PDIA3 knockout abrogate effects of 1,25(OH)2D3 on cellular respiration and glycolysis in squamous cell carcinoma

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Keywords: PDIA3, squamous cell carcinoma, mitochondria bioenergetic, vitamin D

Posted Date: August 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3271679/v1

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Abstract

PDIA3 is an endoplasmic reticulum disulfide isomerase which is involved in the folding and trafficking of newly synthesized proteins. PDIA3 was also described as an alternative receptor for the active form of vitamin D $1,25(OH)_2D_3$. Here, we investigated the impact of PDIA3 in mitochondrial morphology and bioenergetics in squamous cell carcinoma line A431 treated with $1,25(OH)_2D_3$. It was observed that PDIA3 deletion resulted in changes in morphology of mitochondria including a decrease in the percentage of mitochondrial section area, maximal diameter, and perimeter. $1,25(OH)_2D_3$ treatment of $A431\Delta PDIA3$ cells partially reversed the effect of PDIA3 deletion increasing aforementioned parameters, while in $A431WT$ cells only an increase in mitochondrial section area was observed. Moreover, PDIA3 knockout affected mitochondrial bioenergetics and modulated STAT3 signaling. Oxygen Consumption Rate (OCR) was significantly increased, with no visible effect of $1,25(OH)_2D_3$ treatment in $A431\Delta PDIA3$ cells. In the case of Extracellular Acidification Rate (ECAR) rate an increase was observed for glycolysis and glycolytic capacity parameters in the case of non-treated $A431WT$ cells versus $A431\Delta PDIA3$ cells. $1,25(OH)_2D_3$ treatment had no significant effect on glycolytic parameters. Taken together presented results suggests that PDIA3 is strongly involved in the regulation of mitochondrial bioenergetics in cancerous cells and modulation of its response to $1,25(OH)_2D_3$, possibly through STAT3.

Introduction

Protein disulfide isomerases are the key oxidoreductase enzymes that play a role in proper folding and assembling of proteins and their complexes. An oxidoreductase family member, PDIA3 protein, has a broad range of functions from promoting protein folding in ER, participating in signal transduction through STAT3 in nucleus to pro-apoptotic activities in mitochondria. More to that, it was shown that PDIA3 can be localized in the MAM region (mitochondria-associated membranes) of the endoplasmic reticulum closely associated with mitochondria. Several studies have shown that PDIA3 functions as a chaperon to STAT3 protein and can modulate its transcriptional activity by regulating phosphorylation at the Y705 site. Phosphorylation at STAT3 S727 residue alone targets the import of this transcription factor into mitochondria. Moreover, it was suggested, that PDIA3 can suppress mitochondrial bioenergetic functions, by inhibiting phosphorylation of S727 site. PDIA3 has been also linked to various diseases from neurodegenerative to cancer. It seems that PDIA3 can be treated as a chemoprevention target and prognostic marker for an outcome in cancer patients.

An active form of vitamin D, $1,25(OH)_2D_3$, is a steroid hormone that regulates calcium-phosphorus homeostasis along with various cellular processes. Canonically vitamin D acts through the complex of its receptors: VDR and RXR, regulating the expression of hundreds of genes in the human genome. However, not all effects of $1,25(OH)_2D_3$ can be related to the genomic action of VDR-RXR. Consequently, PDIA3 was identified as a membrane-bound receptor for the active form of vitamin D (1,25D$_3$-MARRS) responsible for non-genomic response to hormone. It was shown that PDIA3 can
form a complex with caveolin-1 and subsequently activated phospholipase A2-activating protein (PLAA)\textsuperscript{25,26}. Thus, leading to the rapid action of 1,25(OH)\textsubscript{2}D\textsubscript{3} via PKC\textsuperscript{27}. Our recent studies have shown that genomic activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} strictly depends on VDR and only partially on RXR\textalpha\textsuperscript{28}, while deletion of PDIA3 significantly module the response\textsuperscript{29}. Moreover, it was postulated that VDR can regulate the transcription of mitochondrial genes and directly interacts with mitochondrial DNA\textsuperscript{30}. However, several studies have shown, the direct effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on ion transport\textsuperscript{31,32}, including activity of mitochondrial membrane potassium channels\textsuperscript{33}.

In our previous study, we established that PDIA3 is involved in 1,25(OH)\textsubscript{2}D\textsubscript{3} action in the manner of gene expression profile and range of phenotypic effects such as proliferation or migration\textsuperscript{29}. Here, for the first time, an impact of PDIA3 deletion on mitochondria morphology and bioenergetics in squamous cell carcinoma (A431) and its potential role in vitamin D action on mitochondria was investigated.

**Results**

**Deletion of PDIA3 and 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment affect morphology of mitochondria**

The knockout of PDIA3 in A431 squamous cell carcinoma cell line was generated with the use of CRISPR/Cas9 technology as previously described\textsuperscript{29}. The effects of PDIA3 deletion and 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment on the morphology of mitochondria were investigated by transmission electron microscopy (TEM) (Fig. 1A). A knockout of PDIA3 gene resulted in a two-times decrease in volume of mitochondria in comparison to wild type A431 (A431WT) cells as shown by the percentage of mitochondria section in whole cells observed by TEM. The treatment of A431WT or A431\textDelta PDIA3 with 1,25(OH)\textsubscript{2}D\textsubscript{3} for 24 h resulted in a significant increase in the percentage of mitochondria section (Fig. 1B), but also in a reduction of mitochondria diameter (Fig. 1C) and perimeter (Fig. 1D). 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment of A431\textDelta PDIA3 cells partially reversed the effect of PDIA3 deletion by increasing the aforementioned parameters but there was no visible effect on A431WT cells. Interestingly, elongation factor was not impaired by PDIA3 deletion but was decreased by 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment in the absence of PDIA3 (Fig. 1E). Further investigation, whether 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment affects mitochondrial surface area or mitochondrial membrane potential in A431\textDelta PDIA3 cells fluorescence probe was used, but no statistically significant changes were present (Fig. 2A, B).

**PDIA3 inhibits mitochondrial functions and affects the response to 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment**

The effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment on mitochondrial bioenergetics in A431WT and A431\textDelta PDIA3 was determined using the Seahorse XF24. Oxygen consumption rate (OCR) was monitored in real-time with
the following addition of oligomycin, FCCP, rotenone, and antimycin. It was observed that in A431Δ PDIA3 OCR, expressed in pmoles/min/mg of protein, is significantly higher than in A431WT cells, and 1,25(OH)₂D₃ treatment didn't affect those results (Fig. 3A). Overall, it was shown that deletion of PDIA3 enhances all parameters of oxidative phosphorylation, however despite the clear trends, some results did not reach statistical significance. In order to increase the strength of comparison, data for treated and nontreated cells were combined, and the effect of PDIA3 on cellular bioenergetics was reanalysed (Fig. 3).

In the case of basal respiration (Fig. 3B) and ATP-link respiration (Fig. 3D), a statistically significant increase was observed after deletion of PDIA3, why opposite trends were observed after 1,25(OH)₂D₃ treatment (decrease in A431WT, and increase in A431Δ PDIA3; Fig. 3B). Further PDIA3 deletion increased maximal respiration, but this parameter was not affected by 1,25(OH)₂D₃ treatment (Fig. 3C). Interestingly, for non-mitochondrial oxygen consumption a threefold increase in A431Δ PDIA3 cells was observed, with no further effect 1,25(OH)₂D₃ (Fig. 3E). Similar, an increase of proton leakage was observed, but with adverse trends in A431WT and A431Δ PDIA3 cells after 1,25(OH)₂D₃ addition (decrease in A431WT, and increase in A431Δ PDIA3; Fig. 3F). A mitochondrial spare capacity was increased by twofold in A431Δ PDIA3 cells in comparison to wild-type cells. No effect of 1,25(OH)₂D₃ treatment on this parameter was observed (Fig. 3G). Next, the impact of PDIA3 knockout and/or 1,25(OH)₂D₃ treatment on glycolysis was investigated by glycolytic stress tests. Extracellular Acidification Rate (ECAR) was measured in real-time by adding glucose to the medium on Seahorse XF24. Significant changes in ECAR rate were observed between the 30th and 70th minute of the assay in the case of non-treated A431WT cells versus A431Δ PDIA3 cells (Fig. 4A). Deletion of PDIA3 enhanced levels of glycolysis and other parameters (Fig. 4B, E) except of glycolytic capacity and reserve (Fig. 4C, D). In general, treatment of A431WT cells with 1,25(OH)₂D₃ resulted in a decrease in glycolysis (Fig. 4B), glycolytic capacity (Fig. 4C), glycolytic reserve (Fig. 4D) and non-glycolytic acidification (Fig. 4E) but the results were marginally statistically significant. The tendency was not so pronounced in A431Δ PDIA3 cells.

**PDIA3 knockout affects the expression of mitochondrial genes**

In previous work, the effects of 24h incubation with 1,25(OH)₂D₃ at 100 nM concentration on the transcriptome of A431Δ PDIA3 were studied. To assess the impact of PDIA3 on the expression of the genes related to mitochondria, the dataset of previously obtained differentially expressed genes (DEGs; false discovery rate (FDR) = 0,05) from A431Δ PDIA3 nontreated and 1,25(OH)₂D₃-treated cells, were compared with mitochondria-associated genes (mtDEGs) from MitoCarta 3.0 database by Venn analysis, followed by Gene Ontology (GO) analysis. The data are deposited in Sequence Read Archive (SRA) under accession number PRJNA926032. VENN analysis revealed 5831 DEGs expressed after PDIA3 deletion in A431 cells, and 4372 DEGs after treatment of A431Δ PDIA3 cells with 1,25(OH)₂D₃. Among those, 302 mtDEGs identified in A431Δ PDIA3 were affected solely by PDIA3 deletion, while 149 mtDEGs were changed by 1,25(OH)₂D₃ treatment (Fig. 5A). Interestingly, 111 mtDEGs were commonly regulated after PDIA3 deletion and 1,25(OH)₂D₃ treatment. GO analysis of molecular processes, revealed
that deletion of PDIA3 in A431 cell alone, mainly affected cellular respiration (GO:0045333), aerobic electron transport chain (GO:0019646), and mitochondrial ATP synthesis (GO:0042775) (Fig. 5B). Curiously, 1,25(OH)$_2$D$_3$ treatment of knockout cells changed entirely different molecular processes linked to mitochondrial transcription/translation such as mitochondrial translation (GO:0032543), mitochondrial gene expression (GO:0140053), and mitochondrial transport (GO:0006839) (Fig. 5C). mtDEGs affected by both deletion of PDIA3 and 1,25(OH)$_2$D$_3$ treatment were connected with mitochondrion organization (GO:0007005), Glutamate (GO:0006536) and dicarboxylic acid (GO:0043648) metabolic processes (Fig. 5D).

**PDIA3 or VDR deletion disrupts STAT3 signaling changing response to 1,25(OH)$_2$D$_3$**

As STAT3-PDIA3 interaction is widely described in the context of cell signaling, including regulation of cellular respiratory$^{4,12,37}$; it was checked, whether 1,25(OH)$_2$D$_3$ can affect this signaling, and if so, how PDIA3 is involved in that process. To observe an impact of 1,25(OH)$_2$D$_3$ on STAT3 translocation into the nucleus, immunofluorescent staining was performed (Fig. 6A). In the case of A431WT cells, we observed translocation of STAT3 into the nucleus after 1,25(OH)$_2$D$_3$ treatment, with the highest intensity ratio at 8h time point. Deletion of VDR (Vitamin D Receptor) decreased basal signal, both nuclear and cytoplasm, resulting in a higher nucleus/cytoplasm ratio for STAT3, but the effect of 1,25(OH)$_2$D$_3$ treatment was not observed. Interestingly, deletion of PDIA3 didn’t change basal intensity for STAT3, but similarly to A431ΔVDR cells there was no visible effect of 1,25(OH)$_2$D$_3$ treatment (Fig. 6B). Secondly, levels of STAT3 protein and its two phosphorylation sites (Ser727, Tyr705) were examined by western blot analysis (Fig. 6C). The amount of total STAT3 increased in time, with the highest level observed after 8h of incubation of A431WT cells with 1,25(OH)$_2$D$_3$. The deletion of VDR increased the initial level of STAT3 and abrogated an increase induced by 1,25(OH)$_2$D$_3$ treatment. Similarly, PDIA3 deletion slightly increased the basal amount of STAT3 with no effect from 1,25(OH)$_2$D$_3$ treatment. Finally, phosphorylation of STAT3 at the Y705 site occurred after 4h treatment solely in A431WT cells treated with 1,25(OH)$_2$D$_3$. Interestingly, the second STAT3 phosphorylation site connected to mitochondria (S727) was strongly increased by both VDR/PDIA3 deletion and further by 4h 1,25(OH)$_2$D$_3$ treatment.

**Discussion**

PDIA3 is a pleiotropic protein member of the oxidoreductase enzyme family, which is involved in a broad range of cellular processes: including protein folding and assemble through the formation and remodeling of disulfide bridges$^{38}$. PDIA3 has been strongly associated with cancer as a prognostic biomarker$^{15}$, thus this study focused on a squamous cell carcinoma cell line with deletion of PDIA3 (A431ΔPDIA3) as a model. In our previous study, we had shown that deletion of PDIA3 is not only essential for cellular physiology, but also plays an indispensable role in biological activities of 1,25(OH)$_2$D$_3$ including genomic response$^{29}$. The present study aimed to assess the impact of PDIA3 on morphology and bioenergetics of mitochondria and its role in 1,25(OH)$_2$D$_3$ action on mitochondria in
squamous cell carcinoma. To our knowledge, this is the first study investigating the role of PDIA3 in the mitochondrial activity of 1,25(OH)$_2$D$_3$. Hence, we are presenting data indicating that the deletion of PDIA3 affects the morphology of the A431 cells, especially mitochondria. Knockout of PDIA3 lead to the decrease of total mitochondria surface and size within the cell and 1,25(OH)$_2$D$_3$ treatment reversed the effect of deletion to some extent.

As the deletion of PDIA3 was shown to affect cellular responses to 1,25(OH)$_2$D$_3$ treatment and here we observed changes in morphology of mitochondria we decided to assess the impact of that deletion on mitochondria bioenergetics in A431 cells. All of the respiratory parameters of A431 cells were considerably elevated after PDIA3 deletion. Presented data are in line with results published by Keasey et al., who had shown that PDIA3 inhibits respiratory function in endothelial cells and C.elegans. Previously, PDIA3 was localized within mitochondria, where it associates with mitochondrial μ-calpain, possibly playing a significant role in apoptotic signaling. Moreover, PDIA3 was colocalized with STAT3, suggesting its role in the modulation of STAT3 signaling within cell. As those results suggested the possible involvement of PDIA3 in the modulation of 1,25(OH)$_2$D$_3$-induced STAT3 signaling, we analyzed levels of STAT3 protein together with its two phosphorylation sites at Tyr705 and Ser727. Our results suggest that VDR together with PDIA3 is necessary for the regulation of both phosphorylation sites by 1,25(OH)$_2$D$_3$. In our recent work, we identified the Cyclooxygenase-2 gene (PTGS2), as PDIA3-dependent gene. Interestingly, the expression of PTGS2 is known to be regulated by STAT3. Consequently, we observed that PDIA3 deletion abrogated the induction of the expression of PTGS2 by 1,25(OH)$_2$D$_3$. Here we are presenting results indicating impaired STAT3 phosphorylation at site Y705 in PDIA3 or VDR knockouts, suggesting that both proteins are necessary for the regulation of nuclear STAT3 phosphorylation. Enhanced oxygen consumption rate after PDIA3 deletion, is further supported by increased phosphorylation of STAT3 at S727 residue in A431ΔPDIA3 cells, as PDIA3 is known to inhibit this phosphorylation affecting bioenergetic functions of mitochondria. Recently, Peron and coworkers had shown that both phosphorylation are needed for mito-STAT3 to exert its mitochondrial functions. Here, for the first time, it was shown that phosphorylation of STAT3 at Tyr705 and Ser727, can be induced by 1,25(OH)$_2$D$_3$ and depend on both VDR and PDIA3 presence.

Interestingly, we observed that the lack of PDIA3 abrogated the effects of 1,25(OH)$_2$D$_3$ on energy production parameters, suggesting its involvement in cellular bioenergetics. Furthermore, an increase of glycolytic parameters was acknowledged after PDIA3 deletion, while 1,25(OH)$_2$D$_3$ treatment decreased glycolysis in wild-type A431 cells but no effect was observed in A431ΔPDIA3. This is in agreement with other studies showing reduced glycolysis after vitamin D treatment in breast cancer cells and colorectal cancer.

Recently, we have shown that PDIA3 deletion alters the expression of more than 2000 genes and modulates genomic response to 1,25(OH)$_2$D$_3$. Here we focused on genes related to mitochondria. However, we didn't identify any PDIA3-dependent mtDEGs, even though deletion of PDIA3 alone has
changed basal expression of mtDEGs, regulating different processes connected with cellular respiration. In recent work (Olszewska et al., 2023 under review), it was shown that 1,25(OH)$_2$D$_3$ affects differently morphology and bioenergetics of cancerous and non-cancerous cells through genomic pathways regulated by VDR and partially by RXRA$^{39}$. However, it is clear that PDIA3 somehow modulates the response of cancerous cells to 1,25(OH)$_2$D$_3$ treatment in terms of mitochondrial morphology and bioenergetic, thus it supports our previous founding that PDIA3 possibly functions as a modulator of genomic response to 1,25(OH)$_2$D$_3$. Interestingly, Gezen-Ak and coworkers suggested that VDR affects directly mitochondrial DNA expression after 1,25(OH)$_2$D$_3$ treatment$^{30}$ opening new possibilities for the direct impact of 1,25(OH)$_2$D$_3$ and VDR on mitochondria, however, the presence of VDR in mitochondria is still under debate$^{20}$.

Taken together, PDIA3 is a key enzyme involved in maintaining normal cellular physiology. In this study, we have shown that PDIA3 deletion affects mitochondria morphology and bioenergetics most likely through STAT3 regulation, as well as mitochondrial response to 1,25(OH)$_2$D$_3$. As we didn’t identify any PDIA3-dependent mtDEGs, we suggest that the main effects of 1,25(OH)$_2$D$_3$ are genomic actions mediated by VDR and partially by RXRA (Olszewska et al. 2023, under review). As PDIA3 was found also in mitochondria, the direct impact on mitochondrial structure and function cannot be excluded$^{12,39,46}$. Data presented here, broaden our knowledge about the role of PDIA3 in 1,25(OH)$_2$D$_3$ activities on mitochondria and open new perspectives to explore that topic.

**Methods**

1,25(OH)$_2$D$_3$

1,25(OH)$_2$D$_3$ was purchased from Sigma-Aldrich. Stock solutions of 1,25(OH)$_2$D$_3$ were dissolved in ethanol and stored at -20°C. 1,25(OH)$_2$D$_3$ at 100 nM concentration was used in all experiments (the concentration of solvent (ethanol) was <0.05%).

**Cell cultures**

Immortalized human basal cell carcinoma cell line (A431) was obtained from Synthego Corporation (Menlo Park, CA, USA). PDIA3 knock-out cell line was obtained with CRISPR/Cas9 technology as previously described$^{29}$. Cells were cultured in DMEM high glucose medium (4.5 g/L) with the addition of 10% FBS and penicillin (10000 units/ml) and streptomycin (10mg/ml) (Sigma–Aldrich; Merck KGaA). Cell cultures were performed in the incubator with 5% CO$_2$ at 37°C. Before treatment with 1,25(OH)$_2$D$_3$ medium was changed to DMEM with 2% charcoal-stripped FBS.

**Transmission electron microscopy (TEM)**

The A431 ΔPDIA3 cells were seeded onto a Petri dish at a density of 1 × 10$^6$ cells/plate standard medium and after 24h treated with 100 nM 1,25(OH)$_2$D$_3$. Consequently, the cells were fixed in 2.5% glutaraldehyde
in 0.1 mM sodium-cacodylate buffer, scratched, and centrifuged. The cell pellets were then postfixed in 2% osmium tetroxide, dehydrated in ethanol, and infiltrated with a mixture of propylene. The pelleted cells were subsequently embedded to polymerize. Ultrathin sections (70 nm) were cut and, after dehydration, stained with uranyl acetate (Plano GmbH, Wetzlar, Germany) and lead citrate (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were analyzed with an electron microscope (JEOL JEM-1200 EX II, University Park, PA, USA) at an acceleration voltage of 80 kV. Mitochondria from EM photos were counted in CellSens Olympus Software.

**Seahorse analysis**

The effects of 1,25(OH)$_2$D$_3$ on the mitochondrial function of A431 $\Delta$PDIA3 were measured using the Seahorse Mito Stress Test following the manufacturer's protocol. Briefly, $2 \times 10^4$ cells/well were seeded on a Seahorse plate and after 24h treated with 100 nM 1,25(OH)$_2$D$_3$ for 24h. All essential compounds were diluted to final concentrations of 1 µM for Oligomycin, 1 µM for Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1 µM Antimycin A/ Rotenone and cells were prepared according to Seahorse protocols. The experiment was run with Seahorse XF24 (Agilent Technologies, Santa Clara, CA, USA). After the Seahorse analysis, the cells were lysed with modified RIPA buffer supplemented with Roche (Basel, Switzerland) protease and phosphatase inhibitors cocktail (Roche, Basel, Switzerland), and protein concentration was measured with bicinchoninic acid assay (ThermoFisher Scientific, Waltham, MA, USA) for data normalization. Each experiment was repeated at least three times, independently. The data were analyzed with Wave software (Agilent Technologies, Santa Clara, CA, USA), and the Student's t-test was used to compare the mean fluorescence values between different experimental conditions. Basal respiration was calculated after subtraction of nonmitochondrial respiration (remaining OCR after Antimycin A addition). ATP-linked OCR was derived as the difference between basal and Antimycin A inhibited OCR. Proton leak was calculated as the difference between OCR following Oligomycin A inhibition and OCR following Antimycin A inhibition. Maximal respiration was measured following the addition of FCCP. Spare capacity was calculated based on the difference between basal respiration and maximal respiration.

**Fluorescent probes**

For fluorometric measurements, cells were seeded n 8-well chambers (MoBiTec Molecular Biology, Germany) at a density of 200,000 cells/well and incubated overnight (37 °C, 5% CO2). The next day medium was removed and cells were incubated with diluted to a final concentration of 2 µM JC-1 (ThermoFisher Scientific, Waltham, MA, USA) or 100 nM MitoGreen (ThermoFisher Scientific, Waltham, MA, USA) probes for 20 minutes. Then probe solution was replaced with 100nM 1,25(OH)$_2$D$_3$ medium solution and cells were grown for 24h with live imaging under a microscope Olympus cell Vivo IX83. For JC-1 the ratio of red/green fluorescence intensity was analyzed by the cellSens Olympus software. For Mito-Green calculation fluorescence intensity measurements were normalized against cell numbers before being expressed as percentages of control values.
Western blotting

A431-derived cell lines were treated with 100nM 1,25(OH)$_2$D$_3$ for 4, 8, and 24h. The medium was removed from the plate and cells were washed twice with PBS and were scratched from the plate. The solution was moved to an Eppendorf tube and centrifuged at 16,000×g for 10 min. The received cell sediment was dissolved in 100µl of RIPA buffer (Thermofisher, Waltham, Massachusetts, USA). Concentration was determined by a modified Bradford Assay. For SDS-PAGE electrophoresis 10% bottom gel and 5% upper gel were used. An equal amount of protein (20µg) was loaded into each well. Electrophoresis was run at 90-110V in the Bio-Rad apparatus. Proteins were transferred to PVDF membranes with the use of the Trans-Blot Turbo system (Bio-Rad). After the transfer membranes were blocked in 5% milk dissolved in TBS-T. The membranes were incubated with primary antibodies anti-STAT3 (Abclonal, Woburn, MA 01801, United States), anti-pSTAT3 (Y705) (Abclonal, Woburn, MA 01801, United States), or anti-pSTAT3 (S727) (Abclonal, Woburn, MA 01801, United States), overnight at 4°C. For loading control membranes were stripped and reprobed with anti-β-actin antibodies (Abclonal, Woburn, MA 01801, United States). Then, it was incubated with proper secondary fluorescent antibodies (AlexaFluor® 790 or AlexaFluor® 680 from Jackson ImmunoResearch). Bands were visualized with Odyssey Clx system and densitometry of bands was done with Image Studio Software Ver 5.2.

Immunofluorescence staining

A431 cell lines were seeded in 8-well imaging chambers (MoBiTec Molecular Biology, Germany) at a density of 200.000 cells/well, incubated overnight (37 °C, 5% CO2). The next day cells were treated with 1,25(OH)$_2$D$_3$ in DMEM medium supplemented with 2% charcoal-stripped FBS, and 100U/ml penicillin/streptomycin. After incubation time (4, 8, 24h), cells were rinsed three times with PBS and fixed with 4% (v/v) formaldehyde solution, then washed three times with PBS and permeabilized with 0.1% Triton X-100, blocked with 10% BSA in PBS for 1h at RT and incubated with primary antibodies at 4°C overnight (anti-STAT3, Abclonal, Woburn, MA 01801, United States). Following, the cells were rinsed three times with PBS, incubated with secondary antibodies for 1h at RT (Alexa Fluor 488 anti-rabbit, Invitrogen, USA), then rinsed again with PBS, incubated with DAPI solution and mounted with DAKO fluorescence mounting medium (S3025, Agilent Technologies, USA). The cells were visualized by fluorescence microscopy (Olympus Cell-Vivo IX 83, Japan) with camera ORCA-FLASH 4.0 and 60X objective.

Bioinformatic analysis

Transcriptomic data from a previous study was used to define mitochondrial genes expressed in A431Δ $PDIA3$ cells after 1,25(OH)$_2$D$_3$ treatment $^{29}$. Venn analysis was performed with the online available tool $^{35}$.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.05 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± SD and were analyzed with a Student's t-test (for two
groups) or one-way ANOVA analysis of variance with appropriate posthoc tests (for more than two groups). Statistically significant differences are illustrated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001.

**Declarations**

**Acknowledgments**

The study was supported by a National Science Center OPUS Program under contracts 2017/25/B/NZ3/00431.

**Author contributions statement**

M.A.Z. designed and coordinated the project. M.A.Z., J.I.N. and A.O planned and designing experiments. J.I.N., A.O. and O.K. performed experiments. J.I.N., A.O., and M.A.Z. analyzed data. J.I.N wrote the manuscript together with M.A.Z.; All authors reviewed the manuscript.

**Additional information**

The authors declare that they have no competing interests.

**References**


**Figures**
Figure 1

1,25(OH)₂D₃ treatment and PDIA3 deletion affect the morphology of mitochondria. (A) EM micrographs representing morphology of mitochondria of A431WT and A431ΔPDIA3 cells non-treated/treated with 1,25(OH)₂D₃ at two different magnifications. (B) Percentage of mitochondria section through the cytoplasm of A431WT and ΔPDIA3 cells after 1,25(OH)₂D₃ treatment. Assessment of another
mitochondrial parameter of A431 strains like (C) maximal diameter and (D) perimeter. Data are expressed as mean ± SEM. * p < 0.05, ** p < 0.005, *** p < 0.0005, ****p < 0.00005. (E)

Figure 2

**Mitochondrial surface area and membrane potential in PDIA3 knockout A431 cell line after 1,25(OH)$_2$D$_3$ treatment.** (A) The mitochondrial surface area in A431ΔPDIA3 cells stained with MitoTracker Green dye imaged with live microscopy Olympus cell Vivo IX83. (B) Mitochondrial membrane potential in A431 Δ PDIA3 cells stained with JC-1 fluorescence probe with the use of live microscopy Olympus cell Vivo IX83.
Figure 3

PDIA3 deletion increases mitochondrial bioenergetics and abolishes the effect of 1,25(OH)$_2$D$_3$ treatment in A431 cells. (A) Representative traces of mitochondrial oxygen consumption rate of A431WT and A431ΔPDIA3 cells after 24h 1,25(OH)$_2$D$_3$ treatment. Mitochondrial respiration parameters: (B) basal respiration, (C) maximal respiration, (D) ATP-linked respiration, (E) non-mitochondrial oxygen consumption.
consumption, (F) proton leak, and (G) spare capacity. Data are expressed as mean ± SEM. * p < 0.05, ** p < 0.005, *** p < 0.0005, ****p < 0.00005.

**Figure 4**

PDIA3 deletion disrupts glycolytic functions and response to 1,25(OH)$_2$D$_3$ treatment of A431 strains. (A) Representative traces of mitochondrial extracellular acidification rate of A431WT and A431ΔPDIA3 cells
after 24h 1,25(OH)₂D₃ treatment. Mitochondrial glycolytic parameters: (B) Glycolysis, (C) Glycolytic capacity, (D) Glycolytic reserve, and (E) non-glycolytic acidification. Data are expressed as mean ± SEM. * p < 0,05, ** p < 0,005, *** p < 0,0005, ****p < 0,00005.

Figure 5

PDIA3 deletion alters the expression of mitochondrial genes after 1,25(OH)₂D₃ treatment in A431 cells. (A) Comparison of mitochondrial genes from MitoCarta 3.0, A431WT, and A431Δ PDIA3 cells treated with 1,25(OH)₂D₃. (B) Heatmap of selected mitochondrial genes expressed in A431 A431Δ PDIA3 cell line after 1,25(OH)₂D₃ treatment.
Figure 6

**PDIA3 deletion affects STAT3 signaling in A431 squamous cell carcinoma.** (A) Fluorescence images of A431 cell lines treated with 1,25(OH)_{2}D_{3} for 4, 8, or 24h and stained with anti-STAT3 antibody and DAPI. (B) STAT3 nucleus/cytoplasm ratio in A431 sublines. (C) Analysis of protein levels of STAT3, pSTAT3 (Y705), and pSTAT3 (S727) in A431WT and VDR or PDIA3 deficient knockout cell lines.

Supplementary Files

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