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Tumor suppressor RecQL5 controls recombination induced by DNA crosslinking agents

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ABSTRACT

RecQ family DNA helicases function in the maintenance of genome stability. Mice deficient in RecQL5, one of five RecQ helicases, show a cancer predisposition phenotype, suggesting that RecQL5 plays a tumor suppressor role. RecQL5 interacts with Rad51, a key factor in homologous recombination (HR), and displaces Rad51 from Rad51-single stranded DNA (ssDNA) filaments *in vitro*. However, the precise roles of RecQL5 in the cell remain elusive. Here, we present evidence suggesting that RecQL5 is involved in DNA interstrand crosslink (ICL) repair. Chicken DT40 *RECQL5* gene knockout (KO) cells showed sensitivity to ICL-inducing agents such as cisplatin (CDDP) and mitomycin C (MMC) and a higher number of chromosome aberrations in the presence of MMC than wild-type cells. The phenotypes of *RECQL5* KO cells resembled those of Fanconi anemia gene KO cells. Genetic analysis using corresponding gene knockout cells showed that RecQL5 is involved in the FANCD1 (BRCA2)-dependent ICL repair pathway in which Rad51-ssDNA filament formation is promoted by BRCA2. The disappearance but not appearance of Rad51-foci was delayed in *RECQL5* KO cells after MMC treatment. Deletion of Rad54, which processes the Rad51-ssDNA filament in HR, in *RECQL5* KO cells increased sensitivity to CDDP and further delayed the disappearance of Rad51-foci, suggesting that RecQL5 and Rad54 have different effects on the Rad51-ssDNA filament. Furthermore, the frequency and variation of CDDP-induced gene conversion at the immunoglobulin locus were increased in *RECQL5* KO cells. These results suggest that RecQL5 plays a role in regulating the incidence and quality of ICL-induced recombination.

1. Introduction

Genetic information is stably maintained and accurately inherited through a variety of cellular mechanisms aimed at preventing or repairing DNA damage. The breakdown of genome stability or DNA repair mechanisms could lead to apoptosis, transformation, or premature senescence of cells. Examples of widely known genome instability diseases are RecQ-associated syndromes and Fanconi anemia (FA).

The RecQ family of DNA helicases is highly conserved in evolution and plays important roles in the maintenance of genome stability [1,2]. In higher vertebrates, five RecQ helicases, RecQL1, BLM, WRN, RecQL4, and RecQL5, have been identified. *BLM*, *WRN*, and *RECQL4* are the genes responsible for Bloom syndrome, Werner syndrome, and Rothmund–Thomson syndrome, respectively, which are diseases characterized by cancer predisposition and premature aging [3–5]. Although a genetic disease caused by the *RECQL5* defect has not been identified, *RECQL5* knockout (KO) mice show a cancer prone phenotype, suggesting that RecQL5 functions as a tumor suppressor [6,7]. RecQL5 interacts with PCNA and RNA polymerase II, which are involved in DNA replication and repair, and transcription [8,9]. Furthermore, RecQL5 directly binds to Rad51 recombinase via a BRC variant (BRCv) repeat domain

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of RecQL5 [10] and disrupts the Rad51-single stranded DNA (ssDNA) interaction *in vitro* [6,11]. RecQL5 suppresses sister chromatid exchange (SCE) formation in the cell, and therefore acts as an anti-recombinase *in vivo* [12,13]. RecQL5 has been suggested to be involved in DNA double-strand break (DSB) processing and some aspects of DNA replication and transcription [14]. Despite accumulating information about RecQL5, its cellular role, especially as a tumor suppressor, remains largely elusive.

Besides RecQ-related genome instability diseases, FA is another genome instability disease characterized by cancer predisposition, progressive bone marrow failure, and developmental abnormalities [15–18]. Cells lacking FA-related proteins show high sensitivity to DNA interstrand crosslink (ICL) damage-inducing agents such as cisplatin (CDDP) and mitomycin C (MMC). Sixteen genes have been identified as FA responsible genes, and their gene products function in the ICL repair pathway. Eight FA proteins (FANCA/B/C/E/F/G/L/M) and other proteins form the FA core complex. In response to replication stress during S phase, the FA core complex acts as an E3 ligase catalyzing the monoubiquitination of the FANCI-FANCD2 (ID) complex, and the monoubiquitinated-ID complex promotes downstream ICL repair reactions [15]. By contrast, six FA genes (*FANCD1*, *FANCJ*, *FANCN*, *FANCO*, *FANCP*, and *FANCQ*), which are also known as *BRCA2*, *BRIP1*, *PALB2*, *RAD51C*, *SLX4*, and *XPF*, respectively, are not involved in the monoubiquitination of the ID complex [16,19,20]. Among them, BRCA2, PALB2, and Rad51C, which are important factors for homologous recombination (HR), function in the recruitment of Rad51 to DNA damage sites and the formation of the Rad51-ssDNA filament [16,21]. In vertebrate cells, FA proteins and several others, including those involved in the replication checkpoint, nucleolytic incision, translesion synthesis (TLS), and HR, function in a variety of stages of ICL repair [15–18].

In the present study, analyses performed using various gene knockout DT40 cells suggested that RecQL5 is involved in FANCD1 (BRCA2)-dependent ICL repair and functions after the formation of the Rad51-filament by BRCA2, independently of another regulator of Rad51, Rad54. Loss of RecQL5 increased not only the frequency but also the variation of immunoglobulin gene conversion associated with ICL-inducing agents. The possible function of RecQL5 in regulating the quantity and quality of recombination resulting in tumor suppression *in vivo* will be discussed.

2. Materials and methods

2.1. Cell culture, DNA transfection, and RT-PCR

The chicken DT40 cells used in this study are listed in Supplementary Table S1. Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 1% chicken serum, 2 mM L-glutamine, 10 μM 2-mercaptoethanol and 100 μg/mL kanamycin in 5% CO_2 at 39 °C. DNA transfection and RT-PCR were performed as previously described [22]. Drug-resistant colonies were selected in 96-well plates with medium containing 1 mg/mL zeocin, 10 μg/mL mycophenolic acid, 0.5 μg/mL puromycin, 30 μg/mL blasticidin, or 1 mg/mL L-histidinol. Gene disruption was verified by genomic PCR and RT-PCR. The primers used in RT-PCR are listed in Supplemental Table S2. The targeting vectors for *RECQL5* gene disruption (based on pGEM-T Easy vector) are described in Supplementary Figure S1A. Due to the low transfection efficiency, each construct was digested with NdeI, and the linearized plasmids were used for transfection. The expression vector for human *RECQL5* was previously described [23].

2.2. Assessment of cell growth and sensitivity to DNA-damaging agents

Cell number was determined by flow cytometry using plastic microbeads and propidium iodide (PI). Cell solutions were mixed with the plastic microbead suspension at a rate of 4: 1, and viable cells were counted when a given number of microbeads were detected by flow cytometry. Cells not stained with PI were regarded as viable cells. To assess drug sensitivity, approximately 1×10^4 cells were cultured in 24-well plates containing various concentrations of DNA-damaging agents in 1 mL of medium in duplicate. Cell viability was assessed after 36–48 h by flow cytometry using plastic microbeads and PI (Liquid survival assay). The percent survival was determined by considering the number of untreated cells as 100%. The final concentration of PI was 1 μg/mL.

2.3. Cell cycle analysis by flow cytometry

For two-dimensional cell cycle analysis, cells were cultured in medium containing 1 μM CDDP and treated with 20 μM bromodeoxyuridine (BrdU; BD Biosciences) for 20 min just before harvesting. Cells were fixed in 70% ethanol, treated with 0.5% (v/v) TritonX and 2.5 M HCl, and stained with FITC-labeled anti-BrdU antibody (BD Biosciences) and 1 μg/mL PI. Cell cycle distribution was analyzed by flow cytometry.

2.4. Detection of chromosome aberrations and sister chromatid exchange

To analyze chromosome aberrations, cells were cultured in the presence of 50 ng/mL MMC for 16 h and then treated with colcemid for the last 135 min. Cells were harvested, treated with 75 mM KCl for 15 min, and then fixed with methanol-acetic acid (3:1) for 30 min. The cell suspension was dropped onto wet glass slides, air-dried, and stained with 3% Giemsa solution at pH 6.8 for 20 min and examined with a light microscope. To measure SCE, cells were cultured for two cycle periods in medium containing 10 μM BrdU and then treated with colcemid for the last 135 min. Cells were harvested and fixed as described above. To induce DNA damage, cells were treated with 200 nM CDDP for 8 h just before harvesting. Differential staining was performed as previously described [24].

2.5. Antibodies

The primary antibodies used were anti-chicken FANCD2 [25], anti-Rad51 (a kind gift from Dr. Hitoshi Kurumizaka, Waseda University) [26], anti-phospho-Chk1-Ser^{345} (Cell Signaling), anti-α-tubulin clone DM1A (Sigma), anti-HA clone 3F10 (Roche Applied Science), and anti-Histone H3 ab1791 (Abcam).

2.6. Western blotting

Western blotting was carried out as previously described [27]. The secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit, anti-rat, and anti-mouse IgG (Cell Signaling). Proteins were visualized using ECL prime Western blotting detection reagents (GE Healthcare). Images were captured with an ImageQuant LAS 3000 mini.

2.7. Observation of subnuclear focus formation

After MMC exposure (500 ng/mL, 6 h or indicated periods), cells were harvested and spun onto glass slides using a Cytospin. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% (v/v) NP40 in PBS, and treated with anti-chicken FANCD2 or anti-Rad51 antibodies. Alexa-Fluor 488 goat anti-rabbit IgG (Molecular Probes) was used as secondary antibody and 0.1 μg/mL DAPI was used for counterstaining. We only scored Rad51-foci positive and negative cells with non-apoptotic nuclei. Images were captured with a fluorescent microscope (DM5500B; Leica and BZ-9000; KEYENCE).

2.8. Measurement of immunoglobulin gene conversion frequency

The rate of generation of surface IgM (sIgM) gain variants was monitored as previously described [28]. Cells were treated with 250 nM CDDP every other day, stained with R-Phycoerythrin (R-PE)-conjugated anti-chicken IgM (Southern Biotech), and then analyzed by flow cytometry.

2.9. Nucleotide sequence analysis of rearranged IgVλ segments derived from sIgM-positive cells

Cell sorting was performed using the MiniMACS Starting Kit and Anti-PE MicroBeads (Miltenyi Biotech). Cells were separated into sIgM-positive and negative cell fractions, and genomic DNA for each sample was extracted with PrepMan Ultra (Applied Biosystems). The sorted cells were routinely tested for purity of enrichment by flow cytometry and sorting was repeated until 90% or more cells were sIgM-positive. Nucleotide sequence analysis was performed as described previously [28].

3. Results

3.1. RecQL5 is involved in ICL repair

To elucidate the cellular function of RecQL5, *RECQL5* knock out (KO) DT40 cells were generated using the CL18 subline as the parental cell line. Gene targeting constructs designed to delete exons 3 and 4, including helicase motif Ia, were sequentially transfected into DT40 CL18 cells. *RECQL5* gene disruption was confirmed by genomic PCR and RT-PCR (Supplementary Fig. S1). As previously reported [12,24], lack of *RECQL5* gene function did not affect cell growth (see also Supplementary Fig. S5B).

To examine the involvement of RecQL5 in DNA repair, we initially tested the sensitivity of *RECQL5* KO cells to a variety of DNA-damaging agents. *RECQL5* KO cells were sensitive to the DNA-crosslinking agents cisplatin (CDDP) and mitomycin C (MMC), whereas no sensitivity to camptothecin (CPT), etoposide, hydroxyurea (HU), or X-ray was detected compared with wild-type cells (Supplementary Fig. S2). Complementation of the CDDP and MMC sensitivity of *RECQL5* KO cells by human *RECQL5-FLAG* (Fig. 1A) indicated that the sensitivity was intrinsically caused by the loss of RecQL5. Cell cycle distribution in response to CDDP treatment was visualized by two-dimensional cell cycle analysis using flow cytometry. The proportion of cells in G_1 and S phase was decreased, and that of cells in G_2/M and subG_1 phases, which corresponds to dead cells, was increased in *RECQL5* KO cells compared with wild-type cells (Fig. 1B). Moreover, MMC-treated *RECQL5* KO cells showed a higher number of chromosome aberrations than wild-type cells (Fig. 1C). The loss of factors such as FA proteins, which are involved in ICL repair, leads to the induction of chromosome aberrations by MMC treatment [15,29], suggesting that RecQL5 is involved in ICL repair.

3.2. RecQL5 KO cells show Fanconi anemia pathway and replication checkpoint activation

ICL repair is achieved through a complex mechanism that includes several stages. To identify the specific stage of ICL repair in which

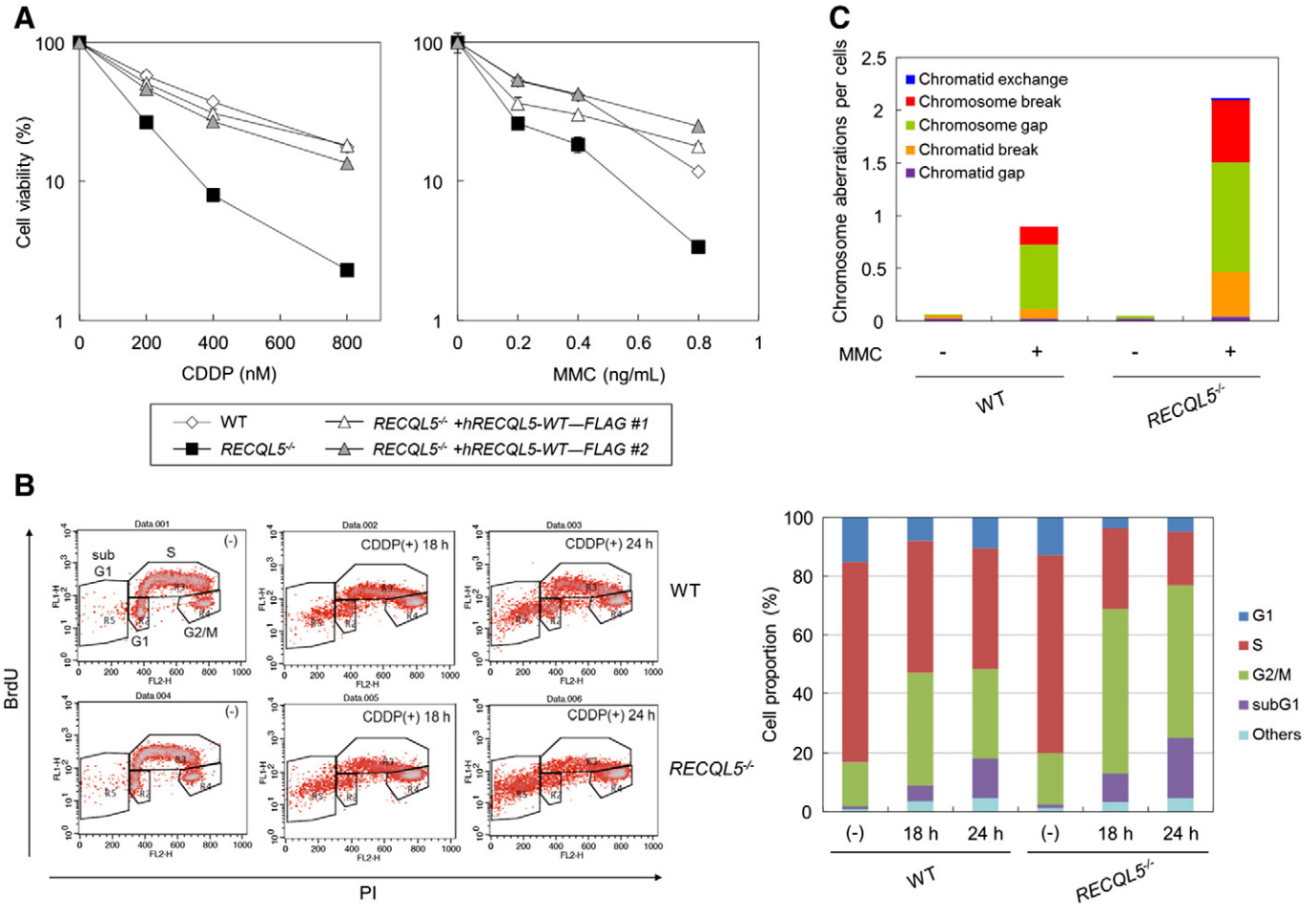

Fig. 1. ICL repair defect in *RECQL5* KO cells. (A) Liquid survival assay. Cells were incubated for 48 h in medium containing cisplatin (CDDP) or mitomycin C (MMC). The error bars indicate standard deviation (SD). (B) Cell cycle analysis. Cell cycle distribution of cells treated with 1 μM CDDP was analyzed by flow cytometry (left) and quantitated (right). (C) Chromosome aberrations. Data are presented as the number of aberrations per 50 metaphases.

RecQL5 plays a role, the function of RecQL5 was examined in well-known ICL repair-related reactions. First, to examine the involvement of RecQL5 in the FA pathway, we generated *RECQL5*/*FANCC* double KO cells (Supplementary Fig. S1C and S3A). FANCC is a component of the FA core complex, and lack of FANCC causes a defect in the FA pathway [16]. *RECQL5*/*FANCC* KO cells grew more slowly than either *RECQL5* or *FANCC* single KO cells (data not shown) and showed a higher proportion of dead cells (Supplementary Fig. S3B). Furthermore, *RECQL5*/*FANCC* KO cells showed higher sensitivity to CDDP than single mutants (Fig. 2A). Monoubiquitinated FANCD2, which is detected as a slow-migrating FANCD2 (FANCD2-L) band and is an indicator of FA pathway activation, was present in *RECQL5* KO cells but not in *FANCC* KO cells (Fig. 2B). Moreover, efficient FANCD2 focus formation was observed in the nuclei of *RECQL5* KO and wild-type cells (Fig. 2C). These results suggest that the FA pathway is normally activated even in the absence of RecQL5.

Next, to examine the involvement of RecQL5 in the replication checkpoint, we generated *RECQL5*/*RAD17* double KO cells (Supplementary Fig. S1C and S4A). Rad17 promotes Chk1 phosphorylation, which is a marker of replication checkpoint activation, in association with Rad9-Rad1-Hus1, TopBP1, and ATR-ATRIP, whereas it does not affect the monoubiquitination of FANCD2 [25,30]. *RECQL5*/*RAD17* KO cells showed higher sensitivity to CDDP than single mutants (Supplementary Fig. S4B). Moreover, phosphorylation of Chk1 was detected in *RECQL5* KO cells in the presence of CDDP, similar to wild-type cells (Supplementary Fig. S4C). These results suggest that activation of the replication checkpoint occurs in the absence of RecQL5.

3.3. The function of RecQL5 in ICL-induced HR repair is dependent on BRCA2

Parallel to the FA pathway, Rad51, a key factor for HR, is recruited to the ICL damage locus [31]. Rad51 interacts with RecQL5, as shown *in vivo* and *in vitro* [6,10,11,32]; therefore, we analyzed the functional relationship between RecQL5 and Rad51. One of the FA proteins, FANCD1 (BRCA2), is required for Rad51-filament formation. Although analysis of *RAD51* KO cells is difficult due to its lethality, *BRCA2* gene KO cells are viable and available for our experiments [33,34]. We generated *RECQL5*/*BRCA2* double KO cells (Fig. 3A) by using $BRCA2^{-/+}$ cells as the parental cell line [33], and converted these to $BRCA2^{-/-}$ null KO cells by exposure to 4-hydroxy tamoxifen (OH-TAM) (Fig. 3A). After re-cloning, $BRCA2^{-/-}$ and $RECQL5^{-/-}/BRCA2^{-/-}$ cells were selected by RT-PCR (Supplementary Fig. S5A). $RECQL5^{-/-}$, $BRCA2^{-/+}$, and $RECQL5^{-/-}/BRCA2^{-/+}$ cells showed the same proliferative capacity as wild-type cells, whereas $BRCA2^{-/-}$ cells had a lower proliferative capacity. $RECQL5^{-/-}/BRCA2^{-/-}$ cells showed a low proliferative capacity similar to that of $BRCA2^{-/-}$ cells (Supplementary Fig. S5B). $RECQL5^{-/-}/BRCA2^{-/+}$ cells were more sensitive to CDDP than $BRCA2^{-/+}$ cells, and $BRCA2^{-/-}$ and $RECQL5^{-/-}/BRCA2^{-/-}$ cells were more sensitive than $RECQL5^{-/-}/BRCA2^{-/+}$ cells (Fig. 3B). Importantly, the sensitivities of $BRCA2^{-/-}$ and $RECQL5^{-/-}/BRCA2^{-/-}$ cells were almost the same. This phenotype of *RECQL5*/*BRCA2* KO cells is obviously different from that of *RECQL5*/*FANCC* or *RECQL5*/*RAD17* KO cells, suggesting that RecQL5 works in the same pathway as BRCA2, but in different pathways as FANCC and RAD17.

As reported, Rad51-foci representing chromatin loading were strongly induced by MMC treatment in wild-type cells [26] but not in $BRCA2^{-/-}$ cells (Fig. 3C). Under the same conditions, Rad51-foci were observed in $RECQL5^{-/-}$ cells but not in $RECQL5^{-/-}/BRCA2^{-/-}$ cells (Fig. 3C). These results indicated that Rad51 chromatin loading after damage induction occurred in a BRCA2-dependent manner regardless of the presence or absence of RecQL5. This speculation was confirmed by an increase in Rad51 association to chromatin upon MMC treatment in wild-type and $RECQL5^{-/-}$ cells that was not observed in $BRCA2^{-/-}$ and $RECQL5^{-/-}/BRCA2^{-/-}$ cells (Supplementary Fig. S5C). Moreover, the increased incidence of SCE, which is an indicator of post-

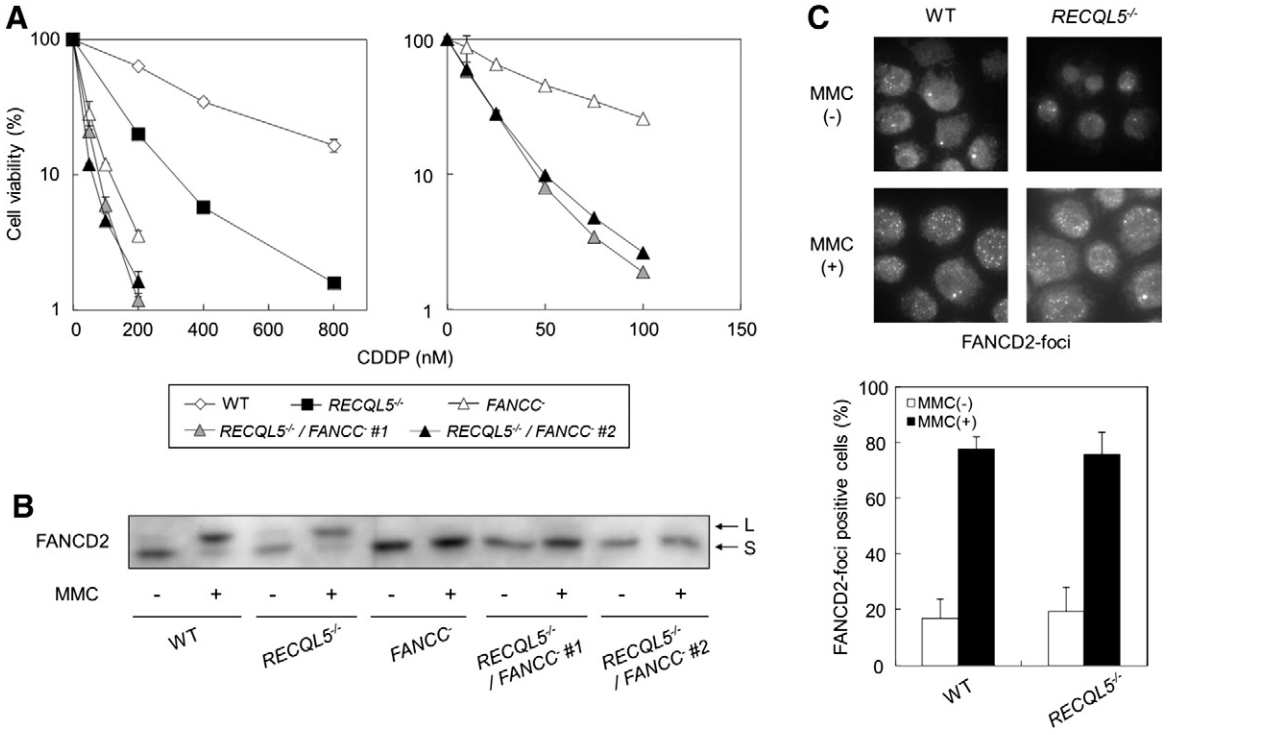

Fig. 2. Genetic analysis of *RECQL5* mutation combined with *FANCC*. (A) Liquid survival assay. Cells were incubated for 48 h in medium containing high (left) or low (right) concentrations of cisplatin (CDDP). The error bars indicate SD. (B) FANCD2 monoubiquitination. Cells were treated with 500 ng/mL mitomycin C (MMC) for 6 h and analyzed by Western blotting. L and S indicate ubiquitin-conjugated FANCD2 and FANCD2, respectively. (C) FANCD2-foci formation. Upper panel, images of cells treated or not treated with 500 ng/mL MMC for 6 h. Lower panel, percentage of FANCD2-foci positive cells. The error bars indicate SD from two independent experiments. At least 100 nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive.

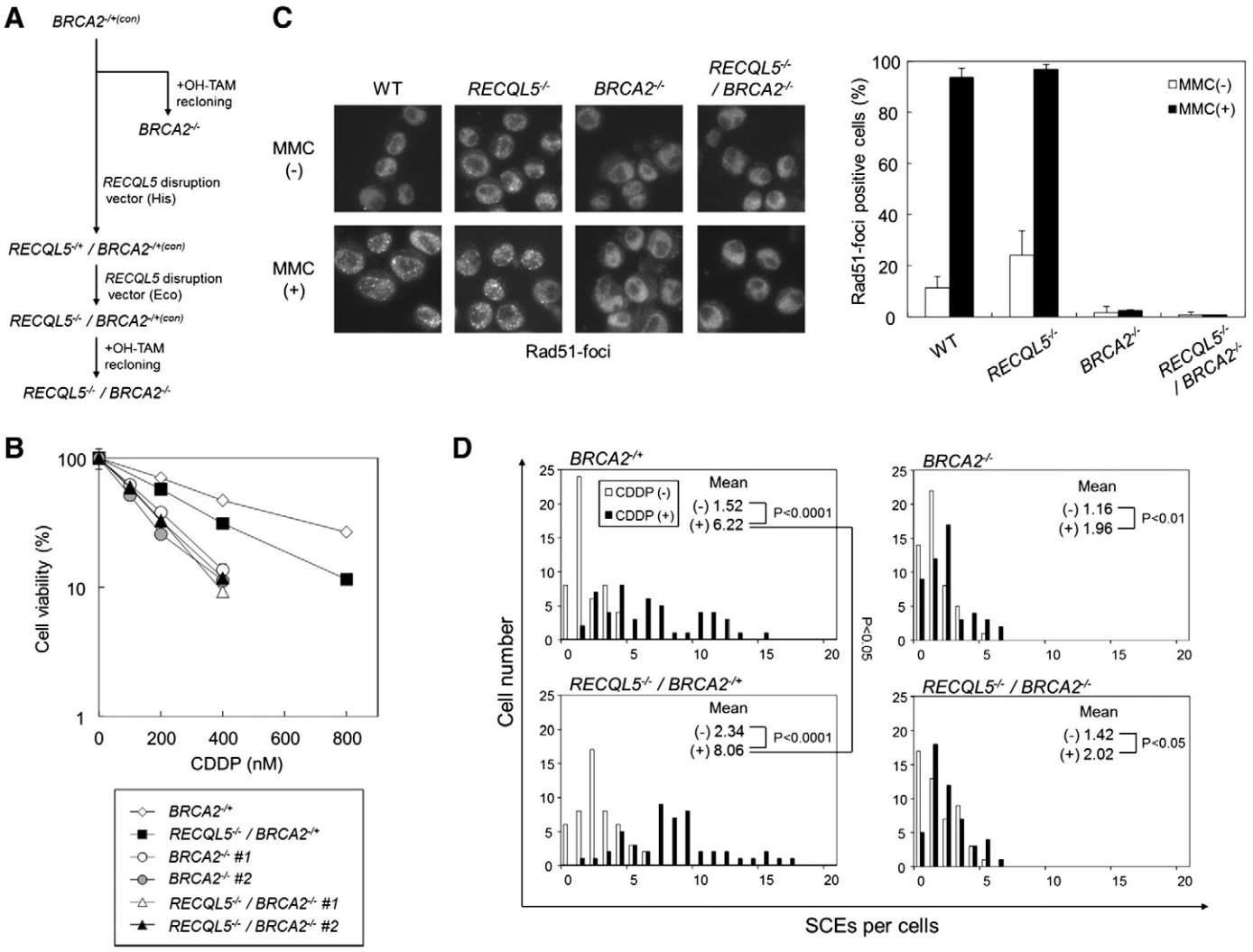

Fig. 3. Genetic analysis of *RECQL5* mutation combined with *BRCA2*. (A) Schematic representation of the gene targeting procedure. Cells were cloned after treatment with 20 nM 4-hydroxy tamoxifen (OH-TAM) for 2 days. (B) Liquid survival assay. Cells were incubated for 48 h in medium containing cisplatin (CDDP). The error bars indicate SD. (C) Rad51-foci formation. Left panel, images of cells treated or not treated with 500 ng/mL mitomycin C (MMC) for 6 h. Right panel, percentage of Rad51-foci positive cells. The error bars indicate SD from two independent experiments. At least 200 nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive. (D) Histograms of SCE. Numbers represent mean of scores from 50 metaphases. *P* values were calculated by Student's *t*-test.

replicative HR repair [35], observed in *BRCA2*$^{-/+}$ cells upon CDDP treatment was almost abolished in *BRCA2*$^{-/-}$ cells (Fig. 3D). *RECQL5*$^{-/-}$/*BRCA2*$^{-/+}$ cells exhibited a slight increase of CDDP-induced SCE compared with *BRCA2*$^{-/+}$ cells (Fig. 3D left). Spontaneous and CDDP-induced SCE were barely detected in *RECQL5*$^{-/-}$/*BRCA2*$^{-/-}$ and *BRCA2*$^{-/-}$ cells (Fig. 3D right). As *RECQL5* and *BRCA2* have an epistatic relationship with regard to ICL repair (Fig. 3B), the data of Fig. 3C and D suggest that RecQL5 is involved in ICL-induced HR repair in association with BRCA2. Additionally, we examined the significance of the interaction between RecQL5 and Rad51 for ICL repair. It was previously revealed that human RecQL5-T668A mutant attenuated to bind to Rad51 [10]. We generated *RECQL5*$^{-/-}$ cells expressing human RecQL5-T668A, and assayed the sensitivity of the cells to CDDP. The expression of the human RecQL5-T668A mutant did not complement the CDDP sensitivity caused by loss of RecQL5 (Supplementary Fig. S6). This result suggests that the interaction between RecQL5 and Rad51 is important for the function of RecQL5 in ICL repair.

3.4. RecQL5 functions in a relatively late step of ICL repair

Monoubiquitination of FANCD2, Chk1 phosphorylation, and Rad51-focus formation were normally induced at relatively early time points after exposure to ICL agents in *RECQL5* KO cells, similar to wild-type cells (Figs. 2B, 3C and Supplementary Fig. S4C), indicating that the absence of RecQL5 has little impact on the early steps of ICL repair. We next examined the effect of lack of RecQL5 on the late steps of ICL repair by monitoring phenomena that occur during the late stages of the damage response after MMC treatment (Fig. 4A). In *RECQL5* KO cells, the timing of the appearance of Rad51-foci in response to MMC treatment was similar to that of wild-type cells (Fig. 4B, 0–8 h). By contrast, the disappearance of Rad51-foci was delayed in *RECQL5* KO cells compared with wild-type cells (Fig. 4B, 8–36 h). At these time points, the proportion of viable cells was more than 60% (Supplementary Fig. S7), and apparently dead cells having apoptotic nuclei were not scored. Thus, it is likely that the persistent Rad51-foci phenotype reflects the phenomenon in viable cells. The sustained presence of Rad51-foci in *RECQL5* KO cells upon MMC exposure has two possible explanations. One is that the amount of DNA damage caused by ICL-inducing agents is increased to a greater extent in *RECQL5* KO cells than in wild-type cells, leading to the prolonged existence of Rad51-foci. The other possibility is that RecQL5 is necessary for the late step of ICL repair. If the former is correct, *RECQL5*/*BRCA2* KO cells would be expected to exhibit higher sensitivity to ICL damage than *RECQL5* KO cells because the increased DNA damage caused by the absence of RecQL5 may be repaired by HR involving

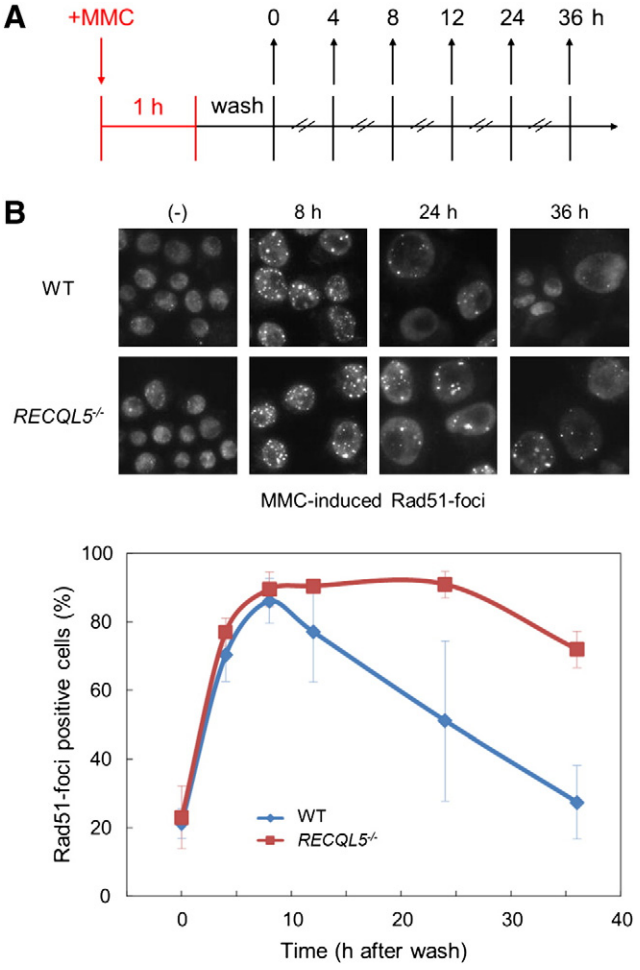

Fig. 4. Change of DNA damage signals in cells. (A) Schematic representation of sampling time after mitomycin C (MMC) (500 ng/mL) exposure. (B) Rad51-foci formation. Upper panel, images of cells treated with MMC. Lower panel, time course of the appearance of Rad51-foci positive cells. The error bars indicate SD from two independent experiments. At least 200 nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive.

BRCA2. Since sensitivity to ICL agents was similar between *RECQL5*/*BRCA2* KO cells and *BRCA2* KO cells (Fig. 3B), we suggest the latter possibility to be more likely.

3.5. Loss of both RecQL5 and Rad54 causes hypersensitivity to ICL agents and Rad51-foci accumulation

Rad54 functions after Rad51-filament formation in HR repair [36]. Because RecQL5 displaces Rad51 from the Rad51-filament *in vitro*, it is interesting to examine *RECQL5*/*RAD54* double KO phenotypes. For this purpose, we generated *RECQL5*/*RAD54* double KO cells using conditional *RAD54 KO* cells (*RAD54*$^{-/-}$ + h*RAD54-HA* cells) as the parental cell line [37], in which expression of human RAD54-HA protein is suppressed by doxycyclin (Dox) treatment, because of the possibility of synthetic lethality (Fig. 5A). The disruption of both alleles of the *RECQL5* gene in *RAD54*$^{-/-}$ + h*RAD54-HA* cells was confirmed by assessing for the disappearance of *RECQL5* mRNA (Supplementary Fig. S8A). The disappearance of hRad54-HA proteins after Dox treatment was verified with an anti-HA tag antibody (Supplementary Fig. S8B). *RECQL5*/*RAD54* KO cells did not show synthetic lethality, but grew at a slower rate than either *RECQL5* or *RAD54* single KO cells (Supplementary Fig. S8C). Cell cycle analysis demonstrated that *RECQL5*/*RAD54* KO cells had a tendency to accumulate in G_2/M phase (Supplementary Fig. S8D). *RECQL5*/*RAD54* KO cells showed higher sensitivity to CDDP than the single mutants (Fig. 5B). In addition, spontaneously formed Rad51-foci showed a greater increase in *RECQL5*/*RAD54* KO cells than in each single mutant, which had more spontaneous Rad51-foci than wild-type cells (Fig. 5C). Moreover, the disappearance of MMC-induced Rad51-foci was slower in *RECQL5*/*RAD54* KO cells than in *RECQL5* or *RAD54* single KO cells (Fig. 5D), suggesting that RecQL5 and RAD54 work in parallel pathways to regulate RAD51 filament disassembly.

3.6. RecQL5 controls the frequency and variation of CDDP-induced gene conversion in the IgVλ locus

In DT40 cells, HR-mediated gene conversion constitutively occurs at the immunoglobulin V gene locus in a process referred to as immunoglobulin gene conversion (IgGC) [38,39]. IgGC uses one of 25 pseudo V (ΨV) gene segments located upstream of the V gene as a donor template for recombination between the DNA sequence and the V gene, resulting in immunoglobulin diversification (Fig. 6A). The cell line used in this study, CL18, does not express surface IgM (sIgM) on the cell membrane because of a frameshift mutation at the V locus in the immunoglobulin light chain (Vλ) (Fig. 6A and Supplementary Fig. S1C). Removing this frameshift mutation by IgGC leads to expression of sIgM on the cell membrane. Surface IgM$^-$ to IgM$^+$ conversion is called sIgM$^+$ gain, which is mainly caused by IgGC. Therefore, the frequency of IgGC can be determined indirectly by measuring the proportion of sIgM-positive cells. Interestingly, loss of BLM, another RecQ helicase, results in a decrease in IgGC frequency in DT40 cells [40]. On the other hand, loss of WRN or BLM leads to an increase in the frequency of class-switch recombination end joining in human B cells [41], indicating that RecQ helicases somehow regulate recombination at the Ig locus.

We first examined the effect of RecQL5 on the frequency of IgGC. Cells were cultured for 30 days under non-perturbed conditions, and then sIgM$^+$ gain was measured with 24 subclones. Under the non-perturbed condition, there was little difference in the average sIgM$^+$ gain between wild-type and *RECQL5* KO cells (Fig. 6B left). However, sIgM$^+$ gain was 6.5-fold higher in *RECQL5* KO cells than in wild-type cells after culture for 8 days in the presence of CDDP (Fig. 6B right). Importantly, sIgM$^+$ gain was not apparently induced by CDDP in wild-type cells (Fig. 6C). These results suggest that RecQL5 suppresses CDDP-induced IgGC. The increase of CDDP-induced sIgM$^+$ gain by the absence of RecQL5 is a novel phenotype that has never been reported in any other DT40 mutants.

Deamination of cytosine on the Ig V gene locus by activation-induced cytidine deaminase (AID) induces spontaneous IgGC [42]. Therefore, we determined whether CDDP-induced IgGC in *RECQL5* KO cells is dependent on the function of AID. We generated *RECQL5*/*AID* double KO cells (Supplementary Fig. S1C and S9A), confirmed that the sensitivity to CDDP in *RECQL5*/*AID* KO cells was the same as that in *RECQL5* KO cells (Supplementary Fig. S9B) and measured the frequency of sIgM$^+$ gain. In *RECQL5*/*AID* KO cells, the frequency of sIgM$^+$ gain was not increased by CDDP (Fig. 6C), suggesting that CDDP-induced IgGC in the absence of RecQL5 depends on AID function.

In addition to measuring the frequency of IgGC, nucleotide sequence analysis of the IgVλ locus in sIgM-positive cells provides information about the variation of IgGC. We therefore analyzed the nucleotide sequence at the IgVλ locus in sIgM-positive cells. In CL18 cells, one nucleotide is inserted in complementary-determining region 1 (CDR1) to generate a frameshift mutation, and sIgM$^-$ to sIgM$^+$ conversion occurs by removing this insertion through IgGC (Fig. 6A). ΨV8, one of 25 ΨV gene segments, has the highest homology to the locus of the Vλ gene containing the insertion [43]. Therefore, ΨV8 is the most frequently used donor template for IgGC among the ΨV gene segments. Under non-perturbed conditions, the ΨV8 segment was used in 100% of the IgGC events in both wild-type and *RECQL5* KO cells (Fig. 6D left). By contrast, in the presence of CDDP, the usage of ΨV segments other than ΨV8 was increased to 22.4% in wild-type cells (Fig. 6D right), although IgGC was not increased by CDDP in wild-type cells (Fig. 6B and C).

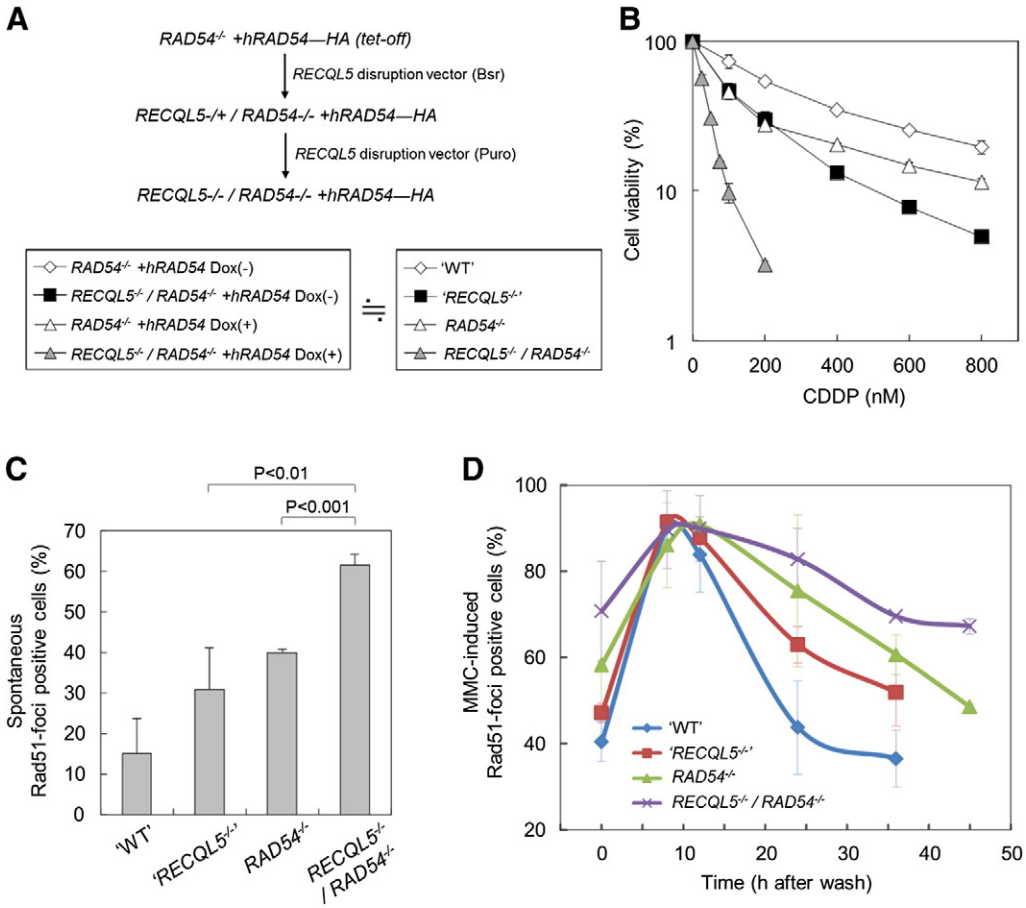

Fig. 5. Genetic analysis of *RECQL5* mutation combined with *RAD54*. (A) Schematic representation of the gene targeting procedure. Cells were used after treatment with 1 μg/mL doxycyclin (Dox) for 5 days. (B) Liquid survival assay. Cells were incubated for 48 h in medium containing cisplatin (CDDP). The error bars indicate SD. (C) Spontaneous Rad51-foci formation. The error bars indicate SD from three independent experiments. At least 250 nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive. *P* values were calculated by Student's *t*-test. (D) Mitomycin C (MMC)-induced Rad51-foci formation. Cells were treated with MMC and washed as described in the figure legend for Fig. 4A, and then harvested at the indicated time points. The plots represent mean and SD from two independent experiments. At least 200 nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive.

Surprisingly, 54.3% of CDDP-induced IgGC used ΨV segments other than ΨV8 in *RECQL5* KO cells (Fig. 6D right), indicating that the donor usage was significantly diversified in *RECQL5* KO cells. These results suggest that RecQL5 regulates the frequency and variation (quality) of CDDP-induced IgGC.

4. Discussion

4.1. Role of RecQL5 in ICL repair

In the present study, we showed that *RECQL5* gene KO chicken DT40 cells showed specific sensitivity to the ICL damage-inducing agents CDDP and MMC. These results are in agreement with a previous study showing that *RECQL5* mutants of *Drosophila melanogaster* were more sensitive to CDDP than wild-type flies [32]. Moreover, RecQL5-foci formed after CDDP treatment co-localize with PCNA in human HeLa cells [9] and RecQL5 was reported to participate in psoralen induced ICL repair in human cells [44]. Taking into account the analyses of function of RecQL5 in fly [32], human [9,44], and chicken (this study), RecQL5 seems to function in ICL repair in many species.

The data presented in this study indicated that the function of RecQL5 in ICL-induced DNA repair is dependent on BRCA2, whereas the monoubiquitination of FANCD2 is performed by FA core-associated proteins (Figs. 2 and 3). The *FANCN* and *FANCO* gene products, PALB2 and Rad51C, associate with BRCA2, and formation of Rad51-foci is suppressed by the lack of PALB2 or Rad51C [21,45]. By contrast, Rad51-foci were efficiently formed and their disappearance was markedly delayed in the absence of RecQL5 (Fig. 4B), suggesting that RecQL5 functions in the step of ICL repair after Rad51-filament formation. Rad54 and the recently identified Mcm8–Mcm9 complex are also involved in the step of ICL repair after Rad51-filament formation [26,36]. However, ICL-induced recombination was increased in *RECQL5* KO cells (Figs. 3D and 6B), whereas various ICL repair-related gene KO cells such as FA core complex, Rad51 paralogs, Rad54, and Mcm8–Mcm9 reportedly show decreased ICL-induced recombination [16,26,36]. Taken together, these data indicate that RecQL5 negatively regulates recombination during ICL repair.

Recently, a mechanism of ICL repair was proposed by analyzing a cell-free ICL repair system using *Xenopus* egg extracts and ICL-containing plasmids [31,46,47]. The proposed model of replication-coupled ICL repair is as follows (Fig. 7A): (i) dual replication forks approach and arrest at ICL sites, and RPA binds a ssDNA gap on the lagging strand; (ii) Rad51 is recruited to RPA coated ssDNA before DSB formation by nucleolytic incision; (iii) the ID complex is monoubiquitinated, which may recruit nucleases SLX4 and XPF to unhook the ICL and allow TLS to occur; (iv) the DNA with a double-strand break is repaired by HR using a sister chromatid. In the present study, we aimed to determine the recombination step in ICL repair in which RecQL5 is involved. Our data indicate that RecQL5 functions after Rad51-filament formation. Furthermore, in prior studies, biochemical analyses have suggested that RecQL5 can dissociate Rad51 from the Rad51-ssDNA filament, inhibiting

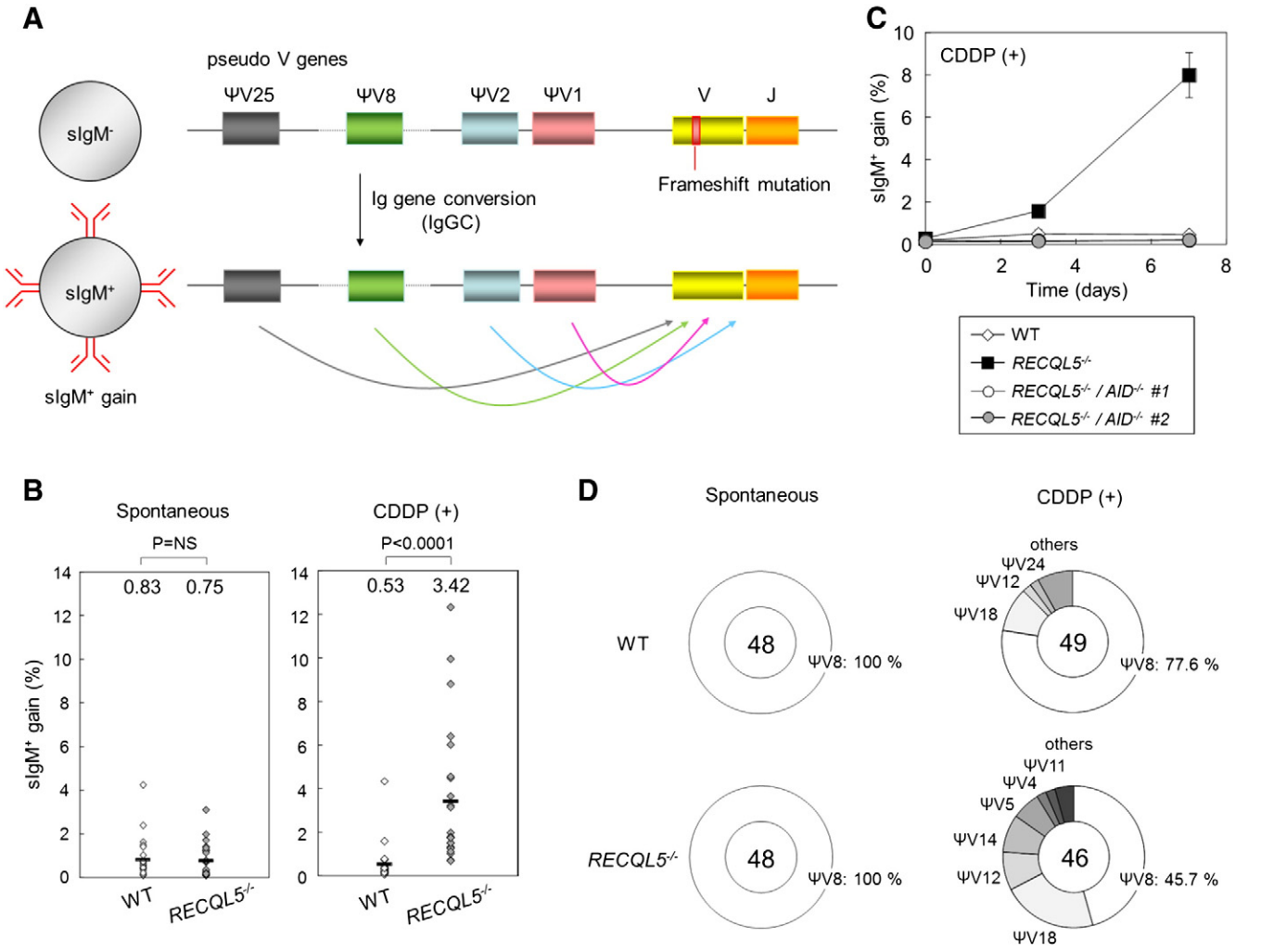

Fig. 6. Analysis of IgGC events at the light chain locus. (A) Schematic representation of $sIgM^-$ to $sIgM^+$ conversion. The detail is described in the text. (B) Fluctuation analysis of $sIgM^+$ gain. Cells were subcloned by limiting dilution ($n = 24$ for each genotype) and kept in culture in the absence of cisplatin (CDDP) for 30 days (left) or in the presence of CDDP for 8 days (right) after subcloning. Diamonds represent percentage of sIgM-positive cells in each clonal subpopulation. Lines and numbers indicate mean of % of $sIgM^+$ gain. *P* values were calculated by Student's *t*-test. NS, statistically not significantly different. (C) Time course of $sIgM^+$ gain. Cells were cultured in medium containing CDDP and the proportion of $sIgM^+$ cells was measured at the time indicated. The error bars indicate SD from two independent experiments. (D) Usage of ΨV genes as a donor for IgGC. Surface IgM-positive fractions were isolated form cells used in Fig. 6B, and the nucleotide sequences of rearranged Vλ genes were analyzed. The total number of Vλ sequences analyzed is indicated in the center of the charts.

D-loop formation [6]. Taken together, these findings indicate that RecQL5 acts on the Rad51-filament before strand invasion (pre-synaptic filament) rather than the filament acting on the D-loop (post-synaptic filament). According to the model, two pre-synaptic filaments are assumed to be possible targets of RecQL5 (Fig. 7A). One is the Rad51-filament formed on the DNA possessing crosslink, which inhibits extension of DNA synthesis after TLS. RecQL5 may function to disrupt the Rad51-filament and promote DNA synthesis. Another possible target is twin Rad51-filament formed on DNA with DSBs. When one Rad51-filament invades a sister chromatid and forms a D-loop structure, another Rad51-filament that is simultaneously formed is unnecessary for the repair process and becomes harmful. RecQL5 may prevent the formation of intricate recombination intermediates by removing the unnecessary Rad51-filament. Although these possibilities are not mutually exclusive, we prefer the latter explanation. If another Rad51-filament is retained because of loss of RecQL5 function, a double Holliday junction (dHJ) is subsequently formed by second end capture or strand invasion to a chromatid other than the sister chromatid containing homologous sequences is induced, leading to a decrease of DNA repair efficiency and an increase of improper recombination (Fig. 7B). This interpretation is not in conflict with the increase of SCE and chromosome aberrations in *RECQL5* KO cells (Figs. 1C and 3D).

4.2. RecQL5 and Rad54 act on different aspects of the Rad51-ssDNA filament

Rad54 plays a role in HR after pre-synaptic filament formation to help strand invasion of the Rad51-filament and to promote branch migration at the D-loop [36]. Since these functions are defective in *RAD54* KO cells, it is likely that *RAD54* KO cells show decreased ICL repair ability and increased Rad51-foci formation associated with the accumulation of pre-synaptic filament. Previously, *BRCA2*/*RAD54* double KO cells showed high sensitivity to CDDP similar to BRCA2 KO cells [33], implying that Rad54 functions after BRCA2 dependent Rad51-filament formation in ICL repair. We found that *RECQL5*/*RAD54* double KO cells were more sensitive to CDDP and accumulated more Rad51-foci than single KO cells (Fig. 5). These phenotypes can be explained by the defects in Rad54 function described above and the removal of unnecessary Rad51-filament by RecQL5 as described in the previous section. Interestingly, *KU70* KO DT40 cells defective in non-homologous end joining (NHEJ) show similar sensitivity to CDDP to that of wild-type cells, whereas *RAD54*/*KU70* double KO cells show higher sensitivity than *RAD54* single KO cells [48]. This finding suggests that some DSBs formed during ICL repair are repaired by NHEJ to complement the defect of HR resulting from the loss of Rad54 (Supplementary

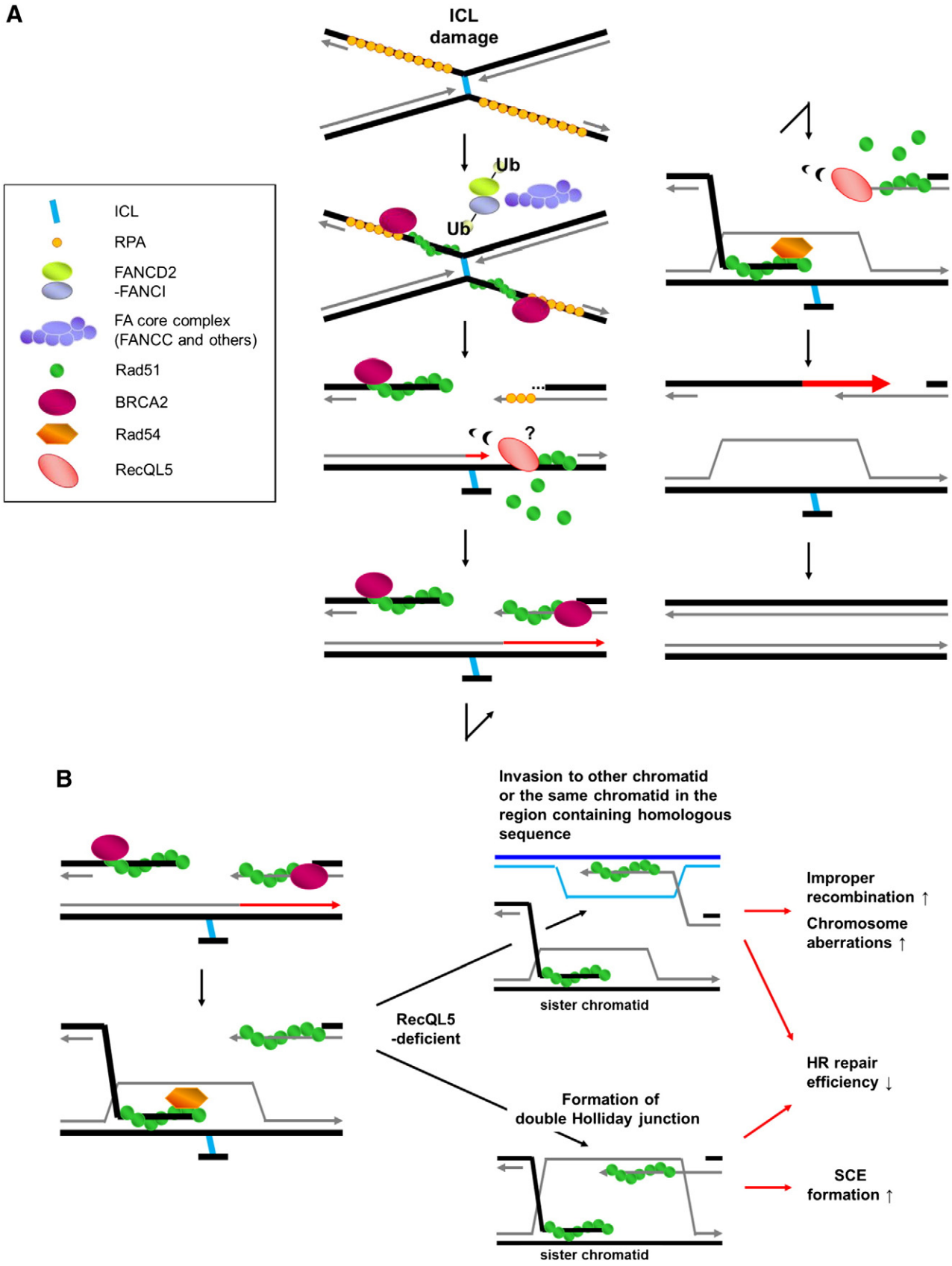

Fig. 7. Model of ICL repair including RecQL5. The detail is described in the Discussion section.

Fig. S10). RecQL5 might play a role in canceling HR by destroying the pre-synaptic filament, whose processing is difficult in *RAD54* KO cells, and promoting NHEJ or other repair pathway. Alternatively, the loss of RecQL5 could induce transcription-associated recombination [49,50] by creating a CDDP-dependent transcription block, and accumulation of excessive recombination intermediates in *RECQL5*/*RAD54* double KO

cells could cause cell toxicity. These possibilities will be investigated in a future study.

4.3. Relation between RecQL5 function and cancer

In the present study, we showed that donor usage in CDDP-induced IgGC was diversified in *RECQL5* KO cells (Fig. 6D), suggesting that RecQL5 affects the quality of recombination by suppressing the usage of a low homology locus as the donor. Although the result concerning IgGC in the Ig locus does not necessarily extend to whole genome events, it may be associated with the cancer predisposition phenotype of *Recql5* KO mice [6,7] if the endogenous ICL damage is processed by RecQL5. Recent studies showed that FA proteins function in damage tolerance to formaldehyde and acetaldehyde [51–55], which are generated in the cell and cause ICL and DNA-protein crosslinks. Therefore, RecQL5 may contribute to the repair of aldehyde-induced ICL damage, similar to FA proteins.

In summary, our systematic analyses using DT40 cells suggest that RecQL5 controls the frequency and quality of ICL-induced recombination to prevent genome instability. Although no clinical syndrome has been associated with defects in *RECQL5* up to present, it is not surprising if Bloom syndrome-like or Fanconi anemia-like genetic disease will be found, which is caused by the abnormality of RecQL5. RecQL5 was found to be overexpressed in mesothelioma in association with resistance to carboplatin, a platinum-containing drug [56]. RecQL5 could be involved in the mechanism of tolerance to carboplatin by promoting ICL repair in mesothelioma, in which case the down-regulation of RecQL5 expression may provide information to counteract the tolerance to ICL agents. Elucidation of the function of RecQL5 could shed light on the mechanisms of tumorigenesis and thus help overcome the drug resistance of tumor cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.01.005.

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