

# *In vitro* analysis of ovarian cancer response to cisplatin, carboplatin, and paclitaxel identifies common pathways that are also associated with overall patient survival

E Bicaku<sup>1,2</sup>, Y Xiong<sup>1,2</sup>, DC Marchion<sup>1,2</sup>, HS Chon<sup>1</sup>, XB Stickle<sup>1</sup>, N Chen<sup>1</sup>, PL Judson<sup>1,3</sup>, A Hakam<sup>3,4</sup>, J Gonzalez-Bosquet<sup>1,3</sup>, RM Wenham<sup>1,2,3</sup>, SM Apte<sup>1,3</sup>, W Fulp<sup>5</sup>, CL Cubitt<sup>6</sup>, D-T Chen<sup>5</sup> and JM Lancaster<sup>\*,1,2,3</sup>

<sup>1</sup>Department of Women's Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA; <sup>2</sup>Experimental Therapeutics Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA; <sup>3</sup>Department of Oncologic Sciences, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA; <sup>4</sup>Department of Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA; <sup>5</sup>Biostatistics Core, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA; <sup>6</sup>Translational Research Core, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA

**BACKGROUND:** Carboplatin and cisplatin, alone or in combination with paclitaxel, have similar efficacies against ovarian cancer (OVCA) yet exhibit different toxicity profiles. We characterised the common and unique cellular pathways that underlie OVCA response to these drugs and analyse whether they have a role in OVCA survival.

**METHODS:** Ovarian cancer cell lines ( $n = 36$ ) were treated with carboplatin, cisplatin, paclitaxel, or carboplatin–paclitaxel (CPTX). For each cell line, IC<sub>50</sub> levels were quantified and pre-treatment gene expression analyses were performed. Genes demonstrating expression/IC<sub>50</sub> correlations (measured by Pearson;  $P < 0.01$ ) were subjected to biological pathway analysis. An independent OVCA clinico-genomic data set ( $n = 142$ ) was evaluated for clinical features associated with represented pathways.

**RESULTS:** Cell line sensitivity to carboplatin, cisplatin, paclitaxel, and CPTX was associated with the expression of 77, 68, 64, and 25 biological pathways ( $P < 0.01$ ), respectively. We found three common pathways when drug combinations were compared. Expression of one pathway ('Transcription/CREB pathway') was associated with OVCA overall survival.

**CONCLUSION:** The identification of the Transcription/CREB pathway (associated with OVCA cell line platinum sensitivity and overall survival) could improve patient stratification for treatment with current therapies and the rational selection of future OVCA therapy agents targeted to these pathways.

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The American Cancer Society ranks ovarian cancer (OVCA) as the fifth leading cause of cancer mortality in women, accounting for more deaths than any other gynaecologic cancer. In 2010 in the United States alone, approximately 22 000 women had been predicted to be diagnosed with OVCA, with an estimated 14 000 women dying of their disease (Jemal *et al*, 2010). This high mortality is attributed not only to the advanced stage (III/IV) at diagnosis observed for most patients (Herrin and Thigpen, 1999) but also to limited treatment options available for patients who develop acquired resistance to chemotherapy.

After cytoreductive surgery, most patients with advanced-stage disease receive systemic treatment with a platinum plus/minus taxane-based regimen. Clinical trials performed in the 1980s and 1990s established the platinum compounds to be superior to alkylating agents, either alone or in combination with other non-platinum drugs (Omura *et al*, 1986). Cisplatin and carboplatin do not differ significantly in their efficacy or effect on survival but

do have markedly different side effects (Ozols *et al*, 2003). Paclitaxel, a compound extracted from Pacific yew tree bark, has been used as an antineoplastic agent since the 1980s and has been shown to improve OVCA progression-free and overall survival when combined with cisplatin or carboplatin in first-line therapy (McGuire *et al*, 1996). Randomised clinical trials comparing the combination of paclitaxel with either cisplatin or with carboplatin in patients with advanced-stage OVCA found no significant differences in efficacy between the two combination treatments, although there was generally an improved tolerability for the carboplatin-containing regimen (Neijt *et al*, 2000; du Bois *et al*, 2003; Ozols *et al*, 2003). Despite substantial clinical data demonstrating the superiority of platinum-based regimens vs non-platinum regimens, the non-inferiority of carboplatin over cisplatin, and the benefit of paclitaxel combined with platinum-based regimens, the molecular basis to OVCA sensitivity to these drugs remains to be fully characterised.

The objectives of this study were to characterise the biological signalling pathways that underlie response to standard of care therapy and to further determine the influence of these pathways on overall survival from OVCA. To accomplish these objectives, we integrated chemo-sensitivity data from OVCA cell lines treated

\*Correspondence: Dr JM Lancaster;

E-mail: Johnathan.Lancaster@moffitt.org.

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with cisplatin, carboplatin, and carboplatin plus paclitaxel with genome-wide expression data to provide insights into the unique and common signalling pathways that may comprise the biological basis of OVCA response to primary therapy agents. Although it is recognised that platinum-based agents induce cell death via initial mechanisms (and molecular pathways) that are distinct from those of the taxanes, it is also accepted that the two groups of drugs likely share a number of final biological and molecular pathways associated with the apoptotic process (Fisher, 1994; Eguchi *et al*, 1997; Zhan *et al*, 1999; Pestell *et al*, 2000; Wang *et al*, 2000), thus underlying the assumptions for this study. The identification of unique and shared determinants of drug response in OVCA has the potential to improve our understanding of OVCA chemo-response and also the stratification of patients for treatment with current therapies and the rational selection of agents targeted to these pathways for future treatment of OVCA.

## MATERIALS AND METHODS

### Cell culture

Ovarian cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) (CAOV3, OV90, OVCAR3, SKOV3, TOV112D); from the European Collection of Cell Cultures, Salisbury, UK (A2780CP, A2780S); or from Kyoto University, Kyoto, Japan (CHI, CHICisR, M41, M41CSR, Tyknu, and TyknuCisR) or were kind gifts from Dr Patricia Kruk, Department of Pathology, College of Medicine, University of South Florida, Tampa, FL, USA, and Susan Murphy, PhD, Department of OBGYN/Division of GYN Oncology, Duke University, Durham, NC, USA (A2008, C13, CAOV2, FUOV1, HeyA8, IGR-OV1, IMCC3, IMCC5, MCAS, OV2008, OVCA420, OVCA429, OVCA432, OVCA433, OVCAR4, OVCAR5, OVCAR8, Dov13, BG1, Ovary1847, OVCAR10, OVCAR2, SK-OV-4).

Cell lines were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA, USA), 1% sodium pyruvate, 1% penicillin/streptomycin (Cellgro, Manassas, VA, USA), and 1% nonessential amino acids (HyClone, Hudson, NH, USA). Mycoplasma testing was performed every 6 months following manufacturer's protocol (Lonza, Rockland, ME, USA).

### RNA extraction and microarray expression analysis

RNA from 36 OVCA cell lines was extracted using RNeasy kit following manufacturer's recommendations (Qiagen, Valencia, CA, USA). Quality of the RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The targets for Affymetrix DNA microarray analysis were prepared according to the manufacturer's instructions, and targets were hybridised to customised Human Affymetrix HuRSTA gene chips (HuRSTA-2a520709), which include 60 607 probe sets and representation of 19 308 genes (Gene Expression Omnibus accession number GSE34615).

### CellTiter-blue cell viability assays

Drug activity was evaluated using a high-throughput CellTiter-Blue cell viability assay. Cells ( $2.5 \times 10^3$  per well) were plated in 384-well plates using complete media with 10% fetal bovine serum and allowed to adhere overnight. After cell adherence, increasing concentrations of cisplatin, carboplatin (Sigma-Aldrich, St Louis, MO, USA), paclitaxel (Sequoia Research Products Ltd., Pangbourne, UK), or carboplatin plus paclitaxel (carbotaxol: constant molar ratio of carboplatin to paclitaxel of 20 000 : 1) were added to appropriate wells using an automated pipetting station. Four replicate wells were used for each drug concentration and vehicle controls. Drug dilutions initially consisted of 1.5-fold serial

dilutions from a maximum concentration of 100  $\mu\text{M}$ . The cells were incubated with the drug for 72 h to ensure all cell lines underwent a minimum of two doublings. After drug incubation, 5  $\mu\text{l}$  of CellTiter-Blue reagent (Promega Corp., Madison, WI, USA) was added to each well. Fluorescence was read at 579 nm excitation/584 nm emission using a Synergy 4 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). IC<sub>50</sub> results were determined using a sigmoidal equilibrium model fit (XLfit 5.2, ID Business Solutions Ltd., Guildford, Surrey, UK). IC<sub>50</sub> was defined as the concentration of drug required for a 50% reduction in growth/viability.

### Statistical analysis

Expression data from 36 OVCA cell lines were subjected to background correction and normalisation using the Robust Multichip Average algorithm in the Affymetrix Expression Console (<http://www.affymetrix.com>). Pearson correlation test was performed on individual gene expression values and IC<sub>50</sub> results. Probe sets with  $P < 0.01$  were considered to have significant correlations with IC<sub>50</sub> results and were uploaded to GeneGo MetaCore for pathway analysis (<http://www.genego.com/metacore.php>). Pathways with  $P < 0.05$  were considered significant, based on the GeneGo MetaCore statistical test for significance.

### Building signatures of pathway activity

The principal component analysis methodology was used to derive a pathway gene expression signature with a corresponding 'pathway score' to represent an overall gene expression level for the pathways of interest. First, data were reduced into a small set of uncorrelated principal components. This set of principal components was generated based on its ability to account for variation. The first principal component analysis is used to represent the overall expression level for the pathway, as it accounts for the largest variability in the data. That is, pathway score is equal to  $\sum w_i x_i$ , a weighted average expression among the BAD pathway genes, where  $x_i$  represents gene  $i$  expression level,  $w_i$  is the corresponding weight (loading coefficient) with  $\sum w_i^2 = 1$ , and the  $w_i$  values maximise the variance of  $\sum w_i x_i$ .

### Validation of signatures in primary OVCA data sets

The pathway gene expression signature scores developed in OVCA cell lines were evaluated in an independent clinico-genomic data set from 142 advanced-stage (III/IV) serous OVCA samples as previously described (Marchion *et al*, 2011). Gene expression data and the corresponding clinical parameters for these 142 OVCA samples are publicly available at [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo), accession number GSE23554. In brief, all 142 patients signed the IRB-approved, written informed consent forms, were known to have advanced-stage (III/IV) serous epithelial OVCA, and underwent primary cytoreductive surgery followed by primary therapy with a platinum-based regimen (with or without taxane or cyclophosphamide). Additional details are provided in the Supplementary Data section. Log-rank tests with Kaplan-Meier survival curves were used to test any association between the pathway scores ('high' vs 'low' based on a median value cutoff) and overall survival for patients with OVCA. None of the data from the 142 OVCA samples was used to identify the principal component analysis signatures; the OVCA ovarian genomic data made up a completely independent evaluation set.

### CREB pathway analysis

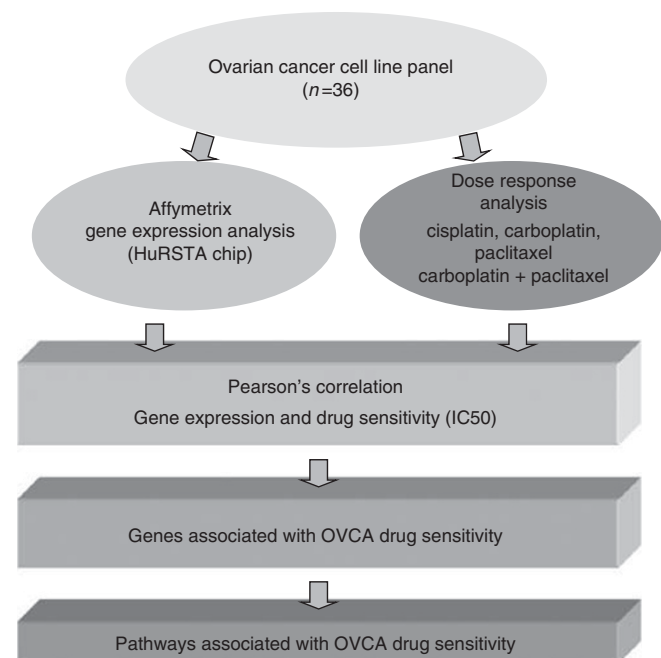
To further elucidate the biological basis to the association between CREB pathway expression and overall survival from OVCA, we performed a series of *in silico* analyses, including Pearson's correlation between growth doubling times for the NCI60 human

cancer cell lines (and CREB pathway expression, quantified by CREB PC1 score). Affymetrix HG-U133A expression and doubling time data for the 60 NCI cancer cell lines (6 leukaemia, 9 melanoma, 9 non-small cell lung, 7 colon, 6 central nervous system, 7 ovarian, 8 renal, 2 prostate, and 6 breast cancer cell lines) were obtained from NCI web sites (<http://discover.nci.nih.gov/cellminer/loadDownload.do> and [http://dtp.nci.nih.gov/docs/misc/common\\_files/cell\\_list.html](http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html)).

In light of the ability of the CREB pathway to influence transcription of other genes and molecular pathways, we evaluated differences in genome-wide expression in OVCAs that demonstrate high *vs* low CREB pathway expression. Using genomic data from 142 advanced-stage (III/IV) serous OVCAs (described above and previously (Marchion *et al*, 2011)), we studied genomic profiles from OVCAs that demonstrated a complete response (CR) to primary therapy for evidence of molecular signalling pathways that may differentially expressed in OVCAs that harbour high *vs* low expression levels of the CREB pathway (based on the median score threshold to define high *vs* low). We performed a SAM *t*-test analysis to identify differentially expressed genes between 'CR high-CREB' *vs* 'CR low-CREB' OVCAs. Differentially expressed genes (FDR = 0) were subjected to GeneGo pathway analysis. In a preliminary analysis, using OVCA data from 23 patients for whom disease-free interval (DFI) information is available, we compared DFI for patients with low and high CREB PC1 scores (median score used as threshold).

## RESULTS

The workflow for this study is summarised in Figure 1. The IC<sub>50</sub> values for 36 OVCA cell lines treated with increasing doses of



**Figure 1** Experimental design flow chart. Gene expression analysis was performed on a panel of ovarian cancer cell lines ( $n = 36$ ) using the custom Affymetrix HuRSTA chip. Each cell line was treated with increasing doses of carboplatin, cisplatin, paclitaxel, or carboplatin plus paclitaxel, and IC<sub>50</sub> values were quantified using CellTiter-Blue cell viability assays. Pearson correlation coefficients were calculated for expression data and drug IC<sub>50</sub> values. Genes associated with OVCA sensitivity (demonstrating expression/IC<sub>50</sub> correlations,  $P < 0.01$ ) were subjected to GeneGo Metacore Pathway analysis.

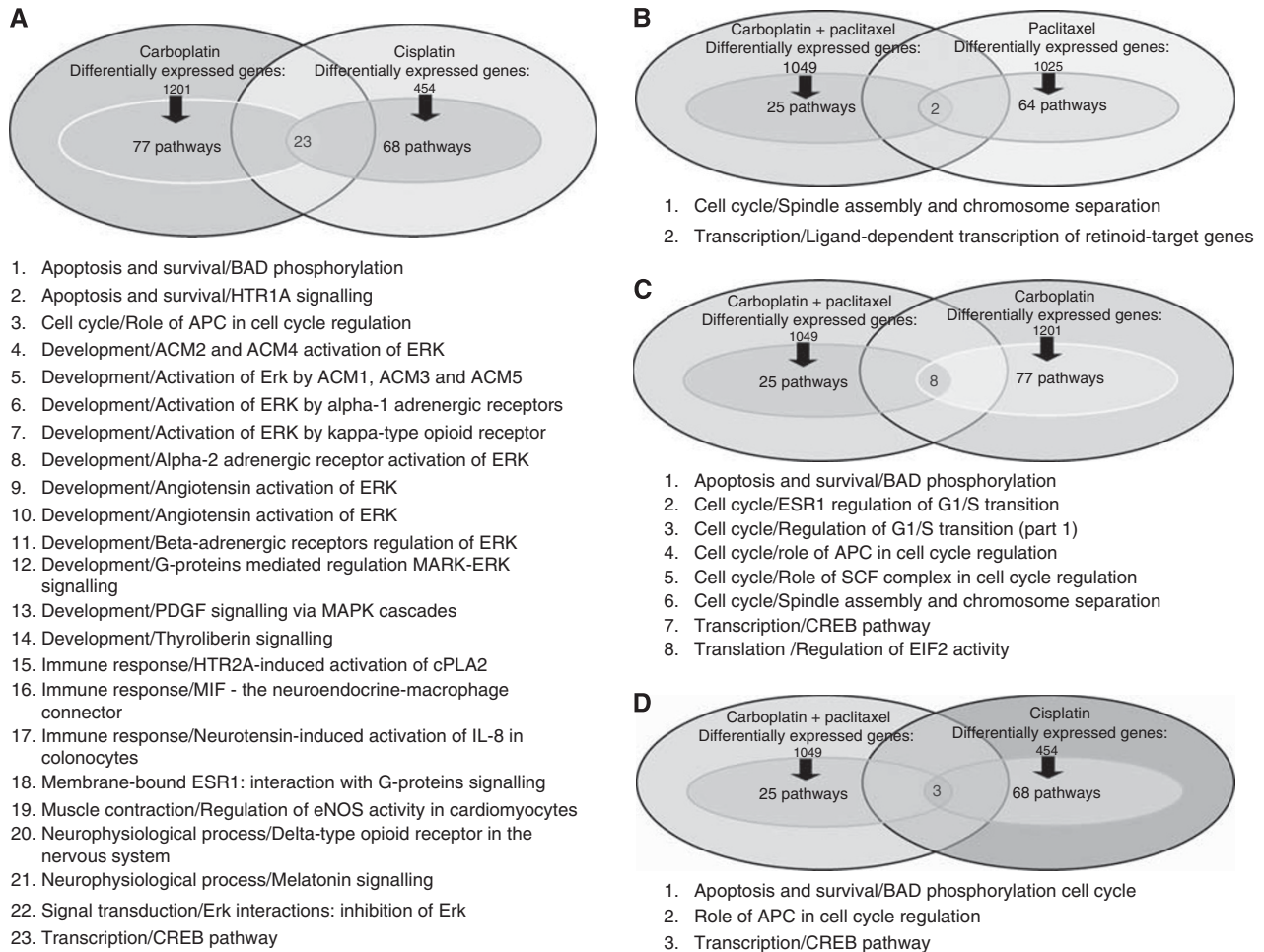
carboplatin, cisplatin, paclitaxel, and carboplatin plus paclitaxel treatment (Supplementary Table 1) were correlated with gene expression data for each drug (Pearson correlation coefficient,  $P < 0.01$ ). Treatment of OVCA cell lines with carboplatin revealed 1201 genes whose differential expression correlated with carboplatin sensitivity ( $P < 0.01$ ). This gene list corresponded to 77 biological pathways identified by GeneGo MetaCore analysis (Figure 2A). Cisplatin sensitivity correlated with the expression of 454 unique genes ( $P < 0.01$ ), corresponding to 68 pathways. Twenty-three of these pathways were also correlated with carboplatin sensitivity (Figure 2A). Treatment with paclitaxel revealed 1025 genes associated with OVCA paclitaxel sensitivity ( $P < 0.01$ ), representing 64 biological pathways (Figure 2B). Pearson correlation of the IC<sub>50</sub> values and gene expression data for the OVCA cell lines treated with a combination of carboplatin and paclitaxel (20 000:1 molar ratio) revealed 1049 differentially expressed genes ( $P < 0.01$ ) and 25 pathways (Figure 2B–D).

To distinguish between pathways that are unique to the mechanism of action of each drug and pathways that are common determinants of OVCA sensitivity, we identified pathways containing genes that were correlated with sensitivity to multiple drugs. Of the 25 pathways linked to sensitivity to carboplatin–paclitaxel combination therapy and the 68 pathways linked to cisplatin sensitivity, only 3 were identified in both analyses: 'Apoptosis and Survival/BAD Phosphorylation', 'Role of APC in Cell Cycle Regulation' (where APC is anaphase-promoting complex) and 'Transcription/CREB' (Figure 2D). Eight pathways were specifically correlated with carboplatin sensitivity, regardless of whether paclitaxel was used (Figure 2C). Two pathways were specifically correlated with sensitivity to paclitaxel treatment in both the presence and absence of carboplatin (Figure 2B).

To further distinguish core pathways involved in the response to chemotherapeutic agents in OVCA, we analysed the union of three individual comparisons: carboplatin sensitivity *vs* cisplatin sensitivity, carboplatin–paclitaxel sensitivity *vs* cisplatin sensitivity, and carboplatin–paclitaxel sensitivity *vs* carboplatin sensitivity. We identified three pathways with a shared role in OVCA sensitivity to carboplatin and cisplatin single-agent treatment and carboplatin and paclitaxel combination treatment: 1) 'Apoptosis and Survival/BAD Phosphorylation' 2) 'Role of APC in Cell Cycle Regulation,' and 3) 'Transcription/CREB' (Figure 3). Diagrams of the genes/proteins involved in these pathways are provided in Figure 3 (GeneGo MetaCore output: September 29, 2010).

### Expression of the Transcription/CREB pathway is associated with OVCA clinical outcome

Based on the above data, we utilised principal component analysis to develop expression signatures for both the Role of APC in Cell Cycle Regulation and the Transcription/CREB pathways. We have previously reported on an Apoptosis and Survival/BAD Phosphorylation pathway signature and its associations with chemosensitivity and overall survival (Marchion *et al*, 2011). A 55-gene APC in Cell Cycle Regulation pathway signature and a 103-gene Transcription/CREB pathway signature were generated and evaluated in an independent OVCA genomic data set (Supplementary Tables 2 and 3). Expression of the Transcription/CREB pathway gene signature was associated with overall survival from OVCA ( $n = 142$ ,  $P \leq 0.0001$ ; Figure 4A). Furthermore, the OVCA genomic data set was evaluated with regard to Transcription/CREB pathway gene expression signature score and surgical cytoreductive (debulking,  $n = 141$ , with debulking status unavailable for 1 of 142 patients) status (optimal:  $< 1$  cm; suboptimal:  $> 1$  cm residual tumour at conclusion of surgery,  $P < 0.001$ ; Figure 4C) and also response to primary platinum-based therapy (complete or incomplete response,  $P < 0.0001$ ; Figure 4B). An association between low Transcription/CREB pathway gene expression signature score and favourable outcome was observed



**Figure 2** Shared biological pathway activation between chemotherapeutic agent treatments. The Pearson correlation test was performed to analyse the relationship between  $IC_{50}$  values and gene expression. Genes whose expression highly correlated with the  $IC_{50}$  value associated with each drug ( $P < 0.01$ ) were uploaded into the GeneGo MetaCore software to identify significantly represented pathways ( $P < 0.05$ ). **(A)** Ovarian cancer cell line sensitivity to carboplatin or cisplatin treatment was associated with 1201 and 454 genes, respectively. Genes associated with OVCA sensitivity to carboplatin and cisplatin were represented in 77 (carboplatin) and 68 (cisplatin) biological pathways. **(B)** Ovarian cancer cell line sensitivity to paclitaxel or carboplatin plus paclitaxel treatment was associated with the expression of 1025 and 1049 genes, respectively. Genes associated with OVCA sensitivity were represented in 64 (paclitaxel) and 25 (carboplatin plus paclitaxel) biological pathways. **(C)** Ovarian cancer cell line sensitivity to carboplatin plus paclitaxel treatment or carboplatin was associated with the expression of 1049 and 1201 genes, respectively. Genes associated with OVCA sensitivity were represented in 25 (carboplatin plus paclitaxel) and 77 (carboplatin) biological pathways. **(D)** Ovarian cancer cell line sensitivity to carboplatin plus paclitaxel treatment or cisplatin treatment was associated with the expression of 1049 and 454 genes, respectively. Genes associated with OVCA sensitivity were represented in 25 (carboplatin plus paclitaxel) and 68 (cisplatin) biological pathways.

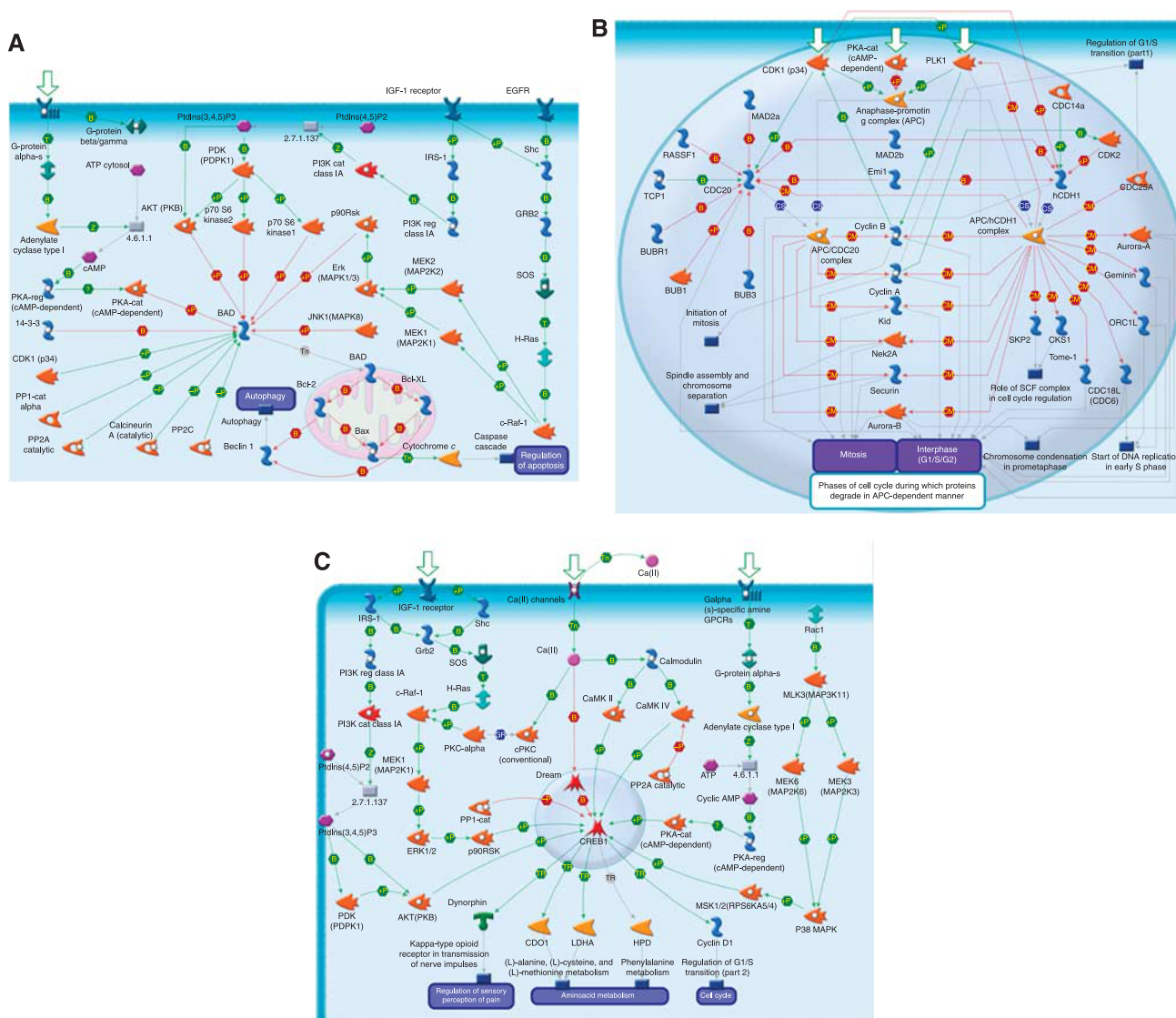
in patients who underwent optimal and suboptimal debulking (optimal: adjusted  $P = 0.002$ , suboptimal: adjusted  $P = 0.02$ ). Most importantly, OVCA patients with a low Transcription/CREB pathway gene expression signature score who underwent suboptimal debulking had survival data that trended toward superiority vs patients with a high Transcription/CREB pathway gene expression signature score who underwent optimal debulking (adjusted  $P = 0.08$ ). Interestingly, patients who demonstrated a complete response to primary platinum-based therapy but had a low Transcription/CREB pathway gene expression signature score had notably superior survival than patients who demonstrated a complete response but had a high Transcription/CREB pathway gene expression signature score (adjusted  $P = 0.0001$ ). Patients who had an incomplete response to primary therapy had no difference in survival associated with their Transcription/CREB pathway gene expression signature score ( $P = 0.27$ ). When evaluated with debulking status and response to primary platinum-based therapy, grade, and age, the Cox proportional hazards multivariable model revealed that the Transcription/CREB

pathway gene expression signature score was an independent variable associated with survival ( $P = 0.01$ ).

The Role of APC in Cell Cycle Regulation pathway gene expression signature score demonstrated a trend toward an association with overall survival from OVCA, although results did not reach statistical significance ( $n = 142$ ,  $P = 0.1$ , Figure 4D). No association between the Role of APC in Cell Cycle Regulation pathway gene expression signature score and survival was observed within the group of patients who had optimal ( $P = 0.1384$ ) or suboptimal ( $P = 0.6192$ ) cytoreductive surgery or who had demonstrated a complete ( $P = 0.2154$ ) or incomplete ( $P = 0.6984$ ) response to primary platinum-based therapy.

### CREB pathway expression and OVCA biology

We identified a correlation between expression of the CREB pathway (quantified by CREB PC1 score) and the doubling times of 60 human cancer cell lines ([http://dtp.nci.nih.gov/docs/misc/common\\_files/cell\\_list.html](http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html)) ( $P = 0.006$ ). As such, cancer cell lines

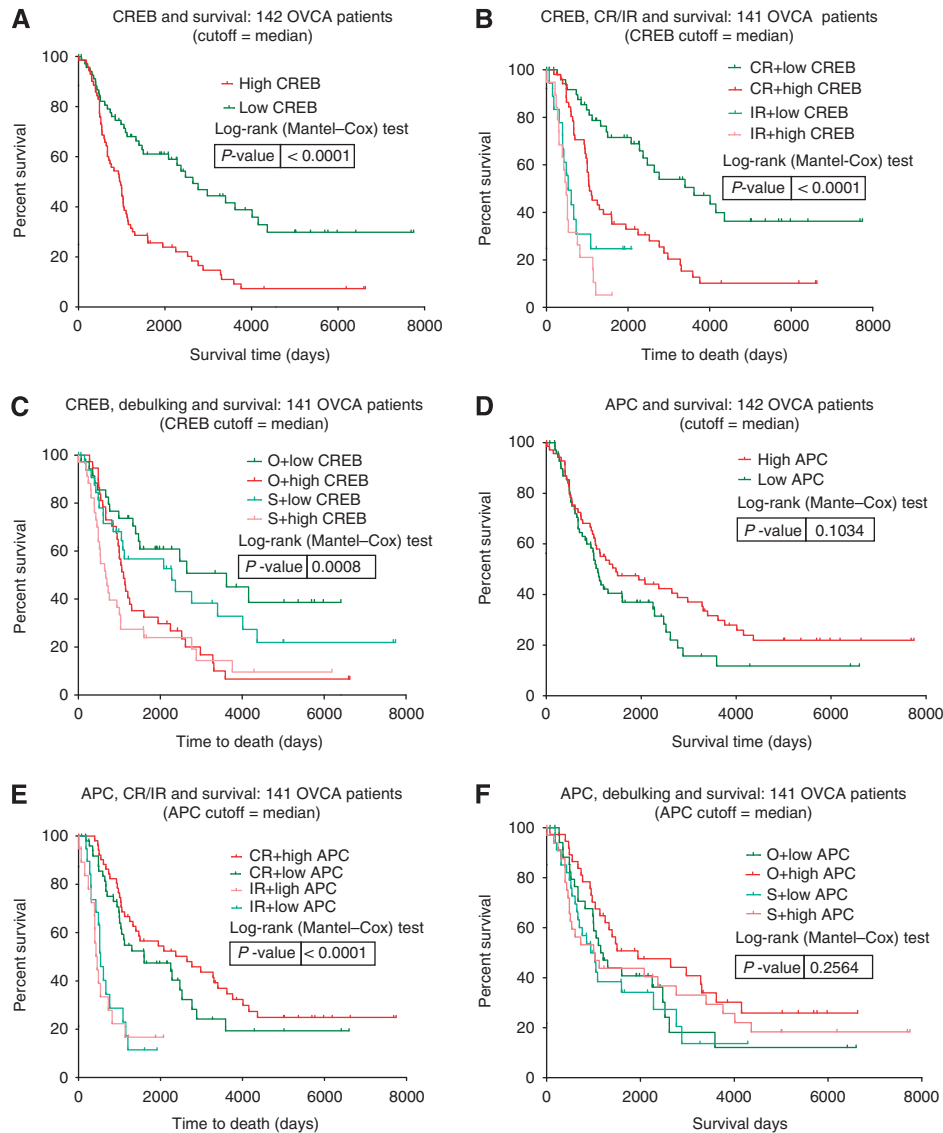


**Figure 3** Common biological pathways activated in OVCA cells in response to chemotherapeutic agents. Three biological pathways are commonly activated in an OVCA cell line panel in multiple comparisons of chemotherapeutic agent response. (A) ‘Apoptosis and Survival/BAD phosphorylation’ pathway. (B) ‘Cell Cycle/Role of APC in Cell Cycle Regulation’ pathway. (C) ‘Transcription/CREB’ pathway.

with low doubling times (using median number as a threshold) demonstrated lower CREB pathway expression (PC1 scores) than cell lines with high doubling times ( $P = 0.003$ ) (Figure 5). Furthermore, study of genomic data from patients who experienced a complete response to primary therapy revealed that OVCA with high vs low CREB pathway expression also reflect differential expression of other pathways that have influence on tumour biology. SAM *t*-test identified differential expression of 4017 probe sets (FDR = 0) between ‘CR low-CREB PC1’ and ‘CR high-CREB PC1’ score OVCA. These differentially expressed genes included representation of 292 pathways ( $P < 0.05$ ; Supplementary Table S4), including cAMP signalling ( $P = 1.0 \times 10^9$ ), CREB pathway ( $P = 1.0 \times 10^6$ ) (both expected given CREB pathway function), IP3 signalling ( $P = 1.0 \times 10^6$ ), apoptosis, survival, and BAD phosphorylation ( $P = 1.0 \times 10^6$ ), G-protein-mediated regulation of MAPK-ERK signalling ( $P = 1.0 \times 10^5$ ), and AKT signalling ( $P = 1.0 \times 10^5$ ). Finally, although the findings did not reach statistical significance, and the number of patients for whom DFI is available is low ( $n = 23$ ), we did observe a trend toward shorter DFI in patients with OVCA harbouring high CREB scores ( $P = 0.6$ , data not shown).

## DISCUSSION

Cancer cells employ a variety of strategies to deregulate apoptosis, cell cycle progression, and cellular metabolism during the course of their evolution (Hanahan and Weinberg, 2011). In this study, integration of OVCA cell line chemo-sensitivity and genome-wide expression data implicated pathways, which include BAD, APC, and CREB, as key mediators of sensitivity to chemotherapeutic agents, that, when deregulated, may contribute to the acquired drug resistance frequently observed in OVCA patients. Furthermore, when we integrated genome-wide expression results with clinical outcomes data from patients with advanced-stage (III/IV) serous OVCA, we found that these pathways are not only associated with *in vitro* drug sensitivity but also with patient survival. It is perhaps not surprising that there are both common and unique pathways that underlie OVCA platinum plus/minus taxane response; those that are associated with the differing initial mechanisms that ultimately lead to apoptosis, and those common (and potentially unique) pathways that may be associated with the apoptotic process. Paclitaxel is a microtubule-stabilising agent with potent anti-proliferative and anti-migratory activity for

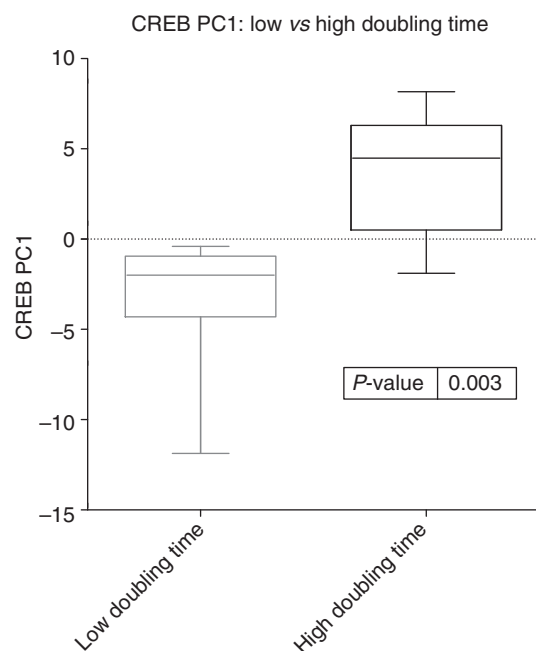


**Figure 4** Low CREB pathway and High APC pathway signature principal component analysis (PCA) scores are associated with favourable clinical outcome. Kaplan–Meier curves depict the association between CREB-pathway signature PCA score (**A–C**) and APC-pathway signature PCA score (**D–F**) and overall survival from cancer for OVCA data sets. Information available for 141 of 142 samples. Log-rank test *P*-values indicate significance. Abbreviations: CR = complete response; IR = incomplete response; O = optimal; S = suboptimal.

multiple cell types, including fibroblasts, epithelial cells and tumour cells (Thyberg, 1984; Hruban *et al*, 1989; Bhalla *et al*, 1993; Donaldson *et al*, 1994; Rowinsky and Donehower, 1995). It has been established that paclitaxel kills cancer cells via microtubule binding, induction of kinetic suppression of microtubule dynamics, and cell cycle arrest in mitosis, that leads to induced apoptosis via activation of cyclin-dependent kinases, and the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (Wang *et al*, 2000). In contrast, the primary mechanism of cisplatin-induced cytotoxicity is generally accepted to be associated with DNA damage that subsequently leads to apoptosis (Fisher, 1994), though apoptosis may also be caused by damage to cytoplasmic or nuclear proteins including molecules involved in cellular energy (i.e., ATP) or proteins directly or indirectly involved in the apoptotic process (i.e., p53, Bax, Bcl, and caspases), leading to necrotic cell death (Perez, 1998). In fact, both apoptosis and necrosis have been observed in cisplatin-treated cells (Eguchi *et al*, 1997; Zhan *et al*, 1999; Pestell *et al*, 2000). In light of the different primary mechanisms of action (and underlying molecular signalling pathways) of platinum-based

agents vs the microtubule-binding taxanes, there also appear to be potential convergences of action via common pathways that lead to—and are associated with—the apoptotic processes. Future studies of both the common and unique pathways associated with OVCA platinum- plus/minus taxane response may also shed light on the relative contributions of identified unique and common pathways to differing mechanisms of cell death.

Our primary finding was regarding the Transcription/CREB pathway. Expression of this pathway was significantly associated with survival in patients who demonstrate a complete response to primary platinum-based therapy (adjusted *P* = 0.0001), suggesting that expression of this pathway not only influences OVCA response to platinum-based therapy but also to the behaviour of *de novo* platinum-sensitive tumours subsequent to a complete response to primary therapy such that survival is impacted. We observed no such effect in patients who experience an incomplete response to platinum, suggesting that, although expression of the pathway is associated with primary chemo-sensitivity, it has no influence on the post-treatment phenotypic behaviour of OVCA that are resistant to platinum. Notably, the influence of the



**Figure 5** Expression of the CREB pathway is associated with the growth rates of cancer cells. Box plot depicting the association between CREB pathway principal component analysis mRNA expression score and cell line doubling times of the NCI60 cancer cell line panel. Median doubling time was used as a cutoff. Boxes indicate 95% confidence.

Transcription/CREB pathway on overall survival also approached statistical significance, despite cytoreductive status (the volume of residual disease at the completion of primary surgery), which is considered one of the most important clinical determinants of survival for patients with OVCA. Our analyses demonstrated a statistically significant correlation between CREB pathway expression and the doubling times of 60 human cancer cell lines, further supporting the view that the influence of the CREB pathway on overall survival from OVCA may be due to an effect on biological processes over-and-above chemo-sensitivity. Indeed, when we explored genes and pathways that are differentially expressed in OVCA that demonstrated complete responses to primary therapy but that harboured high vs low CREB pathway scores, we identified a series of molecular signalling pathways known to influence tumour behaviour, cellular proliferation and apoptosis (e.g., AKT, G-protein signalling, MAP/ERK, BAD). Our findings suggest that CREB pathway activity influence OVCA cell sensitivity to chemotherapeutic agents, but also, perhaps independently, influence cellular proliferation, which can also influence chemo-sensitivity. It is possible, therefore, that expression of the pathway influences clinical outcome from OVCA via direct effects on chemo-sensitivity, but also indirectly via transcriptional influences on additional pathways that impact cellular proliferation and apoptosis, even following a complete response to primary treatment. These findings highlight the importance of the CREB pathway in many aspects of OVCA phenotypic behaviour and may have substantial implications for the future direction of research into disease development, response to therapy, and clinical outcome.

cAMP response element-binding protein (CREB) is a transcription factor that can be activated by multiple pathways that affect cellular cAMP levels, and its many targets have important roles in cellular metabolism, proliferation, and survival (Mayr and Montminy, 2001; Zhang *et al*, 2005). G-protein-coupled receptors, transmembrane proteins that transduce extracellular signals to intracellular effector pathways via heterotrimeric G proteins,

activate the enzyme adenylate cyclase, which catalyses the conversion of ATP to cAMP. The cAMP then binds the regulatory subunits of protein kinase A (PKA), which leads to the release of the PKA catalytic subunits (Lappano and Maggiolini, 2011). Active PKA migrates to the nucleus, where it phosphorylates CREB (Gonzalez and Montminy, 1989). Phosphorylated CREB then binds its coactivator CBP/p300, a histone acetyltransferase, and together CREB and CBP/p300 activate the transcription of genes whose promoters contain a CRE (cAMP response element) sequence (Bannister and Kouzarides, 1996; Parker *et al*, 1996). Overexpression of CREB may be required for the proliferation and survival of human cancers (Bonni *et al*, 1999; Siu and Jin, 2007). Additionally, the CREB coactivator CBP/p300 is mutated in several human cancers, with loss of CBP/p300 histone acetyltransferase activity potentially impacting the expression of several tumour suppressor targets (Iyer *et al*, 2004). Clearly, additional functional analyses are warranted to better define the mechanistic basis to these observations.

BCL-xL/BCL-2-associated death promoter (BAD) was first identified in a yeast two-hybrid screen for BCL-2 interactors (Yang *et al*, 1995). Heterodimerisation between BAD and BCL-2, BCL-xL, or BCL-W promotes apoptosis through the displacement of BAK and BAX (Yang *et al*, 1995; Holmgreen *et al*, 1999). The interaction between BAD and its partners is dependent on the BAD BH3 domain (Zha *et al*, 1997). Upon apoptotic stimuli, BAD translocates from the cytosol to the mitochondria (Zha *et al*, 1996; Jia *et al*, 1999), where it neutralises the anti-apoptotic proteins and promotes mitochondrial membrane permeabilisation (Datta *et al*, 2000; Roy *et al*, 2009). Subversion of BAD-mediated apoptosis may thus be an important mechanism by which tumour cells acquire resistance to apoptotic stimuli. A role of BAD-induced apoptosis in tumour suppression is supported by the identification of BAD mutations in colon cancer that disrupt binding to BCL-2 and BCL-xL and the observation that BAD-deficient mice are prone to diffuse B-cell lymphoma (Ranger *et al*, 2003; Lee *et al*, 2004). Furthermore, because post-translational modification of BAD represents a key control point between cell survival and apoptosis, BAD phosphorylation is frequently deregulated in cancer. Activation of multiple signalling pathways by growth factors, cytokines, or oncogenic mutations can contribute to increased BAD phosphorylation, with aberrant kinase and phosphatase activity leading to BAD inactivation and resistance to cell death signalling in tumours (Danial, 2008).

The anaphase-promoting complex/cyclosome (APC/C) is a multi-subunit ubiquitin ligase required for regulation of cell cycle progression and mitotic exit. The APC/C consists of multiple core subunits and an activator component. The APC/C is tightly regulated during the cell cycle via the cyclical binding of the Cdc20- (cell division cycle 20) and Cdh1- (cell division cycle homologue 1) activating subunits. The major role of APC/C is to target substrates for proteasomal degradation in an orchestrated manner, with Cdc20 and Cdh1 mediating substrate recognition to ensure that mitotic entry and exit occur correctly. The APC/C<sup>Cdc20</sup> is active from anaphase to the end of mitosis, and APC/C<sup>Cdh1</sup> is active from late mitosis to the G<sub>1</sub>-S transition. The APC/C<sup>Cdc20</sup>-mediated destruction of targets such as securin and cyclin B drives chromosome separation and exit from mitosis. As mitotic cyclins are degraded, cyclin-dependent kinase activity decreases. By late M phase, Cdh1 is no longer phosphorylated and can bind to the core APC/C subunits. The APC/C<sup>Cdh1</sup> has multiple targets, including Cdc20, mitotic cyclins, mitotic kinases, and inhibitors of DNA replication origin licensing. Collectively, degradation of APC/C<sup>Cdh1</sup> targets ensures that cells do not duplicate their DNA and/or enter mitosis prematurely (Nakayama and Nakayama, 2006). Because of the critical role of APC/C in regulating chromosome segregation during mitosis, deregulation of the complex could cause genomic instability and contribute to tumourigenesis. Indeed, loss-of-function mutations of core APC/C subunits has been observed in

colorectal cancer cells, upregulation of Cdc20 has been observed in several human cancers, and loss of Cdh1 causes chromosomal instability and tumour formation in mice (Wang et al, 2003; Nakayama and Nakayama, 2006; Garcia-Higuera et al, 2008).

The BAD, CREB, and APC/C may be linked by common signalling molecules, as signalling pathways converge in many ways to regulate cellular proliferation and survival at both the transcriptional and post-transcriptional levels. For example, PKA phosphorylation activates CREB and inactivates BAD and APC/C<sup>Cdc20</sup> (Gonzalez and Montminy, 1989; Lizcano et al, 2000; Harada et al, 2001; Searle et al, 2004). AKT also phosphorylates BAD and CREB, and AKT phosphorylation protects some APC/C<sup>Cdh1</sup> substrates from degradation (Datta et al, 1997; del Peso et al, 1997; Gao et al, 2009). PTEN can also promote the association between APC/C and Cdh1 to prevent S phase entry (Song et al, 2011). Cyclin-dependent kinases phosphorylate BAD, APC/C, and CBP/p300 (Ait-Si-Ali et al, 1998; Zachariae et al, 1998; Konishi et al, 2002). There may also be more direct crosstalk between BAD, CREB, and APC. BCL-xL/BCL-2-associated death promoter (BAD) has been linked to regulation of cell cycle progression and has been found at CREs in cyclin genes (Chattopadhyay et al, 2001; Fernando et al, 2007). The APC/C subunits APC5 and APC7 directly bind CBP/p300, stimulate CBP/p300 histone acetyltransferase activity, and potentiate CBP/p300-dependent transcription (Turnell et al, 2005). cAMP response element-binding protein may also directly impact cell survival by binding to CREs in the promoters of anti-apoptotic factor genes such as BCL-2 to induce their transcription (Wilson et al, 1996).

Based on the known interactions between these proteins, BAD, APC/C, and CREB may potentially interact in a common pathway to induce cell cycle arrest and death in response to mitotic or metabolic stress caused by carboplatin, cisplatin, and paclitaxel. Deregulation of components of these pathways may thus lead to acquired drug resistance. Indeed, abnormal cell cycle progression, defective apoptosis, and metabolic reprogramming have been implicated as key mechanisms of OVCA chemoresistance

(Takahashi et al, 2005; Hajra et al, 2008; Etemadmoghadam et al, 2009; Montopoli et al, 2011).

We have shown that integration of chemo-sensitivity and genome-wide expression data can provide a more comprehensive understanding of the mechanisms involved in drug responses. Our results demonstrate that characterisation of gene expression signatures in OVCA patients may be useful for characterising responses to treatment and also in developing an understanding of the biology that drives clinical outcome and survival. Furthermore, our identification of BAD, APC/C, and CREB as key players in the cytotoxicity of carboplatin, cisplatin, and paclitaxel in OVCA cell lines suggests that targeted therapies against regulators of these proteins may be worthy of study in chemoresistant OVCAs.

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## Disclaimer

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the funding agencies.

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