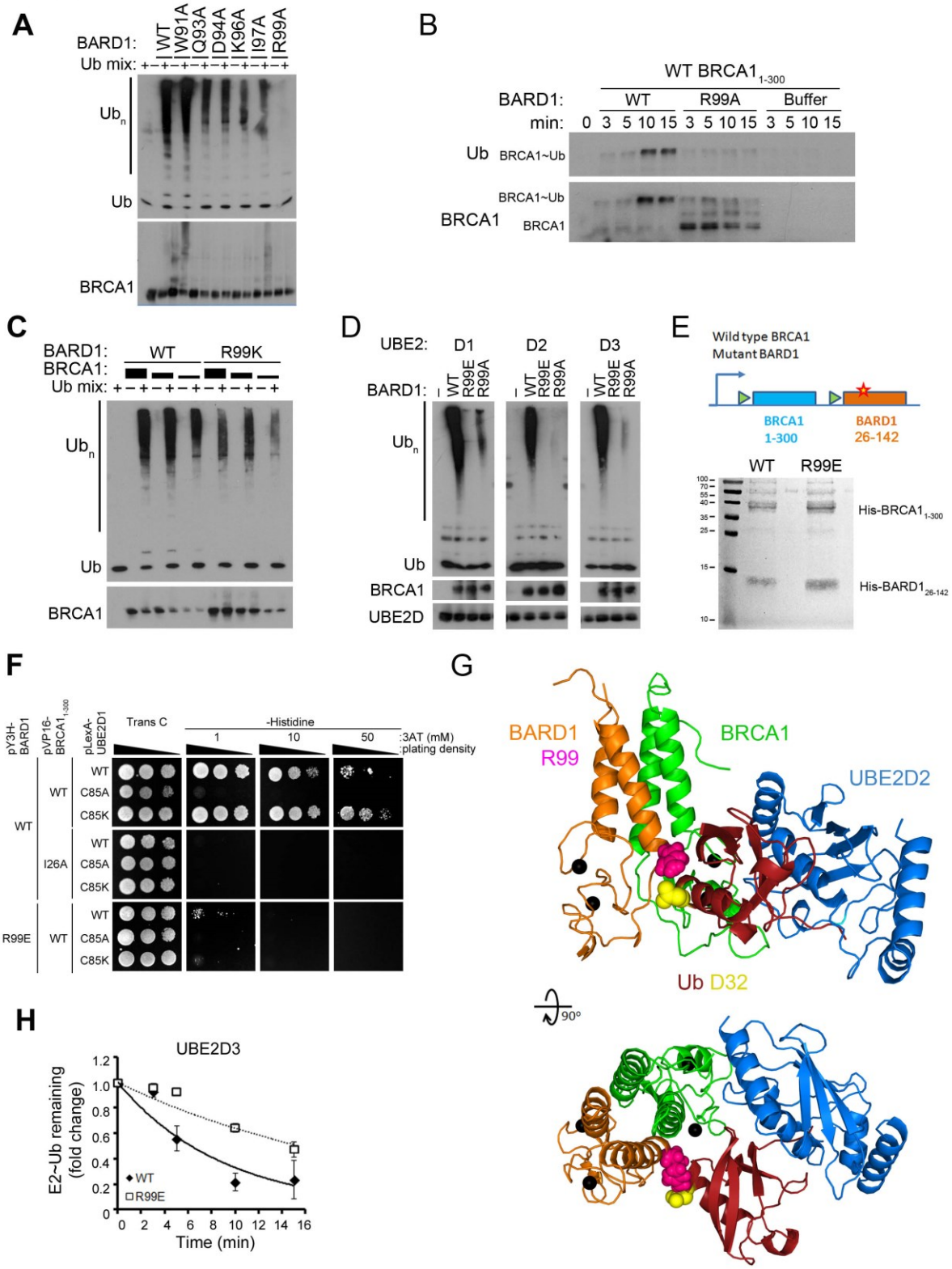
Supplemental Figure 1

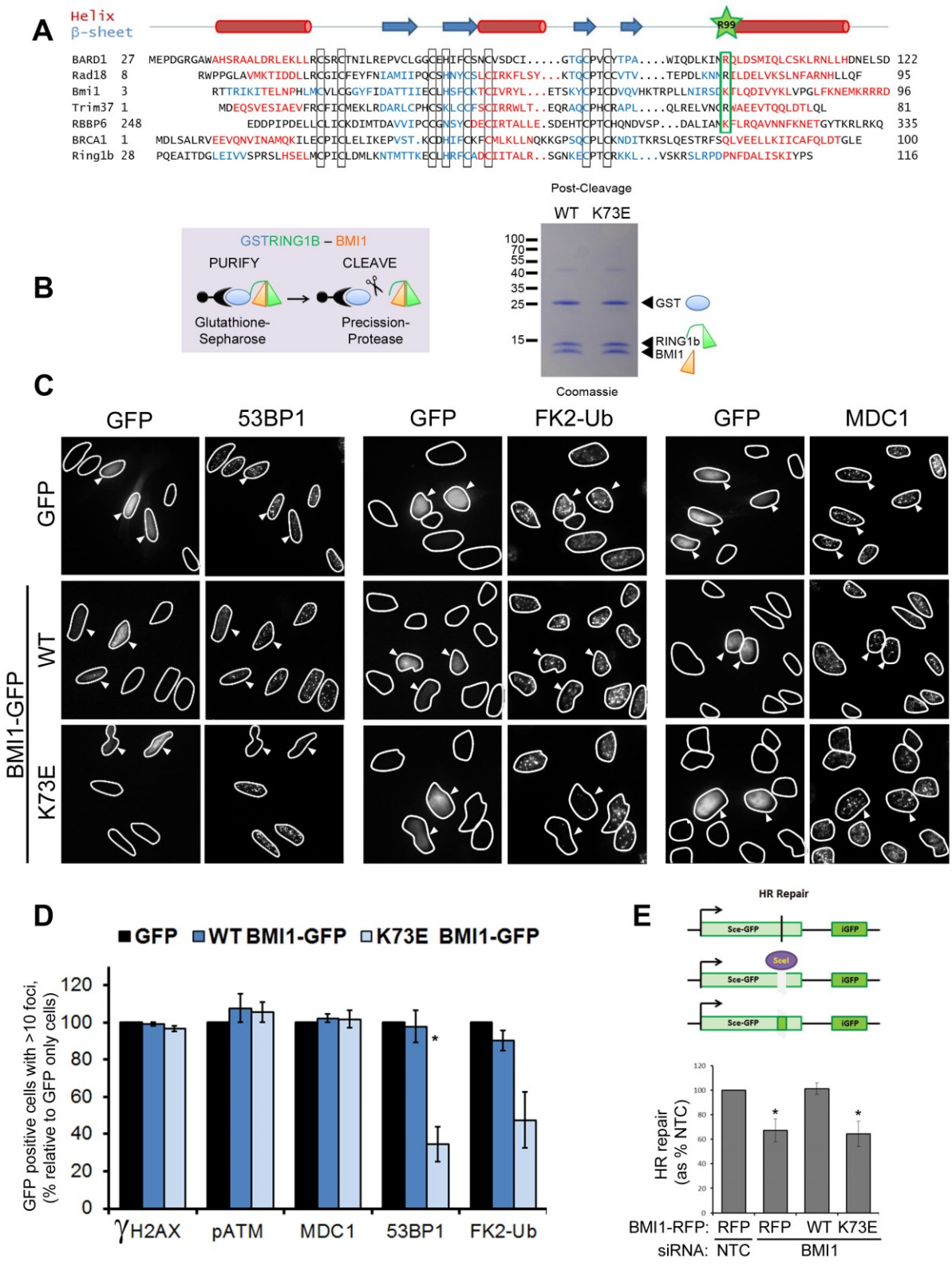


Supplementary Figure 1

A basic residue of BARD1 promotes Ub-transfer from BRCA1-E2~Ub.

A. Scan of external facing BARD1 residues 91-99 for impact on Ub chain formation catalysed by the BRCA1-BARD1 ligase. His-tagged BRCA1 (amino acids 1–300) and either His-tagged WT-BARD1 (amino acids 26–142) or BARD1 bearing the amino acid substitutions shown were co-purified from bacteria, and checked for parity before being subjected to a Ub ligase assay with free Ub and UBE2D1 enzyme.
B. Auto-ubiquitination of BRCA1 is impaired by substitution of R99-BARD1. BRCA1 and WT or mutant BARD1 were co-purified from bacteria, before incubation with UBE2D1 and K-less-Ub. The western shows BRCA1-Ub by blotting for Ub (top) and BRCA1 (bottom).
C. R99K- substitution of BARD1 has little impact on heterodimer ligase activity. BRCA1 and WT or mutant BARD1 were co-purified from bacteria, and checked for parity before being subjected to Ub ligase assay with the UBE2D1 enzyme and Ub.
D. R99E-BARD1 and R99A-BARD1 heterodimers are poorly active with all UBE2D family members. BRCA1 and either WT or mutant BARD1 were co-purified from bacteria, checked for parity before being subjected to a Ub ligase assay with the UBE2D family: D1(UbcH5a), D2 (UbcH5b) and D3 (UbcH5c) enzymes.
E. R99E-BARD1 expressed and purified at a 1:1 ratio with BRCA1. Top panel illustrates the bi-cistronic vector used (green triangles represent 6-histidine tag). Proteins were purified using a nickel column and run on a gel before being Coomassie stained.
F. Contribution of BARD1-R99 to the E2~Ub interaction with BRCA1. VP16-BRCA1$_{1\text{-}300}$ and full length BARD1, were transformed with WT or mutant E2 enzyme LexA-UBE2D1. Yeast also express endogenous Ub. Reduced growth is seen with the E2 catalytic cysteine mutant, C85A, which prevents thioester bonding with Ub, indicating that the heterodimer interacts more strongly with a Ub loaded E2. A slight increase in growth is seen with the C85K mutant, which is reported to increase Ub~E2 stability by preventing Ub transfer (11). I26A-BRCA1 (middle panel) severely impairs E2 interactions (1), and likewise R99E-BARD1 reduces E2 interactions. 'Trans-c' is growth on media lacking leucine, tryptophan and adenine, selecting for all three plasmids. Growth on media also lacking histidine in increasing concentrations of the HIS3 competitive inhibitor, 3AT, is indicative of *HIS3* transcription driven by VP16-BRCA1:UBE2D1-LexA interaction.
G. Modelling of BRCA1-BARD1 on RNF4-RNF4-Ub~E2. The RING domain of BRCA1 (PDB: 1JM7 chain A) was superimposed on the RING domain of RNF4-RNF4 contacting the E2, UBE2D2 (S22R and C85K)–Ub complex (PDB: 4AP4), before removal of the RNF4 structures. BRCA1 is shown in green, BARD1 in orange and UBE2D2 in blue and Ub in brown. The image below is a 90 degree rotation about the horizontal. R99-BARD1 side chain is shown in pink and the Ub D32 side-chain in yellow. Zinc ions are filled spheres (black).
H. Ability of WT and R99E mutant heterodimer to discharge Ub from a loaded E2 (UBE2D3). E2 enzyme was first charged with Ub in the absence of an E3 and substrate, and then incubated with excess lysine and the heterodimer for the times shown before stopping the reaction. E2~Ub dimer was quantified, and the mean across three experiments shown, bars = S.E.

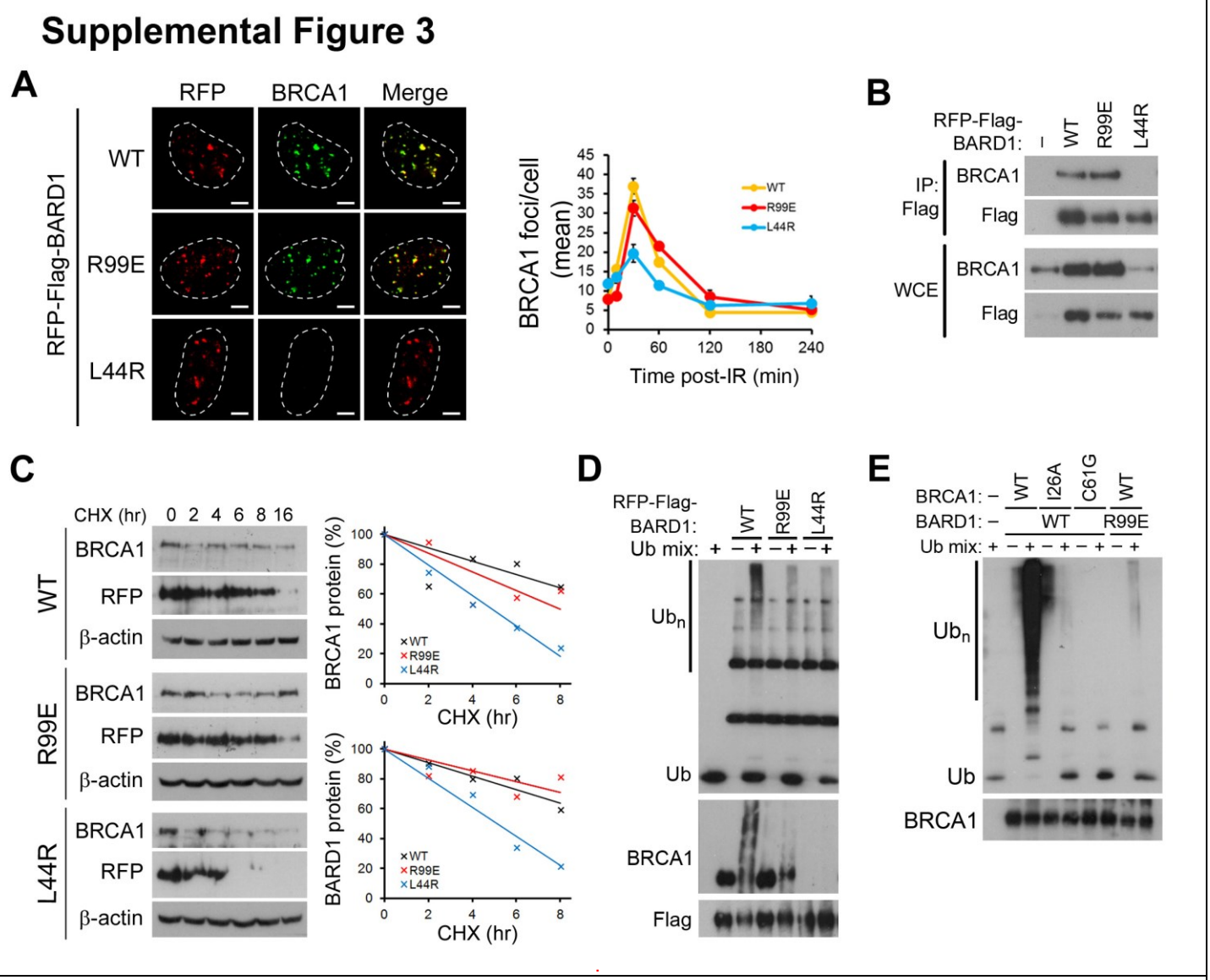
Supplemental Figure 2



Supplementary Figure 2

Other Type-I RING E3 ligases require R or K residues on the partner protomer.

A. Structural sequence alignment of the RING regions highlighting K/R residues (green outline box) and cysteine /histidine residues of the RING (black outline box), α-helices are in red, β-sheets in blue.
B. Purification strategy of RING1B-BMI1, and demonstration of equivalent purification of mutant and WT forms of heterodimer.
C. Inhibition of Ub-signalling in the DNA damage response by ectopic expression of K73E-BMI1 but not WT-BMI1. Cells were transfected with GFP vector, WT-BMI1-GFP or K73E-BMI1-GFP, irradiated with 5 Gy and fixed an hour later before staining with antibodies to γH2AX, pATM, MDC1, 53BP1, and Ubiquitin conjugates (FK2-Ub). Representative images are shown above for GFP vector (top), WT-BMI1 (Middle) or K73E-BMI1 (bottom) for staining for MDC1 (right), where no impact of K73E-BMI1 is seen, FK2-Ub and 53BP1, where expression of K73E-BMI1 is inhibitory to protein recruitment to foci. White arrows indicate GFP positive cells. Data is quantified in C.
D. The % BMI1-GFP positive HeLa cells with >10 foci of each type was quantified relative to % GFP only control (graph below, 100 cells per set, 3 independent experiments, bars = S.E).
E. Complementation of BMI1 siRNA treated cells with siRNA resistant WT BMI1 or with K73E-BMI1 in gene conversion of a substrate integrated into U20S cells after *I-Sce-1* transfection. NTC is non-targeting control siRNA. (DR3-GFP substrate and repair products are illustrated above). (Each assay 3 technical repeats, 3 experiments, bars = S.E. * indicates $p<0.05$)



Supplementary Figure 3

BARD1 L44R and R99E separation of function variants.

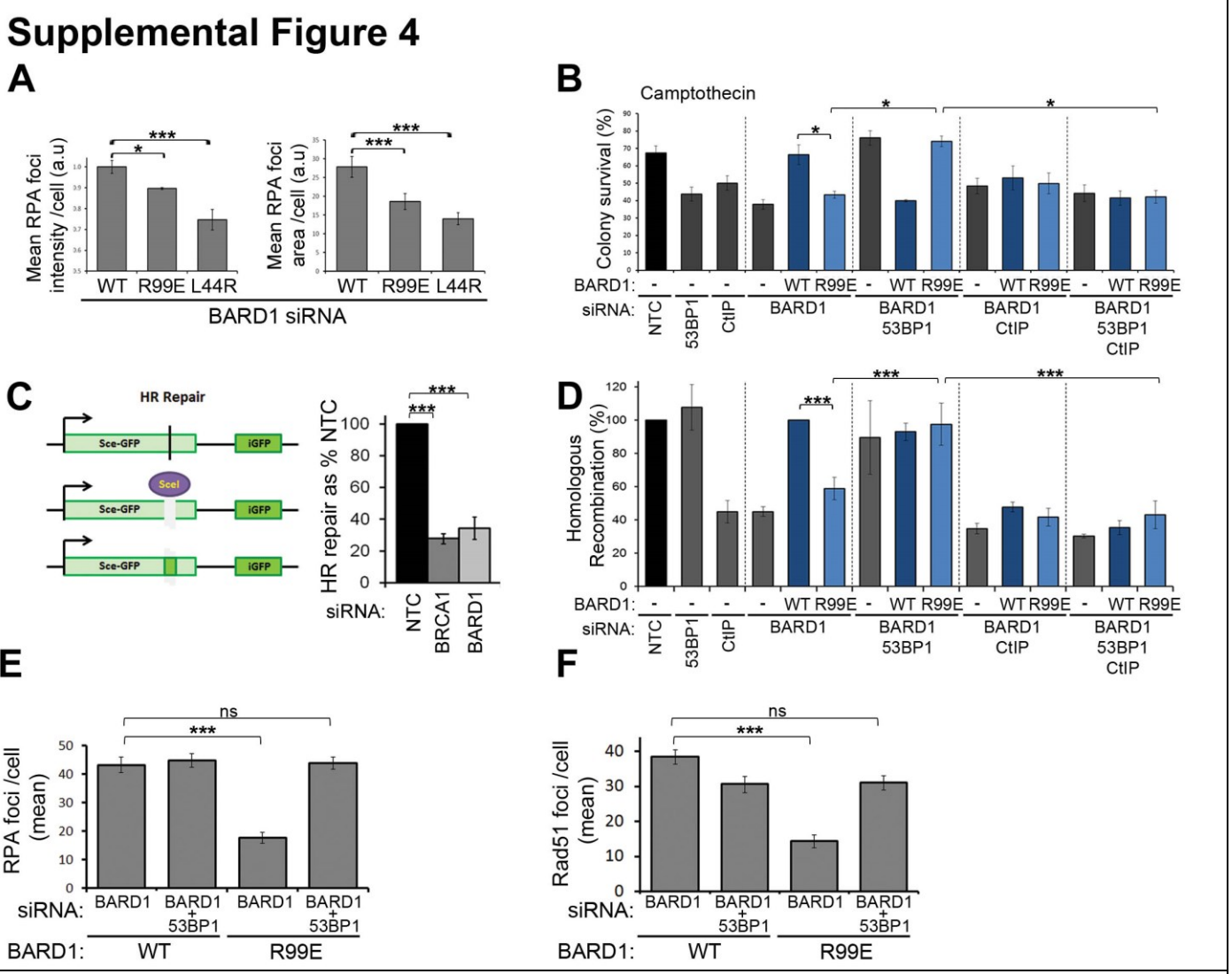
A. Transfection with BARD1 siRNA and complementation with WT-BARD1 or R99E-BARD1 supports the formation of endogenous BRCA1 foci after irradiation (IR). In contrast BRCA1 foci do not form in cells complemented with L44R-BARD1. BARD1 complemented cells were irradiated (5 Gy) and allowed an hour to recover before fixation and staining for BRCA1. Quantification of BRCA1 foci at times shown after IR treatment in cells complemented with BARD1 proteins show in the graph, right (bars = S.E., n=100 cells).

B. WT-BARD1 and R99E-BARD1 stabilize and co-purify endogenous BRCA1, whereas L44R-BARD1 does not. Immunoprecipitation of Flag-bound proteins from 293 cells expressing WT, R99E or L44R substituted RFP-Flag-BARD1, input levels, from Whole Cell Extract (WCE) is shown. (Note the increased endogenous BRCA1 on WT or R99E BARD1 expression).

C. WT-BARD1 and R99E-BARD1 heterodimers are stable. BARD1 variants were expressed in 293 cells and treated with Cycloheximide (CHX) for the times shown. BRCA1 and BARD1 were quantified from immunoblots (graph right).

D. Immunoprecipitated BARD1 complexes were combined with E1, E2 (UBE2D1), Ub and ATP. The complexes purified with WT-BARD1 exhibited ligase activity whereas R99E-BARD1 and L44R-BARD1 precipitated complexes had minimal activity with Ub conjugation components.

E. Comparison of heterodimer bearing R99E-BARD1 or I26A- or C61G –BRCA1 to catalyse the formation of Ub chains *in vitro*. Ub mix refers to E1, E2, Ub, ATP and ligase reaction buffer.



Supplementary Figure 4

BRCA1-BARD1 ligase activity promotes DNA resection in the presence of 53BP1.

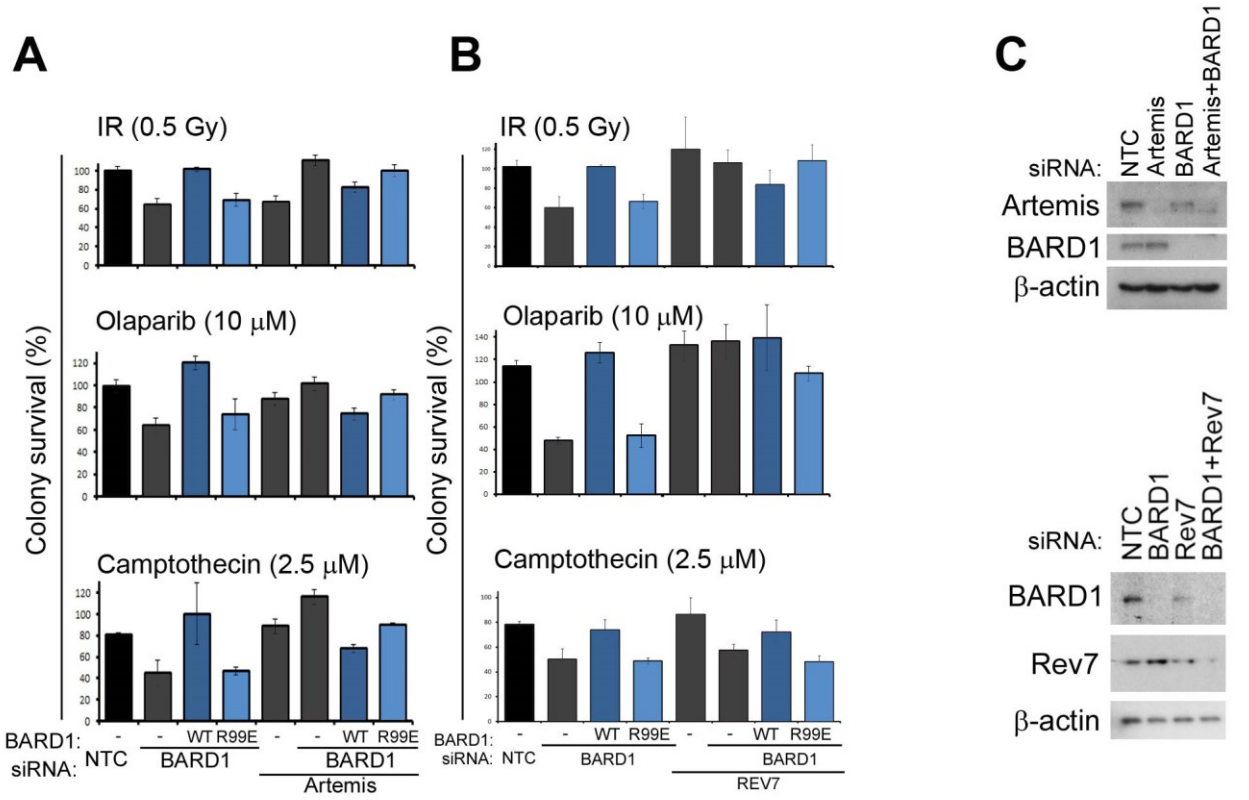
A. Intensity and the area of RPA foci in EdU positive cells treated with BARD1 siRNA and complemented with siRNA resistant WT, R99E- or L44R-BARD1 variants (n= 60 cells, bars= S.E. *= p<0.05, ***= p<0.005 Student t-test, compared to WT). Quantified from data shown in Fig 4a.
B. Camptothecin sensitivity (2.5uM) of cells treated with BARD1 siRNA and complemented with siRNA resistant WT or R99E BARD1. Restoration of resistance requires CtIP (3 replicates per experiment, 3 experiments, error bars = S.E. *= p<0.05, Student t-test).
C. DR3-GFP substrate and repair products are illustrated left, BRCA1 or BARD1 depletion reduce gene conversion of a substrate integrated into U20S cells after *I-Sce-1* transfection, (n=5, 3 technical repeats per experiment, bars = S.E. ***= p<0.005, Student t-test).
D. Gene conversion in cells treated with BARD1 siRNA and complemented with WT or R99E-BARD1.

Restoration of gene conversion by 53BP1 depletion requires CtIP. (n= 4, assays, each assay 3 technical repeats, bars = S.E. ***= p<0.005, Student t-test).

E. 53BP1 depletion increases RPA foci in cells complemented with R99E-BARD1. Cells treated with BARD1 siRNA and complemented with WT-BARD1 or R99E-BARD1, with or without treatment with 53BP1 siRNA. Cells were fixed 2 hours post 5 Gy IR. EdU positive cells were scored for the number of RPA foci. n=>30 cells, bars = S.E. ***= p<0.005, Student t-test.

F. As in E, stained for RAD51.

Supplemental Figure 5

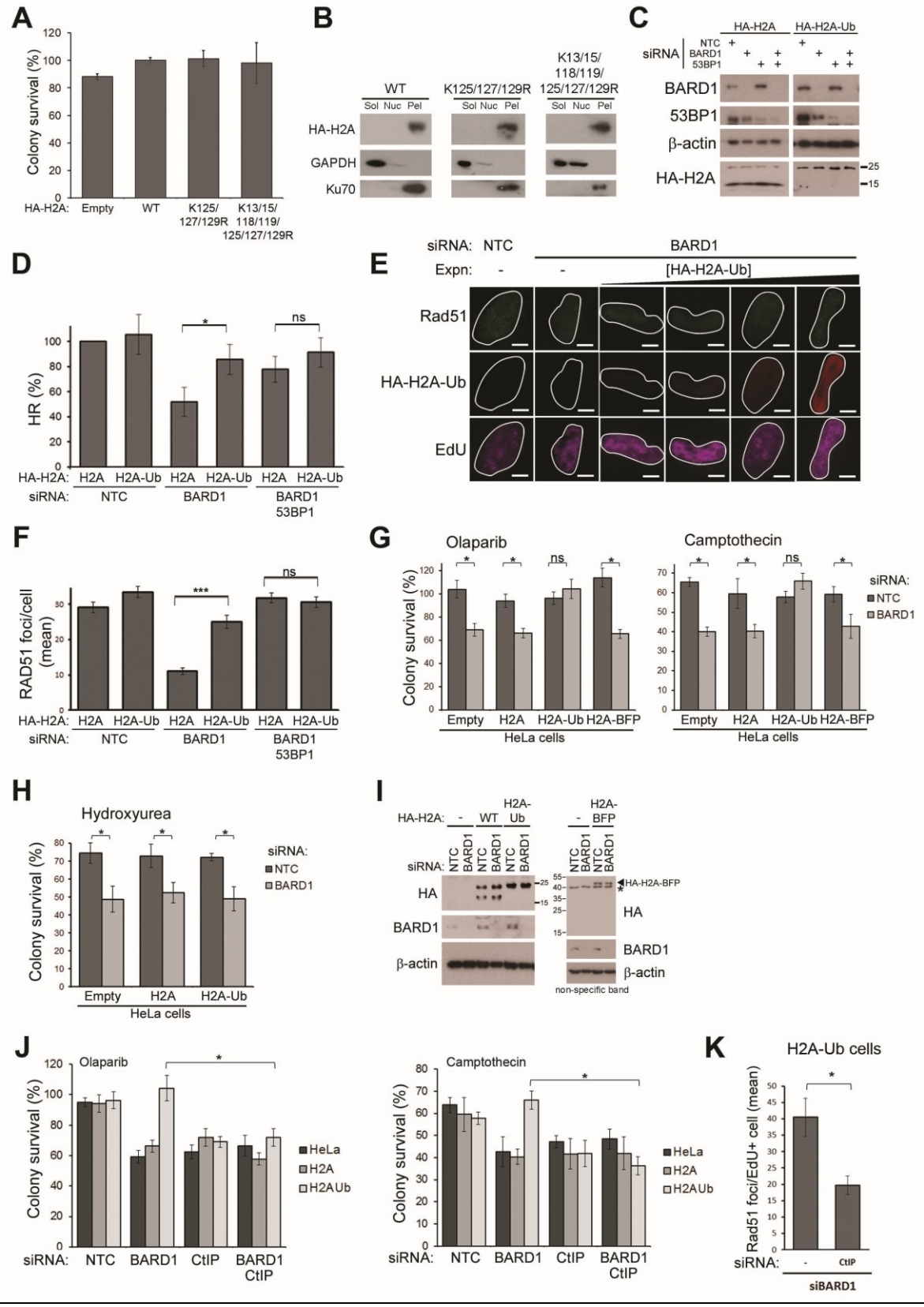


Supplementary Figure 5

53BP1 effector proteins counter BRCA1-BARD1 ligase activity in Olaparib and Camptothecin sensitivity.

A. Artemis depletion promotes resistance of R99E-BARD1 complemented cells and BARD1 depleted cells to Camptothecin, Olaparib and IR. Cells were treated with siRNAs shown plated and clones counted 10-14 days later. Colony numbers are expressed as % of untreated cells. (3 replicates per experiment, 3 experiments, error bars = S.E. *= $p<0.05$, *** $p<0.005$, ns: not significant).
B. As in A but following Rev7 depletion.
C. Lysates from cells treated with siRNAs shown, subject to SDS-PAGE and immunoblotted with the antibodies shown.

Supplemental Figure 6

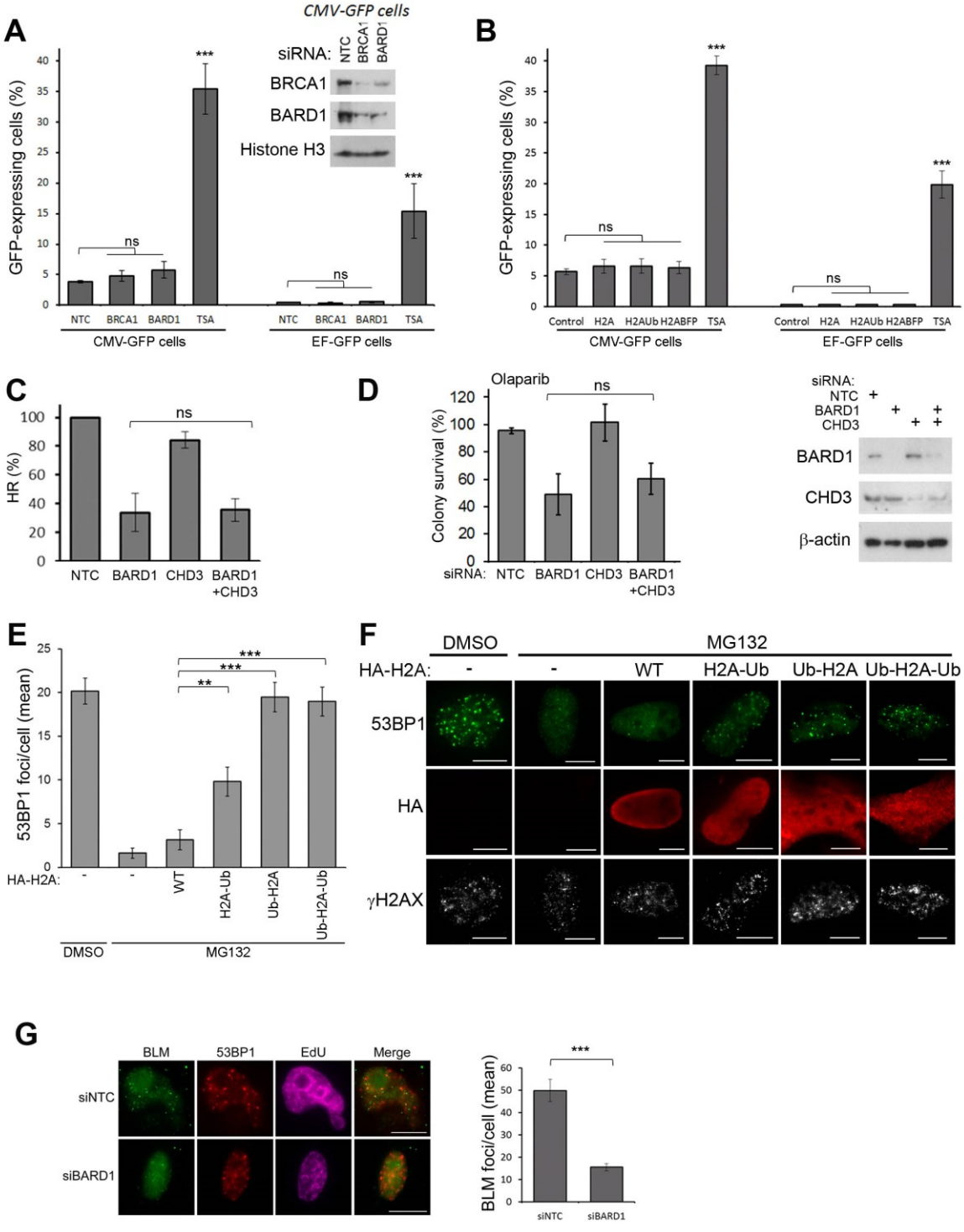


Supplementary Figure 6

An H2A-Ub fusion promotes DNA resection, HR and Olaparib and Camptothecin resistance.

A. H2A-C-terminal K to R mutant had no impact on Olaparib sensitivity in colony survival assays. H2A and H2A mutants were expressed for 72 hours prior to Olaparib exposure (10 μm) n=3, replicates per experiment 3, error bars = S.E
B. Incorporation of H2A WT and H2A-mutants into chromatin. Sol = soluble fraction (100 mM salt), Nuc= nuclear fraction (200 mM salt) and Pel= Pellet (500 mM salt).
C. Western blot for protein levels following BARD1 and 53BP1 depletions and H2A and H2A-Ub expressions for experiments shown in Supplemental Fig. 6D & F).
D. H2A-Ub expression restores gene conversion of an integrated substrate. DR3-GFP-U20S cells were transfected with the siRNAs shown and either WT H2A or H2A-Ub with *I-Sce-1* before assessment of gene conversion (n= 3 assays, each assay 3 technical repeats, bars = S.E. ***= $p<0.005$, ns: not significant).
E. Representative images of HA-H2A-Ub and RAD51 foci quantification (to accompany Fig 6C).
F. RAD51 foci in EdU positive cells treated with BARD1, or BARD1 and 53BP1 siRNA and transfected with either WT H2A or H2A-Ub (n=30 cells, bars = S.E. ***= $p<0.005$.).
G. H2A-Ub, but not H2A or H2A-BFP expression improves survival of BARD1 depleted cells after Olaparib and Camptothecin exposure. Cells were induced with for H2A expression and treated with BARD1 or control siRNA for 72 hours before exposure to Olaparib (10 μM) or Camptothecin (2.5 μM). n=3, replicates per experiment 3, error bars = S.E. *= $p<0.05$, Students T test, ns: not significant.
H. H2A-Ub expression has no impact on cellular sensitivity of BARD1 depleted cells to Hydroxyurea. Inducible cells were doxycycline and siRNA treated as above and exposed to 3mM Hydroxyurea for 16 hours then plated and clones counted 14 days later. 3 replicates per experiment, 3 experiments, error bars = S.E. *= $p<0.05$, Students T test.
I. Western blot for protein levels following BARD1 depletion and H2A, H2A-Ub or H2A-BFP expressions for experiments shown in Supplemental Fig. 6G&H.
J. Restoration of Olaparib (10 μM) and Camptothecin (2.5 μM) resistance of BARD1 depleted cells by H2A-Ub requires CtIP. Inducible H2A-expressing cells were doxycycline and siRNA treated as above and exposed to the agents shown, plated and colonies counted 14 days later. n=>3, replicates per experiment 3, error bars = S.E. *= $p<0.05$, Students T test
K. RAD51 foci quantification in EdU+ cells depleted for BARD1 and expressing H2A-Ub after co-depletion of BARD1 or BARD1 and CtIP. (n=3, 50 cells per replicate, error bars = S.E. *= $p<0.05$, Students T test).

Supplemental Figure 7



Supplementary Figure 7 Text and Legend

Investigation of how BRCA1-BARD1 ligase activity or Ub-modified nucleosomes might impact 53BP1 and resection.

Text.

BRCA1 loss in murine cells has recently been associated with de-repression of heterochromatic silencing (Zhu *et al*., Nature, 2011. 477(7363): p. 179-84.). Similarly H2A mono-ubiquitination at K119 of H2A is associated with transcriptional repression of at least some genes (Pengelly *et al* Genes Dev, 2015. 29(14): p. 1487-92. Scheuermann *et al*., Fly (Austin), 2012. 6(3): p. 162-8). Thus in principle the C-terminal fusion H2A-Ub may counter the loss of BRCA1:BARD1 activity by repressing chromatin and gene expression. We addressed whether epigenetic de-repression is a consequence of BRCA1:BARD1 loss in human cells. We tested two HeLa cell lines harbouring epigenetically silent green fluorescent protein reporter genes (pCMV-GFP and the more tightly silenced pEF1-GFP) which have been extensively used to reveal epigenetic silencing factors (Poleshko *et al*., Biol Chem, 2010. 285(1): p. 422-33. Poleshko *et al*., Epigenetics, 2014. 9(9): p. 1280-9). Neither line exhibited increased GFP expression on BRCA1 or BARD1 depletion (Supplemental Fig 7A), suggesting that epigenetic de-repression is not a general consequence of BRCA1:BARD1 loss at least over the time-frames of expression repression by siRNA knock-down.

Alternatively it is possible that H2A-Ub expression induces chromatin relaxation, although previous examination of global chromatin structure of cells expressing H2A-fusions have found no large scale changes (Kocylowski *et al*., Cell Cycle, 2015; 14(11): 1748–1758.). We nevertheless tested the possibility that the C-terminal H2A-Ub fusion might result in epigenetic de-repression by expressing H2A-Ub in the HeLa cell lines bearing epigenetically silenced GFP genes. No increased expression was observed (Supplemental Fig 7B). We also examined if inducing global chromatin decondensation could rescue gene conversion or Olaparib resistance of BARD1 depleted cells. CHD3 is part of the NuRD complex and its depletion results in global chromatin decondensation (Torchy *et al*., Cell Mol Life Sci, 2015. 72(13): p. 2491-507. Garvin *et al*., Embo Reports, 2013. 14(11): p. 975-83)), however CHD3 depletion did not improve gene conversion or the survival of BARD1 depleted cells (Supplemental Fig 7C & D). These data do not support the view that global chromatin changes as a result of BRCA1:BARD1 loss or H2A-Ub fusion expression are relevant to BRCA1-mediated DNA repair evident in the current study.

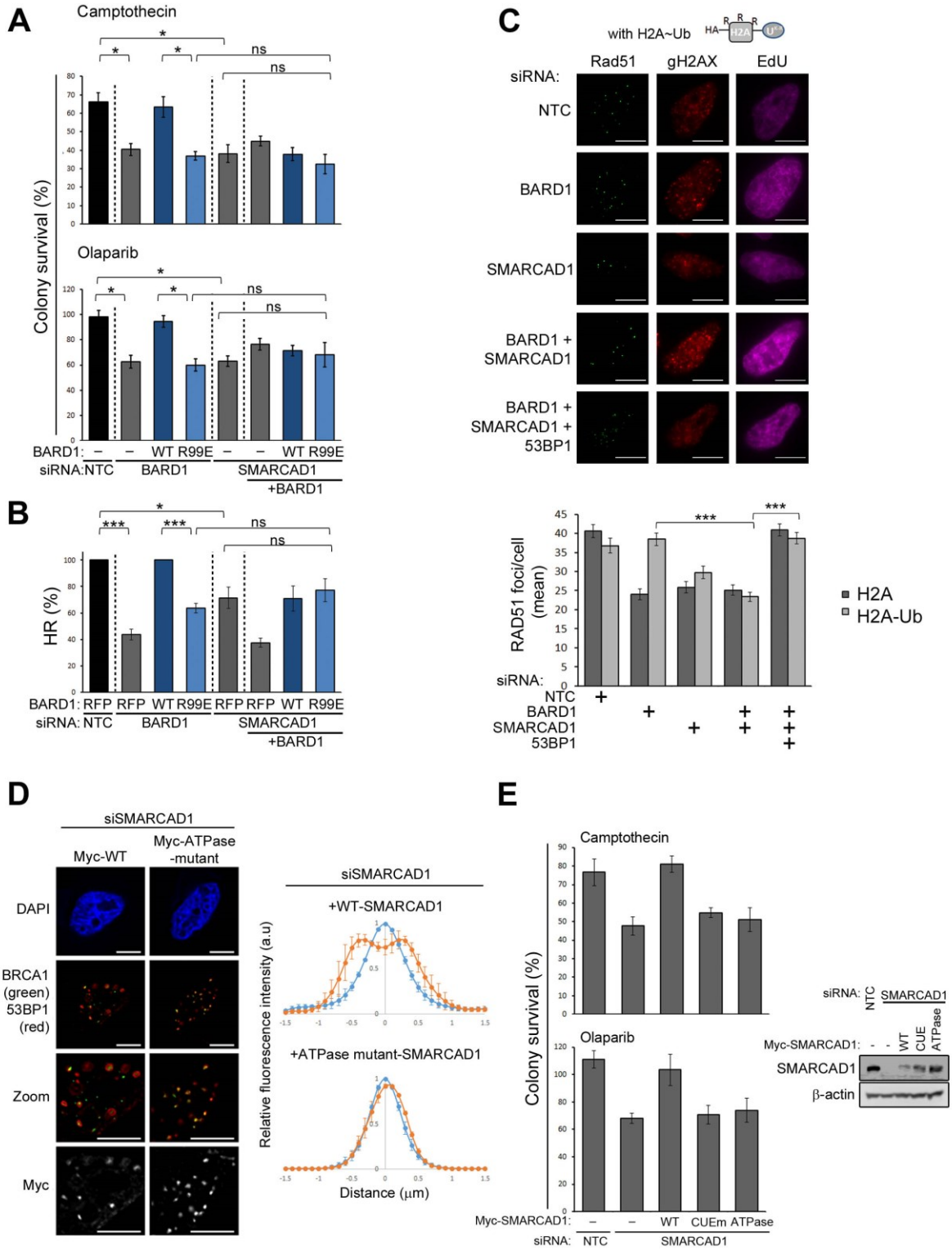
Accumulation of 53BP1 to IRIF in part involves the engagement between 53BP1 and H2A modified by Ub at its N-terminus (H2A K13/15) (Gatti *et al*., Cell Cycle, 2012. 11(13): p. 2538-44. Mattiroli *et al*., Cell, 2012. 150(6): p. 1182-1195 Fradet-Turcotte *et al*., Nature, 2013. 499(7456): p. 50-54). We next speculated that a C-terminal-Ub modification on H2A directed by BRCA1:BARD1 may inhibit 53BP1 recruitment. We tested H2A constructs (mutated K-R at K13, 15, 118, 119, 125, 127 & 129) with Ub genetically fused to the N terminus, C-terminus or both ends for their ability to rescue 53BP1 foci formation in irradiated cells starved of free Ub by proteasome inhibition. As expected expression of a construct in which Ub is fused to the N-terminus of H2A promoted 53BP1 foci formation (Supplemental Fig 7E & F) as previously described for an H2AX-Ub fusion Kocylowski *et al*., Cell Cycle, 2015; 14(11): 1748–1758.). The 53BP1 accumulations observed largely co-localized with γH2AX, and 53BP1 foci were not evident in un-irradiated cells, suggesting they are chromatin associated and not aggregates formed elsewhere. An H2A construct in which Ub was fused to the C-terminus

was able to only partially restore 53BP1 IRIF (Supplemental Fig 7E & F). Moreover H2A bearing Ub fusions both N- and C-terminal restored 53BP1 foci to the same degree as the N-termini Ub-H2A construct, indicating that gross accumulation of 53BP1 to DSBs is not inhibited by a C-terminal Ub modification on the same H2A. The same fusion proteins had the opposite impact on RAD51 foci in BARD1 depleted cells, where the C-terminally-fused H2A promoted greater IRIF formation than an N-terminal fusion, Fig 6D). Thus our data suggest distinct outcomes are determined by Ubiquitin at the N or C terminus of H2A.

Supplemental Figure 7 Legend.

A. Depletion of BRCA1 or BARD1 does not increase GFP expression from epigenetically silenced loci. Cells bearing integrated and epigenetically silenced CMV-GFP and EF-GFP were depleted as shown or treated with 1 mM Trichostatin A (TSA) histone deacetylase inhibitor. 3 replicates per experiment 3 experiments, error bars = S.E. *** $p<0.005$, Students T test, compared to NTC siRNA (left) or untreated control (right)).
B. Expression of H2A-fusions does not increase GFP expression from epigenetically silenced loci.
C. Depletion of CHD3 does not improve gene conversion in BARD1 depleted cells. Mean of 3 assays, each assay 3 technical repeats, bars = S.E. (knockdown shown in D).
D. Depletion of CHD3 does not improve survival of BARD1 depleted cells treated with Olaparib (10 μM). Cells were treated with BARD1 siRNA or BARD1 and CHD3 siRNA for 72 hours before exposure to Olaparib, plated and colonies counted 10-14 days later. Colony numbers are expressed as % of untreated cells. (3 replicates per experiment 3 experiments, error bars = S.E.). Lysates from co-treated cells were immunoblotted for the proteins shown (right).
E. C-terminal fusions of Ub to H2A do not inhibit the restoration of 53BP1 foci in MG132-treated cells mediated by N-terminal H2A Ub fusion. Cells were transfected with HA-H2A expression constructs Ub fusions. The HA-H2A was mutated at K13 15 118 119 and 125 127 129R and the fused Ub moiety carried K to R mutations in all lysines to prevent chain formation. Cells were treated with MG132 (10 μM) for 1 hour prior to irradiation (5Gy) and fixed with PFA prior to permeablization and stained for HA to identify H2A expressing cells, 53BP1 and γH2AX. HA-H2A expressing cells were scored for 53BP1 foci after 3 hours. (30 cells per mutant per technical repeat, 3 experiments repeats, bars =S.E. ** $p<0.01$, *** $p<0.005$, Students T test).
F. Representative images of cell staining used in E.
G. BLM accumulation to IRIF is reduced in cells depleted for BARD1. Cells were transfected with control or BARD1 siRNA, incubated for 72 hours before incubation with EdU for the last half hour, exposure to 5 Gy IR and further incubation for an hour before fixation and staining as shown. 100 cells per condition, 3 experimental repeats, bars =S.E *** $p<0.005$, Students T test.

Supplemental Figure 8



Supplementary Figure 8

The nucleosome remodeller SMARCAD1 is in the same pathway as BRCA1-BARD1 ligase activity.

A. SMARCAD1 knock-down is epistatic with R99E-BARD1 in Camptothecin (2.5 μM) and Olaparib (10 μM) sensitivity. Colony numbers are expressed as % of untreated cells. (n=>3, replicates per experiment 3, error bars = S.E. * $p<0.05$, Student's t test)
B. SMARCAD1 knock-down is epistatic with R99E-BARD1 in gene conversion (mean of n>3 assays, each assay 3 technical repeats, bars = S.E., * $p<0.05$, ** $p<0.05$ Student's t test).
C. Rescue of RAD51 IRIF BARD1 depleted cells by H2A~Ub in requires SMARCAD1. SMARCAD1 is not needed in the absence of 53BP1. The graph below shows quantification. *** $p<0.05$, n>50 cells per repeat, 3 experimental repeats.
D. The ATPase activity of SMARCAD1 influences 53BP1 positioning at IRIF. HeLa cells were transfected with siRNA targeting SMARCAD1 and incubated for 72 hours, exposed to 2 Gy IR and fixed 8 hours later, or transfected with siRNA targeting SMARCAD1 and with siRNA resistant plasmids for WT and ATPase mutant (K528R) myc-SMARCAD1 before treatment. Cells were immunostained for BRCA1 and 53BP1, imaged and deconvolved as in Fig 4D. 30 profiles measured over 3 experimental repeats. Bars =1 standard deviation.
E. SMARCAD1 CUE domains, and ATPase activity promote resistance to Camptothecin (2.5 μM) and Olaparib (10 μM). Colony numbers are expressed as % of untreated cells (3 replicates per experiment, 3 experiments, error bars = S.E. * $p<0.05$).