

Recq15 plays an important role in DNA replication and cell survival following Camptothecin treatment

Yiduo Hu[†], Xincheng Lu, Guangjin Zhou, Ellen L. Barnes and Guangbin Luo^{*}

Department of Genetics, Case Comprehensive Cancer Center and University Hospitals, Case Western Reserve University, Cleveland, OH 44106, USA.

*To whom correspondence should be addressed: Department of Genetics, Case Western Reserve University, BRB-720, 10900 Euclid Avenue, Cleveland, OH 44106, USA.

Tel: 1-(216)-368-4883; Fax: 1-(216)-368-3432; E-mail: guangbin.luo@case.edu

[†]Current address: Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA.

Running title: Recq15 protects CPT-induced stalled fork.

Abstract

Disruption of replication can lead to loss of genome integrity and increase of cancer susceptibility in mammals. Thus, a replication impediment constitutes a formidable challenge to these organisms. Recent studies indicate that homologous recombination (HR) plays an important role in suppressing genome instability and promoting cell survival following exposure to various replication inhibitors, including a topoisomerase I inhibitor, camptothecin (CPT). Here, we report that the deletion of RecQ helicase Recq15 in mouse ES cells and embryonic fibroblast (MEF) cells resulted in a significant increase in CPT sensitivity and a profound reduction in DNA replication following the treatment with CPT, but not other DNA damaging agents. This CPT-induced cell death is replication dependent and occurs primarily after the cells had exited the first cell cycle after CPT treatment. Furthermore, we show that Recq15 functions non-redundantly with Rad51, a key factor for HR to protect mouse ES cells from CPT-induced cytotoxicity. These new findings strongly suggest that Recq15 plays an important role in maintaining active DNA replication to prevent the collapse of replication forks and the accumulation of DSBs in order to preserve genome integrity and to prevent cell death following replication stress as a result of topoisomerase I poisoning.

Introduction

Mammalian RecQ helicases share a high degree of homology with the RecQ helicase of *E. coli* (Nakayama, 2002). These helicases have important roles in maintaining the integrity of the genomes and promoting cell survival in response to various types of genotoxic stress (Hickson, 2003). In addition, some of them have also been implicated in DNA replication (Oakley *et al.*, 2002; Cheok *et al.*, 2005). The only RecQ helicase in *E. coli*, RecQ, plays an important role in the recovery of genomic replication following DNA damage (Courcelle and Hanawalt, 1999, 2001). In both budding yeast and fission yeast, there exist also just a single RecQ helicase. In both species, the RecQ helicase is involved in a number of DNA transaction processes, including repair, recombination and chromosome segregation (Hickson, 2003).

Interestingly, these enzymes do not appear to be essential for processive DNA synthesis. Rather, they are required to prevent genomic instability by coordinating replication and recombination events at stalled replication forks (Lambert *et al.*, 2007). Unlike unicellular organisms which have a single RecQ helicase, mammals possess a family of helicases encoded by five different genes (i.e. human *RECQL*, *BLM*, *WRN*, *RECQL4* and *RECQL5*) (Hickson, 2003). Each of these five genes encodes one or multiple polypeptides with distinctive C- and/or N-terminal domains, suggesting that they have related but non-overlapping functions. In particular, mutations in three of the five RecQ homologues, *RECQL4*, *WRN* and *BLM* give rise to three distinct genomic instability and cancer-prone genetic disorders: Rothmund-Thomson, Werner, and Bloom Syndromes, respectively (Ellis *et al.*, 1995; Yu *et al.*, 1996; Kitao *et al.*, 1999). However, their exact roles during DNA replication remain unclear (Hanawalt, 1966; Lambert *et al.*, 2007).

CPT and its derivatives are potent inhibitors of DNA replications and are very useful for studying DNA replication in mammalian cells. They are a unique group of anticancer agents that target topoisomerase I (Pizzolato and Saltz, 2003; Pommier *et al.*, 2006). Recent studies have shown that Camptothecins kill mammalian cells by interfering with the function of topoisomerase I during DNA replication (Hsiang *et al.*, 1985). Mammalian topoisomerase I facilitates a highly reversible process of incision-religation in front of a replication fork to allow the rapid release of topological constrain. CPT and its derivatives can impede the function of topoisomerase I by blocking the religation step of this process. As a consequence, CPT stabilizes the topoisomerase I-DNA complex, which results in a persistent nick in front of a replication fork. Such a nick, when present on the leading strand of replication, can be converted into a double strand break (DSB) by run-off synthesis, collapsing the fork (Strumberg *et al.*, 2000). It is currently believed that the DSBs associated with such collapsed replication forks represent the main cytotoxic lesion induced by CPT (Ryan *et al.*, 1991; Shao *et al.*, 1999; Furuta *et al.*, 2003). Consistent with this current hypothesis, it has been shown that homologous recombination repair (HRR) and the signal transduction cascade that is involved in this repair process both play critical roles in suppressing genome

instability and promoting cell survival following CPT exposure (Arnaudeau *et al.*, 2001a; Malik and Nitiss, 2004; Sorensen *et al.*, 2005). Therefore, CPT has been widely used to interrogate the mechanisms involved in the stabilization, repair or restoration of impeded replication forks in mammalian cells.

We recently reported that Recq15 deficient mouse cells were prone to gamma H2AX focus formation and gross chromosomal rearrangements (GCRs) following CPT treatment (Hu *et al.*, 2007). These observations strongly suggest that Recq15 plays an important role in preventing the collapse of replication forks following CPT treatment. Here we show that Recq15 functions to ensure that replication forks remain active following CPT treatment. A significantly higher proportion of Recq15-deficient cells, compared with their wild-type counterpart, fail to incorporate BrdU when treated with CPT. Nonetheless, these CPT-treated mutant cells were able to resume replication and exit S phase after the removal of CPT. Moreover, the loss of replication capability upon CPT treatment in the absence of Recq15 is followed by a rapid increase in DSB accumulation, GCRs, and replication-dependent cell death. In addition, Recq15 and Rad51 have non-redundant roles in promoting cell survival after CPT treatment. Collectively, these data demonstrate that Recq15 plays an important role in maintaining the integrity of replication forks when replication is impeded as the result of topoisomerase I poisoning. This function is required for preserving genome stability and for ensuring cell survival in response to the replication stress caused by topoisomerase I poisoning.

Materials and Methods

Establishment and Culture of Mouse ES Cells and MEFs. Culture and genetic manipulation of mouse ES cells were performed as described (Hu *et al.*, 2005). To generate ES cell clones expressing human RECQL5 β , full-length *RECQL5 β* cDNA was amplified by RT-PCR using total RNA prepared from HeLa cells (Sequences of primers used available upon request). The PCR product was cloned into the *pTRE2* vector (Clontech) to obtain *pTRE-hQ5 β* and the identities of the clones were confirmed by sequencing.

PGK-RtTA was obtained by replacing the *CMV* promoter with a *PGK* promoter on the *pTet-On* vector (Clontech) for optimal expression in mouse cells. *pTRE-hQ5 β* , *PGK-RtTA* and *PGKNeo* were then co-electroporated into ES cells and G418 resistant clones were obtained. Clones which contain both *pTRE-hQ5 β* , *PGK-RtTA* were identified by PCR. They were then further screened by quantitative RT-PCR to identify those in which *RECQL5 β* mRNA expression is regulated by doxycycline treatment. The primers used in quantitative RT-PCR are: hQ5e16f (5'-CAGACTGAGGAGTGCCTCAGG-3') and hQ5e17r (5'-CTGCTGGGATCGAGGCCGCTT-3'). *Gapdh* was amplified as internal control by primers: GAPDH1 (5'-GTGCTGAGTATGTCGTGGAGT-3') and GAPDH2 (5'-CACACACCCATCACAAACATG-3'). Rad51 knock-down mouse ES cell lines were generated using a construct expressing shRNA against mouse *Rad51* obtained from the OpenBiosystems (Accession ID NM_011234, clone ID V2MM_77978). The construct was co-electroporated with *PGKNeo* into ES cells and stable cell lines containing this vector were selected in G418-containing medium. G418 resistant clones were further screened by Western blot to identify clones with substantial reduced levels of Rad51. Primary MEF cultures were derived from 13.5 days post coitus embryos of various genotypes as described (Hu *et al.*, 2005; Mann *et al.*, 2005). All MEF cells used in experiments were under passage number four. MEFs were cultured in a low-oxygen incubator (3% O₂, 5% CO₂ and 92% N₂) (Parrinello *et al.*, 2003).

Clonogenic Survival Assay Experiments. ES cells of various genotypes were seeded onto 6-well feeder plates at 1X10⁴ cells/well density. Cells were treated with individual drug or drug combinations for 24 hours. CPT, MMS, aphidicolin were obtained from Sigma-Aldrich. For aphidicolin rescue experiments, 1 μ M aphidicolin was added one hour before the CPT treatment. After the treatments, drugs were removed and treated cells were allowed to grow in drug-free medium for 8 days. Then numbers of colonies in each well were determined as described (Hu *et al.*, 2005). For each treatment, the experiment was repeated three times and the mean values derived from these three experiments were then used to construct the survival curves.

Cell Cycle Analysis and Flow Cytometry. A standard propidium iodide (PI) staining approach was used to analyze the cell cycle profile. Briefly, ES cells were seeded and then treated either with or without 100 nM CPT for 16 hours. At different time point during the treatment, 1×10^6 ES cells were harvested and fixed with 70% ethanol in $-20\text{ }^\circ\text{C}$, washed with PBS and re-suspended in staining solution (50 $\mu\text{g/ml}$ PI (Sigma), 200 $\mu\text{g/ml}$ RNase A (Roche)) for flow analysis. For S phase analysis, cells were initially treated as described above. After the treatment, cells were returned to fresh medium with 1 $\mu\text{g/ml}$ nocodazole and harvested at various time points for analysis. Before each time point, cells were pulse-treated with 10 μM BrdU (Sigma) for 30 minutes and then processed for flow cytometry. BrdU incorporated cells were detected using an FITC conjugated anti-BrdU antibody (Becton Dickinson). TUNEL assay and Annexin V staining experiments were performed with the APO-DIRECT and Annexin V-FITC apoptosis detection kits (BD PharMingen), respectively. The experiments were carried out according to the manufacturer's instructions. All flow cytometry data were collected using a Coulter EPICS XL-MCL Cytometer (Beckman Coulter) or a BD LSR I Cytometer (Becton Dickinson). Data were analyzed using FACSscan (Becton Dickinson) and the WinMDI (J. Trotter, Scripps Institute) software packages.

Western Blot. Western blot experiments were performed with standard procedure. Specifically, the dilutions for each antibody were: Rabbit anti-RAD51 (Santa Cruz), 1:1000; mouse anti-CHK1 (Santa Cruz), 1:500; rabbit anti-phospho-CHK1 (Ser 345, Cell Signaling Technology), 1:1000; and mouse anti-U1-70K (provided by Dr. Hua Lou), 1:2000. HRP conjugated goat anti-rabbit IgG and horse anti-mouse IgG (Cell Signaling Technology) were used as secondary antibodies.

Pulse Field Gel Electrophoresis (PFGE). PFGE experiments were carried out according to the protocol described in the user's manual of the PFGE instrument with some modifications. Briefly, after the designed treatments, ES cells were embedded in 0.1 ml 1.0% low melting point agarose at a concentration of 5×10^6 cells/ml and incubated in 5 ml lysis buffer (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1%

sodium lauryl sarcosine, 1 mg/ml Proteinase K) at 50 °C for 48 hours. Plugs were inserted into 1% megabase agarose gel and electrophoresis was performed in 0.5X TBE buffer using a CHEF-DR II system (Bio-Rad) with the following parameters: 4 V/cm, 120° angle and 60-240 switching time at 10 °C for 28 hours. Gels were stained in 0.5 µg/ml ethidium bromide (EtBr) for 30 min and images were captured on an UV transilluminator equipped with a digital camera (Kodak). For quantification, DNA was transferred onto membranes following a standard Southern hybridization protocol. Membranes were probed with ³²P labelled whole mouse genomic DNA (*Dpn* I digested) and then exposed in a phosphorimager screen cassette (Amershan). Images were captured using a Storm 820 scanner (Amershan) and analyzed using ImageJ software. Background in each lane was removed by subtracting the intensity of an empty lane and then the signal intensity of each lane was normalized to total DNA in both well and lane. The relative amounts of damaged DNA in the non-treated lanes of both wild-type and mutant cells were set to one and the relative intensities of other lanes were expressed as folds increased.

Results

Recq15-deficient cells are hypersensitive to CPT. We have reported recently that Recq15 deficient mouse cells exhibited a hyper-recombination phenotype that is accompanied by an increased accumulations of both gamma H2AX foci and GCRs in response to CPT-induced replication stress (Hu *et al.*, 2007). In mammalian cells, HR plays a critical role in suppressing GCRs and promoting cell survival (Arnaudeau *et al.*, 2001a; Saleh-Gohari *et al.*, 2005). Thus, the observation that Recq15 deficiency resulted in an increase in the accumulation of gamma H2AX foci despite having an elevated rate of HR-mediated repair suggests that, in response to CPT treatment, Recq15 may act either upstream of, or in parallel to HR in response to replication stress to prevent the collapse of replication forks and the accumulation of DSBs. To address this question, we examined the potential effects of Recq15 deletion on the cell survival response towards a number of replication inhibiting agents, including methyl-methanesulfonate (MMS), mitomycin C (MMC),

etoposide, and CPT. We found that Recq15 deletion had no major impact on cell survival after treatment with any of these agents (Figure 1, A-C, and data not shown) except CPT (Figure 2). In particular, the difference in the clonogenic survival capacity between the Recq15 deficient mouse ES cells and their wild-type counterpart was more than 100 folds when cells were treated with 300 nM CPT (Figure 2A). Expression of human RECQL5 β in Recq15-deficient cells restored the CPT resistance to a level similar to that of the wild-type cells (Figure 2B), demonstrating that this enhanced CPT sensitivity is due to the deletion of Recq15. Moreover, primary mouse embryonic fibroblasts (MEFs) derived from Recq15 deficient mice were also significantly more sensitive to CPT than their wild-type counterpart (Figure 2C), indicating that Recq15 plays an important role in CPT resistance not only in ES cells, but also in primary MEFs. Together, these data provide definitive genetic evidence demonstrating that Recq15 has a specific role in promoting cell survival after CPT treatment.

A significant fraction of Recq15 deficient cells lost the ability to incorporate BrdU after CPT treatment. It has been reported previously that the cytotoxic effect of CPT is dependent on the collapse of replication forks (Hsiang *et al.*, 1985; Holm *et al.*, 1989; Ryan *et al.*, 1991) and that HR plays a critical role in promoting cell survival following CPT exposure (Arnaudeau *et al.*, 2001a; Malik and Nitiss, 2004; Sorensen *et al.*, 2005). Therefore, a defect in either preventing forks from collapsing or HR-mediated repair could account for the CPT hypersensitivity observed in Recq15 deficient cells. We showed previously that Recq15-deficiency led to an elevated frequency of HR (Hu *et al.*, 2007), suggesting that a defect in replication, rather than HR-related repair is likely the culprit for the CPT hypersensitivity phenotype. This hypothesis is also consistent with a recent demonstration that homologues of Recq15 bind to replication fork-like structures with high affinities *in vitro* (Kanagaraj *et al.*, 2006). To address this possibility, we examined the effect of Recq15 deletion on the capacity of DNA synthesis after CPT treatment using BrdU incorporation experiments. Specifically, ES cells were treated with 100 nM CPT, which is approximately equivalent to the IC₅₀ for wild-type and IC₉₅ for the mutant cells (Fig, 2A), and then released into fresh

medium containing Nocodazole. CPT causes a cell cycle arrest in late S-phase in both wild-type and mutant cells (data not shown), which is consistent with previous observations (Zhou *et al.*, 2002). Nocodazole-containing medium prevented cells from entering the next cell cycle to produce a new generation of S phase cell, which therefore allows for monitoring of the S phase cells that had been subjected to CPT treatment by standard BrdU pulse labelling experiments. After pulse labelling with BrdU, the percentages of S phase cells (i.e. DNA content between 2n and 4n) as well as the percentages of BrdU-positive S phase cells (i.e. replicating) were determined by flow cytometry analyses. As expected, in untreated wild-type control, most (94.6%) of S phase cells were able to incorporate BrdU (Figure 3A). Interestingly, the percentage of such cells in Recq15 deficient cells was only 88.6%, slight lower than that of the wild-type control (Figure 3A). After the CPT treatment, the fraction of BrdU-positive S phase cells from the wild-type population was reduced by only 7%. In contrast, however, the same treatment to the mutant cells resulted in a 58% reduction in BrdU-positive S phase cells (Figure 3B). By 4 hours after the removal of CPT, however, the percentages of BrdU incorporating S phase cells in both the wild-type and the mutant cells had returned to levels similar to those in respective untreated controls (Figure 3C). Meanwhile, the majority of both wild-type and mutant cells had a 4n DNA content in the absence of a substantial increase of the sub-G1 population (i.e. apoptotic cells) (Figure 3C). These data clearly demonstrate that CPT treatment resulted in a substantial inhibition or loss of the ability to incorporate BrdU in Recq15-deficient cells, but not in wild-type control. However, once CPT was removed, both could resume DNA synthesis and traversed through S phase of the first cell cycle after the treatment without experiencing a substantial amount of apoptosis.

CPT-treated Recq15-deficient cells underwent replication-dependent apoptosis. We showed above that while Recq15 deficient ES cells were hypersensitive to CPT and were unable to retain their ability to synthesize DNA after CPT treatment, they did not experience an extensive cell death within the first S phase after the treatment (Figure 3C). Previously, we have reported that a substantial increase of gamma

H2AX focus formation occurred after a similar treatment with CPT, which peaked at approximately 8 hours after the treatment, when the cells had exited S phase (Hu *et al.*, 2007). Together, these observations suggest that Recq15 deficient cells are hypersensitive to CPT because they are more susceptible to fork collapse than wild-type cells following CPT treatment. To formally test this idea, we first monitored the cell cycle progression and the accumulation of apoptotic cells after CPT treatment. DNA content analysis showed that consistent with the observations from BrdU incorporation experiments (Figure 3C), neither the wild-type or the mutant ES cells exhibited a substantial increase in the sub-G1 population both during the 16 hours incubation and within the first 8 hours after CPT was removed; and that the majority of the cells have exited S phase of the cell cycle (Figure 4A). However, by 16 hours after the treatment an apparent increase in the sub-G1 population was observed in the mutant cells but not in the wild-type control (Figure 4A). TUNEL staining also confirmed that there was no significant increase in apoptosis in both wild-type and mutant cells within 8 hours after the CPT treatment (Figure 4B). However, by 16 hours after the treatment, approximately 10% of mutant cells are TUNEL positive compared to only about 2% in the wild-type control. At later time points, the levels of TUNEL-positive mutant cells continued to increase while those in the wild-type controls remained relatively constant. For example, by 32 hours after the treatment, the percentage of apoptotic mutant cells had reached approximately 22%, while levels in the wild-type remained similar to the background level in the untreated control (Figure 4B). These data clearly show that Recq15 mutant cells suffered a delayed onset type of apoptosis following CPT treatment. We then asked whether this delayed cell death was indeed due to demise in DNA replication. We reasoned that if the enhanced CPT cytotoxicity of Recq15 mutant cells is indeed due to a defect in DNA replication, the observed CPT cytotoxicity would be replication-dependent (Holm *et al.*, 1989; Ryan *et al.*, 1991). We found that co-treatment of CPT together with aphidicolin (APH), an inhibitor of DNA replication (Ishii and Bender, 1980), alleviated the cytotoxic effect of CPT for both wild-type and Recq15-deficient ES cells (Figure 4C). Therefore, together, our data strongly suggest that the enhanced CPT cytotoxicity of Recq15 deficient cells is indeed caused by a defect in DNA replication following CPT treatment.

It is currently believed that DSBs associated with collapsed replication forks is the main cytotoxic lesion generated by CPT. Thus, our finding that Recq15 deficient cells exhibited a replication-dependent hypersensitivity to CPT suggests that Recq15 deficient cells may experience a greater increase in replication fork collapse and DSB accumulation than their wild-type counterpart after the CPT treatment. To formally test this hypothesis, we performed pulse field gel electrophoresis (PFGE) experiment to measure the DNA fragmentation in both untreated and CPT-treated mouse ES cells. In the experiment, the ES cells were treated with the same low dose (100 nM) of CPT, which according to the data from the apoptosis analysis caused a substantial increase in apoptosis in the mutant but not the wild type ES cells (Fig. 4B). We found that this treatment caused a transient and small increase in the amount of fragmented DNA in the wild-type cells immediately after the CPT release. In contrast, the same experiment resulted in a more pronounced effect on the amount of fragmented DNA in the Recq15 deficient cells (Fig. 5A). In particular, the amount of fragmented DNA was higher than the untreated control and was approximately twice that of the wild-type cells (Fig. 5B, NT and 0 hr). Moreover, the level of fragmented DNA continued to increase until it reached a peak level at approximately 12 hours after the treatment (Fig. 5B, 12 hrs). These data, therefore, support the idea that the enhanced CPT cytotoxicity in Recq15 deficient cells is due to, at least partly, the increase of replication fork collapse.

In human cells, cytotoxic doses of CPT activated CHK1-dependent intra-S checkpoint and inhibited both fork elongation and the initiation of new origins of replication (Seiler *et al.*, 2007). Thus, we also analyzed the status of Chk1 in both wild-type and mutant cells before and after treating with 100 nM CPT as before. We found that 100 nM CPT caused only a modest increase in Chk1 phosphorylation in wild type cells (Fig. 5C, left panel). In contrast, the same treatment resulted in a much more pronounced increase in the levels of phosphorylated Chk1 in mutant cells. These relatively high levels of phosphorylated Chk1 persisted for up to 8 hours after the release of the CPT treatment before subsiding at 16 hours after the release (Fig. 5C,

right panel). A significant up-regulation of Chk1 phosphorylation in mutant cells but not in wild-type cells immediately after the CPT treatment is consistent with the significant reduction in the number of BrdU-incorporating S phase cells in the mutant but not wild-type control after the same treatment (Fig. 3). In addition, the prolonged presence of high levels of phosphorylated Chk1 in the CPT-treated mutant cells is also consistent with the sustained high levels of fragmented DNA revealed by PFGE analysis (Fig. 5A). Thus, in aggregate, the data presented thus far strongly suggest that Recq15 is required to prevent the collapse of CPT-induced stalled DNA replication fork to promote cell survival.

Recq15 functions non-redundantly with Rad51 to promote cell survival after CPT treatment. It has been shown that HR plays critical roles in suppressing genome instability and promoting cell survival following CPT exposure (Arnaudeau *et al.*, 2001a; Malik and Nitiss, 2004; Sorensen *et al.*, 2005). Thus, the conclusion reached above also suggests that Recq15 functions in a pathway that has a non-overlapping role with HR with respect to cell survival following CPT treatment. We reasoned that if this is indeed the case, inhibition of HR should have similar effects on CPT sensitivity in both wild-type and mutant ES cells. To test this hypothesis, we investigated the genetic interaction between Recq15 and HR on CPT sensitivity. We first inhibited HR by suppressing the expression of Rad51 in both wild-type and Recq15 deficient ES cells using shRNA-mediated knockdown (Lambert and Lopez, 2000; Stark *et al.*, 2004) and then examined their effects on CPT sensitivity. Suppression of Rad51 expression resulted in an enhanced CPT sensitivity in wild-type cells (Figure 6A, 6B), consistent with previous reports (Thacker and Ganesh, 1990; Arnaudeau *et al.*, 2001b; Saintigny *et al.*, 2001; Lundin *et al.*, 2002). Meanwhile, a similar reduction in Rad51 expression in Recq15-deficient cells resulted in a further enhancement in CPT sensitivity (Figure 6A, 6B). These data support our hypothesis that Recq15 contributes to cell survival following CPT treatment through a mechanism that is non-redundant to the Rad51-dependent mechanism (presumably HR) (Figure. 6C).

Discussion

We report here that Recq15 deficient mouse ES cells were hypersensitive to CPT in a replication dependent manner. Yet, they were not more sensitive than their wild-type counterpart towards a number of other DNA damaging agents that can also impede DNA replication. Moreover, this phenotype is also observed in Recq15 deficient MEF cells. Together, these data demonstrate the unique requirement of Recq15 for the optimal cell survival in both mouse ES and MEF cells following CPT treatment. In addition, we showed that following CPT treatment, a significant fraction of Recq15 deficient cells, but not their wild-type counterpart, lost their ability to incorporate BrdU in S phase. We noticed that this loss of replication competence during the S phase is followed by a rapid accumulation of DSBs, GCRs and then cell death. It has been demonstrated that CPT can cause the collapse of replication forks (Kohn and Pommier, 2000; Strumberg *et al.*, 2000). DSB generated from such fork collapse is believed to represent the main cytotoxic lesion induced by CPT and its derivatives (Pommier *et al.*, 2006). Therefore, our data strongly suggest that Recq15 is required for maintaining the replication competency when forks are impeded following CPT treatment.

We proposed that in CPT-treated wild-type cells, a Recq15-dependent mechanism functions to maintain the integrity and the activity of DNA replication to prevent the collapse of the impeded replication forks. The dilemma caused by the replication impediment can then be resolved either by HR or by other mechanism(s), preventing the accumulation of DSBs. In contrast, in Recq15 deficient cells, the impeded replication forks are more susceptible to collapse, resulted in a significant accumulation of DSBs. This extension is consistent with our previous observation that Recq15 deficient cells exhibited both an elevated HR-mediated repair and an increased in DSB accumulation after CPT treatment (Hu *et al.*, 2005; Hu *et al.*, 2007). Therefore, Recq15 has a non-redundant role with HR with respect to the optimal cell survival following CPT treatment. Thus, defects in either HR or the Recq15-related pathway can significantly increase the cells' sensitivity towards CPT and its derivatives. Importantly, the combined functions of these

two mechanisms as well as other (Pommier *et al.*, 2006) are required to provide an optimal protection against CPT cytotoxicity (Figure 6C).

It has been reported recently that under reconstituted conditions *in vitro*, camptothecin-induced Topoisomerase I-DNA complex formation resulted in the retardation of DNA uncoiling (Koster *et al.*, 2007). Furthermore, in yeast, this retardation of DNA uncoiling is associated with the impediment of both transcription and replication *in vivo* (Koster *et al.*, 2007). The implication that Recq15 can prevent impeded replication forks from collapsing after CPT treatment is consistent with this recent finding and suggests that a similar scenario also exist in mammalian cells. A role of Recq15 in maintaining the integrity of stalled replication forks to prevent fork collapse is consistent with previous reports that link this helicase (Garcia *et al.*, 2004; Kanagaraj *et al.*, 2006) and its homologs (Ozsoy *et al.*, 2003) to DNA replication. Interestingly, we found that Recq15 deficient cells that were initially incapable of incorporating BrdU could eventually resume DNA synthesis and re-enter the next phase of the cell cycle after CPT is removed. This temporally cessation of DNA synthesis could reflect either a delay in the re-activation of the impeded forks, or the inability of impeded forks to resume DNA synthesis, and therefore cells must rely on the firing of new origins of replication in order to complete the duplication of the genome. Future experiments will be needed to distinguish these possibilities. Intriguingly, this resumption of DNA replication in CPT-treated Recq15 deficient cells occurred within 4 hours after CPT removal, when the proper repair or restoration of the collapse forks had not yet been completed, which would be consistent with a current model that the stalled replication fork, but not the specific cues that cause the stalling, provides the necessary signal for intra-S cell cycle arrest in mammalian cells (Zachos *et al.*, 2005). In any cases, this temporally cessation of DNA synthesis is associated with an increase in genome instability and a dramatic decrease in cell survival.

Interestingly, human cells that are deficient in BLM or WRN and mouse cells deficient in Wrn are more sensitive to CPT and other DNA damaging agents than their wild-type counterparts (Hook *et al.*,

1984; Ogburn *et al.*, 1997; Lebel and Leder, 1998; Poot *et al.*, 1999; Rao *et al.*, 2005). Intriguingly, to our knowledge, only Recq15-deficient cells are uniquely hypersensitive to CPT. The molecular basis underlying this specificity of Recq15's function remains unclear. It should be noted that the structures of the stalled forks elicited by CPT and other topoisomerase I inhibitors are likely to be distinct from those induced by other DNA damaging agents or by nucleotide deprivation, the two other major causes of replication stalling. For instance, when a replication fork is stalled by a DNA damage lesion or due to nucleotide deprivation, the progression of the DNA polymerase is impeded, but the MCM replication helicase complex associated with the fork can continue to advance, leading to the creation of an extended stretch of single strand DNA (ssDNA) region and the activation of intra-S checkpoint (Liu *et al.*, 2000; Byun *et al.*, 2005). In contrast, CPT causes the stalling of replication fork by stabilizing DNA-Topo I covalent complexes in front of advancing replication forks. Thus, it impedes the advancement of both the DNA polymerase and MCM helicase complex and hence does not directly create excessive amount of ssDNA. Moreover, DNA-Topo I covalent complexes similar to those induced by CPT also exist under normal physiological conditions. Thus, the formation of a DNA-Topo I covalent complex *per se* may not be consequential. Indeed, CPT causes a peculiar late S-early G2 cell cycle arrest rather than the typical intra-S arrest triggered by many other types of replication inhibiting agents or treatments (Zhou *et al.*, 2002), suggesting that the CPT-induced stalled fork. Therefore, alternative molecular mechanism(s) must exist in order to maintain the integrity of the stalled forks induced by CPT. We hypothesize that Recq15 plays a critical role in such a mechanism. More importantly, when a replication fork is stalled by a DNA damage lesion, both the stabilization of the stalled fork and the subsequent successful repair of the damage lesion are likely to be necessary in order to preserve the integrity of the genome and to promote cell survival (Hook *et al.*, 2007). In contrast, if a replication fork that is stalled as the result of Topoisomerase I inhibition, the removal of the inhibition may be the only key event for reactivating the stalled fork. Thus, we propose that in the event of topoisomerase I poisoning by CPT, stabilization of the stalled replication forks by Recq15 alone provides the critical time that is required for the dissociation of CPT from the stalled

fork and the restoration of the fork before it collapses (Fig. 6C, route 3-4-1R). Therefore, Recq15 is both necessary and sufficient for promoting cell survival following the exposure to CPT, but not necessarily other types of replication fork-stalling agents or treatments. However, such a stalled fork could also collapse, for instance when it is not protected by Recq15 in *Recq15* knockout cells or when the number of the stalled forks outpaced the capacity of Recq15. In this case, faithful HR repair is necessary for preserving the integrity of the genome and promoting survival. Failure to execute such a faithful repair can lead to genome instability or even cell death (Fig. 6C, route 3a-4a).

CPT and its derivatives represent a group of very promising anticancer agents, two of which, Topotecan and Irinotecan, have recently been approved by the FDA for treating patients with several types of malignancies (Pizzolato and Saltz, 2003; Pommier *et al.*, 2006). However, despite a broad range of indication in treating many types of cancers, these drugs, when used in single agent therapy, cannot provide a satisfactory curative effect. Therefore, it has become increasingly evident that combinatorial therapies involving multiple agents and/or treatment modalities is necessary in order to fully realize the potential of these drugs in anticancer treatments (Pommier *et al.*, 2006). A thorough understanding of the molecular determinants of drug sensitivity as well as the molecular pathways that are specific to camptothecins and other topoisomerase I poisoning drugs will be critical in formulating such combinatorial treatment regimens. The mouse Recq15 and human RECQL5 are highly conserved (Ohhata *et al.*, 2001). Thus, it is expected that mutations in *RECQL5* in human cells should also result in an hypersensitivity to CPT. In this context, the finding that Recq15 is an important determinant of CPT resistance can have important implications in improving the use of CPT derivatives and other topoisomerase I inhibitors in anticancer treatment. In particular, we have shown that deletion of *Recq15* in mice resulted in a significant increase in cancer susceptibility (Hu *et al.*, 2007). Thus, it is possible that *RECQL5* mutations may also be associated with some human cancers. In that case, RECQL5 may be a valuable biomarker for determining whether topoisomerase I inhibitors should be used in a patient's treatment regimen. Furthermore, we have noticed

that the combination of Recq15 inactivation and CPT exposure is similar to the effect of PARP inhibitors, that is, both render mammalian cells more dependent on HR for survival in response to agents that can cause replication fork collapse. Thus, HR deficient cancer cells, for example, those with BRCA1 or BRCA2 deficiency, are hypersensitive to PARP inhibitors and PARP inhibitors have been proven very effective in treating tumors that are deficient in either BRCA1 or BRCA2 (Bryant and Helleday, 2004; Bryant *et al.*, 2005; Farmer *et al.*, 2005). By a similar rationale, RECQL5 may be a potential target for developing new anticancer drugs that can enhance the efficacy of topoisomerase I inhibitors and/or those that target HR.

Acknowledgements.

We are grateful to Drs. Nathan Berger, George Stark, Peter Harte and Hua Lou for their comments on the manuscript. This study was supported by grants RO1 CA88939, P20 CA103736 from the US National Institutes of Health and Searle Scholar Award 01-E-109 from the Searle Scholar Program.

References

- Arnaudeau, C., Lundin, C., and Helleday, T. (2001a). DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. *J Mol Biol* 307, 1235-1245.
- Arnaudeau, C., Rozier, L., Cazaux, C., Defais, M., Jenssen, D., and Helleday, T. (2001b). RAD51 supports spontaneous non-homologous recombination in mammalian cells, but not the corresponding process induced by topoisomerase inhibitors. *Nucleic Acids Res* 29, 662-667.
- Bryant, H.E., and Helleday, T. (2004). Poly(ADP-ribose) polymerase inhibitors as potential chemotherapeutic agents. *Biochem Soc Trans* 32, 959-961.
- Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., and Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913-917.
- Byun, T.S., Pacek, M., Yee, M.-c., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev.* 19, 1040-1052.
- Cheek, C.F., Bachrati, C.Z., Chan, K.L., Ralf, C., Wu, L., and Hickson, I.D. (2005). Roles of the Bloom's syndrome helicase in the maintenance of genome stability. *Biochem Soc Trans* 33, 1456-1459.
- Courcelle, J., and Hanawalt, P.C. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated *Escherichia coli*. *Mol Gen Genet* 262, 543-551.
- Courcelle, J., and Hanawalt, P.C. (2001). Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated *Escherichia coli* need not involve recombination. *Proc Natl Acad Sci U S A* 98, 8196-8202.
- 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.

Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., Martin, N.M., Jackson, S.P., Smith, G.C., and Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917-921.

Furuta, T., Takemura, H., Liao, Z.Y., Aune, G.J., Redon, C., Sedelnikova, O.A., Pilch, D.R., Rogakou, E.P., Celeste, A., Chen, H.T., Nussenzweig, A., Aladjem, M.I., Bonner, W.M., and Pommier, Y. (2003). Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. *J Biol Chem* 278, 20303-20312.

Garcia, P.L., Liu, Y., Jiricny, J., West, S.C., and Janscak, P. (2004). Human RECQ5beta, a protein with DNA helicase and strand-annealing activities in a single polypeptide. *Embo J* 23, 2882-2891.

Hanawalt, P.C. (1966). The U.V. sensitivity of bacteria: its relation to the DNA replication cycle. *Photochem Photobiol* 5, 1-12.

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

Holm, C., Covey, J.M., Kerrigan, D., and Pommier, Y. (1989). Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res* 49, 6365-6368.

Hook, G.J., Kwok, E., and Heddle, J.A. (1984). Sensitivity of Bloom syndrome fibroblasts to mitomycin C. *Mutat Res* 131, 223-230.

Hook, S.S., Lin, J.J., and Dutta, A. (2007). Mechanisms to control rereplication and implications for cancer. *Current Opinion in Cell Biology* 19, 663-671.

Hsiang, Y.H., Hertzberg, R., Hecht, S., and Liu, L.F. (1985). Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 260, 14873-14878.

Hu, Y., Lu, X., Barnes, E., Yan, M., Lou, H., and Luo, G. (2005). Recql5 and Blm RecQ DNA helicases have nonredundant roles in suppressing crossovers. *Mol Cell Biol* 25, 3431-3442.

Hu, Y., Raynard, S., Sehorn, M.G., Lu, X., Bussen, W., Zheng, L., Stark, J.M., Barnes, E.L., Chi, P., Janscak, P., Jasin, M., Vogel, H., Sung, P., and Luo, G. (2007). RECQL5/Recql5 helicase regulates homologous recombination and suppresses tumor formation via disruption of Rad51 presynaptic filaments. *Genes Dev* 21, 3073-3084.

Ishii, Y., and Bender, M.A. (1980). Effects of inhibitors of DNA synthesis on spontaneous and ultraviolet light-induced sister-chromatid exchanges in Chinese hamster cells. *Mutat Res* 79, 19-32.

Kanagaraj, R., Saydam, N., Garcia, P.L., Zheng, L., and Janscak, P. (2006). Human RECQ5beta helicase promotes strand exchange on synthetic DNA structures resembling a stalled replication fork. *Nucleic Acids Res* 34, 5217-5231.

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.

Kohn, K.W., and Pommier, Y. (2000). Molecular and biological determinants of the cytotoxic actions of camptothecins. Perspective for the development of new topoisomerase I inhibitors. *Ann N Y Acad Sci* 922, 11-26.

Koster, D.A., Palle, K., Bot, E.S., Bjornsti, M.A., and Dekker, N.H. (2007). Antitumour drugs impede DNA uncoiling by topoisomerase I. *Nature* 448, 213-217.

Lambert, S., Froget, B., and Carr, A.M. (2007). Arrested replication fork processing: interplay between checkpoints and recombination. *DNA Repair (Amst)* 6, 1042-1061.

Lambert, S., and Lopez, B.S. (2000). Characterization of mammalian RAD51 double strand break repair using non-lethal dominant-negative forms. *Embo J* 19, 3090-3099.

Lebel, M., and Leder, P. (1998). A deletion within the murine Werner syndrome helicase induces sensitivity to inhibitors of topoisomerase and loss of cellular proliferative capacity. *Proc Natl Acad Sci U S A* 95, 13097-13102.

Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L.A., and Elledge, S.J. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* *14*, 1448-1459.

Lundin, C., Erixon, K., Arnaudeau, C., Schultz, N., Jenssen, D., Meuth, M., and Helleday, T. (2002). Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. *Mol Cell Biol* *22*, 5869-5878.

Malik, M., and Nitiss, J.L. (2004). DNA repair functions that control sensitivity to topoisomerase-targeting drugs. *Eukaryot Cell* *3*, 82-90.

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.

Nakayama, H. (2002). RecQ family helicases: roles as tumor suppressor proteins. *Oncogene* *21*, 9008-9021.

Oakley, T.J., Goodwin, A., Chakraverty, R.K., and Hickson, I.D. (2002). Inactivation of homologous recombination suppresses defects in topoisomerase III-deficient mutants. *DNA Repair (Amst)* *1*, 463-482.

Ogburn, C.E., Oshima, J., Poot, M., Chen, R., Hunt, K.E., Gollahon, K.A., Rabinovitch, P.S., and Martin, G.M. (1997). An apoptosis-inducing genotoxin differentiates heterozygotic carriers for Werner helicase mutations from wild-type and homozygous mutants. *Hum Genet* *101*, 121-125.

Ohhata, T., Araki, R., Fukumura, R., Kuroiwa, A., Matsuda, Y., and Abe, M. (2001). Cloning, genomic structure and chromosomal localization of the gene encoding mouse DNA helicase RECQL5beta. *Gene* *280*, 59-66.

Ozsoy, A.Z., Ragonese, H.M., and Matson, S.W. (2003). Analysis of helicase activity and substrate specificity of *Drosophila* RECQ5. *Nucleic Acids Res* *31*, 1554-1564.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* *5*, 741-747.

Pizzolato, J.F., and Saltz, L.B. (2003). The camptothecins. *Lancet* *361*, 2235-2242.

Pommier, Y., Barcelo, J.M., Rao, V.A., Sordet, O., Jobson, A.G., Thibaut, L., Miao, Z.H., Seiler, J.A., Zhang, H., Marchand, C., Agama, K., Nitiss, J.L., and Redon, C. (2006). Repair of topoisomerase I-mediated DNA damage. *Prog Nucleic Acid Res Mol Biol* *81*, 179-229.

Poot, M., Gollahon, K.A., and Rabinovitch, P.S. (1999). Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S-phase. *Hum Genet* *104*, 10-14.

Rao, V.A., Fan, A.M., Meng, L., Doe, C.F., North, P.S., Hickson, I.D., and Pommier, Y. (2005). Phosphorylation of BLM, dissociation from topoisomerase IIIalpha, and colocalization with gamma-H2AX after topoisomerase I-induced replication damage. *Mol Cell Biol* *25*, 8925-8937.

Ryan, A.J., Squires, S., Strutt, H.L., and Johnson, R.T. (1991). Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Res* *19*, 3295-3300.

Saintigny, Y., Delacote, F., Vares, G., Petitot, F., Lambert, S., Averbek, D., and Lopez, B.S. (2001). Characterization of homologous recombination induced by replication inhibition in mammalian cells. *Embo J* *20*, 3861-3870.

Saleh-Gohari, N., Bryant, H.E., Schultz, N., Parker, K.M., Cassel, T.N., and Helleday, T. (2005). Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. *Mol Cell Biol* *25*, 7158-7169.

Seiler, J.A., Conti, C., Syed, A., Aladjem, M.I., and Pommier, Y. (2007). The intra-S-phase checkpoint affects both DNA replication initiation and elongation: single-cell and -DNA fiber analyses. *Mol Cell Biol* *27*, 5806-5818.

Shao, R.G., Cao, C.X., Zhang, H., Kohn, K.W., Wold, M.S., and Pommier, Y. (1999). Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. *Embo J* *18*, 1397-1406.

Sorensen, C.S., Hansen, L.T., Dziegielewski, J., Syljuasen, R.G., Lundin, C., Bartek, J., and Helleday, T. (2005). The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol* 7, 195-201.

Stark, J.M., Pierce, A.J., Oh, J., Pastink, A., and Jasin, M. (2004). Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol Cell Biol* 24, 9305-9316.

Strumberg, D., Pilon, A.A., Smith, M., Hickey, R., Malkas, L., and Pommier, Y. (2000). Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5'-phosphorylated DNA double-strand breaks by replication runoff. *Mol Cell Biol* 20, 3977-3987.

Thacker, J., and Ganesh, A.N. (1990). DNA-break repair, radioresistance of DNA synthesis, and camptothecin sensitivity in the radiation-sensitive *irs* mutants: comparisons to ataxia-telangiectasia cells. *Mutat Res* 235, 49-58.

Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G.M., Mulligan, J., and Schellenberg, G.D. (1996). Positional cloning of the Werner's syndrome gene. *Science* 272, 258-262.

Zachos, G., Rainey, M.D., and Gillespie, D.A. (2005). Chk1-dependent S-M checkpoint delay in vertebrate cells is linked to maintenance of viable replication structures. *Mol Cell Biol* 25, 563-574.

Zhou, Y., Gwadry, F.G., Reinhold, W.C., Miller, L.D., Smith, L.H., Scherf, U., Liu, E.T., Kohn, K.W., Pommier, Y., and Weinstein, J.N. (2002). Transcriptional Regulation of Mitotic Genes by Camptothecin-induced DNA Damage: Microarray Analysis of Dose- and Time-dependent Effects. *Cancer Res* 62, 1688-1695.

Figure legends

Figure 1. Clonogenic survival of mouse ES cells in response to DNA damage. Results of clonogenic survival assay of ES cells after exposure to a variety of DNA damaging agents, including methylmethanesulfonate (MMS) (A), Mitomycin C (MMC) (B), etoposide (C), are shown. In each plot of clonogenic survival assay, the Y- and X-axis indicate percentages of cell survival and drug concentrations, respectively. The genotypes of cells used in each experiment are indicated. For all treatments, results from three sets of experiments were used to calculate the mean and standard error of the mean (s.e.m.) for each data point.

Figure 2. *Recq15*-deficient mouse ES and MEF cells are hypersensitive to CPT. Results of clonogenic survival assay of ES cells (A and B) or MEFs (C) after exposure to camptothecin (CPT) are shown. In each plot of clonogenic survival assay, the Y- and X-axis indicate percentages of cell survival and drug concentrations, respectively. The genotypes of cells used in each experiment are indicated. The *Recq15*^{-/-RES} in (B) indicates a cell line derived from the *Recq15*^{-/-} line by introducing a vector expressing human RECQL5 β under the control of a Doxycycline (Dox)-regulated promoter. The expression of human RECQL5 β in this cell line was confirmed by Western blot (data not shown). For all treatments, results from three sets of experiments were used to calculate the mean and standard error of the mean (s.e.m.) for each data point.

Figure 3. A substantial reduction of BrdU-incorporating S phase population in *Recq15* deficient ES cells after CPT treatment. (A) Untreated cells. (B) CPT treated cells at 0 hours after released from the CPT treatment. (C) CPT treated cells at 4 hours after release. For each data point, both a DNA content histogram indicating the profile of cell cycle distribution (on the left) and a BrdU incorporation plot showing the data from the BrdU incorporation experiment (on the right) are shown. For the DNA content plots, the X- and Y-

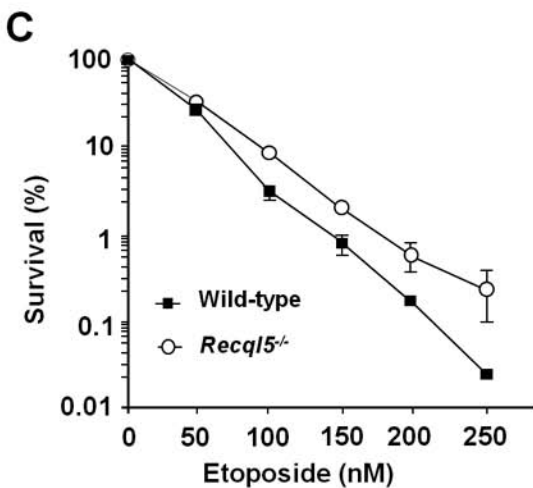
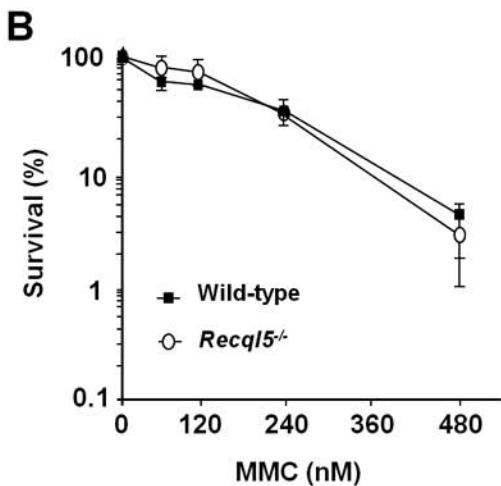
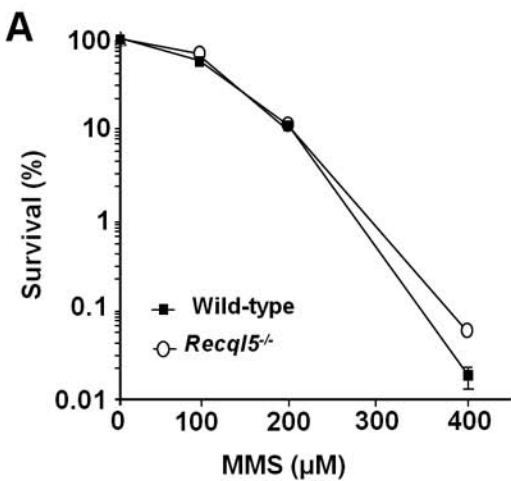
axis indicate the DNA content measured by the intensity of PI staining and the number of cell count, respectively. In each of BrdU incorporation plots, the *X*- and *Y*-axis indicate the DNA content (measured by the intensity of PI staining) and the content of BrdU (the intensity of BrdU signal) in individual cell, respectively. The subpopulation of cells with a BrdU content above the background level is marked within a trapezoidal area and the percentage of this subpopulation among the total S phase population is also shown above the framed area.

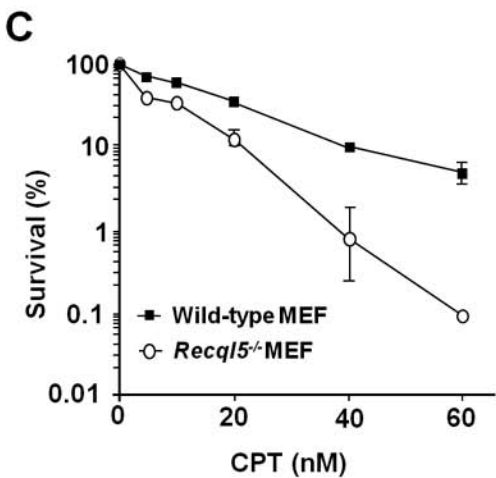
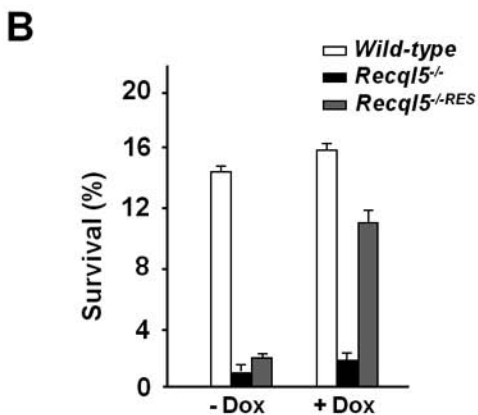
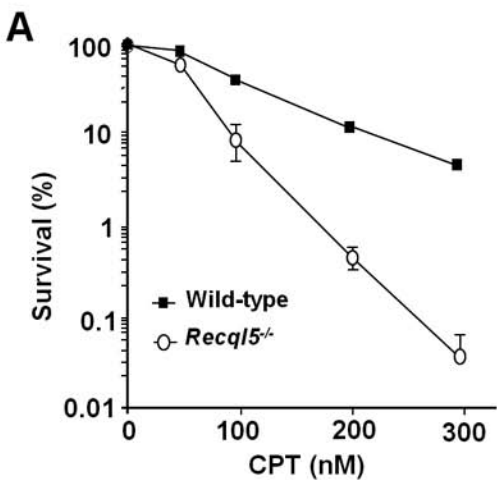
Figure 4. Delayed apoptotic response and replication-dependent cytotoxicity in CPT-treated Recq15-deficient ES cells. (A) Cell cycle profiles of mouse ES cells before and after CPT treatment. The time points (hours) after releasing from CPT treatment are shown to on the left of each plot. The relative positions of the 2n (G1) and 4n (G2/M) peaks of DNA content are shown and the sub-G1 peak in the mutant cells at 16 hours after CPT treatment is indicated. (B) A histogram presentation showing the percentage of TUNEL positive cells of both wild-type and mutant cells at various time points following CPT treatment. (C) A plot summarizing the results of clonogenic survival assay experiment showing the effect of co-treatment of aphidicolin (APH) on the cytotoxicity of CPT.

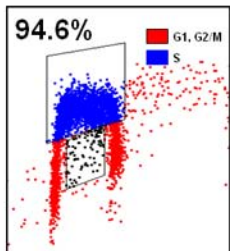
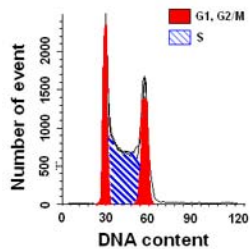
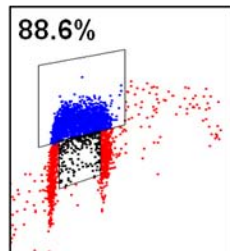
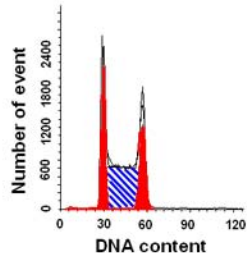
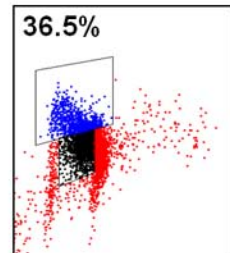
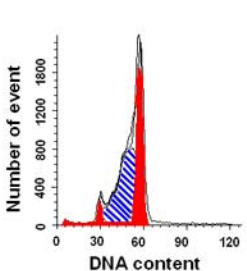
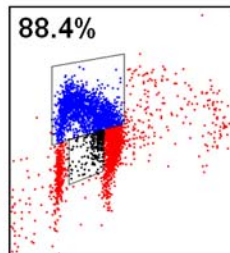
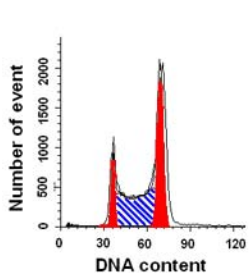
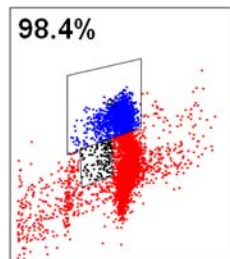
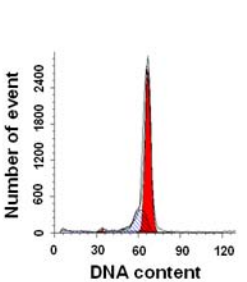
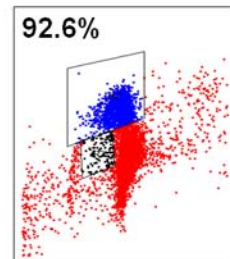
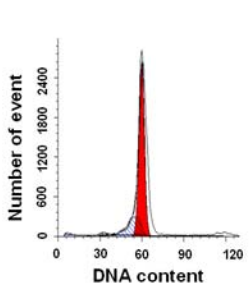
Figure 5. DNA double strand break and Chk1 phosphorylation in CPT-treated ES cells. (A) An image of ethidium bromide-stained gel from a representative pulse field gel electrophoresis (PFGE) experiment. The size range of which fragmented DNA was quantified for individual samples was shown on the right (marked as Fragmented DNA). The sizes of mega-base (Mb) markers were indicated on the left. (B) A plot showing the relative amounts of fragmented DNA in control and CPT-treated ES cells. The vertical and the horizontal axes indicate the relative amount of fragmented DNA and the time after the release from the CPT treatment, respectively. The values on the vertical axis were derived by normalizing to the value of the untreated wild-type which was set as 1. (C) Western blot analysis of the levels of both phosphorylated (top panels) and total (middle panels) Chk1. The identities of the samples are shown on top of the Western blot.

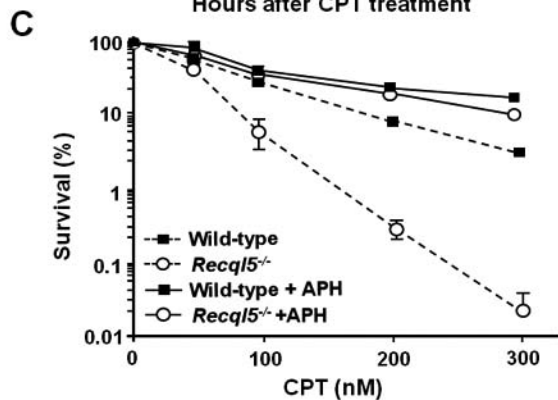
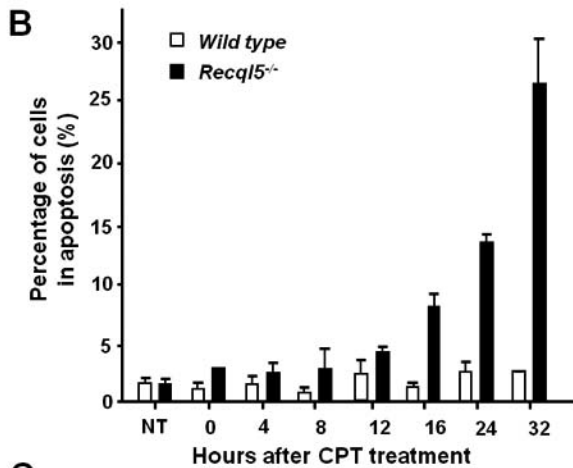
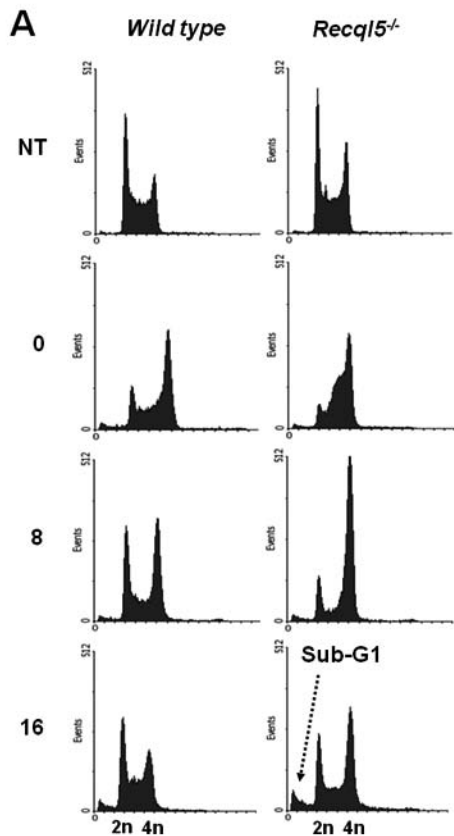
NT: untreated; R0, R2, R4, R8, and R16: 0, 2, 4, 8, 16 hours after releasing from the CPT treatment, respectively. Note that in the top panel, in addition to the specific P^{ser345}-Chk1 band, a non-specific band (indicated by a star sign) was also detected. Levels of U1-70K are shown as control in the bottom panels.

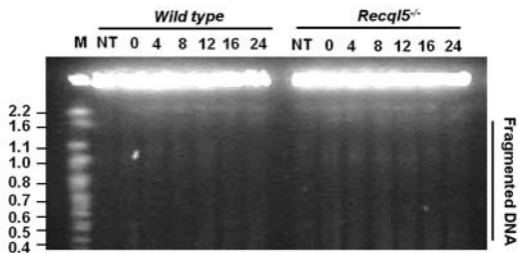
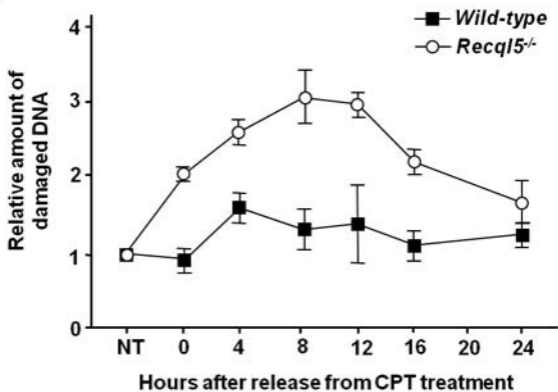
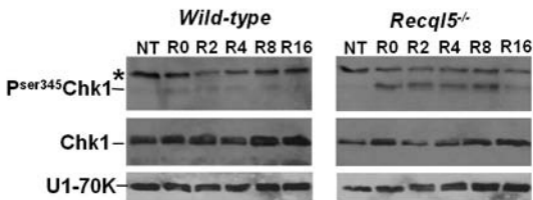
Figure 6. Suppression of Rad51 expression resulted in similar enhancements in CPT cytotoxicity between Recq15-deficient and wild-type cells. The Percentages of Survival after CPT treatment were measured using clonogenic survival assay experiments. (A) The effects on suppressing Rad51 expression by shRNA on CPT sensitivity in wild-type and Recq15 deficient ES cells. (B) Western blot results showing the relative levels of Rad51 in different cell lines. The top and the bottom panels are results of western blots detecting Rad51 and U1-70K (as a loading control) respectively. The relative levels of Rad51 expression after being normalized to that of the wild-type using U1-70K as a control are shown under the top panel. (C) A schematic illustration of a model by which Recq15 and HRR function to promote cell survival in response to topoisomerase I (Topo I) poisoning. First, a pair of arrows of opposite orientations is used to indicate the highly reversible nature of a Topo I-DNA complex associated with an on-going DNA replication fork (1F and 1R). However, camptothecin (CPT, shown as a C inside a black wedged-shape structure) can bind to both the DNA-bound Topo I and the DNA, creating a long-lived CPT-Topo I-DNA complex. This in turn hinders the release of tension in front of the fork (depicted by a thick twisted line), stalling the fork (2). This stalled fork can be stabilized by Recq15 and re-activated once CPT dissociates from the fork (Route 3-4-1R). Alternative, the stalled fork can collapse, creating a single-ended double strand break (DSB) (3a). This DSB must be repaired by HR repair in order to promote cell survival. Otherwise, it could lead to genome instability or even cell death (4a).

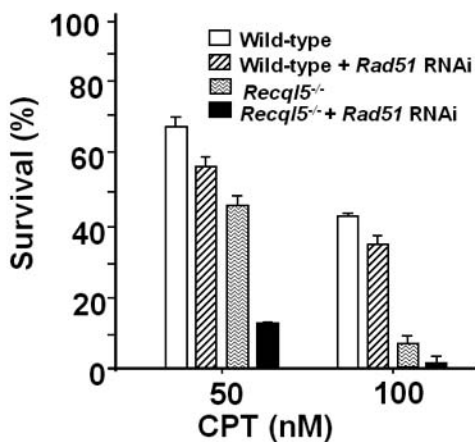
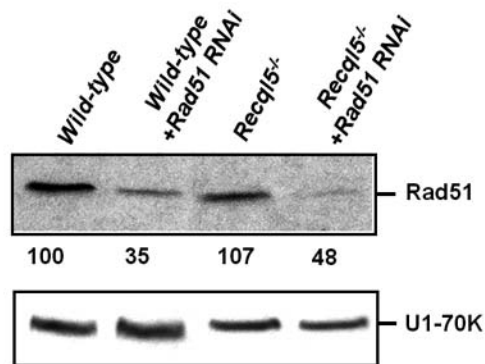




A**Wild-type*****Recql5*^{-/-}****B****C**



A**B****C**

A**B****C**