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2023년 2월

박사학위 논문

PRMT5 regulates Homologous Recombination Repair through CtIP methylation

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의과학과

서 관 우

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PRMT5에 의한 CtIP 메틸화 연구

2023년 2월 24일

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이 논문을 이학 박사학위신청 논문으로 제출함

2022년 10월

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국문초록

PRMT5에 의한 CtIP 메틸화 연구

서 관 우

지도교수 : 이 정 희

조선대학교 일반대학원

의과학과

DNA는 수많은 유전 정보를 포함하고 있지만 매일 내/외인성 요인에 의해서 손상을 받고 있다. 하지만 DNA의 무결성을 유지하기 위한 수많은 단백질들이 세포내에 있다. DNA 손상은 pyrimidine dimers, DNA crosslinks, base oxidation, replication stress, base oxidation, sing‐strand break (SSB), and double‐strand break (DSB)와 같은 몇 가지 유형이 있다. 이 손상들은 염기 절제 복구,

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불일치 복구, 핵산염기 절제 복구, 비상동말단결합, 상동재결합 등에 의해 복구된다. 복구되지 않은 SSB는 DNA 복제를 통해서 DSB가 유도될 수 있기 때문에 DSB가 가장 최악의 DNA 손상이다. DSB를 복구하는 방법에는 상동재결합과 비상동말단결합 두 가지가 있다. 오류가 없는 복구 기전인 상동재결합은 BRCA1이, 오류 발생이 쉬운 복구 기전인 비상동재결합은 53BP1이 각각 중재한다. 상동재결합은 ATM이 활성화되고 나서 순차적으로 RNF8, RNF168, BRCA1, CtIP, Mre11-Rad51-NBS1 (MRN) 복합체가 DSB 병소로 recruit된다. 상동재결합 기전에서 DNA 말단 절제는 중요한 단계이고 개시 (initiation), 신장 (elongation), 확대 (extension) 이 세 가지 단계로 진행된다. DNA 말단 절제가 시작되기 전에 53BP1은 DNA 말단 절제를 막고 있기 때문에 DSB 병소로부터 재 이동되어야 한다. 먼저 53BP1이 재 이동된 후 endonuclease인 Mre11은 DNA 말단으로부터 300 - 400 nucleotide 떨어진 5'가닥에 nick을 만든다. 두 번째 단계에서는 exonuclease인 Mre11이 이전에 만들었던 nick으로부터 DSB 병소 방향으로

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DNA를 절제한다. 마지막으로, EXO1/BLM/DNA2가 3'돌출부의 길이를 확대한다. DNA 말단 절제를 진행하기 위해서 CtIP는 중요한 요소이다. 인산화된 CtIP는 Mre11 endonuclease의 활성(T847)과 BRCA1과의 결합(S327)을 활성화시킨다. 우리는 효모단백질잡종법 (Y2H)를 통해서 PRMT5가 CtIP의 새로운 결합 파트너라는 것을 발견하였다. PRMT5는 기질을 메틸화, 특히 대칭적 디메틸화 (SDMA)를 촉진한다. 우리는 PRMT5가 CtIP의 새로운 조절자라는 것을 증명하기위해 많은 실험을 수행하였다. 우리는 CtIP는 PRMT5의 TIM 도메인에, PRMT5는 CtIP의 801 - 897a.a 부분에 결합한다는 것을 발견하였다. 추가적으로 우리는 PRMT5가 CtIP가 대칭적 디메틸화 (SDMA)를 촉진한다는 것을 PRMT5 저해제 (EPZ105666)와 MEP50 (PRMT5의 효소활성에 필수)을 목표로 하는 siRNA를 사용하여 확인하였다. 게다가, 우리는 LC - MS/MS를 통해 예상되는 8개의 대칭적 디메틸화 (SDMA) 위치를 확인하였고, 이 모든 위치에서 arginine을 alanine으로 돌연변이화를 진행하여 대칭적 디메틸화 (SDMA)가 억제된 단백질이 발현되는 세

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포는 CtIP의 recruitment와 상동재결합 효율이 감소한다는 것을 발견하였다. 종합해보면, 우리는 PRMT5에 의한 CtIP의 대칭적 디멜틸화 (SDMA)는 상동재조합 복구기전에 필수라는 것을 제안한다.

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INTRODUCTION

1. DNA damage and risk of DNA damage

DNA has plenty of genetic information, and the translated proteins from DNA have diverse and specific functions, respectively. Although only a base on DNA is changed to the others, the protein can be lost its function by translating protein including the wrong amino acid. Thus, DNA has to be maintained integrity. But, DNA is damaged every day by endogenous sources (deamination, DNA oxidation, DNA alkylation of DNA, and reactive oxygen species (ROS)) or exogenous source (ionizing radiation (IR), ultraviolet (UV) light, and various chemicals) [1-4], and repaired by DNA repair systems, such as homologous recombination (HR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) [5]. If DNA damage were not repaired, it affects replication or transcription and activates signaling events that alter cell physiology, such as metabolic deregulation, cellular senescence, autophagy, and mitochondrial dysfunction

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[6]. So, unrepaired/misrepaired DNA damage can result in apoptosis, necrosis, and cancer. [7−10]. The human who has a mutant gene, such as Lamin A/C (LMNA), BAF nuclear assembly factor 1 (BANF1), RecQ‐like helicase 4 (RECQL4), BLM RecQ like helicase (BLM), Xeroderma Pigmentosum group A (XPA), and Ataxia telangiectasia mutated (ATM), have genome instability diseases, such as Hutchinson‐Guilford progeria syndrome (associated LMNA), Nestor‐Guillermo progeria syndrome (associated BANF1), Rothmund−Thomson syndrome (associated RECQL4), Bloom syndrome (associated BLM), Xeroderma pigmentosum (associated XPA), and Ataxia telangiectasia (associated ATM).[6, 11−32]. All of these genes are associated with DNA instability and they accelerate aging. Therefore, damaged DNA should be well−repaired through the DNA repair system to prevent DNA instability

2. The type of DNA damage

There are several types of DNA lesions they are pyrimidine dimers, DNA crosslinks, base oxidation, replication stress, single‐strand break (SSB), and double‐strand break (DSB) [33, 34].

Pyrimidine dimers are induced by UV, known as a major genotoxic agent

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in prokaryotic and eukaryotic cells. They are most frequently led from the covalent bonding of two adjacent pyrimidine bases by UVA or UVB. It results in C→T transversion at TC sites and CC→TT tandem mutations. These UV-induced DNA damages are repaired by the nucleotide excision repair (NER) pathway [35].

DNA crosslinks are occurred by crosslinking agents, such as nitrogen mustard, mitomycin, psoralen, and platinum. These agents may react with one or both strands of DNA to form DNA mono-adducts, DNA-protein crosslinks, intra-strand crosslinks, and inter-strand crosslinks (ICL). The resolution of ICL is important among crosslinks because they block DNA replication and transcription that is required DNA strand separation. If these ICLs are failed to remove from DNA, it can inhibit cell cycle progression and induce cell death. The multiple DNA repair pathways participate in the repair of ICLs, and a problem of them can incompletely repair ICLs [36, 37].

DNA is continuously oxidized by reactions with reactive oxygen species (ROS), such as hydroxyl radical, singlet oxygen, peroxynitrite, hydrogen peroxide, and superoxide. They are generated from endogenous processes,

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like metabolism and inflammation, and exogenous factors, like UV radiation, air pollution, and tobacco smoke. All DNA bases can be oxidized, guanine of them is a much more frequently oxidized DNA base. Guanine can be transformed to 8 - oxoG, oxazolone (Ox), 5 - guanindinohydantoin (Gh), spirodihydantoin (Sp), 2,2,4 - triaminooxazolone (Oz), dehydroguanidinohydantoin (DGh), and imidazolone (Iz). by oxidation. These oxidations of guanine lead G to T, and G to C transversions. These problems are fixed by the base excision repair pathway, but unrepaired oxidative DNA damage rises the possibility of neurodegenerative disease, cardiovascular disease, inflammation, aging, and even cancer[38].

Replication stress is caused by faulty DNA replication, which can cause mutations or replication blockage. They can lead to breakage, rearrangement, and the mis - segregation of chromosomes. Prolonged replication stress can lead to a stalled replication fork. They can contribute to DNA damage, mutation, and disease. Failure to resolve stalled replication fork by the defect of some proteins is associated with several diseases, like bloom syndrome, seckel syndrome, and Werner syndrome. [34, 39, 40]

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These damage lesions mentioned above cause SSBs during resolving them. SSBs are fixed by BER, NER, or MMR. The worst critical thing of DNA lesions is DSB because unrepaired SSBs can lead to DSBs through replication [33, 41–43]. The pathway to repair DSBs exists two mechanisms, they are HR and NHEJ [44, 45]. HR as an error‐free DNA repair system repairs DSBs using the sister chromatid as a repair template in S/G2 cells, whereas NHEJ as an error‐prone DNA repair system repairs DSBs by direct ligation of DNA ends without sister chromatid in G1 cells[46–48]. Therefore, HR is essential for maintaining the stability and homeostasis of the genome.

3. Homologous recombination pathway

The DNA damage response (DDR) is an elaborate system for fixing damaged DNA, and it is contributed by a variety of cellular events and many DNA repair factors. DDR is begun by the activation of the ataxia telangiectasia mutated (ATM) and the ataxia telangiectasia and rad3‐related (ATR), which are kinase, being able to phosphorylate substrate. They trigger DNA repair and activate apoptosis and checkpoint through the mechanisms, associated with p53 [49]. ATM and ATR kinases are the starter protein in the DDR pathway. Firstly,

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ATM is autophosphorylated at S1987 in DDR [50]. This activated ATM is accumulated at DNA lesions and phosphorylates H2AX at S139 [51]. The mediator of DNA damage checkpoint protein 1 (MDC1) is recruited to phosphorylated H2AX (γ - H2AX) [52]. The ring finger protein 8 (RNF8) and the ring finger protein 168 (RNF168) are sequentially recruited to the DNA lesion after phosphorylation of MDC1 by ATM [53, 54]. RNF8 and RNF168, act as an E3 ubiquitin ligase, ubiquitylate H2A and H1 for facilitating recruitment of breast and ovarian cancer susceptibility protein 1 (BRCA1), p53-binding protein 1 (53BP1), and the other DSB repair factors [51, 54-56]. Next, the recruitment of 53BP1 and BRCA1 depends on the cell cycle, 53BP1, at the G1 phase cell, and BRCA1, at the S/G2 phase cell, are recruited to DNA lesion [57, 58].

The progression of DNA repair between BRCA1-mediated HR and 53BP1-mediated NHEJ is following the cell cycle. The difference between HR and NHEJ is the existence of the sister chromatid [59]. All repair pathways are regulated for minimizing genomic instability. DNA end resection is considered a DSB repair pathway choice. DNA end resection is enhanced for homologous recombination. DNA end resection functions in three steps,

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initiation, elongation, and extension step. In the first step, Mre11 – Rad50 – NBS1 (MRN) complex, specifically meiotic recombination 11 homolog A (Mre11), initiates end resection by nicking the 5' strands 300~400 nucleotides away from the end. In the initial step of the end resection, 53BP1 has to be repositioned from the DNA lesion for promoting DNA resection because 53BP1 and its effector proteins block DNA resection. For the repositioning of 53BP1, it should be de – phosphorylated, this is catalyzed by BRCA1 for fertilizing HR [60, 61]. Consequently, the role of BRCA1 and 53BP1 antagonize each other in DDR. In the second step, the DNA lesion is elongated from the nick to the DSB (3'→5' direction) by Mre11, stimulated by phosphorylation of CtIP at T847 [47, 62, 63]. Finally, the length of the 3' overhang is extended by exonuclease 1 (EXO1) or Bloom syndrome RecQ helicase (BLM) and DNA replication helicase/nuclease2 (DNA2) [64, 65]. In progressing end resection, replication protein A (RPA) is recruited to single – strand DNA (ssDNA) after phosphorylation of RPA at S4 and S8 [66]. Next, bound RPA to ssDNA is changed to RAD51 by BRCA2 [67]. Then, the DNA legion is fixed through HR using a sister chromatid.

4. CtIP

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CtBP interacting protein (CtIP) functions in largely three areas. there are transcription, DSB repair, and replication stress [68]. CtIP forms complexes with CtBP, BRCA1, LIM-domain only protein 4 (LMO4), retinoblastoma protein (Rb), Nibrin or Nijmegen breakage syndrome protein 1 (NBS1), and exonuclease (EXO1). [69-73]. The CtIP complexes, combined with them, have different functions, respectively. In transcription, the CtIP-Rb complex alleviates repression of *cyclin D1*, an important factor in the control of cell cycle progression, thus promoting G1/S transition [74]. On the other hand, CtIP blocks the G1/S transition by binding to the promoter and inducing the expression of the *p21* in DDR [75]. In addition, the CtIP-CtBP-BRCA1 complex could relieve repression of *p21* and *GADD45A* [76]. Conversely, CtIP-LMO4-BRCA1 complex blocks BRCA1-mediated transcription [70]. In DNA repair, the CtIP-BRCA1 complex prevents the accumulation of 53BP1 and promotes the relocation of 53BP1 to promote HR, when cells face DNA repair choice [60, 77]. In addition, phosphorylation of CtIP controls DNA end resection through the interaction with BRCA1, NBS1, and DNA2. [60, 72, 73, 78]. Similarly, the interactions of CtIP, for example, Mre11, EXO1, DNA2, and BRCA1, are also implicated in replication stress [79, 80]. The phosphorylation

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of CtIP stimulates the activity of Mre11 endonuclease (via pT847) and the interaction with BRCA1 (via pS327). Additionally, CtIP also interacts with FANCD2 for its relocation to damaged replication forks [81]. In this damaged lesion, these complex triggers off the fork restart and block new origin firing during recovering damaged replication forks [81]. Taken together, CtIP plays a critical role in DNA end resection.

5. PRMT5

The protein arginine methyltransferase 5 (PRMT5, also known as Hsl7, Jbp1, Skb1, Capsuleen, or Dart5) is one of nine protein arginine methyltransferases (PRMTs). PRMTs catalyze methylation of substrate arginine residues from S-adenosyl methionine (SAM, methyl donor) [82]. These nine PRMTs are classified into 3 groups, type Ⅰ, type Ⅱ, and type Ⅲ [83]. Type Ⅰ PRMTs, correspond to PRMT1, 2, 3, 4, 6, and 8, catalyze ω-N^G-monomethylarginine (MMA), and asymmetric ω-N^G, N^G-dimethylarginine (ADMA). Type Ⅱ PRMTs, correspond to RPMT5, and 9, catalyze MMA and symmetric ω-N^G, N'^G-dimethylarginine (SDMA). Type Ⅲ

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PRMTs, which correspond to RPMT7, catalyze only MMA [83]. PRMT5 belongs to type Ⅱ. PRMT5 functions in transcription, maintenance of the Golgi architecture, DDR, histone modification, transcriptional elongation, splicing, and translation [82, 84–89]. PRMT5 regulates DNA repair by methylation of histone and nonhistone proteins [90, 91]. The way to regulate DNA repair by methylation of histone protein is through the regulation of transcription of the DNA repair gene. For example, PRMT5 activates *RNF168* transcription by maintaining proteostasis of H2AX in the promoter, then expressed RNF168 (E3 ubiquitin ligase, stabilizer) ubiquitinates H2AX for stabilizing H2AX by blocking the interaction between H2AX and SMURF2 (E3 ubiquitin ligase, destabilizer). Whereas, H2AX is degraded by interaction easily between H2AX and SMUF2 because RNF168 is unexpressed by inactivated *RNF168* promoter in PRMT5 deficient cells [91]. In summary, PRMT5 regulates DDR by enhancing and positively regulating the interaction between H2AX and the transcription factor RNF168, which competes with the interaction of SMURF2. The DDR molecular mechanism is regulated through the non‐histone proteins post‐translational modifications (PTMs). For example, the displacement of 53BP1 is regulated by the acetylation of H4 at K16 by TIP60 getting activity as acetyltransferase by

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RUVBL1. RUVBL1, the cofactor of TIP60, can activate TIP60 after its methylation at R205 by PRMT5. Displacement of 53BP1 is important for DNA repair choice. If PRMT5 is deficient, NHEJ will be activated because the TIP60 – RUVBL1 complex is inactivated [60, 92].

In this study, we inceptively found that PRMT5 interacts with CtIP through yeast two – hybrid screening (Y2H), and PMRT5 regulates homologous recombination through regulating CtIP methylation.

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MATERIALS AND METHODS

1. Cell culture and treatment

HeLa and U2OS cells were obtained from ATCC and grown in DMEM (Dulbecco's modified Eagles' medium, Gibco) supplemented with 10% fetal bovine serum (FBS, YOUNG IN FRONTIER) and antibiotics at 37℃ in a 5% CO2 incubator. U2OS DR-GFP (pHPRT-DRGFP expressing stable cell lines based on U2OS cells) cells were grown in DMEM medium supplemented with 10% FBS and 1% antibiotics at 37℃ in a 5% CO_2 incubator. To induce DNA double - strand breaks, cells were irradiated with 10 Gy from 137Cs source (Gammar cell 3000 Elan radiograph, Best Theratronics) and recovered several times in a 37°C incubator.

2. siRNA transfection

HeLa and U2OS cells were transfected with siRNA oligonucleotide

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duplexes against PRMT5, CtIP, or MEP50 using RNA IMAX (Invitrogen) according to the manufacturer's instructions. The siRNA sequences targeting PRMT5 (PRMT5 siRNA #6: 5'- GCCAUCUAUAAAUGUCUGCUA-3', PRMT5 siRNA #7: 5'- AGGAAUUAGUGCUGGAUUU-3'), CtIP (CtIP siRNA: 5'-CAGAAGGAUGAAGGACAGUUU-3'), and MEP50 (MEP50 siRNA: 5'-GGACUCUGUGUUUCUUUCA-3') designed and synthesized for transient transfection.

3. Immunoprecipitation assay

For immunoprecipitation assay, cells were lysed in NP-40 buffer [50mM Tris PH 8.0, 150mM NaCl, 1% NP-40, and 5mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche Diagnostic Corp)] and incubated at 4 ℃ for 30 minutes. These lysates were cleared by centrifugation and incubated with antibodies or normal IgG (control) at 4℃ for 24 hours. And then, protein A/G

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plus-agarose beads (Santa Cruz Biotechnology), G-sepharose, or A sepharose (GE Healthcare) were added to the lysates, and beads mixtures were incubated at 4℃ for 4 hours with rotating. The beads were washed three times in NP-40 buffer and then samples were boiled in 2 × SDS loading buffer. Then, samples were analyzed by western blotting using the appropriate antibodies.

4. Western blot analysis

For immunoblotting analysis, cells were collected and lysed in RIPA buffer [50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate(NADOC), 0.1% sodium dodecyl sulfate(SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche Diagnostic Corp)]. Protein concentrations were measured using the Bradford assay (Bio-Rad). The same amounts of protein were separated by 6-15% SDS-PAGE and then transferred to PVDF

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(polyvinylidene difluoride) membranes (Millipore, Bedford, MA, USA). PVDF membranes were followed by blocking with 5% milk in TBS-T (10mM Tris-HCl (pH 7.4), 150mM NaCl, and 0.1% Tween-20) and incubated overnight at 4℃ with primary antibodies (1:1000). The membranes were washed 3 times for 10 minutes with TBS-T and then incubated for 2 hours with peroxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were washed 3 times for 10 minutes and developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

5. Clonal survival assay

Control cells and PRMT5 - depleted cells were treated with various doses of a damaging agent (IR) and split into the 60mm dish. After 10 - 14 days, viable cells were fixed with 4% paraformaldehyde and stained with 1% methylene blue in 20% ethanol, and then counted. Every experiment was

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repeated at least three times.

6. Immunofluorescence microscopy

For immunofluorescence studies, cells were seeded onto glass coverslips and irradiated with 10 Gy of ionizing radiation (IR). Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes and ice-cold 100% methanol for 5 minutes, then permeabilized with 0.5% Triton-X for 15 minutes and blocked in 5% bovine serum albumin for 1 hour. Cells were incubated with indicated primary antibodies overnight at 4℃, then incubated Alexaconjugated secondary antibodies for 2 hours at room temperature. After washing, the cells were mounted using Vectashield mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken using confocal microscopy (Zeiss LSM510 Meta: Carl Zeiss) and analyzed with ZEN software.

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7. Yeast two - hybrid screening

Yeast two - hybrid screening of CtIP was performed in yeast PBN204 strain containing 3 reporters (URA3, lacZ, and ADE2) under the control of different GAL promoters with the use of the PanBioNet protocol (PanBioNet corp., Korea).

8. Homologous recombination assay (DR-GFP assay)

To measure the HR repair, stable cell lines expressing DR-GFP reporters were generated by transfection using RNAIMAX (Invitrogen). U2OS-DR-GFP cells were transfected with control or PRMT5 siRNA and then transfected with 0.5 μg of I-SceI-expressing vector. After 72 hours, cells were fixed with 4 % paraformaldehyde and stained 5 μg/ml Hoechst (Sigma) for 1 hour. The images were shown at x20 magnification using an inverted fluorescence microscope (IN Cell Analyzer 2500 HS). The data are presented as the mean

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± SD. value in three independent experiments.

9. In situ proximity ligation assay (PLA)

For PLA assay, cells were seeded onto glass coverslips. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and then blocked using Duolink blocking buffer for 1 hour. Two primary antibody incubation performed overnight at 4℃ with gentle agitation. Samples were washed twice with 0.05% Tween-20 containing PBS, and then incubated with PLA probes MINUS and PLUS (DUO92005, Sigma) for 1 hour at 37℃. After washing using buffer A (0.01M Tris, 0.15M NaCl, 0.05% Tween-20, pH7.4), the probes were ligated with two other circle-forming DNA oligonucleotides by ligation-ligase solution for 30 minutes at 37℃. After washing using buffer A, add the amplification-polymerase solution and incubation over 90 minutes at 37℃. After washing using buffer B (0.2M Tris, 0.1M NaCl, pH7.5) for 10

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minutes twice, the samples were dried for 10 minutes in the dark RT. Then the cells were mounted using Vectachield mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were taken using a fluorescence microscope (IN Cell Analyzer 2500 HS).

10. Chromatin Immunoprecipitation (ChIP)

ChIP was performed using commercially available SimpleChIP Assay Kits (Cell Signaling) according to the manufacturer's instructions. DNA-bound protein was immunoprecipitated using anti-CtIP (Active Motif, mouse, 61141) and anti-Flag (sigma, mouse,) antibody or mouse IgG (Active Motif, Rixensart, Belgium) as a negative control. For quantification of co-precipitated DNA, samples were then subjected to amplification by employing primers (I-SceI forward: 5'-CATGCCCGAAGGCTACGT-3', I-SceI reverse: 5'-CGGCGCGGGTCTTGTA-3').

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11. DNA End Resection

DNA end resection was performed as described in the references [93]. AID-DivA U2OS cells were transfected with siRNA. After 48 hours, AsiSI-mediated DSBs were induced by treatment with 4-OHT for 4 hours. Genomic DNA was isolated using a genomic DNA extraction kit(Bioneer). RNaseH 3ug treated genomic DNA was digested with BamHI, BsrGI, and HindIII digestion at 37℃. The level of resection adjacent to specific DSBs was measured by real-time quantitative polymerase chain reaction(RT-qPCR)

12. Metaphase chromosome spreads

HeLa cells were transfected with siRNA. After 48 hours, the cells were induced DNA damage by IR(2 Gy) and recovered overnight. The cells were treated with colcemid (sigma, D1925, 50 ng/ml final) to synchronize into metaphase. After 3 hours, the cells were detached with trypsin-EDTA and collected in a 15 ml tube. Next, the cells were centrifuged at 1,000 rpm for 5 minutes. The supernatant was completely removed from the tube. The cells were resuspended with 500 ul of PBS. 75 mM KCl was added into the tube

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while gently tapping the tube, then, the tube was incubated at RT for 15 - 30 minutes. The cells were centrifuged at 1,000 rpm for 10 minutes, and the 5 ml of supernatant was removed. The pellet was tapped for resuspension, then 5ml of fixing solution was dropped into the tube while gently tapping the tube to mix. The samples were incubated in RT for 10 minutes and centrifuged at 1,000 rpm for 10 minutes. The 5 ml of supernatant was removed. The steps, dropping fixing buffer, incubation, centrifugation, and removal of the supernatant, were repeated three times. The sample was dropped onto a slide glass and dried. The chromosomes were counterstained using Vectashield mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).

13. Antibody

Anti - CtIP (Active Motif, mouse, 61141), anti - CtIP (Cell Signaling, rabbit, 9201), anti - CtIP (Santa Cruz, mouse, sc - 271339), anti - PRMT5 (Abcam, rabbit, ab109451), anti - PRMT5 (Cell Signaling, rabbit, 79998), Anti - MEP50 (Abnova, mouse, H00079084 - M01), anti - symmetric dimethylarginine

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(SDMA) (Cell Signaling, rabbit, 13222), anti-RPA (Abcam, mouse, ab2175), anti-Rad51 (Abcam, rabbit, ab63801), anti-53BP1 (BD Biosciences, mouse, 612523), anti-GFP (Santa Cruz, mouse, sc-9996) anti-HA (Santa Cruz, mouse, sc-7392), anti-HA (cell signaling, rabbit, C29F4), anti-Flag (Sigma, mouse, F1804), anti-Flag (Sigma, rabbit, F7425), and anti-β-actin (Santa Cruz, mouse, sc-47778) antibodies.

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RESULT

1. PRMT5 interacts with CtIP

CtIP is well known as a key regulator in DNA end resection, but CtIP functions are not fully understood in DNA end resection. To understand the functions of CtIP in DDR, we initially screened for a new regulator of CtIP through a yeast two-hybrid (Y2H) system. Y2H is performed to detect protein-protein interactions (PPIs). We put to use CtIP variant 1 (containing 500a.a-end) as bait and HeLa cDNA library as prey. Every yeast was transformed by CtIP (bait) and one of cDNA (prey) and spread on selection medium, SD-LWU (SD without leucine, tryptophan, and uracil), SD-LWA (SD without leucine, tryptophan, and adenosine) for confirming expression, and we performed filter assay (Fig. 1A). As shown figure 1A and B, No. 20~23 of colonies were not expressed in SD-LWA medium. Through this experiment, we could obtain 19 positive colonies and identified 8 genes. One of them is the protein arginine methyltransferase 5 (PRMT5) (Fig. 1B).

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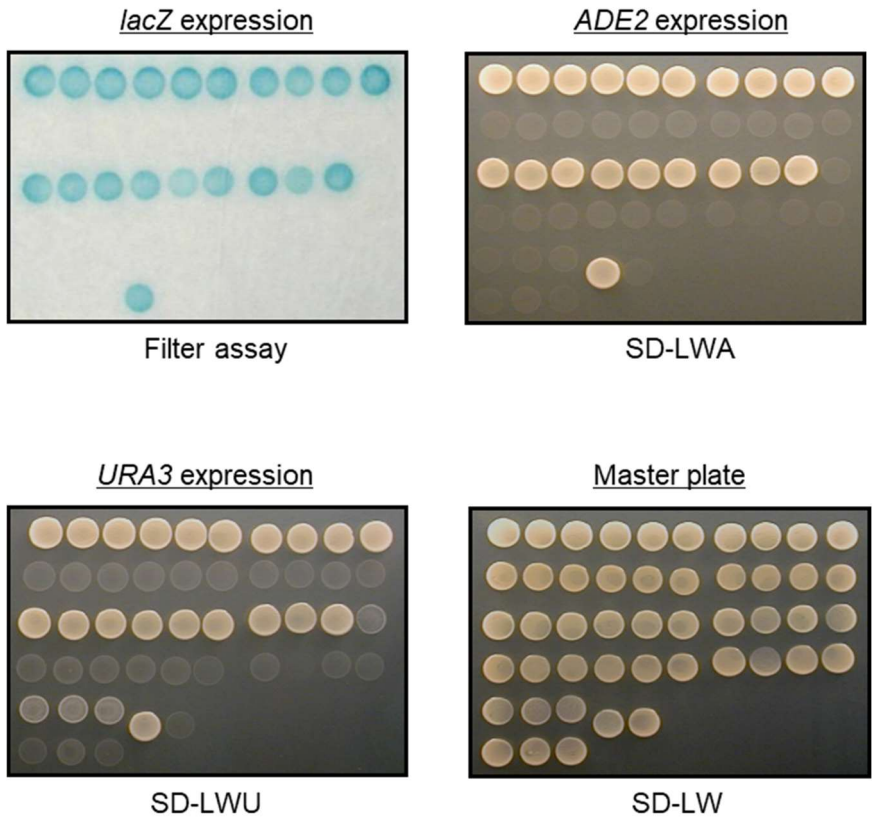
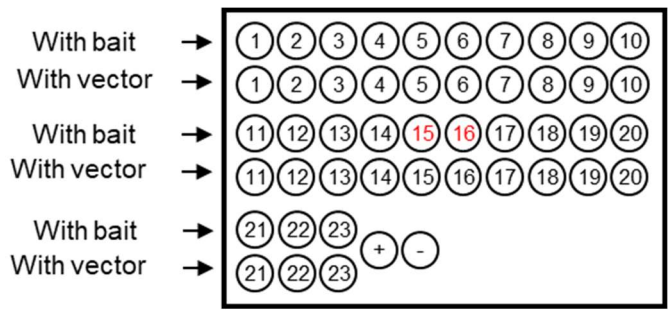
Figure 1

A





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B

Prey ID	Description	Reporter expression		
		acZ	*URA3*	*ADE2*
AD Hybrid – 1 ~ 6	siah E3 ubiquitin protein ligase 1 (SIAH1)	+	+	+
AD Hybrid – 7 ~ 12	siah E3 ubiquitin protein ligase 2 (SIAH2)	+	+	+
AD Hybrid – 13	proliferating cell nuclear antigen (PCNA)	+	+	+
AD Hybrid – 14	of gem nuclear organelle associated protein 4 (GEMIN4)	+	+	+
AD Hybrid – 15 ~ 16	protein arginine methyltransferase 5 (PRMT5),	+	+	+
AD Hybrid – 17	Ribosomal protein L5 (RPL5)	+	+	+
AD Hybrid – 18	centromere protein C (CENPC)	+	+	+
AD Hybrid – 19	eukaryotic translation initiation factor 4A1 (EIF4A1)	+	+	+
AD Hybrid – 20	Nascent polypeptide-associated complex alpha subunit (NACA)	-	+	-
AD Hybrid – 21	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	-	+	-
AD Hybrid – 22	heat shock 60kDa protein 1 (chaperonin) (HSPD1)	-	+	-
AD Hybrid – 23	thioredoxin reductase 2 (TXNRD2)	-	+	-

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Figure 1. Yeast two - hybrid screening identifies proteins that bind to CtIP

(A) Yeast two - hybrid screening to identify CtIP interaction protein. CtIP containing 500a.a - end used as bait and HeLa cDNA library as prey.

(B) A list of proteins identified in the yeast two - hybrid screening. Each clone was determined by DNA sequencing and BLAST search. The 15th and 16th clones are PRMT5

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To confirm the interaction between CtIP and PRMT5, we performed co-immunoprecipitation (co-IP) and proximity ligase assay (PLA). At first, we performed the co-IP assay. HeLa cells were transfected using siRNA against PRMT5 mRNA. In 48 hours after transfection, the lysates performed IP using anti-CtIP antibodies followed by immunoblotting (IB) with CtIP or PRMT5 antibodies. This endogenous IP data showed that they interacted with each other in the normal condition cells (Fig. 2A). In addition, to further confirm their interaction, we performed exogenous IP. HeLa cells were transiently transfected with Flag-tagged CtIP wild type and HA-tagged PRMT5 wild type together. Exogenous co-IP was carried out with anti-Flag antibody. This result showed the same tendency to endogenous IP data (Fig. 1B). Furthermore, we performed PLA which is an antibody-based detection assay for the interaction of two proteins. This assay allows direct visualization, as well as quantification and subcellular localization of the interactome in fixed cells. The interactome is targeted by specific antibodies conjugated with oligonucleotides. If two proteins are close to each other, the ligation of oligonucleotide moieties is caused, then generated a DNA sequence, which can be amplified by PCR. Based on this principle, each interactome generates a detectable fluorescence

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signal under the fluorescence microscope [94]. In the results, we confirmed that the interaction between CtIP and PRMT5 existed (Fig. 2C and D). For checking the knockdown of PRMT5, we performed western blot (WB) analysis (Fig. 2E). Together, all these data showed that PRMT5 is a new binding partner of CtIP. So, we suggest that CtIP interacts with PRMT5.

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Figure 2

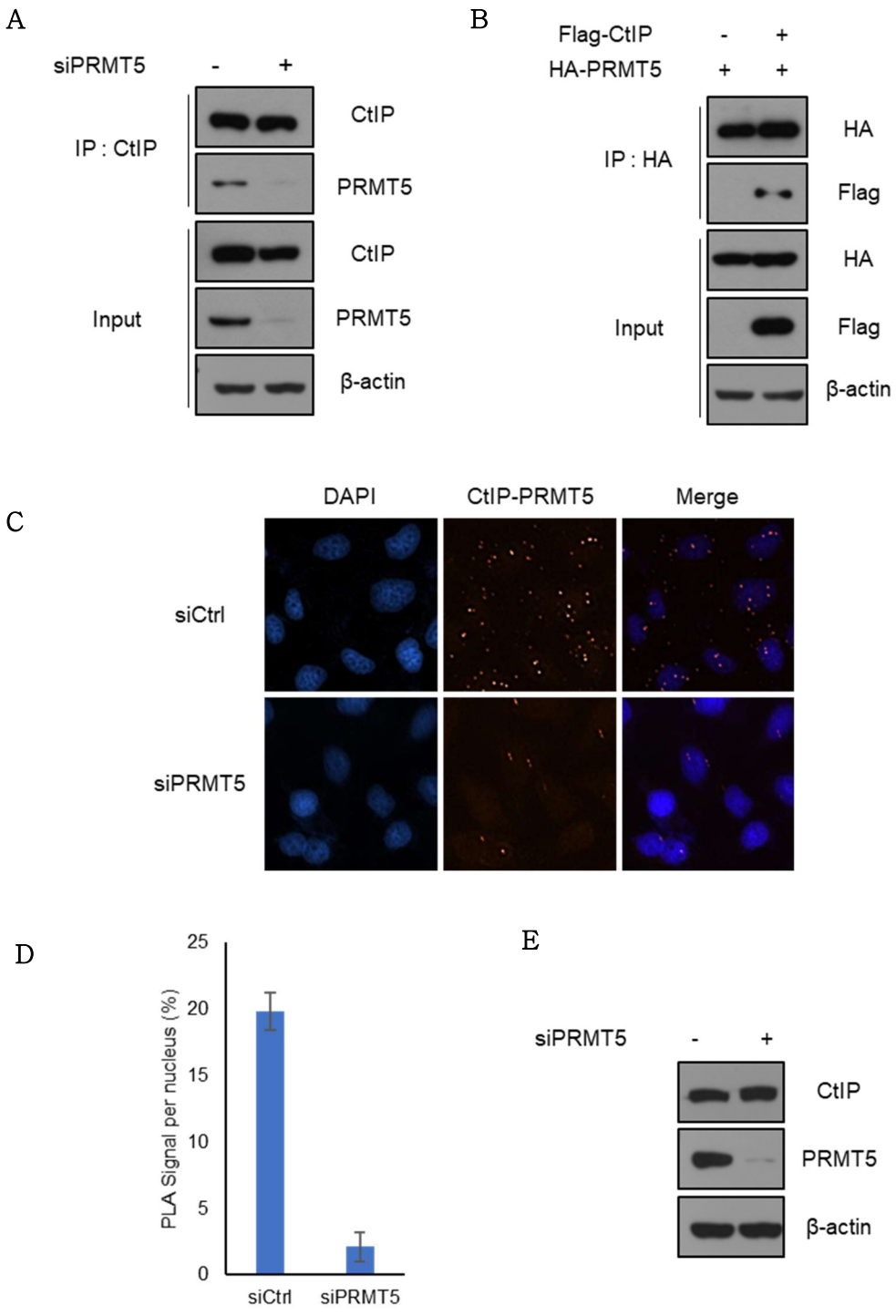

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Figure 2. PRMT5 is a novel binding partner of CtIP

(A) HeLa cells were transfected with siRNA targeting mRNA of PRMT5. After 48 hours, the cells were lysed with NP - 40 buffer, then the lysates were used for immunoprecipitation (IP). The anti - CtIP antibody was used for IP followed by immunoblotting using CtIP or PRMT5 antibodies. (B) HeLa cells were co - transfected with flag - tagged CtIP and HA - tagged PRMT5 construct. After 48 hours, the cells were lysed by NP - 40 buffer, then, the lysates were used for immunoprecipitation (IP). The interaction between flag - tagged CtIP and HA - tagged PRMT5 was determined by immunoprecipitation and immunoblotting with the indicated antibodies. (C) Proximity Ligase Assay (PLA). HeLa cells were transfected with siRNA targeting mRNA of PRMT5. After 48 hours, the cells were fixed with 4% paraformaldehyde (PFA), then followed by the PLA recommendation method. Anti - CtIP and anti - PRMT5 antibodies were used for PLA. DNA was counterstained with DAPI. (D) PLA signals were quantified as foci per nucleus. Data were presented as means ± SD. (E) Batch samples to PLA were used for the western blot for confirming PRMT5 - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of PLA. Then, anti - CtIP and anti - PRMT5 antibodies were used for western blot detection.

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2. PRMT5 induces CtIP recruitments to DSB sites and HR

We wondered whether PRMT5 affects HR because CtIP interacts with PRMT5 and is a key protein in HR. To prove this, we investigated the recruitment of CtIP through immunofluorescence (IF) and ChIP assay in PRMT5 deficient cells. U2OS cells were transfected by siRNA targeting CDS and 3'UTR of PRMT5 mRNA. 48 hours after transfection, PRMT5-deficient U2OS cells were treated with ionizing radiation (IR), and recovered for 6 hours. CtIP is stained with anti-CtIP antibody. Recruitments of CtIP to DSB sites were reduced in CDS or 3'UTR mRNA of PRMT5 deficient cells compared to the control group (Fig. 3A and B). We confirmed the expression level of CtIP and PRMT5 by WB (Fig. 3C). To support this, we additionally performed a ChIP assay for the recruitment of CtIP to DSB sites. DR-GFP U2OS cells were transfected with siRNA targeting mRNA of PRMT5, then 48 hours after transfection, the cells were additionally transfected with the plasmid of I-SceI. After 8 hours, we performed a ChIP assay. In this experiment, we found that the recruitment of CtIP to DNA lesions decreased in PRMT5-deficient cells (Fig. 3D). To check the expression level of PRMT5 and CtIP, we performed WB using a batch sample. The expression level of CtIP was equal, and the

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PRMT5 decreased as we expected (Fig. 3E). Next, we checked the change of RPA and Rad51. We expected that recruitment of RPA and Rad51 would be reduced to the DSB site because CtIP regulates end resection, also RPA and Rad51 are sequentially recruited to ssDNA after initiation of DNA end resection. To prove this, we carried out IF in U2OS cells. The cells were transfected with siRNA against mRNA of PRMT5, then 48 hours after transfection, the cells were treated with IR and took 6 hours for recovery. The cells were stained by anti - RPA and Rad51 antibodies. As we expected, RPA and Rad51 foci were decreased (Fig. 4A - D) and the expression level showed the same level of CtIP and reduced level of PRMT5 compared to the control group by WB (Fig. 4E). Consequently, we guessed that those reasons lead to the defect in HR efficiency. To check this, we carried out a DR - GFP assay. DR - GFP assay is a powerful tool for measuring HR repair. DR - GFP assay is a used repair system of cells. DR-GFP system has two incomplete GFP cassettes that are stably integrated into the genome. In the first cassette, the GFP gene includes a promoter but does not function because it contains a premature stop codon and I-SceI restriction site. The second cassette has a perfect coding sequence but no promoter. The DSBs induced by I - SceI in the first cassette are repaired

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by HR using the second cassette as a template. Finally, a complete GFP gene, including a functional promoter, is produced (Fig. 4F) [95]. Repair efficiency is measured by the percentage of cells expressing GFP using INCell Analyzer 2500HS. The cells were treated with siRNA targeting mRNA of PRMT5 for knockdown and plasmid of I-SceI for inducing DSBs. As we guessed, HR efficiency decreased in deficient PRMT5 cells (Fig. 4G and H). Together, these data showed that PRMT5 controls HR through the regulation of CtIP recruitment to DSBs.

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Figure 3

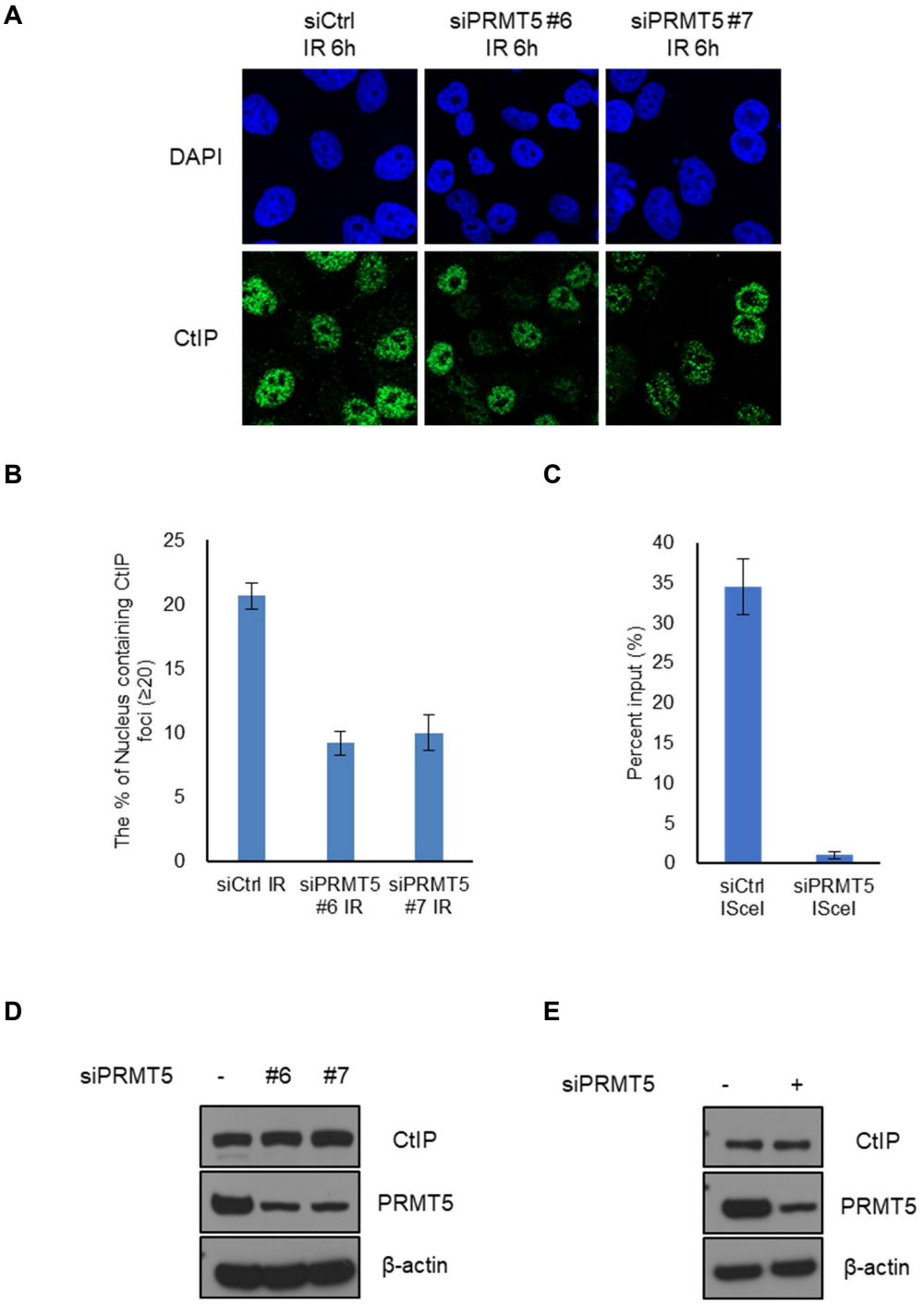

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Figure 3. PRMT5 regulates the recruitment of CtIP to DSB sites

(A) HeLa cells were transfected with siRNA targeting CDS and 3' UTR of PRMT5 mRNA. After 48 hours, the cells were fixed by 4% paraformaldehyde (PFA), then followed immunostained with anti-CtIP antibody. (B) CtIP foci were quantified as foci per nucleus for each group. Data are presented as means ± SD. (C) Batch samples to IF were used for the western blot for confirming PRMT5-knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of IF. Then, anti-CtIP and anti-PRMT5 antibodies were used for western blot detection. (D) We performed chromatin immunoprecipitation (ChIP) for CtIP recruitment to DSB lesion using DR-GFP U2OS cells. The cells were transfected with PRMT5 siRNA. After 48 hours, the cells were transfected again with I-SceI plasmid to induce DSBs. At 7 hours, the cells were fixed and chromatins were extracted. The fractionated chromatins were digested by micrococcal nuclease followed by performed immunoprecipitation with anti-CtIP antibody. The recruitment of CtIP was quantified by RT-qPCR. (E) Batch samples to ChIP were used for western blot for confirming PRMT5-knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of IF. Then, anti-CtIP and anti-PRMT5 antibodies were used for western blot detection.

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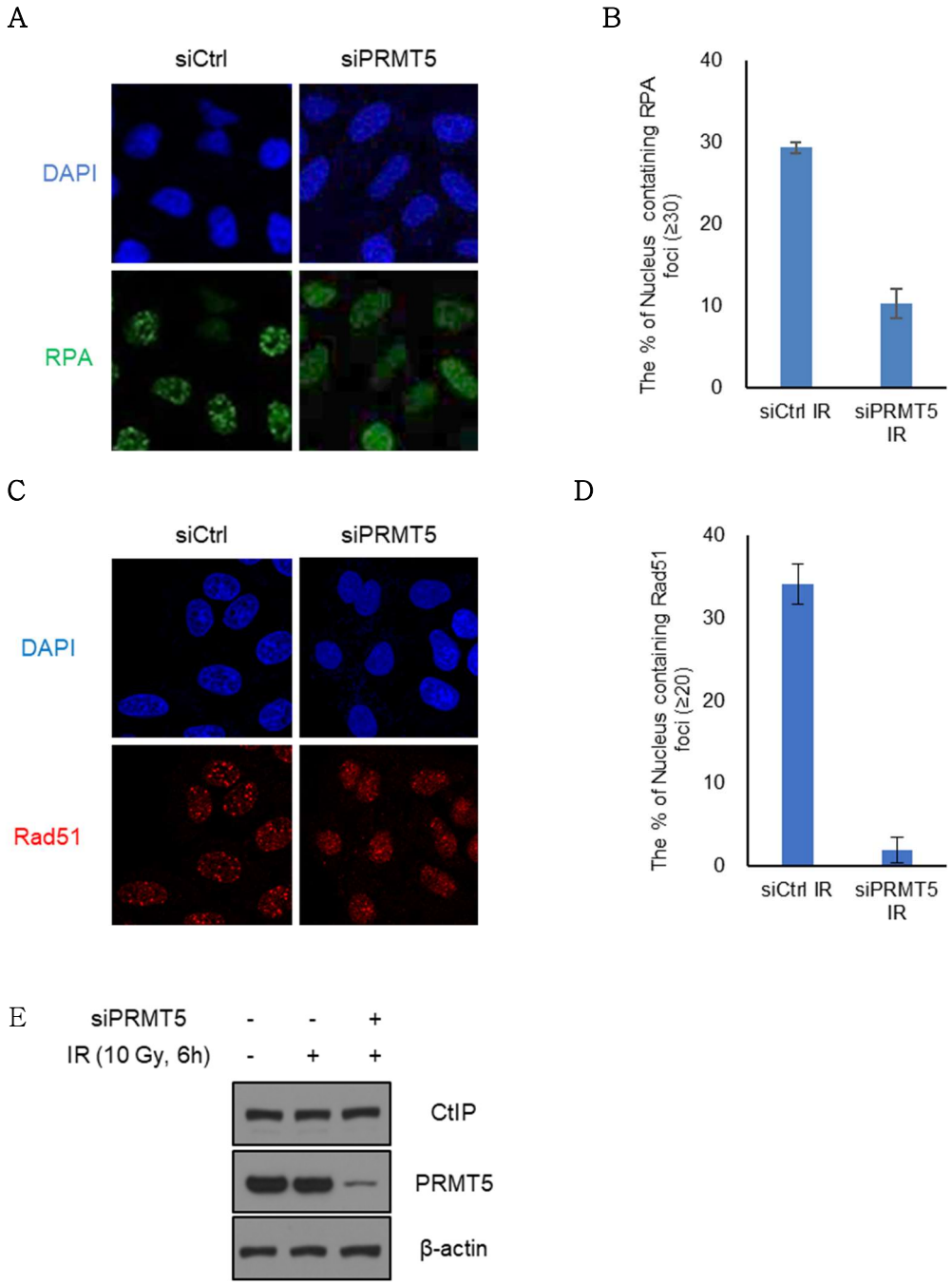
Figure 4



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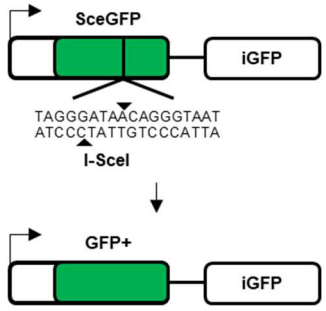
F



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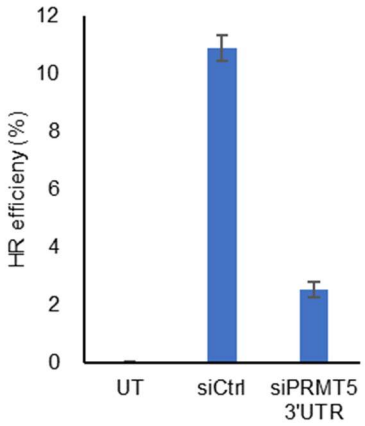

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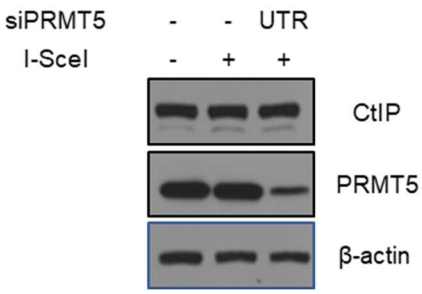

Figure 4. Deficient PRMT5 inhibits homologous recombination by reducing the recruitment of RPA and Rad51 to DSB sites

(A and C) U2OS cells were transfected siRNA targeting mRNA of PRMT5. After 48 hours, the cells were damaged by IR (10 Gy), then recovered for 6 hours. Then, the cells were fixed with 4% paraformaldehyde (PFA). Then, the cells reacted with anti - RPA and Rad51 antibodies. (B and D) RPA and Rad51 foci were quantified as foci per nucleus for each group. Data were presented

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as means ± SD. (E) Batch samples to IF were used for western blot for confirming PRMT5 - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of IF. Then, anti - CtIP and anti - PRMT5 antibodies were used for western blot detection. (F) A schematic of the DR - GFP reporter system. (G) DR - GFP U2OS cells were transfected siRNA targeting mRNA of PRMT5 for 4 hours and then transfected again with I - SceI plasmid for inducing DSBs. After 72 hours, GFP - positive cells were counted by INCell Analyzer 2500HS. The values are the percentage of GFP - expressing cells determined and represent the mean ± SD. (H) Batch samples to DR-GFP assay were used for the western blot for confirming PRMT5 - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of the DR - GFP assay. Then, anti - CtIP and anti - PRMT5 antibodies were used for western blot detection.

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3. PRMT5 promotes DNA end resection

PRMT5 interacts with CtIP and regulates the recruitment of CtIP and its downstream proteins, RPA and Rad51, and CtIP relates to DNA end resection. This analysis is the quantification of ssDNA produced by 5'end resection in AID-DIvA U2OS cells. In addition, 4-hydroxytamoxifen (4-OHT) reacts with estrogen receptor (ER). In this cell line, ER and AsiSI are fused and stably expressed, but they are impossible to enter into nuclear because of inactivated ER. ER usually exists in the cytoplasm, but ER turns to an active form by 4-OHT. So, treatment of 4-OHT allows the fusion protein to enter into nuclear, then AsiSI, restriction endonuclease, induces DSBs in the sequence-specific site (5'-GCGATCGC-3'). Genomic DNA from these cells was prepared and analyzed for ssDNA at DSBs by qPCR (Fig. 5A). To investigate whether PRMT5 is associated with DNA end resection, we performed DNA end resection analysis. The cells were transfected with a siRNA targeting CtIP or PRMT5 or co-transfected with the siRNA targeting CtIP and PRMT5. This result showed reduced DNA end resection efficiency in CtIP or PRMT5 deficient cells. Interestingly, the cell, both gene knockdown of CtIP and PRMT5, showed

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less DNA end resection efficiency compared to the single knockdown (Fig. 5B and C). This means that PRMT5 and CtIP regulate DNA end resection in the different pathways. Together, these data showed that PRMT5 positively regulates HR through DNA end resection following the effective recruitment of CtIP.

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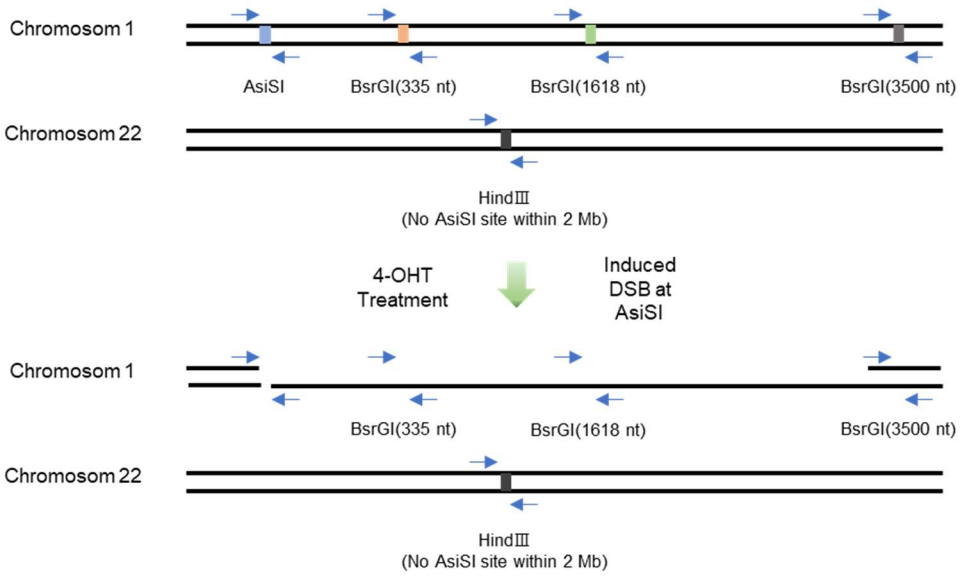
Figure 5

A



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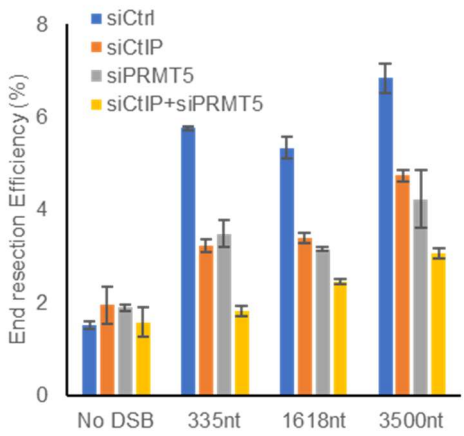

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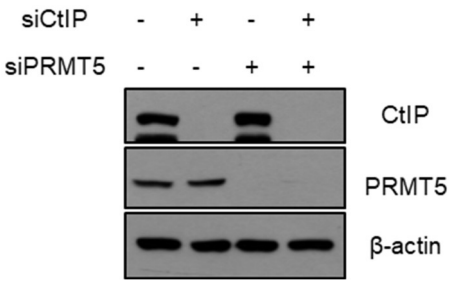

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Figure 5. PRMT5 regulates DNA end resection in the different pathways to CtIP

(A) Schematic representation of DNA end resection experimental procedure. (B) AID - DIvA U2OS cells were treated with 4 - OHT for 4 hours. Genomic DNA was extracted and digested with bamH1, BsrG1, and HindⅢ. DNA resection adjacent to DSB and No DSB site was measured by RT - qPCR. (C) Batch samples to DNA end resection assay were used for the western blot for confirming the knockdown of PRMT5 and CtIP. The cells were lysed with RIPA buffer at the same time. Then, anti - CtIP and anti - PRMT5 antibodies were used for western blot detection.

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4. PRMT5 induces chromosome instability.

In figures 3 and 4, we confirmed that HR decreased through reducing the recruitment of CtIP and its downstream protein, RPA, and Rad51, in PRMT5 deficient cells. We were curious about the condition of chromosomes in the PRMT5 – deficient cells. We can observe the condition of chromosomes in metaphase cells. The metaphase is estimated approximately 30 minutes [96]. The time is lacking for observation. So, we used colcemid for cells to synchronize into metaphase. To confirm this, we performed a metaphase chromosome spread assay. The result showed increasing abnormal chromosomes in PRMT5 deficient cells (Fig. 6A and B). We thought that the increased abnormality of chromosomes in PRMT5 – deficient cells is responsible for the falt of the repair system, and PRMT5 has an essential role in IR – induced DDR. We wondered how sensitive the PRMT5 – deficient cells are to IR. To prove this, we performed a clonal survival assay. HeLa cells were used and transfected with siRNA targeting PRMT5. After 48 hours, the cells were reseeded and induced DSBs by IR, then incubated for 10 – 14 days. Then, the cells were fixed and stained. The outcome result showed the knockdown of PRMT5 led to hypersensitive cell viability to IR (Fig. 6C and D). In this figure

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6, the knockdown of PRMT5 was proved by western blot (Fig. 6E). These data suggest that PRMT5 mediated HR and chromosome integrity by inducing the recruitment of CtIP, RPA, and Rad51 to DSB sites.

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Figure 6

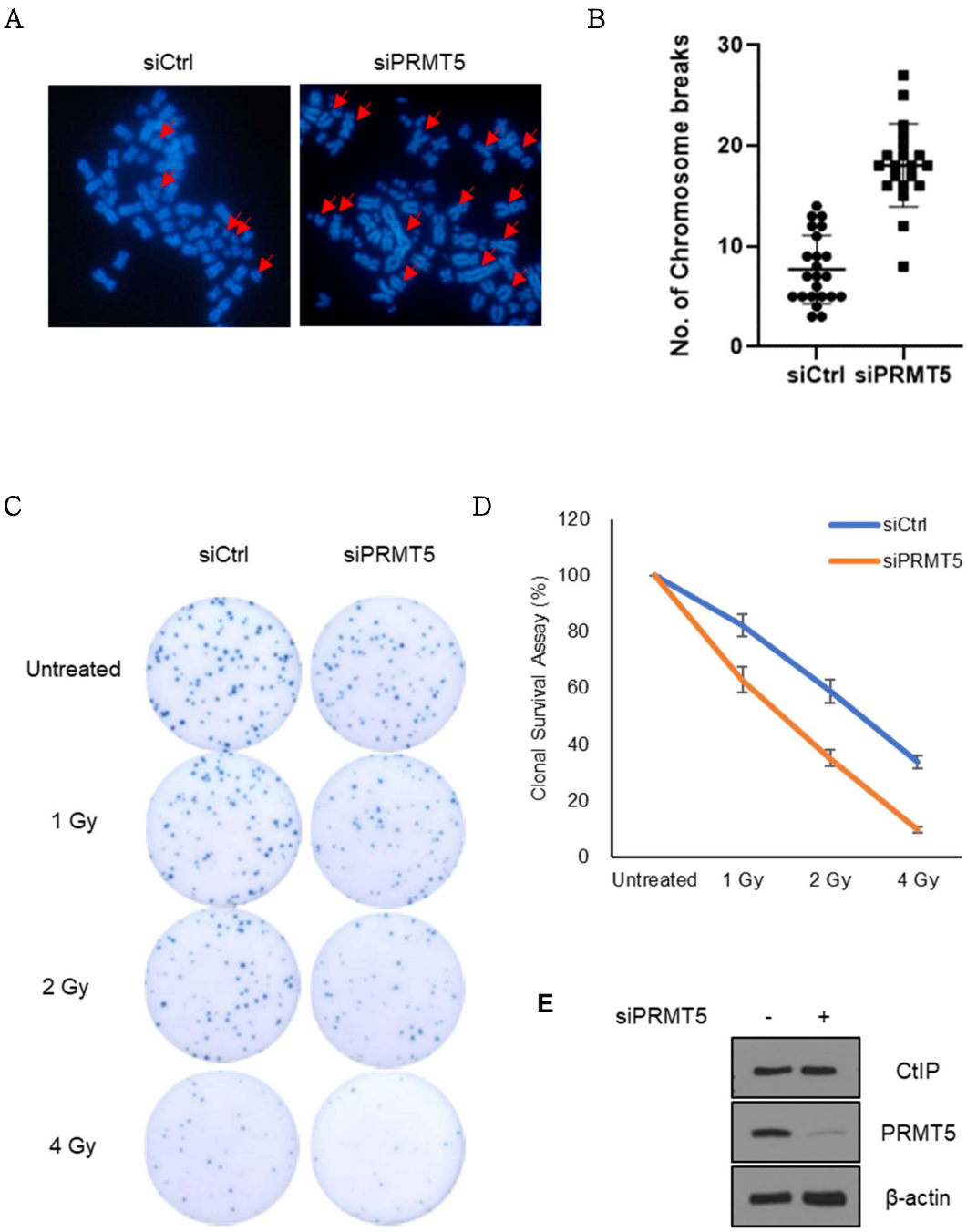

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Figure 6. Deficient PRMT5 impairs chromosomal integrity and IR resistance by decreasing the efficiency of DNA end resection.

(A and B) HeLa cells were transfected siRNA targeting mRNA of PRMT5. After 48 hours, the cells were induced DNA damage by IR (2 Gy) and recovered for 24 hours. After that, the cells were treated with colcemid for synchronizing into metaphase. Next, the cells were treated KCl and fixed with 4% paraformaldehyde (PFA). Then, metaphase chromosomes were screened by confocal. The abnormal chromosomes were counted manually. (C and D) HeLa cells were transfected with siRNA targeting mRNA of PRMT5. Control and depleted HeLa cells were treated with indicated IR dose. The cells were grown for 10 - 14 days, then fixed with 4% paraformaldehyde (PFA). After the fixation, the cells were stained with methylene blue. The colonies were counted in each dish. (E) HeLa cells were used for the western blot for confirming PRMT5 - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of the metaphase chromosome spread assay and clonal survival assay. Then, anti - CtIP and anti - PRMT5 antibodies were used for western blot detection.

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5. PRMT5 methylates CtIP through interaction with the C - terminal of CtIP

To map the binding region of CtIP and PRMT5 to each other, we designed a deletion mutant construct of CtIP (D1; deleted 801 - 897a.a) and PRMT5 (⊿TIM;1 - 299a.a, ⊿SAM;291 - 429a.a, ⊿DMZ;421 - 499a.a, ⊿C;491 - 637a.a) (Fig. 7A and B). To search the binding region, we performed co-IP. The cells were made expressing PRMT5 or CtIP deletion mutants transiently. For the experiments, anti - HA and GFP antibodies were used for IP. In the results, CtIP bound to the ⊿TIM region of PRMT5 (Fig. 7C), and PRMT5 bound to the D1 region of CtIP (Fig. 7D). PRMT5 as type Ⅱ methyltransferase can methylase RGG/RG motif of substrate [97]. We guessed that PRMT5 can catalyze methylation, specifically SDMA, of CtIP. We used siRNA targeting PRMT5 and MEP50, and PRMT5 inhibitor (EPZ015666) for the experiment. Methylosome protein 50 (MEP50) is a cofactor of PRMT5 for methylation, and EPZ015666 as PRMT5 enzymatic inhibitor inhibits the function of PRMT5. Additionally, one of the PRMT5 effects is the regulation of 53BP1 proteostasis through methylation of 53BP1 [90]. To prove this, we carried out IP. The cells were treated with siRNA or PRMT5 inhibitor for 48 hours. The lysate was used for IP with anti - CtIP antibody. In the results, the

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data showed that SDMA decreased in PRMT5 deficient cells (Fig. 8A), and also decreased in MEP50 deficient and PRMT5 inhibitor-treated cells (Fig. 8B). Through this experiment, we found that the enzymatic activity of PRMT5 is responsible for the SDMA of CtIP (Fig. 8B). In summary, PRMT5 catalyzes methylation, specifically SDMA, of CtIP through the interaction between PRMT5 and CtIP. So, we suggest that PRMT5 catalyzes the SDMA of CtIP.

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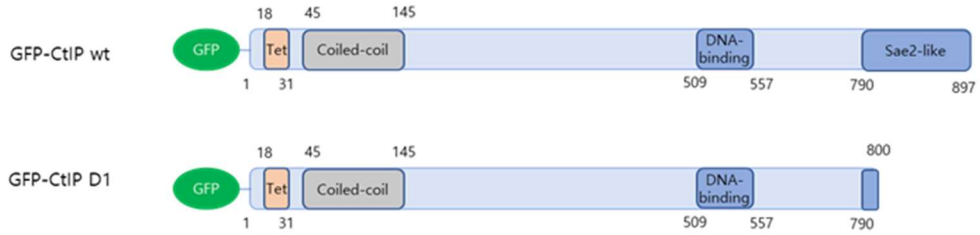
Figure 7

A



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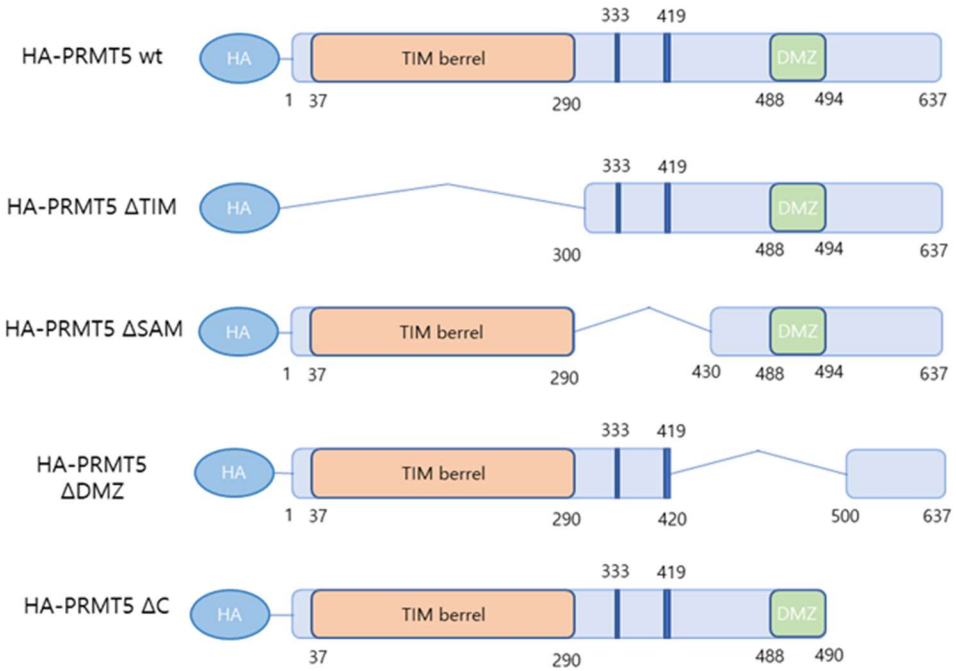

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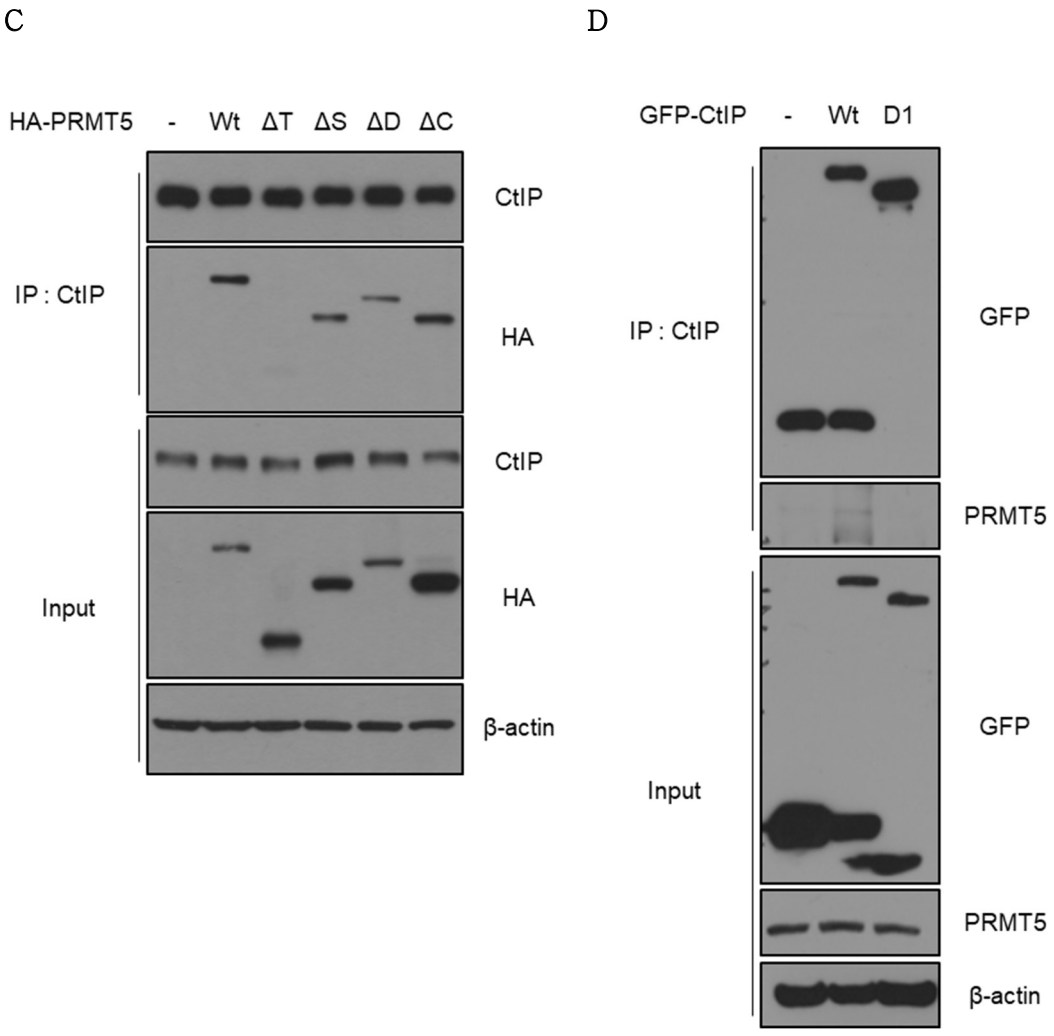

Figure 7. PRMT5 binds to 800 - 897a.a of CtIP and CtIP binds to the TIM domain of PRMT5.

(A) A schematic presentation of the CtIP deletion mutant constructs. (GFP - CtIP wt : 1 - 897 a.a, GFP - CtIP D1 : 1 - 800 a.a). (B) A schematic

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presentation of the PRMT5 deletion mutant constructs. (HA - PRMT5 wt : 1 - 637 a.a, HA - PRMT5 ⊿TIM : 300 - 637 a.a, HA - PRMT5 ⊿SAM : 1 - 290 and 430 - 637 a.a, HA - PRMT5 ⊿DMZ : 1 - 420 and 500 - 637 a.a, HA - PRMT5 ⊿C : 1 - 490 a.a). (C) HeLa cells were transiently transfected by HA - tagged PRMT5 constructs. After 48 hours, the cells were lysed by NP - 40 lysis buffer. The lysates were used for immunoprecipitation using anti - CtIP antibodies and analyzed by western blot using the anti - CtIP and HA antibodies. (D) HeLa cells were transiently transfected by GFP - tagged CtIP constructs. After 48 hours, the cells were lysed by NP - 40 lysis buffer. The lysates were used for immunoprecipitation using anti - GFP antibodies and analyzed by western blot using the anti - GFP and PRMT5 antibodies.

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Figure 8

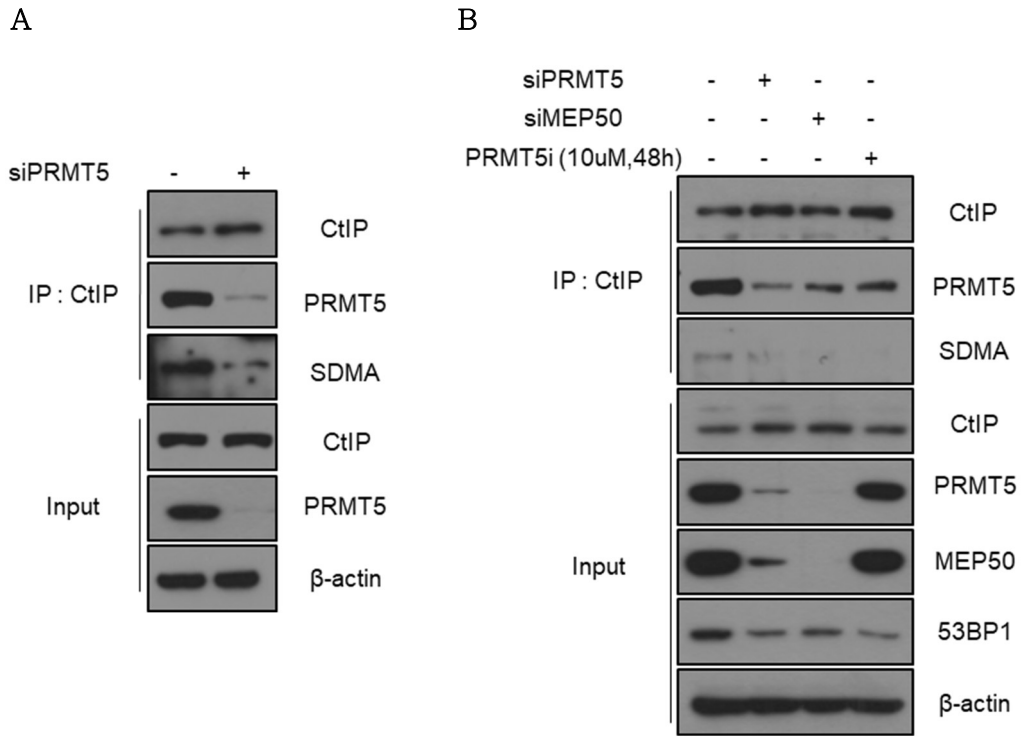

Figure 8. PRMT5 catalyzed symmetric dimethylarginine of CtIP

(A) The HeLa cells were transfected with siRNA targeting mRNA of PRMT5. After 48 hours, the cells were lysed by NP - 40 lysis buffer. Then, the lysates were used for immunoprecipitation using anti - CtIP antibodies and analyzed by western blot using the anti - CtIP, PRMT5, and SDMA antibodies. (B) HeLa cells were transfected with siRNA targeting mRNA of PRMT5 and MEP50, and the other cells were treated with PRMT5 inhibitor (EPZ015666). After 48 hours,

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the cells were lysed by NP - 40 lysis buffer. Then, the lysates were used for immunoprecipitation using anti - CtIP antibodies and analyzed by western blot using the anti - CtIP, PRMT5, MEP50, 53BP1, and SDMA antibodies.

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6. CtIP is methylated at eight Arginine residues.

In figure 8, we discovered that CtIP is methylated by PRMT5. But, we only knew about CtIP is methylated by PRMT5. So, we wondered about specific residues of CtIP methylation by PRMT5. Therefore, we analyzed the putative methylation site of CtIP through LC-MS/MS. To analyze LC-MS/MS, we performed IP. The cells were transfected with plasmid DNA of HA-tagged CtIP. Then, HA-fused proteins were pulled down using anti-HA antibodies. Pulled-down proteins were separated by SDS-PAGE, then SDS-PAGE gel was stained by Coomassie blue for checking the amount of pulled-down CtIP (Fig. 9A). Next, we cut gel for LC-MS/MS analysis. Through LC-MS/MS analysis, we found the putative methylation site, R124, R421, R635, R718, R730, R865, R877, and R881 (Fig. 9B-I). We made all putative methylation residues arginine-to-alanine, which we named 8RA (Fig. 10A). Using this construct, we performed IP for investigating the change in CtIP methylation. The cells were transfected with Flag-tagged-CtIP plasmid wild type or 8RA. As a result of this experiment, SDMA in 8RA CtIP decreased compared to SDMA in wild-type CtIP (Fig. 10B). In summary, there are possible 8 SDMA sites on CtIP.

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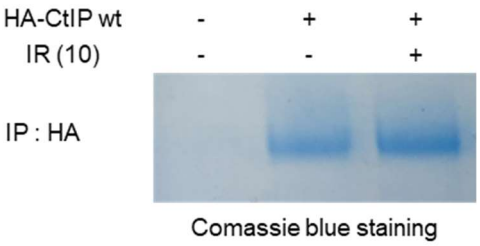
Figure 9

A



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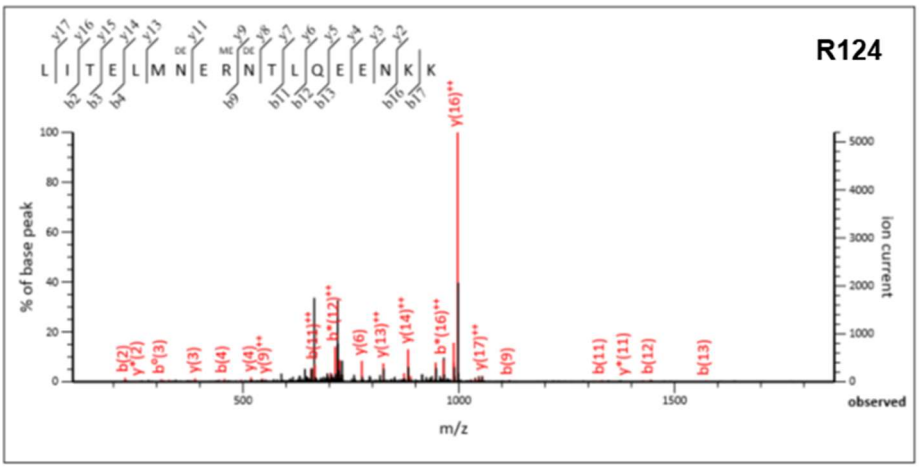

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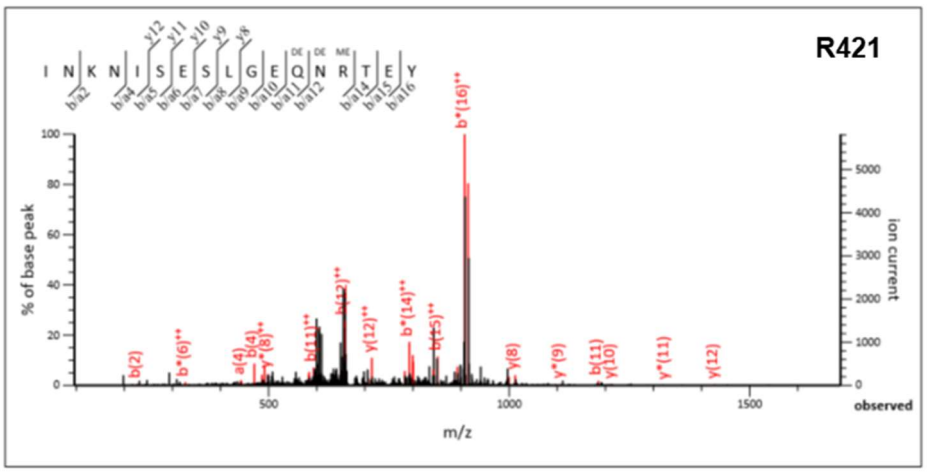

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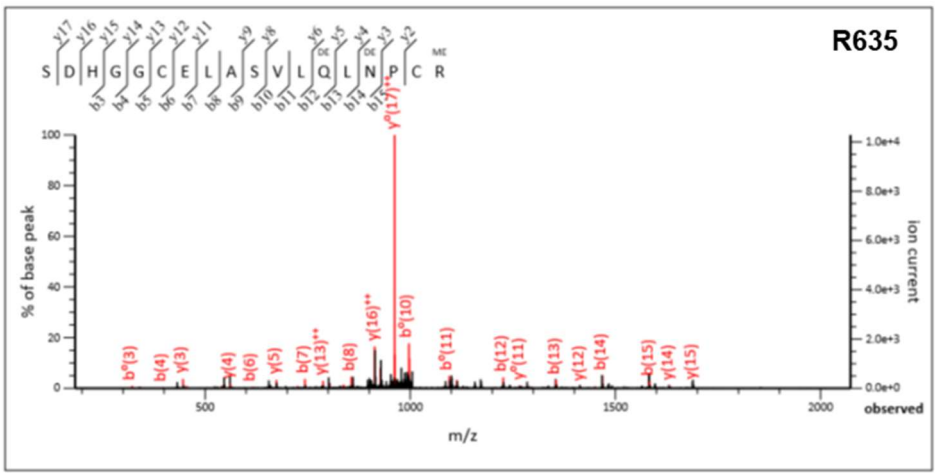

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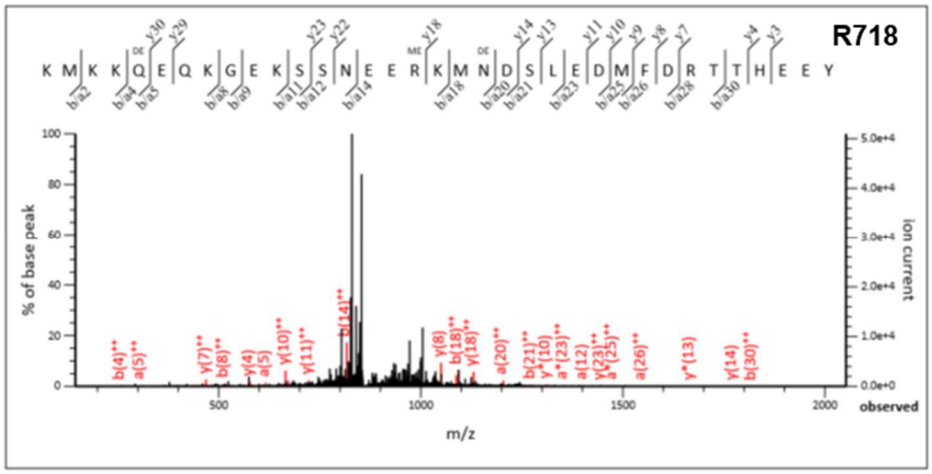

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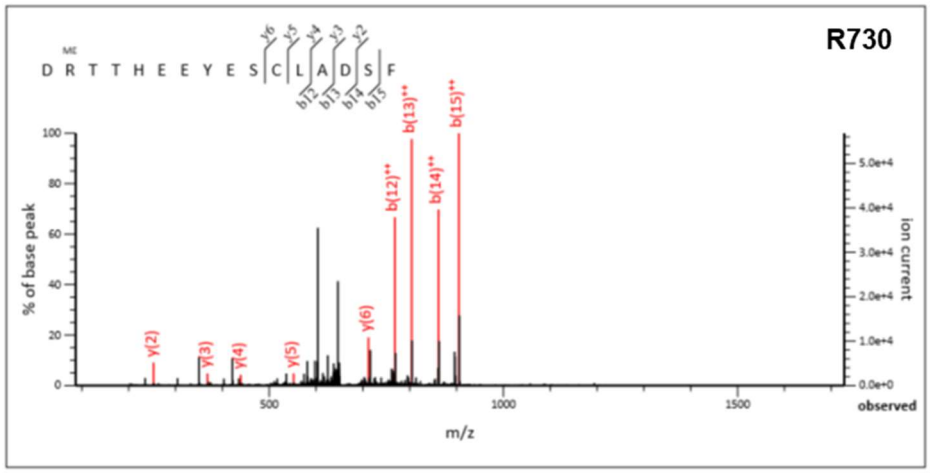

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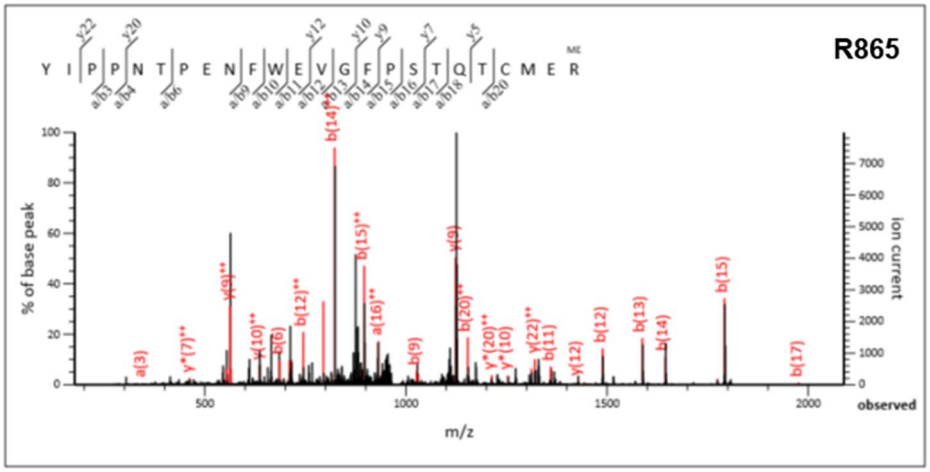
G



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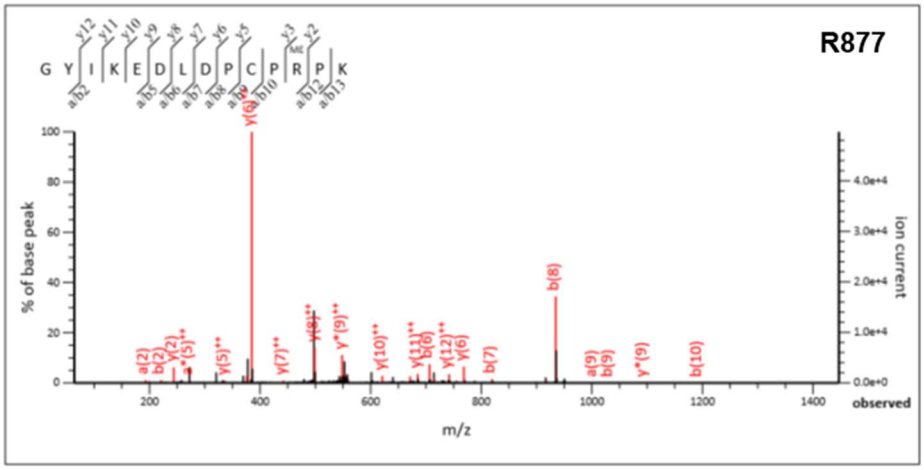

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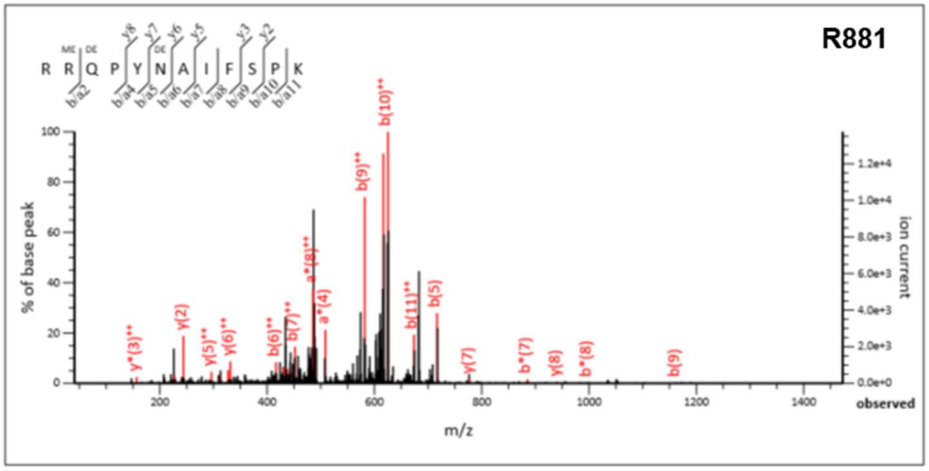

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Figure 9. The data of LC-MS/MS analysis show that SDMA-CtIP can be catalyzed at the 8 putative methylation residues.

(A) HeLa cells were transfected with HA-tagged CtIP construct. After 48 hours, the cells were lysed with NP-40. The lysates were used for immunoprecipitation using anti-HA antibodies. The pulled-down of CtIP was performed SDS-PAGE. Then, the gels were stained with Coomassie blue. (B-I) The data of LC-MSMS. The CtIP has 8 putative methylation sites, at R124, R421, R635, R718, R730, R865, R877, and R881.

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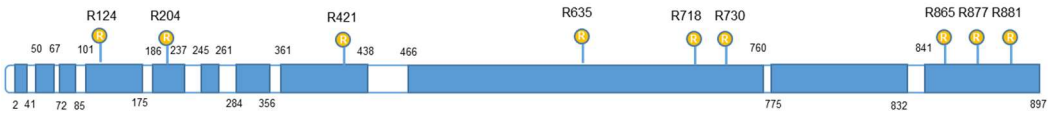
Figure 10

A



Putative methylarginine residues of CtIP

B

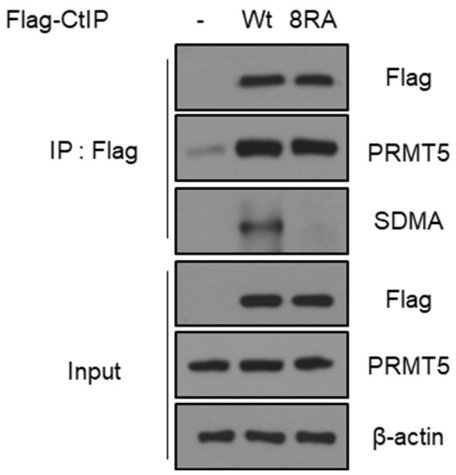


Figure 10. Symmetric dimethylation of CtIP is catalyzed at 8 putative methylarginine residues.

(A) A schematic presentation of the mutant construct of the putative methylarginine residues on CtIP. 8 putative methylarginine residues were mutated from arginine to alanine. The blue areas are reading regions by

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LC-MS/MS. (B) HeLa cells were transfected with Flag-tagged CtIP wild-type or 8RA constructs. After 48 hours, the cells were lysed by NP-40 lysis buffer. Then, the lysate was used for immunoprecipitation using anti-Flag antibodies and analyzed by western blot using the anti-Flag, PRMT5, and SDMA antibodies.

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7. SDMA of CtIP facilitates HR through the recruitment of CtIP to DSB lesions

We found possible 8 SDMA sites on CtIP. Methylation of CtIP is necessary for CtIP recruitment in figures 3 and 4. Based on these data, we guessed that the CtIP mutant is poorly recruited to DSBs. Proving this, we performed IF using Flag-CtIP wild-type and 8RA construct. We expected that CtIP recruitment would decrease in 8RA CtIP. As we expected, the recruitment of CtIP decreased in CtIP 8RA-expressing cells (Fig. 11A and B). To support this, we carried out a ChIP assay. The cells, expressing Flag-CtIP wild type or 8RA, were stained with anti-Flag antibody after fixation and cascade step. We analyzed the data by counting foci numbers in a stained cell. CtIP was recruited to DNA lesions in the cells expressing CtIP wild-type, but reduced recruitment in the cells expressing CtIP 8RA (Fig. 11A-D). We also checked the recruitment of RPA and Rad 51 foci in expressed CtIP mutant cells by IF. The cells were transfected with siRNA, against mRNA of CtIP, and recovered using plasmid DNA, expressing CtIP wildtype or mutant, and then DSBs were induced by IR. RPA and Rad51 foci formation poorly occurred in CtIP-deficient cells but recovered in CtIP wild-type expressed cells. The curious thing, we want to know, is the recruitment of RPA and Rad51 in CtIP mutant expressed

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cells. Following the data, RPA and Rad51 were poorly recruited to DSBs (Fig. 12A - E). This means that PRMT5 is important for the recruitment of CtIP, and it affects the recruitment of RPA and Rad51. Based on this data, we guessed that HR efficiency would decrease. To check it, we performed a DR - GFP assay. As we expected, HR deceased in CtIP mutant expressed cells (Fig. 12G and H). In summary, CtIP interacted with PRMT5 and was methylated by PRMT5 in normal cells, and CtIP was recruited to DSBs when the cells were damaged by IR. Whereas CtIP has no functions in PRMT5 deficient cells under induced DNA damage (Fig. 13). Taken together, we suggest that PRMT5-mediated methylation of CtIP at eight Arginine residues is required for HR repair.

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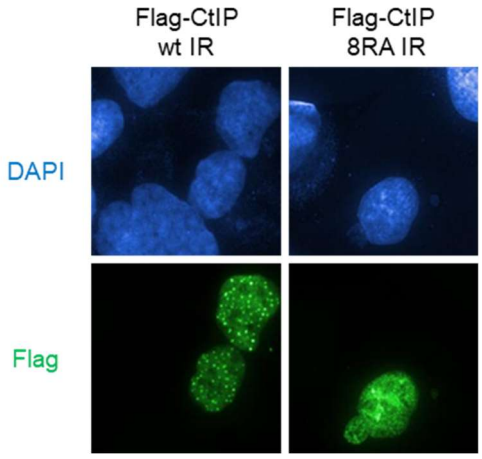
Figure 11

A



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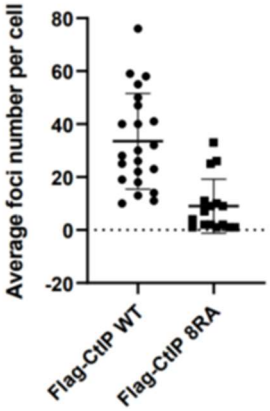

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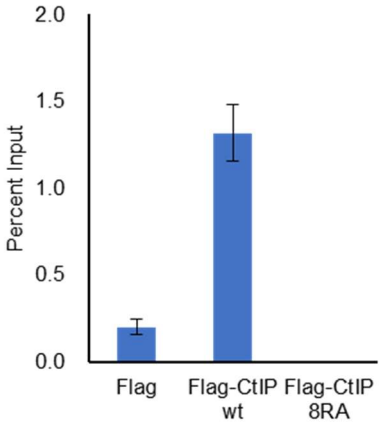

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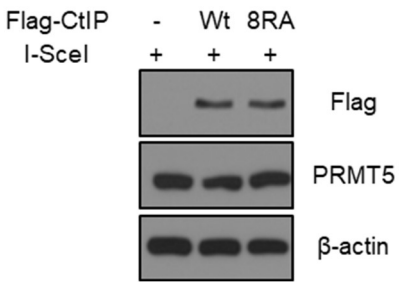

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Figure 11. SDMA - defected CtIP inhibited its recruitment to DSBs.

(A) U2OS cells were transfected with Flag-tagged CtIP wild - type or 8RA constructs. After 48 hours, the cells were induced DNA damage by IR. After 6 hours, the cells were fixed and permeablized. Then, the cells were immunostained with anti-Flag antibodies. (B) The Flag - tagged CtIP foci were quantified as foci number per Flag - positive cells. Data are presented as means ± SD. (C) Chromatin immunoprecipitation (ChIP) was performed for the recruitment of Flag - CtIP wild type or 8RA to DSB lesion using DR - GFP U2OS cells. The cells were transfected with Flag - tagged CtIP wildtype or 8RA constructs. After 48 hours, the cells were transfected again with I - SceI plasmid DNA for inducing DSBs. After 7 hours, the cells were fixed, then, the chromatins were fractionated. The fractionated chromatins were digested by micrococcal nuclease followed by performed immunoprecipitation with anti - CtIP antibody. The recruitment of Flag - tagged CtIP was quantified by RT - qPCR.(D) The batch samples to ChIP were used for the western blot for confirming the expression of Flag - tagged CtIP constructs. The cells were lysed with RIPA buffer at the same time as the initiation time of ChIP. Then, anti - Flag and anti - PRMT5 antibodies were used for western blot detection.

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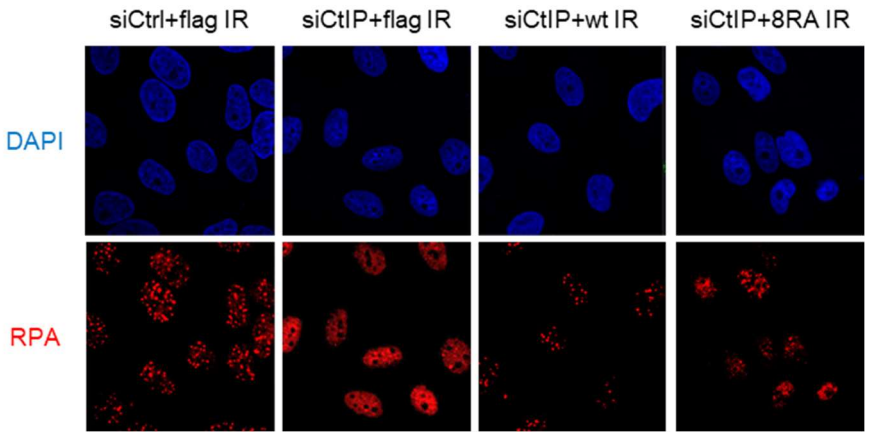
Figure 12

A



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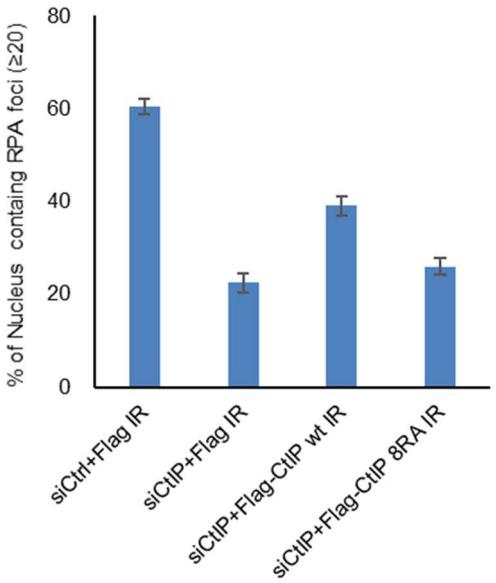

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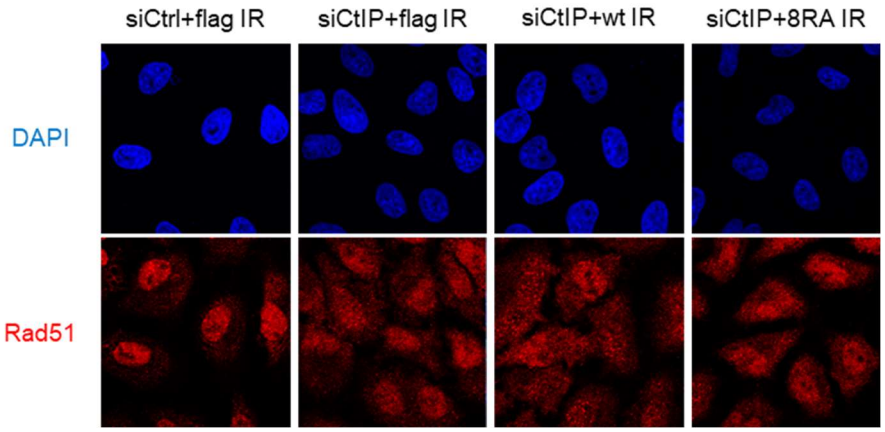

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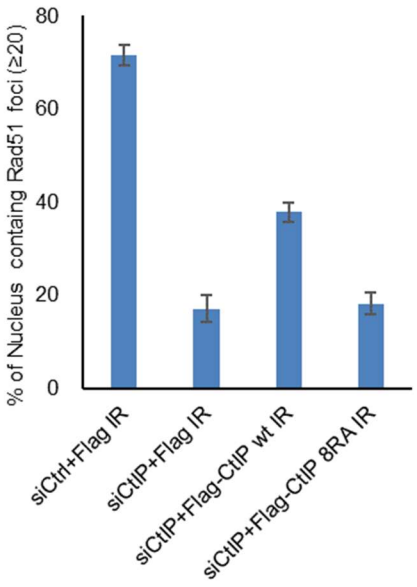

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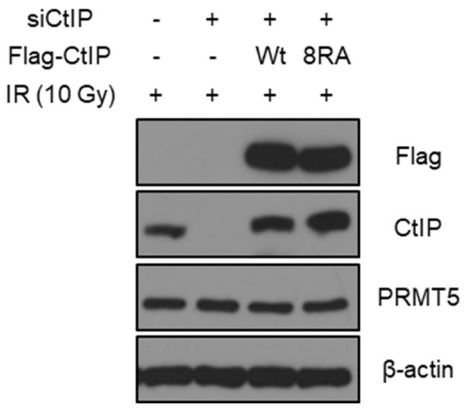

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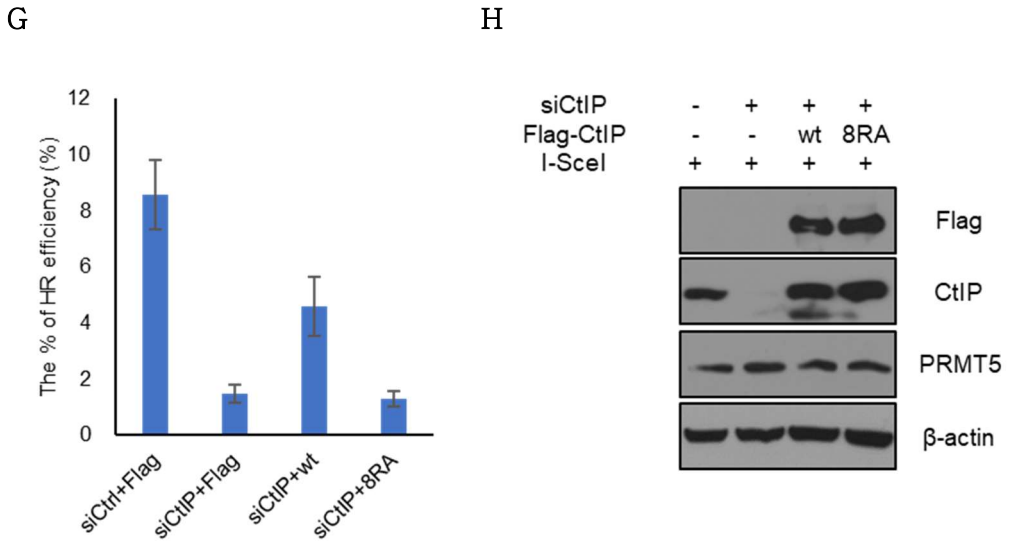

Figure 12. SDMA - Defective CtIP impairs homologous recombination through decreased recruitment of RPA and RAD51.

(A and C) U2OS cells were co - transfected with CtIP construct and siRNA targeting mRNA of CtIP for confirming the effect of CtIP mutant. After 48 hours, the cells were induced DNA damage by IR (10Gy) for 6 hours. Then, the cells were fixed and permeablized. Next, the cells were immunostained with anti - RPA or Rad51 antibodies. (B and D) RPA and Rad51 foci were quantified as foci per nucleus. Data are presented as means ± SD. (E) Batch samples to IF were used for the western blot for confirming the expression of CtIP construct

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and CtIP - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of IF. Then, anti - Flag, CtIP, and PRMT5 antibodies were used for western blot detection. (F) DR - GFP U2OS cells were transfected with CtIP construct and siRNA targeting mRNA of CtIP for 4 hours and then transfected again with I - SceI plasmid DNA for inducing DSBs. After 72 hours, GFP - positive cells were counted by INCell Analyzer 2500HS. The values are the percentage of GFP - expressing cells determined and represent the mean ± SD. (H) Batch samples to DR-GFP assay were used for the western blot for confirming the expression of CtIP constructs and CtIP - knockdown. The cells were lysed with RIPA buffer at the same time as the initiation time of DR - GFP. Then, anti - CtIP, Flag, and PRMT5 antibodies were used for western blot detection.

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Figure 13

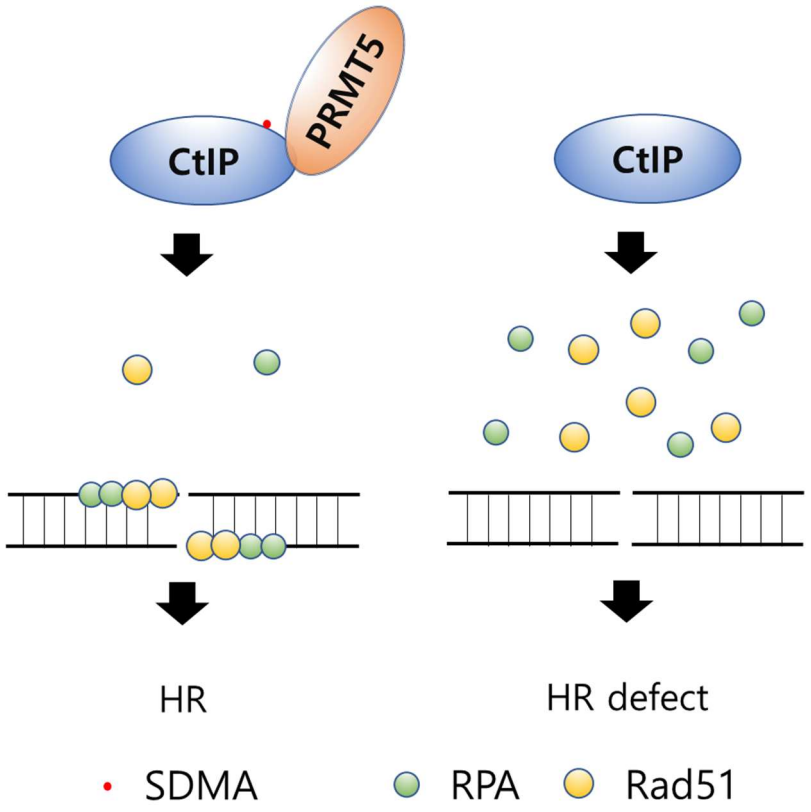

Figure 13. SDMA of CtIP by PRMT5 is essential for Homologous Recombination.

CtIP is recruited to DSBs through its SDMA by PRMT5, consequently, RPA and Rad51 are recruited to DSBs, and promote HR. However, the deficiency of PRMT5 reduced the recruitment of CtIP to DSBs, consequently, the recruitment of RPA and Rad51 is inhibited, and decreased HR efficiency.

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DISCUSSION

Methylation is one of the critical post - translational modifications (PTMs), like phosphorylation, NEDDylation, SUMOylation, ubiquitination, and acetylation. PRMTs catalyze the three types of methylation, which include mono - methylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA), respectively. In this study, we described the function of symmetrically dimethylated CtIP by PRMT5. CtIP interacted with PRMT5 and catalyzed symmetric dimethylation by PRMT5. The inhibited methylation of CtIP by the absence of PRMT5 showed decreased recruitment of CtIP, and consequently, HR efficiency was reduced. To support these data, we used siRNA for MEP50, which is an essential cofactor for the enzymatic activity of PRMT5. So, the methylation of CtIP decreased, but these data were insufficiency proof for the methylation of CtIP by PRMT5 because deficiency of PRMT5 showed a reduced expression level of MEP50, and deficiency of MEP50 also showed a reduced expression level of PRMT5 (Fig. 8B). We treated the PRMT5 inhibitor (PRMT5i), EPZ015666, to block only enzymatic

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activity. The expression level of PRMT5 and MEP50 is still kept although the treatment of PRMT5i. Therefore, we can prove that symmetric dimethylation of CtIP is responsible for PRMT5. Following this, we analyzed CtIP by LC-MS/MS to find specific sites of methylation on CtIP. Thus, we found the 8 putative sites and mutated arginine to alanine all of them. Additionally, we observed a decrease in the SDMA of CtIP and HR efficiency. The data obtained from various experiments strongly suggested and interpreted the SDMA of CtIP by PRMT5. However, we could not specify at which site methylation would occur. To specify the SDMA site, we will mutate each arginine site of CtIP, and check at which site the SDMA band disappears through IP. Next, we will investigate the SDMA of CtIP for its functional regulation in DDR.

In many reports, methylation of protein is mentioned with the other PTMs. So, we expected that methylation of proteins might be involved in the other modification, like ubiquitin ligase and phosphorylation kinase [98, 99]. For example, the phosphorylation of AKT at T308 and S473 has required interaction with PDK1 and SIN1, but these interactions are regulated by the methylation of AKT by PRMT5 [99]. In addition, KLF5 is destabilized by its ubiquitination by Fbw-7γ but inhibited the ubiquitination through the

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methylation by PRMT5 [98]. Therefore, we considered the relationship between methylation and the other modification and tested if decreased PRMT5 led enhance/inhibit phosphorylation of CtIP. In the test, the phosphorylation of CtIP increased by PRMT5 – knockdown (data not shown). it means that PRMT5 has a potential role in the regulation of phosphorylation of CtIP. To make it clear, we will design/perform detailed experiments.

During the study of PRMT5, we were also looking for demethylase, because methylation is a reversible modification. Jing Zhang et all introduced several demethylases [100]. There are 7 arginine – specific demethylases. If we will find demethylase among them, we can discover a detailed mechanism of CtIP in DDR.

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ABSTRACT

PRMT5 regulates homologous recombination repair through CtIP methylation

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Department of Biomedical sciences,

Graduate school of Chosun University

DNA includes plenty of genetic information but is damaged every day by endogenous and exogenous sources. However, there are many proteins in the cells for maintaining DNA integrity. DNA damage has several types of DNA damage, pyrimidine dimers, DNA crosslinks, base oxidation, replication stress, base oxidation, sing - strand break (SSB), and double - strand break (DSB).

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These damages are repaired by base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), non-homologous end joining (NHEJ), and homologous recombination (HR). The worst DNA damage among them is DSB because unrepaired SSBs can lead to DSBs through DNA replication. There are two ways to repair DSBs, they are HR and NHEJ. HR as an error-free repair pathway is mediated by BRCA1 and NHEJ as an error-prone repair pathway is mediated by 53BP1. HR is triggered as activation of ATM, then sequentially recruits RNF8, RNF168, BRCA1, CtIP, Mre11-Rad50-NBS1 (MRN) complex, RPA, and Rad51 to DSBs lesion. In the pathway, DNA end resection is an important step and progressed in the 3 steps, initiation, elongation, and extension. Before the beginning of the DNA end resection, 53BP1 has to reposition from the DSBs, because 53BP1 blocks DNA end resection. At first, after repositioning 53BP1, Mre11 as endonuclease makes a nick on the 5' strands 300~400 nucleotides away from the DNA end. In the second step, Mre11 as an exonuclease processes the DNA from the nick to the DSBs lesion. Finally, EXO1/BLM/DNA2 extends the length of the 3' overhang. To process the DNA end resection, CtIP is a critical factor. p-CtIP stimulates Mre11 endonuclease activity (at T847) and the interaction with

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BRCA1 (at S327). We discovered PRMT5, a new binding partner of CtIP, through yeast-two hybrid (Y2H). PRMT5 catalyzes methylation of the substrate, specifically symmetric dimethylation(SDMA). We performed many experiments for proving that PRMT5 is a new regulator of CtIP. We initially found that CtIP bound on the TIM domain of PRMT5 and PRMT5 bound 801-897a.a legion of CtIP. In addition, we confirmed that PRMT5 catalyzes the symmetric dimethylation (SDMA) of CtIP using PRMT5 inhibitor (EPZ015666) and siRNA targeting mRNA of MEP50 (essential for the enzymatic activity of PRMT5). Moreover, we found 8 putative SDMA sites on CtIP through LC-MS/MS, the cells expressing SDMA-inhibited construct in 8 sites through the mutation arginine to alanine, which reduced both the recruitment of CtIP and the efficiency of HR. Taken together, we suggest that the SDMA of CtIP by PRMT5 is essential for HR.

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