

# ERR $\alpha$ Maintains Mitochondrial Oxidative Metabolism and Constitutes an Actionable Target in PGC1 $\alpha$ -Elevated Melanomas

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## Abstract

The uncontrolled growth of tumors provides metabolic dependencies that can be harnessed for therapeutic benefit. Although tumor cells exhibit these increased metabolic demands due to their rapid proliferation, these metabolic processes are general to all cells, and furthermore, targeted therapeutic intervention can provoke compensatory adaptation that alters tumors' characteristics. As an example, a subset of melanomas depends on the transcriptional coactivator PGC1 $\alpha$  function to sustain their mitochondrial energy-dependent survival. However, selective outgrowth of resistant PGC1 $\alpha$ -independent tumor cells becomes endowed with an augmented metastatic phenotype. To find PGC1 $\alpha$ -dependent components that would not affect metastasis in melanomas, an unbiased proteomic analyses was performed and uncovered the orphan nuclear receptor ERR $\alpha$ , which supports PGC1 $\alpha$ 's control of

mitochondrial energetic metabolism, but does not affect the antioxidant nor antimetastatic regulatory roles. Specifically, genetic or pharmacologic inhibition of ERR $\alpha$  reduces the inherent bioenergetic capacity and decreases melanoma cell growth, but without altering the invasive characteristics. Thus, within this particularly aggressive subset of melanomas, which is characterized by heightened expression of PGC1 $\alpha$ , ERR $\alpha$  specifically mediates prosurvival functions and represents a tangible therapeutic target.

**Implications:** ERR $\alpha$ , a druggable protein, mediates the bioenergetic effects in melanomas defined by high PGC1 $\alpha$  expression, suggesting a rational means for therapeutic targeting of this particularly aggressive melanoma subtype. *Mol Cancer Res*; 15(10): 1366–75. ©2017 AACR.

## Introduction

Tumor cells generally use aerobic glycolysis (Warburg effect) to fuel their growth, yet many also rely on mitochondrial oxidative phosphorylation to support their anabolic needs (1). To this end, we recently identified that a significant fraction of human melanomas (8%–10%) has heightened expression levels of PGC1 $\alpha$  (gene name: *PPARGC1A*), a transcriptional coactivator that promotes mitochondrial biogenesis and respiration, wherein genetic suppression leads to decreased energetic capacity, impaired antioxidant capacity, and subsequent apoptosis (2, 3). Rare melanoma cells that survive chronic depletion of PGC1 $\alpha$  shift their metabolism toward increased aerobic glycolysis (4), and in addition,

adapt invasive and metastatic traits, which is driven by transcriptional upregulation of integrin, TGF $\beta$  and WNT signaling components (5). PGC1 $\alpha$  consequently has opposing functions in melanoma pathogenesis, favoring tumor growth and survival by promoting oxidative metabolism, but also suppressing a transcriptional network that controls invasion and metastatic spread.

Although potentially amenable for therapeutic inhibition, the functional ramifications of inhibiting PGC1 $\alpha$  in melanoma may consequently prove counterproductive. However, PGC1 $\alpha$  is a transcriptional coactivator that is recruited to promoters of genes by certain transcription factors involved in cellular metabolic functions (6). Because our recent data indicated that PGC1 $\alpha$  promotes mitochondrial-dependent survival distinct from the mechanism through which PGC1 $\alpha$  suppresses metastasis (5), it is likely that PGC1 $\alpha$  employs alternate transcriptional cues to regulate these divergent melanoma growth phenotypes. To this end, successful identification of transcription factors that controls each of growth and metastasis may inform selective targets for therapeutic pursuit of PGC1 $\alpha$ -positive melanomas.

The estrogen-related orphan nuclear receptors (ERR $\alpha$ ,  $\beta$ , and  $\gamma$ ) are known PGC1 $\alpha$ -interacting proteins that mediate mitochondrial biogenesis in oxidative tissues (7–9). In addition to participating in normal metabolism, ERRs also play important functions in various malignancies (1, 10), wherein heightened ERR $\alpha$  has been associated with worse prognosis in certain carcinomas, including breast, ovarian, uterine, prostate, and colorectal tumors (10). The ERR $\alpha$ /PGC1 $\alpha$  complex has been mechanistically demonstrated to promote glutamine into *de novo* fatty acid

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biosynthesis, and thereby confer growth advantages to ERBB2-positive breast cancers (11). Increased glutamine metabolism and reactive oxygen species (ROS) detoxification by the ERR $\alpha$ /PGC1 $\alpha$  axis in breast cancers have also been found to promote metabolic adaptation following receptor tyrosine kinase inhibition using lapatinib (12). Furthermore, the ERR $\alpha$ /PGC1 $\alpha$  complex suppresses one-carbon metabolism in response to AMPK stimulation, thus rendering breast cancer cells more vulnerable to antifolate drugs, such as methotrexate (13). On the other hand, in prostate cancer, ERR $\alpha$  has been found to promote catabolic metabolism that subsequently suppresses the metastatic ability (14). In melanoma, it is however not clear to what extent ERR $\alpha$  functionally contributes to tumor growth.

As a nuclear receptor, ERR $\alpha$  is a druggable target, and multiple reverse agonists (antagonists) have been developed that effectively block its transcriptional activation both *in vitro* and *in vivo* (15). Most of these compounds were designed to block the interaction between ERR $\alpha$  and PGC1 $\alpha$ , such as XCT790, compound (Cpd) A and 29, and have been shown to exhibit growth-inhibitory therapeutic effects in certain cancers (16–18). For example, in combination with PI3K inhibition, Cpd29 significantly inhibits the progression of breast cancer in nude mice (18). Cpd29 has also been shown to suppress ERR $\alpha$ -mediated metabolic reprogramming and to overcome drug resistance in a mammary tumor model (12). Hence, ERR $\alpha$  antagonist has shown preclinical activity in certain tumor models, but their potential efficacy has not been characterized in melanoma, with particular emphasis on PGC1 $\alpha$ -positive melanomas that are highly dependent on oxidative metabolism.

In the current study, we have employed a proteomic approach in human melanoma cells to identify functional PGC1 $\alpha$  protein complexes. We characterized ERR $\alpha$  as a critical factor that mediates PGC1 $\alpha$ 's growth and survival functions. Specifically, ERR $\alpha$  physically interacts with PGC1 $\alpha$ , which complex promotes expression of genes involved in mitochondrial oxidative phosphorylation, but does not affect expression of the cellular antioxidant or invasive/metastatic programs. Like suppression of PGC1 $\alpha$ , depletion of ERR $\alpha$  impairs mitochondrial bioenergetic capacity, *in vitro* cell proliferation, and *in vivo* tumor growth in PGC1 $\alpha$ -positive melanoma cells. However, in contrast to genetic manipulation of PGC1 $\alpha$  levels (5), depletion of ERR $\alpha$  did not promote melanoma cell invasion. Strikingly, pharmacologic ERR $\alpha$  antagonism phenocopies genetic ERR $\alpha$  deletion; therefore, ERR $\alpha$  might constitute an attractive and amenable therapeutic target in a subset of PGC1 $\alpha$ -positive melanomas.

## Materials and Methods

### Tissue culture

All melanoma cell lines were obtained from ATCC, and their authentication was confirmed by either DNA fingerprinting with small tandem repeat profiling or in-house PCR testing of melanoma marker genes and BRAF mutation status. Mycoplasma contamination was tested in-house with the PCR Mycoplasma Detection Kit (Lonza). Cells were maintained in DMEM (Sigma-Aldrich) with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were cultured in humidified incubator at 37°C with 5% CO<sub>2</sub>. All cells were used for less than 10 passages upon thawing from liquid nitrogen.

### The Cancer Genome Atlas melanoma tumor analyses

Publicly available RNA sequencing (RNA-seq)-based gene expression, mutation, American Joint Committee on Cancer (AJCC) stage of sample retrieval, and patient outcome data for melanoma tumors were obtained from the TCGA portal ([tcga-data.nci.nih.gov](http://tcga-data.nci.nih.gov)). Using data from the 366 samples obtained at AJCC stage III (local metastasis) and IV (distant metastasis), within-cohort normalized expression levels were calculated for *PPARGC1A* (PGC1 $\alpha$ ) and *ESRRA* (ERR $\alpha$ ) to compare their correlative expression (based on Pearson *r*), relationship between PGC1 $\alpha$  rank-based expression and ERR $\alpha$  levels (unpaired, two-sided *t* test), and mutual (normalized average) expression rank (MER) with patient outcome (survival based on Mantel–Cox log-rank test).

### Reagents and antibodies

Compound 29 (4- $\{4-[(2,4-dioxo-1,3-thiazolidin-5-ylidene)methyl]-2-methoxyphenoxy\}$ -3-(trifluoromethyl)benzotrile, 95%) (17) was synthesized by MolPort. Oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A, and rotenone were all purchased from Sigma-Aldrich. The following antibodies were used: PGC1 $\alpha$  (H300, Santa Cruz Biotechnology; ST1202, EMD Millipore), ERR $\alpha$  (N1, GeneTex), mtCOI (Abcam), actin (Cell Signaling Technology), SOD2 (GeneTex), GPX1 (GeneTex), pFAK-Y397 (Cell Signaling Technology), COX4 (Abcam), COX5A (Abcam), UQCRC2 (Abcam), tubulin (Abcam), histone H3 (Abcam), and Ki-67 (Thermo Fisher Scientific).

### Lentiviral generation and transduction

Lentiviruses encoding shRNAs or sgRNAs (lentiCRISPRv2, Addgene #52961) were produced in HEK293T cells with packaging vectors (pMD2G and psPAX2) using Escort IV Transfection Reagent (Sigma-Aldrich; ref. 19). Lentivirus particles were collected 48 hours posttransfection and used to infect melanoma cells in the presence of 8  $\mu$ g/mL polybrene, and infected cells were selected with 2  $\mu$ g/mL of puromycin, or 7  $\mu$ g/mL blasticidin for at least 4 days prior to experiments. The shERR $\alpha$ -#1 is TRCN0000330256 and #2 is TRCN0000022181 (<http://portals.broadinstitute.org/gpp/public/>). The sgRNA sequences for ERR $\alpha$  are: #1 AGGCTCGGTCTCTGTCTCCG, #2 GACAGAGACCGAG-CCTCCTG, and #3 AGTGGGCTGGGGGCTCACCC.

### Immunoprecipitation, immunoblotting, and qPCR

For coimmunoprecipitation (co-IP), cells were first permeabilized by Swelling Buffer (25 mmol/L HEPES pH 7.6, 1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, 0.5% NP40) on ice for 15 minutes, followed by centrifugation at 2,000 rpm at 4°C for 10 minutes to collect the intact nucleus. The pelleted nucleus was then lysed in RIPA buffer without SDS and sodium deoxycholate but supplemented with 10% glycerol, followed by sonication at low power for 30 seconds and rotation at 4°C for 1 hour. Exogenously expressed Flag/HA-tagged PGC1 $\alpha$  by adenoviruses in the nuclear lysate was subsequently subjected to immunoprecipitation with beads conjugated with anti-Flag M2 antibody (Sigma) and anti-HA antibody (Sigma) at 4°C overnight, followed by wash for 3 times and elution with respective peptides. Elution was performed by 5 mg/mL Flag peptide (Sigma) or 5 mg/mL HA peptide (Sigma) in the same buffer at 37°C with gentle shaking at 100 rpm for 1 hour. Total elution was subjected to mass spec analysis. For endogenous PGC1 $\alpha$  co-IP, 5 mg nuclear lysate with 2  $\mu$ L of

PGC1 $\alpha$  antibody (ST1202) was incubated overnight at 4°C, followed by precipitation using DynaBead A/G (Invitrogen) for 2 hours at 4°C.

For immunoblotting, cells were lysed in RIPA buffer and quantified by DC Protein Concentration Assay Kit (Pierce) before subjecting to SDS-PAGE gel. Total RNA was isolated with TRIzol (Invitrogen) by Direct-zol RNA MiniPrep Kit (Zymo Research), and 2  $\mu$ g of total RNA was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was carried out using SYBR Green PCR Master Mix (Bio-Rad). Experimental  $C_t$  values were normalized to 36B4, and relative mRNA expression was calculated using the  $\Delta\Delta C_t$  method. Sequences for all primers are available upon request.

### Seahorse respirometry

Respirometry was performed using the XFe24 platform (Seahorse Bioscience). Briefly, 4 to 8  $\times 10^4$  cells with indicated genetic manipulations were seeded in the Seahorse plates and allowed to attach overnight. Cells were then washed once with Seahorse buffer (unbuffered DMEM medium without FBS). All the chemicals loaded in the Seahorse cartridge ports were diluted in Seahorse buffer, and the pH was adjusted to 7.4. Following measurement of basal respiration, 2  $\mu$ mol/L oligomycin (complex V/ATP synthase inhibitor) was used to measure ATP production; uncoupled respiration by adding 4  $\mu$ mol/L FCCP was measured to determine the maximal respiration of the cells; nonmitochondrial respiration was recorded by the addition of antimycin A (4  $\mu$ mol/L) and rotenone (2  $\mu$ mol/L). Oxygen consumption values were normalized to cell number (2, 20).

### ATP production assay

Cells were lifted by trypsin, counted, and suspended in PBS. Cells ( $1 \times 10^5$ ) in 50  $\mu$ L were used for ATP assay using CellTiter-Glo Luminescent Kit (Promega), following the manufacturer's instructions.

### In vitro migration assay

Transwell chambers were purchased from Corning Life Science. Generally, A375P ( $1 \times 10^5$ ), G361 ( $5 \times 10^4$ ), or A2058 ( $1 \times 10^4$ ) cells in 0.1 mL of FBS-free medium were seeded into the upper chamber and incubated overnight. Cells that had migrated were then fixed and stained with Crystal violet. The membrane attached with migrated cells was placed on a glass slide; total cells from three images taken from three random fields under  $\times 10$  or  $\times 20$  magnifications as indicate with a Nikon 80i Upright microscope were quantified.

### Cellular ROS measurement by DCF-DA

DCF-DA-based cellular ROS detection was performed according to the manufacturer's instructions (Abcam). Briefly, cells were lifted by trypsin and stained with 20  $\mu$ mol/L DCF-DA at 37°C for 30 minutes, followed by FACS analysis (2, 21). Cells with 1 mmol/L H<sub>2</sub>O<sub>2</sub> treatment were used as positive controls.

### Proliferation assay and clonogenic assay

Melanoma cells with indicated manipulation were seeded in either 6-well or 12-well plates at a density of  $1 \times 10^4$ /well in triplicate. Cell number was counted at indicated time points. For compound treatment experiments, the next day after seeding, cells were exposed to DMSO or indicated concentration of Cpd29, followed by cell counting. For clonogenic assay, cells were seeded

at the density of  $1 \times 10^4$ /well in 6-well plates in triplicate and cultured for 10 to 14 days, followed by fixation with 100% ethanol and staining with Crystal violet. Culture medium was refreshed every other day for all these experiments.

### Animal experiments

All animal experiments were designed and conducted following the protocol approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Eight- to 10-week-old male NCr nude mice (for G361 cell line, purchased from Taconic Biosciences) and female NCI Ath/Nu mice (for MeWo cell line, purchased from Charles River Laboratories) were used for this study. Mice were housed in a controlled environment of 12-hour dark/light cycle and provided food and water *ad libitum*. All mice from a single experiment were at the same age. Tumor cells ( $2 \times 10^6$ ) were injected subcutaneously into the flanks of mice, and the tumor development was monitored three times per week. Tumor volume was calculated on the basis of the equation  $V = [\text{width (in mm)}^2 \times \text{length (in mm)}]/2$  (22). Tumor-bearing mice were given 30 mg/kg Cpd29 (in 10% NMP/90% PEG300; ref. 18) or vehicle via oral administration six times a week.

### Histology

Tissue samples were fixed in 10% buffered formalin overnight and stored in 70% ethanol prior to paraffin embedding, sectioning, and hematoxylin/eosin (H&E) staining (by the Rodent Histopathology Core, Harvard Medical School, Boston, MA; ref. 23). IHC was performed with Ki-67 (SP6, Thermo Fisher Scientific) antibody with protocol described previously (24). Negative control was done by replacing the primary antibody with species-matched total IgG.

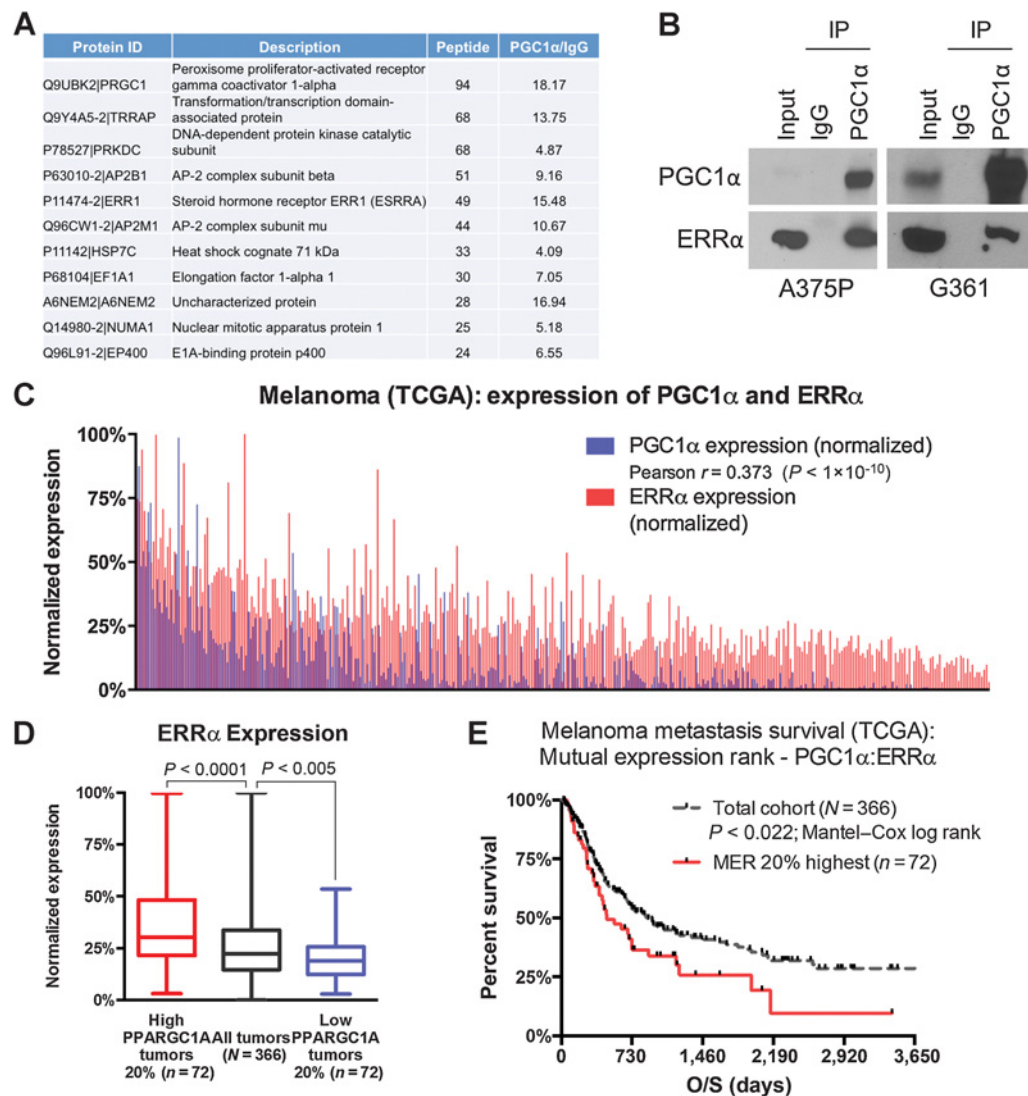
### Statistical analysis

All statistics are described in figure legends and were performed with GraphPad Prism. In general, for two experimental comparisons, a two-tailed unpaired Student *t* test was used. For multiple comparisons, one-way ANOVA was applied. When cells were used for experiments, three replicates per treatment were chosen as an initial sample size. All *n* values defined in the legends refer to biological replicates unless otherwise indicated. Statistical significance is represented by asterisks corresponding to \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001, if not otherwise indicated.

## Results

### ERR $\alpha$ is an interacting partner of PGC1 $\alpha$ in a subset of human melanoma cells

To identify proteins that could selectively mediate PGC1 $\alpha$ -dependent mitochondrial bioenergetics, survival, or metastatic suppression, we set out to analyze PGC1 $\alpha$  proteome complexes in melanoma cells. To accommodate this goal, we ectopically expressed double-FLAG/HA-tagged PGC1 $\alpha$  in the high PGC1 $\alpha$ -expressing melanoma cell line A375P and performed mass spectrometric analyses of associated proteins after sequential immunoprecipitation (Supplementary Fig. S1). Among the top 10 most abundant peptides that were in close stoichiometry with PGC1 $\alpha$ , we identified the estrogen-related receptor  $\alpha$  (ERR $\alpha$ ; gene name *ESRRA*) as the only transcription factor (Fig. 1A). To validate the presence of PGC1 $\alpha$ -ERR $\alpha$  complexes in cells, we confirmed the endogenous interaction between these two proteins using

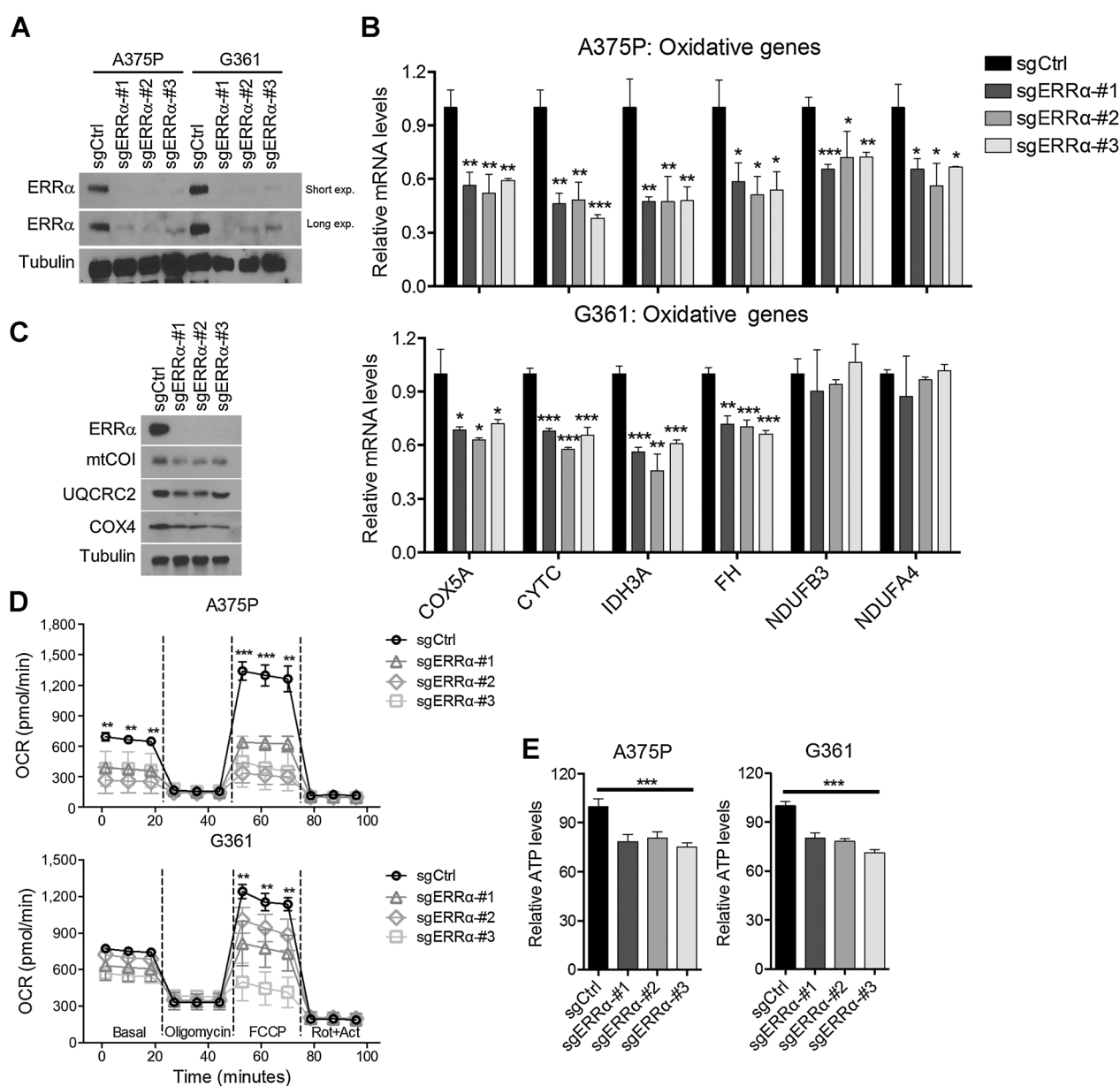
**Figure 1.**

ERR $\alpha$  is associated with PGC1 $\alpha$  in melanoma cells. **A**, A list of the most abundant proteins coimmunoprecipitated with Flag/HA-tagged PGC1 $\alpha$  in A375P melanoma cells as identified by mass spectrometry. **B**, Endogenous PGC1 $\alpha$  is interacting with ERR $\alpha$  in PGC1 $\alpha$ -positive melanoma cell lines A375P and G361. Immunoprecipitation of PGC1 $\alpha$  was followed by immunoblotting using the indicated antibodies. **C**, Pearson-based correlation between PGC1 $\alpha$  and ERR $\alpha$  expression levels across metastatic melanoma samples within TCGA ( $N = 366$ ). **D**, On the basis of two-sample, two-sided  $t$  test statistics, the 20 percentile highest and lowest PGC1 $\alpha$  expression levels associate with highest and lowest ERR $\alpha$  expression levels, respectively. **E**, On the basis of Mantel-Cox log-rank test, the top 20 percentile mutual expression rank (MER) of PGC1 $\alpha$ :ERR $\alpha$  segregates poorer overall survival relative to the metastatic melanoma cohort average ( $P < 0.022$ ).

immunoprecipitation analysis from different PGC1 $\alpha$ -positive melanoma cell extracts (Fig. 1B).

Because of the tight physical interaction between these two proteins, coupled to our recent data indicating that higher PGC1 $\alpha$  expression associates with poor metastatic melanoma survival (2, 3), we examined whether the intersection of high ERR $\alpha$  and PGC1 $\alpha$  levels correlates across melanoma tumors. Using RNA-seq data from the 366 metastatic melanoma lesions within The Cancer Genome Atlas (TCGA), we found a significant association between PGC1 $\alpha$  and ERR $\alpha$  expression levels (Fig. 1C: Pearson  $r = 0.373$ ;  $P < 1 \times 10^{-10}$ ). In addition, each of the highest and lowest 20 percentile PGC1 $\alpha$ -expressing metastatic tumors have higher

versus lower ERR $\alpha$  expression compared with the overall average, respectively (Fig. 1D). Consistent with PGC1 $\alpha$  levels as a means to stratify metastatic disease survival, there is a strong correlation between poor overall survival and the 20 percentile highest PGC1 $\alpha$  and ERR $\alpha$  mutual expression rank, as compared with the cohort average, indicating an improved precision to stratify these aggressive tumors (Fig. 1E;  $P < 0.022$  using Mantel-Cox log-rank test). Together, these biochemical and clinical correlative data suggest that the association between PGC1 $\alpha$  and ERR $\alpha$  may be heightened functional importance, and therefore, may inform therapeutic opportunities in this particularly aggressive subclass of melanoma tumors.

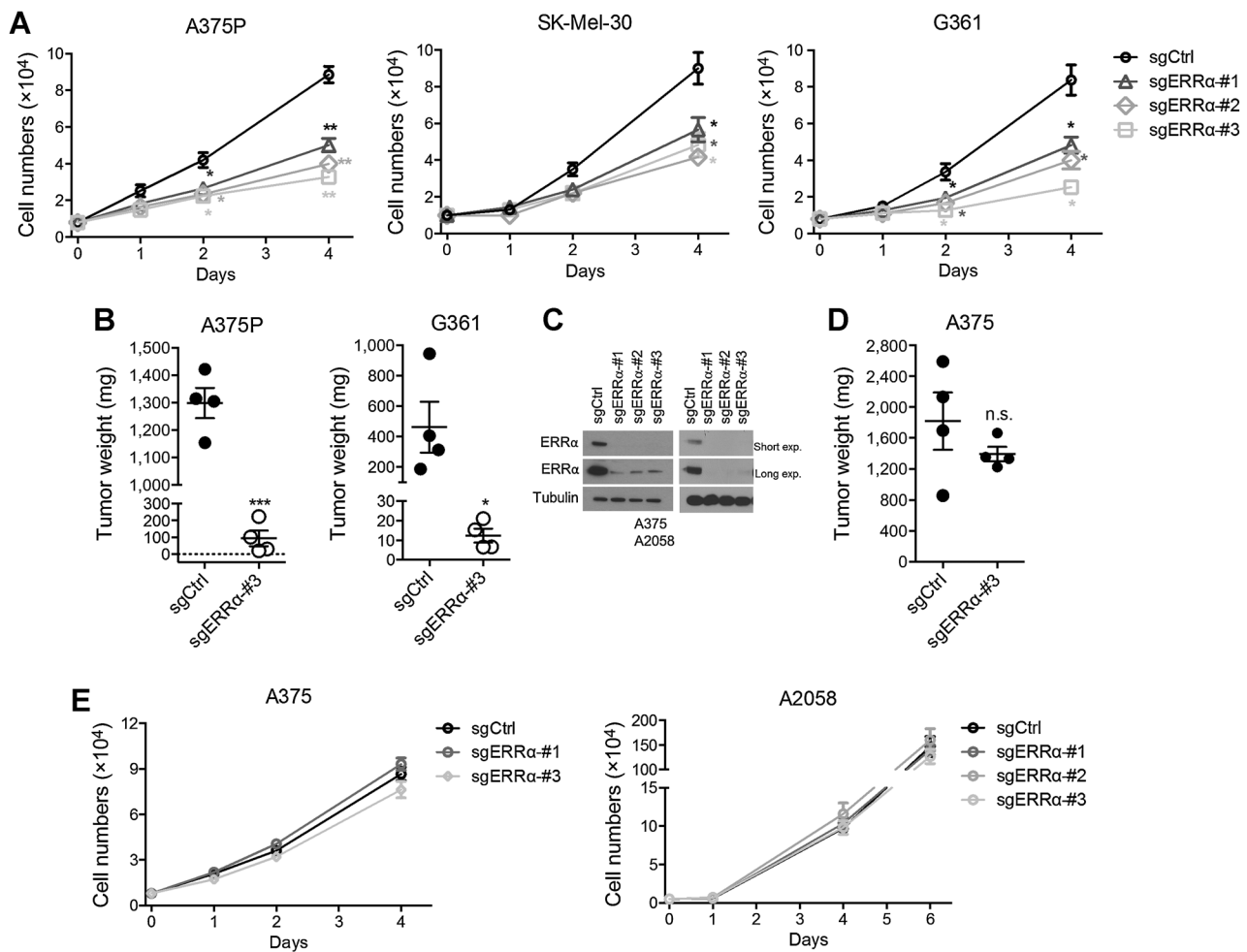
**Figure 2.**

ERR $\alpha$  is required to support mitochondrial oxidative metabolism. **A**, Immunoblotting of melanoma cells upon ERR $\alpha$  depletion by CRISPR. sgCtrl, small guide control vector; sgERR $\alpha$ , small guide RNA for ERR $\alpha$ . **B**, Expression of mitochondria-related genes at the mRNA level in melanoma cells upon ERR $\alpha$  depletion. **C**, Immunoblotting of mitochondria-related genes in ERR $\alpha$ -depleted melanoma cells. **D**, Mitochondrial activity of melanoma cells with ERR $\alpha$  depletion presented as oxygen consumption rate (OCR) measured by Seahorse flux assay. **E**, Intracellular ATP levels in cells with ERR $\alpha$  depletion. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  as determined by  $t$  test (**B**) or one-way ANOVA (**D** and **E**).

### ERR $\alpha$ promotes mitochondrial oxidative metabolism in melanoma cells

ERR $\alpha$  has been shown to promote mitochondrial biogenesis through promoting transcription of genes involved in oxidative phosphorylation and other metabolic pathways (9). Because of the stoichiometric interaction between ERR $\alpha$  and PGC1 $\alpha$  in melanoma cells, we analyzed the transcriptional gene expression programs specifically controlled by this complex. Using targeted CRISPR/Cas9 genome editing, we therefore manipulated ERR $\alpha$

expression in a cohort of melanoma cells with heightened PGC1 $\alpha$  levels, including A375P, G361, and SK-MEL-30. Using this approach, we found that depletion of ERR $\alpha$  significantly decreased the expression of mitochondrial genes, including components of the electron transport chain (COX5A, COX4, CYTC, mtCO1, NDUFB3, NDUFA4, and UQCRC2) and the tricarboxylic acid cycle (IDH3A and FH; Fig. 2A–C; Supplementary Fig. S2A). Similar gene expression pattern was also observed in PGC1 $\alpha$ -positive melanoma cell lines with PGC1 $\alpha$  suppression



**Figure 3.** Depletion of ERR $\alpha$  compromises the growth of human melanomas. **A**, Growth curve of various PGC1 $\alpha$ -positive melanoma cell lines with ERR $\alpha$  depletion. **B**, Endpoint tumor weight of PGC1 $\alpha$ -positive melanoma cells with ERR $\alpha$  depletion after inoculated into nude mice. Data were presented as mean with SD. **C**, Immunoblotting of PGC1 $\alpha$ -negative melanoma cells upon ERR $\alpha$  depletion by CRISPR. **D**, Growth curve of PGC1 $\alpha$ -negative melanoma cell lines with ERR $\alpha$  depletion. **E**, Endpoint tumor weight of PGC1 $\alpha$  melanoma cell line A375 with ERR $\alpha$  depletion in nude mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  and n.s. (not significant) as determined by  $t$  test if not otherwise indicated.

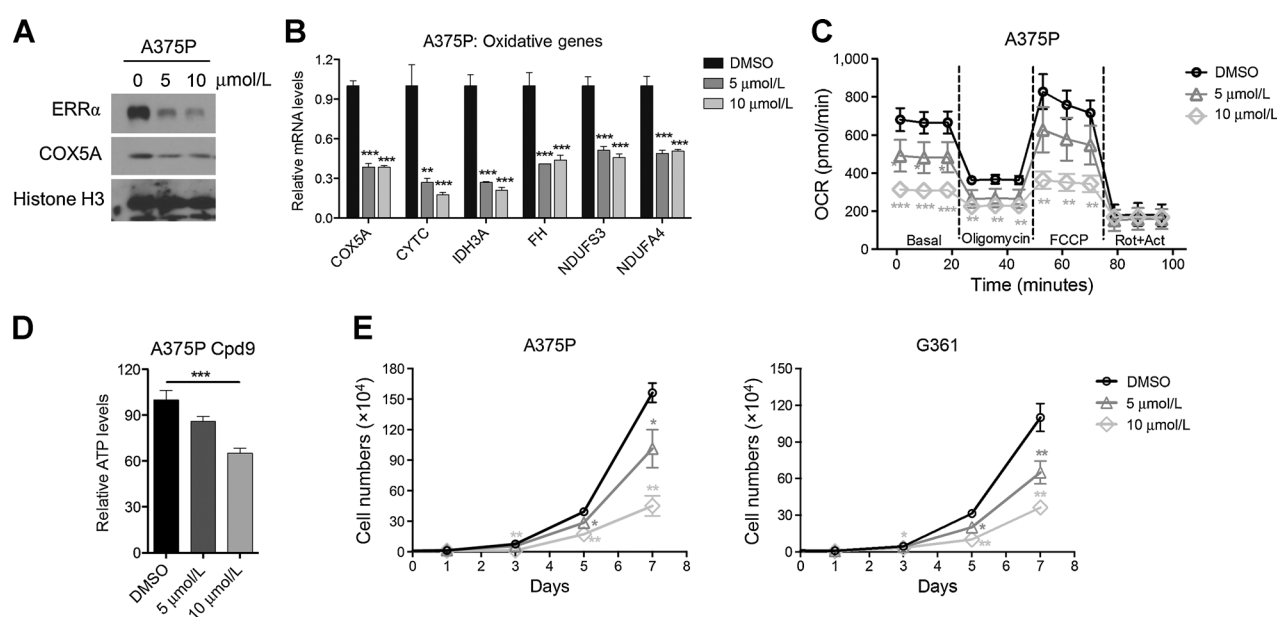
(Supplementary Fig. S2B; ref. 2), or with shRNA-mediated ERR $\alpha$  knockdown (Supplementary Fig. S2C and S2D). In addition, ERR $\alpha$ -depleted cells functionally displayed impaired mitochondrial respiration (Fig. 2D, Supplementary Fig. S2E and S2F) and lower ATP levels (Fig. 2E).

In addition to increasing mitochondrial bioenergetic metabolism, PGC1 $\alpha$  also regulates ROS detoxification capacity in melanoma cells (2, 4). In contrast to PGC1 $\alpha$  knockdown, however, depletion of ERR $\alpha$  did not affect the levels of PGC1 $\alpha$ -controlled antioxidant response (Supplementary Fig. S3A, S3C, and S3D), suggesting that ERR $\alpha$  is not involved in the maintenance of cellular ROS scavenging. Importantly, targeted deletion of ERR $\alpha$  did not alter expression of the genes promoting metastatic spread of melanoma cells that are controlled by PGC1 $\alpha$  (Supplementary Fig. S3B), and we could not detect any significant differences in the resulting migratory ability (Supplementary Fig. S3E). Although PGC1 $\alpha$  overexpression was

able to block the migration of invasive melanoma cells A2085, depletion of ERR $\alpha$  in these cells did not cause significant difference (Supplementary Fig. S3F), further supporting the notion that ERR $\alpha$  is not involved in the regulation of metastasis by PGC1 $\alpha$ . Collectively, our data indicate that ERR $\alpha$  preferentially mediates the mitochondrial functions of PGC1 $\alpha$  in melanoma cells, but does not contribute to ROS detoxification or affect the invasive/metastatic functions. Consequently, and similar to PGC1 $\alpha$ , ERR $\alpha$  supports mitochondrial oxidative metabolism in melanoma cells, but PGC1 $\alpha$  has broader functions.

#### Depletion of ERR $\alpha$ selectively inhibits the growth of PGC1 $\alpha$ -positive but not negative melanomas

We have previously found that PGC1 $\alpha$ -positive melanoma cells depend on mitochondrial oxidative metabolism to maintain their growth and survival (2, 3). Because ERR $\alpha$ , like PGC1 $\alpha$ , also



**Figure 4.** Inhibition of  $ERR\alpha$  activity phenocopies  $ERR\alpha$  depletion in melanoma cells. **A**, Immunoblotting of melanoma cells upon treatment with  $ERR\alpha$  antagonist Cpd29. **B**, Expression of oxidative genes at the mRNA level in melanoma cells upon  $ERR\alpha$  antagonist Cpd29. **C**, Mitochondrial activity of melanoma cells treated with  $ERR\alpha$  antagonist Cpd29 as measured by Seahorse flux assay. **D**, Intracellular ATP levels in cells treated with Cpd29. **E**, Growth curve of various melanoma cell lines treated with Cpd29. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  as determined by  $t$  test (**B** and **E**) or one-way ANOVA (**C** and **D**).

participates in control of mitochondrial bioenergetics, we determined whether  $ERR\alpha$  supports melanoma proliferation. Targeted suppression of  $ERR\alpha$  function using CRISPR/Cas9, or alternatively shRNA, significantly dampened *in vitro* proliferation of  $PGC1\alpha$ -positive melanoma cells, including A375P, G361, SK-MEL-30, MeWo, and KO29A (Fig. 3A; Supplementary Fig. S4A and S4B). In addition, when implanted subcutaneously in athymic (nude) mice,  $PGC1\alpha$ -positive melanoma cells with engineered  $ERR\alpha$  deficiency displayed significantly slower tumor growth (Fig. 3B), consistent with their reduced Ki-67 staining (Supplementary Fig. S4C). Importantly, these growth-suppressive effects of  $ERR\alpha$  depletion were specific to melanoma with heightened  $PGC1\alpha$  expression because melanoma cells with low  $PGC1\alpha$  expression, such as A375, A2058, and M14, were essentially unaffected by targeted deletion of  $ERR\alpha$  (Fig. 3C–E; Supplementary Fig. S4D). Similarly, A375P cells selected to grow with shRNA against  $PGC1\alpha$  were essentially insensitive to  $ERR\alpha$  deletion (Supplementary Fig. S4E), indicating that the proliferative defects following  $ERR\alpha$  depletion are dependent on  $PGC1\alpha$ . Collectively,  $ERR\alpha$  supports growth of melanomas with elevated  $PGC1\alpha$  expression, but is dispensable in those with low  $PGC1\alpha$ . In contrast to  $PGC1\alpha$  depletion, however,  $ERR\alpha$  deficiency did not induce accumulation of cellular ROS, which has been demonstrated to cause stabilization of the master glycolytic regulator HIF1 $\alpha$  (1), suggesting that targeting  $ERR\alpha$  is less likely to trigger compensatory metabolic reprogramming.

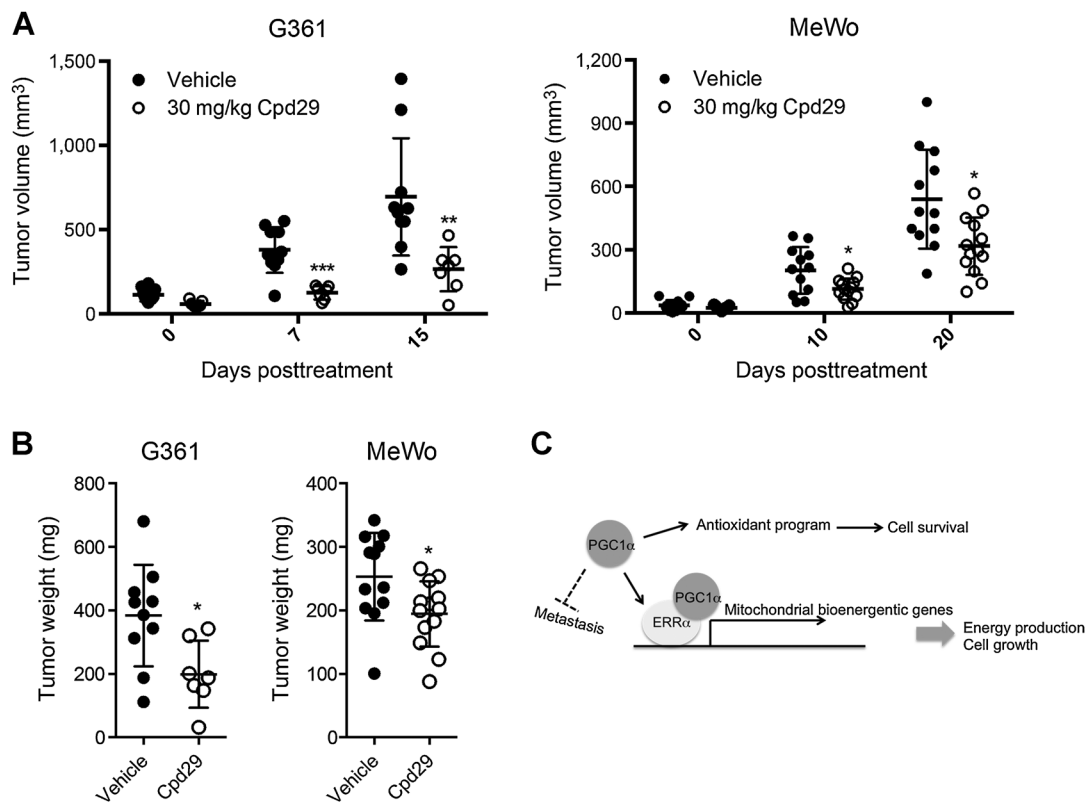
#### Suppression of $ERR\alpha$ activity phenocopies $ERR\alpha$ depletion in melanoma cells

The ligand-binding domain of  $ERR\alpha$  is amenable for therapeutic intervention. To this end, Cpd29 acts as an  $ERR\alpha$  reverse

agonists (antagonist), which interferes with the interaction between  $ERR\alpha$  and  $PGC1\alpha$  to block transcriptional activation (17). Considering that melanoma cells with high  $PGC1\alpha$  expression were particularly susceptible to genetic depletion of  $ERR\alpha$ , we examined whether  $ERR\alpha$  could be pharmacologically targeted using the Cpd29 compound. Similar to  $ERR\alpha$  depletion, inhibition of  $ERR\alpha$  activity by Cpd29 caused a dose-dependent downregulation of  $ERR\alpha$ / $PGC1\alpha$  target genes involved in mitochondrial oxidative metabolism (Fig. 4A and B), leaving the  $PGC1\alpha$ -regulated antioxidant and metastasis-promoting program genes largely unaffected (Supplementary Fig. S5A and S5B). Specifically, depletion of  $ERR\alpha$  in the  $PGC1\alpha$ -positive A375P cells rendered these cells insensitive to the inhibitory effects of Cpd29 (Supplementary Fig. S5C). In addition, Cpd29 treatment affected oxygen consumption activity (Fig. 4C), lowered levels of intracellular ATP (Fig. 4D), and importantly, decreased cell proliferation (Fig. 4E; Supplementary Fig. S5D). Taken together, pharmacologic interference with  $ERR\alpha$  function *in vitro* largely recapitulates  $ERR\alpha$  depletion in  $PGC1\alpha$ -expressing melanoma cells.

#### Pharmacologic inhibition of $ERR\alpha$ activity compromises melanoma growth *in vivo*

On the basis of the results of pharmacologic inhibition of  $ERR\alpha$  on melanoma proliferation *in vitro*, we next assessed whether the antagonist Cpd29 could affect tumor growth *in vivo*. To this end, we established subcutaneous xenografts from the  $PGC1\alpha$ -expressing melanoma cells G361 and MeWo and determined their sensitivity to Cpd29 treatment. Following clearly palpable subcutaneous tumor growth in athymic (nude) mice, Cpd29 or vehicle was administered by oral gavage six times a week. Consistent with the *in vitro* results, Cpd29 administration

**Figure 5.**

Inhibition of ERR $\alpha$  activity suppresses melanoma growth *in vivo*. **A**, Growth curve of melanomas in nude mice upon treatment with 30 mg/kg ERR $\alpha$  antagonist Cpd29 (G361:  $n = 10$  for vehicle,  $n = 7$  for Cpd29; MeWo:  $n = 12$  for vehicle,  $n = 13$  for Cpd29). Data, mean with SD. **B**, Endpoint tumor weight of melanoma xenografts upon Cpd29 treatment. Data, mean with SD. **C**, A schematic model depicting the functional roles of ERR $\alpha$  in mediating PGC1 $\alpha$  effects in melanoma cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  as determined by  $t$  test, if not otherwise indicated.

significantly blocked the tumors growth *in vivo* (Fig. 5A and B) without affecting the body weight (Supplementary Fig. S6A). In agreement with reduced tumor growth, the Ki-67 staining was also lower (Supplementary Fig. S6B), underscoring the efficacy of ERR $\alpha$  inhibition to suppress melanoma growth. These results suggest that ERR $\alpha$  might constitute a valuable therapeutic target to treat melanoma tumors with elevated PGC1 $\alpha$  expression.

## Discussion

Melanoma is recognized as the deadliest among the most common skin cancers because of its highly aggressive nature, as well as its virtual resistance to chemotherapy (25). Despite recent advances in clinical management of melanoma using oncogene-targeted and immune checkpoint therapies, the genetic heterogeneity of melanoma tumors enables treatment resistance that ultimately blunts long-term benefit for patients. To this end, inherent metabolic dependencies in cancer cells represent additional means for therapeutic exploit that may favorably combine with existing anticancer precision medicines to extend patient benefit (26, 27). To this end, a subset of melanomas is defined by heightened expression of the transcriptional coactivator PGC1 $\alpha$  and that is characterized by functional dependence on mitochondrial oxidative metabolism for their growth and survival (2, 3).

However, we and others recently documented that PGC1 $\alpha$  also exerts effects on suppressing metastatic spread, and consequently, PGC1 $\alpha$  does not represent an advantageous target for therapeutic intervention (5). Using an unbiased approach, we have identified ERR $\alpha$  as a key mediator of PGC1 $\alpha$ -dependent mitochondrial oxidative phosphorylation and growth in melanoma cells. Although the amount of ERR $\alpha$  peptides was ranking fourth in the proteomic list, considering the relatively smaller size of ERR $\alpha$  protein (423 amino acids vs. 3,859 for TRRAP, 4,128 for DNA-PK, and 937 for AP2B1), ERR $\alpha$  becomes the most enriched when normalized to molecular weight. Thus, in melanomas with heightened expression of PGC1 $\alpha$ , ERR $\alpha$  offers a metabolic vulnerability that may be exploited as a therapeutic target.

PGC1 $\alpha$  is a transcription coactivator that binds and facilitates the activation of multiple transcription factors and nuclear receptors, which in turn determine the PGC1 $\alpha$  function in different cell types and conditions (6). ERR $\alpha$  is one of the integral transcription factors that binds to a defining set of nuclear-encoded mitochondrial biogenesis genes and utilizes PGC1 $\alpha$  to increase their expression (9). In human melanoma cells, PGC1 $\alpha$  activates mitochondrial bioenergetic metabolism, protects against oxidative stress, and suppresses cellular mobility (2–5), but based on our data mainly employs ERR $\alpha$  to support mitochondrial bioenergetic programs. PGC1 $\alpha$  depletion in melanomas not only causes acute energy deficit due to mitochondrial failure, but also



cause an accumulation of cytotoxic ROS, which stabilizes HIF1 $\alpha$  to attenuate mitochondrial utilization and increase glycolysis that are hallmarks of the Warburg metabolic shift (4). Given that suppression of ERR $\alpha$  does not significantly compromise the antioxidant systems, the compensatory glycolytic reprogramming associated with PGC1 $\alpha$  depletion is likely to be prevented. Although PGC1 $\alpha$ /ERR $\alpha$  complex in prostate cancers has been demonstrated to elicit a catabolic state that compromises metastatic spread (14), yet ERR $\alpha$  in melanoma cells seems to segregate these PGC1 $\alpha$  functions with regard to metastatic regulation. Furthermore, although PGC1 $\alpha$  triggers a metabolism-independent transcription profile that suppresses melanoma metastasis (5), our data indicate that ERR $\alpha$ -mediated control of genes promoting oxidative metabolism is not involved in this regulatory circuit; however, these ERR $\alpha$ -regulated genes are required to support melanoma cell proliferation (Fig. 5C).

Considering that suppression of ERR $\alpha$  selectively compromises cellular bioenergetics but does not affect other PGC1 $\alpha$ -dependent melanoma growth-promoting functions, ERR $\alpha$  represents a potential therapeutic target. A series of small-molecule antagonists have been developed to repress the activity of ERR $\alpha$  and have been applied to both chronic metabolic disorders and cancers (17). As an example, the ERR $\alpha$  antagonist Cpd29 has recently been shown to be effective in some breast cancers either as a single agent or in combination with other chemotherapeutic drugs (12, 18). In the case of melanoma, we observed that Cpd29 mimics the effects of ERR $\alpha$  genetic depletion: decreased mitochondrial gene expression, reduced mitochondrial respiration, and lowered ATP production. Importantly, this decline in bioenergetic capacity translates into significant reduction in tumor progression in animal models, highlighting the efficiency of antagonizing ERR $\alpha$  activity in the therapy of melanomas.

There are subsets of multiple different tumor types that largely depend on mitochondrial oxidative metabolism for their survival and progression (2, 3, 28–34). As an example, it has been shown that a small population of slow-cycling melanoma cells within the bulk tumor predominantly utilizes oxidative phosphorylation to fuel their intrinsic resistance to conventional chemotherapies, such as cisplatin (35), suggesting that blocking mitochondrial respiratory through ERR $\alpha$  inhibition could enhance the efficacy of conventional melanoma chemotherapy. In addition, PGC1 $\alpha$ -mediated mitochondrial biogenesis is involved in the compensatory adaptation to MAP kinase pathway suppression (3, 31). Through improving mitochondrial catabolic programs generally in cells, PGC1 $\alpha$  also plays an important role in promoting tumor-associated M2 macrophage polarization (36). Because most cytolytic lymphocytes, such as effector T cells and natural killer cells, preferably rely on glycolysis (37, 38), suppression of ERR $\alpha$  might therefore modify the microenvironment through decreasing M2 macrophages, while leaving cytolytic lymphocytes unaffected.

Accordingly, ERR $\alpha$  inhibition might consequently represent an approach to improve immune checkpoint treatments for cancer treatment, acting directly by inhibiting the growth of oxidative phosphorylation addicted tumors, such as melanomas with heightened PGC1 $\alpha$  expression, and by altering the balance between tumor cytolytic lymphocytes and immunosuppressing M2 macrophages. Nonetheless, we have here focused our attention to the cell-intrinsic effects on inhibiting melanoma tumor growth, and consequently, future studies will be needed to examine therapeutic effects in the genuine tumor microenvironment.

In summary, our results indicate that the orphan nuclear receptor ERR $\alpha$  selectively mediates the growth-supporting bioenergetic functions of PGC1 $\alpha$ . Hence, ERR $\alpha$  constitutes an appealing therapeutic target to treat melanomas that are addicted to mitochondrial oxidative phosphorylation.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Luo, M. Jedrychowski, H.R. Widlund, P. Puigserver

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Luo, A. Thomas

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