PGC1α suppresses prostate cancer cell invasion through ERRα transcriptional control

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Running title: PGC1α-ERRα suppresses MYC and prostate cancer invasion

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Abstract

The peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) is a prostate tumor suppressor that controls the balance between anabolism and catabolism. PGC1A downregulation in prostate cancer is causally associated with the development of metastasis. Here we show that the transcriptional complex formed by PGC1α and Estrogen related receptor 1 alpha (ERRα) controls the aggressive properties of prostate cancer cells. PGC1α expression significantly decreased migration and invasion of various prostate cancer cell lines. This phenotype was consistent with remarkable cytoskeletal remodeling and inhibition of integrin alpha 1 and beta 4 expression both in vitro and in vivo. CRISPR/Cas9-based deletion of ERRα suppressed PGC1α regulation of cytoskeletal organization and invasiveness. Mechanistically, PGC1α expression decreased MYC levels and activity prior to inhibition of invasiveness. In addition, PGC1α and ERRα associated at the MYC promoter, supporting the inhibitory activity PGC1α. The inverse correlation between PGC1α-ERRα activity and MYC levels was corroborated in multiple prostate cancer datasets. Altogether, these results support that PGC1α-ERRα functions as a tumor suppressive transcriptional complex through the regulation of metabolic and signaling events.

Statement of significance

Findings describe how downregulation of the prostate tumor suppressor PGC1 drives invasiveness and migration of prostate cancer cells.
Introduction

The process of cellular transformation stems from the acquisition of genomic aberrations that altogether change the response of normal cells and enable them with hallmarks of cancer (1,2). The mutational landscape changes within and among tumors and along time following evolutionary principles (3). In addition, non-genomic alterations harness great relevance in the process of cancer progression. Indeed, transcriptional regulation in cancer is an emerging aspect that provides a feasible explanation to the rapid adaptation of transformed cells to hostile environments (4). Yet, the control of oncogenic and tumor suppressive transcriptional programs remains poorly characterized.

Transcriptional co-regulators encompass a family of versatile modulators of gene expression (5). These proteins harbor the capacity of controlling distinct transcriptional programs based on their partner transcription factors. In turn, transcriptional co-regulators operate in a tissue and context-specific manner, thus revealing them as major players in cell and organismal homeostasis. Among this family of genes, the Peroxisome proliferator-activator receptor (PPAR) gamma co-activator 1 alpha (PGC1α) controls biological responses in health and disease (6,7). PGC1α is a tightly regulated protein that interacts with a variety of transcription factors, including Estrogen related receptor 1 alpha (ERRα), PPARs and Nuclear factor erythroid 2 like 2 (NFE2L2, NRF2) (6). As a consequence, PGC1α coordinates metabolic and antioxidant responses, which account for its relevance in diabetes, neurodegeneration, cardiomyopathy and cancer (7,8).

The role of PGC1α in cancer is largely tumor type and context-dependent. On the one hand, this transcriptional co-regulator favors survival, proliferation, stem cell maintenance and therapy resistance in pancreatic tumors, breast cancer and melanoma cells (9-14). On the other hand, we and others have demonstrated that PGC1α expression is reduced in...
renal and prostate carcinoma, as well as in metastatic melanoma, where it opposes the acquisition of aggressive features (15-17). The predominant mechanism of action of PGC1α in cancer biology is ascribed to the regulation of metabolism. This co-regulator promotes the expression of genes that mediate mitochondrial biogenesis, oxidative metabolism and the production of glutathione. In turn, PGC1α enhances the oxidative utilization of nutrients and antioxidant production. However, emerging data suggest that a fraction of the activities of PGC1α neither relies on the regulation of metabolism nor on its main partner, ERRα (16).

In prostate cancer (PCa), PGC1α suppresses cell proliferation, anchorage-independent growth, tumor burden and metastasis (17). This co-regulator is profoundly downregulated in localized PCa, with a further decrease in metastatic specimens (17). Moreover, reduced PGC1α expression is associated to shorter time to biochemical recurrence after surgery, pointing at the relevance of this gene in the control of PCa aggressiveness. Mechanistically, we previously showed that PGC1α requires the presence of ERRα to suppress PCa cell proliferation and metastatic outgrowth, which was consistent with the reduction of biosynthetic capacity of PGC1α re-expressing cells and the elevation of nutrient catabolism (17). Moreover, a recent study revealed that the metabolic control of polyamine synthesis underlies the regulation of prostate cancer aggressiveness by this co-activator (18).

The metastatic process requires the acquisition of discreet capacities beyond cell proliferation. Specifically, the motility and invasive capacity of cancer cells is paramount for the achievement of metastasis (19). Stemming from this notion, in this study we evaluated the contribution of PGC1α to the acquisition of these features in PCa cells. Our analysis uncovers an ERRα-dependent activity of the co-activator that suppresses the acquisition of invasive properties required for PCa aggressiveness.
Materials and Methods

Reagents

Doxycycline hyclate (Sigma #D9891) was used to induce gene expression or silencing in vectors under tetracycline control. Puromycin (Sigma #P8833) and blasticidin (Invitrogen #R210-01) were used for cell selection after lentiviral transfection.

Cell culture

Human prostate carcinoma cell lines PC3 and DU145 were purchased from Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, who provided authentication certificate. Cell lines where periodically subjected to microsatellite-based identity validation. None of the cell lines used in this study were found in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample. 293FT cells were used for lentiviral production. All cell lines were routinely monitored for mycoplasma contamination. DU145, PC3 and 293FT cell lines were maintained in DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. For PGC1A expression, cells were transduced with a modified TRIPZ (Dharmacon) doxycycline inducible lentiviral construct in which the RFP and miR30 region was substituted by HA-Flag-Pgc1a (9). For ESRRA deletion, sgRNA constructs targeting ESRRA (sgERRα#1: 5´CTCCGGCTACCACTATGGTGTTGG3´; sgERRα#2: 3´AGGAACCCTTTGGACTGTCAGGG5´) were designed using Crispor software (crispor.tefor.net) and cloned in a lentiviral vector purchased from addgene LentiCRISPR V2 (a gift from Mohan Babu, Addgene plasmid # 83480). Lentiviral vector expressing a validated shRNA against human MYC from the Mission® shRNA Library (TRCN0000039642) was subcloned in a Plko Tet On inducible system (following the
strategy provided by Dr. Dmitri Wiederschain (20), Addgene plasmid # 21915). Cells were
transfected with lentiviral vectors following standard procedures, and viral supernatant was
used to infect cells. Selection was done using puromycin (2 μg ml⁻¹) or blasticidin (for
LentiCRISPR V2, 10 μg ml⁻¹) for 3 or 5 days, respectively.

Animals

All mouse experiments were carried out following the ethical guidelines established by the
Biosafety and Welfare Committee at CIC bioGUNE. The procedures employed were
carried out following the recommendations from AAALAC. Xenograft experiments were
performed as previously described (17), injecting one million cells per tumor in two flanks
of Hsd:AthymicNude-Foxn1nu “Nude” mouse (Envigo). Once tumors reached an average
of 100mm³, animals were assigned to chow or doxycycline diet regime (Research diets,
D12100402) and tumor volume was monitored with external caliper. After euthanasia,
tumors were weighed, tissue was fresh frozen or paraffin embedded, and histological
evaluation of a Haematoxylin and eosin (H&E) stained sections was performed.
Proliferation was assessed in paraffin embedded tissue samples by using Ki67 antibody
(MA5-14520, Thermo Scientific).

Cellular and molecular assays

Cell number quantification with crystal violet was performed as referenced (21).

Cell morphology and stress fiber content were examined staining the cells with fluorescent
phalloidin (ThermoFisher F432; 1:400 dilution), a high affinity F-actin probe. Images were
taken with AxioImager D1 microscope at 200x for cell area analysis (FiJi Software) or at
400x for stress fiber quantification. Immunofluorescence detection and quantification of p-MLC (Ser19) was performed as referenced (22). Briefly, cells were fixed with 4% formaldehyde, permeabilised with 0.3% Triton and incubated with primary antibody (p-MLC Ser19, CST #3672) overnight. Cells were then stained with secondary Alexa Fluor-488 or 647 anti-rabbit (Life Technologies), Alexa Fluor 546-phalloidin for F-actin detection (Life Technologies) and DAPI (Thermo Fisher D1306; 1:10,000 dilution).

For adhesion assays cells were plated (40,000 cells/well) on a 12-well plate previously coated with rat tail collagen I (Corning 354236) at 50 μg/mL (diluted in 0.02 N of acetic acid) during 1 hour. After 30 minutes, plates were washed twice with PBS, fixed with 10% formalin and stained with crystal violet as previously described (17).

Transwell invasion assay was carried out using matrigel-coated chambers (BD CioCoatTM #354480). Cells (50,000 cells/well) were re-suspended in 0.1 % FBS DMEM and seeded in the upper part of the chamber. In the bottom part of the well 1.4 mL of complete DMEM were added. Plates were maintained at 37 ºC and 5 % CO₂ for 48 hours. Invasion was stopped washing the well twice with PBS and using a cotton bud to remove the remaining cell of the upper part of the membrane, being careful not to compromise the matrigel. The membrane was fixed with 10 % formalin (15 minutes at 4 ºC) and stained with crystal violet (Sigma C3886; 0.1% crystal violet in 20% methanol). Cells were counted under the microscope. For transwell migration, chambers with membranes of 8 μm pores (BD Falcon 351185) were used. Cell plating as well as washing and fixation conditions were the same as in the invasion assay, but cells were fixed after 24 hours.

Spheroid cell culture and 3D invasion assays were performed as previously described (23). Briefly, cells (700 cells/drop) were maintained in drops (25 μL/drop) with DMEM and 6 % methylcellulose (Sigma M0387) on the cover of a 100 mm culture plate. Drops were incubated at 37 ºC and 5 % CO₂ for 48 hours. Once formed, spheroids were collected,
resuspended in collagen I solution (Advanced BioMatrix PureCol®) and added to 12-well plates. After 4h complete media was then added on top of the well and day 0 pictures were taken. For invasive growth quantification, increase in area occupied by the spheroids between day 0 and day 2 was calculated using FIJI software. For 3D invasion assays, cells were resuspended in a FBS-free bovine collagen I solution at 2.3 mg/mL in a 1:1 proportion, to a final concentration of 15000 cells per 100 µL of matrix and spin down in a 96-well plate. After matrix polymerization, 10% FBS-containing media was added on top. Cells were fixed after 24 h. The 3D invasion index was calculated counting the number of cells at 50 µm and 100 µm divided by the number of cells at the bottom. Images for 3D invasion were done using a Zeiss 710 confocal microscope and cell counting was analysed using FIJI Software.

Western blot was performed as previously described (9). Briefly, cells were seeded on 6-well plates and 4 days after seeding cell lysates were prepared with RIPA buffer (50 mM TrisHCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Nonidet P40, 1 % sodium deoxycholate, 1 mM Sodium Fluoride, 1 mM sodium orthovanadate, 1 mM beta-glycerophosphate and protease inhibitor cocktail; Roche). Antibodies: PGC1α H300 (Santa Cruz Technology #sc-13067), ERRα (CST #13826), ITGβ1 (CST #34981S), Caveolin-1 (BD Bioscience ref: 142610059), β-Actin (CST #3700S), phospho-Cofilin (CST #3313), Cofilin (CST #5175), GAPDH (CST #2118), c-MYC (MYC, CST #13987S), ITGβ4 (CST #14803), ITGa3 (Santa Cruz Technology #sc-374242), ITGa6 (CST #3750S), phospho-Src (Life Technologies ref: 44660G; p-Src Tyr419) and Src 36D10 (CST #2109). All were used at a 1:1000 dilution except from β-Actin at 1:2000. Mouse and rabbit secondary antibodies were purchased from Jackson Immuno Research. After standard SDS-PAGE and Western blotting techniques, proteins were visualized using the ECL system in the iBright™ FL1000 Imaging System.
The cytoskeleton phospho-antibody array was performed following Tebu-bio protocol (https://www.tebu-bio.com). Briefly, 5 million induced and non-induced cells were collected and the cell pellet was frozen for further analysis by Tebu-bio services. Over 141 antibodies were present in the screening for phosphorylation rate of main cytoskeleton proteins.

RNA was extracted using NucleoSpin® RNA isolation kit from Macherey-Nagel (Ref: 740955.240C). For xenograft samples, a Trizol-based implementation of the NucleoSpin® RNA isolation kit protocol was used as reported (24). For all cases, 1 μg of total RNA was used for cDNA synthesis using qScript cDNA Supermix from Quanta (Ref: 95048). Quantitative Real Time PCR (qRTPCR) was performed as previously described (9). Universal Probe Library (Roche) primers and probes employed are detailed in supplementary Table S1. All qRTPCR data presented were normalized using GAPDH (Hs02758991_g1 from Applied Biosystems).

**ChIP**

Chromatin Immunoprecipitation (ChIP) was performed using the SimpleChIP® Enzymatic Chromatin IP Kit (Cat: 9003, Cell Signalling Technology, Inc). Four million PC3 TRIPZ-Pgcn1a cells per immunoprecipitation were grown in 150 mm dishes either with or without 0.5 μg mL⁻¹ doxycycline during 16 hours. Cells were cross-linked with 37 % formaldehyde for 10 min at room temperature. Glycine was added to dishes, and cells incubated for 5 min at room temperature. Cells were then washed twice with ice-cold PBS and scraped into PBS+PIC. Pelleted cells were lysed and nuclei were harvested following the manufacturer’s instructions. Nuclear lysates were digested with micrococcal nuclease for 20 min at 37 °C and then sonicated in 500 μl aliquots on ice for 6 pulses of 20 s using a
Branson sonicator. Cells were held on ice for at least 20 s between sonications. Lysates were clarified at $11000 \times g$ for 10 min at 4 °C, and chromatin was stored at -80 °C. HA-Tag polyclonal antibody (CST #3724), anti-ERRα antibody (CST #13826) and IgG antibody (CST #2729), were incubated overnight (4 °C) with rotation and protein G magnetic beads were incubated 2 hours (4 °C). Washes and elution of chromatin were performed following manufacturer’s instructions. DNA quantification was carried out using a Viia7 Real-Time PCR System (Applied Biosystems) with SybrGreen reagents and primers that amplify a PGC1A binding region to MYC promoter (shown in supplementary table S2).

Bioinformatic analysis and statistics

Bioinformatic analysis containing patient data was performed using the web-based interface Cancertool (25). For each available patient dataset, the values of PGC1α-ERRα signature were calculated from the average of the expression signal of those genes that are part of the aforementioned signature. (ACACB, ACSL4, ATP1B1, GSTM4, ISCU, LAMB2, NNT, PPIC, SOD2, SUCLA2). In the case of PPARGC1A /NRIP1 ratio, we calculated the average expression value of PPARGC1A, and, as values are log2 scaled, subtracted the average expression value of NRIP1. R software (https://cran.r-project.org/), version 3.5.1, has been used for these calculations, together with ggplot2 package (https://cran.r-project.org/web/packages/ggplot2) in order to perform the corresponding graphs.

Individual gene expression patterns in Patient dataset, as well as pairwise correlation information can be visualized in the Cancertool interface.

The differential gene expression analysis driven by PGC1α in PC3 cells can be obtained from GEO with reference GSE75193.
In addition, pathway and network enrichment analyses of the significantly regulated genes from GSE75193 (Supplementary Table S3) were performed using MetaCore from GeneGo Inc. (https://portal.genego.com/).

No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. n values represent the number of independent experiments performed, the number of individual mice or patient specimens. For each independent \textit{in vitro} experiment, normal distribution was assumed, and one sample t-test was applied for one component comparisons with control and Student’s t-test for two component comparisons. For \textit{in vivo} experiments a non-parametric Mann-Whitney exact test was used. Two-tail statistical analysis was applied for experimental design without predicted result, and one-tail for validation or hypothesis-driven experiments. The confidence level used for all the statistical analyses was of 95% (alpha value = 0.05). GraphPad Prism 8 software was used for statistical calculations.
Results

In order to address the role of PGC1α in the regulation of PCa features beyond proliferation (17), we carried out a comprehensive evaluation of phenotypes associated to cancer aggressiveness, based on an inducible system previously reported (17). Interestingly, Pgc1α expression elicited a remarkable reduction in the migratory capacity of PC3 and DU145 PCa cells in transwell assays (Fig. 1A; Supplementary Fig. 1A). A similar effect was achieved in matrigel-coated transwell assays as a measure of invasion (Fig. 1B; Supplementary Fig. 1B). In order to further characterize the regulation of invasive properties by PGC1α, we applied two complementary assays in both cell lines. On the one hand, we performed 3D invasion assays. We quantified the number of cells invading at 50 μm and/or 100 μm of distance from the bottom of the plate. The results showed a profound decrease in cells with invasive capacity upon Pgc1α induction (Fig. 1C; Supplementary Fig. 1C, D). On the other hand, we generated spheroids using the hanging drop method in order to measure the invasive growth. The results corroborated that the expression of the co-regulator inhibits the invasive capacity of PCa cells (Fig. 1D; Supplementary Fig. 1E). Of note, this phenotype was observed at time points where proliferation was not significantly influenced by Pgc1α or by the addition of doxycycline (17) (Supplementary Fig. 1F-I). Overall, our results show that, beyond the anti-proliferative capacity of PGC1α in PCa, the transcriptional co-regulator elicits a robust anti-invasive phenotype.

The regulation of cell migration and invasion is intertwined with cell morphology and adhesion (19). Hence, we characterized the effects of PGC1α on these parameters. The expression of the co-regulator in PC3 cells was associated to a remarkable elevation in cell area, with loss of stress fibers and with a modest increase in cell adhesion to collagen I (Fig. 1E-F; Supplementary Fig. 1J). Importantly, Pgc1α induction in subcutaneous
xenografts of PC3 cells confirmed the antitumoral activity of this gene, and its impact on PCa cell size in vivo (Fig. 1G; Supplementary Fig. 1K-M).

We next focused on the molecular alterations underlying the activity of PGC1α. In a previous study, we analyzed a gene expression analysis in PC3 cells upon induction of Pgc1α (Fig. 1) (17) (GSE75193). We sought to extend the analysis of this microarray by taking advantage of bioinformatic tools, such as Metacore (https://clarivate.com/products/metacore/) and Cancertool (25) that enable cancer researchers to perform various functional enrichment analyses. Since functional enrichment allows the integration of larger sets of data in order to identify underlying molecular and functional alterations, we focused our analyses on all genes whose expression was altered with a significant p-value in the transcriptomics analysis (regardless of the adjusted p-value). This led to 1347 upregulated and 990 downregulated unique gene IDs (Supplementary Table S3). Strikingly, functional enrichment of the downregulated genes revealed a significant alteration in cytoskeleton organization, migration, adhesion and integrin and Rho signaling (Fig. 2A; Supplementary Fig. 2A; Supplementary Table S4-S5). Of note, we also identified other pathways with reported activities in the regulation of invasion, such as p27, FAS and RAC, although their prevalence in the analysis and their documented association to this phenotype were minor (26-29). In line with our previous study (17), the enrichment analysis of the genes upregulated upon Pgc1α expression confirmed a significant alteration of catabolic pathways (Supplementary Table S6). We focused our attention in the Metacore analysis of downregulated genes. The results revealed a remarkable alteration in cytoskeletal remodeling upon PGC1α modulation in PCa cells, illustrated by processes regulated by Rho kinase (ROCK). The axes containing ROCK-LIM kinase (LIMK)-Cofilin and ROCK-Myosin Light Chain (MLC) are two key signaling pathways that regulate cytoskeletal


remodeling downstream of the monomeric G protein Rho and integrin signaling (30). The immunostaining and quantification of phosphorylated Myosin-light chain 2 (p-MLC2) revealed a significant reduction in this parameter in Pgc1α-expressing PC3 cells (Figure 2B). This result supports the notion that loss of PGC1α in PCa cells results in changes in the actin-myosin cytoskeleton that are associated with the acquisition of invasive properties. In order to ascertain which signaling pathways were modulated and affecting cytoskeleton organization upon Pgc1α expression, we carried out a cytoskeleton phospho-antibody array (Supplementary Table S7). The phosphorylation of Src protein was among the most prominently reduced in the analysis (Supplementary Fig. 2B). We confirmed this result by western blot analysis, both in vitro and in vivo, together with the reduction in Cofilin phosphorylation, the final effector of actin filament polymerization downstream Src (Fig 2C, D, Supplementary Fig. 2C, D).

Integrins are upstream regulators of the cytoskeleton with well-documented involvement in cancer aggressiveness (19,31,32). The bioinformatics analysis of PGC1α downregulated genes indicated an altered integrin signaling (Fig. 2A, Supplementary Fig. 2A), which would be consistent with the reduction in Src, MLC2 and Cofilin phosphorylation. This, together with the fact that PGC1α controls integrin expression in melanoma (16), prompted us to evaluate integrin expression in our experimental systems. Interestingly, the levels of various integrins and caveolin-1 (CAV1, but not CAV2) were robustly reduced at protein and mRNA levels upon Pgc1α induction, an event that was not influenced by doxycycline treatment (Fig. 2E; Supplementary Fig. 2E-I). Next, we analyzed extracts from xenografts in which Pgc1α expression was activated (Fig. 1G). The western blot and quantitative qRTPCR analysis corroborated the alterations elicited by the co-activator in vivo (Fig. 2F; Supplementary Fig. 2J, K). Our results suggest that PGC1α controls a transcriptional program that results in the alteration of cytoskeleton organization with the
concomitant reduction in integrin expression, an event that is consistent with the observed reduction in migratory and invasive properties of PCa cells.

We then asked which effector of PGC1α could contribute to the negative regulation of invasive properties. Inhibitors of differentiation (ID) are responsible for integrin repression in melanoma (16). We ruled out the potential contribution of ID2-4 to our phenotype, since their expression was not upregulated upon induction of the co-activator (Supplementary Fig. 3A). Then, we applied promoter enrichment analysis (25) to the list of Pgc1α-repressed genes. Strikingly, the results revealed a significant enrichment in MYC within the promoters of the down-regulated genes (p-value = 8.5e-19; Fig. 3A; Supplementary Table S3 and S8). We studied the impact of PGC1α on the expression of MYC and observed that induction of the co-regulator elicited a consistent decrease in MYC expression in PCa cells in a doxycycline-independent manner (Fig. 3B, Supplementary Fig. 3B, C). Importantly, the effect was fully recapitulated at the transcriptional level. In addition, the analysis of previously reported targets or genes contained in the promoter analysis confirmed the reduction in MYC-dependent transcriptional program in the aforementioned conditions (Fig. 3C). We took advantage of our Pgc1α-inducible xenograft analysis to further demonstrate that the reduction in MYC expression and function was not an artifact of in vitro assays (Fig. 3D, E; Supplementary Fig. 3D). These results suggest that MYC repression is upstream of the molecular and cellular alterations elicited by PGC1α associated to PCa invasion. We validated this notion by two different means. On the one hand, a time course experiment upon PGC1α induction showed that MYC repression is prior to the reduction of its targets and integrin gene expression (Fig. 3F, Supplementary Fig. 3E-G). On the other hand, MYC silencing with a validated shRNA (33,34) recapitulated the phenotype of Pgc1a expression in cell area, p-MLC2 and invasive growth (Supplementary Fig. 3H-L).
The rapid repression in MYC mRNA levels prompted us to evaluate whether PGC1α could exert a direct action on MYC promoter. To this end, we performed chromatin immunoprecipitation (ChIP) analysis in Pgc1α-inducible PC3 cells with anti-HA antibody in order to immunoprecipitate ectopic tagged Pgc1α. The ChIP analysis confirmed that the co-regulator is bound to MYC promoter (Fig. 3G), thus suggesting that PGC1α represses MYC expression in PCa. We next sought to ascertain whether the unprecedented regulation of MYC by PGC1α in PCa could be recapitulated in human specimens. We interrogated 5 PCa datasets (25,35-37) and, in agreement with our molecular and mechanistic data, PGC1A expression was inversely correlated with MYC mRNA levels in primary tumors from the majority (4 out of 5) of datasets analyzed (Fig. 3H and Supplementary Fig. 3M).

Our previous studies demonstrated that the anti-proliferative activity of PGC1α in PCa is dependent on its interaction with ERRα (17). In order to ascertain the requirement of ERRα for the anti-invasive activity of PGC1α, we engineered Pgc1α-inducible PCa cells in which ESRRα was deleted using CRISPR/Cas9. ERRα expression was undetectable in PC3 cells in which ESRRα was deleted with two independent short guide RNAs (sgERRα#1, sgERRα#2) (Fig. 4A). ESRRα deletion abolished the induction of target genes of the transcription factor upon induction of Pgc1α, corroborating the functionality of the genetic system (Supplementary Fig. 4A). Of note, we did not recapitulate the regulation of ESRRα by PGC1A observed in vitro (Fig. 4A) in correlative human transcriptomics analyses, suggesting that more complex ERRα-regulatory cues might operate in human disease (Supplementary Fig. 4B). In line with our previous study (17), ESRRα deletion hampered the growth suppressive activity of Pgc1α, rendering PC3 cells insensitive to the action of the co-regulator (Fig. 4B). Strikingly, ESRRα deletion also abolished the effect of Pgc1α on invasive properties and cell morphology at time points
prior to the reduction in cell proliferation, thus demonstrating that the regulation of invasion
by the co-regulator is exquisitely dependent upon its interaction with ERRα (Fig. 4C, D
and Supplementary Fig. 4C, D). The morphological changes and growth suppressive
phenotype elicited by Pgc1α were also absent in tumors in which ESRRα was deleted
(Fig. 4E, Supplementary Fig. 4E, F, G). It is worth noting that despite the requirement of
ERRα for the tumor suppressive activity of PGC1α, deletion of the nuclear receptor alone
negatively influenced the establishment of tumors, suggesting that additional functions of
ERRα may be required for the first stages of tumor establishment (Supplementary Fig. 4H).

We next extended our analysis of ERRα dependency to the reported molecular alterations.
Our results showed that ESRRα deletion abrogated the reduction in protein and/or mRNA
levels of MYC, MYC targets, integrins, CAV1 as well as the reduced phosphorylation of
Src and Cofilin (Fig. 5A, B; Supplementary Fig. 5A, B). Moreover, ESRRα-ablated
tumors exhibited unperturbed MYC, integrin and CAV1 expression, as well as unchanged
Src and Cofilin phosphorylation upon Pgc1α expression (Fig. 5C, D; Supplementary Fig. 5C).
All these data are in line with the association of ERRα to MYC promoter in Pgc1α-
expressing PC3 cells (Supplementary Fig. 5D).

Since we have observed a robust inverse correlation between PGC1A and MYC
expression in various PCa datasets, we asked whether the dependency on ERRα could be
recapitulated in this setting. To this end, we carried out two independent approaches in
PCa patient datasets. On the one hand, we inferred ERRα canonical activity based on the
equilibrium between its main co-activators (PGC1A) and co-repressors (NRIP1). We
calculated the ratio of abundance of PGC1A and NRIP1 transcript (PGC1A/NRIP1), which
provided an estimation of ERRα canonical activity towards its targets, as confirmed
through the analysis of ACACB and LAMB2 expression (Supplementary Fig. 6A). In line
with our mechanistic analysis, ERRα activity, but nor ERRα itself was consistently and inversely correlated with MYC in various PCa datasets (Supplementary Fig. 6B, C). On the other hand, we took advantage from a prognostic PGC1α-ERRα signature that we generated previously (17). This signature was composed of 10 genes that were i) regulated by PGC1α in vitro, ii) predicted to be ERRα targets and iii) correlated with PGC1A in PCa datasets. In full support of our data, this PGC1α-ERRα activity signature was inversely correlated with MYC expression in various PCa patient datasets (Fig. 5E; Supplementary Fig. 6D).

Overall, our results provide solid evidence of the anti-invasive activity of the PGC1α-ERRα transcriptional axis in PCa.
Discussion

Metabolic deregulation is a hallmark of cancer (2), and encompasses a variety of biochemical routes, which must be coordinated in order to result in a phenotypic change. We postulated in the past that this strict requirement for coordination could unveil novel cancer genes. By focusing on transcriptional co-regulators that control the expression of an ample set of metabolic genes, we discovered the predominant perturbation of PGC1α in PCa (7,17). This metabolic regulator orchestrates the activation of catabolic and antioxidant pathways, at the expense of anabolism (8). Interestingly, the contribution of PGC1α to cancer biology is complex. Elegant studies have reported a role of this co-regulator: i) promoting aggressiveness of breast, pancreatic, gastric tumors, cholangiocarcinoma, glioma and melanoma (10-14,38-40), and ii) suppressing cancer aggressiveness in prostate, kidney tumors and melanoma (9,15-18). Moreover, the expression of this co-regulator is associated to the efficacy of anticancer therapies (10,11,14,15,41,42).

PGC1α exhibits a tumor type-dependent activity, ranging from tumor suppressor to advantageous for cancer cells (7). This co-activator is required for the activity of pancreatic cancer stem cells (13) and for the survival of breast cancer cells in circulation (12). In melanoma, the metabolic activity of PGC1α promotes cell proliferation, whereas the non-metabolic function opposes metastatic dissemination (10,11,16). This study together with reports by us and others demonstrate that PGC1α suppresses proliferation and invasion in PCa through presumably distinct molecular pathways emanating from the regulation of ERRα, consistent with its tumor and metastasis suppressive function (17,18) (Fig. 6). Our results mirror the anti-invasive activity of the co-regulator in melanoma, whereas proliferation is regulated in opposite sense in both tumor types. This apparent discrepancy
could be associated to the tissue-specific molecular cues that drive these tumors or the distinct nutrient and metabolic pathways that sustain their growth.

Cancer cell proliferation imposes tremendous pressure to meet the bioenergetics demands and to generate sufficient biomolecules to build new cells. We now possess a more comprehensive view of the metabolic deregulations that sustain or accompany cancer cell proliferation (43). However, beyond the relevance of cell proliferation in cancer, tumor cells need to acquire additional capacities that accounts for the clinical progression of the disease. The process of metastasis is the main cause of mortality in cancer, and only partly depends on cell proliferation, as it requires angiogenesis, intravasation, survival in circulation, extravasation and resuming cell growth in a distant organ (44). Our perspective around the contribution of metabolic regulators to the acquisition of these features is limited. An exciting possibility stems from the notion that factors that control metabolic programs would also regulate molecular cues associated to cancer cell dissemination.

Little is known about the activities of PGC1α in cancer beyond proliferation. This co-regulator inhibits dissemination in melanoma through the regulation of ID2-TCF4-Integrins (16). In gastric cancer, a recent report suggests that PGC1α upregulation supports metastasis though the regulation of SNAI1 (38). Interestingly, none of these effects are ascribed to the regulation of its main transcriptional partner, ERRα. Instead, we demonstrate that the PGC1α-ERRα transcriptional axis in PCa accounts for the invasive phenotype. We demonstrate that PGC1α/ERRα status influences signaling pathways that are important for the regulation of cytoskeletal remodeling. In turn, changes in pathways related to integrin and ROCK signaling provide a feasible explanation for the anti-invasive effects of the co-regulator. Interestingly, the set of genes inhibited in PGC1α-expressing cells that relate to cytoskeletal remodeling are enriched in MYC promoter binding sites.
This data is consistent with the notion that PGC1α/ERRα represses MYC expression and that silencing of this transcription factor partly phenocopies the effect of PGC1α (18).

Similar to PGC1α, ERRα has opposing effects in different tumor types (7). Interestingly, we show that this nuclear receptor is required for the tumor suppressive activity of PGC1α, whereas its deletion delays tumor onset in immunocompromised mice independently of the induction of PGC1α. Our results could be explained by the differential requirement of basal ERRα activity for the establishment of tumors (homing and the initial engagement of cell proliferation in vivo) vs. the proliferation and invasion in later stages. Similar results were reported for LKB1, which is required for the bypass of anoikis and the survival of tumor cells in conditions of energetic stress, despite its tumor suppressive nature in established tumors (45,46).

ERRα functions predominantly as a transcriptional activator, and is rarely reported to repress the expression of target genes (47). However, recent studies demonstrate that a subset of the genes identified by ERRα ChIP-SEQ are repressed by the nuclear receptor (48). In this sense, our results demonstrating that PGC1α/ERRα inhibits the expression of MYC broadens the spectrum of repressed genes by the protein complex. Interestingly, work by the group of Dr. Frederic Bost reports that PGC1α regulates an alternative branch of metabolism (polyamine biosynthesis) through the ERRα-dependent repression of MYC-ODC1 (18), thus opening new molecular avenues connecting this co-activator to metabolic pathways that coordinate proliferation and invasion.

In summary, our study together with recent reports (18) demonstrates that PGC1α/ERRα coordinately control proliferative and invasive features in PCa, thus providing a feasible explanation for its robust clinical association to biochemical recurrence and metastasis.
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**Figure legends**

**Figure 1.** PGC1α expression impacts on invasive properties of PCa in vitro and in vivo. A-B, Effect of Pgc1α expression on transwell migration (n=9 independent experiments) (A) and on transwell invasion (n=4 independent experiments) (B) of PC3 cells. C-D, Effect of Pgc1α expression on 3D invasion (C, n=3 independent experiments) and invasive growth (D, n=3 independent experiments) of PC3 cells. D, the right panel shows one representative experiment of invasive growth and the left panel the quantification. E-F, Quantification of changes in cell area (E) and stress fibers (F) content upon Pgc1α-expression in PC3 cells in vitro (n=3 independent experiments). In F, left panels show representative phalloidin staining of non-expressing (No Dox) and Pgc1α –expressing PC3 cells.
cells and right panel shows the quantification. G, Quantification of changes in cell area upon Pgc1α-expression in PC3 cells in vivo. Left images show representative Hematoxilin&Eosin staining of non-expressing and Pgc1α-expressing xenograft samples (n=4 tumors each condition, No Dox and Dox). Yellow line underlines cell surface. The right panel shows the quantification of number of cells per field. Dox: doxycycline. Dox: Pgc1α induced conditions; No Dox: Pgc1α non-expressing conditions. In A, B, C, D and F data is represented as fold change relative to No Dox condition depicted by a dotted line. Error bars represent the standard error mean (s.e.m). Statistic tests: one sample t-test with a hypothetical value of 1 (A, B, C, D, F), two-tailed Student T test (E) and one-tailed Mann-Whitney U test (G). p, p-value. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 2.** PGC1α expression modulates integrin signalling of PCa in vitro and in vivo. A, Metacore enrichment analysis of the transcriptional program downregulated regulated by PGC1α in PC3 cells. B, Effect of Pgc1α expression on the phosphorylation of Myosin-light chain (MLC) protein in PC3 cells. Left panels show representative images of immunofluorescence staining using p-MLC antibodies. Right panel shows quantification of p-MLC per cell area (n=3 independent experiments). C-D, Representative WB of the effect of Pgc1α on Cofilin and Src phosphorylation in PC3 cells (C) and xenograft samples (D). E-F, Representative WB of the effect of Pgc1α on ITGβ1, ITGβ4, ITGα3 and CAV1 in PC3 cells (E, n=3; independent experiments) and xenograft samples (F, n=4-5 tumors). Dox: doxycycline. Dox: Pgc1α induced conditions; No Dox: Pgc1α non-expressing conditions. Error bars and ± represent the standard error mean (s.e.m). Statistic tests: two-tailed Student T test (B). *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 3.** PGC1α regulates c-Myc expression in prostate cancer. A, Promoter enrichment analysis of the PGC1α transcriptional program in PC3 cells. B, Effect of Pgc1α expression...
on c-Myc protein levels in PC3 cells (n=3 independent experiments). C, Quantification of MYC gene expression and its target genes ODC, FASN, CAD1 and TCF4 by qRTPCR upon Pgc1α expression in PC3 cells (n=4 independent experiments). Data is represented as fold change relative to No Dox, depicted as a dotted line. D, Effect of Pgc1α expression on c-Myc protein levels in xenograft samples (n=5 No Dox tumors, n=4 Dox tumors). E, Quantification of MYC gene expression (and its target genes) by qRTPCR in xenograft samples cells (n=5 No dox tumors, n=4 Dox tumors). F, qRTPCR gene expression analysis of MYC, TCF4, ITGB4, ITGB1 and ITGA3 upon short acute induction of Pgc1α expression (1, 2, 4 and 8h of doxycycline treatment) in PC3 cells. Data is represented as fold change relative to No dox, depicted as a dotted line. G, Chromatin immunoprecipitation (ChIP) of exogenous Pgc1α on MYC promoter in PC3 Pgc1α cells after induction with 0.5 μg mL-1 doxycycline for 16 hours (n=5). Final data was normalized to IgG (negative-immunoprecipitation control) and to No dox condition. H, Correlation analysis between PGC1A and MYC expression in primary tumor specimens of different PCa datasets. Sample sizes: Grasso n=45; Lapointe n=13; Taylor n=131 and TCGA provisional n=495. Dox: doxycycline. Dox: Pgc1α induced conditions; No dox: Pgc1α non-expressing conditions. Error bars and ± represent the standard error mean (s.e.m). Statistic tests: one sample t-test with a hypothetical value of 1 (C, F), one-tailed Student T test (G), one-tailed Mann-Whitney U test (E), Spearman correlation R (H). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. ERRα deletion mediates the effect of Pgc1α on invasive properties and morphology of prostate cancer in vitro and in vivo. A, Representative experiment of ERRα expression in PC3 Pgc1α cells after treatment with 0.5 µg/ml doxycycline (Dox) (n=3; independent experiments). B, Relative cell number quantification upon ERRα deletion.
(sgERRα#1 and sgERRα#2) in PC3 Pgc1α expressing and non-expressing cells. Data is represented as cell number at day 6 relative to day 0 (n=3, independent experiments). C, Effect of ERRα deletion in invasive growth upon Pgc1α expression (n=3 independent experiments). One representative spheroid image of each condition is shown out of 3 biological replicates. D, Quantification of cell area by phalloidin staining after ERRα deletion alone or in combination with Pgc1α expression (n=4 independent experiments) in PC3 cells. E, Effect of ERRα deletion alone or in combination of Pgc1α on the cell content and size in xenograft samples (n=5 per condition). The number of cells per field is an approximate representation of cell area. Dox: doxycycline. sg: short guide RNA. Dox: Pgc1α induced conditions; No Dox: Pgc1α non-expressing conditions. Error bars represent the standard error mean (s.e.m). Dotted line represents No Dox condition. Statistic tests: paired Student t-test between Control -Dox and +Dox conditions (B) unpaired Student t-test between +Dox control and sg conditions (B), one sample t-test with a hypothetical value of 1 (C, D) and one-tailed Mann-Whitney U test (E). p, p-value. */$ p < 0.05, **/$$ p < 0.01, $$$ p < 0.001. In B, C and D asterisks indicate statistical difference between No Dox and Dox conditions and dollar symbol between Control Dox and sgERRα#1/sgERRα#2 Dox.

Figure 5. ERRα mediates the effect of Pgc1α on integrin signalling and MYC expression in vitro and in vivo. A, Representative WB of the effect of ERRα deletion alone or in combination with Pgc1α expression on ITGβ1, ITGβ4, CAV1 and MYC protein expression as well as on Cofilin and Src phosphorylation in PC3 cells (n=3; independent experiments). B, Effect of ERRα deletion alone or in combination with Pgc1α expression in the gene expression (qRTPCR) of MYC, TCF4, ITGB1, ITGA3 and CAV1 (n=4 independent experiments) in PC3 cells. Data is represented by fold change relative to Control No Dox condition that is depicted by a dotted line. C, Effect of ERRα deletion alone or in
combination with Pgc1α expression on ITGβ1, ITGβ4, CAV1, and MYC protein expression as well as on Cofilin and Src phosphorylation in xenograft samples (Control No Dox n=9 tumors, Control +Dox n=9 tumors; sgERRα#1 –Dox n=8 tumors; sgERRα#2 +Dox n=8 tumors). D, Effect of ERRα deletion alone or in combination with Pgc1α expression MYC, TCF4, ITGB1, ITGA3 and CAV1 gene expression analysed by qRTPCR in xenograft samples. (Control No Dox n=4-9 tumors, Control +Dox n=4-9 tumors; sgERRα#1 No Dox n=6-8 tumors; sgERRα#2 +Dox n=5-6 tumors). E, Correlation analysis between MYC and the PGC1α-ERRα transcriptional signature in primary tumor specimens of different PCa datasets. Each dot correspond to a patient. Sample sizes: Grasso n=45; Lapointe n=13; Glinsky n=78 and TCGA provisional n=495. Dox: doxycycline. Dox: Pgc1α induced conditions; No dox: Pgc1α non-expressing conditions. Error bars and ± represent the standard error mean (s.e.m). Statistic tests: one sample t-test (B), unpaired t-test (B, D), Spearman correlation R (E). */p < 0.05, **/$p < 0.01, ***/$$$p < 0.001. Asterisks indicate statistical difference between Control No Dox and the rest of the conditions and dollar symbol between Control Dox and sgERRα#1/ sgERRα#2 Dox.

Figure 6. Schematic summary of the main findings.
Figure 1. Valcarcel et al.

A. Transwell migration PC3

B. Transwell invasion PC3

C. 3D invasion PC3

D. Invasive growth PC3

E. Cell area PC3

F. Stress fibers PC3

G. Number of cells per field (x20)
Figure 2. Valcarcel et al.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3. Valcarcel et al.

A: Promoter analysis

| Promoter                        | Log2(p-val) |
|---------------------------------|-------------|
| GGGCGGR_VSPAX4_03               |             |
| CACGTG_VSMYC_Q2                 |             |
| TGGAAA_VSNFAT_Q4_01             |             |
| CAGGQT_VSE12_06                 |             |
| AACTTTT_UNKNOWN                 |             |
| SCGGAAGY_VSELK1_02              |             |
| CTTTGT_VSLEF1_Q2                |             |
| GGGGARR_VSMAZ_Q6                |             |
| GGCGGGR_VSFP1_06                |             |

B: Gene expression

Dox: +

80KDa PGC1α
70 KDa MYC
35KDa GAPDH

No dox: -

80KDa PGC1α
70 KDa MYC
35KDa GAPDH

C: Gene expression (Fold change relative to No dox)

D: Gene expression

Dox: +

80KDa PGC1α
70 KDa MYC
35KDa GAPDH

No dox: -

80KDa PGC1α
70 KDa MYC
35KDa GAPDH

E: Gene expression (Fold change relative to No dox)

F: Gene expression

| Time (h) | MYC | ODC | FASN | CAD1 | TCF4 |
|----------|-----|-----|------|------|------|
| 0        | 1.0 | 0.5 | 1.0  | 0.5  | 1.0  |
| 1        | 0.5 | 0.2 | 0.2  | 0.2  | 0.2  |
| 2        | 0.2 | 0.1 | 0.1  | 0.1  | 0.1  |
| 3        | 0.1 | 0.05| 0.05 | 0.05 | 0.05 |
| 4        | 0.05| 0.02| 0.02 | 0.02 | 0.02 |
| 5        | 0.02| 0.01| 0.01 | 0.01 | 0.01 |
| 6        | 0.01| 0.005| 0.005| 0.005| 0.005|
| 7        | 0.005| 0.001| 0.001| 0.001| 0.001|
| 8        | 0.001| 0.0005| 0.0005| 0.0005| 0.0005|

G: Fold enrichment relative to Dox

| Time (h) | Log2(p-val) |
|----------|-------------|
| 0        | -1.0        |
| 1        | -0.5        |
| 2        | 0.0         |
| 3        | 0.5         |
| 4        | 1.0         |

H: Correlation analysis

Grasso:
R = -0.3764 p = 0.03371

Lapointe:
R = -0.3295 p = 0.2717

Taylor:
R = -0.2683 p = 0.00203

TCGA:
R = -0.2214 p = 6.5e-7
Figure 4. Valcarcel et al.

(A) Western blot analysis showing the expression levels of ERRα and PGC1α at different molecular weights.

(B) Fold change relative to Day 0.

(C) Invasive growth analysis showing the fold change relative to each No Dox condition.

(D) Cell area analysis showing the fold change relative to each No Dox condition.

(E) Number of cells per field (x20) analysis showing the number of cells under different conditions.

Invasive growth

Control +Dox  sgERRα#1 +Dox  sgERRα#2 +Dox

Fold change relative to each No Dox

Control No Dox  sgERRα#1 No Dox  sgERRα#2 No Dox

Control +Dox  sgERRα#1 +Dox  sgERRα#2 +Dox

Fold change relative to each No Dox

Cell area

Fold change relative to each No Dox

Control +Dox  sgERRα#1 +Dox  sgERRα#2 +Dox

Number of cells per field (x20)

Fold change relative to each No Dox

Control No Dox  sgERRα#1 No Dox  sgERRα#2 No Dox

Control +Dox  sgERRα#1 +Dox  sgERRα#2 +Dox

Control +Dox  sgERRα#1 +Dox  sgERRα#2 +Dox

p=0.059

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Figure 5. Valcarcel et al.

A

| Cont | sgERRα#1 | sgERRα#2 |
|------|----------|----------|
| 80 KDa | ↑ | + | + |
| 45 KDa | ↑ | + | - |
| 160 KDa | + | + | + |
| 140 KDa | + | + | + |
| 17 KDa | + | + | + |
| 70 KDa | + | + | + |
| 75 KDa | + | + | + |
| 35 KDa | - | - | - |

- PGC1α
- ERRα
- ITGβ4
- ITGβ1
- CAV1
- MYC
- p-Cofilin
- GAPDH
- β-actin

B

| MYC | TCF4 |
|-----|------|
| gene expression (Fold change) | gene expression (Fold change) |
| +Dox | +Dox |
| **| **|

- Grasso
- Lapointe

C

| Control | sgERRα#1 |
|---------|----------|
| 80 KDa | + Dox | + Dox |
| 45 KDa | + Dox | + Dox |
| 160 KDa | + Dox | + Dox |
| 140 KDa | + Dox | + Dox |
| 17 KDa | + Dox | + Dox |
| 70 KDa | + Dox | + Dox |
| 75 KDa | + Dox | + Dox |
| 35 KDa | + Dox | + Dox |

- PGC1α
- ERRα
- ITGβ1
- CAV1
- MYC
- p-Cofilin
- GAPDH
- β-actin

D

| MYC | TCF4 |
|-----|------|
| gene expression (Fold change) | gene expression (Fold change) |
| - Dox | + Dox |
| **| **|

E

| Grasso | Lapointe |
|--------|----------|
| R = -0.4883 p = 0.0043 | R = -0.4011 p = 0.175 |
| MYC | TCGA |

- Grasso
- Lapointe

Grasso
Lapointe

R = -0.2464 p = 3e-8
R = -0.2759 p = 0.014

- TCF4
- MYC
- p-Src
- total-Src
- ITGβ4
- CAV1
- MYC
- p-Src
- total-Src
- β-actin

** | **

- control No Dox
- control + Dox
- sgERRα#1 No Dox
- sgERRα#1 + Dox
- sgERRα#2 No Dox
- sgERRα#2 + Dox

- TCF4
- ITGB1
- ITGA3
PGC1α / ERRα

Metastasis

Catabolism

Anabolism

MYC expression
Integrin signaling
Polyamine synthesis
Contractility

• This study
• Torrano et al., Nature Cell Biol. 2016
• Kaminski et al., Cancer Research 2019
PGC1α suppresses prostate cancer cell invasion through ERRα transcriptional control

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