Supporting Online Material for

**DNA Oxidation as Triggered by H3K9me2 Demethylation Drives Estrogen-Induced Gene Expression**

Bruno Perillo,* Maria Neve Ombra, Alessandra Bertoni, Concetta Cuozzo, Silvana Sacchetti, Annarita Sasso, Lorenzo Chiariotti, Antonio Malorni, Ciro Abbondanza, Enrico V. Avvedimento*

*To whom correspondence should be addressed. E-mail: perillo@unina.it (B.P.); avvedim@unina.it (E.V.A.)

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Materials and Methods

**Cells.** Human breast cancer MCF-7 and MDA-231 cells were routinely grown as already described (2). To evaluate the effect of estrogen challenge, cells were first incubated with phenol red-free DMEM with 0.5% dextran-charcoal-stripped FCS for 6-8 h, and then with the same medium containing 5% dextran-charcoal stripped FCS for further 4 days, prior to be challenged with 10 nM E2 for different times according to the experimental needs. To obtain expression of ERα in MDA-231, cells were co-transfected with pSG5-HEGO plasmid expressing human estrogen receptor and the pMACS4-IRES.II vector, used to select transfected cells, according to MACSelect™ System instructions. Human primary fibroblasts were grown in DMEM with 10% FBS prior to be treated as described above for breast cancer cells, in order to evaluate the effects of E2 challenge. In experiments with ICI 182,780, the anti-estrogen (1 µM) was added concomitantly with 10 nM E2. To inhibit LSD1 activity, 3 mM pargyline was added to cells for 14-16 h. To obtain LSD1 knock down with siRNAs, cells were transfected with 37.5 ng of AOF2-1 or AOF2-5 siRNAs targeting protein coding region (Qiagen Inc., USA) or the 3’-untranslated region (customer service, Qiagen, USA), in medium without serum to a final concentration of 5nM and incubation was continued for 72 h. Sch scrambled RNA (1 µl) was used as negative control. The same procedure was used to get OGG1 and topoisomerase IIβ knock down with the specific siRNAs sc-43983 and sc-36697 (Santa Cruz Biotechnology Inc., USA), respectively. To determine rescue of LSD1 activity in knock down experiments with siRNAs, LSD1 full-length cDNA was inserted into the CMV 3xflag expression vector (Sigma-Aldrich, USA). To obtain the mutant, catalytically inactive form, a cDNA encoding a protein lacking the C-terminal tail (BamH1-digested) was used.

**ChIP and sequential ChIP (re-ChIP).** ChIP assays were carried out as previously reported (4). Chromatin was sonicated in order to obtain fragments of 300-400 bp. For each assay, chromatin from a total of 10⁶ cells was used. In re-ChIP experiments, complexes were eluted from the primary
ChIP by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:40 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH8.1) before being re-immunoprecipitated with the secondary antibodies. Re-ChIP of supernatants was performed essentially as for primary immunoprecipitations. All bands from ethidium bromide stained gels were analyzed by densitometry and quantified with two independent programs: Scion Image for Windows and Mac OS (Scion Corporation, USA), and Multi-analyst Biorad version 1.1 (Biorad Laboratories, USA). The antibodies used were as follows: anti-ERα Ab 314 and Ab 1603 were prepared as already described (C. Abbondanza et al. Steroids 58, 4-12 1993). Antiacetyl-H3 06-599, anti-dimethyl-H3 (Lys4) 07-030, anti-dimethyl-H3 (Lys9) 07-441, anti-LSD1 05-939, anti-SET9 07-314, and anti-Suv39H1 05-615 were from Upstate Cell Signaling Solutions. Anti-p300 sc-585, anti-CBP sc-369, anti-RNA pol II sc-899, anti-OGG1 sc-33181, anti-topoisomerase I sc-10783, anti-topoisomerase IIβ sc-13059, and anti-ERK 2 sc-56899 were from Santa Cruz. Anti-HDAC1 Ab 7028, anti-α-tubulin Ab4074 and anti-serine 5 phosphorylated Pol II Ab 5401 were from Abcam. Sequences of primers and PCR conditions are available upon request.

**Nuclear run-on.** Run-on assays were realized as previously reported (4). Briefly, cell nuclei were extracted by addition of NP-40 lysis buffer (10 mM Tris-HCl, pH 7.44; 10 mM NaCl; 3 mM MgCl2; and 0.5% NP-40) and stored in glycerol buffer (50 mM Tris-HCl, pH 8.3; 40% glycerol; 5 mM MgCl2; 0.1 mM EDTA). Transcription was carried-out in the presence of 5 µl of 10 mCi/ml [α-32P]UTP. RNA samples (10 µg) were hybridized with plasmids (pcDNA3) containing cDNAs of bcl-2 or β-actin, immobilized on nylon filters.

**Chromosome conformation capture (3C).** 3C was realized as described previously (10). In brief, transiently interacting chromatin regions were cross-linked with 1% formaldehyde for 15 min at room temperature. The reaction was stopped with 125 mM glycine and plates were maintained for additional 5 min at room temperature. Approximately 2x10^6 cells were then re-suspended in 500 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). Typically, 25 µl of chromatin preparations were digested, in 100 µl final volume, for 2 h with the restriction enzyme Sac I (50-75
U) that cuts DNA within the promoter, downstream from the EREs and once in the intervening region. DNA ends were then ligated in diluted (1:7.5) solutions (750 µl), in order to favor intramolecular ligations. After reversal of protein/DNA cross-links by incubation of samples at 65°C for 6 h in the presence of 100 µg proteinase K, in order to ensure complete removal of RNA, 10 µg of DNase-free RNase were added and incubation was continued for further 15 min at 37°C. Ethanol-precipitated DNA was finally amplified in suitable PCRs to establish transient interactions between distant DNA ends.

**Detection of 8-oxo-guanine by Immunofluorescence.** 2x10⁶ MCF-7 cells were seeded onto glass slides and treated with H₂O₂ (100 µM, 15 min) or 10 nM E₂ for 5 or 45 min, in the absence or presence of pargyline (3 mM, 3 h), NAC (25 mM, 30 min). Control cultures were treated with equivalent ethanol volumes and concentrations. After treatments, the cells were fixed 15 min with 4% paraformaldehyde in PBS. The slides were then washed with TBS/Tween-20 and permeabilized by serial washes in methanol solutions, prior to be washed with TBS/Tween-20, blocked for 1 h at 37°C and incubated with FITC-labeled protein, that binds 8-oxo-guanine, for 15 h at 4°C (Biotrin OxyDNA Test, Biotrin, UK). Cover slips were mounted in Moviol and viewed by fluorescence on a Zeiss axiomatic photomicroscope with a 63x objective. To obtain LSD1 knock down, 10⁵ cells were transfected in 24 multi-well plates with specific or control siRNAs following the protocol described above. After 72 h, cells were subjected to different treatments, according to experimental needs, and processed for fluorescence microscopy, as stated above. For single cell transfection assays, cells were co-transfected with siRNAs and 100 ng pDsRed1-N1 (Clonetech, USA). In both cases the efficiency of transfection was 65%±10.

**Western blotting.** To determine OGG1/2 levels, E₂-deprived cells were treated with 10 nM E₂ for 15 min in the absence or presence of 30 min pre-added MG132 (10 µM) or NAC (25 mM). Treatment with 100 µM H₂O₂ for 15 min was used as control. Thereafter, cells were washed in ice-cold PBS, and dissolved in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet NP-40, 300 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 50 mM NaF, 0.1 mM NaVO₃, 1 mM glycerophosphate, 2.5 mM
sodium pyrophosphate, in the presence of protease inhibitors and 40 U DNase). Eighty µg of protein were electrophoresed on 8% SDS–polyacrylamide gel and transferred onto nitrocellulose membranes (Schleicher and Schuell GmbH, Germany). Western blot analysis was carried out by using anti-OGG1 and anti-ERK 2 antibodies (Santa Cruz Biotechnology Inc., USA), according to manufacturer’s protocols.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6
Supplementary Figure legends

Supplementary Figure 1. H3K4me2 and loss of K9me2 mark the assembly of productive ERα transcription complex. (A) Kinetic ChIPs to show assembly of the positive and negative complexes on bcl-2 estrogen-responsive chromatin. (B) Transcription of bcl-2 and pS2 genes analyzed by nuclear run on in cells stimulated with E2 for different times. β-actin was used as E2-unresponsive control gene. (C) Histograms from three independent experiments showing presence of ERα or H3K4me2 or K9me2 at bcl-2 enhancer (enh.) or 5’ upstream (5’) sites, as assessed by ChIP. (D) Sequential ChIP (re-ChIP) analysis from cells primarily treated with antibodies to ERα. Chromatin from primary ChIPs (1-IP) was subsequently precipitated (2-IP) with the antibodies indicated in the lower panels.

Supplementary Figure 2. The H3K4/K9 methylation switch is spatially restricted to the estrogen-responsive sites. (A) ChIPs with antiH3K4me2 or K9me2 were carried out also with primers located in areas (represented as yellow boxes in the lower insert) surrounding the EREs (red) and downstream from the promoter (green). Sequences and coordinates of primers used are available upon request. (B) The histogram shows the specificity of the PCR analysis. Reported results were derived from at least three independent experiments.

Supplementary Figure 3. Estrogen-induced oxidation of Gs depends by the presence of ERα. Immunofluorescence detection of 8-oxo-Gs by fluorescein-tagged 8-oxo-G-binding protein in MDA231 cells transfected (ERα+) or not (WT) with exogenous estrogen receptor.

Supplementary Figure 4. Estrogen-dependent formation of oxidized guanines (8-oxo-Gs) is mediated by ERα. ChIP analysis of accumulation of 8-oxo-Gs (assessed as recruitment to
chromatin of the BER enzyme OGG1) on the promoter of the estrogen-insensitive PSA gene, notwithstanding the presence of LSD1.

**Supplementary Figure 5. DNA modifications by estrogens preferentially localize around the ERE sites of bcl-2 gene.** (A) Strand-selective PCR on ERE site. The ERE region immunoprecipitated with antibodies to the receptor was pre-amplified (10 cycles) with 1/5 of the final concentration of forward (A) or reverse (B) primers, respectively. 28 cycles were then carried-out with equimolar concentrations of A and B. To reach comparable PCR efficiency, in ChIP reaction where the expected yield of DNA template was very low (ChIP with chromatin from hormone-starved cells) the reaction was continued for further 7 cycles (35 cycles). The samples where the pre-amplification was performed with the primer A or B are indicated by A>B or B>A, respectively. A=B indicates a normal amplification reaction without pre-amplification (28 or 35 cycles) whose product yield has been normalized to 1. The histogram represents the average of at least three independent experiments. (B) Primer extension with primers from bcl-2 ERE region. The primers indicated by the arrows were used to extend the DNA derived from ChIPs of MCF-7 cells challenged or not with E2 for 30 min. The conditions of denaturation, annealing and extension were the following: 95°C for 30 sec, 55°C for 20 sec, 72°C for 30 sec. Sequences and positions of the different primers pairs used for extension are available upon request. The concentrations of nucleotides were 150 µM, including α-P 32-dGTP. At the end of the reaction, 1/5 of the mixture was loaded on 6% polyacrylamide gels in 5 M urea. Labeled size markers were used (300-350 bp). Under these conditions, fragments smaller than 150 bp were not included in the gel. The schematic diagram below the gel shows the extended fragments relative to the ERE. Vertical arrows indicate the putative sites of stop of Taq DNA polymerase.

**Supplementary Figure 6. H2O2 recruits OGG1 and topoisomerase IIβ (Topo IIβ) to chromatin.** (A) Effect of addition of the ROS scavenger NAC on E2-stimulated transcription of
bcl-2 and pS2 genes, measured by run on. Transcription of β-actin was used for normalization. (B-C) The histograms from three independent ChIP experiments show the effect of H₂O₂ addition to cells treated or not with E₂, in the presence or absence of pargyline, on assembly to different chromatin regions of OGG1 and topoisomerase IIβ, respectively.