HHS Public Access

Author manuscript

Aging Cell. Author manuscript; available in PMC 2015 October 28.

Published in final edited form as:

Aging Cell. 2010 June ; 9(3): 358–371. doi:10.1111/j.1474-9726.2010.00562.x.

The involvement of human RECQL4 in DNA double strand break repair

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Summary

Rothmund-Thomson syndrome (RTS) is an autosomal recessive hereditary disorder associated with mutation in *RECQL4* gene, a member of the human RecQ helicases. The disease is characterized by genomic instability, skeletal abnormalities and predisposition to malignant tumors, especially osteosarcomas. The precise role of RECQL4 in cellular pathways is largely unknown, however recent evidence suggest its involvement in multiple DNA metabolic pathways. This study investigates the roles of RECQL4 in DNA double strand break (DSB) repair. The results show that RECQL4-deficient fibroblasts are moderately sensitive to γ-irradiation and accumulate more γH2AX and 53BP1 foci than control fibroblasts. This is suggestive of defects in efficient repair of DSB's in the RECQL4 deficient fibroblasts. Real time imaging of live cells using laser confocal microscopy show that RECQL4 is recruited early to laser induced DSBs and remains for a shorter duration than WRN and BLM indicating its distinct role in repair of DSBs. Endogenous RECQL4 also colocalizes with γH2AX at the site of DSBs. The RECQL4 domain responsible for its DNA damage localization has been mapped to the unique N-terminus domain between amino acids 363–492, which shares no homology to recruitment domains of WRN and BLM to the DSBs. Further, the recruitment of RECQL4 to laser induced DNA damage is independent of functional WRN, BLM or ATM proteins. These results suggest distinct cellular dynamics for RECQL4 protein at the site of laser induced DSB and that it might play important roles in efficient repair of DSB's.

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Author contributions: Conceived and designed the experiments: VB, DKS, PK. Performed the experiments: DKS, PK, MA, SS, AM, LB. Analyzed the data: VB, DKS, PK, DC, SP. Wrote the paper: DKS, VB.

Keywords

RecQ helicase; Rothmund-Thomson syndrome (RTS); Werner syndrome (WRN); Bloom syndrome (BLM); Double strand break repair; Premature aging

Introduction

The RecQ helicase family is a group of evolutionarily conserved DNA unwinding proteins that play diverse roles in multiple DNA metabolic and repair processes. Five RecQ homologues have been identified in humans and mice: RECQL1, BLM, WRN, RECQL4 and RECQL5. Defects in human RecQ helicases are associated with chromosomal and developmental abnormalities, cancer susceptibility and premature aging (Brosh, Jr. & Bohr, 2007), and three loci encoding human RecQ helicases are causally linked to recessive hereditary diseases characterized by genomic instability and cancer predisposition (Bohr, 2008; Hickson, 2003). Werner syndrome (WS) and Bloom syndrome (BS) are caused by defects in WRN and BLM helicase, respectively. Defects in human *RECQL4* are linked to Rothmund Thomson (RTS) Type 2, RAPADILINO and Baller-Gerold syndromes (Kitao *et al.*, 1999; Siitonen *et al.*, 2003; Van Maldergem *et al.*, 2006). Although human WRN and BLM helicases are well characterized, relatively little is known about the biochemistry, cellular biology and function of human RECQL4.

RTS is a rare, autosomal recessive disorder characterized by poikiloderma, growth deficiency, juvenile cataracts, premature aging and predisposition to malignant tumors especially osteosarcomas (Wang *et al.*, 2003;Vennos *et al.*, 1992; Stinco *et al.*, 2008). Approximately two-thirds of RTS patients have mutations in *RECQL4* which are predicted to result in a truncated protein due to premature termination of protein synthesis (Lindor *et al.*, 2000; Wang *et al.*, 2003). These patients are referred to as RTS Type 2 and show strong predisposition to osteosarcoma (Wang *et al.*, 2003). Cells from RTS patients show genomic instability and chromosomal abnormalities such as trisomy, aneuploidy and chromosomal rearrangements (Vennos *et al.*, 1992; Der Kaloustian, V *et al.*, 1990; Orstavik *et al.*, 1994; Durand *et al.*, 2002; Anbari *et al.*, 2000).

RECQL4 is a 1208 amino acid protein (133 kDa) with a central helicase domain, which is characteristic for RecQ helicases (Kitao *et al.*, 1998). However, RECQL4 does not share homology to two other conserved RecQ motifs, RQC and HRDC (helicase and RNaseD C-terminal), which are present in human BLM and WRN helicases. RECQL4 also possesses two N-terminal nuclear localization signal (NLS) sequences (Burks *et al.*, 2007). RECQL4 has an intrinsic ATPase activity and single-strand DNA annealing activity (Yin *et al.*, 2004; Macris *et al.*, 2006). Recent studies have shown that the RECQL4 also has helicase activity *in vitro* (Xu & Liu, 2009; Capp *et al.*, 2009; Suzuki *et al.*, 2009).

The biological functions of RECQL4 are not yet known. However, it has been proposed that the RecQL4 N-terminal region plays a role in recruiting DNA polymerase α to nascent DNA replication forks in *Xenopus* egg extract (Sangrithi *et al.*, 2005; Matsuno *et al.*, 2006). Recently, it has been shown that human RECQL4 is a part of the repliosome complex and interacts with MCM10, MCM2-7, CDC45 and GINS (Xu *et al.*, 2009). Another study

indicates that the assembly of Cdc45-MCM2-7-GINS (CMG) complex at the replication fork requires RECQL4 (Im *et al.*, 2009). These findings suggest an active involvement of RECQL4 in the assembly of the pre-replication complex and initiation of DNA replication.

Recent findings also indicate the involvement of RECQL4 in some DNA repair pathways. Fibroblasts from RTS patients are sensitive to replication-blocking DNA damaging agents including hydroxyurea, camptothecin and doxorubicin (Jin *et al.*, 2008). RTS cells showed sensitivity to H_2O_2 and accumulate more H_2O_2-induced DNA strand breaks than control cells, suggesting that RECQL4 may stimulate repair of H_2O_2-induced DNA damage (Werner *et al.* 2006; Schurman *et al.*, 2009). Biochemically, RECQL4 modulates the intrinsic activities of APE1, FEN1 and DNA polβ, indicating its role in base excision repair (BER) pathway (Schurman *et al.*, 2009). RECQL4 also interacts with poly (ADP-ribose) polymerase 1 (PARP-1), which is implicated in DNA recombination, DNA repair, and transcriptional regulation (Woo *et al.*, 2006). RECQL4 colocalizes with the Xeroderma Pigmentosum Group A (XPA) protein in human cells treated with UV, suggesting that RECQL4 might facilitate nucleotide excision repair (NER)-mediated repair of UV-induced DNA damage in human cells (Fan & Luo, 2008). Furthermore, RECQL4 and Rad51 colocalize in the nuclei of cells treated with etoposide, suggesting that RECQL4 might play a role in homologous recombination (HR)-mediated repair of DSBs (Petkovic *et al.*, 2005). But the mechanism of this interaction is not known. There is also RECQL4 involvement in the repair of DSBs in *Xenopus* egg extracts (Kumata *et al.*, 2007). Moreover, there are inconsistent reports about the sensitivity of RTS cells from patients and from MEFs derived from the RECQL4-deficient mice towards γ-irradiation, which predominantly forms DSB in the cells (Vennos *et al.*, 1992; Cabral *et al.*, 2008; Jin et al., 2008; Hoki *et al.*, 2003).

In this work, we studied the cellular dynamics of RECQL4 in live cells at the site of laser induced DSBs using confocal laser scanning microscopy as well as sensitivity assays. First, we show that the RTS-deficient primary fibroblasts are moderately sensitive to γ-irradiation and accumulate more γ-irradiation induced γ-H2AX and 53BP1 foci that do not get efficiently resolve than control fibroblasts; this suggests that there are DNA repair defects in RTS cells compared to control cells. RECQL4 is recruited early to the laser-induced DNA damage site. Recruitment of RECQL4 to DNA damage requires an N-terminal domain which does not share homology to WRN and BLM proteins. Furthermore, RECQL4 shows distinct cellular dynamics at the site of laser induced DSBs compared to WRN and BLM.

Results

RTS cells are moderately sensitive to γ-irradiation

The importance of RECQL4 in DNA double strand break (DSB) repair was examined by measuring survival of RECQL4-deficient and normal fibroblasts after γ-irradiation. The RTS cells (AG05013 and AG17524) and normal fibroblasts (GM00323 and GM00969) were treated with 0, 1, 2 or 3 Gy of γ-irradiation and survival efficiency was estimated after 14 days by cell proliferation assays. The results showed that both the RTS cells are moderately more sensitive to γ-irradiation than respective normal cells (the RTS and normal fibroblasts were age and sex matched), and that survival decreases with increasing dose of γ-irradiation

(Fig. 1). We also observed that the growth rate of both the RECQL4 deficient fibroblasts was approximately 1.5 to 2-fold slower than the normal fibroblasts (Supplementary Fig S1).

RTS cells are defective in efficient repair of DNA DSBs

Sensitivity of RECQL4-deficient cells to γ-irradiation could be a result of inefficient processing of DSBs. To examine this possibility, two RECQL4-deficient (AG05013 and AG18371) and two normal cells (GM00323 and GM01864) were irradiated with 5 Gy of γ-irradiation and allowed to recover for 1, 3, 6 or 10 hours. Cells were then fixed and immunostained with γ-H2AX and 53BP1 antibodies and examined by confocal laser scanning microscopy. γ-H2AX and 53BP1 foci are widely used as markers for DSBs in the cell (Kinner *et al.*, 2008; Schultz *et al.*, 2000). RTS cells had a higher basal level of γ-H2AX foci than normal cells, suggesting inefficient repair of endogenous DNA DSBs perhaps due to problems at the replication forks (Supplementary Fig S2, lane 1). At early time points (*i.e.*, 1 hour after irradiation), the number of 53BP1 foci in both the RECQL4 deficient cell lines were ~ 2-fold higher than in the control cells (Fig. 2, panels A,B, lane 2 and panels C,D). Furthermore, a significantly higher number of 53BP1 foci (2–3 fold) accumulated in γ-irradiated RTS cells than in normal fibroblasts (Fig. 2, panels A,B, lanes 3–5 and panels E,F) at late time points. The graph shows 53BP1 repair kinetics (percent 53BP1 foci with respect to time) and these focis disappeared more quickly in normal cells than in RTS cells suggesting inefficient repair of DSBs in RTS cells (Fig. 2, panels A,B, upper rows and panels E,F). Similar patterns of foci dispersal was also seen for γ-H2AX at the site of DSBs (Supplementary Fig S2, lanes 3–5). The slight differences in kinetics of γ-H2AX and 53BP1 foci formation and dispersal could be due to differences in recruitment and dissociation properties of γ-H2AX and 53BP1 at the site of DNA damage. These results suggest that RECQL4 deficient cells repair DSBs less efficiently than normal cells, which is consistent with reduced survival of RECQL4 deficient cells after exposure to γ-irradiation.

Characterization of the laser microirradiation

Confocal laser scanning microscopy is a powerful technique for studying the spatiotemporal behaviors of DNA repair proteins in “real time” in living cells. This technique has been used to reveal novel functions of many DNA repair proteins including WRN and BLM (Mailand *et al.*, 2007; Yano *et al.*, 2008; Haince *et al.*, 2008). Previous studies also show that WRN and BLM are recruited very early to laser-induced DSBs via their HRDC domain (Lan *et al.*, 2005; Karmakar *et al.*, 2006). Laser microirradiation produces a variety of DNA lesions, (DSBs, single-strand breaks (SSBs) and base modifications) in a spatially-restricted manner in nuclear DNA. The types of lesions formed after irradiation depends upon laser intensity and wavelength (Dinant *et al.*, 2007; Lan *et al.*, 2005). In this study, DSBs were induced with a 435 nm laser without cellular pre-treatment. We standardized the laser intensity so that SSBs could be distinguished from DSBs. At a laser intensity of <10%, only SSBs were produced, and XRCC1 (both GFP-tagged and endogenous), but not GFP-53BP1 or endogenous 53BP1 were recruited to the microirradiated site (Figure 3A, panels a and b, lanes 1 and 2). At a laser intensity of >10%, both SSBs and DSBs were produced, and both XRCC1 and 53BP1 were recruited to the microirradiated site (Fig. 3A, panels a and b, lanes 4 and 5). Live HeLa cells were also microirradiated using 8% or 18% laser intensity and co-immunostained with antibodies to XRCC1 and γ-H2AX (Fig. 3B). DSBs and γ-H2AX foci

were generated by 18% but not by 8% laser intensity. Under these experimental conditions we then tested for the recruitment of GFP-tagged exogenous and endogenous RECQL4 protein to the site of DNA damage. The results showed that both endogenous and GFP-tagged RECQL4 were recruited to the DNA damage site at high laser intensity (Fig. 3A, compare lanes 3 and 6).

RECQL4 accumulates at laser-induced DNA lesions

Kinetic studies were performed to examine the association and dissociation of RECQL4, WRN and BLM at laser-induced DNA damage in living cells. Similar to previous reports, in most cells, GFP-tagged RECQL4 was localized to the nucleus, but nucleo-cytoplasmic localization was observed in some cells [data not shown,(Burks *et al.*, 2007; Yin *et al.*, 2004)]. For this experiment, HeLa cells were transiently transfected with plasmids expressing GFP-RECQL4, GFP-WRN or GFP-BLM prior to laser-microirradiation, and cells were visualized at different time points after laser treatment. Previously, using a similar approach, WRN and BLM have been shown to be recruited to the DNA damage sites (Lan *et al.*, 2005; Karmakar *et al.*, 2006) and hence were used as a positive controls in this study (Fig. 4, panels A, B, C and D). The results showed that RECQL4 is recruited to DNA damage site within 5 seconds of microirradiation (Fig. 4, panels C and D).

Endogenous γ-H2AX and RECQL4 colocalize after laser-induced DNA damage

Recruitment of endogenous γ-H2AX and RECQL4 to the microirradiation site was also examined in HeLa cells. Cells were fixed and immunostained with γ-H2AX and RECQL4 antibodies after 5 min of laser microirradiation and imaged. The images were analyzed using confocal microscopy and Volocity software. The results showed that endogenous γ-H2AX and RECQL4 colocalize within 5 minutes after laser treatment, suggesting that RECQL4 binds to DSBs (Fig. 5). A significant portion of the endogenous RECQL4 remains in the cytoplasm after irradiation, as reported previously (Yin *et al.*, 2004; Werner *et al.*, 2005).

The NTS2 domain of RECQL4 is sufficient for recruitment to the DSB sites

RECQL4 possesses a characteristic central helicase domain which is a shared feature of RecQ helicases (Kitao *et al.*, 1998). RECQL4 differs from WRN and BLM helicases in that it lacks the C-terminal RQC and HRDC domains. Two unique nuclear localization sequences are also present at the N-terminus of RECQL4 (Burks *et al.*, 2007). To map the domain of RECQL4 responsible for its recruitment to the laser induced DSBs sites, we tested constructs containing either portions of the RECQL4 coding region, or the full length construct with portions deleted. The subcellular localization of each mutant was reported previously (Burks *et al.*, 2007), and we confirmed the expression of each mutant by Western analyses (Supplementary Fig. S3). First, we tested three GFP-tagged constructs which contained portions of the RECQL4 coding region, namely the N-terminus (1-492), the N-terminus with central helicase domain (1-794) and the C-terminus (794-1208) (Figure 6). The results showed that the region spanning from aa 1-794 and aa 1-492 enabled recruitment to the DNA damaged site, whereas the C-terminal aa 794-1208 domain was not recruited, however this construct is cytoplasmic and thus may not have access to the DSB sites. This

collective results suggest that at least one recruitment domain is present within the N-terminus of the RTS protein (Fig. 6, panels A and B, ii, iii, iv, v).

To further map the domain within the N-terminus (1-492) of RECQL4, various mutants within the nuclear targeting sequence (NTS2, aa 363-492) were tested for their recruitment to the microirradiated site. It has been reported that the NTS2 domain of human RECQL4 includes a 130 amino acids segment within exons 5–8 which is necessary and sufficient for nuclear localization (Fig. 6, panel A,B, xi, xii) (Burks *et al.*, 2007). Consistent with this observation, GFP-NTS2 (aa 363-492) was recruited to DNA damage in laser-treated cells, but NTS2-deleted RECQL4 (GFP-RECQL4 ΔNTS2) was not (Fig. 6, panel B, xi and xii). This confirms that NTS2 is sufficient for nuclear localization and for recruitment of RECQL4 to DNA damage. Further, the NTS2 domain was subdivided into $NTS2_A$ (aa 364-426) and $NTS2_B$ (aa 426-492). The $NTS2_A$ is encoded by parts of exons 5–7 of RECQL4, and $NTS2_A$-deleted GFP-RECQL4 shows nuclear or nucleo-cytoplasmic localization pattern similar to wild type RECQL4 (Burks *et al.*, 2007). Deletion of $NTS2_A$ domain (GFP-RECQL4Δ$NTS2_A$), including a highly basic stretch of 22 amino acids named the “basic box” (GFP-RECQL4Δ Basic Box) does not affect the recruitment of RECQL4 to DNA damage (Fig. 6, panels A,B, vi, vii). In contrast, $NTS2_B$ deleted GFP-RECQL4 (GFP-RECQL4Δ$NTS2_B$) show predominantly cytoplasmic localization (Burks *et al.*, 2007). Furthermore, GFP-RECQL4Δ$NTS2_B$ and other mutants with deletions in the $NTS2_B$ domain (*i.e.*, Δexon7 and ΔVLPLY, a highly conserved motif in all orthologues) are cytoplasmic and are not recruited to the site of microirradiation. This suggests their importance in the recruitment of full length RECQL4 to the site of the DNA damage. However, it is possible that these mutants were not recruited to the site of the microirradiation because of their predominant cytoplasmic localization. Thus, in conclusion, these results suggest that the NTS2 domain spanning from aa 363–492 contains at least one domain which is sufficient for the recruitment of RECQL4 to the DSBs. However, the possibility of other domains in the full length RECQL4 can not be ruled out and need further investigation.

GFP tagged-RECQL4 is recruited to DNA damage independent of functional WRN, BLM and ATM proteins

WRN and BLM interact physically and functionally, and BLM inhibits WRN exonuclease (von Kobbe *et al.*, 2002). WRN and BLM also colocalize after cellular stress (von Kobbe *et al.*, 2002). Because these results indicate potential interactions among RecQ helicases, the influence of WRN and BLM on dynamics of RECQL4 at the sites of DNA damage was investigated using cells lacking WRN or BLM. For this purpose, GFP-tagged RECQL4 was transiently expressed in SV40 transformed WRN (AG07066) or BLM (GM08505) and ATM (GM05849) deficient fibroblasts. The cells were microirradiated and analyzed by live cell imaging as described above. The results showed that RECQL4 is recruited efficiently to the microirradiated site in the absence of functional WRN, BLM or ATM proteins (Fig. 7).

The retention of GFP-tagged RECQL4 at the microirradiated site is short compared to WRN and BLM proteins

The efficiency of DNA repair depends on the dynamics of the DNA repair proteins at the site of DNA damage. WRN, BLM and RECQL4 are recruited early, but their rate of dissociation also affects the efficiency of the repair process. While there are many studies addressing the recruitment of DNA repair proteins to DNA damage, there is very little information about the disassociation dynamics of DNA repair proteins from such sites. Such studies require an environmental chamber, which we have utilized in these studies. Kinetics of protein dissociation from DNA damage site was examined in HeLa cells expressing GFP-tagged WRN, BLM or RECQL4. For this experiment, cells were microirradiated using 21% laser intensity, and placed in an environmental chamber, where they could be visualized by confocal microscopy under normal growth conditions throughout the experiment. The graph shows the dynamic association of the various RecQ helicases at the site of a lesion. Interestingly, RECQL4 arrives early and departs within 1 hr (Fig. 8. panels C and D). We also observed that the timing at which the WRN protein reaches its maximum intensity at the DNA damage site is different from BLM and RECQL4. WRN reaches maximal intensity at about one hr and then slowly departs. BLM reaches its maximal intensity at about 2hrs, but its disassociation is faster than for WRN. Collectively, we conclude that the initial recruitment is similar for the different RecQ helicases (Fig.4), but the maximal recruitment and rate of dissociation varies considerably for the different RecQ helicases. Thus, the different RecQ helicases have distinct roles in the response to DNA damage.

DISCUSSION

This report characterizes the role of RECQL4 in DNA DSB repair. Results presented here show that RECQL4-deficient cells are moderately more sensitive to γ-irradiation than normal fibroblasts (Fig. 1). The conflicting results in papers prior to identification of the role of RECQL4 in RTS may reflect that 30% of RTS patients carry mutations in a yet unidentified gene (Kitao *et al.*, 1999; Wang *et al.*, 2003). More recently, there have been divergent reports in the literature regarding the sensitivity of different primary RTS fibroblasts and murine fibroblasts towards ionizing radiation (Jin *et al.*,2008; Cabral *et al.*, 2008, Hoki *et al.*, 2003). Although WRN-deficient primary fibroblasts also demonstrate only modest sensitivity towards ionizing radiation (Yannone *et al.*, 2001), WRN plays important roles in regulating HR- and NHEJ-dependent repair of DSBs. Therefore we propose that RECQL4 might also play important roles in DSB repair with regard to survival and efficient repair of damage.

We then examined γ-H2AX and 53BP1 foci formation and dispersal to show that RECQL4 deficient cells are indeed defective in efficient repair of DSBs. A comparison of the time course kinetics of foci dispersal in normal control fibroblast cells and RECQL4 deficient cells showed that there are approximately 2–3 fold more persistence of 53BP1 foci for >10 hours in RTS cells, suggesting that RECQL4-deficiency impairs efficient repair of DNA DSBs (Figure 2). Similarly, WRN deficient cells also have a higher basal level of γ-H2AX foci than control cells in experiments where WRN and control cells are age-matched. It has been proposed that an age-associated decrease in the efficiency of DSB repair causes

genome instability in the context of both normal and premature aging (Sedelnikova *et al.*, 2008). Also, in *Xenopus* the reduction in DSB induced γ-H2AX was significantly compromised in RECQL4 depleted extracts suggesting that RECQL4 functions in repair of DSB (Kumata *et al.*, 2007).

This study used confocal laser scanning microscopy to image the kinetic behavior of RecQ helicases in response to laser-induced DNA damage in living cells. As previously shown for WRN and BLM protein (Lan *et al.*, 2005; Karmakar *et al.*, 2006), GFP-tagged RECQL4 is recruited rapidly to the site of laser-induced DNA damage (Fig. 4). The endogenous RECQL4 is also recruited to DSBs, where it colocalizes with γ-H2AX at the microirradiated site, but not to SSBs (Fig. 5). A previous study showed that a portion of RECQL4 foci are associated with regions of ssDNA at DSB sites in cells treated with etoposide and IR (Petkovic *et al.*, 2005). A study using Xenopus oocyte extracts also suggested that RECQL4 accumulates in chromatin and promotes repair of DSBs (Kumata *et al.*, 2007). These results suggest that RECQL4 associates with DSBs and promotes efficient processing and repair of DSBs.

This study shows that the NTS2 domain of RECQL4 is required and sufficient for recruitment of RECQL4 to the site of microirradiation (Fig. 6). RECQL4 is structurally unique and distinct from other RecQ helicases homologues, in that it does not possess the RQC and C-terminal HRDC domains, which are present in WRN and BLM. Both WRN and BLM are recruited to laser-induced DSBs via their HRDC domain (Lan *et al.*, 2005; Karmakar *et al.*, 2006). Karmakar *et al.* suggested that the HRDC domain plays a role in recruitment of BLM to DNA damage, because GFP-tagged RECQL1, which lacks an HRDC domain, is not rapidly recruited to DNA damage in irradiated cells (Karmakar *et al.*, 2006). In this context, it is interesting that RECQL4, which also lacks the HRDC domain, is rapidly recruited to the site of microirradiation (this study). RECQL4, unlike other RecQ helicases, carries both nuclear targeting sequences at the N-terminus. Specifically, we show here that the NTS2 region of RECQL4 (aa 363–492) is sufficient for nuclear targeting and for recruitment of RECQL4 to DSB sites. This result is interesting as the N-terminus of RECQL4 is implicated as a critical region important for its essential roles in DNA replication (Sangrithi *et al.*, 2005; Matsuno *et al.*, 2006) and also is deleted through mis-splicing in the RECQL4 associated RAPADILINO syndrome. The N-terminus of the human RECQL4 protein also directly interacts with MCM10 and is important for assembly of pre-initiation complex (Xu *et al.*, 2009). Our results also indicate that the chromatin binding domain resides within the N-terminus of RECQL4 which is also involved in various DNA metabolic pathways such as DNA replication and repair. However, additional studies are needed to determine whether the NTS2 region of RECQL4 mediates binding to DNA damage associated chromatin proteins or to DNA breaks themselves.

While there have been numerous studies examining the recruitment characteristics of DNA repair proteins, very little is known about the retention kinetics of RECQ helicase at DSB sites. In our studies we used an environmental chamber to maintain the cells under normal conditions over the long period of study. Interestingly, RECQL4 is retained at sites of DNA damage for only approximately one hour, while WRN and BLM are retained for longer periods of time (Fig. 8). Thus, RECQL4 might act at an early step in DSB repair, or it could

act on a specialized transient DNA substrate, which is present at a low level in laser treated cells. These results also suggest that different RECQ helicases have distinct and specialized functions in the context of DSB repair which correlates with their specialized biochemical features and their responses to different genotoxic agents.

In cells DSB's are repaired either by homologous recombination (HRR) or non homologous end-joining (NHEJ). NHEJ-mediated DSB repair is largely independent of terminal DNA sequence homology. Therefore, NHEJ is error-prone and produces deletions, insertions and translocations (Thompson & Schild, 2002). The HRR pathway repairs DSBs using homologous sequences on sister chromatids and to a lesser extent, on chromosome homologues (Johnson & Jasin, 2000; Liang *et al.*, 1998). Previous studies have indicated that RECQL4 might be involved in both, HRR and NHEJ pathways. RECQL4 has been shown to form complex with Rad51 which is crucial for the repair of DSBs by the HRR pathway (Petkovik *et al.*, 2005). In another study using *Xenopus*, RECQL4 has been shown to be loaded on to the chromatin in response to DSBs. Further, RECQL4 is loaded adjacent to Ku heterodimer binding site on damaged chromatin suggesting its possible involvement in NHEJ (Kumata *et al.*, 2007). However, the mechanism of RECQL4 involvement in DSB repair is not yet understood. It is possible that RECQL4 participates in the repair process once it is loaded on to the chromatin at the site of DSB. In this complex RECQL4 can interact directly with various proteins involved in the HRR and NHEJ pathway at different stages of DSB repair and modulate their functions. Recent studies have also indicated that the RECQL4 might also be important in replication restart after repair (Xu et al., 2009). However, another likely possibility is that the function of RECQL4 may be indirect. Similar to WRN and BLM helicases, RECQL4 could function in resolving aberrant DNA structures formed during DNA replication and recombination repair processes and facilitate the loading of other repair factors at the site of the break. Therefore, the absence of RECQL4 in the cells would lead to genomic instability and could explain the heterogeneous premature aging phenotype and cancer susceptibility seen in RTS patients.

One of the limitations of this study is that most of the experiments are performed with overexpression of transfected proteins to study the cellular dynamics at the site of DNA damage. Nonetheless, this work strengthens the hypothesis that RECQL4 plays a significant role in the DNA damage response to DSBs. Additional studies are needed to further understand the molecular mechanisms that allow RECQL4 to promote genetic stability and facilitate an efficient response to DNA damage in human cells.

Experimental procedures

Cell lines and transfection

HeLa cells, SV40 transformed normal (GM00637), WRN (AG07066), BLM (GM08505), ATM (GM05849) fibroblasts cell lines, normal primary skin fibroblasts (GM00323, GM00969, GM01864) and RTS-deficient skin primary fibroblasts (AG05013, AG18371, AG17524) cell lines from Coriell Cell Repositories were used in the study. The RTS cell lines have confirmed RECQL4 mutations and do not show RECQL4 proteins in western analysis of the total cell extract (Kitao *et al.*, 1999; Petkovic *et al.*, 2005). The pair of RTS cells and normal fibroblasts cells used for survival assays and the foci formation assays are

age and sex matched. The HeLa cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. SV40 transformed normal, WRN, BLM and ATM fibroblasts were cultured in MEM media supplemented with 10% FBS. Normal primary skin fibroblasts cells and RTS-deficient primary fibroblasts were cultured in Amniomax II Complete media (Invitrogen, CA, USA). For live cell experiments the cells were plated in glass bottom Petri dishes (LabTec corp, USA) at 50% confluence 24 hours before the transfections. For transfection Fugene 6 was used as per manufacturer's instructions (Roche, NJ, USA). For immunofluorescence studies, the cells were plated in 4 well chambered slides 24 hours before the treatments.

Cell proliferation assay

Cell proliferation was assessed using CyQuant (Molecular Probes, OR, USA). Both the normal cell line (GM00323 and GM00969) and the RTS cell line (AG05013 and AG17524) were plated in 60 mm petri dishes at a density of 1000 cells per plate. The cells were treated with 1, 2, 3 Gy of γ irradiation and then allowed to grow in Amniomax-II Complete media for 14 days at 37°C at 5% CO_2. On the day of the analysis, the plates with adherent cells were washed once with 1X PBS and incubated with the CyQuant dye for 30 min in dark at 37 °C with 5% CO_2. Fluorescence intensity, which is proportional to cell number, was measured on a Typhoon 8600 fluorescent scanner (Molecular Dynamics, CA, USA) with filters set at 480 nm excitation and 520 nm emission. Fluorescence data was analyzed and the number of colonies in each plate was counted by Image QuantTL software (Molecular Dynamics).

Analysis of γ-H2AX and 53BP1 foci

The γH2AX and 53BP1 foci were measured in two normal primary fibroblasts cell lines (GM00323 and GM01864) and two RTS cell lines (AG05013 and AG18371). Approximately, 25,000 primary fibroblasts cells were seeded on chambered slides, grown overnight and then treated with 5 Gy of γ-irradiation. Cells were allowed to recover for 1, 3, 6 or 10 hr, under normal growth conditions and then immediately fixed and stained (described in next section) with either mouse monoclonal γH2AX antibody (Upstate Biotechnoligy, 1:200) or rabbit polyclonal 53BP1 antibody (Abcam, 1:200) and Alexa goat anti-mouse 647 or donkey anti-rabbit 647 secondary antibody (Molecular Probes, OR, USA). Ten images representing about 15–20 cells per well were taken using a Nikon TE2000 spinning disk microscope with five laser imaging modules and a CCD camera (Hamamatsu). The data were analyzed using Volocity version 4.3.1 build 6 (Improvision).

Immunocytochemistry and antibodies

The cells were laser microirradiated and stained with antibodies against γH2AX, 53BP1, XRCC1, RECQL4. After microirradiation the cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Then cells were washed 3 times with PBS, permeabilized with 0.2% Triton X 100 in PBS for 5 min at room temperature. The cells were washed with PBS and blocked in 5% fetal bovine serum overnight at 4 °C. The cells were incubated with different primary antibodies for 1 hr at 37 °C in a humidified chamber. Mouse monoclonal anti-phosphorylated H2AX (1:200, Upstate Biotechnology), goat

polyclonal anti-RECQL4 (K16) (1:50, Santa Cruz Biotech), rabbit polyclonal anti-53BP1 (Abcam, 1:200) and rabbit polyclonal anti-XRCC1 (1:200, Santa Cruz Biotech) antibodies were used. Bound antibodies were visualized by incubating the cells with secondary antibodies, namely Alexa FITC (488) conjugated chicken anti-goat, Alexa 647-conjugated goat anti mouse and Alexa-conjugated 647 donkey anti rabbit. The images were captured with a confocal microscope (Nikon eclipse TE2000) and analyzed by volocity software (Improvision).

Western blotting

After separation in SDS-PAGE gel the proteins were transferred to PVDF membrane and the membrane was blocked with 5% fat free milk in 1X TBS-T for 1 hr at room temperature followed by incubation with primary rabbit anti GFP antibody (1: 5000, Novus Biologicals) overnight at 4 °C. The membranes were then washed thrice in 1X TBST, 5 min each, and then incubated in HRP conjugated secondary anti rabbit antibody (1:20,000, Sigma, USA) for 1 hr. After washing the membrane was developed by ECL plus kit following manufacturer's protocol (GE Healthcare, USA).

Laser irradiation and confocal microscopy

We employed a Nikon Eclipse 2000E spinning disk confocal microscope with five laser imaging modules and a CCD camera (Hamamatsu, Tokyo, Japan). The set-up integrated a Stanford Research Systems (SRS) NL100 nitrogen laser by Micropoint ablation system (Photonics Instruments, St. Charles, IL). Site specific DNA damage was induced using the SRS NL100 nitrogen laser that was passed through a dye cell to emit at 435 nm wavelength. The power of the laser was attenuated through Improvison's Volocity software 4.3.1 (Improvision/PerkinElmer, Coventry, England) in terms of percent intensity. Positions internal to the nuclei of either live HeLa cells or HeLa cells transfected with GFP tagged plasmids were targeted via a 40X oil objective lens. The laser intensities of 8% and 21% were used. Images were captured at various time points and analyzed using Volocity version 4.3.1 build 6 (Improvision). Experiments were performed using an environmental chamber attached to the microscope to maintain the normal atmosphere of the cells (i.e. 37°C and 5% CO_2).

Fluorescence Recovery after Photobleaching (FRAP)

FRAP analysis was carried out with live cells transfected with GFP tagged WRN, BLM and RECQL4 plasmids. The same set up as the microirradiation was used to perform the photobleaching. Fluorescence recovery was monitored as described in the figure legends and the data for recovery was corrected for the background intensity and loss of total fluorescence. Each experiment was performed at least three times and the data presented here are mean intensity values obtained in a given experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Drs. Suhasini Avvaru and Venkateswarlu Popuri for critical reading of the manuscript. This work was supported by funds from the Intramural Research Program of the National Institute on Aging, NIH.

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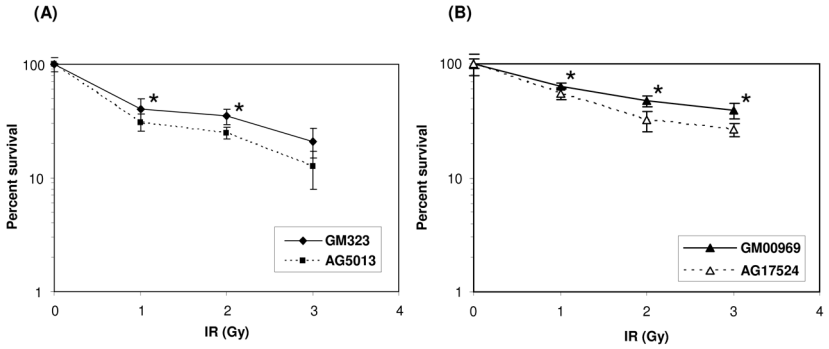

Fig. 1. RECQL4-deficient fibroblasts are sensitive to γ-irradiation compared to normal control fibroblasts

A, Normal (GM00323) and primary RTS fibroblasts (AG05013) cell lines were plated on a 60 mm petri dishes and treated with 0, 1, 2 and 3 Gy of γ-irradiation. Cells were allowed to grow for 14 days and fluorescent intensity was measured as described in Materials and Methods. B, Similar experiments were performed with normal fibroblasts (GM00969) and RTS cell line (AG17524). The experiments were performed in triplicate and the error bars represents the standard deviation (+/−). The degree of significance were calculated by Student's t-Test method and the (*) represent the significant difference from the normal fibroblast with p value < 0.05.

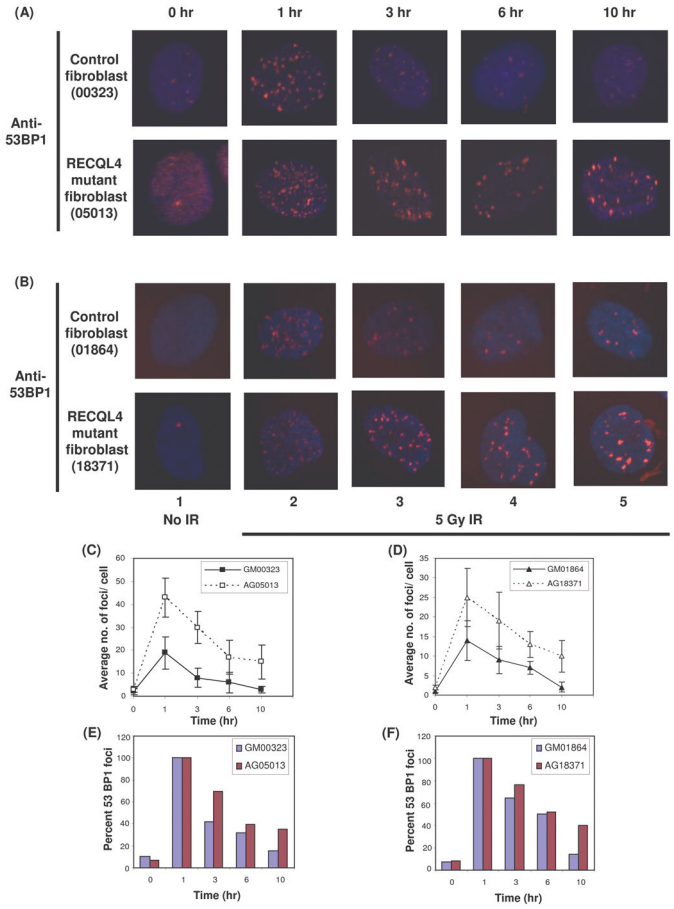

Fig. 2. The processing of DNA double strand breaks is delayed in RECQL4 defective primary fibroblasts

After 5 Gy of γ-irradiation, the cells were allowed to recover under physiological conditions, fixed at indicated time points (0, 1, 3, 6 and 10 hrs) and immunostained with anti rabbit 53BP1 antibodies (as described in materials and methods). The images were captured with confocal microscope. Panel A and B show the persistence of more 53BP1 foci in RECQL4-deficient primary fibroblasts AG05013 and AG18371 (lower rows) compared to normal control fibroblasts GM00323 and GM01864 (upper rows), respectively at different time intervals as indicated. The first column represents the untreated cells (0 hour) as control. The total number of 53BP1 foci's in both the RTS and normal control cell lines for each time point were counted and the average number of foci/ cell with respect to time are represented in panel C and D for two different cell lines, respectively. The repair kinetics of 53BP1 foci in both RTS and normal cell lines in terms of percentage of 53BP1 foci at each time point are represented in panel E and F for two different cell lines, respectively. In both panels, the total number of 53BP1 focis at 1 hr is taken as 100% foci for both normal and RTS cell lines.

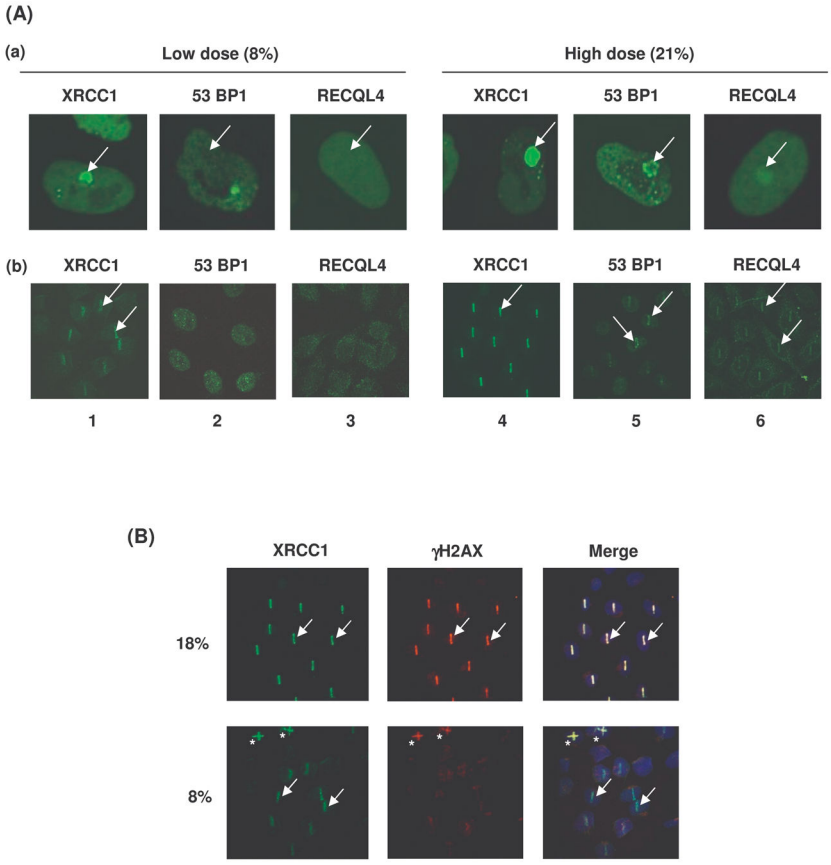

Fig. 3. The formation of DNA double strand breaks at the laser microirradiation site requires higher doses of laser irradiaiton

A. Panel (a) shows the association of GFP tagged XRCC1, 53BP1 and RECQL4 in live HeLa cells at low (8%) and high (21%) doses of laser intensity. Transfected HeLa cells were microirradiated within the nucleus of the cell and images were captured for 2 min after photo bleaching. Panel (b) represents the association of endogenous XRCC1, 53BP1 and RECQL4 proteins at laser induced DNA damage site irradiated with low (8%) and high (21%) doses of laser intensity. Live HeLa cells were microirradiated and fixed after 5 min and immunostained with either XRCC1, 53BP1 or RECQL4 antibodies (see materials and methods). Arrow indicates the exact site of laser DNA damage. B. The live HeLa cells were microirradiated with the laser at 18% and 8% laser intensities. At 18% laser intensity recruitment of both endogenous γ-H2AX and XRCC1 was observed whereas at 8% laser intensity only XRCC1 is recruited and not the γ-H2AX. The arrow indicates the site of exact DNA damage. Asterisks (*) in the lower panel represents the DNA damage at 18% laser intensity.

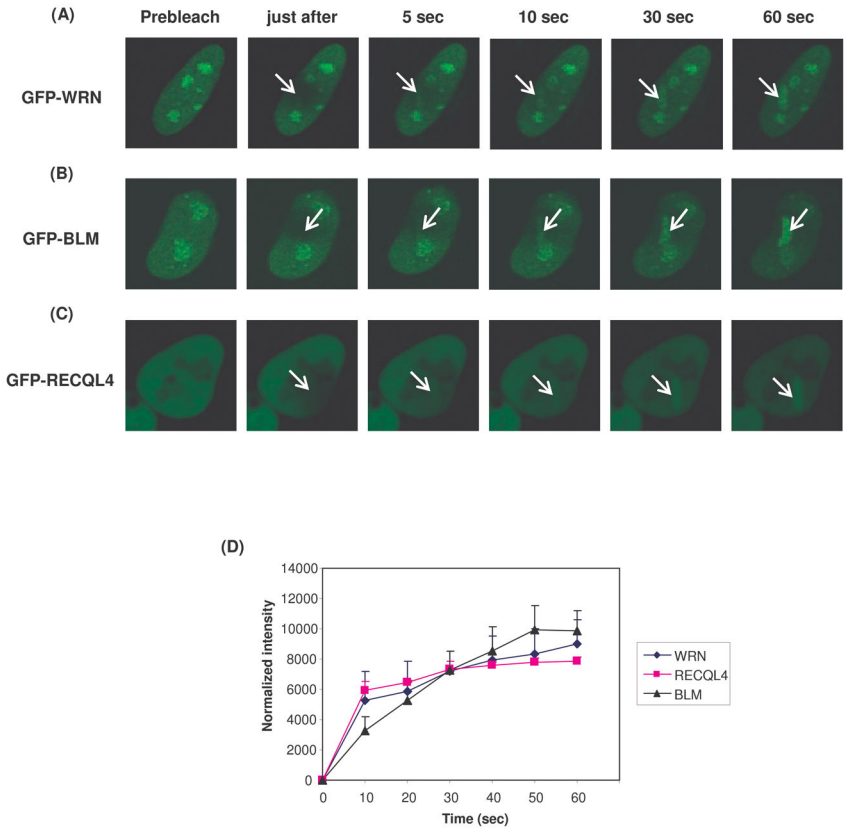

Fig. 4. The recruitment kinetics of GFP tagged WRN, BLM and RECQL4 proteins at the site of laser induced DNA damage

The HeLa cells were transfected with GFP tagged WRN, BLM or RECQL4 plasmids. After 24 hr post transfections, the cells were laser microirradiated within the nucleus with 435 nm laser light at 21% laser intensity. The images were captured for 60 seconds through FRAP channel using confocal microscope. The association of GFP-tagged WRN, BLM and RECQL4 proteins is shown at different time intervals after microirradiation in panel A, B, and C, respectively. Arrow represents the site of laser irradiation. The normalized intensity values of the kinetic of association of different proteins have been represented in panel D.

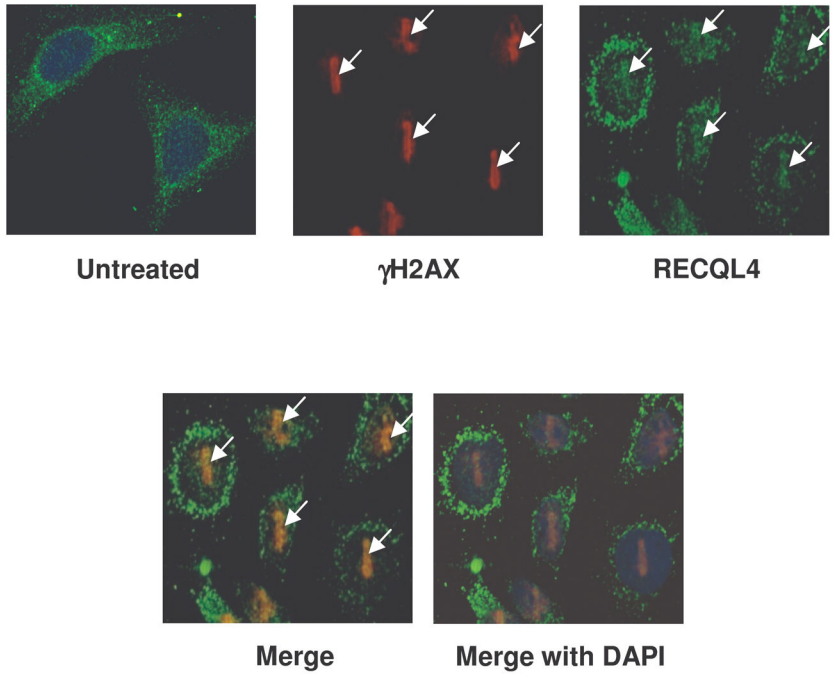

Fig. 5. Endogenous γ-H2AX and RECQL4 colocalizes at the laser induced DNA damage site

HeLa cells were laser microirradiated at 21% laser intensity followed by fixation after 5 min and immunostaining with anti rabbit γ-H2AX and anti goat RECQL4 antibodies. The untreated cell is shown in top left panel, γ-H2AX is shown in red (top middle), RECQL4 is depicted in green (top right) and the merge images of the two showing co-localization is shown in yellow (lower panels).

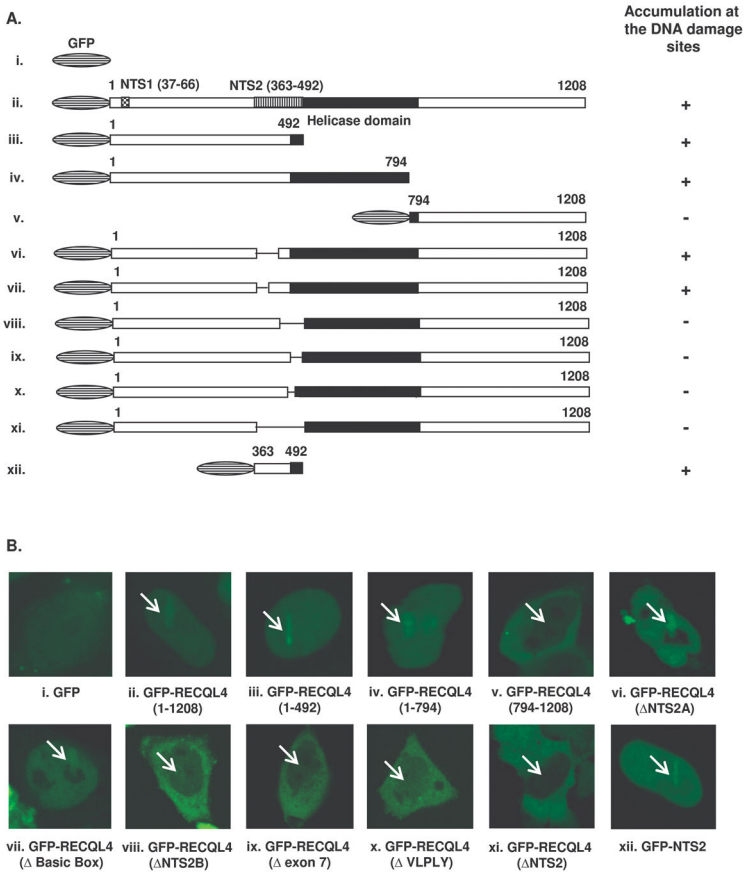

Fig. 6. Analysis of association of GFP tagged RECQL4 mutants at the site of laser DNA damage

The different GFP-RECQL4 mutant constructs were transiently transfected in the HeLa cell and association kinetics were monitored at 21% laser intensity. Panel A, shows the schematics of different mutant and their association at DNA damage site (represented with + sign). Two Nuclear targeting sequences (NTS1 & NTS2) and the helicase domain are shown. Panel B show one representative images of each mutant association at the DNA damaged site.

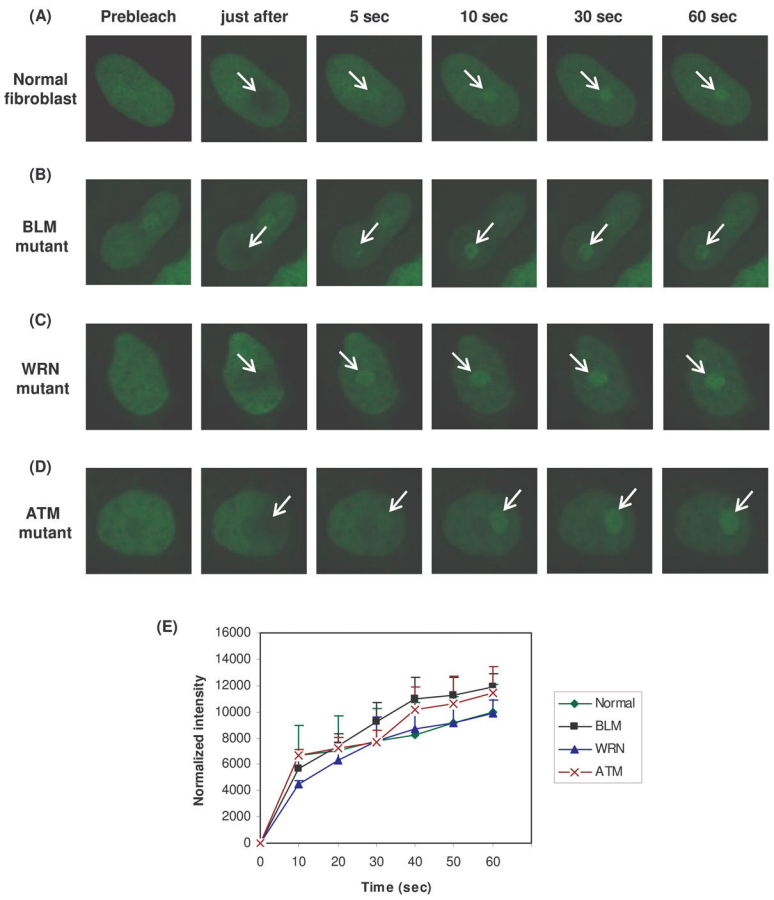

Fig. 7. The recruitment of GFP tagged RECQL4 is independent of functional WRN, BLM and ATM proteins

The association kinetics of transiently transfected GFP-RECQL4 protein at the site of laser induced DNA damage in SV40 transformed Normal fibroblasts (GM00637), BLM (GM08505), WRN (AG07066), and ATM (GM05849) mutant fibroblasts cells are shown in panel A, B, C and D, respectively. The normalized intensity value of the kinetic of association of RECQL4 in normal fibroblasts, BLM, WRN and ATM mutant cells have been represented in panel E.

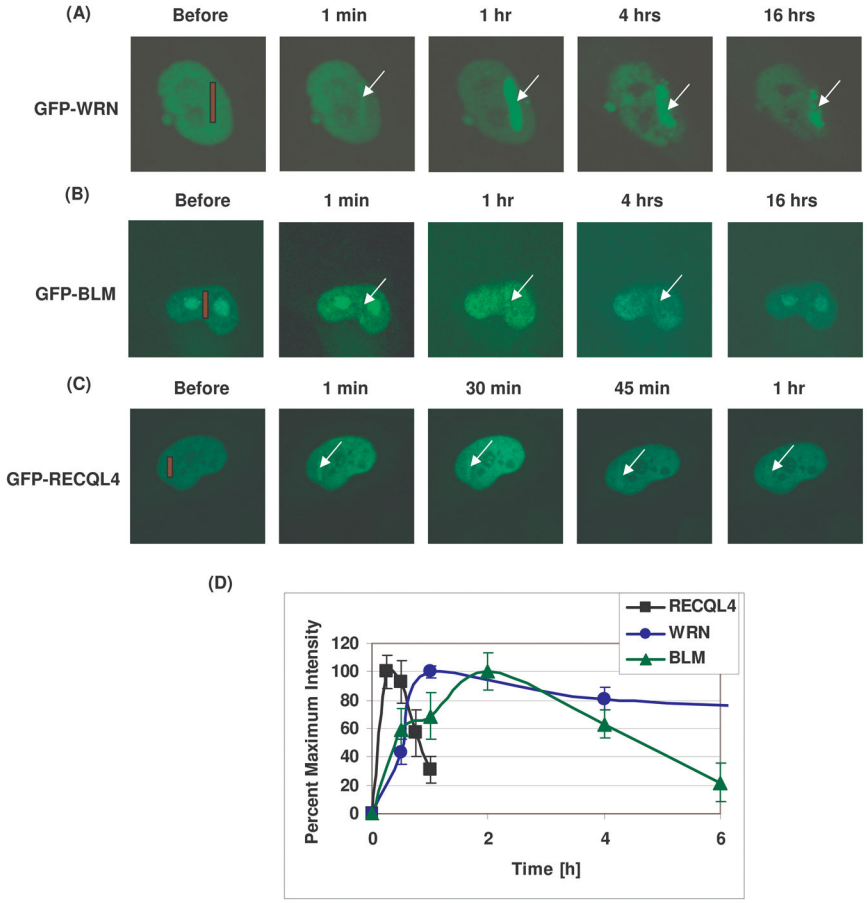

Fig. 8. Retention of RECQL4 at the DNA damaged site is shorter compared to WRN and BLM proteins

The HeLa cells were transiently transfected with GFP tagged WRN, BLM and RECQL4 plasmids and after 24 hr post transfections, the cells were microirradiated at 21% laser intensity and the dissociation kinetics were monitored by live cell imaging. The cells were placed in an environmental chamber and the dissociation kinetics of GFP tagged WRN, BLM and RECQL4 at the site of laser induced DNA damage is shown in panel A, B and C, respectively. The time for image acquisition is shown for each panel. D. The percent maximum intensity values with respect to time of the dissociation of GFP tagged WRN, BLM and RECQL4 in HeLa cells have been represented below.