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Proteomic insight towards key modulating proteins regulated by the aryl hydrocarbon receptor involved in ovarian carcinogenesis and chemoresistance

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ABSTRACT

Gynecological malignancies pose a severe threat to female lives. Ovarian cancer (OC), the most lethal gynecological malignancy, is clinically presented with chemoresistance and a higher relapse rate. Several studies have highly correlated the incidence of OC to exposure to environmental pollutants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a process mainly mediated through activating the aryl hydrocarbon receptor (AhR). We have previously reported that exposure of OC cells to TCDD, an AhR activator, significantly modulated the expression of several genes that play roles in stemness and chemoresistance. However, the effect of AhR activation on the whole OC cell proteome aiming at identifying novel druggable targets for both prevention and treatment intervention purposes remains unrevealed. For this purpose, we conducted a comparative proteomic analysis of OC cells A2780 untreated/treated with TCDD for 24 h using a mass spectrometry-based label-free shotgun proteomics approach. The most significantly dysregulated proteins were validated by Western blot analysis. Our results showed that upon AhR activation by TCDD, out of 2598 proteins identified, 795 proteins were upregulated, and 611 were downregulated. STRING interaction analysis and KEGG-Reactome pathway analysis approaches identified several significantly dysregulated proteins that were categorized to be involved in chemoresistance, cancer progression, invasion and metastasis, apoptosis, survival, and prognosis in OC. Importantly, selected dysregulated genes identified by the proteomic study were validated at the protein expression levels by Western blot analysis. In conclusion, this study provides a better understanding of the the cross-talk between AhR and several other molecular signaling pathways and the role and involvement of AhR in ovarian carcinogenesis and chemoresistance. Moreover, the study suggests that AhR is a potential therapeutic target for OC prevention and maintenance.

Significance: To our knowledge, this is the first study that investigates the role and involvement of AhR and its regulated genes in OC by performing a comparative proteomic analysis to identify the critical proteins with a modulated expression upon AhR activation. We found AhR activation to play a tumor-promoting and chemoresistance-inducing role in the pathogenesis of OC. The results of our study help to devise novel

Abbreviations: ACN, acetonitrile; AhR, Aryl hydrocarbon receptor; ALK, anaplastic lymphoma kinase; ANKMY2, Ankyrin Repeat And MYND Domain Containing 2; ARNT, AhR nuclear translocator; ATG9A, Autophagy Related 9 A; CYP1A1, Cytochrome P450 1A1; CYP1B1, Cytochrome P450 1B1; DDB1, DNA damage-binding protein 1; DNAJC17, DnaJ Heat Shock Protein Family (Hsp40) Member C17; DTT, Dothiothreitol; EMT, Epithelial-to-mesenchymal transition; EP300, E1A Binding Protein P300; FDR, False discovery rate; FKBP4, FK506-binding protein 4; GCLC, glutamate-cysteine ligase catalytic subunit; GO, Gene ontology; GPX4, Glutathione peroxidase 4; GSTM1, Glutathione S-transferase M1; GSTO1, Glutathione S-transferase omega-1; GSTP1, Gutathione S-transferase pi 1; HSP90AB1, Heat shock protein 90 kDa alpha class B member 1; JAK/STAT, Janus kinase/signal transducers and activators of transcription; KEGG, Kyoto Encyclopedia of Genes and Genomes; LFQ, Label-free quantification; MS, Mass spectrometry; PEX5, Peroxisomal Biogenesis Factor 5; PI3K/AKT, phosphoinositide-3-kinase–protein kinase B/Akt; PPI, Protein-protein interaction; RAS/RAF, rat sarcoma virus (Ras)/rapidly accelerated fibrosarcoma; RB1, Retinoblastoma1; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNF, tumor necrosis factor; XRE, xenobiotic responsive element..

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therapeutics for better management and prevention and open the doors to finding novel biomarkers for the early detection and prognosis of OC.

1. Introduction

Ovarian cancer (OC) is a globally lethal gynecological malignancy [1]. As of its late presenting and asymptomatic nature, OC is often called the "whispering cancer" or "silent cancer" [2], and thus its management is challenging [3]. Moreover, despite the efficient treatment regimen, high chemoresistance and recurrence rates are also observed clinically [4]. Though several risk factors have been associated with OC occurrence, such as genetic mutations, family history, lifestyle, and reproductive factors [5,6], exposure to environmental pollutants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), plays a major role in initiating OC [7,8]. TCDD is a highly toxic environmental pollutant and a human group 1 carcinogen. Animal and human epidemiological studies provide sufficient evidence that TCDD exposure is linked to the initiation and progression of various types of cancer [7]. TCDD is frequently released into the environment via air emissions, such as incomplete combustion of vegetation and other organic matter. The emitted TCDD can travel for long distances before settling on soil and water. Because of its stability and resistance to degradation, TCDD can survive in the environment for a long time.

Exposure to TCDD and other environmental pollutants causes activation of a cytoplasmic ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR). Upon activation, AhR translocates into the nucleus, where it heterodimers with AhR nuclear translocator, ARNT, and then binds to specific DNA sequence, xenobiotic responsive element (XRE). This results in the transcription induction of genes that code for proteins involved in xenobiotic metabolism and procarcinogen bioactivation, such as the cytochrome P451A1 (CYP1A1) and CYP1B1 [9]. Even though the role of AhR in several cancer types has been recently established [7,10], studies and reports showcasing its role in ovarian carcinogenesis, progression, and chemoresistance are still in their infancy. It is worth noting here that there is evidence that certain TCDD effects may occur via mechanisms other than AhR, such as interactions with various cellular receptors, direct effects on cellular components, or activation of alternative signaling pathways.

Clinical management of OC through therapeutic procedures includes cytoreductive surgery followed by single or combinational chemotherapy using platinum-based drugs [11]. However, chemoresistance, metastasis, and immune evasion in OC are challenging for current therapy procedures, demanding the development of novel and better therapeutic strategies [12]. A recent study from our group has shown that exposure of different histotypes OC cells to TCDD, an AhR activator, significantly modulated the expression of several genes that play roles in stemness and drug chemoresistance [13]. In this study, we have reported that AhR activation in human ovarian adenocarcinoma A2780 cells promoted cell growth and proliferation by activating the PI3K/AKT pathway, inhibiting apoptosis, and inducing epithelial-to-mesenchymal transition (EMT) and metastasis. In addition, we also observed that cells attained stemness-like characteristics upon AhR activation. These results have encouraged us to explore the effect of AhR ligands on whole OC proteome using proteomics analysis to identify novel druggable targets for prevention and treatment intervention.

Proteomics is an analytical technique that helps identify a whole set of proteins expressed in a cell type or organism at a given time and condition, thus offering the opportunity for a comprehensive analysis of proteins in cells, tissues, or body fluids possible [14,15]. In the current study, we performed a comparative proteomic analysis of OC cell line A2780 treated with TCDD vs. control cells to identify the key proteins with a modulated expression upon AhR activation. We found that AhR activation in A2780 cells resulted in the upregulation of proteins involved in cancer progression, cancer promotion, poor prognosis, worse survival, chemoresistance, metastasis induction, and apoptosis inhibition, whereas caused downregulation of proteins involved in tumor suppression and metastasis inhibition.

2. Materials and methods

2.1. Chemicals and reagents

TCDD was procured from Toronto Research Chemicals (Toronto, ON, Canada). Human OC A2780 cells (ECACC 93112519) were ordered from the European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, UK). Dulbecco's Modified Eagle's Medium (DMEM) was procured from Thermo Fischer Scientific (Waltham, USA). Dithiothreitol (DTT) and indole acetic acid were obtained from Merck KGaA (Darmstadt, Germany). Antibodies against DDB-1, PEX5, Galectin-1, GCLC, FKBP4, GPX4, STAT1, phospho-STAT1, and GAPDH were acquired from Cell Signaling Technologies (Beverly, MA, USA). Antibody against CYP1B1 was purchased from Santacruz Biotechnology (Heidelberg, Germany).

2.2. Ovarian cancer cell culture and TCDD treatment

The human ovarian cancer cell line A2780 was routinely cultured in Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum and 1% antibiotic (penicillin 100 U/mL, streptomycin 10 μg/mL). The cell line was maintained at 37 °C in a humidified 5% CO_2–95% air atmosphere. TCDD stock solution (10 mM) was diluted in cell culture medium to the desired concentrations of 10 nM, a concentration that has been selected from previously published work from our laboratory and others [7,13]. For AhR induction, 5×10^5 A2780 cells were seeded in 6-well culture plates, and then were treated for 24 h with either normal saline, vehicle of TCDD, as control, or 10 nM TCDD.

2.3. Protein extraction (whole proteome)

Protein extraction was carried out, as explained before [16]. The cells were washed with ice-cold PBS and 1× Radioimmunoprecipitation (RIPA) assay lysis buffer was addedwith Halt protease-phosphatase inhibitor cocktail (1×) on ice for thirty minutes. The cells were then scrapped using a cell scraper and pooled together. The pooled cells in 1× RIPA were further incubated on ice for 15 min and centrifuged at 14,000 xg, 4 °C for 15 min. The supernatants were then collected, and the total soluble protein was quantified using a Rapid Gold BCA assay kit (Pierce™, Thermo Scientific, Waltham, MA, United States).

2.4. Sample preparation for mass spectrometry analysis

Approximately 10 μg of protein was first reduced by 5 mM dithiothreitol (DTT) at RT for 40 min and alkylated by 10 mM iodoacetamide for 30 min at RT in the dark. The proteins were then digested overnight at 37°C in ABC buffer containing Mass Spec Grade Trypsin / Lys-C Mix (12.5 ng/μL), Promega (Madison, WI, USA) with trypsin to protein ratio of 1:25. After digestion, trypsin was inactivated by acidification of reaction mixture with 0.1% formic acid. Next, the peptide mixture was desalted and concentrated using the Bond Elut C_{18} cartridges (Agilent Technologies) by solid phase extraction and eluted with different gradients of acetonitrile (ACN) in 0.1% formic acid. The eluate was then dried down in a speed vacuum concentrator to form a clear pellet and reconstituted in 2% ACN in 0.1% formic acid, and the peptides were further subjected to mass spectrometry analysis [17].

2.5. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

MS and MS/MS experiments were performed by trapped ion mobility mass spectrometry using a timsTOF Pro mass spectrometer (Bruker Daltonics, Germany) coupled to nanoelute, a nano-liquid chromatography system, Bruker (Daltonics, Germany). The desalted peptides were first separated on a 15-cm long reversed-phase C18 column with an integrated captive spray emitter (25 cm × 75 μm, 1.6 μm, Ion Optics, Australia) using a gradient of buffer A (Ultrapure water, 1% formic acid) and buffer B (ACN, 1% formic acid) at a flow rate of 400 nL/min. The chromatographic gradient was set to provide a linear increase from 2% to 80% buffer B in 110 min for a total run time of 120 min. MS data were acquired in data-dependent mode, dynamically choosing the top ten most abundant precursor ions from the survey scan (400–1800 m/z) for fragmentation and MS/MS analysis. Precursors with a charged state of +1 were rejected, and the dynamic exclusion duration was 25 s.

2.6. MS and MS/MS data processing and analysis

The MS/MS raw data were processed using MaxQuant software version 2.1.4.0 according to the standard workflow [18] with the built-in search engine Andromeda [19]. Proteins were identified by searching against the UniProtKB/Swiss-Prot human database. Carbamidomethylation of cysteines was set as a fixed modification, while methionine oxidation was defined as variable modifications for peptide search. The first and primary search precursor tolerance was set as 20 ppm and 10 ppm, respectively, while TOF's MS/MS tolerance was set as 0.5 Da (the default setting). The false discovery rates (FDR) for peptide spectrum match and protein identifications were set to 1%, calculated based on the search against the reverse sequence decoy database. A maximum of one missed cleavage was allowed for tryptic digestion. The MaxLFQ label-free quantitation (LFQ) method [20] at an LFQ minimum ratio count of 2 with retention time alignment and match-between-runs feature (a match time window of 0.7 min and a 20-min alignment time window as the default setting) in MaxQuant was applied to extract the maximum of possible quantification information. Protein abundance was then calculated using normalized spectral intensity (LFQ intensity).

Data analysis was performed using the Perseus software (version 2.0.7.0) [21]. The proteins marked as potential contaminants, matched to reverse sequence, and identified only by site were removed. The LFQ intensities from the MaxQuant analysis were imported and transformed to log2(x). The proteins found consistently in all three biological replicates in at least one condition were included for comparative statistical analysis. The missing LFQ intensity values were replaced with the value from the normal distribution (width = 0.3, downshift = 1.8). The protein quantification and the statistical significance were calculated using a two-tailed Student's t-test with a permutation-based FDR of 5%. The adjusted $p\text{-value} < 0.05$ with a 2-fold change indicated significant protein abundance changes. The raw data is available under the Supplementary Materials section.

2.7. Protein-protein interaction and functional enrichment analysis

The protein-protein interaction (PPI) networks of the differentially expressed proteins were generated using STRING database version 11.5 (https://string-db.org/) [22] set at medium confidence (as a default setting) and visualized by Cytoscape software version 3.9.0 (https://cytoscape.org). Their functional enrichment was analyzed based on KEGG and Reactome pathway analysis. Additionally, the ClueGO (v. 2.5.9)/CluePedia (v. 1.5.9) plugins in the Cytoscape software were applied for functional enrichment analysis based on the gene ontology (GO) - Biological Process (BP), KEGG pathway and Reactome pathway. STRING is a precomputed global resource for exploring and analyzing protein-protein associations with a unique scoring framework based on benchmarks of the different types of associations against a standard reference set integrated into a single confidence score per prediction [23,24]. This graphical representation of the network of inferred, weighted protein interactions provides a high-level view of functional linkage, facilitating the analysis of modularity in biological processes. The individual protein association networks were derived from various channels, including neighborhood, co-occurrences, database, co-expression, and experiments. The PPI enrichment p-value indicating the statistical significance was provided by STRING [23].

2.8. Western blot analysis

Forty micrograms of protein were first separated on a sodium dodecyl sulfate polyacrylamide gel followed by immunoblotting as previously described [16]. The protein bands were then identified by ECL chemiluminescent substrate (BioRad Western ECL Substrate) and imaged on ChemiDoc Imaging System, Bio-Rad (Hercules, CA, USA). The protein bands in the figures are a composite of different blots and are representative blots for the indicated proteins and the loading control.

2.9. Statistical analysis

In proteomic analysis, the statistical significance of protein quantification was calculated using a two-tailed Student's t-test with permutation-based FDR of 5% for truncation of all test results. Pearson correlation tests with continuity correction were employed to compare qualitative variables. Statistical analysis was carried out for all other experiments using GraphPad Prism 7.0 software (Boston, USA), and significance was calculated using a non-parametric Student's t-test. Data from two groups were compared using a two-sample t-test, and a p-value <0.05 was considered statistically significant. Data are represented as mean±SD from three independent experiments unless otherwise mentioned.

3. Results

3.1. LC-MS/MS analysis identified differentially expressed proteins upon AhR activation

To cope with the biological and experimental variations, we included triplicate runs in LC-MS/MS analysis with samples from three independent replicates. The Pearson correlation analysis (PCA) of the LFQ intensities of proteins identified from untreated and TCDD-treated replicate samples showed a strong positive correlation accounting to a reproducible, relative label-free quantification between replicates (Fig. 1. A). Upon AhR activation, a total of 2598 proteins were identified, and to explore the unique proteins identified in both groups, as illustrated in Fig. 1. B, we found that approximately 2290 unique proteins, which represent 88% of the total proteins, were identified and overlapped in both untreated and TCDD-treated samples. Further, we compared the differential protein expression in untreated and TCDD-treated cells. We found that out of the whole proteins identified, 795 proteins were significantly upregulated, whereas 611 proteins were significantly downregulated upon AhR induction (Fig. 1. C). Furthermore, the heatmap illustrated in Fig. 1. D explored the significantly dysregulated proteins after TCDD treatment with fold-change >2 or ≤ 1.5 as differential abundance threshold.

3.2. Functional annotation of dysregulated proteins

Based on KEGG pathway analysis, the functional annotation of dysregulated proteins, which potentially interacted with AhR, was performed using the STRING tool. The interactome network of significantly upregulated and downregulated proteins upon TCDD treatment are shown in Fig. 2. A and 2. B, respectively. The KEGG pathway terms are presented in Fig. 2. C. These identified dysregulated proteins were

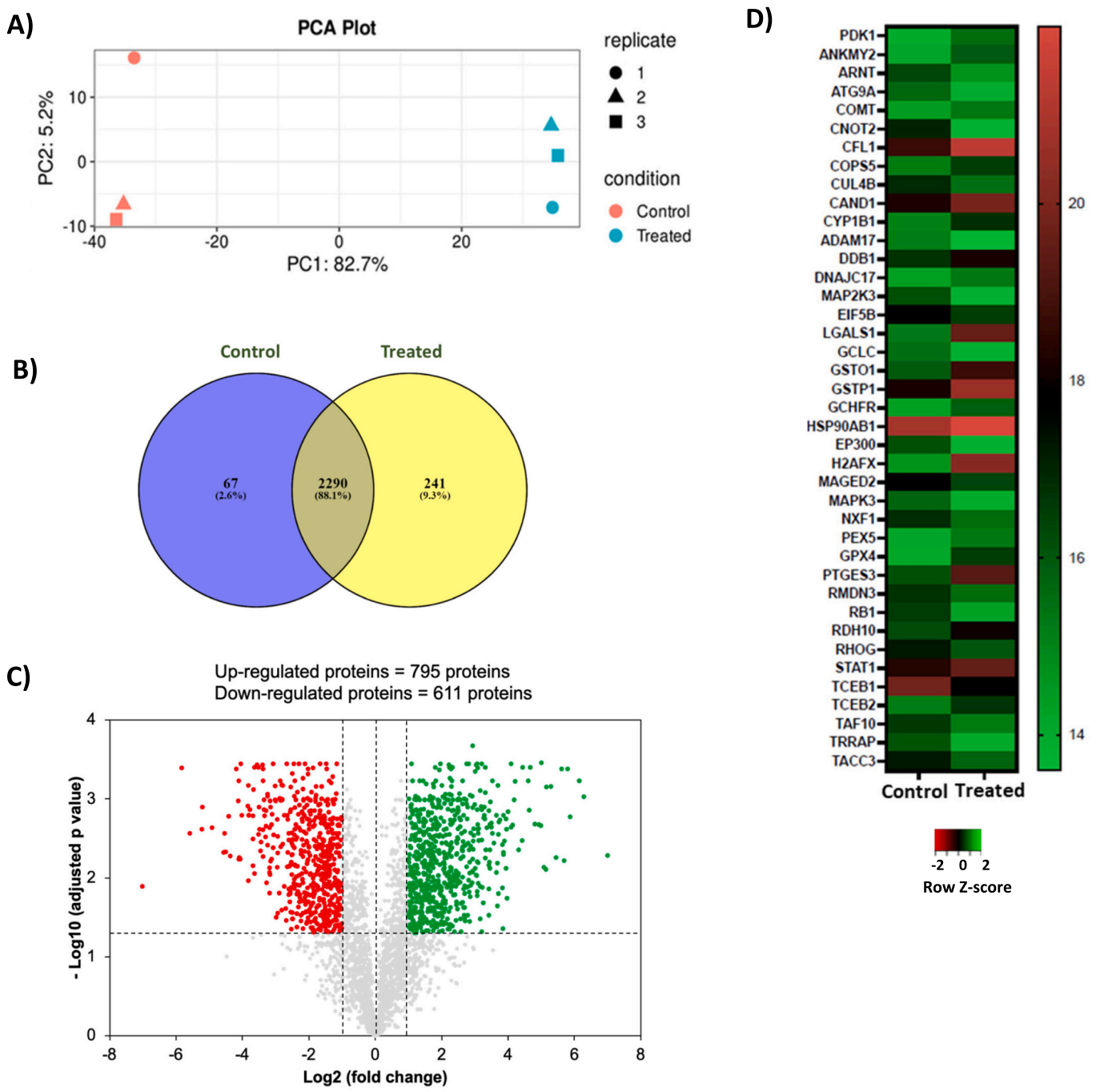

Fig. 1. A) PCA plot showing the cluster of sample similarities in untreated vs. TCDD-treated samples**. B)** Venn diagram showing the unique proteins identified in untreated vs. TCDD treated samples, and the overlapping proteins are present in both. **C)** Volcano plot comparing differential protein expression in untreated and TCDD-treated samples. The proteins significantly upregulated are green dots, and downregulated are red dots, while the grey dots represent the proteins with unaltered expression. The adjusted *p*-value <0.05 was used for this significance cutoff. **D)** Heatmap of the significantly dysregulated proteins after TCDD treatment with FDR (*p*-value) < 0.05 as significance threshold and fold-change >2 or ≤1.5 as differential abundance threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mainly involved in cancer pathways, chemical/viral carcinogenesis, glutathione metabolism, metabolism of xenobiotics by cytochrome P450, nucleotide excision repair, drug metabolism, and platinum drug resistance. In addition, we carried out the ClueGO/CluePedia based on the GO biological process (GO_BP), KEGG pathway, and Reactome pathway analysis of differentially expressed proteins, which potentially interacted with AhR. The data revealed that these differentially expressed proteins were functionally involved in several biological processes and pathways, including chemical carcinogenesis, glutathione metabolism, prostanoid/glutathione/prostaglandin metabolic process, gap-filling DNA repair synthesis, cellular response to heat stress, HSP90 chaperone cycle for steroid hormone receptor, cytochrome P450 arranged by substrate type and AhR signaling (Fig. 2. B).

Moreover, we performed STRING-based reactome pathway analysis of differentially expressed proteins, which interacted with AhR and well-known downstream proteins of the AhR pathway (CYP1B1 and ARNT). Results prove that the proteins involved in DNA repair mechanisms, including nucleotide excision repair and DNA double-strand break

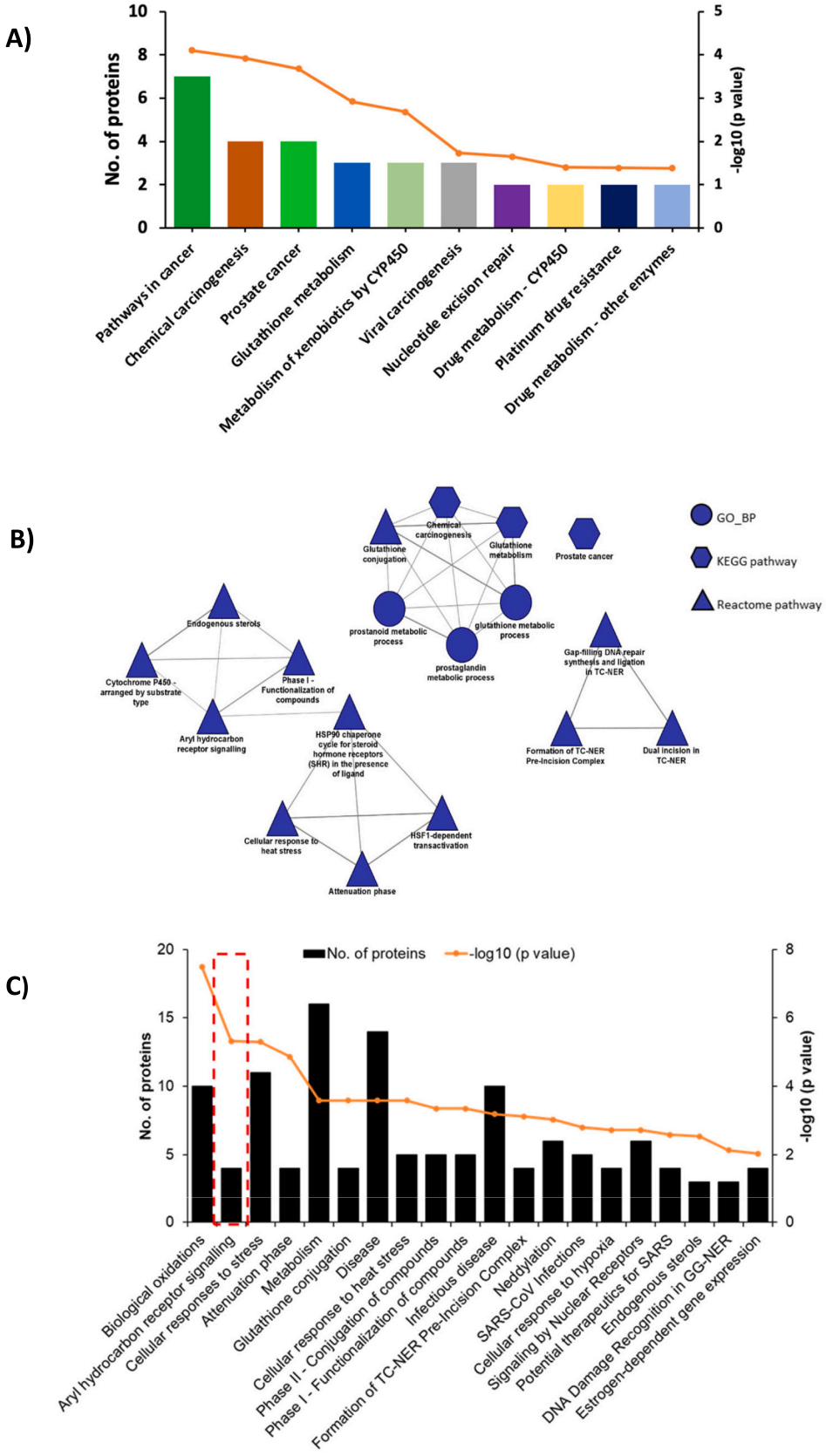

Fig. 2. A) Functional annotation of dysregulated proteins upon TCDD treatment. **B)** ClueGO/CluePedia based on GO_BP + KEGG+Reactome pathway analysis representing the differentially expressed proteins involved in chemical carcinogenesis. **C)** Reactome pathway analysis showing the number of proteins deregulated upon TCDD treatment, categorized as involved in several biological fates of OC cells.

repair, were upregulated upon TCDD treatment and may be related to apoptosis inhibition and autophagy (Fig. 2. C). Additionally, several potential candidate proteins involved in crucial cancer-related pathways, including PI3K/AKT, JAK/STAT, RAS/RAF, TNF, Notch, Hedgehog, P53, and ALK signaling, were upregulated upon TCDD treatment.

3.3. AhR activation in OC cells upregulates proteins involved in chemoresistance and cancer progression

The protein-protein interaction network analysis using STRING identified 17 differentially expressed proteins as interacting partners of AhR. In addition, STRING analysis was also performed to analyze the proteins interacting with CYP1B1 and ARNT, the downstream proteins of the AhR pathway, which also confirmed that AhR regulated several proteins involved in carcinogenesis and chemoresistance (Fig. 3). These proteins from the STRING and Reactome pathway analyses that showed significant deregulation upon AhR induction was further categorized into several cellular fates, including chemoresistance (GSTM1, GSTP1, ATG9A, DDB1, GSTO1, PEX5), cancer progression (Galectin-1, RB1, ARNT, CYP1B1), invasion and metastasis (Cofilin, CYP1B1, EP300), prognosis and survival (ANKMY2, GCLC, HSP90AB1, FKBP4, and DNAJC17) and apoptosis inhibition (GPX4). In addition, the functional activities of these proteins were categorized into cancer-related activities, such as apoptosis, cancer progression, chemoresistance, metastasis, and prognosis (Fig. 4). The most significantly upregulated and downregulated proteins interacting with AhR/CYP1B1/ARNT proteins are listed in Table 1. and Table 2., respectively.

3.4. Validation of key proteins identified by proteomic analysis by Western blot analysis

To further validate the differential expression of proteins upon TCDD treatment in A2780 cells, we validated proteins from each category by immunoblotting. We found that our results are consistent with the proteomic analysis data in which TCDD treatment for 24 h modulated the protein expression of CYP1B1; which is involved in cancer invasion and metastasis (Fig. 5 A), DDB1 and PEX5; which are involved in cancer chemoresistance (Fig. 5 B), GCLC and FKBP4; which are involved in cancer prognosis and survival (Fig. 5 C), GPX4; which is involved in apoptosis evasion (Fig. 5 D), and phospho-STAT1; which is involved in cancer progression (Fig. 5 E).

4. Discussion

The incidence of OC has been elevated in recent years due to its increased recurrence rate and chemoresistant properties. As a major predisposing factor, environmental factors, especially exposure to xenobiotics and environmental pollutants have been studied concerning cancer occurrence in several cancers, including both hematological and solid cancers [25]. For example, we have previously shown that

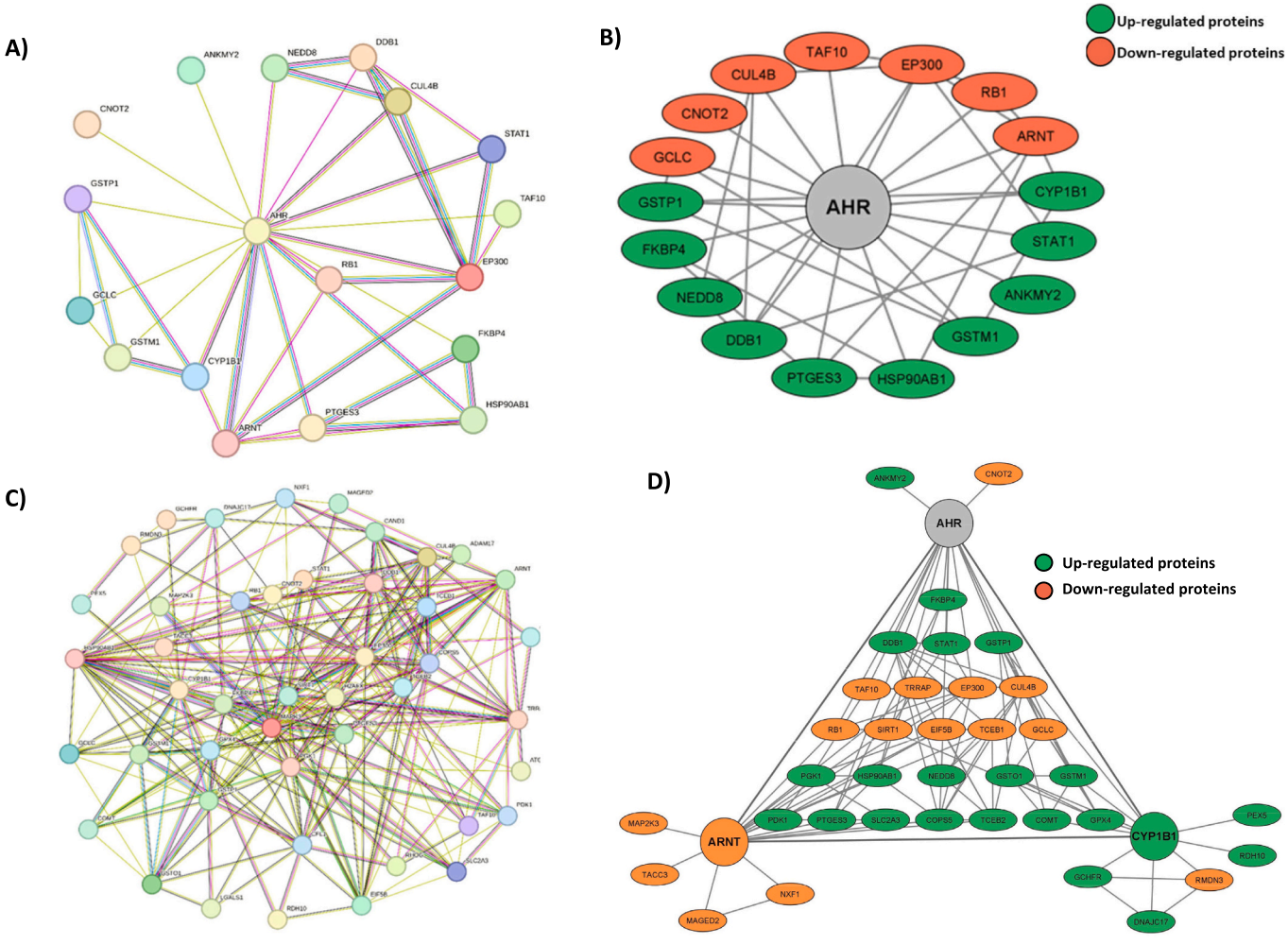

Fig. 3. A) STRING analysis network showing the deregulated proteins interacting with AhR. **B)** Schematic representation of the proteins involved in the STRING AhR network characterized as up/down regulated upon TCDD treatment. **C)** STRING interactome network of AhR/CYP1B1/ARNT. **D)** The schematic representation of the proteins involved in the STRING interactome network of AhR/CYP1B1/ARNT characterized as up/down regulated.

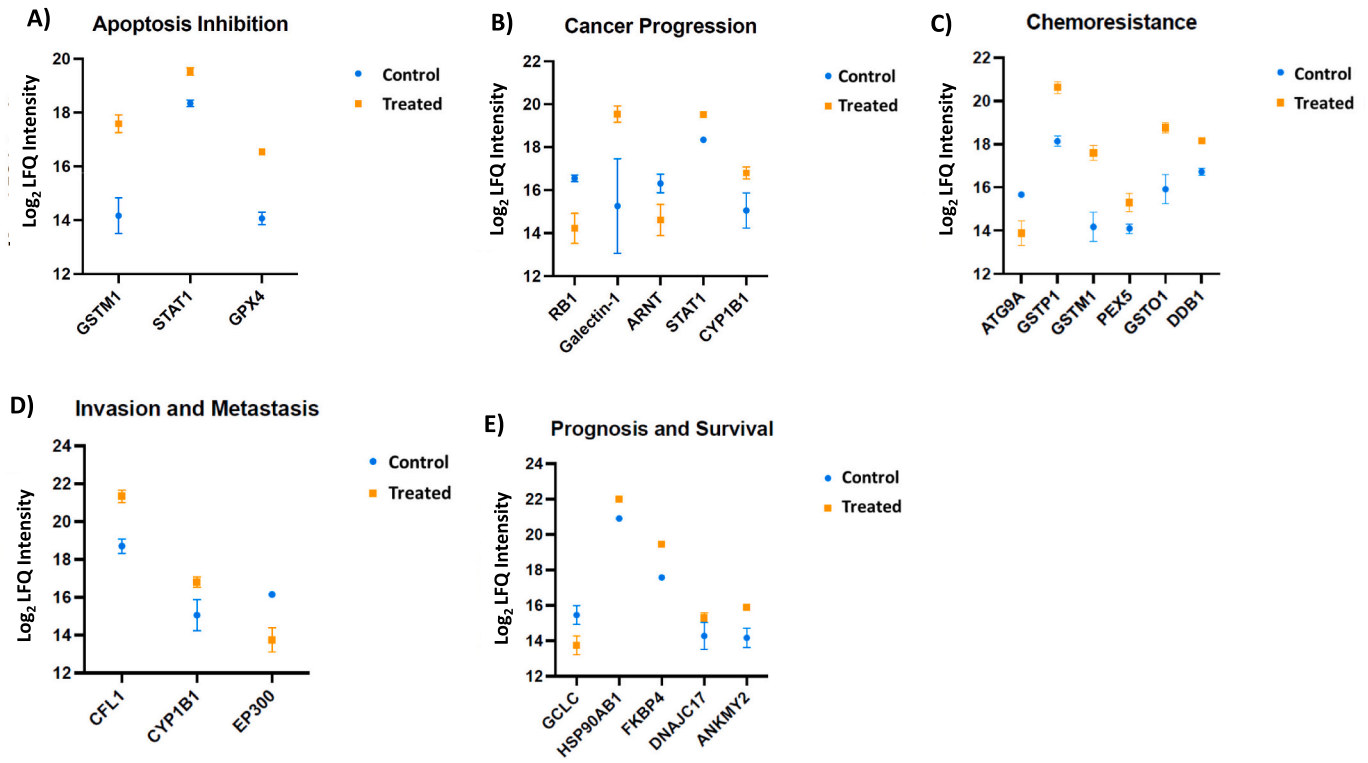

Fig. 4. Functional categorization of significant proteins dysregulated upon TCDD-treated A2780 cells. Box plots representing the differential expression of proteins in untreated vs. TCDD-treated samples of A2780 cells categorized to be involved in **A)** apoptosis inhibition, **B)** cancer progression, **C)** chemoresistance, **D)** invasion and metastasis, and **E)** prognosis and Survival.

Table 1
List of 20 most significantly upregulated proteins upon AhR induction in A2780 cells.

Protein name	Protein ID	Gene name	MW (kDa)	Fold Change	Adjusted *p*-value
Histone H_2AX	P16104	*H2AFX*	15.14	55.6732	<0.0001
Galectin-1	P09382	*LGALS1*	14.72	14.4375	0.0090
Glutathione S-transferase Mu 1	P09488	*GSTM1*	25.71	12.5573	0.0008
Prostaglandin E synthase 3	Q15185	*PTGES3*	18.70	7.7244	0.0179
Glutathione S-transferase omega-1	P78417	*GSTO1*	27.57	7.1425	0.0011
Cofilin-1	P23528	*CFL1*	18.50	6.2181	0.0007
Glutathione S-transferase P	P09211	*GSTP1*	23.36	5.6288	0.0006
Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	P36969	*GPX4*	22.17	5.1108	0.0020
GTP cyclohydrolase 1 feedback regulatory protein	P30047	*GCHFR*	9.70	4.2613	0.0011
Peptidyl-prolyl cis-trans isomerase FKBP4	Q02790	*FKBP4*	51.80	3.7060	<0.0001
Phosphoglycerate kinase 1	P00558	*PGK1*	44.61	3.3669	<0.0001
Cytochrome P450 1B1	Q16678	*CYP1B1*	60.85	3.3535	0.0094
Retinol dehydrogenase 10	Q8IZV5	*RDH10*	38.09	3.3404	0.0006
Cullin-associated NEDD8-dissociated protein 1	Q86VP6	*CAND1*	136.37	3.1535	<0.0001
DnaJ homolog subfamily C member 17	Q9NVM6	*DNAJC17*	34.69	2.8656	0.0010
DNA damage-binding protein 1	Q16531	*DDB1*	126.97	2.7172	0.0007
Ankyrin repeat and MYND domain-containing protein 2	Q8IV38	*ANKMY2*	49.30	2.6630	0.0045
Transcription elongation factor B polypeptide 2	Q15370	*TCEB2*	13.13	2.6482	0.0372
COP9 signalosome complex subunit 5	Q92905	*COPS5*	37.58	2.4784	0.0080
Signal transducer and activator of transcription 1-alpha/beta	P42224	*STAT1*	87.33	2.2667	0.0007
[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1, mitochondrial	Q15118	*PDK1*	49.24	2.1874	0.0011
Solute carrier family 2, facilitated glucose transporter member 3	P11169	*SLC2A3*	53.92	2.1793	0.0023
Heat shock protein HSP 90-beta	P08238	*HSP90AB1*	83.26	2.1300	<0.0001
Peroxisomal targeting signal 1 receptor	P50542	*PEX5*	70.86	2.0575	0.0078
Catechol *O*-methyltransferase	P21964	*COMT*	30.04	2.0433	0.0215

exposure of human breast cancer *in vitro* cell lines and *in vivo* animal models to environmental pollutants such as TCDD and 7,12-dimethylbenzathracene (DMBA) causes activation of cancer stemness markers, chemoresistance mediated proteins, and anti-apoptotic pathways [26]. In addition, a recent study from our laboratory has shown that AhR induction by TCDD promoted OC proliferation and chemoresistance via AKT and EMT pathways [13]. Being a major pathway involved in the metabolism of these compounds, exploring what proteins are targeted is thus crucial to better understanding the role and involvement of AhR in cancer initiation. For this purpose, a mass spectrometry-based label-free proteomics study was conducted to explore the proteomic profiles of OC cell line A2780 exposed to TCDD, aiming to identify a) a novel biomarker for cancer intervention and treatment and b) cancer-favoring mechanisms that could probably be correlated to AhR activation.

Table 2
List of 20 most significantly downregulated proteins upon AhR induction in A2780 cells.

Protein name	Protein ID	Gene name	MW (kDa)	Fold Change	Adjusted *p*-value
NAD-dependent protein deacetylase sirtuin-1	Q96EB6	*SIRT1*	81.68	0.0981	<0.0001
CCR4-NOT transcription complex subunit 2	Q9NZN8	*CNOT2*	59.74	0.1099	0.0006
Retinoblastoma-associated protein	P06400	*RB1*	106.16	0.1988	0.0026
Transformation/ transcription domain-associated protein	Q9Y4A5	*TRRAP*	437.60	0.2277	0.0027
Transcription elongation factor B polypeptide 1	Q15369	*TCEB1*	12.47	0.2508	0.0005
Histone acetyltransferase p300	Q09472	*EP300*	264.16	0.2628	<0.0001
Autophagy-related protein 9 A	Q7Z3C6	*ATG9A*	94.45	0.2698	0.0088
Dual specificity mitogen-activated protein kinase kinase 3	P46734	*MAP2K3*	39.32	0.2719	0.0024
Aryl hydrocarbon receptor nuclear translocator	P27540	*ARNT*	86.64	0.3035	0.0110
Mitogen-activated protein kinase 3	P27361	*MAPK3*	43.14	0.3364	0.0117
Melanoma-associated antigen D2	Q9UNF1	*MAGED2*	64.95	0.3420	0.0008
Transforming acidic coiled-coil-containing protein 3	Q9Y6A5	*TACC3*	90.36	0.3514	0.0011
Cullin-4B	Q13620	*CUL4B*	103.98	0.3818	0.0007
Eukaryotic translation initiation factor 5B	O60841	*EIF5B*	138.83	0.3833	0.0011
Transcription initiation factor TFIID subunit 10	Q12962	*TAF10*	21.71	0.3924	0.0008
Nuclear RNA export factor 1	Q9UBU9	*NXF1*	70.18	0.4080	0.0009
Rho-related GTP-binding protein RhoG	P84095	*RHOG*	21.31	0.4099	0.0007
Glutamate–cysteine ligase catalytic subunit	P48506	*GCLC*	72.77	0.4125	0.0101
Regulator of microtubule dynamics protein 3	Q96TC7	*RMDN3*	52.12	0.4340	0.0283
Disintegrin and metalloproteinase domain-containing protein 17	P78536	*ADAM17*	93.02	0.4590	0.0035

Proteomic profiling identified 2598 proteins in A2780 cells that were differentially regulated, in which 2290 unique proteins, representing 88% of the total proteins, overlapped in both control and TCDD-treated samples. Among these proteins, 795 were significantly upregulated, whereas 611 were significantly downregulated. We used a label-free quantification approach to identify the dysregulated proteins. Functional enrichment analysis was performed to annotate these dysregulated proteins to identify their involvement in biological processes and molecular functions. Functionally, these dysregulated proteins were found to be majorly involved in cancer-related pathways, chemical/viral carcinogenesis, glutathione metabolism, metabolism of xenobiotics by cytochrome P450, nucleotide excision repair, drug metabolism, and platinum drug resistance.

Interactions between different proteins can be functionally linked to or inferred from the associations between the genes encoding them. STRING is a precomputed predictive database used to identify these associations or interactions between proteins against a common gene reference set integrated into a single confidence score per prediction, providing a higher view of functional linkage (http://www.bork.embl-heidelberg.de/STRING/) [24]. The STRING analysis identified 20 significantly dysregulated proteins potentially interacting with AhR and its downstream effectors ARNT and CYP1B1, which are functionally involved in cancer regulation, chemoresistance, cancer progression, invasion and metastasis, prognosis and survival, and apoptosis.

Proteins that play a role in chemoresistance and are found to be upregulated in response to the AhR activator are GSTM1, GSTP1, GSTO1, ATG9A, DDB1, and PEX5. GSTMI, for example, is a glutathione S-transferase (GST) involved in detoxifying metabolites of environmental carcinogens [27]. A comprehensive analysis by Zhang et al. reported that GSTM1 was negatively correlated with OC prognosis and associated with OC chemoresistance and immune escape [28]. GSTP1 is unambiguously reported to be a multidrug-resistant-related gene involved in carcinogenesis and chemoresistance *in vivo* and *in vitro* [29,30]. Moreover, it was reported that GSTP1 knockdown mediated a significant reduction in cell invasion and migration in response to cisplatin and carboplatin [31]. GST omega 1 (GSTO1) is known to be overexpressed in several cancers, and its expression was significantly correlated to chemoresistance [32,33] and cancer progression [34]. In this context, GSTO1 inhibitors have been shown to sensitize cancer cells to cisplatin [35,36]. Taken together, the chemoresistant effect exerted by A2780cis cells could be attributed to the expression of these proteins.

Another protein that is dysregulated upon AhR activation is ATG9A. ATG9A is one of the autophagy markers whose expression is higher in the later stages of OC than in the early stages. Autophagy is an evolutionarily conserved cellular self-digestion mechanism [37] that is extensively studied for its role in different diseases, including cancer [38]. Overexpression of ATG9A was found to be an independent poor prognostic predictor. Furthermore, higher expression of ATG9A is also reported to have a negative impact on overall survival and progression-free survival in OC [39] and oral squamous cell carcinoma [40]. Though the regulation of autophagy by AhR is already reported in triple negative breast cancer cells [41] and in non-small cell lung cancer cells [42], to our knowledge, this is the first report that showcases the mediating effect of AhR induction on autophagy in OC.

DNA Damage Binding Protein Component (DDB1) is another protein modulated in response to the AhR activation. DDB1 is a crucial protein involved in the nuclear excision repair mechanism [43], and a tumor-promoting factor in pancreatic cancer. It plays a role in chemoresistance in pancreatic ductal adenocarcinoma (PDAC) and might serve as a potential predictive marker and therapeutic target for PDAC treatment [44]. However, DDB1 is not a prognostic marker in OC. Though the interaction between AhR and DDB1 has already been reported [45], it was unidentified in OC until now.

The peroxisomal biogenesis factor 5 (PEX5) protein functions as a receptor for transporting peroxisomal matrix proteins in peroxisomes upon intercellular stress to modulate redox homeostasis, and it is predominantly involved in peroxisomal protein import [46]. Peroxisomes mediate the metabolism of reactive oxygen species and have been associated with aberrant metabolic processes in cancer [47]. In addition, PEX5 is reported to induce radioresistance by activating the Wnt/β-catenin pathway in Hepatocellular carcinoma [48]. However, its role in OC remains unidentified.

Several proteins related to cancer progression were found to be dysregulated upon AhR activation. For example, we report that CYPIBI and Galectin-1 are upregulated in OC cells. Higher expression of CYP1B1, that mediates many procarcinogen metabolic bioactivations [49], is observed in multiple malignant tumors, including brain, breast, colon, ovarian, and prostate cancers [26]. Using *in vivo* and *in vitro* models, it is reported that CYP1B1 improved the resistance of epithelial ovarian cancer cells to paclitaxel [50]. On the other hand, Galectin-1 is a

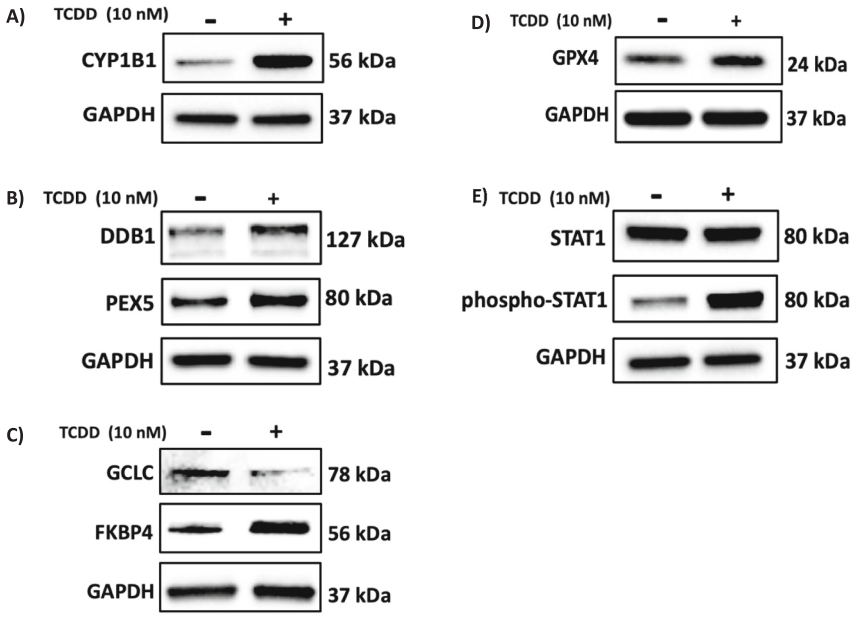

Fig. 5. Validation of significantly dysregulated proteins upon AhR activation in A2780 cells. Protein expression levels of **A)** CYP1B1, **B)** DDB1 and PEX5, **C)** GCLC and FKBP4, **D)** GPX4, and **E)** STAT1 and p-STAT1 were determined by Western blot analysis, GAPDH served as the loading control.

soluble small molecular weight carbohydrate-binding protein that is contributing to OC's invasive, metastatic, chemoresistant, and immunosuppressive properties [51]. It is a prognostic marker for survival in Epithelial OC [52] and a more sensitive predictive biomarker of OC than serum CA-125, which is the most commonly used predictive biomarker for OC [53,54].

On the other hand, several proteins were significantly downregulated in OC upon AhR activation, such as Retinoblastoma transcriptional corepressor 1(RB-1) and ARNT. *RB-1* is a tumor suppressor gene that promotes cancer immunity and regulates cellular activities such as cell progression, differentiation, and DNA damage response [55]. However, the inactivation of RB-1, TP53, and BRCA1/2 in the OSE resulted in the development of metastatic ovarian tumors with HGSC phenotype [56]. However, the interaction between AhR and RB-1 has been previously reported in pituitary adenoma [57]. Our study is the first to report a correlation between AhR and RB-1 in OC. In addition, the downregulation of ARNT could promote cancer metastasis [58], suggesting that ARNT could be potential targets for OC treatment [59].

Our data show that proteins involved in cancer metastasis and invasion were dysregulated. Cofilin, an actin-binding protein that regulates filament dynamics and depolymerization was upregulated after AhR induction [60]. Studies show that cofilin promotes metastasis in cancer cells by regulating EMT [60], and we have already shown that AhR induction promotes EMT. Another significantly downregulated protein in our analysis is EP300, a tumor suppressor gene. Asaduzzaman et al. reported that breast cancer cells lacking EP300 became more resistant to paclitaxel, whereas EP300 overexpression increased their sensitivity to the drug [61].

In our study, several genes associated with prognosis and survival were found to be dysregulated upon AhR induction. These genes include *ANKMY2*, *GCLC*, *HSP90AB1*, and *FKBP4*. *ANKMY2* is a positive regulator of Sonic Hedgehog signal transduction [62]. GCLC is an enzyme involved in glutathione synthesis and was previously reported to play a role in tumor progression and drug resistance. Its role in prognosis has been reported by Sun et al. in Hepatocellular carcinoma [63]. FKBP4 is a co-chaperone molecule involved in the intracellular trafficking of heterooligomeric forms of steroid hormone receptors between the cytoplasm and nuclear compartments. The expression of FKBP4 was highly correlated to tumor size, lymph node metastasis, and patient prognosis in NSCLC patients [64]. HSP90AB1 is a molecular chaperone that stabilizes the functioning of its client proteins, most of which promote cancer cell growth and survival [65]. The role of HSP90AB1 in promoting EMT is also reported in gastric cancer [66]. This study from Wang et al. also reported that targeting HSP90AB1 inhibited cell migration and tumor growth [66]. Another crucial protein that we identified is Glutathione peroxidase 4 (GPX4), a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation and is one of the most important anti-oxidant enzymes [67]. Silencing GPX4 significantly inhibited proliferation and migration ability, induced apoptosis, and arrested cell cycle in endometrial adenocarcinoma cells [68]. In OC cells, the knockdown of GPX4 is believed to interfere with iron metabolism, thereby facilitating cell death [69].

In conclusion, our data suggest that AhR regulates carcinogenesis in OC cells. Several proteins involved in chemoresistance, cancer progression, invasion, metastasis, apoptosis, prognosis, and survival were dysregulated upon AhR activation (Fig. 6). This study further confirms our previous study that reported the cancer-promoting role of AhR in OC. Therefore, we propose that AhR could be a potential therapeutic target for OC prevention and maintenance.

CRediT authorship contribution statement

Lubna Therachiyil: Formal analysis, Investigation, Resources, Software, Writing – original draft, Visualization. **Paleerath Peerapen:** Formal analysis, Investigation, Software. **Shahad Younis:** Formal analysis, Investigation, Software. **Aamir Ahmad:** Formal analysis, Investigation, Writing-review & editing. **Visith Thongboonkerd:** Formal analysis, Writing – review & editing. **Shahab Uddin:** Investigation, Supervision, Validation, Writing – original draft. **Hesham M. Korashy:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We hereby confirm that this is an original work of ours, and neither this work nor a part of it has been submitted or published in any primary scientific journals elsewhere. All the authors are aware of and agree to

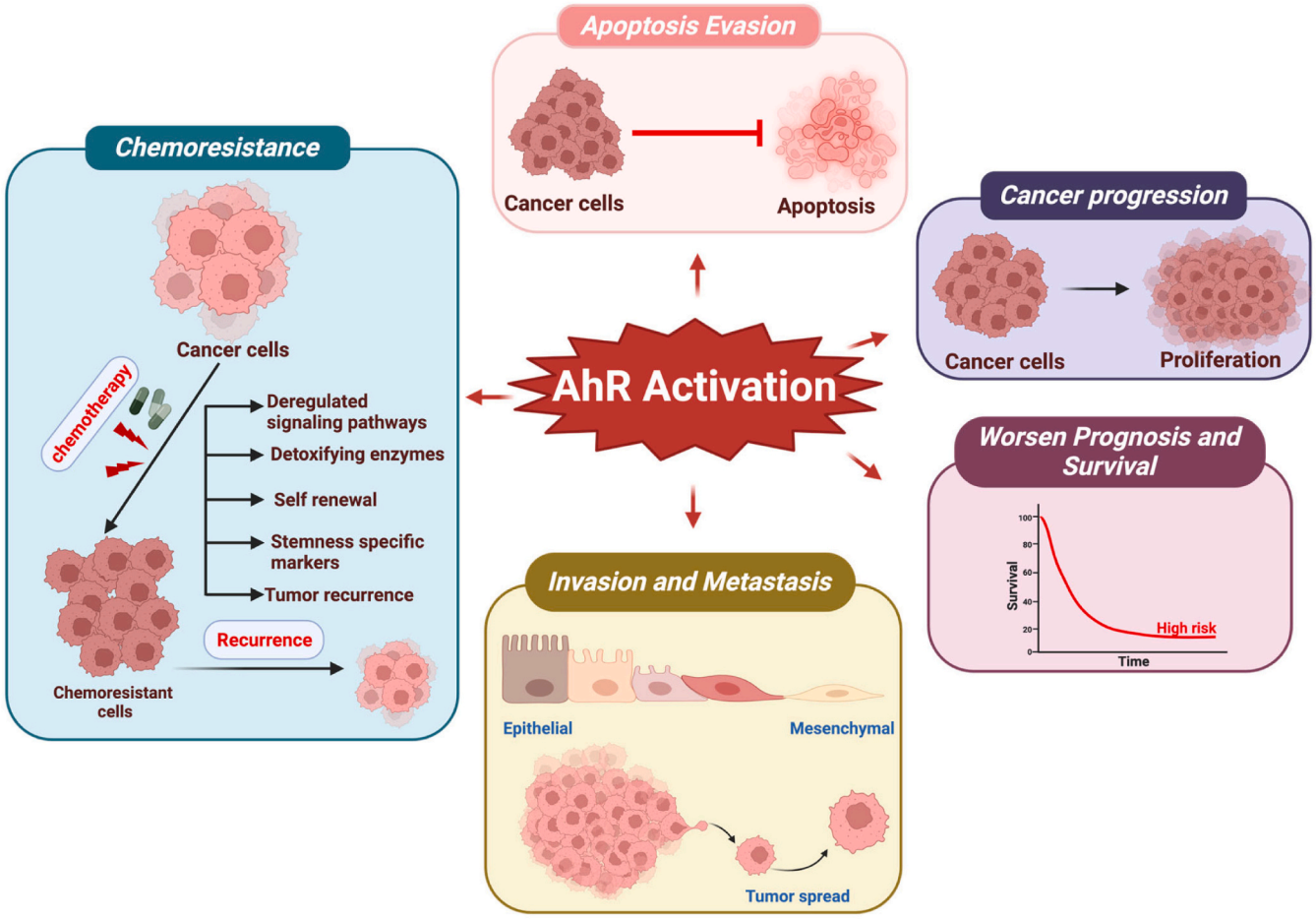

Fig. 6. Illustrative summary of the proposed effects and mechanisms of AhR activation onprogression and chemoresistance in OC.

the article's content and are listed as authors. The authors have no conflicts of interest to disclose.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2024.105108.

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