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The disruption of the CCDC6 – PP4 axis induces a BRCAness like phenotype and sensitivity to PARP inhibitors in high-grade serous ovarian carcinoma

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Abstract

Background: Treatment with PARP inhibitors (PARPi) is primarily effective against high-grade serous ovarian cancers (HGSOC) with BRCA1/2 mutations or other deficiencies in homologous recombination (HR) repair mechanisms. However, resistance to PARPi frequently develops, mostly as a result of BRCA1/2 reversion mutations. The tumour suppressor CCDC6 is involved in HR repair by regulating the PP4c phosphatase activity on γH2AX. In this work, we reported that in ovarian cancer cells, a physical or functional loss of CCDC6 results synthetic lethal with the PARP-inhibitors drugs, by affecting the HR repair. We also unravelled a role for CCDC6 as predictive marker of PARPi sensitivity in ovarian cancer, and the impact of CCDC6 downregulation in overcoming PARPi resistance in these tumours.

Methods: A panel of HGSOC cell lines (either BRCA-wild type or mutant) were treated with PARPi after CCDC6 was attenuated by silencing or by inhibiting USP7, a CCDC6-deubiquitinating enzyme, and the effects on cell survival were assessed. At the cellular and molecular levels, the processes underlying the CCDC6-dependent modification of drugs’ sensitivity were examined. Patient-derived xenografts (PDXs) were immunostained for CCDC6, and the expression of the protein was analysed statistically after digital or visual means.

Results: HGSOC cells acquired PARPi sensitivity after CCDC6 depletion. Notably, CCDC6 downregulation restored the PARPi sensitivity in newly generated or spontaneously resistant cells containing either wild type- or mutant-BRCA2. When in an un-phosphorylated state, the CCDC6 residue threonine 427 is crucial for effective CCDC6-PP4 complex formation and PP4 sequestration, which maintains high γH2AX levels and effective HR. Remarkably, the PP4-dependent control of HR repair is influenced by the CCDC6 constitutively phosphorylated mutant T427D or by the CCDC6 loss, favouring PARPi sensitivity. As a result, the PP4 regulatory component PP4R3α showed to be essential for both

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Background

Members of the poly(ADP-ribose) polymerase (PARP) superfamily of enzymes, specifically PARP1 and PARP2, are quickly recruited to DNA damage foci and initiate DNA damage response (DDR) after exposure to numerous DNA damage insults. Poly(ADP ribosylation), also known as PARylation, is a posttranslational modification that is massively catalysed during DDR by the PARP1 and PARP2 proteins, assisted by the Histone PARylation Factor 1 (HPF1) protein [1, 2]. In response to DNA damaging insults, the transfer of ADP-ribose units from the donor NAD+ onto target proteins is essential for efficient repair, for instance by organising both chromatin rearrangements and the recruitment of DDR factors, such as ALC1, XRCC1, and LIG3 [3–6].

The enzymatic inhibition of DNA repair PARPs by small molecules has pioneered a synthetically lethal target therapy strategy for treatment of tumours characterised by DDR defects, such as breast and ovarian cancer carrying BRCA1 and BRCA2 mutations [7, 8]. Several PARP inhibitors (PARPi) have been developed and currently employed in the clinic or in clinical trials; these include olaparib, niraparib, rucaparib, veliparib, and talazoparib [9].

The inhibition of PARP1/2 enzymatic activity, coupled with PARP trapping, determines cells dependency on parallel pathways to maintain genome integrity, in particular homologous recombination (HR) [6, 7, 10, 11]. When HR is compromised, for example, due to inactivating mutations in BRCA1 or BRCA2, cells are rendered superbly sensitive to PARPi [12–15]. The synthetic lethality strategy has been further extended to additional tumours with DDR defects, such as in ATM, ATR, and PALB2 genes, and has shown impressive efficacy of PARPi in women with high-grade serous ovarian cancer (HGSOC), as maintenance treatment following platinum chemotherapy [7, 16, 17].

The most prevalent and deadly form of ovarian cancer, HGSOC, is frequently identified with extensive peritoneal metastases. Importantly, approximately 50% of HGSOCs are deficient in DDR, where germline or somatic mutations of BRCA1 and BRCA2 account for 20% of the cases, and BRCA1/2 germline and somatic mutations represent clinically validated predictive biomarker of PARPi sensitivity [18]. In addition to BRCA1 and BRCA2 mutations, many other genetic alterations affecting DDR occur; i.e. the epigenetic silencing of BRCA1 (11%), the amplification or mutation of EMSY (8%), the deletion of PTEN (7%), the hypermethylation of RAD51C (3%), mutations in ATM or ATR (2%) or mutations in FANC genes (5%) [19]. Overall, HGSOC has a mutational landscape that makes it susceptible to PARPi treatment.

Nevertheless, 20% of BRCA1/2-mutated ovarian malignancies do not respond to PARPi [20–23], highlighting the urgent need for new treatment approaches for inherently or acquired resistant tumours. The inhibition of poly(ADP-ribose) glycohydrolase (PARG), one of the enzymes necessary for reversing PARylation processes, shows promise as a treatment strategy for PARPi-resistant HGSOC [24–26]. It should be emphasised that HGSOC’s susceptibility to PARPi and PARG inhibitors (PARGi) are mutually exclusive, which raises the possibility of a novel treatment approach for ovarian malignancies that are PARPi-resistant [24]. However, there is still a critical medical need for discovering novel biomarkers that can predict the sensitivity or resistance of HGSOC and/or alter the response of cancer to such medications when biomarkers are lost or acquired in tumours. One potential predictor and susceptibility factor for PARPi is CCDC6.

CCDC6 is a tumour suppressor protein known to be functionally lost due to gene translocations, somatic mutations, and altered protein levels, in several tumours [27]. CCDC6 translocates to the nucleus after DNA damage, where it participates to the HR via controlling H2AX phosphorylation following binding with the protein phosphatase PP4c [28, 29]. Notably, the balance between the E3 ubiquitin ligase FBXW7 and the de-ubiquitinase USP7 activities tightly controls CCDC6 protein levels and functions [30].

Here we unravelled a role for CCDC6 gene product in PARPi sensitivity of ovarian cancer cells via the PP4c-mediated modulation of HR proficiency. The physical or functional loss of CCDC6 could also overcome the acquired resistance to PARPi treatment in ovarian cancer patients. Altogether, our data suggest the potential of...
directly or indirectly targeting CCDC6 to tackle PARPi-resistance in HGSOCs.

**Methods**

**Cell lines, drugs and chemicals**

Ovarian cancer cell lines, OVCAR3, OV-90, PEO1 and PEO4 were purchased from ATCC (American Type Culture Collection) and were maintained, respectively, in RPMI (OVCAR3, PEO1 and PEO4) and DMEM (OV-90) (Gibco, Paisley, UK), supplemented with 20% (OVCAR-3) or 10% (OV-90, PEO1 and PEO4) fetal bovine serum (FBS; Gibco BRL, Italia), 1% L- Glutamine and 1% of penicillin – streptomycin (Gibco, Paisley, UK). Kuramochi cells were purchased from Sekisui XenoTech, LLC and were maintained in RPMI (Gibco, Paisley, UK), supplemented with 10% FBS, 1% L-Glutamine and 1% of penicillin – streptomycin (Gibco, Paisley, UK). Olaparib (AZD2281), and P005091 were provided by SelleckChem. PARG inhibitor (PDD00017273), SB-216763, cycloheximide, MG132 and cisplatin were provided by Merk Millipore. The genetic background of the ovarian cancer cells is reported in Additional File 1: Table S1.

**Generation of olaparib resistant (olaR) ovarian cancer cell lines**

To generate OV-90 and OVCAR3 cells chronically resistant to olaparib (OV-90 olaR and OVCAR3 olaR), the cells were treated with an initial concentration of olaparib [2.5 µM] for 72 h. The treatment was followed by three times cells washing with Dulbecco’s phosphate-buffered saline (DPBS), followed by trypsinization and splitting. The surviving cells were grown to 80% confluence and split once a week over a period of at least two weeks to ensure viability. The concentration of drug was then sequentially increased to [5.0 µM] and [10 µM]. The OVCAR3 olaR and the OV-90 olaR cells viability was assessed by CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega (Madison, WI, USA).

**Reagents and antibodies**

For the biochemical and immunofluorescence analysis the following antibodies were utilised: anti-CCDC6 (ab56353) Abcam, plc (Cambridge, UK), anti-tubulin (T6557), anti-PARG clone D8B10 (MABS61), anti-pan-ADP-ribose (MABE1016) Sigma-Aldrich, Inc, anti-PCNA (NANO3), anti-γH2AX (#05,636) Millipore (Burlington, MA, USA), anti-USP7 (A300-033A) Bethyl, Inc (Montgomery, TX, USA), anti-PARP (#9542) Cell Signaling, Inc (Danvers, MA, USA) and anti-Myc clone 9E10 (sc-40) Santa Cruz Biotechnology, Inc (Dallas, TX, USA). For the immunohistochemical analysis the anti-CCDC6 antibody (HPA 019,051) was from Merk Millipore. The secondary antibodies were from Biorad (Hercules, CA, USA).

**Sensitivity test and design for drug combination**

Antiproliferative activity was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega (Madison, WI, USA), in terms of 50% inhibitory concentration (IC50) values.

The cells were plated in triplicate in 96-well plates at a density of 1000 cells per well, and continuously exposed to each drug for 144 h. Each assay was performed in triplicate and IC50 values were expressed as mean±standard deviation.

The results of the combined treatment were analysed by using the CompuSyn software [31]. The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. CI = 1 denotes additivity, CI > 1 denotes antagonism and CI < 1 denotes synergism.

**Protein extract and western blot analysis**

Total cell extracts (TCE) were prepared with lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Na Deoxycholate, 0.1% SDS) and a mix of protease inhibitors. Protein concentration was estimated by a modified Bradford assay (Biorad). For Western blotting, cell lysates were separated by SDS-PAGE (10% polyacrylamide) and the proteins were transferred to a PVDF membrane. Membranes were blocked with 5% TBS-BSA and incubated with the primary antibodies. Immunoblotting experiments were carried out according to standard procedures and visualised using the ECL chemiluminescence system (Amersham, Little Chalfont, UK/Pharmacia Biotech, Milano, Italy). As a control for equal loading of protein lysates, the blotted proteins were probed with antibody against γ-tubulin.

**Plasmids and transfection**

pcDNA4ToA-myc-CCDC6 plasmids were transfected with FuGene HD (Promega). From the pcDNA4ToA-myc-CCDC6 wild type template two myc-CCDC6 mutants (T427D and T427A), were created using the Quick-Change Site Directed Mutagenesis Kit, Agilent (Santa Clara, CA, USA), with the following primers: T427D Fw 5’-tgctggatatgagcggcgtgctctgcggttaagg-3’, T427D Rv 5’-aattgtccagccgctctgctctgttc-3’, T427D Rv 5’-aatcagacccgcccccc-3’, T427D Rv 5’-attcagacccgcccccc-3’. CCDC6 shRNA (pLKO.1 puro) was from Sigma-Aldrich, Inc. OV-90 and Kuramochi were transfected with a pool of non-targeting vector (ShCTRL) or with a plasmid pool (shCCDC6, NM_005436) for transient CCDC6 silencing by FuGene HD for 48 h.
For production of stable silenced cells, OVCAR-3 were transfected with ShCTRL or with ShCCDC6 plasmid pool by Nucleofection and selected with 0.5 μg/ml of Puromycin.

The siRNAs employed in this study were purchased from Sigma-Aldrich. The RNAi transfections were performed using Oligofectamine Reagent (Invitrogen). The PP4R3α siRNA oligo are: 5’-UGA AUU AAG UCG CUC UGA AUU-3’ and 5’-UUC AAG GCG ACU UAA UUC AUU-3’.

The pDR-GFP reporter plasmid is based on a construct developed by M. Jasin [32] and contains two mutated GFP genes separated by a puromycin drug selection marker.

Homologous recombination transient assay
Ovarian cancer cells were plated in a 60 mm plate and transfected with the pDR-GFP reporter alone (as negative control) or together with the pCAGGS-I-SceI plasmid. After 48 h cells were collected and analysed by FACS analysis with BD Accuri C6 Flow Cytometer (BD Bioscience, Canada).

The schematic representation of the pDR-GFP assay is shown in Additional File 2: Figure S1.

Colony forming assay
10,000 cells (shCTRL or shCCDC6) per well were seeded into 6 well plates and continuously treated with the different drugs. After 10 days of incubation, prior to counting colonies, cells were fixed in methanol and stained with 0.5% Crystal Violet (10 min at room temperature). A population of at least 30 cells was scored as one survivable colony and considered for the count.

The colonies’ counting was performed at the optic microscope and through the open-source software ImageJ-NIH. The relative colony formation (percentage of colonies) was expressed as the number of colonies per treatment versus colonies that appeared in the DMSO control (mean colony counts ± standard errors are reported).

TMA and IHC
A case series of 251 primary ovarian cancers was analysed on TMA. 6 TMAs were prepared with all the analysed samples [33]. Each tissue was examined for representability, and subsequently immunostained with anti-CCDC6 antibody (HPA 019,051, from Sigma-Aldrich), CCDC6 immunoreactivity of tumour cells was annotated. Visual annotation included staining intensity (negative, weak, moderate or strong), fraction of stained cells (% of total counted tumour cells), and subcellular localization. Following acquisition of anti-CCDC6 immunostained glass slides with a digital scanner, we also calculated the H-score on digital images by QuPath image analysis software [34, 35]. Immunohistochemistry was performed as already described [30]. Median H-score was 64.2 (IQR 28.8; 97.2); Mean H-score was 67.5 (sd 47.1). The frequency distribution of digitally evaluated CCDC6 H-Score is summarised in Additional File 3: Table S2. A summary of study population is shown in Additional File 4: Table S3.

Cell blocks
Anti-CCDC6 immunostaining was performed on cell blocks-included OV-90 and OVCAR3 cells, parental (WT) and olaparib-resistant (olaR); PEO1 empty vector and myc-tagged CCDC6 overexpressing cells; PEO4 silenced for CCDC6 (shCCDC6) or control (shCTRL), as in plasmid and transfection paragraph.

Confluent cells, seeded in 100 mm plates, were collected and resuspended in 10% formalin and processed following 12 h fixation. According to manufacturer’s instructions (7401150—Thermo Fisher Scientific, UK), collected samples were centrifuged for 1 min at 2.5 rpm and cell pellet resuspended in Reagent 2. Three drops of Reagent 1 were added into centre of Cytoblock cassette well and spun at 1500 rpm for 5 min. The Cytoblock cassette was processed in a standard tissue processor [36].

Results
CCDC6 protein levels contribute to the differential sensitivity to PARG and PARP inhibitors in high-grade serous ovarian carcinoma cell lines
The identification of tumour biomarkers that can predict sensitivity and/or resistance to PARPi as well as further enhance the selective cytotoxicity of such therapeutic drugs is highly envisaged in order to establish an accurate personalised medicine of HGSOC. According to preclinical research, attenuation of CCDC6 in several cancer cell cultures enhances sensitivity to PARPi, which have the ability to work in concert with cisplatin [36–41].

Here, we investigated if CCDC6 expression would influence how sensitive a panel of ovarian cancer cell lines was to PARPi and PARGi.

To do so, in three ovarian cancer cell lines, namely Kuramochi, OVCAR3 and OV-90 cells, harbouring different genetic backgrounds (Additional File 1: Table S1) [42], we assessed relative sensitivity to the PARPi olaparib in colony formation assays: for these set of experiments
we used a single representative drug concentration, as indicated [shCTRL] (Fig. 1A-C). The Kuramochi cells appeared minimally sensitive to PARPi (Fig. 1A) in comparison to the OVCAR3 and OV-90 cells (Fig. 1B, C), as supported by statistical analysis (Fig. 1A-C). The silencing of CCDC6 (shCCDC6) or pharmacological inhibition of the de-ubiquitinase USP7 (P5091), which results in proteasome-dependent degradation of CCDC6 [30] (Additional File 5: Figure S2A-F), determined a positive modulation of the PARPi sensitivity in all three cell lines (Fig. 1A-C).

Conversely, the Kuramochi cells resulted very responsive to the PARGi (PDD00017273) treatment, while OVCAR3 and OV-90 cells scarcely responded to PARGi (Fig. 1A-C) [24]. The low level of PARG and the high levels of ADP-ribosylated proteins, which are revealed by a pan-ADP-ribose antibody, supported the lower PARPi sensitivity and the higher PARGi sensitivity of Kuramochi cells, in comparison to OVCAR3 and OV-90 cells [6] (Additional File 5: Figure S2G, H). In a 2-D colony-forming assay with one concentration drug, CCDC6 silencing diminished sensitivity to PDD00017273 in Kuramochi cells (Fig. 1A). By contrast, the CCDC6 attenuation minimally affected the PARGi sensitivity in OVCAR3 and OV-90 cells (Fig. 1B-C). Nevertheless, when these ovarian cancer cells were challenged with different concentrations of PARGi and their vitality assessed by MTT assays, the change of PARGi sensitivity upon CCDC6 downregulation appeared evident, as described below.

The relative CCDC6 and USP7 levels in all the analysed ovarian cancer cells are shown in Additional File 5: Figure S2G.

Together these results demonstrate that the genetic or pharmacological modulation of the CCDC6 protein levels in ovarian cancer cells affects sensitivity to the anti-cancer PARPi as well as to the recently developed PARGi.

**CCDC6 depletion affects γH2AX foci formation in response to PARPi and PARG inhibitors and impairs double strand breaks repair by Homologous Recombination**

We evaluated the formation of γH2AX foci, a marker for double strand breaks (DSBs), in order to thoroughly study the molecular mechanisms of CCDC6-dependent effects on the susceptibility of HGSOC cells to PARPi and/or PARGi [43, 44]. In contrast to the untreated cells, foci formation was significantly induced by both PARPi and PARGi in the Kuramochi and OV-90 cell lines, and CCDC6 silencing significantly reduced these effects. In comparison, PARPi and PARGi had minimal effect on γH2AX foci in control OVCAR3 (shCTRL) cells. However, CCDC6 silencing significantly reduced PARPi-induced foci formation in these cells (Fig. 2A, B). Kuramochi cells responded dramatically to the addition of the PARGi therapy, as previously described [24].

Strikingly, silencing of CCDC6 resulted in a considerable reduction in γH2AX foci in all three HGSOC cell lines as seen in the Figure and by relative intensity quantification (Fig. 2A, B). Notably, over-expression of myc-CCDC6 plasmid rescued γH2AX foci formation in CCDC6-downregulated cells, thus excluding off-target issues (Additional File 6: Figure S3, Additional File 7: Figure S4, Additional File 8: Figure S5).

The drugs’ effect observed upon CCDC6 silencing suggested an impairment of DSBs repair by HR in the analysed ovarian cancer cells. In order to assess the efficacy of the HR repair, we employed the DR-GFP reporter system [32]. A schematic representation of the DR-GFP assay is shown in Additional File 2: Figure S1. In these assays, we decided to restrain endogenous CCDC6 activity by using the pharmacologic inhibitor P5091. The ovarian cancer cells, left untreated or after pre-treatment with P5091, were transfected with the DR-GFP reporter plasmid alone, as a control, or together with the I-SceI plasmid able to induce DSBs. The ability to repair the DSBs by HR was measured by flow cytometry and the frequency of HR was reported as a percentage of GFP positive cells. Treatment with USP7 inhibitor determined a significant decrease of the GFP positive cells, compared to non-treated cells, suggesting that the reduction of CCDC6 levels affected the DNA repair by HR in all the ovarian cancer cells analysed, even if the effects were most evident in OVCAR3 and OV-90 cells, with respect to Kuramochi cells (Fig. 2C). The phenotype induced by the P5091 treatment was mainly dependent on the CCDC6 increased turnover, as it was almost completely (Kuramochi) or partially (OVCAR3 and OV-90) reverted following the myc-CCDC6 transient transfection (Fig. 2C). In
Fig. 1 (See legend on previous page.)
ovarian cancer cells the transfection efficiency of HA-IsceI, in presence or absence of the myc-CCDC6 plasmid, was assessed by western blot (Fig. 2D-F).

These data suggest that CCDC6 loss of function confers an HR-deficiency phenotype in HGSOC cancer cells.

**CCDC6 loss sensitises the high-grade serous ovarian carcinoma cell lines to combined treatment with PARPi inhibitors and cisplatinum**

Since the HR deficiency is also accompanied by sensitivity to PARPi, we used cellular metabolic activity as a marker of cell survival to characterize and quantify the effects of CCDC6 downregulation on olaparib sensitivity in HGSOC. To do this, we reduced CCDC6 protein levels in ovarian cancer cells using the USP7 inhibitor P5091 and evaluated sensitivity to various olaparib concentrations. Similarly, PARPi and cisplatin sensitivity were also assessed (Fig. 3A-I). Generally, CCDC6 downregulation by P5091 improved sensitivity to olaparib in all cell lines herein analysed. Specifically, the sensitivity to olaparib in Kuramochi cells (IC50 7.76 μM ± 0.74) was positively modulated to 3.45 μM ± 0.39 by the CCDC6 chemical attenuation (Fig. 3A). Stronger cytotoxic effects were attained in P5091-pretreated OVCAR3 and OV-90 cells when exposed to olaparib compared to Kuramochi cells; these cells’ sensitivity increased by about three and five times, respectively (Fig. 3D, G). Similarly, genetic downregulation of CCDC6 by shRNA leads to overlapping improved sensitivity to olaparib in all cellular models herein analysed (Additional File 9: Figure S6A, D, G).

Platinum containing drugs are widely used for the treatment of ovarian cancer. However, ovarian cancer patients initially responsive to platinum containing drugs invariably relapse becoming resistant. Here we asked whether CCDC6 downregulation may affect the vulnerability of HGSOC to cisplatin alone or in combination with olaparib. While sensitivity to cisplatin was only slightly affected by P5091 (IC50 5.89 μM ± 0.07) in Kuramochi cell lines compared to vehicle (IC50: 6.63 μM ± 0.02) (Fig. 3A), CCDC6 chemical downregulation significantly modifies cisplatin sensitivity in OVCAR3 (IC50 2.42 μM ± 0.03 vs IC50 1.20 μM ± 0.04) and OV-90 cells (IC50 4.49 μM ± 0.05 vs IC50 0.48 μM ± 0.03) (Fig. 3D, G). Strikingly, in these cells the combined treatment of olaparib with cisplatin showed a synergistic effect, magnified by the CCDC6 accelerated turnover, upon P5091 addition (Fig. 3E, H).

Conversely, sensitivity to PARPi in CCDC6 downregulated cells behaved differently compared to olaparib treatment, which is explained by their different mechanism of action (as discussed later in the text). As reported, Kuramochi cells resulted highly responsive to the PARPi treatment (IC50 0.49 μM ± 0.35). Notably, the CCDC6 downregulation by P5091 treatment affected PARPi-induced cytotoxicity (in the presence of P5091 at 2.5 μM, the IC50 raised to 1.33 μM ± 0.32) (Fig. 3A). Similar results were obtained by silencing CCDC6 by shRNA (Additional File 9: Figure S6A). The CCDC6-dependent weak response to PARPi was also visible in intrinsically PARPi-resistant OVCAR3 and OV-90 cell lines, where the low sensitivity to PARPi was enhanced by P5091 (Fig. 3D, G). In detail, following the PARPi addition, the CCDC6-depleted OV-90 cells showed an IC50 of 17.05 μM ± 0.72 compared with an IC50 of 11.02 μM ± 0.43 of control cells. The CCDC6-depleted OVCAR3 cells also exhibited a mild modulation showing an IC50 of 18.94 μM ± 0.59 compared with an IC50 of 17.60 μM ± 0.10 of control cells (Fig. 3D, G). Importantly, all these phenotypes were rescued by CCDC6 exogenous expression in CCDC6 chemically or genetically downregulated cell lines (Additional File 9: Figure S6 A, B, D-E and G-H), thus supporting the specificity of our observations. In detail, in Kuramochi-CCDC6-depleted cells the re-expression of CCDC6 (Additional File 9: Figure S6B) led to an IC50 of 0.76 μM ± 0.10 vs an IC50 of 1.11 μM ± 0.08 detected in control cells (transfected with the empty vector, EV); in OVCAR3 CCDC6 silenced cells the re-expression of CCDC6 (Additional File 9: Figure S6E) determined an IC50 of 18.52 μM ± 0.08 vs an IC50 of 19.36 μM ± 0.05 relieved in control cells; and in OV-90 cells CCDC6-depleted, the re-expression of CCDC6 (Additional File 9: Figure S6F) led to an IC50 of 15.08 μM ± 0.07 vs an IC50 of 17.80 μM ± 0.02 detected in control cells (transfected with the empty vector, EV). By contrast, the overexpression of CCDC6 in wild type Kuramochi, OV-90
Fig. 2 (See legend on previous page.)
and OVCAR3 cells did not determine any variation in the IC50 values upon treatment with different doses of PARPi (Additional File 9: Figure S6B, E, H).

By subjecting HGSOC cells to various PARPi with varied trapping and inhibitory potencies, we further tested whether the increased sensitivity to olaparib could be replicated [45]. Both talazoparib and veliparib, PARP inhibitors with greater PARP activity inhibition and trapping action, have recently received clinical approval for breast cancer treatment, enabling dosage reduction and improving clinical efficacy [17]. Therefore, we examined their effectiveness alone or in conjunction with P5091 in our HGSOC preclinical models. Intriguingly, pharmacological downregulation of CCDC6 enhances the cytotoxic effects of both PARPi in all three cellular models, with P5091-treated OVCAR3 cells showing the most impressive 18.6-fold increase in talazoparib sensitivity (Fig. 3C, F, I).

These findings imply that CCDC6 downregulation enhanced HGSOC cell line sensitivity to various PARPi, regardless of their trapping and inhibitory efficacy.

**CCDC6 downregulation rescues the sensitivity to olaparib in newly generated PARP inhibitor resistant ovarian cancer cells**

Our results suggest the potential of predicting PARPi sensitivity in HGSOCs harbouring CCDC6 loss of functions as well as of downregulating CCDC6 by USP7 inhibitor, even in PARPi-resistant, BRCA wild type (WT), HR competent HGSOC cells. In order to address the intriguing properties of CCDC6 downregulation in re-sensitising PARPi-resistant HGSOC, we generated olaparib-resistant OVCAR3 and OV-90 cell lines, thereafter named OVCAR3olaR and OV-90olaR, respectively, by exposing cell lines to PARPi at IC50 doses and up to 10 μM for two weeks, as described in material and methods section. Compared to the parental OVCAR3 and OV-90 cells, the cytotoxic effects of the PARPi treatment were quantified in the newly generated OVCAR3olaR and the OV-90olaR by cell viability assays (Fig. 4A). Of note, compared to parental cells, CCDC6 protein levels were slightly increased in OV-90olaR at IHC and western blot, while resulted comparable in PARPi-resistant OVCAR3 cells (Additional File 10: Figure S7 D-G). However, the CCDC6 attenuation upon the P5091 treatment, rescued the sensitivity to PARPi in OVCAR3olaR and OV-90olaR HGSOC cells (Fig. 4C, D).

The generation of the olaparib resistance phenotype left nearly unaffected the cisplatin sensitivity in these cells (Fig. 4A). However, while in OV-90olaR cells the combined treatment of cisplatin and olaparib resulted in an antagonistic effect (CI > 1), in OV-90 parental cells it determined a synergistic effect (CI < 1) (Fig. 4B). Interestingly, in the presence of P5091, which increases the CCDC6 degradation, the combined treatment of cisplatin and olaparib turned into an additive effect in OV-90olaR cells (CI = 1) (Fig. 4E). No variations in the combination index between OVCAR3 parental and resistant cells upon cisplatin and olaparib treatment were registered (Fig. 4B).

Then, by hypothesizing CCDC6 as a possible biomarker of cisplatin response, we investigated the CCDC6 expression levels in three ovarian patient-derived xenografts (PDXs) couples, sensitive and with acquired resistance to cisplatin (MNHOC 266, MNHOC 124 and MNHOC 239), obtained after multiple in vivo drug treatment [46–48]. In particular, the PDX MNHOC 266, carries mutations in BRCA1, whereas the PDXs MNHOC 124 and MNHOC 239 are BRCA1/2 WT. The models recapitulate the clinical setting of cisplatin resistance. Following an immunohistochemical staining to assess the levels of CCDC6, a strong expression was found in all PDXs (Fig. 5A, B), with a pattern of CCDC6 staining that was comparable in sensitive and the matching cisplatin resistant PDXs.

Although the CCDC6 staining data on PDXs do not discriminate a primary from a resistant ovarian tumour model, we may hypothesise, based on in vitro evidence, that the CCDC6 positive staining could otherwise predict the response to PARPi, as described in other tumour models [27].

All the PDXs were non-responders to the olaparib treatment. They reproduced the phenotype of the newly generated olaparib-resistant ovarian cancer cells, where CCDC6 showed a high level of expression, mostly mimicking the PDX phenotype (Fig. 5, Additional File 10: Figure S7 D-E). We used the well-known PEO1 and PEO4 cells [49], which represent naturally occurring CCDC6 null and CCDC6 competent ovarian cancer cell types,
**A**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Olaparib [IC₅₀]   |
| Olaparib [IC₅₀]  | 3.45 ± 0.30 | 7.76 ± 0.74 |
| PARGI [IC₅₀]     | 1.33 ± 0.32 | 0.49 ± 0.35 |
| Cisplatin [IC₅₀] | 5.89 ± 0.07 | 6.63 ± 0.02 |

**B**

| Combination Index | CI values | Cell Survival (%) |
|------------------|-----------|-------------------|
| CI values        |           | Olaparib + P5091 [2.5 µM] | 0.11 ± 0.08 |
|                  |           | Cisplatin + Olaparib | 1.07 ± 0.01 |

**C**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Talazoparib [IC₅₀] |
| Talazoparib [IC₅₀]| 0.41 ± 0.07 | 1.38 ± 0.21 |
| Veliparib [IC₅₀] | 0.71 ± 0.14 | 3.55 ± 0.34 |

**D**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Olaparib [IC₅₀]   |
| Olaparib [IC₅₀]  | 1.51 ± 0.07 | 4.70 ± 0.21 |
| PARGI [IC₅₀]     | 18.94 ± 0.59 | 17.60 ± 0.10 |
| Cisplatin [IC₅₀] | 1.20 ± 0.04 | 2.42 ± 0.03 |

**E**

| Combination Index | CI values | Cell Survival (%) |
|------------------|-----------|-------------------|
| Cisplatin + Olaparib + P5091 [2.5 µM] | 0.27 ± 0.14 |
| Cisplatin + Olaparib | 0.51 ± 0.13 |

**F**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Talazoparib [IC₅₀] |
| Talazoparib [IC₅₀] | 0.36 ± 0.03 | 0.67 ± 0.12 |
| Veliparib [IC₅₀] | 0.27 ± 0.01 | 1.75 ± 0.07 |

**G**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Olaparib [IC₅₀]   |
| Olaparib [IC₅₀]  | 0.94 ± 0.02 | 5.09 ± 0.15 |
| PARGI [IC₅₀]     | 17.05 ± 0.72 | 11.02 ± 0.43 |
| Cisplatin [IC₅₀] | 0.48 ± 0.03 | 4.49 ± 0.05 |

**H**

| Combination Index | CI values | Cell Survival (%) |
|------------------|-----------|-------------------|
| Cisplatin + Olaparib + P5091 [2.5 µM] | 0.39 ± 0.01 |
| Cisplatin + Olaparib | 0.97 ± 0.02 |

**I**

| Drug Sensitivity | IC₅₀ [µM] | CI values | Cell Survival (%) |
|------------------|-----------|-----------|-------------------|
| P5091 [2.5 µM]   | +         | -         | Talazoparib [IC₅₀] |
| Talazoparib [IC₅₀] | 0.089 ± 0.01 | 0.27 ± 0.06 |
| Veliparib [IC₅₀] | 0.16 ± 0.01 | 1.33 ± 0.17 |

**Fig. 3** (See legend on previous page.)
respectively, that display differing protein levels despite having equivalent amounts of CCDC6 transcripts, to test this hypothesis (Fig. 5C, D). These cells were from the same patient who had a poorly differentiated serous adenocarcinoma and who after chemotherapy had a status change from platinum sensitivity (PEO1) to platinum resistance (PEO4) [49].

Notably, the PEO1 cells are the first and only human BRCA2 defective ovarian cancer cell line identified thus far and, like the original patient, possess a BRCA2 hemizygous nonsense mutation 5193C > G (Y1655X). PEO4 cells, derived from ascites at the time of relapse with cisplatin resistance, have the secondary mutation (Y1655 mutation spontaneously reverted) and are BRCA2 proficient.

Following CCDC6 knockdown by shRNA in the cisplatin-resistant PEO4 primary cells, the cytotoxic effects of olaparib and the cisplatin sensitivity were measured. The very low sensitivity to olaparib in the PEO4 cells (IC50, 56.15 μM±0.18) was positively modulated by the CCDC6 lowering (IC50 dropped to 36.45 μM±0.06) (Fig. 5E); strikingly, in these cells, the cisplatin response (IC50 35.12 μM±0.15) was also positively modulated by the CCDC6 attenuation (IC50 23.23 μM±0.06) (Fig. 5E, Additional File 10: Figure S7A). Most importantly, upon CCDC6 depletion, the combined treatment of cisplatin and olaparib determined a synergistic effect (CI < 1), while an antagonistic effect (CI > 1) was observed in the PEO4 parental cells (Fig. 5F). Remarkably, the dose response index (DRI) was impressively modulated by the drug combination in PEO4 cells, silenced for CCDC6 (DRI > 1), since the IC50 concentration dropped by more than 30% for both the drugs concentration (Fig. 5F).

Notably, the PEO1 cells resulted naturally null for the CCDC6 protein expression, although CCDC6 mRNA is well expressed in these cells and in PEO4 cells. This suggests that post-translational processes are responsible for CCDC6 deregulation in PEO1 cells. According to preliminary results (Morra F et al., in preparation), the low amount of CCDC6 protein detected in PEO1 cells might be ascribed to GSK3β gain of activity, which

**Fig. 4** CCDC6 downregulation can restore the olaparib sensitivity in resistant ovarian cancer cells. A Drugs sensitivity and surviving fraction in OVCAR3 olaparib Resistant (OVCAR3 olaR) and OV-90 olaparib Resistant (OV-90 olaR) cells, compared to the parental OVCAR3 and OV-90 cells, after 144 h exposure to different doses of Olaparib and Cisplatin. B In the figure, the CI values, according to 1:2 concentration ratio of Cisplatin and Olaparib, are reported. C, D IC50 values and surviving fraction of OVCAR3 olaR and OV-90 olaR cells, compared to parental OVCAR3 and OV-90 cells, after 144 h exposure to Olaparib and Cisplatin, in presence or absence of P5091, are displayed. E In OV-90 olaR, the CI values according to 1:2 concentration ratio of Cisplatin and Olaparib, in presence or absence of P5091 [2.5 μM] are shown.
is common in BRCA2-mutant cells [50], in turn able to sustain the FBXW7-dependent CCDC6 degradation [30]. Importantly, CCDC6 protein levels spontaneously re-established in BRCA2 reverted PEO4 cells (Fig. 5C). The CCDC6 null, cisplatin sensitive, PEO1 cells showed a high PARPi sensitivity (IC50: 2.3 μM ± 0.15). This phenotype was abolished by replenishing the expression of CCDC6, as observed by a 70% increase of the IC50 drug concentration (IC50: 4.11 μM ± 0.17) (Fig. 5E). Moreover, cisplatin sensitivity remained nearly unaffected in the presence of CCDC6 ectopic expression (in PEO1 CCDC6+: IC50 0.78 μM ± 0.11 vs. IC50 1.01 μM ± 0.06 in PEO1 EV cells). However, upon cisplatin and olaparib combined treatment, no significant variations in the combination index between the PEO1 cisplatin sensitive cells, overexpressing either the Myc empty vector (EV) or the Myc CCDC6 plasmid (CCDC6+), were registered. Thus, CCDC6 expression affects olaparib, but not cisplatin sensitivity of these cells. Furthermore, the ability to repair the DNA DSBs by HR was determined in
the PEO1 cells by GFP reporter assays, which revealed a slightly increase in the GFP positive cells following the CCDC6 transient transfection [Myc CCDC6 plasmid (CCDC6 +)], in comparison to the control cells [Myc empty vector (EV)] where a very low percentage of GFP positive cells was detected, mostly ascribed to the presence of BRCA2 inactivating mutation (Additional File 10: Figure S7A-C).

This observation indicates that, in the context of BRCA2 deficiency, CCDC6 activity in HR repair is limited and is epistatic to BRCA2. Nevertheless, in the PEO4 cells, in which BRCA2 functional activity and CCDC6 levels are restored, the CCDC6 depletion determined a significant decrease of GFP positive cells, compared to the control HR proficient cells (Additional File 10: Figure S7B-C).

Overall, our results imply that assessing CCDC6 levels in tumours may provide critical information for therapy choices in HGSOC.

**The CCDC6-PP4c interaction depends on phosphorylation and determines PARP inhibitors sensitivity by modulating γH2AX levels**

To further investigate the molecular mechanisms of PARPi improved sensitivity in HGSOC where CCDC6 is downregulated, we dissected CCDC6 roles in HR response to DNA damage. Indeed, by regulating histone H2AX phosphorylation status, CCDC6 contributes to efficient DDR through HR. Upon DNA damage exposure and in an ATM-dependent manner, CCDC6 moves from cytosol to the nucleus where it binds to the main phosphatase responsible for the maintenance of histone H2AX phosphorylation status, PP4c [28, 51]. The PP4 holoenzyme, consisting of the catalytic subunit (PP4c) and the two major isoforms of PP4R3 (PP4R3α/β, also known as SMEK1/2) binds to the FxxP motif in CCDC6, providing specificity [29]. Moreover, the residue Threonine 427, close to the motif, can modulate the CCDC6 binding to PP4c and determine the CCDC6 intracellular localization through its phosphorylation status [29]. In order to exploit the functional outcome of the Threonine 427, flanking the FxxP interaction motif and preventing, when phosphorylated, the binding to PP4c, we performed site-directed mutagenesis of this residue in alanine (T427A) or aspartate (T427D). We investigated whether the disruption of CCDC6-PP4c interaction might affect the histone H2AX phosphorylation leading to HR deficiency and to PARPi sensitivity.

In HGSOC cells, pharmacologically or genetically silenced for CCDC6, and upon treatment with different concentrations of olaparib, as indicated, the cytotoxic drug effects were quantified by a cell viability assay upon overexpression of myc-CCDC6 WT (wild type), CCDC6 T427A, CCDC6 T427D or empty vector (EV), as control (Fig. 6A). The olaparib sensitivity observed in the OVCAR3 stably silenced for CCDC6, and transiently transfected with the EV, (IC50: 1.90 μM ±0.45), decreased following the transient transfection of myc-CCDC6 WT (IC50: 3.37 μM ±0.23) and myc-CCDC6 T427A (IC50: 3.08 μM ±0.20) plasmids; this data suggests that the T427A mutant, as well as the wild type protein, maintains the ability to interact with PP4c and to inhibit its phosphatase activity toward histone H2AX [29]. However, even after forced expression of the CCDC6 T427D mutant, OVCAR3 remains sensitive to olaparib whether treated with the USP7i P5091 or stably silenced for CCDC6, indicating that this mutant is unable to engage with PP4c phosphatase (Fig. 6A, B). As seen in the western blot analysis, this causes an increase in PP4c activity and a corresponding decrease in histone H2AX phosphorylation, which reproduces the CCDC6 deletion phenotype (Fig. 6C). γH2AX levels were assessed in olaparib-treated (1 μM) or untreated CCDC6-silenced ovarian cancer cells by western blot. This analysis revealed lower γH2AX levels in cells overexpressing the T427D mutant with respect to those expressing the T427A mutant or the wild type CCDC6 protein (Fig. 6C, Additional File 11: Figure S8).

In addition, the suppression of the regulatory component PP4R3α hampered the rescue of the CCDC6 wild type or CCDC6 T427A mutant plasmids overexpression,
### Drug Sensitivity

#### P5091 [2.5 μM]

| Cell line | Olaparib [IC₅₀] | Olaparib [IC₅₀] |
|-----------|-----------------|-----------------|
| OVCAR3 EV | 1.00 +/- 0.13 | 4.24 +/- 0.19 |
| OVCAR3 Myc CCDC6 | 2.91 +/- 0.27 | 4.82 +/- 0.10 |
| OVCAR3 Myc CCDC6 | 1.22 +/- 0.12 | 3.43 +/- 0.15 |
| OVCAR3 Myc CCDC6 | 2.68 +/- 0.04 | 4.19 +/- 0.07 |

#### ShCCDC6

| Cell line | Olaparib [IC₅₀] | Olaparib [IC₅₀] |
|-----------|-----------------|-----------------|
| OVCAR3 EV | 1.90 +/- 0.45 | 4.05 +/- 0.06 |
| OVCAR3 Myc CCDC6 | 3.37 +/- 0.23 | 4.24 +/- 0.29 |
| OVCAR3 Myc CCDC6 | 1.25 +/- 0.18 | 3.31 +/- 0.12 |
| OVCAR3 Myc CCDC6 | 3.08 +/- 0.20 | 4.16 +/- 0.10 |

#### ShCCDC6

| Cell line | Olaparib [IC₅₀] | Olaparib [IC₅₀] |
|-----------|-----------------|-----------------|
| OVCAR3 EV | 1.22 +/- 0.12 | 4.30 +/- 0.09 |
| OVCAR3 Myc CCDC6 | 3.04 +/- 0.15 | 4.49 +/- 0.03 |
| OVCAR3 Myc CCDC6 | 1.29 +/- 0.22 | 3.20 +/- 0.11 |
| OVCAR3 Myc CCDC6 | 2.63 +/- 0.21 | 4.13 +/- 0.25 |

#### ShCCDC6

| Cell line | Olaparib [IC₅₀] | Olaparib [IC₅₀] |
|-----------|-----------------|-----------------|
| OVCAR3 EV | 1.07 +/- 0.18 | 3.15 +/- 0.14 |
| OVCAR3 Myc CCDC6 | 1.41 +/- 0.07 | 3.52 +/- 0.03 |
| OVCAR3 Myc CCDC6 | 1.10 +/- 0.13 | 2.16 +/- 0.16 |
| OVCAR3 Myc CCDC6 | 1.39 +/- 0.08 | 3.68 +/- 0.06 |

### Fig. 6 (See legend on previous page.)
as measured by cell survival experiments, upon olaparib treatment, by blocking the CCDC6-PP4 interaction through the FxxP motif (Fig. 6D, E).

According to our findings, the PP4 complex activity, which is made up of the catalytic subunit PP4c and the regulatory subunit 3α, (PP4R3α), is necessary for the CCDC6-dependent BRCA-ness status.

**Discussion**

The most deadly gynecological cancer and one of the leading causes of cancer death in women is epithelial ovarian carcinoma [52]. Approximately 70% of all epithelial ovarian cancers are HGSOC, while between 85 and 90% of all ovarian malignancies are epithelial in origin [53]. Different histological entities (such as high-grade serous, endometrioid, mucinous, clear cell, and low-grade serous) with different biological, molecular, and clinical features make up epithelial ovarian cancer, which is not a single disease but is treated using a similar strategy that entails debulking surgery followed by adjuvant chemotherapy. The majority of women with advanced-stage epithelial ovarian cancer, nevertheless, will also relapse, necessitating further therapy [18].

Due to genetic and epigenetic changes in HR pathway genes, over 50% of ovarian tumours show poor DNA repair by homologous recombination. The effectiveness of platinum medicines in treating epithelial ovarian cancer and the development of PARP inhibitors, which exhibit synthetic lethality when administered to HR-deficient cells, both demonstrate that defective HR is a relevant therapeutic target in this cancer. Furthermore, the PARP inhibitors’ extraordinary activity in cellular systems lacking HR repair has been demonstrated in preclinical investigations by a synthetic lethality rationale, and this activity has been confirmed in clinical trials in *BRCA1/2* mutation carriers with ovarian carcinomas [7].

The PARP inhibitors olaparib, niraparib, and rucaparib have been authorized and introduced as maintenance treatment in patients with ovarian cancer who have experienced a platinum-sensitive recurrence since their initial admission for clinical usage in 2014 [9, 18, 54–57]. Unfortunately, the *BRCA1/2* mutations or the presence of an HR deficit affected the reported medication response. However, new studies imply that these substances are similarly active in tumours harbouring wild type *BRCA1/2* [45]. The discovery of new biomarkers is therefore highly valued in order to increase the applicability of PARP inhibitors and improve the quality of response [52]. Here, we provide preclinical data demonstrating an association between olaparib sensitivity and the CCDC6 gene product depletion in ovarian cancer, indicating CCDC6 as one of these biomarkers.

Due to gene translocations, somatic mutations, and altered protein levels, the tumour suppressor protein CCDC6 is known to lose its functional properties in several tumour types. When exposed to DNA damage, CCDC6 moves to the nucleus where it interacts with the HR machinery by binding to the protein phosphatase PP4c and controlling the phosphorylation of H2AX [28]. Notably, CCDC6 protein levels and functions are carefully regulated by the balance between the E3 ubiquitin ligase FBXW7 and the de-ubiquitinate USP7 activities [30]. Here, we demonstrate how synthetic lethality is conferred to clinically relevant PARPi in a range of HGSOC cell lines by CCDC6 downregulation, which may be accomplished either genetically, by transfection of short hairpin RNA, or pharmacologically, by using a pharmacological USP7 inhibitor. Interestingly, we discovered that CCDC6 attenuation increased the sensitivity to olaparib and acts synergistically with cisplatin in primary (PEO4) and newly generated (OVCA3 olaR and OV-90 olaR) olaparib-resistant cells of HGSOC. This was accomplished by affecting the formation of H2AX foci and the repair of DNA DSBs by HR. Surprisingly, CCDC6 downregulation predicted susceptibility to many classes of PARP inhibitors, regardless of each compound’s unique capacity to trap or inhibit PARP. Notably, reports of the effectiveness of veliparib and talazoparib in patients with *BRCA* WT, *BRCA* mutation-tested patients, or patients with HR DNA repair abnormalities under investigation, indicate novel additional pathways providing PARPi sensitivity [9, 57].

As a novel treatment strategy for PARPi-resistant HGSOCs, CCDC6 loss of function confers resistance to PARG inhibitor [24]. Comparing the PARPi response to the varied sensitivity to PARG inhibitors in cells with CCDC6 attenuation, it is possible to infer that the two inhibitors have separate mechanisms of action. The significant effects of PARPi, however, are replication stress, replication fork collapse, and double strand breaks [58], all of which can be repaired by HR repair. In this case, CCDC6 depletion might worsen DNA damage by preventing HR repair. By slowing down replication forks, accumulating inverted forks and ssDNA gaps, and inhibiting the RECQ1 helicase, PARG deletion or inhibition, on the other hand, results in cell cycle stalling in the S/G2 phase [24–26, 59–62].

Here, we report that CCDC6 phosphorylation at position T427 altered the activation of H2AX and the repair of DNA DSBs by HR, simulating the loss of CCDC6 or its functional impairment, by inhibiting the connection between CCDC6, the PP4c phosphatase, and the PP4R3 regulatory subunits [29]. This data favours a molecular mechanism that can account for the sensitivity of PARP inhibitors in the absence of CCDC6 activity. The
conserved PP4 holoenzyme’s consensus-binding motif (FxxP) for CCDC6 has recently been characterized, and it has been shown to give PP4 substrate specificity [29]. The rescue experiments carried out with the WT or unphosphorylated point mutant (T427A) CCDC6 isoforms, which are still able to interact and control the phosphatase activity, provide significant support for this observation (Fig. 7). Surprisingly, we also saw a high correlation between the regulatory component PP4R3 and the PARPi sensitivity. In fact, PP4R3 deletion increased the sensitivity to PARPi while also altering the rescue effect of transient transfections of either the WT or T427A mutant CCDC6 isoforms. These findings clearly suggest that the BRCA1-2 mutational phenotype connected to the lack of CCDC6 function depends on the CCDC6-PP4c complex activity. The PP4c functional role and its involvement in multiple aspects of cellular physiology are emerging [63]. Nevertheless, the CCDC6 ability to interact and modulate the activity of the PP4c complex resulted in relevant responses to genotoxic stress and oxidative stress, highlighting the CCDC6 gene product as a stress key player [36, 63–65].

We detected substantial levels of CCDC6 protein expression in olaparib-resistant cell cultures, and most crucially, in olaparib-resistant PDX, as shown by immunoblot or immunohistochemistry (Fig. 5, Additional File 10: Figure S7 D-G). We hypothesized, based on in vitro evidence, that the intensity of the CCDC6 staining could nonetheless predict the response to PARPi, as described in other tumour models [36–41]. However, the CCDC6 staining of PDX cores did not appear sufficient to distinguish a primary sensitive tumour or to predict the cisplatin sensitivity. Relapse-resistant patient-derived xenografts may one day serve as an effective in vivo model to study how various medication combinations affect a patient’s response to therapy. The ovarian PDX may then become susceptible to PARP inhibitors that can work in synergy with cisplatin therapy. The ovarian PDX may then become susceptible to PARPi while also altering the rescue effect of transient transfections of either the WT or T427A mutant CCDC6 isoforms. These findings clearly suggest that the BRCA1-2 mutational status is associated with or determines the sensitivity phenotype connected to the lack of CCDC6 function [305x216].

Platinum resistance in ovarian cancer remains an unmet clinical need and a significant challenge, so it is critical to find molecular biomarkers that can predict how patients will respond to platinum-based therapy. This will enable patient stratification and the development of alternative therapeutic approaches. In addition, soon, it will be possible to prevent or detect tumor relapses sooner thanks to the ability to follow gene product modifications in cells during liquid biopsy [67, 68].

It’s interesting to note that CCDC6 has been immunostained on a collection of primary ovarian tissues (N=251, organized in a Tissue MicroArray (TMA)). Unfortunately, most of the cores showed intense staining of the CCDC6 protein (median H-SCORE was 64.2 (IQR 28.8; 97.2); mean H-SCORE was 67.5 (sd 47.1). (Additional File 12: Figure S9). In addition, nearly 10% of the cores of our research cases (30/251) also had poor expression levels of the CCDC6 protein as well. However, according to the MITO-2 randomised phase III study, all the patient-derived specimens were collected from women who underwent first-line therapy and were not known to have detrimental BRCA1 or BRCA2 mutations (Additional File 2–3: Tables S2 and S3) [33, 69].

We are currently conducting biochemical studies and analysing additional ovarian cancer samples, as well as patient-derived xenografts, enriched for BRCA1/2 mutants, to understand whether the BRCA1/2 mutational status is associated with or determines the CCDC6 increased turnover. This is because in the BRCA2 mutant PEO1 ovarian cancer cells only a barely detection of CCDC6 protein was observed at immunoblot, likely due to an enhanced CCDC6 proteolysis. This finding may have therapeutic significance since it will promote the use of CCDC6 as a biomarker for early-individualized therapy employing targeted treatments. In addition, Patients-Derived Organoids, or PDOs, have also recently been thought to be practical for examining HGSOC’s susceptibility to and resistance to PARPi [70, 71].
Conclusion
As a result of our research, we can say that the disruption of the CCDC6-PP4c axis is related to the phenotype of BRCA1/BRCA2 genotyping by next-generation sequencing, may be advantageous for predicting PARPi sensitivity and customizing HGSOC therapeutic decisions. This is because there are low expression levels of CCDC6 protein have been found in nearly 10% of primary ovarian specimens (30/251) and the increased turnover of CCDC6 may depend on BRCA2 deleterious mutations.

Abbreviations
HGSOC: High-grade serous ovarian cancers; DDR: DNA damage repair; DR-GFP: DNA Repair-GFP reporter plasmid; DSB: Double strand breaks; HR: Homologous recombination; PARP: Poly(ADP-ribose) polymerase; PARP inhibitors; PARylation: Poly(ADP-ribose) polymerase; PARG: Poly(ADP-ribose) glycohydrolase; PARGi: PARG inhibitor; PARPi: PARP inhibitors; PARylation: Poly(ADP-ribose) polymerase; PDDO: Pan-ADP-Ribose; PEO: Pan-ADP-Ribose; PEO1 and PEO4: Pan-ADP-Ribose; PEO1 cells, transfected with short hairpin RNA (ShCCDC6) improved Olaparib sensitivity in HGSOC cells. (A, D, G) Immunofluorescence images showing γH2AX nuclear foci formation in CCDC6-silenced Kuramochi cells, treated with Olaparib [1μM] or PARGi [1μM] for 48 hours and transfected with control (VE) or Myc CCDC6 expression vector. Scale bar 50μm. (B) Graphs represent the percentage of cells with more than 15 foci. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p<0.05; ** p<0.01 and *** p<0.001). (C) The efficacy of CCDC6 silencing and the expression of Myc CCDC6 were assessed at Western Blot by the anti-CCDC6 and anti-Myc antibodies. Anti-Tubulin immunoblots are served as a loading control.

Additional file 6: Figure S5. In CCDC6-silenced Kuramochi cells (Sh-CCDC6), the γH2AX foci formation was rescued by CCDC6 exogenous expression upon Myc CCDC6 transient transfection (Myc CCDC6) vs empty vector (EV) as control. (A) Immunofluorescence images showing γH2AX nuclear foci formation in CCDC6-silenced Kuramochi cells, treated with Olaparib [1μM] or PARGi [1μM] for 48 hours and transfected with control (EV) or Myc CCDC6 expression vector. Scale bar 50μm. (B) Graphs represent the percentage of cells with more than 15 foci. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p<0.05; ** p<0.01 and *** p<0.001). (C) The efficacy of CCDC6 silencing and the expression of Myc CCDC6 were assessed at Western Blot by the anti-CCDC6 and anti-Myc antibodies. Anti-Tubulin immunoblots are served as a loading control.

Additional file 7: Figure S4. In CCDC6-silenced OVCA3 cells (Sh-CCDC6), the γH2AX foci formation was rescued by CCDC6 exogenous expression upon Myc CCDC6 transient transfection (Myc CCDC6) vs empty vector (EV) as control. (A) Immunofluorescence images showing γH2AX nuclear foci formation in CCDC6-silenced OVCA3 cells, treated with olaparib [1μM] or PARGi [1μM] for 48 hours and transfected with control (EV) or Myc CCDC6 expression vector. Scale bar 50μm. (B) Graphs represent the percentage of cells with more than 15 foci. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p<0.05; ** p<0.01 and *** p<0.001). (C) The efficacy of CCDC6 silencing and the expression of Myc CCDC6 were assessed at Western Blot by the anti-CCDC6 and anti-Myc antibodies. Anti-Tubulin immunoblots are served as a loading control.

Additional file 8: Figure S5. In CCDC6-silenced OV-90 cells (Sh-CCDC6), the γH2AX foci formation was rescued by CCDC6 exogenous expression upon Myc CCDC6 transient transfection (Myc CCDC6) vs empty vector (EV) as control. (A) Immunofluorescence images showing γH2AX nuclear foci formation in CCDC6-silenced OV-90 cells, treated with olaparib [1μM] or PARGi [1μM] for 48 hours and transfected with control (EV) or Myc CCDC6 expression vector. Scale bar 50μm. (B) Graphs represent the percentage of cells with more than 15 foci. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p<0.05; ** p<0.01 and *** p<0.001). (C) The efficacy of CCDC6 silencing and the expression of Myc CCDC6 were assessed at Western Blot by the anti-CCDC6 and anti-Myc antibodies. Anti-Tubulin immunoblots are served as a loading control.

Additional file 9: Figure S6. CCDC6 genetic depletion by short hairpin RNA (ShCCDC6) improved Olaparib sensitivity in HGSOC cells. (A, D, G) Immunofluorescence images showing γH2AX nuclear foci formation in CCDC6-silenced Kuramochi cells, treated with Olaparib [1μM] or PARGi [1μM] for 48 hours and transfected with control (EV) or Myc CCDC6 expression vector. Scale bar 50μm. (B) Graphs represent the percentage of cells with more than 15 foci. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p<0.05; ** p<0.01 and *** p<0.001). (C) The efficacy of CCDC6 silencing and the expression of Myc CCDC6 were assessed at Western Blot by the anti-CCDC6 and anti-Myc antibodies. Anti-Tubulin immunoblots are served as a loading control.

Additional file 10: Figure S7. CCDC6 deficiency or chemical downregulation, by impairing the homology-directed repair in PEO1 cells, and the efficacy of CCDC6 silencing, upon ShCCDC6 transient transfection in CCDC6 proficient PEO4 cells, were assessed by anti-Myc and anti-CCDC6 antibodies at Western Blot. (B) In PEO1 cells, transfected with DR-GFP alone, HA-CCDC6 and both HA-CCDC6 and CCDC6 wild type and (C) in PEO4 cells, pre-treated either with vehicle or PS091 [2.5μM] for 4 hours and transfected with the above-mentioned
plasmids, the percentages of GFP positive cells, compared to controls, were plotted as histograms, representative of the mean of three independent experiments. Error bars indicate the measurement of the standard error mean. Statistical significance was verified by 2-tailed Student’s t-test (* p < 0.05; ** p < 0.01 and *** p < 0.001). The Myc CCDC6 and HA-5cnel protein expression were assessed respectively by anti-Myc and anti-HA antibodies, at Western Blot. Anti-Tubulin immunoblots are shown as loading control. (D) CCDC6 protein expression was assayed, following cell block procedure, by immunohistochemistry on the ovarian cancer cell lines (a, b) OV90, parental and olaR, (c-d) OVCAR3, parental and olaR, (e, f) PEO1 transiently expressing Empty Vector (EV) or CCDC6 wild type (Myc CCDC6), respectively, (g, h) PEO4 transfected with ShCTRL and ShCCDC6, respectively. An automatic count of positive cells was performed on digital slides with QuPath image analysis software, and results are shown in the histograms (E). Statistical significance was verified by chi-square test (* p < 0.05, ** p < 0.01 and *** p < 0.001). (F) CCDC6 protein expression was also evaluated at Western Blot, as indicated. Anti-Tubulin immunoblot is shown as loading control. (G) Densitometric analysis have been performed by Image J Software. The histograms represent the relative protein levels of CCDC6 normalised to Tubulin and expressed as relative intensity compared to untreated cells. Error bars indicate the standard error mean derived from three independent experiments. Statistical significance was verified by 2-tailed Student’s t-test (* p < 0.05; ** p < 0.01 and *** p < 0.001).

Additional file 11: Figure S8. Densitometric analysis have been performed by Image J Software. The histograms represent the relative protein levels of yH2AX normalised to total H2AX and expressed as relative intensity compared to control. Error bars indicate the measurement of the standard error mean. Statistical significance was verified by 2-tailed Student’s t-test (* p < 0.05; ** p < 0.01 and *** p < 0.001).

Additional file 12: Figure S9. CCDC6 IHC expression in ovary tumours. Three representative samples of ‘high’ CCDC6 expression (#1, #2, #3) and three representative cases of ‘low’ CCDC6 expression (#4, #5, #6) are shown. For each sample, 10x, 20x, and 40x magnification fields are shown along with the whole TMA core of the digitally scanned glass slide.

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Authors’ contributions
FM1,2, FM3,4, GD, RMM, LP and AC conceived of the study, participated in its design and coordination and helped to draft the manuscript; FM1,2, FM3,4, FR, FR, SV, GI, LA, DC and VN highly contributed to the acquisition and analysis of data; GD, FM1,2, LA, FM3,4, LP and AC made substantial discussion and interpretation of data; all authors read and approved the final manuscript.

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Declarations
Ethics approval and consent to participate
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Competing interests
The authors declare that they have no competing interests.

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