Ligand-induced gene activation is associated with oxidative genome damage whose repair is required for transcription

Shiladitya Sengupta1,2,3, Haibo Wang1,2, Chunying Yang1,4, Bartosz Szczesny1,4, Muralidhar L. Hegde1,5, and Sankar Mitra1,6,7

Departments of Radiation Oncology, Houston Methodist Research Institute, Houston, TX 77030; Department of Neurosurgery, Center for Neurorehabilitation, Houston Methodist Research Institute, Houston, TX 77030; Departments of Ophthalmology and Visual Sciences, The University of Texas Medical Branch, Galveston, TX 77555; Department of Anesthesiology, The University of Texas Medical Branch, Galveston, TX 77555; Weill Cornell Medical College, Cornell University, New York, NY 10065; and Houston Methodist Cancer Center, Houston Methodist Research Institute, Houston, TX 77030

Edited by Sankar Adhya, National Institutes of Health, National Cancer Institute, Bethesda, MD, and approved July 16, 2020 (received for review November 6, 2019)

Among several reversible epigenetic changes occurring during transcriptional activation, only demethylation of histones and cytosine-phosphate-guanine (CpGs) in gene promoters and other regulatory regions by specific demethylase(s) generates reactive oxygen species (ROS), which oxidize DNA and other cellular components. Here, we show induction of oxidized bases and single-strand breaks (SSBs), but not direct double-strand breaks (DSBs), in the genome during gene activation by ligands of the nuclear receptor superfamily. We observed that these damages were preferentially repaired in promoters via the base excision repair (BER)/single-strand break repair (SSBR) pathway. Interestingly, BER/SSBR inhibition suppressed gene activation. Constitutive association of demethylases with BER/SSBR proteins in multiprotein complexes underscores the coordination of histone/DNA demethylation and genome repair during gene activation. However, ligand-independent transcriptional activation occurring during heat shock (HS) induction is associated with the generation of DSBs, the repair of which is likewise essential for the activation of HS-responsive genes. These observations suggest that the repair of distinct damages induced during diverse transcriptional activation is a universal prerequisite for transcription initiation. Because of limited investigation of demethylation-induced genome damage during transcription, this study suggests that the extent of oxidative genome damage resulting from various cellular processes is substantially underestimated.

Significance

The endogenous genome damage induced by mitochondrial/cytosolic reactive oxygen species (ROS) is well recognized. However, similar damage induced by nuclear ROS generated via histone/cytosine-phosphate-guanine (CpG) demethylation during transcription has not been scrupulously investigated. This report documents the formation of genomic oxidized bases and single-strand breaks during ligand-induced gene activation via histone/CpG demethylation. That repair of these damages occurs preferentially in promoters and is essential for transcriptional activation underscores the essentiality of promoter-specific repair for transcription. In contrast, heat shock (HS) induction generates double-strand breaks, the repair of which is essential for the activation of HS-responsive genes. This study thus implies gross underestimation of endogenous oxidative genome damage and highlights the intrinsic diversity of damage and distinct repair processes associated with transcription.

Author contributions: S.S., M.L.H., and S.M. designed research; S.S., H.W., C.Y., B.S., and M.L.H. performed research; S.S., H.W., C.Y., B.S., M.L.H., and S.M. analyzed data; and S.S. and S.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1S.S. and H.W. contributed equally to this work.

2To whom correspondence may be addressed. Email: sengupta.us@gmail.com, mlhegde@houstonmethodist.org, or smitra2@houstonmethodist.org.

3Present address: Texas Heart Institute, Houston, TX 77030.

4Present address: School of Biomedical Informatics, The University of Texas Health Science Center, Houston, TX 77030.
These damages were repaired via the base excision repair (BER)/single-strand break repair (SSBR) pathway (23). We documented the coordination of BER/SSBR with histone/CpG demethylation for promoter-specific repair of these damages during ligand-induced transcriptional activation, in contrast to what we observed during ligand-independent gene activation via heat shock (HS) when double-strand break repair (DSBR) was essential.

Results

Induction of Genome Damage in Diverse Gene Activation Systems.

An earlier study documented the generation of 8-oxoguanine (8-oxoG), the most abundant oxidized DNA base lesion, during activation of the B cell lymphoma 2 (BCL2) gene in MCF7 breast cancer line by 17β-estradiol (E2), a ligand for estrogen receptors (24). The formation of 8-oxoG was also observed during Myc-induced transcriptional activation (25). The authors attributed G oxidation by ROS generated specifically during oxidative demethylation of Lys9 methylated histone H3 in the gene promoters. On the basis of these observations, we postulated that diverse ligand-induced transcriptional reprogramming involving the demethylation of histones and CpGs will cause DNA base oxidation and strand break. The induction of genome damage at a global scale followed by their repair can be monitored by comet assay. To study this global damage induction, we selected E2-induced transcriptional activation because thousands of estrogen response elements are most likely present in the genome; the cognate genes would be activated by E2 involving the demethylation of the promoter-bound histones and CpGs (26). Indeed, in alkaline comet assay, we observed global strand breaks after E2 treatment in MCF7 cells (Fig. 1A). Moreover, these breaks were transient, indicating their efficient repair within 30 min. These results suggest that such genome damages are tightly coordinated with their repair. To explore the universality of this phenomenon, we stimulated two other cell lines, the human embryonic kidney epithelial line HEK293 and the human nonsmall cell lung adenocarcinoma line A549, with all-trans retinoic acid (RA) and tumor necrosis factor-α (TNFα), respectively. Exposure to RA (Fig. 1B) and TNFα (Fig. 1C) induced similar global damage, namely strand breaks, followed by their efficient repair. Together, these results strongly suggest that gene activation-linked genome damage and subsequent repair are universal, although each ligand has distinct kinetics of DNA damage induction and repair.

Generation of ROS due to Histone and CpG Demethylation and Induction of Oxidative Genome Damage during Gene Activation.

While the ligands used above all generate ROS in cells (27–31), we selected RA for in-depth studies of demethylation-associated oxidative genome damage and repair during gene activation. We used the RA-inducible retinoid acid receptor-p2 (RARp2) gene activation in HEK293 cells as the model system. First, we confirmed RA-induced generation of ROS by fluorescence-activated cell sorting (FACS) analysis of 2′,7′-dihydrodichlorofluorescein diacetate (H2-DCFDA) oxidation (Fig. 2A) and then showed the generation of both H2O2 and O2•− by colorimetric assays (Fig. 2B and C). Together, these observations suggest that RA-induced global transcriptional reprogramming generates ROS, presumably because of histone/CpG demethylation. Using chromatin immunoprecipitation (ChIP) analysis, we then showed that RA reduced the Lys9 dimethylated H3 (H3K9me2) level in the RARp2 promoter, which contains the retinoic acid response element (RARE) (Fig. 2D). Similar reduction of the H3K9me2 level was not observed in a nonspecific region lacking RARE (Fig. 2D). Although we observed significant H3K9me2 demethylation not before 60 min of RA treatment, the generation of global strand breaks, estimated by comet assay, peaked at an earlier time point of 15 min (Fig. 1B), thereby implying a range of kinetics of histone demethylation and demethylation at other H3/H4 Lys residues (along with CpG demethylation) across the genome, which would account for the early strand breaks. In any event, this result indicates promoter-specific histone demethylation induced by RA, along with the demethylation of 5-methyl CpGs (5mCpGs), as reported elsewhere (32).

Because the alkaline comet assay (Fig. 1) could not distinguish SSBs and DSBs, we used immunofluorescence microscopy to monitor the formation of phosphorylated (Ser 139) histone H2A.X (γH2AX) foci, a marker of DSB induction. The RA-treated cells showed negligible formation of γH2AX foci, compared with the X-ray irradiated (ionizing radiation [IR]; positive control) cells (Fig. 3A and B). ChIP analysis also confirmed the negligible formation of γH2AX in the RARp2 promoter after RA treatment (Fig. 3C). These observations indicate that demethylation-linked ROS during RA stimulation for the indicated times do not directly induce DSBs. On the other hand, we
observed a striking increase of 8-oxoG level in the RARβ2 promoter after RA treatment (Fig. 3D). Similar increase, also observed elsewhere in the genome (Fig. 3D), may be caused by intranuclear diffusion of ROS across the G-rich genomic regions. Interestingly, the 8-oxoG level rapidly decreased selectively in the promoter (Fig. 3D). Together, these results imply preferential repair of oxidized bases in promoters. We also examined the kinetics of RA-induced RARβ2 activation (Fig. 3E) by measuring the steady-state RARβ2 messenger RNA (mRNA) level by real-time reverse transcription polymerase chain reaction (RT-PCR) assay, which reflected RA-induced RARβ2 transcription. Because optimal RARβ2 transcription occurred after repair completion as suggested by both the comet (Fig. 1A) and 8-oxoG ChIP (Fig. 3D) analyses, we hypothesized that demethylation and damage repair are coordinated during transcriptional activation and that promoter-specific repair is a prerequisite for transcription.

**Association of Demethylases and BER/SSBR Proteins in Chromatin.**

We speculated that to efficiently coordinate histone/CpG demethylation and repair of the demethylation-induced genome damage, the demethylases and BER/SSBR proteins colocalize in chromatin-bound preformed complexes. We extensively characterized chromatin-bound multiprotein complexes that contained BER/SSBR proteins, along with nonrepair proteins; furthermore, we showed that these complexes were competent in repairing the oxidized bases (33–36). Exposure to ROS increased the stability of these unique complexes, which were distinct for various BER subpathways (23, 34, 35, 37). The complexes were stabilized, independent of DNA binding, via pairwise interaction between the components and could be isolated by size fractionation or after immunoprecipitation (IP) of nuclear extracts (33–35). In the present study, we identified BER/SSBR proteins poly (ADP-ribose) polymerase 1 (PARP1), apurinic/apyrimidinic endonuclease 1 (APE1), polynucleotide kinase phosphatase (PNKP), and DNA polymerase β (DNA Polβ) in the chromatin-bound IPs of a histone demethylase LSD1 and a CpG demethylase TET1, even in the control (RA-untreated) cells (Fig. 4A and B). This constitutive association of BER/SSBR proteins in the chromatin-bound LSD1 or TET1 complexes supports our speculation of efficient coordination between histone/CpG demethylation and the repair of the demethylation-induced genome damage. Unexpectedly, we could not detect other BER/SSBR proteins, such as 8-oxoguanine DNA glycosylase 1 (OGG1), Nei-like 1 DNA glycosylase (NEIL1), X-ray repair cross-complementing protein 1 (XRCC1), and DNA ligase 3 (LIG3), in these IPs. The inability to detect these proteins may reflect the sensitivity of detection, which is dependent on many factors, including the quality of antibody and the lability of some complexes. We should also mention that the specific complexes containing repair protein(s) most likely constitute only a very small fraction of the TET1 or LSD1 IPs. In any case, we performed a reciprocal IP with anti-XRCC1 (α-XRCC1) antibody and observed TET1, LSD1, and APE1 (positive control) in the IPs from both control and RA-treated cells (Fig. 4C). Moreover, TET1 IP did not pull down proteins of other DNA repair pathways, such as mutL homolog 1 (MLH1) and postmeiotic segregation increased homolog 2 (PMS2) of the mismatch repair pathway and X-ray repair cross-complementing protein 4 (XRCC4), which is involved in the DSBR pathway via nonhomologous end joining (NHEJ) (Fig. 4D). These results strongly suggest preferential association of specific BER/SSBR proteins with histone/CpG demethylases, which can repair the oxidized bases and SSBs induced by the demethylases.

Sengupta et al.

PNAS Latest Articles | 3 of 10
Essentiality of BER/SSBR Proteins for Ligand-Induced Gene Activation.

Fig. 3. Induction of base oxidation but not DSB by RA. (A and B) Immunofluorescence analysis of γH2AX foci was performed in (A) HEK293 and (B) U2OS cells after 1 μM RA treatment for the indicated times. IR (3-Gy X-rays) was used as a positive control. Representative images are shown, and fraction of cells with more than five foci are plotted. (Scale bars, 10 μm.) (C) Real-time PCR analysis of γH2AX ChIP in the RARβ2 promoter in HEK293 cells collected after 15 min of RA treatment or IR. (D) Real-time PCR analysis of 8-oxoG ChIP in the RARβ2 promoter and the nonspecific region after RA treatment for the indicated times. (E) Real-time RT-PCR analysis of RARβ2 expression as RQ, normalized to HPRT1 expression, in HEK293 cells after 1 μM RA treatment for the indicated times. The data represent the average (with SD) of two or more independent experiments. Statistical analysis was performed in GraphPad Prism by using two-way ANOVA with Tukey’s multiple comparisons test. *P value < 0.05; **P value < 0.01.

DSB Generation and Repair during HS Induction. To examine genome damage and repair during ligand-independent gene activation, we studied gene activation by HS/hyperthermia treatment of HCT116 cells. We observed the formation of γH2AX foci after incubating the cells at 42 °C, reflecting the induction of DSBs, which persisted for at least 24 hr (Fig. 7 A and B). ChIP analysis showed transient increase of the γH2AX level specifically in the promoter of heat shock protein family A member 4 (HSPA4), an HS-responsive gene (Fig. 7C). Interestingly, suppression of the NHEJ-mediated DSBR by a DNA-dependent protein kinase (DNA-PK) inhibitor Nu7441, but not the SSBR by the PARP inhibitor, rucaparib, delayed HS-induced HSPA4 activation (Fig. 7D). Thus, in contrast to the predominant role of BER/SSBR pathways during RA-induced RARβ2 activation, hyperthermia induction of HS-responsive genes may involve DSBR via the NHEJ pathway.

Discussion

In this study, we explored the spatiotemporal regulation of global genome damage and repair during transcriptional activation.
The generation of oxidized bases, predominantly 8-oxoG, was observed earlier during E2-induced activation of the BCL2 gene (24). Similar phenomenon, suggesting coordination between the generation of oxidized bases and transcription, was subsequently reported in many studies (25, 44–48). Here, we showed that during ligand-induced gene activation, both oxidized bases and SSBs were generated by ROS, the by-products of histone/CpG demethylation. 8-oxoG, like most other oxidized base lesions, in promoters or gene bodies does not block transcription. In contrast, SSB, generated directly or as a BER intermediate, prevents RNA chain elongation (39, 49). This study showed that the repair of the ligand-induced SSBs in gene promoters was essential for transcription. On the other hand, during ligand-independent gene activation by HS, the DNA topoisomerases, TOP1 and TOP2, are inhibited (50–52), thereby generating persistent SSBs and DSBs, respectively, which also block transcription (53), warranting their prompt repair. Together, these studies, including our study, imply that the damage in promoter sequences not only inhibits transcription (54, 55) but also promotes a transient competition between repair and transcription machineries (56), warranting repair before transcription. Thus, cells use distinct repair systems to remove diverse damages in promoters before transcription initiation.

The direct connection between active CpG demethylation and BER is well established (12, 57–60). The TET-mediated demethylation of 5mCpG generates 5OH-mC intermediate. The subsequent DNA demethylation products, 5-formyl C and 5-carboxyl C, are excised by the thymine DNA glycosylase (TDG)/single-strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1) family of BER glycosylases, restoring the unmodified C residue. However, BER/SSBR in repairing the oxidized bases and SSBs induced by the ROS by-products of histone/CpG demethylation has not been scrupulously investigated. Although the generation of oxidized bases and SSBs may be global, here we demonstrated that these damages were preferentially repaired in the promoter region, as a prerequisite for transcriptional activation.

Structural relaxation is essential for DNA transcription in condensed chromatin (61). The TOP1 and TOP2 topoisomerases maintain topological states of chromatinized DNA during DNA transactions (62, 63). The TOP1/TOP2-mediated generation of transient strand breaks releases the topological constraint and favors chromatin looping, thereby facilitating cross-talk between distal enhancers and proximal promoters for transcriptional activation (32, 64). However, the demethylation–ROS-induced strand breaks appear to be detrimental to cellular physiology, until repaired, because these strand breaks are not transient, unlike those generated by TOP1 and TOP2. Our study indicates that the strand breaks generated by ligands do not facilitate transcription, rather inhibit, and their prompt repair is essential for transcription. Additionally, during testosterone-induced activation of androgen receptor target genes, TOP1 generates SSBs, which are required for robust enhancer RNA synthesis and enhancer activation (65), a distinct mechanism of transcriptional activation. Although SSBs might favor chromatin looping for transcription, their repair is essential for restoring promoter sequence integrity needed for transcription initiation. Thus, a dynamic balance exists between the induction of strand breaks, chromatin looping, and transcriptional activation (66–68) where subsequent repair of these breaks seems to be essential.

Accumulating evidence indicates the involvement of DNA repair pathway(s) in transcriptional regulation, particularly where TOP2β-mediated generation of DSBs and the involvement of DSBR during signal-dependent gene activation were shown (69, 70). Such signal-dependent “programmed DNA nicks and strand breaks” may promote both transcriptional initiation and elongation by facilitating the assembly of multiprotein regulatory complexes and chromatin looping (71). In this study, we showed that DSBs were not generated directly by RA. However, DSBs could...
be generated during attempted replication of SSB-containing template strands or from bistranded proximal SSBs for which DSBR should be essential to restore promoter integrity. Our study establishes the requirement of BER/SSBR in transcriptional activation. That ROS promote preferential assembly of BER complexes in promoters with open conformation (72), which are vulnerable to oxidation (73), suggests preferential repair of actively transcription gene promoters via BER/SSBR, consistent with our observations. In a different scenario, the DSBs generated during HS induction are repaired via the NHEJ pathway, which is essential for HS-induced gene activation. Collectively, our study highlights the intrinsic diversity of genome damage mechanisms and distinct repair pathways associated with transcriptional activation.

Egly and coworkers (32) showed that the nucleotide excision repair (NER) proteins, XPG and XPF, were essential for RA-induced RARβ2 activation. They concluded that these nucleases induced DNA strand breaks and promoter CpG demethylation, along with posttranslational modifications of histones. These events promoted CCCTC-binding factor recruitment and chromatin looping as prerequisites for RARβ2 activation. However, we propose an alternative interpretation of their results: RA-induced ROS generate cyclopurines and DNA intrastrand cross-links (74), which should be repaired via NER. Furthermore, the NER proteins involved in both transcription-coupled and global genome NER have regulatory roles in oxidized base repair (75). Thus, ligand-induced gene activation involves the generation of a plethora of genome damages: these damages are repaired by distinct repair complexes to restore promoter integrity needed for transcription initiation.

Transcriptional repression also involves demethylation of histones, but not of CpGs. Specifically, Lys4 trimethylated H3, a marker for transcriptionally active promoters, is demethylated by LSD1 and JMJs (7, 76) during repression. However, because
repression does not involve DNA demethylation, the ROS load may be lower during repression than during activation. Thus, it would be interesting to estimate the level of nuclear ROS generated and the extent of genome damage induced during transcriptional activation vs. repression. However, unlike during activation when the repair should precede transcription initiation, repair may not be a prerequisite for repression. In fact, compromised promoter repair may contribute to ligand-induced repression. Thus, future studies exploring differential repair kinetics and complex dynamics that exist across the genomic landscape of active vs. repressed genes should be of significant interest.

Materials and Methods

Cell Lines, siRNAs, Transfection, and Treatment. The human embryonic kidney epithelial HEK293 (ATCC no. CRL-1573), breast adenocarcinoma MCF7 (ATCC no. HTB-22), alveolar adenocarcinoma A549 (ATCC no. CCL-185), and osteosarcoma U2OS (ATCC no. HTB-96) cell lines were maintained in Dulbecco’s modified Eagle medium-high-glucose (HyClone) containing 10% fetal calf serum (FCS; Sigma) and antibiotic mixture of 100 IU penicillin and 100 μg/mL streptomycin (Corning) at 37 °C under 5% CO2 and 95% relative humidity.

Serum (FCS; Sigma) and antibiotic mixture of 100 IU penicillin and 100 μg/mL streptomycin (Corning) were added to the medium, exponentially growing HEK 293 cells were treated with 1 to 10 μM all-trans RA (Sigma; no. R2625), MCF7 cells were treated with 10 nM E2 (Sigma; no. E2257), A549 cells were treated with 1 nM TNF α (Sigma; no. TNF010) for 24 hr before RA treatment or HS induction. The human colorectal adenocarcinoma HCT116 (ATCC no. CCL-247) cell line was grown in McCoy’s 5A medium (Gibco/Life Technologies) supplemented with FCS and antibiotic mixture. After washing thoroughly in serum-free medium, exponentially growing HEK293 cells were treated with 1 to 10 μM all-trans RA (Sigma; no. R2625), MCF7 cells were treated with 10 nM E2 (Sigma; no. E2257), and A549 cells were treated with 1 nM TNFα (a kind gift of Allan R. Brasier, originally at the University of Texas Medical Branch, Galveston, TX, and currently at the University of Wisconsin, Madison, WI) in the absence of serum for the indicated times for various experiments. HS/hyperthermia treatment was performed by incubating HCT116 cells at 42 °C for the indicated times. Down-regulation of endogenous individual repair proteins was achieved by transiently transfecting exponentially growing cells with specific siRNAs: XRCC1 (77), PARP1 (sense: 5′-ACACGUUUCUGUAAAAUCCtt-3′), or PNKP (Sigma; no. SASI_Hs01_00067475), along with a universal negative control siRNA (Sigma; no. SIC001). Transfection was performed with Lipofectamine RNAiMax (Invitrogen; no. 13778-075) per the manufacturer’s protocol (siRNA: 80 nM; Lipofectamine RNAiMax: 10 μL per transfection in 3 mL medium). The cells were harvested at 48 hr after transfection, and down-regulation of target genes was verified by western blot analysis or SYBR Green-based real-time RT-PCR analysis. PARP or DNA-PK inhibition was achieved by treating cells with 1 μM rucaparib (Selleckchem; no. S1098) or 10 μM Nu7441 (Sigma; no. 383406), respectively, for 24 hr before RA treatment or HS induction.

Alkaline Comet Assay. The DNA strand breaks in cells after ligand treatment were analyzed by alkaline comet assay (Comet Assay Kit; Trevigen; no. 4250–050-K) per the manufacturer’s protocol. Briefly, control or ligand-treated cells were lysed on comet slides containing low melting agarose and were electrophoresed in alkaline pH. The DNA in the nucleoid was visualized by SYBR gold staining and imaged with a fluorescence microscope (EVOS FL Auto Imaging System; Life Technologies). The data analysis was performed by using Open Comet/ImageJ (NIH) program in 50 randomly selected cells that were plotted as mean tail moment.

Estimation of Cellular ROS, H2O2, and O2·−. The control and RA-treated cells were loaded with nonfluorescent H2-DCFDA, followed by flow cytometric analysis of 2′, 7′-dichlorofluorescin (excitation and emission peaks at 49 and 529 nm, respectively) fluorescence. DCFDA-labeled tert-butyl hydroperoxide (TBHP; 50 μM) was used as a positive control for ROS generation. RA-induced cellular O2·− and H2O2 levels were measured by using the Superoxide Detection Assay Kit (Abcam; no. ab139476) and Hydrogen Peroxide Assay Kit (Cell Biolabs; no. STA-844), respectively, per the manufacturers’ instructions.

Subcellular Fractionation. Total nuclear and chromatin extracts were isolated from lysed cells, as previously described (37, 79, 80). In brief, proliferating cells on 10-cm plates at ~80% confluence were washed in phosphate-buffered saline (PBS), pH 7.4, and then were lysed in cytoplasmic lysis buffer (500 μL per 10-cm plate) containing 10 mM tris(hydroxymethyl)aminomethane·HCl (Tris·HCl), pH 7.9, 0.34 M sucrose, 3 mM CaCl2, 2 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitor mixture (Thermo Scientific/Pierce; no. 88666). After centrifugation at 3,500 × g for 15 min at 4 °C and discarding the supernatant cytosolic fraction, the nuclear pellets were lysed in nuclear lysis buffer (100 μL per 10-cm plate) containing 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (Hepes), pH 7.9, 1.5 mM MgCl2, 3 mM EDTA, 150 mM K-acetate, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitor mixture.
mixture. After vortexing for 15 min at 4 °C, the soluble fractions were separated from the chromatin pellets by centrifugation at 16,000 × g for 30 min at 4 °C. To isolate the soluble chromatin extracts, the chromatin pellets were dissolved in chromatin lysis buffer (100 μL per 10-cm plate) containing 150 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 150 mM K-acetate, 10% glycerol, and protease inhibitor mixture; incubated with 0.15 units/μL Benzonase (Novagen) at 37 °C for 30 min; and centrifuged at 16,000 × g for 30 min at 4 °C. Total cell extracts were isolated by harvesting PBS-washed cells in whole-cell lysis buffer (700 μL per 10-cm plate) containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture. The total soluble extracts were separated from the debris by vortexing for 15 min at 4 °C and centrifuging at 16,000 × g for 15 min at 4 °C. To isolate the whole nuclear extracts containing chromatin fractions, nuclear pellets were lysed in 500 μL whole-cell lysis buffer per 10-cm plate. All extracts were stored at −80 °C until use.

**Table 1. Primers for real-time ChIP PCR**

| Real-time ChIP PCR amplicon | Sequence in 5’ to 3’ direction; F: forward primer; R: reverse primer |
|-----------------------------|-------------------------------------------------------------------|
| RARβ2 promoter              | F: CTCTGGGCTGCTGCTGTTTG  |
| HSPA4 promoter              | R: CATGGGGGAATTCGCTGCTTC  |
| Nonspecific region from chromosome 17 | F: TACTTCTCCCCCTGCTCCCA  |
|                             | R: CATGAGGAGGGGGCGACAT  |

**Fig. 7.** Generation of DSBs by HS and their repair via NHEJ. (A and B) Immunofluorescence analysis of γH2AX foci in HCT116 cells (A) after HS treatment at 42 °C for the indicated times and (B) at the indicated release times at 37 °C after 3-hr HS treatment. Other details are same as in Fig. 3 A and B. (Scale bars, 10 μm.) (C) Real-time PCR analysis of γH2AX ChIP in the HSPA4 gene promoter and in the nonspecific region after HS induction for the indicated times. (D) Real-time RT-PCR analysis of HS-induced HSPA4 activation, normalized to HPRT1 expression, in PARP inhibitor (1 μM rucaparib)- or DNA-PK inhibitor (10 μM Nu7441)-treated cells as a function of HS induction time. The data represent the average (with SD) of three or more independent experiments. Statistical analysis was performed in GraphPad Prism by using two-way ANOVA with Tukey’s multiple comparisons test. **P value < 0.01.
Commmunoprecipitation Analysis. For communoprecipitation (Co-IP) analysis, chromatin extracts and soluble nuclear extracts were precleared by incubation with control immunoglobulin G (IgG) and Magna ChIP protein A/G magnetic beads (Millipore; no. 16–663) for 1 h at 4 °C with constant shaking. After separating from magnetic beads, the extracts were incubated overnight with 1 μg antibody (α-LSD1, α–SET1, α–XRCC1, or control IgG) per milligram protein in the extracts, together with fresh protein A/G magnetic beads, at 4 °C with constant shaking. The next day, the magnetic beads were washed three times with Tris-buffered saline containing 0.05% Tween 20, followed by sequential washes in low-salt immune complex wash buffer (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 150 mM NaCl), high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8), and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Finally, the proteins were eluted from the beads by boiling in Laemmli buffer and were electrophoresed in SDS-polyacrylamide gel for western blot analysis with the appropriate antibodies.

ChIP Analysis. The cells grown to 80% confluence on 10-cm plates were washed in PBS and then cross-linked with 1% formaldehyde for PBS in 15 min at room temperature. After washing three times with PBS, the cells were scraped off the plates in PBS containing protease inhibitor mixture and pelleted at 1,000 × g (10 min, 4 °C). The cell pellets were lysed in SDS lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and protease inhibitor mixture (1 mL per three 10-cm plates) and incubated on ice for 10 min. The cell lysates were sonicated (XL-2000 QSonica LLC) on ice for five times at a pulse setting of four with 1-min rest intervals in between and then centrifuged (16,000 × g, 15 min, 4 °C) to collect the supernatant containing the sheared chromatin lysate. IP of the chromatin lysates was performed with control IgG or appropriate antibody (5 μg) and 30 μL Magna ChIP Protein A/G magnetic beads. The IP mixture was brought up to 2 mL by 1:10 dilution with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, protease inhibitor mixture) and incubated overnight at 4 °C with constant shaking. The next day, the IPs were washed sequentially with low-salt wash, high-salt wash, LiCl immune complex wash, and TE buffer. The protein–DNA complexes were subsequently eluted in ChIP elution buffer (1% SDS, 0.1 M NaHCO3, and the cross-linking was reversed by incubation with 200 mM NaCl overnight at 65 °C. The ChIP DNA was purified by sequential incubation with RNase A and proteinase K, followed by phenol chloroform extraction and ethanol precipitation by using the standard protocols. Finally, the DNA was dissolved in 10 mM Tris-HCl, pH 8. The ChIP and 10% input DNA were proceeded to SYBR GREEN-based real-time PCR (7500 Real-Time PCR System; Applied Biosystems) with primers (the primer sequences in the 5′ to 3′ direction are given in Table 1) and SYBR Premix Ex Taq (TaKaRa). The data were represented as percentage input per https://www.thermofisher.com/us/en/home/life-science/epigenetics-noncoding-rna-research/chromatin-remodeling-chromatin-immunoprecipitation-chip/chip-analysis.html.

Real-Time RT-PCR Analysis. Total RNA from control and experimental cells was isolated with the Qiagen RNeasy Mini Kit, including on-column DNase1 digestion, and then processed for complementary DNA (cDNA) synthesis by using the SuperScript III First-Strand Synthesis Kit (Invitrogen; no. 18080051) per the manufacturer’s protocol. The expression levels of RARβ2 or HSPA4, along with those of the internal control HPRT1, were analyzed by SYBR GREEN-based real-time RT-PCR. The primer sequences in the 5′ to 3′ direction and other information are given in Table 2. The data were represented as relative quantification (RQ) based on the 2−ΔΔCt (CT: cycle threshold) method with the reference samples set at one.

Amplification of PremRNA and Mature mRNA of RARβ2 by PCR. Total RNA was isolated from HEK293 cells by using the RNeasy Mini Kit (Qiagen) per the manufacturer’s directions. The cDNA was then synthesized by using the SuperScript III Reverse Transcription kit (Invitrogen). Primers (5′ to 3′ sequences: CTTACCTTAAACTGCAATCGATGCACAA and TAAAGATCTTTGGAAACATCAGGAGG) were designed for amplification of the RARβ2 premRNA, containing partial exon 6 (145 nt), intron (812 nt), and partial exon 7 (123 nt), and PCR (95 °C for 2 min; 95 °C for 20 s, 56 °C for 40 s, and 65 °C for 90 s for 27 cycles; followed by 65 °C for 6 min) was performed. The PCR product size of the premRNA amplicon (exon 6 + intron + exon 7) is 1,080 nucleotides (nt), and that of the mature mRNA (without the intron) is 268 nt. The PCR products were separated in ethidium bromide-stained 0.8% agarose gel and visualized by the Gel Logic 2200 imaging system (Kodak), followed by quantitation by using ImageJ program.

Immunofluorescence Analysis. The control, RA-treated, or HS-induced cells, plated on chamber slides, were rinsed with PBS and fixed/permeabilized in cold methanol for 10 min. The cells were then incubated with α-H2AX and diluted (1:1,000) in PBS containing 1% bovine serum albumin (BSA) overnight at 4 °C. The next day, the cells were washed three times with PBS containing 0.1% Tween-20 (PBS-T) and incubated at room temperature in the dark with fluorescence-conjugated secondary antibody diluted (1:500) in 1% BSA for 1 hr. The cells were washed three times with PBS-T, and the slides were mounted by using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized in a Zeiss Axio Observer fluorescence microscope (Olympus).

Statistical Analyses. Statistical analyses were performed in GraphPad Prism by using the unpaired t test with Welch’s correction or one-way or two-way ANOVA with Tukey’s or Dunnett’s multiple comparisons test on the basis of experimental designs.

Data Availability. All data reported in the text have been compiled in the figures. All materials generated in this study are available upon request.

ACKNOWLEDGMENTS. This work was supported by Cancer Prevention & Research Institute of Texas Training Fellowship CPRIT No. RP101489 (to S.S.), NIH Grants R01 NS088645 (to M.L.H.), RF1 NS112719 (to M.L.H.), R01 GM105090 (to S.M.), R01 CA158910 (to S.M.), and P01 CA092584 (to S.M.); NIH Grants R01 NS088645 (to M.L.H.), R01 CA158910 (to S.M.), and P01 CA092584 (to S.M.); and Houston Methodist laboratory start-up funds (to M.L.H. and S.M.). We thank Drs. Tapas K. Hazra of the University of Texas Medical Galveston, and the members of the laboratories of M.L.H. and S.M. for suggestions during preparation of this manuscript.

Table 2. Primers for real time RT-PCR

| Primer Name | Sequence 5′ to 3′ direction | F: forward primer | R: reverse primer |
|-------------|-----------------------------|-------------------|-------------------|
| RARβ2       | F: AACGGACCTTTGCAAGGCA      | R: AGTGCCTCGGATCCTACG |
| HSPA4       | F: GCAACAGACGGACAGGACAGCA   | R: GCCCTCTGTGCTTGGGTGTTG |

https://www.realtimeprimers.com/ https://www.realtimeprimers.com/
37. M. L. Hegde

32. N. Le May, D. Fradin, I. Iltis, P. Bougnères, J. M. Egly, XPG and XPF endonucleases

39. S. D. Kathe, G. P. Shen, S. S. Wallace, Single-stranded breaks in DNA but not oxidative

31. J. J. Kim, S. B. Lee, J. K. Park, Y. D. Yoo, TNF-alpha-induced ROS production triggering

40. A. Campalans

42. K. W. Caldecott, C. K. McKeown, J. D. Tucker, S. Ljungquist, L. H. Thompson, An in-

23. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

45. L. Pan

46. A. M. Fleming, Y. Ding, C. J. Burrows, Oxidative DNA damage is epigenetic by regulat-

48. S. Roychoudhury

50. J. L. Nitiss, DNA topoisomerase II and its growing repertoire of biological functions.

57. N. Bhutani, D. M. Burns, H. M. Blau, DNA demethylation dynamics. Cell 146, 866–872 (2011).

58. M. S. Kim

65. J. Puc

70. M. C. Haffner, A. M. De Marzo, A. K. Meeker, W. G. Nelson, S. Yegnasubramanian,

74. B. R. Berquist, D. M. Wilson 3rd, Pathways for repairing and tolerating the spectrum

75. J. P. Melis, H. van Steeg, M. Luijten, Oxidative DNA damage and nucleotide excision

B. R. Berquist, D. M. Wilson 3rd, Pathways for repairing and tolerating the spectrum

40. J. L. Nitiss, DNA topoisomerase II in cancer chemotherapy. Nat. Rev. Cancer 9, 338–350 (2009).

29. Oncogene 29, 632 (2012).

28. S. Roychoudhury et al., Endogenously oxidized DNA bases and APE1 regulate the

25. M. Dizdaroglu, Oxidatively induced DNA damage and its repair. Mutat. Res. Rev.

26. V. Bourdeau et al., Genome-wide identification of high-affinity estrogen response

27. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

28. S. Amenta et al., LSD1-mediated demethylation of histone H3 lysine 4 triggers Myc-

29. S. D. Kathe, G. P. Shen, S. S. Wallace, Single-stranded breaks in DNA but not oxidative

54. L. Pan

8. J. P. Melis, H. van Steeg, M. Luijten, Oxidative DNA damage and nucleotide excision

11. T. Ismail et al., KDM1A microenvironment, its oncogenic potential, and therapeutic

12. X. Wu, Y. Zhang, TET-mediated active DNA demethylation: Mechanism, function and

13. J. Jan, A. Rao, M. Ko, TET family dioxygenases and DNA demethylation in stem cells

14. P. Melamed, Y. Yosefzon, C. David, A. Tsukerman, L. Prunei, Tet enzymes, variants, and
differential effects on function. Front. Cell Dev. Biol. 6, 22 (2018).

15. M. P. Murphy, How mitochondria produce reactive oxygen species. Biochem. J. 417, 1–15 (2009).

16. K. M. Holstrom, T. Finkel, Cellular mechanisms and physiological consequences of

17. J. J. Kim, S. B. Lee, J. K. Park, Y. D. Yoo, TNF-alpha-induced ROS production triggering

18. Y. Wang, R. Branicky, A. Noé, S. Hekimi, Superoxide dismutases: Dual roles in con-

19. S. Choudhary, I. Boldogh, A. R. Brasier, Inside-out signaling pathways from nuclear

20. J. P. Kehrer, The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149,

21. V. B. Djordjevic, “Free radicals in cell biology” in International Review of Cytology: A

22. N. Le May, D. Fradin, I. Iltis, P. Bougnères, J. M. Egly, XPG and XPF endonucleases

23. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

24. L. Pan

25. M. Dizdaroglu, Oxidatively induced DNA damage and its repair. Mutat. Res. Rev.

26. V. Bourdeau et al., Genome-wide identification of high-affinity estrogen response

27. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

28. S. Amenta et al., LSD1-mediated demethylation of histone H3 lysine 4 triggers Myc-

29. S. Bourdeau et al., Genome-wide identification of high-affinity estrogen response

30. M. S. Kim et al., DNA demethylation in hormone-induced transcriptional derepres-

31. J. J. Kim, S. B. Lee, J. K. Park, Y. D. Yoo, TNF-alpha-induced ROS production triggering

32. N. Le May, D. Fradin, I. Iltis, P. Bougnères, J. M. Egly, XPG and XPF endonucleases

33. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

34. M. L. Hegde

35. S. C.Contextualized results of a genome-wide association study in type-2 diabetes. J. Biol.

36. B. Perillo et al., DNA oxidation as triggered by H3K39me2 demethylation drives
estrogen-induced gene expression. Science 319, 202–206 (2008).

37. S. Amenta et al., LSD1-mediated demethylation of histone H3 lysine 4 triggers Myc-

38. S. Roychoudhury et al., Endogenously oxidized DNA bases and APE1 regulate the

39. S. D. Kathe, G. P. Shen, S. S. Wallace, Single-stranded breaks in DNA but not oxidative

40. A. Campalans

41. J. J. Kim, S. B. Lee, J. K. Park, Y. D. Yoo, TNF-alpha-induced ROS production triggering

42. K. W. Caldecott, C. K. McKeown, J. D. Tucker, S. Ljungquist, L. H. Thompson, An in-

43. M. L. Hegde, T. K. Hazra, S. Mitra, Early steps in the DNA base excision/single-strand

44. L. Pan et al., Oxidized guanine base lesions function in B-oxoguanine DNA glyco-

45. L. Pan

46. A. M. Fleming, Y. Ding, C. J. Burrows, Oxidative DNA damage is epigenetic by regulat-

47. J. P. Melis, H. van Steeg, M. Luijten, Oxidative DNA damage and nucleotide excision

48. S. Roychoudhury

49. R. Ghosh, D. L. Mitchell, Effect of oxidative DNA damage in promoter elements on

50. J. L. Nitiss

51. A. K. Velichko, N. V. Petrova, S. V. Razin, O. L. Kantidze, Mechanism of heat stress-

52. M. L. Hegde

53. L. Pan

54. L. Pan

55. L. Pan

56. L. Pan

57. N. Bhutani, D. M. Burns, H. M. Blau, DNA demethylation dynamics. Cell 146, 866–872 (2011).

58. M. S. Kim et al., DNA demethylation in hormone-induced transcriptional derepres-

59. K. Williams, J. Christensen, K. Helin, DNA methylation: TET proteins-guardians of CpG

60. K. Williams, J. Christensen, K. Helin, DNA methylation: TET proteins-guardians of CpG

61. J. P. Melis, H. van Steeg, M. Luijten, Oxidative DNA damage and nucleotide excision

62. L. Pan

63. L. Pan

64. L. Pan

65. J. Puc

66. V. Bourdeau et al., Genome-wide identification of high-affinity estrogen response

67. F. Kouzine

68. A. Campalans

69. R. Ghosh, D. L. Mitchell, Effect of oxidative DNA damage in promoter elements on

70. M. C. Haffner, A. M. De Marzo, A. K. Meeker, W. G. Nelson, S. Yegnasubramanian,

71. L. Pan

72. P. T. Young

73. A. M. Fleming, Y. Ding, C. J. Burrows, Oxidative DNA damage is epigenetic by regu-

74. B. R. Berquist, D. M. Wilson 3rd, Pathways for repairing and tolerating the spectrum

75. J. P. Melis, H. van Steeg, M. Luijten, Oxidative DNA damage and nucleotide excision

76. A. Chicas

77. L. Pan

78. L. Pan

79. L. Pan

80. J. L. Nitiss

81. J. L. Nitiss, DNA topoisomerase II and its growing repertoire of biological functions.

82. M. S. Kim et al., DNA demethylation in hormone-induced transcriptional derepres-

83. L. Pan

84. L. Pan

85. L. Pan

86. L. Pan

87. L. Pan

88. L. Pan

89. L. Pan

90. L. Pan

91. L. Pan

92. L. Pan

93. L. Pan

94. L. Pan

95. L. Pan

96. L. Pan

97. L. Pan

98. L. Pan

99. L. Pan

100. L. Pan

101. L. Pan

102. L. Pan

103. L. Pan

104. L. Pan

105. L. Pan

106. L. Pan

107. L. Pan

108. L. Pan

109. L. Pan

110. L. Pan

111. L. Pan

112. L. Pan

113. L. Pan

114. L. Pan

115. L. Pan

116. L. Pan

117. L. Pan

118. L. Pan

119. L. Pan

120. L. Pan

121. L. Pan