle, 1.5 mg; intestine, 1.5 mg. **b** Identification of VDR in frog (*Rana catesbeiana*) skin cytosol by shifting its position in sucrose density gradient centrifugation with specific polyclonal and monoclonal antibodies. Sucrose density gradient centrifugation of samples of [^{3}H]1,25D-labeled VDR in tissue cytosols was performed as described elsewhere [125]. **c** Identification of VDR in fish (*Oncorhynchus mykiss*) pituitary gland cytosol by shifting its position in sucrose density gradient centrifugation with specific polyclonal and monoclonal antibodies. Because the epitope for the 4A5 monoclonal antibody is not well conserved in fish VDR, it is not shifted by this antibody; but we demonstrated that it is totally immunoprecipitated when a rabbit anti-rat secondary antibody is employed in conjunction with the 9A7 monoclonal antibody for which the epitope is 100 % conserved between human and fish VDR (data not shown)

decline of VDR in muscle and liver during embryogenesis, we monitored the receptor via high-affinity binding of the radiolabeled hormonal ligand. As shown in Fig. 9a, both skeletal muscle and liver VDR binding activities descend gradually between 9 and 21 days (hatching) to levels that approximate the low concentrations in the adult. The conclusion from these data is that, as expected, the target intestine in avian species primarily expresses VDR after hatching when it is required to signal dietary calcium absorption, whereas "nontarget" tissues unexpectedly express VDR significantly at the early embryonic stage but lose this expression when the cells differentiate and develop into adult tissues.

There are two interpretations to this latter phenomenon. One is that only a select cell type(s) within the "nontarget" tissues expresses VDR, and this cell type is dominant in the embryo but scarce in the adult tissue. The second explanation is that VDR is moderately expressed in stem cells and all cell types, either declining comparably with development if unnecessary for signaling in that tissue or escalating in vitamin D target tissues (e.g., gut) when required to mediate signaling by the calcemic hormone, such as after weaning in rodents. In favor of the first explanation, it is noteworthy that in skeletal muscle VDR is primarily expressed in myoblasts rather than myotubes and that 1,25D initiates the differentiation of myoblasts to myotubes in skeletal muscle [108]. Thus, it may be only the myoblasts in adult muscle that are reactive to 1,25D, and this is consistent with a recent study by Garcia et al. [109] showing that mouse C2C12 myoblastic cells respond to 1,25D to induce markers of differentiation such as MyoD and myogenin, while repressing myostatin. Analogously, it has been recently shown [110, 111] that the minor population of stellate cells in the liver, which are key mediators of progressive hepatic fibrosis following an insult, dramatically express VDR and respond to 1,25D by repressing inflammatory mediators/chemokines and profibrotic collagens/collagenases, not only suppressing hepatic fibrosis but perhaps altering the hepatic microenvironment to lower the risk of malignancy.

The overall conclusion is that rather than "being expressed in every cell in the body," as is touted in the popular press, VDR is expressed in select cells within virtually every tissue in the body. VDR expression is governed by the state of differentiation of the cell, being present in stem cells if needed to control the early development of that line and perhaps cast aside when not required in the end-stage differentiated cell type. Conversely, VDR may not regulate early cell development but instead be induced in differentiated adult tissues to perform signaling for the phenotypic functions of that tissue, as occurs for bone mineral ion transport by enterocytes, renal epithelial cells, and osteoblasts/osteocytes. Because the major cell type in liver is the hepatocyte, the primary cell in skeletal muscle is the myocyte/myotube, and VDR is not expressed significantly in either of these cell types, neither of these bulk tissues is considered a vitamin D target. Yet by initiating differentiation of myoblasts and suppressing inflammation following an insult via hepatic stellate cells, vitamin D is clearly involved in the prevention of sarcopenia and liver fibrosis, respectively.

How evolutionarily ancient is VDR in the eukaryotic animal kingdom? VDR is expressed in neither *Caenorhabditis elegans* nor yeast, but frog and fish tissues express VDR as monitored by its high-affinity binding of radiolabeled 1,25D, sedimentation coefficient (molecular size and shape) in a sucrose density gradient, and immunoreactivity (increased sedimentation coefficient) to specific monoclonal and polyclonal VDR antibodies. Interestingly, the frog expresses VDR in high concentrations in the skin (Fig. 9b), whereas the pituitary gland (Fig. 9c) is a rich source of VDR in fish, where the respective roles of VDR could be analogous to 1,25D signaling cell differentiation and neuroendocrine control in mammals. In support of the receptor protein data in Fig. 9b, c, VDR cDNAs have been cloned from frog [112] and fish [113] species, and VDR homologs appear to be present in all fish and amphibian genomes thus far sequenced (data not shown). Frogs and fish not only express VDR but also synthesize ample amounts of the 1,25D hormonal ligand. Frogs display a circulating level of 14 pg/mL and fish 200 pg/mL compared to the human level of 33 pg/mL [114]. Because of the high bioavailability of calcium in fresh- and seawater, we assert that 1,25D does not necessarily function as a calcemic hormone in lower animals, and VDR likely does not drive skin appendage formation in amphibians and scaleless fish as it does in mammals.

In support of this point, Whitfield et al. [115] cloned *VDR* and demonstrated its expression in the sea lamprey, a very basal vertebrate akin to hagfish, possessing neither calcified tissue nor hair/skin accessory features, and yet this organism biosynthesizes 1,25D [116]. The distribution of VDR in lamprey, showing abundance in the skin and mouth but not intestine [115], suggests that the original function of liganded VDR was protection against the environment by signaling xenobiotic detoxification. In fact, lamprey VDR preferentially activates transcription of CYP3A4 VDRE-containing reporter constructs compared to constructs with calcemic VDREs such as those in osteocalcin and TRPV6 [115]. Thus, an evolutionarily ancient role of liganded VDR appears to be that of detoxification [115], and this detoxification function for vitamin D-VDR is retained in higher vertebrates, as discussed above. Based on the observations in embryos, a second evolutionarily ancient role of VDR may be the fundamental control of cell differentiation/proliferation, a process that could have

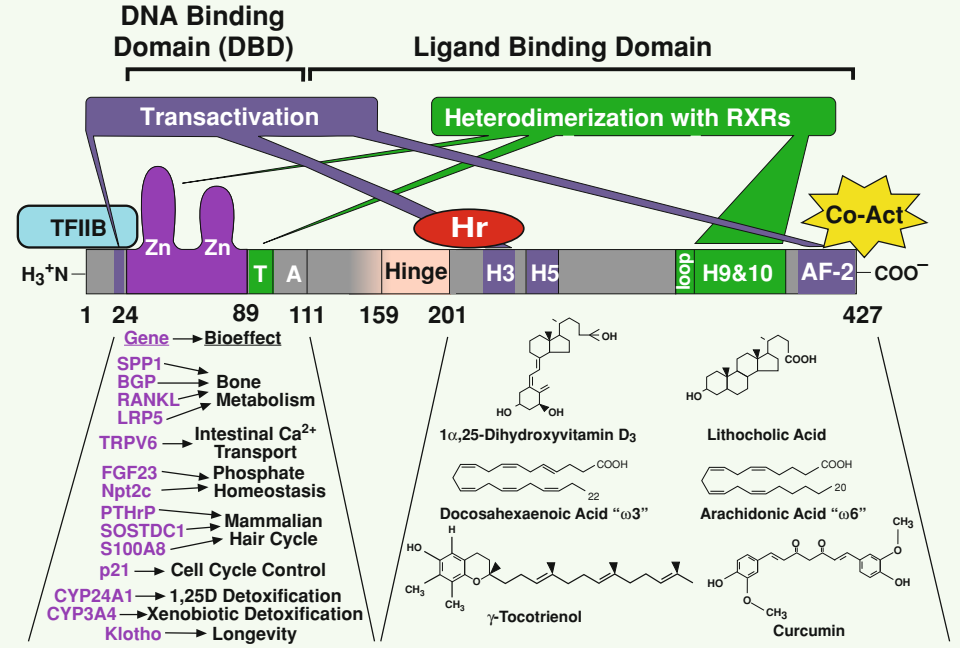

Fig. 10 Novel ligands for VDR shown in the context of the functional domains in human VDR, its interacting comodulators, and a summary of the genes and bioeffects signaled by liganded VDR. *Left* the human VDR zinc finger DNA binding domain, which, in cooperation with the corresponding domain in the RXR heteropartner, mediates direct association with the target genes listed at the *lower left*, leading to the indicated physiological effects. The official gene symbol for BGP is *BGLAP*, that for RANKL is *TNFSF11*, that for Npt2c is *SLC34A3*, that for PTHrP is *PTHLH*, and that for klotho is *KL*. *Right* below the ligand binding domain are illustrated selected VDR ligands, including several novel ligands discussed in the text

morphed into the putative anticancer effects of 1,25D-VDR in humans.

Nonvitamin D Ligands for VDR

One notable feature of the VDR/PXR/CAR subfamily of nuclear receptors involved in detoxification is their ability to recognize multiple ligands. The diversity of ligands for PXR is especially broad and includes not only endogenous steroids but also an array of other lipophilic compounds such as the secondary bile acid LCA, the antibiotic rifampicin, as well as xenobiotics such as hyperforin, the active ingredient of St. John's wort [117]. We have identified several additional nutritional lipids as candidate low-affinity VDR ligands which may function locally in high concentrations. Figure 10 reveals that these novel putative VDR ligands include ω3- and ω6-essential polyunsaturated fatty acids, docosahexaenoic acid and arachidonic acid, respectively [118], the vitamin E derivative γ-tocotrienol, and curcumin [119], the latter of which is a turmeric-derived polyphenol found in curry. Thus, it is now recognized that VDR binds several ligands beyond the 1,25D hormone. Considering the structures for prototypical PXR, CAR, and FXR ligands and comparing these compounds with the ligand binding profile of VDR, expanded to include LCA and its 3-keto derivative, it is evident that VDR exhibits a ligand profile resembling that of the closest VDR relatives in the nuclear receptor superfamily, especially when it is noted that both PXR and FXR [84] are also activated by LCA to some extent. Thus, VDR, PXR, and CAR are three nuclear receptors that bind a host of ligands, heterodimerize with RXR to signal detoxification of xenobiotics, and overlap somewhat in their target gene repertoires, which are laden with CYPs.

Conclusions

In summary, in this review we have highlighted the latest developments in the mechanism of vitamin D action and its biological consequences. A new understanding of the physiology of vitamin D bioactivation in relation to phosphate metabolism and aging has been achieved, with the induction by 1,25D-VDR of FGF23 in bone and klotho in kidney taking center stage as the mechanism whereby vitamin D mediates phosphate homeostasis and possibly delays the chronic diseases of aging. As a DNA-binding chromosomal protein, VDR is a charter member of the nuclear receptor superfamily, specifically a member of the VDR/PXR/CAR subfamily that is rooted evolutionarily in signaling detoxification.

1,25D-VDR functions mainly through genomic mechanisms, although the hormone-receptor complex also acts rapidly via nongenomic mechanisms, as detailed elsewhere [120]. Moreover, there is evidence that for phenomena such as stimulating intestinal calcium transport, 1,25D operates via VDR-independent mechanisms [61]. In the case of genomic mechanisms, new insight into the control of transcription by 1,25D-VDR-RXR has been gained through macromolecular structural studies, which indicate that the vitamin D ligand, the DNA sequence of the VDRE, and the recruited coactivator/corepressor all are capable of allosterically influencing the conformation of the VDR-RXR binary receptor. Functionally important VDREs have been located at remote positions 5′ and 3′ of the transcription start site in 1,25D-regulated genes, implicating DNA-looping and chromatin architecture as major forces in the nucleation of gene-expression regulation by vitamin D. Finally, epigenetic modification of both DNA and histones to modulate gene availability for transcription completes the "painting" of what the authors consider the "molecular masterpiece" of the genomic mechanism of vitamin D action.

The consequences of physiologic (optimal) levels of 1,25D acting directly on the skeleton via VDR genomic signaling appear to comprise a delicate balance of anabolic and catabolic events. Either a deficiency or an excess of 1,25D is deleterious to bone, primarily the result of pathologic resorption, respectively, via PTH signaling in D deficiency and via RANKL action in vitamin D toxicity. However, the paramount physiologic effect of 1,25D-VDR genomic signaling remains the promotion of intestinal absorption of calcium and phosphate, especially when either of these ions is limited in the diet. The bottom line is that the primary mechanism whereby 1,25D-VDR prevents rickets and osteomalacia is the induction of intestinal calcium and phosphate absorption from the gut, thereby providing these ions for proper bone mineralization.

With respect to the appearance of VDR in classic target tissues, for example, small intestine, VDR is induced (in part by 1,25D) in enterocytes when needed to signal calcium absorption, such as at weaning in mammals and at hatching in birds. In "nontarget" tissues such as the liver, VDR (along with CYP27B1) is induced in select cells by an insult, for example, in the minor cell population of stellate cells, where 1,25D-VDR exerts anti-inflammatory effects to lessen hepatic fibrosis. A similar phenomenon of VDR/CYP27B1 induction occurs in macrophages following an infection, where 1,25D-VDR induces cathelicidin and defensins to bolster the innate immune system.

VDR is expressed in stem cells and during the early embryonic period in many tissues, highlighting a possible role for vitamin D in development. This function in cell life may constitute an evolutionarily ancient action of 1,25D-VDR since both the ligand and receptor are present in basal vertebrates such as the lamprey. Sea lampreys possess neither calcified tissues nor hair, demonstrating that the major functions of VDR, namely, calcemia for bone mineralization and hair cycling to provide a shield of protection against UV damage, are relatively modern manifestations of VDR evolution adopted by terrestrial animals. Fortunately, humans appear to have retained the evolutionarily ancient VDR actions of cell growth control and detoxification, rendering 1,25D-VDR a natural cancer chemopreventative. Summarized in Fig. 10 are the actions of 1,25D and alternative (low-affinity) ligands that bind VDR, a macromolecule with the ability to translate small-molecule effectors into biological mediators capable of cell cycle control, initiation of detoxification, driving the hair cycle, and regulation of extracellular calcium and phosphate levels to insure a mineralized, fracture-free skeleton unaccompanied by ectopically calcified tissues.

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