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Self-Renewal Transcription Factor OCT4 Sustains BRCA/Fanconi Anemia Pathway Activity to Suppress R-Loop-Driven Genome Instability in HG-SOC

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Article

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Abstract

High-grade serous ovarian carcinoma (HG-SOC) is characterized by genomic instability, replication stress, and defects in homologous recombination (HR). However, the adaptive transcriptional networks that buffer genome instability and sustain cancer cell fitness and viability remain poorly understood. Here, we report that the embryonic stem cell self-renewal factor OCT4, which is associated with aggressive disease and therapy resistance, plays a central role in suppressing R-loop-mediated replication stress, BRCAness, and Fanconi anemia (FA)-like phenotypes, thereby preserving genome integrity in HG-SOC. OCT4 loss markedly increases R-loop levels, activates ATR signalling, impairs S-phase progression, induces mitotic abnormalities, and triggers broad transcriptional programs associated with replication stress and innate immune activation, a transcriptional pattern that is likewise observed in HG-SOC patients with low OCT4 expression. These defects are accompanied by BRCAness and FA-like phenotypes, including impaired RAD51 foci formation, defective FANCD2 activation, mitotic chromosome abnormalities, and the accumulation of cytoplasmic DNA that activates the cGAS-STING pathway. Notably, all these phenotypes are fully rescued by RNaseH1 overexpression, identifying aberrant R-loop formation as the proximal driver of genome instability in OCT4-depleted cells. Transcriptomic and mechanistic analyses reveal that OCT4 functions as a regulatory hub that promotes the expression of multiple BRCA and FA pathway genes involved in R-loop suppression, replication fork stability, and homologous recombination. Functional studies further identify the BRCAness-associated gene RBBP8/CtIP as a critical OCT4 target required to prevent R-loop-mediated genome instability and innate immune activation. Accordingly, elevated OCT4-RBBP8 expression is associated with reduced disease-free survival in HG-SOC patients. Collectively, these findings uncover a previously unrecognized function of the self-renewal transcription factor OCT4 as a master regulator of BRCA/FA pathway activity, R-loop suppression, genome stability, and restraint of innate immune signalling. These results further highlight the OCT4-CtIP axis as a promising therapeutic target for inducing BRCAness-associated vulnerabilities in HG-SOC.

Introduction:

High-grade serous ovarian carcinoma (HG-SOC) is the most common and aggressive gynaecological malignancy, with an overall survival of ~ 40% and accounting for 70–80% of ovarian cancer–related deaths[1–4]. HG-SOC is marked by extensive genomic instability, > 95% TP53 mutations, frequent defects in homologous recombination repair (~ 50%), and high levels of replication stress [5,6]. This molecular profile underlies the clinical efficacy of platinum-based chemotherapy combined with paclitaxel and Poly (ADP-ribose) polymerase (PARP) inhibitors [7–9]. Despite initial responses, treatment benefits are typically short-lived: 60–80% of patients relapse due to acquired resistance driven by restoration of homologous recombination, remodelling of the tumor microenvironment, and activation of drug-efflux pathways [10,11]. Identifying new therapeutic targets capable of enhancing treatment response therefore remains a critical priority. During early embryogenesis, the transcription factor OCT4, encoded by POU5F1, establishes pluripotency transcriptional programs in self-renewing epiblast cells; thereafter, its expression is largely

extinguished from somatic tissues and becomes restricted to primordial germ cells [12]. Aberrant OCT4 expression has been documented in several tumor types, including HG-SOC, where it promotes oncogenesis, therapy resistance and correlates with poor prognosis [13–16]. Elevated OCT4 levels have also been detected in tumor-initiating cells of HG-SOC, linking self-renewal factors to cancer stemness [17–20]. In ovarian cancer model systems, OCT4 enhances spheroid formation, chemoresistance, cytoskeleton–extracellular matrix remodelling, and tumor aggressiveness in xenograft models [21–23]. Functionally, OCT4 promotes epithelial–mesenchymal transition by inducing N-cadherin and is upregulated via the ERK pathway, demonstrating integration within major oncogenic signalling networks [13,15,24]. Moreover, OCT4 induces the nuclear inhibitor of Protein Phosphatase 1 (NIPP1) and Cyclin F, thereby driving HG-SOC proliferation through retinoblastoma (RB) hyperphosphorylation and supports mitotic stability by promoting the expression of chromosome passenger complex components [14]. In this study, we identify a previously unrecognized role of OCT4 in suppressing R-loop–mediated genome instability. R-loops are three-stranded nucleic acid structures composed of an RNA:DNA hybrid and a displaced ssDNA strand, and act as potent drivers of transcription–replication conflicts and replication stress, a major source of genome instability [25,26]. Our data show that OCT4 functions as a regulatory hub that promotes the expression of multiple genes associated with BRCAness phenotypes, including Fanconi anemia (FA) factors, that antagonize R-loops accumulation, stabilize replication forks, and support homologous recombination–mediated DNA repair. Consistent with this role, OCT4-depleted cells accumulate R-loops, exhibit elevated replication stress, display hypersensitivity to Ataxia telangiectasia and Rad3-related (ATR) and topoisomerase inhibitors, and show defective homologous recombination together with impaired FA pathway activity. These defects lead to genome instability and formation of cytoplasmic DNA species that activate cGAS–STING–dependent innate immunity pathways. All phenotypes are rescued by RNaseH1 overexpression, demonstrating that aberrant R-loop accumulation is the causal driver of genome instability in OCT4-deficient cells. The OCT4 target gene RBBP8/CtIP that is associated with BRCAness rescues R-loop–mediated genome instability and activation of innate-immunity in OCT4 depleted cells, thus confirming the role of OCT4 as regulatory hub for BRCAness/FA family gene expression. This highlights OCT4 as target to exploit BRCAness-associated therapeutic vulnerabilities and modulation of the tumor-microenvironment in HG-SOC via innate immunity pathways.

Material and Methods:

Cell culture

Human cell lines were maintained at 37°C in a humidified 5% CO_2 incubator and cultured for no longer than two months. OVCAR-3 cells (HGSOC, ATCC HTB-161) were cultured in Roswell Park Memorial Institute (RPMI) medium (Euroclone) with 20% fetal bovine serum (FBS, Corning #35-089-CV), 1% L-glutamine (Gibco # ECB3004D), and 1% penicillin/streptomycin (Gibco # ECB3001D). OVCAR-4 cells (provided by Baldassare G.) were cultured in RPMI medium (Euroclone #ECB9006L) with 10% FBS (Euroclone #ECS0183L), 1% L-glutamine (Gibco # ECB3004D), and 1% penicillin/streptomycin (Gibco

#ECB3001D). HEK293 cells (human embryonic kidney, ATCC CRL-1573) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Euroclone #ECM0728L) with 10% fetal bovine serum (Euroclone #ECS0183L), 1% L-glutamine (Gibco #ECB3004D), and 1% penicillin/streptomycin (Gibco # ECB3001D). OVCAR-4 and OVCAR-3 cells were grown with 0.5 µg/ml (OVCAR-4) or 0.25 µg/ml (OVCAR-3) puromycin (Gibco #A11138-03) after transduction with lentiviral vectors. Expression of doxycycline inducible gene expression construct was induced using doxycycline (Sigma # D9891; 2 µg/ml) for 24 hours. When indicated, experimental cells were treated with VE-822 (Selleck # S7102-10MG, 1.5 µM) for 24 hours, or Doxorubicin (Selleck # S1208-10MG, 2 µM) for 24 hours.

Plasmids

Doxycycline inducible vectors expressing RNaseH1 wild type (WT) and RNaseH1 catalytic dead mutant (D210N) were produced in-house. To generate inducible human RNaseH1 expression vectors containing specific epitope tags, target coding sequences were amplified by PCR using custom primers bearing 5' restriction site overhangs. The forward primer pCW57 RNH1 FW (5'-ACTGTGTGTACAatgttctatgccgtg-3') was used in combination with sequence-specific reverse primers to add C-terminal tags: pCW57 RNaseH1 WT MYC REV FW (5'-TGACTGTGTACAtcacagatcctcttctg-3') for Myc-tagged wild-type RNaseH1, and pCW57 RNaseH1 D210N HA REV RV (5'-TGACTGTGTACAtcaagcgtaatctgg-3') for HA-tagged D210N mutant RNaseH1. Primers were engineered to incorporate flanking BsrGI restriction sites (5'-TGTACA-3') directly adjacent to the coding sequences. Following PCR amplification, the amplified fragments were digested with BsrGI restriction endonuclease and subcloned into the linearized pCW57-GFP-2A-MCS backbone (Addgene #71783) to yield pCW57-GFP-2A-HA-RnaseH1 WT and pCW57-GFP-2A-myc-RNaseH1 D210N. Plasmid inserts were confirmed by restriction enzyme mapping and Sanger sequencing prior to transfection. The plasmids pICE (Addgene #46960), pICE-HA-CtIP-siR-WT (Addgene #82030) and pICE-HA-CtIP-siR-N289A_H290A (Addgene #82032) were kindly provided by Pauline Chanut and Sébastien Britton (Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France). All oligonucleotides used for cloning are listed in Supplementary Table 1.

Transfection of siRNAs and plasmids

siGENOME Human POU5F1 (Dharmacon #D-019591-05-0020) and human ON-TARGETplus CONTROL siRNAs (Dharmacon #D-001810-10) were transfected using RNAi-MAX Lipofectamine (Invitrogen #13778150) at a final concentration of 30 nM according to the manufacturer's instructions. Unless specified, OCT4 knockdown was performed for 48 hours. Plasmids were transfected using Lipofectamine 2000 (Invitrogen #11668019) according to manufacturer's instructions.

Lentiviral transduction

For lentiviral transduction, HEK 293 cells were used as packaging cell line. A reaction mix containing DNA was prepared by adding 10 µg of pCW57-turboEGFP-P2A-RNaseH1 wild type or D210N mutant, 7.5 µg psPAX2 (Addgene #12260), 2.5 µg pMD2-env (Addgene #12259), 50 µl Polyethylenimine (PEI, 1mg/ml) (Polysciences #23966-1) and brought to 1ml with OptiMEM (Gibco #31985-070). After incubation for 10 minutes at room temperature, the mix was added dropwise to a 10 cm dish at 70% cell confluence with 9 ml complete medium and left overnight in the incubator. Next, medium was replaced with 6 ml of fresh HEK medium and left for 72 hours. The supernatant containing the virus was collected and aliquots of 1.5 ml were stored at -80°C. For viral transductions 15 µg of Polybrene (10 mg/ml, Sigma-Aldrich #H9268) was added to one aliquot. Then the mixture was added to HG-SOC cells plated on a 3 cm cell culture dish (80% confluence). After 24 hours, puromycin was added to select for stably transduced cells.

Western Blotting

Western Blotting
Cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 250 mM NaCl, 1% Triton-X-100, 1% DOC, 0.1% SDS) and sonicated (Fisherbrand Model 120 Sonic Dismembrator, Fisher Scientific, 20 seconds, 20% amplitude). A Bradford assay (Bio-Rad #5000006) was used to determine protein concentration. 6x Sample buffer (0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue) was added to samples to achieve a final concentration of 1x, followed by denaturation at 95°C for 5 minutes. Samples were loaded onto homemade SDS-PAGE gels and run in Tris-Glycine Running Buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS). Proteins were transferred to a nitrocellulose membrane (Cytiva #10600003) in Transfer Buffer (25 mM Tris, 190 mM Glycine, 0.01% SDS, 20% methanol). Membranes were blocked using Blocking buffer [5% BSA (Sigma-Aldrich #A9647) in 0.1% Tween-20 (Sigma-Aldrich #P1379) in 1x TBS] and incubated overnight with primary antibody diluted in Blocking buffer. The next day, membranes were washed three times in 0.1% Tween-20 in 1xTBS (Sigma-Aldrich #P1379), incubated with HRP-conjugated secondary antibody diluted in Blocking buffer, and washed three times in 1x TBS, 0.1% Tween-20 (Sigma-Aldrich #P1379). The following primary antibodies were used for Western blotting: anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Santa Cruz Biotechnology, cat. sc-365062; RRID:AB_10847862; 1:20000), anti-HSP90 (heat shock protein 90; Santa Cruz Biotechnology, cat. sc-13119; RRID:AB_675659; 1:10000), anti-ATR (Cell Signaling Technology, cat. 2790; RRID:AB_2227860; 1:20000), anti-phospho-ATR (Thr1989) (GeneTex, cat. GTX128145; RRID:AB_2687562; 1:1000), anti-S9.6 (anti-DNA:RNA hybrid monoclonal antibody; homemade; 1:1000), anti-p65/RelA (Cell Signaling Technology, cat. #6956; RRID:AB_10828935; 1:1000), anti-phospho-p65/RelA (Cell Signaling Technology, cat. #3033; RRID:AB_331284; 1:1000), anti-FANCD2 (Thermo Fisher Scientific, cat. MA1-23333; 1:1000), anti-RAD51 (Novus Biologicals, cat. NB100-148; RRID:AB_10002131; 1:1000), anti-phospho-RPA32 (Ser33) (Bethyl Laboratories, cat. A300-246A; RRID:AB_2180847; 1:1000), anti-RPA32 (Bethyl Laboratories, cat. A303-874A; 1:1000), anti-IL8/CXCL8 (Cell Signaling Technology, cat. #57058; 1:1000), anti-phospho-STING (Cell Signaling Technology, cat. #50907; RRID:AB_2827656; 1:1000), anti-STING (Cell Signaling Technology, cat. #13647; RRID:AB_2732796; 1:1000), anti-OCT4/POU5F1 (Abcam,

cat. ab19857; RRID:AB_445175; 1:1000), anti-dsDNA (Abcam, cat. ab27156; RRID:AB_470907; 1:12000), anti-phospho-ATM (Ser1981) (Santa Cruz Biotechnology, cat. sc-47739; RRID:AB_781524; 1:1000), anti-ATM (Santa Cruz Biotechnology, cat. sc-23922; 1:1000), anti-53BP1 (Sigma-Aldrich, cat. MAB3802; RRID:AB_11212586; 1:1000), anti-phospho-CHK1 (Ser345) (Cell Signaling Technology, clone 133D3; RRID:AB_331212; 1:1000), anti-CHK1 (Cell Signaling Technology, cat. 2360S; RRID:AB_2080320; 1:1000), anti-phospho-CHK2 (Thr68) (Cell Signaling Technology, cat. 2661P; RRID:AB_331479; 1:1000), anti-CHK2 (Cell Signaling Technology, cat. 3440S; RRID:AB_2229490; 1:1000), anti-γH2AX (Millipore, cat. 07-164; 1:1000), and anti-phospho-STAT3 (Tyr705) (Cell Signaling Technology, cat. 9145; RRID:AB_2491009; 1:4000). The next day, membranes were washed three times in 0.1% Tween-20 in 1xTBS (Sigma-Aldrich #P1379), incubated with HRP-conjugated secondary antibody diluted in Blocking buffer. The following secondary antibodies were used: goat anti-mouse IgG-HRP (Sigma-Aldrich, cat. A4416; RRID:AB_258167; 1:10000), goat anti-rabbit IgG-HRP (Sigma-Aldrich, cat. A6154; RRID:AB_258284; 1:10000), and goat anti-goat IgG-HRP (Sigma-Aldrich, cat. A5420; 1:50000). Imaging was performed using Clarity Western ECL Substrate (Bio-Rad #1705060) and an iBright FL1500 Imaging System (Invitrogen #A44115). Primary and secondary antibodies are listed in Supplementary Table 2.

Immunofluorescence

Cells were washed with 1x PBS (Gibco #10010023) and fixed in 4% paraformaldehyde (Sigma-Aldrich #158127) for 15 minutes at room temperature. Cells were then washed with 1x PBS (Gibco #10010023) and treated with Citrate Buffer (0.1% sodium citrate (Sigma-Aldrich #S4641), 0.05% Triton X-100 (Sigma-Aldrich #T8787) for 5 minutes at room temperature, followed by washing with 1x PBS (Gibco #10010023) with 0.1% Tween-20 (Sigma-Aldrich #P1379). Cells were blocked for one hour in Blocking Solution (3% BSA (Sigma-Aldrich #A9647), 0.1% Tween-20 (Sigma-Aldrich #P1379) in 1x PBS (Gibco #10010023). Primary antibodies were diluted in the blocking solution, and slides were incubated for 2 hours at room temperature chamber or overnight at 4°C in a wet chamber. The following primary antibodies were used for immunofluorescence: anti-phospho-ATR (Thr1989) (GeneTex, cat. GTX128145; RRID:AB_2687562; 1:250), anti-cGAS (AbClonal, cat. A8335; 1:100), anti-S9.6 (anti-DNA:RNA hybrid monoclonal antibody; homemade, no commercial catalog number; 1:300), anti-phospho-IRF3 (AbClonal, cat. AP1412; RRID:AB_3067078; 1:1000), anti-FANCD2 (Thermo Fisher Scientific, cat. MA1-23333; 1:500), anti-RAD51 (Novus Biologicals, cat. NB100-148; RRID:AB_10002131; 1:100), anti-phospho-RPA32 (Ser33) (Bethyl Laboratories, cat. A300-246A; RRID:AB_2180847; 1:500), anti-RPA32 (Bethyl Laboratories, cat. A303-874A; 1:500), anti-IL8/CXCL8 (Cell Signaling Technology, cat. #57058; 1:5000), anti-phospho-STING (Cell Signaling Technology, cat. #50907; RRID:AB_2827656; 1:5000), anti-STING (Cell Signaling Technology, cat. #13647; RRID:AB_2732796; 1:5000), anti-phospho-ATM (Ser1981) (Santa Cruz Biotechnology, cat. sc-47739; RRID:AB_781524; 1:250), anti-53BP1 (Sigma-Aldrich, cat. MAB3802; RRID:AB_11212586; 1:300), and anti-γH2AX (Millipore, cat. 07-164; 1:500). Cells were washed three times in washing solution [0.3% BSA (Sigma-Aldrich #A9647), 0.1% Tween-20 (Sigma-Aldrich #P1379) in 1x PBS (Gibco #10010023)] for 5 minutes and incubated with Alexa Fluor-conjugated secondary antibodies diluted 1:500 in washing solution in a wet chamber with light protection for 1 hour. The

following secondary antibodies were used: goat anti-mouse Alexa Fluor 555 (Invitrogen, cat. A21422; RRID:AB_141822; 1:500), goat anti-mouse Alexa Fluor 488 (Invitrogen, cat. A11001; RRID:AB_2534069; 1:500), goat anti-rabbit Alexa Fluor 555 (Invitrogen, cat. A21428; RRID:AB_141784; 1:500), goat anti-rabbit Alexa Fluor 488 (Invitrogen, cat. A11008; RRID:AB_143165; 1:500), and goat anti-human Alexa Fluor 555 (Invitrogen, cat. A21433; RRID:AB_1500626; 1:500). Cells were washed three times in washing solution [0.3% BSA (Sigma-Aldrich #A9647), 0.1% Tween-20 (Sigma-Aldrich #P1379) in 1x PBS (Gibco #10010023)] for 5 minutes and incubated with Alexa Fluor secondary antibodies (Invitrogen #A-11001 and #A-11008) diluted 1:500 in washing solution in a wet chamber with light protection for 1 hour. Slides were washed twice in washing solution for 10 minutes at room temperature. For nuclear visualization, slides were incubated with DAPI (1:2000; Sigma-Aldrich #D9542) prepared in 4x SSC buffer at room temperature for 5 minutes with light protection. Slides were mounted with ProLong (cod: P36930, Invitrogen). For S9.6 immunofluorescence, cells were fixed and permeabilized with ice-cold methanol for 10 minutes and ice-cold acetone for 1 minute on ice. All blocking, antibody incubation, and post-staining washes were performed using 4x SSC (Sigma-Aldrich #S6639) supplemented with 0.1% Tween-20 (4x SSCT) and 3% BSA for blocking solutions. Images were captured using a classic immunofluorescence microscope (Leica DM4000B). Quantitative immunofluorescence analysis was performed using ImageJ. The Student's t-test calculated statistical significance. Primary and secondary antibodies are listed in Supplementary Table 2.

Cell proliferation assay

Cells were seeded in 100 µL complete culture medium in triplicate in 96-well plates (Euroclone, flat bottom) (Euroclone #ET3196) at a density of 8000 cells/well and transfected with siRNAs at the day of cell seeding. Cells were allowed to re-attachment overnight. Medium was removed and 100 µL complete culture medium containing drugs was added for two days. Adequate control conditions were selected. After treatment 10 µL of resazurin solution (0.35 mg/ml in ddH20; Sigma-Aldrich #R7017) was added to wells and plates were put into the cell culture incubator for 3 hours. In metabolically active cells, resazurin is reduced to highly fluorescent resorufin, producing a fluorescent signal that is proportional to the number of viable cells. Fluorescence intensity was measured with a POLARstar Omega plate reader (BMG Labtech #415 – 201) with excitation at 560 nm and emission at 590 nm. The average values of the triplicate "blank" wells were subtracted from all other wells and normalized to the average of the vehicle-treated wells. Half-maximal inhibitory concentration (IC50) values were determined from at least three independent experiments.

Metaphase chromosome analysis

To arrest cells in metaphase, cultures were treated with Colcemid (1 µg/mL; Gibco, #15212012) for 4 hours. Both floating cells in the supernatant and adherent cells recovered by trypsinization were pooled and pelleted by centrifugation (200 × g for 5 minutes). The cell pellet was gently resuspended in 5 mL of pre-warmed hypotonic solution (75 mM KCl [#P9333] and 3 mM Sodium Citrate [#S4641]; Sigma-Aldrich)

and incubated at 37°C for 30 minutes. Fixation was initiated by adding ice-cold methanol/acetic acid (3:1 v/v) dropwise to the hypotonic suspension under continuous agitation. The cells were pelleted (1,000 × g for 5 minutes at 4°C) and resuspended in fresh ice-cold fixative; this washing/fixation step was performed a total of three times. The final cell suspension was dropped onto pre-chilled glass slides to yield metaphase spreads and allowed to air-dry. Spreads were stained for 15 minutes using KaryoMAX™ Giemsa Stain Solution diluted 1:3 in 1x PBS (Gibco, #10010023). Seventy-five metaphases per sample were visually inspected using a Leica DM4000B microscope fitted with a Leica DFC420C camera. Images were captured via Leica Application Suite (LAS) software, and chromosomal aberrations were quantified by visual inspection.

DNA:RNA hybrid immunoprecipitation (DRIP)

$1x10^7$ cells were scraped in 1x PBS (Gibco #10010023), pelleted by centrifugation at 4000 g for 5 minutes at 4°C, and lysed in 1x Lysis Buffer (0.5% SDS [Sigma-Aldrich #L4390], 10 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0 [Sigma-Aldrich #E7889], and 50 mM NaCl [Sigma-Aldrich #S9888] in ddH2O) supplemented with Proteinase K (Invitrogen #25530049) to a final concentration of 150 µg/ml. Samples were incubated at 37°C overnight. Genomic DNA was purified by phenol-chloroform extraction, ethanol precipitation, and resuspended in 1x TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA; Ambion #AM9849). Genomic DNA (10–20 µg) was digested overnight at 37°C with a five-enzyme cocktail (EcoRI, HindIII, BsrGI, XbaI, and SspI; NEB; 30–60 U each in 1x CutSmart buffer) to generate ~ 0.5–1 kb fragments. Four micrograms of genomic DNA per sample were treated with recombinant RNase H1 (New England Biolabs #M0297; 3 units per 1 µg of DNA) at 37°C or left untreated. Protein A Dynabeads (Invitrogen #10002D) were blocked overnight in 1x PBS supplemented with 0.5% BSA (Sigma-Aldrich #A9647) and 5 mM EDTA. The following day, 4 µg of S9.6 monoclonal antibody was conjugated to 50 µL of blocked Protein A Dynabeads per sample for 4 hours at 4°C in IP Buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate). Digested genomic DNA was added to antibody-coupled beads and incubated overnight at 4°C. Beads were washed sequentially (5 minutes per wash) with IP Buffer, Wash Buffer I (50 mM HEPES/KOH pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), Wash Buffer II (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and twice with 1x TE buffer. Immune complexes were eluted twice in Elution Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 15 minutes under constant shaking (850 rpm). Eluted DNA was purified via phenol-chloroform extraction and ethanol precipitation, then resuspended in 1x TE buffer. Target region enrichment was quantified by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad #1725121) on a CFX Connect Real-Time PCR Detection System (Bio-Rad #1855201). Data were normalized to input, and background signal from RNase H1-treated samples was subtracted.

RNA extraction and reverse transcription

Cells were washed with 1x PBS (Gibco #10010023) and 500 µl of Trifast) (Euroclone #EMR507100) was added directly on 3 cm wells, pipetted up and down until smooth and left 5 min at room temperature.

After transferring sample to a tube, 100 µl of chloroform (Sigma-Aldrich #C2432) was added and samples were gently agitated for 15' seconds and left at room temperature for 5 minutes. After 10 minutes of centrifugation at 16,000 g at 4°C, the transparent upper phase was collected, an equal amount of Isopropanol (Sigma-Aldrich #I9516) was added and samples were incubated at -20°C for at least 30 minutes. RNA was precipitated by centrifugation at 13,000 rpm at 4°C and pellet was briefly washed twice with 500 µl of 70% EtOH. The pellet was allowed to dry for 40–45 minutes followed by resuspension in 20 µl of RNase free H_2O. Reverse transcription was performed by using QuantiTect Reverse Transcription Kit (Qiagen #205311) following manufacturer's instructions. For RNA-seq experiment, total RNA was prepred using the RNeasy Mini Kit (Qiagen #74104).

Gene expression analysis by RT-PCR

Quantitative real-time PCR (RT-qPCR) was performed in a total reaction volume of 10 µL using 1x iTaq Universal SYBR Green Supermix (Bio-Rad #1725121) and target-specific primers (Eurofins) at a final concentration of 500 nM each. Amplification was carried out on a CFX Connect Real-Time PCR Detection System (Bio-Rad #1855201) according to the manufacturer's instructions. Specificity of PCR amplification was routinely verified by melting curve analysis and 2% agarose gel electrophoresis. Primer sequences are detailed in Supplementary Table 3.

Statistical analyses

Statistical analyses were performed in GraphPad Prism (v8). Results in bar graphs are shown as mean +/- standard deviation, unless otherwise specified. Differences between experimental groups were tested using a Student's t-test, unless a different test is indicated. Results were considered statistically significant at $p < 0.05$; differences were considered not significant (n.s.) for $p >= 0.05$. Precise p-values are reported in the figures; p-values below 0.0001 are indicated as $p < 0.0001$. The number of biological replicates (N) and the number of analysed nuclei/cells/metaphases (n) are reported in the figure legends. No statistical method was used to predetermine sample size, no data were excluded from the analyses, experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

RNA Sequencing (RNA-Seq)

Total RNA was prepared using the Illumina TruSeq Stranded Total RNA LT Sample Prep Kit (Gold) following the manufacturer’s instructions. A TruSeq Stranded Total RNA library was sequenced on an Illumina platform. 2 × 101 bp paired-end sequencing with 18.2 Gbp average throughput per sample was performed by Macrogen, including adapter trimming using Trimmomatic.

Analysis of RNA-seq data:

Read alignment was performed using the STAR (Spliced Transcripts Alignment to a Reference, v2.7.10b) aligner [27]. For the alignment, the Homo sapiens annotation file (GRCh38.111.gtf) and the corresponding reference genome (GRCh38 DNA primary assembly) were obtained from Ensembl (www.ensembl.org). Alignment quality control was carried out using MultiQC, which aggregates FastQC reports. The output

files generated by STAR were imported into R to construct the raw count matrix. Differential gene expression (DGE) analysis was carried out using the DESeq2 R package which was also employed for data normalization [28]. Principal Component Analysis (PCA) was performed to assess sample homogeneity between experimental groups. DGE analysis was conducted separately for the OVCAR-3 and OVCAR-4 cell lines, comparing OCT4 (POU5F1) depleted samples to their respective controls, applying thresholds of |Fold Change| ≥ 1.5 and adjusted p-value ≤ 0.05. Volcano plots were generated using the Enhanced Volcano R package. Identification of shared differentially expressed genes (DEGs) was performed by comparing RNA-seq data from OVCAR3 and OVCAR4 cell lines. A Venn diagram was generated to highlight differentially expressed genes that were commonly upregulated or downregulated between the two cell lines.

Analysis of Microarray Gene Expression Data

OVCAR-3 microarray expression data were obtained from the Gene Expression Omnibus (GEO; accession number GSE76689 [14]. DGE analysis was performed using GEO2R, comparing OCT4 siRNA-treated samples with control siRNA. Genes with an absolute fold change (|FC|) ≥ 1.5 and an adjusted p-value ≤ 0.05 were considered significantly differentially expressed and subsequently downloaded for downstream analyses.

Bioinformatics analyses of transcriptomics data

Heatmaps were generated to visualize the expression of gene signatures related to DNA replication, replication stress, immune system, and DNA repair. Gene sets were retrieved from Gene Ontology (GO), REACTOME databases, and from ref. [29], describing gene signatures associated with defective replication stress response. For the replication stress response signature, representative genes (CDKN1A, GADD45A, BTG2, RRM2B, DDB2, RPA1, RAD9A, HUS1, CLSPN, CHK1, and XPC) were selected to assess the activation of replication stress–induced DNA damage repair pathways in RNA-Seq data obtained from HG-SOC model cells.

Functional annotation was performed on the DEGs obtained from both RNA-seq and microarray analyses using g:Profiler, incorporating terms from Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome. In addition to annotating the full DEG sets, functional enrichment was also performed on the subset of DEGs shared between OVCAR-3 and OVCAR-4. The results were visualized using various plots, including bar plots and concept network plots, which highlight genes shared across different enriched biological terms. Drug signature profiling was performed using the metaLINCS R package, using as input the DEGs derived from RNA-seq data. This analysis evaluated the normalized enrichment scores (NES) for mechanisms of action (MoA) of ATR kinase inhibitors, topoisomerase inhibitors, and DNA replication inhibitors. GSEA plots were generated for drugs within these categories. A positive NES indicates that the transcriptional profile induced by the drug resembles the profile observed upon OCT4 knockdown in OVCAR-3 and OVCAR-4 cell lines, based on the RNA-seq data.

The BRCAness signature was derived from a list of 40 BRCAness related genes [30]. To enhance the biological comprehensiveness of the signature and ensure the inclusion of key components involved in homologous recombination repair, the gene list was further expanded with genes from the “Fanconi anemia pathway” and “Homology directed repair” pathways curated in the Reactome database. These pathways were selected due to their critical involvement in the DNA damage response and their well-established functional interconnection with BRCA-associated repair mechanisms. Comparative heatmaps were generated to visualize the expression of BRCAness-related genes, as well as of immune-related genes. Heatmaps were created using the pathlinkR R package to compare gene expression across OVCAR-4 RNA-seq, OVCAR-3 RNA-seq, and OVCAR-3 microarray datasets.

RNA expression analysis in HG-SOC cell lines

RNA expression data for cell lines were downloaded from the Human Protein Atlas (HPA, https://www.proteinatlas.org/) to assess replication stress across different cell lines. The analysis was restricted to high-grade ovarian cancer cell lines, for a total of 22 cell lines (59M, Caov-3, Caov-4, COV318, COV362, COV504, FU-OV-1, HEY, JHOS-2, JHOS-4, Kuramochi, OAW28, ONCO-DG-1, OV-90, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, OVKATE, OVSAHO, SNU-8, TYK-nu). Replication stress was evaluated using the human gene set “reactome_activation_of_ATR_in_response_to_replication_stress” from MsigDB, which comprises 37 genes [31,32]. The correlation between OCT4 expression and the selected gene signature was visualized using a violin plot, stratifying ovarian cancer cell lines into low- and high-OCT4 groups. The median OCT4 expression across cell lines was used as the threshold to define high and low OCT4 expressing cell lines. Gene signature scores were computed using the singscore method [33,34].

Bioinformatic analysis of TCGA ovarian cancer expression and clinical datasets

Clinical and expression data for ovarian cancer were obtained from The Cancer Genome Atlas (TCGA, https://www.cancer.gov/tcga) via the GDC Data Portal. The correlation between OCT4 expression and the selected gene signature was visualized using boxplots, stratifying high-grade serous ovarian cancer samples into low- and high-OCT4 groups. The 33rd and 66th percentiles of OCT4 expression were used as thresholds to define these groups. Each dot in the plot represents the gene signature expression of an individual patient. Gene signature scores were computed using the singscore method [33,34]. Gene signatures were defined as follows. The BRCAness signature was defined as the top 25 genes correlating with OCT4 expression in TCGA, and an additional BRCAness signature was generated from a subset of four genes (RBBP8, FANCE, XRCC3, CHEK2) showing the strongest consistency across RNA-seq expression data and correlation with OCT4 in TCGA. Similarly, for immune-related genes (Immune System biological pathway, curated in the Reactome database) a signature was defined including all genes showing a positive correlation with OCT4 (24 genes). Further gene expression analyses were performed using GEPIA3, a web-based platform integrating RNA-seq data from TCGA and the Genotype-Tissue Expression (GTEx) project for comparative transcriptomic analyses [35]. Gene expression levels,

normalized as log2(TPM + 1), were compared between ovarian cancer samples from TCGA and normal ovarian tissues from GTEx, and were visualized as boxplots. Correlation analysis between POU5F1 and RBBP8 expression was performed using GEPIA3 on ovarian tissue samples from both the TCGA and GTEx datasets. Correlation strength was evaluated using Spearman's rank correlation coefficient, and the corresponding scatter plot was generated by the platform. Disease-free survival (DFS) analysis was performed using TCGA ovarian cancer clinical and gene expression data accessed through cBioPortal. An expression score was calculated as the mean expression of POU5F1 and RBBP8 for each patient. Patients were stratified into high- and low-expression groups using the optimal cut-off determined by the maximally selected rank statistic implemented in the R package survminer. Kaplan–Meier survival curves were generated to compare DFS between groups, and statistical significance was assessed using the log-rank test. Hazard ratios were estimated using a Cox proportional hazards model. Follow-up was censored at 40 months.

OCT4 target site analysis

OCT4 binding regions for selected BRCAness genes were analysed using the ReMap2022 ChIP-seq database [36]. Human ChIP-seq data from ReMap were downloaded and analysed in R. For each gene, binding regions within 500 nucleotides upstream of the transcription start site (TSS) were considered. The number of OCT4 binding regions per gene was retrieved from the downloaded data, and statistical significance was calculated as the deviation from the mean across all genes using a standard normal distribution. For visualization, a bar chart was used to display both the number of binding regions (bar length) and their statistical significance (bar colour). In addition, ChIP-seq-derived OCT4 binding across the RBBP8 promoter region (-500 nucleotides from the TSS) was examined in greater detail. Individual experiments were annotated by GEO accession, target (POU5F1), and cell type, with genomic coordinates represented on the x-axis. The highest overlap of binding events was highlighted, and an aggregated coverage profile was generated to indicate the density of overlapping OCT4 binding sites along the RBBP8 promoter region.

Patient Specimen

Patient specimens used in this study were obtained from the MICO trial, a prospective monocentric study conducted at the University of Udine, Italy. Ethical approval was granted by the Ethics Committee (CEUR FVG n. 17 380). The MICO trial was registered on ClinicalTrials.gov under the identifier NCT06272240 on 02/14/2024. Written informed consent was obtained from all participants. Fresh specimens were processed to prepare total RNA using Trifast (Euroclone #EMR507100), following the manufacturer’s instructions.

Use of artificial intelligence:

Artificial intelligence tools were used exclusively to improve the clarity and quality of the English writing; no AI assistance contributed to data generation, data analysis, interpretation, or the formulation of scientific conclusions.

Results:

OCT4 represses replication stress in HG-SOC lines

OCT4 depletion in OVCAR-3 cells was previously shown to induce mitotic defects and increase genome instability [14]. To further define the mechanisms involved, we examined activation of major DNA-damage signalling pathways in OVCAR-3 and OVCAR-4 HG-SOC cells following RNAi-mediated depletion of OCT4 (Supplementary Fig. 1A**)**. Immunofluorescence analysis revealed a strong increase of staining for ATR phosphorylated at Thr1989 (Fig. 1A, C; Supplementary Fig. 1B, D**)**, whereas phosphorylation of ATM at Ser1981 remained unchanged **(**Fig. 1A, B; Supplementary Fig. 1B, C**)**. OCT4-depleted OVCAR-3 and OVCAR-4 cells showed elevated phosphorylation of the ATR target CHK1 at Ser345 and RPA32 at Ser33 by both immunofluorescence and western blotting (Fig. 1D-F; Supplementary Fig. 1E–G**)**. CHK2 phosphorylation at Thr68 was variable across the two cell lines (Fig. 1F; Supplementary Fig. 1G**)**. The robust induction of ATR signalling together with increased RPA32Ser33 phosphorylation indicates replication stress in OCT4-deficient cells. Supporting this, cell-cycle analysis of OCT4-depleted OVCAR-4 cells showed accumulation of cell populations in S and G2/M phases and increased apoptosis (Fig. 1G). To obtain transcriptomic evidence for replication stress, we performed RNA-seq 48 hours after OCT4 knock-down. Principal component analysis confirmed substantial transcriptome alterations in both cell lines (Supplementary Fig. 1H, I). In OVCAR-4 cells, 2008 genes were upregulated and 2057 downregulated; in OVCAR-3 cells, 1861 genes were upregulated and 1728 downregulated (Supplementary Fig. 1J, K, Supplementary table 4). Among these, 732 genes were commonly upregulated and 763 commonly downregulated (Supplementary Fig. 1L), indicating shared OCT4-dependent transcriptional programs. Gene-ontology analysis showed consistent upregulation of replication-stress–related pathway and downregulation of genes involved in pre-replication complex assembly and DNA replication in both cell lines (Fig. 1H; Supplementary Fig. 1M). OCT4-depleted cells also displayed enrichment of a replication stress response defect (RSRD) signature identified in CCNE1 oncogene expressing MCF-10 mammary epithelial cells with impaired DNA damage response [29] (Fig. 1H; Supplementary Fig. 1M). Drug-profiling analysis using the metaLINCS R package revealed that transcriptomes of OCT4-depleted OVCAR-3 and OVCAR-4 mirror alterations mediated by replication-stress–generating drug classes, including ATR, topoisomerase and DNA-replication inhibitors (Fig. 1I, Supplementary table 4). Direct comparison confirmed similarity to transcriptional responses triggered by topoisomerase inhibitors Teniposide, Doxorubicin and NVP-BEZ235, an ATR inhibitor with low ATM affinity (Fig. 1J; Supplementary Fig. 1N, Supplementary table 4). In contrast, no similarity was observed with transcriptional profiles induced by selective ATM inhibitors KU-55933 or KU-60019 (Fig. 1J; Supplementary Fig. 1N, Supplementary table 4). Finally, analysis of a panel of 22 HG-SOC cell lines revealed negative correlation between OCT4 expression and activation of ATR-related replication-stress response programs (Fig. 1K, Supplementary table 5). Together, these results indicate a novel function of the self-renewal transcription factor OCT4 in suppressing replication stress and ATR pathway activation in HG-SOC cell models.

OCT4 suppresses replication stress by preventing R-loop formation

We next aimed to identify the molecular basis of replication-stress induction in OCT4-depleted HG-SOC cells. OCT4-depleted cells recapitulate gene-expression patterns of cells treated with ATR, topoisomerase inhibitors that represent major inducers of R-loops (Fig. 1J, Supplementary Fig. 1N)[14]. R-loops preferentially form at GC-rich and repetitive elements, and their RNA:DNA hybrid component can extend up to 2 kb [25,26]. Numerous factors—including helicases, topoisomerases, histone chaperones, epigenetic regulators, DNA-repair and replication proteins, and RNA-binding factors—prevent uncontrolled R-loop accumulation or resolve R-loops to prevent transcription-replication conflicts and maintain genome stability [37,38]. To assess R-loop abundance, we performed immunofluorescence using an in-house purified S9.6 RNA:DNA-hybrid–specific antibody with validated specificity (ref. [39]) using OCT4-depleted OVCAR-4 and OVCAR-3 cells. Transient OCT4 depletion resulted in a strong increase in pan-nuclear S9.6 staining. Pretreatment of fixed cells with recombinant RNaseH1 eliminated RNA:DNA hybrids prior to immunostaining (Fig. 2A, B; Supplementary Fig. 2A, B). In contrast to a catalytically inactive, mutant RNaseH1 version (RNaseH1 D210N), expression of doxycycline-inducible wild-type RNaseH1 (RNaseH1 WT) suppressed R-loop formation in OCT4 depleted OVCAR-4 cells (Fig. 2C; Supplementary Fig. 2C). To obtain molecular evidence for ectopic R-loop formation, we performed DNA:RNA immunoprecipitation (DRIP) followed by quantitative PCR in OCT4-depleted OVCAR-3 and OVCAR-4 cells, focusing on reported R-loop hot spots [39,40]. OCT4 depletion in OVCAR-3 and OCVAR-4 cells increased R-loop levels at centromeric α-satellite repeats, chromosome-1 α-satellite repeats, pericentric SatII (Chr. 1q12) repeats, SatIII repeats, and the RANBP17 gene (Supplementary Fig. 2D, E). Consistent with immunofluorescence results, ectopic expression of wild-type (RNAseH1 WT), but not mutant RNaseH1 (RNaseH1 D210N) suppressed these unscheduled R-loops (Fig. 2E). To demonstrate that R-loops are responsible for replication stress and DNA damage in OCT4-depleted cells, we depleted OCT4 by RNAi and induced expression of wild-type or mutant RNaseH1 D210N 24 hours post-transfetion. Immunofluorescence at 48 hours revealed that wild-type RNaseH1—but not the the catalytic-dead RNAseH1 version—suppressed ATR phosphorylation and γH2AX-foci formation in OCT4 depleted OVCAR-4 and OVCAR-3 cells (Fig. 2F–I; Supplementary Fig. 2F–I). RNA-seq analysis predicted sensitivity to ATR and topoisomerase inhibitors, both strong R-loop inducers (Fig. 1I, J; Supplementary Fig. 1N, Supplementary table 4). In line with this, OCT4 depletion combined with ATR- or topoisomerase-inhibitor treatment led to exacerbated R-loop formation when compared to untreated, OCT4-depleted OVCAR-4 cells (Fig. 2J, K, M, N). This increase in R-loops was accompanied by significantly enhanced sensitivity to VE-822 and Doxorubicin (Fig. 2L, O). As expected, no synergistic effects were observed when OCT4-depleted cells were treated with ATM inhibitors KU-55933 or with the unrelated DNA-crosslinker Cisplatin (Supplementary Fig. 2J–O). Together, these results demonstrate a previously unrecognized function of OCT4 in suppressing R-loop–mediated replication stress and DNA damage, thereby enhancing sensitivity to drugs that interfere with R-loop prevention or resolution.

OCT4 expression promotes DNA damage repair and genome stability pathways

To investigate the biological pathways affected by OCT4 depletion we performed functional annotation analysis using g:Profiler on differentially expressed genes (DEGs) obtained from RNA-seq datasets of OVCAR-3 and OVCAR-4 cells depleted of OCT4 for 48 hours (OVCAR-3–48hrs; OVCAR-4–48hrs, this study), as well as from previously generated microarray data of OVCAR-3 cells after 72 hours of OCT4 depletion (ref. [14] OVCAR-3–72hrs, Supplementary Fig. 3A). Across all datasets, OCT4 depletion consistently downregulated genes involved in cell-cycle regulation, including pathways related to mitosis, sister-chromatid separation, nucleosome organization, centromere function, and chromatin-modifying processes (Fig. 3A, Supplementary Fig. 3B, Supplementary table 6, 8). Upregulation of genes associated with programmed cell death and apoptosis aligned with the increased sub-G0 population observed in cell-cycle profiles of OCT4-depleted cells (Fig. 1G; Fig. 3A; Supplementary Fig. 3B, Supplementary table 8). We also observed a robust impairment of DNA-repair pathways—particularly homologous recombination and DNA replication—in both OVCAR-3 and OVCAR-4 cells after 48 hours of OCT4 depletion (Fig. 3B, **Supplementary table 6, 8**). In parallel, multiple immune-related pathways were activated, including those associated with T- and B-cell function, cytokine production, and innate immunity (Fig. 3B, Supplementary Fig. 3C, Supplementary table 6, 8). These alterations were even more pronounced in OVCAR-3 cells after 72 hours of OCT4 depletion (Supplementary Fig. 3A–C, Supplementary table 8). To refine these observations, we analysed the 1495 genes commonly regulated in OVCAR-3 and OVCAR-4 cells after 48 hours of OCT4 knock-down (Supplementary Fig. 1L). This gene set confirmed downregulation of DNA-damage-repair pathways—particularly homologous recombination—and upregulation of innate-immunity pathways, including cytokine signalling, DNA-sensing pathways, and interferon responses (Fig. 3C, D; Supplementary table 7). Network visualization using cnetplots further supported this transcriptional program. Genes commonly regulated upon OCT4 depletion (48hrs; OVCAR-3 and OVCAR-4) formed coherent functional clusters characterized by reduced expression of DNA-repair and replication genes, and increased expression of genes driving innate immunity, cytosolic DNA sensing, interferon signalling, TNF/NF-κB pathways, and cytokine activity (Fig. 3E, F; Supplementary Fig. 3D–I, Supplementary table 6). Together, these findings indicate that OCT4 expression enhances DNA-repair capacity while suppressing innate-immunity pathways in HG-SOC model cells. This dual role is consistent with the established contribution of OCT4 to ovarian cancer aggressiveness.

Loss of OCT4 expression recapitulates BRCAness and Fanconi anemia phenotypes

R-loop–mediated replication stress, defective DNA-damage repair, and activation of innate-immunity pathways are hallmark features of BRCAness phenotypes, typically associated with mutations in BRCA1, BRCA2 or Fanconi anemia (FA) genes [41–51]. To determine whether OCT4 relates to genes that mediate BRCAness phenotypes we specifically analysed the expression of 69 genes previously linked to

BRCAness phenotypes (ref. [30]) or associated with Fanconi anemia and homology directed repair pathways curated in the Reactome database. Heat-map analysis using RNA-seq datasets from OVCAR-3 and OVCAR-4 cells subjected to 48 hrs of siRNAi mediated knock-down revealed a downregulation of a large set of candidate genes (Fig. 4A, Supplementary table 9), with more pronounced reduction after 72 hours in OVCAR-3 cells (Fig. 4A, Supplementary table 9). Based on their correlation with OCT4 expression in the TCGA ovarian cancer dataset, 25 genes showing the strongest correlations were identified and used to derive a BRCAness-related gene signature, which recapitulated reduced expression in $OCT4^{low}$ patient specimens (Fig. 4B, Supplementary table 10). These findings suggest that OCT4 acts as a central regulator that promotes the expression of genes involved in BRCAness and FA pathways. To test whether OCT4 depletion impairs homologous recombination, we examined RAD51-foci formation following DNA damage induction [52]. Treatment of control OVCAR-4 cells with the topoisomerase inhibitor doxorubicin induced robust RAD51-foci formation. In contrast, OCT4-depleted cells showed severely impaired RAD51-foci formation under the same conditions (Fig. 4C, D). We previously demonstrated that depletion of the R-loop regulator SFPQ induces R-loop mediated DNA damage and RAD51-foci formation [39,53]. Co-depletion of SFPQ and OCT4 blocked RAD51-foci formation and prevented accumulation of FANCD2, a key regulator of replication-fork stability, homologous recombination, and DNA repair (Fig. 4E–G). These results indicate that OCT4 is required to maintain the ability of HG-SOC cells to initiate homologous-recombination repair. A substantial fraction of BRCAness genes downregulated upon OCT4 depletion belong to the FA gene family (Fig. 4A, Supplementary table 9). To test whether OCT4 loss mimics FA-like defects, we examined replication-stress–induced FANCD2 ubiquitination. Hydroxyurea treatment increased FANCD2 ubiquitination in control cells, whereas OCT4 depletion markedly reduced this response (Fig. 4H, I). To further assess FA pathway function, we analysed mitotic chromosome aberrations induced by the interstrand crosslinker Mitomycin C (MMC). OCT4 depletion increased metaphase abnormalities—including chromatid breaks, ring chromosomes, and dicentric chromosomes—in both OVCAR-3 and OVCAR-4 cells (Fig. 4J; Supplementary Fig. 4A). These defects were exacerbated by MMC treatment, supporting a role for OCT4 in sustaining FA pathway activity (Fig. 4J–L; Supplementary Fig. 4A–C). Together, these results demonstrate that the self-renewal transcription factor OCT4 plays a central role in promoting BRCA- and FA-pathway activity in HG-SOC models, thereby ensuring genome stability and DNA-repair competence.

OCT4 suppresses innate immunity by protecting from R-loop mediated genome instability

Gene-expression analysis of OCT4-depleted cells revealed strong upregulation of genes represented by the Reactome Immune System biological pathway, including immune regulation and multiple components of innate immunity (Fig. 5A; Fig. 3B; Supplementary table 6, 11). Based on their correlation with OCT4 expression in the TCGA ovarian cancer dataset, 24 genes showing the strongest negative correlations were identified. These genes recapitulated the upregulation pattern observed in $OCT4^{low}$ patient specimens, supporting their clinical relevance (Supplementary Fig. 5A; Supplementary table 10). Acute OCT4 depletion in OVCAR-3 and OVCAR-4 cells increased expression of interferon-response

markers (INFβ, IFIT3, IFI4), inflammatory mediators (TNFα, IL8, CXCL10, IL6, DDX60), and cGAS, as confirmed by transcriptome and quantitative RT-PCR analyses (Supplementary Fig. 5B–F). Loss of OCT4 in OVCAR-4 cells also led to increased formation of micronuclei, which also stained positive for γH2AX and cGAS (Fig. 5B–E). Due to epigenetic silencing of cGAS–STING signalling in OVCAR-3 cells (ref. [54]) only combining OCT4 depletion with 5-aza-2'-deoxycytidine treatment mediated cGAS localization to micronuclei (Supplementary Fig. 5G–J). The cGAS–STING pathway detects cytosolic DNA and activates type I interferon–driven antitumor immunity, a property increasingly exploited to enhance cancer therapy [55,56]. In line with this, increased micronuclei formation in OCT4-depleted OVCAR-4 and OVCAR-3 cells was accompanied by elevated cytosolic DNA content, as shown by immunostaining with double-stranded DNA-specific antibodies (Fig. 5F, G; Supplementary Fig. 5K, L). Ectopic expression of wild-type RNaseH1—but not the catalytically inactive mutant—suppressed both cytoplasmic DNA accumulation and micronuclei formation in OCT4-depleted OVCAR-4 cells (Fig. 5F–H). To validate downstream consequences of cGAS activation, we examined STAT3 and NF-κB (p65) phosphorylation. OCT4-depleted OVCAR-3 and OVCAR-4 cells induced STAT3 phosphorylation at Tyr705, as shown by immunofluorescence (Fig. 5I, J; Supplementary Fig. 5M, N). Western blotting further confirmed increased levels of phosphorylated STING, phosphorylated NF-κB p65, and γH2AX in OCT4-depleted cells (Fig. 5K; Supplementary Fig. 5O). Consistent with the role of R-loops in driving these phenotypes, ectopic expression of wild-type—but not mutant—RNaseH1 blunted activation of innate-immunity marker genes in OCT4-depleted cells (Fig. 5L; Supplementary Fig. 5P). Together, these findings demonstrate that impaired DNA-repair pathways in OCT4-depleted cells lead to R-loop–mediated genome instability and downstream accumulation of cytoplasmic DNA species that activate innate-immunity and inflammatory pathways. This anticipates a relevant role for OCT4 in modulating the tumor-microenvironment in HG-SOC.

CtIP is an OCT4 dependent suppressor of replication stress in HG SOC cells

Gene-expression analysis of OCT4-depleted cells revealed a subgroup of 11 BRCAness-related genes that showed most consistent downregulation across all transcriptomic datasets obtained from OVCAR-3 and OVCAR-4 cells (Fig. 6A; Supplementary Fig. 6A–C). Within this group, four genes— Retinoblastoma Binding Protein 8 (RBBP8), FA complementation group E (FANCE), X-ray repair cross complementing 3 (XRCC3), and Checkpoint kinase 2 (CHEK2)—displayed a strong and consistent correlation with OCT4 expression in the TCGA ovarian cancer cohort (Fig. 6B, Supplementary Fig. 6D). All four candidates were confirmed to be downregulated following OCT4 knock-down in both OVCAR-3 and OVCAR-4 cells as demonstrated by quantitative real-time PCR (Fig. 6C; Supplementary Fig. 6E). Using fresh - non-fixed - tumor tissues samples from an independent HG-SOC patient cohort, RBBP8, FANCE, and CHEK2 expression positively correlated with OCT4 levels, whereas XRCC3 expression was below detection limit (Fig. 6D). Analysis of ReMap2022 ChIP-seq datasets revealed significant enrichment of OCT4 binding at the promoter regions of RBBP8 and CHEK2 genes (Supplementary Fig. 6F, Supplementary table 13). Notably, multiple independent OCT4 ChIP-seq datasets showed strong OCT4 binding within the proximal

promoter of RBBP8, spanning 47–195 bp upstream of the transcription start site (Supplementary Fig. 6G, Supplementary table 14), supporting a direct regulatory role for OCT4 in driving RBBP8 expression. RBBP8 expression was robustly upregulated in tumor tissue compared with normal ovarian tissue, mirroring OCT4 expression patterns (Fig. 6E, Supplementary table 12). Moreover, OCT4 and RBBP8 levels positively correlated across TCGA and GTEx ovarian tissue datasets and RBBP8 aligned with DNA repair hubs identified in transcriptome analyses from OCT4 depleted HG-SOC cells (Supplementary Fig. 6H; Fig. 3E, Supplementary table 6). These observations prompted us to investigate whether RBBP8 acts as functionally relevant, downstream target of OCT4 in HG-SOC cell models. RBBP8 encodes the CtBP-interacting protein CtIP which holds endonuclease activity and promotes early steps of end-resection during homologous-recombination repair [57]. CtIP also functions as a flap endonuclease capable of processing R-loop structures. Accordingly, loss of endonuclease activity is associated with R-loop accumulation [58–62]. To test whether CtIP can rescue R-loop–mediated genome instability in OCT4-depleted cells, we ectopically expressed CtIP in OVCAR-4 cells. Immunofluorescence analysis revealed that CtIP overexpression in OCT4-depleted OVCAR-4 suppressed R-loop accumulation, replication stress (P-ATR), DNA damage (γH2AX), and micronuclei formation (Fig. 6F-L, Supplementary Fig. 6I-J). Consistent with these effects, CtIP expression also reduced downstream activation of STING and the NF-κB subunit p65 in OCT4-depleted OVCAR-4 cells, thereby blocking activation of innate-immunity pathways (Fig. 6H). Importantly, expression of a CtIP N289A, H290A mutant lacking flap-endonuclease activity (ref. [59]) failed to rescue R-loop–mediated genome instability phenotypes and activation of innate immunity in OCT4-depleted cells (Fig. 6F–L, Supplementary Fig. 6I). In line with this data HG-SOC patients with increased OCT4 and RBBP8 expression show reduced disease-free survival compared to OCT4low/RBBP8 low patients (Fig. 6M). These findings indicate that OCT4 dependent expression of CtIP has a critical role in suppressing R-loop mediated replication stress and exacerbated genome instability, resulting in poor clinical outcome in HG-SOC. Altogether our data identifies an unprecedented function of the self-renewal transcription factor OCT4 in enhancing BRCA and FA pathway activity to suppress R-loop mediated replication stress, genome instability and activation innate immunity pathways in HG-SOC. Thus, OCT4 represent a strategic target to promote BRCAness phenotypes and increase the therapeutic responsiveness to PARP inhibitors and immunotherapy in HG-SOC.

Discussion

Oncogene induced shortening of the G1 cell cycle phase, aberrant replication-origin firing as well as premature S-phase entry leading to transcription–replication conflicts and replication-fork collapse is a typical feature of HG-SOC [63–67]. In this study we provide evidence for a novel function of the self-renewal transcription factor OCT4 in mitigating replication stress in HG-SOC. OCT4 depletion activates ATR-dependent DNA-damage signalling, increases the proportion of cells in S phase, and induces gene-expression programs associated with replication stress and defective DNA replication. Remarkably, OCT4 depletion enhanced a replication stress response defect (RSRD) gene expression signature reported for CCNE1 transformed MCF10A cells with experimentally abrogated DNA damage signalling [29]. This anticipates a genome protective function of OCT4 in the context of oncogenic transformation of

cancer cells. Such a function may be of relevance for HG-SOC tumor initiating cells that display elevated OCT4 expression [17–20]. In this context OCT4 may confer long-term replication stability to this cancer cell compartment. This role is also anticipated to be of relevance in the cancer-unrelated biological context of vertebrate embryonic stem cells. High OCT4 expression levels in self-renewing embryonic stem cell (ESCs) may have an important contribution to the reported ability of rapidly cycling ESCs to efficiently rescue stalled replication forks and suppress replication stress [68,69]. These observations suggest that, in transformed cells, OCT4 may simultaneously promote proliferation, as previously reported by driving CCND1 and CCNE1 expression and assuring RB hyper-phosphorylation, but at the same time enhance pathways that stabilize DNA replication or promote the repair of damaged replication forks [70–73]. Aiming to identify the molecular cause for replication stress, we found that OCT4 depleted HG-SOC cells display massive accumulation of R-loops that are reported to represent a major source of transcription–replication conflicts and replication stress [25,26]. Accordingly, OCT4-depleted cells show increased sensitivity to R-loop-inducing agents such as ATR or topoisomerase inhibitors (ref. [74] and exhibit massive DNA damage under these conditions. Overexpression of RNaseH1 rescues replication stress and DNA damage in OCT4-depleted cells, validating ectopic R-loop accumulation as the causal trigger. Dynamic R-loop patterns were described during embryonic stem cell differentiation that is closely connected to OCT4 function [75,76]. The rapid downregulation of OCT4 in this context may mirror acute, siRNA-mediated OCT4 depletion in HG-SOC cells and contribute to the remodelling of R-loop patterns. On the mechanistic level we provide evidence for an unprecedented function of OCT4 in acting as central hub that promotes the expression of a panel of genes connected to BRCAness phenotypes, including FA family genes and representatives of homology directed repair pathways. This class of genes have a reported role in preventing R-loop formation, promoting replication-fork stability and homologous recombination dependent DNA damage repair [41–49,51,77]. Transcriptome analysis confirmed the downregulation of BRCAness related genes in patient specimen defined by low OCT4 expression. Consistent with identified gene expression alterations, OCT4-depleted cells manifest classic features related to impaired BRCA and FA pathway activity, including impaired homologous recombination, reduced FANCD2 activation, and increased metaphase chromosome aberrations upon drug induced interstrand crosslinking. These findings position OCT4 as a central regulator of FA and BRCAness-associated genes that collectively suppress R-loop-mediated genome instability. R-loop mediated genome instability increased micronuclei frequency and cytoplasmic dsDNA that activated the cytosolic DNA sensing cGAS–STING pathway [78]. Activation of downstream NFκB and STAT3 paired with the expression of pro-inflammatory genes is indicative for the activation of innate-immunity in OCT4 depleted HG-SOC cells. In line with our data, gene mutations driving BRCAness have been reported to trigger innate-immune pathways that enhance immune responses, counteract chemoresistance, and can improve patient outcomes [56,79–83]. Innate immunity in OCT4-depleted cells is suppressed by RNaseH1, demonstrating that R-loop accumulation provokes the formation of cytoplasmic DNA. Given that also cytoplasmic RNA:DNA hybrids can activate innate immunity, we cannot exclude their contribution to cGAS/STING activation [83,84]. We found that amongst BRCAness-related genes monitored in transcriptomics experiments, RBBP8, FANCE, XRCC3 and CHEK2 show robust co-regulation and positive

correlation with OCT4 expression in the TCGA ovarian cancer dataset. We identified RBBP8 as OCT4 target in multiple ChIP-seq datasets and found robust RBBP8 downregulation in OCT4-depleted HG-SOC models but also $OCT4^{low}$ patient samples. Significant upregulation in HG-SOC tissue compared to normal tissue was paralleled by reduced disease-free survival of $OCT4^{high}$, $RBBP8^{high}$ patients, anticipating clinical relevance of OCT4 dependent regulation of RBBP8. RBBP8 encodes CtIP, whose endonuclease activity that drives early steps in end-resection of double stranded DNA breaks to promote homologous recombination and holds flap-endonuclease activity reported to target and remove R-loops in yeast and human cells [57–62]. Importantly, the endonuclease activity of CtIP was found to be essential for rescuing all R-loop mediated phenotypes of OCT4-depleted cells in CtIP overexpression experiments. CtIP is thought to recognize the unpaired ssDNA strand within R-loops as a DNA flap, introducing single-strand breaks enabling to remove of RNA trapped in RNA:DNA hybrids via recruited helicases or DNA damage-based repair [61]. A connection of RBBP8/CtIP to cancer formation and replication stress is supported by reports on germline variants and single nucleotide polymorphisms (SNPs) in the RBBP8 gene that are linked to early-onset breast and ovarian cancers and defects in replication-fork stability [85,86]. Mechanistic data link the transcription factor OCT4 to CtIP, as representative BRCAness associated gene in our study, thus providing a novel pathway for the suppression of R-loop mediated replication stress with relevance to HG-SOC. Additional work is required to gain a complete insights into the role of other BRCAness and FA genes in an OCT4 guided regulatory network for R-loop resolution.

Overall, our study demonstrates a new function of the self-renewal transcription factor OCT4 by promoting the expression of multiple BRCAness-related genes and FA family genes to suppress R-loop-mediated replication stress and genome instability. This identifies OCT4 as a potential therapeutic target to increase R-loop-mediated genome instability but also modify the tumor-microenvironment by activating innate immunity. Such a strategy is supported by synthetic lethality between R-loop suppressors and oncogenes (ref. [87,88] but also the synergistic action of R loop–inducing drugs such as PARP or ATR inhibitors in the context of mutations in R-loop antagonizing factors including FANCA [89,90]. Targeting OCT4 may also drive R-loop mediated activation of innate immunity pathways, generating an immunogenic tumor microenvironment as recently reported for the R-loop antagonizing DExD/H-box helicase 9 (DHX9)[91]. Employing more advanced preclinical model systems in future studies will enable the exploration of the therapeutic value of promoting BRCAness by targeting OCT4 using novel or reported compounds [92–94].

Declarations

Data availability statement:

RNA sequencing data generated in this study are publicly available in NCBI's Gene Expression Omnibus with the GEO Series accession number GSE343752. Data will be accessible at the date of publication. This study analysed existing, publicly available data from the TCGA Research Network.

Source data are provided with this paper. Any additional information required to reanalyse the data reported in this work article is available from the lead contact upon request.

Code Availability:

This study did not involve the development of any new computational code or software.

Authors' Disclosure: The authors declare that they have no conflicts of interest.

Authors' contributions:

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Inclusion and Ethics Statement:

We are committed to fostering an inclusive and ethical research environment. This study was conducted in accordance with institutional and international ethical guidelines. Our research team values diversity in scientific collaboration. Contributors to this work represent a range of career stages, disciplines, and cultural backgrounds. Authorship was determined based on substantial intellectual contribution, and all authors have reviewed and approved the final manuscript. We recognize the importance of equitable access to scientific knowledge and have made efforts to ensure that our data, methods, and findings are openly available and reproducible.

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