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Analyses of functional interaction between RECQL1, RECQL5, and BLM which physically interact with DNA topoisomerase IIIα

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Abstract

RECQL1 and RECQL5 as well as BLM reportedly interact with TOP3α whose defect is lethal for the cell. Therefore in this study, we characterized *recql5*/*recql1*/*blm* triple mutants from DT40 cells to determine whether the triple mutants show a *top3α* disrupted cell-like phenotype. The triple mutants are viable. Moreover, both *blm*/*recql1* and *recql5*/*blm* cells, and *recql5*/*recql1*/*blm* cells grew slightly slower than *blm* cells, that is, triple mutant cells grew almost the same rate as either of the double mutant cells. The *blm* cells showed sensitivity to methyl methanesulfonate (MMS) and ultraviolet light (UV), about a 10-fold increase in sister chromatid exchange (SCE), and about a 3-fold increase in damage-induced mitotic chiasma compared to wild-type cells. The triple mutants showed the same sensitivity to MMS or UV and the same frequency of damage-induced mitotic chiasma compared to those of *blm* cells, indicating that unlike BLM, RECQL1 and RECQL5 play a little role in the repair of or tolerance to DNA damages. However, *recql5*/*blm* cells showed higher frequency of SCE than *blm* cells, whereas the *RECQL1* gene disruption had no effect on SCE in *blm* cells and even in *recql5*/*blm* cells.

Keywords: RECQL1; RECQL5; BLM; RECQ; DT40

1. Introduction

Five genes encoding RecQ helicase homologues have been identified in human cells, and three are the causative genes for Bloom syndrome (BLM), Werner syndrome (WRN), and Rothmund–Thomson syndrome (RTS) [1–3]. Bloom syndrome is a rare genetic disorder characterized by retarded growth, sunlight sensitivity, immunodeficiency, male infertility, and a predisposition to a wide variety of malignant tumors [4]. The most characteristic feature of BS cells is genomic instability, which is manifested as an elevated frequency of chromosome breaks, interchange between homologous chromosomes, and sister chromatid exchange (SCE) [4]. Mutations in the *WRN* and *RTS* (*RECQL4*) genes have been found in patients with Werner syndrome and Rothmund–Thomson syndrome that are rare genetic disorders characterized by premature aging and a predisposition to specific types of cancer [5,6]. Cells containing mutations in either *WRN* or *RTS*, also show genomic instability [5,6]. The two remaining RecQ homologues, RECQL1 and RECQL5 [7–9] have not been linked to any disease.

Before cloning a cDNA encoding the RECQL1 [7], we purified this protein and designated it as DNA-dependent ATPase Q1 [10] and later as DNA helicase Q1 because of its DNA helicase activity [11]. Puranam and Blackshear named the gene encoding this protein as *RECQL* [8]. To eliminate confusion over the nomenclature of the multiple RecQ homologues, we proposed to designate this gene *RECQL1* [12]. The fourth and fifth *RECQ* homologues, originally called *RecQ4* and *RecQ5* and recently called *RECQL4* and *RECQL5*, were cloned in 1998 after a search for sequences similar to the RecQ helicase motifs in the EST (expressed sequence tag) database [9].

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Although little is known about the functions of RECQL1 and RECQL5 in the cell, biochemical analyses of these proteins revealed that they have DNA helicase activity [11–14] and strand annealing activity like BLM [15,16]. RECQL1 was found as a Holliday junction processing enzyme [17] and RECQL5 was shown to promote strand exchange on synthetic DNA structure [18]. In addition, RECQL1 and RECQL5 as well as BLM interact with DNA topoisomerase IIIα (TOP3α) [19,20]. Recently, several studies to pursue the functions of RECQL1 and RECQL5 have been performed using a variety of model cell lines including human cells, mouse embryonic fibroblasts and ES cells, fruit fly, and chicken DT40 cells. We previously generated and characterized *recql1*, *recql5*, *recql5*/*recql1*, *blm*/*recql1*, and *recql5*/*blm* cells from the chicken B lymphocyte line, DT40 cells [21]. Although *blm* DT40 cells showed a slow growth phenotype, a higher sensitivity to methyl methanesulfonate (MMS), and an increase in the frequency of sister chromatid exchange (SCE) compared with wild-type cells, *recql1*, *recql5* and *recql5*/*recql1* cells were not significantly different from the wild-type cells in cell growth, sensitivity to MMS, and the frequency of SCE. We have found that both *blm*/*recql1* and *recql5*/*blm* cells grew slower than *blm* cells because of the increase in the population of dead cells, indicating that RECQL1 and RECQL5 play some roles for viability in *blm* cells. In addition, *recql5*/*blm* cells had a higher frequency of SCE than *blm* cells, suggesting that RECQL5 suppresses SCE when BLM-function is impaired.

Furthermore, we previously generated and characterized the cells whose expression of TOP3α can be switched off to circumvent the *top3α* lethality phenotype [22]. TOP3α depletion caused accumulation of cells in the G_2 phase, enlargement of nuclei, and chromosome gaps and breaks that occurred at the same position in sister chromatids. The transition from metaphase to anaphase was also inhibited. In *blm*/*top3α* double mutant cells, all of these phenomena caused by TOP3α depletion except cell lethality were suppressed by *BLM* gene disruption. Considering that BLM, RECQL1, and RECQL5 interact with TOP3α, we hypothesized that deletion of BLM, RECQL1, and RECQL5 would result in cell lethality because of complete dysfunction of TOP3α. To examine this possibility and to detemine the functional relationship among BLM, RECQL1, and RECQL5, we generated *recql5*/*recql1*/*blm* triple gene knockout cells and compared it to corresponding double and single gene disrupted cells.

2. Materials and methods

2.1. Cell culture and generation of recql5/recql1/blm triple mutants

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum (Sigma, St. Louis, MO, USA), and 100 μg/ml kanamycin at 39.5 °C. *recql1*, *recql5*, *recql5*/*recql1*, *recql1*/*blm*, and *blm*/*recql5* cells were previously generated from chicken DT40 cells [21]. *recql5*/*recql1*/*blm* triple mutants were generated from *recql5*/*recql1* cells using *BLM* targeting constructs containing the drug resistance marker *histidinol* or *blastidin* [21,23]. For gene targeting, 10^7 DT40 cells were electroporated with 30 μg of linearized targeting constructs using a Gene Pulser apparatus (BioRad, Hercules, CA, USA) at 550 V and 25 μF. Drug-resistant colonies were selected in 96-well plates with medium containing 1 mg/ml histidinol or 20 μg/ml blastidin. Genomic DNA was isolated from drug-resistant cells. Gene disruption was confirmed by Southern blot and RT-PCR. We named multiple gene knockout cells according to the order of gene disruption as shown in Fig. 1A such as *recql5*/*recql1*/*blm*.

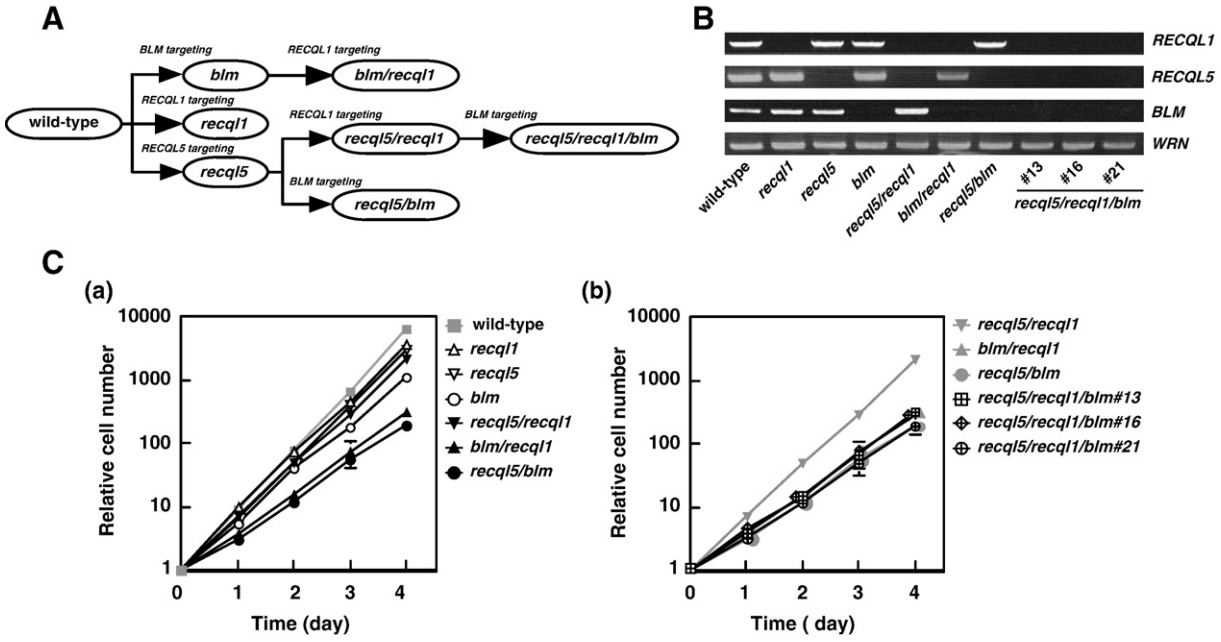

Fig. 1. Generation of *recql5*/*recql1*/*blm* triple mutant cells and growth property of various *RECQL* genes disrupted cells. (A) Schematic representation of the generation of various *recql* mutants including *recql5*/*recql1*/*blm* triple mutants. (B) Confirmation of gene disruption by RT-PCR in *recql* mutants. (C) Growth rate of various *recql* mutants. #13, #16 and #21 are independently isolated *recql5*/*recql1*/*blm* triple mutant clones. Cells not stained with trypan blue were counted. The bar indicates standard deviation.

Table 1
DT40 strains used in this study

Genotype	Disrupted gene (selective marker[a])	Reference
wild-type		
recql1	*HPRT*$^{+/-}$ (Neo), *RECQL1* (His/Bsr)	[21]
recql5	*HPRT*$^{+/-}$ (Neo), *RECQL5* (His/Bsr)	[21]
blm	*HPRT*$^{+/-}$ (Neo), *BLM* (His/Bsr)	[23]
recql5/*recql1*	*HPRT*$^{+/-}$ (Neo), *RECQL5* (His/Bsr), *RECQL1* (Hyg/Bleo)	[21]
blm/*recql1*	*HPRT*$^{+/-}$ (Neo), *RECQL1* (His/Bsr), *BLM* (Hyg/Puro)	[21]
recql5/*blm*	*HPRT*$^{+/-}$ (Neo), *BLM* (Hyg/Puro), *RECQL5* (His/Bsr)	[21]
recql5/*recql1*/*blm*	*HPRT*$^{+/-}$ (Neo), *RECQL5* (His/Bsr), *RECQL1* (Hyg/Bleo), *BLM* (Eco/Puro)	This study

[a] Selective markers; Neo, neomycin; His, histidinol; Bsr, blasticidin; Hyg, hygromycin; Puro, puromycin; Eco, ecogpt; Bleo, bleomycin.

2.2. Growth rate

Cells were inoculated into 24-well plates at $1\times10^5\sim3\times10^5$ cells/ml and cultured at 39.5 °C for 4 days. To maintain exponential cell growth, cultures were diluted appropriately every day. The cells were counted and growth rates were determined.

2.3. Measurement of sensitivity to MMS and UV-irradiation

To determine sensitivity to MMS, $4\times10^2\sim6\times10^2$ cells were inoculated into 60 mm dishes containing various concentrations of MMS in a medium supplemented with 1.5% (w/v) methylcellulose, 15% fetal bovine serum, and 1.5% chicken serum. To determine sensitivity to UV light, 4×10^3 cells were suspended in 1 ml of PBS, inoculated in 6-well plates, and irradiated with various doses of UV. The $4\times10^2\sim6\times10^2$ cells were inoculated into 60 mm dishes containing a medium supplemented with 1.5% (w/v) methylcellulose, 15% fetal bovine serum, and 1.5% chicken serum. Colonies were enumerated after 7 to 10 days, and the percent survival was determined relative to the number of colonies of untreated cells.

2.4. Measurement of Spontaneous and UV-induced SCE

Cells (2×10^6) were cultured for two cycle periods with medium containing 10 μM BrdU and pulsed with 0.1 μg/ml colcemid for 2 h. The cells were harvested and treated with 75 mM KCl for 12 min at room temperature and then fixed with methanol-acetic acid (3:1) for 30 min. The cell suspension was dropped onto ice-cold wet glass slides and air-dried. The cells on the slides were incubated with 10 μg/ml Hoechst 33258 in phosphate buffer (pH 6.8) for 20 min and rinsed with MacIlvaine solution (164 mM Na_2HPO_4, 16 mM citric acid, pH 7.0). The cells were exposed to a black light (λ=352 nm) at a distance of 1 cm for 30 min and incubated in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) at 58 °C for 20 min and then stained with 3% Giemsa solution for 25 min.

To measure UV-induced SCE, cells were suspended in PBS, inoculated in 6-well plates, and irradiated with UV at 0.25 J/m^2. Next, those cells were cultured for two cycle periods with medium containing 10 μM BrdU and pulsed with 0.1 μg/ml colcemid for 2 h.

2.5. Detection of MMS- or UV-induced mitotic chiasma

Cells were cultured in the presence of 8×10^{-4} (%) MMS for 4 h and then transferred to fresh medium without MMS. The cells were then harvested after 12 h and stained with Giemsa. Two hundred first metaphase cells were analyzed in each case. In the case of UV-irradiation, samples were prepared after 8 h following irradiation with 8 J/m^2 UV. Two hundred metaphase cells were analyzed in each case. Cells were treated with 0.1 μg/ml colcemid for 2 h and harvested. The cells were treated with 75 mM KCl for 12 min at room temperature and then fixed with methanol-acetic acid (3:1) for 30 min. The cell suspension was dropped onto ice-cold wet glass slides and air-dried. The cells on the slides were stained with 3% Giemsa solution at pH 6.8 for 25 min and examined with a light microscope.

3. Results

3.1. Generation of recql5/recql1/blm triple mutant cells

The *recql1*, *blm*, *recql5*, *recql5*/*recql1*, *blm*/*recql1*, and *recql5*/*blm* cells had been constructed from chicken DT40 cells as described previously [21]. The *recql5*/*recql1*/*blm* triple mutant cells were constructed by transfecting two *BLM* targeting vectors sequentially into *recql5*/*recql1* double mutant cells (Fig. 1A). The cells used in this study are listed in Table 1 and disruption of genes in the mutants was confirmed by RT-PCR (Fig. 1B).

3.2. recql5/recql1/blm triple mutant grows at a similar rate to that of blm/recql1 or recql5/blm double mutant

We examined proliferation properties of various gene knockout cells. As we reported previously [21], *recql1*, *recql5*, and *recql5*/*recql1* cells proliferated at a similar rate to that of wild-type cells (Fig. 1Ca), suggesting that both RECQL1 and RECQL5 play no essential role in cell growth [21]. However, *blm* cells proliferated at a slightly slower rate than wild-type cells, and *blm*/*recql1* and *recql5*/*blm* cells proliferated more slowly than *blm* cells, that is, disruption of either *RECQL1* or *RECQL5* increases the proliferation deficiency observed in cells lacking a functional BLM as reported previously [21]. Since RECQL1, BLM, and RECQL5 interact with TOP3α [19,20], and depletion of TOP3α leads to cell death [22], we hypothesized that *recql5*/*recql1*/*blm* triple mutant should be lethal or grows extremely slowly. Surprisingly, we found that the *recql5*/*recql1*/*blm* triple mutant was viable, and it grew at a similar rate as *blm*/*recql1* or *recql5*/*blm* double mutant (Fig. 1Cb). These data indicate that disruption of *RECQL1* or *RECQL5* does not increase the proliferation deficiency observed in *blm*/*recql5* cells or *blm*/*recql1* cells.

3.3. Frequencies of spontaneous and UV-induced SCE in various RECQL mutants

The high incidence of sister chromatid exchange is a hallmark feature of BS cells [4]. Therefore, we examined the frequency of SCE in various *RECQL* mutants, which is highly increased in BS cells. The frequencies of spontaneous SCE in *recql1*, *recql5* and *recql5*/*recql1* cells were approximately equal to that of wild-type cells. In contrast, previous studies have shown that *Recql1*$^{-/-}$ mouse embryonic fibroblasts [24] and *Recql5*$^{-/-}$ mouse ES cells [25] show higher frequency of SCE than wild-type cells. Although the reason for a discrepancy between these cells and chicken DT40 cells is not clear at present, it may be due to species or cell type variation in the requirement of RECQLs. The disruption of *RECQL1* gene in *blm* cells did not affect the spontaneously elevated frequency of SCE in *blm* cells. In contrast, the frequency of spontaneous SCE in *recql5*/*blm* cells was increased by 1.7-fold over *blm* cells and 22-fold

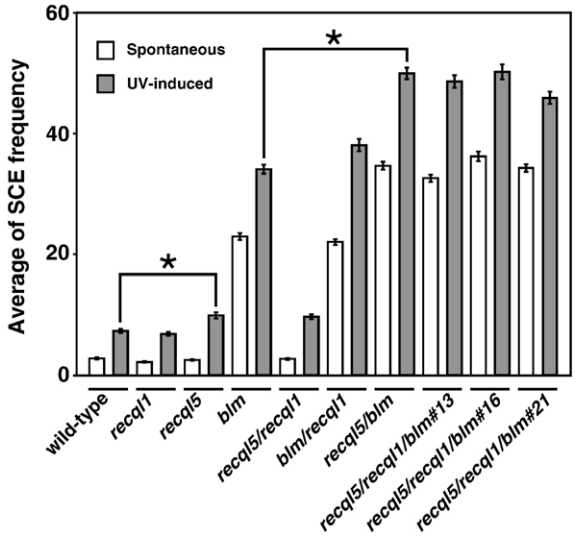

Fig. 2. Frequencies of spontaneous and UV-induced SCE in various *recql* mutants. The number of spontaneous and UV-induced SCE in the macro-chromosomes was counted with 100 metaphase cells of various *recql* mutants. The bar indicates standard error. There were statistically significant differences between UV-induced SCE in wild-type and that in *recql5* cells (Student's *t*-test $*p<0.001$), and between UV-induced SCE in *blm* and that in *recql5*/*blm* cells (Student's t-test $*p<0.001$).

over wild-type cells, respectively and these data are consisted with our previous findings [21]. The disruption of *RECQL1* gene in *recql5*/*blm* cells did not affect the spontaneously elevated frequency of SCE in *recql5*/*blm* cells (Fig. 2).

We next examined the frequencies of SCE in various *RECQL* mutants under damage-induced conditions by treating the cells with UV light. As shown in Fig. 2, the frequency of SCE in UV-irradiated *recql1* cells was similar to that of UV-irradiated wild-type cells. A slight increase in SCE was observed in both *recql5* and *recql5*/*recql1* cells compared with wild-type cells, suggesting that RECQL5 is important in the suppression of SCE after following UV-irradiation even in the presence of BLM. Increase in the SCE frequency by following UV-irradiation was observed in *blm*, *blm*/*recql1*, *recql5*/*blm*, and *recql5*/*recql1*/*blm* cells, and the SCE frequencies in *recql5*/*blm* and *recql5*/*recql1*/*blm* cells were slightly higher than that of *blm* cells. As observed during spontaneous SCE, the SCE frequencies in *recql5*/*blm* and *recql5*/*recql1*/*blm* cells following UV treatment were approximately equivalent, suggesting that RECQL1 does nothing in the suppression of SCE even after damage induction by UV in the absence of BLM and RECQL5.

3.4. Sensitivity of various RECQL mutants to DNA damaging agents

The sensitivity of various *RECQL* mutants to methylmethanesulfonate (MMS) and UV-irradiation was examined. As reported previously [21], *blm* cells showed a moderate sensitivity to MMS, while *recql1* and *recql5* single mutants and *recql5*/*recql1*

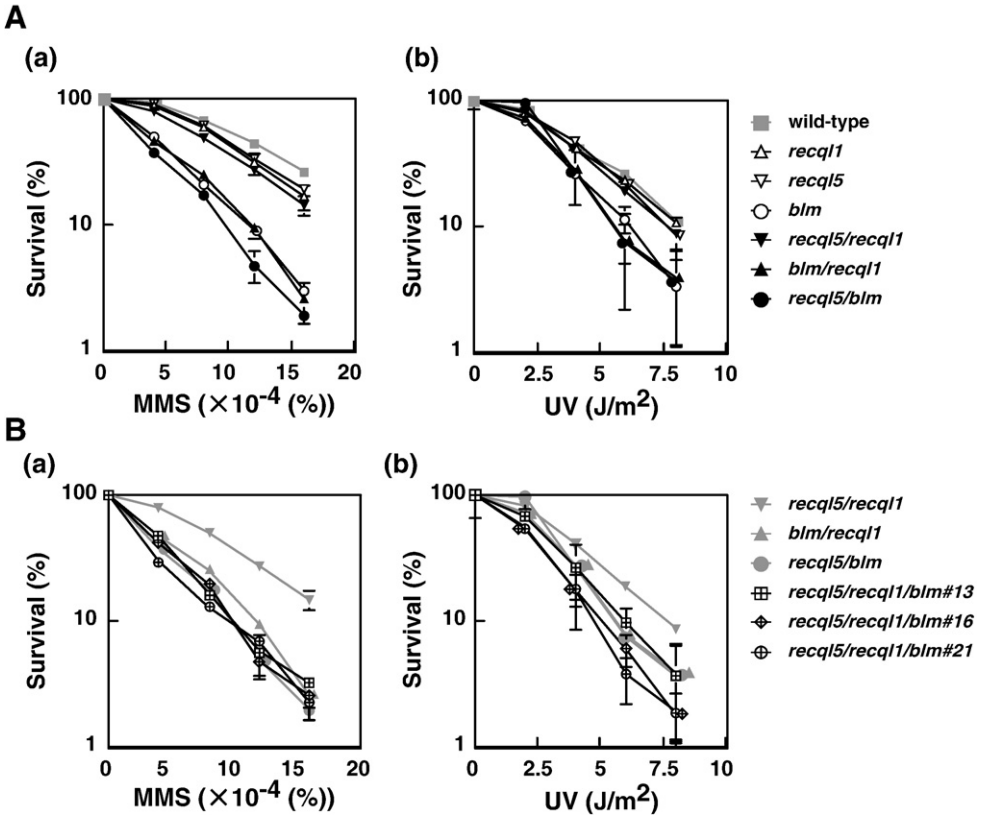

Fig. 3. Sensitivity of various *recql* mutants to MMS and UV. Cells were treated with the indicated concentration of MMS or irradiated with the indicated doses of UV as described in Materials and methods. The bar indicates standard deviation. In some cases, the standard deviation of each point is too small to see because it is within the symbol.

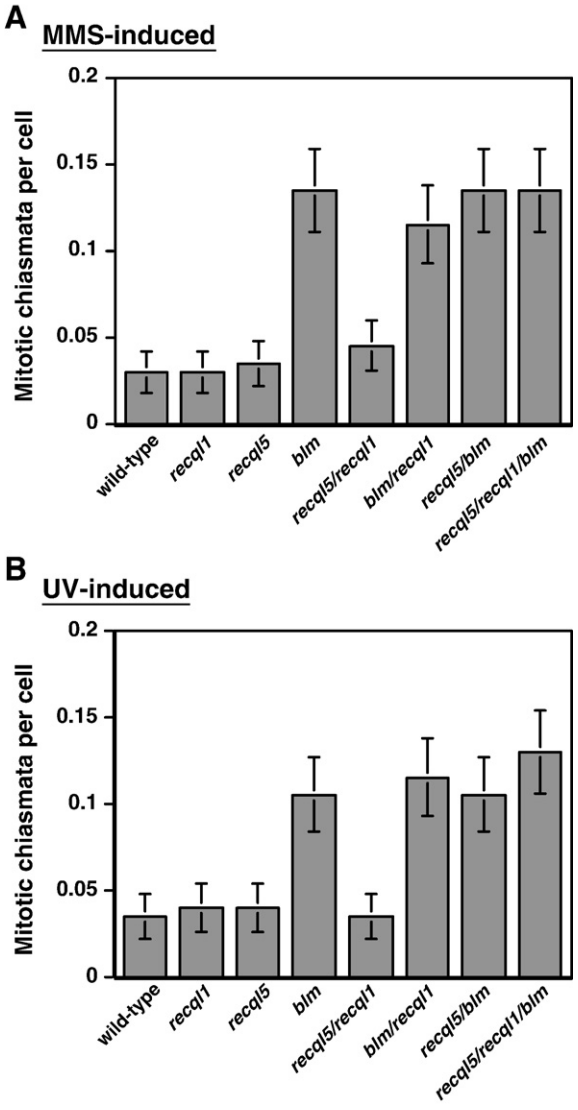

Fig. 4. Frequency of damage-induced mitotic chiasma in various *recql* mutants. (A) MMS-induced mitotic chiasma. (B) UV-irradiation-induced mitotic chiasma. Cells were treated as described in Materials and methods. The macro-chromosomes of 200 metaphase cells were monitored. The bar indicates standard error.

double mutant showed almost the same sensitivity to MMS or UV compared with that of wild-type cells (Fig. 3A and B). Although *blm*/*recql1*, *recql5*/*blm*, and *recql5*/*recql1*/*blm* cells showed a slightly greater growth defect than *blm* cells, the sensitivity of these mutants to MMS or UV, was almost equivalent to *blm* cells (Fig. 3). These data suggest that RECQL1 and RECQL5 cannot replace BLM upon induction of DNA damage even in the absence of BLM.

3.5. Deletion of RECQL1 or RECQL5 does not affect the increase of damage-induced mitotic chiasma in blm cells

Symmetrical quadriradial chromosomes that consist of homologous chromosomes, are often observed in BS cells [4]. This type of chromosome aberration is called mitotic chiasma because it resembles the chiasma structure seen in meiosis, which is formed by interchange between homologous chromosomes at homologous sites [26,27]. Consistent with these earlier studies, the frequencies of MMS- or UV-induced mitotic chiasma in *blm* cells were increased by about 3-fold compared to that of wild-type cells (Fig. 4A and B) [28]. In contrast, mitotic chiasma levels in *recql1*, *recql5* and *recql5*/*recql1* cells were similar to that of wild-type cells. Disruption of either *RECQL1* or *RECQL5* gene in *blm* cells did not affect the frequency of damage-induced mitotic chiasma observed in *blm* cells. Moreover, deletion of both *RECQL1* and *RECQL5* genes simultaneously also did not alter the frequency of mitotic chiasma in *blm* cells (Fig. 4).

4. Discussion

In our previous study using TOP3α depleted cells and TOP3α depleted *blm* cells [22], we suggested that BLM/TOP3α functions to dissolve sister chromatids at the late stage of DNA replication (Fig. 5A). Although disruption of *RECQL1* or *RECQL5* gene showed no effect on cell growth in wild-type cells, it caused slow growth in *blm* cells. Thus, it seems likely that RECQL1 and RECQL5 together with TOP3α partially replace the function of BLM in the dissolution of sister chromatids in *blm* cells (Fig. 5, closed arrows toward panel A). Very recently, it was reported that BLM-defective human cells display a higher frequency of anaphase bridges than do isogenic corrected derivatives that ectopically express the BLM protein [29]. Moreover, in normal cells undergoing mitosis, BLM protein localizes to anaphase bridges, where it colocalizes with its cellular partners, TOP3α and BLAP75 suggesting the role of BLM in ensuring complete sister chromatid decatenation in anaphase [29]. These data are consistent with our notion deduced from the data obtained by using TOP3α depleted cells and TOP3α depleted *blm* cells [22]. In this context, it is interesting to examine whether RECQL1 and RECQL5 also exist in anaphase bridges in the future study.

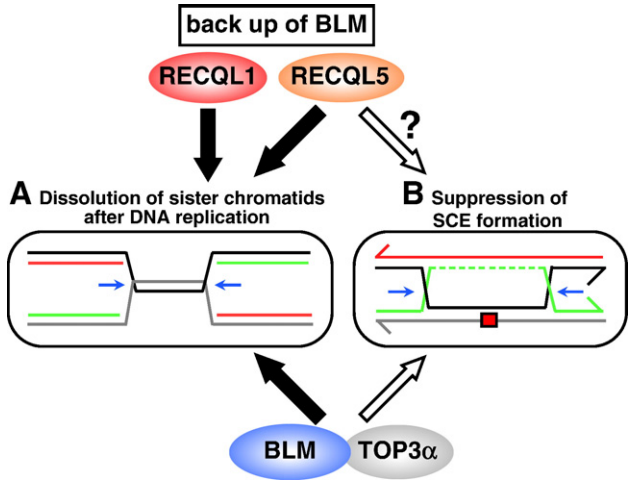

Fig. 5. Possible roles of RECQL1 or RECQL5 in BLM impaired condition. (A) A model for the dissolution of the structures arisen during DNA replication by BLM and TOP3α [22]. BLM and TOP3α process the termination intermediates that are arisen when replication forks converge. (B) Model for suppression of SCE by BLM/TOP3α. This model is based on the fact that purified BLM and TOP3α have activity to dissolve double Holliday junctions not to form crossover [30].

A characteristic feature of BS cells is a high incidence of SCE. A possible explanation for this property is that BLM with TOP3α dissolves double Holliday junctions in a manner that does not produce crossovers [30] (Fig. 5, open arrow toward panel B), while the BLM defect alone results in crossovers that are detected as SCE. Despite earlier findings that *Recql5*$^{-/-}$ mouse cells showed an increase in spontaneously occurring SCE [25], we did not observe this phenomenon in DT40 *recql5* cells. However, the spontaneously occurring SCE in *blm* cells was increased by disruption of *RECQL5* gene. In addition, *recql5* and *recql5*/*blm* cells showed a slightly higher frequency of SCE following UV exposure than wild-type cells and *blm* cells, respectively. Considering these results with the fact that RECQL5, like BLM, interacts with TOP3α [20], it is conceivable that RECQL5 also functions with TOP3α to dissolve double Holliday junctions in a manner that does not produce crossovers (Fig. 5, open arrow toward panel B) and that this interaction is especially critical in the absence of BLM. However, recent biochemical studies using purified RECQL5 and TOP3α seem to indicate that the combination of these proteins is not able to dissolve double Holliday junctions [31]. Since BLAP75, that associates with BLM and TOP3α, greatly stimulates the dissolution of Holliday junctions by BLM and TOP3α [32–34], it seems likely that in the presence of some missing factor, RECQL5 and TOP3α are able dissolve double Holliday junctions. Nonetheless, we cannot exclude the possibility that the increase of SCE by disruption of *RECQL5* is due to the increase in DNA lesions that cause SCE.

It must be noted that SCE frequency does not increase in *recql1* cells, and the SCE frequency in *recql5*/*recql1*/*blm* cells is approximately the same as that of *recql5*/*blm* cells, suggesting that RECQL1 does nothing in the suppression of SCE even in the absence of BLM and RECQL5. These results conflict with the previous findings indicating the involvement of RECQL1 in the suppression of SCE, that *Recql1*$^{-/-}$ embryonic fibroblasts show higher frequency of SCE than wild-type cells [24], and treatment of *RECQL1*siRNA increases SCE in HeLa cells [17]. These discrepancies may be due to the different requirements of individual RECQL between species or cell types.

Our systematic genetic analyses of various *RECQL* mutant cell lines, including *blm* cells suggested that BLM functions probably with TOP3α in a template-switching type of lesion bypass pathway which follows when cells are exposed to DNA damaging agents such as MMS and UV and suppresses the formation of mitotic chiasma [28]. The results shown in Figs. 3 and 4 indicate that RECQL1 and RECQL5 are not involved in these processes. Interestingly, the disruption of *FBH1* (F-box DNA helicase 1) gene in DT40 *blm* cells leads to additive increases in both SCE and mitotic chiasma [35]. Thus, it seems likely that vertebrate cells harbor parallel systems of BLM other than RECQLs to control homologous recombination-mediated reactions.

Finally, it is possible that the inability to detect a clear phenotype by disrupting *RECQL1* gene in DT40 cells in the assays used in this study may be due to the likelihood that the main function of RECQL1 is not related to DNA replication or repair but to an unknown process. In this context, it is interesting that the characterization of the piRNA complex from rat testis, which may suppress gene expression, revealed the existence of RECQL1 in this complex, suggesting involvement of RECQL1 in the suppression of gene expression [36]. In fact, we observed a very high expression of *Recql1*mRNA and stage-dependent increase of the expression of *Recql1*mRNA in the mouse testis [37]. The function of RECQL1 must be addressed in the future study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2007.11.003.

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