Supplementary Information for

The VEGF receptor Neuropilin 2 promotes homologous recombination by stimulating YAP/TAZ-mediated Rad51 expression

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Supplementary Information Text

Materials and Methods

Reagents, antibodies and cell culture. Verteporfin and cisplatin were purchased from Tocris, olaparib was purchased from Selleckchem and human VEGFA was purchased from R & D Systems. The NRP2 function blocking antibody was provided by Genentech (1). Bevacizumab was provided by the UMASS Medical School oncology pharmacy. Cisplatin was used at a concentration of 10 μM for 24 hours, verteporfin was used at a concentration of 2 μM for 24 hours, the NRP2 function blocking antibody and bevacizumab were used at a concentration of 10 μg/mL for 24 hours and olaparib was used at a concentration of 5, 10 and 20 μM for 5 days. Immunoblotting antibodies were acquired as follows: Actin (MA5-15739, Thermo Fisher Scientific), TAZ (560235, BD Biosciences), YAP/TAZ (8418S, Cell Signaling Technologies), Rad51 (8875S, Cell Signaling Technologies), NRP2 (AF2215, R & D Systems), γH2AX (05-636, Millipore), HA-tag (3724S, Cell Signaling Technologies), FLAG-tag (F3165, Sigma-Aldrich) and Myc-tag (2278S, Cell Signaling Technologies). MDA-MB-231 and Hs578t were obtained from the American Type Culture Collection and SUM-1315 cells were provided by Dr. Stephen Ethier.

Constructs, transfection and siRNA knockdown. shNRP2, shVEGF, shTAZ, S89A TAZ pcDNA 3.1 and Myc-tagged dominant negative TEAD4 were used as previously described (2). Retroviral S127A YAP (plasmid #33092) and lentiviral S89A TAZ (plasmid #52084) were purchased from Addgene. Rad51 was provided by Dr. Maria Jasin (Memorial Sloan Kettering Cancer Cancer) (3). Lipofectamine 3000 (Thermo Fisher Scientific) was used for plasmid expression and DharmaFect 4 (Dharmacon) was used for siRNA knockdown. YAP/TAZ siRNA has been previously described (4).

Cell viability assay and organoid culture. To assess viability, cells were seeded and subsequently treated with cisplatin, the NRP2 function blocking antibody, bevacizumab, verteporfin or combinations for 24 hours as described in the figure legends as well as 2, 4 and 6 Gy of IR. Cells were then washed with 1X phosphate-buffered saline (PBS) and complete medium was added. Cells were counted 3 days following treatment using trypan blue exclusion. For the olaparib experiment, cells were seeded and subsequently treated with 5, 10 and 20 μM concentrations of olaparib for 5 days, and then counted after trypan blue exclusion to assess cell viability. Cell number was normalized to 1 based on the control sample. BRCA proficient mammary tumor organoids were provided by Dr. Jos Jonkers (Netherlands Cancer Institute) and cell viability was assessed as previously described (5).

Immunoblotting. Cells were scraped on ice in RIPA buffer with EDTA and EGTA (BP-115DG, Boston Bioproducts) supplemented with protease inhibitor cocktail (Roche, 04693132001). Subsequently, laemmli buffer (BP-111R, Boston Bioproducts) was added to each sample and the lysate was boiled and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Luciferase reporter assay. The Rad51 promoter luciferase construct was provided by Dr. Vera Gorbunova (University of Rochester) and Dual-Luciferase Reporter Assay System (#E2940, Promega) was used to asses Rad51 luciferase activity, which was measured as the average ratio of firefly to Renilla luciferase.

Real-time qPCR. An RNA isolation kit (BS88133, Bio Basic Inc) was used to extract RNA and cDNAs were synthesized using qScript cDNA kit (#95047, Quantabio). The qPCR master mix used was SYBR green (Applied Biosystems). Experiments were performed with three technical replicates and normalized to GAPDH. Rad51 qPCR primer sequence has been previously described (6).

Chromatin immunoprecipitation. ChIP-IT Express Chromatin Immunoprecipitation kit (Active Motif) was used for TEAD4 antibody (N-G2, Santa Cruz Biotechnology) ChiP experiments. The following qPCR primer sequence was used to amplify the region of the TEAD4 signal in the Rad51 promoter identified in ENCODE: Forward primer 5'-TTGCTCCAGGAATGCGAGTA-3' Reverse primer 5'-AGCGCTCTTGTGGTTTGTTT-3'.

DR-GFP assays. Puromycin resistant DR-GFP MCF7 cells were provided by Dr. Sharon Cantor (University of Massachusetts Medical School). Stable expression of S89A TAZ (Addgene plasmid #52084) was accomplished by selecting cells in blasticidin and stable expression of S127A YAP (Addgene plasmid #33092) was accomplished by selecting cells in hygromycin. DR-GFP reporter plasmid was purchased from Addgene and was electroporated into Hs578t cells (Addgene plasmid #26475). Subsequently, cells were transfected with I-SceI (Addgene plasmid #26477) and processed for flow cytometry 72 hours later using two-color fluorescence analysis (3). Specifically, a green/GFP (FL1) vs. red/DsRed (FL2) fluorescent plot was utilized so that GFP-negative cells that may have autofluorescence fall along the green/red diagonal, and GFP positive cells are shifted above the diagonal towards higher FL1. The DsRed signal is derived from transfecting control DR-GFP cells with DsRed (provided by Dr. Vera Gorbunova, University of Rochester) to generate the two-color plot to be used in our analysis. GFP positive cells were normalized to 1 and are depicted as HR efficiency.

Immunofluorescence microscopy. Rad51 and γH2AX immunofluorescence microscopy was performed by fixing cells with paraformaldehyde (4%) and permeabilizing them with triton X-100 (0.1%). Cells were blocked with 0.5% BSA and incubated with Rad51 antibody (ab63801, Abcam) or γH2AX antibody (05-636, Millipore) overnight at 4°C. The following morning, cells were washed with 1X PBS and incubated with fluorochrome-conjugated secondary antibodies at

room temperature for 45 minutes. A confocal microscope (Zeiss) was used to capture images at 20X magnification. FociCounter http://focicounter.sourceforge.net/ was used to quantify γH2AX positive cells.

ENCODE data analysis. TEAD4 binding signals were downloaded from www.encodeproject.org in bigwig format. The signals of duplicate samples were pooled and then plotted along the promoter region of the Rad51 gene using trackViewer package (7).

Metabric analysis. cBioPortal (www.cbioportal.org) was used to compare the mRNA expression of Rad51, TAZ, CTGF, Cyr61, VEGFA and NRP2 using the Metabric breast cancer dataset (8, 9). To determine if the expression of two genes exhibit an inverse correlation, we performed the mutual exclusivity analysis with a z-score threshold of ± 2 as expressed, and calculated the log odds ratio between two genes and *P* value using Fisher exact *t*-test. We also stratified breast cancer patients in the Metabric dataset based on their ER status and tumor grade, and Welch *t*-test was used to compare Rad51 expression in ER^+ and ER^- and tumor grade 1-3 patient groups.

Microarray analysis. The Bioconductor package *GEOquery* (version 2.41.0) (10) was used to download the microarray dataset from GEO (GSE59230) (11). Differentially expressed genes between MDA-MB-231 cells transfected with siRNA control and two different siRNAs targeting YAP/TAZ were identified using moderated *t*-test with limma package (12). Genes with an adjusted *P*-value of ≤ 0.05 using Benjamini-Hochberg method were considered significant (13).

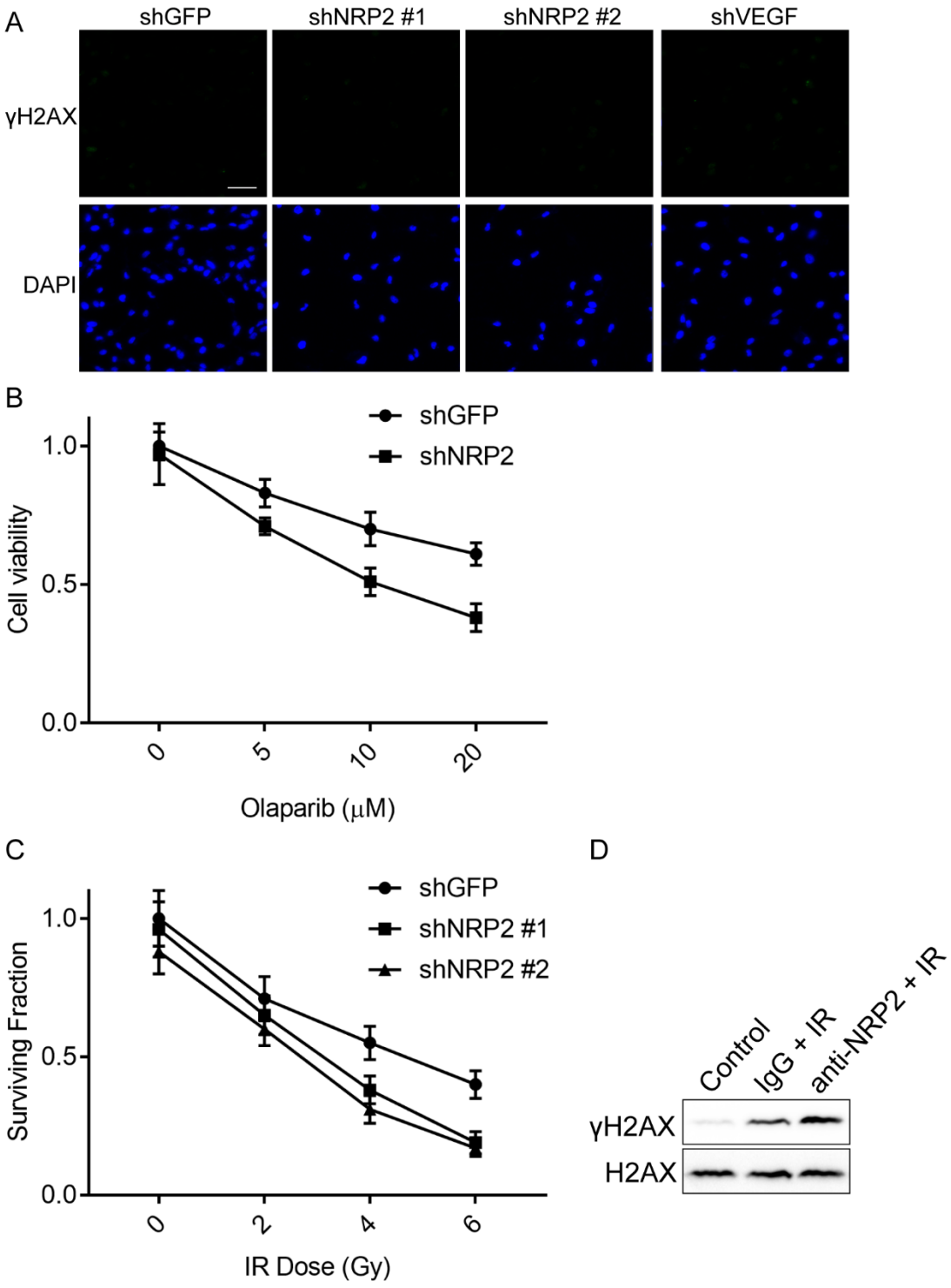


Fig. S1, related to Fig. 1. *(A)* Expression of NRP2 and VEGF was diminished with shRNAs in MDA-MB-231 cells, which were subsequently processed for immunofluorescence microscopy to assess baseline γH2AX. Scale bar represents 50 μm. *(B)* Cell viability in control and NRP2-depleted Hs578t cells treated with the indicated concentrations of olaparib was assessed. *(C)* Cell viability in control and NRP2-depleted Hs578t cells treated with the indicated doses of IR was

assessed. *(D)* Hs578t cells were pre-treated with a control IgG or a NRP2 function blocking antibody. Subsequently, they were treated with 6 Gy of IR and the impact on γH2AX abundance was quantified by immunoblotting 30 minutes following IR.

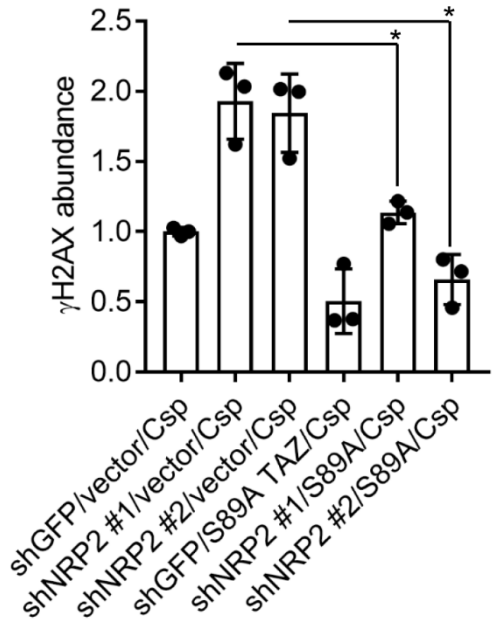


Fig. S2, related to Fig. 2. NRP2-depleted Hs578t cells expressing either S89A TAZ or a control vector were treated with cisplatin and the impact on γH2AX abundance was quantified by immunoblotting. Densitometry was assessed using ImageJ and corresponds to **Fig. 2A**. Dot plot (mean ± standard deviation) represents three independent experiments. * $P \leq 0.05$ by 2-tailed *t* test.

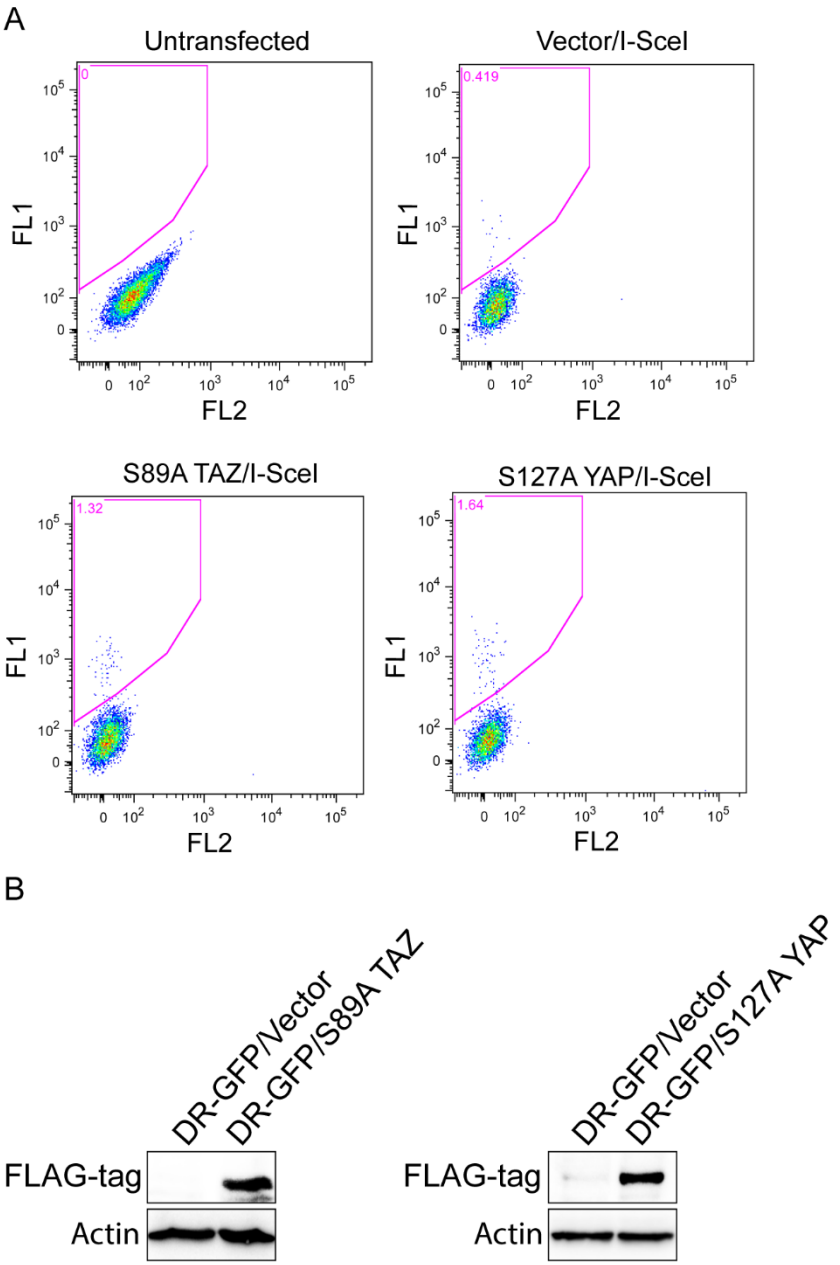
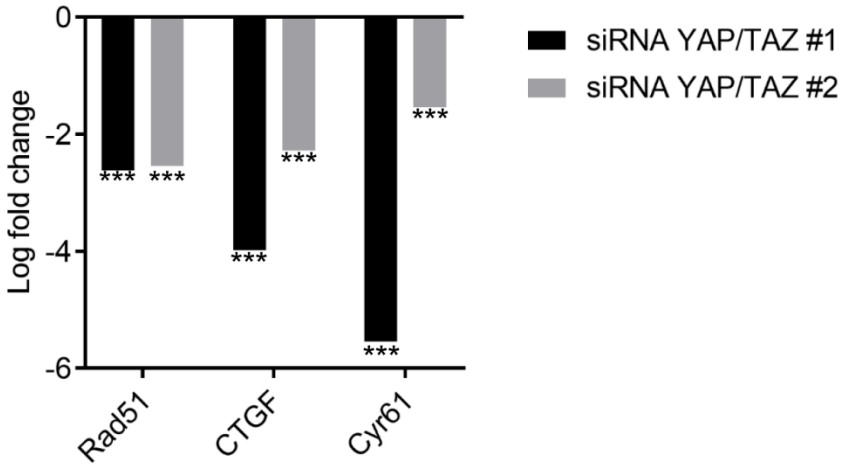
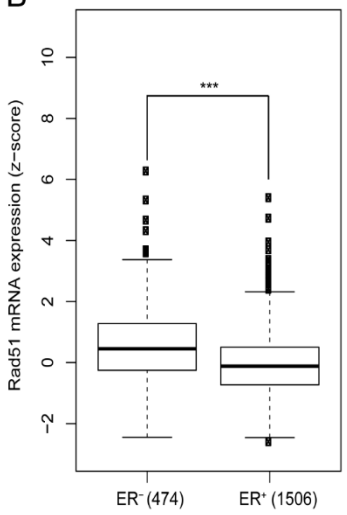


Fig. S3, related to Fig. 3. *(A)* Representative flow cytometry of MCF7 DR-GFP cells expressing a control vector, S89A TAZ or S127A YAP. *(B)* Expression of FLAG-tagged S89A TAZ and S127A YAP in MCF7 DR-GFP cells was assessed by immunoblotting.

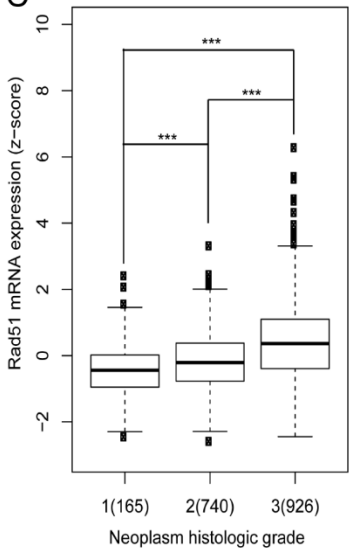
A



B



C



D

Gene analysis	Log odds ratio	*p* value	No. samples
Rad51 vs. TAZ	+1.3	< 0.001	2509
Rad51 vs. CTGF	+1.4	< 0.001	2509
Rad51 vs. Cyr61	+1.4	0.002	2509
Rad51 vs. VEGFA	+1.6	< 0.001	2509
Rad51 vs. NRP2	+0.9	0.02	2509

Fig. S4, related to Fig. 3. *(A)* The indicated genes from a microarray (accession number GSE59230 (11)) using siRNA to deplete YAP/TAZ in MDA-MB-231 cells were analyzed. Log fold change in expression from four biological replicates is shown relative to siRNA control, and adjusted *P* value of ≤ 0.0005 is indicated by ***. cBioPortal for cancer genomics was used to analyze the expression of Rad51 based on expression of the ER *(B)* and tumor grade *(C)* in breast cancer patients from the Metabric database. *** $P < 0.0005$ by Welch *t* test. *(D)* The expression of Rad51 was compared with TAZ, the YAP/TAZ target genes CTGF and Cyr61, VEGFA and

NRP2 in the Metabric database. Log odds ratios were generated and *P* values were calculated using fisher exact *t* test.

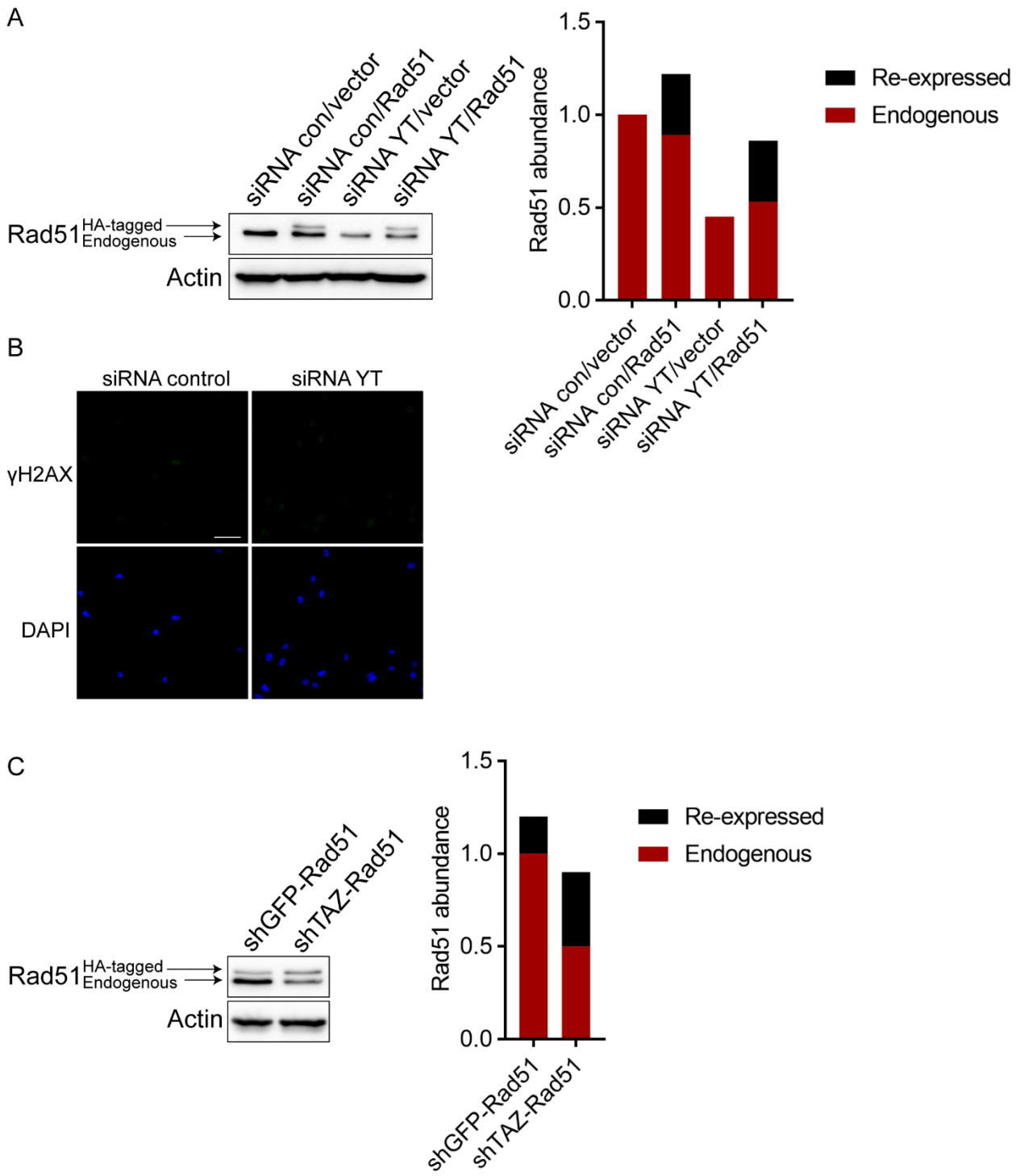


Fig. S5, related to Fig. 5. *(A)* Expression of YAP/TAZ was diminished with siRNA in Hs578t cells (siRNA YT). Cells were then transfected with HA-tagged Rad51 or empty vector and processed for immunoblotting to quantify the degree of Rad51 rescue. Densitometry was assessed using ImageJ (right bar graph). *(B)* Expression of YAP/TAZ was diminished with siRNA in Hs578t cells, which were subsequently processed for immunofluorescence microscopy to assess

baseline γH2AX. Scale bar represents 50 μm. *(C)* Expression of Rad51 was quantified in control and TAZ-depleted MDA-MB-231 cells by immunoblotting to determine the degree of Rad51 rescue. Densitometry was assessed using ImageJ (right bar graph).

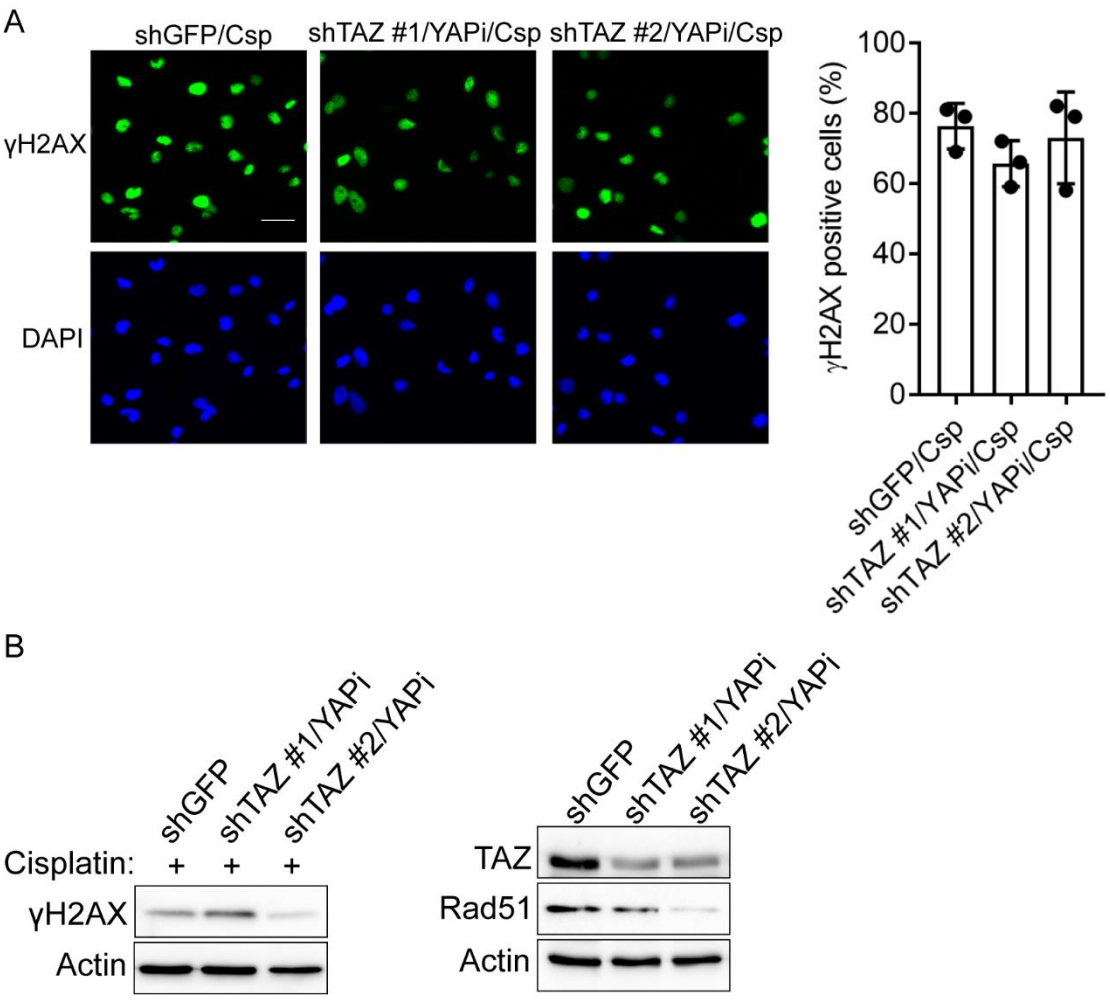


Fig. S6, related to Fig. 5. Expression of TAZ was depleted in SUM-1315 cells, which were treated with verteporfin and cisplatin and processed for *(A)* immunofluorescence microscopy to assess γH2AX and *(B)* immunoblotting to assess γH2AX and Rad51. Scale bar represents 50 μm. Dot plot (mean ± standard deviation) represents three independent experiments.

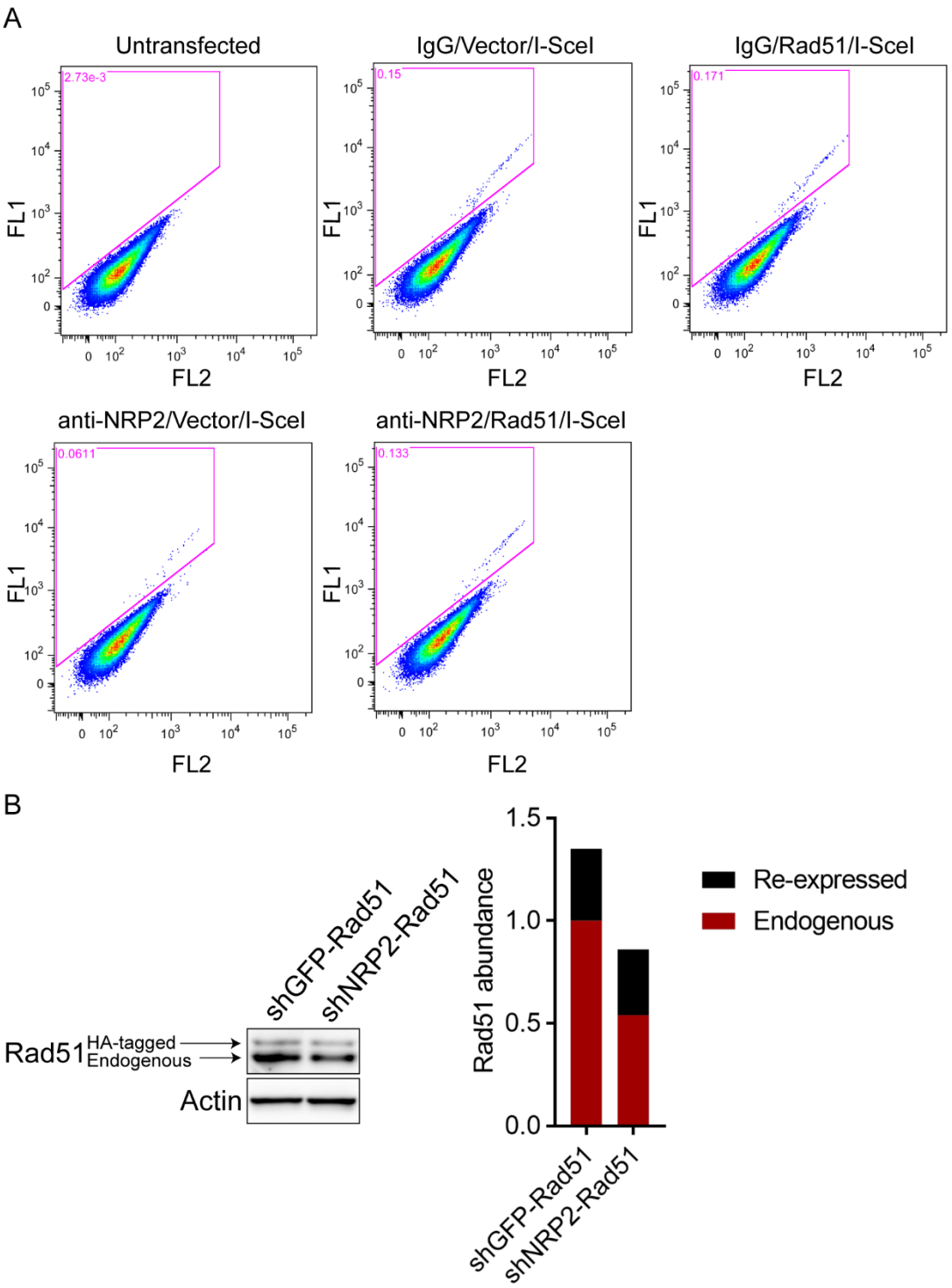


Fig. S7, related to Fig. 6. *(A)* Representative flow cytometry of Hs578t cells transfected with empty vector or Rad51 and subsequently treated with a control IgG or a function blocking NRP2 antibody. *(B)* Expression of Rad51 was quantified in control and NRP2-depleted cells by immunoblotting to determine the degree of Rad51 rescue. Densitometry was assessed using ImageJ (right bar graph).

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