

Supplementary Information

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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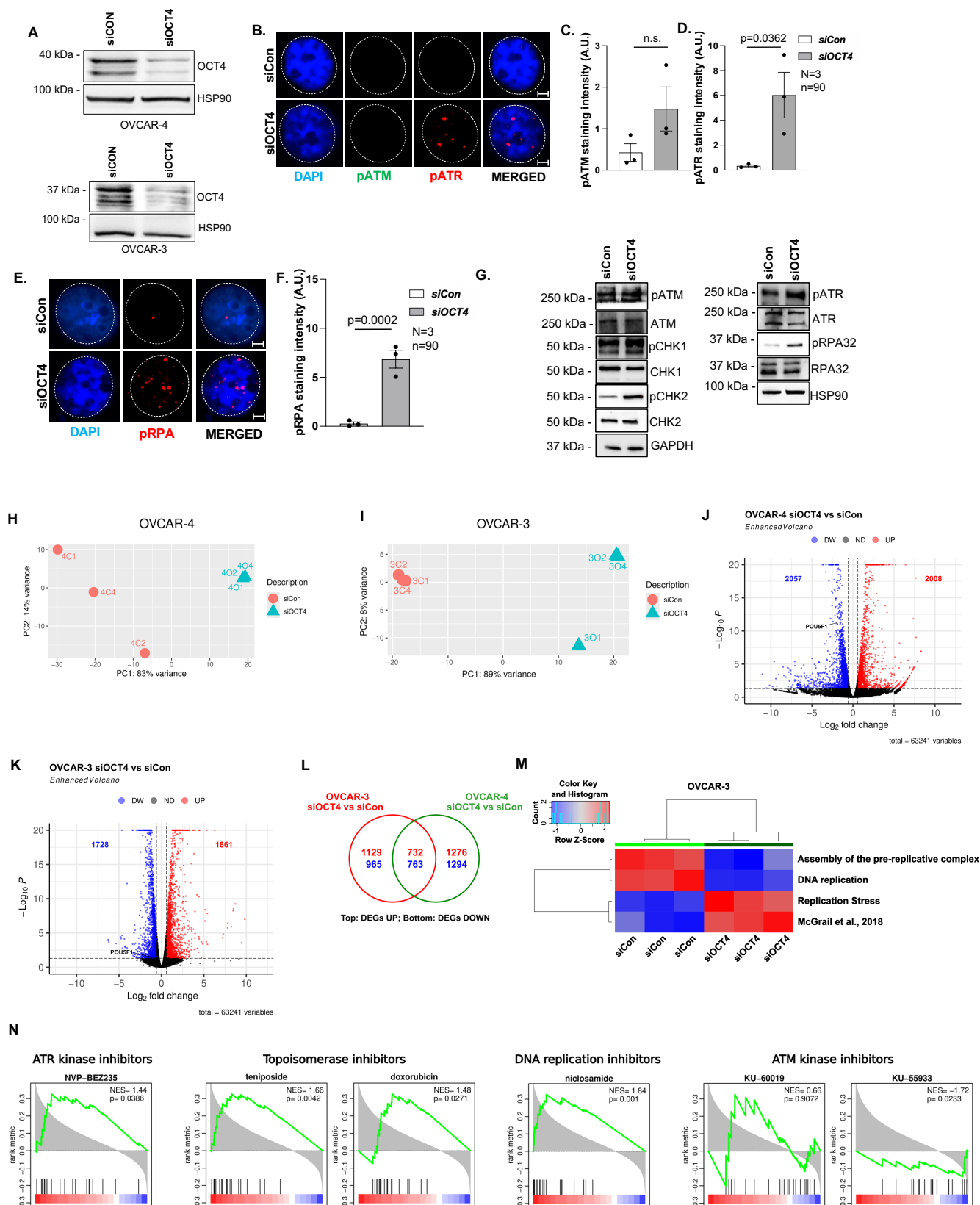
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Supplementary Figures:



Supplementary figure 1

Supplementary Figure 1. OCT4 knockdown triggers replication stress and alters global transcriptomic profiles in HG-SOC cells.

a) Western blot analysis of OVCAR-4 (top) and OVCAR-4 (bottom) cells transiently transfected with the indicated siRNAs. HSP90 was used as loading control.

b) Immunofluorescence on OVCAR-3 cells after transient transfection with control (siCon) and OCT4 specific siRNAs (siOCT4). Representative images of pATM and pATR staining are shown.

c) Quantification of pan-nuclear pATM staining intensity of cells shown in b).

d) Quantification of pan-nuclear staining pATR intensity of cells shown in b).

e) Immunofluorescence for pRPA on control and OCT4 knock-down cells. Representative images are shown.

f) Quantification of pRPA pan-nuclear staining intensity of cells shown in e).

g) Western blot analysis on OVCAR-4 cells under the indicated conditions for pATM, ATM, pATR, ATR, pRPA32, RPA32, pCHK1, CHK1, pCHK2, CHK2; GAPDH was used as loading control.

h, i) Principal Component Analysis (PCA) plots obtained from RNA-seq data of OVCAR-4 (h) and OVCAR-3 (i) transiently transfected with control and OCT4 specific siRNAs. PC1 (First Principal Component) is the direction that shows the highest amount of spread or variation in the data. PC2 (Second Principal Component) shows the next highest spread. Variance values are indicated.

j-k) Volcano plot of RNA-seq showing differentially expressed genes (DEGs) in OVCAR-4 (j) and OVCAR-3 (k) cells upon siRNA mediated OCT4 depletion. Red, upregulated genes; blue, downregulated genes; grey, not significantly altered.

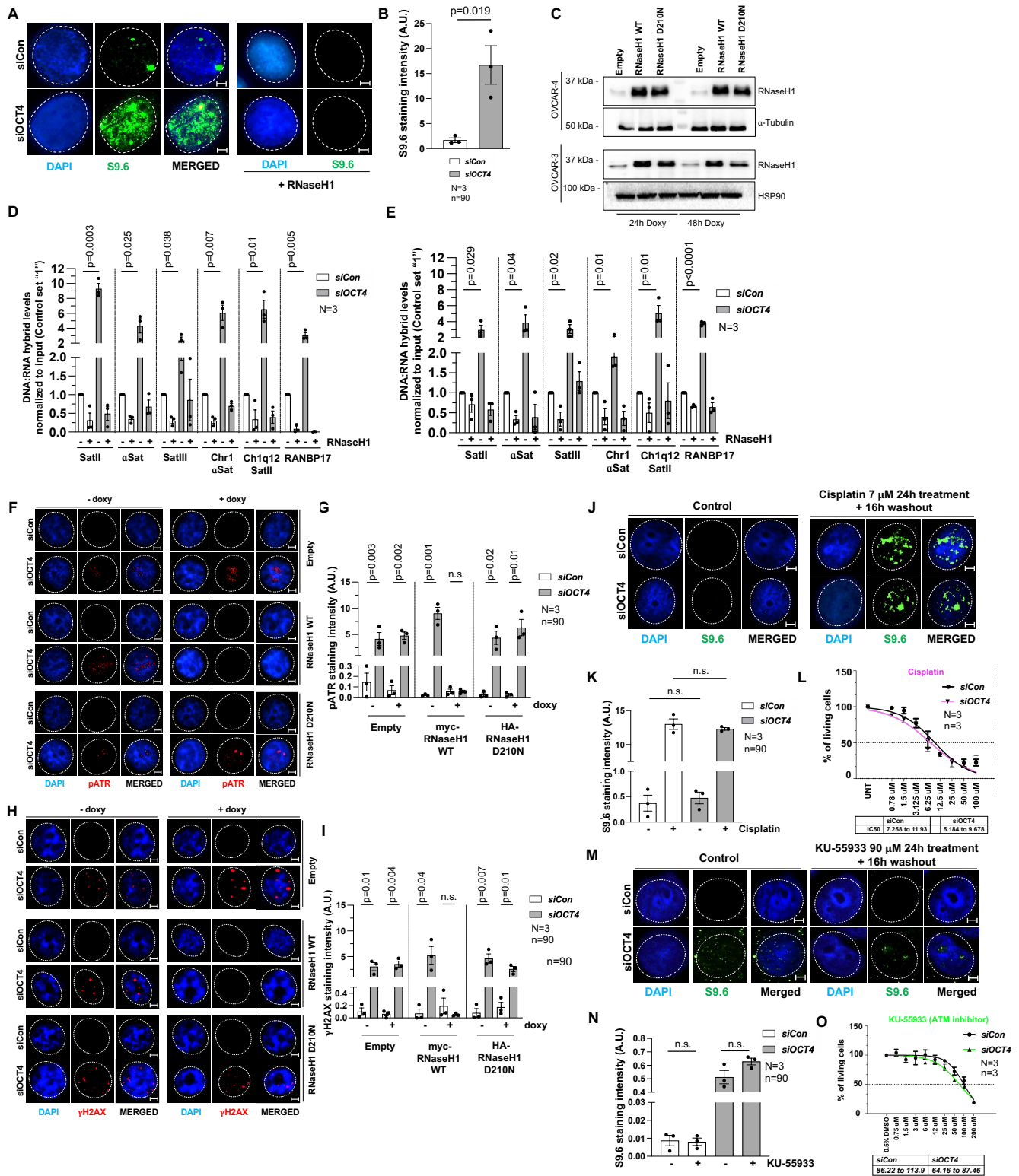
l) Venn diagram showing the overlap of up- and down-regulated DEGs between OVCAR-3 and OVCAR-4 cells upon OCT4 knockdown.

m) Heatmap displaying gene signatures related to the assembly of the pre-replicative complex, DNA replication, replication stress and RSRD (1

) signatures in OCT4 depleted OVCAR-4 cells. Colour code indicates row Z-score.

n) GSEA plots for ATR kinase inhibitors, ATM kinase inhibitors, topoisomerase inhibitors, and DNA replication inhibitors in OVCAR-3 RNA-seq data.

Data represent mean values; error bars indicate standard deviation. N, number of independent biological experiments. n, number of analysed nuclei. Signal intensity if immunofluorescence experiments are quantified as arbitrary fluorescence units (A.U.) c, d, f, an unpaired, two-sided Student's t-test was used to calculate statistical significance; p-values are shown. Scale bar refers to 10 μ m. DNA was stained using DAPI.



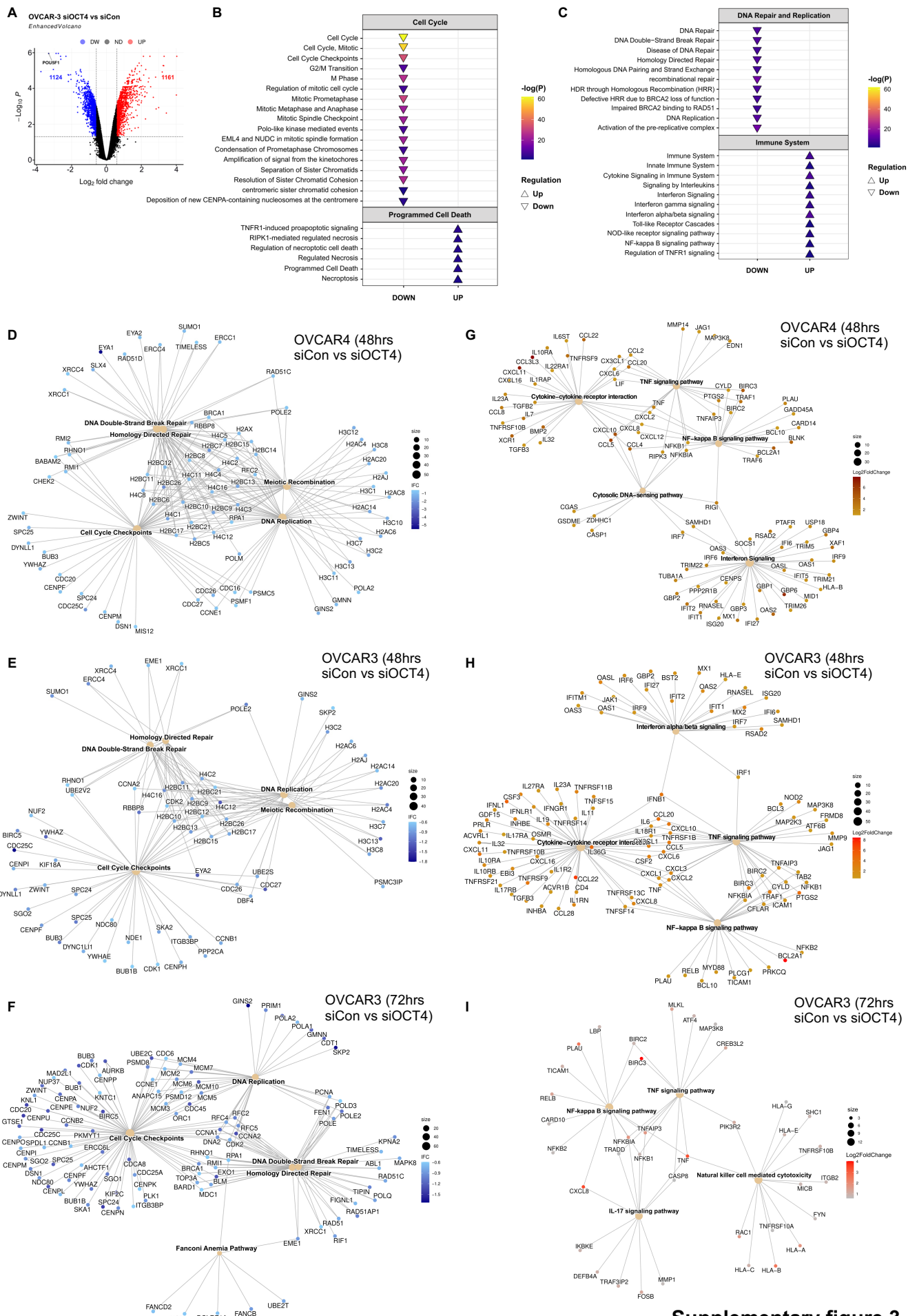
Supplementary figure 2

Supplementary Figure 2. R-loop accumulation and DNA damage responses in OVCAR-4 and OVCAR-3 cells.

- a)** Immunofluorescence on control and OCT4 depleted OVCAR-3 cells using S9.6 monoclonal antibodies detecting DNA:RNA hybrids. +RNaseH1, *in vitro* pre-treatment of fixed cells with recombinant RNase H1. Representative images are shown.
- b)** Quantification of experiment shown in a).
- c)** Western blot analysis validating doxycycline-inducible expression of wild type RNase H1 (RNaseH1 WT) and mutant RNaseH1 (RNaseH1 D210N) constructs. OVCAR3 and OVCAR4 cells were stably transfected with expression vectors.
- d)** DRIP-qPCR analysis was performed in OVCAR-4 cells under the indicated conditions. Where indicated (+) genomic DNA prepared from experimental cells was pre-treated with recombinant RNaseH1 prior to immunoprecipitation.
- e)** DRIP-qPCR analysis was performed in OVCAR-3 cells under the indicated conditions. Where indicated (+) genomic DNA prepared from experimental cells was pre-treated with recombinant RNaseH1 prior to immunoprecipitation.
- f)** Immunofluorescence for pATR was performed in OVCAR-3 cells carrying doxycycline (doxy) inducible expression constructs for wild-type (RNaseH1 WT) or mutant (RNaseH1 D210N) RNaseH1. Empty, control vector without insert. Representative images are shown.
- g)** Quantification of pATR staining intensity related to f).
- h)** Immunofluorescence for γ H2AX was performed in OVCAR-3 cells carrying doxycycline (doxy) inducible expression constructs for wild-type (RNaseH1 WT) or mutant (RNaseH1 D210N) RNaseH1. Empty, control vector without insert. Representative images are shown.
- i)** Quantification of γ H2AX staining intensity related to h).
- j)** Immunofluorescence using S9.6 monoclonal antibodies was performed on control and OCT4 depleted OVCAR-4 cells following Cisplatin treatment (24 hrs) and drug washout for 16hrs. Representative images are shown.
- k)** Quantification of S9.6 staining intensity related to experiment described in j).
- l)** Cell viability assay was performed using indicated Cisplatin concentrations. IC50 values are indicated.
- m)** Immunofluorescence using S9.6 monoclonal antibodies was performed in OVCAR-4 cells following KU-55933 treatment. Representative images are shown.
- n)** Quantification of S9.6 staining intensity of experiment shown in m).

o) Cell viability assay was performed under indicated KU-55933 concentrations. IC₅₀ values are indicated.

Data represent mean values; error bars indicate standard deviation. N, number of independent biological experiments. n, number of analysed nuclei. An unpaired, two-sided Student's t-test was used to calculate statistical significance; p-values are shown. Scale bar refers to 10 μ m. DNA was stained using DAPI. IC₅₀, half maximal inhibitory concentration.



Supplementary figure 3

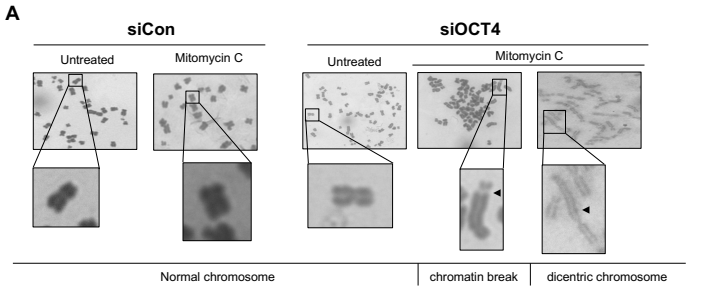
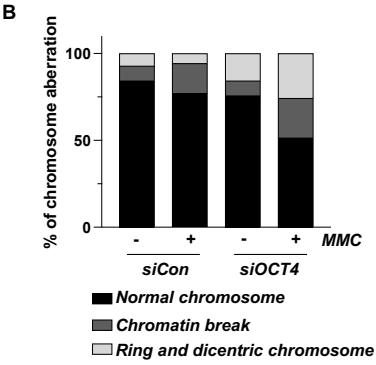
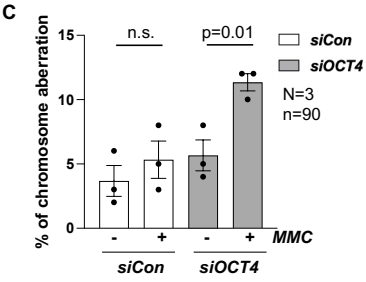
Supplementary Figure 3. Detailed networks of transcriptomic alterations following OCT4 depletion.

a) Volcano plot of Microarray data showing DEGs in OVCAR-3 cells upon siRNA mediated depletion of OCT4 silencing for 72hrs. Original data was obtained from ref. (2). Red, upregulated genes; blue, downregulated genes; grey, not significantly altered.

b-c) GO pathway enrichment analysis for Cell Cycle, Programmed Cell Death, DNA Repair and Replication, and Immune System categories in OCT4 depleted OVCAR-3 cells from Microarray data (ref. (2)). Colours indicate statistical significance as $-\log_{10}(P\text{-adj})$ values, triangle shape orientation indicates direction of regulation.

d-f) Concept network plot of downregulated genes associated with homology directed repair, DNA double strand break repair, meiotic recombination, DNA replication and cell cycle checkpoints. Data was obtained from RNA-seq data of control and OCT4 siRNA transfected OVCAR-4 (d) and OVCAR-3 (e) cells (48 hrs knock-down). Data shown in (f) refer gene expression array data of OVCAR-3 cells after 72hrs of OCT4 depletion (ref. (2)). The size of each biological term circle is proportional to the number of downregulated DEGs (DEGs DW) associated with that term. DEGs DW are shown.

g-i) Concept network plot of downregulated genes associated with NF κ B signalling, cytokine-cytokine receptor interaction, TNF signalling, cytosolic DNA-sensing and Interferon signalling. Data was obtained from RNA-seq data of control and OCT4 siRNA transfected OVCAR-4 (g) and OVCAR-3 (h) cells (48 hrs knock-down). Data shown in (i) refer gene expression array data of OVCAR-3 cells after 72hrs of OCT4 depletion (ref. (2)). The size of each biological term circle is proportional to the number of upregulated DEGs (DEGs UP) associated with that term. DEGs UP are shown.



Supplementary figure 4

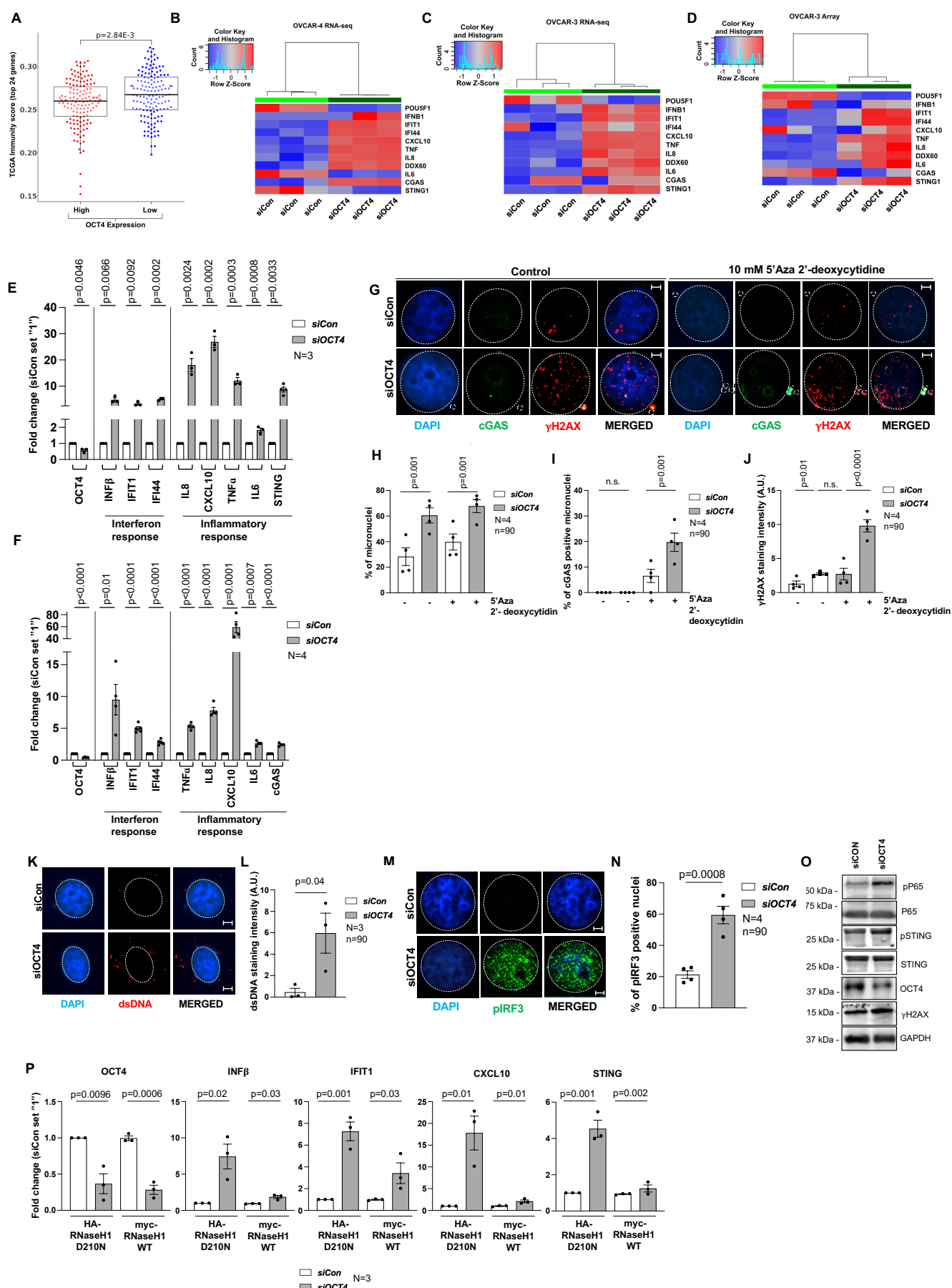
Supplementary figure 4. OCT4 depletion induces a BRCAness phenotype and impairs homologous recombination.

a) Metaphase spread analysis in in control and OCT4 depleted OVCAR-3 cells treated with the interstrand crosslinking agent Mitomycin C (MMC). Representative images of normal chromosomes, chromatin breaks, and dicentric chromosomes are shown. Arrowheads indicate chromosomal aberrations.

b) Quantification of the percentage of specific chromosome aberrations related to a).

c) Quantification of the total percentage of chromosome aberrations related to a).

Data represent mean values; error bars indicate standard deviation. N, number of independent biological experiments. n, number of analysed nuclei or metaphase spreads. The following statistical tests were applied: an unpaired, two-sided Student's t-test. Scale bars refer to 10 μ m. DNA was stained using DAPI.



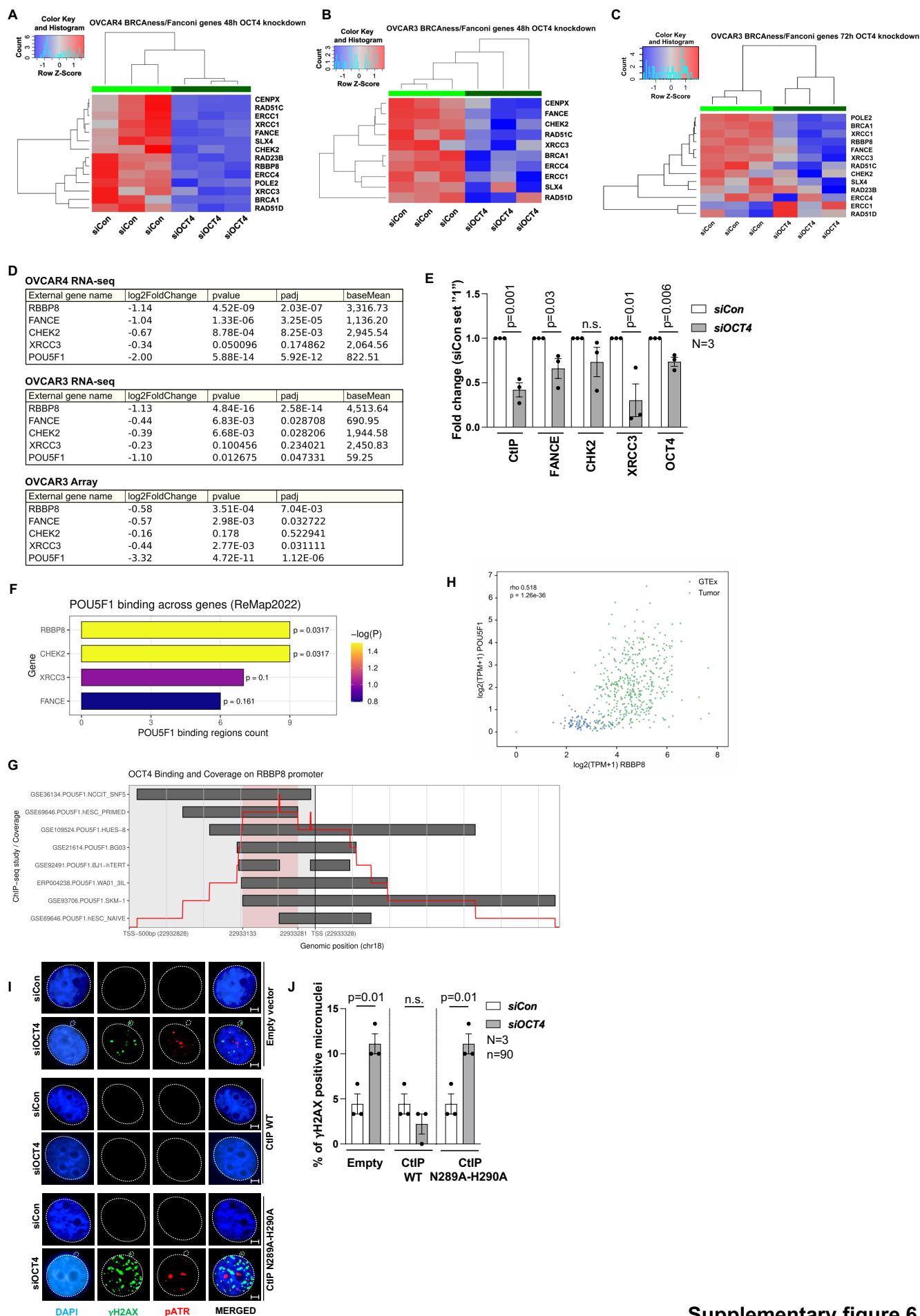
Supplementary figure 5

Supplementary Figure 5. OCT4 depletion activates interferon and inflammatory responses via cGAS-STING

- a)** Boxplot displaying the gene expression-based score of the top 24 genes represented in figure 5A (REACTOME Immune System biological pathway) ranked by their reverse correlation with OCT4 expression in HG-SOC patients from the TCGA Ovarian Cancer data. Patients were stratified into high- and low-OCT4 expression groups. Gene expression signature scores were calculated using the singscore method. A two-sided Welch's t-test was used to assess statistical significance.
- b-d)** Heatmap representing selected interferon and inflammatory response gene expression profiles in RNA-seq OVCAR-4 (b), RNA-seq OVCAR-3 (c) and microarray OVCAR-3 (d, ref. (2)) cells upon OCT4 silencing. Colour code indicates Row Z-Score values.
- e)** Quantitative RT-qPCR analysis was performed in OVCAR-4 cells transiently transfected with control and OCT4 specific siRNAs. ACTIN was used as reference gene.
- f)** Quantitative RT-qPCR analysis was performed in OVCAR-3 cells transiently transfected with control and OCT4 specific siRNAs. ACTIN was used as reference gene.
- g)** Immunofluorescence was performed in control and OCT4 knock-down OVCAR-3 cells treated with control (1x PBS) or 10 mM 5-Aza-2'-deoxycytidine (in 1x PBS). Antibodies specific for cGAS and γ H2AX were used.
- h)** Quantification of the percentage of micronuclei of experiment shown in g).
- i)** Quantification of the percentage of cGAS positive micronuclei of experiment shown in g).
- j)** Quantification of γ H2AX staining intensity of experiment shown in g).
- k)** Immunofluorescence staining for double-stranded DNA (dsDNA) was performed in OVCAR-3 subjected to siRNA mediated depletion of OCT4. Representative images are shown.
- l)** Quantification of dsDNA staining intensity related to experiment shown in k).
- m)** Immunofluorescence for pIRF3 was performed in OVCAR-3 cells under the indicated conditions. Representative images are shown.
- n)** Quantification of the percentage of pIRF3 positive nuclei is shown. Data relates to experiment shown in l).
- o)** Western blot analysis was performed in control and OCT4 depleted OVCAR-3 cells. Protein expression levels of phosphorylated P65 (pP65), P65, pSTING, STING, OCT4, γ H2AX are shown. GAPDH was used to control for equal loading of protein extracts.
- p)** Quantitative RT-qPCR analysis was performed on control and OCT4 knock-down OVCAR-3 cells containing doxycycline (doxy) inducible expression constructs for wild-type (WT) or mutant

(RNaseH1D210N) RNaseH1. Fold changes of the indicated genes are shown. ACTIN was used for normalization.

Data represent mean values; error bars indicate standard deviation. N, number of independent biological experiments. n, number of analysed nuclei. The following statistical tests were applied: panel a) a two-sided Welch's t-test; panel e, f, l, n, p) an unpaired, two-sided Student's t-test. Scale bars refer to 10 μ m. DNA was stained using DAPI.



Supplementary figure 6

Supplementary Figure 6. Extended analysis of BRCAness pathway downregulation upon OCT4 depletion.

a-c) Heatmap showing expression profiles of the most down-regulated BRCAness and Fanconi anemia genes determined by RNA-seq in OCT4 depleted (48hrs) OVCAR-4 (a) and OVCAR-3 (b) cells. Data in (c) was obtained from gene expression array data from OVCAR-3 cells after depletion of OCT4 for 72 hrs (ref. (2)). Colour code indicates Row Z-Score values

d) Tables displaying log2 fold change, p-values, p-adjusted, and base mean values for the indicated genes in control and OCT4 depleted OVCAR-4 and OVCAR-3. Analysis is based on RNA-seq and microarray datasets (ref. (2)).

e) Quantitative RT-qPCR analysis was performed on OVCAR-3 cells under the indicated conditions. Fold change of indicated genes is shown. ACTIN was used for normalization.

f) Bar chart displaying the number of studies reporting OCT4 binding regions for selected BRCAness genes based on the ReMap2022 ChIP-seq database. Bar length represents the number of regions, while colour indicates statistical significance ($-\log(P)$), computed as deviation from the mean across all genes using the standard normal distribution.

g) ChIP-seq-derived OCT4 binding across the RBBP8 promoter region (-500 nt from the TSS) from ReMap2022 datasets. Y-axis represents individual experiments annotated by GEO accession, target (POU5F1), and cell type; x-axis shows genomic coordinates. The red region indicates the highest overlap of binding events, and the red line shows the aggregated coverage profile, indicating the density of overlapping OCT4 binding sites along RBBP8 promoter region.

h) GEPIA3 scatter plot showing the correlation between POU5F1 and RBBP8 expression normalized as $\log_2(\text{TPM} + 1)$ in ovarian tissue samples from both the TCGA and GTEx datasets. Spearman's rank correlation coefficient and p-value are reported (ρ : 0.518, p : 1.26×10^{-36}).

i) Immunofluorescence was performed in OVCAR-4 cells after knock-down of OCT4 and transiently transfected with a control vector lacking an insert (Empty vector) and vectors encoding wild-type CtIP (CtIP WT) or a catalytic dead CtIP (CtIP N289A-H290A). Specific antibodies detecting pATR and γ H2AX were used. Representative images are shown.

j) Quantification of the percentage of γ H2AX positive micronuclei. Data relates to experiment shown in i).

Data represent mean values; error bars indicate standard deviation. N, number of independent biological experiments. n, number of analysed nuclei. j, an unpaired, two-sided Student's t-test

was used to calculate statistical significance; p-values are shown. Scale bars refer to 10 μ m. DNA was stained using DAPI.

Supplementary references,

References are related to Supplementary Table 3 and Supplementary figure legends.

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