Conjugation of Human Topoisomerase 2α with Small Ubiquitin-like Modifiers 2/3 in Response to Topoisomerase Inhibitors: Cell Cycle Stage and Chromosome Domain Specificity

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Abstract

Type 2 topoisomerases, in particular the α isoform in human cells, play a key role in cohesion and sister chromatid separation during mitosis. These enzymes are thus vital for cycling cells and are obvious targets in cancer chemotherapy. Evidence obtained in yeast and *Xenopus* model systems indicates that conjugation of topoisomerase 2 with small ubiquitin-like modifier (SUMO) proteins is required for its mitotic functions. Here, we provide biochemical and cytologic evidence that topoisomerase 2α is conjugated to SUMO-2/3 during interphase and mitosis in response to topoisomerase 2 inhibitors and "poisons" (ICRF-187, etoposide, doxorubicin) that stabilize catalytic intermediates (cleavage complexes, closed clamp forms) of the enzyme onto target DNA. During mitosis, SUMO-2/3–modified forms of topoisomerase 2α localize to centromeres and chromosome cores/axes. However, centromeres are unresponsive to inhibitors during interphase. Furthermore, formation of topoisomerase 2α–SUMO-2/3 conjugates within mitotic chromosomes strongly correlates with incomplete chromatid decatenation and decreases progressively as cells approach the metaphase-anaphase transition. We also found that the PIASy protein, an E3 ligase for SUMO proteins, colocalizes with SUMO-2/3 at the mitotic chromosomal cores/axes and is necessary for both formation of SUMO-2/3 conjugates and proper chromatid segregation. We suggest that the efficacy of topoisomerase inhibitors to arrest cells traversing mitosis may relate to their targeting of topoisomerase 2α–SUMO-2/3 conjugates that concentrate at mitotic chromosome axes and are directly involved in chromatid arm separation. [Cancer Res 2008;68(7):2409–18]

Introduction

Topoisomerases are complex multifunctional enzymes that are required to resolve topological complexities that arise in the genome as a result of DNA-based cellular activities. In eukaryotic cells, type II enzymes act as dimers that use the energy of ATP hydrolysis to introduce a transient double-stranded cleavage in the DNA helix, through which the passage of a separate intact helix is promoted. The nicked DNA double strand is subsequently (re)ligated under ATP hydrolysis, followed by dissociation of topoisomerase 2 from target DNA. This capacity to cleave and religate both DNA strands explains why type II topoisomerases are required for diverse aspects of DNA metabolism, such as transcription, replication, recombination, chromosome condensation/decondensation and segregation, and possibly DNA repair (1–3). Indeed, certain types of topological complexities, such as knots, tangles, and catenanes require for their resolution that DNA be cleaved on both strands (4).

Whereas yeasts and *Drosophila* contain only a single type II topoisomerase, mammals possess two isoforms of the enzyme, α and β (5). Although both isoforms may facilitate transcription of chromatin templates (6–8), only the α isoform is absolutely required for full removal of catenanes remaining in DNA after replication in S phase (2, 3), which is essential for chromatid separation during anaphase (9, 10).

Small ubiquitin-like modifier (SUMO) proteins, which in mammals comprise three isoforms (1–3), are distantly related to ubiquitin (20% identity), and their covalent binding to target proteins occurs through a stepwise process closely resembling that used by the ubiquitin conjugation pathway (11, 12).

Topoisomerase 2 inhibitors that trap catalytic intermediates of these enzymes onto target DNA may stabilize conjugates of topoisomerase 2β with either SUMO-1 or SUMO-2/3 and of topoisomerase 2α with SUMO-1 (13, 14). However, it remains unknown whether conjugation of topoisomerase 2α with SUMO-2/3 is also promoted by these drugs. Interestingly, these drugs interfere with chromatid separation during anaphase, and recent genetic and biochemical evidence obtained from yeast and *Xenopus* model systems implicate the SUMO conjugation pathway in chromatid cohesion and separation during M phase (15–17). Data obtained using mammalian cells corroborate these conclusions (18, 19).

Because the α isoform of topoisomerase 2 plays a major role in chromosomal events occurring during mitosis, namely in chromatid cohesion and separation, it is important to determine (*a*) whether in mammalian cells traversing mitosis topoisomerase 2α conjugates with SUMO proteins and whether SUMO paralogs are differentially used in M phase and interphase and (*b*) whether in mitosis components of the SUMO conjugation pathway localize to chromosomal domains involved in chromatid cohesion/segregation, and if so, whether they modulate topoisomerase 2–dependent chromatid separation. We have, herein, addressed these issues making use of well-characterized topoisomerase 2 inhibitors that stabilize specific catalytic intermediates of the enzyme.

Materials and Methods

Cell culture and cell cycle synchronization procedures. HeLa cells were obtained from American Type Culture Collection and cultured as

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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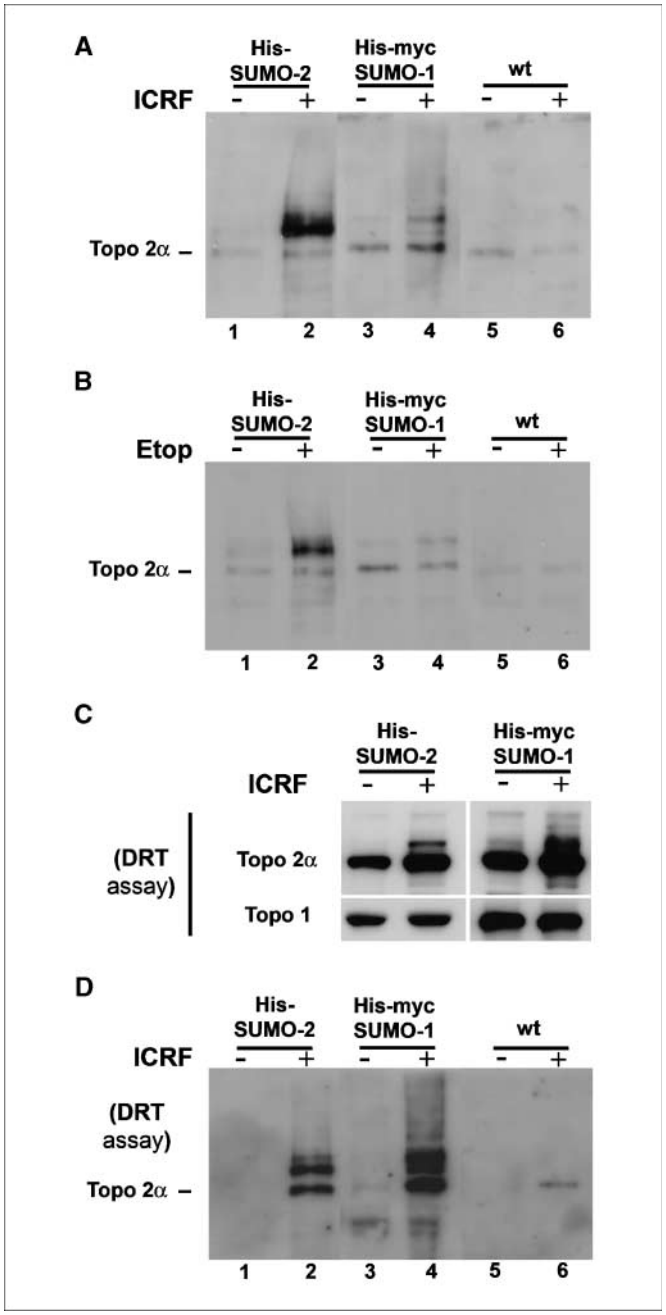

Figure 1. Topoisomerase 2 (*Topo 2*) inhibitors stabilize catalytically committed conjugates of topoisomerase IIα with SUMO-2/3. *A,* a similar number ($\sim 20 \times 10^6$) of asynchronously growing ($\sim$96% in interphase) HeLa$^{His\text{-}SUMO\text{-}2}$, HeLa$^{His\text{-}myc\text{-}SUMO\text{-}1}$, or HeLa wt cells was treated with 50 μg/mL ICRF-187 (+) or solvent alone (−) for 20 min. Cells were lysed in 6 mol/L guanidium-HCL buffer and processed for Ni^{2+}-affinity purification of His-SUMO–conjugated forms. Purified forms were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with topoisomerase 2α–specific antibodies. *B,* experimental design was as delineated above except that cell lines were exposed to 25 μmol/L etoposide (+) for 20 min or solvent alone (−). *C,* HeLa$^{His\text{-}SUMO\text{-}2}$ and HeLa$^{His\text{-}myc\text{-}SUMO\text{-}1}$ cells exposed to ICRF-187(+) (50 μg/mL, 20 min) or solvent(−) were subjected to salt (350 mmol/L NaCl)–detergent extraction according to the DRT protocol; blots of gel resolved proteins present in the insoluble remnants were probed with antibodies specific for either topoisomerase 2α or topoisomerase 1; note that topoisomerase 2α, but not topoisomerase 1, is enriched in the insoluble fraction of ICRF-187(+) cells. *D,* HeLa$^{His\text{-}SUMO\text{-}2}$, HeLa$^{His\text{-}myc\text{-}SUMO\text{-}1}$, and HeLa wt cells were treated with ICRF-187(+) or solvent(−) and extracted according to the DRT protocol, as above, before affinity (Ni-NTA) purification of salt-detergent insoluble SUMO conjugates; shown are blots of gel resolved SUMO conjugates probed for topoisomerase 2α.

described (20). Cell lines stably expressing His-tagged isoforms of SUMO proteins (SUMO-2 or SUMO-1), respectively HeLa$^{His6\text{-}SUMO\text{-}2}$ and HeLa$^{His6\text{-}myc\text{-}SUMO\text{-}1}$, were described previously (21, 22) and were cultured as indicated. Purification of mitotic cells and cell synchronization procedures (G_1-S transition and G_2 stages) were as described (20).

Antibodies and chemicals. The following antibodies were used: mouse monoclonals against topoisomerase 2α, clone Ki-S1 (Chemicon International), and clone 3D4 (Abcam); mouse monoclonal anti–β-actin (clone ac-15; Sigma-Aldrich). Rabbit polyclonal antisera specific for human topoisomerase 2α, human topoisomerase 2β, human topoisomerase 1, SUMO-2+3, SUMO-1, SMC-2, and PIASy were from Abcam. Antikinetochore autoimmune antisera specific for CENP-A/C was a kind gift from W. van Venrooij (Katholieke Universiteit, Nijmegen, the Netherlands). Anti-hemagglutinin (HA) epitope monoclonal antibody (clone 16B12) was from Covance Research Products. FITC-conjugated, Alexa 488–conjugated, Cy3-conjugated, and Cy5-conjugated affinity-purified secondary antibodies for immunofluorescence procedures were purchased from Jackson ImmunoResearch Laboratories. Chemicals used in this research were ICRF-187 from Chiron Laboratories, merbarone from Calbiochem/Merck Biosciences, and etoposide (VP-16), doxorubicin (Adriamycin), aclarubicin, and roscovitine from Sigma-Aldrich.

Affinity purification of His-tagged SUMO conjugates, immunoprecipitation, and Western blotting. Purification of His_6-SUMO conjugates and detection of proteins by Western blotting were performed as described previously (20–23). Immunoprecipitation of topoisomerase 2α and SUMO-2/3 proteins from mitotic cells was conducted after initial alkaline lysis as described elsewhere (24), except that immunoprecipitation buffer was supplemented with 10 mmol/L *N*-ethylmaleimide.

Differential retention of topoisomerase assay. The differential retention of topoisomerase (DRT) assay was performed as detailed in a previous publication (20).

Topoisomerase 2α and PIASy small interfering RNA transfection and plasmid construction. For topoisomerase 2α RNA interference (RNAi) experiments, the following oligonucleotides targeting topoisomerase 2α have been used: OLIGO 1 (5-GCA CAU CAA AGG AAG CUA A-3) and OLIGO 2 (5-GCC AUC CAC UUC UGA UGA U-3) from Eurogentech S.A. As controls, both a scramble sequence (5-GGC AAG ACU CAA GAA UCA A-3) and mock transfection (no oligo) were used. Oligonucleotides for PIASy RNAi experiments were used as previously reported (19). dsRNA oligonucleotides were transfected with Oligofectamine (Invitrogen) as per the manufacturer's instructions. Culture medium was changed 24 h after transfection, and cells were incubated for an additional 24 h before collection. pcDNA3/HA-huPIASy was constructed according to standard recombinant DNA techniques.

Immunofluorescence, microscopy, and image analysis. For immunofluorescence analysis, cells growing on coverslips were routinely fixed in freshly prepared 3.7% paraformaldehyde in HPEM buffer [30 mmol/L HEPES, 65 mmol/L Pipes, 10 mmol/L EGTA, 2 mmol/L $MgCl_2$ (pH 6.9)] plus 0.5% Triton X-100 for 10 min at room temperature before immunostaining (20). To enhance detection of SUMO proteins coverslips were placed in 10 mmol/L sodium citrate (pH 7) and subjected to microwave treatment (700 W, 30 s) before immunostaining.

Confocal microscopy analysis, image segmentation procedures and quantification of fluorescence intensities were performed as described in detail elsewhere (20).

Results

Human topoisomerase 2α is conjugated to SUMO-2 during interphase in response to ICRF-187 and etoposide. Previous studies in mammalian cells have shown that conjugation of topoisomerase 2α and β to SUMO-1 is induced by catalytic inhibitors of topoisomerase 2 activity, such as ICRF-193 (14). Here, we tested whether human topoisomerase 2α is also modified with SUMO-2 in response to different classes of topoisomerase 2 inhibitors. To this end, we used stable cell lines expressing either His_6-SUMO-2 or His_6-myc-SUMO-1 (21, 22), which allow

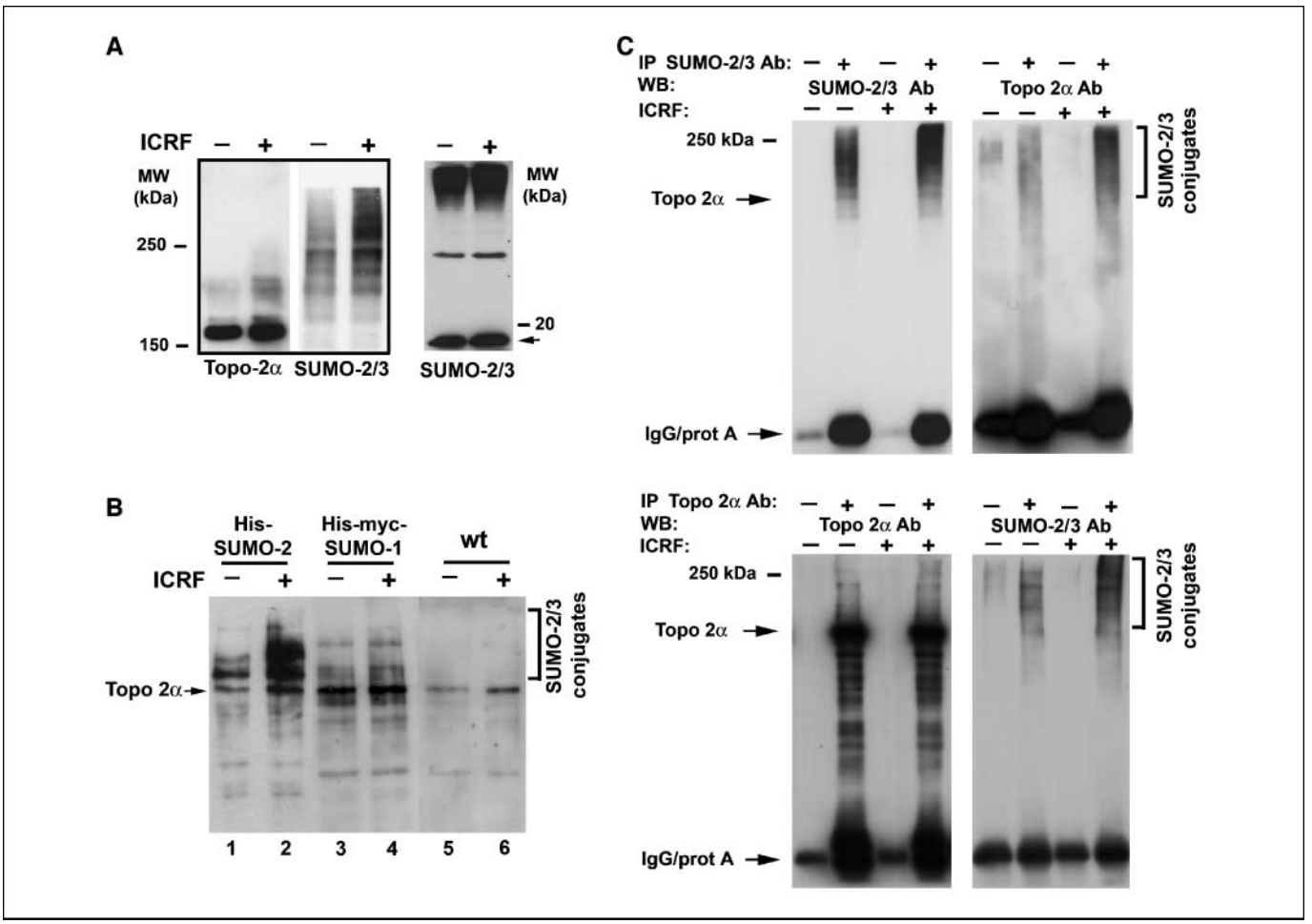

Figure 2. During mitosis human topoisomerase 2α is conjugated to SUMO-2 in response to ICRF-187. *A, left*, HeLa wt mitotic cells were purified by mechanical shake-off (≥95% mitotics), replated in poly-L-lysine coated dishes, and given either solvent(−) or ICRF-187(+) (50 μg/mL, 20 min) before lysis in Laemmli's sample buffer; proteins were resolved by SDS-PAGE in low acrylamide (6%) gels before electro-blotting and probing with antibodies specific for topoisomerase 2α and SUMO-2/3 proteins. *Right,* the same whole mitotic cell extracts were resolved in high acrylamide (12%) gel before labeling of immunoblots for SUMO-2/3 proteins; *arrow,* unconjugated/free SUMO-2/3 proteins. *B,* purified mitotic HeLa$^{His\text{-}SUMO\text{-}2}$, HeLa$^{His\text{-}myc\text{-}SUMO\text{-}1}$, and HeLa wt cells were treated with 50 μg/mL ICRF-187(+) or solvent(−) for 20 min before lysis in 6 mol/L guanidium-HCL buffer and processing for Ni^{2+}-affinity purification of His-SUMO–conjugated forms. Immunoblots of SDS-PAGE resolved conjugates were probed with topoisomerase 2α–specific antibodies. *C, top,* whole mitotic cell extracts obtained from purified HeLa wt cells (ICRF +/−; 50 μg/mL, 20 min) were subjected to immunoprecipitation (*IP*) with SUMO-2/3–specific antibodies (4 μg antiserum/ ~ 20 × 10^6 cells), separated in 7% acrylamide gels and processed for Western blotting (*WB*) with SUMO-2/3–specific and topoisomerase 2α–specific (mouse monoclonal plus rabbit polyclonal) antibodies. For each experimental group (ICRF +/−), equal amounts of mitotic extract were mock-precipitated in parallel (no antibody controls; *bottom*) and whole mitotic cell extracts obtained and treated as above (ICRF +/−; 50 μg/mL, 20 min) were subjected to immunoprecipitation with anti–topoisomerase 2α monoclonal antibody (clone KiS1; 5 μg antibody/ ~ 20 × 10^6 cells) or else mock-precipitated. Western blots were probed with anti–topoisomerase 2α and anti–SUMO-2/3 antibodies as described above.

purification by affinity procedures of proteins conjugated with SUMO-2 or SUMO-1, respectively. Asynchronous cell populations (~ 96% interphasic) were thus exposed to ICRF-187 (50 μg/mL, 20 minutes) or solvent alone (controls), and whole-cell lysates were subsequently used for affinity purification of SUMO conjugates with Ni-NTA agarose. HeLa cells not expressing His_6-tagged SUMO isoforms ("HeLa wt") were processed in parallel as a negative control. Probing Western blots with anti–topoisomerase 2α antibodies revealed an enrichment in topoisomerase 2α–SUMO-1 conjugates in ICRF-187–treated cells, as expected (Fig. 1*A, lanes 3 and 4*), but also prominent modification of topoisomerase 2α with SUMO-2 (Fig. 1*A, lanes 1 and 2*). We next tested whether a prototypical topoisomerase 2 poison, etoposide (25 μmol/L, 20 minutes), which stabilizes cleavage complexes similarly induced conjugation of topoisomerase 2α with SUMO-2. Interestingly, treatment with etoposide resulted in significantly higher levels of topoisomerase 2α–SUMO-2 conjugates (Fig. 1*B, lanes 1 and 2*). Etoposide, as previously described for teniposide (14), also led to increased conjugation of topoisomerase 2α with SUMO-1 (Fig. 1*B, lanes 3 and 4*). Together, these results indicate that two different classes of topoisomerase 2–specific drugs, which stabilize topoisomerase 2 dimers at distinct stages of the catalytic cycle, result in increased conjugation of the enzyme with SUMO-2 during interphase.

Catalytically committed topoisomerase 2α is conjugated to SUMO-2 and SUMO-1 during interphase. We have described previously the DRT assay that allows an enrichment in catalytically committed forms of topoisomerase 2 (20). In this procedure, a topoisomerase 2–specific drug is used to selectively trap onto target DNA topoisomerase 2 molecules that entered the catalytic cycle, thus rendering this fraction insoluble upon subsequent exposure to salt plus detergent; controls exposed to drug solvent alone are processed in parallel. Consequently, increments in salt-detergent insoluble topoisomerase 2 that are observed in

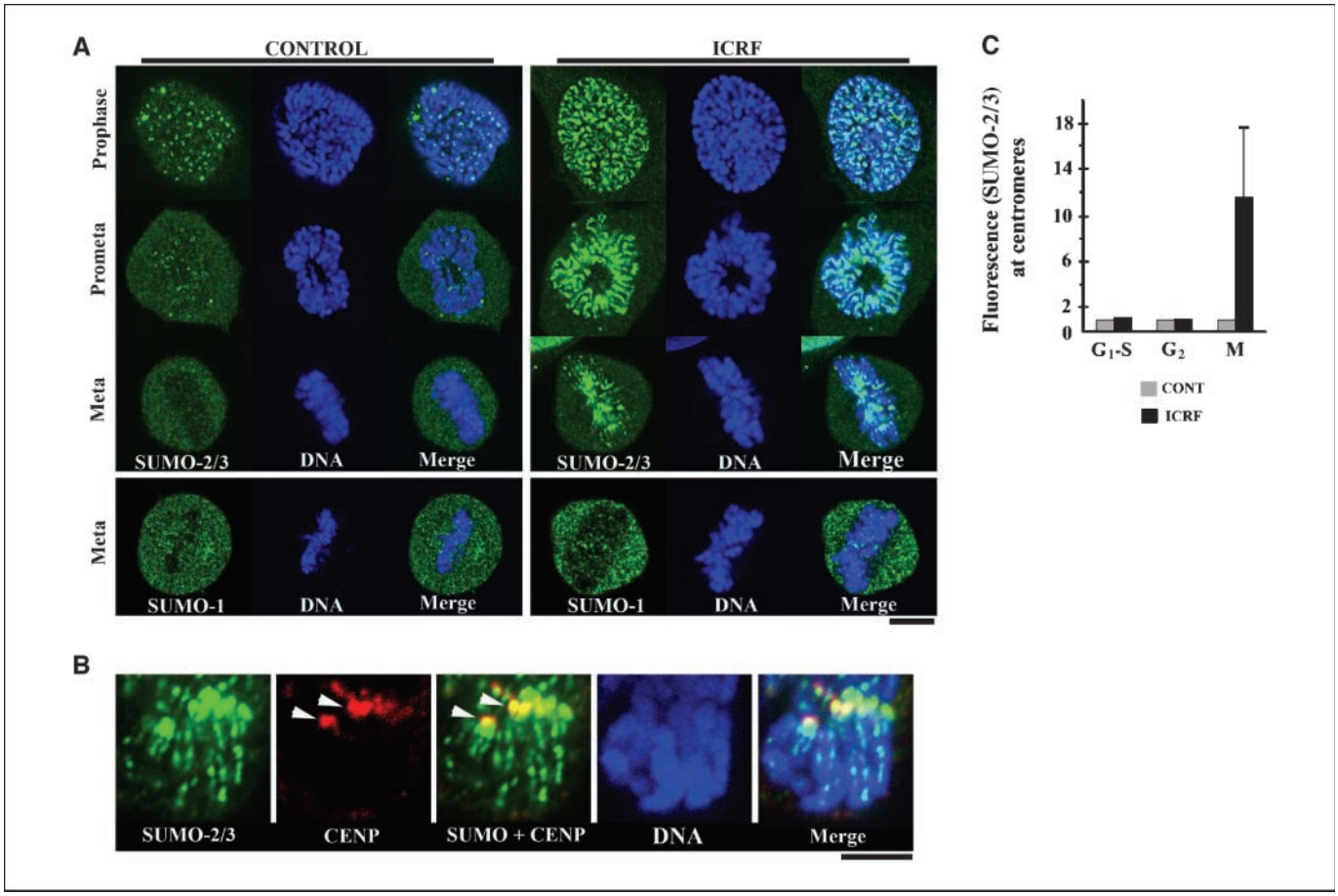

Figure 3. ICRF-187 induces association of SUMO-2/3 with mitotic chromatin at stages preceding sister chromatid separation. *A,* cells exposed to solvent alone (*left*; controls) or ICRF-187 (*right*; 50 μg/mL, 15 min) were stained for either SUMO-2/3 or SUMO-1 with specific polyclonal antisera and imaged while traversing mitotic stages preceding anaphase; DNA was stained with DAPI (*bar,* 7 μm). *B,* close-up view of a metaphase cell exposed to ICRF-187 and stained for SUMO-2/3 and centromeres (CENP A/C staining; *arrowheads*); colocalization of the two patterns appears yellow in the merged image (SUMO + CENP; *arrowheads*). Additional superimposition of DNA (DAPI) staining shows that SUMO-2/3 also delineates chromosome arm cores/axes in a dotted, discontinuous pattern (*Merge*); *bar,* 4 μm. *C,* cells synchronized at the G_1-S border (hidroxyurea block), traversing G_2 (10 h after release from a hidroxyurea block) or M stage cells (prometaphase/metaphase) were given solvent (controls) or ICRF-187 and costained for SUMO-2/3 and CENP A/C. Cells were imaged by confocal microscopy using identical, high sensitivity, image capture settings. For G_1-S and G_2 stages, 25 cells were analyzed per experimental group. For M stage, 50 cells per experimental group were analyzed in each of a triplicate set of experiments. The intensity of the SUMO-2/3 signal that colocalized with centromeric regions (CENP A/C staining) was quantified. For each cell cycle substage (G_1-S, G_2, M), the average intensity of centromere-associated SUMO-2/3 signals in the ICRF-187–treated groups was divided by the average intensity of the centromeric SUMO-2/3 signals obtained in the corresponding matched controls (normalized to value of 1); thus, the bar corresponding to the ICRF-187 group represents the fold increase relative to the normalized control.

drug-treated cells relative to controls mostly comprise catalytically committed forms of the enzyme (20). Here, we used the DRT assay to address whether topoisomerase 2α that is modified by SUMO proteins is predominantly catalytically committed. To do so, HeLa cells were exposed to ICRF-187 (50 μg/mL, 20 minutes) and briefly (2 minutes) extracted in ice-cold buffer containing detergent (Triton X-100) plus 350 mmol/L NaCl (cf. Materials and Methods). The insoluble remnant was lysed in guanidium and used for affinity purification (Ni-NTA agarose) of SUMO-1 or SUMO-2 conjugates before gel separation and immunoblotting for topoisomerase 2α. Western blots of proteins remaining in extracted cells before undertaking the affinity purification step revealed that ICRF-187 treatment, as previously shown for HeLa wt cells (20), readily induced retention of additional topoisomerase 2α, but not topoisomerase 1, in $HeLa^{His\text{-}SUMO\text{-}2}$ and $HeLa^{His\text{-}myc\text{-}SUMO\text{-}1}$ cell lines (Fig. 1*C*). Analysis of purified SUMO-1 and SUMO-2 conjugates revealed that exposure to ICRF-187 dramatically increased retention of topoisomerase 2α forms modified with SUMO-1 (Fig. 1*D, lanes 3 and 4*) and SUMO-2 (Fig. 1*D, lanes 1 and 2*). These data are consistent with SUMO-2 and SUMO-1 modifications occurring on catalytically committed topoisomerase 2α molecules that were stabilized onto DNA by the inhibitor.

During mitosis, human topoisomerase 2α is conjugated to SUMO-2 in response to ICRF-187. Previous reports have provided evidence that in the *Xenopus* egg system SUMO-2/3 is the preferred isoform for conjugation with topoisomerase II during mitosis (16, 17). To address if the same occurs in mammalian cells, highly purified mitotic HeLa cells expressing wt SUMO proteins were given ICRF-187 (50 μg/mL, 20 minutes) or solvent (controls), and whole-cell extracts were prepared for SDS-PAGE. Western blots of gel-resolved proteins were subsequently probed with antibodies specific for either topoisomerase 2α, SUMO-2/3, or SUMO-1. The results showed that in ICRF-187–treated cells, there was a modest, but consistent, increase in SUMO-2/3 conjugates of high molecular mass (≥170 kDa) in relation to controls (SUMO-2/3; Fig. 2*A, left*); a similar change in SUMO-1 conjugates could not be detected (data not shown). Of note, bands of topoisomerase 2α that became

upper-shifted in response to ICRF-187 were barely visible, suggesting only a minor fraction of the enzyme might engage in conjugation with SUMO proteins (topoisomerase 2α; Fig. 2*A, left*). Accordingly, ICRF-187 did not induce any significant reduction of free SUMO-2/3 proteins (Fig. 2*A, right, arrow*).

To ascertain whether topoisomerase 2α is indeed a mitotic substrate for SUMO-2/3 conjugation, HeLa$^{His\text{-}SUMO\text{-}2}$ and HeLa$^{His\text{-}myc\text{-}SUMO\text{-}1}$ cell lines were incubated with ICRF-187 before affinity purification of His_6-SUMO forms. As shown in Fig. 2*B* (*lanes 1 and 2*), ICRF-187 induced a strong increment in the amount of topoisomerase 2α–SUMO-2 while not significantly changing topoisomerase 2α–SUMO-1 levels (Fig. 2*B, lanes 3 and 4*). Finally, immunoprecipitation of mitotic extracts obtained from HeLa wt cells (plus/minus ICRF-187) revealed that topoisomerase 2α and high molecular mass SUMO-2/3 conjugates (≥170kDa) coprecipitated when either anti–SUMO-2/3 or anti–topoisomerase 2α antibodies were used, with coprecipitation being enhanced by the presence of ICRF-187 (Fig. 2*C*). An indifferent anti-p53 antibody (p53 is mostly absent in HeLa cells) did not precipitate detectable amounts of either topoisomerase 2α or SUMO-2/3 (data not shown). These results show that human topoisomerase 2α is conjugated to both SUMO-2 and SUMO-1 during mitosis, but ICRF-187 only significantly affects conjugation with the SUMO-2 isoform.

ICRF-187 induces accumulation of SUMO-2/3 in mitotic chromosomes. In the yeast and *Xenopus* model systems, conjugation of topoisomerase 2 with SUMO paralogs during mitosis is required for proper sister chromatid cohesion and segregation (15, 17, 25). Therefore, we were interested in determining whether topoisomerase 2α–SUMO conjugates targeted chromosomal domains involved in cohesion and separation of chromatids. To this end, we costained HeLa cells for SUMO-2/3, centromeres (CENP A/C antiserum), and DNA [4′,6-diamidino-2-phenylindole (DAPI) staining] and searched for cells traversing specific substages of M phase. The results revealed a weak, but consistent, staining for SUMO-2/3 within mitotic chromatin in prophase and prometaphase cells (Fig. 3*A, left*). As reported previously in HeLa cells (26), staining for SUMO-2/3 was mostly absent from chromosomes at metaphase and anaphase stages, but was obvious in the reforming nuclei of telophase cells (Fig. 3*A*; data not shown). Exposure to ICRF-187 dramatically increased association of SUMO-2/3 proteins with mitotic chromosomes in prophase and prometaphase cells, and in a fraction of cells traversing metaphase (Fig. 3*A, right*; data not shown); typically, staining was less intense during metaphase than at preceding stages (cf. Fig. 3*A*). Within chromosomes, distribution of SUMO-2/3 proteins displayed a distinctive concentration at centromeres and the

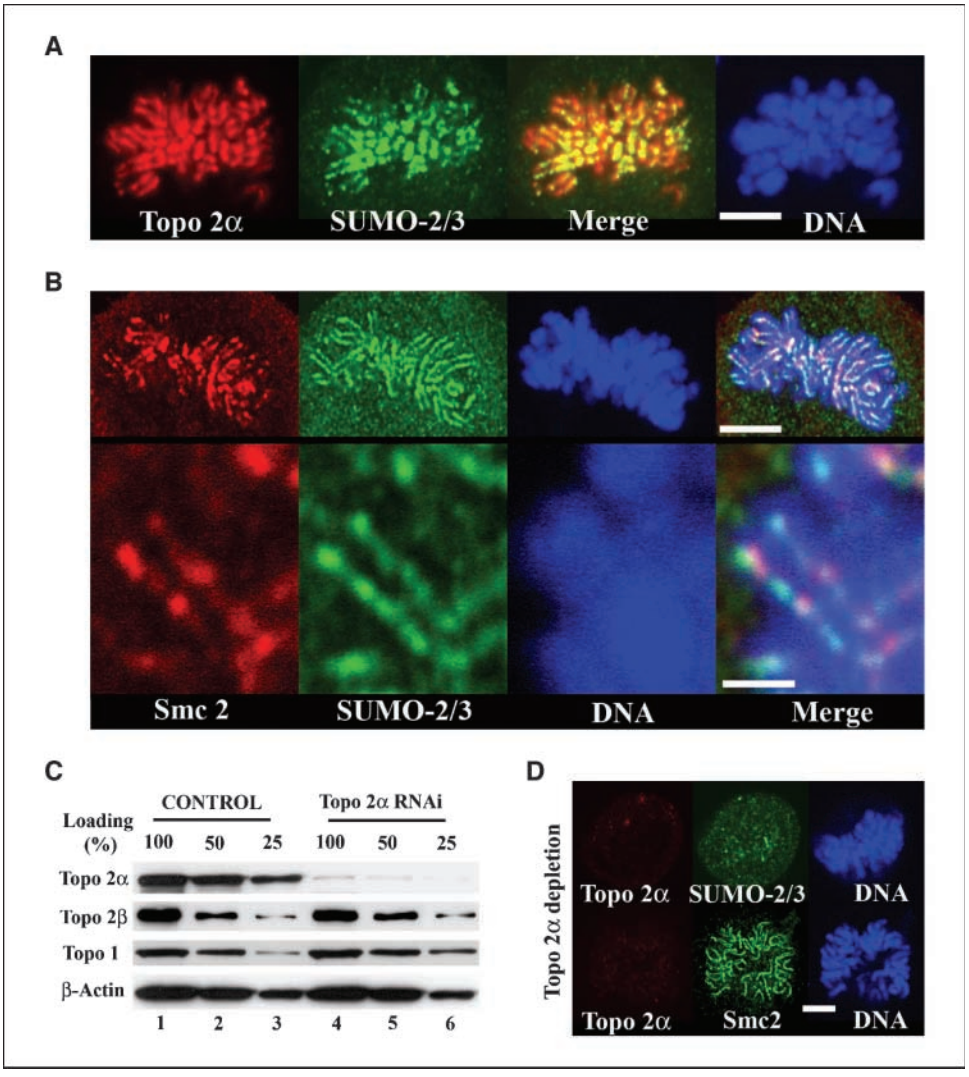

Figure 4. ICRF-187 induces concentration of SUMO-2/3 at mitotic chromosome cores/axes in a topoisomerase 2α–dependent fashion. *A,* cells exposed to ICRF-187 (50 μg/mL, 15 min) were costained for topoisomerase 2α and SUMO-2/3. Regions of colocalization of the two staining patterns are depicted in yellow in the merged image; DNA is stained with DAPI. *Bar,* 5 μm. *B,* colabeling of ICRF-187–treated cells for smc2 (condensin subunit) and SUMO-2/3; DNA is stained with DAPI. *Bar,* 5 μm. *Bottom,* high-magnification images; *bar,* 2 μm. *C,* protein extracts from HeLa cells that were either pseudodepleted (scramble sequence; controls) or depleted of topoisomerase 2α with specific small interfering RNA (topoisomerase 2α RNAi) were probed for topoisomerase 2α, topoisomerase 2β, topoisomerase 1, and β-actin (loading control) by Western blotting; to better judge the degree of specific depletion, different amounts of protein extract were loaded per experimental group (expressed in percentage). Note that only topoisomerase 2α is specifically depleted. *D,* cells depleted of topoisomerase 2α with specific small interfering RNA were given ICRF-187 and costained for either topoisomerase 2α and SUMO-2/3 (*top*) or topoisomerase 2α and smc 2 (*bottom*); DNA is stained with DAPI. *Bar,* 5 μm.

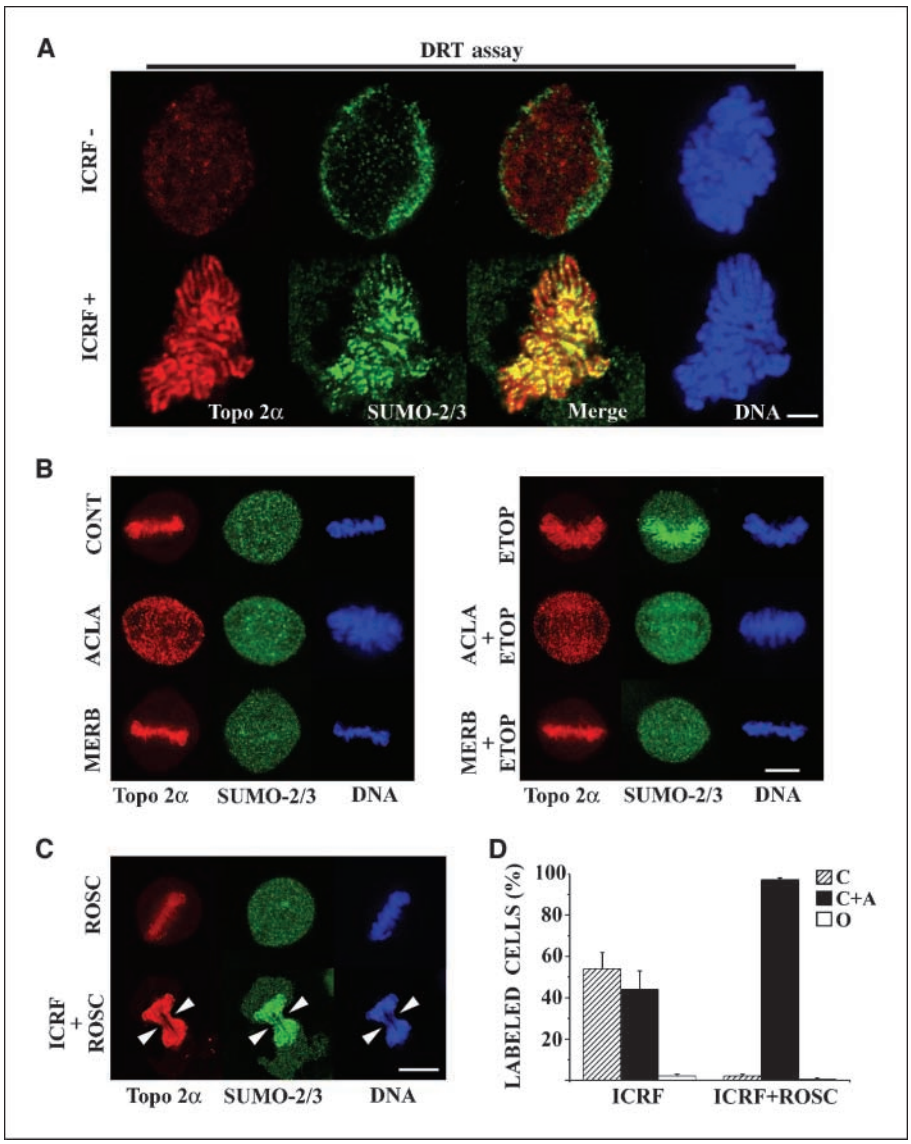

Figure 5. Retention of SUMO-2/3 onto mitotic chromatin correlates with catalytic commitment of topoisomerase 2α preceding full chromatid resolution. *A,* cells exposed to either solvent (controls) or ICRF-187 were extracted with salt (350 mmol/L NaCl) plus detergent (DRT procedure) and fixed in formaldehyde and immunostained for topoisomerase 2α and SUMO-2/3; regions of colocalization appear yellow in the merged image. DNA is stained with DAPI. *B,* cells exposed to drug solvent (control) or aclarubicin (ACLA; 2 μmol/L, 15 min), merbarone (*MERB*; 40 μmol/L, 15 min), etoposide (*ETOP*; 200 μmol/L, 15 min) were stained for topoisomerase 2α, SUMO-2/3, and DNA (DAPI). Cells exposed to aclarubicin or merbarone preceding addition of etoposide (*ACLA* + *ETOP*; *MERB* + *ETOP*) were similarly stained. *C,* cells exposed to either roscovitine (*ROSC*; 50 μmol/L, 7 min) or ICRF-187 (50 μg/mL, 15 min) plus roscovitine (*ROSC* + *ICRF*; roscovitine added 7 min before cell collection) were stained for SUMO-2/3 and DNA (DAPI). A metaphase cell (*top*) and a cell arrested at the metaphase/anaphase transition (*bottom*). Note the continuity between lagging chromosome arms (*arrowheads*). *Bars,* 5 μm. *D,* cells exposed to either ICRF-187 (*ICRF*) or else ICRF-187 plus roscovitine (*ICRF* + *ROSC*) as described above were colabeled for SUMO-2/3 and CENP A/C (to highlight centromeric domains). Prometaphase and metaphase cells were scored according to the pattern of staining for SUMO-2/3 as either no staining (*O*), staining restricted to centromeric regions (*C*), or staining at both centromeres and chromosome arms (*C* + *A*). Histograms depict the distribution of staining patterns in each experimental group (ICRF and ICRF + ROSC) evaluated in triplicate experiments (≥80 cells analyzed per experiment).

cores (axes) of chromosome arms (Fig. 3*B*). A detailed analysis of anaphase was hampered possibly because ICRF-187 blocks transition from metaphase to anaphase, and cells already at anaphase upon exposure to the drug might have reached telophase by the end of the experiment. SUMO-1, however, did not concentrate at mitotic chromosomes in response to ICRF-187 (Fig. 3*A, bottom*). Interestingly, ICRF-187–dependent accumulation of SUMO-2/3 at centromeres did not occur in interphase cells (G_1-S transition, G_2 stage) and was exclusive to M phase as confirmed by quantitative analysis of the SUMO-2/3–specific fluorescent signals (Fig. 3*C*).

If accumulation of SUMO-2/3 in response to ICRF-187 corresponds, at least partially, to SUMO–topoisomerase 2α conjugates then colocalization between the staining patterns of these two proteins should occur; this was indeed observed (Fig. 4*A*, merge).

Topoisomerase 2α is known to concentrate at a chromosome axis that is intertwined with, but mostly separate from, the axis that concentrates components of the condensin complex (27, 28). Thus, we subsequently analyzed the spatial relationship between the SUMO-2/3 axis induced by ICRF-187 and the staining pattern of the smc2 component of the condensin complex. The axis delineated by SUMO-2/3, as expected from its association with topoisomerase 2α, was mostly distinct from that decorated with condensin (Fig. 4*B*). To further ascertain whether the association of SUMO-2/3 with mitotic chromosomes in response to ICRF-187 was indeed dependent on topoisomerase 2α, we depleted this enzyme from HeLa cells using small interfering RNA (siRNA) technology; mock-depleted cells served as controls (cf. Materials and Methods). The results showed that after depletion of >75% to 80% of topoisomerase 2α (Fig. 4*C*), a fraction of mitotic cells failed to concentrate topoisomerase 2α at chromosomes by immunofluorescence analysis (Fig. 4*D*). Importantly, these cells also displayed little to none SUMO-2/3 onto chromosomes in response to ICRF-187 (Fig. 4*D*). Noteworthy, the reduction of SUMO-2/3 at chromosome cores in cells severely depleted for topoisomerase 2α does not relate to absence of an axial structure because the condensin axis persists in these cells (Fig. 4*D*). Taken together, these data indicate that minor amounts of SUMO-2/3 proteins associate normally with chromatin during mitotic stages that

precede chromosome separation. However, association of SUMO-2/3 with chromosomes (centromeres plus axes) increases sharply in presence of a topoisomerase 2 inhibitor (ICRF-187) and normal levels of topoisomerase 2α.

Association of SUMO-2/3 with mitotic chromatin requires catalytic commitment of topoisomerase 2α. The data presented above are consistent with SUMO-2/3 conjugation targeting a subpopulation of catalytic intermediates of topoisomerase 2α (closed clamps) that are trapped onto DNA by ICRF-187. Thus, both SUMO-2/3 and a fraction of topoisomerase 2α should resist the salt-detergent extraction used in the DRT protocol and remain colocalized in the mitotic chromosomes. To test this prediction, cells given ICRF-187 (50 μg/mL, 15 minutes) or solvent (controls) were subjected to extraction with the DRT buffer before fixation with formaldehyde. Costaining for topoisomerase 2α and SUMO-2/3 revealed little topoisomerase 2α and essentially no SUMO-2/3 over mitotic chromatin in control cells. By contrast, in ICRF-187–treated populations, fluorescent signals from both proteins were notoriously more intense and colocalized at centromeres and chromosome cores/axes (Fig. 5*A*).

To further test whether association of SUMO-2/3 with mitotic chromatin requires entry of topoisomerase 2α into catalysis, we next used a battery of clinically relevant and well-characterized inhibitors of topoisomerase 2 that block its catalytic cycle at distinct steps. This set of drugs comprised two topoisomerase 2 inhibitors, aclarubicin and merbarone, that abrogate catalysis at the early steps preceding DNA cleavage (29–31) and two poisons, etoposide and doxorubicin (Adriamycin), that covalently stabilize topoisomerase 2–DNA complexes at the cleavage complex stage (30, 32, 33), which precedes the closed clamp conformation stabilized by ICRF-187. Cells were thus exposed to either aclarubicin (2 μmol/L, 15 minutes), merbarone (40 μmol/L, 15 minutes), etoposide (200 μmol/L, 15 minutes), or doxorubicin (50 μmol/L, 15 minutes) before fixation and immunostaining with anti–SUMO-2/3 and anti–topoisomerase 2α antibodies. Microscopic analysis revealed that only drugs that stabilize catalytic intermediates, i.e., etoposide and doxorubicin, allow retention of SUMO-2/3 onto mitotic chromosomes (Fig. 5*B*; data not shown). In subsequent experiments, cells were exposed to aclarubicin (2 μmol/L, 30 minutes) or merbarone (40 μmol/L, 30 minutes), which impede initiation of catalysis, preceding addition of either etoposide (200 μmol/L, last 15 minutes) or ICRF-187 (50 μg/mL, last 15 minutes) to the cultures; note that aclarubicin and merbarone remained present after subsequent addition of the other drugs. Staining for SUMO-2/3 and topoisomerase 2α showed that prior exposure to aclarubicin or merbarone abrogated etoposide-induced and ICRF-187–induced accumulation of SUMO-2/3 onto mitotic chromosomes (Fig. 5*B*; data not shown). Together, these results support the hypothesis that only topoisomerase 2 complexes that have engaged into DNA cleavage/religation activities become targets for conjugation with SUMO-2/3 during mitosis.

Accumulation of SUMO-2/3 in response to ICRF-187 correlates with incomplete decatenation at chromosome arms. We reasoned that if concentration of SUMO-2/3 at chromosome cores correlates with topoisomerase 2α–dependent catalytic activity, then failure to accumulate SUMO-2/3 at chromosome arms in response to ICRF-187 (~50% of metaphase cells show staining for SUMO-2/3 restricted to centromeres; Fig. 5*D*) might highlight full catenane resolution; conversely, retention of SUMO-2/3 should imply incomplete decatenation. We have tested these predictions by forcing exit from mitosis with roscovitine, a cdk inhibitor, in presence of ICRF-187. Because ICRF-187 impedes passage through anaphase of cells with incomplete chromatid decatenation, we anticipated a positive correlation between ability to retain SUMO-2/3 and trapping in the preanaphase compartment. Indeed, treatment with ICRF-187 (15 minutes) plus roscovitine (last 7 minutes, 50 μmol/L) before collection led to appearance of abundant cells at metaphase-anaphase transition with chromosomes showing intense staining for SUMO-2/3 and lagging arms, the hallmark of insufficient decatenation (Fig. 5*C*); centromeric domains (CENP A/C staining), however, were fully separated as previously shown for ICRF-187–treated cells (ref. 34; data not shown). Of note, roscovitine per se did not induce retention of SUMO-2/3 in mitotic chromatin (Fig. 5*C*). Remarkably, metaphase cells without SUMO-2/3 staining of the arms (i.e., centromeric staining only) were now virtually absent (Fig. 5*D*; data not shown). Therefore, we favor the interpretation that cells that failed to retain SUMO-2/3 at chromosome cores in response to ICRF-187 must have completed chromatid arm resolution and thus escaped the metaphase block imposed by ICRF-187.

PIASy localizes to mitotic chromosome cores and promotes accumulation of SUMO-2/3 in response to ICRF-187. The PIASy protein, an E3 ligase for SUMO-2/3, was shown to be responsible for the concentration of SUMO-2 conjugates at mitotic centromeric domains and mediation of chromatid segregation in *Xenopus* (17). Here, we asked whether PIASy was required for the concentration of SUMO-2/3 conjugates at the mitotic chromosome cores in mammalian cells. We first searched for the localization of PIASy in interphase and mitotic HeLa cells expressing HA-PIASy by immunostaining with anti-HA antibodies. This showed that during interphase PIASy is nucleoplasmic with occasional concentration in nuclear bodies that accumulate PML and SUMO proteins, as described (ref. 35; Fig. 6*A*; data not shown). PIASy staining sharply delineates chromosome cores/axes during prometaphase and metaphase and dissociates from chromatin during anaphase to reappear in the reforming nuclei at telophase (Fig. 6*A*; data not shown). Note that global levels of PIASy (Western blotting) are not affected by ICRF-187 (Fig. 6*A*). Although PIASy localizes to centromeric domains, there is no obvious accumulation within these regions (data not shown). Double staining experiments revealed that in ICRF-187–treated cells, PIASy and SUMO-2/3 proteins colocalize extensively (Fig. 6*A; middle-bottom*). These results, revealing for the first time the localization of PIASy within mitotic chromosomes, suggested a role for PIASy in the formation of SUMO-2/3 conjugates at chromosomal cores. To further test this idea, we used siRNA technology to efficiently deplete HeLa cells of PIASy protein (Fig. 6*B*). Staining of PIASy-depleted cells for topoisomerase 2α showed diffuse staining over mitotic chromosomes with ill-defined axes, as reported (Fig. 6*B*; ref. 19). Also, we noticed that misaligned chromosomes at metaphase plates (Fig. 6*B, arrow*) increased significantly after depletion of PIASy (Fig. 6*C*). More importantly, depletion of PIASy almost completely abrogated the ICRF-187–induced concentration of SUMO-2/3 proteins within mitotic chromosomes (Fig. 6*B*). Finally, we checked whether PIASy-depleted cells displayed chromatid segregation defects upon roscovitine-induced forced mitotic exit in presence of ICRF-187. Results revealed that, under steady-state conditions (no drug), the distribution of mitotic populations between the preanaphase (prometaphase plus metaphase) and postmetaphase (anaphase plus telophase) compartments was similar in PIASy-depleted and nondepleted controls (Fig. 6*D*). However, upon addition of

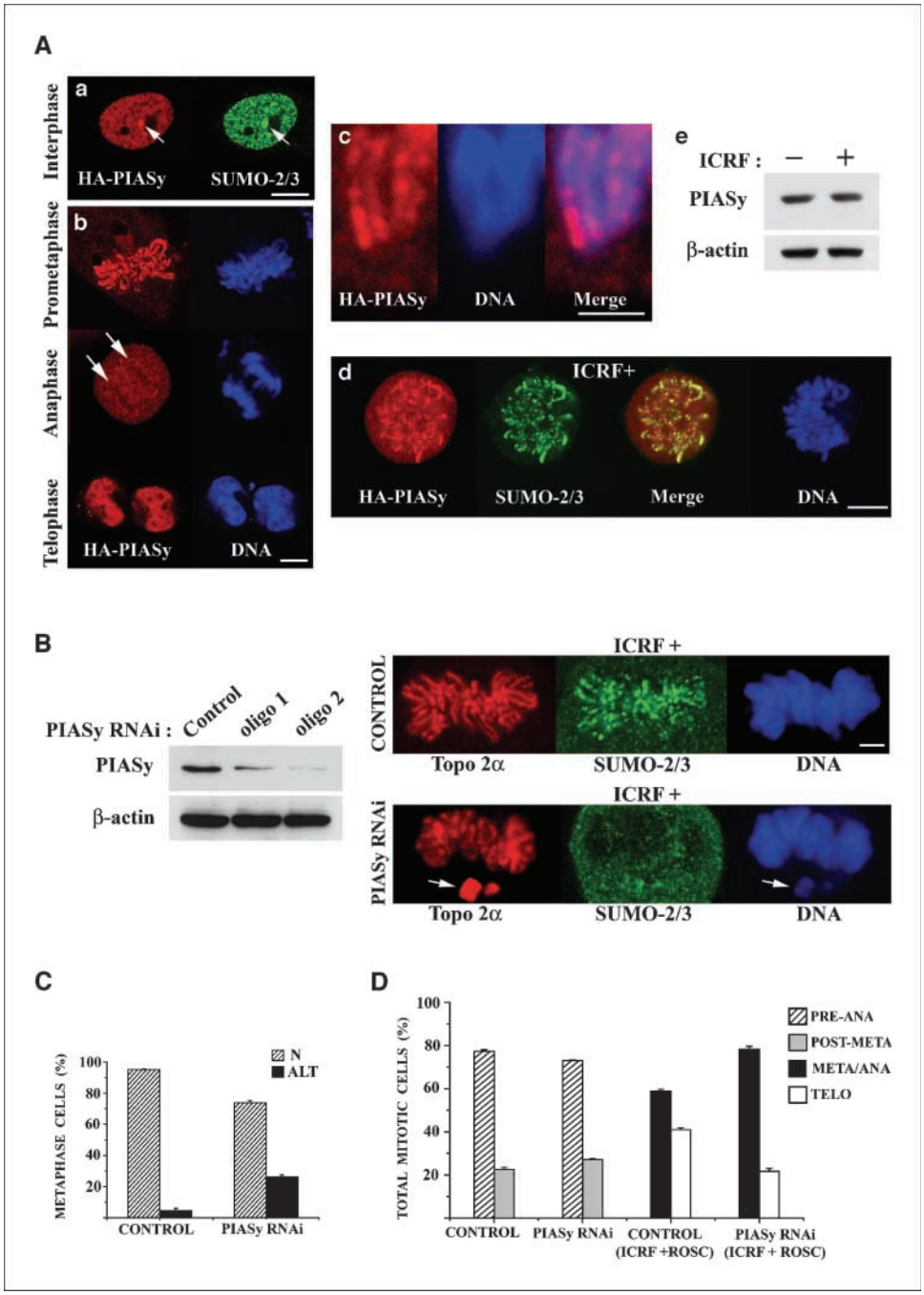

Figure 6. PIASy localizes to mitotic chromosome cores/axes to promote accumulation of SUMO-2/3 conjugates and sister chromatid resolution. *A,* HeLa cells expressing HA-PIASy (~24 h after transfection) were costained for PIASy (anti-HA antibody) and SUMO-2/3. Note that in interphase, PIASy distributes in the nucleoplasm with occasional concentration in nuclear bodies (arrows) that also accumulate SUMO-2/3 (*a*). *Bar,* 5 μm. *b,* shown are cells traversing different stages of mitosis stained for PIASy (anti-HA antibody) and DNA (DAPI). Note absence of HA-PIASy over chromosome regions (*arrows*) at anaphase. *Bar,* 5 μm. *c,* close-up view of a metaphase cell expressing HA-PIASy and immunolabeled with the anti-HA antibody; DNA is stained with DAPI. Note localization of PIASy at chromosome cores in the merged image. *Bar,* 2 μm. *d,* cells expressing HA-PIASy were exposed to ICRF-187 (50 μg/mL, 20 min) and double-immunolabeled for PIASy and SUMO-2/3. A prometaphase cell; note that regions of colocalization appear yellow in the merge. DNA is stained with DAPI. *Bar,* 5 μm. *e,* levels of PIASy in nontransfected mitotic HeLa cells (ICRF −/+) were determined by Western blot analysis; β-actin levels serve as loading controls. *B, left,* Western blots of whole-cell protein extracts obtained from mock-depleted (control) and PIASy-depleted populations (two different silencing oligonucleotides: *oligo 1, oligo 2*) were probed with antibodies specific for PIASy and β-actin (loading control); *right,* HeLa cells were depleted of PIASy with specific siRNA (PIASy RNAi) or mock-depleted (control) and given ICRF-187 (50 μg/mL, 20 min) before staining for topoisomerase 2α and SUMO-2/3. Note the reduced amounts of SUMO-2/3 proteins within mitotic chromosomes in the PIASy-depleted cell; also shown in this confocal section are two chromosomes that did not incorporate into the metaphase plate (*arrows*). DNA is stained with DAPI. *Bar,* 2 μm. *C,* PIASy-depleted (PIASy RNAi) and mock-depleted (control) HeLa cell populations were stained with DAPI, and cells at metaphase stage were classified as having either normal (*N*) metaphase plates (fully congregated chromosomes) or altered (*ALT*) metaphase plates with misincorporated chromosomes; ≥250 cells per experimental group were analyzed in each of a triplicate set of experiments. *D,* PIASy-depleted (PIASy RNAi) and mock-depleted (control) HeLa cell populations were stained with DAPI and cells at the preanaphase (*preANA*; prometaphase + metaphase) and postmetaphase (*postMETA*; anaphase + telophase) compartments were quantified. In parallel experiments, control and PIASy-depleted populations were exposed to ICRF-187 (50 μg/mL, 20 min) plus roscovitine (50 μmol/L) and cells trapped at the metaphase-anaphase transition (*META/ANA*) and traversing telophase (*TELO*) were scored; ≥250 cells per experimental group were analyzed in each of a triplicate set of experiments.

roscovitine (plus ICRF-187, 20 minutes), PIASy-depleted cells became significantly more trapped at the metaphase-anaphase transition than the nondepleted controls (Fig. 6*D*).

Together, these data are consistent with PIASy localizing at mitotic chromosome cores and acting locally to promote chromatid separation and the formation of SUMO-2/3 conjugates that may become stabilized by ICRF-187.

Discussion

In this work, we show that topoisomerase 2–specific drugs (e.g., etoposide and ICRF-187) that stabilize catalytic intermediates of topoisomerase 2, namely cleavage complexes and closed clamp forms, promote accumulation of topoisomerase 2α–SUMO-2/3 conjugates during both interphase and mitosis. During mitosis SUMO-modified topoisomerase 2α localizes to chromosome domains that are involved in chromatid cohesion and separation, i.e., the centromeres and chromosome axes. We propose that the sumoylation of this specific subpopulation of topoisomerase 2α may explain, at least partially, the mitotic arrest induced by some topoisomerase 2–specific drugs.

The functional relevance of sumoylation of topoisomerase 2 during mitosis seems preserved from simple to higher eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, which harbors a single SUMO species (smt3/SUMO-1) and a single gene encoding for topoisomerase 2, mutation of the smt4 isopeptidase responsible for the removal of smt3/SUMO-1 led to cohesion defects at centromere-proximal regions during mitosis (15). This defect was corrected in strains containing a mutant topoisomerase 2 that was resistant to Smt3/SUMO-1 modification, suggesting an important role for sumoylation of topoisomerase 2 in chromatid cohesion (15). Using the *Xenopus* egg extract system, it was shown that topoisomerase 2 conjugates exclusively with SUMO-2/3 during mitosis and that this conjugation was required for proper separation of chromatids during anaphase (16). As expected for a role of sumoylation in chromatid cohesion and segregation, SUMO proteins were found at centromeres of mitotic chromosomes in both *S. cerevisiae* and *Xenopus* (15, 17). Intriguingly, mammalian SUMO paralogs were not detectable over mitotic chromosomes during the metaphase to anaphase transition in HeLa cells (26). Herein, we have performed a detailed analysis of the distribution of SUMO-2/3 proteins in HeLa cells from prophase to early G_1 stage. In agreement with previously reported data (26), we also found that SUMO-2/3 was mostly undetectable at chromatin during metaphase and anaphase (Fig. 3*A* and *B*; data not shown). However, during prophase and prometaphase, centromeres and chromosome arms were weakly, but consistently, labeled for SUMO-2/3 but not SUMO-1 (Fig. 3*A* and *B*). These findings indicate that under normal circumstances SUMO-2/3 proteins are present onto mitotic chromatin at low levels preceding chromatid separation. A brief exposure of cells to ICRF-187 sufficed to dramatically increase staining of mitotic chromosomes for SUMO-2/3 during prophase and prometaphase and, to a lesser extent, metaphase (Fig. 3*A*). Our data strongly support the notion that this drug-induced increment in SUMO-2/3 proteins reflects the cumulative retention of sumoylated catalytic intermediates of topoisomerase 2α onto mitotic chromatin, as discussed below. First, this accumulation of SUMO-2/3 is observed when cells are treated with topoisomerase 2–specific inhibitors and poisons that stabilize catalytic intermediates of topoisomerase 2, but not with inhibitors that abrogate initiation of catalysis (aclarubicin, merbarone). Second, preexposure of cells to aclarubicin and merbarone prevents accumulation of SUMO-2/3 when either inhibitor (ICRF-187) or poison (etoposide) are used subsequently. Third, topoisomerase 2α and SUMO-2/3 colocalize extensively within mitotic chromatin at the centromere and the chromosomal axis. Fourth, depletion of topoisomerase 2α using siRNA technology results in a sharp decrease in the concentration of SUMO-2/3 over mitotic chromosomes in response to topoisomerase 2–specific drugs (ICRF-187, etoposide, doxorubicin). Finally, using the DRT assay (20), we showed that SUMO-2/3 becomes salt-detergent insoluble along with catalytic intermediates of topoisomerase 2α (Fig. 5*A*).

Based on these data, we suggest that from prophase to metaphase a subpopulation of topoisomerase 2α that localizes to the chromosomal axis and centromeres becomes transiently conjugated with SUMO-2/3 during catalysis (Supplementary Fig. S1). The transient nature of this modification should explain the small amount of SUMO proteins that normally associate with chromosomes under steady-state conditions (Supplementary Fig. S1). The peculiar localization of sumoylated topoisomerase 2α within the mitotic chromosome substructure (centromeres plus axes), however, places it in a position of privilege to carry out functions in chromatid cohesion and separation (15, 17, 19, 36). We cannot, however, exclude that other proteins besides topoisomerase 2α become sumoylated at the axes and centromeres.

According to a current model, the mitotic chromosome axis functions as a scaffold to tether chromatin loops via their AT-rich regions (27, 37–39). Topoisomerase 2α and the 13S condensin are major components of the axial scaffolding, which distribute as two mostly independent, yet closely juxtaposed, intertwined chains. Typically, optical sectioning of chromosomes double-stained for topoisomerase 2α and condensin generates the appearance of a row of beads that concentrate either topoisomerase 2α or condensin in an alternate manner; overlap between the two stainings does occur, but is minimal (27). Available evidence indicates that the axial scaffold harbors insoluble topoisomerase 2α (38, 39), which is mostly catalytically inert (40). Data presented here indicate that the low amounts of SUMO-2/3 proteins that normally associate with mitotic chromosomes are readily solubilized/removed by salt (350 mmol/L NaCl) plus detergent if cells are not exposed to topoisomerase 2 inhibitor (Fig. 5*A*). This is inconsistent with SUMO-2/3 proteins binding preferentially to a pool of insoluble topoisomerase 2α with scaffolding functions. Instead, as reasoned above, our data favor the idea that conjugation with SUMO proteins targets a subpopulation of topoisomerase 2α that enters catalysis within the chromosome axis. Thus, the chromosome axis may harbor minor amounts of both catalytically committed and catalytically inert/insoluble topoisomerase 2α, as previously suggested (20).

Sumoylation of topoisomerase 2 was shown to require the E3 SUMO ligase PIASy in the *Xenopus* egg extract system and was predicted to influence the targeting of the enzyme to mitotic chromatin (17). Very recently, it was reported that in mitotic human cells PIASy is required for proper localization of topoisomerase 2α at the centromeres and, to a lesser extent, to the chromosome cores. Importantly, targeting of topoisomerase 2 to the centromere mediated by PIASy was shown to promote a DNA decatenation–dependent and cohesion-independent mechanism for sister chromatid cohesion (19). This revealed an important role for the SUMO conjugation pathway in cohesion regulation via topoisomerase 2α but it remained, however, unknown whether topoisomerase 2α was the direct substrate for sumoylation. In this

research, we showed for the first time in human cells that topoisomerase 2α conjugates with SUMO-2/3 during mitosis and that sumoylated topoisomerase 2α distributes, along with PIASy, through chromosomal domains involved in chromatid cohesion and separation. We have also shown that PIASy modulates both the formation of SUMO-2/3 conjugates within mitotic chromosomes and the decatenation defects imposed by topoisomerase 2 inhibitors. We have additionally shown that the capacity of mitotic chromosomes to retain SUMO-2/3 proteins in response to pharmacologically relevant topoisomerase 2–specific drugs correlated with incomplete decatenation (Fig. 5*D*). This may highlight retention of SUMO-2/3 proteins as a signature for insufficient chromatid decatenation and further add useful insight into the mechanism of action of topoisomerase 2–specific drugs.

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Correction: Human Topoisomerase 2α Conjugates with SUMO-2

The article on human topoisomerase 2α conjugates with SUMO-2 in the April 1, 2008 issue of *Cancer Research* (1) appeared in the incorrect section of the journal. The article should have appeared in the Cell, Tumor, and Stem Cell Biology section.

1. Agostinho M, Santos V, Ferreira F, Costa R, Cardoso R, Pinheiro I, Rino J, Jaffray E, Hay RT, Ferreira J. Conjugation of human topoisomerase 2α with small ubiquitin-like modifiers 2/3 in response to topoisomerase inhibitors: cell cycle stage and chromosome domain specificity. Cancer Res 2008;68:2409–18.

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Conjugation of Human Topoisomerase 2α with Small Ubiquitin-like Modifiers 2/3 in Response to Topoisomerase Inhibitors: Cell Cycle Stage and Chromosome Domain Specificity

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Cancer Res 2008;68:2409-2418.