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REPORT

Estrogen induces RAD51C expression and localization to sites of DNA damage

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ABSTRACT

Homologous recombination (HR) is a conserved process that maintains genome stability and cell survival by repairing DNA double-strand breaks (DSBs). The RAD51-related family of proteins is involved in repair of DSBs; consequently, deregulation of RAD51 causes chromosomal rearrangements and stimulates tumorigenesis. RAD51C has been identified as a potential tumor suppressor and a breast and ovarian cancer susceptibility gene. Recent studies have also implicated estrogen as a DNA-damaging agent that causes DSBs. We found that in ERα-positive breast cancer cells, estrogen transcriptionally regulates RAD51C expression in ERα-dependent mechanism. Moreover, estrogen induces RAD51C assembly into nuclear foci at DSBs, which is a precursor to RAD51 complex recruitment to the nucleus. Additionally, disruption of ERα signaling by either anti-estrogens or siRNA prevented estrogen induced upregulation of RAD51C. We have also found an association of a worse clinical outcome between RAD51C expression and ERα status of tumors. These findings provide insight into the mechanism of genomic instability in ERα-positive breast cancer and suggest that individuals with mutations in *RAD51C* that are exposed to estrogen would be more susceptible to accumulation of DNA damage, leading to cancer progression.

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Introduction

Homologous recombination (HR) is a conserved process that maintains genome stability and cell survival by repairing DNA double-strand breaks (DSBs). DSBs arise from V(D)J recombination, meiosis, class switch recombination, exposure to reactive oxygen species, ionizing radiation (IR), ultraviolet light or chemotherapeutic agents as well as DNA replication. Unrepaired DSBs represent the most detrimental form of DNA damage and lead to accumulation of chromosome aberrations that may lead to immunodeficiency, neurodegeneration and cancer susceptibility. The importance of proper HR to DSB repair is further supported by findings that impaired HR is associated with genetic diseases such as ataxia telangiectasia, Nijmegen breakage syndrome, Fanconi anemia (FA) and Bloom's syndrome.[1,2] Importantly, mutations in HR-related genes are associated with tumorigenesis and cause accumulation of unrepaired DSBs leading to increased genomic instability and cancer. Breast cancer is the most common cancer affecting women, and ~5% of cases are due to inherited mutations in *BRCA1* and *BRCA2* genes, encoding for tumor suppressors that are involved in HR and DSB repair. Heterozygous mutation of *BRCA1* or *BRCA2* increases the risk of developing breast cancer by 80% and ovarian cancer by 40%.[3-6]

RAD51 is a small monomeric protein that assembles into long helical polymers on single strand DNA at the break site during HR and is important for DSB repair.[7] Deregulation of RAD51 in human cells causes chromosome rearrangements and stimulates tumorigenesis.[8] RAD51 paralogs is a family of proteins that help RAD51 recruitment to the DNA break sites and include RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3.[9-13] These paralogs share 20–30% of identity at the amino acid level and can assemble into 2 distinct complexes: RAD51B/RAD51C/RAD51D/XRCC2 complex and RAD51C/XRCC3 complex, with RAD51C being the only member that is part of both complexes.[8,14,15] Mutation of *Rad51* paralogs in mice causes early embryonic lethality and accumulation of unrepaired DNA damage,[16,17] highlighting their function in preserving genomic integrity. Abrogation of their function in Chinese hamster ovary (CHO) cells or chicken DT40 B-lymphocytes sensitizes cells to ionizing radiation (IR) and DNA damaging agents such as mitomycin C, cisplatin and camptothecin.[18-26] Additionally, RAD51-deficient cells exhibit increased chromosomal aberrations, abnormal centromere number, reduced frequency of DSB repair and reduced sister chromatid exchanges. Knockdown of RAD51 expression was able to inhibit cancer cell migration as well as tumor growth and metastasis,[27] highlighting the role of RAD51 in triple negative breast cancers (TNBC). Specifically, in TNBC, it has been shown that co-inhibition of RAD51 together with p38 reduced cell proliferation and may be a novel clinical strategy.[28]

RAD51C, which has a role in HR and RAD51 recruitment to DSBs, has been recently identified as a potential tumor suppressor[29] and a breast and ovarian cancer susceptibility gene.[30-32] RAD51C appears to have a uniquely important role in breast cancer. *RAD51C* is localized to the chromosomal region 17q23, an amplicon present in high copy number in breast cancer.[33-35]

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An additional link between RAD51C and breast cancer stems from direct interaction of BRCA2 with RAD51 complex, which directs BRCA2 to the sites of DNA damage.[36,37] BRCA1 localizes with RAD51 into nuclei foci,[38] further implicating RAD51 paralogs in both DSB repair and breast cancer.

Upon DNA damage caused by IR, RAD51C accumulates into nuclear foci and co-localizes with RAD51. Moreover, RAD51C is thought to regulate the translocation of RAD51 from the cytoplasm to the nucleus via the nuclear localization signal.[39,40] *Rad51c* deletion in mouse embryonic fibroblasts (MEFs) also results in reduction in sister chromatid exchanges and failure to proliferate, indicating that RAD51C is required for inter-sister chromatid recombination repair in mice.[41] Furthermore, *RAD51C*-mutated breast and ovarian cancers have similar histopathology to that of *BRCA2*-defective cancers indicating the involvement of RAD51C in recombinational repair.[30] Additionally, recent clinical studies linked germline mutation in *RAD51C* with development of FA-like disorder,[42] further underscoring the crucial role of RAD51C in DSB repair.

In addition to its role in DSB repair, RAD51C also acts as a mediator of checkpoint signaling. In response to DNA damage, RAD51C facilitates phosphorylation of checkpoint kinase 2 (CHK2) by ataxia-telangiectasia mutated (ATM) and delays cell cycle progression.[43] *CHK2* is a cancer susceptibility gene mutations in which have been identified in familial breast and prostate cancers[44-47] that relays DNA damage signaling and regulates cell cycle progression, DNA repair and progression to senescence or apoptosis.[48] Additionally, in HR-mediated DSB repair, CHK2 phosphorylates BRCA1[49] further connecting the proper regulation of DSB repair and checkpoint signaling in cancer.

Recent studies implicated estrogen as a DNA-damaging agent that causes DSBs. It was found that treatment of breast cancer cells with estrogen induced DSBs and caused co-localization with RAD51,[50] suggesting that defects in DSB repair could contribute to ERα-positive breast cancer pathogenesis. *RAD51C* is localized at the chromosomal region 17q23, which appears to be a hotspot for estrogen-driven gene expression.[51] Importantly, expression of several genes within this amplicon have been shown to be regulated by estrogen, including *RPS6KB1, PPM1D* and *MIR21* - all of which have oncogenic roles in breast cancer.[52-54] Based on these findings, we hypothesized that estrogen may regulate RAD51C expression and DSB repair. Indeed, we found that in ERα-positive breast cancer cells, estrogen transcriptionally regulates RAD51C expression in ERα-dependent mechanism. Moreover, estrogen induces RAD51C assembly into nuclear foci at DSBs, which is a precursor to RAD51 recruitment to the nucleus. Finally, we found a prognostic correlation between ERα-positive breast cancer and RAD51C expression, further establishing the clinical significance of these findings.

Results

Estrogen induces RAD51C expression in ERα-positive but not ERα-negative breast cancer cells

To confirm that estrogen induces DNA damage, ERα-positive and ERα-negative breast cancer cells were treated with estrogen for 30 minutes, which was sufficient to induce DNA damage response signaling as analyzed by phosphorylation of ATM on S1981 and Chk2 on T68 (Fig. 1A). Although phosphorylation of ATM on S1981 was preferentially induced in ERα-positive breast cancer cells, phosphorylation of Chk2 on T68 was activated irrespective of the ERα status of the cell, indicating that this process does not require estrogen receptor signaling. Estrogen effect was specific to activation of DNA-damage response pathway and not general cytoplasmic kinase signaling as levels of phospho-ERK remained unchanged. Previous work has shown that estrogen induces localization of RAD51 to the sites of DNA damage, and RAD51C regulates nuclear translocation of RAD51.[39,40] Because *RAD51C* is localized to the 17q23 region, which contains estrogen regulated genes, we wanted to test whether estrogen can induce RAD51C expression in ERα-dependent manner. As shown in Fig. 1B and quantified in Fig. 1C, estrogen treatment caused upregulation of RAD51C expression in the ERα-positive MCF7, T47D, and ZR-75-1 breast cancer cells. As expected, expression of TFF1, a known estrogenically-regulated gene is induced solely in ERα-positive MCF7 and ZR 75-1 cells and not ERα-negative cells (Fig. 1B). T47D cells express TFF1 at much lower levels reflecting heterogeneity among ER-positive cell lines. Most importantly, the effect of estrogen on RAD51C expression appeared dependent on the presence ERα, as in ERα-negative MDA-MB-231, −436, and −468 cells, RAD51C levels were not affected by estrogen treatment (Fig. 1B).

Estrogen induces RAD51C focus formation in ERα-dependent manner

Since estrogen has previously been shown to induce DNA double-strand breaks, and we observed that estrogen regulates RAD51C protein expression, we hypothesized that estrogen can also induce nuclear RAD51C focus formation in ERα-dependent manner. Using immunofluorescence, we observed that estrogen induces γH2A.X foci in the nucleus of T47D breast cancer cells, a marker of DNA double-strand breaks, as previously shown.[50] Importantly, similar to estrogen's induction of γH2A.X foci formation, RAD51C foci were also induced by estrogen treatment (Fig. 2A). This finding was further observed in another ERα-positive breast cancer cell line, MCF7 (Fig. 2B). To test for the requirement of ERα in estrogen-induced RAD51C focus formation, MDA-MB-231 cells, an ERα-negative breast cancer cell line was used. As expected, MDA-MB-231 cells did not show induction of γH2A.X or RAD51C foci upon estrogen treatment (Fig. 2C), indicating that RAD51C localization to the DNA double-strand breaks requires ERα.

Estrogen directly regulates RAD51C expression via ERα

To confirm that the regulation of RAD51C expression is mediated by ERα, we downregulated ERα expression using a combination of 2 siRNA against ERα (Supplementary Fig. 1) and treated the cells with estrogen for 24 hr. RT-qPCR analysis of MCF7 cells showed upregulation of mRNA for RAD51C (Fig. 3A) and TFF1 (Fig. 3B) in the presence of estrogen, while cells transfected with siRNA against ERα showed reduced

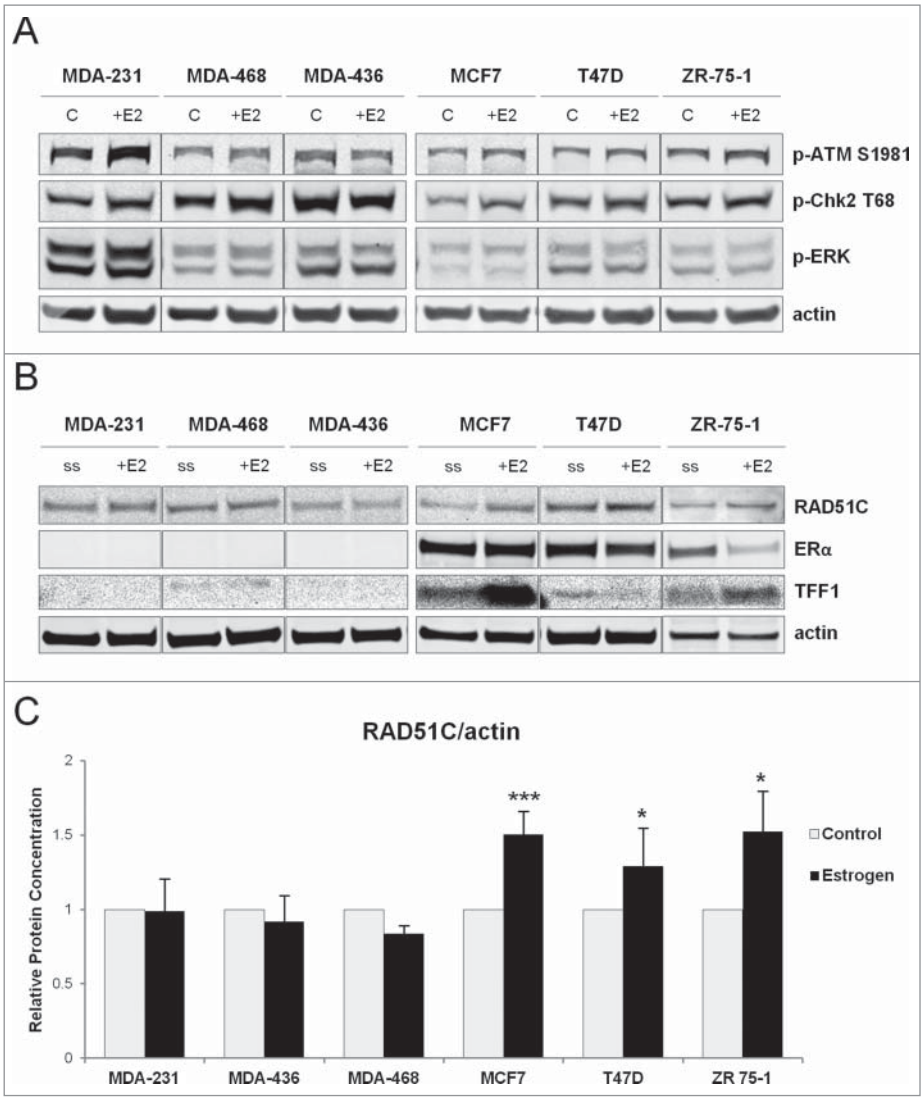

Figure 1. ERα regulates RAD51C expression in an estrogen dependent manner. (A) MDA-MB-231, −468, −436, MCF7, T47D and ZR 75-1 cells were grown in phenol red-free media with 10% charcoal-stripped FBS for 3 d and either serum starved or treated with estrogen for 30 min as indicated. Lysates were generated[67] and the indicated proteins were detected by immunoblot. (B) MDA-MB-231, −468, −436, MCF7, T47D and ZR 75-1 cells were grown in phenol red-free media with 10% charcoal-stripped FBS for 3 d and either serum starved or treated with 10nM estrogen for 24 hr as indicated. Lysates were generated as described in "*Materials and Methods*" and the indicated proteins were detected by immunoblot. (**C**) Quantification of RAD51C protein levels normalized to actin from (*B*) was performed using Odyssey Image Studio Version 4.0 and graphed using Excel.

upregulation of RAD51C when stimulated with estrogen. This effect on RAD51C was further observed on the level of protein in MCF7, ZR-75-1 cells and T47D cells, indicating that RAD51C expression is regulated by estrogen resulting in increased protein product, while ERα knockdown reduced RAD51C protein induction (Fig. 3C).

To confirm that estrogen regulates RAD51C on the transcriptional level, MCF7 cells were transfected with a construct whereby expression of renilla luciferase is driven by the proximal *RAD51C* promoter. A construct encoding for firefly luciferase under the control of 3 estrogen response elements (EREs) was used as an internal control for ER-mediated transcription. As shown in Fig. 3D, estrogen stimulation significantly increased the expression of luciferase controlled by the *RAD51C* promoter. Additionally we observed that downregulation of ERα expression using siRNA significantly reduced expression of luciferase controlled by the *RAD51C* promoter and the addition of estrogen was not able to counteract this effect (Fig. 3E), indicating that estrogen transcriptionally regulates RAD51C via ERα.

To further confirm the role of ERα mediated regulation of RAD51C, MCF7 and MDA-MB-231 cells were treated with estrogen in conjunction with either tamoxifen, a selective estrogen receptor modulator, or fulvestrant, a selective estrogen receptor degrader, to disrupt the action of estrogen. Treatment of MCF7 cells with either tamoxifen (Fig. 3F) or fulvestrant (Fig. 3G) was able to block estrogen mediated upregulation of RAD51C. Furthermore, this effect was dependent on ERα, as RAD51C levels remained unchanged in MDA-MB-231 cells. Induction of TFF1 expression served as control.

To validate that ERα is necessary for estrogen induced RAD51C focus formation, the expression of ERα was downregulated in MCF7 cells using siRNA, and cells were stimulated with estrogen for 24 hr. Compared to cells transfected with scrambled siRNA that showed RAD51C and γH2A.X focus formation upon treatment with estrogen, MCF7 cell transfected

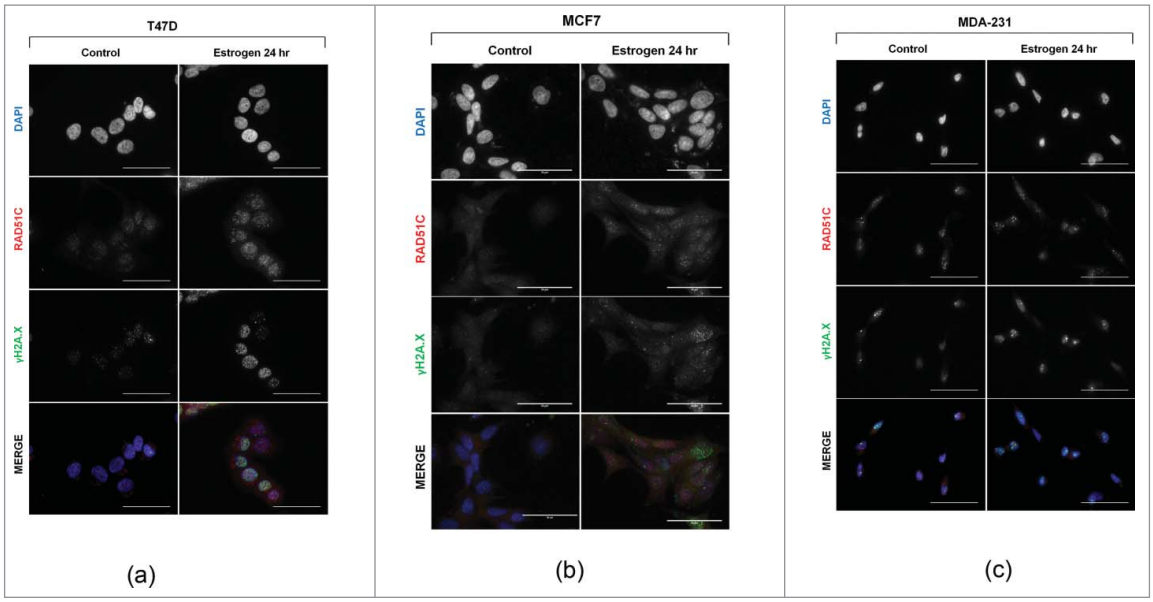

Figure 2. Estrogen induces RAD51C foci assembly. (A) T47D cells were serum-starved or stimulated with estrogen for 24 hr as indicated. Immunofluorescence was performed as described in "*Materials and Methods*." Scale bar represents 50 μm. (B) MCF7 cells were treated and processed as described in (*A*). (**C**) MDA-MB-231 cells were treated and processed as described in (*A*).

with siRNA against ERα did not exhibit formation of either RAD51C or γH2A.X foci upon estrogen treatment (Fig. 4A). This finding was also confirmed in another ERα-positive cell line, T47D (Fig. 4B).

RAD51C expression is prognostic of poor clinical outcome in ERα-positive breast cancer

Owing to the fact that RAD51C is a breast cancer susceptibility gene, we wanted to investigate the effect of estrogen in cells transfected with siRNA against RAD51C (Supplementary Fig. 2). As expected, estrogen treatment upregulated RAD51C levels in ERα-positive and not ERα-negative breast cancer cells (Fig. 5A) and this effect was diminished in cells transfected with siRNA against RAD51C. Interestingly, γH2A.X levels were elevated in cells with reduced levels of RAD51C, indicating that these cells experience DNA damage. To explore the clinical significance between RAD51C expression and ERα status of breast tumors, we used the Gene expression-based Outcome for Breast cancer Online (GOBO) database to examine the association between the RAD51C expression in ERα-positive breast tumors and patient outcome. The GSA-Tumor analysis of RAD51C expression revealed a worse clinical outcome in ER-positive tumors with regard to Distant Metastasis Free Survival (DMSF) endpoint, as shown in Figure 5B. The poor prognostic value of RAD51C was further confirmed using Relapse-Free Survival (RFS) endpoint when tumors where stratified based on the luminal A and B (ERα-positive like) breast cancer molecular subtype[55] (Figs. 5C and 5D, respectively).

Discussion

Breast cancer is a heterogeneous disease with involvement of different cellular mechanisms and signaling pathways.[56] Identification of the molecular targets that are altered is a crucial challenge for our understanding of breast cancer pathogenesis and facilitating the design of tailored treatments that are effective at stopping breast cancer progression and preventing cancer recurrence. ERα status of breast cancer is an important marker for selecting patients for treatment with endocrine therapy such as selective estrogen receptor modulators (e.g- tamoxifen), selective estrogen receptor degraders (e.g. fulvestrant) or aromatase inhibitors (such as letrozole). However, only 35–70% of patients respond to endocrine therapy and resistance develops in most cases, in part due to activation of growth factor signaling pathways.[57] Another pathway that is often attenuated in breast cancer is the DSB repair with main focus on germline loss of function of tumor suppressors *BRCA1* and *BRCA2* leading to hereditary breast and ovarian cancer progression. Mutations in DSB repair genes lead to accumulation of DSBs, genome instability and tumorigenesis.

RAD51C is a recently identified high penetrance cancer susceptibility gene,[30-32] and germline mutations in *RAD51C* predispose to breast and ovarian cancer with high similarity to patients carrying *BRCA1* and *BRCA2* mutations. In the current work we identified a novel mechanism of estrogen dependent DSB repair, whereby estrogen regulates RAD51C via ERα on a transcriptional level, demonstrating a direct link between ERα signaling and HR pathways. A recent study describing *Rad51c*- and *Trp53*-double-mutant mice showed that these mice have an accelerated production of mammary carcinomas.[58] Similar to our results, these tumors exhibited high levels of genomic instability and DNA damage and most interestingly, the tumors retained expression of ERα, which is similar to the luminal-like phenotype described in *RAD51C* mutant breast cancers.[59] Therefore, the estrogen-dependent DNA damage and repair mechanism we described here could be responsible for the phenotype observed *in vivo*. Paradoxically, estrogen appears to induce DNA damage, either by direct oxidative damage or indirectly, by promoting progression into S-phase, while simultaneously activating the expression of DNA damage repair genes, such as RAD51C, which may be a homeostatic

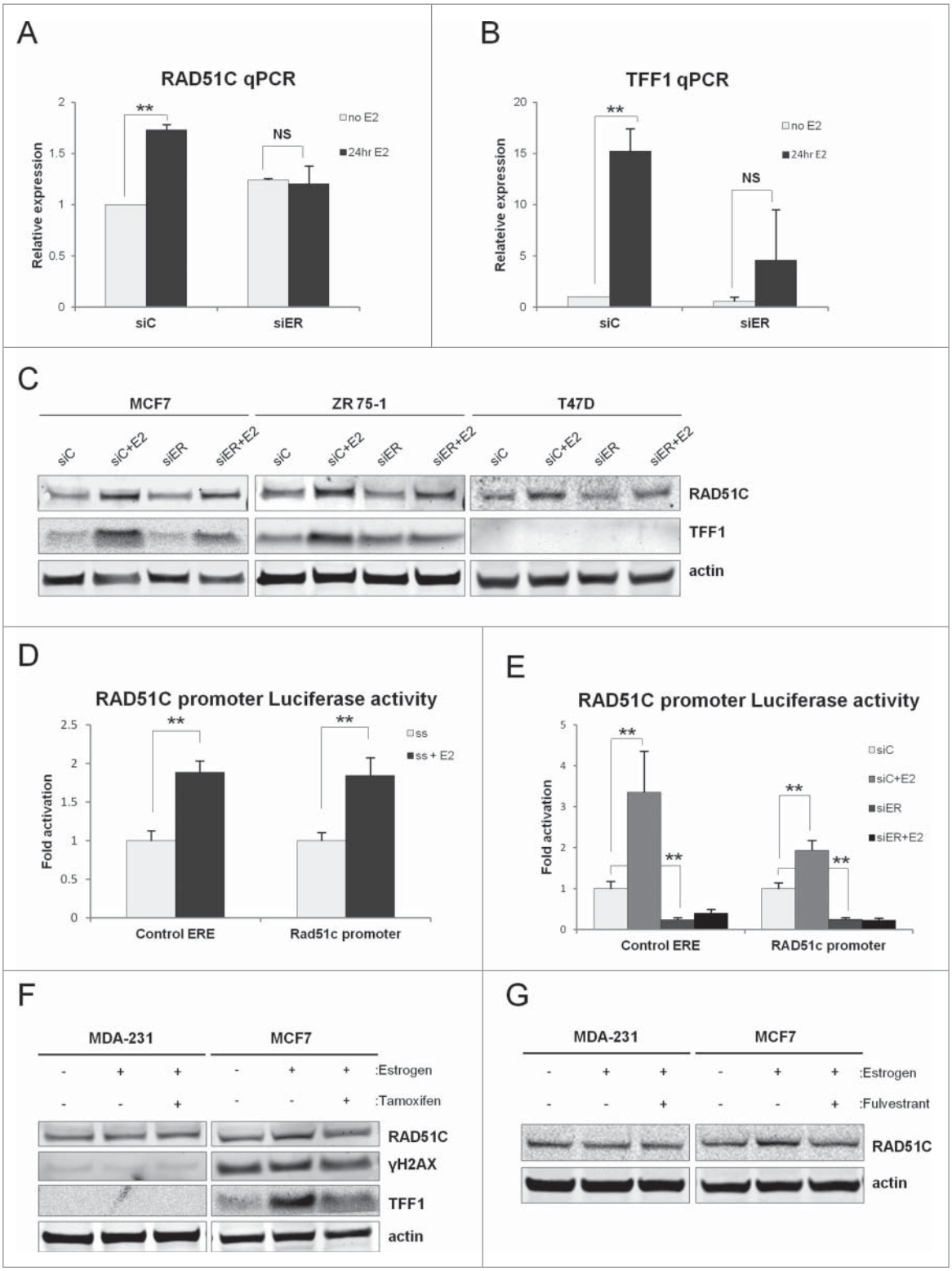

Figure 3. ERα transcriptionally regulates RAD51C expression in estrogen-dependent manner. (A) MCF7 cells were transfected with scambled siRNA or siRNA against ERα and treated with estrogen for 48 hr, as indicated. RT-qPCR was performed as described in "*Materials and Methods*" and data was plotted using Excel. $^{**}p < 0.001$; NS, non-statistically significant; n = 3. (B) MCF7 cells were treated as described in (*A*), RT-qPCR was performed as described in "*Materials and Methods*" and data was plotted using Excel. $^{**}p < 0.001$; NS, non-statistically significant; n = 3. (C) MDA-MB-231, −468, −436, MCF7, T47D and ZR 75-1 cells were serum starved or treated with estrogen for 24 hr, as indicated. Lysates were generated as described in "*Materials and Methods*" and the indicated proteins were detected by immunblot. (D) MCF7 cells were transfected, stimulated with estrogen for 24 hr as indicated and Luciferase reporter assay was performed as described in "*Materials and Methods*." Data was plotted using Excel. $^{**}p < 0.001$. (E) MCF7 cells were transfected, stimulated with estrogen for 24 hr as indicated and Luciferase reporter assay was performed as described in "*Materials and Methods*." Data was plotted using Excel. $^{**}p < 0.001$; n = 3. (F) MDA-MB-231 and MCF7 cells were either serum starved, treated with estrogen for 24 hr or pre-treated with estrogen for 30 min following treatment with tamoxifen for 24 hr. Lysates were generated as described in "*Materials and Methods*" and indicated proteins were detected by immunoblot. (G) MDA-MB-231 and MCF7 cells were either serum starved, treated with estrogen for 24 hr or pre-treated with estrogen for 30 min following treatment with fulvestrant for 24 hr. Lysates were generated as described in "*Materials and Methods*" and indicated proteins were detected by immunoblot.

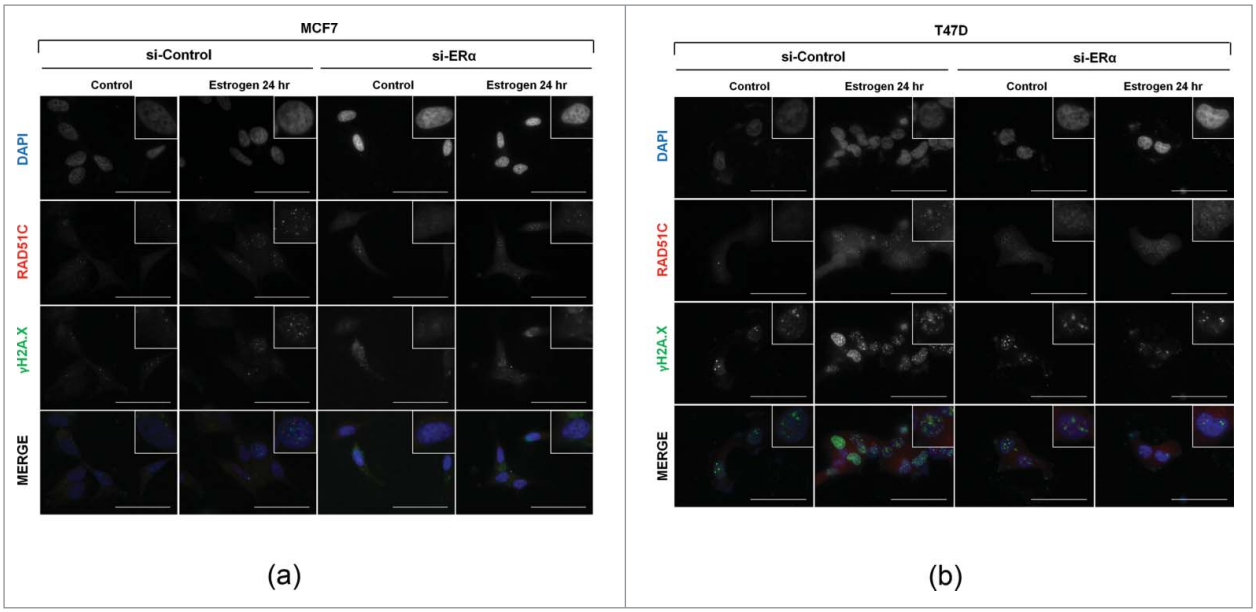

Figure 4. Estrogen induces RAD51C foci assembly in ERα dependent manner. (A) T47D cells were transfected with scrambled siRNA, or siRNAs against ERα and either serum-starved or stimulated with estrogen for 24 hr. Immunofluorescence was performed as described in "*Materials and Methods*." Scale bar represents 50 μm. (B) MCF7 cells were treated and processed as described in (*A*).

mechanism that prevents accumulation of estrogen-induced DNA damage. This finding adds a potential new link to the relationship between lifetime estrogen exposure and breast malignancy. Clinically, these findings may be extremely significant because ERα-positive and estrogen dependent breast cancer tumors would incur estrogen-induced DNA damage, thus increased expression of RAD51C could be indicative of increased genomic instability and worse disease outcome. Moreover, individuals with inactivating mutations in *RAD51C* would be more susceptible to accumulation of unrepaired estrogen-induced DNA damage, increased genomic instability and cancer progression, similar to deficiencies in BRCA1/2 and RAD51.[60-62] Estrogen is sometimes used clinically for treatment of breast cancer because of its apoptosis-inducing capacity.[63] It would be interesting to determine whether in this setting, high-dose estrogen may contribute to synthetic lethality in combination with DNA damaging agents such as cisplatin and PARP inhibitors.[64,65] Thus RAD51C may serve as a prognostic marker for disease severity in ER-positive breast cancer, as well as a marker for sensitivity to DNA damaging chemotherapeutics.

Materials and methods

Cell culture and treatment

MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, ZR 75-1 and T47D cells were cultured in a humidified incubator with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). For experiments, cells were grown in phenol red-free media with 10% charcoal-stripped FBS for 3 d. Where indicated, cells were starved in serum-free media for 24 hr and stimulated with 10nM estrogen (in ethanol), 100 nM 4-hydroxy-tamoxifen (in ethanol), or 100 nM fulvestrant (in DMSO).

siRNA transfection: siRNAs against ERα (HSC.RNAI.N000125.12.5 and HSC.RNAI.N000125.12.6), RAD51C (HSC.RNAI.N058216.12.1 and HSC.RNAI.N058216.12.2) and scrambled controls were ordered from Integrated DNA technologies. 25 pmol of siRNA was transfected using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to manufacturer's protocol. Cells were lysed 48 h post-transfection.

Constructs: Firefly luciferase-3ERE reporter plasmid was previously described.[66] Renilla luciferase-RAD51C promoter was purchased from SwitchGear Genomics.

Immunofluorescence

Cells were plated on 18mm Poly-L-Lysine coated cover slips (Fisher Scientific). Following treatment, cells were fixed in 4% parafalmaldehyde and permeabilized in 0.2% Triton-X. After blocking and incubation with primary (RAD51C from Abcam, ab72063 and ERα from Santa Cruz Biotechnology, sc-8005) and secondary antibodies (Alexa Fluor 55 donkey anti-rabbit IgG, A31572 and Alexa Fluor 488 donkey anti-mouse IgG, A21202; all from Invitrogen), coverslips were mounted using DAPI-Fluoromount G mounting media (Southern Biotech, 0100-20). Images were collected using EVOS FL Auto microscope (Invitrogen) under 60X magnification.

Cell lysis

Cells were lysed in ice-cold buffer containing 10 mM KPO_4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40 [NP-40], 0.1% Brij 35, 1 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin. Lysates were cleared of insoluble material by centrifugation at 15,000*g* for 10 min at 4°C. Protein concentration in cell extracts was measured by Bradford reagent (BioRAD) according to the manufacturer's protocol using Eppendorf BioPhotometer (Eppendorf). Samples were equalized for protein concentration and denatured using LDS Sample buffer and

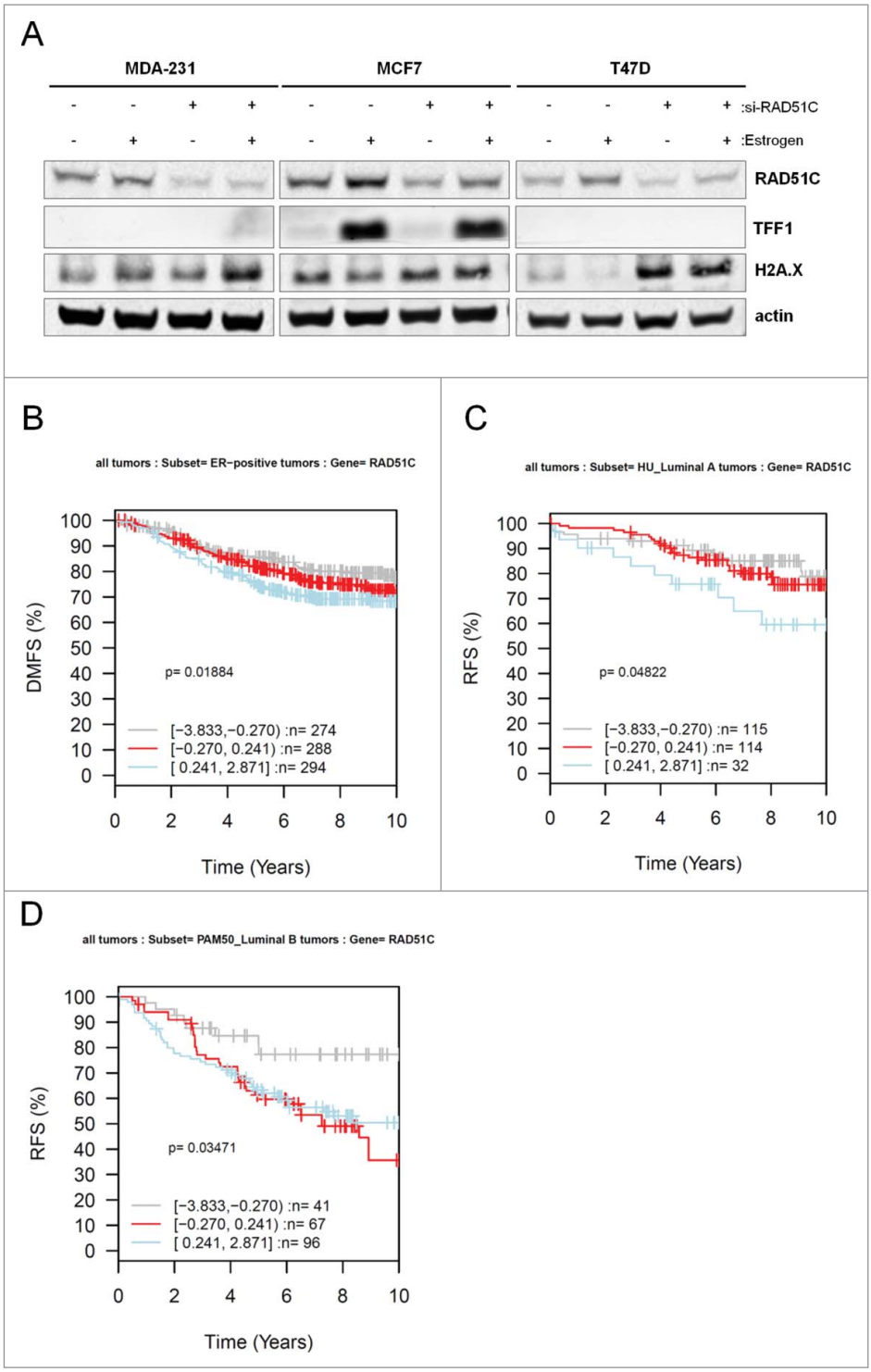

Figure 5. GSA-Tumor analysis of *RAD51C* shows worse clinical prognosis for ERα-positive tumors. (A) MDA-MB-231, MCF7 and T47D cells were transfected with scrambled siRNA or siRNA against RAD51C and treated with estrogen for 48 hr, as indicated. Lysates were generated as described in "*Materials and Methods*" and the indicated proteins were detected by immunblot. (B) Kaplan-Meier analysis using DMFS of ERα-positive tumors separated into 3 categories with respect to *RAD51C* expression. (B) Kaplan-Meier analysis using RFS of Luminal A tumors separated into 3 categories with respect to *RAD51C* expression. (C) Kaplan-Meier analysis using RFS of Luminal A tumors separated into 3 categories with respect to *RAD51C* expression.

Reducing agent (Invitrogen) at 70°C for 10 min. Samples were resolved using Bis-Tris Plus gels (Invitrogen) and transferred onto nitrocellulose membrane (GE Healthcare). Membranes were probed with the following primary antibodies: RAD51C (sc56214 Santa Cruz Biotechnology), p-ATM S1981 (5883 Cell Signaling Technologies), p-Chk2 T 68 (2197 Cell Signaling Technologies) p-ERK (4695 Cell Signaling), γH2A.X (05–636 Millipore), ERα (sc543 Santa Cruz Biotechnology), actin (sc1615 Santa Cruz Biotechnology), and TFF1 (12419S Cell Signaling Technologies).

Signal detection and quantification was accomplished using IRDye-conjugated anti-rabbit (LI-COR, 827-08365), anti-

mouse (LI-COR, 926-68070) or anti-goat (LI-COR, 926-68074) secondary antibodies using Odyssey infrared detection instrument (LI-COR). All immunoblots were performed at least thrice to ensure reproducibility.

Luciferase reporter assay

MCF7 cells were transiently transfected with a Rad51c-promoter *Renilla* luciferase construct (SwitchGear Genomics) and a reporter plasmid containing 3 estrogen response elements (ERE) controlling expression of firefly luciferase, as previously described.[66] siRNA against ERα was co-transfected, as indicated in figure legends. Luciferase expression following cell treatment with estrogen was assayed using a dual luciferase kit and GloMax® 20/20 Luminometer (Promega). Assays were performed in triplicates and results were analyzed and plotted using Excel.

Quantitative RT-PCR

RNA was isolated using RNeasy® Mini Kit (Qiagen) and 1 μg of RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (BioRAD) and C1000 thermal cycler (BioRAD). For qPCR, cDNA was amplified with iQ™ SYBR® Green Supermix (BioRAD) in CFX96™Real-Time PCR Detection System (BioRAD, Hercules, CA) with CFX Manager analysis on-board software. TFF1 F: 5′ ATC GAC GTC CCT CCA GAA GAG 3′; TFF1 R: 5′ CTC TGG GAC TAA TCA CCG TGC TG 3′; 18s F: 5′ TTC GAA CGT CTG CCC TAT CAA 3′; 18s R: 5′ ATG GTA GGC ACG GCG ACT A 3′; Rad51c F: 5′ GGA TTT GGT GAG TTT CCC GC 3′; Rad51c R: 5′ TCT TTG CTA AGC TCG GAG GG 3′

Statistical analysis

Data are presented as mean ± S .D. and n = 3. Statistical significance was determined by paired Student's t-test using Microsoft Excel.

GOBO analysis

Gene expression-based Outcome for Breast cancer Online database was used to identify prognostic validation of *RAD51C* expression and ERα status of breast tumors collected from pooled breast cancer data sets http://co.bmc.lu.se/gobo/. The Gene Set Analysis (GSA) of tumors were further subdivided based on *RAD51C* expression with gray representing low expressing, red representing intermediate expressing and blue representing high expressing.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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