Dach1-Variant 4 Plays an Oncogenic Role and Promotes Radioresistance in Prostate Cancer

Ilenia Giordani

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Carlo M. Scornajenghi

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Francesco Marampon

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Antonella Stoppacciaro

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Silvia Di Agostino

University of Catanzaro: Università degli Studi Magna Graecia di Catanzaro

Cosimo De Nunzio

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Andrea Tubaro

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Zhiping Li

University of Pennsylvania

Richard G. Pestell

University of Pennsylvania

Paola De Cesaris (✉ paola.decesaris@univaq.it)

University of L'Aquila Department of Clinical Medicine Life Health and Environmental Sciences: Università degli Studi dell'Aquila Dipartimento di Medicina Clinica Sanita Pubblica Scienze della Vita e dell'Ambiente <https://orcid.org/0000-0002-3802-9103>

Anna Riccioli

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Antonio Filippini

Sapienza University of Rome: Università degli Studi di Roma La Sapienza

Primary research

Keywords: prostate cancer, RDGN, isoforms, radiotherapy, DACH1, radioresistance

Posted Date: September 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-892536/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Human Dachshund homologue 1 (DACH1) is involved in carcinogenesis with opposite roles reported in different tumor types. Four alternatively spliced transcripts encoding different DACH1 isoforms were described but their specific role in human cancers is still unknown. Prostate cancer (PCa) is a heterogeneous disease with a very wide variability, so there is yet a relevant need to find new diagnostic and therapeutic biomarkers to make a safe clinical evaluation. It is well known that the differential expression of protein isoforms can induce distinct transcriptional programs with opposing effects on tumor progression and therapy. Thus, in this study we aimed to correlate the functional role of DACH1 with its splicing variants expression in PCa.

Methods: The expression and functional role of DACH1 splicing variants in PCa were investigated using tumor (PC3) and normal (RWPE-1) cell lines, patient biopsies and TCGA dataset. Flow-cytometry, western blots and RT-qPCR were used for *in vitro* molecular characterization; invasion, adhesion, clonogenic assays and cell cycle analysis for functional characterization. Immunohistochemistry and western blot were performed on human PCa biopsies.

Results: RT-qPCR and Western Blot revealed that DACH1-positive PC3 cells predominantly expressed DACH1 variant 4 (DACH1-v4), whereas RWPE-1 cells mostly expressed DACH1 variant 3. Stable DACH1-v4 overexpression enhanced the transformed phenotype of PC3 cells by inducing proliferation, colony formation, invasion ability, epithelial to mesenchymal transition. Given its intrinsic radioresistance, PCa frequently recurs after radiotherapy. Of note, DACH1-v4-overexpressing PC3 cells displayed higher radioresistant behavior. Overexpression of DACH1-v4 also transformed RWPE-1 cells to oncogenic phenotype, suggesting a pro-oncogenic role for this specific isoform. PCa biopsies analysis showed DACH1 nuclear staining enhanced throughout the increase of the tumor grade. Remarkably, tumor glands were found to express a long DACH1 variant, while normal prostate tissue expressed the short DACH1 isoform, in line with data from TCGA-PRAD analysis and our data in RWPE-1 cells.

Conclusions: Our findings highlight the oncogenic role of DACH1-v4 in PCa and suggest that the longer DACH1 variants could be associated to pro-tumor function, while the shortest DACH1 variant would perform tumor suppression. The expression of specific DACH1 isoforms could represent a novel diagnostic/prognostic marker in PCa.

Background

Prostate cancer (PCa), the second leading form of cancer-related death in men worldwide [1], is a heterogeneous disease with a very wide variability. Thus, nowadays, new diagnostic and therapeutic biomarkers are urgently needed to better stratify the tumor risk level and choose the most personalized therapy. Active surveillance, surgery, and radiation therapy (RT) are the standard therapeutic choices for men with PCa. Particularly, radiotherapy, more frequently delivered with external beam radioactive sources (EBRT), has been shown to offer cure rates similar to surgery, with fewer long-term sexual side effects and little effects on urinary continence [2]. However, PCa local and distant recurrence frequently occurs and treatment failure is attributable to the intrinsic radioresistance of cancer cells. Furthermore, acquisition of radioresistance has been reported during cancer treatment with fractionated irradiation [3]. Notably, the mechanisms underlying intrinsic or acquired radioresistance remain unclear. RT kills cancer cells by means of ionizing radiations (IR) that damage DNA both directly, by inducing DNA single (SSB) or double (DSB)-strand breaks, and indirectly, through the formation of reactive-oxygen-species (ROS) [4]. This results in cell cycle arrest and the activation of DNA repair mechanisms to protect genomic integrity even though, upon irreversible DNA damage, the apoptotic response is activated. Nevertheless, cancer cells often activate several mechanisms to overcome the therapeutic efficiency of EBRT including the aberrant expression of anti-apoptotic genes, the activation of the epithelial-mesenchymal transition program (EMT), the enhanced DNA repair system and the increased intracellular levels of ROS [5, 6]. These mechanisms are finely regulated and identifying the upstream molecules orchestrating these systems could permit the identification of new patient-based radiosensitizing strategies.

The Human Dachshund homologue 1 (DACH1) is a key component of the Retinal Determination Gene Network (RDGN), originally identified in *Drosophila Melanogaster* eye specification. In mammals DACH1 governs morphogenetic processes at early growth stages, cell differentiation and tissue homeostasis [7] and participates in physiological functions of the liver [8], kidney [9], and mammary gland development, where DACH1 also enhanced mammosphere formation in the LA-7 rat mammary gland stem cells and promoted mammary gland ductal branching during mammary gland development in transgenic mice [10]. Recent studies demonstrated that aberrant expression of RDGN members is involved in the tumorigenic process of different human cancers. The molecular mechanisms governing the RDGN have been investigated in order to identify potential therapeutic targets in different cancer types [11, 12].

DACH1 expression was reduced in human breast tumors, correlating with poor prognosis [13] and breast cancer cell lines enriched in cancer stem cells (CSC) also showed a reduction in DACH1 expression, while DACH1 over-expression reduced the percentage of CSC in an *in vivo* murine model [14]. DACH1 restrained breast cancer invasion and metastasis via interleukin-8 [15]. Furthermore, the high levels of DACH1 expression seen in normal lung tissues were reduced in lung adenocarcinoma, and DACH1 over-expression inhibited lung cancer cell proliferation, invasion, colony formation *in vitro* and tumor growth *in vivo* involving p53 [16] and pro-inflammatory chemokines CXCL5, CXCL1 and CXCL8 [17–19]. Furthermore, DACH1 was target of the oncogenic miR-194 in pancreatic cancer, consistent with a tumor suppressor role [20], and DACH1 reduced cell proliferation and migration and functioned as a sensitizer to docetaxel in different colorectal cancer cell lines [21, 22].

However, the role of DACH1 in cancer onset and progression is controversial. In contrast with a tumor suppressor role, DACH1 knockdown inhibited cell proliferation and invasion in Capan-1 pancreatic cancer cells [23]. In ovarian cancer, DACH1 protein levels increased with tumor invasiveness and was highly expressed in metastatic ovarian cancer compared with that of normal, benign, and borderline ovarian tissues [24]. Hu et al. reported that DACH1 was expressed in intestine crypt basal cells and that DACH1 was necessary for the formation of tumor organoids, which were considered by the authors to serve as a functional assay of tumorigenesis. Moreover, high expression of DACH1 in colorectal cancer at all stages of tumor progression correlated with poor prognosis [25].

Collectively this evidence suggests that DACH1 may act a tumor suppressor or oncogene in different kinds of cancer and tumoral microenvironment [19, 26].

Four alternatively spliced transcripts encoding different isoforms have been described for human DACH1 gene [27], even though no studies have been conducted so far on the role of different DACH1 isoforms in human cancers. It is well known that alternative splicing changes are frequently observed in cancer and are starting to be recognized as important signatures for tumor progression and therapy [28]. It was also shown that the expression of different isoforms of specific proteins can induce distinct transcriptional programs with opposing effects on clinical outcome [29, 30]. Although DACH1 is considered a tumor-suppressor for PCa where it has been reported to restrain the transformed phenotype in cell lines [31, 32], the specific DACH1 isoform responsible for the antitumor function was not determined. Notably, no studies have been yet performed to dissect the different role of DACH1 isoforms in PCa onset, progression and radioresistance.

In this study, we aimed to correlate the functional role of DACH1 with its splicing variants expression in the androgen-independent metastatic prostate cancer PC3 cell line and in primary prostate normal and tumor tissues of PCa patients. In particular, we asked whether the specific DACH1 variant 4 (DACH1-v4) plays a role in enhancing or rather abating radioresistance, which most PCa patients develop after first-line radiation treatment.

Methods

Cell culture

PC3 and RWPE-1 cells were purchased from the American Type Culture Collection (ATCC, Manassan, VA). PC3 cells (ATCC n. CRL-1435 Lot n. 61777391) were routinely checked for mycoplasma and were maintained in RPMI-1640 (Sigma, Saint Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma), 200 U/ml penicillin–streptomycin (Sigma), sodium pyruvate 1 mM (Sigma), Hepes 10 mM (Sigma) and 10% fetal bovine serum (FBS) (Life Technologies-Gibco Eugene, OR, USA). RWPE-1 cells were maintained in Keratinocyte serum-free medium (Gibco, Grand Island, NY) supplemented with bovine pituitary extract, 10 ng/mL epidermal growth factor (EGF), and 0.5% Gentamicin (Invitrogen).

Cells were maintained at 37°C in a humidified incubator with 5% CO₂. Corning T25 ultra low attachment flasks were used to culture the cells in anchorage-independence conditions in basal growth medium.

Bacterial Transformation And Cell Transfection

Plasmids used are: CMV10 expression vector encoding an N-terminal FLAG peptide linked to DACH1-v4 isoform (1 ng of this vector was subjected to PCR with primers shown in Supplementary Fig. 1 and Supplementary Table 1, where the pair of primers 1 and 5 significantly produced an amplicon compared to PCR on the CMV empty vector); DACH1 shRNA pGIPZ vector and the respective empty vectors (kind gifts from Dr. Richard Pestell, Pennsylvania Cancer and Regenerative Medicine Research Center, Baruch S. Blumberg Institute, Pennsylvania Biotechnology Center, Wynnwood, PA, 19096, USA).

For the transfection, 3x10⁵ PC3 and 2.5x10⁵ RWPE-1 cells were plated in 6-well plates on the day prior to transfection in RPMI complete medium with 10% FBS and Opti-MEM medium (Gibco), respectively. After 24 hours, cells reaching the 80% confluence were transfected with 1 µg vector expressing DACH1-v4 isoform or the empty vector by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To obtain stable cell lines, the previous vectors were cotransfected with 0.5 µg of pcDNA3.1 carrying puromycin resistance (kind gift from Prof. Lorenzo Puri), as selection marker. 24 h after transfection, complete medium was substituted and puromycin (2µg/ml) was added, repeating this passage every day until all the non-transfected cells, used as control, died. The fold effect of expression vector was determined by comparison to the effect of the empty vector and statistical analyses were performed using a t-test.

Cell Proliferation Assay And Cell Cycle Analysis

For proliferation assay, 3x10⁵ PC3 cells and 2x10⁴ RWPE-1 cells, transfected or not with DACH1-v4 vector, were seeded into Corning flask T-25 and Corning 12 well plate respectively, and cells were counted daily for three days using a Thoma cell counting chamber. Sh DACH1-v4 cells were plated in 96-well plates at a seeding density of 4x10³ cells/well, crystal violet-stained daily for three days, diluted with acetic acid 10% and then a spectrophotometer was used to measure the absorbance of solutions at 550 nm.

To analyze cell cycle, 2x10⁵ cells were plated in Corning flask T-25 in complete growth medium and after 48h, all samples except the controls were treated with a 4 Gray (Gy) dose of IR. At 24h and 48h after radiation treatment, both untreated and treated cells were trypsinized, fixed with 70% ethanol and incubated in a solution containing 20µg/ml Propidium Iodide and 100 µg/ml DNase-free RNaseA (Sigma) for 3 h at room temperature. Samples were analyzed by CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA). When cell DNA content was lower than 2N (sub-G₁ cell population), cells were considered apoptotic.

Radiation Treatment And Colony Formation Assay

Radiation treatment was delivered at room temperature using an x-6 MV photon linear accelerator.

For colony formation assay, 5x10⁵ cells were plated in Corning flask T-25 in complete growth medium and after 48h, all samples except the controls were treated with different doses of IR (from 2 to 6 Gy). 3h after radiation, all treated and untreated cells were trypsinized and counted. 400 untreated cells were plated for the controls, while 400 irradiated cells for each dose of Gy were plated to determine their ability to undergo cell division after treatment and maintained at 37°C in a humidified incubator with 5% CO₂. After ten days, the colonies were visualized after fixation with 4% paraformaldehyde at room temperature for 15 minutes and stained with 0.05% crystal violet for 1h. Only colonies of at least 50 cells were counted. The Surviving Fraction was calculated

as the ratio between the number of colonies formed after treatment and the number of colonies formed without treatment. The same procedure was performed for clonogenic assay in basal condition both on PC3 and RWPE-1 cells.

Immunohistochemistry

Tumor samples obtained for diagnostic purposes were used after personal consent, following consent procedures approved by International Review Board of Department of Clinical and Molecular Medicine, Sant'Andrea Hospital, Sapienza University of Rome (Prot. n. 228 SA_2016, 12.12.2016 RIF. CE: 4208_2016).

Immunohistochemistry was performed on formalin fixed, paraffin included biopsies from 30 patients with clinically localized prostate cancers, representative of the 5 differentiation group grades as classified by ajcc 2017, 6 cases for each group grade.

5 µm sections were deparaffinized, antigen retrieved using a pH6 DAKO Retrievier solution and immunostained by a DAKO autostainer using EnVision™ FLEX + revelation system (Dako, Colorado Inc, Fort Collins, Colorado, USA). Anti-Human DACH1 primary antibody (NBP2-04080, Novus Biological) was used at the optimal dilution 1:1000. The immunogen recognized by this antibody maps to a region between residue 720 and 740 of human DACH1, that is included in all isoforms.

Western Blot

PC3 and RWPE-1 cells were collected and lysed with a buffer containing a protease and phosphatase inhibitors cocktail (Lysis Buffer, Cell Signaling Technology CST, MA, USA) for total protein extraction. Lysates were sonicated, centrifuged at 1400 RPM for 15 minutes at 4°C and boiled for 10 minutes.

Primary biopsies protein extraction was performed first by mechanical mince with sterile scissors and tweezer for tissue dissociation and then in lysis buffer with a potter to lyse and homogenize samples. They were kept on ice for 15 minutes and then centrifuged at 1400 RPM for 15 minutes at 4°C. Cell lysates, normalized for protein content, were resolved by SDS-PAGE on 10–15% polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, UK) and immunoblotted. Rabbit polyclonal anti-DACH1 antibody (NBP2-04080) was purchased from Novus Biologicals (Centennial, Colorado, USA).

Anti-PCNA (rabbit pAb #ab 2426, abcam, Cambridge, UK). Anti-E-cadherin (24E10 mAb #3195), anti-Snail (C15D3 mAb #3879), anti-cleaved Notch1 (D3B8 mAb #4147), anti total-β-catenin (rabbit pAb #9562), anti total-AKT (rabbit mAb #4961), anti phospho-AKT (ser 473 rabbit mAb #9271), anti phospho-GSK3β (rabbit pAb #9336), anti total GSK3β (rabbit mAb #9315), anti-Vimentin (Rabbit mAb #5741) antibodies were purchased from Cell Signaling Technology (CST, MA, USA). Anti-Cytokeratin 18 (DC-10 #sc6259), anti-N-cadherin (H-2 #sc393933) anti-phospho H2AX (ser 139 #sc517348), total H2AX (C-20 #sc54606), anti-Ku70 (A-9 #sc5309), anti-Bax (2D2 #sc20067), anti-c-Myc (9E10 #sc40), anti-cyclin D1 (DCS-6 #sc20044), anti-p27 (F-8 #sc1641) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Primary antibody incubations were followed by peroxidase-conjugate secondary antibody (Goat anti-Rabbit and goat anti-mouse IgG HRP-conjugated from Bio-Rad Laboratories (Berkeley, CA). The blots were re-probed with α-Tubulin or α-Actin (Sigma-Aldrich) for normalization of protein content. Precision Plus Protein All Blue Standards (Bio-Rad) was used and the intensity of western blot bands was visualized with ECL (Western nova 2.0, Cyanagen, Italy) and quantified by Chemidoc Gel Imaging System Image Lab 5.2.1. software (Bio-Rad) from three independent experiments. Densitometric analysis of immunoblots was performed by ImageJ.

Flow Cytometry

1x10⁵ cells were trypsinized, washed with PBS 0.1% BSA and incubated with: anti-human EpCAM antibody (clone: EBA-1, BD PE-mouse anti-human), anti-human CD44 antibody (clone: G44-26, BD Pharmingen PE-Cy[™] 7-mouse anti-human), polyclonal anti-DACH1 antibody (SC-398706, from Santa Cruz Biotechnology).

For intracellular antigen detection, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100/PBS for 3 minutes at room temperature before incubation with the specific antibody. After 45 minutes of incubation at 4°C, cells were washed twice in PBS 0.1% BSA and incubated with the secondary antibody (PE-labeled Goat anti-mouse IgG (H + L) (Invitrogen) for DACH1 staining.

Cells were washed again and unfixed cells were stained with Sytox Blue Dead Cell (Life Technologies, Eugene, OR, USA) to exclude dead cells. The analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA).

Rna Extraction And Rt-qpcr Analysis

Total RNAs were purified from cells by using TRIzol reagent (Invitrogen, USA) and quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). cDNA synthesis was obtained using SuperScript[™] II Reverse Transcriptase kit (Thermo Scientific, Pittsburgh, PA, USA) following the manufacturer's instructions. RT-qPCR was performed using Luna[®] Universal qPCR Master Mix (New England Biolabs) in a QuantStudio5 and QuantStudio7 Fast Real-Time PCR Systems (Applied Biosystems). The 2^{-ΔΔCT} method for relative quantitation of gene expression was used to determine mRNA expression levels. *Beta-actin* and *H3 histone* gene expression was used as endogenous controls to standardize mRNA expression. p-values were calculated with two-tailed Student's t-test from at least three experiments. Statistically significant results are indicated by a p-value < 0.05.

Ros Detection

1x10⁵ cells were seeded into T-25 Corning flask in complete growth medium. After 48 hours, cells were detached with trypsin/EDTA (Sigma), washed with PBS 0,1% BSA and incubated at 37°C for 30 minutes with the cell permeant reagent 2',7'- dichlorofluorescein diacetate (DCFDA) (Sigma), a fluorogenic dye that measures hydroxyl, peroxy and other reactive oxygen species (ROS) activity within the cell. ROS value has been detected by flow cytometry analysis using a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA) and data were analyzed employing FlowJo express Software.

Adhesion And Invasion Assay

Adhesion assay was performed using Corning 12-well plates pre-coated with 10 µg/ml of type1 collagen and seeding 2x10⁵ PC3 cells. After 90 minutes at 37°C, adherent cells were washed with PBS for 3 times, fixed in 4% paraformaldehyde at room temperature for 15 minutes and stained with 0,05% crystal violet for 1 hour. The plates were left to dry overnight and after 24 h stained cells were re-suspended with 10% acetic acid to solubilize Crystal Violet. The absorbance at 550 nm was read by a spectrophotometer.

Invasion assay was performed by using *transwell* inserts of 8 µm pore size membrane (Falcon) coated with Corning Matrigel. 2x10⁵ cells were re-suspended in 1% FBS complete medium and seeded in the upper chamber, while 20% FBS medium was added to the bottom chamber. 48 hours after plating, the membrane was cut away to fix and to stain the invasive cells adhering to the bottom surface of the membrane with 4% paraformaldehyde and 300 µM DAPI (ThermoFisher Scientific, Waltham, MA, USA).

Five random fields per well were acquired by fluorescence microscope and DAPI-stained nuclei were counted and values averaged.

Tcga Patient Analysis

GEPIA2 (<http://gepia2.cancer-pku.cn/#index>) is an interactive web resource and database for analyzing cancer transcriptome and patients' survival. In this study, we utilized this online tool to analyze DACH1 expression isoforms associated with normal and cancer tissues. In the box-plot analysis, the method for differential analysis is one-way ANOVA. A t-test was used to compare the expression difference between tumor and normal tissue; $p < 0.05$ was considered statistically significant. * $p < 0.05$ based on the Student's t test. Values are mean $\pm$ SEM. TPM, transcript per million.

Statistical Analysis

All experiments were repeated at least three times. All numerical data are reported as mean $\pm$ SEM. Data were analyzed by using the two-tailed student t-test or Anova test. A probability value less of 0.05 was considered significant.

Results

DACH1 variant 4 (DACH1-v4) increases proliferation and clonogenic potential in PC3 cells

Flow cytometry analysis performed to investigate the expression level of DACH1 in PC3 cells showed that DACH1 is expressed in a cell population, accounting for about 37% of cells (Fig. 1A).

To further study which isoform of DACH1 was expressed in PC3 cells, we analyzed DACH1 gene annotations in Santa Cruz (UCSC) Genome Browser [http://genome-euro.ucsc.edu/cgi-bin/hgTracks?](http://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr13%3A71437966%2D71867)

[db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr13%3A71437966%2D71867](http://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr13%3A71437966%2D71867)
In the **Supplementary Figure S1**, we reported the structure of DACH1 gene where ENSEMBL ID of DACH1 transcripts are indicated. We designed specific oligonucleotides to discriminate the expression levels of each DACH1 isoform, as indicated by red arrows, by RT-qPCR analysis (**Suppl. Fig. S1 and Suppl. TABLE 1**). Real-Time PCR revealed that PC3 cells predominantly express DACH1 variant 4 (DACH1-v4) (Fig. 1B). To explore the role of DACH1-v4-positive cells in this metastatic cell line, we stably over-expressed and down-regulated DACH1-v4 in PC3 cells. (Fig. 1C and 1E). Firstly, we analyzed the effects of DACH1-v4 stable over-expression and down-regulation on the proliferation rate and colony formation. Compared to control cells (PC3^{EV}), DACH1-v4-transfected PC3 cells (PC3^{DACH1-v4}) showed an increased proliferation rate and a greater ability to form colonies (Fig. 1D), while DACH1 knockdown (PC3^{sh DACH1}) reduced proliferation rate and colony number (Fig. 1F). Western blot analysis showed that the variant knocked-down after shDACH1 was the variant 4 (Fig. 1E). These results strongly suggest that DACH1-v4 expressed in PC3 cell line is involved in oncogenic activities such as hyper-proliferation and clonogenic ability.

Dach1-v4 Over-expression Induces Radioresistance In Pc3 Cell Line

The potential correlation between DACH1-v4 expression and response to ionizing radiations (IR) was investigated by colony formation assay. PC3^{EV} and PC3^{DACH1-v4} cells were treated with increasing doses of IR and the value of the surviving fraction calculated. DACH1-v4 over-expression significantly induced radioresistance as indicated by the increased surviving fraction in PC3^{DACH1-v4} cells irradiated with 4 and 6 Gy (Fig. 2A). To characterize DACH1-v4-induced radioresistance, the cell cycle distribution after a single radiation dose of 4 Gy was investigated by flow cytometry. As shown in Fig. 2B, contrary to PC3^{EV} cells that underwent a persistent G2 phase arrest, no significant arrest in G2 phase was observed in PC3^{DACH1-v4}. The expression of PCa radioresistance markers

EpCAM [33], cyclin D1 [34] and c-myc [35] was then investigated. As shown in Fig. 3, compared to PC3^{EV}, PC3^{DACH1-v4} showed an increased expression of EpCAM (Fig. 3A), cyclin D1 and c-myc (Fig. 3B). Altogether, our evidence indicates that DACH1-v4 over-expression enhances PC3 cells radioresistance.

Oncogenic Phenotype Is Enhanced In Pc3 Cells

Since an increased radioresistance generally parallels a more aggressive phenotype, we analyzed the effects of DACH1-v4 stable over-expression on the activation of epithelial to mesenchymal transition (EMT). In comparison with PC3^{EV}, PC3^{DACH1-v4} expressed lower levels of E-cadherin and cytokeratin-18, known to be epithelial markers, lower N-cadherin and higher Snail, mesenchymal markers. No modulation of vimentin expression was found (Fig. 4A). These results are in accordance to the literature data [36], that state that suppression of E-cadherin and the loss of the cell to cell contact markers, such as N-cadherin, are the prerequisite for EMT. Based on this data, cell invasion assay was performed and showed that DACH1-v4 induced a 5-fold increase in PC3 cell invasion ability (Fig. 4B).

Moreover, PC3^{DACH1-v4} showed a reduced ability to adhere to type-1 collagen (Fig. 5A) associated to reduced levels of membrane protein CD44, the receptor of hyaluronic acid which is the main component of extracellular matrix (Fig. 5B). This evidence suggests that increasing DACH1-v4 expression enhances the transformed phenotype of PC3 cells.

DACH1-v4 over-expression promotes oxidative stress and DNA damage in PC3 cells

Oxidative stress promotes DNA damage, genetic instability and chemoresistance [37, 38]. We therefore evaluated endogenous ROS levels as well as the expression levels of the DNA damage marker p-H2AX, a known biomarker of DNA double-strand breaks (DSBs). Compared to PC3^{EV}, PC3^{DACH1-v4} showed increased endogenous ROS levels (Fig. 6A) and phosphorylation of H2AX (Fig. 6B). Notably, DACH1-v4 over-expression down-regulated the expression levels of Ku-70, indicating a weaker ability of PC3^{DACH1-v4} to activate DNA repair machinery (Fig. 6B). Nevertheless, DACH1-v4 over-expression did not affect the basal apoptosis (Fig. 6C), rather PC3^{DACH1-v4} cells inhibited Bax expression (Fig. 6D), over-expressed the cell cycle promoter PCNA and down-regulated the cell cycle inhibitor p27 (Fig. 6E). Altogether, these findings indicate that DACH1-v4 seems to increase oxidative stress-mediated genomic instability of PC3 cells.

Dach1-v4 Induces Downstream Pro-oncogenic Signaling

Notch1 signaling promotes onset, progression and radioresistance of several cancers, including PCa [39–44]. It has been further reported that AKT signaling can act as a parallel and synergic pathway with Notch1 as well as downstream target of Notch1 [45–47]. As shown in Fig. 7A, DACH1-v4 over-expression increased the active cleaved form of Notch1 and enhanced the activation of AKT pathway, by both upregulating total AKT expression and its phosphorylation (Fig. 7B). Furthermore, DACH1-v4 over-expression increased GSK3 β phosphorylation with consequent stabilization of total β -catenin levels (Fig. 7C). Thus, DACH1-v4 increased expression seems to enhance oncogenic phenotype of PC3 cells by activating pro-oncogenic signals.

Restoring DACH1-v4 expression in normal prostate RWPE-1 cells triggers a pro-oncogenic phenotype

DACH1 expression levels were investigated in RWPE-1 cells, a cell line derived from luminal epithelium of normal human prostate. Flow cytometry analysis showed that DACH1 is more expressed in RWPE-1 cells in comparison with PC3 cells (85% vs 37% DACH1-positive cells) (Fig. 8A and 1A). However, the analysis of different DACH1 isoforms by quantitative PCR (Fig. 8B) and western blot analysis (Fig. 8C) showed that RWPE-1 cells mostly expressed DACH1 variant 3 (DACH1-v3), while PC3 cells only expressed DACH1-v4 (Fig. 8C).

In order to evaluate the functional effect of DACH1-v4 in a normal prostate cell line, we transfected DACH1-v4 vector in RWPE-1 cells. After confirming DACH1-v4 over-expression by western blot analysis (Fig. 9A) and by Real-Time PCR (Fig. 9B), we observed that DACH1-v4 behave as dominant negative on the DACH1-v3 isoform increasing the expression of proliferation marker PCNA in RWPE-1 cells (Fig. 9A and 9B), according to a higher proliferation rate (Fig. 9C), a higher ability to form colonies and an increase in colony size (Fig. 9D). In order to mimic the anchorage-independence of intravascular condition, we cultured RWPE^{EV} and RWPE^{DACH1-v4} cells in ultra-low attachment flasks with growth basal medium. After three days, only RWPE^{DACH1-v4} cells were able to form spheroids as opposed to RWPE^{EV} (Fig. 9E). We also found that this isoform induced activated AKT through its phosphorylation (Fig. 9F). Taken together, our results indicate that over-expression of DACH1-v4 in normal prostate cells induces some features of malignant cells.

Dach1 Isoforms In Prostate Adenocarcinoma Patients (Prad)

In order to correlate the *in vitro* data obtained in PC3 cells with clinical data, we interrogated TCGA dataset analyzing the expression of DACH1 isoforms in 492 prostate adenocarcinoma tissues from patients (PRAD). The analysis, shown as violin plot, highlighted that the variants expressed in healthy and tumor prostate are different, being DACH1-v1(long variant) significantly higher in tumor tissue and DACH1-v3 (short variant) in normal prostate. The other long isoform, DACH1-v4, differs from DACH1-v1 only for the presence of exon 4 (Fig. 10). We confirmed that also DACH1-v1 has oncogenic potential by analyzing the rectal cancer TCGA dataset (READ), in line with the data previously published where DACH1 was found to be overexpressed in all stages of human colorectal cancer (CRC) and its high expression predicted a poor prognosis in CRC patients [25]. Interestingly, rectal cancer tissues (T = 92) expressed high

levels of both long isoforms of DACH1, DACH1-v1 and DACH1-v4, and very low levels of DACH1-v2 and DACH1-v3 (short isoforms) (Supplementary Fig. 2). Furthermore, DACH1-v1 and DACH1-v4 are significantly associated with tumoral tissues compared to their expression in normal prostate samples (Supplementary Fig. 2). Collectively, these analysis in colorectal tissues suggested that Hu and colleagues have brought to light oncogenic activities that were attributable to both DACH1-v1 and DACH1-v4 long variants [25]. In light of these data, the PC3 cell line preferentially expresses DACH1-v4 and PRAD patients DACH1-v1, however both isoforms have oncogenic potential.

To further address the oncogenic role of DACH1-v1 in PRAD patients, we analyzed its expression in the iClusters by TCGA dataset [48]. We reported that DACH1-v1 expression was significantly higher in the iCluster2 and iCluster3 which represents the groups including the gene signatures most associated with the aggressive phenotype (Fig. 10). In particular, iCluster3 gene signature is associated with EMT-related pathways, genomic instability and poor prognosis of the patients. On the contrary, DACH1-v3 variants was significantly associated with normal prostate tissues (Fig. 10).

Normal and tumor prostate tissues show a different sub-cellular distribution of DACH1 and different DACH1 splicing variants expression

In order to investigate DACH1 expression and distribution in cancer prostate tissue, we performed immunohistochemical analysis of PCa specimens from 30 patients. The analysis of the normal and tumor prostate tissues examined showed that DACH1 expression was limited to the epithelial compartment. In normal tissue the expression was more evident in the nuclei of the luminal cells while basal cells were frequently negative or faintly positive (Fig. 11 panel A).

As reported in Table 1, almost all the tumor tissues present a nuclear positivity although we found a great variability of nuclear staining intensity among the cases of the same group. Two cases in group grade 1 were completely unstained. Normal nuclei may be more (Fig. 11 panel B) or less (Fig. 11 panel C) intense than the tumor counterpart. Moreover, the intensity of the nuclear staining was basically enhanced throughout the increase of the tumor grade, reaching the highest score in Group Grade 5 (Table 1). Interestingly, the increase of the tumor grade of differentiation is associated with the tendency to gain a cytoplasmic expression (Fig. 11 panels D and E), particularly intense in tumors with invasion of the periprostatic fat (Fig. 11 panel F shows a representative section).

Based on these data, we decided to analyze some of these primary tumor and normal samples by western blot to identify the presence of different DACH1 splicing variants. We found that a ~ 100-kDa DACH1 protein was strongly expressed in tumor glands (Fig. 11). In line with our data in RWPE-1 cells, we also observed that normal glands mostly expressed a ~ 65 kDa DACH1 isoform, and that we can observe also in the tumor samples because of the mixed nature of tumor specimens (Fig. 11).

Table 1
Expression of DACH1 protein in prostate cancer tissues.

N°of cases	Gleason score	Group Grade	Tumors				Normal parenchyma			
			Nuclei		Cytoplasm		Nuclei		Cytoplasm	
			N°positive	Score	N°positive	Score	N°positive	Score	N°positive	Score
				Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD
6	5(2 + 3) 6(3 + 3)	1	4	1.5 ± 1.4	0		6	4.8 ± 3.3	0	
6	7(3 + 4)	2	6	3.5 ± 2	2	1 ± 1.5	6	4 ± 2.2	0	
6	7(4 + 3)	3	6	6.8 ± 2,3	4	2.16 ± 2	6	4.3 ± 1.8	0	
6	8(3 + 5) (4 + 4)	4	6	7.0 ± 2.9	5	3.9 ± 3.3	6	4.8 ± 2.5	0	
6	9(4 + 5) 10(5 + 5)	5	6	9 ± 0	6	5.8 ± 3.6	4/4	4.7 ± 1.2	0	

DACH expression was evaluated in tumor and normal peritumoral gland using immunohistochemistry on formalin fixed prostate biopsies. Multiple biopsies (from 1 to 4) were stained for each of the patients and reported as number of cases with of positive nuclear and cytoplasm staining.

Tumors were considered positive when more than 10% of the epithelial cells showed a consistent nuclear staining and % positive cells was evaluated as follows: 11-30% = 1; 31-60% = 2 and >61 = 3. Stain intensity was determined as low (1), medium (2), high (3) and protein expression score from 1 to 9 as % positive cells x Stain intensity. Group Grade as classified by ajcc 2017.

Discussion

DACH1 is a key master regulator of tumorigenesis [11, 12, 49] even though its role remains still controversial. DACH1 has been considered to function as a tumor suppressor in lung [17, 18], breast [15, 50], prostate [31, 32], pancreas [20], colon [21, 22], whereas other studies suggest DACH1 as an oncogene in pancreas [23], colon [25] or ovary [24]. However, despite the availability of databases reporting variant sequences and molecular weights of DACH1 isoforms, no study so far has mentioned any possible different role of DACH1 isoforms in human cancers. We investigated herein the role of DACH1 splicing variant expression in prostate cancer cells. We used an *in vitro* cellular model of castration-resistant PCa, the PC3 cell line derived from bone metastasis, which is very aggressive and also insensitive to the anticancer effect of immunomodulators [51, 52]. Data herein collected show that DACH1-positive PC3 cells (~ 37%) predominantly express DACH1 variant 4 (DACH1-v4). Stably DACH1 v4-transfected PC3 cells (PC3^{DACH1 - v4}) more efficiently proliferated, also in low density

condition, and were more able to form colonies compared to control cells. Confirming these results, DACH1-v4 knockdown reduced proliferation rate and colony number in PC3 cells.

PC3^{DACH1-v4} also showed increased radioresistance and expression of a more aggressive phenotype and related-markers, such as EpCam, c-myc and cyclin D1, known to sustain oncophenotype and radioresistance of PCa cells [33–35].

We observed that DACH1-v4 over-expression in PC3 cells increased the endogenous reactive oxygen species (ROS) levels and DNA damage, inhibited DNA repair thereby escaping the physiological apoptotic response to the ROS increase, indicating the presence of radioresistance traits. Our data are in accordance with published evidence, showing that ROS represent an endogenous source of DNA-damaging agents that promotes genetic instability, therapies resistance and pro-tumorigenic signaling [37, 38]. Cancer cells have higher ROS compared to normal cells and an even higher level of ROS and antioxidants are detected in radioresistant PCa [6].

In accordance with evidence showing that acquiring a more aggressive phenotype frequently parallels the activation of an epithelial to mesenchymal transition (EMT) program [53], PC3^{DACH1-v4} showed a mesenchymal-like phenotype molecularly characterized by the reduction of E-cadherin and cytokeratin-18 expression and increased Snail-1 levels, while decreasing N-cadherin expression. This is in accordance with several data supporting the notion that suppression of E-cadherin and the loss of the cell-cell contact markers such as N-cadherin are the prerequisite for EMT [36]. Moreover, evidence demonstrated that Snail-1 is a key regulator of EMT in PCa with low E-cadherin and it controls stemness, invasiveness and metastasis [54, 55]. *In vitro* invasion assay showed that DACH1-v4 induced a 5 fold-increase in invasion ability, hence our results suggest that these mesenchymal and invasiveness traits could enable a subpopulation of bone metastasis to enter the blood system and metastasize again. Moreover, the loss of E-cadherin is associated with radioresistance by attenuating radiation-induced DNA damage [56]. Conversely, DACH1 has been also described as negative regulator of CSC markers, such as CD44, Klf4 and Myc, and EMT markers in Met-1 breast cancer cells line [57], while DACH1 knockdown induced EMT in MCF-7 breast cancer cells by repressing E-cadherin, cell-cell contact and by inducing cell migration and invasion [15, 50].

PC3^{DACH1-v4} cells also showed a lower ability to adhere to type-1 collagen and to hyaluronic acid, consistent with the reduction in CD44 expression. Detachment of epithelial cells from matrix usually induces an apoptotic response known as anoikis, which prevents metastasis. However, many scientific data showed that cellular sensitivity to anoikis is compromised in cancer cells and during the oncogenic EMT, both by directly regulating apoptotic genes and by suppressing E-cadherin expression, confirming that EMT confers anoikis resistance [58, 59].

To clarify the oncogenic signaling pathways involved in DACH1-v4-induced radioresistance and aggressiveness traits, we investigated Notch-1 and AKT pathways, known to be key regulators of EMT, radioresistance, apoptosis inhibition and cancer survival, by acting in parallel or in synergistic way [39–42, 44, 46, 47]. Consistent with previous outcomes, DACH1-4 over-expression in PC3 cells results in increased Notch-1 and induction of AKT pathway with phosphorylation/inactivation of GSK3 β and consequent increased levels of free β -catenin into the cytoplasm, which can move to the nucleus, where it binds to target genes responsible for cell proliferation and survival, such as c-Myc and cyclin D1 [60, 61].

We have also performed immunohistochemical analysis of PCa specimens from 30 patients and we found a positive correlation between DACH1 nuclear staining in tumor cells and grade group. Interestingly, only high grade primary tumors acquired also a cytoplasmic DACH1 positivity compared to normal tissue. In line with our results, DACH1 cytoplasmic localization directly correlates with proliferation and angiogenesis in osteosarcoma [62] for which the authors hypothesized that cytoplasmic and nuclear DACH1 proteins might be one the mutant form of the other, playing different roles in cell proliferation and angiogenesis. Moreover, ovarian cancers over-express DACH1, preferentially localized in the cytoplasm when compared to normal tissues [24]. Therefore, this line of evidence strongly suggests that DACH1 could move from the nucleus to the cytoplasm to play its oncogenic role.

Interestingly, Real-Time PCR and western blot analysis showed that normal RWPE-1 cells mostly expressed the short DACH1 variant 3, while the longer variants 1/4 were expressed at low levels. When RWPE-1 cells were transfected with DACH1-v4, they displayed oncogenic traits, such as increased proliferation rate, propensity to form spheroids in anchorage-independent culture and higher ability to form colonies. These results confirm that DACH1 variant 4 plays an oncogenic role when overexpressed in normal prostate cells and suggest that it could be critically involved in the origin of PCa as well as its progression. This hypothesis is sustained by our analysis of PCa patient specimens, showing for the first time that normal and tumor prostate tissues differ in DACH1 splicing variants expression: notably, tumor tissues strongly express a ~ 100 kDa protein, while normal tissues mostly express a ~ 65 kDa protein, that we can presume to be respectively DACH1-v1 and DACH1-v3 as suggested by the TCGA-PRAD analysis. This assumption is further corroborated by the expression analysis that we conducted in colorectal cancer patients, where Hu and colleagues demonstrated that DACH1 was associated with cancer and poor prognosis of CRC patients [25]. We observed that both the long DACH1 isoforms, DACH1-v1 and v4, are significantly associated with READ cancer tissues and with aggressive features of this kind of tumor.

Taken together, our results indicate that the longer DACH1 variants (DACH1-v1 and v4) are associated to pro-tumor function, while the shortest DACH1 variant (DACH1-v3) could act as tumor suppressor. The preferential expression of DACH1-v4 and DACH1-v1 in bone metastatic and primary tumors remains to be elucidated. One significant difference between the isoforms is the loss in DACH1-v2 and DACH1-v3 of the region including amino acids S439/S441 representing phosphorylation sites, that may play different crucial roles in governing tumor growth, the epithelial to mesenchymal transition (EMT) program and the metastatic process, depending on specific signaling pathways activated into the tumor microenvironment [63].

In conclusion, the present results definitely open a completely new research field in which the longer variants DACH1-v1 and DACH1-v4 could eventually be established as new diagnostic and therapeutic markers in PCa patients: the use of specific antibodies against different DACH1 isoforms during the diagnostic phase in PCa patients would represent a useful novel tool for a clinical approach.

Abbreviations

DACH1: Human Dachshund homologue 1; PCa:prostate cancer; RDGN:Retinal Determination Gene Network; DACH1-v4:DACH1 variant 4; RT:radiation therapy; EBRT:external beam radioactive sources; IR:ionizing radiations; ROS:reactive-oxygen-species; EMT:epithelial-mesenchymal transition; SSB or DSB:DNA single or double-strand breaks; CSC:cancer stem cells.

Declarations

Ethics approval and consent to participate

This study was conducted under approval by the S. Andrea Hospital (n°Prot. n. 228 SA_2016, 12.12.2016 RIF. CE: 4208_2016). Histology specimens were obtained from the histoteque of Sant'Andrea Hospital Pathology Section of Clinical and Molecular Department of 'Sapienza' University of Rome, among the PCa blocks.

Consent for publication

not applicable

Availability of data and materials

The datasets analysed during the current study are available in the Gepia2 repository [<http://gepia2.cancer-pku.cn/#index>] and in UCSC Genome Browser [<http://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr13%3A71437966%2D71867>]

Competing interests

The authors declare that they have no conflict of interest. R.G.P. holds ownership interests in CytoDyn, EcoGenome and LightSeed, Inc. R.G.P. additionally holds ownership interests (value unknown) for several patents and submitted patent applications.

Funding

This work was supported by Ateneo Sapienza University (Ref. Number RP11816427B97420 and RG11916B7AF0C02D) to A.R. This work was supported in part by R01 CA132115, R21 CA235139-01-A1 and a Breakthrough Breast Cancer Research Program award # W81XWH1810605 (R.G.P.).

Authors' contributions

AR, AF and FM designed and coordinated the study. IG, AS, CMS and SDA performed experiments, investigation and analyzed data. AR and PDC took care of the original draft preparation and AF, FM, ZL and RGP reviewed & edited the manuscript. The study was conducted under the supervision of AT and CDN.

Acknowledgements

The Authors thank Fioretta Palombi for critical reading and revision of the article.

References

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2019. *CA Cancer J Clin* 2019, 69(1):7-34.
2. Vanneste BG, Van Limbergen EJ, van Lin EN, van Roermund JG, Lambin P: Prostate Cancer Radiation Therapy: What Do Clinicians Have to Know? *Biomed Res Int* 2016, 2016:6829875.
3. Murata K, Saga R, Monzen S, Tsuruga E, Hasegawa K, Hosokawa Y: Understanding the mechanism underlying the acquisition of radioresistance in human prostate cancer cells. *Oncol Lett* 2019, 17(6):5830-5838.
4. Sia J, Szmyd R, Hau E, Gee HE: Molecular Mechanisms of Radiation-Induced Cancer Cell Death: A Primer. *Front Cell Dev Biol* 2020, 8:41.
5. Tsao T, Beretov J, Ni J, Bai X, Bucci J, Graham P, Li Y: Cancer stem cells in prostate cancer radioresistance. *Cancer Lett* 2019, 465:94-104.
6. Chaiswing L, Weiss HL, Jayswal RD, Clair DKS, Kyprianou N: Profiles of Radioresistance Mechanisms in Prostate Cancer. *Crit Rev Oncog* 2018, 23(1-2):39-67.
7. Davis RJ, Shen W, Sandler YI, Amoui M, Purcell P, Maas R, Ou CN, Vogel H, Beaudet AL, Mardon G: Dach1 mutant mice bear no gross abnormalities in eye, limb, and brain development and exhibit postnatal lethality. *Mol Cell Biol* 2001, 21(5):1484-1490.
8. Ozcan L, Ghorpade DS, Zheng Z, de Souza JC, Chen K, Bessler M, Bagloo M, Schroppe B, Pestell R, Tabas I: Hepatocyte DACH1 Is Increased in Obesity via Nuclear Exclusion of HDAC4 and Promotes Hepatic Insulin Resistance. *Cell Rep* 2016, 15(10):2214-2225.
9. Doke T, Huang S, Qiu C, Liu H, Guan Y, Hu H, Ma Z, Wu J, Miao Z, Sheng X *et al*: Transcriptome-wide association analysis identifies DACH1 as a kidney disease risk gene that contributes to fibrosis. *J Clin Invest* 2021, 131(10).
10. Jiao X, Li Z, Wang M, Katiyar S, Di Sante G, Farshchian M, South AP, Cocola C, Colombo D, Reinbold R *et al*: Dachshund Depletion Disrupts Mammary Gland Development and Diverts the Composition of the Mammary Gland Progenitor Pool. *Stem Cell Reports* 2019, 12(1):135-151.
11. Liu Y, Han N, Zhou S, Zhou R, Yuan X, Xu H, Zhang C, Yin T, Wu K: The DACH/EYA/SIX gene network and its role in tumor initiation and progression. *Int J Cancer* 2016, 138(5):1067-1075.

12. Blevins MA, Towers CG, Patrick AN, Zhao R, Ford HL: The SIX1-EYA transcriptional complex as a therapeutic target in cancer. *Expert Opin Ther Targets* 2015, 19(2):213-225.
13. Wu K, Li A, Rao M, Liu M, Dailey V, Yang Y, Di Vizio D, Wang C, Lisanti MP, Sauter G *et al*: DACH1 is a cell fate determination factor that inhibits cyclin D1 and breast tumor growth. *Mol Cell Biol* 2006, 26(19):7116-7129.
14. Wu K, Jiao X, Li Z, Katiyar S, Casimiro MC, Yang W, Zhang Q, Willmarth NE, Chepelev I, Crosariol M *et al*: Cell fate determination factor Dachshund reprograms breast cancer stem cell function. *J Biol Chem* 2011, 286(3):2132-2142.
15. Wu K, Katiyar S, Li A, Liu M, Ju X, Popov VM, Jiao X, Lisanti MP, Casola A, Pestell RG: Dachshund inhibits oncogene-induced breast cancer cellular migration and invasion through suppression of interleukin-8. *Proc Natl Acad Sci U S A* 2008, 105(19):6924-6929.
16. Chen K, Wu K, Cai S, Zhang W, Zhou J, Wang J, Ertel A, Li Z, Rui H, Quong A *et al*: Dachshund binds p53 to block the growth of lung adenocarcinoma cells. *Cancer Res* 2013, 73(11):3262-3274.
17. Han N, Yuan X, Wu H, Xu H, Chu Q, Guo M, Yu S, Chen Y, Wu K: DACH1 inhibits lung adenocarcinoma invasion and tumor growth by repressing CXCL5 signaling. *Oncotarget* 2015, 6(8):5877-5888.
18. Yu S, Yi M, Xu L, Qin S, Li A, Wu K: CXCL1 as an Unfavorable Prognosis Factor Negatively Regulated by DACH1 in Non-small Cell Lung Cancer. *Front Oncol* 2019, 9:1515.
19. Liu Q, Li A, Yu S, Qin S, Han N, Pestell RG, Han X, Wu K: DACH1 antagonizes CXCL8 to repress tumorigenesis of lung adenocarcinoma and improve prognosis. *J Hematol Oncol* 2018, 11(1):53.
20. Zhang J, Zhao CY, Zhang SH, Yu DH, Chen Y, Liu QH, Shi M, Ni CR, Zhu MH: Upregulation of miR-194 contributes to tumor growth and progression in pancreatic ductal adenocarcinoma. *Oncol Rep* 2014, 31(3):1157-1164.
21. Cao J, Yan XR, Liu T, Han XB, Yu JJ, Liu SH, Wang LB: MicroRNA-552 promotes tumor cell proliferation and migration by directly targeting DACH1 via the Wnt/beta-catenin signaling pathway in colorectal cancer. *Oncol Lett* 2017, 14(3):3795-3802.
22. Yan W, Wu K, Herman JG, Brock MV, Fuks F, Yang L, Zhu H, Li Y, Yang Y, Guo M: Epigenetic regulation of DACH1, a novel Wnt signaling component in colorectal cancer. *Epigenetics* 2013, 8(12):1373-1383.
23. Bu XN, Qiu C, Wang C, Jiang Z: Inhibition of DACH1 activity by short hairpin RNA represses cell proliferation and tumor invasion in pancreatic cancer. *Oncol Rep* 2016, 36(2):745-754.
24. Liang F, Lu Q, Sun S, Zhou J, Popov VM, Li S, Li W, Liu Y, Jiang J, Kong B: Increased expression of dachshund homolog 1 in ovarian cancer as a predictor for poor outcome. *Int J Gynecol Cancer* 2012, 22(3):386-393.
25. Hu X, Zhang L, Li Y, Ma X, Dai W, Gao X, Rao X, Fu G, Wang R, Pan M *et al*: Organoid modelling identifies that DACH1 functions as a tumour promoter in colorectal cancer by modulating BMP signalling. *EBioMedicine* 2020, 56:102800.
26. Cheng Q, Ning D, Chen J, Li X, Chen XP, Jiang L: SIX1 and DACH1 influence the proliferation and apoptosis of hepatocellular carcinoma through regulating p53. *Cancer Biol Ther* 2018, 19(5):381-390.
27. Ayres JA, Shum L, Akarsu AN, Dashner R, Takahashi K, Ikura T, Slavkin HC, Nuckolls GH: DACH: genomic characterization, evaluation as a candidate for postaxial polydactyly type A2, and developmental expression pattern of the mouse homologue. *Genomics* 2001, 77(1-2):18-26.
28. Climente-Gonzalez H, Porta-Pardo E, Godzik A, Eyraes E: The Functional Impact of Alternative Splicing in Cancer. *Cell Rep* 2017, 20(9):2215-2226.
29. Bankhead A, 3rd, McMaster T, Wang Y, Boonstra PS, Palmboos PL: TP63 isoform expression is linked with distinct clinical outcomes in cancer. *EBioMedicine* 2020, 51:102561.
30. Aigner P, Just V, Stoiber D: STAT3 isoforms: Alternative fates in cancer? *Cytokine* 2019, 118:27-34.
31. Wu K, Katiyar S, Witkiewicz A, Li A, McCue P, Song LN, Tian L, Jin M, Pestell RG: The cell fate determination factor dachshund inhibits androgen receptor signaling and prostate cancer cellular growth. *Cancer Res* 2009, 69(8):3347-3355.
32. Chen K, Wu K, Jiao X, Wang L, Ju X, Wang M, Di Sante G, Xu S, Wang Q, Li K *et al*: The endogenous cell-fate factor dachshund restrains prostate epithelial cell migration via repression of cytokine secretion via a cxcl signaling module. *Cancer Res* 2015, 75(10):1992-2004.
33. Ni J, Cozzi P, Beretov J, Duan W, Bucci J, Graham P, Li Y: Epithelial cell adhesion molecule (EpCAM) is involved in prostate cancer chemotherapy/radiotherapy response in vivo. *BMC Cancer* 2018, 18(1):1092.
34. Marampon F, Gravina GL, Ju X, Vetusch A, Sferra R, Casimiro MC, Pompili S, Festuccia C, Colapietro A, Gaudio E *et al*: Cyclin D1 silencing suppresses tumorigenicity, impairs DNA double strand break repair and thus radiosensitizes androgen-independent prostate cancer cells to DNA damage. *Oncotarget* 2016, 7(39):64526.
35. Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O: The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene* 2004, 23(34):5748-5758.
36. Friedl P, Wolf K: Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003, 3(5):362-374.
37. Okon IS, Zou MH: Mitochondrial ROS and cancer drug resistance: Implications for therapy. *Pharmacol Res* 2015, 100:170-174.
38. Moloney JN, Cotter TG: ROS signalling in the biology of cancer. *Semin Cell Dev Biol* 2018, 80:50-64.
39. Shao S, Zhao X, Zhang X, Luo M, Zuo X, Huang S, Wang Y, Gu S, Zhao X: Notch1 signaling regulates the epithelial-mesenchymal transition and invasion of breast cancer in a Slug-dependent manner. *Mol Cancer* 2015, 14:28.
40. Hassan WA, Yoshida R, Kudoh S, Hasegawa K, Niimori-Kita K, Ito T: Notch1 controls cell invasion and metastasis in small cell lung carcinoma cell lines. *Lung Cancer* 2014, 86(3):304-310.

41. Jue C, Lin C, Zhisheng Z, Yayun Q, Feng J, Min Z, Haibo W, Youyang S, Hisamitsu T, Shintaro I *et al*: Notch1 promotes vasculogenic mimicry in hepatocellular carcinoma by inducing EMT signaling. *Oncotarget* 2017, 8(2):2501-2513.
42. Zhang L, Sha J, Yang G, Huang X, Bo J, Huang Y: Activation of Notch pathway is linked with epithelial-mesenchymal transition in prostate cancer cells. *Cell Cycle* 2017, 16(10):999-1007.
43. Ye QF, Zhang YC, Peng XQ, Long Z, Ming YZ, He LY: siRNA-mediated silencing of Notch-1 enhances docetaxel induced mitotic arrest and apoptosis in prostate cancer cells. *Asian Pac J Cancer Prev* 2012, 13(6):2485-2489.
44. Zhang H, Jiang H, Chen L, Liu J, Hu X, Zhang H: Inhibition of Notch1/Hes1 signaling pathway improves radiosensitivity of colorectal cancer cells. *Eur J Pharmacol* 2018, 818:364-370.
45. Wu X, Xu K, Zhang L, Deng Y, Lee P, Shapiro E, Monaco M, Makarenkova HP, Li J, Lepor H *et al*: Differentiation of the ductal epithelium and smooth muscle in the prostate gland are regulated by the Notch/PTEN-dependent mechanism. *Dev Biol* 2011, 356(2):337-349.
46. Atala A: Re: Activation of Notch1 Synergizes with Multiple Pathways in Promoting Castration-Resistant Prostate Cancer. *J Urol* 2017, 197(6):1458-1459.
47. Pal D, Tyagi A, Chandrasekaran B, Alattasi H, Ankem MK, Sharma AK, Damodaran C: Suppression of Notch1 and AKT mediated epithelial to mesenchymal transition by Verrucarin J in metastatic colon cancer. *Cell Death Dis* 2018, 9(8):798.
48. Cancer Genome Atlas Research N: The Molecular Taxonomy of Primary Prostate Cancer. *Cell* 2015, 163(4):1011-1025.
49. Popov VM, Zhou J, Shirley LA, Quong J, Yeow WS, Wright JA, Wu K, Rui H, Vadlamudi RK, Jiang J *et al*: The cell fate determination factor DACH1 is expressed in estrogen receptor-alpha-positive breast cancer and represses estrogen receptor-alpha signaling. *Cancer Res* 2009, 69(14):5752-5760.
50. Zhao F, Wang M, Li S, Bai X, Bi H, Liu Y, Ao X, Jia Z, Wu H: DACH1 inhibits SNAI1-mediated epithelial-mesenchymal transition and represses breast carcinoma metastasis. *Oncogenesis* 2015, 4:e143.
51. Paone A, Galli R, Gabellini C, Lukashev D, Starace D, Grolach A, De Cesaris P, Ziparo E, Del Bufalo D, Sitkovsky MV *et al*: Toll-like receptor 3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor 1 alpha. *Neoplasia* 2010, 12(7):539-549.
52. Galli R, Paone A, Fabbri M, Zanasi N, Calore F, Cascione L, Acunzo M, Stoppacciaro A, Tubaro A, Lovat F *et al*: Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RARbeta and tumor regression. *Proc Natl Acad Sci U S A* 2013, 110(24):9812-9817.
53. Liao TT, Yang MH: Revisiting epithelial-mesenchymal transition in cancer metastasis: the connection between epithelial plasticity and stemness. *Mol Oncol* 2017, 11(7):792-804.
54. Deep G, Jain AK, Ramteke A, Ting H, Vijendra KC, Gangar SC, Agarwal C, Agarwal R: SNAI1 is critical for the aggressiveness of prostate cancer cells with low E-cadherin. *Mol Cancer* 2014, 13:37.
55. Smith BN, Odero-Marah VA: The role of Snail in prostate cancer. *Cell Adh Migr* 2012, 6(5):433-441.
56. Theys J, Jutten B, Habets R, Paesmans K, Groot AJ, Lambin P, Wouters BG, Lammering G, Vooijs M: E-Cadherin loss associated with EMT promotes radioresistance in human tumor cells. *Radiother Oncol* 2011, 99(3):392-397.
57. Xu H, Yu S, Yuan X, Xiong J, Kuang D, Pestell RG, Wu K: DACH1 suppresses breast cancer as a negative regulator of CD44. *Sci Rep* 2017, 7(1):4361.
58. Frisch SM, Schaller M, Cieply B: Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *J Cell Sci* 2013, 126(Pt 1):21-29.
59. Kumar S, Park SH, Cieply B, Schupp J, Killiam E, Zhang F, Rimm DL, Frisch SM: A pathway for the control of anoikis sensitivity by E-cadherin and epithelial-to-mesenchymal transition. *Mol Cell Biol* 2011, 31(19):4036-4051.
60. Mosimann C, Hausmann G, Basler K: Beta-catenin hits chromatin: regulation of Wnt target gene activation. *Nat Rev Mol Cell Biol* 2009, 10(4):276-286.
61. Song GY, Lee JH, Cho M, Park BS, Kim DE, Oh S: Decursin suppresses human androgen-independent PC3 prostate cancer cell proliferation by promoting the degradation of beta-catenin. *Mol Pharmacol* 2007, 72(6):1599-1606.
62. Ren P, Gong MZ, Wang ZY, Zhang P, Chen P, Ma WL, Zhou CJ: DACH1 Expression in Osteosarcoma and Its Relationship with Proliferation and Angiogenesis. *Indian J Surg* 2015, 77(3):200-205.
63. Wu K, Chen K, Wang C, Jiao X, Wang L, Zhou J, Wang J, Li Z, Addya S, Sorensen PH *et al*: Cell fate factor DACH1 represses YB-1-mediated oncogenic transcription and translation. *Cancer Res* 2014, 74(3):829-839.

Figures

Figure 1

DACH1-v4 over-expression and downregulation in PC3 cell line. (A) Representative dot plot of FACS analysis for DACH1 expression in PC3 cells. (B) Expression analysis of DACH1 variants by RT-qPCR in PC3 cell line. Specific oligonucleotides shown in the Supplementary Table 1 are used in the PCR reactions. ** $p=10^{-3}$, *** $p=10^{-4}$ Western blot analysis showed DACH1-v4 increase and down-regulation respectively in the DACH1-v4 vector-transfected PC3 cells (DACH1-v4) (C) and in the short hairpin DACH1 vector-transfected PC3 cells (sh DACH1) (E), compared to PC3 cells transfected with the empty vector (EV). β -actin was used as control for equal amounts of proteins loaded. The histograms are densitometric analysis of western blot and data represent the mean $\pm$ SEM derived from three independent experiments; ** $p < 0.01$, Student's paired t-test. (D) Analysis of proliferation rate through cell count at 24, 48 and 72 hours after plating (upper panel) and colony formation assay by crystal violet staining (bottom panel) in PC3Dach1-v4 cells. Data represent the mean $\pm$ SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.005$, Student's paired t-test. (F) Time course proliferation (upper panel) and colony formation assay (bottom panel) assessed by crystal violet staining in PC3sh DACH1 cells. Data represent the mean $\pm$ SEM of three independent experiments. ** $p=10^{-5}$, *** $p=10^{-6}$, Student's paired t-test.

Figure 2

Role of DACH1-v4 in radiation therapy of PC3 cells. (A) Surviving fraction of PC3 cells after 2, 4, and 6 Gy was evaluated by means colony formation assay, determined as described in Materials and Methods. Data represent the mean $\pm$ SEM derived from three independent experiments. (B) Representative images of cell cycle analysis by propidium staining at 24h and 48 h after 4 Gy treatment. Histogram represents the G2 phase after IR treatment in PC3Dach1-v4 cells compared with PC3EV cells. Data are from three independent experiments. * $p < 0.05$, ** $p < 0.01$, Anova test.

Figure 3

Analysis of radioresistance markers in PC3Dach1-v4. (A) Representative dot plot of flow cytometry analysis for EpCAM expression in PC3Dach1-v4 and PC3EV cells (left panel). (B) Representative Western blot for c-Myc and Cyclin D1 expression. β -actin or tubulin were used as control for equal amounts of proteins loaded (left panels). Data shown in the right panels represent the mean $\pm$ SEM derived from three independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, Student's paired t-test

Figure 4

EMT markers and invasion ability in PC3Dach1-v4. (A) Representative Western blots for the indicated proteins (left panels), β -actin or tubulin were used as control for equal amounts of proteins loaded. Densitometric analysis of three separate experiments is shown (right panels). (B) Representative images of DAPI-stained nuclei of invading cells acquired by fluorescence microscopy (left) and relative increase in cell number assessed by nuclear counts performed in five microscopic fields for each sample of three independent experiments (right) **p < 0.01, ***p < 0.005, Student's paired t-test.

Figure 5

Adhesion ability to extracellular matrix of PC3Dach1-v4. (A) Representative image of crystal-violet stained cells adhering on type-1 collagen matrix (left) and the relative absorbance read by a spectrophotometer (right). (B) Representative flow cytometry histogram of CD44 expression (left) and statistical analysis of three independent experiments (right). ** $p < 0.01$, *** $p < 0.005$, Student's paired t-test.

Figure 6

Analysis of ROS levels and DNA damage in PC3Dach1-v4 (A) Representative histogram of flow cytometry analysis for ROS endogenous levels (left) and graphic showing fold increase of ROS level in PC3Dach1-v4 cells (right). (B) Western blot analysis for total and phosphorylated-H2AX and Ku70 expression (D) Bax, (E) PCNA and p27. β -actin or tubulin were used as control for equal amounts of proteins loaded. Graphic representation of densitometric analysis of three separate experiments is shown for each blot. (C) Time course of basal apoptosis in cultured PC3Dach1-v4 and PC3EV cells. Histograms represent the mean $\pm$ SEM derived from three independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, Student's paired t-test.

Figure 7

Signal transduction pathways triggered by DACH1-v4 overexpression Representative Western blots for the indicated proteins (left panels). β-actin or tubulin were used as control for equal amounts of proteins loaded. Densitometric analyses of three separate experiments are shown (right panels). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, Student's paired t-test.

Figure 8

DACH1 expression in normal RWPE-1 cell line. (A) Representative flow cytometric dot plot for DACH1 expression in RWPE-1 cells (left) and statistical analysis for DACH1 positive cells in RWPE-1 cells compared to PC3 cells in three independent experiments (right). ***p < 0.005, Student's paired t-test. (B) Expression analysis of DACH1 variants by RT-qPCR in RWPE-1 cell line. Specific oligonucleotides shown in the Supplementary Table 1 are used in the PCR reactions *p = 0.01, **p = 10-3, ***p=10-4 (C) Representative western blot analysis for expression of different DACH1 isoforms in PC3 and RWPE-1 cells. β-actin was used as control for equal amounts of proteins loaded.

Figure 9

Malignant traits induced by DACH1-v4 over-expression in RWPE-1 cells. (A) Western blot analysis for DACH1 and PCNA in RWPE-1 cells stably transfected with DACH1-v4 expression vector (DACH1-v4) RWPEDACH1-v4 cells or empty vector (EV). β-actin was used as control for equal amounts of proteins loaded. (B) Expression analysis of DACH1-v4 and PCNA by RT-qPCR in RWPEDACH1-v4 cells and RWPEEV. Specific oligonucleotides shown in the Supplementary Table 1 are used in the PCR reactions (C) Analysis of proliferation rate through cell count at 24, 48 and 72 hours after plating of RWPEDACH1-v4 cells or RWPEEV. (D) Representative images of colony formation by crystal violet staining (left) and evaluation of colony formation ability as fold induction, setting as 1 the number of colonies in RWPEEV (right). (E) Spheroids formed by RWPEEV compared to RWPEDACH1-v4 cells after 3 days in anchorage-independent conditions. (F) Representative Western blot analysis for p-AKT expression in RWPEDACH1-v4 cells or RWPEEV. β-actin was used as control for equal amounts of proteins loaded. In the right histogram densitometric analysis of three separate experiments was shown.

Figure 10

Gene expression analysis of DACH1 variants in prostate adenocarcinoma (PRAD TCGA) patients. Gene expression analysis of DACH1 variants in prostate adenocarcinoma (PRAD TCGA) patients using GEPIA2 based on the TCGA and GTEx database. (Upper panel) Violin plots represent log₂(TPM+1) of DACH1 variants in 492 prostate cancer tissue samples. (Bottom panel) Box plots, in terms of log₂(TPM+1), represent the gene expression level of oncogenic variant 1 and the less expressed variant 3 in the tumor (red) and normal (grey) samples, respectively, analyzing the iCluster2 and 3 that are associated with clinical and molecular features of patients with invasion and metastases and who showed poor prognosis. Normal tissues are matched TCGA adjacent tissue and GTEx data. The method for differential analysis is one-way ANOVA. A t-test was used to compare the expression difference between tumor and normal tissue; $p < 0.05$ was considered statistically significant. * $p < 0.05$ based on the Student's t test. Values are mean $\pm$ SEM. TPM, transcript per million.

Figure 11

DACH1 expression in normal and tumor prostate specimens. (A-F) Immunohistochemical staining for DACH1 in human prostate specimens. (A) DACH1 expression in prostate cancer sample with normal tissue showing positive nuclei of luminal cells (blue arrow) and negative nuclei of basal cells (red arrow). DACH1 expression in normal cells (blue asterisk) may be higher (B), or lower (C) than in tumor cells (red asterisk). B and C represent sections of group grade 1 and 2, respectively. (D) The expression of DACH1 appears limited to the nucleus in a group grade 2 tumor; (E) a group grade 4 tumor displays nuclear and cytoplasm positivity; (F) tumor invasion of peri-prostatic fat (star). Immunohistochemistry: anti-human DACH1 primary antibody with EnVision™ FLEX+ revelation system brown staining. H&E Counterstaining: 400x enlargement. (G) Western blot analysis for DACH1 expression in 3 specimens enriched (T) or not (N) in tumor tissue. #1, Gleason 4+4; #2, Gleason 4+4; #3, Gleason 4+5. β -actin was used as control for equal amounts of proteins loaded.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFig1.tif](#)
- [SupplementaryFig2.tif](#)
- [SupplementaryTab1.docx](#)