SUPPLEMENTARY INFORMATION

DNA topoisomerase I inhibition by camptothecin induces escape of RNA polymerase II from promoter-proximal pause site, antisense transcription, and histone acetylation at the human HIF-1α gene locus.

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MATERIALS AND METHODS

Cell lines and treatments.

Human HCT116 cell lines were cultured in Dulbecco's modified Eagle's medium (Euroclone) containing 10% heat-inactivated fetal calf serum (Invitrogen). HCT116(top1 siRNA) cells (24) were cultured in the same medium added with 500 µg/ml hygromycin B. Human MRCV fibroblast cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated foetal calf serum. Human Jurkat cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine (Invitrogen). Cells were maintained at 37°C in a humidified incubator containing 5% CO2 in air (referred to as normoxic conditions, 20% O2). Hypoxia treatments were performed by placing cells in a modular incubator chamber

(HERAcell^{R}150, Kendro Laboratory Products), and then flushing with a mixture of 1% O2, 5% CO2, and 94% nitrogen for 20 minutes. The chamber was then placed at 37°C.

All drug treatments were performed on exponentially-growing cells at 37°C. In case of co-treatments, cells were first incubated with aphidicolin (3 μM) or DRB (50 μM) for 15 minutes, or caffeine (5 mM) for 30 minutes, and then, CPT was added to the medium for the indicated time. All drugs were purchased from Sigma.

RNA purification and primer-specific cDNA preparation.

After drug treatments, $5x10^7$ cells were washed twice with cold PBS and collected through centrifugation. The pellet was resuspended and well mixed in 3.6 ml AE buffer [50 mM NaOAc (pH 5.2), 10 mM EDTA], 240 μl of SDS 25% and 3.6 ml of acid phenol (pH 4.5). Samples were then incubated for 10 minutes at 65°C mixing vigorously every minute. After a short incubation on ice, samples were centrifuged for 15 minutes at 12,000 g. The upper phase was collected, 3.9 ml of chloroform/isoamylic alcohol was added to it, then mixed and centrifuged for 10 minutes at 1,800 g. Final RNA precipitation was with isopropanol and NaOAc. RNA pellets were resuspended in TE, and DNA was digested with DNAse I.

Total RNA (1 μg) was used to prepare cDNA using random primers and SuperScript III (Invitrogen) with reaction buffers suggested by the manufacturer, for 5 minutes at 65°C, 5 minutes at 25°C, and 60 minutes at 50°C, followed by incubation with RNase H. When specific primers were used for reverse transcription with SuperScript III, conditions for the reaction were as follows: 5 minutes at 65°C and 50 minutes at 55°C. As negative controls, we used cDNAs prepared with no primer during reverse transcription reactions. Primer sequences are listed in Table 1S.

Northern blotting.

For Northern blot analysis, we used the procedures of (25) with minor modifications. 20 μg RNA per sample were separated by 1.5% agarose-formaldehyde gel electrophoresis in MOPS

buffer [20 mM MOPS (pH 7.0), 2 mM NaOAC, 1 mM EDTA (pH 8.0), in DEPC-treated water]. Then, RNA was transferred to a Hybond-N membrane in 20X SSC buffer (3 M NaCl, 0.3 M sodium citrate), and then fixed to the membrane by UV-crosslinking and 1 hour baking at 80°C. Strand-specific probe was designed to be 40 nucleotides in length and was synthesized by Intregrated DNA Technologies (IDT) with the 3' StarFire extension (5'- CAG CCC CAA TTC TAA ATA AGC TCT TAG ATT TTC CTC AGC C/NNNNNN/ -3'). The probe was then 3'-end ^{32}P-labeled at high specific activity with the IDT StarFire kit (IDT) using the manufacturer's instructions. Hybridization was performed for 16 hours in 7% SDS, 500 mM Sodium Phosphate (pH 7.0), 1 mM EDTA, 25% formamide at 42°C followed by two washes in 2X SSC, 0.5% SDS at 25°C for 20 minutes.

The HIF-1α mRNA exon 2 (corresponding to the 25-kb amplicon shown in Figure 1) was cloned by PCR fragment into the TOPO-TA vector (Invitrogen). Plasmid DNA was isolated with QIAGEN kit according to manufacturer protocol, and digested with EcoRI (New England Biolabs), and the exon 2 insert was then purified with gel electrophoresis and electroelution. The probe was labeled using Ready-To-Go DNA Labelling Beads (GE Healthcare) using manufacturer's instructions. Hybridization was performed for 16 hours in 7% SDS, 500 mM Sodium Phosphate (pH 7.0), 1mM EDTA at 65°C followed by two washes [1% SDS, 40 mM Sodium Phosphate (pH 7.0)], at room temperature for 30 minutes and then at 65°C for 45 minutes. Membranes were then exposed to Phospho Screen (Biosciences Amersham) for 1-2 days and then developed using Storm 860 (Biosciences Amersham).

ChIP procedure.

The ChIP protocol was essentially as reported already (6). To prepare chromatin, 10^7cells were fixed with 1% formaldehyde for 15 minutes. The reaction was stopped with 0.125 M glycin and cells were washed twice with ice-cold PBS followed by TEET [10 mM Tris-HCl (pH 8.0) 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100], TEEN [10 mM Tris-HCl (pH 8.0) 10 mM

EDTA, 0.5 mM EGTA, 200 mM NaCl], and resuspended in TEE [10 mM Tris-HCl (pH 8.0) 10 mM EDTA, 0.5 mM EGTA]. Protease inhibitors [aprotinin, leupeptin and pepstatin (Sigma)] were added to the buffers immediately before use. Chromatin was then sheared by sonication with a Bioruptor (Diagenode) to an average DNA fragment size of 300–400 bp.

Immunoprecipitations were performed at 4°C in RIPA buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS]. Amounts of chromatin, equivalent to 0.4 OD at 260 nm were taken for each immunoprecipitation. Samples were precleared for 2 hour with 4 µg of non-immune rabbit IgG and 20 µl of 50% suspension of a 1:1 mix of Protein A- and Protein G-Sepharose beads. Then, chromatin was recovered by centrifugation. One tenth of the supernatants were saved as "10% input." Supernatants were incubated overnight with 4 µg of specific antibody or nonimmune rabbit IgG (to measure background recovery). ChIP-grade Abs against anti-acetylated K9 and K14 of H3 histone or anti-acetylated K5, K8, K12, K16 of H4 histone were purchased from Upstate (Lake Placid, NY), and Abs against anti dimeK9 of H3 histone or anti H4 histone were purchased from Abcam. The H-224 Ab Antibody against the N-terminal of Pol II large subunit and the SI-1 against TBP were from Santa Cruz Biotechnology (Santa Cruz, CA). Non-immune rabbit IgG were from Cedarlane (Hornby, Canada). Immunocomplexes were recovered by addition of 40 µl of Protein A-/Protein G-Sepharose beads blocked with DNase-free BSA (9.95 mg/ml) and salmon testes DNA (10.5 mg/ml). Then, the beads were washed four times with RIPA buffer; once with RIPA buffer containing 0.5 M NaCl; once with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 M LiCl, 0.5% Na-deoxycholate, and 0.5% NP40; twice with TE [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]; and finally resuspended in TE. Each wash was performed for 10 minutes by rocking at 20 rpm. The pellets were then were adjusted to 0.5% SDS and incubated overnight at 65°C to reverse cross-links. Sample were then digested with proteinase K (500 µg/ml sigma) for 4 hours at 52°C and phenol extracted. DNAs were then ethanol-precipitated in the presence of 20 µg of glycogen (Roche Diagnostics, Manheim) and dissolved in TE. Recovered DNA was quantified by real-time PCR. At

least four dilutions of input DNA were run to generate the standard curve. DNA recovery was measured as input DNA fraction.

RIP procedure.

The RNA-ChIP (RIP) method was as the ChIP protocol with some modifications. After drug-treatments, $5x10^7$ cells were cross-linked with a final concentration of 1% formaldehyde added directly to the medium for 15 minutes. The reaction was stopped with 0.125 M glycin. Cells were washed twice with cold PBS, scraped and collected. Cell pellets were resuspended in 1 ml of RIPA buffer [50 mM Tris-HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl] containing protease inhibitors [aprotinin, leupeptin and pepstatin (Sigma)] and RNasin (50U/500ml). Extracts were sonicated with a Branson 250 sonifier (Branson Ultrasonic Corp., Danbury, CT) at 30% amplitude with 8 x 10-s bursts and 30-s pauses, resulting in an average fragment size of 1000 nucleotides. Chromatin was then centrifuged for 10 minutes at 15000 g to remove insoluble materials. Amounts of chromatin equivalent to 4 OD at 260 nm were taken for each immunoprecipitation.

Chromatin samples were then pre-cleared and immunoprecipitated with specific antibody following the ChIP protocol. The final pellets were washed by rocking for 4 minutes once in each of the following buffers: low-salt immune complex wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, (pH 8.1), 150 mM NaCl], high-salt immune complex wash buffer (the previous buffer with 500 mM NaCl) and LiCl immune complex wash buffer [0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)]; and twice in TE (10 mM Tris-HCl, 1 mM EDTA). The immunecomplexes were eluted in TE-SDS 1% and digested with proteinase K for 1 hour at 52°C. Cross-links were reversed at 65°C for 5 hours. RNA was extracted with phenol-chloroform and treated extensively with DNase I. RNA was ethanol-precipitated in the presence of 20 µg of glycogen and treated again with DNase I. cDNA was then prepared from 66% of the RNA sample using SuperScript III and random primers (Invitrogen). The conditions used for

retrotranscription were as follows: 5 minutes at 65°C, 5 minutes at 25°C, and 60 minutes at 50°C. RNA was hydrolyzed with 0.2 N NaOH and 0.1 M EDTA at 65°C for 7 minutes. HEPES buffer was then added to a final concentration of 0.3 M.

cDNA were analyzed by quantitative PCR with primer pairs spanning the HIF 1α gene (Figure 1A). As negative controls, we used cDNA prepared with no primers (NP), and non-retrotrascribed samples. At least four dilutions of genomic DNA were run to generate the standard curve. For all specific antibodies, the RNA recovered values were at least 10-fold more enriched than non-immune controls, and background levels set by NP and α-satellite DNA. Moreover, recovered RNA values were at least 100-fold more enriched than non-retrotranscribed samples.

Quantitative real-time PCR.

Quantitative real-time PCR (qrt-PCR) was performed as described already (6) by using the LightCycler and the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim). Quantification and melting curve analyses were performed using the Roche LightCycler software by the crossing point method as indicated by the supplier. PCR reactions contained 1x FastStart DNA SYBR Green I Master Mix, 2.08 mM $MgCl_2$ and 350 nM each of primers. Specificity of PCR products was routinely controlled by melting curve analysis and agarose gel electrophoresis.

Protein extracts and Western blotting.

For total extracts, cells were lysed with RIPA buffer, briefly sonicated and then pelleted by centrifugation. 20 μg of proteins were separated by 6.5% SDS-PAGE, and then blotted onto a Hybond ECL-nitrocellulose membrane. Equal loading was checked by Ponceau staining. Then, membranes were probed with specific Abs and detected with ECL Plus Western blot imaging system (GE Healthecare). Abs specific for Top1 or Actin (Santa Cruz, CA) were used at a 1:400 and 1:2,000 dilution, respectively. Ab specific for total p53 or CHK2 were used at a 1:1,000 dilution (Cell Signaling Technology Inc., Beverly, MA). To determine γ-H2AX, histones were

extracted as reported (Sedelnikova 2003, 278 /id). Histone aliquots, corresponding to 10-15 μg, were loaded onto a 12% SDS-PAGE gel, and then transferred to a Hybond ECL–nitrocellulose membrane. The membranes were then probed with anti-γ-H2AX or H1 (Santa Cruz, CA) Abs, used at 1:1,000 and 1:500 dilution, respectively, and specific bands were detected with ECL.

Table 1S

Name/Position	**Fw Primer 5' > 3'**	**Rv Primer 5' > 3'**
HIF 1α - 0.5 kb	TGAACAGAGAGCCCAGCAGAG	CCTGGTCCCAAACATGCATC
HIF 1α 0.05 kb	AGCTCCTCAGTGCACAGTGC	AGACTAGAGAGAAGCGGGCG
HIF 1α 0.1 kb	AGGATCACCCTCTTCGTCGC	AAGGCAAGTCCAGAGGTGGG
HIF 1α 0.2 kb	AGGATCACCCTCTTCGTCGC	CCGAGGGAATGGGCTTACTT
HIF 1α 2.1 kb	CTCTTAGATTTTCCTCAGCC	GCTGAGTAACCACCACTTAT
HIF 1α 7.9 kb	GAGGGGAAAATGTAGTCATTGGC	CGTCCTCTTCCACACCATACAGA
HIF 1α 25 kb	AGCCAGATCTCGGCGAAGTA	CCAGAAGTTTCCTCACACGC
HIF 1α 45kb	CCAGTTACGTTCCTTCGATCAGT	TTTGAGGACTTGCGCTTTCA
3'aHIF 1α	TTTGTGTTTGAGCATTTTAATAGGC	CCAGGCCCCTTTGATCAGCTT
Myc 0 kb	AGAAGGGCAGGGCTTCTCAGA	TCTGCCTCTCGCTGGAATTAC
Myc 1.9 kb	TAGCTTCACCAACAGGAACT	AGCTCGAATTTCTTCCAGAT
GAPD 0 kb	TAGCTCAGGCCTCAAGACCTT	AAGAAGATGCGGCTGACTGTC
GAPD 2 kb	CTTGCCTCTTGTCTCTTAGAT	TGTAGCACTCACCATGTAGTT
α-sat DNA	CTTTTTCATCATAGGCCTCAA	AGCTCACAGAGCTGAAACATT