A polycomb group protein, PHF1, is involved in the response to DNA double-strand breaks in human cell

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ABSTRACT

DNA double-strand breaks (DSBs) represent the most toxic DNA damage arisen from endogenous and exogenous genotoxic stresses and are known to be repaired by either homologous recombination or nonhomologous end-joining processes. Although many proteins have been identified to participate in either of the processes, the whole processes still remain elusive. Polycomb group (PcG) proteins are epigenetic chromatin modifiers involved in gene silencing, cancer development and the maintenance of embryonic and adult stem cells. By screening proteins responding to DNA damage using laser micro-irradiation, we found that PHF1, a human homolog of Drosophila polycomb-like, Pcl, protein, was recruited to DSBs immediately after irradiation and dissociated within 10 min. The accumulation at DSBs is Ku70/Ku80-dependent, and knockdown of PHF1 leads to X-ray sensitivity and increases the frequency of homologous recombination in HeLa cell. We found that PHF1 interacts physically with Ku70/Ku80, suggesting that PHF1 promotes non-homologous end-joining processes. Furthermore, we found that PHF1 interacts with a number of proteins involved in DNA damage responses, RAD50, SMC1, DHX9 and p53, further suggesting that PHF1, besides the function in PcG, is involved in genome maintenance processes.

INTRODUCTION

DNA double-strand breaks (DSBs) can be caused by both cell-intrinsic sources, such as replication errors or reactive oxygen species, and a variety of extrinsic factors, including ionizing radiation (IR) and radiomimetic chemicals. DSBs representing the most toxic DNA lesions, if left unrepaired, may cause cell death and genomic instability. Inefficient or inaccurate repair may lead to mutation and/or chromosome rearrangement, and predisposition to cancer (1–5). DSBs also represent obligatory intermediates of physiological DNA rearrangement processes taking place during the development and maturation of the adaptive immune system, V(D)J recombination and immunoglobulin (Ig) heavy-chain class switch recombination (CSR) (6). Therefore, defects in the repair of these DNA breaks can cause profound immuno-deficiencies (7).

Eukaryotes cells have evolved two major pathways for repairing DSBs, homologous recombination (HR) and nonhomologous end joining (NHEJ). Both pathways are conserved from yeast to mammals and function in complementary ways to repair DSBs (1,5,8). During HR, DSBs are repaired through a precise pathway that uses homologous sequence usually provided by the sister chromatid during replication as for template. In contrast, NHEJ is an error-prone repair pathway that joins ends together without the requirement for significant sequence homology (1,5,8). Once DSBs are produced, cells trigger a series of signaling pathway including cycle regulation, transcription, histone modification and apoptosis that have direct or indirect effect on DSB repair. Following DNA damage, the DNA damage sensors ATM/ATR and DNA-PK phosphorylate CHK1 and CHK2 to regulate cell cycle checkpoint, phosphorylate P53 to activate apoptosis signal pathway, phosphorylate H2AX and a number of proteins involved in DSB repair such as NBS1 and SMC1 (5,9). Besides phosphorylation of H2AX, recently, histone ubiquitinations, acetylations and methylations have been implicated in the DNA damage checkpoint and DSBs repair pathways (10). Although the last few years a wealth of new information has been produced about DSBs damage response and DNA repair, and many novel proteins involved in the process have been identified, the process still remains elusive.

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With the aim of identifying new factors involved in DSBs damage response and repair of mammalian cells, we screened a number of proteins involved in chromatin remodeling and regulation by using laser micro-irradiation system (11–13). PcG proteins are epigenetic chromatin modifiers involved in transcription regulation, maintenance of embryonic and adult stem cells and cancer development (14,15). PcG genes were first identified by their requirement for the maintenance of the stable repression of Hox genes during the development of Drosophila melanogaster and are highly conserved throughout evolution. In mammals, PcG genes are also implicated in Homeobox (Hox) gene regulation. Their biological activity lies in stable silencing of specific sets of genes through chromatin modifications. Recently, emerging evidence implicates the PcG proteins in cellular proliferation and tumorigenesis (15–18). Furthermore, overexpression of a PcG protein, EZH2, in breast epithelial cells reduced Rad51 paralogs both in the mRNA and protein levels which are required for proper HR DNA repair (19), and heterozygosity for mutations in either extra sex combs (Esc) or Enhancer of Polycomb [E[PC]] increases the lever of HR and enhances genome stability in somatic cells of D. melanogaster (20).

Laser micro-irradiation makes it possible to introduce various types of DNA damage at restricted regions in the nucleus of a single cell and to analyze the response of proteins to the damage with antibody by immuno-staining or with transfected GFP-tagged proteins under microscope in a real-time image. So far as laser light dose and exposed time are extremely limited, there is no effect of heat production. The major product of irradiation with UVA laser light is DNA damage, because UVA laser light is either absorbed directly by DNA or by photosensitizers around DNA creating radicals, which attack DNA. Therefore, major types of DNA damage produced by UVA laser irradiation are oxidative ones, and with increasing laser dose and by addition of photosensitizers, DNA single-strand breaks, DSBs and base damage can be produced effectively (11,12,21).

For the reason that both PcG proteins play important roles in tumorigenesis, and some of PcG proteins are shown by genetic analysis to be involved in DSBs damage response, we analyzed whether or not PcG proteins are directly involved in the response to DSBs. We found that PHD finger Protein 1 (PHF1) is recruited rapidly to DSBs sites, that is dependent on Ku70/Ku80, and demonstrated that PHF1 is associated with Ku70/Ku80, Rad50, DHX9, SMC1 and P53 besides PcG proteins. Furthermore, knockdown of PHF1 leads to X-ray sensitivity and increases HR frequency. These data suggest that PHF1 is involved in DSBs damage response and may play an important role in NHEJ and maintaining genome stability.

MATERIALS AND METHODS

Construction of plasmids for expression of various genes

Plasmids expressing human genes encoding KU70, KU80, XRCC4, LigaseIV, Artemis, XL, RAD52 and NBS1 were constructed by cloning cDNA amplified from HeLa cells. PHF1 (isoform 2) was amplified from a human cDNA clone (no. MGC: BC008834). We modified the multiple cloning sites of vectors EGFP-C1, EGFP-N1 to introduce various cDNAs attached with an in-frame XhoI or SalI site at the start and NotI site at the stop codons. Deletion fragments of PHF1 were generated by PCR amplification, and then cloned into vectors. All constructs were verified by sequencing.

Cell lines, culture and transfection

The following cell lines were used in this study: HeLa; V79B; XR-V15B (Ku80−/− CHO cell line); CHO9; XR-C1 (DNA-PKcs−/− CHO cell line); XR-1 (XRCC4−/− CHO cell line); 1022QVAP81 (NBS1-deficient human cell line); Flp-In-293 (Invitrogen), All cell lines were propagated in Dulbecco's modified-MEM (Nissui), supplemented with 1 mM t-glutamine and 10% fetal bovine serum at 37°C and 5% CO₂. For suspension culture, cells were grown in Joklik medium (Sigma) supplemented with 5% fetal bovine serum at 37°C in spinner flask. Caps of spinner flask should be tightly closed and density of cells was kept between 2 × 10⁶/ml. For UVA-laser irradiation, cells were plated in glass-bottomed dishes (Matsumani Glass) and transfected with expression vectors using fuGene6 (Roche), according to the manufacturer’s protocol.

Microscopy and UVA-laser irradiation

Fluorescence images were obtained and processed using a confocal scanning laser microscopy system (FV-500, Olympus). UVA-laser irradiation was used to induce DSBs in cultured cells as described previously (11–13). Briefly, cells in glass-bottomed dishes were micro-irradiated with a 405 nm pulse laser (Olympus) along a user-defined path. Laser was focused through a 40× objective lens and the treatment dose was controlled by number of scans used. A single laser scan at full power delivers about 1600 nW. Cells were treated with 10 nM 5-bromo-2-deoxyuridine (BrdU) during 24 h prior to irradiation. Given the low treatment doses and the wavelength used, the influence of the laser light on DNA is mainly indirect via photosensitization of natural or added (BrdU) during 24 h prior to irradiation. The number of DSBs was determined by comparison of that produced by X-Ray irradiation, based on the method reported before (48).

Immunofluorescence

Cells were fixed in cold methanol/acetone (1:1) for 10 min at −20°C and probed with mouse anti-PHF1 (1:30; M01, Abnova), mouse anti-γH2AX (1:400; jbw103, Upstate). The secondary antibody used was Alexa fluor 594 anti-mouse IgG (1:400; molecular Probes). Nuclear DNA was stained with 4’,6’-diamidino-2-phenylindole (DAPI; 0.5 μg/ml, Wako). Fluorescence microscopy was
performed using the same microscopy as used in laser micro-irradiation.

**Immunoblotting**

Cells were sonicated in lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% NP-40, 1 mM EDTA) supplemented with protease inhibitor (complete, EDTA-free, Roche) and cleared by centrifugation. Proteins were separated by SDS/PAGE, electroblotted and detected with the following antibodies: mouse anti-PHF1 (1:1000; M01, Abnova), mouse anti-Ku70 (1:3000; N3H10, Sigma), mouse anti-FLAG (1:3000; F-3165, Sigma), goat anti-actin (1:2000; I-19, Santa Cruz Biotechnology), mouse anti-P53 (1:1000; DO-1, Santa Cruz Biotechnology), rabbit anti-RbAP46 (1:1000; PA1-868, ABR) and rabbit anti-SMC1 (1:1000; BL308, Bethyl). The secondary antibodies used were from Santa Cruz Biotechnology: anti-rabbit IgG-HRP (1:3000, sc-2004), anti-mouse IgG-HRP (1:3000, sc-2005) and anti-goat IgG-HRP (1:3000, sc-2056).

**Stable cell lines**

Stable isogenic cell lines expressing either PHF1 or Ku80 tagged with a FLAG-HA were established using Flp-In system (Invitrogen), according to the manufacturer's protocol. Briefly, we modified pcDNA5/FRT vector system (Invitrogen) tagged with a FLAG-HA were established using Flp-In Stable isogenic cell lines expressing either PHF1 or Ku80 Stable cell lines

**Immunoprecipitation and nanoLC/MS/MS**

Cells were sonicated in lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% NP-40, 1 mM EDTA) supplemented with protease inhibitor (complete, EDTA-free, Roche). Lysates were incubated on ice in the presence of 20 μg/ml ethidium bromide (EtBr) for 1 h and cleared by centrifugation, and then the supernatants were collected. Immunoprecipitation was carried out with anti-FLAG M2 affinity gel (A2220, Sigma), according to the manufacturer’s protocol. Proteins were eluted by lysis buffer containing 100 μg/ml FLAG peptide (F3290, Sigma). Eluted proteins were detected by immunoblotting. For mass spectrometry, a large-scale suspension culture was carried out. Cells were grown in Joklik medium (Sigma) to 1 l kept between 2 and −6 × 10^6/ml cells were collected and lysated for immunoprecipitation. Eluted proteins were separated on a 12.5% polyacrylamide gel and stained using a Wako Mass silver stain kit.

Gel slippage was reduced by 100 mM DTT and alkylated by 100 mM iodoacetamide. After washing, the gels were incubated with trypsin overnight at 30°C. Recovered peptides were desalted by ZipTip c18 (Millipore). Samples were analyzed by nanoLC/MS/MS systems (DiNa HPLC system KYA TECH Corporation/QSTAR XL Applied Biosystems). Mass data acquisitions were piloted by Mascot software (30).

**Generation of a stable PHF1 knockdown cell line and colony formation assay**

Short hairpin siRNA constructs with 21 ribo-nucleotide sequences from PHF1. Oligo-ribo-nucleotides were synthesized and cloned into the psiRNA-h7SKzeo G1 vector (Invivogen). The PHF1 siRNA vector and control vector (psiRNA-h7SKz-Luc, Invivogen) were transfected into HeLa cells by fuGene6 (Roche). Stable transfectants were selected in the presence of 500 μg/ml zeocin. Knockdown of PHF1 was detected by immunoblotting and real-time PCR. Among the siRNA constructs only one sequence (122- GGACTGATGGGCTGCTATACT) had an effect on the expression of PHF1 in HeLa cell. For the colony formation assay, cells were plated in duplicate at 400 cells/6 cm dishes. Eight hours after plating, cells were irradiated with X-ray. Eight days later, colonies were fixed and stained with 0.3% crystal violet in methanol for counting. Three independent experiments were carried out and the standard errors were indicated with an error bar.

**Recombination assay**

Recombination assay was carried out as described previously (29). The pCMV3nls-I-sceI expression vector and the HeLa cell line, which contain a stably integrated copy of recombination reporter vector Scneo, were kind gifts from Dr Maria Jasin. Double strand siRNA for PHF1 and scramble were synthesized and purified by a Silencer siRNA construction kit (Ambion) (PHF1: 5'-AA GCTTTTCCTGCCCATATGGA. Scramble: 5'-AAGCT TACCGTCTTAAACGA). The HeLa cells in 3.5 cm dishes were transfected with 2 nM siRNA for PHF1 and control by using OligofectAMINE (Invitrogen). The cells were trypsinized and counted 48 h after transfection. With 2 μg pCMV3nls-I-SceI 1 × 10^6 cells were electroporated according to manufacturer's protocol (Digital Bio. 950 V, 35 ms and 2 pulse), and cells were plated in 10 cm dishes. Cells were selected in 1 mg/ml of G418 beginning 24 h after electroporation. The cloning efficiency was determined by plating 1000 cells in 10 cm dishes. Colonies were fixed and stained with 0.3% crystal violet in methanol for counting. Three independent experiments were carried out and the standard errors were indicated by an error bar.

**Real-time PCR**

Total RNA was extracted from cells by using a High Pure RNA Isolation Kit (Roche). cDNA were synthesized by using a First Strand cDNA synthesis Kit (Roche). Real-time PCR was performed in triplicate using Thermal Cycler Dice Real Time System (TaKaRa). The primer of PHF1 used in quantitative PCR were: 5'-TTACTGTTTACT GTGGTG GCC, 5'-GGTGATACAGGACAAGATGG.
RESULTS

PHF1 was recruited to DSB sites induced by laser micro-irradiation

We established a laser micro-irradiation system that can induce different types of DNA damage (SSBs, DSBs and base damage) in living cells and have investigated proteins responding to DNA damage (11–13). We found that proteins involved in NHEJ (Ku70, Ku80, XRCC4, Ligase4, XLF and Artemis) and HR factors (Rad52 and NBS1) are recruited to the sites by laser micro-irradiation (Supplementary Figure 1). In an effort to find new components in the cellular process responding to DSBs, we used this system to screen a group of GFP-tagged mouse PcG proteins, which are highly conserved throughout evolution and involved in transcription regulation, development, cell proliferation and tumorigenesis. We found that mPcl1, a component involved in PcG complex PRC2, was recruited to damage site immediately after irradiation with 405 nm laser in the presence of BrdU (data not shown). We observed no recruitment of other PcG proteins including mPcl2, mPcl3, PLZF, Scom1, Ring1A, Ring1B, Mel18 and EED (data not shown). We isolated a human homolog of mPcl1, PHF1 cDNA, attached it with GFP and expressed in HeLa cell. We found that both endogenous PHF1 and GFP-tagged PHF1 accumulated at the irradiated sites and co-localized with γH2AX or with GFP-tagged Ku70 only after irradiation with 500 scans in the presence of BrdU (Figure 1A and B, see Materials and methods section). Although GFP-PHF1 accumulates at the laser irradiated site after 100 scans with BrdU pretreatment, the accumulation was significantly weak (data not shown). As shown in Figure 1C, GFP-PHF1 accumulates rapidly at the irradiated sites within 1 min after irradiation and dissociates before 10 min. Since PHF1 dissociates very rapidly from DSBs sites, and NHEJ and HR factors remain at damage sites more than 2–4 h after irradiation (data not shown, 22), these data suggest that PHF1 is an early factor involved in DSBs damage response.

PHF1 is recruited to the sites of laser micro irradiation via two independent domains of the N-terminus

Tudor domain and the central region

PHF1 contains two zinc finger-like PHD (derived from the name ‘plant homeodomain’) domains and a Tudor domain (Figure 2A). PHD domain is found in a number of nuclear proteins and thought to be involved in chromatin-mediated transcriptional regulation (23); the Tudor domain is found in several RNA-binding factors and believed to be chromatin-binding domain. To determine which domain is responsible for the recruitment of PHF1 to laser-irradiated sites, we constructed several GFP-tagged PHF1 deletion mutants and analyzed the recruitment of these deletion mutants (Figure 2A). We found that three deletion mutants of PHF1 (aa 2–349, aa 81–349 and aa 340–567) are all recruited to irradiation sites (Figure 2B), suggesting that more than one domain are responsible for the recruitment of PHF1. More detailed analysis showed that both Tudor domain and central region (aa 340–431) are recruited to damaged sites, but PHD domain and C-terminal domain are not (Figure 2B). To further address the importance of Tudor domain and central region (aa 340–431) for the recruitment of PHF1 to laser-irradiated sites, we examined the recruitment of deletion mutants of PHF1 (aa 81–349 and 422–567). As expectedly, deletion mutants of PHF1 (aa 81–349 and 422–567) lacking both Tudor domain and central region (aa 340–431) showed no recruitment to damaged sites (Figure 2B). It has been reported that the two tandem Tudor domains of 53BP1 binds to methylated lysine 79 of histone H3 in response to DNA damage (24,25). Thus, our data show another example that Tudor domain plays an important role in response to DNA damage. Besides Tudor domain, the central region of PHF1 (aa 310–431) is also recruited to laser-irradiated sites, suggesting that PHF1 possesses complexity in response to DNA damage, which is not related to PHD domain.

Recruitment of PHF1 is Ku70/Ku80 dependent

We tested whether or not the recruitment of PHF1 to laser-irradiated sites is influenced by factors involved in NHEJ or HR. The idea is that, if PHF1 is involved in either NHEJ or HR pathway, its recruitment may be influenced by the absence of upstream factors of either pathway. PHF1 showed normal recruitment in human cell line 1022QVA (Nijmegen patient cells) mutated in NBS1, which is essential for HR (Figure 3D). PHF1 was also recruited to laser-irradiated sites in cells derived...
from the Chinese hamster ovary (CHO) cell lines of XR-C1 (DNA-PKcs-deficient), XR-1 (XRCC4-deficient) (Figure 3B and C). However, PHF1 was not recruited to laser-irradiated sites in XR-V15B (Ku80-deficient), while PHF1 is recruited in XR-V79B (Ku80 proficient) (Figure 3A). We generated a V15B cell line expressing His-ku80, in which expression of Ku80 as well as recruitment of Ku70 to laser-irradiated site is restored. In this cell line, recruitment of EGFP-PHF1 is also restored (Figure 3A). Thus, this indicates that PHF1 is recruited to the sites of DSBs in a Ku70/80-dependent manner. Like NHEJ factors, PHF1 is recruited to DSBs sites both in G1 and S/G2 phases (Figure 3E) without cell-cycle dependency.

**PHF1 is associated physically with ku70/ku80**

Having known that the recruitment of PHF1 is Ku70/Ku80-dependent, we tested whether or not PHF1 is associated with Ku70/Ku80 proteins. In order to check the interaction, we generated two human 293 cell lines stably expressing either PHF1 or Ku80 fusion protein containing FLAG-HA tags. For prevention of any DNA-dependent association, coimmunoprecipitation was carried out in the presence of ethidium bromide (26,27). PHF1 was immunoprecipitated from 293 cells extracts by using anti-FLAG antibody and the immunoprecipitated products were analyzed by western blotting with anti-Ku70 antibody. As shown in Figure 4A, Ku70 was communoprecipitated with Flag-HA-PHF1, but was not present in control immunoprecipitants from the extracts of cells transfected with a blank vector. Moreover, PHF1 also communoprecipitaited with FLAG-HA-Ku80 from extracts of cells transfected with FLAG-HA-Ku80, but was not present in control immunoprecipitants (Figure 4B). These results indicate that PHF1 is associated physically with Ku70/Ku80 in the cells.

**RNA interference of PHF1 causes cell sensitivity to X-Ray and increases HR frequency**

In light of the above data, we speculated that PHF1 might be involved in NHEJ. Either NHEJ or HR deficiency will lead cells to sensitivity to X-Ray or chemical reagent. In order to analyze this, we generated a stable PHF1 knockdown cell line using a vector-based siRNA approach. Characterization of the established cell line indicates that about 77% knockdown is achieved at mRNA level by quantitative RT–PCR (Figure 4C), but only 50% knockdown at the protein level (Figure 4D). Downregulation of PHF1 causes mild cell sensitivity to X-ray (Figure 4E). It is probably due to low efficiency of
PHF1 knockdown. It has been also shown that deficiency in NHEJ components such as Ku70, XRCC4 and DNA-PKcs results in increased levels of HR (28). Therefore, we tested whether or not downregulation of PHF1 affects HR using recombination assay described previously (29). As expectedly, downregulation of PHF1 increases HR frequency several folds (Figure 4F). These data strongly suggest that PHF1 is involved in NHEJ pathway and supports its efficiency.

PHF1 is associated with proteins responding to DNA damage besides Ku and those from Polycomb group (PcG)

If PHF1 is involved in damage response besides transcriptional regulation in human cell, PHF1 can be identified in a proteome other than PcG group and may have other interacting proteins of damage response than Ku. Therefore, a proteomics approach was undertaken to identify interacting proteins of PHF1 within human cell. A 293-cell line stably expressing PHF1 fused with FLAG-HA tags was used to immunoprecipitate the PHF1 complex by anti-FLAG antibody in the presence of ethidium bromide for exclusion of DNA-associated nonspecific proteins. Immunoprecipitated products were analyzed by SDS–PAGE, and then silver stained (Figure 5A); bands were excised, treated as described (30) and tryptic peptides were analyzed by mass spectrometry (Supplementary Table 1).

It was not surprising that Suz12, EZH2 and RBBP7 (RbAp46) were identified, consistent with a previous report about Drosophila Pcl protein. More interestingly, Rad50, SMC1, DHX9 and P53, which are proteins involved in DNA damage response and repair, were also identified with high confidence as coimmunoprecipitants of PHF1. The data of mass analysis were further confirmed by western blotting with SMC1, p53 and RbAp46 antibodies (Figure 5B). Rad50 forms a complex with Mre11 and NBS1 that is essential in maintaining DNA integrity by functioning in DSBs repair, meiotic recombination and telomere maintenance (31–33).

DISCUSSION

PcG proteins are epigenetic chromatin modifiers involved in gene silencing, cancer development and the maintenance of embryonic and adult stem cells. During screening of proteins responding to DNA damage by using laser micro-irradiation we found that mPcl1, a mouse homolog of polycomb-like protein (Pcl) of the D. melanogaster, accumulates at laser-irradiated site. It was previously shown that the Drosophila Pcl protein is associated with
PHF1 interacts with Ku proteins, suggesting that accumulation of PHF1 at DSBs requires Ku protein and the two domains have individual binding activities to DSBs. Further analysis is necessary to understand the mechanisms behind the recruitment of PHF1 to DSBs.

Besides Ku70/Ku80, PHF1 is also associated with various proteins involved in the response to DSBs and other genomic maintenance mechanisms, Rad50, DHX9, SMC1 and p53 (Figure 5). Rad50 forms a complex with Mre11 and NBS1 that is essential in maintaining DNA integrity by functioning in DSBs repair, meiotic recombination and telomere maintenance (31–33). SMC1 and SMC3 constitute the core of the cohesion complexes which play an important role in the repair of DNA DSBs from yeast to human, and SMC1 and SMC3 also interact with Mre11-Rad50 (41–44). DHX9 is a DNA- and RNA-dependent helicase associated directly with γ-H2AX (45). As a tumor suppressor, p53 plays a central role in the DNA damage response involved in multiple signaling pathways (46,47). Rad50, SMC1 and p53 were also demonstrated to be recruited to DSBs sites induced by laser micro-irradiation (44,48). The SMC1/SMC3 cohesion complex facilitates DSBs repair by HR and holds sister chromatids together locally at DSBs to allow strand invasion and exchange with the sister chromatid template (41–43). Defect in Rad50 influences phosphorylation of SMC1 and reduces HR (31,49). Moreover, another binding protein of PHF1, RbAp46 is a component of histone deacetylase complexes and is involved in chromatin remodeling, interacts with BRCA1 (50). These data suggest that PHF1 may play a role in HR as well. Further analysis is required to understand possible functions of PHF1 in the damage response. While we do not know the exact function of PHF1 in the DSBs repair yet, we demonstrated for the first time in mammalian cells that PHF1, as known as a PcG protein, is involved in DSBs response and possibly in its repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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