JVI Accepted Manuscript Posted Online 13 March 2019
J. Virol. doi:10.1128/JVI.02230-18

Cellular DNA topoisomerases are required for the synthesis of hepatitis B virus covalently closed circular DNA

Muhammad Sheraz[1], Junjun Cheng[2], Liudi Tang[1], Jinhong Chang[2] and Ju-Tao Guo[2*].

[1]Microbiology and Immunology Graduate Program, Drexel University College of Medicine, 2900 West Queen Lane, Philadelphia, PA 19129, USA.

[2]Baruch S. Blumberg Institute, 3805 Old Easton Road, Doylestown, PA 18902. USA.

Running title: Topoisomerases are required for HBV cccDNA synthesis

* Corresponding author's email address: ju-tao.guo@bblumberg.org

Abstract

In order to identify host cellular DNA metabolic enzymes that are involved in the biosynthesis of hepatitis B virus (HBV) covalently closed circular (ccc) DNA, we developed a cell-based assay supporting synchronized and rapid cccDNA synthesis from intracellular progeny nucleocapsid DNA. This was achieved by arresting HBV DNA replication in HepAD38 cells with phosphonoformic acid (PFA), a reversible HBV DNA polymerase inhibitor, at the stage of single-stranded DNA, and followed by removal of PFA to allow the synchronized synthesis of relaxed circular (rc) DNA and subsequent conversion into cccDNA within 12 to 24 h. This cccDNA formation assay allows for systematic screening of small molecular inhibitors of DNA metabolic enzymes on cccDNA synthesis, but avoiding cytotoxic effects upon long term treatment. Using this assay, we found that all the tested topoisomerase I and II poisons as well as topoisomerase II DNA binding and ATPase inhibitors significantly reduced the levels of cccDNA. It was further demonstrated that these inhibitors also disrupted cccDNA synthesis during *de novo* HBV infection of HepG2 cells expressing sodium taurocholate cotransporting polypeptide (NTCP). Mechanistic analyses indicate whereas TOP1 inhibitor treatment prevented the production of covalently closed negative-strand rcDNA, TOP2 inhibitors reduced the production of this cccDNA synthesis intermediate to a lesser extent. Moreover, siRNA knockdown of topoisomerase II significantly reduced cccDNA amplification. Taken together, our study demonstrates that topoisomerase I and II may catalyze distinct steps of HBV cccDNA synthesis and pharmacologic targeting of these cellular enzymes may facilitate the cure of chronic hepatitis B.

44 **Importance**

45

46 Persistent HBV infection relies on stable maintenance and proper functioning of a nuclear
47 episomal form of viral genome called cccDNA, the most stable HBV replication intermediate.
48 One of the major reasons for the failure of currently available antiviral therapeutics to cure
49 chronic HBV infection is their inability to eradicate or inactivate cccDNA. We reported herein a
50 chemical genetics approach to identify host cellular factors essential for the biosynthesis and
51 maintenance of cccDNA and discovered that cellular DNA topoisomerases are required for both
52 *de novo* synthesis and intracellular amplification of cccDNA. This approach is suitable for
53 systematic screening of compounds targeting cellular DNA metabolic enzymes and chromatin
54 remodelers for their ability to disrupt cccDNA biosynthesis and function. Identification of key
55 host factors required for cccDNA metabolism and function will reveal molecular targets for
56 developing curative therapeutics of chronic HBV infection.

57

58 **Introduction**

59

60 Hepatitis B virus (HBV), the prototype member of *Hepadnaviridae* family, chronically
61 infects 257 million people worldwide (1) and approximately one-third of these individuals will
62 die from severe liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC), if left
63 untreated (2, 3). Therapies with currently available antiviral regimens, including pegylated
64 interferon (IFN)-alpha and nucleos(t)ide analog viral DNA polymerase inhibitors, can improve
65 liver diseases and reduce hepatocellular carcinoma morbidity and mortality in a portion of
66 treated patients (4, 5). However, HBV surface antigen (HBsAg) loss or seroconversion, the
67 hallmark of a successful immunological response to HBV with complete and durable control of
68 infection, or a "functional cure", is rarely achieved with the current therapies (6, 7), and a life-
69 long antiviral therapy is thus required to maintain the therapeutic benefits (8, 9).

70 HBV contains a relaxed circular (rc) partially double stranded DNA (3.2kb in length)
71 genome, but replicates its genomic DNA *via* reverse transcription of a RNA intermediate called
72 pre-genomic (pg) RNA (10, 11). However, unlike classical retroviruses where viral RNAs are
73 transcribed from integrated proviral DNA within host cellular chromosomes, HBV RNAs are
74 transcribed from episomal covalently closed circular (ccc) DNA minichromosomes in the nuclei
75 of infected hepatocytes (12). Briefly, HBV infects hepatocytes by binding to its cellular receptor,
76 sodium taurocholate cotransporting polypeptide (NTCP), on cell surface and delivers
77 nucleocapsid into the cytoplasm *via* endocytosis (13, 14). The viral rcDNA genome in
78 nucleocapsid is then transported into the nucleus and converted into cccDNA to serve as a
79 template for transcription of viral RNA. Binding of viral DNA polymerase to the stem-loop

structure at the 5' terminus of pgRNA initiates their packaging by core protein dimers to form a nucleocapsid where viral DNA polymerase converts the pgRNA first to a single-stranded DNA and then to rcDNA. The rcDNA-containing mature nucleocapsid can either acquire an envelope and be secreted out of cells as an infectious virion or deliver the rcDNA into the nucleus to amplify cccDNA pool, a process termed cccDNA intracellular amplification (15).

Although the reasons for the failure of current antiviral agents to cure chronic HBV infection after long-term therapy are not completely understood, clinical studies as well as studies in animal models suggest that the intrinsic stability of cccDNA is one of the key determining factors for viral persistence and outcomes of antiviral therapy (16-20). Therefore, development of antiviral agents to eliminate or functionally inactivate cccDNA should facilitate the cure of chronic hepatitis B (21). However, although recent studies indicated that several host cellular DNA repair proteins, such as tyrosyl-DNA phosphodiesterase 2 (TDP2) (22), DNA polymerase κ (23), flap endonuclease 1 (FEN 1) (24) and DNA ligases (25), are required for cccDNA synthesis in *de novo* infection and intracellular amplification, the molecular mechanism of cccDNA metabolism and structure organization in the nuclei of infected cells remains to be thoroughly investigated. These studies will identify and validate molecular targets for development of therapeutics that can eradicate or functionally inactivate cccDNA and thus cure chronic hepatitis B (26). To achieve this goal, we take a chemical genetics approach to identify host cellular metabolic enzymes required for HBV cccDNA biosynthesis with a newly developed cell-based assay that allows for synchronized and rapid cccDNA synthesis (27). Our initial efforts revealed that the cellular DNA topoisomerase I (TOP1) and II (TOP2) are required for both *de novo* synthesis and intracellular amplification of cccDNA.

Results

Characterization of cccDNA synthesis in HepAD38 cells HepAD38 is a HepG2-derived stable cell line in which HBV pgRNA transcription is controlled by a tetracycline (tet)-inducible promoter and thus supports pgRNA transcription and DNA replication upon removal of tet from culture medium (28). In order to detect HBV cccDNA and all the possible biosynthesis intermediates, HepAD38 cells were cultured in the absence of tet for 12 days to allow extensive HBV replication and cccDNA synthesis. Cellular DNA was extracted with Hirt DNA extraction method and HBV DNA species in the Hirt DNA preparations were detected by Southern blot hybridization with or without prior heating at 88°C to denature rcDNA species and/or digested with EcoRI to linearize cccDNA into a unit-length double-stranded linear (dsl) DNA. As shown in Fig. 1A, the results indicated that the Hirt DNA preparation contains three major DNA species, supercoiled cccDNA, protein-free (PF) or deproteinized (DP) double-stranded linear DNA (DP-dslDNA) and relaxed-circular DNA (DP-rcDNA) (lane 1). While the cccDNA is resistant to heat denaturalization, the heat denaturalization at 88°C converts DP-dslDNA and DP-rcDNA into single-stranded DNA (Lane 2). As expected, EcoRI digestion of heat denatured Hirt DNA converts cccDNA to unit-length dslDNA (Lane 3). Moreover, to demonstrate the existence of the covalently closed negative strand rcDNA species, or cc(-)rcDNA for short, a putative intermediate of cccDNA synthesis from DP-rcDNA (29), Hirt DNA preparations were digested with 3' → 5' exonuclease-I and III (Lane 4) to remove DNA species or strands with free 3'-terminus and followed by EcoRI digestion (Lane 5) before electrophoresis. In agreement with the previous report (29), a DNA species migrated faster than cccDNA with negative polarity and resistance to EcoRI digestion was revealed (Lanes 4 and 5). The property of this DNA species is

126 consistent with that of the covalently closed negative strand DNA, or cc(-)DNA, derived from
127 exonuclease digestion of gapped positive strand of cc(-)rcDNA. As expected, cccDNA was
128 resistant to exonuclease digestion (Lane 4) and converted into unit-length dslDNA by EcoRI
129 digestion (Lane 5). Because EcoRI linearization of cccDNA after heat denaturalization of
130 protein-free rc and dslDNA increased Southern blot hybridization signal and resulted in more
131 accurate quantification of cccDNA, we used this cccDNA validation method as our routine
132 cccDNA assay in this study. Consistent with our previous report (30, 31), upon removal of tet
133 from culture medium, HBV DNA replication intermediates gradually accumulated and cccDNA
134 became detectable at sixth day after tet removal and slowly increased thereafter (Fig. 1D).
135
136 **Establishment of a synchronized and rapid cccDNA synthesis assay** RNA interference and
137 CRISPR/Cas9 gene editing technologies have been used to identify host cellular factors required
138 for viral replication in general (32) (33) and HBV cccDNA biosynthesis in particular (23, 25). In
139 order to reveal "druggable" molecular targets for pharmacological intervention of cccDNA
140 metabolism and function, we intended to systematically test the effects of commercially
141 available small molecular compounds that target cellular DNA metabolic enzymes on HBV
142 cccDNA synthesis and maintenance. However, the slow kinetics of cccDNA synthesis in
143 HepAD38 cells requires prolonged compound treatment that usually results in cytotoxicity. To
144 overcome this problem, we took the advantage of foscarnet, or phosphonoformate acid (PFA), a
145 pyrophosphate analogue, to reversibly inhibit HBV DNA polymerase thereby synchronizing
146 HBV cccDNA synthesis (34). Unlike that observed in duck hepatitis B virus (DHBV) replicating
147 cells where PFA treatment efficiently arrests DHBV DNA replication at a step immediately after
148 the priming of minus strand DNA synthesis (34, 35), PFA less effectively inhibits HBV DNA

149 synthesis and particularly, does not efficiently inhibit the synthesis of the minus strand of HBV
150 DNA and predominantly arrests HBV replication at the single–strand DNA (27). In agreement
151 with the prior observation, PFA treatment of HepAD38 cells arrested HBV DNA replication at
152 the stage of full-length negative strand DNA (Fig, 2B, core DNA panel, Time 0). Upon addition
153 of tet to shut off pgRNA transcription from HBV transgene within host cellular chromosome and
154 removal of PFA to resume viral DNA synthesis, rcDNA was gradually accumulated and
155 cccDNA became detectable at 12 h and gradually increased in the following 12 h. As expected,
156 synthesis of rcDNA and cccDNA can be completely inhibited by HBV DNA polymerase
157 inhibitor lamivudine (3TC). Therefore, the PFA-arresting and releasing of HBV DNA replication
158 in HepAD38 cells created a condition for synchronized and rapid cccDNA synthesis within a
159 short period of time and thus allowed for screening of compounds that disrupt cccDNA synthesis
160 or stability.
161
162 **TOP inhibitors inhibit cccDNA amplification** Using the above assay, we screened compounds
163 that target DNA replication and repair enzymes as well as enzymes catalyzing post-
164 transcriptional modifications of histones to identify cellular functions that are required for or
165 regulate cccDNA synthesis (Fig. 3A). We consistently found that all the human DNA TOP1 and
166 TOP2 inhibitors tested, including topotecan (Fig. 3B and C), camptothecin (Fig. 3D and E),
167 idarubicin (Fig. 3F and G) and doxorubicin (Fig. 3H and I), reduced the amounts of HBV
168 cccDNA in a dose-dependent manner, but did not affect the levels of core DNA and DP-rcDNA.
169 As a positive control, 3TC treatment efficiently inhibited the production of DP-rcDNA and
170 cccDNA under this experimental condition. The cytotoxicity of those compounds was tested
171 under the same treatment condition with extended incubation period of time, *i.e.* 48 h, instead of

24 h as for efficacy assay. As shown in Table 1, all TOP inhibitors significantly reduced HBV cccDNA at the concentration much lower than their maximal non-cytotoxic concentrations. Moreover, as shown in Fig. 3, all the TOP inhibitors tested did not apparently reduced HBV core DNA and cellular mitochondrial DNA (mtDNA). In addition, we further demonstrated that treatment of the cells with topotecan or doxorubicin started at 16 h after removal of PFA for only 8 h significantly inhibited the increase of cccDNA (Fig. 4). The results indicate that these compounds rapidly blocked cccDNA synthesis.

Because all those TOP inhibitors examined are mechanistically TOP poisons, they freeze TOP1 and TOP2 covalently attaching to the 3'- and 5'- end of cleaved DNA, and thus result in single-strand and double-strand DNA breaks, respectively (36, 37). It is, therefore, possible that the observed reduction of cccDNA amounts in the TOP poison-treated cells is not due to the inhibition of cccDNA synthesis, but because of the cleavage of already formed cccDNA. To distinguish those two possibilities, we further examined the effects of those compounds on established cccDNA in HepAD38 cells. To this end, as illustrated in Fig. 5A and C, the cells were cultured for 48 h after removal of PFA to allow for establishment of cccDNA pool and followed by treatment with indicated concentrations of topotecan (Fig. 5B) or doxorubicin (Fig. 5D) for an additional 24 h. The results demonstrated that treatment of both compounds did not apparently alter the levels of HBV core DNA and cccDNA. These results thus imply that both TOP1 and TOP2 poisons inhibited HBV cccDNA synthesis, but did not induce the decay or cleavage of cccDNA.

Moreover, it had been shown recently that treatment of cells with TOP1 or TOP2 poisons can induce cellular DNA damage response and subsequently activate an innate immune response to inhibit viral replication (38, 39). To rule out this possibility, we demonstrated that

195 under our experimental conditions, treatment of both TOP1 and TOP2 poisons did not induce
196 any detectable inflammatory cytokine response (Fig. 6). Taken together, the results presented
197 above indicate that the TOP1 and TOP2 poisons most likely directly inhibit HBV cccDNA
198 synthesis.
199
200 **TOP2 DNA binding and ATPase inhibitors also inhibit cccDNA amplification** As illustrated
201 in Fig. 7A, in addition to TOP2 poisons that inhibit TOP2 catalytic cycle after DNA is cleaved
202 but before DNA re-ligation and result in double-stranded DNA break, TOP2 can be inhibited at
203 other points of its catalytic cycle with different biochemical and biological consequences (37,
204 40). For instance, inhibition of TOP2 binding to DNA by aclarubicin or competitive inhibition of
205 ATP binding to TOP2 and thus preventing strand passage by merbarone should not produce
206 DNA damage (41). TOP2 can also be inhibited after strand passage is completed but before ATP
207 hydrolysis and dissociation of amino terminal dimerization. ICRF-187 and ICRF-193 inhibits
208 ATP hydrolysis and maintains the TOP2 structure as a closed clamp (42). To further investigate
209 the role and molecular mechanism of TOPs in cccDNA synthesis, we tested the effects of
210 additional TOP2 inhibitors and a TOP1 enzymatic inhibitor β-lapachone (43) on HBV cccDNA
211 synthesis. As shown in Fig. 7B to E, in addition to another TOP2 poison mitoxantrone (MTX),
212 both aclarubicin and merbarone reduced HBV cccDNA levels in a concentration-dependent
213 manner at concentrations that did not affect the levels of mtDNA. The maximal non-cytotoxic
214 concentrations of aclarubicin and merbarone are 1000 nM and 100 μM, respectively. However,
215 treatment with TOP2 releasing inhibitors (ICRF-193 or ICRF-187) or etoposide as well as TOP1
216 enzymatic inhibitor β-lapachone did not apparently inhibit HBV cccDNA synthesis (Fig. 7E).
217 This later result could be due to either the failure of these compounds to reach their effective

concentrations in the cells under this assay condition or the unique interaction between TOPs and HBV DNA to evade the inhibition of those compounds. In summary, the results presented above suggest that inhibition of several distinct steps of TOP2 catalytic cycle can efficiently reduce the amounts of HBV cccDNA synthesis.

TOP inhibitors disrupt *de novo* cccDNA synthesis in C3A^{hNTCP} cells. Having shown that TOP inhibitors inhibit cccDNA formation through the intracellular amplification pathway in HepAD38 cells, we next tested whether cccDNA synthesis from *de novo* HBV infection can also be inhibited. As shown in Fig. 8, as anticipated, treatment of C3A^{hNTCP} cells with Myrcludex B (MyrB), an acylated peptide derived from the HBV large envelope protein, that blocks virus entry (44), completely inhibited HBV infection and consequential cccDNA synthesis. Interestingly, treatment of the cells starting at HBV infection for 36 h with the indicated TOP1 and/or TOP2 inhibitors significantly reduced the amounts of HBV cccDNA. These results thus suggest that both TOP1 and TOP2 are required for cccDNA synthesis in *de novo* HBV infection and intracellular amplification pathway. Both the pathways are essential for establishment and maintenance of cccDNA pool in virally infected hepatocytes (45).

TOP1 and TOP2 inhibitors block distinct steps of HBV cccDNA synthesis While it is very clear that the rcDNA in the infected virion particles or progeny mature nucleocapsids are the precursors of cccDNA synthesis from *de novo* infection and intracellular amplification, respectively, the DP-rcDNA had been postulated as a potential intermediate of cccDNA synthesis (31, 46, 47). Interestingly, during further characterization of PF rcDNA by exonuclease digestion, a covalently closed negative strand rcDNA, or cc(-)rcDNA, was revealed (29) (Fig.

1A). Existence of this novel rcDNA species implies that the gap of negative strand DNA in rcDNA was repaired before the gap of positive strand (Fig. 9A). While the precursor → product relationship of DP-rcDNA, cc(-)rcDNA and cccDNA has not been firmly established in the field, it will be interesting to dissect their relationship by inhibition of cccDNA synthesis with topoisomerase inhibitors. To this end, the effects of TOP1 and TOP2 inhibitors on this newly identified putative intermediate of cccDNA synthesis were determined. Due to the lower levels of cc(-)rcDNA in the synchronized and fast cccDNA synthesis condition, the numbers of cells for Hirt DNA extraction were doubled in this experiment as compared with the experimental results presented in Fig. 1A. As shown in Fig. 9B to D, treatment of Hirt DNA preparations with Exo I and III revealed a ladder of 9 DNA species migrating between cccDNA and DP-rc DNA. All those DNA species can be linearized into a single species of dslDNA by EcoRI digestion and thus represent the different topological isoforms of cccDNA (48, 49). Moreover, an additional DNA species migrating faster than supercoiled cccDNA band is resistant to EcoRI digestion and thus the cc(-)DNA derived from exonuclease digestion of the gapped positive strand of cc(-)rcDNA (Fig. 1A and Fig. 9). Interestingly, while TOP1 inhibitor treatment significantly reduced the amounts of cc(-)DNA and cccDNA (Fig. 9B and 9E), TOP2 inhibitor treatment decreased the amounts of cc(-)DNA to a lesser extent, but significantly reduced the amounts of cccDNA (Fig. 9C, 9D and 9E). These results suggest that while TOP1 inhibitors disrupted cccDNA synthesis at a step in the repair of negative strand DNA gap, TOP2 inhibitors more efficiently inhibited the repair of the gap in positive strand DNA, but was less effective in suppression of repairing the gap of negative strand DNA.

Knockdown TOP2 expression significantly reduce cccDNA synthesis While the pharmacological evidence obtained so far clearly indicated that TOP1 and TOP2 may have played a distinct and non-redundant roles in HBV cccDNA synthesis, we desired to validate their roles with genetic approaches. However, due to their essential role in cell proliferation and survival, we could only utilize siRNA knockdown technology to determine the role of TOP1, TOP2α and TOP2β in cccDNA synthesis in HepAD38 cells. As shown in Fig. 10A, siRNA transfection of HepAD38 cells specifically reduced the levels of targeted TOP mRNA, but not the mRNA of untargeted TOPs. The reduction of the respective TOP proteins in TOP siRNA transfected cells was confirmed by Western blot assays (Fig. 10B and D). Interestingly, while knocking down the expression of TOP2α and TOP2β, alone (Fig. 10C) or in combination (Fig. 10E), significantly reduced the level of cccDNA, transfection of TOP1 siRNA slightly, but statistically significantly, increased the level of cccDNA (Fig. 10C, E and F). Whereas our results clearly demonstrated that both TOP2α and TOP2β play an essential role in HBV cccDNA synthesis, the role and mechanism of TOP1 in cccDNA synthesis remains to be further investigated.

Discussion

Although a small fraction of HBV cccDNA can be synthesized from dslDNA *via* NHEJ DNA repair pathway (50), the vast majority of functional HBV cccDNA is synthesized from rcDNA (51, 52). Biochemically, conversion of rcDNA into cccDNA requires the involvement of at least four classes of cellular enzymes, including DNA repair nucleases to process the ends, DNA polymerases to fill in the gaps, DNA ligases to ligate the ends and topoisomerases for the

286 winding or unwinding of rcDNA and cccDNA. In order to assemble a functional cccDNA
287 minichromosomes, histones and their modification enzymes as well as chromatin structure
288 remodelers are also required (15). It is thus conceivable that many cellular DNA metabolic
289 proteins are involved in cccDNA biosynthesis, maintenance and functioning. While recent
290 studies identified a few cellular DNA repair enzymes, including TDP2, Pol κ, DNA ligases and
291 FEN1, that participate in cccDNA synthesis, the molecular pathways that repair rcDNA into
292 cccDNA remains elusive (15, 21). Because many of these host cellular DNA metabolic enzymes
293 are essential for cell proliferation and survival, genome-wide CRISPR/Cas9 gene knockout
294 technology is not a suitable approach to identify host cellular proteins required for cccDNA
295 synthesis and functioning. RNA interference screening of DNA repair genes for their roles in
296 cccDNA synthesis has only achieved limited success (25). We report herein the development of
297 a rapid cccDNA synthesis assay that is suitable for targeted screening of small molecules that
298 inhibit the function of cellular DNA metabolic and epigenetic modification enzymes for their
299 activities to inhibit cccDNA synthesis (Fig. 2). Among approximately 200 compounds tested
300 thus far, compounds that target several host cellular proteins had been discovered to modulate
301 cccDNA synthesis and their modes of action are currently under investigation. We report herein
302 that TOP1 and TOP2 poisons as well as TOP2 DNA binding and ATPase inhibitors significantly
303 reduced the amounts of cccDNA (Fig. 3, 4 and 7). It was further demonstrated that all these
304 inhibitors also disrupted cccDNA synthesis during *de novo* HBV infection of C3A^{hNTCP} cells
305 (Fig. 8). Interestingly, we were able to show that TOP1 and TOP2 inhibitors rapidly inhibited
306 cccDNA synthesis (Fig. 4) and while TOP1 inhibitors blocked the repair of the gap in negative
307 strand DNA, TOP2 inhibitors disrupted the repair of the gap of positive strand DNA in the
308 conversion of rcDNA to cccDNA (Fig. 9). Finally, using RNAi technology, we demonstrated

that both TOP2α and TOP2β are required for cccDNA synthesis, but a contradictive result of TOP1 on cccDNA synthesis was revealed (Fig. 10).

DNA TOPs regulate the function of genomic DNA by changing its topology. Biochemically, DNA TOPs cleave the phosphodiester bond in a coordinated way and re-ligate the ends of DNA to unwind or wind DNA to control the supercoiling (53) and resolve disordered DNA entanglements and knots (54, 55). Because the dynamics of topological conformation of genomic DNA is an integral part of its functions, such as replication, repair, transcription, chromatin assembly, remodeling and segregation, DNA TOPs are thus essential for all living organisms (56, 57). Except for a few large DNA viruses that encode their own DNA TOPs (58-60), many DNA viruses, including herpesviruses (61) (62) (63), vaccinia virus (64), adenovirus (65) and polyomavirus (66, 67), recruit host cellular DNA TOPs for their replication and transcription. Moreover, it has been shown that both cellular TOP1 and TOP2 are associated with human immunodeficiency virus-1 (HIV-1) or recruited to viral DNA replication complex to promote reverse transcriptional viral DNA synthesis (68, 69). TOPs have also been demonstrated to play a role in the replication of other retroviruses (70). Thus far, even though the functional roles of TOPs in viral DNA replication and RNA transcription as well as the recruitment of TOPs to viral genome replication complex have been clearly demonstrated, the biochemical mechanism of TOPs to facilitate viral replication and transcription for most of those viruses remains to be illustrated.

Concerning the role of TOPs in HBV replication, an early study suggested that TOP1 can cleave DHBV rcDNA *in vitro* at specific sites of both negative and positive strands and linearize rcDNA (71). Based on this biochemical study, Pourquier and colleagues postulated that TOP1 may play a role in the circularization of negative stranded DNA in cccDNA synthesis and viral

DNA integration into host cellular chromosome. Interestingly, our result that TOP1 poisons inhibited the production of covalently closed circular negative strand DNA is consistent with this hypothesis (Fig. 9B). However, the slight increase of cccDNA levels in HepAD38 cells, when TOP1 expression was reduced by siRNA knockdown, is apparently contradictive to an essential role of TOP1 in cccDNA synthesis (Fig. 10). A possible explanation to these contradictive results is that while camptothecin treatment efficiently arrests TOP1 catalyzed negative strand DNA religation and prevents its circularization, reduction of the amount of TOP1 protein by siRNA may result in the recruitment of alternative cellular enzymes or repair pathway that can more efficiently catalyze cccDNA synthesis. Nevertheless, despite the discrepancy between the results obtained from TOP1 inhibitor treatment and siRNA knockdown of TOP1 expression, the results indicate that TOP1 does involve in or regulate cccDNA synthesis. Further analyses of DNA repair intermediates under these specific experimental conditions should resolve the molecular basis of the discrepancy. In contrast to TOP1, both pharmacological and genetic evidence obtained in this study support that TOP2 plays an essential role in HBV cccDNA synthesis. Mechanistically, our results indicate that unlike TOP1 that catalyzes the negative strand DNA circularization, TOP2 appears to be required for the circularization of both strands of rcDNA (Fig. 9B and E). In conclusion, our results provide evidence suggesting that TOP1 and TOP2 play non-redundant roles in HBV cccDNA synthesis. Further investigation to understand the biochemical mechanism of those cellular enzymes in cccDNA synthesis will advance our knowledge on HBV biology and establish molecular basis for development of therapeutics to suppress cccDNA synthesis and transcriptional function and ultimately cure chronic hepatitis B.

355 **Materials and Methods**

356

357 **Cell culture** HepAD38, a HepG2-derived cell line supporting HBV replication in a tet-inducible
358 manner, was obtained from Dr. Christoph Seeger at Fox Chase Cancer Center, Philadelphia,
359 USA (28) and cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine
360 serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 1µg/mL tetracycline. C3A^{hNTCP} is C3A,
361 a sub-clone of HepG2 (ATCC HB-8065), derived cell line stably expresses human NTCP (72)
362 and maintained in DMEM/F12 medium supplemented with 10% FBS and 100 U/ml penicillin
363 and 100 µg/ml streptomycin.

364

365 **Chemical reagents** Topoisomerase inhibitors doxorubicin (Cat. No. S1208), camptothecin
366 (S1288), etoposide (S1225), dexrazoxane (ICRF-187) HCl (S1222) and mitoxantrone 2HCl
367 (S1889) were purchased from Selleckchem. Topotecan (HY-13768A) and idarubicin (HY-
368 17381) were purchased from MedChemExpress (MCE). Aclarubicin (A-101-5) was obtained
369 from GoldBio. Merbarone (M2070), ICRF-193 (I4659) and β-lapacone (L2037) were purchased
370 from Sigma-Aldrich.

371

372 **Analyses of HBV DNA.** HBV core DNA and Hirt DNA extraction from HepAD38 or C3A^{hNTCP}
373 cells as well as analyses by Southern blot hybridization were described previously (73-75).
374 Human mitochondrial DNA, as a loading control for Hirt DNA, was also detected with Southern
375 blot hybridization after stripping of HBV probes. Briefly, a 2709 bp long of mtDNA from hepG2
376 cells was amplified with the primers HuMt NotI-F: ACACAC GCGGCCGC
377 ctgctggcatcactatactacta and HuMt NdeI-R: ACACAC CATATG gattggtgggtcattatgtgttg by

standard PCR and cloned into pGEM T Easy Vector (Promega). Recombinant plasmid was amplified in DH5α competent cells (Invitrogen), purified with Midiprep (QIAGEN) and linearized with Sph1-HF (NEB, Cat. No. R3182). An α-^{32}P-UTP labeled riboprobe was prepared by *in vitro* transcription of the linearized recombinant plasmid with RiboProbe System-SP6 (Promega, Cat. No. P1420) and used for Southern blot hybridization of human mtDNA. A mtDNA fragment of 7366 nt in length can be detected in EcoRI-digested Hirt DNA preparations.

Restriction enzyme and exonuclease treatment of Hirt DNA For Southern blot detection of HBV cccDNA, Hirt DNA preparations were either left untreated or heated at 88^{o}C for 8 min to denature DP-rcDNA and dslDNA into single-stranded DNA; and cccDNA species were subsequently digested by EcoRI at 37 °C for 1 h to convert them into unit-length double-stranded linear DNA (27). For detection of single-stranded closed circular rcDNA, Hirt DNA preparations were digested with Exonuclease I (NEB, Cat. No. M0293S) and Exonuclease III (NEB, Cat. No. M0206S) for 2 h to remove DNA species with free 5'- and 3' ends (29). After Exo I and III digestion, the remaining DNA in reactions were extracted by phenol and precipitated with isopropanol. If needed, the purified DNA samples can be digested by EcoRI to convert cccDNA into unit-length double-stranded linear DNA as described above.

siRNA knockdown in PFA-arrested HepAD38 cells. HepAD38 cells were cultured in the absence of tetracycline (tet) for two days and 2 mM PFA was added into culture media and cultured for another two days. The cells were then re-seeded into 12-well plates at a density of 4×10^5 cells per well and transfected with 10 nM of indicated siRNA at 6 h post-transfection in OptiMEM with equal amount of Lipofectamine RNAiMax (Invitrogen, Cat. No. 13778150). Human topoisomerase siRNAs were bought from origene (TOP1 Human siRNA Oligo Duplex

(Locus ID 7150, CAT# SR304897), TOP2A Human siRNA Oligo Duplex (Locus ID 7153, CAT# SR322074), TOP2B Human siRNA Oligo Duplex (Locus ID 7155, CAT# SR304899). At 48 h post transfection, the cells were refreshed with DMEM/F12 supplemented with tet to stop HBV pgRNA transcription from transgene and without PFA to resume HBV DNA synthesis and cccDNA formation. The cells were harvested 24 h later for analyses of topoisomerase mRNA and HBV DNA.

Quantification of inflammatory cytokine topoisomerase mRNA by qRT-PCR assay. Total cellular RNA was extracted by using TRIzol reagent (Invitrogen). cDNA was synthesized by using SuperScript III Platinum one-step qRT-PCR Kit (Invitrogen). Real-time PCR assays were performed using a LightCycler 480 II. Primer sequences for topoisomerases mRNA levels by qRT-PCR analyses are provided in Table 2. Primer sequences for analyses of cytokine mRNA were reported previously (72).

Western blot assay Cells in a well of 12-well plate were lysed with 200μL NuPAGE® SDS sample buffer (Thermo Fisher Scientific) supplemented with 2.5% 2-Mercaptoethanol (Sigma). Cell lysate was subjected to denaturing gel electrophoresis with NuPAGE 4-12% Bis-Tris Gel and NuPAGE MOPS SDS Running Buffer (Thermo Fischer Scientific). Proteins were transferred from the gel onto a PVDF membrane using iBlot 2 Dry Blotting System (Thermo Fischer Scientific). Membranes were blocked with TBST (TBS containing 0.1% Tween 20) containing 5% nonfat milk for 1h and incubated with desired antibody overnight at 4°C. We used anti-TOP1 (abcam, Cat. No. ab3825), anti-TOP2α (Santa Cruz, Cat. No. sc-166934) and anti-TOP2β, (Bethyl Laboratories, Inc., Cat. No. A300-949A) primary antibodies. After washing with

TBST, the membrane was incubated with LI-COR® IRDye® secondary antibodies. Membranes were again washed with TBST and imaged with LI-COR Odyssey system (LI-COR Biotechnology).

HBV infection of C3A^{hNTCP} cells. For HBV infection, C3A^{hNTCP} cells were seeded into collagen-coated 12-well plates at a density of 1.5×10^6 cells per well and cultured in complete DMEM medium containing 3% dimethyl sulfoxide (DMSO). One day later, the cells were infected with HBV prepared from HepAD38 cell culture media at a MOI of 250 genome equivalents per cell in DMEM containing 4% PEG-8000. The inoculums were removed at 24 h and cells were washed three times with PBS. The infected cultures were maintained in complete DMEM medium containing 3% DMSO until harvesting.

Acknowledgement

We thank Dr. Eain A. Murphy for critical reading and comments on the manuscript.

Funding information

This work was supported by a grant from the National Institutes of Health, USA (AI113267), Arbutus Biopharma Inc. and the Commonwealth of Pennsylvania through the Hepatitis B Foundation.

Table 1. Effects of topoisomerase poisons on cccDNA synthesis

Compound	Target	Concentration (μM) *	cccDNA (% of control)	Maximum non-toxic concentration (μM)#
Doxorubicine	TOP2	1.00	22	2.0
Idarubicin	TOP2	0.25	19	2.5
Camptothecin	TOP1	1.00	37	2.0
Topotecan	TOP1	0.25	30	2.5

*Compound concentration used in screening test.

#Cytotoxicity was determined by MTT assay and visual inspection under microscopy after 48 of compound treatment.

458 **Table 2. Sequence of the primers for qPCR**

459
460
461 Target Gene | Species | Sequence (s)
462
463

TOP1 Origene (Gene ID 7150)	Human	Sense: GAACAAGCAGCCCGAGGATGAT Antisense: TGCTGTAGCGTGATGGAGGCAT
TOP2A Origene (Gene ID 7153)	Human	Sense: GTGGCAAGGATTCTGCTAGTCC Antisense: ACCATTCAGGCTCAACACGCTG
TOP2B Origene (Gene ID 7155)	Human	Sense: GGTCAGTTTGGAACTCGGCTTC Antisense: AGGAGGTTGTCATCCACAGCAG
TOP3A Origene (Gene ID 7156)	Human	Sense: GCATCGACTCTTTAACCACACGG Antisense: CTCCACAGTGTCCAAGGCTTGA
TOP3B Origene (Gene ID 8940)	Human	Sense: GATGCTGGAGAAGCAGACGAAC Antisense: CTCTCCACCGTGACATAGTTGC
TOP1MT Origene (Gene ID 116447)	Human	Sense: GACCTACAACGCCTCCATCACT Antisense: TGCTCGCTGATGGTTGCAGAGA
β-actin	Human	Sense: CACCATTGGCAATGAGCGGTTC Antisense: AGGTCTTTGCGGATGTCCACGT
Core DNA	HBV	Sense: GGCTTTCGGAAAATTCCTATG Antisense: AGCCCTACGAACCACTGAAC
cccDNA	HBV	Sense: GGGGCGCACCTCTCTTTA Antisense: CCACCCAGGTAGCTAGAGTCATTAG

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659 **Figure Legend**

660

661 **Fig. 1. Characterization of protein-free HBV DNA species in HepAD38 cells**. **(A** and **B)**
662 HepAD38 cells cultured for 12 days in the absence of tet. Hirt DNA was extracted and detected
663 by Southern blot hybridization. The size markers of HBV DNA in length are denoted. Hirt DNA
664 without prior treatment (lane 1), after heat denaturalization without (lane 2) or with following
665 EcoRI digestion (lane 3), or treated with Exo I and III without (lane 4) or with following EcoRI
666 digestion (lane 5) were resolved by agarose gel electrophoresis and transferred on Nylon
667 membranes. The membranes were hybridized with a full-length riboprobe specifically
668 hybridizing to negative (**A**) and positive strand (**B**) of HBV DNA, respectively. Red arrow
669 indicates covalently closed circular negative strand HBV DNA. **(C)** Schematic presentation of
670 experimental schedule. HepAD38 cells were maintained in culture in the presence of tet and
671 harvested at the indicated time post removal of tet. **(D)** HBV core DNA (upper panel) and Hirt
672 DNA after heat denaturalization and EcoRI digestion (lower panel) were resolved by agarose gel
673 electrophoresis and detected by Southern blot hybridization with a full-length riboprobe
674 specifically hybridizing to negative strand HBV DNA. Mitochondrial DNA (mtDNA) serves as a
675 loading control for Hirt DNA. Biological duplicate samples were obtained and analyzed for each
676 of the indicated time points. rc, relaxed circular DNA; DP-rc, deproteinized rc DNA; *DP-rc,
677 denatured deproteinized rc DNA; dsl, double-stranded linear DNA; ss, single-stranded DNA;
678 ccc, covalently closed circular DNA; ccc*, EcoRI linearized cccDNA.

679

680 **Fig. 2. A synchronized and fast cccDNA synthesis assay in HepAD38 cells. (A)** Schematic
681 presentation of experimental schedule. HepAD38 cells were cultured in the absence of tet and 2

mM of PFA was added in culture medium 2 days after tet removal to arrest viral DNA synthesis. Four days later, while PFA was withdrawn, tet was added back to culture medium to stop viral pgRNA transcription from transgene. Cells were harvested at the indicated time points. **(B)** HBV core DNA (upper panel) and Hirt DNA after heat-denaturalization at 88°C for 8 min and EcoRI digestion (lower panel) were resolved by agarose gel electrophoresis and HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. (C) The amounts of HBV rcDNA and cccDNA were quantified by phosphoimager and plotted as the ratio over the amount of the corresponding DNA species at time 0 of PFA removal. Mean and standard deviation from two biological duplicates were presented.

Fig. 3. TOP1 and TOP2 inhibitors reduce the level of cccDNA. (A) Schematic presentation of experimental schedule. HepAD38 cells were cultured in the absence of tet and HBV DNA replication were arrested by PFA treatment between day 3 to 6 after tet removal. The cells were immediately harvested (0 h) or cultured in the presence of tet and absence of PFA and mock-treated (UT) or treated with 10 μM of 3TC or the indicated concentrations of DNA TOP poisons for 24 h. (**B**, **D**, **F** and **H**) Intracellular HBV core DNA (upper panel) and Hirt DNA after heat-denaturalization at 88°C for 8 min and EcoRI digestion (lower panel) were resolved by agarose gel electrophoresis and HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. mtDNA served as loading control of Hirt DNA analysis. (**C**, **E**, **G** and **I**) Core DNA (including ss and rcDNA), DP-rcDNA and cccDNA were quantified by phosphoimager and normalized by the mtDNA. The average level

Journal of Virology

704 from two biological duplicates under compound treatment were plotted as the percentage of that
705 in the mock-treated cells (UT) at 24 h post PFA removal.
706
707 **Fig. 4. TOP1 and TOP2 inhibitors block cccDNA synthesis**. **(A)** Schematic presentation of
708 experimental schedule. HepAD38 cells were cultured in the absence of tet and 2 mM of PFA was
709 added in culture medium 2 days after tet removal to arrest viral DNA synthesis. Four days later,
710 while PFA was withdrawn, tet was added back to culture medium to stop viral pgRNA
711 transcription from transgene. Cells were left untreated or treated with toptecan (1 μM) or
712 doxorubicin (1 μM) at 16 h after PFA removal and harvested at the indicated time points. **(B)**
713 Hirt DNA after heat-denaturalization at 88°C for 8 min and EcoRI digestion (lower panel) were
714 resolved by agarose gel electrophoresis and HBV DNA species were detected by Southern blot
715 hybridization with a riboprobe specifically hybridizing to negative strand DNA. mtDNA served
716 as loading control. **(C)** The amounts of cccDNA were quantified by phosphoimager, normalized
717 by the mtDNA and plotted as the percentage of that in the mock-treated cells (UT) harvested at
718 16 h post PFA removal. Mean and standard deviation (n = 4) are presented.
719
720 **Fig. 5. TOP1 and TOP2 inhibitors do not alter the level of pre-existing cccDNA**. **(A and C)**
721 Schematic representation of experimental schedule. (**B and D**) Intracellular HBV core DNA
722 (upper panel) and Hirt DNA after heat-denaturalization at 88°C for 8 min and EcoRI digestion
723 (lower panel) were resolved by agarose gel electrophoresis and HBV DNA species were detected
724 by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA.
725 mtDNA served as loading control of Hirt DNA analysis. As illustrated in panel C, in the results
726 presented in panel D, treatment with 3TC alone was started at day 6 and the cells were harvested

727 at Day 9, whereas mock-treatment (UT) or treatment with the indicated concentrations of
728 doxorubicin were started at day 8 and the cells were harvested at day 9. The amounts of cccDNA
729 were quantified by phosphoimager, normalized by the mtDNA. The average levels of cccDNA in
730 compound treated cells were denoted as the percentage of that in the mock-treated cells (UT).
731
732 **Fig. 6. TOP1 and TOP2 inhibitors did not induce prominent cytokines.** HepAD38 cells
733 were mock-treated (UT) or treated with 250 nM camptothecin (CTP), or 250 nM doxorubicin
734 (Doxo) for 6 h or 24 h. Intracellular IFN-β, IL-6, IL-29, IL-28A, IL-28B and TNF-α transcripts
735 were quantified by RT-qPCR assays and normalized by the level of β-actin mRNA. Mean and
736 standard deviation (n = 3) are presented.
737
738 **Fig. 7. Effects of distinct TOP1 and TOP2 inhibitors on HBV cccDNA synthesis**. (**A**)
739 Illustration of DNA TOP2 catalytic cycle. Briefly, TOP2 enzyme binds to the DNA molecule
740 (Step 1). In the presence of Mg++, two ATP molecules bind to the ATPase domain and result
741 in its dimerization and cleavage of one double-stranded DNA (blue) (Step 2). The second DNA
742 molecule (orange) is transported through the break (Step 3). Upon transport of the DNA segment
743 through the break, one molecule of the ATP is hydrolyzed (Step 4) and followed by the re-
744 ligation of the cleaved DNA segment along with hydrolysis of another ATP molecule (Step 5)
745 and releasing of DNA fragment (Step 6). Compounds that inhibit each of these steps are
746 indicated. (**B** to **E**) HepAD38 cells were cultured in the absence of tet and HBV DNA replication
747 were arrested by PFA treatment between day 3 to 6 after tet removal. The cells were immediately
748 harvested (0 h) or cultured in the presence of tet and absence of PFA and mock-treated (UT) or
749 treated with 10 μM of 3TC or the indicated concentrations of aclarubicin and merbarone (**B**) or

500 nM ICRF-187, 500 nM etoposide (Etop), 500 nM mitoxanthrone (MXT), 500 nM ICRF-193, 500 nM β-lapacone (β-Lap) and 50 μM merbarone (Merb) (**E**) for 24 h. Hirt DNA were resolved by agarose gel electrophoresis after heat-denaturalization at 88°C for 8 min and EcoRI digestion. HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. mtDNA served as loading control of Hirt DNA analysis. The amounts of cccDNA were quantified by phosphoimager and normalized to the mtDNA. The levels of cccDNA in compound treated cells were plotted (**C** and **D**) or denoted (E) as the percentage of that in the mock-treated cells (UT).

Fig. 8. TOP1 and TOP2 inhibitors inhibited HBV cccDNA synthesis in *de novo* infection. **(A)** Schematic representation of experimental schedule. C3A^{hNTCP} cells were infected with HBV at a MOI of 250 genome equivalents (GEQ) for 24 h. The cells were mock-treated or treated with 200 nM doxorubicin (Doxo), 200 nM topotecan (TPT), 200 nM doxorubicin and 200 nM topotecan (Doxo + TPT), 200 nM aclarubicin (Acla) or 1 μg/mL Myrcludex-B (Myr-B) starting from HBV infection for total 36 h. (**B**) Hirt DNA were resolved by agarose gel electrophoresis after heat-denaturalization at 88°C for 8 min and EcoRI digestion. HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. mtDNA served as loading control of Hirt DNA analysis. (**C**) cccDNA were quantified by a phosphoimager. The data were presented from three independent experiments. *p* values calculated by student t test are presented.

Fig. 9. TOP1 and TOP2 inhibitors disrupted distinct steps of HBV cccDNA synthesis. (A) Schematic illustration of cccDNA synthesis from DP-rcDNA *via* an intermediate, cc(-)rcDNA,

and production of cc(-)DNA by exonuclease I and III digestion of cc(-)rcDNA. **(B to E)** HepAD38 cells were cultured in the absence of tet and HBV DNA replication were arrested by PFA treatment between day 3 to 6 after tet removal. The cells were then cultured in the presence of tet and absence of PFA and mock-treated (UT) or treated for 24 h with 500 nM camptothecin (CPT) (**B**), 500 nM doxorubicin (Doxo) **(C)**, 40 μM merbarone (Merb) **(D)**. Hirt DNA without prior treatment, digested with Exo I and III without or with following EcoRI restriction were resolved by agarose gel electrophoresis. HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. (**E**) The amounts of cc(-)DNA were quantified by a phosphoimager and normalized to that in mock (DMSO)-treated cells. Mean and standard derivations from six (CPT and Doxo) or three (Merb) independent experiments are presented. *p* values calculated by student t test are presented.

Fig. 10. Knockdown of TOP2 mRNA reduced cccDNA formation. HepAD38 cells were mock-transfected or transfected with the indicated siRNA and cultured in the absence of tet and presence of PFA for 2 days. The cells were then cultured in the presence of tet and absence of PFA for additional 24 h. **(A)** Total cellular RNAs were extracted and TOP mRNAs were quantified by qRT-PCR assay, normalized to the level of β-actin mRNA and expressed as the ratio over the level of respective TOP mRNA in cells transfected with scrambled siRNA. **(B** and **D)** The levels of TOP proteins were determined by Western blot assays. TBP served as a loading control. **(C** and **E)** Hirt DNA was resolved by agarose gel electrophoresis after heat-denaturalization at 88°C for 8 min and EcoRI digestion. HBV DNA species were detected by Southern blot hybridization with a riboprobe specifically hybridizing to negative strand DNA. mtDNA served as loading control of Hirt DNA analysis. (**F**) cccDNA were quantified by a

phosphoimager. Mean and standard deviations are presented (n = 8). *p* values calculated by student t test are presented.

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Figure 1

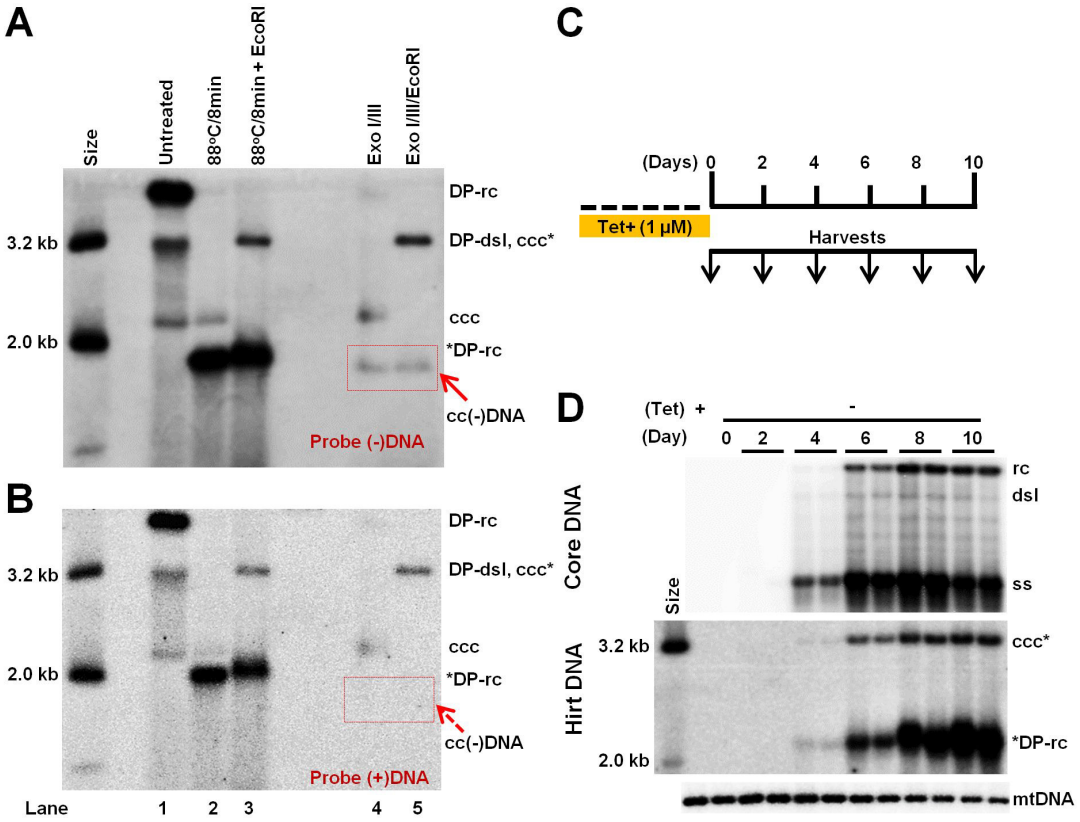
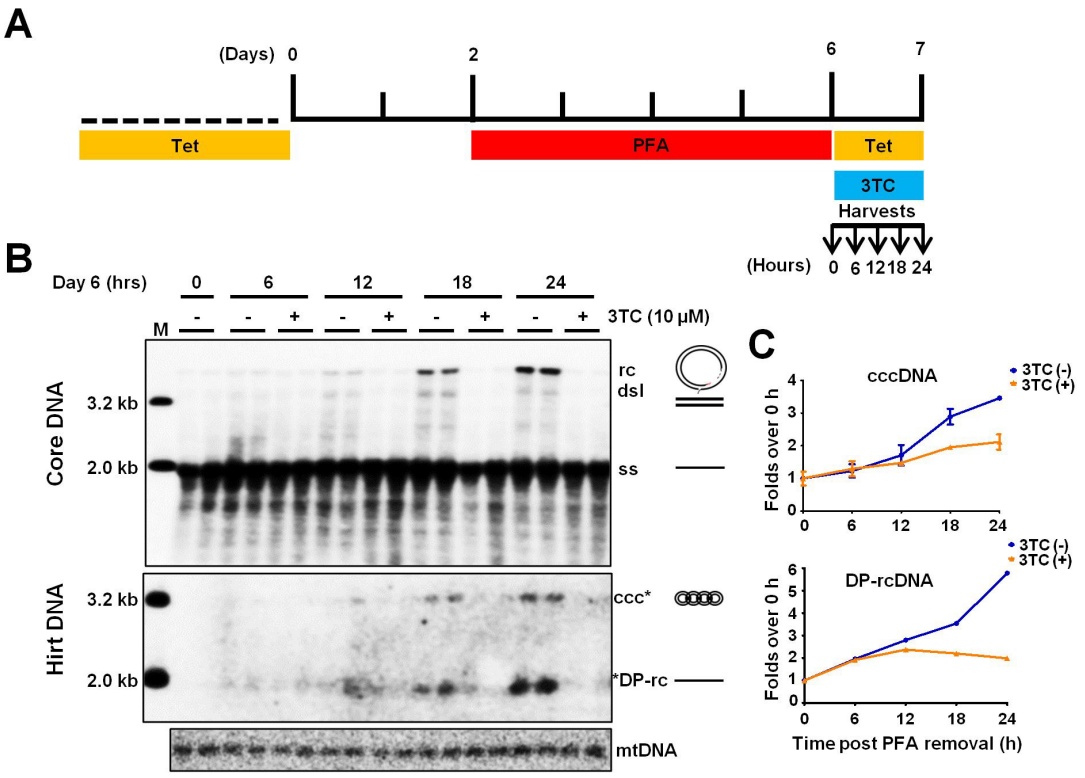


Figure 2



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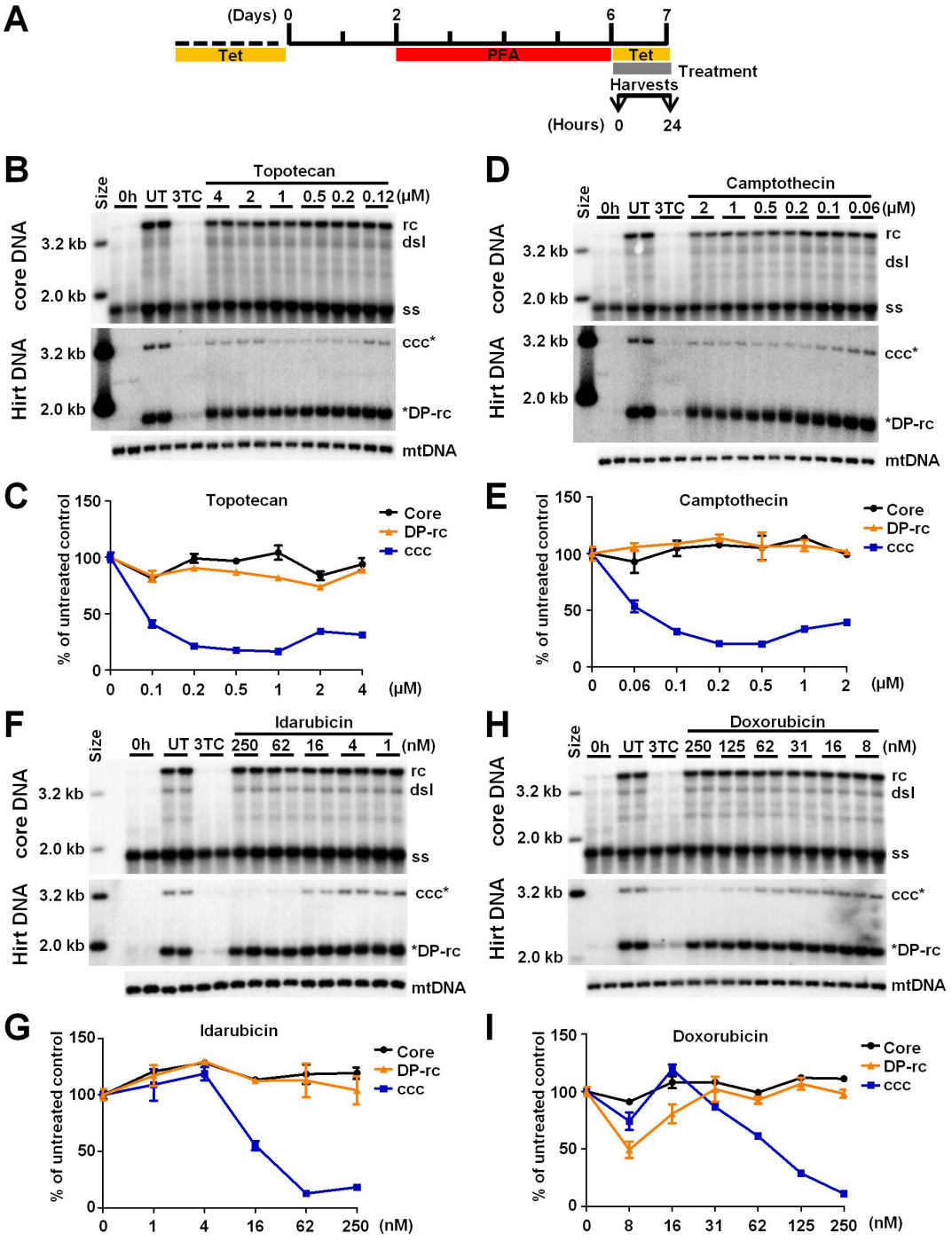

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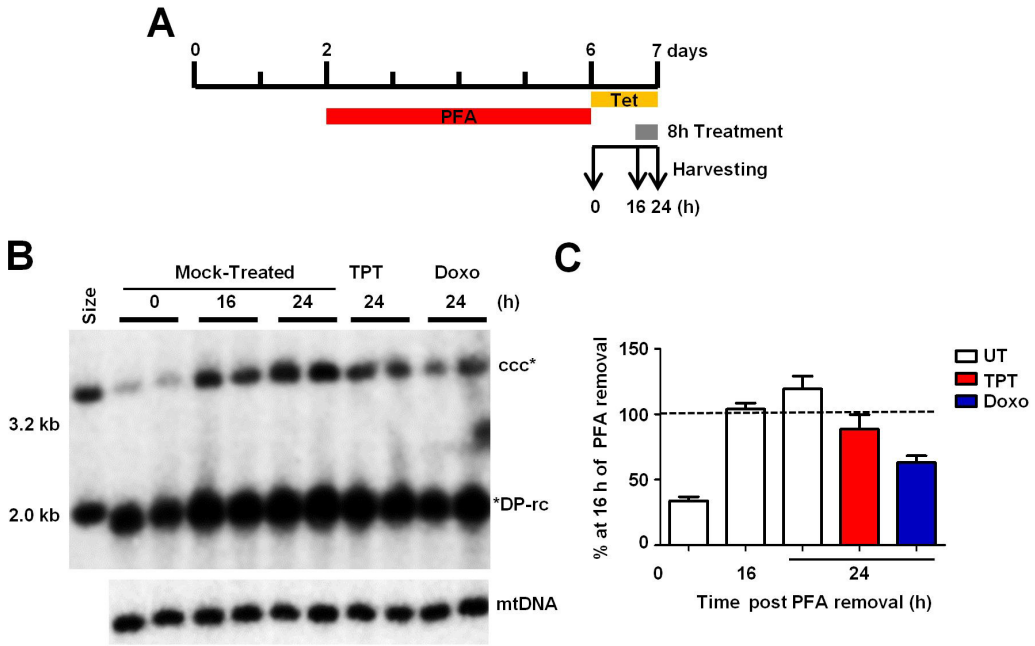

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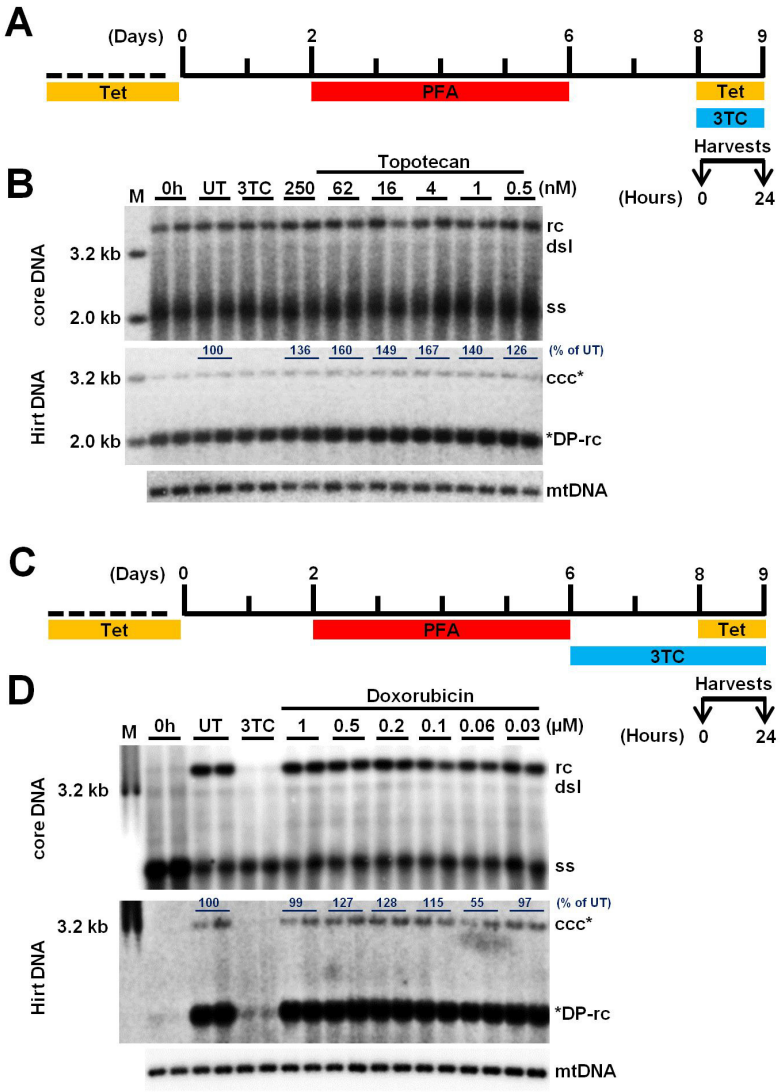

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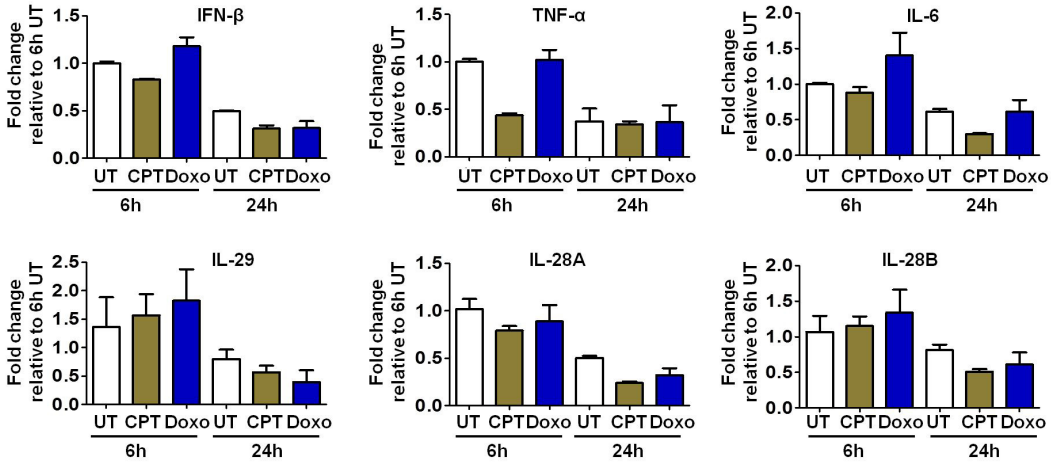

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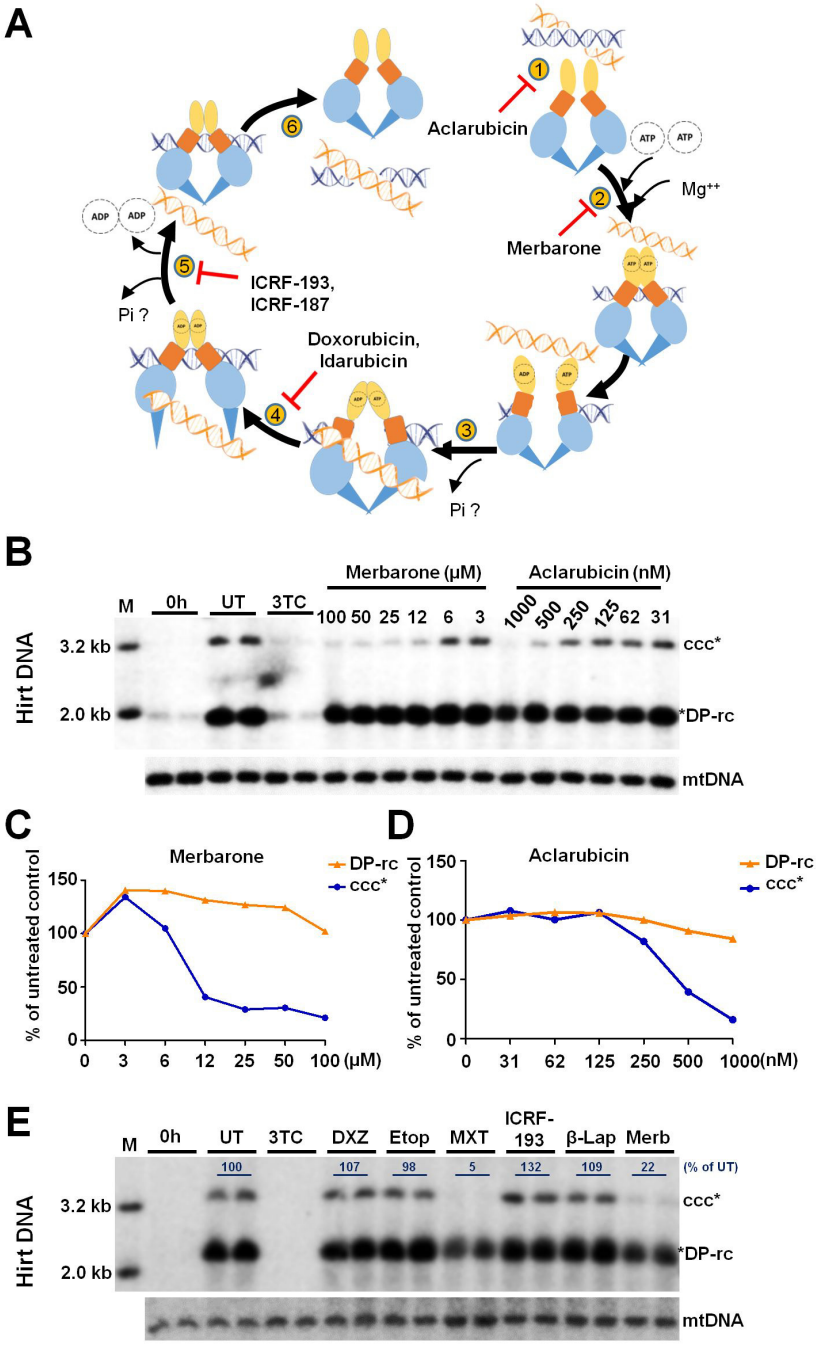

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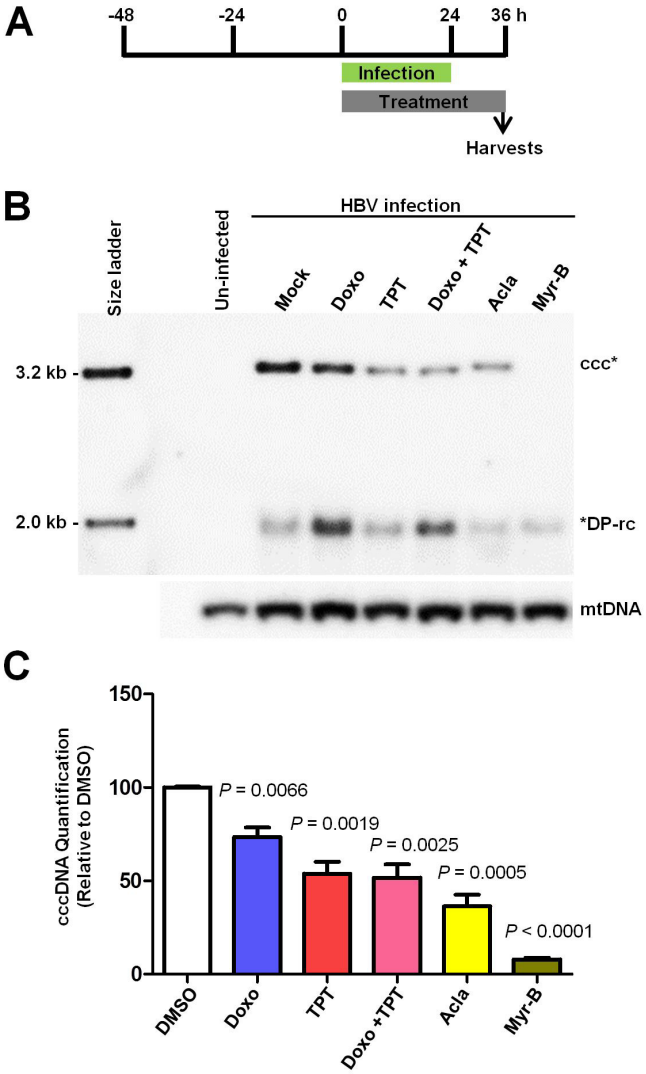

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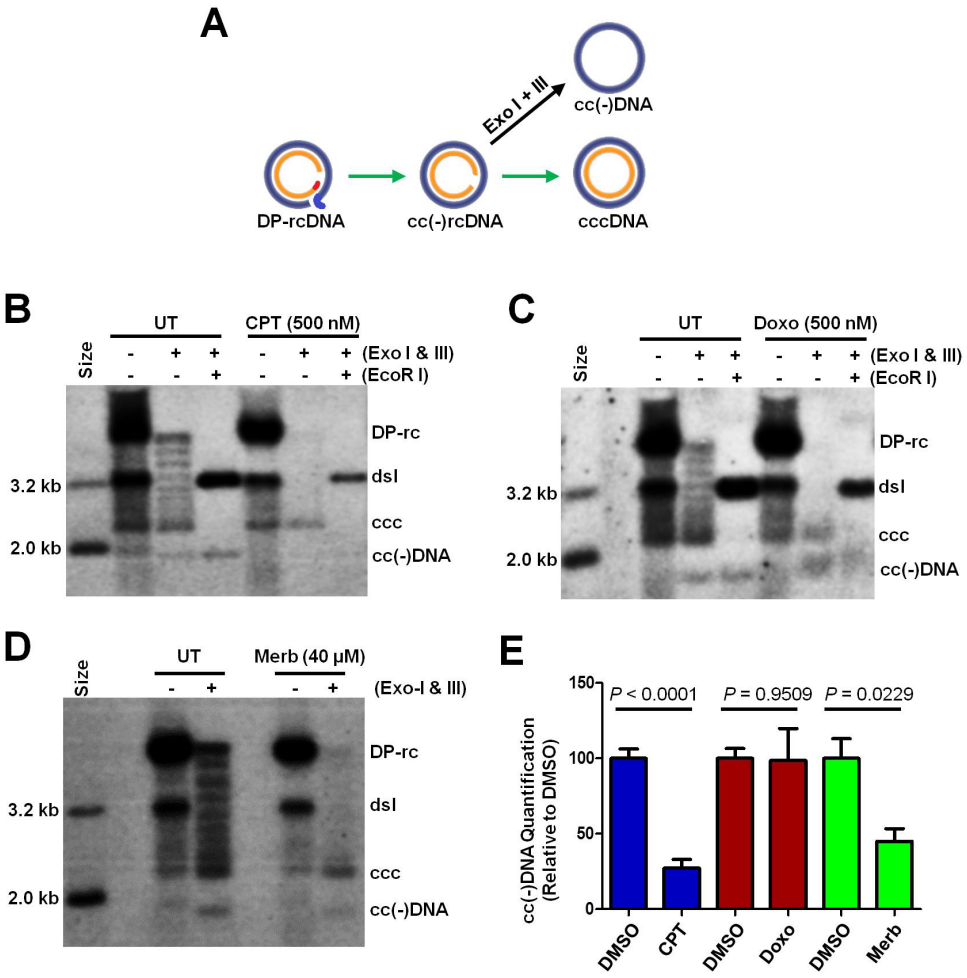

Figure 10