

Loss of expression of the E3 ligase and tumour suppressor FBW7 attenuates vitamin D signaling

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Abstract

Hormonal 1,25-dihydroxyvitamin D (1,25D) and its analogues signal through the nuclear vitamin D receptor (VDR). While numerous preclinical studies have provided evidence for antiproliferative and antitumour activities of 1,25D (analogues), such compounds have failed in clinical trials because of acquired tumour resistance. We previously found that the E3 ligase FBW7 interacts with the VDR. FBW7 regulates turnover of cell cycle drivers and is a tumour suppressor that is mutated in multiple malignancies, including head and neck squamous cell carcinoma (HNSCC). 1,25D accelerates the proteasomal degradation of FBW7 target proteins in a VDR- and FBW7-dependent manner. Surprisingly, FBW7 is also required for 1,25D-dependent transactivation. Here, we investigated the effects of *FBW7* ablation by CRISPR/Cas9 editing on gene expression in 1,25D-sensitive human SCC25 HNSCC cells. We find that FBW7 loss induces the expression of several genes implicated in tumour progression and represses expression of those inhibiting tumour growth. Fold induction of the vast majority of the top 50 genes induced or repressed by 1,25D is attenuated by FBW7 ablation, suppressing 1,25D-driven regulation of genes implicated in cell proliferation and immune responses. Collectively, these data reveal that FBW7 is a critical cofactor of 1,25D signaling and provide a mechanism of acquired tumour resistance.

Introduction

While originally identified for its critical role in calcium homeostasis, vitamin D is now known to exert a range of physiological functions ^{1,2}. These include regulation of the innate and adaptive arms of the immune system. Vitamin D has also been investigated for its potential roles in cancer prevention and therapy. It is obtained from the diet or by photochemical and thermal conversion of the cholesterol precursor 7-dehydrocholesterol in the presence of sufficient solar ultraviolet B (UVB) irradiation ^{1,2}. While it may seem that vitamin D should be readily available, deficiency is a widespread problem. Dietary sources are limited and in many populations sun exposure is the main source of vitamin D. However, UVB is absorbed during passage through the ozone layer, and at sea level substantial cutaneous vitamin D synthesis occurs only when the sun is above 45°C; so-called vitamin D winter can last several months at temperate latitudes ³. In the absence of sufficient supplementation, circulating vitamin D metabolite levels thus vary seasonally ⁴. Moreover, cutaneous vitamin D production is dependent on skin color and declines with age ⁵. These factors, coupled with sun avoidance and conservative dress, can lead to vitamin D deficiency in several populations worldwide ⁶⁻⁹.

Vitamin D obtained from the sun, diet or supplementation must undergo sequential hydroxylations, first at the 25 position of the cholesterol sidechain followed by tightly

regulated 1 α hydroxylation to produce the biologically active form 1,25-dihydroxyvitamin D (1,25D). 1,25D binds to and regulates the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-regulated transcription factors. In the presence of 1,25D, the VDR heterodimerizes with related retinoid X receptors and transactivates gene transcription by binding to cognate vitamin D response elements (VDREs). The hormone-bound VDR can also repress gene transcription by several, heterogeneous mechanisms¹⁰

The VDR and the critical 25-hydroxyvitamin D 1 α -hydroxylase enzyme CYP27B1 are expressed in numerous tissues throughout the body¹¹. Notably, 1,25D signaling has pro-differentiating and antiproliferative activities in a number of cell types and there is epidemiological evidence that maintaining vitamin D sufficiency is cancer preventive^{12,13}. Typical of many findings, a recent scoping review provided evidence of links between vitamin D deficiency and an increased risk of oral cancer¹². Intervention trials have given mixed results but are fraught with complications because of limited dosing in some cases and the fact that vitamin D is an essential nutrient, thus complicating placebo wings. Prevention trials are also compromised by their relatively short duration. Nonetheless, a meta-analysis of randomized, placebo-controlled trials concluded that vitamin D supplementation significantly reduced total cancer mortality but did not reduce total cancer incidence¹⁴.

The potential of the anticancer properties of vitamin D signaling are supported by numerous preclinical studies of several cancer models¹. While 1,25D or its analogues have shown encouraging efficacy in such studies, they have failed as cancer therapeutics because of acquired tumour resistance even though in most instances VDR expression is maintained. We have been interested in studying the anticancer properties of 1,25D (analogues) using mouse and human head and neck squamous cell carcinoma (HNSCC) as models¹⁵⁻¹⁸. In human SCC25 HNSCC cells, 1,25D is antiproliferative and induces a more differentiated state^{15,19}. Biochemical studies have revealed a striking functional relationship between the VDR and the F box protein FBW7 (FBXW7/Sel-10/Ago/hCDC4) in HNSCC cells¹⁸. FBW7 is a subunit of SCF E3 ligase complexes (Skp1, Cullin-1, F box protein), which are key components of the ubiquitin proteasome system (UPS) as they catalyze the ubiquitination of substrates destined for degradation by the 26S proteasome. The UPS regulates numerous fundamental biological processes, including cell cycle progression, antigen processing, cellular defense, signaling pathways and gene transcription²⁰⁻²². FBW7 is expressed in multiple isoforms of which α and γ are nuclear²³. Our initial interest was in the role of FBW7 in the accelerated proteasomal turnover of the oncogenic transcription factor c-MYC observed in 1,25D-treated cells^{17,18}. While 1,25D reduces expression of c-MYC in a manner partially dependent on FBW7, c-Myc accumulates in *Vdr*-null mice¹⁷. Unexpectedly, we also found that FBW7 is required for

optimal transactivation by the hormone-bound VDR; FBW7, along with proteasomal subunits, was recruited to the DNA-bound VDR and FBW7 ablation substantially reduced transactivation of selected VDR target genes¹⁸. While expression of numerous transcription factors is controlled by proteasomal turnover²⁴, the VDR does not appear to be a substrate of FBW7 even though the two proteins interact¹⁸.

The best characterized FBW7 target is c-MYC; downregulation of FBW7 results in accumulation of cellular and chromatin-bound c-MYC²⁵. In addition, FBW7 controls the turnover of several other proteins that drive cell cycle progression through recognition of “phosphodegron” motifs (consensus T/S-P-X-X-S/T/E), which are phosphorylated by GSK3 β ²⁶. Consistent with this, FBW7 is a tumour suppressor and is one of the most frequently dysregulated UPS components in cancer²⁷, including HNSCC^{28,29}. Its functional relationship with the VDR is thus fascinating because its mutation may provide a mechanism of acquired resistance to 1,25D or its analogues. Indeed, ablation of FBW7 strongly impaired the antiproliferative activity of 1,25D in HNSCC cells¹⁸. Here, we have investigated broadly the interplay between FBW7 and vitamin D signaling by generating an SCC25 HNSCC variant in which FBW7 expression has been ablated by CRISPR/Cas genome editing and have analyzed its effects on 1,25D-regulated gene expression by RNAseq. We find that FBW7 ablation has multiple effects on SCC25 gene expression, consistent with its role as a tumour suppressor. We also find that FBW7 loss attenuates the induction of the vast majority of genes regulated by 1,25D, both activated and repressed. Accordingly, several signaling mechanisms regulated by 1,25D are attenuated in FBW7-deficient cells. Our results suggest that loss of tumour suppressor FBW7 function during cancer progression provides a mechanism of acquired resistance to vitamin D signaling.

Results

Ablation of FBW7 expression in SCC25 cells

To disrupt FBW7 expression we used CRISPR/Cas genome editing (see Materials and Methods for details). The gene encoding FBW7 has multiple 5' exons specific to each isoform (Fig. 1A). Guide RNAs were generated that targeted exon c7, which encodes sequence lying in the WD40 domains and is common to all isoforms (Fig. 1A). The WD40 domains contain the phosphodegron binding repeats and are hotspots for mutations in cancer³⁰. The clone selected for RNAseq is homozygous for a 37 bp deletion and exhibits strongly reduced *FBW7* mRNA expression (Fig. 1B). Note that we did not test for endogenous protein expression because available antibodies against FBW7 are unreliable. Consistent with siRNA knockdown¹⁸, *FBW7* ablation led to severely compromised induction of VDR target genes *CYP24A1* and *CAMP* in the presence of 1,25D (Fig. 1C). In

control experiments, using control genes *CYP24A1* and *CAMP*, we found was no substantial effect on 1,25D-regulated gene expression in transduced cell lines where *FBW7* gene editing failed (Fig. S1). Moreover, the attenuated response to 1,25D in *FBW7*-ablated cells was not due to loss of VDR gene or protein expression under any of the conditions tested (Figs. 1D,E).

To determine as broadly as possible the effects of *FBW7* ablation on gene expression in SCC25 cells and on 1,25D signaling we performed in triplicate comparative RNAseq analyses in control and *FBW7*-ablated cells cultured in the presence of vehicle or 100nM 1,25D for 16h. Principal component analysis (Fig. 1F) showed that gene expression patterns were highly similar between triplicates and that *FBW7* ablation and/or 1,25D treatment produced distinct sets of regulated genes. Several hundred genes were differentially regulated by at least 1.5-fold when comparing vehicle- to 1,25D-treated control cells or control cells to those lacking *FBW7* (Fig. 1G). In contrast, treatment of *FBW7* KO (knockout) cells with 1,25D produced a more limited set of differentially up- or downregulated genes (Fig. 1G), with substantial but incomplete overlap with those regulated by 1,25D in control cells (Fig. 1H). In both control and *FBW7*-depleted cells, 1,25D treatment led to activation of the vitamin D receptor pathway as determined by GSEA Wiki Pathways analysis (normalized enrichment scores in control and *FBW7* cells are 2.79 [p-adjusted value: 3.64e-10] and 2.43 [p-adjusted value: 1.0e-05], respectively).

Upon examination of DEGs regulated within the VDR pathway by a heatmap, 1,25D treatment in *FBW7* KO cells led to attenuated regulation of several 1,25D-VDR target genes relative to 1,25D-treated control cells (Fig. S2). To probe the effects of *FBW7* loss further, we compared the fold regulation of the top 50 most 1,25D-inducible genes in control and *FBW7* KO cells. This revealed that, for 45 of the 50 genes, fold induction by 1,25D was partially or strongly reduced (Fig. 2A). A similar inhibitory effect of *FBW7* ablation was observed on genes downregulated by 1,25D signaling (Fig. 2B). Differential regulation of a subset of upregulated and downregulated genes was validated independently by RT/qPCR (Fig. 2C, D). As expected, one of the top upregulated genes whose expression was diminished was *CAMP* (Figs. 1C, 2A). *FBW7* ablation decreased both the basal expression and induction by 1,25D of *CAMP*. It can be argued that the loss of effect of 1,25D on *CAMP* expression may be due to the loss of expression/activity of a cooperating transcription factor given the reduced basal expression. However, depletion of *FBW7* had a wide range of effects on basal expression of 1,25D target genes. Notably, fold regulation by 1,25D of the *ALP 1* and *FEZF1* genes was also diminished in the absence of *FBW7* even though their basal expression increased substantially (Fig. 2C).

Although KO does not substantially affect VDR expression (Fig. 1), another possible mechanism underlying attenuated 1,25D signaling would be suppressed expression of RXR heterodimeric partners. While *RXRG* encoding RXR γ is listed as a downregulated DEG,

analysis of RNAseq data revealed that its expression is very weak (Fig. S3A). Western blotting of RXR α , by far the most strongly expressed VDR heterodimeric partner (*RXRA*, Fig. S3A), revealed that its expression was not affected by knockdown (Fig. S3B). Taken together, the above results indicate that attenuation of 1,25D-dependent gene regulation does not appear to be due to loss of the VDR or its major heterodimeric partner. They also show that FBW7 ablation broadly inhibits 1,25D-regulated gene expression in SCC25 cells.

FBW7 loss induces pro-tumourigenic signaling pathways

To further examine the consequences of FBW7 loss on SCC25 cell gene expression and on signaling by 1,25D, we performed pathways analyses to identify changes in gene expression signatures associated with molecular signaling pathways. We focused initially on the effects of FBW7 ablation. Gene set enrichment analysis (GSEA) for Gene Ontology biological processes revealed downregulated pathways related to innate immune responses, calcium ion transport into cytosol, migration and chemotaxis when FBW7 was ablated (Fig. 3A). Similarly, Ingenuity Pathway Analysis (IPA) of enriched diseases and functions uncovered suppression of cell movement and calcium ion transport with FBW7 loss (Table S5). Interestingly, cancer cells evade apoptosis via decreasing calcium influx into the cytosol³¹. Conversely, epithelial cell development appeared as upregulated in FBW7-depleted cells. To further investigate this, we produced a heatmap of differentially expressed genes (DEGs) within the epithelial cell development category, which included the effects of 1,25D (Fig. 3B). FBW7 ablation induced expression of a subset of genes (enclosed in red) that were not substantially regulated by 1,25D. These include several genes that encoded proteins involved in tumour promotion such as *VIM* and *PODXL*, associated with the epithelial-to-mesenchymal transition and accelerated tumour growth³²⁻³⁴, *FSRL1*, a biomarker for cervical cancer diagnosis^{35,36}, *EPAS1*, *AMPD2* and *XBP1*, biomarkers of colorectal cancer³⁷⁻⁴², as well as *RFX3* and *IL6ST*, which are molecular drivers of breast cancer and drug resistance to tumours^{43,44}. The only exceptions were *TMOD1* and *ABCB1*, which are implicated in tumourigenesis^{45,46} and are induced with 1,25D but not suppressed with FBW7 loss. Conversely, FBW7 depletion repressed a subset of genes that are not similarly regulated by 1,25D (enclosed in blue). These include genes that encoded proteins involved in tumour suppression such as the metastatic suppressors *BMP4* and *NFX6-1*^{47,48} and the tumour suppressors *RAB25*, *RARG*, *EDNRB* and *PAX6*⁴⁹⁻⁵². There were a few exceptions with *NR5A2* and *RARB*, nuclear receptors that function as negative regulators of tumour growth^{53,54}, which were similarly downregulated with 1,25D and by FBW7 loss.

We also found that pathways involved in actin-based motility, histone and DNA methylation, phosphoinositide 3-kinase (PI3K)/AKT signaling, integrin signaling and

SPINK1 pancreatic cancer were activated in FBW7 KO cells as demonstrated by IPA enrichment for canonical pathways (Fig. 3C). Aberrant activation of PI3K/AKT signaling is associated with cellular transformation, tumourigenesis, cancer progression, and drug resistance⁵⁵. Increased signaling of the extracellular matrix through its receptors, the integrins, was previously shown to contribute to cancer cell survival and resistance to chemotherapy⁵⁶⁻⁵⁸. SPINK1 is a growth factor and inhibitor of apoptosis in several cancers⁵⁹, and in human pancreatic cancer, SPINK1 promotes proliferation by inducing EGFR phosphorylation and subsequent downstream activation of the mitogen-activated protein kinase pathway⁶⁰. In contrast, multiple pathways related to immune signaling and signaling by thyroid hormone (TR), PPAR and LXR nuclear receptors were strongly downregulated by *FBW7* ablation (Fig. 3C). While these receptors and the VDR share common heterodimeric partners, RXRs, as stated above it is unlikely that their attenuated signaling is due to loss of RXR expression.

To investigate regulation of genes encoding histones, we generated a heatmap of DEGs within the histone category, which included the effects of 1,25D (Fig. 3D). Interestingly, nearly all genes encoding histones were induced when FBW7 was ablated, and these effects were independent of 1,25D (Fig. 3D). This is noteworthy as genes encoding histones are overexpressed in various cancers, which indicate a requirement for higher amounts of histones in proliferating cells undergoing high rates of replication, such as in cancer cells⁶¹⁻⁶⁴. The sole exception was with *H2AC25*, which is linked with shorter survival in oral squamous cell carcinoma⁶⁵. Consistent with its induction of tumour promoting and proliferative histone genes, several enriched diseases and functions related to various cancers, which include development of head and neck tumours, were activated in FBW7 depleted cells (Table S4).

Regulation of cell cycle drivers by 1,25D signaling

A similar analysis was performed to identify 1,25D-regulated pathways. 1,25D appeared to broadly suppress transcription of Myc target genes (Fig. 4A). However, this effect was attenuated in FBW7-depleted cells. cMYC protein levels in the absence and presence of 1,25D were elevated relative to controls in FBW7 KO cells and expression of cMYC antagonist MXD1 induced by 1,25D was lost in the absence of FBW7 (Fig. 4B). While 1,25D broadly suppressed transcription of Myc target genes in control cells (Figs. 4C), the degree of their repression in the presence of 1,25D was generally reduced in FBW7 KO cells (Fig. 4C). The only exception is the *EXOSC5* gene, which encodes a protein correlated with advanced clinical stage of head and neck squamous cell carcinoma⁶⁶. Its expression was downregulated by 1,25D or FBW7 ablation and further inhibited by 1,25D in KO cells (Fig. 4C). In the absence of 1,25D, expression of roughly half of cMYC target

genes was modestly or substantially upregulated in FBW7 KO cells, whereas expression of the remainder was lower, consistent with effects on FBW7 loss on cMYC target genes independent of cMYC expression.

1,25D treatment and FBW7 KO both strongly affect the expression of cyclins and cyclin-dependent kinases (Fig. 4D). Expression of several of these genes whose elevated levels are associated with tumourigenesis was inhibited by 1,25D (for example, the *CCNA1* and *CCND2* genes encoding cyclins A and D2, respectively), consistent with antiproliferative properties of 1,25D. Effects of 1,25D on genes encoding cyclins or related proteins that are negative regulators of the cell cycle was more mixed. *CCNG2*, encoding inhibitory cyclin G2, was strongly upregulated, whereas other related genes were modestly downregulated. FBW7 KO substantially disrupted basal expression of this gene set, with expression of 14 of the 23 genes strongly upregulated while that of 9 of 23 genes was downregulated. Upregulated genes encoded several proteins implicated in tumourigenesis (e.g. cyclins A1, D2 and J). However, expression of *CDKN1A*, encoding CDK inhibitor p21^{WAF1/CIP1} was also strongly upregulated.

Both 1,25D treatment and FBW7 KO also robustly affect expression of genes within the interferon alpha (IFN- α) response (Fig. 4E). Expression of most of these genes were downregulated by 1,25D and even more inhibited in the KO (Fig. 4E). The antitumourigenic and antiproliferative functions of IFN- α are documented^{67,68} and it has been used as monotherapy or adjuvant treatment before chemotherapy to improve efficacy against HNSCC tumours⁶⁸. Suppression of the antitumour IFN- α response in KO cells is consistent with the previously mentioned enriched tumour promoting pathways and genes. However, it is unclear why the IFN- α response is inhibited in response to antiproliferative 1,25D. One explanation may be due to well-known role of 1,25D signaling in suppressing inflammation^{69,70}, and the IFN- α pathway is often upregulated and promotes pathogenesis of autoimmune conditions such as systemic lupus erythematosus (SLE)⁷¹. Notably, there is a negative correlation between circulating 25D levels and IFN- α serum level in SLE patients^{72,73} and 1,25D was shown to suppress expression of IFN- α induced genes in these patients⁷⁴.

Effects of 1,25D signaling are suppressed with FBW7 ablation

Next, we evaluated the effects of 1,25D signaling in FBW7 depleted cells. Disease ontology enrichment revealed loss of enrichment of several pathways related to infectious and autoimmune conditions in 1,25D-treated FBW7 depleted cells relative to 1,25D-exposed control cells (Fig. 5A). Consistent with this, FBW7 loss decreased regulation (activation and suppression) of 1,25D-regulated canonical pathways (Figs. 5B, C). As 1,25D is anti-

inflammatory and anti-microbial ⁶⁹, we were interested in determining how 1,25D-regulated immune and inflammatory pathways were affected in the KO. Interestingly, FBW7 loss dampened 1,25D-induced signaling of innate immune responses and 1,25D-attenuated inflammation (Fig. 5D). For instance, the pathways included KO-mediated suppression of 1,25D-induced expression of anti-microbial *CAMP*, *ALPI*, an anti-inflammatory metalloenzyme that attenuates TLR4 agonist activity ⁷⁵ previously shown to be regulated by 1,25D in Caco-2 cells ⁷⁶, and *TREM1*, a triggering receptor that demonstrates antiviral activity ⁷⁷.

Discussion

A combination of preclinical data and epidemiological studies have provided evidence that vitamin D deficiency increases the risk of cancer development ⁷⁸⁻⁸⁰. Notably, a large prospective study showed that vitamin D sufficiency correlated with reduced total incidence and mortality from leukemias and digestive cancers ⁷⁸. The findings are significant as vitamin D deficiency remains a widespread problem. Most western diets are vitamin D-poor and combinations of inadequate solar UVB, sun avoidance, conservative dress and dark skin colour contribute to vitamin D deficiency in several populations in the absence of adequate supplementation ⁶⁻⁹.

Collectively, the above observations along with earlier findings spurred our interest in studying the anti-cancer properties of vitamin D signaling. We originally chose to study SCC25 cells as a model to test the potential cancer-preventive actions of vitamin D. Although derived from a tumour of the oral cavity, SCC25 cells are well differentiated and sensitive to the antiproliferative activity of 1,25D and its analogues ^{15,19}. Findings presented above substantiate the potential of vitamin D as an anticancer agent. For example, pathways analysis revealed that 1,25D signaling suppressed the expression of several genes regulated by the oncogenic transcription factor cMYC and regulated the expression of cyclins and cyclin-dependent kinase genes in manner consistent with cell cycle arrest. Previous work also showed that 1,25D signaling induced a more differentiated phenotype in SCC25 cells, notably by suppressing the expression of N-cadherin a marker of epithelial-mesenchymal transition ¹⁹.

Despite substantial evidence that maintenance of vitamin D sufficiency reduces cancer risk, 1,25D analogues have failed in cancer monotherapies because of acquired tumour resistance. Our present results are consistent with loss of *FBW7* expression being one of the mutations contributing to vitamin D resistance. The *FBW7* gene encodes a well-characterized tumour suppressor that is frequently dysregulated in cancer ²⁷, including HNSCC ^{28,29}. Other findings have noted that *FBW7* mutations also correlate with resistance to other chemotherapeutic agents ⁸¹. Our previous studies showed that the VDR

and FBW7 function as cofactors; they interact in a partially 1,25D-dependent manner and FBW7 and other proteasomal subunits are recruited to genes whose expression is stimulated by 1,25D. We initially identified FBW7 as a VDR cofactor that contributed to the accelerated proteasomal turnover of cMYC observed in 1,25D-treated SCC25 cells^{17,18}. We provided evidence that this occurs via 1,25D-dependent recruitment of FBW7 and the proteasome to DNA-bound cMYC. These findings provided a novel but highly specific mechanism of 1,25D-dependent repression of gene transcription. Unlike transactivation, which is generally initiated by VDR/RXR heterodimers binding to VDREs in regulatory regions of target genes, mechanisms of transrepression by the VDR are much more heterogeneous¹⁰. Therefore, in studies presented above, the observation that repression of gene transcription by 1,25D was generally attenuated in cells lacking FBW7 was unexpected (Fig. 2). The widespread reduction of fold regulation of 1,25D-induced genes was less of a surprise given that mechanisms of direct target gene regulation by the VDR are more homogeneous.

Bioinformatic analyses presented above of the effects of FBW7 loss on gene expression and 1,25D signaling in SCC25 cells are consistent with its role as a tumour suppressor and a key cofactor of vitamin D signaling. Regulation of canonical pathways up- or downregulated by 1,25D was generally reduced in FBW7-deficient cells. Unsurprisingly, given the role of the VDR and FBW7 in regulating cMYC, inhibition by 1,25D of expression of target genes of cMYC, a driver of cell cycle progression, was generally attenuated by knockout of FBW7. There was also dysregulation of several genes encoding cyclins and cyclin-dependent kinases, largely consistent with enhanced cell proliferation (Fig. 4). In addition, the major GO biological pathway upregulated in FBW7-depleted cells was “epithelial cell development”, in which expression of several genes associated with tumour progression was enhanced, whereas that of genes associated with tumour suppression was inhibited (Fig. 3). FBW7 depletion also upregulated the expression of several genes encoding histones in a manner that was essentially independent of vitamin D signaling.

Much of the focus of the current study was on the potential effects of FBW7 loss on the anticancer properties of 1,25D signaling. However, 1,25D is also a major regulator of innate immune pathways in epithelial cells and other innate immune cell types, notably through the induction of antimicrobial peptide genes such as *CAMP*. It is also anti-inflammatory⁸². Induction of *CAMP* expression by 1,25D in FBW7-deficient cells is strongly inhibited (Fig. 1). Moreover, pathways analysis revealed that the degree of upregulation by 1,25D of innate immune pathways and downregulation of inflammatory pathways was suppressed in the absence of FBW7 (Fig. 5).

In conclusion, results presented above show that FBW7 depletion has widespread effects on gene expression in SCC25 cells, most of which are consistent with its loss being a marker of cancer progression. The results also strongly support FBW7 being a cofactor of

the VDR that contributes broadly to both induction and repression of gene transcription observed in the presence of 1,25D. Finally, our findings are consistent with *de novo* FBW7 mutations being a plausible mechanism of acquired resistance to 1,25D analogue therapy.

Materials and Methods

CRISPR/Cas9 genome editing

CRISPR/Cas9 genome editing was done using the protocol of Malina et al (1). The target sequence, AGATGCATACAACGCACAGTGG, was designed using the website <https://chopchop.cbu.uib.no/> to target the human *FBXW7* gene and then cloned in the LeGO-U6LTR-iGCas9-GFP plasmid. The plasmid is a gift from Dr. Jerry Pelletier, McGill University. To produce viral particles containing LeGO-U6LTR-iGCas9-GFP, HEK293T/17 cells (CRL-11268, ATCC) were transfected along with packaging plasmids using Calfectin (SL100478, Signagen) reagent. To transduce SCC25 cells (CRL-1628, ATCC), the medium from HEK293T/17 cells containing lentiviral particles was collected, filtered, and added to SCC25 cells. The cells were grown for 3 weeks and then a single-cell green fluorescence protein selection was made by fluorescence-activated cell sorting. Different clonal cell lines were then tested via *FBXW7* genomic DNA PCR on the site of Crisper/Cas9-gRNA cut using AGGAAGCTGACAACACTAGCA and TGTCTGAGAACATTAGTGGGACA.

Cell culture and treatment.

We performed gene expression profiling studies by RNAseq in triplicate in wild-type and FBW7-depleted SCC25 to determine the effects of FBW7 ablation on nuclear 1,25D-dependent transactivation and repression. Cells were treated with 100nM 1,25D (BML-DM200, Enzo Life Sciences), or vehicle (dimethyl sulfoxide) for 16 hours to generate expression profiles enriched in primary target genes.

RNA sequencing

RNA sequencing was conducted essentially as described^{83,84}. Total RNA was extracted using the FavorPrep Blood/Cultured Cell Total RNA Mini Kit (FABRK 001, Favorgen) according to the manufacturer's protocol. RNA samples with an OD 260/280 ratio higher than 1.7 and an RNA integrity number greater than 7 were kept for subsequent analysis. Next, these samples were submitted to Génome Québec for paired-end sequencing with 100M reads on an Illumina NovaSeq PE100 sequencer. Library preparation was performed using the polyA Enriched RNA Library Preparation. All samples were included in the analysis as they met quality standards by QC reports from Génome Québec. The quality of sequence reads was then verified using FastQC, with poor-quality reads determined based on the Phred score, which is logarithmically related to base calling error probabilities. The

Phred offset quality score was greater than 30, and the minimum fragment size for alignment was set to 50. Using HISAT2⁸⁵, reads were mapped to the human GRCh38 genome assembly. Gene expression was quantified by counting uniquely mapped reads with featureCounts, using default settings. Normalization of gene counts and differential gene expression analysis were executed with the edgeR Bioconductor R package⁸⁶. Genes with $\geq|1.5|$ fold-change and adjusted p-values ≤ 0.05 were considered significant. Differentially expressed genes from the RNAseq analysis are available in Tables S1-S3.

Data availability

The data presented in this study are deposited in the Gene expression omnibus (GEO) repository, accession number GSE312616 and are available at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE312616>

Bioinformatics and pathways analysis

The overlap between conditions is illustrated using the DeepVenn website <https://www.deepvenn.com/>⁸⁷. Principal component analysis (PCA) was performed using the R function plotMDS and visualized with the limma R package. Pathways analyses enrichment was carried out using clusterProfiler, Disease Ontology (DOSE) and Gene set enrichment analysis (GSEA) R packages⁸⁸⁻⁹⁰. In addition, QIAGEN's Ingenuity Pathway Analysis software (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to probe for additional enriched pathways. For visualisation of expression level per gene by heatmaps, using edgeR, the logCPM (counts per million) was calculated for each gene using TMM (trimmed mean of M values) normalization method to calculate effective library sizes which are subsequently used as part of the per-sample normalization⁹¹. After this, a z-score normalization was performed to render features comparable⁹². Heatmaps with hierarchical clustering were constructed using the heatmap.2 package in R.

RNA extraction, reverse transcription and qPCR

RNA extraction was performed using the FavorPrep™ Tissue Total RNA Mini Kit (FATRK 001, Favorgen) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using the 5× All-in-One RT Mastermix (G485, abm) and diluted 5 times. Quantitative polymerase chain reaction (qPCR) was conducted with BrightGreen 2×qPCR MasterMix (MasterMix-LR-XL, abm) on a Roche Applied Science LightCycler 96 machine. Gene expression was normalized to Actin.

Statistics

A one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons was used using the dplyr R package. A p value of less than or equal to 0.05 was considered

significant. To denote p values, symbols were used as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and ns ≥ 0.05 .

Declarations

Funding Declaration

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Author Contribution

Wrote the paper: RST, JHW, AI. Conceived and designed experiments: JHW, RST, AI. Performed experiments (RST, ZC) and analysis of experimental data: RST, JHW. Methodology and analysis of RNAseq (bioinformatics): AI, RST. Supervision: JHW.

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Data Availability

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Figures

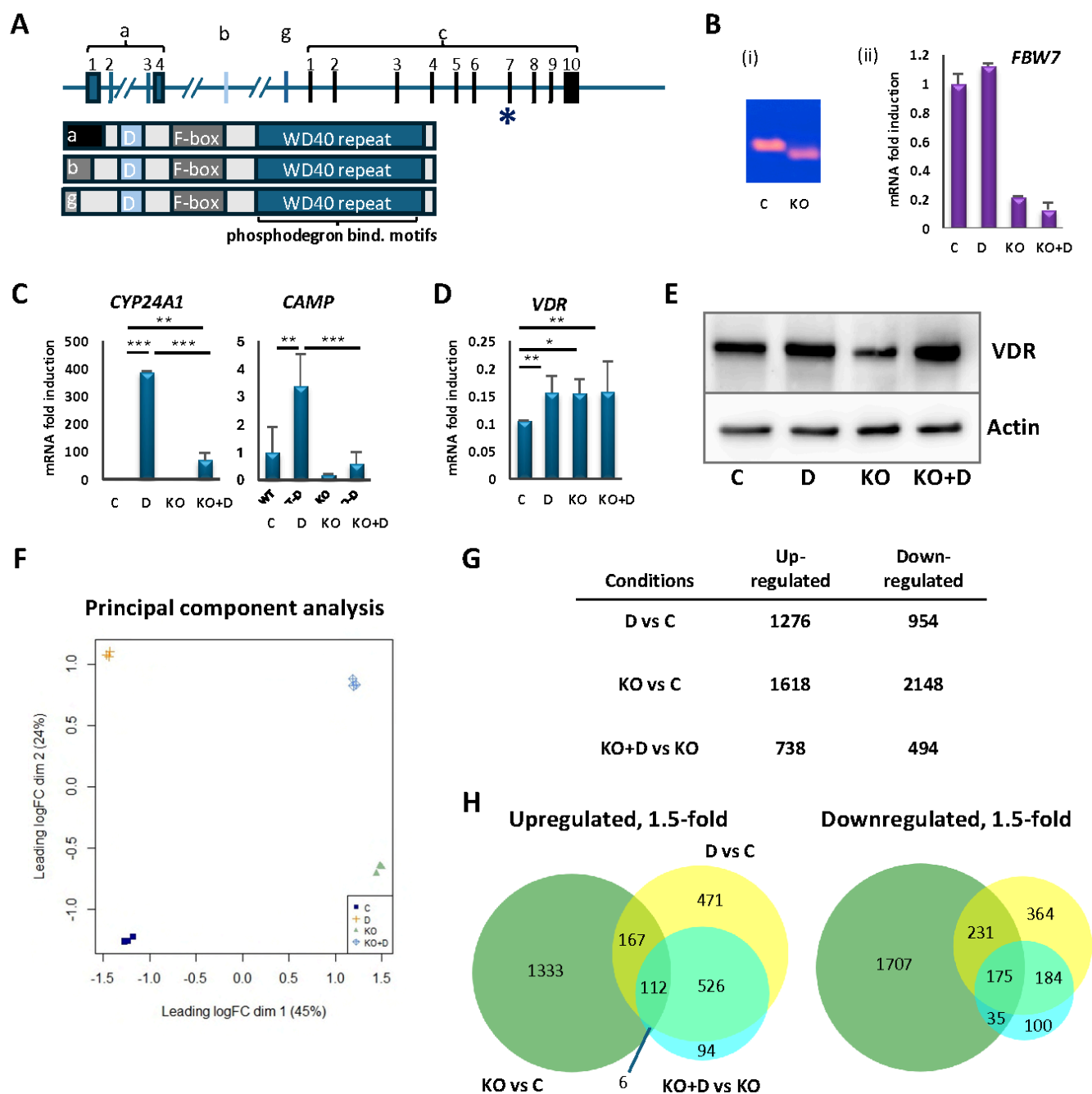


Figure 1

A. Schematic representation of the *FBW7* gene structure and protein domains. The CRISPR/Cas9-generated mutation was in exon c7 (asterisk) in sequence encoding the WD40 domains. **B.** (i) Agarose gel analysis of PCR fragments amplified from wild-type and *FBW7*-mutated SCC25 cells (see Fig. 1A). (ii) RT/qPCR analysis of *FBW7* mRNA expression in wild-type and *FBW7*-mutated cells in the absence and presence of 1,25D (D). RT/qPCR analysis of *CYP24A1*, *CAMP* (**C**) and *VDR* (**D**) in control-treated (C), 1,25D-treated (D), *FBW7* depleted (KO) and 1,25D-treated *FBW7* depleted cells (KO+D).

Graphics representative of 3 biological replicates. Graphics are mean $\pm$ SD from 3 technical replicates from a representative sample and one-way ANOVAs were used to determine significance (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and ns ≥ 0.05). **E.** Western blotting of VDR expression in wild-type and *FBW7*-depleted cells. **F.** Principal component analysis of triplicate untreated control cells, 1,25D-treated control cells, FBW7 depleted and 1,25D-treated FBW7 depleted cells. (See uncropped gel images in Supplementary Fig. S4) **G.** Table of differentially expressed genes (DEGs) regulated 1.5-fold ($p < 0.05$) by 1,25D and KO alone relative to control as well as by 1,25D-treated KO cells relative to KO. **H.** Venn diagrams of 1.5-fold regulated gene expression changes in cells treated by 1,25D (yellow), FBW7-depleted cells (green), and 1,25D-treated KO cells (aqua).

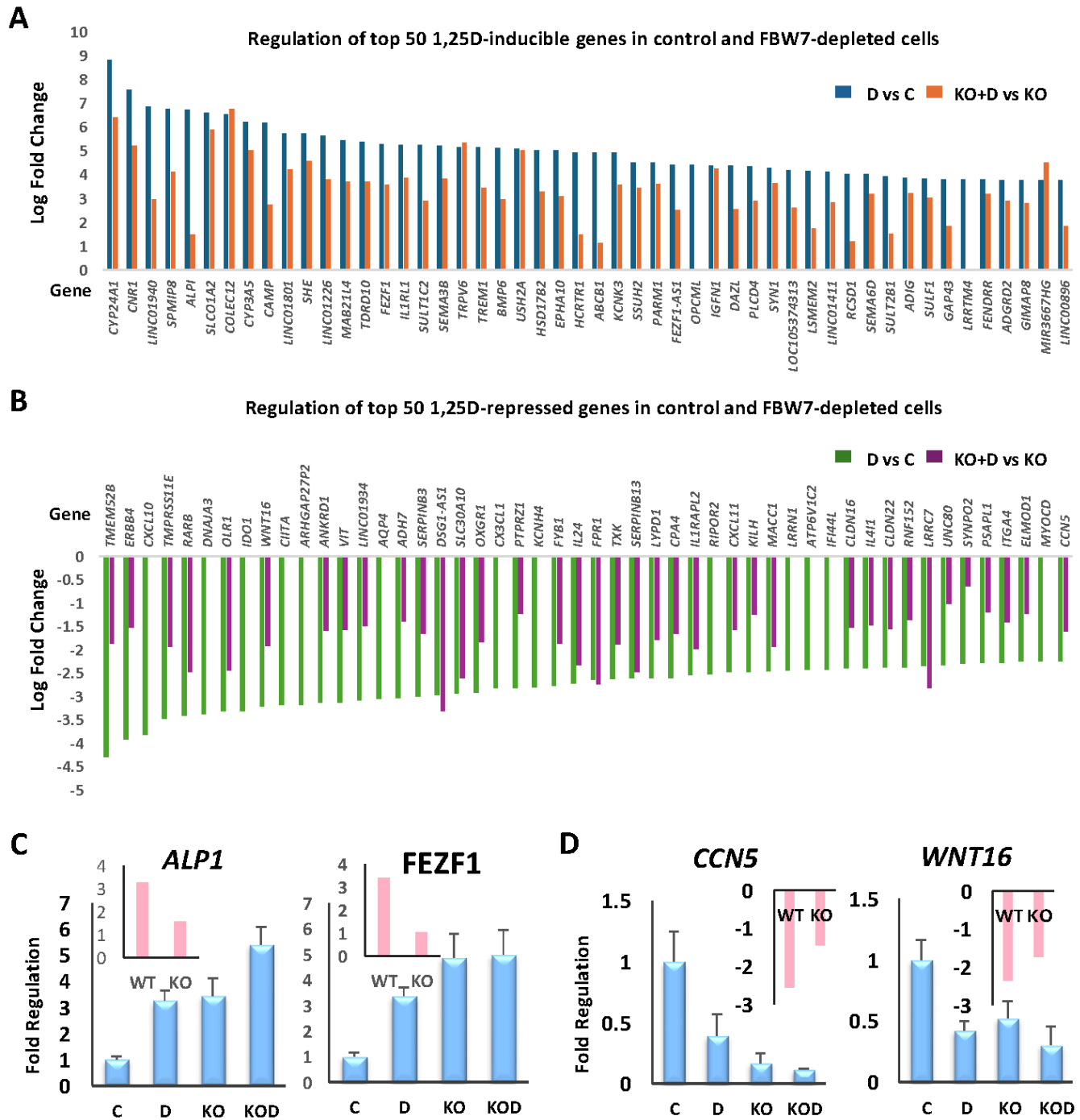


Figure 2

A. Regulation of top 50 1,25D-inducible genes in control and FBW7-depleted cells. **B.** Regulation of top 50 1,25D-repressed genes in control and FBW7-depleted cells. **C, D.** RT/qPCR analysis of a subset of genes from A-B. The changes in fold regulation are shown as insets in each figure.

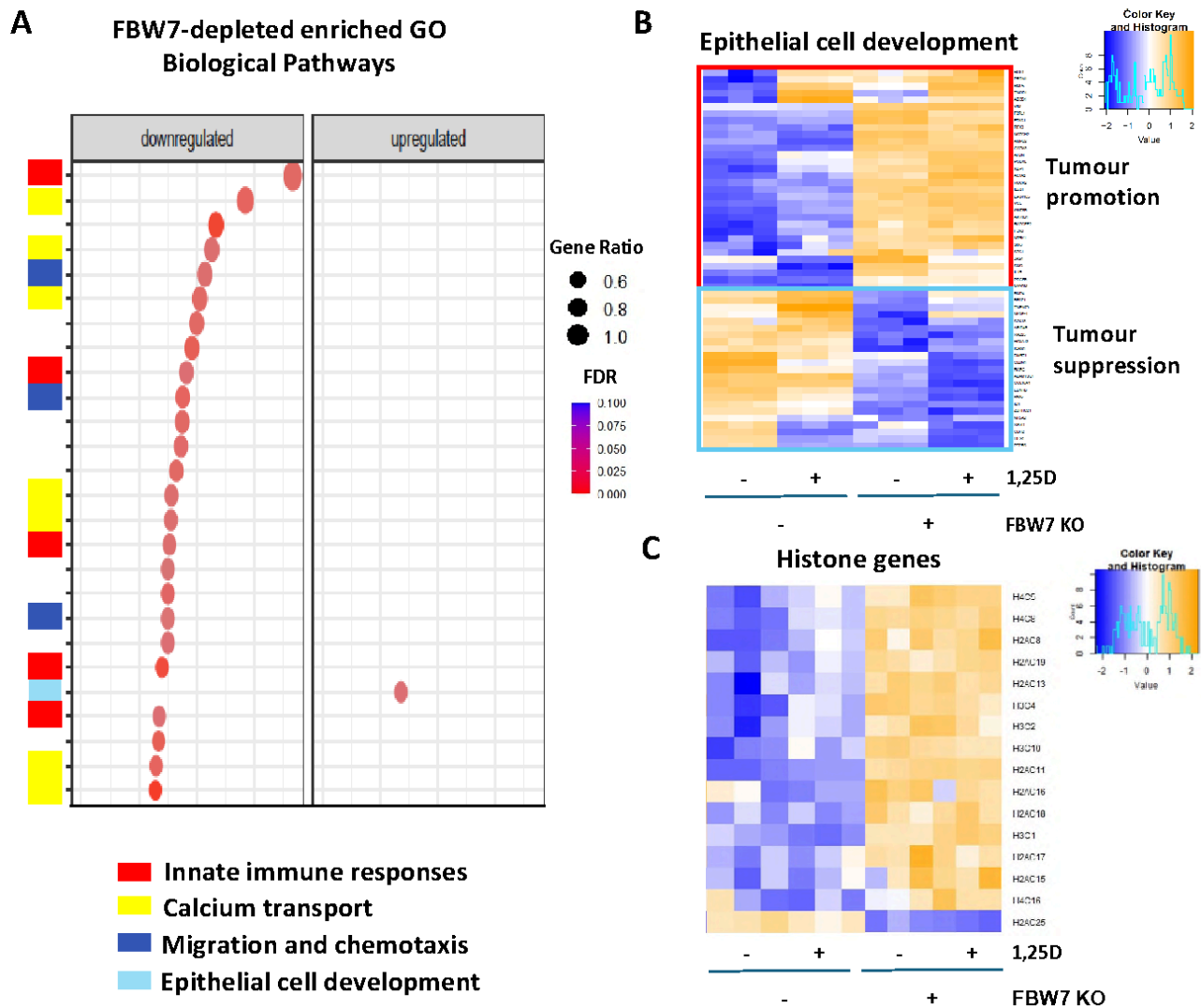


Figure 3

A. Dotplot of enriched GO biological pathways as determined by Gene set enrichment analysis (GSEA) in FBW7-depleted cells relative to control. FDR = False discovery rate. Gene ratio refers to the ratio of differentially expressed genes (DEGs) being up or downregulated relative to the total number of DEGs within a pathway. **B.** Heatmap of regulation of genes within the 'Epithelial cell development' category in the absence and presence of 1,25D and in control and FBW7 depleted cells. Upregulated DEGs within the category are depicted as orange, and those downregulated are blue. **C.** Heatmap of regulation of genes encoding histones in the absence and presence of 1,25D and in control and FBW7 depleted cells.

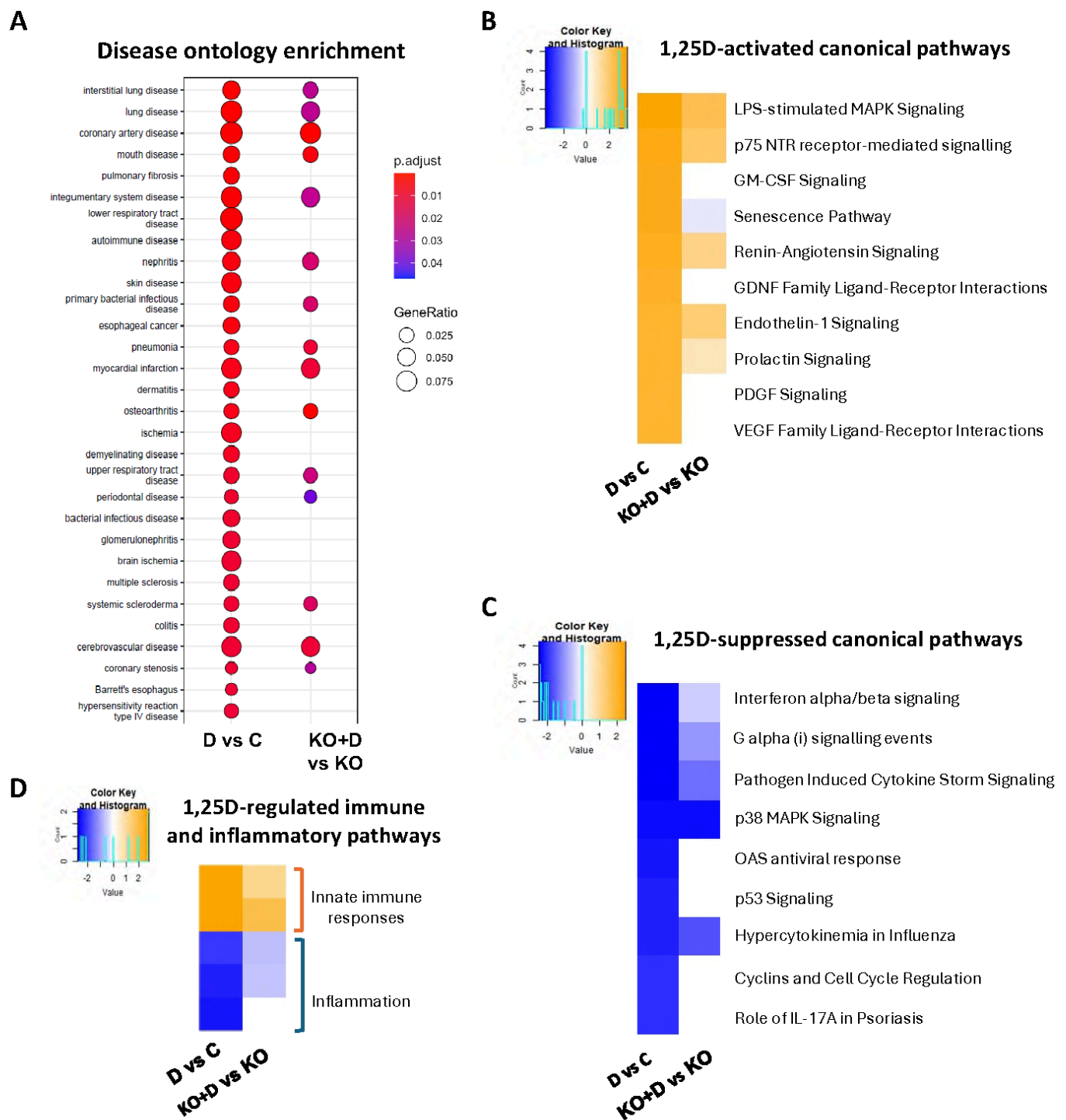


Figure 5

A. Enriched disease ontologies in 1,25D-treated control and FBW7-depleted cells relative to control and FBW7 KO, respectively. **B-D.** Heatmaps of 1,25D-regulated pathways in control and FBW7 KO cells.

Supplementary Files

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- [SupplementaryDatasetFileTableS2DEGsFBW7KOvs.WT.xlsx](#)
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