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Topoisomerase IIα promotes activation of RNA polymerase I transcription by facilitating pre-initiation complex formation

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Type II DNA topoisomerases catalyse DNA double-strand cleavage, passage and re-ligation to effect topological changes. There is considerable interest in elucidating topoisomerase II roles, particularly as these proteins are targets for anti-cancer drugs. Here we uncover a role for topoisomerase IIα in RNA polymerase I-directed ribosomal RNA gene transcription, which drives cell growth and proliferation and is upregulated in cancer cells. Our data suggest that topoisomerase IIα is a component of the initiation-competent RNA polymerase Iβ complex and interacts directly with RNA polymerase I-associated transcription factor RRN3, which targets the polymerase to promoter-bound SL1 in pre-initiation complex formation. In cells, activation of rDNA transcription is reduced by inhibition or depletion of topoisomerase II, and this is accompanied by reduced transient double-strand DNA cleavage in the rDNA-promoter region and reduced pre-initiation complex formation. We propose that topoisomerase IIα functions in RNA polymerase I transcription to produce topological changes at the rDNA promoter that facilitate efficient de novo pre-initiation complex formation.

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To substantiate an association of Top2α with Pol Iβ in cells, we immunoprecipitated Pol I from nuclear extracts of HeLa cells transiently expressing Flag-tagged Pol I subunit CAST23, using Flag-specific antibodies, then immunoblotted the immunoprecipitates using antibodies specific for Top2α, RN3 and Pol I subunits PAF53 and AC19. Top2α co-immunoprecipitated with Pol I in this experiment (Fig. 1b). We also passed purified Pol Iβ over a Top2α-antibody column and analysed proteins eluted from the column and proteins in the flow-through in immunoblot and promoter-specific transcription assays (Supplementary Fig. S1b–d). Intriguingly, the majority of promoter-specific transcription activity was lost and most of the RN3 protein was depleted from Pol Iβ and retained on the Top2α-antibody column. Therefore, the specific Top2α antibody used disrupted Top2α and RN3 interactions with Pol I. These data indicate that the majority of Pol Iβ complexes purified from human cell nuclei include Top2α.

**Top2α interacts directly with Pol Iβ-specific component RN3.** We reasoned that a direct interaction between Top2α and Pol Iβ might involve Pol Iβ-specific component RN3. Two polypeptides unique to Pol Iβ bound to RN3, the larger of which migrated similarly to full-length Top2α protein, in a Far-western analysis in which *in vitro*-translated 35S-labelled hRN3 was used to probe renatured Pol Iα or Pol Iβ (Fig. 1c). Moreover, there was a direct interaction between RN3 and Top2α in a recombinant protein-binding assay, in which *in vitro*-translated RN3 was incubated with *in vitro*-translated green fluorescent protein (GFP)-Top2α23 on GFP-antibody beads (Fig. 1d). To test the likely involvement of the isoform-specific CTD in the interaction of Top2α with RN3, we used Top2α CTD-mutant proteins lacking the terminal 42 or 180 amino acids (st1 or st5, respectively). These were the shortest and longest of a series of CTD truncations shown to impair the ability of GFP-Top2α to rescue the conditional Top2α-mutant cell line HTETOP33 from lethal Top2α depletion (Supplementary Fig. S2). A C-terminal-truncated version of Top2α (st5, 180 amino-acid deletion) displayed significantly reduced interaction with RN3 in the recombinant protein-binding assay (Fig. 1d). To further assess Top2α–RN3 interactions, nuclear extracts of HTETOP cells depleted of endogenous Top2α33 and stably expressing GFP-Top2α-wild type (WT) or -st1 (CDT 42 amino-acid deletion) were incubated with GFP-specific antibodies, and immunoprecipitates were immunoblotted using antibodies specific for Top2α, RN3 and Pol I subunit PAF53. Endogenous RN3 and Pol I subunit PAF53 co-immunoprecipitated with GFP-Top2α-WT (Fig. 1e). There was a significant decrease (~sixfold) in the amount of RN3 that co-immunoprecipitated with the Top2α-st1 mutant, compared with GFP-Top2α-WT. There was also a decrease (~twofold) in the amount of PAF53 co-immunoprecipitated; the smaller magnitude of this decrease might reflect interactions between Top2α and other components of the initiation-competent Pol Iβ complex, such as CK223. Collectively, these data demonstrate that Top2α interacts directly with RN3 and that residues 1491-1531 in the Top2α CTD are important for such interaction. Therefore, there is evidence, both *in vitro* and in cells, to support an interaction between Top2α and RN3 as components of initiation-competent Pol Iβ.
Top2α occupies the rDNA promoter in an SL1-dependent manner. The association of Top2α with initiation-competent Pol I predicts the presence of Top2α at the rDNA promoter. Top2α was detectable at the rDNA promoter by chromatin immunoprecipitation (ChIP) analysis in all cell types tested (Fig. 1f and Supplementary Fig. S3); elsewhere, along the rDNA repeat, Top2α association varied according to cell type (Supplementary Fig. S3). Small interfering RNA-mediated depletion of the TAF1D subunit of SL1 in cells, which leads to the disappearance of SL1 and Pol I from the rDNA promoter and reduces Pol I transcription, as expected for a stalled Pol I complex (Fig. 2a, lane 2). Note that in control transcription reactions supplemented with all four NTPs, Pol I transcribes to the end of the template, whereupon it dissociates in the presence of excess competitor DNA (Fig. 2a, lane 3) and, consequently, Pol I and Pol I-associated factors were present in the reaction supernatant (Fig. 2a, lane 4). Therefore, both Top2α and RRN3 dissociate from Pol I following initiation of transcription in vitro (Fig. 2b), consistent with a tethering of Top2α to Pol I, at least in part, through RRN3. Should Top2α indeed dissociate from Pol I at initiation or during/immediately following promoter escape in cells, any role for Pol Iβ-associated Top2α in Pol I transcription would be predicted to be at an early step in transcription.

Top2α and RRN3 dissociate from Pol I following initiation. RRN3 dissociates from Pol I at an early step following initiation of transcription and we have used a stalled Pol I transcription system to assess whether Top2α and RRN3 both dissociate from Pol I following initiation of transcription. Pol I can be stalled at the position of the first T (+31) on a ‘T-less’ template when the transcription reaction is carried out in the absence of UTP. Immobilization of the template allows the template-associated proteins to be separated from proteins that dissociate from the transcription complex after initiation of transcription. We demonstrate that Pol I subunit PAF53 was still associated with the DNA template following initiation of transcription, as expected for a stalled Pol I complex (Fig. 2a, lane 1), whereas RRN3 and Top2α were present in the reaction supernatant (Fig. 2a, lane 2). Note that in control transcription reactions supplemented with all four NTPs, Pol I transcribes to the end of the template, whereupon it dissociates in the presence of excess competitor DNA (Fig. 2a, lane 3) and, consequently, Pol I and Pol I-associated factors were present in the reaction supernatant (Fig. 2a, lane 4). Therefore, both Top2α and RRN3 dissociate from Pol I following initiation of transcription in vitro (Fig. 2b), consistent with a tethering of Top2α to Pol I, at least in part, through RRN3. Should Top2α indeed dissociate from Pol I at initiation or during/immediately following promoter escape in cells, any role for Pol Iβ-associated Top2α in Pol I transcription would be predicted to be at an early step in transcription.

**Figure 1 | Top2α is a component of initiation-competent Pol Iβ and interacts directly with RRN3 and occupies the rDNA promoter.** (a) Pol Iβ-associated decatenation activity is ATP-dependent and sensitive to non-hydrolysable ATP and Top2 inhibitor. Pol Iβ peak fractions were incubated +/− ATP, AMP-PNP and etoposide in a kinetoplast DNA decatenation assay. (b) Top2α co-immunoprecipitates with Pol Iβ. Pol I was immunoprecipitated from nuclear extracts of HeLa cells expressing Flag-CAST (Pol I), using Flag-antibodies, and then immunoblotted for Top2α, Pol Iβ subunit RRN3 and Pol I subunits PAF53 and AC19. Purified Pol Iα (lane 1); IgG immunoprecipitate (lane 2). (c) RRN3 interacts with Top2α of Pol Iβ in far-western analysis. Pol Iα and Iβ were probed with 35S-Flag-RRN3 and Top2α-antibodies. (d) Top2α C-terminus is important for RRN3 interaction. In vitro-translated and Flag-immunopurified 35S-Flag-RRN3 was incubated with in vitro-translated 35S-GFP Top2α WT and C-terminal-truncated (st5; 180 amino-acid deletion) proteins on GFP-antibody beads. Input RRN3 (1%, lane 1); RRN3 wt and GFP-antibody beads (lane 4); *contaminating translation product. (e) Efficient co-IP of Top2α with Pol Iβ requires its C-terminal 42 amino acids. Top2α was immunoprecipitated from HTETOP cells depleted for endogenous Top2α and stably expressing GFP-Top2α WT and C-terminal-truncated (st1, 42 amino-acid deletion) proteins, using GFP antibodies. IgG control (lane 1). Immunoprecipitates were immunoblotted using antibodies for Top2α, PAF53 and RRN3. Representative immunoblot is shown. Quantification and normalization of the RRN3 and PAF53 immunoblot signals to those of Top2α-WT and -st1, in three independent experiments, revealed that C-terminal 42 amino-acid truncation of Top2α reduced RRN3 and PAF53 signals ~ sixfold and ~ twofold, respectively. (f) Top2α occupancy of the rDNA promoter is reduced in TAF1D-depleted cells with decreased rRNA transcription. HEK293 cells were transfected with scrambled or SL1 subunit TAF1D (TAF1D) small interfering RNAs (siRNAs). rDNA-promoter occupancy by Top2α, Pol I subunit A135 and SL1 subunit TAF110 (TAF1C) in these cells was analysed by ChIP using Top2α, A135 and TAF110 antibodies; data in bar graphs are from three independent ChIP experiments, normalized to control IgG samples; s.d. is shown. (g) Control for f showing pre-rRNA levels from cells transfected with scrambled siRNA (lane 1) or TAF1D siRNA (lane 2) as analysed by 51 nucleic acid protection.
Effects of Top2 inhibition on Pol I transcription. To investigate a role for Top2α in early Pol I transcription events, we first tested the effects of inhibition of Top2 activity on Pol I transcription, using \(^{3}\text{H}\)-uridine pulse-chase labelling of (pre-)rRNA in cells. We observed a negative effect on Pol I transcription of etoposide treatment of U2OS cells (Supplementary Fig. S4). However, there is evidence to suggest that this was likely to be indirect and as a consequence of ataxia telangiectasia mutated (ATM) and p53-dependent DNA-damage signalling, activated by double-strand DNA breaks arising from trapped Top2–DNA cleavage complexes. Therefore, to assess whether inhibition of Top2 activity could induce any effects on Pol I transcription independent of DNA-damage signalling, we treated cells with alternative Top2 inhibitors and/or used cell lines that could not elicit a p53-dependent DNA-damage response.

Crucially, treatment for up to 15 h of U2OS cells with merbarone (a Top2 catalytic inhibitor blocking DNA cleavage) and of p53-null H1299 cells with etoposide (Fig. 3a–c) produced no significant effects on Pol I transcription. As inhibition of Top2 activity for up to 15 h does not have a detectable direct effect on Pol I transcription in actively growing cell populations, this suggests that Top2 activity is not essential for transcription (re-)initiation or elongation of rRNA transcripts. Notably, Top2 inhibitor treatments for 24 and 48 h, of U2OS cells with merbarone or HCT116 (p53 null) cells with etoposide, resulted in significant decreases in Pol I transcription (Fig. 3d–f). These findings imply a potential role for Top2 in Pol I transcription, outside of (re-)initiation or elongation.

**Top2α depletion negatively affects Pol I β assembly/stability.** To further explore the possibility of a role for Top2α in Pol I transcription, we analysed rRNA transcripts from HTETOP cells specifically depleted of the α-isoform of Top2 by treatment with tetracycline (Tet) for 48 h (Fig. 4a and b). In common with other cell types depleted of Top2α protein or treated with Top2 catalytic inhibitors (reviewed in Nitiss), this impairs sister chromatid segregation causing aberrant anaphases and cytokinesis. After 48 h in Tet, the only mRNA transcripts to be dramatically depleted in HTETOP cells are those encoding Top2α itself. Nevertheless, we detected an ~ two-fold reduction in Pol I synthesis of the 47S pre-rRNA transcript, with no effect on pre-rRNA processing (Fig. 4c). Pol I was immunoprecipitated from the Top2α-depleted and control cells in equivalent amounts, as determined by the non-specific Pol I transcription activities of the immunoprecipitates (Fig. 4d). Yet, there was significantly less promoter-specific transcription activity associated with Pol I immunoprecipitates from the Top2α-depleted cells (Fig. 4d) and a reduced amount of RRN3 protein in these immunoprecipitates (Fig. 4e), compared with those of the control cells. These data suggest the presence of fewer initiation-competent Pol I β complexes in Top2α-depleted cells. Such a decrease could account for the observed two-fold reduction in Pol I transcription in Top2α-depleted cells. Taken together, these results suggest that Top2α can influence the assembly and/or stability of initiation-competent Pol I β at the rDNA promoter, and thereby PIC formation, in cells.

We reasoned that in a population of actively growing cells, at any one time, most of the active rDNA promoters are engaged in multiple-round transcription, with relatively few requiring de novo PIC formation and activation of transcription. De novo PIC formation is required at actively transcribing rDNA genes following DNA replication (on one set of duplicates). Lack of de novo PIC assembly would lead to a predicted ~ 50% reduction in Pol I transcription with each cell cycle. Our data (Figs 3 and 4) suggest that in the absence of Top2α activity, there may be a gradual accumulation of rDNA promoters requiring de novo PIC formation to achieve transcription.

**Top2α facilitates assembly of Pol I PICs.** To investigate the involvement of Top2α in de novo PIC formation, we sought a system in which de novo functional PIC formation was required for Pol I transcription at the majority of rDNA promoters. Pol I transcription can be downregulated by serum starvation of cells and activated by serum refeeding. Starved U2OS cells exhibit decreased levels of Pol I transcription (Fig. 5a), accompanied by reduction of SL1 and Pol I from the rDNA promoter (Fig. 5b) and the disappearance of Top2α from the rDNA promoter.
Figure 3 | Effects of Top2 inhibition on Pol I transcription. (a) Schematic of pulse-chase labelling of cells with 3H-uridine to determine effects of Top2 inhibition on rRNA by Pol I (used in b and c). (b) Pre-rRNA (47/45S), 28S and 18S rRNA transcript levels in actively growing U2OS cells were unaffected by treatment with Top2 inhibitor merbarone (Me) for up to 15 h. 3H-uridine was added 1 h after Me; non-radioactive uridine was added after an additional 2 h. Total RNA was extracted after 0, 4 and 12 h, separated by formaldehyde agarose gel electrophoresis and transferred to membrane. Newly synthesized pre-rRNA and rRNA transcripts were detected by autoradiography (representative experiment; upper panel); total 18S and 28S rRNAs were detected by ethidium bromide staining (lower panel). Relative efficiencies of rRNA synthesis and transcript processing are shown (bar graphs: – Me, dark-blue bars; + Me, light-red bars) for 47S/45S pre-rRNA (top), 28S rRNA (middle) and 18S rRNA (bottom), expressed as percentage of highest value (set at 100%). s.d. is shown, n = 3; *P < 0.05. (c) Pre-rRNA (47/45S), 28S and 18S rRNA transcript levels in actively growing H1299 (p53 null) cells were unaffected by treatment with etoposide (Et) for up to 15 h. Experimental details as in a and b, except H1299 instead of U2OS cells; Et instead of Me. (d) Schematic of 3H-uridine labelling of cells to determine effects of Top2 inhibition on rRNA synthesis (used in e and f). (e) Pre-rRNA (47/45S) and 32S rRNA transcript levels in actively growing U2OS cells were reduced by treatment with Me for 24 or 48 h. Newly synthesized pre-rRNA and rRNA transcripts from cells treated with Me for 24 or 48 h, then incubated with 3H-uridine for 1 h, were detected, quantitated and expressed as in b: representative experiment (upper panel); total 18S and 28S rRNAs (lower panel); and 47/45S pre-rRNA bar graphs (– Me, dark-blue bars; + Me, light-red bars). s.d. is shown, n = 3; ***P < 0.001. (f) Pre-rRNA (47/45S) and 32S rRNA transcript levels in HCT116 (p53 null) cells were reduced by treatment with Et for 24 or 48 h. Experimental details as in e.
There was a marked (~twofold) reduction in activation of Pol I transcription in starved H1299 and U2OS cells treated with Top2 inhibitors etoposide and merbarone, respectively, then resupplied with serum, as determined by S1 nuclease protection of the first 40 nucleotides of the pre-rRNA (Fig. 6a,b) and by 3H-uridine pulse-chase labelling (Supplementary Fig. S6a,b). Top2 inhibitor ICRF-193 similarly reduced activation of Pol I transcription in U2OS cells (Supplementary Fig. S6d,e). These data suggested a defect in the early stages of transcription. There was a corresponding reduced occupancy of SL1, UBF and Pol I, with little Top2x detectable, at the rDNA promoter in the H1299 cells (Fig. 6d), suggesting that, inhibition of Top2 activity reduces the efficiency of PIC formation and, thereby, the efficiency of Pol I transcription activation. This defect in activation is likely to be independent of DNA-damage signalling through p53 and ATM as it occurred in p53-null cells (H1299) (Fig. 6a) and could not be rescued by incubation of cells with caffeine (an inhibitor of ATM/ATR [ataxia telangiectasia and Rad3-related] signalling) (Fig. 6g).

HTETOP cells depleted of Top2α protein, serum-starved, then resupplied with serum, also showed reduced activation of Pol I transcription and a corresponding reduced promoter occupancy of SL1, UBF and Pol I (Fig. 6c,e and Supplementary Fig. S6c), complementing the results obtained by pharmacological inhibition of Top2.

Taken together, these results suggest that Top2α activity, specifically that of the α isoform of Top2, facilitates the efficient de novo assembly of PICs in Pol I transcription.

**Figure 4 | Top2α depletion downregulates Pol I transcription and the level of the initiation-competent Pol IIj complex in cells.** (a) Schematic of 3H-uridine pulse-chase labelling of cells to determine effects of Top2α depletion on rRNA synthesis (used in c). (b) Control for Top2α-depletion. HTETOP cells were incubated for 48 h with 1 μg ml−1 Tet (+ Tet) or without (− Tet). Proteins were immunoblotted using Top2α and actin antibodies. (c) rRNA synthesis is reduced in Top2α-depleted cells. Cells depleted of Top2α, as in b, were 3H-uridine-labelled for 2 h and cold-chased with uridine, and RNA was extracted after 0, 4 and 12 h. Newly synthesized transcripts were detected by autoradiography (representative experiment; upper panel); 18S and 28S rRNAs were ethidium bromide stained (lower panel). Relative efficiencies of rRNA synthesis and transcript processing are shown (bar graphs: − Tet, dark-blue bars; + Tet, light-red bars) for 47S/45S pre-rRNA (top), 28S rRNA (middle) and 18S rRNA (bottom), expressed as percentage of highest value (set at 100%). s.d. is shown, n = 3; **P < 0.001, *P < 0.01 and *P < 0.05. (d) Pol I from Top2α-depleted cells supports a reduced level of rDNA promoter-specific Pol I transcription activity. HTETOP cells, transfected with Flag-CAST (Pol I subunit), were incubated with or without Tet for 48 h. Pol I complexes immunoprecipitated using Flag-antibody were analysed for non-specific transcription and rDNA promoter-specific transcription activity in a run-off transcription assay. Relative levels of non-specific and promoter-specific transcription in Top2α-depleted (+ Tet, light-red bars) or non-depleted (− Tet, dark-blue bars) cells are indicated in the graph. s.d. is shown, n = 3; **P < 0.001 and *P < 0.05. The non-specific transcription activities of Pol I immunoprecipitated from Tet− and Tet+ cells were similar, reflecting that similar amounts of Pol I were immunoprecipitated. (e) The amount of RRN3 co-immunoprecipitating with Pol I is reduced in Top2α-depleted cells. Pol I complexes, immunoprecipitated from nuclear extracts of Flag-CAST-transfected HTETOP cells incubated with (+ Tet) or without Tet (− Tet) for 48 h, were analysed by immunoblotting, using Top2α and RRN3 antibodies. Immunoblots of the nuclear extract inputs (upper panels) and Pol I immunoprecipitates (lower panels) from two independent experiments (NE1 and NE2) are shown.
PIC formation in activation of Pol I transcription by producing topological changes at the rDNA promoter that would support efficient assembly of the PIC. We reasoned that double-strand DNA (dsDNA) cleavage would arise at the rDNA promoter from such Top2a activation, since Top2a-mediated DNA cleavage, strand passage and re-ligation activity. Taken together, our data suggest that Top2a activity at the rDNA promoter facilitates efficient de novo assembly of functional PICs, which include SL1, UBF and Pol Iβ (Fig. 7c).

**Discussion**

This study identifies a novel function for a Top2a in facilitating de novo PIC formation and activation of Pol I transcription of the rRNA genes in human cells.

We present evidence of a role for the Top2a isoform in Pol I transcription. Our data suggest that active Top2a is a component of the initiation-competent Pol Iβ complex, targeted to the rDNA promoter, at least in part, through the interaction of its isoform-specific C terminus with the RRN3 component of Pol Iβ, which interacts with promoter-bound transcription factor SL1. Depletion of Top2a negatively affects the assembly and/or stability of initiation-competent Pol Iβ and decreases Pol I transcription in cells, implying that Top2a can influence the assembly and/or stability of initiation-competent Pol Iβ at the rDNA promoter and, thereby, PIC formation in cells. De novo PIC formation is an event expected to occur at the active rDNA gene promoters following DNA replication (on one set of the duplicates) during each cell cycle. De novo functional PIC formation is also required for activation of Pol I transcription at the majority of rDNA promoters upon refeeding of serum-starved cells, and we discovered that Top2a facilitates efficient activation of Pol I transcription from such promoters and that this is accompanied by Top2a-dependent DNA cleavage and accumulation of PIC components and Top2a at the rDNA-promoter region. Our data suggest a role for Top2a in de novo PIC formation, and we propose that Top2a facilitates efficient activation of Pol I transcription through its ability to cleave, passage and re-ligate dsDNA and, thereby, to alter the topology of the rDNA promoter, alleviating topological constraints to PIC assembly and stability (Fig. 7c).

At the rDNA promoter, the local topology or higher-order structure of the DNA can influence transcription of the RNA gene and can be affected by chromatin context, including binding of the architectural protein UBF, which bends and supercoils the promoter and nucleosome positioning. TBP–TFI complex SL1 directs Pol I PIC formation and stabilizes UBF at the rDNA promoter. We envisage that, in activation of Pol I transcription, SL1 binds to the rDNA promoter, RRN3 binds to SL1 and Top2a is recruited through its interaction with RRN3, and then Top2a-mediated cleavage, passage and re-ligation of dsDNA at the rDNA promoter creates a topological state conducive to the efficient de novo assembly and stabilization of a functional PIC, including SL1, UBF and initiation-competent Pol Iβ, so that transcription can now be initiated and re-initiated. Lack of Top2a catalytic activity during de novo PIC formation would reduce Pol I transcription activation by affecting the equilibrium of SL1 and UBF binding to the promoter and, thereby, the efficiency of Pol I recruitment.
**Figure 6 | Top2α facilitates formation of PICs in Pol I transcription activation.** (a,b) Top2 inhibition reduces activation of Pol I transcription in serum-refed cells. H1299 cells (p53 null) (a) or U2OS cells (b) were serum-starved for 20 h and (starting at –1h) incubated with Top2 inhibitors etoposide (Et) or merbarone (Me), respectively, or without inhibitors, for 1h. At 0h, serum was added to the cells. RNA was extracted 15, 30, 60 and 90 min after serum addition. Pre-rRNA levels were measured by S1 nuclease protection (complementary assay, Supplementary Fig. S6a,b); s.d. is shown. (c) Top2α depletion reduces activation of Pol I transcription in serum-refed cells. HTETOP cells were incubated for 48 h with or without inhibitors, for 1h. At 0h, serum was added to the cells. RNA was extracted 15, 30, 60 and 90 min after serum addition. Pre-rRNA levels were measured by S1 nuclease protection (complementary assay, Supplementary Fig. S6c). (d,e) Inhibition of Top2 or depletion of Top2α reduces the accumulation of PIC components SL1, UBF and Pol I at the rDNA promoter in serum-refed cells. ChIP assays were performed on chromatin from cells treated as in a and c, respectively, using antibodies for Top2α, TAF63 (TAF1B; SL1), UBF and A135 (Pol I), and normalized to control IgG samples. s.d. is shown, n = 3. ***P < 0.001 and *P < 0.05. (f) Top2α co-localises with Pol I upon activation of Pol I transcription. U2OS cells serum-starved for 20 h were serum-refed for 2h, then analysed by indirect immunofluorescence, using antibodies for A194 (Pol I; red) or Top2α (green), and laser scanning confocal microscopy. Scale bar, 10.4 μm (expanded version, Supplementary Fig. S5b). (g) The reduction in activation of Pol I transcription detected in serum-refed cells treated with Top2 inhibitor is not affected by treatment with caffeine, an inhibitor of ATM/ATR. U2OS cells were serum-starved (20 h) and incubated with or without Et (25 μM) and without (left panel) or with caffeine (125 μM) (right panel) 1h before addition of serum. RNA was extracted at 0, 15, 30, 60 and 90 min following serum addition, and pre-rRNA levels were measured as in a.

Top2α could also, potentially, facilitate efficient de novo PIC formation upon activation of rDNA transcription by stimulating promoter escape and Pol I processivity in the pioneering round of transcription. In actively growing cells, a relatively high density of Pol I complexes facilitates Pol I clustering. Consequently, positive supercoils ahead of the transcribing complex and...
negative supercoils behind\textsuperscript{7,12} could potentially be dissipated by the actions of the adjacent polymerases\textsuperscript{26,27}, such that topoisomerase activity would not be required, except perhaps ahead of a stalled polymerase or in regions where the density of polymerases is sparse. A polymerase pioneering the first round of activated transcription, without the advantage of an adjacent polymerase to dissipate the supercoiling it generates, as it transcribes the rDNA, would require topoisomerase cleavage. In the absence of Top2α, the observed decrease in occupancy by PIC components of the rDNA promoter might be accounted for if pioneering polymerases were stalled, due to the topological constraints imposed by failure to resolve supercoiling at or before promoter escape, thereby preventing the productive interaction of incoming PIC components with the rDNA promoter.

We considered the possibility that Top2α might affect serum-activated \textit{de novo} PIC formation by influencing the assembly of nucleosome remodelling machineries for repositioning of nucleosomes through a mechanism similar to that proposed for DNA topoisomerase IIβ (Top2β) in ligand (hormone)-stimulated activation of Pol II promoters\textsuperscript{4}, which involves recruitment of DNA-damage response proteins. However, such a mode of action seems unlikely, as key components of the repair machineries (such as Ku70, Ku80 and DNA-PK) were not detectable by ChIP analysis at the activated rDNA promoters (our unpublished results), suggesting that, if Top2α affects nucleosome positioning in activation of Pol I transcription, then it achieves this through alternative means.

Our findings reveal a novel dimension to the efficacy of Top2 inhibitors used in cancer treatment\textsuperscript{4–6} and, potentially, to the search for Top2α-specific anti-cancer agents\textsuperscript{5,8}. \textit{De novo} PIC formation and activation of Pol I transcription occur during each cell cycle at newly replicated rRNA genes and might also be required for the upregulation of Pol I transcription linked to cancer\textsuperscript{5,6,27}. We have demonstrated that the Top2 inhibitor etoposide, an effective anti-cancer drug, can reduce \textit{de novo} PIC assembly and activation of Pol I transcription, independently of the p53 status of cells and the ATM/ATR-dependent DNA-damage response pathways. This suggests that this Top2 inhibitor might function in part to restrict Pol I transcription by limiting \textit{de novo} activation of rRNA genes, which, ultimately, could lead to the abrogation of Pol I transcription, even in p53-null cells. This would have devastating consequences for protein synthesis, constraining the runaway growth associated with cancers. Indeed, maintenance of elevated levels of Pol I activity in cancer cells appears critically important for the process of malignant transformation and cancer cell survival. For instance, CX-5461, a selective inhibitor of Pol I transcription, induced p53-dependent apoptotic cell death in the majority of Eμ-Myc lymphoma cells at concentrations that reduced Pol I transcription about 50% (ref. 54). Recent studies have illustrated the
effectiveness of targeting Pol I transcription in anti-cancer therapy for haematological malignancies and solid tumours. Therefore, we speculate that inhibitors specifically designed to target Top2α in Pol I transcription (which may be less likely to cause secondary cancers than those targeting the β-isom form) could be effective non-genotoxic tools for use in the battle against cancer.

Methods

Cell-culture conditions and Top2 depletion or inhibition. U2OS cells in McCoy’s 5A medium plus 10% FBS, H1299 cells (homologous partial deletion of p53) in RPMI plus 10% FBS and HTETOP cells (derivative of human fibrosarcoma cell line HT1080) in DMEM high glucose (4.5 g l⁻¹) plus 10% FBS and other additives were grown to ~60–70% confluency, washed twice with Dulbecco’s PBS and then serum-starved for 20 h in DMEM low glucose (1 g l⁻¹). For activation of Pol I transcription, serum-starved cells were incubated in DMEM low glucose (1 g l⁻¹) containing 20% FBS. For Top2 inhibition, Top2 poison etoposide (100 μM final concentration; Merck) or catalytic inhibitors ICRF-193 (50 μM) and merbarone (100 μM; Merck) were added (except Fig. 6g). For Top2 depletion, HTETOP medium was supplemented with 1 μg ml⁻¹ Tet for 48 h.

Cell and in vitro expression of GFP-Top2α fusion proteins. HTETOP cells expressing GFP-Top2α were cultured (in 75 cm² flasks; stop codon was introduced into pGFP-Top2α by Quickchange site-directed mutagenesis (Agilent Technologies; see Supplementary Fig. S2). pGFP-Top2α-WT (full-length human, 1,531 amino acids) or pGFP-Top2α-st1 (stop-codon mutant) plasmids (Novi-linearized) were electroporated into HTETOP cells, puromycin-resistant colonies were selected and single GFP-positive colonies were expanded. In vitro transcription/translation experiments used pGFP-Top2α-WT and -st1.

Immunocytochemistry. Cells were fixed (10 min, 4% paraformaldehyde), permeabilized (10 min, 1% Triton X-100) and blocked (10 min, 1% donkey serum in PBS), and then incubated (1 h) with antibodies (Supplementary Table S1) in the blocking buffer, washed X3 for 10 min in PBS and incubated (1 h) with labelled secondary antibodies (Supplementary Table S1). After washes, cells were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole and visualized under confocal microscopy.

RNA labelling in cells and RNA analysis. Labelling of RNA in cells (~70% confluent or ~50% for starved/refed) involved 1 μCi ³H-uridine for ~0.2–0.4 × 10⁵ cells per well of a six-well plate. In pulse-chase labelling, cells were incubated for 2 h with ³H-uridine, washed and incubated in unlabelled medium containing 0.5 mM uridine (+/- Top2 inhibitors). RNA was extracted (RNeasy Mini Kit (Qiagen)). An amount of 2 μg of ³H-labelled total RNA was run on a 1% formaldehyde agarose gel at 130 V for 90 min in Xio MOPS, blotted onto Hybond-N membrane (Amersham), cross-linked (ultraviolet cross-linker; UVP), analysed by tritium imaging using Fuji Tritium image plate (or following PerkinElmer EnHance spray, exposed to Kodak Biomax XAR film at ~80°C) and then quantified using Aida software.

S1 nuclease protection analysis of pre-RNA levels. Total RNA isolation from cells (~70% confluent or ~50% for starved/refed) and S1 nuclease protection analysis were performed with a 5′ end-labelled oligonucleotide probe complementary to the first 40 nucleotides of the 47S pre-rRNA transcript 44.

In vitro transcription assays. Non-specific transcription assays were performed as described 37. Run-off transcription reactions were performed essentially as described 37, using immobilized rDNA fragments containing the human rRNA gene promoter, supplemented with purified SL1 and recombinant UBF56. Reactions were terminated by addition of RT buffer and RNA transcripts (purified using RNeasy mini kits (Qiagen)) were electrophoresed on denaturing 4% acrylamide/8 M urea gels, visualized by phosphorimaging FLA-7000 (Fuji) and analysed with Aida software.

Decatenuation assay. DNA topoisomerase II activity was assayed by decatenuation of kinetoplast DNA (TopoGen), according to the manufacturer’s protocols. AMP-PNP was substituted for ATP (200 and 100 μM) or Top2 inhibitor etoposide (250 and 100 μM) was included in some reactions. Reaction products were resolved on a 1% agarose gel in TBE buffer.

Immunoblotting and immunoprecipitation. Antibodies used for immunoblotting and immunoprecipitations (IPs) are listed (Supplementary Table S1). IP of nuclear extracts was performed as described 37, from U2OS and HTETOP cells (1 × 10⁶ cells) and DNA double-strand break detection assay. Cells were subjected to cross-linking (1% formaldehyde, room temperature, 10 min; terminated by addition of glycine to a final concentration of 0.1 M for 5 min) and then chromatin isolated as described 37 and sheared to ~300 bp of average-size fragments (Bioruptor; Diagenode). A single ChIP used chromatin from 2.5 × 10⁶ or 1 × 10⁷ cells and antibodies as in Supplementary Table S2, incubated overnight at 4°C, and then for 2 h with Protein A/G beads (Invitrogen) at room temperature. Beads were washed five times in 450 μl volume of RIPA ChiP buffer 58 and twice with 200 μl TE buffer with aid of the Precipitor (Abnova). DNA on washed beads 59 was eluted, and cross-link reversal was performed in one stage as described 40. DNA was purified (IPure kit; Diagenode with Precipitor; Abnova) and analysed by qPCR (QuantiFast Multiplex PCR Mix; Qiagen) in triplicates using primer combinations and probes covering regions of rDNA repeat (Supplementary Table S3) on Light Cycler 480-II (Roche). Results were expressed as percentage of input chromatin and normalized to control IgG levels. Bar graphs show the average from three independent experiments (n = 3); s.d. and statistical significance (probability values; ***P<0.001; **P<0.01; and *P<0.05, for drug-treated versus control cells) have been calculated using one- and two-way analysis of variance on R software (open-source statistical computing and graphics software).

ChIP assay. Cells were subjected to cross-linking (1% formaldehyde, room temperature, 10 min; terminated by addition of glycine to a final concentration of 0.1 M for 5 min) and then chromatin isolated as described 37 and sheared to ~300 bp of average-size fragments (Bioruptor; Diagenode). A single ChIP used chromatin from 2.5 × 10⁶ or 1 × 10⁷ cells and antibodies as in Supplementary Table S2, incubated overnight at 4°C, and then for 2 h with Protein A/G beads (Invitrogen) at room temperature. Beads were washed five times in 450 μl volume of RIPA ChiP buffer 58 and twice with 200 μl TE buffer with aid of the Precipitor (Abnova). DNA on washed beads 59 was eluted, and cross-link reversal was performed in one stage as described 40. DNA was purified (IPure kit; Diagenode with Precipitor; Abnova) and analysed by qPCR (QuantiFast Multiplex PCR Mix; Qiagen) in triplicates using primer combinations and probes covering regions of rDNA repeat (Supplementary Table S3) on Light Cycler 480-II (Roche). Results were expressed as percentage of input chromatin and normalized to control IgG levels. Bar graphs show the average from three independent experiments (n = 3); s.d. and statistical significance (probability values; ***P<0.001; **P<0.01; and *P<0.05, for drug-treated versus control cells) have been calculated using one- and two-way analysis of variance on R software (open-source statistical computing and graphics software).

DNA double-strand break detection assay. Cells were fixed, permeabilized in situ and DNA breaks were labelled with biotin-11–dUTP and terminal TdT dUTP as described 37. In brief, cells (15 cm dish per single experimental point) were washed twice with PBS and fixed for 20 min with 2% PBS with 30% methanol/0.125 M for 5 min) and then chromatin isolated as described 37 and sheared to ~300 bp of average-size fragments (Bioruptor; Diagenode). A single ChIP used chromatin from 2.5 × 10⁶ or 1 × 10⁷ cells and antibodies as in Supplementary Table S2, incubated overnight at 4°C, and then for 2 h with Protein A/G beads (Invitrogen) at room temperature. Beads were washed five times in 450 μl volume of RIPA ChiP buffer 58 and twice with 200 μl TE buffer with aid of the Precipitor (Abnova). DNA on washed beads 59 was eluted, and cross-link reversal was performed in one stage as described 40. DNA was purified (IPure kit; Diagenode with Precipitor; Abnova) and analysed by qPCR (QuantiFast Multiplex PCR Mix; Qiagen) in triplicates using primer combinations and probes covering regions of rDNA repeat (Supplementary Table S3) on Light Cycler 480-II (Roche). Results were expressed as percentage of input chromatin and normalized to control IgG levels. Bar graphs show the average from three independent experiments (n = 3); s.d. and statistical significance (probability values; ***P<0.001; **P<0.01; and *P<0.05, for drug-treated versus control cells) have been calculated using one- and two-way analysis of variance on R software (open-source statistical computing and graphics software).

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Author contributions
S.R., K.I.P., T.P. and G.M. carried out the experiments. A.V. and A.C.G.P. constructed the conditional Top2α expressing cell line (HTETOP) derivatives and Top2α expression constructs. K.I.P, J.R. and J.C.B.M.Z. conceived the project, designed the experiments, analysed the data (with input from other authors) and, with the help of A.C.G.P., wrote the paper.

Additional information
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