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Article

Proteomic Analysis Reveals Major Proteins and Pathways That Mediate the Effect of 17-β-Estradiol in Cell Division and Apoptosis in Breast Cancer MCF7 Cells

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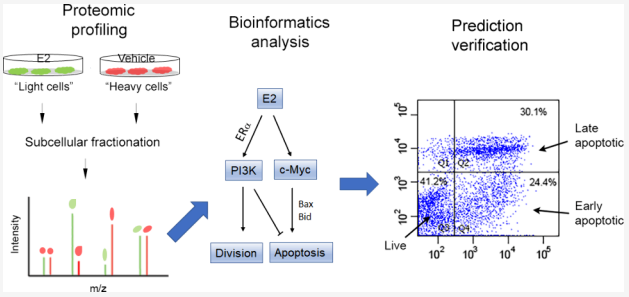
ABSTRACT: Despite extensive research, the genes/proteins and pathways responsible for the physiological effects of estrogen remain elusive. In this study, we determined the effect of estrogen on global protein expression in breast cancer MCF7 cells using a proteomic method. The expression of 77 cytosolic, 74 nuclear, and 81 membrane/organelle proteins was significantly altered by 17-β-estradiol (E2). Protein enrichment analyses suggest that E2 may stimulate cell division primarily by promoting the G1 to S phase transition and advancing the G2/M checkpoint. The effect of E2 on cell survival was complex, as it could simultaneously enhance and inhibit apoptosis. Bioinformatics analysis suggests that E2 may enhance apoptosis by promoting the accumulation of the pore-forming protein Bax in the mitochondria and inhibit apoptosis by activating the PI3K/AKT/mTOR signaling pathway. We verified the activation of the PI3K signaling and the accumulation of Bax in the membrane/organelle fraction in E2-treated cells using immunoblotting. Treatment of MCF7 cells with E2 and the PI3K inhibitor Ly294002 significantly enhanced apoptosis compared to those treated with E2 alone, suggesting that combining estrogen with a PI3K inhibitor could be a promising strategy for treating ERα-positive breast cancer. Interestingly, many of the E2-upregulated proteins contained the HEAT, KH, and RRM domains.



KEYWORDS: *Quantitative proteomics, proteome profiling, SILAC, estrogen, breast cancer, cell division, apoptosis*

INTRODUCTION

Estrogen plays an essential role in the proliferation and differentiation of normal breast tissue and is linked to the development of breast cancer. The main form of estrogen in the cells is 17-β-estradiol (E2). E2 stimulates breast cell proliferation by binding to estrogen receptor alpha (ERα) thereby altering estrogen-responsive gene expression that regulates the cell cycle and apoptosis.[1,2] E2 can influence cell behavior through genomic and nongenomic actions. In the genomic mechanism, the E2-bound ERα translocates into the nucleus, where it binds estrogen response elements (EREs) and regulates the expression of estrogen-responsive genes.[3] Alternatively, ERα can also regulate gene expression by interacting with other transcription factors such as AP1.[4,5] In the nongenomic mechanism, E2 binds the plasma membrane-bound ERα and the G-protein-coupled receptor GPR30 leading to rapid activation of several signaling pathways.[3]

The effect of estrogen on cell survival is complex. On the one hand, estrogen can induce apoptosis in long-term estrogen-deprived (LTED) cells or in cells treated exhaustively with antiestrogen by activating mitochondrion-dependent and receptor-activated pathways, such as upregulation of cytochrome c and Fas/FasL.[6] On the other hand, estrogen has been shown to inhibit apoptosis by activating the phosphoinositide 3-kinase (PI3K) signaling pathway via the binding of ERα to the p85α regulatory subunit of PI3K[7] and inducing the expression of antiapoptotic genes.[8]

For most ERα-positive breast cancer patients, endocrine therapy is the first choice of treatment, which includes the use of selective ER modulators (SERMs; e.g., tamoxifen and raloxifene), selective ER down-regulators (SERDs; e.g., fulvestrant), and/or aromatase inhibitors (e.g., anastrozole); all of which disrupt E2 signaling through the disruption of E2-ERa binding or by inhibiting E2 production.[9] Although the antiestrogen drugs tamoxifen and raloxifene have been successfully used to treat ERα-positive breast cancer for decades, approximately 40% of patients treated with antiestrogen therapy for five to ten years develop resistance toward tamoxifen or raloxifene and 50% of women with metastatic breast cancer do not respond to antiestrogen

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treatment,[10] creating a major issue in the treatment of ERα-positive breast cancers. Because E2 can induce apoptosis, E2 was successfully used in high concentrations to treat ERα-positive breast cancer in postmenopausal women[11] before tamoxifen was used as an antiestrogen drug.[12] After the development of antiestrogen drugs, E2 has been exploited as an alternative drug to overcome antiestrogen resistance in the treatment of ER-positive breast cancer.[13,14] Although the results are not conclusive, there is potential for E2 as a viable therapy for breast cancer patients.[15]

Due to the profound biological and pathological effects of estrogen on normal and breast cancer cells, researchers have undertaken extensive efforts to identify ERα target genes associated with its physiological effects. DNA microarray and RNA sequencing analyses, for instance, have revealed the impact of estrogen on the expression of hundreds of genes.[16−20] Additionally, chromatin immunoprecipitation (Chip)-sequencing and Chip-microarray analyses have uncovered thousands of ERα-binding sites in breast cancer cells.[21−24] Moreover, proteomic methods have been employed to identify ERα-regulated proteins in breast cancer cells.[25] Despite these efforts, the genes and pathways responsible for the physiological effects of estrogen remain elusive.

To gain a better understanding of the key proteins and pathways that mediate the global effects of estrogen in breast cancer cells, we employed a SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture)-based quantitative proteomic method[26,27] coupled with subcellular fractionation and subsequent bioinformatics analysis to assess the major pathways affected by E2 in human breast cancer MCF7 cells—a model widely used for studying hormone response in breast cancer.[5,17,18,24] Our results suggest that E2 may promote cell division primarily by enhancing the G1 to S phase transition and advancing the G2/M checkpoint. Moreover, our bioinformatics analysis suggests that E2 has a dual effect on apoptosis, enhancing and inhibiting apoptosis simultaneously. By leveraging the dual effect of estrogen on apoptosis, we tested the possibility of enhancing the pro-apoptotic effect of estrogen by blocking the inhibitory pathway identified in this study. Our findings suggest that coadministration of estrogen and a PI3K inhibitor could be a promising strategy for treating ERα-positive breast cancer.

MATERIALS AND METHODS

Cell Culture, Proteome Labeling, and E2 Treatment

The MCF7 cell line was maintained in α-MEN with 5% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. For proteome labeling, the MCF7 cells were cultured in unlabeled α-MEN with 5% dialyzed FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin (light medium) or the α-MEN containing arginine-$^{13}C_6$ and lysine-$^{13}C_6{}^{15}N_2$ with 5% dialyzed FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin (heavy medium) for 2 weeks. The unlabeled and the labeled cells were then cultured in the unlabeled and labeled, phenol red-free α-MEN with charcoal treated 5% dialyzed FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin for 6 days. After the 6-day hormonal starvation, the cells in the unlabeled medium were treated with 100 nM E2 (Sigma, St. Louis, MO), and the cells in the labeled medium were treated with an equivalent amount of vehicle (ethanol) for 18 h.

Subcellular Fractionation

After the E2 (or ethanol for control) treatment, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS). The cells were then resuspended in 5 packed cell pellet volumes of hypotonic buffer [20 mM Hepes-NaOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, and protease inhibitor mixture (Roche Applied Science)], incubated on ice for 30 min, and lysed with approximately 20 strokes of a tight-fitting pestle (type B) in Dounce homogenizer (Kontes Glass Co., Vineland, NJ) until >95% of cells were ruptured. The cell lysate was then centrifuged at 1,000 × g at 4 °C for 10 min, and the pellet was collected as the nuclear fraction. The supernatant was centrifuged at 10,000 × g at 4 °C for 15 min. The resulting supernatant was designated as the cytosolic protein, and the pellet was collected as the membrane/organelle fraction. The nuclear fraction and the membrane/organelle fraction were then respectively dissolved in a modified RIPA buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, and protease inhibitor mixture). After centrifugation at 21,000 × g at 4 °C for 10 min, the resulting supernatant from the nuclear fraction was designated as nuclear protein, and the supernatant from the membrane/organelle fraction was designed as membrane/organelle protein. The concentrations of the cytosolic, nuclear, and membrane/organelle proteins were determined by the Bio-Rad RC DC method (Hercules, CA). It is important to note that the subcellular fractionation procedures were designed for simplicity and consistency, with the goal of increasing the coverage of the proteomes rather than accurately characterizing the subproteomes.

Gel Electrophoresis, in-Gel Digestion, and LC-MS/MS

Equal amounts of unlabeled cytosolic, nuclear, and membrane/organelle proteins were mixed separately with equal amounts of labeled cytosolic, nuclear, and membrane/organelle proteins. The mixed protein was then fractionated with a 12% SDS-PAGE gel, and the fractioned proteins were visualized by Coomassie brilliant blue G-250. Each protein lane of the SDS-PAGE gel was cut into 13−15 slices. Proteins in gel slices were digested with trypsin (Promega, Madison, WI) overnight at 37 °C and the resulting peptides were dissolved in 20 μL 0.1% formic acid and then analyzed with an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA) operated in a data-dependent mode for tandem MS. The LC-MS/MS analysis was performed as previously described in detail.[28]

Protein Identification and Quantification

Raw data from the LC-MS/MS analysis were processed by MaxQuant (version 1.6.2.10)[29] with the built-in search engine Andromeda and searched against a target-decoy human SwissProt protein database (20,408 entries) retrieved from UniProt (www.uniprot.org) as described previously.[28,30] Specifically, the false discovery rates (FDRs) for peptide and protein identification were set to 1%. The MS error tolerance was set to 4.5 ppm, and the MS/MS error tolerance was set to 20 ppm. The minimum required peptide length was set to 7 amino acids, and a maximum of 2 missed cleavages was allowed. Variable modifications of acetylation at peptide N-terminus and oxidation on methionine, and fixed modification of cysteine carbamidomethylation were included. SILAC ratios (light/heavy ratios) were calculated using unique and razor peptides with a minimum ratio of 2.

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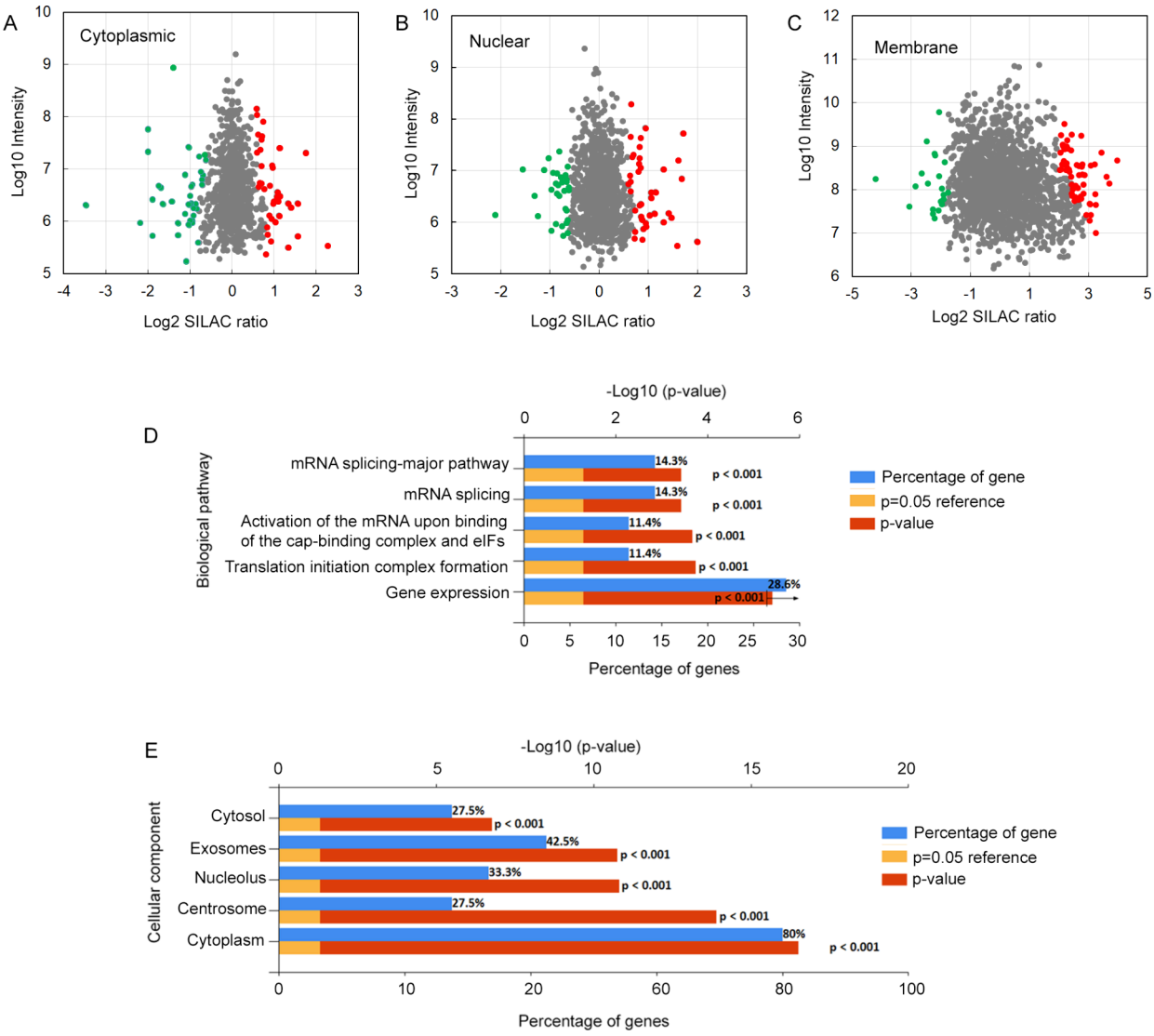

Figure 1. E2-regulated proteins and enrichment analysis of biological pathways and cellular components in the E2-upregulated proteins. SILAC proteomic analyses after E2 treatment and fractionation of MCF7 cells identified the upregulated (red) and downregulated proteins (green) in the cytosolic (A), nuclear (B), and membrane/organelle (C) fractions. FunRich analyses of the E2-upregulated proteins revealed the biological pathways (D) and cellular components (E) enriched in the E2-treated MCF7 cells compared to vehicle-treated control cells.

The proteins matched to the reverse database, identified only by site or single peptide, and common contaminants were removed. The remaining proteins were analyzed by Perseus (version 2.0.3.0),[31] and the Significance B score was obtained for the quantified proteins. The Significance B score is a significance score for protein SILAC ratios and identifies outliers based on the standard deviation of the protein SILAC ratios of the main distribution and signal intensity.[32] A protein was considered a differentially expressed protein when its ratio was significant by the Significance B score with a $p < 0.05$, and the L/H ratio was >1.5 or <0.65.

Bioinformatics Analysis of the E2-Regulated Proteins

The cytosolic, nuclear, and membrane/organelle proteins that were identified and quantified with at least two peptides were separately analyzed with GSEA using the module of PreRanked genes.[33] The proteins were first analyzed using the Hallmark gene collection and then the Pathway Interaction Database (PID) to identify the pathways affected by the E2 treatment. A gene set with an FDR < 0.25, which was the preferred criterion for GSEA analysis,[33] was considered enriched. The E2-regulated proteins (up or downregulated) were also analyzed by DAVID, a web server for functional annotation and enrichment analyses of gene lists.[34] For the DAVID analysis, the differentially expressed cytosolic, nuclear, and membrane/organelle proteins were pooled together. During the pooling process, proteins identified in two or more fractions (i.e., shared proteins) with conflicting SILAC ratios (e.g., one up and others down) were removed from the pooled protein list. In the DAVID enrichment analysis, the E2-regulated proteins were cluster-analyzed with GOTERM_BP_DIRECT and GOTERM_MF_DIRECT to determine the functional enrichment within the pooled list of proteins. The biological pathways and molecular function categories with $p < 0.05$ were considered significant. The E2-regulated proteins were also cluster-analyzed with UP_SEQ_FEATURE to determine the sequence features of the E2-regulated proteins. The E2-regulated (up or downregulated) proteins were further loaded to FunRich[35] to identify the enriched biological pathways and cellular compartments. Biological pathways and cellular

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Table 1. Short List of Top E2-Regulated Proteins in the Cytosolic, Nuclear, and Membrane/Organelle Fractions

	UniProt ID	gene name	SILAC L/H ratio	no peptide	protein name
cytosolic fraction	Q04637	EIF4G1	4.83	2	eukaryotic translation initiation factor 4 gamma 1
	P00918	CA2	3.37	4	carbonic anhydrase 2
	Q3YEC7	RABL6	2.94	2	RAB, member RAS oncogene family like 6
	Q8TD06	AGR3	2.94	3	anterior gradient 3
	P05161	ISG15	0.25	4	ISG15 ubiquitin like modifier
	P61019	RAB2A	0.22	3	RAB2A, member RAS oncogene family
	Q9Y6U3	SCIN	0.09	6	scinderin
nuclear fraction	Q6UXG2	ELAPOR1	3.99	2	endosome-lysosome associated apoptosis and autophagy regulator 1
	P38159	RBMX	3.27	10	RNA binding motif protein X-linked
	Q8TD06	AGR3	3.20	4	anterior gradient 3
	P00918	CA2	3.05	5	carbonic anhydrase 2
	Q16629	SRSF7	0.34	3	serine and arginine rich splicing factor 7
	P05161	ISG15	0.23	2	ISG15 ubiquitin like modifier
membrane/organelle fraction	Q6ZRV2	FAM83H	15.58	18	family with sequence similarity 83 member H
	O95747	OXSR1	12.99	6	oxidative stress responsive kinase 1
	Q6P996	PDXDC1	12.13	8	pyridoxal dependent decarboxylase domain containing 1
	Q15365	PCBP1	10.83	8	poly(rC)-binding protein 1
	Q9Y6U3	SCIN	0.14	7	scinderin
	Q08380	LGALS3BP	0.12	4	galectin 3 binding protein
	P32004	L1CAM	0.05	8	L1 cell adhesion molecule

compartments with $p < 0.05$ were considered significantly enriched.

Western Blotting

After culturing MCF7 cells in phenol red-free α-MEM with charcoal-treated 5% dialyzed FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin for 3 days, the cells were treated with E2 to detect AKT Ser-473 phosphorylation or Bax protein levels using Western blotting. For the detection of AKT Ser-473 phosphorylation, the estrogen-starved cells were either mock-treated or treated with 10 nM E2 for 10 min. Subsequently, the cells were harvested, lysed, and the resulting proteins were fractionated on a 4−12% Bis-Tris NuPAGE gel under reducing conditions. Western blotting was then performed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). To determine Bax protein levels in the membrane/organelle fractions, the estrogen-starved cells were either mock-treated or treated with 100 nM E2 for 18 h. Following treatment, the cells were harvested, and membrane/organelle proteins were prepared as described above for LC-MS/MS analysis. The resulting membrane/organelle proteins were then analyzed using Western blotting as previously described.[30,36] The Western blot images were acquired using a Li-COR 9120 scanner (Li-COR Inc., Lincoln, NE). The anti-AKT (Cat#: 10176−2-AP), anti-F1-β-ATPase (Cat#: 17247−1-AP), antihistone H4 (16047−1-AP), and anti-Bax (Cat#: 50599−2-Ig) antibodies were purchased from Proteintech (Rosemont, IL). The antiphospho-AKT (Ser-473) antibody (Cat#: 9271) was from Cell Signaling (Danvers, MA), anti-EGFR (A19002A) was from Biolegend (San Diego, CA), and the antitubulin antibody (Cat#: T9026) was from Sigma (St. Louis, MO).

Treatment of MCF7 Cells with E2 and a PI3K Inhibitor and Flow Cytometry Analysis of the Cells

MCF7 cells (2.5×10^5 cells) grown in α-MEM medium supplemented with 5% charcoal-treated FBS were either vehicle-treated or treated with 25 μM PI3K inhibitor Ly294002 for 1 h. E2 was then added to the mock-treated or Ly294002-treated cells to a final concentration of 0, 0.1, 1, or 10 μM. Subsequently, the cells were incubated with the inhibitor and E2 for another 18 h. After the treatments, the cells were incubated with 0.05% trypsin, harvested, and washed twice with cold PBS. The cells were then resuspended in 0.1 mL of binding buffer (10 mM Hepes-NaOH, pH7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) and stained with annexin V using a FITC Annexin V Apoptosis Detection kit I (BD Pharmingen 556547) according to the manufacturer's instructions. Briefly, 5 μL of annexin V and 5 μL of propidium iodide were added to the cell suspension, and the mixture was incubated at room temperature in the dark for 20 min. After adding 400 μL of PBS to the cells, the cells were analyzed with a FACSAria Fusion flow cytometer (BD Biosciences, San Jose, CA). While the annexin V and PI negative cells were defined as viable cells, the annexin V positive and PI negative were defined as early apoptotic cells, and the annexin V and PI positive cells as late apoptotic cells.[37]

Statistical Analysis

The statistical analyses for GSEA, DAVID, and FunRich enrichment analyses were conducted using the default settings in the respective software. To analyze differences in apoptosis and viability between various pairs of conditions in Figure 3, we employed a t test.

RESULTS

Identification of E2-Induced Changes in Protein Expression in MCF7 Cells

We used a SILAC-based quantitative proteomic method[26,27] to identify the E2-regulated proteins in MCF7 cells. To increase the chances of detecting novel/low abundant proteins, we fractionated total cellular protein into the cytosolic, nuclear, and membrane/organelle fractions (Supporting Figure S1) and analyzed each fraction independently. We quantified 776 cytosolic, 936 nuclear, and 1,703 membrane/organelle proteins by at least 2 unique peptides (Supporting Tables S1−S3). Cut-offs of $p < 0.05$ in the Significance B score in Perseus analysis and an L/H fold change in SILAC ratios (E2 treated/vehicle-

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Table 2. List of the Gene Sets That Are Overrepresented in the E2 Treated MCF7 Cells Compared to the Vehicle-Treated Control Cells[a]

	collection: Hallmark		collection: PID pathway	
cellular fractions	enriched gene set	FDR	enriched gene set	FDR
cytosolic	estrogen response late	0.099	PID_PDGFRB pathway	0.164
			PID_VEGFR1/2 pathway	0.222
nuclear	estrogen response late	0.001		
	E2F targets	0.115		
	Heme metabolism	0.121		
	Complement	0.162		
membrane/organelle	E2F targets	0.078	PID_REG_GR pathway	0.001
	G2/M checkpoint	0.096	PID_LKB1_pathway	0.007
	PI3K AKT mTOR signaling	0.145	PID_NFAT_3 pathway	0.016
			PID_mTOR_4 pathway	0.015
			PID_Hedgehog_Gli pathway	0.015
			PID_beta_Catenin_Nuc pathway	0.014
			PID_Telomerase pathway	0.012
			PID_PDGFRB pathway	0.018
			PID_FOXO pathway	0.037
			PID_ERBB1_Downstream pathway	0.040
			PID_VEGFR1/2 pathway	0.053
			PID_MET pathway	0.060
			PID_A6B1_A6B4_Integrin pathway	0.056
			PID_ILK pathway	0.212
			PID_TRKR pathway	0.248
			PID_Thrombin_Par1 pathway	0.240
			PID_Myc_Activ pathway	0.233

[a]Only the gene sets with FDR less than 0.25 are listed.

treated) of >1.5 or <0.65 resulted in 77 cytosolic, 74 nuclear, and 81 membrane/organelle proteins that were considered differentially expressed between the E2-treated cells and the vehicle-treated control cells (Figure 1A−C; Supporting Tables S4−S6). Some E2-regulated proteins were consistently identified in two of the three fractions. For instance, carbonic anhydrase 2 (gene name: CA2) and anterior gradient protein 3 (AGR3) were upregulated, whereas ubiquitin-like protein ISG15 (ISG15) was downregulated by E2 in both the cytosolic and the nuclear fractions. Similarly, scinderin (SCIN) exhibited downregulation by E2 in both the nuclear and the membrane/organelle fractions (Table 1). Furthermore, the expression of many of the identified E2-regulated proteins has previously been shown to be associated with breast cancer. For example, the expression of all of the top 2−4 E2-upregulated or E2-downregulated proteins (Table 1) has been shown to be associated with breast cancer,[38,39] except for endosome/lysosome-associated apoptosis and autophagy regulator 1 (ELAPOR1), protein FAM83H (FAM83H), and pyridoxal-dependent decarboxylase domain containing 1 (PDXDC1), whose roles in breast cancer remain unreported.

E2 Regulates the Expression of the Proteins That Affect the Transition From the G1 to S Phase and the G2/M Checkpoint

We uploaded the 776 cytosolic, 936 nuclear, and 1703 membrane/organelle proteins that were quantified by at least two unique peptides to the GSEA and analyzed the proteins using the module of PreRanked genes[33] using the Hallmark and the PID gene collections. GSEA is a software that determines whether members of a gene set defined based on prior biological knowledge are overrepresented within the experimental data set,[33] and the Hallmark gene collection was generated by a computational methodology identifying overlap between gene sets in other MSigDB collections to summarize well-established biological states and processes that display coherent expression among them. As expected, the "Estrogen response late" gene set was overrepresented in both the cytosolic and nuclear proteins in the GSEA analysis (Table 2). Identification of the enrichment of the estrogen response gene set in two of the three fractions serves as validation of the appropriate cellular responses to E2 treatment and our experimental procedures.

In addition to the estrogen response late gene set, the GSEA analysis revealed that the "E2F targets" and "G2/M checkpoint" gene sets associated with cell cycle progression were enriched in the nuclear and/or membrane/organelle fractions (Table 2). The enrichment of the "E2F targets" and "G2/M checkpoint" gene sets in the E2-treated cells relative to vehicle-treated cells implies that the E2F transcriptional activity and G2/M checkpoint are regulated by E2 in MCF7 cells. The transition of cells from the G1 phase to the S phase is regulated at the restriction point during the late G1 phase, where cells commit to cell division if the cellular environment supports it. Beyond the restriction point, the transition from S to G2 to M to G1 becomes autonomous and no longer relies on environmental factors. Transcription of the target genes of the E2Fs is necessary for the cells to pass through the restriction point.[40] The significance of E2F's transcriptional activity in cell proliferation is evident from the observation that many proliferation signature genes contain binding sites for the E2Fs.[41] The predicted regulation of E2Fs by E2 in the GSEA analysis is supported by changes in the expression of some well-known E2F target genes and proliferation signature genes.[42] For example, the expression of minichromosome maintenance (MCM)2 and MCM7 was significantly upregu-

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lated by E2 (Supporting Table S5), while MCM3, MCM4, and MCM6 approached the significance threshold in the nuclear fraction (Supporting Table S2). The expression of MCM genes is under control of E2Fs and is critical for DNA replication in the S phase.[42,43] Through the regulation of the expression of E2F target genes, estrogen may drive the transition from the G1 phase to the S phase of the cell cycle, facilitating DNA replication and promoting cancer cell division in estrogen-responsive breast cancers. During the G2/M checkpoint in the cell cycle, the cell undergoes checks and balances to ensure that DNA replication and cell division occur accurately. This checkpoint prevents DNA-damaged cells or the cells in which DNA replication is not completed from entering the M phase, reducing the risk of mutations. The effect of estrogen on the G2/M checkpoint has been previously reported.[44] The identification of the enrichment of the "E2F targets" and "G2/M checkpoint" in the E2-treated MCF7 cells at the proteome levels, suggests that these two molecular processes are likely the primary points at which E2 drives the cell cycle progression in ERα-positive breast cancer cells.

Consistent with the report that ERα interacts with the p85α regulatory subunit of PI3K,[7] the GSEA analysis revealed the enrichment of the proteins that regulate the PI3K signaling following E2 treatment (Table 2). To confirm the predicted changes in the PI3K signaling in the E2-treated cells relative to the control cells, we assessed the phosphorylation and activation of AKT/PKB, a key downstream kinase of PI3K, in E2-treated MCF7 cells using Western blotting. Our Western blot analysis results (Figure 2A-B) were consistent with the GSEA prediction, confirming the activation of PI3K signaling by E2 in MCF7 cells. The phosphorylation and activation of AKT/PKB by E2 in MCF7 cells has also been shown by others.[45] The PI3K/AKT/mTOR signaling is a key regulator of cell growth.[46] The activated PI3K/AKT/mTOR signaling may contribute to the expression of the E2F target genes,[47] along with other factors such as upregulation of cyclin D1 and activation of CDK4/6, which are known to be associated with the induction of E2F-mediated transcription.[48] The E2-regulated cytosolic, nuclear, and membrane/organelle proteins were also analyzed using the PID gene sets in GSEA.[33] Most PID gene sets that were overrepresented in the E2-treated cells relative to the control cells supported cell division (Table 2). Thus, our proteomic data support the notion that one of the primary effects of E2 in ERα-positive breast cancer cells is to promote cell division.

The Expression of the Proteins Involved in Nuclear Import, Protein Translation, and mRNA/Protein Stability Was Enriched in the E2-Treated Cells

Among the 77 cytosolic proteins, 74 nuclear proteins, and 81 membrane/organelle proteins significantly regulated by E2 (Figure 1A–C; Supporting Tables S4–S6), 24 proteins were quantified in two of the three fractions (bold in Supporting Tables S4–S6). Of the 24 proteins, 20 were consistently upregulated or downregulated by E2 in both fractions, and 4 exhibited upregulation in one fraction but downregulation in another. We pooled the differentially expressed cytosolic, nuclear, and membrane/organelle proteins for further bioinformatics analysis. During the pooling, we included the 20 proteins that changed in the same direction and excluded the 4 proteins that changed in the opposite direction. The data pooling resulted in a list of 204 E2-regulated proteins (Supporting Table S7). Among these 204 proteins, E2

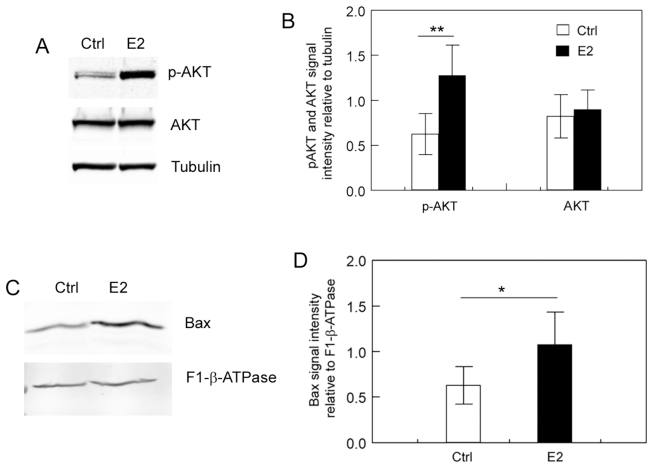

Figure 2. Validation of PI3K signaling activation and the elevation of Bax in the membrane/organelle fraction of E2-treated MCF7 cells. (A) Validation of PI3K signaling activation. MCF7 cells were either mock-treated or treated with 10 nM E2 for 10 min. AKT Ser-473 phosphorylation was analyzed using Western blotting with the indicated antibodies. The experiments were performed in five replicates using independently prepared biological samples (n = 5). Panel A shows one of the Western blots, and uncropped images of the five Western blot replicates are shown in Supporting Figure S2. (B) Quantification of p-AKT and AKT signal intensities. Signal intensities were quantified relative to tubulin in Western blots (n = 5). As depicted, while the AKT protein level remained unchanged, AKT Ser-473 phosphorylation significantly increased in E2-treated cells compared to mock-treated cells. (C) Validation of the elevation of Bax protein in the membrane/organelle fraction of the E2-treated MCF7 cells. MCF7 cells were either mock-treated or treated with 100 nM E2 for 18 h. Bax protein levels in the membrane/organelle fraction were analyzed using Western blotting with an anti-Bax antibody. The experiments were performed in five replicates using independently prepared biological samples (n = 5). Panel C shows one of the Western blots, and uncropped images of the five Western blot replicates are shown in Supporting Figure S3. (D) Quantification of Bax signal intensities. Bax signal intensities were quantified relative to F1-β-ATPase in Western blots (n = 5). Tubulin and the mitochondrion inner membrane protein F1-β-ATPase served as loading controls for the whole-cell lysate and membrane/organelle proteins, respectively. *p < 0.05; **p < 0.01.

upregulated 124 and downregulated 80 proteins. Thus, the number of E2-upregulated proteins was 55% higher than that of E2-downregulated proteins, suggesting that E2 influences MCF7 cells primarily through protein upregulation rather than downregulation.

To understand how the E2-regulated proteins influence breast cancer cell division and apoptosis, we analyzed the E2-upregulated and E2-downregulated proteins with DAVID, a web server for functional annotation and enrichment analyses of gene/protein lists.[34] In this analysis, we first determined the sets of genes/proteins overrepresented in the E2-upregulated and E2-downregulated proteins by performing Gene Ontology (GO) enrichment analysis. Cluster analysis of the E2-upregulated proteins using the GO biological processes (BP) (GOTERM_BP_DIRECT) and GO molecular functions (MF) (GOTERM_MF_DIRECT) revealed that the proteins involved in protein nuclear import, protein translation, mRNA stability, protein folding/stability, and nucleosome assembly were enriched in the E2-upregulated proteins (Table 3). All of these changes appear to facilitate cell division. To strengthen this notion, we further analyzed the E2-upregulated proteins

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with FunRich, a stand-alone software tool for functional enrichment and interaction network analysis of genes and proteins.[35] Consistent with the results from the DAVID analysis, when the E2-upregulated proteins were analyzed with FunRich for biological pathways, the proteins involved in gene expression, protein translation, and mRNA splicing were significantly enriched in the E2-upregulated proteins (Figure 1D). Thus, it is likely that E2 may promote MCF7 cell division (Table 2) by enhancing various molecular processes that are supportive of cell division, including protein nuclear import, protein translation, mRNA stability, protein folding, and nucleosome assembly (Table 3; Figure 1D). Interestingly, when the E2-upregulated proteins were analyzed with FunRich to determine the enrichment of cellular components, proteins related to the centrosome were enriched (Figure 1E). Centrosomes are prominent during the M phase when the two daughter cells are forming, which happens in the last hour of the cell cycle.[49] The enrichment of centrosome proteins in the E2-upregulated proteins suggests that the E2-treated cells are more active in cell division compared to vehicle-treated cells. This observation is consistent with the results from the functional annotation analysis (Table 3; Figure 1D) and the GSEA analysis (Table 2).

Different from the E2-upregulated proteins, only the actin/actin filament binding and actin/actin filament-based motor activities were enriched in the E2-downregulated proteins (Supporting Table S8). Actin and actin filaments play key roles in both the division of the nucleus (karyokinesis) and the division of the cytoplasm (cytokinesis). The cellular actin network undergoes dynamic changes during cell division, including vigorous disassembly and reassembly of actin filaments.[50] Whether E2 can promote cell division by inhibiting actin/actin filament binding and actin/actin filament-based motor activity warrants further investigation.

Many E2-Upregulated Proteins Contain the HEAT, KH, and RRM Domains

We also determined the sequence features of E2-regulated proteins through Functional annotation analysis using UP_SEQ_FEATURES in DAVID. Interestingly, many of the E2-upregulated proteins contained the HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1), KH (K homology) domains, and RRM (RNA recognition motif) (Table 4). The HEAT repeat is a structural motif found in proteins and was named after the four proteins in which it was initially identified: Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and TOR1. The HEAT domain is characterized by a tandem repeat of two α-helices connected by a short loop. These repeats can vary in number, ranging from 3 to 30 in a single protein. HEAT domain-containing proteins participate in various cellular processes, including protein−protein interactions, cellular organization, and cell cycle regulation. Proteins with HEAT domains are often associated with cellular growth and proliferation.[51] The enrichment of HEAT domains in the E2-upregulated proteins suggests that, apart from breast cancer, estrogen may also be related to other diseases, such as neurodegenerative conditions like Huntington's Disease and Alzheimer's Disease.[52]

The KH (K homology) domain is a protein domain that is named after the first protein in which it was identified, the human heterogeneous nuclear ribonucleoprotein K (hnRNP K). The KH domain is a small, highly conserved RNA-binding domain found in various proteins involved in RNA metabolism and regulation. The KH domain is approximately 70 amino acids long and adopts a compact, globular structure. It is characterized by a three-stranded β-sheet and an α-helix, forming a characteristic fold.[53] The domain contains two conserved motifs, the GxxG, and the KH box, contributing to RNA binding. The GxxG motif stabilizes the RNA-binding site, while the KH box interacts directly with the RNA molecule.[53] KH domains are known to bind single-stranded RNA, and their specificity and affinity for RNA can vary depending on the protein context. Proteins containing KH domains are involved in various RNA-related processes, including RNA splicing, mRNA stabilization and localization, translation regulation, and microRNA processing.[53]

The RNA recognition motif (RRM), also known as the RNA-binding domain (RBD), is a common and versatile protein domain in many proteins involved in RNA binding and processing. RRMs are approximately 90 amino acids long and are characterized by a conserved RNA-binding fold. This fold consists of four antiparallel beta strands and two alpha helices, forming a compact structure with a characteristic beta-alpha-beta−beta-alpha-beta topology.[54] The RRM domain typically contains conserved aromatic residues involved in RNA recognition. RRM domains are crucial in various RNA-related processes, including pre-mRNA splicing, mRNA stability and localization, translation regulation, and RNA processing.[54] Proteins containing RRM domains can recognize specific RNA sequences or structures and interact with RNA molecules to mediate their function. Many RNA-binding proteins, such as heterogeneous nuclear ribonucleoproteins (hnRNPs), splicing factors, RNA helicases, and poly(A)-binding proteins, contain one or multiple RRM domains.[54] The versatility of the RRM domain allows it to interact with RNA in a modular and specific manner, enabling it to participate in diverse RNA-related processes within the cell.

The observation that many E2-upregulated proteins contain the HEAT, KH, and RRM domains is consistent with the results from the DAVID analysis, which suggested that E2 may promote MCF7 cell proliferation mainly by affecting the expression of the proteins involved in protein nuclear import, protein translation, protein folding, and mRNA stability (Table 3; Figure 1D). In contrast, among the E2-downregulated proteins, only the RRM domains showed moderate enrichment, and no other domains displayed significant enrichment (Supporting Table S9).

E2 Regulates the Abundance of the Proteins That Promote or Inhibit Apoptosis in MCF7 Cells

In addition to affecting cell division, the DAVID analysis revealed that the proteins involved in cytochrome c release were enriched in the E2-upregulated proteins (Table 3). Cytochrome c release from mitochondria is a critical step in mitochondrion-dependent apoptosis. Consistent with this, our proteomic data revealed that the levels of two pro-apoptotic proteins, Bax and Bid, in the membrane/organelle fraction were significantly higher in E2-treated cells compared to vehicle-treated cells (Supporting Table S6). Bax is known to translocate from the cytosol to the mitochondria to initiate mitochondrion-dependent apoptosis by inducing cytochrome c release from the intermembrane space of mitochondria.[55−57] We validated the higher level of Bax protein in the membrane/organelle fraction in the E2-treated MCF7 cells relative to vehicle-treated cells by performing Western blot analysis (Figure 2C−D). Thus, it is likely that the changes in the

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Table 3. Functional Annotation Analysis by DAVID of the Proteins Whose Expression Was Significantly Upregulated by E2: with GOTERM_BP_DIRECT and GOTERM_MF_DIRECT[a]

category	term	count	UniProt ID	*p* value	fold enrichment
cluster 1	enrichment score: 4.78				
GOTERM_MF_DIRECT	GO:0031267 ~ small GTPase binding	13	Q14974, O00429, O14980, Q07960, Q8TEX9, Q9Y3P9, Q96P70, O95373, O60610, Q92973, Q13492, O14787, P55060	3.76×10^{-07}	6.9
GOTERM_MF_DIRECT	GO:0061608 ~ nuclear import signal receptor activity	5	Q14974, Q92973, O14787, Q8TEX9, Q96P70	7.09×10^{-06}	38.8
GOTERM_BP_DIRECT	GO:0006606 ~ protein import into nucleus	7	Q14974, Q92973, O14787, P55060, Q8TEX9, Q96P70, O95373	4.17×10^{-05}	11.0
GOTERM_MF_DIRECT	GO:0008139 ~ nuclear localization sequence binding	4	Q14974, Q92973, O14787, Q8TEX9	6.65×10^{-04}	23.0
cluster 2	enrichment score: 3.66				
GOTERM_BP_DIRECT	GO:0006413 ~ translational initiation	7	O15371, Q04637, Q99613, O75821, O00571, Q15056, P60842	9.59×10^{-07}	20.9
GOTERM_MF_DIRECT	GO:0003743 ~ translation initiation factor activity	6	O15371, Q04637, Q99613, O75821, Q15056, P60842	5.52×10^{-05}	14.6
GOTERM_BP_DIRECT	GO:0001732 ~ formation of cytoplasmic translation initiation complex	3	O15371, Q99613, O75821	0.0046	29.0
GOTERM_MF_DIRECT	GO:0008135 ~ translation factor activity, RNA binding	3	Q04637, Q15056, P60842	0.0094	20.2
cluster 3	enrichment score: 2.69				
GOTERM_BP_DIRECT	GO:1900152 ~ negative regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3	P67809, P11940, O60506	0.0013	54.8
GOTERM_BP_DIRECT	GO:0070934 ~ CRD-mediated mRNA stabilization	3	P67809, P11940, O60506	0.0019	44.9
GOTERM_BP_DIRECT	GO:2000767 ~ positive regulation of cytoplasmic translation	3	P67809, P11940, O60506	0.0036	32.9
cluster 4	enrichment score: 2.67				
GOTERM_BP_DIRECT	GO:0061077 ~ chaperone-mediated protein folding	6	P50990, O75190, P78371, P49368, P04792, Q02790	3.57×10^{-06}	25.3
GOTERM_MF_DIRECT	GO:0051082 ~ unfolded protein binding	7	P50990, O00170, O75190, Q8N163, P78371, P49368, P04792	1.88×10^{-04}	8.4
GOTERM_MF_DIRECT	GO:0044183 ~ protein binding involved in protein folding	5	P50990, O75190, P78371, P49368, P04792	3.14×10^{-04}	15.2
GOTERM_BP_DIRECT	GO:0032212 ~ positive regulation of telomere maintenance via telomerase	4	P50990, P78371, P49368, P09651	0.0011	19.4
GOTERM_BP_DIRECT	GO:1904851 ~ positive regulation of establishment of protein localization to telomere	3	P50990, P78371, P49368	0.0016	49.4
GOTERM_BP_DIRECT	GO:1904871 ~ positive regulation of protein localization to Cajal body	3	P50990, P78371, P49368	0.0019	44.9
GOTERM_BP_DIRECT	GO:1904874 ~ positive regulation of telomerase RNA localization to Cajal body	3	P50990, P78371, P49368	0.0036	32.9
GOTERM_BP_DIRECT	GO:0050821 ~ protein stabilization	6	P50990, P78371, P46379, P49368, P83436, O60716	0.0122	4.3
GOTERM_MF_DIRECT	GO:0016887 ~ ATPase activity	8	P50990, P78371, P33993, O00571, P49368, P49736, O00148, P60842	0.0181	3.0
GOTERM_BP_DIRECT	GO:0007339 ~ binding of sperm to zona pellucida	3	P50990, P78371, P49368	0.0242	12.3
GOTERM_BP_DIRECT	GO:0006457 ~ protein folding	5	P50990, O75190, P78371, P49368, Q02790	0.0253	4.5
GOTERM_BP_DIRECT	GO:1901998 ~ toxin transport	3	P50990, P78371, P49368	0.0265	11.8
cluster 5	enrichment score: 1.75				
GOTERM_BP_DIRECT	GO:0001836 ~ release of cytochrome c from mitochondria	3	O00429, Q07812, P55957	0.0084	21.5
GOTERM_BP_DIRECT	GO:0090200 ~ positive regulation of release of cytochrome c from mitochondria	3	O00429, Q07812, P55957	0.0114	18.3
GOTERM_BP_DIRECT	GO:2001244 ~ positive regulation of intrinsic apoptotic signaling pathway	3	O00429, Q07812, P55957	0.0209	13.3
GOTERM_BP_DIRECT	GO:0043065 ~ positive regulation of apoptotic process	6	O00429, P50570, Q8N163, Q07812, O00571, P55957	0.0480	3.0
cluster 6	enrichment score: 1.58				
GOTERM_BP_DIRECT	GO:0006334 ~ nucleosome assembly	5	P49321, Q01105, P16403, P49736, P16402	0.0144	5.4
GOTERM_MF_DIRECT	GO:0042393 ~ histone binding	5	P49321, Q01105, Q96P70, P49736, O95373	0.0342	4.1
GOTERM_BP_DIRECT	GO:0006260 ~ DNA replication	4	P49321, Q01105, P33993, P49736	0.0368	5.4
cluster 7	enrichment score: 1.19				
GOTERM_BP_DIRECT	GO:0071902 ~ positive regulation of protein serine/threonine kinase activity	3	P36507, O00571, Q02750	0.0265	11.8

[a]Only the categories and terms with $p < 0.05$ are listed.

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Table 4. Functional Annotation Analysis by DAVID Software of the Proteins Whose Expression Was Significantly Upregulated by E2: with UP_SEQ_FEATURE[a]

category	term	count	UniProt ID	*p* value	fold enrichment
cluster 1	enrichment score: 6.37				
UP_SEQ_FEATURE	DOMAIN:Importin N-terminal	8	Q14974, O14980, Q92973, O14787, P55060, Q8TEX9, Q96P70, O95373	2.13×10^{-12}	84.9
UP_SEQ_FEATURE	REPEAT:HEAT 4	9	Q14974, Q6AI08, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	4.68×10^{-10}	30.6
UP_SEQ_FEATURE	REPEAT:HEAT 3	9	Q14974, Q6AI08, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	1.39×10^{-09}	26.8
UP_SEQ_FEATURE	REPEAT:HEAT 6	8	Q14974, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	1.73×10^{-09}	36.7
UP_SEQ_FEATURE	REPEAT:HEAT 1	9	Q14974, Q6AI08, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	4.63×10^{-09}	23.2
UP_SEQ_FEATURE	REPEAT:HEAT 2	9	Q14974, Q6AI08, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	4.63×10^{-09}	23.2
UP_SEQ_FEATURE	REPEAT:HEAT 5	8	Q14974, O14980, Q92973, P53618, Q86VP6, P30153, O14787, Q8TEX9	5.27×10^{-09}	31.6
UP_SEQ_FEATURE	REPEAT:HEAT 10	6	Q14974, O14980, Q92973, Q86VP6, P30153, O14787	2.43×10^{-07}	42.4
UP_SEQ_FEATURE	REPEAT:HEAT 9	6	Q14974, O14980, Q92973, Q86VP6, P30153, O14787	3.03×10^{-07}	40.8
UP_SEQ_FEATURE	REPEAT:HEAT 8	6	Q14974, O14980, Q92973, Q86VP6, P30153, O14787	5.52×10^{-07}	36.4
UP_SEQ_FEATURE	REPEAT:HEAT 7	6	Q14974, O14980, Q92973, Q86VP6, P30153, O14787	1.11×10^{-06}	31.8
UP_SEQ_FEATURE	REPEAT:HEAT 15	5	Q14974, Q92973, Q86VP6, P30153, O14787	1.44×10^{-06}	56.6
UP_SEQ_FEATURE	REPEAT:HEAT 14	5	Q14974, Q92973, Q86VP6, P30153, O14787	2.48×10^{-06}	49.9
UP_SEQ_FEATURE	REPEAT:HEAT 12	5	Q14974, Q92973, Q86VP6, P30153, O14787	3.18×10^{-06}	47.2
UP_SEQ_FEATURE	REPEAT:HEAT 13	5	Q14974, Q92973, Q86VP6, P30153, O14787	3.18×10^{-06}	47.2
UP_SEQ_FEATURE	REPEAT:HEAT 11	5	Q14974, Q92973, Q86VP6, P30153, O14787	4.99×10^{-06}	42.4
UP_SEQ_FEATURE	REPEAT:HEAT 17	4	Q14974, Q92973, Q86VP6, O14787	2.26×10^{-05}	67.9
UP_SEQ_FEATURE	REPEAT:HEAT 18	4	Q14974, Q92973, Q86VP6, O14787	2.26×10^{-05}	67.9
UP_SEQ_FEATURE	REPEAT:HEAT 19	4	Q14974, Q92973, Q86VP6, O14787	2.26×10^{-05}	67.9
UP_SEQ_FEATURE	REPEAT:HEAT 16	4	Q14974, Q92973, Q86VP6, O14787	3.10×10^{-05}	61.7
UP_SEQ_FEATURE	REPEAT:HEAT 20	3	Q92973, Q86VP6, O14787	0.0012	56.6
UP_SEQ_FEATURE	REPEAT:HEAT	3	Q14974, P30153, Q8TEX9	0.0072	23.1
cluster 2	enrichment score: 5.97				
UP_SEQ_FEATURE	DOMAIN:KH 1	6	Q96AE4, Q96I24, P61978, Q15366, P51114, Q15365	2.43×10^{-07}	42.4
UP_SEQ_FEATURE	DOMAIN:KH 2	6	Q96AE4, Q96I24, P61978, Q15366, P51114, Q15365	2.43×10^{-07}	42.4
UP_SEQ_FEATURE	DOMAIN:KH 3	5	Q96AE4, Q96I24, P61978, Q15366, Q15365	1.06×10^{-06}	60.6
UP_SEQ_FEATURE	DOMAIN:K Homology	5	Q96AE4, Q96I24, P61978, Q15366, Q15365	2.03×10^{-05}	30.3
cluster 3	enrichment score: 4.87				
UP_SEQ_FEATURE	DOMAIN:RRM	11	P52597, P31942, Q13148, O75821, P98179, P11940, Q8N684, P09651, O60506, P26599, Q15056	1.43×10^{-06}	7.8
UP_SEQ_FEATURE	DOMAIN:RRM 1	8	P52597, P31942, Q13148, Q13151, P11940, P09651, O60506, P26599	2.76×10^{-06}	12.9
UP_SEQ_FEATURE	DOMAIN:RRM 2	8	P52597, P31942, Q13148, Q13151, P11940, P09651, O60506, P26599	2.76×10^{-06}	12.9
UP_SEQ_FEATURE	DOMAIN:RRM 3	4	P52597, P11940, O60506, P26599	0.0031	13.6
cluster 4	enrichment score: 1.29				
UP_SEQ_FEATURE	DOMAIN:DEAD-box RNA helicase Q	3	O00571, O00148, P60842	0.0132	17.0
UP_SEQ_FEATURE	MOTIF:Q motif	3	O00571, O00148, P60842	0.0228	12.7

[a]Only the categories and terms with $p < 0.05$ are listed.

abundance of the proteins related to cytochrome c release in the E2-treated cells relative to the control cells are linked to the elevated levels of the pore-forming protein Bax in the mitochondria. These results suggest that induction of mitochondrion-dependent apoptosis is potentially an important physiological function of E2. Although E2 has been reported to induce apoptosis through the death receptor-activated apoptotic pathway,[6,58] we did not observe this at the proteome level. This discrepancy could potentially be due to the fact that death receptor-activated cell death has been observed in cells that are typically long-depleted of estrogen, such as triple-negative breast cancer cells or in patients undergoing antiestrogen therapy.[58,59] Contrary to the effect of E2 on the abundance of the proteins related to cytochrome c release (Table 3), the GSEA analysis revealed that the proteins involved in enhancing the PI3K/AKT/mTOR signaling pathway, a major apoptosis-inhibitory pathway in mammalian cells,[60] were enriched in the E2-treated cells compared to the control cells (Table 2). It is known that the activated PI3K leads to activation of AKT, which in turn inactivates the proapoptotic protein Bad and other factors.[61] Inhibition of PI3K leads to cell apoptosis.[62,63] Thus, our proteomic and subsequent bioinformatics analyses suggest that E2 potentially has a dual effect on apoptosis in MCF7 cells. It may promote

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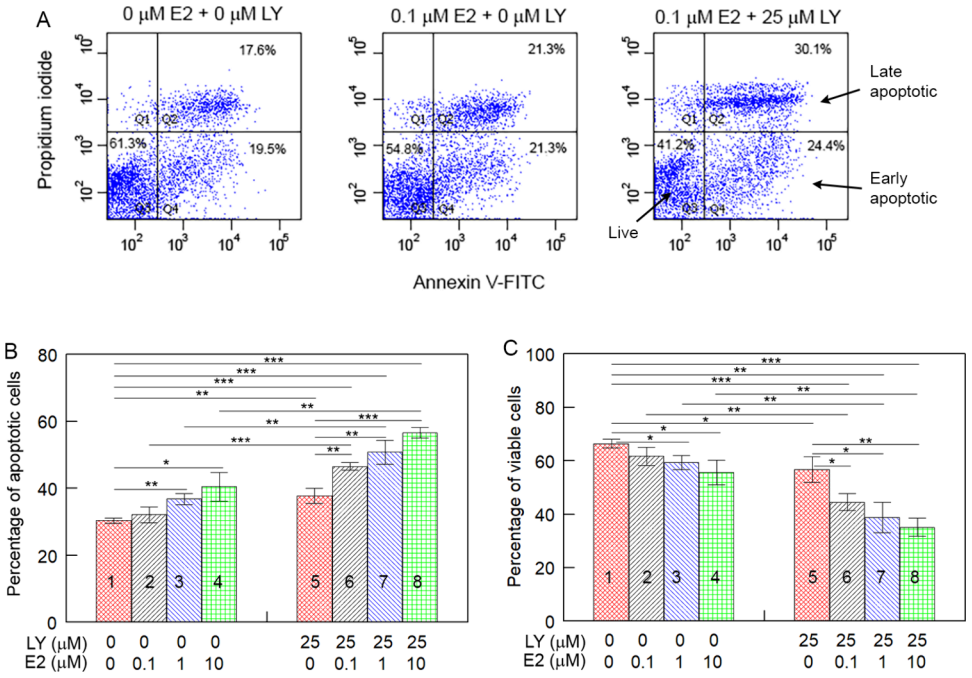

Figure 3. Inhibition of the PI3K enhances the stimulatory effect of E2 on apoptosis in MCF7 cells. (A) Representative flowcharts showing the effects of E2 (compare the middle chart to the left chart) or a combination of E2 and the PI3K inhibitor Ly294002 (compare the right chart to the middle and left charts) on apoptosis in MCF cells. (B and C) The effects of E2 or a combination of E2 and the PI3K inhibitor Ly294002 on apoptosis (sum of early and late apoptosis; B) and viability (C) in MCF cells. MCF7 cells were treated with indicated concentrations of E2 in the presence or absence of the PI3K inhibitor Ly294002. The cells were then stained with annexin V-FITC and propidium iodide, and apoptosis was assessed using a flow cytometer. Values in B and C are the mean ± SD of three separate sample preparations. LY: Ly294002. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

apoptosis through affecting the abundance of proteins related to cytochrome c release(Table 3) and simultaneously inhibit apoptosis by regulating the PI3K signaling (Table 2). We verified the predicted activation of PI3K in E2-treated MCF7 cells by determining the phosphorylation of the key PI3K downstream kinase, AKT/PKB, using Western blotting (Figure 2A-B). Additionally, we indirectly assessed cytochrome c release from the mitochondria in the cells by determining the levels of the pore-forming protein Bax in the membrane/organelle fraction, also using Western blotting. The Western blot results (Figure 2C−D) were consistent with the proteomic results (Supporting Table S6). Our results align with previous molecular and cellular studies that demonstrate the complex effects of estrogen on apoptosis, which can be both stimulatory and inhibitory.[6,58]

A Combination of E2 and a PI3K Inhibitor Significantly Increases Apoptosis in MCF7 Cells

Since E2 may promote and inhibit apoptosis simultaneously (Tables 1 and 2),[6,58] we hypothesized that blocking the inhibitory effect of E2 could significantly enhance its stimulatory effect on apoptosis. To assess this possibility, we treated MCF7 cells with varying concentrations of E2 in the presence or absence of the PI3K inhibitor Ly294002. We then stained the cells with annexin V and propidium iodide and assessed apoptosis using a flow cytometer (Figure 3A). In the absence of Ly294002, while apoptosis in cells treated with 1 μM and 10 μM E2 were 21.4% (p = 0.0034) and 31.6% (p = 0.016) higher than in vehicle-treated cells, respectively (Figure 3B, compare columns 3 and 4 with 1), 0.1 μM E2 did not induce significant apoptosis when compared to the vehicle control (compare column 2 with 1). In the presence of Ly294002, apoptosis in cells treated with 0.1 μM, 1 μM, and 10 μM E2 were 23.4% (p = 0.0000), 34.7% (p = 0.0006), and 50.0% (p = 0.0000) higher than in vehicle-treated cells, respectively, with all differences being statistically significant (compare columns 6, 7, and 8 with 1). Furthermore, apoptosis in cells treated with both E2 and Ly294002 was significantly higher than in cells treated with E2 alone at all three E2 concentrations used (compare columns 6 with 2, columns 7 with 3, and columns 8 with 4) (p = 0.0007, 0.0036, and 0.0037, respectively). Additionally, apoptosis in cells treated with E2 and Ly294002 was also significantly higher than in cells treated with Ly294002 alone (p = 0.0039, 0.0060, 0.0003, respectively; compare columns 6, 7, and 8 with 5). The effect of E2 on the percentage of viable cells in the presence of Ly294002 compared to its absence was consistent with the results on apoptosis (Figure 3C). These results demonstrate that by blocking the PI3K pathway, the effect of E2 on apoptosis in MCF7 cells can be substantially enhanced.

DISCUSSION

In this study, we used a stable isotope labeling-based quantitative proteomics method coupled with subcellular fractionation to comprehensively assess the impact of E2 on protein expression in MCF7 cells. Our findings suggest that E2 may promote MCF7 cell division primarily by inducing the expression of E2F target genes and enhancing the G2/M checkpoint transition (Table 2). The stimulatory effect of estrogen on the G1 to S phase transition has been reported.[64,65] Therefore, it appears that one key mechanism by which estrogen drives cell division in ERα-positive cells is by promoting progression through the restriction point. While the effect of estrogen on the G2/M checkpoint has been

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reported,[44] its physiological impact remains elusive. Notably, the G2/M checkpoint is a DNA damage checkpoint, and unchecked progression at this point may increase the risk of mutations in the cell. Additional investigation into the stimulatory effect of estrogen on the G2/M checkpoint may be warranted, as the results could provide insights into the potential association between estrogen's influence at this stage and its pathogenic effects.

Our proteomic and bioinformatics analyses suggest a complex role of E2 in apoptosis in MCF7 cells. On the one hand, the abundance of the proteins involved in cytochrome c release from the mitochondria, including the levels of the pore-forming protein Bax in the membrane/organelle fraction, was altered by E2 (Table 3, Figure 2C−D, and Supporting Table S6). Cytochrome c release is a crucial event in the mitochondrion-dependent apoptotic pathway. On the other hand, E2 treatment led to the enrichment of the proteins related to the PI3K/AKT/mTOR pathway among the E2-regulated proteins (Table 2). E2 has been shown to regulate PI3K through the binding of ERα with the p85α regulatory subunit of PI3K.[7] We assessed the PI3K signaling in the E2- and vehicle-treated MCF7 cells by determining the phosphorylation of the key PI3K downstream kinase AKT/PKB using Western blotting. Our Western blot results (Figure 2A-B) were consistent with the proteomic and bioinformatics prediction (Table 2). The phosphorylation and activation of AKT/PKB in MCF7 have also been reported by others.[45] Since the PI3K pathway is a major antiapoptotic pathway in mammalian cells,[60] it is evident that the antiapoptotic effect of E2 is also discernible at the proteome level. Therefore, in addition to its mitogenic property of stimulating ERα-positive breast cancer cell division, E2 can also fundamentally influence apoptosis. Given its dual role in enhancing and inhibiting apoptosis (Tables 2 and 3),[6] we conducted experiments to determine whether blocking the apoptosis-inhibitory PI3K could enhance the stimulatory effect of E2 on apoptosis in MCF7 cells. Our results demonstrate that apoptosis in MCF7 cells treated with a combination of E2 and the PI3K inhibitor Ly294002 was significantly higher than that in cells treated with E2 alone, suggesting that by blocking the E2-induced PI3K pathway, the effect of E2 on apoptosis in MCF7 cells can be substantially enhanced. It might be worthwhile to investigate further the effects of a combination of E2 and a PI3K inhibitor on apoptosis *in vivo* in the future, as the results could shed light on the potential application of this strategy in treating ERα-positive cancer. Furthermore, several practical issues must be addressed in future studies. For instance, what is the lowest concentration of E2 that is effective in inducing apoptosis when E2 and a PI3K inhibitor are used in the treatment? If the lowest required concentration is higher than the typical pharmacological concentrations of E2 achieved by conventional means, is it possible to increase the local E2 concentration in tumor cells?

The PI3K pathway may play a pivotal role in mediating the physiological effects of estrogen. Once activated by estrogen, presumably via the binding of ERα to the p85 subunit of PI3K,[7] the activated PI3K catalyzes the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PIP3), resulting in the phosphorylation of AKT/PKB. On the one hand, the phosphorylated and activated AKT/PKB mediates the stimulatory effect of estrogen on breast cancer cells by inhibiting GSK3β, leading to the activation of the Wnt-β-catenin pathway.[66] In line with this expected effect, our GSEA analysis revealed that the abundance of the proteins related to the Wnt-β-catenin pathway was enriched in E2-treated MCF7 cells compared to vehicle-treated control cells (Table 2). Thus, in addition to other molecular processes that are stimulated by estrogen during the cell cycle, for example, upregulation of cyclins A, B, D1, and E, and cyclin-dependent kinases (CDKs),[64] and the interaction of activated ERα with cyclins, CDKs, CDK inhibitors, and the retinoblastoma protein,[67] the PI3K signaling appears to contribute significantly to the mitogenic effect of estrogen in breast cancer cells. On the other hand, the activated AKT/PKB can also inhibit apoptosis by inactivating the proapoptotic protein Bad.[61] In this study, we demonstrated that E2 treatment led to the enrichment of the proteins involved in the PI3K signaling among the E2-regulated proteins (Table 2) and to the phosphorylation of AKT/PKB (Figure 2A-B), a finding also reported by others.[45] Therefore, the estrogen-activated PI3K signaling pathway likely plays a major role in mediating the effects of estrogen on both cell division and apoptosis. Inhibition of PI3K activity, on the one hand, can suppress the estrogen-induced G1 to S phase transition in the cell cycle, mimicking the effect of CDK inhibitors.[68,69] On the other hand, it can enhance estrogen's apoptosis-inducing effect in ERα-positive breast cancer cells.

Another cellular factor that may play a pivotal role in mediating the physiological effects of estrogen in ERα-positive breast cancer cells is the proto-oncogene c-Myc. c-Myc is a transcription factor involved in various cellular processes, including cell growth, proliferation, metabolism, differentiation, stress responses, apoptosis, and drug resistance.[70] Dysregulation of c-Myc is implicated in the development of multiple types of cancer, including breast cancer.[70] c-Myc is a target gene of ERα.[71] Consistent with this, our GSEA analysis showed that the proteins involved in c-Myc were enriched among the E2-regulated proteins (Table 2). If this holds true, the estrogen-activated c-Myc can lead to downstream pathway activation, including the G1 to S phase transition in the cell cycle,[64] which was a significant molecular event predicted to be influenced by E2 in MCF7 cells in this study (Table 2). Therefore, in addition to the PI3K/AKT/mTOR pathway, c-Myc may also play a pivotal role in mediating the mitogenic effect of estrogen in breast cancer cell division.

Furthermore, c-Myc has also been shown to regulate apoptosis. For instance, c-Myc can induce apoptosis by upregulating the expression of pro-apoptotic genes including Bax.[72,73] Upregulated c-Myc can also disrupt mitochondrial membrane potential, promoting mitochondrion-dependent apoptosis.[74] Consistent with these expected effects, our proteomic data revealed that the protein levels of Bax in the membrane/organelle fraction of the E2-treated cells were significantly elevated compared to vehicle-treated cells (Supporting Table S6); the subsequent bioinformatics analysis revealed that the proteins involved in cytochrome c release from mitochondria was enriched among the E2-upregulated proteins in MCF7 cells (Table 3). It is noticeable that Bax protein levels in E2-treated cells were higher than their counterparts in the membrane/organelle fraction (Supporting Table S6), but not in the cytosolic and nuclear fractions (Supporting Tables 4 and 5). It is well established that the Bax protein migrates from the cytosol to the mitochondria upon apoptosis induction.[55−57] While the potential role of c-Myc in upregulating Bax expression is known,[72,73] it is more likely that the higher levels of Bax in the membrane/organelle fraction of the E2-treated MCF7 cells result from the translocation of Bax

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from the cytosol to the mitochondria. Regardless of the underlying mechanism for the higher levels of Bax in the membrane/organelle fraction of E2-treated cells, it is expected that the higher levels of the pore-forming protein Bax could significantly contribute to the mitochondrion-dependent apoptosis. In summary, PI3K and c-Myc likely play pivotal roles in mediating the physiological effects of estrogen, including its impact on cell division and apoptosis, in ERα-positive breast cancer cells.

■ ASSOCIATED CONTENT

Data Availability Statement

The MS proteomic data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRoteomics IDEntifications (PRIDE) partner repository with the data set identifier PXD049343.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.4c00102.

Supporting Figures: Supporting Figure S1, fractionation of cellular proteins into cytosolic, nuclear, and membrane/organelle fractions. Supporting Figure S2, uncropped images of the Western blots showing AKT Ser-473 phosphorylation in E2-treated MCF7 cells (E2) compared to vehicle-treated control cells (Ctrl). Supporting Figure S3, uncropped images of the Western blots showing elevated Bax protein levels in the membrane/organelle fraction of E2-treated MCF7 cells (E2) compared to vehicle-treated control cells (Ctrl) (PDF)

Supporting Table S1, list of quantified cytosolic proteins (XLSX)

Supporting Table S2, list of quantified nuclear proteins (XLSX)

Supporting Table S3, list of quantified membrane/organelle proteins (XLSX)

Supporting Table S4, list of E2-regulated cytosolic proteins (XLSX)

Supporting Table S5, list of E2-regulated nuclear proteins (XLSX)

Supporting Table S6, list of E2-regulated membrane/organelle proteins (XLSX)

Supporting Table S7, list of E2-regulated cytosolic, nuclear, and membrane/organelle proteins (XLSX)

Supporting Table S8, functional annotation analysis by DAVID of the proteins whose expression was significantly downregulated by E2: with GOTERM_BP_DIRECT and GOTERM_MF_DIRECT (PDF)

Supporting Table S9, functional annotation analysis by DAVID of the proteins whose expression was significantly downregulated by E2: with UP_SEQ_FEATURE (PDF)

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Notes

The authors declare no competing financial interest.

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