The synaptonemal complex protein SYCP3 impairs mitotic recombination by interfering with BRCA2

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Supplementary Information

Contents

Supplementary Methods

Cell lines. Telomerase-immortalized RPE cells and HepG2 cells were obtained from Clontech and the Riken Cell Bank, respectively. HT1080 cells and Capan-1 cells were from American Type Culture Collection. RPE cells were cultured in Dulbecco's modified Eagle's and Ham's F12 medium containing 0.25% additional sodium bicarbonate and 10% fetal bovine serum. HepG2 cells, HT1080 cells and MCF7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Samples. Total RNA and protein from normal human testis were purchased from Clontech and BioChain, respectively.

Antibodies. Antibodies against SYCP3 (FL-254), Cdk2 (M-2), and BRCA2 (H-299) were obtained from Santa Cruz Biotechnology. Antibodies against RAD51 (Ab-1), BRCA1 (Ab-1) and BRCA2 (Ab-1) were obtained from Calbiochem. Antibodies against gamma-H2AX (JBW301) and NBS1 (NB100-143) were from Upstate and Novus, respectively. Antibodies against FLAG (M2) and cyclin A (CY-A1) were from Sigma. As for BRCA2, the anti-BRCA2 (Ab-1) antibody was used for immunoprecipitation and immunoblotting, and the anti-BRCA2 (H-299) antibody was used for immunofluorescence.

Immunoprecipitation and western blot analyses. Cells were lysed in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40, 1 mM DTT) supplemented with 1 mM phenylmethylsulphonyl fluoride and 10 μg/ml aprotinin. Immunoprecipitation and western blot analyses were carried out as described previously (Yoshihara *et al*, 2004; Date *et al*, 2006).

Reverse transcriptase-polymerase chain reaction (RT-PCR). One microgram of total RNA was reversely transcribed in a 20 μl reaction mixture with 2 units of Moloney murine leukemia virus reverse-transcriptase (Takara) using a random hexamer. Five microliter of the synthesized cDNA was analyzed for *SYCP3* expression by PCR as previously described (Kalejs *et al*, 2006). One microliter of the synthesized cDNA was also analyzed for *GAPDH* expression by PCR using primers GAPDH-F/GAPDH-R (supplementary Table S2 on line) as a control experiment. The sequences of all the RT-PCR products were verified by sequencing.

Expression analysis using the tumor/normal tissue mRNA array. The mRNA Array-Human Tumor Tissue Array II (BioChain) was used to analyze *SYCP3* expression. This array contains mRNA from 47 tumor tissues and 47 normal tissues from unmatched donors (supplementary Table S1 online). One hundred nanograms of DNA probes recognizing

either full-length *SYCP3* or *β-actin* transcripts were labeled with [α-^{32}P]-dCTP using the Megaprime DNA labeling system (GE Healthcare). After overnight hybridization at 65°C in a buffer (0.5 M $NaPO_4$ (pH 7.2), 1 mM EDTA, 1% BSA, 7% SDS) containing the labeled probe, the array membrane was washed and exposed to an imaging plate (Fujifilm), which was subsequently scanned using the BAS-2500 system (Fujifilm). The scanned images were analyzed with the Multi Gauge Ver 3.0 software (Fujifilm) to extract *SYCP3* and *β-actin* signals for each spot, and after local background signals were subtracted, the signal ratios of *SYCP3*/*β-actin* were calculated for all spots. A spot was eliminated from the analysis if the signal intensity of *β-actin* after the background subtraction was less than zero, which was applied to the spots for one normal liver sample (E15 & E16) and one normal ovary sample (H15 & H16). The average *SYCP3*/*β-actin* signal ratios of the two duplicated spots were calculated for the remaining spots.

cDNA probes for full-length *SYCP3* and *β-actin*. The cDNA probe for *β-actin* was provided with the human tissue mRNA array (BioChain). The human full-length *SYCP3* cDNA fragment was amplified from human testis cDNA by PCR using ClaI-SYCP3-F/ApaI-SYCP3-R primers (supplementary Table S2 online) and cloned to the pCR2.1 vector using the TA cloning kit (Invitrogen). After verification of the sequence, the inserted full-length *SYCP3* cDNA was extracted by digesting the vector with ClaI and ApaI.

Vector constructions. Human *SYCP3* and FLAG-tagged *SYCP3* cDNAs were amplified

from human testis cDNA by PCR using SYCP3-F/SYCP3-R1 and FLAG-SYCP3-F/SYCP3-R2 primers, respectively (supplementary Table S2 online), followed by insertion into an expression vector containing the MMTV promoter. The FLAG-tagged *SYCP3* cDNA was also inserted into the pcDNA3.1-zeo expression vector (Invitrogen) and used in the rescue experiments using RNA interference and transfection in HepG2 cells.

Knockdown experiments. Transfection of the siRNA alone was performed using DharmaFECT 4 (Dharmacon), whereas co-transfection of the siRNA with the expression vector was performed using DharmaFECT Duo (Dharmacon). Cells were subjected to immunofluorescence, western blot analysis or cell survival assay, at 48 hours post-transfection, and to the DR-green fluorescent protein (GFP) assay at 72 hours post-transfection.

Immunofluorescence. The cells were cultured on coverslips. They were either nontreated or irradiated, and at 2 hours after irradiation, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were blocked with 10% horse serum and incubated with primary antibodies at 37°C for 1 hour and with secondary antibodies at 37°C for 30 minutes. Finally, cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and mounted. For the analyses of foci formation of RAD51, NBS1 and BRCA1, a total of 100 cells per each cell type were counted in randomly selected fields using an Olympus BX51 fluorescence microscope at 1,000 magnification. Images were

captured with an Olympus DP 70 digital camera using Olympus DP controller and DP manager softwares.

FISH analyses. Chromosome-specific centromeric probes were obtained from Vysis. DNA in cells was denatured in 70% formamide/2 x SSC at 73°C for 5 minutes. Hybridization was performed in CEP Hybridization Buffer (Vysis) at 42°C for 1 hour. Slides were washed in 0.4 x SSC/ 0.3% NP-40 at 73°C for 3 minutes and then in 2 x SSC/0.3% NP-40 at room temperature for 2 minutes. Finally, cellular DNA was stained with DAPI. A total of 500 cells were examined for each cell clone using an Olympus BX51 fluorescence microscope.

Cell survival assays. To examine the sensitivity to radiation and cisplatin, the RPE cell clones in suspension were subjected to X-ray irradiation or incubation in the presence of cisplatin (Nihon-Kayaku) for 1 hour and washed three times with phosphate-buffered saline. The cells were plated at a density of 1,000 cells per 60 mm dish and grown for 7 to 10 days and, after fixing and staining, colonies were counted. To examine the sensitivity to a PARP inhibitor, a total of 1,000 cells for RPE cells and a total of 2,000 cells for HepG2 cells, HT1080 cells, MCF7 cells and Capan-1 cells were plated in triplicate onto 60 mm dishes with the addition of the PARP inhibitor NU1025 (Sigma). Original media containing the inhibitor was left on cells until colonies were stained and counted. All measurements were performed in triplicate.

Homologous recombination assay (the DR-GFP assay). For HeLa-DRGFP cells stably expressing FLAG-SYCP3 or stably transfected with the empty vector, the DR-GFP assay was performed as previously reported (Pierce & Jasin, 2005; Sakamoto *et al*, 2007). In knockdown experiments using RPE-FLAG-SYCP3-DRGFP cells, MCF7-DRGFP cells and HepG2-DRGFP cells, 1 μg of the I-SceI expression vector was introduced to 1.5×10^5 cells using DharmaFECT Duo (Dharmacon) together with either control siRNA or the siRNA for *SYCP3*. To determine the amount of HR repair, the percentage of GFP-positive cells was quantified by flow cytometry 3 days after electroporation using FACSCanto II (Becton Dickinson).

Analysis of sister chromatid exchanges (SCEs). Cells were either nontreated or irradiated with 2 Gy, and cultured in 6 μg/ml 5-bromodeoxyuridine. The mock cells were cultured for 48 hours, whereas the two independent RPE cell clones expressing FLAG-SYCP3 were cultured for 56 and 60 hours, respectively, because they showed slow growth. Cells were pulsed with 0.1 μg/ml colcemid for the last 12 hours. Then, cells were harvested and treated with 75 mM KCl for 20 minutes and fixed with methanol:acetic acid (3:1). Cells were fixed on slides and incubated with 5 μg/ml Hoechst 33258 for 20 minutes. The slides were irradiated with ultraviolet radiation (= 352 nm) for 20 minutes at 50°C, washed in 2 x SSC at 65°C for 20 minutes, and stained with 4% Giemsa solution.

Cell cycle analysis. Cell cycle analysis was performed with EPICS XL (Beckman Coulter) using the CycleTEST™ PLUS DNA Reagent Kit (Beckton Dickinson).

Supplementary References

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Yoshihara T, Ishida M, Kinomura A, Katsura M, Tsuruga T, Tashiro S, Asahara T, Miyagawa K (2004) XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. *EMBO J* **23**: 670-680

Supplementary Table S1. Tumor and normal tissue samples spotted on the mRNA array

	1&2	3&4	5&6	7&8	9&10	11&12	13&14	15&16
A	control (*)	control (**)	adrenal tumor	normal adrenal	adipose tumor	normal adipose	bladder tumor	normal bladder
B	bladder tumor	normal bladder	breast tumor	normal breast	breast tumor	normal breast	breast tumor	normal breast
C	breast tumor	normal breast	colon tumor	normal colon	colon tumor	normal colon	colon tumor	normal colon
D	esophagus tumor	normal esophagus	esophagus tumor	normal esophagus	gall bladder tumor	normal gall bladder	kidney tumor	normal kidney
E	kidney tumor	normal kidney	kidney tumor	normal kidney	liver tumor	normal liver	liver tumor	normal liver
F	liver tumor	normal liver	liver tumor	normal liver	lung tumor	normal lung	lung tumor	normal lung
G	lung tumor	normal lung	lung tumor	normal lung	lymphoma	normal lymph node	lymphoma	normal lymph node
H	ovary tumor	normal ovary	ovary tumor	normal ovary	ovary tumor	normal ovary	ovary tumor	normal ovary
I	prostate tumor	normal prostate	prostate tumor	normal prostate	prostate tumor	normal prostate	rectum tumor	normal rectum
J	rectum tumor	normal rectum	rectum tumor	normal rectum	small intestine tumor	normal small intestine	soft tissue tumor	normal soft tissue
K	soft tissue tumor	normal soft tissue	stomach tumor	normal stomach	stomach tumor	normal stomach	stomach tumor	normal stomach
L	thymus tumor	normal thymus	thyroid tumor	normal thyroid	thyroid tumor	normal thyroid	uterus tumor	normal uterus

*** normal placenta; ** water**

Supplementary Table S2. Sequences of the primers used in RT-PCR experiments

primer name	sequence
SYCP3-F	5'-GGATCCTGTCGACGACCAGTAACT-3'
SYCP3-R1	5'-GGGCCCTCAGAATAACATGGATTGAA-3'
FLAG-SYCP3-F	5'-AAGAATTCACCATGGACTACAAGGACGAC GATGACAAGTCCGTGTCCTCCGGAAAAAA-3'
SYCP3-R2	5'-AAGAATTCTCAGAATAACATGGATTGAA-3'
ClaI-SYCP3-F	5'-ATCGATATGGTGTCCTCCGGAAAAAA-3'
ApaI-SYCP3-R	5'-GGGCCCTCAGAATAACATGGATTGAA-3'
GAPDH-F	5'-TGTTGCCATCAATGACC-3'
GAPDH-R	5'-TCTCATGGTTCACACCCA-3'

Supplementary Legends for Figures

Supplementary Figure S1 The meiosis-specific protein SYCP3 is expressed in various cancer cell lines. (**A**) Western blot analysis of the SYCP3 protein using human cancer cell lines (upper panel). The membrane was re-blotted with the Cdk-2 antibody as a control for protein integrity (lower panel). Proteins from normal testis and RPE and HME cell lysates were used as positive and non-cancerous normal controls, respectively. (**B**) Induction of *SYCP3* expression in DLD1 cells by treatment with 1 μM 5-azacytizine for 2 days. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis results for the *SYCP3* (upper panel) and *GAPDH* genes (lower panel) are shown.

Supplementary Figure S2 The meiosis-specific protein SYCP3 is expressed in various primary tumors. (**A**) The mRNA array of 47 tumors and 47 normal tissues. The mRNA for each sample is spotted in duplicate. The array was probed for *SYCP3* (upper panel) and then reprobed for *β-actin* (lower panel). (**B**) The *SYCP3*/*β-actin* signal ratios in 47 tumors and 45 normal tissues. The columns represent the averaged signal ratios of *SYCP3*/*β-actin* for the two duplicated spots in each sample, and the bars represent the two data points of the duplicated spots. The colored columns represent the signal ratios for tumor samples, while the white ones represent the signal ratios for normal tissues.

Supplementary Figure S3 Generation of RPE clones stably expressing SYCP3 or FLAG-SYCP3. (**A**) Identification of forced expression of SYCP3 in RPE-SYCP3 clones 1 and 2 by western blot analysis (upper panel). The membrane was re-blotted with the anti-Cdk2 antibody which served as an internal control (lower panel). (**B**) Western blot analysis showing expression of FLAG-SYCP3 in RPE-FLAG-SYCP3 clones A and B at levels comparable to endogenous expression in HepG2 cells (upper panel). The membrane was re-blotted with the anti-Cdk2 antibody as an internal control (lower panel).

Supplementary Figure S4 SYCP3 inhibits ionizing radiation (IR)-induced RAD51 foci formation in cells in S and G2 phases. (**A**) Western blot analysis of RAD51 (upper panel) in

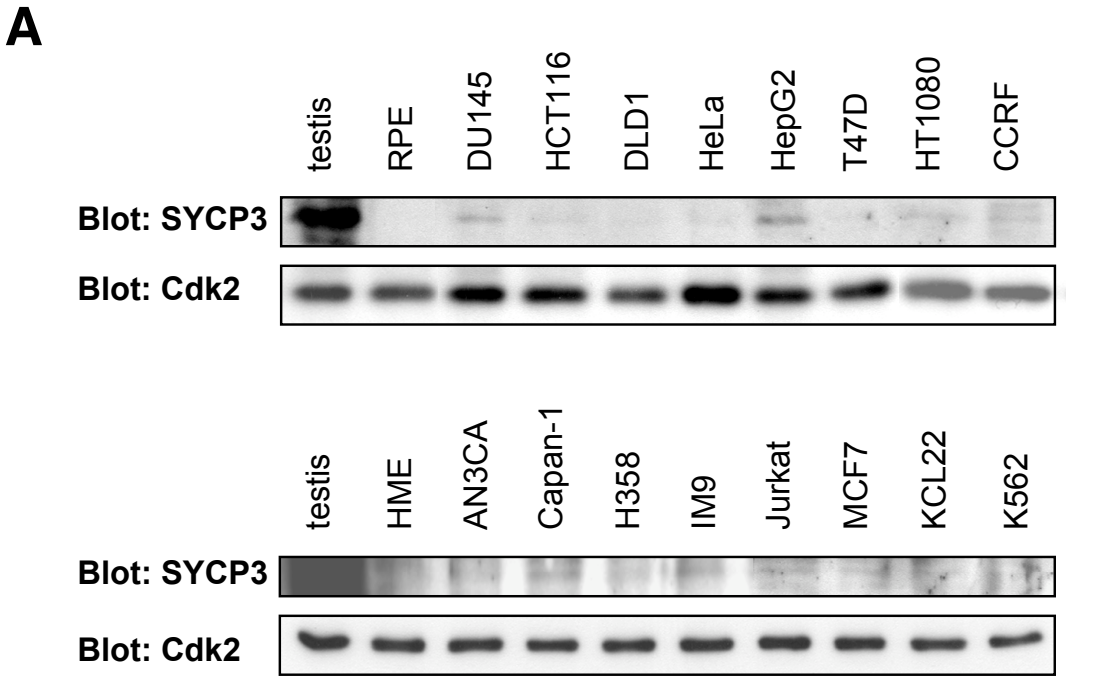
two independent RPE-FLAG-SYCP3 clones. Cdk2 expression (lower panel) is shown as a loading control. (**B**) Cell cycle distribution of the two independent RPE-FLAG-SYCP3 clones and mock cells analyzed by fluorescent activated cell sorting (FACS). (**C** and **E**) Immunofluorescence visualization of NBS1 and BRCA1 foci formation. Cells were untreated or treated with 8 Gy X-ray, followed by staining with the anti-NBS1 antibody (red) (**C**) or the anti-BRCA1 antibody (green) (**E**) 2 hours later. Scale bar, 10 mm. (**D** and **F**) Percentage of cells containing more than five damage-induced NBS1 foci (**D**) or BRCA1 foci (**F**). In **D** and **F**, columns and bars represent the mean of three independent experiments and the standard deviation (SD), respectively. A total of 100 cells were examined for each cell line.

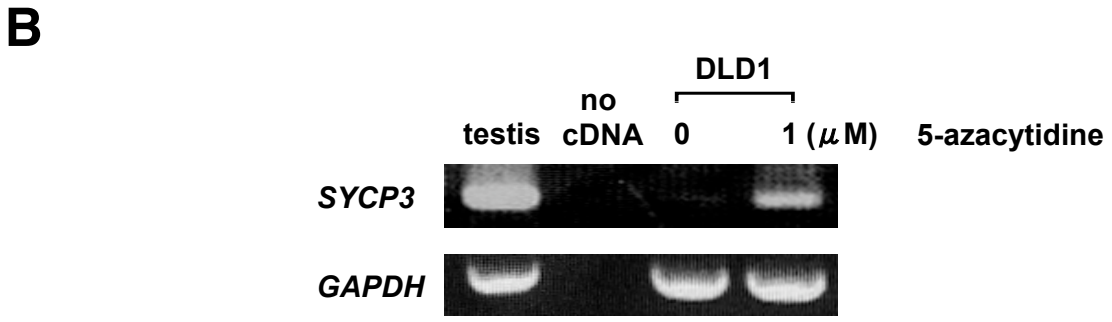
Supplementary Figure S5 IR-induced foci formation of RAD51 was recovered after knockdown of endogenous SYCP3 in HepG2 cells. (**A**) Schematic representation of *SYCP3* mRNA and the siRNA-resistant *FLAG-SYCP3* expression vector. The target site of *SYCP3* siRNA existing in the 3' UTR region of *SYCP3* mRNA but not existing in the siRNA-resistant *FLAG-SYCP3* expression vector is indicated by a horizontal bar. (**B**) Western blot analysis showing that the endogenous SYCP3 protein was silenced by *SYCP3*-targeting siRNA and rescued by expression of the siRNA-resistant FLAG-SYCP3 construct in HepG2 cells. HepG2 cells transfected with a nontargeting siRNA control were used as controls. (**C** and **E**) Immunofluorescence visualization of NBS1 and BRCA1 foci formation. Cells were untreated or treated with 8 Gy X-ray, followed by staining with the anti-NBS1 antibody (**C**) or the anti-BRCA1 antibody (**E**) 2 hours later. Scale bar, 10 mm. (**D** and **F**) Percentage of cells containing more than five damage-induced NBS1 foci (**D**) or BRCA1 foci (**F**). In **D** and **F**, columns and bars represent the mean of three independent experiments and SD, respectively. A total of 100 cells were examined for each cell line.

Supplementary Figure S6 Stable expression of FLAG-SYCP3 and knockdown of SYCP3 in various cells. (**A**) Western blot analysis showing expression of FLAG-SYCP3 in HeLa-DRGFP cells (upper panel). The membrane was re-blotted with the anti-Cdk2 antibody as an internal control (lower panel). (**B**) Knockdown of the exogenous *SYCP3* gene in RPE-FLAG-SYCP3-DRGFP cells, and knockdown of endogenous *SYCP3* gene in MCF7-

DRGFP cells and HepG2-DRGFP cells. (**C**) Knockdown of the endogenous *SYCP3* gene in HT1080 cells, HepG2 cells, MCF7 cells and Capan-1 cells. In **B** and **C**, the *SYCP3* gene was knocked down in each cell line by small interfering RNA (siRNA) transfection and subjected to RT-PCR analysis. Cells transfected with a nontargeting siRNA control were used as controls.

Supplementary Figure S1

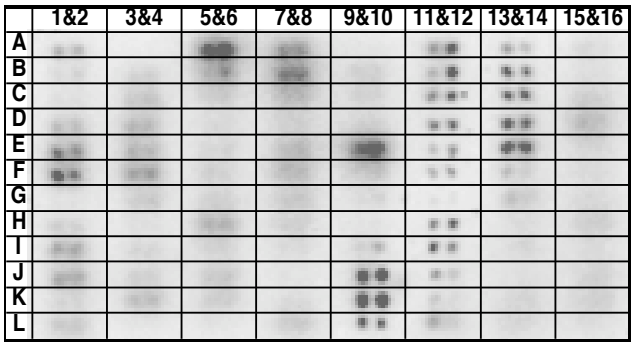




Supplementary Figure S2

A

SYCP3



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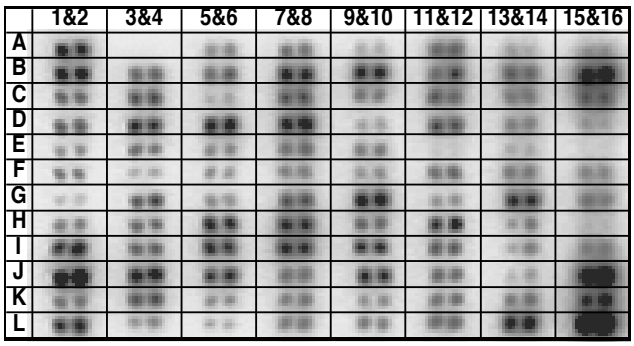

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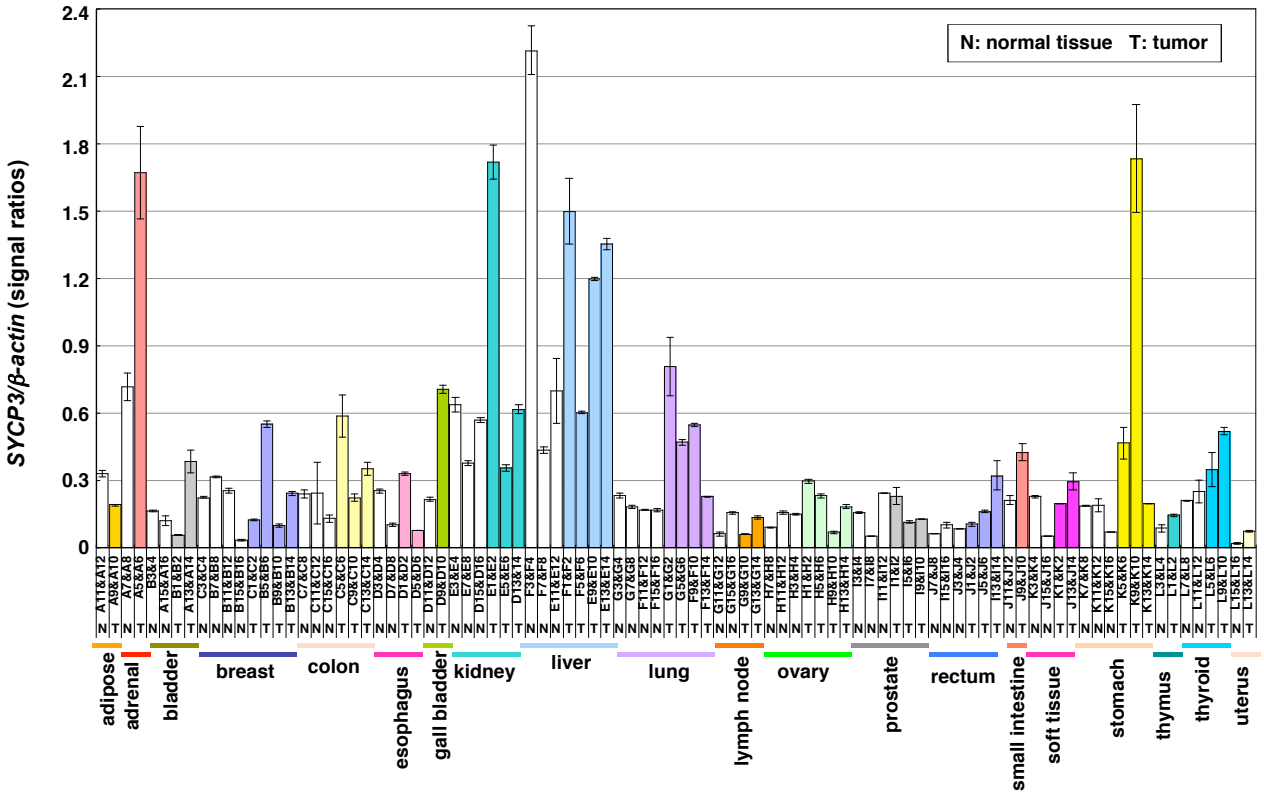
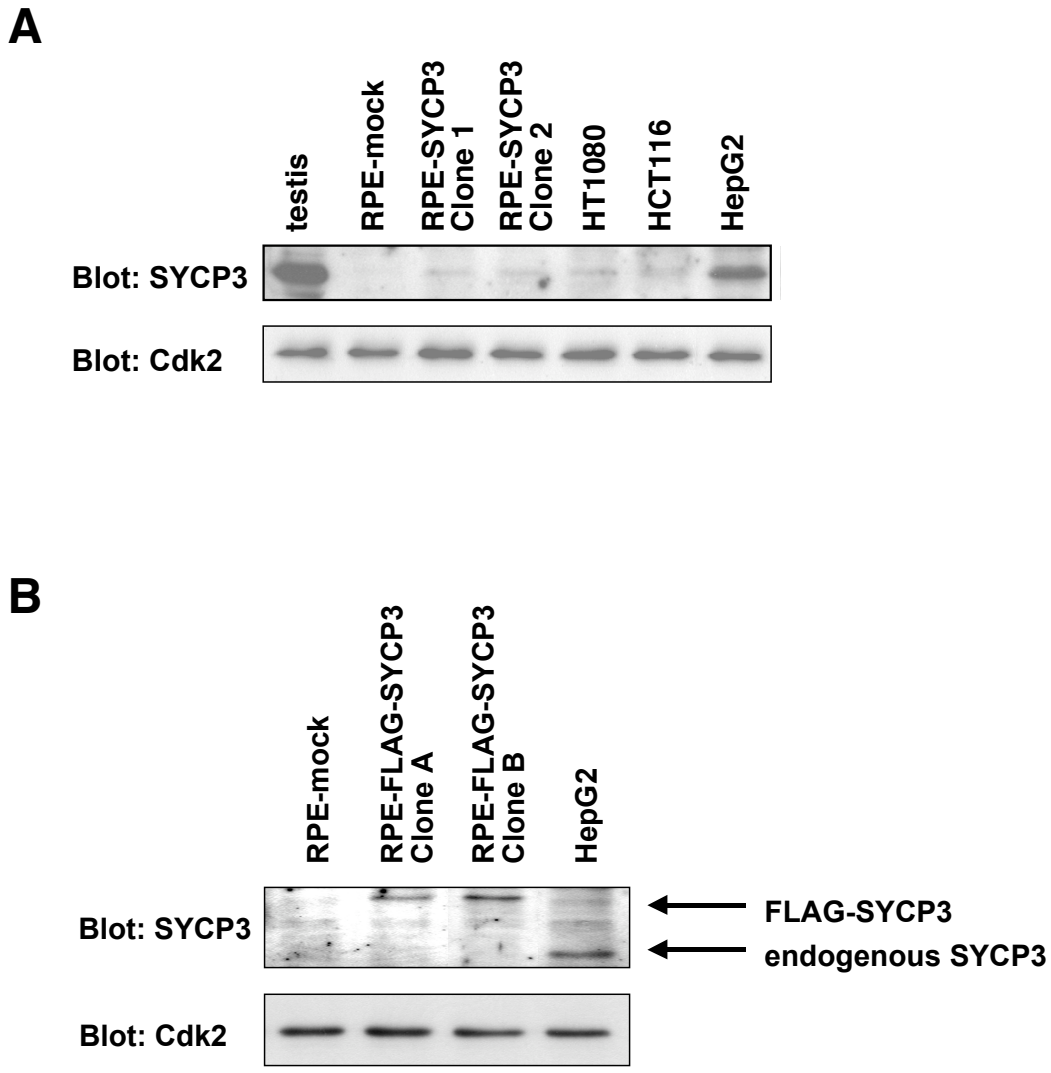
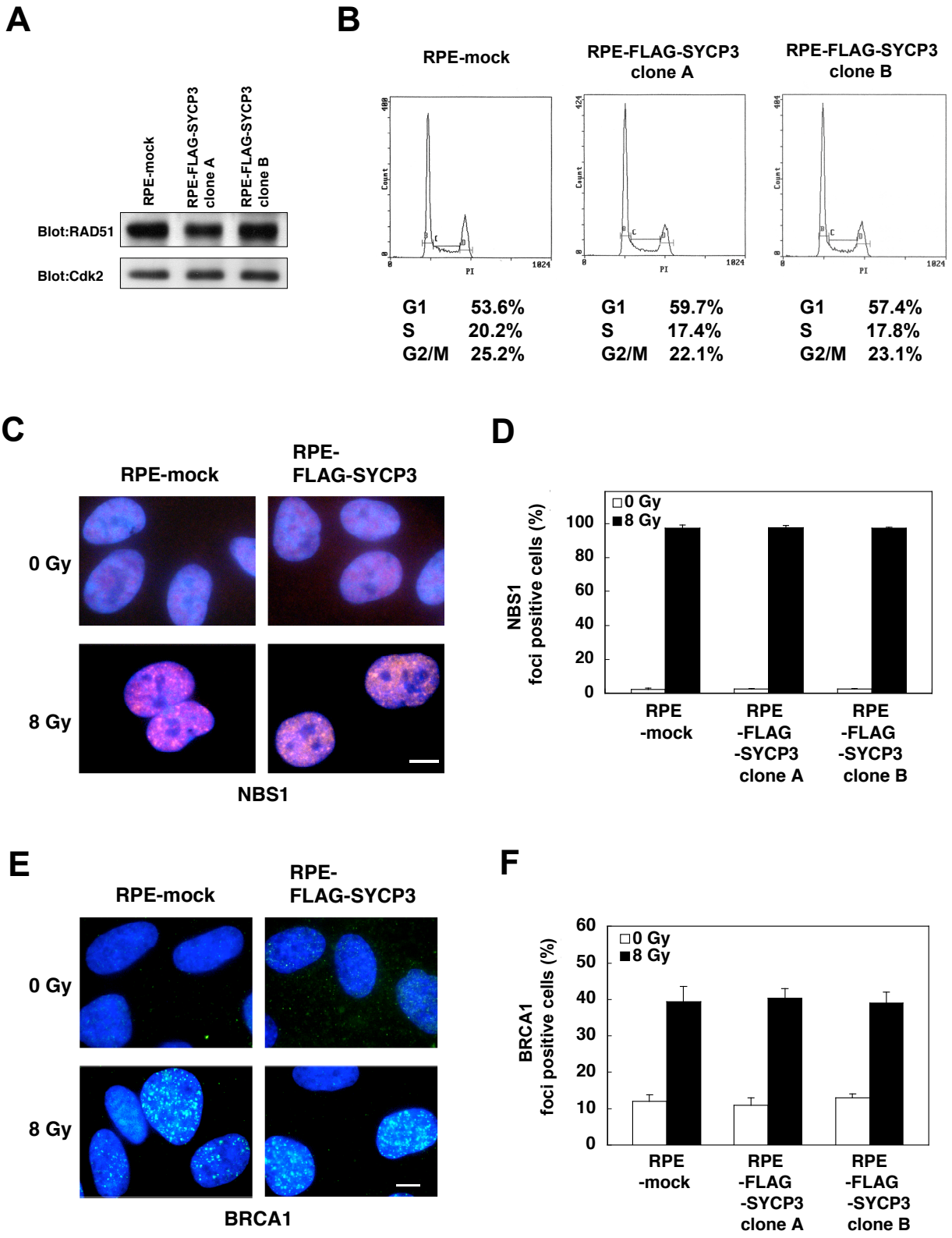


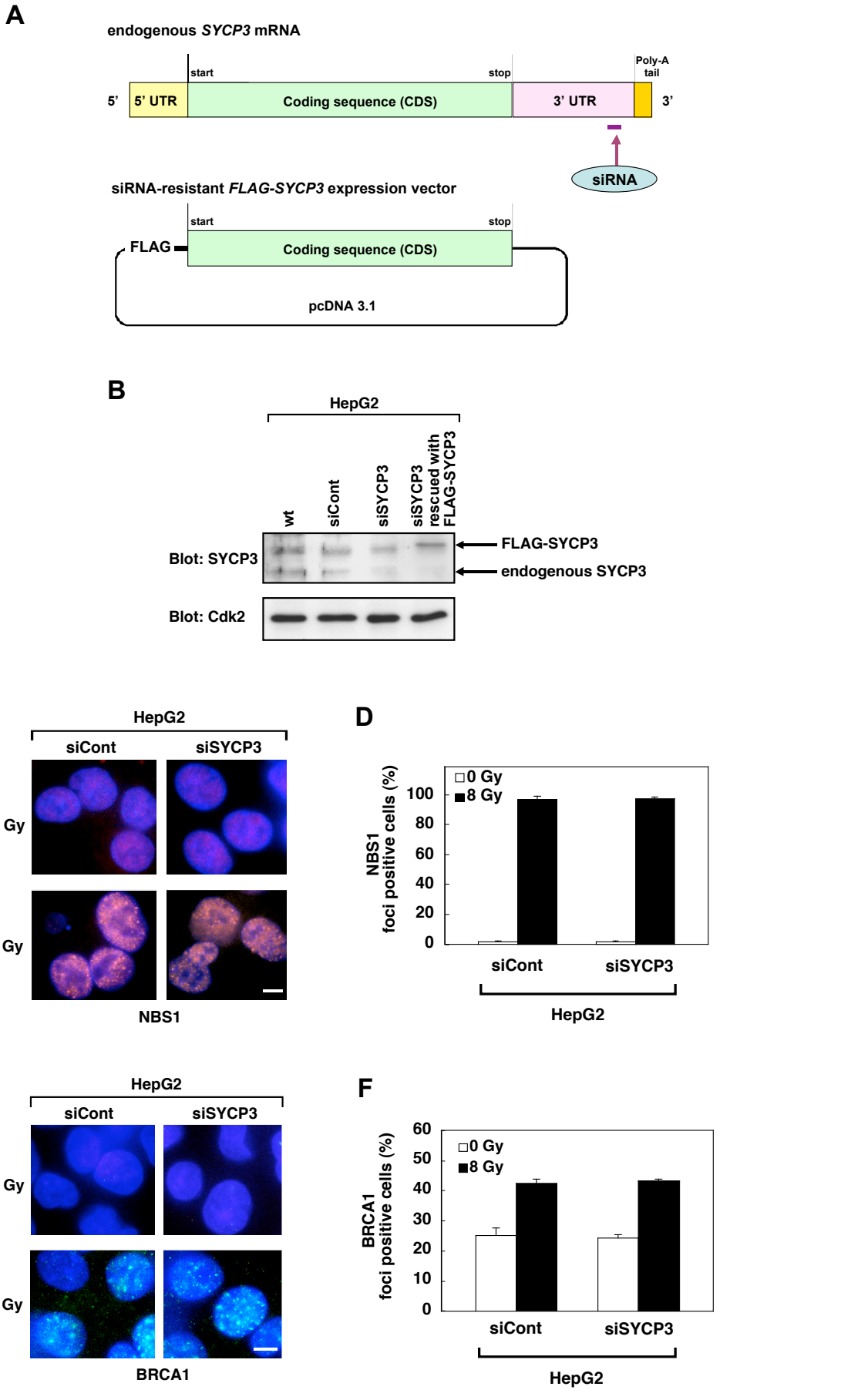
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6