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IMPACT OF CHROMATIN ORGANIZATION ON THE REGULATION OF VITAMIN D RECEPTOR TARGET GENES EXPRESSION IN HUMAN PROSTATE CELLS

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ABBREVIATIONS

15-PGDH	15-hydroxyprostaglandin dehydrogenase
$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
3C	chromosome conformation captures
AF-1	activation function-1 domain
AF-2	activation function-2 domain
APO	apolipoprotein
AR	androgen receptor
ARE	AR response elements
ATP	adenosine-5'-triphosphate
BAF	brahma-associated factor
BAK	BCL2-antagonist 1
BAX	BCL2-associated X protein
BCL6	B-cell CLL/lymphoma 6
bp	base pair
Ca^{2+}	calcium
CAMP	cathelicidin
CBP	CREB-binding protein
CD	Crohn’s disease
CD14	CD14 molecule
CDH1	cadherin 1
CDK	cyclin-dependent kinase
CDKI	CDK inhibitor
c-fos	FBJ murine osteosarcoma viral oncogene homolog
ChIP	chromatin immunoprecipitation
ChIP-Seq	ChIP massively with parallel DNA sequencing
CNS	central nervous system
CoA	co-activator
COX2	cyclooxygenase-2
CREB1	cAMP responsive element binding protein 1
Ct	cycle threshold
CTCF	CCCTC-binding factor

CTD	c-terminal domain
Ctrl	control
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1
CYP3A4	cytochromeP450, family3, subfamilyA, polypeptide4
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBD	DNA-binding domain
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DR	direct repeat
DRIP	vitamin D receptor – interacting proteins
DUSP	dual specificity phosphatase
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERG	ETS-related gene
EtOH	ethanol
ETS	erythroblast-transformation specific gene
FBS	fetal bovine serum
FDR	false discovery rate
GADD45	growth arrest and DNA damage-inducible
GO	gene ontology
GR	glucocorticoid receptor
H12	twelfth helix
H3K27Ac	histone 3 lysine 27 acetylation
H3K4me	histone 3 lysine 4 monomethylation
H3K4me1	histone 3 lysine 4 methylation
H3K4me3	histone 3 lysine 4 trimethylation
H4Ac	acetylation of histone 4
HAT	histone acetyltransferase
Hbb	hemoglobin β-chain complex

HBD2	human β defensin 2
HDAC	histone deacetylase
HDM	histone demethylases
Hox	homeobox
HSP	Heat-shock protein
IGFBP3	insulin growth factor binding protein 3
IgG	immunoglobulin G
IL8	interleukin 8
IP	immunoprecipitate
K	lysine
KLK/hK	kallikrein
LBD	ligand-binding domain
LCoR	ligand dependent co-repressor
LiCl	lithium chloride
LXR	liver X receptor
MBD4	methyl-CpG binding domain 4
M-MuLV	M-MuLV reverse transcriptase
mRNA	messenger RNA
MS	multiple sclerosis
$NaHCO_3$	sodium bicarbonate
NCoR	nuclear receptor co-repressor
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NKX3.1	NK3 homeobox 1
NOD2	nucleotide oligomerization domain 2
NR	nuclear receptor
NSAID	Non-Steroidal Anti-inflammatory Drugs
p	p-value
p21/ CDKN1A	cyclin-dependent kinase inhibitor 1A
p27/ CDKN1B	cyclin-dependent kinase inhibitor 1B
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PCa	prostate cancer
PG	prostaglandin

PIA	proliferative inflammatory atrophy
PIN	prostate intraepithelial neoplasia
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptors
PR	progesterone receptor
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
PTGS2	prostaglandin-endoperoxide synthase 2
PTH	parathyroid hormone
QPCR	quantitative real-time polymerase chain reaction
RAD21	double-strand-break repair protein rad21 homolog
RANKL	Receptor Activator of Nuclear Factor-κB Ligand
RAR	retinoic acid receptor
Rb	retinoblastoma
RE	response element
RIP-140	receptor-interacting protein-140
RNA	ribonucleic acid
RNA pol II	polymerase II RNA
RPLPO	riboprotein PO
RT	reverse transcription
RXR	retinoic X receptor
SDS	sodium dodecyl sulfate
SHE	splicing factor 1
SEM	standard error of mean
SERPINB2	plasminogen activator inhibitor-2
SFM	serum free medium
siRNA	short-interfering RNA
SMAD3	mothers against decapentaplegic homolog 3
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SWI/SNF	SWItch Sucrose NonFermentable
TB	mycobacterium tuberculosis

TBP	TATA box binding protein
TE buffer	Tris EDTA buffer
TF	transcription factor
TFIID	transcription factor II D
TGFβ	transforming growth factor β
Th1	T helper 1
TMPRSS2	trans-membrane protease serine 2
TR	thyroid receptor
TRPV6	transient receptor potential Ca^{2+} channel V6
TSS	transcription start site
uPA	urokinase plasminogen activator
UV-B	ultraviolet B
VDIR	VDR interacting repressor
VDR	vitamin D receptor
VDRE	VDR response element
VDRWT	wild-type Vdr
VEGF	vascular endothelial growth factor

SUMMARY

Vitamin D Receptor (VDR) belongs to the superfamily of nuclear receptors that are in total 48 ligand-activated transcription factors that bind to the DNA and are involved in the regulation of gene expression. The active form of vitamin D_3, hormone 1α,25-dihydroxyvitaminD_3 (1α,25$(OH)_2D_3$), is a natural ligand for VDR. The generally described physiological functions of 1α,25$(OH)_2D_3$-activated VDR target genes are stimulation of metabolism as well as differentiation and inhibition of inflammation and cellular proliferation. In this study we focus primarily on the target genes of VDR that could potentially have a positive role in cancer prevention. Microarrays were performed of human non-malignant prostate RWPE1 cells after 4 h and 24 h treatment with 100 nM 1α,25$(OH)_2D_3$ and among several hundred responsive genes, we identified multiple members of the *kallikrein* (*KLK*) gene family as putative primary VDR targets. The KLKs are serine proteases that have been shown to be deregulated in various cancers. The 15 genes in the *KLK* family cluster together on human chromosome 19 span roughly 270 kB. *KLK6* was the most responsive gene (16-fold induction after 4h treatment), followed by its neighboring *KLK5*, *KLK7*, *KLK8* and *KLK9*. This effect of 1α,25$(OH)_2D_3$ was confirmed by real-time quantitative PCR and loss-of-function experiments. Interestingly, the VDR-mediated induction of the *KLK* genes was less pronounced in the cancer than in non-malignant prostate cells. In addition, we analyzed the genomic sequence of the KLK cluster *in silico* and identified a number of putative VDR binding sites (VDREs) as well as putative insulator CTCF binding sites. Chromatin regions containing putative binding sites were analyzed by chromatin immunoprecipitation assays to assess their functionality in RWPE1 cells. Six VDREs were associated with VDR whereas validation of detected CTCF binding sites showed increased occupancy of CTCF upon 1α,25$(OH)_2D_3$ stimulation. These results allow describing the changes in chromatin architecture on the *KLK* locus after VDR activation. Taken together, our study shows that 1α,25$(OH)_2D_3$-activated VDR has an impact on the regulation of the whole *KLK* gene cluster in prostate cells.

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1. INTRODUCTION

1.1. Chromatin architecture and its relevance in regulation of gene expression

Chromatin refers to the complex of DNA and proteins that form the chromosomes during cell division. It plays a crucial role in the cell-specific gene regulation. Its organization structure is directly involved in the process of allowing or denying transcription factors access to DNA. The basic units of chromatin structure are the nucleosomes. Each of them is composed of four histones proteins wrapped by 147 base pairs (bp) of DNA and is separated from the neighboring nucleosome by linker DNA. Their disassembly and reassembly abilities are necessary for the proper maintenance of the genome (Deal, 2010). Consequently, nucleosomes are involved in DNA packaging and accessibility, which is basically directly, related to two chromatin states of relaxation (euchromatin) and of compaction (heterochromatin). In the open state of the chromatin the promoter and enhancers of a certain gene are available for the transcription complexes whereas they are inaccessible in the densely packed, heterochromatin state (Fig. 1).

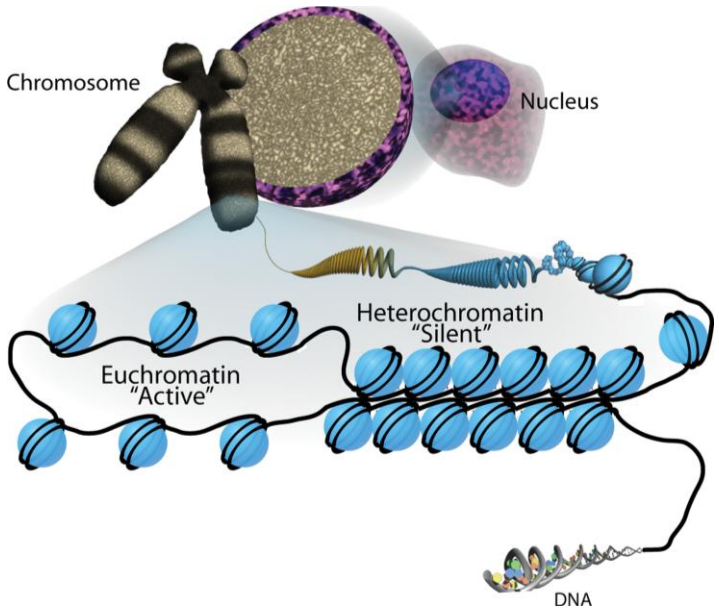


Figure 1. Chromatin organization.

The DNA of a cell is located in the nucleus and organized in chromatin, the material of chromosomes. The nucleosome, the basic unit of the chromatin, comprises 147 bp of DNA wrapped around the core of histones. Euchromatin is associated with an open state of the chromatin that is permissive for transcription, whereas heterochromatin is compact and inaccessible for the factors involved in transcription (Taken form Sha, 2009).

1.1.1 Control of chromatin structure

There are at least three processes that are responsible for regulation and assembly of the chromatin: DNA methylations, histone modifications and adenosine-tri-phosphate (ATP)-dependent chromatin remodeling (Ho, 2010).

DNA methylation is a mechanism of transcription repression that occurs via addition of a methyl group to the cytosine (C) in cytosine guanine (GC)-rich regions, called CpG islands. This modification on a promoter or enhancer induces histone deacetylation and chromatin remodeling resulting in the inactivation of the gene. In addition, abnormal DNA methylation serves as a marker of cancer (Schones, 2008).

Post-translational histone modifications are crucial for chromatin organization. They modulate nucleosome stability and therefore control DNA accessibility and gene transcription. Several modifications have been described, including acetylation and methylation of the *N*-terminal histone tails (Suganuma, 2008). Acetylation, which is reflected by the presence of acetyl groups in histones H3 and H4, increases chromatin flexibility, while deacetylation is correlated with locally compacted chromatin. Acetylation is controlled by two kinds of enzymes. Histone acetyltransferases (HATs), like steroid receptor co-activator 1 (SRC1/p160) modify histones by adding acetyl groups to their tails, which correlates to the open state of the chromatin, and is a marker for active transcription. The second group, the histone deacetylases (HDACs), on the contrary remove acetyl groups from targeted histones and are associated with transcriptional repression (Wu, 2009). Histone methylation is characterized by the presence or removal of a methyl group from the histone tails. The methyl group is delivered by histone methyltransferases (HMTs) and removed by histone demethylases (HDMs) (Chi, 2010). Histone H3 methylation has been observed at many lysine (K) sites e.g. H3K4, K9 and K27 with four different states: unmethylated, monomethylated, dimethylated and trimethylated. Each of the states has a different impact on the gene expression. For instance, H3K4 trimethylation (H3K4me3) is associated with an open state of chromatin and its highest level has been observed at the transcription start sites (TSSs) of the active genes. Interestingly, the H3K4 dimethylation (H3K4me2) is related not only to the TSS but also to the enhancers that modulate gene expression. In contrast, H3K27 trimethylation (H3K27me3) is correlated with condensed chromatin and gene silencing. In addition, methylation and acetylation may also neutralize the positive charge of histones and by that reduce the interactions with the negatively charged DNA. Consequently nucleosomes may be repositioned allowing the recruitment of regulatory complexes, including transcription factors (TFs), to interact with the DNA (Chi, 2010).

Interestingly, if a binding site is located at nucleosome-free regions or between positioned nucleosomes, transcription factors may access it easily. However, if the regulatory sequence is hidden "inside" the nucleosome TF association requires engagement of chromatin remodeling complexes (Cairns, 2009). These are specific units that use the energy of ATP hydrolysis to regulate nucleosome mobility by destabilization, restructuring or even their ejection. Thus, recruited complexes like SWItch Sucrose NonFermentable (SWI/SNF)-like ATPases or brahma-associated factor (BAF) may enable TFs to access to respective binding sites on DNA, in order to recruit co-activators as well as the general transcription machinery. Therefore, they are crucial for rapid induction of gene expression (Weake, 2010). There are more than 30 SWI/SNF-like ATPase subunits involved in chromatin remodeling complexes, which suggests that the function of this class of proteins is very broad and specific (Wu, 2009). Intriguingly, SWI/SNF "remodellers" contain a domain that may bind to acetylated histone tails and promote their targeting or activity of gene promoters (Cairns, 2009).

Interestingly, Chodavarapu *et al.* have shown that nucleosome positioning influences DNA methylation, i.e. the level of methylation was higher on nucleosome-bound DNA than in flanking regions (Chodavarapu, 2010). Moreover, He *et al.* noticed that changes in nucleosome occupancy are associated with the presence of enhancers. They performed genome-wide analyses, which reveled that upon androgen stimulation of a prostate cancer cell line, nucleosomes present at androgen receptor binding sites are dismissed not only from the TSS regions but also from enhancers. In addition, histone modifications were linked with those observations, since H3K4me2 was very efficient for detection of TF binding to TSSs and enhancers (He, 2010).

1.1.2 Chromatin loop formation

Enhancers can be marked by H3K27ac, H3K9ac, H3K4 monomethylation (H3K4me) and H3K4me2 but not by H3K4me3. They contribute to gene transcriptional activation from upstream or downstream, in distance or close proximity to the TSS of the targeted genes (Heintzman, 2007). Together with other regulatory elements like insulators, silencers and locus control regions (LCR), enhancers are mainly situated in great distances, up to hundreds of kilobases (kb) away from the TSS of targeted gene. Two ways of distant transcriptional regulation have been discovered, the spreading and the looping. The spreading model suggests that the initial signal starts at an enhancer and is passed along on the chromatin fiber to the promoter that finally results in modulation of gene expression. The expanse can also be

reduced by formation of chromatin loops, which bring enhancer and promoter together to the active gene. The proteins associated with loop formation are under investigation and may involve transcription factors, nuclear receptors, insulators, chromatin remodelers and possibly others (Hakim, 2010). For instance, the activation of the mouse hemoglobin β-chain complex (*Hbb*) genes occurs with the cooperation of their distal enhancers located around 50 kb away from the promoters. Gene transcription is triggered by formation of a loop structure between the enhancer and the TSSs of the genes, thus composing a so-called hub (Chakalova, 2005) (Fig. 2).

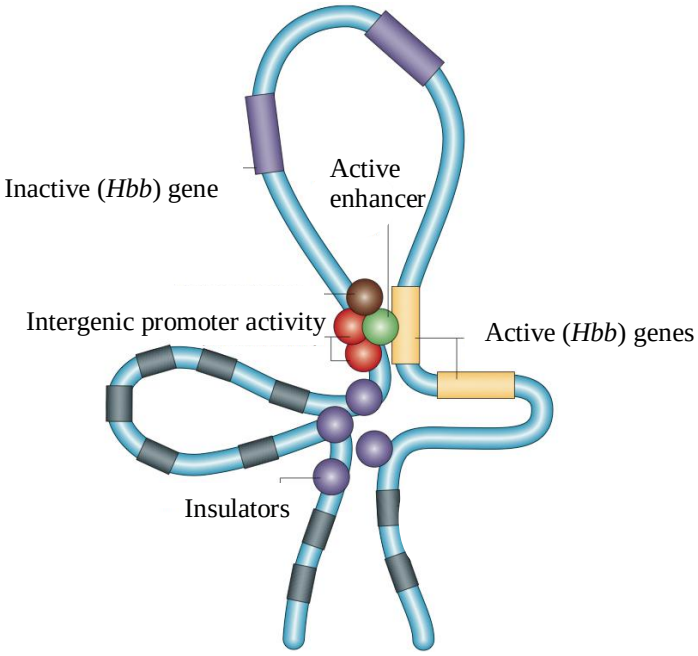


Figure 2. A chromatin hub. The figure represents a hub formation between the *Hbb* gene cluster and enhancer. The *Hbb* complex shares a distal enhancer that leads to transcriptional activation (Adapted from Chakalova, 2005).

A similar gene regulation was found for the *Hox* (Homeobox) genes. In higher vertebrates, *Hox* genes are clustered into four groups: *Hox*A, *Hox*B, *Hox*C and *Hox*D, where each cluster of genes is arranged in the same linear order. In mouse, the activated *Hoxb* gene cluster forms a loop with distal enhancers that may be located even at other chromosomes. Local decondensation of the chromatin, together with transcription activation might lead to chromatin mobility that facilitates formation of loops, thus enabling contacts between the genes and distal regulatory elements. In this way, genes located in the same chromatin loop may share a common regulation of their expression (Fraser, 2007).

In addition, chromatin fiber may create a loop through interaction with the CCCTC-binding factor (CTCF), an insulator that contains eleven zinc finger DNA-binding domains that allow

binding to a variety of sequences and to specific co-regulators. CTCF is known to have many functions, including promoter activation/repression, enhancer blocking and barrier isolation (Cuddapah, 2009). Moreover, CTCF has ability to separate euchromatin from heterochromatin state (Felsenfeld, 2004). It can mediate chromatin loop formation via the contact of two CTCF insulators (Philips, 2009).
Interestingly, Kim *et al*. observed that CTCF-depleted domains contain clusters of co-regulated genes or gene families that share similar patterns of expression, suggesting that segregated clusters undergo intra-domain transcriptional regulation (Kim, 2007).

Thus, the state of nucleosome positioning, histone modification, DNA methylation, chromatin organization, activity of DNA-binding proteins and co-regulator proteins are determining the transcriptional expression of a gene (Sproul, 2005).

1.2. The nuclear receptor superfamily

Nuclear receptors (NRs) are proteins belonging to the transcription factors that mediate the transcriptional response to lipophilic hormones like vitamins D_3 and A, thyroid and endocrine steroids as well as to metabolic molecules. These ligands are specifically regulating the transcriptional activity of NRs (Nagy, 2006). NRs can regulate a number of genes involved in many processes in the body, like reproduction, development, growth, mineral homeostasis and nutrition utilization, as well as in the immune response (Bookout, 2006).
In the 1960s, steroid receptors were identified and analyzed, but only in the mid 1980s nuclear receptors were classified as a superfamily of transcription factors. In the beginning of the 1990s, the regulation of most NRs by their ligands was identified as a general phenomenon (Tata, 2002).

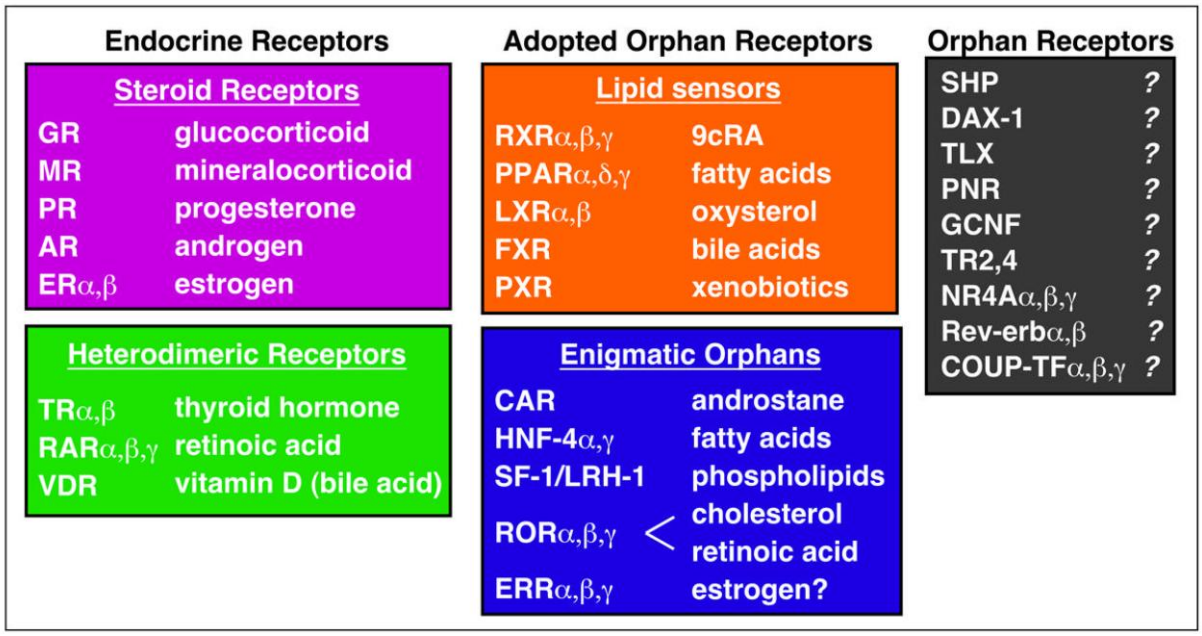
1.2.1 Classification

Since NRs have the ability to bind directly to DNA and influence the expression of their target genes, they are assigned as TFs. In human the NR superfamily consists of 48 members, which share a common protein structure (Yang, 2006). The activity of a significant number among the 24 NRs is modulated by ligands, small lipophilic molecules (Gronemeyer, 2004). The presence of the ligand triggers conformational changes of the receptor that allows the NR to interact with many different proteins like co-regulators and chromatin modifiers, resulting in changes in the transcriptional expression of a targeted gene. However, some NRs can decrease the gene expression in a ligand-independent or dependent manner, by repressing it through its interaction with co-repressor complexes on the gene promoter (Germain, 2006).
There are several ways for classifying the superfamily. One is to divide them into three categories according to their ligand-binding affinities: classic endocrine receptors bind with high affinity (e.g. vitamin D receptor (VDR), androgen receptor (AR), glucocorticoid receptor (GR)); adopted orphan receptors – have a low affinity to their ligands (e.g. retinoic X receptor (RXR), liver X receptor (LXR)); and orphan receptors, for which the kind of activation and ligands are still unknown (e.g. SHP) (Germain, 2006) (Table 1).
Another way of classification refers to the DNA binding: NRs of type 1 bind to DNA as homodimer; after ligand activation in the cytoplasm is transported to the nucleus (e.g. estrogen receptor (ER), progesterone receptor (PR), AR); NRs of type 2 usually bind to DNA as heterodimers with RXR (e.g. VDR, retinoic acid receptor (RAR), thyroid receptor (TR));

type 3 NRs include some "orphan" receptors which bind to DNA as monomers (see, www.nursa.org).

The third and most recent way of division is due to shared physiology and function (Bookout, 2006) (Fig. 3). That analysis points out relations of NRs in various processes. Besides the importance of single receptors this classification also revealed the interconnection of NRs that together regulate the expression of specific gene networks (McKenna, 2009).

Table 1. Classification of the superfamily comprising 48 nuclear receptors in human.
The natural ligands of the NRs are shown on the right (taken from Sonoda, 2008).



1.2.2. Role of nuclear receptors

Bookout and colleagues divided NRs according to their expression, function and physiology. They segregated two major classes into six subclasses: three for nutrient metabolism and three for reproduction. Cluster IA represents NRs involved in steroidogenesis. Cluster IB contains all steroid hormone receptors involved in reproduction (e.g. AR, ER, PR). It also contains retinoic acid receptors (RAR) participating in development. Custer IC comprises the largest number of NRs. They are expressed in the CNS (e.g. RORα, ERR ß) and are responsible for controlling the basal metabolism (e.g. LXRß, RXRα, RXRß). Cluster IIA represent NRs regulating nutrient intake (e.g. VDR, CAR, PXR). Cluster IIB and cluster IIC contain NRs that govern utilization of lipid nutrients as fuel and are thereby responsible for energy

homeostasis (e.g. peroxisome proliferator-activated receptors (PPARs), LXRα) (Bookout, 2006) (Fig.3).

Notably, as NRs have a big impact on a wide range of biological processes, the loss of control of NR signaling pathways provokes deregulation of their normal function and thus, contribute to many diseases such as chronic inflammation, metabolic (Lonard, 2007), cardiovascular diseases and cancer (Payne, 2011; Beer, 2007). For that reason, NRs can be used as drug targets to develop ligand-based therapies to treat and prevent important disorders. Since they were discovered, many NR ligands were evaluated in clinical trials and several have been approved for use in therapy. The endocrine receptors are cancer drug targets and their ligands, agonist or antagonist, are commonly used in pharmacology: anti-estrogens to treat breast (Veer, 2002) and ovarian cancer, androgens and anti-androgens for the treatment of prostate cancer (Sharifi, 2010). Vitamin D is used in fortified food to prevent inflammatory and bone diseases (Evans, 2003).

Adopted orphans receptors are the most promising drug targets for the metabolic syndrome, because of their functions in the lipid – glucose homeostasis and inflammation. Synthetical ligands for PPARγ, for instance, are commonly used drugs for treatment of for type II diabetes (Sonoda, 2008).

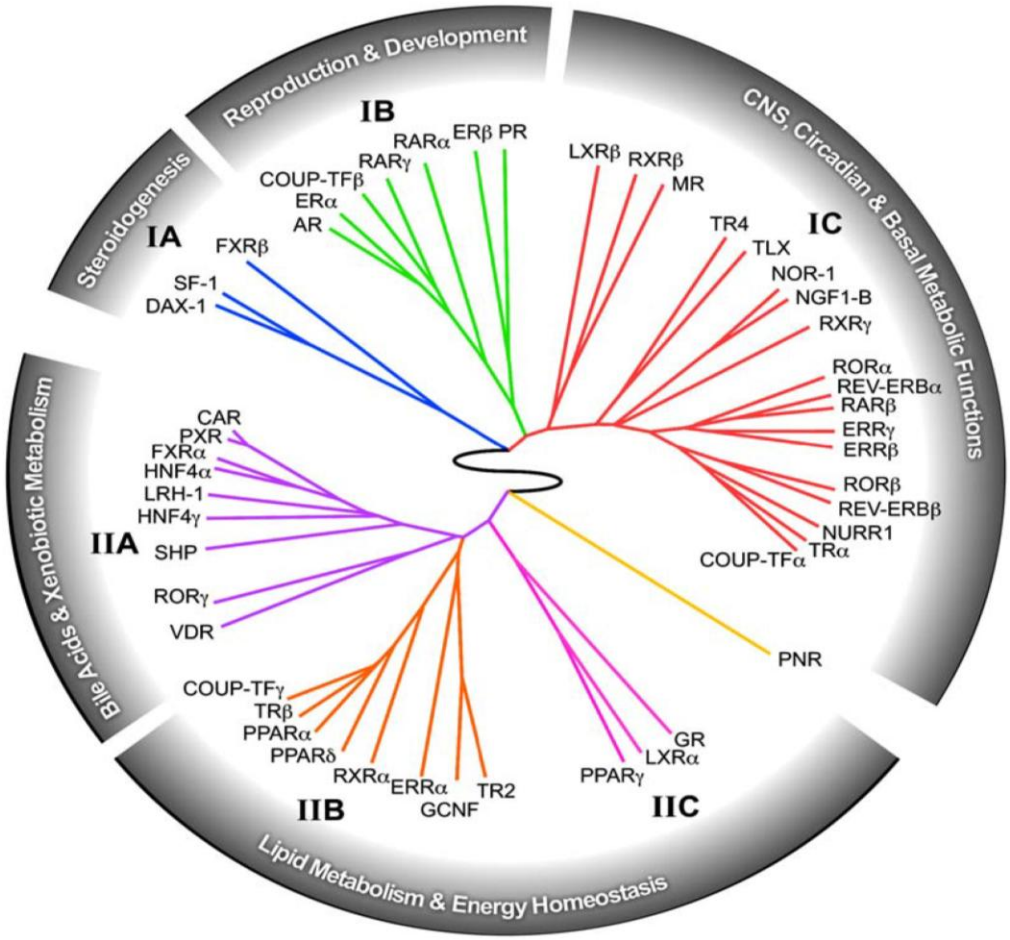


Figure 3. The nuclear receptor ring of physiology

Classification of the NRs according to their expression, function and physiology (taken from Bookout, 2006).

1.2.3. General structure of nuclear receptors

All NRs contain specific domains in their protein structure. These are: the *N*-terminal activation function-1 domain (AF1), a central DNA-binding domain (DBD), a hinge region, a ligand-binding domain (LBD), and the C-terminal activation function-2 domain (AF2).

Figure 4. Structure of the nuclear receptors

NRs contain five domains: the *N*-terminal AF1 domain, the DNA-binding domain (DBD), the hinge region (Hinge), the ligand-binding domain (LBD), and the *C*-terminal AF2 domain (taken from Jonker, 2009).

The AF1 domain has a different length and sequences across different NRs and can be recognized by co-regulators. The DBD is the most conserved domain. It contains two zinc fingers motifs, that are responsible for the binding to a specific DNA sequence of a target gene, the so-called hormone response elements (HREs). The response elements (REs) of NR are described as two hexameric half-site motifs. The consensus sequences of these motifs is RGGTCA (R = A/G) and may be configured as direct, inverted or everted repeats with a different number of spacing nucleotides between the motifs. These modifications form REs selective for specific receptors (Gronemeyer, 2006). Ligand-induced steroid hormone receptors, like AR or ER, form homodimers and bind to DNA in the inverted repeat manner. Others form heterodimers with RXR in a direct repeat manner e.g. VDR, RAR, thyroid receptor (TR) and several orphan NRs. The last group binds as monomers; this class comprises of some orphan NRs (Mangelsdorf, 1995).

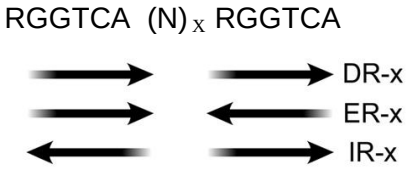


Figure 5. Types of nuclear receptor binding sites

There are three types of NRs binding to the response elements: in direct repeat (DR), in everted repeat (ER), or in inverted repeat (IR). (Transformed from Jonker, 2009).

Between the DBD and the LBD lies a less conserved hinge region, which enables to separate those two domains. In addition it allows the DBD and the LBD to perform conformational changes (Germain, 2003). The LBD mediates ligand-binding, dimerization, the binding of co-regulators and transactivation (Germain, 2006). The LBD is composed of twelve helices; eleven of those create a structure consisting of a specific ligand-binding pocket (differs between family members of the NRs). The twelfth helix (H12) that contains transactivation domain AF2 rests on the top of the pocket. It changes its conformations upon the LBD interaction with the ligands. Those specific, lipophilic molecules are able to pass through the lipid bilayer of the cell membrane and interact with the receptors. In most cases, binding of a ligand leads to allosteric effects that cause the H12 repositioning which promotes co-repressors displacement and the recruitment of co-activators that interact with the LBD surface via specific short LxxLL-like motifs (L = leucine and x = any amino acid). As a result

of ligand-mediated action the transactivation function of a NR is switched on (Germain, 2006).

This is the classical way of the LBD ligand-dependent function. Nevertheless, it has been shown that other mechanisms exist. Some co-factors like receptor-interacting protein-140 (RIP-140) and ligand dependent co-repressor (LCoR) act as NR co-repressors in a ligand-dependent manner and replace co-activators. Co-repressors can trigger the recruitment of transcriptional complexes with histone deacetylases, which leads to the deacetylation of histones that closes the local chromatin and may result in gene repression (Gurevich, 2007).

1.2.4. Mechanism of action

The specific regulation of transcriptional activation upon ligand binding to its NR, facilitates recruitment of the transcriptional machinery compounds, including RNA polymerase II, co-regulators and histone modifiers, and induces the expression of the targeted gene (Germain, 2006).

The principle concerning the function of classical mechanism of the steroid receptors (e.g. GR, ER, AR) is that in the non-active state they are located in cytoplasm and are associated with HSPs. Upon hormone activation HSPs dissociate, thus allowing NRs to target their specific genes as homodimers, and recruits series of co-regulators.

Another group of NRs binds, upon ligand activation, to the REs in the promoter or enhancer regions of targeted genes as heterodimers with RXR, and leads to transcriptional activity. Prominent example of those receptors is vitamin D receptor, which may generate biological response after interaction with hormonally active vitamin D (Fig. 6).

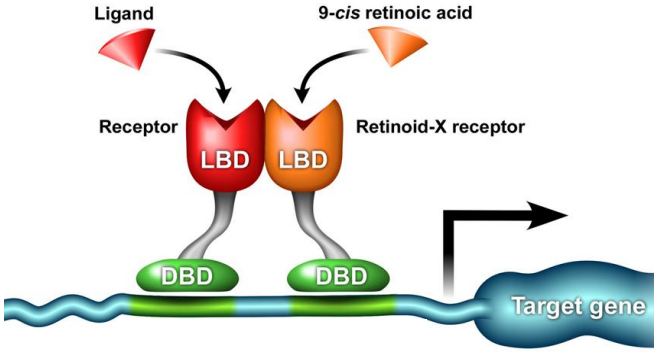


Figure 6. Activation of nuclear receptors

Classical example of NR heterodimerization with RXR. The LBD changes its conformation due to ligand action; the DBD recognizes a specific DNA sequence (Taken from Jonker, 2009).

Two types of mechanisms for repression can be distinguished. The first one refers to the ligand-independent action by attracting a complex of co-repressors leading to the repression of the genes. The second kind, called transrepression, happens in a ligand-dependent manner. Transrepression applies to the process where a NR represses the activity of another TF by protein – protein interaction and therefore inhibits expression of specific genes. For instance, it was shown that ligand-activated GR has the capability to physically bind to NFkappaB and to block its activity (De Bosscher, 2003). Moreover, an example for repression in the ligand-dependent manner is the negative regulation of the *CYP27B1* gene by VDR upon vitamin D stimulation. This gene encodes an enzyme that plays a crucial role in vitamin D biosynthesis. Therefore, due to recruitment of specific co-repressors involved in chromatin remodeling and histone modification, the gene is negatively regulated, which results in suppressing the vitamin D synthesis. Details of that homeostatic regulation are described later (Germain, 2006).

1.2.5. Interaction with co-regulators

Transcriptional regulation requires a number of co-regulators with different functions and enzymatic activities (Perissi, 2005). As mentioned before, liganded NRs interact with co-activators/co-repressors and mediate target gene transactivation/transrepression. Co-activators (as well as co-repressors) form complexes that are recruited to NRs. The complexes mediating transactivation include proteins involved in histone modifications and chromatin remodeling (e.g. HATs, SWI/SNF complexes), initiation of the transcription (e.g. SRC-1), elongation of RNA chains, mRNA splicing, and proteolytic termination of the transcriptional response (E3 ligases). To date, more than 300 co-regulators have been discovered (O'Maley, 2009).

In contrast, unliganded NRs may interact with co-repressors leading to repression of targeted gene. These include nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT), which are essential for binding smaller complexes containing HDAC activity. This complex increases the histones' ability to bind to DNA, thereby tightening it up and repressing transcription. Interestingly, there are many other co-repressors like LCoR that compete with co-activators and replace them in the presence of ligand (Perissi, 2005; Germain, 2006).

Transcriptional regulation is a highly dynamic event. Many scientists argue whether regulating complexes are temporarily recruited to each active site or, on contrary, if DNA migrates to "ready" transcriptional factories (Perissi, 2005). Because of the complex, three-

dimensional nuclear organization and limited molecular techniques for studying the kinetics of nuclear factors, the dilemma remains unsolved (Sutherland, 2009).

Therefore, the gene expression is controlled by tightly regulated bulk of proteins and their small disruption, like under- or over-expression can lead to many diseases including cancer, possibly because of their pleiotropic capabilities (Lonard, 2007).

1.3. Vitamin D and the vitamin D receptor (VDR)

1.3.1. The history of Vitamin D

In mid 1920s, Windaus, Hess, Rosenheim and colleagues found that skin exposure to ultraviolet B (UV-B) light or sunlight results in vitamin D synthesis, thus restoring a positive calcium balance (Feldman, 2011). In the 1970s it was already well known that vitamin D has to be turned into its active form of the steroid hormone, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), also known as calcitriol, to have biological function, and that its intake involves three organs: intestine, bone and kidney which are involved in calcium homeostasis (Norman, 2006). During the next 15 years scientists discovered that steroid hormones like $1\alpha,25(OH)_2D_3$ serve as regulators of gene expression (Mangelsdorf, 1995) through interaction with their corresponding receptors. Together they form a high affinity ligand – receptor complex, such as VDR with its affiliated ligand $1\alpha,25(OH)_2D_3$.

Today we know that $1\alpha,25(OH)_2D_3$ is not only essential for mineral homeostasis and bone integrity, but it has functions in many biological processes like regulation of growth, differentiation and apoptosis in a broad variety of normal and malignant tissues like prostate, breast, skin, adipose tissue, brain, bone marrow, colon, muscle, as well as in activated B and T lymphocytes. There are more than 30 target tissues where VDR is present (Norman, 2006; Reichrath, 2007). This suggests a global transcriptomic reaction upon stimulation with $1\alpha,25(OH)_2D_3$ across may different types of cells.

1.3.2. Vitamin D metabolism

Vitamin D is mainly synthesized in the epidermal layers through exposure to UV-B or sunlight and less in intestine from food and supplements. Vitamin D synthesis is a multistep process. The first step is conversion of pro-Vitamin D_3 (7-dehydrocholesterol) into pre-Vitamin D_3, which is isomerized into Vitamin D_3 (cholecalcitriol). This form is transported with the bloodstream to the liver, by binding to vitamin D binding protein (DBP). There it is hydroxylated, by a group of 25- hydroxylases from the cytochrome P450 family (encoded by *CYP27A1, CYP3A4* and *CYP2R1*) (Prosser, 2004) to 25-hydroxycholecalcitriol ($25(OH)D_3$). In the kidney, that form is a substrate for 1α-hydroxylase (encoded by *CYP27B1*), which turns it into the biologically active form of vitamin D_3 called 1α,25-dihydroxycholecalciferol - $1\alpha,25(OH)_2D_3$ (calcitriol) (Deeb 2007). The major inducers of 1α-hydroxylase are parathyroid

hormone (PTH) as well as low body levels of phosphate and calcium (Prosser, 2004). Degradation of $1\alpha,25(OH)_2D_3$ is promoted by 24-hydroxylase, product of the *CYP24A1* gene. This constitutes a negative feedback loop, since $1\alpha,25(OH)_2D_3$ strongly induces the expression of the *CYP24A1* gene. The consequence is formation of the blood metabolites $1\alpha,24,25(OH)_2D_3$ from $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$ from $25(OH)D_3$ (Deeb 2007) (Fig. 7).

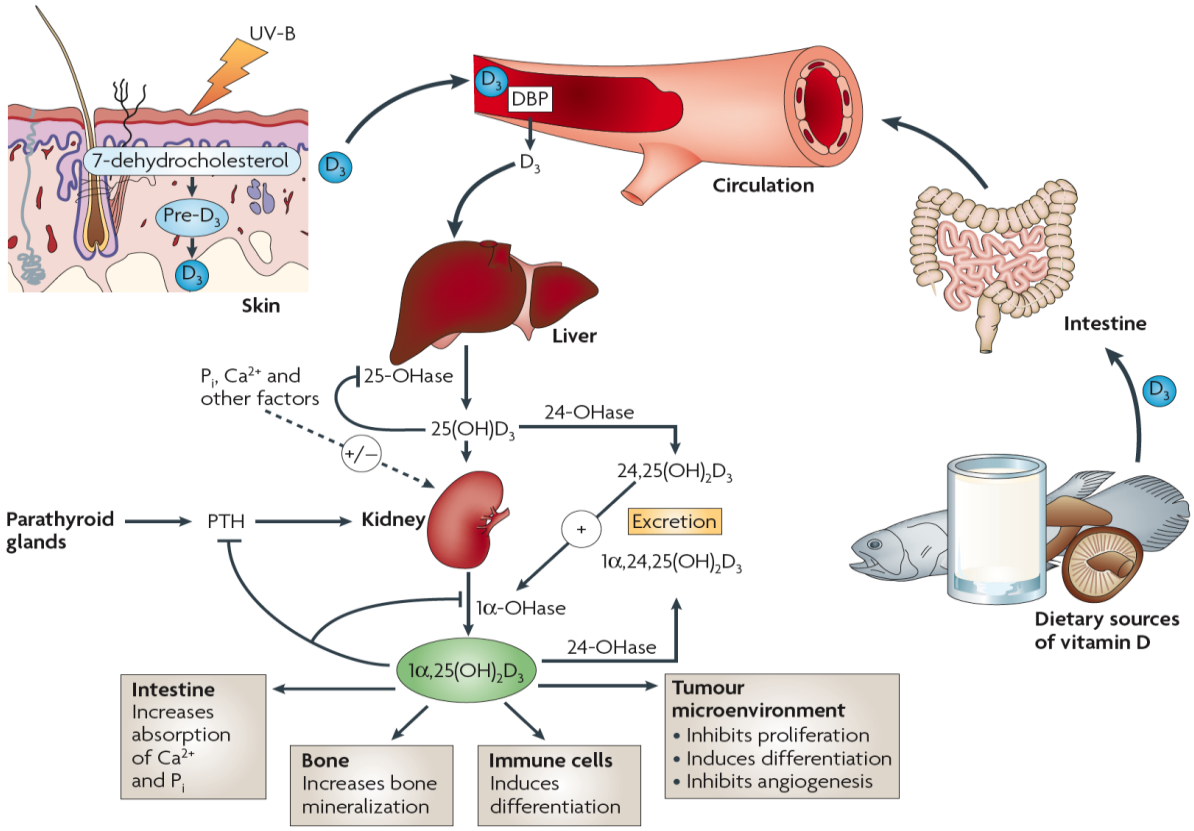


Figure 7. The metabolism of Vitamin D

Explanation of the figure in the text (Taken from Deeb, 2007).

1.3.3. Discovery of the Vitamin D Receptor

The VDR was identified in 1969 as a binding protein for an unknown vitamin D metabolite (Haussler, 1969). In 1975 Brumbaugh and Haussler discovered that VDR forms a complex with the active form of vitamin D - $1\alpha,25(OH)_2D_3$ with a high affinity. Eventually, experiments with VDR knock-out mice proved a crucial role of this receptor in the action of vitamin D, including growth and bone formation (Yoshizawa, 1997).

VDR is a member of the nuclear receptor superfamily; it belongs to the subgroup of endocrine receptors. Upon 1α,25$(OH)_2D_3$ stimulation VDR can trigger a variety of biological processes via genome-wide modification of the expression of a high number of genes within many different types of tissues. The major requirement for its effect on gene modulation is the binding of the VDR protein close to the transcription start site (TSS) of the respective gene.

1.3.4. Principles of VDR action

The structure of VDR resembles that of a typical NR (Fig. 4). Ligand occupancy leads to the formation of two specific protein interaction sites within the VDR. One of them allows it to associate with its heterodimer partner RXR in order to bind to DNA, and the second one is required to engage a large number of co-regulator proteins that are essential for regulation of genes, encoding proteins which mediate 1α,25$(OH)_2D_3$ response at transcriptional level (Fig 8).

VDR binds to DNA through specific regulatory sequences called "vitamin D response elements" (VDREs). These are 2 hexanucleotides repeats with RGKTCA motifs (R = A/G, K = G/T) to which VDR binds in a direct repeat (DR) orientation, usually with a spacing of three nucleotides. The two half-sites of VDREs are occupied by a single VDR and a single RXR protein.

These specific response elements may be located at distant or proximal sites from the promoter of the gene regulated by VDR. Therefore, VDREs can act as *cis* or *trans* elements to interact with the basal transcriptional machinery, and mediate the transcription process. *Cis*-elements are located close to the transcription start site (TSS) of the targeted genes. *Trans*-elements, on the other hand, are distant and therefore VDR may bind far away up- or down-stream from the TSS of a targeted gene. Interestingly, there are genes that are regulated by both proximal and distant response elements, e.g. the best-known VDR target, the gene *CYP24A1*. Two VDREs are located proximal to its promoter, but its induction is additionally correlated with a cluster of distal enhancers that also show association with VDR after 1α,25$(OH)_2D_3$ stimulation (Meyer, 2010). Likewise, several enhancers seem to regulate the gene of the Receptor Activator of Nuclear Factor-κB Ligand (*RANKL*) required for accurate osteoclast maturation and function, and is involved in bone remodeling. It is induced by 1α,25$(OH)_2D_3$, and its expression is controlled by multiple functional VDREs of which the most distal from the TSS was located -96 kb in human (Nerenz, 2008).

1.3.5. Protein-protein interactions that mediate VDR transactivation

Local changes in chromatin structure are essential for proper transcriptional action. For instance, when chromatin is in a condensed state VDR-RXR heterodimers are not able to bind to their response elements. Only a local chromatin relaxation allows functional VDREs to associate with the liganded VDR. Therefore, changes in gene expression through VDR-RXR binding to the VDREs are accompanied by the involvement of a set of large complexes of co-regulators mediating chromatin decondensation/condensation. These co-regulatory complexes seem to be gene-specific, conduct a serious of actions in specific environment manner and they also contain many proteins with enzymatic activities (Pike, 2010). These are mediator complexes called vitamin D receptor – interacting proteins (DRIPs), as well as complexes responsible for chromatin remodeling, histone modification, and the general transcription complex. Hence, gene regulation is a tightly regulated process that needs certain proteins to interact with each other to mediate the regulation (Fig. 8). The complexes are listed below:

The chromatin remodeling complex

Proteins involved in this process have the ability to effectively shift nucleosomes in an ATP-dependent fashion. Thereby, they may assemble and reassemble the nucleosome core (Cairns, 2009).

The histone modifier complex

A complex responsible for the chromatin state encompasses co-regulators that can trigger histone acetylation/deacetylation and methylation/demethylation, and it is related to an open or close state of chromatin structure.

The Mediator complex

While the chromatin is open and VDR is able to interact with VDREs freely, the Mediator complex also known as the Vitamin D Receptor Interacting Protein (DRIP) forms a bridge between the General Transcription complex on the TSS of a targeted gene through binding to the C-terminal domain (CTD) of the polymerase II RNA (RNA pol II) and the VDR.

The General Transcriptional complex

The RNA pol II is part of the General Transcriptional complex, which additionally encompasses transcription factor II D (TFIID), a subunit that contains the TATA-binding protein (TBP), which interacts with DNA close to the promoter. TBP is responsible for recruitment of RNA pol II, which is followed by joining the five remaining subunits of TFs to the whole complex.

VDR transactivation

Taken together, 1α,25(OH)$_2$D$_3$ stimulation is a process that involves recruitment of co-activators. The Mediator complex binds to VDR via DRIP205, the complex with HAT and HMT activities binds to VDR through SRC1, 2 or 3. SRC recruits CREB-binding protein (CBP) or p300 and other HATs and HMTs, which destabilize the DNA – histone connection and thereby enable transcription to take place. The Mediator with no HAT or HMT functions participates in RNA pol II transportation to the TSS of the gene (Bikle, 2010) (Fig.8).

Interestingly, DRIP205 has the ability to bind directly to the VDR also at an enhancer position, which leads to recruitment of RNA pol II not only at TSS. This implies involvement of a series of co-regulators including SRC1, CBP (causing H4Ac), SMRT also to the regulatory regions (Meyer, 2010).

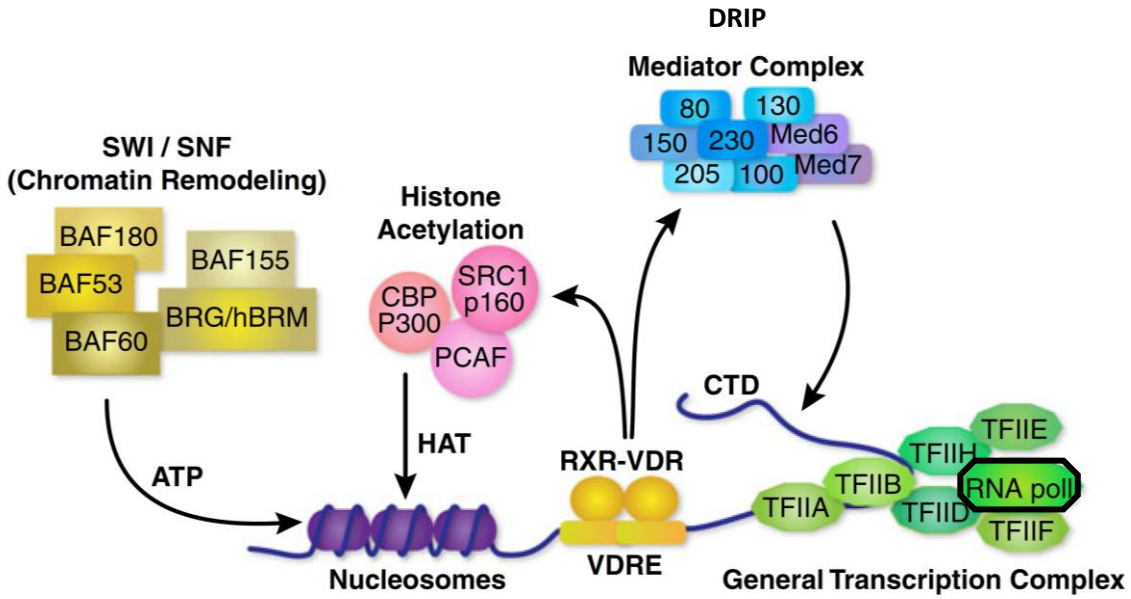


Figure 8. Effect of VDR on chromatin remodeling and transcription initiation

The 1α,25(OH)$_2$D$_3$ mediated response regulates the action of several complexes of co-regulatory proteins. There is a General Transcriptional Complex located at the TSS, a VDR-RXR heterodimer at the VDRE, a Mediator complex that binds to the CTD thereby interacting with RNA pol II, a histone acetylation complex with HAT activity and a chromatin remodeling (SWI/SNF) complex with ATPase activity (Adapted from Pike, 2011).

In contrast, VDR is able to repress gene transcription in a ligand-dependent (transrepression) or independent (derepression) manner. For example, *CYP27B1*, a gene encoding a critical enzyme in vitamin D synthesis, is induced by PTH via activation of protein kinase A and C

(PKA and PKC), but is suppressed by $1\alpha,25(OH)_2D_3$ (Fig. 9). In the absence of ligand, VDR interacting repressor (VDIR) binds to negative vitamin D response elements (nVDREs) of *CYP27B1,* interacts with HAT co-activators and leads to transactivation. Upon $1\alpha,25(OH)_2D_3$ stimulation, the VDR-RXR complex blocks VDIR binding leading to the dissociation of HATs and recruitment of HDAC co-repressors which changes the chromatin structure. That, in turn, leads to transrepression of *CYP27B1*. Two DNA methyltransferases are involved in this process, i.e. DNMT1 and DNMT3B. They methylate cytosine residues at CpG sites close to the gene promoter, thereby turning it off. In the presence of PTH and the absence of $1\alpha,25(OH)_2D_3$, VDR-RXR, DNMT and HDAC complexes are dismissed and the gene is activated (Kim, 2007; Kim 2009).

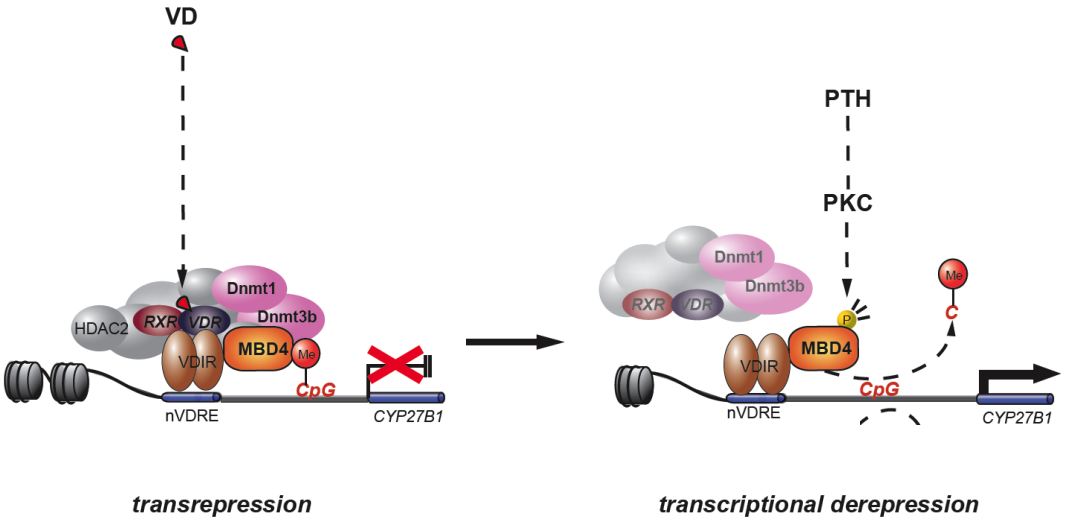


Figure 9. Mechanism of VDR-mediated gene repression

Representation of ligand-induced transrepression of the *CYP27B1* gene by VDR and derepression of the same gene via PTH action. Transrepression is a result of VDR-RXR heterodimer interaction with VDIR connected to VDREs. It leads to HDAC and DNMT recruitment and inhibition of gene expression. Derepression is an effect of PTH that stimulates PKC and PKA, which leads to DNA demethylation via methyl-CpG binding domain 4 (MBD4) phosphorylation, and results in gene activation (Adapted from Kim, 2009).

In the past few years, a series of genome-wide analyses uncovered great numbers of new VDR target genes as well as new binding sites located in distant and close proximity from the TSSs after stimulation with $1\alpha,25(OH)_2D_3$ in many different cell types. Moreover, a large number of recruitments of co-activator and co-repressors, occupancy of RNA pol II and modifications of histones across the genome were discovered. This enables researchers to analyze variable interactions within the nucleus. Interestingly, it has been shown that many

VDR target genes stimulated by 1α,25$(OH)_2D_3$ did not harbor VDREs close to the promoter, suggesting that rather distal regulatory regions, rich in VDR-RXR occupancy are involved in gene induction or repression. Furthermore, Meyer *et al.* have shown that MED1, a protein from the Mediator complex is present at many distal response elements, associated also with VDR (Meyer, 2010; Pike, 2011). The fact that distant response elements from promoters of 1α,25$(OH)_2D_3$ target genes are functional and associate with VDR-RXR heterodimer and co-regulators, suggested the formation of chromatin loops which facilitate the contact between enhancers and the TSSs, thus enabling transcription regulation (Hakim, 2010).

1.3.6. 1α,25$(OH)_2D_3$ and its analogs

Since 1α,25$(OH)_2D_3$ was shown to play an important role in many biological processes, including the prevention of disorders, its therapeutic use has gained considerable attention. Considering the fact that intake of the natural ligand for VDR may lead to some side effects like hypercalcemia as a result of excessive accumulation of calcium ions in the body, more than 3000 analogs of 1α,25$(OH)_2D_3$, with lower calcemic activity have been synthesized (Eduardo-Canosa, 2010). Many of the proposed analogs of 1α,25$(OH)_2D_3$ may also be used to prevent cancer development due to their enhanced anti-proliferative effect (Masuda, 2006).

1.3.7. 1α,25$(OH)_2D_3$ effects in biological processes

There are several areas in which 1α,25$(OH)_2D_3$ and its receptor elicit biological responses. These include calcium homeostasis, the immune system, glucose and fat metabolism, the cardiovascular, muscle and brain systems, and the control of the cell cycle, thus affecting also the process of cancer development. Responses to 1α,25$(OH)_2D_3$ are very broad since the VDR is present in more than 30 tissues in human (Norman, 2006; Norman, 2010). Insufficient sunlight exposure is a major factor for vitamin D deficiency. Since VDR plays an important role in cell growth and differentiation and has anti-proliferative activities (Bouillon, 2006), inadequate vitamin D levels may contribute to a series of abnormalities including bone diseases, multiple sclerosis (MS) (Holick, 2008), autoimmune diseases (van Etten, 2005), diabetes, cardiovascular diseases, hypertension, metabolic syndrome, infectious diseases and cancer (Norman, 2010; Liu, 2007; Tokar, 2005). According to M. Holick, A. Norman and R. Boullion the general recommendation of vitamin D intake, 200 International Unit (IU) per day, is much too low. It should be increased at least five times (Holick 2008, Holick 2011, Norman 2010) in order to prevent the development of above-mentioned disorders. Moreover,

Holick *et al*. propose that a high dosage of Vitamin D could have a beneficial impact on those diseases states. Three systems in which VDR and its ligands play a crucial are highlighted below.

1.3.7.1 Functions of the VDR in calcium homeostasis

Calcium (Ca^{2+}) homeostasis is dependent on vitamin D. Ca^{2+} is one of the major components of the bone and is absorbed from the small intestine, passing through the epithelial transient receptor potential Ca^{2+} channel V6 (TRPV6) and the plasma membrane Ca^{2+} ATPase PMCA pump (Bouillon, 2006; Fleet 2006) regulated by $1\alpha,25(OH)_2D_3$, thus enabling Ca^{2+} transportation. Its absorption, reduced renal Ca^{2+} excretion and elevated bone resorption is related to elevated levels of circulating $1\alpha,25(OH)_2D_3$ (Xue, 2009). Low levels of vitamin D are related to rickets and low bone density. $1\alpha,25(OH)_2D_3$ together with parathyroid hormone has a crucial impact on proper bone mineralization, due to its genomic effects in regulation in osteoblasts and osteoclasts (Jones, 1998). These are the two types of cells in the bone remodeling process that control the bone formation and the bone resorption, respectively. The VDR regulates differentiation and function of osteoclasts and leads to bone resorption and release of the Ca^{2+} from the bone due to direct regulation of *RANKL* gene expression. *RANKL* is expressed in osteoblasts and is necessary for a proper osteoclast maturation and function (Nerenz, 2008).

1.3.7.2. Functions of the VDR in the immune system

It is well known that VDR regulates a number of genes implicated in the immune system and thus has a crucial role in the innate and adaptive immune system. Interestingly, the immune system participates in cancer prevention by protecting the host against viral infection (virus-induced tumors), and by suppressing inflammation, by defeating pathogens that contribute to carcinogenesis (Fleet, 2012). VDR is expressed in several immune-related cells like monocytes, macrophages, activated T cells and dendritic cells (DCs). It has been found to stimulate phagocytosis and the synthesis of antimicrobial factors (Schauber, 2007). Macrophages infected with *Mycobacterium tuberculosis* (TB) when exposed to $1\alpha,25(OH)_2D_3$ elicit signal events leading to the expression of toll-like receptors and expression of the cathelicidin (*CAMP*) gene, a defensive, antimicrobial protein, that removes TB from the cell (Liu, 2006).

In addition, cytokines that have a pivotal role in T cell activation are regulated by VDR. Interleukin 12, crucial in T helper 1 (Th1) development, is repressed in the presence of

1α,25$(OH)_2D_3$ together with NF-κB. Thus, VDR suppresses the Th1-response (D'Ambrosio, 1998).

In addition, the chronic bowel inflammatory disease called Crohn's disease (CD) caused by chronic inflammation due to intestinal bacteria's may be a result of vitamin D deficiency. Genes encoding nucleotide oligomerization domain 2 (NOD2) and human β defensin 2 (HBD2), proteins involved in innate immune response to pathogens in CD, are up-regulated upon 1α,25$(OH)_2D_3$ stimulation (White, 2011).

1.3.7.3. Functions of the VDR in cell cycle and cancer

VDR exerts anti-proliferative and pro-differentiation effects on many cells, which suggests, together with its pro-apoptotic functions, that it could decrease cancer progression. Already in the 1990s it was discovered that the functions of vitamin D are not only confined to mineral homeostasis. Abe and colleagues showed that in murine myeloid leukemia cells activation of VDR stopped their proliferation and enhanced their differentiation into macrophages (Abe, 1998). Since then a bulk of genes important for regulation of proliferation and differentiation were identified in the context of VDR action (Holick, 2008; Peehl, 2003; Feldman, 2000). Moreover, studies with model systems of cancers of prostate (Banach-Petrosky, 2006), ovary (Zhang, 2005) or lung (Nakagawa, 2005) demonstrated that vitamin D had anti-cancer effects. These VDR functions are related to cell cycle arrest, a process strictly regulated by number of events to prevent uncontrolled division and pre-maturation of a cell. Progression of cells from G_1 to S phase directs the phosphorylation of retinoblastoma (Rb) protein through G_1 cyclins in complex with their cyclin-dependent kinases (CDKs) (Feldman, 2000). C-myc, a transcription factor with an oncogene function, regulates the progression into the S phase of the cell cycle. In general, c-myc, among many targets, induces cyclin D expression thereby leading to increased CDK4 activity due to the formation of cyclin D-CDK4 complexes that can phosphorylate Rb. The active Rb dissociates from the transcription factor E2F and allows becoming active; this results in the transcription of various genes that enable the cell to enter S phase. VDR leads to dephosphorylation of Rb via the up-regulation of CDK inhibitors (CDKIs) including p21 $^{(waf1/cip1)}$ and p27 $^{(kip1)}$ (Liu, 1996; Thorne, 2010; Li, 2004). Studies have identified several VDREs close to the *p21* $^{(waf1/cip1)}$ promoter, indicating direct action of VDR on that gene (Liu, 1996; Saramaki, 2006). VDR also mediates G_2/M-phase cell cycle arrest by the induction of the growth arrest and DNA damage-inducible (*GADD45*) gene involved in growth arrest and DNA repair mechanism (Campbell, 2006).

Interestingly, 1α,25$(OH)_2D_3$ affects the activity of prostaglandins (PGs). In some cells, especially in prostate cancer (PCa) cells, PGs can trigger cell growth. Vitamin D suppresses this by inhibiting gene encoding PG synthesizing cyclooxygenase-2 (*COX2*), also known as prostaglandin-endoperoxide synthase 2 (*PTGS2)*, and increasing the gene encoding the expression of the PG inactivating dehydrogenase (*15-PGDH*). Thus, calcitriol influences the proliferation of PCa cell growth (Moreno, 2005).

Moreover, vitamin D seems to have indirect effects on cell cycle regulation via different pathways; e.g. studies revealed an interplay between 1α,25$(OH)_2D_3$ action and some of the growth factors. For instance, it stimulates expression of key components of the transforming growth factor β2 (*TGFβ*2)-SMAD3 signaling pathway, which is involved in cell growth and differentiation. 1α,25$(OH)_2D_3$ induce *TGFβ,* and by that mediates activation of the SMAD proteins that are co-activators for regulation of gene expression involved in growth inhibition like *E-cadherin* (Wu, 1999; Murthy, 2004). Also, members of the insulin-like growth factor (IGF) binding proteins (IGFBPs) family like *IGFBP3* and *IGFBP5* are gene targets for VDR (Matilainen, 2005). The function IGFBPs is to control the IGFs - mitogens that participate in cell division, differentiation and apoptosis. IGFBPs bind IGFs and thus reduce their interactions with cell surface receptors (Fleet, 2012). Moreover, IGFBPs mediate IGF-independent effects like cell growth arrest by stimulation of the p21 $^{(waf1/cip1)}$ gene (Peng, 2007).

Furthermore, VDR may decrease proliferation through the induction of differentiation of cancer cells. Larriba *et al*. have shown that in the presence of 1α,25$(OH)_2D_3$, VDR promotes cell differentiation due to inhibition of the Wnt/β-catenin pathway. Active β-catenin induces expression of a series of factors important for promoting proliferation (e.g. c-myc). Over-expression of β-catenin is related with skin tumors (Bikle, 2011) and is found in most colon cancers. VDR is able to control and block β-catenin by the induction of *E-cadherin*, which has the function to transport nuclear β-catenin to the plasma membrane, thus blocking its action (Larriba, 2011).

Additional VDR has the ability to induce apoptosis. It was shown that 1α,25$(OH)_2D_3$ represses the expression of the anti-apoptotic gene *BCL2* in breast cancer cells and that it induces the expression of pro-apoptotic genes like *BAK* and *BAX* in prostate and colorectal cancer cells (Deeb, 2007).

1.4. Prostate cancer and vitamin D

1.4.1. Phenomenon of cancer

The nineteenth century discovery that all cells of the body derive from the fertilized egg, suggested that a cancer cell developed from a single cell that lost its ability for creation of tissues of a normal form and function. More than one hundred years of studies revealed that cancer involves dynamic changes in the genome as well as in "tumor environment". The complexity of cell malformation and the consequent implications of metastasis have inspired Hanahan and Weinberg in 2000 to propose the six basic hallmarks that most, if not all, types of cancer demonstrate during their progression. This unique set of cancer cell features include: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and finally resisting cell death. All cancer types fulfill those steps to complete tumorigenesis, though they may do so at different times and via different mechanisms (Hanahan and Weinberg, 2000) (Fig. 11).

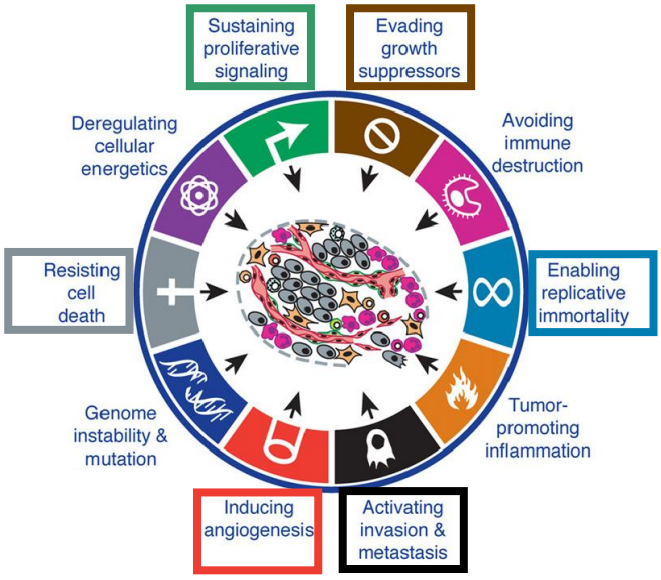


Figure 11. The hallmarks of cancer

The core of the cancer hallmarks is presented within the boxes, along with the emerging hallmarks (the deregulating cellular energetics and avoiding immune destruction) and with enabling characteristics (genome instability and mutations with tumor promoting inflammation) (Adapted from Hanahan & Weinberg, 2011).

Recently, Hanahan and Weinberg expand their circle of hallmarks with two additional once: avoiding immune destruction (especially for detection by T, B lymphocytes and macrophages) and modifying or deregulating of the energy metabolism to promote cell

growth and proliferation. Those new hallmarks are considered to be emerging, since their functions are not yet fully validated. The genome instability and mutation (marking genetic alternation) as well as tumor-promoting inflammation, are the "enabling characteristics" for achieving the core and emerging hallmarks (Hanahan and Weinberg, 2011).
Additionally, the new insights into chromatin organization together with the complexity of gene regulation have given a new perspective into the development of cancer, which acquires the capabilities presented in the above-mentioned hallmarks.

1.4.2. Prostate cancer (PCa) genesis

PCa is one of the most common non-cutaneous malignancies in the Western world. In 2010 more than 200,000 new cases were diagnosed and the disease causes around 32,000 deaths per year in the United States (Berger, 2011). Data from the National Cancer Institute show that there are differences in the PCa incidence among the races. Mostly it affects Afro-American men. Thus, per each 100,000 men from diverse ethnicity there are ~234 Black, ~150 White and ~88 Asian men diagnosed with PCa (data taken from 2004-2008, see www.seer.cancer.gov). As seen above, the probability of getting PCa in Southeast and East Asian populations is much lower, but interestingly De Marzo *et al.* noticed that it increases when Chinese/Japanese men move to the west. This suggests that geographic variation associated with insufficient sun exposure is a potential risk factor, along with diet, older age, family history and race (De Marzo, 2007).

1.4.3. PCa and inflammation

Chronic inflammation is one of the risk factors for the development growth of many cancers. It is often related to environmental exposures. In PCa, inflammation is linked to infectious agents (viruses, bacteria), urine reflux and physical trauma, which might be caused by development of corpora amylacea (small nodules in the lumen and ducts), dietary habits (consumption of red meat with carcinogens, vitamin deficiency), and finally hormonal changes (De Marzo, 2007). The last phenomenon is particularly interesting, since the AR is one of the major gene regulators in prostate cells. In addition, the over-expression of estrogens in prostate cells is linked to the development of PCa (Harkonen, 2004). The inflammation-related factors lead to malformations of the epithelial secretory cells in the peripheral prostate gland (Polek, 2003). They start to form proliferative inflammatory atrophy (PIA) lesions that are related to the emerging cancer hallmark, the avoidance of immune

destruction. Through the following proliferation prostate intraepithelial neoplasia (PIN) develop, which is genetically instable and often leads to invasive prostate carcinoma (De Marzo, 2007) (Fig. 12).

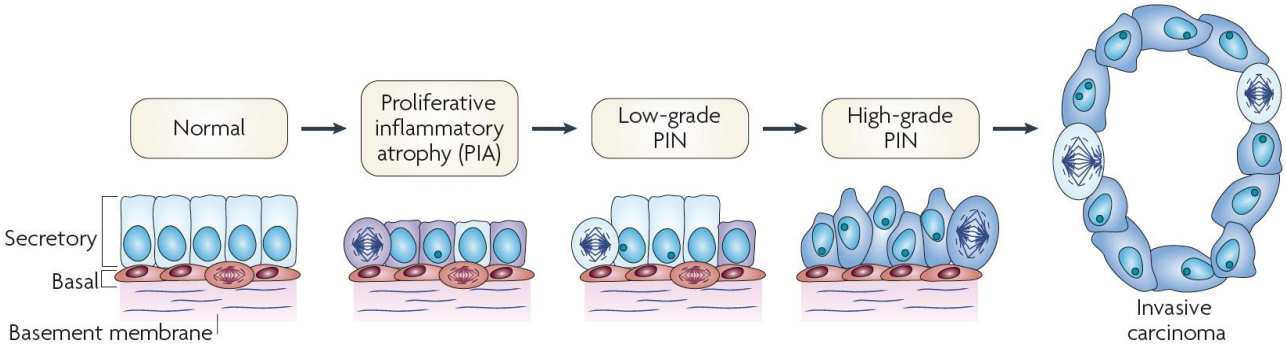


Figure 12. Model of early prostate carcinogenesis

Four stages of PCa progression are presented. The first stage is the formation of PIA with chronic inflammation being the crucial agent. Continuous cell growth and genetic changes lead to massive proliferation of genetically unstable intraepithelial cells (PIN) and accumulation of new genomic alternations that finalizes in the formation of an invasive carcinoma (Taken from De Marzo, 2007).

1.4.4. The cancer hallmarks distinguished in PCa

The AR, as a nuclear receptor activated by androgens, plays an important role in the development and maintenance of non-malignant prostate via specific gene regulation, but being deregulated, it also takes part in initiation and progression of prostate cancer. Tumor growth is initially androgen-dependent. Ablation of androgens (e.g. dihydrotestosterone (DHT)) causes regression of "androgen-dependent" tumor and is often used in cancer therapy (Pheel, 2004). However, the symptomatic relapse frequently occurs 2-3 years later with so-called "androgen-independent" disease and the mechanism for this transition is not yet clear. The activation of the AR in androgen-independent states leads to the up-regulation of genes that are implicated in the M-phase of the cell cycle, and therefore cell growth is promoted (Wang, 2009). Additionally, two proteins of the kallikrein (KLK) family, KLK3 and KLK2, which are encoded by neighboring genes on chromosome 19, are up-regulated after androgen treatment in an advanced, androgen-dependent PCa cell line called LNCaP. AR seems to have a direct regulating effect on these genes as the proteins of AR, RNA pol II and a set of co-factors were detected on the promoters of these genes (Kang, 2004). KLK3, also known as

prostate specific antigen (PSA), is a serine protease that is commonly used for the screening and monitoring of PCa (see also chapter 1.5).

1.4.4.1. Evasion of growth suppressors

Several genes have already been correlated with the development of PCa. In advanced PCa tumor suppressor genes are lost. For example, the single copy deletion of the *NKX3.1* gene that encodes the prostate-restricted homeobox protein leads to increased cell growth and oxidative DNA damage (De Marzo, 2007). Moreover, in primary tumor cells including PCa, the phosphatase and tensin homologue encoded by the *PTEN* gene that suppresses proliferation and increases apoptosis, is frequently lost (Li, 1997). Also the cyclin-dependent kinase inhibitor p27 encoded by the *CDKN1B* (p*27*) gene, a cell cycle suppressor gene, is silent in PCa (Taylor, 2010).

1.4.4.2. Sustained proliferative signals, death resistance and immortality

C-myc as an oncogene and transcription factor, together with the pro-apoptotic *BCL2* gene were shown to be over-expressed in many cancers including PCa. Thereby, their main functions of regulation of cell proliferation and apoptosis are deregulated in tumors (Sinha, 1995). Similarly, the proper cell growth and apoptosis are affected in advanced PCa by inappropriate integrin expression, which additionally influences cell adhesion and migration (Altieri, 2010). Gene expression arrays identified novel target genes as well as variety of mutations, including gene fusions. The best-known gene fusion in PCa is the one of the androgen-regulated trans-membrane protease serine 2 (*TMPRSS2*) gene with the erythroblast-transformation specific (ETS)-related gene (*ERG*) (Witte, 2009). Merged, they play a role of an oncogene. The *TMPRSS2* gene under AR action mediates over-expression of the transcription factor *ERG*, which eventually contributes to deregulation of many genes and promotes cell growth and invasion in PCa (Tomlins, 2007; Yu, 2010). In addition, Berger and colleagues performed a ChIP-Seq analysis in seven prostate tumors. The genomes were divided according to the presence of the *TMPRSS2-ERG* fusion. The location of the fusion was correlated with an open state of the chromatin, thus with the presence of ERG, the AR, the co-regulators and the RNA pol II that mediates gene expression. In contrast, the lack of *TMPRSS2-ERG* fusion was associated with a closed chromatin state and therefore results in gene repression (Berger, 2011).

1.4.4.3. Induced angiogenesis

A fast developing tumor needs a blood connection for nutrition and oxygen supply. The process of new blood vessel formation is called angiogenesis (Polek, 2002). Many pro-inflammatory agents are able to activate that process, by controlling the vascular endothelial growth factor (VEGF), a stimulus of angiogenesis. As a growth factor, VEGF is critical in cell proliferation, migration and it is therefore crucial for vessel formation (Benjamin, 1999).

Furthermore, PTGS2 was shown to increase angiogenesis, whereas its inhibitors block that process. It is a gene responding fast to stress-related factors like mitogens, growth factors, cytokines and tumor promoters. Moreover, PTGS2 is over-expressed in PCa (Hussain, 2003). A further study revealed connection between PTGS2 and VEGF. A selective PTGS2 inhibitor reduces the growth of advanced, metastatic prostate cancer cell line (PC-3) implanted into mice, through apoptosis and down-regulation of VEGF. As a consequence, microvessel density decreases and angiogenesis is inhibited. PTGS2 therefore facilitates tumor progression via angiogenesis, tumor invasion and resistance to apoptosis (Liu, 2007).

Interestingly, the transforming growth factor (TGF)-β pathway that is involved in cell growth and differentiation, mediates the inhibition of an immune response and promotes angiogenesis in cancer. Lu *et al.* have shown that over-expressed TGFβ1 in PCa up-regulate the expression of potent pro-angiogenic interleukin 8 (*IL8*) (Lu, 2006).

1.4.4.4. Metastasis

Metastasis is a process of cell invasion leading to the growth of tumors that might be detected far away from the original location. The epithelial-mesenchymal transition (EMT) mediates the process of metastasis. The initial steps of invasion include the degradation of extracellular matrix, adhesion and degradation of the basement membrane, with invasion into vessels often termed as intravasation and angiogenesis essential for tumor cells translocation to distinct organs where they proliferate (Sung, 2000). The EMT involves fundamental alternations in the gene expression profiles. This includes repression of E-cadherin, encoded by the *CDH1* gene, that enables epithelial cells to adhere to one another as well as the repression of the β-catenins that allow binding to actin fibers. Moreover, as mentioned above, the TGFβ signaling besides the role in angiogenesis is also able to induce EMT in cancer cells together with effects mediated by mutations in the *RAS* oncogene. Reduced expression of E-cadherin, β-catenins and most α, β integrins (with exception of α6 and β4 that are over-expressed in androgen independent tumor (Baust, 2010)) responsible for cell-cell and cell-matrix adhesion

and participation in control of cell proliferation and apoptosis, was also observed in PCa. Additionally, matrix metalloproteinases (MMPs), the most important effectors of EMT formation that degrade the extracellular matrix, are secreted in cancer including PCa (Weinberg, 2007).

1.4.5. Vitamin D and its function in non-malignant prostate cells

The VDR is expressed in both, non-malignant and tumor prostate cells. Studies with immortalized non-malignant human prostate epithelial cell line (RWPE1), revealed after $1\alpha,25(OH)_2D_3$ treatment an up-regulation of the infection/inflammation gene *CD14* and inhibition of the pro-angiogenic *VEGF* gene. Moreover, several transcripts related to control of the cell cycle were repressed in the long-term treatment. These results indicate that vitamin D may inhibit cell growth and has anti-angiogenic and anti-inflammatory actions (Kovalenko, 2010).

Furthermore, the enzyme CYP27B1 catalyzing the conversion of $25(OH)D_3$ into its active hormone, is present in non-malignant human prostatic epithelial cells, indicating the local production of $1\alpha,25(OH)_2D_3$ derived from $25(OH)D_3$. Interestingly, CYP27B1 activity is reduced in PCa cells compared with non-malignant cells, leading to a local reduction of the $1\alpha,25(OH)_2D_3$ production and the excessive, intrinsic growth of cancer cells. As the prostatic *CYP27B1* gene is not regulated by PTH or calcium, the treatment with vitamin D might be a useful chemoprevention, without undesirable side effects of increased circulating hormone levels (Chen, 2003; Swami, 2011). Intriguingly, the anti-proliferative and differentiating cytostatic rather than a cytolytic one, action of vitamin D, can be more effective in prevention therapy or in the treatment of early disease, with doses not exceeding toxicity level (Krishnan, 2010).

1.4.6. VDR and PCa epidemiology

A variety of modified genes involved in PCa, including the deficiency of tumor suppressors and the up-regulation of oncogenes, are closely related to inflammation, proliferation and differentiation, the processes in which vitamin D signaling plays a crucial role. Thus, it is crucial to investigate the relationship between PCa and VDR function.

Another connection between prostate cancer development and vitamin D intake may be, as above-mentioned, ethnicity. The disease affects mainly Afro-American men; white men have a nearly twice lower risk and Asian men, the lowest. Epidemiological studies implied that the

chance of suffering from PCa is also associated with residence in northern countries. Thereby, a strong correlation between sunlight exposure and cancer growth was monitored (Polek, 2002; John, 2007). Since vitamin D synthesis occurs via the action of UV-B light in the skin, a low level of $1\alpha,25(OH)_2D_3$ was correlated with an increased probability of getting PCa (Moreno, 2005).

1.4.7. Role of VDR in prostate cancer development

1.4.7.1. The anti-proliferative function of the VDR

It is well known that $1\alpha,25(OH)_2D_3$ controls the cell cycle through regulation of expression of genes involved in that process (Norman, 2010). It was reported that VDR action arrests the cell cycle in the G_0/G_1 phase in LNCaP advanced prostate cancer cell line due to up-regulation of *p21* and *p27* gene expression (Zhuang, 1998; Lou, 2004). Interestingly, the transgenic over-expression of human c-myc in the murine prostate has been shown to lead to the development of neoplasia, which transformed into invasive adenocarcinomas (Ellwood-Yen, 2005). JoyAnn *et al.* reported that stimulation of androgen-independent prostate cancer cells (C4-2) with $1\alpha,25(OH)_2D_3$ reduces *MYC* expression on the mRNA level and decreases protein stability. Moreover, after *MYC* gene silencing, cell growth inhibition and G_1 phase accumulation was observed. These findings suggest importance of $1\alpha,25(OH)_2D_3$ in cell cycle arrest in PCa studies. Nevertheless, the exact mechanism by which VDR controls cell growth in PCa is still not fully understood (JoyAnn, 2009; Stewart, 2005).

One of known targets of VDR, *IGFBP3* encoding a protein that binds to the IGF, as mentioned above, interestingly is reduced in PCa (Jerome, 2003). IGFs are essential for proper cell proliferation and development (Kriebitzsch, 2009). Microarray analysis has shown that *IGFBP3* is the highest induced gene in LNCaP cells after stimulation with $1\alpha,25(OH)_2D_3$ (Krishnan, 2004). Moreover, in the same cell line it was noticed that *IGFBP3* expression is directly regulated by VDR through binding to the VDRE located close to the promoter of that gene. IGFBP3 activates pathways including the activation of p21 that participates in cell cycle arrest. Thus, IGFBP3 contributes to the growth inhibitory action of $1\alpha,25(OH)_2D_3$ and is associated with the induction of apoptosis (Peng, 2004).

A pro-apoptotic function of $1\alpha,25(OH)_2D_3$ was confirmed by many studies on different cancer cells including prostate (Blutt, 2000). For example, the activation of VDR is lowering the

expression level of the anti-apoptotic gene *BCL2* (Samuel, 2008) that is normally highly expressed in PCa (Moreno, 2005), and thus enhances apoptosis.

Moreover, genes involved in PG metabolism and action were shown to be regulated by VDR in primary prostatic cells and in PCa cell lines. Interestingly PGs, along with having effect on growth promotion and PCa progression, are involved in the inhibition of apoptosis. The effects of $1\alpha,25(OH)_2D_3$ may reduce the levels of biologically active PGs and therefore decrease a PG-related cell proliferation in PCa. Microarray data from an advanced prostate cancer cell line (LNCaP and PC3) stimulated with $1\alpha,25(OH)_2D_3$ confirmed this hypothesis (Moreno, 2005). The analysis revealed significant induction of the NAD-dependent 15-hydroxyprostaglandin dehydrogenase (*15-PGDH*) gene, a putative tumor suppressor gene, product of which is important in inactivation of PGs synthesis. In addition, the expression of *PTGS2* gene, which catalyzes PG synthesis and is induced in many cancer cells (Greenhough, 2009), is down-regulated after $1\alpha,25(OH)_2D_3$ treatment. (Moreno, 2006; Kriebitzsch, 2009).

In addition, relations between androgens and vitamin D action were reported in association with the control of cell growth. A recent genomic study on the LNCaP cell line revealed over 250 genes that were synergistically regulated by both, the AR and the VDR, in a co-treatment experiment. Interestingly, neither hormone alone did not significantly enrich those genes (Wang, 2011). This study indicates a relation between VDR signaling and AR signaling.

The co-operation between VDR and AR was also investigated *in vivo*. A mouse model for PCa with and without *Vdr* was compared, indicating that *Vdr* ablation is correlated with PCa progression. *Vdr* knockout mice showed increased cell proliferation and faster prostate tumor progression than the control wild-type *Vdr* (VDRWT). Moreover, supplementation of both types of mice with testosterone abrogated the protective role of vitamin D, suggesting a cross talk between VDR and AR (Mordan-McCombs, 2010).

Furthermore, other animal models validated the postulated function of vitamin D in growth regulation of PCa (Thorne, 2008; Chen, 2003). For instance, Banach-Petrosky *et al.* have used a mouse model with a loss of function of *Nkx3.1* and *Pten* tumor suppressor genes; these mutations are known to be relevant in PCa development. They reported that, upon $1\alpha,25(OH)_2D_3$ stimulation, the progression of PIN formation from low to high level was significantly reduced, especially when administrated before, rather than subsequent to, the initial occurrence of PIN (Banach-Petrosky, 2006). These findings support the role of vitamin D in chemoprevention of PCa, and suggest that clinical trials for patients with PIN or earlier stage of disease may show a slowdown of PCa development (Swami, 2011; De Marzo, 2007).

1.4.7.2. Anti-angiogenic functions of VDR

It is already known, that vitamin D may inhibit angiogenesis. $1\alpha,25(OH)_2D_3$ does not only reduces proliferation, but also moderates the formation of endothelial cell tube-like structures induced by the VEGF. $1\alpha,25(OH)_2D_3$ decreases expression of *VEGF* gene in several malignant cells including PCa. Moreover, suppression of *PTGS2* sustains vitamin D's anti-angiogenic action (Krishnan, 2010; Kundu, 2008). Mantell *et al.* have performed an *in vivo* study in mice implanted with transfected breast cancer cells with *VEGF* over-expression. The tumor was less vascularized in the presence of $1\alpha,25(OH)_2D_3$ compared to the vehicle control, due to VDR-dependent reduction of *VEGF* gene expression (Mantell, 2000).

1.4.7.3. Anti-metastatic function of the VDR

$1\alpha,25(OH)_2D_3$ inhibits the angiogenesis and controls expression of genes involved in the progression to invasion and metastasis (Krishnan, 2004). For instance, through stimulation of an up-regulation of $\alpha6$ and $\beta4$ integrins, the receptors for the basement membrane matrix protein laminin involved in cell migration and adhesion in metastatic PC-3 and DU145 prostate cancer cell lines, $1\alpha,25(OH)_2D_3$ shows association with PCa metastasis suppression (Sung, 2000). Moreover, in PCa vitamin D increases the level of the tumor suppressor E-cadherin, which down-regulation is correlated with cell proliferation, progression to invasion and metastasis (Krishnan, 2010).

1.4.8. Clinical studies of vitamin D in PCa

In general, the care of common prostate cancer patients is based on prostatectomy combined with radiation and hormonal therapy. Clinically two basic stages of PCa are distinguished: early (androgen-dependent) and late (androgen-independent) (Krishnan, 2007). Since the growth of prostate cells depends on androgen, its deprivation is used for treatment of an early-detected PCa. This therapy is effective as palliation. However during PCa progression, the tumor is no longer androgen-dependent and stops therefore responding to a hormonal therapy (Schwartz, 2009; Vijjan, 2007). Considering the relation between PCa and vitamin D deficiency, $1\alpha,25(OH)_2D_3$ appeared to be beneficial in slowing down the tumor progression in clinical trials. Nevertheless, the promising long time-scale administration of high dosage of $1\alpha,25(OH)_2D_3$ to the patients with advanced cancer, brought side effects, like hypercalcemia (Schwartz, 2009). Thus, the question arose how to get a response without inducing hypercalcemia. Some clinical studies in androgen-independent PCa showed that intermittent

application of very high doses of $1\alpha,25(OH)_2D_3$ alone or in combination with dexamethasone, an immunosuppressant and an agonist of GR, enhanced the anti-proliferative effects of $1\alpha,25(OH)_2D_3$ and caused only a transient hypercalcemia. In 28% of those patients the PSA serum level was halved (Trump, 2006).

Another study on androgen-independent PCa reported the relation between high dosage of $1\alpha,25(OH)_2D_3$ in combination with the chemotherapy drug docetaxel and tumor progression. The PSA doubling time was prolonged in 63% of the patient treated with $1\alpha,25(OH)_2D_3$ and docetaxel (Beer, 2007).

Also, analogs of $1\alpha,25(OH)_2D_3$ with enhanced anti-proliferative function and reduced calcemic action have been created and administrated to patients (Masuda, 2006). Further, using low doses of vitamin D or its analogs in combination with low doses of other drugs, could minimize the side effect of the latter ones and augment their response on cancer. For instance, long intake of the Non-Steroidal Anti-inflammatory Drugs (NSAIDs) like aspirin, ibuprofen or naproxen may block tumor progression (Hussain, 2003), by inhibiting PTGS2 activity and thereby leading to a reduction of PG synthesis. However, it also lead to the development of cardiovascular complications. Cocktails containing lower concentrations of $1\alpha,25(OH)_2D_3$ and NSAIDs induced equally strong inhibition of PCa growth when compared to NSAID alone, but without the side effects (Moreno, 2006). In early recurrent PCa the administration of $1\alpha,25(OH)_2D_3$ in combination with naproxen slowed down the PCa progression and prolonged the PSA doubling time in 75% of the patients (Srinivas and Feldman, 2009).

Interestingly, there is a controversy whether PSA is a good biomarker for PCa. On the one hand it is the best-known indicator of risk for PCa, its initial diagnosis and in monitoring the cancer progression, but it is also elevated in benign prostate diseases. Therefore, many cases of over-diagnosis and over-treatment have occurred due to PSA screening (Lilja, 2008; Croswell, 2011).

1.5. The Kallikrein gene family

1.5.1. General overview on the Kallikrein gene family

PSA, being used as a biomarker for routine screening of PCa, is a member of the largest, contiguous gene cluster family in the human genome, encoding so called kallikrein-related peptidases (KLKs). It is a family of highly conserved, trypsin- or chymotrypsin-like serine protease enzyme that consists of 15 members (KLK1-15) (Sardana, 2009). First KLK1 was discovered, which was followed by the detection of elevated levels of KLK3 in PCa in the 1970s. A few years later the localization of *KLK1, KLK2* and *KLK3* was assigned to chromosome 19q13.4. The other family members were detected in the 1990s because of similarities in their gene structure (Borgono, 2004). The KLKs encode pre-pro-enzymes that are up to 40% identical in all family members. The KLK genes are often co-expressed in normal tissues (prostate, breast, ovarian, skin, central nervous system (CNS), pancreas) and biological fluids, which suggest that these secreted proteins may have a common mechanism of action (Paliouras, 2007). The activation of KLK pro-enzymes, produced as zymogens, is a consequence of a complex proteolytic cascade resulting in the activation of multiple KLKs. Together they are involved in various processes like skin desquamation, degradation and remodeling of ECM, semen liquefaction, innate immunity, among others (Sotiropoulou, 2009). Intriguingly, KLKs can act individually or in a complex that promotes the proteolytic cascade. However, the diversity of KLKs functions and altered expression in various types of cancer/diseases is broad and still not well understood (Emami, 2007).

1.5.2. Organization and structure of the KLKs and their genes

The *KLK* family is located on chromosome 19q13.3-13.4 and forms a cluster, with the length of 265 kb. Except *KLK2* and *KLK3* all genes are transcribed from telomere to centromere. 34 - 52% of the locus consists repetitive elements mainly located outside the protein coding regions (only 4.3% were found inside) (Yousef, 2001). Moreover, the “classical kallikreins” – *KLK1, KLK2* and *KLK3* are highly similar in the amino acid sequence. *KLK2* and *KLK3* show 62-67% of identity with the *KLK1,* whereas the other KLKs (4-15) are less conserved and show only about 27-39% homology.

All genes contain five exons, conserved in size and arrangement. In addition, each *KLK* shows several transcript variants, as a result of alternative transcription start sites (TSS) derived from

additional upstream exons, usually between one and three (Clements, 2004). Tandem genomic organization of the 15 *KLK* genes is shown in the figure bellow (Fig. 13).

Interestingly, *KLK1* and *KLK4-15* are present in all placental mammals, whereas *KLK2* and *KLK3* are not, suggesting a different evolutionary background. *KLK2* and *KLK3* show high similarities to *KLK1*. Probably, *KLK1* duplicated to give rise to *KLK2* from which in primate evolution *KLK3* has arisen (Yousef, 2003).

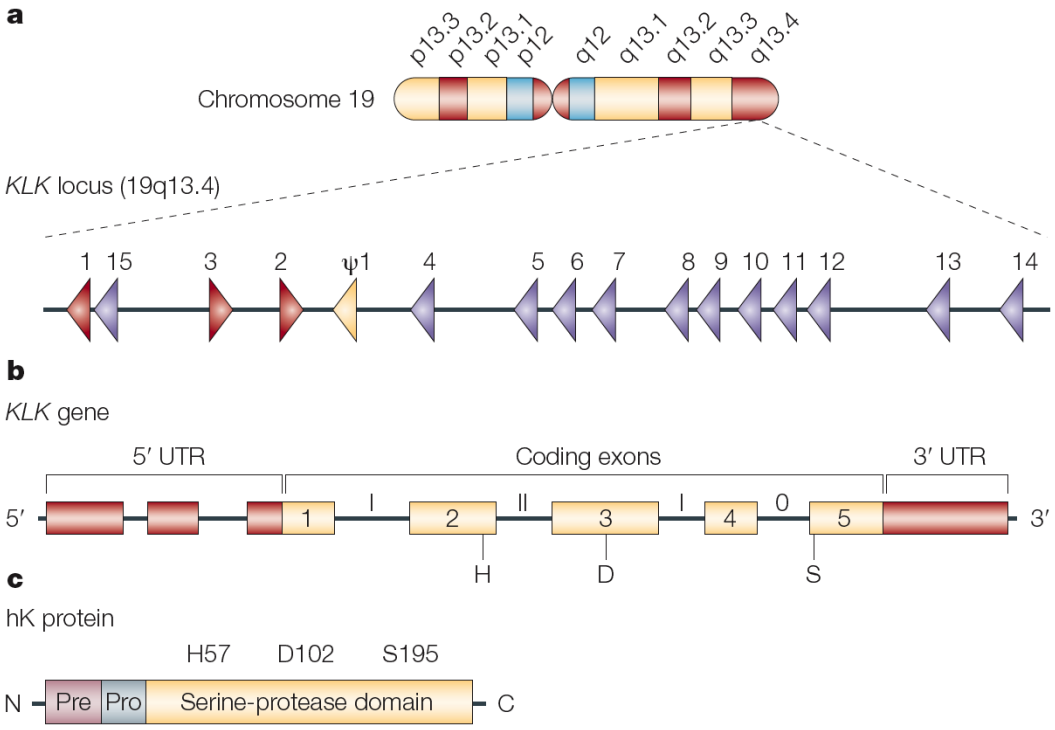


Figure 13. The kallikrein gene locus organization

A. *KLKs* are located on chromosome 19q13.4 in a cluster of 265 kb length. The "classical kallikreins" (*KLK1, KLK2* and *KLK3)* are marked by red arrows, the pseudogene *ΨKLK1* in yellow and the *KLK4-KLK15* in purple B. All *KLK* genes are similar and have usually five exons and three introns C. The human KLK proteins contain an amino-terminal signal sequence (Pre), a pro-peptide (Pro), and a serine-protease domain responsible for catalytic activity (Taken from Borgono, 2004).

All human KLKs (hK) are synthesized as inactive or minimally active pre-pro-enzymes. The pre-domain (Pre) enables insertion of the KLKs into the endoplasmatic reticulum for later secretion. The emerged pro-enzymes (Pro), called zymogens, stay in latency until the pro-domain is discarded, which lead to conformational changes in the enzyme and activation of

the catalytic activity of the serine-protease domain. Thus, they are able to cleave bonds in targeted polypeptide chains and to start protein catabolism (Lawrence, 2010).

Studies have shown that some hKs undergo self-activation, which means that they are activated by other hKs, e.g. pro-hK3 is activated by hK2, hK4, hK5 and hK15 (Borgono, 2004). Moreover, they may lose their activity via internal cleavage by the same or different, mature hKs (Sotiropoulou, 2009). For instance, hK6 maintains an autoactivation / autoinactivation process of its own (Magklara, 2003), whereas pro-hK2 and pro-hK3 activated by hK5 are inhibited by the same enzyme, after longer incubation (Michael, 2006). Moreover, as other serine proteases, hKs interact with endogenous inhibitors like the serpins in two ways. One, known as the "inhibitory pathway", results in the irreversible inactivation of the kallikrein when the inhibitory covalent hK-serpin complex is formed, and triggers permanent hK deformation. In the second way, the so called "substrate pathway", the hK cleaves and inactivates the serpin, whereas the hK remains active (Sotiropoulou, 2009).

The regulation of the enzymatic activity of KLKs requires a series of feedback loops and inhibitors. Therefore, it involves a complex network of events that occurs at multiple levels.

1.5.3. Regulation of kallikrein genes

Kallikreins are present in most of the tissues and body fluids in human (Yousef, 2001). Intriguingly, many of the *KLKs* and their different transcript variants, are simultaneously expressed in a cell-specific manner. For instance, sets of *KLKs* are found in breast (*KLK3*, *KLK4*, *KLK5*, *KLK6*, *KLK8*, *KLK10*, *KLK13*, *KLK14*), in the skin (*KLK5*, *KLK7*, *KLK8*, *KLK9*, *KLK11*, *KLK13*, and *KLK14*), in the CNS (*KLK5-KLK9*, *KLK11*, *KLK14*) and in prostate (*KLK2-KLK6*, *KLK11-KLK13*, and *KLK15)* (Lose, 2011; Paliouras, 2007). This way of expression suggests a complex mechanism of regulation that may be common for a cluster of genes.

The majority of the *KLK* genes are regulated by steroid hormones, like androgens, estrogens, glucocorticoids and mineralocorticoids (Lawrence, 2010). The most studied, in terms of hormonal regulation of gene expression, are *KLK3* and lately *KLK2*. They are related to PCa and regulated by androgens through several AR response elements (ARE) located proximal and distant from the TSSs of the genes. This suggests a possible formation of a chromatin loop in order to bring the ARE enhancer and the promoter together (Jia, 2006). Interestingly, anti-androgen treatment resulted in AR occupancy in the promoter but not at the enhancer of *KLK3*, which was correlated with the recruitment of co-repressors, e.g. NCoR and SMRT,

instead of RNA pol II and co-activators (Kang, 2002; Kang, 2004). Moreover, several polymorphisms that occur within the *KLK3* promoter and its enhancer were associated with a different response to androgen stimulation. They could either increase or decrease the AR affinity to its AREs (Lawrence, 2010).

It was also reported that *KLKs* might be additionally regulated by a series of other factors, including progestin, thyroid hormone, retinoic acid, and vitamin D. Yet, the exact mechanism of the transcriptional regulation by these ligands for specific nuclear receptors of the whole KLK cluster is not yet known (Lawrence, 2010).

1.5.4. The role of KLKs in cancer

The expression of many KLKs is altered in hormonally regulated cancers. In general, the expression of these extracellular proteases was thought to be correlating with cancer progression, as they are capable of the degradation of the extracellular matrix (ECM), which is necessary for tumor metastasis and invasion. However, recent studies have shown that specific proteases might have anti-tumor properties. The over-expression of KLKs in cancer is linked with poor clinical prognosis. Nevertheless the increased level of some of the KLKs is associated with their role in cancer prevention (Lopez-Otin, 2007).

For instance, *KLK6* is silenced in metastatic breast cancer. Pampalakis *et al*. analyzed non-malignant vs. tumor breast cells and found that loss of *KLK6* is correlated with the presence of hypermethylation of specific CpGs located near the *KLK6* promoter, and with the deacetylation of histones that led to formation of condensed chromatin in cancer but not in non-malignant breast cells. Moreover, treatment with inhibitors of DNA methyltransferase and histone deacetylase, re-activated expression of the *KLK6* gene. This caused a recruitment of histone acetyltransferase CBP co-activator together with RNA pol II and TATA-binding protein (Pampalakis, 2009). Interestingly, re-expression of the KLK6 protein results in reduced proliferation and motility, and thus inhibits tumorigenicity in breast tumor cells. The potential substrates for KLK6 found after its reactivation include vimentin, a major mesenchymal marker of cancer aggressiveness, that was down-regulated in parallel with the up-regulation of the epithelial markers that are associated with tumor reversion, e.g. anti-angiogenic activity. In this way the KLK6 action shows a protective role against cancer development by inhibition of EMT (Pampalakis, 2009).

Additionally, a similar pattern of promoter hypermethylation and, as follows gene inhibition was observed for *KLK10*. This gene was also characterized by its anti-proliferative action, and was proposed to have a role as tumor suppressor in breast cancer (Borgono, 2004).

Furthermore, in breast cancer cells, hormonal steroids may induce the expression of multiple *KLKs* including *KLK2, -3, -8, -10, -11, 13-15.* The promoters of *KLK3*, *KLK10* and *KLK11* recruit AR upon androgen stimulation. *KLK5, -6, -10, -14* are up-regulated upon estrogen stimulation, though it is not clear whether these genes are ER-dependent since they are also expressed in ER-negative tumors (Lawrence, 2010; Luo, 2002). In addition, loss of *KLK10* in breast cancer is linked with poor prognosis and does not respond on the ER action (Paliouras, 2007).

Interestingly, the lack of KLK8 is correlated with the poor prognosis in patients with non-small cell lung cancer. KLK8 is able to repress cell invasiveness and motility through targeting and cleaving the fibronectin, a protein of the ECM responsible for integrin signaling. By that KLK8 may suppress tumor progression (Fortier, 2003).

Moreover, PSA (KLK3) known from its role as biomarker for screening, diagnosis and monitoring of prostate cancer, can promote metastasis from the prostate to the bone and may have immunosuppressive function that can result in inhibition of T cell proliferation. However, its putative functions are widely heterogeneous. KLK3 may regulate oxygen stability in cancer; enhance expression of androgen target genes in castrate resistant prostate cancer cells by binding to a specific co-activator. It can also cleave semenogelins that forms seminal clots and encases spermatozoa (Lawrence, 2010). Moreover, KLK3 together with KLK2, KLK4, KLK5, KLK11 and KLK14 are able to target the IGFBPs. The cleavage of those proteins through the protease activity of KLK proteases results in a reduced affinity for IGF leading to abnormal cell growth stimulation in malignant prostate cells (Borgono, 2004). Additional data shows that KLK3 and KLK4 participate in the EMT of the prostate cancer, i.e. the transformation of stable epithelial cells into migratory mesenchymal cells (Lawrence, 2007). Moreover, some KLKs can promote angiogenesis, by cleavage of ECM components or through the pathways, in which they are involved i.e. the kallikrein-kinin, urokinase plasminogen activator (uPA) and/or PARs pathways that facilitate tumor invasion and migration (Emami, 2007).

1.5.5. Signaling pathways and other functions

Since KLKs are characterized by various expression and activation profiles, they are believed to be involved in a variety of signaling pathways, as well as biological and physiological processes.

1.5.5.1. The kallikrein-kinin pathway

The best understood signaling cascade involving KLKs is the kallikrein-kinin pathway. Here, KLK1 and KLK2 can cleave the precursor called kininogen to release kinins, like bradykinin, via a specific KLK protein loop that contains kininogenase activity. Active kinin peptides mediate a series of actions by binding to their receptors. These G protein-coupled, transmembrane receptors induce the production of prostaglandins, nitric acids and other mediators that are involved in a wide range of processes including the regulation of blood pressure, inflammation, the stimulation of angiogenesis and the development of cardiovascular diseases (Costa-Neto, 2008).

1.5.5.2. The urokinase plasminogen activator (uPA)

Moreover, some KLKs like KLK2, KLK4, and KLK12 are able to activate uPA. This enzyme cleaves a variety of extracellular substrates like plasminogen into active plasmin. Later on, the plasmin proceeds to cleave and activate other pro-enzymes, like the pro-MMPs that are involved in ECM degradation and in the release and/or activation of tumor growth factors. Alternatively, uPA may activate some pro-MMPs directly (Borgono, 2004).

1.5.5.3. The PARs

KLKs are also activators of PARs that belong to the G protein-coupled receptor family and are activated by cleavage of part of their extracellular domain. Upon KLK1, KLK2, KLK4 – KLK6 and KLK14 cleavage, PARs initiates downstream signaling taking part in the inflammatory response as well as in cellular migration (Lawrence, 2010; Hollenberg, 2007).

1.5.5.4. Additional functional roles of KLK6

Besides its tumor suppressor function, KLK6 is also associated with the inflammation of the CNS and in MS. For instance, KLK6 is abundantly expressed in the normal CNS and altered levels of the KLK6 in the brain are correlated with neurological disorders including Alzheimer's and Parkinson's disease. Importantly, this protein is widely induced by inflammatory cells (T cells) in human brain at damaged myelin sheaths suggesting that KLK6 participate in the demyelination by the cleavage of myelin. Nevertheless, the physiological role of KLK6 in the brain is poorly understood (Blaber, 2004). Alzheimer's disease brain cells

reveal a low level of KLK6 compared to healthy control cells (Ashby, 2010). Moreover, KLK6 might have a role in the pathogenesis of Parkinson's disease, since it degrades α-synuclein, the major component of the so-called Lewy bodies, abnormal aggregates of proteins. Upon a stress signal KLK6 translocates from the mitochondria to the cytoplasm, where it cleaves α-synuclein, thereby preventing the accumulation of aggregates (Iwata, 2003; Sotiropoulou, 2009).

Intriguingly, *KLK6* expression may be modulated by vitamin D action. Up-regulation was seen in normal skin keratinocytes (Lu, 2005) as well as in colon cancer (Palmer, 2003). Also one VDRE located up-stream from the *KLK6* TSS was found in colon cancer (Wang, 2005). Yet, the exact function of its hormonal regulation is not well defined and need to be further explored.

2. OBJECTIVES

The main objective of my thesis was to explore in a broad approach the action of VDR in human prostate cells. Many studies have shown that VDR affects pathways crucial for proper cell and tissue function by regulating the expression levels of its target genes. It has been documented that many of these genes are fundamental to the immune system and to the regulation of proliferation and differentiation. As they were found to be deregulated across various tumor cells (reviewed by Deeb, 2007), an important role of the VDR in cancer prevention can be assumed. Therefore, additional investigations are required to fully understand the mechanisms underlying the effects elicited by $1\alpha,25(OH)_2D_3$. For that reason, the aims of my study were the following:

1. To identify primary and secondary VDR target genes on a transcriptome-wide level in human non-malignant prostate cells.

2. To compare the inducibility of a selected group of genes (*Kallikreins*) between human non-malignant and malignant cell lines.

3. To examine the effect of VDR suppression on the mRNA expression of *KLKs* in non-malignant prostate cells.

4. To correlate the results of *in silico* screening for putative VDR binding sites with the mRNA regulation of identified *KLK* genes.

5. To investigate the functionality of identified VDR binding sites within the genomic region of VDR-regulated *KLK* genes.

3. MATERIALS AND METHODS

3.1. Cell culture

Human immortalized non-malignant adherent epithelial prostate RWPE1 cells were derived from the peripheral zone of a histologically healthy 54-year old male's prostate and malignant RWPE2 cells were derived from RWPE1 cells via transformation with Ki-ras by the Kirsten murine sarcoma virus (Bello, 1997). Both cells were obtained from American Type Culture Collection (ATCC). Cells were cultured in Keratinocyte serum free medium (SFM) containing L-glutamine, 25 mg bovine pituitary extract, 2.5 μg human recombinant epidermal growth factor, and 100 U/ml of penicillin-streptomycin mixture. For mRNA and chromatin extractions cells were seeded in the culture medium except short-interfering RNA (siRNA) transfection experiments medium did not contain the antibiotics. PC3 human adherent from a bone metastasis prostatic adenocarcinoma cells from 62-year old male were cultured in RPMI medium containing 10% fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml penicillin-streptomycin mixture. DU145 human adherent brain-derived prostatic carcinoma cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml of penicillin-streptomycin mixture. Around 24h prior to treatment PC3 and DU145 cells were seeded into medium with lower (5%) charcoal-treated fetal bovine serum (FBS). All cells were maintained in a humidified 95% air / 5% CO_2 incubator. Cells were maintained according to recommendations from ATCC.

For all experiments cells were treated either with solvent (ethanol, with final concentration of 0.1%) or 100 nM $1\alpha,25(OH)_2D_3$.

3.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted using the Invisorb Spin Cell RNA Mini Kit (Invitek GmbH, Berlin, Germany) according to the manufacturer's instructions. cDNA was synthesized for 1h at 37 ^{O}C using 1 μg of total RNA as a template, 100 pmol oligodT_{15} primer and 40U of M-MuLV reverse transcriptase (Fermentas). The

reverse transcription was terminated by 10 min incubation at 70 ^{O}C. The cDNA was diluted 1:10 and served as a template for real-time quantitative PCR.

3.3. Microarray analysis

RNA samples quality was checked with BioRad Experion Automated Electrophoresis Systems (Biorad). Samples were analyzed with Human HT-12 Expression Beadchip Arrays from Illumina (San Diego, USA). The microarray data pre-processing and differential expression analysis were performed using the R statistical software, version 2.11 (R Development Core Team, 2011). Lumi package (Du, 2008) was used for processing raw data normalization. Data was normalized using VST transformation function, which was followed by RSN normalization used as a standard approach for Illumina arrays. In order to eliminate probes without detected signal normalized data was filtered. Probes that had a detection p-value < 0.05 were used in the statistical analysis using linear Models for Microarray Data (limma) package (Smyth, 2004) for detection of differentially expressed genes. The adjusted p-value corrected for multiple testing by the Benjamini–Hochberg method (Benjamini, 1995) was set to 0.01. Significantly differentially expressed genes for a selected time-point in response to $1\alpha,25(OH)_2D_3$-treatment were detected using the cut-off that was set for log2 fold change > 0.5 or less than < -0.5.

3.4. Real-time quantitative PCR

Real-time quantitative PCR was performed using iCycler (BioRad) with Absolute SYBR Green Fluorescein (Thermo Scientific) and in Applied Biosystems 7500 Fast Real-Time PCR System using Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific, Surrey UK). The PCR cycling conditions were as follows: polymerase activation time of 15 min at 95^{O}C, followed by 40 cycles performed with 30s at 95^{O}C, 30 s at primer specific temperatures, 30s at 72 ^{O}C and final elongation for 5 min at 72 ^{O}C. We performed 2 technical replicates per sample on a PCR plate.
Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is the $\Delta Ct_{(ligand)}$-$\Delta Ct_{(vehicle)}$, ΔCt is the $Ct_{(target\ gene)}$-$Ct_{(house\ keeping\ gene)}$ and Ct is the cycle at

which the threshold is crossed. Quality of the PCR product was monitored using post-PCR melt curve analysis. The values were normalized to acidic riboprotein *PO* (*RPLPO*) gene. The sequences of the primers are shown in Appendix I.

3.5. siRNA silencing

RWPE1 cells were transfected with non-specific control siRNA oligonucleotides or with specific for human siRNAs targeting the *VDR* or *CTCF*, by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The sequences of double-stranded siRNAs were obtained from Eurogentec. RWPE1 cells were seeded in 6-well plates in Keratinocyte serum free medium (SFM) containing L-glutamine, 25 mg bovine pituitary extract, 2.5 μg human recombinant epidermal growth factor, and 100 U/ml of penicillin-streptomycin mixture for 24 h. The specific siRNAs and control siRNAs were incubated with RNAiMAX in a total volume of 500 μl of antibiotic-free medium. The mixture was added to the cells and the transfection was continued for 48 h, and then cells were treated with 100 nM $1\alpha,25(OH)_2D_3$ or ethanol for 4 h and 24 h for cells transfected with siRNA against VDR and 4 h for cells transfected with siRNA against CTCF.

3.6. Chromatin immunoprecipitation (ChIP) assays

Cells were grown in 60 mm dish at confluence for 24 h. Next they were treated for 60 min with 100 nM $1\alpha,25(OH)_2D_3$. The nuclear proteins were cross-linked to genomic DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 5 min at room temperature. Cross-linking reaction was stopped by adding glycine to a final concentration of 125 mM and incubation for 5 min at room temperature. Next, medium was aspirated; cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and collected by scraping. After centrifugation pellet was resuspended in 600μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, protease inhibitors) and incubated on ice for 10 min. Next, chromatin was sonicated by a Bioruptor (Diagenode, Liege, Belgium) with 30 pulses of 30 s on and 30 s off to obtain DNA fragments of 300-700bp in length. Cellular debris was

removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl). Chromatin mixture was incubated overnight at 4^{O}C with rotation with 1 μg of antibodies against VDR (sc-1008X) or control IgG (sc-2027) and 5 μl H4Ac (06-866), CTCF (07-729). The immuno-complexes were collected with 60 μl of protein A agarose slurry (Millipore) for 2h at 4^{O}C with rotation. The beads were washed consecutively for 3 min on rotating platform with 1 ml of each solution: ChIP wash 1 (20 mM Tris-HCl pH 8, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl), ChIP wash 2 (20 mM Tris-HCl pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl), ChIP wash 3 (0.25 M LiCl, 1%NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8) and finally twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA pH8). For each wash, samples were incubated 3 min and then centrifuged for 1 min at room temperature with 400x g. The immuno-complexes were next eluted twice using 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature with rotation. Proteins from the obtained supernatant were digested overnight at 65 ^{O}C using proteinase K (Fermentas, final concentration = 40 μg/ml). Genomic DNA fragments were extracted using the ChIP DNA Clean & Concentrator^{TM} isolation kit (Zymo Research Cooperation, HiSS Diagnostics, Freiburg, Germany) according to manufacturer's instructions. For anti-VDR and anti-IgG antibodies collections of more than 50 immunoprecipitations were pooled in 40 μl nuclease-free water.

3.7. PCR of chromatin templates

Genomic primers for the *KLKs* TSSs and regions containing putative VDRE were designed and real-time PCR was performed with Absolute Blue qPCR SYBR low Rox Mix (Thermo Scientific) using 7500 Fast Real-Time PCR System (Applied Biosystems). The cycling conditions were: pre-incubation for 15 min at 95^{O}C, followed by amplification steps: 40 cycles of 30 s at 95^{O}C, 30 s at 60 ^{O}C, 30 s at 72 ^{O}C and final elongation for 10 min at 72 ^{O}C. Relative association of chromatin bound proteins or histone modifications were calculated using the formula $2^{-(\Delta Ct)}$, where ΔCt is the $Ct_{(output)}$-$Ct_{(input)}$. Output is the immuno-precipitated DNA and input is the purified DNA from the starting material of the ChIP assay. Relative fold changes were calculated against the control IgG.

3.8. RNA degradation rate experiment

RWPE1 cells were seeded in 6-well plates in Keratinocyte serum free medium (SFM) containing L-glutamine, 25 mg bovine pituitary extract, 2.5 μg human recombinant epidermal growth factor, and 100 U/ml of penicillin-streptomycin mixture for 24 h. Next, cells were treated with 100 nM $1\alpha,25(OH)_2D_3$ for 2h and then new RNA synthesis was blocked by treating the cells with Actinomycin D (final concentration: 10 μg/ml).

3.9. Gene annotation analysis

Genes were annotated according to Gene Ontology (GO) biological processes and enriched pathways analysis were determined by publicly available Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/), using default parameters with selected background as a reference (Huang, 2009). The performed statistics for GO was generated as expression analysis systematic explorer (EASE) score. Significant p-values and the false discovery rate (FDR) were less than 0.05 and 25%, respectively.

3.10. The *in silico* screening analysis

The identification of putative VDR binding sites was performed using publicly available JASPAR database, using default parameters (Sandelin, 2004). By employing the positional weight matrix (PWM) with cut-off of 80%, 30 putative binding sites were selected within KLK locus. Datasets from the ENCODE (The ENCODE Project Consortium, 2007) regulation tracks in the UCSC Genome Browser version hg18, were applied to detection of putative active regions within genomic KLK region. TSS marked by H3K4me3 and enhancers marked by H3K4me1 together with H3K27Ac were displayed from the Regulation Super-track. Chromatin boundary elements i.e. CTCF were displayed from Open Chromatin Track using HeLA cells. The sequence conservation profile across species was obtained from the UCSC Human Genome Browser, version hg18 (Kent, 2002).

4. RESULTS

4.1. Profiling of VDR target genes in non-malignant prostate cells

VDR is essential for the control of growth and differentiation in non-malignant and cancer cells from tissues such as skin, bone, prostate, breast, and more (Norman, 2010). However, the exact mechanism underlying the function of this nuclear receptor is continuously under investigations. VDR stimulation with $1\alpha,25(OH)_2D_3$ ligand increases cell differentiation, apoptosis and exhibits anti-angiogenic and anti-metastatic effects in a mouse model, whereas $1\alpha,25(OH)_2D_3$ deficiency may increase the risk of certain cancers (Deeb, 2007). After activation, VDR gains the abilities to mediate the inhibition of cell proliferation and promote cell cycle arrest. Hence, since VDR is expressed in non-malignant and malignant prostatic cells, it is considered to be an anti-proliferative agent and can be tested as a preventive factor against cancer development.

In order to identify potential target genes of VDR in human non-malignant epithelial prostate cells (RWPE1), a transcriptome-wide expression analysis using Illumina Human Expression BeadChips array version 3 was performed, following stimulation with 100 nM $1\alpha,25(OH)_2D_3$ and its solvent (EtOH) for 4 and 24 h (Fig. 14).

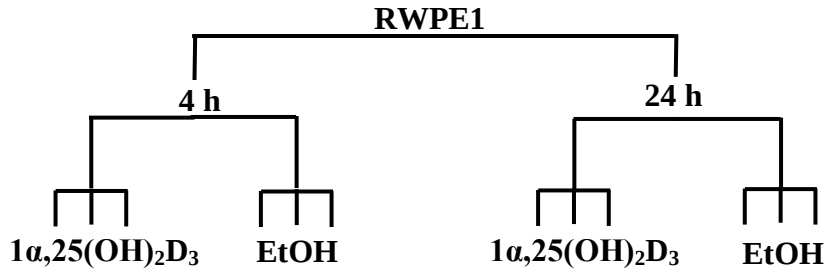


Figure 14. Experimental setup for microarray analysis
Human non-malignant epithelial prostate RWPE1 cells were treated with either solvent (EtOH) or 100 nM $1\alpha,25(OH)_2D_3$ for 4 and 24 h. Technical triplicates were performed for each time point. After incubation RNA was extracted and analyzed on the Illumina human Expression BeadChips arrays.

Based on the applied cut-offs set for logarithmic2 fold change (FC) > 0.5 or <-0.5 (equivalent to FC >1.4 or <0.7), statistical analysis revealed 87 regulated genes (p value (p) < 0.01) after 4 h of treatment, 76 of which were up-regulated and 11 were down-regulated (see Appendix II for the list of 87 early regulated genes). Interestingly, VDR activation significantly altered the expression of 663 target genes after 24 h of stimulation, with 467 induced and 196 repressed genes (see Appendix II for top 100 up- and down-regulated genes after 24 h of $1\alpha,25(OH)_2D_3$ stimulation).

Table 2. Number of genes significantly altered after 4 and 24 h of stimulation with $1\alpha,25(OH)_2D_3$ in RWPE1 cells

TIME	UP (P VAL < 0.01)	DOWN (P VAL< 0.01)	TOTAL
4 h	76	11	87
24 h	467	196	663

The top 15 genes that were most induced by $1\alpha,25(OH)_2D_3$ treatment compared to the solvent control are listed in table 3 and table 4.

Table 3. Top 15 genes significantly up-regulated after 4 h of stimulation with $1\alpha,25(OH)_2D_3$ in RWPE1 cells

GENE SYMBOL	GENE NAME	FOLD CHANGE
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	32.35
HAS3	hyaluronan synthase 3	5.27
CD14	CD14 molecule	4.35
IGFBP3	insulin-like growth factor binding protein 3	3.48
KLK6	kallikrein-related peptidase 6	3.34
PADI3	peptidyl arginine deiminase 3	3.25
C9ORF169	chromosome 9 open reading frame 169	3.11
FAM83A	family with sequence similarity 83 A	2.85
ANGPTL4	angiopoietin-like 4	2.83
PTGES	prostaglandin E synthase	2.77
SERPINB1	serpin peptidase inhibitor clade B (ovalbumin) 1	2.75
DHRS3	dehydrogenase/reductase (SDR family) 3	2.71
DUSP1	dual specificity phosphatase 1	2.45
ABCA1	ATP-binding cassette, sub-family A, 1	2.43
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	2.42

Table 4. Top 15 genes significantly up-regulated after 24 h of stimulation with 1α,25(OH)$_2$D$_3$ in RWPE1 cells

GENE SYMBOL	GENE NAME	FOLD CHANGE
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	60.21
KLK6	kallikrein-related peptidase 6	38.28
PADI3	peptidyl arginine deiminase 3	31.99
CD14	CD14 molecule	28.18
CA9	carbonic anhydrase 9	15.63
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin) 1	15.44
DHRS9	dehydrogenase/reductase (SDR family) 9	10.74
BAIAP2L2	BAI1-associated protein 2-like 2	9.09
TMEM91	transmembrane protein 91	7.51
TCEA2	transcription elongation factor A	7.00
IGFBP3	insulin-like growth factor binding protein 3	6.18
MGC102966	keratin 16 pseudogene 3	5.18
DUSP10	dual specificity phosphatase 10	5.18
CALB2	calbindin 2	5.13
KLK5	kallikrein-related peptidase 5	5.07

Comparing the top 15 genes up-regulated at the two time points of 1α,25(OH)$_2$D$_3$ stimulation we observed five genes that overlapped. The gene expression of *CYP24*, a well-known VDR target (Deeb, 2007), was the most elevated one at both time points. Moreover, the previously reported 1α,25(OH)$_2$D$_3$-dependent regulation of the expression of the VDR targets *IGFBP3*, *CD14*, *DUSP10* (Kovalenko, 2010) and *KLK6* (Lu, 2005) was also observed in our analysis.

Microarray data from non-malignant prostate cells stimulated with 1α,25(OH)$_2$D$_3$ demonstrated numbers of differentially regulated genes that can be considered as primary (4 h stimulation) or secondary (24 h stimulation) targets of VDR. In order to get an overview on the short- and long-term 1α,25(OH)$_2$D$_3$-dependent genes regulation, the VDR targets were segregated accordingly to the implemented fold change and statistical limits ($p<0.01$, $\log2FC>0.5$ or <-0.5). The expression levels of early responding (4 h) genes were sorted according to their regulation by 1α,25(OH)$_2$D$_3$ treatment after 24 h, as they are displayed on the heat maps, in Figure 15. This manner of sorting genes revealed different patterns regarding the VDR-dependent regulation.

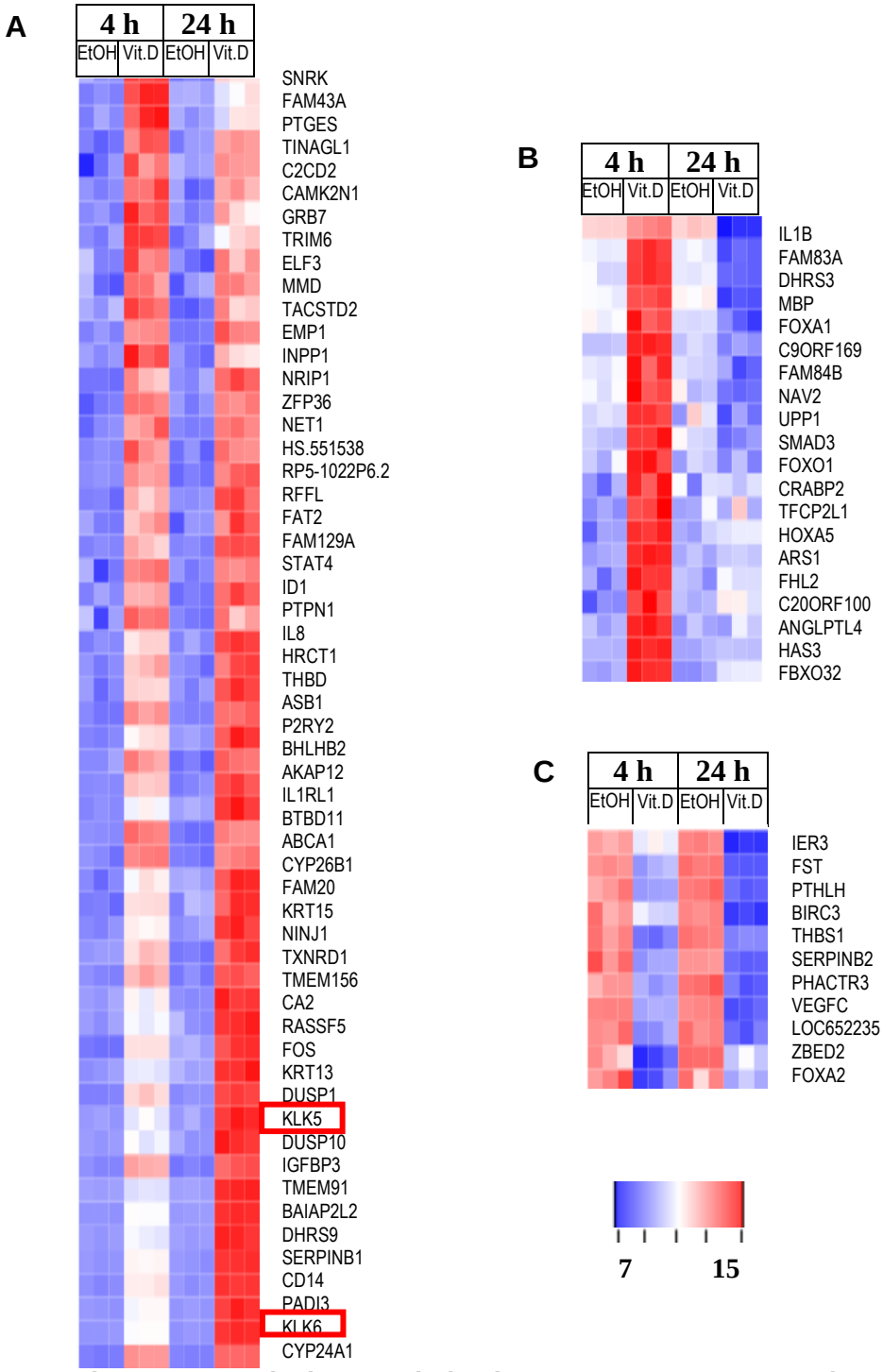
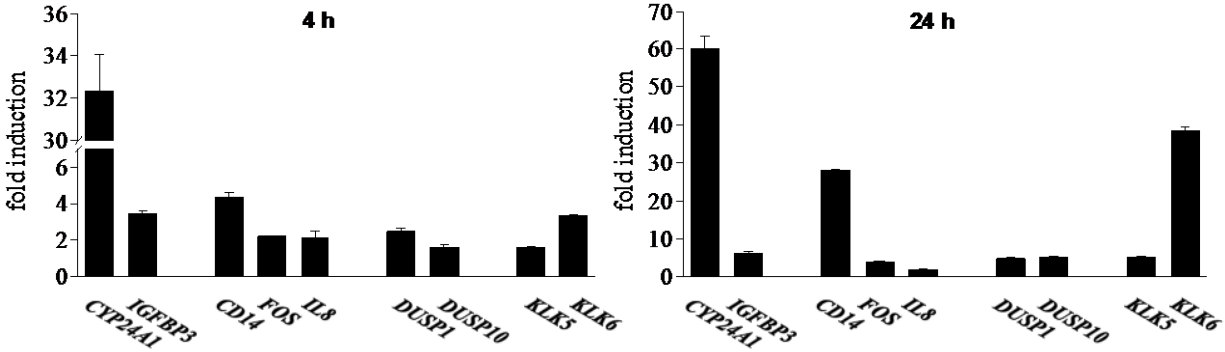


Figure 15. Microarray analysis revealed primary VDR target genes in RWPE1 cells. (A) Heat maps displaying the gene expression profile of differentially expressed genes according to applied cut offs ($p<0.01$, log2FC>0.5 or <-0.5). **(A)** Common up-regulated genes: 56 genes significantly up-regulated upon 4 h of $1\alpha,25(OH)_2D_3$ stimulation. Genes were sorted according to their induction after 24 h of treatment. (**B)** Oppositely regulated genes: depicted are 20 genes, that were significantly up-regulated after 4 h but down-regulated after 24 h of $1\alpha,25(OH)_2D_3$ treatment. **(C)** Common down-regulated genes: depicted are 11 significantly down-regulated genes upon 4 and 24 h of $1\alpha,25(OH)_2D_3$ stimulation. Red indicates up-regulation and blue down-regulation. *KLK5* and *KLK6* are highlighted as their regulation was later investigated in greater detail.

The microarray results showed that 56 genes were significantly up-regulated after 4 h of stimulation and remained induced after 24 h of treatment with VDR ligand (Fig. 15A). Interestingly, we found a set of 20 genes that was enhanced after 4 h but repressed after 24 h of stimulation. Among these genes were the *IL1B* and *SMAD3* genes (Fig. 15B). Moreover, 11 significantly down-regulated genes were identified after 4 and 24 h of treatment (Fig. 15C).

Interestingly, among top list of significantly early induced VDR targets there were genes involved in fast immune response (*CD14*, *FOS*, *IL8*) and members of two gene families i.e. *DUSP1* and *DUSP10* that are part of the dual specificity phosphatase (DUSP) family and members of the *KLK* family of genes, with *KLK6* being the second highest induced gene after 24 h of $1\alpha,25(OH)_2D_3$ stimulation (Figure 16A, B). Genes down-regulated at both time points were e.g. involved in angiogenesis (*THBS1*, *VEGFC*), with anti-apoptotic (*IER3*, *BIRC3*) or proliferative (*FST*) functions. Moreover, the *PTHLH* gene that encodes parathyroid hormone-like hormone protein, which increases activity of CYP27B1 that converts vitamin D into its active form, was decreased after $1\alpha,25(OH)_2D_3$ treatment (Fig. 16C, D).

UP-REGULATED GENES



DOWN-REGULATED GENES

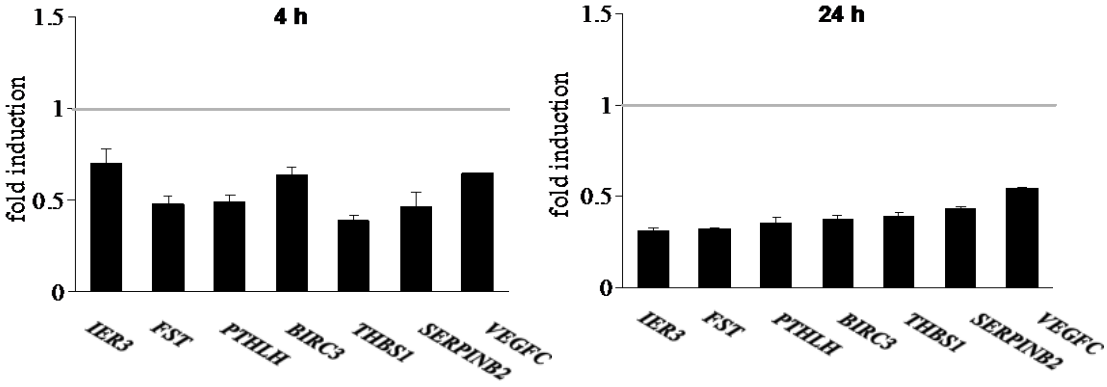


Figure 16. Representation of VDR-regulated genes in RWPE1 cells. The fold changes of significantly ($p<0.01$) regulated VDR target genes were obtained from the microarray study. The inducibility of selected genes after 4 (**A**) and 24 h (**B**) of stimulation with 1α,25(OH_2D_3 are shown in upper panel, whereas genes down-regulated after 4 (**C**) and 24 h (**D**) treatment with 1α,25(OH_2D_3 are shown in lower panel. A column represents the average of triplicates and a bars represents SD.

Several of the significantly up-regulated genes were linked to the function of immune response, which would correlate with the identification of prostate cells as early sensors of infections (De Marzo, 2007). Significantly down-regulated genes after 4 h of stimulation indicated pro-apoptotic, anti-angiogenic and anti-proliferative VDR functions crucial in cancer prevention.

4.2. Physiological role of VDR in non-malignant prostate cells

In order to understand the potential function of genes induced by VDR activation in non-malignant prostate cells, we identified overrepresented groups of genes that share common biological processes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang, 2009). For the analysis we selected significantly regulated ($p < 0.01$) genes both induced and repressed, from the array corresponding to 4 and 24 h of $1\alpha,25(OH)_2D_3$ treatment. Of the identified gene ontology (GO) terms some were matching to known VDR functions like "regulation of cell cycle", "defense response", "positive regulation of cell differentiation", and "regulation of apoptosis". The list of canonical biological processes in which VDR plays an important role is presented in the Table 5. The genes from each GO term were divided into two groups corresponding to the ones up- and down-regulated by $1\alpha,25(OH)_2D_3$ in prostate cells. In addition, using the DAVID database we selected the pathways defined from Kyoto Encyclopedia of Genes and Genomes (KEGG) in which VDR target genes may be also involved. One of the most enriched groups was the "p53 signaling pathway" known from its role in cell cycle arrest and apoptosis. Also, the "arachidonic acid metabolism" pathway taking part in prostaglandin production, which may affects processes like cellular proliferation and inflammation was enriched. Interestingly, we could also assign significantly VDR-regulated genes to the "cancer pathway" where 22 genes were up-regulated and 9 gene were down-regulated after 4 or 24 h of $1\alpha,25(OH)_2D_3$ stimulation. Among the significantly induced genes we distinguished tumor suppressors (*RASSF5, RAC2* and *CDH1*) and transcription factors that control proliferation (*JUN, FOS*) or respond to hypoxia like *HIF1A*. The list of down-regulated genes contained the ones encoding angiogenic factor VEGFC and HSP90AA2, which is required for its activation. Moreover, we detected inhibitors of apoptosis (*BIRC3, BIRC5*) as well as transcription factors which, when over-expressed, have been associated with tumorigenesis (*ETS1, MYC*) (Table 6).

These results indicate an important role of VDR in controlling the expression of genes involved in "cancer pathways".

Table 5. Enrichment analysis of the transcriptome profile of VDR-dependent genes in its canonical biological processes.

ID	GO TERM	No. of genes regula-ted by VDR	GENES		P Value	FDR
			Up-regulated	Down-regulated		
GO:0051726	regulation of cell cycle	42	*BCL6, SERTAD1, SMAD3, BMP2, c13orf15, CCNB1, CCND2, CDKN1A, GADD45A, ID3, IL1A, IL1B, JUNB, JUN, NGF, PTGS2, PRKCQ, SPHK1*	*BCCIP, CKS1B, CKS2, MAD2L1, NEK2, SMAD3, TTK, ASNS, BIRC5, BUB1, CAV2, CDK1, CDC25C, CENPE, CENPF, CCNA2, CCNB1, CCND2, CDKN3, DLGAP5, HERC5, IL1A, IL1B, KIF20B, PLK4, PTPRK, UBE2C, ETS1, MYC*	1.12E-07	2.00E-04
GO:0006952	defense response	32	*BNIP3, BNIP3L, CEBPB, CD14, CD24L4, CD97, ELF3, ALOX5, BMP2, BMP6, CAMP, HIF1A, IL1RL1, IL1A, IL1B, IL8, MGLL, NGF, PRDX5, PTAFR, PDPN, TPST1, FOS*	*CD83, ELF3, CCL20, CCL5, F2R, F3, CYP27B1, GAL, LOC100131909, IL1A, IL1B, THBS1*	3.05E-04	5.44E-01
GO:0045597	positive regulation of cell differentiation	20	*SH3PXD2B, SMAD3, BMP2, BMP6, CA2, CCNE1, FOXA1, HIF1A, IGFBP3, JUNB, JUN, NGF, PLA2G10*	*CD83, SMAD3, CCL5, CYP27B1, FOXA1, FOXA2, IL7R, ETS1, VEGFC*	5.02E-04	8.94E-01
GO:0042981	regulation of apoptosis	52	*BCL6, BOK, BNIP3, BNIP3L, BTG2, CEBPB, CD24L4, FGD3, PERP, PYCARD, ARHGEF16, SMAD3, ACTN4, ABR, ALDH1A3, ANGPTL4, CDH1, CLCF1, CIDEA, CDKN1A, DUSP1, FOXO1, ID3, IGFBP3, IL1A, IL1B, JUN, NGF, NET1, NFKBIA, PRDX5, PHLDA3, PTGS2, SPHK1, TP53I3*	*SMAD3, ANGPTL4, ASNS, BIRC3, BIRC5, CDK1, F2R, F3, GAL, IER3, IL1A, IL1B, JAG2, PMAIP1, PHLDA1, SERPINB2, SOCS2, THBS1, TNFRSF6B, MYC*	8.46E-03	1.41E+01

Table 6. Enrichment analysis of VDR-dependent genes involved in the cancer pathway.

P value	GENES				FDR
	Up-regulated		Down-regulated		
	ID	Name	ID	Name	
4.33E-03	*JUN*	jun oncogene	*BIRC5*	baculoviral IAP repeat-containing 5	5.05E+00
	CSF1R	colony stimulating factor 1 receptor	*BIRC3*	baculoviral IAP repeat-containing 3	
	EPAS1	endothelial PAS domain protein 1	*HSP90AA2*	heat shock protein 90kDa alpha (cytosolic), class A 2	
	CDKN1A	cyclin-dependent kinase inhibitor 1A	*VEGFC*	vascular endothelial growth factor C	
	RASSF5	Ras association (RalGDS/AF-6) domain family member 5	*PLCG2*	phospholipase C, gamma 2	
	SLC2A1	solute carrier family 2 (facilitated glucose transporter) 1	*SMAD3*	SMAD family member 3	
	IL8	interleukin 8	*ETS1*	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	
	RAC2	ras-related C3 botulinum toxin substrate 2	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	
	PDGFA	platelet-derived growth factor alpha polypeptide	*MYC*	v-myc myelocytomatosis viral oncogene homolog (avian)	
	FOXO1	forkhead box O1			
	PTGS2	prostaglandin-endoperoxide synthase 2			
	HIF1A	hypoxia inducible factor 1, alpha			
	BMP2	bone morphogenetic protein 2			
	FGFR3	fibroblast growth factor receptor 3			
	FGF11	fibroblast growth factor 11			
	LAMB3	laminin, beta 3			
	SMAD3	SMAD family member 3			
	MAPK3	mitogen-activated protein kinase 3			
	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog			
	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha			
	CCNE1	cyclin E1			
	CDH1	cadherin 1, type 1, E-cadherin (epithelial)			

Taken together, we found that the significantly regulated genes after 4 or 24 h of VDR ligand stimulation are enriched in various biological processes that indicate pro-differentiative, pro-apoptotic, and anti-proliferative function of VDR in non-malignant prostate cells.

4.3. Regulation of the *KLK* genes in non-malignant and malignant prostate epithelial cells by VDR

The microarray analysis revealed that upon 1α,25$(OH)_2D_3$ treatment, the second most responsive and up-regulated gene after *CYP24A1*, was *KLK6*. Interestingly, eight members of the *KLK* gene family (*KLK3, KLK5, KLK6* and *KLK7-11)* were found to be expressed in RWPE1 cells (Fig.17). The most prominent ones were *KLK5* and *KLK6*, while other *KLKs* showed a similar modest inducibility profile. Stimulation with 1α,25$(OH)_2D_3$ for 4 h led to up-regulation of *KLK5* with more than 1.5-fold induction and of *KLK6* with more than 3-fold induction. After 24 h of 1α,25$(OH)_2D_3$ treatment we noticed that *KLK5* and *KLK6* were induced over 5-fold and more than 38-fold, respectively. In addition, the *KLK7* gene was ~1.4-fold up-regulated, the *KLK8* gene ~1.4-fold, and the *KLK9* gene ~1.4-fold. A similar observation was previously published for keratinocyte cells where it was shown that *KLK5-8, -10* and *-13* were up-regulated by 1α,25$(OH)_2D_3$ stimulation (Lu, 2005).

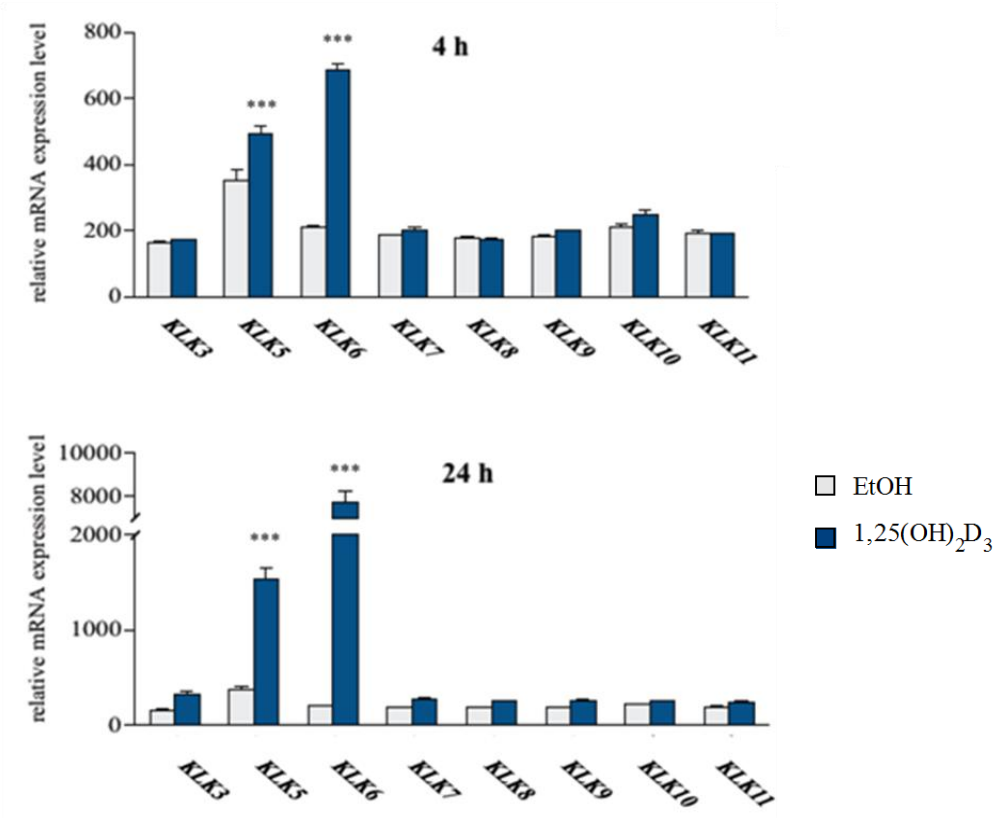


Figure 17. Microarray analysis identified several 1α,25$(OH)_2D_3$-responsive genes from the kallikrein family. The relative expression of the *KLK* family genes after 4 and 24 h of 1α,25$(OH)_2D_3$ stimulation detected in the microarray study shows their inducibility by VDR activity. Each bar represents the averages and indicates SD of triplicates.

The fact that several members from the kallikrein family were strongly up-regulated and that these genes are located next to each other within the central region of the cluster on chromosome 19, can indicate a common transcriptional gene regulation.

In order to validate the VDR-dependent up-regulation of the *KLK* genes that were identified as VDR targets by the microarray data, a series of real-time quantitative PCR (qPCR) experiments was performed for different prostate cell lines.

First, we tested the responsiveness of *KLK* genes to VDR ligand stimulation in the non-malignant prostate cell line RWPE1 that was also used for the microarray experiment. The cells were treated for 2, 4, 6, or 24 h with 1α,25$(OH)_2D_3$ or with its solvent control (EtOH). Expression of the *KLK* genes was measured in relation to the control gene acidic riboprotein PO (*RPLPO*). The *bona fide* VDR target gene *CYP24* served as a positive control and showed that VDR was activated at each time point. We observed significant up-regulation of five *KLKs*, *KLK5, -6, -7, -8* and *-9* (Fig. 18A). The most prominent induction was observed for the expression of *KLK6* with a more than 5-fold change already after 2 h, which was followed by an increasing expression, up to 160-fold after 24 h of treatment. *KLK5* and *KLK7* were induced over 2-fold after 4 h and more than 7-fold after 24 h, whereas *KLK8* and *KLK9* were modestly up-regulated at 6 h and more than 3-fold induced after 24 h (Fig. 18A). Next, we tested whether the central region of the *KLK* locus would also be a VDR target in human malignant prostate cell lines such as RWPE2, PC3, and DU145. Therefore, we treated these cancer cell lines for 2, 4, 6, or 24 h with 1α,25$(OH)_2D_3$ or solvent. We observed that the effect of VDR ligand on the *KLK* locus in malignant cell lines was only slight and not significant in the PC3 cells and was even absent in DU145 cells (Fig. 18C and D). In contrast, in RWPE2 cells a significant, but delayed induction of the *KLK* genes could be observed (Fig. 18B).

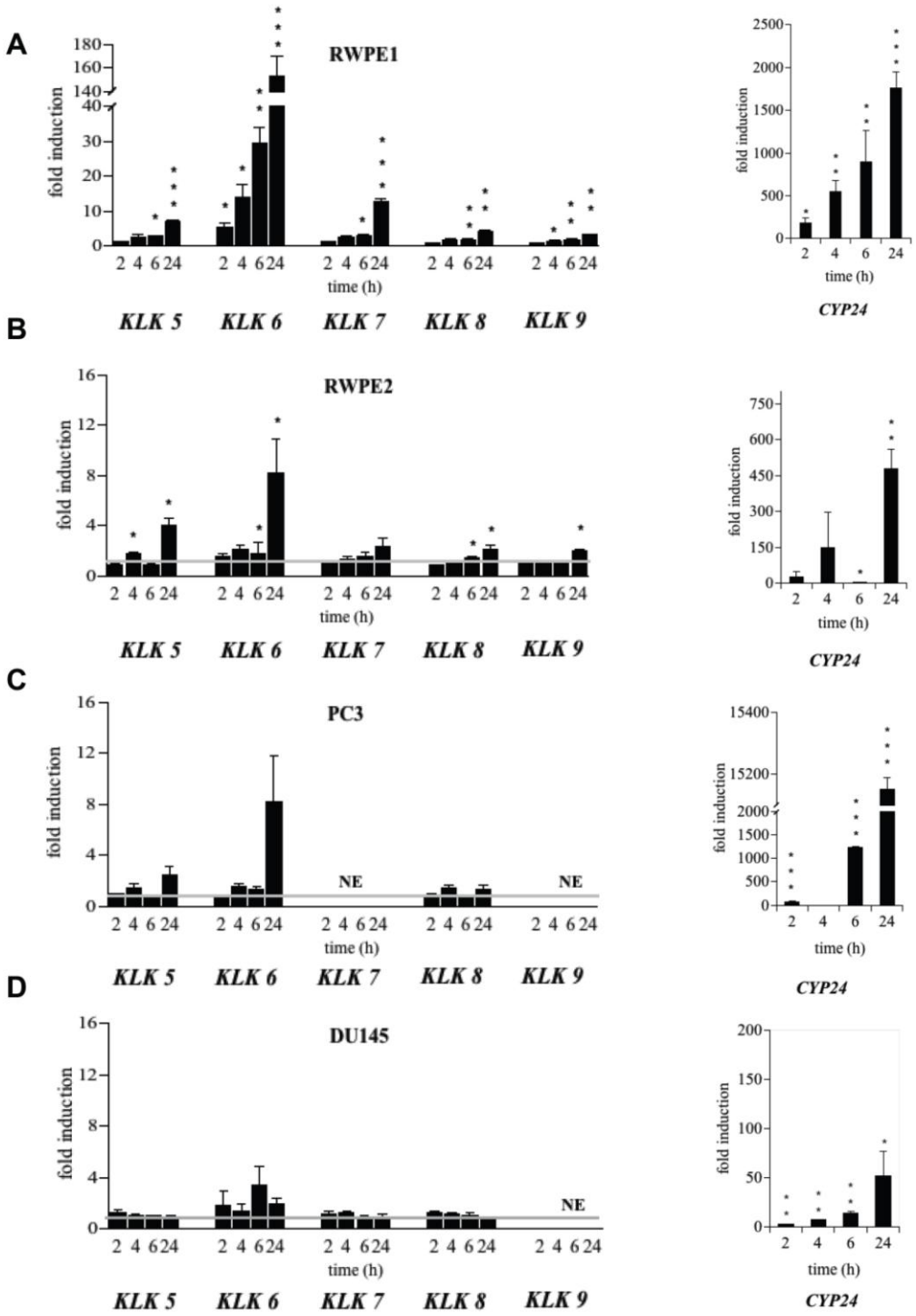


Figure 18. Several members of the *KLK* gene family are responsive to 1α,25(OH)$_2$D$_3$ stimulation in prostate cells and exhibit a distinct profile in cancer cell lines. The mRNA expression profiles of the *KLK* genes in the non-malignant RWPE1 cell line (**A**) and in malignant cell lines RWPE2 (**B**), PC3 (**C**) and DU145 (**D**) were determined by qPCR. The *KLK*s that showed a significant response in at least one cell line are shown. All cells were stimulated with 1α,25(OH)$_2$D$_3$ or with solvent control (EtOH). Values were normalized to the *RPLPO* control gene. The fold inductions of 1α,25(OH)$_2$D$_3$ treatment relative to solvent control (EtOH) over a 4-time point series are displayed with significance, as calculated using a paired student's t-test from three independent experiments, each performed in triplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and the error bars indicate SEM. The known VDR target gene *CYP24* served as a positive control.

Comparing the different cell lines, the non-malignant RWPE1 cell line was clearly most responsive to $1\alpha,25(OH)_2D_3$. It is possible that an already high basal expression of the *KLK*s could explain the low inducibility by VDR in the cancerous prostate cell lines. Surprisingly, the basal expression was reduced for most of the *KLK* genes in the cancer cell lines compared to the non-malignant prostate cell line. In the non-malignant RWPE1 cells *KLK8* was the highest, while *KLK7* was the lowest expressed *KLK* gene. The relative expression of *KLK6* was comparable in all types of prostate cells (Fig. 19). Therefore the capacity of VDR activation to induce the *KLK* genes is not correlating to the basal expression of these genes.

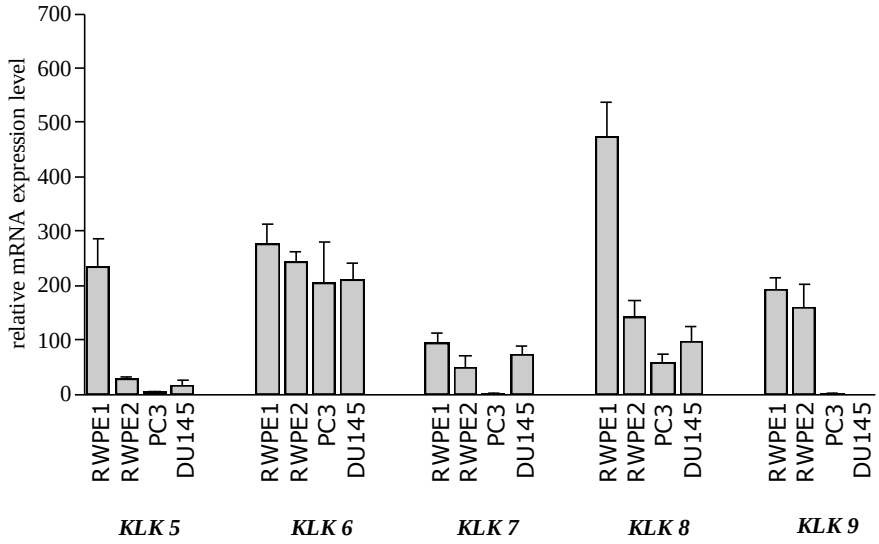


Figure 19. The relative mRNA expression level of *KLKs* across four cell lines. The relative mRNA expression of the *KLKs* (shown as separate panels) was quantified by qPCR in relation to *RPLP0* from the non-malignant RWPE1 cell line and from the malignant prostate cell lines RWPE2, PC3 and DU145. Data points show the means of three independent experiments, each performed in triplicate and the error bars indicate SEM.

In order to investigate whether the reduced inducibility of VDR target genes in cancer cell lines relative to non-malignant prostate cells is confined to *KLKs*, we analyzed the expression of another *bona fide* VDR target, *IGFBP3*, in these cell lines. *IGFBP3* was highly induced in non-malignant prostate RWPE1 cells, whereas in the malignant RWEP2 cells its up-regulation was delayed and less pronounced (Fig. 20).

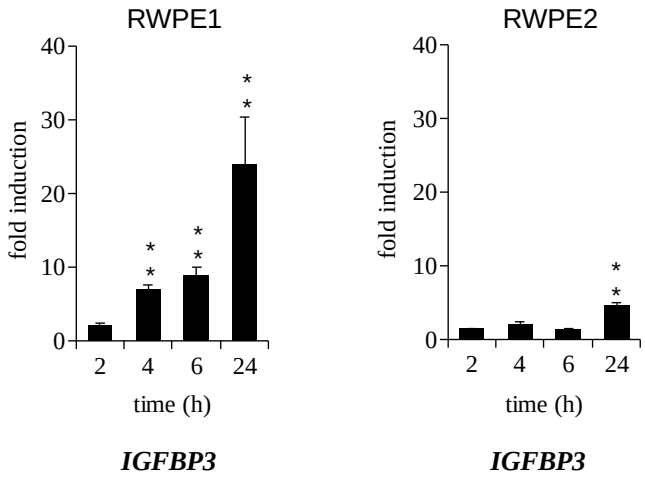


Figure 20. *IGFBP3* expression in both non-malignant and cancer prostate cells after treatment with 1α,25(OH)$_2$D$_3$. Real-time quantitative PCR was performed to analyze the mRNA expression of *IGFBP3* relative to the housekeeping *RPLPO* gene in RWPE1 cells after treatment with 1α,25(OH)$_2$D$_3$; the fold inductions were determined in relation to solvent control (EtOH). Data points show the means of three independent experiments, each performed in triplicate and the error bars indicate SEM. Paired student's t-test was performed to specify the significance of ligand-dependent regulation of *IGFBP3* gene (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In summary, the mRNAs of five *KLKs* genes were higher expressed and regulated by VDR in non-malignant cells, whereas the prostate cancer cells showed only minor VDR-mediated effects. Moreover, the mRNA level of *KLK6* was the highest induced one compared to the other *KLKs* in RWPE1 cells. The early induction profile observed suggests a direct regulation of *KLK* genes by ligand-bound VDR.

4.4. Effect of VDR silencing on the central region of the *KLK* gene family

In order to exclude that the regulation of the *KLK* gene family expression is due to an unspecific ligand effect and to verify that the up-regulation of the genes is dependent on the presence of VDR, we performed RNA interference experiments in RWPE1 cells. Cells were transfected with siRNAs against *VDR* or control siRNA for 48 h, which was followed by 4 or 24 h treatment with 1α,25(OH)$_2$D$_3$ or solvent. The successful significant knockdown of around 80 % of *VDR* mRNA, compared to unspecific control siRNAs as determined by qPCR (Fig. 21A), was confirmed by the reduced induction of mRNA expression of the best-known VDR target gene *CYP24*.

We noticed that the effective silencing of *VDR* led to a ~90% reduced induction of *CYP24* after 4 and ~70% after 24 h with 1α,25(OH)$_2$D$_3$ stimulation (Fig. 21B).

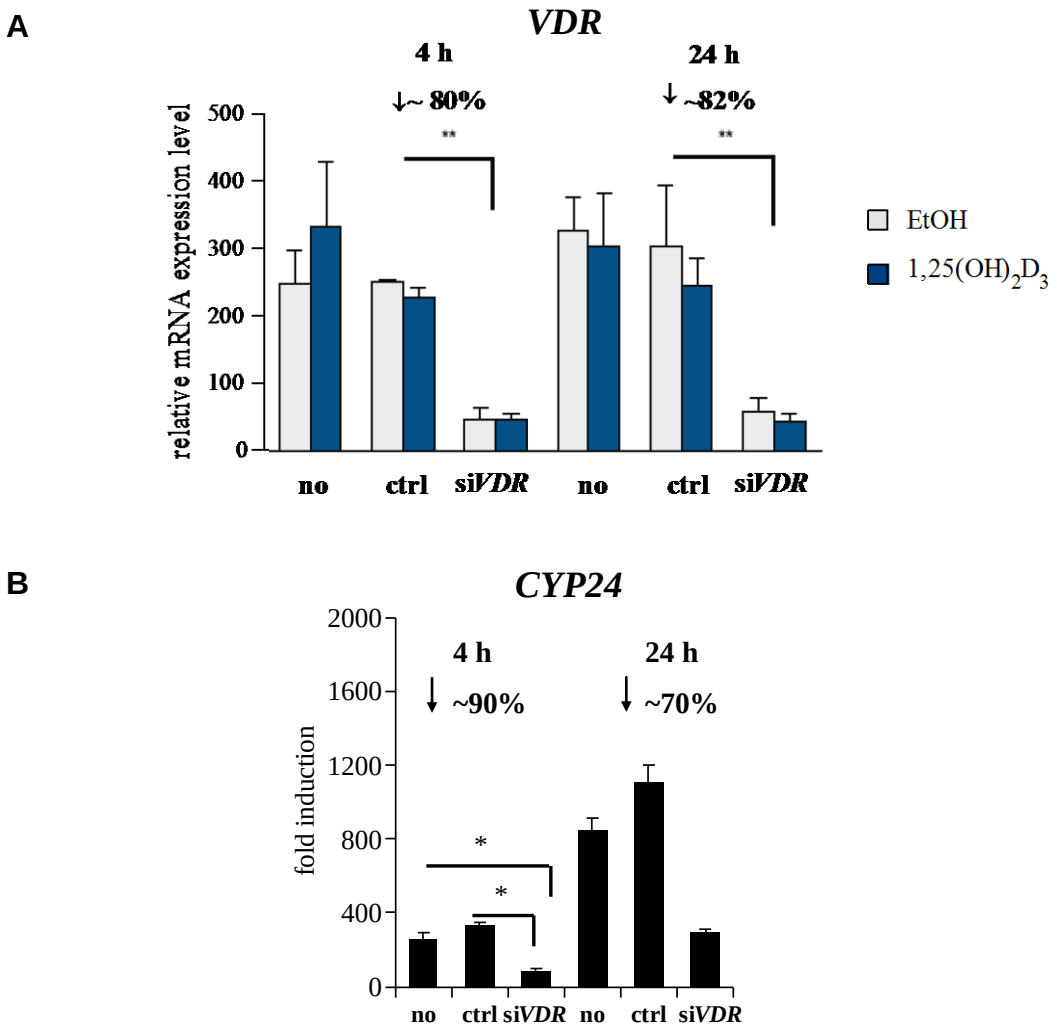


Figure 21. *VDR* silencing reduces the inducibility of *CYP24*. RWPE1 cells transfected for 48 h with specific siRNA oligonucleotides against *VDR* or unspecific siRNA were stimulated with 1α,25(OH)$_2$D$_3$ for 4 and 24 h to determine (**A**) the effect of VDR silencing. The known VDR target gene (**B**) *CYP24* serves as a positive control for the effectiveness of *VDR* depletion. The mRNA expression of both genes relative to the control gene *RPLPO* was determined by qPCR. The *CYP24* fold induction was determined in relation to solvent control (EtOH). Data points show the means of three independent experiments, each performed in triplicate and the error bars indicate SEM. Paired student's t-test was performed to specify the significance of ligand-dependent regulation of *CYP24* gene (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Next, we tested whether the *VDR* knockdown has an effect on induction of *KLK* genes by VDR ligand treatment. After 4 h of stimulation with 1α,25(OH)$_2$D$_3$ approximately 90% of the most responsive *KLK6* gene expression was abolished and subsequently after 24 h of stimulation it was reduced about 74%. The decreased expression affected the induction of genes, which is presented on Figure 22.

Furthermore, the *VDR* silencing diminished the *KLK5* gene inducibility after 4 h of 1α,25(OH)$_2$D$_3$ stimulation and decreased the mRNA expression by 45%, and the ones of *KLK7* by ~72%, of *KLK8* gene by ~49% and of *KLK9* by ~60%, respectively (Fig. 22A). This effect was maintained or even increased after 24 h of VDR ligand incubation (Fig. 22B).

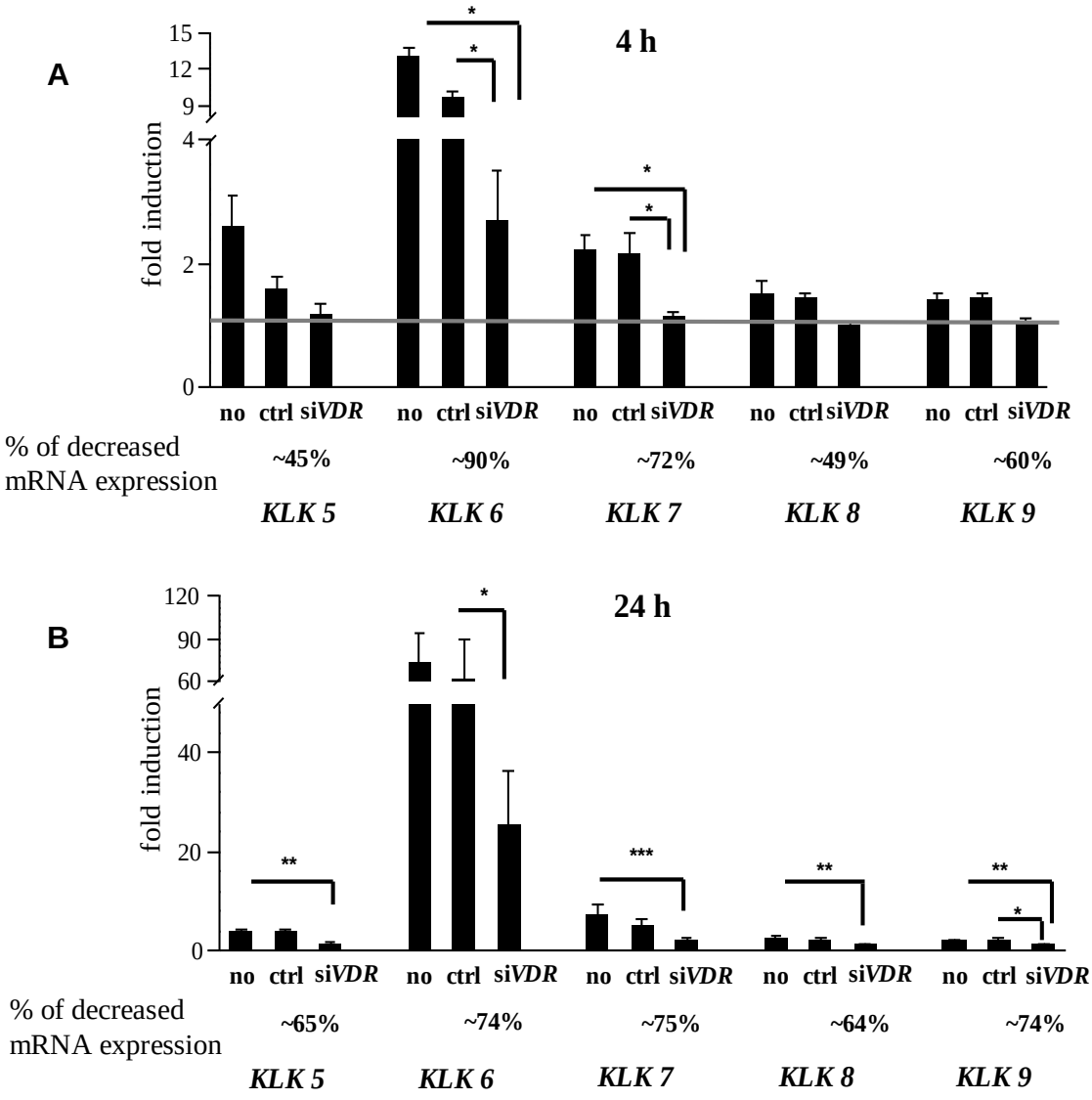


Figure 22. Effect of *VDR* depletion on the induction of the 1α,25(OH)$_2$D$_3$-responsive *KLKs.* To determine the impact of *VDR* knockdown on the *KLK* genes expression in RWPE1 cells, qPCR was performed and the data were analyzed in relation to the control *RPLPO* gene. The induction profiles of the responsive *KLK* genes after (**A**) 4 h and (**B**) 24 h of 1α,25(OH)$_2$D$_3$ treatment are shown. The fold change of *KLK genes* was determined in relation to solvent control (EtOH). Data points show the means of three independent experiments, each performed in triplicate and the error bars represent SEM. Paired student's t-test was performed to determine the significance for the siVDR silencing effect on the ligand-inducibility in relation to unspecific control siRNA (ctrl) or not transfected (no) samples (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The % represents the reduced gene expression level after *VDR* knockdown and it was calculated in relation to the ctrl siRNA.

Taken together, the silencing of VDR confirms the receptor-dependent up-regulation of the *KLK5, 6, 7, 8, 9* genes by 1α,25$(OH)_2D_3$ in human non-malignant prostate cells.

4.5. The early VDR-dependent, dynamic response of *KLK6* gene expression in non-malignant prostate cell line

In order to investigate how fast mRNA of the *KLK6* is produced after VDR activation, for the subsequent analysis of the expression of the *KLK6* gene in response to 1α,25$(OH)_2D_3$, we performed a detailed time course experiment with RWPE1 cells. Therefore, mRNA was isolated every 15 min up to 285 min and the levels of *KLK6* and *CYP24* genes were determined by qPCR. *CYP24* showed significant induction already after 45 min of treatment. Level of induction was higher than 2-fold (Fig. 23B). Interestingly, also the expression of *KLK6* changed very early and was doubled already after 45 min of 1α,25$(OH)_2D_3$ treatment and significantly increased after 75 min, suggesting a direct regulation of *KLK6* by VDR. The gene expression increased constantly with the time in a linear fashion, while after 285 min an induction of 25-fold was achieved (Fig. 23B).

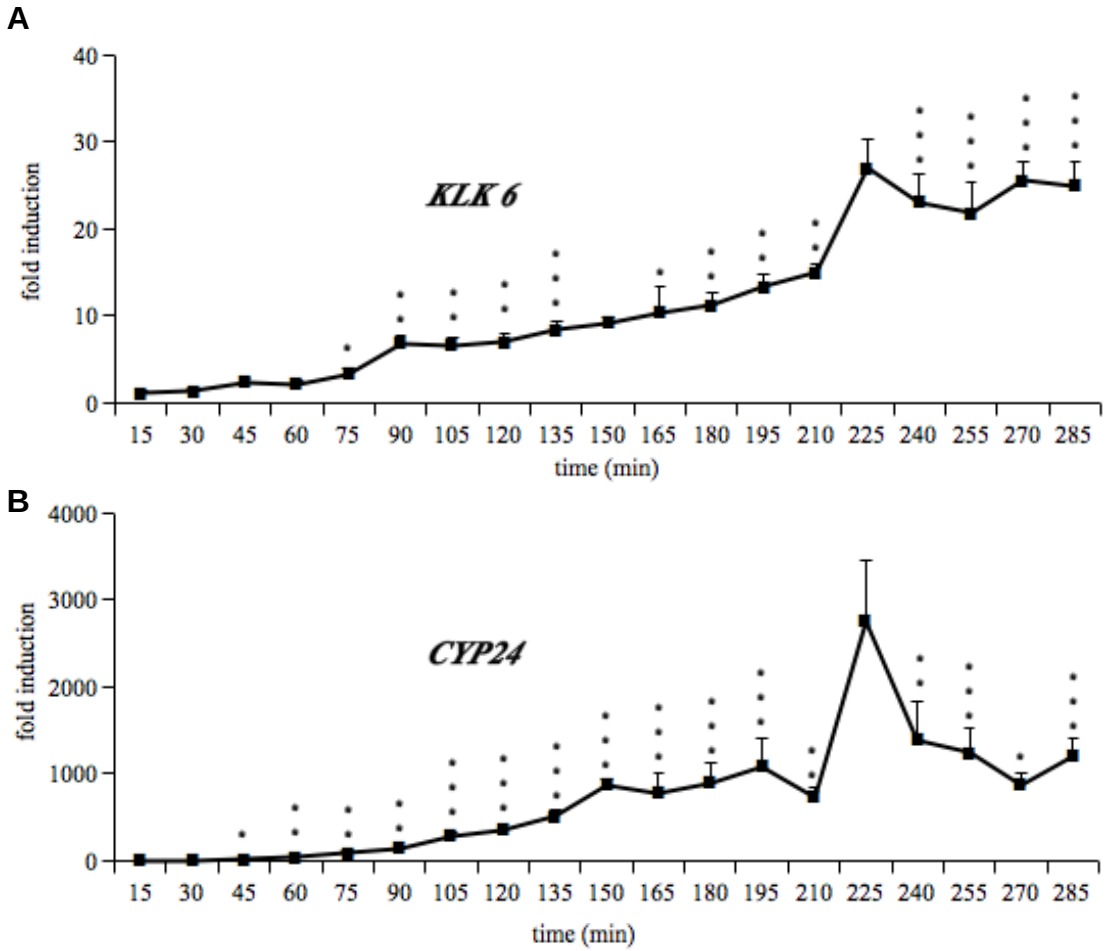


Figure 23. The early dynamic response of the *KLK6* gene. The induction of the genes *KLK6* (**A**) and *CYP24* (**B**) was measured by qPCR from an extended time series after stimulation of RWPE1 cells with 1α,25(OH)$_2$D$_3$. The mRNA expression of *KLK6* was calculated relative to the housekeeping *RPLPO* gene. The fold inductions compared to control (EtOH) stimulated cells are displayed with significance using a two-tailed students t-test from three independent experiments as shown in the figure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The mRNA levels may depend on regulation of their decay rate and altered RNA production can activate the process of its degradation and thus may prevent its translation into a functional protein (Doma, 2007). Therefore, in order to analyze whether VDR-dependent, highly induced mRNA expression of *KLK6* is stable, after analyzing the mRNA synthesis of this gene upon 1α,25(OH)$_2$D$_3$ stimulation we looked on the degradation of *KLK6* mRNA. To determine the stability of *KLK6's* mRNA non-malignant prostate cells were stimulated with 1α,25(OH)$_2$D$_3$ or with EtOH for 2 h. This was followed up by incubation with a transcriptional inhibitor that blocks RNA Pol II activity, actinomycin D, for 2, 3, 4, 5, 6 h, respectively, and mRNA levels were measured by qPCR. We observed that the *KLK6* expression level remained unchanged upon actinomycin D treatment at selected time intervals, both in

solvent-and 1α,25(OH)$_2$D$_3$-treated samples (Fig. 24A). As a positive control for the actinomycin D treatment we also tested the stability of the *IL8* gene. IL8 is a fast response immune cytokine and its mRNA is quickly degraded. In the microarray this gene was more than 2-fold induced at 4 h by 1α,25(OH)$_2$D$_3$. Already after 2 h of actinomycin D treatment (Fig. 24B) the mRNA of *IL8* was reduced in both, the EtOH and 1α,25(OH)$_2$D$_3$-treated samples. Therefore we can conclude that the mRNA of *KLK6* is a stable mRNA, giving a possible explanation of the consistent accumulation of *KLK6* mRNA in the long-term 1α,25(OH)$_2$D$_3$ treatment.

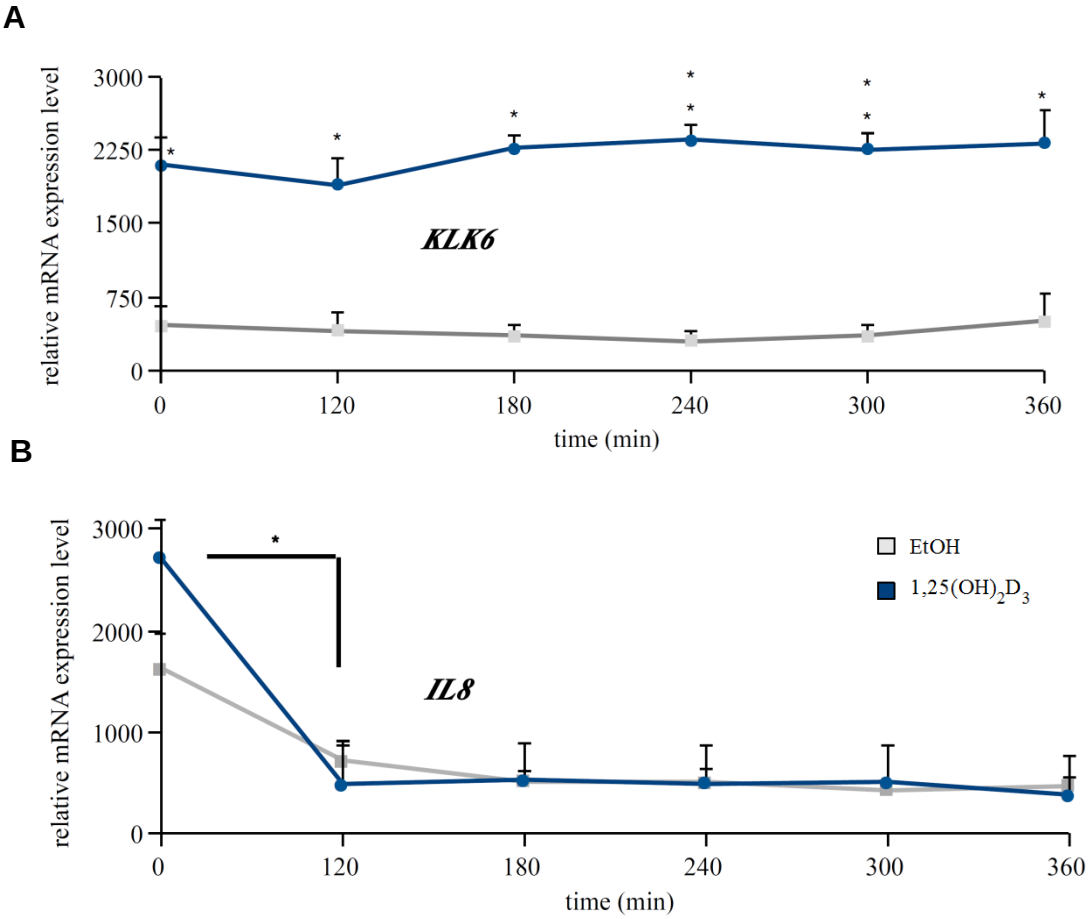


Figure 24. mRNA stability of *KLK6* gene. (**A**) Measurement of the *KLK6* mRNA stability was performed for RWPE1 cells stimulated for 2 h with 1α,25(OH)$_2$D$_3$ or solvent control (ETOH) followed by treatment with 1 μM actinomycin D for different periods of time. The level of *KLK6* mRNA normalized to the control gene *RPLPO* was determined by qPCR. Data points show the means of three independent experiments and the error bars indicate SEM. A two-tailed student's t-test was performed to specify the significance of ligand-dependent regulation of *KLK6* gene compared to ETOH control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The mRNA expression of (**B**) *IL8* gene served as a reference.

Taken together, *KLK6* was rapidly and strongly induced by the VDR ligand in the RWPE1 cell line, indicating that *KLK6* is a direct target gene of VDR. Moreover, we showed that the mRNA of *KLK6* was stable for more than 5 h, indicating that this gene has long-term rather than short-term functions.

4.6. Identification of putative binding sites for VDR within the human *KLK* cluster

In order to investigate potential VDREs one can use *in silico* screening to predict the affinity of the binding of VDR to DNA. Several programs based on biological data have been established and are available online, e.g. JASPAR (www.jaspar.cgb.ki.se). According to the specificity of VDR for its response elements, these algorithms screen the genome based on alignments of known binding sites. Nevertheless, the most accurate method to locate NR regulatory elements are *in vitro* assays like the chromatin immunoprecipitation assay (ChIP), combined with *in silico* analysis (Meyer, 2010). The ChIP technique enables to cross-link the protein of interest (e.g. VDR) directly to the associated chromatin using formaldehyde; the protein-chromatin complex is subjected to fragmentation of the DNA to fragments of 300-1000 base pairs and the protein of interest is then selectively immunoprecipitated. The associated DNA fragments are then purified and serve for further quantitative analysis. Since the *in silico* screening methods deliver putative binding sites and *in vitro* studies verify them, it is important to combine these two methods as they complement one another. In this way it is possible to discover VDREs across the whole genome in a cell-specific manner.

4.6.1. *In silico* screening for putative VDREs in the human *KLK* cluster based on datasets tracks

The analysis of mRNA expression levels of five of the *KLKs* after stimulation with 1α,25$(OH)_2D_3$ in RWPE1 cells showed their regulation by VDR. Especially the rapid up-regulation of *KLK6* gene that was noticeable already after 45 min of treatment, which suggested that they are likely to be direct target genes for VDR. Thus, our results implied that there should be VDR response elements (RE) within the regulatory regions of the *KLK* genes. In order to get an overview of the possible functional elements in *KLK* locus we analyzed different datasets from the ENCODE

regulation tracks in the UCSC Genome Browser (Kent, 2002) through utilization of selected ENCODE tracks that represent biological data from 8 different cell lines (The ENCODE Project Consortium, 2007). To investigate the open chromatin regions we have chosen three "open" chromatin markers: H3K4me3 associated with transcriptional initiation that is found near TSSs and H3K4me1 together with H3K27Ac that are more linked to enhancer rather than to promoter activity. We observed that H3K4me3 was distributed in surroundings of *KLK1, KLK15, KLK5, KLK8* and *KLK10*, whereas the H3K4me1 and H3K27Ac were found at the regions close to *KLK1, KLK15, KLK6* and *KLK9* and in between *KLK2* and *KLK4, KLK4* and *KLK5, KLK5* and *KLK6, KLK7* and *KLK8* (Fig. 25B).

Thus, relying on the supplied data we selected regions from the potential enhancers marked by H3K4me1 and H3K27Ac to find putative VDREs (chosen VDR REs across the selected regions are shown as red vertical lines in Figure 25A). Using ChIP assays with a specific antibody against VDR we analyzed eight potential VDREs on chromatin extracted from RWPE1 cells that were either untreated or incubated for 60 min with $1\alpha,25(OH)_2D_3$. The experiment was based on pooling over 50 independent collections of immunoprecipitates (IPs) in order to obtain sufficient amount of DNA and to minimize the error between IPs. Analysis was conducted by real-time quantitative PCR. Five of the eight tested REs showed a binding of VDR: the RE1, RE2, RE3, RE4 and RE5 (Fig. 25C). The activation of VDR for one hour led to increase of VDR binding on these response elements. At the same time, negative control IgG did not show enriched binding after $1\alpha,25(OH)_2D_3$ treatment indicating specificity of VDR antibody. For three REs (RE6, RE7, RE8) no binding of VDR could be detected. As a positive control, we could identify a ligand-dependent VDR enrichment on a well-described VDRE located in close proximity to another VDR target, the *IGFBP3* gene (Fig. 25D).

Therefore, the *in silico* analysis followed by ChIP analysis revealed 8 putative VDREs from which 5 were VDR-enriched in the *KLK* cluster region of RWPE1 cells.

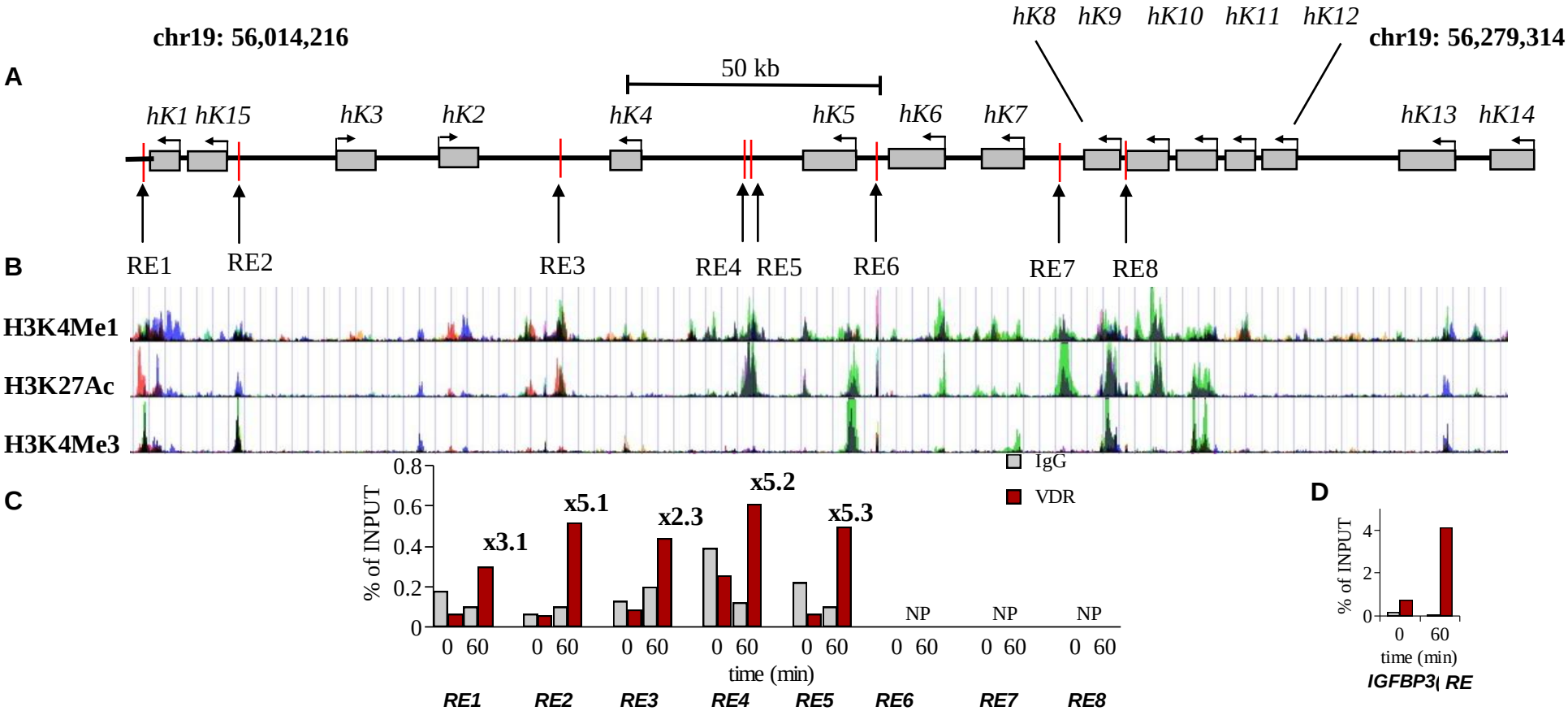


Figure 25. An overview of the *KLK* genes densely clustered on chromosome 19. (A) The genomic region of *KLK* locus spans ~265 KB uninterrupted with non-*KLK* genes. (**B**) The UCSC genome browser was used to display several regulatory regions of this genomic area using data from the ENCODE consortium: enhancer regions identified by H3K4me1 and H3K27ac markers and active promoters identified by H3K4me3 are displayed from the regulation track. The peaks represent data from 8 different cell lines, each associated with a specific color. Putative active regions (RE1-8) labeled by chromatin activity markers were selected (A) and marked as red vertical line. (**C)** Regions were tested in follow-up experiments using ChIP assay in RWPE1 cells treated with 1α,25$(OH)_2D_3$ for 60 min. RE1-RE5 showed binding of VDR whereas at RE6-RE8 the binding could not be detected. ChIP was performed by pooling over 50 independent IPs with the antibody against VDR and analyses were conducted by qPCR. The non-specific antibody IgG served as a specificity control. Numbers represent fold enrichments relative to IgG. NP, not present, *n*=1. (**D**) The VDRE near the *IGFBP3* gene was used as a positive control.

4.6.2. Recruitment of VDR to the TSS regions of *KLK* genes

The closer look into the genomic region of the *KLKs* revealed that the 1α,25(OH)$_2$D$_3$-responsive *KLKs* are positioned at a short distance from each other (Fig. 25A). As we observed in the ENCODE tracks an association of the H3K4me3 marker of transcriptional initiation close to *KLKs'* promoters, we proceeded to verify VDR binding on the TSS regions of the *KLK* genes using ChIP assays. Performed qPCR on obtained chromatin templates enabled us to detect the presence of VDR upon 60 min of ligand treatment on the TSS regions of *KLK5*, *KLK6*, *KLK7A* and *KLK9* in RWPE1 cells which was in consistence with the mRNA data. The most prominent enrichment of VDR was identified on the three regions of *KLK6* located in surroundings of its TSSs, with an up to 15-fold enrichment over unspecific IgG. Other regions associated with VDR showed enrichments as follows: *KLK5* 3.4-fold, *KLK7* 6.3-fold and *KLK9* 3.6-fold. The rest of the analyzed regions did not show significant enrichment for VDR binding after 1α,25(OH)$_2$D$_3$ treatment (*KLK4, KLK7B, KLK8, KLK10-KLK12*) (Fig. 26).

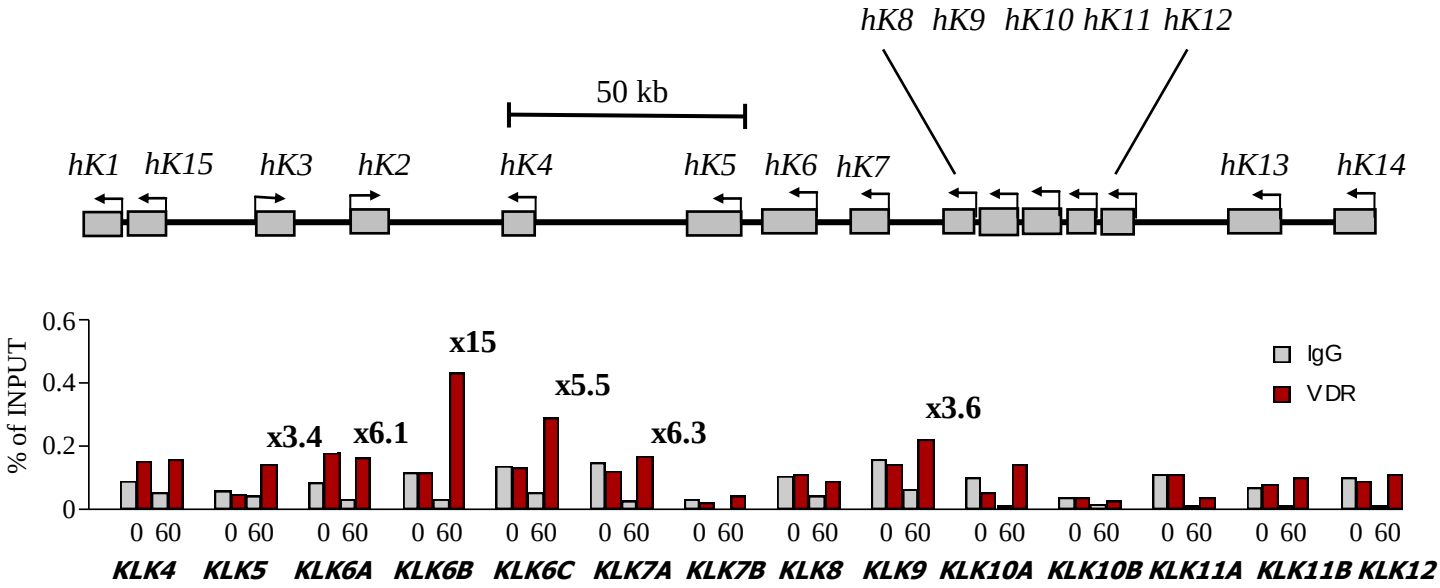


Figure 26. The analysis of VDR association with *KLK* gene promoters. Ligand-dependent recruitment of VDR to *KLK* genes TSSs in RWPE1 cells was tested using ChIP assay. Chromatin was extracted from RWPE1 cells treated with 1α,25(OH)$_2$D$_3$ for 60 min. More than 50 independent immunoprecipitations with anti-VDR antibody or control non-specific anti-IgG antibody were pooled and the analysis of VDR recruitment to the *KLK* TSSs was performed by qPCR. A published VDRE close to *IGFBP3* gene served as a positive control (see Fig. 25D). Columns represents fold enrichments relative to IgG. Note, that *n*=1.

We concluded that the TSSs of the genes from the central part of the *KLK* region exhibited association with VDR and that the *KLK6* TSS regions were the most enriched ones for binding of VDR.

4.6.3. Identification of functional VDREs in close proximity to the *KLK6* promoter

As we identified binding of the VDR to the selected chromatin regions marked by modified histones, next we scanned *in silico* those "open" genomic sequences in the *KLK* locus using the JASPAR VDR specific weight matrix (with 80% cut-off) to look for the presence of further putative VDR binding sites. We selected 7 potential VDREs that overlapped with the histone marks (Fig. 27A): one was corresponding to the VDR-associated region carrying RE1, two were in surroundings of VDR enriched binding sites RE2 and RE3, and three were found close to the regions not associated with VDR in RWPE1 cells: RE4, RE5 and RE7. Interestingly, one VDRE was situated in close proximity to the most prominent *KLK6* promoter, located -101 bp away from TSSs of *KLK6* variant B and C, and +781 bp from variant A. This region was marked by H3K4me1 and H3K27Ac in ENCODE tracks, suggesting the presence of an enhancer (Fig. 27A). Moreover, it was located 50 bp away from the amplified by qPCR region of *KLK6B* TSS, which suggested functionality of the identified VDR binding site. In addition, the recognized VDRE was evolutionary conserved across multiple mammalian genomes, i.e. human, rhesus, mouse and dog. The sequence and the conservation of the VDRE are given in Figure 27B.

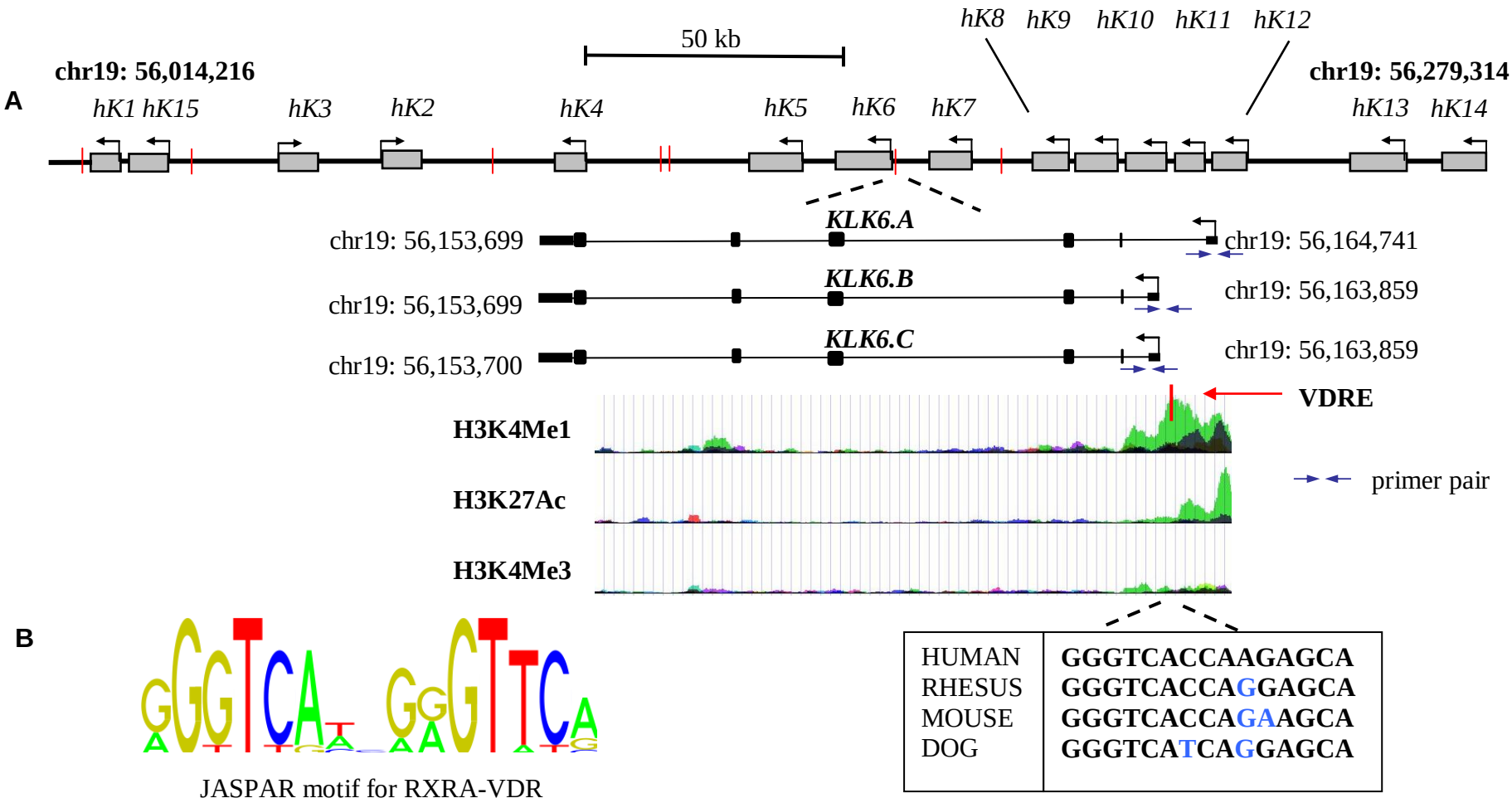


Figure 27. Association of VDR with the genomic region containing a highly conserved putative VDRE near *KLK6* TSS variants. (**A**) The region with 7 putative VDREs selected from the JAPSAR database (red vertical line indicated by red arrow) overlapped with active chromatin markers identified from the ENCODE tracks. One VDRE was located near the most prominent TSS of *KLK6.* (**B**) The conservation of the selected VDRE between human and multiple vertebrate species is shown from the UCSC multi conservation track. Letters in blue indicate differences in the sequence across species.

To validate direct binding of VDR to the selected regulatory region located in the vicinity of the TSSs of the *KLK6* transcripts B and C variants, we conducted additional ChIP assays followed by qPCR from five independent experiments on RWPE1 cells. 60 min after stimulation with $1\alpha,25(OH)_2D_3$ the *KLK6* promoter showed increased enrichment of VDR. This result was highly reproducible and clearly significant compared to the non-specific IgG control (Fig. 28A-B). The VDRE located near the *IGFBP3* promoter served as positive control (Fig. 28A, C).

This observation leads us to conclude that the identified conserved VDRE next to the *KLK6* promoter must be active and functional in RWPE1 prostate cells.

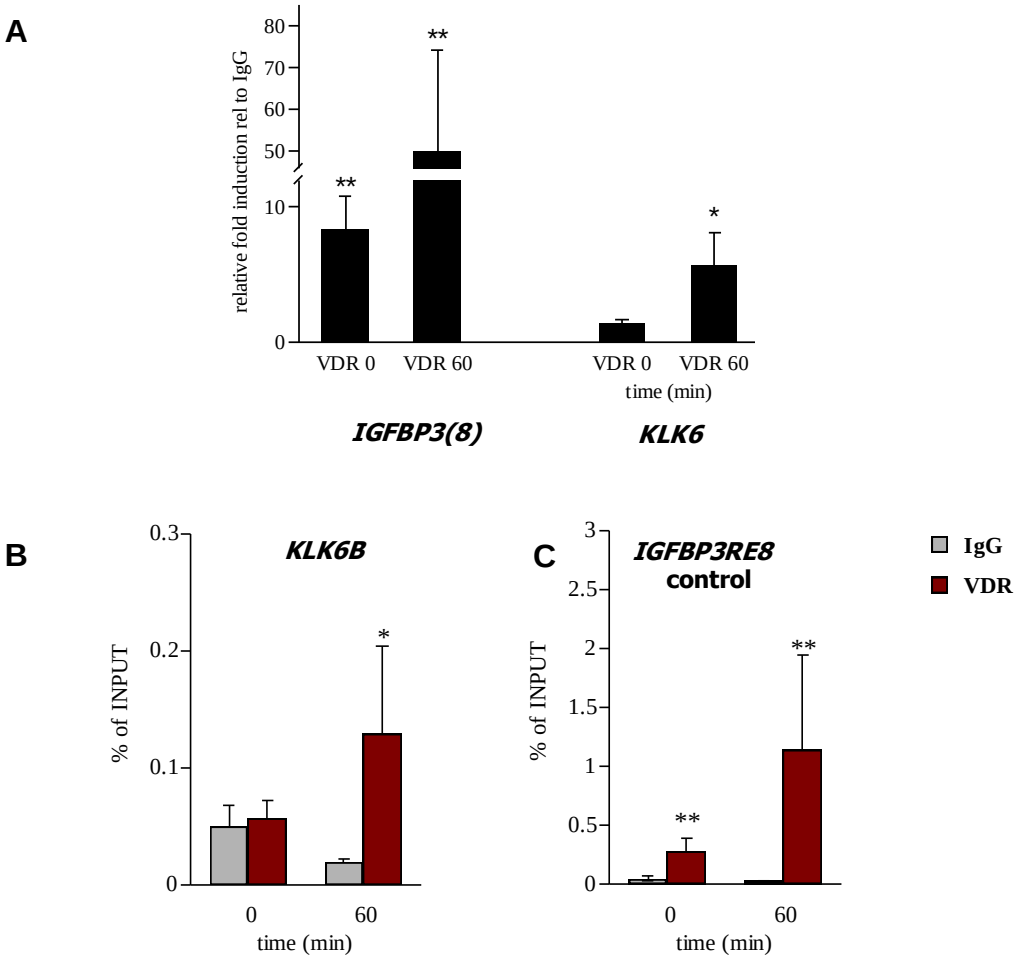


Figure 28. Recruitment of VDR to the proximal VDRE at the *KLK6* promoter in a ligand-dependent manner. The region with a putative VDRE near the TSS of *KLK6* transcript variant B and C was selected for further validation by ChIP assay with antibody against VDR. Chromatin was extracted from RWPE1 cells stimulated with $1\alpha,25(OH)_2D_3$ for 60 min. Analyses were conducted by qPCR and the association of VDR at 0 and 60 min after ligand stimulation was calculated relative to the control, non-specific antibody IgG (**A**) or in comparison to the control IgG (**B, C**). The previously verified VDRE near the *IGFBP3* gene served as a positive control. Significance as calculated using a paired students t-test from five independent experiments is shown in the figure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars indicate SEM.

Since H3K4me1 and H3K27Ac marked the VDRE next to the *KLK6* promoter region we also analyzed the presence of acetylation of histone 4 (H4Ac) at the *KLK6* TSS in order to confirm the authenticity of an open state of chromatin in this region. For this purpose, we performed additional ChIP assays with a specific antibody against H4Ac in RWPE1 cells using the same parameters as for VDR analysis. The level of general H4Ac antibody compared to the non-specific IgG control was significantly high in both 0 and 60 min time points upon $1\alpha,25(OH)_2D_3$ stimulation (Fig. 29). Our results suggest that acetylated H4 is present at the *KLK6* promoter and is increased in ligand-dependent manner.

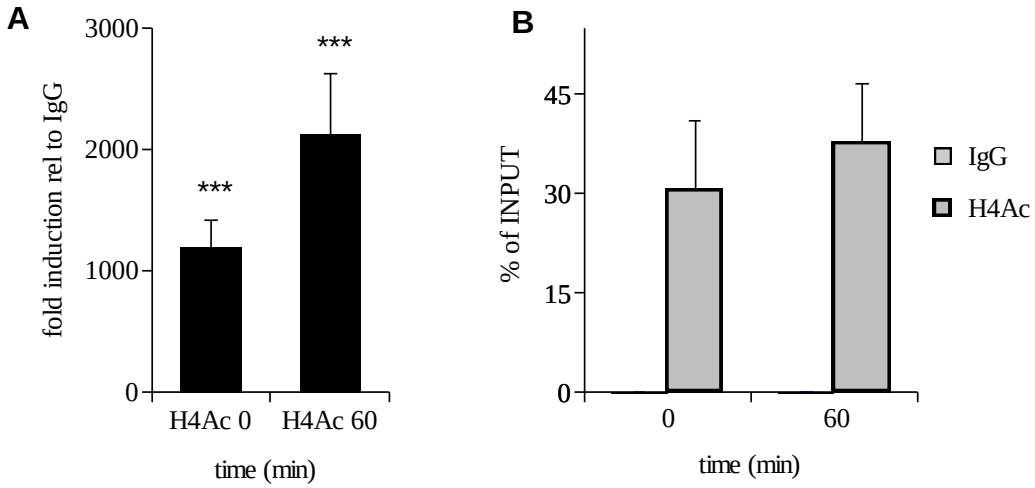


Figure 29. Ligand-dependent and -independent association of acetylated H4 with the *KLK6* promoter. For ChIP analysis with an anti-H4Ac antibody, chromatin was extracted from RWPE1 cells treated for 60 min with $1\alpha,25(OH)_2D_3$ and the results were determined by qPCR. Data points were analyzed as the mean of three independent biological replicates and the error bars indicate SEM. VDR recruitment at 0 and 60 min after ligand stimulation was calculated relative to the control IgG antibody (**A**) or in comparison to the control IgG (**B**) and was used for significance calculation with the paired students t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Taken together, we conducted *in silico* screening for putative regulatory regions within the *KLK* locus and performed ChIP analysis from non-malignant prostate cells. Thereby, we validated *KLK* TSSs and 8 putative VDREs for the presence of VDR. Our results showed VDR recruitment to the TSSs of the *KLKs* from the central region of their locus with the highest enrichment for *KLK6*. The VDRE located near the *KLK6* promoter was found to be highly conserved. Moreover, this genomic region was associated with the presence of H3K4me1 and H3K27Ac and the validation of the presence of H4Ac showed VDR-dependent increased occupancy.

4.7. Association of VDR and H4Ac with the *KLK6* promoter in malignant prostate cells

We deduced that the different mRNA expression levels of *KLKs* in non-malignant and cancer prostate cell lines are linked with regulation of their transcription. Therefore, we analyzed the promoter of *KLK6* in RWPE2 prostate cancer cells using ChIP assay with antibodies against VDR and H4Ac compared to non-specific IgG control. The chromatin was isolated after 60 min of treatment with $1\alpha,25(OH)_2D_3$. We observed that the level of VDR binding to the *KLK6* promoter was minor and not significant, in fact it did not change after stimulation with $1\alpha,25(OH)_2D_3$ (Fig. 30A). The acetylation of H4 upon VDR ligand treatment as well remained unchanged (Fig. 30B). In addition, due to differences in the induction of *IGFBP3* mRNA by VDR ligand in both types of prostate cell lines, we tested the VDR association to the known *IGFBP3* VDRE in RWPE2 cells. Interestingly, we observed that in contrast to RWPE1 cells the recruitment of VDR in RWPE2 was minor and was not influenced by treatment with $1\alpha,25(OH)_2D_3$ (Fig. 30C).

These results suggest that the binding of VDR to its regulatory regions occurs in a cell-specific manner and that it is enhanced in non-malignant compared to malignant prostate cells.

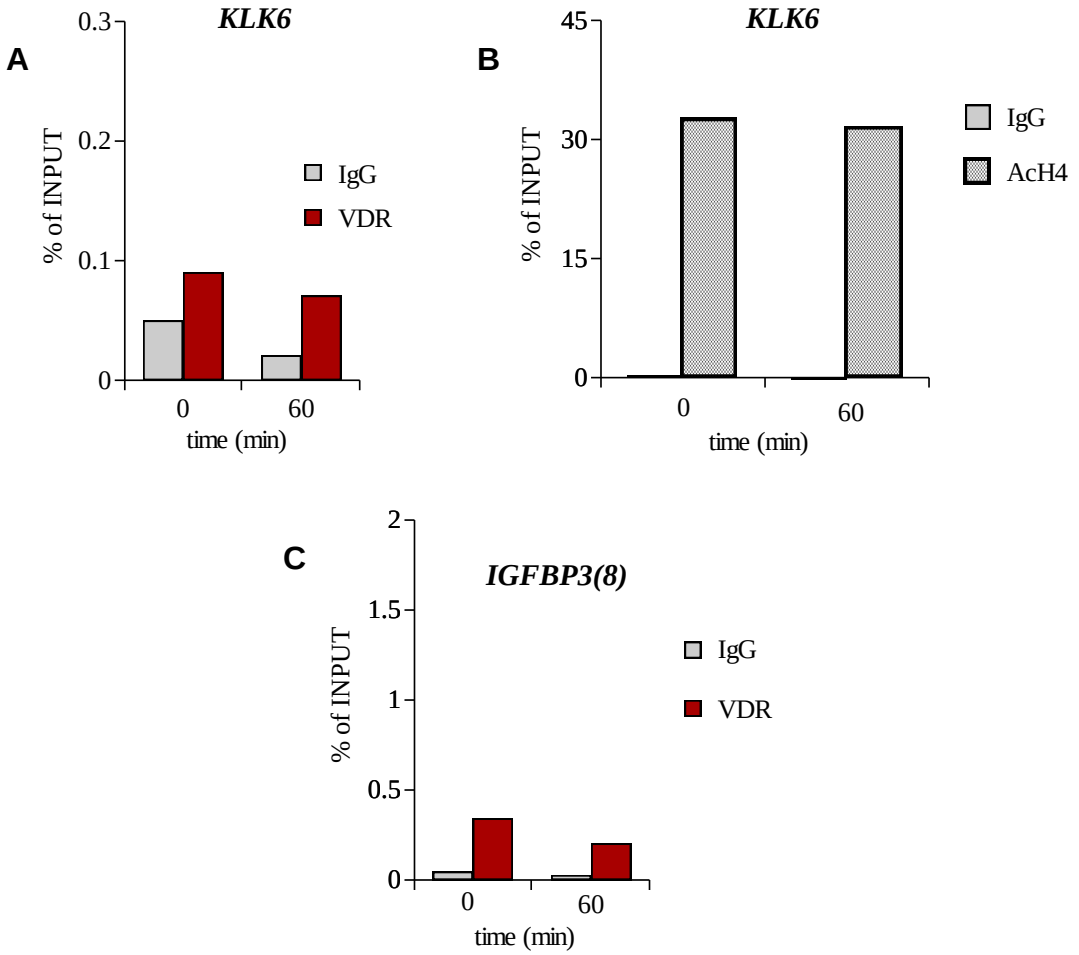


Figure 30. Analysis of the *KLK6* promoter activity in the RWPE2 cancer cell line. RWPE2 cells were treated with 1α,25$(OH)_2D_3$ for 60 min and subjected to ChIP analysis using antibodies against VDR, acetylated H4 or non-specific IgG, which served as a control. Analysis was performed by qPCR and the VDR association with the *KLK6* TSS was determined by comparison to the control IgG (**A, C**), whereas acetylation of H4 was calculated relative to the control, non-specific IgG (**B**). The VDRE located close to the *IGFBP3* gene serves as a control. Data points stand for one biological replicate that represent 9 pooled independent immunoprecipitations.

4.8. Distribution of CTCF binding sites and their activity within the *KLK* locus

Next, we were interested in possible cause of the common mRNA expression pattern of the five *KLK*s located next to each other on chromosome 19. To investigate the presence of putative chromatin boundary insulators we used the ENCODE tracks using the UCSC Genome Browser. Interestingly, we found that five potential CTCF binding sites located inside the *KLK* locus separated the central region from the rest of the *KLKs* (Fig. 31A). Three of them were located around the *KLK4* gene (CTCF1, CTCF2, CTCF3) and two were in the vicinity of the *KLK10* gene (CTCF4 and CTCF5). Using ChIP-qPCR analysis with antibody against CTCF or a non-specific IgG control, we demonstrated that the insulators surround the *KLK5*, *-6*, *-7*, *-8* and *-9* in RWPE1 cells. Surprisingly, stimulation with the $1\alpha,25(OH)_2D_3$ for 60 min enhanced association of CTCF with the binding sites CTCF1, CTCF2, CTCF4 and CTCF5 (Fig. 31B), whereas a negative control region (*KLK6* TSS) shows no binding (Fig. 31C).

Taken together, the up-regulated *KLK*s were situated between the two boundaries of CTCFs that were additionally enriched in a VDR-dependent manner. Thus, we conclude that CTCF distribution agrees with the regulation of *KLKs* genes from the central region of their locus by $1\alpha,25(OH)_2D_3$ stimulation.

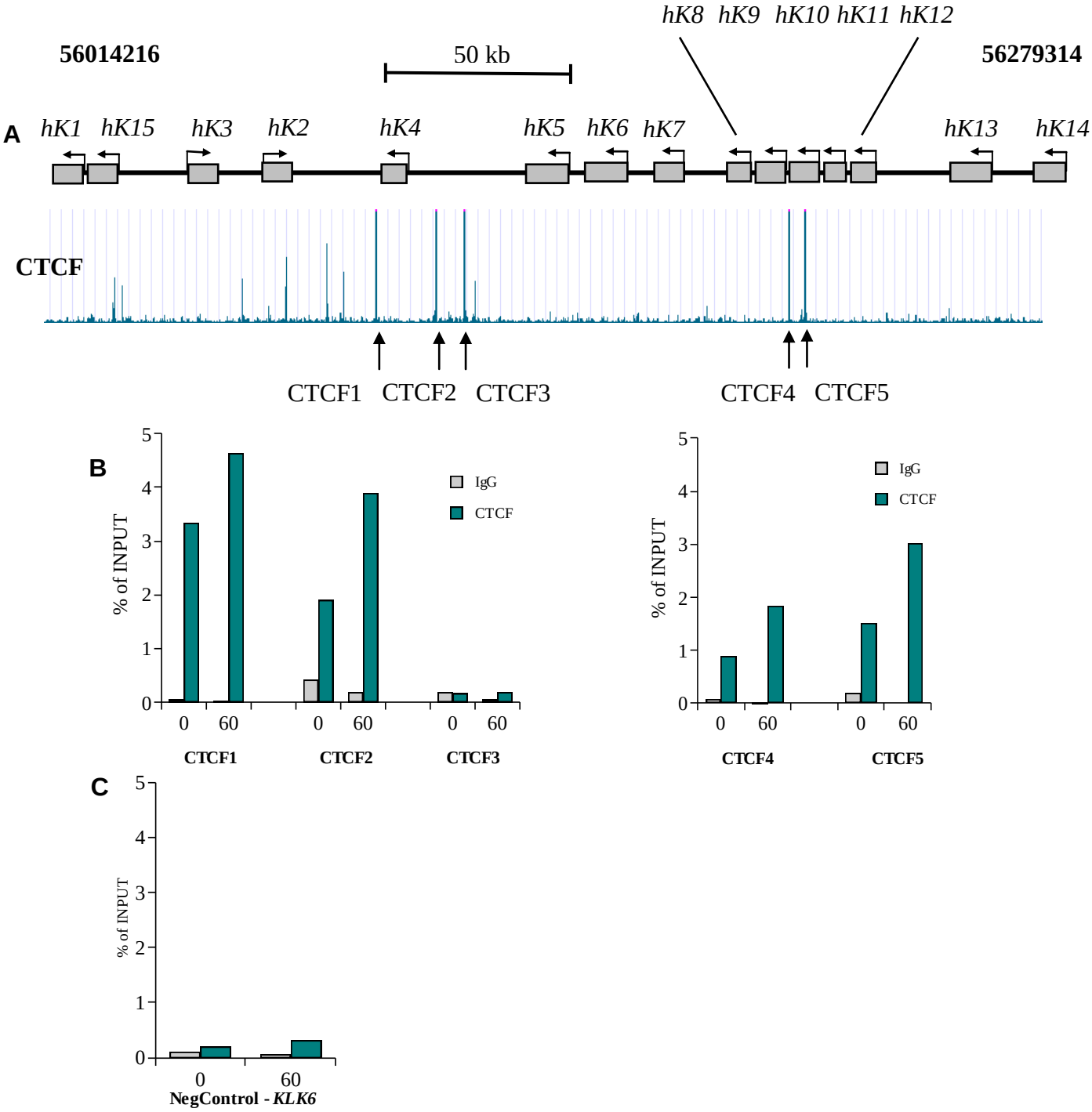


Figure 31. Distribution of insulators identified within the KLK cluster from multiple cell types indicate a separation of *KLK5-KLK9* to an isolated chromatin domain. (**A**) Extracted track from the ENCODE datasets displayed at the *KLK* gene cluster area represent ChIP-seq peak data from HeLa cells for CTCF binding sites, which indicate potential chromatin boundaries. Selected putative active regions (CTCF1-5) were labeled by insulators occupancy. (**B**) Regions were tested using ChIP assay in RWPE1 cells treated with 1α,25(OH)$_2$D$_3$ for 60 min. ChIP was performed by pooling over 10 independent collections with antibody against CTCF and analysis was performed by qPCR. A non-specific IgG served as a specificity control. Columns represent fold enrichments relative to IgG. Note that *n*=1 and was consisting of more than 10 independent immunoprecipitations with anti-CTCF antibody. (**C**) A region empty in CTCF binding sites, the *KLK6* TSS, was used as a negative control.

4.9. Effect of CTCF silencing on 1α,25(OH)$_2$D$_3$ stimulation

CTCF is one of the proteins involved in formation of a higher-order chromatin structures and influence on regulation of gene expression (Philips, 2009). Loss of CTCF can be related to concomitantly increased mRNA expression of specific genes like the pro-apoptotic *PUMA* gene (Gomes, 2010). To test whether the VDR-dependent regulation of *KLKs* located in the central part of their locus can be extended to the entire cluster, we knocked-down *CTCF* and validated the response to VDR ligand treatment for all *KLKs*. RWPE1 cells transfected for 48 h with CTCF-specific siRNAs or control siRNAs were stimulated for 4 h with 1α,25(OH)$_2$D$_3$. CTCF mRNA was successfully knocked-down by 84% (Fig. 32A). However, its depletion did not alter the pattern of *KLK* inducibility by VDR ligand. Indeed, the expression of the *KLK6* gene was similarly elevated after 4 h of 1α,25(OH)$_2$D$_3$ treatment. Likewise, the mRNA level of the other genes continued to be modestly up-regulated or did not change upon CTCF suppression, i.e. the *KLKs* situated at the borders of the locus remained inert after stimulation with 1α,25(OH)$_2$D$_3$ (Fig. 32B).

These data showed, that CTCF depletion did not affect the induction of the *KLK* genes both inside the central area and on the border of the locus, suggesting the influence of additional factors taking part in the maintenance of barriers in the *KLK* locus in RWPE1 cells.

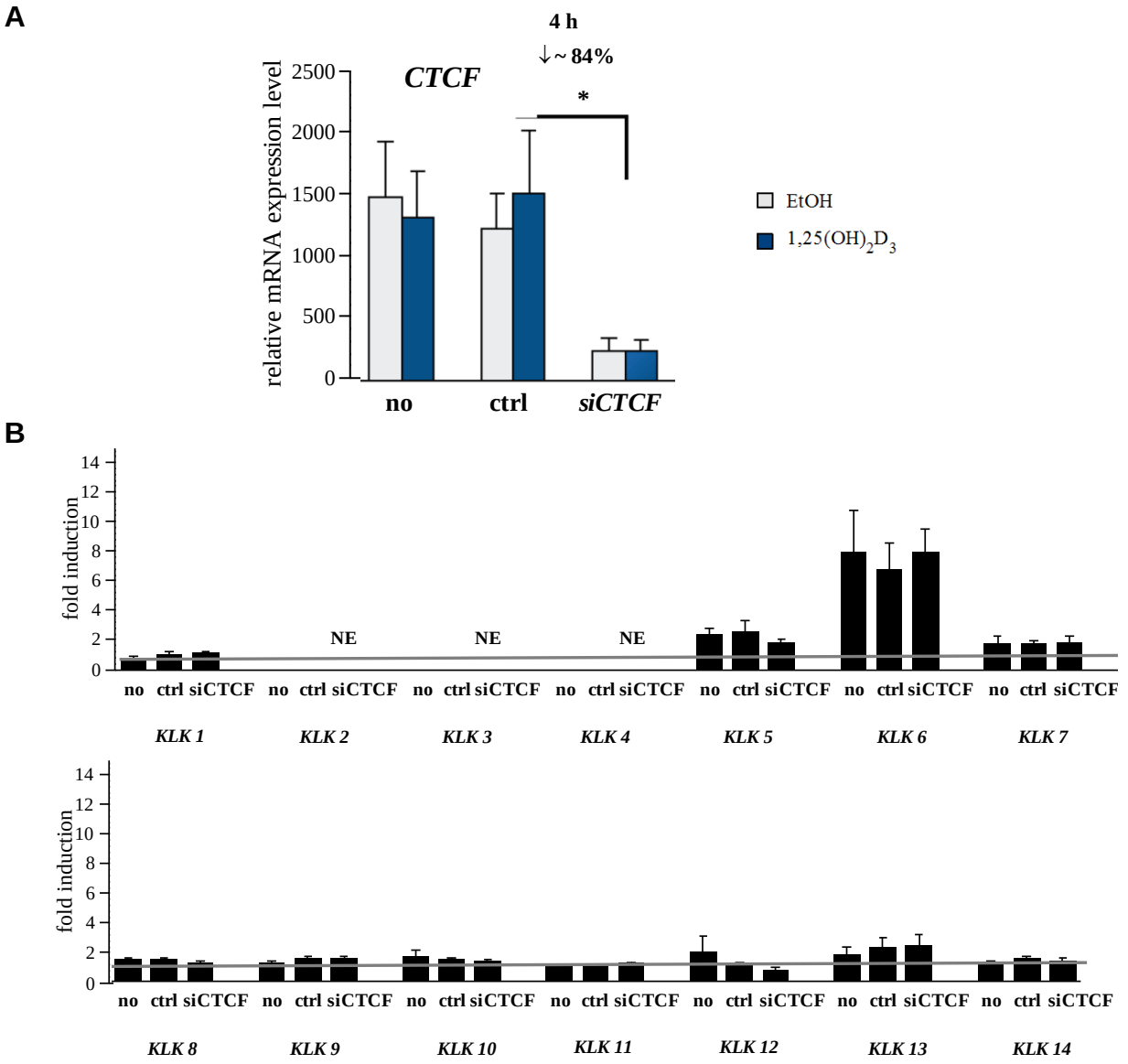


Figure 32. Effect of CTCF depletion on *KLKs* stimulated with VDR ligand. To determine whether CTCF knockdown expands the ability of VDR to induce the whole KLK cluster, RWPE1 cells were transfected with specific siRNA oligonucleotides against CTCF or control siRNAs, and were stimulated with 1α,25(OH)$_2$D$_3$ for 4 h. qPCR was performed to verify the effect of CTCF inhibition (**A**) and to analyze the expression of the *KLK* gene family (**B**) in the insulator-depleted condition. All genes were normalized to the control *RPLPO* gene and the fold inductions were determined in relation to solvent control (EtOH). Data points show the means of three independent experiments, each done in triplicate and the error bars represent SEM. The significance was calculated using the paired student's t-test for the siRNA silencing effect on the ligand-inducibility in relation to unspecific control siRNA (ctrl) or not transfected (no) samples (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and no significant changes was observed. NE, not expressed.

4.10. The correlation between KLK substrates and VDR action.

To explore the relation between VDR-regulated genes and possible KLK enzymatic effects in the prostate we selected, based on the literature (Lawrence, 2010), around 100 known KLK substrates and compared their expression in our microarray data. Next, we identified and selected the GO terms referring to the biological processes to which both, genes significantly regulated by VDR as well as genes expressed in RWPE1 cells encoding potential substrates for the KLK enzymes were assigned (Table 7). Interestingly, we could distinguish enriched GO terms like "cell adhesion", "regulation of cell motion" and also "response to wounding", "defense response" or "regulation of cell proliferation" that suggest a role of KLK enzymes in important VDR-related vital processes. Regulation of *KLK* gene expression by VDR may contribute to indirect, secondary or tertiary effects of this NR. The KLKs as proteases can activate or deactivate their substrates that may belong to the biological process in which VDR directly controls expression of different set of genes. Thus, VDR through up-regulation of KLKs from the central part of their cluster, additionally may influence the selected biological process. Moreover, we noticed several overlapping genes when comparing VDR target genes and the KLK substrates group within a given GO terms. We were able to select 15 genes encoding ascribed KLK substrates significantly up- or down-regulated by $1\alpha,25(OH)_2D_3$ after 4 or 24 h of stimulation.

Table 7. The enrichment analysis of the relation between genes significantly regulated by VDR and putative KLK substrates expressed in the RWPE1 cells

ID	GO TERM	VDR-REGULATED GENES		KLKs SUBSTRATES		OVERLAPPING GENES
		Count	P Value	Count	P Value	
GO:0007155	cell adhesion	39	2.25E-04	18	1.56E-11	*LAMB3, CDH1*
GO:0051270	regulation of cell motion	20	6.06E-04	9	2.06E-06	*IGFBP3, F3*
GO:0007398	ectoderm development	25	1.19E-07	8	8.41E-06	*KLK5, LAMB3, PTHLH, KLK7*
GO:0032989	cellular component morphogenesis	23	3.07E-02	8	6.84E-04	
GO:0009611	response to wounding	49	1.80E-11	11	7.19E-06	*KLK6, PLAT, IL1B, F3*
GO:0042127	regulation of cell proliferation	59	2.74E-06	11	1.29E-03	*PTHLH, IGFBP3, F3*
GO:0048878	chemical homeostasis	30	6.52E-04	9	1.68E-04	*PTHLH, KLK6, IL1B, MBP*
GO:0006952	defense response	32	3.05E-04	7	6.26E-03	*IL1B, F3, CAMP*
GO:0007166	cell surface receptor linked signal transduction	70	1.84E-05	11	1.03E-02	*PTHLH, PLAT*

Data taken from DNA microarray

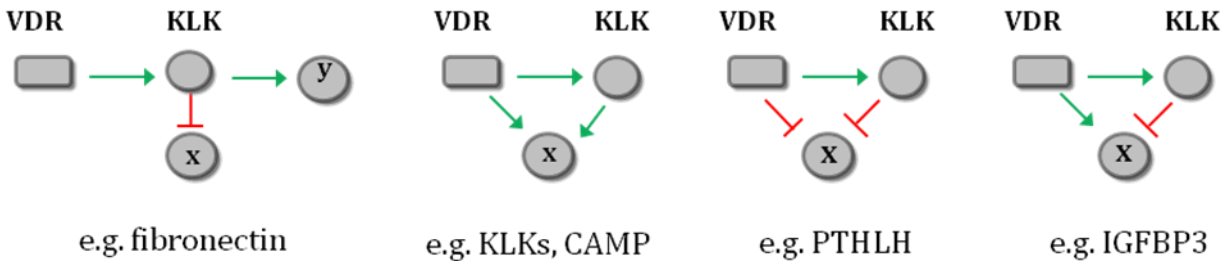


Figure 33. Models representing a link between VDR action and KLK activity. KLKs as VDR targets may contribute to effects elicted by this NR. Several genes were both VDR target genes and KLKs substrates. There are four interactions between VDR and KLK enzymes. These proteins can act independently, or both can activate/deactivate the same targets (coherent feedforward loop), or they can act in opposition (incoherent feedforward loop). A green arrow represents up-regulated/activated targets while red marks show down-regulated/inactivated targets.

Among the identified overlapping genes there were members of the KLK family, and two genes from the insulin-like growth factor binding protein family. Our data suggest a correlation between KLK substrates and VDR effects elicited after ligand stimulation in non-malignant prostate cells. The observed four types of interactions between KLK substrates and their regulation by VDR in common biological processes could be associated with the transcriptional dynamic (Fig. 33). Thereby, expression of some substrates activated by KLKs like KLK5, KLK6, CAMP (Morizane, 2010) is increased by 1α,25(OH)2D3 treatment, and the expression of substrate inactivated by KLKs like PTHLH is decreased by stimulation with 1α,25(OH)2D3. These patterns are called coherent feedforward loops. They consist of regulators like VDR or KLKs that together regulates same targets (Alon, 2007). In addition, we noticed that identified VDR target genes, *IGFBP3* and *IGFBP6* are known to be inactivated by the KLKs including KLK5. These are incoherent feedforward loop motifs that comprise a pulse generator like VDR and a response accelerator like KLKs, where one activates a target while the second one represses it (Alon, 2007). This shows that VDR and KLKs may also act in opposition.

Moreover, these genes were associated with GO terms such as: “extracellular region and space”, “homeostatic process” and “cell-cell signaling” (Table 8).

Table 8. GO analysis of putative KLK substrate genes significantly regulated by VDR

ID	GO TERM	Count	GENES		P Value	FDR
			Up-regulated	Down-regulated		
GO:0005576	extracellular region	11	*KLK9, IGFBP6, CAMP, KLK5-KLK7, IGFBP3, PLAT, IL1B, COL4A5*	*IL1B*	2.17E-07	1.03E-04
GO:0005615	extracellular space	6	*IGFBP3, IGFBP6, KLK5, PLAT*	*IL1B, PTHLH*	1.17E-04	2.25E-04
GO:0042592	homeostatic process	5	*KLK6, MB*	*IL1B, MBP, PTHLH*	1.42E-04	1.21E-01
GO:0007267	cell-cell signaling	4	*PLAT*	*IL1B, MBP, PTHLH*	4.90E-04	7.32E-01

Data taken from DNA microarray

Taken together, the VDR ligand rapidly induces the *KLK6* gene and influences the expression of *KLKs* from the central part of their locus. We showed, based on GO analysis, the noticeable communication between KLK enzyme substrates and VDR-regulated genes in several biological processes. Moreover, the microarray data showed the impact of $1\alpha,25(OH)_2D_3$ on known KLKs substrates, implying a crosstalk between regulation of mRNA expression of KLK substrates by VDR ligand and potential functions of KLKs as proteases.

5. DISCUSSION

5.1. Identification of VDR-regulated genes in human prostate cells

The activation of VDR triggers a cascade of cellular events resulting in various effects involved in anti-inflammatory, anti-proliferative, pro-apoptotic and differentiative functions (Sproul, 2005). These features can be correlated with a protective role of $1\alpha,25(OH)_2D_3$ against cancer development. As the tumor develops progressively, it is very important to study the ways to prevent its growth already in non-malignant cells.

In order to explore the role of VDR in human prostate epithelial cells, we performed a microarray analysis in the human prostate RWPE1 cell line. To conduct a classification of the genes in early-responding, primary and late-responding, secondary target genes, we treated these cells for 4 or 24 h with $1\alpha,25(OH)_2D_3$. Among the 87 genes regulated at an early time point, only 11 genes were down-regulated, suggesting that VDR preferentially controls genes by their stimulation rather than inhibition after a short-term treatment. The broad action of VDR across many different genes was seen at the late time point of treatment where a bulk of more than 400 genes was significantly regulated. Among the induced genes at both time points we recognized well-known VDR targets like *CYP24A1* and *IGFBP3*, members of the *DUSP* and *KLK* gene families, but also genes involved in the immune response (*CD14, IL8, FOS*). Among the early down-regulated targets, genes were identified that are implicated in promotion of angiogenesis (*VEGFC, THBS1*), inhibition of apoptosis (*IER3, BIRC3*) and TGFβ signaling (*FST*). These data indicate that the activation of VDR and the reduction in expression of these target genes could contribute to the possible anti-cancer effects by inhibiting the angiogenesis of a developing tumor and by the reduced inhibition of apoptosis. Furthermore, the GO enrichment analysis revealed biological processes in which VDR plays an important role also in prostate cells. We noted that VDR activation influences the "regulation of cell cycle" by inducing genes such as *p21* and *GADD45A* known as growth arrest and DNA-damage-inducible gene, or *BCL6* encoding a protein that acts as transcription repressor suppressing genes like *CDK1, MYC, UBEC2* that are crucial in cell cycle progression, pointing at the role of $1\alpha,25(OH)_2D_3$ in cell cycle arrest. In addition, the up-

regulated genes involved in "cell differentiation" including *BMP2, BMP6*, and *JUN*, may potentially induce differentiation, suggesting that VDR also promotes cell differentiation in prostate cells. Interestingly, "regulation of apoptosis" was assigned to 52 genes from which 60% were induced by $1\alpha,25(OH)_2D_3$ such as *BOK, BNIP3* and *BNIP3L,* genes with pro-apoptotic functions that belong to the BCL2 protein family. On the other hand, the inhibitors of apoptosis like *ANGPTL4, BIRC3, BIRC5* were down-regulated, which clearly indicates a VDR effect also on this process. These results suggest that activation of VDR may possibly act as a preventive guard against uncontrolled proliferation.

Interestingly, the transcriptome analysis revealed that the second most induced gene in the non-malignant prostate cell line after $1\alpha,25(OH)_2D_3$ stimulation was *kallikrein 6* (*KLK6*) that belongs to the largest continuous cluster of genes encoding serine proteases. This information was exciting as another member of this family, KLK3, also known as prostate specific antigen (PSA), is widely used as a prostate cancer biomarker (Lilja, 2008). A detailed look on the *KLK* genes revealed that several additional *KLKs (KLK5, KLK7, KLK8, KLK9)* were also expressed in non-malignant prostate cells and can be up-regulated by the activation of VDR. Aberrant regulation of KLKs has been associated with many diseases like inflammation, skin disorders, neurodegeneration and cancer (Sotiropoulou, 2009). It has been suggested that the *KLK5, -6* and *-8* genes in non-malignant keratinocyte cells (Lu, 2005) and the *KLK6* gene in breast cancer cells (Vannoirbeek, 2009) can be regulated by $1\alpha,25(OH)_2D_3$ stimulation. As the exact mechanism of their activation by VDR remained unsolved, this study presents for the first time a detailed analysis of VDR function in the regulation of the kallikrein gene cluster.

5.2. Selectively regulated genes from the *KLK* cluster in prostate cells

Several members of the *KLK* gene family were expressed (*KLK3* and *KLK5-11*) in the non-malignant prostate cell line but few of these genes were directly regulated by $1\alpha,25(OH)_2D_3$ (*KLK3, KLK5-9*) based on the microarray results. Interestingly, the induced genes are located in the central region of the *KLK* locus. However, the PSA was not located in this central part of the cluster, which might separate this gene from the VDR-specific transcriptional regulatory network. This may probably explain why the *KLK3* gene was slightly responding to $1\alpha,25(OH)_2D_3$ in the microarray analysis which however, could not be confirmed by qPCR. Interestingly, *KLKs* can be co-expressed together in many different tissues, like in breast (*KLK5-8, KLK10, KLK11,* and *14*) or cervix (*KLK4-11*), which suggest that they may be

regulated in a coordinated manner (Debela, 2008, Borgono, 2004). The presented results showed that several genes from central part of *KLK* locus can be also jointly induced upon 1α,25$(OH)_2D_3$ stimulation in prostate, which implies a common transcriptional mechanism.

As 1α,25$(OH)_2D_3$ could differently affect the induction of the *KLK* genes in RWPE1 cells the question raised whether mRNA expression of genes from the central *KLK* region could also be stimulated by the VDR ligand in the malignant prostate cells. KLKs are often deregulated in tumor and their expression pattern differs from cancer to cancer (Borgono, 2004). For instance, in prostate cancer mRNA levels of some *KLK* genes like *KLK5, KLK6, KLK8, KLK10, KLK12* was found to be decreased in relation to non-malignant prostate (Lawrence, 2010; Yousef, 2002; Petraki, 2003).

Intriguingly, our series of time course experiments revealed that the highest induction of the *KLK6* gene upon 1α,25$(OH)_2D_3$ stimulation was more than 10 times lower in cancer cell lines compared to the non-malignant prostate cell line. *KLK7, KLK8* and *KLK9*, which were modestly but significantly induced in non-malignant prostate cells by VDR activation, could not be significantly induced in the cancerous PC3 and DU145 cells. Only the RWPE2 cancer cells showed a small significant induction of *KLK5, -7, -8* and -9 upon 1α,25$(OH)_2D_3$ treatment. These observations led to the assumption that the *KLKs* may be a direct target of VDR in non-malignant prostate cells but not in cancer cells. Moreover, the relative expression of the *KLK* genes from the central part of their cluster was lower in cancer when compared to a non-malignant prostate cells. The exception was the *KLK6* gene; here the expression level remained unchanged across different prostate cell lines. *KLKs* expression in malignant versus non-malignant prostate was also studied by others, and the results have shown a similar outcome. For example, *KLK5* gene expression was lower in cancer than in normal cells (Yousef, 2002). Perhaps the accessibility of these genes to the transcriptional machinery is limited in cancer cells. We observed a similar effect for the known VDR target gene *IGFBP3* that showed decreased up-regulation by VDR ligand in malignant prostate cells compared to non-malignant cells. Interestingly, Zhang *et al.* have shown that the VDR protein levels in RWPE1 and RWPE2 cells were equal upon 1α,25$(OH)_2D_3$ treatment. However, the transcription in RWPE2 after VDR stimulation was blunted as a result of phosphorylation of RXRα in the AF1 domain that decreased binding of co-activators. It would possibly explain lower response of the VDR to its ligand in malignant prostate cells (Zhang, 2010).

To further deduce the role of VDR in the regulation of the *KLKs* in non-malignant prostate cells, we analyzed the central region of the *KLKs* also under conditions of depleted VDR. We verified the effectiveness of VDR silencing via gene-specific siRNAs by showing inhibition

of *CYP24A1* expression, the best-known direct VDR target gene, by around 90% after 1α,25(OH)$_2$D$_3$ treatment. Silencing of VDR alters the transcription of *KLKs* differently, depending on their localization, confirming the idea that only the *KLKs* of the central region are VDR-responsive. The highest responsive gene was *KLK6* whose expression was impaired up to 90% upon 1α,25(OH)$_2$D$_3$ stimulation, whereas the second most up-regulated gene, *KLK5*, showed ~45% of knockdown suggesting that it might be regulated by additional factors. The slight up-regulation of *KLK7, -8* and *-9* was completely abolished after VDR silencing, showing that VDR may have been involved in their expression. Thus, VDR depletion in the non-malignant prostate cell line demonstrated that the up-regulation of the *KLK6, -7, -8* and *-9* genes is dependent on the presence of VDR and not induced through an unspecific ligand effect.

These results are particular interesting since *KLK6* was assigned as a tumor suppressor gene that plays a protective role against breast cancer development (Pampalakis, 2009).

In addition, the mRNA of *KLK6* after 1α,25(OH)$_2$D$_3$ treatment in RWPE1 cells was continuously accumulating in a time-dependent manner. The induction of *KLK6* was detectable already after 45 min of 1α,25(OH)$_2$D$_3$ stimulation and was accumulating thereafter in a linear fashion. The time and rate of the mRNA transcription should harmonize with the mRNA degradation rate since both determine the actual amount of mRNA designated for protein production (Houseley, 2009). The consistent accumulation of *KLK6* mRNA was in agreement with the stability of its mRNA in certain time interval. The *KLK6* mRNA half-life was higher than 5 hours, the time span covered by the experimental set-up. The rapid and high VDR-dependent induction of the *KLK6,* correlated with rather stable mRNA indicates that the *KLK6* gene is a primary VDR target gene in non-malignant prostate cells.

5.3. Organization of the *KLK* gene locus, and implications of the VDR in its regulation

In order to further investigate the gene regulation of the *KLK* locus we looked at the whole *KLK* gene region, first to determine sites enriched with a marker of chromatin decondensation. The UCSC Genome Browser tools provided us a set of diverse binding patterns of the H3K4me1, H3K4me3 and H3K27Ac markers from 8 different cell lines. These histone modifications were associated with an open chromatin state (Heintzman, 2009). Thereby, we were able to identify the location of these indicators of chromatin relaxation at the TSSs of some genes and in intergenic sites that indicate potential accessibility for the

transcriptional machinery at the *KLK* locus. On this basis, 8 putative VDREs located inside the cluster and in proximity to *KLKs* TSSs were analyzed, in non-malignant prostate cells upon $1\alpha,25(OH)_2D_3$ stimulation, using the chromatin immunopecipitation (ChIP) assay. The results revealed the strongest binding of VDR to the TSS region of the *KLK6*, which indicates the presence of VDRE close to the promoter of this gene. In contrast, the rest of analyzed TSSs showed minor or no association with VDR. Intriguingly, the potential VDREs, selected based on histone modification markers also demonstrated diverse association with the VDR protein. Since genes located close to these binding sites were not regulated by VDR, one can assume that predicted response elements may be involved in transcription of other, perhaps distinct VDR target genes. However, detailed analyses are required to verify their function. It would be especially valuable for the RE4 and RE5 located between *KLK4* and *KLK5,* since the mRNA expression of the second gene was increased after $1\alpha,25(OH)_2D_3$ treatment. In general, the REs were considered to be located in close proximity to the promoter of targeted gene to activate transcription. However, emerging studies have shown that distal REs can also participate in gene regulation. For instance, it was shown that multiple ER binding sites situated both distal and in close proximity from the regulated gene, can cooperate via long-range chromosomal interactions (Pan, 2008). Moreover, the well-known VDR target gene, *CYP24A1* was shown to be regulated by a several VDREs located both at distant and proximal sites from the promoter, connected by a chromatin loop formed in a ligand-dependent manner (Matilainen, 2010). Nonetheless, the exact mechanism on how multiple REs act jointly is not yet clear.

For further analysis, we conducted an *in silico* screening with "the high-quality transcription factor binding profile database" called Jaspar that revealed a number of putative VDR response elements inside the *KLK* locus, one of which was located at position -101 bp of the *KLK6* promoter. Despite the presence of a considerable number of putative VDREs within the *KLK* cluster, majority of TSSs in the locus except *KLK6* showed minor or no VDR recruitment, which suggests selectivity of VDR for its response elements in prostate cells, perhaps due to differential attraction to the VDREs. One binding site flanking the TSS of *KLK6* gene showed significant association with the VDR after $1\alpha,25(OH)_2D_3$ stimulation. Interestingly, this VDRE was evolutionary conserved and showed similarities across multiple species. The 15 nucleotides are highly similar between mammalian species (human, rhesus, mouse and dog) with only one nucleotide change in human and two changes in dog. The high conservation may reflect the functionality of this VDRE across species. The VDRE sequence inside the *KLK* cluster remained unchanged throughout the evolution, which suggests its

unique value. Moreover, the *KLK6* gene together with the other kallikrein family members (except *KLK2* and *-3*), are highly conserved, since they are present in all placental mammals. That suggests they have an important biological function (Lawrence, 2010). Nevertheless, their potential roles in co-operation with VDR are poorly understood. Intriguingly, Wang *et al.* using their *in silico* screening for predicting VDREs have found another binding site located -489 bp away from the promoter of *KLK6* gene (Wang, 2005). However this RE could not be confirmed by the Jaspar screening.

The comparison of the *KLK6* promoter binding site in non-malignant and cancer cells regarding presence of the VDR, reflected differences in the mRNA expression level of the *KLK6* gene between those two kinds of cells. The smaller enrichment for VDR binding to its promoter resulted in a lower mRNA expression of the *KLK6* that was seen in the cancer cell line, whereas strong and significant VDR binding to the *KLK6* promoter in the non-malignant prostate cell line may be a sign of a higher mRNA expression of the gene. The differences between induction of *KLK6* might be due to variations in chromatin remodeling affecting the genes' accessibility for the transcriptional machinery and ability of the VDR to bind to the potential VDREs, which might differ in non-malignant and cancer cells. Moreover, possible loss-of-function mutations in VDR may impair ligand binding, heterodimerization and thus transactivation in cancer (Haussler, 2011).

In order to confirm the authenticity of the regulatory regions in non-malignant and cancer cells near the TSS of *KLK6* gene, we examined the presence of H4Ac, which reflects the activation state of transcription. It is a marker for the relaxation and open state of chromatin structure, and therefore its accessibility for the transcriptional machinery (Zella, 2009). Interestingly, its high levels in the absence and increased levels in the presence of $1\alpha,25(OH)_2D_3$ in non-malignant prostate cells suggested that histone acetyltransferases are associated with the *KLK6* gene induction. This underlines the authenticity of the regulatory region and suggests that the *KLK6* TSS is permissive and allows VDR to bind. The histone 4 was also acetylated at the promoter site of *KLK6* in malignant cells, but its level remained unchanged after induction with $1\alpha,25(OH)_2D_3$. Nevertheless, this also can be a possible sign of euchromatin formation, which might suggest that VDR cooperates with other transcription factors and thereby takes part in chromatin decondensation, allowing other proteins bind to the DNA. However, the presence of H4Ac may also relate to the situation observed with trimethylated histones, which reside at silent promoters, and take part in maintenance or priming the chromatin for the future activation or act as a memorizer of a previous transcriptional activation (Heintzman, 2007).

In addition, data taken from the UCSC Genome Browser showed that among several different cell lines, the surrounding of the *KLK6* TSS is covered with H3K4 monomethylation, which is supposed to be enriched in active genes and to have possibly a function in transcriptional activation (Barski, 2007). Also presence of acetylated H3K27 was detected, which is thought to augment the transcription (Kim, 2011). These results also suggest that the *KLK6* gene may be active across many cell lines.

5.4. Role of insulators in the *KLK* cluster

The datasets from the ENCODE tracks in the UCSC Genome Browser helped us to notice that the *KLK* cluster is separated by putative insulators marked by CTCF binding sites. The different expression patterns for the regions of the *KLK* family members were compatible with the presence of CTCFs on the borders of the central region of the *KLK* cluster that would separate *KLK5, -6, -7, -8, -9* from the rest of the family. In general, insulators may regulate the transcription of specific genes by several mechanisms including restricting the enhancer-promoter interactions (Gomes, 2010) and by obstructing heterochromatin from spreading (Witcher, 2009). Interestingly, upon $1\alpha,25(OH)_2D_3$ stimulation of the prostate cells, the strength of CTCF binding in the *KLK* locus increased. This indicate VDR role in recruitment of CTCFs, which in turns may augment the transcription, limited to the central region of the *KLK* locus. Intriguingly, three out of five VDREs, predicted based on "open" chromatin markers, were located outside the CTCF-restricted region, while the other two were positioned in close proximity to the CTCF binding sites. These results would indicate the possibility of *KLK* genes regulation by distal and close (situated near *KLK6* gene promoter) response elements to happen only inside the loop, formed by flanking insulators (Philips, 2009). Perhaps VDR-dependent recruitment of CTCFs to their binding sites in the *KLK* locus could also influence the accessibility of functional VDREs, located outside the loop, to the active *KLK* genes.

CTCF, being a highly conserved protein, has also a crucial role in the chromatin architecture. It was proposed that CTCF can be "a leading candidate as a global genome organizer" to control chromatin organization and gene expression (Handoko, 2011). The VDR-related enriched association of CTCF with the *KLK* locus suggests a link between these two proteins in regulation of *KLK* genes. Since insulators may modify the chromatin conformation to control gene regulation we inhibited *CTCF* mRNA expression to investigate whether VDR

may then expand its action on the whole *KLK* gene cluster possibly by utilizing the active VDREs located on the border of the *KLK* locus. Surprisingly, our data did not show changes in the VDR-mediated regulation of the *KLK* locus. The genes were similarly induced in control samples as well as in samples treated with siRNA against *CTCF*, which indicate that loss of this insulator did not disturb the *KLK* genes induction by $1\alpha,25(OH)_2D_3$ in non-malignant prostate cells.

Perhaps, additional mechanisms might be involved in the control of the *KLK* gene expression e.g. the promoters of inactive genes may contains CpG islands that can be hypermethylated and result in formation of transcriptionally inactive state of chromatin (Esteller, 2007). An *in silico* analysis has shown that the *KLK* locus hold 16 CpG islands, interestingly absent in the region of *KLK6, -7, -8* and *-9* genes (Pampalakis, 2006, Pasic, 2012). The presence of CpG island in the regions of the *KLKs* not regulated by VDR may suggest their association with the DNA methylation and formation of heterochromatin structure.

Another argument why the *CTCF* inhibition did not broaden the VDR-mediated induction of the whole *KLK* cluster can be related to the fact that cohesin proteins may reside in CTCF sites and can have a role in insulator functions. Cohesin has been implicated to participate in gene regulation and this may also contribute to the CTCF positioning (Wendt, 2008). For instance, RAD21, a cohesin protein, was found to co-occupy many CTCF binding sites.

Mishiro *et al*. have shown that inhibition either of *CTCF* or *RAD21* was followed by disturbance in the chromatin structure and by expression of the apolipoprotein (*APO*) gene cluster (Mishiro, 2009).

Preliminary data have shown that *CTCF* silencing did not alter the expression profile of *RAD21* in non-malignant prostate cells, however upon $1\alpha,25(OH)_2D_3$ stimulation in CTCF-depleted conditions, *RAD21* gene was induced (see Appendix III).

Another study showed that *CTCF* silencing also did not reduce chromatin-bound cohesin level; likewise *CTCF* was also not reduced by depletion of *RAD21* or other cohesins. This led to the conclusion that CTCF may not be required for binding of RAD21 on DNA regions (Wendt, 2008), which could also suggest an involvement of cohesin protein complex in maintaining the DNA boundaries in the *KLK* locus. However, the exact mechanism of insulator-dependent chromatin loop formation that enables selective gene regulation through connection with distant regulatory elements to their promoters is not well known and is still under investigation.

5.5. Potential functions of VDR and KLKs in human non-malignant prostate cells

The enrichment analysis performed on data from non-malignant prostate cells stimulated with $1\alpha,25(OH)_2D_3$ provided also a representation of different pathways in which VDR may be involved, and the most enriched ones were "arachidonic acid metabolism", "p53 signaling pathway" and "cancer pathway". These data were consistent with the observation of Kovalenko *et al.*, showing that $1\alpha,25(OH)_2D_3$ affects processes involved in cancer development in prostate cells. The $1\alpha,25(OH)_2D_3$ target genes may have biological functions related to VDR's preventive impact on tumor growth (Kovalenko, 2010). Thus, we identified significantly up-regulated genes in the "cancer pathway" that act as tumor suppressors or transcription factors that control proliferation. Down-regulated genes were involved in angiogenesis, inhibition of apoptosis or they belonged to transcription factors over-expressed in cancer. Thus, the functional analysis of genes significantly regulated by $1\alpha,25(OH)_2D_3$ pointed at biological processes that encompass the anti-proliferative, pro-apoptotic and pro-differentiating role of VDR, which may be a prominent preventive factor against prostate cancer development.

Regarding the interactions between VDR-regulated genes and the potential KLKs substrates (obtained from the literature (Lawrence, 2010)), we identified several common and overlapping biological processes that might indicate a synergistic relationship between VDR and KLK proteins. The association of both groups of the selected genes with "cell adhesion", "response to wounding" or "regulation of cell proliferation" may implicate a common function in controlling these processes. For instance, KLKs are known for their role in degradation or remodeling of extracellular matrix by digestion of its components like collagen IV, fibronectin or laminin (Lawrence, 2010). This may affect the adhesion of cells, especially since KLKs cleave cell-cell adhesion molecules like desmoglein or E-cadherin (Borgono, 2007). Interestingly, Sung *et al.* have shown that in prostate cancer cells $1\alpha,25(OH)_2D_3$ can decrease the tumor invasion by reducing cell adhesion to the basement membrane protein laminin, in part via suppression of laminin receptors i.e. α6 and β4 integrins (Sung, 2000). Our study implicates that VDR-dependent activation of *KLKs* from the central part of their locus, triggers a cascade of events that may be also correlated with a function of a direct VDR target genes in a cellular processes. It would suggest a possibility of secondary or tertiary effect of this NR.

Further, we wondered if VDR, additionally to the induction of *KLKs*, also regulates KLK substrates in order to boost the KLK processes. Indeed, we observed that some of potential KLK substrates overlapped with the VDR-regulated genes. Moreover, we have noticed that VDR may intensify the KLKs function by inducing or repressing their substrates and VDR up-regulates substrates that are deactivated by KLK proteolysis. This observation implies a broad cellular response to $1\alpha,25(OH)_2D_3$ treatment. The conducted GO analysis showed relation to "the extracellular space and region" where we identified two members of IGFBP family. Intriguingly, the IGFBP3 protein encoded by a well-known VDR target gene may be cleaved and inactivated along with other IGFBP members by the KLK5 enzyme (Michael, 2006). Both, the *IGFBP3* and *KLK5* genes, are elevated upon $1\alpha,25(OH)_2D_3$ stimulation in prostate cells. The simultaneous up-regulation of IGFBP3 and its deactivator indicate an important function since this gene has a multifunctional role e.g. in promoting apoptosis or inhibiting the growth of prostate cancer cells (Liu, 2007). The IGFBP3 is also regulating the bioactivity of IGFs and its proteolysis may result in reduced affinity for IGF, which can make IGFs more available to the cell (Jerome, 2003). Perhaps KLK5 cleaves IGFBP3 in order to liberate the IGFs to exercise control of normal regulation of cell differentiation and proliferation in non-malignant prostate cells.

Importantly, KLK pro-proteins can be cleaved by other KLKs. For example, KLK7 is a substrate of KLK5. In our microarray analyses we identified *KLK5* as an early responding gene, while its substrate *KLK7* was late responding to $1\alpha,25(OH)_2D_3$. This data indicate that the activation of VDR is boosting the KLK5-KLK7 biochemical process. Furthermore, the KLK activity may be enhanced by the activation of VDR that mediates the mRNA down-regulation of the serine protease inhibitor *SERPINB2*.

Deregulated in tumor environment KLKs, excessive activate substrates like MMPs, PAR, uPA or TGFβ1 that are correlated with cancer development and progression (Avgeris, 2012; Kessenbrock 2011). Moreover, the KLKs ability to degrade ECM proteins (e.g. fibronectin) that act as a barrier for tumor cells to spread enables to facilitate angiogenesis and metastasis (Borgono, 2004). In accordance with these studies it was thought that extracellular proteases including MMPs and KLKs are involved in tumorigenesis (Egeblad, 2002). However, clinical studies on MMP inhibitors failed to show anti-cancer effect, on contrary, in some cases it was associated with promoting of tumor growth (Overall, 2002). This suggest more complex role of extacellular proteases in biological processes. Since then studies have shown that proteases can exhibit anti-tumor functions (Rodriguez, 2010). Interestingly, it was previously shown that the expression of *KLK6* in non-malignant and in cancer breast cell lines differs.

Moreover, this serine protease has anti-tumor properties that inhibit epithelial-mesenchymal transition in tumor cells and was proposed to be classified as a tumor suppressor (Pampalakis, 2009).

Our closer look into the KLK6 substrates has revealed some of the genes regulated by VDR in prostate. Intriguingly, myelin that may be cleaved by KLK6 is down-regulated by VDR in prostate cells. However, KLK6 may also take part in E-cadherin shedding in epidermal keratinocytes, which is a mark for the extracellular matrix degradation. In contrast, the gene encoding E-cadherin is induced upon VDR activation in prostate cells. The diversity of effects elicited by the KLK6 enzyme and members of its family may suggest that they can demonstrate a variety of functions. Therefore, the functional meaning of KLK6 up-regulation by $1\alpha,25(OH)_2D_3$ in non-malignant prostate cells needs to be further explored.

5.6. Conclusions

In this study we conducted a genome-wide expression profiling to reveal the most responsive target genes of VDR in non-malignant human prostate cells. Our data suggest that the second most responsive target under these conditions was the *KLK6* gene, a member of the largest contiguous cluster of genes encoding peptidases in human. Moreover, several other genes belonging to this family were also regulated by $1\alpha,25(OH)_2D_3$ in non-malignant prostate cells, while their induction in cancer cells was decreased or was abolished.

We showed that the expression of the central region of the *KLK* gene cluster depends on VDR activation and that *KLK6* is a direct target of VDR. Moreover, the transcriptional activation of *KLK6* gene begins rapidly upon $1\alpha,25(OH)_2D_3$ stimulation and the mRNA stability remained unchanged for indicated time.

The central region of the *KLK* cluster in prostate cells is separated from the other *KLK* members by CTCF insulators, whose occupancy is enhanced after $1\alpha,25(OH)_2D_3$ stimulation. The CTCF insulators may create boundaries allowing the selective regulation of the central *KLK* genes. Furthermore, from several putative VDREs we identified six functional ones from which three were located on the border of the *KLK* locus and the other three were situated in the central part of the *KLK* cluster and were encompassed by CTCFs. One evolutionary conserved VDRE was located close to the *KLK6* promoter, which indicated the direct action of VDR on this gene. This was also reflected in chromatin decondensation that occurred in a presence of histone modification like H4Ac, in non-malignant prostate cells. In contrast, the selected VDRE was not active in the cancer cell line, which affects the mRNA

expression profile. Looking on genomic context, basal expression level, responsiveness to VDR ligand we proposed a model that present formation of chromatin loop in the nucleolus.

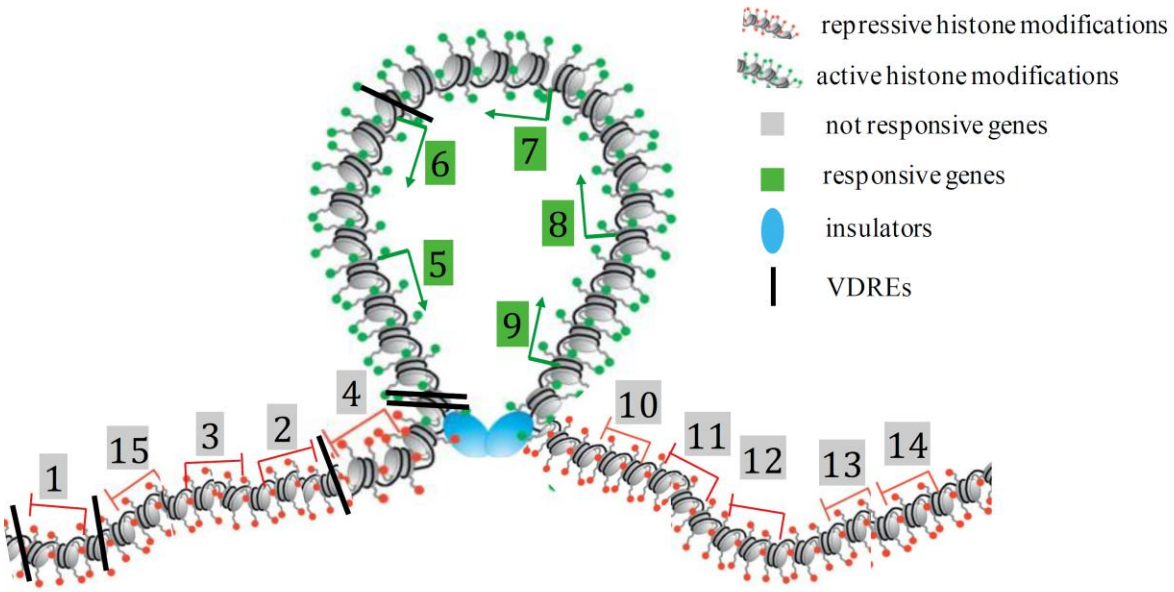


Figure 34. Model of kallikrein cluster organization in prostate cells upon 1α,25(OH)$_2$D$_3$ treatment in non-malignant prostate cells (adapted from Yang & Corces, 2012).

Therefore, our study for the first time provides insight into the transcriptional regulation of the expression on *KLK* gene family after VDR activation in prostate cells.

5.7. Future perspective

To further explore the regulation of expression of the whole *KLK* cluster, the silencing of *RAD21* alone and together with *CTCF* could provide detailed insights into the boundaries within the *KLK* locus. Additional ChIP experiments under *CTCF* and *RAD21* depletion conditions along with the analysis of chromatin markers such as H3K4me3 would possibly show abolition of VDR restriction in the *KLK* gene regulation. To investigate the functionality of VDREs located at the border of a cluster and close to the CTCF binding sites it would be necessary to perform chromosome conformation capture (3C) assays in order to verify long-distance interactions between regulatory elements and TSS regions. In addition, it would be a great of interest to analyze the responsiveness to 1α,25(OH)$_2$D$_3$ treatment of the

KLK genes located outside the central part of their locus and to analyze the presence of insulators in a malignant prostate cells.

Furthermore, it would be very interesting if the VDR-dependent mRNA regulation of the central *KLK* genes is also translated on the KLK protein level and whether the augmented proteins stay in the cell or are secreted immediately.

To broaden the investigation of gene regulation by VDR in human prostate, one could perform a genome-wide profiling of DNA-binding proteins and histone modifications as an epigenetic study, in both non-malignant and cancer cells, using ChIP-Seq experiments. The data would contribute to delineate the number of binding sites for selected nuclear receptors that might assist in regulation of genes involved in prostate cell function. Hence, the identification of RNA pol II, VDR, AR, histone acetylation (H4Ac, H3K27Ac), methylation (H3K4me1, H3K4me3) and of the presence of insulators would help to discover the complexity of the regulation of the human genome and its expression in prostate cells. For instance, it could allow identifying the functional distal and proximal response elements to which attracted VDR would bind and thus modulate expression of target genes.

In order to understand wide connections of VDR effects on the protein level in non-malignant and cancer prostate cells, it would be of great interest to perform a proteome analysis to investigate proteins interactions upon $1\alpha,25(OH)_2D_3$ treatment, using mass spectrometry technique, and compare them with both prostate cell lines. This challenging approach would also reveal possible differences in the modulated expressions of the genes mRNA and modulated expressions of their corresponding proteins.

6. REFERENCES

Altieri, D. C., Languino, L. R., Lian, J. B., Stein, J. L., Wijnen, A. J. V., Jiang, Z., & Stein, G. S. (2010). Prostate Cancer Regulatory Networks. *Cell*, *107*(5), 845-852.

Banach-Petrosky, W., Ouyang, X., Gao, H., Nader, K., Ji, Y., Suh, N., DiPaola, R. S., et al. (2006). Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1; Pten mutant mice. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *12*(19), 5895-901.

Baust, J. G., Ph, D., Klossner, D. P., Ph, D., Vanbuskirk, R. G., Ph, D., Gage, A. A., et al. (2010). Integrin involvement in freeze resistance of androgen-insensitive prostate cancer. *Prostate cancer and prostatic diseases*, *13*(2), 151-161.

Beer, T. M., Christopher, R., Venner, P., Daniel, P., Gurkamal, C., & Al., E. (2007). Double-Blinded Randomized Study of High-Dose Calcitriol Plus Docetaxel Compared With Placebo Plus Docetaxel in Androgen-Independent Prostate Cancer : A Report From the ASCENT Investigators. *Journal of Clinical Oncology*, *25*(6), 669-674.

Bello, D., Webber, M. M., Kleinman, H. K., Wartinger, D. D., & Rhim, J. S. (1997). Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis*, *18*(6), 1215-23.

Birney, E., Stamatoyannopoulos, J. a, Dutta, A., Guigó, R., Gingeras, T. R., Margulies, E. H., Weng, Z., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, *447*(7146), 799-816.

Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., & Keshet, E. (1999). Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *The Journal of clinical investigation*, *103*(2), 159-65.

Benjamini Y & Hochberg Y., E. (1995). Controlling of False Discovery Rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, *57*(1), 289-300.

Berger, M. F., Lawrence, M. S., Demichelis, F., Drier, Y., Cibulskis, K., Sivachenko, A. Y., Sboner, A., et al. (2011). The genomic complexity of primary human prostate cancer. *Nature*, *470*(7333), 214-20.

Birney, E., Stamatoyannopoulos, J. a, Dutta, A., Guigó, R., Gingeras, T. R., Margulies, E. H., Weng, Z., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, *447*(7146), 799-816.

Bikle, D. D. (2010). Vitamin D3: an ancient hormone. *Experimental Dermatology*, *20*(1), 7-13.

Bikle, D. D. (2011). Vitamin D and the skin: Physiology and pathophysiology. *Reviews in endocrine & metabolic disorders*, (08).

Blaber, M., Rodriguez, M., & Scarisbrick, I. A. (2004). Targeting kallikrein 6-proteolysis attenuates CNS inflammatory disease. *The FASEB Journal*, *1*, 1-25.

Blutt, S. E., McDonnell, T. J., Polek, T. C., & Weigel, N. L. (2000). Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. *Endocrinology*, *141*(1), 10-7.

Bookout, A. L., Jeong, Y., Downes, M., Yu, R. T., Evans, R. M., & Mangelsdorf, D. J. (2006). Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell*, *126*, 789-99.

Bouillon, R., Eelen, G., Verlinden, L., Mathieu, C., Carmeliet, G., & Verstuyf, A. (2006). Vitamin D and cancer. *The Journal of steroid biochemistry and molecular biology*, *102*, 156-162.

Campbell, M. J., & Adorini, L. (2006). The vitamin D receptor as a therapeutic target. *Expert Opinion Ther. Targets*, *10*(5), 735-748.

Cairns, B. R. (2009). The logic of chromatin architecture and remodelling at promoters. *Nature*, *461*, 20-22.

Chakalova, L., Debrand, E., Mitchell, J. A., Osborne, C. S., & Fraser, P. (2005). Replication and transcription: shaping the landscape of the genome. *Genetics*, *6*, 669-678.

Chen, T., & Holick, M. (2003). Vitamin D and prostate cancer prevention and treatment. *Trends in Endocrinology and Metabolism*, *14*(9), 423-430.

Chi, P., Allis, C. D., & Wang, G. G. (2010). Covalent histone modifications — miswritten, misinterpreted and mis-erased in human cancers, Nature Publishing Group *10*, 457-469.

Chodavarapu, R. K., Feng, S., Bernatavichute, Y. V., Chen, P.-yang, Stroud, H., Yu, Y., Hetzel, J. A., et al. (2010). Relationship between nucleosome positioning and. *Nature*, *466*(07), 388-392.

Clements, J. A., Willemsen, N. M., & Myers, S. A. (2004). The Tissue Kallikrein Family of Serine Proteases: Functional Roles in Human Biomarkers. *Life Sciences*, *41*(3), 265-312.

Costa-Neto, C. M., Dillenburg-Pilla, P., Heinrich, T. a, Parreiras-e-Silva, L. T., Pereira, M. G. a G., Reis, R. I., & Souza, P. P. C. (2008). Participation of kallikrein-kinin system in different pathologies. *International immunopharmacology*, *8*, 135-42.

Croswell, B. J. M., Kramer, B. S., & Crawford, E. D. (2011). Screening for Prostate Cancer With PSA Testing: Current Status and Future Directions. *Oncology*, *25*, 1-14.

Cuddapah, S., Jothi, R., Schones, D. E., Roh, T.-Y., Cui, K., & Zhao, K. (2009). Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. *Genome research*, *19*(1), 24-32.

De Bosscher, K & Haegeman, G (2003). The Interplay between the Glucocorticoid Receptor and Nuclear Factor- B or Activator Protein-1 : Molecular Mechanisms for Gene Repression. *Endocrine Reviews*, *24*(4), 488-522.

Debela, M., Beaufort, N., Magdolen, V., Schechter, N. M., Craik, C. S., Schmitt, M., Bode, W., et al. (2008). Structures and specificity of the human kallikrein-related peptidases KLK 4, 5, 6, and 7. *Biological chemistry*, *389*(6), 623-32.

Deeb, K. K., Trump, D. L., & Johnson, C. S. (2007). Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Cancer*, *7*, 684-700.

D'Ambrosio, D., Cippitelli, M., Cocciolo, M. G., Mazzeo, D., Di Lucia, P., Lang, R., Sinigaglia, F., et al. (1998). Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *The Journal of clinical investigation*, *101*(1), 252-62.

Du, P., Kibbe, W. a, & Lin, S. M. (2008). lumi: a pipeline for processing Illumina microarray. *Bioinformatics (Oxford, England)*, *24*(13), 1547-8.

Eduardo-Canosa, S., Fraga, R., Sigüeiro, R., Marco, M., Rochel, N., Moras, D., & Mouriño, A. (2010). Design and synthesis of active vitamin D analogs. *The Journal of steroid biochemistry and molecular biology*, *121*, 7-12.

Egeblad, M., & Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews Cancer*, *2*(March), 161-172.

Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., Iruela-Arispe, M. L., Zhang, J., Matusik, R., Thomas, G. V., et al. (2003). Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer cell*, *4*(3), 223-38.

Emami, N., & Diamandis, E. P. (2007). Human tissue kallikreins: a road under construction. *Clinica chimica acta; international journal of clinical chemistry*, *381*, 78-84.

Evans, R. M. (2003). An interview with Ronald M. Evans, Ph.D. Investigator, the Salk Institute for Biological Studies. *Assay and drug development technologies*, *1*(6), 749-54.

Feldman, D., Pike, W., Adams, J. (2011). Vitamin D. Third edition.

Fleet, J C. (2006). Molecular regulation of calcium and bone metabolism through the vitamin D receptor. *Journal of musculoskeletal & neuronal interactions*, *6*(4), 336-7.

Fleet, J. C., Smet, M. D. E., Johnson, R., & Li, Y. (2012). Vitamin D and cancer : a review of molecular mechanisms. *Biochemistry*, *76*, 61-76.

Fortier, A. H., Holaday, J. W., Liang, H., Dey, C., Grella, D. K., Vu, H., Plum, S. M., et al. (2003). Recombinant Prostate Specific Antigen Inhibits Angiogenesis InVitro and InVivo. *Prostate, The*, *56*, 212-219.

Germain, P., Altucci, L., Bourguet, W., Rochette-Egly, C., & Gronemeyer, H. (2003). Nuclear receptor superfamily: Principles of signaling. *Pure and Applied Chemistry*, *75*(11-12), 1619-1664.

Germain, Pierre, Staels, B., Dacquet, C., Spedding, M., & Laudet, V. (2006). Overview of Nomenclature of Nuclear Receptors. *Pharmacological Reviews*, *58*(4), 685-704.

Gregg, J. L., Brown, K. E., Mintz, E. M., Piontkivska, H., & Fraizer, G. C. (2010). Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection. *BMC cancer*, *10*(165), 1-14.

Greenhough, A., Smartt, H. J. M., Moore, E., Roberts, H. R., & Williams, A. C. (2009). The COX-2 / PGE 2 pathway : key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcenogenesis*, *30*(3), 377-386.

Gronemeyer, Hinrich, Gustafsson, J.-A., & Laudet, V. (2004). Principles for modulation of the nuclear receptor superfamily. *Nature reviews. Drug discovery*, *3*, 950-64.

Hakim, O., Sung, M.-hee, & Hager, G. L. (2010). 3D shortcuts to gene regulation. *Current Opinion in Cell Biology*, *22*, 305-313.

Hanahan, D., Weinberg, R. A., & Francisco, S. (2000). The Hallmarks of Cancer Review University of California at San Francisco. *Cell*, *100*, 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Review Hallmarks of Cancer : The Next Generation. *Cell*, *144*(5), 646-674.

Haussler, M. R., Ph, D., Jurutka, P. W., & Ph, D. (2011). Vitamin D receptor (VDR) - mediated actions of mechanisms. *Best Practice & Research Clinical Endocrinology & Metabolism*, *25*, 543-559.

Heintzman, N. D., Stuart, R. K., Hon, G., Fu, Y., Ching, C. W., Hawkins, R. D., Barrera, L. O., et al. (2007). Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nature Genetics*, *39*, 311-318.

Holick, M. F. (2011). Vitamin D deficiency in 2010: health benefits of vitamin D and sunlight: a D-bate. *Nature reviews. Endocrinology*, *7*, 73-5. Nature Publishing Group.

Holick, M. F., & Chen, T. C. (2008). Vitamin D deficiency: a worldwide problem with health consequences. *The American journal of clinical nutrition*, *87*, 1080S-1086S.

Huang, C.-Y., Beer, T. M., Higano, C. S., True, L. D., Vessella, R., Lange, P. H., Garzotto, M., et al. (2007). Molecular alterations in prostate carcinomas that associate with in vivo exposure to chemotherapy: identification of a cytoprotective mechanism involving growth differentiation factor 15. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *13*(19), 5825-5833.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Access*, *37*(1), 1-13.

Hussain, T., Gupta, S., & Mukhtar, H. (2003). Cyclooxygenase-2 and prostate carcinogenesis. *Cancer Letters*, *191*, 125-135.

Index, R., Development, T. R., & Team, C. (2008). *R: A Language and Environment for Statistical Computing* (Vol. 2).

Iwata, A., Maruyama, M., Akagi, T., & Hashikawa, T. (2003). Alpha-synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. *Human molecular genetics*, *12*(20), 2625-2635.

Jerome, L., & Shiry, L. (2003). Deregulation of the IGF axis in cancer : epidemiological evidence and potential therapeutic interventions. *Endocrine-Related Cancer*, *10*, 561-578.

Jia, L., Shen, H. C., Wantroba, M., Khalid, O., Liang, G., Wang, Q., Gentzschein, E., et al. (2006). Locus-Wide Chromatin Remodeling and Enhanced Androgen Receptor-Mediated Transcription in Recurrent Prostate Tumor Cells. *Molecular and cellular biology*, *26*(19), 7331-7341.

John, E. M., Schwartz, G. G., Koo, J., Berg, D. V. D., Ingles, S. a, Van Den Berg, D., & Ingles, S. a. (2005). Sun exposure, vitamin D receptor gene polymorphisms, and risk of advanced prostate cancer. *Cancer research*, *65*(12), 5470-9.

Jones, G, Strugnell, S. & DeLuca, H. F. (1998). Current understanding of the molecular actions of vitamin D. *Physiological reviews*, *78*(4), 1193-231.

Jonker, J, Stedman, C., Liddle, C., Downes, M. (2009). Hepatobiliary ABC transporters: physiology, regulation and implications in disease. *Frontiers in Bioscience*, *17*, 4904-4920.

Kang, Z., Jänne, O. a, & Palvimo, J. J. (2004). Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Molecular endocrinology (Baltimore, Md.)*, *18*(11), 2633-48.

Kang, Z., Pirskanen, A., Jänne, O. a, & Palvimo, J. J. (2002). Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. *The Journal of biological chemistry*, *277*(50), 48366-71.

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., & Haussler, D. (2002). The Human Genome Browser at UCSC. *Genome Research*, 996-1006.

Kim, M.-sun, Fujiki, R., Murayama, A., Kitagawa, H., Yamaoka, K., Yamamoto, Y., Mihara, M., et al. (2007). 1Alpha,25(OH)2D3-induced transrepression by vitamin D receptor through E-box-type elements in the human parathyroid hormone gene promoter. *Molecular endocrinology*, *21*(2), 334-42.

Kovalenko, P. L., Zhang, Z., Cui, M., Clinton, S. K., & Fleet, J. C. (2010). 1,25 dihydroxyvitamin D-mediated orchestration of anticancer , transcript-level effects in the immortalized , non-transformed prostate epithelial. *BMC Genomics*, *11*, 1-15.

Kriebitzsch, C., Verlinden, L., Eelen, G. U. Y., Tan, B. K., Camp, M. V. A. N., Bouillon, R., Verstuyf, A., et al. (2009). The Impact of 1,25(OH)2D3 and its Structural Analogs on Gene Expression in Cancer Cells – A Microarray Approach. *Anticancer research*, *29*, 3471-3483.

Krishnan, A. V., & Feldman, D. (2010). Molecular pathways mediating the anti-inflammatory effects of calcitriol: implications for prostate cancer chemoprevention and treatment. *Endocrine-Related Cancer*, 19-38.

Krishnan, A. V., Moreno, J., Nonn, L., Swami, S., Peehl, D. M., & Feldman, D. (2007). Calcitriol as a Chemopreventive and Therapeutic Agent in Prostate Cancer: Role of Anti-Inflammatory Activity. *Journal of Bone and Mineral Research*, *22*, 74-80.

Krishnan, A. V., Shinghal, R., Raghavachari, N., Brooks, J. D., Peehl, D. M., & Feldman, D. (2004). Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. *The Prostate*, *59*, 243-51.

Kundu, J. K., & Surh, Y.-J. (2008). Inflammation: gearing the journey to cancer. *Mutation research*, *659*, 15-30.

Larriba, M. J., Ordóñez-Morán, P., Chicote, I., Martín-Fernández, G., Puig, I., Muñoz, A., & Pálmer, H. G. (2011). Vitamin D Receptor Deficiency Enhances Wnt/β-Catenin Signaling and Tumor Burden in Colon Cancer. (M. Campbell, Ed.)*PLoS ONE*, *6*(8), e23524-e23524.

Lawrence, M. G., Lai, J., & Clements, J. a. (2010). Kallikreins on steroids: structure, function, and hormonal regulation of prostate-specific antigen and the extended kallikrein locus. *Endocrine reviews*, *31*, 407-46.

Li, J, Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, *275*, 1943-1947.

Li, P., Li, C., Zhao, X., Zhang, X., Nicosia, S. V., & Bai, W. (2004). p27(Kip1) stabilization and G(1) arrest by 1,25-dihydroxyvitamin D(3) in ovarian cancer cells mediated through down-regulation of cyclin E/cyclin-dependent kinase 2 and Skp1-Cullin-F-box protein/Skp2 ubiquitin ligase. *The Journal of biological chemistry*, *279*(24), 25260-7.

Lilja, H., Ulmert, D., & Vickers, A. J. (2008). Prostate-specific antigen and prostate cancer: prediction , detection and monitoring. *Nature*, *8*, 268-278.

Liu, B., Lee, K.-w, Anzo, M., Zhang, B., Zi, X., Tao, Y., Shiry, L., et al. (2007). Insulin-like growth factor-binding protein-3 inhibition of prostate cancer growth involves suppression of angiogenesis. *Oncogene*, *26*, 1811-1819.

Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., & Freedman, L. P. (1996). Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes & Development*, *10*, 142-153.

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., et al. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science (New York, N.Y.)*, *10.1126*, 1-8.

Lonard, D. M., Lanz, R. B., & O'Malley, B. W. (2007). Nuclear receptor coregulators and human disease. *Endocrine reviews*, *28*(5), 575-87.

Lou, Y.-R., Qiao, S., Talonpoika, R., Syvälä, H., & Tuohimaa, P. (2004). The role of Vitamin D3 metabolism in prostate cancer. *The Journal of steroid biochemistry and molecular biology*, *92*, 317-25.

López-Otín, C., & Matrisian, L. M. (2007). Emerging roles of proteases in tumour suppression. *Nature reviews. Cancer*, *7*(10), 800-8.

Lu, S., & Dong, Z. (2006). Characterization of TGF-b-Regulated Interleukin- 8 Expression in Human Prostate Cancer Cells. *The Prostate*, *66*, 996-1004.

Luo, L.-y, Diamandis, E. P., Look, M. P., Soosaipillai, A. P., & Foekens, J. A. (2002). Higher expression of human kallikrein 10 in breast cancer tissue predicts tamoxifen resistance. *British Journal of Cancer*, *86*, 1790-1796.

Magklara, A., Mellati, A. A., Wasney, G. A., Little, S. P., Sotiropoulou, G., Becker, G. W., & Diamandis, E. P. (2003). Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors. *Biochemical and biophysical research communications*, *307*, 948-955.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schiitq, G., Umesono, K., Blumberg, B., et al. (1995). The Nuclear Receptor Superfamily: The Second Decade. *Cell*, *83*, 835-839.

Mantell, Owens, Bundred, Mawer, & Canfield. (2000). 1a,25-Dihydroxyvitamin D3 inhibits angiogenesis in vitro and in vivo. *Circulation Research*, *87*, 214-220.

Masuda, S., & Jones, G. (2006). Promise of vitamin D analogues in the treatment of hyperproliferative conditions Review Promise of vitamin D analogues in the treatment of hyperproliferative conditions. *Molecular Cancer Therapeutics*, *5*, 797-808.

Matilainen, M., Malinen, M., Saavalainen, K., & Carlberg, C. (2005). Regulation of multiple insulin-like growth factor binding protein genes by 1alpha,25-dihydroxyvitamin D3. *Nucleic acids research*, *33*(17), 5521-32.

McKenna, N. J., Cooney, A. J., DeMayo, F. J., Downes, M., Glass, C. K., Lanz, R. B., Lazar, M. a, et al. (2009). Minireview: Evolution of NURSA, the Nuclear Receptor Signaling Atlas. *Molecular endocrinology*, *23*(6), 740-746.

Meyer, M. B., Goetsch, P. D., & Pike, J. W. (2010a). A downstream intergenic cluster of regulatory enhancers contributes to the induction of CYP24A1 expression by 1alpha,25-dihydroxyvitamin D3. *The Journal of biological chemistry*, *285*(20), 15599-610.

Meyer, M. B., Goetsch, P. D., & Pike, J. W. (2010b). Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. *The Journal of steroid biochemistry and molecular biology*, *121*(1-2), 136-41.

Mordan-McCombs, S., Brown, T., Wang, W.-L. W., Gaupel, A.-C., Welsh, J., & Tenniswood, M. (2010). Tumor progression in the LPB-Tag transgenic model of prostate cancer is altered by vitamin D receptor and serum testosterone status. *The Journal of steroid biochemistry and molecular biology*, *121*, 368-71.

Moreno, J., Krishnan, A. V., & Feldman, D. (2005). Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *The Journal of steroid biochemistry and molecular biology*, *97*, 31-6.

Moreno, J., Krishnan, A. V., Peehl, D. M., & Feldman, D. (2006). Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer research*, *26*, 2525-30.

Murthy, S., & Weigel, N. L. (2004). 1a , 25-Dihydroxyvitamin D 3 Induced Growth Inhibition of PC-3 Prostate Cancer Cells Requires an Active ransforming Growth Factor Beta Signaling Pathway. *The Prostate*, *59*, 282-291.

Nagy, L., Schüle, R., & Gronemeyer, H. (2006). Twenty years of nuclear receptors: Conference on Nuclear Receptors: from Chromatin to Disease. *EMBO reports*, *7*(6), 579-84.

Nakagawa, K., Kawaura, A., & Kato, S. (2005). 1a, 25-Dihydroxyvitamin D 3 is a preventive factor in the metastasis of lung cancer. *Carcinogenesis*, *26*(2)

Nerenz, R. D., Martowicz, M. L., & Pike, J. W. (2008). An enhancer 20 kilobases upstream of the human receptor activator of nuclear factor-kappaB ligand gene mediates dominant activation by 1,25-dihydroxyvitamin D3. *Molecular endocrinology*, *22*(5), 1044-56.

Norman, A. W. (2006). Minireview: vitamin D receptor: new assignments for an already busy receptor. *Endocrinology*, *147*(12), 5542-8.

Norman, A. W., & Bouillon, R. (2010a). Vitamin D nutritional policy needs a vision for the future. *Experimental biology and medicine (Maywood, N.J.)*, *235*, 1034-45.

Norman, A. W., & Bouillon, R. (2010b). Vitamin D nutritional policy needs a vision for the future. *Experimental Biology and Medicine*, 1034-1045.

Overall, Mark, & López-otín, C. (2002). Strategies for MMP inhibition in cancer :innovations for the post-trial era. *Nature*, *2*(September), 657-672.

Paliouras, M., Borgono, C., & Diamandis, E. P. (2007). Human tissue kallikreins: the cancer biomarker family. *Cancer letters*, *249*, 61-79.

Pampalakis, G., Diamandis E., & Sotiropoulou, G. (2006). The epigenetic basis for the aberrant expression of kallikreins in human cancers *Biological Chemistry*, *387*, 795-799.

Pampalakis, G., Arampatzidou, M., Amoutzias, G., Kossida, S., & Sotiropoulou, G. (2008). Identification and analysis of mammalian KLK6 orthologue genes for prediction of physiological substrates. *Computational Biology and Chemistry*, *32*, 111-121.

Pampalakis, G., Prosnikli, E., Agalioti, T., Vlahou, A., Zoumpourlis, V., & Sotiropoulou, G. (2009). A tumor-protective role for human kallikrein-related peptidase 6 in breast cancer mediated by inhibition of epithelial-to-mesenchymal transition. *Cancer research*, *69*, 3779-3787.

Pasic, M. D., Olkhov, E., Bapat, B., & Yousef, G. M. (2012). Epigenetic regulation of kallikrein-related peptidases: there is a whole new world out there. *Review Literature And Arts Of The Americas*, *393* (May), 319-330.

Payne, H., & Mason, M. (2011). Androgen deprivation therapy as adjuvant / neoadjuvant to radiotherapy for high-risk localised and locally advanced prostate cancer: recent developments. *British Journal of Cancer*, *105*, 1628-1634.

Peehl, D M, & Feldman, D. (2003). The role of vitamin D and retinoids in controlling prostate cancer progression. *Endocrine-related cancer*, *10*(2), 131-40.

Peng, L., Malloy, P. J., & Feldman, D. (2004). Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. *Molecular endocrinology (Baltimore, Md.)*, *18*(5), 1109-19.

Peng, L., Wang, J., Malloy, P. J., & Feldman, D. (2008). The role of insulin-like growth factor binding protein-3 in the growth inhibitory actions of androgens in LNCaP human prostate cancer cells. *Growth (Lakeland)*, *566*(May 2007), 558-566.

Perissi, V., & Rosenfeld, M. G. (2005a). Controlling nuclear receptors: the circular logic of cofactor cycles. *Nature Reviews Molecular Cell Biology*, *6*(7), 542-554. doi:10.1038/nrm1680

Perissi, V., & Rosenfeld, M. G. (2005b). Controlling Nuclear Receptors: The Circular Logic of Cofactor Cycles. *Nature Reviews Molecular Cell Biology*, *6*(J), 542-554.

Pike, J. W. (2011). Genome-wide principles of gene regulation by the vitamin D receptor and its activating ligand. *Molecular and cellular endocrinology*, *347*, 3-10.

Pike, J. W., Meyer, M. B., Martowicz, M. L., Bishop, K. a, Lee, S. M., Nerenz, R. D., & Goetsch, P. D. (2010). Emerging regulatory paradigms for control of gene expression by 1,25-dihydroxyvitamin D3. *The Journal of steroid biochemistry and molecular biology*, *121*, 130-5.

Polek, Tara C, & Weigel, N. L. (2002). Vitamin D and prostate cancer. *Journal of andrology*, *23*(1), 9-17.

Prosser, D. E., & Jones, G. (2004). Enzymes involved in the activation and inactivation of vitamin D. *Trends in biochemical sciences*, *29*(12), 664-73.

Reichrath, J., Lehmann, B., Carlberg, C., Varani, J., & Zouboulis, C. C. (2007). Vitamins as Hormones. *Hormone and Metabolic Research*, *00*, 1 - 14.

Rodríguez, D., Morrison, C. J., & Overall, C. M. (2010). Matrix metalloproteinases : What do they not do ? New substrates and biological roles identified by murine models and proteomics. *BBA - Molecular Cell Research*, *1803*(1), 39-54.

Rohan, J. N. P., & Weigel, N. L. (2009). 1Alpha,25-dihydroxyvitamin D3 reduces c-Myc expression, inhibiting proliferation and causing G1 accumulation in C4-2 prostate cancer cells. *Endocrinology*, *150*(5), 2046-54.

Samuel, S., & Sitrin, M. D. (2008). Vitamin D's role in cell proliferation and differentiation. *Nutrition reviews*, *66*, S116-24.

Sandelin, A., Alkema, W., Engström, P., Wasserman, W. W., & Lenhard, B. (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic acids research*, *32*(Database issue), D91-4.

Saramäki, A., Banwell, C. M., Campbell, M. J., & Carlberg, C. (2006). Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic acids research*, *34*(2), 543-54.

Sardana, G., & Diamandis, E. P. (2009). The kallikrein family of proteins as urinary biomarkers for the detection of prostate cancer. *Clinical Biochemistry*.

Schauber, J., Dorschner, R. A., Coda, A. B., Büchau, A. S., Liu, P. T., Kiken, D., Helfrich, Y. R., et al. (2007). Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D – dependent mechanism. *The Journal of clinical investigation*, *117*(3), 803-811.

Schones, D. E., & Zhao, K. (2008). Genome-wide approaches to studying chromatin modifications. *Nature reviews. Genetics*, *9*, 179-191.

Schwartz, G. G. (2009). Vitamin D and intervention trials in prostate cancer: from theory to therapy. *Annals of epidemiology*, *19*, 96-102.

Sharifi, N., Gulley, J. L., & Dahut, W. L. (2010). An update on androgen deprivation therapy for prostate cancer. *Endocrine-Related Cancer*, 305-315.

Smyth, G. (2004). Limma Package Vignette, (October), 2004.

Sonoda, J., Pei, L., & Evans, R. M. (2008). Nuclear receptors: decoding metabolic disease. *FEBS letters*, *582*(1), 2-9.

Sotiropoulou, G., Pampalakis, G., Diamandis, E. P., & Hospital, M. S. (2009). Functional roles of human kallikrein-related peptidases. *Journal of Biological Chemistry*, *284* (48), 32989 -32994.

Sproul, D., Gilbert, N., & Bickmore, W. A. (2005). The role of chromatin expression of chromatin expression of clustered genes. *Nature reviews. Genetics*, *6*, 775-782.

Srinivas, S., & Feldman, D. (2009). A Phase II Trial of Calcitriol and Naproxen in Recurrent Prostate Cancer. *Anticancer research*, *29*, 3605-3610.Stewart, L. V., Lyles, B., Lin, M.-fong, & Weigel, N. L. (2005). Vitamin D receptor agonists induce prostatic acid phosphatase to reduce cell growth and HER-2 signaling in LNCaP-derived human prostate cancer cells. *Journal of Steroid Biochemistry*, *97*, 37-46.

Suganuma, T., & Workman, J. L. (2008). Minireview Crosstalk among Histone Modifications. *Cell*, *135*, 604-607.

Sung, V., & Feldman, D. (2000). 1,25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration. *Molecular and cellular endocrinology*, *164*, 133-43.

Sutherland, H., & Bickmore, W. a. (2009). Transcription factories: gene expression in unions? *Nature reviews. Genetics*, *10*, 457-66.

Swami, S., Krishnan, A. V., & Feldman, D. (2011). Vitamin D metabolism and action in the prostate: Implications for health and disease. *Molecular and cellular endocrinology*, *347*, 61-69.

Tata, J. R. (2002). Signalling through nuclear receptors. *Nature reviews. Molecular cell biology*, *3*, 702-10.

Taylor, W., Mathias, A., Ali, A., Ke, H., Stoynev, N., Shilkaitis, A., Green, A., et al. (2010). p27(Kip1) deficiency promotes prostate carcinogenesis but does not affect the efficacy of retinoids in suppressing the neoplastic process. *BMC cancer*, *10*, 541-553.

Thorne, J. L., Maguire, O., Doig, C. L., Battaglia, S., Fehr, L., Sucheston, L. E., Heinaniemi, M., et al. (2010). Epigenetic control of a VDR-governed feed-forward function in non-malignant prostate cells. *Nucleic Acids Research*, *21*, 1-12.

Thorne, J., & Campbell, M. J. (2008). The vitamin D receptor in cancer. *The Proceedings of the Nutrition Society*, *67*, 115-27.

Tokar, E. J., & Webber, M. M. (2005). Chemoprevention of prostate cancer by cholecalciferol (vitamin D3): 25-hydroxylase (CYP27A1) in human prostate epithelial cells. *Clinical & Experimental Metastasis*, *22*, 265-273.

Tomlins, S. a, Laxman, B., Dhanasekaran, S. M., Helgeson, B. E., Cao, X., Morris, D. S., Menon, A., et al. (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature*, *448*, 595-601.

Trump, D., Potter, D., Muindi, J., Brufsky, A., & Johnson, C. S. (2006). Phase II Trial of High-Dose, Intermittent Calcitriol (1 , 25 Dihydroxyvitamin D3) and Dexamethasone in Androgen-Independent Prostate Cancer. *Cancer*, (April), 2136-2142.

Veer, L. J. V., Dai, H., Vijver, M. J. V. D., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, *415*(345).

Venning, G. (2005). Clinical review weakness among elderly people. *Journal of Bone and Mineral Research*, *330* (March).

Vijjan, V., & Dubey, D. (2007). New therapeutic targets in the treatment of prostate cancer. *Indian Journal Of Urology*, *23*, 61-66.

Wang, T.-T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., Bourdeau, V., et al. (2005). Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Molecular endocrinology (Baltimore, Md.)*, *19*(11), 2685-95.

Wang, Wei-Lin W, Chatterjee, N., Chittur, S. V., Welsh, J., & Tenniswood, M. P. (2011). Effects of 1α,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. *Molecular cancer*, *10*, 58-73.

Wang, Qianben, Li, W., Zhang, Y., Yuan, X., Xu, K., Yu, J., Chen, Z., et al. (2009). Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell*, *138*, 245-56.

Weake, V. M., & Workman, J. L. (2010). Inducible gene expression: diverse regulatory mechanisms. *Nature reviews. Genetics*, *11*, 426-37.

Weinberg, R. (2007). The biology of cancer. *Garland Science, Taylor & Francis Group, LLC*.

White, J. H. (2011). Vitamin D metabolism and signaling in the immune system. *Reviews in endocrine & metabolic disorders*.

Wu, J. I., Lessard, J., & Crabtree, G. R. (2009). Understanding the words of chromatin regulation. *Cell*, *136*, 200-206.

Yang, X., Downes, M., Yu, R. T., Bookout, A. L., He, W., Straume, M., Mangelsdorf, D. J., et al. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell*, *126*, 801-810.

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, et al. (1997). Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning *Nature Genetics*, *16*, 391-396.

Yousef, G M, & Diamandis, E. P. (2001). The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocrine reviews*, *22*(2), 184-204.

Yousef, G. M., Scorilas, A., Chang, A., Rendl, L., Diamandis, M., Jung, K., & Diamandis, E. P. (2002). Down-Regulation of the Human Kallikrein Gene 5 (KLK5) in Prostate CancerTissues. *Analysis*, *132*(July 2001), 126-132.

Yousef, George M, & Diamandis, E. P. (2003). Human Kallikreins: Common Structural Features , Sequence Analysis and Evolution. *Current Genomics*, *4*, 147-165.

Yu, Jindan, Yu, J., Mani, R.-shankar, Cao, Q., Brenner, C. J., Cao, X., Wang, X., et al. (2010). An Integrated Network of Androgen Receptor, Polycomb, and TMPRSS2-ERG Gene Fusions in Prostate Cancer Progression. *Cancer Cell*, *17*, 443-454.

Zella, L. A., Meyer, M. B., Nerenz, R. D., Lee, S. M., Martowicz, M. L., & Pike, J. W. (2009). Multifunctional enhancers regulate mouse and Human Vitamin D Receptor Gene Transcription. *Molecular Endocrinology*, *23* (December), 1-20.

Zhuang, S.-hong, & Burnstein, K. L. (1998). Antiproliferative Effect of 1a,25-Dihydroxyvitamin D3 in Human Prostate Cancer Cell Line LNCaP Involves Reduction of Cyclin-Dependent Kinase 2 Activity and Persistent G1 Acumulation. *Endocrynology*, *139*(3), 1197-1207.

7. APPENDIX I

A tables that representing primer list of selected genes (Table 9) and the predicted VDREs using JASPAR dataset (Table 10) are shown below.

Table 9. A primer list of selected genes

Primer pair	Sequence
KLK3	F - TGCTGTGAAGGTCATGGACC R - CCAGCACACAGCATGAACTTG
KLK4	F - CGGTGGTGTCTGAGGAGGTCTGC R - TTCCTTCCGCAGGATGTATTTGG
KLK5	F - CTGGATGTGGGTGCTCTGTGCTC R - GATGCGGCTGCTGCTGTCATC
KLK6	F - GTGATTTCCCTGACACCATCCAGTG R - TGGCTTCTCCTTTGATCCACAGG
KLK7	F - TGGCATCCCCGACTCCAAGA R - TTAACTCAGTGTGGCGTTAGCGATG
KLK8	F - ATGGTCTGTGCAGGCAGCAGC R - TGCTTATCCTAGAATCAGCCCTTGCT
KLK9	F - GCATCCTGGAGAACAAACTCTGTCACT R - CCAGTCAAGGTAGTGGCATACGCTG
KLK10	F - CAGCATCACTATCCTGAGCCCTAAAGA R - AGCTGGAGCGTAGCATCTGGATC
KLK11	F - ACCATCATTGAGCACCAGAAGTGTGAG R - CCAGTCCACATATTTGCAGACTTTCGTG
KLK12	F - CTCCATCGTCTCCCATGCCACCT R -CCAGGGATGCCATCTTGTCCACAG
KLK13	F - CGGCACAAAAGAGGGTGGCAAAGAC R - AGGGCTAAGCAGACCATGTGAGGAAG
KLK14	F - TGGTCTGTGCAGGAGTTCCCCAGG R - CTCCATTCCCCAAGACACGAGGC
KLK15	F - CTCCCAGATACGTTGCATTGTGCCA R - CAGGCTTGGTGGTGTTGTCACAAGG
RPLPO	F - AGATGCAGCAGATCCGCAT R - GTGGTGATACCTAAAGCCTG

IGFBP3	F - AAGTTGACTACGAGTCTCAG R - AATCAGTTCACCACAAACAGA
CYP24	F - GACTACCGCAAAGAAGGCTAC R - CATCACTTCCCCTGGTTTCATTA
IL8	F - TAGCAAAATTGAGGCCAAGG R -AAACCAAGGCACAGTGGAAC
VDR	F - AGATGACCCTTCTGTGACCC R - AGCTTCTTCAGTCCCACCTG
CTCF	F - CAG-ATC-TGG-ACG-ACA-ATG-AGG R - AAC-ACA-GCC-CAG-AGA-AGT-CC
RAD21	F - GAGTCAGCTATGCCTCCAC R -CCTGATGCATCTTCATCCTC
KLK4_TSS	F - CCCAGACACGGCTAAGTCTC R -AGATACCTGCGACACCAAGG
KLK5_TSS	F - GCTATTGCTAAGGCCCGATA R -CTCAGCCGCAGACTTCTCAG
KLK6_TSS1	F - CTGCTGGGAGCATGGCACTG R -CTCTGTGTGCTGCCTGCCGA
KLK6_TSS2	F - GCCTGGGTAAGGAGGAAAAG R -TGGAAGAGGCTGGTTCTGTC
KLK6_TSS3	F - AAAGGACGTTCCAGAAGCAT R - GCCGTGAGGAGAGAAGACAC
KLK7_TSS1	F - CCAGCAGAGGGATGAAGAT R - GAGCAGAGTCAGGCTTGGAG
KLK7_TSS2	F - CCAGCAGAGGGATGAAGAT R - CCCAAATTGCAGACTTCCAG
KLK8_TSS	F - TCCTCCCTCAAGATTTCACG R - ACTGGGAATCTCCACCTCC
KLK9_TSS	F - GAAGGAGATGGCATGGCTTA R - CAGCAGAGAGAGCAGAGCAC
KLK10_TSS1	F - TCCCATTGATTTCCTCTTGC R -GCCCTGGAATACAGGCTTC
KLK10_TSS2	F - CCTCACCCACGGTACAATTAG R - GAGTCGCCGATAGGAAGGAG
KLK10_TSS3	F - CACACACAGAGCTCACTCACG R - CAGGTTGAGTAGGAGGGACAG
KLK11_TSS1	F - GGGACGTGGCTTTGTTCTAA

	R - TCCCAGTGGAGACAGAATCC
KLK11_TSS2	F - CCCACGTCATAAGGGTAGTCA R -ATTCCTGCTTCTCCCAGCTTG
KLK12_TSS	F - GCCAGGTGCAGAGTGGTAG R - CCTCTACGTGGCTGTCACTG
KLK13_TSS	F - GAGTGAATGAGGCGAGGAGA R - CGCATTCTTACCTCCTGACAA
KLK14_TSS	F - GCATCTCCAGCTCTCACTTTG R - AGATACCTGGACCTGGAGCA
IGFBP3_RE8	F - CTGGAGTGACTCACCAGAGTC R - CTTGCCTGCCTCTCTCAGCTG
KLK_RE1	F - GAGCCGTGTCCTACCTAAGC R - AGACCTCGGGTGCTATTCCT
KLK_RE2	F - GAGGAAACTGAGGCCCAGAC R - GTGTCCCCGTCTGTATCAGG
KLK_RE3	F - CCCATACACACGTCATACGG R - CACCCTCACAACCTCAGCTA
KLK_RE4	F - CCACTCTCCCTCAGTCCTACA R - CGCCATGAAGTTCCTATGC
KLK_RE5	F - GATCTTCAGCCAATTCTGTGC R - GGCCACCTTCTGATGCTGTT
KLK_RE6	F - GGCTCACGCCTGCTATCCCAAC R - GTCTCAGAGTCTCACTCTGTCACC
KLK_RE7	F - GGTTCTGATGGCCCATTGTTTC R - CACTGATCTTGTTGACTTGGA
KLK_RE8	F - CGAGACTGGTCACTGCACTC R - GACTACAGGTGTGCAACAGCA
CTCF_1	F - CCAGATAGGGAAATGCGACC R - GCGGTCCAATGCCACCTAG
CTCF_2	F - GGAGTCATCCATCACCCTGAC R - CACAGTTGTAGGCGCACTTG
CTCF_3	F - CTGACCTCAAGTGATCCAC R - ATAAGATCCCCTGGTGTTTCG
CTCF_4	F - CTAATCCAGCCACCACCAGT R -AGCAAGGATCTGAAGCAAGG
CTCF_5	F - GCCATCTAACTCAGTGCTGCT R - AAGAAGTCAGTTAGCCTGCACA

Table 10. A list of potential VDREs predicted by JASPAR inside the *KLK* locus. According to the JASPAR VDR weight matrix (80% cut offs) there were detected 30 putative VDREs, one of which was located in close proximity to *KLK6* TSS.

JASPAR VDRE sequences	Position at chromosome 19		
GGGTCACAGGGATTC	56033320	56033338	
GGGTGTCTGAGTTCA	56039257	56039275	
GGGTCATAGAGTCAA	56045445	56045463	
GGGTCTTGGAGTGCA	56049642	56049660	
AGGTGACCAAGTTCA	56053606	56053624	
GGGTCCACGAGGACA	56054785	56054803	
AGGGCACATGGTTCA	56055103	56055121	
GGGTTGATAGGTACA	56057298	56057316	
AGGTCAGGAGTTTCA	56060696	56060714	
GGGTCACTGACTACA	56062825	56062843	
GGGACACTGGATTCA	56066602	56066620	
GGGTCAGGGGGATCA	56062825	56069777	
AGGTGACAGAGTTCA	56072019	56072037	
GGGTCCTGAGTTTCA	56079652	56079670	
AGGTCACTGGATACA	56069759	56087294	
GGGCCACAGAGTTTC	56101291	56101309	
GGGTCATAGAGCTTA	56103489	56103507	
GTGTCAAATGGTTCA	56121828	56121846	
GGGTGAAGGGGTTCT	56122078	56122096	
GGGTCTAAAGGTTGA	56139382	56139400	
AGGTAAATGAGTTAA	56141351	56141369	
GGGTCACCAAGAGCA	56163960	56163978	VDRE close to the *KLK6* TSS
GGGTCATTGGGTTGG	56172672	56172690	
GAGTCATCAGCTTCA	56174257	56174275	
GGGTCACTGAGGCCA	56175289	56175307	
GGGTCACAGTGTTCA	59187219	56187237	
GGATCAAGAAGATCA	56191145	56191163	
GAGTTATAGGGTTCA	56250950	56250968	
GGGTCATCCAGTAGA	56257290	56257308	
AGGTCCATGGGTTCC	56276369	56276387	

APPENDIX II

The Volcano Plots showed below visualize the fold change of genes stimulated with 1α,25(OH)$_2$D$_3$ for 4 h (Fig. 35A) or 24 h (Fig. 35B) in non-malignant prostate cells. Data are taken from the RWPE1 microarray.

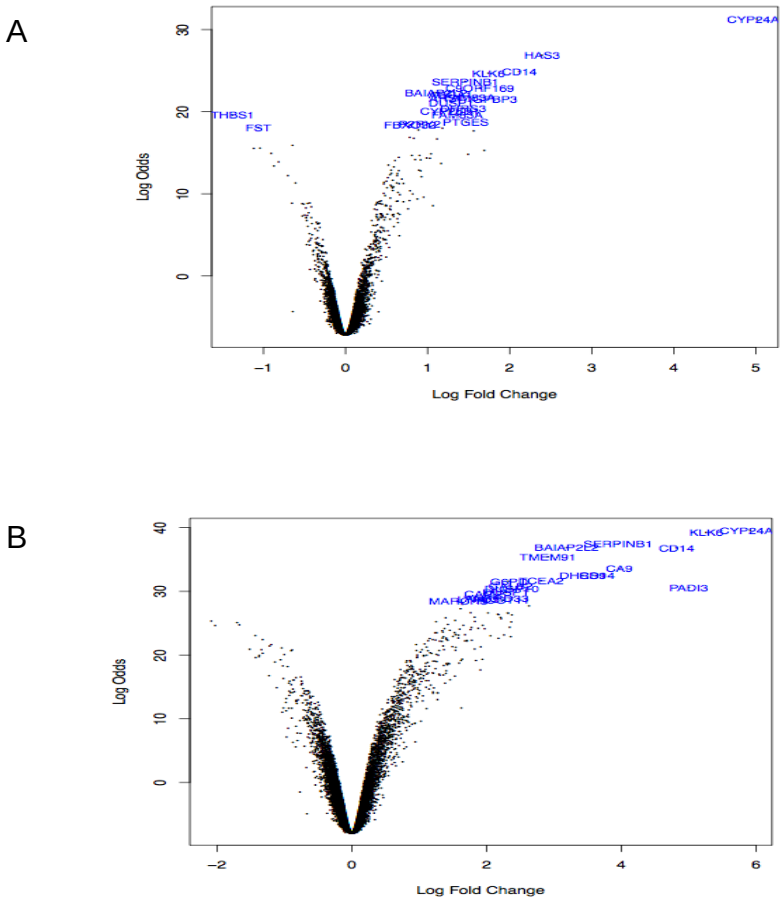


Figure 35. Volcano plot of significantly expressed genes after 4 h (A) or 24 h (B) of 1α,25(OH)$_2$D$_3$ stimulation of non-malignant prostate RWPE1 cells.

Tables presented below represents fold changes of genes regulated by $1\alpha,25(OH)_2D_3$ after 4 h (Table 11) or 24 h (Table 12) of treatment in RWPE1 cells. Table 12 shows top 100 genes both significantly up- or down-regulated.

Table 11. List of top significantly up- or down-regulated genes after 4 h of treatment with $1\alpha,25(OH)_2D_3$ in RWPE1 cells

Gene symbol	Gene name	Fold change
Up-regulated genes		
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	32.35
HAS3	hyaluronan synthase 3	5.27
CD14	CD14 molecule	4.35
IGFBP3	insulin-like growth factor binding protein 3	3.48
KLK6	kallikrein-related peptidase 6	3.34
PADI3	peptidyl arginine deiminase, type III	3.25
C9ORF169	chromosome 9 open reading frame 169	3.11
FAM83A	family with sequence similarity 83, member A	2.85
ANGPTL4	angiopoietin-like 4	2.83
PTGES	prostaglandin E synthase	2.77
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	2.75
DHRS3	dehydrogenase/reductase (SDR family) member 3	2.71
DUSP1	dual specificity phosphatase 1	2.45
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	2.43
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	2.42
ARSI	arylsulfatase family, member I	2.32
TMEM156	transmembrane protein 156	2.27
TINAGL1	tubulointerstitial nephritis antigen-like 1	2.25
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	2.17
BAIAP2L2	BAI1-associated protein 2-like 2	2.15
IL8	interleukin 8	2.09
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	2.06
NRIP1	nuclear receptor interacting protein 1	2.04
NET1	neuroepithelial cell transforming 1	1.94
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	1.90
DHRS9	dehydrogenase/reductase (SDR family) member 9	1.89
TRIM6	TRIM6-TRIM34 readthrough transcript; tripartite motif-containing 6	1.89
P2RY2	purinergic receptor P2Y, G-protein coupled, 2	1.87
KRT15	keratin 15	1.83
AKAP12	A kinase (PRKA) anchor protein 12	1.80
TXNRD1	thioredoxin reductase 1; hypothetical LOC100130902	1.77
IL1RL1	interleukin 1 receptor-like 1	1.75
FBXO32	F-box protein 32	1.72
FAM84B	family with sequence similarity 84, member B	1.70
RP5-1022P6.2	glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)	1.63
FAM20C	family with sequence similarity 20, member C	1.63
HS.551538		1.61
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	1.58
DUSP10	dual specificity phosphatase 10	1.58
NINJ1	ninjurin 1	1.57
FHL2	four and a half LIM domains 2	1.57
NAV2	neuron navigator 2	1.56
KLK5	kallikrein-related peptidase 5	1.56
EMP1	epithelial membrane protein 1	1.56
THBD	thrombomodulin	1.56
SMAD3	SMAD family member 3	1.55
TFCP2L1	transcription factor CP2-like 1	1.55
C20ORF100	chromosome 20 open reading frame 100	1.55
FOXQ1	forkhead box Q1	1.54

TMEM91	transmembrane protein 91	1.52
FAM129A	family with sequence similarity 129, member A	1.51
C2CD2	C2 calcium-dependent domain containing 2	1.50
IL1B	interleukin 1, beta	1.50
FOXA1	forkhead box A1	1.50
PTPN1	protein tyrosine phosphatase, non-receptor type 1	1.49
ZFP36	zinc finger protein 36, C3H type, homolog (mouse)	1.49
GRB7	growth factor receptor-bound protein 7	1.49
TACSTD2	tumor-associated calcium signal transducer 2	1.49
FAT2	FAT tumor suppressor homolog 2 (Drosophila)	1.48
RFFL	ring finger and FYVE-like domain containing 1	1.48
MBP	myelin basic protein	1.47
KRT13	keratin 13	1.47
CA2	carbonic anhydrase II	1.46
SNRK	SNF related kinase	1.46
ASB1	ankyrin repeat and SOCS box-containing 1	1.46
HOXA5	homeobox A5	1.46
MMD	monocyte to macrophage differentiation-associated	1.46
BHLHB2	basic helix-loop-helix family, member e40	1.45
UPP1	uridine phosphorylase 1	1.45
FAM43A	family with sequence similarity 43, member A	1.44
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	1.43
STAT4	signal transducer and activator of transcription 4	1.43
CRABP2	cellular retinoic acid binding protein 2	1.43
HRCT1	histidine rich carboxyl terminus 1	1.42
INPP1	inositol polyphosphate-1-phosphatase	1.41
BTBD11	BTB (POZ) domain containing 11	1.41
Down-regulated genes		
ZBED2	zinc finger, BED-type containing 2	0.70
FOXA2	forkhead box A2	0.69
IER3	immediate early response 3	0.69
LOC652235	thioredoxin reductase 1; hypothetical LOC100130902	0.66
VEGFC	vascular endothelial growth factor C	0.64
BIRC3	baculoviral IAP repeat-containing 3	0.64
PHACTR3	phosphatase and actin regulator 3	0.61
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	0.57
FST	follistatin	0.55
PTHLH	parathyroid hormone-like hormone	0.53
THBS1	thrombospondin 1	0.39

A heat map shown in a Figure 36 represents genes regulated after 24 h of 1α,25(OH)$_2$D$_3$ treatment in RWPE1 cells. There were 663 genes significantly up- or down-regulated in a VDR-dependent manner.

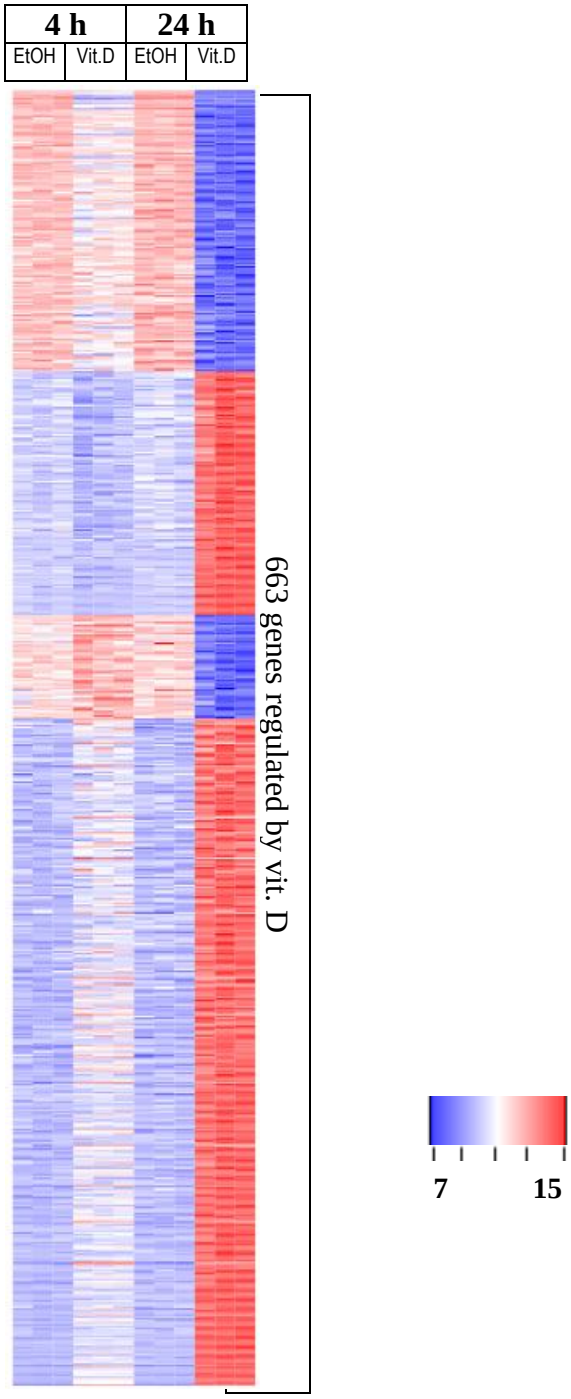


Figure 36. Microarray analysis revealed secondary VDR target genes in RWPE1 cells. Heat map displaying the gene expression profile of differential expressed genes according to applied cut offs (p<0.01, log2FC>0.5 or <-0.5). There were 467 genes significantly up-regulated and 196 significantly down-regulated genes upon 24 h of 1α,25(OH)$_2$D$_3$ stimulation. Red indicates up-regulation and blue down-regulation.

Table 12. List of top 100 significantly up- or down-regulated genes after 24 h of treatment with 1α,25(OH)$_2$D$_3$ in RWPE1 cells.

Gene symbol	Gene name	Fold change
Up-regulated genes		
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	60.21
KLK6	kallikrein-related peptidase 6	38.28
PADI3	peptidyl arginine deiminase, type III	31.99
CD14	CD14 molecule	28.18
CA9	carbonic anhydrase IX	15.63
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	15.44
DHRS9	dehydrogenase/reductase (SDR family) member 9	10.74
BAIAP2L2	BAI1-associated protein 2-like 2	9.09
TMEM91	transmembrane protein 91	7.51
TCEA2	transcription elongation factor A (SII), 2	7.00
IGFBP3	insulin-like growth factor binding protein 3	6.18
MGC102966	keratin 16 pseudogene 3	5.18
DUSP10	dual specificity phosphatase 10	5.18
CALB2	calbindin 2	5.13
KLK5	kallikrein-related peptidase 5	5.07
G6PD	glucose-6-phosphate dehydrogenase	5.03
DNER	delta/notch-like EGF repeat containing	5.00
DUSP1	dual specificity phosphatase 1	4.87
ACOT11	acyl-CoA thioesterase 11	4.74
CTSD	cathepsin D	4.65
CST6	cystatin E/M	4.62
MALL	mal, T-cell differentiation protein-like	4.51
TMEM79	transmembrane protein 79	4.49
ANKRD33	ankyrin repeat domain 33	4.44
KRT13	keratin 13	4.06
CAMP	cathelicidin antimicrobial peptide	3.90
FTH1	ferritin, heavy polypeptide 1; ferritin	3.84
FTHL3	ferritin, heavy polypeptide-like 3 pseudogene	3.83
FTHL8	ferritin, heavy polypeptide-like 8	3.76
LYPD5	LY6/PLAUR domain containing 5	3.68
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	3.66
FTHL12	ferritin, heavy polypeptide-like 12	3.59
HES5	hairy and enhancer of split 5 (Drosophila)	3.54
ZNF185	zinc finger protein 185 (LIM domain)	3.54
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	3.44
AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	3.39
CDA	cytidine deaminase	3.27
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	3.26
NDRG1	N-myc downstream regulated 1	3.20
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3.20
BGN	biglycan	3.15
SHE	Src homology 2 domain containing E	3.11
TXNRD1	thioredoxin reductase 1; hypothetical LOC100130902	3.11
FTHL2	ferritin, heavy polypeptide-like 2	3.09
FTHL11	ferritin, heavy polypeptide-like 11	3.08
ALOX5	arachidonate 5-lipoxygenase	3.06
AKR1C4	aldo-keto reductase family 1, member C4 (dihydrodiol dehydrogenase 4)	2.99
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2)	2.99
CA12	carbonic anhydrase XII	2.92
LOC729252	keratin 16 pseudogene 1	2.91
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	2.90
NINJ1	ninjurin 1	2.90
PLA2G10	phospholipase A2, group X	2.90
CA2	carbonic anhydrase II	2.88
LOX	lysyl oxidase	2.88
FAM116B	family with sequence similarity 116, member B	2.83
PDPN	podoplanin	2.82

KRT15	keratin 15	2.74
SEMA4B	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	2.72
ETNK2	ethanolamine kinase 2	2.69
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	2.69
MVK	mevalonate kinase	2.68
IL1RL1	interleukin 1 receptor-like 1	2.65
MTSS1	metastasis suppressor 1	2.64
SGPP2	sphingosine-1-phosphate phosphotase 2	2.64
ADM	adrenomedullin	2.64
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2.61
SLC47A2	solute carrier family 47, member 2	2.59
ASS1	argininosuccinate synthetase 1	2.57
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.56
COL16A1	collagen, type XVI, alpha 1	2.56
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	2.56
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	2.55
ADSSL1	adenylosuccinate synthase like 1	2.54
TRIML2	tripartite motif family-like 2	2.53
BTBD11	BTB (POZ) domain containing 11	2.53
TNNI2	troponin I type 2 (skeletal, fast)	2.47
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	2.47
PTK7	PTK7 protein tyrosine kinase 7	2.45
FADS1	fatty acid desaturase 1	2.45
LOC400578	keratin 1 pseudogene 2	2.44
KRT6B	keratin 6B	2.42
TUBB3	tubulin, beta 3; melanocortin 1 receptor	2.41
LAMB3	laminin, beta 3	2.41
KRT80	keratin 80	2.39
ERRFI1	ERBB receptor feedback inhibitor 1	2.39
IRX4	iroquois homeobox 4	2.38
MFGE8	milk fat globule-EGF factor 8 protein	2.38
GDPD3	glycerophosphodiester phosphodiesterase domain containing 3	2.36
BMP6	bone morphogenetic protein 6	2.36
PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	2.32
PYCARD	PYD and CARD domain containing	2.31
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	2.31
ULK1	unc-51-like kinase 1 (C. elegans)	2.30
TMEM40	transmembrane protein 40	2.30
TRPV6	transient receptor potential cation channel, subfamily V, member 6	2.30
FAM46A	family with sequence similarity 46, member A	2.30
RHPN2	rhophilin, Rho GTPase binding protein 2	2.27
C14ORF78	chromosome 14 open reading frame 78	2.25
CD9	CD9 molecule	2.21
Down-regulated genes		
IL1A	interleukin 1, alpha	0.24
IL1B	interleukin 1, beta	0.25
FST	follistatin	0.31
IER3	immediate early response 3	0.31
PTHLH	parathyroid hormone-like hormone	0.35
BIRC3	baculoviral IAP repeat-containing 3	0.37
CAV1	caveolin 1, caveolae protein, 22kDa	0.37
NUDT1	nudix (nucleoside diphosphate linked moiety X)-type motif 1	0.39
THBS1	thrombospondin 1	0.39
PHLDA1	pleckstrin homology-like domain, family A, member 1	0.4
F2R	coagulation factor II (thrombin) receptor	0.43
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	0.43
PHACTR3	phosphatase and actin regulator 3	0.45
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	0.46
LEPREL1	leprecan-like 1	0.48
DUSP6	dual specificity phosphatase 6	0.49

ALDH1A3	aldehyde dehydrogenase 1 family, member A3	0.49
HERC5	hect domain and RLD 5	0.5
CCL20	chemokine (C-C motif) ligand 20	0.5
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	0.51
AXL	AXL receptor tyrosine kinase	0.51
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.51
DKK3	dickkopf homolog 3 (Xenopus laevis)	0.52
LOC653820	family with sequence similarity 72, member B	0.52
CAV2	caveolin 2	0.52
CPA4	carboxypeptidase A4	0.52
FAM83A	family with sequence similarity 83, member A	0.53
FGFBP1	fibroblast growth factor binding protein 1	0.53
CCNA2	cyclin A2	0.54
CDC20	cell division cycle 25 homolog C (S. pombe)	0.54
VEGFC	vascular endothelial growth factor C	0.54
FJX1	four jointed box 1 (Drosophila)	0.54
GAL	galanin prepropeptide	0.55
DLK2	delta-like 2 homolog (Drosophila)	0.55
CCNB1	cyclin B1	0.55
DHRS3	dehydrogenase/reductase (SDR family) member 3	0.56
HMMR	hyaluronan-mediated motility receptor (RHAMM)	0.56
STAMBPL1	STAM binding protein-like 1	0.56
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	0.57
LPAR1	lysophosphatidic acid receptor 1	0.57
ODC1	ornithine decarboxylase 1	0.57
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	0.58
CKS2	CDC28 protein kinase regulatory subunit 2	0.59
CKS1B	CDC28 protein kinase regulatory subunit 1B	0.59
SLC39A10	solute carrier family 39 (zinc transporter), member 10	0.59
CLEC2D	C-type lectin domain family 2, member D	0.59
PAAF1	proteasomal ATPase-associated factor 1	0.6
LOC652235	hypothetical protein LOC652235	0.6
CENPA	centromere protein A	0.6
LHX6	LIM homeobox 6	0.6
CKAP2L	cytoskeleton associated protein 2-like	0.6
HS3ST3A1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	0.61
CDKN3	cyclin-dependent kinase inhibitor 3	0.61
OASL	2'-5'-oligoadenylate synthetase-like	0.61
DKK1	dickkopf homolog 1 (Xenopus laevis)	0.61
SERPINB13	serpin peptidase inhibitor, clade B (ovalbumin), member 13	0.61
IL7R	interleukin 7 receptor	0.61
TRIP13	thyroid hormone receptor interactor 13	0.61
KIF14	kinesin family member 14	0.61
PBK	PDZ binding kinase	0.62
CCNB2	cyclin B2	0.62
CTGF	connective tissue growth factor	0.62
KIF20A	kinesin family member 20A	0.62
CEP55	centrosomal protein 55kDa	0.62
ADORA2B	hypothetical LOC100131909; adenosine A2b receptor	0.62
DDX10	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	0.63
CLCC1	chloride channel CLIC-like 1	0.63
PTTG3P	pituitary tumor-transforming 3	0.63
CCDC86	coiled-coil domain containing 86	0.63
CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	0.63
BIRC5	baculoviral IAP repeat-containing 5	0.63
CCND2	cyclin D2	0.63
FAM3C	family with sequence similarity 3, member C	0.63
TIMM10	translocase of inner mitochondrial membrane 10 homolog (yeast)	0.63
ITPRIPL2	inositol 1,4,5-triphosphate receptor interacting protein-like 2	0.63
TTK	TTK protein kinase	0.63
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1); karyopherin alpha-2 subunit like	0.64
CENPF	centromere protein F, 350/400ka (mitosin)	0.64
AURKB	aurora kinase B	0.64

CENPN	centromere protein N	0.64
RABEPK	Rab9 effector protein with kelch motifs	0.64
GAS6	similar to growth arrest-specific 6; growth arrest-specific 6	0.64
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	0.64
DLEU2	deleted in lymphocytic leukemia 2 (non-protein coding); deleted in lymphocytic leukemia 2-like	0.64
F3	coagulation factor III (thromboplastin, tissue factor)	0.64
FAM83D	family with sequence similarity 83, member D	0.65
DEPDC1B	DEP domain containing 1B	0.65
MND1	meiotic nuclear divisions 1 homolog (S. cerevisiae)	0.65
SOCS2	suppressor of cytokine signaling 2	0.65
GPSM2	G-protein signaling modulator 2 (AGS3-like, C. elegans)	0.65
ARL9	ADP-ribosylation factor-like 9	0.65
PLD6	phospholipase D family, member 6	0.65
FEZ1	fasciculation and elongation protein zeta 1 (zygin I)	0.65
C13ORF34	chromosome 13 open reading frame 34	0.65
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	0.65
PDCD2L	programmed cell death 2-like	0.65
LOC731049	similar to Ubiquitin-conjugating enzyme E2S	0.65
FAM64A	family with sequence similarity 64, member A	0.65
PTTG1	pituitary tumor-transforming 1; pituitary tumor-transforming 2	0.65
RAB3IL1	RAB3A interacting protein (rabin3)-like 1	0.66

APPENDIX III

Interestingly, the reported data illustrate that CTCF and cohesin may bind the same DNA regions independently and thus may share the function as insulators (Wendt, 2008). To analyze the differences in *CTCF* and *RAD21* (that is a subunit of cohesin complex and encodes the double-strand-break repair protein) we compared their relative expression levels in non-malignant prostate cells using qPCR analysis. As shown in the Figure 37A, the expression of *RAD21* gene was more than 25-times higher than that of the *CTCF* gene. Based on our previous data showing the relation between CTCF stronger associations to its binding sites upon VDR ligand stimulation, we tested whether VDR influences the expression of *RAD21* gene in *CTCF* depletion condition. Therefore, after CTCF knockdown we stimulated non-malignant prostate cells with $1\alpha,25(OH)_2D_3$ for 4 h and we noticed that *RAD21* was induced (Fig. 37B). Inhibition of *CTCF* had no effect on the transcript level of *RAD21* (Fig. 37C).

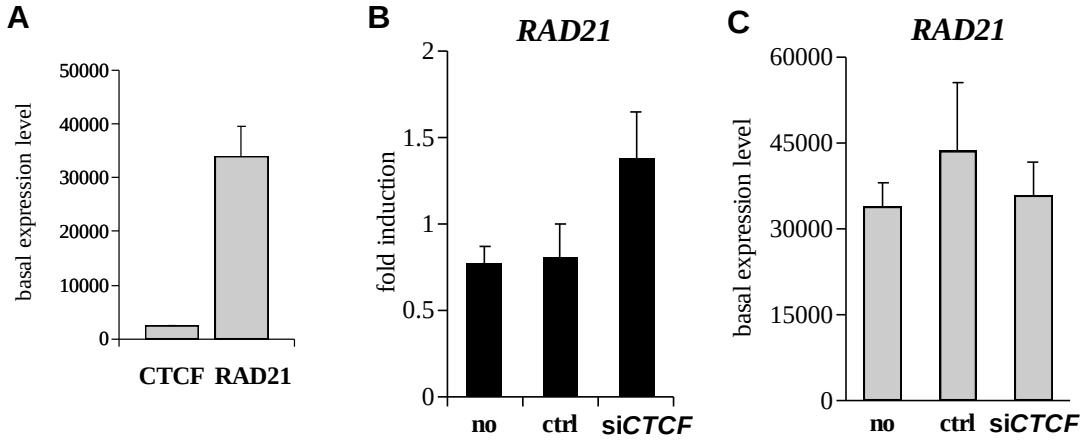


Figure 37. CTCF knockdown causes $1\alpha,25(OH)_2D_3$-dependent changes in mRNA expression level of *RAD21* gene. The basal expression levels of *RAD21* and *CTCF* (**A**) were analyzed by qPCR in relation to housekeeping gene *RPLPO.* RWPE1 cells transfected with specific siRNAs oligonucleotides against *CTCF* and control siRNA or not transfected, were stimulated for 4 h with $1\alpha,25(OH)_2D_3$ **(B)** and the fold inductions were determined relative to the solvent (EtOH). (**C**) The basal expression levels of *RAD21* after *CTCF* depletion were analyzed according to the *RPLPO* gene. Columns indicate a mean of three independent experiments, each done in triplicate and the error bars stand for SEM.

Taken together, our data suggests that in non-malignant prostate cells the mRNA level of *RAD21* gene was strikingly higher than the level of *CTCF* gene. Moreover, after knockdown of *CTCF* gene, relative expression level of *RAD21* did not change however this gene was induced by $1\alpha,25(OH)_2D_3$ in CTCF-depleted condition, which may suggest VDR contribution in regulation of that gene.