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Review

Regulation of the vitamin D receptor gene by environment, genetics and epigenetics

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ABSTRACT

The vitamin D receptor (VDR) plays a pivotal role as a mediator of 1α,25$(OH)_2D$ signalling. Besides its role in calcium homeostasis, ligand bound VDR supports immunity, cell proliferation and differentiation, and cell cycle control. While VDR regulates numerous genes across the genome, much remains to be learned about the regulation of VDR itself. Hindered VDR expression and function have a broad impact, contributing to diverse diseases, including cancer, multiple sclerosis, type 1 diabetes and tuberculosis. A better understanding of the three main factors regulating the *VDR*, namely environment, genetics and epigenetics, may facilitate the development of improved strategies for treatment and prevention of diseases associated with impaired VDR function. This review aims to illuminate the complex interaction and contributions of the three levels of *VDR* gene regulation to endorse consideration of all three regulatory factors when studying gene regulation.

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1. Introduction

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily of transcriptional regulators and mediates the diverse biological effects of calcitriol (1α,25$(OH)_2D_3$) and its analogues. VDR have been suggested to originate from duplication of an ancestral gene, along with the pregnane X receptor (PXR — both NR1I subfamily members) (Reschley and Krasowski, 2006). Conservation of 18 of the 22 ligand binding residues in the VDR has been shown across vertebrate species, from the lamprey to humans (Krasowski et al., 2005). The varied roles of vitamin D in immunity, cell proliferation and differentiation (Samuel and Sitrin, 2008), phosphate absorption and calcium homeostasis (DeLuca, 2004) are most likely the cause of VDR abundance across species (Hochberg and Templeton, 2010).

Liganded VDR in complex with retinoid X receptor acts as a promiscuous transcription factor (Haussler et al., 2013). It transactivates or represses numerous target genes by binding to positive or negative vitamin D responsive elements (VDREs and nVDREs, respectively) present in promoters, enhancers or suppressors of these genes (Chen and DeLuca, 1995; Meyer et al., 2014). In this capacity VDR regulates the expression of genes involved in diverse biological functions, including organ development, cell cycle control, calcium and phosphate homeostasis in bone metabolism, and xenobiotic detoxification (Haussler et al., 2013). The VDR also plays a role in both the innate and adaptive arms of the immune system, and has thus been implicated in a range of diseases. Non-communicable diseases associated with vitamin D and VDR include cancers as well as autoimmune disorders such as systemic lupus erythematosus, Crohn's disease, type I diabetes mellitus, multiple sclerosis, and rheumatoid arthritis (Holick, 2004a). VDR-related infectious diseases most notably include HIV, tuberculosis (TB) and leprosy (White, 2008).

A total of six genome-wide VDR-binding ChIP-seq experiments have been performed on six separate cell lines (reviewed in (Carlberg, 2014)). A combined analysis of all six experiments was performed using identical peak calling settings to harmonize the results (Tuoresmäki et al., 2014). When allowing a distance of up to 250 bp between peak summits, the six VDR ChIP-seq datasets specified 21,776 non-overlapping VDR binding sites (Tuoresmäki et al., 2014). Gene ontology (GO) analysis of 11,031 putative VDR target genes revealed that these target genes were involved in a number of diverse functions namely, metabolism (43%), cell and tissue morphology (19%), cell junction and adhesion (10%), differentiation and development (10%), angiogenesis (9%), and epithelial to mesenchymal transition (5%) (Ding et al., 2013). The involvement of VDR in such a large number of diverse diseases and physiological roles makes it a strong focal point for studying the underlying mechanisms of diseases and their possible prevention (Andress, 2006; Wang et al., 2008; Holick, 2004b). Consequently the importance of VDR function, and by extension *VDR* expression,

Abbreviations: AZA, 5′deoxy-azacytidine; CDGE, common disease genetic epigenetic; CGI, CpG island; DBP, vitamin-D binding protein; DMH, dimethylhydrazinedihydrochloride; E_2, estradiol; HMR, human mouse and rat; LD, linkage disequilibrium; MRE, miRNA recognition element; TF, transcription factor; TFBS, transcription factor binding site; TSS, transcription start site; UTR, untranslated region; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

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warrants an understanding of the underlying mechanisms of the regulation of the *VDR* gene.

The regulation of *VDR* under basal conditions and upon induction is multifaceted; shaped by environment, genetics and epigenetics. Examining the interactions and combined roles of these three facets of gene regulation would facilitate a greater overall understanding of the predisposition and progression of VDR-related diseases such as cancer and TB. This approach to studying gene regulation in relation to disease was put forward as the common disease genetic epigenetic (CDGE) hypothesis by Bjornsson et al. (2004). The principles by which environmental factors influence *VDR* regulation, as well as the mechanisms of its genetic and epigenetic regulation are illustrated in Fig. 1. Rather than detailing how VDR regulates other genes, this review aims to summarize literature on the regulation of the *VDR* itself. To the knowledge of the authors, this article is the first to review the *VDR* as a paradigm of gene-environment interaction through epigenetics. It highlights the inextricable nature of environmental, genetic and epigenetic factors in *VDR* regulation, and encourages a holistic approach when studying gene regulation to uncover the molecular basis of disease.

2. Environmental regulation

Diverse environmental factors regulate the VDR, among which are diet (Lamberg-Allardt, 2006), sun exposure (Holick, 2003), age (Hagenau et al., 2009), pollution (Agarwal et al., 2002) and infection (Liu et al., 2006). The majority of these factors exert their effects on *VDR* regulation by altering levels of vitamin D. Vitamin D is the collective name for cholecalciferol (D_3) and ergocalciferol (D_2), both of which are precursors of the active VDR ligand, $1\alpha,25(OH)_2D$. The vitamin-D binding protein (DBP) translocates vitamin D from the skin (Holick et al., 1980a) or intestines into circulation, where it remains bound while circulating in the blood (Fig. 1). DBP delivers vitamin D to the liver for activation (Haddad et al., 1993), where 25-hydroxyvitamin D (25(OH)D) is synthesized from vitamin D and again delivered to DBP in circulation. The multifunctional endocytic clearance receptor megalin then mediates the absorption of the DBP-25(OH)D complex into the proximal tubules of the kidney via endocytosis (Nykjaer et al., 1999). This process is facilitated by the membrane-associated coreceptor cubilin, which colocalizes with megalin (Nykjaer et al., 2001). Production of the active $1\alpha,25(OH)_2D$ takes place in the kidneys or other target

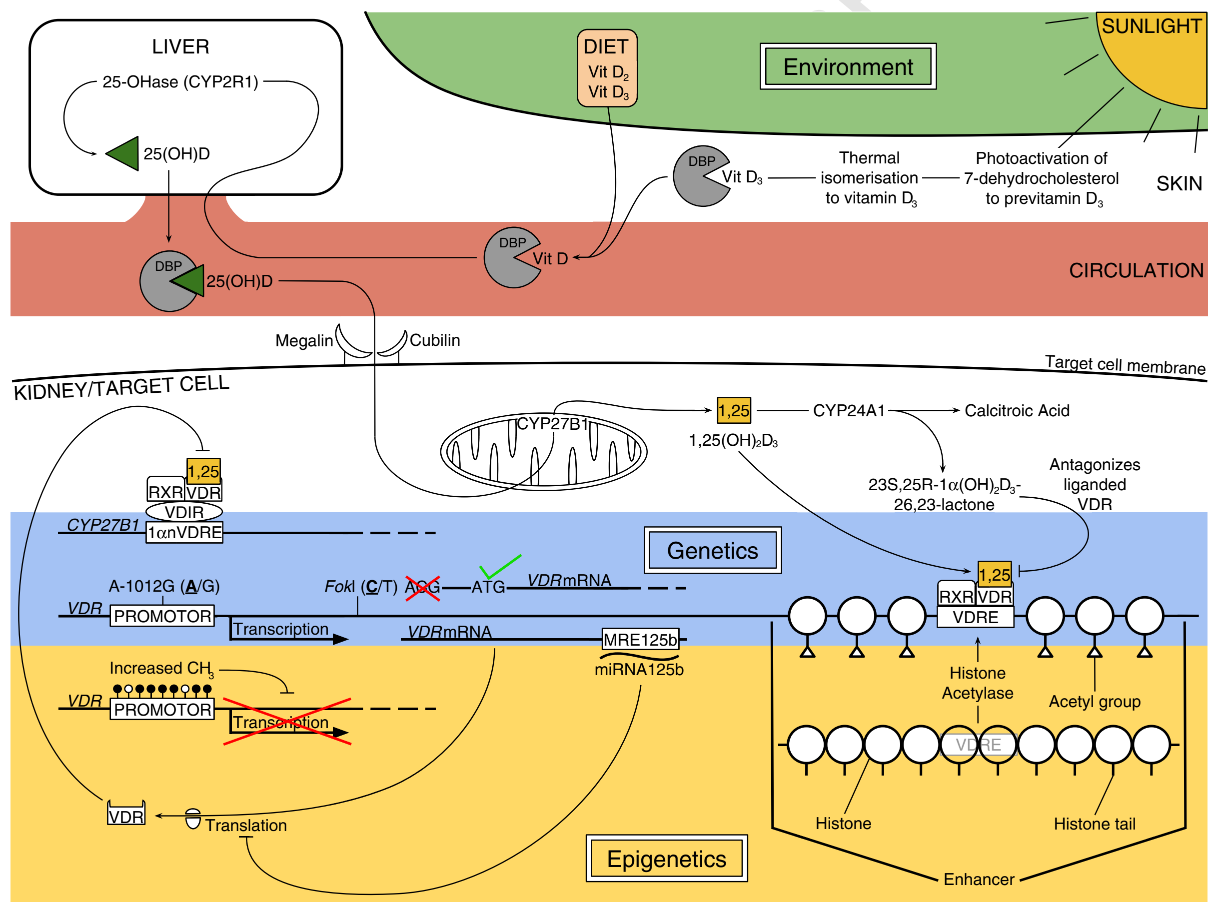

Fig. 1. Schematic overview of the three main factors influencing *VDR* regulation; environment, genetics and epigenetics, and the interaction between them. The main environmental influences on vitamin D status are sunlight exposure (Holick, 2003) and dietary vitamin D intake (Lamberg-Allardt, 2006). The white portions of the diagram illustrate the metabolism of vitamin D, which is linked to *VDR* autoregulation and is influenced by environmental factors. The endocytic clearance receptor megalin, in conjunction with the coreceptor cubilin, transfers DBP-bound vitamin D into the proximal tubules of the kidney, the major site of hydroxylation to the active $1,25(OH)_2D_3$. The genetics of the *VDR* in the form of promoter and enhancer sequence also play a major role in gene regulation. The effect of polymorphisms as part of the genetic mode of regulation of *VDR* is shown using examples such as the promoter SNP A-1012G and the translation start site SNP *Fok*I, though other SNPs with regulatory impact exist. The C allele of *Fok*I results in use of an alternate start codon and a truncated protein that has higher transactivational capacity. The A allele of A-1012G allows for improved binding of the GATA TF, resulting in increased expression. Most SNPs are excluded from the figure given the complexity of illustrating the numerous polymorphic sites in this region, however, their locations and some of their effects on *VDR* expression and VDR function can be found in (Fang et al., 2005). All three nucleic acid-related modes of epigenetic regulation, i.e. DNA methylation, histone modification and ncRNA, have been found to modulate *VDR* regulation. Hypermethylation of the promoter will shut down gene expression. High levels of miRNA125b are known to post-transcriptionally regulate *VDR* mRNA levels, negatively affecting VDR protein levels (Mohri et al., 2009). Lastly, histone modifications such as acetylation of histone 4 tails occurs in *VDR* enhancers (Zella et al., 2010), thus modulating chromatin structure and the availability of key TFBS. The diagram is not to scale, and is given only as a representation of the mechanisms of regulation.

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tissue, catalysed by CYP27B1, expressed in most tissues including immune cells (Zehnder et al., 2001). The latter supports paracrine and autocrine function in immune related processes (Fig. 1).

When bound to VDR, active vitamin D ($1\alpha,25(OH)_2D$) regulates *VDR* expression through VDREs in its own enhancers (Zella et al., 2006, 2007, 2010), thus vitamin D autoregulates *VDR*. Although the exact mechanism in the *VDR* gene is not yet known, this type of regulation is often achieved by modifying nuclear chromatin through histone modification and DNA methylation or demethylation, done in conjunction with co-repressors and co-activators (Murayama et al., 2004; Kim et al., 2009; Fetahu et al., 2014). The acquisition of vitamin D precursor, as well as production and bioavailability of active ligand, is therefore essential for *VDR* regulation and VDR activity. Stabilisation of the VDR protein by its ligand, extending its half-life, is another mechanism through which environmentally acquired vitamin D may modulate VDR levels (Wiese et al., 1992).

Besides a number of genetic determinants of circulating 25(OH)D (Wang et al., 2010; Ahn et al., 2010), vitamin D status is influenced by environmental factors including UVB photosynthesis, dietary intake, age, infection, and pollution such as airborne particulates and cigarette smoke (Sundar and Rahman, 2011).

Photosynthesis produces 90 to 100% of required vitamin D (Holick, 2003). Exposure to UVB in the 295–300 nm range photochemically transforms 7-dehydrocholesterol (7-DHC) to previtamin D_3 in the skin (Holick et al., 1980b), which is followed by thermal isomerisation to vitamin D_3 (MacLaughlin et al., 1982). The high melanin content of darker skin types blocks UVB, producing less vitamin D, and the lower melanin content of lighter skin allows for more UVB penetration, producing more vitamin D. Loomis (Loomis, 1967) proposed that migration of early hominids to higher latitudes facilitated a reduction in skin pigmentation, driven by the need for increased vitamin D production to prevent rickets. Skin types of the world have been categorized, with types I–IV being fair-skinned individuals that have a decreasing tendency to get sunburnt, and increasing tendency to get tanned. Skin types V and VI are brown and black individuals, respectively, both types getting tanned but rarely getting sunburnt (Fitzpatrick, 1988). Production of vitamin D in the skin is proposed to be inversely related to latitude and skin pigmentation (Norman, 1998). However, a meta-analysis comprising 394 studies found no correlation between vitamin D levels and latitude, but showed lower vitamin D levels in non-Caucasians than Caucasians (Hagenau et al., 2009). Gujarati Indians in West London (Wilkinson et al., 2000) as well as African- and Mexican-Americans (Prentice, 2008) have been found to have lower levels of vitamin D in plasma than Caucasians. This supports the role of skin pigmentation in determining plasma vitamin D levels, and consequently *VDR* autoregulation.

ncRNA is an alternative mechanism through which UVB may regulate VDR at the post-transcriptional level. The 3′ untranslated region (UTR) of VDR contains a target site for the narrow band UVB induced miR-125b (Gu et al., 2011), leading to VDR down regulation (Mohri et al., 2009).

As of 2011, deficient serum 25(OH)D levels are defined by the Institute of Medicine of the National Academies (United States) as <50 nmol/L in adults; with ≥ 50 nmol/L being considered as normal, and >125 nmol/L as excessive. Maasai and Hadzebe populations have been shown to have a mean 25(OH)D levels of 115 nmol/L (Luxwolda et al., 2012). This is considerably higher than most studies have reported for individuals of African descent. This may however be due to the intensity of UV exposure of these populations due to latitude (2–4° south of the equator). Seasonal variations in UVB radiation also influences vitamin D levels (Norman, 1998) and through autoregulation, may affect VDR levels (Selvaraj et al., 2009). Clothing and personal habits may also have an effect on the amount of cutaneous vitamin D photosynthesis. Photoproduction of previtamin D_3 in the skin when covered by garments made from fabrics such as black or white cotton, wool or polyester (Matsuoka et al., 1992). Even increasing the light exposure to six times the minimal erythema dose (MED) did not significantly increase serum 25(OH)D levels in garment-clad individuals (Matsuoka et al., 1992). In addition, sunscreens may also be contributing to lower serum levels of 25(OH)D by blocking UVB rays from penetrating the skin. Mean serum 25(OH)D concentration in individuals that applied para-aminobenzoic acid (sun protection factor 8) before being exposed to one MED of UV radiation increased by an average of 2.9 ng/mL, compared to 24.1 ng/mL in individuals that applied no sun protection (Matsuoka et al., 1992).

While photosynthesis provides the majority of required vitamin D for most individuals, a vitamin D-rich diet, as well as vitamin D supplementation can influence vitamin D serum levels. Both vitamin D_3 and D_2 can be obtained in the diet; D_3 from animal sources, D_2 from plants and fungi. However, most foods except meat, oily fish and eggs are low in vitamin D unless fortified (Lamberg-Allardt, 2006; Nowson and Margerison, 2002). Dietary vitamin D is largely obtained through fortified edibles (Nowson and Margerison, 2002) and vitamin supplements (Lamberg-Allardt, 2006). A study of cholecalciferol supplementation in adult men at doses of 0, 25, 125 and 250 μg daily for 5 months during winter showed that serum 25(OH)D concentrations changed in direct proportion to dose. Serum concentrations increased with a slope of approximately 0.7 nmol/L for each additional 1 μg of cholecalciferol administered (Heaney et al., 2003). Daily supplementation of generally healthy adults with 100 μg/d of vitamin D_3 for 5 months increased 25(OH)D to normal-high physiological levels and remained safe (Vieth et al., 2001). Although the debate on safe supplementation levels continues (Lamberg-Allardt, 2006), evidence clearly indicates that dietary vitamin D supplementation increases serum 25(OH)D levels. Increased levels of serum 25(OH)D through dietary intake may contribute to higher levels of active $1,25(OH)_2D$, thus affecting *VDR* autoregulation through VDREs in its enhancers (Zella et al., 2006, 2007, 2010).

The true complexity of VDR autoregulation as influenced by environment can only be appreciated when considering the network of regulation that exists between *VDR*, *CYP24A1*, *CYP27B1*, vitamin D and its metabolites. An indirect form of negative regulation of the *VDR* may also play a role in this autoregulation. CYP27B1 is a hydroxylase that catalyses the addition of a hydroxyl group to the 1α position of $25(OH)D_3$ in the kidney producing $1\alpha,25(OH)_2D_3$ (Cheng et al., 2004). A negative VDRE, called 1αnVDRE, is present in the *CYP27B1* promoter, to which a VDR interacting repressor (VDIR) binds and activates expression of *CYP27B1*. This results in increased levels of active ligand, which in turn activates the VDR protein. However, $1\alpha,25(OH)_2D$ liganded VDR appears to suppresses VDIR activation of the *CYP27B1* gene by switching the coregulator from p300 histone acetylase coactivator complexes to histone deacetylase co-repressor complexes (Murayama et al., 2004) (Fig. 1). Repression of CYP27B1 thus reduces the levels of active vitamin D, indirectly suppressing VDR function.

While environmental determinants of VDR regulation have their origin in personal, social and cultural aspects, their impact is often mediated by epigenetic mechanisms and modulated by genetic variation. For example, VDR *Taq*I genotype influences regional DNA methylation of a 3′ end CpG island (Andraos et al., 2011), single nucleotide polymorphisms (SNPs) in transcription factor binding sites (TFBSs) of environmentally-activated transcription factors (TFs) determine their binding efficiency (Arai et al., 2001; Fang et al., 2005) and polymorphisms in genes determining substrate for D synthesis (Wang et al., 2010), its transport (Sinotte et al., 2009) and hydroxylation (Cheng et al., 2004) during activation and breakdown greatly impacts circulating 25(OH)D levels. Thus, environment, genetics and epigenetics seldom acts in isolation, as further outlined below.

3. Genetic regulation

3.1. Promoters and enhancers

To facilitate the diverse functions of VDR, the complex set of coding and non-coding exons of the *VDR* are under the control of four promoters (Table 1), some of which are tissue-specific. The gene contains a TATA-

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Table 1
Spatial characteristics of regulatory elements in the *VDR* gene. Details of elements illustrated in the enclosed gene diagram are given in the table below. The diagram was created using the UCSC Genome Browser, and depicts the approximately 102 kb *VDR* gene, as well as approximately 8.8 kb upstream of exon 1f, and 3.9 kb downstream of the 3′ UTR. The promoter regions were obtained from Gene2Promoter on Genomatix (v3.2). Enhancer regions indicated are according to Zella et al. (2010), characterized by ChIP-chip analysis for VDR, CREB, GR, C/EBPβ, Runx2 factors, and H4ac histone mark. The enhancer originally named S1 by Zella et al. (2010) was expanded in this review to the newly termed S1 +, which includes an adjacent region shown by Zella et al. to bind VDR, important in VDR autoregulation. All coordinates correspond to the hg19 build of the human genome (UCSC). *Bona fide* CGIs were mapped according to an algorithm based on both CGI sequence criteria for CpG density, as well as epigenomic datasets from large scale experiments described by Bock et al. (2007). These epigenomic datasets included DNA methylation, PolyII PIC (pre-initiation complex) binding, histone marks (H3K4me2, H3K4me3 and H3K9/14 ac), DNase I hypersensitivity and Sp1 binding.

Gene

Genomatix promoters									
Code[a]	GXP_3654261		GXP_168257		GXP_3654258			GXP_168256	
Position[b]	48,336,731–48,337,434		48,298,687–48,299,314		48,276,618–48,277,218			48,237,996–48,238,596	
Length	703 bp		627 bp		600 bp			600 bp	
Enhancers									
Code	I2		U3		S3			S1 +	
Position	48,342,213–48,344,773		48,339,493–48,341,893		48,275,013–48,277,093			48,261,253–48,265,573	
Length	2560 bp		2400 bp		2080 bp			4320 bp	
Bona fide CGIs									
Code	1067	1066	1065	1064	1063	1062	1061	1060	1059
Position	48,343,769–48,344,090	48,340,624–48,340,878	48,336,576–48,336,888	48,335,999–48,336,347	48,335,764–48,335,963	48,298,319–48,299,653	48,258,829–48,259,046	48,238,552–48,238,840	48,237,082–48,237,288
Length	321 bp	254 bp	312 bp	348 bp	199 bp	1334 bp	217 bp	288 bp	206 bp

[a] Code of the element corresponding to the image of the gene.
[b] Position of the element within the hg19 build (UCSC) of the human genome.

less, Sp1-driven primary promoter (GXP_168257; Gene2Promoter, Genomatix) encompassing exon 1a (Miyamoto et al., 1997). Characterization of the structure of the *VDR* gene revealed constitutive TFBS in the primary promoter, supporting the constitutive expression of *VDR* from this promoter (Miyamoto et al., 1997). Three additional promoters have been identified; one each at non-coding exons 1c (GXP_3654258) (Fetahu et al., 2014) and 1f (GXP_3654261) (Crofts et al., 1998), and one at exon 9 (GXP_168256), controlling expression of a long non-coding RNA in the 3′ UTR (lncRNA; discussed in Section 4.3.2).

Crofts et al. (1998) first identified fourteen alternatively spliced *VDR* transcripts originating from the primary promoter and the distal 5′ promoter upstream of exon 1 f. Confirming the tissue or disease-specific nature of *VDR* promoters, certain of these transcripts were found only in kidney, parathyroid adenoma and intestinal carcinoma tissues; targets specifically of the calcitropic functions of VDR (Crofts et al., 1998). More recent information on *VDR* transcripts was gathered from GenBank via the UCSC Human Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway) and via the Gene2Promoter tool from the Genomatix Software Suite (v3.2; (http://www.genomatix.de), as seen in Supplementary Fig. 1 and Supplementary Table 1, respectively. As of early October 2014, ten experimentally verified and two annotated but non-confirmed VDR transcripts were recorded on the Genomatix database. Eight experimentally verified transcripts originate from the primary promoter (GXP_168257) according to Genomatix, all of which are expressed in a wide variety of tissues (Supplementary Table 1). The number of exons spliced into the mature transcripts originating from the primary promoter ranges from 6 to 11 exons. Only one experimentally verified transcript originates from the 5′ promoter (GXP_3654261). This transcript contains only exons 1f and 9, and has only been found in cancerous tissues and ascites. A single non-verified transcript is shown by Genomatix to originate from the promoter at exon 1c (GXP_3654258), containing four exons. This transcript is expressed in cancerous tissues, as well as germinal centre B cells, pancreatic islets and in ascites. The 3′ promoter (GXP_168256) expresses only one experimentally verified transcript, containing only exon 9. This transcript, which is a potential lncRNA, has as yet only been found in ascites according to Genomatix. The high number of splice variants generated by the four different *VDR* promoters in such a large variety of tissues illustrates the necessary complexity of this gene's regulation.

A VDR isoform known as VDRB1 was also discovered, which makes use of a start codon within exon 1d, and includes part of exon 1d and the whole of exon 1c in its transcript (Gardiner et al., 2004). This VDRB1 isoform therefore possesses an additional 50 aa N-terminal extension. VDRB1 showed greater transactivation activity than the originally described VDR in transfections of the *CYP24* promoter in COS-1 cells (Gardiner et al., 2004). Evidence was also found for the conservation of exon 1d in other mammalian and avian species (Gardiner et al., 2004). The VDRB1 isoform was detected in human kidney tissue as well as in osteoblastic (MG63), intestinal (Int-407, DLD-1, and COLO 206F), and kidney epithelial (786) human cell lines (Sunn et al., 2001). The differential functional activity of this isoform of VDR may indicate that it mediates specific physiological roles in certain tissues. In fact, it was found that tissue-specific transcriptional activity and alternative splicing of *VDR* mRNA takes place in osseous, cartilaginous and paravertebral muscle tissue in cases of idiopathic scoliosis (Nowak et al., 2012). In this study, abundance of VDRB1 isoform mRNA was significantly different between juvenile and adolescent idiopathic scoliosis in paravertebral muscles (Nowak et al., 2012). The tissue- and disease-specific expression profiles found in literature and in databases highlight the complex genetic regulation of *VDR*.

Comparative genetics revealed that exon 1a, the main initiating exon for the primary *VDR* transcript, exhibits a high degree of human, mouse and rat (HMR) conservation and exists within a strong CpG island (CGI; 1062 in Table 1) (Halsall et al., 2007). Conservation of this CGI suggests that it plays a crucial role in *VDR* regulation. Combined with the findings of constitutive TFBS in the exon 1a (primary) promoter (Miyamoto et al., 1997), the presence of this conserved CGI suggests that regulation of transcripts from the primary promoter results from a synergism between TF binding and differential CGI methylation. The influence of altered methylation at this primary promoter CGI is discussed in greater detail in Section 4.1. Exons 1d and 1c also showed high HMR conservation, which is consistent with the fact that these exons are encoding alternative VDR proteins (Halsall et al., 2007). There is little conservation of the 1c promoter region, suggesting that initiation from this promoter may occur exclusively in a tissue-specific manner, or during abnormal VDR signalling e.g., in tumour cells (Halsall et al., 2007).

Four enhancer elements in, and upstream of the human *VDR* gene (Table 1) have been characterized to date (Zella et al., 2010); two upstream of exon 1f (U3 and I2), one at exon 1c (S3), and one upstream of exon 3 (S1 +; Table 1). The term enhancer is used throughout the review in accordance with the designation as such by Zella et al. (2010), and is supported by these regions' classification as 'strong enhancer' or 'weak/poised enhancer' in the ENCODE 'chromatin state segmentation by HMM' tracks in Supplementary Figs. 2 to 7. These enhancers were identified via in silico analysis and ChIP-chip analysis of VDR binding at basal levels and after treatment with 1α,25-$(OH)_2D_3$ (Zella et al., 2010). Binding of liganded VDR to VDREs in the S1 enhancer of mice (conserved in humans) increased luciferase activity for mouse *VDR* (m*VDR*) S1 constructs in MC3T3-E1 cells (Zella et al., 2006). This shows that *VDR* transcription can be directly autoregulated via enhancers in a positive manner in line with 1α,25$(OH)_2D_3$ levels (Fig. 1). The enhancers identified by Zella et al. (2010) correspond closely with enhancer regions predicted by ENCODE/Broad institute. Enhancer prediction for ENCODE was done using chromatin state segmentation by hidden Markov-model based on data from ChIP-seq of CTCF and 8 distinct histone modification marks (Supplementary Figs. 2 to 10).

In the m*VDR* ortholog, RNA polymerase II presence at S1 and S3 enhancers (both conserved in the human gene) was significantly increased upon induction by 1α,25$(OH)_2D_3$ (Zella et al., 2010). RXR was also bound to these enhancers, indicating that RXR/VDR co-binding plays a regulatory role in enhanced *VDR* transcription. Inducible binding of common transcription factors such as CREB (cAMP response element-binding factor) and GR (glucocorticoid receptor) to these enhancers (Zella et al., 2010) indicates that the S1 and S3 conserved regions downstream of the TSS are fully fledged enhancers, rather than single VDREs. Increased binding of C/EBPβ (CCAAT/enhancer-binding protein beta involved in regulation of immune response genes) and Runx2 (Runt-related transcription factor 2 involved in osteoblastic differentiation and skeletal morphogenesis) to the mouse S1 enhancer induced by 1α,25$(OH)_2D_3$ was also shown by Zella et al. (2010), who proposed a regulatory role for C/EBPβ and Runx2 in basal and inducible expression of the *VDR*.

3.2. Sequence polymorphisms

Certain genetic variants provide ideal examples of how genetics and environment work in concert to influence *VDR* autoregulation by affecting serum vitamin D levels. Two genome-wide association studies (GWAS) identified polymorphisms in or near genes involved in cholesterol synthesis (*DHCR7*: rs1790349 and rs12785878), hydroxylation (*CYP2R1*: rs2060793 and rs10741657; *CYP24A1*: rs6013897) and vitamin D transport (*GC*: rs2282679, rs7041 and rs1155563) that were highly significantly associated with circulating 25(OH)D levels (Wang et al., 2010; Ahn et al., 2010). It is interesting to note that no polymorphisms in the *VDR* itself are associated with 25(OH)D levels in either of these studies. *Fok*I has been shown to interact with 25(OH)D levels and modify prostate cancer risk (Li et al., 2007), presumably by influencing VDR protein level and VDR transactivation capacity (Arai et al., 1997). However, any such effects these polymorphisms may have on 25(OH)D levels would be indirect, and may be too weak to be identified by GWAS.

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Although genetic variants in the *VDR* itself do not seem to contribute strongly to 25(OH)D levels, they have a profound effect on VDR expression and function in their own right. *VDR* primary promoter polymorphisms alter transcription factor binding for Cdx-2 (G-1739A: rs11568820) (Arai et al., 2001) and GATA (A-1012G: rs4516035) (Fang et al., 2005). The 'A' nucleotide alleles of both G-1739A and A-1012G have been shown to markedly increase binding capacity compared to their allelic counterparts for the Cdx-2 and GATA TFs, respectively. In addition, functional experiments show that the two weak-binding alleles translate into lower transcriptional activity for the *VDR* primary promoter (Arai et al., 2001; Fang et al., 2005). However, these transcription factors are tissue-specific, particularly in the case of Cdx-2 (Fang et al., 2005), and may therefore regulate *VDR* expression only in specific cell types and upon certain environmental stimuli. The *Fok*I (rs2228570) SNP is a 'T' (f) to 'C' (F) transition in the translation initiation codon of *VDR*. A *VDR* gene containing the F allele produces a three amino acid-truncated form of the VDR, known to have a significantly higher transactivational capacity than the non-truncated form (Arai et al., 1997). *Fok*I may therefore indirectly affect *VDR* regulation through autoregulation.

To better understand differential disease susceptibility, studies of the past 15 years have become increasingly focused on SNPs in the *VDR* associated with regulation of gene expression and protein production. This is in contrast to the preceding period, which focussed on SNPs influencing protein structure. Recent studies include those on infectious diseases (Selvaraj et al., 2009), osteoporosis (Arai et al., 1997), and diabetes mellitus type II (Ogunkolade et al., 2002) in different populations. SNPs such as *Fok*I as well as G-1739A and A-1012G in the promoter appear to consistently influence VDR expression. However, functional findings on the commonly studied 3′ UTR SNPs *Bsm*I (rs1544410), *Apa*I (rs7975232) and *Taq*I (rs731236) and their haplotypes have been conflicting. It has been suggested though that the 3′ UTR SNPs may play a role in *VDR* gene regulation, as variants of these SNPs included in a reporter assay have shown differential luciferase activity in COS-7 cells (Morrison et al., 1994). The functional impact of these three SNPs remains unclear, but given their 3′ location it is likely they are involved in *VDR* mRNA stability (Fang et al., 2005). Though they are non-coding or silent SNPs, they could also mark yet unidentified functional variants with which they are in linkage disequilibrium (LD). Breakdown in LD among some populations likely contributes to their inconsistent marking of function.

Length of a singlet 'A' repeat in the 3′ UTR of *VDR* has been shown to influence the transcriptional activity of VDR (Whitfield et al., 2001; Fang et al., 2005). This singlet repeat is classified as either long (L) or short (S) alleles depending on how many consecutive 'A' repeats exist, with an intermediate LS heterozygote. The Rotterdam study (Fang et al., 2005) grouped risk alleles in the 3′ UTR haplotype block into haplotype risk alleles hap1 and hap 2, which contained the L and S alleles of the singlet 'A' repeat, respectively. Reporter constructs of functional *VDR* containing either hap1 or hap2 3′ segments were transfected into 5 separate cell lines and mRNA was determined by qPCR. They found that on average across the cell lines *VDR* mRNA levels were 15% lower in hap1 (L allele) than in hap 2 (S allele) (Fang et al., 2005). It should be noted however that this effect was contributed to by the 'BAt' haplotype of *Bsm*I-*Apa*I-*Taq*I (A–T–C nucleotides). Length of the repeat most probably affects mRNA stability, as it was shown that mRNA decay rate for hap1 was 30% higher than for hap2 in the cell line studied (Fang et al., 2005).

Functional data on VDR level in peripheral blood mononuclear cells of normal healthy subjects from Chennai, India, shows that homozygosity for the 'B' allele of *Bsm*I and the 't' allele of *Taq*I is associated with lower levels of VDR protein (Selvaraj et al., 2009). Although no difference in VDR protein was observed for variant genotypes of *Apa*I, the TT (C/C) genotype of *Taq*I did show a trend towards higher VDR protein levels. Regarding haplotypes in the 3′ UTR region of the gene, increased VDR levels were observed in individuals with the bbaaTT genotype (G–G–T haplotype) compared to those with BBAAtt (A–T–C haplotype) (Selvaraj et al., 2009).

The 3′ *Taq*I SNP is of particular interest as it is located in a CpG site. Its genotype has been found to influence not only methylation at this site, but also regional methylation of CGI 1060 at the 3′ end of the *VDR* (Andraos et al., 2011). This relationship is discussed in greater detail in Section 4.1, but is worth mentioning here as it illustrates the need to consider the effect SNPs may be having not only on genetic regulation, but also epigenetic regulation of the *VDR*.

4. Epigenetic regulation

Broadly, epigenetics refers to heritable and transient changes in gene expression not caused by nucleotide sequence variation, but collectively instigated by epigenetic marks classified as DNA methylation, histone modification and non-coding RNA (O'Neill et al., 2012). However, the definition of this term is still a matter of contention, and many distinct variations exist (Ledford, 2008). Epigenetic regulation of gene function may occur on four levels; DNA methylation, histone modifications, non-coding RNA (ncRNA) and prion-mediated variation in protein folding (O'Neill et al., 2012). *VDR* is regulated by at least the three nucleic-acid-related epigenetic mechanisms, albeit in a tissue-specific manner.

4.1. DNA methylation

DNA methylation occurring at Cytosine-phosphate–Guanine dinucleotides (CpGs) in CGIs in promoters, as well as aberrant methylation of the gene body, may alter gene expression. In the classical model, normal gene expression is linked to hypomethylation of the promoter and enhancer regions, and hypermethylation of the gene body (Chen and Riggs, 2011). Aberrant methylation such as hypermethylation in the promoter or enhancer regions usually results in abrogation of gene expression (Chen and Riggs, 2011). Based on the majority of current literature, it would seem that VDR follows this classical model, in accordance with its constitutive expression in most tissues. Although one study did find non-classical effects of gene-body methylation on *VDR* expression (Smirnoff et al., 1999), to the knowledge of the authors no evidence exists yet suggesting that VDR is regulated by a unique DNA methylation mechanism.

Smirnoff et al. (1999) found *VDR* expression to be lower in the colonic mucosa of rats treated with dimethylhydrazinedihydrochloride (DMH, a carcinogen) compared to controls. Treatment with DMH in combination with estradiol (E_2), however, resulted in higher expression than with DMH alone. Hypermethylation of the *VDR* was found in exons 2 and 3 in DMH-treated normal tissue as well as tumour tissue, which coincided with lower *VDR* mRNA levels. However, rats treated with DMH together with E_2 showed lower levels of methylation in these regions, and higher levels of *VDR* mRNA. The results indicate that *VDR* was expressed at higher levels due to protection by estradiol against induced aberrant hypermethylation and carcinogenesis. Interestingly, this is inconsistent with the classical model where hypermethylation in the gene body corresponds to increased expression (Chen and Riggs, 2011). However this study only focussed on gene body methylation, and gave no indication of the effect of DMH treatment on promoter methylation.

In an almost opposite approach compared to Smirnoff et al. (1999), Marik et al. (2010) showed that treatment of breast cancer cell lines with 5′ deoxy-azacytidine (AZA, a DNA methyltransferase inhibitor) reduced aberrant *VDR* promoter hypermethylation in these cell lines. Decreasing promoter methylation increased cellular responsiveness to $1\alpha,25(OH)_2D_3$, while decreasing viability (possibly via VDR-mediated induction of tumour-suppressor genes and pro-apoptotic genes). These findings suggest that hypermethylation in the *VDR*, specifically in the primary promoter, greatly influences *VDR* expression and function (Fig. 1). In addition, it was found that the variation of transcripts

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produced via alternative splicing and the use of alternate transcription start sites can also be affected by such methylation in diseased tissue (Marik et al., 2010). These two studies not only demonstrate the capacity for *VDR* to be regulated via DNA methylation in both a classical and non-classical manner, but also the role of *VDR* epigenetics in cancer susceptibility and prevention.

Aberrant *VDR* DNA methylation plays a role in *VDR* regulation not only in cancer, but infectious disease as well. HIV has been shown to induce hypermethylation in the region −28 to −512 bp upstream of the ATG start codon (chr12:48,272,923–48,273,407; hg19) of the *VDR* in healthy primary T cells infected with primary X4 strain HIV-$1_{HT/92/599}$ (Chandel et al., 2013). Bisulfite pyrosequencing revealed that that 45% of CpGs in the studied promoter region were methylated in HIV infected T cells. CpG DNA methylation qPCR also revealed a 2.5-fold increase in DNA methylation in the gene body of HIV infected T cells compared to controls (Chandel et al., 2013). This study demonstrates the power that environmental factors such as infectious agents wield over *VDR* regulation via epigenetic modifications.

According to the predictive model for CGIs by Bock et al. (2007) used in the USCS Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), the *VDR* contains three *bona fide* CGIs at promoter regions; CGI 1065, 1062 and 1060 (Table 1). These were defined as *bona fide* as per the criteria set forth by Bock et al. predicted to be unmethylated, with promoter activity and open chromatin structure (Bock et al., 2007). Of these three CGIs, 1060 exhibited the highest regional methylation variability in Venda TB cases and controls, EBV-transformed B lymphocytes and a monocytic cell line (Andraos et al., 2011). The disease-associated SNP, *Taq*I, creates or destroys a CpG site. Although not a classic methylation variable position, if the CpG site is present it is methylated and associated with decreased regional methylation of CGI 1060 (Andraos et al., 2011). Methylation of CGI 1060 may affect regulation of the 3′ promoter, driving transcription of the proposed lncRNA in this region and potentially regulating *VDR* expression post-transcriptionally. Two recent studies showed that liganded VDR is capable of inducing DNA methylation in the vicinity of nVDREs, which appears to be the causal factor for the down-regulation of associated genes (Kim et al., 2009; Fu et al., 2013). This may have implications for VDR autoregulation, however, no nVDREs have as yet been identified in the *VDR*. DNA methylation in the *VDR* gene for primary cells and transformed/diseased cell lines from the Roadmap Epigenomics and ENCODE projects are shown in Supplementary Fig. 2. These large-scale mapping projects show that in both primary cells and transformed/diseased cell lines, the primary promoter of the *VDR* remains unmethylated and the gene body is methylated, indicative of active expression. Both the 5′ and 3′ promoters are methylated in all tissues studied, suggesting that expression from these promoters is shut down for these tissues. However, the promoter at exon 1c is unmethylated in stem cells, immune cells and in foetal pulmonary tissue, while being methylated in neural and skin cells. The discrepancy in methylation at this alternative promoter demonstrates the tissue-specific nature of *VDR* regulation.

4.2. Histone modification

Histones are post-transcriptionally modified, primarily on their N terminal tails. Various modifications on different residues give histones distinct roles in facilitating active transcription or repression of genes or gene regions to which they are bound. Specific modifications are linked to particular effects on transcription (activation or repression), and are enriched at different regulatory regions depending on the modification (Supplementary Figs. 4 to 7).

One study reported that treatment of the $1\alpha,25(OH)_2D_3$ resistant malignant melanoma cell line IGR with trichostatin-A, an inhibitor of histone deacetylase, caused a significant increase in *VDR* expression (Essa et al., 2012). Using ChIP-seq Zella et al. (2010) found that $1\alpha,25(OH)_2D_3$ treatment of MG63 osteosarcoma cells caused an increase of H4 acetylation in VDR enhancers (Fig. 1). Coupled with the fact that histone acetylation is involved in chromatin remodelling conducive to active transcription, the results of Zella et al. (2010) provide further evidence of *VDR* autoregulation. These studies confirm the substantial contribution of histone modification to *VDR* regulation. However, the effects of specific histone modifications on transcriptional regulation of the *VDR* remain largely unclear, especially regarding the dynamics linked to tissues and developmental stages.

Nonetheless, some work on histone modification marks and chromatin conformation in the *VDR* has been covered by the Roadmap Epigenomics and ENCODE projects, and is shown in Supplementary Figs. 2 to 7. H3K4me1 is associated with transcriptional elongation (Kouzarides, 2007), and in both primary and transformed/diseases cell lines is largely found at and directly downstream of the primary promoter and the 1c promoter. Although this modification is found at the 5′ and 3′ promoters in some tissue types, there is a distinct lack of consistency between tissues for these two promoters (Supplementary Fig. 4). H3K4me3 is found near TSSs and correlates with active transcription (Kolasinska-Zwierz et al., 2009). It is found enriched at the primary promoter of the *VDR* throughout all tissues, reflecting the constitutive expression from this promoter. The mark is also present at the 1c promoter, but only for select tissue types (Supplementary Fig. 5). H3K27ac is a modification associated with active enhancers and enhanced proximal gene activity (Creyghton et al., 2010). This mark is present at the S3 and S1+ gene body enhancers in most primary cell types, while being present at the 5′ enhancers I2 and U3 in only a few primary cell types (Supplementary Fig. 6). This suggests that U3 and I2 are tissue-specific enhancers, while S3 and S1+ are general enhancers promoting constitutive *VDR* expression. The H3K27me3 modification has been associated with inhibition of transcription when covering a broad domain in the gene body (Young et al., 2011). This mark is mainly found at and downstream of the 5′ promoter in most primary cell types as well as in carcinomatous cell lines, which may indicate that expression from the 5′ promoter is repressed in most tissue types (Supplementary Fig. 7).

The observed tissue-specific presence of H3K4me3 at promoter 1c, combined with a lack of DNAse activity (Supplementary Fig. 3) and the presence of methylation at this promoter supports the notion that expression from the 1c promoter is tissue-specific rather than constitutive. An extreme version of a similar pattern is seen for the 5′ and 3′ promoters, suggesting that these promoters are active in even fewer tissues than promoter 1c.

4.3. Non-coding RNA and mRNA stability

The stability of mRNA is a deciding factor in the ultimate levels of protein produced. Non-coding RNAs (ncRNAs) are RNA molecules which do not code for protein products, but which may have profound effects on the stability of target mRNAs. ncRNA include among others, miRNA (micro RNA) and lncRNA, both of which may affect *VDR* mRNA stability.

4.3.1. Micro RNA

miRNA target sequences are usually 3′-end located, giving rise to miRNA recognition elements (MREs) in the 3′-untranslated region of transcripts, facilitating control of mRNA stability via miRNA (Bartel, 2004). miRNA is a class of endogenous ncRNA about 22 nucleotides in length, considered to be gene regulatory molecules. They can play a key role in the regulation of gene expression by either cleavage of mRNA, or repression of translation.

VDR mRNA has three experimentally verified MREs located in the 3′ UTR (Mohri et al., 2009; Pan et al., 2009). One of these is an 8 nucleotide element known as MRE125b, occurring at chr12:48,238,160-48,238,167 (hg19) and is a target for miR125b (Mohri et al., 2009). Inhibition of miR125b using anti-sense oligonucleotides confirmed the recognition

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of the MRE by this miRNA. MRE125b has been shown to be functional in a regulatory capacity by the use of a luciferase reporter system in MCF-7 and KGN cells transfected with the miR125b precursor and the MRE. Furthermore, over-expression of mature miR125b results in significantly decreased levels of in vitro synthesized VDR protein (Mohri et al., 2009).

A reiteration of this form of VDR regulation was reported by Essa et al. (2010). In this study, two calcitriol responsive melanoma cell lines, MeWo and SK-Mel28, were shown to express high levels of *VDR* mRNA when compared with calcitriol resistant cells. The high VDR expression levels were inversely proportional to miR125b levels. These results confirm the post-transcriptional regulation of VDR by miR125b (Fig. 1).

The second and third experimentally verified MREs are those of miR27b and mmu-miR298, which fall at chr12:48,236,776-48,236,783 and chr12:48,238,408-48,238,414, respectively (hg19). Luciferase reporter assays on *VDR* 3′ UTR segments cloned into the *Renilla* luciferase gene system and transfected into HEK293 cells confirmed that mmu-miR298 and miR27b lowered *VDR* 3′ UTR-luciferase activities by 40 and 50%, respectively (Pan et al., 2009). In addition, western blotting revealed that LS-180 colon adenocarcinoma cells and PANC1 pancreatic cancer cells transfected with miR27b and mmu-miR298 plasmids separately, had reduced VDR protein levels compared to controls (Pan et al., 2009). miR27b levels were shown to be significantly reduced in response to 1,25(OH)$_2$D$_3$ in conjunction with AZA treatment in the human melanoma cell lines SK-Mel28, SK-Mel5 and IGR (Essa et al., 2012). Reduced miR27b levels coincided with *VDR* mRNA induction in these cells, strongly suggesting that miR27b specifically targets *VDR* mRNA, and that its expression is controlled by a synergy of methylation and vitamin D levels (Essa et al., 2012).

Two more putative MREs (chr12:48,237,724-48,237,731 and chr12:48,236,695-48,236,701 [hg19], recognized by miR124/506 and miR544, respectively) have been identified downstream from MRE125b in the *VDR* 3′ UTR by TargetScanHuman 5.1. However, none of these has been experimentally verified.

4.3.2. Long non-coding RNA

lncRNAs are RNA molecules larger than 200 nucleotides (Ponting et al., 2009) which do not code for proteins. The known mechanisms by which lncRNAs modulate transcriptional regulation are reviewed comprehensively by Ponting et al. (2009). The GXP_168256 promoter (Table 1) controls the expression of a proposed lncRNA of 1687 nucleotides in length (Transcript GXT_2780949 from Genomatix v3.2, ElDorado; GenBank accession no. AK024830), for which a corresponding protein has not been identified. It may be that the expression of this lncRNA is dependent on methylation levels in the 3′ promoter, which is in turn affected by *Taq*I genotype (Andraos et al., 2011). This possibility highlights the potential of the integrated nature of genetics and epigenetics for control of *VDR* regulation. However, the expression patterns and role of this putative lncRNA in *VDR* regulation remain unknown. Possible additional roles for lncRNAs are reviewed by Baker (2011), where lncRNA is suggested to play a role in protein stability by acting as a scaffold.

4.3.3. HuR and mRNA structure

Another factor that may influence *VDR* mRNA stability is the presence of a binding motif for HuR, a member of the Hu family of RNA-binding proteins (López de Silanes et al., 2004). Hu proteins bind specifically, and with high affinity, to mRNAs containing AU- and U-rich sequences, altering their stability and translation usually in a positive manner. Although HuR did not show binding to *VDR* mRNA in a biotin pull-down assay, immunoprecipitation coupled with low cycle RT-PCR did detect binding (López de Silanes et al., 2004). This discrepancy indicates that *VDR* mRNA structural conformation may be important in its post-transcriptional regulation.

5. Concluding remarks

The complex and tight regulation of VDR via environmental, genetic and epigenetic factors supports its important regulatory role in numerous critical physiological systems. Given the important role VDR plays in metabolism, homeostasis and immunity, the understanding of its regulation is of the utmost importance in the fight against infectious diseases and cancer. Although previous studies have excelled in exposing the influence of environmental, genetic and epigenetic components of *VDR* regulation in isolation, focused functional studies of larger scope are required to fully illuminate this complex gene's multifaceted regulation.

Acknowledgements

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Appendix A. Supplementary data

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