

The helicase domain and C-terminus of human RecQL4 facilitate replication elongation on DNA templates damaged by ionizing radiation

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The vertebrate *RECQL4* (*RECQ4*) gene is thought to be the ortholog of budding yeast *SLD2*. However, RecQL4 contains within its C-terminus a RecQ-like helicase domain, which is absent in Sld2. We established human pre-B lymphocyte Nalm-6 cells, in which the endogenous *RECQL4* gene was homozygously targeted such that the entire C-terminus would not be expressed. The *RECQL4*(Δ C/ Δ C) cells behaved like the parental cells during unperturbed DNA replication or after treatment with agents that induce stalling of DNA replication forks, such as hydroxyurea (HU). However, after exposure to ionizing radiation (IR), the *RECQL4*(Δ C/ Δ C) cells exhibited hypersensitivity, inability to complete S phase and prematurely terminated or paused DNA replication forks. Deletion of *BLM*, a gene that also encodes a RecQ helicase, had the opposite phenotype; an almost wild-type response to IR, but hypersensitivity to HU. Targeting both *RECQL4* and *BLM* resulted in viable cells, which exhibited mostly additive phenotypes compared with those exhibited by the *RECQL4*(Δ C/ Δ C) and the *BLM*($-/-$) cells. We propose that RecQL4 facilitates DNA replication in cells that have been exposed to IR.

Introduction

Maintenance of genomic stability in eukaryotes involves a multitude of evolutionarily conserved proteins. Among these proteins, the RecQ DNA helicases are conserved from bacteria to mammals (1,2). There are five RecQ DNA helicase genes in humans: *RECQL1*, *BLM* (*RECQL2*), *WRN* (*RECQL3*), *RECQL4* and *RECQL5*. Mutations in three of these genes, *BLM*, *WRN* and *RECQL4*, are linked to human syndromes characterized by genomic instability, cancer predisposition and premature aging.

The RecQ DNA helicase proteins function in processes related to DNA replication, DNA recombination and DNA repair (1–4). *BLM*, a well-characterized member of the RecQ family, facilitates dissolution of potential recombinogenic DNA substrates behind stalled replication forks (5–8). Consequently, cells from patients with Bloom syndrome, in which the *BLM* gene is mutated, have an increased rate of sister chromatid exchanges (SCEs), particularly in response to agents, such as hydroxyurea (HU), that induce replication fork stalling. The *WRN* helicase, another well-characterized RecQ helicase, has been linked to DNA replication and branch migration of Holliday junctions, particularly at telomeres, suggesting functional similarities to *BLM* (9,10).

Abbreviations: 53BP1, p53-binding protein 1; BLM, Bloom syndrome protein; HU, hydroxyurea; RecQL4, RecQ protein-like 4; UV, ultraviolet; WRN, Werner syndrome protein.

The RecQL4 helicase, which is the focus of this study, differs from all other RecQ helicases in that it contains an N-terminal domain with homology to the budding yeast Sld2 protein (11). When Sld2 is phosphorylated by cyclin-dependent kinases, it interacts with the BRCT domain-containing protein Dpb11; this interaction is essential for initiation of DNA replication (12–15). The role of Sld2 in replication initiation is apparently conserved in all eukaryotes (11,16–19). Depletion of RecQL4 from *Xenopus* egg extracts inhibits loading of replication factors on chromatin and initiation of DNA replication and knockout of the entire *RECQL4* gene or of the part of the gene encoding the Sld2-like N-terminus leads to embryonic lethality in mice and flies (20–24).

While the function of the N-terminal domain of RecQL4 is becoming more clear, the function of its C-terminus, which includes the RecQ helicase domain, is less well understood. Mutations targeting the C-terminus of RecQL4 in humans lead to the Rothmund–Thomson (RTS), RAPADILINO and Baller–Gerold syndromes, which are characterized by skin and skeletal abnormalities, juvenile cataracts, premature aging and cancer predisposition, albeit with some variation in the extent to which these phenotypes are expressed (25–30). Interestingly, many of the mutations linked to RTS target the helicase domain itself, but many others target the protein sequences further C-terminal to the helicase domain (26,27). The common phenotype, in RTS patients, elicited by the mutations targeting the helicase domain itself or the region C-terminal to the helicase domain suggests that the entire C-terminus acts as one functional unit.

Experimental systems designed to address the role of the RecQL4 C-terminus, including its helicase domain, on cell proliferation have not converged on a clear answer. DT40 cells with homozygous deletions targeting the endogenous *RECQL4* gene fail to proliferate, but the proliferation defect is rescued by ectopic expression of only the N-terminal Sld2-like domain, suggesting that the C-terminus is not required for cell proliferation (31). However, other experiments suggest that the C-terminus and its helicase domain are required for DNA replication. In frog extracts depleted of their endogenous RecQL4 protein, the DNA replication defect cannot be rescued by adding a recombinant RecQL4 protein carrying an amino acid substitution that inactivates the helicase domain (20). Furthermore, a RecQL4 mutant that lacks helicase activity cannot rescue a null mutant in *Drosophila* (32). Nevertheless, knockout mice lacking one of the exons that encode the helicase domain (exon 13) are viable but exhibit severe growth retardation, perinatal lethality (at a frequency of 95%) and tissue atrophy, particularly of tissues that normally have high proliferation rates (24).

Cells isolated from patients with RTS typically exhibit sensitivity to DNA damaging agents, implicating RecQL4 in the DNA damage response. However, the spectrum of agents to which RTS cells are sensitive differs from study to study. Some studies report sensitivity to agents that stall DNA replication forks, but not to agents that induce DNA double-strand breaks (DSBs), whereas other studies report the exact opposite spectrum (29,33–36). Furthermore, surprisingly, mouse embryo fibroblasts with a homozygous deletion of exon 13 are not hypersensitive to DNA damaging agents (24).

In an effort to better understand the function of the C-terminus of RecQL4, we generated human NALM-6 cells, in which a termination codon was introduced in both *RECQL4* alleles just upstream of the exons encoding the helicase domain. These cells express the N-terminal Sld2-like domain of RecQL4 under the control of the endogenous promoter, thus allowing us to study the consequences of deleting the C-terminus of RecQL4. Furthermore, to explore the possibility of functional redundancy between RecQL4 and BLM, we also generated NALM-6 cells in which both the *RECQL4* and *BLM* genes were homozygously targeted.

Materials and methods

Vector construction

The human *RECQL4* genomic DNA sequence was amplified with primers: 5'-ATGGAGCGGCTGCGGGACGT-3' and 5'-TGGTCCTCCAATCCTG-GAG-3'. The 6-kb PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen). The *RECQL4* sequence was subsequently inserted into the EcoRI site of the pCR2.1 vector and then the blasticidin (*bsr*) and puromycin (*puro*) selection cassettes were cloned into the BamHI site of the *RECQL4* insert to generate the *RECQL4*-*bsr* and *RECQL4*-*puro* gene disruption constructs, respectively. The BamHI site in the *RECQL4* gene is present within exon 8 (RefSeq file: NG_016430) and the *bsr* and *puro* cassettes had termination codons in all three frames, immediately downstream of their BamHI site. Thus, the *RECQL4* targeting constructs directed the expression of the N-terminal 493 amino acids of the human RecQL4 protein. After linearization with PvuI, the targeting constructs were transfected into Nalm-6 cells. The 0.9-kb fragment generated by PCR amplification of genomic DNA using the primers 5'-CAGCGAGCCTTCATGCAGGG-3' and 5'-GTCCTCAGG-CAGTGTGGG-3' was used as a probe for Southern blot analysis to screen gene-targeting events.

Cell culture and DNA transfection

The human pre-B cell line Nalm-6 was maintained in ES medium (Nissui Seiyaku Co., Tokyo) supplemented with 50 μ M 2-mercaptoethanol, 10% fetal bovine serum (Gibco) at 37°C. Cells (2×10^6) were resuspended with 2 μ g linearized DNA and electroporated using an Amaxa Nucleofector II set at program C-05. Following electroporation, the cells were transferred into 5 ml fresh medium; 24 h later, the cells were resuspended in 80 ml medium containing 0.5 μ g/ml puromycin (Sigma) or 6 μ g/ml blasticidin (Sigma) and aliquoted into four 96-well plates. After a 2- to 4-week incubation, the drug-resistant colonies were isolated and screened for gene targeting by Southern blot analysis.

Flow cytometry analysis, immunofluorescence and western blot analysis

Flow cytometry analysis, immunofluorescence and western blot analysis were performed as described previously (37) using antibodies against human RecQL4 (25470002; Novus), Chk1 (G-4; Santa Cruz), Chk1 phosphorylated on Ser317 (2344; Cell Signaling), Chk2 phosphorylated on Ser33/35 (2665; Cell Signaling) or on Thr68 (2661; Cell Signaling), γ -H2AX (JBW301; Millipore), α -actinin (AT6172; Millipore) and hybridoma supernatants recognizing Chk2 or p53-binding protein 1 (53BP1) (38).

Proliferation assay

Cells were cultured at a starting concentration of 2×10^6 cells/ml in a total volume of 5 ml. The cell number was determined every 24 h using a hemacytometer. After cell counting, the cells were diluted to their original concentration of 2×10^6 cells/ml to maintain exponential growth.

Clonogenic assay

To determine sensitivity to genotoxic agents, 1×10^2 to 2×10^5 cells were plated in 6-well plates containing 4 ml of ES medium supplemented with 50 μ M 2-mercaptoethanol, 20% fetal bovine serum and 0.15% agarose (agarose medium) with various concentrations of etoposide/VP16 (100–400 nM), 4-nitroquinoline 1-oxide/4NQO (50–200 nM), methyl methanesulfonate/MMS (50–125 μ M), camptothecin/CPT (3–5 nM), cisplatin/CDDP (1.2–4.5 μ M) or hydroxyurea/HU (50–200 μ M). To determine sensitivity to IR, the cells, already plated in 6-well dishes containing 4 ml agarose medium, were exposed to IR using an X-Rad 320 irradiator (Precision X-Ray, North Branford, CT) operating at 320 kV and 12.5 mA. To determine sensitivity to ultraviolet (UV) light, the cells were exposed to different doses of UVB irradiation, inoculated in agarose medium and cultured as above. To determine sensitivity to H₂O₂, the cells were exposed to different H₂O₂ concentrations for 1 h at 37°C and then plated into 6-well dishes containing 4 ml agarose medium. Colonies were counted 2–3 weeks later. For each assay, at least three independent experiments were performed and results are presented as means \pm 1 SE.

Analysis of SCEs

The frequency of SCEs was performed as described previously (39) with slight modifications. Briefly, Nalm-6 cells were cultured for 34–48 h in medium containing 5'-bromo-2'-deoxyuridine (BrdU; Sigma) at a final concentration of 3 μ g/ml. Colcemid (0.1 μ g/ml; Gibco) was added for 1 h prior to cell harvest. Metaphase spreads were prepared by conventional cytogenetic methods; chromosome preparations were stained with Hoechst 33258 (0.5 μ g/ml; Sigma), incubated in 2 \times standard saline citrate (Invitrogen) for 20 min at room temperature and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 40 min. The slides were then dehydrated through a cold ethanol series (70, 85 and 100%) and air-dried. All preparations were mounted and counter-

stained with VectaShield antifade medium (Vector), containing 0.1 μ g/ml 4',6-diamidino-2-phenylindole (Sigma).

Radio-resistant DNA synthesis assay

To monitor inhibition of DNA synthesis after exposure to IR, 5×10^4 cells were plated in 96-well tissue culture plates with 200 μ l medium and exposed to either no IR or 8 Gy IR. [³H]thymidine (2.5 μ Ci/ml) was added 15 min after irradiation. The cells were fixed in 70% methanol 15 min later and incorporated [³H]thymidine was determined by liquid scintillation counting. The experiment was performed in quadruplicate.

DNA molecular combing

Genomic DNA was prepared and combed onto silanized coverslips (Matsunami Glass), as described previously with modifications (40). Briefly, 2×10^6 Nalm-6 cells were pulse labeled for 20 min with 20 μ M IdU (Sigma), washed with medium twice and then pulse labeled for 40 min with 100 μ M CldU (Sigma). To remove the mitochondrial genome, the nuclei were extracted with buffer A [250 mM sucrose, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminoethyl ether)-tetraacetic acid, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride] before resuspension into low melting point agarose (InCert agarose; Lonza). DNA molecules were denatured in 2.5 N HCl for 45 min, combed on coverslips under constant speed using an apparatus custom-made for this purpose and then incubated with mouse anti-BrdU monoclonal antibody (Beckton Dickinson) to detect DNA that had incorporated IdU, rat anti-BrdU monoclonal antibody [BU1/75(ICR1) Abcam] to detect DNA that had incorporated CldU and rabbit anti-single strand DNA antibody (JP18731, IBL) to detect the DNA fibers that had not incorporated IdU or CldU. After incubation at 37°C for 1 h, the fibers were washed three times with phosphate-buffered saline and 0.05% Tween-20. After washing, the fibers were incubated with Alexa Fluor 555-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated rabbit anti-rat IgG and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) at 37°C for 30 min. All antibodies were diluted in blocking buffer (BlockAid, Invitrogen). After washing with phosphate-buffered saline and 0.05% Tween-20, coverslips were mounted in Fluoromount-G (Southern Biotech). To calibrate the lengths of the measured DNA molecules, coverslips were prepared with lambda-DNA (N3011; New England Biolabs).

Results

Establishment of *RECQL4* Δ C-terminus/ Δ C-terminus (Δ C/ Δ C) Nalm-6 cells

To examine the function of the C-terminus of RecQL4, we designed a knock-in construct that introduces a termination codon after exon 8 of the endogenous human *RECQL4* gene. This termination codon leads to expression of a truncated protein that includes the N-terminal Sld2-like domain and the nuclear localization signal, but which lacks the entire C-terminus, including the helicase domain and the sequences further C-terminal to the helicase domain (41–44). To disrupt both alleles of the *RECQL4* gene, we generated two targeting constructs that contain blasticidin- or puromycin-resistance genes, respectively (Figure 1A). First, the blasticidin-resistance construct was transfected into Nalm-6 cells, a diploid pre-B cell acute lymphoblastic leukemia cell line that is highly proficient for homologous recombination-mediated gene targeting (45). Analysis of blasticidin-resistant clones by Southern blot analysis led to the identification of two targeted heterozygous clones (Figure 1B). The puromycin-resistant construct was subsequently transfected into these two clones, leading to the generation of several homozygously targeted clones (Figure 1C). The successful disruption of both *RECQL4* alleles was further confirmed by western blot analysis, which showed expression of a truncated RecQL4 protein, in place of the full-length protein (Figure 1D). All together, several independently derived clones were obtained, allowing us to verify that any observed effects were due to deletion of the RecQL4 C-terminus rather than to clonal variations.

Proliferation and DNA damage sensitivity profile of *RECQL4*(Δ C/ Δ C) cells

Deletion of the *RECQL4* gene or depletion of the RecQL4 protein is incompatible with cell proliferation, reflecting the requirement of the N-terminal Sld2-like domain for initiation of DNA replication

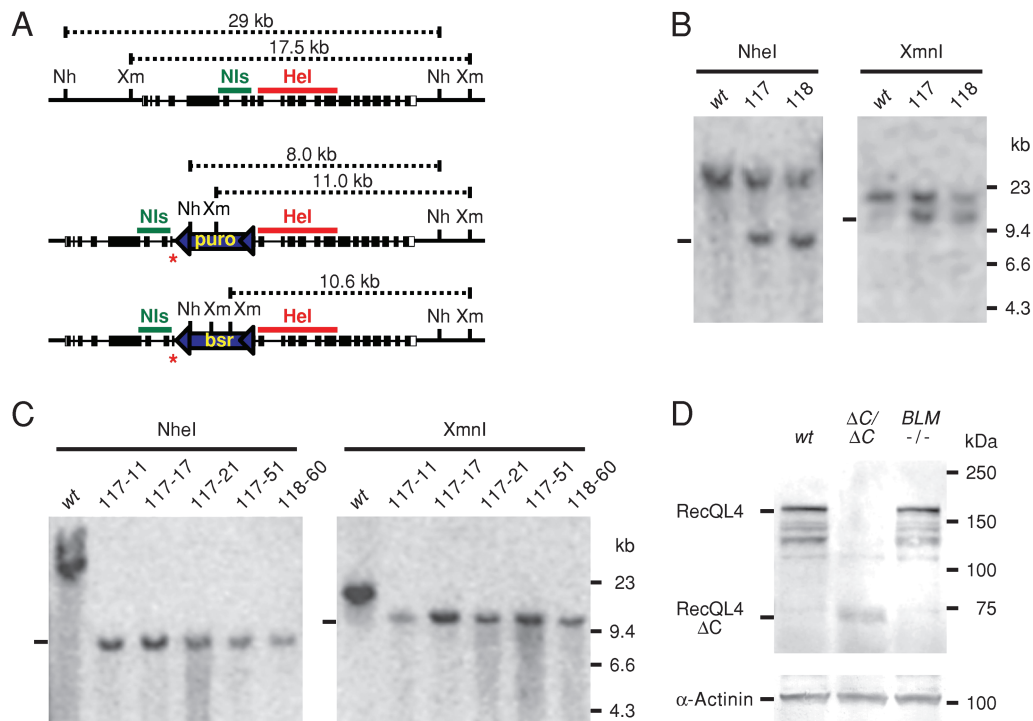


Fig. 1. Establishment of *RECQL4*($\Delta C/\Delta C$) Nalm-6 cells. (A) Diagram of the wild-type *RECQL4* genomic locus (top) and of the same locus after integration of the knock-in constructs that introduce a termination codon, indicated by the red asterisk, after exon 8. The nuclear localization signal (NLS) spans exons 5–8; the helicase domain (He) spans exons 8–14. Puro and Bsr indicate the puromycin- and blasticidin-resistance genes, respectively. Nh and Xm indicate the NheI and XmnI restriction enzyme cleavage sites, respectively. (B) Southern blot analysis showing targeting of one of the *RECQL4* alleles by the blasticidin-resistance construct in the independently derived clones 117 and 118. Horizontal lines indicate the bands corresponding to the targeted alleles. (C) Southern blot analysis showing targeting of the second *RECQL4* allele by the puromycin-resistance construct in several independently derived clones. Horizontal lines indicate the bands corresponding to the targeted alleles. (D) Western blot analysis showing expression of N-terminally truncated RecQL4 protein in Nalm-6 cells with targeting of both *RECQL4* alleles ($\Delta C/\Delta C$). Parental Nalm-6 cells (wt) and Nalm-6 cells with knockout of both *BLM* alleles (*BLM*^{-/-}) serve as controls and reveal the band corresponding to full-length RecQL4, as well as bands corresponding to lower molecular weight degradation products of full-length RecQL4. α -Actinin serves as a loading control.

(20–24). However, the *RECQL4*($\Delta C/\Delta C$) cells proliferated and, in fact, did so at rates that approached those of the parental wild-type cells (Figure 2A). For comparison, we also examined *BLM*($-/-$) Nalm-6 cells (46) since both RecQL4 and BLM are RecQ helicases. The *BLM*($-/-$) cells proliferated at a considerable lower rate than the wild-type and *RECQL4*($\Delta C/\Delta C$) cells (Figure 2A). These results suggest that the C-terminus of RecQL4 is not critical for unperturbed DNA replication.

To further explore the function of the C-terminus of RecQL4, we compared the DNA damage sensitivity profiles of wild-type, *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) cells. *RECQL4*($\Delta C/\Delta C$) cells were hypersensitive to ionizing radiation (IR), whereas the IR sensitivity of *BLM*($-/-$) cells was similar to that of the wild-type cells (Figure 2B). Remarkably, the opposite pattern was observed with agents that stall DNA replication forks, such as HU and UV light: *RECQL4*($\Delta C/\Delta C$) cells had the same sensitivity as wild-type cells, whereas *BLM*($-/-$) cells were hypersensitive (Figure 2B, Supplementary Figure 1, available at *Carcinogenesis* Online). Finally, both the *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) cells were sensitive to agents that induce both fork stalling and DNA DSBs, such as cisplatin (Supplementary Figure 1, available at *Carcinogenesis* Online).

Consistent with the C-terminus of RecQL4 not being involved in the response of cells to agents that induce stalling of DNA replication forks, *RECQL4*($\Delta C/\Delta C$) cells did not have an elevated frequency of spontaneous or aphidicolin-induced SCEs, in agreement with a previous report (47), whereas the frequency of SCEs in Nalm-6 *BLM*($-/-$) cells was elevated (Figure 2C, Supplementary Figure 2, available at *Carcinogenesis* Online).

All the effects described above were reproduced with several independently derived *RECQL4*($\Delta C/\Delta C$) clones, suggesting that the

effects were due to deletion of the C-terminus of RecQL4 and not due to clonal variations or other mutations that the cells might have acquired during the process of targeting the endogenous *RECQL4* alleles. Nevertheless, to further support this conclusion, we transiently transfected *RECQL4*($\Delta C/\Delta C$) cells with plasmids expressing full-length wild-type RecQL4 or a mutant RecQL4 protein containing an amino acid substitution of Lys508 with Asn that disrupts the function of the helicase domain (Figure 2D). The cells expressing full-length wild-type RecQL4 were no longer hypersensitive to IR, whereas the cells expressing the helicase domain mutant retained IR sensitivity (Figure 2E). We conclude that the IR sensitivity of *RECQL4*($\Delta C/\Delta C$) cells is due to loss of the C-terminus of RecQL4 and more specifically to the loss of helicase activity. Of course, we cannot rule out the possibility that the sequences C-terminal to the helicase domain also contribute to the function of RecQL4 in the response to IR. In fact, the mutation spectrum of *RECQL4* in RTS patients suggests that the helicase domain and the sequences C-terminal to the helicase domain constitute one functional unit.

Absence of a DNA replication checkpoint defect in RECQL4($\Delta C/\Delta C$) cells

The sensitivity of *RECQL4*($\Delta C/\Delta C$) cells to IR could reflect checkpoint and/or DNA repair defects. To explore for a possible checkpoint deficit, we exposed wild-type and *RECQL4*($\Delta C/\Delta C$) cells to IR and monitored phosphorylation of the checkpoint kinases Chk1 and Chk2. These two kinases are downstream of ATR and ATM, respectively, and are good molecular indicators of activated DNA damage checkpoint pathways (48). There was no difference in Chk1 or Chk2 phosphorylation after irradiation between the wild-type and

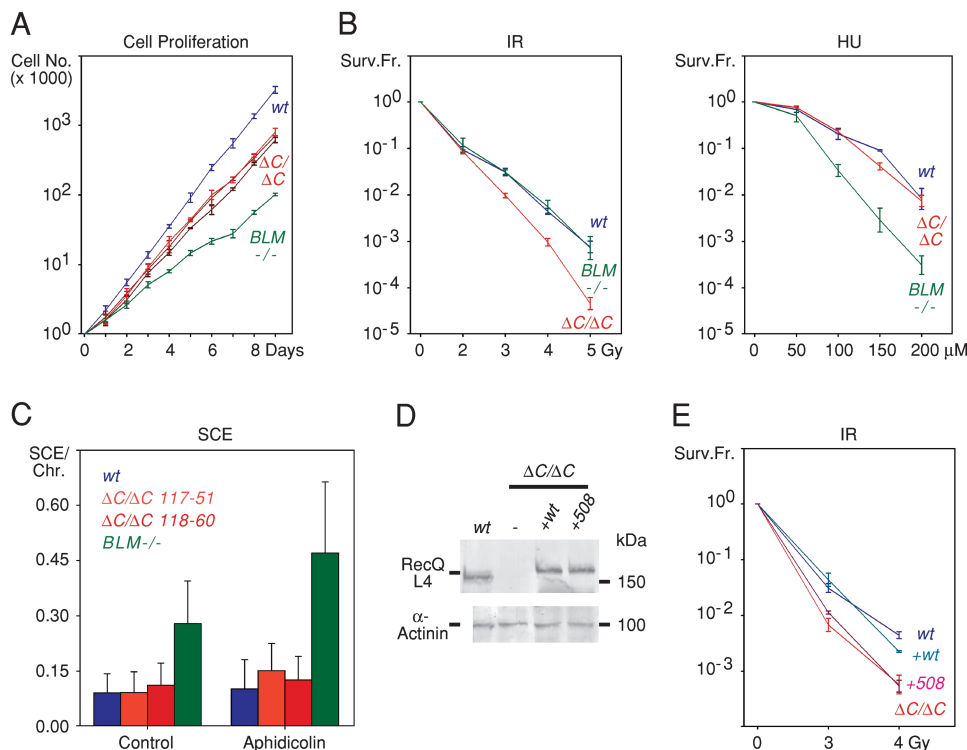


Fig. 2. Growth kinetics and DNA damage sensitivity of *RECQL4*($\Delta C/\Delta C$) Nalm-6 cells. **(A)** Cell proliferation of wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) Nalm-6 cells. Results from three different *RECQL4*($\Delta C/\Delta C$) clones are shown. **(B)** Sensitivity of wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) Nalm-6 cells to IR and HU. The surviving fraction (Surv. Fr.) is plotted relative to the dose of the DNA damaging agent. The *RECQL4* curves were generated by averaging the data from four independently derived *RECQL4*($\Delta C/\Delta C$) clones. **(C)** Frequencies (means and standard deviations) of spontaneous and aphidicolin-induced SCEs in wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) Nalm-6 cells. Data from two different *RECQL4*($\Delta C/\Delta C$) clones (117–51 and 118–60) are shown. For each condition, 47 metaphase spreads, corresponding to 2002–2096 chromosomes, were examined. **(D)** Expression of full-length wild-type (+*wt*) and K508N mutant (+508) *RecQL4* in transiently transfected *RECQL4*($\Delta C/\Delta C$) cells compared with non-transfected ($-$) *RECQL4*($\Delta C/\Delta C$) cells and to wild-type (*wt*) Nalm-6 cells, as determined by immunoblotting with an anti-*RecQL4* antibody. The transiently expressed *RecQL4* proteins contain two N-terminal FLAG tags, which explain their slow migration compared with endogenous *RecQL4*. α -Actinin serves as a loading control. **(E)** Rescue of the IR sensitivity of *RECQL4*($\Delta C/\Delta C$) cells by transient expression of wild-type (+*wt*), but not mutant (+508), full-length *RecQL4*. Parental Nalm-6 cells (*wt*) serve as controls.

RECQL4($\Delta C/\Delta C$) cells (Figure 3A). Subsequently, we examined whether deletion of the C-terminus of *RecQL4* compromised the intra-S-phase checkpoint. In mammals, this checkpoint inhibits both DNA replication initiation and elongation and is dependent on activation of the Chk1 kinase (49). We ascertained intra-S-phase checkpoint integrity by monitoring tritiated thymidine incorporation either before or 30 min after exposure to 8 Gy IR. In both wild-type and *RECQL4*($\Delta C/\Delta C$) cells, tritiated thymidine incorporation was reduced in response to IR suggesting that the checkpoint was intact (Figure 3B). Analysis of the G2 DNA damage checkpoint by monitoring mitotic entry at several time points after irradiation also revealed no checkpoint defect in *RECQL4*($\Delta C/\Delta C$) cells (data not shown).

The absence of an apparent checkpoint defect prompted us to consider a possible role of *RecQL4* in DNA DSB repair. Analysis of the function of *RecQL4* in human cells and in frog extracts has already revealed a role of this protein in DNA repair (36,50,51). We performed a DNA repair analysis in the wild-type and *RECQL4*($\Delta C/\Delta C$) cells, monitoring DNA DSB repair indirectly by counting IR-induced 53BP1 and γ -H2AX foci at 3 and 24 h after irradiation, as described previously (36). Consistent with the previous analyses of RTS cells, we observed a DNA repair defect, which was statistically significant. However, the magnitude of the defect, as revealed by comparing the mean values, was very small (Figure 3C and D).

S phase progression defect in irradiated *RECQL4*($\Delta C/\Delta C$) cells

Since *RecQL4* has a clear role in DNA replication, via its N-terminal Sld2-like domain, we reasoned that we might obtain more clear phenotypes, if we examined specifically cells that were in S phase. For

this purpose, wild-type and *RECQL4*($\Delta C/\Delta C$) cells were pulse labeled with EdU, a thymidine analog, to allow cells in S phase to be distinguished from G₁/G₂ cells. Then, the cells were irradiated (4 Gy IR) and progression through S phase was monitored 8 and 12 h later by flow cytometry (Figure 4A). EdU incorporation could readily distinguish S phase cells from those in G₁/G₂ (Figure 4B, Supplementary Figure 3, available at *Carcinogenesis* Online), thus allowing us to monitor whether cells that were in S phase at the time of irradiation progressed through S to G₂/M. A significant fraction of wild-type and *BLM*($-/-$) cells that had been irradiated during S phase completed DNA replication over the study period and accumulated in G₂/M. In contrast, practically all the *RECQL4*($\Delta C/\Delta C$) cells irradiated during S phase remained in S phase during the study period (Figure 4C).

To better understand the nature of the S phase progression defect, we performed a DNA combing assay to monitor the dynamics of DNA replication at the level of single molecules. Irradiated and non-irradiated wild-type, *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) cells were pulse-labeled with IdU for 20 min and then with CldU for 40 min (Figure 4D). DNA fibers were prepared from these cells and incubated with antibodies that recognize single-stranded DNA, IdU and CldU (Figure 4E). We focused our analysis on DNA molecules that had incorporated sequentially both IdU and CldU and tabulated the ratios of the lengths of the fibers labeled with each nucleoside analog. In the absence of irradiation, the ratios of the lengths of the fibers incorporating IdU and CldU were similar in the three cell types, suggesting that deletion of the *RecQL4* C-terminus does not affect DNA replication elongation rates in the absence of exogenous DNA damaging agents (Figure 4F). However, after irradiation, the CldU/IdU length ratios for the *RECQL4*($\Delta C/\Delta C$) cells decreased, suggesting stochastic

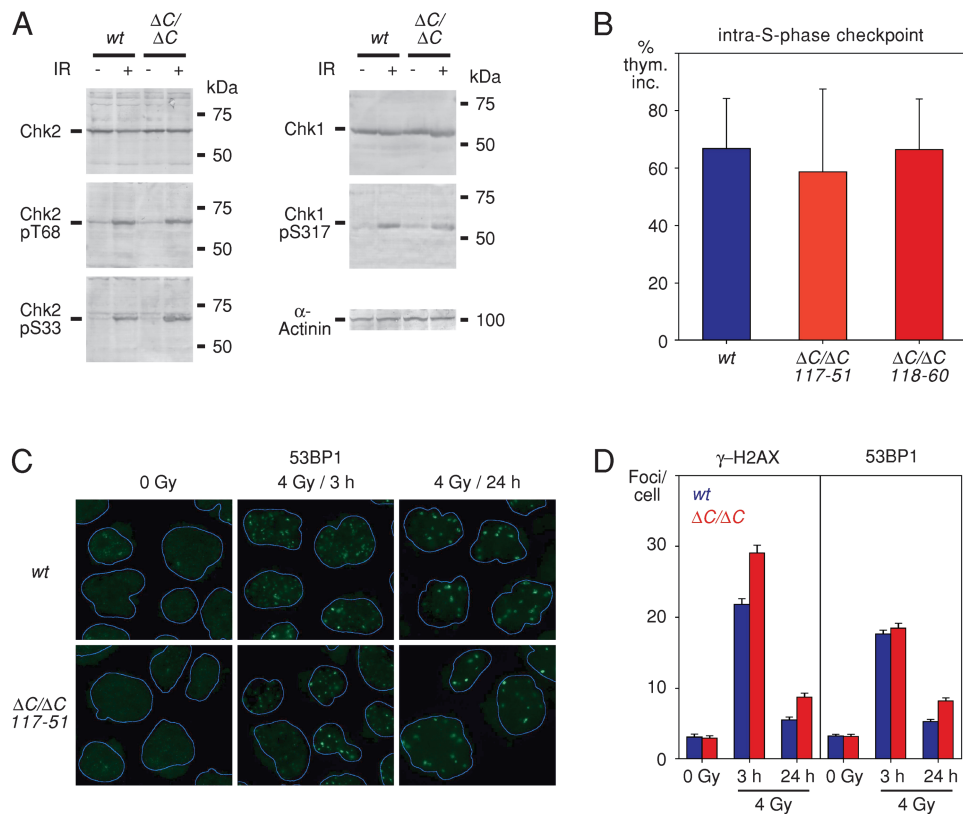


Fig. 3. IR-induced DNA damage responses of *RECQL4(ΔC/ΔC)* Nalm-6 cells. **(A)** Phosphorylation of Chk1 and Chk2 protein kinases in wild-type (*wt*) and *RECQL4(ΔC/ΔC)* Nalm-6 cells 1 h after exposure to 6 Gy IR. pT68, phospho-Thr68; pS33, phospho-Ser33; pS317, phospho-Ser317. α -Actinin serves as a loading control. **(B)** Intact intra-S-phase checkpoint in *RECQL4(ΔC/ΔC)* Nalm-6 cells. Thymidine incorporation (thym. inc.) 30 min after exposure to 8 Gy IR is expressed as a percentage relative to thymidine incorporation in the absence of exogenous DNA damage. Means and standard deviations are derived from four independent experiments. **(C)** IR-induced 53BP1 focus formation in wild-type (*wt*) and *RECQL4(ΔC/ΔC)* Nalm-6 cells, as ascertained by immunofluorescence analysis. The peripheries of the nuclei are indicated by blue lines. **(D)** DNA DSB repair defect in *RECQL4(ΔC/ΔC)* Nalm-6 cells (clone 117-51). The average number of γ -H2AX and 53BP1 foci per cell was determined in untreated cells and at the indicated timepoints after exposure to 4 Gy IR. For each condition, the number of foci in >200 cells was counted. Results are shown as means and standard errors. The differences between the means of wild-type and *RECQL4(ΔC/ΔC)* Nalm-6 cells were statistically significant for the number of γ -H2AX foci at 3 h ($P < 0.05$) and 24 h ($P < 0.01$) and for the number of 53BP1 foci at 24 h ($P < 0.05$), as determined by the Student's *t*-test.

pausing and/or collapse of the replication forks, whereas no such decrease was observed with the wild-type and *BLM(-/-)* cells (Figure 4F). We conclude that in irradiated cells, the RecQL4 C-terminus allows replication forks to negotiate DNA templates that have been damaged by irradiation.

Phenotype of *RECQL4(ΔC/ΔC);BLM(-/-)* cells

The distinct phenotypes of the *RECQL4(ΔC/ΔC)* and *BLM(-/-)* cells suggest that these genes function in distinct DNA damage response pathways. To examine the validity of this prediction, we generated human NALM-6 cells, in which both alleles of the *RECQL4* and *BLM* genes were targeted by homologous recombination. To our knowledge, targeting both these genes has not been examined in any system. Several independently derived clones of *RECQL4(ΔC/ΔC);BLM(-/-)* cells were obtained by targeting both *RECQL4* alleles in *BLM(-/-)* cells (Figure 5A). This indicates that concurrent inactivation of the function of the C-terminus of RecQL4 and of BLM is compatible with cell viability. The doubly targeted cells exhibited proliferation rates that were reduced compared with those of wild-type and *RECQL4(ΔC/ΔC)* cells but similar to those of *BLM(-/-)* cells (Figure 5B). The number of SCEs in *RECQL4(ΔC/ΔC);BLM(-/-)* cells was elevated compared with wild-type and *RECQL4(ΔC/ΔC)* cells; however, it was somewhat lower than the number of SCEs in *BLM(-/-)* cells (Figure 5C).

In terms of sensitivity to DNA damaging agents, the *RECQL4(ΔC/ΔC)* and *BLM(-/-)* cells showed a somewhat complex profiles

consistent with RecQL4 and BLM functioning in distinct, but related, pathways. Thus, the *RECQL4(ΔC/ΔC);BLM(-/-)* cells were less sensitive to IR than the *RECQL4(ΔC/ΔC)* cells, although more sensitive than the wild-type and *BLM(-/-)* cells (Figure 5D). Thus, deletion of *BLM* rescues in part the IR sensitivity of *RECQL4(ΔC/ΔC)* cells. The opposite effect was observed with HU and other DNA damaging agents, such as cisplatin (Figure 5D and data not shown). In this case, deletion of *BLM* enhanced the sensitivity of the *RECQL4(ΔC/ΔC)* cells. In these cases, the effects were mostly additive, with the *RECQL4(ΔC/ΔC);BLM(-/-)* cells exhibiting the sensitivity expected by adding the sensitivities of the *RECQL4(ΔC/ΔC)* and *BLM(-/-)* cells.

Discussion

Prior studies have established a role for RecQL4 in initiation of DNA replication (17–24). This function is mediated by the N-terminal Sld2-like domain. Here, we addressed the function of the C-terminus by targeted mutagenesis of the endogenous *RECQL4* alleles in human Nalm-6 cells. A significant body of evidence already indicated that the C-terminus plays a role in the response to DNA damage (25–31,33–36,50,51). However, some studies suggested a function in the response to agents that induce replication fork stalling, whereas others suggested a role in the response to agents that induce DNA DSBs. There are many ways to explain these apparent discrepancies. First, most studies focused on analysis of cells obtained from RTS patients;

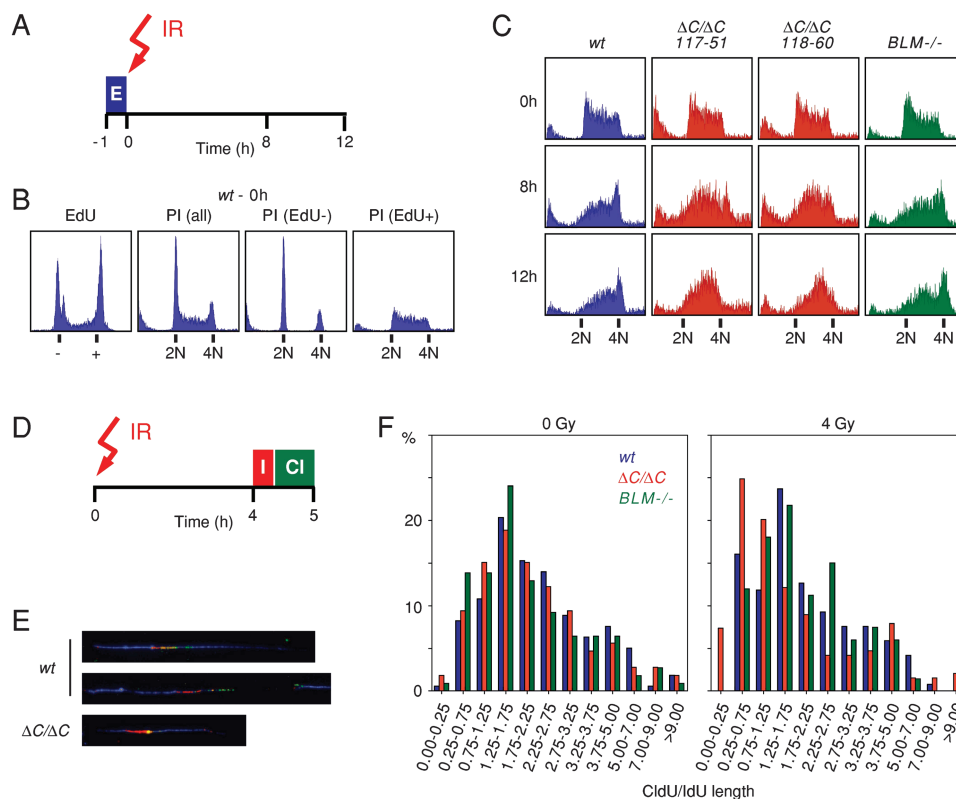


Fig. 4. S phase progression defect in irradiated *RECQL4*($\Delta C/\Delta C$) cells. **(A)** Outline of the experiment to monitor cell cycle progression of wild-type and *RECQL4*($\Delta C/\Delta C$) cells irradiated in S phase. Cells were exposed to EdU (E) for 1 h and then irradiated (4 Gy). **(B)** Efficiency of gating of S phase versus G_1/G_2 cells by EdU incorporation. The propidium iodide (PI) profiles of the entire cell population (all) and of the EdU-negative and EdU-positive subpopulations are shown for wild-type (*wt*) Nalm-6 cells at the 0-h timepoint. **(C)** Cell cycle progression of wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) Nalm-6 cells after exposure to 4 Gy IR. The PI profiles of the EdU-positive cells are shown. **(D)** Outline of the experiment to monitor DNA replication at the single-molecule level by DNA combing. Cells were untreated or exposed to 4 Gy IR and 4 h later were incubated sequentially with IdU (I; for 20 min) and CldU (Cl; for 40 min). **(E)** Images of DNA replication fibers from wild-type (*wt*) and *RECQL4*($\Delta C/\Delta C$) cells exposed to 4 Gy IR. IdU incorporation, red; CldU incorporation, green; single-stranded DNA, blue. **(F)** Ratios of lengths of DNA fibers incorporating CldU over IdU from wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$) (clone 118-60) and *BLM*($-/-$) cells. For each condition, 150–200 DNA replication forks were counted. The results are expressed as percentages. The profile of the irradiated *RECQL4*($\Delta C/\Delta C$) cells is statistically significantly different from the profiles of the irradiated wild-type and *BLM*($-/-$) cells ($P < 0.01$), as determined by the Mann–Whitney *U*-test.

the mutations present in these cells differ from patient to patient and may not fully inactivate function, making comparisons among studies difficult (25–30). Second, some mutations may facilitate protein unfolding, thereby targeting the mutant RecQL4 protein for degradation or sequestering it in inactive complexes with protein chaperones. As a result, these mutations have the potential to interfere with the function of the N-terminal domain.

To study the function of the C-terminus of RecQL4 in a well-defined system, we introduced in human Nalm-6 cells a termination codon in the endogenous *RECQL4* gene just upstream of the coding sequence for the helicase domain. This mutation resulted in expression of a truncated RecQL4 protein that contained the essential N-terminal Sld2-like domain and the nuclear localization signal, but which lacked the entire C-terminus, including the helicase domain. Thus, the deleted segment corresponds to the part of the human RecQL4 protein that is missing in budding yeast Sld2. Several independently derived Nalm-6 clones expressing the truncated RecQL4 protein were analyzed.

Deletion of the RecQL4 C-terminus did not have a major effect on unperturbed DNA replication, as ascertained by cell proliferation rates and by analysis of DNA replication at the single-molecule level. This conclusion appears to contradict some previously published results. For example, knockout mice lacking exon 13 of *RECQL4* exhibit severe growth retardation, tissue atrophy and perinatal lethality and mouse embryo fibroblasts prepared from these mice have a proliferation defect *in vitro* (24). However, because the helicase domain is encoded by exons 8–14, deletion of exon 13 will almost certainly result in expression of an unfolded protein, thereby potentially also

compromising the function of the N-terminal Sld-2 like domain. A similar rationale may also explain why RecQL4 proteins with mutations in the helicase domain cannot rescue a null mutant in *Drosophila* and the apparent conflicting results in *Xenopus* regarding whether the helicase domain of RecQL4 is needed for unperturbed DNA replication (20,21,32). Consistent with our conclusion that the RecQL4 C-terminus is not critical for unperturbed DNA replication, the replication defect of chicken DT40 cells with homozygous *RECQL4* deletions is rescued by ectopic expression of the RecQL4 N-terminus (31).

Even though the C-terminus of RecQL4 appears dispensable for unperturbed DNA replication, it is apparently required for the cellular response to IR. Previously, other groups have reported conflicting results regarding the sensitivity of RTS cells to agents that induce stalling of DNA replication forks, such as UV light and HU, and to agents that induce DNA DSBs (25–31,33–36,50,51). These conflicting results could be explained by the fact that the mutant RecQL4 proteins expressed in RTS cells may retain partial activity and/or be defective in the activity of their N-terminal Sld2-like domain. However, our analysis of the sensitivity profiles of wild-type, *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) Nalm-6 cells to IR, UV light and HU provides a very clear result. The *RECQL4*($\Delta C/\Delta C$) cells were hypersensitive to IR, but their response to UV light and HU was not different from that of wild-type cells. The wild-type response cannot be attributed to an inherent resistance of Nalm-6 cells to these agents since deletion of the *BLM* gene led to sensitivity to UV and HU, as expected.

The sensitivity of *RECQL4*($\Delta C/\Delta C$) cells to IR could be due to a checkpoint defect, a general DNA DSB repair defect and/or a defect of replication forks in negotiating IR-induced DNA damage. Previous

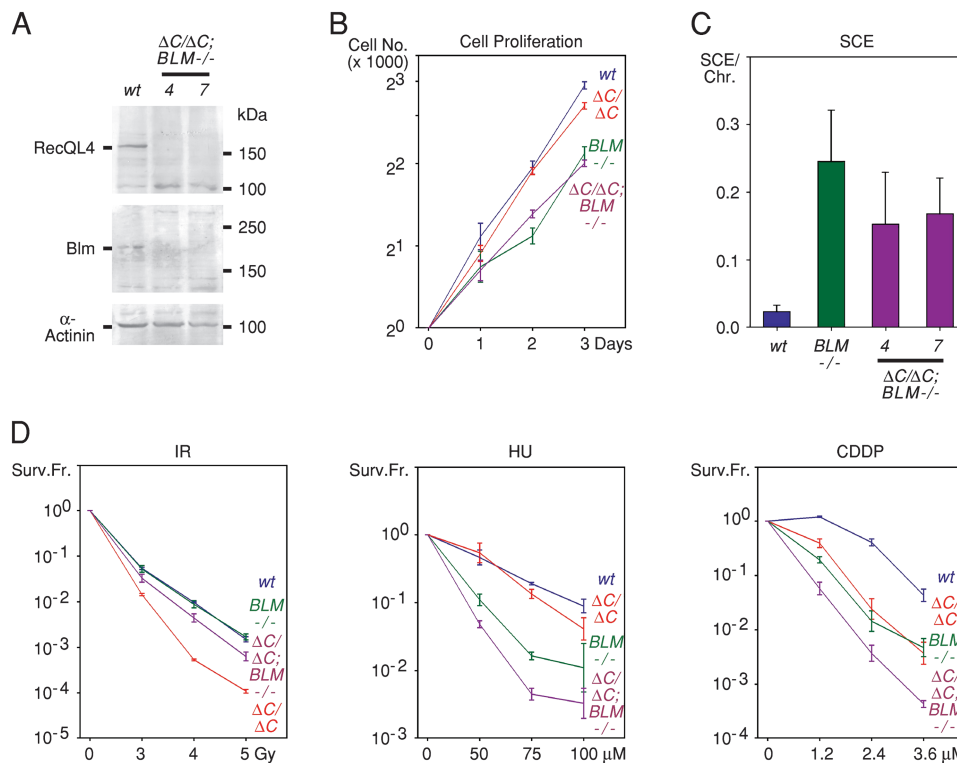


Fig. 5. Phenotype of *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) cells. (A) Lack of expression of full-length RecQL4 and BLM proteins in two independently derived *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) clones, as determined by immunoblotting. Wild-type (*wt*) Nalm-6 cells serve as controls. α -Actinin serves as a loading control. (B) Cell proliferation of wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$), *BLM*($-/-$) and *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) Nalm-6 cells. Results from three different *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) clones are shown. (C) Frequencies (means and standard deviations) of spontaneous SCEs in wild-type (*wt*), *BLM*($-/-$) and *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) Nalm-6 cells. Data from two different *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) clones (4 and 7) are shown. For each condition, 25 metaphase spreads were examined. (D) Sensitivity of wild-type (*wt*), *RECQL4*($\Delta C/\Delta C$), *BLM*($-/-$) and *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) Nalm-6 cells to IR, HU and cisplatin (CDDP). The surviving fraction (Surv. Fr.) is plotted relative to the dose of the DNA damaging agent. Data from three independently derived *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) clones were averaged.

studies have reported checkpoint defects in RTS cells or in *Drosophila* cells in which the endogenous RecQL4 protein was depleted by small interfering RNA (52,53). We did not observe a checkpoint defect in the *RECQL4*($\Delta C/\Delta C$) cells when we monitored Chk1 and Chk2 phosphorylation, the intra-S-phase checkpoint or the G₂/M checkpoint. We attribute the previously observed checkpoint defects to suppression of DNA replication resulting from compromised RecQL4 function since suppression of DNA replication compromises ATR activation (54).

A general defect in DNA DSB repair could also have explained the sensitivity of *RECQL4*($\Delta C/\Delta C$) cells to IR. Previous studies have reported a DNA DSB repair defect in RTS cells, as ascertained by monitoring the number of 53BP1 foci at various timepoints after irradiation (36). We also observed a subtle defect in resolution of γ -H2AX and 53BP1 foci in irradiated *RECQL4*($\Delta C/\Delta C$) cells over time. However, the magnitude of the defect, although statistically significant, appears insufficient to explain the profound sensitivity of these cells to IR. Thus, instead of a general defect in DNA DSB repair, we propose a DNA replication defect in cells that have been exposed to IR. More specifically, one could envision a defect in the ability of DNA replication forks to negotiate IR-induced DNA damage. Consistent with this proposal, *RECQL4*($\Delta C/\Delta C$) cells, that were irradiated while in S phase, failed to complete DNA replication. In addition, by DNA combing analysis, these cells displayed stochastic premature termination of DNA replication forks. It has been proposed that RecQL4 travels with the DNA replication fork (19,22), which would be consistent with a role of its helicase domain in facilitating the ability of forks to negotiate IR-induced DNA damage. It is noteworthy that, compared with the other RecQ helicases, RecQL4 appears to stand out in its role in the response to IR, whereas all other RecQ helicases appear to be more important for the response to agents that stall DNA replication forks, such as HU and UV light (1,2,55,56).

Prokaryotes and budding yeast have only one RecQ helicase, whereas mammals have five such helicases, including RecQL4 and BLM (1,2). The presence of multiple RecQ helicases in higher eukaryotes raises the question whether these helicases have distinct or overlapping functions. In DT40 chicken cells, double knockout mutants, such as *BLM*($-/-$);*WRN*($-/-$), *BLM*($-/-$);*RECQL1*($-/-$), *BLM*($-/-$);*RECQL5*($-/-$) and *RECQL1*($-/-$);*RECQL5*($-/-$), have been generated and, in general, show more severe phenotypes after induction of DNA damage, as compared with the single mutants (55,56). However, to our knowledge, no double mutant of *RECQL4* with any other RecQ helicase gene has been described. We were surprised to see that *RECQL4*($\Delta C/\Delta C$);*BLM*($-/-$) cells were viable, given that both the single *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) mutants had strong phenotypes. The epistasis analysis of the *RECQL4*($\Delta C/\Delta C$) and *BLM*($-/-$) mutants described here is consistent with RecQL4 and BLM having distinct functions. Thus, we conclude that the function of the RecQ helicases has diverged during evolution, with RecQL4 acquiring a function that allows cells to negotiate DNA replication templates that have been damaged by IR.

Supplementary material

Supplementary Figures 1–3 can be found at <http://carcin.oxfordjournals.org/>

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