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Recruitment of the cohesin loading factor NIPBL to DNA double-strand breaks depends on MDC1, RNF168 and HP1γ in human cells

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ABSTRACT

The cohesin loading factor NIPBL is required for cohesin to associate with chromosomes and plays a role in DNA double-strand break (DSB) repair. Although the NIPBL homolog Scc2 is recruited to an enzymatically generated DSB and promotes cohesin-dependent DSB repair in yeast, the mechanism of the recruitment remains poorly understood. Here we show that the human NIPBL is recruited to the sites of DNA damage generated by micro-irradiation as well as to the sites of DSBs induced by homing endonuclease, I-PpoI. The recruitment of NIPBL was impaired by RNAi-mediated knockdown of MDC1 or RNF168, both of which also accumulate at DSBs. We also show that the recruitment of NIPBL to the sites of DNA damage is mediated by its C-terminal region containing HEAT repeats and Heterochromatin protein 1 (HP1) interacting motif. Furthermore, NIPBL accumulation at damaged sites was also compromised by HP1γ depletion. Taken together, our study reveals that human NIPBL is a novel protein recruited to DSB sites, and the recruitment is controlled by MDC1, RNF168 and HP1γ.

1. Introduction

Genome integrity is constantly challenged by DNA damage, resulting from a variety of genotoxic insults. DNA double-strand breaks (DSBs) are the most hazardous lesions because they can lead to cell death or cancer development if unrepaired or repaired incorrectly. DSBs trigger a conserved cellular response including DNA repair and cell cycle checkpoint to protect genome integrity. In fact, the defects of these cellular responses are observed in many tumor cells [1]. In response to DSBs, sensor proteins directly recognize damaged regions and recruit DNA damage response proteins to DSBs. The accumulation of these proteins occurs in a hierarchical fashion and is regulated by sequential signal transduction via protein–protein interaction and post-translational modifications. In human cells, DSBs are recognized by MRE11/RAD50/NBS1 complex, which triggers ATM-dependent phosphorylation cascade. At the vicinity of DSBs, ATM phosphorylates the variant histone H2AX. MDC1 binds directly to phosphorylated H2AX (γH2AX) via the BRCT domains, and then ATM-dependent phosphorylation of MDC1 recruits an E3 ubiquitin ligase, RNF8. RNF8-dependent histone ubiquitylation surrounding DNA lesions is further facilitated by another E3 ligase, RNF168. It is believed that such a DNA damage signaling cascade promotes further accumulation of proteins related to DNA repair or cell cycle checkpoint [2,3].

Cohesin, which is composed of four evolutionarily conserved subunit SMC1, SMC3, Rad21 and SA1/2 in human cells, is a chromosome-associated protein and mediates cohesion between replicated sister chromatids [4]. In *Saccharomyces cerevisiae,* the loading of cohesin to chromosome depends on the heterodimeric cohesin loading factor, Scc2 and Scc4 [5]. Scc2 is evolutionally conserved from yeast to human [6], and the human Scc2 homolog *NIPBL* was identified as a responsible gene for about 60% individuals with Cornelia de Lange syndrome [7,8]. Introducing mutation or depletion of homologs of NIPBL from yeast to human results in defects of sister chromatid cohesion [6], however the exact mechanism of cohesin loading to chromosomes by NIPBL is largely unknown.

Scc2/NIPBL has also a critical role in DNA damage response. In yeast, Scc2 is recruited to a HO endonuclease-induced DSB and facilitates DSB repair via Scc2-dependent accumulation of cohesin around the DSB [9–11]. In addition to Scc2, the loading of cohesin to the DSB requires DNA damage signaling factors, Tel1/Mec1 (orthologous to the mammalian ATM/ATR), phosphorylation of H2A (H2AX in mammals) and Mre11 (a subunit of MRE11/RAD50/NBS1 in mammals) [9]. In human, NIPBL was identified in comprehensive RNAi screenings for genes that are involved in sensitivity to ionizing radiation, and cells derived from Cornelia de Lange syndrome patients exhibited high frequency of chromosomal abnormalities following ionizing radiation [12–14]. However, unlike Scc2 in yeast, whether NIPBL accumulates around DSBs in human is unclear.

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To address this question, we investigate NIPBL accumulation in response to DSBs in human cells. In this study, we show the recruitment of NIPBL to sites of DNA damage generated by UV-C micro-irradiation in the presence of BrdU or homing endonuclease I-PpoI. Furthermore, we also demonstrate that the recruitment of NIPBL depends on MDC1, RNF168 and HP1.

2. Materials and methods

2.1. Cell culture

HT1080 cells and normal human diploid cells were cultured in a minimal essential Eagle's medium supplemented with 10% fatal bovine serum (ThermoTrace Ltd.) at 37 °C in a humidified atmosphere containing 5% CO_2. HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fatal bovine serum. HT1080 cells expressing MDC1 shRNA in a Doxycycline (Dox)-responsive manner were selected and cultured in a medium with 5 μg/ml puromycin. For generation of stable cell lines, HT1080 cells were infected with the HA-ER-I-PpoI retrovirus according to a previous report [15].

2.2. γ-irradiation, micro-irradiation and generation of DNA double-strand breaks by I-PpoI

Cells were irradiated with γ-rays from 137Cs source (PS-3100SB, Pony). Micro-irradiation assay was performed as described previously [16]. To generate I-PpoI-dependent DSBs in HT1080 cells infected with retroviruses expressing HA-ER-I-PpoI, cells were treated with 1 μM 4-OHT (Sigma).

2.3. Expression plasmid constructs and cell transfection

Total RNA was extracted from normal human diploid cells using ISOGEN reagent (Nippon Gene), and cDNA synthesis was performed using SuperScript III First-strand Synthesis SuperMix (Invitrogen) according to manufacturer's instructions. The 5535-bp NIPBL cDNA corresponding to Met-1 to Lys-1845 region (N-terminal NIPBL) and the 2889-bp NIPBL cDNA corresponding to Pro-1843 to Ser-2804 region (C-terminal NIPBL) were amplified with PCR using primers which introduce AsisI and PmeI sites (N-terminal NIPBL: forward primer, 5′-TAAAGCGATCGCCATGAATGGGGATATGCCCCATGT-3′; reverse primer, 5′-TTCTGTTTAAACAAGCTGAGGTCGACAAAGGA-3′), (C-terminal NIPBL: forward primer, 5′-CACCGCGATCGCCATGCCTCAGCTTGCTGAACAGTATTATGATATGC-3′; reverse primer, 5′-GATTGTTTAAACGCTGGAAGTCCCATCCTTGGC-3′). PCR products were digested with AsisI and Pmei and subcloned into the cloning site of pFN21A HaloTag CMV Flexi Vector (Promega). These constructs were verified by sequencing. The C-terminal NIPBL cDNA was excised with BbvcI and PmeI from the C-terminal NIPBL in pFN21A HaloTag CMV Flexi Vector and ligated to the N-terminal NIPBL in pFN21A HaloTag CMV Flexi Vector to generate the full-length NIPBL cDNA expression vector. Deletion fragments of NIPBL were generated using PCR amplification and cloned into pFN21A HaloTag CMV Flexi Vector. Point and deletion mutations were introduced into the vectors using QuikChange® II site-directed mutagenesis (Agilent) following manufacturer's instructions. Plasmid transfections to cells were performed using Lipofectamine 2000 (Invitrogen).

2.4. RNA interference

The MDC1 shRNA constructs was purchased from Open Biosystems (Cat #: RHS4696-99361078 and RHS4696-99706479). The MDC1 shRNA 22-mer targeting sequences is CGCCTTTCCACCCAAAGCCTAA. siRNA transfections were performed with 100 nM siRNA duplexes using Lipofectamine 2000 (Invitrogen). Control siRNA was purchased from Qiagen (AllStars Negative Control siRNA, Cat. 1027281). RNA oligonucleotides corresponding to RNF168 #1 (sense: 5′-GGCGAAGAGCGAUGGAGGATT-3′; antisense: 5′-UCCUCCAUCGCUCUUCGCCTT-3′), RNF168 #2 (sense: 5′-GACACUUUCUCCACAGAUAUU-3′; antisense: 5′-UAUCUGUGGAGAAAGUGUCUU-3′), MDC1 (sense: 5′-GGAUCACACAAAGAUUAGATT-3′; antisense: 5′-UCUAAUCUUUGUGUGAUCCTT-3′), HP1α #1 (sensen: 5′-CACAAAUUGUGAUAGCAUUTT-3′; antisense: 5′-AAUGCUAUCACAAUUUGUGTT-3′), HP1α #2 (sense: 5′-GCUUUGAGAGAGGACUGGAACTT-3′; antisense: 5′-GUUCCAGUCCUCUCUCAAAGCTT-3′), HP1β #1 (sense: 5′-AGCUCAUGUUCCUGAUGAATT-3′; antisense: 5′-UUCAUCAGGAACAUGAGCUTT-3′), HP1β #2 (sense: 5′-GACUCCAGUGGAGAGCUCAUGTT-3′; antisense: 5′-CAUGAGCUCUCCACUGGAGUCTT-3′), HP1γ #1 (sense: 5′-AUCUGACAGUGAAUCUGAUTT-3′; antisense: 5′-AUCAGAUUCACUGUCAGAUTT-3′) and HP1γ #2 (sense: 5′-GAGGCAGAGCCUGAAGAAUTT-3′; antisense: 5′-AUUCUUCAGGCUCUGCCUCTT-3′) were synthesized (Gene Design Inc., Japan).

Methods of chromatin immunoprecipitation-quantitative PCR, immunofluorescence staining, Halo Tag ligand labeling, western blot analysis, reverse transcription-PCR and co-immunoprecipitation assay are presented in the Supplementary data.

3. Results

3.1. Recruitment of NIPBL to sites of DNA damage

To examine NIPBL accumulation at DNA damage sites in human cells, we applied UV-C irradiation through a micro-porous filter in the presence of photo-activating reagent BrdU to create local DNA damage [16], because we observed no recruitment of Halo-tagged NIPBL to DSBs generated by conventional γ-rays under the fluorescence microscopic technique (data not shown). Human fibrosarcoma cell line, HT1080, was transfected with the plasmid encoding Halo-tagged full-length *NIPBL* gene (Supplementary Fig. 1). We observed localized signals of Halo-NIPBL, which were colocalized with γH2AX foci as well as those of MDC1 or 53BP1 after UV-C micro-irradiation (Fig. 1A), suggesting that NIPBL is recruited to DSB sites.

In order to confirm that NIPBL accumulation was independent of UV-damage, such as cyclobutane pyrimidine dimers and (6-4) photoproducts, we took advantage of the I-PpoI system to visualize NIPBL localization to clustered DSBs in the region of ribosomal DNA (rDNA) repeats [15]. The homing endonuclease I-PpoI recognizes a 15 base pair sequence and cleaves endogenous DNA target sites containing 28S rDNA in the human genome [15]. In human cells, rDNA genes, which are composed of 300–400 copies, are tandemly repeated and located on different five chromosomes [17].

We established a stable cell line, HT1080 expressing HA-ER-I-PpoI, and confirmed phosphorylation of ATM in response to 4-hydroxytamoxifen (4-OHT) (Supplementary Fig. 2). Furthermore, we observed foci formation of 53BP1 around Nucleophosmin, which is a surrogate maker for a nucleolus, and induction of γH2AX near the I-PpoI recognition sequence in the region of 28S rDNA at 6 h after addition of 4-OHT by chromatin immunoprecipitation assay (Fig. 1B, C). These data suggest that DSBs occur in the region of rDNA in an I-PpoI-dependent manner in this cell line. To investigate whether NIPBL accumulates at I-PpoI-induced DSBs, cells were transfected with the plasmid encoding Halo-tagged full-length *NIPBL* gene, and localization of NIPBL to DSBs were detected using anti-Halo antibody or ligand that binds covalently to Halo-tag. As shown Fig. 1D, we observed colocalized signals of NIPBL with γH2AX, MDC1 or 53BP1 foci at 6 h after addition of 4-OHT.