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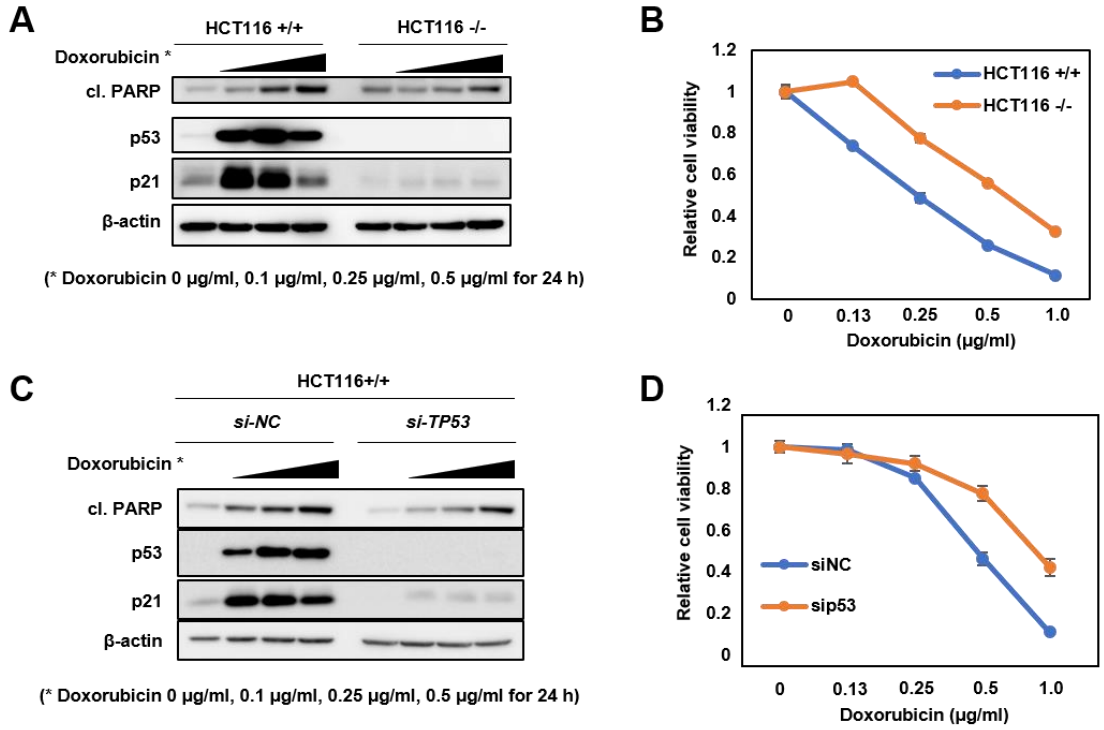
Supplemental Information

miR-1293, a Candidate for miRNA-Based Cancer Therapeutics, Simultaneously Targets *BRD4* and the DNA Repair Pathway

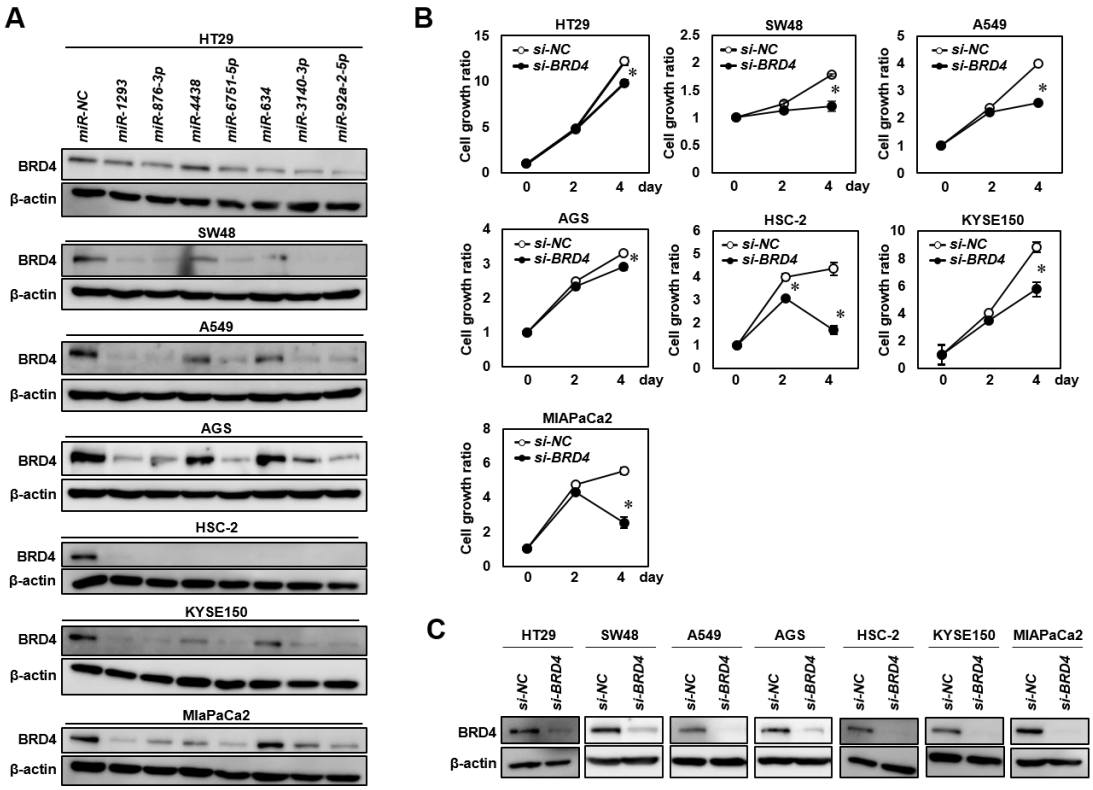
Yuki Takagawa, Yasuyuki Gen, Tomoki Muramatsu, Kousuke Tanimoto, Jun Inoue, Hiroyuki Harada, and Johji Inazawa

Figure S1



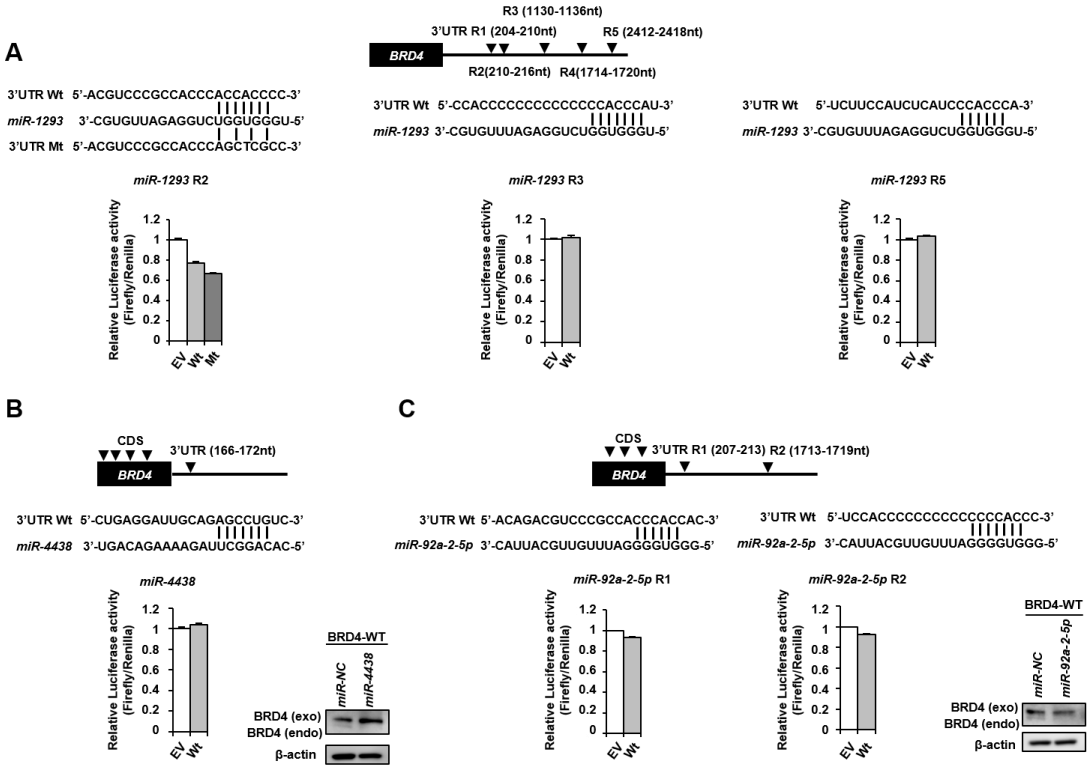
(A) Western blot analysis of cleaved PARP, p53 and p21 in HCT116+/+ and HCT116-/- cells 24 hours after treatment with doxorubicin hydrochloride at the indicated concentrations. **(B)** Dose response curve of doxorubicin at 48 hours after treatment with drug in the HCT116+/+ and HCT116-/- cell lines. The cell growth ratio was assessed by a crystal violet staining assay using a relative ratio compared with DMSO-treated cells. **(C)** Western blot analysis of cleaved PARP, p53 and p21 in HCT116+/+ cells transfected with *si-NC* and *si-TP53* 24 hours after treatment with doxorubicin hydrochloride at the indicated concentrations. **(D)** Dose response curve of doxorubicin at 48 hours after treatment with drug in HCT116+/+ cells transfected with *si-NC* and *si-TP53*. The cell growth ratio was assessed by a crystal violet staining assay using a relative ratio compared with DMSO-treated cells.

Figure S2



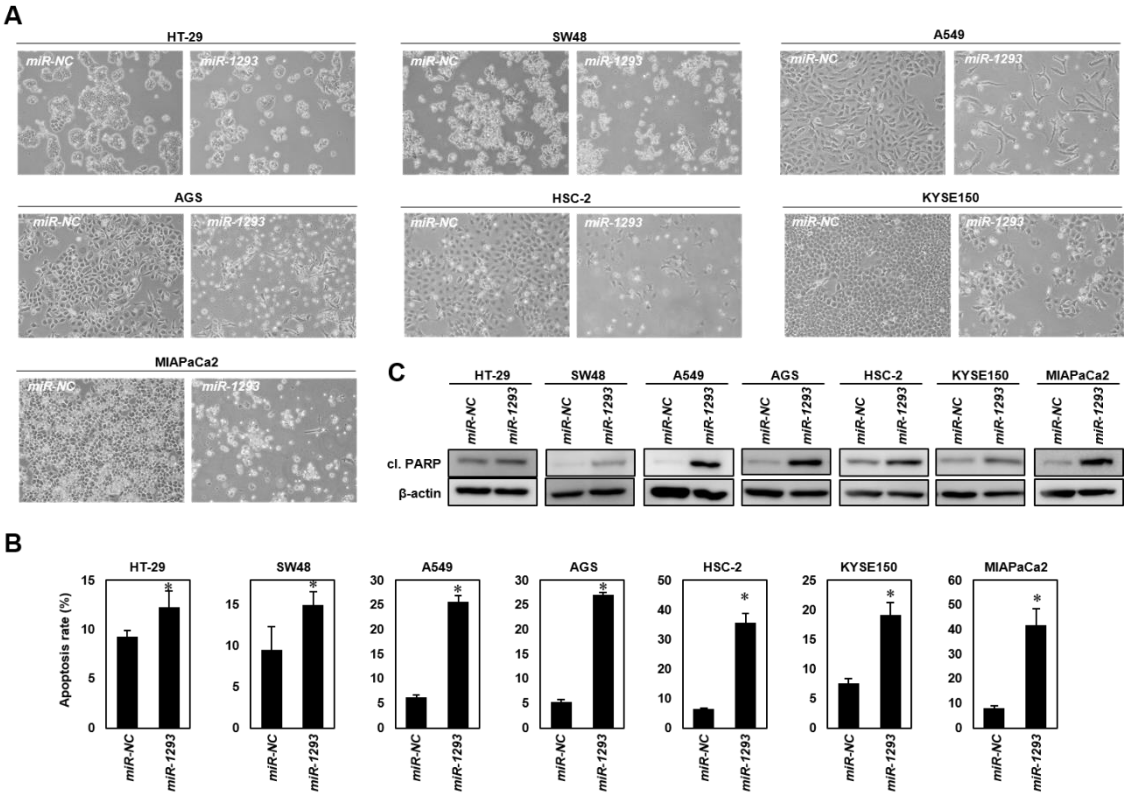
(A) Western blot analysis of BRD4 in HT29, SW48, A549, AGS, HSC-2, KYSE150, and MIAPaCa2 cells 48 hours after transfection with 10 nmol/L *miR-NC* or the indicated 7 miRNAs. **(B, C)** Knocking down *BRD4* suppressed *in vitro* cell proliferation in indicated cells. Cell growth assay (B) and western blot analysis (C) in indicated cells after transfection with 20 nmol/L negative control siRNA (*si-NC*) or siRNA targeting *BRD4* (*si-BRD4*). * $P < 0.05$.

Figure S3



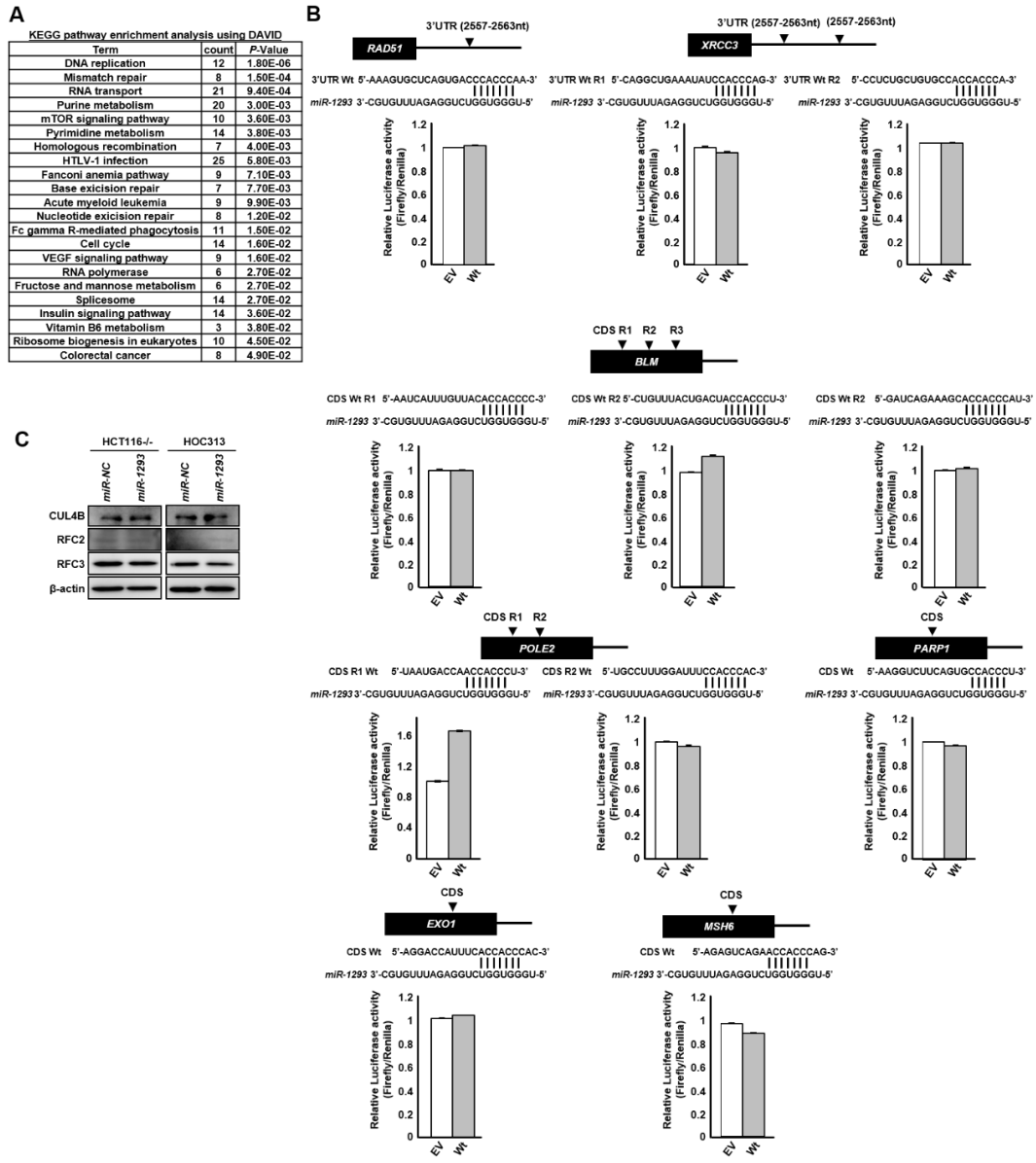
(A-C) Luciferase reporter assay. HOC313 cells were cotransfected with pmirGLO dual-luciferase vectors containing wild-type (WT) *BRD4* or mutant variants of *BRD4* and *miR-NC*, *miR-1293*, *miR-4438* or *miR-92a-2-5p*. Top, putative binding sequence of each miRNA in the 3'UTR or CDS of *BRD4* and mutant sequences are indicated. Bottom, the results of the luciferase assay. Western blot analysis of BRD4 in HOC313 cells. Cells were transfected with the WT BRD4 expression vector, and after 24 hours, 10 nmol/L *miR-NC*, *miR-4438* or *miR-92a-2-5p* was additionally transfected. The expression of exogenous BRD4 was not reduced after transfection with *miR-4438* or *miR-92a-2-5p*.

Figure S4



(A) Phase contrast images of indicated cells transfected with 10 nmol/L *miR-NC* or *miR-1293*. Images were obtained 48 hours after transfection. **(B)** The percentage of apoptotic cells in indicated cells transfected with *miR-NC* or *miR-1293*. Cells were double stained with Annexin V and propidium iodide (PI) 48 hours after transfection and analyzed by flow cytometry. The percentage of cells indicates late apoptotic cells (Annexin V+/ PI+). Bar, SD for triplicate experiments. * $P < 0.05$. **(C)** Western blot analysis of cleaved PARP (cl. PARP) in indicated cells 48 hours after transfection with 10 nmol/L *miR-NC* or *miR-1293*.

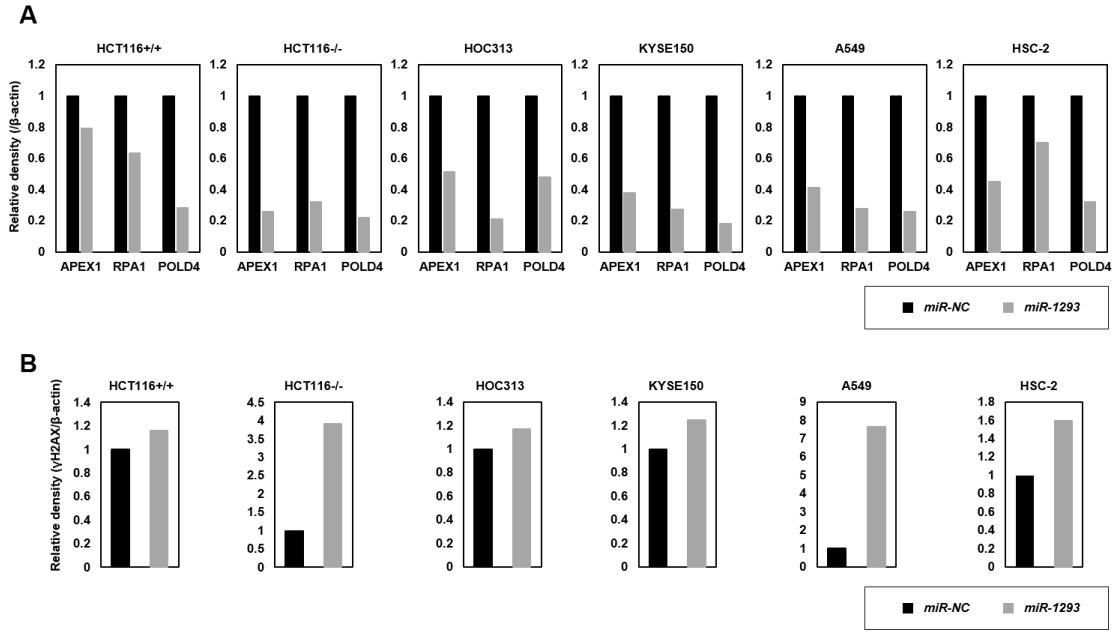
Figure S5



Term	count	*P*-Value
DNA replication	12	1.80E-06
Mismatch repair	8	1.50E-04
RNA transport	21	9.40E-04
Purine metabolism	20	3.00E-03
mTOR signaling pathway	10	3.60E-03
Pyrimidine metabolism	14	3.80E-03
Homologous recombination	7	4.00E-03
HTLV-1 infection	25	5.80E-03
Fanconi anemia pathway	9	7.10E-03
Base excision repair	7	7.70E-03
Acute myeloid leukemia	9	9.90E-03
Nucleotide excision repair	8	1.20E-02
Fc gamma R-mediated phagocytosis	11	1.50E-02
Cell cycle	14	1.60E-02
VEGF signaling pathway	9	1.60E-02
RNA polymerase	6	2.70E-02
Fructose and mannose metabolism	6	2.70E-02
Spliceosome	14	2.70E-02
Insulin signaling pathway	14	3.60E-02
Vitamin B6 metabolism	3	3.80E-02
Ribosome biogenesis in eukaryotes	10	4.50E-02
Colorectal cancer	8	4.90E-02

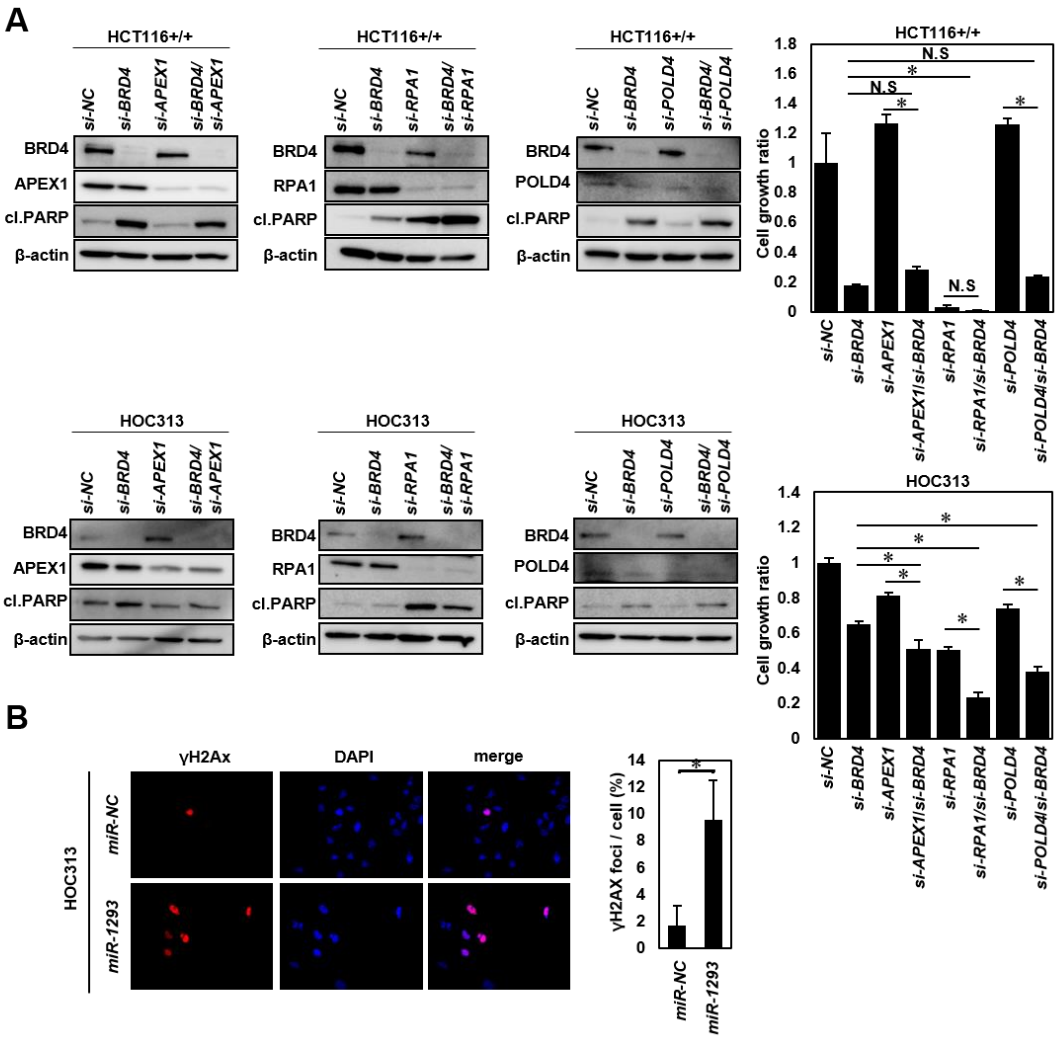
(A) Table showing the results of the pathway analysis of the 1248 commonly downregulated genes using DAVID Bioinformatics Resources 6.8. Fisher's exact test was used for genes selection. $P < 0.05$ were considered significant. **(B)** Luciferase reporter assays. HOC313 cells cotransfected with pmirGLO dual-luciferase vectors containing the wild-type (WT) 3'UTR or CDS of *RAD51*, *XRCC3*, *BLM*, *POLE2*, *PARP1*, *EXO1* and *MSH6*, and *miR-NC* or *miR-1293*. Top, putative binding site of *miR-1293* within the 3'UTR or CDS of each gene and mutant sequences. Bottom, the results of the luciferase assay. **(C)** Western blot analysis of CUL4B, RFC2 and RFC3 in HCT116-/- and HOC313 cells 48 hours after transfection with 10 nmol/L *miR-NC* or *miR-1293*.

Figure S6



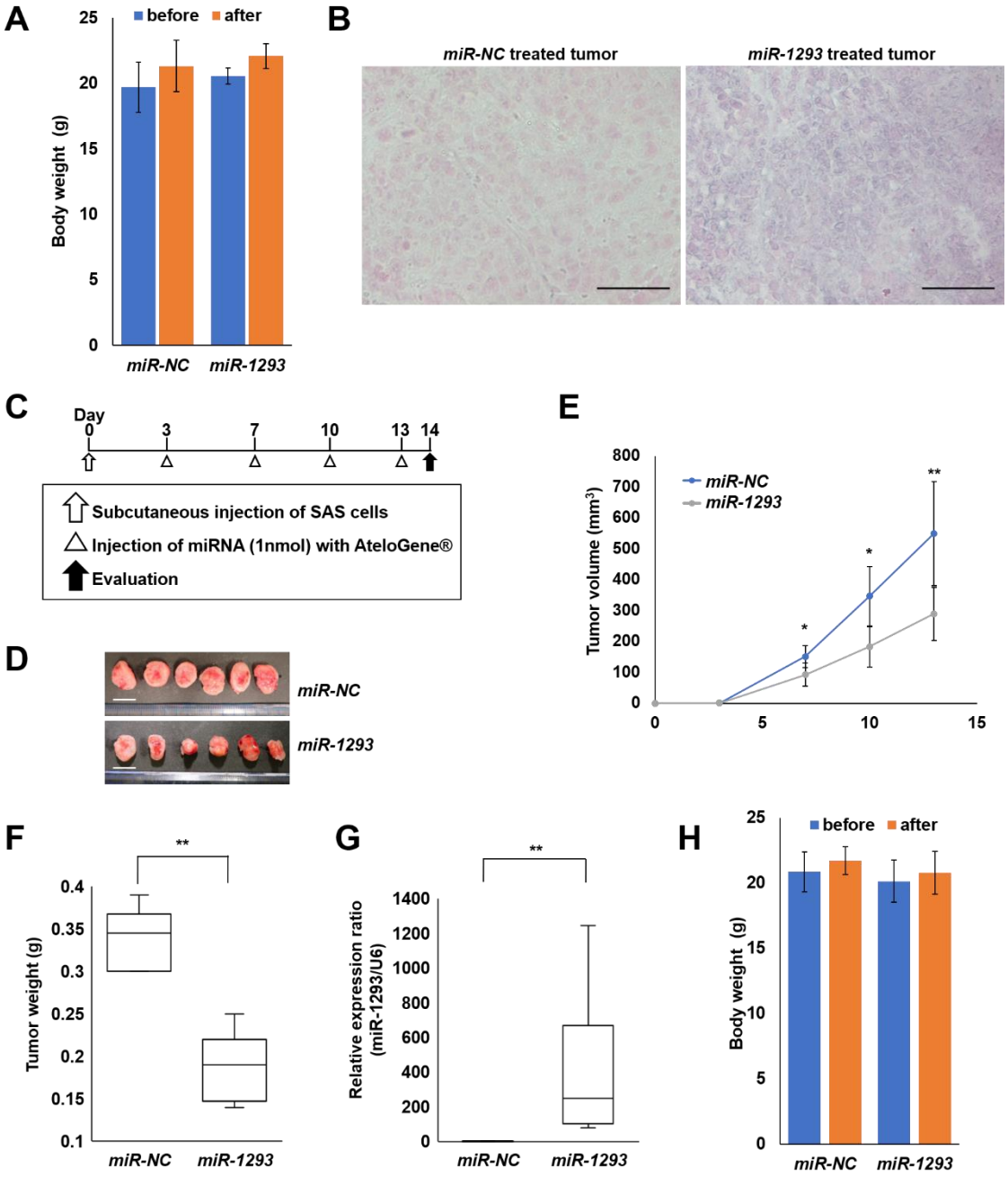
(A, B) Densitometric quantification of the bands in **Figure 4B**. Band intensities of APEX1, RPA1, POLD4 (A), and ɤH2AX (B) were analyzed by Image J software (National Institutes of Health, Bethesda, MA, USA). The values were normalized against β-actin for each sample.

Figure S7



(A) Evaluation of the effect of *si-BRD4* and *si-APEX1*, *si-RPA1* or *si-POLD4* in HCT116+/+ and HOC313 cells. Western blot analysis (left) and cell growth assay (right) in indicated cell lines after transfection with siRNA (*si-NC* (40 nmol/L); *si-BRD4*, *si-APEX1*, *si-RPA1*, and *si-POLD4* (each 20 nmol/L)). After 120 hours of transfection with siRNA, the cell growth rate was assessed with the crystal violet staining assay using a relative ratio compared with the growth of *si-NC*-treated cells. Bar, SD for triplicate experiments. **P* < 0.05. **(B)** Immunofluorescence analysis of HOC313 cells that were double labeled with anti-γH2AX (red) and DAPI (blue; nuclei). Cells were transfected with 10 nmol/L *miR-NC* or *miR-1293* for 6 hours following treatment with neocarzinostatin (200 ng/mL) for 30 minutes.

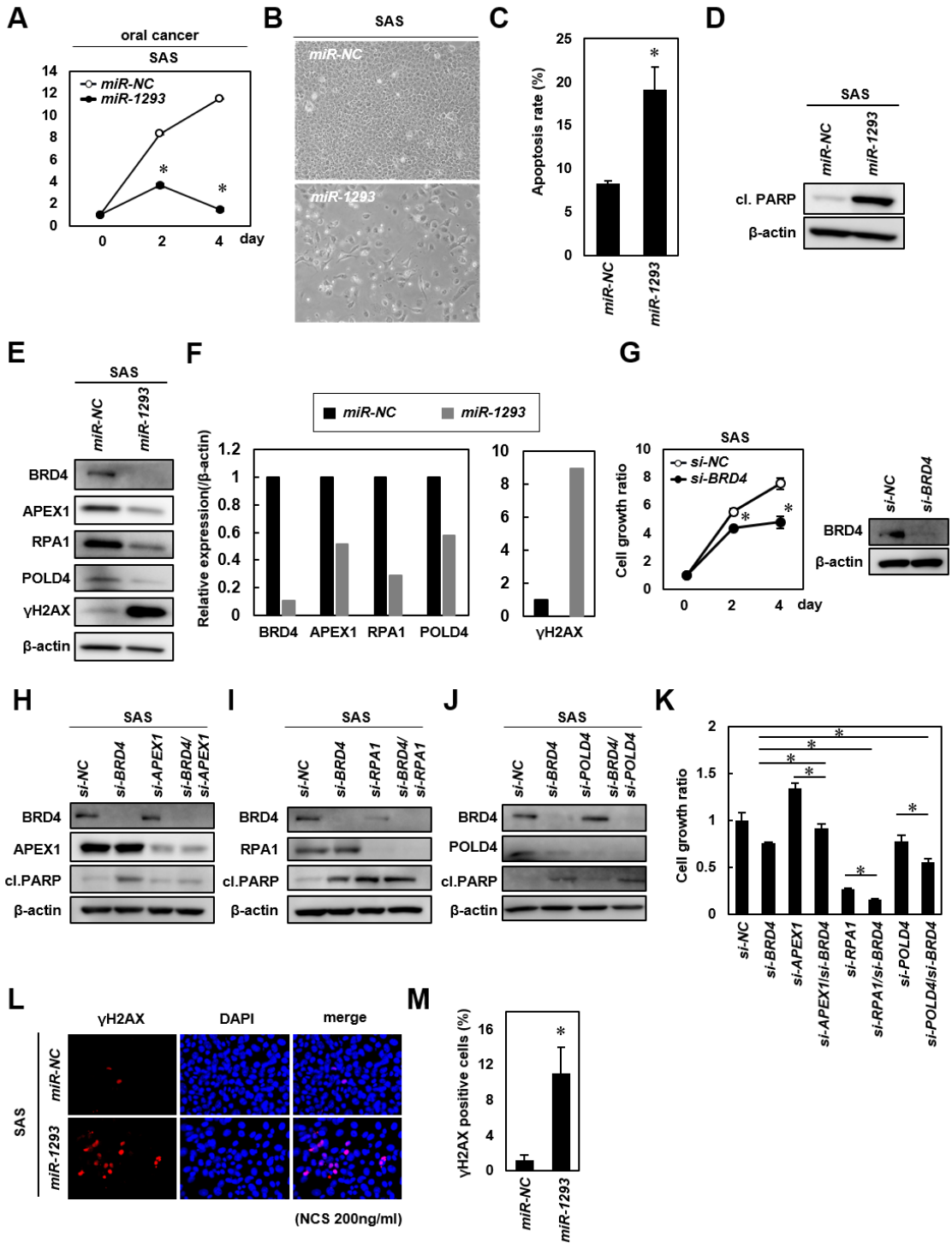
Figure S8



(A) Body weights on day 3 (before the treatment) and on day 19 (after the treatment) were measured. **(B)** Representative images of *in situ* hybridization (ISH) in tumors from mice treated with *miR-NC* or *miR-1293*. The *miR-1293*-specific probe was visualized in purple in the cytoplasm, and the nucleus was counterstained with nuclear fast red. Scale bars, 50 μm. **(C)** The experimental schedule for *miR-1293* treatment in nude mice.

Tumors were formed by subcutaneous injection of SAS cells in nude mice. *miR-NC* or *miR-1293* was administered subcutaneously around the tumors derived from the SAS for a total of 4 times (3, 7, 10 and 13 days after the injection of cells). **(D)** Representative images of tumor-bearing nude mice and resected tumors at 14 days after the injection of HCT116-/- cells. Scale bar, 10 mm. **(E)** Tumor growth curves of xenograft mouse models treated with *miR-NC* or *miR-1293* (n=6, each). Tumor volume was calculated using the following formula: $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. Bar, SD for 6 mice; $*P < 0.05$, $**P < 0.01$. **(F)** Weights of the resected tumors. Tumor weights are shown as a box plot. Bar, SD for 6 mice; $**P < 0.01$. **(G)** Expression analysis of *miR-1293* in the resected tumors. The expression level of *miR-1293* was measured by qRT-PCR. Each experiment was performed in duplicate. The relative ratio was normalized based on the expression of *RNU6B*. Bar, SD for 6 mice; $**P < 0.01$. **(H)** Body weights on day 3 (before the treatment) and on day 14 (after the treatment) were measured.

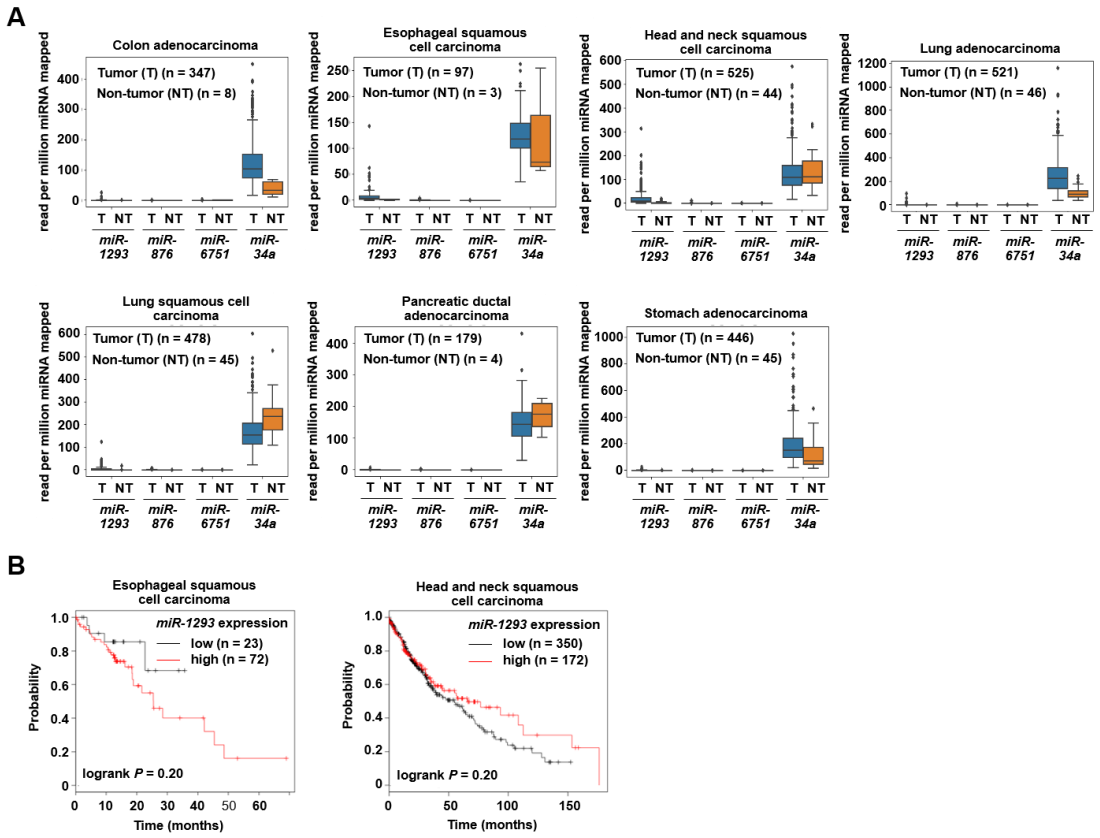
Figure S9



(A) *In vitro* cell growth assay in SAS cells. Cells were transfected with 10 nmol/L *miR-NC* or *miR-1293*. Cell growth rate was assessed with the crystal violet staining assay using a relative ratio compared with cell growth on day 0. Bar, SD for triplicate experiments. *

$P < 0.05$. **(B)** Phase contrast images of SAS cells transfected with 10 nmol/L *miR-NC* or *miR-1293*. Images were obtained 48 hours after transfection. **(C)** The percentage of apoptotic cells in SAS cells transfected with 10 nmol/L *miR-NC* or *miR-1293*. Cells were double stained with Annexin V and propidium iodide (PI) 48 hours after transfection and analyzed by flow cytometry. The percentage of cells indicates late apoptotic cells (Annexin V+/ PI+). Bar, SD for triplicate experiments. * $P < 0.05$. **(D)** Western blot analysis of cleaved PARP (cl. PARP) in SAS cells 48 hours after transfection with 10 nmol/L *miR-NC* or *miR-1293*. **(E)** Western blot analysis of BRD4, APEX1, RPA1, POLD4 and ɣH2AX in SAS cells 48 hours after transfection with 10 nmol/L *miR-NC* or *miR-1293*. **(F)** Western blot band intensities of (E). The band intensities of indicated proteins were analyzed by densitometry and relative protein amounts through normalization against β-actin. **(G)** Knock-down of BRD4 suppressed *in vitro* cell proliferation in SAS cells. Cell growth assay (left) and western blot analysis (right) in SAS cells after transfection with 20 nmol/L negative control siRNA (*si-NC*) or siRNA targeting BRD4 (*si-BRD4*). Bar, SD for triplicate experiments. * $P < 0.05$. **(H-K)** Evaluation of the effect of *si-BRD4* and *si-APEX1*, *si-RPA1* or *si-POLD4*. Western blot analysis (H-J) and cell growth assay (K) in indicated cell lines after transfection with siRNA (*si-NC* (40 nmol/L); *si-BRD4*, *si-APEX1*, *si-RPA1*, and *si-POLD4* (each 20 nmol/L)). One hundred twenty hours following transfection, the cell growth ratio was assessed with the crystal violet staining assay using a relative ratio compared with that of *si-NC*-transfected cells. Bar, SD for triplicate experiments. *$P < 0.05$. knockdown of BRD4 in combination with knockdown of *RPA1*, or *POLD4* led to a more pronounced suppression of *in vitro* cell growth with increased expression of cl. PARP in SAS cells, whereas combination of *si-APEX1* with *si-BRD4* antagonized the growth-suppressive effects of *si-BRD4*. **(L)** Immunofluorescence analysis of SAS cells that were double-labeled with anti-γH2AX (red) and DAPI (blue; nuclei). Cells were transfected with 10 nmol/L *miR-NC*, or *miR-1293* for 6 hours following treatment with neocarzinostatin (200 ng/ml) for 30 minutes. **(M)** The percentage of ɣH2AX-positive cells in (L). *$P < 0.05$.

Figure S10



(A) Expression level of *miR-1293*, *miR-876*, and *miR-6751* in primary colon adenocarcinoma, esophageal squamous cell carcinoma (ESCC), head and neck squamous cell carcinoma (HNSCC), lung adenocarcinoma, lung squamous cell carcinoma, pancreatic ductal adenocarcinoma, and stomach adenocarcinoma in TCGA database. In contrast with the expression of *miR-34a*, a representative tumor suppressive miRNA, the expression of *miR-1293*, *miR-876*, and *miR-6751* are extremely low in both tumor tissue (T) and non-tumor tissue (NT), although the expression of *miR-1293* is slightly upregulated in the tumor tissue of ESCC, and HNSCC.

(B) Kaplan-Meier curves for overall survival rates of patients with ESCC and HNSCC According to the Kaplan-Meier Plotter (http://kmplot.com/analysis/). The expression of *miR-1293* is not correlated with overall survival in a corresponding cohort of 95 patients with ESCC, and 522 patients with HNSCC (both $P = 0.20$, log-rank test).