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RNA polymerase II is Hyperphosphorylated in Response to Topoisomerase I-DNA Cleavage Complexes and is Associated with Transcription- and BRCA1-Dependent Degradation of Topoisomerase I

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SUMMARY

The progression of RNA polymerase II can be blocked by lesions on the DNA template. In this study, we focused in 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 and its derivatives used in cancer treatment. We find that, within a few minutes, camptothecin produces the complete hyperphosphorylation of Rpb1 in both primary and transformed cancer cells.

Hyperphosphorylation is rapidly reversible following camptothecin 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 TFIIH-associated kinase, Cdk7.

Hyperphosphorylated Rpb1 is not primarily targeted for proteosomal degradation, but instead is subjected to cycles of phosphorylation and dephosphorylation as long as Top1 cleavage complexes are trapped by camptothecin. 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

INTRODUCTION

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II, Rpb1, (Pol II) contains an evolutionary conserved and repeated heptapeptide, Tyr¹-Ser²-Pro³-Thr⁴-Ser⁵-

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Pro⁶-Ser⁷, which is subjected to reversible phosphorylations during each transcription cycle. ¹ Hypophosphorylated Pol II-CTD (Pol II_a) corresponds to the transcriptionally inactive Pol II, whereas hyperphosphorylated Pol II-CTD (Pol II_o) is the transcriptionally active Pol II. A variety of kinases have been identified, with phosphorylation activity directed toward the amino acids tyrosine-1 (Abl1/2), serine-2 (Cdk9, 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 TFIIF (Cdk7) and P-TEFb (Cdk9), respectively. The kinase(s) involved in serine-7 phosphorylation (Ser⁷-P) remains to be identified. Dephosphorylation of Ser⁵ is preferentially carried out by SSU72 and SCPs (small CTD phosphatases), whereas FCP1 (TFIIF-associating CTD-phosphatase-1) dephosphorylates both Ser² and Ser⁵.¹ While Ser⁵-P peaks early in the transcription cycle and decreases towards the 3'-end of the gene, Ser²-P predominates in the body and towards 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-induced pyrimidine dimers, carcinogenic adducts, adducts induced by anticancer drugs, DNA-protein crosslinks, oxidative DNA lesions, abasic sites and DNA strand breaks. ³ Transcription-blocking DNA lesions are primarily repaired by transcription-coupled repair (TCR), a specialized nucleotide-excision repair (NER) pathway elicited by transcription-blocking lesions located on the transcribed strand.^{4,5} In order to repair the DNA, two main mechanisms exist to displace the Pol II-DNA complexes: Pol II backtracking or Pol II degradation. Pol II backtracking enables the DNA repair complexes to access the damaged sites, after which transcription elongation resume to finish the transcript.⁶ In the second mechanism, Pol II is removed following ubiquitinylation and proteosomal degradation⁷⁻⁹ 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), 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 it can relax DNA on both sides of transcription complexes. However, Top1 seems more efficient at removing positive DNA supercoiling, but also tends to form more cleavage complexes in positively versus negatively supercoiled DNA.¹⁴ The rapid resealing of Top1cc 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 can also be trapped with exquisite selectivity by camptothecin (CPT), a plant alkaloid whose semi-synthetic 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 reversibly. Indeed, the Top1cc reverse fully within minutes after washing out CPT. Trapped Top1cc are potent transcription-blocking DNA lesions.¹⁹⁻²¹ Transcription complexes may be blocked physically a few base pair upstream of the Top1cc,^{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 are removed following ubiquitinylation and proteosomal degradation of Top1.²⁰

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

RESULTS

Top1 cleavage complexes induce rapid, complete and reversible Pol II hyperphosphorylation

Hyperphosphorylated Pol II (Pol Ilo) can be distinguished from hypophosphorylated Pol II (Pol Ila) because of its reduced electrophoretic mobility in SDS polyacrylamide gels.²⁵ Under normal conditions, both Pol Ilo and Pol Ila can be detected (Fig. 1a, left lane). In contrast, Top1cc stabilization by CPT produced a rapid disappearance of Pol Ila with coincident increase of Pol Ilo (Fig. 1a and b). Within 30 min of CPT treatment, Pol II became completely hyperphosphorylated (Ilo form; Fig. 1a). Quantitatively, the increase in Pol Ilo corresponded to the disappearance of Pol Ila (Fig. 1c). Experiments performed in the presence of inhibitors of the proteasome (MG132) or other proteases (TPCK and thimerosal) confirmed that the decrease of Pol Ila was not due to its degradation (Supplementary Fig. 1a). Moreover, experiments performed with cycloheximide (CHX) demonstrated that the increased Pol Ilo 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 (Fig. 1–Fig. 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 Ila becomes rapidly phosphorylated and fully converted into Pol Ilo in response to Top1cc.

Because Top1cc are reversible,²⁶ 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 reversed (Fig. 1e). Thus, Pol II hyperphosphorylation is closely associated with the presence of Top1cc.

Top1cc-induced Pol II hyperphosphorylation is mediated by cyclin-dependent kinases

To determine whether Pol II hyperphosphorylation is related to its normal phosphorylation by Cdk, ^{1,27} 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)²⁸ and flavopiridol (FLV).²⁹ 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 (Fig. 2c).

A well-known pathway by which Top1cc activate cellular stress and checkpoint pathways is in 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, which prevents CPT-induced replication-dependent DNA double-strand breaks.³⁰ Figure 2d shows that aphidicolin did not affect CPT-induced Pol II hyperphosphorylation. In accordance with this observation, Pol II hyperphosphorylation was also induced by CPT in non-replicating human lymphocytes isolated from peripheral blood (Fig. 2e). Together, the above experiments indicate that Pol II is hyperphosphorylated, most likely by Cdk, independent of DNA replication.

Cdk7 contributes to Top1cc-mediated Pol II hyperphosphorylation

To gain insight into which kinases were involved in Pol Ilo 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 phosphorylation 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).³¹ This mutation allows Cdk7 to be specifically and quickly inhibited by ATP analogs without affecting other Cdk7s.³¹ 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 siRNA did not prevent CPT-induced Ser⁵ phosphorylation and Pol II hyperphosphorylation (Fig. 3e). From these results, we conclude that Cdk7 participates in Pol II hyperphosphorylation in response to Top1cc. CPT-induced Ser⁵-P and Pol II hyperphosphorylation were also prevented in Top1-deficient cells (Supplementary Fig. 2), which is consistent with Top1cc 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 hyperphosphorylated Pol II arrested by Top1cc was still subjected to cycles of phosphorylation and dephosphorylation. To answer this question, we tested the ability of the Cdk inhibitors (FLV and DRB) to cause dephosphorylation of Pol II while it was arrested by trapped Top1cc (see protocol in Fig. 4a).

Fully hyperphosphorylated Pol II after 1 h 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 to 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 II_o levels together with the increase of Pol II_a 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 is 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 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 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 aphidicolin 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 with the elongating Pol II³² 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 to *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 is currently limited regarding the molecular processes involved in and resulting from the stalling of transcription complexes by Top1cc.

Our finding that Top1cc selectively increase Ser⁵-P without affecting Ser²-P is consistent with studies showing that Top1cc 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 (Fig. 3). Other Cdk7s are likely involved as complete and selective inhibition of Cdk7 kinase activity in *Cdk7*^{as/as} HCT116 cells³¹ 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 Ser⁵40 and DRB and FLV are somewhat more selective for Cdk9 than for Cdk7.^{28,29} 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,³¹ 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 involves Cdk7 activity.

The absence of Ser²-P in response to Top1cc might protect Pol II from proteasomal degradation as Ser²-P (but not Ser⁵-P) signals Pol II for ubiquitinylation and subsequent degradation.⁹ In contrast to Top1cc, UV leads to the degradation of Pol II phosphorylated at Ser².⁹ Cisplatin⁸ and hydrogen peroxide⁷ also induce Pol II degradation. In response to Top1cc, 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 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 is prematurely released from the DNA and then recycled to re-initiate 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 compared to other types of DNA lesions (UV, cisplatin, hydrogen peroxide) is plausible considering that the mechanisms of Pol II arrest are different. In the case of UV, Pol II is directly arrested at cyclobutane pyrimidine dimer sites.

⁴² In the case of Top1cc, several interference mechanisms can be envisaged. First, transcription complexes may be blocked physically a few base pair upstream of the Top1cc.^{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-loops⁴⁷ and therefore also impair transcriptional elongation.^{44,48}

Our study (Fig. 5b) as well as 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 whether it is first removed from the DNA and then degraded. It has been reported that Top1 degradation was defective in most cancer cells as compared to normal cells³⁴ 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 cells³⁴ 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 damage⁵⁰ and Brca1-deficient cells are hypersensitive to CPT.⁵¹ Further studies will be required to determine how transcription-blocking Top1cc lead to Brca1-dependent degradation of Top1.

In conclusion, our study demonstrates that Top1cc are remarkably efficient at inducing cycles of Pol II phosphorylation-dephosphorylation. Such transcriptional response appears unique to Top1cc as other bulky adducts such as those induced by UV 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 is of particular interest as the CPT derivatives irinotecan and topotecan are used to treat human cancers.¹⁸ Moreover, transcriptional responses to Top1cc may occur spontaneously under normal conditions as Top1cc are readily stabilized by endogenous DNA alterations,^{15,52,53} which may lead, if not repaired, to neurological diseases such as SCAN1.⁵⁴

MATERIALS AND METHODS

Drugs and Antibodies

The Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, National Institutes of Health (NIH), provided camptothecin (CPT) and flavopiridol (FLV). Aphidicolin (APD), 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB), cycloheximide (CHX), and α -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) was a gift from Dr. Yung-Chi Cheng (Yale University, New Haven, CT). The results in Fig. 3a were confirmed with other phospho-serine 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 Developmental Therapeutics Program (NCI, NIH, Bethesda, MD). *Cdk7^{as/as}* HCT116 cells were generated previously as described.³¹ 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 above cells lines were cultured in Dulbecco's modified Eagle's medium. Lymphocytes isolated from peripheral blood of healthy donors were provided by the Blood Bank at the NIH and maintained in RPMI 1640 medium.

Cell extracts and immunoblotting

Whole cell extracts were prepared by lysing cells at in buffer containing 1% SDS, 1 mM sodium vanadate, 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) were complemented with loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol 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 anti-rabbit or anti-mouse (1:10,000 dilution) antibody (GE Healthcare BioSciences, Piscataway, NJ) for 1 h and then washed three times in TPBS. Immunoblots were revealed using enhanced chemiluminescence detection kit (Pierce, Rockford, IL) followed by autoradiography.

Cellular fractionation

Fractionation of cellular proteins was performed as described⁵⁵ with some modifications. HCT116 cells were extracted in cytoskeleton buffer (CSK) (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100) supplemented with 1 mM dithiothreitol, protease inhibitors (Complete; Roche Diagnostics) and phosphatase inhibitors (Cocktail I; Sigma). After 10 min at 4°C, the cytoskeletal frameworks were separated from soluble proteins by centrifugation at 7,500 × g for 3 min. Chromatin was solubilized by DNA digestion with 0.1 mg/ml DNase I in CSK buffer (containing 50 mM NaCl instead of 100 mM) for 30 min at RT. Then, ammonium sulfate was added to a final concentration of 0.25 M and, after 5 min at 4°C, the lysate was centrifuged again at 7,500 × 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, 10 mM Tris-HCl, pH 7.4, supplemented with protease and phosphatase inhibitors and was considered the nuclear matrix-containing fraction.

Detection of Cellular Top1 Cleavage Complexes

Top1cc were detected using the *in vivo* complex of enzyme (ICE) 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 (vol/vol) 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, MA) using a slot-blot vacuum manifold. Top1cc 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 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'; mouse-*gapdh*-FW, 5'-TCA CCA TCT TCC AGG AGC-3' and mouse-*gapdh*-RV, 5'-CTG CTT CAC CAC CTT CTT GA-3'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations footnote

CPT, Camptothecin; CTD, Carboxy-terminal domain; Cdk, Cyclin-dependent kinase; FLV, Flavopiridol; Pol II, RNA polymerase II; Top1, Topoisomerase I; UV, Ultraviolet light; DRB, 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside.

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Fig 1.

CPT induces Pol II hyperphosphorylation. (a and b) CPT induces a 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 μ M CPT (panel a), and for the indicated concentrations of CPT for 1 h (panel b). Pol IIa (hypophosphorylated CTD) and Pol Ilo (hyperphosphorylated CTD) forms are indicated. Tubulin was used as a loading control. (c) Bottom panel: representative Western blotting of Pol II in HCT116 cells treated with 25 μ M CPT for 1 h. Top panel: quantification of the data shown in the bottom panel (mean $\pm$ S.D. of 5 independent experiments). Pol IIa is shown in gray and Pol Ilo is shown in black. (d and e) HCT116 cells were treated with 25 μ M 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 by

slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μ g DNA) with an antibody against Top1.

Fig 2.

Pol II hyperphosphorylation induced by Top1cc is prevented by cyclin-dependent kinase inhibitors. (a and b) The Cdk inhibitors 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) and flavopiridol (FLV) both prevent Pol II hyperphosphorylation. Western blotting analyses of Pol II in HCT116 cells treated for 1 h with 50 μM DRB (panel a) or with the indicated concentrations of FLV (panel b) before the addition of CPT (25 μM, 1 h). Pol Ila (hypophosphorylated CTD) and Pol Ilo (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 by CPT. HCT116 cells were treated as in panels a and b, and Top1cc were detected by slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μg DNA) with an antibody against Top1. (d) The DNA polymerase inhibitor aphidicolin (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 non-replicating primary lymphocytes.

Fig 3.

Top1cc induce Cdk7-mediated Pol II's CTD phosphorylation at residues serine-5. (a) Western blot analysis of Pol II phosphorylated at serine-5 (Ser⁵-P) and serine-2 (Ser²-P) in HCT116 cells treated with 25 μ M CPT for 1 h. (b) Quantification of the data shown in panel *a* (mean $\pm$ S.D. of 3 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 μ M) or FLV (0.5 μ M) before the addition of CPT (25 μ M, 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-hour incubation with 1 μ M 3-MBPP1. Then, cells were treated with 25 μ M CPT for 1 h and assayed for Ser⁵-P, Ser²-P and Pol II by Western blotting. Pol Ila (hypophosphorylated CTD) and Pol Ilo (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.³¹ (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 μ M CPT and assayed for Cdk9, Cdk7, Ser⁵-P, Ser²-P and Pol II by Western blotting.

Fig 4.

Pol II dephosphorylation in CTP-treated cells. (a) Cell treatment protocol. HCT116 cells were treated with 25 μ M CPT for 1 h prior to and during the flavopiridol (FLV, 0.5 μ M) or DRB (50 μ M) 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 and f) Panel e is quantifications of Pol IIo data shown in panels b and c. Panel f is quantifications of Pol IIo data shown in panels b and d. Dashed line indicates Pol IIo's levels in untreated cells. Diamonds: CPT; circles: CPT + FLV in panel e and CPT + DRB in panel f; squares: FLV in panel e and DRB in panel 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 immunoblotted with the indicated antibodies. Tubulin and lamin A/C were used as controls for *Soluble* and *Nuclear matrix* fractions, respectively.

Fig 5.

Top1cc induce Brca1-dependent degradation of Top1 without substantial degradation of Pol II. (a) CPT induces proteasomal degradation of Top1 in MEF cells. Cells were treated with the proteasome inhibitor MG132 (50 μ M) for 1 h before the addition of CPT (25 μ M) for the indicated times. Top1cc were detected by slot blot after probing the DNA-containing fractions at three concentrations (10, 3, and 1 μ g 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 μ M) or for 15 min with the replication inhibitor aphidicolin (APD, 1 μ M) before the addition of CPT (25 μ M) 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) MEFs cells of the indicated

genotype were treated with 25 μ M CPT for the indicated time periods. 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. Top panel: quantification of Top1 expression shown in this panel. (e) Model showing Brca1-dependent degradation of Top1 in response to hyperphosphorylated Pol II arrested by a Top1cc.