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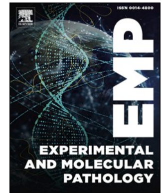

FANCD2 expression affects platinum response and further characteristics of high grade serous ovarian cancer in cells with different genetic backgrounds

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ABSTRACT

High-grade serous ovarian cancer (HGSOC) is the most prevalent subtype of ovarian cancer and demonstrates 5-year survival of just 40%. One of the major causes of mortality is the development of tumour resistance to platinum-based chemotherapy, which can be modulated by dysregulation of DNA damage repair pathways. We therefore investigated the contribution of the DNA interstrand crosslink repair protein FANCD2 to chemosensitivity in HGSOC. Increased FANCD2 protein expression was observed in some cell line models of platinum resistant HGSOC compared with paired platinum sensitive models. Knockdown of FANCD2 in some cell lines, including the platinum resistant PEO4, led to increased carboplatin sensitivity. Investigation into mechanisms of FANCD2 regulation showed that increased FANCD2 expression in platinum resistant cells coincides with increased expression of mTOR. Treatment with mTOR inhibitors resulted in FANCD2 depletion, suggesting that mTOR can mediate platinum sensitivity via regulation of FANCD2. Tumours from a cohort of HGSOC patients showed varied nuclear and cytoplasmic FANCD2 expression, however this was not significantly associated with clinical characteristics. Knockout of FANCD2 was associated with increased cell migration, which may represent a non-canonical function of cytoplasmic FANCD2. We conclude that upregulation of FANCD2, possibly mediated by mTOR, is a potential mechanism of chemoresistance in HGSOC and modulation of FANCD2 expression can influence platinum sensitivity and other tumour cell characteristics.

1. Introduction

Ovarian cancer is among the most common cancers in women, with 300,000 new cases identified globally per year (Torre et al., 2018). The majority of these are of the high-grade serous histotype, which accounts for 70% of cases and carries a particularly poor prognosis, with 5-year survival of just 40% (Berns and Bowtell, 2012; Hollis, 2023). This is largely due to frequent advanced stage disease at diagnosis and development of resistance to standard of care platinum-based chemotherapy (Taylor et al., 2020). While most HGSOCs are initially highly sensitive to platinum-based chemotherapy, they typically recur with increasing levels of resistance, which eventually leads to treatment failure (Bowtell et al., 2015). Identifying the mechanisms behind platinum resistance is therefore key to reducing HGSOC mortality.

A prevailing mechanism by which platinum sensitivity can be modulated in HGSOC tumours is via the *BRCA1* (breast cancer 1) and *BRCA2* (breast cancer 2) genes, which are collectively mutated in 22% of cases (Bell et al., 2011). BRCA1 and BRCA2 participate in the repair of double-strand breaks via the homologous recombination (HR) pathway, a critical process in Fanconi anaemia (FA) mediated repair of DNA

Abbreviations: HGSOC, high grade serous ovarian cancer; FANCD2, Fanconi anaemia complement group D2; mTOR, mammalian target of rapamycin; BRCA1, breast cancer 1; BRCA2, breast cancer 2; HR, homologous recombination; FA, Fanconi anaemia; ICL, interstrand crosslink; PALB2, partner and localizer of BRCA2; BCA, bicinchoninic acid; SRB, sulforhodamine B; sgRNA, small guide RNA; IHC, immunohistochemistry; siRNA, small interfering RNA; FANCF, Fanconi anaemia complement group F.

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interstrand crosslinks (ICLs) induced by platinum (Taylor et al., 2020). Tumours with inactivating *BRCA1* or *BRCA2* mutations are highly platinum sensitive (Tan et al., 2008). However, during treatment, further mutations can be acquired which restore gene function and DNA repair capacity, leading to the generation of platinum resistance (Sakai et al., 2009; Swisher et al., 2008; Hollis et al., 2017). Mutation and hypermethylation of other genes associated with the FA pathway, such as *RAD51* family members and *PALB2* has also been observed in HGSOC and is associated with improved response to chemotherapy (Liu et al., 2015; Guffanti et al., 2018; Potapova et al., 2008; Kanchi et al., 2014). However, the contribution of other FA proteins to platinum sensitivity in HGSOC is generally less well established (Taylor et al., 2020).

FANCD2 functions in recruitment of downstream repair factors to ICL sites (Alcon et al., 2020) and is a key component of ICL repair, with deficiency of FANCD2 causing more severe phenotypes than other FA pathway proteins (Kalb et al., 2007). Loss of FANCD2 in mouse models has been previously linked to the development of epithelial cancers including ovarian, breast and lung cancers (Houghtaling et al., 2003). Reduced FANCD2 expression has also been observed in both ovarian tumours and epithelial cells from women at high risk of ovarian cancer, where it is associated with enhanced cytogenetic instability and sensitivity to DNA crosslinking agents (Pejovic et al., 2006). Conversely, increased expression of FANCD2 has been identified as a negative prognostic factor for ovarian tumours (Moes-Sosnowska et al., 2019), although this may be dependent on subcellular localization, with cytoplasmic FANCD2 expression reported to confer a favourable prognosis (Joshi et al., 2020). In other cancer types, overexpression of FANCD2 has also been observed in glioma, where it is associated with advanced disease stage (Metselaar et al., 2019), and malignant oesophageal cancer (Lei et al., 2020). Moreover, depletion of FANCD2 leads to enhanced sensitivity to DNA damaging agents including platinum chemotherapy in glioma and lung cancer (Metselaar et al., 2019; Wang et al., 2015).

As a central component of the FA pathway, FANCD2 is necessary for ICL repair (Knipscheer et al., 2009), and changes in its expression may therefore be highly relevant to platinum resistance. However, the importance of FANCD2 in HGSOC remains poorly defined. While low expression of FANCD2 may represent an early driver of genomic instability and lead to the development of ovarian cancer (Houghtaling et al., 2003; Pejovic et al., 2006), the effect of tumour FANCD2 expression on patient prognosis is uncertain, with different studies describing an unfavourable prognosis for ovarian cancer cases with high mRNA levels and those with loss of cytoplasmic FANCD2 respectively (Moes-Sosnowska et al., 2019; Joshi et al., 2020). We therefore aimed to further characterise the impact of FANCD2 expression changes on chemoresistance in HGSOC.

2. Methods

2.1. Cell culture

Human OC cell lines ES2, 59 M, COV318, PEO1 and PEO4 were obtained from in-house liquid nitrogen frozen stocks, originally purchased from the American Type Culture Collection, ATCC (ES2) or the European collection of cell cultures, ECACC (59 M, COV318) via Sigma. These have been previously shown to have copy number profiles and TP53 mutations characteristic of HGSOC (Domcke et al., 2013). PEO1 and PEO4 cell lines were previously established from tumour samples taken from the same patient before (PEO1) and after (PEO4) the development of platinum resistant HGSOC disease as described in (Langdon et al., 1988). Cell lines were authenticated via short tandem repeat profiling prior to experimentation using the Promega GenePrint 10 System (Promega, Southampton, UK), and were regularly tested for mycoplasma contamination using the Lonza MycoAlert Detection Kit (Lonza, Slough, UK). Cells were grown in RPMI-1640 (Thermo Fisher, Loughborough, UK) supplemented with 10% fetal bovine serum (Thermo Fisher), 1% penicillin streptomycin (Thermo Fisher), and 2 mM L-glutamine (Thermo Fisher).

2.2. Western blot analysis of protein expression

Lysis buffer was added to cells on ice. Lysates were centrifuged at 15,000 *g*, and supernatant collected. Protein content was quantified using the BCA assay. Lysates were loaded on 7.5% acrylamide gels and proteins were separated by SDS-page, then transferred to PVDF membrane. Membrane was incubated for 1 h with blocking buffer (LI-COR, Cambridge, UK) and overnight with blocking buffer containing primary antibody. Primary antibodies targeting FANCD2 (Abcam, Cambridge, UK; ab108928; 1:1000 dilution), mTOR (Proteintech, Manchester, UK; 66888–1; 1:2500 dilution) and tubulin (Abcam; ab7291; 1:5000 dilution), were used. Membranes were incubated with fluorophore conjugated secondary antibody (LI-COR) diluted in blocking buffer, and imaged using the Odyssey Infrared Imaging System (LI-COR). Band intensity was quantified using ImageJ software. All antibody quantifications were normalised to tubulin loading controls.

2.3. PCR analysis of mRNA expression

RNA was extracted from cells using a RNeasy Mini Kit (Qiagen, Manchester, UK), quantified using the Nanodrop 2000c (Thermo Fisher) and reverse transcribed using a high capacity cDNA reverse transcription kit (Thermo Fisher) following manufacturer's instructions. Quantitative PCR was carried out using the commercially available Taqman gene expression assay for FANCD2 (assay ID Hs00276992_m1, Thermo Fisher) with Taqman fast advanced master mix (Thermo Fisher). The GAPDH assay was used as an internal control (assay ID Hs03929097_g1, Thermo fisher). PCR was performed using the StepOne PCR system (Applied Biosystems). PEO1 and PEO4 expression values were normalised to GAPDH expression, and FANCD2 expression in the 59 M cell line was used as a reference to enable comparison across assay plates.

2.4. Sulforhodamine B assay

Drug cytotoxicity was measured using the sulforhodamine B (SRB) assay. 250–3000 cells were plated per well in 96-well plates, treated with 1:4 or 1:2 dilution series of carboplatin (Sigma-Aldrich, Gillingham, UK) and incubated for 5 days at 37 °C. These were fixed using 25% trichloroacetic acid (Sigma-Aldrich), and stained with 0.4% *w/v* sulforhodamine B dye (Sigma-Aldrich) in Tris pH 10.5. Optical density was measured at 540 nM using a BP800 Microplate Reader (Biohit, Cheshire, UK). Absolute IC_{50} values were interpolated from concentration-response curves using Graphpad Prism.

2.5. mTOR inhibitor treatment

Cells were seeded in 6 well plates and treated with the mTOR inhibitor AZD8055 (Selleckchem, Houston, TX, USA) at a concentration of 1-5 μM for 48 h prior to lysis.

2.6. Generation of platinum resistant cell lines

Carboplatin resistant cell lines were generated from 59 M, ES2, COV318, PEO1 and PEO4 cell lines by culturing with media complemented with FBS and L-glutamine, containing low concentrations of carboplatin for up to 236 days. Initial carboplatin concentrations of 1/10th the IC_{50} were used, and concentration was increased once cells had regained confluence and been passaged a minimum of 3 times. A minimum of 5 times the initial carboplatin concentration was reached for all cell lines.

2.7. Transient siRNA transfection of cell lines

Transfection of cell lines with siRNA was performed using

Lipofectamine 3000 (Thermo Fisher) according to manufacturer's instructions. Transfections were carried out using FANCD2 targeting siRNA1 (UUUUAGUUGACUGACAAUGag; Integrated DNA Technologies (IDT), Ilkeston, UK), siRNA2 (UUGUACUUGAAUACGGUGCta; IDT) and negative control siRNA (Qiagen).

2.8. Sanger sequencing of BRCA2

DNA extractions were carried out using an Allprep DNA/RNA kit (Qiagen) following manufacturer's instructions. DNA was amplified for sequencing via PCR, and PCR product was Sanger sequenced around the expected *BRCA2* mutation site. Forward primer sequence TAGCATGT-GAGACCATTGAGATCACA, reverse primer sequence TGGTAGGAA-TAGCTGTTAGACATGCTA (IDT).

2.9. CRISPR/cas9 mediated gene editing

sgRNA sequences targeting two distinct sequences in the FANCD2 gene (CACCGCATCCTCAATGTAAGACTCC and CACCGGA-TAGGAAGGGTGTCTCCTC; IDT) were cloned into pSpCas9(BB)-2 A-Puro (PX459) V2.0 plasmid backbones (Ran et al., 2013). Cells were nucleofected with the resulting plasmids using a Cell Line Nucleofector Kit T (Lonza) according to manufacturer's instructions. Selection of nucleofected cells was carried out for 48 h using 5 μg/mL puromycin, and individual cells were subcloned to generate monoclonal cell lines. Monoclonal cell lines were evaluated for FANCD2 expression via Western blot.

2.10. Cell migration analysis

Analysis of cell migration was carried out using the Incucyte S3 live imaging system (Essen, Royston, UK). 96 well Imagelock plates (Essen) were coated with 50 μg/mL collagen I (prepared in-house according to (Timpson et al., 2011)). 1000 cells were seeded per well. Wells were imaged every 15 min, and image sequences were analysed using the mTrackJ plugin on ImageJ v1.53. Individual cells were tracked for 10 h, and mean migration velocity per track was calculated using mTrackJ. 30 cells were tracked per biological replicate.

2.11. Tumour tissue microarrays

Tumour tissue microarrays of HGSOC patient samples were constructed as previously described (Hollis et al., 2022). Briefly, from ovarian cancer cases treated at the Edinburgh Cancer Centre between 1984 and 2007, 362 high-grade serous ovarian cancer cases were identified following expert pathological review. All had a minimum of 3-year follow-up and were treated with first line platinum-containing chemotherapy. Samples of treatment naïve tumour were taken at primary surgery, fixed in formalin and embedded in paraffin. 4 μm sections were cut for IHC staining. Ethical approval was obtained from South East Scotland Human Annotated Bioresource (Lothian NRS Bioresource Ethics Committee reference (Joshi et al., 2020)/ES/0061-SR705 and SR1518). The need for consent was waived by the ethics committee due to the retrospective nature of the study. The authors did not have access to patient identifiable information during or after data collection. This study was carried out in accordance with the principles of the Declaration of Helsinki.

2.12. Immunohistochemistry staining

Slides were dewaxed and rehydrated using the Leica Autostainer XL (Leica, Newcastle, UK), and treated with 3% hydrogen peroxide solution (Sigma-Aldrich). Antigen retrieval was performed by heating slides in a pressure cooker containing Tris-EDTA buffer for 12 min. Slides were permeabilised using 0.5% Triton-X100. Blocking was with 5% goat serum for 1 h. Antibody staining was with anti-FANCD2 antibody (Abcam; ab108928) diluted 1:100 overnight at 4 °C. Antibody specificity was confirmed using both Western blot and immunohistochemistry (IHC) methods. Slides were then incubated with peroxidase conjugated secondary antibody (Sigma-Aldrich) and 3, 3′-diaminobenzidine (DAB) solution (Agilent, Wokingham, UK) for 10 min. Slides were then dehydrated and counterstained with haematoxylin on the Leica Autostainer XL before mounting. Slides were imaged in the brightfield channel using the Nanozoomer XR (Hamamatsu, Welwyn Garden City, UK).

2.13. Scoring of immunostained tumour tissue microarrays for FANCD2

FANCD2 IHC staining was scored by manual assessment of digitized slides for nuclear and cytoplasmic staining. For each core, staining intensity of tumour cells was scored as negative (0), weak (1), moderate (2) or strong (3), and the proportions of each were used to generate histoscores ranging from 0 to 300. Two histoscores were generated for each core, one for the nucleus and one for the cytoplasm. Where available, triplicate (53/259) and duplicate (99/259) cores for each case were analysed, and mean values were used for statistical analysis. Good concordance was observed between duplicate and triplicate cores (median coefficient of variation 12.3 nuclear, 12.1 cytoplasmic).

2.14. Statistical analysis

All graphs and statistical analyses were generated using Graphpad Prism v8. Statistical testing for significance was carried out using *t*-tests, or ANOVA with post-hoc testing adjusted for multiple comparisons, as appropriate. Univariate survival analysis was carried out using the log-rank test.

3. Results

3.1. Expression of FANCD2 is increased in a cell line model of platinum resistant high-grade serous ovarian cancer

Expression of FANCD2 was initially examined in the paired PEO1/PEO4 HGSOC cell line models of chemoresistance. The cell lines were assessed for carboplatin sensitivity, and PEO4 was confirmed to be significantly more resistant to carboplatin in vitro than PEO1 (mean IC_{50} 6.5 μM PEO4 VS 1.2 μM PEO1, $p = 0.0005$) (Fig. 1A-B). PEO4 demonstrated significantly higher levels of FANCD2 than PEO1 at both the mRNA ($p = 0.002$) and protein ($p = 0.037$) levels (Fig. 1C-E). Therefore, transcriptional upregulation of *FANCD2* expression may occur in response to platinum treatment, and could have a role in chemoresistance.

3.2. Expression of FANCD2 modulates response to platinum in high-grade serous ovarian cancer cell lines

To further investigate the impact of FANCD2 expression changes on chemosensitivity, knockdown of FANCD2 was performed using 2 distinct siRNAs in the PEO1 and PEO4 cell lines, and response to carboplatin was assessed. Both *FANCD2* targeting siRNAs successfully reduced FANCD2 expression in the PEO1 and PEO4 cell lines to below 50% of negative control siRNA transfected cells (Fig. 2A-C). For PEO4, reduced expression of FANCD2 led to significantly increased sensitivity to carboplatin (mean IC_{50} 1.2 μM control VS 0.5 μM knockdown, $p = 0.005$) (Fig. 2D-G). In contrast, knockdown of FANCD2 had no effect on the carboplatin sensitivity of PEO1 (mean IC_{50} 0.45 μM control VS 0.34 μM knockdown, $p > 0.999$) (Fig. 2D-G). It was noted that the transfection process appeared to affect the carboplatin sensitivity of the cell lines, as both non-targeting siRNA transfected cell lines showed lower IC50 values than the original cell lines. Altered FANCD2 expression may therefore contribute to changes in sensitivity to platinum in HGSOCs.

FANCD2 expression was then examined in further OC cell line

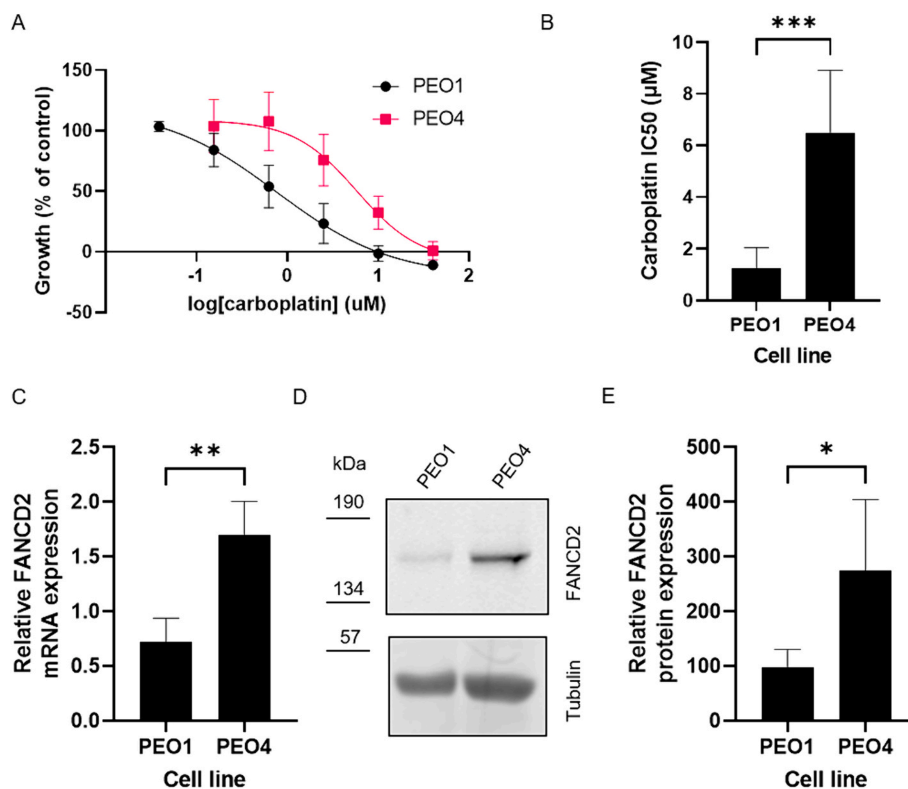

Fig. 1. FANCD2 is upregulated in chemoresistant OC cells. (A) Concentration response curves for PEO1 and PEO4 cell lines treated with 40–0.039 μM carboplatin. Error bars show SD. 6 biological replicates. (B) Carboplatin IC_{50} values for PEO1 and PEO4 cell lines. Error bars show SD. Mean values of 6 biological replicates. (C) Expression of FANCD2 mRNA in PEO1 and PEO4 cells, described as ΔΔCT values compared to a reference cell line. Error bars show SD. Mean values of 4 biological replicates. (D) Representative Western blot (cropped) showing expression of FANCD2 protein in PEO1 and PEO4 cell lines. Molecular weights in kDa. Upper panel FANCD2, lower panel tubulin. (E) Quantification of FANCD2 expression from Western blot in PEO1 and PEO4 cells. Error bars show SD. Mean values of 4 biological replicates. Significance calculated via unpaired *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

models (59 M, ES2, COV318) of varying platinum sensitivities (mean IC_{50} values 10.2 μM, 5.4 μM, 2.6 μM respectively) (Supplementary S1 Fig). The PEO1 cell line expressed similar levels of FANCD2 to COV318 and 59 M; however, PEO4 and ES2 showed significantly higher expression of FANCD2 (Fig. 3A/B). This suggests that the FANCD2 expression range observed in PEO1 and PEO4 is typical for OC cells. FANCD2 was knocked down using siRNA in the 59 M and COV318 cell lines (Fig. 3C-E). This resulted in increased carboplatin sensitivity in 59 M (mean IC_{50} 2.0 μM control VS 0.5 μM knockdown, $p = 0.039$) (Fig. 3F/G) but not COV318 (mean IC_{50} 0.89 μM control VS 0.98 μM knockdown, $p = 0.879$) (Fig. 3H/I). Therefore, the differences in the way that the cell lines respond to FANCD2 knockdown are not due to initial differences in FANCD2 expression, but may be a result of differences in genetic background. This again suggests that FANCD2 expression is an important factor influencing chemosensitivity only in certain cellular contexts. As the PEO1 cell line has been reported previously to harbour a truncating mutation in *BRCA2* (Sakai et al., 2009), but exhibits clonal variation (Ng et al., 2012), the mutation site was sequenced to determine whether this may be a factor affecting the differences in response to FANCD2 knockdown. However, this clone of PEO1 was found to lack the *BRCA2* truncating mutation at this site, instead harbouring a missense mutation which restored full length *BRCA2*, as occurs in PEO4 (S2 Fig).

3.3. FANCD2 expression is upregulated in a platinum resistant cell line generated in vitro

To determine whether FANCD2 expression could be upregulated in response to platinum treatment in other contexts, carboplatin resistant cell lines were generated from the previously used cell line models in vitro. Resistant cell lines, defined as those with over two-fold higher carboplatin IC_{50} values than parental cells, were generated for the 59 M (mean IC_{50} 9.1 μM parental VS 30.3 μM resistant), COV318 (mean IC_{50} 1.2 μM parental VS 8.4 μM resistant) and PEO1 (mean IC_{50} 0.7 μM parental VS 5.7 μM resistant) cell lines (Fig. 4A). FANCD2 expression was then compared in the platinum resistant and parental cell lines. Platinum resistant 59 M cells were found to express significantly more FANCD2 than the parental cell line ($p = 0.032$), however this was not the case for the other cell lines (Fig. 4B-C). This confirms the ability of OC cell lines to upregulate FANCD2 as a mechanism of chemoresistance in response to platinum treatment in some contexts.

3.4. FANCD2 expression is regulated by mTOR in PEO1 and PEO4 cells

As both FANCD2 mRNA and protein expression were upregulated in the PEO4 cell line, potential mechanisms of regulation were investigated. As mechanistic target of rapamycin (mTOR) has been shown to regulate FANCD2 expression in haematopoetic cells and sarcoma (Shen et al., 2013; Guo et al., 2013), the impact of mTOR on FANCD2 expression was investigated in PEO1 and PEO4 cells. Expression of mTOR in PEO1 and PEO4 cells was analysed via Western blot and was found to be significantly higher in PEO4 than PEO1 ($p = 0.045$) (Fig. 5A-B), consistent with the increased FANCD2 expression. The cell lines were then treated with AZD8055, a selective inhibitor of mTOR kinase activity (Chresta et al., 2010). Expression of FANCD2 was significantly reduced as a result of mTOR inhibitor treatment in both cell lines ($p < 0.0001$) (Fig. 5C-F). In these models of HGSOC, FANCD2 expression therefore appears to be modulated by mTOR. Increased mTOR expression in PEO4 may be responsible for the increased levels of FANCD2.

Fig. 2. Knockdown of FANCD2 increases carboplatin sensitivity of chemoresistant PEO4 cells. (A) Representative Western blots (cropped) showing FANCD2 expression in PEO1 and PEO4 cells transfected with negative control siRNA (con) or two different FANCD2 targeting siRNAs (si1 and si2). Upper panel FANCD2, lower panel tubulin. Molecular weights indicated in kDa. (B) and (C) bar charts showing FANCD2 expression of PEO1 and PEO4 cell lines transfected with FANCD2 targeting siRNA or non-targeting control siRNA. (B) siRNA1, (C) siRNA2. Normalised to tubulin loading control, expressed as a percentage of control transfection. (D) Concentration response curves for control siRNA or FANCD2 siRNA1 transfected cell lines, treated with 40–0.16 μM carboplatin for PEO4 or 10–0.039 μM carboplatin for PEO1. Error bars represent SD. Mean of 3 biological replicates. (E) Carboplatin IC_{50} values for PEO1 and PEO4 cells transfected with control siRNA or FANCD2 siRNA1. Error bars represent SD. Mean of 3 biological replicates. (F) Concentration response curves for control siRNA or FANCD2 siRNA2 transfected cell lines, treated with 40–0.16 μM carboplatin for PEO4 or 10–0.039 μM carboplatin for PEO1. Error bars represent SD. Mean of 3 biological replicates. (G) Carboplatin IC_{50} values for PEO1 and PEO4 cells transfected with control siRNA or FANCD2 siRNA2. Error bars represent SD. Mean of 3 biological replicates. Statistics by ANOVA and post hoc testing with adjustment for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

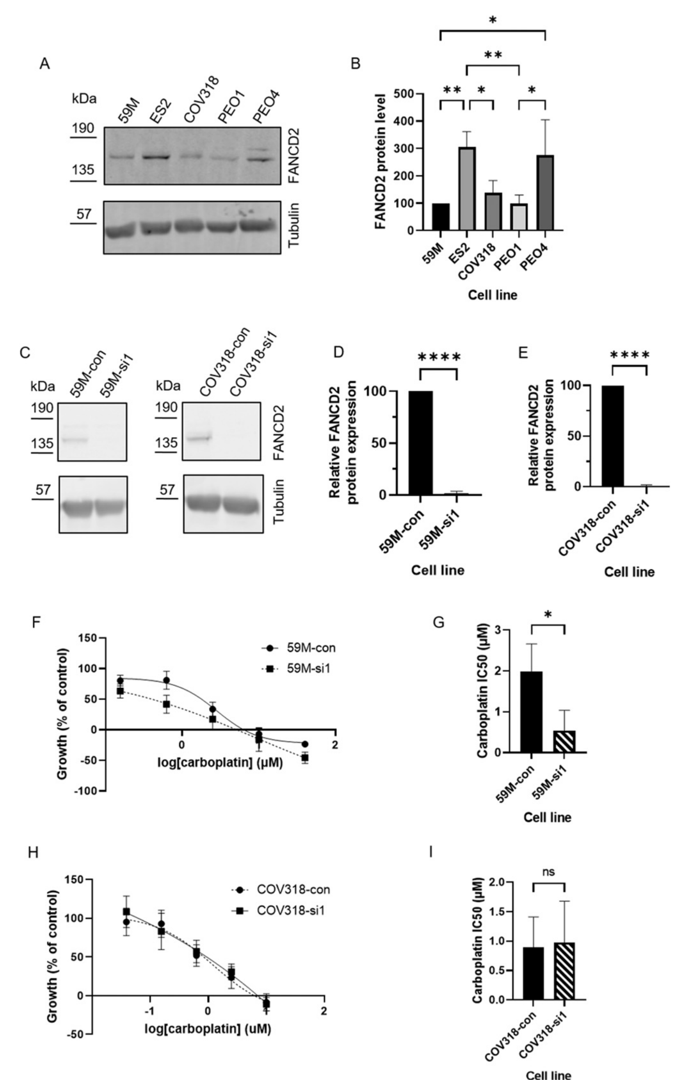

Fig. 3. FANCD2 knockdown enhances platinum sensitivity of an OC cell line. (A) Representative Western blot showing expression of FANCD2 protein in a panel of OC cell lines. Molecular weights in kDa. Upper panel FANCD2, lower panel tubulin. (B) Carboplatin IC_{50} values for OC cell lines. Error bars show SD. Mean values of 4 biological replicates. Significance calculated using ANOVA and post-hoc testing. (C) Representative Western blots (cropped) showing FANCD2 expression in 59 M and COV318 cells transfected with negative control siRNA (con) or FANCD2 targeting siRNA (si1). Upper panel FANCD2, lower panel tubulin. Molecular weights indicated in kDa. (D) and (E) Quantification of FANCD2 expression from Western blot of (D) 59 M and (E) COV318 cell lines transfected with control (con) or FANCD2 targeting (si1) siRNA. Error bars show SD. Mean values of 3 biological replicates. (F) Concentration response curves for control siRNA or FANCD2 siRNA1 transfected 59 M cells, treated with 40–0.16 μM carboplatin. Error bars represent SD. Mean of 3 biological replicates. (G) Carboplatin IC_{50} values for 59 M cells transfected with control siRNA or FANCD2 targeting siRNA1. Error bars represent SD. Mean of 3 biological replicates. (H) Concentration response curves for control siRNA or FANCD2 siRNA1 transfected COV318 cells, treated with 10–0.039 μM carboplatin. Error bars represent SD. Mean of 3 biological replicates. (I) Carboplatin IC_{50} values for COV318 cells transfected with control siRNA or FANCD2 targeting siRNA1. Error bars represent SD. Mean of 3 biological replicates. Significance calculated by unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

3.5. Expression of FANCD2 in a high-grade serous ovarian cancer patient cohort is not associated with clinical outcomes

To explore potential relationships between FANCD2 expression patterns at the protein level and outcomes in HGSOC, tumour

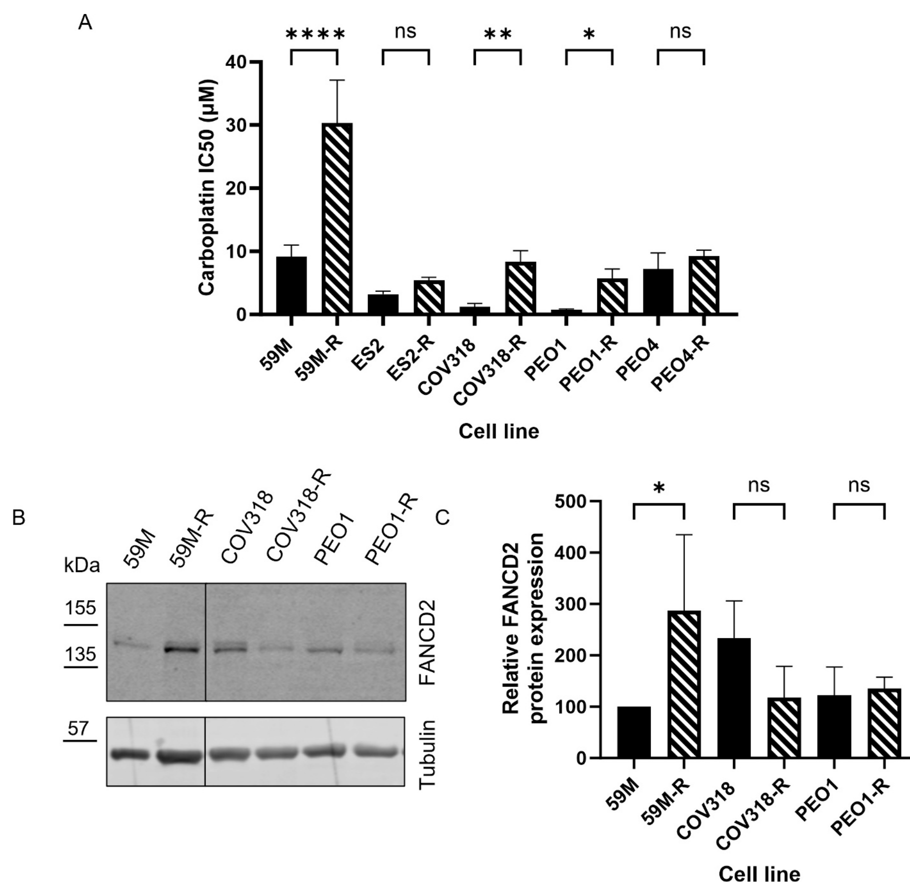

Fig. 4. FANCD2 is upregulated in an in vitro generated platinum resistant cell line. (A) Bar chart showing carboplatin IC_{50} values for in vitro generated platinum resistant cell lines and parental cell lines. Platinum resistant lines are indicated with "R". Error bars represent SD. Mean of 3 biological replicates. (B) Representative Western blot (cropped) showing expression of FANCD2 protein in platinum resistant and parental cell lines. Molecular weights in kDa. Upper panel FANCD2, lower panel tubulin. (C) Quantification of FANCD2 expression from Western blot in platinum resistant cell lines. Error bars show SD. Mean values of 3 biological replicates. Statistics performed using ANOVA and post-hoc testing with adjustment for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

microarrays were immunostained for FANCD2. Microarrays consisted of 362 tumour cores sampled at primary surgery from a cohort of chemo-naïve HGSOC patients, 259 of which were evaluable.

FANCD2 expression occurred in both the tumour cell nuclei and cytoplasm to varying degrees (Fig. 6). Cytoplasmic staining was significantly stronger than nuclear staining across the HGSOC cases (median histoscore 150 cytoplasmic VS 107 nuclear, $p < 0.0001$) (Fig. 6). Most cases also showed greater cytoplasmic than nuclear histoscores (209/259), although individual tumours exhibited distinct staining patterns (Fig. 6). Consistent with this, no correlation was observed between nuclear and cytoplasmic staining (S3 Fig). Therefore, the associations between nuclear and cytoplasmic staining with clinical characteristics were considered independently.

Histoscores were generated for both nuclear and cytoplasmic staining. Cases were then split into "high", "mid" and "low" expression groups based on quartiles, with the two central quartile groups combined to form the "mid" expression group. No associations were found between nuclear FANCD2 expression and stage, residual disease, or *BRCA1/2* mutation status (S1 Table), but a significant difference in age at diagnosis between the groups was observed. Post-hoc testing showed this was driven by significantly lower patient age in the high expression group compared to the mid expression group (median age 58y VS 64y, Bonferonni-adjusted $p = 0.003$).

No significant associations were found between FANCD2 expression in the cytoplasm and tumour stage, age or *BRCA1/2* mutation status (S2 Table). However, a significant difference in minimum residual disease status was observed, with post-hoc testing showing significantly fewer cases of macroscopic disease remaining following surgery in the low expression group compared to the mid expression group (Bonferonni-adjusted $p = 0.043$).

Univariate survival analysis was carried out using the same FANCD2 expression groups. No significant differences in progression-free (PFS) or overall survival (OS) were observed based on either cytoplasmic (median PFS 439 days low VS 411 days mid VS 402 days high, $p = 0.733$; median OS 972 low VS 873 mid VS 958 days high, $p = 0.725$) or nuclear (median PFS 420 days low VS 479 days mid VS 361 days high, $p = 0.562$; median OS 753 days low VS 1125 days mid VS 870 days high, $p = 0.676$) FANCD2 expression (S4 Fig). As a significant association between FANCD2 expression and other clinical parameters influencing survival, such as age and residual disease status, was observed, multivariate survival analysis was carried out using the Cox proportional hazards model including age and minimum residual disease status as covariates, and stratified by stage. Again, no significant changes in progression-free or overall survival were associated with cytoplasmic or nuclear FANCD2 expression groups (S3 Table).

3.6. Loss of FANCD2 enhances migration of high-grade serous ovarian cancer cells

FANCD2 has been previously reported to carry out non-canonical roles in different cancer types (Zheng et al., 2022; Li and Liu, 2023), including functions in migration, invasion and metastasis (Zheng et al., 2022; Romick-Rosendale et al., 2016). As FANCD2 staining was stronger in the cytoplasm than the nucleus in HGSOC tumours (Fig. 6), it was thought that other functions of FANCD2 aside from its role in ICL repair may be relevant to HGSOC. The impact of FANCD2 modulation on cell

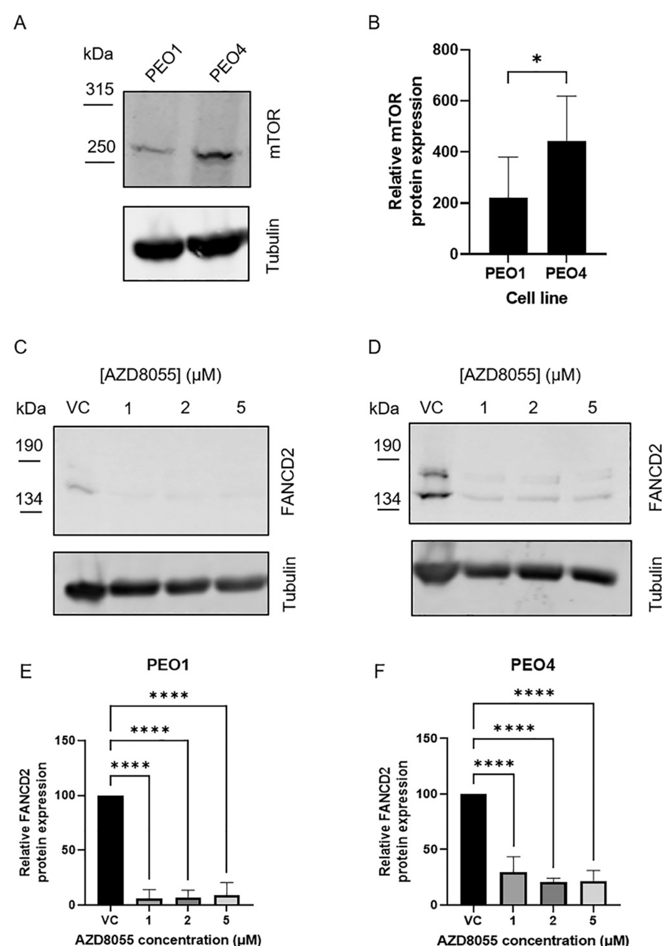

Fig. 5. FANCD2 expression is regulated by mTOR in PEO1 and PEO4 cells. (A) Representative Western blot showing mTOR expression in PEO1 and PEO4 cell lines. Molecular weights in kDa. Upper panel mTOR, lower panel tubulin. (B) Quantification of mTOR expression in PEO1 and PEO4 cell lines from Western blot. Error bars show SD. 6 biological replicates. Significance calculated by unpaired t-test. (C) and (D) Representative Western blots (cropped) showing FANCD2 expression in (C) PEO1 and (D) PEO4 cell lines treated with mTOR inhibitor AZD8055 at 1-5 μM or vehicle control (VC) for 96 h. Molecular weights in kDa. Upper panel FANCD2, lower panel tubulin. (E) and (F) Quantification of FANCD2 from Western blot of (E) PEO1 and (F) PEO4 following treatment with 1-5 μM AZD8055 or vehicle control (VC) for 96 h. Significance calculated by ANOVA and post-hoc testing with adjustment for multiple comparisons. * $p < 0.05$, **** $p < 0.0001$.

migration was therefore assessed. Stable FANCD2 knockout cell lines were generated from PEO1 cells using CRISPR-cas9 gene editing, which were confirmed via Western blot to lack FANCD2 protein (S5 Fig). The 2D migration of these cell lines on collagen I was then assessed. Wild type PEO1 cells showed minimal migration, at low velocity, however knockout of FANCD2 resulted in significantly enhanced cellular migration (mean migration velocity 0.21 μm/min control VS 0.55 μm/min FANCD2 knockout clone 1, $p = 0.0003$; 0.21 μm/min control VS 0.39 μm/min FANCD2 knockout clone 2, $p = 0.012$) (Fig. 7, S6–9 Fig). Interestingly, the PEO4 cell line with higher FANCD2 expression showed no migration on collagen I (S10 Fig). This indicates that FANCD2 may function in non-canonical roles, such as in inhibiting cell migration, alongside the classical role it plays in ICL repair.

4. Discussion

Due to its role as an FA protein, FANCD2 is generally associated with repair of DNA ICLs, such as those induced by platinum (Lopez-Martinez et al., 2016). Although mutations in non-BRCA components of the FA pathway are rare in HGSOC (Bell et al., 2011), modulation of FA protein expression does occur, and can be associated with differences in platinum sensitivity (Taniguchi et al., 2003). Previous studies have shown that FANCD2 expression may be of value as a prognostic biomarker in HGSOC (Moes-Sosnowska et al., 2019; Joshi et al., 2020). However, the dynamics of FANCD2 regulation in HGSOC have not been thoroughly explored. Although it is known that platinum treatment can lead to temporary changes in FANCD2 expression as part of the immediate cellular response to DNA damage (Konstantinopoulos et al., 2009; Koussounadis et al., 2014), whether this can lead to more permanent changes in the landscape of HGSOC tumours was previously unknown. However, both FANCD2 and other FA pathway proteins have been shown to be upregulated in platinum resistant lung cancer cells (Chen et al., 2016).

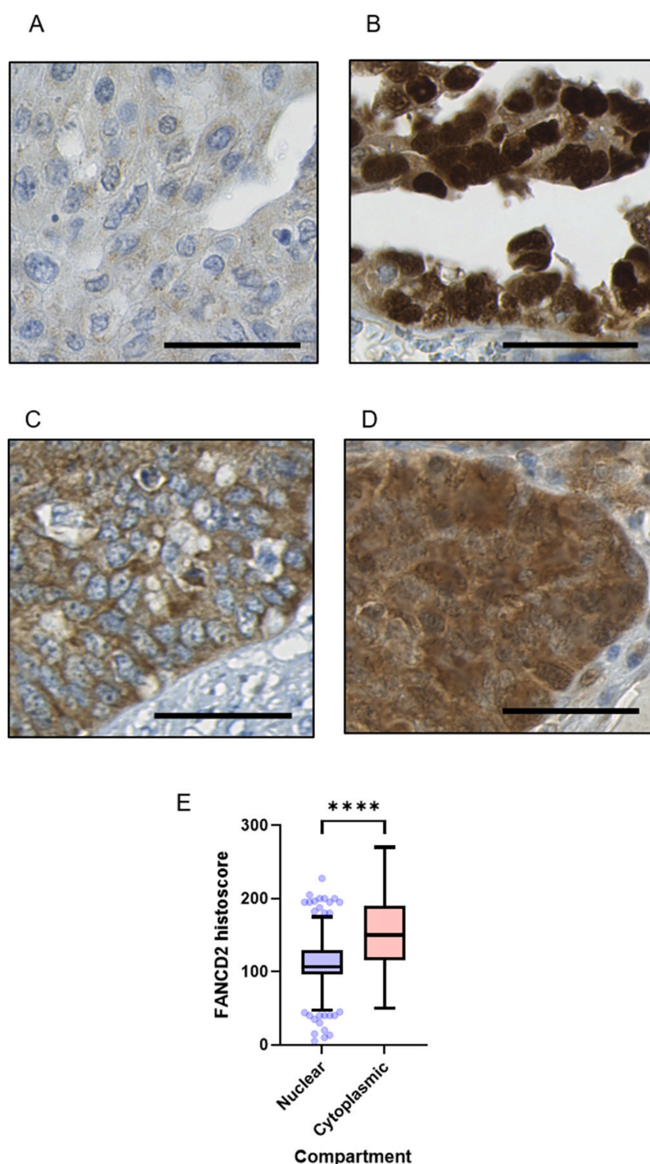

Fig. 6. FANCD2 expression occurs in both the nuclear and cytoplasmic compartments in HGSOCs. (A-D) Representative images of HGSOC tumours stained for FANCD2 by immunohistochemistry. Scale bars 50 μM. (A) Weak staining of both nucleus and cytoplasm, (B) Predominantly nuclear staining, (C) predominantly cytoplasmic staining, (D) strong nuclear and cytoplasmic staining. (E) Tukey's box plot of nuclear and cytoplasmic staining intensity across HGSOC tumours ($n = 259$). Significance calculated by unpaired t-test. **** $p < 0.0001$.

We have shown that FANCD2 is upregulated in a cell line model of

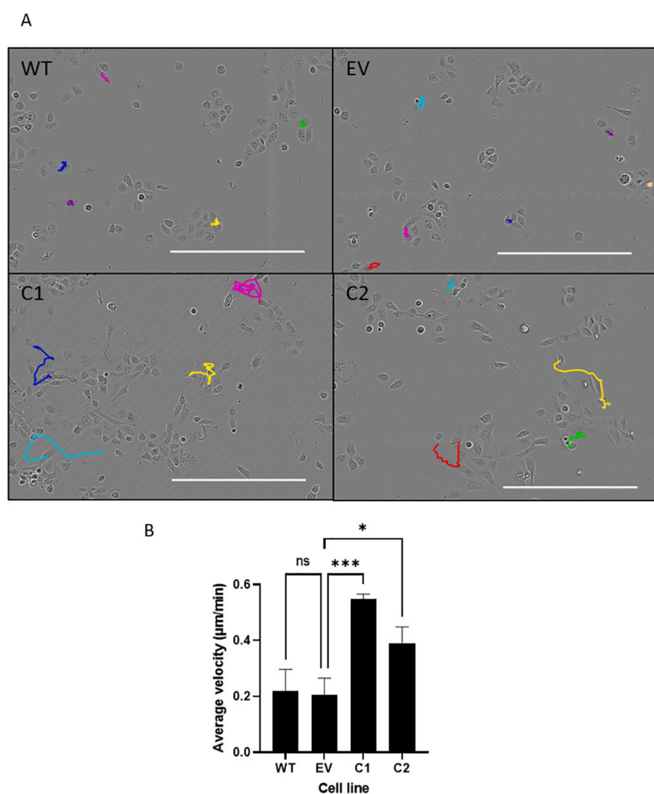

Fig. 7. Knockout of FANCD2 enhances migration of PEO1 cells. (A) Representative images of PEO1 wild type (WT), empty vector (EV) control and FANCD2 knockout clones 1 and 2 (C1 and C2) on collagen I. Coloured tracks show cell motility over 10 h. Scale bars 250 μm. (B) Migration velocity of PEO1 wild type (WT), empty vector (EV) control and FANCD2 knockout clones 1 and 2 (C1 and C2) on collagen I. 30 cells tracked per biological replicate over 3 wells, Mean of 3 biological replicates. Error bars show SD. Significance calculated by ANOVA and post-hoc testing with adjustment for multiple comparisons. *p < 0.05, ***p < 0.001.

chemoresistance generated following the development of clinical chemoresistance in an OC patient, compared with paired cells from the initial platinum sensitive tumour. When further platinum resistant cell lines were generated in vitro, 1/3 of these also demonstrated upregulation of FANCD2. Therefore, FANCD2 expression can be regulated in response to platinum chemotherapy. Interestingly, low levels of FANCD2 expression are a frequent occurrence in ovarian epithelial cells from women at high risk of ovarian cancer and have been linked to cytogenetic instability (Pejovic et al., 2006). Knockout of FANCD2 also leads to increased incidence of epithelial cancers in mouse models (Houghtaling et al., 2003). Initial low expression of FANCD2 may therefore be a favourable factor for the development of HGSOC, but under the selection pressure of platinum treatment, expression within individual cells could be dynamically regulated to promote tumour cell survival. This has been previously reported for another FA protein, FANCF, whereby expression is initially suppressed, but can be upregulated over the course of long-term platinum treatment leading to the development of platinum resistance (Taniguchi et al., 2003). Another alternative is that in a clonally diverse population of cells, those with higher expression of FANCD2 are selected for by platinum chemotherapy, resulting in expansion of these clones and higher overall expression of FANCD2 in the population (Schwarz et al., 2015), as has previously been proposed to account for differences in the PEO1 and PEO4 cell lines (Cooke et al., 2010).

To further explore the role of FANCD2 in chemosensitivity, the effect of modulating FANCD2 expression on sensitivity to carboplatin was tested in OC cell lines. FANCD2 knockdown was confirmed to increase the platinum sensitivity of the PEO4 cell line, indicating that upregulation of FANCD2 contributes to the chemoresistant phenotype observed in this model. This is consistent with reports from other cancer types, where FANCD2 mutation or depletion is associated with increased sensitivity to a range of platinum-based chemotherapies (Metselaar et al., 2019; Kachnic et al., 2010; Matsushita et al., 2005; Bretz et al., 2016). Interestingly, knockdown of FANCD2 did not affect the chemosensitivity of the PEO1 cell line. FANCD2 was therefore depleted in further OC cell lines, which was found to increase platinum sensitivity in half of cell lines tested. Therefore, although FANCD2 expression is highly relevant to platinum resistance of HGSOC, the effect is not universal. The reason for the lack of impact of FANCD2 modulation on some cell lines is not known. However, factors influencing chemosensitivity are diverse, and it is possible that another element of the genetic background is of more importance in these cell lines. This may be due to existing impairment of the FA pathway via a different mechanism, which is common in HGSOC, with approximately 50% of tumours thought to be HR defective (Bell et al., 2011), or another element of the genetic background unrelated to DNA damage repair. This is consistent with our finding that while FANCD2 was upregulated in one of the platinum resistant cell lines generated, the others showed no significant changes in FANCD2 expression, and therefore other mechanisms must be responsible for the enhanced platinum resistance, highlighting the heterogeneity in platinum resistance mechanisms. Indeed, a previous study of in vitro generated platinum resistant PEO1 cells showed that expression of 51 of 1185 genes were upregulated 2-fold, and 36 genes were similarly decreased compared with parental cells, highlighting the diverse mechanisms that can contribute to chemoresistance (Macleod et al., 2005). Notably, the cell lines which FANCD2 knockdown did sensitize to platinum were the more platinum resistant ones tested. Another potential explanation for the increased carboplatin sensitivity associated with FANCD2 expression in PEO4 cells is that FANCD2 expression is a marker of proliferating cells (Hözel et al., 2003), and reduced cellular proliferation could lead to increased drug resistance. However, knockdown experiments suggest that, at least in some contexts, reduced FANCD2 expression can directly affect cellular carboplatin sensitivity. A limitation of our work is that we did not consider activation status of FANCD2, which may also differ between cell lines and affect platinum sensitivity.

The potential mechanism behind the differences in FANCD2 expression in PEO1 and PEO4 cells was also investigated. We found that expression of mTOR is significantly higher in PEO4 cells than PEO1, corresponding with the higher levels of FANCD2 expression. Inhibition of mTOR led to suppression of FANCD2 protein in both cell lines. mTOR is therefore a positive regulator of FANCD2 in this setting, and the increased expression observed in PEO4 cells may contribute to increased resistance to platinum. mTOR has previously been implicated as a positive regulator of FANCD2 in diverse cell types including haematopoietic cells (Guo et al., 2013), hepatocellular carcinoma (Komatsu et al., 2017) and rhabdomyosarcoma (Shen et al., 2013), consistent with our findings. In haematopoietic cells, this occurs via increased phosphorylation of nuclear factor (NF)-κB in the absence of mTOR, which leads to binding of NF- κB to FANCD2 promoter regions and suppression of gene transcription. Regulation of FANCD2 by mTOR, and the contribution of this to chemoresistance, has not previously been documented in OC. However, chemoresistant ovarian cancer has been associated with reduced levels of MiR-497, a negative regulator of mTOR (Xu et al., 2015). Our study suggests a mechanism by which this may alter DNA damage repair pathway activation through modulation of FANCD2, resulting in the observed chemoresistance. Notably, the PI3K/AKT/mTOR pathway is activated in approximately 70% of OCs, making it an attractive therapeutic target (Li et al., 2014). However, clinical trials of inhibitors targeting this pathway have yielded disappointing results, with low response rates in the general patient population, few complete or partial responses to therapy (van der Ploeg et al., 2021) and frequent failure to meet primary survival endpoints (Behbakht et al., 2011).

Clinical trials combining PI3K/AKT/mTOR pathway inhibition with chemotherapy have demonstrated more promising response rates than monotherapy, and may help to overcome resistance in tumours with high levels of FANCD2 expression. However overlapping drug toxicity profiles, particularly haematological toxicities, can limit tolerability (Kollmannsberger et al., 2012), and no inhibitors of this pathway have yet progressed to late phase clinical trials in ovarian cancer. Inclusion of relevant stratification biomarkers may help identify patients who benefit from such inhibitors (van der Ploeg et al., 2021). Further work is however required to define the effect of mTOR inhibitors on platinum sensitivity in FANCD2 high and low expressing cells, and whether this, like the effect of FANCD2 knockdown, is dependent on genetic background. Given the wide range of downstream signalling targets of mTOR, consideration should be given to the compound effects of inhibiting this pathway, and whether this results in the same effects as FANCD2 ablation. For example, a previous study noted that treatment of OC cell lines with mTOR inhibitors resulted in heterogeneous effects on cellular proliferation and migration across the cell line panel. Notably, despite our finding that FANCD2 knockout enhanced migration in PEO1 cells, mTOR inhibitors typically inhibit migration, which may be due to effects of these inhibitors on additional targets (Xiao et al., 2020).

Previous studies have shown that differential FANCD2 expression can impact patient outcomes in ovarian and other cancers, in a localization-dependent manner. High expression of nuclear FANCD2 has been associated with poor survival in endometrial and breast cancers (Zheng et al., 2022; van der Groep et al., 2008). However, in a non-small cell lung cancer cohort, no associations between nuclear FANCD2 expression and survival were observed (Ferrer et al., 2005). Expression of cytoplasmic FANCD2 was associated with good prognosis in ovarian cancer (Joshi et al., 2020), and loss of cytoplasmic FANCD2 linked to increased risk of death in breast cancer (Rudland et al., 2010). In our HGSOC patient cohort, we found no association between FANCD2 expression in either the nucleus or cytoplasm and patient outcomes, contrary to previous work (Moes-Sosnowska et al., 2019; Joshi et al., 2020). This could be due in part to the complexity of interpreting different cellular staining patterns of FANCD2, as it has been previously speculated that FANCD2 may perform different functions in different subcellular locations (Moes-Sosnowska et al., 2019; Rudland et al., 2010). Although one of the aforementioned studies reported that increased FANCD2 expression was associated with poor prognosis in ovarian cancer, expression was only analysed at the mRNA level, and protein localisation was not taken into account (Moes-Sosnowska et al., 2019). The subcellular distribution of FANCD2 in these samples is therefore unknown, and could be either nuclear, cytoplasmic, or both, leading to different outcomes. Interestingly, the association they found between high FANCD2 expression and poor prognosis is more in line with the effect of high nuclear FANCD2 expression in other cancer types (Zheng et al., 2022; van der Groep et al., 2008). While cytoplasmic expression of FANCD2 was previously associated with good prognosis in ovarian cancer in another study, the study focused on tumours with total loss of expression of cytoplasmic FANCD2, whereas these were rare in the cohort we analysed, with only 5/259 cases demonstrating histoscores of under 100 for cytoplasmic FANCD2. Therefore, complete FANCD2 loss as a parameter may have limited utility in HGSOC. Another possible explanation for the lack of association between FANCD2 expression and survival is that, as the samples stained in this study were obtained at primary debulking surgery and were chemonaïve, the FANCD2 levels observed may not have been representative of those present post chemotherapy. This is in agreement with the cell line data presented here, in which FANCD2 levels were higher following chemotherapy. In future work, it would be interesting to assess FANCD2 expression of patient tumours pre- and post-chemotherapy, and determine whether any differences in expression level or protein localisation occur. A further point to consider is that if the effect of FANCD2 expression is reliant on other genetic factors, as is supported by cell line data, a general trend in a patient cohort may not be observed.

In our patient cohort, overall levels of cytoplasmic staining were high, and most individual cases exhibited stronger cytoplasmic than nuclear staining. High levels of cytoplasmic FANCD2 were also observed in previous studies of breast and ovarian tumours (Joshi et al., 2020; Rudland et al., 2010), but non-small cell lung cancers showed strong staining in the nuclear compartment, yet absence of staining in the cytoplasm (Ferrer et al., 2005). These distinct localization patterns may be representative of different functions of FANCD2 in different subcellular contexts (Joshi et al., 2020), and could indicate the relevance of cytoplasmic FANCD2, as well as nuclear FANCD2, in HGSOC. It is possible that cytoplasmic FANCD2 is able to interact with binding partners in the cytoplasm that it is normally spatially separated from, causing activation of distinct signalling pathways. Alternative functions for nuclear and cytoplasmic FANCD2 may also explain why knockdown of FANCD2 in some OC cell lines had no effect on platinum sensitivity.

A proposed alternative function of cytoplasmic FANCD2 is in cell migration and metastasis. However, reports on how FANCD2 expression may affect this vary between cancer types. In breast cancer, loss of cytoplasmic FANCD2 is associated with upregulation of markers of migration, invasion and metastasis OPN, S100A4, S100P and AGR2 (Rudland et al., 2010). Loss of cytoplasmic and nuclear FANCD2 alongside significant expression of the aforementioned markers has also been observed in brain metastases (Zakaria et al., 2016). FANCD2 deficiency in head and neck squamous cell carcinoma is also associated with activation of non-canonical signalling pathways and cytoskeletal rearrangement, resulting in enhanced cell motility (Romick-Rosendale et al., 2016). In contrast, in melanoma and endometrial tumour cells, knockdown of FANCD2 has been reported to supress invasion and migration (Zheng et al., 2022; Bourseguin et al., 2016), and in colorectal cancer low expression of FANCD2 is linked with poor prognosis and metastasis, with the majority of FANCD2 expression occurring in the cytoplasm (Ozawa et al., 2010). These apparently conflicting studies may indicate different roles for FANCD2 in the context of different cancer types. A role for FANCD2 in invasion and metastasis of OC has not previously been described. However, we observed loss of FANCD2 causing increased migration and that is consistent with the positive effect on cell survival linked to high cytoplasmic staining for ovarian cancer previously reported (Joshi et al., 2020).

In conclusion, we have demonstrated a novel mechanism of chemoresistance in OC cells, involving upregulation of the FA protein FANCD2. This can be mediated by mTOR, which positively regulates FANCD2 expression. Modulation of FANCD2 can also enhance chemosensitivity of OC cell lines, although this effect may be context dependent, and a prognostic effect was not observed in our patient cohort. However, FANCD2 also demonstrates non-canonical functions in OC, such as in suppression of migration, which may be dependent on subcellular localization. As FANCD2 is expressed in both the cytoplasm and nucleus of HGSOCs, and can be modulated in response to platinum treatment, interpretation of FANCD2 expression as a biomarker or target for platinum sensitization is therefore complex and may benefit from further study.

Additional information

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CRediT authorship contribution statement

Sarah J. Taylor: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Robert L. Hollis:** Writing – review & editing, Resources, Methodology. **Charlie Gourley:** Writing – review & editing, Resources, Methodology. **C. Simon Herrington:** Writing – review & editing, Resources, Methodology. **Simon P. Langdon:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Mark J. Arends:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Data availability

Relevant data are within the manuscript and its supporting Information files. Case-level data for the clinical cohort, excluding immunohistochemistry data for FANCD2, is not available due to constraints imposed by our ethical framework.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2024.104916.

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