Proteomic analysis of 1 α ,25-Dihydroxyvitamin D₃ action on human colon cancer cells reveals a link to splicing regulation

Iván Cristobo¹⁺, María Jesús Larriba^{2,3+}, Vivian de los Ríos⁴, Francisco García⁴, Alberto Muñoz^{2,3} and J. Ignacio Casal^{1*}

1. Functional Proteomics Laboratory. Centro de Investigaciones Biológicas. Consejo Superior de Investigaciones Científicas. Madrid. Spain

2. Instituto de Investigaciones Biomédicas “Alberto Sols”. Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid. Madrid. Spain

3. Instituto de Investigación Biosanitaria del Hospital Universitario La Paz (IdiPaz)

4. Proteomics Facility. Centro de Investigaciones Biológicas. Consejo Superior de Investigaciones Científicas. Madrid. Spain

⁺. Both authors contributed equally to this work

Running title: Proteomic analysis of vitamin D action in cancer cells

* Corresponding author

Functional Proteomics Laboratory

Centro de Investigaciones Biológicas. CSIC.

Ramiro de Maeztu 9

28040 Madrid. Spain

e-mail: icasal@cib.csic.es

FAX: 34-915360432

ABSTRACT

1 α ,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) and other vitamin D compounds are promising molecules in the prevention and therapy of colon cancer and other neoplasias. To study the mechanism of action of 1,25(OH)₂D₃ in colon cancer cells, we carried out a comparative proteomic analysis of the nuclear fractions of SW480-ADH cells treated with 1,25(OH)₂D₃ or vehicle during 8 or 48h. 2D-DIGE analysis combined with MALDI-TOF-TOF mass spectrometry interrogation led to the identification of 59 differentially-expressed unique proteins. Most identified proteins were nuclear, but several cytoskeleton-associated proteins were also detected. A good concordance between changes in expression at protein and RNA levels was observed for the validated proteins. A large group of identified proteins, such as SFPQ, SMARCE, KHSRP, TARDBP and PARP1, were involved in RNA processing or modification and have been ascribed to the spliceosome compartment of human cells. In addition, a smaller group of proteins (ERM (Ezrin, Radixin, Moesin) family, VCL, CORO1C, ACTB) were cytoskeleton-associated and played a role in cell adhesion and morphology. These results confirm the induction of epithelial phenotype by 1,25(OH)₂D₃ and suggest a role for vitamin D compounds in the regulation of the spliceosome and thus, in alternative splicing and possibly microRNA synthesis in colon cancer cells.

Keywords: Vitamin D, colon cancer, spliceosome, nuclear proteins, 2D-DIGE

1 INTRODUCTION

2 Many epidemiological studies have shown an inverse relation between exposure to
3 sunlight, vitamin D₃ (cholecalciferol) intake or 25-hydroxyvitamin D₃ (calcidiol) blood
4 levels and the incidence of several neoplasias, particularly colorectal cancer (CRC) [1-4].
5 Preclinical data obtained in cultured cells and experimental animals support a protective
6 effect of vitamin D compounds (vitamin D₃, its most active metabolite 1 α ,25-
7 dihydroxyvitamin D₃ (calcitriol), or less calcemic derivatives) against a variety of cancers
8 [5-7]. Clinical trials have been scarce and inconclusive [7-9], and several are in progress
9 using different vitamin D compounds alone or in combination in patients with different
10 neoplasms (see *ClinicalTrials.gov*).

11 1 α ,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) has anti-proliferative, pro-apoptotic and
12 pro-differentiation effects on many tumor cell lines. In human SW480-ADH colon cancer
13 cells that express vitamin D receptor (VDR), 1,25(OH)₂D₃ induces *CDH1*/E-cadherin
14 expression, an epithelial differentiated adhesive phenotype and antagonizes the Wnt/ β -
15 catenin signaling pathway [10]. A global transcriptomic study showed that 1,25(OH)₂D₃
16 changes significantly the RNA expression profile of these cells: about two-thirds of the
17 target RNAs were upregulated and one-third were downregulated [11]. These RNAs
18 encoded for proteins involved in many different cell functions including transcription, cell
19 adhesion, DNA synthesis, apoptosis, redox status and intracellular signaling. In-depth
20 functional studies of some of the reported target genes such as *DICKKOPF-1*,
21 *CST5/Cystatin D* or *SPROUTY-2* confirmed their role mediating the protective effect of
22 1,25(OH)₂D₃ against colon cancer [12-14].

23 Several transcriptomic studies searching for RNAs regulated by 1,25(OH)₂D₃ have

1 been done in different cell types [11, 15-17]. However, studies at the protein level in
2 human cancer cells are lacking. In order to get a more comprehensive understanding of the
3 molecular mechanisms of action of $1,25(\text{OH})_2\text{D}_3$ we have used 2D-DIGE electrophoresis
4 combined with MALDI-TOF-TOF mass spectrometry to identify proteins regulated by
5 $1,25(\text{OH})_2\text{D}_3$ in SW480-ADH cells. We used nuclear extracts from $1,25(\text{OH})_2\text{D}_3$ - and
6 vehicle-treated cells based on the results of previous transcriptomic studies, which showed
7 that $1,25(\text{OH})_2\text{D}_3$ regulates many transcription factors and other nuclear proteins [11].
8 Moreover, the wide gene regulatory effects of $1,25(\text{OH})_2\text{D}_3$ cannot be exclusively
9 mediated by VDR binding to their promoters and should require additional changes in
10 transcription regulators. Finally, the pre-fractionation of the cellular content should
11 facilitate the identification of non-abundant proteins by decreasing those of preferential
12 cytosolic and membrane location. Here, a number of novel $1,25(\text{OH})_2\text{D}_3$ -regulated proteins
13 were identified. Validation of candidate target proteins was performed by Western blot,
14 immunofluorescence and quantitative PCR analyses. Interestingly, several $1,25(\text{OH})_2\text{D}_3$ -
15 regulated proteins were related with the spliceosome compartment and RNA metabolism,
16 suggesting a role of $1,25(\text{OH})_2\text{D}_3$ on alternative splicing, a process frequently altered in
17 cancer cells [18-19].

MATERIALS & METHODS

Cell culture

SW480-ADH human colon cancer cells were grown in DMEM supplemented with 10% FCS (Invitrogen). The active vitamin D metabolite 1,25(OH)₂D₃ was used for cell treatment. Briefly, cells were washed twice with PBS and incubated with 100 nM 1,25(OH)₂D₃ (with isopropanol as vehicle) in DMEM supplemented with liposoluble hormone-depleted FCS for the indicated times.

Protein extraction and subcellular fractioning

To obtain nuclear extracts, cell monolayers were washed in PBS and lysed for 15 min in hypotonic buffer (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with 1 mM DDT, 1 mM ortovanadate, 1 mM PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin. Just before centrifugation (13,000 rpm at 4°C for 1 min), 10% NP40 was added to the tubes. Supernatants (cytosolic extracts) were conserved at -80°C until analysis. Pellets containing nuclei were lysed by incubation for 30 min in hypertonic buffer (20 mM HEPES pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) supplemented as above, and then centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants (nuclear extracts) were conserved at -80°C until analysis. Protein expression of cytosolic (β-Tubulin and Rho-GDI) and nuclear (HDAC, Lamin B and VDR) markers was assessed by Western blot to confirm a correct fractioning (Fig. 1A).

2D-DIGE electrophoresis

Nuclear extracts were precipitated using methanol/chloroform [20]. Briefly, samples were

1 incubated with 0.1% deoxycolic acid at 4°C for 10 min. Just before centrifugation (13,000
2 rpm at 4°C for 30 min), cold methanol and chloroform were added to the sample tubes.
3 Protein-interphase was washed and centrifuged twice with cold methanol. Pellets were
4 dried and resuspended in 2D lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl pH 8.5,
5 4% CHAPS). 2D-DIGE experiments were carried out as previously described [21-23].
6 Briefly, after protein quantification with RC DC Protein Assay (Bio-Rad), 50 µg of each
7 nuclear protein extract was DIGE labeled (CyDye DIGE Fluor, minimal labeling kit, GE
8 Healthcare) with 400 pmol CyDyes on ice for 30 min and then blocked with L-Lysine for
9 10 min (incubations were done in the dark on ice). Nuclear protein extracts from SW480-
10 ADH control and 1,25(OH)₂D₃-treated cells were labeled with Cy3 and Cy5
11 fluorochromes, respectively. A pool containing equal amounts of extract from each cell
12 condition was labeled with Cy2 dye and was included in all gel runs. First dimension was
13 run on IPG strips (pH 3–10 NL, 18 cm length) (GE Healthcare) in a Multiprotean IEF cell
14 (Bio-Rad) with a 7 steps program (50 V for 10 h, 100 V for 1 h, 250 V for 1 h, 500 V for 1
15 h, 1000 V for 1 h, 8000 V for 1 h and 8000 V until voltage reached 50 kVh). Second
16 dimension was run overnight on 11% SDS-PAGE in a cooled Protean Plus Dodeca (Bio-
17 Rad). Samples were run for 1 h more after the dye-front run out of the gel. Protein
18 expression patterns were visualized with a Typhoon 9400 scanner (GE Healthcare). Images
19 were processed using two different softwares for analysis: Redfin Analysis Center-Pro
20 (Ludesi) and Progenesis SameSpots (NonLynear Dymanics). Cy3: Cy2 and Cy5: Cy2 ratios
21 for each individual protein were used for quantification. After quantification and
22 normalization of spots, statistical analysis was applied to find significant differences
23 between groups of samples. The 12 spot maps corresponding to 4 replica gels/treatment
24 were used to calculate average abundance changes and paired ANOVA *p*-values and fold-

change for each protein across the different gels. Protein spots that showed a significant change in abundance between control and treatment (ANOVA-test $p < 0.05$; fold-change > 1.25 or < 0.80) were selected for further characterization by mass spectrometry.

Identification of proteins by MALDI-TOF-TOF peptide mass fingerprinting

A preparative gel containing 500 μg of each nuclear extract was run and stained with the Colloidal Blue Staining Kit (Invitrogen) for protein visualization and spot picking after matching against the analytical gels. Spots of interest were automatically excised using a ProteomeWorks Spot Cutter (Bio-Rad) and subjected to manual tryptic digestion. For digestion, gel pieces were washed first with 50 mM ammonium bicarbonate (Sigma-Aldrich) and second with ACN (Scharlau). Trypsin (Promega), at a final concentration of 12.5 ng/ μl in 50 mM ammonium bicarbonate solution, was added to the gel pieces for 8 h at 37 °C. Finally, 70% ACN containing 0.5% TFA (Sigma-Aldrich) was added for peptide extraction. Tryptic eluted peptides were dried by speed-vacuum centrifugation and resuspended in 4 μl of 30% ACN - 0.1% TFA. One μl of each peptide mixture was deposited onto an 800 μm AnchorChip (Bruker-Daltonics) and dried at RT. One μl of matrix solution (3 mg/ml α -cyano-4-hydroxycinnamic acid) in 33% ACN - 0.1% TFA was then deposited onto the digest and allowed to dry at room temperature.

Samples were analyzed with an Autoflex III TOF/TOF mass spectrometer (Bruker-Daltonics). Typically, 1000 scans for peptide mass fingerprinting (PMF) and 2000 scans for MS/MS were collected. Automated analysis of mass data was performed using FlexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z 842.510 and m/z 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra

1 obtained for the proton adducts of a peptide mixture covering the m/z 800–3200 region.
2 The typical error observed in mass accuracy for calibration was usually below 20 ppm. We
3 verified this accuracy intra-experiment with ACTB peptides. MALDI-MS and MS/MS
4 data were combined through the BioTools 3.0 program (Bruker-Daltonics) to interrogate
5 the NCBI non-redundant protein database (NCBI: 20100306) using MASCOT software
6 v2.3 (Matrix Science). Relevant search parameters were set as follows: enzyme, trypsin;
7 fixed modifications, carbamidomethyl (C); oxidation (M); 1 missed cleavage allowed;
8 peptide tolerance, 20 ppm; MS/MS tolerance, 0.5 Da. Protein scores greater than 75 were
9 considered significant ($p < 0.05$).

11 *Western blot*

12 Nuclear protein extracts were separated by SDS-PAGE electrophoresis and transferred to
13 Transblot nitrocellulose membranes (Bio-Rad). Membranes were incubated with the
14 appropriate primary and secondary horseradish peroxidase-conjugated (Sigma-Aldrich) or
15 fluorescence Alexa-conjugated (Invitrogen) antibodies. The antibody binding was
16 visualized using SuperSignal Substrate (Pierce) or ECL Western Blot Detection Reagents
17 (GE Healthcare) with a fluorescent image analyzer FLA-3000 (Fuji Photo Film Co. Ltd.).
18 The following primary antibodies were used: LMNB, PARP1, NCL, HDAC, Rho-GDI
19 (Santa Cruz Biotechnology); SFPQ, KHSRP (Abnova Corporation); PPIA, VDR
20 (Millipore); TARDBP, RBBP4, NPM1, PHB (Abcam), β -Tubulin (Sigma-Aldrich). EZR
21 and SMARCE1 antibodies were kindly provided by Dr JM Serrador (Centro Nacional de
22 Investigaciones Cardiovasculares, Madrid) and Dr B Belandia (Instituto de Investigaciones
23 Biomédicas “Alberto Sols”, Madrid), respectively. Quantification was done by

densitometry using Quantity One software (Bio-Rad) and LMNB was used as a loading control.

Immunofluorescence and confocal microscopy

Cells were rinsed once with PBS, fixed in 4% paraformaldehyde for 10 min at RT and rinsed twice with PBS. Afterward, cells were permeabilized with 0.2% Triton X-100 for 10 min and then washed three times with PBS. Non-specific sites were blocked with 5% BSA in PBS for 1 h at RT. Then, cells were incubated with primary antibodies diluted in PBS-5% BSA for 1 h at 37°C in a humidity chamber. After three washes in PBS, cells were incubated with Alexa-conjugated antibodies diluted 1/250 for 1 h at 37°C, and then washed three times with PBS. Finally, cells were mounted in fluorescent media (Dako). Confocal microscopy was performed with a TCS SP5 laser scanning microscope (Leica), using a 63x oil objective (2x zoom) and excitation at 488/633 nm to detect Alexa 488 or Alexa 647, respectively. We used primary antibodies against: EZR, KHSRP, NPM1, PARP1, RBBP4, SFPQ, SMARCE1, TARDBP (same as for Western blot), NCL (Abcam) and CDH1/E-cadherin (BD Biosciences). Anti-rabbit Texas Red-conjugated was used for NCL as secondary antibody.

RNA extraction and quantitative RT-PCR

RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen) and retrotranscribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Target genes RNA expression was measured by quantitative RT-PCR in relation to that of 18S rRNA applying the comparative C_T method [24] and using RNA TaqMan probes (Applied Biosystems) for *KHSRP*, *PARP1* and *SFPQ* or oligonucleotides for *SMARCE1* (sense 5'-

GTCGGTTGTCACAACAGCTAGAA-3', antisense 5'-TCTAGTTTCCGCTGATGAACCA-3') and *CDH1*/E-cadherin (sense 5'-AGAACGCATTGCCACATACACTC-3', antisense 5'-CATTCTGATCGGTTACCGTGATC-3'). The quantitative PCR reaction was performed in an ABI Prism 7900 HT thermal cycler using TaqMan or SYBR Gene Expression Master Mix (both from Applied Biosystems). Thermal cycling consisted of a denaturing step at 95°C for 10 min and 40 cycles of denaturing at 95°C for 15 s and annealing and elongation at 60°C for 60 s. Statistical significance was assessed by two-tailed unpaired Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Bioinformatic analysis

GO-term analysis and functional annotation was carried out with DAVID [25]. DAVID uses a modified Fisher Exact *p*-value to determine whether a GO-term is over- or under-represented in a given proteomic data set. Enrichment scores denote the geometric mean of all the enrichment *p*-values for each annotation term associated with the gene members in the group [26]. Enrichment scores above 1.3 were statistically significant (1.3 is equivalent to p -value < 0.05). Protein interaction analysis was carried out with the free web-based tool STRING [27]. STRING is a database of known and predicted protein interactions. We used iHOP [28] for text mining and KEGG for pathway studies [29]. Moreover, individual characteristics of the identified proteins (location, function, etc) were assigned or confirmed using bioinformatic tools like GeneCards (www.genecards.org). All these resources are freely available for academic users.

RESULTS

Identification of proteins regulated by 1,25(OH)₂D₃ using 2D-DIGE analysis

Nuclear extracts from SW480-ADH cells treated with 1,25(OH)₂D₃ or vehicle for 8 or 48 h were analyzed by 2D-DIGE, for early and late events, respectively. These time points were selected to discriminate between E-cadherin-dependent or -independent 1,25(OH)₂D₃ target genes, as *CDH1*/E-cadherin protein increases 10-12 h after 1,25(OH)₂D₃ treatment and is responsible for a drastic morphological change in SW480-ADH cells [10]. Also, they may permit to differentiate between direct and indirect 1,25(OH)₂D₃ targets. Changes in the pattern of protein expression were quantitatively and statistically analyzed by using Ludesi Redfin and Progenesis SameSpots considering the 12 spot-maps included in the study for each time point. Each gel was analyzed individually to calculate the volume ratios between individual samples and the internal pool, which was used as the standard reference. Then, all the images were combined and analyzed together by using both softwares. 115 spots differentially-expressed between 1,25(OH)₂D₃- and vehicle-treated cells were selected based on the fold-change (<0.80 or >1.25) and the ANOVA *p*-value (<0.05). The spots of interest were picked from a preparative gel for protein identification. Using this strategy 59 differentially-expressed unique proteins were identified in nuclear extracts from 1,25(OH)₂D₃-treated SW480-ADH cells (Tables 1a and 1b). In addition, a number of proteins was identified as different isoforms or fragments (ACTB (4), PP1A (4), EZR (3), XRCC6 (2), YWHAE (2), SMARCE1 (2), ATP5A1 (2), PHB (2), being the number of isoforms or fragments shown among parentheses), which raised the total number of identifications to 72. At 8 h, only 12 changes in protein abundance were observed between 1,25(OH)₂D₃-treated and control cells, all of them downregulated (Supplementary information Fig. 1, Table 1a); whereas 47 protein changes were found at

48 h treatment (*Fig. 1B, Table 1b*). 1,25(OH)₂D₃ might act primarily as repressing gene/protein expression because nuclear VDR binds to the promoter of their target genes, recruits transcriptional co-repressors and so inhibits gene expression. Then, 1,25(OH)₂D₃ increases VDR DNA binding and induces the replacement of VDR-bound co-repressors by transcriptional co-activators, altogether favouring gene induction. This may explain, at least in part, why the percentage of induced genes augments with time after 1,25(OH)₂D₃ treatment.

Downregulated proteins included SFPQ, SMARCE1, RBBP4, RBBP7, G3BP1, and various members of 14-3-3 protein family (YWHAE, YWHAG, YWHAQ). However, some proteins implicated in epithelial morphology and cell adhesion (ERM (Ezrin, Radixin, Moesin) family, VCL, ACTB, KRT8) were increased by 1,25(OH)₂D₃. Other proteins whose levels were upregulated were TARDBP, KHSRP and LMNA. A substantial number of identified proteins such as SFPQ, SMARCE, KHSRP, TARDBP and PARP1, among others, were involved in RNA maturation (splicing, modification) as constituents of the spliceosome and show predominant nucleolar location. Other proteins, such as EZR, although having a major role in cell adhesion or morphology and being mainly described as cytosolic, membrane or cytoskeletal proteins, have also been found to be partially nuclear [30].

Correlation between alterations at the protein and RNA level

Nuclear extracts of SW480-ADH cells treated with 1,25(OH)₂D₃ or vehicle for 8 or 48 h were analyzed by Western blot using specific antibodies against a panel of altered proteins (*Fig. 2*). Three independent experiments were done. We confirmed that the nuclear content of NCL, EZR, KHSRP and TARDBP increased by 1,25(OH)₂D₃ treatment. By contrast,

1 the levels of RBBP4, SFPQ, NPM1, PARP1, PHB, SMARCE1 and PPIA were lower in
2 the nuclear extracts of 1,25(OH)₂D₃-treated cells than in control cells. Ratios of expression
3 between 1,25(OH)₂D₃- and vehicle-treated cells were similar to those found by 2D-DIGE.

4 Furthermore, we selected a few proteins to study whether the changes in their
5 protein expression correlated with alterations at the RNA level. This was the case for all
6 four cases studied: similarly to protein levels, the RNA expression of *PARP1*, *SMARCE1*
7 and *SFPQ* decreased by 1,25(OH)₂D₃ treatment and those of *KHSRP* were induced (*Fig.*
8 *3*). These results suggest a transcriptional regulation of these genes. As a positive control,
9 we confirmed the induction by 1,25(OH)₂D₃ of *CDH1*/E-cadherin RNA expression, a well
10 known 1,25(OH)₂D₃ target gene [10, 31]. To further study the correlation between
11 alterations at the protein and RNA and level, we compared the results of the proteomic
12 study with those of our own transcriptomic analyses [11 and unpublished results]. This
13 analysis revealed that only 13 out of the 57 proteins were found as regulated by
14 1,25(OH)₂D₃ in SW480-ADH cells in both types of studies (*Table 2*). In all these cases,
15 regulation followed the same trend.

16 17 *Validation of differentially-regulated proteins by immunofluorescence analysis*

18 Immunofluorescence and confocal microscopy analysis of cells treated with 1,25(OH)₂D₃
19 or vehicle was used to further confirm the regulation and to examine the subcellular
20 location of the identified proteins (*Fig. 4*). A good correlation was observed with the
21 Western blot analyses. NCL pattern of expression was similar in vehicle- or treated-cells,
22 strong signal in nucleolus and diffuse in nucleoplasm, but more intense in 1,25(OH)₂D₃-
23 treated cells. TARDBP and KHSRP levels were higher in both nucleus and cytosol in
24 SW480-ADH treated with 1,25(OH)₂D₃, showing a punctuate pattern with a few defined

nuclear foci. In contrast, and in agreement with Western blot data, RBBP4, SFPQ, PARP1, NPM1 and SMARCE1 expression was lower in 1,25(OH)₂D₃-treated SW480-ADH cells than in vehicle-treated cells. For RBBP4, SFPQ and PARP1, the expression was exclusively nuclear; whereas NPM1 and SMARCE1 were located at both the nucleus and cytoplasm. As expected, EZR was preferentially located close to the plasma membrane and its expression was higher in 1,25(OH)₂D₃-treated cells (*Fig. 4*).

Cellular localization and biological function of 1,25(OH)₂D₃-regulated proteins

Gene ontology and functional classification of the identified proteins was carried out with DAVID bioinformatics tools [26]. Molecular function of the identified proteins according to GO ontology is represented in *Fig. 5A*. There was a predominant enrichment in functional terms like RNA and nucleotide binding, RNA processing, telomeric DNA binding, cytoskeleton organization, cell adhesion and protein kinase binding. These terms should indicate the biological functions where 1,25(OH)₂D₃ treatment exert its effects. In particular, there was enrichment in nuclear proteins (enrichment factor: 6.55, *Supplementary information Fig. 2A*). Some of these proteins were previously identified as nucleolar [32]. By functional annotation clustering, one of the main groups was related to RNA binding and processing (enrichment factor: 2) (*Fig. 5B*). This group includes 17 proteins, either up- or down-regulated, which stresses the relevance of 1,25(OH)₂D₃/VDR as a regulator of RNA maturation and RNA metabolism/editing. Proteins as KHSRP, TARDBP or SFPQ work as splicing factors and participate in the formation of the spliceosome [33-34]. Ribosomal proteins (RPLP2, RPSA, MRPS23), ATPases (ATP5B), helicases (XRCC6), PARP1 and chaperones (HSPA9) are also common components of the nucleolar fraction and many of them have also been reported as components of the

1 proteome of spliceosomes assembled *in vitro* [35]. In addition, it has recently been
2 described the ability of PARP1 to modulate the splicing process [36].

3 Another functional group was composed by proteins related with cytoskeleton
4 organization, actin binding and cell adhesion (LMNA, VCL, SEPT2, KRT8, KRT9,
5 ACTB, ERM family) (enrichment score: 1.96, *Supplementary information Fig. 2B*).

6 7 *Alterations in the spliceosome compartment and cellular adhesion proteins in* 8 *1,25(OH)₂D₃-treated colon cancer cells*

9 Proteins identified as 1,25(OH)₂D₃ targets were classified and clustered using several
10 algorithms. STRING was used to find interaction groups. In order to expand the initial
11 interaction network, STRING added 10 more partners. By means of MCL algorithm, two
12 major clusters of interacting proteins were highlighted by STRING (*Fig. 6*). A large cluster
13 corresponded to proteins previously identified as spliceosome components [35]. Within
14 this group were TARDBP, KHSRP, SFPQ and the family of DNA-directed RNA
15 polymerases I, II, and III, which synthesize ribosomal RNA precursors, mRNA precursors
16 and a large number of non-coding RNAs and small RNAs, respectively. The presence of
17 RNA polymerase II and other transcription factors in the spliceosome was previously
18 described [35], suggesting a tight coupling of transcription with splicing [37].

19 Up-regulated KHSRP is a well-known regulator of splicing, among its targets being
20 the c-*SRC* proto-oncogene [38]. KHSRP plays also a role in the processing of microRNAs
21 as a component of both Drosha and Dicer complexes and regulates the biogenesis and
22 maturation of a subset of miRNAs [39]. Additionally, KHSRP interacts with single-strand
23 AU-rich-element-containing mRNAs and is a key mediator of mRNA decay [40]. It targets
24 the expression of mRNAs that affect specific biological programs, including proliferation,

1 apoptosis and differentiation. TARDBP is also up-regulated by $1,25(\text{OH})_2\text{D}_3$, it displays a
2 similar functionality to KHSRP in the regulation of microRNAs, i.e. let7b [41]. Other
3 interaction platforms (iHOP) showed interactions between TARDBP and TMLHE and
4 RNA polymerases I, II, and III.

5 According to the text-mining program iHOP (data not shown), LMNA, CORO1C,
6 XRCC6 or NCL proteins act as a hub to interconnect other proteins such as NPM1,
7 PPP1CB, EEF1G or RBBP7. RBBP4 interacts with HDAC1 and both mediate chromatin
8 remodeling and transcription regulation. In line with this, a recent genome-wide analysis of
9 VDR binding sites has shown a significant enrichment of sites in regions associated with
10 active chromatin such as DNase I-hypersensitive sites and regions with specific histone
11 modifications [42]. Another interaction node was centered on NCL and PPP1CB (protein
12 phosphatase 1, beta isoform), which might cooperate to downregulate NPM1 and HMGB2
13 that are involved in cell proliferation. A few small groups including elongation factors,
14 mitochondrial proteins and the 14-3-3 protein family were also observed (*Fig. 6*).

15 A second cluster was composed of cytoskeleton-associated proteins (ERM family,
16 VCL, ACTB, CORO1C, PPP1CB (also known as MLCP) and IQGAP3) (*Fig. 6*). KEGG
17 pathways analysis showed that increases in the abundance of ERM family, VCL and
18 ACTB contribute to the increase of focal adhesions in $1,25(\text{OH})_2\text{D}_3$ -treated cells and,
19 therefore, to cell-matrix adhesion [43] (*Supplementary information Fig. 3*). It also
20 suggested the involvement of phosphatidylinositol-5-phosphate 4-kinase type II β
21 (PI4P5K) and phosphatidylinositol-4,5-bisphosphate (PIP2) signalling in VCL and ACTB
22 expression and focal adhesion assembly. Accordingly, PI4P5K has been recently
23 implicated in the induction of E-cadherin and the inhibition of cell motility by
24 $1,25(\text{OH})_2\text{D}_3$ in colon cancer cells [44]. A decrease in IQGAP3 expression should increase

1 the adherens junctions and cell-cell contacts in the treated cells. Collectively, these data
2 support the observations that 1,25(OH)₂D₃ modulates cell morphology through the RhoA-
3 ROCK pathway [31]. An increase of PPP1CB phosphatase could reduce actomyosin
4 assembly contraction. Interestingly, PPP1 phosphatases have also been located in the
5 nucleolus and are rapidly exchanging between the nucleolar, nucleoplasmic and
6 cytoplasmic compartments [45]. Likewise, regarding down-regulation of CORO1C by
7 1,25(OH)₂D₃, is worth mentioning that CORO1C-depleted cells have been reported to
8 display increased cell-matrix adhesion and enhanced cell spreading [46]. These findings
9 related to epithelial markers are in agreement with the observed effect of 1,25(OH)₂D₃ and
10 several derivatives regulating the morphology and phenotype of colon carcinoma cells [10,
11 31].

DISCUSSION

Vitamin D compounds are promising molecules for the prevention and possibly for the therapy of cancer. They have pleiotropic effects affecting many intracellular pathways and cell compartments. Although several transcriptomic studies have identified genes whose RNA expression respond to $1,25(\text{OH})_2\text{D}_3$ or derivatives, little was known about changes at the protein level in cancer cells. In this report we describe for the first time the proteomic alterations induced by $1,25(\text{OH})_2\text{D}_3$ in the nucleus of human colon cancer cells. 2D-DIGE quantitative analysis enabled the identification of a number of target proteins that were predominantly down-regulated by $1,25(\text{OH})_2\text{D}_3$, particularly after 8 h treatment. In contrast, previous transcriptomic studies revealed that two thirds of $1,25(\text{OH})_2\text{D}_3$ -regulated RNAs were up-regulated and one third was down-regulated [11]. However, when we compared our transcriptomic and proteomic data, we recovered 13 proteins that were present in both analyses as regulated by $1,25(\text{OH})_2\text{D}_3$ and these proteins showed the same trend in regulation. The reasons for the apparent discrepancy between proteomic and transcriptomic data are unclear and, probably, multifactorial. Among other reasons, the differences might arise from comparing a transcriptomic profile, that was not genome-wide [11], with the more partial nuclear proteome regulated by the hormone. Also, a different post-translational regulation, alternative splicing or stability issues of the proteins could explain the differences between mRNA and protein levels. In addition, as 2D-DIGE analysis is biased towards the detection of relatively abundant proteins, it can be assumed that additional changes affecting low-level expressed proteins were not detected. The use of more powerful LC-MS systems could lead to a deeper insight into the regulatory effects of vitamin D compounds on the proteome.

Despite nuclear extracts were used in the study, only 27 out of 59 proteins were

pre-assigned to the nucleus (*Table 1*), while the remaining were preferentially cytosolic, cytoskeletal, mitochondrial or membrane proteins. Although we cannot discard a slight contamination of nuclear extracts with cytosolic proteins, the latter proteins may be partially or temporarily present in the nucleus due to chemical modifications and/or transport mechanisms induced by specific signals. Accordingly, nuclear localization has been described for classical cytoskeleton-associated proteins as actin, myosin, filamin A and coronin 2A. Among the proteins identified in our study, EZR, in addition to its well-known location at the subcortical region linking actin filaments to the plasma membrane, has been reported to be present within the nucleus of osteosarcoma cells in a phosphorylated form [30].

We are also interested in the regulation of the epithelial-mesenchymal transition in colon cancer by vitamin D analogues. SNAIL1 and SNAIL2/SLUG are transcriptional repressors that induce epithelial-mesenchymal transition by inhibiting the expression of E-cadherin and other adhesion proteins [47-48], and that also repress VDR and inhibit 1,25(OH)₂D₃ action [49-50]. A recent proteomic study allowed the identification of SNAIL1 target proteins in SW480-ADH cells [21]. Among others, two proteins involved in RNA processing and splicing, CPSF6 and SFPQ, were found to be upregulated. Interestingly, we have now found that SFPQ was downregulated in 1,25(OH)₂D₃-treated cells, opposite to its upregulation in SNAIL1-transfected cells [21], which agrees with the antagonism between SNAIL1 and VDR, and further supports the involvement of 1,25(OH)₂D₃/VDR in RNA biology. Giving the opposite effects of 1,25(OH)₂D₃ and SNAIL1 on the phenotype of colon epithelial cells, it is tempting to speculate that the splicing regulators here identified might play a role in the control of the epithelial-mesenchymal transition process. SFPQ is involved in some cancer-related gene

translocations [51], and it is located in paraspeckles, a subnuclear compartment for pre-mRNA processing and retention [52]. Moreover, SFPQ was shown to interact with RAD51 and XRCC2 proteins of the DNA repair complex and involved in the maintenance of chromosome integrity [53]. We have identified XRCC6 DNA helicase and PARP1 Poly (ADP-ribose) polymerase as 1,25(OH)₂D₃-regulated proteins, which together suggest a role for SFPQ also in DNA repair.

Conclusions

Many of the proteins identified in this study correspond to the nucleolar compartment and have been assigned to the spliceosome machinery. Interestingly, a significant number (8) of the proteins identified as regulated by 1,25(OH)₂D₃ showed a variable number of isoforms, which might result from a differential splicing. Cell nucleolus plays important roles in ribosome subunit biogenesis, maturation of RNAs, spliceosomal machinery and the regulation of specific regulatory factors [32]. Alternative splicing is an important point of control of gene expression that may render proteins with distinct and sometimes opposite activity and is frequently altered in cancer cells [19]. Remarkably, in malignancies of the digestive tract including colon cancer both aberrant splice variants and altered expression of splice variants have been described [18]. Thus, the panel of target proteins here identified may constitute a good starting point for the characterization of the effect of vitamin D compounds on the spliceosome machinery, which may in turn be relevant to understand their anti-tumoral effects.

ACKNOWLEDGEMENTS

We thank Dr J Valcárcel for helpful discussions on the role and functionality of splicing factors, Dr M. Lafarga for his help with confocal microscopy analyses, Drs JM Serrador and B Belandia for kindly providing us reagents for EZR and SMARCE1, respectively, and T. Martinez for technical assistance. This research was supported by grants from Colomics Programme Comunidad de Madrid (S-GEN-0266/2006), Ayudas a grupos estables de investigación Fundación Científica de la Asociación Española contra el Cáncer to J.I.C., Ministerio de Ciencia e Innovación de España (BIO2009-08818 (J.I.C.), SAF07-60341(A.M.) and SAF2010-18302 (A.M.)), Proteored platform (J.I.C.), and Red Temática de Investigación Cooperativa en Cáncer (RD06/0020/0009, ISCIII) (A.M.).

REFERENCES

- [1] Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, et al. Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis. *Am J Prev Med.* 2007;32:210-6.
- [2] Yin L, Grandi N, Raum E, Haug U, Arndt V, Brenner H. Meta-analysis: longitudinal studies of serum vitamin D and colorectal cancer risk. *Aliment Pharmacol Ther.* 2009;30:113-25.
- [3] Giovannucci E. Epidemiology of vitamin D and colorectal cancer: casual or causal link? *J Steroid Biochem Mol Biol.* 2010;121:349-54.
- [4] Mezawa H, Sugiura T, Watanabe M, Norizoe C, Takahashi D, Shimojima A, et al. Serum vitamin D levels and survival of patients with colorectal cancer: post-hoc analysis of a prospective cohort study. *BMC Cancer.* 2010;10:347.
- [5] Ordóñez-Morán P, Larriba MJ, Pendás-Franco N, Aguilera O, González-Sancho JM, Muñoz A. Vitamin D and cancer: an update of *in vitro* and *in vivo* data. *Front Biosci.* 2005;10:2723-49.
- [6] Eelen G, Gysemans C, Verlinden L, Vanoirbeek E, De Clercq P, Van Haver D, et al. Mechanism and potential of the growth-inhibitory actions of vitamin D and ana-logs. *Curr Med Chem.* 2007;14:1893-910.
- [7] Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer.* 2007;7:684-700.
- [8] Plum LA, DeLuca HF. Vitamin D, disease and therapeutic opportunities. *Nature reviews.* 2010;9:941-55.
- [9] Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr.* 2007;85:1586-91.
- [10] Pálmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D₃ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β -catenin signaling. *J Cell Biol.* 2001;154:369-87.
- [11] Pálmer HG, Sanchez-Carbayo M, Ordóñez-Morán P, Larriba MJ, Cordon-Cardo C, Muñoz A. Genetic signatures of differentiation induced by 1 α ,25-dihydroxyvitamin D₃ in human colon cancer cells. *Cancer Res.* 2003;63:7799-806.

- [12] Aguilera O, Peña C, García JM, Larriba MJ, Ordóñez-Morán P, Navarro D, et al. The Wnt antagonist DICKKOPF-1 gene is induced by 1 α ,25-dihydroxyvitamin D₃ associated to the differentiation of human colon cancer cells. *Carcinogenesis*. 2007;28:1877-84.
- [13] Álvarez-Díaz S, Valle N, García JM, Peña C, Freije JM, Quesada V, et al. Cystatin D is a candidate tumor suppressor gene induced by vitamin D in human colon cancer cells. *J Clin Invest*. 2009;119:2343-58.
- [14] Barbachano A, Ordóñez-Moran P, Garcia JM, Sánchez A, Pereira F, Larriba MJ, et al. SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumorigenicity. *Oncogene*. 2010;29:4800-13.
- [15] Akutsu N, Lin R, Bastien Y, Bestawros A, Enepekides DJ, Black MJ, et al. Regulation of gene expression by 1 α ,25-dihydroxyvitamin D₃ and its analog EB1089 under growth-inhibitory conditions in squamous carcinoma cells. *Mol Endocrinol*. 2001;15:1127-39.
- [16] Peehl DM, Shinghal R, Nonn L, Seto E, Krishnan AV, Brooks JD, et al. Molecular activity of 1,25-dihydroxyvitamin D₃ in primary cultures of human prostatic epithelial cells revealed by cDNA microarray analysis. *J Steroid Biochem Mol Biol*. 2004;92:131-41.
- [17] Zhang X, Li P, Bao J, Nicosia SV, Wang H, Enkemann SA, et al. Suppression of death receptor-mediated apoptosis by 1,25-dihydroxyvitamin D₃ revealed by microarray analysis. *J Biol Chem*. 2005;280:35458-68.
- [18] Miura K, Fujibuchi W, Sasaki I. Alternative pre-mRNA splicing in digestive tract malignancy. *Cancer Sci*. 2011;102:309-16.
- [19] David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev*. 2011;24:2343-64.
- [20] Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*. 1984;138:141-3.
- [21] Larriba MJ, Casado-Vela J, Pendás-Franco N, Pena R, García de Herreros A, Berciano MT, et al. Novel snail1 target proteins in human colon cancer identified by proteomic analysis. *PLoS One*. 2010;5:e10221.
- [22] Alfonso P, Cañamero M, Fernandez-Carbonie F, Núñez A, Casal JI. Proteome analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. *Journal of proteome research*. 2008;7:4247-55.

- [23] Alfonso P, Nuñez A, Madoz-Gurpide J, Lombardía L, Sánchez L, Casal JI. Proteomic expression analysis of colorectal cancer by two-dimensional differential gel electrophoresis. *Proteomics*. 2005;5:2602-11.
- [24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-8.
- [25] Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003;4:P3.
- [26] Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
- [27] Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic acids research*. 2009;37:D412-6.
- [28] Hoffmann R, Valencia A. A gene network for navigating the literature. *Nat Genet*. 2004;36:664.
- [29] Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. 2008;36:D480-4.
- [30] Di Cristofano C, Leopizzi M, Miraglia A, Sardella B, Moretti V, Ferrara A, et al. Phosphorylated ezrin is located in the nucleus of the osteosarcoma cell. *Mod Pathol*. 2010;23:1012-20.
- [31] Ordóñez-Morán P, Larriba MJ, Pálmer HG, Valero RA, Barbachano A, Dunach M, et al. RhoA-ROCK and p38MAPK-MSK1 mediate vitamin D effects on gene expression, phenotype, and Wnt pathway in colon cancer cells. *J Cell Biol*. 2008;183:697-710.
- [32] Andersen JS, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, et al. Directed proteomic analysis of the human nucleolus. *Curr Biol*. 2002;12:1-11.
- [33] Patton JG, Porro EB, Galceran J, Tempst P, Nadal-Ginard B. Cloning and characterization of PSF, a novel pre-mRNA splicing factor. *Genes Dev*. 1993;7:393-406.
- [34] Min H, Turck CW, Nikolic JM, Black DL. A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. *Genes Dev*. 1997;11:1023-36.
- [35] Rappsilber J, Ryder U, Lamond AI, Mann M. Large-scale proteomic analysis of the human spliceosome. *Genome Res*. 2002;12:1231-45.

- [36] Ji Y, Tulin AV. Poly(ADP-ribosyl)ation of heterogeneous nuclear ribonucleoproteins modulates splicing. *Nucleic Acids Res.* 2009;37:3501-13.
- [37] Bentley D. The mRNA assembly line: transcription and processing machines in the same factory. *Curr Opin Cell Biol.* 2002;14:336-42.
- [38] Hall MP, Huang S, Black DL. Differentiation-induced colocalization of the KH-type splicing regulatory protein with polypyrimidine tract binding protein and the c-src pre-mRNA. *Mol Biol Cell.* 2004;15:774-86.
- [39] Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, et al. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature.* 2009;459:1010-4.
- [40] Gherzi R, Trabucchi M, Ponassi M, Ruggiero T, Corte G, Moroni C, et al. The RNA-binding protein KSRP promotes decay of beta-catenin mRNA and is inactivated by PI3K-AKT signaling. *PLoS Biol.* 2006;5:e5.
- [41] Buratti E, Baralle FE. The multiple roles of TDP-43 in pre-mRNA processing and gene expression regulation. *RNA Biol.* 2010;7:420-9.
- [42] Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. *Genome Res.* 2010;20:1352-60.
- [43] Pokutta S, Weis WI. The cytoplasmic face of cell contact sites. *Curr Opin Struct Biol.* 2002;12:255-62.
- [44] Kouchi Z, Fujiwara Y, Yamaguchi H, Nakamura Y, Fukami K. Phosphatidylinositol 5-phosphate 4-kinase type II beta is required for vitamin D receptor-dependent E-cadherin expression in SW480 cells. *Biochem Biophys Res Commun.* 2011;408:523-9.
- [45] Trinkle-Mulcahy L, Sleeman JE, Lamond AI. Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells. *J Cell Sci.* 2001;114:4219-28.
- [46] Samarin SN, Koch S, Ivanov AI, Parkos CA, Nusrat A. Coronin 1C negatively regulates cell-matrix adhesion and motility of intestinal epithelial cells. *Biochem Biophys Res Commun.* 2010;391:394-400.
- [47] Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer.* 2007;7:415-28.

- 1 [48] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin
2 Invest. 2009;119:1420-8.
- 3 [49] Larriba MJ, Martin-Villar E, García JM, Pereira F, Pena C, de Herreros AG, et al.
4 Snail2 cooperates with Snail1 in the repression of vitamin D receptor in colon cancer.
5 Carcinogenesis. 2009;30:1459-68.
- 6 [50] Pálmer HG, Larriba MJ, Garcia JM, Ordóñez-Morán P, Pena C, Peiro S, et al. The
7 transcription factor SNAIL represses vitamin D receptor expression and responsiveness in
8 human colon cancer. Nat Med. 2004;10:917-9.
- 9 [51] Hidalgo-Curtis C, Chase A, Drachenberg M, Roberts MW, Finkelstein JZ, Mould S, et
10 al. The t(1;9)(p34;q34) and t(8;12)(p11;q15) fuse pre-mRNA processing proteins SFPQ
11 (PSF) and CPSF6 to ABL and FGFR1. Genes Chromosomes Cancer. 2008;47:379-85.
- 12 [52] Bond CS, Fox AH. Paraspeckles: nuclear bodies built on long noncoding RNA. J Cell
13 Biol. 2009;186:637-44.
- 14 [53] Rajesh C, Gruver AM, Basrur V, Pittman DL. The interaction profile of homologous
15 recombination repair proteins RAD51C, RAD51D and XRCC2 as determined by proteomic
16 analysis. Proteomics. 2009;9:4071-86.

Table 1a. Proteins differentially-expressed in SW480-ADH cells after 8 h 1,25(OH)₂D₃-treatment

UNIPROT			COVERAGE						FOLD	
SPOT	AC #	PROTEIN	GENE	SCORE	(%)	MW (Da)	pI	p	CHANGE	LOCALIZATION
19	Q09028	Histone-binding protein RBBP4	RBBP4	334	35	46,656	4.74	0.003	0.78	Nucleus
1276	P14618	Pyruvate kinase isozymes M1/M2	PKM2	107	36	57,937	7.95	0.015	0.78	Cytosol
93	Q969G3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	SMARCE1	252	20	46,649	4.85	0.024	0.78	Nucleus
1776	P30040	Endoplasmic reticulum protein ERp29	ERP29	82	21	28,993	6.08	0.003	0.76	Endoplasmatic reticulum
97	Q0QEN7	ATP synthase subunit beta	ATP5B	100	26	48,113	4.95	0.023	0.69	Mitochondrial inner membrane
14	Q86VI3	Ras GTPase-activating-like protein IQGAP3	IQGAP3	80	18	184,699	7.34	0.019	0.66	Intracellular
15	Q9ULV4	Coronin-1C	CORO1C	108	12	53,249	6.65	0.003	0.64	Actin cytoskeleton
1121	P25705	ATP synthase subunit alpha, mitochondrial	ATP5A1	300	28	59,751	8.28	0.003	0.56	Mitochondrion inner membrane
1360	P35232	Prohibitin	PHB	484	42	29,804	5.57	0.009	0.56	Mitochondrion inner membrane, nucleoplasm
52	P05387	60S acidic ribosomal protein P2	RPLP2	122	64	11,665	4.42	0.008	0.52	Cytosolic large ribosomal subunit
32	P13645	Keratin, type I cytoskeletal 10	KRT10	88	18	59,511	5.13	0.000	0.48	Intermediate filament
1347	P09661	U2 small nuclear ribonucleoprotein A'	SNRPA1	192	52	28,416	8.72	0.009	0.41	Nucleus

Table 1b. Proteins differentially-expressed in SW480-ADH cells after 48 h 1,25(OH)₂D₃-treatment

SPOT	UNIPROT AC #	PROTEIN	GENE	SCORE	COVERAGE (%)	MW (Da)	pI	<i>p</i>	FOLD CHANGE	LOCALIZATION
283	P38646	Stress-70 protein, mitochondrial	HSPA9	259	18	73,680	5.44	0.005	2.50	Mitochondrion
642	Q5TCI8	Lamin A/C	LMNA	141	23	55,762	6.55	0.003	1.73	Nuclear matrix
127	P60709	Actin, cytoplasmic 1	ACTB	103	27	41,137	5.29	0.003	1.72	Cytoskeleton
561	P19338	Nucleolin	NCL	121	22	76,614	4.60	0.001	1.71	Nucleus
625	P15311	Ezrin, Moesin, Radixin, Cytovillin 2	EZR / MSN / RDX / VIL2	98,79,74,71	16	69,413	5.95	0.002	1.63	Cytoskeleton, cell membrane
627	P15311	Ezrin	EZR	182	29	69,413	5.95	0.001	1.61	Cytoskeleton, cell membrane
802	P18206	Vinculin	VCL	82	11	123,779	5.51	0.035	1.55	Cell membrane
1349	P05787	Keratin, type II cytoskeletal 8	KRT8	119	28	53,704	5.52	0.030	1.55	Intermediate filament

907	Q92945	Far upstream element-binding protein 2	KHSRP	187	33	73,146	6.84	0.017	1.53	Nucleus
516	P62140	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB	117	23	37,187	5.85	0.003	1.52	Cytoplasm
1154	P12956	ATP-dependent DNA helicase 2 subunit 1	XRCC6	206	17	69,843	6.23	0.015	1.46	Nucleus
493	Q13148	TAR DNA-binding protein 43	TARDBP	83	4	44,740	5.85	0.042	1.44	Nucleus
1086	Q9NVH6	Trimethyllysine dioxygenase, mitochondrial	TMLHE	74	32	49,518	7.64	0.016	1.42	Mitochondrion
1319	P51991	Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3	193	21	39,595	9.10	0.026	1.39	Nucleus
688	Q15019	Septin-2	SEPT2	460	38	41,487	6.15	0.039	1.38	Nucleus
1746	P09661	U2 small nuclear ribonucleoprotein A'	SNRPA1	120	39	28416	8.72	0.0008	0.79	Nucleus
163	P25705	ATP synthase subunit alpha, mitochondrial	ATP5A1	112	23	59751	8.28	0.028	0.76	Nucleus
1074	Q9BSV4	SFPQ protein	SFPQ	143	23	68,631	8.89	0.019	0.75	Nucleus
1797	P26583	High mobility group protein B2	HMGB2	103	39	24,034	9.45	0.043	0.75	Nucleus
1195	Q13283	Ras GTPase-activating protein-binding protein 1	G3BP1	156	30	52,164	5.36	0.042	0.74	Nucleus
23	P08865	40S ribosomal protein SA	RPSA	354	59	32,854	4.79	0.001	0.73	Cytoplasm, nucleus
296	P31930	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	323	37	52,646	5.43	0.021	0.72	Mitochondrial inner membrane
952	Q0QEN7	ATP synthase subunit beta	ATP5B	475	55	48,113	4.95	0.021	0.69	Mitochondrial inner membrane
1623	P40926	Malate dehydrogenase	MDH2	159	45	35,503	8.54	0.017	0.69	Mitochondrial inner membrane
682	P26641	Elongation factor 1-gamma	EEF1G	255	26	50,119	6.27	0.022	0.68	Cytosol
1867	Q9Y3D9	28S ribosomal protein S23, mitochondrial	MRPS23	97	36	21,711	8.94	0.017	0.67	Mitochondrion
15	Q9ULV4	Coronin-1C	CORO1C	108	12	53,249	6.65	0.002	0.66	Actin cytoskeleton
1549	P06748	Nucleophosmin	NPM1	140	40	32,575	4.64	0.027	0.66	Nucleus
574	P09874	Poly [ADP-ribose] polymerase 1	PARP1	327	34	113,084	8.99	0.008	0.66	Nucleus
1810	P19388	DNA-directed RNA polymerases I, II, and III subunit RPABC1	POLR2E	109	31	24,551	5.69	0.032	0.65	Nucleus
1625	P29692	Elongation factor 1-delta	EEF1D	387	50	31,122	4.90	0.008	0.64	Cytosol
1387	Q16576	Histone-binding protein RBBP7	RBBP7	144	21	47820	4.89	0.028	0.61	Nucleus
1358	Q12849	G-rich sequence factor 1	GRSF1	220	28	53,126	5.83	0.012	0.60	Cytoplasm
704	Q3B7T1	Erythroid differentiation-related factor 1	EDRF1	107	53	138,528	5.93	0.036	0.60	Nucleus
27	P35527	Keratin, type I cytoskeletal 9	KRT9	90	35	62,064	5.14	0.040	0.59	Intermediate filament
175	P13693	Translationally-controlled tumor protein	TPT1	201	45	19,595	4.84	0.011	0.59	Cytoplasm
695	Q01518	Adenylyl cyclase-associated protein 1	CAP1	68	12	51,855	8.29	0.013	0.57	Cell membrane
1203	P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA	278	50	18,012	7.82	0.020	0.56	Cytoplasm, nucleus
695	Q96IZ6	Methyltransferase-like protein 2A	METTL2A	78	18	43,521	5.69	0.018	0.56	Nucleus
14	Q86VI3	Ras GTPase-activating-like protein IQGAP3	IQGAP3	80	18	184,699	7.34	0.005	0.52	Intracellular
1794	Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	242	26	31,362	4.32	0.035	0.48	Mitochondrion
337	P35232	Prohibitin	PHB	690	66	29,804	5.57	0.026	0.46	Mitochondrial inner

37	P27348	14-3-3 protein theta	YWHAQ	123	29	27,764	4.68	0.000	0.42	membrane, nucleoplasm
39	Q969G3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	SMARCE1	89	20	46,649	4.85	0.000	0.42	Cytoplasm, nucleus
39	P62258	14-3-3 protein epsilon	YWHAE	136	43	29,174	4.63	0.000	0.42	Nucleus
34	P61981	14-3-3 protein gamma	YWHAG	75	21	28,303	4.80	0.000	0.41	Cytoplasm
52	P05387	60S acidic ribosomal protein P2	RPLP2	122	64	11,665	4.42	0.007	0.41	Cytoplasm
										Cytosolic large ribosomal subunit

Table 2. Proteins identified by both, proteomic and transcriptomic, studies as differentially-expressed in 1,25(OH)₂D₃- vs vehicle-treated SW480-ADH cells

PROTEIN	PROTEOMIC		TRANSCRIPTOMIC	
	FOLD CHANGE	1,25(OH) ₂ D ₃ TREATMENT (h)	FOLD CHANGE	1,25(OH) ₂ D ₃ TREATMENT (h)
RBBP4	0.78	8	0.50	4
			0.43	48
PKM2	0.78	8	0.14	48
SMARCE1	0.78	8	0.43	4
	0.42	48		
ATP5A1	0.56	8	0.33	48
SNRPA1	0.41	8	0.31	48
ACTB	1.72	48	2.83	48
RDX	1.63	48	2.14	48
EZR	1.61	48	2.46	48
VCL	1.55	48	2.83	48
SFPQ	0.75	48	0.38	48
POLR2E	0.65	48	0.33	48
EEF1D	0.64	48	0.50	48
YWHAE	0.42	48	0.38	48

FIGURE LEGENDS

Figure 1. (A) Western blot analysis showing the expression of markers in nuclear (N; HDAC, Lamin B and VDR) and cytosolic (C; β -Tubulin and Rho-GDI) extracts of SW480-ADH cells treated with 1,25(OH) $_2$ D $_3$ or vehicle for the indicated times. Data from three independent experiments (Exp.) are shown. (B) Representative 2D map obtained by 2D-DIGE analysis of nuclear proteins from SW480-ADH cells treated with 1,25(OH) $_2$ D $_3$ or vehicle for 48 h. Nuclear extracts from SW480-ADH cells treated with 1,25(OH) $_2$ D $_3$ or vehicle for 48 h were differentially labeled with Cy5 and Cy3 dyes. An internal standard, combining proteins from both extracts, was included in all gels after labeling with Cy2 dye. Samples were separated in 3-10 NL IPG strips for IEF and standard SDS-PAGE for the second dimension. The representative spot map corresponds to the internal standard, which is common to all gels analyzed, at 48h. Only those spots whose levels of expression were significantly different between both conditions (ANOVA $p < 0.05$; fold-change < 0.80 or > 1.25) were considered for the analysis. Those proteins that were unequivocally identified by MALDI-TOF-TOF analysis are named and indicated by arrows (black: overexpressed proteins; white: repressed proteins).

Figure 2. Validation of 1,25(OH) $_2$ D $_3$ target proteins in SW480-ADH cells by Western blot analysis. Representative Western blots showing the expression of several proteins identified by 2D-DIGE. Nuclear extracts from SW480-ADH cells treated with 1,25(OH) $_2$ D $_3$ or vehicle for 8 h (RBBP4) or 48 h (all others). Numbers are fold-change values after normalization using LMNB as control. Experiments were performed in triplicate using extracts from three independent assays. The volume density of the

experiments and the standard deviation was calculated and represented in bar graphs.

Statistical significance was assessed by two-tailed unpaired Student's t-test (* $p<0.05$, ** $p<0.01$).

Figure 3. Quantitative RT-PCR analysis of the RNA expression corresponding to the proteins regulated by 1,25(OH)₂D₃ in SW480-ADH cells.

Quantitative RT-PCR study of *KHSRP*, *PARP1*, *SMARCE1* and *SFPQ* in 1,25(OH)₂D₃- or vehicle-treated SW480-ADH cells at 48h. 18S rRNA expression was used for normalization. *CDH1*/E-cadherin was included as a positive control. Statistical significance was assessed by two-tailed unpaired Student's t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 4. Subcellular distribution of several target proteins in SW480-ADH cells treated with 1,25(OH)₂D₃ or vehicle. Representative immunofluorescence and confocal microscopy images showing the expression of the target proteins in SW480-ADH cells treated with 1,25(OH)₂D₃ or vehicle for 48 h. Nuclei were counterstained with DAPI. Confocal microscopy was performed with a TCS SP5 laser scanning microscope (Leica). Bars, 25 μ m.

Figure 5. Protein classification according to localization and biological function.

Functional annotation of the identified proteins was carried out with DAVID, a web-accessible tool. A. Pie chart indicating the number of identified proteins involved in each molecular function. Gene ontology numbers are provided. EF: Enrichment scores for each term. B. Gene-term 2D heat map view for functional annotation clustering of the identified

proteins involved in RNA processing and binding. *Green box*. Corresponding gene-term association positively reported. *Black box*. Corresponding gene-term association not reported yet.

Figure 6. 1,25(OH)₂D₃ treatment of colon cancer cells regulates spliceosome and cell-adhesion proteins. *In silico* protein interaction analysis for the proteins identified in 1,25(OH)₂D₃-treated SW480-ADH cells was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins). STRING was required to add 10 more predicted functional partners to facilitate the creation of networks. These proteins are indicated by red boxes. Most of the added proteins were members of the RNA Polymerase II complex. Two functional sets of proteins were highlighted manually with circles: proteins involved in gene expression regulation and RNA processing, which are related to the spliceosome (yellow circle), and those involved in cell-cell and cell-matrix interactions in relation to the cytoskeleton (green circle). Different line colors indicate different types of evidence for the association: Neighborhood (green), co-expression (dark blue), experimental (red), association in curated databases (blue) or co-mentioned in PubMed abstracts (light green).