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Estrogen receptor α-mediated transcription induces cell cycle-dependent DNA double-strand breaks

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Prolonged exposure to estrogen increases breast cancer risk. Estrogen is known to induce chromosomal aberrations, yet the mechanisms by which estrogen promotes genomic instability are not fully understood. Here, we show that exposure of MCF-7 cells to 17β-estradiol (E2) induces DNA double-strand breaks (DSBs), as determined by the formation of γH2AX foci. Foci formation was dependent upon estrogen receptor-α (ERα) and the catalytic activity of the type II topoisomerase, topoisomerase IIβ (topoIIβ). Moreover, we show by chromatin immunoprecipitation that topoIIβ-dependent E2-induced γH2AX localizes to the promoter of the estrogen-inducible gene, trefoil factor 1. E2-induced foci were associated with cyclin A expression and inhibited by pre-incubation with the DNA polymerase inhibitor aphidicolin suggesting that E2-induced DSBs are mediated by progression through S phase. Furthermore, E2-induced γH2AX foci colocalized with Rad51, suggesting that E2-induced DSBs are repaired by homologous recombination. We propose that DNA DSBs formed by the strand-cleaving activity of the topoIIβ-DNA cleavage complex at estrogen-inducible genes can present a barrier to DNA replication, leading to persistent DNA DSBs in ERα-positive breast cancer cells.

Introduction

Prolonged exposure to estrogen correlates with increased breast cancer risk. In addition to its role as a mitogen, estrogen is also a genotoxic agent. Exposure to estrogen or its catechol metabolites, 2- and 4-hydroxyestradiol, results in oxidative DNA damage and DNA strand breaks (1–4). Furthermore, estrogen quinone metabolites interact directly with DNA resulting in abasic sites (1,4). Exposure to estrogen increases the frequency of chromosomal aberrations, a phenotype associated with early stages of breast cancer (1). Maintenance of genome stability is essential for sustained cellular viability and for the prevention of transformation (5). Although estrogen-induced DNA damage has been the subject of investigation for many years, a full understanding of how estrogen results in genomic instability has yet to be achieved.

Estrogen exerts its proliferative function through interaction with and activation of the steroid hormone receptor, estrogen receptor-α (ERα). Recently, a novel mechanism of ERα-mediated transcription regulation that requires the decatenating enzyme topoisomerase IIβ (topoIIβ) has been described (6). An intermediary step of the topoIIβ-dependent decantenating activity involves covalent interaction with the DNA backbone resulting in generation of transient DNA double-strand breaks (DSBs) (7,8). Indeed, transient, topoIIβ-mediated DSBs are generated as a result of multiple normal cellular processes (7); however, stabilization of topoIIβ–DNA covalent complexes can result in persistent DSBs that can lead to genomic instability (9,10).

Here, we investigated the effect of estrogen as a DNA-damaging agent in human breast cancer cells. Treatment of estrogen-responsive MCF-7 breast cancer cells with 17β-estradiol (E2) induced ERα-dependent DSBs as measured by γH2AX foci, a sensitive marker of DSB formation (11). E2-induced DSBs occurred at the promoter of the estrogen-responsive gene, trefoil factor 1 (*TFF1*) and required topoIIβ. Foci appeared 6–24 h after exposure to E2, suggesting that they represent persistent DSBs and not transient topoIIβ cleavage complexes previously ascribed to ERα-mediated transcription (6). Interestingly, E2-induced DNA damage occurred in S and G_2 phases of the cell cycle and colocalized with Rad51, implicating the homologous recombination repair (HRR) pathway in the repair of E2-induced breaks. We propose that estrogen-induced transcription results in the formation of stable topoIIβ–DNA cleavage complexes leading to DNA DSBs and initiation of HRR.

Materials and methods

Reagents and antibodies

Microcystin-LR, bovine serum albumin, phenylmethylsulfonyl fluoride, Tris base, 1,4-piperazinediethane-sulfonic acid, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, ethyleneglycol-bis(aminoethylether)-tetraacetic acid, leupeptin, pepstatin, RNase A, sodium dodecyl sulfate, Triton X-100, XK-469 and 17β-E2 were purchased from Sigma–Aldrich (St Louis, MO). Aphidicolin and merbarone were purchased from EMD Chemicals (Gibbstown, NJ). KU55933 and NU7441 were kind gifts from Drs Graeme Smith and Mark O'Connor (KuDos Pharmaceuticals, Cambridge, UK). Small interfering RNAs (siRNAs) (nonspecific, ataxia-telangiectasia mutated and Rad3-related (ATR), topoIIβ and ERα siRNA ON-TARGETplus SMARTpools) were purchased from Dharmacon (Lafayette, CO). 2- and 4-Hydroxyestradiol were purchased from MP Biomedicals LLC (Solon, OH).

Antibodies were purchased from the following suppliers: poly-ubiquitin (FK2) and γH2AX [immunofluorescence: cat # 05-636; chromatin immunoprecipitation (ChIP): cat# 07-164], Millipore (Billerica, MA); 53BP1 and SMC1, Novus Biologicals LLC (Littleton, CO); pS1981 ataxia-telangiectasia mutated (ATM), Cell Signaling Technology (Danvers, MA); MDC1, Abcam (Cambridge, UK) and ATR, cyclin A and Rad51, Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines

Cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (Hyclone, Logan, UT), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen) at 37°C under a humidified atmosphere of 5% CO_2. Prior to addition of E2, cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (vol/vol) charcoal/dextran-treated fetal bovine serum (Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin 48–72 h.

Ionizing radiation treatment of cells

Irradiation of cells was carried out as described in (ref. 12).

Immunofluorescence

Cells were cultured on poly-L-lysine-coated coverslips and treated with 2 Gy ionizing radiation (IR), E2 or vehicle control (ethanol). Slides were prepared and images captured as in (12).

Quantification of γH2AX foci

Incremental slices (0.5 μm) were taken of each image and deconvolved using Openlab 5.0.2 software (Improvision, Coventry, UK). The number of γH2AX foci from samples containing at least 100 cells was quantified using Imaris ×64 version 5.5.1 (Bitplane, Zurich, Switzerland) using constant intensity and size thresholds (≥0.5 μm) in at least three independent experiments. Standard error of the mean between the three independent experiments was calculated and plotted on the corresponding graphs. One-way analysis of variance with Newman–Keuls post-hoc test was applied and statistical significance of $P < 0.05$ (95% confidence interval) is indicated by asterisk.

Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia mutated and Rad3-related; ChIP, chromatin immunoprecipitation; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; E2, estradiol; ERα, estrogen receptor-α; HRR, homologous recombination repair; IR, ionizing radiation; IRIF, ionizing radiation-induced foci; siRNAs, Small interfering RNAs; topoIIβ, topoisomerase IIβ; TFF1, trefoil factor 1.

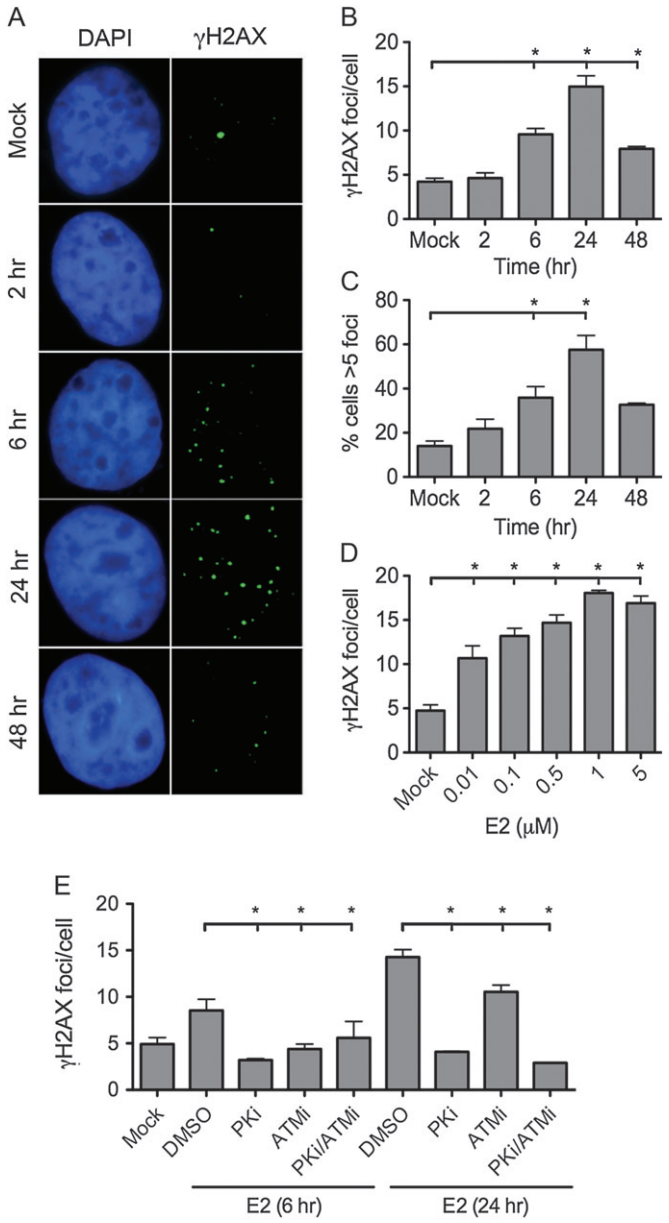


Fig. 1. E2 induces γH2AX foci in MCF-7 cells. (**A**) Cells were treated with E2 (1 μM) or vehicle control (mock) for the indicated times and stained for γH2AX foci (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**B**) Quantification of the mean number of γH2AX foci per cell in E2-treated cells as in panel (A). (**C**) Percentage of cells that displayed >5 γH2AX foci after exposure to E2 (1 μM). (**D**) Quantification of the mean number of γH2AX foci per cell in cells treated with E2 for 24 h at the indicated concentrations. (**E**) Cells were pretreated with the ATM inhibitor 55933 (ATMi) (5 μM) and/or the DNA-PK inhibitor NU7441 (PKi) (5 μM) or dimethyl sulfoxide (DMSO) control for 1 h followed by treatment with E2 (1 μM) or vehicle control (mock) for 6 or 24 h and γH2AX foci were quantified.

siRNA transfections

MCF-7 cells were plated in antibiotic-free medium for 24 h prior to transfection and target siRNA or a scrambled siRNA control (100 nM each) was transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions. For ERα and ATR siRNA, cells were incubated for 55 h prior to addition of E2. For topoIIβ siRNA, cells were incubated for 72 h prior to addition of E2.

ChIP

ChIP was performed essentially as described (13). Briefly, MCF-7 cells were treated with E2 (1 μM, 24 h), merbarone (10 μM, 24 h) or vehicle controls then were fixed with 1% (vol/vol) formaldehyde, collected, washed with phosphate-buffered saline and lysed by incubation in cell lysis buffer (see supplementary Material, available at *Carcinogenesis* Online, for details), homogenized with a manual Dounce homogenizer and incubated in nuclei lysis buffer. Lysates were sonicated using a Diagenode Bioruptor^{TM} sonicator (Sparta, NJ) for 7 × 15 s. Sonicates were diluted and incubated with 5 μg anti-γH2AX (Millipore; cat # 07-164) or non-immune serum control overnight at 4°C and antibody complexes were washed, collected and cross-links reversed as in (13). DNA was purified by phenol–chloroform extraction. Quantitative real time polymerase chain reaction was performed using SYBR green qPCR mastermix (Applied Biosystems, Carlsbad, CA) and primers to regions within the *GAPDH* gene and three regions up- and downstream of the transcription start site of the *TFF1* promoter. Percent input was used to quantify the quantitative real time polymerase chain reaction results and fold enrichment was derived by normalizing E2-treated to mock-treated results. Errors bars represent standard error of the mean. Additional details and primer sequences are provided in supplementary Material, available at *Carcinogenesis* Online.

Other methods

Methods for preparation of cell extracts, immunoblotting and cell cycle analysis are provided in supplementary Material, available at *Carcinogenesis* Online.

Results

E2 induces DNA-dependent protein kinase- and ATM-dependent DNA repair foci

To determine whether E2 induces DSBs, we first investigated whether exposure to E2 induces γH2AX foci formation. Incubation of MCF-7 cells with E2 induced a time-dependent increase in the mean number of γH2AX foci per cell as well as an increase in the percentage of cells displaying ≥5 γH2AX foci (Figure 1A–C; supplementary Figure 1A, available at *Carcinogenesis* Online). Moreover, the mean number of E2-induced γH2AX foci increased with increasing concentrations of E2, reaching a maximum at 1 μM (Figure 1D). E2-induced γH2AX foci were also observed in the breast cancer cell line T47D (supplementary Figure 1B and C is available at *Carcinogenesis* Online). As with ionizing radiation-induced foci (IRIF), 53BP1, MDC1, serine 1981-phosphorylated ATM and poly-ubiquitin localized to foci in response to E2 (Figure 2). Together, these data suggest that exposure to E2 creates DSBs in a time- and dose-dependent manner and that E2-induced DNA repair foci contain a similar complement of proteins to those observed in cells following exposure to IR.

ATM and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) are members of phosphatidyl inositol kinase-like-kinase family of protein kinases and both contribute to phosphorylation of H2AX in response to IR (12,14,15). To determine if ATM and/or DNA-PK were responsible for γH2AX foci formation in response to E2, MCF-7 cells were incubated with the ATM-specific inhibitor KU55933 (16) and/or the DNA-PK-specific inhibitor NU7441 (17) prior to addition of E2. Pre-incubation with either KU55933 or NU7441 significantly decreased E2-induced foci formation, with the DNA-PK inhibitor having a greater effect following 24 h of exposure to E2 (Figure 1E). Moreover, pretreatment with both inhibitors together reduced E2-induced foci formation to background levels indicating that ATM and DNA-PK play major roles in induction of γH2AX in response to E2. The related phosphatidyl inositol kinase-like-kinase family member ATR has also been reported to phosphorylate H2AX in response to DNA damage, specifically under conditions of replication stress (18). To determine whether ATR was also involved in E2-induced γH2AX foci, ATR was silenced using siRNA and foci were analyzed. Knock down of ATR did not affect E2-induced γH2AX foci (supplementary Figure 2, available at *Carcinogenesis* Online) consistent with the major kinases responsible for E2-induced γH2AX foci being ATM and DNA-PK.

2- and 4-Hydroxyestradiol do not induce γH2AX foci formation

Given that estrogen metabolites have been implicated in genotoxicity, we treated cells with 2- and 4-hydroxyestradiol at

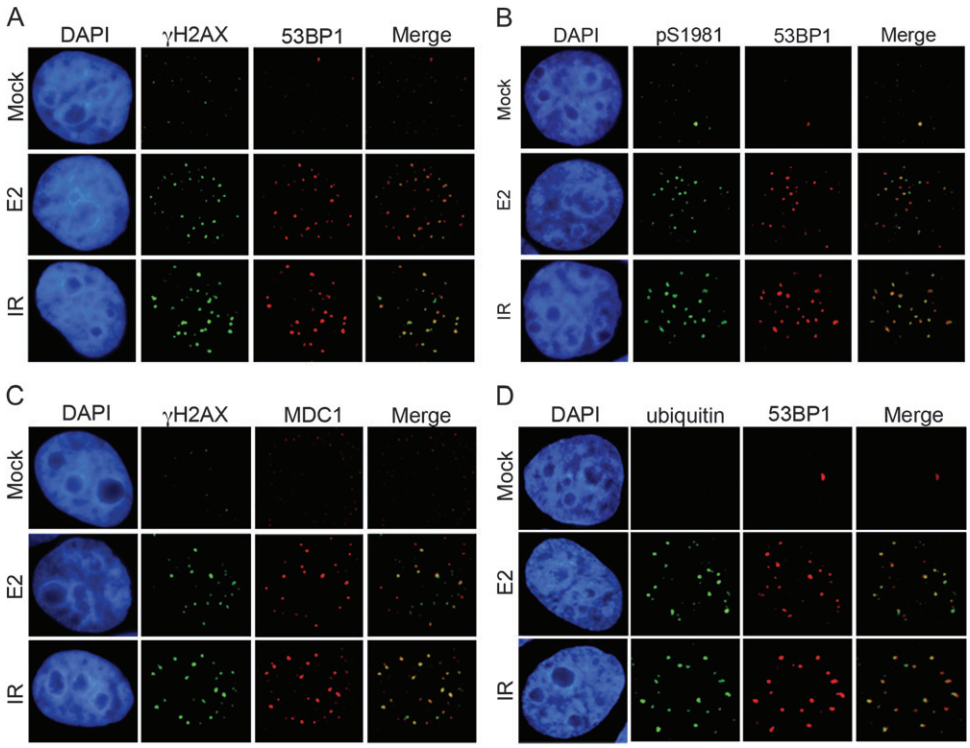


Fig. 2. DNA damage response proteins colocalize with γH2AX in E2-treated cells. Cells were treated with E2 (1 μM) or vehicle control (mock) for 24 h or 2 Gy IR followed by 1 h recovery and stained for (**A**) γH2AX (green) and 53BP1 (red); (**B**) pS1981 ATM (green) and 53BP1 (red); (**C**) γH2AX (green) and MDC1 (red) (**D**) poly-ubiquitin (green) and 53BP1 (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Merged images are shown on the right in each panel.

concentrations shown previously to be capable of inducing reactive oxygen species as well as depurinating adducts (19,20) and measured γH2AX foci formation. However, treatment of MCF-7 cells with either 2- or 4-hydroxyestradiol did not induce γH2AX foci formation (supplementary Figure 3, available at *Carcinogenesis* Online).

E2-induced foci require ERα-mediated transcription

During the course of these experiments, we also analyzed ERα-negative MDA-MB-231 cells for γH2AX foci in response to E2. Although, like MCF-7 cells, MDA-MB-231 cells were capable of forming IRIF, no increase in γH2AX foci over background levels in response to E2 was observed (Figure 3A), suggesting a possible role for ERα in E2-induced γH2AX foci. To test this hypothesis, we depleted ERα in MCF-7 cells using siRNA (supplementary Figure 4, available at *Carcinogenesis* Online). Significantly, knock down of ERα abrogated E2-induced foci but not IRIF (Figure 3B and C). Moreover, E2-induced γH2AX foci were also suppressed by pretreatment with the ERα antagonist ICI 182780 (21) (Figure 3D). To determine whether the transcriptional activity of ERα played a role in γH2AX foci formation, MCF-7 cells were treated with the RNA polymerase II inhibitor α-amanitin. Significantly, α-amanitin also suppressed E2-induced γH2AX foci formation (Figure 3E) suggesting a role for transcription.

We next asked whether E2-induced DSBs occurred at sites of ERα-mediated transcription by assaying for enrichment of γH2AX in the promoter of the E2-responsive gene, *TFF1*, using ChIP. γH2AX was significantly enriched in the *TFF1* promoter in E2-treated cells when compared with mock-treated control cells but not in the *GAPDH* control region (Figure 3F). Taken together, these results suggest that ERα-mediated transcription leads to the production of DSBs, a subset of which occur at sites of E2 inducible genes, leading to γH2AX foci formation.

TopoIIβ is required for E2-induced foci formation

Given that topoIIβ has been shown to function at transcription start sites in nuclear receptor target genes (6,22), we next investigated the role of topoIIβ in the formation of DSBs observed following exposure to E2. Pre-incubation of MCF-7 cells with the general type II topoisomerase inhibitor merbarone (23) reduced the formation of E2-induced foci (Figure 4A and B) while having no effect on IRIF (Figure 4A). Moreover, siRNA depletion of topoIIβ (supplementary Figure 5, available at *Carcinogenesis* Online) reduced E2-induced foci by ∼50% compared with cells transfected with nonspecific siRNA (Figure 4C and D). Additionally, pre-incubation with the topoIIβ inhibitor, XK469 (24), also abrogated E2-induced γH2AX foci (supplementary Figure 6, available at *Carcinogenesis* Online), clearly implicating topoIIβ in this process. To determine whether E2-induced H2AX phosphorylation at the *TFF1* promoter was also dependent on the catalytic activity of topoIIβ, we incubated cells with merbarone followed by E2 and analyzed γH2AX by ChIP. Merbarone significantly reduced γH2AX at the *TFF1* promoter suggesting topoIIβ catalytic activity is required for γH2AX at sites of ERα-mediated transcription (Figure 4E). We conclude that E2-induced DSBs are mediated by the catalytic activity of topoIIβ.

E2-induced γH2AX foci are associated with S and G_2 phase and colocalize with Rad51

As expected, in response to IR, 100% of cells displayed γH2AX foci. In contrast, treatment with E2 for 24 h induced γH2AX foci in only 60–70% of cells (Figure 1C), prompting us to investigate a potential correlation between γH2AX foci and cell cycle. Indeed, we observed an association between the expression of cyclin A, which is upregulated upon onset of S phase, and E2-induced γH2AX foci (Figure 5A and B). Significantly, the percentage of cyclin A-expressing cells with ≥5 γH2AX foci increased in response to E2 treatment (Figure 5B).

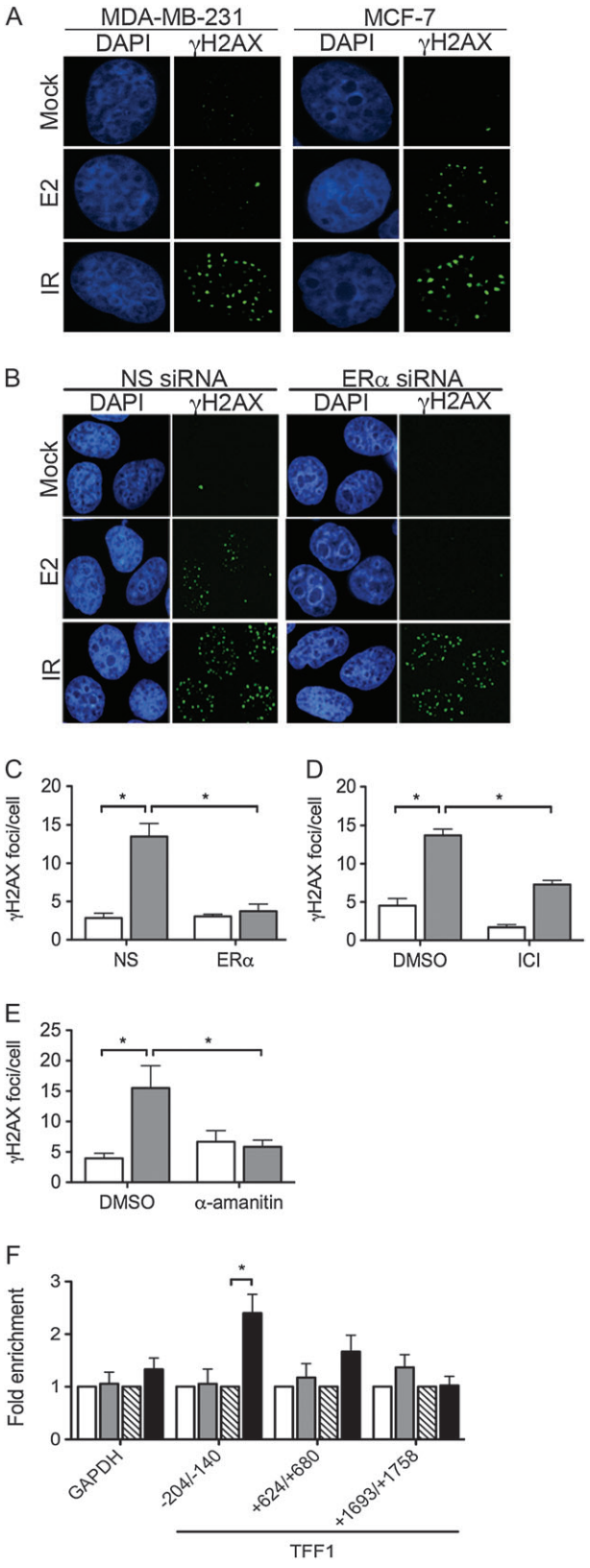


Fig. 3. E2-induced foci require ERα-mediated transcription. (**A**) ERα-positive MCF-7 and ERα-negative MDA-MB-231 breast cancer cell lines were treated with E2 (1 μM, 24 h), 2 Gy IR followed by 30 min incubation or vehicle control (mock) and stained for γH2AX foci (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**B**) MCF-7 cells transfected with nonspecific (NS) siRNA or ERα siRNA were treated with E2 (1 μM) or vehicle control (mock) for 24 h or 2 Gy IR followed by a 1 h recovery then stained for γH2AX foci (green) and DAPI (blue). (**C**) Quantification of γH2AX foci from panel (B), E2 (gray bars), vehicle control (white bars). (**D**) MCF-7 cells were pretreated with the ERα antagonist ICI 182780 (ICI) (100 nM) or dimethyl sulfoxide (DMSO) control (mock) for 1 h followed by E2 (1 μM) (gray bars) or vehicle control (white bars) for 24 h, then stained for γH2AX foci and quantified. (**E**) MCF-7 cells were pretreated with the RNA polymerase II inhibitor α-amanitin (10 μg/ml) or DMSO control and E2 (1 μM) (gray bars) or mock control (white bars) for 6 h and then γH2AX foci was quantified. (**F**) Cells were treated with E2 (1 μM) or vehicle control for 24 h and fold enrichment of ChIP with anti-γH2AX or non-immune serum control at three regions of the *TFF1* gene (−204 to −140; +624 to +680 and +1693 to +1758) or GAPDH was analyzed. Mock, non-immune serum, (white bars); E2-treated, non-immune serum, (gray bars); mock, γH2AX antibody, (hashed bars) and E2 treated, γH2AX antibody, (black bars). Two-way analysis of variance with a Bonferroni post-hoc test was performed and statistical significance $P < 0.05$ (95% confidence interval) is indicated with asterisk.

MCF-7 cells were grown in phenol red-free Dulbecco's modified Eagle's medium supplemented with charcoal/dextran stripped fetal bovine serum for at least 48 h prior to estrogen treatment, resulting in accumulation of cells in G_1 (supplementary Figure 7, available at *Carcinogenesis* Online), and, consistent with its known effect on proliferation, addition of E2 stimulated progression through the cell cycle (supplementary Figure 8 is available at *Carcinogenesis* Online). To hinder the progression of E2-treated cells through S phase, we treated G_1-arrested MCF-7 cells with the DNA polymerase inhibitor aphidicolin prior to addition of E2. Significantly, addition of aphidicolin attenuated the E2-induced increase in γH2AX foci (Figure 5C), suggesting that progression through S phase is required for E2-induced DSBs and consequently γH2AX foci.

Given that unperturbed S phase cells have been reported to contain γH2AX foci as a result of endogenous replication stress (25,26), we wanted to eliminate the possibility that the dose-dependent increase in E2-induced γH2AX foci observed (Figure 1) was due to an increase in the percentage of cells undergoing DNA replication. As shown, 10, 100 or 1000 nM E2 stimulated cell cycle progression to the same extent (supplementary Figure 8, available at *Carcinogenesis* Online) while inducing increasing amounts of γH2AX foci (Figure 1). From these experiments, we conclude that the dose-dependent increase in E2-induced γH2AX foci is not due to an increase in the percentage of replicating cells and thus is not due to endogenous replication stress.

In contrast to nonhomologous end joining, which is considered to be error prone and is active throughout the cell cycle (27), HRR is an error-free DSB repair pathway that utilizes the homologous sister chromatid for a template during repair and is active only in S and G_2 phases of the cell cycle (28). E2-induced γH2AX foci were therefore analyzed for colocalization with Rad51, a key protein required for the HHR. Significantly, γH2AX and Rad51 colocalize in E2-treated cells (Figure 5D). Moreover, all cells that displayed ≥5 γH2AX foci also had ≥5 Rad51 foci (Figure 5D and E). Together, these results suggest that E2-induced damage occurs in S and/or G_2 phase cells and implicates HHR as a repair pathway for E2-induced DSBs.

Discussion

Here, we show that exposure of ERα-expressing breast cancer cells to E2 induces γH2AX foci, which are markers of DNA DSBs. We show that γH2AX foci require ERα, transcription and the catalytic activity of topoIIβ and occur at the promoter of the E2 inducible gene *TFF1*. Furthermore, E2-induced γH2AX foci only appear following progression through S phase and colocalize with Rad51, suggesting that HRR is utilized for repair of E2-induced DSBs. Based on these results, we propose that transient DSBs generated at ERα-responsive genes as a result of the activity of topoIIβ can become stabilized and that, upon progression of cells into S phase, the topoIIβ cleavage complex can pose a barrier to DNA replication and lead to replication stress and DSB formation. This in turn leads to H2AX phosphorylation by ATM and DNA-PK and initiation of Rad51-mediated HRR (Figure 6). Interestingly, DSBs induced by topoIIβ-dependent androgen receptor-mediated

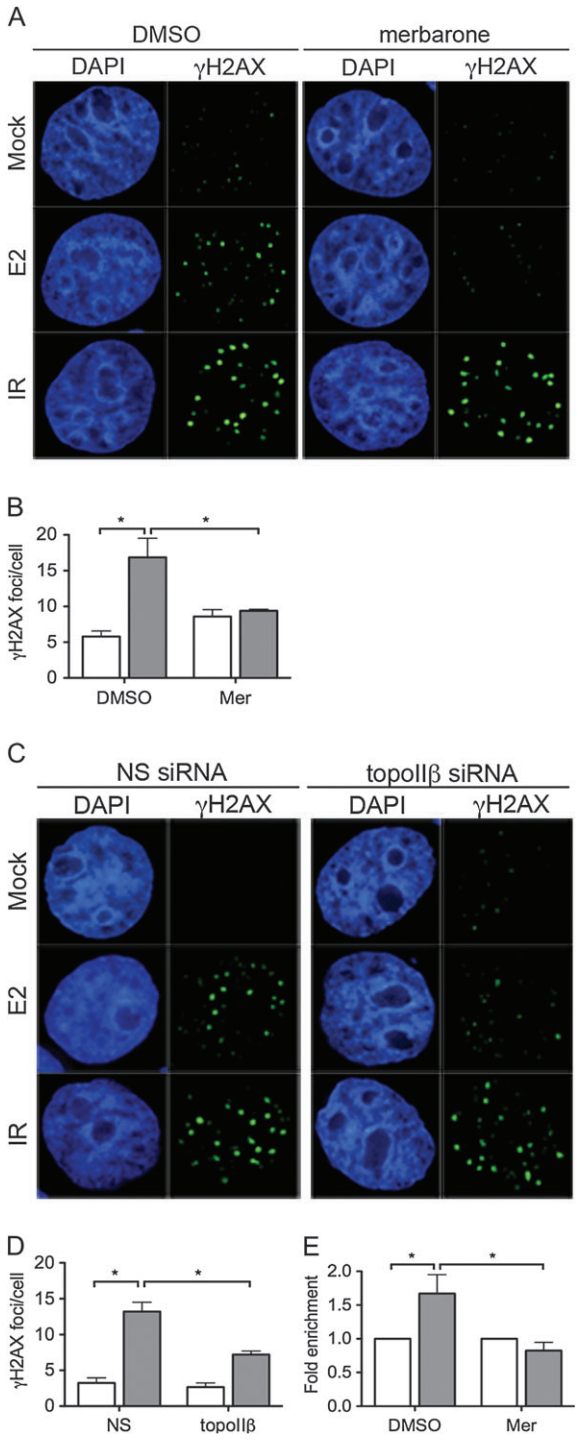


Fig. 4. E2-induced γH2AX foci require the catalytic activity of topoIIβ (**A**) MCF-7 cells were pretreated with dimethyl sulfoxide (DMSO) (mock) or merbarone (Mer) (10 μM) for 1 h and then incubated with E2 (1 μM) for 24 h or irradiated at 2 Gy followed by a 1 h recovery as indicated. Cells were stained for γH2AX foci (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**B**) Quantification of γH2AX foci from (A), E2 (gray bars) or vehicle control (white bars). (**C**) MCF-7 cells transfected with NS siRNA or topoIIβ siRNA were treated with E2 (1 μM) or vehicle control for 24 h or 2 Gy IR followed by a 1 h recovery and then stained for γH2AX foci (green) and DAPI (blue). (**D**) Quantification of γH2AX foci from (C), E2 (gray bars) or vehicle control (white bars). (**E**) Cells were treated with merbarone (10 μM) (gray bars) or DMSO control (white bars) for 1 h followed by E2 (1 μM 24 h) or vehicle control and fold enrichment of ChIP with anti-γH2AX at the TFF1 promoter was analyzed. Students unpaired, two-tailed *t*-test was performed and statistical significance $P < 0.05$ (95% confidence interval) is indicated with asterisk.

transcription in response to testosterone have been shown to lead to translocations involving activated gene targets in prostate cancer cells, suggesting that lesions associated with aberrant steroid hormone receptor signaling can lead to genomic instability (29,30).

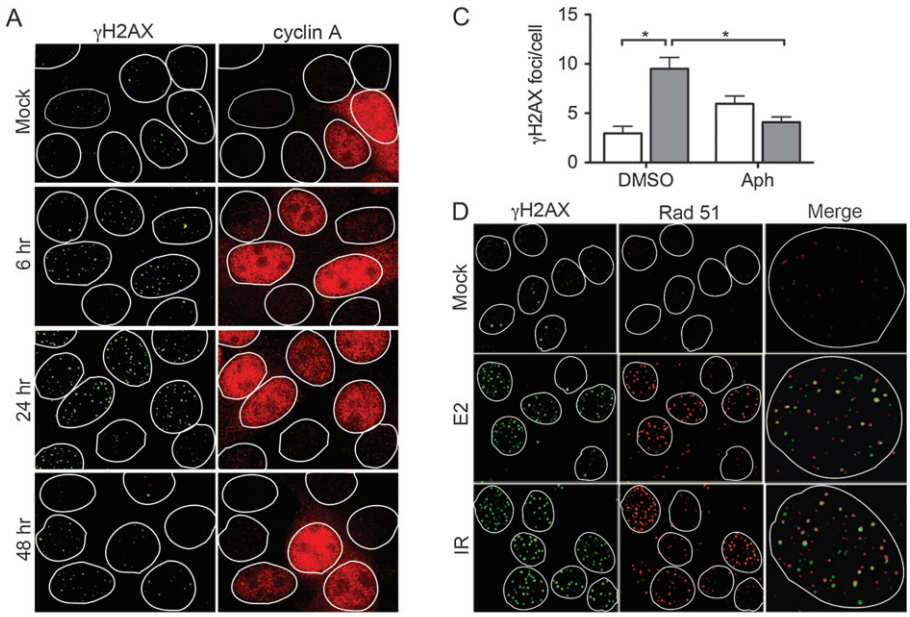
Interestingly, we did not detect a significant increase in γH2AX foci formation when MCF-7 cells were exposed to the estrogen catechol metabolites 2- and 4-hydroxyestradiol. These estrogen metabolites are genotoxic and can promote cellular transformation mediated by the production of reactive oxygen species during redox cycling and by the formation of depurinating DNA adducts (1,4). DNA damage as a result of these processes could lead to mutagenesis and transformation; however, we speculate that the majority of DNA damage caused by reactive oxygen species and depurinating adducts would be single stranded in nature and thus would not lead to a dramatic increase in H2AX phosphorylation or γH2AX foci.

As for IR, E2-induced γH2AX foci formation required the activity of both DNA-PK and ATM (Figure 1E). In addition to its well-established role in phosphorylation of H2AX (12,14,15), DNA-PK also forms a complex with topoIIβ and poly-ADP ribose polymerase-1 at sites of transcription (6,22) (Figure 6) and may be involved in regulating E2-mediated transcription (31). We speculate that the presence of DNA-PK at transcription sites might allow for efficient signaling and repair in response to transcription-induced DSBs.

Given that ATR is responsible for activation of the DNA damage response in response to replication stress (18) and that E2-induced foci required passage through S phase, it might be expected that ATR would be required for H2AX phosphorylation in response to E2 treatment. However, although we cannot exclude the possibility that residual ATR remaining after siRNA knock down contributes to H2AX phosphorylation, our data suggest that ATR is not involved in E2-induced γH2AX foci formation (supplementary Figure 2, available at *Carcinogenesis* Online). Interestingly, E2 signaling has been shown to inhibit DNA damage induced activation of ATR (32). Given the role for ATR in stabilization of the replication fork in response to replication stress (33), we speculate that inhibition of ATR by E2 could suppress fork stabilization upon encountering replication barriers. Inhibition of ATR by E2 signaling could therefore promote fork collapse and DSB formation. Accordingly, ATM and DNA-PK have previously been reported to phosphorylate H2AX and promote Rad51 foci formation in response to replication stress in an ATR-deficient background (34).

Our work also raises several interesting questions regarding the mechanism of formation of E2-induced DSBs. E2-stimulated transcription is initiated within 1 h (35). However, E2-induced foci occurred over 6–24 h, subsequent to ERα ubiquitylation and degradation (data not shown). One possible explanation for this paradox is that breaks only occur following the attempted replication of transcription-associated lesions, a notion that is supported by our results. Interestingly, it has been recently reported that actively transcribed genes are refractory to H2AX phosphorylation suggesting that transcriptional activity may also influence the response to transcription-induced DBSs (36). The inhibitory effect of transcription on H2AX phosphorylation may provide insight into our observation that regions downstream of the *TFF1* promoter do not show significant enrichment of γH2AX in ChIP assays (Figure 3F).

In conclusion, our results provide new insights into the influence of transcription and replication on the induction of, and response to, DNA DSBs in response to estrogen. Moreover, our study links E2-induced DNA damage to pathways known to be required for the detection and repair of DNA DSBs and maintenance of genomic stability. Chromosomal aberrations are associated with early stage breast cancer and are also observed in cells exposed to estrogen (1,4). Furthermore, mutations in genes encoding for proteins involved in the DNA damage response, including ATM (37), the DSB sensing proteins Nbs1 and Rad50 (38) and HRR factors BRCA1/2 (39), BLM and Rad51 (40) are associated with increased risk of breast cancer. Our results suggest a plausible link between estrogen exposure, endogenous DSB formation and increased risk of cancer of the breast.



B

	Mock	6 hr	24 hr	48 hr
% of cyclin A pos. cells with ≥5 γH2AX foci	60	83	72	60

E

	IR	E2
% of cells with ≥5 γH2AX foci	100	68
% of cells with ≥5 Rad51 foci	53	68
% of γH2AX pos. cells with Rad51 foci	53	100

Fig. 5. E2-induced foci are associated with S/G_2 phase and contain Rad51. (**A**) MCF-7 cells were treated with E2 (1 μM) for 6, 24 and 48 h and stained for γH2AX foci (green) and cyclin A (red). White circles represent 4′,6-diamidino-2-phenylindole boundaries. (**B**) Percent of cyclin A-positive cells that have ≥5 γH2AX foci. (**C**) Cells were pretreated with aphidicolin (Aph, 0.3 μM) or dimethyl sulfoxide (DMSO) control (mock) for 1 h followed by incubation with E2 (1 μM) (gray bars) or vehicle (white bars) control for 6 h were stained for γH2AX foci and the number of foci per cell was quantified (**D**) Cells were treated with E2 (1 μM) or vehicle control (mock) for 24 h or 2 Gy IR followed by a 1 h recovery and then stained for γH2AX (green) and Rad51 (red). Individual nuclei are circled in white. Merged γH2AX and Rad51 from a representative cell is shown on the right. (**E**) Percentage of cells displaying >5 γH2AX foci (top row), >5 Rad51 foci (middle row) and both >5 γH2AX and Rad51 foci (bottom row) following exposure to 2 Gy IR followed by a 1 h recovery or E2 (1 μM) for 24 h.

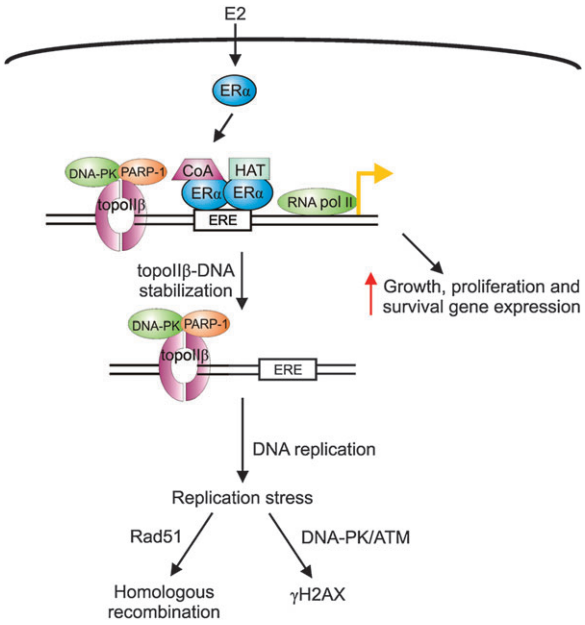


Fig. 6. Model for E2-induced DSBs. In response to E2 exposure, ligand-bound ERα, histone acetyltransferases (HAT), transcription co-activators (CoA), topoIIβ in complex with DNA-PK and poly-ADP ribose polymerase-1 localize to estrogen-inducible genes facilitating the expression of growth- and proliferation-associated genes. Upon DNA replication, stabilized topoIIβ-DNA cleavage complexes can result in replication stress, ATM and DNA-PK-dependent H2AX phosphorylation and Rad51-mediated HRR.

Supplementary material

Supplementary Material and Figures 1–8 can be found at http://carcin.oxfordjournals.org/

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