Construction, Characterization, and Complementation of a Conditional-Lethal DNA Topoisomerase II α Mutant Human Cell Line[□] [▽]

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DNA Topoisomerase II α (topoII α) is a DNA decatenating enzyme, abundant constituent of mammalian mitotic chromosomes, and target of numerous antitumor drugs, but its exact role in chromosome structure and dynamics is unclear. In a powerful new approach to this important problem, with significant advantages over the use of topoII inhibitors or RNA interference, we have generated and characterized a human cell line (HTETOP) in which >99.5% topoII α expression can be silenced in all cells by the addition of tetracycline. TopoII α -depleted HTETOP cells enter mitosis and undergo chromosome condensation, albeit with delayed kinetics, but normal anaphases and cytokineses are completely prevented, and all cells die, some becoming polyploid in the process. Cells can be rescued by expression of topoII α fused to green fluorescent protein (GFP), even when certain phosphorylation sites have been mutated, but not when the catalytic residue Y805 is mutated. Thus, in addition to validating GFP-tagged topoII α as an indicator for endogenous topoII α dynamics, our analyses provide new evidence that topoII α plays a largely redundant role in chromosome condensation, but an essential catalytic role in chromosome segregation that cannot be complemented by topoII β and does not require phosphorylation at serine residues 1106, 1247, 1354, or 1393.

INTRODUCTION

Type II topoisomerases are ubiquitous, highly conserved proteins that catalyze the passage of one DNA duplex through another, creating a transient double-strand break (DSB) in the latter (Watt and Hickson, 1994; Wang, 2002). Studies in a variety of systems indicate that topoII is essential for cellular viability and that it plays a key role in chromosome segregation. Thus, the abnormal cell divisions seen in yeast top2 mutants (Holm *et al.*, 1985; Uemura and Yanagida, 1986), and in vertebrate cells treated with topoII inhibitors (Downes *et al.*, 1991; Ishida *et al.*, 1991, 1994; Haraguchi *et al.*, 2000) are consistent with the unique ability of topoII to resolve the sister chromatids catenations generated when DNA replication forks meet (Wang, 2002). TopoII has been implicated in mammalian centromere function (Florida *et al.*, 2000; Spence *et al.*, 2002), and it has been suggested that topoII in yeast may play a role in centromeric chromatin structure (Bachant *et al.*, 2002). Further work is required to understand the relationship between any such structural role, topoII-mediated chromatid decatenation, and the role of the cohesin complex (Nasmyth, 2002) in maintaining sister chromatid cohesion until anaphase (Bernard and Allshire, 2002).

TopoII also has been implicated in DNA recombination, replication, transcription, and chromosome condensation

(Watt and Hickson, 1994; Wang, 2002). Of these, chromosome condensation is the only process in which topoII is widely reported to play an essential role (Koshland and Strunnikov, 1996; Losada and Hirano, 2001; Swedlow and Hirano, 2003), but the data are equivocal. Genetic analyses suggest that topoII is required for chromosome condensation in *Schizosaccharomyces pombe* (Uemura *et al.*, 1987) but not in *Saccharomyces cerevisiae* (Lavoie *et al.*, 2002). In vitro studies of chromosome condensation in mitotic extracts (Newport and Spann, 1987; Adachi *et al.*, 1991; Hirano and Mitchison, 1991, 1993), in which topoII is immunodepleted or inactivated by inhibitors, showed varying requirements for topoII. In the absence of suitable topoII mutants, in vivo studies in higher eukaryotes have made use of topoII inhibitors (Andoh *et al.*, 1993; Downes *et al.*, 1994; Gorbsky, 1994; Ishida *et al.*, 1994; Gimenez-Abian *et al.*, 1995; Andreassen *et al.*, 1997; Gimenez-Abian *et al.*, 2000) or RNA interference (Chang *et al.*, 2003; Sakaguchi and Kikuchi, 2004). Such studies mostly support a role for topoII in chromosome condensation, but again, condensation was impaired to varying degrees, and in some cases (Andreassen *et al.*, 1997; Chang *et al.*, 2003; Sakaguchi and Kikuchi, 2004) impairment was surprisingly mild or absent. Thus, although both structural (Mirkovitch *et al.*, 1988; Hart and Laemmli, 1998) and catalytic (Koshland and Strunnikov, 1996; Hirano, 2000) models for how topoII may be involved in chromosome condensation have been proposed, the issue of whether topoII is required for condensation remains controversial.

Vertebrates have two very similar topoII isoforms (α and β) encoded by distinct genes. Although both human isoforms can complement yeast TOP2 mutants (Jensen *et al.*, 1996), only the α isoform is abundantly associated with chromosomes throughout mitosis (Christensen *et al.*, 2002), particularly at centromeres (Rattner *et al.*, 1996; Christensen

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Table 1. Antibodies used in this study

Name	Antigen	Type	Use (dilution) ^a	Source
1HIC7	topoII α Residues 1370-end	Rabbit	IF 1° (1:100) western 1° (1:1000)	I.D Hickson
06-570	Histone H3P (Ser10)	Rabbit	IF 1° (1:200)	Upstate (Milton Keynes, United Kingdom)
F9887	Rabbit Ig	Goat-FITC	IF 2° (1:100)	Sigma Chemical
347583	BrdU	Monoclonal-FITC	IF (1:12)	BD Biosciences (San Jose, CA)
A2066	Actin	Rabbit	western 1° (1:200)	Sigma Chemical
M7001	p53	Monoclonal	western 1° (1:1000)	Dako (Ely, United Kingdom)
K2882	Ku86	Monoclonal	western 1° (1:5000)	Sigma Chemical
P0448	Rabbit Ig	Goat-HRP	western 2° (1:1000)	Dako
P0447	Mouse Ig	Goat-HRP	western 2° (1:1000)	Dako

^a For immunofluorescence (IF) dilutions were in PBS-wash; for Western blots (W), dilutions were in blocking solution.

et al., 2002). Furthermore, the toxic effects of topoII inhibitors and the viability of topoII β -deficient cells (Khelifa *et al.*, 1994; Yang *et al.*, 2000) together suggest that only the α isoform is essential for cellular viability. Consistent with this, development of mouse *TOP2II α* ^{-/-} embryos was found to terminate at the four- to eight-cell stage with chromosome segregation defects (Akimitsu *et al.*, 2003). A detailed genetic approach to topoII α function therefore requires conditional mutagenesis.

Conditional mutagenesis offers several advantages over the use of topoII inhibitors for the analysis of topoII α function. First, it specifically inactivates only the α isoform and is certain to remove any structural role. Second, it avoids the complication of side effects, especially DNA DSBs, which were initially thought to be limited to topoII “poisons” such as etoposide, but more recently linked to catalytic inhibitors such as ICRF-193 (van Hille *et al.*, 1999; Huang *et al.*, 2001; Kobayashi *et al.*, 2001; Wang and Eastmond, 2002; Adachi *et al.*, 2003). Third, conditional mutagenesis allows complementation studies to be done. Although RNA interference can be used to deplete individual topoII isoforms (Sakaguchi and Kikuchi, 2004), this approach has not yet been applied to topoII in a way that avoids significant residual expression or allows for complementation analyses. Complementation would be particularly valuable for the analysis of the numerous phosphorylation sites in topoII α , which have variously been implicated in controlling topoII α activity, resistance to inhibition, and subcellular localization, but whose true physiological significance remains untested (Isaacs *et al.*, 1998; Chikamori *et al.*, 2003). Similarly, the ability of biofluorescent topoII α (topoII α GFP) derivatives to complement topoII α -deficient cells could be tested, providing a necessary adjunct to recent important studies of topoII α GFP dynamics (Christensen *et al.*, 2002; Tavormina *et al.*, 2002).

We describe here the use of conditional gene targeting in somatic cells (Porter, 1998; Hudson *et al.*, 2002) to generate a cell line, HTETOP, in which endogenous *TOP2II α* genes are disrupted and transcription of an exogenous *TOP2II α* cDNA is controlled by the tetracycline transactivator (tTA) (Gossen *et al.*, 1994). TopoII α expression in HTETOP cells is dependent on the absence of tetracycline, or its analogue doxycycline, in the growth medium. Analyses of HTETOP cells provide new and direct evidence, with considerable advantages over previous studies based on inhibitors or RNA interference (RNAi), showing topoII α to be essential for chromosome segregation but not for chromosome condensation. They show further that a highly dynamic green fluorescent protein (GFP)-topoII α fusion protein is a valid reporter for topoII α dynamics in vivo and that essential

cellular topoII α functions do not require phosphorylation at any of four serine residues (1106, 1247, 1354, or 1393) previously shown to subject cell cycle-regulated phosphorylation.

MATERIALS AND METHODS

Plasmids, Cell Lines, and Polymerase Chain Reaction (PCR)

See Supplementary Material for details of plasmid construction, in vitro mutagenesis, generation of cell lines, and PCR assays to identify targeted clones.

Cell Culture and Transfection

All cells were grown and stably transfected by electroporation, as described previously (Itzhaki and Porter, 1991). Transient transfection was as described previously (Porter *et al.*, 1988). To select for colonies stably transfected with *neo*, *hygro*, *gpt*, *zeo*, *puro*, and *blast* cassettes, G418 (200 μ g/ml), hygromycin (200 μ g/ml), MPA/X/IFN (5 μ g/ml mycophenolic acid, 100 μ g/ml xanthine, 100 IU/ml type I interferon [IFN] Wellferon; GlaxoSmithKline, Uxbridge, Middlesex, United Kingdom), zeocin (100 μ g/ml), puromycin (0.4 μ g/ml), and blasticidin (5 μ g/ml), respectively, were added to the medium 48 h after electroporation, and colonies were picked 10–16 d later. Unless stated otherwise, dox and tet concentrations were 1 μ g/ml. When required, ICRF-193 (kindly supplied by A. Creighton, St Bartholomew's Hospital, London, United Kingdom), nocodazole, colcemid, and caffeine were used at final concentrations of 2 μ g/ml, 0.1 μ g/ml, 0.1 μ g/ml, and 2 mM, respectively, unless indicated otherwise. Growth curves were obtained by trypsinizing subconfluent cultures every 2–3 d, counting, and replating at 3000–6000 cells/cm².

RNase Protection Assays

Total cellular RNA was prepared by lysis in guanidine isothiocyanate and density gradient centrifugation. The riboprobe system-T7 kit (Promega, Madison, WI) was used, following manufacturer's specifications, to transcribe a [³²P]CTP-labeled RNA probe from the *A*/III-linearized probe template plasmid (p13.3TOPmaPr), hybridize it to cellular RNA, and RNase-digest unprotected RNA. The digested RNA samples were ethanol-precipitated and separated by electrophoresis on a 4% (wt/vol) polyacrylamide-8 M urea sequencing gel. Gels were dried and exposed to film.

Blots and Flow Cytometry

Genomic DNA analysis was as described previously (Itzhaki *et al.*, 1997). The probe was a 300-base pair *Eco* R1-*Hind*III fragment from the *TOP2II α* promoter region. Southern blots were washed with 2 $\times$ SSC (1 $\times$ SSC is 150 mM NaCl, 15 mM trisodium citrate), 0.5% (wt/vol) SDS for 10 min at room temperature followed by 0.2 $\times$ SSC, 0.5% (wt/vol) SDS for 10 min at 68°C. Western blots were as described previously (Yáñez and Porter, 1999). Antibodies are described in Table 1. Flow cytometry analysis was performed as described previously (Itzhaki *et al.*, 1997).

Fixing Cells for Microscopy

Cells were grown on glass coverslips in six-well plates. For immunolabeling of topoII α or GFP, cells were fixed in 4% (vol/vol) in phosphate-buffered saline [PBS]-A paraformaldehyde for 15 min and then washed three times with PBS-A. For immunolabeling of histone H3P, cells were fixed in methanol

at -20°C for 30 min and then acetone at -20°C for 5 s, and then washed three times with PBS-A. For immunolabeling of 5-bromo-2-deoxyuridine (BrdU)-labeled DNA, cells were fixed with paraformaldehyde as described above, permeabilized in 0.2% (vol/vol) Triton X-100, and the DNA denatured in 2 N HCl, 0.1% (vol/vol) Triton X-100 for 10 min.

Chromosome Spreads

Confluent cells (50–75%) were treated with or without 0.1 $\mu\text{g}/\text{ml}$ colcemid (Sigma Chemical, Poole, Dorset, United Kingdom) for 2 h, swollen in hypotonic solution (75 mM KCl, prewarmed to 37°C) for 20 min, and three washes in Carnoy's fixative [3:1(vol/vol) methanol:acetic acid]. Condensation was scored as "low" if there was poor axial condensation and no detectable sister chromatid individualization; as "medium" if there was moderate axial condensation, often with detectable individualization; or as "high" if condensation seemed similar to that seen in a normal metaphase. All spreads were assigned to one of these categories and similar results were obtained when samples were analyzed "blind" by a second person using the same scoring system.

Immunocytochemistry

Antibodies are summarized in Table 1. Cells (except BrdU-labeled cells) or chromosome spreads fixed on coverslips were processed in the following sequence: 20 min at 20°C in 0.1 M glycine (in PBS-A); three washes in PBS-A; 30 min at 20°C in PBS-block [PBS-wash [0.05% (wt/vol) saponin in PBS-A] supplemented with 5% (vol/vol) fetal calf serum (FCS) and 5% (vol/vol) goat serum]; three washes in PBS-wash; 1 h at 20°C in primary antibody (100 μl of a dilution in PBS-block, between Nescio film and coverslip); three washes in PBS-wash; 1 h at 20°C in secondary antibody (as for primary, but in the dark and with 0.1 $\mu\text{g}/\text{ml}$ 4,6-diamidino-2-phenylindole [DAPI]); three washes in PBS-wash; and one wash in distilled water. Coverslips were mounted onto glass slides with mounting medium (Vectorshield) and sealed with nail varnish. BrdU-labeled cells were processed similarly except that the PBS block was PBS-A containing 5% (vol/vol) goat serum, 0.2% (wt/vol) fish skin gelatin and 0.2% (vol/vol) Tween 20, antibody was added for 30 min at 37°C , and no secondary antibody was required.

Microscopy

Phase contrast microscopy used the Olympus OM system including an Olympus CK2 inverted microscope, with SPlan 4PL, A10 PL or A20 PL objectives, and an Olympus OM-4 Ti camera. Images were captured on film and digitally scanned.

Immunocytochemistry images (other than chromosome spreads) were generated with a Leica TCS SP1 confocal microscope, 100 $\times$ HCX PLAN APO 1.35 oil objective, and Leica confocal software.

Images of chromosome spreads were generated with a Leica DMRB microscope, 100 $\times$ PL Fluotar1.30 oil objective, Photometrics CoolSnap HQ charge-coupled device camera, and Scanalytics IP Lab version 3.6.1 and MultiProbe version 2.1.4 imaging software.

Live cell images were obtained with a Leica TCS SP2 confocal microscope, 100 $\times$ PL APO 1.40 oil PH3 objective and Leica confocal software. Cells were grown on 40-mm-diameter circular glass coverslips and mounted onto an FCS2 closed system live cell chamber (Bioptechs, Butler, PA). The cell chamber contained DMEM 10% (vol/vol) FCS with 10 mM HEPES and appropriate supplements. Except for fluorescence recovery after photobleaching (FRAP) assays, for which cells were at room temperature, cells were maintained at 37°C .

Online Supplementary Material

Supplementary material (videos and supplemental materials and methods) is available. Videos (Fig 5video1.mov, Fig 5video2.mov and Fig 5video3.mov) are of the anaphases described in Figure 5, D–F, and are supplied with legends.

RESULTS

Construction of a Human Cell Line (HTETOP) in Which *topoII α* Can Be Depleted

The fibrosarcoma cell line HT1080 was stably transfected with a tTA expression plasmid (pUHC13.3). One of the tTA-expressing clones (HTET) was then stably transfected with a plasmid (pUHC13.3hygTOP) in which *topoII α* cDNA is linked to a tTA-responsive promoter. An RNase protection assay was used to detect pUHC13.3hygTOP-encoded *topoII α* transcripts and so identify a clone (HTETOPwt) in which the latter were tightly regulated by doxycycline (Figure 1, A and B). The endogenous *TOPOII α* genes in clone HTETOPwt were then disrupted by two rounds of gene

Figure 1. Construction and initial characterization of HTETOP cells. (A) Principal of RNase protection assay used to detect endogenous (top) and exogenous (bottom) *topoII α* transcripts. The 5' ends of transcripts are shown as shaded boxes, numbered according to exons. The exogenous transcript has non-*topoII α* -derived sequences at its 5' end (unshaded box). Protected and unprotected regions of the RNA probe are shown above each transcript (solid and broken lines, respectively). (B) Products of RNase protection assay outlined in A were separated by denaturing PAGE. Protecting RNA was from the indicated cell lines grown in the presence or absence of doxycycline for 2 d. (C) Gene targeting strategy. Bold lines represent 5' regions of *TOPOII α* genes with exons 1–4 (left to right) shown as black boxes. Pairs of *TOPOII α* alleles in HTETOPwt (+/+), HTETOPhet (−/+), and HTETOP (−/−) cells are shown with relevant sites for *PstI* (P), and the resulting fragment sizes are indicated. Linearized targeting constructs are shown with *TOPOII α* DNA aligned to *TOPOII α* target alleles, and the position (disrupting exon 4) and orientation (opposite to *TOPOII α*) of their *gpt* and *zeo* selection cassettes are indicated; remaining plasmid DNA is shown as a thin line. The vertical dotted line indicates the limit of homology between targeting constructs and *TOPOII α* alleles. The probe (Pr) used for Southern analysis (gray bar) and oligonucleotides (O1, O2, and O3) used for PCR assays (arrowheads) are shown aligned with homologous DNA. (D) Southern analysis. *PstI*-digested genomic DNA isolated from HTETOPwt (+/+), HTETOPhet (−/+), and HTETOP (−/−) cells, and labeled marker DNA (M), were separated, blotted, and probed with the probe shown in C. (E) HTETOPhet doubling times during continuous culturing (see *Materials and Methods*) with (open circles) or without (squares) added tetracycline at 1 $\mu\text{g}/\text{ml}$. (F) HTETOP doubling times, grown as in E but with tetracycline at 0 (squares), 1 (diamonds), or 2 (circles) ng/ml .

targeting according to the scheme outlined in Figure 1C. PCR screens were used to detect targeted clones at frequencies of $\sim 1/300$ and $1/500$ stably transfected clones for the first and second rounds of targeting, respectively. Southern analysis (Figure 1D) confirmed that the desired targeting events had occurred: disruption of a single allele in clone HTETOPhet

and both alleles in clone HTETOP. Proliferation of HTETOP was not affected by the addition of tetracycline (Figure 1E), but doubling times for HTETOP were elevated at low concentrations of tetracycline (Figure 1F), whereas proliferation ceased at tetracycline concentrations of 10 ng/ml or higher (our unpublished data).

Polyploidy, Nuclear Abnormalities, and Cell Death Follow topoII α Depletion

The response of HTETOP cells was followed for several days after addition of tetracycline or doxycycline at 1 μ g/ml (the concentration used throughout this article, unless stated otherwise). Western analyses (Figure 2A) showed that the amount of topoII α protein was drastically reduced 24 h after doxycycline addition and that by 48 h, it was barely detectable. In the same samples, an accumulation of p53 was detected, while amounts of actin and Ku70, a protein involved in nonhomologous DNA end-joining, were unaffected. In long exposures of Western blots, a faint topoII α signal was detectable 3 d after doxycycline addition, and by comparison with diluted samples of untreated HTETOP cells, topoII α was estimated to be expressed at <0.5% of control levels (Figure 2B). After 2 d in tetracycline, cell numbers ceased to increase, and after 3–4 d, cell numbers began to fall (Figure 2C). Similar results were obtained with doxycycline (our unpublished data). In doxycycline, HTETOP cells gradually developed abnormal morphologies, including enlargement and flattening, and the accumulation of cytokines with intercellular bridges of various thicknesses and lengths (Figure 2D). Giant cells, as big as 250 μ m in diameter, were detectable within a week of topoII α depletion. Nuclei also became enlarged, distorted, or both, with evidence of DNA bridging (Figure 2E). After 4 d in doxycycline, 70% of nuclei had a grossly abnormal appearance (Figure 2F). Flow cytometric analysis of nuclear DNA content in doxycycline-treated cells indicated that cells become increasingly polyploid (Figure 2G). A sub2N peak of cells, often indicative of apoptotic cells, also increased with time after doxycycline addition. 2N cells detected at late time points cannot be recovered by removal of tetracycline from the medium and probably represent cells that have undergone aberrant cytokinesis followed by breakage of the intercellular DNA bridge. Thus, in the absence of topoII α , cells cease to proliferate and eventually die, probably by apoptosis, a significant proportion developing various combinations of distorted, bridged, and enlarged/polyploid nuclei in the process.

HTETOP Cells Enter Mitosis after topoII α Depletion

HTETOP cells continue to enter into mitosis after topoII α has been depleted (Figure 3A). Thus, the mitotic index, which actually rises after doxycycline-treatment, does not fall below the control value until 4 d of doxycycline-treatment, by which time abnormal nuclei unlikely to be capable of entering mitosis are accumulating in substantial numbers. More importantly, a 7-h treatment of cells with the microtubule polymerization inhibitor nocodazole, which blocks cells in metaphase, causes a substantial increase in mitotic indices at times when topoII α is absent (Figure 3A). Furthermore, the percentage of mitoses pulse-labeled with BrdU (8.5-h pulse, the last 3.5 h including nocodazole) was not altered by a 2-d pretreatment with doxycycline: 61% (19/31) for doxycycline-treated cells and 54% (15/28) for control cells.

Because a caffeine-sensitive, topoII-dependent G2 cell cycle checkpoint has been described previously (Downes *et al.*, 1991), we were interested to know the effect of caffeine in

Figure 2. TopoII α depletion is accompanied by loss of proliferation, cell death, abnormal cell and nuclear morphology, and increased ploidy. (A) Western analysis of HTETOP cells grown in doxycycline for the indicated number of days. Duplicate blots were probed with antibodies to topoII α and actin or Ku80 and p53. (B) The indicated dilutions of lysates from untreated HTETOP cells (lanes 1–5) or undiluted lysates from HTETOP cells treated with doxycycline for 1 (lane 7) or 3 (lane 6) d, were probed with antibodies to topoII α (top) or actin (bottom). (C) Growth curves for HTETOP cells after addition (open circles) or mock addition (squares) of tetracycline at day 0. (D) Phase contrast microscopy of HTETOP grown in doxycycline for the indicated number of days. Bar, 250 μ m. (E) Fluorescence microscopy of DAPI-stained nuclei of HTETOP cells treated with doxycycline for the indicated number of days. Bars, 10 μ m. (F) Frequency of abnormal (enlarged and/or distorted as in E) nuclei in HTETOP cells grown in doxycycline for the indicated times. Cultures were treated with (diamonds) or without (open circles) caffeine for 7 h before fixing. The experiment was done in triplicate, scoring ~1000 cells for each; means and standard deviations are shown. (G) Flow cytometric analysis of DNA content in nuclei prepared from HTETOP at the indicated number of days after addition of doxycycline. x-axis, fluorescence (log scale); y-axis, frequency; and z-axis, time (days).

Figure 3. Mitosis after topoII α -depletion. (A) Mitotic indices in HTETOP cells were determined at the indicated times after doxycycline addition. Cells were fixed for microscopic analysis after no further treatment (circles; green) or 7-h treatments with nocodazole (triangles; blue), caffeine (diamonds; brown), or nocodazole and caffeine (squares; black). The experiment was done in triplicate, scoring ~ 1000 cells in each; means and standard deviations are shown. The same samples were used in Figures 2E, 3C, and 5A. (B) Fluorescent and immunofluorescent confocal microscopy of DNA (DAPI; blue) and topoII α (fluorescein isothiocyanate; green), respectively, in mitotic HTETOP cells grown with (+) or without (–) doxycycline for 2 d. Bars, 10 μ m. No topoII α -positive cells were detected in many fields (representing $\gg 1000$ cells) of such doxycycline-treated cells. (C) Frequency of normal anaphases (open symbols) or anaphases with DNA bridges (closed symbols) in HTETOP cells after addition of doxycycline. Samples were treated with (diamonds; brown) or without (circles; green) caffeine for 7 h before harvesting and were the same as those describe in A. (D) Fluorescent confocal microscopy of mitotic HTETOP/H2B-GFP cells grown without (–) or with (+) doxycycline for 0 (a), 1 (b), or 3 (c) d. Bars, 10 μ m.

HTETOP cells. Despite clear evidence for active entry into mitosis in the absence of topoII α , mitotic indices could be increased by a 7-h treatment with caffeine, with or without the addition of nocodazole (Figure 3A). TopoII α depletion is therefore sufficient to activate the caffeine-sensitive G2 checkpoint, but the checkpoint is only partially effective at delaying mitosis. Such leakiness has been noted in cells treated with topoII inhibitors (Downes *et al.*, 1994; Deming *et al.*, 2001) and may be indicative of checkpoint attenuation.

Mitotic Chromosome Condensation in topoII α -depleted HTETOP Cells

Because topoII α has been reported to be required for chromosome condensation, initial measurements of mitotic index were made on the basis of a signal for ser-10 phosphorylated Histone H3 (H3P), a marker for mitosis (Crosio *et al.*, 2002). For each time point after the addition of doxycycline, however, all cells that were positive for H3P also contained condensed chromosomes (our unpublished data). Furthermore, immunofluorescence of fixed cells confirmed that 100% of cells treated with doxycycline for 2 d, including those with condensed chromosomes, were negative for topoII α , whereas untreated cells were all positive (examples are shown in Figure 3B). It has been shown *in vitro* that condensed chromosomes remain condensed after addition of topoII α antibodies or inhibitors or after topoII α extraction (Hirano and Mitchison, 1993). It was therefore conceivable that condensed chromosomes lacking topoII α had undergone condensation before topoII α depletion and then become trapped in mitosis, suffering topoII α depletion but not decondensation. Such a process, however, is inconsistent with our observations that topoII α -depleted cells actively enter mitosis and that no mitotic doxycycline-treated chromosomes stain positively for topoII α . We conclude that condensed chromosomes, such as those in the doxycycline-treated cell of Figure 3B, not only lack topoII α but also have undergone condensation in its absence.

Chromosome condensation also was examined in chromosome spreads from HTETOP cells treated with doxycy-

cline, ICRF-193, and colcemid in various combinations (Figure 4). The extent of condensation varied in all samples; some examples are shown in Figure 4A. The degree of condensation for each spread was scored as low, medium, or high (see *Materials and Methods*), and frequencies determined for each sample are shown in Figure 4, B and C. This analysis showed that the proportion of partially condensed chromosomes was increased by doxycycline treatment. A similar, although more pronounced, increase was observed in ICRF-193-treated cells. Significantly, however, neither doxycycline nor ICRF-193 treatment completely prevented the detection of condensed chromosomes or their accumulation after a short (2-h) treatment with the microtubule-destabilizing agent colcemid (Figure 4B). Thus, both topoII α depletion and, to a greater extent, ICRF-193 treatment, seem to impair the kinetics of chromosome condensation, but without completely preventing condensation from proceeding to apparent completion. Interestingly, the more marked effect of ICRF-193 on condensation was still observed when cells had been depleted of topoII α by treatment with doxycycline (Figure 4C). This excludes any active role for ICRF-193-inhibited topoII α (e.g., DNA breakage via topoII α poisoning, or structural interference) in the effect of ICRF-193 on condensation.

As further confirmation that condensed chromosomes from doxycycline-treated HTETOP cells lacked topoII α , immunofluorescence was used in chromosome spreads (Figure 4D). Although topoII α labeling was detectable in only a proportion ($\sim 1/3$) of control cells, indicating some incompatibility between the spreading and immunocytochemical methods used, topoII α labeling of the chromosomes from doxycycline-treated cells was never detected ($\gg 1000$ spreads).

Aberrant Chromosome Segregation in topoII α -depleted HTETOP Cells

Although the effects of topoII α depletion on premetaphase events were subtle, the effects on postmetaphase events

Figure 4. Chromosome spreads from HTETOP cells. (A) DAPI-stained spreads from untreated cells (a and b) or cells treated with doxycycline for 2–3 d (c–e), or ICRF-193 for 2 h (f–h). These were placed in the low (h and f), medium (b[lower spread], c, and g), or high (a, b[upper spread], d, and e) categories of chromosome condensation. (B) Frequencies of spreads with high, medium, or low levels of chromosome condensation (as defined in methods). Spreads were prepared from untreated cells or cells treated with colcemid (Colc) for 2 h, doxycycline (Dox) for 2–3 d, or ICRF-193 (ICRF) for 2 h, in the indicated combinations. The number of mitotic spreads categorized (n) and mitotic indices (MI) for each treatment are indicated. (C) As for B, but this experiment was done in triplicate and error bars show standard deviations. (D) Fluorescent and immunofluorescent microscopy of DNA and topoiII α , respectively, in chromosome spreads of cells grown with (+) or without (–) doxycycline for 3 d. Bars, 10 μ m.

were profound. First, abnormal anaphases/telophases, involving varying degrees of lagging chromosomes, were detected, examples of which are shown in Figure 3, B and D. The frequency of abnormal anaphases rose sharply for the first 2 d of doxycycline treatment and fell thereafter (Figure 3C). Although the absolute frequencies of anaphases and telophases were low, reflecting their short lifetime, it was notable that, from day 3 of doxycycline treatment onwards, 100% of those detected had lagging chromosomes. Second, DAPI staining of accumulating cytokinesis revealed structures in which DNA seems to have become trapped and “squeezed” in the cleavage furrow to form DNA bridges

(Figure 5). The frequency of such bridged cytokinesis (Figure 5A), which was reduced by microtubule disruption with nocodazole, was much higher than the frequency of bridged anaphases and telophases (Figure 3C), but both frequencies peaked at ~2 d of doxycycline treatment. This indicates that anaphase progresses rapidly to cytokinesis whose completion is delayed or prevented. Similar bridged cytokinesis, sometimes referred to as “butterfly” or “teardrop,” have been observed in vertebrate cells treated with topoiII inhibitors (Downes *et al.*, 1991; Ishida *et al.*, 1991; Gorbsky, 1994; Haraguchi *et al.*, 2000; Tavormina *et al.*, 2002) and in *TOPOII α –/–* mouse embryos (Akimitsu *et al.*, 2003), and seem

Figure 5. Abnormal chromosome segregation in *topoII α* -depleted cells. (A) Frequency of abnormal cytokinesis after addition of doxycycline. The samples were the same as those analyzed in Figure 3A: no further treatment (circles; green) or 7-h treatments with nocodazole (triangles; blue), caffeine (diamonds; brown), or nocodazole and caffeine (squares; black). (B) Fluorescence and immunofluorescence confocal microscopy of DNA (DAPI; blue) and phosphorylated Histone H3 (green), respectively, in normal (a) and abnormal (b–d) cytokinesis in cultures treated with doxycycline for 1 (a and b), 2 (c), or 3 (d) d. (C) Confocal fluorescence microscopy of an abnormal cytokinesis in HTETOP/H2B-GFP cells treated with doxycycline for 3 d. (D) Time-lapse confocal microscopy showing an abnormal cytokinesis arising from an abnormal anaphase in HTETOP/H2B-GFP cells treated with doxycycline for 1 d. (E) Time-lapse confocal microscopy showing normal anaphase in HTETOP/H2B-GFP cells not treated with doxycycline. (F) Time-lapse confocal microscopy showing abortive anaphase in HTETOP/H2B-GFP cells treated with doxycycline for 2 d. All bars, 10 μ m. Images in D, E, and F were taken from videos (Fig 5video1.mov, Fig 5video2.mov and Fig 5video3.mov, respectively) that can be seen as online Supplementary Material.

to be the vertebrate equivalent of the *S. pombe* “cut” phenotype (Yanagida, 1998).

Apart from the formation of DNA bridges and impaired completion, cytokinetic events seem normal. Thus, the mid-body was highlighted by immunofluorescence with an H3P antibody (Figure 5B), as is typical for normal cytokinesis, and consistent with the location of Aurora B, the kinase responsible for H3 phosphorylation (although it is unknown why H3P is detected in the absence of chromatin). Single cells with tails (Figure 2D) and single nuclei with DNA tails (Figure 2E) also were detected, suggesting that the bridged structures eventually break into daughter cells with distorted nuclei. The kinetics with which bridged cytokinesis disappear (Figure 5A) and abnormal single nuclei accumulate (Figure 2F) is certainly consistent with this interpretation.

To study chromosome behavior in live cells depleted for *topoII α* , HTETOP cells were stably transfected with a plasmid (pBOS-H2BGFP) encoding enhanced GFP (EGFP) fused to histone H2B. When one such clone (HTETOP/H2GFP) was treated with doxycycline and observed by confocal microscopy, abnormal anaphases (Figure 3D) and cytokine-

sis (Figure 5C), similar to those seen in fixed cells (Figures 3B and 5B) were seen. Furthermore, time-lapse analyses provided direct evidence that the abnormal cytokinesis are derived from aberrant anaphases with lagging chromosomes (Figure 5D). Despite the clear evidence of lagging chromosomes, the bulk of chromosomes became clearly separated within $\sim$ 6 min after the beginning of anaphase (Figure 5D), similar to the corresponding time for a normal anaphase (Figure 5E). We also found examples of anaphases that initiate but never reach a stage where two groups of chromosomes are clearly separated (Figure 5F). Such abortive anaphases may easily have been overlooked in single time-point analyses and may account for at least some of the polyploid cells that accumulate after *topoII α* depletion. Further time-lapse studies should reveal the full range and frequencies of abnormal chromosome segregations.

We confirmed that *topoII* inhibition (by ICRF-193) also gives rise to cytokinesis with DNA bridges in HT1080 cells (Table 2). It was notable that the frequency of DNA bridges was zero in untreated HT1080 but nonzero in untreated HTETOP cells. This implies that the exogenous *topoII α* expression is not able to fully compensate for the absence of

endogenous protein in HTETOP cells. This may partly explain the increased doubling times of HTETOP cells (~30 h) compared with parental HT1080 cells (~20 h).

GFP-topoIIα Rescues HTETOP Cells in Doxycycline and Is Highly Dynamic

HTETOP cells were stably transfected with a plasmid (pGFP-topoIIα) carrying, in addition to a puromycin resistance cassette, the full-length topoIIα open reading frame fused, in frame, to the C terminus of EGFP. Selection in doxycycline generated four colonies (A–D) from 10⁶ electroporated cells, two of which (B and D) also were resistant to puromycin. Selection in puromycin generated six colonies (E–K) from 10⁶ electroporated cells, two of which (H and J) also were resistant to doxycycline. All six doxycycline-resistant colonies expressed GFP, as judged by fluorescence microscopy. None of these colonies is likely to be spontaneously drug resistant because selection of $>2 \times 10^6$ untransfected HTETOP cells in either drug generated no colonies. As was shown with pBOS-H2BGFP (see above), HTETOP cells can be stably transfected to drug resistance at frequencies of $\sim 10^{-4}$, which is typical for HT1080 cells under our electroporation conditions. Thus, although the majority of pGFP-topoIIα transfectants seem to lose viability, presumably because excess topoIIα is harmful, a significant minority are rescued from endogenous topoIIα depletion. Five HTETOP/GFP-topoIIα clones, resistant to both puromycin and doxycycline, were shown by Western blot, to express a topoIIα protein of the expected size (200 kDa) for the fusion protein, with some degradation products, in quantities similar to those for normal protein (Figure 6A). As a control, a puromycin-resistant, doxycycline-sensitive clone (clone I) failed to express the fusion protein and associated degradation products (Figure 6A).

These results show that the GFP-topoIIα fusion protein is able to carry out all the essential functions of normal topoIIα protein when expressed at appropriate levels. One of the doxycycline-resistant transfectants (clone B) was expanded and shown to contain a pattern of GFP fluorescence, in both fixed samples (Figure 6B) and live cells (Figure 6C), typical for topoIIα: distributed throughout the nucleus in interphase nuclei (our unpublished data) and on chromosomes during mitosis, with regions of intense staining that probably correspond to centromeres. The same clone was analyzed by FRAP. Recovery of chromosomal GFP-topoIIα fluorescence from photobleaching was rapid compared with its recovery in the presence of ICRF-193 or to recovery of chromosomal H2B-GFP fluorescence (Figure 7). Similar results were obtained for nucleolar GFP-topoIIα (our unpublished data). Thus, as previously observed in human (Christensen

Figure 6. GFP-topoIIα rescues HTETOP from doxycycline sensitivity. (A) Western analysis of six puromycin-resistant HTETOP/GFP-topoIIα clones, treated with or without doxycycline for 2 d, with antibodies to topoIIα (top) and actin (bottom). All clones except clone I were doxycycline resistant. (B) Confocal fluorescence microscopy of GFP-topoIIα (green) and DNA (DAPI, blue) in fixed nuclei of clone B grown continuously in doxycycline. (C) Confocal fluorescence microscopy of GFP-topoIIα in nuclei of live clone B cells; a and b are separate mitoses; b' is a single z-section of b.

et al., 2002) and pig (Tavormina *et al.*, 2002) kidney cell lines (but with the important difference that in the present study biofluorescent topoIIα was expressed in the absence of endogenous topoIIα) chromosomal, nucleolar, and nucleoplasmic GFP-tagged topoIIα is in a state of rapid dynamic equilibrium that is sensitive to catalytic inhibition.

Complementation with GFP-topoIIα Requires Y805 but Not Phosphorylation at Residues S1106, S1247, S1354, or S1393

To investigate the role of topoIIα-specific residues, in vitro mutagenesis of pGFP-topoIIα was carried out and the resulting plasmids tested for complementation of doxycycline-sensitivity in HTETOP cells. Four serine residues (S1106, S1247, S1354, and S1393), previously shown to be phosphorylated by proline-directed kinase(s) in a cell cycle-dependent manner (Wells and Hickson, 1995), were individually mutated to alanine. Each of the resulting plasmids was able to rescue HTETOP cells from doxycycline-sensitivity. Fluorescence microscopy of such clones indicated that they all expressed GFP-topoIIα (Table 3), and expression was still nuclear (our unpublished data). Mutation of the catalytic

Table 2. Effects of topoII inhibition and depletion in HT1080 and HTETOP cells, respectively, on DNA bridge formation and mitotic index

	HT1080		HTETOP	
	–ICRF-193	+ICRF 193 ^a	–doxycycline	+doxycycline ^b
DNA bridges (%)	0	4.7	2.7	17.9
MI (%)	1.9	0.4	2.4	1.9

^a 8 μg/ml for 24 h.

^b 1 μg/ml for 48 h.

Figure 7. FRAP of GFP-topoII α -rescued HTETOP cells shows topoII α to be highly mobile. (A) Left. Relative fluorescence at 1.6-s intervals after photobleaching of mitotic chromosomes in HTETOP/H2B-GFP cells (triangles) or clone B (GFP-topoII-rescued HTETOP) cells untreated (closed squares) or treated (open squares) with ICRF-193 for 1 h before bleaching. Right, examples of images analyzed. Dotted white squares (7 μm^2) indicate areas of analysis. (B) Analysis as in A but for nucleoplasmic fluorescence of interphase cells.

tyrosine to phenylalanine (Y805F) prevented rescue from doxycycline sensitivity: the only doxycycline-resistant colony recovered, like that recovered after a mock transfection, did not express GFP-topoII α . Such colonies are presumably the result of rare reversion events that allow expression of the topoII α minigene in HTETOP cells. All of the plasmids generated puromycin-resistant colonies in the absence of doxycycline, and for the S1106A, S1247A, and S1354A mutations, a proportion of these (10–20%) also expressed GFP-

Table 3. HTETOP complementation with topoII α mutants

Plasmid ^a	Dox ^R ^b	Dox ^R /GFP ⁺ ^b	Puro ^R ^b	Puro ^R /GFP ⁺ ^b
pGFP-topoII α	62, 27	62, 27	33, 24	5, 5
pGFP-topoII α +	30, 20	30, 20	30, 30	6, 4
pGFP-topoII α -S1106A	24	24	26	3
pGFP-topoII α -S1247A	9	9	35	6
pGFP-topoII α -S1393A	27	27	5	0
pGFP-topoII α -S1354A	22	22	20	3
pGFP-topoII α -Y805F	1	0	53	0
No DNA	1, 0	0, 0	0, 0	0, 0

^a All plasmids were based on pGFP-topoII α . pGFP-topoII α was unchanged but subjected to the same *Spe* I/*Cla* I subcloning procedure used for introducing mutations (see Supplementary Material).

^b Each number represents the number of colonies obtained after transfection of 2.5–3 million HTETOP cells and selection in puromycin or doxycycline, before and after discounting those that did not express detectable GFP.

topoII α . The higher frequency of GFP⁺ colonies recovered in the presence of doxycycline than in its absence probably indicates that GFP-topoII α is more toxic when endogenous HTETOP topoII α is already expressed. For the Y805F mutation, however, 0/53 puromycin-resistant colonies were GFP⁺, suggesting that GFP-topoII α -Y805F exerts a strong dominant-negative effect on cell viability. Further work is required to determine whether the relatively low frequencies of doxycycline- and puromycin-resistant colonies generated by plasmids with the S1247A and S1393A mutations, respectively, are significant.

DISCUSSION

The HTETOP cells described here provide a unique system for the analysis of human topoII α function, allowing the effects of sustained topoII α depletion (to <0.5% normal levels) to be studied and complementation analyses to be carried out. As discussed below, our observations during such studies help to define various controversial aspects of topoII function.

Minimal Requirement for topoII α (and topoII β) in Chromosome Condensation

Previous attempts to demonstrate a role for mammalian topoII in chromosome condensation have produced conflicting results (see Introduction). Our experiments represent a new and particularly precise approach to this problem and indicate that topoII α is not required for chromosome condensation but can contribute to its timely completion. TopoII α depletion and ICRF-193, which inhibits both topoII isoforms, both reduced the incidence of fully condensed chromosomes, although the latter was more potent (Figure 4). The simplest interpretation is that both topoII α and β play facilitating but nonessential roles in chromosome condensation. The possibility that only the α isoform contributes to condensation is unlikely because it requires that depletion of >99.5% of topoII α has less effect on condensation than inactivation by ICRF-193 of the residual <0.5%. Indeed, the continued formation of individualized chromatids in topoII α -depleted or ICRF-193-treated cells (Figure 4A) suggests that some decatenation can occur independently of either topoII isoform. Type IA topoisomerases (topoII α and β) can catalyze decatenation at sites of duplex nicking (Tse and Wang, 1980) and may therefore be responsible. Our results exclude any active role for ICRF-193-inhibited topoII α (e.g., DNA breakage via topoII α poisoning, or structural interference) in the effect of ICRF-193 on condensation, because topoII α depletion does not affect this (Figure 4C).

Our conclusion that topoII α is not essential for chromosome condensation, although consistent with some studies (Andreassen *et al.*, 1997; Chang *et al.*, 2003; Sakaguchi and Kikuchi, 2004), seems at odds with reports that topoII inhibitors prevent condensation (see Introduction) which, combined with the fact that *TOPOII β -/-* cells divide normally (Yang *et al.*, 2000), imply that topoII α is essential for condensation. These discrepancies may reflect differences in interpretation, however, because there clearly is an effect on the timing of condensation, and this may be more or less pronounced depending on cell type and whether microtubule inhibitors are used to allow further time for condensation to occur. A nonessential role in chromosome condensation has recently been described for the condensin subunit SMC2/ScII (Hudson *et al.*, 2003). In that study, the condensed chromosomes were shown to be structurally compromised, and it will be interesting to determine whether

condensed chromosomes in topoII α -depleted cells have similar defects. Nevertheless, there is now clear genetic evidence that neither of the two major components of the chromosome scaffold (topoII α and SMC2/ScII) is essential for the formation of cytogenetically normal condensed chromosomes. The molecules required for this key mitotic process therefore remain to be identified.

Essential Role for topoII α Activity in Chromosome Segregation

The role of vertebrate topoII in chromosome segregation has previously been studied by the use of inhibitors, gene disruption, or RNA interference. Inhibitor studies revealed the importance of topoII activity in segregation but could not reveal whether a single isoform was responsible and were complicated by possible side effects of inhibitors, such as DNA damage from topoII poisoning. Gene targeting in mice showed that segregation was dependent on topoII α (Akimitsu *et al.*, 2003) and not topoII β (Yang *et al.*, 2000), with the qualification that topoII α requirements could only be analyzed during the initial segregations of the mouse embryo that may be exceptional. TopoII α depletion by RNAi in HeLa cells also impaired segregation, but some residual segregation that was sensitive to topoII β depletion was interpreted as evidence that topoII β can compensate for topoII α depletion (Sakaguchi and Kikuchi, 2004). This discrepancy could reflect differences between mouse embryo and HeLa cells or limitations in the extent and specificity of topoII depletion by RNAi. Our analysis therefore provides the clearest evidence to date that topoII α plays an essential role in chromosome segregation of differentiated vertebrate cells.

The lethal nature of topoII α depletion is readily explained in terms of lagging chromosomes that, if cytokinesis proceeds, become trapped in the midbody. The resulting structures most likely degenerate into nonviable cells with distorted nuclei. Alternatively, cytokinesis may abort to generate polyploidy progeny. In principle, polyploid cells could be caused by the suppression of CDC2, the major cyclin-dependent kinase controlling the G2/M transition. TopoII α inactivation has been shown to result in the cytoplasmic sequestration of CDC2 (Deming *et al.*, 2001), and down-regulation of CDC2 causes G2-arrested cells to reset in G1, bypassing mitosis (Itzhaki *et al.*, 1997). The fact that topoII α -depleted cells enter mitosis (Figures 3 and 5), however, favors abortive cytokinesis as the best explanation for polyploidy.

Although lagging chromosomes in topoII α -depleted HTETOP cells are sufficiently frequent to be lethal, it is striking that only a minority of chromosomes seem to be lagging in any given anaphase. This implies that the bulk of chromosome decatenation can occur independently of topoII α , possibly requiring topoII β or topoII α activity. Conversely, lagging chromosomes presumably reflect residual catenations with the particular properties of being resolvable only by the action of topoII α and of producing little or no impediment to chromosome condensation. What is the nature of such catenations and why do they seem to affect only a subset of chromosomes? Given the persistent association of sister centromeres after chromatid arms have separated (Losada and Hirano, 2001; Bernard and Allshire, 2002), and the known accumulation of topoII α protein (Rattner *et al.*, 1996; Sumner, 1996) and topoII activity (Florida *et al.*, 2000; Andersen *et al.*, 2002; Spence *et al.*, 2002; Agostinho *et al.*, 2004) at centromeres, one may reasonably speculate that centromeric catenations are uniquely resolved by topoII α and persist after anaphase onset in topoII α -depleted cells. In

the absence of cohesin-dependent centromeric cohesion, poleward forces might then be sufficient to allow near normal separation of sister kinetochores, at the expense of severe chromosomal distortions and DNA damage, resulting in the dissociation of many, but not all, sister chromosomes. Some chromosomes, because of their size or sequence organization, might be more prone to lag under these circumstances than others. The observed induction of p53 is consistent with this model, although DNA damage also could be associated with events earlier in the cycle that are sensitive to topoII α -depletion (see below). It will therefore be interesting to determine the timing of p53 induction and any underlying DNA damage, and the nature of the lagging chromatin and damaged DNA.

TopoII α and the Nature of the Catenation Checkpoint

Evidence for a G2 catenation checkpoint depends heavily on the belief that catalytic inhibitors such as ICRF-193 have no topoII poisoning activity (Downes *et al.*, 1994), and because recent data challenge this belief (see Introduction), it was important to test for G2 delay after topoII α depletion in HTETOP cells, where topoII poisoning is not an issue. The observed stimulation by caffeine of mitotic entry after topoII α depletion (Figure 3) is certainly consistent with G2 delay caused by the proposed catenation checkpoint, which is known to be caffeine sensitive. Nevertheless, the observation (Figure 2A) that p53 is induced after topoII α depletion, combined with the fact that caffeine is an established radiosensitizer and inhibitor of kinases intimately involved in the response to DNA damage (Sarkaria *et al.*, 1999), suggest that G2 delay after topoII α depletion, and perhaps after topoII inhibition, may be a response to DNA damage. DNA damage may be caused by mechanical stresses that are likely to accumulate in overly catenated and supercoiled sister chromatids even before anaphase (e.g., during replication, transcription, and condensation), and in this sense the G2 delay could be considered as both a catenation and a DNA damage checkpoint. Regardless of the exact nature of the checkpoint pathway, it is clear that the G2 delay after topoII α depletion is insufficiently robust to prevent HTETOP cells from entering mitosis and suffering catastrophic consequences. Presumably the response has evolved to deal with less severe perturbations than those caused by extensive topoII α depletion.

Physiological Significance of topoII α Phosphorylation Sites

The ability to carry out complementation analyses in HTETOP cells has allowed us to begin an analysis of the physiological significance of topoII α phosphorylation sites. Two sites in the catalytic domain were mutated: Y805, the tyrosine residue to which DNA becomes covalently linked as part of the catalytic cycle, and S1106, a residue whose phosphorylation was previously shown to be required for full catalytic activity and sensitivity to topoII inhibitors (Chikamori *et al.*, 2003). As expected, the Y805F mutation failed to rescue HTETOP from doxycycline sensitivity, confirming that catalysis is essential. Although the S1106A protein was able to complement yeast TOP2 mutants (Chikamori *et al.*, 2003), it was possible that its deficiencies were more severe in mammalian cells. The S1106A protein was able to complement HTETOP cells, however, indicating that this phosphorylation site, on its own, is dispensable for essential topoII functions. Three further phosphorylatable serine residues, 1247, 1354, and 1393, were mutated to alanine. Each of these is in the C-terminal region and, like S1106, has previously been shown to be phosphorylated by

proline-directed kinase preferentially in G2/M, suggestive of roles in regulating topoi functions in preparation for and during mitosis (Wells and Hickson, 1995). None of these mutations, however, was found to prevent rescue from doxycycline sensitivity or nuclear localization. Thus, although phosphorylations of these residues may yet prove to be essential under certain physiological conditions, they are not individually essential during the normal cell cycle.

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