Expanded View Figures

Figure EV1. Related to Fig 1. DEAD-box proteins identified in the proteomics mass spectrometry screen.

A Amylose pull-down from HEK293T nuclear cell extracts expressing 2xMBP-BRCA2NT ($BRCA2_{NT}$) and 2XMBP, detected by immunoblot, showing the samples for mass spectrometry experiment. The loading for input and unbound fractions is 1%, for the elution fraction is 8%, and for boiled bead fraction is 35%.

B DEAD-box helicases enriched in the $BRCA2_{NT}$ interactome. Label-free protein quantification. (Left) BRCA2 (in italic) and DDX Protein ID present in the proteomics mass spectrometry screen. (Center) Heat-map showing fold enrichment of each protein in BRCA2NT/2xMBP. Infinite-fold indicates proteins that are only present in BRCA2NT sample and not in pull-down performed with the 2xMBP. (Bottom) Heat-map log_2 color scale. (Right) Bar graph showing protein abundance in molar fraction percentage (mol %) in each pull-down (yellow in 2xMBP, blue in $BRCA2_{NT}$) based on label-free emPAI quantification (see Materials and Methods section).

C Immunoprecipitation (IP) of endogenous BRCA2 from benzonase-treated HEK293T whole cell lysates treated or not with IR (6 Gy), as indicated. Normal mouse IgG was used as negative control. Immunoblot of DDX5, DDX21 and RBMX and BRCA2. Stain-Free images of the gels before transfer were used as loading control (cropped images are shown).

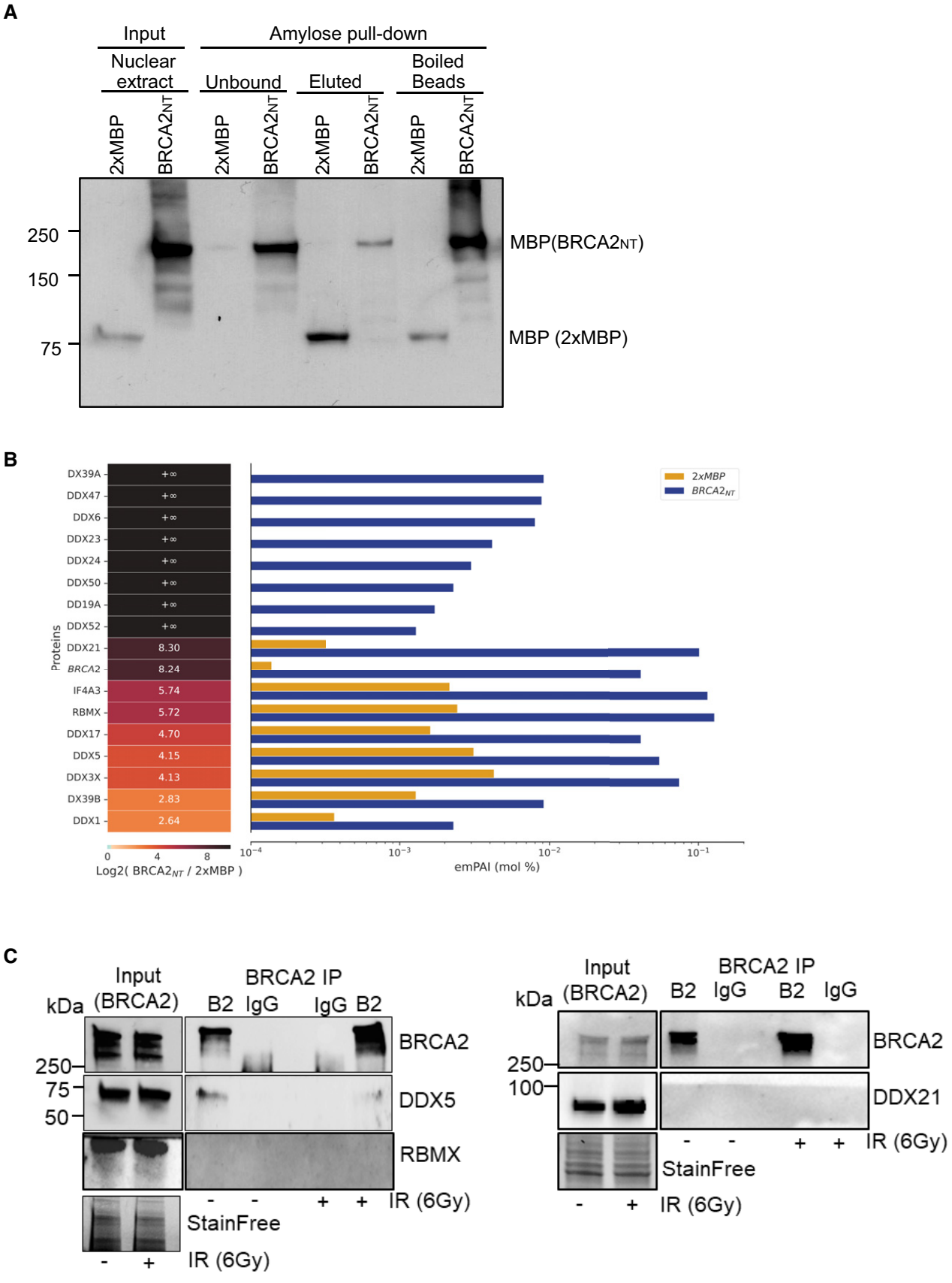
A
Input
Amylose pull-down
Nuclear extract
Unbound
Eluted
Boiled Beads
2xMBP
BRCA2NT
250
150
75
MBP(BRCA2NT)
MBP (2xMBP)
B
DX39A
DDX47
DDX6
DDX23
DDX24
DDX50
DD19A
DDX52
DDX21
BRCA2
IF4A3
RBMX
DDX17
DDX5
DDX3X
DX39B
DDX1
Proteins
2xMBP
BRCA2NT
emPAI (mol %)
Log2(BRCA2NT / 2xMBP)
C
Input (BRCA2)
BRCA2 IP
kDa
B2
IgG
BRCA2
DDX5
RBMX
DDX21
StainFree
IR (6Gy)

Figure EV1.

Figure EV2. Related to Figs 1 and 2. DNA-RNA hybrids levels in BRCA2- and DDX5-depleted cells and localization of DDX5 at DNA-RNA hybrids.

A Left: Quantification of the relative intensity of S9.6 staining. The data represent at least 500 cells per condition from three independent experiments. The red line in the scatter plot represents the median. For statistical comparison of the differences between the samples, we applied Kruskal–Wallis test followed by Dunn's multiple comparison test and the *P*-values show significant differences. Right: Representative immunofluorescence images of U2OS cells depleted of DDX5 (siDDX5), BRCA2 (siBRCA2), or control cells (siC) and stained with S9.6 antibody (DNA-RNA hybrids) and counterstained with DAPI. When indicated, cells were transfected/treated with RNase H1 (RH) 24 h before fixation. Scale bar indicates 10 μm.

B Left: Representative images of S9.6 immunofluorescence of U2OS cells depleted of DDX5 (siDDX5) or control cells (siC) expressing RNaseH1-GFP and/or DDX5-GFP. Scale bar indicates 10 μm. Right: Quantification of S9.6 average nuclear intensity of U2OS cells depleted of DDX5 (siDDX5) or control cells (siC) expressing RNaseH1-GFP and/or DDX5-GFP. The red line in the plot indicates the median, and each symbol represents the value of a single cell. The statistical significance of the difference was calculated with Mann–Whitney *U*-test; the *P*-values show the significant difference. The data represent at least 170 cells per condition from one single experiment.

C Left: Representative images of *in situ* PLA experiment performed between DDX5 and S9.6 antibodies in U2OS cells. When indicated, cells were treated with cordycepin (Cordy) for 2 h at 37°C before fixation. Single antibody controls from untreated cells are shown. Scale bar indicates 10 μm. Nuclei as defined by auto threshold plugin on the DAPI image (ImageJ) are outlined in yellow. Right: Quantification of the number of PLA spots per nucleus in different conditions, as indicated. The data represent at least 200 cells per condition from three independent experiments. For statistical comparison of the differences between the samples, we applied a Kruskal–Wallis test followed by Dunn's multiple comparison test and the *P*-values show significant differences. The red line in the plot indicates the median, and each symbol represents a single PLA spot.

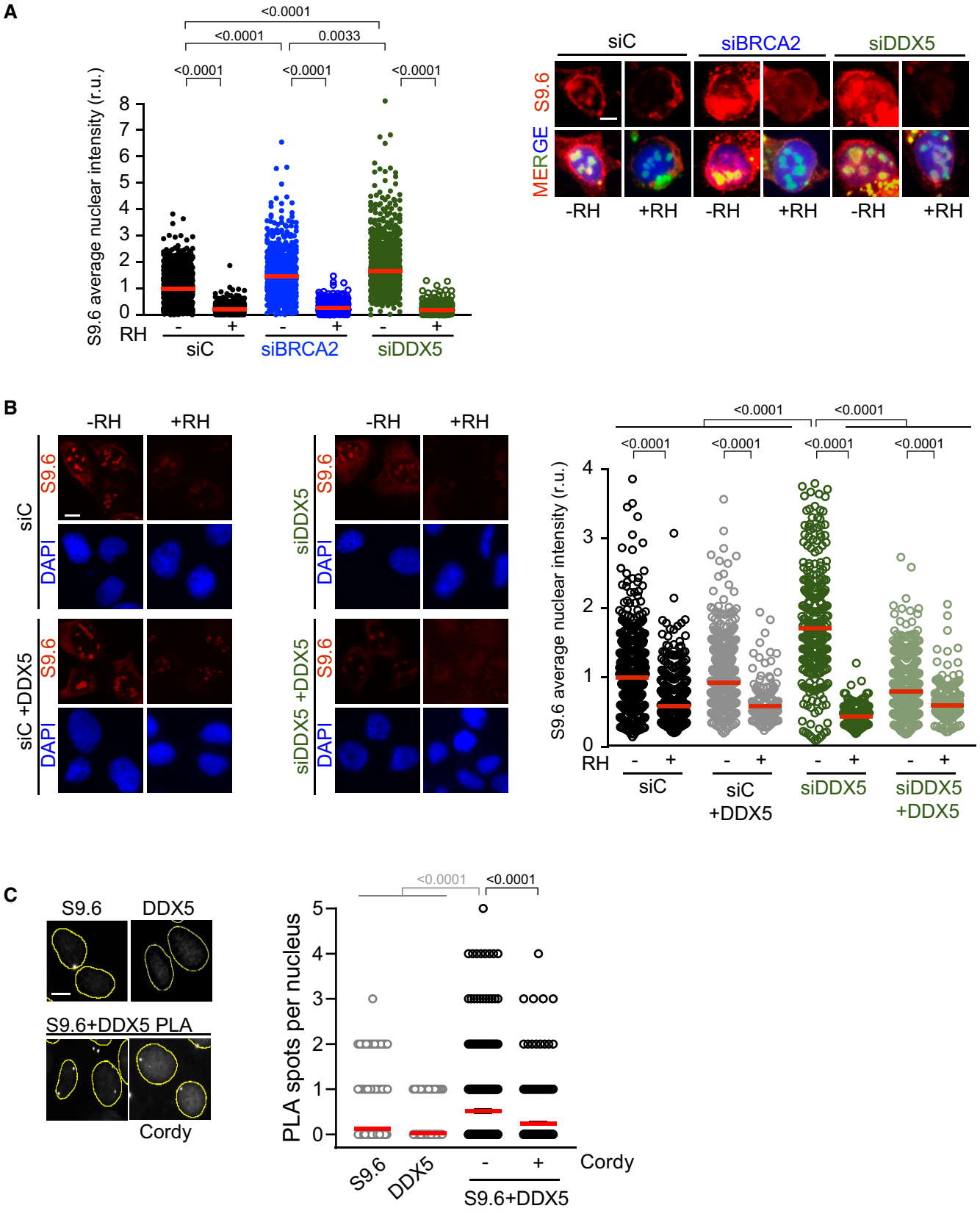
A
<0.0001
<0.0001
0.0033
<0.0001
<0.0001
<0.0001
S9.6 average nuclear intensity (r.u.)
RH
siC
siBRCA2
siDDX5
siC
siBRCA2
siDDX5
S9.6
MERGE
-RH
+RH
B
-RH
+RH
siC
S9.6
DAPI
siC +DDX5
siDDX5
siDDX5 +DDX5
<0.0001
<0.0001
S9.6 average nuclear intensity (r.u.)
siC
siC +DDX5
siDDX5
siDDX5 +DDX5
C
S9.6
DDX5
S9.6+DDX5 PLA
Cordy
<0.0001
<0.0001
PLA spots per nucleus
S9.6
DDX5
S9.6+DDX5
Cordy

Figure EV2.

Figure EV3. Related to Fig 2. Reproducibility of the DRIPc-seq data.

A Relative DRIP-qPCR signal values (respect to the siC level at each locus) at the *APOE*, *HIST1H2BG*, and *WDR90* loci in K562 cells transfected with the indicated siRNAs and treated *in vitro* with RNase H1 (RH) pre-immunoprecipitation where indicated. The data represent the mean ± SEM from two independent experiments. The statistical significance of the difference was calculated with paired Student *t*-test, and the *P*-values show the significant difference.

B Representative screenshots of specific genomic regions showing the DRIPc-seq profiles without DNA strand separation in K562 cells depleted of DDX5 (siDDX5) from three independent experiments as compared to data from control cells (siC). Two regions with different Alu sequence density are shown for comparison.

C *xy* correlation plot between the DRIPc signal intensity from two DRIPc-seq independent experiments performed in DDX5-depleted K562 cells (PCC, Pearson correlation coefficient).

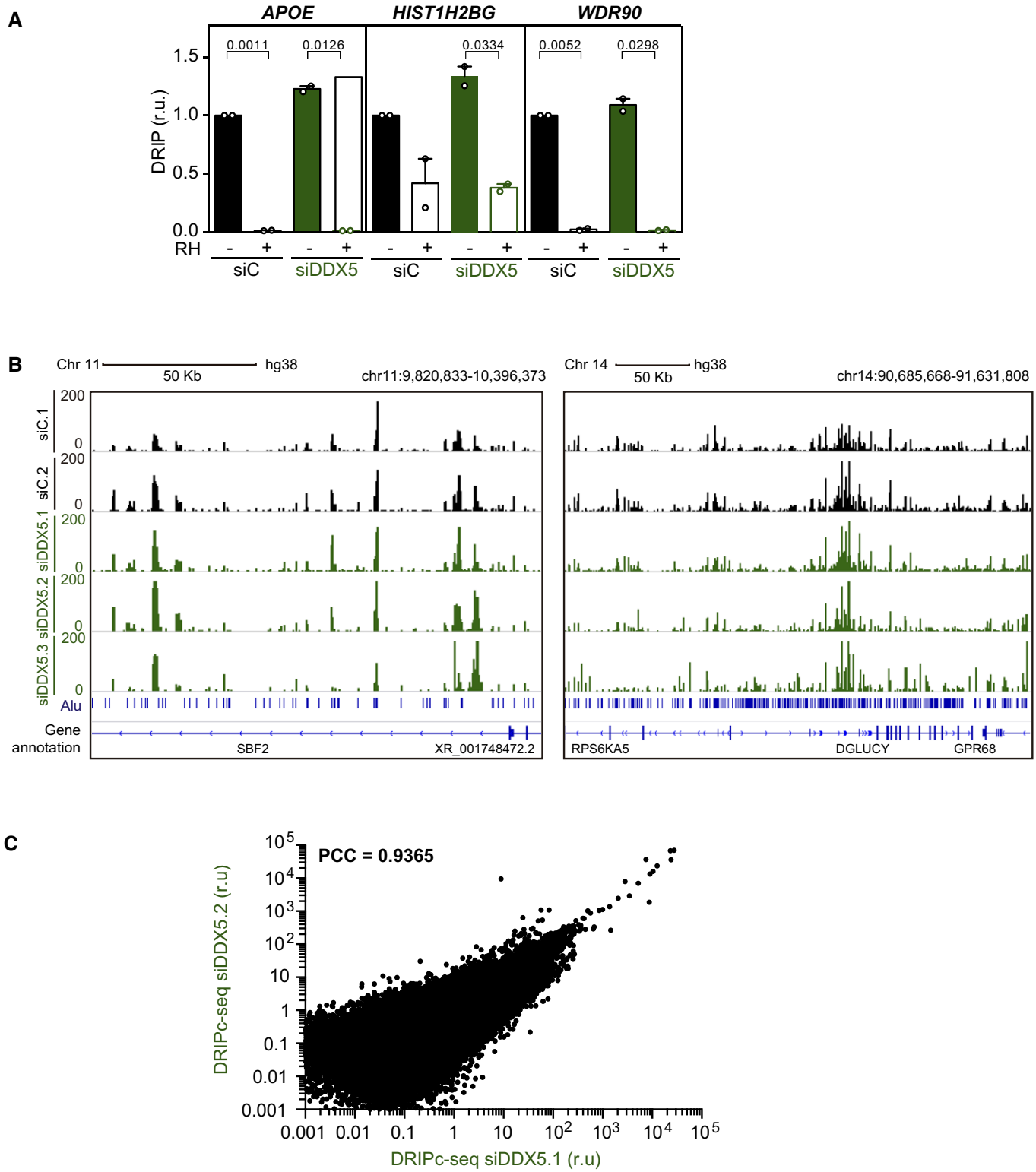
A
APOE
HIST1H2BG
WDR90
0.0011
0.0126
0.0334
0.0052
0.0298
DRIP (r.u.)
1.5
1.0
0.5
0.0
RH
siC
siDDX5
B
Chr 11
50 Kb
hg38
chr11:9,820,833-10,396,373
Chr 14
chr14:90,685,668-91,631,808
siC.1
siC.2
siDDX5.1
siDDX5.2
siDDX5.3
Alu
Gene annotation
SBF2
XR_001748472.2
RPS6KA5
DGLUCY
GPR68
C
PCC = 0.9365
DRIPc-seq siDDX5.2 (r.u)
DRIPc-seq siDDX5.1 (r.u)

Figure EV3.

Figure EV4. Related to Fig 3. NBS1 co-localization with DNA-RNA hybrids in U2OS depleted of DDX5 or BRCA2 and DNA-RNA hybrids, transcription levels, and DDX5 occupancy at different genomic locations in U2OS DIvA cells.

A Left: Representative images of *in situ* PLA experiment performed between NBS1 and S9.6 antibodies in U2OS cells transfected with either siRNA control (siC) or siRNA specific for DDX5 (siDDX5). When indicated, cells were transfected with RNAseH1 (RH) 24 h before fixation. Single antibody controls from untreated cells are shown. Scale bar indicates 10 μm. Nuclei as defined by auto threshold plugin on the DAPI image (ImageJ) are outlined in yellow. Right: Quantification of the number of PLA spots per nucleus in different conditions, as indicated. The data represent at least 400 cells per condition from three independent experiments. For statistical comparison of the differences between the samples, we applied a Kruskal–Wallis test followed by Dunn's multiple comparison test and the *P*-values show significant differences. The red line in the plot indicates the median, and each symbol represents a single PLA spot.

B DRIP-qPCR signal values at *RBMXL1*, *ASXL1*, *HIST1H2BG*, *WDR90*, and *SNPRN* loci in U2OS DIvA cells transfected with the indicated siRNAs and treated *in vitro* with RNase H1 (RH) pre-immunoprecipitation where indicated. The experiment was performed in both untreated cells (−OHT) and after tamoxifen addition (+OHT). The data represent the mean ± SEM from at least four independent experiments. The statistical significance of the difference was calculated with unpaired one-tailed Student *t*-test, and the *P*-values show the significant differences.

C Relative *RBMXL1*, *ASXL1*, *HIST1H2BG*, *WDR90*, and *SNPRN* gene expression levels in U2OS DiVA cells transfected with the indicated siRNAs. The data represent the mean ± SEM from two or three independent experiments. The statistical significance of the difference was calculated with unpaired Student *t*-test; the *P*-values show the significant differences.

D γH2AX (top) and DDX5 (bottom) ChIP-qPCR signal values at *RBMXL1*, *ASXL1*, *HIST1H2BG*, *WDR90*, and *SNPRN* loci in U2OS DiVA cells transfected with the indicated siRNAs and either left untreated (−OHT) or treated with tamoxifen (+OHT). The green line represents the background levels of DDX5 signal. The data represent the mean ± SEM from at least three independent experiments. The statistical significance of the difference was calculated with unpaired one-tailed Student *t*-test, and the *P*-values show the significant differences.

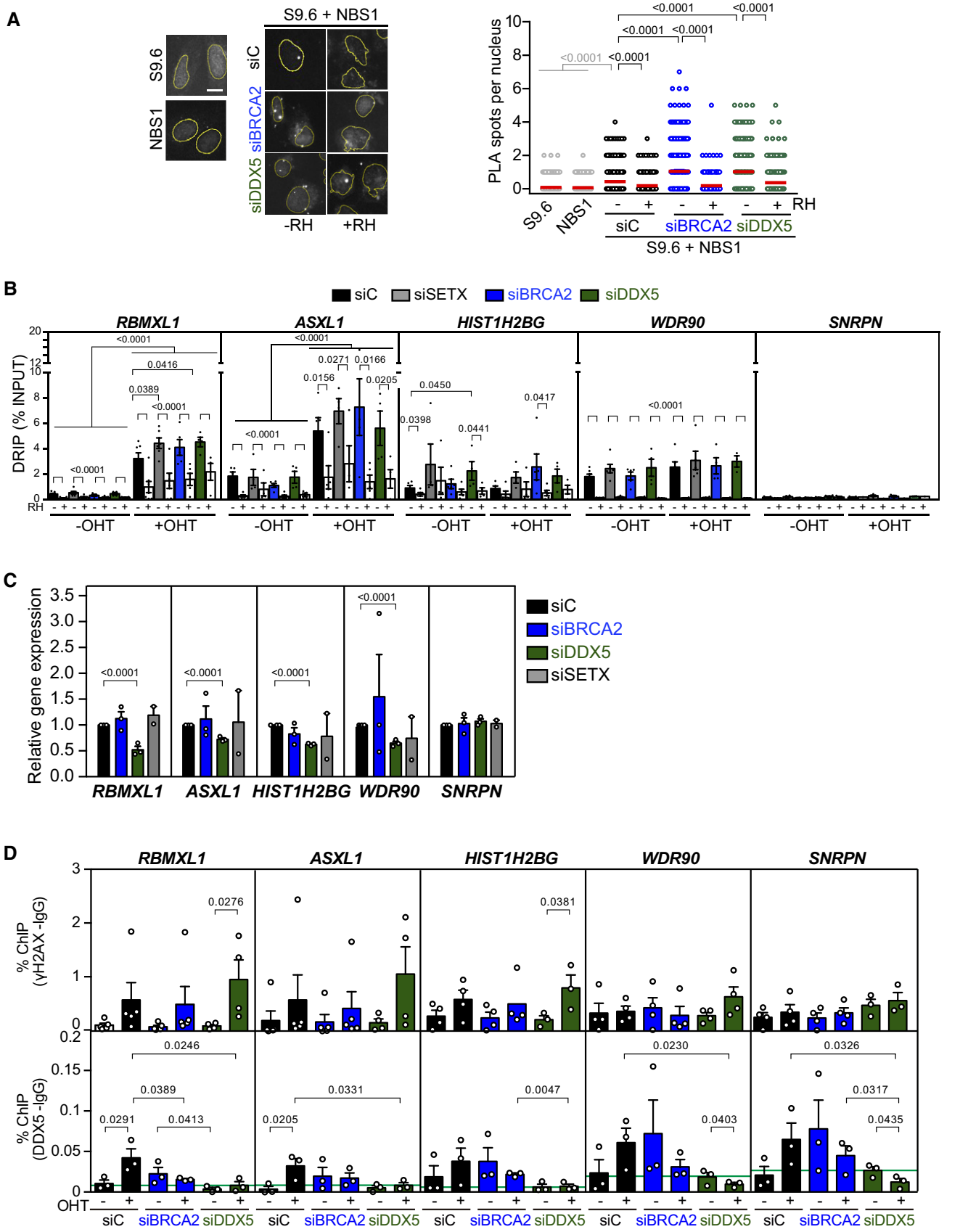
A
S9.6 + NBS1
S9.6
NBS1
siC
siBRCA2
siDDX5
-RH
+RH
PLA spots per nucleus
S9.6
NBS1
siC
siBRCA2
siDDX5
RH
S9.6 + NBS1
B
siC
siSETX
siBRCA2
siDDX5
RBMXL1
ASXL1
HIST1H2BG
WDR90
SNRPN
DRIP (% INPUT)
-OHT
+OHT
C
Relative gene expression
siC
siBRCA2
siDDX5
siSETX
RBMXL1
ASXL1
HIST1H2BG
WDR90
SNRPN
D
RBMXL1
ASXL1
HIST1H2BG
WDR90
SNRPN
% ChIP (γH2AX -IgG)
% ChIP (DDX5 -IgG)
OHT
siC
siBRCA2
siDDX5

Figure EV4.

Figure EV5. Related to Figs 5–8. Effect of $BRCA2_{T2}$ and BRCA2 on DDX5 R-loop and DNA-RNA hybrid-unwinding activity; accumulation of DNA-RNA hybrids in BRCA2-deficient DLD1 cells; interaction of $BRCA2_{T1}$-T207A with DDX5; and kinetics of DNA damage accumulation in cells depleted of DDX5 or in cells bearing BRCA2-T207A mutation.

A Top: PAGE gel showing a representative unwinding assays in which purified MBP-DDX5-GST (1–5 nM) was incubated with ^{32}P-labeled synthetic R-loop substrate in the presence or absence of 50 nM purified 2xMBP-$BRCA2_{T2}$. Bottom: Quantification of the unwinding experiments showing the percentage of free RNA relative to the R-loop substrate (unwound product) as a function of DDX5 concentration alone (black) or in the presence of $BRCA2_{T2}$ (red). Bottom right: SDS–PAGE gel showing 1 μg of purified 2xMBP-$BRCA2_{T2}$ used for the unwinding assay. The data represent the mean ± SD of two independent experiments.

B Top: PAGE gel showing a representative unwinding assays in which purified MBP-DDX5-GST (1–5 nM) was incubated with ^{32}P-labeled synthetic DNA-RNA hybrid substrate in the presence or absence of 2 nM purified EGFP-MBP-BRCA2. Bottom: Quantification of the unwinding experiments showing the percentage of free RNA relative to the DNA-RNA hybrid substrate (unwound product) as a function of DDX5 concentration alone (black) or in presence of BRCA2 (red). The data represent the mean ± SD of three independent experiments.

C Quantification of the average nuclear intensity of S9.6 antibody in DLD1 $BRCA2^{+/+}$ (+/+) and $BRCA2^{-/-}$ (−/−) cells. The red line in the plot indicates the median, and each symbol represents the value of a single cell. The data represent at least 2,000 cells per condition from four independent experiments. The statistical significance of the difference was calculated with Mann–Whitney *U*-test; the *P*-values show the significant difference.

D Relative DRIP-qPCR signal values (respect to the siC level at each locus) at the *MALAT1*, *RRPH1*, and *HIST1H2BG* loci in DLD1 $BRCA2^{+/+}$ (+/+) and $BRCA2^{-/-}$ (−/−) cells and treated *in vitro* with RNase H1 (RH) pre-immunoprecipitation where indicated. The data represent the mean ± SEM from five independent experiments. The statistical significance of the difference was calculated with unpaired Student *t*-test, and the *P*-values show the significant difference.

E GST pull-down assay using purified $BRCA2_{T1}$ (T1) or $BRCA2_{T1}$-T207A (T1-T207A) and DDX5. MBP antibody was used for the detection of both proteins. UB: unbound; E: eluate. Right: Quantification of the GST pull-down experiments calculated as the pulled-down $BRCA2_{T1}$ (WT and T207A) with DDX5 relative to the input levels of $BRCA2_{T1}$ (WT and T207A) and the amount of pulled-down MBP-DDX5-GST. Results are presented as the fold change compared to the BRCA2 WT clone. The data represent the mean ± SD of three independent experiments. The statistical significance of the difference was calculated with unpaired *t*-test, and the *P*-values show the significant difference.

F Top: Representative immunofluorescence images of cells hybridized with anti-γH2AX antibody in U2OS cells depleted of DDX5 (siDDX5) and in control cells (siC) in cells left untreated (−) or at different time points after exposure to IR (6 Gy), as indicated. Scale bar indicates 10 μm. Nuclei as defined by auto threshold plugin on the DAPI image (ImageJ) are outlined in yellow. Bottom: Graph showing the average number of γH2AX foci. The data represent the mean ± SEM of three independent experiments.

G Top: Representative immunofluorescence images of three independent experiments of nuclear γH2AX foci in BRCA2-deficient DLD1 cells ($BRCA2^{-/-}$) or $BRCA2^{-/-}$ bearing BRCA2 WT (WT) or BRCA2-T207A (T207A) variant in cells left untreated or at different time points after exposure to IR (6Gy), as indicated. Scale bar indicates 10 μm. Nuclei as defined by auto threshold plugin on the DAPI image (ImageJ) are outlined in yellow. Bottom: Graph showing the average number of γH2AX foci. The data represent the mean ± SEM of three independent experiments.

H Top: Representative immunofluorescence images of DLD1 BRCA2-T207A stained for γH2AX and RAD51 in non-treated conditions. When indicated, cells were transfected with a plasmid expressing RNaseH1 (+RH) 48 h prior fixation. Right: Quantification of the number of γH2AX foci (left) or RAD51 foci (right) per nucleus. The data shown are from at least 400 cells per condition from three independent experiments. For statistical comparison of the differences between the samples, we applied a Kruskal–Wallis test followed by Dunn's multiple comparison test and no statistically significant differences were found. The red line in the plot indicates the median, and each symbol represents a single focus.

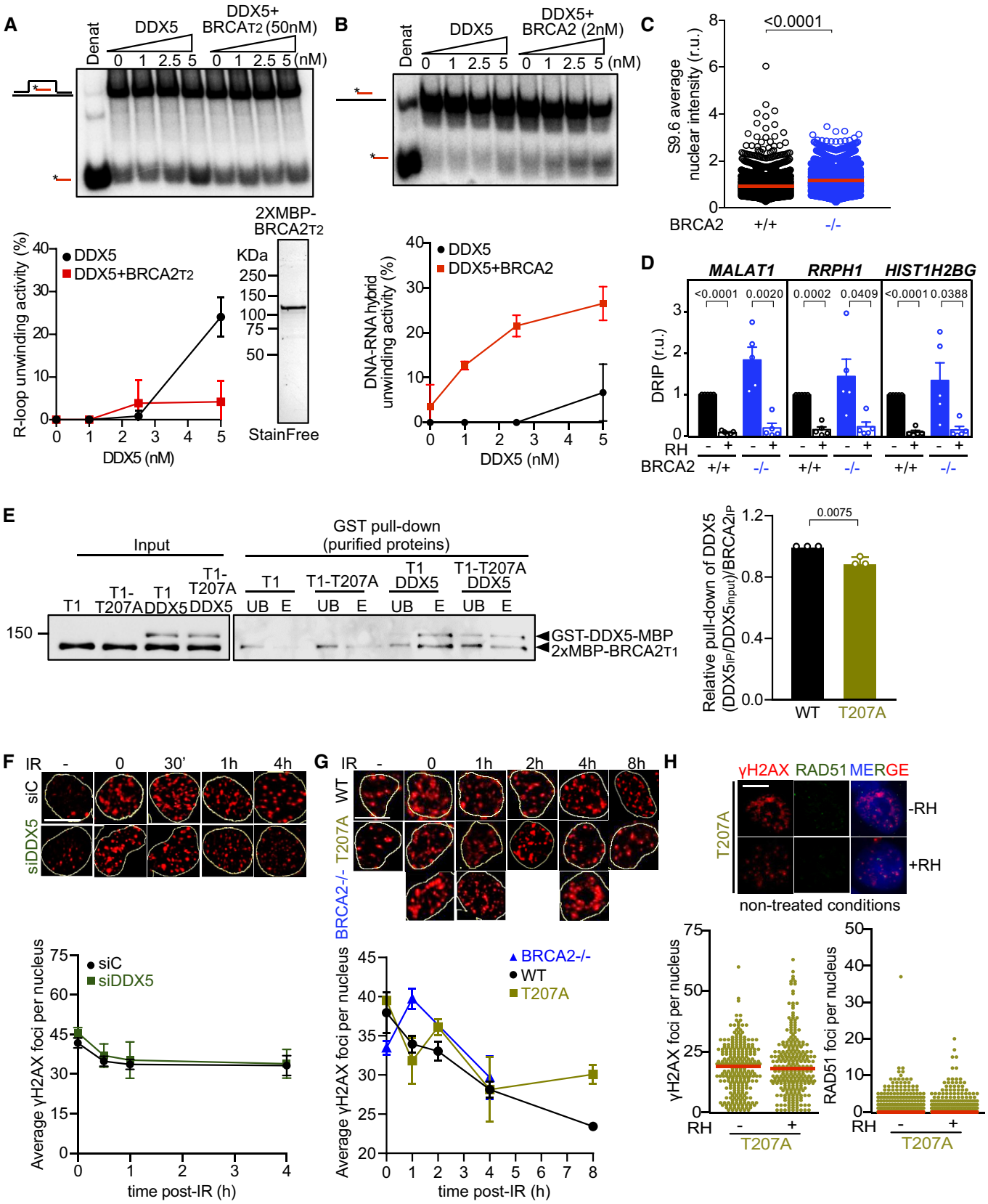
A
Denat
DDX5
DDX5+ BRCAT2 (50nM)
0 1 2.5 5 0 1 2.5 5 (nM)
R-loop unwinding activity (%)
DDX5
DDX5+BRCA2T2
DDX5 (nM)
2XMBP-BRCA2T2
KDa
250
150
100
75
50
StainFree
B
Denat
DDX5
DDX5+ BRCA2 (2nM)
0 1 2.5 5 0 1 2.5 5 (nM)
DNA-RNA hybrid unwinding activity (%)
DDX5
DDX5+BRCA2
DDX5 (nM)
C
<0.0001
S9.6 average nuclear intensity (r.u.)
BRCA2 +/+ -/-
D
MALAT1 RRPH1 HIST1H2BG
<0.0001 0.0020 0.0002 0.0409 <0.0001 0.0388
DRIP (r.u.)
RH - + - + - + - + - + - +
BRCA2 +/+ -/- +/+ -/- +/+ -/-
E
Input
GST pull-down (purified proteins)
T1 T1-T207A T1 DDX5 T1-T207A DDX5
T1 T1-T207A T1 DDX5 T1-T207A DDX5
UB E UB E UB E UB E
150
GST-DDX5-MBP
2xMBP-BRCA2T1
Relative pull-down of DDX5 (DDX5IP/DDX5Input)/BRCA2IP
0.0075
WT T207A
F
IR - 0 30' 1h 4h
siC
siDDX5
Average γH2AX foci per nucleus
siC
siDDX5
time post-IR (h)
G
IR - 0 1h 2h 4h 8h
WT
BRCA2-/- T207A
Average γH2AX foci per nucleus
BRCA2-/-
WT
T207A
time post-IR (h)
H
γH2AX RAD51 MERGE
T207A
-RH
+RH
non-treated conditions
γH2AX foci per nucleus
RAD51 foci per nucleus
RH - +
T207A

Figure EV5.