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Vitamin D_3 signalling in the brain enhances the function of phosphoprotein enriched in astrocytes – 15 kD (PEA-15)

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Abstract

In spite of growing evidence linking vitamin D_3 levels to mental health disorders, little is known about its direct targets in the brain. This study set out to investigate targets of vitamin D_3 in a human brain stem cell line. We employed arrays with antibodies directed against more than 600 structural and signalling proteins, including phospho-variants. Over 180 proteins responded to vitamin D_3, such as cyclin-dependent protein-serine kinase 1/2, epidermal growth factor receptor-tyrosine kinase, protein kinase A, protein-serine kinase Bγ and protein-serine kinase Cα. PEA-15 (phosphoprotein enriched in astrocytes-15 kD, also known as PED), known to be involved in various anti-proliferative and anti-apoptotic effects, was strongly up-regulated. In silico promoter analysis revealed conserved binding sites for vitamin D_3 receptor, suggesting a strong vitamin D_3 dependency of the PEA-15 promoter. PEA-15 up-regulation by vitamin D_3 could be confirmed by Western blot in two different cell lines. Analysis of mRNA and protein phosphorylation status of PEA-15 suggests that increased PEA-15 promoter activity and increased protein stabilization contribute to the overall rise of PEA-15 protein. In a functional test of this novel pathway, we demonstrated that vitamin D_3 was able to rescue cells from TRAIL-induced apoptosis through regulation of the PEA-15 expression and function. Summarized, our study presents novel targets of vitamin D_3 relevant for apoptosis and cell proliferation, and thus strongly supports a function of vitamin D_3 in the brain that impacts on processes highly relevant for major neurological disorders.

Keywords: vitamin D_3 • brain • signalling • apoptosis • PED/PEA-15 • human neuronal stem cells • protein antibody array

Introduction

The vitamin D_3 receptor (VDR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors and was first identified as a key player maintaining calcium and phosphate homeostasis [1]. VDR is activated upon binding of its ligand calcitriol ($1,25(OH)_2$ vitamin D_3, throughout the text referred to as 'vitamin D_3'). This leads to homodimerization of VDR or heterodimerization with other nuclear receptors, binding to vitamin D_3 responsive elements (VDREs) and activation of the transcription of VDR target genes [2–5].

There is accumulating evidence that actions of VDR are not confined to processes related to calcium homeostasis and skeletal development, but extend to cell survival, differentiation [6, 7], proliferation [8, 9] and cell death [10]. VDR expression in the brain was first evidenced by autoradiographic hormone-binding studies in the 1980s, together with the report that effects of sunlight or equivalent artificial light on behavioral processes could at least partially be mediated through vitamin D_3-endocrine system [11, 12]. Prenatal vitamin D_3 deficiency was demonstrated to impair brain development in rats [13] and cause subtle alterations in their learning and memory abilities [14]. Together with dexamethasone, vitamin D_3 down-regulated microglial activation in the rat hippocampus, thus reversing various neuroinflammatory age-related changes [15]. Furthermore,

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a low vitamin D_3 status is associated with low mood and impaired measures of cognitive performance in humans [16], and could possibly contribute to the aetiology of schizophrenia [17, 18]. Recent findings suggest that regular use of cod liver oil (major natural source of vitamin D_3) decreases depressive symptoms in the general population [19]. In addition, genetic variance in the VDR gene apparently influences the susceptibility to age-related changes in cognitive functioning and in depressive symptoms [20].

Although there is growing evidence linking vitamin D_3 levels to mental health [21–23], little is known about its direct effects, genetic targets and modes of action in the brain. With this study, we aimed to search for signalling pathways affected by vitamin D_3 in the human CNS. As a model, we chose the human neuronal stem cell line HNSC.100, derived from a 10–10.5 weeks gestational age human Caucasian embryo and immortalized by v-myc expression [24]. These cells can be differentiated (by addition of CNTF) to up-regulate markers of three lineages (astrocytes $\geq$ 85%, neurons 10–15% and oligodendrocytes $\leq$ 1%; [24, 25]).

We first employed antibody microarrays (featuring over 600 different antibodies) to detect proteins that were changed in their expression and/or activation upon treatment with vitamin D_3. The most prominent candidates were then further tested by Western blot analysis. Several structural and signalling targets of vitamin D_3 were detected, with particularly strong up-regulation of PEA-15 (Phosphoprotein Enriched in Astrocytes-15 kD, also known as PED). This was highly interesting, because PEA-15 is a major phosphoprotein and an endogenous substrate for protein kinase C in astrocytes. It contains the death effector domain (DED) and is predominantly expressed in the central nervous system [26]. Among other functions, PEA-15 regulates the sub-cellular localization of ERK MAP kinase [27] and is linked to various anti-apoptotic and anti-proliferative effects [28–31]. For example, PEA-15 expression protected astrocytes from PEA-15 knockout mice from TNF-induced apoptosis [32] and transfection of PEA-15 cDNA in TRAIL (TNF-related apoptosis inducing ligand)–sensitive glioma cells rendered these cells resistant to TRAIL-induced apoptosis [33].

These important features of PEA-15 prompted us to characterize its up-regulation further and to examine the effects of vitamin D_3 on processes known to be mediated by PEA-15, such as TRAIL-induced apoptosis. We revealed that (*i*) the promoter of PEA-15 contains a conserved VDR response element, (*ii*) vitamin D_3 leads to an up-regulation of the mRNA of PEA-15, which precedes the rise in protein level, (*iii*) vitamin D_3 increases the level of phosphorylated PEA-15, possibly *via* AKT1, (*iv*) vitamin D_3 treatment significantly rescued A549 cells from TRAIL-induced apoptosis and (*v*) this effect was strongly dependent on the presence of PEA-15, as revealed by RNAi experiments. In summary, this study provides insight into novel targets of vitamin D_3 in HNSC.100 human brain cells and defines a novel pathway of vitamin D_3 that counteracts apoptosis *via* up-regulation of PEA-15.

Materials and methods

Cell culture and treatments

The human neuronal stem cell line (HNSC.100) was propagated in DMEM:F-12 (1:1) medium supplemented with 0.5% FCS, 1% N2 (Invitrogen, Karlsruhe, Germany), 1% BSA (Sigma, Taufkirchen, Germant), 1% penicillin/streptomycin (Invitrogen) and human recombinant growth factors EGF and FGF-β (Tebu Biochem, Offenbach, Germany, 20 nM each). For differentiation, mitogens were removed and replaced with CNTF (100 nM) for 2–3 weeks. Differentiated HNSC.100 were treated for 24 hrs with ethanol vehicle (Sigma), or 10^{-7} M vitamin D_3 (Biomol, Hamburg, Germany) and lysates prepared with ice-cold lysis buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 3.5 mM EDTA, 30 mM sodium fluoride, 60 mM glycerophosphate pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, 1% Triton X-100).

Transfection and siRNA design

To knock-down PEA-15, two siRNA duplexes were designed (21 nt double-stranded RNA containing 19 base pairs and 3' T- overhanging ends) using the Dharmacon siDesign Center software (UCACUAUGGUGGUUGACUATT and UGCAAGACCUGACCAACAATT, synthesized by Invitrogen). Transfections were performed with Lipofectamine according to the manufacturer's instructions (Gibco).

Antibody protein arrays

Over 600 antibodies (≥350 pan- and ≥280 phospho-site-specific, specification available upon request) were deposited in adjacent, duplicate spots on the glass slides (Kinexus Bioinformatics proprietary methodology). To ensure the most reliable comparison possible, both samples (control and treatment) were labelled with the same fluorescent dye (unlike DNA, proteins display strong individual differences in their relative affinities for dyes) and incubated on the same microarray (details available at www.kinexus.ca). Unbound dye-labelled lysate proteins were washed away and captured proteins quantified (Kinexus KAM-1.1 software). Analysis of the fluorescence signal strength for each target protein was provided in duplicates (Table 3) and includes the (average) percent change from the control sample, the percent range in error and fold-changes.

Western blot quantification

For validation, 500 μg of protein lysate derived from HNSC.100 was subjected to Western blot expression analysis. This analysis combines proprietary methodology (Kinexus Bioinformatics Corporation, Vancouver, BC, Canada) with analytical techniques, including SDS-PAGE gel electrophoresis, immunoblotting and enhanced chemoluminescence (ECL) protein visualization. First, proteins were separated by conventional gel electrophoresis based on their molecular weights, followed by detection with highly validated panel of human antibodies (the same as applied on the KAM1.1 chip).

Quantification of the bands was performed with a highly sensitive imaging system with a 16-bit camera in combination with the quantification software Immuno-Reactivity Identification System (IRIS) converting

the light signals from the sample into digital data. From this, an intensity profile for the band is generated from summing each pixel's intensity for that band (only the bands whose clustered pixels were higher than the intensity of the pixels that make up the background of the image were considered for quantification). The raw data (a resulting trace quantity for each band scanned at the maximum scan time) was normalized to the average of all signals detected on the immunoblots of the screen. This was accomplished by summing the trace quantity of all the bands detected (known and unknown) on each immunoblot, finding the average total intensity per screen in any given normalization group, and then using a coefficient or scaling factor to multiply each trace quantity in each sample (this procedure helps to safeguard against inaccuracies in protein determination, protein loading and the inconsistencies as when normalizing the results to common internal references, which often vary significantly between samples). Therefore, the normalization was based on the same principle, but utilizing many proteins as references instead of one internal standard, possibly giving a more conservative estimate of the expression changes occurring. Data are listed as percent change from control (%CFC).

For the Western blots in Figs 4–6, cells were lysed as described earlier. Fifty micrograms of cell extract were resolved on 15% SDS–polyacrylamide and transferred to Hybond-C extra nitrocellulose. Membranes were blocked for 1 hr with 5% non-fat dry milk in TBS containing 0.05% Tween-20 incubated with primary antibodies (directed against: total AKT, pAKT (S473), total PEA-15, pPEA-15 (S116) and β-actin as indicated in the figures) and visualized by chemiluminescence [28].

Real-time PCR

Total RNA was extracted from A549 cells using the TRIZOL reagent protocol (Invitrogen). Quality and quantity of the RNA were determined by measuring the absorbance at 260 and 280 nm, and by 1% agarose gel electrophoresis under denaturing conditions. RNA (1 μg) from each sample was reverse transcribed and one-step RT-PCR was performed by using SuperScript® III First-Strand system (Invitrogen) according to manufacturer's instructions. Primers for PEA-15 were 5′-GCAGTGCCTGGTTTAGCTTC-3′ (forward), 5′-TACGGGTTAGCTTGGTGTCC-3′ (reverse) and were synthesized commercially (PRIMM, Milan, Italy). Real-time PCR was performed by using iQ™ SYBR® Green Supermix (BIO-RAD, Munich, Germany). Reactions were performed in triplicate and β-Actin used as an internal reference cycling (conditions are available upon request).

Proliferation assays and cell death

Proliferation was evaluated with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). A549 (lung carcinoma) cells (2×10^5) were plated in 96-well plates (triplicates), stimulated with ligands and incubated at 37°C (at 5% CO_2). Cell death was induced by using Super*Killer*TRAIL™ (Alexis Biochemicals, Lausanne, Switzerland) for 24 hrs at 100 ng/ml. Metabolically active cells were detected by adding 20 μl of methyl tetrazolium salt (MTS) to each well. After 2 hrs of incubation, the plates were analysed on a Multilabel Counter (BIO-RAD) according to the manufacturer's instructions. Apoptosis was measured by FACS (propidium iodide assay). The cells (2×10^5) were washed with PBS and re-suspended in 200 μl buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide (Sigma). Following incubation at 4°C for 30 min. in the dark, nuclei were analysed with a Becton Dickinson FACScan flow cytometer. Cellular debris was excluded from analyses by raising the forward scatter threshold, and the DNA content of the nuclei registered on a logarithmic scale.

Gene ontology sorting

The Gene Ontology data annotation (version 161) http://www.geneontology.org; http://www.ebi.ac.uk/GOA/) was used to classify and characterize the genes that resulted from the antibody microarray analysis. Regulated genes were imported into BiblioSphere software (Version 7.13; Genomatix, Munich, Germany) and mapped to Gene Ontology (GO) trees in order to identify their biological function. In addition, the number of genes discovered as regulated by vitamin D_3 in a certain class was related to the total number of genes to which antibodies were spotted on the chip.

Computational promoter analysis

To identify conserved transcription factor binding sites for PEA-15, promoter sequences from three different species (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*) were first extracted by use of ElDorado program in the Genomatix software suite. The proximal promoter regions used were generally defined as about 500 nt upstream and 100 nt downstream of the transcriptional start side (TSS). Promoter sequences of the three different species were then compared by alignment with the help of DiAlign to evaluate overall promoter similarity. To find transcription factor–binding sites in the input sequences arranged by defined order, orientation and certain distance range between adjacent binding sites, the FrameWorker program (Genomatix) has been applied. For the promoter analysis position weight, matrices according to Matrix Family Library Version 7.0 (October 2007) were used.

Results

Proteomic search indicates numerous targets of vitamin D_3 in brain-derived cells

To further elucidate the actions of vitamin D_3 in human brain–derived cells, we started out by using antibody microarrays. They have the advantage of detecting the functional entity (protein) directly and also the added benefit of detecting protein isoforms, including in some cases phosphorylation isoforms. The antibody array used in our study (Kinexus) features more than 600 antibodies (≥350 pan-specific and ≥250 phospho-site-specific) deposited in duplicate spots.

As a model, we used the human neuronal stem cell line HNSC.100 [24]. Because of the sTable expression of v-myc, it can be perpetuated in the presence of the mitogens FGF-2 and EGF. Upon removal of the mitogens and in the presence of CNTF, cells undergo spontaneous morphological differentiation [24, 25]. After 2 weeks of differentiation, cultures were exposed to vitamin D_3 for 24 hrs (10^{-7} M, Fig. 1A). Lysates of treated and untreated cells

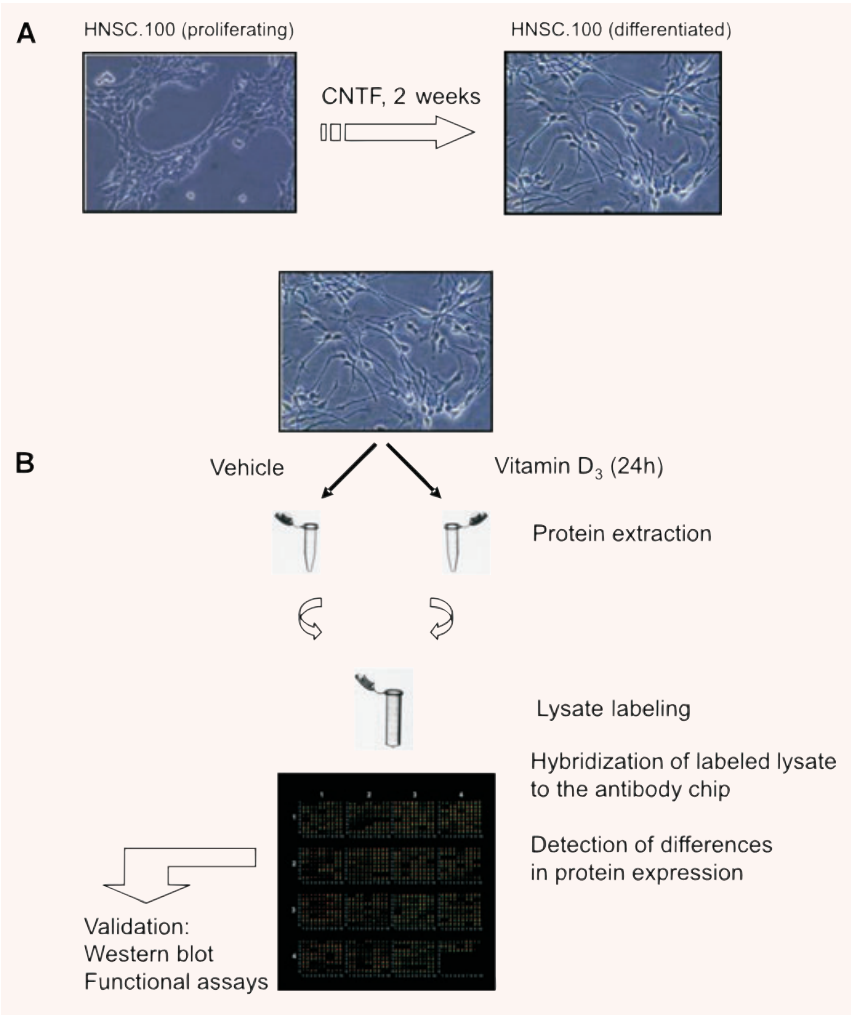

Fig. 1 Procedure of the proteomic search for vitamin D_3-regulated targets. HNSC.100 brain stem cells were cultivated and differentiated and treated with vitamin D_3 for 24 hrs, and proteins were extracted from treated and untreated cells. After labelling proteins were bound to the antibody chips, which were then processed to yield candidate targets for further validation and characterization.

were then applied to the antibody array (Fig. 1B, complete list of antibody target proteins are referenced in supplementary Table 1).

More than 180 antibodies indicated altered expression and/or phosphorylation after vitamin D_3 treatment, as compared with the vehicle-treated control (Table 1). Using Gene Ontology (GO, *via* BiblioSphere), we analysed regulated targets according to their functional classes and calculated, which percentage of proteins represented on the chip for any given category was regulated by the treatment. As summarized in Table 2, a high percentage of regulated proteins was found in the categories neuron development, neurogenesis, neurological processes and organelle organization and biogenesis (55.5%, 58.3%, 75.0% and 51.1% of the targets corresponding to the respective category). Like in the case of RNA microarrays, it is important to test some of these screening results by independent methods. Therefore, several candidates were selected (Table 3) according to their percentage change from the control (%CFC with respective error range) for validation by conventional Western blot.

Western blot validation

Independent samples of differentiated HNSC.100 cells were lysed in SDS-PAGE sample buffer as described (Materials and Methods) and resolved on a SDS-PAGE gel followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were probed with antibodies identical to those used on the protein array (Kinexus), visualized with the ECL detection system and quantified by using IRIS identification software (Fig. 2). For approximately 40% of examined targets, changes measured by the antibody microarray could be reproduced by Western blot, such as CDK1/2, EGFR, PKA, Akt3 and PKCα. GSK3α/β yielded partially conflicting results (down-regulated -59.8% and -43.04% on the array and -28% and -10% on Western blot), as well as PI3K/p100 delta (up-regulated 75.9% on the array and down-regulated 17% in the Western blot). Notably, the phosphorylated form of PEA-15 remained the most strongly vitamin D_3-regulated protein after the Western blot validation (Figs 2 and 3). This confirms the

Table 1 List of vitamin D_3-regulated proteins*

Protein name	%CFC	Protein name	%CFC	Protein name	%CFC	Protein name	%CFC
Abl (Y412)	48.39	JIK [TAO3]	–53.38	PKBa (Akt1) (S473)	–56.65	Rb (T821)	–47.07
ACK1[ACK]	49.40	JNK	–60.27	PKBa (Akt1) (S473)	1.37	Rb (T826)	–48.51
AIF	58.18	Jun (S63)	–64.43	PKBa (Akt1) (S473)	47.91	Rb (S780)	–61.39
Alk	59.02	MAPKAPK2 (T222)	–48.57	PKBa (Akt1) (T308)	44.07	Ret (S696)	–50.89
ANKRD3	52.82	MEK1[MAP2K1] (T385)	160.80	PKBg (Akt3)	60.60	RIPK	–54.19
Arrestin b1 (S412)	49.79	MEK7[MAP2K7]	39.93	PKBg (Akt3)	104.38	Rb (T356)	–39.08
ASK1[MAP3K5]	42.07	MEKK1[MAP3K1]	42.01	PKCa	59.75	ROKa[ROCK2]	–52.16
Bad (S99)	76.14	MKP1	–37.80	PKCa/b2 (T638/T641)	–54.75	ROR2	–41.76
Bak	69.32	Mnk1 (T209 + T214)	–52.98	PKCb2	74.40	RSK2	–43.86
Bcl-xS/L	62.51	Mnk2	–65.50	PKCd (S664)	70.96	RSK1/2 (S380/S386)	78.34
Bid	68.12	Mos	–44.47	PKCd (Y313)	87.09	RSK1/2 (S221/S227)	–59.42
Btk	115.73	MRLC2 (S18)	–49.26	PKCe (S729)	111.75	RSK1/2 (S363/S369)	–41.89
CAS	69.55	Msk1 (S376)	–69.50	PKCe (S729)	55.77	RSK1/2 (S380/S386)	–44.83
Catenin b	55.47	MST1	–57.57	PKCg	87.89	S6Ka [p70 S6Ka]	–42.53
CDK2	101.83	MST1	–46.41	PKCg (T514)	76.71	SIRPa1	–42.01
CDK1/2 (Y15)	104.61	MYPT1 (T696)	–48.44	PKCg (T674)	79.58	STAT1 (S727)	–53.87
CDK10	92.90	Nek2	–53.10	PKCq (S676)	68.89	STAT1 (Y701)	–51.38
Chk1	68.90	Nek2	–62.32	PKCq (S695)	116.97	STAT3 (S727)	–45.76
Crystallin aB (S19)	49.62	Nek2	–50.27	PKD (PKCm)	52.85	STI1	41.78
Crystallin aB (S45)	60.01	Nek7	–49.49	PKD (PKCm) (S910)	–63.08	STK33	85.79
DNAPK	56.89	NFkappaB p50	–44.64	PKM2	62.55	Syk	102.15
Dok2 (Y142)	96.20	NFkB p65 (Rel A) (S276)	–47.40	PKN	45.04	Synapsin 1 (S9)	79.19
EGFR	50.89	Nip1	–46.25	Plk1	72.54	TAK1	25.86
EGFR	53.24	NME6	–48.11	PP1/Ca (T320)	56.56	Tau (S738)	59.37
EGFR (Y1068)	53.23	NME7	–53.78	PP2Cab	45.78	Tau (S518)	25.20
eIF2a (S51)	66.64	NR1 (S896)	–68.80	PP5C	94.37	Tau (S530)	58.44
eIF2Be (S540)	61.39	p16 INK4	–50.85	PP6C	49.63	Tau (S578)	93.33
Erk4	–48.65	p18 INK4c	–45.99	PRAS40 (T246)	41.71	Tau (S712)	–50.11
Erk6[p38g]	54.02	p35	–41.17	PRK1/2[PKN1/2] (T774)	–53.59	Tau (T547)	64.83
FAK (Y576)	54.89	p38a MAPK	–48.49	PRKWNK4	–53.20	TBK1	127.12
FAK (Y577)	–56.74	p38a MAPK (T180 + Y182)	–62.98	Progesterone Receptor (S294)	–50.71	Tlk1	130.92
GCK	52.03	p53 (S392)	–45.00	PTEN	–51.11	TRADD	95.57

Continued

Table 1 Continued

Protein name	%CFC	Protein name	%CFC	Protein name	%CFC	Protein name	%CFC
GFAP (S8)	–49.22	PAK1/2/3 (S144/S141/S154)	–52.47	PTEN (S370)	–41.89	TrkA	81.99
GRK3[BARK2]	–35.17	PARP1	–48.38	PTEN (S380 + S382 + S385)	–57.24	TTK	105.00
GSK3a/b (S21/S9)	–59.81	Pax2 (S394)	–41.20	PTP1B	–58.39	Tyk2	78.70
GSK3a/b (Y279/ Y216)	–43.04	Paxillin 1 (Y118)	–61.03	PTP1C	–55.86	TH (S19)	122.06
hHR23B	–52.41	Paxillin 1 (Y118)	56.45	PTP1D	–57.37	TH (S71)	73.38
Histone H2A.X (S139)	–75.10	Paxillin 1 (Y31)	–47.29	PKR	–43.59	VEGFR2[KDR] (Y1054)	62.03
Hpk1	58.84	PCTK1[PCTAIRE1]	–44.85	PTP-PEST	–98.46	Vimentin (S33)	87.10
Hsp27 (S78)	177.00	PDK1 (S244)	–41.46	Pyk2 (Y579)	–43.64	Vrk1	117.51
Hsp60	133.04	PEA-15 (PED15) (S116)	953.61	Rad17 (S645)	–53.78	Wee1	44.52
IKKa/b (S180)	–50.72	PI3K p110 delta	75.96	Raf1	–51.29	XIAP	55.94
IKKb	–56.26	PKA	49.52	Rb (S612)	–50.50	ZAP70 (Y315+Y319)	48.60
IRAK4	–50.42	PKA Cb (S338)	55.65	Rb (S807)	–58.72	ZAP70 (Y319)	51.94
JAK2	–62.42	PKBa (Akt1) (T308)	75.72	Rb (S807 + S811)	–67.14	ZIPK	44.78

*Listed are the results of antibody microarray analysis (duplicate spots, complete list). In cases where the antibodies were directed against the phosphorylated form of the protein, the site of phosphorylation is given in parentheses after the protein name. %CFC, percent change from control.

responsiveness of PEA-15 to vitamin D_3, although the two methods indicated different degrees of up-regulation (953% antibody array *versus* 268% Western blot up-regulation).

PEA-15 promoter analysis and mRNA induction by vitamin D_3

Given the strong response of PEA-15 to vitamin D_3 indicated by two independent samples/methods and its prominent expression in the CNS (particularly abundant in astrocytes [31]), we focussed our further experiments on this candidate. First, we searched by bioinformatics tools for VDR-binding sites in the PEA-15 promoter of the human, mouse and rat genomes. The sequence upstream of the transcription site contains several transcription factor binding motifs, including those for NFκB, EGRF, EKLF and SP1 (Fig. 4A). One of these sites is conserved between human, mouse and rat promoter, thus strongly indicating a functional vitamin D_3 – dependency of the PEA-15 gene promoter.

To experimentally test the up-regulation of the PEA-15 gene promoter by vitamin D_3, we treated cells with vitamin D_3 and measured the mRNA of PEA-15 by real-time PCR. We observed indeed a rise of the PEA-15 mRNA already after 3 and 6 hrs (Fig. 4B). Using an antibody that recognizes PEA-15 irrespective of its phosphorylation status we also detected an up-regulation of the total amount of PEA protein after 12 hrs (Fig. 4C).

Vitamin D_3 regulates PEA-15 function

We next investigated whether the effect of vitamin D_3 on PEA-15 expression changes cellular processes that depend on the function of PEA-15. Numerous studies suggest the involvement of PEA-15 in a broad range of anti-apoptotic processes in cultured cells [32–35]. TRAIL (tumour necrosis factor apoptosis-inducing ligand) is an efficient inducer of apoptosis and in TRAIL-sensitive cells; transfection of PEA-15 cDNA resulted in cell resistance, whereas inhibition of PEA-15 expression changed the TRAIL-resistant phenotype to sensitive [29, 33]. Given that normal astrocytes are generally TRAIL-resistant [33], we chose the TRAIL-sensitive cell line A549 to examine VDR and PEA-15 expression in the presence and absence of vitamin D_3.

First, we verified by Western blot that A549 cells express VDR, indeed (data not shown). To test whether vitamin D_3 influences TRAIL-induced apoptosis, we pre-treated A549 cells with vitamin D_3 (10^{-7} M, as in the previous experiments) for 12 hrs before apoptosis was induced with TRAIL (100 ng/ml for the next 24 hrs). Cell nuclei were visualized with propidium iodide and

Table 2 Gene Ontology data sorting*

Gene ontology classes of vitamin D_3 targets	Percentage of regulated proteins per GO class
Cell cycle	36.6
Cell differentiation	44.2
Cellular development	42.8
Cellular communication	38.8
General regulation of biological processes	38.6
Intracellular signalling cascade	40.1
MAPKKK cascade	37.9
NS development	41.9
Phosphorylation	40.2
Primary metabolic processes	38.5
Programmed cell death	38.7
Protein modification	42.0
Regulation of programmed cell death	32.9
Response to stimulus	42.5
Signal transduction	38.0
Intracellular transport	61.9
Neurogenesis	58.3
Neurological processes	75.0
Neuron development	55.5
Organelle organization and biogenesis	51.1

*Results of the antibody array were sorted using Gene Ontology (http://www.geneontology.org, http://www.ebi.ac.uk/GOA/). The percentage of regulated proteins was calculated as the proportion of affected proteins from the total number of proteins for the given category.

Table 3 Selected protein targets for validation by conventional Western blot*

Protein name	%CFC
CDK2	101.83
EGFR	50.89
GSK3a/b	–61.19
GSK3a/b (S21/S9)	–59.81
GSK3a/b (Y279/ Y216)	–43.04
MAPK14	–18.77
p38a MAPK (T180 + Y182)	–62.98
PEA-15 (PED15) (S116)	953.61
PI3K p110 delta	75.96
PKA	49.52
PKBg (Akt3)	60.60
PKCa	59.75

*In cases where the antibodies were directed against the phosphorylated form of the protein, the site of phosphorylation is given in parentheses after the protein name.

subjected to FACS analysis to monitor late-stage apoptosis, and MTS proliferation assays were performed to measure cell viability. Treatment with vitamin D_3 alone did not have a significant effect on apoptosis, as compared with vehicle-treated cells (Fig. 5A, right panel, grey bar), whereas treatment with TRAIL resulted in approximately 50% apoptotic cells, as expected (Fig. 5A, right panel, black bar). Notably, cells pre-treated with vitamin D_3 prior to TRAIL administration exhibited markedly decreased levels of TRAIL-induced apoptosis (Fig. 5A, right panel, dark grey bar). MTS assays paralleled the observed changes, indicating that a decrease in cell proliferation/viability induced by TRAIL could be significantly reverted by pre-treating cells with vitamin D_3 (Fig. 5A, left panel).

To answer the crucial question of whether the effect of vitamin D_3 is dependent on PEA-15, we silenced the PEA-15 gene with siPEA RNA, prior to treating cells with vitamin D_3 and TRAIL (reduced PEA-15 expression level are documented below the graphs of Fig. 5B). Interestingly, the attenuating effect of vitamin D_3 on the actions of TRAIL in apoptosis and cell viability was completely abolished in PEA-15 silenced cells (Fig. 5B, dark grey bars). These data suggest that PEA-15 is mediating the effects of vitamin D_3 in cell survival.

Vitamin D_3 increases phosphorylation of AKT1 and PEA-15

The strong up-regulation observed using an antibody directed against PEA-15 phosphorylated at serine 116 raised the question of whether the increase in the level of total protein is achieved in part by increasing protein stability. It has been reported that PEA-15 is phosphorylated by protein kinase Bα/AKT1, which leads to stabilization of the protein [36]. Therefore, we used Western blot analysis to measure the levels of phosphorylated AKT1, total AKT1, phosphorylated PEA-15 and total PEA-15 after treatment of the cells with vitamin D_3. Phosphorylation of AKT1 is a measure of the activity of this kinase [37]. We observed an activation of AKT1 3 and 6 hrs after addition of vitamin D_3, whereas the level of total AKT1 protein was unchanged (Fig. 6). Interestingly, 6 hrs after addition of vitamin D_3, we found an increase in phosphorylation of PEA-15 but no change in the level of total PEA-15 yet (Fig. 6),

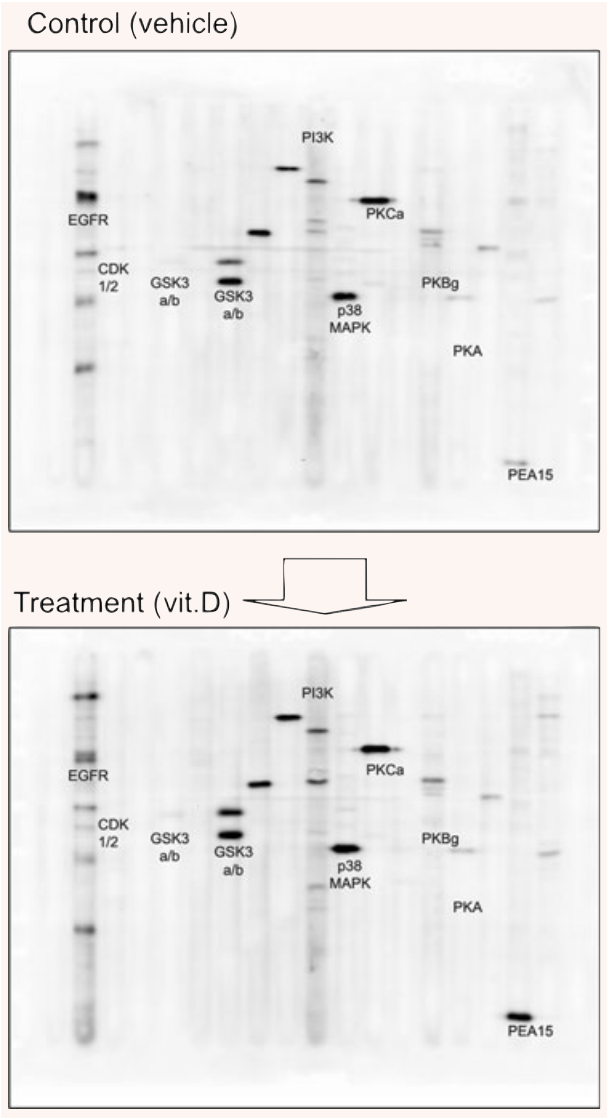

Fig. 2 Western blot testing of candidate targets of vitamin D_3. Differentiated HNSC.100 cells were treated with vitamin D_3 or vehicle for 24 hrs, and Western blots were performed with cell lysates.

which rises only after 12 hrs (Fig. 4). These data support the view that vitamin D_3 uses two mechanisms to increase the function of PEA-15, stimulation of gene transcription and stabilization of the protein through phosphorylation, possibly *via* activation of AKT1.

Discussion

In this study we searched for vitamin D_3-regulated targets in the proteome of human brain–derived cells. Several expression changes indicated by the antibody array paralleled the ones described previously by other authors. Among them are up-regulation of EGFR [38–40], down-regulation of activated p38 MAPK [41], up-regulation of tyrosine hydroxylase [42] and down-regulation of GSK3β mRNA in differentiated HIB5 rat hippocampal cells (own unpublished observations). Although this congruence with other published data in general attests to the reliability of the array, we also observed inconsistencies for some of the proteins examined on the array, for example, AKT1 or paxillin 1. We do not know whether the reason for this is accidental variation or due to unknown differences in the spotted antibodies, which are supposed to recognize the same modified protein. In any case, this reinforces the notion that results of screening methods such as mRNA or protein microarrays should be validated by independent methods.

Up-regulation of PEA-15 was the most prominent feature on the antibody array. We investigated this up-regulation further and conclude from our experiments with different cell lines that vitamin D_3 leads to an increase in promoter activity of PEA-15 already after 3–6 hrs (Fig. 4B), and with a little delay to an increase in protein phosphorylation after 6 hrs (Fig. 6), most likely *via* activation of AKT1, which is known to phosphorylate PEA-15 [36]. The increase in mRNA and the protein stabilization through phosphorylation in combination lead to an increase in total PEA-15 protein level after 12 hrs (Fig. 4B). On the microarray, five antibodies were directed against activated (*i.e.* phosphorylated) AKT1. Most of them actually indicated the increased activation of AKT1, but one of them detected less phosphorylated AKT1 (see paragraph above).

A bioinformatics search revealed a conserved VDR-binding site in the promoter of PEA-15, in line with a direct regulation of the promoter activity by VDR. In addition, we identified a conserved Sp1 binding site. Considering the finding of a functional VDR/SP1 complex [43], this SP1 site could contribute further to the VDR dependency of PEA-15. PEA-15 is also on the list of about 50 differentially expressed genes in the mouse brain revealed by two-dimensional gel electrophoresis in a model of developmental vitamin D deficiency [44]. Although this finding was not discussed or followed-up, it is in line with the vitamin D dependency of PEA-15 outlined in our study.

Significant knowledge about the function of PEA-15 has already been accumulated, such as its anti-apoptotic and anti-proliferation features [32, 34, 45, 46]. The interaction of PEA-15 with DED containing molecules such as FADD and caspase-8 in the death inducing signalling complex is considered to play an important role in the inhibition of cell death–induced activation of TNFR1 [45]. The mechanism of the anti-proliferative action of PEA-15 involves binding to the extracellular signal receptor-activated kinases (ERK1/2) and their export out of the nucleus [47]. This shift of ERK activity from nuclear to cytosolic targets then results in attenuated cell proliferation [46]. Our finding of increased AKT1 activity after vitamin D_3 exposure not only provides a potential mechanism for activation of PEA-15 but also adds another pathway through which vitamin D_3 exerts anti-apoptotic effects. AKT1 has been established as a central node promoting cell survival downstream of growth factors, oncogenes and cell stress [48].

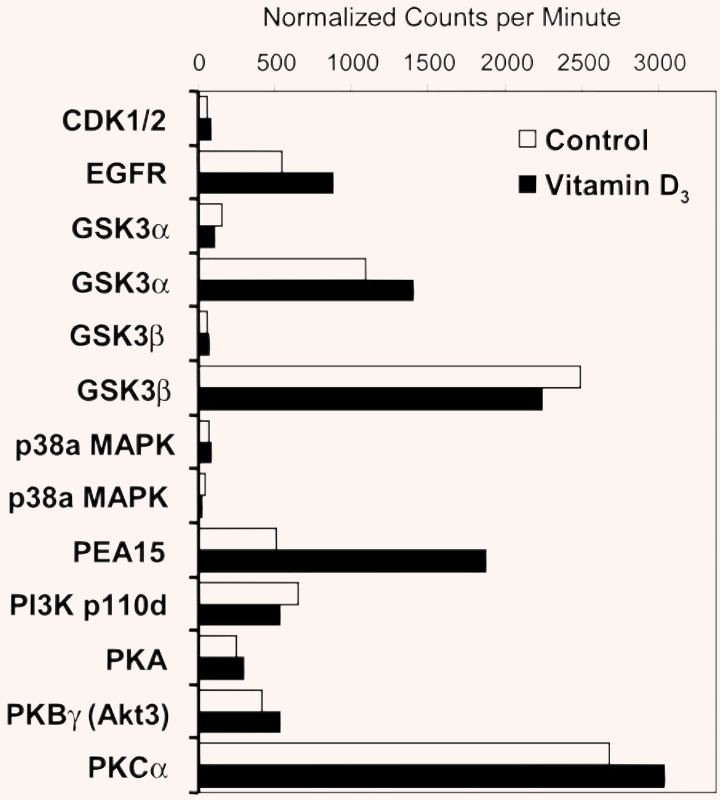

Fig. 3 Quantification of the changes in protein level induced by vitamin D_3 for the selected proteins. Quantification of the bands shown in Fig. 2 was performed with an imaging system in combination with the quantification software IRIS (Immuno-Reactivity Identification System, see Materials and Methods).

Proteins validated by Western-blot	Abbreviation	% Difference
Cyclin-dependent protein-serine kinase ½	CDK1/2	47%
Epidermal growth factor receptor-tyrosine kinase	EGFR	62%
Glycogen synthase-serine kinase 3 alpha	GSK3α	-31%
Glycogen synthase-serine kinase 3 alpha	GSK3α	28%
Glycogen synthase-serine kinase 3 beta	GSK3β	35%
Glycogen synthase-serine kinase 3 beta	GSK3β	-10%
Mitogen-activated protein-serine kinase	p38a MAPK	20%
Mitogen-activated protein-serine kinase	p38a MAPK	-50%
Phosphoprotein-enriched in astrocytes/diabetes 15	PEA15	268%
PI3K p110 delta	PI3K p110d	-17%
Protein kinase A (cAMP-dependent protein kinase)	PKA	17%
Protein-serine kinase B gamma (Akt3)	PKBγ (Akt3)	29%
Protein-serine kinase C alpha	PKCα	13%

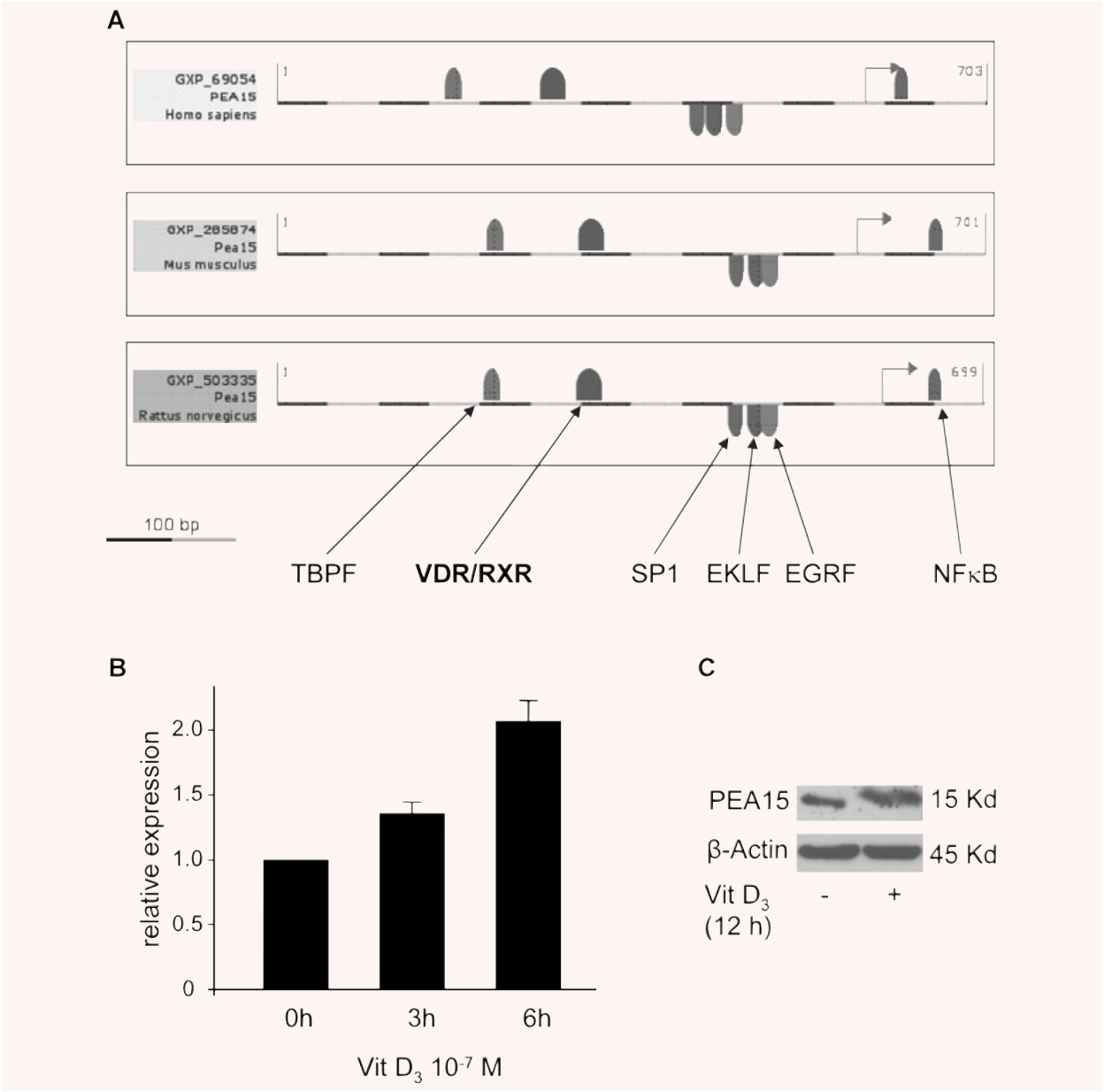

Fig. 4 Conserved transcription factor binding sites in the promoter of PEA-15 and up-regulation of PEA-15 mRNA and protein. (**A**) Several transcription factor binding motifs were identified, including those for VDR, NFκB, EGRF, EKLF and SP1. (**B**) Effect of vitamin D_3 on PEA-15 mRNA levels. A549 cells were treated with vitamin D_3 (10^{-7} M), and the mRNA levels were determined by real-time PCR at the times indicated. (**C**) Verification of induction of total PEA-15 after 12 hrs by Western blot.

Important for understanding the function of PEA-15 in the brain was its immunohistochemical mapping in the mouse brain [31]. Although early *in vitro* studies suggested its expression mainly in astrocytes [26], immunostaining revealed that PEA-15 was expressed in defined astrocyte sub-populations as well as neurons throughout the brain [31]. Interestingly, it is also expressed in areas known for neurogenesis in the murine embryonic and adult brain [31, 49, 50]. Therefore, vitamin D_3 impact on PEA-15 expression and function described in this study may be important in this context to contain further proliferation and allow differentiation.

Our discovery that PEA-15 is strongly responsive to vitamin D_3 potentially links vitamin D_3 to any of the actions of PEA-15. Although

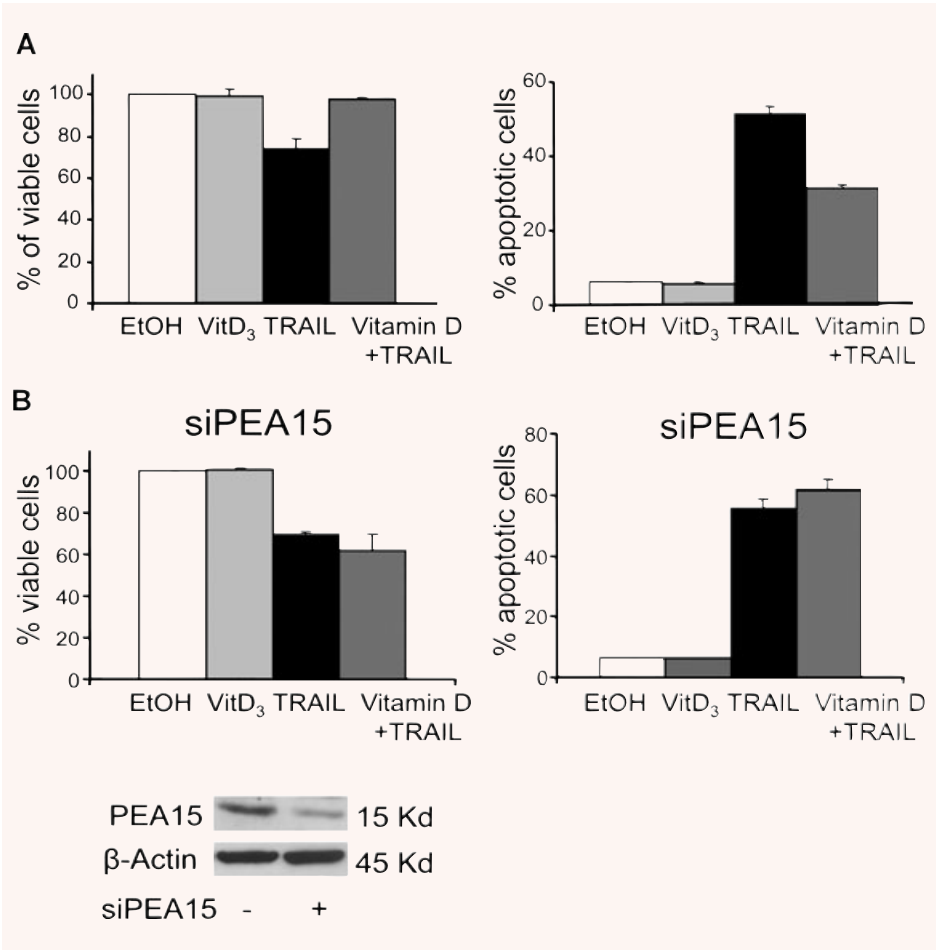

Fig. 5 Effect of vitamin D_3 on TRAIL-induced apoptosis and cell survival in A549 cells. (**A**) Cells were treated with vehicle, vitamin D_3 and TRAIL in the combinations indicated. Metabolic activity (as a measure of cell viability and apoptosis [FACS analysis] were determined. (**B**) Same treatment as in (**A**), but in the presence of RNAi directed against PEA-15. Data represent mean values + S.E.M. of three independent experiments performed in triplicates each. Panel below (**B**), verification of the efficacy of the siRNA directed against PEA-15 by Western blot using an antibody detecting total PEA-15.

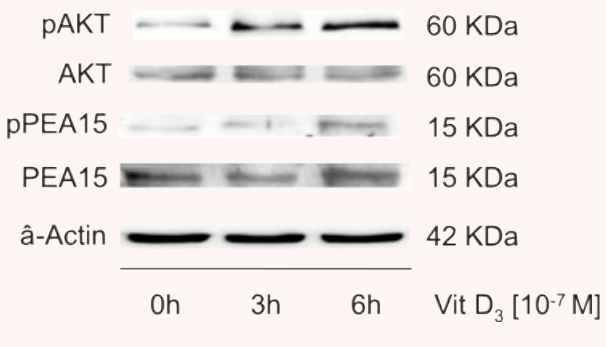

Fig. 6 Effect of vitamin D_3 on AKT and PEA-15. A549 cells were treated with vitamin D_3 and harvested at the times indicated. Western blot analysis was performed to detect the levels of total AKT (AKT), phosphorylated AKT (pAKT), total PEA-15, (PEA15), phosphorylated PEA-15 (pPEA15) and β-actin as loading control.

extensive work needs to be done to test the degree of overlapping activities, we already showed in this work that vitamin D_3 significantly affected apoptosis and cell survival in A549 cells in a PEA-15-dependent manner. The differentiation-inducing and anti-proliferative effects of vitamin D_3 in various tissues are well described [51–54]. Therefore, we propose that the pathway revealed in this study contributes to these effects and constitutes the molecular basis for these actions of vitamin D_3. This probably also applies to the positive effects of vitamin D_3 in cancer [55, 56], most recently highlighted by a widely noticed study outlining positive effects of sun exposure *via* improving the vitamin D_3 status [57].

Since VDR activity was reported to be induced by stress [58], it is tempting to speculate that up-regulation of PEA-15 by VDR plays a role in the stress response in the brain. It could, for example, contribute to the suppression of neurogenesis by stress [59] as well as counteracting the damage stress is executing on some brain cells [60], in particular because PEA-15 is found in brain areas sensitive to stress [61]. In this respect, it is interesting to note that in the ventral tegmental area AKT1, another target of vitamin D_3 we could confirm in this study, has very recently been

shown to be involved in the regulation of vulnerability to social defeat and of depression-related behaviours in rats [62].

With respect to brain function in general, numerous studies indicated the importance of regulating apoptosis for normal brain development and function [63, 64]. Thus, the positive role of vitamin D_3 reported for brain development [22, 65] and mental health [16, 20] may operate at least in part through its effect on apoptosis *via* inducing PEA-15.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Antibody array – full list.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2009.00753.x
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