

Proximity Mapping of the ER Proteome Reveals Metastasis-specific Candidate Biomarkers in Breast Cancer Cell Lines

MEHMET SARIHAN, ELIFCAN KOCYIGIT, MURAT KASAP and GURLER AKPINAR

Proteomics Laboratory, Department of Medical Biology, Faculty of Medicine, Kocaeli University, Kocaeli, Türkiye

Abstract

Background/Aim: Breast cancer is the most frequently diagnosed cancer among women. While biomarkers are critical for early detection and therapy, current markers lack sufficient specificity and sensitivity. The endoplasmic reticulum (ER) plays a central role in protein folding, post-translational modification, and lipid metabolism, and its alterations are linked to tumor progression. This study aimed to map ER proteome changes associated with breast cancer invasiveness and identify novel candidate biomarkers.

Materials and Methods: We compared the ER proteomes of non-invasive MCF-7 and invasive MDA-MB-231 breast cancer cell lines using an ER-targeted TurboID proximity labelling approach, followed by LC-MS/MS analysis. Bioinformatic analyses were performed to determine functional associations and differential expression related to invasion and metastasis for the candidate biomarkers.

Results: A total of 2,079 proteins were identified, including 1,378 ER proteins. Analysis revealed that more than four hundred ER-resident or associated proteins were differentially regulated in invasive MDA-MB-231, many of which were linked to invasion and metastasis. Upregulated proteins were involved in cellular localization, ECM remodeling, cell mobility, adhesion, vesicle trafficking, and ER stress, whereas downregulated proteins were primarily associated with energy metabolism. Additionally, in this study, 36 ER-associated proteins were identified for the first time as candidates linked to breast cancer, highlighting their potential as novel biomarkers and therapeutic targets.

Conclusion: ER-targeted TurboID proximity labelling effectively maps the proteomic landscape of breast cancer cells, revealing functional adaptations that support invasive and metastatic phenotypes. Notably, 36 ER proteins were identified as novel candidates not previously linked to breast cancer, highlighting new potential biomarkers and therapeutic targets. These findings provide valuable insights into ER proteome remodeling, offering avenues for understanding breast cancer progression and strategies to prevent metastasis.

Keywords: Breast cancer, ER-proteome, biomarker, invasion and metastasis, biotinylation.



Mehmet Sarihan, Department of Medical Biology, Medical Faculty of Kocaeli University, 41380 Umuttepe, Kocaeli, Türkiye.
Tel: +90 5318475929, e-mail: mehmet.sarihan@kocaeli.edu.tr

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Introduction

Cancer remains one of the leading causes of morbidity and mortality worldwide, despite extensive efforts and scientific advancements. According to 2022 statistical data, nearly twenty million new cancer cases were reported globally (1). Among all cancer types in women, breast cancer has the highest incidence and approximately 2.3 million new cases are diagnosed each year. It ranks after lung, colorectal and liver cancers in mortality, but remains the leading cause of cancer-related deaths among women, with an estimated 685,000 deaths worldwide in 2020 (1). Breast cancer incidence is highest in high-income regions such as Northern America, Western Europe and it is lowest in parts of Asia and Africa (2, 3). Conversely, despite a lower incidence, low- and middle-income regions experience higher mortality rates due to limited access to healthcare, delayed diagnosis and inadequate treatment, resulting in increased mortality (4, 5). In recent decades, some high-income countries with developed healthcare systems have reported decreased mortality rates attributed to screening, early diagnosis and better treatments, while both incidence and mortality continue to rise in many transitioning countries (6). In addition, the global burden of breast cancer will continue to grow, with projections of more than three million new cases and about one million deaths per year by 2040-2050, increasing the need for prevention, early detection and equitable cancer treatment strategies (7, 8).

Despite all advances in breast cancer diagnostics and therapeutics, currently available and candidate biomarkers exhibit significant limitations in sensitivity and specificity. For example, HER2 is a clinically important target, but it lacks breast cancer specificity and is also highly expressed in other cancer types (9). Similar limitations also apply to other breast cancer biomarkers, including BRCA1, BRCA2, CCND1 and Ki-67 (10, 11). Therefore, identification of novel, highly specific and robust biomarkers is critically important for early detection and also for targeting and monitoring breast cancer, assessing invasive potential and predicting therapeutic response.

The endoplasmic reticulum (ER) is one of the largest organelles in the cell and plays a critical role in cellular homeostasis, including protein synthesis and folding, post-translational modifications and lipid and steroid biosynthesis (12, 13). Many important protein families such as membrane, secretory, signaling and extracellular matrix proteins are processed, classified and targeted to their respective destinations in the ER (14). ER occupies a central role in cells because it regulates protein synthesis, secretion and stress responses (15, 16). Currently, more than 60% of drugs on the market target ER-processed proteins, highlighting the importance of understanding ER proteome alterations for disease diagnosis and therapy (17). Since changes in the ER proteome of cancer cells have been shown to be closely associated with tumor progression, invasion and the development of aggressive phenotypes, advanced biomarker studies focusing on the ER proteome are critical for understanding cancer-related processes (18). This makes the ER proteome a valuable source for biomarker research (19, 20).

Cancer progression involves uncontrolled proliferation together with the gradual development of invasive and metastatic traits (21, 22). During this process, cancer cells exhibit extensive molecular reprogramming, including alterations in signaling pathways, cytoskeletal dynamics, membrane composition, adhesion molecules and extracellular matrix interactions (23-25). These molecular changes enable cells to detach from the primary tumor, migrate through surrounding tissues, intravasate into the bloodstream and metastasize to distant organs (26). Dysregulation of key pathways, such as epithelial-to-mesenchymal transition, integrin signaling and matrix metalloproteinase activity, has been shown to play a central role in promoting invasiveness and metastasis (27). Understanding these molecular alterations is critical, as they not only provide insights into the mechanisms underlying tumor metastasis and aggressiveness but also offer potential biomarkers for early detection, prognostic evaluation and the development of targeted therapies. The pivotal role of ER in protein metabolism makes it a valuable source for studying the invasion and metastasis

processes during carcinogenesis, as well as the molecular alterations that occur throughout these processes.

In this study, we performed a comparative analysis of the ER proteome to elucidate molecular alterations associated with breast cancer invasion and metastasis and to identify potential novel biomarkers. For this, MCF-7 cells, representing non-invasive breast cancer and MDA-MB-231 cells, which display invasive and metastatic traits, were used to assess differences in cellular aggressiveness (28, 29). Traditional approaches for ER proteome analysis are based on centrifugation techniques or on labelling ER proteins, but these methods are often limited by high material requirements, contamination and poor reproducibility (30). In recent years, proximity labelling approaches, such as TurboID biotin ligase, have presented a rapid, controlled and minimally perturbing alternative (31). Due to these advantages, the TurboID-based labelling approach was used to enrich the ER proteome. This study aimed to elucidate the proteomic mechanisms underlying invasion and metastasis in breast cancer and identify novel ER-based biomarkers.

Materials and Methods

Cell culture. To initiate cell cultures, MCF-7 and MDA-MB-231 cells were rapidly thawed from liquid nitrogen in a 37°C water bath until partially thawed. Thawed cells were transferred to 15-ml sterile tubes containing pre-warmed recovery medium (20% FBS in DMEM) and centrifuged at 400×g for 10 min. The supernatant was removed and cell pellets were resuspended in fresh DMEM supplemented with 10% heat-inactivated FBS, 1× penicillin/streptomycin and 2 mM glutamine (Gibco, Grand Island, NY, USA). Cells were transferred to culture plates, cultured at 37°C in a 5% CO₂ atmosphere and passaged upon reaching approximately 80% confluency.

Determination of selection dose for colony selection. To select transfected cells with the plasmid harboring the ER-directed TurboID gene, pDisplay-TurboID-KDEL, Geneticin (G-418) was used. Prior to transfection, the

cytotoxic concentration of Geneticin for each cell line was determined. The optimal concentration had been defined in previous experiments; however, the assay was repeated because a new batch of the antibiotic was used. Cells were seeded at 50,000 cells per well in 12-well plates, with three replicates per concentration. After 24 h, the medium was replaced with fresh medium containing varying concentrations of Geneticin. The medium was renewed every two days, and the effective lethal concentration was determined. For selection, cells were treated with Geneticin at concentrations of 0, 100, 200, 400, 800 and 1,200 µg/ml.

Plasmid transfection and establishment of stable cell lines. For transfection of breast cell lines with the pDisplay-TurboID-KDEL plasmid, cells were cultured to 70-80% confluence. They were then trypsinized and 1×10⁶ cells were mixed with 15 µg of plasmid DNA in the R buffers. Transfection was performed using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommended protocols and conditions for each cell line. Following transfection, cells were resuspended in antibiotic-free medium, plated into 10-cm culture dishes and subsequently subjected to antibiotic selection. Colonies were isolated using cloning rings, transferred into 96-well culture plates and scaled up to larger culture plates for further culture. Colony validation was performed by assessing enzyme expression and localization using an anti-BirA antibody via Western blot (WB) (1:1,000 dilution) and immunofluorescence (IF) (1:100 dilution) analysis.

Assessment of the effects of endogenous TurboID expression on cell viability and ER stress. The effect of endogenously expressed TurboID on cell metabolism under basal biotin conditions was evaluated by assessing both cell viability and ER stress. For this purpose, the viability of stable cell lines transfected with the pDisplay-TurboID-KDEL plasmid was compared by WST-1 assay. ER stress levels were also determined by analyzing cellular GRP78 expression by WB.

Protein isolation. For protein isolation, cells were placed on ice and washed with cold PBS. Cells were then removed from the plate by scraping in PBS and collected by centrifugation at $1,500 \times g$ for 5 min at 4°C. Cell pellets were resuspended in RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail and incubated on ice for 20 min. Lysis was achieved by homogenization using a bead beater with 0.2 mm stainless-steel beads (NextAdvance, Troy, NY, USA). Cell lysates were clarified by centrifugation at $15,000 \times g$ for 20 min at 4°C. Protein concentrations were measured using the modified Bradford assay (Bio-Rad, Hercules, CA, USA). Protein extracts were snap-frozen in liquid nitrogen and stored at -80°C.

Western blot. Proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry electrophoretic transfer system (Bio-Rad) at 25 V for 30 min. Membranes were blocked with 5% non-fat dry milk in TBS-T for 1 h at room temperature and then incubated overnight at 4°C with the following antibodies: anti-BirA antibody (Novus, Centennial, CO, USA, 5B11C3-3), HRP-Conjugated Streptavidin (Invitrogen, Carlsbad, CA, USA, N100, 1:5,000 dilution) and anti-Beta Actin (Santa Cruz, CA, USA, sc-69879, 12,000 dilution). For detection, a goat anti-mouse secondary antibody (Bio-Rad, 170-5047, 1:10,000 dilution) was used. Signals were visualized using ultrasensitive X-ray film (GE Healthcare, Chicago, IL, USA).

Immunofluorescence staining. Cells were grown on coverslips to 70-80% confluency and washed with PBS twice before fixation with 4% formaldehyde. After the fixation step, cells were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum. To detect biotinylation and TurboID localization, cells were incubated with Texas Red-conjugated neutroavidin (Invitrogen, A2665, 1:750 dilution) and with anti-BirA followed by FITC-conjugated anti-mouse secondary antibody (Abcam, Cambridge, UK, ab6785, 1:750 dilution). Nuclei were stained with DAPI (1 µg/ml) for 10 min. Slides

were mounted using Mowiol mounting medium (Sigma Merck, St. Louis, MO, USA, Mowiol® 4-88). The cells were visualized using an Olympus CKx41 microscope equipped with a DP74 digital camera system.

Biotin labelling of endoplasmic reticulum proteome. For each experimental group, cells were cultured in 100 mm dishes under standard conditions as three independent biological replicates to ensure statistical reproducibility. The cells were grown to 80% confluency, washed with PBS and then incubated in protein-free medium containing 1 mM ATP, 3 mM MgCl₂ and 50 µM biotin (for biotinylation groups only). Cells were incubated for 30 min at 37°C, washed three times with ice-cold PBS, scraped with a cell scraper and then subjected to protein isolation.

Enrichment of biotinylated endoplasmic reticulum proteins. Prior to the enrichment process, 1 mg of protein from three biological replicates per group were combined to create the group pooled samples. All subsequent analyses were performed using these pooled samples. Enrichment of biotinylated proteins was performed as described by Cho *et al.* (15). Briefly, 75 µl of streptavidin-coated magnetic beads (Thermo Fisher Scientific) were washed twice with RIPA lysis buffer and 750 µg of pooled protein sample was mixed with the beads and incubated overnight at 4°C with rotation. Following incubation, the beads were washed sequentially: twice with RIPA lysis buffer, once with 1 M KCl, once with 0.1 M Na₂CO₃, once with 2 M urea in 10 mM Tris (pH 8.0) and twice with RIPA lysis buffer. Biotinylated proteins were eluted with an elution buffer containing 30 mM biotin, 300 mM NaCl, 2% SDS and 25 mM Tris. To evaluate enrichment of biotinylated proteins, an SDS-PAGE analysis was performed. For this purpose, five µl of the eluted fractions ($\approx 1-2$ µg) were loaded onto two gels and then transferred to nitrocellulose membranes for WB to visualize biotinylated proteins. Signal detection was performed using HRP-conjugated streptavidin.

Tryptic digestion. For tryptic digestion, the filter-aided sample preparation (FASP) method was used as described

by Rencher *et al.* (32). For this, 80 µl of the eluted fractions were mixed with 200 µl of 8 M urea in 100 mM Tris (pH 8.0) and transferred to Microcon Ultracel 30 kDa centrifugal units (Millipore, Darmstadt, Germany). After tryptic digestion, the peptides were collected in 50 mM ammonium bicarbonate and 500 mM NaCl by centrifugation at 15,000×g for 15 min. Peptides were dried using a SpeedVac centrifuge (Eppendorf, Hamburg, Germany) and resuspended in 0.1% formic acid (FA). Peptide concentrations were measured using the Qubit assay (Invitrogen, Q33211).

Label-free protein identification and quantification using nHPLC-MS/MS. The peptide samples were analyzed using an Ultimate 3000 RSLC nano system (Dionex, Thermo Scientific) coupled to a Q-Exactive mass spectrometer (Thermo Scientific). High-performance liquid chromatography (HPLC) separation was performed using mobile phases A (0.1% FA) and B (80% acetonitrile + 0.1% FA). Digested peptides were pre-concentrated and cleaned on a trap column. A multi-step gradient (6-90% B) was applied over a 120-minute run at 300 nl/min. The scanning parameters for data acquisition were those described by Sarihan *et al.* and each sample was injected as three technical replicates to ensure analytical consistency (33).

Analysis of mass spectrometry data. The data were analyzed using Proteome Discoverer 2.2 software (Thermo Fisher Scientific) with the following parameters for protein identification: peptide mass tolerance of 4 ppm, MS/MS mass tolerance of 0.2 Da, mass accuracy of 2 ppm, maximum missed cleavages of 1, minimum peptide length of 6 amino acids, fixed modification of carbamidomethylation on cysteine residues and variable modifications of methionine oxidation, asparagine deamination and lysine biotinylation. All extracted spectra were searched separately against UniProt databases containing human reference proteome sequences and against the ER proteome database (GO:0005783). Peptide and protein identifications were filtered using FDR thresholds of 0.01 (strict) and 0.05 (relaxed). Differentially abundant proteins were defined based on

their abundance ratios using a significance cut-off of a two-fold change ($\log_2 \text{fold change} \geq 1$) and a statistically significant *p*-value < 0.05 (determined by ANOVA). Based on these criteria, proteins were categorized as upregulated (abundance ratios ≥ 2) or downregulated (abundance ratios ≤ 0.5).

Bioinformatics analysis. The regulated proteins were analyzed based on the expression trends. Proteins were subjected to bioinformatics analysis using g:Profiler and STRING to evaluate regulated molecular functions, biological pathways (Reactome) and protein-protein interactions. Additionally, to identify breast cancer-associated proteins, the databases GEPIA (<http://gepia.cancer-pku.cn>), UniProt (<https://www.uniprot.org>), Human Protein Atlas (<https://www.proteinatlas.org>) and DisGeNET (<https://disgenet.com>) were used. The DGIdb (Drug-Gene Interaction Database) was used to identify drug-gene interactions and to evaluate the status of proteins in drug studies. Proteins previously reported to be associated with breast cancer in these databases were integrated into a unified reference pool. The candidate marker proteins identified in this study were then compared with both the literature and the pooled database for evaluation. For hierarchical clustering, the online Heatmapper tool was used (<http://www.heatmapper.ca/expression>).

Statistical analysis. WB data were analyzed with IBM SPSS software version 20.0 (IBM Co., Armonk, NY, USA). The Kolmogorov – Smirnov test with skewness and kurtosis values were used to assess the normality of data distributions. WST-1 results were analyzed by one-way ANOVA with Tukey's *post hoc* correction, and Western blot data were compared using independent *t*-tests. The data are expressed as mean±standard deviation. A value of $p < 0.05$ was considered statistically significant.

Results

Generation of ER-localized TurboID-expressing cell lines. To generate ER-localized TurboID-expressing

cell lines firstly the selection doses of Geneticin (G418) were determined. Based on the results from kill-curve experiments, the optimal selection doses were 400 and 800 $\mu\text{g}/\text{ml}$ for the MCF-7 and MDA-MB-231 cell lines, respectively (Figure 1A). Both cell lines were transfected with the pDisplay-TurboID-KDEL plasmid using the Neon transfection system and colony formation was monitored. Following antibiotic selection, single-cell-derived colonies were obtained from both cell lines (Figure 1B). The selected colonies were further cultured and subjected to verification analyses; three colonies from each cell line were screened to assess TurboID expression. The results showed that three MCF-7 and two MDA-MB-231 colonies exhibited detectable TurboID expression (Figure 1C). Accordingly, colony C1 from MCF-7 cells and colony C3 from MDA-MB-231 cells were selected for further experiments. After confirming TurboID expression by WB analyses, IF analyses were performed to examine TurboID's subcellular localization and enzymatic activity. IF analyses demonstrated that TurboID was specifically localized to the ER and exhibited high biotin ligase activity (Figure 1D).

Effect of TurboID expression on cell viability and ER stress. To evaluate the effect of TurboID expression on cellular homeostasis, we assessed cell viability and ER stress levels. We first monitored the cells for morphological changes, and no significant differences were observed before and after transfection (Figure 2A). To assess cell viability, WST-1 assays were performed and the results indicated that the expression of the TurboID enzyme did not significantly affect the viability of MCF-7 cells. Notably, biotin supplementation resulted in a slight increase in viability, whereas Geneticin treatment led to a modest reduction in viability (Figure 2B). In MDA-MB-231 cells, transfection resulted in a slight decrease in viability, but this decrease was not statistically significant (Figure 2B). Additionally, to assess ER stress levels, the expression of GRP78 was evaluated. The results showed no alterations, indicating that TurboID expression did not induce detectable ER stress (Figure 2C).

Biotinylation of the ER proteome in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were cultured in 100mm dishes for ER proteome biotinylation. When cells reached approximately 80% confluency, ER protein biotinylation was performed. The results showed that TurboID enzyme expression was detected in plasmid-transfected cells but not in negative controls (Figure 3A). The localization and intracellular levels of the enzyme and of biotinylated proteins were assessed by IF. Analyses confirmed that negative-control cells showed only basal biotinylation and no enzyme expression, whereas plasmid-transfected cells exhibited enzyme expression and low levels of basal biotinylation in the absence of biotinylation components. The addition of biotinylation components resulted in ER-specific biotinylation co-localized with enzyme expression (Figure 3B). Following confirmation of enzyme expression and intracellular biotinylation activity, biotinylation levels were further assessed by WB analyses using Streptavidin-HRP. Analysis confirmed ER-specific biotinylation in both cell lines and while negative controls showed only signals from endogenous biotinylated proteins, the enzyme expressed biotinylation group exhibited strong signals (Figure 3C).

The Streptavidin-coated magnetic beads were used to enrich biotinylated proteins. To validate the enriched fractions, 5 μl of each sample (approximately 1-2 μg) were subjected to WB analysis and biotinylation levels were assessed using streptavidin-HRP. Although high background noise and low protein loading limited exposure times, WB analysis confirmed successful enrichment of biotinylated proteins, with no detectable signal observed in unbiotinylated negative controls (Figure 3D).

Identification of ER proteins. LC-MS/MS analyses were performed using peptide samples obtained from both the negative control and biotinylated groups of the cell lines. The raw data were analyzed separately using the UniProt human proteome database (UP000005640, reviewed) and curated human ER protein databases. Evaluation of the results revealed that after exclusion of negative controls, 2,079 proteins were identified in the

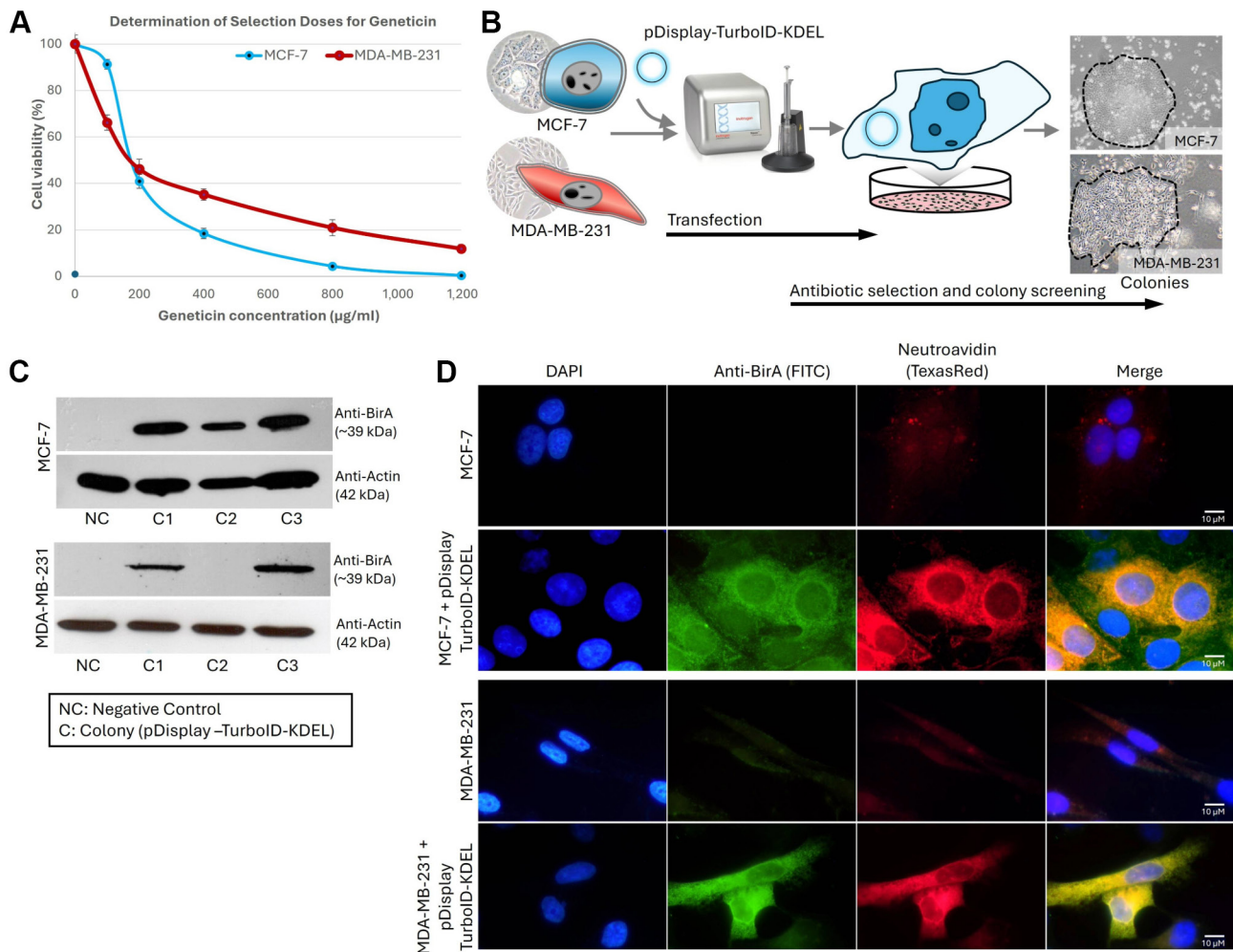


Figure 1. Generation of ER-localized TurboID-expressing MCF-7 and MDA-MB-231 cell lines. (A) The optimal geneticin concentration for selection was determined by subjecting cells to a range of concentrations (0, 200, 400, 800 and 1,200 $\mu\text{g/ml}$). Cell viability was monitored and the results are shown in the graph. (B) Representative colonies formed during the selection process. (C) TurboID expression in selected colonies was confirmed with WB analysis using HRP-conjugated streptavidin. (D) Cellular validation of TurboID expression, Endoplasmic Reticulum (ER) localization and enzymatic activity in MCF-7 (C1) and MDA-MB-231 (C3) cell lines were performed by IF analysis using anti-BirA (FITC) antibody and streptavidin-Texas Red staining; negative controls (non-transduced cells) were included. Scale bars are indicated on the images.

biotinylation groups (Figure 4A). Of these, 675 proteins belonged to the ER proteome (Figure 4A). In addition, the analysis of ER-associated proteins such as transmembrane and secreted proteins, potentially biotinylated while passing through the ER, demonstrated the presence of an additional 703 proteins (Figure 4A). In total, 1,378 proteins were identified as ER-resident or ER-associated (Figure 4A). To improve the reliability of the analyses, the dataset was restricted to proteins identified by at least

two unique peptides. Of a total of 1,190 proteins identified with at least two unique peptides, 403 were classified as ER proteins, including 332 that overlapped with the ER-associated group (Figure 4A). The latter contained 771 proteins, of which 439 were exclusively ER-associated (Figure 4A). For further analysis, only proteins with two or more unique peptides were used.

Comparative analysis of ER proteins revealed that 185 proteins were upregulated and 28 were

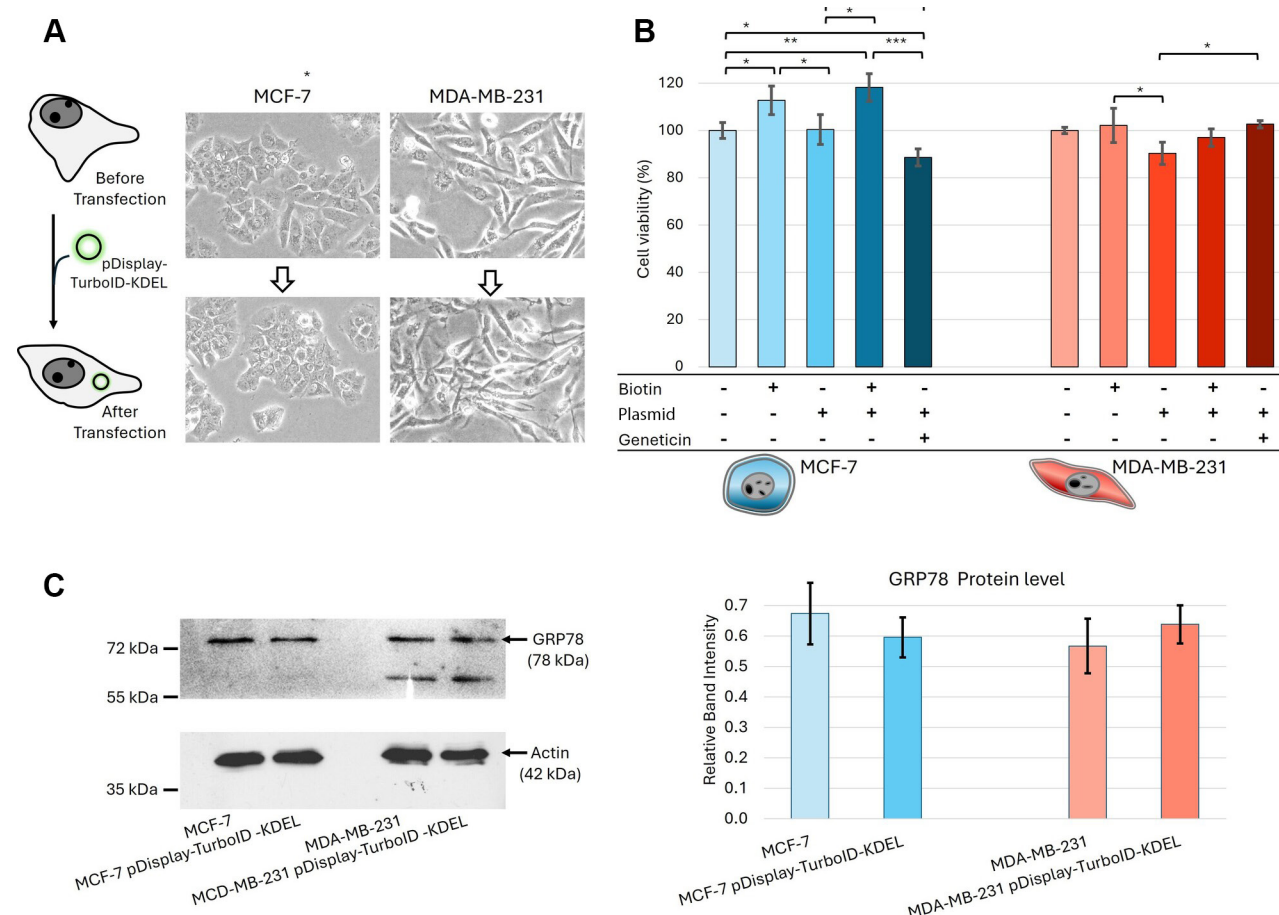


Figure 2. Effect of ER-directed TurboID expression on cell morphology, viability and ER stress in MCF-7 and MDA-MB-231 cells. (A) Cell morphology after transfection with the pDisplay-TurboID-KDEL plasmid. (B) Effects of plasmid transfection and excess biotin supplementation on cell viability of MCF-7 and MDA-MB-231, assessed using the WST-1 cell viability assay. (C) Effect of ER-directed TurboID expression on ER stress was analyzed by WB of the ER stress marker GRP78. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test to evaluate WST-1 results and by independent t-tests for WB analyses. All statistical analyses were performed using SPSS version 20. * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

downregulated in MDA-MB-231 cells (Figure 4B). Among the most prominent differences between the two cell lines are their invasive and metastatic characteristics. Therefore, proteins associated with invasion and metastasis were evaluated. Results revealed that 116 upregulated proteins were linked to these processes, while 12 downregulated proteins were associated with invasion and metastasis (Figure 4C). These findings highlight key molecular differences underlying the aggressive phenotype of MDA-MB-231 cells.

Functional analysis of differentially expressed proteins.

To investigate the functional roles of the differentially expressed proteins, STRING and g:Profiler analyses were conducted to identify associated molecular functions, biological processes, Reactome pathways and protein-protein interaction networks. STRING analysis was conducted on 185 upregulated proteins identified in MDA-MB-231 cells that exhibited invasive and metastatic characteristics (Figure 5A). Functional enrichment analysis revealed that the upregulated proteins were significantly associated with biological

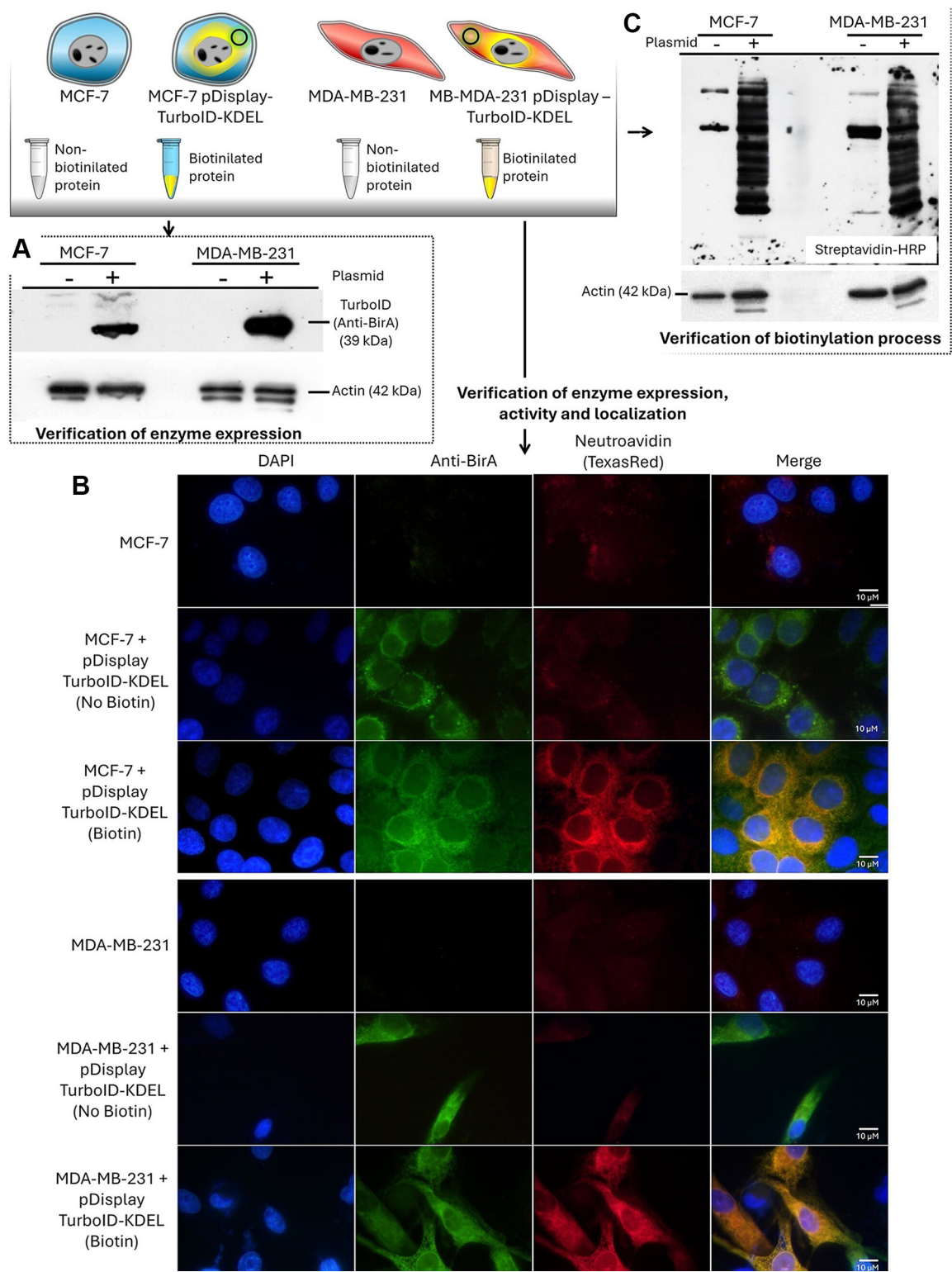


Figure 3. Continued

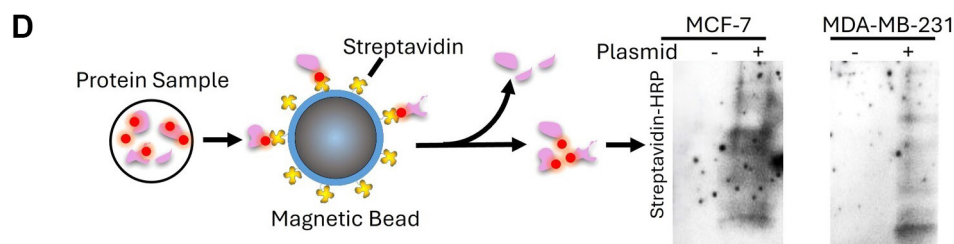


Figure 3. Biotinylation and enrichment of the ER proteome of MCF-7 and MDA-MB-231 cells. (A) WB analysis demonstrates TurboID expression in MCF-7 and MDA-MB-231 cells. (B) IF analyses were performed to validate TurboID expression, ER localization and enzymatic activity in MCF-7 and MDA-MB-231 cell lines using an anti-BirA (FITC) antibody and streptavidin–Texas Red staining. Non-transduced cells were included as negative controls. (C) Cellular biotinylation levels were analyzed by Western blotting using streptavidin–HRP. (D) Enrichment of biotinylated proteins was assessed by WB analysis. Due to the limited amount of loaded biotinylated protein and background signal interference, weak and low-intensity signals were observed.

processes, including endoplasmic reticulum stress, protein folding, extracellular matrix organization, membrane organization, apoptosis and cell motility. In parallel, g:Profiler analysis evaluated the same protein set in terms of biological processes, molecular functions and reactome pathways (Figure 5B). Consistent with the STRING results, the g:Profiler analysis highlighted similar underlying mechanisms, with particular enrichment in processes associated with cellular localization, cell migration, regulation of gene expression and hydrolase activity (Figure 5B). Furthermore, analysis of the 28 downregulated proteins showed that processes related to cellular localization, endoplasmic reticulum stress, post-translational modifications and lipid metabolism were affected (Figure 5C, D).

Analysis of the remaining ER-associated proteins revealed 146 upregulated targets with functional profiles that corroborated the pathways identified by the ER-resident protein analysis. These upregulated proteins were primarily involved in cellular localization, migration, translation, vesicle-mediated transport, hydrolase activity, gene expression, angiogenesis and cell adhesion (Figure 6A, B). Additionally, analysis of the downregulated proteins pointed to mitochondrial aerobic respiration, oxidoreductase activity and mitochondrial transport (Figure 6C, D).

Candidate ER-resident and ER-associated biomarkers involved in breast cancer metastasis and invasion. The

association between the regulated proteins and breast cancer was investigated using GEPIA (TCGA), UniProt, the Human Protein Atlas (HPA) and the Drug–Gene Interaction Database (DGIdb) to assess their potential relevance for drug target development. Due to the input requirements of the analysis programs used, gene names were preferred over accession numbers. The results indicated that among ER-resident proteins (165 upregulated and 25 downregulated genes corresponding to 213 regulated proteins), no database evidence was found linking 64 upregulated and 12 downregulated proteins to either breast cancer or drug-related studies (Figure 7A). Additionally, analysis of 199 ER-associated proteins (143 upregulated and 53 downregulated) revealed that 59 upregulated and 18 downregulated proteins had no reported association with breast cancer drug studies (Figure 7B).

Combining proteins that had neither previously been associated with breast cancer nor annotated as drug targets in existing databases, a total of 153 candidate biomarkers were identified (Figure 7C). These proteins were analyzed with respect to their plasma membrane and extracellular localizations, which are particularly relevant to the development of prognostic and diagnostic biomarkers and to therapeutic targeting. The analysis showed that a substantial proportion of the candidate biomarkers can localize to these compartments. Of the 153 identified proteins, 86 were annotated as extracellular region and 50 as plasma membrane proteins. Among these, 27 proteins were common to both localizations.

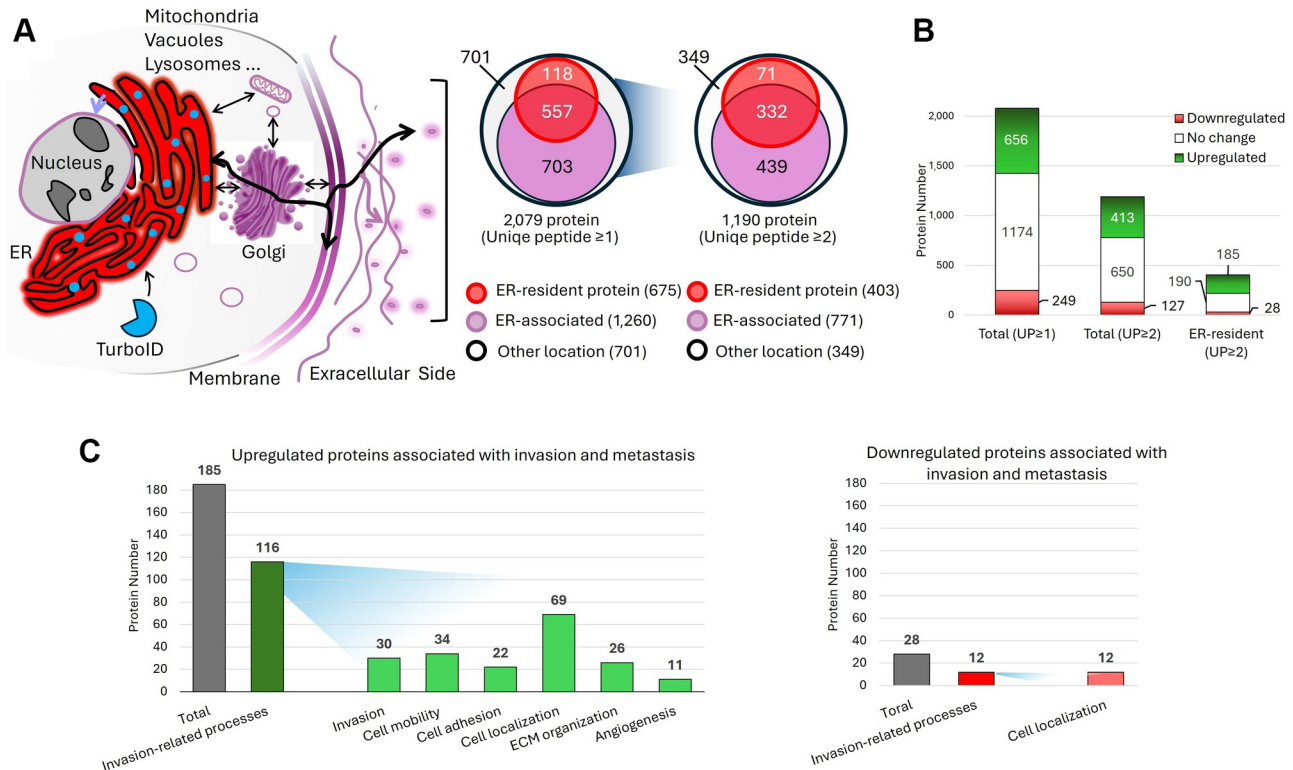


Figure 4. Identification of ER proteins. (A) Subcellular localization distribution of identified proteins. Venn diagrams show the overlap between ER, ER-associated and other subcellular proteins identified with ≥ 1 or ≥ 2 unique peptides. (B) Expression-based distribution of proteins among total identified proteins with ≥ 1 or ≥ 2 unique peptides. (C) Distribution of upregulated and downregulated proteins associated with invasion- and metastasis-related biological processes. Bar graphs depict the total number of invasion-related proteins and their classification into specific processes, including invasion, cell mobility, cell adhesion, cellular localization, extracellular matrix (ECM) organization and angiogenesis.

The remaining 44 proteins were associated with other cellular compartments.

A literature review of the 109 proteins localized to the extracellular region and/or the plasma membrane revealed that the majority had previously been associated with breast cancer. However, a substantial proportion of these studies were primarily based on transcript-level data or bioinformatic predictions. In contrast, the present study directly interrogated candidate proteins at the protein level. Notably, 28 of the regulated proteins are reported here for the first time to be associated with breast cancer (Table I).

Thirty-six novel biomarker candidates were subjected to survival analyses. The analyses revealed that almost none of the investigated proteins were significantly associated with survival in breast cancer. In contrast, the majority

exhibited significant associations with survival in multiple other cancer types (Supplementary File 2). Furthermore, a comprehensive literature review regarding their biological functions and disease associations confirmed that while these candidates are established regulators or biomarkers in various malignancies, their role in breast cancer remains largely unexplored (Supplementary File 2, Table I). Based on the literature review, 36 proteins were classified according to their biological functions and presented along with their fold change values (Figure 8).

Discussion

In this study, ER-targeted TurboID-expressing MCF-7 and MDA-MB-231 cell lines were successfully generated.

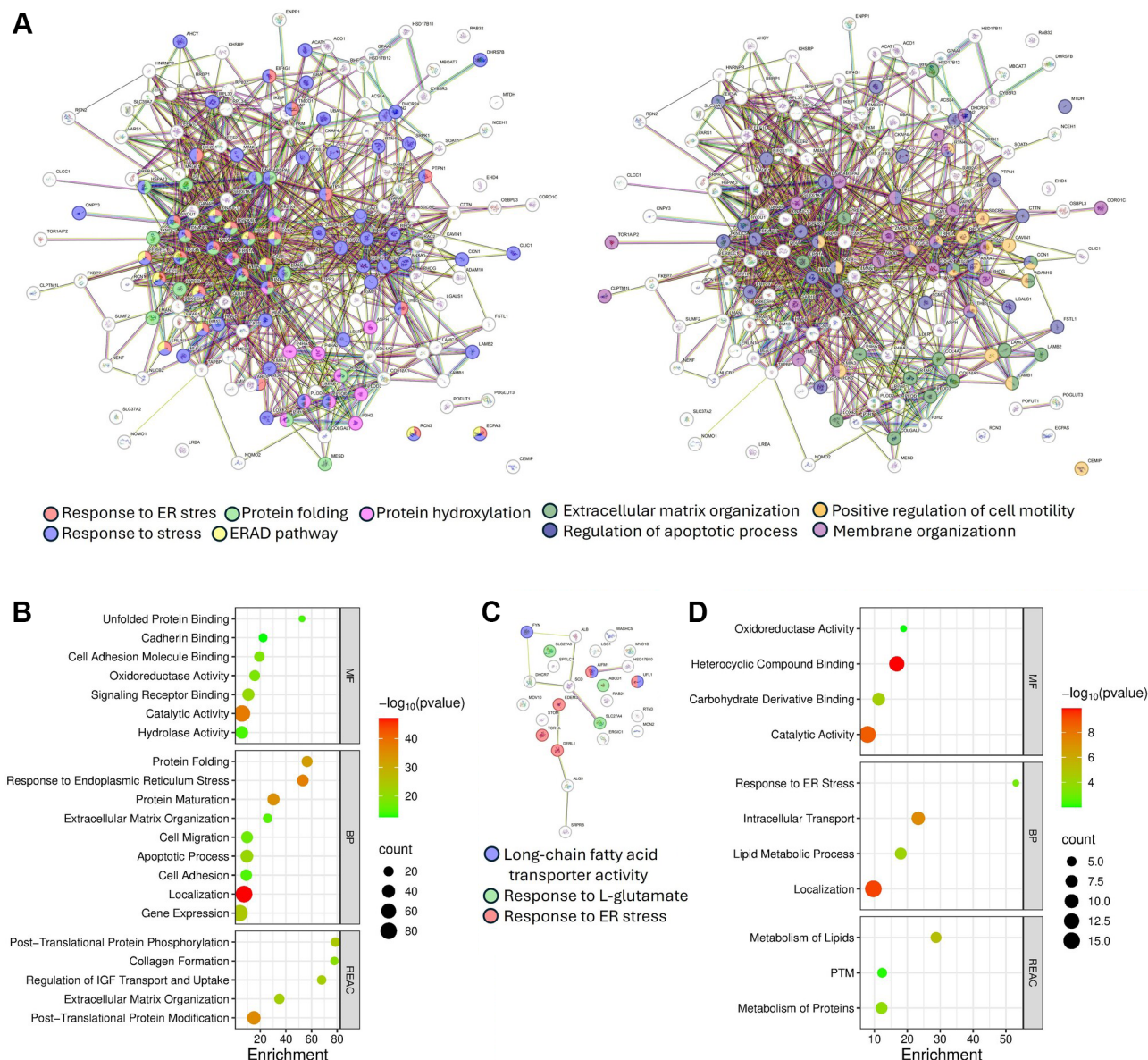


Figure 5. *STRING* and *g:Profiler* analysis of differentially expressed ER proteins in MDA-MB-231 cells compared with MCF-7 cells. (A, C) *STRING* protein-protein interaction network of upregulated (A) and downregulated (C) proteins with functional clusters. (B, D) *g:Profiler* functional enrichment analysis of upregulated (B) and downregulated (D) proteins showing molecular function (MF), biological process (BP) and reactome pathways (REAC).

Analyses showed no significant differences between the control and plasmid-transfected groups, indicating that TurboID expression and the subsequent labelling process did not induce discernible adverse effects. Additionally, we demonstrated that the enzyme was efficiently expressed

in both cell lines and localized within the ER in an active form. Although the lack of co-localization studies with established ER markers is considered a limitation, the highly specific ER proteomic profile obtained was deemed sufficient to support ER localization (12, 34). Furthermore,

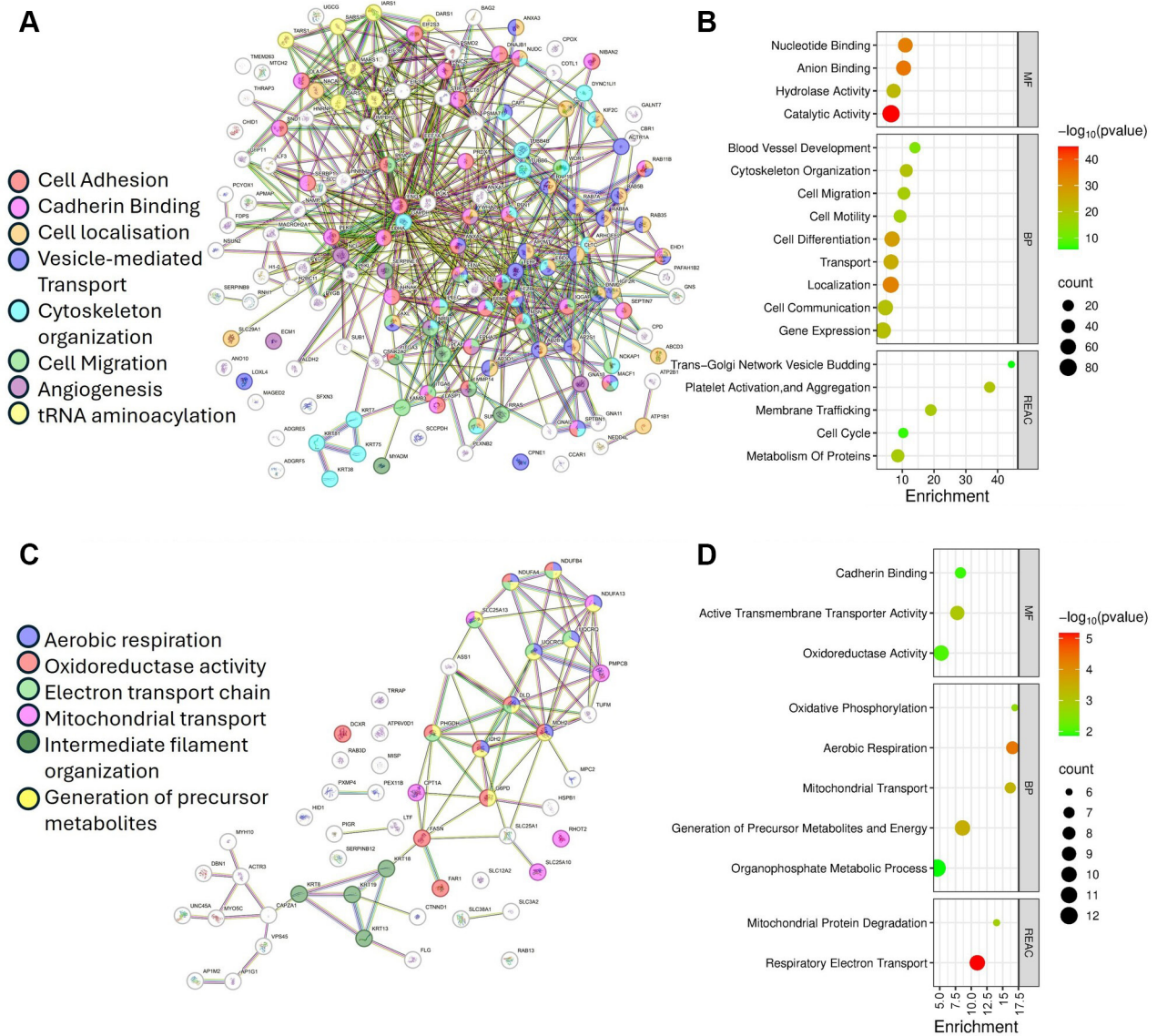


Figure 6. *STRING* and *g:Profiler* analysis of differentially expressed ER-associated (excluding ER proteins) in MDA-MB-231 cells compared with MCF-7 cells. (A, C) *STRING* protein-protein interaction network of upregulated (A) and downregulated (C) proteins with functional clusters. (B, D) *g:Profiler* functional enrichment analysis of upregulated (B) and downregulated (D) proteins showing molecular function (MF), biological process (BP) and reactome pathways (REAC).

the ER biotinylation was successfully validated by both IF and WB analyses. The enrichment of biotinylated proteins was confirmed by detectable WB signals that clearly differed from those of the negative controls, despite relatively weak Streptavidin-HRP signal intensity. This situation resulted from technical constraints, including

limited protein loading ($\approx 1\text{--}2\ \mu\text{g}$) and background noise that prevented prolonged exposure.

The LC-MS/MS analyses revealed that approximately 70% of the identified proteins were associated with ER. In addition, during the transition from a non-invasive cellular phenotype to an invasive and metastatic phenotype, a

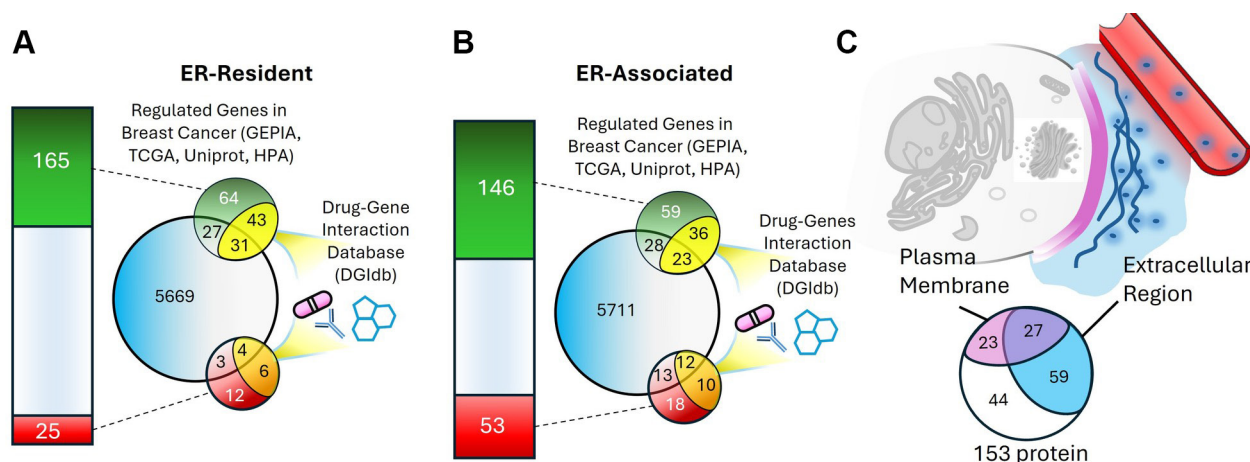


Figure 7. Bioinformatic analysis of ER-resident and ER-associated genes in breast cancer and their comparison with drug databanks. (A, B) ER-resident and ER-associated proteins were analyzed and compared with breast cancer-related genes in the UniProt, HPA and GEPIA (TCGA) databases and their potential as drug targets was evaluated. (C) Additionally, 153 ER-resident and ER-associated proteins that were neither linked to breast cancer nor regulated were assessed for their biomarker potential, with a focus on extracellular and plasma localization.

substantial increase was observed in the levels of proteins associated with invasion and metastasis. The STRING and g:Profiler analyses indicated that the ER-resident and ER-associated upregulated proteins in MDA-MB-231 cells were associated with cellular localization, protein folding, ER stress, vesicle-mediated transport, cell migration and adhesion, hydrolase activity and extracellular matrix organization. Hydrolases, such as MMP14, MMP2 and MMP13, drive tumor progression and metastasis by degrading the ECM and releasing pro-tumorigenic factors and are linked to aggressive tumors and poor prognosis (33, 35). ECM is a dynamic scaffold that undergoes continuous remodeling to maintain tissue homeostasis. In tumors, the ECM is extensively reorganized, becoming stiffer and biochemically distinct, which directly promotes cancer cell proliferation, survival, migration and differentiation (36). Furthermore, ECM organization reprograms stromal cells, sustains cancer stem cells and enhances drug resistance and relapse (37). Cancer cells exhibit markedly increased adhesion to the ECM, forming stable focal adhesions that allow them to withstand mechanical stress and enhance survival and proliferation (38). Additionally, organ-specific adhesion to endothelial cells directs metastatic colonization, with inducible

endothelial adhesion molecules further facilitating tissue-specific metastasis (39). Vesicle-mediated transport is deeply involved in cancer initiation, progression, metastasis, immune escape and drug resistance through both intracellular trafficking and extracellular vesicles (38-41). Additionally, ER-resident and ER-associated proteins involved in aerobic respiration, oxidoreductase activity and mitochondrial transport were downregulated in MDA-MB-231 cells. These observations are consistent with previous studies and align with the Warburg hypothesis (42, 43). Results showed strong concordance with the literature and indicated that ER-targeted proximity labelling is a powerful and reliable approach for uncovering functionally relevant alterations in ER-resident and ER-associated protein landscapes linked to invasive and metastatic phenotypes.

In addition to established cellular mechanisms, a significant outcome of our study involves a distinct group of proteins with no previously reported links to breast cancer. One notable finding of this study is observed during the analyses of ER-resident and ER-associated proteins association with breast cancer. The search for regulated proteins in relevant databases showed that most of these proteins had no previously reported association

Table I. 109 ER-resident or associated proteins are shown. While most appear in transcriptomic or bioinformatic data, this proteomic study identified 36 new associations: 28 localized to the plasma membrane/extracellular space and 8 ER-associated proteins (highlighted in bold). Proteins found in both locations are framed in black.

Proteins with no breast cancer association in reference databases and no identification in DrugBank

Extracellular region				Plasma membrane			
SLC37A2	NOMO1	NUDC	MACROH2A1	PEX11B	CPNE1	H2BC11	ITGA3
P3H1	HSD17B12	GNS	HNRNPL	UNC45A	PAFAH1B2	DCXR	PLXNB2
ERO1A	HEL-S-30	ABCD3	SND1	MYO5C	KRT75	ITPR3	EHD1
TXNDC5	SAR1B	PCYOX1	SARS1	OSBPL3	ARHGEF2	CORO1C	CAP1
RAB32	VARS1	QARS1	NSUN2	CAPN2	RAB35	CLIC1	MYADM
PLOD3	LSG1	NACA	THRAP3	SDCBP	PLEC	MESD	ADGRE5
ERP29	WASHC5	CHID1	IARS1	HSPA13	AHNAK	MAGT1	ANO10
SIL1	MON2	ACTR1A	PFKL	HEL-S-89N	RAP1B	LRPAP1	RAB5B
NOMO2	BAG2	SERBP1	CCT8	HEL-S-99N	NIBAN2	PSAP	SLC25A13
PLOD2	KRT38	APMAP	RAB11B	ARF4	RNH1	EHD4	ATP6V0D1
DNAJC3	SUB1	PFKP	SLC25A1	VAPA	WDR1	SLC27A4	SERPINB12
GPAA1	COTL1	EIF3B	AP1G1	RAB21	DYNC1L1	SLC27A3	
SRPRA	TARS1	MARS1	TUFM	MSN	NCKAP1	RTN3	
RAB2A	SERPINB9	EIF2S3	CAPZA1	TLN1	AP2S1	MYO1D	
Other ER-associated							
RCN3	LMAN2L	ERLIN1	HEL-S-164	ECPAS	DERL1	AP3D1	PMPCB
POGLUT3	SUMF2	MIA3	OS9	CLCC1	EDEM3	TRRAP	FAR1
GPX8	POFUT1	HEL-S-97n	SEL1L	DHRS7B	L1RE1	RHOT2	
COLGALT1	CNPY3	HEL117	TOR1AIP2	PRKCSH	SUN1	UQCRQ	
IKBIP	ERLEC1	YIPF5	DNAJC10	SEC61A1	TMEM263	VPS45	
FKBP7	RCN2	LBR	HNRNPR	ALG5	SFXN3	MPC2	

with breast cancer. This discrepancy may result from the relatively limited amount of verified data in existing databases compared to the literature and delays in integrating findings from the literature into the databases (44, 45). On the other hand, when the drug associations of the regulated proteins were analyzed, a significant portion was found to be involved in target drug studies. This underscores the relevance of the identified protein set for drug development and highlights their potential as important targets for therapeutic intervention.

Proteins not previously linked to breast cancer or to drug-targeting studies were systematically evaluated, focusing on extracellular and plasma membrane compartments because of their high potential as biomarkers and therapeutic targets. We identified 109 proteins in these compartments and conducted a detailed literature review for each protein to examine possible associations with breast cancer. The results indicate that

most of these proteins have been associated with breast cancer in recent studies. However, most of these studies are based on transcriptomic and bioinformatics analyses. The identification of these associations at the protein level in this study may provide critical insights into the underlying molecular mechanisms.

Moreover, this study provides the first protein-level evidence supporting potential associations between numerous proteins and aggressive molecular phenotypes in breast cancer. Another significant outcome of this work is the first demonstration of the regulation of 36 ER-resident or ER-associated candidate biomarker proteins during breast carcinogenesis. We further analyzed these 36 proteins in GEPIA to assess associations with survival.

ERLEC1 is an ER-resident protein that plays a pivotal role in N-glycan recognition and ER-associated degradation which are critical for tumor invasion and metastasis (46). While ERLEC1 has not yet been directly linked to breast cancer

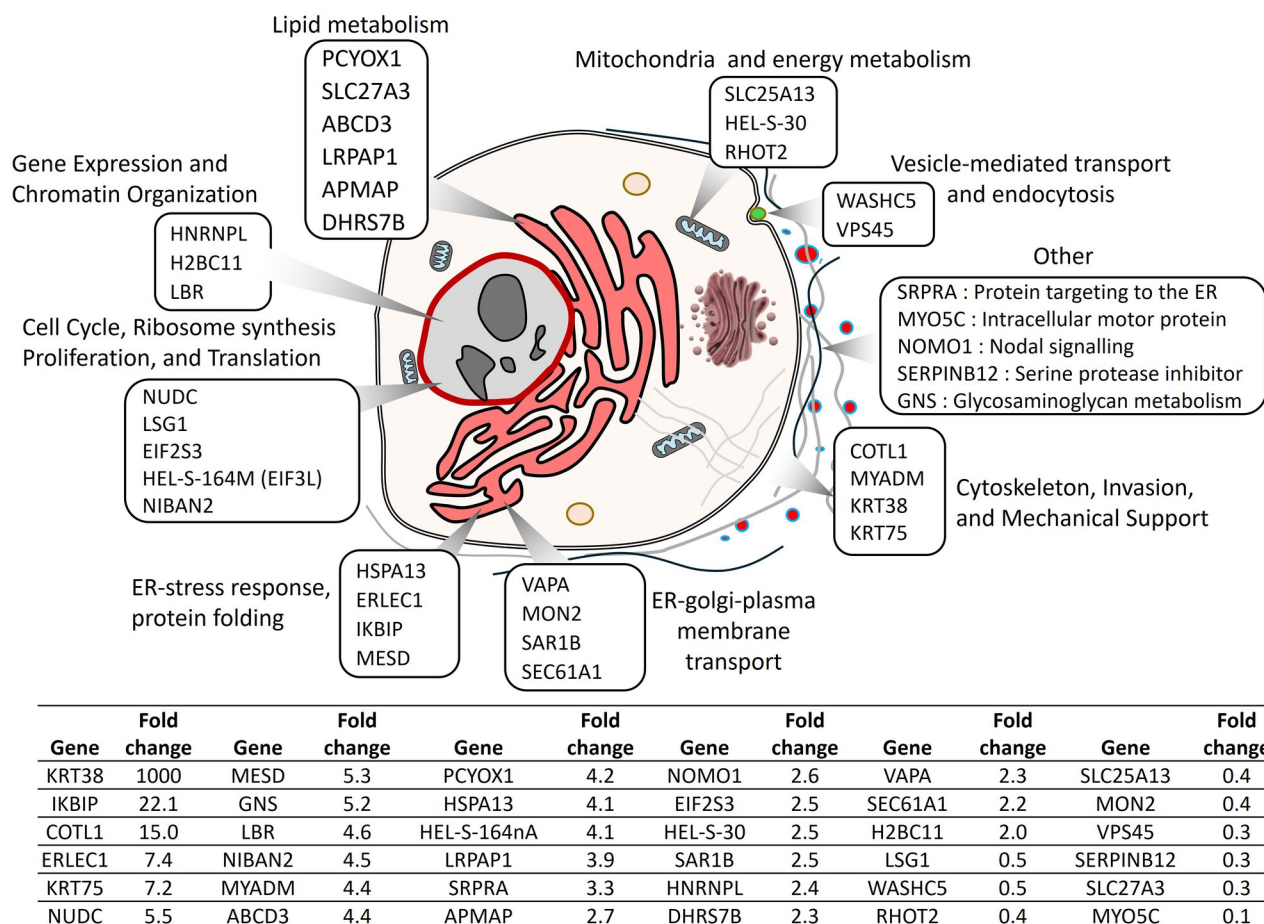


Figure 8. Functional distribution and fold-change ratio of 36 proteins not previously associated with cancer or drug studies.

in the current literature, ER stress and redox homeostasis related proteins such as ERO1 have been associated with cancer progression (47). These proteins promote tumor aggressiveness, metastasis, and therapy resistance through mechanisms of hypoxia response and immune evasion. SEC61G is highly expressed in breast cancer tissues and is associated with poor prognosis. SEC61G promotes breast cancer cell proliferation, migration, invasion, and inhibits apoptosis by modulating glycolysis (48). VAPA is another ER-resident membrane protein involved in lipid transport, peroxisome maintenance, cholesterol regulation, membrane contact site tethering and stabilizes ER-plasma membrane contact sites which important for cell motility and focal adhesion dynamics (49, 50). VAPA also appears to play

a role in metastasis and cancer cell motility (51), though VAPA itself has not been directly linked to breast cancer in current studies. COTL1 acts as a tumor suppressor in breast cancer by inhibiting cell proliferation and tumor growth. It is highly expressed in epithelial breast cancer cells and its knockdown increases proliferation, while overexpression reduces tumor growth in mouse models (52, 53). COTL1 promotes the expression of the growth-suppressor interleukin-24 (IL-24). Additionally, COTL1 enhances the inhibitory effects of TGFβ1 which include inhibition of MAPK/ERK phosphorylation. Loss of COTL1 expression is common in breast and other cancers, and its presence also sensitizes breast cancer cells to chemotherapy, highlighting its potential as a target molecule (53). Despite

the well-established roles of the broader HSP70 family in tumor survival and stress responses, HSPA13 remains poorly characterized in breast cancer (54-56). SAR1B, a small GTPase mediating ER-to-Golgi vesicle transport, is limited direct linking to breast cancer (57, 58). Keratins are a big family with numerous members that fulfill various biological roles beyond their structural functions (59). These proteins play role in oncogenic processes, including cellular signaling, ECM remodeling, metastasis, and cell differentiation. While some isoforms, such as KRT16 and KRT17, have established roles in cancer progression, there is limited information in the literature regarding KRT38 and KRT75 (60). Notably, our observations of significant upregulation of KRT38 and KRT75 proteins during metastasis in breast cancer cells suggest that these specific keratins may play an important role in breast cancer malignancy. Taken together, ERLEC1, HSPA13, SAR1B, VAPA, KRT38, KRT75, and other candidate biomarkers not discussed here, such as NUDC and IKBIP, show significant potential in breast cancer; however, much more research is needed to determine their precise roles in tumor progression and therapy response.

While most of these proteins have not yet been linked to survival in breast cancer, some have been correlated with survival in other cancer types. These findings suggest that these proteins may also play a role in cancer progression, prognosis and metastasis and have the potential to be biomarkers for breast cancer. In this regard, further investigation and comprehensive validation studies are needed.

Conclusion

This study shows that ER-targeted TurboID proximity labelling is a powerful approach for mapping the proteomic landscape of cells. Analysis of the ER-associated proteome highlighted extensive alterations in key oncogenic drivers such as cellular localization, ECM remodeling, cell mobility and migration, cell adhesion, vesicle trafficking and energy production. By analyzing these important pathways at the protein level, our results provide a clearer understanding of how the ER

environment adapts to support invasive and metastatic phenotypes. Beyond these well-known mechanisms, our results provide a comprehensive list of proteins showing significant differential expression, many of which are known to play roles in cancer biology but have not been specifically mapped to the ER compartment in this context. Furthermore, one of the main contributions of this work is the identification of 36 ER-associated proteins that were previously unlinked to breast cancer and drug studies. Several of these novel candidate biomarkers represent potential therapeutic targets, underscoring the clinical value of our findings and providing new avenues for understanding and preventing metastasis.

Supplementary Material

Supplementary material can be found at: <https://doi.org/10.6084/m9.figshare.31329109>.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

GA: Formal analysis. EK: Investigation, Formal analysis, Visualization. MK: Investigation, Methodology. MS: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Software, Validation.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (PoolText) was used solely for language editing and stylistic improvements in select paragraphs.

No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual data were generated or modified using generative AI or machine learning-based image enhancement tools.

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