Cell, Vol. 121, 887–898, June 17, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.05.015

# Initiation of DNA Replication Requires the RECQL4 Protein Mutated in Rothmund-Thomson Syndrome

**Mahesh N. Sangrithi,[1,2] Juan A. Bernal,[1,2] Mark Madine,[2] Anna Philpott,[1] Joon Lee,[3] William G. Dunphy,[3] and Ashok R. Venkitaraman[1,2,*]**
[1]Cancer Research UK Department of Oncology
University of Cambridge
[2]The Medical Research Council Cancer Cell Unit
Hutchison/MRC Research Centre
Hills Road
Cambridge CB2 2XZ
United Kingdom
[3]Division of Biology
California Institute of Technology
Pasadena, California 91125

## Summary

**How the replication machinery is loaded at origins of DNA replication is poorly understood. Here, we implicate in this process the *Xenopus laevis* homolog (xRTS) of the RECQL4 helicase mutated in Rothmund-Thomson syndrome. xRTS, which bears homology to the yeast replication factors Sld2/DRC1, is essential for DNA replication in egg extracts. xRTS can be replaced in extracts by its human homolog, while RECQL4 depletion from mammalian cells induces proliferation failure, suggesting an evolutionarily conserved function. xRTS accumulates on chromatin during replication initiation, after prereplication-complex (pre-RC) proteins, Cut5, Sld5, or Cdc45 but before replicative polymerases. xRTS depletion suppresses the loading of RPA, the ssDNA binding protein that marks unwound origins before polymerase recruitment. However, xRTS is unaffected by xRPA depletion. Thus, xRTS functions after pre-RC formation to promote loading of replication factors at origins, a previously unrecognized activity necessary for initiation. This role connects defective replication initiation to a chromosome-fragility disorder.**

## Introduction

Mutations inactivating the human *RECQL4* gene occur in some cases of Rothmund-Thomson syndrome (RTS), a rare genetic disorder characterized by chromosome fragility, developmental abnormalities, and predisposition to cancers, including osteogenic sarcoma (Kitao et al., 1998; Kitao et al., 1999; Vennos and James, 1995). Distinct *RECQL4* mutations also occur in RAPADILINO syndrome, where they are associated with skeletal malformations but not cancer predisposition (Siitonen et al., 2003). Homozygosity for an extensive disruption of the murine homolog of *RECQL4* results in early embryonal lethality accompanied by defective cell proliferation (Ichikawa et al., 2002), suggesting an indispensable role in normal cell growth. A hypomorphic mutation deleting a single exon in the murine gene causes growth retardation, developmental anomalies, and impeded cell division in vitro (Hoki et al., 2003), while a gene deletion truncating the carboxyl-terminal half of the encoded protein triggers centromeric anomalies, aneuploidy, and cancer predisposition (Mann et al., 2005). Thus, correlations between genotype and phenotype in different *RECQL4* mutant alleles are complex and uncertain, providing little insight into the biological functions of the encoded protein.

Although *RECQL4* possesses a helicase domain characteristic of the RecQ family, it lacks many of the conserved features present in other members, such as the domains involved in DNA binding (Hickson, 2003). Indeed, the human diseases connected with *RECQL4* mutations appear distinct in their clinical phenotypes from Bloom or Werner's syndrome, caused by inactivation of the RecQ helicases *BLM* or *WRN*, respectively (Ellis, 1997).

We have cloned the *X. laevis* homolog, xRTS, of *RECQL4*, and report here that it has a novel function in the initiation of DNA replication not so far ascribed to other RecQ helicases. Our findings suggest that xRTS functions after prereplication-complex (pre-RC) assembly to make origins of replication accessible for loading of subsequent replication factors—an essential, early step in the initiation of eukaryotic DNA replication.

## Results

### Features of the xRTS Protein

xRTS encodes a novel protein of 1500 residues with 60% overall identity to its human counterpart. It, too, includes the ~380 amino acid helicase domain that defines it as a member of the RecQ family, but it lacks domains present in other vertebrate RecQ helicases (Figure 1A). A phylogenetic dendrogram of the known eukaryotic homologs of xRTS suggests early divergence from other members of the RecQ family (data not shown). Indeed, the amino-(N-)terminal region of xRTS (and its homologs in the human and other eukaryotic genomes) bears a hitherto unrecognized homology (Figure 1B) to the yeast proteins Sld2 (*S. cerevisiae)* and DRC1 (*S. pombe*), not found in other RecQ helicases. Sld2/DRC1 are essential for establishment of DNA replication forks in yeast but have no known homologs in vertebrates (Masumoto et al., 2002; Noguchi et al., 2002; Wang and Elledge, 1999). Pairwise comparisons between the N-terminal 210 residues of Sld2, DRC1, and xRTS (see Figure S1 in the Supplemental Data available with this article online) reveal that this region of xRTS is as similar (~20% identity) to the corresponding sequences in the yeast proteins as they are to one another. To characterize the function of xRTS, we raised a polyclonal rabbit antiserum directed against its N-terminal 260 residues. The antiserum recognizes a single 168 kDa protein in Western blot analysis of *X. laevis* egg extracts (Figure 1C) that corresponds to the predicted molecular weight of xRTS.

*Correspondence: arv22@cam.ac.uk

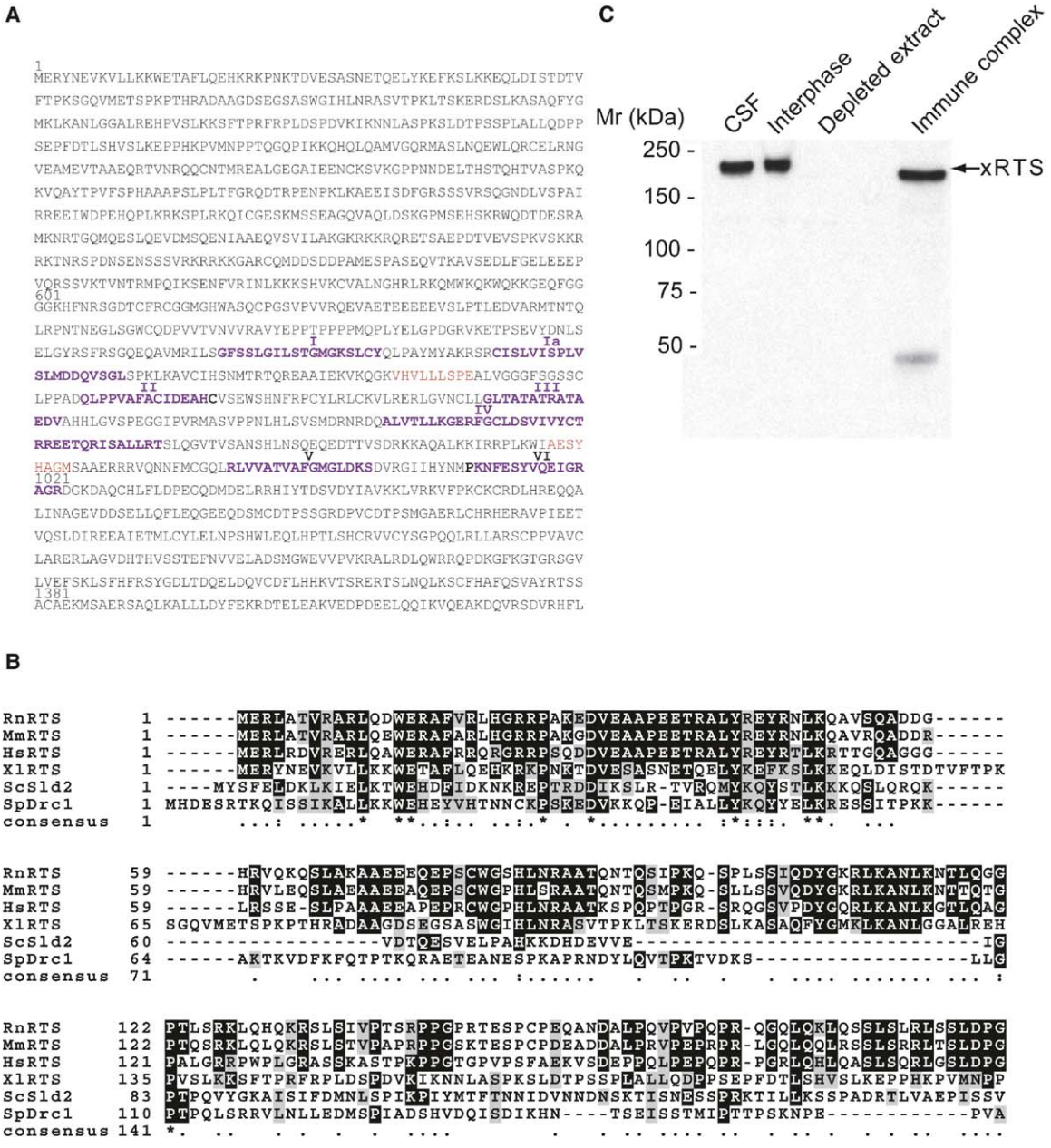


Figure 1. Cloning of the *Xenopus laevis* Homolog of the Rothmund-Thomson Helicase

(A) Protein sequence of the *Xenopus laevis* homolog of the Rothmund-Thomson helicase (xRTS). Numbers indicate codons. Signature motifs for the DNA helicase domain (I–VI) are in purple. Motifs characteristic of the RecQ family are in red.

(B) Alignment of an N-terminal region of xRTS with the corresponding region from the Sld2 and DRC1 proteins of *S. cerevisiae* and *S. pombe*, respectively. Identical residues are marked with an asterisk and conservative changes with double or single dots, proportional to the similarity of the two residues. Pairwise alignments are shown in Figure S1.

(C) Specificity of a rabbit antiserum against xRTS. The first two lanes show CSF or interphase extracts from *X. laevis* eggs immunoblotted with anti-xRTS. In the third lane, the interphase extract was immunodepleted of xRTS before Western blotting. The final lane contains eluted immune complexes from the immunodepletion reactions.

**xRTS Is Essential for Chromosomal DNA Replication**

xRTS is essential for chromosomal DNA replication in *X. laevis* egg extracts. Replication of demembranated sperm chromatin is reduced and delayed in extracts depleted of xRTS using affinity-purified anti-xRTS antibodies coupled to protein A-conjugated Dynabeads (Figure 2A). Control reactions performed with extracts mock depleted with a control antibody show no such reduction. Moreover, supplementation of xRTS-depleted extract with mock-depleted (but not xRTS-depleted) extract restores DNA replication. Together, these controls confirm that the immunodepletion procedure does not itself impair extract activity.

The requirement for xRTS can be complemented by

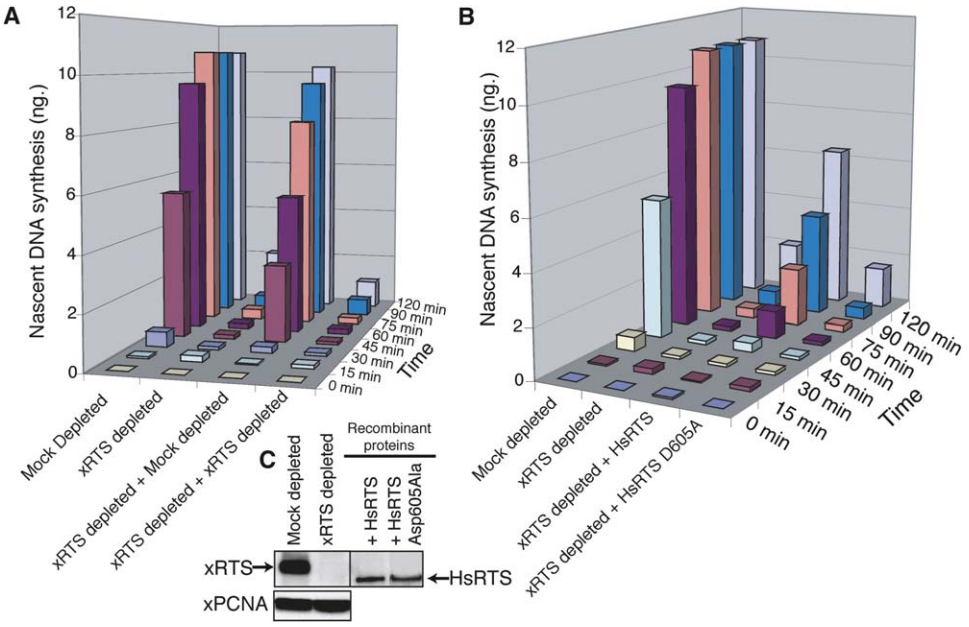


Figure 2. xRTS Is Required for DNA Replication in *Xenopus laevis* and Can Be Complemented by Recombinant Human RECQL4 Protein

(A) Egg extracts were immunodepleted with either a control antiserum (mock depleted) or anti-xRTS (xRTS depleted). Nascent DNA synthesis measured in ng/μl of egg extract is plotted on the vertical axis against time for each experiment. As positive and negative controls, xRTS-depleted extracts were supplemented with either one-twentieth volume of mock-depleted (xRTS-depleted + mock-depleted) or xRTS-depleted (xRTS-depleted + xRTS-depleted) extract preparations, respectively.

(B) In similar but independent DNA replication assays, xRTS-depleted extracts were supplemented with either recombinant wild-type human RTS protein (xRTS depleted + HsRTS depleted) or the recombinant Asp605Ala mutant (xRTS depleted + HsRTS D605A) predicted to inactivate DNA helicase activity. Recombinant human RECQL4 (HsRTS) and HsRTS Asp605Ala proteins were partially purified from *E. coli* as described and were used at 10 ng/μl. Their purity is demonstrated in Figure S2.

(C) Western blot analysis of mock-depleted or xRTS-depleted and recombinant HsRTS or HsRTS Asp605Ala (D605A) proteins used in the replication experiments, with antibodies against xRTS or xPCNA. Results shown are typical of at least five independent experiments.

adding recombinant human RECQL4 (HsRTS) protein, purified from *E. coli* by affinity chromatography, to xRTS-depleted egg extracts (Figures 2B and 2C). In contrast, a mutant protein (HsRTS Asp605Ala, D605A), in which an Asp residue in the Walker B motif, thought critical for the helicase activity of RecQ domains, has been replaced, fails to complement in this way. A silver-stained gel (Figure S2) confirms the purity of the recombinant proteins.

## RECQL4 Depletion in Mammalian Cells Leads to Proliferation Failure

The ability of recombinant human RECQL4 protein to compensate for its *Xenopus* homolog in vitro, when taken together with the considerable level of sequence homology in the N-terminal (Figure 1B) and RecQ domains of the two proteins, suggests conservation of function during evolution. To test this further, we employed RNA interference mediated by short-hairpin RNAs (shRNAs) to deplete RECQL4 (RTS) from primary murine embryo fibroblasts (MEFs) before analyzing cell proliferation and DNA replication (Figure 3A). Retroviral delivery of an RTS-specific shRNA decreases the steady-state level of RNA expression detected by a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method by up to 73% compared to cells treated with control shRNA (Figure 3B). Protein levels could not be measured directly in these experiments. We find that RTS is not abundant enough, using limited numbers of cells in shRNA experiments, to be detected in whole-cell extracts, or in nuclear or chromatin fractions, using several different antibodies that can readily detect heterologously expressed protein (Figure S3), consistent with the work of others (Yin et al., 2004). RTS depletion by shRNA decreases the proliferation of primary MEFs in culture when compared to cells treated with control shRNA (Figure 3C). In contrast to the control cells, which undergo one to two population doublings between 24 and 72 hr after shRNA treatment, RTS-depleted MEFs do not increase in number. Moreover, impeded proliferation is accompanied by the failure of DNA replication, marked by the absence of [$^3$H]thymidine incorporation into nascent DNA, normalized to genomic DNA content uniformly labeled with $^{14}$C (Figure 3D). Analysis by propidium-iodide staining and flow cytometry reveals an increase from 63% to 70% in the fraction of RTS shRNA-treated cells with 2n DNA content indicative of G1 or early S phase relative to controls (Figure 3E). There is no increase in 4n cells, ruling out an impediment to G2/M progression. Although the fraction of cells with >2n, <4n DNA content does not change after RTS shRNA exposure, there is a decrease in the percentage of cells strongly positive for BrdU, a marker of DNA synthesis (control shRNA, 11.9% ± 5% [n = 271]; RTS shRNA, 4.4% ± 5% [n = 139]). Thus, taken together, our findings

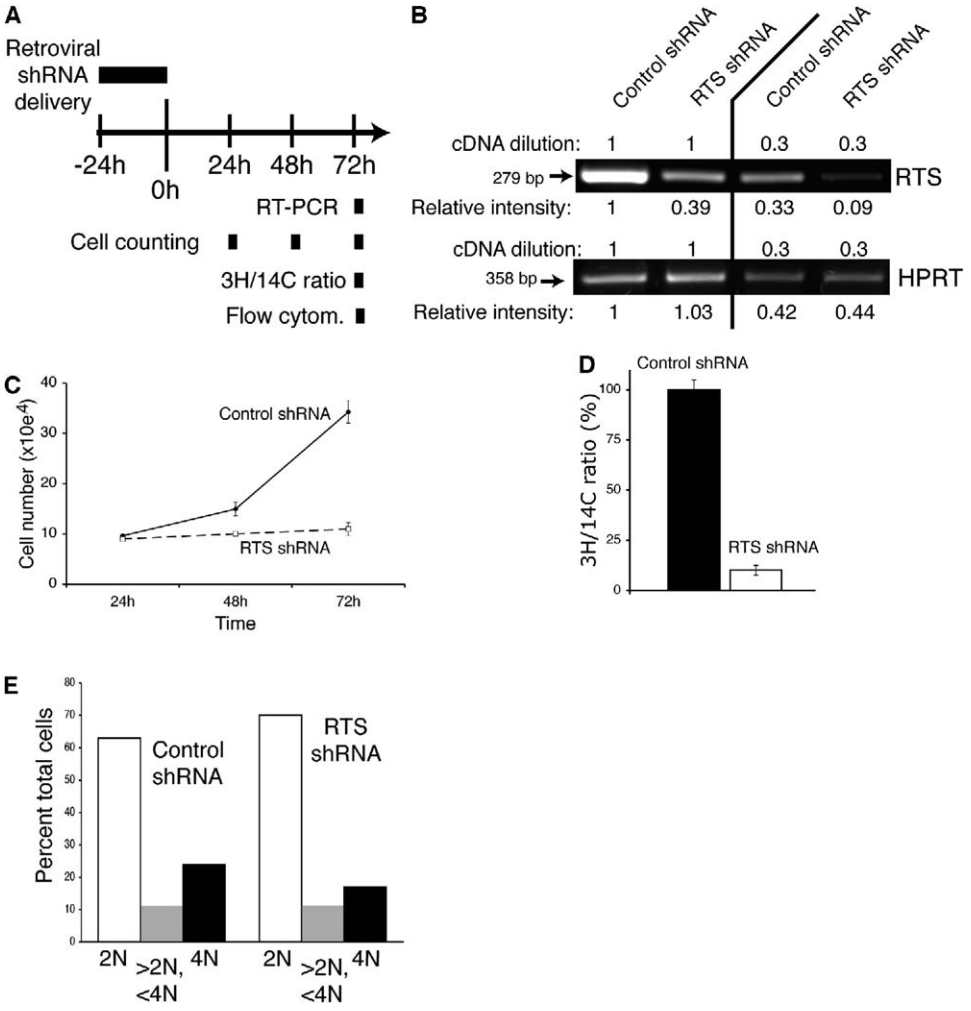


Figure 3. Depletion of RECQL4 in Mammalian Cells Leads to Proliferation Failure

(A) Experimental timeline. shRNA delivery by retroviral gene transfer was started 24 hr before equal numbers of cells were plated (0 hr). Experimental analyses were performed at the indicated times.

(B) RT-PCR analysis of RNA expression 72 hr after shRNA delivery. PCR products were resolved on agarose gels before staining with ethidium bromide and quantitation by densitometry. Reactions were also performed with a 1:3 dilution of cDNA to ensure a linear relationship between the amount of RNA in the samples and its RT-PCR product. The relative intensity of each product is shown as densitometric ratios, normalized to that in control shRNA-treated cells, taken as 1. HPRT was used as a control to verify that equal amounts of mRNA were used in the reactions. The primer pairs used in amplification reactions span introns in the RECQL4 (RTS) or HPRT genes, excluding genomic DNA contamination.

(C) Viable cell counts at the indicated times after shRNA delivery are plotted. Each data point represents the mean of three independent observations. Standard errors from the mean are shown.

(D) Incorporation of [$^3$H]thymidine into nascent DNA at 72 hr after shRNA delivery. Genomic DNA was uniformly labeled with $^{14}$C as previously described (Lomonosov et al., 2003), prior to a 1 hr pulse with [$^3$H]thymidine 72 hr after shRNA delivery. The $^3$H/$^{14}$C ratio therefore measures nascent DNA synthesis normalized to cellular DNA content. Values are shown as a percentage of nascent DNA synthesis in cells exposed to control shRNA (taken as 100%). Each data point shows the mean and standard error of three independent observations.

(E) DNA content of shRNA-treated cells 72 hr after exposure. Samples were gated on the FSC/SSC channels to exclude subcellular debris (which was more abundant in cells treated with RTS shRNA) and on the FL2-A/FL2-W channels to exclude cell aggregates. The percentage of cells with 2n, 2n to 4n (marked ">2n, <4n"), or 4n DNA content was calculated from histogram plots of PI staining against relative cell number. At the same time point, the percentage of cells incorporating BrdU, a marker of nascent DNA synthesis, was decreased after RTS shRNA treatment (control shRNA, 11.9% ± 5% [n = 271]; RTS shRNA, 4.4% ± 5% [n = 139]). An experiment typical of two independent repeats is shown.

show that depletion of the murine homolog of xRTS impedes proliferation and DNA replication in primary cell cultures, consistent with our studies using *Xenopus* egg extracts.

Why, then, might cells from patients with Rothmund-Thomson (Kitao et al., 1998; Kitao et al., 1999; Vennos and James, 1995) or RAPADILINO (Siitonen et al., 2003) syndrome, or from viable mice carrying targeted truncations or deletions in *RecQL4* (Hoki et al., 2003; Mann et al., 2005), exhibit defective proliferation and chromo-

somal instability but not failure to replicate? Our preliminary results suggest a possible explanation. We noted that mutations compatible with cell growth generally occur 3′ to the Sld2/DRC1 homology region encoded in exons 1 to 4 and the 5′ end of exon 5 (Figure S3A). This prompted us to test if the N-terminal region of human RECQL4 could promote DNA replication in egg extracts depleted of xRTS. This region, from residues 1 to 118 encoded in exons 1 to 4 (termed HsRTS[Exon1-4]), was expressed and purified in *E. coli* (Figure S3B). Supplementation of xRTS-depleted extract with HsRTS [Exon1-4] consistently increases nascent DNA synthesis by 3- to 5-fold over the same extract without supplementation (Figure S3C). But replication is restored to no more than ~20% of full activity. Thus the Sld2/DRC1 homology region at the N terminus of human RECQL4, independent of the RecQ domain, might possess a weak activity in promoting chromosomal DNA replication. In this light, it is puzzling that the D605A mutant of RTS does not also weakly stimulate replication (Figure 2B). However, its lower abundance in the replication reactions (10 ng/μl of D605A mutant versus 50 ng/μl of HsRTS[Exon1-4]), predicated by the difficulty in purifying the full-length D605A protein at a high concentration, might account for the observed difference in activity. Alternatively, the short HsRTS[Exon1-4] fragment might exhibit an activity absent from the full-length mutant protein. Further investigation is required to clarify these possibilities.

### Chromatin Association during Replication Initiation

To define the function of xRTS during replication, we studied its association with chromatin during DNA replication in egg extracts (Figure 4). xMCM3 and xORC2, components of the prereplication complex of proteins that bind to origins of replication during the G1 phase of cell cycle, associate with chromatin 20 min after the addition of nuclei to extract; xORC2 remains chromatin associated throughout, and xMCM3 levels decline during the course of replication. The accumulation of xRTS on chromatin occurs after pre-RC formation, peaking around 40 min and declining as replication proceeds to completion. The peak overlaps with that of xRPA70, a single-strand DNA (ssDNA) binding protein, which loads at origins after pre-RC formation. Blocking elongation by addition of the polymerase inhibitor aphidicolin does not prevent (but, in fact, enhances) the accumulation of xRTS on chromatin. Collectively, these observations suggest that xRTS is recruited to chromatin early during replication initiation, after pre-RC formation but before or during the establishment of active replication forks.

### A Role for xRTS in Replication Initiation

Consistent with this proposal, the recruitment of xRTS to chromatin is dependent on the formation of a pre-RC at origins of replication (Figure 5A). Geminin, an inhibitor of replication in multicellular eukaryotes, prevents pre-RC formation by blocking Cdt1, a factor essential for loading the MCM proteins onto chromatin (Wohlschlegel et al., 2000). When recombinant geminin is added to replication reactions from the outset (Figure 5A, 0 min), it prevents the accumulation of xRTS (as

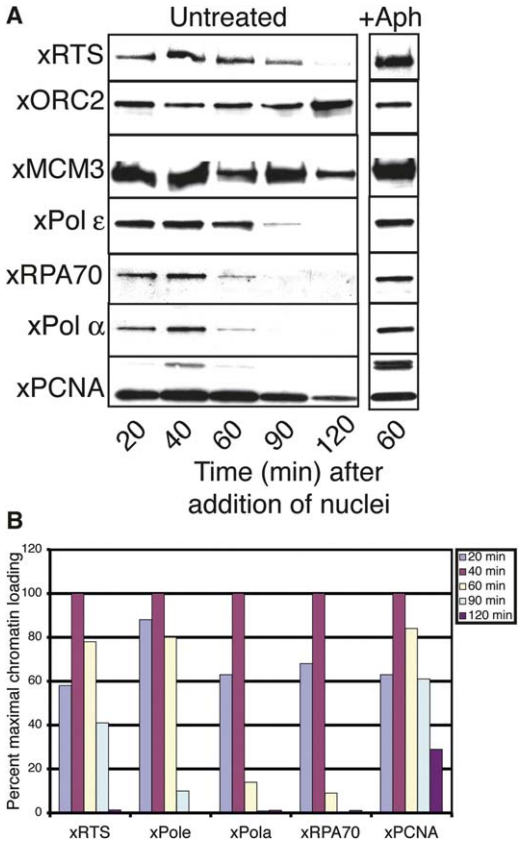


Figure 4. Regulated Loading of xRTS onto Chromatin during DNA Replication

(A) Time course of xRTS loading. Chromatin was extracted at different times after the assembly of replication reactions for the detection of chromatin bound proteins by immunoblotting.

(B) A semiquantitative plot of the time course of chromatin loading, derived from a typical immunoblotting experiment. The relative amount of chromatin bound protein at each time point was determined by densitometry, taking the maximal value to be 100%, and plotted on the vertical axis against time.

well as MCM3) on chromatin. Addition of geminin to the reactions after pre-RC formation (at 20 min) has no effect on xRTS accumulation. Taken together, these experiments suggest that xRTS requires the presence of mature pre-RCs for binding to chromatin.

The activity of cyclin/cyclin-dependent-kinase (cdk) complexes mediates the transformation of the pre-RC into an active replication fork during the G1-to-S phase transition. Cyclin/cdk complexes appear to be first required at a step that follows the chromatin loading of MCM proteins but occurs before Cdc45 loading or the unwinding and stabilization of replication origins for recruitment of the elongation machinery (Walter and Newport, 2000; Zou and Stillman, 2000). We find that inhibition of cyclin/cdk activity by the addition of an N-terminal fragment of the cdk inhibitor p21 (p21N) to replication reactions (Chen et al., 1995) does not prevent xRTS accumulation on chromatin (Figure 5A), suggesting that the recruitment of xRTS to chromatin follows pre-RC formation but precedes origin unwinding and the establishment of active replication forks.

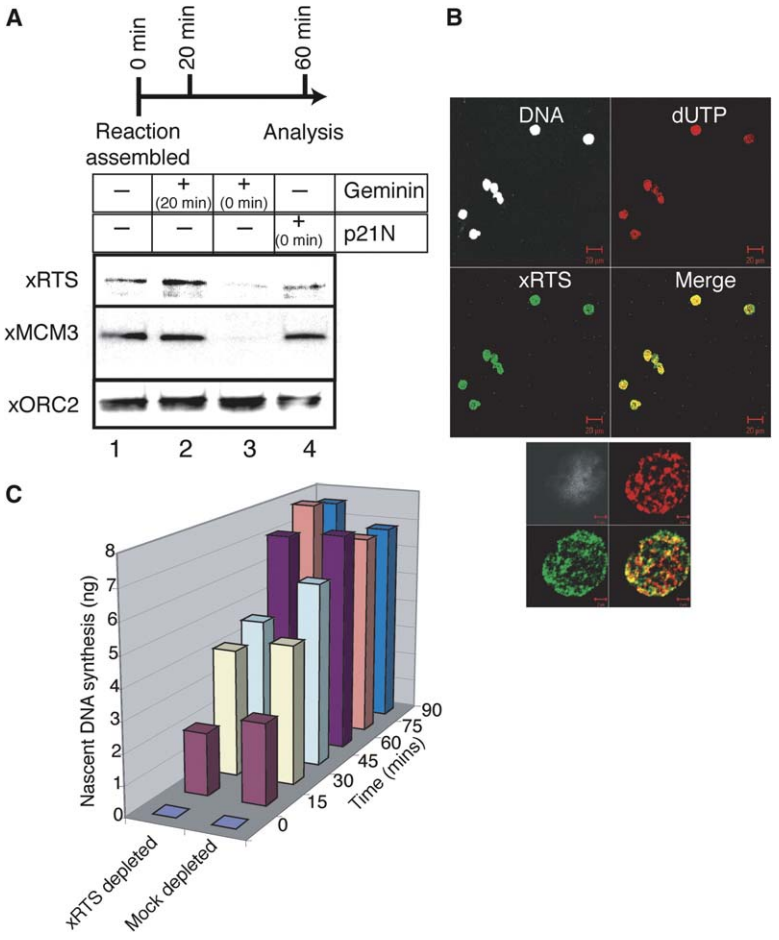


Figure 5. xRTS Functions after Pre-RC Assembly and Localizes to Sites of Replication Initiation

(A) Loading of xRTS onto chromatin requires pre-RC assembly but not cdk activity. DNA replication reactions were assembled (0 min) in the presence or absence of recombinant geminin (which blocks pre-RC assembly) or p21N (which blocks cdk activity) before analysis of chromatin bound proteins 60 min afterwards. In one reaction (lane 2), geminin was added 20 min after the reaction was assembled, and analysis was carried out 40 min later. Immunoblots show the presence or absence of xMCM3—a component of the pre-RC complex—or xRTS. Addition of geminin but not p21N at 0 min prevents chromatin loading of xRTS (compare lanes 1, 3, and 4). Addition of geminin after pre-RC assembly (20 min, lane 2) is without effect.

(B) xRTS localizes to sites of nucleotide incorporation during replication initiation. Sperm nuclei (1000 sperm heads/μl extract) were incubated in interphase egg extract with aphidocolin (50 μg/ml) for 30 min. Incubations were pulsed with biotin-dUTP for a further 10–15 min before being fixed and stained with appropriate antibodies and streptavidin conjugate. xRTS and dUTP incorporation were visualized by confocal microscopy.

(C) xRTS is dispensable for complementary-strand synthesis on M13 ssDNA templates. Circular M13 ssDNA was added instead of sperm nuclei to replication reactions in xRTS-depleted or mock-depleted egg extracts as previously described. Nascent DNA synthesis determined by incorporation of [$^{32}$P]dATP is plotted on the vertical axis against time. Unlike chromosomal DNA, the complementary strand of M13 ssDNA can be synthesized in egg extracts by the replication machinery independent of replication origins or initiation complexes.

To examine the intranuclear localization of xRTS during replication initiation, we allowed nuclei to initiate replication by incubation in egg extracts containing aphidicolin, labeling sites of nascent DNA synthesis by incorporation of biotin-dUTP. The localization of xRTS was then determined by indirect immunofluorescence (Figure 5B). There is significant overlap between xRTS staining and sites of biotin-dUTP incorporation, suggesting that xRTS is located at sites of DNA synthesis during replication initiation. In this respect, its behavior differs from that of the MCM complex, which performs its functions during initiation at a distance from sites of DNA synthesis (Laskey and Madine, 2003).

Because xRTS remains associated with chromatin throughout replication elongation (Figure 4), it is not possible to ascertain using chromosomal DNA templates whether xRTS is required solely for initiation or has additional functions during elongation. However, we find that xRTS-depleted extracts are competent for complementary-strand synthesis using a circular ssDNA template derived from the M13 bacteriophage (Figure 5C). In egg extracts, this reaction proceeds in a manner akin to DNA-strand elongation during chromosomal DNA replication (Cox and Leno, 1990; Jenkins et al., 1992; Mechali and Harland, 1982). It utilizes components of the replication machinery but occurs independently of pre-RC formation and the conventional events required to initiate replication.

Taken together, our results identify an essential role for xRTS during chromosomal DNA replication. That xRTS is recruited to chromatin after pre-RC formation—but before the unwinding of replication origins and the establishment of active replication forks—provides evidence that it participates in replication initiation. Our results also indicate that xRTS is dispensable for complementary-strand synthesis using M13 ssDNA templates. Because this is a limited model for replication elongation, we cannot formally exclude the possibility that xRTS has additional functions during elongation beyond its role in DNA replication initiation demonstrated here.

### Recruitment of RPA

A critical but poorly understood requirement for replication initiation is that origins of replication, defined by pre-RC formation, be rendered accessible for loading

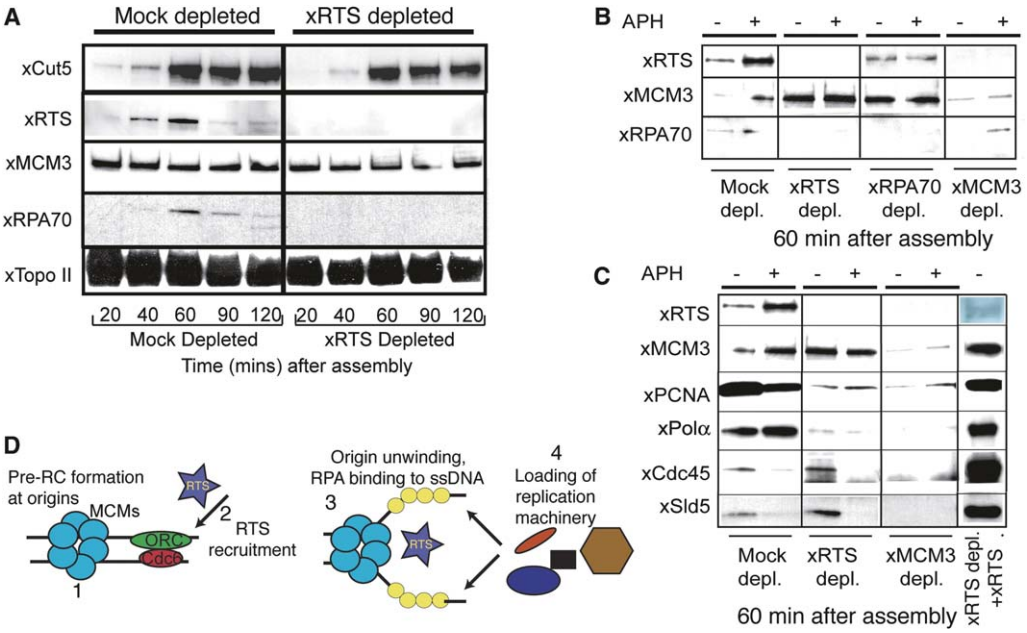


Figure 6. xRTS Functions during Replication Initiation to Promote RPA Loading onto Chromatin

(A) xRTS depletion prevents loading of xRPA70 onto chromatin. Chromosomal DNA replication reactions were assembled using mock-depleted or xRTS-depleted extracts as described in Figure 2, and chromatin bound proteins were extracted at 20–120 min after assembly for analysis by immunoblotting. xRTS loads onto chromatin somewhat after xMCM3, but coincident with xRPA70, in reactions using the mock-depleted extract. xRPA—but not xMCM3 or xCut5—fails to load onto chromatin after xRTS depletion.

(B and C) A hierarchy for chromatin loading of proteins during replication initiation. Replication reactions were assembled (0 min) as described in Figure 2 using mock-depleted, xRTS-depleted, xRPA70-depleted, or xMCM3-depleted egg extracts. Chromatin bound proteins were analyzed by immunoblotting 60 min afterwards. xRTS depletion suppresses the loading of xRPA70 and components of the polymerase machinery (PCNA, pol α) but not xMCM3 or replication factors like xSld5 or xCdc45. xRPA depletion is without effect on xMCM3 or xRTS. Addition of in vitro-translated xRTS to RTS-depleted extracts (column marked "xRTS depl. + xRTS") restores their ability to support chromatin association of RPA70, Cdc45, and pol α. In this column, the blot showing in vitro-translated xRTS has been deliberately overexposed.

(D) Hypothetical model for the function of xRTS during replication initiation. Pre-RC formation (1) defines origins of replication (although MCM complexes need not afterwards remain in proximity to forks as shown). Recruitment of xRTS to the origins (2) promotes unwinding and the stabilization of ssDNA by RPA (3), enabling loading of the replication machinery (4).

of the replication machinery (Walter and Newport, 2000; Wohlschlegel et al., 2002). This process involves the unwinding of DNA at origins through a helicase activity that remains to be definitively identified but is proposed to include the MCM2–7 protein complex (Pacek and Walter, 2004). Origin unwinding is marked by the recruitment of RPA to the exposed ssDNA, thus stabilizing these structures for polymerase recruitment (Walter and Newport, 2000). To test if xRTS is involved in this process, we asked if xRTS depletion could suppress the recruitment of RPA to chromatin. This is indeed the case (Figure 6A). xMCM3 continues to be recruited to chromatin during DNA replication in xRTS-depleted egg extracts. However, recruitment of the 70 kDa component of RPA (xRPA70) is markedly suppressed in comparison to control reactions performed using mock-depleted extracts.

The hierarchy and sequential nature of the recruitment of these proteins to chromatin provides further evidence that xRTS recruitment follows pre-RC formation but precedes—and is required for—xRPA recruitment (Figure 6B). Depletion of xRTS prevents the association of xRPA but not xMCM3 with chromatin. In contrast, xRPA depletion has no effect on recruitment of either xMCM3 or xRTS.

Inhibition of DNA polymerase with aphidicolin is reported to uncouple origin-unwinding activity from the replication fork, resulting in enhanced RPA recruitment (Pacek and Walter, 2004). That aphidicolin treatment increases the chromatin association of both xRTS and xRPA (Figures 4 and 6B) is consistent with this view.

*Xenopus* Cut5 (Hashimoto and Takisawa, 2003), a homolog of yeast Dpb11, has been hypothesized to function during replication initiation after MCM2–7 loading and is itself required for the recruitment of replication factors like Cdc45 (Walter and Newport, 2000) and components of the GINS complex such as Sld5 (Kubota et al., 2003). We find (Figure 6A) that xCut5 continues to load onto chromatin during replication reactions even after xRTS depletion, placing xRTS downstream of Cut5 in the sequence of recruitment to replication origins.

To further narrow down the window in which xRTS exerts its function, we examined the hierarchy of chromatin association of additional replication factors (Cdc45, Sld5) or polymerase machinery components (PCNA, polymerase α) in extracts depleted of xRTS or, as a control, xMCM3 (Figure 6C). As expected, MCM3 depletion prevents or greatly decreases the recruitment of all four factors. Recruitment of Cdc45 or Sld5 is unaf-

fected by xRTS depletion. In contrast, xRTS depletion effectively suppresses the loading of RPA70 and polymerase α. Recruitment of these factors can be restored by addition of in vitro-translated xRTS protein, further evidence that it is RTS, and not a physically associated molecule, that is active in these assays.

Thus, collectively, our findings implicate xRTS in a previously unrecognized, early step during the initiation of DNA replication (Figure 6D). xRTS is recruited to origins of replication after pre-RC formation and the loading of Cut5, GINS, or Cdc45, and its recruitment is in turn required for the chromatin association of xRPA and assembly of the polymerase machinery. Because xRPA is proposed to act in concert with uncharacterized helicase activities, which may include the MCM complex, to unwind and stabilize DNA replication origins, our findings reveal a hitherto unrecognized role for xRTS in this process.

## Discussion

Our work extends the known cellular functions of the RecQ-type DNA helicases, mutations that trigger human genetic diseases such as Bloom or Werner's syndrome that are characterized by premature aging, developmental abnormalities, and cancer predisposition. There is abundant evidence that members of this family participate in reactions that underlie DNA recombination (Hickson, 2003; Prince et al., 2001; Saintigny et al., 2002) and recovery from replication arrest (Davies et al., 2004; Pichierri et al., 2001). However, the participation of RecQ helicases in processive DNA replication remains controversial and the nature of their contribution unclear (Li et al., 2004; Liao et al., 2000).

Hence, the novel role of xRTS in the initiation of DNA replication is unique among the RecQ family. Consistent with this role is the presence of an evolutionarily conserved N-terminal homology to the Sld2/DRC1 proteins, which are essential for the establishment of DNA replication forks in yeast, that is not found in other RecQ family members. Conversely, other members of the RecQ family contain conserved motifs not present in xRTS. These observations suggest divergence of function during the evolution of this family of DNA helicases, signified by the fusion of distinct functional modules to a conserved catalytic domain.

It should be emphasized, however, that xRTS is not a precise functional homolog of Sld2/DRC1 in vertebrates. The region of homology is limited and is confined to a small area of a large protein (Figure 1B). Moreover, unlike Sld2/DRC1, xRTS is recruited to chromatin independent of cyclin/cdk activity (Figure 5A), although we cannot rule out that this activity is necessary for its function after chromatin loading.

Interestingly, unlike xRTS, Cdc45 and the Sld5 component of the GINS complex require cyclin/cdk activity for their recruitment to chromatin (Hashimoto and Takisawa, 2003; Kubota et al., 2003). This raises the possibility that xRTS might be loaded to replication origins by a pathway independent of Cdc45 or GINS, even though it appears to come after them in the temporal sequence of events.

Two lines of evidence indicate that the function of xRTS is conserved during evolution. Recombinant human RECQL4 can replace the requirement for xRTS in DNA replication reactions carried out in *Xenopus* egg extracts. Conversely, depletion of RECQL4 from mammalian cells leads to failure of cell proliferation and nascent DNA synthesis, consistent with our in vitro studies.

However, these results also raise a salient question. If human RECQL4, like its counterpart in *Xenopus*, is essential for replication in cell culture, how are germline mutations inactivating the protein compatible with viability in Rothmund-Thomson syndrome? Our preliminary findings suggest one possible explanation. Many of the truncating mutations associated with Rothmund-Thomson syndrome leave varying portions of the N-terminal end of the protein intact, including the Sld2/DRC homology region. A fragment of human RECQL4 from residues 1 to 118, spanning much of this region, weakly stimulates chromosomal DNA replication in *Xenopus* egg extracts depleted of xRTS. This raises the possibility that the mutations in *RECQL4* associated with human genetic diseases might be hypomorphic with respect to replication initiation. For this explanation to be tenable requires, however, that truncated RTS proteins be stably expressed in Rothmund-Thomson syndrome cells and be able to be transported to the nucleus, neither of which has yet been established.

A nonexclusive, alternate explanation is that the effects of germline *RECQL4* inactivation could be compensated during embryonal development, as has previously been reported for tumor suppressors like pRb, where acute versus germline inactivation engenders different phenotypes (Sage et al., 2003). For instance, other molecules implicated in origin unwinding, such as the MCM complex, might conceivably counteract the chronic deficiency of RTS function following germline mutations but not, perhaps, its acute depletion.

The phenotypes of mouse strains carrying three distinct deletions in murine *RecQL4* lend some support to these possibilities (Ichikawa et al., 2002; Hoki et al., 2003; Mann et al., 2005). Extensive gene disruption by replacement of exons 5–8 with a *lacZ-PGKneo* cassette results in embryonal lethality by day 3–6 and is incompatible with embryonal stem cell viability (Ichikawa et al., 2002). Less extensive genetic alterations, which are known to permit expression of transcripts encoding the Sld2/DRC1 homology region at the N terminus, permit viability to differing degrees. Deletion of exon 13, which encodes a segment of the RecQ domain that is often mutated in humans with Rothmund-Thomson syndrome, is compatible with viability but causes embryonal growth retardation, death of over 90% of newborns within 2 weeks of birth, and severe defects in cell proliferation in primary MEF cultures (Hoki et al., 2003). Disruption of exons 9–14, which encompass most of the RecQ domain, not only allows viability but also predisposes to cancer (Mann et al., 2005). The complexity of the correlations between genotype and phenotype associated with germline *RECQL4* mutations in humans and mice suggests the existence of redundant pathways and, possibly, additional functions.

Our results do not unequivocally establish whether the helicase activity of RECQL4 is required for its function in replication initiation. Indeed, it is unclear whether RECQL4 purified from HeLa cells has intrinsic activity

(Yin et al., 2004). From our findings, on the one hand, the D605A mutant fails to complement replication in xRTS-depleted extracts, suggesting that helicase activity is necessary. On the other hand, the N-terminal Sld2/DRC1 region, when used at high concentrations in vitro, can weakly stimulate replication apparently independently of the RecQ domain. Whether or not these represent distinct, physiologically relevant activities of RECQL4 remains to be determined.

Dividing fibroblasts from Rothmund-Thomson syndrome patients are reported to acquire trisomies and isochromosomes (Lindor et al., 2000) but not the aberrant chromatid exchanges or rearrangements typical of Bloom syndrome. Indeed, sister chromatid exchange is normal in MEFs homozygous for a germline deletion in *RecQL4*, but premature release of centromeric cohesion has been reported (Mann et al., 2005). How this might be connected to defective replication is a matter for conjecture. However, we note that in yeast, the establishment of chromosome cohesion occurs during DNA replication (for example, Skibbens et al., 1999; Tanaka et al., 2000; Toth et al., 1999).

Our work provides fresh insight into the events that initiate DNA replication in vertebrate cells. One major unresolved question concerns the nature of the steps that lead to the unwinding of replication origins on chromosomal DNA prior to the loading of replication factors. Our results suggest that xRTS is essential for this process, working after pre-RC assembly but before the establishment of active replication forks in a mechanism responsible for the stabilization of unwound origins by the ssDNA binding protein, RPA. In this context, the fact that xRTS, like its ortholog in humans, putatively encodes a helicase activity through its RecQ domain suggests a direct role, but this awaits verification. This caveat notwithstanding, our observations define an essential and previously unrecognized step during the initiation of DNA replication in vertebrate cells that requires a molecule mutated in a human disease. The regulation of xRTS and its orthologs in other species may determine how origins, marked by pre-RC assembly, can be transformed into active replication forks in a timely and coordinated manner during the S phase of the cell cycle.

## Experimental Procedures

### Cloning of xRTS

Expressed sequence tags (ESTs) encoding xRTS were identified with TblastN from the *Xenopus* EST database against the human RECQL4 and *S. pombe* DRC1 protein sequences. The predicted coding sequence (CDS) including the putative codons for translation initiation and termination was assembled from four ESTs. A cDNA clone spanning the entire CDS was isolated by RT-PCR from oligo-dT-primed, reverse-transcribed total RNA using the primers 5′-TGCCCATGGAGATGGAGCGCTATAATGAGGTTAAGG-3′ and 5′-ACTCTCGAGCAACATCCTCTGCTGCTCACGGACT-3′ and a proofreading polymerase (Accuprime *Pfx*, Invitrogen). The veracity of the nucleotide sequence was established in multiple independent RT-PCR reactions.

### Constructs

Human *RECQL4* cDNA was cloned by RT-PCR using the primers 5′-ATAGCGGCCGCTATGGAGCGGCTGCGGGACGTG-3′ and 5′-ATATCTAGATCAGCGGGCGACCTGCAGGAGCTCTT-3′ and directionally cloned into pcDNA3.1-HisA. pET30a-HsRecQL4-$His_6$ constructs were made by PCR amplification of the HsRecQL4 cDNA using the primers 5′-GTCGATCATATGGAGCGGCTGCGGGACGTG-3′ and 5′-CGTCTCGAGGCGGGCCACCTGCAGGAGCTCTTCCGT-3′, followed by cloning into pET30a. For the Asp605Ala mutant, the plasmid was subjected to site-directed mutagenesis (QuikChange XL, Stratagene) using the primer 5′-GTTGCTTTTGCCTGCATTGCT GAGGCCCACTGCCTCC-3′. After nucleotide sequencing, the insert was recloned into pET30a to ensure integrity of the vector sequence.

### Anti-RTS Antisera

Polyclonal rabbit antiserum was raised against the N-terminal 260 residues of xRTS expressed as a GST fusion in *E. coli* and affinity purified against the antigen or the corresponding region from human RECQL4 after preadsorption on GST-Sepharose 4B. Antibodies against human (sc-16924, sc-16925) and murine RECQL4 (sc-16927) were purchased (Santa Cruz).

### *Xenopus* Egg Extracts

CSF extracts were prepared as stated in Murray (1991), with minor modifications. Briefly, dejellied *Xenopus* eggs were crushed at 10K rpm for 10 min at 16°C. The golden cytosolic fraction was separated and supplemented with protease inhibitors, cytochalasin D to 10 μg/ml, and 1/20 v/v energy mix. Extracts were diluted 1/10 v/v in 2 M sucrose, and clarified at 10,000 rpm for 20 min. All experiments were with freshly prepared extracts. CSF extracts were activated by the addition of $CaCl_2$ to 0.4 mM and incubated at room temperature before the addition of DNA template after 20 min. When necessary, cycloheximide (100 μg/ml final) was added at the time of activation to prevent entry into mitosis.

### Immunodepletion

xRTS was depleted from egg extract by three rounds of immunodepletion. Saturating amounts of anti-xRTS (or rabbit IgG for mock depletion) were preincubated with protein A-conjugated Dynabeads (Dynal Biotech) for 90 min at 4°C, washed once in HEPES-buffered saline, three times in SDB (10 mM HEPES-KOH [pH 7.4], 100 mM KCl, 1 mM $MgCl_2$, 150 mM sucrose, with protease inhibitors and cytochalasin D), and divided into three equal aliquots. For each immunodepletion, one aliquot was completely resuspended in the extract by gentle pipetting and incubated for 35–40 min. Dynabeads were removed by two passes through a magnetic particle separator (Dynal, MPC-S). For complementation of depleted extracts in Figure 6, xRTS mRNA was transcribed from the T7 promoter and in vitro translated using rabbit reticulocyte lysate in a coupled reaction according to manufacturer's instructions (Promega). Five microliters of the reaction product was used to supplement 50 μl of xRTS-depleted egg extract.

### Replication Assays

Replication assays using demembranated sperm nuclei were performed as described by Mills et al. (1989), using 3600 nuclei/μl in the presence of cycloheximide.

### Isolation of Chromatin Bound Proteins

Sperm nuclei were incubated at 4000 nuclei/μl in 50 μl of egg extract for the indicated times, then diluted 10-fold with ice-cold SDB supplemented with 0.1% Triton X-100; 10 μg/ml leupeptin, pepstatin, chymostatin mix (LPC); 1 mM DTT; 1 mM orthovanadate; 1 mM NaF; 0.2 mM PMSF and incubated on ice for 5 min after gentle mixing. The mixture was layered carefully onto a 1 ml cushion of MMR/40% glycerol and spun to 6800 × g at 4°C for 20 min. The aspirated chromatin pellet was washed in the same buffer again and centrifuged for 5 min. Pellets were resuspended in 50 μl of SDS-PAGE sample buffer, subjected to SDS-PAGE, transferred onto PVDF membranes (Millipore), and processed for Western blot analysis with the indicated antibodies.

### Biotin-dUTP Incorporation and Microscopy

Sperm nuclei were added to a final concentration of 1000 nuclei/μl in 20 μl of interphase extract plus aphidicolin (50 μg/ml). Biotin-dUTP was added at the indicated times. Reactions were incubated for 10 min before diluting in 500 μl of MMR and 500 μl of 8% form-

aldehyde (Agar Scientific). After 10 min at room temperature, nuclei were spun onto poly-L-lysine-coated coverslips, washed in PBS, permeabilized in PFB (0.75× PBS/0.1% T×100/0.02% SDS), blocked in PFB/2% BSA, and labeled with anti-xRTS. Biotin was detected with Alexa568-streptavidin and anti-xRTS with Alexa488-conjugated anti-rabbit (Molecular Probes) before visualization on a Zeiss LSM confocal microscope equipped with AxioVision software.

### RTS Depletion with shRNAs

Primary cultures of MEFs were established from 129×CBA embryos as previously described (Patel et al., 1998). shRNAs against RTS (RTS-F, 5′-GATCCCCGAGACCCGCGCGCTCTACCTTCAAGAGAGGTA GAGCGCGCGGGTCTCTTTTTGGAAA-3′; RTS-R, 5′-AGCTTTTCCAAAAAGAGACCCGCGCGCTCTACCTCTCTTGAAGGTAGAGCGCGCGGGTCTCGGG-3′) or a null control (Oligoengine) were cloned into the pSUPER-Retro vector with a puromycin-resistance marker. Virion production and transduction of MEFs were carried out as described (Lee et al., 1999) using 3–5 × $10^6$ cells per experiment. Twenty-four hours after retroviral delivery, puromycin (2 μg/ml) was used to select the shRNA-expressing cells.

### RT-PCR Reactions

Total RNA was isolated using the Trizol-RNA isolation kit (Invitrogen) and transcribed into cDNA using the first-strand SuperScriptII cDNA synthesis kit, priming with a poly-dT nucleotide (Invitrogen). PCR was carried out on equal amounts of each sample (neat or at 1:3 dilution) with the Accuprime *Pfx* enzyme (Invitrogen), using cDNA-specific primers to RTS or HPRT for 35 or 20 cycles, respectively. The primers span exon-intron boundaries in the respective genes, ensuring that the results are not affected by genomic DNA contamination. For murine RTS, the primers were Sense 5′-CATGCTGGCATGAGCAGCCAGGA-3′ and Antisense 5′-AGTGCTGTCAGCGTGGGCATGTCT-3′, and for murine HPRT, they were Sense 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and Antisense 5′-CTACCAGAGGGTAGGCTGGCCTAT-3′. Each amplification cycle consisted of 95°C, 30 s; 60°C, 30 s; and 72°C, 45 s, with a final extension period of 10 min at 72°C.

### Cell Proliferation

Viable cell numbers were enumerated every 24 hr by trypan-blue exclusion as previously described (Patel et al., 1998). Each data point in the results shown is the mean ± standard deviation from at least three independent measurements.

### DNA Replication Assay

Measurements were performed in shRNA-treated MEFs as previously described (Lomonosov et al., 2003). Genomic DNA was labeled to saturation over 96 hr with [$^{14}$C]thymidine (Amersham) at 50 nCi/ml. Seventy-two hours after exposure to control or RTS shRNAs, MEFs were washed extensively in phosphate-buffered saline (PBS). Nascent DNA synthesis was labeled with [$^3$H]thymidine (Amersham) at 2 μCi/ml per 5 × $10^4$ cells for 1 hr. Cells were harvested and centrifuged and the media aspirated, and the pellet was washed extensively in PBS. Cell pellets were lysed in 0.2 ml of 0.25 M NaOH and 7 ml of scintillation cocktail (Ultima Gold, Perkin Elmer) before liquid scintillation counting (dual DPM mode). Nascent DNA synthesis was calculated as the ratio of $^3$H/$^{14}$C incorporation, thus normalizing it to genomic DNA content (Lomonosov et al., 2003). Values shown are expressed as a percentage of nascent DNA synthesis in cells exposed to control shRNAs.

### Measurement of DNA Content by Flow Cytometry

Both attached and floating cells from cultures treated with shRNA were collected and washed in ice-cold PBS. The cells were fixed for 2 hr in ice-cold 70% ethanol, washed once with PBS, and stained in 0.2 μg/ml propidium iodide, 0.1% Triton-X100, and 0.02 mg/ml RNase A before further washing. Samples were analyzed on a Becton-Dickinson LSRII flow cytometer. Analysis of DNA content was with FACSDiva software.

### Purification of Recombinant Wild-Type and Mutant HsRTS Proteins

HsRTS proteins were expressed as C-terminal-$His_6$-tagged polypeptides in *E. coli* (Rosetta(DE3)pLysS, Novagen) from the plasmid pET30a-HsRTS-$His_6$ encoding wild-type or mutant forms. Cells were lysed in buffer A (10 mM phosphate buffer [pH 8], 300 mM NaCl, 10 mM imidazole with protease inhibitors), and the soluble fraction was applied onto Ni-NTA resin (Qiagen). After washing with buffer A (plus 10 mM imidazole), bound proteins were eluted in buffer B (buffer A with 200 mM imidazole). Eluates were dialyzed into buffer C (10 mM phosphate buffer [pH 8], 150 mM NaCl) and applied onto a HiTrap-Heparin column (Amersham) pre-equilibrated in the same buffer. The column was washed with buffer containing 300 mM NaCl, and bound protein was eluted using a linear salt gradient extending from 0.3–1.2 M NaCl. HsRTS protein typically eluted between 0.45–0.75 M NaCl. Peak fractions containing HsRTS protein were pooled and concentrated 5- to 10-fold using a spun-concentrator device (Vivascience) before dialysis into HKM buffer (10 mM HEPES-KOH [pH 7.7], 100 mM KCl, 1 mM $MgCl_2$ with 10% glycerol). Silver-stained gels (Figure S1) demonstrate the purity of the recombinant protein preparations.

### Expression and Purification of HsRTS[Exon1–4]

The coding sequence from human *RECQL4* exons 1 to 4 (see Figure S3) was cloned by PCR from a cDNA clone using the primers 5′-GAGATATACATATGGAGCGGCTGCGGGACG-3′ and 5′-CTGGCTCGAGCTGCAGGGTGCCTTTCAGATTG-3′ before directional ligation into the pGEX4T3 vector (Pharmacia). The fragment was expressed as a C-terminal fusion to GST in *E. coli* BL21-CodonPlus (DE3)-RIPL (Stratagene). Bacteria expressing the fusion protein were lysed in PBS, 5 mM DTT, 0.5% NP40 with protease inhibitors, and the soluble fraction was applied onto glutathione Sepharose 4B beads (Amersham). After extensive washing, HsRTS[Exon1–4] was cleaved away from the GST moiety using thrombin protease (Amersham) in PBS buffer at 22°C for 16 hr, leaving the residues Arg-Gly-Ser-Pro-Asn-Ser-Arg-Val-Asp at its N terminus. Thrombin was removed from the sample by chromatography with benzamidine-Sepharose 4B (Amersham).

### Silver Staining

Recombinant RTS proteins purified as described above were separated by SDS-PAGE. Gels were stained with Coomassie brilliant blue, fixed, and destained in 10% acetic acid/30% methanol. Silver staining of the gels using a kit (Invitrogen) was according to the manufacturer's guidelines.

### Supplemental Data

Supplemental Data include four figures and are available with this article online at http://www.cell.com/cgi/content/full/121/6/887/DC1/.

### Acknowledgments

The experiments reported here were performed by M.N.S., J.A.B., M.M., and A.R.V. We thank two anonymous referees for constructive criticism that has strengthened this paper, Dr. H. Takisawa for generously providing antibodies against xSld5 and xCut5, Dr. S. Waga (Kyoto University, Japan) for antibodies against pol α and pol ε, Dr. A. Mills for anti-xORC2, Dr. Kiku-e Tachibana for recombinant human geminin protein, Dr. J. Pines (Wellcome-CRUK Institute, Cambridge) for the p21N plasmid, and Dr. R. Laskey for advice on establishing the *Xenopus* replication system. M.N.S. was supported by a Clinical Training Fellowship from Cancer Research UK (CR UK) and J.A.B. by an EMBO postdoctoral fellowship. W.G.D. was supported by National Institutes of Health grant GM070891. Work in A.R.V.'s laboratory is funded by CR UK and the Medical Research Council.

Received: October 13, 2004
Revised: March 21, 2005
Accepted: May 5, 2005
Published: June 16, 2005

## References

Chen, J., Jackson, P.K., Kirschner, M.W., and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature *374*, 386–388.

Cox, L.S., and Leno, G.H. (1990). Extracts from eggs and oocytes of Xenopus laevis differ in their capacities for nuclear assembly and DNA replication. J. Cell Sci. *97*, 177–184.

Davies, S.L., North, P.S., Dart, A., Lakin, N.D., and Hickson, I.D. (2004). Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. Mol. Cell. Biol. *24*, 1279–1291.

Ellis, N.A. (1997). DNA helicases in inherited human disorders. Curr. Opin. Genet. Dev. *7*, 354–363.

Hashimoto, Y., and Takisawa, H. (2003). Xenopus Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. EMBO J. *22*, 2526–2535.

Hickson, I.D. (2003). RecQ helicases: caretakers of the genome. Nat. Rev. Cancer *3*, 169–178.

Hoki, Y., Araki, R., Fujimori, A., Ohhata, T., Koseki, H., Fukumura, R., Nakamura, M., Takahashi, H., Noda, Y., Kito, S., and Abe, M. (2003). Growth retardation and skin abnormalities of the Recql4-deficient mouse. Hum. Mol. Genet. *12*, 2293–2299.

Ichikawa, K., Noda, T., and Furuichi, Y. (2002). Preparation of the gene targeted knockout mice for human premature aging diseases, Werner syndrome, and Rothmund-Thomson syndrome caused by the mutation of DNA helicases. Nippon Yakurigaku Zasshi *119*, 219–226.

Jenkins, T.M., Saxena, J.K., Kumar, A., Wilson, S.H., and Ackerman, E.J. (1992). DNA polymerase beta and DNA synthesis in Xenopus oocytes and in a nuclear extract. Science *258*, 475–478.

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., Shimamoto, A., Goto, M., Miller, R.W., Smithson, W.A., Lindor, N.M., and Furuichi, Y. (1999). Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. Nat. Genet. *22*, 82–84.

Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H., and Takisawa, H. (2003). A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. Genes Dev. *17*, 1141–1152.

Laskey, R.A., and Madine, M.A. (2003). A rotary pumping model for helicase function of MCM proteins at a distance from replication forks. EMBO Rep. *4*, 26–30.

Lee, H., Trainer, A.H., Friedman, L.S., Thistlethwaite, F.C., Evans, M.J., Ponder, B.A., and Venkitaraman, A.R. (1999). Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, Brca2. Mol. Cell *4*, 1–10.

Li, W., Kim, S.M., Lee, J., and Dunphy, W.G. (2004). Absence of BLM leads to accumulation of chromosomal DNA breaks during both unperturbed and disrupted S phases. J. Cell Biol. *165*, 801–812.

Liao, S., Graham, J., and Yan, H. (2000). The function of Xenopus Bloom's syndrome protein homolog (xBLM) in DNA replication. Genes Dev. *14*, 2570–2575.

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.

Lomonosov, M., Anand, S., Sangrithi, M.N., Davies, R., and Venkitaraman, A.R. (2003). Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. Genes Dev. *17*, 3017–3022.

Mann, M.B., Hodges, C.A., Barnes, E., Vogel, H., Hassold, T.J., and Luo, G. (2005). Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund-Thomson syndrome. Hum. Mol. Genet. *14*, 813–825.

Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature *415*, 651–655.

Mechali, M., and Harland, R.M. (1982). DNA synthesis in a cell-free system from Xenopus eggs: priming and elongation on single-stranded DNA in vitro. Cell *30*, 93–101.

Mills, A.D., Blow, J.J., White, J.G., Amos, W.B., Wilcock, D., and Laskey, R.A. (1989). Replication occurs at discrete foci spaced throughout nuclei replicating in vitro. J. Cell Sci. *94*, 471–477.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Noguchi, E., Shanahan, P., Noguchi, C., and Russell, P. (2002). CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. Curr. Biol. *12*, 599–605.

Pacek, M., and Walter, J.C. (2004). A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. EMBO J. *23*, 3667–3676.

Patel, K.J., Yu, V.P., Lee, H., Corcoran, A., Thistlethwaite, F.C., Evans, M.J., Colledge, W.H., Friedman, L.S., Ponder, B.A., and Venkitaraman, A.R. (1998). Involvement of Brca2 in DNA repair. Mol. Cell *1*, 347–357.

Pichierri, P., Franchitto, A., Mosesso, P., and Palitti, F. (2001). Werner's syndrome protein is required for correct recovery after replication arrest and DNA damage induced in S-phase of cell cycle. Mol. Biol. Cell *12*, 2412–2421.

Prince, P.R., Emond, M.J., and Monnat, R.J., Jr. (2001). Loss of Werner syndrome protein function promotes aberrant mitotic recombination. Genes Dev. *15*, 933–938.

Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M., and Jacks, T. (2003). Acute mutation of retinoblastoma protein is sufficient for cell cycle re-entry. Nature *424*, 223–227.

Saintigny, Y., Makienko, K., Swanson, C., Emond, M.J., and Monnat, R.J., Jr. (2002). Homologous recombination resolution defect in Werner syndrome. Mol. Cell. Biol. *22*, 6971–6978.

Siitonen, 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.

Skibbens, R.V., Corson, L.B., Koshland, D., and Hieter, P. (1999). Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev. *13*, 307–319.

Tanaka, K., Yonekawa, T., Kawasaki, Y., Kai, M., Furuya, K., Iwasaki, M., Murakami, H., Yanagida, M., and Okayama, H. (2000). Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. Mol. Cell. Biol. *20*, 3459–3469.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., and Nasmyth, K. (1999). Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Vennos, E.M., and James, W.D. (1995). Rothmund-Thomson syndrome. Dermatol. Clin. *13*, 143–150.

Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. Mol. Cell *5*, 617–627.

Wang, H., and Elledge, S.J. (1999). DRC1, DNA replication and checkpoint protein *1*, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *96*, 3824–3829.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science *290*, 2309–2312.

Wohlschlegel, J.A., Dhar, S.K., Prokhorova, T.A., Dutta, A., and Walter, J.C. (2002). Xenopus Mcm10 binds to origins of DNA replication after Mcm2–7 and stimulates origin binding of Cdc45. Mol. Cell *9*, 233–240.

Yin, J., Kwon, Y.T., 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.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol. Cell. Biol. *20*, 3086–3096.

## Accession Numbers

The nucleotide sequence of a cDNA encoding *Xenopus laevis* RECQL4 has been deposited in GenBank with the accession number DQ059311.

## Note Added in Proof

While our paper was in press, we learned of the independent discovery by Dr. H. Takisawa (Osaka University, Japan) and his colleagues that the *Xenopus laevis* homolog of *RECQL4* contains an N-terminal homology to yeast Sld2/DRC1 and is essential for chromosomal DNA replication in egg extracts.