Supplementary information

Materials & Methods

Cell lines and cell culture

Breast cancer cell lines MCF7, BT20 and BTB474 were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI1640 (GIBCO, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin–streptomycin (P/S; Nacalai tesque, Inc., Kyoto, Japan). HEK293T cells (ATCC) were cultured for virus production in Dulbecco's Modified Eagle Medium: Nutrient Mixture (DMEM) (GIBCO) supplemented with 10% FBS and 1% P/S. The cells were maintained in a humidified incubator with 5% CO_2 at 37°C. They are routinely tested for contamination of mycoplasma by using PCR Micoplasma Test Kit (Takara Bio Inc., Shiga, Japan) and confirmed to be negative before performing experiments.

RNA extraction, cDNA amplification, library preparation, and sequencing

Total RNA was extracted from cells using the NucleoSpin RNA XS kit (Clontech, Moutain View, CA). The Smarter Ultra low RNA input kit (Clontech) was used for the synthesis and amplification of cDNA using up to 10 ng of total RNA following the manufacturer's instructions and performing no more than 12 cycles of PCR in order to minimize amplification biases. The quality of cDNA was verified by Agilent 2100 Bioanalyzer using High Sensitivity DNA Chips (Agilent Technologies, Santa Clara, CA). Truseq DNA Illumina libraries were prepared and sequenced to obtain approximately 90 million reads (101 bp paired-end reads) per library using the Hiseq 2000/2500 Illumina sequencer (San Diego, CA).

RNA-sequence data analysis

Sequences were trimmed to remove adaptors and low-quality bases. Trimmed reads were mapped

onto the hg19 genome (UCSC human genome 19, version:20150519) using Tophat 2.0.10 and transcripts were assembled by Cufflinks 2.1.1 based on RefSeq gene annotation. Transcript expression levels were quantified by Cuffdiff 2.1.1 using the fragments per kilobase of transcript per million mapped fragments (FPKM) method.

GEO accession number is GSE127264.

Real-time PCR analysis

Total RNAs were extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to prepare the cDNA solution. For real-time PCR analyses, Taqman probes of *MCM10* and *Nanog* were purchased from Applied Biosystems. Reactions were performed using the pre-set program of the ABI ViiA 7 Real-Time PCR System (Thermo Fisher Scientific).

Immunocytochemistry

Cells in adherent and sphere culture condition were plated on BioCoat Culture Slide (Corning, Corning, NY) after trypsinization, and incubated for 6 h. To detect expression of proteins, cells were fixed with 4% paraformaldehyde (PFA) (Wako, Osaka, Japan) or 100% methanol (Wako, Osaka, Japan), 6 h after seeding, the shortest period for cell attachment. Cells were incubated with 0.2% Triton X-100 (Wako, Osaka, Japan) to permeabilize membranes, and stained overnight with primary antibodies and for 1 h with secondary antibodies. Immunofluorescent visualization of Nuclei was counterstained with DAPI (Thermo Fisher Scientific). Coverslips were mounted with Fluorescence Mounting Medium (Dako, Glostrup, Denmark). Immunofluorescence was detected using an Olympus IXplore pro microscope (Tokyo, Japan) or Nikon confocal microscopy (A1

HD25) (Tokyo, Japan) with the ANDOR software. Acquired images were analyzed by ImageJ software. Antibodies against Nanog (4903S) and c-Myc (5605S) were purchased from Cell Signaling Technology. MCM10 antibody was purchased from Invitrogen (PA5-67218). S9.6 antibody that binds to RNA/DNA hybrid was purchased from Millipore (MABE1095).

RNaseH treatment

Cells were incubated with Ribonuclease H RNaseH (60 U/μL) (Takara Bio Inc., Code No. 2150A) for 4 hours before immunocytochemistry assay.

DNA fiber assay

Adherent and sphere-cultured cells were pulsed-labeled with 25 μM ldU (Sigma) for 30 min, followed by 250 μM CIdU (Sigma) for 30 min. The cells were trypsinized and resuspended in 100 μL PBS (GIBCO)(10^5–10^6 cells/mL). Then, a 2 μL cell suspension was placed at the end of a glass slide. After air drying for 8 min, 7 μL of fiber lysis solution (50 mM EDTA, 0.5% SDS, 200 mM Tris-HCl [pH 7.5]) was pipetted on top of the cell suspension and mixed. Cell lysis proceeded for 5 min, and then the slides were tilted at 15° to allow the DNA spread down the slide. Slides were air-dried for 15 min and fixed in methanol/acetic acid (3:1). After washing with distilled water, DNA was denatured in 2.5 M HCl for 80 min. The slides were washed with PBS three times, and blocked for 1 h in 5% bovine serum albumin (BSA) (Sigma) in PBS (GIBCO). After blocking, the slides were incubated with primary antibodies (anti-CldU, Abcam ab6326; anti-IdU, BD 347580) followed by secondary antibodies (Alexa Fluor 594–conjugated anti–rat IgG and Alexa Fluor 488–conjugated anti–mouse IgG)(Molecular Probes).

siRNA

We purchased two different siRNA duplexes of *MCM10* (#1, HSS124480 and #2, HSS124482), two different siRNA duplexes of *Myc* (#1, VHS40785 and #2, VHS40789) and a nonspecific control siRNA duplex with similar GC content (siCtrl; Medium GC Duplex #2) from Invitrogen. siRNAs against *MCM5* were designed according to a previous report [1]; target sequences were 5'-GGAGGUAGCUGAUGAGGUGTT-3' (#1) and 5'-AAGCAGUCGCAGUGAAGAUUG-3' (#2). siRNAs were transfected using RNAiMAX (Invitrogen).

Generation of ovarian cancer spheroid cells with inducible-CRISPR/Cas9 targeting *MCM10*

Inducible-Cas9 lentiviral plasmid (Edit-R Inducible lentiviral Cas9; CAS11229) were purchased from Horizon Discovery (Cambridge, UK). For production of lentivirus encoding inducible-Cas9 nuclease, the lentiviral plasmids and packaging plasmids were transfected into lentiX-293T cells using Lipofectamine 2000 (Invitrogen, Carlsbud, CA) and lentivirus-containing supernatants were harvested after 3 days. The lentivirus-containing media was transferred onto OVN62 cells (Ovarian cancer spheroid cells) to generate cells expressing inducible-Cas9 and incubated with blasticidin (5 μ g/mL) (Nakarai tesque, Inc.) for 3 days. After selection, OVN62 cells heterogeneously expressing inducible-Cas9 were performed single-cell sorting using a FACS Aria Ⅲ Cell Sorter (BD Bioscience, San Jose, CA) to pick up stably expressing inducible-Cas9 construct.

We performed a modification of pLenti-sgRNA plasmid (Addgene #71409) with the sgRNA scaffold with the sgRNA sequence targeting *MCM10* (MCM10 sgRNA #1: 5'-CGGTGAATCTTATACAGAAG-3', MCM10 sgRNA #2: 5'-GAGGGTGGCTCGAACACCAA-3', MCM10 sgRNA #3: 5'-CGGTGAATCTTATACAGAAG-3' and 5'-GAGGGTGGCTCGAACACCAA-3'). OVN62 with stably expressing inducible-Cas9 and targeting *MCM10* were selected by puromycin selection (2 μg/mL) (Nacalai tesque, Inc.).

Cell viability assay for ovarian cancer spheroid cells

OVN62 cells stably expressing inducible-Cas9 nuclease and *MCM10*-targeting sgRNA (Non-target sgRNA, MCM10 sgRNA #1, and #2) were treated with Doxycycline (Dox) (Nacalai tesque, Inc.) for 3 days to express Cas9 and induce *MCM10* knockout. After DOX treatment, cells were dissociated to single cells and seeded 3000 cells in each well of the 96-well plates. After 0, 3, 7 days incubation, cell viability was measured by using CellTiter-Glo Assay (Promega, Madison, WI).

Transient overexpression of MCM10

Cells were transfected with pCMV6-Myc-DDK-MCM10 (OriGene, Rockville, MD) and control vector using ViaFect Transfection Reagent (Promega, Madison, WI).

Western blot analysis

Immunoblotting was performed using standard procedures as described [2]. Antibodies against Nanog (4903S), Oct-4 (2750S), ATR (2790S), p-ATR (2853S), Chk1 (2360T), p-Chk1 (2349T), c-Myc (5605S) and Myc-tag (2278) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against MCM10 (3733) and MCM5 (17967) were purchased from Abcam (Cambridge, UK). Anti-actin antibody (MAB150) was purchased from Millipore. Cas9 antibody was purchased from Active Motif. Proteins were detected with horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare Life Sciences, Marlborough, MA).

Proliferation assay for breast cancer cells

Cells were seeded in 12-well plates at low density (5000–10000 cells/well), cultured in RPMI1640 supplemented with 10% FBS and 1% P/S or DMEM with 10% FBS and 1% P/S. HU (Wako, Tokyo, Japan) was added to the medium as necessary. After 4–6 days, cells were harvested and counted.

Flow cytometry analysis

To identify the breast CSC population, cells were stained with Alexa Fluor 647–conjugated anti–human CD24 and APC-H7 labeled anti–human CD44 antibodies (BD Pharmingen, San Jose, CA) at 4°C for 20 min. The cells were then analyzed with a FACSAria II flow cytometer (BD Bioscience, San Jose, CA). Dead cells were excluded by propidium iodide (PI; Sigma, St. Luis, MO) staining. Data were analyzed using the FlowJo software (TreeStar, San Carlos, CA).

To detect DNA-binding MCM3, collected cells were first treated with 750 μL low-salt extraction buffer (0.1% Igepal CA-630, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 10 mM potassium phosphate buffer [pH 7.4]) for 5 min on ice. Then, the cells were fixed by adding 250 μL 10% formalin (SIGMA). After incubation at 4°C for 1 h, the cells were washed with phosphate buffered saline (PBS)(GIBCO). Extracted cells were then incubated with anti-MCM3 antibody (Abcam) and anti–rabbit IgG secondary antibody (Alexa Fluor 488) (Molecular Probes, Eugene, OR) in flow buffer (0.1% Igepal CA-630, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.5 mM EDTA [pH 7.5]). Cells were analyzed on a FACSAria II flow cytometer (BD Bioscience) after staining with Hoechst 33258 (Sigma) to detect nuclear DNA (1 μg/mL).

Plasmid construction

The pLKO shRNA vector was used for knockdown experiments. Target sequences for human *MCM10* were 5’-TCATCCTCAGAAGGTCTTAAT-3’ (#1) and 5’-

GGACTTAACAGATGAAGAAGA-3' (#2). Lentiviral plasmids were transduced into HEK293T cells along with ViraPower Lentiviral Packaging Mix (Invitrogen) using the Lipofectamine 3000 Transfection Reagent (Invitrogen). The medium was changed after 16 h.

Transduction of patient-derived cancer cells with lentiviral vectors

Culture supernatant from HEK293T cells containing virus particles was applied to patient-derived cancer cells. The cells were incubated at 37°C in 5% CO_2 for 48 h, and then virus-infected cells were selected using 2.5 μg/mL puromycin for breast cancer cells and 2 μg/mL puromycin (Nakarai tesque, Inc.) for ovarian cancer spheroid cells.

***In vivo* limiting dilution assay**

Seven-week-old female immunodeficient NSG mice were anesthetized with isoflurane (Abbott, Lake Bluff, IL). Patient-derived breast cancer cells infected with lentivirus (shMCM10 #1, #2, and shCtrl), or cells cultured in adherent and sphere condition were suspended in 50 μL Matrigel (BD Biosciences) in a dilution series (10^3, 10^4, and 10^5 cells). Suspended samples were then injected subcutaneously into the mammary fat pads of NSG mice. Tumor volume was measured twice a week using the following formula: $V = 1/2(L \times W^2)$, where L equals length and W equals width. Tumors larger than 50 mm^3 were counted.

Statistical analysis

All data are presented as means ± SEM or means ± SD. The unpaired Student *t*-test was used to compare differences between two samples, and values of $p < 0.01–0.05$ (*), $p < 0.001–0.01$ (**), or $p < 0.001$ (***) were considered significant. Tumor-initiating frequency was calculated using the ELDA Software. Kaplan-Meier survival curves were analyzed by log-rank test. To statistically

analyze tumor growth rate (shown as means ± SEM), two-way analysis of variance (ANOVA) was used.

References

1 Ge XQ, Jackson DA, Blow JJ. Dormant origins licensed by excess Mcm2–7 are required for human cells to survive replicative stress. *Genes Dev*. 2007; 21: 3331–3341.

2 Hinohara K, Kobayashi S, Kanauchi H, et al. ErbB receptor tyrosine kinase/NF–kappaB signaling controls mammosphere formation in human breast cancer. *Proc Natl Acad Sci U S A*. 2012; 109: 6584–6589.