

Activation of Holliday Junction–Recognizing Protein Involved in the Chromosomal Stability and Immortality of Cancer Cells

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Abstract

We identified a novel gene *HJURP* (Holliday junction–recognizing protein) whose activation seemed to play a pivotal role in the immortality of cancer cells. *HJURP* was considered a possible downstream target for ataxia telangiectasia mutated signaling, and its expression was increased by DNA double-strand breaks (DSB). *HJURP* was involved in the homologous recombination pathway in the DSB repair process through interaction with hMSH5 and NBS1, which is a part of the MRN protein complex. *HJURP* formed nuclear foci in cells at S phase and those subjected to DNA damage. *In vitro* assays implied that *HJURP* bound directly to the Holliday junction and rDNA arrays. Treatment of cancer cells with small interfering RNA (siRNA) against *HJURP* caused abnormal chromosomal fusions and led to genomic instability and senescence. In addition, *HJURP* overexpression was observed in a majority of lung cancers and was associated with poor prognosis as well. We suggest that *HJURP* is an indispensable factor for chromosomal stability in immortalized cancer cells and is a potential novel therapeutic target for the development of anticancer drugs. [Cancer Res 2007; 67(18):8544–53]

Introduction

Cellular crisis, which is characterized by chromosomal instability, chromosomal end-to-end fusions, and subsequent cell death, is a potent barrier to make cells immortal. On the other hand, the genetic instability itself may also be the mechanism by which some cells rarely overcome this critical stage and acquire a group of genetic alterations that are essential for malignant transformation and uncontrolled cell growth (1). The cells that survive this cellular crisis are likely to have activated chromosome stabilization mechanisms such as activation of the telomerase function. In chromosomes, telomeres protect chromosomal ends from misrecognition by DNA repair machinery or by DNA damage checkpoint systems. TRF2, a dimeric DNA-binding protein, is known to bind mammalian telomeric TTAGGG repeats and play a key role in telomere protection (2). The mechanism by which TRF2 protects telomeres from nonhomologous end-joining (NHEJ) can be explained by the formation of t-loops (3). When telomeres are in the t-loop configuration, the NHEJ repair machinery is unable to

access chromosomal ends, resulting in the prevention of end-to-end fusion caused by telomeres.

Recent studies in *Saccharomyces cerevisiae* have suggested that the nucleolus, rather than telomeres, is a key factor in cellular aging (4). Although rDNA is very highly transcribed, accounting for ~60% of total RNA synthesis in active cells, not all copies of rDNA genes are active at any given time. The mechanism responsible for this repression involves Sir2 protein that is shown to cause gene silencing by inducing condensation of DNA into a heterochromatin state (5). Heterochromatic repression of rDNA is likely to reflect a general protective mechanism that restrains homologous recombination (HR) at genomic regions containing multiple tandem repeats (6). In fact, uncontrolled recombination of yeast nucleolus generates extrachromosomal copy of rDNA, which on accumulation shortens the life span of yeast cells (7).

In most organisms, HR is essential for repairing double-strand DNA breaks (DSB), chromosome segregation, and the rescue of stalled replication forks as well as the strict regulation of the copy number of rDNA (8, 9). Impairment of the proper recombination could cause genomic instability and subsequent DNA damage response (DDR) that can arrest cell cycle or induce cell death. Recent studies revealed that higher expression of various DSB repair proteins in cancer cells could be associated with poor prognosis for patients (10–14). In addition, HsRAD51 recombinase was reported to mediate elevated recombination in immortal human cells (15). These data suggested that cancer cells might have some maintenance mechanisms against DDR for protecting cancerous cells from excessive chromosomal instability and for promoting the malignant nature of the cells.

Through genome-wide expression profile analysis of non-small cell lung cancer (NSCLC) using cDNA microarray and subsequent analysis to identify therapeutic targets (16, 17), we identified a novel gene, *HJURP*, whose overexpression was observed in majority of human lung tumors. By biologically analyzing this gene, we have found that the activation of *HJURP* plays a critical role in HR as well as in rDNA and contributes to immortality and chromosomal stability of cancer cells.

Materials and Methods

Cancer cell lines and tissue samples. The 20 human NSCLC, 4 small cell lung cancer cell lines, a cervical carcinoma cell line (HeLa), 2 osteosarcoma cell lines (U2OS and Saos2), and 2 testicular cancer cells (Tera-1 from teratocarcinoma and NEC8 from germ cell tumor) as well as human small airway epithelial cells were used in this study. Two ataxia telangiectasia mutated (ATM)–deficient AT cell lines (AT-2KY-TERT and AT-5KY-TERT) were kindly provided by Dr. O. Niwa (Kyoto University, Kyoto, Japan). In all, 252 NSCLC and adjacent normal lung tissue samples were obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan). This study and the use of all clinical materials mentioned were approved by individual

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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institutional Ethical Committees (see Supplementary Materials and Methods).

Coimmunoprecipitation and matrix-assisted laser desorption/ionization-time of flight mass spectrometry mapping of HJURP-associated proteins. Cell extracts from lung cancer cell lines A549 and LC319 were precleared by incubation at 4°C for 1 h with 100 µL of protein G-agarose beads in a final volume of 2 mL of immunoprecipitation buffer (0.5% NP40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of proteinase inhibitor. After centrifugation at 1,000 rpm for 5 min at 4°C, the supernatant was incubated at 4°C with anti-HJURP polyclonal antibody or normal rabbit IgG for 2 h. The beads were then collected by centrifugation at 5,000 rpm for 2 min and washed six times with 1 mL of each immunoprecipitation buffer. The washed beads were resuspended in 50 µL of Laemmli sample buffer and boiled for 5 min, and the proteins were separated using 5% to 20% SDS PAGE gels (Bio-Rad). After electrophoresis, the gels were stained with silver. Protein bands specifically found in extracts immunoprecipitated with anti-HJURP polyclonal antibody were excised and served for matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis (AXIMA-CFR plus, Shimadzu Biotech).

Immunofluorescence studies. A549 and U2OS cells were plated on collagen-coated coverslips (IWAKI) in six-well plates. For immunostaining, cells were pre-permeabilized with 0.1% Triton X-100 for 10 min for detecting only DNA binding protein and fixed in 4% paraformaldehyde for 20 min followed by permeabilization in 0.5% Triton X-100 for 10 min. Cells were then blocked using 0.1% bovine serum albumin and 0.1% skim milk with PBS for 1 h and incubated with primary antibody in blocking solution for 2 h and Alexa488 and Alexa594-conjugated affinity-purified goat anti-mouse, anti-rabbit, and donkey anti-goat IgG (Molecular Probes; 1:1,000 dilution) for 1 h (see Supplementary Materials and Methods).

Imaging of HJURP proteins in living cells. Plasmids expressing enhanced green fluorescent protein (EGFP)-fused HJURP protein (pEGFP-HJURP) were prepared using pEGFP-N2 vectors (BD Biosciences Clontech). Time-lapse images of the stable transformant of U2OS cells were captured for 32 h using Live Cell Imaging System (Power IX81, Olympus; see Supplementary Materials and Methods).

Immunofluorescence fluorescence *in situ* hybridization, fluorescence *in situ* hybridization, and chromosomal analysis in metaphase cells. Immunofluorescence fluorescence *in situ* hybridization (IF-FISH) was done according to the protocol reported by Chen et al. (18). Briefly, after protein detection, the antibodies were cross-linked using 50 mmol/L ethylene glycol bis(succinimidyl succinate) for 30 min at 37°C. Samples were incubated with RNase (100 ng/mL in 2× SSC) for 60 min at 37°C. The chromosomal DNA was denatured in 0.1 mol/L NaOH for 2 min followed by immersion in cold PBS. The DIG-labeled 18S rDNA probe was hybridized at 37°C overnight, followed by standard immunofluorescence detection. Fluorescence images represent a single optical section. In metaphase FISH, *in situ* hybridization was executed according to the manufacturer's instructions (TSA-Plus Fluorescein System, Perkin-Elmer Life Sciences; see Supplementary Materials and Methods).

Chromatin immunoprecipitations. We did chromatin immunoprecipitations (ChIP) assay using a ChIP Assay kit (Upstate) according to the supplier's protocol and a reported protocol (19) with minor modification (see Supplementary Materials and Methods).

Gene conversion and recombination analysis. Gene conversion analysis was done as described elsewhere (20, 21). The SCneo construct, kindly provided by Dr. M. Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY), was used for site-specific DNA repair analysis. For this study, the hygromycin-resistant marker for stable transformant selection was used. The plasmid was transfected into A549 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions, and stable transformants with one copy of the SCneo were isolated. For induction of DSBs at the *I-SceI* site, 2 µg of pCMV-3xnlx *I-SceI* (ref. 20; *I-SceI*) were introduced into the cells by transfection. To investigate the effect of HR by HJURP gene knockdown, SCneo-stably expressing A549 cells were transfected with 6 µg of pSUPER control vector [pSUPER-SCR (Scramble) and pSUPER-LUC (Luciferase)], pSUPER-si-HJURP-#A and pSUPER-si-HJURP-#B,

and pSUPER-si-RAD51-#A and pSUPER-si-RAD51-#B with *I-SceI* using 24 µL of LipofectAMINE 2000 (Invitrogen). The target sequences of the synthetic oligonucleotides for RNA interference (RNAi) were as follows: control siRNA-1 (EGFP), 5'-GAAGCAGCAGACTTCTC-3'; control siRNA-2 (LUC), 5'-CGTACGCGGAATACTTCGA-3'; control siRNA-3 (SCR: gene coding for 5S and 16S rRNAs in chloroplasts of *Euglena gracilis*), 5'-GCGCGTTGTAG-GATTGCG-3'; si-HJURP-#A, 5'-GTTGGAAGGAGCGCAAAGT-3'; si-HJURP-#B, 5'-GAGCGATTTCATCTTCATCA-3'; si-RAD51-#A, 5'-GCAGCTAAATTAGTTC-CAA-3'; si-RAD51-#B, 5'-GCAGTGATGTCCTGGATA-3'. The transfected cells were then incubated for 48 h and subsequently grown in medium containing 0.4 mg/mL of G418 (Invitrogen). Cell viability was measured by triplicate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Down-regulation of HJURP and HsRAD51 expression by functional siRNA, but not by controls, was confirmed by Western blotting using anti-HJURP polyclonal antibody and anti-RAD51 monoclonal antibody (Calbiochem/Merck KGaA).

Generation of synthetic Holliday junction and gel-shift assay. We did gel-shift assay using synthetic Holliday junction as described elsewhere (ref. 22; see Supplementary Materials and Methods).

Results

Identification of HJURP and its overexpression in human cancer. Through expression profile analysis of primary NSCLCs using cDNA microarray consisting of 27,648 genes, and pure populations of tumor cells prepared from NSCLC tissues by laser microdissection, we identified a novel gene that showed a 5-fold or higher expression level in cancer cells than in normal lung cells (control) in >50% of the NSCLC cases examined. This unannotated gene was later termed *HJURP* based on the evidences shown below. The *HJURP* full-length cDNA sequences of 2,529 nucleotides included an open reading frame of 2,244 nucleotides encoding a 748-amino acid peptide (the sequence will appear in the Genbank with the accession no. AB101211). The analysis using the SMART program⁴ identified no known motifs except one coiled-coil structure at the NH₂-terminal end. We confirmed transactivation of *HJURP* by semiquantitative reverse transcription-PCR (RT-PCR) experiments in 10 of 14 additional NSCLC cases (adenocarcinomas and squamous cell carcinomas; Supplementary Fig. S1A). In addition, up-regulation of *HJURP* was observed in majority of the 23 lung cancer cell lines examined, whereas no expression was found in small airway epithelial cells derived from normal bronchial epithelium (Supplementary Fig. S1B). We subsequently generated rabbit polyclonal antibody specific for human HJURP and by Western blot analysis confirmed a high level of expression of ~83-kDa HJURP protein in various cancer cell lines of lung and other organs, and no expression in small airway epithelial cells (Supplementary Fig. S1C). Northern blot analysis using *HJURP* cDNA as a probe identified a 2.7-kb transcript; a strong signal was seen in the testis and weak signals were seen in thymus and bone marrow among the 23 normal human tissues examined (Supplementary Fig. S1D). To verify the clinicopathologic significance of HJURP, we examined the protein expression in clinical lung tumors using tissue microarrays containing NSCLC tissues from 252 patients. Positive staining for HJURP was observed in 139 of the 252 surgically resected NSCLCs, whereas no staining was observed in any of the corresponding normal lung tissues examined (Supplementary Fig. S2A). Kaplan-Meier analysis indicated that NSCLC patients with HJURP-positive tumors showed shorter

⁴ <http://smart.embl-heidelberg.de/>

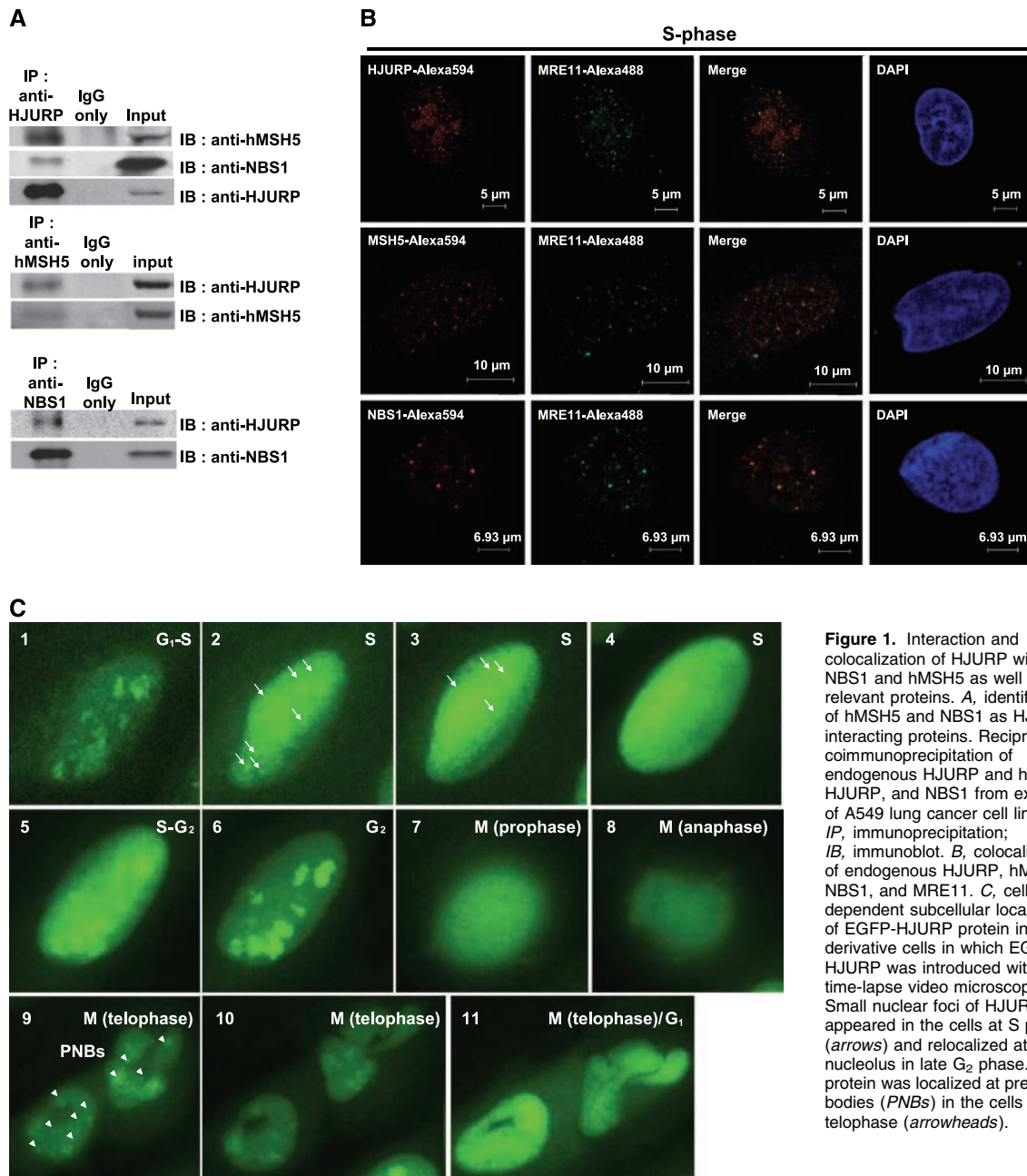


Figure 1. Interaction and colocalization of HJURP with NBS1 and hMSH5 as well as their relevant proteins. **A**, identification of hMSH5 and NBS1 as HJURP-interacting proteins. Reciprocal coimmunoprecipitation of endogenous HJURP and hMSH5, HJURP, and NBS1 from extracts of A549 lung cancer cell lines. *IP*, immunoprecipitation; *IB*, immunoblot. **B**, colocalization of endogenous HJURP, hMSH5, NBS1, and MRE11. **C**, cell cycle-dependent subcellular localization of EGFP-HJURP protein in U2OS-derivative cells in which EGFP-HJURP was introduced with time-lapse video microscopy. Small nuclear foci of HJURP appeared in the cells at S phase (*arrows*) and relocalized at the nucleolus in late G₂ phase. HJURP protein was localized at prenucleolar bodies (*PNBs*) in the cells at telophase (*arrowheads*).

survival times than those with HJURP-negative tumors ($P = 0.0089$ by the log-rank test; Supplementary Fig. S2B).

Interaction and colocalization of HJURP with hMSH5 and NBS1. To elucidate the function of HJURP, we screened a protein(s) that interacts with HJURP. Lysates of A549 and LC319 cells were extracted and immunoprecipitated with affinity-purified anti-HJURP polyclonal antibody. The protein complex including HJURP was stained with SilverQuest (Invitrogen) on the SDS-PAGE gel. A 95-kDa band, which was seen in cell lysates of both A549 and LC319 immunoprecipitated with anti-HJURP polyclonal antibody, but not in those with normal rabbit IgG, was extracted. The peptide sequence determined by MALDI-TOF-MS sequencing indicated the

protein to be human MSH5 [mutS homologue 5 (*Escherichia coli*); hMSH5]. We then generated rabbit polyclonal antibody against hMSH5 and confirmed the cognate interaction between endogenous HJURP and hMSH5 by coimmunoprecipitation experiments (Fig. 1A). Because hMSH5 was recently identified as a sliding clamp in Holliday junction of HR intermediate by forming heterodimer complex with hMSH4 during meiosis (23), we further investigated the interactions between HJURP and the various HR-related proteins by immunoprecipitation experiments. We confirmed coimmunoprecipitation of endogenous HJURP with NBS1, which is a member of the MRN (MRE11-RAD50-NBS1) protein complex, using antibodies to HJURP and NBS1 (anti-NBS1 polyclonal antibody;

Novus Biologicals) using extracts from A549 cells (Fig. 1A). We further found the interaction of human hMSH5 and MRE11 by immunoprecipitation experiments; an interaction of the corresponding yeast homologues, Msh5 and Mre11, was shown using a large-scale yeast two-hybrid screening system (ref. 24; see Supplementary Fig. S2C). We subsequently did immunofluorescence analysis using A549 cells and found colocalization of endogenous HJURP with each of these interacting proteins and their related proteins, hMSH5, NBS1, and MRE11, in the subnuclear foci at the expected time in S phase, suggesting that HJURP was associated with hMSH5 and MRN complexes in the cancer cells (Fig. 1B). To further clarify the detailed intracellular dynamics of HJURP at various cell cycle phases, we cloned full-length HJURP into an EGFP-fused protein expression vector (pEGFP-N2) and established U2OS cells stably expressing EGFP-HJURP. We synchronized these cells with aphidicolin treatment and monitored their subcellular localization using time-lapse video microscopy. After release from the aphidicolin block, HJURP protein dominantly localized at the nucleolus in G₁ phase and gradually formed discrete subnuclear foci at S phase (Fig. 1C), independently confirming our immunocytochemical observation of HJURP localization at the nucleus. Interestingly, HJURP spread into the

whole nucleus and relocalized at the nucleolus in late G₂ phase. Finally, HJURP mainly localized at prenucleolar bodies immediately after mitosis (telophase) and subsequently distributed at the reassembled nucleolus in G₁ phase (Fig. 1C).

Involvement of HJURP in ATM signaling. The ATM kinase gene, responsible for ataxia telangiectasia, plays a critical role in DDR and is known to phosphorylate downstream targets including the components of HR-related proteins, such as NBS1 and WRN (25). In addition, HJURP contains three possible S/TQ sites that matched the minimal recognition sites for ATM (ref. 26; AA152-3, AA247-8, and AA316-7). Therefore, we first incubated the extracts from A549 cells in the presence and absence of λ -phosphatase and analyzed the molecular weight of HJURP protein using Western blot analysis. Expectedly, the measured weight of the majority of HJURP protein in the extracts treated with phosphatase was smaller than that in the untreated cells (Fig. 2A). The data indicated that HJURP protein was mainly present as a phosphorylated form. We then checked the status of endogenous HJURP protein in two ATM-deficient cell lines (AT-2KY-TERT and AT-5KY-TERT) and U2OS cells expressing ATM as a positive control. Although we detected a high level of HJURP protein in U2OS cells as observed by Western blotting of a panel of various cancer cell

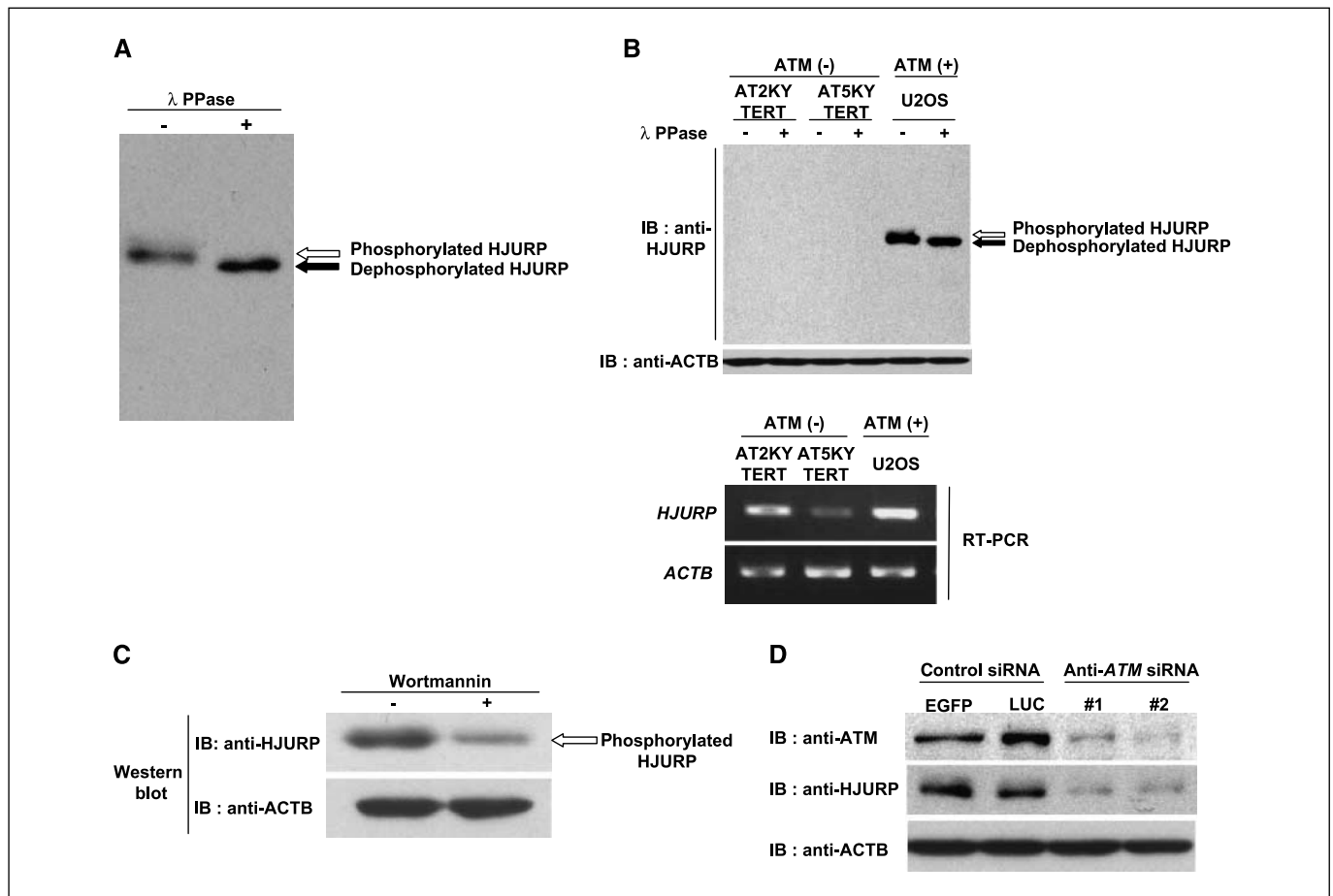
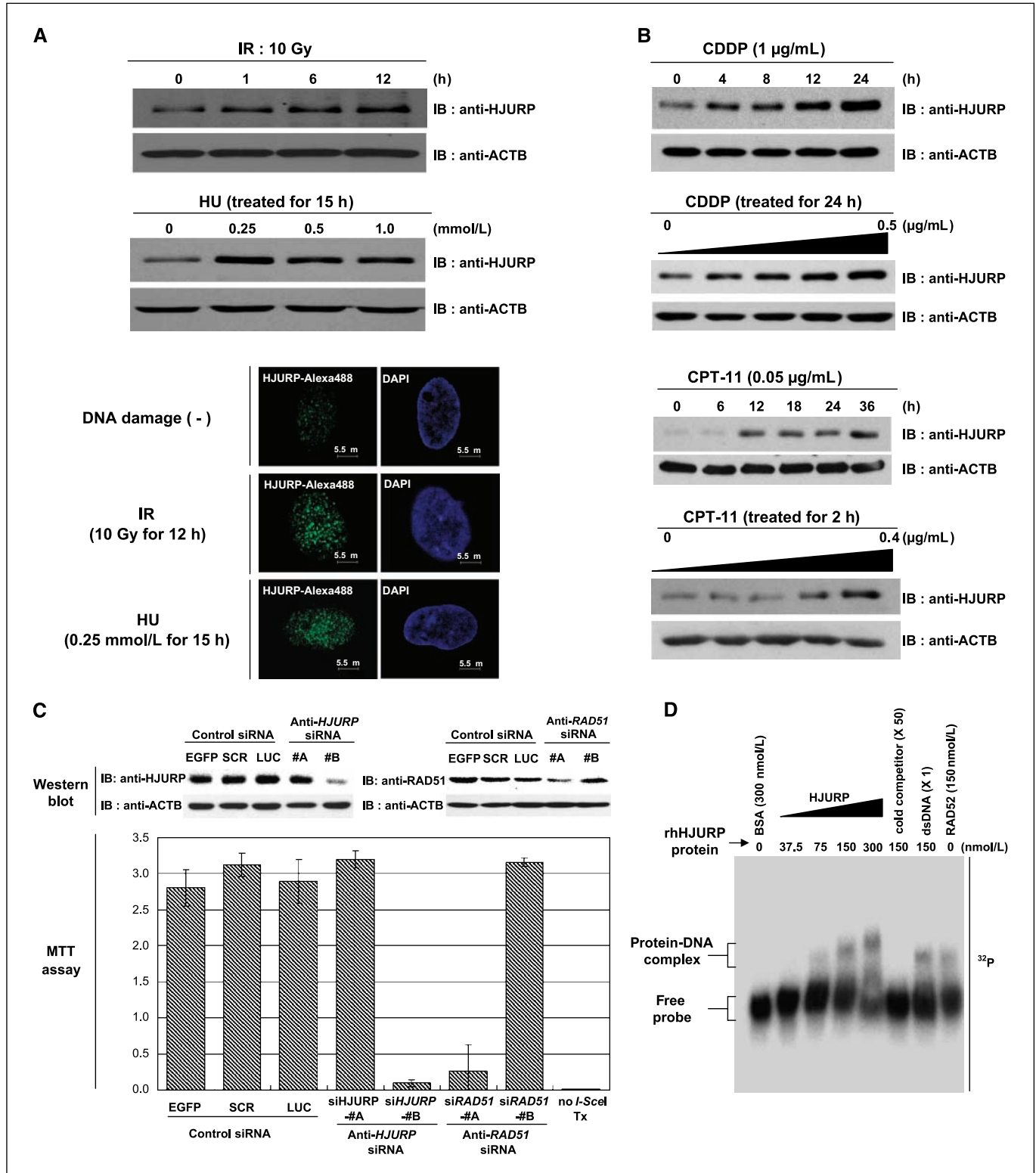


Figure 2. Involvement of HJURP in ATM signaling. *A*, phosphorylated levels of HJURP detected by λ -phosphatase assay coupled with Western blot analysis. The shifted band was detected in λ -phosphatase-treated extracts of A549 cells. *B* (top), protein levels of HJURP in the ATM-deficient cells and U2OS cells by Western blot analysis. HJURP protein was not detected in the ATM-deficient cells by Western blot analysis. Bottom, transcriptional levels of HJURP in the ATM-deficient cells and U2OS cells by semiquantitative RT-PCR. Expression of *HJURP* mRNA in AT-2KY-TERT cells was almost the same level compared with that in U2OS cells. *C*, inhibition of HJURP phosphorylation by wortmannin. U2OS cells were pretreated with 50 μ g/mL wortmannin for 30 min before collecting cell lysates from treated and control samples, and were immunoblotted with anti-HJURP antibodies. *D*, reduction of the level of HJURP protein by treatment of U2OS cells with anti-ATM siRNA (#1 and #2), detected by Western blot analysis.

lines (Supplementary Fig. S1C), no HJURP protein was detected in the two ATM-deficient cells (Fig. 2B, top). However, we identified a high level of *HJURP* transcripts in AT-2KY cells as similar to those in U2OS cells, and a low level (but not absent) of the transcript in AT-5KY cells (Fig. 2B, bottom). Thus, we hypothesized that HJURP protein may be stabilized when phosphorylated and/or incorpo-

rated in a complex with its interacting proteins, some of which are also regulated by ATM. Inhibition of the ATM kinase activity in U2OS cells using wortmannin or siRNA against ATM kinase showed the significant decrease in the level of endogenous HJURP protein (Figs. 2C and D). On the other hand, the level of HJURP transcripts in the same cells was not influenced by ATM



suppression (data not shown). Therefore, we further hypothesized that HJURP may be a downstream target for ATM signaling *in vivo*, although further analyses are required to clarify whether this was because of the direct effect of the inhibition of ATM-dependent HJURP phosphorylation or because of suppression of other ATM-target proteins, which could associate with HJURP.

Increase in HJURP protein levels by DNA damage and its involvement in HR. Several proteins, including phosphorylated ATM and members in the MRN complex, were known to form DNA damage-regulated subnuclear foci. To investigate whether HJURP participates in DNA damage-signaling pathways, we first examined the levels of HJURP protein after the treatment of cells with various types of DNA-damaging agent. Exposure of A549 cells to γ -irradiation (0 Gy) and treatment of the cells with hydroxyurea strongly increased the levels of HJURP protein (Fig. 3A, *top* and *middle*). HJURP formed distinct nuclear foci in the nucleoplasm by exposure of the cells to two different genotoxic agents (γ -irradiation and hydroxyurea) that generate DSBs (Fig. 3A, *bottom*). Treatment of A549 cells with other DNA-damaging agents, cisplatin and camptothecin, also revealed an increase in HJURP proteins in a time- and dose-dependent manner (Fig. 3B, *top* and *bottom*). No HJURP protein was detected in ATM-deficient cells (AT-5KY) before and after exposure to γ -irradiation, whereas the same treatment of A549 cells, in which wild-type ATM was expressed, significantly increased the level of HJURP protein (Supplementary Fig. S3A). The result independently confirmed that the HJURP levels in cells with DNA damage were likely to be influenced by the presence and absence of the ATM signaling. Interestingly, the binding between endogenous HJURP and endogenous hMSH5/NBS1 was strongly enhanced after exposure of A549 cells to the DNA damage agents (representative data are shown in Supplementary Fig. S3B), suggesting that cognate interactions of HJURP with hMSH5 and NBS1 could play an important role for the DNA repair process after DNA damage.

Because HJURP seemed to be involved in cellular DDR, we examined involvement of HJURP in the HR process using the artificial recombination substrate SCneo (20, 21) in combination with the siRNA method. We established A549-derivative cells stably expressing a modified SCneo reporter construct (20), and transiently coexpressed the *I-SceI* enzyme (pCMV-3xnlx *I-SceI*) and siRNA expression vector against HJURP in the cells. After DSB induction in a nonfunctional *neo* segment of SCneo by expression of *I-SceI*, G418 selection was applied for 2 weeks until the colonies were clearly visible. In this system, cells were G418-resistant only if the DSB at the upstream *neo* fragment was repaired correctly by HR. The levels of endogenous HJURP protein in the A549 cells cotransfected with pSUPER-*HJURP*-siRNA-#B (si-*HJURP*-#B) were significantly decreased in comparison with those cotransfected

with the control siRNAs (pSUPER-EGFP, pSUPER-SCR, and pSUPER-LUC-siRNA) and pSUPER-*HJURP*-siRNA-#A (si-*HJURP*-#A; Fig. 3C, *top left*). The number of G418-resistant colonies was significantly reduced in si-*HJURP*-#B-transfected cells compared with others (Fig. 3C, *bottom*). The cells cotransfected with a positive control of pSUPER-*RAD51*-siRNA-#A also revealed reduction of HsRAD51 expression and resulted in reduction of the number of colonies (Fig. 3C, *top right* and *bottom*). Cells without *I-SceI* enzyme showed no visible colonies throughout observation (Fig. 3C, *bottom*). The result implied that HJURP could play a significant role for HR in DSB repair process.

Because HJURP was supposed to have some function in HR, we tested the binding affinity of HJURP to DNAs with four-way junction structures, including a double-stranded 49-mer oligonucleotide (dsDNA 49-mer); a synthetic nucleotide with Holliday junction structure that has a 12-bp homologous region at the center as well as at 5' ends of four oligonucleotides was constructed as shown by Yokoyama et al. (22). A four-way junction of DNA molecules is a key intermediate structure in the HR pathway. When 32 P-labeled synthetic DNAs with Holliday junction were incubated with recombinant HJURP protein, we observed the band corresponding to the HJURP-Holliday junction complex, which was clearly shifted from HJURP-free probes, in a dose-dependent manner (Fig. 3D). These shifted bands disappeared on the addition of a 50-fold higher concentration of cold competitor, and were not decreased by the addition of the same amount of cold double-stranded DNA oligonucleotides (Fig. 3D). The data suggested that HJURP had a direct-binding affinity to the synthetic Holliday junction *in vitro*. Interestingly, because higher amounts of recombinant protein not only increased the amount of shifted band, but also made the shifted band to the higher molecular weight (the same tendency was observed by electrophoresis on both polyacrylamide and agarose gels), we speculated that multiple HJURP molecules could bind to the DNA complex.

Binding of HJURP to rDNA array. Endogenous HJURP was mainly located at the interphasic nucleoli (data not shown). In addition, our time-lapse video microscopic observation using aphidicolin-treated synchronized U2OS cells that stably expressed EGFP-HJURP further revealed disappearance of HJURP from nucleoli before mitosis and formation of small round structures very similar to prenucleolar bodies immediately after mitosis (Fig. 1C). The prenucleolar bodies were described as mobile nuclear bodies that appear in the newly formed nuclei and participate in the delivery of the processing machinery at the sites of rDNA transcription. Subcellular localization of HJURP and the possible relevance of HJURP in the HR pathway suggested its potential role in proper recombination at genomic regions that contain multiple tandem repeats such as rDNA. Therefore, we next investigated the

Figure 3. Induction of HJURP after having DNA damage and its possible relevance to HR. *A* (*top*), the level of HJURP was elevated in A549 cells after exposure to γ -irradiation (*IR*). The indicated substrates were examined in the absence of irradiation and at 1, 6, and 12 h after exposure to 10 Gy γ -irradiation. ACTB was used as expression controls. *Middle*, increased HJURP protein levels in A549 cells after treatment with hydroxyurea (*HU*). *Bottom*, subcellular localization of HJURP in A549 cells before and after exposure to γ -irradiation and DNA-damaging agent (*HU*). *DAPI*, 4',6-diamidino-2-phenylindole. HJURP formed distinct nuclear foci in the nucleoplasm after having DNA damage. *B* (*top* and *bottom*), increased HJURP protein levels in A549 cells after treatment with cisplatin (*CCDP*) and camptothecin (*CPT-11*) in a time- and dose-dependent manner. *C*, HR level of A549 cells transfected with SCneo construct after suppression of HJURP with RNAi. SCneo-introduced A549 cells were transfected with *I-SceI* (2 μ g) and each of pSUPER plasmid (6 μ g). *Top*, transfection with pSUPER-*HJURP*-#B, but not control (pSUPER-EGFP, pSUPER-SCR, and pSUPER-LUC) and pSUPER-*HJURP*-#A resulted in reduction of the HJURP protein level. *RAD51* siRNA was used as a positive control. No *I-SceI* Tx indicates *I-SceI*-untransfected cells. *Bottom*, MTT values reflecting the HR rate in the SCneo-expressing A549 cells were measured after treatment of the cells with G418 for 14 d (each in triplicate). *D*, specific binding of HJURP to the synthetic Holliday junction. Increasing amounts of HJURP (0–300 nmol/L) were incubated with the DNA mixture containing the 32 P-labeled synthetic Holliday junction (5 μ mol/L). The same series of HJURP were incubated in the presence of 250 μ mol/L of cold synthetic Holliday junction, or 5 μ mol/L of dsDNA. *RAD52* (150 nmol/L) was used as a positive control, and bovine serum albumin (300 nmol/L) was used as a negative control. The samples were separated by electrophoresis on a 5% polyacrylamide gel in 0.5% Tris-borate EDTA buffer and were transferred by the standard protocol.

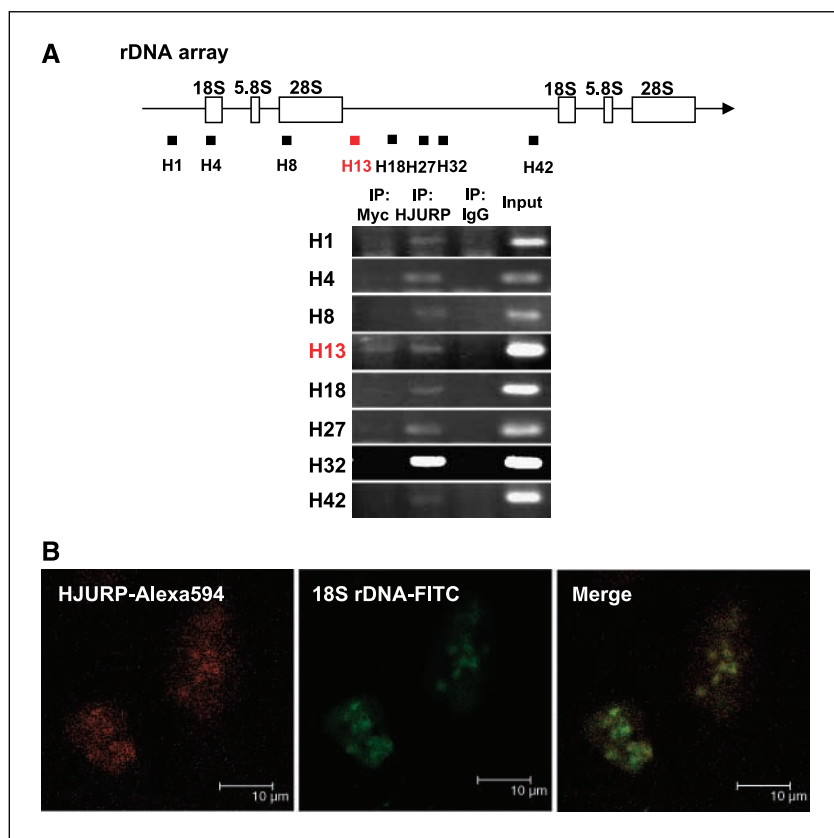


Figure 4. Binding of HJURP to rDNA array. **A**, immunoprecipitations were done with each indicated antibody using U2OS cells, and genomic PCR was carried out using primers for the indicated region (H1, H4, H8, H13, H18, H27, H32, and H42). ChIP analysis using primers for H13 and immunoprecipitated rDNA by anti-c-Myc antibody as templates was done as a positive control assay (*red*). **B**, HJURP foci in U2OS cells were colocalized with 18S rDNA signals as detected by IF-FISH analysis.

association of HJURP with rDNA array by ChIP assays using extracts prepared from U2OS cells. Cross-linked protein-DNA complexes were immunoprecipitated by anti-HJURP antibodies or two control antibodies (anti-c-Myc antibody as a positive control and normal rabbit IgG) and served as PCR templates using eight sets of primer (19). We found that the anti-HJURP polyclonal antibody pulled down the genomic DNA within the rDNA region (Fig. 4A). We also detected the colocalization of HJURP protein and rDNA in the nucleoli by IF-FISH analysis (Fig. 4B).

Suppression of HJURP-induced chromosomal instability and senescence in cancer cells. To investigate the functional role of HJURP in cancer cells, we attempted to inhibit HJURP expression using siRNAs. Using Western blot analysis, we found that transfection of siRNA against HJURP (defined as anti-*HJURP* siRNA-#1 and siRNA-#2) into U2OS cells caused a remarkable decrease of HJURP at protein levels, compared with control siRNAs. This knockdown effect was very specific for HJURP, and no off-target effect was detected in other HJURP-related MRN proteins (Fig. 5A). Subsequent microscopic observation detected the localization of hMSH5, NBS1, and MRE11 in the nuclear foci of the nucleoplasm, suggesting that HJURP inhibition was unlikely to affect the levels and subcellular localization of these HJURP-related proteins (data not shown). The cell viability of the U2OS cells transfected with *HJURP*-siRNA was significantly decreased compared with that of the cells with control siRNA, as measured by MTT assay (Fig. 5B). To clarify the molecular mechanism(s) of this growth-suppressing effect, using flow cytometry, we analyzed U2OS cells that had been transfected with *HJURP*-siRNA, and found that the proportion of cells in G₂-M phase and with 4n-16n were significantly larger when the cells were transfected with anti-HJURP siRNA than with control

siRNA (Fig. 5C, *top* and *bottom*). We suspect that HJURP may usually protect damaged DNA from exposure to a DNA-damage sensor as part of the DSB checkpoint mechanism through its binding to damaged DNA. Microscopic analysis of 4',6-diamidino-2-phenylindole-stained U2OS cells transfected with *HJURP*-siRNA revealed the enlargement of the nuclei, the increase in chromosome number in metaphase and anaphase, and the anaphase and telophase bridges, which were very similar to a well-known indicator of "chromosomal instability" (Fig. 5D; refs. 1, 2). The data indicated that HJURP might protect the immortalized cancer cells from a deleterious level of chromosomal instability. In addition, using FISH analysis with 18S rDNA probes, we detected a significant increase of chromosomal fusions in metaphase spreads of cells treated with *HJURP*-siRNA. Colcemid-treated cells showed a variety of chromosomal aberrations including dicentric chromosomes fused at chromosomal ends, multiple-fused chromosomes, and chromosomal fusions with rDNA signal at the fusion site (Fig. 6A). To examine whether HJURP regulates the immortality of cancer cells, we did the senescence analysis using acidic β -galactosidase (SA- β -Gal) staining, and found that anti-*HJURP*-siRNA-treated cells underwent premature senescence, as proven by positive SA- β -Gal staining (Fig. 6B). The evidence suggests that HJURP is an important molecule for the immortality of cancer cells.

Discussion

HJURP was significantly overexpressed in majority of lung cancer samples, compared with normal lung tissues. In addition, we detected a high level of expression of this gene in testis, and also at relatively low levels in thymus and bone marrow where frequent

HR is physiologically required (Supplementary Fig. S1D); for example, during the first step of meiosis in testis as well as in the maturation process related with V(D)J recombination occurred in T cells in thymus and B cells in bone marrow. HJURP physically interacted with hMSH5, which was related to HR during meiosis through heterodimeric formation with hMSH4 (23). hMSH5 and hMSH4 are members of the seven proteins belonging to the mismatch repair genes (MSH1-7) and their expression is largely

confined to testis and ovary (27). Genetic studies in various species suggested that hMSH4 and hMSH5 were required for the formation of viable gametes during meiosis, but not for mismatch repair (27, 28). Recently, it was shown that hMSH4-hMSH5 complex recognizes Holliday junctions and forms a hydrolysis-independent sliding clamp that embraces adjacent homologous duplex arms for Holliday junctions (23). In addition to its involvement in meiotic recombination, experimental evidence has also suggested that

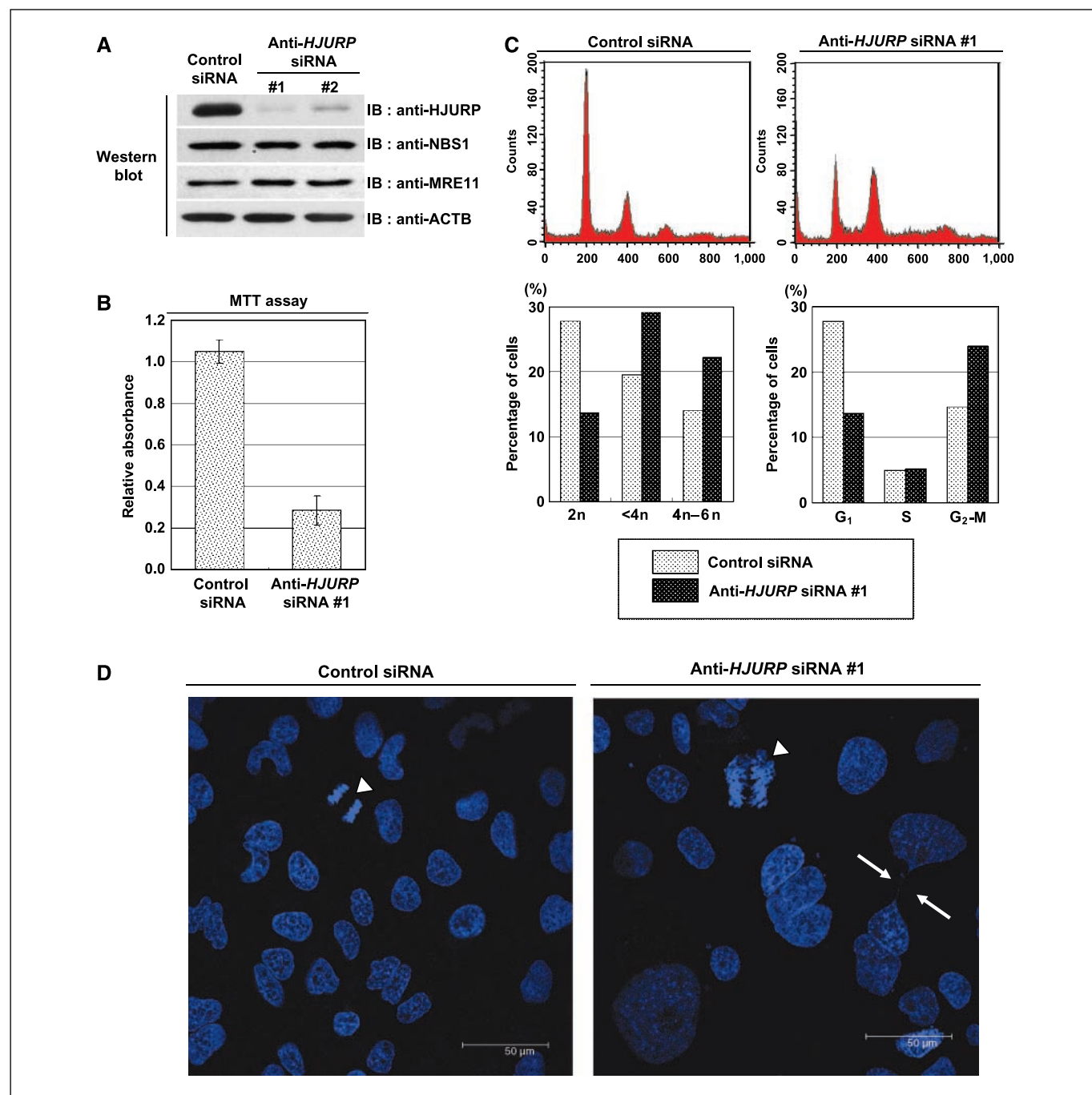


Figure 5. Chromosomal instability by the treatment of cancer cells with siRNA against HJURP. *A*, HJURP protein knockdown effect on U2OS cells in response to anti-HJURP-#1, anti-HJURP-#2, and control siRNAs, analyzed by Western blotting. *B*, MTT assay of cells transfected with the anti-HJURP siRNA-#1 and control siRNAs. *C* (top and bottom), U2OS cells treated with anti-HJURP siRNA-#1 and control siRNA were analyzed by flow cytometric analysis. Suppression of HJURP expression increased the proportion of cells with 4n-16n and G₂-M phase. *D*, treatment of U2OS cells with siRNA against HJURP that resulted in chromosomal instability, accompanying nuclear enlargement, telophase bridges (arrows), and increase in chromosome number at anaphase (arrowheads).

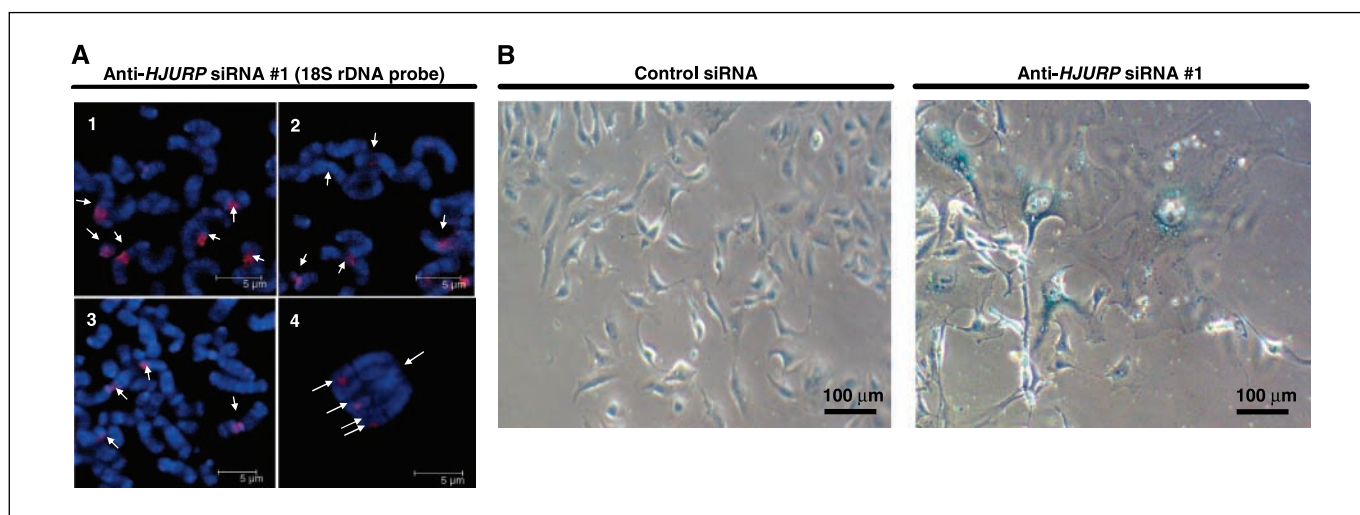


Figure 6. Chromosomal instability, chromosomal fusion at rDNA signals, and premature senescence by treatment of cancer cells with siRNA against HJURP. A, FISH analysis with 18S rDNA probes (red), detecting chromosomal fusions at rDNA (arrows) in metaphase chromosomes from U2OS cells transfected with anti-HJURP siRNA-#1. B, SA-β-Gal staining of anti-HJURP siRNA-#1-treated cells.

Msh5 could possess multiple cellular functions. For instance, mouse Msh5 played a critical role in chromosome synapsis, and Msh5 deficiency resulted in testicular and ovarian degeneration due to the massive induction of apoptosis (27, 29). Furthermore, the involvement of MSH5 in DDR has also been implicated in *S. cerevisiae* (30, 31). In particular, a gain-of-function of yeast MSH5 Y823H amino acid substitution has been shown to possess a dominant effect in mediating tolerance to DNA alkylating agents (31). It also physically interacts with a non-receptor tyrosine kinase c-Abl and this interaction manipulates cellular responses to ionizing radiation-induced DNA damage (32). Our results in lung cancer implied that the interaction between HJURP and hMSH5 could play an important role in HR not only in meiosis, but also in immortalized cancer cells.

In mammalian cells, DNA DSBs trigger activation of the ATM protein kinase, which is known to phosphorylate downstream targets that initiate cell cycle arrest and DNA repair for cell survival, or induce apoptosis for eliminating cells (33). In this study, we found that HJURP protein was likely to be involved in the ATM signaling and its expression was increased by DSB damage. We also detected that HJURP is likely to be involved in the HR pathway with NBS1, a member of the MRN (MRE11-RAD50-NBS1) complexes, at Holliday junctions. Interestingly, HJURP was activated by the exposure to γ -irradiation or DNA-damaging agent, and formed distinct nuclear foci in the nucleoplasm. The association between HJURP and hMSH5/NBS1 in cancer cells was significantly enhanced by DNA damage. In addition, we found that HJURP directly bound to Holliday junctions and the suppression of HJURP reduced recombination rates. A combination of these evidences strongly implies that HJURP was indispensable for HR along with its interacting proteins in the DSB repair process.

The t-loop structure in the telomeric region resembles a DNA recombination intermediate of a stalled replication fork observed during HR (2). Both telomeric and rDNA regions contain highly multiple tandem repeat sequences and were usually silenced. Although the total number of these chromosomal rDNA repeats seems to be maintained at an appropriate level for each organism, genomic regions with the repeated structure are in general considered unstable due to the high incidence of recombinational

events (34). Thus, DSBs accompanied by recombinational events may also be harmful in the absence of appropriate repair systems. A strong evidence suggesting a linkage between rDNA recombination and aging was provided by studies done on Werner syndrome, a human recessive disorder caused by mutations in the *WRN* gene that encodes a DNA helicase belonging to the RecQ family. WRN protein is principally localized in the nucleolus where it could modulate rDNA metabolism (4, 35, 36). Mutants for *sgs1*, the yeast homologue of *WRN*, accumulate extrachromosomal copies of rDNA more rapidly than the wild-type and lead to premature aging and a shorter life span (4, 7, 37). Extrachromosomal copies of rDNA have not been described in mammals, but age-associated changes in the nucleolus and rDNA have been described. For example, the rDNA of mice would be hypermethylated according to aging (38), and decreased rRNA transcription and other structural changes in the nucleolus of cells with aging were also reported (39, 40). In the present study, we showed that HJURP bound to rDNA array and that suppression of HJURP in cancer cells using siRNA resulted in chromosomal fusions at the rDNA regions and induction of premature senescence. Hence, HJURP might function in a nucleolus as a repressor for deregulation of recombination in the rDNA region and maintain the chromosomal stability, resulting in cancer cell immortalization (see Supplementary Fig. S4). Interestingly, we also found that HJURP interacted with nucleophosmin (B23) that have diverse molecular functions, including ribosome biogenesis, centrosome duplication, and maintenance of genomic stability (41–44), and that inhibition of HJURP using siRNA resulted in translocation of nucleophosmin to the perinucleoli and caused the abnormal number of centrosomes.⁵ HJURP may have an important function in centrosome and ribosome biogenesis as well as genomic stability through its interaction with nucleophosmin, although the detailed mechanism of this pathway remains to be elucidated.

In an early process of carcinogenesis, oncogenic stimuli cause accelerated cell division that may trigger the cellular DDR because of aberrant DNA replication as well (45). The DDR arrests cell cycle or induces cell death, and this DDR process becomes a hurdle to

⁵ Unpublished data.

sustained proliferation of cells. Hence, transformation of normal cells to malignant cells needs to overcome this DDR process. Inactivation of the DDR would generate genetic instability and may be one of the ways to accelerate malignant transformation (45). However, genomic instability caused by inactivation of the DDR itself has both advantages and disadvantages for the cells to be immortalized and proliferate continuously. The genomic instability may increase the chance of transformation of normal cells to cancer cells and cause the cancer cells to have more malignant phenotypes. However, it is also true that cells without some surveillance mechanisms have a higher risk of cell death, because an uncontrolled HR pathway generates the accumulation of DSBs that become targets for the DNA repair machinery (in particular, the NHEJ machinery). As mentioned, inhibition of HJURP in U2OS cells by siRNA leads to the excess of chromosomal instability, the G₂-M arrest, and premature senescence. Hence, we suspect that a HJURP-dependent HR pathway may be used for the maintenance mechanisms against DDR that protect the immortalized cancer cells from a dangerous level of chromosomal instability (Supplementary Fig. S4). To support this idea, we found through our tissue-microarray experiments that NSCLC patients with HJURP-positive

tumors showed shorter cancer-specific survival times than patients whose tumors were negative for HJURP (Supplementary Fig. S2B).

In summary, we showed that HJURP is involved in the HR pathway in the DSB repair process as a member of a protein complex including hMSH5 and/or NBS1 proteins. Treatment of cancer cells with siRNA against HJURP resulted in G₂-M arrest and chromosomal instability, and also caused premature senescence. Because HJURP is an indispensable factor for chromosomal stability in immortalized cancer cells, we suggest that it could be a novel therapeutic target for development of anticancer drugs.

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