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Adipocyte/Tumor cell crosstalk via IGF-1/TXNIP axis promotes malignancy and endocrine resistance in breast cancer

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Abstract

Background Despite significant improvements in the outcome of Estrogen Receptor (ER) α-positive breast cancer (BC) following the use of endocrine therapies, resistance remains a major challenge. Clinical studies proved that obesity, in addition to promote BC progression, is associated with a reduced efficacy to these treatments, but mechanisms remain unclear.

Methods We used co-culture systems followed by validation through an ‘ex vivo’ model of human mammary obese (Ob) adipocytes and obese endocrine-resistant metastatic Patient-Derived Organoids (PDOs). Transcriptomics with MixOmics-MINT and MetaCore Functional Tools along with lentiviral and pharmacological approaches provide insights into mechanisms. Clinical relevance was investigated using public datasets, transcriptome-based ($n=375$), and immunohistochemistry-based ($n=65$) evaluations.

Results In a model of co-culture, we demonstrated that conditioned media (CM) released by 3T3-L1A adipocytes reduced the sensitivity of parental MCF-7 BC cells to the inhibitory effects of Tamoxifen (Tam) on growth, motility and invasion and significantly increased the proliferative, motile and invasive phenotype of Tam-resistant (TR) BC cells. Transcriptomics identified TXNIP (Thioredoxin-interacting protein), a known tumor suppressor gene, as a network central hub, that was significantly down-regulated in CM-treated MCF-7 and TR cells. Accordingly, TXNIP expression was negatively correlated with Body Mass Index (BMI) in BC patients. Lentiviral TXNIP overexpression and pharmacological induction of TXNIP (i.e. SAHA) or the blockade of insulin-like growth factor-I (IGF-1) signaling, an

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obesity hallmark able to affect TXNIP expression, reversed CM-mediated effects. TXNIP down-regulation, proliferation and motility in TR cells were exacerbated by CM derived from Ob 3T3-L1A, and combination of an IGF-1 inhibitor and SAHA abrogated Ob-CM activities. Results were also validated in aromatase inhibitor-resistant BC cells. The effectiveness of IGF-1/TXNIP axis inhibition was confirmed using an 'ex vivo' model of human mammary obese adipocytes and PDO models. Finally, retrospective analyses demonstrated that an IGF-1_{high}/TXNIP_{low} signature was correlated with poorer survival in endocrine-treated BC patients.

Conclusions In conclusion, our study sheds new light on adipocyte/BC cell crosstalk, underscoring the potential of targeting IGF-1/TXNIP axis to block this harmful connection, especially in the context of obesity.

Keywords Breast Cancer, Endocrine resistance, Adipocytes, Tumor microenvironment, Obesity, IGF-1, TXNIP

Background

The global obesity epidemic, or “globesity”, poses a major public health and economic burden. Obesity is a significant risk factor for diet-related non-communicable diseases (e.g., diabetes, cardiovascular diseases) and is linked to ~30% of new cancer cases and 15–20% of cancer-related deaths [1]. Increasing evidence associates high body fat with elevated risk and poor outcomes in breast cancer (BC), particularly in postmenopausal women with ER α /PR-positive tumors [2, 3]. High Body Mass Index (BMI) correlates with large-sized tumors, metastatic lymph nodes, recurrences, and worse prognosis [4, 5]. Conversely, healthy lifestyles (i.e. healthy dietary patterns, and physical activity) were associated with a reduced BC morbidity and mortality [6]. In the adjuvant setting, data also highlighted low clinical benefits of anti-tumor therapies in women with obesity, including endocrine treatments in ER α -positive BC patients [7]. Although literature data in this area are not conclusive, high BMI was significantly associated with poorer outcome in women treated with the antiestrogen Tamoxifen (Tam) and/or Aromatase Inhibitors (AIs) [7]. Recently, BMI in the obese range was associated with a significant enrichment of endocrine therapy-resistant tumors [8].

Several experimental studies have attempted to define the complex molecular interplay between obesity, breast carcinogenesis and endocrine therapy resistance [7]. Important host extrinsic mediators, encompassing adipokines, IGF-I (Insulin-like Growth Factor I) signaling, hormone pathways, lipid substrates, and inflammatory burden, may interact with cancer cell-intrinsic signalings (i.e., MAPK, PI3K/Akt) and transcriptional factors (i.e., NF- κ B, HIF-1 α) to boost BC progression and therapy resistance [7]. In this context, adipocytes are progressively taking center stage for their role in systemic obesity-associated changes and tumor-stroma crosstalk. Using co-culture models, Bougaret and colleagues demonstrated that human mammary obese adipocytes interfere with the anti-proliferative effects of Tam in MCF-7 BC cells [9]. Co-culturing MCF-7 cells with adipocytes exposed to high glucose increased CTGF (Connective Tissue Growth Factor) expression and decreased Tam

responsiveness in terms of BC cell viability [10]. Adipose microenvironment increased MCF-7 cell proliferation in the presence of Tam, Fulvestrant [11] or the AI Letrozole [12], and stromal cells from obese women affected AI response in an organotypic mammary duct model [13].

Despite this knowledge, the influence of adipocytes and their secretome on endocrine therapy efficacy was not properly addressed. Our findings bring novel insights on adipocytes and mammary cancer cell crosstalk during endocrine therapy, highlighting IGF-1/TXNIP (Thioredoxin Interacting Protein) axis as a potential heavyweight player to be targeted to block this harmful connection, particularly in the context of obesity.

Methods

Reagents, and antibodies

The following antibodies and reagents were used: anti-GAPDH (sc-47724, Santa Cruz Biotechnology), anti- β -Actin (sc-69879, Santa Cruz Biotechnology), anti-TXNIP (D5F3E, #14715S, Cell Signaling Technology), anti-IGF-1R (#9750, Cell Signaling Technology), anti-phospho-IGF1R (#80732, Cell Signaling Technology), anti-IGF-1 antibody (#28530-1-AP, Proteintech), anti-Myc and anti-DDK (TA150014, OriGene), (Z)-4-Hydroxytamoxifen (H7904, Sigma-Aldrich), IGF-1 (I3769, Sigma-Aldrich), SAHA (SML0061, Sigma-Aldrich), DZNep (5.06069, Sigma-Aldrich), GSK1838705A (S2703, Aurogene), Everolimus (S1120, Aurogene), Anastrozole (A2736, Sigma-Aldrich), Insulin-like growth factor I (#I3769, Sigma-Aldrich).

Cell culture

MCF-7 BC cells, and 3T3-L1 pre-adipocytes were purchased from American Type Culture Collection, cultured and authenticated following supplier's instruction. MCF-7 Tam-resistant (TR) [14], MCF-7 aro and Anastrozole-resistant MCF-7 aro (AnaR) [15, 16], and long-term estrogen deprived (LTED) [17, 18] BC cells were generated as described. 3T3-L1A obese (Ob) adipocytes were obtained by incubating fully-differentiated 3T3-L1A adipocytes in mature-adipocyte growth medium supplemented with 1mM of a 1:2:1 palmitate/oleate/linoleate

(Sigma-Aldrich) mixture for 24 h [19]. Cells were authenticated every 6 months by short-tandem-repeat-analysis (PowerPlex 4 C Matrix Standard, and GenePrint 10 system, Promega), morphology, doubling times, and tested for mycoplasma-negativity (MycoAlert, Lonza).

Lipid droplet visualization and lipid content

Lipid droplets were fixed and incubated with Oil Red O solution (Bio Optica) according to the manufacturer's instructions and visualized under a light microscope (Olympus). Lipid content was measured as described [20]. Briefly, cells were treated with 100% isopropanol, and lipid content was quantified at 510 nm using a Multiskan SkyHigh microplate reader (Thermo Fisher Scientific).

Real-time RT-PCR assay

Gene expression was evaluated by reverse transcription (RT)-PCR method as described [21], using SYBR Green Universal PCR Master Mix (Thermo Fisher Scientific). Each sample was normalized on 18 S mRNA content. Final results were expressed as n-fold differences in gene expression relative to 18 S (or 36B4) and calibrator, calculated using the DDCT method. Primers are listed in Supplementary Table S1.

Conditioned medium (CM) systems

Mature (3T3-L1A) or obese (3T3-L1A Ob) adipocytes were incubated in 3% Charcoal-stripped Fetal Bovine Serum (CS, Sigma-Aldrich) phenol-red free media (36–48 h). CM was collected, centrifuged, and used in respective experiments.

IGF-1-immunodepleted conditioned media

IGF-1 immunodepletion from 3T3-L1A adipocytes was performed as previously described [22]. Briefly, CM was collected, centrifuged (1200×g, 5 min) and the supernatant incubated with 2 µg/mL IGF-1 antibody under gentle rotation (overnight, 4 °C). The following day, Protein A/G-agarose beads (Santa Cruz Biotechnology; 25 µL/mL CM) were washed with PBS, centrifuged (10000×g, 10 min, at 4 °C), and incubated with antibody-treated CM under gentle rotation (1 h, 4 °C). Then, the mixture was centrifuged (10,000×g, 10 min), and supernatant filtered through a 0.22 µm membrane. IGF-1 immunodepletion was confirmed by enzyme-linked immunosorbent assay (ELISA).

MTT cell proliferation assay

Cell proliferation was assessed using 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reagent (MTT; Sigma) and expressed as fold change relative to control MCF-7 BC cells. Data represent two independent experiments, performed in quadruplicate. The IC50

values were calculated using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA).

Trypan-blue cell count assay

After 48 h of treatment, cell numbers were evaluated by trypsin suspension of samples followed by counting with Countess® II Automated Cell Counter (Thermo Fisher), following manufacturer's instructions. Data represent three independent experiments, performed in triplicate.

Soft agar growth assay

Cells were plated in 2 mL of 0.35% agarose in phenol red-free medium. Two days after plating, medium with or without the indicated treatments was added to the top layer and refreshed every two days. After 14 days colonies > 50 µm diameter were counted [23]. Data represent three independent experiments, performed in duplicate or triplicate.

Wound-healing assay

Cell monolayers were scraped and treated as indicated. Cell migration was monitored over 16–24 h and then cells were fixed, and stained with Coomassie Brilliant Blue. Pictures are representative of wound closure in three independent experiments taken at 10× magnification using phase-contrast microscopy. The rate of wound-healing was quantified as reported [24].

Transmigration and Matrigel-based invasion assay

Cells were seeded in the upper compartments of Boyden-chamber (8 µm membranes, Corning Costar). For invasion assay, Boyden-chamber were coated with Matrigel (BD Biosciences, 2 mg/mL). Regular growth medium was used in the lower chambers. After 5–8 h, migrated/invaded cells were DAPI-stained, and quantified by viewing five separate fields per membrane at 10 × magnification, using Image J software (NIH). Migration/invasion is expressed as numbers of migrated/invaded cells. Data represent three independent experiments, assayed in triplicate.

RNA library Preparation and sequencing

RNA Library Preparation and Sequencing were conducted at Genomix4Life Srl (Baronissi, SA, Italy). After RNA extraction, the quality was assessed with Agilent TapeStation4200 (Agilent, CA, USA). High quality RNA from three-independent purifications for each experimental point was used for library preparation. Indexed libraries were prepared from 1 µg/ea of purified RNA with TruSeq Stranded mRNA Library Prep (Illumina, CA, USA) following supplier's instructions. Libraries were quantified using the TapeStation 4200 and Qubit fluorometer (Thermo Fisher), then pooled such that each index-tagged sample was present in equimolar amounts.

The pooled samples were subject to cluster generation and sequencing using an Illumina NovaSeq6000 (Illumina) in a 2×75 paired-end format. RNA sequencing data have been deposited in the EBI ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>, RRID: SCR_002964) with Accession Numbers GSE223853 and GSE254882. Transcriptional comparative analyses between MCF-7 vs. CM-treated MCF-7 and MCF-7 TR vs. CM-treated MCF-7 TR were performed separately through the DeSeq2 tool on R environment (Version 1.45.0) [25]. The variables presenting an absolute fold-change higher than 1.5 and a significant Benjamini-Hochberg False Discovery Rate (FDR) (≤ 0.05) were considered as differentially expressed genes.

MixOmics-MINT tool

All the common (~17400) genes between the MCF-7 and MCF-7 TR Datasets were analysed by MixOmics-MINT tool on R environment (Version 6.22.0) [26], a multivariate integrative approach to identify reproducible signatures of conditioned medium across the two experiments. The appropriate number of components and variables was optimized by a preliminary model according to the Balance Error Rate of misclassification. Through Sparse Partial Least Squares - Discriminant Analysis (sPLS-DA) the samples were finally supervised, classified and the common variables between the two datasets were selected according to their contribution in discriminating between conditioned and control media. The selected genes of the model were represented by heatmap of scaled correlation clustering distances and tree-clusterized according to Ward's method.

Enrichment analysis

Enrichment analysis was performed by EnrichR R package (Version 3.2). Selected reference datasets were Biological Process Gene Ontology/Kegg/Reactome/MSigDB. Only terms presenting lower adjusted p-value than 0.05 and more than one included element were considered in the analysis.

MetaCore functional analysis

DEGs presenting similar deregulation trends in CM treated MCF-7 and MCF-7 TR BC cells, according to MixOmics analysis, were functionally processed by applying the MetaCore™ integrated software suite for functional analysis of experimental data (Clarivate Analytics, London). By using the network building tool and its shortest path algorithm and by allowing a maximum of 2 steps in connecting seed nodes, proteins coded by the 27 investigated DEGs were functionally crosslinked to generate shortest path networks (SPN). The latter were built limiting protein processes to individual proteins, excluding their involvement in multimeric complexes,

and avoiding canonical pathways. According to the manually curated MetaCore in-house database of physiological and pathological protein-protein and protein-nucleic acid interactions, signaling and metabolic pathways, from scientific literature, only those proteins known to be closely related were consequently included in the resulting net(s). The obtained SPNs represent an interactomic overview on the investigated process and are prioritized according to their statistical significance ($p \leq 0.001$). The most relevant SPN is then graphically visualized by nodes (proteins) and edges (interconnections among proteins) and named in relation to its five most significant nodes, i.e., central hubs. Since they establish the highest number of interactions with the other components of the net, central hubs acquire crucial roles in defining the biochemical and molecular properties of the investigated systems. In fact, they are usually recognised as highly significant biomarkers of physiological and pathological states as well as in drug design and pharmacological attempts, as we previously widely proved [27].

Immunoblotting

Cell extracts were resolved by SDS-PAGE, as described [28]. Odyssey FC (Licor) and Image Studio Lite softwares were employed to acquire and quantify the band of interest. Immunoblots show a single representative of two or three separate experiments. The uncropped blots are reported in the Supplementary Fig. S1.

TRX activity

Thioredoxin (TRX) activity assay was evaluated by Thioredoxin Reductase Assay Kit (Sigma-Aldrich), following recommendations. Briefly, cells were washed with cold PBS and lysed in the supplied extraction buffer. Lysates were centrifuged at 12,000 × g for 10 minutes at 4°C, and supernatants were transferred to a 96-well plate. TRX activity was determined by monitoring the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB), which produces a colorimetric change detectable at 412 nm using a microplate reader. Activity values were normalized to total protein concentration measured by BCA protein assay (Thermo Fisher Scientific).

Gene reporter assays

Cells were transfected with firefly luciferase reporter TXNIP plasmid (Addgene) and *Renilla* luciferase (internal control) as described [29]. Briefly, cells were plated in 24-well plates at a density of 1.5×10^5 cells/well and allowed to adhere overnight. The following day, cells were co-transfected with 500 ng of a firefly luciferase reporter plasmid containing the human TXNIP promoter region (Addgene plasmid #37084) and 50 ng of a *Renilla* luciferase control plasmid (pRL-TK, Promega) using

Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's recommendations. After 24 h, cells were lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a luminometer. Firefly luciferase activity was normalized to Renilla to control for transfection efficiency. All experiments were performed in triplicate and repeated independently three times.

Lentiviral transfection

Stable MCF-7 and MCF-7 TR cells expressing TXNIP (NM_006472) Human Tagged ORF Clone Lentiviral Particle, and Lenti-ORF Control Particles (OriGene, RC210804L3V and PS100092V, respectively) were generated following manufacturer's instructions with 2 µg/mL puromycin (Sigma-Aldrich). Viral transduction was carried out in the presence of 5 µg/mL polybrene to enhance infection efficiency. TXNIP overexpression was confirmed by real-time RT-PCR assay and immunoblotting.

IGF-1 measurement by ELISA

IGF-1 was measured by ELISA (R&D System) following manufacturer's protocol. Briefly, cells were seeded in 6-well plates at a density of 3×10^5 cells/well and incubated until ~80% confluency. After treatment, media were collected, centrifuged to remove debris (1,000 × g, 10 min), and stored at -80 °C until analysis. Standards and samples were run in duplicates, and absorbance was measured at 450 nm using a microplate reader. IGF-1 concentrations were calculated from a standard curve.

Human mammary adipocytes

Human mammary adipocytes were isolated from mammary adipose tissue biopsies of obese women (BMI = 30–40 kg/m²), prior informed consent [30]. CM were collected after washing twice with PBS and incubation with 3% CS for 24 h.

Patient-derived organoid (PDO) culture

Metastatic BC fluid specimens derived from ascites were collected from metastatic BC patients via the Manchester Cancer Research Centre Biobank (Ethical Approval Study Ref: 05-Q1402-25 and 12-ROCL-01) prior patients' informed consent [31]. BC cells derived from metastatic samples were pelleted, washed twice in ice-cold PBS and embedded in 75% (v) growth-factor-reduced Matrigel (Corning). Domes of 30 µL were plated in 24-well plates, polymerized for 15 min at 37 °C and overlaid with 500 µL organoid media (DMEM F-12, 10% FBS, 1% pen/strep, 10 µg/mL insulin, 1 µg/mL hydrocortisone, 10 ng/mL EGF, 10 µM ROCK inhibitor Y-27632). For passaging organoids, Matrigel was mechanically disaggregated and centrifuged (500 g/5 min). The bottom was resuspended in 500 µL Dispase II 1 U mL⁻¹ (Stem Cell Technologies)

for 1 h at 37 °C and 1 mL pre-warmed Accutase 1X (Stem Cell Technologies) for 5 min at 37 °C to degrade organoid cellular junctions. Finally, organoids were washed in PBS, centrifuged (10 min/500 g), counted and resuspended, in fresh Matrigel at 5×10^3 cells per 30 µL dome (~1:3–1:4 split relative to the previous passage).

PDO viability assay

PDOs were plated in Matrigel and cultured in growth media, refreshed every 3 days. After 9 days, organoid viability was assessed by CellTiter-Glo® 3D Cell Viability Assay (Promega). The luminescence was measured by Varioskan Lux (Thermo Fisher, 1000 ms/well).

PDO growth assay

PDOs were plated in Matrigel and cultured in growth medium with different conditions. The number of organoid structures was measured after 10 days using EVOS XL CORE (Thermo Fisher). The number of organoids per 100x microscope field were averaged. Three fields were counted around the edge of the matrix dome.

Survival Kaplan-Meier analysis

Kaplan-Meier plots were generated using the Kaplan-Meier plotter online tool (<https://kmplot.com/analysis/>) for TXNIP correlation with recurrence-free (RFS), overall (OS), distant metastasis-free (DMFS), or post-progression (PPS) survival in endocrine-treated BC patients, choosing auto-select best cutoff, and the JeSet best probe set. These same parameters were applied when stratifying patients into Luminal A and Luminal B BC subtypes, according to the St. Gallen criteria. Kaplan-Meier survival plots were also generated to evaluate correlation of IGF-1/TXNIP levels with RFS in endocrine-treated patients in TCGA, GEO, and Metabric datasets. In this, each available cutoff value between the lower and upper quartiles of expression were evaluated and the best performing cutoff was used in the Kaplan-Meier plots. False discovery rate (FDR) was computed to correct for multiple hypothesis testing, and was uniformly applied across the datasets. Hazard ratio with 95% confidence intervals and log-rank P value were calculated and plotted in R as described [32].

Immunohistochemistry (IHC)

Fresh tissues were formalin-fixed/paraffin-embedded after surgical removal. Sections were stained with H&E to select samples consisting of at least 50% tumor cells and to establish the histologic type, and grade. The study included a total of 65 primary breast carcinomas with ER-positive, ERBB2-negative breast cancer and known BMI values, enrolled in 2 hospitals. The clinical investigation conformed the Declaration of Helsinki of 1975 and was approved by ethics and institutional human subject

committees at the Annunziata Hospital (Cosenza, Italy), and at the Istituto Oncologico Del Mediterraneo (Catania, Italy). Breast cancer sections were stained with customized immunohistochemistry (IHC) protocols from Dako Omnis and Ventana Benchmark Ultra with the primary TXNIP antibody (clone EPR14774, Abcam, Cambridge, UK). The protocol for IHC has been previously described [33]. Interpretation of immunoreactivity is based on evaluation of both staining intensity and the percentage of cells stained at each intensity level. The intensity values ranged from 0 (no evidence of staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). Cell values ranged from 0 to 49% to 50–100%. Specifically, high immunoreactivity was associated to a strong staining (score 3) observed in $\geq 50\%$ of cells, while low immunoreactivity includes all other combinations, such as weak or moderate staining, or strong staining in $< 50\%$ of cells. Immunohistochemical scoring was performed without prior knowledge of BMI, and independently evaluated by two independent pathologists in a blinded fashion based on the staining intensity and the percentage of positive cells. Positive staining controls (human tonsil) were included.

Literature-based validation of TXNIP-obesity signaling relations using INDRA (Integrated network and dynamical reasoning Assembler)

To evaluate literature-supported causal interactions between TXNIP and upstream regulators implicated in obesity-related signaling, we utilized the Integrated Network and Dynamical Reasoning Assembler (INDRA) framework (<https://indra.bio>) [34, 35]. All analyses were performed through a custom-built Python 3.10 pipeline executed within a Jupyter Notebook environment (Anaconda 3 distribution). INDRA aggregates mechanistic statements extracted from published literature via multiple machine reading systems (e.g., REACH, Eidos, TRIPS) and assembles them into unified biological relations. A curated panel of obesity-associated molecular effectors was defined a priori, including IGF-1, LEP, PPARG, MTOR, IL6, TNF, HIF1A, and SREBF1, among others. For each gene pair (regulator-TXNIP), we retrieved mechanistic statements using the `indra_db_rest.get_statements()` API function. Extracted data included: (i) the type of mechanistic relationship (e.g., Activation, Inhibition), (ii) source of evidence (reader engine and PubMed ID), and (iii) supporting textual excerpt. Each relationship was subsequently mapped to a standardized ontology (activates, deactivates, interacts, modulates) using fuzzy semantic matching.

Statistical analysis

Data were analyzed for statistical significance ($P < 0.05$) using a two-tailed student's Test, performed by

GraphPad-Prism 7 software program (GraphPad Inc). All data are shown as mean $\pm$ S.E.M. For RNA sequencing bioinformatics Analysis, Fastq underwent Quality Control using the FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). The tool cutadapt (version 2.5) was used to remove the adapter sequence and the very short reads (reads length < 20). The mapping of paired-end reads was performed using STAR (version 2.7.5c) with the standard parameters for paired reads on reference genome assembly hg38 obtained from GenCode (Release 37 GRCh38.p13). The quantification of transcripts expressed for each sample was performed using the FeatureCount (version 2.0) algorithm. DESeq2 was used to perform the normalization matrix, and differentially expressed gene of all samples were considered. The significant differentially expressed genes were determined using false discovery rate (FDR) cutoff ≤ 0.01 , $\text{padj} < 0.05$ and at least 1.5-fold change between conditions. Spearman correlation was computed to compare the association between TXNIP expression, and BMI in BC patients stratified as normal ($\text{BMI} < 25 \text{ kg/m}^2$), overweight ($\text{BMI} = 25\text{--}29.9 \text{ kg/m}^2$), or obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) [36]. TXNIP expression was also analyzed in another public dataset, comparing normal weight (NW) and obese (Ob) patients using a one-sided Mann-Whitney U test after back-transformation of log2 values [37]. For IHC, BMI and TXNIP correlation was examined with Mann-Whitney-U test (GraphPad-Prism7).

Results

Impact of 3T3-L1 mature adipocyte-derived conditioned medium on growth, motility, and invasion of MCF-7 and MCF-7 Tam-resistant (TR) BC cells

As experimental models to evaluate adipocyte/BC cell crosstalk and Tam response, we used fully differentiated 3T3-L1A cells, a widely used 'in vitro' model of white adipocytes [28], and parental human ER α -positive MCF-7 BC cells along with their TR counterpart in co-culture experiments. We first evaluated the effects of increasing doses of Tam on MCF-7 cell proliferation using MTT growth assays, in the presence or absence of conditioned media (CM) derived from 3T3-L1A cells. Tamoxifen significantly inhibited cell proliferation in a dose-dependent manner under both conditions (Supplementary Fig. S2). However, it is noteworthy that the inhibitory effect of Tam was observed at higher concentrations in 3T3-L1A CM-treated MCF-7 cells compared to control cells, as indicated by the increased half-maximal inhibitory concentration (IC₅₀) values (6.8 μM in MCF-7 cells versus 10.1 μM in MCF-7 TR cells). Then, we assessed the effects of 3T3-L1A CM on both anchorage-dependent and -independent growth of MCF-7 and TR cells (Fig. 1A, B). As expected, Tam treatment significantly reduced the proliferation of MCF-7 cells, and 3T3-L1A

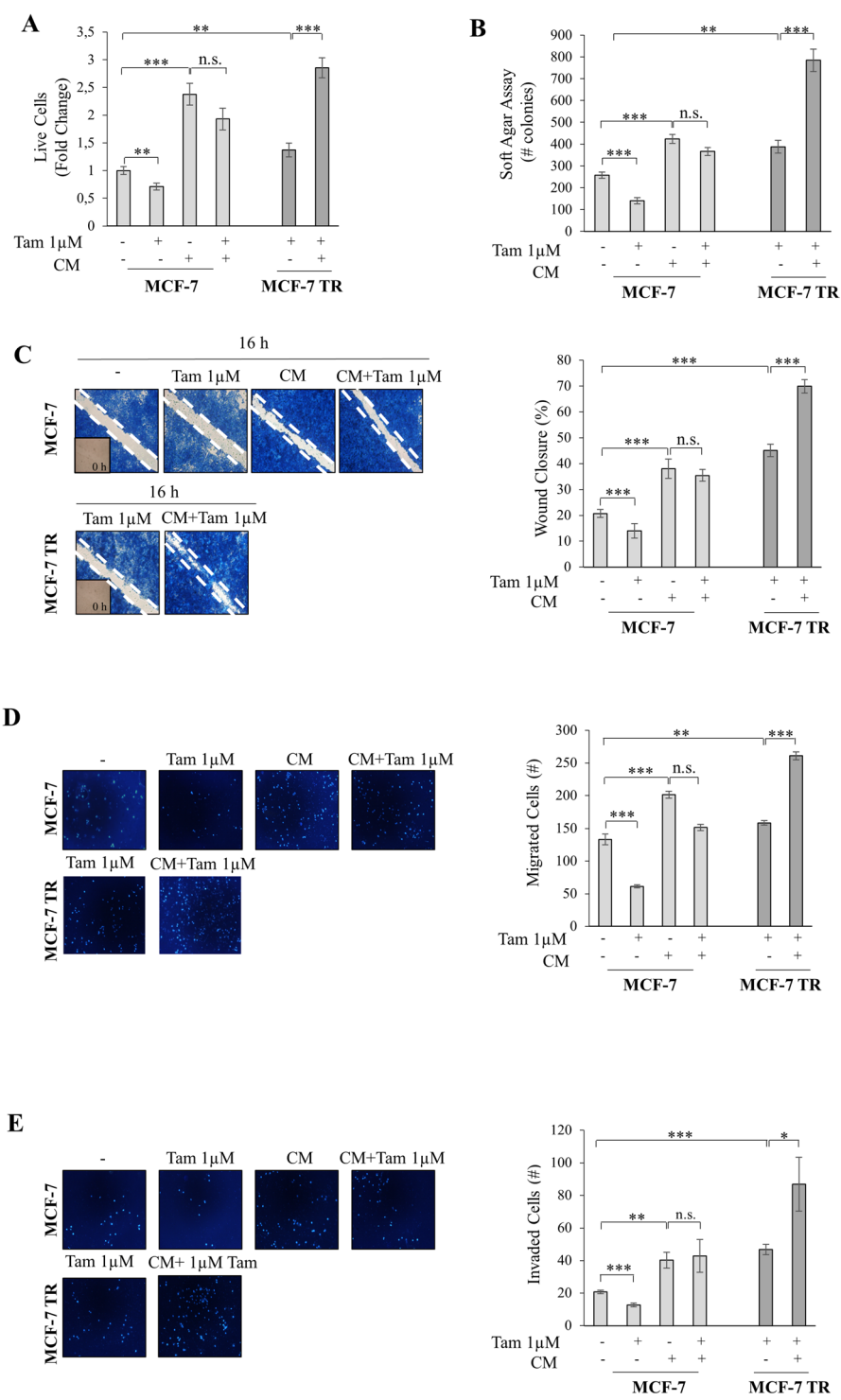


Fig. 1 (See legend on next page.)

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Fig. 1 Effects of 3T3-L1A conditioned medium (CM) on growth, motility, and invasion of MCF-7 and MCF-7 TR BC cells. **(A)** Trypan blue cell count assay in MCF-7 and MCF-7 TR BC cells treated (+) or not (-) with Tam and/or 3T3-L1A-derived CM for 48 h. **(B)** Soft agar growth assays in cells treated as indicated. After 14 days of growth, colonies $\geq 50 \mu\text{m}$ were counted. **(C)** Wound healing assay in MCF-7 and MCF-7 TR cells treated as indicated for 16 h. Inset, time 0. *Left panel*, representative pictures. *Right panel*, the histograms represent the relative percentage of wound closure calculated by image analysis using Scion Image software. Transmigration **(D)** and invasion **(E)** assays in MCF-7 and MCF-7 TR cells treated as indicated for 5 h. The migrated/invaded cells were DAPI-stained, counted, and images were captured at 10X magnification. Representative images of cell transwell migration and invasion are shown. Mean $\pm$ S.E.M. n.s., non significant; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$

CM increased cell growth in both assays, whereas the anti-proliferative effects of Tam were counteracted by 3T3-L1A CM. Incubation of MCF-7 cells with 3T3-L1A CM also reduced their sensitivity to the inhibitory effects of Tam on motility and invasion (Fig. 1C-E). Interestingly, the proliferative, motile and invasive phenotype of MCF-7 TR cells was significantly increased in the presence of 3T3-L1A CM (Fig. 1), further suggesting a supportive role of adipocyte secretome in the development and progression of endocrine-resistant BC phenotypes.

3T3-L1A-derived conditioned medium affects TXNIP expression and activity in MCF-7 and MCF-7 TR BC cells

To gain insights into the molecular mechanisms underlying the phenotypic modulations induced by 3T3-L1A CM, a wide transcriptomics study (17400 investigated genes) was performed in MCF-7 and MCF-7 TR cells treated with 3T3-L1A CM. The transcript-abundance differential-analysis unraveled a consistent modulation in gene expression (Supplementary Tables S3 and S4). The differentially expressed gene (DEG) datasets from CM-treated MCF-7 and MCF-7 TR cells were then analyzed through the MixOmics-MINT tool to define reproducible signatures of 3T3-L1A CM effects on recipient cells. MixOmics analysis detected 27 DEGs with similar modulation trends, despite at different intensity levels, in MCF-7 and MCF-7 TR cells exposed to 3T3-L1A CM (Supplementary Table S5). The unsupervised hierarchical clustering analysis of the 27 DEGs is shown in Fig. 2A, while the mean value of contribution in criterion assessments of each gene is visualized by the horizontal bar plot in Fig. 2B.

A functionally predictive evaluation about the impact of the 27 CM-affected genes on BC cells was then performed by enrichment analysis and protein-network generation. The enrichment analysis, performed in Gene Ontology-Biological Process, Kegg pathway, Reactome, and MSigDB hallmark ontologies, recognized “negative regulation of IRE1-mediated unfolded protein response” and “PERK-mediated unfolded protein response” as the most significant enriched terms, among the 25 top-scored ones (Fig. 2C). The co-processing of the 27 DEGs by the MetaCore-network-building-tool led to the construction of a highly significant shortest path network (SPN), which included the 27 canonical-proteins, coded by the MixOmics designated DEGs. According to the number of interconnections, five most relevant nodes,

i.e. central hubs, were delineated: COUP transcription factor 2 (protein short name: COUP-TFI; I included into the SPN COUP-TF-group; gene name: NR2F2), endoplasmic reticulum chaperone BiP (GRP78 in MetaCore; included into the SPN HSP70-group; gene name: HSPA5), E3 SUMO-protein ligase CBX4 (CBX4), Thioredoxin-interacting protein (TXNIP), and Ras GTPase-activating protein-binding protein 1 (G3BP1; G3bp1 (hdhVIII) in MetaCore) (Fig. 2D). Among these 27 genes, the most commonly and significantly down-regulated gene in response to 3T3-L1A CM was TXNIP (fold change_{WT CM/WT} = -21.2 and fold change_{TR CM/TR} = -5.13, Supplementary Table S5). TXNIP, a tumor suppressor gene participating in the metabolic reprogramming and oxidative stress in various solid cancers, particularly by inhibiting or limiting the bioavailability of the thioredoxin (TRX, TXN) system [38, 39], has been found to be regulated by Endoplasmic Reticulum stress through IRE-1 and PERK pathways [40], which are, as above detailed, the most enriched Pathway Ontology used to annotate the 27 investigated DEGs. Interestingly, retrospective analyses showed that low TXNIP expression in endocrine therapy-treated BC patients was associated with a statistically significant poorer recurrence-free (RFS), overall (OS), distant metastasis-free (DMFS), and post-progression (PPS) survival compared to high TXNIP-expressing patients (Supplementary Fig. S3), highlighting a role of TXNIP for identifying patients at high risk of progression during endocrine therapy. Low TXNIP levels were correlated with poorer outcomes in patients with both Luminal A and Luminal B BC subtypes, undergoing endocrine therapy (Supplementary Fig. S3). To validate the TXNIP gene expression modulation identified by RNA-sequencing, realtime RT-PCR and immunoblotting analyses were performed (Fig. 2E, F). We found low TXNIP mRNA and protein levels in MCF-7 TR compared to MCF-7 cells. Importantly, 3T3-L1A CM treatment resulted in a significant reduction of TXNIP expression in both cell lines, although to a slightly higher extent in TR cells. In line with these results, a decreased TXNIP transcriptional activity (Fig. 2G), and an increased TRX activity (Fig. 2H), which is negatively regulated by TXNIP, were observed in cells treated with 3T3-L1A CM, especially in MCF-7 TR cells. By a transcriptome-based analysis, we also found a significant inverse correlation between TXNIP expression and BMI in a cohort of BC patients stratified according to patients’ obesity phenotype defined as

normal ($\text{BMI} < 25 \text{ kg/m}^2$), overweight ($\text{BMI} = 25\text{--}29.9 \text{ kg/m}^2$), or obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) (Fig. 2I). Consistently, analysis of an independent transcriptomic dataset confirmed a significant reduction of TXNIP expression in obese (Ob, $n = 141$) compared to normal-weight (NW, $n = 131$) BC patients ($p = 0.036$) (Fig. 2L). To strengthen these results, we further investigated TXNIP expression levels in normal weight, overweight and obese patient-derived tissues ($n = 65$) by immunohistochemistry. The mean BMI was $21.6 \pm 2.2 \text{ kg/m}^2$ in normal weight group and $31.2 \pm 3.3 \text{ kg/m}^2$ in overweight and obese group. The participant's clinical and pathologic characteristics are summarized in Supplementary Table S6. Of note, TXNIP expression was negatively associated with BMI values (Fig. 2M). The rate of low expression of TXNIP protein in BC tissues was 27%, and 73% in normal weight, and overweight/obese patients, respectively, indicating how TXNIP levels may decrease in BC tissues of patients with increased adiposity.

Inducing TXNIP re-expression reverses 3T3-L1A CM-mediated effects in MCF-7 and MCF-7 TR BC cells

To assess the role of TXNIP expression/activity in adipocyte/BC cell interaction, we evaluated whether enforced TXNIP expression by pharmacological or genetic approaches may reverse adipocyte-induced effects. Thus, the following drugs that were shown to be able to re-express TXNIP in BC cells [41] were used: (i) Suberoyl-anilide hydroxamic acid (SAHA, Vorinostat), a histone deacetylase inhibitor (HDAC) approved by food and drug administration (FDA) for the treatment of cutaneous T-cell lymphoma, and (ii) 3-Deazaneplanocin A (DZNep), a histone methyltransferase EZH2, approved by FDA in hematological malignancies. As shown in Fig. 3A, SAHA and DZNep were able to abrogate CM-mediated reduction of TXNIP expression in MCF-7 and MCF-7 TR cells in a dose-dependent manner. Concomitantly with TXNIP re-expression, SAHA and DZNep significantly counteracted the effects of 3T3-L1A CM on anchorage-independent proliferation (Fig. 3B) and migration (Fig. 3C) in both cell lines. We then stably overexpressed TXNIP (o.e.) in MCF-7 and MCF-7 TR cells by using lentivirus vector system with c-Myc expression tag. The expression product of the exogenously added TXNIP gene tagged with c-Myc was detected in TXNIP o.e. cells by immunoblotting analysis using TXNIP and c-Myc antibodies (Fig. 3D). We used these experimental models to validate the specific role of TXNIP in MCF-7 and MCF-7 TR cells upon exposure to 3T3-L1A CM. In line with the well-known role of TXNIP as a tumor suppressor gene, the lentiviral overexpression of TXNIP (o.e.) was associated with a reduction in the number of colonies and migrated MCF-7 and MCF-TR cells (Fig. 3E, F). Importantly, 3T3-L1A CM effects were reversed in

TXNIP o.e. cells. Therefore, our data suggest that TXNIP activity may be required for controlling proliferation and migration processes mediated by adipocyte secretome in malignant cells.

IGF-1 signaling inhibition counteracts 3T3-L1A CM-induced effects on TXNIP Expression, Growth and Migration in MCF-7 and MCF-7 TR BC cells.

TXNIP was previously identified as an important downstream target of IGF-1 (Insulin-like growth factor-1) action in patients with Laron syndrome [42]. On the other hand, IGF-1, highly released by adipocytes, has been widely shown to contribute to obesity-associated BC progression and endocrine resistance [7]. Thus, we investigated whether IGF-1/TXNIP axis may have a role in 3T3-L1A CM-mediated effects in our model systems. Multiple studies showed altered IGF-1R expression and signaling dynamics in TR BC cell models, although findings across the literature remain heterogeneous. Some reports described a downregulation of IGF-1R in long-term tamoxifen-treated MCF-7 cells, associated with a shift toward IGF-1R-independent survival pathways; while others demonstrated an hyperactivation of downstream effectors of the IGF-1 signaling cascade (e.g., IRS-1/PI3K/AKT), even with unchanged or reduced IGF-1R levels [43–50]. Our results showed that IGF-1R levels were reduced in MCF-7 TR cells compared to parental MCF-7 cells; however, IGF-1R phosphorylation levels remained comparable between the two cell lines (Supplementary Figure S4A). Functionally, MCF-7 TR cells were responsive to the growth-promoting effects of IGF-1 in both MTT and soft agar growth assays (Supplementary Figure S4 B and C), and this proliferative induction was significantly counteracted by the IGF-1R inhibitor GSK7818305A. Thus, co-culture experiments involving MCF-7 or MCF-7 TR cells and 3T3-L1A CM in the presence or not of the IGF-1R inhibitor GSK1838705A were performed. The IGF-1R downstream mTOR inhibitor Everolimus was also included in the experiments. First, we evaluated the effects of GSK1838705A and Everolimus alone and in combination on TXNIP expression and found that both drugs were able to reverse the reduction of TXNIP expression induced by 3T3-L1A CM exposure (Fig. 4A). However, the recovery of TXNIP expression in MCF-7 cells was more pronounced following treatment with GSK1838705A compared to Everolimus, while MCF-7 TR cells exhibited the opposite effect. This difference may be related to the distinct points of action within the signaling pathways: GSK1838705A targets upstream signaling through IGF-1R inhibition, while Everolimus inhibits mTOR downstream signaling, a key convergence node of multiple intracellular cascades that may be hyperactivated in MCF-7 TR cells [51]. However, it is important to highlight that combined treatments appear to be slightly more effective in inducing TXNIP

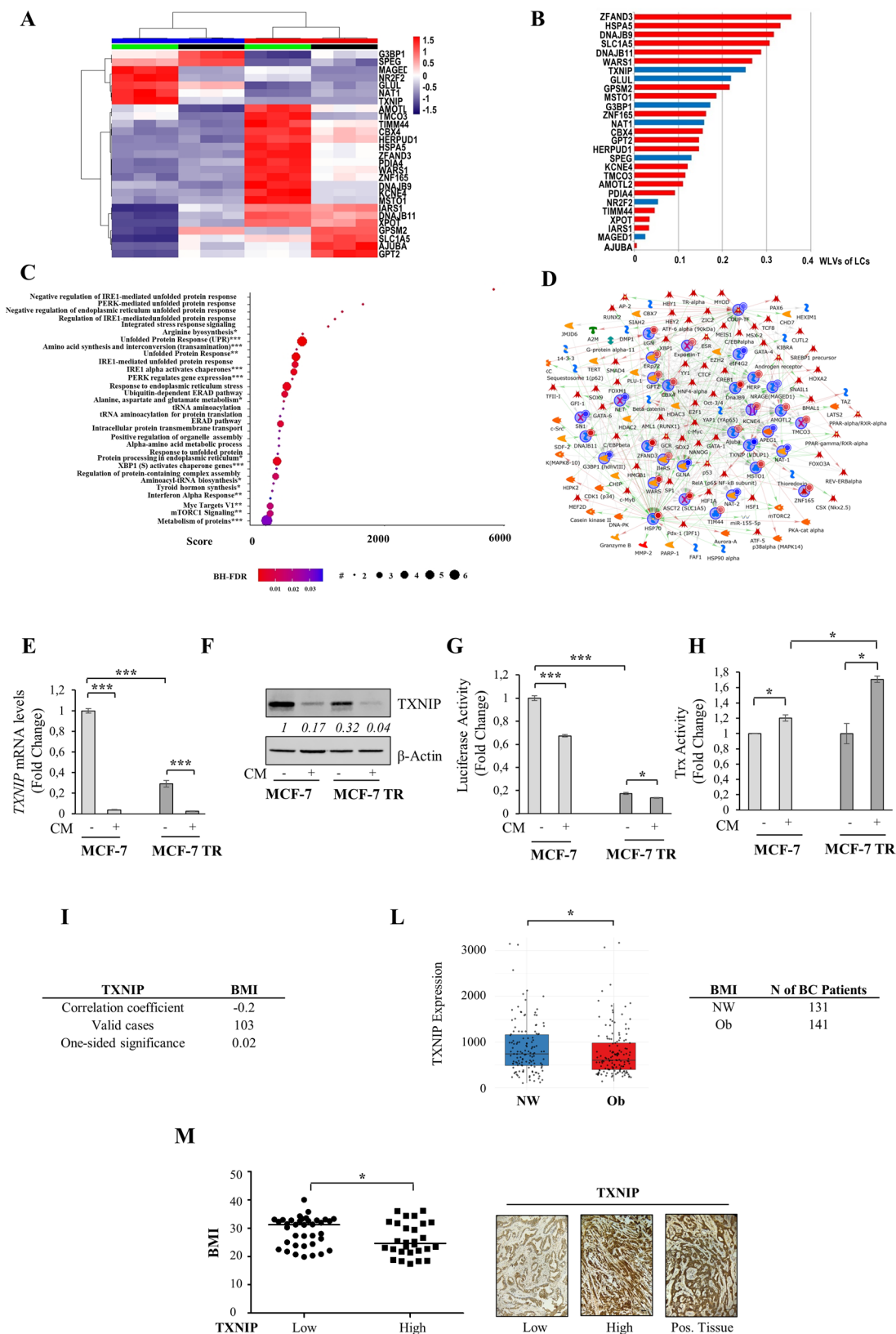


Fig. 2 (See legend on next page.)

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Fig. 2 Effects of 3T3-L1A CM on TXNIP expression and activity in MCF-7 and MCF-7 TR cells. **(A)** Correlation heatmap of 27 selected genes of integrative MixOmics model. Tree-clustering was obtained according Ward's method. 3T3-L1A CM and Control media **(C)** were shown in column annotation as red and blue, respectively. Similarly, the MCF-7 and MCF-7 TR cells were reported as green and black, respectively. **(B)** Horizontal bar plot visualizing the mean weight of each selected gene in the classification of a given condition in PLS-DA integrated model. The red and blue loadings represent the contribution of 3T3-L1A CM and C, respectively, as final outcome of interest. WLVs of LCs: weight of each loading vectors of latent components. **(C)** Enrichment analysis on terms from Biological Process Gene Ontology, KEGG, Reactome, and MSigDB annotating the 27 genes presenting similar modulation trends in both MCF-7 and MCF-7 TR cells treated with 3T3-L1A CM. The size and colour of each dot indicate number of genes per term and the BH-FDR value, respectively. The reference databases are: GO Biological Process (no star), Kegg (*), MSigDB (**), and Reactome (***). Score: combined score of EnrichR. **(D)** Shortest Path Network (SPN) built by processing the 27 genes with similar deregulation trends in 3T3-L1A CM-treated MCF-7 and 3T3-L1A CM-treated MCF-7 TR cells. Edge colours and arrowheads indicate the type and the direction of protein interconnections (green arrow = positive effect; red arrow = negative effect; and grey arrow = unspecified interaction). **(E)** Real-time RT-PCR for TXNIP mRNA levels in MCF-7 and MCF-7 TR cells treated (+) or not (-) for 48 h with 3T3-L1A CM. **(F)** Immunoblotting showing TXNIP protein expression in cells treated as indicated for 48 h. β -Actin was used as a control for equal loading and transfer. Numbers below the blots represent the mean of the band optical density expressed as fold over CM-untreated MCF-7 cells. **(G)** TXNIP promoter activity in cells treated as indicated for 24 h. **(H)** TRX activity in cells treated as indicated for 48 h. Mean $\pm$ S.E.M. *, $P < 0.05$; ***, $P < 0.0005$. **(I)** Spearman rank correlation between TXNIP and BMI in 103 BC patients with known BMI. **(L)** One-sided Mann-Whitney U test after back-transformation of log2 values for evaluating TXNIP expression in Normal Weight (NW, $n = 131$) and Obese (Ob, $n = 141$) patients. **(M)** Immunohistochemical detection of TXNIP expression in normal weight, overweight and obese patient-derived breast cancer tissues ($n = 65$). *Left Panel*, statistically significant difference between TXNIP expression and BMI values was evaluated using the Mann-Whitney U test (*, $P = 0.0382$). *Right Panel*, representative IHC images showing low and high cytoplasmic TXNIP expression in breast cancer epithelial tissues. Pos. Tissue, IHC staining in tonsil, used as positive control tissue. Magnification: 20X. Scale bar: 50 μ m

expression. As a positive control, we treated cells with IGF-1, which - as previously demonstrated in other cell lines [42] - strongly reduced TXNIP protein expression in both MCF-7 and MCF-7 TR cells, further supporting the hypothesis that IGF-1 may act as an up-stream regulator of TXNIP repression. To evaluate the direct involvement of IGF-1 in mediating adipocyte-derived effects, IGF-1 was immunodepleted from 3T3-L1A CM using an IGF-1 specific antibody, and the resulting media were tested for their ability to induce anchorage-independent growth and migration of MCF-7 breast cancer cells in the presence and absence of Tam. As expected, treatment with 3T3-L1A CM pre-incubated with a nonspecific IgG (control) promotes cell growth and migration, while counteracting TAM effects. In contrast, IGF-1 immunodepletion significantly reduced the growth- and migration-promoting activities of 3T3-L1A CM and, notably, restored Tam inhibitory activity (Supplementary Fig. S5).

According to these results, treatment with GSK1838705A and Everolimus was able to significantly reduce the increased cell growth and migration induced by 3T3-L1A CM in MCF-7 and MCF-7 TR cells, with comparable efficacy, although the overall inhibitory effects on proliferation ($\sim 49\%$ with GSK1838705A and $\sim 41\%$ with Everolimus in MCF-7 TR cells vs. $\sim 30\%$ with GSK1838705A and with 20%-Everolimus in MCF-7 cells) and migration ($\sim 80\%$ with GSK1838705A and $\sim 72\%$ with Everolimus in MCF-7 TR cells vs. $\sim 50\%$ with GSK1838705A and $\sim 50\%$ with Everolimus in MCF-7 cells) were more pronounced in MCF-7 TR (Fig. 4B, C). Notably, the combination of GSK1838705A and Everolimus resulted in a higher reduction compared to either treatment alone, suggesting that dual targeted therapy could be beneficial in mitigating CM-mediated growth and migration of BC cells. These data highlight the potential role of IGF-1/TXNIP axis in mediating

adipocyte-derived effects, suggesting that targeting this signaling could hamper the crosstalk between adipocytes and BC cells, with particular relevance in Tam-resistant settings.

IGF-1/TXNIP axis and Tam resistance in an obesity 'in vitro' experimental model

Based on the results obtained, we wondered whether the impact of adipocytes in endocrine therapy resistant BC cells may be amplified by obesity. To this aim, we used an 'in vitro' model of obese (Ob) adipocytes obtained from mature 3T3-L1A adipocytes (3T3-L1A Ob), as described in 'Material and Methods' section. 3T3-L1A Ob displayed a hypertrophic and hyperplastic phenotype, a higher content of neutral lipids, expression of specific adipocyte markers and different inflammatory factor secretion patterns compared to 3T3-L1A (Supplementary Fig. S6). Interestingly, IGF-1 content was significantly higher in CM from 3T3-L1A Ob than in that of 3T3-L1A (1520.95 ± 31.45 ng/mL vs. 1154 ± 2.8 ng/mL, $P = 0.007$). First, we found that TXNIP mRNA (Fig. 5A) and protein abundance (Fig. 5B) were further reduced in MCF-7 TR BC cells treated with 3T3-L1A Ob CM compared to cells treated with 3T3-L1A CM. Concomitantly, the increase in the number of colonies (Fig. 5C) and migrated cells (Fig. 5D) was significantly more evident upon exposure to 3T3-L1A Ob CM, and the treatment with SAHA or the IGF-1R inhibitor GSK1838705A reduced the 3T3-L1A Ob CM-mediated effects. Importantly, this reduction was significantly higher upon combined drug treatments (Fig. 5C, D). In line with these data, lentiviral overexpression of TXNIP (o.e.) counteracted 3T3-L1A Ob CM-induced proliferation (Fig. 5E) and migration (Fig. 5F) of TR cells, further supporting the role of TXNIP in controlling obese adipocyte/tumor cell crosstalk.

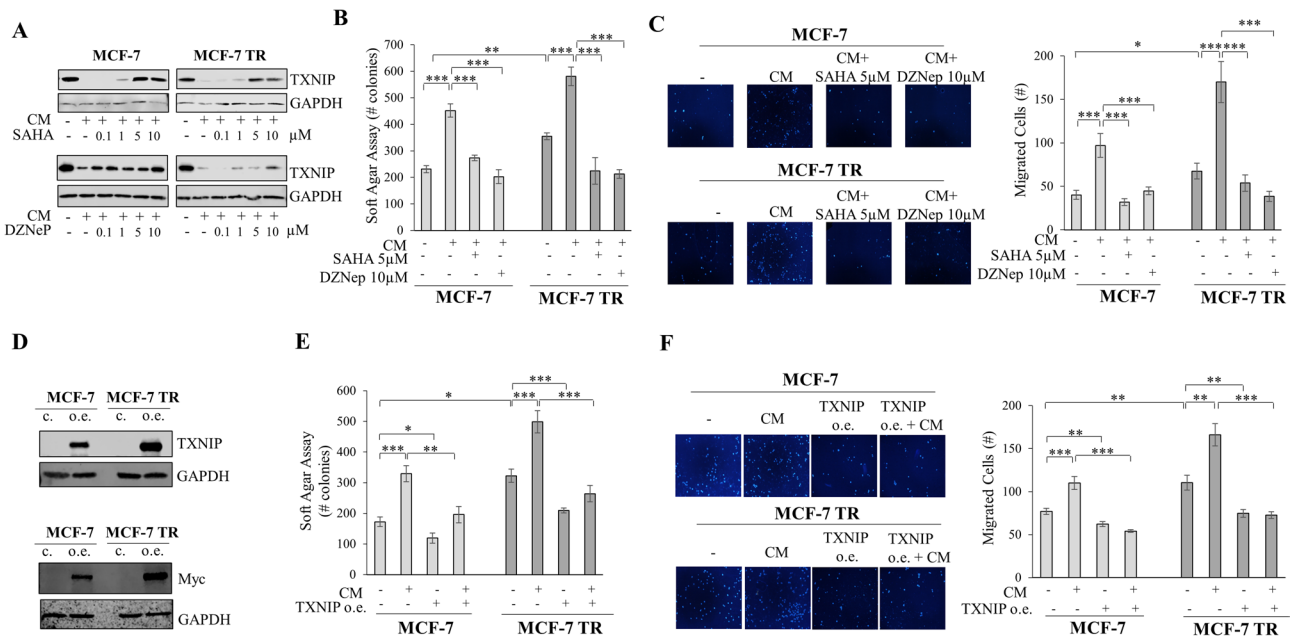


Fig. 3 Pharmacological and genetic re-expression of TXNIP counteract 3T3-L1A CM effects on MCF-7 and MCF-7 TR BC cells. **(A)** Immunoblotting showing TXNIP protein expression in MCF-7 and MCF-7 TR cells treated (+) or not (-) with 3T3-L1A CM with/without the histone deacetylase inhibitor (HDAC) SAHA or the histone methyltransferase EZH2 inhibitor DZNep for 48 h at 0.1, 1, 5, or 10 μ M concentration. GAPDH was used as a control for equal loading and transfer. Soft agar growth **(B)** and Transmigration **(C)** assays in cells treated (+) or not (-) with 3T3-L1A CM $\pm$ SAHA (5 μ M) or DZNep (10 μ M). **(D)** Immunoblotting showing TXNIP protein expression in cells stably overexpressing Myc-tagged human TXNIP protein (o.e.) or empty vector (c) using TXNIP (upper panel) and c-Myc (lower panel) antibodies. GAPDH was used as a control for equal loading and transfer. Soft agar growth **(E)** and Transmigration **(F)** assays in c and TXNIP o.e. cells treated (+) or not (-) with 3T3-L1A CM. Representative images of cell transwell migration are shown. Mean $\pm$ S.E.M. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$

To extend these results, we also used Anastrozole-Resistant (AnaR), and Long-term Estrogen Deprived (LTED) BC cells, modeling Aromatase-Inhibitor resistance. As previously shown for MCF-7 TR cells, we observed a significant reduction in TXNIP mRNA and in its corresponding protein expression in AnaR (Supplementary Fig. S7 A, and B) and LTED (Supplementary Fig. S7 E, and F) cells after 3T3-L1A CM exposure. Again, TXNIP down-regulation was amplified upon treatment with CM isolated from 3T3-L1A Ob in resistant cells. Similarly, 3T3-L1A Ob CM resulted in a significantly increased colony numbers as well as cell migration in both AnaR (Supplementary Fig. S7 C and D), and LTED BC cells (Supplementary Fig. S7 G, and H), while treatment with SAHA or GSK1838705A strongly reduced the proliferative and migratory promoting activities of 3T3-L1A Ob CM in resistant cells. Moreover, the combined treatments induced a greater decrease of anchorage-independent growth and motility (Supplementary Fig. S7 C, D, G, and H), thus indicating that IGF-1/TXNIP axis may be a common player in mediating adipocyte/tumor cell interaction in different endocrine-resistant backgrounds.

Role of IGF-1/TXNIP axis in BC patients

To evaluate the clinical significance of this study, we first validated our results by using an 'ex vivo' model of human mammary adipocytes from obese (hAdipo-Ob) women undergoing surgical mammary reduction (Fig. 6A). In our previous publication [30], we compared the effects of CM from obese (Ob) and normal-weight (NW) human adipocytes on breast cancer cell behavior, and found that Ob-CM significantly enhanced the migratory and invasive properties of breast cancer cells compared to both NW-CM and controls, whereas NW-CM induced only minimal or non-significant changes compared to control treatment. In the present study, we again found that CM from hAdipo-Ob increased the proliferative and migratory invasive properties of TR BC cells compared to control treatment (Fig. 6B, C), and that SAHA as well as GSK1838705A exposure reversed these effects. Again, this inhibition was significantly higher in the presence of the combined drugs. Since establishing animal models that simultaneously recapitulate both obesity and tumor progression remains a significant challenge [52], we validated our findings by using Patient-Derived Organoid (PDO) models. PDOs were generated from overweight or obese patients with endocrine-resistant metastatic BC to assess the effectiveness of IGF-1/TXNIP axis inhibition

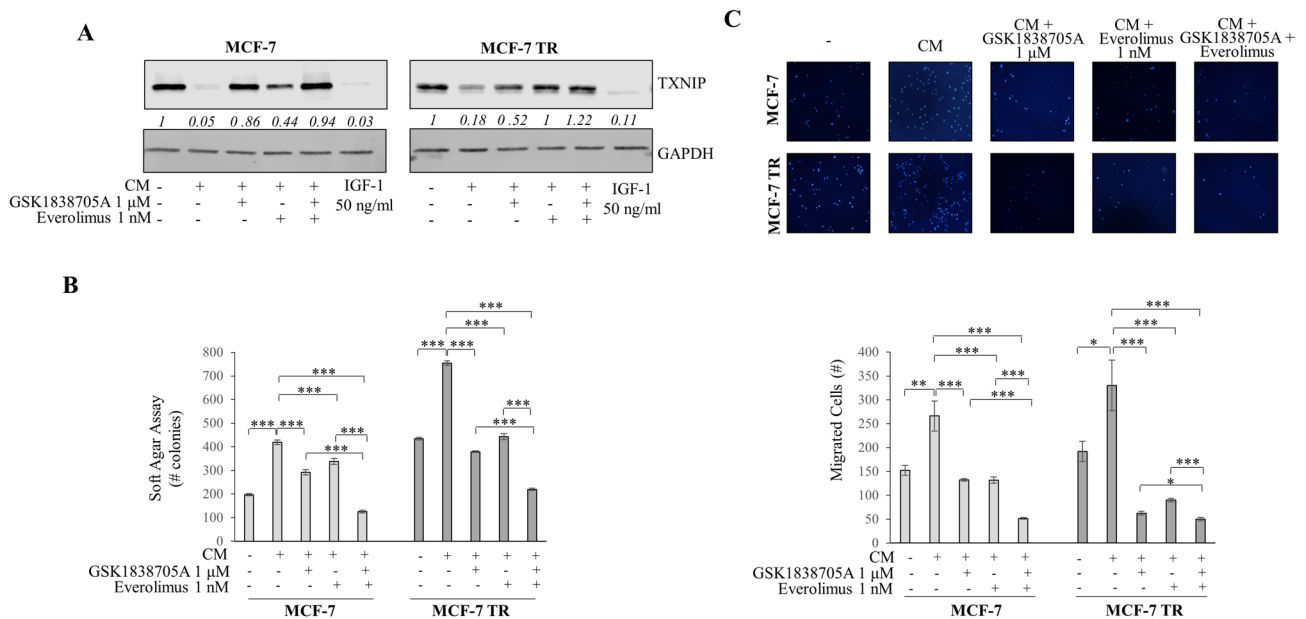


Fig. 4 IGF-1 signaling inhibitors affect 3T3-L1A CM-mediated effects in MCF-7 and MCF-7 TR BC cells. **(A)** Immunoblotting showing TXNIP protein expression in MCF-7 and MCF-7 TR cells treated (+) or not (-) with 3T3-L1A CM in the presence or not of the IGF-1R inhibitor GSK1838705A (1 μ M) and the mTOR inhibitor Everolimus (1 nM) for 48 h. IGF-1 (50 ng/mL) was used as a positive control. GAPDH was used as a control for equal loading and transfer. Numbers below the blots represent the mean of the band optical density expressed as fold over CM-untreated cells. Soft agar growth **(B)** and Transmigration **(C)** assays in cells treated as indicated. Representative images of cell transwell migration are shown. Mean $\pm$ S.E.M. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$

in a more relevant obese setting (Fig. 6D). As shown in Fig. 6E and F, organoid viability and the number of organoid structures were significantly reduced after treatment with SAHA or GSK1838705A and further suppressed upon their combination.

Finally, the potential relevance of the reciprocal relationship between IGF-1 and TXNIP was evaluated by conducting retrospective analyses of the correlation between their expression levels and outcomes of BC patients treated with endocrine therapies. Kaplan-Meier plots demonstrated that an IGF-1_{high}/TXNIP_{low} gene signature correlated with poorer survival in endocrine-treated BC patients in three independent datasets (Fig. 6G), hence confirming a role for IGF-1 and TXNIP in BC tumors resistant to hormone therapy. Notably, when stratifying patients by Luminal A and Luminal B subtypes, this gene signature retained its prognostic significance for Luminal B breast cancer in both the TCGA and GEO datasets. Although a similar trend was observed for Luminal A tumors in the GEO dataset, statistical significance was maintained only in the TCGA cohort (Supplementary Fig. S8).

Discussion

Despite advances in ER α -positive BC treatment with endocrine therapies, resistance and relapse remain common. Obesity, in addition to promoting BC aggressiveness, reduces the efficacy of endocrine therapies, although the mechanisms remain unclear. Our findings identify the IGF-1/TXNIP axis as a novel mechanistic pathway involved in adipocyte/tumor cell crosstalk, which could support obesity-related endocrine resistance. In experimental BC cell lines, adipocyte-derived conditioned medium reduced TXNIP expression/activity and promoted an endocrine-resistant phenotype that was amplified by obesity. Enforced TXNIP overexpression and/or inhibiting IGF-1 signaling reversed adipocyte-mediated effects. The effectiveness of IGF-1/TXNIP axis inhibition was confirmed using an 'ex vivo' model of human mammary obese adipocytes and obese endocrine-refractory metastatic BC PDO models. Importantly, an IGF-1_{high}/TXNIP_{low} signature correlated with poor survival in BC patients treated with endocrine therapies, suggesting the prognostic/predictive and therapeutic potential of IGF-1/TXNIP axis in ER α -positive BC.

TXNIP, initially identified as a redox regulator, is now recognized for its wide-ranging patho-physiological effects and as an important tumor suppressor gene [53]. TXNIP expression is lower in several cancers than in

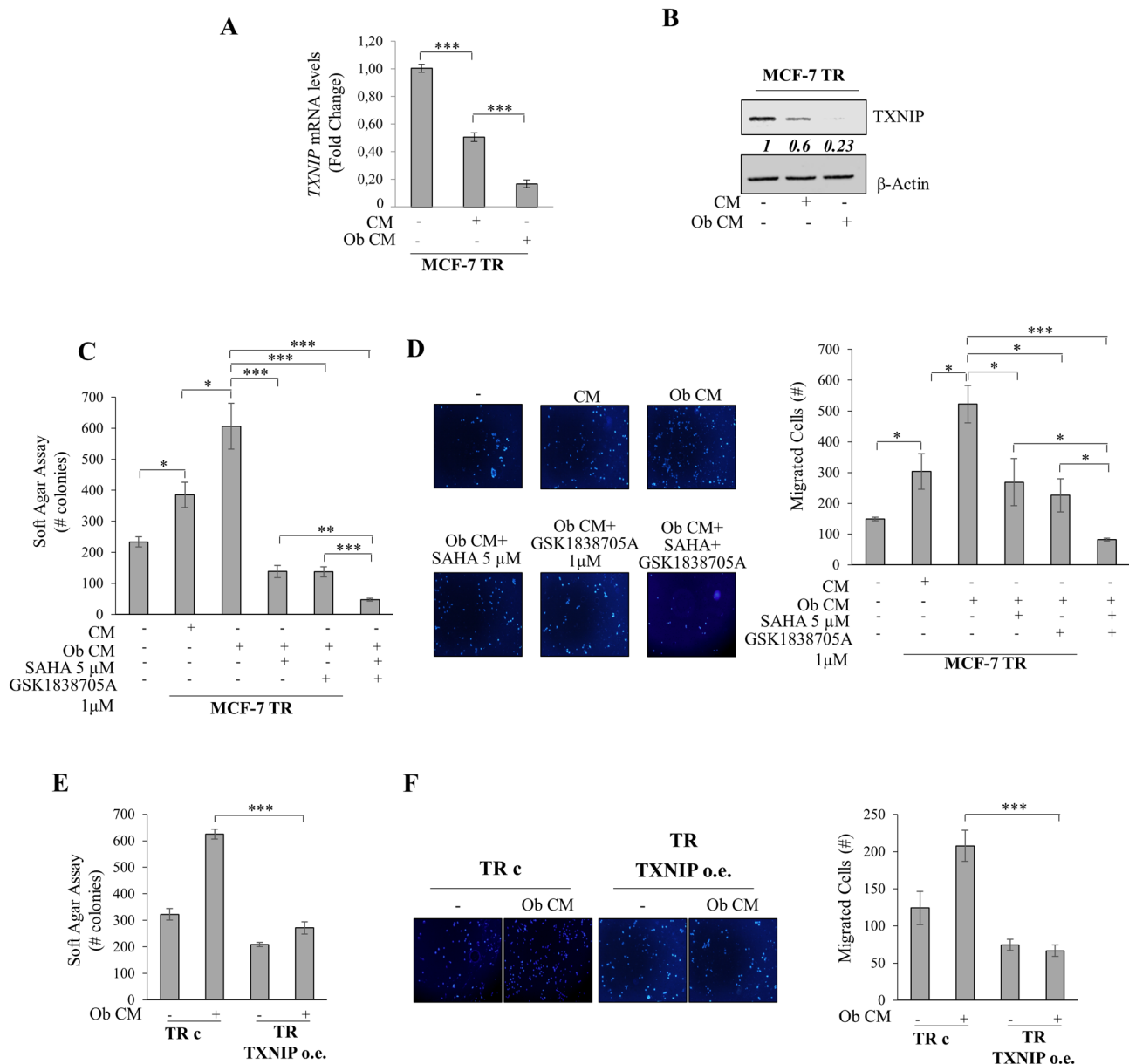


Fig. 5 Targeting IGF-1/TXNIP axis in obesity-induced Tam resistance. **(A)** Real-time RT-PCR for TXNIP mRNA levels in MCF-7 TR cells treated (+) or not (-) with 3T3-L1A CM (CM) and obese 3T3-L1A CM (Ob CM), generated by supplementation with 1 mM of a 1:2:1 palmitate (C16:0), oleate (C18:1), and linoleate (C18:2) mixture for 24 h. **(B)** Immunoblotting showing TXNIP protein expression in cells treated as indicated for 48 h. β -Actin was used as a control for equal loading and transfer. Numbers below the blots represent the mean of the band optical density expressed as fold over CM-untreated MCF-7 TR cells. Soft agar growth **(C)** and Transmigration **(D)** assays in MCF-7 TR cells treated with 3T3-L1A CM and Ob CM $\pm$ SAHA (5 μ M) and/or GSK1838705A (1 μ M). For combination treatments, the same concentrations used for single-agent exposures were applied. Soft agar growth **(E)** and Transmigration **(F)** assays in vector (c) and TXNIP overexpressing MCF-7 TR cells (o.e.) treated with Ob CM. Representative images of cell transwell migration are shown. Mean $\pm$ S.E.M. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$

normal-matched tissues, including lymphoma, breast, colorectal, lung, ovarian, liver tumors [54], being low TXNIP expression an independent risk factor associated with poor prognosis [54–56], and features of aggressiveness [54, 55]. Accordingly, TXNIP deficiency promoted

BC growth [57], while its overexpression inhibited tumor progression ‘in vivo’ [58]. ‘In vitro’ studies indicated that TXNIP may impact BC progression by affecting metabolic reprogramming [59]; down-regulating core factors for Epithelial-Mesenchymal Transition (EMT) [60];

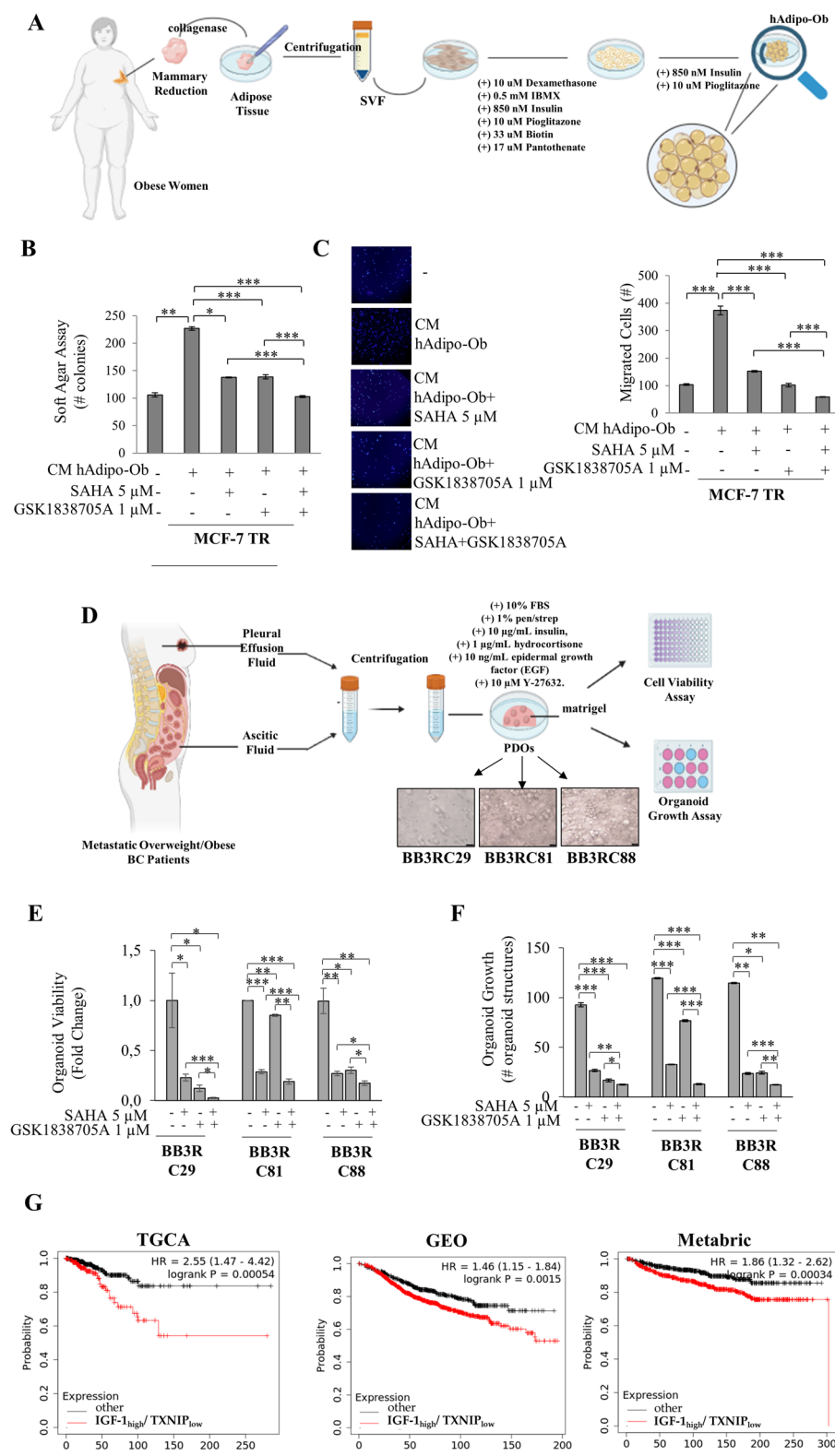


Figure 6

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Fig. 6 IGF-1/TXNIP axis in patients with BC. **(A)** A flow scheme showing the isolation of primary mammary adipocytes from obese women (h Adipo-Ob). Soft agar growth **(B)** and Transmigration **(C)** assays in MCF-7 TR cells treated with CM isolated from h Adipo-Ob $\pm$ SAHA (5 μ M) and/or GSK1838705A (1 μ M). Representative images of cell transwell migration are shown. **(D)** A flow scheme showing the isolation of Patient-derived Organoids (PDOs) from overweight/obese metastatic endocrine resistant BC patients. Representative images of PDOs are shown. Scale bar: 20 μ m **(E)** Relative luminescence of PDOs treated (+) or not (-) with SAHA (5 μ M) and/or GSK1838705A (1 μ M). The combination regimens were administered at the same doses as the individual treatments. Spheroids were cultured for 9 days before their luminescence was measured. **(F)** Number of organoids treated as indicated. Organoids were counted after 12 days. Mean $\pm$ S.E.M. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. **(G)** Kaplan-Meier survival analysis relating IGF-1/TXNIP levels and recurrence-free survival (RFS) in TGCA, GEO, and Metabric datasets, including ER α -positive BC patients treated with endocrine therapy

increasing ROS-mediated DNA damage for cell apoptosis [58], and inhibiting the activation of BC-associated fibroblasts [61]. A role for TXNIP in affecting treatment efficacy has also been suggested. Indeed, TXNIP was lowly expressed in triple-negative chemotherapy-resistant BC tissues and cells, and knocking-down TXNIP reduced cell sensitivity to doxorubicin [58] or fulvestrant [57]. We demonstrated reduced TXNIP expression in multiple endocrine resistant-models, and lentiviral TXNIP overexpression reduced colony formation and migration in MCF-7 and TR BC cells. In endocrine therapy-treated BC patients, low TXNIP expression was associated with a statistically-significant poorer RFS, OS, DMFS, and PPS, outlining the ability of TXNIP to identify patients at high risk of progression during endocrine therapy.

TXNIP expression could be modulated by several biological processes/pathways, including common regulatory mechanisms (such as transcriptional factors, microRNAs, and epigenetic regulators), oncogenes, tumor suppressor genes, cytokines and growth factors [61]. For instance, in BC, c-Myc antagonized TXNIP expression in MondoA-dependent pathway [59]. TNF α [62], and IL-1 β [63] trigger TXNIP down-regulation. Evidence of a regulatory link between IGF-1 signaling and TXNIP was also proven [42]. Indeed, a literature-based causal extraction using the INDRA framework revealed a broader regulatory landscape surrounding TXNIP, including inflammatory mediators (e.g., TNF- α , IL-6), major transcription factors regulating lipid metabolism (e.g., SREBF1, PPAR γ , HIF1A), adipokines (e.g., leptin) and growth factors (e.g., IGF-1). Multiple interactions between TXNIP and these regulators were identified, exerting both activating or inhibitory effects on TXNIP depending on cellular context and stress conditions (Supplementary Table S7). The dataset also comprised multiple independent statements supporting an inhibitory interaction between IGF-1 and TXNIP across various biological systems, associated with high belief scores. Notably, a pathway enrichment analyses concerning TXNIP-correlated proteins/genes across tumors also identified 'regulation of cellular response to insulin stimulus', 'regulation of insulin receptor signaling pathway', and 'insulin receptor signaling pathway' as the most enriched biological processes, and insulin resistance, a common feature of metabolic unhealthy obesity, ranked among the enriched pathways [54]. We found that treatment with

adipocyte-derived CM resulted in a significant reduction of TXNIP expression alongside with a decreased TXNIP transcriptional activity, and an increased TRX activity in MCF-7 BC cells and at a higher extent in TR BC cells. We then extended our investigation by using an 'in vitro' model of obese adipocytes, previously well characterized for key obesity-associated hallmark features (i.e. increased lipid droplet accumulation, TAG content, and basal lipolysis) as well as for their impact on breast cancer cell behaviour, including enhanced uptake of fatty acids, lipid storage, and mitochondrial oxidation compared to lean adipocytes in co-culture experiments [19]. Interestingly, we observed that TXNIP levels were further reduced in MCF-7 TR BC cells treated with CM derived from these obese adipocytes. Moreover, a significant negative correlation between TXNIP expression and BMI values was found in BC patients in transcriptome-based and IHC-based evaluations. As epigenetic mechanisms, particularly DNA methylation and histone acetylation, are involved in TXNIP downregulation in BC [41], we found that the FDA-approved HDAC inhibitor SAHA abrogated CM-mediated reduction of TXNIP expression in cells. In line with data highlighting the release of IGF-1 by adipocytes, and its contribution to obesity-associated BC progression, inhibition of IGF-1 signaling using the IGF-1 receptor (IGF-1R) inhibitor GSK1838705A and/or the IGF-1R downstream mTOR inhibitor Everolimus reversed the reduction of TXNIP expression induced by adipocyte-CM. We also found that IGF-1_{high}/TXNIP_{low} gene signature correlated with poorer survival in endocrine-treated BC patients, confirming IGF-1 and TXNIP's roles in endocrine resistance.

Dysregulation of the IGF-1 system represents a key mechanism in BC endocrine resistance, with IGF-1R up-regulation and/or increased IGF-1 sensitivity facilitating estrogen independence [64], bypassing Tam or AI effects [65, 66], and driving endocrine resistance in ESR1 mutant BC cells [15, 67, 68]. Despite these concepts, clinical studies have failed to ascertain a significant effect of IGF-1R inhibition in therapeutic settings, most probably due to the lack of patient selection biomarkers. Here, we found that treatment with IGF-1R signaling inhibitors significantly reduced the increased cell growth and migration induced by adipocytes in TR cells. Of note, treatment with SAHA, able to re-express TXNIP in BC cells, plus an IGF-1R inhibitor strongly reduced growth

and motility induced by human obese adipocytes. The viability and the number of structures of PDOs derived from overweight/obese patients with endocrine-resistant metastatic BC were significantly reduced after treatment with SAHA or GSK1838705A and further suppressed upon their combination. Consistent with these observations, combined HDAC/mTOR inhibitors killed RAS-driven tumors, that are largely unresponsive to current therapies [69]. Thus, as previously suggested [42], TXNIP may constitute a biomarker to predict and/or monitor responsiveness to anti-IGF-1R therapy.

Conclusion

This study offers fresh molecular and functional insights into the involvement of the IGF-1/TXNIP axis as a pivotal regulator of the cross-talk between adipocytes and BC cells, holding particular relevance in endocrine-resistant settings. Given the growing prevalence of obesity, these findings highlight the potential for novel biomarkers and innovative targets that deserve further investigation for personalized treatment strategies of BC patients with increased adiposity.

Abbreviations

AI	Aromatase Inhibitors
AnaR cells	Anastrozole-resistant cells
BC	Breast Cancer, CTGF: Connective Tissue Growth Factor
DEGs	differentially expressed genes
DMFS	distant metastasis-free survival
DZNep	3-Deazaneplanocin A
BMI	Body Mass Index
ER	Estrogen Receptor
HIF-1 α	Hypoxia-Inducible Factor-1
IGF-I	Insulin-like Growth Factor I
IHC	immunohistochemistry
LTED cells	long-term estrogen deprived cells
MAPK	mitogen-activated protein kinase
NF- κ B	Nuclear factor kappaB
Ob	obese
OS	overall survival
PI3K/Akt	phosphoinositide 3-kinase/ Protein kinase B
PDO	Patient-derived organoids
PPS	post-progression survival
PR	Progesterone Receptor
RFS	recurrence-free survival
SAHA	Suberoylanilide hydroxamic acid
SPN	shortest path network
Tam	Tamoxifen
TR cells	Tamoxifen-resistant cells
TRX	Thioredoxin
TXNIP	Thioredoxin Interacting Protein

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-025-02262-4>.

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
Supplementary Material 10
Supplementary Material 11
Supplementary Material 12
Supplementary Material 13
Supplementary Material 14
Supplementary Material 15

Author contributions

IB, Conceptualization, Visualization, Methodology, Supervision, Writing – original draft, Writing - Review & Editing, Funding acquisition. SC, Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition. SA, Supervision, Writing - Review & Editing, Funding acquisition. AC (Caruso), and FMA, Investigation, Methodology, Writing – original draft. LG, PDC, MFF, LM, CC, EA, and FC, Investigation. BG, LB, AC, RDS, Formal Analysis. CG, DB, CM, and DS, Data Curation, Visualization, Validation. SAWF, BMS, RBC, SAWF, MT, and GS, Resources, Visualization. All authors have reviewed and approved the paper.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki. All clinical specimens and information involved in this study provided informed consent before collection and were approved by The Ethics Committee as specified in Methods' section.

Consent for publication

N/A.

Competing interests

The authors declare no competing interests.

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