

Research Paper

Tumor Suppressor RARRES1 Regulates DLG2, PP2A, VCP, EB1, and Ankrd26

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Published: 2010.06.02

Abstract

Retinoic Acid Receptor Responder (RARRES1) initially identified as a novel retinoic acid receptor regulated gene in the skin is a putative tumor suppressor of unknown function. RARRES1 was knocked down in immortalized human prostatic epithelial cell line PWR-IE cells and differential protein expression was identified using differential in-gel electrophoresis (DIGE) followed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and western Blot analysis excluding highly abundant proteins routinely identified in almost all proteomics projects. Knock-down of RARRES1: 1- down-regulates PP2A, an enzyme involved in the negative regulation of the growth hormone-stimulated signal transduction pathways; 2- down-regulates Valosin-containing protein causing impaired autophagy; 3- up-regulates the tumor suppressor disks large 2; 4- up-regulates Ankrd26 that belongs to the POTE family of genes that are highly expressed in cancer patients with poor outcome; and 5- down-regulates EB1, a protein that is involved in spindle dynamics and chromosome alignment during mitosis.

Key words: Retinoic Acid Receptor Responder, RARRES1, tumor suppressor

1. Introduction

Retinoic acids (RA) are ligands which signal through a family of six nuclear ligand-activated receptors, termed retinoic acid receptors (RAR α , β , and γ) and retinoid X receptors (RXR α , β , and γ) (1). These receptors form RXR/RXR homodimers or RAR/RXR heterodimers and bind to retinoic acid response elements (RARE) in DNA (1, 2). In the absence of RA, the RAR/RXR heterodimers bind to RARE and mediate a transcriptional repression of target genes (3). In contrast, when stimulated with physiological levels of RA, the ligand binding induces a conformational change in the dimer and causes a release of the corepressor complex and a subsequent recruitment of transcriptional coactivators, thereby stimulating transcription of target genes such as HOX family

members. Interestingly, RXRs have proven quite promiscuous, having been shown to facilitate binding of thyroid hormone receptor (TR) and the vitamin D receptor (VDR) to their respective response elements in DNA, indicating a broad role in the transcriptional events of many cell types (2). RA has a profound impact on vertebrate embryogenesis. RA has been described as a morphogen which is fundamental in proper embryonic patterning, especially in the rhombomeric region of the developing brain (4). Embryonic RA signaling is also crucial in the augmentation of cell survival, proliferation and differentiation (5). These observations have made RA the focus of research into its use as a front-line therapy in the treatment of human cancers.

RARRES1 was initially identified as a novel retinoic acid receptor (RAR β/γ) regulated gene in the skin (6). Several reports have implicated RARRES1 as a putative tumor suppressor genes based largely on the hypermethylation of its promoter in many tumor types and ageing normal tissues (7-14). Studies involving the re-expression of RARRES1 have also pointed to its tumor suppressive function, as it decreased the growth of aggressive PC-3M prostate cancer cells and Ishikawa endometrial cancer cells (10, 16). RARRES1 also greatly reduced the in vitro invasiveness and in vitro tumor growth of the PC-3M prostate cancer cells in nude mice (16). Recently, RARRES1 expression has been linked to the proliferation and differentiation of adult adipose-derived mesenchymal stem cells (17). RARRES1 expression was substantially reduced in the majority of cancer cell lines and was undetectable in 7 of them; furthermore, a significant reduction of RARRES1 has been observed in advanced and poorly differentiated tumors (18).

Here we report the mimicking of RARRES1 hypermethylation by knocking it down in PWR-1E cells. Proteins were then extracted from the knocked-down and control samples, were subjected to 2-D DIGE followed by MALDI-TOF/TOF analysis to identify the differentially expressed proteins secondary to the knock down. Since proteins don't act individually but rather in a network, we identified the proteins that are affected by the down-regulation of RARRES1 to be: Disk-large-2, PP2A, VCP, EB1, and Ankrd26.

2. Materials and Methods

2.1 Cell Culture and Nucleofection

PWR-1E cells passage 18 (ATCC CRL-11611) were cultured in Keratinocyte Serum Free Medium (K-SFM) supplemented with 0.05mg/mL of bovine pituitary extract (BPE) and 5ng/mL epidermal growth factor (EGF). Cells were nucleofected using the Cell line Nucleofector Kit V (Amaxa inc., Gaithersburg, MD), program T-20, and 200 picomole of RARRES1 or scrambled siRNA per million cells according to the manufacturer's instructions. A total of four biological replicates were performed for each sample and control.

2.2 Protein Extraction and Quantitation

Cells were harvested 48 hrs after nucleofection. Cells that were cultured in 100mm dishes were briefly rinsed with PBS, incubated with 3mL of a 0.05% trypsin - 0.53 mM EDTA solution, diluted 1:1 with PBS and incubated for 5 minutes at 37°C. Cells were then transferred to a centrifuge tube, washed 3 times with

PBS, each time centrifuging and discarding the supernatant. Cells were then collected and lysed with a buffer composed of 30 mM Tris-HCl, 7 M Urea, and 4% CHAPS. The lysates were then vortexed for 1 hour at 4°C followed by a centrifugation at 15,000xg for 15 minutes. Insoluble pellets were discarded. Proteins were then quantified as described previously (19) using a Protein Assay (cat # 500-0006) according to the manufacturer's description (Bio-Rad, Hercules, CA). Protein concentrations were then diluted with the same lysis buffer to a final concentration of 5mg/mL.

2.3 Protein-Dye Labeling for 2D-DIGE Analysis

CyDye DIGE Fluor minimal dyes were reconstituted in water free DMF to a final concentration of 200pmole/ μ L. 25 μ g of each of the 4 biological replicates of RARRES1 Knockdown or control samples were labeled with 200 pmoles of either Cy3 or Cy5. Cy3 and Cy5 were equally swapped among RARRES1 Knockdown and control samples. Upon addition of the dyes, samples were vigorously vortexed and kept on ice in the dark for 30 min. The labeling reaction was quenched by adding 10 μ L of a 10 mM lysine solution followed by vortexing. An internal standard sample composed of equal amounts of both conditions was labeled with Cy2 dye using the same procedure. Labeled proteins were pooled into 4 different fractions according to Table 1. Each fraction was diluted with rehydration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 4% CHAPS (w/v), 1% DTT (w/v), 1% Bio-lyte 3/10 Ampholyte) to a final volume of 450 μ L.

2.4 2D DIGE

Isoelectric focusing (IEF) was performed by rehydrating the 24 cm IPG strips pH 3-10 non-linear for 8 hours at 50 V in 450 μ L of sample in rehydration buffer. The rehydration step was followed by focusing the proteins using the following series of step and hold voltages: 250 V for 30 min, 500 V for 30 min, 1000 V for 1 hr, 3000 V for 3 hrs. An 8000 V was then maintained for a total of 65,000 Vh. IPG strips were then incubated in a reducing buffer composed of 50 mM Tris-HCl pH 8.8, 6 M Urea, 2% SDS (w/v), and 1% DTT (w/v) for 15 min. A second incubation was performed in an alkylating buffer composed of 50 mM Tris-HCl pH 8.8, 6 M Urea, 2% SDS (w/v), and 4% iodoacetamide (w/v) and 0.01% bromophenol blue for 15 min. Strips were then placed onto a 5-20% gradient gel that has 2 built-in spot picking references (Nextgensciences, Ann Arbor, MI). Gels were then loaded into an Ettan DALTsix buffer tank (GE Healthcare) filled with SDS electrophoresis buffer. Proteins were electrophoresed overnight at 8 W until the solvent front reached the bottom of the gel. The

buffer temperature was maintained at 10°C throughout the separation. The analytical gels (Gels 1 to 4 Table 1) were scanned right after the end of the separation as described below while the “pick gel” (Gel 5, Table 1) was fixed for 6 hrs with a solution containing

30% methanol and 7.5% acetic acid followed by overnight incubation with Sypro Ruby stain (Bio-Rad, Hercules, CA). The gel was then incubated in destaining solution (10% methanol, 7% acetic acid) for 3 hrs before proceeding with the scanning step.

Table 1. Sample mixture loaded on each gel: Each sample was composed of equal amounts of RARRES1 knock-down PWR-1E and control lysates. Cy3 and Cy5 dyes were equally swapped among RARRES1 knock-down and control samples. The internal standard was labeled with Cy2 and consisted of 12.5 µg of each knock-down and control. The sample loaded on the pick-gel was composed of 300 µg of each sample and control.

	RARRES1 KNOCKDOWN SAMPLE	CONTROL	INTERNAL STANDARD
Fraction 1/GEL 1	25 µg labeled with Cy3	25 µg labeled with Cy5	25 µg labeled with Cy2
Fraction 2/GEL 2	25 µg labeled with Cy3	25 µg labeled with Cy5	25 µg labeled with Cy2
Fraction 3/GEL 3	25 µg labeled with Cy5	25 µg labeled with Cy3	25 µg labeled with Cy2
Fraction 4/GEL 4	25 µg labeled with Cy5	25 µg labeled with Cy3	25 µg labeled with Cy2
PICK GEL	300 µg non-labeled	300 µg non-labeled	

Table 2. Identified proteins: Table of proteins that were differentially expressed across the 4 biological replicates with a t value < 0.05 and that were identified with a significant MASCOT interval of confidence ($p < 0.05$). Among the identified proteins, 31 highly abundant, structural proteins, HSPs as well as proteins that are repeatedly identified as being differentially-expressed were omitted from this list.

T-test	Av. Ratio	Protein Name
0.0054	-1.36	P55072 TERA_HUMAN Transitional endoplasmic reticulum ATPase - Homo sapiens (Human)
0.044	-1.36	P13798 ACPH_HUMAN Acylamino-acid-releasing enzyme - Homo sapiens (Human)
0.0072	1.21	Q15700 DLG2_HUMAN Disks large homolog 2 - Homo sapiens (Human)
1.20E-05	-1.73	P30153 2AAA_HUMAN Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform - (Human)
0.031	-1.14	O00303 EIF3F_HUMAN Eukaryotic translation initiation factor 3 subunit F - Homo sapiens (Human)
0.048	1.19	A5A3E0 A26CB_HUMAN ANKRD26-like family C member 1B - Homo sapiens (Human)
0.047	-1.19	Q15293 RCN1_HUMAN Reticulocalbin-1 precursor - Homo sapiens (Human)
0.0032	-1.49	Q9UNZ2 NSF1C_HUMAN NSF1 cofactor p47 - Homo sapiens (Human)
0.042	-1.23	P08700 IL3_HUMAN Interleukin-3 precursor - Homo sapiens (Human)
0.0039	1.17	Q99829 CPNE1_HUMAN Copine-1 - Homo sapiens (Human)
0.0041	1.18	P06748 NPM_HUMAN Nucleophosmin - Homo sapiens (Human)
0.034	-1.18	Q15691 MARE1_HUMAN Microtubule-associated protein RP/EB family member 1 - Homo sapiens (Human)
0.042	-1.24	P49411 EFTU_HUMAN Elongation factor Tu, mitochondrial precursor - Homo sapiens (Human)
0.049	1.1	P63104 1433Z_HUMAN 14-3-3 protein zeta/delta - Homo sapiens (Human)
0.02	-1.27	P62714 PP2AB_HUMAN Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform - Homo sapiens (Human)
0.039	-1.15	P21796 VDAC1_HUMAN Voltage-dependent anion-selective channel protein 1 - Homo sapiens (Human)
0.048	1.15	Q9HCY8 S10AE_HUMAN Protein S100-A14 - Homo sapiens (Human)
0.027	1.25	P31949 S10AB_HUMAN Protein S100-A11 - Homo sapiens (Human)
0.035	1.44	P31949 S10AB_HUMAN Protein S100-A11 - Homo sapiens (Human)

2.5 Gel Scanning and Image Analysis

Gels were scanned using an Ettan DIGE Imager (GE Healthcare) at 100 μ m size. Appropriate laser exposure times were used so that no protein spot is saturated and all spot intensities fall within the linear range. The analytical gel images were cropped using ImageQuant TL (GE Healthcare) to get rid of the smears on gel edges. Image analysis was then performed using DeCyder™2D 6.5 software. Cropped images of the analytical gels were loaded using the Image Loader tool of the software and spot detection and matching was performed using the Batch Processor tool while applying the following spot filtering parameters: Slope > 1.1 ; Area < 100 ; Peak Height < 100 ; and Volume < 65,000. Automatic spot matching was followed by a manual confirmation and re-matching of unmatched or mistakenly matched spots. Inter-gel analysis and calculation of average protein abundance ratios were performed using the Biological Variation Analysis (BVA) tool. A Student's *t*-test was applied to generate a list of differentially-expressed spots between control and RARRES1 Knockdown PWR-1E protein extracts.

2.6 Spot Excision and Digestion

The "pick gel" image was matched with the analytical gel images using the BVA tool of the DeCyder™2D v.6.5 software. A spot pick list coordinates was generated for proteins that were differentially-expressed within the 4 biological replicates with a *p* value of 0.05 or less. The coordinates of the 2 internal references were included in the pick list that was exported into the Spot Picker v1.2 which is the driver for the Ettan Spot Picker instrument (GE Healthcare). The "Pick Gel" was then placed in the gel holder plate. The spot picker was calibrated using the internal references and spots were then excised in ultrapure water and transferred into a 96-well ZiplateC₁₈ (Cat # ZPC180010, Millipore, Bedford, MA). Protein digestion and peptide recovery procedures were modified from the manufacturer's instructions as follows: Gel pieces were dehydrated by adding 200 μ L of acetonitrile to each well followed by 10 minute incubation. Full vacuum was then applied to elute the acetonitrile through the C₁₈ resin forming the bottom of the Ziplate. Gel pieces were then rehydrated by adding 15 μ L of a 25 mM ammonium bicarbonate containing 5 ng/ μ L of modified trypsin. After overnight incubation at 37°C, 8 μ L of acetonitrile was added to the resin. After 12 minutes of incubation, 100 μ L of 0.2% TFA ultrapure water solution was added to each well and incubated for 30 minutes. The 96-well plate was then placed on a vacuum plate holder to empty wells. A final washing step was performed with a 100 μ L of

0.2% TFA ultrapure water solution followed by vacuum to empty wells. The Ziplate was then placed upon a low retention 96-well "V" bottom plate (cat# 2897, Corning Inc., Corning, NY) and tryptic digests were eluted by adding 8 μ L of acetonitrile followed by centrifugation at 3000 \times g. The bottom plate was left to air dry. Peptides were reconstituted with 2 μ L of a 2.5 mg/mL α -cyano-4-hydroxycinnamic acid matrix solution. The matrix solvent was composed of 49.95% water, 49.95% acetonitrile, and 0.1% TFA (v/v/v). The peptide-matrix mixture was then deposited onto a MALDI target plate and allowed to air dry.

2.7 MALDI TOF and TOF/TOF Analysis

MS and MS/MS analysis were performed using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster city, CA). The instrument was calibrated using Applied Biosystems calibration standards. MALDI-TOF spectra were acquired by accumulating 1000 laser shots in reflector mode for positive ion detection between 800 and 4000 *m/z*. The most intense 15 peaks with S/N of 10 or higher were selected for MS/MS analysis excluding the commonly observed peaks for trypsin. Argon was used as the collision gas.

2.8 Protein Identification

Protein were identified as described previously (20). Briefly, MS and MS/MS Mass lists were picked by the GPS Explorer v3.5 software and submitted to the MASCOT v.2.0.00 search engine. The settings that were used were the following: database: Swiss-Prot; Taxonomy: *homo sapiens*; Enzyme: trypsin; Variable modifications: carbamidomethyl (C) and Oxidation (M); MS/MS fragment tolerance: 0.3Da; Precursor mass tolerance: 75 ppm; Maximum missed cleavage allowed: 1. Only proteins with MASCOT confidence interval higher than 95% (*p* < 0.05) were considered. Experimental molecular mass and *pI* were used to confirm protein identities.

2.9 Western Blotting

Western Blotting was performed as described previously (21) using the following antibodies: Rabbit monoclonal to PP2A (cat. ab32141, Abcam, Cambridge, MA); Rabbit polyclonal to PSD93 (cat. ab2930, Abcam, Cambridge, MA); Goat polyclonal to RARRES1 (R&D systems, Mineapolis, MN); Mouse monoclonal to VCP (cat. ab11433, Abcam, Cambridge, MA).

3. Results and Discussion

RARRES1 knockdown in PWR-1E cells was validated using western blot that shows the significant decrease in RARRES1 expression as a result of

RARRES1 siRNA nucleofection when compared to RARRES1 expression in the scrambled siRNA nucleofected cells. Relative protein expression change that resulted from RARRES1 knockdown in PWR-1E cells was assessed by DIGE. A total of 8121 spots were detected on the 2-dimensional gels (Figure 1) of which 97 spots were consistently differentially-expressed with a $p < 0.05$ across the 4 biological replicates. An advantage of DIGE over regular 2-D gel techniques is the elimination of gel to gel variations since both sample and control are run on the same gel and normalized based on the internal standard pool which is also run on the same gel. Only spots with level changes of 10% or more were considered. These spots

were matched and excised from the pick gel (Figure 2) and processed for MALDI-MS/MS analysis (21) that allowed for the identification of 50 proteins. Differentially-expressed proteins that were identified included highly abundant proteins (e.g. tubulin), heat shock proteins (e.g. HSP 90) in addition to other proteins that are repeatedly identified as being differentially-expressed (e.g. peroxiredoxins, enolases, piruvate kinases) (22). These proteins were not considered in the study. Emphasis was given to proteins that are involved in the regulation of cell cycle. Of particular interest were Disks large homolog 2 (Dlg-2), Serine/threonine-protein phosphatase (PP2A), and Valosin containing protein (VCP) (Figure 3).

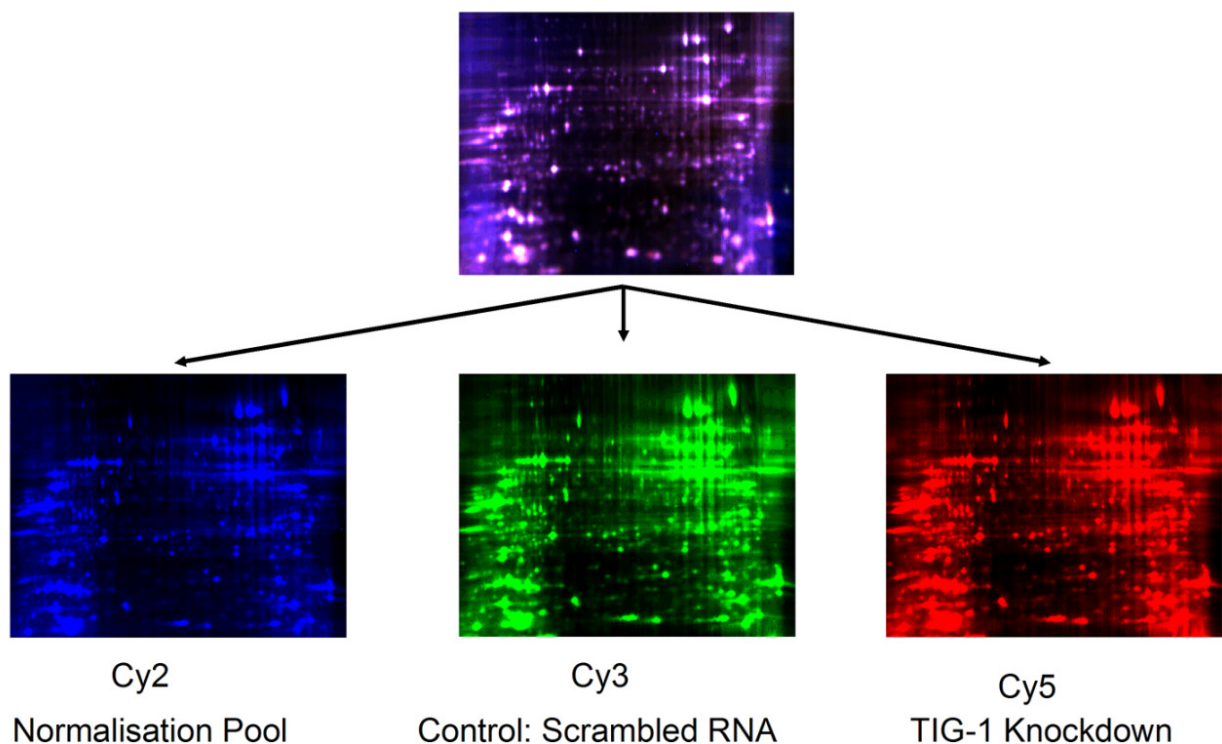


Figure 1. DIGE data: Representation set of one of the 4 biological replicates. 25 μ g of control PWR-1E cells lysates labeled with 200 pmoles of Cy3 (green); 25 μ g of RARRES1 knock-down PWR-1E cells labeled with 200 pmoles of Cy5 (red) and a normalization pool composed of a 50:50 mixture of sample and control labeled with Cy2 (blue). The first dimension separation was performed on a 24 cm non-linear IPG strips and the second dimension separation was accomplished on a 5-20% gradient polyacrylamide gel with 2 built-in spot picking references. Gels were scanned at a 100 μ m resolution using excitation and emission wavelength that corresponds to each dye. Combined Cy2, Cy3, and Cy5 signals are represented in the top gel.

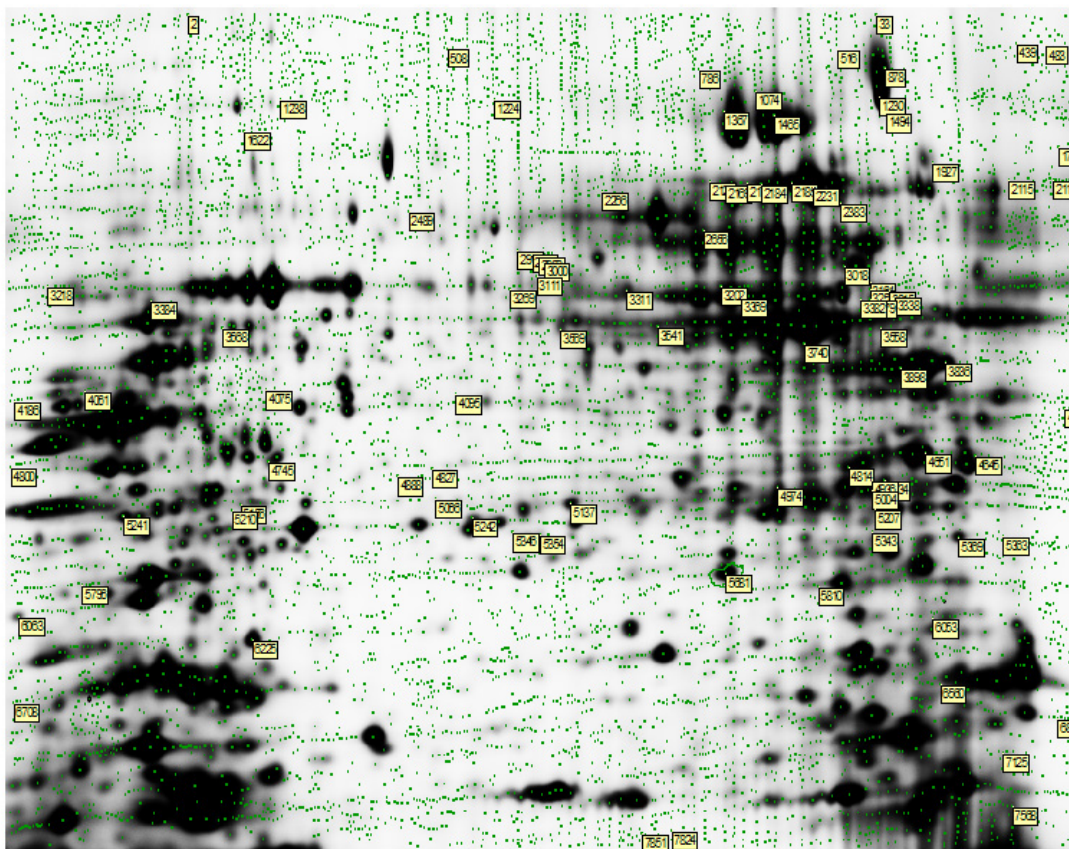


Figure 2. Sypro Ruby Pick gel: loaded with 300 μ g of PVR-IE RARRES1 knock-down protein extracts and 300 μ g of control sample. The green dots represent the proteins that were detected by the Decyder software. Annotated proteins are those that were picked for mass spectrometry analysis. These spots were identified as differentially-expressed across the 4 biological replicates in the analytical gels. The coordinates of these spots were recorded, matched, and excised from the Pick gel for identification.

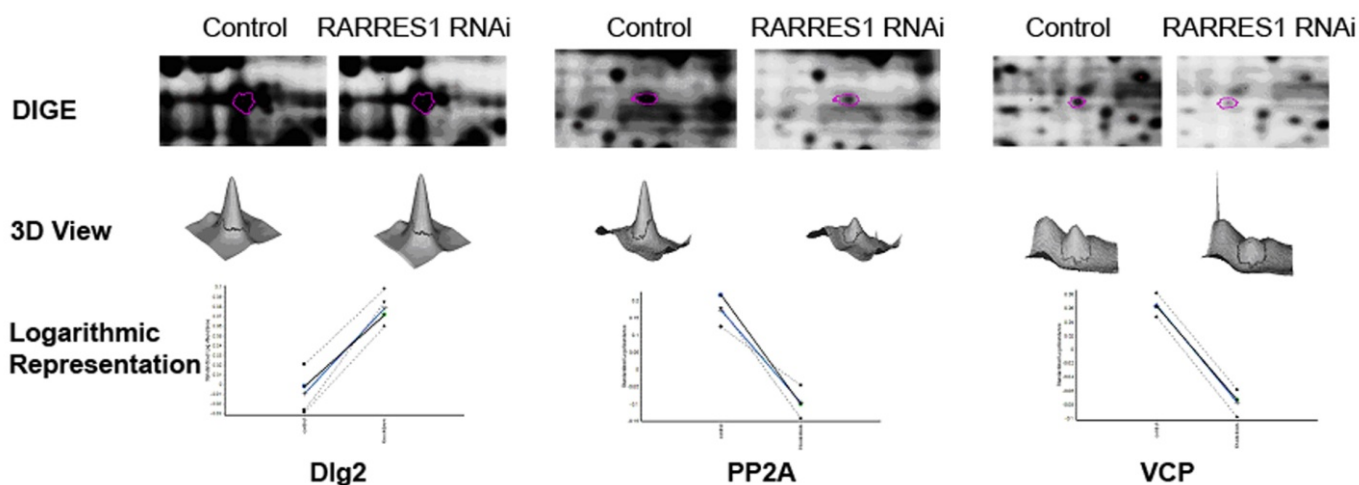


Figure 3. Differentially expressed proteins: a) 2- Cropped DIGE; b) three-dimensional view, and c) logarithmic representation of the 4 four biological replicates of Dlg-2 upregulation, PP2A and VCP down-regulation as a result of RARRES1 knock-down in PVR-IE cells.

Retinoic Acid Receptor Responder 1 (RARRES1) is a putative carboxypeptidase inhibitor and tumor suppressor which gene is frequently silenced in cancer and aging normal cells. RARRES1 knock-down in PWR-1E cells resulted in the upregulation of disks large 2 (Dlg2), a neoplastic tumor suppressor that acts as a scaffold at cell-cell junctions (23-25). Dlg2 consists exclusively of protein-protein interaction domains and PDZ domains implicated in cell polarity control and is localized basal to the adherens junctions (26, 27). Dlg2 homologue was found to be upregulated in oncocytoma, a benign tumor of the kidney and is therefore considered an oncogene (28). RARRES1 knock-down resulted also in the down-regulation of serine/threonine-protein phosphatase 2A (PP2A) in addition to its catalytic subunit. PP2A has broad substrate specificity. It targets proteins of oncogenic signaling cascades including Raf, Mek, and Akt (29). PP2A is a negative regulator of the growth hormone stimulated signal transduction pathways. Mutation of this enzyme has been identified in several types of cancer including lung (30, 31) and breast (32-36). Its down-regulation secondary to RARRES1 knock-down results in a decreased targeting of Raf, Mek, and Akt. Furthermore Valosin-containing Protein (VCP) was also down-regulated as a result of RARRES1 knock-down. Mutation of VCP causes inappropriate activation of NF κ B signaling cascade (37) as well as impaired autophagy (38). End-binding protein-1 (EB1) was found to be down-regulated as a result of RARRES1 knock-down. EB1 is mainly involved in the regulation of spindle dynamics and chromosome alignment during mitosis (39) and promotes microtubule growth by suppressing catastrophes (40). It was recently shown to promote colony formation and enhancing tumor growth in nude cells while its knock-down resulted in the inhibition of cancer cell proliferation suggesting an oncogenic role (41). As for Ankrd26, it belongs to the POTE family of genes containing ankyrin repeat and coiled coil domains (42). Ankrd1 has recently been found to be expressed in the majority of ovarian adenocarcinomas and found at high levels in patients with worse outcome (43). Western blotting was performed to validate the upregulation of Dlg-2 and down-regulation of PP2A and VCP (Figure 4) as a result of RARRES1 knock-down.

While the down-regulation of VCP and PP2A and the up-regulation of Ankrd26 are consistent with the knock-down of tumor suppressor RARRES1, oncogenic EB1 down-regulation and tumor suppressor dlg2 up-regulation following RARRES1 knock-down are thought to have occurred to compensate for the loss of RARRES1 expression.

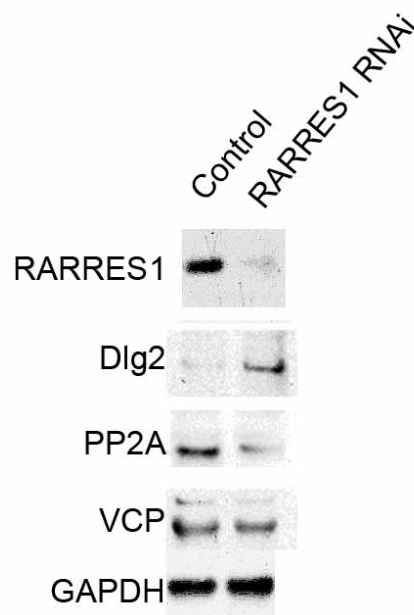


Figure 4. Western Blot Validation: of a) RARRES1 knock-down, b) Dlg2 homologue, c) PP2A, d) VCP and e) GAPDH loading control. Lane 1 scrambled siRNA nucleofected PWR-1E lysates; lane 2 RARRES1 siRNA nucleofected PWR-1E lysates.

Conclusion

Our analysis shows that knock-down of RARRES1 exhibits a consistent change in the expression level of several proteins notably up-regulation of Dlg2, a neoplastic tumor suppressor, down-regulation of VCP that results in the activation of NF κ B signaling cascade, down-regulation of PP2A that results in a decreased targeting of oncogenes Raf, Mek, and Akt, up-regulation of Ankrd26, a member of the POTE family of genes, and down-regulation of EB1. This data highlights the role of RARRES1 and as a consequence that of retinoic acid as a tumor suppressor. More studies are required to test which one of these molecules interacts directly with RARRES1 to elucidate its mechanism of tumor suppressing.

Acknowledgment

This study was funded by NIH R01CA129813, NIH 1 P01 CA130821. The authors wish to acknowledge the support of the following Lombardi Cancer Center Core Facilities (NIH P30 CA51008): Tissue culture and proteomics and metabolomics.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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