Assessment of protein dynamics and DNA repair following generation of DNA double-strand breaks at defined genomic sites

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The formation of protein aggregates (foci) at sites of DNA double-strand breaks (DSBs) is mainly studied by immunostaining and is hence limited by the low resolution of light microscopy and the availability of appropriate and selective antibodies. Here, we describe a system using enzymatic creation of site-specific DNA DSBs within the human genome combined with chromatin immunoprecipitation (ChIP) that enables molecular probing of a DSB. Following induction of the I-*Ppo*I enzyme and generation of DSBs, cellular DNA and proteins are crosslinked and analyzed by ChIP for specific proteins at the site of the break. The system allows the direct detection of protein and chromatin dynamics at the site of the break with high resolution, as well as direct measurement of DNA repair defects in human cells. Starting with fragmented chromatin, results can be achieved in 2–3 d.

INTRODUCTION

DSBs are problematic genomic lesions that, if misrepaired, can lead to mutations, chromosomal rearrangements and cell death. DSBs can be generated by many different processes, ranging from by-products of normal metabolic pathways to environmental agents such as ionizing radiation. Thus, all organisms have developed elegant strategies to respond to and repair these lesions[1]. One of the key phenomena observed after damage is the localization of hundreds to thousands of molecules at and around the site of the break. Until recently[2–4], there has been no way to directly identify proteins bound to defined DSBs in intact mammalian cells. The main method used to study the binding of proteins to DSBs in mammalian cells uses fluorescent microscopy to analyze this formation of protein aggregates, termed foci, after DNA damage[5]. Analyses of damage-induced foci have provided a wealth of information on the kinetics and colocalization of proteins in response to DNA damage. However, the interpretive power of this approach has been hampered by the low resolution of light microscopy, and by the lack of evidence that foci directly reflect proteins recruited and maintained at sites of discrete DNA strand breaks.

An alternative method that has facilitated the high-resolution analysis of the recruitment of proteins to DSB sites in yeast uses the HO mating-type switch endonuclease to introduce a DSB at a specific genomic site[6]. This system has been widely used to study the kinetics of the recruitment of proteins to DSB sites, response to DSBs and molecular steps involved in DSB repair[7–9]. This alternative approach is advantageous as the site and chemical nature of the DSBs are known, and other intracellular changes caused by exposure to ionizing radiation are avoided. Introduction of a target sequence for the budding yeast I-*Sce*I homing endonuclease into human cells was used for several studies of DSB response and repair upon expression of I-*Sce*I (see refs. 10,11). I-*Sce*I was also used for studying responses to a single DSB occurring at an integrated site in the human genome[3] and for differential fluorescent tagging of broken DNA ends to show that broken ends are positionally stable and unable to roam the cell nucleus[4].

To improve and further use the approach of generating DSBs at defined sites in human cells, we used an endonuclease that targeted endogenous genomic sequences to generate site-specific DSBs in mammalian cells and examined the resultant responses at high resolution. This approach permits examination of the recruitment of proteins to DSB sites in living cells independent of the modification status of the proteins. The procedure consists of two parts. First, the eukaryotic homing endonuclease I-*Ppo*I is introduced into and induced in human cells. I-*Ppo*I is an intron-encoded homing endonuclease from the myxomycete *Physarum polycephalum* that cuts with high specificity at a 15-bp recognition sequence in 28s rDNA[12,13]. The human 28s rDNA consists of about 300 copies, with about 10% of these sites available for cutting by the I-*Ppo*I enzyme. Several other potential sites can be found in the human genome because of degeneracy of the I-*Ppo*I restriction sequence[14]. A major advantage of using I-*Ppo*I over I-*Sce*I is the fact that the I-*Sce*I restriction sequence needs to be introduced into the genome of the specific cell line to be used in the assay, whereas the I-*Ppo*I sites are available and conserved in the genome, ranging from yeast to human. To more effectively control DSB initiation in this system, we fused a mutant estrogen receptor (ER) hormone-binding domain to the open reading frame of I-*Ppo*I. The mutant hormone-binding domain of this fusion protein promotes rapid nuclear localization of preformed ER-I-*Ppo*I in response to 4-hydroxytamoxifen (4-OHT), but not in response to estrogen[15,16]. The expression of I-*Ppo*I in human cells results in specific cleavage of the target site in human 28s rDNA[14] and a few other genomic sites outside the rDNA cluster, and is accompanied by the activation of DNA damage response, phosphorylation of known damage-response proteins and formation of immunofluorescent foci at the sites of the break[2]. No evidence of toxicity has been detected during the first 12 h of induction of I-*Ppo*I, although a small amount of

PARP cleavage, potentially indicating some toxicity, can be detected after 24–48 h (data not shown).

As I-*Ppo*I-induced DSBs occur at defined DNA target sequences, it was possible to analyze proteins bound to these DSBs by ChIP and PCR analysis[17]. Indeed, applying ChIP to proteins known to be present at the break resulted in pull-down of the specific DNA sequence where I-*Ppo*I cuts[2]. One of the advantages of this protocol compared with immunostaining for focus formation, where many of the antibodies used must be directed against modified forms of the protein (i.e., phosphorylation, acetylation, etc.), is that this protocol is not limited to using modification-specific antibodies. In addition, as analysis of the site uses PCR amplification, relatively low amounts of proteins can be detected at the DSB. Finally, the high resolution achieved by the use of specific PCR oligos allows scanning of the flanking DNA around the damage site, resulting in the ability to analyze the precise distribution of DNA damage proteins around the break at various times after damage. Currently, the main limitation of using this system is the inability to stop the enzyme from cutting after the lesion has been properly repaired; thus, continuous cutting may occur at later time points than suggested in this protocol.

Another important aspect of this assay is the ability to measure DNA repair defects by quantitative real-time PCR or Southern blot analysis. The repair defect assay, which uses specific oligos flanking the I-*Ppo*I single site at chromosome I (see ref. 2) and real-time PCR to assess the creation and repair of a DSB, can be carried out using various conditions/genomic defects perturbing the DNA damage-response pathway enabling the determination of each genetic component's contribution to the repair process. Using this protocol, we have been able to interrogate chromatin changes, protein dynamics and repair of DSBs while determining the genetic and biochemical dependencies of the DNA damage-response processes. The target site for I-*Ppo*I is conserved from yeast to human, theoretically permitting the use of this protocol in multiple genetic systems, thus exploiting the availability of different genetic backgrounds.

Experimental design

Overview of steps. Cells are infected with the HA-ER-I-*Ppo*I retrovirus (HA, hemagglutinin tag used in a western blot with anti-HA antibody to detect the fusion protein HA-ER-I-*Ppo*I; ER, estrogen receptor hormone-binding domain) and serum-starved for synchronization in G1. Induction of the I-*Ppo*I enzyme is achieved by the addition of 4-OHT, followed by harvesting the induced cells at intervals of 2 h for several time points (6–7 collections, 0–12 h). This is followed by crosslinking with formaldehyde and preparation of fragmented chromatin for immunoprecipitation (IP) with antibodies directed to proteins suspected to be present at the damage site (see Steps 1–22). Following IP, the crosslinked protein/DNA complex is eluted and DNA is purified (see Steps 22–40). PCR analysis of the purified DNA allows the relative change in protein binding to the site of damage to be estimated by comparing the PCR band intensity before and after the induction of the enzyme (see Steps 41–43); an increase in band intensity compared with zero time point (no induction of the enzyme, therefore no DSBs) indicates recruitment of the specific protein to the site of damage. Real-time PCR can alternatively be used to follow the creation and repair of I-*Ppo*I-mediated DSBs, using specific oligos near the DSB site (as described in **Box 1**). Finally, Southern blotting, using genomic DNA prepared from cells collected after the induction of the enzyme, can also be used to examine rDNA DSB repair (see **Box 2**).

Vector design and construction. The open reading frame of I-*Ppo*I was amplified by PCR and the *Bgl*II fragment was ligated into pBABE-HA-ER-E7 (ref. 16) cut with *Bam*HI to maintain the reading frame with HA-ER.

Target cells and their preparation. The I-*Ppo*I consensus site is conserved from yeast to human, thereby permitting the usage of various cell lines. Adjustment of the number of cells, formaldehyde concentration and time of crosslinking is needed for each cell line to be used. Cells are grown in their appropriate growth media and need to be cycling to be infected by the I-*Ppo*I retroviruses. For performing the repair defect assay, cells need to be synchronized by serum starvation. This procedure needs to be tested on each cell line as not all cell lines arrest at low serum.

BOX 1 | REALTIME PCR REPAIR DEFECT ASSAY

Perform a SYBR Green reaction in 96-well plates on ABI-7900 real-time PCR machine in a total reaction volume of 25 µl (1 µl template input DNA (from Step 21), 12.5 µl SYBR Green Master mix, 1 µl primer (10 pmol each) and 10.5 µl PCR-H_2O) (95 °C, 30 s; 60 °C, 1 min). Real-time PCR is done with oligos no. 4 and no. 5 (Supplemental Table 1 in ref. 2). Oligos 4 are flanking the I-*Ppo*I site on chromosome 1, whereas oligos 5 are the calibration for an undamaged site in the human genome (GAPDH). Use a dissociation curve step to verify amplification of a single PCR product, add a sample without a DNA template as a negative control.

▲ **CRITICAL STEP** In this assay, cells need to be synchronized before induction (as described in Step 2).

An increase in the CT value over time points indicates loss of the PCR product due to cutting by I-*Ppo*I, whereas a decrease in the CT value over time points represents repair of the lesion. Analysis is done by the ΔΔCT method, as explained in great detail and by examples in http://www3.appliedbiosystems.com/sup/URLRedirect/index.htm?xDoD=4371095.

MATERIALS

REAGENTS

- 37% (wt/vol) formaldehyde solution (Sigma, cat. no. F1635) ! **CAUTION** Very toxic if inhaled, ingested or absorbed through skin.
- 4-OHT (Sigma, cat. no. H7904) ! **CAUTION** Harmful.
- Polybrene (hexadimethrine bromide; Sigma, cat. no. H9268)
- Phenylmethanesulfonylfluoride (PMSF; Sigma, cat. no. P-7626) ! **CAUTION** Toxic if absorbed through skin or ingested.
- RNase A solution (Qiagen, cat. no. 19101 or any other)
- ProteinA beads for IP (Oncogene, cat. no. IP06 or any other beads)
- tRNA (Sigma, cat. no. R8505)
- BSA (Sigma, cat. no. A4378)
- QIAquick PCR purification kit (Qiagen, cat. no. 28106)
- Proteinase K solution (Roche, cat. no. 03115828001 or any other)
- Aprotinin (Sigma, cat. no. A6279)
- Leupeptin (Sigma, cat. no. L2884)
- Phosphatase inhibitor cocktail 1 (Sigma, cat. no. P2850)
- Phosphatase inhibitor cocktail 2 (Sigma, cat. no. P5726)
- 10% (wt/vol) SDS solution

BOX 2 | SOUTHERN BLOT ANALYSIS OF rDNA REPAIR

Southern blotting, using genomic DNA prepared from cells collected after induction of the enzyme, can alternatively be used to examine rDNA DSB repair.

1. Prepare genomic DNA from cells synchronized, induced and collected at several time points after induction (from Step 3) by using a Puregene kit, according to the manufacturer's instructions.
2. Cut the genomic DNA with *Nco*I (50 µl total reaction volume, 20 µg genomic DNA, 2 µl *Nco*I (10 U/µl), cut over-night at 37 °C).

▲ CRITICAL STEP It is important to load equal amounts of cut genomic DNA to be able to compare between lanes for the appearance of the I-*Ppo*I cut 0.8 kb fragment. To achieve equal loading, it is suggested to cut excess genomic DNA (10–20 µg) with *Nco*I and measure the DNA content after the restriction reaction without DNA cleaning; this can be done by use of a Nanodrop measurement. The blank reaction consisting of water, enzyme buffer and *Nco*I enzyme is used as a base line for the measurement.

▲ CRITICAL STEP This results in an ~2.6 kb rDNA fragment in which the I-*Ppo*I site is located 0.8 kb 5′ to the 3′ of the rDNA fragments.

3. For each time point, fractionate 5 µg of digested genomic DNA in an individual lane of a 1% (wt/vol) agarose gel (8 cm × 10 cm) run at 20 V in 1× TAE for ~17 h to achieve separation of the cut genomic DNA.
4. Blot the DNA onto a Biodyne membrane by the alkaline transfer method[20].
5. Detect membrane-bound rDNA fragments using the Detector AP chemiluminescent blotting kit, according to the manufacturer's instructions. The probe for detection is prepared by PCR amplification of genomic DNA with oligos 1B (Supplemental Table 1 in ref. 2).

▲ CRITICAL STEP In our assay, we label the probe with biotin using a PCR DNA biotinylation kit to use the Detector AP chemiluminescent blotting kit. Any other method can be used to label the probe and detect the rDNA fragment on the membrane.

▲ CRITICAL STEP Upon induction of the I-*Ppo*I enzyme, a fragment of ~0.8 kb is detected, indicating cutting at the rDNA site. An increase in the 0.8 kb band indicates increase in DSB formation, whereas reduction in band intensity indicates repair of the DSB at the rDNA site.

- $NaHCO_3$ (Sigma, cat. no. S5761)
- Ready beads for PCR (GE Healthcare PuReTaq Ready-To-Go PCR beads, cat. no. 27-9557-02)
- ATM AB3 (Calbiochem, cat. no. PC116)
- H2AX pS139 (Millipore, cat. no. 07-164)
- XRCC4 (Abcam, cat. no. ab145)
- H2B (Millipore, cat. no. 07-371)
- NBS1 pS343 (Cell Signaling, cat. no. 3001)
- Chk2 T68 (Cell Signaling, cat. no. 2661)
- p53 pS15 (Cell Signaling, cat. no. 9284)
- HA tag (Covance, cat. no. MMS-101R)
- Rabbit IgG (Jackson ImmunoResearch Lab, cat. no. 011-000-002)
- SYBR Green master mix (Applied Biosystems, cat. no. 4309155)
- Oligos (see Supplemental Table 1 in ref. 2)
- Puregene DNA-extraction kit (Gentra, cat. no. D-5000A)
- *Nco*I (Invitrogen, cat. no. 15421-050)
- Detector AP chemiluminescent blotting kit (KPL, cat. no. 54-30-02)
- PCR DNA biotinylation kit (KPL, cat. no. 60-0101)

EQUIPMENT

- Sonicator with fine tip for chromatin fragmentation (Branson Sonifier S-450A, 101-063-198; may use any other brand)
- Rotating platform for IP at 4 °C
- Heated vortex for Eppendorf tubes
- PCR machine for 96-well plates or tubes (Eppendorf MasterCycler or any other brand)
- Real-time PCR machine (ABI 7900 or other brand)
- DNA gel-running apparatus
- Low-retention 1.5-ml Eppendorf tubes (most of the companies sell tubes treated with silicon)
- Nanodrop spectrophotometer (Nanodrop, cat. no. ND-1000)
- Biodyne membrane (KPL, cat. no. 60-00-50)

REAGENT SETUP

4-OHT Dissolve 5 mg in 1.3 ml ethanol for a final concentration of 10 mM. Before use, dilute sufficient amount from the 10 mM stock in ethanol (1:10) to a final concentration of 1 mM. Use 1 µl per 1 ml of medium for a final concentration of 1 µM. Stock solution should be stored at −20 or −80 °C for up to 6 months.

Cell lysis buffer I (Prepared fresh) 10 mM HEPES (pH 6.5), 10 mM EDTA, 0.5 mM EGTA and 0.25% Triton X-100. For 50 ml, add 500 µl HEPES (pH 6.5; 1 M), 1 µl EDTA (0.5 M), 125 µl EGTA (0.2 M) and 1.25 ml Triton X-100 (10% vol/vol). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh in the following concentrations: PMSF 1:200 (0.2 M), aprotinin 1:200 (5–10 TIU ml^{-1}), leupeptin 1:10,000 (10 mg ml^{-1}), phosphatase inhibitor cocktail 1 (1 ml per 100 ml of buffer) and phosphatase inhibitor cocktail 2 (1 ml per 100 ml of buffer).

Cell lysis buffer II (Prepared fresh) 10 mM HEPES (pH 6.5), 1 mM EDTA, 0.5 mM EGTA and 200 mM NaCl. For 50 ml, add 500 µl HEPES (1 M), 100 µl EDTA (0.5 M), 125 µl EGTA (0.2 M) and 2 ml NaCl (5 M). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh.

Nuclei lysis buffer (Prepared fresh) 50 mM Tris-Cl (pH 8.1), 10 mM EDTA and 0.5% SDS. For 10 ml, add 0.5 ml Tris-Cl (pH 8.1; 1 M), 200 µl EDTA (0.5 M) and 0.5 ml SDS (10% wt/vol). Protease and phosphatase inhibitors are added fresh.

IP dilution buffer (Can be prepared and stored for several weeks at 4 °C) 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl (pH 8.1) and 150 mM NaCl. For 50 ml, add 5 ml Triton X-100 (10%), 200 µl EDTA (0.5 M), 1 ml Tris-Cl (pH 8.1; 1 M) and 1.5 ml NaCl (5 M). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh.

IP wash buffer (RIPA), stringent (Can be prepared and stored for several weeks at 4 °C) 50 mM HEPES (pH 7.6), 1 mM EDTA, 0.7% deoxycholic acid, 0.5 M LiCl and 1% NP40. For 50 ml, add 5 ml HEPES (pH 7.6; 1 M), 100 µl EDTA (0.5 M), 0.35 g deoxycholic acid, 3.125 ml LiCl (8 M) and 5 ml NP40 (10% vol/vol). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh.

IP wash buffer (SDS-RIPA), less stringent (Can be prepared and stored for several weeks at 4 °C) 0.1% SDS, 1% NP40, 2 mM EDTA, 20 mM Tris-Cl (pH 8) and 0.5 M NaCl. For 50 ml, add 0.5 ml SDS (10% vol/vol), 200 ml EDTA (0.5 M), 1 ml Tris-Cl (pH 8; 1 M) and 5 ml NaCl (5 M). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh.

TE (Prepared fresh) 10 mM Tris-Cl (pH 8) and 1 mM EDTA. For 50 ml, add 0.5 ml Tris-Cl (pH 8; 1 M) and 100 µl EDTA (0.5 M). Cool the buffer to 4 °C. Protease and phosphatase inhibitors are added fresh.

Elution buffer (Prepared fresh) 0.1 M $NaHCO_3$ and 1% SDS. For 50 ml, add 0.42 g $NaHCO_3$ and 5 ml SDS (10% wt/vol).

PROCEDURE

Generation of DSBs; introduction and induction of I-*Ppo*I in human cells

1| After packaging the retroviral vector pBABE-HA-ER-I-*Ppo*I, use 4 ml of virus supernatant per 10-cm dish to infect the target cells. Add polybrene (1 µl ml^{-1} medium from a stock of 8 mg ml^{-1} in PBS). Place the cells in a humidified CO_2 incubator for 4–5 h.

▲ CRITICAL STEP Infection may be repeated to increase efficiency by removing the previous viral medium and addition of fresh 4 ml of virus supernatant for an additional 4 h.

2| At the end of the infection, add 6 ml of fresh growth medium on top of the viral supernatant and place cells in a humidified CO_2 incubator overnight (ON).
▲ CRITICAL STEP (optional) Before proceeding to Step 3, it is suggested to synchronize the cells to achieve a more uniform induction of the enzyme and limit the cells to nonhomologous repair. Most of the synchronization protocols[18,19] use some form of DNA damage, which is not applicable to this system. Serum synchronization can be done in MCF7 cells by washing the cells twice with serum-free medium and then adding medium with reduced serum level (0.1–0.5%) for 24–48 h.

3| Induce the enzyme by addition of 1 μM 4-OHT (1 μl ml^{-1} medium from a 4-OHT stock of 1 mM). Uninduced cells serve as zero time point, induced cells are harvested every 2 h for 6–7 time points (0–12 h).

4| Activation of DNA damage response can be detected after 2–4 h by western blotting with a Chk2 pT68 or p53 pS15 antibody (see ref. 2), by Southern blotting (see **Box 2**) or by ChIP, as described in the following steps.
? TROUBLESHOOTING

Preparation of samples for analysis of proteins bound to DSBs by ChIP

5| Crosslink bound proteins and DNA in the cells by directly adding 27 μl of formaldehyde (37% stock, wt/vol) per 1 ml of tissue culture media, thereby reaching a final concentration of 1%.
▲ CRITICAL STEP Generally use 5×10^6 to 1×10^7 cells per ChIP antibody per time point. Fewer cells can be used but usually results in a lower signal-to-noise ratio.
▲ CRITICAL STEP Add formaldehyde dropwise while gently agitating the plate on an orbital shaker. It is important not to create a local high concentration of formaldehyde.

6| Incubate adherent cells for 10 min at 25 °C (room temperature (RT)) with continued agitation.
▲ CRITICAL STEP The crosslinking time and formaldehyde concentration will determine the efficiency of the IP.

7| Stop the crosslinking reaction by adding glycine to a final concentration of 0.125 M (50 μl ml^{-1} medium from a 2.5 M stock). Incubate at RT for 5 min with continuous agitation.

8| Pour off media and rinse plates twice with cold PBS.

9| To obtain a nuclear pellet, scrape cells in cold PBS. Centrifuge cells (5 min, 750*g*, 4 °C) and wash pellet once with 10 ml of cold 1× PBS containing PMSF (5 μl ml^{-1} from a stock of 0.2 M). Centrifuge cells (5 min, 750*g*, 4 °C).
▲ CRITICAL STEP Steps 9–19 should be carried out on ice.
■ PAUSE POINT The cell pellet can be stored at −80 °C for several weeks.

10| Resuspend the cell pellet in 1 ml cell lysis buffer I including protease and phosphates inhibitors by pipetting up and down several times. The final volume of the cell lysis buffer I should be sufficient so that there are no clumps of cells. Incubate on ice for 10 min.

11| Centrifuge at 1,700*g* for 5 min at 4 °C to pellet the nuclei.

12| Aspirate the supernatant and resuspend the pellet in 1 ml cell lysis buffer II including protease and phosphatase inhibitors.

13| Centrifuge at 1,700*g* for 5 min at 4 °C.

14| Aspirate the supernatant and resuspend nuclei in 0.3–1 ml nuclei lysis buffer including protease and phosphatase inhibitors. Incubate on ice for 10 min.

15| Sonicate chromatin to an average length of about 1,000 bp. In between sonications, keep samples on ice for 1 min (the time and number of pulses will vary depending on the volume, tube and sonicator being used (calibration needed), cell type and extent of crosslinking. For MCF7 cell in a Branson 450 sonicator, it is suggested to do 7× 10 s sonications at level 3 output, constant).

16| Treat a 5 μl sample with RNaseA (1 μl from 10 mg ml^{-1} stock) by incubating for 15–30 min at 37 °C, and run on a gel (1% agarose in 1× TAE buffer) with the suggested loading dye (see Step 42) at 150 V for 15–30 min to see the size of the chromatin. A bulk of fragmented DNA should run at the 1,000-bp marker.

17| Centrifuge fragmented chromatin (from Step 15) at 20,000*g* for 10 min at 4 °C. Using a pipette collect the supernatant (fragmented chromatin) to a new, clean tube, being careful not to touch the pellet. This supernatant is the fragmented chromatin to be used in the following ChIP assay.

18| Dilute the fragmented chromatin 1:5 with the IP dilution buffer.
▲ CRITICAL STEP It is suggested to measure the protein concentration after dilution as well, using the standard assay available in most labs according to the manufacturer's instructions (Bradford or Lowry method kits are available from Bio-Rad). Knowing the protein concentration can save time in future experiments, as there is a correlation between DNA and protein amount for a specific cell line. Therefore, in future experiments, the measurement of protein will suffice to determine the amount of fragmented chromatin to be used for each IP (instead of using the method described in Steps 19–22).

19| To determine the DNA content of the chromatin sample, and therefore the volume of sample needed per IP reaction, take out 1% of the fragmented chromatin to serve as the input sample to measure the total DNA content of the chromatin sample. This input sample will also serve as a positive control in the PCR.
■ PAUSE POINT At this point, the rest of the chromatin sample (from Step 18) can be stored at −80 °C for up to several months.

20| Incubate the input sample from Step 19 at 65 °C after adding 1 µl of proteinase K (from 20 µg µl^{-1}) for 4 h to ON for reversal of the formaldehyde crosslinking.

21| Purify the input sample from Step 20 using QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, resuspend in 30 µl.
▲ CRITICAL STEP The creation and repair of I-*Ppo*I DSBs can alternatively be analyzed at this point using a real-time PCR repair defect assay (see **Box 1**), or ChIP can be used, as described in the following steps.

22| Read concentration of input DNA (from Step 21) without dilution; this amount equals 1% of the total DNA from the fragmented chromatin. Accordingly calculate the volumes for IP for each sample; take up to 1 ml chromatin for containing at least 50 µg DNA for IP. Adjust the final volume of each sample with IP dilution buffer.

Analysis of proteins bound to DSBs by ChIP

23| Prepare blocked proteinA beads: calculate the amount of beads needed for the ChIP assay according to the number of samples in the experiment (see Steps 25–26 and 30–31), wash the beads three times with 1 ml of IP dilution buffer, add 1 bead's volume of IP dilution buffer, 100 µg ml^{-1} tRNA, 1 mg ml^{-1} BSA and incubate on a rotating platform at 4 °C for 4–5 h or ON.
▲ CRITICAL STEP Do not use sonicated salmon sperm DNA for this assay as the I-*Ppo*I site occurs in the salmon DNA and results in higher background in the control IP.

24| Wash twice with 1 ml of IP dilution buffer. Add 1 bead's volume of IP dilution buffer to get a 50% suspension of beads volume/buffer volume.
▲ CRITICAL STEP Beads can be prepared (Steps 23 and 24) during the incubation of the sample in Step 20.

25| Preclear chromatin by adding blocked proteinA beads. This procedure clears nonspecific binding of proteins and DNA to the beads. Use 25–30 µl of beads per sample. Rotate at 4 °C for 1–2 h.

26| Centrifuge at 20,000*g* for 5 min. Transfer the supernatant and repeat preclearing for an additional 2 h.

27| Centrifuge at 20,000*g* for 10 min.

28| Transfer the supernatant to a clean tube for IP.

29| Add 1–3 µg of antibody to each sample; antibody amount should be in excess of the protein being immunoprecipitated. Be sure to include a nonimmune IgG ChIP sample as a background control.
▲ CRITICAL STEP In general, preferentially use a polyclonal antibody, as formaldehyde mildly denatures the protein and a polyclonal antibody has several epitopes on the target protein. It is suggested to test the suitability of each antibody to be used in a ChIP assay under formaldehyde conditions by western blotting. Varying the formaldehyde concentration and time of crosslinking will indicate the desired combination to be used in the ChIP.

30| Rotate at 4 °C for at least 3 h (ON is better). A volume of 30 µl of blocked proteinA beads may be added for the ON incubation.

31| Add 30 µl of blocked proteinA beads to each sample. Rotate at 4 °C for 3 h. Addition of the beads at this time point may reduce the background from nonspecific binding of DNA to the beads.

32| Centrifuge samples at 950*g* for 1 min at 4 °C.
▲ CRITICAL STEP Be sure not to use very high r.p.m./r.c.f. that will crush the beads.

33| Wash the pellets seven times with 1.4 ml IP wash buffer (RIPA or SDS-RIPA) and once with 1.4 ml TE. For each wash, aspirate the supernatant, add wash buffer to resuspend the beads, rotate the samples for 3 min at RT and then centrifuge at 950*g* for 1 min at 4 °C.
▲ CRITICAL STEP Efficient washing is critical to reduce background. RIPA buffer is more stringent than the RIPA-SDS buffer, but may result in low detection due to loss of the immunoprecipitated protein.

34| After the last wash, centrifuge and remove the last traces of the TE buffer.

35| Elute the antibody/protein/DNA complexes by the addition of 100 µl of elution buffer. Shake on a thermal mixer at 65 °C for at least 15 min. Centrifuge at 950*g* for 1 min at 25 °C. Transfer the supernatants to clean tubes.

36| Repeat and combine both elutions in the same tube.
▲ CRITICAL STEP Be careful not to carry over beads when transferring the elution supernatant.

37| Add 1 µl of high-concentration RNase A (10 mg ml^{-1}). Incubate samples at 37 °C for 1 h.

38| Add 2 µl of 1 M Tris-Cl (pH 8), 4 µl of 0.5 M EDTA (pH 8) and 1 µl of proteinase K (from 20 µg $µl^{-1}$) to each sample. Incubate at 55 °C for 1 h.

39| Incubate samples at 65 °C ON for reversal of formaldehyde crosslinking.

40| Spin down the condensate and use QIAquick PCR purification kit (Qiagen) to clean the DNA, according to the manufacturer's instructions. Resuspend in 30 µl of the final buffer.

PCR amplification of immunoprecipitated DNA and analysis by gel electrophoresis

41| To PCR-amplify the immunoprecipitated DNA, and monitor protein dynamics at the I-*Ppo*I DSB, use 1–2 µl of the ChIP DNA (from Step 40) and approximately 50 ng of the input sample DNA (from Step 21, as a positive control) for the PCR. Perform PCR in a 25-µl reaction (1 µl DNA, 1 µl primer (10 pmol each) and 23 µl H_2O on ready beads), using 32–36 cycles for a single site (95 °C, 30 s; 52.8 °C, 30 s; 72 °C, 30 s) and 26–28 cycles for the rDNA multiple sites (95 °C, 30 s; 55.6 °C, 30 s; 72 °C, 30 s). For rDNA multiple I-*Ppo*I site detection, use oligos 1B, and for detection of a single I-*Ppo*I site on chromosome 1, use oligos 2 (oligos 1B and 2 are listed in the Supplemental Table 1 in ref. 2). Use the input DNA (from Step 21) as a positive control and a PCR without DNA template as a negative control in parallel to the IgG ChIP sample (see Step 29), which serves as the background control.

42| Run the samples on a 2% EtBr agarose gel (1× TAE at 150 V for 15–30 min) with 6× DNA-loading buffer containing only xylene cyanol.
▲ CRITICAL STEP Xylene cyanol migrates at approximately 4 kb equivalence and the PCR products run at around 200–300 bp, which can be obscured by bromophenol blue, which migrates at 200–400 bp.
? TROUBLESHOOTING

43| Estimate the relative change in protein binding to the site of damage by comparing the PCR band intensity before (zero time point) and after induction of the enzyme (induction of DNA damage). Upon recruitment of proteins to the site of damage, band intensity of ChIPed DNA damage proteins is increased after the induction of the enzyme. Removal of histones from the break site results in reduction of histone ChIP band intensity after induction of I-*Ppo*I compared with zero time point.
? TROUBLESHOOTING

● TIMING
Day 1: Steps 5–9, crosslinking and harvesting cells: 1 h; Steps 10–14, obtaining nuclear pellet: 1 h; Steps 15–18, DNA shearing by sonication: 1 h
Day 2: Steps 19–22, input cleaning and IP sample preparation: 1 h; Steps 23–31, preclearing and IP: 4–6 h with intermissions
Day 3: Steps 32–39, wash and elution: 1–2 h
Day 4: Step 40, DNA cleaning: 30 min; Steps 41–43, PCR: 2–4 h

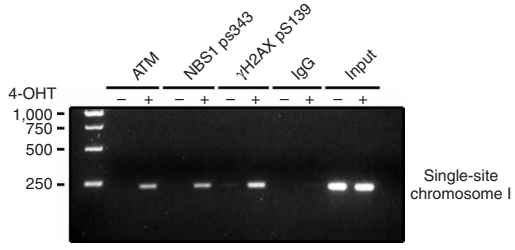

Figure 1 | Agarose gel picture of a ChIP assay for protein recruitment to DNA DSBs. MCF7 cells were infected with HA-ER-I-*Ppo*I and starved for 24 h in 0.1% serum. The cells were then either noninduced (–) or induced with 4-OHT (+) for 6 h. Following crosslinking and sonication, fragmented chromatin was used for IP with the indicated antibodies. IgG — non-specific background control, Input — positive PCR reaction control. Purified DNA was used in a PCR with oligos amplifying the single I-*Ppo*I site at chromosome I. PCR samples were electrophoresed on an EtBr agarose gel and visualized by a UV illuminator (Image Master VDS; Pharmacia Biotech).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Reason	Solution
4	Low or undetectable DNA damage response after enzyme induction	Problem with virus production or infection	Make sure packaging and target cells are growing and are not infected with mycoplasma
			Add a control virus that expresses a fluorescent marker to asses the packaging and infection efficiency (Step 1)
			Infect the cells twice (every 3–4 h) (Step 1)
			Extend the induction time with 4-OHT (Step 3)
42	No bands appearing in the PCR	PCR did not work	Run PCR reaction of input DNA as a positive control (Step 41)
			Increase PCR cycles until bands appear (Step 41)
43	No difference in specific PCR band intensity after damage	Antibody fails to IP after crosslinking	Test antibody on crosslinked cells extract by western blot to make sure it is suitable for a ChIP assay (use fragmented chromatin sample from Step 18)
		Formaldehyde concentration and time of crosslinking not suitable for specific protein IP	Vary formaldehyde concentration and crosslinking time until detection of IPed protein (Step 5)
		Low amount of fragmented chromatin used for IP	Use 5–10 million cells per IP or more than 50 μg DNA per IP (Step 1 or Step 22)
		High background in nonimmune IgG ChIP	Wash with more stringent RIPA buffer (see reagent setup) (Step 33)
			Reduce amount of beads used and time of IP, be sure to take the same amount of beads per IP—use cut tips and resuspend beads thoroughly (Step 31)
		Insufficient induction	Induce the enzyme for up to 24 h to reach a steady state of breakage/repair compared with uninduced cells (Step 3)

ANTICIPATED RESULTS

In general, 1 × 10 cm dish of MCF7 (90% confluence, 2 × 10^7 cells) should be sufficient for ChIP using 2–3 specific antibodies. An increase in PCR band intensity is detected when using antibodies for proteins relocated to the sites of damage, such as ATM, NBS1 pS343 and H2AX pS139 (see **Fig. 1**). A decrease in band intensity is detected on loss of histones at the site of the break. During a time course, sampling every 2 h for a total of 10 h, gradual increase of ATM and gradual loss of histone H2B should be detected at the site of the break.

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