Interaction Proteomics Identifies ERbeta Association with Chromatin Repressive Complexes to Inhibit Cholesterol Biosynthesis and Exert An Oncosuppressive Role in Triple-negative Breast Cancer

Authors
Elena Alexandrova, Giorgio Giurato, Pasquale Saggese, Giovanni Pecoraro, Jessica Lamberti, Maria Ravo, Francesca Rizzo, Domenico Rocco, Roberta Tarallo, Tuula A. Nyman, Francesca Collina, Monica Cantile, Maurizio Di Bonito, Gerardo Botti, Giovanni Nassa, and Alessandro Weisz

Correspondence
aweisz@unisa.it; gnassa@unisa.it

In Brief
Triple-negative breast cancers (TNBCs) are characterized by low overall survival and poor response to therapy because of their aggressiveness and limited available treatments. We found a subset of TNBC expressing the master regulator estrogen receptor beta (ERβ1) and characterized the effect of this nuclear receptor in human TNBC cells by a multiomics approach. Results highlight transcriptome deregulation by ERβ involving association with chromatin remodeling complexes, including PRC1/2, and provide an explanation for its oncosuppressive effects on TNBC cells.

Highlights
- ERβ inhibits cell growth, migration and clonogenicity in TNBC cells.
- In TNBC ERβ deregulates the transcriptome and cholesterol biosynthesis pathway.
- ERβ interacts with multiple chromatin remodeling complexes including PRC1/2.
Interaction Proteomics Identifies ERbeta Association with Chromatin Repressive Complexes to Inhibit Cholesterol Biosynthesis and Exert An Oncosuppressive Role in Triple-negative Breast Cancer* 

Elena Alexandrova‡§¶¶, Giorgio Giurato‡§¶¶, Pasquale Saggese‡¶, Giovanni Pecoraro‡, Jessica Lamberti‡, Maria Ravo‡§, Francesca Rizzo‡, Domenico Rocco‡, Roberta Tarallo‡, Tuula A. Nyman||, Francesca Collina**, Monica Cantile**, Maurizio Di Bonito**, Gerardo Botti‡‡, Giovanni Nassa‡¶, and Alessandro Weiszत

Triple-negative breast cancer (TNBC) is characterized by poor response to therapy and low overall patient survival. Recently, Estrogen Receptor beta (ERβ) has been found to be expressed in a fraction of TNBCs where, because of its oncoseuctive actions on the genome, it represents a potential therapeutic target, provided a better understanding of its actions in these tumors becomes available. To this end, the cell lines Hs 578T, MDA-MB-468 and HCC1806, representing the claudin-low, basal-like 1 and 2 TNBC molecular subtypes respectively, were engineered to express ERβ under the control of a Tetracycline-inducible promoter and used to investigate the effects of this transcription factor on gene activity. The antiproliferative effects of ERβ in these cells were confirmed by multiple functional approaches, including transcriptome profiling and global mapping of receptor binding sites in the genome, that revealed direct negative regulation by ERβ of genes, encoding for key components of cellular pathways associated to TNBC aggressiveness representing novel therapeutic targets such as angiogenesis, invasion, metastasis and cholesterol biosynthesis. Supporting these results, interaction proteomics by immunoprecipitation coupled to nano LC-MS/MS mass spectrometry revealed ERβ association with several potential nuclear protein partners, including key components of regulatory complexes known to control chromatin remodeling, transcriptional and post-transcriptional gene regulation and RNA splicing. Among these, ERβ association with the Polycomb Repressor Complexes 1 and 2 (PRC1/2), known for their central role in gene regulation in cancer cells, was confirmed in all three TNBC subtypes investigated, suggesting its occurrence independently from the cellular context. These results demonstrate a significant impact of ERβ in TNBC genome activity mediated by its cooperation with regulatory multiprotein chromatin remodeling complexes, providing novel ground to devise new strategies for the treatment of these diseases based on ligands affecting the activity of this nuclear receptor or some of its protein partners. Molecular & Cellular Proteomics 19: 245–260, 2020. DOI: 10.1074/mcp.RA119.001817.

Breast cancer (BC)¹ is one of the most common malignancies in women, accounting for about 25% of all new cancer cases and representing the leading cause of cancer death in women. BC is a heterogeneous pathology classified based on status determination of the following receptors: Estrogen Receptor alpha (ERα), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2/neu), because they represent known targets for BC treatment. The molecular subtype of BC lacking the expression of these three receptors is defined as triple-negative breast cancer (TNBC). It accounts for ~15–20% of all breast carcinomas and is associated with earlier
onset, aggressive clinical phenotype, and poor prognosis compared with other BCs (1). Moreover, because of the limited number of therapies applicable for its treatment, the need for novel molecular targets identification becomes critical.

Estrogen receptors belong to the nuclear receptor superfamily and comprise two main members: Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ). Although much is known about ERα and its proliferative effect on breast epithelium, the role of ERβ is still debated and not fully understood (2, 3). Nevertheless, according to different studies, ERβ appears to be a tumor suppressor gene with higher expression in normal breast tissue when compared with the cancerous one, thus representing an appealing clinical target for anti-cancer treatment because of the possibility of its selective activation with ERβ-specific agonists (4, 5). In the TNBC context, several studies, including a meta-analysis, reported receptor expression in a small fraction (15–20%) of patients and its presence was correlated to improved patient outcomes (6, 7). It has been demonstrated that ERβ exerts its action in TNBC by targeting genes involved in cell cycle, proliferation, death and development (8). In basal-like BC it regulates epithelial to mesenchymal transition (EMT) either by up-regulation of miR-200a/b/429 (9) or by EGFR downregulation (9). Moreover, the presence of liganded ERβ in TNBC cells causes a reduction of proinflammatory cytokines production and an increased synthesis of cystatin superfamily members, whose expression generally correlates with better relapse-free survival in TNBC patients and is linked to a concomitant decrease of migration and invasion of TNBC cells (10). In any case, the molecular mechanisms underlying ERβ tumor-suppressive effects in TNBC are still not fully characterized, thus being a crucial point in therapy response evaluation and further classification of patients who might benefit from therapies targeting this estrogen receptor subtype.

Here, we show that ERβ is detectable in a fraction of primary TNBCs and that its expression in TNBC cells in vitro leads to reduced cell proliferation by the increase of G1 cell cycle phase. Transcriptome analysis combined with genomewide ERβ binding sites mapping revealed the involvement of the receptor in cholesterol biosynthesis downregulation through its recruitment to regulatory elements of the gene encoding for sterol regulatory element-binding transcription factor 1 (SREBF1), an upstream regulator of cholesterol biosynthesis pathway. Interactional proteomics, performed to unveil the molecular bases of ERβ action, revealed its nuclear association with protein complexes involved in several key biological events, such as DNA replication, transcription regulation, post-transcriptional mRNA expression, and small molecule biochemistry control. Multiple complexes, such as polycomb repressor complexes 1 and 2, known to be involved in negative epigenetic regulation of transcription by chromatin remodeling, were found to be a part of ERβ interactome.

These data allow us to suggest an immediate contribution of ERβ and its molecular partners in the downregulation of key pathways in TNBC, including those involved in cholesterol metabolism.

**Experimental Procedures**

*Tissue Microarray (TMA) Construction—* A breast Tissue MicroArray (TMA) was constructed using 217 samples of triple-negative breast cancer collected from 2003 to 2013 and 5 normal breast tissues from the Pathology Unit of the National Cancer Institute Fondazione G. Pascale of Naples. Informed consent was obtained from all patients. All tumors and controls were reviewed according to WHO classification criteria, using standard tissue sections and appropriate immunohistochemical slides. TMA was built using the most representative neoplastic areas of each sample by semi-automated tissue arrayer (Galileo TMA) as described previously (11).

**Immunohistochemistry (IHC) and TUNEL Assay—** Formalin-Fixed Paraffin-Embedded (FFPE) sections were deparaffinized in an organic solvent (Bio-Clear, Clodia Laboratori, Chioggia, Italy), in order to remove the including agent and rehydrated following a normal desiccating alcohol scale. Then, the endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. Antigen unbleaching was conducted using 10x citrate buffer (0,01M) in a decloaking chamber at 110 °C for 20 min. After that, the slides were cooled, washed in TBS buffer solution (Tris buffer saline)/Tween and protein blockade was performed (5% BSA in 1× PBS). The slides of TMA were incubated with two different primary antibodies that recognize ERβ: PPG5/10 (1:15; GeneTex, Irvine, CA) and PPZ0506 (1:60; ThermoFisher Scientific, Waltham, MA) overnight at 4 °C. The binding of the primary antibody to the antigen was visualized by incubation with a secondary antibody (anti-mouse) associated with horseradish peroxidase molecules (HRP) by a dextran polymer for 30 min at 4 °C and followed by washing in TBS/Tween buffer (2 steps of 5 min each). The peroxidase activity was visualized by the addition of a chromogenic substrate (DAB, 3,3'-Diaminobenzidine and 2,5-3% hydrogen peroxide). The reaction with peroxidase produces a visible brown precipitate at the antigenic site. The tissue sections were immersed in 0.02% hematoxylin for about 30 s, to contrast the cores and dehydrated following an ascending scale of alcohol clarified by a passage in Bio-Clear and mounted using a non-aqueous permanent medium. Finally, the prepared slides were interpreted using a standard light field optical microscope by two expert pathologists. For each core sample, at least five fields and more than 500 cells were analyzed. Using a semi-quantitative scoring system, under the microscope, the observer evaluated the intensity, extent and subcellular distribution of the marker, for which there are no standardized criteria for assessing the intensity of the reaction. For the definition and evaluation of the score both qualitative and quantitative parameters were considered. For the qualitative criteria, we considered the intensity of the reaction subdividing it into “mild,” “moderate,” and “intense.” For the quantitative criteria, the percentage of positive tumor cells was considered. The following antibodies were used for immunohistochemistry assay: rabbit polyclonal C-terminal anti-ERβ (PPG5/10, Thermo Fisher Scien-
entific), mouse monoclonal N-terminal anti-ERβ (PPZ0506, Thermo Fisher Scientific).

Cell Culture and Clone Generation—HCC1806 (CRL-2335), MDA-MB-468 (HTB-132) and Hs 578T (HTB-126) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All experiments relative to cell lines were performed under conditions of exponential growth and each cell line was grown in the appropriate cell culture medium, according to manufacturer protocol, and kept in an incubator at 37 °C in the presence of 5% CO2. HCC1806 cells (CRL-2335) were grown in phenol red-containing Roswell Park Memorial Institute (RPMI) 1640 Medium (Euroclone, Milano, Italy) complemented with HEPES (pH 7.3; Euroclone) to a final concentration of 2.383 g/L, d-Glucose (Lonza, Basel, Switzerland) to a final concentration of 4.5 g/L and Sodium Pyruvate (Euroclone) to a final concentration of 0.11 g/L. MDA-MB-468 (HTB-132) and Hs 578T (HTB-126) were maintained in phenol red-containing Dulbecco’s modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO). For all cell lines, medium was complemented with 10% FBS (GE Healthcare, Chicago, IL), 1 x Pen-Strep (Lonza), 2 mM L-Glutamine (Lonza) and 0.25 µg/ml of Amphotericin B (Sigma-Aldrich). Medium for Hs 578T cell line was complemented with 0.01 mg/ml bovine insulin (Sigma-Aldrich) according to vendor’s instructions. ERβ expression was induced using doxycycline (Sigma-Aldrich). Cells were STR authenticated and periodically tested for the presence of mycoplasma contamination using ABM mycoplasma PCR detection kit. All experiments were performed in media complemented with non-steroid depleted FBS. The indicated cell lines were used to generate clones stably expressing full-length-3xFlag-ERβ using the Clontech Lentil-X Tet-On Advanced Inducible Expression System (Takara-Clontech Europe, Göteborg, Sweden). First, the 3xFlag-ERβ DNA fragment, amplified from CSIL-ESR2 vector, kindly provided by Dr. P. Dotto (12), was cloned into a pLVX-Tight-Puro vector downstream to a tetracycline-inducible promoter. The cloning procedure was performed using Clontech InFusion HD Cloning Kit. Subsequently, two lentiviral particle types, one containing Tet-On Advanced and another ERβ-encoding RNAs, were produced using the Clontech Lentivector Packaging Single Shots (SVS-G) according to manufacturer protocol. Selected TNBC cell lines were infected first with Tet-On Advanced lentiviral particles, encoding for a transcription factor that in the presence of doxycycline binds to a tightly regulated inducible promoter, and separate clones were produced. One Tet-On Advanced clone for each cell line was then chosen and transduced with 3xFlag-ERβ particles. Single ERβ clones were grown and tested for receptor protein expression on doxycycline (Sigma-Aldrich) induction by Western blotting. For each cell line, one ERβ clone was chosen for further experiments.

Total Protein Extraction—Cells were seeded in 60-mm plates and grown in the presence of doxycycline or vehicle for 9 days. The following doxycycline concentrations were used to induce ERβ expression: 0.035 µg/ml, 1.0 µg/ml and 2.0 µg/ml for HCC1806, Hs 578T and MDA-MB-468-derived cell lines respectively. For total protein extraction, cells were harvested, washed twice with ice-cold PBS-EDTA (0.5 mM EDTA), lysed using RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% SDS, 0.5% C12H22O4, 1% NP-40, 2 mM EDTA, 50 mM NaF) for 15 min on ice and centrifuged at 13,000 rpm for 30 min at +4 °C. Resulting protein extracts were quantified using Bradford protein assay followed by analysis by SDS-PAGE and Western blotting.

Cell Proliferation and Cell Cycle Analysis—Cell proliferation of ERβ-expressing clones was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)-based colorimetric assay (M8494, Invitrogen, Carlsbad, CA), according to the manufacturer instructions. Cells were seeded in 96-well plates and exposed to doxycycline or vehicle for 12 days. The same experiments were simultaneously performed also on the parental Tet-On Advanced cells (see Supporting Information). Absorbance was measured by a microplate reader at 570 nm, and corresponding background values read at 620 nm were subtracted for each sample. Cell cycle assay was performed in both ERβ and Tet-On Advanced TNBC cell lines. To this aim, cells, grown in the presence or absence of the aforementioned doxycycline concentrations for 9 days, were detached using 1 x trypsin-EDTA in PBS (Aurogene), washed twice with ice-cold PBS, fixed with 70% Ethanol (300 µl of PBS + EDTA 20 mM and 700 µl of Ethanol 100%), and stored at ~80 °C for 1 h. Once fixed, cells were washed with ice-cold PBS, centrifuged, resuspended in RNaseA-containing PBS (0.05 µg/µl) and incubated for 10 min at room temperature. Finally, cells were stained with Propidium Iodide (0.05 µg/µl; Sigma Aldrich) and analyzed using BD FACSVerse (Becton Dickinson, Franklin Lakes, NJ). For each sample, the scatterplots were analyzed using ModFit LT 5.0 software (Verity Software House). Functional validation of ERβ effects and the RNA profiling experiments described below were performed using the above-indicated doxycycline concentrations.

Total RNA Extraction, Profiling, and Data Analysis—Total RNA was extracted from HCC1806, MDA-MB-468 and Hs 578T ERβ clones grown for 9 days in the presence and absence of doxycycline, by using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer instructions. As a control for doxycycline effect on RNA expression, total RNA was extracted also from MDA-MB-468 Tet-On Advanced cell line, cultured at the same conditions as the corresponding ERβ clone. Once extracted, RNA concentration and purity were assayed by NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific) whereas its integrity was evaluated using TapeStation 2200 (Agilent, Santa Clara, CA) instrument through RNA ScreenTape Assay. Then, RNA was treated with DNase, using TURBO™ DNase kit (Thermo Fisher Scientific), and 0.5 µg of each sample were used as input for sequencing libraries preparation with TruSeq Stranded Total RNA Sample Prep Kit (Illunima Inc., San Diego, CA). HCC1806, MDA-MB-468 and Hs 578T ERβ clones’ libraries were sequenced (single read, 1 x 50 cycles) at a concentration of 8 pJ/pi/sample on the HiSeq 2500 platform (Illumina Inc.) whereas control libraries of MDA-MB-468 Tet-On Advanced clone were sequenced (single read, 1 x 75 cycles) at a concentration of 1.8 pJ/pi/sample on the NextSeq 500 platform (Illumina Inc.). Bioinformatics analysis was performed as described by Tarallo et al. (13). In brief, quality check of the reads was done using FASTQC [https://www.bioinformatics.babraham.ac.uk/projects/fastqc] and STAR (14) software was used for reads alignment on human genome (assembly version hg38, GeneCode version 29). The list of ERβ-regulated transcripts in MDA-MB-468 cells was compared with that of deregulated RNAs in response to doxycycline in Tet-On Advanced MDA-MB-468 cell line. Transcripts showing a fold-change ≥2 between the two conditions were filtered out from the list of ERβ-regulated RNAs. The final data set of receptor-influenced transcripts in MDA-MB-468 cells was compared with that of ERβ-modulated RNAs in response to doxycycline in Tet-On Advanced MDA-MB-468 cell line. Transcripts showing a fold-change ≥2 between the two conditions were filtered out from the list of ERβ-regulated RNAs. The final data set of receptor-influenced transcripts in MDA-MB-468 cells was compared with that of ERβ-modulated RNAs in response to doxycycline in Tet-On Advanced MDA-MB-468 cell line. Transcripts showing a fold-change ≥2 between the two conditions were filtered out from the list of ERβ-regulated RNAs. The final data set of receptor-influenced transcripts in MDA-MB-468 cells was compared with that of ERβ-modulated RNAs in response to doxycycline in Tet-On Advanced MDA-MB-468 cell line.
Co-immunoprecipitation conditions for ERβ interactors (EZH2, SUZ12, EED and RING1) are described in Supporting Information. ERβ− cells were used as negative controls in Estrogen Receptor immunoprecipitation experiments, whereas rabbit IgG (Cat. 02–6102, Thermo Fisher Scientific) was used for this purpose in PRC1/2 complex components immunoprecipitations. After overnight incubation with rotation at 4 °C, beads were sequentially washed 4 times with IPP150 buffer (7.14 mM HEPES pH 7.5, 8.93% glycerol, 150 mM NaCl, 0.53 mM MgCl₂, 0.07 mM EDTA, 18 mM TRIS - HCl pH 7.5, 0.18% Triton X-100, 1 mM PMSF and 1X Protease Inhibitor Mixture (Sigma-Aldrich)) and 3 times with wash buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM PMSF and 1X Protease Inhibitor Mixture (Sigma-Aldrich)). Proteins were eluted from beads by resuspension in sample buffer (0.167 mM Tris-HCl pH 6.8, 10% glycerol, 4% SDS, 3.1% DTT and 0.004% Bromphenol blue) and boiling at 100 °C for 10 min. The following primary antibodies were used for the co-immunoprecipitation reaction: rabbit polyclonal C-terminal anti-ERβ (PA1–313, Thermo Fisher Scientific), anti-EED (17–10034, Millipore, Burlington, MA), anti-SUZ12 (ab12073, Abcam) and anti-RING1 (ab32644, Abcam, Cambridge, UK); rabbit monoclonal anti-EZH2 (D2C9) (5246, Cell Signaling Technology, Danvers, MA).

**Mass Spectrometry and Data Analysis—**MDA-MB-468 and HCC1806 nuclear ERβ+ and ERβ− extracts were used for interaction proteomics experiments. In brief, ERβ was immunoprecipitated in both ERβ+ and ERβ− cells (cultured with and without doxycycline induction respectively) as it is described in Co-Immunoprecipitation section using same amounts of nuclear protein extracts, with the only difference that after the last wash, beads were resuspended in 100 mM ammonium bicarbonate buffer. On beads trypsin digestion was carried out by 0.5 µg trypsin (Promega, Madison, WI) addition to each replicate followed by samples incubation at 37 °C overnight. Additionally, 0.2 µg of trypsin was added to the samples the next day followed by incubation at 37 °C for another 2 h. Peptides were acidified with 1% trifluoroacetic acid, desalted, concentrated with C18 zip tips (Thermo Fisher Scientific) and eluted twice with 0.1% trifluoroacetic acid/50% acetonitrile before drying and solubilization in 7 µl 0.1% formic acid for the following mass spectrometry analysis. Each peptide mixture was analyzed on an Easy nLC1000 nano-LC system connected to a quadrupole-Orbitrap mass spectrometer (QExactive Plus, ThermoElectron, Langenselbold, Germany) equipped with a nanoelectrospray ion source (EasySpray; Thermo Fisher Scientific) as described previously (18). The resulting MS raw files were submitted to the MaxQuant software (version 1.6.1.10) for protein identification and quantitation using the Andromeda search engine. MaxQuant search was done against the UniProt Human database (October 2017, containing 20,239 entries). Carbamidomethyl was set as a fixed modification and protein N-acetylation and methionine oxidation were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages.

**Functional Analyses—**The lists of differentially expressed transcripts were submitted to Ingenuity Pathway Software (IPA, Ingenuity System, www.ingenuity.com; QIAGEN, Hilden, Germany) and investigation of ERβ-modulated disease and disorder gene networks, cellular processes and canonical pathways was carried out. Circos plot was generated using GOPlot (19). Molecular type and function enrichment analysis of the ERβ-interacting protein sets was performed by IPA. Receptor partners were further associated with known protein complexes annotated in CORUM database (20) if at least two complex members were present in the protein data set. For functional interaction networks visualization, FunRich v3.0 was used (http://www.funrich.org).

**Western Blotting—**SDS-PAGE and Western blotting analyses were performed using standard protocols. The following primary antibodies were used for protein detection: mouse monoclonal N-terminal anti-ERβ PPZ0506 (MA5–24807, Thermo Fisher Scientific), anti-FLAG (F3165, Sigma-Aldrich) and anti-β-actin (A1978, Sigma-Aldrich); rabbit polyclonal anti-EED (17–10034, Millipore), anti-SUZ12 (ab12073, Abcam) and anti-RING1 (ab32644–100, Abcam); rabbit monoclonal anti-EZH2 (D2C9) (5246, Cell Signaling Technology), anti-JARID2 (D6H9X) (13594, Cell Signaling Technology) and anti-PHF1 (ab184951, Abcam). WB images shown for each given immunodetection derive from the same autoradiographic film with identical exposure times, and when cropped for representation purposes this is indicated by a dotted line.

**Experimental Design and Statistical Rationale—**Froportion was evaluated at different time points: 0 days (start point), 3, 6, 9 and 12 days (end point) using six biological replicates for each condition tested. Cell cycle assay was performed in triplicate for each condition, 15,000 events were acquired. Colony formation and migration assays were performed in triplicate for each of the conditions tested. One-tailed Student’s t test was applied in all cases to assess statistical significance (p value).

For total RNA profiling experiment, three independent biological replicates were prepared for each treatment. Differentially expressed RNAs (fold change cutoff 1.5 and adjusted p value (p-adj)<0.05) were identified using DESeq2 (21). For alternative splicing events, an Inclusion Level cut-off ≥ 0.1 and FDR<0.05 have been considered.

For interaction proteomics experiment, three biological replicates of each ERβ+ and ERβ− control samples were analyzed. After MaxQuant search against the UniProt Human database, the minimal unique+ razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Statistical analysis was performed using a permutation t test that was applied to MaxQuant protein “Intensities” values to obtain statistically significant difference (FDR<0.05) between ERβ+ and ERβ− samples. ERβ− cells (cultured in the absence of doxycycline) were used as a control for ERβ+ cells (where ERβ expression was induced by doxycycline addition to the culture medium), in order to avoid potential antibody cross-reactivity and strengthen specificity of the interactions. Proteins identified and supported by statistical analysis and showing a fold-change ratio greater than 1.5 when compared with the controls were considered as potential ERβ interactors. The indicated cutoff was chosen based on the average value of the median distribution of the fold-changes, computed in two generated data sets.

In the case of IPA cellular processes and canonical pathways analysis, only networks, processes and pathways showing a p value ≤0.05 were considered statistically significant.

**RESULTS**

**ERβ Detection in Primary TNBC Tissues—**TNBCs are known to lack ERα expression, but several studies reported that in a fraction of patients the tumor expresses its counterpart ERβ. Among the five known ERβ isoforms, only full-length isoform ERβ1 has an intact C-terminal ligand-binding domain and exerts antiproliferative effects, whereas other variants are instead associated with early disease recurrence (22, 23). Recently, two studies focusing on validation of anti-ERβ antibody specificity demonstrated that some commercially available antibody might not be specific for ERβ in different assays (2, 3), in particular by immunohistochemistry (IHC), raising concerns about the conclusions of previous studies focusing on the role of this nuclear receptor in TNBCs.
**Fig. 1.** ERβ1 expression in TNBC biopsies and effect on cell proliferation and cell cycle in TNBC cells. 

**A,** Schematic representation of ERβ1 protein (top) showing the regions recognized by PPZ0506 and PPG5/10 antibodies, respectively, and (lower panels) micrographs of the results of an immunocytochemistry assay performed with PPZ0506 in HCC1806 cells expressing (+Dox) or not (-Dox) ERβ1 (left) or representative ERβ1- and ERβ- TNBCs stained with the same antibody (right). The histogram (center) shows the percentage of TNBCs samples scoring positive for ERβ with PPZ0506, alone (light blue bar) or in combination with PPG5/10 (dark blue bar), or negative to both antibodies (gray). 

**B,** Western blot analysis of ERβ1 expression in HCC1806, MDA-MB-468 and Hs 578T TNBC cells, performed after 9 days of doxycycline exposure.

**C,** Proliferation rate evaluation of ERβ1-expressing HCC1806, MDA-MB-468 and Hs 578T cells, validated by MTT assay in
The monoclonal antibody PPZ0506, recognizing the N terminus of the protein, was demonstrated instead to specifically detect ERβ by IHC ERβ by IHC (2). Therefore, PPZ0506 was selected to evaluate ERβ expression in primary TNBC biopsies. As shown in the left panel of Fig. 1A, this antibody indeed recognizes ERβ1 by immunocytochemistry in fixed HCC1806, showing a positive signal only when expression of this protein is induced in the cells by doxycycline (Fig. 1B). IHC analysis with PPZ0506 of tissue microarrays containing 217 TNBC tissue samples revealed that 27% of these tumors are positive for ERβ that, as shown in the micrographs displayed in the right panels of Fig. 1A, is detectable mainly in the cell nucleus. Interestingly, nearly half of the PPZ0506+ tumors (15% of total; light blue bar) scored positive only with this antibody, whereas the remaining PPZ0506+ (12% of total; dark blue bar) were stained also by PPG5/10, an anti-ERβ antibody directed against the C terminus of the protein, whereas 31% of tumors were positive only for PPG5/10 and the remaining 42% were negative for both antibodies (Fig. 1A and data not shown). Immunohistochemistry staining was performed on sequential slices from the same fixed tissue sample, using each antibody separately.

**ERβ Expression in TNBC Reduces Cell Proliferation and Inhibits Cell Cycle Progression**—To our knowledge, there are no available TNBC cell lines that express detectable full-length ERβ1 levels. For this reason, its activity and functions are generally investigated in stably ERβ expressing cell models, where receptor sequence is introduced by exogenous transfer of its cDNA. In order to study the role of ERβ1 (from now on ERβ) in TNBC, three inducible ERβ-expressing cell models, belonging to different breast cancer subtypes, were generated. In particular, the following cell lines were used: MDA-MB-468, HCC1806 and Hs 578T that belong to basal-like 1, basal-like 2 and claudin-low subtypes, respectively. Clones were produced as described in the experimental procedures section, to obtain ERβ expression in all cell lines generated. To this aim, ERβ-expressing cell lines, together with MDA-MB-468 Tet-On Advanced cell line, were cultured in the presence and absence of doxycycline for 9 days. This experimental condition was chosen as far as we observed pronounced ERβ effect on proliferation and cell cycle in all three cell lines at the indicated time point and at the same time it allows us mimicking constitutive protein expression. Total RNA was isolated and RNA-Se- quencing (RNA-Seq) was carried out as described in the Experimental Procedures section. For each cell line, ERβ-specific changes were determined. Background doxycycline effect was taken into consideration by analyzing MDA-MB-468 Tet-On Advanced cells and modulated transcripts (fold-change ratio ≥2) were excluded from the list of ERβ-regulated genes as described in the Experimental Procedures section.

In order to characterize the generated cell lines and to determine ERβ effect on cell proliferation, MTT assay was applied for cells grown in the presence (ERβ+) and absence (ERβ-) of selected doxycycline concentrations. We demonstrated that ERβ+ cells grow considerably slower than ERβ- ones (Fig. 1C), whereas the parental Tet-On Advanced-expressing cell lines, used for generation of ERβ-expressing clones, did not show reduced proliferation on doxycycline exposure (supplemental Fig. S1A). Interestingly, the basal-like 1 cell line MDA-MB-468 demonstrated the highest degree of growth inhibition (about 70% at 12th day) on ERβ induction (Fig. 1C). As shown in Fig. 1C, growth inhibition by ERβ shows slow kinetics, reaching significant values between days 6–9 of Doxy treatment for Hs578T and days 9–12 for MDA-MB-468 and HCC1806 cells.

To check whether this growth inhibition may be caused by a reduction of cell cycle kinetics because of ERβ activity, cell cycle phase distribution analysis was performed before and after induction of ERβ expression by doxycycline and results confirmed this hypothesis. We observed an accumulation of ERβ+ cells in G1 phase, accompanied by a parallel reduction of S and G2/M phases in the cultures (Fig. 1D). It is worthwhile to note that MDA-MB-468 and Hs 578T cell lines exhibited a slight increase (p-val<0.05) of cell number in sub-G0 cell cycle phase, indirectly indicating ERβ-mediated increase of programmed cell death. Again, cell cycle profiles of Tet-On Advanced-expressing cell lines were not influenced by doxycycline exposure (supplemental Fig. S1B). The results recapitulate the ability of Estrogen Receptor beta to inhibit cell proliferation by cell cycle arrest, indicating that these cellular models are suitable for the investigation of ERβ role in TNBC cells.

**ERβ Expression Modulates Transcriptome of TNBC Cells, Determining Cholesterol Biosynthesis Pathway Downregulation**—To further investigate the molecular bases of ERβ action in TNBC, we assessed receptor-specific modulation of mRNA expression in all cell lines generated. To this aim, ERβ-expressing TNBC cells, together with MDA-MB-468 Tet-On Advanced cell line, were cultured in the presence and absence of doxycycline for 9 days. This experimental condition was chosen as far as we observed pronounced ERβ effect on proliferation and cell cycle in all three cell lines at the indicated time point and at the same time it allows us mimicking constitutive protein expression. Total RNA was isolated and RNA-Se- quencing (RNA-Seq) was carried out as described in the Experimental Procedures section. For each cell line, ERβ-specific changes were determined. Background doxycycline effect was taken into consideration by analyzing MDA-MB-468 Tet-On Advanced cells and modulated transcripts (fold-change ratio ≥2) were excluded from the list of ERβ-regulated genes as described in the Experimental Procedures section. As far as ERβ effect was more pronounced in MDA-MB-468 respect to the other cell lines tested, it was chosen as the headliner in further analyses. A total number of 2397 and 2205 RNAs were found up- and downregulated respectively as a result of ERβ expression in MDA-MB-468 (fold-change cut-off 1.3 , padj≤0.05) (supplemental Table S1). Signaling pathway analysis revealed statistically significant deregulation of several pathways, among which estrogen and cholesterol biosynthesis pathways demonstrated the highest level of deregulation (z-score <-2.0). Two other pathways (Wnt/Ca+ and

exponentially growing conditions (–: no doxycycline; +: + doxycycline). D, Cell cycle phase distribution in exponentially growing HCC1806, MDA-MB-468 and Hs 578T ERβ expressing cell cultures before (-) and after (+) doxycycline induction. Percentages of cells, present in sub-G0, G1, S, and G2/M phases, determined by flow cytometry after propidium iodide (PI) staining. Stars indicate p values (* < 0.05; ** < 0.01; *** < 0.001).
Sirtuin), instead, demonstrated a tendency to be up-regulated (z-score >1) (supplemental Fig. S2A). To evaluate our results, we compared our list of differentially expressed genes with the one published by Shanle et al., where the same TNBC cell line was used for inducible ERβ clone generation (8). Despite different experimental settings (the use of estradiol-free medium for cell cultivation and shorter time of ERβ induction), the two data sets revealed 942 commonly deregulated genes, among which 735 (78%) showed the same behavior (up or downregulation), indicating the presence of a high degree of correlation between the two studies. To further characterize ERβ impact on TNBC transcriptome, we evaluated if the receptor expression induces alternative splicing events in this breast cancer subtype, like it does in case of hormone-responsive breast cancer (15). To this aim, we compared RNA profiles of MDA-MB-468 ERβ+ and ERβ- cells and identified a total of 440 alternative splicing events, among which the following ones were present: exon skipping, intron retention, the use of alternative 5’ donor and 3’ acceptor sites and mutually exclusive exons (supplemental Fig. S2B). Splicing pattern was like the one observed in MCF-7 cell line, with exon skipping being the most frequent event (supplemental Fig. S2B).

Comparison of differentially expressed genes in MDA-MB-468 (fold-change cut-off ≥ 1.3, FDR < 0.05) with ERβ-regulated transcripts (FDR < 0.05) in the other cell lines revealed that HCC1806 and Hs 578T are characterized by less marked changes of mRNA profiles on ERβ expression (Fig. 2A, supplemental Tables S2 and S3 for HCC1806 and Hs 578T respectively). A total number of 1820 (857 up and 963 down-regulated) transcripts showed a common response to ERβ in at least two cell lines with several of them, shown in Fig. 2B, displaying the same behavior (fold-change cut-off 1.2, FDR < 0.05) in all three cell lines (supplemental Table S4). The fact that a higher number of differentially expressed transcripts in the presence of ERβ were down and not up-regulated, reflects the well-known repressive effect of ERβ on gene expression. Among commonly deregulated transcripts in all three cell lines, we found some known to be hallmarks of TNBC, such as IGFBP3, ID1, TM4SF1, TSPO, and ABAT. Interestingly, all the above-mentioned mRNAs were down-regulated on ERβ expression, with the notable exception of ABAT. Functional annotation by Ingenuity Comparative Analysis performed on differentially expressed transcripts, revealed that apart from regulation of proliferation and cell cycle progression, ERβ controls the expression of genes involved in tumor progression, invasion, angiogenesis, cell death, apoptosis and metabolism of steroids and cholesterol (Fig. 2C). Comparison of influenced signaling pathways (supplemental Fig. S2C) corroborated the result obtained for MDA-MB-468 cell line alone (supplemental Fig. S2A) and confirmed that sirtuin, cholesterol and estrogen biosynthesis pathways are characterized by the same behavior in all three TNBC experimental models, indicating that their deregulation represents a common effect of ERβ expression in TNBC cells. Importantly, all three branches of cholesterol biosynthesis pathway were downregulated (supplemental Fig. S2C). Analysis of genes known to participate in cholesterol biogenesis revealed down-regulation also of SREBF1 gene, encoding for an upstream regulator of cholesterol signaling pathway (supplemental Fig. S2D). Indeed, it encodes for a transcription factor driving the expression of both cholesterol biosynthesis and fatty acid synthesis genes, indicating a profound regulation of this signaling pathway by ERβ (supplemental Fig. S2E). On the other hand, some genes displayed discordant response to ERβ between the three cell lines, suggesting subtype-specific effects of the receptor (supplemental Table S4 and supplemental Fig. S3). Finally, considering the possibility that the activity of ERβ may influence the global TNBC character of the cell, a deep learning-based framework subtype classification was performed before and after ERβ induction with DeepCC that employs PAM50 for intrinsic subtype classification and whose classification performance has been trained on TCGA RNA-Seq data sets (24). This method, that represents a biological knowledge-based framework for cancer molecular subclassification, considers the expression patterns of a given tumoral cell or tissue to classify it according to defined parameters. The results obtained indicate that the presence of ERβ causes changes leading to re-classification of all three cell lines, with a repositioning from a TNBC-associated subtype to one like Luminal A (probability score > 99%).

**ERβ Inhibits TNBC Cell Clonogenic and Migratory Properties**—Functional analysis of the RNA-seq results suggested that a significant number of genes and signaling pathways downregulated by ERβ in all three TNBC cell lines studied are involved in cell migration, invasion and viability. This was confirmed experimentally by measuring the clonogenic and migratory potential of these cells before and after ERβ induction. The results obtained show that this is indeed the case, as the expression of the receptor caused a significant reduction of clonogenic potential (supplemental Fig. S4A) and migratory capability (supplemental Fig. S4B) in all three cell lines.

**ERβ Binds Promoter of Downregulated Genes Including SREBF1**—As far as ERβ is a transcription factor, able for DNA binding with further regulation of gene activity, we hypothesized that transcriptional downregulation, including transcripts belonging to cholesterol biosynthesis pathway, may be directly controlled by receptor binding to regulatory elements of its genes. To investigate it and to further characterize the functional role of ERβ in TNBC, ChIP-Sequencing (ChIP-Seq) was performed with an anti-Flag antibody in MDA-MB-468 ERβ+ and ERβ- cells as control. Results showed the ability of ERβ to interact with the TNBC cell genome and led us to identify a total number of 15843 binding sites (supplemental Table S5), as displayed in supplemental Fig. S5A. The density plot reported in supplemental Fig. S5B shows the signal distribution profile of ERβ-binding sites, indicating transcription start site-specific association of the receptor and highlighting the prevalence of its positioning in gene promoter regions. An
Fig. 2. Transcriptome analysis of ERβ-expressing TNBC cells. A, Venn diagram of specific and common downregulated (upper panel) and up-regulated (lower panel) transcripts in ERβ-expressing MDA-MB-468 cells (fold-change cutoff 1.3, false discovery rate (FDR) ≤ 0.05), HCC1806 and Hs 578T cells (FDR ≤ 0.05) compared with controls; B, Heatmap of common downregulated (green) and up-regulated (red) transcripts in the cell lines analyzed (FC ≥ 1.2, FDR ≤ 0.05 in at least two cell lines). C, Heatmap depicting Z-score activation/inhibition of the most significantly influenced functional processes in ERβ-expressing MDA-MB-468, HCC1806 and Hs 578T cells.
analysis of overrepresented transcription factor binding motifs among the binding sites revealed, as expected, the highest accumulation of EREs (estrogen response elements) and ERE-like sequences. Enrichment for the following transcription factor binding sequences was observed: RARA, RARB, PPARG, ESRRSA, ESRRB, nuclear receptors NR2F2, NR2F6, and NR4A1 (Supplemental Fig. S5C), that are known to recognize DNA motifs similar to ERE. Genome distribution analysis of the receptor binding sites showed preferential ERβ positioning in gene introns (~47%) and intergenic regions (~42%) with around 6% sites mapping to gene promoters (supplemental Fig. S5D). We further analyzed if commonly deregulated transcripts in all tested TNBC cell lines represent primary ERβ-responsive genes, namely hold a receptor binding site within their promoter region. In this way, we found that out of 843 ERβ-responsive RNAs, 280 represent direct targets of this nuclear receptor, 68% binding the receptor via one or more EREs. IPA functional annotation analysis performed on this gene set corroborated the results previously obtained by functional assays and RNA profiling, again suggesting the involvement of this receptor in regulation of cell cycle, growth and proliferation, cell death and survival, metabolic processes (supplemental Fig. S5E). Here, many novel ERβ-influenced processes were present, among which molecular transport, cell signaling and interaction, indicating a possible role of the receptor in intercellular communication. Interestingly, among downregulated genes in all three TNBC cell lines, having ERβ binding sites in their gene body, we found, TM4SF1 and ABAT, whose association with TNBC was previously described by others, as it was mentioned above. Interestingly, expression of several components of the cholesterol biosynthesis and signaling pathway present in the list of ERβ-regulated genes is inhibited by the receptor in all TNBC cell lines analyzed and, in several cases, the transcription unit harbors a receptor binding site. CYP51A1, DHCR7, PMVK, and SREBF1 genes show an ERβ binding site in the promoter region (supplemental Fig. S5F and data not shown), whereas DHCR24 and LSS carry it in the second and third intron respectively. Sequence analysis of the ERβ binding sites of all aforementioned genes revealed the presence of ERE motifs in DHCR7, LSS, PMVK, and SREBF1. Altogether, these data provide mechanistic evidence of cholesterol biosynthesis regulation by ERβ in TNBC cells. Further, as the SREBF mRNA isoform SREBP1a, known to activate both fatty acid and triglyceride biosynthesis together with the cholesterol pathway (25, 26), it has been shown to be highly expressed in proliferating cells, including particular cancer cells (27), confirming that direct downregulation of the corresponding gene transcription by ERβ may underlie the anti-proliferative actions of the receptor in TNBC cells.

Mapping Nuclear ERβ Interactome Reveals Its Association with Polycomb Repressor Complexes 1 and 2—To unveil molecular bases of ERβ action in TNBC, and thus its impact on transcriptome deregulation, characterization of receptor interactome in MDA-MB-468 cell line was performed. To this aim, native nuclear protein complexes were purified by immunoprecipitation, analyzed by nano LC-MS/MS and mapped (Fig. 3A). Potential contaminants, such as keratins and immunoglobulins, were excluded from further analysis. Data analysis allowed us to identify 1023 potential ERβ interactors in this cell line (supplemental Table S6). The following known receptor molecular partners were present among ERβ interactors: NRP1 (also called RIP140) (28), NCOA5 and NCOA6 (also known as CIA and RAP250 respectively (29, 30), DNT-TIP2 (otherwise known as ERBP) (31), KAT5 (also referred to as TIP60) (32), mediator complex (33) and RBM39 (which alternative name is CAPER) (34). Molecular type classification of receptor-associated proteins revealed the prevalence of enzymes and transcription factors within ERβ interactome (Fig. 3B). Functional enrichment analysis performed by IPA, elucidated the involvement of receptor partners in multiple molecular functions, both relevant to cancer and functional processes known to be fulfilled by this nuclear receptor, such as RNA post-transcriptional regulation, gene expression, DNA replication, cell cycle, cell death and survival and protein synthesis (Fig. 3B). Comparative analysis of the present ERβ interactome with the one of hormone-responsive cell line MCF7 (35), revealed the significant similarity of the two data sets. To support the results obtained for ERβ interactome in MDA-MB-468, we performed the same experiment in another TNBC cell line: the HCC1806. Here, we successfully identified 462 ERβ molecular partners (supplemental Table S7), among which 174 were common for two cell lines analyzed (supplemental Fig. S6A). Classification of HCC1806 ERβ interactome demonstrated enrichment of enzymes and transcription factors also in this data set (supplemental Fig. S6B). A comparison of molecular functions exerted by ERβ partners in these two cell lines was basically the same (supplemental Fig. S6C), demonstrating the high similarity of ERβ functional activity in both cell lines.

The ERβ partner proteins identified here are likely to be involved in multiple pathways controlled by the receptor in TNBC cell nuclei, such as those controlling cell proliferation and migration. An upstream regulator analysis based on the gene expression changes detected in the three cell lines profiled here supported this possibility, as it highlighted the following receptor interactors: CTNNB1, a coactivator of CREB, the transcription factors MTA1, E2F6, STAT3, RUNX1, and EGR1 and the chromatin remodeling factors HDAC1, HDAC2, NCOA6, SMARCA2, and EZH2, all connected to transcription regulatory pathways known to control these cellular functions in cancer cells. The generated data sets were further screened for the presence of protein complexes annotated in CORUM database (20), leading to identification of multicomponent assemblies associated to ERβ in TNBC cells nuclei involved in DNA replication, transcription regulation (RNA polymerase and mediator complex), RNA splicing and post-transcriptional regu-
FIG. 3. Characterization of ERβ interactome in MDA-MB-468 cells. A, Summary of the experimental workflow applied to generate the protein data sets. B, Classification and Functional enrichment analysis of ERβ molecular partners performed by IPA (B-H: Benjamini-Hochberg)

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lation (spliceosome and exosome complex, supplemental Fig. S7 and supplemental Table S8). These results let us suggest that association with ERβ could drive these proteins to DNA regulatory elements (e.g. enhancers and promoters) or RNAs to exert cooperative regulation of transcriptional activity and post-transcriptional RNA processing. Functionality of the observed protein associations was confirmed by the fact that ERβ expression induces alternative splicing events, as described above (supplemental Fig. S2B). Among ERβ molecular partners, we identified several proteins belonging to different chromatin remodelling assemblies, involved in both transcriptional activation (COMPASS and SWI/SNF) and repression (NCOR1, LSD1, Polycomb Repressor Complexes 1 and 2 (PRC1/2)) (Fig. 3C). Interestingly, both transcriptional activator complexes identified are known to antagonize PRC1/2 function, whereas transcriptional repressor LSD1 interacts with PRC2 via the long non-coding RNA HOTAIR and functions through demethylation of the H3K4 histone mark of transcriptionally active chromatin, whereas at the same time PRC2 methylates H3K27 leading to chromatin condensation (36–38). Moreover, the core PRC2 component EZH2 is a histone methyltransferase known as a master regulator of chromatin rearrangements, involved in cell survival, proliferation, epithelial to mesenchimal transition, invasion and drug resistance of cancer cells (39). EZH2 high expression was also found in a wide range of cancer types, such as lymphoma, sarcoma, breast (including TNBC), prostate, bladder, colon, lung, and pancreatic cancers, where it correlates with advanced disease stages and poor prognosis (40, 41). Thus, EZH2 inhibitors represent potential drugs for anticancer therapy, some of them being currently validated in pre-clinical and clinical trials (39). On the other hand, PRC1 is required for stabilizing PRC2-introduced epigenetic silencing and has been found recruited to oncogenic active enhancers in breast cancer cells to regulate their activity (42).

Given the key roles of PRCs in breast cancer, we further focused on the interaction between ERβ and PRC1/2 complexes. Components of PRC2 and of canonical and non-canonical PRC1 (cPRC1 and ncPRC1) complexes were found among ERβ interactors in both cell lines. In particular, the core PRC2 components SUZ12, EED, and EZH2 and the accessory proteins JARID2, PHF1, and RBBP7 were present at least in one of the two data sets, together with the cPRC1 and ncPRC1-participating proteins RING1, PHF1, and RBBP7 were present in both experimental conditions in both cell lines and the results, reported in Fig. 4 and supplemental Fig. S6D, confirmed ERβ association with PRC2 in MDA-MB-
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468 and HCC1806 cell lines. Interestingly, ERβ+ samples were enriched in both PHF1 and JARID2 proteins, that according to Holoch et al. (44) represent PRC2.1 and PRC2.1-specific components, leading us to conclude that ERβ interacts with both PRC2 complex subtypes in both cell lines analyzed. The interaction was further confirmed in the same cell lines by immunoprecipitation of key PRC2 components EED, EZH2 and SUZ12 (Fig. 4 and supplemental Fig. 6D).

Similarly, ERβ-PRC1 association was validated by RING immunoprecipitation (Fig. 4 and supplemental Fig. S6D). EED and RING1 association with ERβ was also confirmed by co-immunoprecipitation (Fig. 4 and supplemental Fig. S6D). Finally, PRC1 and PRC2.1, but not PRC2.2, association with ERβ were detectable also in Hs 578T cells probably reflecting subtle differences between TNBC subtypes (supplemental Fig. S8). Finally, it is worth mentioning that among potential ERβ interactors we found several proteins involved in biosynthetic processes, including the lamin B receptor (LBR), essential for cholesterol biosynthesis (45).

Finally, because our analyses indicate EZH2 as a potential regulator of gene expression changes via association with ERβ, we evaluated if the interaction of this protein with the receptor indeed occurs on the chromatin, leading to both proteins binding together to regulatory elements of ERβ-responsive genes. To this aim, ChIP-Western blotting and ChIP-qPCR were performed in ERβ- and ERβ+ MDA-MB-468 cells. Results, shown in supplemental Fig. S9A, demonstrated that the association between the two proteins can be detected also on isolated chromatin. Further, ChIP-qPCR focusing on the ERβ binding sites identified by ChIP-Seq on SREBF1, CYP51A1 and DHCR7 transcription units (supplemental Fig. S5F) revealed co-recruitment of both factors to the first intron of SREBF1 and the promoter regions of CYP51A1 and DHCR7 (supplemental Fig. S9B). On the other hand, EZH2 was not found associated with the receptor in the SREBF1 promoter region, in correspondence of one of the strongest ERβ binding sites mapped in this cell line. This could indicate that the strength of ERβ binding to chromatin might be a factor influencing its ability to interact with other proteins or, alternatively, that the nature/composition of its interacting complex could affect the kinetics of receptor association to the genome.

DISCUSSION

TNBC is a heterogeneous group, characterized by the lack of ERα, PR, and HER2/neu, high proliferative rate, low response to current therapies and elevated risk of recurrence (46). In this study, using validated antibodies, we demonstrated that full-length ERβ is expressed in a sizeable fraction of TNBCs, in agreement with other studies showing that these tumors can carry this receptor subtype, where it exerts oncosuppressive activities (47, 48). Thus, the presence of ERβ expression may represent a potential advantage for TNBC patients, because ERβ activation with agonist ligands could enhance its antiproliferative activity and, in early cancer stages, amplify the receptor anti-tumoral effects (49). A good example of such drug is the selective ERβ agonist LY500307, currently tested in phase 2 clinical trials for the treatment of estradiol withdrawal-induced mood symptoms in women with post-perimenopausal depression, raising the possibility of its use also in TNBC patients carrying ERβ+ neoplasms. We investigated here the effects of ERβ in TNBC cells by combining interactional proteomics and genomics, in order to functionally elucidate the molecular mechanism of receptor action. ERβ was found to inhibit proliferation, migration and clonogenicity of TNBC cells belonging to three distinct subtypes by reducing cell cycle kinetics with an accumulation of cells in G1 cell cycle phase, as suggested by previous studies (8, 50). Interestingly, ERβ-induced growth-inhibition occurs in all TNBC cell lines tested, although with slightly different kinetics, suggesting that the oncosuppressive effects of the receptor may be independent from the cellular background and, more important, from the TNBC subtype.

Characterization of the gene expression changes induced by ERβ led to the identification of genes whose expression is greatly influenced by the receptor in TNBC cells, including several genes known to be critical in TNBC biology, such as IGFBP3, ID1, NRP2, MAPK12, KCNN1, TM4SF1, Tsps, and ABAT, all of which (except ABAT) result to be downregulated by the receptor. IGFBP3 was the most significantly ERβ-downregulated gene in all three TNBC cell lines (fold-change in the range of −3.69 to −9.69). Interestingly, its role in basal-like TNBC cells was previously reported, showing that targeting of oncogenic signaling exerted by this protein with SpHK and EGFR kinase inhibitors enhances mouse survival and increased apoptosis (51). In another study, targeting IGFBP3-mediated DNA repair function was shown to enhance chemosensitivity of basal-like TNBC (52). On the other hand, a high level of ID1 expression has been associated with stemness and EMT transition, and this protein is known as a mediator of lung metastatic colonization of TNBCs, together with its closely related family member ID3 (53, 54). Further, NRP2 was shown to be expressed preferentially in tumor-initiating cells, whereas MAPK12 activation is known to promote cancer development and progression by stimulation of cancer stem-like cell expansion. These two proteins were proposed as novel therapeutic targets in TNBC (55, 56). Inhibition of KCNN4 expression by specific siRNAs significantly inhibited cell proliferation, migration and promoted apoptosis, whereas its enhanced expression was correlated to EMT in MDA-MB-231 cells (57), whereas high levels of TM4SF1 mRNA correlated with poor TNBC prognosis (58). The gene encoding TSPO, a protein generally associated with advanced breast cancer stages and expressed at higher levels in ER-negative compared with ER-positive tumors, was also found to be downregulated by ERβ in all three TNBC cell lines studied here. Interestingly, TSPO overexpression significantly altered cell migration and combined treatment of TSPO li-
gands with the antiglycolytic drug lonidamine lead to decreased viability of ER-negative breast cancer cell lines (59), whereas a synergistic inhibition in TNBC cell and tumor growth was achieved by combining these ligands with drugs targeting the cannabinoid receptor CB2R (60). Among the genes up-regulated by ERβ in all three cell lines, we noticed ABAT, whose decreased expression was correlated with shortened recurrence-free survival in both ER+ and ER-breast cancer patients and induced tumorigenic and metastatic advantages to basal-like breast cancer by activating the GABA-mediated Ca2+-NFAT1 axis (61). All these gene expression changes in response to ERβ induction support the oncosuppressive role of this transcription factor in TNBC, marking its significance in TNBC biology. ERβ expression causes also inhibition of all three branches of the cholesterol biosynthesis pathway, as demonstrated by a strong down-regulation of the genes encoding most of the enzymes catalyzing different steps of this process, as summarized in supplemental Fig. S2E. Supporting this possibility, genome-wide mapping of ERβ binding to MDA-MB-468 cell genome revealed that SREBF1 and other genes participating in cholesterol biosynthesis are directly regulated by receptor binding to their promoter and/or other regions (supplemental Table S5 and supplemental Fig. S5). More interestingly, activation of the cholesterol biosynthesis pathway was found relevant for BC responses to extracellular stimuli (62) and disruption of cholesterol-containing lipid rafts induced apoptosis, expression of WNT receptor LRP6, survivin and common apoptotic markers in TNBC cells (63). Moreover, TNBC cells treatment with natural dietary compounds exerting anti-cancer activity resulted in reduced accumulation of esterified cholesterol caused by decreased SREBP1, SREBP2, FASN and ACAT-1 levels (64). Higher expression of genes involved in cholesterol biosynthesis has been found associated with shorter relapse-free survival in basal-like breast cancer, whereas patient-derived mammospheres exhibit increased de novo cholesterol synthesis and their formation is reduced by genetic or chemical inhibition of this pathway (65). Interestingly, another epigenetic target of ERβ is represented by the histone deacetylases sirtuins, whose effects on lipid metabolism in normal and cancer cells are well-known. SIRT1 directly deacetylates SREBPs and thereby impacts on SREBP ubiquitination, protein stability and activity on its target genes. SIRT1 chemical activators inhibit SREBP target gene expression, leading to a decrease of lipid and cholesterol levels in mice liver (66). Moreover, small molecule activators of sirtuins are considered as promising therapeutic agents for the treatment of metabolic diseases (67). The molecular mechanisms mediating the above described effects of ERβ on gene activity and, as a consequence, on TNBC cell functions find an explanation in the nature and composition of the nuclear interactome of this receptor identified by interaction proteomics (Fig. 3B–3C, supplemental Fig. S6 to S8 and supplemental Tables S6 and S7), in particular when combined with distribution of ERβ cistrome in the TNBC cells genome.

Among potential ERβ interactors, we identified a network of transcription factors and chromatin remodeling proteins involved in the regulation of proliferation, cell cycle and migration that the IPA upstream transcriptional regulator module revealed to be potentially involved in transcriptome reprogramming by ERβ-mediated in TNBC cells. These include, among others: the CREB/CTNNB1 complex, considered important for TNBC biology and response to therapy (68), whose association with FOXM1 modulates cancer stem cells phenotype of TNBC (69); the tumor suppressor CDKN2A that acts through interaction with cyclin-dependent kinases to inhibit cell cycle progression leading to growth inhibition (70); the transcription cofactor MTA1, that is known to bind ERα in hormone-responsive BC cells thereby inhibiting the ability of estradiol to stimulate estrogen receptor-mediated transcription (71); the oncogenic transcription factor STAT3, whose activity has been linked with cancer initiation, progression, metastasis, chemoresistance and immune evasion and plays a critical role in TNBC, where its inhibitors have shown efficacy in inhibiting tumor growth and metastasis (72); the core binding factor RUNX1, already shown to play an important role in BC (73). Interestingly, the interaction proteomics results described here comprise several known ERβ-partners present in the BioGRID database (ca 54%), that although identified in other cell types provide indirect confirmation of our findings.

One of the most interesting findings provided here is ERβ association with key components of the multiprotein epigenetic complexes COMPASS, SWI/SNF, NCO1/HDAC3, LSD1 and PRC1/2 (Fig. 3C, 5 and supplemental Fig. S6D and S8), all known to be involved in chromatin remodeling and transcriptional and post-transcriptional gene regulation in normal and transformed cells including TNBC. It was recently shown that the H3K4 histone methyltransferase MLL4, a component of one of the COMPASS complexes, regulates a cohort of oncogenes and pro-metastatic genes linked to BC, including TNBC, cell proliferation and invasion in association with the H3K27 demethylase UTX (also known as KDM6A) (74). Knock-down of either one of these two genes significantly decreased cell proliferation and invasiveness, both in vitro and in vivo, whereas their high expression was found to associate with poor prognosis. On the other hand, the BRG1 and BRM ATP-dependent chromatin remodeling enzymes, key catalytic subunits of the SWI/SNF complex, were found to be overexpressed in most primary BCs, including TNBCs, where inactivation of the corresponding genes affects tumor formation in vivo and cell proliferation in vitro (75). The importance of another two ERβ partners - HDAC5 and complex LSD1 in TNBC progression was demonstrated by the fact that combinational treatment with HDAC5 and LSD1 inhibitors acted synergically to inhibit the growth of TNBC xenografts in mice (76).
Among the protein complexes identified in the ERβ interactome mapped here, of particular importance appear the polycomb repressive complexes (PRC) 1 and 2, as these are well known to control key features of TNBCs, mediated by their activity on chromatin repression (42) resulting in increased cell proliferation, tumor invasiveness, poor differentiation and aggressiveness in BC, typical signatures of TNBCs (73–75, 77–79). The ability of EZH2 to interact with ERβ to control gene transcription, namely to inhibit expression of cholesterol biosynthesis pathway genes, is supported by the finding that both proteins can be recruited to regulatory elements of at least three genes involved in cholesterol biosynthesis and downregulated in ERβ+ cells. When combined, these evidences point to the possibility that ERβ association with regulatory factors may likely cause their repositioning within TNBC cell genome, leading to reprogramming of the cell fate program to a less aggressive phenotype.

It should be mentioned that exogenous protein expression in cell models do not allow to reproduce an exact expression range of the expressed product that matches the one present in tumor samples, so that validations in alternative experimental models are generally required. For this reason, the most relevant results obtained here will need now further investigation in in vivo in animal models, patient-derived xenografts and tumor biopsies. Another interesting direction of future research, guided by results obtained here on chromating repressive complex association with the receptor and inhibition of cholesterol biosynthesis, will concern the effects of other known ERβ isoforms in TNBCs.

In conclusion, the results reported here provide for the first time a comprehensive catalogue of ERβ effects in the three most representative TNBC subtypes, that helps elucidate the oncosuppressive actions of this nuclear receptor in this aggressive cancer type. Further, available genetic and biochemical data point out to several molecular targets potentially exploitable to interfere with specific features of TNBC cells.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
The study protocol received approval by the Ethics Committee of the Istituto Nazionale Tumori ‘Fondazione Giovanni Pascale’ (protocol n.er CEI/393/15) before the beginning of the study, in accordance with The Code of Ethics of the Declaration of Helsinki, and informed consent was obtained from all patients involved.

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DATA AVAILABILITY
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (73) with the data set identifier PXD014488 for interaction proteomics data sets comprising CTRLs and ERβ samples from MDA-MB-468 and HCC1806 cell lines. The mass spectrometry proteomics data have been also deposited in MS-Viewer (87) with Search Keys identifiers: 9fyu9hs85b and qojtj2yzd9b for the HCC1806 and MDA-MB-468 data sets respectively. RNAseq data from MDA-MB-468, HCC1806 and Hs 578T and ChIP-seq data from MDA-MB-468 cells were deposited to ArrayExpress with the accession numbers E-MTAB-8055 and E-MTAB-8056 respectively.

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This article contains supplemental Figures and Tables.

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