Supporting Information

Motegi *et al.* 10.1073/pnas.0805685105

SI Methods

Cell Culture, Reagents, and Antibodies. HEK293T, HCT116, and SW480 cells were cultured as described previously (1, 2). MEFs were cultured in DMEM supplemented with 15% FBS, 2% glutamine, 0.1 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. U2OS/DR-GFP cells were grown in McCoy's media supplemented with 10% FBS. Antibodies used here are anti-HLTF polyclonal (Bethyl Laboratories), anti-SHPRH polyclonal (a gift from Dr. R. Sood, National Human Genome Research Institute), anti-PCNA (PC10, Santa Cruz Biotechnology), anti-ubiquitin (P4D1, Santa Cruz Biotechnology), anti-α-tubulin (ab21058, Abcam), anti-myc (9E10, Santa Cruz Biotechnology), anti-HA monoclonal (12CA5, Roche), and anti-FLAG monoclonal (M2, Sigma). MMS, hydroxyurea, aphidicolin, and 5-azacytidine and were purchased from Sigma. Cells were treated with 5-azacytidine as described in ref. 3.

Construction of Various Expression Plasmids. FLAG-tagged HLTF mammalian expression plasmid has been described (2). A full-length cDNA of *HLTF* was cloned into yeast plasmids (a low-copy plasmid with a weak CYC promoter or a high-copy plasmid with a TEF promoter). Plasmids expressing SHPRH-myc-His, RAD18-myc-His, 3XFLAG-PCNA (wild-type or K164R mutant), UBC13-HA (wild-type or C87A mutant), MMS2-HA, and HR6B-HA have been described (1).

Yeast UV Sensitivity Assay. Wild-type or *rad5*-deficient strains transformed with plasmids expressing yeast Rad5, SHPRH, or HLTF or empty vectors were serially diluted, and the cells were spotted on 2 synthetic drop-out plates without tryptophan and with 200 μg/ml G418 to keep plasmids. One plate was treated with UV irradiation (50 J/m^2), and both plates were incubated at 30°C for 3 days before pictures were taken.

Coimmunoprecipitation Analysis and GST Pull-Down Assays. Coimmunoprecipitation and interaction determined by various GST fusion proteins, including GST-UBC13, GST-MMS2, GST-RAD6, and GST were performed as described in ref. 1.

Lentiviral shRNA-Mediated Gene-Silencing. *HLTF*- or *SHPRH*-silencing lentivirus vectors were purchased from Open Biosystems. Viruses were produced as described in ref. 1.

MMS Sensitivity and MMS-Induced Chromosome Breakage Analysis. *HLTF*-silenced cells were treated with various concentrations of MMS for 1 h and plated onto 6-well plates at defined cell densities. Surviving colonies were counted and the surviving fraction (%) was calculated by comparing colony numbers of untreated cells. For MMS-induced chromosome breakage assay, wild type and *Hltf*$^{-/-}$ MEFs were treated with 0.01% MMS for 1 h, cultured for 24 h, then chromosomes in metaphase spreads were examined as described in ref. 1.

siRNA and Recombination Reporter Assay. U2OS human osteocarcinoma cell lines stably transfected with a single copy of an intact DR-GFP reporter gene (gift from Dr. M. Jasin at the Memorial Sloan-Kettering Institute) were used to measure the HR frequency (4). siRNA duplexes specifically targeting *SHPRH*, *HLTF*, *RAD18*, and *MMS2* (SMARTpool) and control siRNAs were purchased from Dharmacon and transfected as the manufacturer suggested. On-target plus nontargeting siRNA was used as a control. HR frequency was determined by the number of cells expressing GFP divided by the number of cells expressing DsRed (as an indicator for transfection). Experiments were repeated at least 3 times, and the average values are reported.

Mutation Frequency Analysis. The Burkitt cell line, Ramos (gift from Dr. R. Harris at the University of Minnesota) was used to determine mutation frequencies as described in ref. 5.

1. Motegi A, *et al.* (2006) Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. *J Cell Biol* 175:703–708.
2. Moinova HR, *et al.* (2002) *HLTF* gene silencing in human colon cancer. *Proc Natl Acad Sci USA* 99:4562–4567.
3. Veigl ML, *et al.* (1998) Biallelic inactivation of hMLH1 by epugenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci USA* 95:8698–8702.
4. Nakanishi K, *et al.* (2005) Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc Natl Acad Sci USA* 102:1110–1115.
5. Harris RS, Sale JE, Petersen-Mahrt SK, Neuberger MS (2002) AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr Biol* 12:435–438.

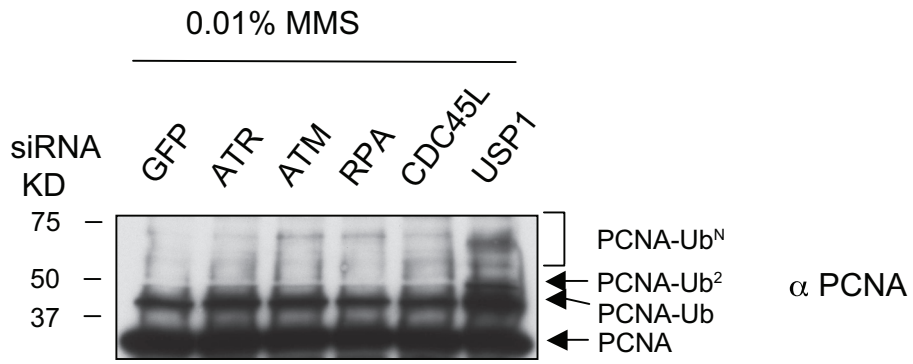

Fig. S1. PCNA polyubiquitination was enhanced by the knockdown of USP1, whereas it was not affected by the knockdown of *ATR, ATM, RPA*, or *CDC45L*. Ub, Ub^2, and Ub^N indicate mono-, di-, and polyubiquitination of PCNA, respectively.

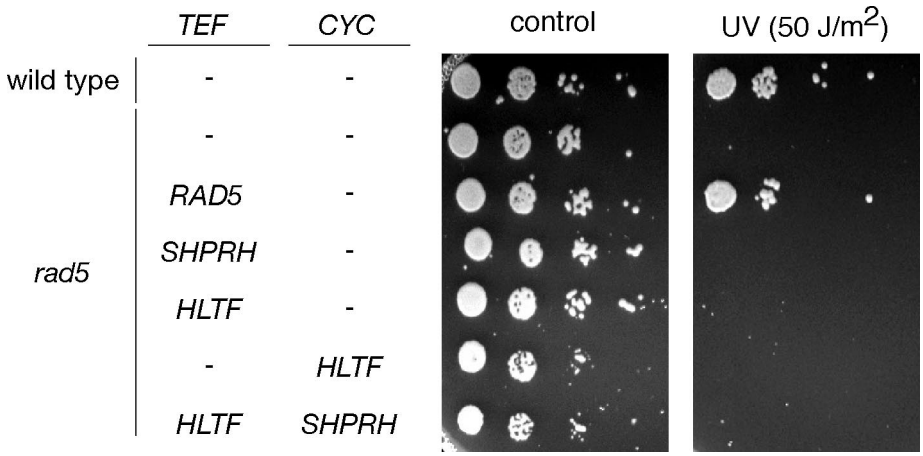

Fig. S2. *HLTF* and/or *SHPRH* expression does not rescue the UV sensitivity of the yeast *rad5*Δ strain. The human *HLTF*, *SHPRH*, or both were expressed under weak (*CYC*) or strong (*TEF*) promoter in the yeast *rad5*Δ strain. Yeast *RAD5* gene was expressed as a positive control.

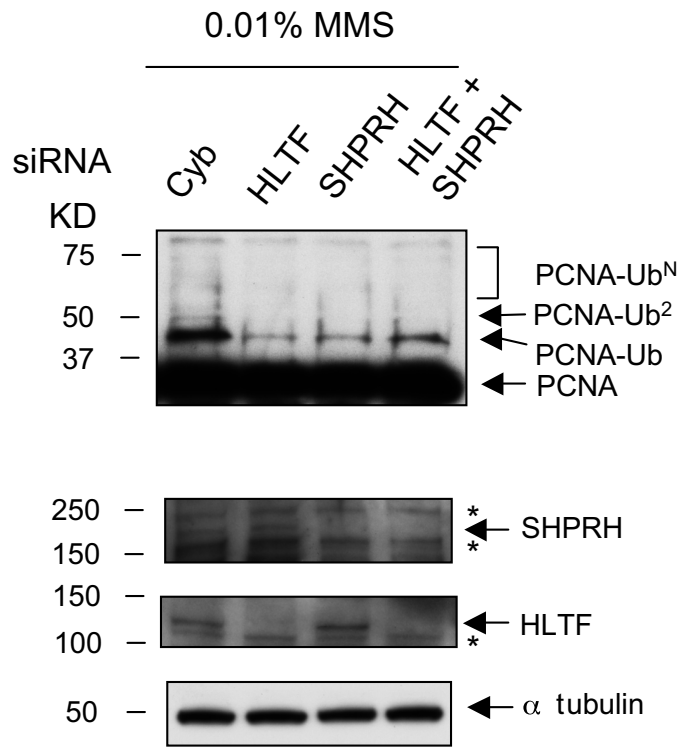

Fig. S3. The reduction of *HLTF* or *SHPRH* expression by siRNA-reduced PCNA polyubiquitination. Depletion of HLTF and SHPRH was achieved by siRNA in HEK293T cells. 72 h after transfection, cells were either mock treated or treated with 0.01% MMS. Cells were harvested 1.5 h later. Anti-PCNA immunoprecipitation were performed as in Fig. 1. Cyb is a control siRNA transfection targeting *cyclophilin B* that does not have any known function in DNA metabolism. The siRNA knockdown of HLTF and SHPRH was checked by Western blot. Asterisks indicate nonspecific bands.

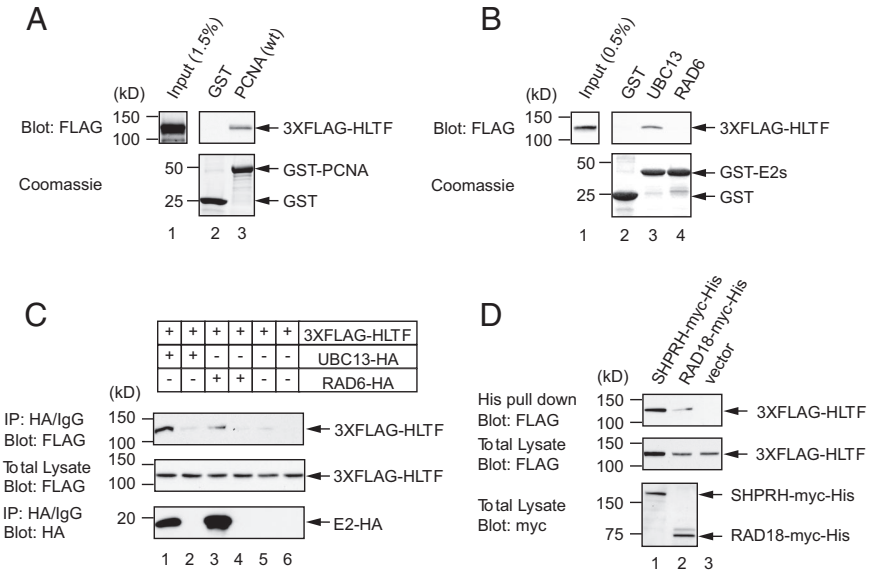

Fig. S4. HLTF associates with PCNA, UBC13, RAD18, and SHPRH. (*A*, *B*) HLTF associates with PCNA and UBC13 *in vitro*. Bacterially expressed GST-PCNA (*A*), GST-UBC13, GST-RAD6 (*B*), or GST alone was incubated with 3XFLAG-HLTF. GST-bound HLTF was detected with an anti-FLAG antibody. (*C*) HLTF coimmunoprecipitates with UBC13. 3XFLAG-HLTF was expressed alone or with UBC13-HA or MMS2-HA and anti-HA (lanes 1, 3, 5) or control IgG (lanes 2, 4, 6), and immunoprecipitates were blotted with an anti-FLAG antibody. Note that HLTF showed a weak, probably indirect, interaction with RAD6 *in vivo*. (*D*) HLTF interacts with SHPRH and RAD18 *in vivo*. 3XFLAG-HLTF was coexpressed with SHPRH-myc-His or RAD18-myc-His, and anti-myc immunoprecipitates were blotted with an anti-FLAG antibody.

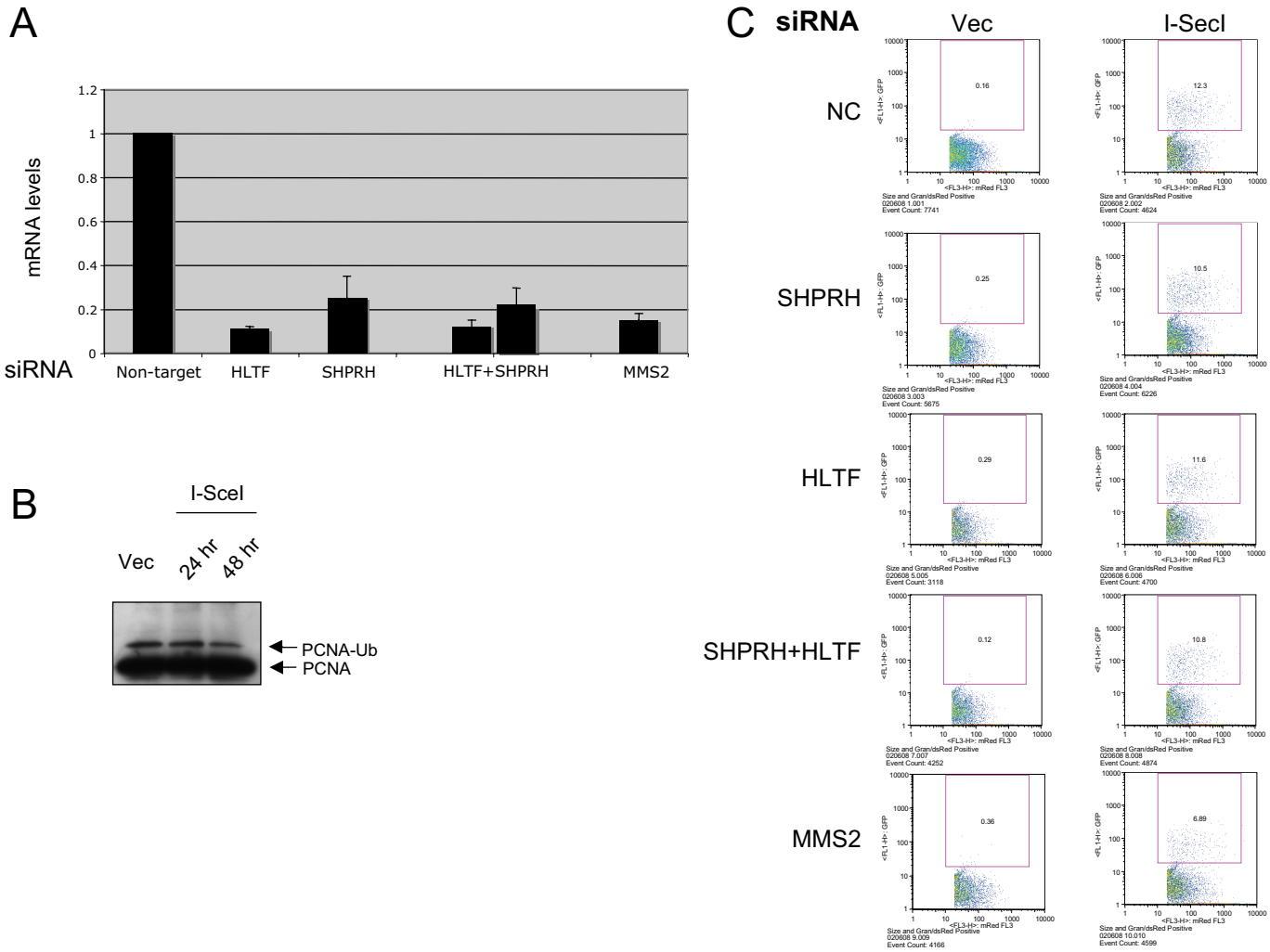

Fig. S5. The reduced expression of *HLTF* or *SHPRH* does not affect homologous recombination (HR). (*A*) The siRNA gene knockdown of each gene was checked by quantitative RT-PCR. The data were normalized to the nontargeting siRNA control. Error bars represent the standard deviation. (*B*) A single DSB does not enhance PCNA ubiquitination. U2OS/DR-GFP cells were transfected with either empty vectors (Vec) or I-SceI plasmids. Cells were harvested 24 h or 48 h after transfection. Detection of PCNA ubiquitination was performed as described in Fig. 1. (*C*) The actual FACS profile of GFP-positive cells representing cells undergoing HR before (Vec) and after (I-SceI) DSBs. The *x* and *y* axes represent the red fluorescent cells and the GFP-positive cells, respectively. HR-positive cells are gated, and the HR frequency is shown inside red rectangles.

Table S1. Sequence comparisons between yeast and human ubiquitin ligases

		HsSHPRH	HsHLTF
ScRad5	Full length	17.2 (27.5)	21.1 (34.5)
	RING	36.4 (47.3)	35.3 (51.0)
	SWI2/SNF2	45.5 (62.1)	59.8 (75.8)

Sequence identities and similarities (in parenthesis) between budding yeast (Sc) and human (Hs) ubiquitin ligases involved in PCNA modifications were calculated by using the NeedleN program (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+Launch+-id+19son1NKD7a+-appl+needleN+-launchFrom+top) and expressed in %. Comparisons of full-length, RING, or SWI2/SNF2 domains are shown.