The Cancer Chemo-Preventive Properties of Vitamin D are Due, at Least in Part, to the Transcriptional Regulation of Genes Implicated in Cell Cycle, DNA Replication and Apoptosis, and Activation of FoxO3a Transcription Factor

Vassil Dimitrov
Division of Experimental Medicine
McGill University
Montréal, Québec
December, 2011

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement of the degree of Masters in Sciences.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. John White for his support and guidance. He was always available when I needed advice. His patience and optimistic attitude during our fruitful discussions provided me not only with exciting new ideas, but also cheered me up when things were not going as planned. Thank you, John!

Another person I would like to thank is Dr. Beum-Soo An, with whom I was working closely on my project. An provided me with a day-to-day guidance and assistance when I needed it. I am also grateful to Hui-Jun Wang, who taught me the basic techniques I used and the other members of the lab: Mark – for his discussions and advice, Reyhaneh – with whom we keep exchanging experience regarding experimental conditions, and Mario, Manuella and Tian-Tian – for being there when I need some help.

LIST OF ABBREVIATIONS

Abbreviation	Description	Abbreviation	Description
1,25D	1,25-dihydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_2	IBD	Inflammatory Bowel Disease
1,25-$(OH)_2D_3$	1,25-dihydroxyvitamin D_3	IPA	Ingenuity Pathway Analysis
7-DHT	7-dehydrocholesterol	JNK	c-Jun N-terminal Kinase
25-OHD_2	25-hydroxyvitamin D_2	IKK	IκB Kinase
25-OHD_3	25-hydroxyvitamin D_3	KD	Knock-Down
25D or 25-OHD	25-OHD_2 or 25-OHD_3	KO	Knock-Out
CaR	Ca^{2+} Sensing Receptor	LBD	Ligand Binding Domain
CBP	CREB-Binding Protein	MARRS	Membrane-Associated Rapid Response Steroid binding protein
CC	Compound C	MDM2	Murine Double Mutant 2
CCNB1	Cyclin B1	MnSOD	Manganese Superoxide Dismutase
CCNB2	Cyclin B2	MS	Multiple Sclerosis
CCND1	Cyclin D1	NAD^+	Nicotinamide Adenine Dinucleotide
CCND2	Cyclin D2	NCI	National Cancer Institute
CCNG2	Cyclin G2	NCoR	Nuclear Corepressor
cGMP-1	Cyclic GMP-dependent kinase-1	NCX1	Sodium-Potassium Exchanger 1
CDK	Cyclin-Dependent Kinase	NES	Nuclear Export Signal
ChIP	Chromatin Immunoprecipitation	NaPi-II	Sodium-Phosphate co-transporter 2
CK1	Casein Kinase 1	NR	Nuclear Receptor
CNT	Connecting Tubule	O-GlcNAc	O-linked β-N-Acetylglucosamine
CoIP	Co-Immunoprecipitation	OGT	O-linked N-acetylglucosamine Transferase
CVD	Cardio-Vascular Disease	PCAF	P300/CBP-Associated Factor
DBE	Daf-16 Response Element	PDK1	Phosphoinositide-Dependent Kinase 1
DBP	Vitamin D Binding Protein	PGC1α	PPAR-Gamma Coactivators -1 alpha
DCT	Distal Convoluted Tubule	PI	Propidium Iodide
DNMT	DNA (cytosine-5)-methyltransferase	PI3K	Phosphoinositide-3-Kinase
DR3	Direct Repeats separated by 3bp	PIP2	Phosphatidylinositol 4,5-bisPhosphate, PtIns(4,5)P2
DYRK1A	Dual Tyrosine Phosphorylated Regulated Kinase-1A	PIP3	Phosphoinositide-3,4,5-trisPhosphate
ER	Endoplasmic Reticulum	PKB	Protein Kinase B

Abbreviation	Description	Abbreviation	Description
ER6	Everted Repeats separated by 6bp	PMCA1b	Plasma Membrane Ca^{2+} ATPase 1b
ERp57	Endoplasmic Reticulum protein 57kDa	PTEN	PI3K/phosphatase and Tensin homologue deleted on chromosome Ten
ERp60	Endoplasmic Reticulum protein 60kDa	PTH	Parathyroid Hormone
FACS	Fluorescence-Activated Cell Sorting	PTHR	Parathyroid Hormone Receptor
FASL	Fas Ligand	RXR	Retinoid X Receptor
FGF23	Fibroblast Growth Factor 23	SCC25	Squamous Cell Carcinoma
FGFR	Fibroblast Growth Factor Receptor	SGK	Serum and Glucocorticoid-inducible Kinase
FHRE	Fork-Head Response Element	SIRT1	Sirtuin 1
FoxO	Fork-head Box O transcription factor	Skp2	S-phase Kinase-associated Protein 2
GADD45a	Growth Arrest and DNA-Damage-inducible protein 45 α	SLE	Systemic Lupus Erythematosus
GRP58	Glucose Responsive Protein 58kDa	SMRT	Silencing Mediator for Retinoid and Thyroid Hormone Receptors
HAT	Histone Acetyl-Transferase	SRC	Steroid Receptor Co-activator
HDAC	Histone Deacetylase	T1D	Type 1 Diabetes
H&N SCC	Head And Neck Squamous Cell Carcinoma	T2D	Type 2 Diabetes
HRE	Hormone Response Element	TB	Tuberculosis
HSC	Hematopoietic Stem Cells	TBP	TATA box binding protein
TNFα	Tumour Necrosis Factor Alpha	TFIIB	Transcription Factor 2 B
TRADD	Tumour Necrosis Factor Receptor-Associated Death Domain	VD3	Vitamin D_3, cholecalciferol, calciol,
TRAIL	Tumour Necrosis Factor-Related Apoptosis Inducing Ligand	VDR	Vitamin D Receptor
TRPV	Transient Receptor Potential Vanilloid	VDRE	Vitamin D Response Element
UVB	Ultra-Violet B light	VDR_{mem}	Membrane Vitamin D Receptor
VD	Vitamin D	VDR_{nuc}	Nuclear Vitamin D Receptor, Vitamin D Receptor
VD_2	Vitamin D_2, ergocalciferol	WB	Western Blot

ABSTRACT

Vitamin D is produced in the skin or obtained from limited dietary sources. Two sequential hydroxylation reactions in the liver and kidney produce the active form of the hormone, 1,25-$(OH)_2D_3$, which binds to and activates VDR to regulate gene expression (genomic effects) or to VDR_{mem} or MARRS to trigger certain signalling pathways (non-genomic effects). 1,25-$(OH)_2D_3$ is best known for its effects on calcium and phosphate metabolism, but it also possesses chemopreventive properties for many types of cancers, particularly those of the digestive tract.

The anti-proliferative effects of 1,25-$(OH)_2D_3$ on SCC25, a head and neck squamous cell carcinoma cell line derived from the floor of the mouth, have previously been demonstrated in this lab. Here, I extend these finding by investigating mechanisms of gene regulation and by discovering novel genes implicated in proliferation and apoptosis regulated by 1,25-$(OH)_2D_3$.

CCND2, *CCNG2*, *MMP3* and *BNIP3* are FoxO target genes, whose expression is controlled by 1,25-$(OH)_2D_3$. FoxO transcription factors are *bona fide* tumour suppressors regulating expression of genes implicated in cell cycle arrest, apoptosis and oxidative stress resistance. I show here that the four FoxO target genes are suppressed (*CCND2*) or stimulated (*CCNG2*, *BNIP3* and *MMP3*) by 1,25-$(OH)_2D_3$ at the transcription level through activation of FoxO3a. This activation is achieved through deacetylation and dephosphorylation of the transcription factor mediated by SIRT1 and PP1, respectively. Additionally, the crucial role FoxO3a plays in 1,25-$(OH)_2D_3$-mediated regulation of these genes is demonstrated by the fact that ablation of FoxO3a attenuates or completely blocks gene regulation.

To extend the list of VD-regulated genes implicated in cancer, a microarray analysis was performed. Ingenuity pathway analysis software was used to determine pathways and networks regulated by 1,25-$(OH)_2D_3$. Notably, one of the canonical pathways is VDR/RXR activation. Another canonical pathway displays down-regulation of genes responsible for chromosomal replication. In addition, a network of interacting genes constructed by IPA also shows that VD reduces DNA replication

and progression through the cell cycle. Several VD-regulated genes (*OLR1*, *MACC1*, *BIRC3*, *POLE2*, *PCNA*, *CCNB1*, *CCNA2*, *APPL1* and *BIRC5*) were selected and validated by qPCR. Interestingly, all of these genes are down-regulated by 1,25-$(OH)_2D_3$ and are over-expressed in various cancer types relative to normal tissues, as assessed using Oncomine. These results support potential mechanisms of VD actions in cancer.

RÉSUMÉ

La vitamine D est synthétisée dans l'organisme à partir du rayonnement UV au niveau de la peau, ou bien produite à partir de certains produits spécifiques issus de l'alimentation. Deux réactions d'hydroxylation séquentielles, qui ont respectivement lieu dans le foie et les reins permettent la synthèse de la forme active de l'hormone, 1,25-$(OH)_2D_3$. Cette hormone régule l'expression de nombreux gènes *via* sa fixation au VDR (Récepteur à la Vitamine D) (effets génomiques) et cible également certaines voies de signalisation via sa fixation au VDR_{mem} ou MARRS (effets non génomiques). 1,25-$(OH)_2D_3$ est plus connue pour ses effets sur le métabolisme du calcium et du phosphate, mais cette hormone possède également des propriétés chimio-préventives dans de nombreux types de cancers, particulièrement ceux concernant le système digestif.

L'effet antiprolifératif de 1,25-$(OH)_2D_3$ dans la lignée cellulaire SCC25, (établie à partir de cellules dérivées d'une cavité buccale d'un carcinome de cellules squameuses du cou et de la tête), avait déjà été étudié dans le laboratoire. Durant ce projet, j'ai étendu ces résultats en m'intéressant aux mécanismes de régulation génique et en mettant en évidence de nouveaux gènes impliqués dans la prolifération et/ou l'apoptose régulée par 1,25D-$(OH)_2D_3$.

CCND2, *CCNG2*, *MMP3* et *BNIP3* sont des gènes cibles de la famille des protéines FoxO, dont l'expression est contrôlée par 1,25-$(OH)_2D_3$. Les facteurs de transcription FoxO sont des suppresseurs de tumeurs validés, qui régulent l'expression de gènes impliqués dans l'arrêt du cycle cellulaire, l'apoptose et la résistance au stress oxydatif. J'ai mis en évidence ici, que les gènes cibles de FoxO sont régulés par 1,25-$(OH)_2D_3$ au niveau transcriptionnel, via l'activation de FoxO3a. En effet, l'expression transcriptomique de *CCND2* est inhibée, tandis que celles de *CCNG2*, *BNIP3* et *MMP3* sont stimulées. L'activation a lieu grâce à la déacétylation et la déphosphorylation des facteurs de transcription FoxO, assurées respectivement par SIRT1 et PP1. De plus, le rôle central que joue FoxO3a dans la régulation

génique assurée par 1,25-$(OH)_2D_3$ est mise en évidence, par le fait que cette régulation est atténuée ou complètement abolie par l'extinction de FoxO3a par siRNA. Afin d'élargir la liste de gènes régulés par la vitamine D impliqués dans le cancer, nous avons réalisé une analyse par microarrays. Le logiciel d'analyse « Ingenuity » a été utilisé pour déterminer quels sont les voies de signalisation et les réseaux régulés par 1,25-$(OH)_2D_3$, notamment la voie canonique de l'activation du VDR/RXR. Une autre voie signalisation impliquée dans la réplication chromosomique est inhibée dans nos conditions. Par ailleurs, un réseau de régulation de gènes réalisés par IPA, a mis en évidence que la vitamine D réduit la réplication de l'ADN et la progression du cycle cellulaire. De nombreux gènes régulés par la vitamine D (*OLR1*, *MACC1*, *BIRC3*, *POLE2*, *PCNA*, *CCNB1*, *CCNA2*, *APPL1* et *BIRC5*) ont été identifiés et validés par qPCR. De façon intéressante, le logiciel Oncomine montre que l'ensemble de ces gènes est inhibé par 1,25-$(OH)_2D_3$ et est surexprimé dans de nombreux types de cancers comparativement à des tissus sains. L'ensemble de ces résultats coincident avec nos observations montrant que 1,25-$(OH)_2D_3$ induit l'arrêt du cycle cellulaire et inhibe la prolifération cellulaire.

LITERATURE REVIEW

Vitamin D

Vitamin D in Health and Disease

The beneficial effects of vitamin D (VD) for human health have been known or at least suspected since antiquity. Hippocrates claimed that the southern face of a hill (receiving the most sunlight in the northern hemisphere) is the healthiest place to live and found that sunlight, or heliotherapy, can be used to treat phthisis (tuberculosis) [6]. In 1793, cod liver oil, which was later found to contain vitamin D [7], was used to treat rheumatism and later gout and scrofula. It was, however, the Polish physician Sniadecki, who at around the same time established the link between VD, or sunlight, and rickets, which in the 1800s was an epidemic in Northern Europe, North America and parts of Northern Asia [8]. In 1890, Theodore Palm made the observation that children living near the Equator display much lower incidence of rickets compared with Europeans [9]. Sir Edward Mellanby was able to cure rickets in dogs kept indoors and fed oats by exposing them to sunlight in the beginning of the nineteenth century [10], followed by Huldshinsky's demonstration that ultraviolet (UV) light, which, as proved by Steenbock, induces the production of vitamin D in skin and fatty portions of the diet, can cure rickets in humans [8]. This led to the addition of ingestion of UV-irradiated foods to the arsenal of anti-rachitic treatments [11]. The use of this latter strategy and its implications (only foods containing cholesterol could be employed as an anti-rachitic agent) led to the discovery of the cholesterol precursor of vitamin D, 7-dehydrocholesterol (7-DHT) [11-13] by Windaus, for which he received the noble price in 1937.

Rickets is the classical and best characterized example of a disease cured by VD. However, there are others, such as tuberculosis (TB). Sanatoria were used starting the mid-1800s for the treatment of TB, which was also curable by cod liver oil or UV light, as demonstrated by Nobel Prize winner Niels

Finsen for cutaneous TB (lupus vulgaris) [14, 15]. Although not as definitive as the case of TB, VD has a preventive potential against other infectious diseases. For example, elderly women receiving VD supplements had significantly lower incidence of *Helicobacter pylori* infections [16]. Moreover, VD deficiency or insufficiency (less than 50nmol/l of circulating VD) is associated with increased risk of lower respiratory tract infections [17-22], and even HIV infection [23-25]. Regarding adaptive immunity, VD has tolerogenic effects on T-lymphocytes [26], beneficial for a number of autoimmune disorders [27, 28]. One example is Multiple Sclerosis (MS). Its incidence increases 200-fold from the equator to north of the 50^{th} parallel [29] and 77% of patients with MS were found to be VD insufficient (43nmol/L) [30]. Consistently, VD supplementation decreased MS relapse rate and MS risk in general by 41% for every 20ng/l increase of circulating VD [31, 32].

VD, along with Ca^{2+} consumption, was also suggested to prevent inflammatory bowel disease (IBD) [33]. Higher incidence of IBD is associated with populations inhabiting regions that receive less sunlight and IBD patients have lower circulating VD [34, 35]. Two other conditions associated with autoimmunity are arthritis and systemic lupus erythematosus (SLE). In the case of the former, not only there is an inverse correlation with VD status [36-38], but supplementation seems to decrease pain and C-reactive protein levels[39]. On the other hand, despite insufficient VD status of SLE patients, causative relationship has not been demonstrated [40-42].

VD could also have preventive effects for diabetes, especially type 1 (T1D). Apart from increased incidence of the disease in northern latitudes combined with seasonality of onset, studies have demonstrated the beneficial effect of increased circulating VD levels to prevent T1D and has been proposed to increase glucose tolerance and incidence and severity of T2D and the metabolic syndrome [43].

Most, but not all, studies about VD supplementation and cardio-vascular disease (CVD) point to beneficial effects especially with regards to blood pressure. Part of these are due to parathyroid

hormone (PTH) and renin-angiotensin system down-regulation. No association was found between VD status and coronary artery disease, but the risk of myocardial infarction, heart failure and fatal stroke for patients with levels lower than 30ng/ml was significantly increased compare to VD-sufficient controls [44].

The effects of VD on the musculo-skeletal system were introduced earlier with rickets in children. In adults, VD deficiency and low dietary calcium result in similar consequences (osteomalacia). In this sense, it is not surprising that increases in circulating VD prevent fractures in the elderly. One of the reasons is increased bone density due to efficient Ca^{2+} absorption. The other is decreased risk of falling [45, 46], due to enhanced muscle strength and power, specifically in muscle fibre type IIa [47].

VD has also been associated with decreased cancer risk since 1936 when Peller made the observation that US Navy soldiers suffering from skin cancer had a much lower incidence of other forms of cancer [48]. In 1942, Apperly noticed that lower internal cancer incidence correlates with sunnier places in the USA [49]. The increased awareness for this chronic disease led to the declaration of "war on cancer" by the US government in 1970, prompting the generation of maps by the National Cancer Institute (NCI) showing cancer mortality rates according to geographical regions. This led to the discovery made by Cedrik and Frank Garland that VD correlates with lower colon cancer risk and mortality [50-52]. Groham and colleagues established a link in 1989 between colon and breast cancer incidence and mortality in 20 Canadian cities and air pollution (UVB absorption, which blocks VD synthesis) [53]. The same group applied this analysis to the Soviet union in 1990 to find the same correlation. Epidemiological data about the preventive properties of VD for colon cancer were extended to other types of cancer such as ovarian [54], breast [55], renal [56], endometrial [57] and lung [58], eventually leading to the Women Health Initiative clinical trial consisting of 60,000 female subjects. This study investigated the effects of 400 IU VD per day taken orally on colon cancer. The amount of VD, however was argued to be too small and had no effect on tumorigenesis [59].

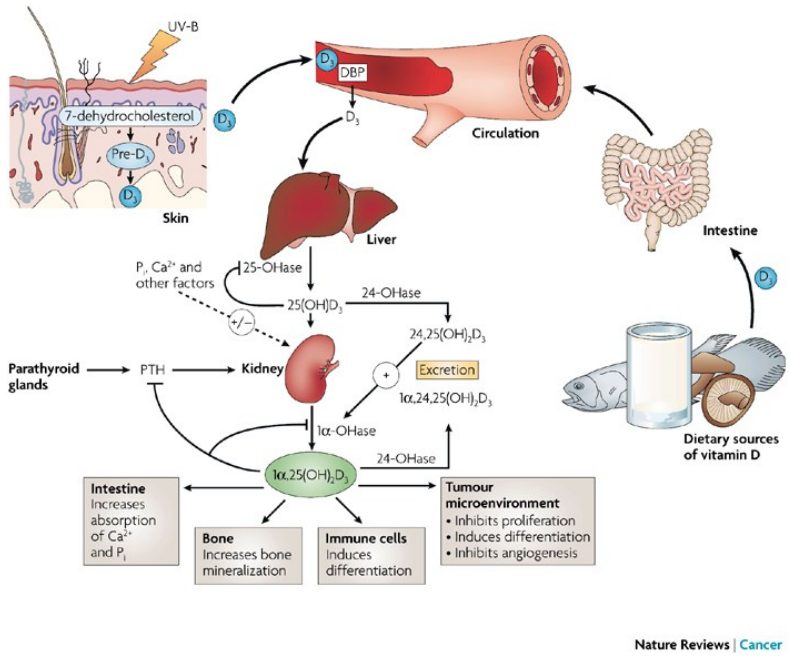


Figure 1: VD production in the skin or from diet, its metabolism and effects [2]

The effects of VD on prostate cancer seem to be controversial and no definitive correlation has been established [60]. The same appears to be valid for pancreatic cancer [61]. However, VD may have an indirect effect since it is beneficial for conditions such as glucose intolerance, diabetes and insulin resistance, all of which may play a role in the development and progression of pancreatic cancer. Skin cancer and VD is also a controversial topic. Sunlight has been used to treat psoriasis (although the exact mechanism is unknown) for years [62] and VD induces block in proliferation and increased differentiation of keratinocytes [63]. Anti-proliferative and pro-differentiation properties are also displayed by VD in the context of lymphoma and leukemia [64]. It must be noted that sunlight (especially its UVB spectrum) has been used in the discussion so far as a source for VD (**fig. 1**), as will be discussed below.

Vitamin D Endocrine System

Vitamin D3 is also referred to as cholecalciferol or calciol. It should be noted that it is not really a vitamin since it can be produced in basal or supra-basal levels of the skin in a reaction catalyzed by UVB

[65]. The wavelength of UVB is 280-315nm, but wavelengths of 297-302nm are optimal for this reaction[66]. The precursor for vitamin D_3 is 7-DHC, also known as pro-vitamin D_3. In keratinocytes and spinous cell layers [67], UVB stimulates the relatively rapid photolysis of 7-DHC to produce pre-vitamin D_3 [68] in an intensity-dependent manner [69] (**fig. 1**). There are several factors that influence the production of pre-vitamin D_3 in the skin. Firstly, the amount of 7-DHC present in the epidermis (65%) and dermis (35%), of which 80% is in the membrane fraction and 20%, in the cytosolic fraction, is crucial. 7-DHC production is controlled by 7-DHC-Δ7-reductase, which interconverts cholesterol and 7-DHC [70]. 7-DHC is an important factor influencing the production of VD and its levels decrease with age [71]. Another essential factor is the availability of UVB irradiation to drive the isomerization of 7-DHC leading to carbons 9-10 bond cleavage. In this context, the skin pigmentation [72] and solar zenith angle [69] are critical for vitamin D_3 production, as well as other similar factors such as clothing and sunscreen [73, 74]. Pre-vitamin D_3 then undergoes heat-dependent isomerization to produce vitamin D_3, which takes up to several hours [68]. Temperature is therefore also an essential aspect of VD synthesis. It should be noted that under prolonged UV irradiation, the pre-vitamin D_3 can be further isomerized to two inert compounds, lumisterol and tachysterol, or converted back to 7-dehydrocholesterol [65]. It is therefore important that UVB exposure is at levels that are lower than erythemogenic for optimal vitamin D_3 production. Vitamin D_3 can also be obtained, along with supplements or fortified foods, from limited food sources such as oily fish (mackerel, salmon, tuna and herring) and fish oils [75]. Smaller amounts are present in beef liver, egg yolk and cheese. Some mushrooms provide vitamin D_2 (VD_2), or ergocalciferol [76]. VD_2, together with vitamin D_3 (VD_3), are collectively referred to as VD. Vitamin D_2 is also found in some plants. It is produced in yeast from the steroid precursor ergosterol [77]. In terms of metabolism in humans, VD_2 follows the exact same pathway, undergoes identical reactions as VD_3 and a randomized control study demonstrated similar effects of 1000IU of VD_2 or VD_3 in maintaining the levels of circulating VD [78] although there are reports about ergocalciferol being less effective in maintaining

circulating VD levels [79-81]. The physiological effects of these two forms, however, seem to be similar [82] and from this point on, only VD_3 will be considered with the understanding that the same applies for VD_2. Interestingly, there are four other naturally occurring vitamin D compounds (vitamin D_4 to D_7) [83], which are less studied, and around 2000 analogues, which differ in their effects and tissues of accumulation [84]

Once VD_3 has been produced in the skin, it is exported into the circulation by the vitamin D binding protein (DBP) [85] as further UV will cause its degradation [86]. DBP is a group-specific, Gc-globulin of the alpha-albumin, albumin and alpha-fetoprotein gene family [87], that has a half-life of 2.5 days in the plasma [87, 88] and does not only act to transport VD in the circulation, but also as a storage site for the latter [89]. It is synthesized predominantly in the liver, has a molecular weight of 52-59 kDa [90, 91] and its gene is located on chromosome 4. There are two domains of the protein capable of VD metabolites and actin [90, 92-94]. DBP is also implicated in the transport of fatty acids and macrophage activation and chemotaxis [95]. Alternatively, chylomicrons can also bind VD_3 in the circulation [89]. The VD_3/DBP complex is thus transported through the circulation to the liver. Its whole-body half-life is about 2 months and the circulating half-life is about 4-6h [96]. Once in the liver, VD_3 undergoes hydroxylation at position 25 to give the major circulating form of vitamin D, 25-hydroxyvitamin D_3 (25-OHD_3) (calcidiol, calcifediol, or 25-hydroxycholecalciferol) [97] or 25-OHD_2 collectively referred to as 25-OHD. 25-OHD is used as a measure for the VD status in the body [98]. It is considered to be representative of VD status in the body due to the fact that 25-hydroxylation is a rapid reaction that is not subjected to any regulation allowing for virtually all VD_3 to be converted to 25-OHD_3 [66]. The half-life of 25-OHD_3 is about 12 days. Different threshold levels of 25-OHD define VD sufficiency or deficiency with intermediate values indicating insufficiency, severe or sub-clinical deficiency. In biochemical terms, vitamin D sufficiency is defined as the levels of 25-OHD for which PTH production and secretion is stable, usually achieved at a concentration of ~75nmol/l (30ng/ml; 1nmol/l=2.5*1ng/ml) or more [99]. In this

context, VD sufficiency is defined as circulating 25-OHD_3 levels of 75-80nmol/l or more, insufficiency – between 50 and 74nmol/l and 25OHD levels lower than 50nmol/l are reflective of a vitamin D deficiency or hypovitaminosis D [27, 100]. In order to achieve vitamin D sufficiency, a recommended supplementation of at least 45-100µg per day (1800-4000IU; 1IU=1µg*40) [101] is required.

The enzymes responsible for the 25-hydroxylation step in the liver are members of the cytochrome p450 family. CYP27A1 is a mitochondrial enzyme. It is most highly expressed in liver and is believed to be important in hepatic vitamin D3 hydroxylation [102]. It was observed, however, that the microsomal CYP2R1, which is almost ubiquitously expressed, contributes more to the this hydroxylation reaction since a defect in its gene causes rickets [103]. It should be noted that other microsomal enzymes such as CYP3A4 [104] and CYP2J2 [105] also significantly contribute to vitamin D 25-hydroxylation. CYP2R1, however, has the highest specificity and binds vitamin D with the highest affinity compared to the other enzymes (Km of 0.45 and 0.67 µM for VD_3 and VD_2, respectively) [106]. 25-OHD_3 exits the liver and enters the circulation, bound by DBP. DBP has the highest affinity to 25-OHD_3 compared to all other VD_3 metabolites (Ka=$5*10^{-8}$ M) [94] and is in a large molecular excess (5.52-7.93 * 10^{-6} M) compared to its main binding substrate (5 * 10^{-8} M) [87] allowing it to act as a reservoir for calcidiol and potentially protect against vitamin D toxicity. 25-OHD_3 is still not the active form of VD and has to be further hydroxylated at position 1, although several studies argue that calcidiol can produce some of the effects of hormonal VD, as well as bind the vitamin D receptor (VDR). This activation occurs in the kidney, after the 25-OHD_3/DBP complex leaves the liver and travels through the circulation [97]. It was initially believed that unbound 25-OHD_3 diffuses freely across the basolateral membrane of the renal proximal tubule epithelium – "the free hormone hypothesis" [107, 108]. The high affinity of DBP for calcidiol, however, allows for very small amounts of 25-OHD_3 to be present in a free form. A subsequent study, therefore, provides a more plausible mechanism for transport of 25-OHD_3 into cells of the proximal tubule. Notably, the 25-OHD_3/DBP complex was shown to be filtered through the

glomerulus and internalized from the luminal side of the proximal tubules by the megalin/cubulin receptor [109]. There, 1-alpha hydroxylation is catalyzed by the mitochondrial CYP27B1 [110, 111] to produce the active vitamin D, also referred to hormonal VD, calcitriol, or 1,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$]. It was discovered between 1968 and 1971 as the result of studies performed by several groups [112, 113]. Unlike the VD_3 25-hydroxylases, the renal 25-OHD_3 1-alpha-hydroxylase is tightly regulated by phosphate and calcium homeostatic signals, as will be discussed later. Interestingly, Inuit populations, which receive very small amounts of UVB light, seem to have an enhanced conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ in the kidneys [114]. It is noteworthy to mention that CYP27B1 could be expressed in a variety of other tissues, such as the gastrointestinal tract, pancreas, endothelial cells, brain, placenta, epidermis, adipose tissue, activated leukocytes and macrophages [115-117]. CYP27B1 in the aforementioned tissues, however, is not subject to the same regulation as renal CYP27B1 and could provide sufficient hormonal vitamin D in autocrine and paracrine fashion. In fact, certain tissues possess the full set of cytochrome p450 enzymes necessary for the synthesis of hormonal VD from its precursor 7-DHC. Such tissues include prostate epithelium [118, 119], osteoblasts [120], macrophages [121] and keratinocytes [122-125]. In this context, skin is of obvious interest due to the presence of high levels of 7-DHC and, therefore, its capacity to produce 1,25$(OH)_2D_3$ in a completely independent fashion. CYP27B1 seems to be the only enzyme capable of 25-OHD_3 1alpha-hydroxylation discovered so far and mutation of its gene causes symptoms of VD deficiency, which are easily reversed by administration of 1,25-$(OH)_2D_3$ [110]. Once produced in the kidney, 1,25D enters the circulation and is transported, again by DBP (Ka = $4 * 10^{-7}$ M) [94], to various tissues where free 1,25-$(OH)_2D_3$ diffuses through the plasma membrane to exert its many effects. The latter can be subdivided into two categories: non-genomic (fast) and genomic (slower) [126].

Non-genomic effects of 1,25D

As suggested by the name, the non-genomic effects of 1,25D do not require modulation of gene expression and usually consist of regulating signal transduction pathways, such as peptide and growth factor receptor activation, as well as ion channel function. Examples of the former include activation (or suppression in a cell-dependent context) of ERK [127-133], PKC [134-139], PLC [136, 140-144]and PLD [145], production of 1,3,4-triphosphate (IP3) and 1,2-diacylglycerol (DAG) as a result of increased phosphoinositide turnover, release of intracellular calcium stores [144, 146-149] and PKA stimulation through increased adenylate cyclase activity [150-154]. In terms of ion channel regulation, there is a substantial amount of studies demonstrating the rapid increase in absorption of Ca^{2+} from the extracellular space or its release from intracellular reservoirs as a result of the non-genomic actions of hormonal VD [143, 144, 146, 154-163]. Another effect of the rapid actions of calcitriol is the regulation of chloride channels resulting in an outward ionic flux [164-168] and regulatory volume changes.

The genomic effects of hormonal vitamin D are mediated through the vitamin D receptor, which is localized in the nucleus (VDR_{nuc} or just VDR) of target cells. VDR was discovered in the mid-1970s [169, 170] by Mark R. Hausler and cloned in 1988 [171]. The non-genomic effects, on the other hand, are believed to be initiated at the plasma membrane by a unique receptor for calcitriol or other vitamin D metabolites. Studies in this field have provided evidence for the existence of two such receptors: membrane-localized VDR (VDR_{mem}) and membrane-associated rapid response steroid binding protein (MARRS) [172]. MARRS – also referred to as glucose responsive protein 58kDa (GRP58) and endoplasmic reticulum (ER) 57-60kDa (ERp57 or ERp60) [172] – is a multi-functional thioredoxin-like protein anchored to the plasma membrane by a myristoylation sequence and can also be found at the ER [173]. Its discovery was triggered by the observations that calcitriol binds to the basolateral membrane of chick and rat enterocytes to a protein that is distinct from the classical VDR [174, 175]. In addition, VD analogues that are unable to bind the classical VDR are capable of initiating rapid responses [176] such

as PKC activation and Ca^{2+} and PO_4^- uptake[134, 172] – effects which are blocked by an anti-MARRS antibody[132]. Similarly, rapid responses are not entirely abolished in VDR-null mice [177]. Interestingly, binding of hormonal VD to MARRS was also shown to trigger translocation of the complex to the nucleus [173].

VDR_{mem} is the classical VDR that is localized to the plasma membrane [178], in particular lipid rafts and caveolae in cells of several tissues (intestine, kidney, lung, osteoblasts) [179], and has been shown to mediate the rapid effects of calcitriol in osteoblasts and fibroblasts [180] as they are abolished in VDR-null mice. Additionally, analogues which only induce rapid effects were shown do bind to an alternative pocket of the VDR [181], suggesting that VDR_{mem} participates in mediating the non-genomic effects of vitamin D.

Genomic effects of 1,25D

The VDR_{nuc} mediates the genomic effects of calcitriol. VDR is a member of the nuclear receptor

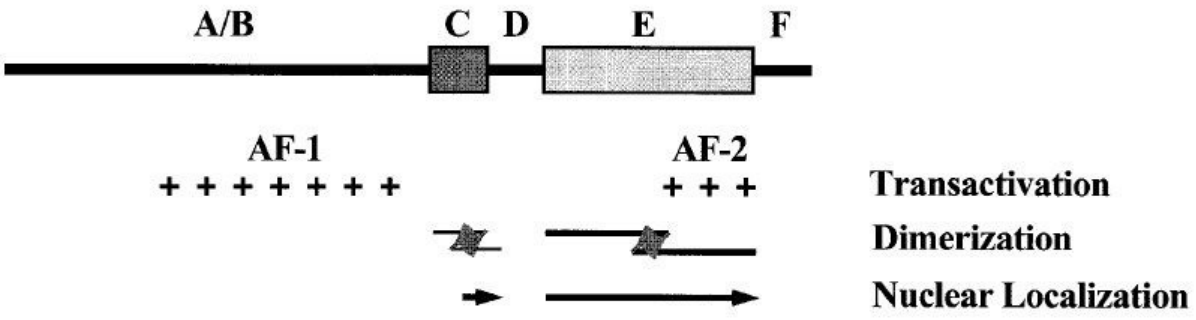


Figure 2: NR members are composed of 6 regions. The positions of motifs implicated in transactivation, dimerizaion and NLSs are also marked [185].

(NR) superfamily. The latter represents a class of transcription factor, whose function is induced by ligand binding [182-184]. Structurally, they are composed of six regions, conserved between members of the seven subfamilies [185] (**fig. 2**). These regions are designated A through F and feature functional domains crucial for the function of NR: DNA binding domain (DBD) (C), C-terminal ligand binding domain

(LBD) (E/F) and hinge domain (D) [186-188] (**fig. 2**). Ligand binding induces NRs to associate with their cognate DNA response elements (also known as hormone response elements, HREs) in the regulatory regions of target genes. The DBD contains two C4 zinc fingers of which many amino acids establish non-specific contacts with the DNA backbone and only a few within the first zinc finger – residues 2-6 in the so called P box region [189, 190] – are required to confer specificity for the cognate HRE [191, 192]. Binding of agonists or antagonists to NRs results in the recruitment of coactivators, such as p160 or steroid receptor coactivators (SRC), or corepressors, such as silencing mediator for retinoid and thyroid hormone receptors (SMART) and nuclear corepressor (NCoR) [193, 194], respectively, to stimulate or inhibit gene expression. The LBD is composed of 12 (11-13) α-helices in an anti-parallel sandwich arrangement [195]. Helices 4, 5, 8 and 9 enclose a cavity and ligand binding generally stabilizes a specific conformation of the NR by interacting with residues from helices 3, 5, 6, 7 and 10, as well as the loop N-terminal to activation function 2 domain (AF-2 located C-terminally to the LBD and important for

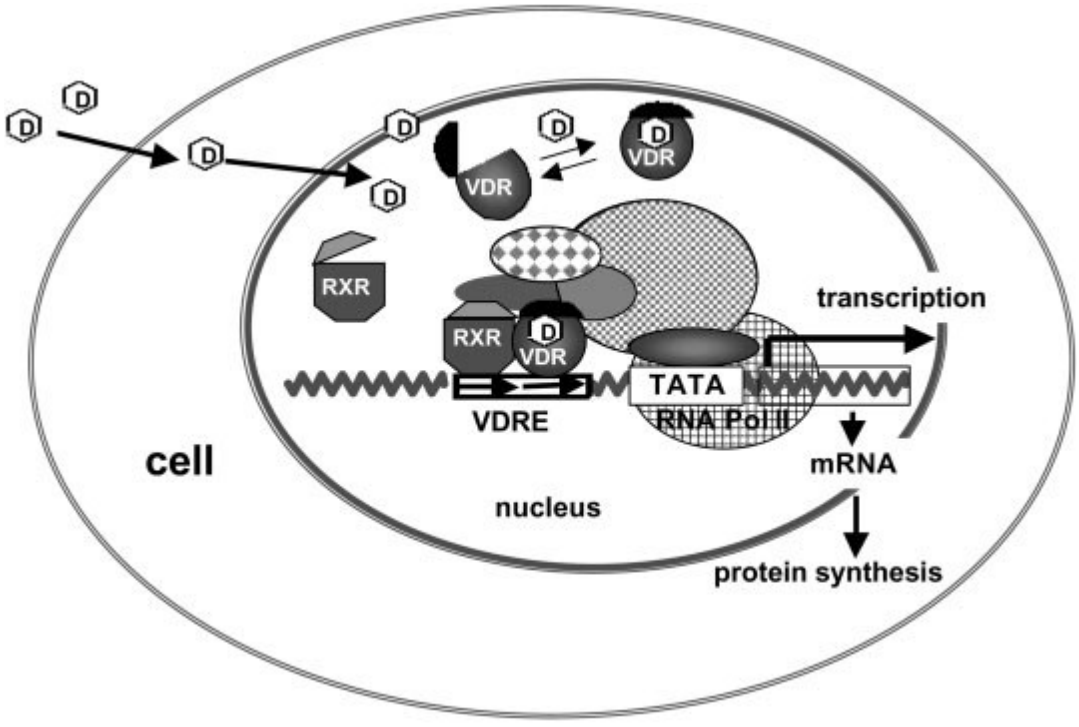


Figure 3: VDR/RXR gene regulation. Ligand-bound VDR and unbound RXR interact with co-activators and the mediator complex to recruit RNA Polymerase II and stimulate gene expression [4].

transcriptional regulation) [195]. There are some NRs with no known ligands. These are known as orphan receptors (e.g. ERR) [196, 197]. In addition, SHP1 and DAX1 constitute a NR family characterized by the lack of a DBD and they function through binding to other NRs and modulating their activity [198-200]. The NRs can bind their HREs as monomers (retinoic acid receptor-related orphan receptor γ, RORγ) [201], homodimers – retinoid X receptor (RXR) [202] – or heterodimers – RXR can function as a homo- or heterodimer [203]. VDR is also part of the latter category. It is generally accepted that upon ligand binding, VDR associates with ligand-free RXR in the nucleus to bind to vitamin D response elements and stimulate gene expression through interaction with co-activators [4] (**fig. 3**). It was recently demonstrated, however, that RXR stabilizes the otherwise unstable VDR through direct interaction even in the absence of calcitriol [204]. Residues 317-325 of the VDR and 347-353 and 419-432 of the RXRα are important for this association[203]. Binding of 1,25D to VDR triggers some important conformational changes. Hormonal vitamin D, as well as its analogs, associate with VDR through a part of the LBD called ligand binding pocket (LBP). LBP is delimited by helix 11 (H11), the beta-turn between H3-H7, and H12. H12 is particularly noteworthy since its position determines the properties of VDR: transcriptional activation or repression. The LBP is hydrophobic with certain charged residues that establish contacts with the ligand. They are located at two distinct sites within the LBP, termed site 1 – narrower, and site two – wider [205]. Residues within site 1 interact with the 1α- hydroxyl and site 2 bind the 25-hydroxyl group of the hormone. Once 1,25D is in the LBP, H12 covers the LBP, thus forming the AF-2 surface with the charged E420 and K246 positioned at opposite ends of the hydrophobic region of AF-2. This conformation of AF-2 is suitable for interacting with coactivators, whereas the open H12 is interacting with nuclear co-repressors NCoR [206]. It was shown that ligand binding to VDR does not increase its affinity towards RXR, but rather stabilizes further the VDR/RXR heterodimer and induces conformational changes in the DBD domain of VDR and H3 of RXR [203] suggesting the presence of allosteric communication between the co-receptors. Binding of either 9-cis retinoic acid or 1,25D to their cognate

receptors appears to destabilize the DBD of VDR independently of the presence of the other ligand [203]. Two models have been proposed to mechanistically explain this phenomenon: (1) the flexibility of the DBD domain is increased, which allows for a bigger capture radius – the fly-cast model [207]; (2) the increased flexibility allows the heterodimer to screen faster the DNA for the presence of vitamin D response elements (VDREs) [208].

Not only does ligand binding affect the DBD, but the reverse is also true. Binding to DNA has an effect on the LBD and co-activator-interacting surfaces of the NR. Association with a VDRE results in stabilization of the dimer interaction: H7, H8, H9 and H10 for VDR and H7 and H10 of RXR, which are part of the dimerization interface [203]. The VDR/RXR heterodimer binds to direct repeat response elements (DR3), which consists of 2 identical sites of the consensus sequence 5'-AGGTCA-3' (or a more general 5'-PuG(G/T)TCA-3' motif), separated by 3 base-pairs or everted repeats spaced by 6 base-pairs (ER6) [209-211]. The heterodimer establishes contact with the major groove of each repeat of the VDRE through both DBDs such that the LBDs are located 5' relative to the DBDs and rotated around an axis perpendicular to DNA [203, 212]. The LBDs are connected to the DBDs by a small segment, the hinge, which determines the positioning of the LBD relative to the DBD [212]. Moreover, it has been suggested that the hinge region also binds DNA [213]. VDR makes stronger contact with the 3' repeat, compared to RXR, which interacts with the 5' repeat [203]. Moreover, DNA binding stimulates further association between VDR and RXR in the DBDs and the region upstream of it. It also induces conformational changes in the co-activator binding cleft, including AF-2, to enhance the ligand-dependent co-activator recruitment [203, 214]. The rotation of the heterodimer's LBDs upon DNA binding also may play a role in orienting the co-regulators [212]. Interestingly, binding of 9-cis-retinoic acid to the RXR can synergize with calcitriol in recruiting co-activators to the heterodimer [215].

The VDR/RXRα heterodimer binds to co-activators such as SRC-1 in a 1:1 stoichiometry with SRC-1 associating only with the binding cleft of VDR [212, 216, 217], which is created through conformational

changes induced by ligand and DNA binding [203, 212, 218] (**fig. 3**). Co-activators associate with VDR or other NRs through a specific motif termed NR box (LXXLL) [219]. In the coactivators-interacting cleft of the VDR, lysine in H3-H4 and glutamate in H12 of the AF-2 domain form a charge clamp which binds and orients SRC-1 via its NR box [220]. SRC-1 [221] belongs to a family of NR co-activators named p160. Other members of this family are TIF-2 (SRC-2) [222, 223]and AIB-1 (SRC-3) [224-228]. P160 co-activators along with enzymes that possess histone acetyl-transferase (HAT) activity (CBP/p300) [229] constitute a class of VDR co-activators that modify covalently histone tails in order to activate gene transcription and facilitate the recruitment of general transcription factors and RNA Polymerase II that form the pre-initiation complex (PIC). SRC-1 has a HAT activity [230] and was shown to associate with CBP/p300 [231, 232], as well as transcription factor 2 B (TFIIB) and TATA box binding protein (TBP) [233] thus bridging VDR with the basal transcriptional machinery. Another class of co-activators for VDR is the ATP-dependent chromatin remodelling complexes such as WINAC [234]. Yet a third class of co-activators consist of proteins that regulate gene transcription through recruitment of GTFs and RNA polymerase II at target gene promoters. In the case of VDR, this is mediated by the DRIP/TRAP complex [235]. It has been demonstrated that DRIP and p160 co-activators are involved in the regulation of different aspects of cellular behaviour in keratinocytes. Notably, DRIP co-activators are implicated in the regulation of proliferation, whereas SRC – in genes important for differentiation [236]. In addition, the preferential binding of one or the other class of co-activators to the VDR is dependent on the differentiation state of the cell [236-239]. Other co-activators have been described that do not belong to any of the above classes, such as SMAD3 [240] and NCoA-62 [241]. NCoA-62 (Nuclear Receptor Co-activator, 62kDa) does not belong to the p160 family of co-activators and its mode of action is quite different. It is bound to unliganded VDR (but does not interact with the AF-2 domain) and calcitriol enhances this interaction 2-3-fold and modestly augments gene transactivation. Importantly, calcitriol also triggers the formation of a ternary complex of VDR/SRC-1/NCoA-62, in which NCoA-62 and SRC-1 synergize in gene activation

[230]. It should be mentioned that co-activator recruitment does not result in proteins being permanently docked to the VDR. Rather, it is a cyclic process that allows for continuous assessment of ligand presence in the LBD of VDR [242]. Thus, when the ligand is no longer present, the co-activators are released giving co-repressors a chance to bind so that target genes are repressed. Nuclear receptor co-repressor (NCoR) [243] and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) [244] are the founding members of the co-repressor family for VDR. It has grown to include RIP13δ1 [245, 246], SMRTe [247, 248], SUNCoR [249] and Alien [250]. NCoR and SMRT mediate repression through N-terminal HDAC activities [251-253] and associate with VDR through a C-terminal NR box [254], which contains a motif called co-repressor NR box (CoRNR box) of consensus sequence L/I XX I/V I and LXX I/H IXXX I/L [255-257]. The sequence of the CoRNR box allows for the formation of an α-helix one turn longer than the LXXLL motif present in the co-activators, which permits binding to the open conformation of AF-2 of the VDR [256, 257]. Ligand binding, therefore results in release of co-repressors as they are no longer able to bind the open AF-2 [258]. Alternatively, the co-repressor hairless (Hr) was found to repress gene transcription in a ligand-dependent manner. It is mainly expressed in hair follicles, brain and epidermis. Its association with unliganded VDR is weak, but present (mediated by its LDSII motif) and is greatly increased upon ligand binding (through its LSELL motif) and so are its gene repression actions [259, 260]. Alien, on the other hand, does not bind to the AF-2 domain of VDR and is released upon $1,25D_3$ binding [251]. Ligand-bound VDR is also able to repress gene transcription through squelching of TF [261, 262]. These modes of gene regulation, along with the non-genomic responses elicited by VD mediate its beneficial for human health effects, the classical example of which is the regulation of skeletal and mineral homeostasis.

Skeletal and Mineral Homeostasis Effects of VD

Calcium is an important element that plays an essential role in skeletal mineralization [263] and a variety of physiological, extracellular and intracellular signalling events. It can only be obtained from the diet and must be kept within a narrow range in human serum [264]. As a result, a complex regulatory system has evolved to control calcium homeostasis, in which VD plays a crucial role [265, 266]. Calcitriol is associated with increased Ca^{2+} absorption from the intestine and reabsorption from the kidney.

Intestinal Calcium Absorption

There are two modes of calcium transport in the intestine: saturable (transcellular) and non-saturable (paracellular) [267, 268]. The former is energy-dependent, mostly present in the lower bowel [269] and accounts for close to 60% of total calcium absorption. It is stimulated by 1,25D [270-272]. Specifically, VD sufficiency is required for the transport of Ca^{2+} from the brush border across the cell to the basolateral membrane [273] of the enterocytes. Its effects are noticeable after 2-4h, suggesting that they are mediated by ligand-bound VDR-stimulated gene expression. The fact that VDR is essential for Ca^{2+} absorption is supported by the following observations: (1) *In vivo*, VDR gene deletion is associated with Ca^{2+} malabsorption phenotypes such as osteomalacia, poor growth and elevated serum PTH [274-276]; (2) *In vitro*, overexpression of VDR in the intestinal cell line CaCo-2 increases 1,25D-stimulated Ca^{2+} transport [277]. Its absorption in the intestine can be attributed to the effects of VD on four modes of transportation: facilitated diffusion, vesicular transport, transcaltachia and paracellular entry through tight junctions. In the context of facilitated diffusion, VD was shown to regulate both uptake (through an apical membrane calcium channel TRPV6) [278-284] and the rate-limiting [280, 281] transport to the basolateral membrane (by calbindin D_{9k})[271, 274, 278, 284, 285] in mice, rats and humans. Finally, once the calcium has been ferried from the brush border to the basolateral membrane, where the plasma

membrane Ca^{2+} ATPase 1b (PMCA1b), a vitamin D target gene [286] , exports Ca^{2+} against the concentration gradient from the cytoplasm to the serum [287, 288]. Calcitriol is able to enhance intestinal calcium absorption also through stimulating vesicular transport [289]. There is evidence that 1,25D increases lysosome formation [290, 291]. Vesicles thus formed act as $Calbindin_{9k}$ surrogates to ferry Ca^{2+} across the cell and blocking their formation limits calcium absorption [290].

A very interesting mode of intestinal calcium absorption regulation by VD is transcaltachia. It occurs only minutes after 1,25D exposure implicating non-genomic effects. The fact that only serosal 1,25D stimulates transcaltachia [292] points to the existence of a 1,25-$(OH)_2D_3$ binding protein at the basolateral side of enterocytes [176]. Two such receptors have been identified: membrane VDR [179] and MARRS [172]. Notably, deletions of MARRS in mice limit 1,25D-induced calcium absorption [293]. There is also evidence that 1,25D increases paracellular Ca^{2+} flux [268, 294, 295] through upregulation of claudin 2 and 12 mRNA and protein [296]. They participate in the formation of tight junctions and their levels are significantly lower in VDR KO mice. In line with these observations, knockdown of these claudins leads to reduced Ca2+ permeability in CaCo-2 cell line.

Renal Calcium Reabsorption

Apart from the well characterized effects on intestine, VD is also know to increase renal calcium reabsorption in the distal convoluted (DCT) and connecting tubules (CNT) [280, 297]. The mechanism of transport is quite similar to that in enterocytes: Ca^{2+} enters the renal epithelial cell via the calcium channel TRPV5 at the apical membrane, is ferried by $calbindinD_{28k}$, and is released into the serum at the basolateral side via PMCA1b and the sodium/calcium exchanger, NCX1. In a rat model, vitamin D deficiency resulted in a decreased expression of TRPV5 [298], $calbindinD_{28k}$ and NCX1, and the physiological manifestation was hypocalcemia. Supplementation with 1,25-$(OH)_2D_3$ restored expression of the three renal proteins mentioned above and normalized serum Ca^{2+} levels [299]. Similar

observations were documented in mice where TRPV5 KO is associated with malfunction of the Ca^{2+} reabsorption pathway in the DCT and the CNT [300].

Endocrine Regulation

The kidney is also important for production of the hormonally active form of vitamin D, which is regulated by Ca^{2+} homeostatic signals such as Ca^{2+} itself and PTH (**fig. 1**). *CYP27B1* is expressed in the proximal tubule of the kidney [301-303]. The expression of this enzyme is inhibited by its own end product through the VDR in a negative feedback loop [304] and is activated by calciotropic stimuli such as PTH and calcitonin [305, 306]. *CYP27B1* repression is VDR/RXR- and 1,25-$(OH)_2D_3$-dependent without there being any consensus VDREs [307, 308]. Binding of the activated VDR/RXR heterodimer to the negative gene regulatory sequence was found to occur indirectly through tethering to a transcription factor called vitamin D interacting receptor (VDIR) [307, 308]. There is dissociation of p300 and a subsequent reduction of HAT with a concomitant upregulation of HDAC activity and binding of co-repressors NCoR, HDAC2 and Sin3A. Moreover, there is increased methylation of CpG islands at the promoter as a result of DNMT1 and DNMT3B methyltransferase activity. 1,25-$(OH)_2D_3$, therefore, represses the expression of renal 25-OHD_3 1α-hydroxylase through histone deacetylation and promoter DNA methylation [309]. Alternatively, calcitriol also stimulates its own degradation by strongly enhancing the expression of *CYP24A1* – the enzyme that catalyzes the limiting step of vitamin D catabolism (**fig. 1**)– through binding of VDR/RXR to several regulatory elements of the gene. PTH induces HAT activity association with VDIR in a PKA and cAMP dependent manner [310, 311] resulting in increased renal *CYP27B1* expression. Circulating PTH levels are increased as a consequence of low serum Ca^{2+}, which activates several pathways in order to correct the hypocalcemia. The Ca^{2+} sensing receptor (CaR) is normally active when bound to Ca^{2+} and is inhibited at low serum Ca^{2+} concentrations. Active

CaR decreases PTH secretion and parathyroid cell growth [312]. It acts in the kidney, on the other hand, to decrease calcium reabsorption [313, 314].

Under conditions of low serum Ca^{2+}, PTH increases gene expression of renal 25-OHD_3 1α-hydroxylase through its cognate receptor, PTHR, resulting in upregulation of intestinal Ca^{2+} absorption and kidney reabsorption [299, 315]. Increased 1,25-$(OH)_2D_3$ and Ca^{2+} in turn act in the parathyroid gland to decrease PTH production [316] and secretion [317] via VDR [318] and CaR [315], respectively. It was also demonstrated that ligand-bound VDR inhibits proliferation of parathyroid cells [319, 320] where it upregulates its own expression. PTH and calcitriol, as Ca^{2+} homeostatic hormones, also have an effect on bone, which acts as a reservoir of calcium and phosphate. Acting together through their cognate receptors, they increase resorption in order ameliorate hypocalcemia. PTH by itself is associated with increased bone formation and osteoblastic resorption [321], whereas ligand-bound VDR stimulates net bone resorption through increased osteoclast size [321]. The bone anabolic effects of PTH appear to be mediated, at least in part through canonical Wnt/β-catenin signaling pathway in osteoblasts [322]. In terms of VD-induced bone accretion, there is the obvious indirect effect of calcitriol on elevation of circulating Ca^{2+} levels. In addition, 1,25-$(OH)_2D_3$ seems to have direct effects on bone mineralization as was shown in mouse models [323].

Vitamin D and Phosphate Homeostasis

The role of VD in phosphate homeostasis has also generated considerable interest. Phosphate is involved in bone mineralization, macromolecule synthesis and intracellular signalling. It is readily available from the diet and its intestinal absorption is mostly unregulated [324]. Renal reabsorption, therefore, is the convergence point for the majority of phosphate homeostatic signals [325], the two main players being PTH and fibroblast growth factor 23 (FGF23). Increased serum phosphate upregulates PTH secretion directly by increasing its mRNA stability and indirectly through down-

regulating free Ca^{2+} in the serum [298]. PTH then binds its receptor (PTHR1) at the basolateral and apical membrane of the proximal tubule in the kidney inducing PKA and PKC activation, respectively, which both result in internalization of the membrane sodium-phosphate co-transporters NaPi-IIa and NaPi-IIc responsible for phosphate reabsorption. The net effect is phosphate wasting [326]. The same effect is attained through increased FGF23 production – mainly by osteocytes and osteoblasts [327] – stimulated by increase in phosphate [328]. FGF23 binds to its receptor (FGFR1) in the proximal tubule and results in decreased phosphate reabsorption [329] by down-regulating NaPi-IIa and NaPi-IIc expression [330]. It also inhibits *CYP27B1* and up-regulates *CYP24A1* expression, thus reducing active VD levels [331]. 1,25-$(OH)_2D_3$ has several effects on phosphate homeostasis. Firstly, it increases phosphate uptake from the diet by Pit-2 upregulation [332]. Secondly, it enhances the production and secretion of FGF23 in osteocytes and osteoblasts [333, 334], thus acting as a negative feedback mechanism to reduce serum phosphate levels.

In conclusion, the complex endocrine interaction between PTH, hormonal VD and FGF23 implies that disruption of signalling pathways implicating any of these hormones will affect the others and negatively impact calcium or phosphate homeostasis, or both.

Molecular effects of VD in cancer

The anti-cancer effects of 1,25D at the cellular level appear to be tissue specific and no universal mechanism or pathway could be used to explain them. However, a common feature is that VD seems to promote quiescence and induce a more differentiated phenotype in most cancer cells. Here, three such cancer cell types will be discussed, as the VD effects on these cells are well described.

As mentioned earlier, 1,25D has beneficial effects for skin cancer patients. At the molecular level, this is due to inhibition of proliferation and is associated with the expression of differentiation markers such as involucrin, loricrine, flaggrin and transglutaminase [236]. VD was also demonstrated to

confer protection against UV-induced DNA damage by stimulating mechanisms resulting in increased p53 expression, down-regulated stress-induced kinases activation and inhibition of nitric oxide production.

The anti-cancer effects of VD are best described and the most extensively studied in colon cancer. In terms of the molecular mechanisms, the most prominent pathway affected is the Wnt/β-catenin pathway. It is inhibited by ligand-bound VDR via binding to β-catenin (via its AF-2 domain) [335]. VD is also able to increase the expression of cystatin D, which inhibits cysteine proteases from the cathepsin family and is thus needed to block Wnt signalling and confer differentiated phenotype [336].

VD has also been shown to trigger cellular events leading to breast cancer prevention. Most breast cancer cell lines, especially the less aggressive and estrogen receptor-positive ones are growth inhibited by 1,25-$(OH)_2D_3$ [337]. This is achieved through regulation of a broad range of targets. Some of these targets are implicated in the cell cycle: down-regulation of cyclins, cyclin-dependent kinases and upregulation of their inhibitors [338-340]. Other targets consist of apoptotic and autophagy-related genes such as members of the Bcl-2 family, caspases and cathepsins . In addition, differentiation markers such as casein, adherent proteins (E-cadherin) and lipid droplets are also induced as a result of 1,25D treatment [341, 342].

FoxO

The forkhead box family of transcription factors was first discovered in *Drosophila melanogaster* [343] where the FOX gene product was found to play an important role in development. In mammals, there are several families of forkhead transcription factors, one of them being the forkhead box O (FoxO) subfamily. It is composed of 4 members: FoxO1, FoxO3a, FoxO4 and FoxO6. The first three share a great degree of homology and are ubiquitously expressed. Still, FoxO1 appears to be present at higher levels in adipose tissue, FoxO3a – in ovaries, kidneys, heart and brain, whereas FoxO4 is mainly found in

muscle and heart [344-346]. FoxO6, however appears to have a neuronal pattern of expression [347] and is not regulated by nuclear-cytoplasmic shuttling following growth factor-induced phosphorylation [348]. FoxOs are *bona-fide* tumour suppressors as their ablation results in transformation and tumorigenesis at the cellular [349-356] and organismal level [357]. In the same line of evidence, overexpression of FoxOs in various cell types strongly blocks cell proliferation [358-361]. Their functions of regulating apoptosis, cell proliferation, differentiation and stress (especially oxidative) resistance are conserved in *Caenorhabditis elegans*, *D. melanogaster* and vertebrates [362, 363].

Structure-Function Relationship

Due to the importance of these transcription factors, it is crucial that they are tightly regulated.

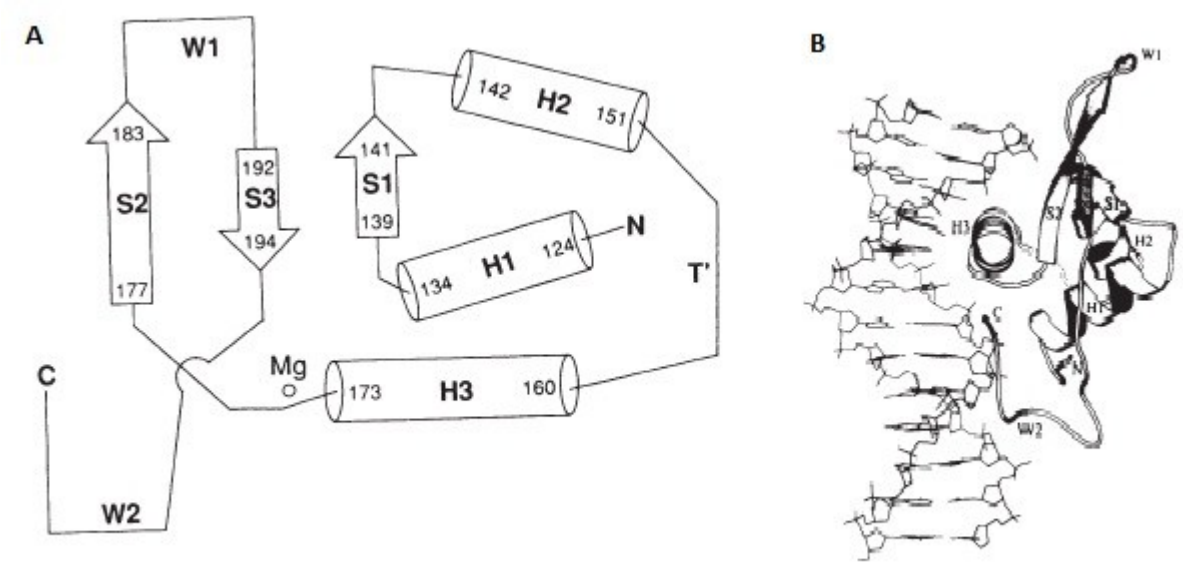


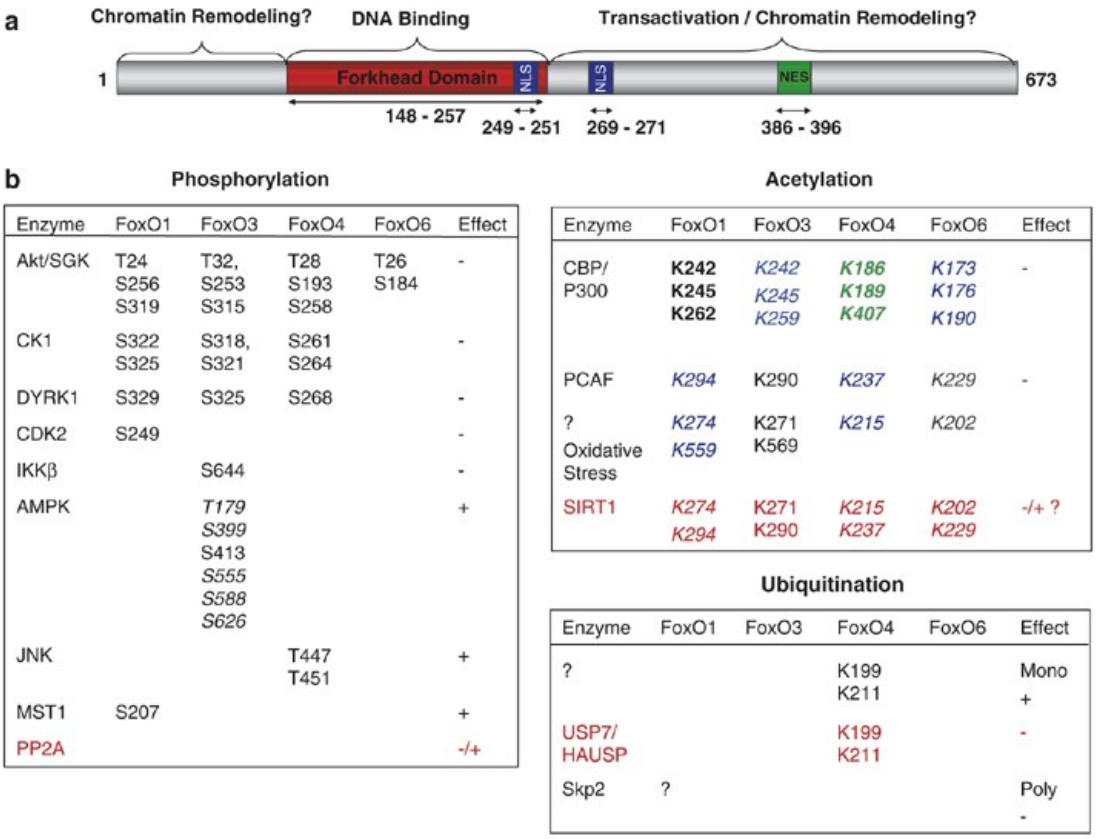
Figure 4: Structure of the Fork-head Domain. (A) S designates β-strands, H refers to helices and W, wings or loops. The relative position of the amino-acids delimiting each segment are also represented. (B) The fork-head domain as it interacts with DNA. The DNA is represented by a stick figure, whereas the winged helix domain, as a ribbon structure. The importance of H3 for establishing contact with DNA is apparent [3].

Based on what have been described so far, this regulation is mostly done through post-transcriptional mechanisms such as post-translational modifications. In order to better understand how these mechanisms can impact the function and stability of FoxOs, one should first become familiar with their

structure (**fig. 4**). A unifying feature of all Fox proteins is their DNA binding domain [3, 364], which is composed of around 110 amino-acids arranged in a winged helix motif [365-367] . Generally, it contains three alpha-helices (H1, H2 and H3), three beta-strands (S1, S2 and S3) and two loops or wings (W1 and W2) [3](**fig. 4**) such that the alpha-helical cluster (H1, H2 and H3) is C-terminal relative to the β-strands portion (S2 and S3) and the two protruding wings (W1 and W2). In the context of the whole molecule, the DBD is located closer to the N-terminus and is followed by a nuclear localization signal, nuclear export sequence and the transactivation domain at the C-terminus. The core sequence recognized by the DBD of Fox transcription factor is 5'-(A/C)AA(C/T)A-3' [343, 368]. H3 (especially Asn165 and His169) is crucial for sequence recognition and binding of the Fox monomer to the major groove of DNA [3]. W1 and W2 also interact with the DNA duplex (both bases and backbone) via van der Waals forces, hydrogen bonding and electrostatic forces thus establishing specific and non-specific interactions with major and minor grooves [3, 369, 370].

Members of each subfamily of Fox transcription factors, however harbor minor variations in the DBD motif, possibly allowing them to preferentially bind to different consensus sequences. FoxO4, for example contains another short helix between H2 and H3 [343]. The FoxO subfamily members bind preferentially to the consensus sequence 5'-GTAAACAA-3' referred to as the DAF-16, the *C. elegans* orthologue of FoxO, binding element (DBE) or fork-head response element (FHRE) [371, 372]. FoxOs were also shown to bind with a smaller affinity to the insulin-response sequence, 5'-(C/A)(A/C)AAA(C/T)AA-3' [371-379]. Studies of FoxO3a bound to a FHRE demonstrated that Arg211, His212 and Ser215 of the H3 are required for binding [380]. Additionally, the W2 adopts a coil structure exposing the basic Arg248, Arg249 and Arg250 to interact directly with the DNA backbone [380]. It was also observed that the N-terminal region of the DBD, upstream of H1, is important for DNA binding as well [381].

Post translational modifications have a great impact on FoxO function and stability and it is important to understand their role in the structural context of the proteins. Phosphorylation is the best



Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
Akt/SGK	T24 S256 S319	T32, S253 S315	T28 S193 S258	T26 S184	-
CK1	S322 S325	S318, S321	S261 S264		-
DYRK1	S329	S325	S268		-
CDK2	S249				-
IKKβ		S644			-
AMPK		*T179* *S399* S413 *S555* *S588* *S626*			+
JNK			T447 T451		+
MST1	S207				+
PP2A					-/+

Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
CBP/ P300	**K242** **K245** **K262**	*K242* *K245* *K259*	*K186* *K189* *K407*	*K173* *K176* *K190*	-
PCAF	*K294*	K290	*K237*	*K229*	-
? Oxidative Stress	*K274* *K559*	K271 K569	*K215*	*K202*	
SIRT1	*K274* *K294*	K271 K290	*K215* *K237*	*K202* *K229*	-/+ ?

Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
?			K199 K211		Mono +
USP7/ HAUSP			K199 K211		-
Skp2	?				Poly -

Figure 5: FoxO translational modifications. (A) Schematic of human Foxo3a. Different domains are represented with the amino acids delimiting them. (B) Post-translational modifications of the members of the FoxO family. Left panel: phosphorylation. Upper right panel: acetylation. Bottom right panel: ubiquitination [1].

studies and seems to have the greatest effect (both positive and negative) on function. FoxOs are phosphorylated by protein kinase B (PKB) and serum and glucocorticoid-inducible kinase (SGK) on three sites (**fig. 5**) [372, 374, 377, 382-384]: the N-terminus upstream of the DBD; the W2 of the fork-head domain itself in a region proposed to play the role of NLS [3, 380, 385-387] and between the NLS and the NES. There is evidence that phosphorylation at the second PKB/SGK site interferes with DNA binding [380, 381, 388, 389]. The third site – Ser319 for FoxO1 – results in the stimulation of casein kinase 1 (CK1) binding to further phosphorylate the protein at Ser322 and subsequently, Ser325 [390], followed by phosphate modification at a fourth site by the dual tyrosine phosphorylated regulated kinase-1A

(DYRK1A)[391]. These four latter modifications result in the creation of a negatively charged region promoting nuclear export in an Exportin/Crm1- and Ran-dependent manner [390]. In addition, the first two PKB phosphorylation sites (the N-terminus and in W2/NLS) represent a 14-3-3 consensus binding motif [382, 392-395]. Moreover, as suggested by their position, disruption of DNA binding has been demonstrated [396, 397]. In addition, 14-3-3 binding induces a conformational change that exposes FoxOs' NES (leucine-rich) to promote, along with 14-3-3 itself, Exportin/Crm1-mediated nuclear export [394]. Regulation of subcellular localization of FoxOs by 14-3-3 is also controlled by masking the NLS thus preventing nuclear re-import [382, 384, 398-401]. Apart from PKB, other kinases are also able to phosphorylate FoxOs resulting in various outcomes regarding their function. Cyclic GMP-dependent kinase-1 (cGMP-1) phosphorylates Ser152-155 upstream of H1 and Ser184 in H2 of FoxO1 and inhibits DNA binding [380, 387, 402]. This, however, is unique to FoxO1. MST1 phosphorylates a highly conserved serine residue in H3 that disrupts interaction with 14-3-3 potentiating FoxO activity [3]. Again specific to FoxO1 only, is the Cdk-2-mediated phosphorylation of Ser249 in W2 that results in reduced DNA binding [380].

Although not as extensively studied, acetylation also plays an important role in regulating FoxO function. Out of the several acetylation sites, two occur at W2 of the fork-head domain and were shown to disrupt DNA binding by neutralizing the negative charge of lysine residues [5, 380]. Generally, however, not much is known about effects of acetylation and it is accepted that acetylation has gene-specific effects on FoxOs. Mono-ubiquitination, on the other hand, has an opposing effect to acetylation - nuclear localization and activation – and occurs, at least in part, at the same lysine residues as acetylation [403]. USP7/HAUSP catalyzes the removal of single ubiquitin molecules thus reversing the effect. Conversely, poly-ubiquitination triggers degradation of FoxO by the proteasome [349].

In conclusion, one could claim that there exists a FoxO code (**fig. 5**) that, analogous to the histone code, fine-tunes the function of these transcription factors according to the various signals that converge on them.

Regulation of FoxOs and Effects on Their Function

FoxO proteins are transcription factors normally found in the nucleus. They induce or repress target gene expression to control cellular processes involved in stress resistance, cell cycle, programmed cell death and metabolism. FoxOs induce arrest at the G1 phase of the cell cycle by enhancing $p27^{kip}$ [360, 361, 404-406], p15 and p19 [407] gene expression, and by inhibiting c-Myc through upregulation of its transcriptional inhibitor Mxi1 [408]. In addition, cyclin D1 (CCND1) [409] and cyclin D2 (CCND2) [410] are transcriptionally suppressed, which, along with stimulation of p130 [359], delays or blocks entry into S phase. FoxOs also up-regulate cyclin G2 (CCNG2) [411] and growth arrest and DNA-damage-inducible protein 45 α(GADD45a) [412, 413], which results in arrest in the G2 phase of the cell cycle [413]. The latter occurs mostly under conditions of stress [412]. The G2 arrest is believed to be important in DNA repair.

In the context of ROS detoxification, FoxOs induce the expression of enzymes such as manganese superoxide dismutase (MnSOD) [414], peroxiredoxin III (Prx-3) [415], sestrins [416], selenoprotein P [417], PINK1 [418] and Catalase [419], and down-regulate thioredoxin interacting protein (TXNIP) to enhance thioredoxin activity [420] thus decreasing oxidative stress and increasing cell survival [345]. In the advent of high oxidative stress level with which the cell cannot cope, FoxOs contribute to the induction of cell cycle arrest followed by programmed cell death [421, 422]. FoxOs are capable of inducing apoptosis by stimulating genes involved in triggering both the intrinsic [404, 406] and extrinsic pathways. Upregulation of Bim [404], PUMA [423], BNIP3 [424] and Bcl-6 [425] stimulates cytochrome c release and activation of the intrinsic apoptotic pathway, whereas induction of tumour

necrosis factor-related apoptosis inducing ligand (TRAIL) [426], tumour necrosis factor receptor-associated death domain (TRADD) [427] and Fas-ligand (FasL) [382] stimulate the extrinsic apoptotic pathway.

In the liver, FoxO enhances hepatic gluconeogenesis by inducing the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxikinase (PEPCK) [428, 429]. In muscle cells, on the other hand, FoxO transcription factors stimulate the expression of antrogin-1 and MuRF-1, which are ubiquitin ligases essential for muscle atrophy and muscle wasting [429, 430]. Similarly, autophagy is enhanced by FoxO3a in muscle cells by up-regulating relevant genes such as *MAP1LC3*, *GABARAP*,

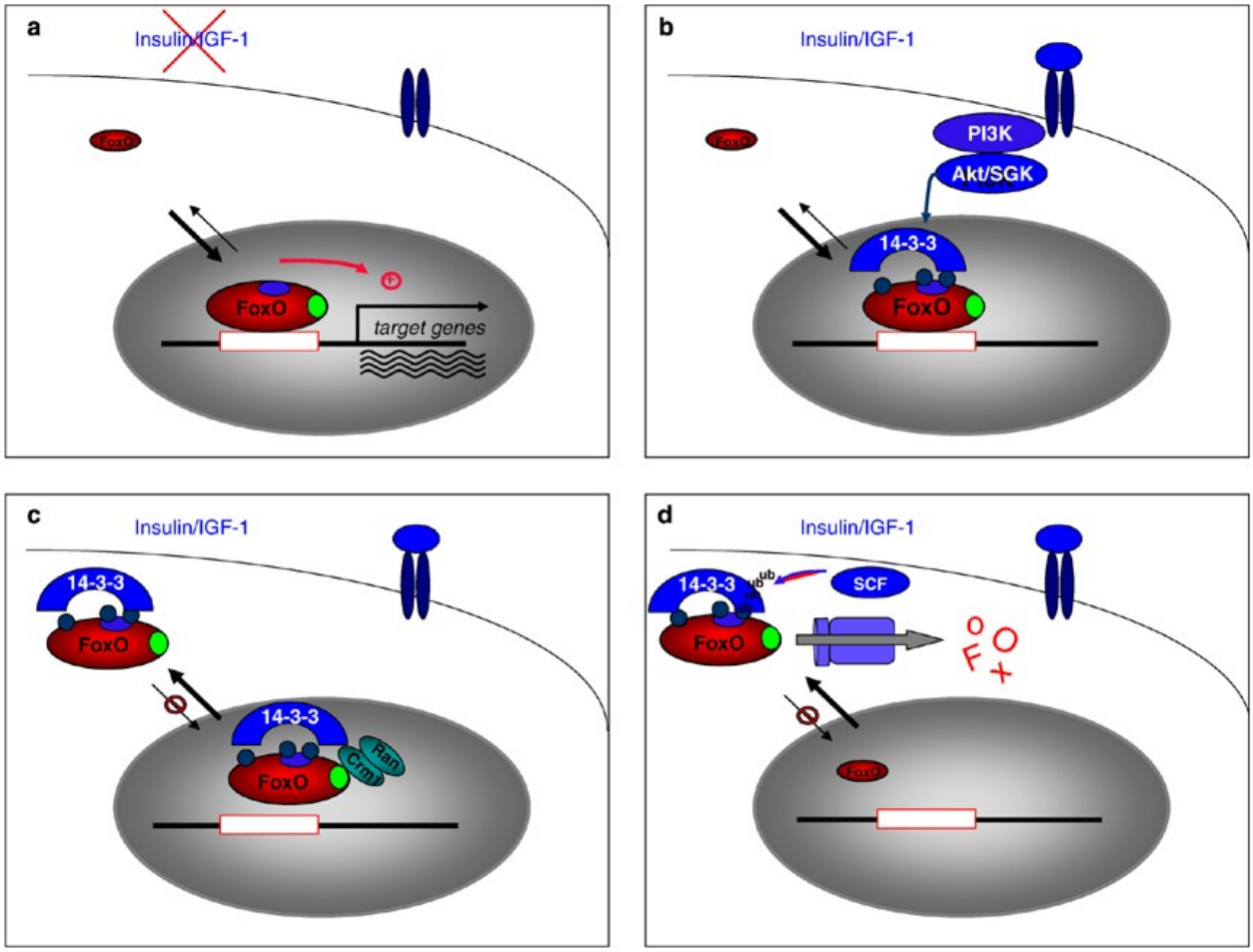


Figure 6: Regulation of FoxO subcellular localization and function by the PI3K/PKB pathway. (A) In the absence of growth factor stimulation, FoxO proteins are in the nucleus and regulates target genes through binding to FHREs. (B) Upon mitogenic stimulation, PKB (designated here as Akt) and SGK are stimulated and are able to phosphorylate FoxO at three conserved sites. (C) This results in release of FoxO from FHREs and binding to 14-3-3 resulting in nuclear export. (D) Upon prolonged growth factor stimulation, FoxOs are poly-ubiquitinated by SCF and degraded by the proteasome [1].

ATG12, *BNIP3* and *BNIP3L* [431-435]. Both proteasomal degradation, autophagy and the upregulation of myostatin [436] are known to cause muscle atrophy [432].

FoxOs are also implicated in tissue homeostasis. They maintain hematopoietic stem cell (HSC) pool by keeping the latter in a quiescent state and loss of FoxOs results in increased levels of myeloid progenitors [345] and a concomitant decrease in HSC due to increased ROS levels and apoptosis [345]. These transcription factors are also implicated in maintenance of the naïve T cell pool through inducing IL7-R production [437] and differentiation of chronic myeloid leukemia cells into erythrocytes through upregulation of ID1 [438]. All these important functions of FoxO transcription factors imply tight regulation.

In this context, the best studied and probably one of the most important pathways is the phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathway (**fig. 6**). Upon insulin and growth factor stimulation, a receptor tyrosine kinase is activated to subsequently recruit and activate the phosphoinositide-3-kinase (PI3K). PI3K transfers a phosphate group from ATP to phosphatidylinositol 4,5-bisphosphate [PtIns(4,5)P2; PIP2] in the cell membrane to generate phosphoinositide-3,4,5-P3 (PIP3), which serves as a secondary messenger [439]. This action is reversed by the phosphatase PI3K/phosphatase and tensin homologue deleted on chromosome ten (PTEN), which removes a phosphate group from PtIns (3,4,5)P3 essentially blocking the whole pathway [440]. PKB is recruited to the PIP3 at the membrane via its pleckstrin homology domain [441]. Once at the cell membrane, PKB is phosphorylated by another kinase – phosphoinositide-dependent kinase 1 (PDK1), which is localized to the cell membrane by the same mechanism as PKB [442]. This phosphorylation at Thr308 results in activation of PKB and translocation to the nucleus where it can phosphorylate and thus modify the activity of a plethora of substrates. In this context, it was demonstrated that FoxO proteins are phosphorylated on all three PKB sites [373, 374, 376, 382] as discussed earlier upon growth factor stimulation [359, 382] with insulin and insulin-like growth factor (IGF-1) having the most prominent

effect. Conversely, mitogens and cytokine withdrawal results in activation of FoxOs and the transcription of their target genes [404-406, 414]. Notably, the same effect is achieved by treating cells with the PI3K inhibitor LY294002 demonstrating the importance of the PI3K/PKB signaling pathway in inhibition of FoxO transcription factors. SGK, which is highly homologous to PKB, is able to phosphorylate FoxOs at the same sites (Thr32, Ser253 and Ser315 for FoxO3a) [443], but has preference for Thr32 and Ser315, whereas PKB, for Thr32 and Ser253. As mentioned earlier, phosphorylation by PKB/SGK results in nuclear export through the 14-3-3 chaperone. Interestingly, phosphorylation at Ser319 of FoxO1 creates a consensus binding motif for CK1, which further phosphorylates it on Ser322 and Ser325 [390] to increase its sequestration in the cytoplasm [444]. This sequestration could be further enhanced by binding of FoxOs to the protein Melted found in *Drosophila*, which is found at the plasma membrane [445]. The PI3K/PKB-mediated regulation FoxO transcription factors is conserved in *C. elegans* [446-451] and *Drosophila* [452-454] emphasizing the importance of this pathway. Additionally, in these two model organisms, DAF16 and dFOXO, respectively, significantly extend the lifespan of the organism [455]. A similar role of FoxOs has been suggested for humans [456-458].

DYRK1A is another serine/threonine kinase that constitutively modifies FoxO1 at Ser329 and FoxO4 at Ser268 to enhance PKB/SGK- and CK-1-mediated nuclear export [390, 391], as discussed earlier. IκB kinase (IKK) is able to phosphorylate FoxO3a on Ser644 in response to tumour necrosis factor alpha (TNFα) resulting in inhibition of transcriptional activity, nuclear export and proteasomal degradation following poly-ubiquitination [349]. Unlike DYRK1A, this pathway is not complementary to PKB, but rather functions independently [349]. It has been shown also that cyclin-dependent kinase 1 and 2 (CDK1 and CDK2) synchronize cell cycle progression with FoxO inhibition by direct phosphorylation of FoxO1 at Ser249 inducing its cytoplasmic translocation [459, 460]. When DNA damage triggers Chk1- and Chk2-mediated inhibition of CDK2, however, FoxOs are able to up-regulate CCNG2 [461] and down-

regulate CCNB1 and CCNB2 expression [462]. In the advent of too extensive DNA damage, FoxOs trigger cell death through the induction of apoptotic genes, notably Bim, BNIP3, BNIP3L, FASL and TRAIL [424].

Phosphatases also play an important role in the regulation of FoxO function, although they have not been studied as extensively as the kinases and are not as well defined at this time. One phosphatase that was demonstrated to remove phosphates off FoxO protein, is PP2A, which essentially activates the transcription factors [463-465]. PTEN, as mentioned earlier, does not directly act on FoxO, but rather inhibits the signaling pathway leading to FoxO deactivation. Interestingly, oxidative stress was shown to inhibit PTEN, which results in increased phosphorylation and nuclear export by PKB [440].

There are certain kinases which are induced under stress conditions – oxidative and metabolic – that activate FoxOs. Oxidative stress-induced kinases generally overpower the inhibitory effects of PKB. One example is the c-Jun N-terminal kinase (JNK), which, following increased ROS levels, phosphorylates FoxO4 at Thr447 and Thr451 inducing its nuclear re-localization and target gene regulation [466-469]. In addition, JNK can phosphorylate 14-3-3, which greatly reduces the affinity of the latter for FoxO [469], and PKB, thus inhibiting its activity [470]. It was also shown that phosphorylation by JNK increases FoxO acetylation and pro-apoptotic gene expression [471]. The mammalian homologue of yeast Sir2- (SIRT1) mediated deacetylation, on the other hand, targets FoxOs to genes increasing the defenses against oxidative stress [471] as outlined above. The mammalian Ste-20-like protein kinase (MST1) is another kinase induced by oxidative stress. It targets FoxO3a to the nucleus via phosphorylation of Ser207 and subsequent release from 14-3-3 [472]. This mode of regulation has also been demonstrated for FoxO1 [473, 474]. However, phosphorylation by MST1 interferes with binding of the transcription factors to DNA, which raises the point that a phosphatase is required to remove the phosphate group prior to gene regulation [473]. Additionally, MST1 activates JNK unless the latter is inhibited via phosphorylation by PKB at Thr120 [475, 476]. PKB also directly phosphorylates MST1 on Thr387 thus blocking activation of JNK [475, 477]. Regulation of DAF16 by the JNK and MST1 homologues – JNK-1 and CST-1,

respectively – in *C. elegans* is conserved: both kinases are activated as a result of increased oxidative stress and phosphorylate DAF16 inducing its activation [467, 472]. These pathways are also present in *Drosophila* where, Hippo, the homologue of MST1, and JNK activate dFoxO in response to oxidative stress [468, 478], which, as in vertebrates, induces the expression of genes with antioxidant function such as *l(2)efl*, *hsp68*, *fer1HCH*, *Sestrin* and *jafrac1* [468, 479, 480]. Mitochondrial ROS are also able to activate AMPK [481]. Normally, AMPK activity is triggered by metabolic stress, which is sensed via increase AMP/ATP or ADP/ATP ratio. In this context, ROS and energy depletion is coupled to FoxO3a activity through AMPK, which phosphorylates this transcription factor on 6 novel sites to increase their transactivation activity, but not DNA binding or nuclear import [482]. These sites are Thr179, Ser399, Ser413, Ser555, Ser321, Ser528 and Ser626 [482]. The effect of activation of FoxO3a by AMPK is changes in expression of genes implicated in energy metabolism and stress resistance ultimately leading to health span and life span extension as a result of dietary restriction [483]. This pathway is conserved in *C. elegans* [482, 484] where the beneficial effects of caloric restriction were shown to be mediated by DAF16. Under conditions of lack of nutrients, however, autophagy is triggered in a FoxO-dependent fashion [433-435].

The fact that FoxOs are induced by metabolic stress is consistent with their role in the regulation of metabolism and changes during fasting at the organismal level [485-487]. For example, FoxO1 expression was found to be higher in the liver of diabetic mice [486] with a concomitant increase in the enzymes G6Pase and PEPCK, as well as PGC1α [488] and increased gluconeogenesis [487], which is conserved in *Drosophila* [455, 489, 490]. FoxO1 is also responsible for the impairment of pancreatic beta cells proliferation and function [491], and promotes food intake [492]. At the cellular level, the response of forkhead box O transcription factors to metabolic stress is mediated not only by AMPK, but also by SIRT1, a mechanism conserved in *C. elegans* . SIRT1 is class III HDAC enzyme which requires nicotinamide adenine dinucleotide (NAD^+) to function. The NAD^+/NADH ratio is also a sensor of energy status, higher

numbers signifying energy deficiency. AMPK is implicated in increasing this ratio, and therefore, SirtT1 activity [493]. Acetylation of FoxO transcription factors is mediated by several HAT enzymes: CREB-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF). PCAF binds to phospho-Ser253 of FoxO1 (PKB phosphorylation site) [494]. CBP/p300 binding were shown to occur directly through disulfide bridges that form as a result of increased ROS. Acetylation of FoxO1 prevents its poly-ubiquitination and degradation although the acetylated lysines are different from the ones that are polyubiqitinated [5, 495, 496]. On the other hand, acetylation at Lys242, Lys245 and Lys262 inhibits DNA binding [5] resulting in the ability of PKB to phosphorylate and inhibit the function of the transcription factor [5] (**fig. 7**). Deacetylation of FoxOs by SIRT1 enhances DNA binding [403, 471, 496-500], thus

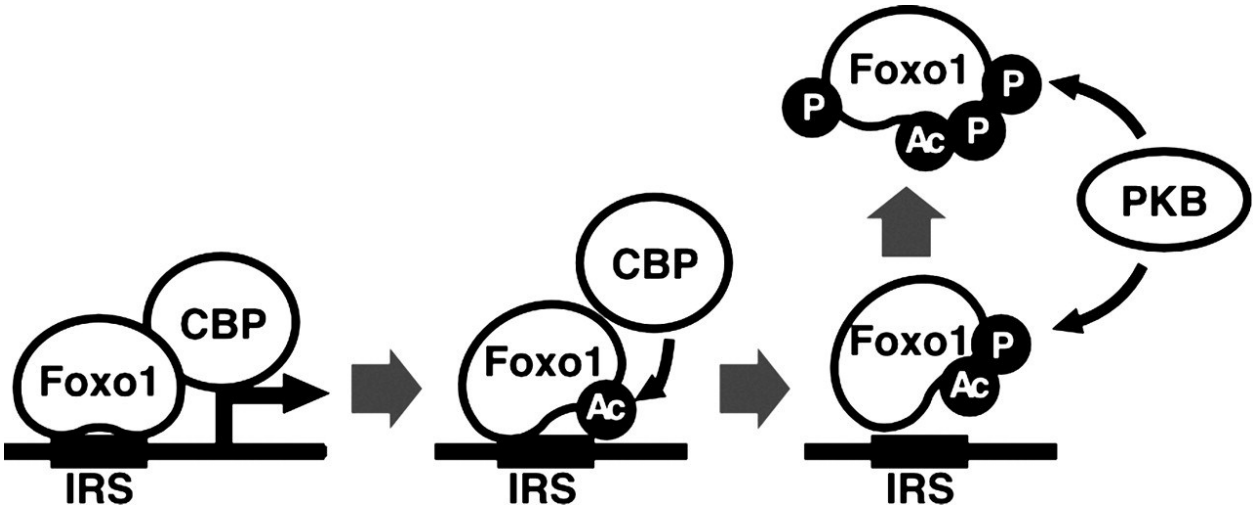


Figure 7: Effect of acetylation on DNA binding and phosphorylation by PKB. CBP is required to stimulate gene expression through histone acetylation. It acetylates FoxO which induces its release from DNA. Once FoxO-DNA interaction is compromised, PKB is able to phosphorylate and inhibit its function [5].

blocking PKB phosphorylation [5], and promotes antioxidant and cell cycle arrest gene transcription [1, 403, 471, 498, 499]. The effect of SIRT1 on FoxO proteins is conserved in *C. elegans* where 14-3-3 bridges Sir-2.1 to DAF16 in response to stress. This leads to increased longevity and expression of oxidative stress genes [501-505].

There exist other less well studied PTMs. One of them is ubiquitination. Poly-ubiquitination targets FoxO proteins to proteasomal degradation. There are two such E3 ubiquitin ligases that have

been defined so far: murine double mutant 2 (MDM2) and SCF^{skp2} (composed of Skp1, Cul1 and Skp2 [506]) . S-phase kinase-associated protein 2 (Skp2) acts on PKB-phosphorylated FoxOs [5, 350]. Moreover, phosphorylation by PKB stabilizes Skp2 proteins themselves [371, 506]. MDM2 also poly-ubiquitinates PKB-phosphorylated FoxO1 and FoxO3a [351, 507, 508]. However, MDM2 can act as a mono-ubiquitinase. It mono-ubiquitinates FoxO4 at residues Lys199 and Lys211 under oxidative stress, which increases its activity [509, 510], overriding all other inhibitory PTMs [510]. The deubiquitinase USP7/HAUSP is able to remove these ubiquitines from the lysine residues, which can also be acetylated [510]. It therefore seems to play an important role in regulating FoxO function.

Methylation of FoxOs is another way to prevent nuclear export. Protein arginine N-methyltransferase 1 (PRMT1) methylates FoxO1 at Arg248 and Arg259 in response to oxidative stress thus blocking phosphorylation by PKB at Ser253 [511]. Interestingly, FoxOs are also modified by the addition of O-linked β-N-acetylglucosamine (O-GlcNAc) via O-linked N-acetylglucosamine transferase (OGT) [512] in response to glucose [513] resulting in transcriptional activation of target genes implicated in gluconeogenesis and ROS detoxification – a pathway conserved in *C. elegans*, where it is important in *dauer* formation [514]. This process is dependent on binding of the FoxO cofactor PPAR-gamma coactivators -1 alpha (PGC1α), which directs the activity of OGT towards FoxO transcription factors [512]. PGC1α binding to FoxO1 stimulates the expression of gluconeogenic genes in the liver [487]. FoxOs also bind beta-catenin through disulfide bridge formation under conditions of oxidative stress to stimulate FoxO activity while inhibiting the proliferation-stimulating TCF-β-catenin complex formation [515, 516]. Again, this pathway is conserved in *C. elegans* and other mammals [515]. FoxOs also interact with other transcription factors. FoxO and RUNX3 bind together to the promoter of BIM to cooperatively up-regulate its expression [517]. Similarly, the close positioning of SMAD3/SMAD4 and FoxO at the regulatory regions of $p21^{cip1}$ and other common genes results in the formation of a complex between the transcription factors and stimulation of gene expression [356, 518]. Conversely, FoxO4 was shown to

interact with myocardin – a transcriptional coactivator important in smooth muscle gene expression – thus inhibiting its activity and preventing smooth muscle differentiation [519].

In conclusion, as hinted earlier, many regulatory pathways can converge on FoxO proteins to create a PTM code [1] in order to fine-tune the activity of these important transcription factors (**fig. 5**).

PROJECT PROPOSAL

The anticancer properties of VD in the digestive tract were previously described. In this lab, studies have shown the anti-proliferative effects 1,25-$(OH)_2D_3$ on SCC25 cells [520, 521]. My project aimed at extending these findings in two directions.

(1) Based on the microarray analysis performed by Wang *et al*, 1,25D was found to regulate FoxO target genes [522]. Genes selected for further analysis were four: *CCNG2*, *CCND2*, *BNIP3* and *MMP3*. Two sub-aims were considered. (1-i) Investigate whether FoxO transcription factors are activated by 1,25D and determine the mechanism through which that activation takes place. This could be achieved through assessment of the post-translational modifications of these transcription factors and determination of the enzymes responsible. Based on the literature, a candidate enzyme for deacetylation was found to be SIRT1 and for dephosphorylation - PP1 and PP2A. (1-ii) Determine the importance of FoxOs for 1,25D-regulated gene expression of *CCND2*, *CCNG2*, *BNIP3* and *MMP3*.

(2) Select new genes, regulated by 1,25D and related to cell proliferation, differentiation, apoptosis and cancer. In order to accomplish this, a gene expression profiling of SCC25 cells treated with 1,25D or vehicle for 24h hours in the absence of CHX had to be performed. It also had to be demonstrated that 1,25D is generally involved in cell cycle regulation and proliferation pathways in SCC25 cells.

The experiments described below were designed in an effort to elucidate the anti-cancer effects of VD in SCC25 cells following the directions outlined above.

MATERIALS AND METHODS

Cell Culture

SCC25 cells were cultured in 10cm tissue culture dishes (BD Biosciences) or 6-welled plates (BD Biosceinces). The medium used was DMEM/F12 (Wisent) containing 10% heat-inactivated FBS (Wisent), 1% Penicillin/Streptomycin (Wisent) and 0.2% Normocin (Invitrogen). Cells were split when around 80% confluent and treated 24h after splitting. Cells were treated with 10^{-7}M 1,25-$(OH)_2D_3$ (Calbiochem), 1mM AICAR (Toronto Research Chemicals), 2.5µM CC (EMD Chemicals), or DMSO (Sigma-Aldrich). Treatments were done for the time indicated. When co-treatments were performed, the compounds were introduced into the medium at the same time, unless specified otherwise.

Co-Immunoprecipitation Assays

CoIPs were performed as described in [523]. Briefly, A/G plus agarose beads (Santa Cruz Biotechnology) were washed 3 times and used to pre-clear the protein samples by incubation for 1h. The supernatant was then incubated with new beads and the appropriate antibody overnight at 4^{o}C, after which the beads were washed three times at 4^{o}C and then boiled in 70µl of 3x Laemmli buffer for 7min. The supernatant was used for WB and the beads were discarded. VDR was immunoprecipitated using a rabbit polyclonal VDR antibody (Santa Cruz Biotechnology) and WB was done using mouse monoclonal antibodies (Santa Cruz Biotechnology). SIRT1 was immunoprecipitated with a polyclonal rabbit antibody (Cell Signaling) and immonoblot was performed using monoclonal mouse antibody (Sigma-Aldrich).

FACS Analysis

Cells were trypsinized for 5min using 1x Trypsin/EDTA [0.05% Trypsin/0.53mM EDTA(Wisent)] followed by 2 washes with PBS. Fixation using 70% ethanol was performed by adding it drop-by-drop to

the cell while gently vortexing. Cells were then left in a -20^{o}C freezer for at least 30min. Just before cells were subjected to analysis using flow cytometry, they were washed with PBS and stained using 1ml of PI staining solution. The PI staining solution is made fresh and consists of 0.25g Sodium citrate, 0.75ml Triton X 100, 0.025g PI and distilled water for a total volume of 250ml. Once the PI solution is added, cells are kept in the dark at 37^{o}C for 15min and brought to the FACScan where cell cycle analysis was performed.

Microarray Analysis

The raw data obtained from the Affymetrix Human Exon 1.0ST Array was processed using a software package for statistical analysis and visualization of microarray expression data called FlexArray. The statistical test used was Wright and Simon's implementation of the Empirical Bayes method. No threshold was selected. The results obtained were imported into a Microsoft Excel spreadsheet. The expression values were based on probes for regions located in various exons. In this context, several expression values were present for each gene. The average of these values was taken as the final expression for each gene. This final value was then used to filter the genes for 1.6 or higher fold change of 1,25D-treated, relative to control group.

This gene list was uploaded into IPA and a core analysis was performed.

PCR

RNA was extracted from cells using TRIZOL reagent (Invitrogen) and cDNA was generated. qPCR was performed using a kit, SsoFast Eva Green Supermix (Bio-Rad) and the Eco Real-Time PCR System instrument (Illumina). Primers for each gene were designed using Primer3 at http://frodo.wi.mit.edu/primer3/ and are represented bellow:

Gene Symbol	Left Primer	Right primer
MMP3	GCAGTTTGCTCAGCCTATCG	GAGTGTCGGAGTCCAGCTTC
BNIP3	GTCAAGTCGGCCGGAAAATA	TTCATGACGCTCGTGTTCCT
CCNG2	TCCTGAGCTGCCAACGATAC	ACTGGGAGGAGAGCTGCTGA
CCND2	GTGATGCCCTGACTGAGCTG	CGATATCCCGCACGTCTGTA
FOXO3A	ATATGGCAGGCACCATGAAT	CTGGCGTAGGGAGTTCAGAG
CYP24A1	CCCAACTTCATGCGGAAAAT	TCTCTGGAAAGGGGGTCTCA
FOXO4	TCGAGTTCTTCCATCCTGCT	TGTAACAGGTCCTCGGAAGG
NOD2	CTCCATGGCTAAGCTCCTTG	CACACTGCCAATGTTGTTCC
ALOX5	AGTCGGCGAAGTCATTCC	TCCTACACGGTCACCGTGG
AREG	TCCACTCGCTCTTCCAACAC	CATTGAGGTCCAATCCAGCA
TRPV6	GATCGACTCCTCAGGGGATG	AGGCTCACCAGCTCCTTCAC
G6PD	GAGGCCGTGTACACCAAGAT	AGCAGTGGGGTGAAAATACG
OLR1	GTGCTGGGCATGCAATTATC	GGCTGAGATCTGTCCCTCCA
MACC1	GGCTGGAGGGAACTAGCTGA	TCTGTCCAAAGCTGACTGAAGG
BIRC3	CACATCAGACAGCCCAGGAG	CGGCAGCATTAATCACAGGA
POLE2	ACTGCGTCCGTTTTCCTAGC	CCCAATACACTGGGCAGACA
PCNA	TGAGGGCTTCGACACCTACC	GCCAAGGTATCCGCGTTATC
CCNB1	CCAGAACCTGAGCCAGAACC	TGGGCTTGGAGAGGCAGTAT
CCNA2	CGGTACTGAAGTCCGGGAAC	CCAAGGAGGAACGGTGACAT
APPL1	GCAGCAACACACCTGACCTC	CATCATCACCTCCCAATGGA
BIRC5	TGTCTTGAAAGTGGCACCAG	GCCTTCTTCCTCCCTCACTT
GAPDH	ACCGTCAAGGCTGAGAACG	ACTGTGGTCATGAGTCC

Transfections

Cells were split and plated in antibiotic-free medium containing 10% FBS. 24h later, the transfection was done using Lipofectamine 2000 (Invitrogen) reagent and protocol. The final concentration of the siRNA used for the KDs was 60piM. AllStar negative control, as well as siRNA specific for FOXO3A (5'-CTGAATGATGGGCTGACTGAA-3') was purchased from Quagen. Cells were incubated with transfection solution containing Lipofectamine 2000 with siRNA for 24h, followed by the appropriate treatments.

Western Blot

WBs were performed as described elsewhere [523]. The antibodies for FoxO3a (goat), VDR (rabbit), PP1 (rabbit), Sirt1 (rabbit), and Ccnd2 (rabbit) were from Santa Cruz Biotechnology, antibodies against FoxO4 (rabbit) and p53 acetylated on Lys382 (rabbit) were purchased from Cell Signaling. Anti-Ph-Th32 FoxO3a (rabbit) was obtained from Millipore. Mouse anti-actin antibody (Santa Cruz) was used for loading controls.

RESULTS

A head and neck squamous cell carcinoma (HNSCC) cell line, SCC25, was used for all of the experiments described hereafter. SCC25 cells are derived from the base of the tongue or floor of the mouth tumour origin, are relatively differentiated [524] and appear to behave similarly to non-neoplastic oral cavity epithelial cells. However, the appearance of second primary carcinomas after surgical removal of tumours in patients with head and neck cancer [525] necessitates the discovery of agents effective in chemoprevention or treatment. In the case of SCC25, one such agent is 1,25D, which induces G0/G1 cell cycle arrest [520]. AT84 is the mouse equivalent of SCC25 and is also responsive to growth inhibition by calcitriol, making the two cell lines appropriate not only for investigating the effects of 1,25D at the cellular level, but also allowing the prospective of investigating its effects at the organismal level in a mouse model of HNSCC.

Effects of 1,25D on proliferation of SCC25 cells

In order to assess the responsiveness of SCC25 cells to calcitriol and confirm the findings that 1,25D induces cell cycle arrest [520], cells were treated with 10^{-7}M 1,25D or vehicle for 16, 24 and 48 hours. After staining the DNA with propidium iodide (PI), samples were subjected to cell cycle analysis by fluorescence-activated cell sorting (FACS). Figure 8 shows that there is a time-dependent accumulation of 1,25D-treated cells in the G1 phase of the cell cycle compared to vehicle, as expected.

1,25D regulates genes implicated in cell cycle regulation and apoptosis

The effect of 1,25D on cell proliferation is likely caused by the genomic effects elicited by calcitriol through VDR. Four genes, among others, are known to be regulated by hormonal vitamin D in SCC25 cells. Two of them, *CCNG2* and *CCND2*, are implicated in cell cycle regulation, and the two others, in apoptosis. Previous data from microarray analysis suggests that 1,25D up-regulates the expression of

CCNG2 and down-regulates that of *CCND2* [522]. CCND2 is essential for G1/S transition of the cell cycle. It binds to the cyclin-dependent kinase 4 [526], 6 [527] and even 2 (11) (CDK4/6/2). CCND2/CDK4 or CCND2/CDK6 are then able to phosphorylate and inactivate pRB, p107 and p130 (12,13). CCNG2 is an atypical cyclin. It is localized primarily in the cytoplasm and is associated with G0/G1 cell cycle arrest-induced apoptosis [528, 529]. Apoptosis is also induced by the other two genes that are transcriptionally activated by 1,25D, notably *MMP3* [530, 531]and *BNIP3* [532]. The expression of *CCND2*, *CCNG2*, *MMP3* and *BNIP3* was assessed by real-time quantitative PCR analysis (qPCR) (**fig. 9**). The house-keeping gene *GAPDH* was used as a negative or loading control, since its expression is not altered by 1,25D.

The 1,25D-dependent regulation of the four genes is in agreement with previous observations based on gene expression profiling [522]. In addition, bearing in mind these four genes' properties, one can conclude that these results are consistent with the cancer chemopreventive effects of calcitriol and suggest that the latter could be mediated at the gene level.

1,25D upregulates FoxO activity

Strikingly, the four genes transcriptionally regulated by 1,25D described above are also targets for FoxO transcription factors [461, 531-533]. This suggested that regulation of these genes by ligand-bound VDR could be taking place through FoxO, in particular FoxO3a function activation. In order to test this hypothesis 1,25D-dependent binding of FoxOs to the response elements in the promoters of these genes had to be determined. A post-doctoral fellow in the lab, Dr. Beum-Soo An, performed chromatin immunoprecipitation (ChIP) assays using antibodies against FoxO3a and testing for the enrichment of FHREs from the regulatory regions of *CCND2* (**fig. 10B**) and *BNIP3* (**fig. 10C**). A transient 1,25D-enhanced binding of FoxO3a was observed, peaking at 4h after treatment and dropping at 8h to the level of vehicle-treated cells. The obvious effect of hormonal vitamin D raised questions regarding the extent of involvement of the VDR in the regulation of these two genes. Another ChIP (done by Dr. An) (**fig. 10D**)

showed that VDR can be found at the same DNA regions to which FoxO3a was bound (**fig. 10A**). VDR and FoxO3a, therefore, can be found at the FHRE in the promoter region of *CCND2* and *BNIP3* and binding of both transcription factors increases at 4h after 1,25D treatment.

These results point to the possibility that VDR forms a complex with FoxO3a and activates the latter upon 1,25D binding, inducing its interaction with a FHRE in the promoter of the four target genes. Activation of FoxOs can be achieved by removing phosphates from phosphorylated residues associated with inhibition of transcription factors' function, particularly PKB sites, or by deacetylation of acetylated sites associated with disruption of protein-DNA interactions (**fig. 5**). Phosphatases that are able to remove inhibitory phosphate groups from FoxOs are PP1 and PP2. SIRT1, on the other hand, deacetylates and activates FoxO proteins [5]. In order to test the role of these enzymes in 1,25D-mediated FoxO activation, I performed a series of co-immunoprecipitations (CoIPs). First, I immunoprecipitated VDR, followed by a western blot (WB) for PP1c (the catalytic subunit of PP1) and SIRT1. As shown on figure 11A, SIRT1 interacts with VDR in a partially ligand-dependent manner peaking at 4h after 1,25D treatment, whereas PP1c binding to VDR appears to be ligand-independent (**fig. 11A**). Notably, highest association of SIRT1 with VDR occurs at 4h post treatment, consistent with enhanced FoxO and VDR binding to the FHREs in the promoter regions of *CCND2* and *CCNG2*. Another CoIP experiment, where Sirt1 was immunoprecipitated and WB for FoxO3a was performed, clearly shows 1,25D-dependent association of SIRT1 with FoxO3a (**fig. 11B**). Alternatively, Dr. An demonstrated a ligand-independent association between VDR and transcription factors FoxO3 and FoxO4. These data suggests that activated VDR directs the activities of PP1 and SIRT1to activate FoxO3a. To test this hypothesis, I assessed the phosphorylation and acetylation status of FoxO3a following time-course 1,25D treatment of SCC25 cells. One of the three residues phosphorylated by PKB in FoxO3a is Thr32. It is also crucial for interaction with 14-3-3 and nuclear export. As, shown on figure 12A, 1,25D induces a decrease in the phosphorylation status at Thr32, which is detectable at 4h and lowest at 8h, but goes

back to control levels at 24h. Since commercial antibodies against any of the acetylated residues of FoxO3a are not available, FoxO3a was immunoprecipitated and subsequently immunoblotted using Acetyl-Lysine antibody. Acetylation is drastically reduced at 4h post-treatment with 1,25D. As a control experiment, the acetylation status of p53, another target for SIRT1, was assayed using anti-acetyl-p53 antibody (**fig. 12B**). This suggests that ligand-bound VDR directs the activity of SIRT1 specifically to FoxO3a. In conclusion, FoxO3a appears to be activated by ligand-bound VDR through deacetylation and dephosphorylation, which could be due to PP1 activity or indirectly the result of SIRT1-mediated deacetylation, which decreases release of DNA-bound FoxO and the subsequent PKB-mediated phosphorylation (**fig. 7**).

1,25D induces cell cycle arrest and apoptosis through the regulation of genes in a FoxO3a-dependent manner

In order to demonstrate the importance of FoxO3a in the regulation of the four target genes implicated in cell cycle arrest and apoptosis, SCC25 cells were transfected with siRNA against FoxO3a (siFOXO3). The levels of FOXO3A mRNA was reduced after KD to about 30% that of scrambled siRNA-(siCtrl) transfected cells (**fig. 13A**), whereas FoxO3a appeared to be completely absent at the protein levels (**fig. 13B**). The expression of the four FoxO target genes – *CCNG2*, *CCND2*, *BNIP3* and *MMP3* – regulated by 1,25D was then assessed by q PCR (**fig. 14**). Ablation of FoxO3a abolished (*CCNG2*) or significantly attenuated (*CCND2*, *BNIP3* and *MMP3*) the regulation of these genes. In addition, FoxO3a KD did not alter gene regulation of *CYP24A1* by 1,25D. The expression of the latter is controlled directly by activated VDR/RXR heterodimers bound to several VDREs in the regulatory region of the *CYP24A1* gene. Knockdown of FoxO4 had no effect on the regulation of these four genes (**fig. 15**) allowing me to focus only on FoxO3a as a potential mediator of the anticancer effects of vitamin D. Moreover, EdU proliferation assay performed by Dr. An (data not shown) [523] in control and FoxO3a or FoxO4

knockdown cells treated with 1,25D or vehicle showed that only FoxO3a ablation attenuated the effect of VD on reduction of cell proliferation [523].

Interestingly, activation of AMPK by the pharmacological agonist AICAR appeared to enhance the 1,25D-mediated transcription of the FoxO3a target gene *CCNG2*, *BNIP3* and *MMP3* (**fig. 16A**). Alternatively, inhibition of AMPK by Compound C (CC) blunts the 1,25D-induced gene regulation (**fig. 16B**). This is consistent with the reports showing enhancement of the transactivation activity of FoxO3a by AMPK-mediated phosphorylation [482] and suggests that part of the 1,25D effects on FoxO3a target gene expression may be mediated by AMPK that is upstream of FoxOs. AMPK inhibition, on the other hand, had no effect on the direct VDR/RXR target gene *CYP24A1* (**fig. 16C**).

Gene Expression Microarray

The 1,25D-regulated FoxO3a target genes *CCNG2*, *CCND2*, *BNIP3* and *MMP3* described above were selected from a gene expression profiling done in the lab using SCC25 cells cultured in cycloheximide (CHX) [522]. In this sense, all the differentially regulated genes in this microarray are considered to be primary vitamin D target genes, as their expression or repression does not require *de novo* protein synthesis. In order to extend the findings of Wang *et al.,* I generated samples for a microarray, where cells were cultured in the absence of CHX and treated with vehicle or 1,25D for 24h, as opposed to 12h [522]. The experiment was set up this way in order to identify biological pathways and cellular functions and networks controlled by calcitriol in SCC25 cells, in which secondary 1,25D target genes that require protein synthesis may also be important. The Affymetrix Human Exon 1.0 ST Array chip contains probes for 22 000 genes. Out of these, 1940 were up- or down-regulated by 1,25D 1.6-fold or more compared to vehicle (**fig. 17**). Comparison with results from the 2005 +CHX microarray revealed that a number of genes re-appeared in the –CHX profiling of calcitriol target genes. Some of these are represented in table 1. The genes up-regulated the most by 1,25D appear in both microarrays

consistent with the ability of ligand-bound VDR to induce transcriptional activation. Conversely, only 255 genes were found to be under-expressed (1.6-fold or more) in cells treated with 1,25D and CHX, compared to CHX and vehicle-treated cells. 155 out of these 255 genes also appeared in my microarray analysis with similar fold-change relative to control.

Validation of positive control genes appearing in the microarray by qPCR

Some of the primary 1,25D target genes (apart from *CYP24A1*) that appeared to be regulated in my microarray in a similar fashion as in that performed by Wang and colleagues in 2005 have previously been shown to be activated VDR/RXR targets [522]. Their transcriptional regulation, as assessed by the microarray, was validated by qPCR (**fig. 18**). In addition, other genes that are known to be ligand-bound VDR targets were also validated, such as *NOD2* [534]. The results on figure 18 show not only that the results from the microarray correspond to those obtained by qPCR, but also demonstrate that they are similar to previously published data and imply that the values obtained from the gene expression profiling represent a valid reference to explore pathways affected by vitamin D ultimately resulting in gene regulation.

Cellular functions and networks affected by 1,25D

Next, I asked the question: what are the cellular functions affected by 1,25D. Ingenuity Pathway Analysis (IPA) software was used for this purpose. Genes that were up- or down-regulated at least 1.6 fold by 1,25D relative to control were uploaded to IPA and a core analysis was performed. As a reference, the available microarray results (1.7-fold change or more) by Wang and colleagues [522]were also analysed separately. One of the sets of results returned as output is the functions which are associated with the uploaded gene lists. I subdivided them into two categories: cellular functions and diseases. Consistent with the growth inhibitory and pro-differentiation effects of calcitriol, the largest

groups of genes were implicated in cellular functions such as cell death, growth and proliferation, cell cycle and DNA replication (**fig. 19A**). Interestingly, the same functional groups were identified by IPA based on the data of Wang and colleagues with fewer genes present in each functional category. This is consistent with the fact that (1) no secondary 1,25D target genes can be expressed in the presence of cycloheximide and (2) data was obtained from cells treated with calcitriol or vehicle for 12h [522] as opposed to 24h. In terms of diseases, the 1,25D-regulated genes from the microarray analysis that I performed are implicated in gastrointestinal disease (the majority of which is various gastrointestinal cancers), cancer, musculo-skeletal disorders, cardiovascular disease and hematological disease (again, mostly disorders associated with hyperproliferation and reduced differentiation). Similar results, but with less genes in each group, are observed for the gene expression profiling performed in the presence of CHX and treatment with vehicle/1,25D for 12h [522] (**fig. 19B**).

I then focused on the pathways that are altered by 1,25D. IPA provides two pathway analyses: canonical pathways and networks. The canonical pathways are well defined signalling pathways, whereas the networks are generated based on at least one published source describing the interaction between a certain proteins and a certain gene, and are more exploratory in nature. As expected, the genes that were found to be up- or down-regulated at least 1.6 fold by a 24h treatment with 1,25D in the absence of CHX were associated with activation of the VDR/RXR, heterodimer (**fig. 20**). Another canonical pathway that was identified to be influenced by 1,25D is cell cycle control of chromosomal replication (**fig. 21**). The effect of 1,25D regarding down-regulating components of this canonical pathway responsible for DNA replication is not surprising given its anti-proliferative effects. Similar conclusions can be drawn from the constructed networks after core analysis of the microarray data using IPA. Two of these networks are designated “DNA Replication, Recombination and Repair, Cell Cycle, Cancer,” based on the functions and diseases they are implicated in. The network represented on figure 22 shows genes involved in cell cycle and some involved in DNA recombination and repair,

whereas the other network with the same name consists predominantly of genes required for chromosomal replication, DNA recombination and repair, and less genes involved in cell cycle regulation are present. Interestingly, there is no overlap of genes used to construct both networks.

Validation of selected genes from the microarray, implicated in cancer

Obvious cancer-related genes were selected from the gene expression profiling I performed (**table 2**). Their expression was validated by qPCR (**fig. 23**). Although gene expression, as assessed by qPCR did not produce the same values as those obtained after analysis of the microarray data, the pattern of expression was the same (**fig. 23**). The role of some of these genes in proliferation and apoptosis is apparent. For example, *BIRC3* (cIAP2) is an inhibitor of apoptosis and its down-regulation results in increased apoptosis following efficient caspase 3/7 activation [535]. PCNA is necessary for DNA replication, whereas CCNB1 – involved in the early events of mitosis [536], and CCNA2 – an E-type cyclin used as a marker of proliferation [537], are associated with increased cell division. *BIRC5* (Survivin) is a member of the inhibitor of apoptosis family, to which *BIRC3* (cIAP2) belongs and whose expression is high during development, but low in the majority of adult tissues. The fact that it is usually re-expressed in cancer have prompted certain researches to call *BIRC5* the "Achilles' heel of cancer" [538]. The other genes' association with cancer is not as salient. *OLR1* (oxidized lipid receptor 1) was found to be part of a cancer gene signature [539]. It is implicated in the development of diabetes, hypertension and atherosclerosis, which is consistent with the cellular functions (lipid metabolism) and diseases (cardiovascular disease) (**fig. 19**) associated with 1,25D-regulated genes. In order to assess its expression in cancer cells relative to normal tissues, I used a bioinformatics tool called Oncomine. As shown in table 5, OLR1 was significantly overexpressed in a number of different cancer cells, notably, in head and neck squamous cell carcinomas (**fig. 24A**).

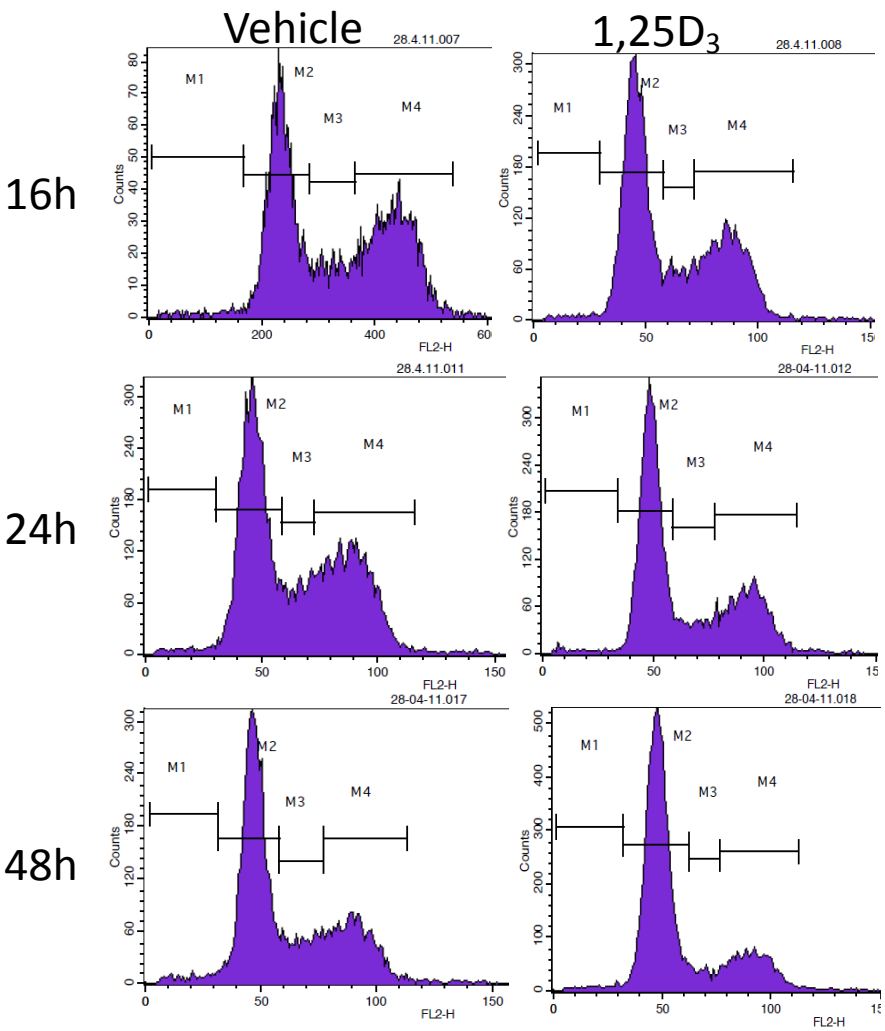
MACC1, metastasis-associated colon cancer 1, is predominantly expressed in various types of colon or colorectal cancer (**fig. 24B**) and is associated with low survival in patients and a more invasive phenotype [540]. Not much is known about the mechanism of action of MACC1, but it seems to transcriptionally activate the expression of the receptor tyrosine kinase Met, resulting in increased Met signaling and subsequent gain of cell motility, leading to invasion and metastasis. Interestingly, higher expression of *MACC1* was also observed in ovarian, pancreatic, breast and renal cancers (table 5), emphasizing its importance in tumorigenesis.

Another 1,25D-regulated gene selected from the microarray is DNA polymerase epsilon B-subunit (*POLE2*). POLE2 is known for its role in DNA replication, specifically in the elongation step of the leading or lagging strand, and is essential for viability of *Saccharomyces cerevisiae* [541]. In this context, POLE2 appears to be indispensable for cell proliferation and could play a role in neoplasia. Assessment of its expression in cancer relative to normal tissue using Oncomine confirms the role of POLE2 in tumorigenesis – *POLE2* was found to be overexpressed 2-fold or more in the greatest number of cancers compared to all the other genes from table 3, notably, in esophageal squamous cell carcinoma (**fig. 24C**) [542].

Contrary to OLR1, MACC1 and POLE2, Adaptor Protein, Phosphotyrosine Interaction, PH Domain, And Leucine Zipper-Containing Protein 1 (*APPL1*) was not found to be expressed in head and neck or digestive system cancers (**table 3** and **fig. 24D**). However, its function attracts a great deal of interest, especially regarding the PI3K/PKB/FoxO signalling pathway. APPL1 is able to bridge PI3K and inactive PKB thus enhancing activation of the latter upon mitogenic signalling [543, 544]. Moreover, APPL1 was shown to be essential for cell proliferation and survival [544, 545], and its ablation leads to increased apoptosis [544].

In conclusion, any or all of the genes from table 3 could be important for SCC25 cells survival, proliferation and evasion of apoptosis. Therefore, 1,25D could mediate its anti-cancer effects via any or all of these genes.

Figures and Tables



	Vehicle	1,25D
sub-G1	1.34	1.37
G1	44.08	53.1
S	14.51	10.29
G2	39.93	35.26

	Vehicle	1,25D
sub-G1	1.56	1.68
G1	48.13	56.35
S	13.03	12.75
G2	38.26	30

	Vehicle	1,25D
sub-G1	2.5	1.88
G1	55.44	72.29
S	15.55	6.21
G2	27.25	19.63

Figure 8: **FACS analysis of the effects of 1,25D on the cell cycle of SCC25 cells**. SCC25 cells were treated with vehicle or 1,25-(OH)2D3 for 16h (top) 24h (middle) or 48h (bottom) and subjected to FACS analysis after PI staining. The tables on the left indicate the measurements obtained from the graphs (right).

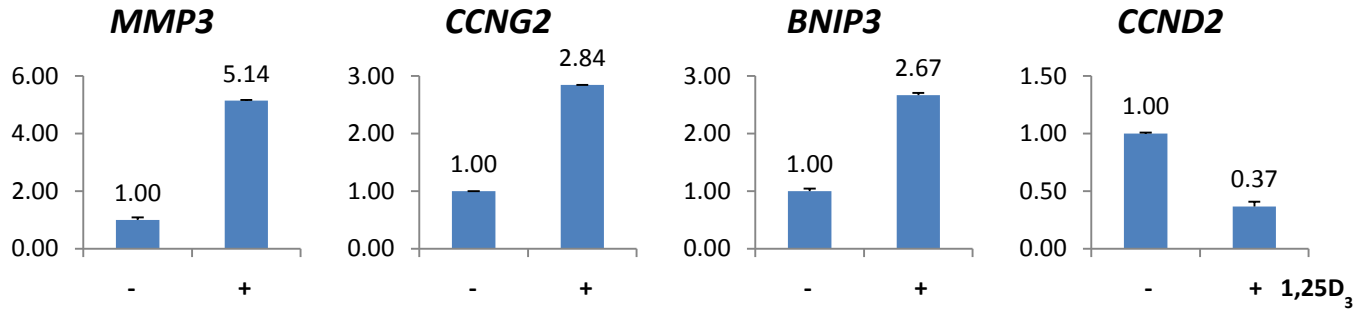


Figure 9: 1,25D$_3$-regulated expression of genes implicated in cell cycle and apoptosis. MMP3 and BNIP3 induce apoptosis. CCND2 is required for G1/S transition and CCNG2 induces cell cycle arrest. The cells were treated with vehicle (-) or 1,25D$_3$ (+) for 24h as indicated.

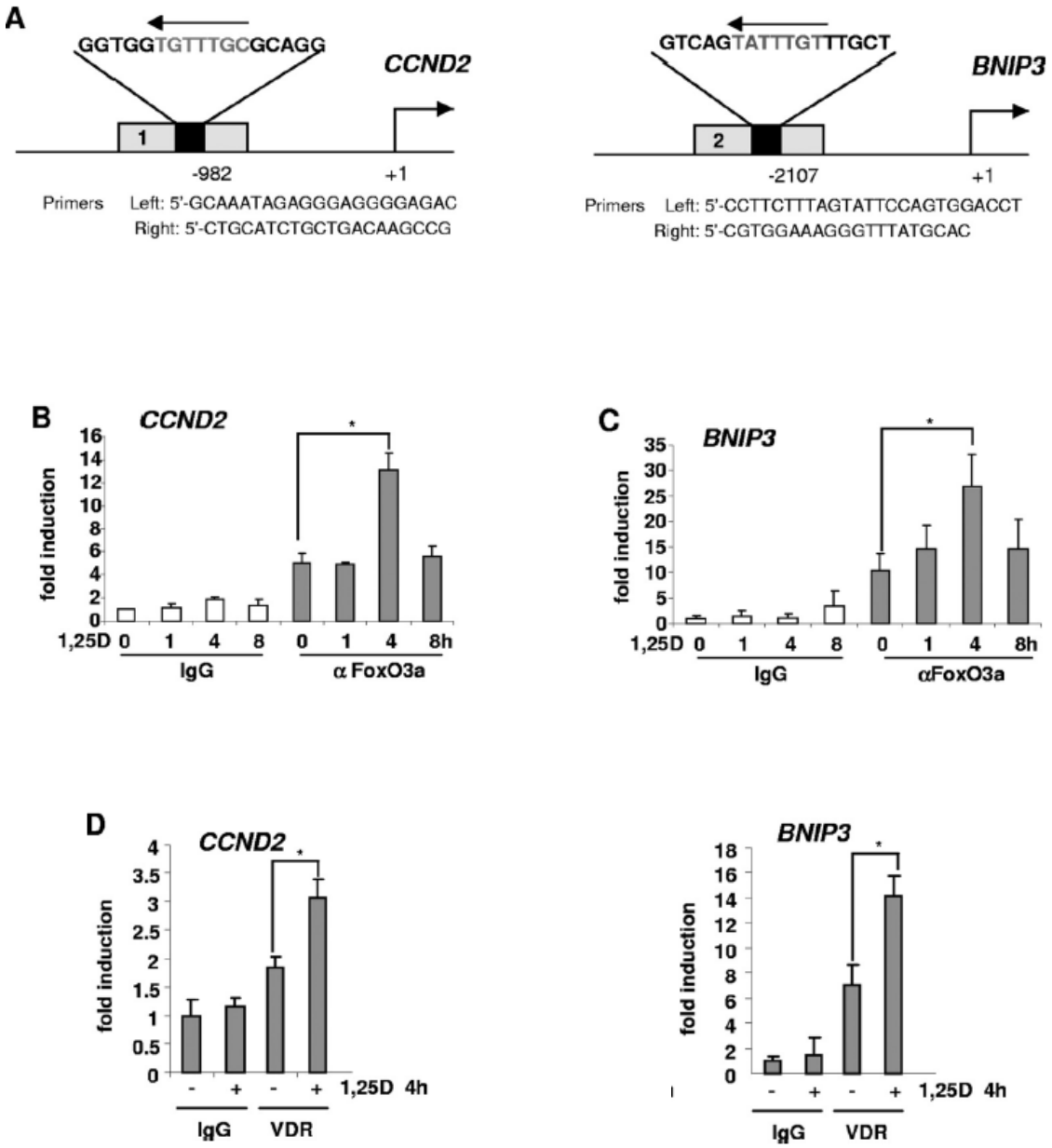


Figure 10: **1,25D$_3$-dependent binding of FoxO3a and VDR to *CCND2* and *BNIP3* promoters (performed by Dr. An).** (A) A schematic representation and the sequence of the FHRE found in the promoter of *CCND2* (left) and *BNIP3* (right). The primers designed to detect these sequences by qPCR are indicated below. (B) ChIP for FoxO3a at the FHRE in the *CCND2* promoter. The numbers at the x-axis indicate the hours during which SCC25 cells were treated with 1,25D$_3$. (C) ChIP for FoxO3a at FHRE in the *BNIP3* promoter. (D) ChIP for VDR at the FHREs in the promoters of *CCND2* (left) and *BNIP3* (right). + or – indicate treatment with 1,25D or vehicle, respectively for 4h.

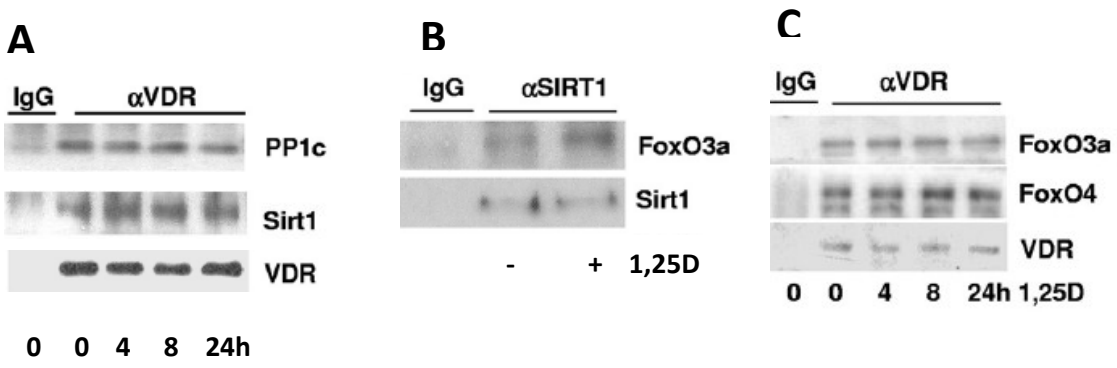


Figure 11: VDR interacts with PP1c, SIRT1, FoxO3a and FoxO4. (A) Immunoprecipitation of VDR and immunoblot for PP1c, SIRT1 and VDR. Cells treatment was done as indicated at the x-axis. (B) Immunoprecipitation of SIRT1 and WB for FoxO3a and SIRT1. The + and – sign indicates treatment with 1,25D or vehicle, respectively, for 4h. (C) This experiment was done by Dr. An. Immunoprecipitation of VDR and WB for FoxO3a, FoxO4 and VDR. Normal non-specific IgG antibodies were used as a negative control in order to demonstrate the specificities of the antibodies used for the immune-precipitations.

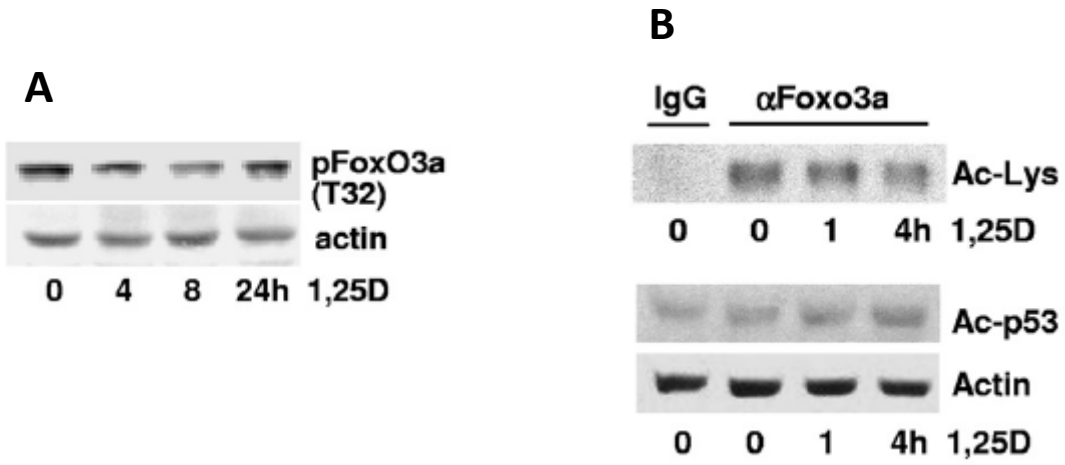


Figure 12: Reduction in the phosphorylation and acetylation status of FoxO3a. (A) Phosphorylation at Thr32 is reduced at 4 and even more at 8h, but not after 24h of 1,25D_3 treatment, as assessed by immunoblotting with phospho-Thr32 specific antibody. (B) Immunoprecipitation of FoxO3a followed by WB with anti-acetyl-lysine (Ac-Lys) antibody. As a negative control, acetylation using anti-acetyl-p53 antibody was performed. Actin was included as a loading control.

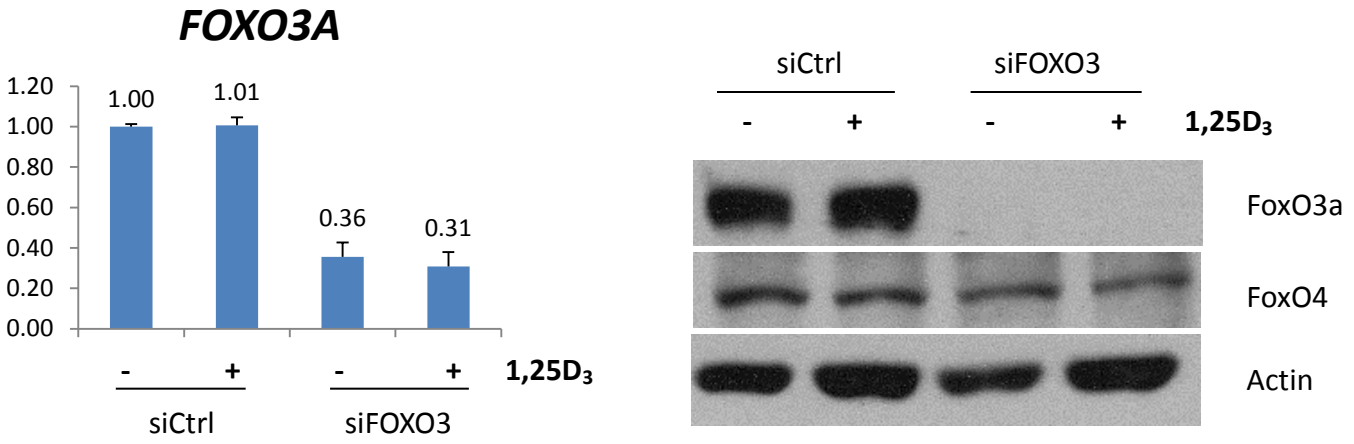


Figure 13: Knockdown of FoxO3a. KD efficiency of FoxO3a as assessed by qPCR (A) and by WB (B). siCtrl refers to scrambled control siRNA, whereas siFOXO3 is siRNA directed against *FOXO3A*. Treatment with 1,25D_3 (+) or vehicle (-) was done for 24h. FoxO4 protein levels were also assessed (B) as a control for the specificity of siFOXO3. Actin was used as a loading control.

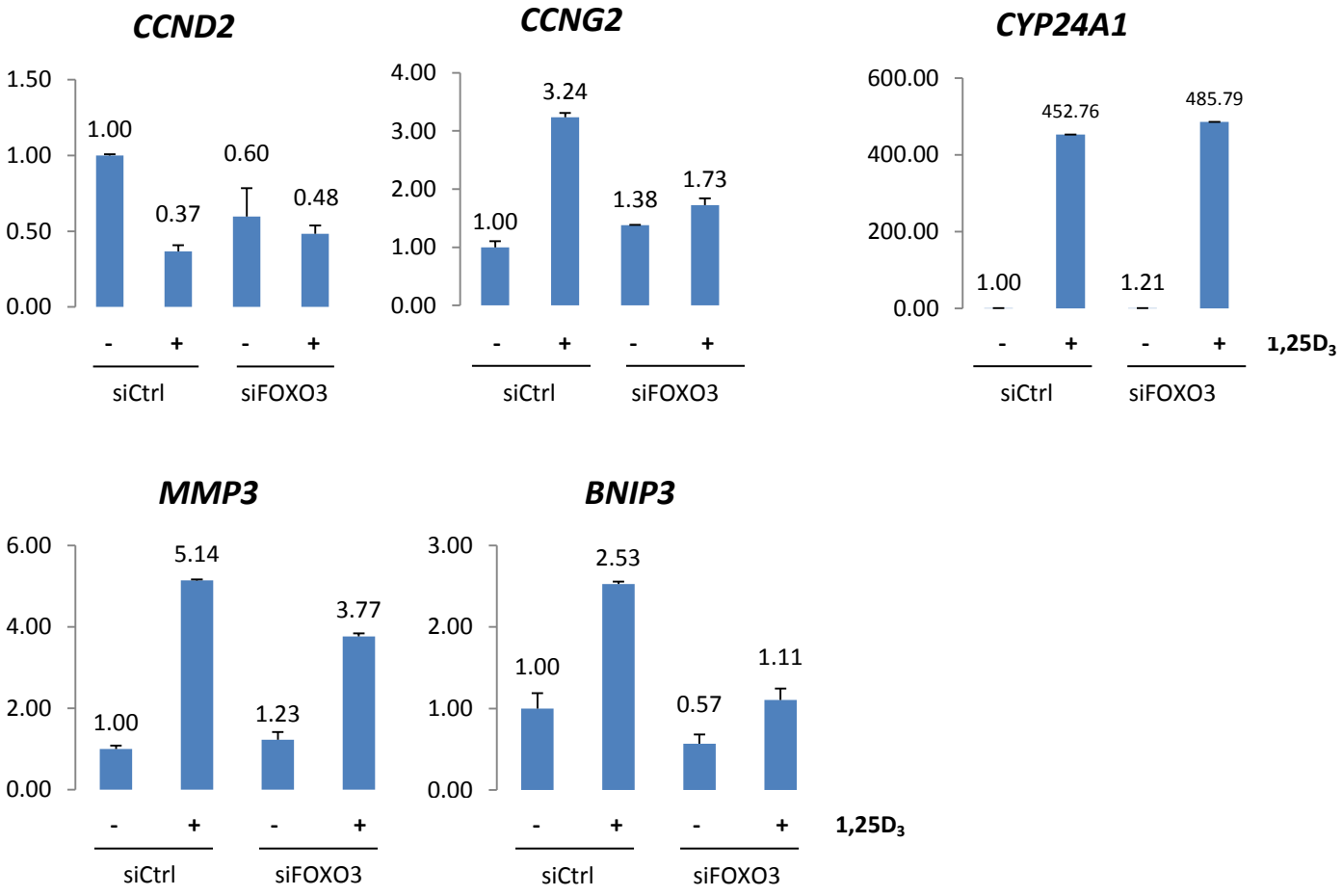
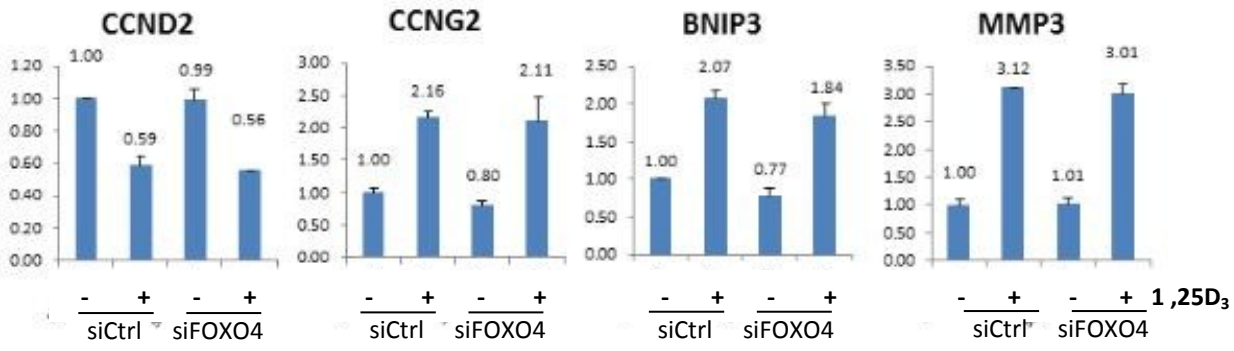


Figure 14: **FoxO3a ablation attenuates the 1,25D_3-dependent regulation of FoxO target genes (*CCND2*, *CCNG2*, *BNIP3* and *MMP3*), but not of VDR/RXR target gene *CYP24A1*.** Cells were treated with 1,25D_3 (+) or vehicle (-) for 24h after being transfected with siRNA against *FOXO3A* (siFOXO3) or a scrambles non-specific siRNA (siCtrl).



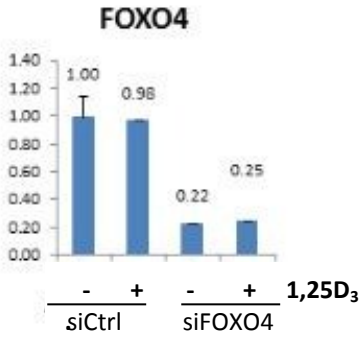


Figure 15: **Effect of FoxO4 ablation on FoxO target genes regulated by 1,25D$_3$.** Cells were treated with 1,25D3 (+) or vehicle for 24h after transfection with siRNA against *FOXO4* (siFOXO4) or scrambled non-specific siRNA (siCtrl).

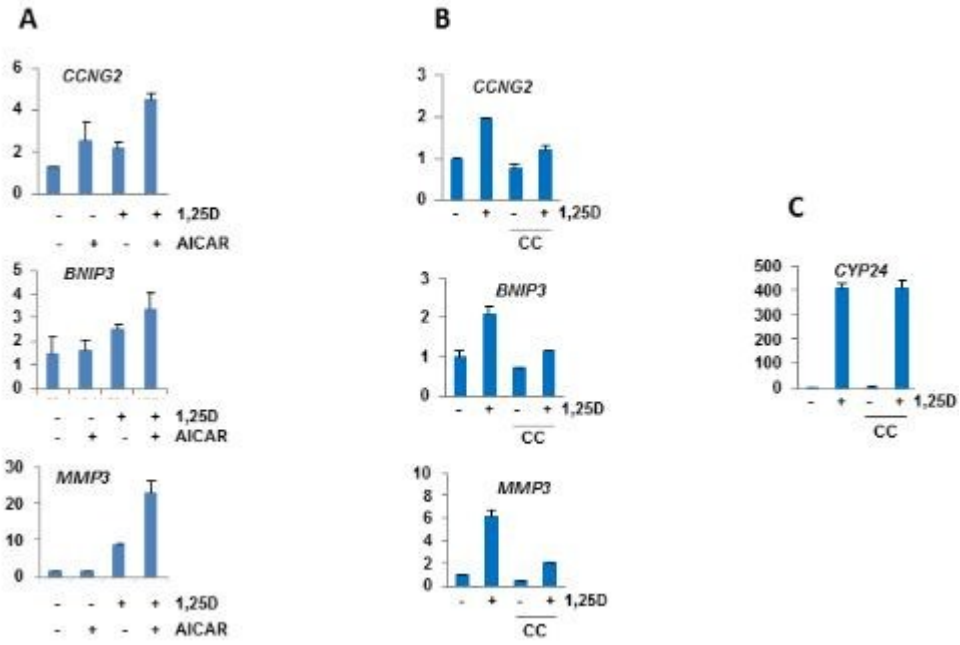


Figure 16: **AMPK activation enhances the effect of 1,25D on the regulation of FoxO3a target genes**. (A) Cells treated with vehicle, AICAR, 1,25D or both AICAR and 1,25D were tested for *CCNG2*, *BNIP3* or *MMP3* gene expression by qPCR. (B) Cells were treated with vehicle, CC, 1,25D, or both CC and 1,25D for 24h and gene expression for *CCNG2*, *BNIP3* and *MMP3* was assessed by qPCR. (C) Expression of *CYP24A1* after treatemnt with vehicle, CC, 1,25D or both CC and 1,25D for 24h and gene expression of *CCNG2*, *BNIP3* and *MMP3* was assayed by qPCR.

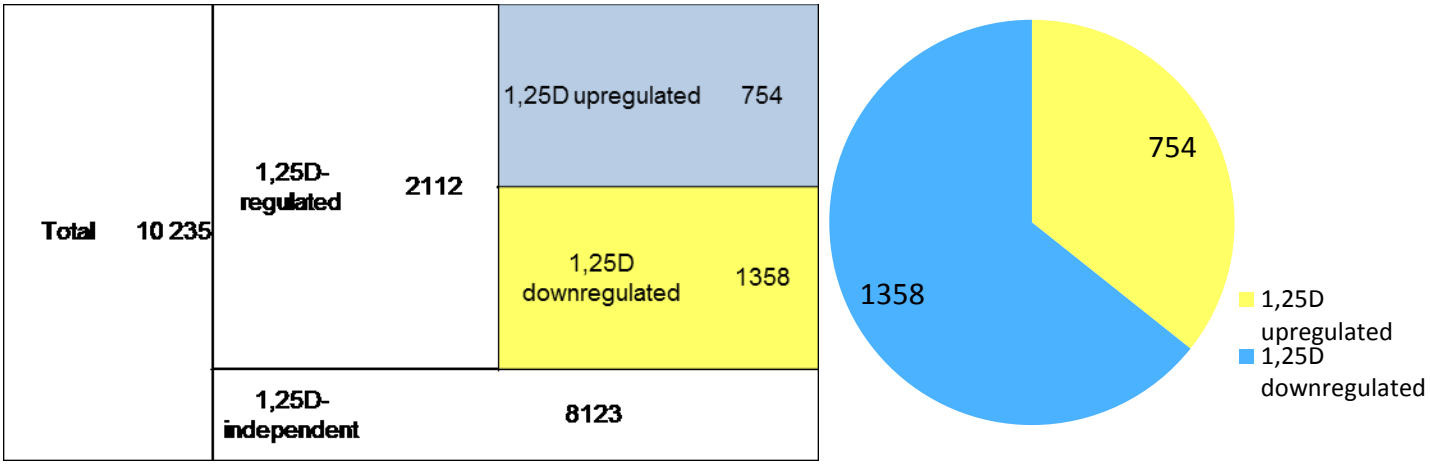


Figure 17: **Gene expression profiling in cell treated with 1,25D$_3$ or vehicle for 24h in the absence of CHX.** The yellow colour represents up-regulated, whereas the blue colour, down-regulated genes. The threshold fold-change was taken to be at least 1.6.

Gene Symbol	Fold-Change (+CHX)	Fold-Change (-CHX)
CYP24A1	18.514	20.382
IL1RL1	12.264	11.482
ALOX5	10.868	9.393
SLCO1A2	9.323	9.721
COLEC12	2.263	7.414
HSD17B2	3.141	5.695
CYP3A5	4.042	5.073
SEMA3B	6.540	4.516
DNER	4.597	3.356
IGFBP3	1.748	2.715
COL13A1	2.568	2.584
KLK6	6.763	2.564
TRPV6	1.938	2.529
KLK13	4.481	2.421
G6PD	4.423	2.296
AREG	3.225	2.285

Table 1: **Comparison of results from +CHX and –CHX gene expression profiling of 1,25D-regulated genes.** +CHX refers to the microarray analysis performed by Wang and colleagues, whereas the –CHX column represents results from my microarray.

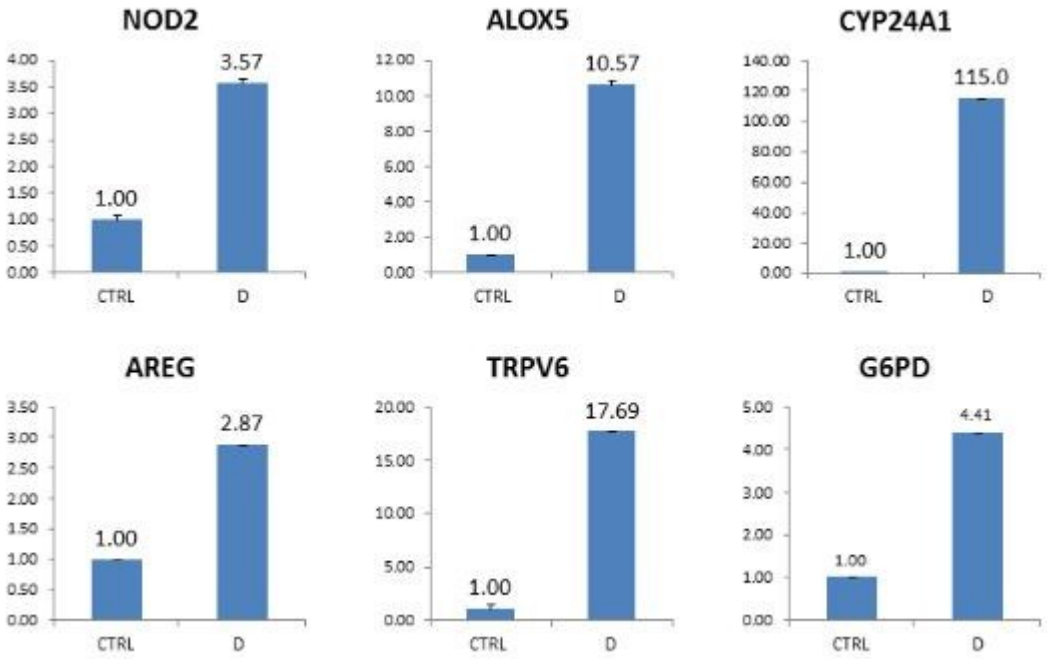


Figure 18: **qPCR validation of 1,25D$_3$ target genes.** Selected genes (positive controls) regulated by 1,25D$_3$ in both microarrays (+CHX or –CHX) were validated using samples from SCC25 cells treated with vehicle (CTRL) or 1,25D$_3$ (D) for 24h.

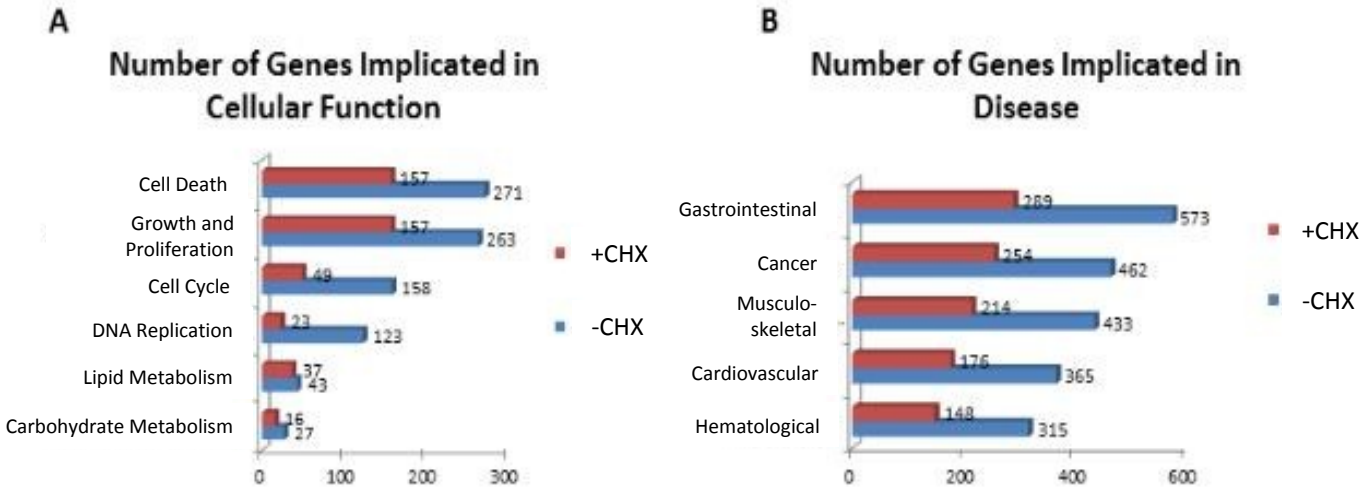


Figure 19: **Cellular functions and diseases associated with 1,25D$_3$-regulated genes.** Both primary (regulated in the presence of CHX, red), obtained from microarray analysis by Wang et al, and primary and secondary (regulated in the absence of CHX, blue) genes from that the gene expression profiling that I performed, were analyzed using IPA for the associated cellular functions (A) and diseases (B).

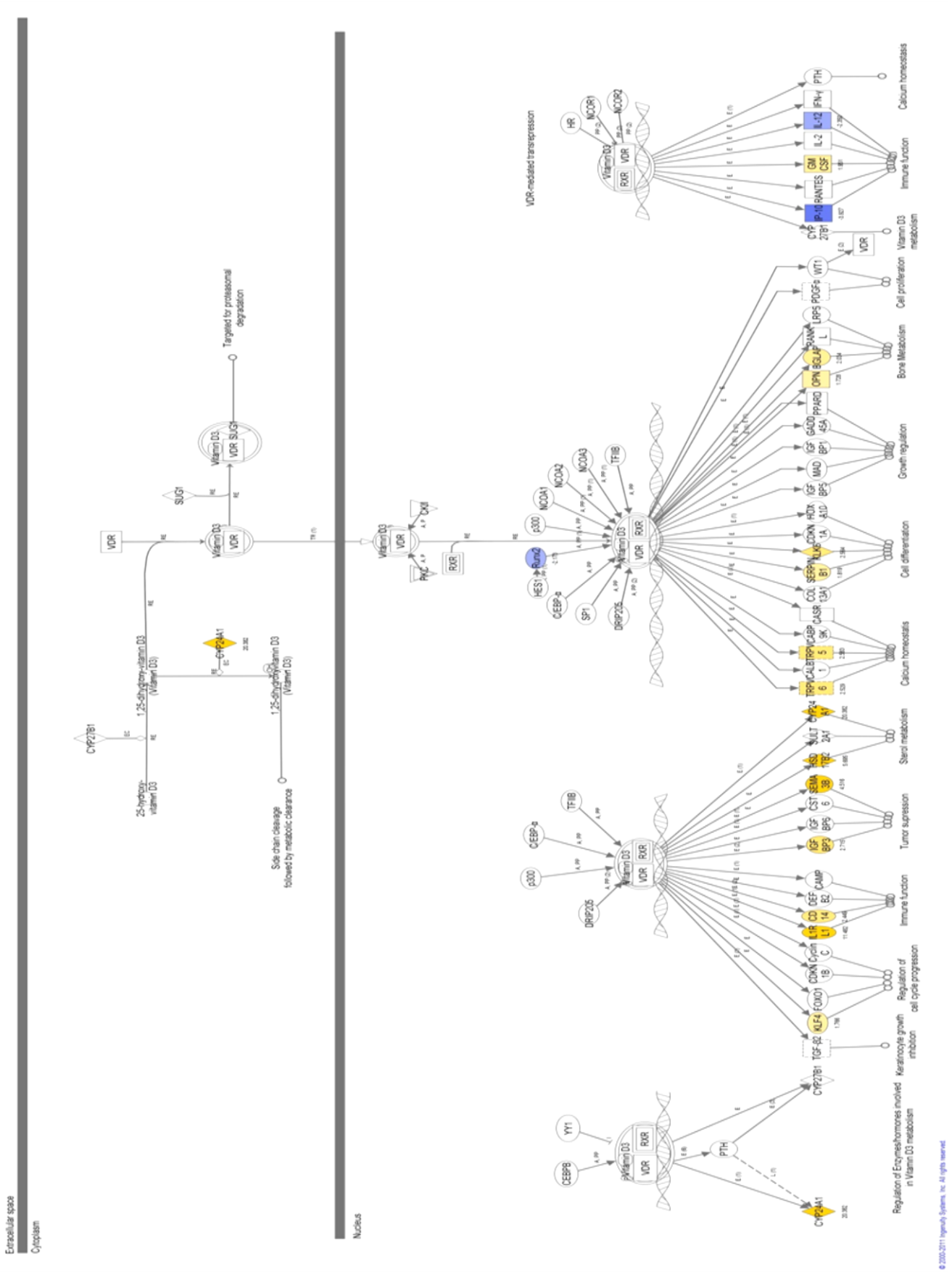


Figure 20: **Canonical pathways: VDR/RXR activation**. This canonical pathway shows genes that are regulated as the result of VDR/RXR activation. Down-regulate are blue and up-regulated, yellow.

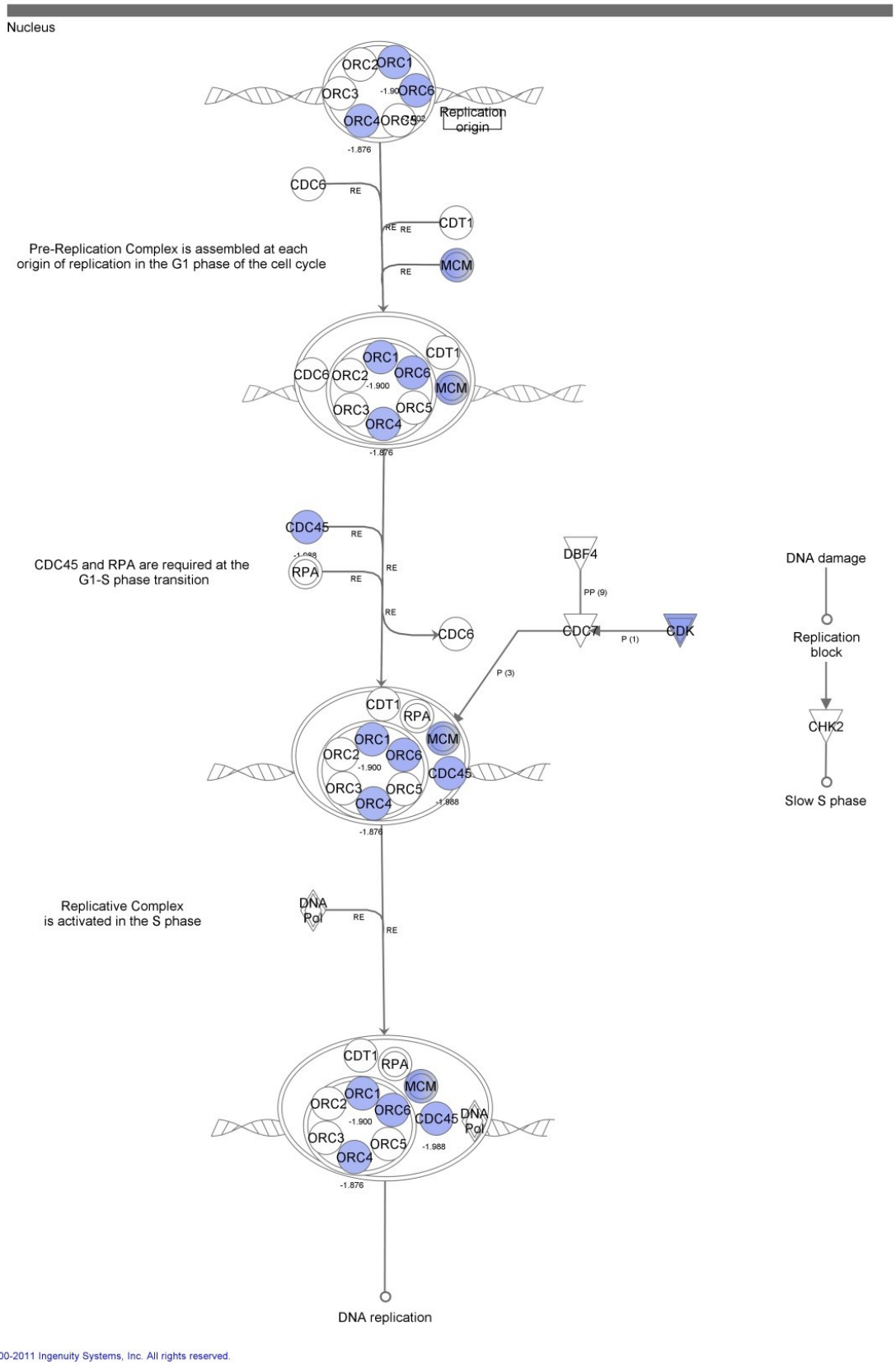


Figure 21: **Canonical pathways: Cell Cycle Control of Chromosomal Replication**. Some of the genes regulated directly or indirectly by 1,25D_3 are implicated in cell cycle-dependent control of chromosomal replication. Note that all these genes appear to be downregulated (blue).

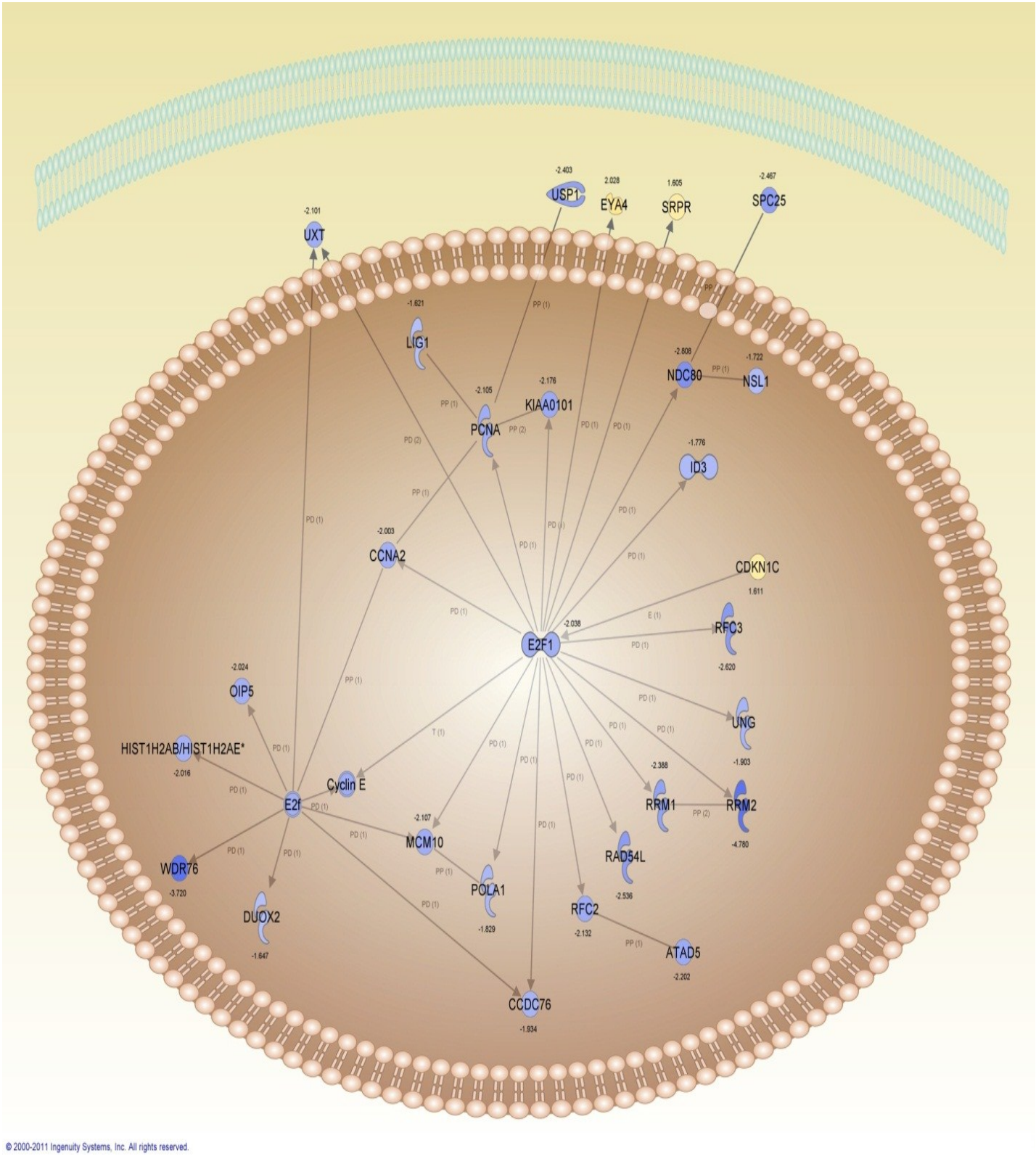


Figure 22: **Network: DNA Replication, Recombination and Repair, Cell Cycle, Cancer**. Published interactions represented in this diagram are shown for genes from the microarray analysis in the absence of CHX - both down-regulated (blue) or up-regulated (yellow) by 1,25D_3.

Gene Name	Fold-Change	RefSeq.
OLR1	-3.565	NM_002543
MACC1	-2.633	NM_182762
BIRC3	-2.393	NM_001165
POLE2	-2.296	NM_002692
PCNA	-2.105	NM_002592
CCNB1	-2.027	NM_031966
CCNA2	-2.003	NM_001237
APPL1	-1.973	NM_012096
BIRC5	-1.837	NM_001168

Table 2: Cancer related genes. 1,25D_3-regulated genes were selected from the microarray (-CHX) that are related to cancer and expression fold-change after the microarray analysis is indicated for each gene.

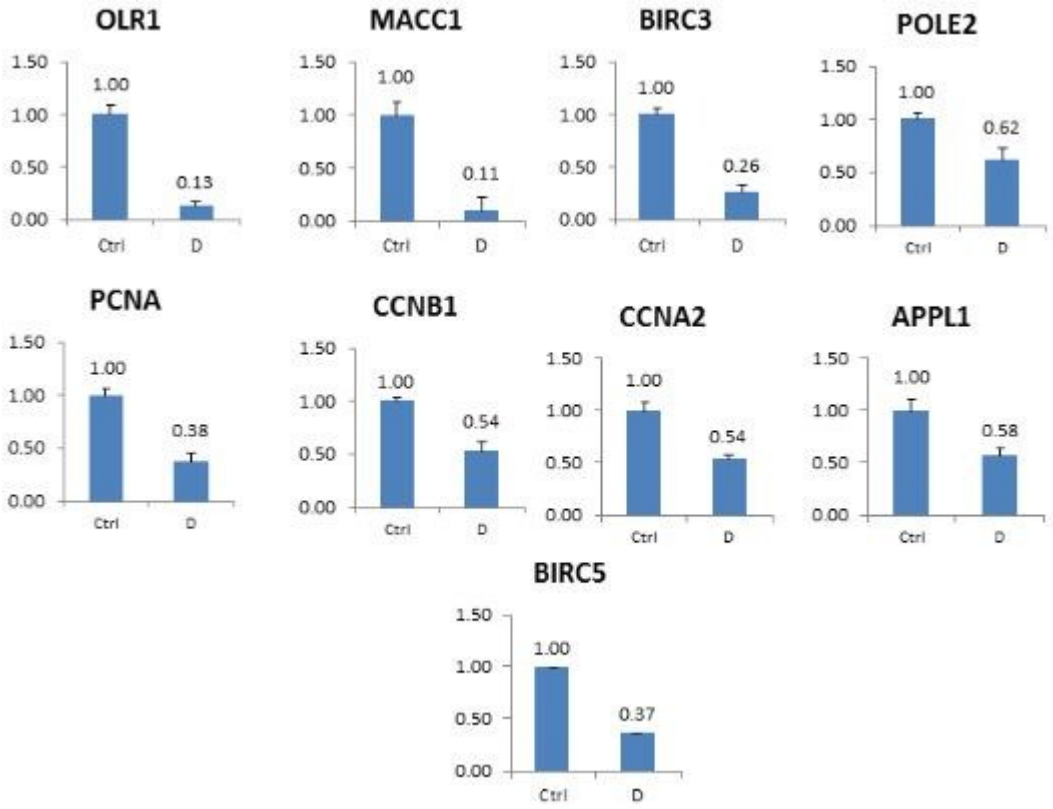


Figure 23: **Validation of genes from table 2 by qPCR**. Ctrl refers to vehicle- and D, to 1,25D_3-treated SCC25 cells for 24h.

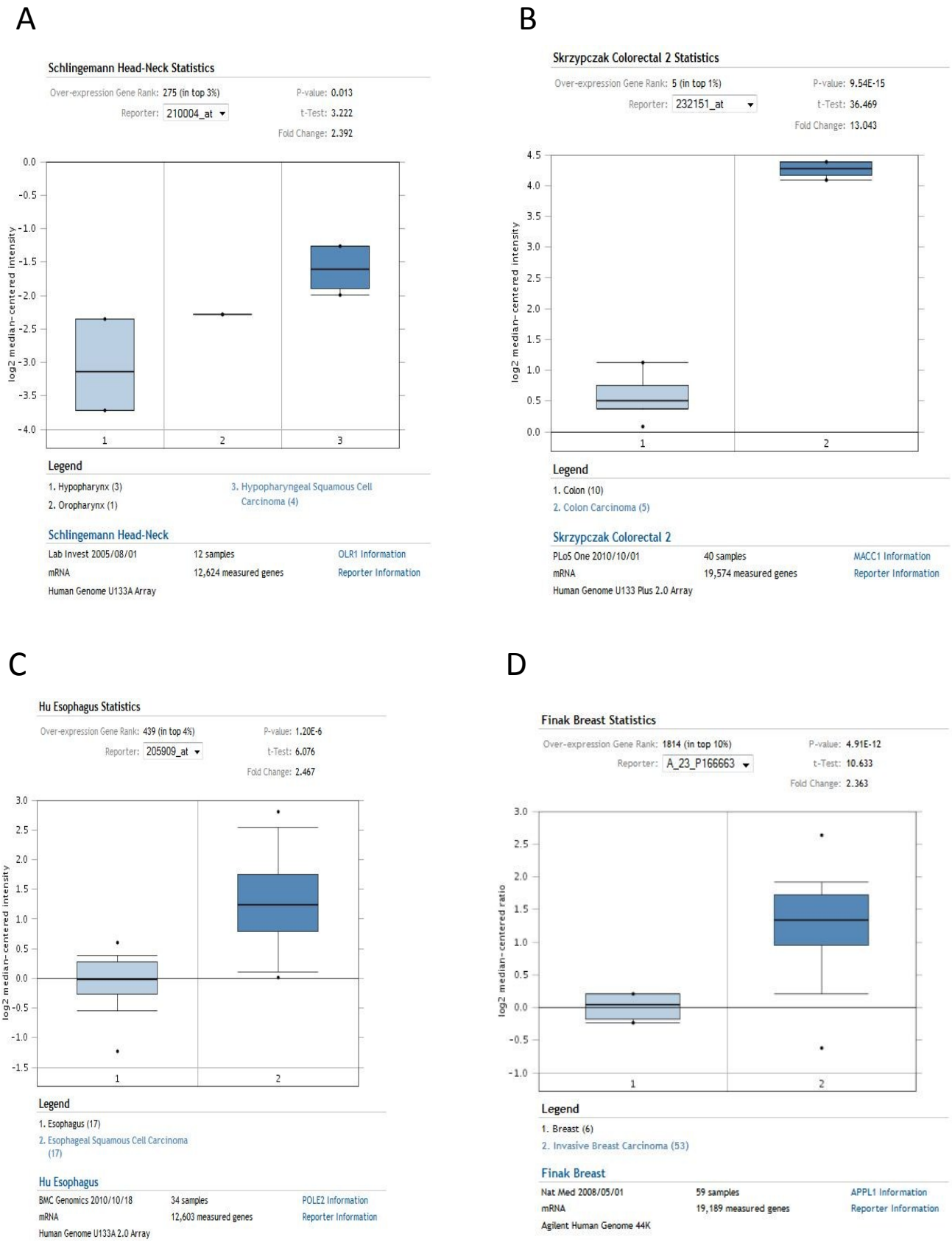


Figure 24: **Overexpression of *OLR1*, *MACC1*, *POLE2* and *APPL1* in cancer**. Oncomine was used to compare the expression levels of *OLR1* (A), *MACC1* (B), *POLE2* (C) and *APPL1* (D) in various cancers relative to normal tissue.

Organ	Cancer Type	Fold-Change	P-value	Reference
OLR1				
Pancreas	Pancreatic Ductal Adenocarcinoma	8.295	2.50E-16	[546]
	Pancreatic Adenocarcinoma	4.263	0.003	[547]
Breast	Invasive Lobular Breast Carcinoma	4.698 to 5.733	3.00E-19 to 0.009	TCGA, [548]
	Invasive Breast Carcinoma	5.271 to 6.700	9.95E-29 to 0.006	TCGA, [548]
	Intraductal Cribriform Breast Adenocarcinoma	6.464	5.93E-6	TCGA
	Invasive Ductal Breast Carcinoma	2.812 to 11.112	9.81E-31 to 0.013	TCGA, [548], [549]
	Mucinous Breast Carcinoma	5.555	0.002	TCGA
	Lobular Breast Carcinoma	3.644	0.004	[550]
	Ductal Breast Carcinoma	2.503-3.670	7.50E-4 to 0.028	[548], [550], [551], [552]
Bladder	Infiltrating Bladder Urothelial Carcinoma	2.877	1.26E-4	[553]
Colon	Colon Adenocarcinoma	2.047 to 3.148	3.93E-15 to 1.31E-11	TCGA, [554]
	Cecum Adenocarcinoma	4.233	1.70E-6	[554]
	Colon Mucinous Adenocarcinoma	2.951 to 6.404	6.14E-13 to 5.91E-5	TCGA, [554]
Head-Neck	Hypopharyngeal Squamous Cell Carcinoma	2.392	0.013	[555]
	Head and Neck Squamous Cell Carcinoma	2.042	6.03E-7	[556]
Brain	Glioblastoma	10.017	8,29E-6	[557]
Cervix	Cervical Squamous Cell Carcinoma	2.700	2.51E-8	[558]
Kidney	Papillary Renal Cell Carcinoma	6.941	6.03E-7	[559]
	Clear Cell Renal Cell Carcinoma	2.042	3.50E-4	[560]
MACC1				
Colon	Colon Carcinoma	13.043	9.54E-15	[561]
	Colon Carcinoma Epithelia	8.712	4.74E-12	[561]
	Colon Adenoma Epithelia	2.760	2.66E-7	[561]
	Colon Adenoma	2.288 to 2.860	6.55E-14 to 8.30E-7	[561], [562]
	Colorectal Carcinoma	2.446 to 3.733	6.62E-15 to 1.57E-15	[561], [563]
	Cecum Adenocarcinoma	2.724 to 8.712	1.20E-8 to 7.02E-8	TCGA, [554]
	Rectosigmoid Adenocarcinoma	6.693	9.96E-7	[554]
	Colon Adenocarcinoma	3.369	1.22E-9	[554]
	Rectal Mucinous Adenocarcinoma	22.820	1.28E-4	[554]
	Colon Mucinous Adenocarcinoma	2.467 to 5.751	2.83E-7 to 3.41E-6	TCGA, [554]

	Rectal Adenoma	3.554	1.83E-6	[562]
	Rectal Adenocarcinoma	3.390	7.92E-12	TCGA
Ovary	Ovarian Serous Adenocarcinoma	2.543	2.86E-7	[564]
	Ovarian Mucinous Adenocarcinoma	2.817	0.006	[564]
Brain	Oligoastrocytoma	3.356	0.007	[565]
	Diffuse Astrocytoma	2.707	2.09E-4	[566]
Pancreas	Pancreatic Carcinoma	3.372	3.72E-7	[567]
Breast	Invasive Lobular Breast Carcinoma	4.558	0.031	[548]
Kidney	Papillary Renal Cell Carcinoma	2.872	0.003	[559]
APPL1				
Myeloma	Smoldering Myeloma	2.167	1.01E-7	[568]
Sarcoma	Dedifferentiated Liposarcoma	2.049	0.0014	[569]
Breast	Invasive Lobular Breast Carcinoma	2.285	0.035	[570]
	Invasive Breast Carcinoma Stroma	2.363	4.9E-12	[571]

Table 3: Expression of selected genes in cancer relative to normal tissues. TCGA= The cancer Genome Atlas: http://tcga-data.nci.nih.gov/tcga/.

DISCUSSION

Based on the results presented here (**fig. 8** and **fig. 9**), one can firmly conclude that $1,25D_3$ has an inhibitory effect on proliferation of SCC25 cells. It is not clear, however, whether these effects are due to cell cycle arrest and entry into a quiescent state, apoptosis or both. It is likely that, based on the calcitriol-dependent transcriptional activation of pro-apoptotic genes such as *BNIP3* and *MMP3*, along with the regulation of cell cycle genes *CCND2* and *CCNG2*, both mechanisms contribute to a decrease in cell numbers, which can be properly quantified using viability and apoptosis assays. Moreover, the role of FoxO3a – traditionally associated with both the induction of programmed cell death and cell cycle arrest – in the regulation of these genes also supports this possibility. It has to be explained here why the focus is on FoxO3a. Firstly, comparing the effect of FoxO3a (**fig. 14**) or FoxO4 (**fig. 15**) ablation on $1,25D_3$-dependent gene regulation, it becomes apparent that FoxO4 is far less important. Secondly, proliferation assays performed by Dr. An demonstrated that FoxO3a ablation negatively affects the 1,25D-induced block of insulin-stimulated cell proliferation, whereas the effect of FoxO4 is much less pronounced [523]. This is the reason why ChIPs at the promoters of *CCND2* and *BNIP3* were only performed for FoxO3a (**fig. 10**). The results from these ChIPs are very interesting: 1,25D-enhanced binding of FoxO3a is highest at 4h and back to control levels at 8h. Dephosphorylation, on the other hand, is detectable at 4h, but is highest at 8h, after which it returns to control levels at 24h. This is consistent with the model proposed by Matsuzaki [5]: 1,25D induces quick deacetylation of FoxO3a (**fig. 12B**) at 4h, resulting in increased binding of FoxO3a to the promoter region of *BNIP3*; gene induction by FoxO3a is usually associated with the recruitment of CBP/p300 and histone acetylation, potentially resulting in FoxO3a acetylation by the coactivators and release from DNA (**fig. 10C** – 8h); once released from the DNA, FoxO3a can be phosphorylated by PKB, which is still low at 8h, but reaches control levels at 24h (**fig. 12A**). The deacetylation of FoxO3a following VD treatment is not striking. However, it should be noted that the totality of acetylated lysine residues of FoxO3a were detected and the latter could be

acetylated at several sites. In this context, VD may stimulate the removal of the acetyl group only from a specific lysine residue leaving the others intact or even stimulating their acetylation.

Another interesting point is the association of the VDR with FoxO3a and the FHRE in gene promoters (of *BNIP3* and *CCND2*), to which FoxO3a binds in a ligand-independent and ligand-dependent manner, respectively. This raises the possibility that VDR is tethered to these FHREs by FoxO3a. In order to test this possibility, ChIPs for VDR at these sites must be performed for up two 8h post 1,25D treatment, similar to FoxO3a. Similar 1,25D-dependent pattern of VDR association with FHREs as FoxO3a could mean regulation of binding of these two transcriptional factor by the same pathway. In addition, ChIP-re-ChIP experiments can definitively demonstrate association of VDR with FoxO3a at the FHREs in the regulatory regions of *BNIP3* and *CCND2*. Irrespective of the mechanism, however, FoxO3a is important for mediating the effects of 1,25D on the expression of certain genes. It is curious that the 1,25D-induced expression of *CYP24A1* is unaffected by the ablation of FoxO3a. *CYP24A1* contains multiple VDREs, to which ligand-bound VDR can associate directly. In this context, FoxO3a may be required for the regulation of a subset of vitamin D target genes implicated in tumorigenesis, but the extent of this list is currently unknown. *CYP24A1* regulation by calcitriol is also unaffected by the inhibition of a FoxO activator, AMPK. Activation of the latter appears to synergize with 1,25D in stimulating or inhibiting the expression of FoxO target genes, whereas its inhibition attenuates the effect of hormonal vitamin D (**fig. 16**). It therefore seems plausible that 1,25D induces AMPK activation. This is not surprising, given the fact that AMPK increases SIRT1 activity [572], which results in activation of FoxO3a through deacetylation and inhibition of phosphorylation by PKB.

FoxO3a could also be directly dephosphorylated by the phosphatase PP1, which was found to be bound to VDR in a ligand-independent manner(**fig. 11A**). GST pull-down experiment can extend the findings from the CoIP for VDR and show whether this interaction is direct or not. The same can be applied for SIRT1's and FoxO3a's association with VDR.

Apart from the four FoxO target genes, 1,25D is potentially capable of regulating others that are important in controlling cell proliferation. *Wang et al.* and I investigated this possibility by performing gene expression profiling. Consistent with the role of ligand-bound VDR in transcriptional activation rather than repression, the vast majority of 1,25D-regulated genes were up-regulated after 12h of treatment in the presence of CHX. The microarray that I performed, however, shows that more than half of the calcitriol-regulated genes are down-regulated. The fact that cells were incubated with vehicle or 1,25D for 24h in the absence of cycloheximide implies that secondary 1,25D target genes that require *de novo* protein synthesis will be detected. This also allows for the detection of pathways and cellular functions dependent on vitamin D signalling. Similar functions and disease were identified by IPA software in both 24h treatment –CHX and 12h treatment +CHX microarrays, except for, naturally, higher number of genes involved in these for the 24h –CHX gene expression profiling (**fig. 19**). With respect to the diseases identified, the data is consistent with already published studies, as discussed in the literature review section. Strikingly, some of the gene functions of the 1,25D-regulated genes were associated with metabolism – carbohydrate and lipid – that could be relevant to cancer (e.g. *OLR1*) [539]. This is supported by the effect of AMPK – normally associated with changes in cellular metabolism – on the regulation of 1,25D-regulated FoxO target genes (**fig. 16**).

In terms of the pathways affected by 1,25D, the obvious is VDR/RXR activation (**fig. 20**). This serves as a control for the responsiveness of SCC25 cells to calcitriol and for the effectiveness of IPA to identify relevant pathways. The genes that are known to be induced by activated VDR/RXR (e.g. *CYP24A*) are up-regulated in this diagram, based on their microarray expression values. Alternatively, genes such as *RUNX2*, whose expression was shown to be inhibited by 1,25D [136], as well as IP-10 and IL-12 appear as down-regulated in figure 20. Interestingly, the canonical pathway predicts that VDR/RXR activation will result in the regulation of certain genes implicated in cell growth (*IGFBP5*, *MAD*, *IGFBP1*, *GADD45A* and *PPARD*)and proliferation (*PDGF* and *WT1*) which was not the case in SCC25 cells based on the

microarray results. This is not surprising considering that the inhibitory effects of calcitriol on proliferation and growth are mediated by different mechanism in different cellular contexts. Supporting this statement is the observation that other 1,25D-regulated genes playing a role in cell growth and proliferation were discovered. Some of them are indicated on figure 21 depicting another canonical pathway detected by IPA called Cell Cycle Control of Chromosomal Replication. Here, genes that code for proteins involved in the pre-replication and replicative complex in G1 and S phase, respectively, are inhibited by 1,25D, which is in agreement with the anti-proliferative effects of vitamin D (**fig. 21**). Block in the replicative capacity of the cell is also suggested by the more exploratory network analysis. As mentioned earlier, two networks were identified to be implicated in DNA replication, recombination and repair, cell cycle and cancer. The network depicted on figure 22 contains elements related to cell cycle progression (e.g. CCNA2, Cyclin E, E2F1), chromosomal segregation (e.g. NDC80, SPC25, NSL1) and DNA replication (e.g. RFC3, RRM1/2, POLA1, MCM10, LIG1). Whether proteins related to chromosomal segregation comes as a cause of 1,25D-induced proliferation inhibition or as a result is not clear. It seems more probable, however, that this down-regulation of genes involved in chromosomal segregation comes as a result of cell cycle arrest. This claim is reinforced by the other network identified with the same designation, DNA replication, recombination and repair, cell cycle and cancer (not shown). Many of the genes present in this network are required for DNA repair and are down-regulated by 1,25D. This likely comes as a consequence of decreased DNA replication resulting from inhibition of the cell cycle. It should be noted here that there are mouse genes (Cyclin E and E2f) that are represented on figure 22. These genes are human homologues, which are regulated by 1,25D. The reason they appear as mouse genes is that their interaction with other genes has only been experimentally demonstrated in mice. In general, the canonical pathways and networks associated with 1,25D-regulated genes solidify the idea that calcitriol has anti-cancer properties and induces cell cycle arrest.

The genes selected from the microarray also support this hypothesis. The values obtained from their validation by qPCR do not exactly correspond to those from the microarray. This can be explained by the way the gene expression profiling results were analyzed. First of all, the samples were run on the Affymetrix Exon 1.0 ST Array chip. The main idea behind the development of this chip is the ability to make inferences about alternative splicing events. In this sense, an average of 22 probes per gene are designed in order to detect expression of different exons. The results from different probes were averaged to obtain the final fold-change displayed in table 1. It should be noted here that averaging the expression levels of individual probes may not be representative of the gene expression, specifically if there are splice variants. Nevertheless, the qPCR results seem to roughly agree with these obtained from the gene expression profiling analysis. Another interesting fact about these genes is that they are all transcriptionally inhibited by 1,25D, which is not in agreement with its conventional role of gene activation. This could be due to two reasons. First, some genes can be regulated by tethering the ligand-bound VDR through a DNA response element by another transcription factor, such as FoxO3a. Such genes could be *OLR1* and *BIRC3* which also appear to possess predicted FHREs in their promoters, as assessed by Genomatix. Their expression is similarly down-regulated by 1,25D after 12h of treatment and in the presence of CHX [136, 522]. Another possibility is the squelching of transcription factors required for gene expression or the active repression, which could take place even in the absence of VDRE as is the case with CYP27B1 and VDIR [307, 308]. Alternatively, some genes can simply be secondary 1,25D target genes that require *de novo* protein synthesis and lie downstream of a primary VDR/RXR targets. The lack of regulation by calcitriol of *MACC1*, POLE2, PCNA, CCNB1, CCNA2, APPL1 and BIRC3 in the presence of CHX is compatible with the latter description. In order to dissect the mechanism through which the genes from table 1 are regulated, several experimental approaches can be adopted. Since there is no published data about their regulation by $1,25D_3$, *in silico* screen for potential VDREs can be performed, followed by ChIP assays to determine primary hormonal vitamin D

target genes. If these genes do not contain VDREs, it is possible that ligand-bound VDR still contributes to their regulation through binding to other transcription factors, such as FoxOs or VDIR. This possibility could be explored, again, through ChIP experiments designed to detect binding of VDR to the promoter region in general. This could be done by designing primers for every 500 bp of the promoters and combining them together to test the DNA fragments immunoprecipitated using anti-VDR antibodies.

Whether they are VD primary targets or not, the genes from table 1 are clearly down-regulated by $1,25D_3$ and play a role in cell growth and division, and DNA replication. In order to test their importance in vitamin D-mediated cell cycle arrest and apoptosis, a conditionally-induced transgene can be stably integrated into the SCC25 cell chromatin. If the anti-proliferative effects of 1,25D are attenuated when the transgene is induced, the gene in question is an important mediator of the effects of calcitriol in SCC25 cells. APPL1 appears to be very interesting as its down-regulation may be able to disrupt PI3K/PKB signalling. In this context, PKB activation can be tested using anti-phospho-Thr308 or anti-phospho-Ser473 antibodies, phosphorylation of Thr308 and Ser473 being indicative of PKB activation.

Finally, various vitamin D analogues can also be tested for the effect on these genes. These analogues are designed to produce specific effects by binding to VDR or MARRS, which stimulates non-genomic or genomic effects and the recruitment of a different cohort of co-regulators resulting in the regulation of different sets of genes. This information can be used to further investigate the cellular pathways affecting by vitamin D responsible for its anti-cancer properties.

CONCLUSION

The results presented above are a clear indication of the fact that 1,25D regulates processes related to the cell cycle(fig. **19A** and **fig. 22**), DNA replication (**fig. 21**), proliferation and ultimately cancer (**fig. 19B**), in line with its known cancer chemopreventive effects . However, these findings have to be extended in several directions in order to better define the cellular pathways leading to and the associated phenotypes resulting from the 1,25D-mediated cell cycle arrest.

First, the role of PP1 and SIRT1 as inducers of 1,25D-dependent FoxO3a activation can be better defined by ablation of these two enzymes using RNAi and assessment of proliferation or cell death of SCC25 cells. Techniques such as FACS and Click-iT® EdU Alexa Fluor® 488 HCS Assay (cat. # C10350, Life Technologies) could be employed to assess cell proliferation. EdU is thymidine analogue and is incorporated into the replicating DNA of dividing cells. Its subsequent detection, therefore, provides a measure of the proliferative status of the cells. Regarding cell death, the ELISA-based QIA20 Cell Death Detection (Nuclear Matrix Protein) kit from Calbiochem (cat. # QIA20-1EA) provides an easy way of assessing apoptosis. The principle behind this assay is that the nuclear matrix protein NuMa is released from the nucleus of apoptotic cells to be subsequently detected by a specific antibody. The result will determine the importance of SIRT1 and PP1c in (1) the anti-proliferative effects of VD and (2) regulation of FoxO function. The latter could be tested using several strategies such as nuclear localization, target gene transcription regulation, including binding to FHREs, and phosphorylation and acetylation status. There are other PTMs, however that are known to regulate FoxO3a function, such as ubiquitination and methylation. Unfortunately, antibodies available for ubiquitinated or methylated FoxOs are not available rendering the detection of these modification more difficult, but still feasible via immunoprecipitation of the transcription factors, followed by WB using anti-methyl-lysing or anti-ubiquitin antibodies.

FoxO3a ablation was shown to limit 1,25D-mediated gene suppression (*CCND2*) and gene activation (*CCNG2*, *BNIP3* and *MMP3*), and its capacity to inhibit cell proliferation [523]. The importance

of repressed (*CCND2* and genes from table 1) or activated (*BNIP3*, *CCNG2* and *MMP3*) genes in this latter process can be determined through constitutively expressed transgenes or siRNA-mediated KDs, respectively, and assessment of cell proliferation in the presence and absence of FoxO3a in SCC25 cells treated with vehicle or 1,25-$(OH)_2D_3$.

All of the genes that appear to be regulated by VD in the microarray I did, can be scanned using bio-informatics approaches for the presence of FHREs. Alternatively, a ChIP-Seq technique can be employed to determine all the genes regulated by FoxO3a in a VD-dependent fashion. This, combined with the results from the gene expression profiling, could be used to test the hypothesis that many of the cancer-chemopreventive effects of vitamin D are mediated through FoxO-dependent gene expression. It should be noted here that FoxO1 activity after vitamin D stimulation was not determined and its implication in the anti-proliferative effects of VD has to be assessed using the same approach as for FoxO3a.

The results discussed here only apply to SCC25 cells. In order to generate a model for the cancer chemopreventive actions of VD, however, other cell lines have to be tested. Interestingly, two other HNSCC cell lines exist that are partially responsive (SCC9) or unresponsive (SCC4) to VD. The partial conservation (SCC9) or the absence (SCC4) of regulation of the pathways controlled by VD in these cells will determine which one(s) of them are relevant to VD-induced cell cycle arrest and/or apoptosis in HNSCC. Their conservation in other cancer cell types may contribute to finding a universal mechanism through which VD exerts its anti-cancer effects, which could be used in the development of new therapeutics.

Overall, the results provided in this work demonstrate that VD down-regulates genes implicated in cell-cycle progression, proliferation and DNA replication and underline the importance of FoxO3a in stimulation (*CCNG2*, *BNIP3* and *MMP3*) or repression (*CCND2*) of another set of genes involved in cell cycle arrest and apoptosis, and G1/S phase transition, respectively.

REFERENCES

1. Calnan, D.R. and A. Brunet, *The FoxO code.* Oncogene, 0000. **27**(16): p. 2276-2288.
2. Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nat Rev Cancer, 2007. **7**(9): p. 684-700.
3. Clark, K.L., et al., *Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5.* Nature, 1993. **364**(6436): p. 412-420.
4. Yamada, S., M. Shimizu, and K. Yamamoto, *Structure–function relationships of vitamin D including ligand recognition by the vitamin D receptor.* Medicinal Research Reviews, 2003. **23**(1): p. 89-115.
5. Matsuzaki, H., et al., *Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(32): p. 11278-11283.
6. Masten, A.R., *Sunlight in tuberculosis.* Chest, 1935. **1**: p. 16.
7. E. McCollum, N.S., J. Becker, P. Shipley, *An experimental demonstration of the existence of a vitamin which promotes calcium deposition.* J Biol Chem, 1922. **53**: p. 19.
8. DeLuca, H., *The vitamin D story: a collaborative effort of basic science and clinical medicine.* The FASEB Journal, 1988. **2**(3): p. 224-236.
9. Palm, T., *The geographical distribution and etiology of rickets.* The Practitioner, 1890. **45**: p. 9.
10. Mellanby, T., *The part played by an 'accessory factor' in the production of experimental rickets.* J Physiol Sci, 1918. **52**: p. 3.
11. Goldblatt, H., *A study of rats on a normal diet irradiated daily by the mercury vapor quartz lamp or kept in darkness.* Biochem J, 1924. **17**: p. 3.
12. Windaus, A., F. Schenck, and F. von Werder, *The antirachitis active irradiation product from 7-dehydrocholesterol.* Hoppe-Seylers Zeitschrift Fur Physiologische Chemie, 1936. **241**: p. 100-103.
13. H. Steenbock, A.B., *Fat soluble vitamins XVII. The induction of growth-promoting and calcifying properties of a ration by exposure to ultraviolet light.* J Biol Chem, 1924. **61**: p. 17.
14. R., G., *Cod and the consumptive: a brief history of cod-liver oil in the treatment of pulmonary tuberculosis.* Pharm. Hist., 2004. **46**: p. 15.
15. Martineau, A.R., et al., *Vitamin D in the treatment of pulmonary tuberculosis.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 793-798.
16. Kawarau A., T.E., Tanida N, Nakagawa K., Yamamoto H., Sawada K., *Inhibitory effect of lonh term 1 alpha-hydroxyvitamin D3 administration on Helicobacter pylori infection.* J. Clin. Biochem. & Nutr., 2006. **38**: p. 4.
17. Wayse, V., et al., *Association of subclinical vitamin D deficiency with severe acute lower respiratory infection in Indian children under 5[thinsp]y.* Eur J Clin Nutr, 2003. **58**(4): p. 563-567.
18. Muhe, L., et al., *Case-control study of the role of nutritional rickets in the risk of developing pneumonia in Ethiopian children.* The Lancet, 1997. **349**(9068): p. 1801-1804.
19. Laaksi, I., et al., *An association of serum vitamin D concentrations < 40 nmol/L with acute respiratory tract infection in young Finnish men.* The American Journal of Clinical Nutrition, 2007. **86**(3): p. 714-717.
20. Juzeniene, A., et al., *The seasonality of pandemic and non-pandemic influenzas: the roles of solar radiation and vitamin D.* International Journal of Infectious Diseases, 2010. **14**(12): p. e1099-e1105.
21. Hughes D.A., N.R., *Vitamin D and respiratory health.* Clin. Exper. Immunol, 2009. **158**: p. 6.
22. Cannell, J., et al., *On the epidemiology of influenza.* Virology Journal, 2008. **5**(1): p. 29.
23. Villamor, E., *A Potential Role for Vitamin D on HIV Infection?* Nutrition Reviews, 2006. **64**(5): p. 226-233.
24. Mehta, S., et al., *Perinatal Outcomes, Including Mother-to-Child Transmission of HIV, and Child Mortality and Their Association with Maternal Vitamin D Status in Tanzania.* Journal of Infectious Diseases, 2009. **200**(7): p. 1022-1030.
25. Mehta, S., et al., *Vitamin D Status of HIV-Infected Women and Its Association with HIV Disease Progression, Anemia, and Mortality.* PLoS ONE, 2010. **5**(1): p. e8770.
26. Smolders, J., et al., *Vitamin D as an immune modulator in multiple sclerosis, a review.* Journal of Neuroimmunology, 2008. **194**(1-2): p. 7-17.
27. Holick, M.F., *Vitamin D Deficiency.* New England Journal of Medicine, 2007. **357**(3): p. 266-281.
28. Guillot, X., et al., *Vitamin D and inflammation.* Joint Bone Spine, 2010. **77**(6): p. 552-557.
29. Hayes, C.E., *Vitamin D and Multiple Sclerosis.* Proceedings of the Society for Experimental Biology and Medicine, 1997. **216**(1): p. 21-27.
30. Nieves, J., et al., *High prevalence of vitamin D deficiency and reduced bone mass in multiple sclerosis.* Neurology, 1994. **44**(9): p. 1687-1692.
31. Munger, K.L., et al., *Vitamin D intake and incidence of multiple sclerosis.* Neurology, 2004. **62**(1): p. 60-65.
32. Munger, K.L., et al., *Serum 25-Hydroxyvitamin D Levels and Risk of Multiple Sclerosis.* JAMA: The Journal of the American Medical Association, 2006. **296**(23): p. 2832-2838.

33. Cantorna, M.T., J. Humpal-Winter, and H.F. DeLuca, *Dietary calcium is a major factor in 1,25-dihydroxycholecalciferol suppression of experimental autoimmune encephalomyelitis in mice.* Journal of Nutrition, 1999. **129**(11): p. 1966-1971.
34. Podolsky, D.K., *Inflammatory Bowel Disease.* New England Journal of Medicine, 1991. **325**(13): p. 928-937.
35. Jahnsen, J., et al., *Vitamin D Status, Parathyroid Hormone and Bone Mineral Density in Patients with Inflammatory Bowel Disease.* Scandinavian Journal of Gastroenterology, 2002. **37**(2): p. 192-199.
36. Aguado, P., et al., *Low Vitamin D Levels in Outpatient Postmenopausal Women from a Rheumatology Clinic in Madrid, Spain: Their Relationship with Bone Mineral Density.* Osteoporosis International, 2000. **11**(9): p. 739-744.
37. Merlino, L.A., et al., *Vitamin D intake is inversely associated with rheumatoid arthritis: Results from the Iowa Women's Health Study.* Arthritis & Rheumatism, 2004. **50**(1): p. 72-77.
38. Patel, S., et al., *Association between serum vitamin D metabolite levels and disease activity in patients with early inflammatory polyarthritis.* Arthritis & Rheumatism, 2007. **56**(7): p. 2143-2149.
39. Andjelkovic, Z., et al., *Disease modifying and immunomodulatory effects of high dose 1α(OH) D3 in rheumatoid arthritis patients.* Clinical and Experimental Rheumatology, 1999. **17**(4): p. 453-456.
40. Alarcón, G.S., et al., *Systemic lupus erythematosus in three ethnic groups: III A comparison of characteristics early in the natural history of the LUMINA cohort.* Lupus, 1999. **8**(3): p. 197-209.
41. Kamen, D.L., et al., *Vitamin D deficiency in systemic lupus erythematosus.* Autoimmunity Reviews, 2006. **5**(2 SPEC. ISS.): p. 114-117.
42. Amital, H., et al., *Serum concentrations of 25-OH vitamin D in patients with systemic lupus erythematosus (SLE) are inversely related to disease activity: is it time to routinely supplement patients with SLE with vitamin D?* Annals of the Rheumatic Diseases, 2010. **69**(6): p. 1155-1157.
43. Takiishi, T., et al., *Vitamin D and Diabetes.* Endocrinology and metabolism clinics of North America, 2010. **39**(2): p. 419-446.
44. Geleijnse, J.M., *Vitamin D and the Prevention of Hypertension and Cardiovascular Diseases: A Review of the Current Evidence.* Am J Hypertens, 2011. **24**(3): p. 253-262.
45. Ceglia, L., *Vitamin D and its role in skeletal muscle.* Current Opinion in Clinical Nutrition & Metabolic Care, 2009. **12**(6): p. 628-633 10.1097/MCO.0b013e328331c707.
46. Bischoff, H.A., et al., *Effects of Vitamin D and Calcium Supplementation on Falls: A Randomized Controlled Trial.* Journal of Bone and Mineral Research, 2003. **18**(2): p. 343-351.
47. BOLAND, R., *Role of Vitamin D in Skeletal Muscle Function.* Endocrine Reviews, 1986. **7**(4): p. 434-448.
48. Peller, S., *Skin irritation and cancer in the U.S. Navy.* Am J Med Sci, 1937. **194**: p. 7.
49. Apperly, F.L., *The relationship of solar radiation to cancer mortality in North America.* Cancer Res, 1941. **1**: p. 4.
50. GARLAND, C.F. and F.C. GARLAND, *Do Sunlight and Vitamin D Reduce the Likelihood of Colon Cancer?* International Journal of Epidemiology, 1980. **9**(3): p. 227-231.
51. Garland, C., et al., *DIETARY VITAMIN D AND CALCIUM AND RISK OF COLORECTAL CANCER: A 19-YEAR PROSPECTIVE STUDY IN MEN.* The Lancet, 1985. **325**(8424): p. 307-309.
52. Garland, C., et al., *SERUM 25-HYDROXYVITAMIN D AND COLON CANCER: EIGHT-YEAR PROSPECTIVE STUDY.* The Lancet, 1989. **334**(8673): p. 1176-1178.
53. Gorham, E.D., et al., *Vitamin D and prevention of colorectal cancer.* The Journal of Steroid Biochemistry and Molecular Biology, 2005. **97**(1-2): p. 179-194.
54. LEFKOWITZ, E.S. and C.F. GARLAND, *Sunlight, Vitamin D, and Ovarian Cancer Mortality Rates in US Women.* International Journal of Epidemiology, 1994. **23**(6): p. 1133-1136.
55. Garland, F.C., et al., *Geographic variation in breast cancer mortality in the United States: A hypothesis involving exposure to solar radiation.* Preventive Medicine, 1990. **19**(6): p. 614-622.
56. Grant, W.B., *An estimate of premature cancer mortality in the U.S. due to inadequate doses of solar ultraviolet-B radiation.* Cancer, 2002. **94**(6): p. 1867-1875.
57. Salazar-Martinez, E., et al., *Dietary factors and endometrial cancer risk. Results of a case–control study in Mexico.* International Journal of Gynecological Cancer, 2005. **15**(5): p. 938-945.
58. Mohr, S.B., et al., *Could ultraviolet B irradiance and vitamin D be associated with lower incidence rates of lung cancer?* Journal of Epidemiology and Community Health, 2008. **62**(1): p. 69-74.
59. J. Wactawski-Wende, J.M.K., G.L. Anderson, A.R. Assaf, R.L. Brunner and M.J. O'Sullivan, et al., *Calcium plus vitamin D supplementation and the risk of colorectal cancer.* N Eng J Med, 2006. **354**: p. 12.
60. Gilbert, R., et al., *Associations of circulating and dietary vitamin D with prostate cancer risk: a systematic review and dose–response meta-analysis.* Cancer Causes and Control, 2011. **22**(3): p. 319-340.
61. Stolzenberg-Solomon, R.Z., *Vitamin D and Pancreatic Cancer.* Annals of Epidemiology, 2009. **19**(2): p. 89-95.
62. Howard, H.R.a.M., *Informa Healthcare.* Psoriasis, 1998: p. 7.
63. Wolpowitz, D. and B.A. Gilchrest, *The vitamin D questions: How much do you need and how should you get it?* Journal of the American Academy of Dermatology, 2006. **54**(2): p. 301-317.

64. Hughes, P.J., et al., *Vitamin D3-driven signals for myeloid cell differentiation--Implications for differentiation therapy.* Leukemia Research, 2010. **34**(5): p. 553-565.
65. Webb, A.R., *Who, what, where and when--influences on cutaneous vitamin D synthesis.* Progress in Biophysics and Molecular Biology, 2006. **92**(1): p. 17-25.
66. Lehmann, B., *Role of the vitamin D3 pathway in healthy and diseased skin – facts, contradictions and hypotheses.* Experimental Dermatology, 2009. **18**(2): p. 97-108.
67. Norman, A.W., *Sunlight, season, skin pigmentation, vitamin D, and 25-hydroxyvitamin D: Integral components of the vitamin D endocrine system.* American Journal of Clinical Nutrition, 1998. **67**(6): p. 1108-1110.
68. Holick, M.F., et al., *Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin.* Biochemical and Biophysical Research Communications, 1977. **76**(1): p. 107-114.
69. WEBB, A.R., L. KLINE, and M.F. HOLICK, *Influence of Season and Latitude on the Cutaneous Synthesis of Vitamin D3: Exposure to Winter Sunlight in Boston and Edmonton Will Not Promote Vitamin D3 Synthesis in Human Skin.* Journal of Clinical Endocrinology & Metabolism, 1988. **67**(2): p. 373-378.
70. Bonjour JP, T.U., Granzer E, Kloepffer G, Mueller K, Scholler D., *The increase in skin 7-dehydrocholesterol induced by an hypocholesterolemic agent is associated with elevated 25-hydroxyvitamin D3 plasma level.* Pflugers Arch, 1987. **410**(1-2): p. 4.
71. MacLaughlin, J. and M.F. Holick, *Aging decreases the capacity of human skin to produce vitamin D3.* The Journal of Clinical Investigation, 1985. **76**(4): p. 1536-1538.
72. Clemens, T.L., et al., *INCREASED SKIN PIGMENT REDUCES THE CAPACITY OF SKIN TO SYNTHESISE VITAMIN D3.* The Lancet, 1982. **319**(8263): p. 74-76.
73. MATSUOKA, L.Y., et al., *Sunscreens Suppress Cutaneous Vitamin D3 Synthesis.* Journal of Clinical Endocrinology & Metabolism, 1987. **64**(6): p. 1165-1168.
74. Matsuoka, L.Y., et al., *Chronic Sunscreen Use Decreases Circulating Concentrations of 25-Hydroxyvitamin D: A Preliminary Study.* Arch Dermatol, 1988. **124**(12): p. 1802-1804.
75. Ross, A.C., et al., *The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know.* Journal of Clinical Endocrinology & Metabolism, 2011. **96**(1): p. 53-58.
76. Mattila PH, P.V., Uusi-Rauva EJ, Koivistoinen PE., *Vitamin D contents in edible mushrooms.* Journal of Agriculture and Food Chemistry, 1994. **42**: p. 5.
77. White, J.H., *Vitamin D Signaling, Infectious Diseases, and Regulation of Innate Immunity.* Infect. Immun., 2008. **76**(9): p. 3837-3843.
78. Levin, A., et al., *Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: Results of the study to evaluate early kidney disease.* Kidney Int, 2006. **71**(1): p. 31-38.
79. Armas, L.A.G., B.W. Hollis, and R.P. Heaney, *Vitamin D2 Is Much Less Effective than Vitamin D3 in Humans.* Journal of Clinical Endocrinology & Metabolism, 2004. **89**(11): p. 5387-5391.
80. Houghton, L.A. and R. Vieth, *The case against ergocalciferol (vitamin D2) as a vitamin supplement.* The American Journal of Clinical Nutrition, 2006. **84**(4): p. 694-697.
81. Trang, H., et al., *Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2.* The American Journal of Clinical Nutrition, 1998. **68**(4): p. 854-858.
82. Holick, M.F., et al., *Vitamin D2 Is as Effective as Vitamin D3 in Maintaining Circulating Concentrations of 25-Hydroxyvitamin D.* Journal of Clinical Endocrinology & Metabolism, 2008. **93**(3): p. 677-681.
83. N, K., *A new look at the most successful prodrugs for active vitamin D (D hormone): alfacalcidol and doxercalciferol.* Molecules, 2009. **10**(14): p. 12.
84. Choi M, M.M., *Therapeutic applications for novel nonhypercalcemic vitamin D receptor ligands.* Expert Opin Ther Patents, 2009. **19**: p. 14.
85. Daiger, S.P., M.S. Schanfield, and L.L. Cavalli-Sforza, *Group-specific component (Gc) proteins bind vitamin D and 25-hydroxyvitamin D.* Proceedings of the National Academy of Sciences, 1975. **72**(6): p. 2076-2080.
86. WEBB, A.R., B.R. DECOSTA, and M.F. HOLICK, *Sunlight Regulates the Cutaneous Production of Vitamin D3 by Causing Its Photodegradation.* Journal of Clinical Endocrinology & Metabolism, 1989. **68**(5): p. 882-887.
87. COOKE, N.E. and J.G. HADDAD, *Vitamin D Binding Protein (Gc-Globulin).* Endocrine Reviews, 1989. **10**(3): p. 294-307.
88. Haddad, J.G., D.R. Fraser, and D.E. Lawson, *Vitamin D plasma binding protein. Turnover and fate in the rabbit.* The Journal of Clinical Investigation, 1981. **67**(5): p. 1550-1560.
89. Boullata, J.I., *Vitamin D supplementation: a pharmacologic perspective.* Current Opinion in Clinical Nutrition & Metabolic Care, 2010. **13**(6): p. 677-684 10.1097/MCO.0b013e32833de911.
90. Cooke, N.E. and E.V. David, *Serum vitamin D-binding protein is a third member of the albumin and alpha fetoprotein gene family.* The Journal of Clinical Investigation, 1985. **76**(6): p. 2420-2424.
91. KAWAKAMI, M., et al., *Turnover of the Plasma Binding Protein for Vitamin D and Its Metabolites in Normal Human Subjects.* Journal of Clinical Endocrinology & Metabolism, 1981. **53**(6): p. 1110-1116.

92. Braun, A., et al., *Sequence and organization of the human vitamin D-binding protein gene.* Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1993. **1216**(3): p. 385-394.
93. Cooke, N.E., et al., *Vitamin D binding protein: Genomic structure, functional domains, and mRNA expression in tissues.* The Journal of Steroid Biochemistry and Molecular Biology, 1991. **40**(4-6): p. 787-793.
94. White, P. and N. Cooke, *The Multifunctional Properties and Characteristics of Vitamin D-binding Protein.* Trends in Endocrinology and Metabolism, 2000. **11**(8): p. 320-327.
95. Speeckaert, M., et al., *Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism.* Clinica Chimica Acta, 2006. **372**(1-2): p. 33-42.
96. Jones, G., *Pharmacokinetics of vitamin D toxicity.* The American Journal of Clinical Nutrition, 2008. **88**(2): p. 582S-586S.
97. Christakos, S., et al., *Vitamin D: Metabolism.* Endocrinology & Metabolism Clinics of North America, 2010. **39**(2): p. 243-253.
98. Ilahi, M., L.A. Armas, and R.P. Heaney, *Pharmacokinetics of a single, large dose of cholecalciferol.* The American Journal of Clinical Nutrition, 2008. **87**(3): p. 688-691.
99. Kampman, M.T. and L.H. Steffensen, *The role of vitamin D in multiple sclerosis.* Journal of Photochemistry and Photobiology B: Biology, 2010. **101**(2): p. 137-141.
100. Lips, P., *Which circulating level of 25-hydroxyvitamin D is appropriate?* The Journal of Steroid Biochemistry and Molecular Biology, 2004. **89-90**: p. 611-614.
101. Bischoff-Ferrari, H., *Health effects of vitamin D.* Dermatologic Therapy, 2010. **23**(1): p. 23-30.
102. Usui, E., et al., *Unique property of liver mitochondrial P450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation.* FEBS Letters, 1990. **274**(1-2): p. 175-177.
103. Cheng, J.B., et al., *De-orphanization of Cytochrome P450 2R1.* Journal of Biological Chemistry, 2003. **278**(39): p. 38084-38093.
104. Gupta, R.P., et al., *CYP3A4 is a Human Microsomal Vitamin D 25-Hydroxylase.* Journal of Bone and Mineral Research, 2004. **19**(4): p. 680-688.
105. Aiba, I., et al., *Characterization of rat and human CYP2J enzymes as Vitamin D 25-hydroxylases.* Steroids, 2006. **71**(10): p. 849-856.
106. Shinkyo, R., et al., *Metabolism of vitamin D by human microsomal CYP2R1.* Biochemical and Biophysical Research Communications, 2004. **324**(1): p. 451-457.
107. Haddad, J.G., *Plasma vitamin D-binding protein (Gc-globulin): Multiple tasks.* The Journal of Steroid Biochemistry and Molecular Biology, 1995. **53**(1-6): p. 579-582.
108. MENDEL, C.M., *The Free Hormone Hypothesis: A Physiologically Based Mathematical Model.* Endocrine Reviews, 1989. **10**(3): p. 232-274.
109. Nykjaer, A., et al., *An Endocytic Pathway Essential for Renal Uptake and Activation of the Steroid 25-(OH) Vitamin D3.* Cell, 1999. **96**(4): p. 507-515.
110. Sawada, N., et al., *Structure–function analysis of CYP27B1 and CYP27A1.* European Journal of Biochemistry, 2001. **268**(24): p. 6607-6615.
111. Panda, D.K., et al., *Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: Evidence for skeletal, reproductive, and immune dysfunction.* Proceedings of the National Academy of Sciences, 2001. **98**(13): p. 7498-7503.
112. Lawson DE, F.D., Kodicek E, Morris HR, Williams DH, *Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism.* Nature, 1971. **230**(5291): p. 3.
113. Holick MF, S.H., DeLuca HF, *Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D3 metabolically active in the intestine.* Proc Natl Acad Sci U S A., 1971. **4**: p. 2.
114. Rejnmark, L., et al., *Vitamin D Insufficiency in Greenlanders on a Westernized Fare: Ethnic Differences in Calcitropic Hormones Between Greenlanders and Danes.* Calcified Tissue International, 2004. **74**(3): p. 255-263.
115. Adams, J.S., et al., *Metabolism of 25-hydroxyvitamin D3 by cultured pulmonary alveolar macrophages in sarcoidosis.* The Journal of Clinical Investigation, 1983. **72**(5): p. 1856-1860.
116. Hewison, M., et al., *1alpha-Hydroxylase and the action of vitamin D.* Journal of Molecular Endocrinology, 2000. **25**(2): p. 141-148.
117. Townsend, K., et al., *Biological actions of extra-renal 25-hydroxyvitamin D-1[alpha]-hydroxylase and implications for chemoprevention and treatment.* The Journal of Steroid Biochemistry and Molecular Biology, 2005. **97**(1-2): p. 103-109.
118. Tokar, E. and M. Webber, *Cholecalciferol (Vitamin D₃) Inhibits Growth and Invasion by Up-regulating Nuclear Receptors and 25-Hydroxylase (CYP27A1) in Human Prostate Cancer Cells.* Clinical and Experimental Metastasis, 2005. **22**(3): p. 275-284.
119. Tokar, E. and M. Webber, *Chemoprevention of Prostate Cancer by Cholecalciferol (Vitamin D₃): 25-hydroxylase (CYP27A1) in Human Prostate Epithelial Cells.* Clinical and Experimental Metastasis, 2005. **22**(3): p. 265-273.

120. Ichikawa F, S.K., Nanjo M, Nishii Y, Shinki T, Takahashi N, Suda T, *Mouse primary osteoblasts express vitamin D3 25-hydroxylase mRNA and convert 1 alpha-hydroxyvitamin D3 into 1 alpha,25-dihydroxyvitamin D3.* Bone, 1995. **16**(1): p. 7.
121. Gottfried, E., et al., *Monocyte-derived cells express CYP27A1 and convert vitamin D3 into its active metabolite.* Biochemical and Biophysical Research Communications, 2006. **349**(1): p. 209-213.
122. Vantieghem, K., et al., *UVB-induced production of 1,25-dihydroxyvitamin D3 and vitamin D activity in human keratinocytes pretreated with a sterol Δ7-reductase inhibitor.* Journal of Cellular Biochemistry, 2006. **98**(1): p. 81-92.
123. Lehmann B, M.M., *Extrarenal sites of calcitriol synthesis: the particular role of the skin.* Recent Results Cancer Res, 2003. **164**: p. 11.
124. Schuessler, M., et al., *Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D3: CYP27 in epidermis completes the set of essential vitamin D3-hydroxylases.* Steroids, 2001. **66**(3-5): p. 399-408.
125. Lehmann, B., et al., *UVB-Induced Conversion of 7-Dehydrocholesterol to 1[agr],25-Dihydroxyvitamin D3 in an In Vitro Human Skin Equivalent Model.* 2001. **117**(5): p. 1179-1185.
126. Fleet, J.C., *Rapid, Membrane-Initiated Actions of 1,25 Dihydroxyvitamin D: What Are They and What Do They Mean?* The Journal of Nutrition, 2004. **134**(12): p. 3215-3218.
127. Hughes, P.J. and G. Brown, *1α,25-dihydroxyvitamin D3-mediated stimulation of steroid sulphatase activity in myeloid leukaemic cell lines requires VDRnuc-mediated activation of the RAS/RAF/ERK-MAP kinase signalling pathway.* Journal of Cellular Biochemistry, 2006. **98**(3): p. 590-617.
128. J, Y., *The establishment of a HeLa cell demonstrating rapid mitogen-activated protein kinase phosphorylation in response to 1alpha,25-dihydroxyvitamin D3 by stable transfection of a chick skeletal muscle cDNA library.* Biosci. Biotechnol. Biochem, 2006. **70**(1): p. 4.
129. Ji Y, K.A., Verstuyf A, Verlinden L, Studzinski GP, *Derivatives of vitamins D2 and D3 activate three MAPK pathways and upregulate pRb expression in differentiating HL60 cells.* Cell Cycle, 2002. **6**(1): p. 6.
130. Narayanan, R., et al., *The Functional Consequences of Cross-talk between the Vitamin D Receptor and ERK Signaling Pathways Are Cell-specific.* Journal of Biological Chemistry, 2004. **279**(45): p. 47298-47310.
131. Rossi, A.M., et al., *MAPK inhibition by 1[alpha],25(OH)2-Vitamin D3 in breast cancer cells.: Evidence on the participation of the VDR and Src.* The Journal of Steroid Biochemistry and Molecular Biology, 2004. **89-90**: p. 287-290.
132. Schwartz, Z., et al., *1α,25-Dihydroxyvitamin D3 and 24R,25-Dihydroxyvitamin D3 Modulate Growth Plate Chondrocyte Physiology via Protein Kinase C-Dependent Phosphorylation of Extracellular Signal-Regulated Kinase 1/2 Mitogen-Activated Protein Kinase.* Endocrinology, 2002. **143**(7): p. 2775-2786.
133. Wang, X. and G.P. Studzinski, *Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells.* Journal of Cellular Biochemistry, 2001. **80**(4): p. 471-482.
134. Nemere, I., et al., *Identification of a Membrane Receptor for 1,25-Dihydroxyvitamin D3 Which Mediates Rapid Activation of Protein Kinase C.* Journal of Bone and Mineral Research, 1998. **13**(9): p. 1353-1359.
135. Wali, R.K., et al., *Vitamin D receptor is not required for the rapid actions of 1,25-dihydroxyvitamin D3 to increase intracellular calcium and activate protein kinase C in mouse osteoblasts.* Journal of Cellular Biochemistry, 2003. **88**(4): p. 794-801.
136. Boyan, B.D., et al., *Plasma membrane requirements for 1[alpha],25(OH)2D3 dependent PKC signaling in chondrocytes and osteoblasts.* Steroids, 2006. **71**(4): p. 286-290.
137. Li, J., J.C. Fleet, and D. Teegarden, *Activation of rapid signaling pathways does not contribute to 1α,25-dihydroxyvitamin D3-induced growth inhibition of mouse prostate epithelial progenitor cells.* Journal of Cellular Biochemistry, 2009. **107**(5): p. 1031-1036.
138. Wang, Y., et al., *Disruption of Pdia3 gene results in bone abnormality and affects 1[alpha],25-dihydroxy-vitamin D3-induced rapid activation of PKC.* The Journal of Steroid Biochemistry and Molecular Biology, 2010. **121**(1-2): p. 257-260.
139. Boland, R.L., *VDR activation of intracellular signaling pathways in skeletal muscle.* Molecular and Cellular Endocrinology, 2011. **In Press, Corrected Proof**.
140. Schwartz, Z., et al., *1[alpha],25(OH)2D3 causes a rapid increase in phosphatidylinositol-specific PLC-[beta] activity via phospholipase A2-dependent production of lysophospholipid.* Steroids, 2003. **68**(5): p. 423-437.
141. Buitrago, C., V. González Pardo, and A.R. de Boland, *Nongenomic action of 1α,25(OH)2-vitamin D3.* European Journal of Biochemistry, 2002. **269**(10): p. 2506-2515.
142. Schwartz, Z., et al., *The effect of 24R,25-(OH)2D3 on protein kinase C activity in chondrocytes is mediated by phospholipase D whereas the effect of 1[alpha],25-(OH)2D3 is mediated by phospholipase C.* Steroids, 2001. **66**(9): p. 683-694.
143. Vazquez, G., A.R. de Boland, and R.L. Boland, *1α,25-Dihydroxy-vitamin-D3-induced Store-operated Ca2+ Influx in Skeletal Muscle Cells.* Journal of Biological Chemistry, 1998. **273**(51): p. 33954-33960.
144. Le Mellay, V., B. Grosse, and M. Lieberherr, *Phospholipase C β and Membrane Action of Calcitriol and Estradiol.* Journal of Biological Chemistry, 1997. **272**(18): p. 11902-11907.

145. Khare, S., et al., *1,25-Dihydroxyvitamin D3 and TPA activate phospholipase D in Caco-2 cells: role of PKC-α.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1999. **276**(4): p. G993-G1004.
146. Boyan, B.D., et al., *Membrane mediated signaling mechanisms are used differentially by metabolites of vitamin D3 in musculoskeletal cells.* Steroids, 2002. **67**(6): p. 421-427.
147. Facchinetti, M.M., R. Boland, and A.R. de Boland, *Age-related loss of calcitriol stimulation of phosphoinositide hydrolysis in rat skeletal muscle.* Molecular and Cellular Endocrinology, 1998. **136**(2): p. 131-138.
148. Facchinetti, M.M., R. Boland, and A.R. de Boland, *Calcitriol transmembrane signalling: regulation of rat muscle phospholipase D activity.* Journal of Lipid Research, 1998. **39**(1): p. 197-204.
149. de Boland, A.R., et al., *Age-associated decrease in inositol 1,4,5-triphosphate and diacylglycerol generation by 1,25(OH)2-vitamin D3 in rat intestine.* Cellular Signalling, 1996. **8**(3): p. 153-157.
150. Khanal, R.C., et al., *Membrane receptor-initiated signaling in 1,25(OH)2D3-stimulated calcium uptake in intestinal epithelial cells.* Journal of Cellular Biochemistry, 2008. **105**(4): p. 1109-1116.
151. Santillán, G.E. and R.L. Boland, *Studies Suggesting the Participation of Protein Kinase A in 1,25(OH)2-Vitamin D3-Dependent Protein Phosphorylation in Cardiac Muscle.* Journal of Molecular and Cellular Cardiology, 1998. **30**(2): p. 225-233.
152. Vazquez, G., A.R. de Boland, and R.L. Boland, *1[alpha],25-(OH)2-Vitamin D3Stimulates the Adenylyl Cyclase Pathway in Muscle Cells by a GTP-Dependent Mechanism Which Presumably Involves Phosphorylation of G[alpha]i.* Biochemical and Biophysical Research Communications, 1997. **234**(1): p. 125-128.
153. Vazquez G, B.R., de Boland A, *Modulation by 1,25(OH)2-vitamin D3 of the adenylyl cyclase/cyclic AMP pathway in rat and chick myoblasts.* Biochim Biophys Acta, 1995. **1269**(1): p. 7.
154. Marinissen M, S.J., Boland R, *Involvement of protein kinase C in 1,25(OH)2-vitamin D3 regulation of calcium uptake by cultured myocytes.* Cell Signal, 1994. **6**(5): p. 8.
155. Uchida, Y., et al., *1α,25-dihydroxyvitamin D3 Rapidly Modulates Ca2+ Influx in Osteoblasts Mediated by Ca2+ Channels.* The Bulletin of Tokyo Dental College, 2010. **51**(4): p. 221-226.
156. Morelli, A., et al., *The vitamin D receptor agonist elocalcitol upregulates L-type calcium channel activity in human and rat bladder.* American Journal of Physiology - Cell Physiology, 2008. **294**(5): p. C1206-C1214.
157. Lajdova, I., D. Chorvat Jr, and A. Chorvatova, *Rapid effects of 1[alpha],25(OH)2D3 in resting human peripheral blood mononuclear cells.* European Journal of Pharmacology, 2008. **586**(1-3): p. 14-23.
158. Xiaoyu, Z., et al., *1[alpha],25(OH)2-vitamin D3 membrane-initiated calcium signaling modulates exocytosis and cell survival.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 457-461.
159. Liu, R., et al., *Ribozyme Ablation Demonstrates That the Cardiac Subtype of the Voltage-sensitive Calcium Channel Is the Molecular Transducer of 1,25-Dihydroxyvitamin D3-stimulated Calcium Influx in Osteoblastic Cells.* Journal of Biological Chemistry, 2000. **275**(12): p. 8711-8718.
160. Nakagawa K, T.N., Okamoto T, Kishi T, Ono T, Kubodera N, Okano T, *Rapid control of transmembrane calcium influx by 1alpha,25-dihydroxyvitamin D3 and its analogues in rat osteoblast-like cells.* Biol Pharm Bull, 1999. **22**(10): p. 6.
161. Vazquez G, d.B.A., *Involvement of protein kinase C in the modulation of 1alpha,25-dihydroxy-vitamin D3-induced 45Ca2+ uptake in rat and chick cultured myoblasts.* Biochim Biophys Acta, 1996. **1310**(1): p. 5.
162. Billaudel B, B.P., Sutter B, Faure-Dussert A, *Regulatory effect of 1,25-dihydroxyvitamin D3 on insulin release and calcium handling via the phospholipid pathway in islets from vitamin D-deficient rats.* J Endocrinol Invest, 1995. **9**(18): p. 12.
163. Tien X, K.C., Qasawa B, Sitrin M, Nelson D, Brasitus T, *Characterization of the 1,25-dihydroxycholecalciferol-stimulated calcium influx pathway in CaCo-2 cells.* J Membr Biol, 1993. **136**(2): p. 9.
164. Menegaz, D., et al., *Vitamin D Receptor (VDR) Regulation of Voltage-Gated Chloride Channels by Ligands Preferring a VDR-Alternative Pocket (VDR-AP).* Molecular Endocrinology, 2011. **25**(8): p. 1289-1300.
165. Menegaz, D., et al., *1[alpha],25(OH)2-Vitamin D3 stimulation of secretion via chloride channel activation in Sertoli cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2010. **119**(3-5): p. 127-134.
166. Zanello, L.P. and A. Norman, *1[alpha],25(OH)2 Vitamin D3 actions on ion channels in osteoblasts.* Steroids, 2006. **71**(4): p. 291-297.
167. Zanello, L.P. and A.W. Norman, *Stimulation by 1α,25(OH)2-Vitamin D3 of Whole Cell Chloride Currents in Osteoblastic ROS 17/2.8 Cells.* Journal of Biological Chemistry, 1997. **272**(36): p. 22617-22622.
168. Zanello, L.P. and A.W. Norman, *1[alpha],25(OH)2Vitamin D3-Mediated Stimulation of Outward Anionic Currents in Osteoblast-like ROS 17/2.8 Cells.* Biochemical and Biophysical Research Communications, 1996. **225**(2): p. 551-556.
169. P Brumbaugh, M.H., *Nuclear and cytoplasmic binding components for vitamin D metabolites.* Life Sciences, 1975. **16**(3): p. 9.
170. B Kream, R.R., J Knutson, J Eisman, H DeLuca, *Intestinal cytosol binders of 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D.* Archives of Biochemistry and Biophysics, 1976. **176**(2): p. 8.
171. Baker, A.R., et al., *Cloning and expression of full-length cDNA encoding human vitamin D receptor.* Proceedings of the National Academy of Sciences, 1988. **85**(10): p. 3294-3298.

172. Nemere, I., et al., *Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(19): p. 7392-7397.
173. Nemere, I., R. Ray, and W. McManus, *Immunochemical studies on the putative plasmalemmal receptor for 1,25(OH)2D3. I. Chick intestine.* American Journal of Physiology - Endocrinology And Metabolism, 2000. **278**(6): p. E1104-E1114.
174. Dormanen, M.C., et al., *Nonnuclear Effects of the Steroid Hormone 1[alpha],25(OH)2-Vitamin D3: Analogs Are Able to Functionally Differentiate Between Nuclear and Membrane Receptors.* Biochemical and Biophysical Research Communications, 1994. **201**(1): p. 394-401.
175. Norman, A.W., et al., *Demonstration that 1 beta,25-dihydroxyvitamin D3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of 1 alpha,25-dihydroxyvitamin D3.* Journal of Biological Chemistry, 1993. **268**(27): p. 20022-20030.
176. Nemere, I., et al., *Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia.* Journal of Biological Chemistry, 1994. **269**(38): p. 23750-23756.
177. Boyan, B.D., et al., *Membrane actions of vitamin D metabolites 1α,25(OH)2D3 and 24R,25(OH)2D3 are retained in growth plate cartilage cells from vitamin D receptor knockout mice.* Journal of Cellular Biochemistry, 2003. **90**(6): p. 1207-1223.
178. Capiati, D., S. Benassati, and R.L. Boland, *1,25(OH)2-vitamin D3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells.* Journal of Cellular Biochemistry, 2002. **86**(1): p. 128-135.
179. Huhtakangas, J.A., et al., *The Vitamin D Receptor Is Present in Caveolae-Enriched Plasma Membranes and Binds 1α,25(OH)2-Vitamin D3 in Vivo and in Vitro.* Molecular Endocrinology, 2004. **18**(11): p. 2660-2671.
180. Zanello, L.P. and A.W. Norman, *Rapid modulation of osteoblast ion channel responses by 1α,25(OH)2-vitamin D3 requires the presence of a functional vitamin D nuclear receptor.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(6): p. 1589-1594.
181. Norman, A.W., M.T. Mizwicki, and D.P.G. Norman, *Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model.* Nat Rev Drug Discov, 2004. **3**(1): p. 27-41.
182. Bertrand, S., et al., *Evolutionary Genomics of Nuclear Receptors: From Twenty-Five Ancestral Genes to Derived Endocrine Systems.* Molecular Biology and Evolution, 2004. **21**(10): p. 1923-1937.
183. Chute, J.P., J.R. Ross, and D.P. McDonnell, *Minireview: Nuclear Receptors, Hematopoiesis, and Stem Cells.* Molecular Endocrinology, 2010. **24**(1): p. 1-10.
184. Sonoda, J., L. Pei, and R.M. Evans, *Nuclear receptors: Decoding metabolic disease.* FEBS Letters, 2008. **582**(1): p. 2-9.
185. Green, S. and P. Chambon, *Nuclear receptors enhance our understanding of transcription regulation.* Trends in Genetics, 1988. **4**(11): p. 309-314.
186. Beato, M. and J. Klug, *Steroid hormone receptors: an update.* Human Reproduction Update, 2000. **6**(3): p. 225-236.
187. Li, Y., M.H. Lambert, and H.E. Xu, *Activation of Nuclear Receptors: A Perspective from Structural Genomics.* Structure, 2003. **11**(7): p. 741-746.
188. Robinson-Rechavi, M., H.E. Garcia, and V. Laudet, *The nuclear receptor superfamily.* Journal of Cell Science, 2003. **116**(4): p. 585-586.
189. Mader, S., et al., *Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element.* Nature, 1989. **338**(6212): p. 271-274.
190. Nguyen, D., et al., *Rational design of an estrogen receptor mutant with altered DNA-binding specificity.* Nucleic Acids Research, 2007. **35**(10): p. 3465-3477.
191. Luisi, B.F., et al., *Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA.* Nature, 1991. **352**(6335): p. 497-505.
192. Schwabe, J.W.R., et al., *The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: How receptors discriminate between their response elements.* Cell, 1993. **75**(3): p. 567-578.
193. Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors.* Genes & Development, 2000. **14**(2): p. 121-141.
194. Nettles, K.W. and G.L. Greene, *LIGAND CONTROL OF COREGULATOR RECRUITMENT TO NUCLEAR RECEPTORS.* Annual Review of Physiology, 2005. **67**(1): p. 309-333.
195. Jin, L. and Y. Li, *Structural and functional insights into nuclear receptor signaling.* Advanced Drug Delivery Reviews, 2010. **62**(13): p. 1218-1226.
196. Deblois, G. and V. Giguère, *Functional and physiological genomics of estrogen-related receptors (ERRs) in health and disease.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2011. **1812**(8): p. 1032-1040.
197. Zhao Y, B.D., *NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology.* Arterioscler Thromb Vasc Biol, 2010. **30**(8): p. 6.

198. Seol, W., M. Chung, and D. Moore, *Novel receptor interaction and repression domains in the orphan receptor SHP.* Mol. Cell. Biol., 1997. **17**(12): p. 7126-7131.
199. Seol W, C.H., Moore D, *An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors.* Science, 1996. **272**: p. 3.
200. Zanaria, E., et al., *An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita.* Nature, 1994. **372**(6507): p. 635-641.
201. Xue, Y., et al., *Crystal Structure of the Pregnane X Receptor-Estradiol Complex Provides Insights into Endobiotic Recognition.* Molecular Endocrinology, 2007. **21**(5): p. 1028-1038.
202. Egea, P.F., A. Mitschler, and D. Moras, *Molecular Recognition of Agonist Ligands by RXRs.* Molecular Endocrinology, 2002. **16**(5): p. 987-997.
203. Zhang, J., et al., *DNA binding alters coactivator interaction surfaces of the intact VDR–RXR complex.* Nat Struct Mol Biol, 2011. **18**(5): p. 556-563.
204. Zhang, J., et al., *Hydrogen/Deuterium Exchange Reveals Distinct Agonist/Partial Agonist Receptor Dynamics within Vitamin D Receptor/Retinoid X Receptor Heterodimer.* Structure, 2010. **18**(10): p. 1332-1341.
205. Yamada, S., et al., *Three-dimensional structure-function relationship of vitamin D and vitamin D receptor model.* Steroids, 2001. **66**(3-5): p. 177-187.
206. Mizwicki, M.T. and A.W. Norman, *The Vitamin D Sterol-Vitamin D Receptor Ensemble Model Offers Unique Insights into Both Genomic and Rapid-Response Signaling.* Sci. Signal., 2009. **2**(75): p. re4-.
207. Shoemaker, B.A., J.J. Portman, and P.G. Wolynes, *Speeding molecular recognition by using the folding funnel: The fly-casting mechanism.* Proceedings of the National Academy of Sciences, 2000. **97**(16): p. 8868-8873.
208. von Hippel, P.H., *Completing the View of Transcriptional Regulation.* Science, 2004. **305**(5682): p. 350-352.
209. Thompson, P.D., et al., *Liganded VDR induces CYP3A4 in small intestinal and colon cancer cells via DR3 and ER6 vitamin D responsive elements.* Biochemical and Biophysical Research Communications, 2002. **299**(5): p. 730-738.
210. Drocourt, L., et al., *Expression of CYP3A4, CYP2B6, andCYP2C9 Is Regulated by the Vitamin D Receptor Pathway in Primary Human Hepatocytes.* Journal of Biological Chemistry, 2002. **277**(28): p. 25125-25132.
211. Thummel, K.E., et al., *Transcriptional Control of Intestinal Cytochrome P-4503A by 1α,25-Dihydroxy Vitamin D3.* Molecular Pharmacology, 2001. **60**(6): p. 1399-1406.
212. Rochel, N., et al., *Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacings.* Nat Struct Mol Biol, 2011. **18**(5): p. 564-570.
213. Hamuro, Y., et al., *Hydrogen/deuterium-exchange (H/D-Ex) of PPARγ LBD in the presence of various modulators.* Protein Science, 2006. **15**(8): p. 1883-1892.
214. Gampe Jr, R.T., et al., *Asymmetry in the PPARγ/RXRα Crystal Structure Reveals the Molecular Basis of Heterodimerization among Nuclear Receptors.* Molecular Cell, 2000. **5**(3): p. 545-555.
215. Sanchez-Martinez, R., et al., *The retinoid X receptor ligand restores defective signalling by the vitamin D receptor.* EMBO Rep, 2006. **7**(10): p. 1030-1034.
216. Wright, E., J. Vincent, and E.J. Fernandez, *Thermodynamic Characterization of the Interaction between CAR−RXR and SRC-1 Peptide by Isothermal Titration Calorimetry†.* Biochemistry, 2006. **46**(3): p. 862-870.
217. Pogenberg, V., et al., *Characterization of the Interaction between Retinoic Acid Receptor/Retinoid X Receptor (RAR/RXR) Heterodimers and Transcriptional Coactivators through Structural and Fluorescence Anisotropy Studies.* Journal of Biological Chemistry, 2005. **280**(2): p. 1625-1633.
218. Rachez, C. and L.P. Freedman, *Mechanisms of gene regulation by vitamin D3 receptor: a network of coactivator interactions.* Gene, 2000. **246**(1-2): p. 9-21.
219. Heery, D.M., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**(6634): p. 733-736.
220. Nolte, R.T., et al., *Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-[gamma].* Nature, 1998. **395**(6698): p. 137-143.
221. Onate S, T.S., Tsai M, O'Malley B, *Sequence and characterization of a coactivator for the steroid hormone receptor superfamily.* Science, 1995. **24**(270): p. 3.
222. Hong, H., et al., *GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors.* Proceedings of the National Academy of Sciences, 1996. **93**(10): p. 4948-4952.
223. Voegel J, H.M., Zechel C, Chambon P, Gronemeyer H, *TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors.* EMBO, 1996. **15**(14): p. 8.
224. Anzick, S.L., et al., *AIB1, a Steroid Receptor Coactivator Amplified in Breast and Ovarian Cancer.* Science, 1997. **277**(5328): p. 965-968.
225. Chen, H., et al., *Nuclear Receptor Coactivator ACTR Is a Novel Histone Acetyltransferase and Forms a Multimeric Activation Complex with P/CAF and CBP/p300.* Cell, 1997. **90**(3): p. 569-580.
226. Li, H., P.J. Gomes, and J.D. Chen, *RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2.* Proceedings of the National Academy of Sciences, 1997. **94**(16): p. 8479-8484.

227. Takeshita, A., et al., *TRAM-1, A Novel 160-kDa Thyroid Hormone Receptor Activator Molecule, Exhibits Distinct Properties from Steroid Receptor Coactivator-1.* Journal of Biological Chemistry, 1997. **272**(44): p. 27629-27634.
228. Torchia, J., et al., *The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function.* Nature, 1997. **387**(6634): p. 677-684.
229. Yamaoka, K., et al., *Multiple co-activator complexes support ligand-induced transactivation function of VDR.* Archives of Biochemistry and Biophysics, 2007. **460**(2): p. 166-171.
230. MacDonald, P.N., et al., *Vitamin D receptor and nuclear receptor coactivators: crucial interactions in vitamin D-mediated transcription.* Steroids, 2001. **66**(3-5): p. 171-176.
231. Kamei, Y., et al., *A CBP Integrator Complex Mediates Transcriptional Activation and AP-1 Inhibition by Nuclear Receptors.* Cell, 1996. **85**(3): p. 403-414.
232. Yao, T.P., et al., *The nuclear hormone receptor coactivator SRC-1 is a specific target of p300.* Proceedings of the National Academy of Sciences, 1996. **93**(20): p. 10626-10631.
233. Takeshita, A., et al., *Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator.* Endocrinology, 1996. **137**(8): p. 3594-7.
234. Kitagawa, H., et al., *The Chromatin-Remodeling Complex WINAC Targets a Nuclear Receptor to Promoters and Is Impaired in Williams Syndrome.* Cell, 2003. **113**(7): p. 905-917.
235. Rachez, C., et al., *A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system.* Genes & Development, 1998. **12**(12): p. 1787-1800.
236. Bikle, D.D., et al., *Differential regulation of epidermal function by VDR coactivators.* The Journal of Steroid Biochemistry and Molecular Biology, 2010. **121**(1-2): p. 308-313.
237. Schauber, J., et al., *Histone Acetylation in Keratinocytes Enables Control of the Expression of Cathelicidin and CD14 by 1,25-Dihydroxyvitamin D3.* J Invest Dermatol, 2007. **128**(4): p. 816-824.
238. Oda, Y., et al., *Differential role of two VDR coactivators, DRIP205 and SRC-3, in keratinocyte proliferation and differentiation.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 776-780.
239. Oda, Y., et al., *Two Distinct Coactivators, DRIP/Mediator and SRC/p160, Are Differentially Involved in Vitamin D Receptor Transactivation during Keratinocyte Differentiation.* Molecular Endocrinology, 2003. **17**(11): p. 2329-2339.
240. Yanagisawa, J., et al., *Convergence of Transforming Growth Factor-β and Vitamin D Signaling Pathways on SMAD Transcriptional Coactivators.* Science, 1999. **283**(5406): p. 1317-1321.
241. Baudino, T.A., et al., *Isolation and Characterization of a Novel Coactivator Protein, NCoA-62, Involved in Vitamin D-mediated Transcription.* Journal of Biological Chemistry, 1998. **273**(26): p. 16434-16441.
242. Kim, S., N.K. Shevde, and J.W. Pike, *1,25-Dihydroxyvitamin D3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts.* Journal of Bone and Mineral Research, 2005. **20**(2): p. 305-317.
243. Horlein, A.J., et al., *Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor.* Nature, 1995. **377**(6548): p. 397-404.
244. Chen, J.D. and R.M. Evans, *A transcriptional co-repressor that interacts with nuclear hormone receptors.* Nature, 1995. **377**(6548): p. 454-457.
245. Tagami, T., et al., *The Interaction of the Vitamin D Receptor with Nuclear Receptor Corepressors and Coactivators.* Biochemical and Biophysical Research Communications, 1998. **253**(2): p. 358-363.
246. Dwivedi, P., et al., *Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13delta1.* Journal of Molecular Endocrinology, 1998. **20**(3): p. 327-335.
247. Ordentlich, P., et al., *Unique forms of human and mouse nuclear receptor corepressor SMRT.* Proceedings of the National Academy of Sciences, 1999. **96**(6): p. 2639-2644.
248. Seol, W., et al., *Two receptor interacting domains in the nuclear hormone receptor corepressor RIP13/N-CoR.* Molecular Endocrinology, 1996. **10**(12): p. 1646-55.
249. Zamir, I., et al., *Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex.* Proceedings of the National Academy of Sciences, 1997. **94**(26): p. 14400-14405.
250. Dressel, U., et al., *Alien, a Highly Conserved Protein with Characteristics of a Corepressor for Members of the Nuclear Hormone Receptor Superfamily.* Mol. Cell. Biol., 1999. **19**(5): p. 3383-3394.
251. POLLY, P., et al., *VDR-Alien: a novel, DNA-selective vitamin D3 receptor-corepressor partnership.* The FASEB Journal, 2000. **14**(10): p. 1455-1463.
252. Li, Q., et al., *Modification of Chromatin Structure by the Thyroid Hormone Receptor.* Trends in Endocrinology & Metabolism, 1999. **10**(4): p. 157-164.
253. Guido, J., *Coactivators and corepressors as mediators of nuclear receptor function: An update.* Molecular and Cellular Endocrinology, 1998. **143**(1-2): p. 1-7.
254. Kim, J.Y., Y.L. Son, and Y.C. Lee, *A role of helix 12 of the vitamin D receptor in SMRT corepressor interaction.* Biochemical and Biophysical Research Communications, 2009. **379**(3): p. 780-784.

255. Perissi, V., et al., *Molecular determinants of nuclear receptor–corepressor interaction.* Genes & Development, 1999. **13**(24): p. 3198-3208.
256. Nagy, L., et al., *Mechanism of corepressor binding and release from nuclear hormone receptors.* Genes & Development, 1999. **13**(24): p. 3209-3216.
257. Hu, X. and M.A. Lazar, *The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors.* Nature, 1999. **402**(6757): p. 93-96.
258. Robyr, D., A.P. Wolffe, and W. Wahli, *Nuclear Hormone Receptor Coregulators In Action: Diversity For Shared Tasks.* Molecular Endocrinology, 2000. **14**(3): p. 329-347.
259. Xie, Z., et al., *Hairless Suppresses Vitamin D Receptor Transactivation in Human Keratinocytes.* Endocrinology, 2006. **147**(1): p. 314-323.
260. Hsieh, J.-C., et al., *Physical and Functional Interaction between the Vitamin D Receptor and Hairless Corepressor, Two Proteins Required for Hair Cycling.* Journal of Biological Chemistry, 2003. **278**(40): p. 38665-38674.
261. Alroy, I., T. Towers, and L. Freedman, *Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor.* Mol. Cell. Biol., 1995. **15**(10): p. 5789-5799.
262. Towers, T.L., T.P. Staeva, and L.P. Freedman, *A Two-Hit Mechanism for Vitamin D3-Mediated Transcriptional Repression of the Granulocyte-Macrophage Colony-Stimulating Factor Gene: Vitamin D Receptor Competes for DNA Binding with NFAT1 and Stabilizes c-Jun.* Mol. Cell. Biol., 1999. **19**(6): p. 4191-4199.
263. Wang L, N.G., Henneman ZJ, Klein E, Weiner S, *Nanosized particles in bone and dissolution insensitivity of bone mineral.* Biointerphases, 2006. **1**(3): p. 5.
264. Robertson WG, M.R., *Calcium measurements in serum and plasma--total and ionized.* CRC Crit Rev Clin Lab Sci, 1979. **11**(3): p. 33.
265. R, N., *Studies upon the mode of action of vitamin D: The absorption of calcium chloride, xylose and sodium sulphate from isolated loops of the small intestine and of calcium chloride from the abdominal cavity in the rat.* Biochem J, 1937. **31**(2): p. 5.
266. Gershoff, S.N. and D.M. Hegsted, *Effect of Vitamin D and Ca:P Ratios on Chick Gastrointestinal Tract.* American Journal of Physiology -- Legacy Content, 1956. **187**(2): p. 203-206.
267. Pansu, D., C. Bellaton, and F. Bronner, *Effect of Ca intake on saturable and nonsaturable components of duodenal Ca transport.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1981. **240**(1): p. G32-G37.
268. Sheikh MS, S.L., Fordtran JS, *In vivo intestinal absorption of calcium in humans.* Miner Electrolyte Metab, 1990. **16**(2-3): p. 16.
269. Grinstead, W.C., C.Y. Pak, and G.J. Krejs, *Effect of 1,25-dihydroxyvitamin D3 on calcium absorption in the colon of healthy humans.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1984. **247**(2): p. G189-G192.
270. Pansu, D., et al., *Duodenal and ileal calcium absorption in the rat and effects of vitamin D.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1983. **244**(6): p. G695-G700.
271. Chandra, S., et al., *Ion microscopic imaging of calcium transport in the intestinal tissue of vitamin D-deficient and vitamin D-replete chickens: a 44Ca stable isotope study.* Proceedings of the National Academy of Sciences, 1990. **87**(15): p. 5715-5719.
272. Giuliano, A.R. and R.J. Wood, *Vitamin D-regulated calcium transport in Caco-2 cells: unique in vitro model.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1991. **260**(2): p. G207-G212.
273. Fullmer, C., et al., *Ion microscopic imaging of calcium during 1,25-dihydroxyvitamin D-mediated intestinal absorption.* Histochemistry and Cell Biology, 1996. **106**(2): p. 215-222.
274. Van Cromphaut, S.J., et al., *Duodenal calcium absorption in vitamin D receptor–knockout mice: Functional and molecular aspects.* Proceedings of the National Academy of Sciences, 2001. **98**(23): p. 13324-13329.
275. Song, Y., S. Kato, and J.C. Fleet, *Vitamin D Receptor (VDR) Knockout Mice Reveal VDR-Independent Regulation of Intestinal Calcium Absorption and ECaC2 and Calbindin D9k mRNA.* The Journal of Nutrition, 2003. **133**(2): p. 374-380.
276. Xue, Y. and J.C. Fleet, *Intestinal Vitamin D Receptor Is Required for Normal Calcium and Bone Metabolism in Mice.* Gastroenterology, 2009. **136**(4): p. 1317-1327.e2.
277. Shao, A., R.J. Wood, and J.C. Fleet, *Increased Vitamin D Receptor Level Enhances 1,25-Dihydroxyvitamin D3-Mediated Gene Expression and Calcium Transport in Caco-2 Cells.* Journal of Bone and Mineral Research, 2001. **16**(4): p. 615-624.
278. Bronner, F.B.M., *The molecular nature of 1,25-(OH)2-D3-induced calcium-binding protein biosynthesis in the rat.* Adv Exp Med Biol, 1982. **151**: p. 5.
279. Cui, M., et al., *Effects of MAPK signaling on 1,25-dihydroxyvitamin D-mediated CYP24 gene expression in the enterocyte-like cell line, Caco-2.* Journal of Cellular Physiology, 2009. **219**(1): p. 132-142.
280. Fleet, J.C., et al., *Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2002. **283**(3): p. G618-G625.

281. Meyer, M.B., et al., *Characterizing Early Events Associated with the Activation of Target Genes by 1,25-Dihydroxyvitamin D3 in Mouse Kidney and Intestine in Vivo.* Journal of Biological Chemistry, 2007. **282**(31): p. 22344-22352.
282. Song, Y., et al., *Calcium Transporter 1 and Epithelial Calcium Channel Messenger Ribonucleic Acid Are Differentially Regulated by 1,25 Dihydroxyvitamin D3 in the Intestine and Kidney of Mice.* Endocrinology, 2003. **144**(9): p. 3885-3894.
283. Walters, J.R.F., et al., *Calcium Channel TRPV6 Expression in Human Duodenum: Different Relationships to the Vitamin D System and Aging in Men and Women.* Journal of Bone and Mineral Research, 2006. **21**(11): p. 1770-1777.
284. Wasserman, R.H.T.A., *Vitamin d3-induced calcium-binding protein in chick intestinal mucosa.* science, 1966. **152**: p. 2.
285. Feher, J.J., C.S. Fullmer, and R.H. Wasserman, *Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption.* American Journal of Physiology - Cell Physiology, 1992. **262**(2): p. C517-C526.
286. Favus, M.J., et al., *Effects of trifluoperazine, ouabain, and ethacrynic acid on intestinal calcium transport.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 1983. **244**(2): p. G111-G115.
287. Cai, Q., et al., *Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression.* Proceedings of the National Academy of Sciences, 1993. **90**(4): p. 1345-1349.
288. Wasserman RH, S.C., Brindak ME, De Talamoni N, Fullmer CS, Penniston JT, Kumar R, *Vitamin D and mineral deficiencies increase the plasma membrane calcium pump of chicken intestine.* Gastroenterology, 1992. **102**(3): p. 8.
289. Warner, R.R. and J.R. Coleman, *Electron probe analysis of calcium transport by small intestine.* The Journal of Cell Biology, 1975. **64**(1): p. 54-74.
290. Nemere, I., V. Leathers, and A.W. Norman, *1,25-Dihydroxyvitamin D3-mediated intestinal calcium transport. Biochemical identification of lysosomes containing calcium and calcium-binding protein (calbindin-D28K).* Journal of Biological Chemistry, 1986. **261**(34): p. 16106-16114.
291. NEMERE, I. and A.W. NORMAN, *1,25-Dihydroxyvitamin D3-Mediated Vesicular Transport of Calcium in Intestine: Time-Course Studies.* Endocrinology, 1988. **122**(6): p. 2962-2969.
292. NEMERE, I., Y. YOSHIMOTO, and A.W. NORMAN, *Calcium Transport in Perfused Duodena from Normal Chicks: Enhancement within Fourteen Minutes of Exposure to 1,25-Dihydroxyvitamin D3.* Endocrinology, 1984. **115**(4): p. 1476-1483.
293. Nemere, I., et al., *Intestinal Cell Calcium Uptake and the Targeted Knockout of the 1,25D3-MARRS (Membrane-associated, Rapid Response Steroid-binding) Receptor/PDIA3/Erp57.* Journal of Biological Chemistry, 2010. **285**(41): p. 31859-31866.
294. Karbach, U., *Paracellular Calcium Transport across the Small Intestine.* The Journal of Nutrition, 1992. **122**(3 Suppl): p. 672-677.
295. Tudpor K, T.J., Jantarajit W, Krishnamra N, Charoenphandhu N, *1,25-dihydroxyvitamin D(3) rapidly stimulates the solvent drag-induced paracellular calcium transport in the duodenum of female rats.* J Physiol Sci, 2008. **58**(5): p. 10.
296. Fujita, H., et al., *Tight Junction Proteins Claudin-2 and -12 Are Critical for Vitamin D-dependent Ca2+ Absorption between Enterocytes.* Molecular Biology of the Cell, 2008. **19**(5): p. 1912-1921.
297. Bindels, R.J., et al., *Active Ca2+ transport in primary cultures of rabbit kidney CCD: stimulation by 1,25-dihydroxyvitamin D3 and PTH.* American Journal of Physiology - Renal Physiology, 1991. **261**(5): p. F799-F807.
298. Dardenne, O., et al., *Targeted Inactivation of the 25-Hydroxyvitamin D3-1α-Hydroxylase Gene (CYP27B1) Creates an Animal Model of Pseudovitamin D-Deficiency Rickets.* Endocrinology, 2001. **142**(7): p. 3135-3141.
299. Hoenderop, J.G.J., B. Nilius, and R.J.M. Bindels, *Calcium Absorption Across Epithelia.* Physiological Reviews, 2005. **85**(1): p. 373-422.
300. Hoenderop, J.G.J., et al., *Renal Ca2+ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5.* The Journal of Clinical Investigation, 2003. **112**(12): p. 1906-1914.
301. David R, N., *Cytochrome P450 and the Individuality of Species.* Archives of Biochemistry and Biophysics, 1999. **369**(1): p. 1-10.
302. Tanaka, Y. and H.F. Deluca, *STIMULATION OF 24,25-DIHYDROXYVITAMIN-D3 PRODUCTION BY 1,25-DIHYDROXYVITAMIN-D3.* science, 1974. **183**(4130): p. 1198-1200.
303. Ikeda, K., et al., *Associated autoimmune diseases following complete remission of myasthenia gravis.* Acta Neurologica Scandinavica, 1995. **92**(6): p. 503-504.
304. Yoshizawa, T., et al., *Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning.* Nature Genetics, 1997. **16**(4): p. 391-396.
305. Kawashima, H., S. Torikai, and K. Kurokawa, *CALCITONIN SELECTIVELY STIMULATES 25-HYDROXYVITAMIN D3-1ALPHA-HYDROXYLASE IN PROXIMAL STRAIGHT TUBULE OF RAT-KIDNEY.* Nature, 1981. **291**(5813): p. 327-329.
306. Beckman, M.J., et al., *THE ROLE OF DIETARY CALCIUM IN THE PHYSIOLOGY OF VITAMIN-D TOXICITY - EXCESS DIETARY VITAMIN-D-3 BLUNTS PARATHYROID-HORMONE INDUCTION OF KIDNEY 1-HYDROXYLASE.* Archives of Biochemistry and Biophysics, 1995. **319**(2): p. 535-539.

307. Murayama, A., et al., *Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching.* Embo Journal, 2004. **23**(7): p. 1598-1608.
308. Vierra, C.A. and C. Nelson, *THE PAN BASIC HELIX-LOOP-HELIX PROTEINS ARE REQUIRED FOR INSULIN GENE-EXPRESSION.* Molecular Endocrinology, 1995. **9**(1): p. 64-71.
309. Kim, M.S., et al., *DNA demethylation in hormone-induced transcriptional derepression.* Nature, 2009. **461**(7266): p. 1007-1012.
310. HENRY, H.L., *Parathyroid Hormone Modulation of 25-Hydroxyvitamin D3 Metabolism by Cultured Chick Kidney Cells Is Mimicked and Enhanced by Forskolin.* Endocrinology, 1985. **116**(2): p. 503-510.
311. Rost, C.R., D.D. Bikle, and R.A. Kaplan, *INVITRO STIMULATION OF 25-HYDROXYCHOLECALCIFEROL 1 ALPHA-HYDROXYLATION BY PARATHYROID-HORMONE IN CHICK KIDNEY SLICES - EVIDENCE FOR A ROLE FOR ADENOSINE-3',5'-MONOPHOSPHATE.* Endocrinology, 1981. **108**(3): p. 1002-1006.
312. Norman, A.W., et al., *Update on biological actions of 1α,25(OH)2-vitamin D3 (rapid effects) and 24R,25(OH)2-vitamin D3.* Molecular and Cellular Endocrinology, 2002. **197**(1-2): p. 1-13.
313. Lienhardt, A., et al., *Activating Mutations of the Calcium-Sensing Receptor: Management of Hypocalcemia.* Journal of Clinical Endocrinology & Metabolism, 2001. **86**(11): p. 5313-5323.
314. Uçkun-Kitapçi, A., et al., *A novel mutation (E767K) in the second extracellular loop of the calcium sensing receptor in a family with autosomal dominant hypocalcemia.* American Journal of Medical Genetics Part A, 2005. **132A**(2): p. 125-129.
315. BOUILLON, R., W.H. OKAMURA, and A.W. NORMAN, *Structure-Function Relationships in the Vitamin D Endocrine System.* Endocrine Reviews, 1995. **16**(2): p. 200-257.
316. Russell, J., J. Silver, and L.M. Sherwood, *The effects of calcium and vitamin D metabolites on cytoplasmic mRNA coding for pre-proparathyroid hormone in isolated parathyroid cells.* Transactions of the Association of American Physicians, 1984. **97**: p. 296-303.
317. Chan, Y.-L., et al., *The effect of 1,25 dihydroxycholecalciferol on parathyroid hormone secretion by monolayer cultures of bovine parathyroid cells.* Calcified Tissue International, 1986. **38**(1): p. 27-32.
318. McKenna, M.J. and R. Freaney, *Secondary Hyperparathyroidism in the Elderly: Means to Defining Hypovitaminosis D.* Osteoporosis International, 1998. **8**(0): p. S3-S6.
319. KREMER, R., et al., *Influence of Calcium and 1,25-Dihydroxycholecalciferol on Proliferation and Proto-Oncogene Expression in Primary Cultures of Bovine Parathyroid Cells.* Endocrinology, 1989. **125**(2): p. 935-941.
320. Sudhaker, R., et al., *Reduced vitamin D receptor expression in parathyroid adenomas: implications for pathogenesis.* Clinical Endocrinology, 2000. **53**(3): p. 373-381.
321. Panda, D.K., et al., *Inactivation of the 25-Hydroxyvitamin D 1α-Hydroxylase and Vitamin D Receptor Demonstrates Independent and Interdependent Effects of Calcium and Vitamin D on Skeletal and Mineral Homeostasis.* Journal of Biological Chemistry, 2004. **279**(16): p. 16754-16766.
322. Lee, M. and N.C. Partridge, *Parathyroid hormone signaling in bone and kidney.* Current Opinion in Nephrology and Hypertension, 2009. **18**(4): p. 298-302 10.1097/MNH.0b013e32832c2264.
323. Xue, Y., et al., *Exogenous 1,25-Dihydroxyvitamin D3 Exerts a Skeletal Anabolic Effect and Improves Mineral Ion Homeostasis in Mice that Are Homozygous for Both the 1α-Hydroxylase and Parathyroid Hormone Null Alleles.* Endocrinology, 2006. **147**(10): p. 4801-4810.
324. Wilz, D., et al., *Plasma 1,25-(OH)2-vitamin D concentrations and net intestinal calcium, phosphate, and magnesium absorption in humans.* The American Journal of Clinical Nutrition, 1979. **32**(10): p. 2052-2060.
325. Murer, H., I. Forster, and J. Biber, *The sodium phosphate cotransporter family SLC34.* Pflugers Archiv-European Journal of Physiology, 2004. **447**(5): p. 763-767.
326. Forster, I.C., et al., *Proximal tubular handling of phosphate: A molecular perspective.* Kidney International, 2006. **70**(9): p. 1548-1559.
327. Yamashita, T., M. Yoshioka, and N. Itoh, *Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain.* Biochemical and Biophysical Research Communications, 2000. **277**(2): p. 494-498.
328. Burnett, S.A.M., et al., *Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women.* Journal of Bone and Mineral Research, 2006. **21**(8): p. 1187-1196.
329. Gattineni, J., et al., *FGF23 decreases renal NaPi-2a and NaPi-2c expression and induces hypophosphatemia in vivo predominantly via FGF receptor 1.* American Journal of Physiology-Renal Physiology, 2009. **297**(2): p. F282-F291.
330. Strom, T.M. and H. Juppner, *PHEX, FGF23, DMP1 and beyond.* Current Opinion in Nephrology and Hypertension, 2008. **17**(4): p. 357-362.
331. Bai, X.Y., et al., *The autosomal dominant hypophosphatemic rickets R176Q mutation in fibroblast growth factor 23 resists proteolytic cleavage and enhances in vivo biological potency.* Journal of Biological Chemistry, 2003. **278**(11): p. 9843-9849.

332. Katai, K., et al., *Regulation of intestinal Na+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D-3.* Biochemical Journal, 1999. **343**: p. 705-712.
333. Kolek, O.I., et al., *1 alpha,25-Dihydroxyvitamin D-3 upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2005. **289**(6): p. G1036-G1042.
334. Nishi, H., et al., *Intravenous calcitriol therapy increases serum concentrations of fibroblast growth factor-23 in dialysis patients with secondary hyperparathyroidism.* Nephron Clinical Practice, 2005. **101**(2): p. C94-C99.
335. Shah, S., et al., *The Molecular Basis of Vitamin D Receptor and β-Catenin Crossregulation.* Molecular Cell, 2006. **21**(6): p. 799-809.
336. Álvarez-Díaz, S., et al., *Vitamin D: Proteases, protease inhibitors and cancer.* Cell Cycle, 2010. **9**(1): p. 32-37.
337. Matthews, D., et al., *Genomic vitamin D signaling in breast cancer: Insights from animal models and human cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2010. **121**(1-2): p. 362-367.
338. Byrne, B. and J. Welsh, *Identification of novel mediators of Vitamin D signaling and 1,25(OH)2D3 resistance in mammary cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2007. **103**(3-5): p. 703-707.
339. Swami, S., et al., *Vitamin D Growth Inhibition of Breast Cancer Cells: Gene Expression Patterns Assessed by cDNA Microarray.* Breast Cancer Research and Treatment, 2003. **80**(1): p. 49-62.
340. Lee, H.J., et al., *Gene expression profiling changes induced by a novel Gemini Vitamin D derivative during the progression of breast cancer.* Biochemical Pharmacology, 2006. **72**(3): p. 332-343.
341. Lazzaro, G., et al., *Induction of differentiation by 1α-hydroxyvitamin D5 in T47D human breast cancer cells and its interaction with vitamin D receptors.* European Journal of Cancer, 2000. **36**(6): p. 780-786.
342. Pendás-Franco, N., et al., *Vitamin D regulates the phenotype of human breast cancer cells.* Differentiation, 2007. **75**(3): p. 193-207.
343. Weigelt, J., et al., *Solution Structure of the DNA Binding Domain of the Human Forkhead Transcription Factor AFX (FOXO4)†.* Biochemistry, 2001. **40**(20): p. 5861-5869.
344. Arden, K.C., *FOXO animal models reveal a variety of diverse roles for FOXO transcription factors.* Oncogene, 0000. **27**(16): p. 2345-2350.
345. Tothova, Z., et al., *FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress.* Cell, 2007. **128**(2): p. 325-339.
346. Shackelford, D.B. and R.J. Shaw, *The LKB1-AMPK pathway: metabolism and growth control in tumour suppression.* Nat Rev Cancer, 2009. **9**(8): p. 563-575.
347. Maswood, N., et al., *Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease.* Proceedings of the National Academy of Sciences, 2004. **101**(52): p. 18171-18176.
348. Jacobs, F.M.J., et al., *FoxO6, a Novel Member of the FoxO Class of Transcription Factors with Distinct Shuttling Dynamics.* Journal of Biological Chemistry, 2003. **278**(38): p. 35959-35967.
349. Hu, M.C.T., et al., *IκB Kinase Promotes Tumorigenesis through Inhibition of Forkhead FOXO3a.* Cell, 2004. **117**(2): p. 225-237.
350. Huang, H., et al., *Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(5): p. 1649-1654.
351. Yang, J.-Y., et al., *ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation.* Nat Cell Biol, 2008. **10**(2): p. 138-148.
352. Jagani, Z., A. Singh, and R. Khosravi-Far, *FoxO tumor suppressors and BCR–ABL-induced leukemia: A matter of evasion of apoptosis.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2008. **1785**(1): p. 63-84.
353. Trotman, L.C., et al., *Identification of a tumour suppressor network opposing nuclear Akt function.* Nature, 2006. **441**(7092): p. 523-527.
354. Kikuno, N., et al., *Knockdown of astrocyte-elevated gene-1 inhibits prostate cancer progression through upregulation of FOXO3a activity.* Oncogene, 2007. **26**(55): p. 7647-7655.
355. Cornforth, A.N., et al., *FOXO3a mediates the androgen-dependent regulation of FLIP and contributes to TRAIL-induced apoptosis of LNCaP cells.* Oncogene, 2008. **27**(32): p. 4422-4433.
356. Seoane, J., et al., *Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation.* Cell, 2004. **117**(2): p. 211-223.
357. Paik, J.-H., et al., *FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis.* Cell, 2007. **128**(2): p. 309-323.
358. Collado, M., et al., *Inhibition of the Phosphoinositide 3-Kinase Pathway Induces a Senescence-like Arrest Mediated by p27Kip1.* Journal of Biological Chemistry, 2000. **275**(29): p. 21960-21968.
359. Kops, G.J.P.L., et al., *Control of Cell Cycle Exit and Entry by Protein Kinase B-Regulated Forkhead Transcription Factors.* Molecular and Cellular Biology, 2002. **22**(7): p. 2025-2036.

360. Medema, R.H., et al., *AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1.* Nature, 2000. **404**(6779): p. 782-787.
361. Nakamura, N., et al., *Forkhead Transcription Factors Are Critical Effectors of Cell Death and Cell Cycle Arrest Downstream of PTEN.* Molecular and Cellular Biology, 2000. **20**(23): p. 8969-8982.
362. Myatt, S.S. and E.W.F. Lam, *The emerging roles of forkhead box (Fox) proteins in cancer.* Nat Rev Cancer, 2007. **7**(11): p. 847-859.
363. Ho, K.K., S.S. Myatt, and E.W.F. Lam, *Many forks in the path: cycling with FoxO.* Oncogene, 0000. **27**(16): p. 2300-2311.
364. Gajiwala, K.S. and S.K. Burley, *Winged helix proteins.* Current Opinion in Structural Biology, 2000. **10**(1): p. 110-116.
365. Weigel, D. and H. Jäckle, *The fork head domain: A novel DNA binding motif of eukaryotic transcription factors?* Cell, 1990. **63**(3): p. 455-456.
366. Kaestner, K.H., W. Knochel, and D.E. Martinez, *Unified nomenclature for the winged helix/forkhead transcription factors.* Genes & Development, 2000. **14**(2): p. 142-146.
367. Mazet, F., et al., *Phylogenetic relationships of the Fox (Forkhead) gene family in the Bilateria.* Gene, 2003. **316**(0): p. 79-89.
368. Overdier, D.G., A. Porcella, and R.H. Costa, *THE DNA-BINDING SPECIFICITY OF THE HEPATOCYTE NUCLEAR FACTOR 3/FORKHEAD DOMAIN IS INFLUENCED BY AMINO-ACID-RESIDUES ADJACENT TO THE RECOGNITION HELIX.* Molecular and Cellular Biology, 1994. **14**(4): p. 2755-2766.
369. Shiyanova, T. and X. Liao, *The Dissociation Rate of a Winged Helix Protein–DNA Complex Is Influenced by Non-DNA Contact Residues.* Archives of Biochemistry and Biophysics, 1999. **362**(2): p. 356-362.
370. van Dongen, M.J.P., et al., *Solution structure and dynamics of the DNA-binding domain of the adipocyte-transcription factor FREAC-11.* Journal of Molecular Biology, 2000. **296**(2): p. 351-359.
371. Furuyama, T., et al., *Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues.* Biochem. J., 2000. **349**(2): p. 629-634.
372. Biggs, W.H., et al., *Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1.* Proceedings of the National Academy of Sciences, 1999. **96**(13): p. 7421-7426.
373. Guo, S., et al., *Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence.* Journal of Biological Chemistry, 1999. **274**(24): p. 17184-17192.
374. Kops, G.J.P.L., et al., *Direct control of the Forkhead transcription factor AFX by protein kinase B.* Nature, 1999. **398**(6728): p. 630-634.
375. Nakae, J., B.-C. Park, and D. Accili, *Insulin Stimulates Phosphorylation of the Forkhead Transcription Factor FKHR on Serine 253 through a Wortmannin-sensitive Pathway.* Journal of Biological Chemistry, 1999. **274**(23): p. 15982-15985.
376. Rena, G., et al., *Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B.* Journal of Biological Chemistry, 1999. **274**(24): p. 17179-17183.
377. Tang, E.D., et al., *Negative Regulation of the Forkhead Transcription Factor FKHR by Akt.* Journal of Biological Chemistry, 1999. **274**(24): p. 16741-16746.
378. O'Brien, R.M. and D.K. Granner, *Regulation of gene expression by insulin.* Physiological Reviews, 1996. **76**(4): p. 1109-1161.
379. Streeper, R.S., et al., *A Multicomponent Insulin Response Sequence Mediates a Strong Repression of Mouse Glucose-6-phosphatase Gene Transcription by Insulin.* Journal of Biological Chemistry, 1997. **272**(18): p. 11698-11701.
380. Tsai, K.-L., et al., *Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification.* Nucleic Acids Research, 2007. **35**(20): p. 6984-6994.
381. Boura, E., et al., *Both the N-terminal Loop and Wing W2 of the Forkhead Domain of Transcription Factor Foxo4 Are Important for DNA Binding.* Journal of Biological Chemistry, 2007. **282**(11): p. 8265-8275.
382. Brunet, A., et al., *Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor.* Cell, 1999. **96**(6): p. 857-868.
383. Takaishi, H., et al., *Regulation of nuclear translocation of Forkhead transcription factor AFX by protein kinase B.* Proceedings of the National Academy of Sciences, 1999. **96**(21): p. 11836-11841.
384. Brownawell, A.M., et al., *Inhibition of Nuclear Import by Protein Kinase B (Akt) Regulates the Subcellular Distribution and Activity of the Forkhead Transcription Factor AFX.* Molecular and Cellular Biology, 2001. **21**(10): p. 3534-3546.
385. Jin, C. and X. Liao, *Backbone Dynamics of a Winged Helix Protein and its DNA Complex at Different Temperatures: Changes of Internal Motions in Genesis upon Binding to DNA.* Journal of Molecular Biology, 1999. **292**(3): p. 641-651.
386. Stroud, J.C., et al., *Structure of the Forkhead Domain of FOXP2 Bound to DNA.* Structure, 2006. **14**(1): p. 159-166.
387. Tsai, K.-L., et al., *Crystal Structure of the Human FOXK1a-DNA Complex and Its Implications on the Diverse Binding Specificity of Winged Helix/Forkhead Proteins.* Journal of Biological Chemistry, 2006. **281**(25): p. 17400-17409.
388. Zhang, X., et al., *Phosphorylation of Serine 256 Suppresses Transactivation by FKHR (FOXO1) by Multiple Mechanisms.* Journal of Biological Chemistry, 2002. **277**(47): p. 45276-45284.

389. van der Heide, L.P., et al., *FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling.* Biochem. J., 2005. **391**(3): p. 623-629.
390. Rena, G., et al., *Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion.* EMBO J, 2002. **21**(9): p. 2263-2271.
391. Woods, Y.L., et al., *The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser(329) in vitro, a novel in vivo phosphorylation site.* Biochemical Journal, 2001. **355**: p. 597-607.
392. Alessi, D.R., et al., *Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase.* FEBS Letters, 1996. **399**(3): p. 333-338.
393. Yaffe, M.B., et al., *The Structural Basis for 14-3-3:Phosphopeptide Binding Specificity.* Cell, 1997. **91**(7): p. 961-971.
394. Brunet, A., et al., *14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport.* The Journal of Cell Biology, 2002. **156**(5): p. 817-828.
395. Rittinger, K., et al., *Structural Analysis of 14-3-3 Phosphopeptide Complexes Identifies a Dual Role for the Nuclear Export Signal of 14-3-3 in Ligand Binding.* Molecular Cell, 1999. **4**(2): p. 153-166.
396. Cahill, C.M., et al., *Phosphatidylinositol 3-Kinase Signaling Inhibits DAF-16 DNA Binding and Function via 14-3-3-dependent and 14-3-3-independent Pathways.* Journal of Biological Chemistry, 2001. **276**(16): p. 13402-13410.
397. Obsil, T., et al., *Two 14-3-3 Binding Motifs Are Required for Stable Association of Forkhead Transcription Factor FOXO4 with 14-3-3 Proteins and Inhibition of DNA Binding.* Biochemistry, 2003. **42**(51): p. 15264-15272.
398. Rena, G., et al., *Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting.* Biochem. J., 2001. **354**(3): p. 605-612.
399. Michael B, Y., *How do 14-3-3 proteins work? – Gatekeeper phosphorylation and the molecular anvil hypothesis.* FEBS Letters, 2002. **513**(1): p. 53-57.
400. Obsil, T., et al., *Crystal Structure of the 14-3-3ζ:Serotonin N-Acetyltransferase Complex: A Role for Scaffolding in Enzyme Regulation.* Cell, 2001. **105**(2): p. 257-267.
401. Dingwall, C. and R.A. Laskey, *Nuclear targeting sequences — a consensus?* Trends in Biochemical Sciences, 1991. **16**(0): p. 478-481.
402. Bois, P.R.J., et al., *FoxO1a-Cyclic GMP-Dependent Kinase I Interactions Orchestrate Myoblast Fusion.* Molecular and Cellular Biology, 2005. **25**(17): p. 7645-7656.
403. van der Horst, A., et al., *FOXO4 Is Acetylated upon Peroxide Stress and Deacetylated by the Longevity Protein hSir2SIRT1.* Journal of Biological Chemistry, 2004. **279**(28): p. 28873-28879.
404. Dijkers, P.F., et al., *Forkhead Transcription Factor FKHR-L1 Modulates Cytokine-Dependent Transcriptional Regulation of p27KIP1.* Molecular and Cellular Biology, 2000. **20**(24): p. 9138-9148.
405. Hideshima, T., et al., *Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma.* Blood, 2001. **98**(11): p. 639A-639A.
406. Stahl, M., et al., *The Forkhead Transcription Factor FoxO Regulates Transcription of p27Kip1 and Bim in Response to IL-2.* The Journal of Immunology, 2002. **168**(10): p. 5024-5031.
407. Katayama, K., et al., *FOXO transcription factor-dependent p15INK4b and p19INK4d expression.* Oncogene, 2007. **27**(12): p. 1677-1686.
408. Delpuech, O., et al., *Induction of Mxi1-SRα by FOXO3a Contributes to Repression of Myc-Dependent Gene Expression.* Molecular and Cellular Biology, 2007. **27**(13): p. 4917-4930.
409. Schmidt, M., et al., *Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D.* Molecular and Cellular Biology, 2002. **22**(22): p. 7842-7852.
410. Ramaswamy, S., et al., *A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR.* Cancer Cell, 2002. **2**(1): p. 81-91.
411. Chen, J., et al., *FOXO Transcription Factors Cooperate with δEF1 to Activate Growth Suppressive Genes in B Lymphocytes.* The Journal of Immunology, 2006. **176**(5): p. 2711-2721.
412. Furukawa-Hibi, Y., et al., *FOXO Forkhead Transcription Factors Induce G2-M Checkpoint in Response to Oxidative Stress.* Journal of Biological Chemistry, 2002. **277**(30): p. 26729-26732.
413. Tran, H., et al., *DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein.* science, 2002. **296**(5567): p. 530-534.
414. Kops, G.J.P.L., et al., *Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress.* Nature, 2002. **419**(6904): p. 316-321.
415. Chiribau, C.B., et al., *FOXO3A regulates peroxiredoxin III expression in human cardiac fibroblasts.* Journal of Biological Chemistry, 2008. **283**(13): p. 8211-8217.
416. Nogueira, V., et al., *Akt Determines Replicative Senescence and Oxidative or Oncogenic Premature Senescence and Sensitizes Cells to Oxidative Apoptosis.* Cancer Cell, 2008. **14**(6): p. 458-470.
417. Walter, P.L., et al., *Stimulation of selenoprotein P promoter activity in hepatoma cells by FoxO1a transcription factor.* Biochemical and Biophysical Research Communications, 2008. **365**(2): p. 316-321.

418. Mei, Y., et al., *FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation.* Proceedings of the National Academy of Sciences, 2009. **106**(13): p. 5153-5158.
419. Nemoto, S. and T. Finkel, *Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway.* science, 2002. **295**(5564): p. 2450-2452.
420. de Candia, P., et al., *A Combination of Genomic Approaches Reveals the Role of <italic>FOXO1a</italic> in Regulating an Oxidative Stress Response Pathway.* PLoS ONE, 2008. **3**(2): p. e1670.
421. Essafi, A., et al., *Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells.* 2005. **24**(14): p. 2317-2329.
422. Fernández de Mattos, S., et al., *FoxO3a and BCR-ABL Regulate cyclin D2 Transcription through a STAT5/BCL6-Dependent Mechanism.* Molecular and Cellular Biology, 2004. **24**(22): p. 10058-10071.
423. You, H., et al., *FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal.* The Journal of Experimental Medicine, 2006. **203**(7): p. 1657-1663.
424. Fu, Z. and D.J. Tindall, *FOXOs, cancer and regulation of apoptosis.* Oncogene, 0000. **27**(16): p. 2312-2319.
425. Tang, T.T.-L., et al., *The Forkhead Transcription Factor AFX Activates Apoptosis by Induction of the BCL-6 Transcriptional Repressor.* Journal of Biological Chemistry, 2002. **277**(16): p. 14255-14265.
426. Modur, V., et al., *FOXO Proteins Regulate Tumor Necrosis Factor-related Apoptosis Inducing Ligand Expression.* Journal of Biological Chemistry, 2002. **277**(49): p. 47928-47937.
427. Rokudai, S., et al., *Involvement of FKHR-Dependent TRADD Expression in Chemotherapeutic Drug-Induced Apoptosis.* Molecular and Cellular Biology, 2002. **22**(24): p. 8695-8708.
428. Nakae, J., et al., *The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression.* The Journal of Clinical Investigation, 2001. **108**(9): p. 1359-1367.
429. Schmoll, D., et al., *Regulation of Glucose-6-phosphatase Gene Expression by Protein Kinase Bα and the Forkhead Transcription Factor FKHR.* Journal of Biological Chemistry, 2000. **275**(46): p. 36324-36333.
430. Stitt, T.N., et al., *The IGF-1/PI3K/Akt Pathway Prevents Expression of Muscle Atrophy-Induced Ubiquitin Ligases by Inhibiting FOXO Transcription Factors.* Molecular Cell, 2004. **14**(3): p. 395-403.
431. Mammucari, C., et al., *FoxO3 Controls Autophagy in Skeletal Muscle In Vivo.* Cell Metabolism, 2007. **6**(6): p. 458-471.
432. Zhao, J., et al., *FoxO3 Coordinately Activates Protein Degradation by the Autophagic/Lysosomal and Proteasomal Pathways in Atrophying Muscle Cells.* Cell Metabolism, 2007. **6**(6): p. 472-483.
433. Chiacchiera, F., et al., *p38[alpha] blockade inhibits colorectal cancer growth in vivo by inducing a switch from HIF1[alpha]- to FoxO-dependent transcription.* Cell Death Differ, 2009. **16**(9): p. 1203-1214.
434. Chiacchiera, F. and C. Simone, *Inhibition of p38α unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism.* Autophagy, 2009. **5**(7): p. 1030-1033.
435. Matrone, A., et al., *p38[alpha] Is Required for Ovarian Cancer Cell Metabolism and Survival.* International Journal of Gynecological Cancer, 2010. **20**(2): p. 203-211 10.1111/IGC.0b013e3181c8ca12.
436. Allen, D.L. and T.G. Unterman, *Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors.* American Journal of Physiology - Cell Physiology, 2007. **292**(1): p. C188-C199.
437. Ouyang, W., et al., *An Essential Role of the Forkhead-Box Transcription Factor Foxo1 in Control of T Cell Homeostasis and Tolerance.* Immunity, 2009. **30**(3): p. 358-371.
438. Birkenkamp, K.U., et al., *FOXO3a Induces Differentiation of Bcr-Abl-transformed Cells through Transcriptional Down-regulation of Id1.* Journal of Biological Chemistry, 2007. **282**(4): p. 2211-2220.
439. Altomare, D.A. and J.R. Testa, *Perturbations of the AKT signaling pathway in human cancer.* Oncogene, 0000. **24**(50): p. 7455-7464.
440. Lee, S.-R., et al., *Reversible Inactivation of the Tumor Suppressor PTEN by H2O2.* Journal of Biological Chemistry, 2002. **277**(23): p. 20336-20342.
441. Calleja, V., et al., *Intramolecular and Intermolecular Interactions of Protein Kinase B Define Its Activation In Vivo.* PLoS Biol, 2007. **5**(4): p. e95.
442. Toker, A. and A.C. Newton, *Akt/Protein Kinase B Is Regulated by Autophosphorylation at the Hypothetical PDK-2 Site.* Journal of Biological Chemistry, 2000. **275**(12): p. 8271-8274.
443. Brunet, A., et al., *Protein Kinase SGK Mediates Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHRL1 (FOXO3a).* Molecular and Cellular Biology, 2001. **21**(3): p. 952-965.
444. Zhao, X., et al., *Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms.* Biochem. J., 2004. **378**(3): p. 839-849.
445. Teleman, A.A., Y.-W. Chen, and S.M. Cohen, *Drosophila Melted Modulates FOXO and TOR Activity.* Developmental Cell, 2005. **9**(2): p. 271-281.
446. Kenyon, C., et al., *A C. elegans mutant that lives twice as long as wild type.* Nature, 1993. **366**(6454): p. 461-464.
447. Vowels, J.J. and J.H. Thomas, *GENETIC-ANALYSIS OF CHEMOSENSORY CONTROL OF DAUER FORMATION IN CAENORHABDITIS-ELEGANS.* Genetics, 1992. **130**(1): p. 105-123.

448. Gottlieb, S. and G. Ruvkun, *DAF-2, DAF-16 AND DAF-23 - GENETICALLY INTERACTING GENES-CONTROLLING DAUER FORMATION IN CAENORHABDITIS-ELEGANS.* Genetics, 1994. **137**(1): p. 107-120.
449. Hertweck, M., C. Gobel, and R. Baumeister, *C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span.* Developmental Cell, 2004. **6**(4): p. 577-588.
450. Ogg, S. and G. Ruvkun, *The C-elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway.* Molecular Cell, 1998. **2**(6): p. 887-893.
451. Li, J., et al., *The 14-3-3 protein FTT-2 regulates DAF-16 in Caenorhabditis elegans.* Developmental Biology, 2007. **301**(1): p. 82-91.
452. Puig, O., et al., *Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway.* Genes & Development, 2003. **17**(16): p. 2006-2020.
453. Nielsen, M.D., et al., *14-3-3ε antagonizes FoxO to control growth, apoptosis and longevity in Drosophila.* Aging Cell, 2008. **7**(5): p. 688-699.
454. Puig, O. and R. Tjian, *Transcriptional feedback control of insulin receptor by dFOXO/FOXO1.* Genes & Development, 2005. **19**(20): p. 2435-2446.
455. Giannakou, M.E., et al., *Long-lived Drosophila with overexpressed dFOXO in adult fat body.* science, 2004. **305**(5682): p. 361-361.
456. Wilicox, B.J., et al., *FOXO3A genotype is strongly associated with human longevity.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(37): p. 13987-13992.
457. Flachsbart, F., et al., *Association of FOXO3A variation with human longevity confirmed in German centenarians.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(8): p. 2700-2705.
458. Suh, Y., et al., *Functionally significant insulin-like growth factor I receptor mutations in centenarians.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(9): p. 3438-3442.
459. Huang, H., et al., *CDK2-Dependent Phosphorylation of FOXO1 as an Apoptotic Response to DNA Damage.* science, 2006. **314**(5797): p. 294-297.
460. Liu, P., T.P. Kao, and H. Huang, *CDK1 promotes cell proliferation and survival via phosphorylation and inhibition of FOXO1 transcription factor.* Oncogene, 2008. **27**(34): p. 4733-4744.
461. Martínez-Gac, L., et al., *Control of Cyclin G2 mRNA Expression by Forkhead Transcription Factors: Novel Mechanism for Cell Cycle Control by Phosphoinositide 3-Kinase and Forkhead.* Molecular and Cellular Biology, 2004. **24**(5): p. 2181-2189.
462. Takano, M., et al., *Transcriptional Cross Talk between the Forkhead Transcription Factor Forkhead Box O1A and the Progesterone Receptor Coordinates Cell Cycle Regulation and Differentiation in Human Endometrial Stromal Cells.* Molecular Endocrinology, 2007. **21**(10): p. 2334-2349.
463. Yan, L., et al., *PP2A Regulates the Pro-apoptotic Activity of FOXO1.* Journal of Biological Chemistry, 2008. **283**(12): p. 7411-7420.
464. Singh, A., et al., *Protein Phosphatase 2A Reactivates FOXO3a through a Dynamic Interplay with 14-3-3 and AKT.* Molecular Biology of the Cell, 2010. **21**(6): p. 1140-1152.
465. Rinner, O., et al., *An integrated mass spectrometric and computational framework for the analysis of protein interaction networks.* Nat Biotech, 2007. **25**(3): p. 345-352.
466. Essers, M.A.G., et al., *FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK.* EMBO J, 2004. **23**(24): p. 4802-4812.
467. Oh, S.W., et al., *JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(12): p. 4494-4499.
468. Wang, M.C., D. Bohmann, and H. Jasper, *JNK Extends Life Span and Limits Growth by Antagonizing Cellular and Organism-Wide Responses to Insulin Signaling.* Cell, 2005. **121**(1): p. 115-125.
469. Sunayama, J., et al., *JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3.* The Journal of Cell Biology, 2005. **170**(2): p. 295-304.
470. Sunters, A., et al., *Paclitaxel-Induced Nuclear Translocation of FOXO3a in Breast Cancer Cells Is Mediated by c-Jun NH2-Terminal Kinase and Akt.* Cancer Research, 2006. **66**(1): p. 212-220.
471. Brunet, A., et al., *Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase.* Sci. Aging Knowl. Environ., 2004. **2004**(8): p. or2-.
472. Lehtinen, M.K., et al., *A Conserved MST-FOXO Signaling Pathway Mediates Oxidative-Stress Responses and Extends Life Span.* Cell, 2006. **125**(5): p. 987-1001.
473. Brent, M.M., R. Anand, and R. Marmorstein, *Structural Basis for DNA Recognition by FoxO1 and Its Regulation by Posttranslational Modification.* Structure, 2008. **16**(9): p. 1407-1416.
474. Yuan, Z., et al., *Regulation of Neuronal Cell Death by MST1-FOXO1 Signaling.* Journal of Biological Chemistry, 2009. **284**(17): p. 11285-11292.

475. Jang, S.-W., et al., *Akt Phosphorylates MstI and Prevents Its Proteolytic Activation, Blocking FOXO3 Phosphorylation and Nuclear Translocation.* Journal of Biological Chemistry, 2007. **282**(42): p. 30836-30844.
476. Yuan, Z., et al., *Phosphoinositide 3-Kinase/Akt Inhibits MST1-Mediated Pro-apoptotic Signaling through Phosphorylation of Threonine 120.* Journal of Biological Chemistry, 2010. **285**(6): p. 3815-3824.
477. Bi, W., et al., *c-Jun N-terminal Kinase Enhances MST1-mediated Pro-apoptotic Signaling through Phosphorylation at Serine 82.* Journal of Biological Chemistry, 2010. **285**(9): p. 6259-6264.
478. Harvey, K.F., C.M. Pfleger, and I.K. Hariharan, *The Drosophila Mst Ortholog, hippo, Restricts Growth and Cell Proliferation and Promotes Apoptosis.* Cell, 2003. **114**(4): p. 457-467.
479. Lee, J.H., et al., *Sestrin as a Feedback Inhibitor of TOR That Prevents Age-Related Pathologies.* science, 2010. **327**(5970): p. 1223-1228.
480. Lee, K.-S., et al., *JNK/FOXO-mediated Neuronal Expression of Fly Homologue of Peroxiredoxin II Reduces Oxidative Stress and Extends Life Span.* Journal of Biological Chemistry, 2009. **284**(43): p. 29454-29461.
481. Colombo, S.L. and S. Moncada, *AMPKα1 regulates the antioxidant status of vascular endothelial cells.* Biochemical Journal, 2009. **421**(2): p. 163-169.
482. Greer, E.L., et al., *The Energy Sensor AMP-activated Protein Kinase Directly Regulates the Mammalian FOXO3 Transcription Factor.* Journal of Biological Chemistry, 2007. **282**(41): p. 30107-30119.
483. Greer, E.L., M.R. Banko, and A. Brunet, *AMP-activated Protein Kinase and FoxO Transcription Factors in Dietary Restriction–induced Longevity.* Annals of the New York Academy of Sciences, 2009. **1170**(1): p. 688-692.
484. Katic, M. and C.R. Kahn, *The role of insulin and IGF-1 signaling in longevity.* Cellular and Molecular Life Sciences, 2005. **62**(3): p. 320-343.
485. Altomonte, J., et al., *Foxo1 mediates insulin action on apoC-III and triglyceride metabolism.* The Journal of Clinical Investigation, 2004. **114**(10): p. 1493-1503.
486. Altomonte, J., et al., *Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice.* American Journal of Physiology - Endocrinology And Metabolism, 2003. **285**(4): p. E718-E728.
487. Puigserver, P., et al., *Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1[alpha] interaction.* Nature, 2003. **423**(6939): p. 550-555.
488. Daitoku, H., et al., *Regulation of PGC-1 Promoter Activity by Protein Kinase B and the Forkhead Transcription Factor FKHR.* Diabetes, 2003. **52**(3): p. 642-649.
489. Hwangbo, D.S., et al., *Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body.* Nature, 2004. **429**(6991): p. 562-566.
490. Bluher, M., B.B. Kahn, and C.R. Kahn, *Extended longevity in mice lacking the insulin receptor in adipose tissue.* science, 2003. **299**(5606): p. 572-574.
491. Kitamura, T., et al., *The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic β cell growth.* The Journal of Clinical Investigation, 2002. **110**(12): p. 1839-1847.
492. Kim, M.-S., et al., *Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis.* Nat Neurosci, 2006. **9**(7): p. 901-906.
493. Fulco, M., et al., *Glucose Restriction Inhibits Skeletal Myoblast Differentiation by Activating SIRT1 through AMPK-Mediated Regulation of Nampt.* Developmental Cell, 2008. **14**(5): p. 661-673.
494. Yoshimochi, K., H. Daitoku, and A. Fukamizu, *PCAF represses transactivation function of FOXO1 in an acetyltransferase-independent manner.* Journal of Receptors and Signal Transduction, 2010. **30**(1): p. 43-49.
495. Dansen, T.B., et al., *Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity.* Nat Chem Biol, 2009. **5**(9): p. 664-672.
496. Kitamura, Y.I., et al., *FoxO1 protects against pancreatic β cell failure through NeuroD and MafA induction.* Cell Metabolism, 2005. **2**(3): p. 153-163.
497. Fukuoka, M., et al., *Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation.* International Journal of Molecular Medicine, 2003. **12**(4): p. 503-508.
498. Daitoku, H., et al., *Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(27): p. 10042-10047.
499. Motta, M.C., et al., *Mammalian SIRT1 Represses Forkhead Transcription Factors.* Cell, 2004. **116**(4): p. 551-563.
500. Wang, F., et al., *SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction.* Aging Cell, 2007. **6**(4): p. 505-514.
501. Kaeberlein, M., M. McVey, and L. Guarente, *The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms.* Genes & Development, 1999. **13**(19): p. 2570-2580.
502. Tissenbaum, H.A. and L. Guarente, *Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans.* Nature, 2001. **410**(6825): p. 227-230.
503. Rogina, B. and S.L. Helfand, *Sir2 mediates longevity in the fly through a pathway related to calorie restriction.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(45): p. 15998-16003.

504. Wood, J.G., et al., *Sirtuin activators mimic caloric restriction and delay ageing in metazoans.* Nature, 2004. **430**(7000): p. 686-689.
505. Berdichevsky, A., et al., *C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span.* Cell, 2006. **125**(6): p. 1165-1177.
506. Mamillapalli, R., et al., *PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27KIP1 through the ubiquitin E3 ligase SCFSKP2.* Current Biology, 2001. **11**(4): p. 263-267.
507. Asada, S., et al., *Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1.* Cellular Signalling, 2007. **19**(3): p. 519-527.
508. Fu, W., et al., *MDM2 Acts Downstream of p53 as an E3 Ligase to Promote FOXO Ubiquitination and Degradation.* Journal of Biological Chemistry, 2009. **284**(21): p. 13987-14000.
509. Brenkman, A.B., et al., *Mdm2 Induces Mono-Ubiquitination of FOXO4.* PLoS ONE, 2008. **3**(7): p. e2819.
510. van der Horst, A., et al., *FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP.* Nat Cell Biol, 2006. **8**(10): p. 1064-1073.
511. Yamagata, K., et al., *Arginine Methylation of FOXO Transcription Factors Inhibits Their Phosphorylation by Akt.* Molecular Cell, 2008. **32**(2): p. 221-231.
512. Housley, M.P., et al., *A PGC-1α-O-GlcNAc Transferase Complex Regulates FoxO Transcription Factor Activity in Response to Glucose.* Journal of Biological Chemistry, 2009. **284**(8): p. 5148-5157.
513. Housley, M.P., et al., *O-GlcNAc Regulates FoxO Activation in Response to Glucose.* Journal of Biological Chemistry, 2008. **283**(24): p. 16283-16292.
514. Hanover, J.A., et al., *A Caenorhabditis elegans model of insulin resistance: Altered macronutrient storage and dauer formation in an OGT-1 knockout.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(32): p. 11266-11271.
515. Hoogeboom, D., et al., *Interaction of FOXO with β-Catenin Inhibits β-Catenin/T Cell Factor Activity.* Journal of Biological Chemistry, 2008. **283**(14): p. 9224-9230.
516. Almeida, M., et al., *Oxidative Stress Antagonizes Wnt Signaling in Osteoblast Precursors by Diverting β-Catenin from T Cell Factor- to Forkhead Box O-mediated Transcription.* Journal of Biological Chemistry, 2007. **282**(37): p. 27298-27305.
517. Yamamura, Y., et al., *RUNX3 Cooperates with FoxO3a to Induce Apoptosis in Gastric Cancer Cells.* Journal of Biological Chemistry, 2006. **281**(8): p. 5267-5276.
518. Gomis, R.R., et al., *A FoxO–Smad synexpression group in human keratinocytes.* Proceedings of the National Academy of Sciences, 2006. **103**(34): p. 12747-12752.
519. Liu, Z.-P., et al., *Phenotypic Modulation of Smooth Muscle Cells through Interaction of Foxo4 and Myocardin.* Developmental Cell, 2005. **9**(2): p. 261-270.
520. Akutsu, N., et al., *Regulation of Gene Expression by 1α,25-Dihydroxyvitamin D3 and Its Analog EB1089 under Growth-Inhibitory Conditions in Squamous Carcinoma Cells.* Molecular Endocrinology, 2001. **15**(7): p. 1127-1139.
521. Lin, R., et al., *Expression Profiling in Squamous Carcinoma Cells Reveals Pleiotropic Effects of Vitamin D3 Analog EB1089 Signaling on Cell Proliferation, Differentiation, and Immune System Regulation.* Molecular Endocrinology, 2002. **16**(6): p. 1243-1256.
522. Wang, T.-T., et al., *Large-Scale in Silico and Microarray-Based Identification of Direct 1,25-Dihydroxyvitamin D3 Target Genes.* Molecular Endocrinology, 2005. **19**(11): p. 2685-2695.
523. An, B.-S., et al., *Stimulation of Sirt1-Regulated FoxO Protein Function by the Ligand-Bound Vitamin D Receptor.* Molecular and Cellular Biology, 2010. **30**(20): p. 4890-4900.
524. Hu, L., et al., *Abnormal Expression of Retinoic Acid Receptors and Keratin 19 by Human Oral and Epidermal Squamous Cell Carcinoma Cell Lines.* Cancer Research, 1991. **51**(15): p. 3972-3981.
525. Vokes, E.E., et al., *Head and Neck Cancer.* New England Journal of Medicine, 1993. **328**(3): p. 184-194.
526. Matsushime, H., et al., *Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins.* Cell, 1992. **71**(2): p. 323-334.
527. Meyerson, M. and E. Harlow, *Identification of G1 kinase activity for cdk6, a novel cyclin D partner.* Molecular and Cellular Biology, 1994. **14**(3): p. 2077-2086.
528. Bennin, D.A., et al., *Cyclin G2 Associates with Protein Phosphatase 2A Catalytic and Regulatory B' Subunits in Active Complexes and Induces Nuclear Aberrations and a G1/S Phase Cell Cycle Arrest.* Journal of Biological Chemistry, 2002. **277**(30): p. 27449-27467.
529. Jensen, M.R., et al., *Reduced hepatic tumor incidence in cyclin G1-deficient mice.* Hepatology, 2003. **37**(4): p. 862-870.
530. Kim, E.-M., et al., *Matrix Metalloproteinase-3 Is Increased and Participates in Neuronal Apoptotic Signaling Downstream of Caspase-12 during Endoplasmic Reticulum Stress.* Journal of Biological Chemistry, 2010. **285**(22): p. 16444-16452.
531. Lee, H.-Y., et al., *Forkhead Factor, FOXO3a, Induces Apoptosis of Endothelial Cells Through Activation of Matrix Metalloproteinases.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(2): p. 302-308.

532. Boyd, J.M., et al., *Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins.* Cell, 1994. **79**(2): p. 341-351.
533. Schmidt M, F.S., van der Horst A, Klompmaker R, Kops G, Lam E, Burgering B, Medema R, *Cell Cycle Inhibition by FoxO Forkhead Transcription Factors Involves Downregulation of Cyclin D.* Molecular and Cellular Biology, 2002. **22**(22): p. 10.
534. Wang, T.-T., et al., *Direct and Indirect Induction by 1,25-Dihydroxyvitamin D3 of the NOD2/CARD15-Defensin β2 Innate Immune Pathway Defective in Crohn Disease.* Journal of Biological Chemistry, 2010. **285**(4): p. 2227-2231.
535. Miura, K., H. Karasawa, and I. Sasaki, *cIAP2 as a therapeutic target in colorectal cancer and other malignancies.* Expert Opinion on Therapeutic Targets, 2009. **13**(11): p. 1333-1345.
536. Miyazaki, T. and S. Arai, *Two Distinct Controls of Mitotic Cdk1/Cyclin B1 Activity Requisite for Cell Growth Prior to Cell Division.* Cell Cycle, 2007. **6**(12): p. 1418-1424.
537. Yasmeen, A., et al., *E- and A-type cyclins as markers for cancer diagnosis and prognosis.* Expert Review of Molecular Diagnostics, 2003. **3**(5): p. 617-633.
538. Lladser, A., et al., *Is Survivin the Potential Achilles' Heel of Cancer?*, in *Advances in Cancer Research, Vol 111*, G.F. VandeWoude and G. Klein, Editors. 2011, Elsevier Academic Press Inc: San Diego. p. 1-37.
539. Hirsch, H.A., et al., *A Transcriptional Signature and Common Gene Networks Link Cancer with Lipid Metabolism and Diverse Human Diseases.* Cancer Cell, 2010. **17**(4): p. 348-361.
540. Stein, U., M. Dahlmann, and W. Walther, *MACC1 — more than metastasis? Facts and predictions about a novel gene.* Journal of Molecular Medicine, 2010. **88**(1): p. 11-18.
541. Wada, M., et al., *The Second Largest Subunit of Mouse DNA Polymerase ε, DPE2, Interacts with SAP18 and Recruits the Sin3 Co-Repressor Protein to DNA.* Journal of Biochemistry, 2002. **131**(3): p. 307-311.
542. Hu, N., et al., *Genome wide analysis of DNA copy number neutral loss of heterozygosity (CNNLOH) and its relation to gene expression in esophageal squamous cell carcinoma.* BMC Genomics, 2010. **11**(1): p. 576.
543. Mitsuuchi Y, J.S., Sonoda G, Tanno S, Golemis E, Testa J, *Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2.* Oncogene, 1999. **18**(35): p. 7.
544. Schenck, A., et al., *The Endosomal Protein Appl1 Mediates Akt Substrate Specificity and Cell Survival in Vertebrate Development.* Cell, 2008. **133**(3): p. 486-497.
545. Miaczynska, M., et al., *APPL Proteins Link Rab5 to Nuclear Signal Transduction via an Endosomal Compartment.* Cell, 2004. **116**(3): p. 445-456.
546. Badea L, H.V., Dima SO, Dumitrascu T, Popescu I, *Combined gene expression analysis of whole-tissue and microdissected pancreatic ductal adenocarcinoma identifies genes specifically overexpressed in tumor epithelia.* Hepatogastroenterology, 2008. **55**(88): p. 11.
547. Iacobuzio-Donahue, C.A., et al., *Exploration of Global Gene Expression Patterns in Pancreatic Adenocarcinoma Using cDNA Microarrays.* The American Journal of Pathology, 2003. **162**(4): p. 1151-1162.
548. Radvanyi, L., et al., *The gene associated with trichorhinophalangeal syndrome in humans is overexpressed in breast cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(31): p. 11005-11010.
549. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis.* Nature, 2007. **449**(7162): p. 557-563.
550. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-752.
551. Sørlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proceedings of the National Academy of Sciences, 2003. **100**(14): p. 8418-8423.
552. Sørlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proceedings of the National Academy of Sciences, 2001. **98**(19): p. 10869-10874.
553. Blaveri, E., et al., *Bladder Cancer Outcome and Subtype Classification by Gene Expression.* Clinical Cancer Research, 2005. **11**(11): p. 4044-4055.
554. Kaiser, S., et al., *Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer.* Genome Biology, 2007. **8**(7): p. R131.
555. Schlingemann, J., et al., *Patient-based cross-platform comparison of oligonucleotide microarray expression profiles.* Lab Invest, 2005. **85**(8): p. 1024-1039.
556. Ginos, M.A., et al., *Identification of a Gene Expression Signature Associated with Recurrent Disease in Squamous Cell Carcinoma of the Head and Neck.* Cancer Research, 2004. **64**(1): p. 55-63.
557. Lee, J., et al., *Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines.* Cancer Cell, 2006. **9**(5): p. 391-403.
558. Biewenga, P., et al., *Gene expression in early stage cervical cancer.* Gynecologic Oncology, 2008. **108**(3): p. 520-526.
559. Yusenko, M., et al., *High-resolution DNA copy number and gene expression analyses distinguish chromophobe renal cell carcinomas and renal oncocytomas.* BMC Cancer, 2009. **9**(1): p. 152.

560. Gumz, M.L., et al., *Secreted Frizzled-Related Protein 1 Loss Contributes to Tumor Phenotype of Clear Cell Renal Cell Carcinoma.* Clinical Cancer Research, 2007. **13**(16): p. 4740-4749.
561. Skrzypczak, M., et al., *Modeling Oncogenic Signaling in Colon Tumors by Multidirectional Analyses of Microarray Data Directed for Maximization of Analytical Reliability.* PLoS ONE, 2010. **5**(10): p. e13091.
562. Sabates-Bellver, J., et al., *Transcriptome Profile of Human Colorectal Adenomas.* Molecular Cancer Research, 2007. **5**(12): p. 1263-1275.
563. Hong, Y., et al., *A 'metastasis-prone' signature for early-stage mismatch-repair proficient sporadic colorectal cancer patients and its implications for possible therapeutics.* Clinical and Experimental Metastasis, 2010. **27**(2): p. 83-90.
564. Lu, K.H., et al., *Selection of Potential Markers for Epithelial Ovarian Cancer with Gene Expression Arrays and Recursive Descent Partition Analysis.* Clinical Cancer Research, 2004. **10**(10): p. 3291-3300.
565. Liang, Y., et al., *Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(16): p. 5814-5819.
566. Sun, L., et al., *Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain.* Cancer Cell, 2006. **9**(4): p. 287-300.
567. Pei, H., et al., *FKBP51 Affects Cancer Cell Response to Chemotherapy by Negatively Regulating Akt.* Cancer Cell, 2009. **16**(3): p. 259-266.
568. Zhan, F., et al., *Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis.* Blood, 2007. **109**(4): p. 1692-1700.
569. Detwiller, K.Y., et al., *Analysis of Hypoxia-Related Gene Expression in Sarcomas and Effect of Hypoxia on RNA Interference of Vascular Endothelial Cell Growth Factor A.* Cancer Research, 2005. **65**(13): p. 5881-5889.
570. Turashvili, G., et al., *Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis.* BMC Cancer, 2007. **7**(1): p. 55.
571. Finak, G., et al., *Stromal gene expression predicts clinical outcome in breast cancer.* Nat Med, 2008. **14**(5): p. 518-527.
572. Ruderman, N.B., et al., *AMPK and SIRT1: a long-standing partnership?* American Journal of Physiology - Endocrinology And Metabolism, 2010. **298**(4): p. E751-E760.