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Essential gene screening identifies the bromodomain-containing protein BRPF1 as a new actionable target for endocrine therapy-resistant breast cancers

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Abstract

Identifying master epigenetic factors controlling proliferation and survival of cancer cells allows to discover new molecular targets exploitable to overcome resistance to current pharmacological regimens. In breast cancer (BC), resistance to endocrine therapy (ET) arises from aberrant Estrogen Receptor alpha (ERα) signaling caused by genetic and epigenetic events still mainly unknown. Targeting key upstream components of the ERα pathway provides a way to interfere with estrogen signaling in cancer cells independently from any other downstream event. By combining computational analysis of genome-wide 'drop-out' screenings with siRNA-mediated gene knock-down (*kd*), we identified a set of essential genes in luminal-like, ERα + BC that includes BRPF1, encoding a bromodomain-containing protein belonging to a family of epigenetic readers that act as chromatin remodelers to control gene transcription. To gather mechanistic insights into the role of BRPF1 in BC and ERα signaling, we applied chromatin and transcriptome profiling, gene ablation and targeted pharmacological inhibition coupled to cellular and functional assays. Results indicate that BRPF1 associates with ERα onto BC cell chromatin and its blockade inhibits cell cycle progression, reduces cell proliferation and mediates transcriptome changes through the modulation of chromatin accessibility. This effect is elicited by a widespread inhibition of estrogen signaling, consequent to ERα gene silencing, in antiestrogen (AE) -sensitive and -resistant BC cells and pre-clinical patient-derived models (PDOs). Characterization of the functional interplay of BRPF1 with ERα reveals a new regulator of

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estrogen-responsive BC cell survival and suggests that this epigenetic factor is a potential new target for treatment of these tumors.

Keywords Breast cancer, Estrogen signaling, BRPF1, Endocrine therapy resistance

Background

Epigenetic dysregulation can affect chromatin structure contributing to various diseases, including cancer, where alterations in chromatin accessibility to transcription factors result, among other, also in unbalanced oncogene or tumor-suppressor activities. These changes cause in fact abnormal gene expression patterns that play a crucial role in cancer initiation, progression up to response to treatments [1]. Luminal Breast Cancer (LBC) subtypes, characterized by the expression of ERα, comprise up to 75% of diagnosed BCs. ERα, being a key regulator of the estrogenic signaling within cancer cells is a predictor of responsiveness to Endocrine Therapy (ET), that involves blockade of the mitogenic effect of estrogens by selective ERα modulators/disruptors (SERMs/SERDs) or aromatase inhibitors (AIs), that all inhibit ERα activity directly or reducing estrogen biosynthesis [2]. De novo or acquired resistance to ET arises in 30–40% of LBC patients and represents a clinical challenging problem for treatment of this aggressive disease, driving research toward identification of genetic and molecular mechanisms causing ET failure [2]. Characterization of ERα interactome landscapes in cancer cells helped in highlighting the pivotal role of essential ER molecular partners, that include chromatin remodelers [3, 4] crucial in biological processes associated with BC progression [5]. This is particularly relevant, since targeting these chromatin-modifying enzymes with small molecule inhibitors is already applied in clinical trials for cancer treatment and represents a new avenue for a more effective therapeutic management of BC [6]. In particular, bromodomain protein inhibitors emerged as a promising class of epigenetic drugs, since these epigenetic factors are crucial in regulating multiple cellular processes, including gene transcription, protein interactions and cellular signaling. These findings arise from ongoing clinical trials concerning the use of these compounds for the treatment of different types of human cancers, such as acute leukemia and other solid tumors, ranging from early to advanced stages [7]. In this context, siRNA-mediated gene-screening approach was applied in luminal ERα positive (ER+) BC cell lines, to assess the functional impact of 36 ER interactors in LBC cell chromatin endowed with high fitness properties, measured in loss-of-function genome-wide screenings [8]. Among these, the bromodomain and PHD finger containing 1 (BRPF1) caught our attention being among the top fitness genes in ER+LBC cells, whose silencing caused a significant arrest of cell proliferation rate and activation of apoptotic mechanisms, associated with strong inhibition of ERα transactivation capability. Being BRPF1 a member of the family of bromodomain-containing proteins, it acts as epigenetic reader, modifying post-translationally histones through its two domains, PHD fingers and a PWWP. It is also the scaffold protein in the MOZ/MORF complex, allowing the formation of a tetrameric structure with MOZ (monocytic leukemia zinc finger protein) or MORF (MOZ-related factor) enzymes and two non-catalytic proteins, ING5 (Inhibitor of Growth Family Member 5) and MEAF6 (MYST/Esa1 Associated Factor 6), that modulate chromatin accessibility and gene expression [9]. While alterations in BRPF1 have been associated with several pathological conditions, including intellectual development disorders [9], and some types of tumors [9], its functional role in BC is completely unknown. Starting from the evidence that BRPF1 overexpression correlates with a poor survival rate in LBC patients, we characterized the functional interplay of ERα and BRPF1, revealing that this factor is a functional component of receptor cistromes in AE-responsive BC cell nuclei. The co-recruitment of both factors on MCF-7 cell chromatin regulates the transcription of genes involved in several cellular functions, including ER-mediated signaling, glucose metabolism, and Vascular Endothelial Growth Factor signaling, contributing to ET resistance in BC [2]. Transcriptome profiling after siRNA-mediated BRPF1 gene *kd* or cell treatment with specific small molecule inhibitors (GSK6853; GSK5959) demonstrate that BRPF1 inhibition causes growth inhibition by cell cycle arrest in G1, followed by massive apoptotic cell death, in several hormone-responsive and ET-resistant BC cells and in ex vivo preclinical patient-derived models (PDOs). Mechanistically, BRPF1 controls the expression of multiple key effectors of the estrogen pathway, disrupting ER-mediated BC transcriptional programs by reshaping chromatin accessibility through ERα silencing. Results obtained here suggest that BRPF1 represents a key functional component of the estrogen signaling pathway in BC and it is a new actionable therapeutic target against resistance to currently used ETs.

Results

BRPF1 is an essential gene in AE-sensitive and -resistant ER+BC cells

Genome-wide 'drop-out' screenings led to the identification of several genetic dependencies of human diseases, focused analyses are now required to highlight cancer-specific molecular signatures involved in the deregulation

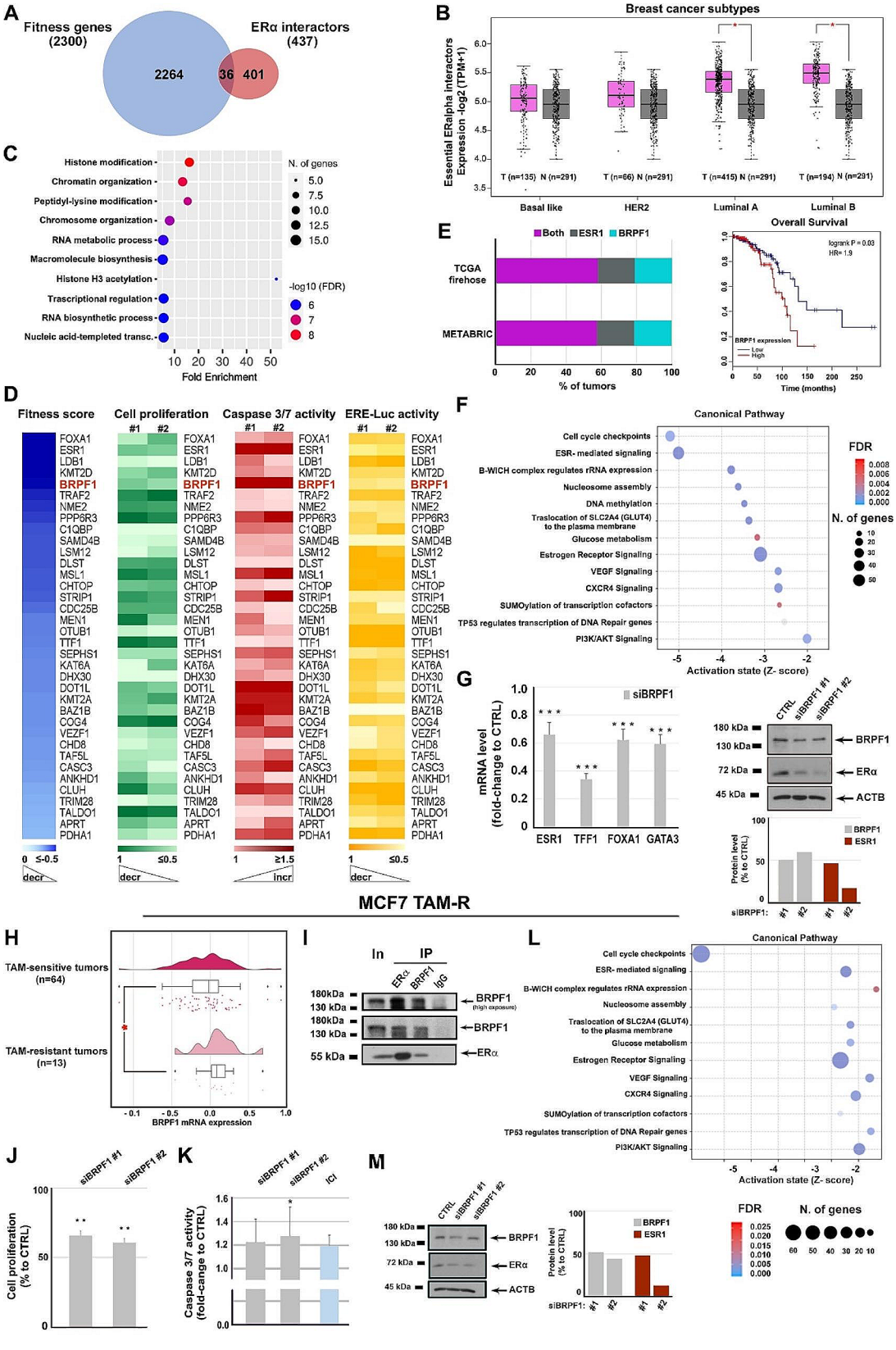

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Fig. 1 BRPF1 gene essentiality in AE-sensitive and -resistant BC cells. **(A)** Venn diagram showing the overlap between luminal-like BC fitness genes and ERα interacting partners. **(B)** Box plots showing the mRNA expression levels of the 36 ERα essential interactors in BC subtypes obtained from datasets deposited in The Cancer Genome Atlas (TCGA) and analyzed with GEPIA2. **(C)** Graphical display showing functional enrichment analysis of statistically significant molecular function encoded in the 36 essential gene encoding ERα interactors. **(D)** Heatmaps showing the fitness (essentiality) score and impact of siRNA mediate *kd* of the 36 ERα essential interactors on cell proliferation, caspase, and ERE-Luc reporter gene activity. Data related to fitness score represent the median of essentiality value of each molecule in BC cell analyzed [5]. Data related to cell proliferation, caspase, and ERE-Luc activity are analyzed with respect to the scramble siRNA (CTRL). Data are presented as the mean ± SD of determinations from a representative experiment performed with 2 siRNAs (#1 and #2) in six independent replicates after 72 h of silencing. Triangle forms indicate the orientation of each scale in terms of decrease (decr) or increase (incr) of the value respect to the CTRL. **(E)** Histogram (left panel) showing BRPF1 and ERα mRNA co-expression from two additional luminal-like BC patient datasets from TCGA and Kaplan-Meier curves (right panel) showing the probability of overall survival, according to BRPF1 mRNA expression levels, of luminal-like BCs contained in TCGA database analyzed by GEPIA2. **(F)** Graphical display summarizing functional enrichment analysis of statistically significant modulated pathways by RNA-seq following BRPF1 *kd* for 72 h in MCF-7 cells. **(G)** Left panel: Bar chart showing mRNA expression levels of ESR1 (ERα) and its genomic partners FOXA1 and GATA3 and downstream target TFF1 following BRPF1 *kd* for 72 h in MCF-7 cells. Data from RNA-seq analysis were analyzed with respect to a scramble siRNA (CTRL); asterisks indicate statistically significant differences (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.005$) respect to CTRL. Right panel: Representative western blot and relative densitometry showing BRPF1 and ERα protein levels following BRPF1 *kd* in MCF-7 cells for 72 h. β-actin (ACTB) was used as loading control and images were processed with ImageJ software (https://imagej.Net) for densitometry readings. **(H)** Graphical display of BRPF1 mRNA expression in TAM-sensitive and -resistant BC samples from TCGA datasets. **(I)** Representative western blot showing BRPF1 and ERα co-immunoprecipitation in TAM- resistant MCF-7 (MCF7 TAM-R) nuclear extracts. IgG was used as negative control. **(J)** MCF7 TAM-R cell proliferation rate measured by MTT assay following BRPF1 silencing in MCF-7 cells. **K)** Caspase 3/7 activity assay following BRPF1 silencing in MCF-7 cells. Data are presented as the mean ± SD of determinations from a representative experiment performed in six independent replicates after 72 h of silencing All data are analyzed with respect to the scramble siRNA (CTRL). Asterisks indicate statistically significant differences (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.005$) to CTRL. **L)** Graphical display summarizing functional enrichment analysis of statistically significant modulated pathways by RNA-seq following BRPF1 *kd* for 72 in MCF-7 TMA-R BC cells. **(M)** Representative western blot and relative densitometry showing BRPF1 and ERα protein levels following BRPF1 silencing or treatment with ICI (100 nM) in MCF7 TAM-R BC cells. β-actin (ACTB) was used as loading control and images were processed with ImageJ software (https://imagej.Net) for densitometry readings

of essential pathways in cancer progression and response to therapy. Considering the relevance of ERα as therapeutic target in hormone-dependent BC, and the biological key role of the components of its associated multiprotein complexes for cancer development [3, 4], we focused our investigation to ER-interacting partners essential for LBC subtype, uncovering novel exploitable therapeutic targets to prevent and/or overcome ET-resistance (Fig. 1A). We compared the subset of ER + BC cells fitness genes, essential for BC cell growth and proliferation, obtained from a focused investigation of ERα co-essentiality from DepMap and ProjectScore [5], with a subgroup of proteins retrieved from databases of ERα interacting partners [3, 10, 11], identifying a subset of 36 estrogen receptor-associated essential proteins whose corresponding mRNAs result highly expressed in BCs (Additional File 1: Fig. S1A), specifically in Luminal A and B subtypes (Fig. 1B), when compared to normal mammary gland, in TCGA BC datasets. These proteins, which are by functional enrichment analysis mainly involved in histone modifications and chromatin organization (Fig. 1C), include ERα (ESR1) and previously characterized ER partners such as BAZ1B, DOT1L, KMT2D, LBD1, MEN1 and the pioneering factor FOXA1 [3, 4, 12, 13]. To validate our *in silico* results, we performed a small scale siRNA screening in MCF-7 cells by using two different siRNAs against each gene target and assessing the consequences of gene *kd* on key cellular functions, such as cell proliferation and death, and on ERα-mediated activation of estrogen signaling (Fig. 1D). Results demonstrate that the candidate genes selected for this study are involved in BC cell proliferation, apoptotic cell death and efficient receptor-mediated transactivation of target genes (in this case a reporter gene: ERE-luc) already after 72 h of siRNA transfection and without showing significant toxicity under these experimental conditions (Additional File 1: Fig. S1B). Of note, these analyses, together with the above-mentioned known receptor coregulators revealed new and previously uncharacterized BC cell factors, including in particular BRPF1 (Bromodomain and PHD Finger Containing 1). This gene caught our attention as it scored fifth in the essential gene list according to fitness score and its inhibition exerted a strong effect on all the three cellular parameters analyzed, comparable to those elicited by ESR1 *kd* (Additional File 1: Fig. S1C-E). Moreover, a protein complex assembly analysis, performed to gather information on possible complex formation among our candidates, revealed the MOZ-complex, of which BRPF1 is a functional component, as the highest enriched (p-value = 0.0316) in our dataset of estrogen receptor-associated essential proteins (Additional File 1: Fig. S1F). Given the ability of this epigenetic factor to influence cellular behaviors through transcriptional control events mediated by its chromatin remodeling activity, we further characterized functional role of BRPF1 in ER + BC cells. BRPF1-ERα interaction was experimentally validated by co-IP in nuclear lysates from MCF-7 cells (Additional File 1: Fig. S1G). Analyzing TCGA datasets, we observed that both genes are often co-expressed in the same ER + tumors (Fig. 1E left), with a positive correlation (R = 0.53, p-value = 0,) between each other (Additional File 1: Fig. S1H). Interrogation of CPTAC BC dataset revealed that BRPF1 is expressed at a higher level in luminal-like BC compared to normal mammary

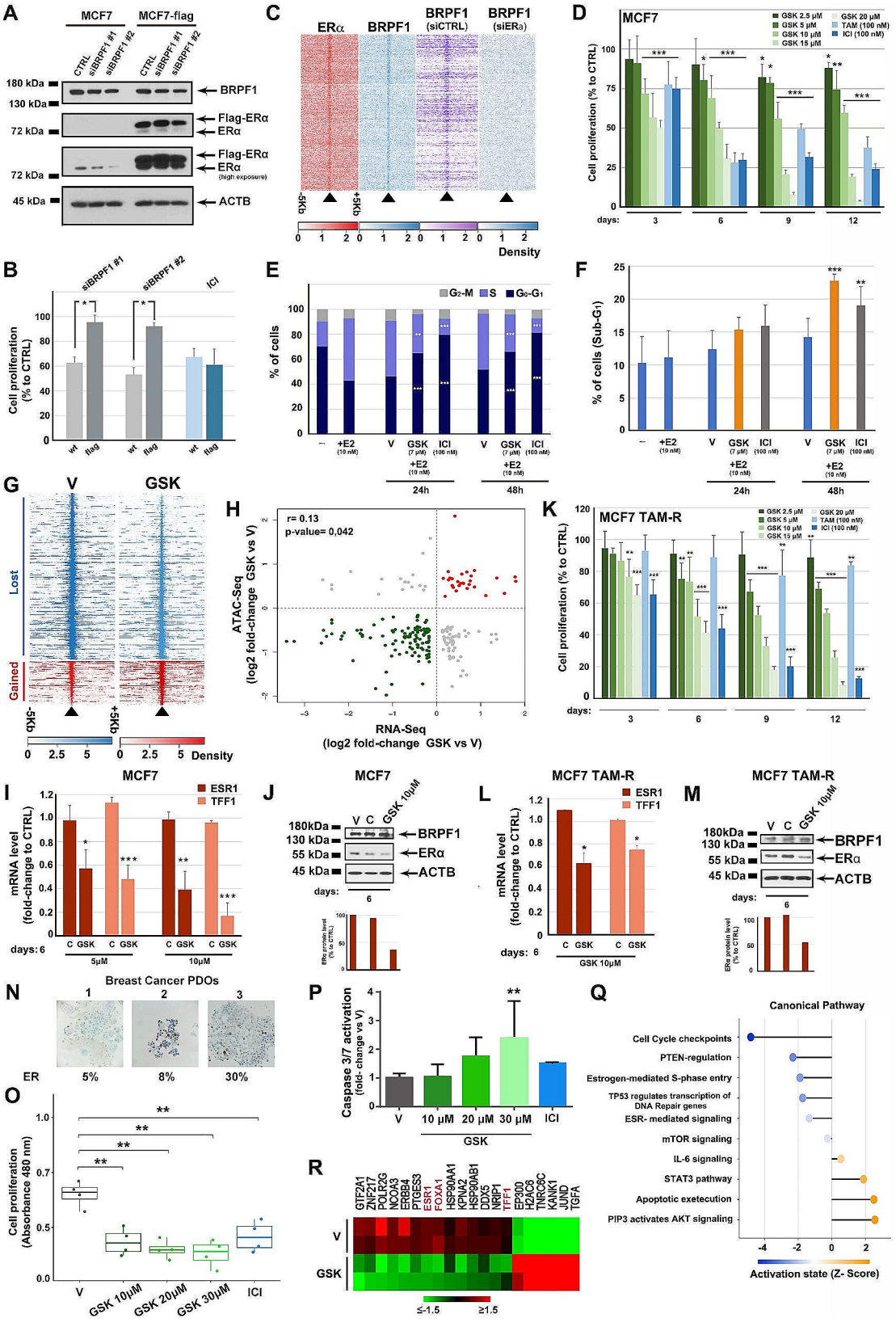

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Fig. 2 Impact of BRPF1 pharmacological inactivation on cell functions in AE-sensitive and -resistant BC cells and PDOs. **(A)** Western blot analysis and **(B)** cell proliferation rate in MCF-7 and MCF-7-flag cells before and after BRPF1 silencing or treatment with ICI (100 nM). For western blot analysis β-actin (ACTB) was used as loading control. For MTT assays all data are analyzed with respect to scramble siRNA (CTRL) and presented as the mean ± SD of determinations from a representative experiment performed in six independent replicates. Both experiments were performed after 72 h of silencing. Asterisks indicate statistically significant differences (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.005$) to CTRL. **(C)** Heatmap showing the effects of ERα silencing on ERα-BRPF1 shared binding sites in MCF-7 genome. **(D)** Effect of BRPF1 pharmacological blockade on AE-sensitive (MCF-7) BC cells following increasing concentrations of GSK, TAM (100nM) and ICI (100 nM) after 3, 6, 9 and 12 days. DMSO (vehicle) was used as control. Data are presented as mean ± SD from six independent replicates. **(E)** Cell cycle phase distribution (Percentages of G1, S, and G2/M) in hormone-deprived MCF-7 cell cultures before (-) or after treatment with E2 alone (24 h) or in combination with GSK or ICI at the indicated times and concentrations determined by flow cytometry after PI staining. **(F)** Cell cycle sub-G phase analyzed as described above. Results shown represent the means ± SD of multiple determinations from a representative experiment performed at least in triplicate. **(G)** Heatmap shoving ATAC-seq (normalized) signals at sites of increasing ("opening", gained: red) and decreasing ("closing", lost: blue) chromatin accessibility following GSK treatment compared to vehicle (DMSO, V) in MCF-7 cells. **(H)** Correlation graph between RNA expression and accessibility changes following GSK treatment in MCF-7 cells showing that the transcriptome changes identified positively correlate with changes in chromatin accessibility of the corresponding transcription units. **I)** RT-qPCR analysis of ESR1 (ERα) and TFF1 mRNA levels following treatment with GSK9311 (negative control inhibitor of GSK: C) or GSK in MCF-7 cells. Data are analyzed with respect to vehicle (DMSO: CTRL) and presented as the mean ± SD of triplicate determinations from a representative experiment. **J)** Representative western blot and relative densitometry showing BRPF1 and ERα protein levels following treatment with vehicle (DMSO: V), GSK9311 (negative control inhibitor of GSK: C) or GSK in MCF-7 cells. β-actin (ACTB) was used as loading control and images were processed with ImageJ software (https://imagej.Net) for densitometry readings. **K)** Effect of BRPF1 pharmacological blockade on AE-resistant (MCF-7 TAM-R) BC cells after 3, 6, 9 and 12 days of treatment with the indicated concentrations of GSK, TAM or ICI. DMSO (vehicle) was used as control. Data are presented as mean ± SD from six independent replicates. **L)** RT-qPCR analysis of ESR1 (ERα) and TFF1 mRNA levels following treatment with GSK9311 (control inhibitor, C) or GSK in MCF-7 TAM-R cells. Data are analyzed with respect to vehicle (DMSO: CTRL) and presented as the mean ± SD of triplicate determinations from a representative experiment. **M)** Representative western blot and relative densitometry showing BRPF1 and ERα protein levels following treatment with vehicle (DMSO, V), GSK9311 (control inhibitor, C) or GSK in MCF-7 cells treatment in MCF-7 TAM-R cells. β-actin (ACTB) was used as loading control and images were processed with ImageJ software (https://imagej.Net) for densitometry readings. **N)** Representative microscope photographs of ERα immuno-staining on organoids from $n = 3$ ER + BCs (see also Additional file 1: Table 1). **O)** Box plot showing proliferation rate in the 3 PDOs following treatment with vehicle (DMSO: V) and the indicated concentrations of GSK or with 100 nM ICI for 10 days. Results shown represent the means ± SD of multiple determinations of biological and technical replicates obtained from independent experiments performed on each PDO. **P)** Caspase activity assay in 2 PDOs following treatment with vehicle (DMSO, V) and the indicated concentrations of GSK or with 100 nM ICI for 10 days. Results shown represent the means ± SD of multiple determinations of biological replicates obtained from independent experiments performed on each PDO. **Q)** Graphical display of functional enrichment analysis of statistically significant deregulated pathways following BRPF1 pharmacological blockade in BC PDOs analyzed by RNA-seq. **R)** Heatmap showing differentially expressed genes identified by RNA-seq following pharmacological blockade of BRPF1 with GSK compared to vehicle (DMSO, V) in BC PDOs

gland (Additional File 1: Fig. S1I), and this associates to worse overall survival in BC patients (Fig. 1E right). When considering the mutational status of the BRPF1 in BCs from TCGA, this gene results mutated in only 2% and 0.7% of ER + LBCs. All these results suggest that, within the gene set identified here, BRPF1 might represent a novel prognostic biomarker and actionable target against ER-expressing BCs. To this end, after assessing BRPF1 protein expression level in a panel of ER + and ER- BC cells (Supp. Fig. S1J), we selected exponentially growing MCF-7 for further analyses. Cells were subjected to transient BRPF1 *kd* with two siRNAs, a reduction of up to 80% of mRNA level (Additional File 1: Fig. S2A, left panel), followed by gene expression profiling by RNA-Seq. As shown in Fig. 1F, BRPF1 silencing had a significant effect on MCF-7 cells transcriptome (196 up- and 957 down-regulated genes, Additional File 2: Table S1), affecting signal transduction pathways known to control key cellular processes involved in cancer, such as cell cycle checkpoints, glucose metabolism, VEGF signaling and above all, those controlled by ERα in BC (Fig. 1F). Indeed, BRPF1 silencing significantly downregulates well-known receptor target and pioneering genes, like TFF1, GATA3 and FOXA1, probably because of a reduction of ERα mRNA (Fig. 1G left panel and Additional File 1: Fig S2A right panel) and protein (Fig. 1G right panel) levels in the cell, confirming that the activity of this protein is essential to allow efficient estrogen signaling. This evidence suggested that this enzyme could also represent a candidate target for silencing ER-mediated pathway in AE-resistant breast tumors, such as Tamoxifen-resistant (TAM-R) ones, that maintain hormone responsiveness and express mutated and/or constitutively active receptors, where a significantly higher BRPF1 expression was found when compared to AE-sensitive ones (Fig. 1H). Thus, we validated the association between ERα and BRPF1 also in TAM-R MCF-7 cells (Fig. 1I). The effects of BRPF1 *kd* were assessed on cell proliferation, death and the transcriptome of TAM-R MCF-7 cell lines (Additional File 1: Fig. S2B left panel). Consistently with the results obtained in AE-sensitive cells, BRPF1 *kd* in TAM-R cells resulted in reduction of cell proliferation (Fig. 1J), increased apoptosis (Fig. 1K), and deregulation of receptor signaling (Fig. 1L; Additional File 3: Table S2), primarily driven by downregulation of ERα mRNA and protein levels (Fig. 1M and Additional File 1, Fig. S2B right panel). Comparable results on cell proliferation and death were detected in Fulvestrant/ICI-resistant (ICI-R) BC cells (Additional File 1: Fig. S2C). Also here, RNA-seq showed a deregulation of receptor signaling (Additional File 1: Fig. S2D; Additional File 4: Table S3), downregulation of ERα mRNA and protein levels (Additional File 1:

Fig. 2E). A significant inhibition of cell proliferation and ER downregulation following BRPF1 *kd* was observed also in T47D and ZR75.1 ERα+BC cells, further supporting the direct involvement of BRPF1 in ER mediated mitogenic signaling (Additional File 1: Fig S2F-G).

Pharmacological inhibition of BRPF1 induces deregulation of chromatin accessibility, causing growth arrest and cell death, via inhibition of ERα-mediated signaling, in both AE-sensitive and -resistant BC cells and in preclinical BC models

Given the results described above, and the response of ET-sensitive and -resistant cells to BRPF1 *kd*, we sought then to elucidate the involvement of this enzyme on ER-cistrome and ERα-mediated transcriptional program. To characterize the mechanistic and functional interplay of the BRPF1-ERα nuclear complex, we used first an MCF-7 cell line stably expressing full-length-3xFlag-ESR1 (ERα-flag) [4]. These clones carry double ERα expression, from the endogenous and the transfected genes, the first under the control of the natural promoter and the second from the exogenous one. The effects of BRPF1 silencing on cell proliferation and ERα protein expression were assessed in this complemented (ERα+ERα-flag) cellular model, compared to the same in *wt* MCF-7 cells (ERα only). Results reported in Fig. 2A and B confirmed a role of BRPF1 on ERα gene, as expression of exogenous ERα and proliferation rate of cells expressing it were not affected by BRPF1 *kd*, that caused instead downregulation of endogenous ER, demonstrating that the endogenous ER gene is the target of this epigenetic factor. Subsequently, we mapped BRPF1 binding to MCF-7 cell genome by Chromatin Immunoprecipitation coupled to sequencing (ChIP-seq). As shown in Supp. Fig. S3A, ChIP-western blotting showed an association of the two proteins on MCF-7 cell chromatin and their colocalization [3] in a sizable number of chromatin sites (6332 binding sites). In addition, analysis of the BRPF1 cistrome (20049 binding sites) revealed, among others, enriched binding motifs for HOX13 and GFLI factors (Additional File 1: Fig. S3B), while co-occupied ER+BRPF1 binding sites showed a prevalence of ERE (estrogen response element) or ERE-like sequences (Additional File 1: Fig. S3C). ER+BRPF1 binding sites resulted distributed within heterochromatin, enhancers, and promoters (Additional File 1: Fig. S3D left panel) and with a significant enrichment in these regulatory regions when considering the observed/expected distribution, also in comparison to ERα binding sites (Additional File 1: Fig. S3D right panel). Considering that BRPF1 does not bind DNA directly, our results suggest that the association with ERα targets this bromodomain protein to specific chromatin locations, where the two proteins act together to regulate gene activity. In fact, when performing BRPF1 ChIP-seq following ER *kd*, we observed that this factor lost the ability to bind to the subset of binding sites shared with ERα but not to those independent form the presence of the receptor, confirming the physical/functional cooperation between these two proteins in BC cell chromatin (Fig. 2C). This relationship between the receptor and BRPF1 was then further investigated through pharmacological inhibition of the latter with specific small molecule inhibitors: GSK5959, GSK6853, namely GSK, and its less active analogue, GSK9311, used as negative control [14]. This is of particular interest since bromodomain inhibitors represent a novel class of epigenetic drugs which hold great promise for anti-cancer therapy, although their therapeutic potential in BC remains still unexplored. To this end, the effects of GSK on proliferation, death, and transcriptome changes in a panel of ER+AE-sensitive and -resistant and, as control, in ER- BC cells was evaluated in detail. Considering MCF-7 cells, results showed that these compounds cause a dose-dependent inhibition of cell growth to levels comparable to, or even stronger than, those observed with the AEs (TAM or ICI), both in exponential growth conditions or following treatment of hormone-deprived cells with a mitogenic dose of estrogen (E2) (Fig. 2D and Additional File 1: Fig. S3E). This response resulted absent and comparable with the vehicle (DMSO; V) when considering the negative control inhibitor GSK9311 (Additional File 1: Fig. S3F), inducing us to use the vehicle (DMSO) in other or more complex experiments. The effect observed results from blockade of cell cycle progression in G1 (Fig. 2E and Additional File 1: Fig. S3G left panel), massive induction of apoptotic cell death (Fig. 2F and Additional File 1: Fig. S3G right panel and H) and interference with receptor-mediated transactivating functions resulting in deregulation of ER target genes expression (Additional File 1: Fig. S3I), all comparable to what induced by cell treatment with the pure AE ICI. This confirms the data obtained after BRPF1 *kd*, and further support the functional link between these two regulatory factors.

BRPF1 is a chromatin remodeler [9], and chromatin accessibility exerts a functional role in gene regulation. We therefore hypothesized that BRPF1 blockade may alter chromatin structure, thereby influencing receptor-mediated BC cell transcriptional regulatory networks, as determined by RNA-seq analyses (Additional File 1: Fig. S3J and Additional File 5: Table S4). To characterize changes in chromatin accessibility upon BRPF1 inhibition, we thus performed chromatin accessibility ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) test. In MCF-7 cells, BRPF1 inhibition resulted in 615 differentially accessible chromatin regions, 80% of which consisting of less accessible chromatin sites following BRPF1 inhibition (Fig. 2G and Additional File 6: Table S5A-B). Of note, most of

the chromatin regions detected following BRPF1 blockade were similarly influenced also by ICI, although this compound induced more pronounced chromatin accessibility changes in the same BC cell line (data not shown). Integrating ATAC- and RNA-seq data following GSK treatment, we observed a positive correlation between chromatin remodeling and transcriptome changes (Fig. 2H). Moreover, several deregulated genes associated with modulated chromatin regions are involved in key survival signal transduction pathways, such as TP53 transcription and DNA repair and PIK/AKT signaling (Additional File 1: Fig. S3J), and harbor BRPF1/ERα-BRPF1 binding sites in their regulatory elements. These include genes involved in ER-mediated signaling harboring ERα-BRPF1 binding sites, and of note, the ESR1 gene itself. Moreover, closed (lost, blue) regions resulted statistically significant enriched by ERα/ERα-BRPF1 binding sites (*p*-value 0,000099; z-score 145.80) when compared to open (gained, red) ones. These data provided mechanistic evidence to explain the effects of the drug on downregulation of estrogen signaling, which was achieved through ESR1 silencing confirmed both at mRNA and protein levels and in comparison, also, to the control (C) inhibitor GSK9311 (Fig. 2I and J). Results, consistent with those described above, were also obtained with the use of an additional BRPF1 specific small molecule inhibitor, GSK5959, in MCF-7 (Additional File 1: Fig. S3K-N), and with both GSK and GSK5959 in T47D BC cells (Additional File 1: Fig. S4A-F). The availability of an already approved drug to block this enzyme, mimicking the effects of gene silencing in AE-sensitive BC cells, led us to translate our findings also in AE-resistant cells. We therefore tested the effects of GSK in TAM-R and ICI-R BC cell models. As shown in Fig. 2K, GSK administration to MCF-7 TAM-R cells determined a marked growth inhibition not observable with the control (C) inhibitor GSK9311 (Additional File 1: Fig. S4G), coupled with a transcriptome deregulation (Supp. Fig. S4H and Additional File 7: Table S6), *via* blockade of cell cycle progression accompanied by increased apoptotic BC cell death (Additional File 1: Fig. S4I and J). This effect was observed under both, exponential growth conditions and after E2 stimulation, and it is due to decreased ER mRNA and protein levels in comparison, also, to the control (C) inhibitor GSK9311 (Fig. 2, L and M). MCF-7 TAM-R responded to BRPF1 inhibition similarly to wild-type MCF-7 cells (Fig. 2D-F). Likewise, GSK administration to ICI-R cells inhibited cell growth and ER expression via deregulation of essential BC cell pathways (Additional File 1: Fig. S4 K-N and Additional File 8: Table S7). The antiproliferative effects of GSK were also observed in several other E2-responsive and TAM-R BC cell lines, namely T47D-TAM, ZR-75.1 and ZR-75.1 TAM-R, BT-474 and BT474 TAM-R. At same time, significant responses to GSK or GSK6959 were not detected in ER-negative, E2-unresponsive MDA-MB 231 and HS578T BC cells and in ER- mammary epithelial MCF10 cells (Additional File 1: Fig S5 A to I). The results demonstrate that ERα gene silencing through BRPF1 inhibition, and the downstream effects on estrogen signaling, occur in LBC independently from the cellular background and the molecular mechanism responsible for ET resistance. Given the practical implications of these findings for cancer therapy, we challenged them in preclinical models derived from ER+BC patients (PDOs), since these have been demonstrated to hold molecular characteristics of the original tumors, including tumor heterogeneity [15, 16]. PDOs are also consolidated forecaster models for ex vivo patient's drug response, mirroring those clinically observed [16]. We therefore tested our findings in organoids derived from three ER-positive breast carcinoma biopsies, from three different patients, all retaining ERα expression to different extents and being estrogen responsive (Fig. 2N and Additional File 1: Table 1). Using GSK at concentrations comparable and also higher than those employed previously in 2D BC cells, we analyzed the effect of BRPF1 pharmacological inhibition on proliferation and death in PDOs. As shown in Fig. 2O and P, GSK was able to dramatically decrease cell growth and induce apoptotic events in a dose-dependent manner in all PDOs tested after 9 days of treatment, at an extent comparable to or higher than that elicited by ICI. To confirm that the observed phenotype would resemble, mechanistically, the results obtained in 2D models, we performed transcriptome analyses on two independent PDOs after BRPF1 pharmacological blockade. Results show that also in these preclinical models, attenuation of BC growth was significantly mediated by cell cycle and ER-mediated signaling, coupled to an enhancement of apoptosis (Fig. 2Q and Additional file 9: Table S8), by downregulation of ESR1 gene and additional key ER pioneering factors and co-activators, such as FOXA1 and NCOA3 and with a consequent impact on expression of ER target genes such as TFF1 (Fig. 2R). Finally, considering that a combinatorial therapeutic approach, using more than one compound targeting different cellular targets, could represent a novel strategy to improve treatment of ER+BCs resistant to actual adopted therapies (AE and CDK4/6 inhibitors) and help maximize the therapeutic efficacy, we examined the effect of combinatory treatment with AE (TAM and ICI) or CDK4/6 inhibitor Palbociclib (Palb) [17] in combination with GSK. Interestingly, the test revealed a synergistic effect of suboptimal concentrations of the pairs of drugs used on growth inhibition of both AE-sensitive and -resistant LBC cells (Additional file 1: Fig. S6 A–B). When combined, the results described above demonstrate a pivotal role of BRPF1 in regulation of ERα activity in E2-responsive BC

cells, due both to a functional interplay of the encoded protein with the receptor itself and to enhancement of its gene expression, both resulting in efficient regulation of the estrogen signaling pathway in LBC cells. Interestingly, this occurs also in AE-resistant BC cells, suggesting that this epigenetic factor is a molecular target exploitable to silence ERα signaling in ET-resistant tumors, particularly, in the majority of these that retain responsiveness to estrogen.

Discussion

Lack or loss of response to current therapies is a critical issue in clinical management of malignant tumors. For this reason, research leading to new ways to treat aggressive cancers is extensively pursued, mainly by identification of key molecules in cancer cells that can be targeted by specific and effective drugs. In case of ERα+LBC, lack of response to ET is particularly critical, due to the high incidence of these diseases and the paucity of predictive markers of resistance. Indeed, despite the effectiveness of ET in the clinical management of these tumors, intrinsic and acquired resistance occurs in one-third of patients, resulting in relapse or in progression of this aggressive disease generally to the *exitus* of the patient. Mechanistically, it has been proposed that ET-resistance arises from multiple genetic and epigenetic mechanisms, such as deregulation or mutations of key molecules involved in E2-mediated signaling, conferring to ERα a constitutive activation able to escape to the inhibitory effects of AE drugs. Thus, the possibility of shutting down ER-mediated signaling in ET-resistant BCs, by drugs able to regulate key mechanisms influencing cancer vulnerabilities, is a consolidated and promising approach to overcome resistance phenomena [2]. Here, we discovered BRPF1 among the top 5 essential genes in ER+BCs, whose inactivation causes rapid decline of cell proliferation and indices cells death. The ability of BRPF1 to promote cancer cell growth and tumor progression has been postulated to depend upon its ability to facilitate H3K14 acetylation of gene promoters through the MOZ/MORF complex, thereby controlling the expression of multiple key oncogenes [18]. Given the known role of epigenetic chromatin remodelers on ET resistance in BC [2], we characterized here in detail the functional interplay between ERα and BRPF1 in AE-sensitive and -resistant luminal-like ER+BC cell lines representing the best characterized and informative in vitro model of these conditions. Results show that this epigenetic "reader" needs ERα to be located at specific regulatory elements that control the activity of early and late estrogen-responsive BC genes, including the receptor itself. BRPF1 blockade by both siRNA-mediated gene silencing or pharmacological inhibition induces a specific reprogramming of E2-responsive BC cell transcriptome with impairment of critical functions such as growth, mainly by cell cycle arrest, as it was shown that MOZ is involved in regulating cell-cycle arrest in the G1 phase [19], and by apoptotic cell death. These phenotypic consequences observed are mechanistically mediated by the effects of BRPF1 inhibition on accessibility of selective chromatin regions endowed by shared BRPF1+ERα binding sites, that control the transcriptional program of several essential genes involved the hormonal signaling in BC. These include the pioneering factor FOXA1 [20] and the gene encoding the receptor itself. Thus, targeting BRPF1 to overcome failure of ET has, in our opinion, substantial clinical relevance and, for this reason, it was tested also in ET-resistant BC models, where this "reader" was able to induce "AE-like" responses. It is worth mentioning also that analysing data from AE-insensitive tumors present in TCGA, we observed higher BRPF1 expression compared to responsive ones, further supporting the usefulness of targeting this epigenetic factor for ET-resistant BCs. Indeed, when this was tested here in several ET-resistant (TAM and ICI) BC cell models results show a rapid and massive effect of BRPF1 inhibition in inhibiting cell proliferation, and survival, mediated by transcriptome changes consequent to cell desensitization to estrogen signaling and ERα downregulation. ET-resistance has been shown to arise by ESR1 gene mutations and /or posttranslational modification that allow an abnormal activation of the receptor, that drive tumor relapse and metastatic progression [17]. All these cases, which account for a large fraction of ET-resistant tumors, would benefit from stripping ERα from cancer cells. In this respect, we demonstrate here that targeting BRPF1 fits within this aim, since in ER+and functionally active BC PDOs, inhibitors of this protein strongly reduce tumor growth by silencing ESR1 gene together with several components of its signaling machinery. Of note, the promising results of clinical trials with bromodomain protein inhibitors and several tumors [21], sustain the idea of the use of BRPF1 inhibitors for treatment of ET-resistant LBC. We previously reported that combinatorial treatment with epidrugs enhance the effect of single small molecule inhibitors in LBC cell models [3, 4]. A similar combinatorial approach using BRPF1 inhibitors and other epidrugs could also represent, as demonstrated here, an additional strategy for treatment of aggressive LBCs that escape ETs.

Conclusions

The results reported here describe for the first time and provide mechanistic insights in the functional interplay between ERα and BRPF1 in receptor-positive LBC cells and reveal a new therapeutic vulnerability of ET-resistant tumors through pharmacological inhibition of this epigenetic factor.

Abbreviations

AE	Anti estrogen
AI	Aromatase Inhibitor
ATAC	Assay for Transposase-Accessible Chromatin
BRPF1	Bromodomain And PHD Finger Containing 1
BC	Breast cancer
CPTAC	Clinical Proteomic Tumor Analysis Consortium
ChIP	Chromatin immunoprecipitation
E2	17-beta estradiol/estrogen
ERα	Estrogen receptor alpha
ESR1	Estrogen receptor alpha
ET	Endocrine therapy
GSEA	Gene Set Enrichment Analysis
ICI	ICI 182780/Fulvestrant
kd	Knock-down
TAM	4-hydroxytamoxifen
TCGA	The Cancer Genome Atlas

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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Author contributions

Conceptualization: A.W. and G.N.; investigation: A.S., J.L., I.T., L.C., V.M., L.P, F.S. G.R, C.Q.; Bioinformatic analyses: G.G and A.G.; writing and original draft preparation A.S., G.G., G.N. and A.W.; writing, review, and editing: G.C., G.G., G.N., F.R., R.T., and A.W; funding acquisition: C.Q., G.C., G.N. and A.W. All the authors approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

All data supporting the findings of this study are available from the corresponding authors upon reasonable request. RNA-seq sequencing data related to CTRL and BRPF1 siRNA, Vehicle and BRPF1 pharmacological blockade in AE-sensitive and -resistant cells and ER+ BC PDOs and ATAC-seq in AE-sensitive BC cells have been deposited in the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with the following accession number: E-MTAB-13876, E-MTAB-13884, E-MTAB-13887 and E-MTAB-13885, respectively.

Declarations

Ethical approval and consent to participate

The study was approved by the Research Ethics Committee of the AUO-University of Naples Federico II n° 119/15ES1. Samples (PDO) were collected according to the declaration of Helsinki and each subject signed an informed consent before participating in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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