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Hyperphosphorylation of RNA Polymerase II in Response to Topoisomerase I Cleavage Complexes and Its Association with Transcription- and BRCA1-dependent Degradation of Topoisomerase I

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The progression of RNA polymerase II can be blocked by lesions on the DNA template. In this study, we focused on the modifications of the largest subunit of RNA polymerase II, Rpb1, in response to stabilized topoisomerase I (Top1)–DNA cleavage complexes. In addition to DNA modifications (base damages and strand breaks), Top1 cleavage complexes can be trapped by camptothecin (CPT) and its derivatives used in cancer treatment. We found that, within a few minutes, CPT produces the complete hyperphosphorylation of Rpb1 in both primary and transformed cancer cells. Hyperphosphorylation is rapidly reversible following CPT removal. Hyperphosphorylation occurs selectively on the serine 5 residue of the conserved heptapeptide repeats in the Rpb1 carboxy-terminal domain and is mediated principally by the transcription factor IIH-associated cyclin-dependent kinase Cdk7. Hyperphosphorylated Rpb1 is not primarily targeted for proteosomal degradation and instead is subjected to cycles of phosphorylation and dephosphorylation as long as Top1 cleavage complexes are trapped by CPT. Finally, we show that transcription-induced degradation of Top1 is Brca1 dependent, suggesting a role for Brca1 in the repair or removal of transcription-blocking Top1–DNA cleavage complexes.

Keywords: RNA polymerase II; topoisomerase I; transcription; Cdk7; Brca1

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Abbreviations used: APD, aphidicolin; Cdk, cyclin-dependent kinase; CPT, camptothecin; CSK, cytoskeleton; CTD, carboxy-terminal domain; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; FLV, flavopiridol; Pol II, RNA polymerase II; Pol IIa, hypophosphorylated Pol II; Pol IIo, hyperphosphorylated Pol II; RT, reverse transcription; Ser2-P, serine 2 phosphorylation; Ser5-P, serine 5 phosphorylation; siRNA, small interfering RNA; Top1, topoisomerase I; Top1cc, Top1 cleavage complex.

Introduction

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II), Rpb1, contains an evolutionary conserved and repeated heptapeptide, Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7, that is subjected to reversible phosphorylations during each transcription cycle.[1] Hypophosphorylated Pol II (Pol IIa) CTD corresponds to the transcriptionally inactive Pol II, whereas hyperphosphorylated Pol II (Pol IIo) CTD is the transcriptionally active Pol II. A variety of kinases have been identified, with

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phosphorylation activity directed toward the amino acids tyrosine 1 (Abl1/2), serine 2 [cyclin-dependent kinase (Cdk) 9, CTDK1, and DNA-PK], serine 5 (Cdk7, Cdk9, and Erk1/2), and serine 7.[1] During the transcription cycle, serine 2, serine 5, and serine 7 are the main residues subjected to regulatory phosphorylation–dephosphorylation.[1,2] Serine 5 phosphorylation (Ser5-P) and serine 2 phosphorylation (Ser2-P) are thought to be principally mediated by the kinase activities of transcription factor IIH (Cdk7) and P-TEFb (Cdk9), respectively. The kinase or kinases involved in serine 7 phosphorylation remain to be identified. Dephosphorylation of Ser5 is preferentially carried out by SSU72 and SCPs (small CTD phosphatases), whereas FCP1 (transcription factor IIF-associating CTD-phosphatase 1) dephosphorylates both Ser2 and Ser5.[1] While Ser5-P peaks early in the transcription cycle and decreases toward the 3′-end of the gene, Ser2-P predominates in the body and toward the 3′-end of transcribed genes.[1]

Elongating Pol II can be arrested by a broad range of endogenous and exogenous DNA lesions, including UV light-induced pyrimidine dimers, carcinogenic adducts, adducts induced by anticancer drugs, DNA–protein cross-links, oxidative DNA lesions, abasic sites, and DNA strand breaks.[3] Transcription-blocking DNA lesions are primarily repaired by transcription-coupled repair, a specialized nucleotide-excision repair pathway elicited by transcription-blocking lesions located on the transcribed strand.[4,5] In the repair of DNA, two main mechanisms exist to displace the Pol II–DNA complexes: Pol II backtracking and Pol II degradation. Pol II backtracking enables the DNA repair complexes to access the damaged sites, after which transcription elongation resumes to finish the transcript.[6] In the second mechanism, Pol II is removed following ubiquitinylation and proteosomal degradation[7–9] and a new Pol II complex transcribes the repaired gene.

DNA topoisomerase I (Top1) is required to remove DNA superhelical tensions generated during transcription.[10,11] Top1 relaxes DNA supercoiling by producing transient Top1 cleavage complexes (Top1cc's), which are Top1-linked DNA single-strand breaks.[10–12] Both positive and negative DNA supercoiling are produced ahead of and behind the elongating Pol II, respectively.[13] Top1 can remove both positive and negative supercoiling[10,11] and therefore can relax DNA on both sides of transcription complexes. However, Top1 seems more efficient at removing positive DNA supercoiling and tends to form more cleavage complexes in positively *versus* negatively supercoiled DNA.[14] The rapid resealing of Top1cc's is inhibited by a wide range of common DNA base alterations (oxidation, alkylation, base mismatch, base loss), carcinogenic DNA adducts, and DNA backbone nicks.[15,16] Top1cc's can also be trapped with exquisite selectivity by camptothecin (CPT), a plant alkaloid whose semisynthetic derivatives, topotecan and irinotecan, are used to treat human cancers.[17,18] From a mechanistic standpoint, CPT is a sharp molecular tool since it has no other cellular target besides Top1. CPT also has the advantage of trapping Top1cc's reversibly. Indeed, Top1cc's reverse fully within minutes after washing out CPT. Trapped Top1cc's are potent transcription-blocking DNA lesions.[19–21] Transcription complexes may be blocked physically a few base pairs upstream of the Top1cc's,[22,23] or their progression may be arrested by the accumulation of positive DNA supercoiling ahead of the transcribing Pol II along the chromatin template.[24] Consequent to Top1cc-mediated Pol II arrest, Top1cc's are removed following ubiquitinylation and proteosomal degradation of Top1.[20]

In the present study, we used CPT to trap Top1cc's and investigated the cellular posttranslational modifications of Pol II itself, as well as the mechanism involved in transcription-dependent Top1 degradation.

Results

Top1cc's induce rapid, complete, and reversible Pol II hyperphosphorylation

Pol IIo can be distinguished from Pol IIa because of its reduced electrophoretic mobility in SDS polyacrylamide gels.[25] Under normal conditions, both Pol IIo and Pol IIa can be detected (Fig. 1a, left lane). In contrast, Top1cc stabilization by CPT produced a rapid disappearance of Pol IIa with coincident increase of Pol IIo (Fig. 1a and b). Within 30 min of CPT treatment, Pol II became completely hyperphosphorylated (IIo form; Fig. 1a). Quantitatively, the increase in Pol IIo corresponded to the disappearance of Pol IIa (Fig. 1c). Experiments performed in the presence of inhibitors of the proteasome (MG132) or other proteases (L-1-tosylamido-2-phenylethyl choloromethyl ketone and thimerosal) confirmed that the decrease of Pol IIa was not due to its degradation (Supplementary Fig. 1a). Moreover, experiments performed with cycloheximide demonstrated that the increased Pol IIo was not due to enhanced protein synthesis (Supplementary Fig. 1b). CPT-induced Pol II hyperphosphorylation was observed in all cell lines examined [human colon cancer HCT116 cells (Figs. 1–4), primary human lymphocytes isolated from peripheral blood (Fig. 2e), mouse fibroblast cells (Fig. 5d), and human prostate cancer DU145 cells (Supplementary Fig. 3b)]. These experiments indicate that Pol IIa becomes rapidly phosphorylated and fully converted into Pol IIo in response to Top1cc's.

Because Top1cc's are reversible,[27] we examined Pol II phosphorylation following CPT removal. After termination of the CPT treatment, Pol II hyperphosphorylation returned to its baseline levels (Fig. 1d) as Top1cc's reversed (Fig. 1e). Thus, Pol II hyperphosphorylation is closely associated with the presence of Top1cc's.

Top1cc-induced Pol II hyperphosphorylation is mediated by Cdks

To determine whether Pol II hyperphosphorylation is related to its normal phosphorylation by Cdks,[1,28]

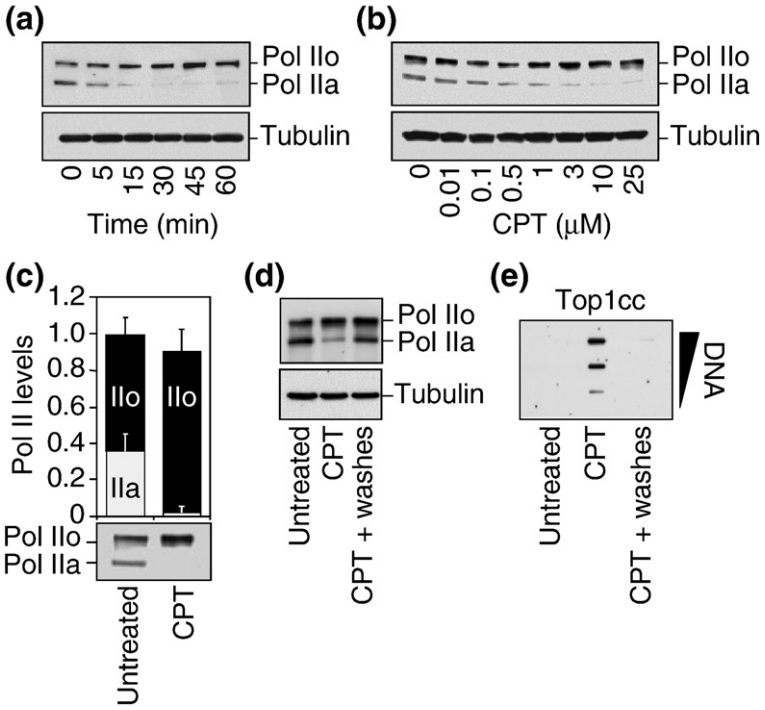

Fig. 1. CPT induces Pol II hyperphosphorylation. (a and b) CPT induces time- and concentration-dependent hyperphosphorylation of Pol II. Western blotting analyses of Pol II in whole extracts from HCT116 cells treated for the indicated times with 25 μM CPT (a) and for the indicated concentrations of CPT for 1 h (b). Pol IIa (hypophosphorylated CTD) and Pol IIo (hyperphosphorylated CTD) forms are indicated. Tubulin was used as a loading control. (c) The bottom panel shows representative Western blotting of Pol II in HCT116 cells treated with 25 μM CPT for 1 h. The top panel shows quantification of the data shown in the bottom panel (mean±SD of five independent experiments). Pol IIa is shown in gray, and Pol IIo is shown in black. (d and e) HCT116 cells were treated with 25 μM CPT for 1 h, washed, and cultured in CPT-free medium for 1 h. (d) Western blotting analyses of Pol II. (e) Detection of cellular Top1cc's by slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μg of DNA) with an antibody against Top1.

we examined whether CPT-induced Pol II hyperphosphorylation would be prevented by the commonly used Cdk inhibitors 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB)[29] and flavopiridol (FLV).[30] Figure 2a and b shows that both DRB and FLV prevented CPT-induced Pol II hyperphosphorylation. The suppressive effect of DRB and FLV was not due to a reduction of CPT-induced Top1cc's (Fig. 2c).

A well-known pathway by which Top1cc's activate cellular stress and checkpoint pathways is in

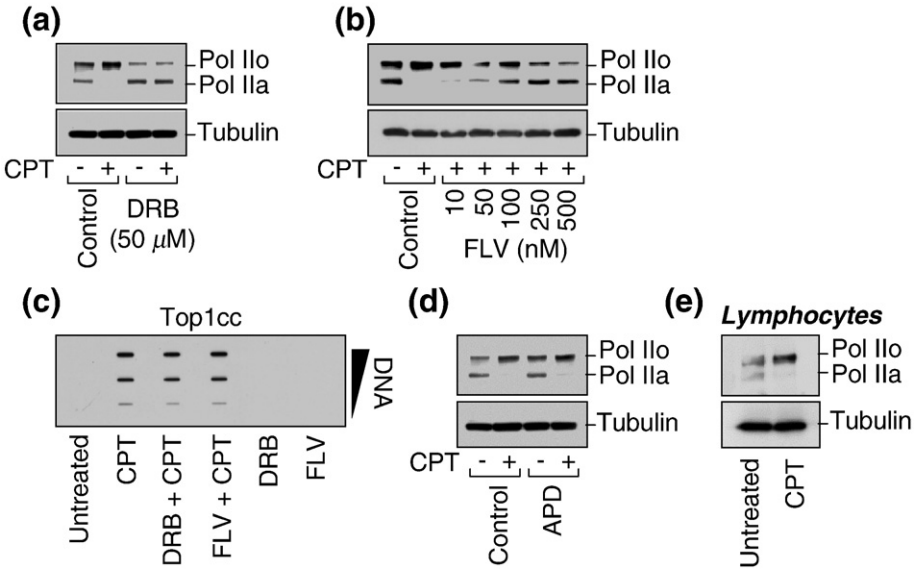

Fig. 2. Pol II hyperphosphorylation induced by Top1cc's is prevented by Cdk inhibitors. (a and b) The Cdk inhibitors DRB and FLV both prevent Pol II hyperphosphorylation. Western blotting analyses of Pol II in HCT116 cells treated for 1 h with 50 μM DRB (a) or with the indicated concentrations of FLV (b) before the addition of CPT (25 μM, 1 h). Pol IIa (hypophosphorylated CTD) and Pol IIo (hyperphosphorylated CTD) forms are indicated. Tubulin was used as a loading control. (c) DRB (50 μM) and FLV (500 nM) do not affect the induction of Top1cc's by CPT. HCT116 cells were treated as in (a) and (b), and Top1cc's were detected by slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μg of DNA) with an antibody against Top1. (d) The DNA polymerase inhibitor APD does not prevent CPT-induced Pol II hyperphosphorylation. HCT116 cells were treated with 1 μM APD for 15 min before the addition of CPT (25 μM, 1 h). (e) CPT (25 μM, 1 h) induces Pol II hyperphosphorylation in nonreplicating primary lymphocytes.

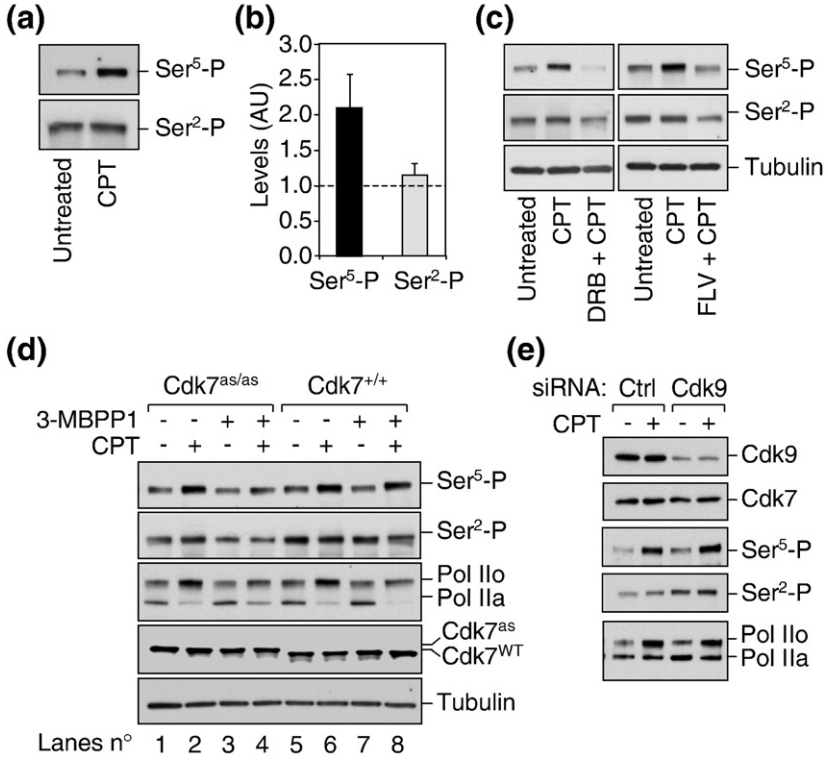

Fig. 3. Top1cc's induce Cdk7-mediated Pol II CTD phosphorylation at residue serine 5. (a) Western blot analysis of Pol II phosphorylated at serine 5 (Ser5-P) and that at serine 2 (Ser2-P) in HCT116 cells treated with 25 μM CPT for 1 h. (b) Quantification of the data shown in (a) (mean±SD of three independent experiments). (c) The Cdk inhibitors DRB and FLV both prevent CPT-induced Ser5-P. Western blotting analyses of Ser5-P and Ser2-P in HCT116 cells treated for 1 h with DRB (50 μM) or FLV (0.5 μM) before the addition of CPT (25 μM, 1 h). Tubulin was used as a loading control. (d) Inhibition of Cdk7 decreases Ser5-P and Pol II hyperphosphorylation. Cdk7 was inhibited in *Cdk7*$^{as/as}$ HCT116 cells by a 1-h incubation with 1 μM 3-MBPP1. Then, cells were treated with 25 μM CPT for 1 h and assayed for Ser5-P, Ser2-P, and Pol II by Western blotting. Pol IIa (hypophosphorylated CTD) and Pol IIo (hyperphosphorylated CTD) forms are indicated. Wild-type HCT116 cells (*Cdk7*$^{+/+}$) were examined in parallel. Cdk7^{as} expression can be distinguished from Cdk7^{WT} expression because of its decreased electrophoretic mobility.[26] (e) Knocking down Cdk9 does not prevent CPT-induced Ser5-P and Pol II hyperphosphorylation. HCT116 cells were transfected with a duplex siRNA against Cdk9 (siRNA-Cdk9) or a negative control sequence (siRNA-Ctrl), treated for 1 h with 25 μM CPT and assayed for Cdk9, Cdk7, Ser5-P, Ser2-P, and Pol II by Western blotting.

response to replication-induced DNA double-strand breaks.[18] To examine whether replication-induced DNA double-strand breaks induced Pol II hyperphosphorylation, we used the DNA polymerase inhibitor aphidicolin (APD), which prevents CPT-induced replication-dependent DNA double-strand breaks.[31] Figure 2d shows that APD did not affect CPT-induced Pol II hyperphosphorylation. In accordance with this observation, Pol II hyperphosphorylation was also induced by CPT in nonreplicating human lymphocytes isolated from peripheral blood (Fig. 2e). Together, the abovementioned experiments indicate that Pol II is hyperphosphorylated, most likely by Cdks, independent of DNA replication.

Cdk7 contributes to Top1cc-mediated Pol II hyperphosphorylation

To gain insight into which kinases were involved in Pol IIo formation, we determined which residues in the heptapeptide repeats of the Pol II CTD were phosphorylated. In a prevalent model of the transcription cycle,[1] Pol II is initially phosphorylated at Ser5 by Cdk7. As Pol II leaves the proximal end of the gene, it becomes phosphorylated at Ser2 by Cdk9. Toward the 3′-end of the gene, Pol II tends to lose its Ser5-P and to be mainly phosphorylated at Ser2. Using phospho-specific antibodies, we found that CPT induced DRB- and FLV-sensitive Ser5-P, while it did not affect levels of Ser2-P (Fig. 3a–c). These results, which were reproducible with antibodies from different sources, suggest that Cdk7 is involved in Top1cc-induced Pol II hyperphosphorylation.

To assess directly the involvement of Cdk7, we used HCT116 cells expressing an analog-sensitive (as) mutant version of Cdk7 (*Cdk7*$^{as/as}$ cells).[26] This mutation allows Cdk7 to be specifically and quickly inhibited by ATP analogs without affecting other Cdks.[26] In *Cdk7*$^{as/as}$ HCT116 cells, inhibition of Cdk7 with the ATP analog 3-MBPP1 reduced CPT-induced Ser5-P and Pol II hyperphosphorylation (Fig. 3d, compare lanes 2 and 4). By contrast, knocking down Cdk9 by small interfering RNA (siRNA) did not prevent CPT-induced Ser5-P and Pol II hyperphosphorylation (Fig. 3e). From these results, we conclude that Cdk7 participates in Pol II hyperphosphorylation in response to Top1cc's. CPT-induced Ser5-P and Pol II hyperphosphorylation were also prevented in Top1-deficient cells (Supplementary Fig. 2), which is consistent with Top1cc's being the initiating events for the selective hyperphosphorylation of Pol II at Ser5.

Pol II hyperphosphorylation results from steady-state phosphorylation–dephosphorylation

Next, we examined whether Pol IIo arrested by Top1cc's was still subjected to cycles of phosphorylation and dephosphorylation. To answer this question, we tested the ability of the Cdk inhibitors (FLV

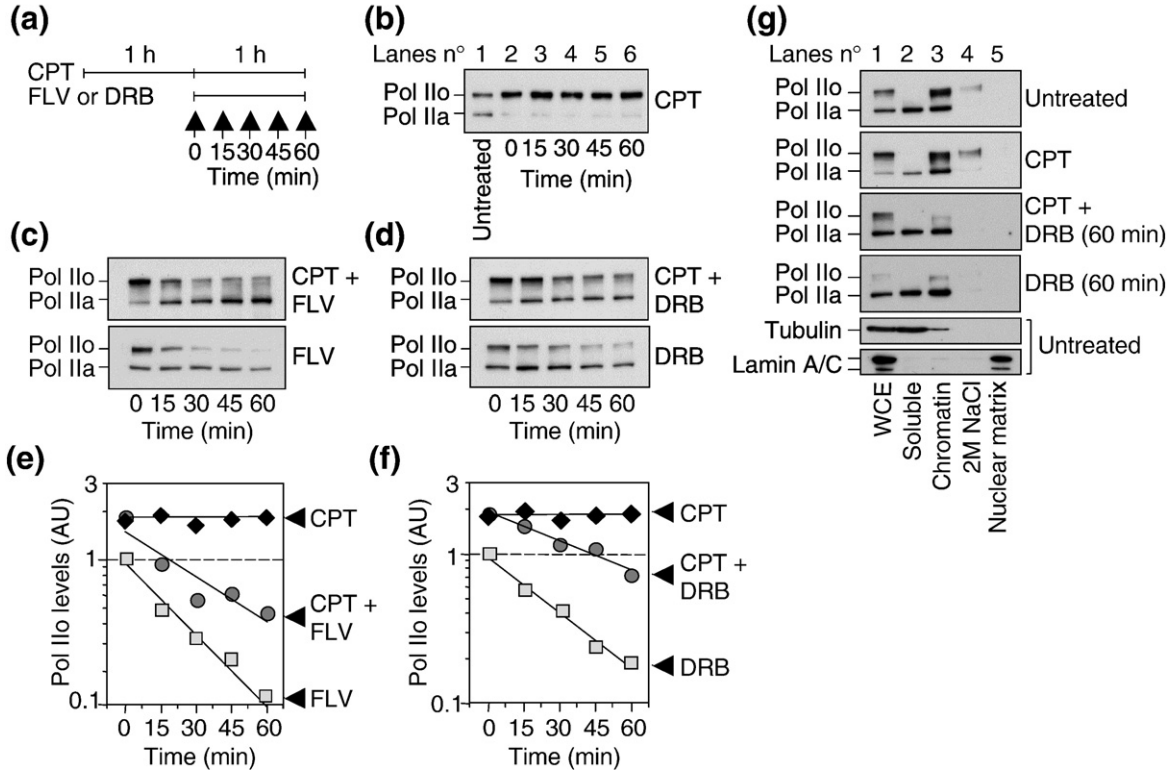

Fig. 4. Pol II dephosphorylation in CPT-treated cells. (a) Cell treatment protocol. HCT116 cells were treated with 25 μM CPT for 1 h prior to and during the FLV (0.5 μM) or DRB (50 μM) exposure for the indicated times. Arrowheads indicate sampling times. (b–d) Western blotting analyses of Pol II in whole cell extracts. Pol IIa (hypophosphorylated CTD) and Pol IIo (hyperphosphorylated CTD) forms are indicated. (e) Quantification of Pol IIo data shown in (b) and (c). (f) Quantification of Pol IIo data shown in (b) and (d). Dashed line indicates Pol IIo levels in untreated cells. Diamonds, CPT; circles, CPT + FLV in (e) and CPT + DRB in (f); squares, FLV in (e) and DRB in (f). (g) Cells were subjected to fractionation (see Materials and Methods). Proteins from whole cell extract (*WCE*), 0.5% Triton X-100 supernatant (*Soluble*), DNase I and ammonium sulfate supernatant (*Chromatin*), 2 M NaCl supernatant (*2 M NaCl*), and pellet (*Nuclear matrix*) that were prepared from equivalent cell numbers were immunoblotting with the indicated antibodies. Tubulin and lamin A/C were used as controls for the *Soluble* and *Nuclear matrix* fractions, respectively.

and DRB) to cause dephosphorylation of Pol II while it was arrested by trapped Top1cc's (see protocol in Fig. 4a).

Pol II that was fully hyperphosphorylated after 1 h of CPT exposure (Fig. 4b, compare lanes 1 and 2) remained hyperphosphorylated for an additional hour as CPT was kept in the cell culture (Fig. 4b, lanes 3–6). However, addition of FLV while CPT was kept in the culture led to a rapid dephosphorylation of Pol II, demonstrated by the decrease of Pol IIo levels together with the increase of Pol IIa levels within minutes (Fig. 4c). Quantitative analyses showed that the kinetics of Pol II dephosphorylation by FLV in CPT-treated cells was similar to that of cells that were not exposed to CPT (Fig. 4e), indicating that CPT does not inhibit the dephosphorylation of Pol II. Similar results were obtained with DRB (Fig. 4d and f).

To investigate whether the cycles of phosphorylation and dephosphorylation are related to abortive cycles of transcription, we examined the binding of Pol II to chromatin. At the end of normal transcription cycles, Pol II is released from the DNA and dephosphorylated to recycle the polymerase to an initiating form.[1] Cellular fractionation experiments show that, upon CPT treatment, there are a decrease in soluble Pol II and an increase in chromatin-bound Pol II (chromatin and 2 M salt fractions) (Fig. 4g), suggesting that Top1cc's trigger the accumulation of Pol II complexes tightly bound to chromatin. By contrast, dephosphorylation of Pol II by addition of DRB during CPT treatment induced a decrease in chromatin-bound Pol II with an increase in soluble Pol II (Fig. 4g). These experiments suggest that transcribing Pol II arrested by trapped Top1cc's can dissociate from chromatin upon DRB treatment.

Transcription-induced Top1 degradation is dependent on Brca1

Top1cc-mediated stalling of Pol II complexes has been shown to induce Top1 degradation as demonstrated by lack of Top1 degradation in cells treated with DRB during CPT exposure.[20] By contrast, the DNA polymerase inhibitor APD did not impede CPT-induced Top1 degradation.[20] Thus, similar to Pol II hyperphosphorylation (Fig. 2), Top1 degradation is selectively transcription dependent.[20] Because the DNA damage-responsive protein Brca1 is associated

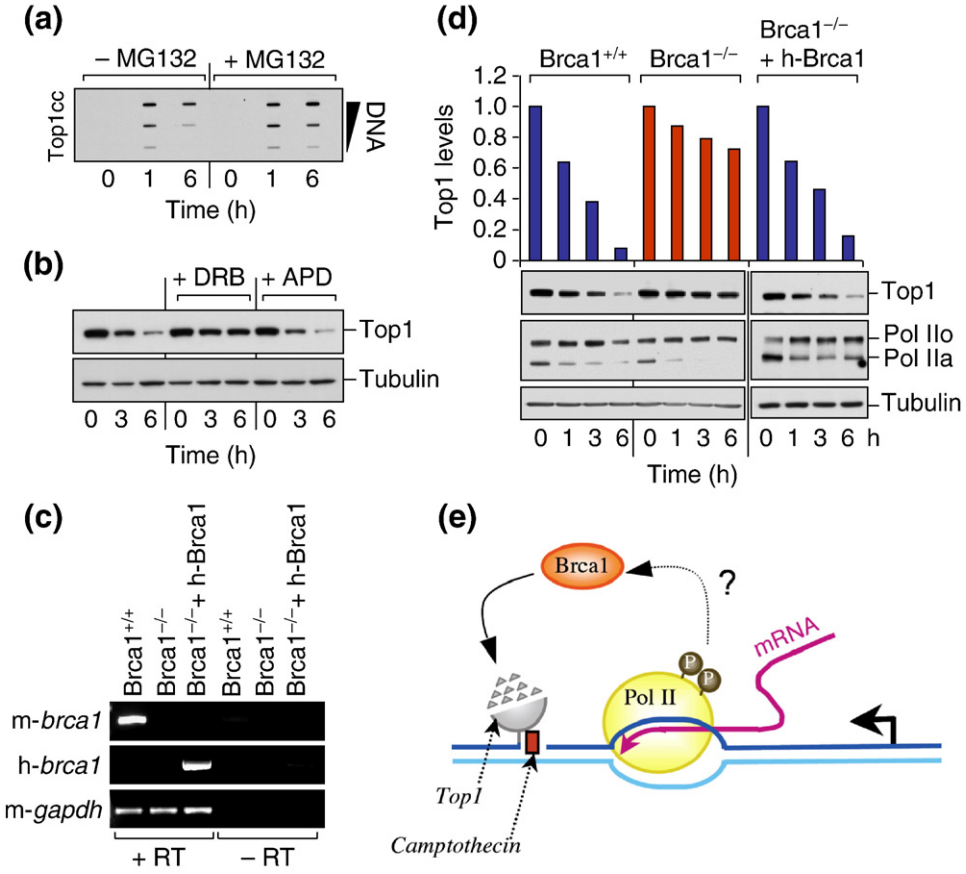

Fig. 5. Top1cc's induce Brca1-dependent degradation of Top1 without substantial degradation of Pol II. (a) CPT induces proteosomal degradation of Top1 in MEF cells. Cells were treated with the proteasome inhibitor MG132 (50 μM) for 1 h before the addition of CPT (25 μM) for the indicated times. Top1cc's were detected by slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μg of DNA) with an antibody against Top1. (b) CPT-induced Top1 degradation is transcription dependent. Western blotting analysis of Top1 in MEF cells treated for 1 h with the transcription inhibitor DRB (100 μM) or for 15 min with the replication inhibitor APD (1 μM) before the addition of CPT (25 μM) for the indicated times. Tubulin was used as a loading control. (c) Total RNAs were purified from MEF cells of each genotype (*Brca1*$^{+/+}$, *Brca1*$^{-/-}$, and *Brca1*$^{-/-}$ complemented with human *Brca1*), and the indicated transcripts were examined by RT-PCR. Right lanes (−RT) show the absence of DNA contamination in the RNA samples. m, mouse; h, human. (d) MEF cells of the indicated genotype were treated with 25 μM CPT for the indicated times. Western blotting analyses of Top1 and Pol II. Pol IIa (hypophosphorylated CTD) and Pol IIo (hyperphosphorylated CTD) forms are indicated. Tubulin was used as a loading control. The top panel (d) shows quantification of Top1 expression shown. (e) Model showing Brca1-dependent degradation of Top1 in response to Pol IIo arrested by a Top1cc.

with the elongating Pol II[32] and possesses ubiquitin E3 ligase activity,[33] we examined the possibility that Brca1 could promote Top1 degradation. As expected,[20] CPT induced proteosomal degradation of Top1 in MEF cells (Fig. 5a) in a transcription-dependent manner (Fig. 5b). Then, we examined Top1 degradation in MEF cells of each genotype: *Brca1*$^{+/+}$, *Brca1*$^{-/-}$, and *Brca1*$^{-/-}$ complemented with human *Brca1* (Fig. 5c). Figure 5d shows that Top1 degradation is reduced in *Brca1*$^{-/-}$ cells as compared with *Brca1*$^{+/+}$ cells and *Brca1*$^{-/-}$ cells complemented with human *Brca1*.

CPT-induced Top1 degradation was not associated with Pol II degradation as neither *Brca1*-proficient nor *Brca1*$^{-/-}$-deficient cells showed significant Pol II degradation (Fig. 5d). Experiments performed with cancer cells that degrade Top1 (DU145 cells) or not (HCT116 cells) following CPT treatment[20,34] confirmed the uncoupling between Top1 and Pol II degradation (Supplementary Fig. 3). Together, these experiments indicate that transcription-induced Top1 degradation is Brca1 dependent and is independent of Pol II degradation.

Discussion

Although Top1 is critical for transcription,[10,11,19,35–38] our understanding regarding the molecular processes involved in and resulting from the stalling of transcription complexes by Top1cc's is currently limited.

Our finding that Top1cc's selectively increase Ser5-P without affecting Ser2-P is consistent with results of studies showing that Top1cc's can block elongation while stimulating initiation of Pol II transcription.[36,39] Our analyses using *Cdk7*$^{as/as}$ HCT116

cells further demonstrate that Cdk7 hyperphosphorylates Pol II–Ser5 in response to Top1cc's (Fig. 3). Other Cdks are likely involved as complete and selective inhibition of Cdk7 kinase activity in *Cdk7*$^{as/as}$ HCT116 cells[26] only partially prevented Pol II–Ser5-P (Fig. 3d), whereas the pan-Cdk inhibitors DRB and FLV completely abrogated Pol II–Ser5-P (Fig. 3c). Besides Cdk7, Cdk9 (and Cdk8) can phosphorylate Pol II at residue Ser5,[40] and DRB and FLV are somewhat more selective for Cdk9 than for Cdk7.[29,30] However, downregulation of Cdk9 by siRNA failed to reduce CPT-induced Ser5-P (Fig. 3e). It is possible that the remaining pool of Cdk9 after siRNA is sufficient to phosphorylate Ser5.[41] In line with this possibility, downregulation of Cdk9 by siRNA did not decrease steady-state levels of Ser2-P (Fig. 3e), although it is possible that Ser2-P can be compensated by other kinases. Likewise, inhibition of Cdk7 did not decrease steady-state levels of Ser5-P, as shown previously,[26] suggesting that other kinases can compensate to maintain those levels under normal growth conditions. Nevertheless, our results demonstrate that increased Ser5-P in response to transcription arrest by Top1cc's involves Cdk7 activity.

The absence of Ser2-P in response to Top1cc's might protect Pol II from proteosomal degradation as Ser2-P (but not Ser5-P) signals Pol II for ubiquitinylation and subsequent degradation.[9] In contrast to Top1cc's, UV light leads to the degradation of Pol II phosphorylated at Ser2.[9] Cisplatin[8] and hydrogen peroxide[7] also induce Pol II degradation. In response to Top1cc's, instead of being degraded, Pol II is subjected to phosphorylation–dephosphorylation cycles. Our kinetic experiments in the presence of Cdk inhibitors (Fig. 4) suggest that Top1cc's do not significantly affect the Pol II dephosphorylation rate. Thus, the net increase of Pol II phosphorylation is likely to be due to increased kinase rather than decreased phosphatase activity. Our cellular fractionation analyses suggest that the cycles of phosphorylation and dephosphorylation could correspond to abortive cycles of transcription in response to CPT. Indeed, in the presence of CPT, DRB was able to induce both the dephosphorylation of Pol II and its release from chromatin (Fig. 4g). Therefore, it is possible that Pol II arrested by trapped Top1cc's is prematurely released from the DNA and then recycled to reinitiate transcription. In which case, unfinished RNAs could be released after such abortive transcription cycles. In line with this possibility, it has been shown that CPT triggers the accumulation of 5′-end abortive RNA.[36]

The differential response of Pol II to Top1cc's compared with other types of DNA lesions (UV light, cisplatin, hydrogen peroxide) is plausible considering that the mechanisms of Pol II arrest are different. In the case of UV light, Pol II is directly arrested at cyclobutane pyrimidine dimer sites.[42] In the case of Top1cc's, several interference mechanisms can be envisaged. First, transcription complexes may be blocked physically a few base pairs upstream of the Top1cc's.[22,23] Second, enzymatic inactivation of Top1 may lead to an accumulation of DNA torsional stress.[10,11] Consequently, positive supercoiling ahead of the transcribing Pol II could inhibit Pol II translocation along the chromatin template.[24] Also, negative supercoiling behind the transcription complex could promote the formation of R-loops (DNA:RNA hybrids),[43] which are known to impair transcriptional elongation.[44] However, CPT seems to preferentially inhibit Top1-mediated relaxation of positive *versus* negative DNA supercoiling.[14,45] Finally, CPT may also interfere with RNA splicing by blocking the SR kinase activity of Top1,[46] which would promote the formation of R-loops[47] and therefore impair transcriptional elongation.[44,48]

Our study (Fig. 5b) and previous reports[20,34] indicate that Top1 degradation is transcription dependent following CPT exposure. However, it is still unclear whether Top1 is degraded directly on DNA or is first removed from the DNA and then degraded. It has been reported that Top1 degradation was defective in most cancer cells as compared with normal cells,[34] but this differential response has remained unexplained. Our finding that Top1 degradation is reduced in Brca1-deficient cells (Fig. 5d) suggests that the Brca1–Bard1 (Brca1-associated RING domain 1) complex, which possesses ubiquitin E3 ligase activity,[33] might be involved in the transcription-induced degradation of Top1 (see scheme in Fig. 5e). It is therefore possible that the lack of Top1 degradation in cancer cells[34] could be related to Brca1-inactivating mutations that occur frequently during tumorigenesis.[49] Other parallel pathways besides Brca1 probably also contribute to Top1 degradation as *Brca1*$^{-/-}$ cells are not completely defective in Top1 degradation (Fig. 5d, top panel). Top1 degradation has been suggested to promote resumption of RNA synthesis,[20] although it remains unclear whether it is mediated by the previously stalled elongating Pol II complex or after reinitiation of a new Pol II complex at the promoter. In accord with the role of Brca1 in promoting Top1 degradation, Brca1 also stimulates resumption of RNA synthesis following DNA damage[50] and Brca1-deficient cells are hypersensitive to CPT.[51] Further studies will be required to determine how transcription-blocking Top1cc's lead to Brca1-dependent degradation of Top1.

In conclusion, our study demonstrates that Top1cc's are remarkably efficient at inducing cycles of Pol II phosphorylation–dephosphorylation. Such transcriptional response appears unique to Top1cc's as other bulky adducts, such as those induced by UV light and cisplatin, trigger Pol II degradation rather than sustained phosphorylation. Moreover, we show that Top1cc-mediated Pol II arrest leads to Brca1-dependent degradation of Top1. Understanding the transcriptional response to Top1cc's is of particular interest as the CPT derivatives irinotecan and topotecan are used to treat human cancers.[18] Moreover, transcriptional responses to Top1cc's may occur spontaneously under normal conditions as Top1cc's are readily stabilized by endogenous DNA alterations,[15,52,53] which may lead, if not repaired, to neurological diseases such as SCAN1 (spinocerebellar ataxia with axonal neuropathy).[54]

Materials and Methods

Drugs and antibodies

The Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health (NIH), provided CPT and FLV. APD, DRB, cycloheximide, and z-Leu-Leu-Leu-al (MG132) were obtained from Sigma (St. Louis, MO). The ATP analog 3-MBPP1 was provided by Dr. Kevan M. Shokat (University of California, San Francisco, CA). The following antibodies were used: Pol II (clone N20), Ser5-P Pol II (clone 8A7), Cdk7 (clone C4), Cdk9 (clone C20), and lamin A/C (clone 346) from Santa Cruz Biotechnology (Santa Cruz, CA); Ser2-P Pol II (clone H5) from Covance (Emeryville, CA); tubulin (Ab-4) from NeoMarkers (Fremont, CA); and Top1 (C21), which was a gift from Dr. Yung-Chi Cheng (Yale University, New Haven, CT). The results in Fig. 3a were confirmed with other phosphoserine antibodies against Pol II: Ser5-P (clone H14) from Covance and Pol II Ser2-P (ab5095) from Abcam (Cambridge, MA) (data not shown).

Cell culture

The human colon carcinoma HCT116 cell line was obtained from the NCI Developmental Therapeutics Program (NIH, Bethesda, MD). *Cdk7*$^{as/as}$ HCT116 cells were generated as described previously.[26] MEF cells of each genotype (*Brca1*$^{+/+}$, *Brca1*$^{-/-}$, and *Brca1*$^{-/-}$ stably expressing human *Brca1*) were kind gifts from W.H. Lee (University of California, Irvine, CA). All the abovementioned cell lines were cultured in Dulbecco's modified Eagle's medium. Lymphocytes isolated from peripheral blood of healthy donors were provided by the NIH Blood Bank and maintained in RPMI 1640 medium.

Cell extracts and immunoblotting

Whole cell extracts were prepared by lysing cells in buffer containing 1% SDS, 1 mM sodium vanadate, and 10 mM Tris–HCl, pH 7.4, supplemented with protease inhibitors (Complete, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1, Sigma). Viscosity of the samples was reduced by brief sonication, and 20 μg of protein (Bio-Rad DC Protein Assay) was complemented with loading buffer (125 mM Tris–HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromphenol blue), separated by SDS polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA). After blocking nonspecific binding for 1 h with 5% milk in TPBS (phosphate-buffered saline, Tween 20 0.2%), membranes were incubated for 2 h with primary antibody. After three washes in TPBS, membranes were incubated with horseradish peroxidase-conjugated goat antirabbit or antimouse (1:10,000 dilution) antibody (GE Healthcare BioSciences, Piscataway, NJ) for 1 h and then washed three times in TPBS. Immunoblots were revealed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL) followed by autoradiography.

Cellular fractionation

Fractionation of cellular proteins was performed as described previously[55] with some modifications. HCT116 cells were extracted in cytoskeleton (CSK) buffer [10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid, and 0.5% Triton X-100] supplemented with 1 mM dithiothreitol, protease inhibitors (Complete, Roche Diagnostics), and phosphatase inhibitors (Cocktail 1, Sigma). After 10 min at 4 °C, the cytoskeletal frameworks were separated from soluble proteins by centrifugation at 7500*g* for 3 min. Chromatin was solubilized by DNA digestion with 0.1 mg/ml of DNase I in CSK buffer (containing 50 mM NaCl instead of 100 mM) for 30 min at room temperature. Then, ammonium sulfate was added to a final concentration of 0.25 M; after 5 min at 4 °C, the lysate was centrifuged again at 7500*g* for 3 min. This treatment released chromatin-associated proteins almost completely,[55] including the transcribing Pol IIo.[56] The remaining chromatin-associated proteins in the pellet were further extracted with 2 M NaCl in CSK buffer for 15 min at 4 °C and centrifuged at 20,000*g* for 15 min. The remaining pellet was solubilized in buffer containing 1% SDS, 1 mM sodium vanadate, and 10 mM Tris–HCl, pH 7.4, supplemented with protease and phosphatase inhibitors, and was considered the nuclear matrix-containing fraction.

Detection of cellular Top1cc's

Top1cc's were detected using the ICE (*in vivo* complex of enzyme) bioassay.[57] Briefly, cells were lysed in 1% Sarkosyl and homogenized with a Dounce homogenizer. The cell lysates were centrifuged on cesium chloride step gradients at 165,000*g* for 20 h at 20 °C. Twenty 0.5-ml fractions were collected and diluted (v/v) into 25 mM potassium phosphate buffer, pH 6.6. The DNA-containing fractions (fractions 7–11) were pooled and applied to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a slot-blot vacuum manifold. Top1cc's were detected by immunoblotting.

Cdk9 silencing by RNA interference

Cdk9 expression was knocked down by transfection with an siRNA duplex (Qiagen, Valencia, CA) against the sequence CCACGACTTCTTCTGGTCC of the Cdk9 mRNA.[58] Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. A negative control siRNA duplex from Qiagen (target DNA sequence: TTCTCCGAACGTGTCACGT) was used. The experiments were carried out 72 h after transfection.

Reverse transcription PCR

RNAs (250 ng) from MEF cells of each genotype (*Brca1*$^{+/+}$, *Brca1*$^{-/-}$, and *Brca1*$^{-/-}$ stably expressing human *Brca1*) were subjected to reverse transcription (RT) PCR (Onestep RT-PCR kit, Qiagen). The primers used are mouse-*brca1*-FW, 5′-AAC AGC CTG GCA TAG CAG TGA GCC A-3′ and mouse-*brca1*-RV, 5′-TTG CGG GTG AGT CCA CTT CTC TCT A-3′; human-*brca1*-FW, 5′-CAG CTT GAC ACA GGT TTG GAG-3′ and human-*brca1*-RV, 5′-GGC ATG AGT ATT TGT GCC AC-3′; and mouse-*gapdh*-FW, 5′-TCA CCA TCT TCC AGG AGC-3′ and mouse-*gapdh*-RV, 5′-CTG CTT CAC CAC CTT CTT GA-3′.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.06.028

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