Defective p53 engagement after the induction of DNA damage in cells deficient in topoisomerase 3β

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The type IA topoisomerases have been implicated in the repair of dsDNA breaks by homologous recombination and in the resolution of stalled or damaged DNA replication forks; thus, these proteins play important roles in the maintenance of genomic stability. We studied the functions of one of the two mammalian type IA enzymes, Top3β, using murine embryonic fibroblasts (MEFs) derived from top3β−/− embryos. top3β−/− MEFs proliferated more slowly than TOP3β+/+ control MEFs, demonstrated increased sensitivity to DNA-damaging agents such as ionizing and UV radiation, and had increased DNA double-strand breaks as manifested by increased γ-H2AX phosphorylation. However, incomplete enforcement of the G1-S cell cycle checkpoint was observed in top3β−/− MEFs. Notably, ataxia-telangiectasia, mutated (ATM)/ATM and Rad3-related (ATR)-dependent substrate phosphorylation after UV-B and ionizing radiation was impaired in top3β−/− versus TOP3β+/+ control MEFs, and impaired up-regulation of total and Ser-18-phosphorylated p53 was observed in top3β−/− cells. Taken together, these results suggest an unanticipated role for Top3β beyond DNA repair in the activation of cellular responses to DNA damage.

cell cycle checkpoint | DNA repair | radiation sensitivity

The type I and type II DNA topoisomerases catalyze the sequential breakage and rejoining of single DNA strands or double helices, respectively, and play crucial functions in the maintenance of genomic stability (1). Type I enzymes break and rejoin DNA strands one at a time, whereas type II enzymes break and rejoin DNA strands two at a time in an ATP-dependent manner (2). In particular, the type IA topoisomerases, characterized by an active site tyrosyl residue linked to a DNA 5′ phosphoryl group (3), have emerged as potential key players in the resolution of intermediates of recombination/repair (6). The authors declare no conflict of interest.


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Fig. 2. Increased γ-H2AX phosphorylation in top3β−/− cells. Shown are flow cytometry profiles (A) and Western analyses (B) of acid-extracted nuclear proteins from top3β−/− and TOP3β−/+ control (WT) MEFs before and 30 min after irradiation with 10 Gy from a 137Cs source, in the presence or absence of 10 nM of the phosphatase inhibitor Calyculin A. For Western analyses, TATA binding protein (TBP) was used as a nuclear loading control.
point responses appeared grossly unperturbed. In S. cerevisiae, an inappropriate acceleration in S-phase progression was observed in top3 cells after the induction of DNA damage, also suggesting a role for Top3 in the enforcement of cell cycle checkpoints (4, 30). The effect of Top3β deficiency on enforcement of the G1/S checkpoint may reflect differential roles for Top3α and Top3β and the activity of p53 in mediating the G1/S checkpoint in mammalian cells of S. cerevisiae. In contrast, the fundamental role of p53 in mediating the G1/S checkpoint in mammalian cells led us to evaluate p53 engagement after the induction of DNA damage in top3 cells (31). Because top3β cells have increased levels of DNA damage as manifested by phosphorylation of γ-H2AX, we expected to find intact or increased p53 engagement in such cells, instead, we observed that the up-regulation of both total and Ser-18 phosphorylated p53 was defective in top3β−/− cells treated with both ionizing and, particularly, UV-B radiation (Fig. 5). Moreover, we observed markedly decreased ATM/ATR-dependent phosphorylation after the induction of DNA damage in top3β−/− cells, although the recruitment of H2AX to sites of DNA double-strand breaks appeared intact and indeed enhanced in such cells (Figs. 2 and 4). Taken together, these results suggest that Top3β plays an unanticipated role in facilitating the transduction of signals resulting from DNA damage to the p53 effector response. Decreased p53 activation in this setting could result from several potentially interrelated mechanisms. Because Ser-15/18 phosphorylation of p53 is ATM- and ATR-dependent (32–34), it is attractive to speculate that the decreased phosphorylation we have observed is a consequence of decreased ATM and ATR kinase activity. However, it is also possible that Top3β-mediated repair of a DNA damage intermediate may be required for optimal activation of ATM and ATR, and subsequent p53 engagement. Alternatively, the diminished immunofluorescence observed by using the ATM/ ATR substrate antibody in top3β−/− cells after DNA damage could also suggest a potential function for Top3β in mediating the formation of nuclear foci indicative of ATM and ATR transduction complexes. In this context, the reduced number of positive nuclei in top3β−/− cells (see Fig. 4) likely is an underestimate of ATM/ATR substrate phosphorylation, because cells were scored as positive regardless of the intensity of fluorescence. Finally, a direct interaction between Top3β and p53 remains a possibility, although at present we have no evidence for such an association.

Although our results are consistent with a function for Top3β in DNA repair processes, they also suggest a previously unanticipated role for Top3β in the signal transduction response to DNA damage, and thereby provide evidence for a plausible link between Top3β and regulation of the G1/S transition in the maintenance of genomic stability. Whether our findings reflect a combination of differential roles for Top3β and Top3α, the two mammalian type IA topoisomerases, and/or compensatory functions mediated by Top3α in the absence of Top3β activity remains to be determined in future studies.

Materials and Methods

Mice. Mice lacking Top3β were maintained in a specific pathogen-free environment at the Yale Animal Resource Center, Yale University School of Medicine. These mice were originally generated in a 129 × C57BL/6 background.
Ground and had been backcrossed to C57Bl/6 for five generations. Genotyping of mice was carried out via a PCR-based assay as described (26).

Culture of MEFs. Embryos were harvested on embryonic day 13.5 and placed separately in PBS. The fetal liver was removed by forceps to avoid contamination from hepatocytes and was used for genotyping of the embryos. The remaining portion of the embryo was minced, subsequently placed in a 15-ml conical tube carrying 0.25% trypsin EDTA (GIBCO), and incubated at 37°C for 1 h with intermittent pipetting to generate single-cell suspensions and allow debris to settle. The suspended cells were transferred to a fresh 50-ml tube containing 25 ml of DMEM (supplemented with 10% FBS, penicillin, streptomycin, 40 mM L-glutamine, 2 mM nonessential amino acids, 20 mM sodium pyruvate, 200 mM Hepes buffer, and 0.6% β-mercaptoethanol), washed twice with PBS, and finally plated in a 10-cm culture dish. The next day, the medium was changed with pipetting to remove nonadherent cells. Cells at the second passage (P2) were grown to near confluence, harvested by trypsinization, and frozen in 10% DMSO in liquid nitrogen.

Such P2 MEFs were thawed and seeded as described for all analyses described below. For analysis of cell growth, TOP3β++ control and TOP3β−/− MEFs (2 × 10⁴ cells in DMEM) were seeded onto six-well plates in duplicate; on days 2, 4, 6, and 8, cells were harvested by trypsinization, and live cells were counted by using the trypan blue dye exclusion method.

Radiation Sensitivity Assay. Briefly, TOP3β++ control and TOP3β−/− MEFs were seeded onto 6-cm dishes in triplicate. The next day, MEFs were irradiated with differing dosages of ionizing radiation (ranging from 0 to 500 rads) by using a 137Cs γ-ray source. After irradiation, medium in each plate was replaced with fresh warm medium, and cultures were incubated for 7 days at 37°C with a change in media at day 3. At the end of the assay period, MEFs were harvested by trypsinization, and viable cells were counted as described above.

Sensitivity to UV-B radiation was assessed as described, with some modifications (35). TOP3β++ control and TOP3β−/− MEFs were seeded in triplicate onto six-well plates at 2 × 10⁴ cells per well in DMEM and allowed to grow to ~70% confluence. The medium was replaced with PBS, and MEFs were irradiated with varying dosages of UV-B (ranging from 0 to 1,000 J/m²). After irradiation, the MEFs were washed twice with fresh DMEM without HU, and cultures were incubated at 37°C with a change in media at day 3. At the end of the assay period, MEFs were harvested by trypsinization, and viable cells were counted as described above.

For sensitivity to hydroxyurea (HU), TOP3β++ control and TOP3β−/− MEFs were allowed to attach to plates at a density of 2 × 10⁴ per well in six-well plates in triplicate. At ~60-70% confluence, the medium was replaced with DMEM containing varying concentrations of HU (from 0 to 500 μM) for 24 h. After incubation with HU, MEFs were supplied with fresh DMEM without HU and allowed to grow for 2 more days, and the viable cells were counted as outlined above.
Analysis of p53 After Genotoxic Stress. MEFs were seeded in 6-cm dishes, grown to 80% confluence in DMEM, and subjected to either 10 Gy of ionizing radiation from a 137Cs γ-ray source or 1,000 J/m² UV-B. At specific time points (30, 120, and 360 min) MEFs were harvested by trypsinization and washed twice with cold PBS. Cells were lysed in 20 mM Tris/HCl buffer (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 1 mM Na₂VO₄, 1 mM PMSF, and 1 × Complete protease inhibitors (Roche) on ice. After lysis, the samples were centrifuged at 9,279 × g at 4°C for 20 min. The cell extract was then boiled in SDS sample buffer for 5 min, fractionated by 10% SDS/PAGE, and transferred to PVDF membranes (Millipore). Membranes were blocked for 1 h at room temperature with 1 × PBS containing 0.1% Tween-20 and 5% nonfat milk powder and incubated overnight at 4°C with the primary antibody (anti-p53 mA b (clone IC12), rabbit anti-phospho-Ser-15-p53 Ab (both from Cell Signaling Technology) and monoclonal anti-p21 (clone SX118) from BD PharMingen). After washing, the membranes were incubated with HRP-conjugated anti-mouse and anti-rabbit IgG (H + L) secondary antibodies (Zymed) for 1 h at room temperature, washed, and developed by using a chemiluminescent reagent (Amersham) followed by exposure to Kodak Biomax XAR film.

For the luminescent reagent (Amersham) followed by exposure to Kodak Biomax XAR film. The β-actin control, membranes were stripped of the bound antibodies and probed with anti-mouse β-actin mAb (clone AC74; Sigma) for 1 h at 1 × TBS, 0.1% Tween-20, and 5% milk powder followed by an HRP-conjugated anti-mouse secondary antibody.

Cell Cycle Checkpoint Analyses. For assessment of the G1/S checkpoint, MEFs were incubated with medium containing 0.1% FBS for 72 h, then replated overnight in 6-cm dishes containing fresh growth medium supplemented with 10% FBS. MEFs were then either mock-treated or irradiated with 10 Gy from a 137Cs γ-ray source. Eighteen hours after irradiation, MEFs were pulsed with 10 μM BrdU for 1 h at 37°C and then harvested and processed for FACS analysis. BrdU incorporation was visualized using the FITC BrdU Flow Kit (BD Pharmingen) according to the manufacturer’s instructions. Acquisition of data was made with a BD FACS Calibur machine, and analysis used FlowJo software (Treestar).

For evaluation of the intra-S-phase checkpoint, MEFs were irradiated with 350 J/m² UV-B in PBS, pulsed with BrdU as described above 2 h postirradiation, and then processed for FACS analysis. Assessment of radio-resistant DNA synthesis was performed as described (36, 37) with slight modification. Briefly, 70% confluent MEFs were incubated with growth medium containing 0.0125 μCi/methyl-14C-thymidine (GE Healthcare) for 24 h, then harvested and re-plated in 6-cm dishes in equal number. At 24 h after the MEFs were either mock-treated or irradiated with 10 Gy from a 137Cs γ-ray source, one hour after BRIT treatment, MEFs were pulsed with 1 μCi of methyl-14C-thymidine (GE Healthcare) for 30 min. Cells were then washed with medium/PBS and trypsinized in solutions containing 2.5 mM cold thymidine. Dual-labeled cells were processed in a cell harvester, and radioactivity was measured using a 1450 MicroBeta Trilux scintillation counter (PerkinElmer); the 14C/12C ratio was calculated after correction for channel cross-over as described (36).

For interrogation of the G2/M checkpoint, MEFs at 60–70% confluence were treated with 5 Gy of ionizing radiation and at the timepoints indicated, they were fixed and permeabilized by using Cytofix/Cytoperm buffer (BD PharMingen). The cells were stained with Alexa Fluor 647 antibody phosphohistone H3 (1:10) antibody and 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry. For assessment of the G1/S checkpoint, MEFs were treated with 10 Gy or with a polyclonal rabbit anti-phospho-Ser/Thr ATM/ATR substrate antibody (Cell Signaling Technology). MEFs were plated on glass coverslips for attachment before irradiation. After irradiation with either ionizing radiation or UV-B, cells were fixed with 3% paraformaldehyde at room temperature for 20 min, washed with DMEM containing 10 mM Hepes (pH 7.4) three times for 5 min each, and then incubated with permeabilization buffer (DMEM containing 10% FBS, 10 mM Hepes (pH 7.4), 10 mM glycine, and 0.05% saponin (Sigma)) for 15 min at room temperature. Then permeabilized and fluorophore-labeled cells were incubated with the ATM/ATR-phosphorylated substrate antibody (diluted in permeabilization buffer) overnight at 4°C, washed three times for 5 min each with permeabilization buffer, and incubated with Alexa Fluor 546-conjugated anti-rabbit IgG (Invitrogen) for 1 h at room temperature. Cells were washed three times with permeabilization buffer and mounted with VectaShield (Vector Laboratories) containing DAPI. Images were acquired under dark field with an automated Olympus BX-61 microscope, and analysis was carried out with Adobe Photoshop CS2 software.

For statistical analyses comparing pATM/pATR phosphorylation in TOP3β−/− control and top3β−/− MEFs after either ionizing or UV-B radiation, we used an exact Wilcoxon nonparametric test. The proportion of pATM/pATR substrate-positive fluorescence to the total number of DAPI-positive cells in three microscopic fields was assessed in three independent experiments with independently derived WT and top3β−/− MEFs. All statistical tests were two-tailed, and P < 0.05 was considered to indicate statistical significance. All analyses were performed with SAS version 9.1 (SAS Institute).

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Ionizing Radiation-Induced Phosphorylation of Histone H2AX. MEFs grown in the presence or absence of 10 nM Calyculin A (Sigma) were treated with 10 Gy of ionizing radiation from a 137Cs γ-ray source; 30 min later, cells were harvested, fixed, permeabilized, and stained with FITC-conjugated anti-phospho Ser-139 histone H2-AX antibody (Upstate) and propidium iodide per the manufacturer’s protocol for analysis by flow cytometry.

For Western analysis, cells were harvested, washed in PBS, and lysed in 20 mM Tris/HCl buffer (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 1 mM Na₂VO₄, 1 mM PMSF, and 1 × Complete protease inhibitors (Roche) on ice. After lysis, the samples were centrifuged at 9,279 × g at 4°C for 20 min. The pellet was washed with cold lysis buffer, resuspended in 0.2 M HCl, and incubated overnight at 4°C. Western analysis of acid-extracted proteins was carried out by using a rabbit anti-phospho-Ser-139 H2-AX antibody (Cell Signaling Technology), with a mAb against TBP (clone 17; BD Biosciences) used as a control.

Immunofluorescence Microscopy. Immunofluorescence microscopic analysis of irradiated and nonirradiated TOP3β−/− control and top3β−/− MEFs was carried out as described (38) with a polyclonal rabbit anti-phospho-(Ser/Thr) ATM/ATR antibody (BD PharMingen). MEFs were plated on glass coverslips for attachment before irradiation. After irradiation with either ionizing radiation or UV-B, cells were fixed with 3% paraformaldehyde at room temperature for 20 min, washed with DMEM containing 10 mM Hepes (pH 7.4) three times for 5 min each, and then incubated with permeabilization buffer (DMEM containing 10% FBS, 10 mM Hepes (pH 7.4), 10 mM glycine, and 0.05% saponin (Sigma)) for 15 min at room temperature. Then permeabilized and fluorophore-labeled cells were incubated with the ATM/ATR-phosphorylated substrate antibody (diluted in permeabilization buffer) overnight at 4°C, washed three times for 5 min each with permeabilization buffer, and incubated with Alexa Fluor 546-conjugated anti-rabbit IgG (Invitrogen) for 1 h at room temperature. Cells were washed three times with permeabilization buffer and mounted with VectaShield (Vector Laboratories) containing DAPI. Images were acquired under dark field with an automated Olympus BX-61 microscope, and analysis was carried out with Adobe Photoshop CS2 software.

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