MATERIALS AND METHODS

Luciferase reporter assays. The Rad51 promoter driven luciferase reporter (500 ng) was assessed in MCF-7 cells with co-transfected expression plasmid for cyclin D1 (pCMV10-cyclin D1 30 ng, 100 ng, and 300 ng) using Lipofectamine 2000 (Qiagen, Valencia, CA). A CMV-β-gal plasmid was co-transfected as a control of transfection efficiency. 48 hrs post transfection, luciferase assays were performed as previously described (1). In the experiments with E_2 and the protein kinase inhibitors MCF-7 cells were cultured in phenol red-free DMEM with 10% charcoal/dextran–treated FBS and 2 mM glutamine for 24 hrs. 24 hrs post transfection the cells were treated with different dosage of E_2 (0.1 nM, 1 nM, and 10 nM) or vehicle (ethanol) control plus treatments as follows. PI3K inhibitor (100 nM LY294002, EMD), HDAC inhibitor (100 nM Trichostatin A (TSA), Sigma), JNK inhibitor (5 μM SP600125, EMD), ATM inhibitor (10 μM KU55933, EMD), ATM/ATR inhibitor (5 mM Caffeine, Fisher Scientific), DNA-PK inhibitor (10 μM NU7026, Sigma), casein kinase-2 (CK2) inhibitor (100 μM TBB, Santa Cruz Biotechnology), or vehicle (DMSO) control. 24 hrs after treatment with E_2 plus the protein kinase inhibitors luciferase assays were performed.

siRNA knockdown of endogenous cyclin D1 in MCF-7 cell. MCF-7 cells were transfected with the cyclin D1 siRNAs or negative control siRNA (Qiagen) using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in phenol red-free DMEM with 10% charcoal/dextran–treated FBS and 2 mM glutamine. 48 hrs later the cells were treated with 17β-estrodial (E_2, 10 nM) or vehicle (ethanol) control. One hour later the cells were treated with UV irradiation (100 J/m^2). 2, 4, and 8 hrs later after UV the cells were processed for immunoflurescencence staining. 24 hrs later the cells were harvested for Western blot analysis.

Western blot. The antibodies used in Western blot analysis were cyclin D1 (DCS-6), Rad51 (H-

92), β-actin (C4), β tubulin (H-235), ERα (H-184), phosphorylated Akt1/2/3 (Ser473) (sc-7985-R), GFP (FL) (All were from Santa Cruz Biotechnology (Santa Cruz, CA)). Mouse anti-FLAG (M2), mouse anti- vinculin (hVIN-1) antibodies were from Sigma (St. Louis, MO). Mouse anti-Akt1 (2H10) antibody was from Cell Signaling Techology (Danvers, MA). Antibody to γH2AX (Ser139) (clone JBW301) was from Millipore Corporation (Billerica, MA).

Immunostaining. The cells in four-well chamber slides were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and cold methanol for 5 min at -20°C. The slides were then treated with 0.2% Triton X-100 for 5 min at RT and blocked with 2%BSA overnight at 4^{o}C. The primary antibodies used were rabbit polyclonal anti-cyclin D1 (Ab-3) (Thermo Fisher Scientific, Fremont, CA) (1/100) and mouse monoclonal anti-phospho-Histone H2A.X (Ser139) (clone JBW301) (Millipore Corporation, Billerica, MA) (1/1,000), mouse monoclonal anti-cyclin D1 (DCS-6) (1:100) and rabbit polyclonal anti-Rad51 (clone H-92) (Santa Cruz Biotechnology, Santa Cruz, CA) (1/100). The secondary antibodies used were Alexa Fluor 488-conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Inc.) (1/500), Rhodamine-conjugated F(ab')2 fragment of goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Labiratories, Inc.) (1/500). The samples were visualized on a Zeiss LSM 510 META Confocal Microscope with a 63× objective.

RNA isolation, quantitative real-time PCR. Total RNA was isolated from MCF-7 cells treated with three individual cyclin D1 siRNAs or control siRNA using Trizol (2). RNA samples were treated with RQ1 DNase I (Promega Inc, Madison, WI) to remove contaminating DNA from RNA preparations followed by re-purification using RNeasy Mini Kit (Qiagen, Valencia, CA). DNA-free RNA was subjected to reverse transcription reactions, performed using SuperScript™ III reverse transcriptase kit (Invitrogen, Carlsbad, CA). Following preparation of cDNA, SYBR

Green based real-time PCR reactions were performed using Power SYBR Green Master Mix (Invitrogen, Carlsbad, CA) on an ABI Prism 7900HT system (Applied Biosystems Inc., Foster City, CA). Specific Real-time PCR primers of human Rad51 were as follows: Forward: 5'-CGAGCGTTCAACACAGACCA, Reverse: 5'- GTGGCACTGTCTACAATAAGCA. Amplification of 18s rRNA (Forward: 5'- GTAACCCGTTGAACCCCATT. Reverse: 5'-CCATCCAATCGGTAGTAGCG) (3) was performed in every sample and the obtained Ct values for each sample were used for normalization of data for Rad51 expression. To calculate the fold change of Rad51 gene expression between various treatments, Ct values obtained from amplification of Rad51 transcripts in control siRNA and vehicle control treated cells were used for calibration.

REFERENCES

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