MCM10 mediates RECQ4 association with MCM2-7 helicase complex during DNA replication

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Mutations in RECQ4, a member of the RecQ family of DNA helicases, have been linked to the progeroid disease Rothmund–Thomson Syndrome. Attempts to understand the complex phenotypes observed in recq4-deficient cells suggest a potential involvement in DNA repair and replication, yet the molecular basis of the function of RECQ4 in these processes remains unknown. Here, we report the identification of a highly purified chromatin-bound RECQ4 complex from human cell extracts. We found that essential replisome factors MCM10, MCM2-7 helicase, CDC45 and GINS are the primary interaction partner proteins of human RECQ4. Importantly, complex formation and the association of RECQ4 with the replication origin are cell-cycle regulated. Furthermore, we show that MCM10 is essential for the integrity of the RECQ4–MCM replicative helicase complex. MCM10 interacts directly with RECQ4 and regulates its DNA unwinding activity, and that this interaction may be modulated by cyclin-dependent kinase phosphorylation. Thus, these studies show that RECQ4 is an integral component of the MCM replicative helicase complex participating in DNA replication in human cells.

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Introduction

The human RECQ family helicases have non-redundant roles in maintaining genome integrity, shown by the fact that mutations in three of the five RECQ homologues, BLM, WRN and RECQ4, have been implicated in distinct clinical diseases (Mohaghegh and Hickson, 2002; Singh et al, 2009). In particular, mutations in the RECQ4 gene have been associated with Rothmund–Thomson Syndrome (RTS), RAPADILINO and Baller–Gerold Syndrome. RTS patients exhibit various physical and mental developmental abnormalities and show signs of premature aging, such as early development of cataracts, hair loss and increased risk of osteosarcoma.

Most of the mutations associated with RTS lie within or after the conserved helicase domain located at the centre of the RECQ4 protein, and RTS symptoms were further recapitulated in mouse models containing deletion or nonsense mutations within the helicase domain (Kitao et al, 2002; Hoki et al, 2003; Mann et al, 2005). Cells derived from these recq4-deficient mice showed phenotypes ranging from aneuploidy to slow cell growth. The defect in cellular proliferation may be because of a cell-cycle block or problems with DNA damage repair. The latter possibility remains controversial, however, as mouse embryonic fibroblasts derived from the RECQ4 helicase-deficient mutants do not exhibit sensitivity to DNA damaging agents (Hoki et al, 2003). In addition, unlike other RECQ mutants, such as cells deficient in BLM and RECQ5 helicases, recq4-deficient mouse cells fail to show increased sister chromatid exchanges, chromosome breakages or fusions (Mann et al, 2005). These observations indicate that the primary function of RECQ4 is not a role in DNA damage repair. Nevertheless, cells from some, but not all RTS patients, are indeed sensitive to UV and ionizing radiation and show reduced DNA repair synthesis (Kitao et al, 2002; Singh et al, 2009).

An alternative explanation for the growth retardation and genome instability phenotypes of recq4 mutants is a defect in cell-cycle progression. Interestingly, sequence comparisons show that the N-terminus of Xenopus RECQ4 shares sequence homology with the yeast replication factor, Sld2 (Figure 1A; Sangrithi et al, 2005; Matsuno et al, 2006). Genetic studies show that yeast Sld2 interacts with Dpb11 to allow the onset of S phase (Kamimura et al, 1998; Tak et al, 2006), and this interaction is indeed mirrored between RECQ4 and the corresponding Dpb11 homologue in Xenopus, known as Cut5 (Matsuno et al, 2006). The potential of RECQ4 as the Sld2 homologue in Xenopus was further shown by the fact that DNA replication initiation is compromised in RECQ4-depleted Xenopus extracts (Sangrithi et al, 2005; Matsuno et al, 2006). It is possible that RECQ4 is the Sld2 homologue in vertebrates, in which case it would be expected to be essential for cell growth. Consistent with this prediction, deletion of the N-terminus of RECQ4 results in embryonic lethality in mice (Ichikawa et al, 2002). However, unlike Xenopus RECQ4, in which the sequence homology with Sld2 spans the entire 453a.a. region of Sld2, only the first 70a.a. of mammalian RECQ4 show detectable sequence homology with yeast Sld2 (Figure 1A). Additionally, the proposed Cut5 interaction domain in Xenopus RECQ4 is missing in the mammalian homologues. Presently, there is little mechanistic insight available to define the biochemical properties of the Sld2 protein and suggest a precise role for Sld2 in DNA replication initiation in yeast (Tanaka et al,
This very limited sequence homology, restricted to a small region of the Sld2 N-terminus, makes it difficult to sufficiently conclude whether RECQ4 functions as a true Sld2 homologue in DNA replication in mammalian cells.

To understand the unique function of each of the RECQ helicases in the DNA metabolism and their relationships with different clinical diseases, recent efforts have focused on elucidating specific protein–protein interactions that provide insight into the cellular processes of a particular RECQ helicase (for review see Liu and West, 2008). In this work, we show that human RECQ4 is an integral part of the DNA replisome. Importantly, our data provide novel molecular insights into the regulation of human RECQ4 and its communication with the DNA replication machinery that is distinct from our current understanding on the proposed homologues in lower organisms.

Results

Purification and identification of the human RECQ4 complex

To facilitate purification of the RECQ4 complex, a 293T cell line stably expressing FLAG-tagged RECQ4 was established. The exogenous FLAG–RECQ4 expression was approximately two-fold of the endogenous RECQ4 protein in 293T cells. The nucleoplasmic fraction was prepared from these cells and chromatin-bound proteins were solubilized by digesting the chromatin pellet with benzonase to remove nucleic acids as described earlier (Aygun et al., 2008). FLAG–RECQ4 complex was then purified by M2-agarose affinity chromatography and subjected to mass spectrometric analysis to identify co-purifying polypeptides. Although very few proteins are associated with nucleoplasmic RECQ4, SDS–PAGE showed several prominent bands for the RECQ4 complex purified from the soluble chromatin fraction (Figure 1B). After excluding the contaminants commonly found during FLAG immunopurification (Supplementary Figure 1; Guo et al., 2009), mass spectrometric analysis showed that the most abundant co-purified polypeptides in the chromatin-bound RECQ4 complex were MCM10, MCM4 and SLD5 (Figure 1B and C). All of these factors are known to interact together to form the MCM replicative helicase complex important for DNA replication initiation and progression (for review see Sclafani and Holzen, 2007). In addition, within the RECQ4 complex, we identified the human homologue of yeast Tof1-Csm3, the TIMELESS (TIM)/ TIPIN heterodimer, which is a known MCM2-7 helicase interacting protein important for fork progression and replication stress response (Errico et al., 2007; Gotter et al., 2007).
The interaction between RECQ4 and the MCM replicative helicase complex was further confirmed by the co-immunoprecipitation of MCM10, MCM7 and SLD5 with endogenous RECQ4 protein from 293T cells without ectopic expression using a rabbit anti-RECQ4 antibody (Figure 1D).

Surprisingly, in contrast to the studies with Xenopus RECQ4, we failed to detect the presence of the human Dpb11/Cut5 homologue, TOPBP1, in the purified RECQ4 complex by both mass spectrometry analysis and western blotting. Moreover, though Xenopus RECQ4 has been shown to co-immunopurify with Cut5, other replication factors such as MCM2-7 helicase and CDC45 were reported not to associate with Xenopus RECQ4 (Matsuno et al., 2006). Our identification of the MCM proteins as the primary RECQ4 interacting partners on human chromatin provides the first evidence of a physical link between human RECQ4 and the DNA replication machinery that is distinct from the Xenopus homologue.

**Cell cycle regulated RECQ4–MCM complex formation**

During our purification, we observed that the interaction of RECQ4 with MCM replicative complex only exists in actively dividing cells, but not in quiescent cells, leading us to hypothesize that RECQ4–MCM complex formation is cell-cycle regulated. Consistent with this proposal, complex formation among DNA replication factors is known to be highly regulated by post-translational modification or by cell cycle-dependent protein expression. Indeed, we found that both endogenous RECQ4 and MCM10 are predominantly expressed during G1 and S phases, in contrast to the constitutive expression of MCM2-7 helicase (represented by MCM7 expression) and GINS (represented by SLD5 expression; Figure 1D). To examine the dynamics of the RECQ4 complex during G1 and S phases, in which all the major components of the complex are present, 293T cells stably expressing FLAG–RECQ4 were synchronized in G2/M phase by serum starvation followed by nocodazole treatment to arrest the cells. At different time points after nocodazole release, cells were collected and their stage in the cell cycle was monitored by flow cytometry (Figure 2A, upper) and Cyclin A expression, which is required for S-phase progression (Figure 2A, lower; Yam et al., 2002). We established that the mitotic phase takes place between 1–4 h after nocodazole release, followed by G1 phase (5–8 h) and S phase (9–14 h). Unlike endogenous RECQ4, FLAG–RECQ4 was constitutively expressed in these cells and could be detected in the chromatin-bound fraction at all cell-cycle stages (Figure 2C, top panel). When FLAG–RECQ4 was immunoprecipitated from the soluble chromatin-bound fraction at each time point, we found that MCM10 indeed associates with RECQ4 only during G1 and S phases because of the cell cycle regulated MCM10 expression. The kinetics of the association of MCM7 with RECQ4 was similar to that of MCM10, whereas SLD5 was only enriched significantly in the RECQ4–MCM complex starting at the G1/S-phase transition. The latter interaction is consistent with observations made in yeast that GINS initiates its interaction with MCM2-7 helicase at the G1/S-phase transition (for review see Sclafani and Holzen, 2007).
Cell cycle-dependent association of RECQ4 with origins of replication

Given that RECQ4 associates with MCM2-7 helicase during G1 phase and that MCM2-7 helicase is part of pre-RC complex (for review see Sclafani and Holzen, 2007), we further established that RECQ4 is recruited to the origins of replication before replication initiation. To do this, we analysed the association of FLAG–RECQ4 with a 225 bp LAMIN B sequence containing one of the most well-characterized mammalian replication origins (Todorovic et al., 1999). Experiments containing an equal amount of DNA input from G1 (7 h post-nocodazole release) and S-phase cells (12 h post-nocodazole release; Figure 3A, top panel) were incubated with M2-agarose to isolate FLAG-tagged proteins at each time point. The 225 bp fragment from the M2-bound protein–DNA complex was amplified by PCR showing that the LAMIN B Ori sequence was five-fold enriched in the purified RECQ4–DNA complex from G1 cells compared to those from S phase (Figure 3A, lanes a and c, and Figure 3B). In contrast, this cell cycle-dependent enrichment at the LAMIN B Ori was not seen in a 309 bp-control PCR at the p53 gene locus (Figure 3A, 3rd panel). In addition, a control chromatin-bound protein, FLAG–XRCC2, failed to exhibit similar cell cycle-dependent LAMIN B Ori association (Figure 3A, lanes b and d, and Figure 3B). Our observations indicate that RECQ4 is recruited to the origin of replication before replication initiation.

The recruitment of RECQ4 to the origin of replication led us to ask whether RECQ4 has a function in pre-RC establishment on human chromatin. For this, RECQ4 siRNA knockdown 293T cells were separated into cytosolic, nucleoplasmic and soluble chromatin-bound fractions (Figure 3C). The lysates were probed with antibodies to detect RECQ4, MCM10, MCM7, SLD5 and CDC6. Consistent with earlier observation (Yin et al., 2004), RECQ4 was found in both the cytoplasm and the nucleus (Figure 3C, top panel, lanes a–c). In contrast, MCM10, MCM7 and CDC6 were enriched in the chromatin fraction. When RECQ4 was downregulated, neither the protein stability nor the chromatin association of CDC6, MCM10 and MCM7 was affected (Figure 3C, lanes d–f), suggesting that RECQ4 is downstream of MCM10 and MCM2-7 in pre-RC establishment. On the other hand, the amount of chromatin-bound SLD5 was significantly reduced in the RECQ4 knockout cells (Figure 3C, 5th panel from top, comparing lanes c and f). This observation not only is consistent with our cell-cycle analysis indicating that RECQ4 associates with MCM2-7 helicase before GINS during G1 (Figure 2C), but also suggests that RECQ4 is required for the recruitment of GINS to the human chromatin and its interaction with MCM2-7 helicase.

MCM10 mediates RECQ4 association with MCM2-7 helicase/GINS complex

Surprisingly, even though MCM7 and SLD5 are present throughout the cell cycle (Figure 2B, 3rd and 4th panels from top), their interactions with constitutively expressed FLAG–RECQ4 were still limited to G1 and S phases, when MCM10 was expressed (Figure 2C and D). To determine whether the RECQ4 interactions with MCM2-7 helicase and GINS require the presence of MCM10, we analysed FLAG–RECQ4 complex in MCM10 siRNA knockdown cells (Figure 4A). In the absence of MCM10, neither the level of RECQ4 nor that of MCM7 in the chromatin-bound fraction was affected. The latter was in agreement with the earlier report that MCM10 is not required for the chromatin binding of MCM2-7 helicase (Wohlschlegel et al., 2002). On the other hand, similar to the RECQ4 knockout cells (Figure 3C), the amount of chromatin-bound SLD5 was noticeably reduced in the MCM10 knockdown cells, consistent with a function of
MCM10 upstream of GINS and CDC45 during DNA replication initiation (Figure 4A, lane b, bottom panel; Wohlschlegel et al., 2002). Nevertheless, in the absence of MCM10, RECQ4 failed to co-purify with both MCM7 and SLD5 (Figure 4A, comparing lanes c and d). This result indicates that MCM10 is required for the formation and the integrity of the RECQ4–MCM replicative complex.

Direct interaction between RECQ4 and MCM10

As MCM10 has a crucial role in the complex formation of RECQ4 with the DNA replication factors, we next determined whether RECQ4 directly interacts with MCM10 using purified recombinant proteins. For this, we overexpressed and purified full-length RECQ4 and RECQ4 fragments as N-terminal His-tagged and C-terminal FLAG-tagged (Xu and Liu, 2009). We also cloned and purified MCM10 as GST-tagged to homogeneity. We found that GST–MCM10, but not GST alone, bound to glutathione resin, efficiently pulled down full-length RECQ4 and RECQ4 fragments. His-tagged RECQ1 was used as control. After washing, the beads were boiled, and bound proteins were analysed by SDS–PAGE followed by western blotting using anti-His antibodies. (Upper panel) 30% of total input of each His-tagged proteins. (Bottom panel) His-tagged proteins after pull-down. (C) MCM10 specifically interacts with RECQ4 but not with other RECQ family helicases. FLAG–RECQ complexes were immunopurified from 293T cells using M2-agarose and analysed for the presence of MCM10 by western blotting using rabbit anti-MCM10 antibody. (D) Schematic diagram of the full-length RECQ4 and RECQ4 fragments. The conserved SFII helicase domain is shown in dark grey, whereas the Sld2-like domain is shown in light grey. (E) GST pull-down experiment as described in (B), except different His–RECQ4 N-terminal fragments were used. (F) GST pull-down experiment as described in (B) to test MCM10 interaction with the RECQ41–100 fragment.

MCM10 inhibits RECQ4 helicase activity. Helicase activity of the recombinant RECQ4 proteins on 32P-labelled duplex oligos in the presence of increasing amounts of purified MCM10 proteins. 32P-labelled single-stranded DNA products were visualized by autoradiography following neutral PAGE.
analysis failed to detect the presence of MCM10 in any of the RECQ complexes except RECQ4. The unique interaction of MCM10 with RECQ4 was further confirmed by western blotting of the purified RECQ complexes (Figure 4C).

Interestingly, using different RECQ4 fragments, the interaction domain was mapped to the N-terminus of RECQ4 (Figure 4B, lane b). When smaller fragments containing sequences derived from the N-terminus were generated and analysed (Figure 4D), we found that all RECQ4 fragments containing the first 99a.a. with the Sld2-like sequence showed a positive interaction with MCM10, suggesting that this region is crucial for the MCM10 interaction (Figure 4E, lanes a, b and d). However, the Sld2-like domain alone is not sufficient for the interaction, as shown by the negative interaction using a RECQ4 fragment containing only the first 100a.a. (Figure 4F, lane c). These data together indicate that MCM10 interacts with the first 200a.a. of RECQ4 containing the Sld2-like sequence.

**MCM10 regulates RECQ4 helicase activity**

Earlier, we showed that RECQ4 is an active DNA helicase capable of unwinding DNA structures including splayed arms, bubbles and blunt-end duplex DNA (Xu and Liu, 2009). We further showed that the first 99a.a. containing the Sld2-like domain is required for the efficient DNA unwinding activity by promoting protein–DNA interaction. As this Sld2-like domain is also involved in the interaction with MCM10, we next tested the effect of MCM10 interaction on RECQ4 helicase activity. Interestingly, the addition of GST–MCM10 to the RECQ4 reaction efficiently inhibits RECQ4 helicase activity (Figure 5, lanes c–f). Under the same condition, GST showed no effect on the RECQ4 helicase reaction (Figure 5, lane g). The inhibition may be because of the competition for DNA substrate binding by MCM10; however, this is unlikely because the reaction contains excess of unlabelled ssDNA relative to protein concentration (Xu and Liu, 2009). Most likely, the inhibition is due to the inability of MCM10-associated RECQ4 to directly bind to DNA substrate. Our result suggests that RECQ4 is subject to regulation through its direct interaction with MCM10.

**Regulation of RECQ4–MCM10 interaction through cyclin-dependent kinase phosphorylation sites**

In mammalian cells, excess of MCM2-7 molecules are found on chromatin, and the majority of these molecules are kept inactive during an unperturbed S-phase progression (Ge et al, 2007; Ibarra et al, 2008). Only a limited number of MCM2-7 helicases are needed for origin activation and replication progression. Most of the MCM2-7 helicases associate with dormant origins that serve as backup origins for re-starting DNA synthesis when cells are challenged with DNA replication stress that either stalls or slows down normal replication forks. Consistent with the dormant origin model, we found that MCM7, MCM10 and RECQ4 siRNA knockdown cells showed little defect in cell cycle progression compared with the mock-controlled cells (Figure 6A). In contrast, depletion of CDC6, a protein important for pre-RC establishment, resulted in cell cycle arrest at G2/M phase (Figure 6A).

We next examined the ability of RECQ4 and MCM knockdown cells to restart DNA synthesis after DNA replication stress. For this, we treated the knockdown cells with hydroxyurea for 48 h (Figure 6B). After the cells were released from hydroxyurea, we measured the rate of DNA synthesis by in vivo ³H-thymidine labelling. We found that MCM7, MCM10 and RECQ4 siRNA knockdown cells all showed much reduced efficiency in DNA synthesis. This observation suggests that similar to the MCM proteins, RECQ4 is involved in replication fork restart and/or initiation of dormant origins for the efficient recovery from DNA replication stress (Figure 6C).

In yeast, both MCM2-7 helicase and Sld2 are activated by hyperphosphorylation to engage in DNA replication (Masumoto et al, 2002; Sheu and Stillman, 2006; Zegerman and Diffley, 2007; Tanaka et al, 2007a,b). Human RECQ4 may also be subjected to cell cycle control by phosphorylation. Indeed, we found that a purified recombinant GST–RECQ41–200 fragment containing the Sld2-like sequence was phosphorylated in vitro by human cell extracts (Figure 7A, left lane). The kinase activity on the GST–RECQ41–200 was significantly reduced by the addition of roscovitine, a cyclin-dependent kinase (CDK) inhibitor, to the extracts (Figure 7A, right lane). RECQ41–200 contains three S/T/P CDK target sites (S89, T93, T139; Figure 7B, dark grey boxes) and two CDK-binding motifs (RxL; Figure 7B, light grey boxes). Phospho-mimicking mutations of any of the CDK target S/T sites to glutamate (E) reduce the amount of ³²P-label on the GST–RECQ41–200 fragment (Figure 7C). The reduction is most profound in the fragment containing mutations at all three CDK target sites (e.g. triple mutant, Figure 7C), suggesting that all the three CDK target sites are potentially phosphorylated in vivo. Interestingly, even though the RECQ4 triple-glutamate mutant is proficient in DNA unwinding (Supplementary Figure 2), the mutations significantly weaken the interaction of RECQ4 with MCM10 in a salt-dependent manner compared with WT RECQ4 (Figure 7D). Overall, our data suggest that though MCM10 negatively regulates RECQ4 helicase activity through direct protein–protein interaction, CDK phosphorylation at the Sld2-like domain of RECQ4 may serve as a switch to activate RECQ4 helicase activity during replication by allowing a transient dissociation of RECQ4 from MCM10.

**Discussion**

DNA replication is essential for cell proliferation. Improper DNA replication may compromise chromosome integrity, leading to apoptosis or cell transformation. Studies in mice indicate that the N-terminal domain of RECQ4 is crucial for embryonic development and cell survival, whereas the conserved helicase domain is required for preventing premature aging and cancer predisposition (Ichikawa et al, 2002; Hoki et al, 2003; Mann et al, 2005). In this study, we report that RECQ4 is a part of the replication initiation complex and forms a stable association with essential DNA replication factors, MCM10, MCM2-7 helicase, CDC45 and GINS in a cell cycle-dependent manner. After replication initiation, RECQ4 may also be a part of the replication progression complex along with the replication progression factors, TIM/TIPIN heterodimer. Among these replication factors, we showed that MCM10 directly interacts with RECQ4 and regulates its DNA helicase activity. This interaction is specific to RECQ4 but not other RECQ helicases, suggesting a unique role of RECQ4 in DNA replication. Importantly, we further showed that MCM10 is required for the interactions of RECQ4...
with MCM2-7 helicase and GINS. As MCM10 is known to directly interact with MCM2-7 (Homesley et al., 2000), it is possible that RECQ4 indirectly associates with MCM2-7 and GINS through MCM10. Alternatively, we cannot rule out the possibility that a direct interaction between RECQ4 and MCM2-7 helicase requires the MCM10-dependent post-translational modification events (Lee et al., 2003).

Our identification of a cell cycle regulated human RECQ4–MCM replicative helicase complex indicates that the primary role of RECQ4 is in DNA replication, and allows us to suggest a model for the recruitment and activation of RECQ4 in DNA replication. After cell division, RECQ4 establishes a stable interaction with MCM10 and MCM2-7 helicase and is recruited to the origins of replication at G1 phase before replication initiation. Evidence in the *Xenopus* study suggests that RECQ4 helicase activity is required for efficient origin melting (Sangrithi et al., 2005); therefore, we postulate that RECQ4 activity requires tight regulation by MCM10 to prevent unlicensed replication initiation. Similar to MCM2-7 helicase, RECQ4 may be activated temporarily for the limited number of origin firings during the G1/S transition and S phases, which could be achieved through CDK phosphorylations that release RECQ4 from MCM10, allowing RECQ4 to directly interact with DNA at origins and unwind DNA. Supporting this model, we found that the RECQ4 fragment containing the first 200 a.a. was phosphorylated in vitro in a CDK-dependent manner. Importantly, phospho-mimicking mutations at the CDK target sites within this region of RECQ4 greatly reduce its affinity to MCM10 at physiological salt concentrations (e.g. 150 mM). The interesting question that remains to be addressed is why do two DNA helicases (e.g. MCM2-7 and RECQ4) associate with each other during S phase? One possibility is that RECQ4 helicase activity is required to facilitate the opening of duplex DNA at the origin by the MCM2-7 helicase to allow the subsequent loading of RPA and DNA polymerase onto chromatin (Sangrithi et al., 2005).

The data presented here indicate that human RECQ4 is an integral component of the MCM replicative helicase complex during DNA replication. The implication that RECQ4 acts in DNA replication initiation has been suggested from studies of *Xenopus* extracts, based on its sequence similarity to the yeast replication initiation factor, Sld2 (Sangrithi et al., 2005; Matsuno et al., 2006). However, doubts that this function is evolutionally conserved in mammals were raised. First, not only there is less than 20% sequence identity found between the N-termini of human and *Xenopus* RECQ4, but also only the first 70 a.a. of the human RECQ4 protein sequence can be aligned with a small fragment of the yeast Sld2 protein.

**Figure 6** RECQ4–MCM complex is required for replication-restart after hydroxyurea treatment. (A) Flow cytometry of 293T cells 48 h after transfecting with mock, CDC6, MCM7, MCM10 and RECQ4 siRNA. (B) Outline of 3H-thymidine labelling of the siRNA knockdown cells after hydroxyurea treatment. (C) DNA synthesis of 293T siRNA knockdown cells after hydroxyurea treatment was monitored by [3H]-thymidine labelling. Count per minute (CPM) was divided by the actual number of cells of each of the siRNA knockdowns to obtain CPM per cell (y-axis).
Second, no interaction between human RECQ4 and TOPBP1 was found in normal cycling cells in our study. Our findings suggest that though the participation of RECQ4 in DNA replication is evolutionally conserved in vertebrates, the molecular basis of the role of RECQ4 in DNA replication may likely have diverged among different organisms.

Although yeast Mcm10 and Sl2 are both important factors for DNA replication initiation, no reports have suggested a direct interaction between Mcm10 and Sl2 in lower eukaryotes. Nonetheless, we cannot exclude the possibility that an association between Sl2 and Mcm10 may also exist but is yet to be shown in lower organisms. Indeed, in humans, MCM10 interacts with RECQ4 through its Sl2-like region. The failure to detect an interaction between RECQ4 and MCM complex in Xenopus egg extracts may be because of the fact that this interaction primarily forms on chromatin rather than in chromatin-free extracts. In addition, although cumulative genetic studies so far demonstrate the requirement of Sl2 for DNA replication initiation, it is not clear how Sl2 communicates with pre-RC in licensing replication (Tanaka et al., 2007a). Our data presented here not only show the complex interaction between human RECQ4 and DNA replication machinery but may also provide an important clue to how Sl2 in yeast potentially interacts with the replication complex containing MCM2-7 helicase, GINS and Cdc45 during replication initiation.

Materials and methods

Cell culture and siRNA

Human 293T cells were cultured in DMEM medium supplemented with 10% v/v foetal bovine serum (FBS) and streptomycin/penicillin (100 U/ml). For synchronization, cells were cultured in DMEM without FBS for 22 h and released from serum starvation by the addition of FBS to a final concentration of 10%. After 2 h incubation at 37°C supplemented with 5% CO2, nocodazole was added to the medium to a final concentration of 50 ng/ml. After 16 h of incubation, cells were released by washing 2 × with complete DMEM medium. FACs analysis was carried out using a standard propidium iodide method. siRNA knockdown cells were generated using SMARTpool siRNAs (Dharmacon) for RECQ4, MCM10 and MCM7. CdC6 siRNA was generated as described (Malland and Diffley, 2005). siRNAs were transfected into 293T cells using RNAiMax transfection reagent (Invitrogen) according to the manufacturer’s protocol. For hydroxyurea treatment, 48 h after siRNA knockdown, 293T cells were incubated with 0.75 mM hydroxyurea for 48 h. Cells were released from hydroxyurea treatment by washing twice with pre-warm medium and further incubated for 1 h in complete medium before the addition of 5 µCi/ml [3H]thymidine (76 Ci/mmol, Perkin Elmer) for 2 h. Cells were processed as described (Liu et al., 1999).

Cell fractionation, immunoprecipitation and ChIP

293T parental cell line and 293T cells stably expressing FLAG-tagged RECQ constructs were fractionated to cytosolic, nucleoplasmic and benzonase-treated soluble chromatin-bound fractions performed as described earlier (Aygun et al., 2008) with the exception that MgCl2 and KCl were used in lysis and nuclease buffers. To immunopurify FLAG-tagged proteins, chromatin or nucleoplasmic extracts were incubated overnight with M2-agarose (Sigma) at 4°C. After binding of the protein complexes, beads were washed extensively with FLAG-A-binding buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.3 M NaCl, 10 mM KCl, 0.2% Triton X-100, 10% glycerol). The purified FLAG-tagged protein complexes were eluted by using FLAG elution A buffer (10 mM HEPES 7.9, 0.2 M NaCl, 0.2 mM EDTA, 0.05% Triton-X, 0.3 mg/ml FLAG peptide, 10% glycerol), and subjected to western blotting by standard methods or mass spectrometric analysis at the Taplin Biological Mass Spectrometry Facility at Harvard University. For ChIP analyses, chromatin pellet was obtained as described (Aygun et al., 2008) but was re-suspended in chromatin buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 150 mM KCl, 10% glycerol, protease and phosphatase inhibitors) followed by sonication. FLAG-tagged protein–DNA complex was immunopurified on M2-agarose, eluted with FLAG elution A buffer. After proteinase K digestion, the co-purified DNA was cleaned up by PCR purification kit (Qiagen) before PCR analysis using primers, 5’-CTGCAGTGGGGCTTGCCATG-3’ and 5’-GACATCGCT
TCATAGGGCAC-3' for LAMIN B Ori locus. For the control PCR, 5'CAGCTCCTCTCCTCTTTCC-3' and 5'GTTCTCTCTTTGCGTG CTG GG-3' were used to amplify the p53 gene locus. For immunopreparation of endogenous RECQ4, rabbit anti-RECQ4 antibody (SDI) was conjugated to protein-A beads, incubated with 293T extracts and washed with FLAG-A-binding buffer to remove non-specific proteins. For rabbit, anti-MCM10 (ProteinTech Group), rabbit anti-RECQ4 (4–11), goat anti-RECQ4 (Santa Cruz), rabbit anti-SLD5 (SDI) and rabbit anti-CD6 (Santa Cruz) were used.

**Proteins**

Recombinant RECQ4 FL and fragments were prepared as described (Xu and Liu, 2009). GST–MCM10 and GST in pGEX-4T-1 vector (GE Healthcare) were expressed in Rosetta (DE3) pLYsS cells by induction with 0.1 mM isopropyl-β-D-thio-galactoside overnight at 16 °C. Cell pellets were suspended in buffer H (1 × PBS, pH7.4, 10% glycerol, 0.1 mM EDTA, 0.5 % Triton X-100), plus 1× protease inhibitor cocktail (Roche) and lysed by sonication. The supernatant after centrifugation was incubated with glutathione agarose beads (PierceNet) overnight at 4 °C. The beads were washed five times with buffer H and eluted with 10 mM glutathione. For GST–MCM10 containing a C-terminal 2XFLAG tag, the eluate from glutathione agarose beads was further purified with anti-FLAG M2 beads (Sigma) as described for the RECQ4 proteins.

**In vitro protein interactions and helicase assays**

GST–MCM10/GST bound to the glutathione beads were first blocked with 5 mg/ml of BSA in binding buffer (40 mM Tris, pH 7.4, 10% glycerol, 0.1 mM EDTA, 0.5 % Triton X-100), plus 1× protease inhibitor cocktail (Roche) and lysed by sonication. The supernatant after centrifugation was incubated with glutathione agarose beads (PierceNet) overnight at 4 °C. The beads were washed five times with buffer H and eluted with 10 mM glutathione. For GST–MCM10 containing a C-terminal 2XFLAG tag, the eluate from glutathione agarose beads was further purified with anti-FLAG M2 beads (Sigma) as described for the RECQ4 proteins.

**References**


**Conflict of interest**

The authors declare that they have no conflict of interest.