Research paper

Integrative proteogenomic analyses of human tumours identifies ADNP as a novel oncogenic mediator of cell cycle progression in high-grade serous ovarian cancer with poor prognosis

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ABSTRACT

Background: Despite toxic side effects and limited durable response, the current standard-of-care treatment for high grade serous ovarian cancer (HGSOC) remains platinum/taxane-based chemotherapy. Given that the overall prognosis has not improved drastically over the past several decades, there is a critical need to understand the underlying mechanisms that lead to tumour development and progression.

Methods: We utilized an integrative proteogenomic analysis of HGSOC tumours applying a poor prognosis gene expression signature (PPS) as a conceptual framework to analyse orthogonal genomic and proteomic data from the TCGA (n = 488) and CPTAC (n = 169) studies. Genes identified through in silico analyses were assessed in vitro studies to demonstrate their impact on proliferation and cell cycle progression.

Findings: These analyses identified DNA amplification and overexpression of the transcription factor ADNP (Activity Dependent Neuroprotector Homeobox) in poorly prognostic tumours. Validation studies confirmed the prognostic capacity of ADNP and suggested an oncogenic role for this protein given the association between ADNP expression and pro-proliferative signalling. In vitro studies confirmed ADNP as a novel and essential mediator of cell proliferation through dysregulation of cell cycle checkpoints.

Interpretation: We identified ADNP as being amplified and overexpressed in poor prognosis HGSOC in silico analyses and demonstrated that ADNP is a novel and essential oncogene in HGSOC which mediates proliferation through dysregulation of cell cycle checkpoints in vitro.

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1. Introduction

Ovarian cancer is the fifth leading cause of cancer-related deaths among women in the United States in 2019 [1]. The most common histological subtype of epithelial ovarian cancer is high-grade serous ovarian cancer (HGSOC). Although most patients initially respond to platinum–taxane based chemotherapy and surgical resection, most tumours recur and become increasingly resistant to chemotherapy [2].

HGSOC tumours express a relatively homogenous somatic or germline mutation profile and are characterized by TP53 mutations in ~90% of tumours as well as frequent BRCA1 and BRCA2 mutations [3]. Although these mutations occur at a high frequency, HGSOC tumors have been shown to be C class tumors characterized by recurrent DNA copy number alterations and few other common mutations. [4]. As was shown by the Cancer Genome Atlas (TCGA) project [3], these alterations manifest as dysregulated Rb/E2F, Ras/PI3K, FoxM1 and Notch signalling; however clinical trials have generally demonstrated a lack of response in these tumours to inhibition of these pathways [5,6]. A number of previous studies, including those from the TCGA and Clinical Proteomic Tumour Analysis Consortium (CPTAC) projects have demonstrated that HGSOC can be classified into multiple transcriptome or proteome-based classes [3,7,8]. While these subtypes do exhibit unique genomic and/or proteomic patterns, the prognostic capacity of these groups remains unclear as several conflicting studies have been reported [3]. While the TCGA initially demonstrated no significant prognostic difference between these groups, more recent studies have suggested that the proliferative and
Research in context

Evidence before this study

Ovarian cancer is the fifth-leading cause of cancer death among women in the United States. Despite extensive multi-omics characterization of high grade serous ovarian cancer (HGSOC) and improved treatment strategies, the overall 5-year survival rate continues to trail most other malignancies. Thus, it is urgent to identify novel therapeutic targets and biomarkers.

Added value of this study

In this study, integrative proteogenomic analyses of HGSOC tumours identified ADNP as a potential novel driver of HGSOC. We confirmed the prognostic capacity of ADNP in multiple independent datasets and in vitro studies showed the essentiality of this protein in regulating cell proliferation and survival. Our analyses demonstrate that ADNP regulates HGSOC tumorigenesis by promoting dysregulation of cell cycle checkpoints.

Implications of all the available evidence

Our findings indicated that ADNP is poor prognostic marker in multiple datasets. Importantly, we validated that ADNP mediates cell proliferation through dysregulation of cell cycle checkpoints in ovarian cancer. Our findings supported ADNP as a novel oncogenic driver of HGSOC growth and survival.

2. Materials and methods

2.1. Human tumour and cell line multi-omics data

Affymetrix HT-HG-U133A microarray data for HGSOC samples was obtained from the Firehose data portal (https://gdac.broadinstitute.org/). Affymetrix U133Plus2.0 microarray data for the 285 patients Tothill (GSE8981) [7] and 260 patients Yoshihara [21] (GSE2062) datasets as well as 29 ovarian cancer cell lines (GSE36139) [22] were acquired from the Gene Expression Omnibus (GEO). RNASeq data of HCT116 colorectal cancer cells treated with siControl or siADNP (n = 6) were obtained from GEO (GSE79395) [23].

GISTIC 2.0 [24] segmentation scores as well as threshold copy number calls (i.e. –2, –1, 0, 1, or 2) for the 488 TCGA samples with corresponding mRNA expression data were acquired (April 2015) from the Firehose data portal. Reverse Phase Protein Array (RPPA) data for 190 proteins and phosphoproteins from 338 HGSOC samples in the TCGA cohort were acquired (June 2015) from the Firehose data portal. In addition, we acquired mass spectrometry data (n = 3586 proteins) for 169 samples from the CPTAC study [8].

2.2. Gene expression signatures

A panel of 62 previously published gene expression signatures was used to examine patterns of pathway activity and/or microenvironmental states (Table S1). To implement each signature, the methods detailed in the original studies were followed as closely as possible. The list of signatures is shown in Table S1 and the scores for the TCGA data set (Table S2), Tothill (Table S3) and Yoshihara (Table S4) are reported.

2.3. Statistical analyses of signature scores

To quantify differences in patterns of signature scores across subtypes, a two-way ANOVA followed by Tukey’s post-test for pairwise comparisons was used (Fig. 1b and Table S5). Principal component analysis (PCA) was performed to assess the distribution of TCGA tumour samples across transcriptomic subtypes [3] using pathway activity scores. A Pearson correlation was used to determine the correlation coefficient (R-value) for each pairwise relationship between each signature and the Poor Prognosis Signature (PPS) for samples in the TCGA (Table S6), Tothill (Table S7) and Yoshihara (Table S8) studies. The top 10 most consistently positively correlated signatures associated with the PPS score are reported in Fig. 1 and Fig. S1.

2.4. Identification of genomic and proteomic alterations associated with poor prognosis

Pathway-specific copy number alterations (CNA), were identified using a previously described strategy [25,26] by using a Spearman rank correlation and Fisher’s exact test. For each analysis, the Benjamin-Hochberg (BH) adjusted P values are reported and a threshold of P < 0.05 (BH corrected) was set (Table S9). A Spearman rank correlation was used to evaluate the association between poor prognosis and mRNA and protein; these data are reported in Table S10-11.

2.5. Analysis of genome-wide RNAi proliferation screen data

To assess the essentiality of ADNP relative to the PPS signature score, ovarian cancer cell line shRNA abundance data was acquired from the Broad Institute Project Achilles dataset [27]. These data growth and survival thereby supporting a role for ADNP in HGSOC tumorigenesis.
Fig. 1. Patterns of pathway activity in HGSOC. (a) Patterns of pathway activity (n = 62) were determined for each sample (n = 488) in the TCGA high-grade serous ovarian cancer cohort and plotted relative to the previously described molecular subtypes. Expression signatures (y-axis) are median centered and clustered by complete linkage hierarchical clustering (b) ANOVA test followed by Tukey pairwise comparison was used to demonstrate statistically significant difference in signature score across the molecular subtypes. (c) Principal Component Analysis (PCA) was used to demonstrate the spatial distribution of subtypes based on the expression patterns of the 62 signature scores. Subtype colouring is the same as in (a). (d) Pearson Correlation coefficient (r-values) for the top 10 most strongly and consistently correlated pathways are shown relative to the Poor Prognosis Signature (PPS) for the TCGA cohort.
were filtered to include only those 29 cell lines for which gene expression data (GSE36139) were also available [22]. A Spearman rank correlation was used to calculate the negative correlation between ADNP shRNA abundance and PPS score.

2.6. Survival analyses

To confirm the prognostic capacity of the PPS signature, samples from the TCGA (n = 564), Tothill (n = 256) and Yoshihara (n = 260) studies were assessed. Clinical data from each study was acquired as detailed above. Overall survival (OS) in each dataset was assessed by comparing patients with high (PPSHigh; top quartile) and low PPS score (PPSLow; bottom quartile) by log-rank test; p-value and hazard ratio are reported in Fig. S1. To demonstrate the relationship between ADNP copy number status or mRNA expression level and overall survival (OS), three datasets were assessed. For copy number-based analyses, OS was determined for TCGA samples with a low level copy number gain or amplification as compared to all other samples (i.e. diploid, LOH or deletion). To assess difference in OS based on ADNP mRNA level, samples were dichotomized into ADNPHigh (top quartile) and ADNPLow (bottom quartile) in the Tothill and Yoshihara datasets. For each analysis, significance was calculated by a log-rank test and the hazard ratio (HR) is reported.

2.7. Cell culture and shRNA knockdown

Ovarian cancer cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA) and cultured according to the suggested guidelines. OVCAR3 or OVCAR5 cell lines expressing one of two tetracycline (tet)-inducible shRNA expressing cell lines were created using the pTRIPZ Inducible Lentiviral shRNA system (GE Dharmacon). The catalogue number for shRNA(1) is: VSTHS_313782 and for shRNA(2) is: VSTHS_313783. The shRNA expression was induced using 1.0 μg/mL of doxycycline and ADNP silencing was verified by qRT-PCR and/or western blot analyses.

2.8. Western blot analysis

50 μg of protein was loaded on 4–20% Mini-protein TGX gradient gel (BioRad). Proteins were separated at 100 V for 2 h at room temperature and then transferred onto nitrocellulose membrane at 100 V for 1 h at 4 °C. The membranes were blocked using 5% milk solution, incubated with primary antibody against total ADNP or beta-actin (Cell Signalling Technology) overnight at 4 °C followed by incubation with HRP-conjugated secondary antibodies (Cell Signalling Technology) for 1 h at room temperature. The signal was developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific), digitally imaged using the ChemiDoc Touch Imaging System (BioRad).

2.9. Quantitative real-time PCR

Total RNA was isolated using RNeasy plus Mini Kit (Qiagen) and cDNA was synthesized using the Quantitect Reverse Transcription Kit (Qiagen). Quantitative PCR (qPCR) was performed and analysed using the Applied Biosystems QuantStudio3 real time thermal cycler system. Primer utilized for human genes are as follow: ADNP Forward: 5'-GAATTTTGGCTTCTTCTCAG-3', ADNP Reverse: 5'-AGCCGAGCA-CAAGAAGC-3', GAPDH Forward: 5'-TCTGACTCAACAGGACAC-3', GAPDH Reverse: 5'-CCAGCACATACAGGAAAT-3', CDC25A Forward: 5'-GGAGATCTGTCACAGGGAA-3', CDC25A Reverse: 5'-GCCCAGTGCACTCATACA-3', C33A Forward: 5'-ATGACATGTGCTCTTCTCC-3', C33A Reverse: 5'-CAATGCCGATGGATATG-3'. CCNE1 Forward: 5'-CAAACCTGAAGGAAAGGACC-3', CCNE1 Reverse: 5'-GCCCAAGCTCAGTACGGCAAG-3', CCNE2 Forward: 5'-AATATACAAACACCTTTAGAAGG3', and CCNE2 Reverse: 5'-GTGCTTTCCGCGTGTGTCATAA-3'.

2.10. Cell proliferation assay and colony formation assay

For cell proliferation assay, OVCAR3 and OVCAR5 cells were mock treated or treated with doxycycline (1 μg/mL) for 96 h. Cell proliferation was assessed by the CellTiter 96 AQueous One Cell proliferation assay (Promega BioSciences) according to the manufacturer’s protocol. For colony formation assay, OVCAR3 and OVCAR5 cells were mock treated or treated with doxycycline (1 μg/mL) and grown for 14 days for OVCAR3 or 7 days for OVCAR5. The cells were stained with 0.2% crystal violet in 95% ethanol and photographed; colonies were manually quantified for each experimental replicate and normalized to the untreated control.

2.11. Cell cycle assay

OVCAR3 and OVCAR5 cells were treated either mock or doxycycline treated for 96 h. DNA content was assessed by Sytox Green staining (50 μg/mL) for 30 min in the dark. The cell cycle distribution was analysed by Beckman-Coulter Cytomics FC500 Flow Cytometer. The percentage of cells in G0/G1 and G2/M were determined for a minimum of 3 independent experiments.

2.12. Apoptosis assay

OVCAR3 and OVCAR5 cells were either mock treated or treated with doxycycline for 96 h before apoptosis assays. After 96 h of treatment, the percentage of apoptotic cells were determined by Annexin V-FITC/Hoechst 33342 staining using the Dead Cell Apoptosis Kit with Annexin V-FITC and Hoechst 33342 (Invitrogen) according to the manufacturer’s instructions.

3. Results

3.1. Identification of subtype-specific patterns of oncogenic activity

In order to identify genetic drivers of HGSOC that contribute to tumour aggressiveness and are associated with poor overall survival, we first examined patterns of oncogenic signalling, the tumour microenvironment, immune infiltration, and other essential tumorigenic characteristics in human ovarian tumours. A panel of 62 previously published gene expression signatures (Tables S1 and S2), including a Poor Prognosis Signature (PPS) [3], was applied to HGSOC gene expression microarray data (n = 488) from the TCGA study (Table S3) for which the molecular subtype had been determined [3] (Fig. 1a). While the PPS signature was only modestly enriched in the proliferative subgroup, patterns of pathway activity were able to recapitulate some known features of each molecular subtype. For instance, immune signatures including T cells [28], B cells [28], macrophage [28], HCK [29], LCK [29], and other immune-related signatures were shown to be consistently up-regulated in the immunoreactive (IR) subtype (Fig. 1a). Likewise, signatures associated with epithelial-to-mesenchymal transition including the cancer stem cell [30], stroma-associated signalling [31] and TGFβ [25,32] signatures were found to be activated in the mesenchymal subtype. Finally, several proliferation-associated signatures were found to be modestly up-regulated in the proliferative subtype.

In contrast to previous studies in breast cancer [25] which showed robust and uniform subtype-specific patterns of pathway activity, with the above noted exceptions, consistent patterns of oncogenic pathway activity, including MYC, RAS, AKT and RB/E2F1 were not observed across HGSOC subtypes. This observation is more readily
apparent when differences in patterns of pathway activity are quantitatively assessed by an analysis of variance (ANOVA) test followed by Tukey’s test for pairwise comparison (Fig. 1b and Table S4). Further analysis of subtype-specific patterns of pathway activity by Principal Component Analysis (PCA) indicated that while molecular subtypes could be distinguished amongst the entire dataset based on pathway patterns, HGSOC tumours appear to be largely homogeneous in nature with respect to these specific molecular characteristics (Fig. 1c). This observation is consistent with previous studies indicating a higher degree of homogeneity between HGSOC tumours relative to the diversity observed in other tumour types [3].

Given the lack of subtype-specific oncogenic signalling, we next sought to identify oncogenic signalling or other tumour features associated with prognosis. To do so, we took advantage of the previously published Poor Prognosis Signature (PPS) [3]. We first validated the prognostic capacity of this signature in three independent datasets. As illustrated in Fig S1, the PPS signature was applied to the TCGA (Fig S1a), Tothill [7] (Fig S1b) and Yoshihara [21] (Fig S1c) datasets. Patients were then dichotomized into PPSHigh (top quartile) and PPSLow (bottom quartile) subgroups (Fig S1d–f) to examine differences in overall survival. As expected, the PPSHigh subgroup of patients consistently showed an overall worse prognosis (Fig S1g–i) thereby validating the prognostic capacity of the PPS signature.

A Pearson Correlation was next used to calculate the concordance between the PPS signature and all other signatures in the TCGA, Tothill [7] and Yoshihira [21] datasets (Tables S5-S6). As illustrated in Fig. 1d (and Fig S1j–l) the top 10 most consistent and strongly concordant signalling pathways across each dataset associated with the PPS signature were identified. These analyses demonstrated that poor prognosis may be associated with altered cell cycle progression, chromosome instability and proliferation as illustrated by the observed correlation between PPS signature and RB LOH [33], RB Loss [34], CMYB [35], bMYB [36], and Chromosome Instability 70 gene signature (Q70) [37] signatures as well as two independent proliferation signatures [38,39]. Although these associations are statistically significant (p < 0.05), modest correlation coefficients (r = 0.1 to 0.6) suggest that other genomic events or altered genes not directly identified by these analyses may also contribute to HGSOC development and poor prognosis.

3.2. Identification of genomic and proteomic alterations associated with PPS activity

We next identified genomic and proteomic alterations directly associated with poor prognosis in HGSOC, including potential novel drivers of HGSOC oncogenesis. To do so, we utilized an integrated proteogenomics strategy based on the use of the previously discussed PPS signature as a conceptual framework to interrogate orthogonal genomic and proteomic data from the TCGA [3] (n = 488) and CPTAC [8] (n = 169) studies. We have outlined the scheme used for our analysis to identify DNA copy number alterations as well as altered mRNA expression and protein expression associated with the PPS signature in Fig 2a.

Given the high level of chromosomal instability in HGSOC, we first identified DNA copy number alterations that were unique to tumours with a high PPS score using a previously published approach [25]. To identify PPS-specific copy number gains or losses, we used two approaches to minimize potential biases that might be associated with either strategy alone. First, using DNA copy number (CN) data from TCGA samples (n = 488), we identified those genes that showed an increased in CN gains (either a low level gain or high-level amplification) or losses (either loss of heterozygosity or deletion) in the PPSHigh (top quartile) tumours compared to PPSLow (all other samples) tumours using an FDR-corrected (Benjamini – Hockberg) Fisher’s Exact test (Fig. 2b). To confirm the association between CN frequency and PPS score, an FDR-corrected Spearman rank correlation (Benjamini – Hockberg) between DNA segment score and PPS score was used as a secondary analysis. Genes that demonstrated an increase in CN gains in PPSHigh samples and had a positive Spearman rank correlation were considered PPS-specific gains whereas those genes that showed increased CN losses in PPSHigh samples and a negative correlation were considered PPS-specific losses. Requiring that PPS-specific copy number alterations meet each of these criterion (q < 0.05) reduces potential false-positives associated with either strategy alone; chromosomal regions that met these thresholds are illustrated in Fig. 2c and summarized in Table S7.

Consistent with previous studies reporting that HGSOC tumours often exhibited alterations in one or more components of the RAS/PI3K/AKT signalling cascade, these analyses identified increased CN gains in multiple members of this pathway including: EGFR (qFisher = 4.6 x 10−3, qSpearman = 1.7 x 10−3), KRAS (qFisher = 1.7 x 10−2, qSpearman = 6.1 x 10−3), PIK3R2 (qFisher = 2.1 x 10−2, qSpearman = 2.4 x 10−3), and AKT2 (qFisher = 5.9 x 10−4, qSpearman = 2.1 x 10−3) (Fig. 2b). In addition to identify oncogenic genes that are known to promote HGSOC tumorigenesis, our analyses also identified a number of chromosomal regions that are frequently amplified in HGSOC, including chromosome 20q12 and 1q22 but do not contain genes that have been reported to drive tumour development or progression.

To further prioritize potential candidate oncogenes in each amplification, we postulated that amplified oncogenic genes must also be overexpressed at the mRNA and protein levels in PPSHigh tumours to be functionally significant. Therefore, we employed a Spearman rank correlation to identify positively (or inversely) correlated genes (Fig. 2d) and proteins (Fig. 2d) associated with poor prognosis (Table S10 and S11). By integrating these analyses, we identified 131 genes which were characterized as PPS-specific CN gains and which were overexpressed at the transcript and protein levels in PPSHigh tumours. By assessing individual genes, we identified a number of known oncogenes including: BADEN [41,42], TFB1M [43], NDRG2 [43], NFYB [44], PAX8 [45,46], PSM1D1 [47]. A number of other genes that represent FDA approved drug targets including: TOP1, PARP1, HDAC2, and BRD4 were also included on this list of genes. Moreover, GSEA analysis [48] determined that these genes are associated with Myc or E2F activated genes, Insulin Receptor signalling, mitotic regulation, DNA replication and mRNA processing (Fig. 2f).

To confirm the association between the 131 identified candidate genes and PPS, we next examined this relationship in both the Tothill [7] and Yoshihira [21] validation datasets to identify the subset of genes that were consistently associated with PPS score (Fig. 3a). Our analyses confirmed that a subset of 39 genes were consistently associated with PPS score (Fig 3b, Figs S2a, S2b). In order to further prioritize genes for functional analyses, we assessed the essentiality of each of the 39 genes by analysing data from a genome-wide RNA-sequencing [45,46], copy number [43], PI3K/AKT signalling cascade, these analyses identified increased CN gains in multiple members of this pathway including: EGFR (qFisher = 4.6 x 10−3, qSpearman = 1.7 x 10−3), KRAS (qFisher = 1.7 x 10−2, qSpearman = 6.1 x 10−3), PIK3R2 (qFisher = 2.1 x 10−2, qSpearman = 2.4 x 10−3), and AKT2 (qFisher = 5.9 x 10−4, qSpearman = 2.1 x 10−3) (Fig. 2b). In addition to identify oncogenic genes that are known to promote HGSOC tumorigenesis, our analyses also identified a number of chromosomal regions that are frequently amplified in HGSOC, including chromosome 20q12 and 1q22 but do not contain genes that have been reported to drive tumour development or progression.

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3.3. ADNP is a putative oncogene associated with PPS score

As noted above, among the candidate genes associated with PPS, the transcription factor ADNP (Activity Dependent Neurprotector Homeobox) was selected as being of particular interest. Our analyses
identified ADNP DNA copy number gains ($q = 2.8 \times 10^{-15}$), increased mRNA expression ($p = 6.9 \times 10^{-22}$), and increased protein expression ($p = 4.3 \times 10^{-04}$) relative to PPS score (Fig. 4a). Importantly, ADNP mRNA expression was strongly associated with both DNA CN status ($p = 4.1 \times 10^{-36}$) and protein expression ($p = 5.1 \times 10^{-18}$) indicating that this is not a silent amplification and that ADNP over-expression
may be functionally relevant in this subset of tumours. Consistent with this argument, analyses of clinical data in both the Tothill (Fig. 4b, p = 0.02; HR: 1.9) and Yoshihara (Fig. 4c, p = 0.04; HR: 1.7) datasets demonstrated that patients whose tumours have high levels of ADNP mRNA expression have a worse prognosis.

Given these associations, we next interrogated the relationship between ADNP mRNA expression and oncogenic signalling as measured by gene expression signatures (Fig. 1). As illustrated in Fig. 4d for the TCGA dataset and Fig. S3a and b for the Tothill and Yoshihara datasets, ADNP mRNA expression is consistently and strongly correlated with proliferation signatures (UNC, p = 4.4 × 10^-7 and Wirapati, p = 1.2 × 10^-11) [38,39], bMY8 (p = 1.8 × 10^-08) [36], cMY8 (p = 6.4 × 10^-22) [35] as well as RB loss (p = 8.2 × 10^-08) [34], PI3K (p = 1.8 × 10^-7) [25], MYC (p = 7.3 × 10^-16) [25] and HER1 (p = 2.0 × 10^-15) [49]. As would be expected from analysis of the TCGA samples, ADNP mRNA levels were strongly correlated with PPS score in the Tothill and Yoshihara datasets (Fig. S2). Further analysis of proteomic data from 338 TCGA samples demonstrated that ADNP mRNA levels are strongly correlated with the expression of a cell cycle proteins including Cyclin D1 (p = 1.2 × 10^-3), Chk1 (p = 2.6 × 10^-2), Chk2 (p = 4.7 × 10^-2), pChk2 (p = 7.4 × 10^-3), pRb1 (p = 6.5 × 10^-3) and FoxM1 (p = 3.8 × 10^-2) (Fig. 4e). Collectively, these analyses suggest that ADNP is associated with cell cycle progression and proliferation in HGSOC tumours.

3.4. ADNP is essential for cell proliferation

Given that ADNP was found to be an essential gene for cancer cell line viability specifically in those cell lines with a gene expression profile associated with poor prognosis as illustrated in Fig. 3c (p = 0.02, F = -0.4) we next examined the effect of ADNP on tumour cell proliferation and growth in order to begin to investigate the mechanisms by which ADNP affects HGSOC genesis and progression. To confirm the essential role of ADNP in ovarian cancer proliferation, we next identified HGSOC cell lines that are characterized by high ADNP protein expression; OVCA5 and OVCA5 were selected for further in vitro experiments (Fig. 3a). We engineered OVCA3 and OVCA5 cell lines to express one of two tetracycline (tet)-inducible shRNA against ADNP. Validation studies demonstrate a consistent 60–80% reduction in ADNP mRNA and protein expression in OVCA3 and OVCA5 (Fig. 3b and c) cells following doxycycline (dox) treatment (1 μg/ml; 48 h); dox had no effect on ADNP expression in the parental cell line.

We next performed MTT and colony formation assays to determine the effect of ADNP on cell proliferation and survival. We determined that shRNA-mediated silencing of ADNP following dox treatment (1 μg/ml; 96 h) resulted in a 18.8% or 25.4% reduction in cell proliferation as measured by Cell T1itre Glow Assay in OVCA3 cells expressing either shRNA(1) (p = 0.0004) or shRNA(2) (p = 0.0001), respectively (Fig. 5a). Likewise, OVCA5 cells expressing either shRNA(1) (p = 0.0001) or shRNA(2) (p = 0.0005) treated with dox (1 μg/ml; 48 h) showed similar 16.9% and 26.0% reduction in cell proliferation relative to untreated control cells (Fig. 5e); neither OVCA3 nor OVCA5 parental cell line growth was affected by dox.

We extended these studies to assess the impact of ADNP silencing on long-term colony forming capabilities of these cells. While a modest increase in OVCA3 (p = 0.0105) and OVCA5 (p = 0.1215) parental cell colony formation was observed in response to dox treatment (1 μg/ml), we determined that OVCA3 (Fig. 5b) or OVCA5 (Fig. 5f) cells expressing either shRNA demonstrated a significant decrease in colony formation over a 14-day time course relative to the untreated control cells. Quantification of colonies relative to untreated control cells determined that shRNA(1) and shRNA(2) expressing OVCA3 (Fig. 5c) and OVCA5 (Fig. 5g) cells expressing either shRNA demonstrated a significant decrease in colony formation over a 14-day time course relative to the untreated control cells. Furthermore, OVCA5 cells expressing either shRNA(1) (p = 0.0012) and 57.5% (p = 0.0019) reduction in colony formation relative to untreated shRNA expressing cells, respectively. Similarly, OVCA5 cells expressing shRNA(1) had a 26.5% reduction (p = 0.0032) in colonies while shRNA(2) expressing cells had a 37.5% reduction (p = 0.0044) in colony formation relative to untreated cells. A more significant decrease in colony formation was observed when comparing dox-treated shRNA expressing cells to the dox treated parental cells suggesting that these data may underestimate the effect of ADNP silencing on colony formation.

Finally, since our data indicate that ADNP silencing results in reduced cell growth and colony formation, we examined the impact
of ADNP silencing on induction of apoptosis. We determined that OVCAR3 cells demonstrated a significant 5.8-fold (p = 0.0017) and 3.3-fold (p = 0.0019) increase in the combined percentage of early and late apoptotic cells following shRNA-mediated silencing of ADNP for 96 h (Fig. 5d). This corresponded with an increase from 4.6% of apoptotic cells (combining early and late apoptosis) to 25.8% in shRNA(1) expressing cells (Figs. S5b and S5e) and from 5.9% to 18.8% in shRNA(2) expressing cells (Figs. S5c and S5f); no change in the percentage of apoptotic cells was observed in dox-treated parental cells (Fig. 5d, S5a and S5d). Interestingly, when these studies were repeated in OVCAR5 cells, we observed a modest decrease in apoptosis levels following ADNP silencing. OVCAR5 cells expressing shRNA(1) showed a 1.1-fold reduction (p < 0.0001) while shRNA(2) expressing cells showed a 1.42-fold reduction (p = 0.021) (Fig. 5h and Fig. S5g). This corresponded with a 1.9% decrease apoptotic cells in shRNA(1) expressing cells and a 5.5% decrease in shRNA(2) expressing OVCAR5 cells. Collectively, these data indicate that ADNP is essential for cell proliferation and survival.

3.5. ADNP regulates cell cycle gene expression and ADNP loss induces cell cycle arrest

Finally, analyses of publicly available gene expression data [23] demonstrated that siRNA-mediated silencing of ADNP results in down-regulation of 432 genes (p < 0.05) (Fig. S6a). Functional enrichment analysis through GSEA [48] showed that these genes play a predominant role in cell cycle checkpoints and cell cycle related pathways (Fig. S6b). As expected, further analyses of these genes identified multiple key cell cycle regulators including CDC25A, CDC25B, CCNE1, CCNB1, CCND1, CDK6, and WEE1 among others (Fig. 6a). Thus, we next validated the impact of shRNA-mediated silencing of ADNP on the expression of a subset of these cell cycle
genes in ovarian cancer cell lines. Our analyses demonstrated that shRNA-mediated silencing of ADNP (1 μg/ml, 96 h) resulted in a significant and reproducible reduction in CDC25A, WEE1, CCNE1 and CCNE2, as well as CCNB1 and CCNB2 expression in OVCAR3 (Fig. 6b) and OVCAR5 (Fig. 6c) cells. Consistent with these findings, we confirmed that Cdc25a, a key regulator of both CDK2/Cyclin E activity at the G1/S transition and CDK1/Cyclin B activity at the G2/M checkpoint is significantly reduced at the protein level following ADNP silencing in both OVCAR3 and OVCAR5 cell lines (Fig. 6d).

Given the effect of ADNP on these key regulators of cell cycle progression, as well as our previous data (Fig. 4) which suggests an association between ADNP expression and cell cycle-related proteins and signalling pathways, we determined the impact of ADNP silencing on the cell cycle. As illustrated in Fig. 6e, OVCAR3 cells, including parental and shRNA(1) or shRNA(2) expressing cells, have a normal cell cycle distribution. To assess changes in the cell cycle, cells were treated with dox (1 μg/ml) for 48 h to obtain optimal silencing of ADNP, dox (1 μg/ml) containing medium was replaced and the effect on the cell cycle examined 48 h later. We determined that ADNP silencing in shRNA(1) (p = 0.0005) or shRNA(2) (p = 0.0018) expressing cells resulted in a significant arrest at the G1/S checkpoint as shown by the 28.3% and 26.0% increase in cells in G0/G1 (Fig. 6e and f). This corresponded with a similar 32.7% and 27.3% reduction of cells in the G2/M phase (Fig. 6e and g).

To confirm these observations, we examined the impact of ADNP silencing on the cell cycle in OVCAR5 cells. Similar to OVCAR3 cells, OVCAR5 cells showed a normal cell cycle distribution in the parental as well as shRNA(1) and shRNA(2) expressing cells (Fig. 6h). However, while silencing of ADNP in OVCAR5 cells had no effect on the G1/S checkpoint (Fig. 6h and i), we observed a significant 24.7% and 21.3% increase in cells in the G2/M phase in shRNA(1) (p = 0.0061) and shRNA(2) (p = 0.0014) expressing cells. These data indicate that ADNP is a key regulator of cell cycle genes, including CDC25A which has been shown to mediate both G1/S and G2/M checkpoints as well as CCNE, which mediates the G1/S transition and CCNB which is required for progression through the G2/M checkpoint.

4. Discussion

Although recent advances in ovarian cancer research have resulted in improved treatment strategies leading to increased overall survival of HGSC patients, the 5-year survival rate of this disease still trails those of the vast majority of other malignancies. This highlights the need to develop approaches to identify novel, essential genes that regulate signalling networks and tumour characteristics that are required for tumour development and progression, and to develop strategies to identify the subsets of patients who will best respond to a given treatment. In this study, we undertook an integrative proteogenomic analysis of HGSC tumours utilizing a previously published prognostic gene expression signature [3] as a conceptual framework to identify novel and essential regulators of ovarian cancer.

Our integrative in silico analyses of HGSC tumour DNA copy number, mRNA and proteomic data identified ADNP as a potential novel driver of HGSC tumorigenesis based on the association between poor prognosis and the expression of this gene/protein. Further analyses confirmed the prognostic capacity of ADNP in multiple independent datasets and suggested that ADNP expression is not only required for cell viability but is strongly associated with proliferation and cell cycle related signalling pathways. Indeed, in vitro studies confirmed the essentiality of this protein in regulating cell proliferation and survival and demonstrate a role for this gene in modulating cell cycle progression through altered expression of key cell cycle genes including CDC25A.

ADNP is a Homeobox transcription regulator which includes nine zinc-fingers that play a role in neuroprotective responses to cellular growth, chromatin remodelling, microtubule/autophagy regulation and cancer cell proliferation [9,13–15]. The vast majority of studies have focused on the role of ADNP in neurological diseases including...
ADNP expression in HGSOC tumours is strongly associated with altered cell cycle progression and altered cell cycle checkpoints and these relationships are confirmed by analyses of down-regulated genes following ADNP silencing. Interestingly however, while shRNA-induced silencing of ADNP in either OVCAR3 or OVCAR5 cells results in loss of CDC25A, WEE1, CCNE1 and CCNE2, and CCNB1 and CCNB2, loss of ADNP results in G1/S checkpoint arrest in OVCAR3 cells and G2/M checkpoint arrest in OVCAR5 cells. While it is unclear why these cells arrest at different phases of the cell cycle, it is clear that loss of ADNP expression results in decreased CDC25A expression, at both the mRNA and protein levels, which has been shown to directly regulate CDK2/Cyclin E signalling at the G1/S checkpoint and CDK1/ Cyclin B activity at the G2/M checkpoint [52,53]. These data would lead us to speculate that CDC25A activation may play a significant role in ADNP regulation of the cell cycle and that additional co-factors and/or cell-specific genomic alterations, including differences in p53 mutational status, may contribute to the differential response observed in OVCAR3 and OVCAR5 cells. Clearly additional studies will be required to fully delineate the mechanisms by which ADNP mediates the cell cycle in HGSOC.

Finally, ADNP mRNA expression was found to be predictive of poor overall survival in multiple independent HGSOC datasets [72,21]. While these results are contradictory to recent observations in colorectal cancer and triple negative breast cancer [23,54], which indicate that ADNP may play a tumour suppressive role in these cancers, they are
supported by more recent studies that indicate that ADNP is oncogenic in colorectal cancer [51], pan-cancer analyses which demonstrate that ADNP is part of a SWI/SNF containing oncogenic sub-network in human cancers [44] as well as previously published described mechanistic studies in multiple tissue types indicating that ADNP mediates aspects of cell proliferation and growth [13,40,41,44,47]. As such our data also suggest a tissue specific or dichotomous role may exist for ADNP in tumorigenesis.

Collectively, our cross-platform analyses of proteogenomic data, together with in vitro experiments, have identified and validated ADNP as a novel mediator of cell proliferation in HGSOc. Although the exact mechanisms by which ADNP modulates ovarian cancer tumorigenesis remains to be determined, our data, in combination with previous studies, demonstrate that ADNP mediates its effects on HGSOc tumorigenesis, in part, by promoting dysregulation of cell cycle checkpoints. How ADNP abrogates this process, the identification of co-factors required for ADNP activity, the down-stream signalling network activated by ADNP in HGSOc as well as other tumour characteristics impacted by ADNP overexpression remain unclear. As such, defining these mechanisms will be paramount for determining the therapeutic and/or biomarker potential of ADNP in high-grade serous ovarian cancer as well as for other tumour types.

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Declaration of Competing Interests

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi: 10.1016/j.ebiom.2019.11.009.

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