

The human Rothmund-Thomson syndrome gene product, RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability

Maja Petkovic¹, Tobias Dietschy¹, Raimundo Freire², Renjie Jiao^{1,*} and Igor Stagljär^{1,‡,§}

¹Institute of Vet. Biochemistry and Molecular Biology, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland

²Unidad de investigación, Hospital Universitario de Canarias, Ofra s/n, La Cuesta, 38071 Tenerife, Spain

*Present address: Institute of Biophysics, The Chinese Academy of Sciences, Datun Road 15, Chaoyang District, Beijing 100101, China

‡Present address: Department of Biochemistry and Department of Medical Genetics and Microbiology, The Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, 1 Kings College Circle, Toronto, ON, Canada, M5S 1A8

§Author for correspondence (e-mail: stagljär@utoronto.ca)

Accepted 14 June 2005

Journal of Cell Science 118, 4261–4269 Published by The Company of Biologists 2005

doi:10.1242/jcs.02556

Summary

Rothmund-Thomson syndrome (RTS) is a human genetic disorder characterized by genome instability, cancer susceptibility and premature aging. The gene defective in a subset of RTS cases, *RECQL4*, encodes a member of the RecQ family of DNA helicases. To better define the function of the RECQL4 protein, we have determined its subcellular localization. We have raised antibodies against the N- and C-terminal parts of RECQL4 and could show that in various human cells endogenous RECQL4 forms discrete nuclear foci that colocalize with promyelotic leukaemia protein (PML). The number of foci and their colocalization with PML does not significantly change after induction of different types of DNA damages. Silencing of RECQL4 expression by siRNA causes a significant reduction in RECQL4 nuclear foci formation. Furthermore, we

demonstrate that RECQL4 foci coincide with foci formed by human Rad51 and regions of single-stranded DNA after induction of DNA double-strand breaks. In agreement with this, we also show that RECQL4 and Rad51 form a complex in human cells. Our findings suggest a role for RECQL4 in the repair of DNA double-strand breaks by homologous recombination and shed new light onto RECQL4's function in human cells.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/118/18/4261/DC1>

Key words: Genome stability, Rothmund-Thomson syndrome (RTS), RecQ helicases, Immunofluorescence, Promyelotic leukaemia protein (PML), Rad51

Introduction

Genome instability is thought to play a major role in the development and progression of cancer, and has also been implicated in the aging process. One important family of proteins required to maintain genome stability is the RecQ family of DNA helicases (Hickson, 2003). This highly-conserved protein family includes *Escherichia coli* RecQ (Nakayama et al., 1984), *Saccharomyces cerevisiae* Sgs1p (Gangloff et al., 1994), *Schizosaccharomyces pombe* Rqh1p (Enoch et al., 1992), and at least five human homologs named *RECQL1* (Puranam and Blackshear, 1994; Seki et al., 1994), *WRN* (Yu et al., 1996), *BLM* (Ellis et al., 1995), *RECQL4* and *RECQL5* (Kitao et al., 1998). Common to all RecQ family members is a helicase domain that comprises seven highly conserved motifs of the DEXH-box family that are found in many DNA and RNA helicases (Singleton and Wigley, 2002). During the past decade, there has been increasing interest in the RecQ family of DNA helicases because a group of rare, autosomal recessive human disorders has been linked to mutations in some of its members. These disorders include Werner's syndrome (WS), the premature aging disease caused

by mutations in *WRN* gene (Yu et al., 1996); Bloom's syndrome (BS), a cancer predisposition disease caused by mutations in *BLM* gene (Ellis et al., 1995), and a subset of cases of RTS characterized by mutations in the *RECQL4* gene (Kitao et al., 1999b).

RTS displays heterogeneous clinical profiles that include growth-deficiency, photosensitivity with poikiloderma, cataracts, early graying and loss of hair, as well as the early development of cancers of many types, in particular osteosarcoma (Lindor et al., 2000). The gene mutated in a subset of RTS patients, *RECQL4*, contains 21 exons spanning only 6 kb of genomic DNA sequence and is located on human chromosome 8q24.3 (Kitao et al., 1999a). In addition to RTS, mutations in the *RECQL4* gene can also cause an RTS-similar disease termed RAPADILINO syndrome (Siitonen et al., 2003). The latter is an autosomal recessive disorder characterized by short stature, radial ray defects and other malformations, as well as infantile diarrhoea, but not by a significant cancer risk (Jam et al., 1999). The most common mutations of the *RECQL4* gene in RAPADILINO patients represent in-frame deletions of exon 7 (Siitonen et al., 2003). On the other hand, most alternations

of *RECQL4* found in RTS patients represent nonsense or frameshift mutations, resulting in a truncated polypeptide and map to the helicase domain (Kitao et al., 1999b; Wang et al., 2003). Somatic cells isolated from RTS individuals are sensitive to ionizing radiation (IR) (Vennos and James, 1995) and show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements (Der Kaloustian et al., 1990). In agreement with this notion, targeted disruption of *RECQL4* in mice results in abnormalities similar to those in RTS patients (Hoki et al., 2003). During G1 arrest, the promoter of the *RECQL4* gene is repressed by p53 that forms a complex with the transcription factor SP1 in concert with histone deacetylase 1 (HDAC1) (Sengupta et al., 2005). In human cells, *RECQL4* is associated with ubiquitin ligases UBR1 and UBR2, thus indicating a potential role of *RECQL4* in the N-end rule pathway (Yin et al., 2004).

Numerous studies on Sgs1p, Rqh1, BLM and WRN have revealed that RecQ helicases are involved in the maintenance of genome stability by functioning at the interface between DNA replication, recombination and repair (Hickson, 2003). However, the biochemical properties, interaction partners and subcellular localization of *RECQL4* have to date been largely unexplored. In this study, we have carried out a cellular localization analysis of the human *RECQL4* protein. To achieve this goal, we have raised two different anti-*RECQL4* antibodies that recognize a *RECQL4*-specific band from human cell extracts. We show that *RECQL4* forms nuclear foci in several human cell lines, the number of which does not significantly change after induction of different types of DNA damages. Importantly, downregulation of *RECQL4* by siRNA causes reduction in *RECQL4* nuclear foci formation. The *RECQL4* foci coincide with those formed by PML nuclear bodies and the number of colocalizing foci slightly decreases after induction of DNA double-strand breaks (DSBs). In addition, *RECQL4* forms a complex with the human Rad51 protein and colocalizes with Rad51 and regions of single-stranded DNA (ssDNA) upon induction of DNA DSBs. These findings suggest a role for *RECQL4* in the repair of DSBs by homologous recombination and indicate a novel function for *RECQL4* in human cells.

Materials and Methods

Cell culture and indirect immunofluorescence assays

The HeLa, 293T and WI38/VA13 cells were from the American Type Culture Collection (ATCC), AG18371 cells from Coriell and the primary skin fibroblasts were a kind gift from Silvio Hemmi, University of Zurich, Switzerland. Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) and 10% FBS. For some experiments cells were exposed to 10 μ M etoposide for 1 hour at 37°C, washed, given drug-free medium, and collected 10-12 hours later. For detection of ssDNA after damage, cells were grown on slides in medium containing 10 μ g/ml BrdU, exposed to 10 μ M etoposide for 1 hour in BrdU-free medium and fixed after 10 hours. For HU treatment cells were incubated with 10 mM HU for 24 hours, UV treatment was performed with 50 J/m² at 254 nm, cells were fixed 16 hours after the treatment; for ionizing-radiation treatment cells were exposed to 5 Gy and fixed 4 hours later. For the indirect immunofluorescence assays, cells growing on glass slides were incubated with 0.5% Triton X-100 in PBS for 5 minutes, fixed with 2% paraformaldehyde in PBS (20 minutes at RT), and permeabilized with 0.5% Triton X-100 in PBS (20 minutes at RT). After the blocking

step (0.1% Tween-20, 5% BSA in PBS, 20 minutes at RT), slides were incubated with a mixture of different rabbit and mouse antibodies. Rabbit antibodies were detected with Cy3-conjugated goat anti rabbit IgG (Jackson Laboratories; 1:200 in blocking buffer), and mouse antibodies were detected with FITC-conjugated goat anti mouse IgG (Jackson Laboratories; 1:100 in blocking buffer). To visualize nuclear DNA, slides were incubated with DAPI (0.4 μ g/ml). After washing, slides were mounted in Vectashield (Vector Laboratories) and viewed under confocal microscope Leica TCS 4D. Images were processed by Imaris software. For statistical analysis a minimum of 200 cells was counted in two independent experiments.

Antibody production

The rabbit polyclonal *RECQL4* N-t and *RECQL4* C-t antisera were raised against recombinant human *RECQL4* fragments comprising amino acid residues 359 to 478 and 911 to 1208, respectively. For that, the corresponding cDNA fragments were cloned in pET-30 (*RECQL4* N-t) or pET-28 (*RECQL4* C-t) expression vectors (Novagen) and recombinant proteins fused to a histidine tag were purified using Ni-NTA resin (Qiagen) following the manufacturer's instructions.

RECQL4 N-t and C-t antibodies were affinity-purified using hexahistidine-tagged *RECQL4* N-t and C-t recombinant proteins as an affinity matrix, respectively. The purified proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with Ponceau S, and the corresponding 6xHis-*RECQL4* band was excised. The strips were blocked for 1 hour with 2% bovine serum albumin in TBS (10 mM Tris, pH 7.4, 150 mM NaCl) containing 0.1% Tween 20. The strips were then incubated overnight with rabbit antiserum and washed with TBS containing 0.1% Tween 20 and 1 M NaCl. Anti-*RECQL4* antibodies were eluted by incubation in 100 mM glycine pH 2.3, for 5 minutes. The eluate was then immediately neutralized with 1 M Tris-HCl pH 8.8.

Antibodies

In immunofluorescence experiments, anti-PML antibody (Santa Cruz, PG-M3) was used in a 1:50 dilution, anti-Rad51 antibody (abcam, ab1837) in a 1:50 dilution, anti-BrdU antibody (Pharmingen, 3D4) in a 1:50 dilution, anti-*RECQL4* N-t antibody in a 1:200 dilution. For western blots, anti-*RECQL4* N-t and anti *RECQL4* C-t antibodies were used in a 1:2000 dilution, anti-tubulin antibody (Santa Cruz, TU-02) in a 1:1000 dilution, anti-PARP1 antibody (Petrilli et al., 2004) in a 1:2000 dilution, anti-BLM antibody (Wu et al., 2000) in a 1:1500 dilution, anti-GRB2 antibody (BD Transduction, G10111) in a 1:2000 dilution, anti-FLAG antibody (Sigma, M2) in a 1:2000 dilution and anti-Rad51 antibody (Tan et al., 1999) in a dilution 1:20,000.

Total cell and nuclear extracts

Total cell extracts were prepared from exponentially growing HeLa cells by lysis in lysis buffer (50 mM Hepes, pH 7.6; 500 mM NaCl; 1% Triton X-100, protease inhibitors) for 15 minutes with rolling at 4°C and centrifugation at 14,000 rpm for 15 minutes at 4°C. Nuclear extracts were prepared from HeLa cells, which were washed in PBS and re-suspended in buffer A (10 mM Hepes, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; protease inhibitors) and then centrifuged for 1 minute at 2000 rpm. The cell pellet was re-suspended in buffer A+ (buffer A; 0.1% Nonidet P 40) for 5 minutes on ice and centrifuged at 10,000 rpm, for 5 minutes. The supernatant contained cytosolic extract. The pellet was washed in buffer A and then re-suspended in buffer C (20 mM Hepes, pH 7.9; 420 mM NaCl; 1.5 mM MgCl₂; 25% glycerol, protease inhibitors) for 15 minutes, with rolling at 4°C. The samples were then centrifuged at 14,000 rpm, for 5 minutes. The supernatant contained nuclear extract and the NaCl concentration was adjusted to 120 mM with buffer D (20 mM Hepes, pH 7.9, protease inhibitors).

Transfection of cells and immunoprecipitation assay

293T cells were transiently transfected with p3xFLAG-myc-CMV-23 (Sigma) expressing RECQL4 or vector alone. Preparation of nuclear extracts was described in previous section. Aliquots (120 μ g) of nuclear extracts were incubated with 3 μ g of anti-FLAG antibody coupled to magnetic tosylactivated Dynabeads (DynaL Biotech, M-280) according to manufacturer's instructions in immunoprecipitation buffer (20 mM Hepes, pH 7.5; 120 mM NaCl; 5 mM MgCl₂; 0.1% Nonidet P 40; protease inhibitors) at 4°C overnight. As a control, nuclear extracts from untransfected cells were used. The beads were washed three times in immunoprecipitation buffer before the protein complexes bound to the beads were eluted and analysed by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis. A 12 μ g aliquot of nuclear extract was used as input control. For the reverse immunoprecipitation assay, an aliquot of nuclear extracts (120 μ g) was incubated with 3 μ l (approximately 2 μ g) of polyclonal anti-Rad51 antibody (kind gift of Roland Kanaar, Erasmus University, Rotterdam, The Netherlands) in immunoprecipitation buffer for 3 hours, to which 20 μ l of protein G sepharose 4 fast flow (Amersham Biosciences) beads were added and incubated for an additional 2 hours. As a control, nuclear extracts were incubated with 1.2 μ g of rabbit IgG (Santa Cruz). The beads were washed three times in immunoprecipitation buffer before the protein complexes bound to the beads were eluted and analysed by SDS gel electrophoresis.

RNA-mediated interference

For short interfering RNA (siRNA) experiments, RECQL4-1 5'-aacuucgacuccugugagu-3' and control-1 5'-aacuagcucuccugaggggu-3' with dTdT overhang (Qiagen, Operon) were used. The siRNAs were transfected in HeLa cells using a 200 nM siRNA concentration with Oligofectamine (Invitrogen) according to manufacturer's instruction and harvested after 24 hours.

Results

Generation of anti-RECQL4 antibodies

Antibodies against N- and C-termini of RECQL4 were raised in rabbits using chimeric proteins consisting of 6xHis-tag fused to residues 359-478 and 911-1208 of RecQL4. These regions

of RECQL4 were chosen because they show no sequence homology to other human RecQ helicases. These sera, designated anti-RECQL4 N-t and anti-RECQL4 C-t, respectively, were affinity-purified as described in Materials and Methods. In western blots, both sera recognized their protein fragments used for immunization (data not shown), and a major protein band in HeLa nuclear, cytosolic and total cell extracts with a molecular mass of approximately 150 kDa (Fig. 1A). To control our cell fractionation experiments, we examined the distribution of the endogenous human nuclear proteins PARP1 and BLM as well as a cytosolic protein GRB2. As expected, a band corresponding to PARP1 and BLM was detected in the nuclear extract only, whereas the band corresponding to GRB2 was detected only in the cytosolic extract (Fig. 1A, three lower panels).

In addition, both RECQL4 antisera also recognized FLAG-epitope-tagged RECQL4, expressed in 293T cells, with the expected molecular mass (Fig. 1B). No such protein was detected in HeLa nuclear and total cell extracts with preimmune serum (data not shown) or cell in extracts derived from the RTS-patient cell line AG18371, using both of the anti-RECQL4 sera (Fig. 1A). Likewise, both RECQL4 antibodies did not cross-react with purified recombinant BLM and WRN helicases (data not shown). These results, and the results from the siRNA experiments (discussed below), show that both antisera are specific for the RECQL4 protein.

RECQL4 forms discrete nuclear foci in various human cells

Next, we used the affinity purified anti-RECQL4 N-t antiserum to visualize the in situ distribution of RECQL4 in human cells. Indirect immunofluorescence staining analysis by confocal microscopy of exponentially growing HeLa, WI38/VA13 and primary skin fibroblast cell lines revealed a punctate nuclear staining in all three cell lines, which was observed in the majority of the cells (Fig. 2A-C). No cytoplasmic staining was evident in any of the examined cells, which is contradictory

to our western blot results. This can be explained by the difference in sample preparation and detection methods used in these two assays, and that the protocol used for our immunofluorescence assays presumably removes the RECQL4 protein – which is not bound to the nuclear matrix. The calculated average number of RECQL4 foci formed per cell is 25. Furthermore, no specific fluorescence was seen in the cells with pre-immune serum (Fig. 2D).

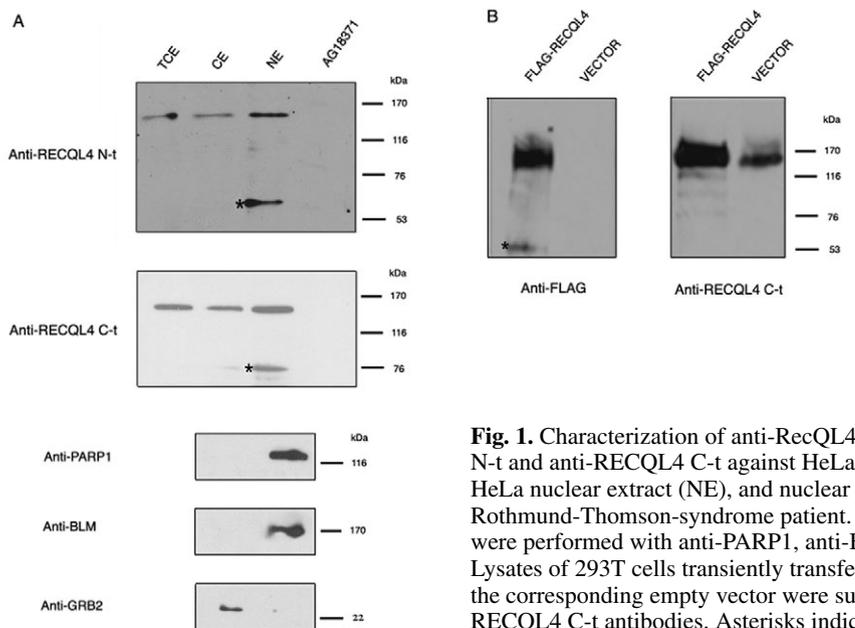


Fig. 1. Characterization of anti-RecQL4 antibodies. (A) Western blot using the anti-RECQL4 N-t and anti-RECQL4 C-t against HeLa total cell extract (TCE), HeLa cytosolic extracts (CE), HeLa nuclear extract (NE), and nuclear extracts from AG18371 fibroblasts derived from a Rothmund-Thomson-syndrome patient. Control western blots against HeLa CE and HeLa NE were performed with anti-PARP1, anti-BLM and anti-GRB2 antibodies, respectively. (B) Lysates of 293T cells transiently transfected with a vector expressing FLAG-RECQL4 or with the corresponding empty vector were subjected to western blot with anti-FLAG and anti-RECQL4 C-t antibodies. Asterisks indicate breakdown products.

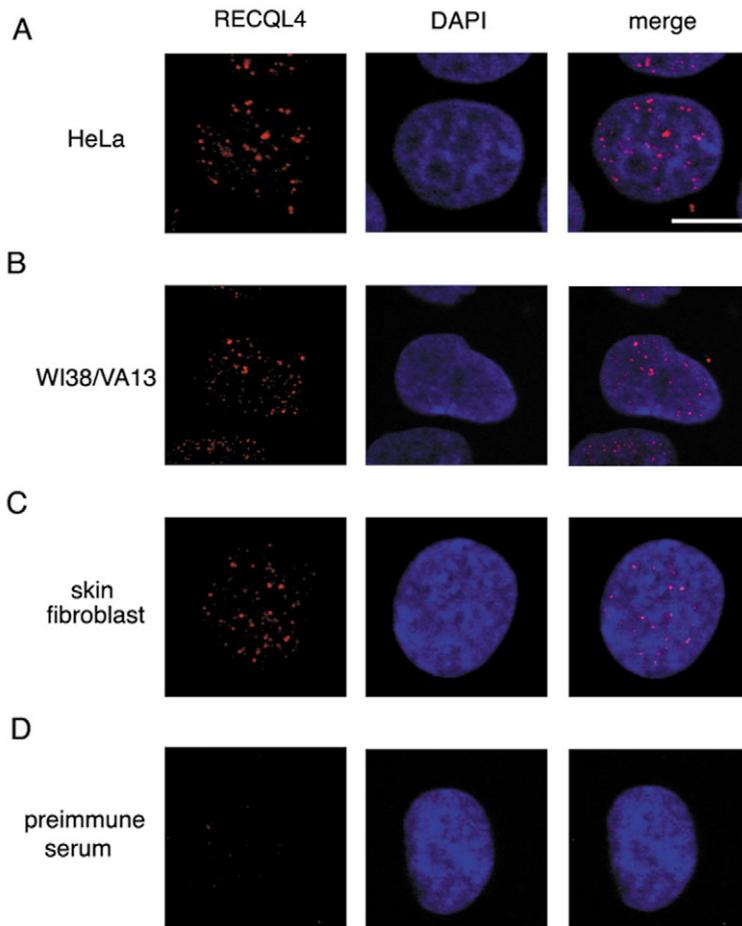


Fig. 2. RECQL4 localizes to nuclear foci in different cell lines. The foci were detected with anti-RECQL4 N-t antibody (red). DAPI-staining shows nuclear DNA. Bar, $\sim 10 \mu\text{m}$. (A) HeLa cell. (B) WI38/VA13 cell. (C) Primary skin fibroblast. (D) Preimmune serum, HeLa cell.

To further confirm the specificity of RECQL4 nuclear foci staining, we silenced RECQL4 using RNA interference. We transiently transfected HeLa cells with siRNA RECQL4-1 directed against the RECQL4 mRNA, and control-1 siRNA containing a scrambled sequence of RECQL4-1. We first examined the efficiency of silencing of RECQL4 by immunoblotting. As shown in Fig. 3A, silencing of RECQL4 occurred only in cells transfected with RECQL4-1 siRNA, in which approximately a 70% decrease in the level of endogenous RECQL4 was detected compared with the cells transfected with a control-1 siRNA. An even more pronounced effect was observed after immunofluorescence staining analysis (Fig. 3B). We detected a significant reduction of RECQL4 nuclear foci in the cells transfected with siRNA RECQL4-1, which was not the case for the cells treated with the control-1 siRNA (Fig. 3B,C).

We also examined whether the RECQL4 nuclear foci staining changes after induction of DNA damage. For that purpose, HeLa and WI38/VA13 human fibroblasts were treated with different DNA damaging agents including ionizing radiation (IR), which produces DSBs; ultraviolet light (UV), which generates predominantly pyrimidine dimers and 6-4 photoproducts; and hydroxyurea (HU), an inhibitor of DNA

replication. As shown in supplementary material Fig. S1, indirect immunofluorescence analysis showed that there was no significant change in the number of RECQL4 foci after these treatments.

RECQL4 colocalizes with promyelotic leukaemia protein (PML) nuclear bodies

Previous reports have identified a dynamic association of BLM with promyelotic leukaemia protein (PML) (Bischof et al., 2001; Zhong et al., 1999). PML functions as a tumor suppressor, and localizes to nuclear foci termed PML nuclear bodies (PML NBs) (Hodges et al., 1998). It has been suggested that these localization sites function as protein-storage sites, sites where multi-subunit complexes form and post-translational modification of regulatory factors occur, and that are modified in response to a variety of stresses including oncogenes (Dellaire and Bazett-Jones, 2004), and DNA breaks (Carbone et al., 2002). We have therefore analyzed whether the degree to which RECQL4 foci colocalized with PML in exponentially growing, untreated HeLa, WI38/VA13 and primary skin fibroblast cells. In these cells, we observed a partial colocalization between RECQL4 and PML (Fig. 4, upper panel). On average, 14% of RECQL4 foci colocalized with PML foci and 27% of PML foci colocalized with RECQL4 foci, an observation seen in 90% of cells. We then wanted to test whether the number of colocalizing RECQL4 and PML foci changes after treatment of cells with DNA-damaging agents. Upon treatment with a DNA-DSB-inducing agent, etoposide, the number of colocalizing foci was slightly reduced (Fig. 4, lower panel). In 90% of treated cells, 10% of RECQL4 foci colocalized with PML foci and 18% of PML foci colocalized with RECQL4 foci. Collectively, these data indicate that at least a portion of RECQL4 foci is associated with PML NBs and that, after induction of DNA DSBs, a minor portion of RECQL4 foci relocalizes from PML NBs.

RECQL4 colocalizes with regions of ssDNA

We next wanted to determine whether RECQL4 is present at regions of ssDNA after the induction of DSBs. For that purpose, 5-bromo-2'-deoxyuridine (BrdU), was used to visualize sites of ssDNA that are often associated with DSB repair. BrdU is inaccessible to an anti-BrdU antibody when present in duplex DNA, but readily accessible to the antibody when the DNA is denatured or single-stranded (Raderschall et al., 1999). HeLa cells that were grown in medium containing BrdU, then fixed with paraformaldehyde and stained under non-denaturing conditions, showed no significant staining, confirming that the antibody does not detect BrdU in duplex DNA (Fig. 5A, upper panel). The same cells, however, showed the expected pattern of RECQL4 foci, indicating that BrdU does not perturb RECQL4 localization. After exposing BrdU-labelled cells to etoposide, BrdU foci appeared in the majority of the cells (Fig. 5A, lower panel). These foci formed because of DNA DSBs, where they presumably identify sites of repair. To determine whether RECQL4 localizes with BrdU, we co-

stained for RECQL4 and BrdU 10 to 12 hours after etoposide treatment. We could detect partial colocalization between RECQL4 and regions of ssDNA as determined by anti-BrdU staining (Fig. 5A, lower panel). There were 9% of RECQL4 foci colocalizing with BrdU foci and 18% of BrdU foci colocalizing with RECQL4 foci. The RECQL4/BrdU colocalization was evident in 46% of cells. Similar results were obtained after cell treatment with IR (data not shown). These results suggest that a portion of RECQL4 foci can associate

with regions of ssDNA after exposure to DSB-inducing agent, and might represent sites of ongoing DNA repair.

RECQL4 forms a complex in human cells and colocalizes with human Rad51 after the induction of DNA double-strand breaks

It has been previously reported that Rad51 foci, which are important for DNA repair by homologous recombination, accumulate at sites of ssDNA after induction of DSBs (Raderschall et al., 1999). The colocalization of RECQL4 and regions of ssDNA after induction of DSBs prompted us to test whether RECQL4 is a component of Rad51 foci after the same treatment. In exponentially growing, untreated asynchronous WI38/VA13 cells, no Rad51 foci were observed (Fig. 5B, upper panel). This is in agreement with previous studies, which showed that Rad51 foci are detectable only in S phase and/or after induction of DSBs (Tashiro et al., 1996; Thompson and Schild, 2001). However, after we treated the cells with etoposide, the percentage of cells with focally concentrated Rad51 increased. We found that in 43% of these cells, 29% of Rad51 foci colocalized with RECQL4 foci and 10% of RECQL4 foci colocalized with Rad51 foci, indicating that at least a portion of RECQL4 nuclear foci is recruited to sites of DNA-damage-induced ssDNA (Fig. 5B, lower panel). Similar results were obtained with HeLa cells and primary skin fibroblast cells after exposure to etoposide (data not shown). Additionally, in etoposide-treated HeLa cells transfected with RECQL4-1 siRNA, but not in cells transfected with the control siRNA, there was a significant reduction in the formation of RECQL4 foci and thus no colocalization of RECQL4 with Rad51 (supplementary material Fig. S2).

We next analysed whether RECQL4 and Rad51 interact in human cells using a co-immunoprecipitation assay. To that end, the FLAG-RECQL4 was transiently transfected into 293T cells. Nuclear extracts were then immunoprecipitated with anti-FLAG resin and analysed by immunoblotting using the anti-Rad51 antibody. As can be seen in the left panel of Fig. 5C, endogenous Rad51 was efficiently co-precipitated by the anti-FLAG antibody (lane 3), but was not detected when the control IgG antibody was used (lane 2). The reciprocal co-immunoprecipitation experiment was also carried out with an anti-Rad51 polyclonal antibody, to immunoprecipitate FLAG-RECQL4 from 293T nuclear extracts. As shown in the right panel of Fig. 5C, RECQL4 could be specifically co-immunoprecipitated with endogenous anti-Rad51 (lane 6), but not with the control IgG antibody (lane 5). Taken together, these data indicate that RECQL4 and Rad51 form a complex in human cells and that, in response to the induction of DSBs, a portion of RECQL4 and Rad51 nuclear foci colocalize to sites that represent sites of undergoing repair by homologous recombination.

BRCA1, a multifunctional protein with proposed roles in the repair of DSBs by homologous recombination, transcriptional activation, cell-cycle regulation and apoptosis (Narod and Foulkes, 2004), has been shown to colocalize with Rad51 in S-phase foci (Scully et al., 1997)

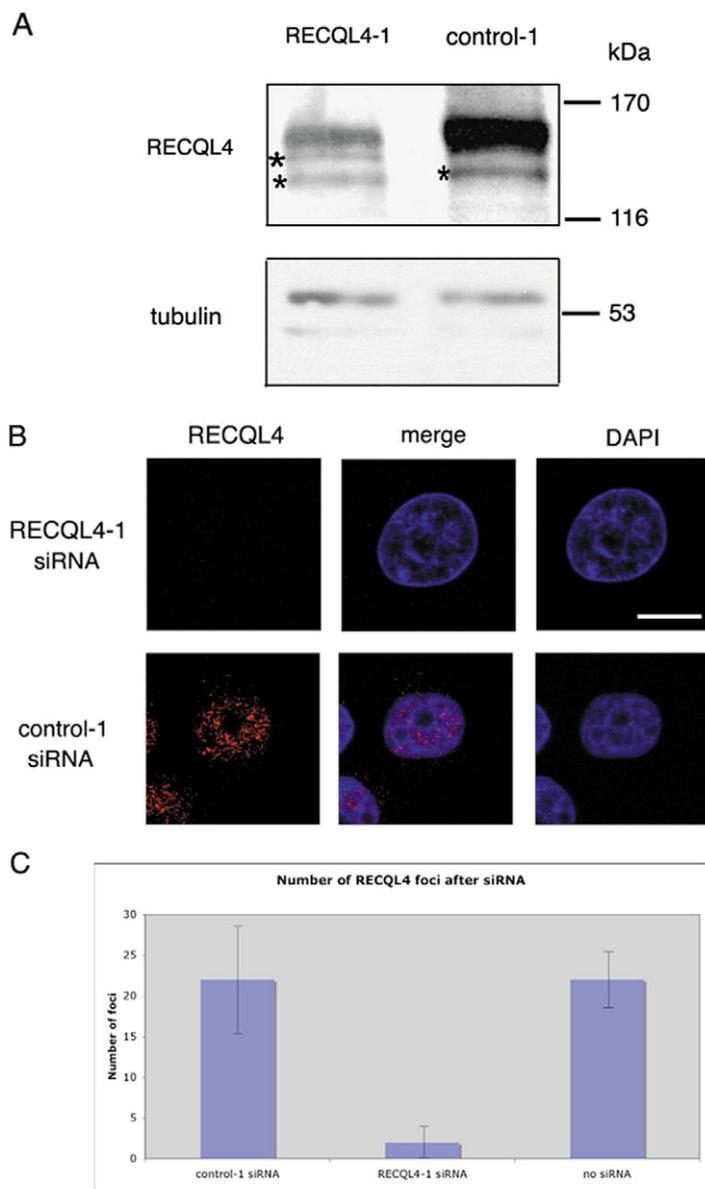


Fig. 3. Determination of the specificity of the RECQL4 antibodies by siRNA. (A) Western blot with anti-RECQL4 C-t antibody (upper panel) or anti-tubulin antibody (lower panel) using total cell extracts from HeLa cells transfected with 200 nM RECQL4-1 or control-1 siRNA. Asterisks indicate breakdown products. (B) RECQL4 knock-down leads to a loss of RECQL4 nuclear foci. Immunofluorescence analysis with anti-RECQL4 N-t antibody (red) on exponentially growing HeLa cells transfected with 200 nM RECQL4-1 or control-1 siRNA, DAPI-staining shows nuclear DNA (blue). Bar, ~10 μ m. (C) The mean number of RECQL4 foci per cell after siRNA treatment is displayed in histograms (standard error of the mean is indicated in the columns).

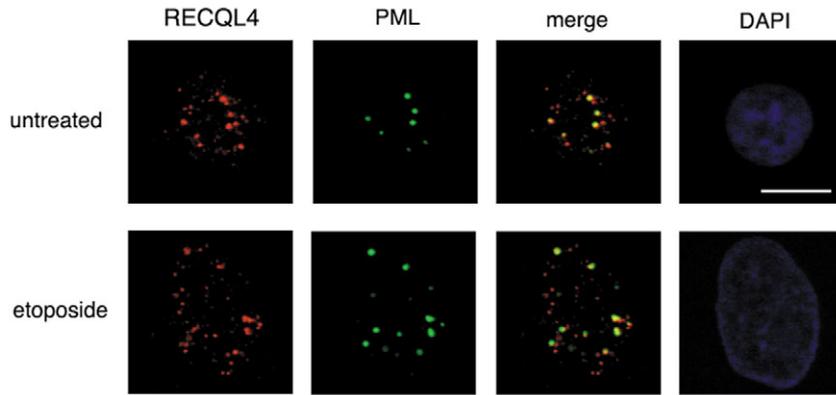


Fig. 4. RECQL4 colocalizes with PML. HeLa cells were stained with anti-RECQL4 (red) and anti-PML (green) antibodies. Where red and green signals overlap, a yellow pattern is seen, indicating colocalization of RECQL4 and PML. DAPI-staining shows nuclear DNA. Bar, $\sim 10 \mu\text{m}$. (Upper panel) Colocalization of RECQL4 and PML in untreated HeLa cells. (Lower panel) Colocalization of RECQL4 and PML after $10 \mu\text{M}$ etoposide treatment of HeLa cells.

and IR-inducible foci (Bhattacharyya et al., 2000). We therefore wanted to determine whether RECQL4 protein colocalizes with BRCA1, by performing double immunolabeling in untreated HeLa cells and cells treated with $10 \mu\text{M}$ etoposide. In contrast to the partial RECQL4/Rad51 colocalization, a very weak colocalization of RECQL4 with BRCA1 was observed (Fig. 5D). Namely, 2% of RECQL4 foci colocalized with BRCA1 foci and 5% of BRCA1 foci colocalized with RECQL4 foci, an observation made in only 11% of cells. We conclude from this result that RECQL4 foci differ from BLM foci, which colocalize with the BRCA1 protein (Jiao et al., 2004; Wang et al., 2000).

Discussion

Numerous genetic, biochemical and cell biological studies in yeast and human cells have suggested that the members of the RecQ helicase family play a pivotal role in maintaining genome stability (Hickson, 2003). Along with this notion, mutations in the *RECQL4* gene have been shown to cause some cases of the Rothmund-Thomson and RAPADILINO syndromes, which have severe physiological consequences in humans, the most prominent of which are cancer susceptibility and premature aging. At the cellular level, defects in *RECQL4* cause genome instability particularly trisomy, aneuploidy, deletions, translocations and high frequencies of chromosomal rearrangements. Despite this knowledge, the molecular function of RECQL4, as well as the cellular pathways in which it is involved, remain poorly understood. In the current study, we have shown for the first time that endogenous RECQL4 localizes to distinct nuclear foci in various human cell lines. This nuclear foci staining pattern is specific for RECQL4 because silencing of RECQL4 through RNA interference markedly decreases the RECQL4 nuclear foci formation. In contrast to previous studies involving BLM and WRN helicases, which have shown their presence in the nucleus (Bischof et al., 2001; Jiao et al., 2004; Pedrazzi et al., 2001; Sakamoto et al., 2001; Yankiwski et al., 2000), our cellular fractionation experiments showed that, although a majority of RECQL4 is present in the nucleus, a small amount of it can be found in the cytosolic fraction of HeLa cell extracts. A similar result has also been recently reported by Yin and colleagues, who showed that the greater portion of RECQL4 protein is present in the cytosol compared to the nucleus of transformed human cells (Yin et al., 2004). At the present time, the precise

role of such 'cytosolic RECQL4' is not known and remains to be determined. Our data that RECQL4 forms nuclear foci seemingly contrasts with the data of Yin and colleagues, who reported that RECQL4 is present in both the cytoplasm and the nucleus of human cells, with a punctate and uniform distribution of RECQL4 in both compartments (Yin et al., 2004). These differences are probably the result of different cell fixation approaches and antibodies that have been employed in these two studies.

Furthermore, we have provided evidence that the number of RECQL4 foci does not significantly change upon cellular treatment with different DNA damaging agents including IR, UV light and HU. Interestingly, these data differ from those obtained for BLM and WRN helicases, and may thus suggest that RECQL4 has a different function in human cells. Namely, previous studies have shown that BLM assembles into nuclear foci specifically in response to agents that cause DNA DSBs (Bischof et al., 2001; Davalos and Campisi, 2003) and block replication (Jiao et al., 2004; Sengupta et al., 2004). However, WRN was shown to traffic between the nucleolus and nucleoplasm, and to form nuclear foci in response to treating cells with various DNA-damaging agents (Gray et al., 1998; Sakamoto et al., 2001).

We have also found that a portion of RECQL4 foci colocalizes with PML NBs in untreated human cells. PML NBs have been shown to be the sites of BLM, RAD51, MRE11 and p53 assembly during late S-G2 phase and following the induction of DSBs (Bischof et al., 2001; Carbone et al., 2002). It has therefore been suggested that these foci represent sites of presumptive homologous recombinational repair after induction of DSBs (Bischof et al., 2001). Given the fact that a portion of RECQL4 foci colocalizes with PML NBs, our findings suggest that RECQL4 plays an active role in DSB repair. Furthermore, we have observed a slight decrease in the number of RECQL4- and PML-colocalizing foci after treatment of cells with the DSB-inducing agent etoposide. Similarly to this observation, it has recently been reported that, BLM helicase relocalizes from PML NBs after replication stall due to HU treatment (Davalos et al., 2004). Thus, our results also suggest that RECQL4 leaves PML foci and, after induction of DNA damage, relocalizes in a similar manner as BLM. In other words, there are different pools of RECQL4 foci in the cell that have a different function after induction of DNA damage.

We have also demonstrated that RECQL4 is a component of

a portion of Rad51 foci that is thought to be crucial for the repair of DSBs by homologous recombination. We found that RECQL4 forms a complex with Rad51 in human cells and partially colocalizes with Rad51 and regions of ssDNA after

induction of DSBs. Nuclear foci containing the Rad51 protein were shown to include other recombination proteins, such as Rad52, Rad54, the single-strand-binding-protein RPA, and the tumor suppressors BRCA1 and BRCA2 (Tarsounas et al., 2004). Given the fact that the processing of DSBs frequently involves the formation of long 3' single-stranded regions and that Rad51 is tightly associated with these structures in vivo, which promote pairing and strand-exchange between ssDNA and homologous dsDNA (Raderschall et al., 2002; Raderschall et al., 1999), one possible interpretation of the RECQL4/Rad51 complex formation, and RECQL4/PML as well as RECQL4/Rad51 colocalization is that, these structures represent the physical sites where the repair of DSBs by homologous recombination is coordinated and monitored. In agreement with this observation it has previously been shown that cultured cells from RTS patients are sensitive to IR, which further suggests that RECQL4 might participate in DSB repair (Vennos and James, 1995). However, it should be noticed that, it remains to be determined whether RECQL4 has a direct or indirect role in DSB

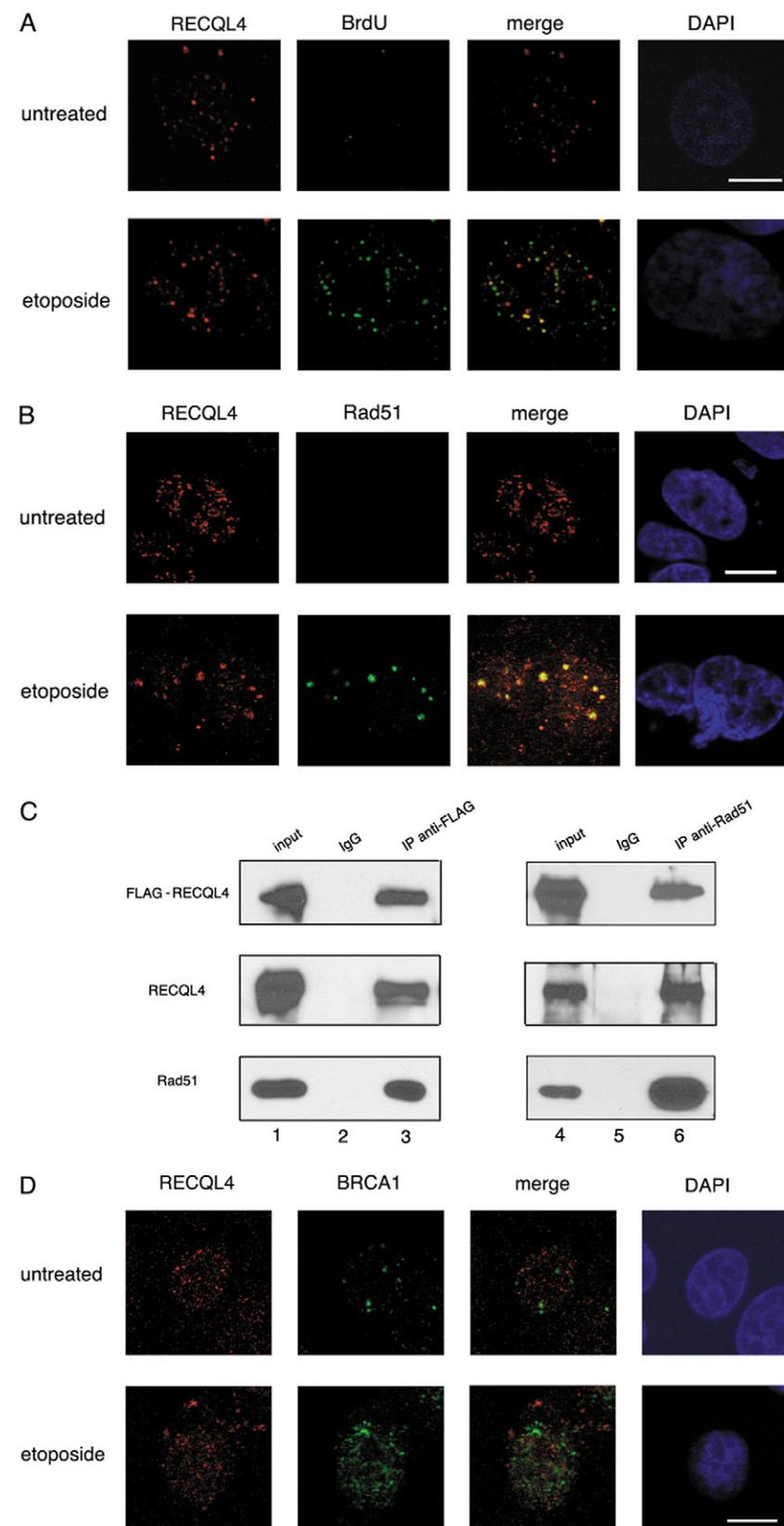


Fig. 5. RECQL4 is associated with regions of ssDNA and colocalizes with Rad51, but it does not colocalize with BRCA1. (A) HeLa cells were grown in BrdU-containing medium and stained for RECQL4 (red), BrdU (green) and nuclear DNA (blue). Bar, ~10 μ m. (Upper panel) RecQL4 and BrdU staining in untreated cells. (Lower panel) RECQL4 colocalizes with ssDNA after treatment with 10 μ M etoposide. (B) RECQL4 colocalizes with Rad51. WI38/VA13 cells were stained with anti-RECQL4 (red) and anti-Rad51 antibodies (green), and DAPI (blue). RECQL4 and Rad51 antibody-staining in untreated cells (upper panel) and after 10 μ M etoposide treatment (lower panel). Bar, ~10 μ m. (C) RECQL4 and Rad51 form a complex in human cells. 293T cells were transiently transfected with FLAG-RECQL4, nuclear extracts derived from these cells were immunoprecipitated with the anti-FLAG antibody, and analysed by SDS-polyacrylamide gel electrophoresis. One-tenth of the same nuclear extract was used as input control (lane 1). Immunoprecipitated FLAG-RECQL4 (upper and middle panels) and Rad51 (lower panel) were detected by western blotting with the anti-FLAG, anti-RECQL4 C-t and anti-Rad51 antibodies, but not with the control IgG (lane 2). Reciprocal co-immunoprecipitation is shown in lanes 4-6; lane 4, input; lane 5, immunoprecipitation with the control IgG; lane 6, immunoprecipitation with an anti-Rad51 antibody. (D) Colocalization of RECQL4 with BRCA1 in untreated HeLa cells (upper panel) and in HeLa cells treated with 10 μ M etoposide (lower panel). RECQL4 (red), BRCA1 (green), DAPI-staining shows nuclear DNA. Bar, ~10 μ m.

repair by homologous recombination. Similarly to BLM and WRN helicases, RECQL4's function could be to resolve aberrant DNA structures that are formed during DNA replication and recombinational repair processes. Alternatively, RECQL4 could have a signalling function during the same processes through interactions with other proteins. Either hypothesis or a combination of the two would explain the genome instability displayed by RTS cells and the high incidence of cancer seen in RTS patients.

Our results also show that RECQL4 foci, unlike BLM foci, do not coincide with BRCA1 (Jiao et al., 2004). Previous work has revealed that BRCA1 colocalizes and resides in a so-called 'BASC' (BRCA1-associated genome surveillance complex) that consists of 40 different proteins, including BLM, RAD50, ATM, RAD50/MRE11/NBS1 complex, MSH6, MSH2, MLH1 and three subunits of the replication factor C (RFC) complex (namely p140, p37 and p34) (Wang et al., 2000). BLM and BRCA1 foci do not coincide in untreated human cells; however, their colocalization is greatly enhanced in those cells that are in mid-to-late S phase or in G2 phase after treatment with HU or IR, which suggests a specific requirement for BLM/BRCA1 in replication and/or repair of late-replicating DNA (Wang et al., 2000). In this way, our results imply that RECQL4 foci are distinct from the BLM foci, suggesting that RECQL4 does not participate in the same damage response pathway as BLM.

In conclusion, we have shown that endogenous human RECQL4 localizes to distinct nuclear foci that coincide with PML NBs, Rad51 and regions of ssDNA, suggesting that RECQL4 plays a role in the repair of DSBs by homologous recombination. Further confirmation of the above mentioned results will come from genetic, biochemical and protein-protein-interaction assays performed on RECQL4. These approaches, along with the cell biological approach described in this study, will ultimately help to understand the function of RECQL4 protein in human cells and to understand how genome instability influences cancer predisposition and aging.

We thank Silvio Hemmi for the gift of primary skin fibroblast cell line, Roland Kanaar for the gift of the anti-Rad51 antibody, Paul Hassa and Michael Hottiger for the gift of the anti-PARP1 antibody, Veronique Smits for the gift of the anti-GRB2 antibody, Ian Hickson for the gift of the anti-BLM antibody and Vilhelm Bohr, Michael Fetchko, Ian Hickson, Orlando Schärer, Caroline Zbinden and other members of the Stagljar group for helpful discussions. We are grateful to the Elektronenmikroskopisches Zentrallaboratorium der Universität Zürich for providing confocal facilities for this study. I.S.'s group is financed by grants from the Union Bank of Switzerland (im Auftrag eines Kunden), Gebert Ruff Foundation, EU Grant HPRN-CT-2002-00240, Schweizer Bundesamt für Bildung und Wissenschaft BBW Nr. 01-0528, the Swiss Cancer League (OCS-01310-02-2003) and the Swiss National Science Foundation (Nr. 3100A0-100256/1). R.F. is supported by the EU Grant HPRN-CT-2002-00240 and the GEN2003-20243-C08-07 from the Spanish Ministry of Education and Science.

References

Bhattacharyya, A., Ear, U. S., Koller, B. H., Weichselbaum, R. R. and Bishop, D. K. (2000). The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J. Biol. Chem.* **275**, 23899-23903.

- Bischof, O., Kim, S. H., Irving, J., Beresten, S., Ellis, N. A. and Campisi, J. (2001). Regulation and localization of the Bloom syndrome protein in response to DNA damage. *J. Cell Biol.* **153**, 367-380.
- Carbone, R., Pearson, M., Minucci, S. and Pelicci, P. G. (2002). PML NBs associate with the hMre11 complex and p53 at sites of irradiation induced DNA damage. *Oncogene* **21**, 1633-1640.
- Davalos, A. R. and Campisi, J. (2003). Bloom syndrome cells undergo p53-dependent apoptosis and delayed assembly of BRCA1 and NBS1 repair complexes at stalled replication forks. *J. Cell Biol.* **162**, 1197-1209.
- Davalos, A. R., Kaminker, P., Hansen, R. K. and Campisi, J. (2004). ATR and ATM-dependent movement of BLM helicase during replication stress ensures optimal ATM activation and 53BP1 focus formation. *Cell Cycle* **3**, 1579-1586.
- Dellaire, G. and Bazett-Jones, D. P. (2004). PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. *BioEssays* **26**, 963-977.
- Der Kaloustian, V. M., McGill, J. J., Vekemans, M. and Kopelman, H. R. (1990). Clonal lines of aneuploid cells in Rothmund-Thomson syndrome. *Am. J. Med. Genet.* **37**, 336-339.
- Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M. and German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* **83**, 655-666.
- Enoch, T., Carr, A. M. and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Dev.* **6**, 2035-2046.
- Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L. and Rothstein, R. (1994). The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. *Mol. Cell. Biol.* **14**, 8391-8398.
- Gray, M. D., Wang, L., Youssoufian, H., Martin, G. M. and Oshima, J. (1998). Werner helicase is localized to transcriptionally active nucleoli of cycling cells. *Exp. Cell Res.* **242**, 487-494.
- Hickson, I. D. (2003). RecQ helicases: caretakers of the genome. *Nat. Rev. Cancer* **3**, 169-178.
- Hodges, M., Tissot, C., Howe, K., Grimwade, D. and Freemont, P. S. (1998). Structure, organization, and dynamics of promyelocytic leukemia protein nuclear bodies. *Am. J. Hum. Genet.* **63**, 297-304.
- Hoki, Y., Araki, R., Fujimori, A., Ohhata, T., Koseki, H., Fukumura, R., Nakamura, M., Takahashi, H., Noda, Y., Kito, S. et al. (2003). Growth retardation and skin abnormalities of the Recql4-deficient mouse. *Hum. Mol. Genet.* **12**, 2293-2299.
- Jam, K., Fox, M. and Crandall, B. F. (1999). RAPADILINO syndrome: a multiple malformation syndrome with radial and patellar aplasia. *Teratology* **60**, 37-38.
- Jiao, R., Bachrati, C. Z., Pedrazzi, G., Kuster, P., Petkovic, M., Li, J. L., Egli, D., Hickson, I. D. and Stagljar, I. (2004). Physical and functional interaction between the Bloom's syndrome gene product and the largest subunit of chromatin assembly factor 1. *Mol. Cell. Biol.* **24**, 4710-4719.
- Kitao, S., Ohsugi, I., Ichikawa, K., Goto, M., Furuichi, Y. and Shimamoto, A. (1998). Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. *Genomics* **54**, 443-452.
- Kitao, S., Lindor, N. M., Shiratori, M., Furuichi, Y. and Shimamoto, A. (1999a). Rothmund-thomson syndrome responsible gene, RECQL4: genomic structure and products. *Genomics* **61**, 268-276.
- Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M. and Furuichi, Y. (1999b). Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat. Genet.* **22**, 82-84.
- Lindor, N. M., Furuichi, Y., Kitao, S., Shimamoto, A., Arndt, C. and Jalal, S. (2000). Rothmund-Thomson syndrome due to RECQ4 helicase mutations: report and clinical and molecular comparisons with Bloom syndrome and Werner syndrome. *Am. J. Med. Genet.* **90**, 223-228.
- Nakayama, H., Nakayama, K., Nakayama, R., Irino, N., Nakayama, Y. and Hanawalt, P. C. (1984). Isolation and genetic characterization of a thymineless death-resistant mutant of Escherichia coli K12: identification of a new mutation (recQ1) that blocks the RecF recombination pathway. *Mol. Gen. Genet.* **195**, 474-480.
- Narod, S. A. and Foulkes, W. D. (2004). BRCA1 and BRCA2: 1994 and beyond. *Nat. Rev. Cancer* **4**, 665-676.
- Pedrazzi, G., Perrera, C., Blaser, H., Kuster, P., Marra, G., Freire, R., Ryu, G.-H., Jiricny, J. and Stagljar, I. (2001). Direct association of Bloom's syndrome gene product with the human mismatch repair protein MLH1. *Nucleic Acids Res.* **29**, 4378-4386.

- Petrilli, V., Herceg, Z., Hassa, P. O., Patel, N. S., Di Paola, R., Cortes, U., Dugo, L., Filipe, H. M., Thiemermann, C., Hottiger, M. O. et al. (2004). Noncleavable poly(ADP-ribose) polymerase-1 regulates the inflammation response in mice. *J. Clin. Invest.* **114**, 1072-1081.
- Puranam, K. L. and Blackshear, P. J. (1994). Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ. *J. Biol. Chem.* **269**, 29838-29845.
- Raderschall, E., Golub, E. I. and Haaf, T. (1999). Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proc. Natl. Acad. Sci. USA* **96**, 1921-1926.
- Raderschall, E., Bazarov, A., Cao, J., Lurz, R., Smith, A., Mann, W., Ropers, H. H., Sedivy, J. M., Golub, E. I., Fritz, E. et al. (2002). Formation of higher-order nuclear Rad51 structures is functionally linked to p21 expression and protection from DNA damage-induced apoptosis. *J. Cell Sci.* **115**, 153-164.
- Sakamoto, S., Nishikawa, K., Heo, S. J., Goto, M., Furuichi, Y. and Shimamoto, A. (2001). Werner helicase relocates into nuclear foci in response to DNA damaging agents and co-localizes with RPA and Rad51. *Genes Cells* **6**, 421-430.
- Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D. M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425-435.
- Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., Okumura, K. et al. (1994). Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res.* **22**, 4566-4573.
- Sengupta, S., Robles, A. I., Linke, S. P., Sinogeeva, N. I., Zhang, R., Pedoux, R., Ward, I. M., Celeste, A., Nussenzweig, A., Chen, J. et al. (2004). Functional interaction between BLM helicase and 53BP1 in a Chk1-mediated pathway during S-phase arrest. *J. Cell Biol.* **166**, 801-813.
- Sengupta, S., Shimamoto, A., Koshiji, M., Pedoux, R., Rusin, M., Spillare, E. A., Shen, J. C., Huang, L. E., Lindor, N. M., Furuichi, Y. et al. (2005). Tumor suppressor p53 represses transcription of RECQ4 helicase. *Oncogene* **24**, 1738-1748.
- Sittonen, H. A., Kopra, O., Kaariainen, H., Haravuori, H., Winter, R. M., Saamanen, A. M., Peltonen, L. and Kestila, M. (2003). Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of RECQL diseases. *Hum. Mol. Genet.* **12**, 2837-2844.
- Singleton, M. R. and Wigley, D. B. (2002). Modularity and specialization in superfamily 1 and 2 helicases. *J. Bacteriol.* **184**, 1819-1826.
- Tan, T. L., Essers, J., Citterio, E., Swagemakers, S. M., de Wit, J., Benson, F. E., Hoeijmakers, J. H. and Kanaar, R. (1999). Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation. *Curr. Biol.* **9**, 325-328.
- Tarsounas, M., Davies, A. A. and West, S. C. (2004). RAD51 localization and activation following DNA damage. *Philos. Trans. R. Soc London Ser. B* **359**, 87-93.
- Tashiro, S., Kotomura, N., Shinohara, A., Tanaka, K., Ueda, K. and Kamada, N. (1996). S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes. *Oncogene* **12**, 2165-2170.
- Thompson, L. H. and Schild, D. (2001). Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutat. Res.* **477**, 131-153.
- Vennos, E. M. and James, W. D. (1995). Rothmund-Thomson syndrome. *Dermatol. Clin.* **13**, 143-150.
- Wang, L. L., Gannavarapu, A., Kozinets, C. A., Levy, M. L., Lewis, R. A., Chintagumpala, M. M., Ruiz-Maldonado, R., Contreras-Ruiz, J., Cunniff, C., Erickson, R. P. et al. (2003). Association between osteosarcoma and deleterious mutations in the RECQL4 gene in Rothmund-Thomson syndrome. *J. Natl. Cancer Inst.* **95**, 669-674.
- Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J. and Qin, J. (2000). BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.* **14**, 927-939.
- Wu, L., Davies, S. L., North, P. S., Goulaouic, H., Riou, J. F., Turley, H., Gatter, K. C. and Hickson, I. D. (2000). The Bloom's syndrome gene product interacts with topoisomerase III. *J. Biol. Chem.* **275**, 9636-9644.
- Yankiwski, V., Marciniak, R. A., Guarente, L. and Neff, N. F. (2000). Nuclear structure in normal and Bloom syndrome cells. *Proc. Natl. Acad. Sci. USA* **97**, 5214-5219.
- Yin, J., Tae Kwon, Y., Varshavsky, A. and Wang, W. (2004). RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Hum Mol Genet.* **13**, 2421-2430.
- Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S. et al. (1996). Positional cloning of the Werner's syndrome gene. *Science* **272**, 258-262.
- Zhong, S., Hu, P., Ye, T. Z., Stan, R., Ellis, N. A. and Pandolfi, P. P. (1999). A role for PML and the nuclear body in genomic stability. *Oncogene* **18**, 7941-7947.