[UCI]I804:24011-200000267058

2019 년 2 월

박사학위 논문

Effect of ubiquitin E3 ligase SIAH1/2 in the DNA double-strand break repair

조선대학교 대학원

의과학과

정 서 연

Effect of ubiquitin E3 ligase SIAH1/2 in the DNA double-strand break repair

2019 년 2 월 25 일

조선대학교 대학원

의과학과

정 서 연

Effect of ubiquitin E3 ligase SIAH1/2 in the DNA double-strand break repair

지도교수 이 정 희

공동지도교수 유 호 진

이 논문을 이학박사학위신청 논문으로 제출함

2018 년 10 월

조선대학교 대학원

의과학과

정 서 연

정서연의 박사학위논문을 인준함

위원장 조선대학교 교 수 장인엽(인)

위 원 부산대학교 교 수 김치대(인)

위 원 조선대학교 부교수 온탁범(인)

위 원 조선대학교 교 수 유호진(인)

위 원 조선대학교 부교수 이정희(인)

2018년 12월

조선대학교 대학원

CONTENTS

ABSTARACT

REFERENCES

CONTENTS OF FIGURES

PART I

Figure 19. Effect of SIAH1 on the clonal survival of Hela cells after IR

국문초록

유비퀴틴 E3 효소 SIAH1/2 가 이중가닥절단 복구에 미치는 영향

정 서 연

지도교수 : 이 정 희

공동지도교수 : 유 호 진

조선대학교 일반대학원

의과학과

DNA에 이중나선절단에 의한 DNA손상시 세포는 상동재결합과 비상동말단결합으로 손상이 복구되어 세포는 항상성을 유지하게 된다. Ubiquitination은 타겟단백질에 ubiquitin을 결합함으로써 프로테오좀에 의한 타겟단백질의 분해를 촉진시키고, 이로 인

해 세포주기, 신호전달, 전사, 세포사멸 및 DNA손상복구등의 다양한 세포내 과정에 관여함이 알려져 있다. 그러나 최근엔 이런 단백질 Ubiquitination이 단백질 분해 촉진이 아니라 가역적 반응으로 타겟단백질의 활성을 조절함이 보고되고 있다. 따라서 본 연구에서는 대표적인 E3 ubiquitin ligase인 SIAH1/2 가 타겟단백질의 Ubiquitination을 통한 단백질 분해가 아니라 가역적으로 활성을 조절함으로써 타겟단백질에 의한 세포내 신호전달 기전을 규명하고자 하였다. Yeast two hybrid 실험을 통해 SIAH1,2 에 결합하는 DNA손상복구단백질 BRCA2와 CtIP를 동정하였고, SIAH1,2가 표적단백질의 Ubiquitination을 매개함을 보았다. 또한 SIAH1/2가 결핍된 세포에서 방사선 조사에 의한 DNA 이중나선절단시 손상 부위에 CtIP, BRCA1, BRCA2 및 Rad51 foci가 감소함을 확인하였다. SIAH1,2의 결핍은 정상세포에 비해 상동재조합에 의한 DNA손상복구 및 DNA end resection 이 저하되었으며, 방사선손상에 의한 세포 손상 민감도 또한 증가됨을 관찰하였다. 따라서 본 연구는 E3 ubiquitin ligase SIAH1/2가 DNA손상복구에 중요한 두가지 단백질인 BRCA2와 CtIP의 ubiquitination을 통해 단백질 분해가 아니라 그 활성을 조절함을 증명한 연구로 이는 SIAH단백질이 DNA손상복구 및 세포 안정성 유지에 중요한 역할을 하는 새로운 단백질 임을 밝힌 최초의 보고이다.

INTRODUCTION

DNA double-strand breaks(DSBs) are generated by ionizing radiation and various DNA damage agents. DSBs are deleterious DNA lesions and if left unrepaired result in severe genomic instability, chromosomal rearrangements, cellular senescence, and cell death[1]. Therefore, it is of the utmost importance that cells have a mechanism to quickly repair DSBs. Cells use two main mechanisms to repair DSBs; non-homologous end-joining (NHEJ) and homologous recombination(HR). Whereas NHEJ repair predominates in cells at the G0/G1 phase in the cell cycle and it occurs through a ligation of the broken DNA ends in the absence of homology, error-prone, HR repair occurs in cells at the S and G2 phases and it ensures accurate, error-free repair of DNA damage(Figure1)[2, 3].

HR is initiated following 3' -5' DNA end resection coordinated by MRN complex and the resulting single strand DNA (ssDNA) is stabilized through RPA (replication protein A) coating[4, 5]. Following recruitment of MRN complex at

DSB site, CtIP is recruited to DSB site and promotes DNA end resection[5].

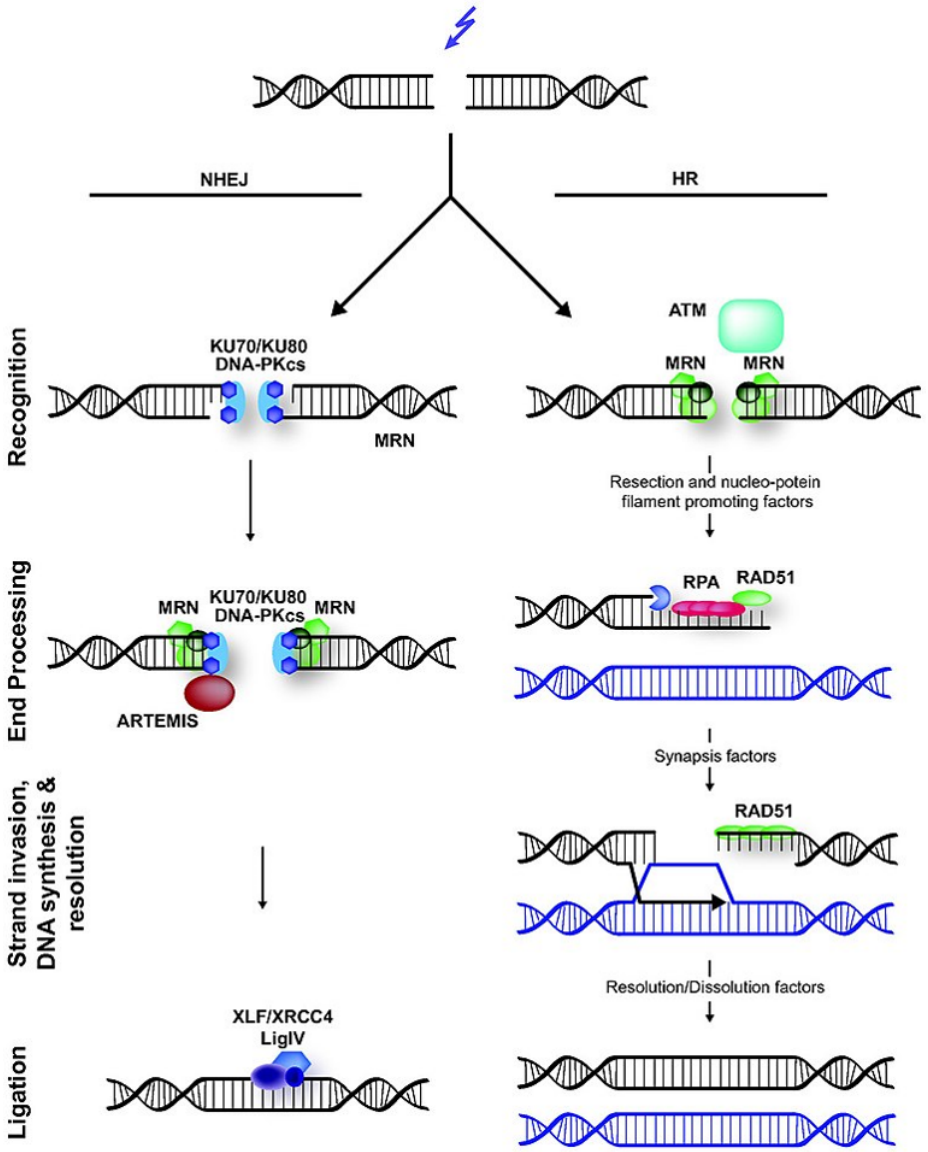


Figure 1. Non-homologous end joining and Homologous recombination in during DNA double-strand break.

BRCA2 (breast cancer type 2 susceptibility protein), with the help of BRCA1 (breast cancer type 1 susceptibility protein) and PALB2 (partner and localizer of BRCA2), promotes the loading of RAD51 onto RPA-coated ssDNA, which then enables strand invasion of a homologous DNA sequence in a sister chromatid and formation of a complex DNA arrangement[7, 8].

DDR process is tightly controlled by reversible protein post-translational modifications(PTMs), including phosphorylation, sumoylation, ubiquitination, methylation, acetylation that combine to propagate the DNA damage signal to elicit cell cycle arrests, DNA repair, apoptosis, and senescence. Ubiquitination is process involving the covalent attachment of 76 a.a ubiquitin (Ub) protein to target molecules, and regulates DNA damage signal[9-11]. The formation of covalently linked ubiquitin-protein conjugates via a three-step enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3), which cooperate to transfer the ubiquitin moiety to a lysine residue in the target protein[12]. Mono-ubiquitination plays a role in recognizing

DNA double-strand breaks (DSBs), K63-linked Ub chains are involved in the generation of signaling platforms during DNA repair[13] and poly-ubiquitin chains covalently connected via K48 linkages mainly target proteins for degradation by the proteasome, whereas non-proteolytic ubiquitination via other chain linkages has an important regulatory role in double-strand break (DSB) signaling and repair[12, 14].

SIAH (Seven in Absentia Homolog) is a mammalian homolog of Seven in Absentia (SINA), a Drosophila protein that has a function in eye development[15]. Two SIAH homologs have been identified in the human genome, SIAH1 and SIAH2, both of which encode functional proteins. These two proteins share high sequence similarity and presumably high structural homology. Also, SIAH1 and SIAH2 protein are ring finger E3 ubiquitin ligases composed of a catalytic RING domain, zinc finger domain, and a substrate binding domain(SBD)[16]. They mediate the ubiquitination and subsequent proteasomal degradation of biologically important target proteins; these include proteins that regulate general functions such as cell

cycle control, apoptosis, hypoxia, tumorigenesis and DNA repair[17-20].

Interestingly, we found that SIAH interacts with homologous recombination repair-related protein, CtIP and BRCA2, through yeast two hybrid screening. We identified in this study that CtIP binds to SIAH2, and BRCA2 binds to SIAH1, respectively. This suggests that SIAH may be involved in the DNA damage repair, and it is aussumed that CtIP and BRCA2 proteins can be ubiquitinated by E3 ubiquitin ligase SIAH.

MATERIALS AND METHODS

1. Cell culture and treatment

The human osteosarcoma bone morphogenetic cell line U2OS and human cervix adenocarcinoma cell line HeLa, and human embryonic kidney cell line HEK293T were cultured in DMEM medium supplemented with 10 % fetal bovine serum(FBS) and streptomycin (0.1 mg/ml), penicillin (100 units/ml). Cell growth was monitored under an inverted microscope. Upon reaching 70-80 % confluency, cells were digested with 0.5 % trypsin-EDTA before being passaged. All cell lines were maintained at 37 ° C and in 5 % CO2 at saturated humidity. Cells in exponential growth were harvested for subsequent experiments. To induce DNA double strand breaks, exponentially growing cells were irradiated from ^{137}Cs source (Grammacell 3000 Elan irradiator, Best Theratronics) and allowed to recover at 37 ℃ incubator for various times. 4-Hydroxytamoxifen (4-OHT) (Sigma catalog#H7904).

2. Plasmid construct

Indicated cDNAs were cloned into pcDNA3.1 HA-ubiquitin, pcDNA3.1 flag-CtIP, pcDNA3.1-HA-CtIP, BRCA2 cDNA, pcDNA3.1 HA-SIAH1, Myc-DDk-SIAH2(origene cat#RC203802). The full length SIAH1 cDNA was amplified from cDNA of HeLa cells by PCR using the SIAH1 primers 5'-ACCGAATTCATGAGCCGTCAGACTGCTACA-3' (Forward primer) and 5'-GGGCTCGAGACACATGGAAATAGTTAC-3' (Reverse primer). The amplified SIAH1 cDNA construct was cloned into the expression vector pcDNA3.1 in frame with the HA tag

3. Antibody

Anti-HA(F-7)sc-7392, anti-BRCA2 A303-434A_bethyl, anti-SIAH2(N-14)SC-5507, anti-SIAH1(N-15)sc-5505, anti-Rad51(H-92)sc-8349, anti-RPA(Ab-2) NA18_Calbiochem, anti-CtIP(T-16)sc-5970, anti-BRCA1(D-9) sc-6954, anti-γH2AX(ser139 05-363_millipore), anti-FLAG(sigma F1804),

anti-Cyclin A(C-19)sc-596, anti-beta action(C-4)sc-47778, anti-CENP-F(H260)sc-22791, anti-Histone H3 (#4499P), anti- P-Histone H3 (cell signaling 9701S)

4. Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP40, 0.5 % sodium deoxycholate(NaDOC), 0.1 % sodium dodecyl sulfate(SDS), 1 % TritonX-100) with protease inhibitors (Roche Diagnostic Corp.). Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1hr with TBS-t (10mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20) containing 5 % skim milk and then incubated at 4° C with primary antibodies(1:1000). The blots were washed six times for 10min with 0.1 % Tween 20 containing TBS-t and then incubated for 2hrs with peroxidase-conjugated secondary antibodies (1:4000) at RT. The

membranes were washed six times for 10min, and developed using an enhanced chemiluminescence detection system.

5. Co-immunoprecipitation and ubiquitination assay.

For the immunoprecipitation assay, Cell lysates were prepared by lysing cells in NP-40 buffer[1% NP40, 50mM Tris HCL(pH7.5), 150mM NaCl, 5mM EDTA] with protease inhibitors, followed by pre-clearing with protein G Sepharose(GE Healthcare). The precleared lysates were incubated with either antibodies for overnight at 4 °C After the addition of fresh protein G sepharose beads, the reaction was incubated for 4hrs at room temperature. The resulting complex with antibody and sepharose G bead was centrifuged at 3000 rpm for 5 min and then washed with NP40 buffer three times. The proteins were eluted from the beads by boiling in an SDS sample buffer. They were then analyzed using a western blotting with the corresponding antibodies. For the ubiquitination assay, cells were transfected with indicating plasmids and siRNA. Cell lysates were prepared by lysing cells in ubiquitinaion buffer[1% NP40, 150mM NaCl. 50mM Tris

HCL(pH8.0), 10mM NaF, 1mM Na_3 VO_4 , 5mM EDTA, 1mM EGTA, 1mM DTT] with protease inhibitors, followed by pre-clearing with protein G Sepharose(GE Healthcare) .The precleared lysates were incubated with either antibodies for overnight at 4 ° C After the addition of fresh protein G sepharose beads, the reaction was incubated for 4hr at room temperature. The resulting complex with antibody and sepharose G bead was centrifuged at 3000 rpm for 5 min and then washed with NP40 buffer three times. The proteins were eluted from the beads by boiling in an SDS sample buffer. They were then analyzed using a western blotting with the corresponding antibodies.

6. DNA end resection

DNA end resection was performed as described in references.[21] AID-DivA U2OS cells were transfected with siRNA. After 48hr, AsiSI-mediated DSBs were induced by treatment with 4-OHT for4hr. Genomic DNA was isolated using 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 quantitative polymerase chain reaction(qPCR)

7. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were irradiated with ionizing radiation. Cells were then washed twice with PBS, fixed with 4 % paraformaldehyde for 10min and ice-cold 98 % methanol for 5min, followed by permeabilization with 0.3 % Triton X-100 for 15min at room temperature. Next, the cover slips were washed three times with PBS and then blocked with 5 % BSA in PBS for 1hr. The cells were single or double immunostained with primary antibodies against various proteins overnight at 4° C. Next, the cells were washed with PBS and then stained with Alexa Fluor 488 (green, Molecular Probes) or Alexa Fluor 594 (red, Molecular Probes) conjugated secondary antibodies, as appropriate. After washing, the cells were mounted using mounting medium with 4, 6-diamidino-2-phenylindole (Vector Laboratories,

Burlingame, CA, USA). Fluorescence images were taken using a confocal microscopy (Zeiss LSM510 Meta; Carl Zeiss) analyzed with ZEN software.

8. Chromatin Fractionation

HeLa and HEK293T cells were treated with IR and harvested. The pellet was resuspended and incubated for 10min in ice-cold buffer containing 10mM Hepes-KOH (pH 7.9), 1.5mM MgCl2, 10mM KCl, 0.2 % Triton X-100 and complete protease inhibitor cocktail (Roche Diagnostic Corp). The suspension was homogenized and the supernatant containing the cytoplasmic fraction was collected after centrifugation 15min at 400*g* at 4° C. The pellet was washed in ice-cold PBS, resuspended in cold buffer containing 420mM NaCl, 20mM Hepes-KOH (pH 7.9), 20 % glycerol, 2mM MgCl2, 0.2mM EDTA, 0.1 % triton, 0.5mM DTT, and complete protease inhibitor cocktail (Roche Diagnostic Corp) and incubated on ice for 1hr. The supernatant containing the nuclear fraction was collected after 15min centrifugation at 18000*g* at 4° C. The chromatin-containing pellet was

resuspended in cold PBS supplemented with 600mM NaCl, 1 % N-octyl glucoside, and 125 units of DNase, incubated for 30min in an ultrasonic bath and centrifuged for 15min at 18000*g* at 4° C. Chromatin proteins were collected with the supernatant.

9. Clonal survival assay

After treatment with IR , $1x10^3$ cells were immediately seeded onto a 60 mm dish in duplicated and grown for 2weeks at 37° C to allow colony formation. Colonies were fixed with 95 % methanol for 10min and stained with 2 % methylene blue in 50 % ethanol. The fraction of surviving cells was calculated as the ratio of the plating as efficiencies of treated cells to untreated cells.

10. comet assay

The alkaline version of the comet assay (single cell agarose gel electrophoresis) was performed as described previously. For the comet assay, HeLa cell was treated with 10 Gy IR by followed by incubation in culture medium

at 37° C. Cells were then harvested ($1x10^5$cells/ml), homogenized with low-melting point agarose, spread on a microscope slide pre-coated with normal-melting-point agarose and covered with a coverslip. After 30min at 4° C, the coverslip was removed from the slides. Slides were submerged in cold lysis solution(2.5M NaCl, 0.1M EDTA, 10mM Tris-HCl(pH 10), 10 % dimethylsulfoxide and 1 % Triton-X100) for 1hr. After lysis, the slides were placed in an electrophoresis chamber, covered with electrophoresis buffer (300mM NaOH, 200mM EDTA) and left for 30min for the DNA to unwind. The electrophoresis an for 30min (1V/cm tank length), after which the slides were and fixed in 70 % ethanol for 5min. air-dried and neutralized slides were stained with 30-50 ul ethidium bromide (20 mg/ml). Cells were screened per sample in a fluorescent microscope. Average comet tail moment was scored for 40-50 cells/slide using a computerized image analysis system(Komet 5.5; Andor Technology, South Windsor, CT, USA).

11. Homologous recombination assay (DR-GFP assay)

The recombination repair substrate DR-GFP is composed of two differentially mutated GFP genes, SceGFP and iGFP when I-sce1 endonuclease is expressed in cells containing the DR-GFP substrate in their genome, a DSB will be introduced at the I-Sce1 site in the SceGFP gene. Repair of the DSB by a noncrossover gene conversion with the downstream iGFP gene results in reconstitution of functional GFP gene, invoving loss of the I-Sce1 site and gain of the Bcgl site. Because the I-SceI site mutation in the SceGFP gene entails 11bp changes including the introduction of two sop codons, homologous recombination between SceGFP and iGFP is necessary to resore a functional GFP gene. Hela containing a stably integrated copy of the DR-GFP reporter were used to measure the repair of I-SceI-induced DSBs by HR. cells were transfected with control or siRNA, and then transfected with the I-SceI expression vector. After 48hr, GFP-positive cells measured by flow cytometry. Hela containing a stably integrated copy of the DR-GFP reporter were used to measure the repair of I-SceI-induced DSBs by HR.

Cells were transfected with control or siRNA, and then transfected with the I-SceI expression vector. After 48hr, GFP-positive cells measured by flow cytometry.

RESULT

PART I .

The ubiquitinated CtIP by SIAH2 controls DSB repair choice between non-homologous end joining and homologous recombination

1. Identification of E3 ubiquitin ligase SIAH2 as a CtIP-associated protein

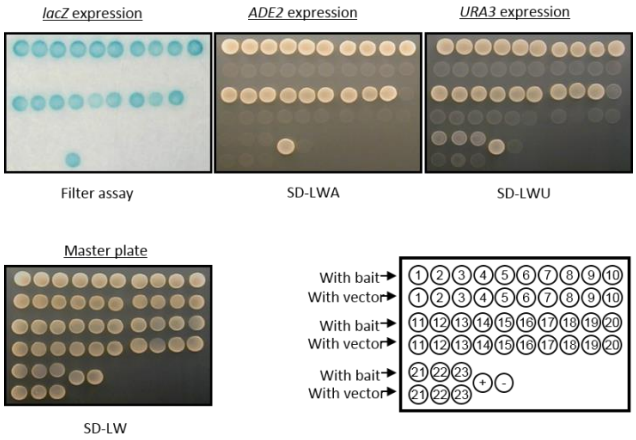
HR activity is based on CtIP which has function of initiation of DNA end resection and BRCA1 recruitment to DSB site. In a yeast two-hybrid screening using CtIP(containing 500a.a-end) as a bait, we identified CtIP-binding proteins, including SIAH2(Figure2A). We validated this interaction by co-immunoprecipitation with antibody against SIAH2 or CtIP. We found that SIAH2 associated with CtIP(Figure2B). To examine further this, we transfected with flag-tagged SIAH2 and HA-tagged CtIP cDNA into HEK293T cells and performed

co-immunoprecipitation with anti-flag antibody(Figure2C). We confirmed that CtIP associates with SIAH2 in vivo.

To identify the region of SIAH2 that are responsible for the CtIP-SIAH2 interacting, we generated deletion mutants of SIAH2, encoding flag-tagged full length(WT), RING domain(△RING), ZINC domain(△ZINC), SBD domain(△SBD) deletion mutants of SIAH2(Figure2D). HEK29T cells were transiently co-transfection with these constructs and HA-tagged CtIP, co-IP and western blotting were performed using HA and flag antibodies. We found that the △RING and △ZINC mutant of SIAH2 were weakly bound to the CtIP, but the △SBD mutant of SIAH2 was not bound to the CtIP(Figure2E). Though series of deletions separating functional domains in SIAH2, we identified the SBD domain(residue 130-322 a.a) as important for binding to CtIP.

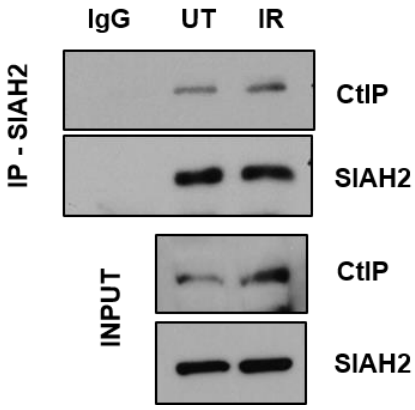
Figure 2

A.

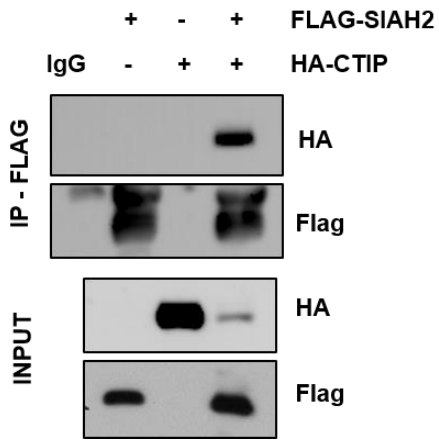


Prey ID	Description
AD Hybrid 1-35	SIAH1 (NM_003031)
AD Hybrid 36-43	SIAH2 (NM_005067)
AD Hybrid 44, 46	PCNA (NM_002592)
AD Hybrid 47	GEMIN4 (NM_015721)
AD Hybrid 48,49	PRMT5 (NM_001282954)
AD Hybrid 50	RPL5 (NM_000969)
AD Hybrid 51	CENPC (NM_001812)
AD Hybrid 52	EIF4A1 (NM_001416)
AD Hybrid 53	NACA (NM_001113202)
AD Hybrid 54	GAPDH (NM_001256799)
AD Hybrid 55	HSPD1 (NM_002156)
AD Hybrid 56	TXNRD2 (NM_006440).

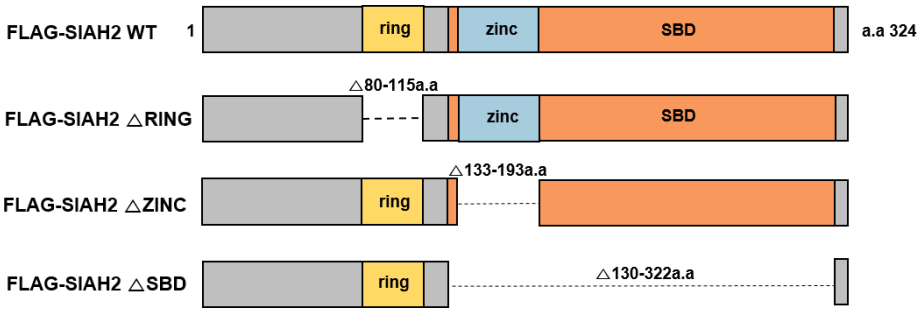
B.



C.



D.



E.

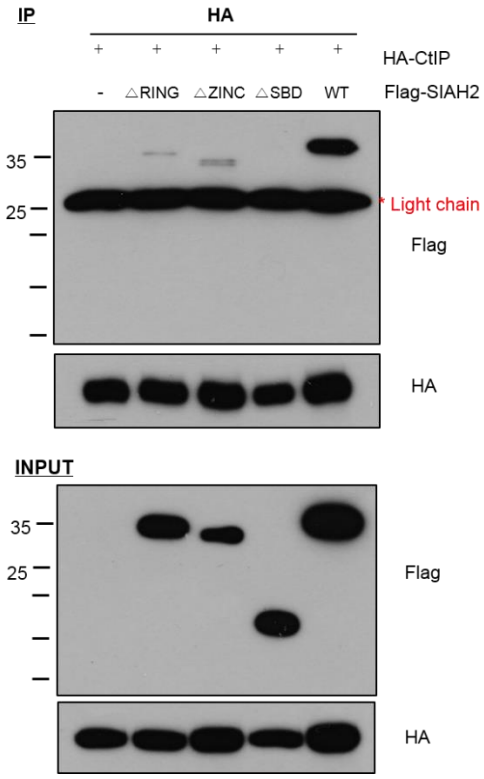


Figure 2. E3 ubiquitin ligase SIAH2 interacts with CtIP

(A) Yeast two-hybrid assay using CtIP as bait. (B) HEK293T cells were untreated or treated with IR, and cell lysates were subjected to immunoprecipitation using an anti-SIAH2 antibody followed by immunoblotting using anti-SIAH2 or anti-CtIP antibodies. (C) HEK293T cells were co-transfected with flag-SIAH2 and with HA-CtIP construct. After 48hr, the interaction between exogenously expressed flag-SIAH2 and HA-CtIP was determined by immunoprecipitation and western blotting with the indicated antibodies. (D) Schematic representation of SIAH2. FLAG-SIAH2-WT(1-324a.a), FLAG-SIAH2△RING(Ring figer domain deletion mutant), FLAG-SIAH2 △ZINC(zinc figer domain deletion mutant), FLAG-SIAH2△SBD(substrate binding domain deletion mutant) (E) HEK293T cells were co-transfected with the indicated flag-tagged SIAH2 constructs along with those encoding HA-tagged CtIP. After 48hr, cells were lysed and subjected to co- immunoprecipitation (IP) using anti-HA antibody followed by western blotting with the indicated antibodies.

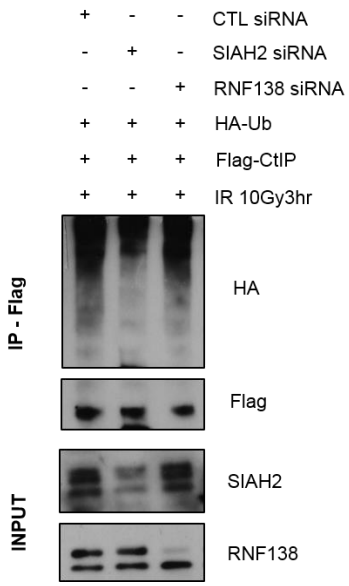
2. E3 ubiquitin ligase, SIAH2 promotes ubiquitination of CtIP

To investigate the physiological relevance of CtIP-SIAH2 interaction, we first asked whether SIAH2 ubiquitinates CtIP. Previous reports have shown that RNF138 mediate ubiquitination of CtIP as an E3 ubiquitin ligase on DNA damage response[22]. Since SIAH2 is also known as an E3 ubiquitin ligase, we investigated to CtIP ubiquitination by SIAH2 on DNA damage response. We co-transfected with either SIAH2 siRNA or RNF138 siRNA, HA-tagged ubiquitin and flag-tagged CtIP into HEK293T cells, co-IP was performed using flag antibody. In SIAH2 or RNF138 depleted cells, CtIP ubiquitination was decreased compared with control cells. Moreover, CtIP ubiquitination was lower in the absence of SIAH2 and was comparable to that of a RNF138 knockdown. These results suggest that SIAH2 may act as a more potent E3 ubiquitin ligase than RNF138 in CtIP ubiquitination(Figure3A). To further investigation the SIAH2-mediated CtIP ubiquitination, we examined co-IP in the presence or absence of flag-tagged SIAH2 cDNA. We observed that ubiquitination of endogenous CtIP was more

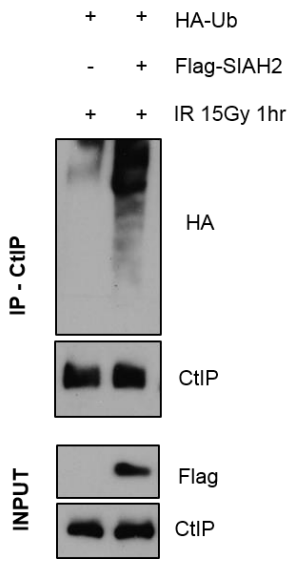
increase than control cells(Figure3B). Next, we examined whether ubiquitination of CtIP is regulated by DSB. As previously reported that CtIP ubiquitination is increased by IR treatment[23], exogenous HA-tagged CtIP ubiquitination was significantly induced following IR exposure(Figure3C). These results suggest that SIAH2 plays a critical role in regulating the DNA damage-induced CtIP ubiquitination.

Figure 3

A.



B.



C.

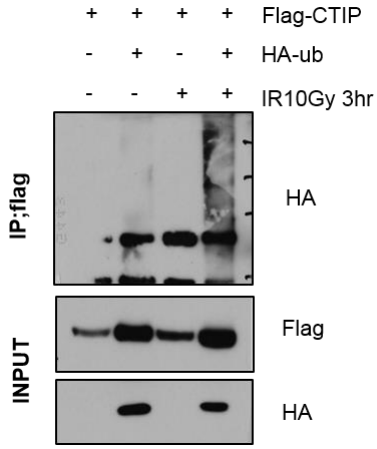


Figure 3. SIAH2 mediates ubiquitination of CtIP in IR-induced DNA damage response

(A) HEK293T cells were co-transfected with the indicated siRNA, flag-tagged CtIP and HA-tagged Ub. Cell Lysates were co-immunoprecipitated with anti-flag antibody followed by immunoblotting using anti-flag or anti-HA antibodies. (B) HEK293T cells were co-transfected with indicated. Cells were treated with IR(15Gy) for 1hr and immunoprecipitated with anti-CtIP antibody and performed western blotting with indicated antibody. (C) HEK293T cells were transiently co-transfected with flag-tagged-CtIP and HA-tagged-Ub construct. After 48hr, cells were treated with IR(10Gy) for 3hr. Total cell lysates were subjected to immunoprecipitation with anti-flag and analyzed by western blot with HA-antibody.

3. SIAH2 is required for the recruitment of CtIP and BRCA1 to DSB sites

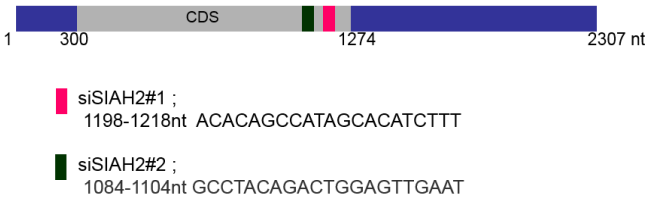
SIAH2 is a key E3 ubiquitin ligase of CtIP ubiquitination, we thus hypothesized that SIAH2 is also contributed to DNA damage response through regulation of CtIP ubiquitination. First, we designed two type of siRNA, recognizes CDS sequence of SIAH2 mRNA. We named this siRNA to siSIAH2#1 and siSIAH2#2(Figure4A). To check of siRNA efficiency, we performed western blotting using anti-SIAH2 antibody following each siRNA treatment (Figure4B).

To investigate the role of SIAH2 in DDR, first, we examined whether SIAH2 affects the foci formation of CtIP and BRCA1. U2OS cells were transfected with SIAH2 siRNA and induced IR during indicated time points, and Immunostaining was performed using anti-CtIP and anti-BRCA1 antibodies. SIAH2-depleted U2OS cells had significantly fewer CtIP and BRCA1 foci following IR exposure when compared to control cells, and the percentage of cells with more

than ten foci remained low throughout the entire time course, indicating that SIAH2 facilitates recruitment of CtIP and BRCA1 to the damage foci(Figure5A,B).

Figure 4

A.



B.

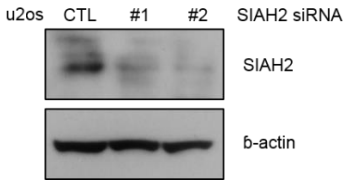
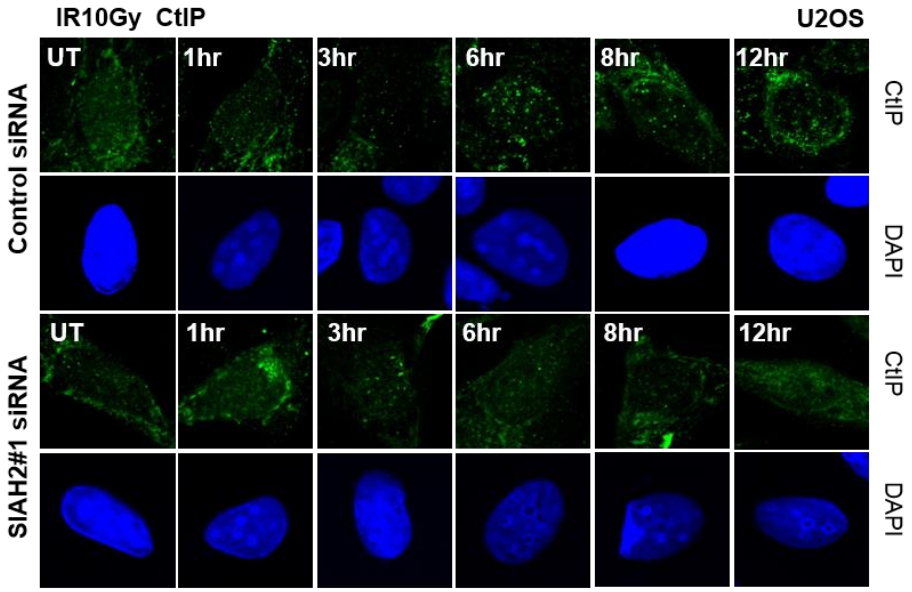
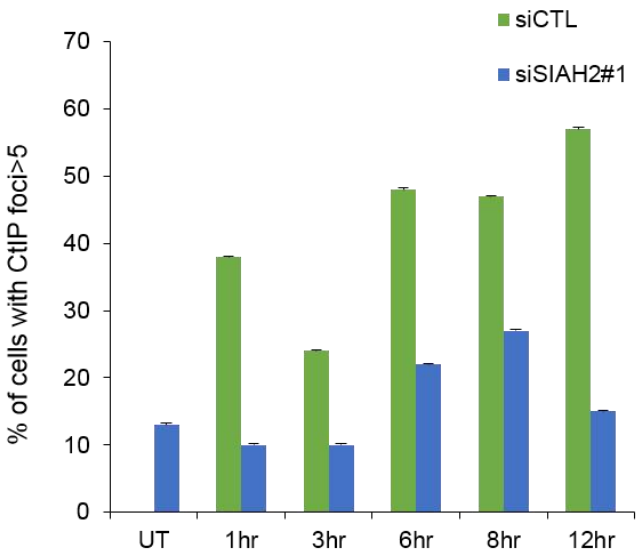


Figure 4. Transfection efficiency of two SIAH2 siRNAs

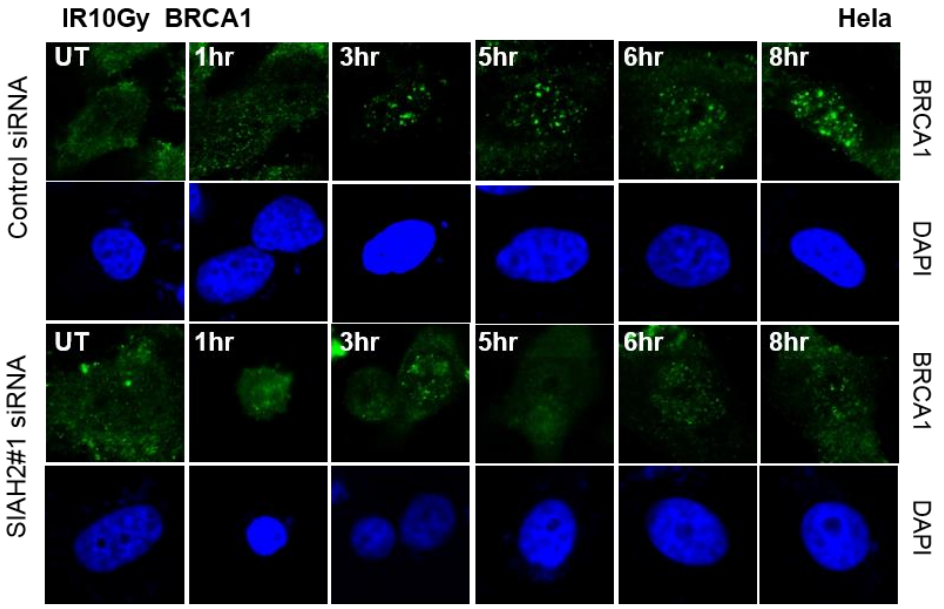
(A) Schematic representation of SIAH2 siRNA sequence. (B) The levels of endogenous SIAH2 in control and SIAH2 siRNA treated cells were analyzed by western blotting.

Figure 5 A.





B.



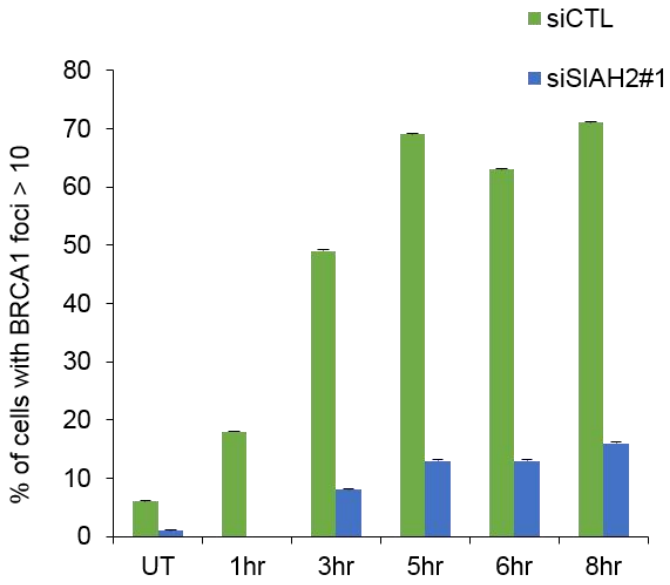


Figure 5. SIAH2 knockdown cells exhibit impaired IR-induced CtIP and BRCA1 foci formation

Control- and SIAH2-depleted U2OS cells were untreated or treated with IR(10Gy) and fixed at the indicated time point. Immunostaining experiments were performed using an anti-CtIP(A) or anti-BRCA1(B) antibodies. Representative images (upper panel) and quantification(lower panel) of CtIP or BRCA1 foci in control and SIAH2 depleted U2OS cells.

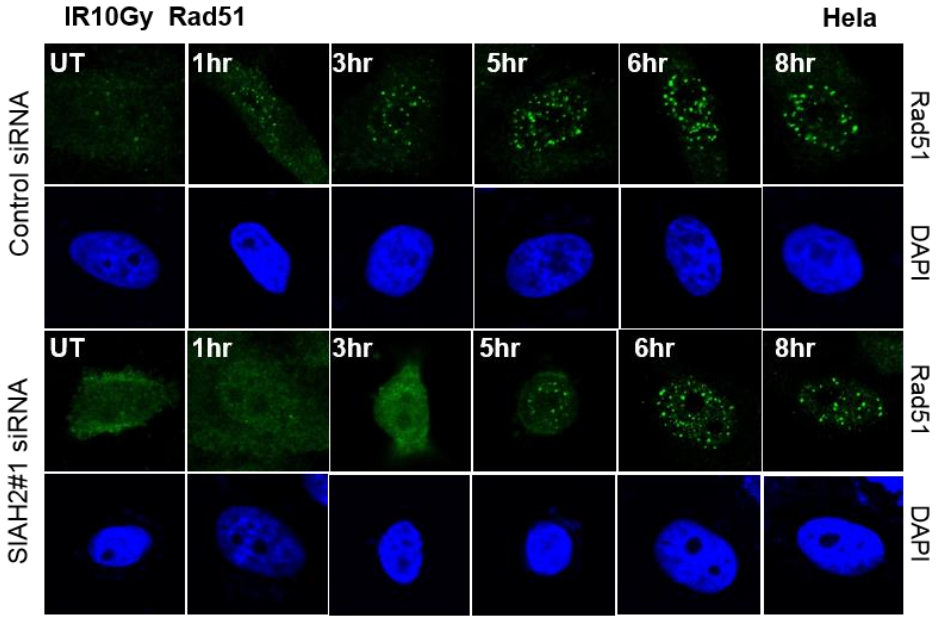
4. SIAH2 depletion reduces HR

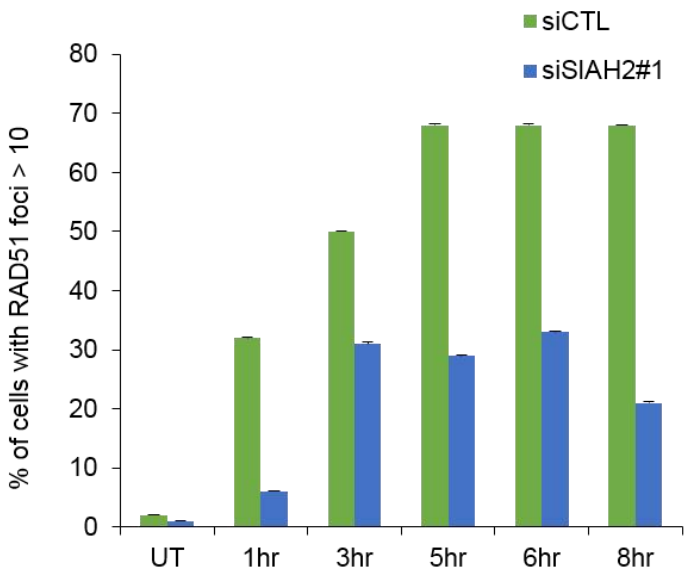
Previously reported that BRCA1, CtIP and Rad51 are important for DNA repair in particular homologous recombination, which enables repair of double-stranded DNA breaks[24-26]. In the above result, SIAH2 regulated CtIP and BRCA1. Therefore, we hypothesized that SIAH2 would also affect recruitment of Rad51 and DNA repair. Hela cells were transfected with SIAH2 siRNA and induced IR during indicated time points, and Immunostaining was performed using anti-Rad51 antibody. SIAH2 depleted HeLa cells were decreased Rad51 foci following IR exposure when compared to control cells(Figure6A). In the opposite concept, we observed foci formation of Rad51 in HeLa cells overexpressed SIAH2. As a result, foci formation of Rad51 increased dependent to concentration of FLAG-tagged SIAH2(Figure6B). To further confirm these effect of SIAH2, we performed re-transfection of SIAH2 into SIAH2-knockdown Hela cells. We found that SIAH2 knockdown decreased Rad51 foci formation, reintroduction of SIAH2 cDNA into cells depleted of endogenous SIAH2 recovered Rad51 foci

formation(Figure6C,D). These results suggest that SIAH2 required for recruitment of Rad51 to the damage foci.

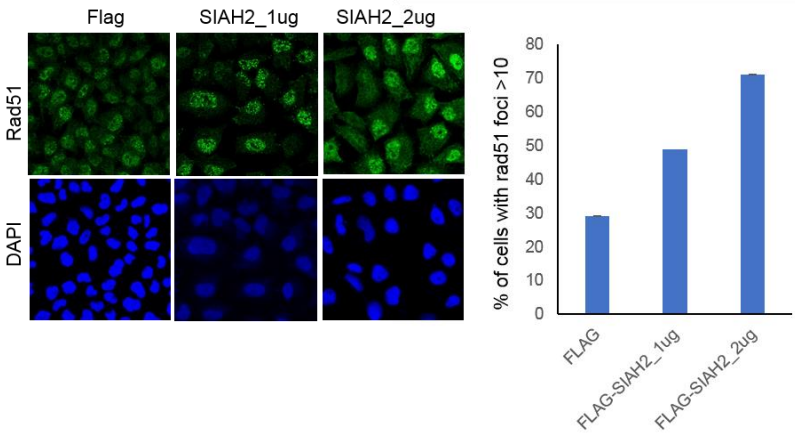
Because Rad51 directly mediates DSB repair through HR, and because SIAH2 regulates foci formation of Rad51, we predicted that HR of DSBs would be affected in SIAH2 knockdown cells. To determine whether SIAH2 play a role in regulation of HR and NHEJ repair, DR-GFP-U2OS cells were transiently transfected with either contol siRNA or SIAH2 siRNA. After 4hr, these cells were transfected with the I-SceI expressing construct and then measured GFP-positive cells, followed by flow cytometry analysis. The HR efficiency was significantly reduced in the SIAH2 siRNA relative to control siRNA-trnsfected cells(Figure6E). Knockdown of SIAH2 caused an increased NHEJ repair(Figure6F), indicating that SIAH2 stimulates HR and restricts NHEJ. These results suggest that SIAH2 promotes CtIP-mediated HR and counters NHEJ. Thereby, SIAH2 regulate to between HR and NHEJ balance.

Figure6 A.



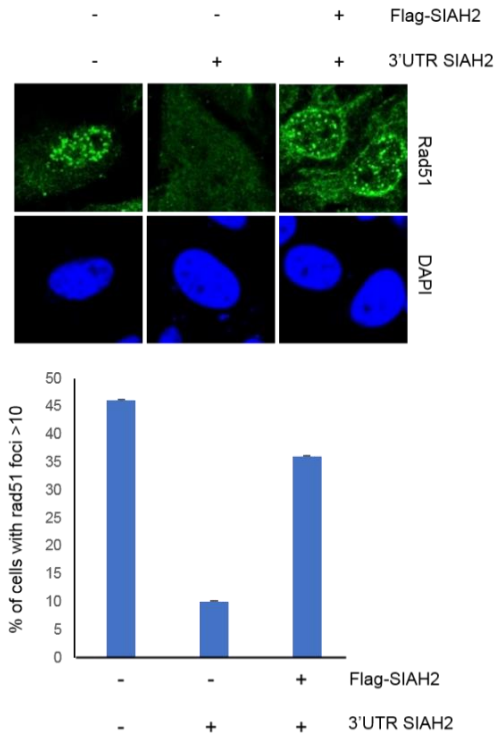


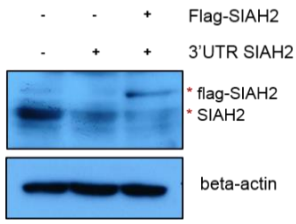
B.



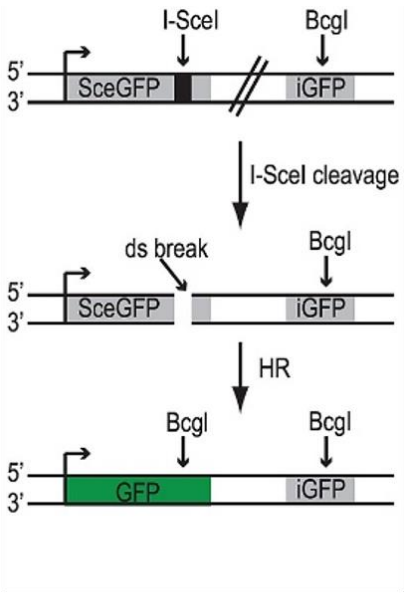
C.

D.

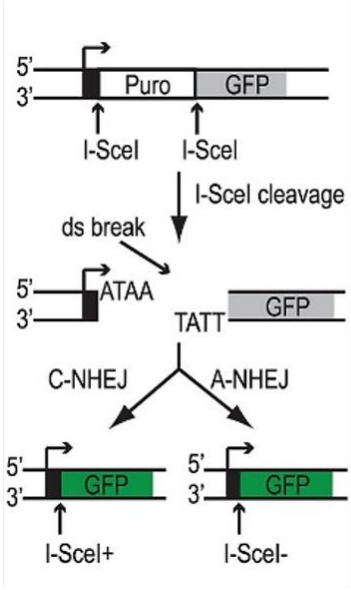


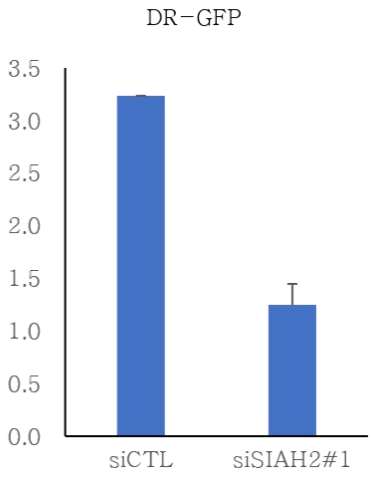


E.



F.





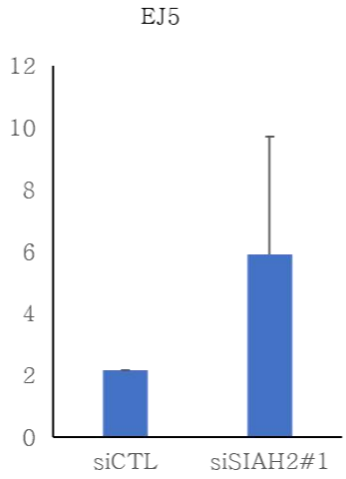


Figure 6. SIAH2 functions during DSB repair

(A) Control and SIAH2-depleted Hela cells were left untreated or treated with IR

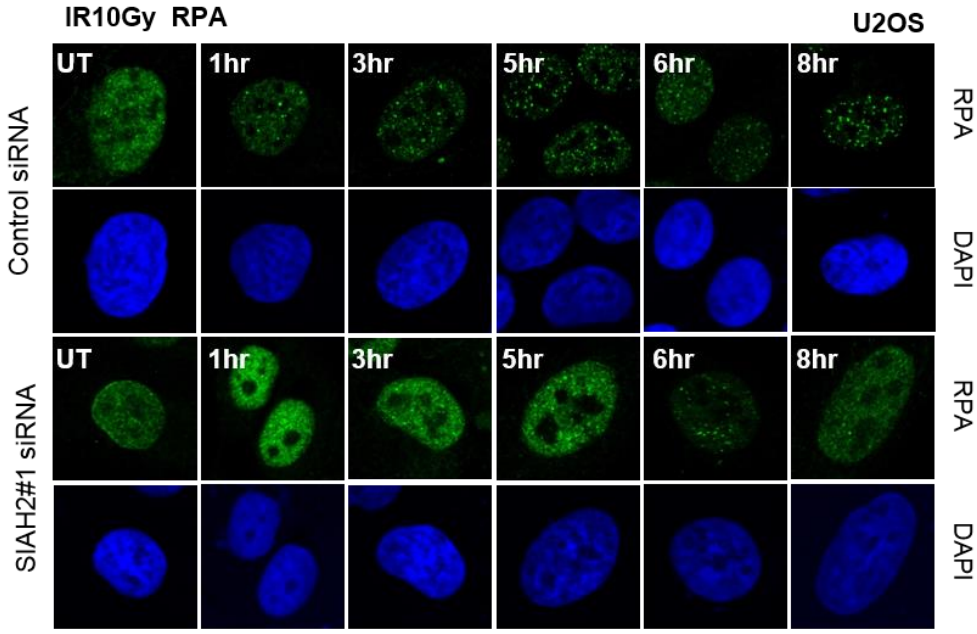
for indicated times and then fixed for immunofluorescence staining of Rad51. DAPI was used for nuclear staining. Representative images(upper panel) and quantification(lower panel) of Rad51 foci in control and SIAH2-depleted Hela cells. (B) Hela cells were transfected with indication dose of flag-SIAH2 vector and then fixed and immunostained following IR treatment. (C. D) SIAH2-depleted Hela cells were transfected with flag-tagged siRNA-resistant SIAH2 construct. After 48hr, cells were treated with IR(10Gy). (C) Cells were fixed and immunostained with Rad51 antibody. Quantification of cells with Rad51 foci in nuclei. The histogram shows the percentage of cells containing >10 nuclear Rad51 foci. (D) Hela cells were transiently transfected with the indicated siRNA and/or vector. (E. F) A diagram of the fluorescence-based assay for HR(E) and NHEJ(F) is shown(upper panel). (E) The efficiency of HR repair was measured by FACS analysis in DR-GFP-U2OS cells transfected with either control or SIAH2 siRNA. (F) The efficiency of NHEJ repair was measured by FACS analysis in EJ5-GFP-U2OS cell transfected with either control or SIAH2 siRNA.

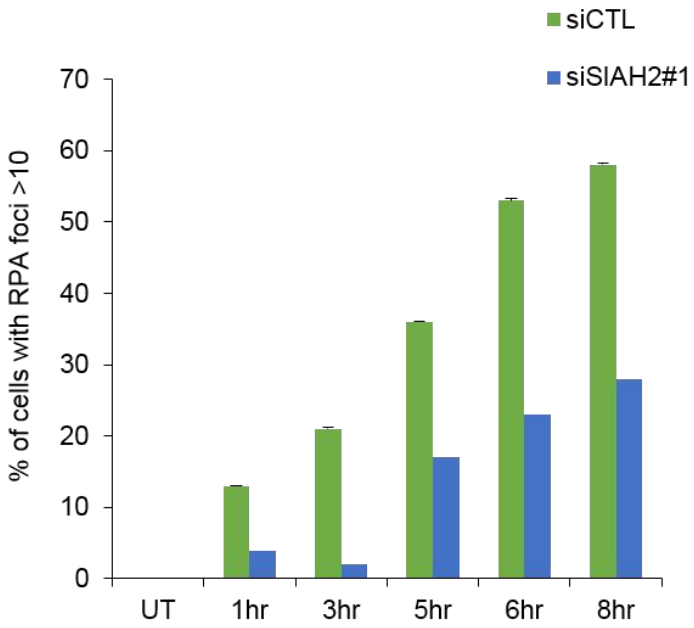
5. SIAH2 promotes CtIP-mediated end resection

CtIP is important for DSB resection, resected DNA is immediately coated by the single-stranded DNA (ssDNA)-protecting complex RPA, and then RAD51 replaces RPA to promote strand invasion[5] [27] [28]. We wondered if SIAH2 might be directly involved in DNA resection. First, we observed foci formation of RPA in control and SIAH2 depleted cells after IR treatment. RPA foci formation was significantly decreased in SIAH2 depleted cells(Figure7A). Next, to investigate the role of SIAH2 in end resection, we used ER-AsiSI system. The ER-AsiSI system is the quantification of ssDNA produced by 5'end resection in AsiSI-induced DSB in AID-DIvA U2OS cells. Addition, 4-hydroxytamoxifen (4-OHT) 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, as previously described [21](Figure7B). AID-DIvA U2OS cells were co-transfected with control siRNA, SIAH2 siRNA, CtIP siRNA, and 53BP1 siRNA. As reported, CtIP depletion was decreased end resection and knockdown of 53BP1

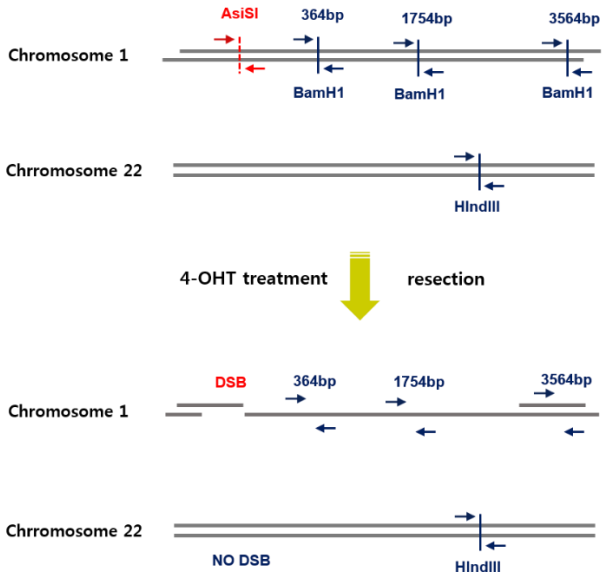
was increased end resection[29]. Interestingly, SIAH2 depletion cells were lower ssDNA than control cells, to similar to CtIP deficient cells(Figure7C). 'No DSB' was used as a negative control. These result indicated that SIAH2 is important for 5'end resection of DSBs. DNA end-resection is well known as the initial and essential step of DNA double-strand breaks repair by Homologous recombination[30]. Taken together, we suggested that SIAH2 promotes the DNA end resection through binding with CtIP and ubiquitination.

Figure7 A.





B.



C.

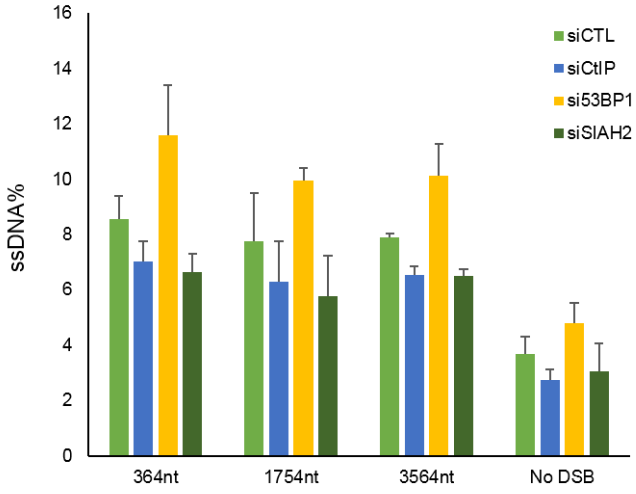


Figure 7. SIAH2 is essential for DNA end-resection

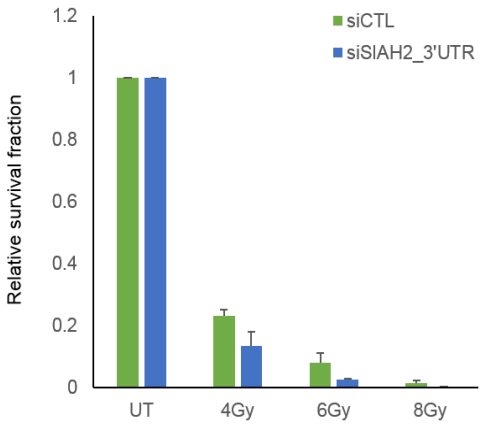
(A) Control and SIAH2-depleted Hela cells were left untreated or treated with IR(10Gy) and fixed at the indicated time point. Immunofluorescence experiments were performed using an anti-RPA antibody. DAPI was used for nuclear staining. Representative images(upper panel) and quantification(lower panel) of RPA foci in control and SIAH2-depleted U2OS cells. (B) Schematic representation of the end resection experimental procedure. (C) AID-DIvA U2OS cells were treated with 500nM 4-OHT for 4hr. Genomic DNA was extracted digested or mock digested with bamH1, BsrGI and HindIII. DNA end resection adjacent to DSB and No DSB site was measured by qPCR.

6. SIAH2 deficiency is hypersensitive to IR

In order to investigate the effect of SIAH2 depletion on cell survival to DNA damage, we performed a clonal survival assay. Hela cells were transfected with 3' UTR SIAH2 siRNA or control siRNA. The next day cells were reseeded and exposed to indicated dose of IR. SIAH2-knockdown cells were more sensitive to IR exposure than the control cells(Figure8A). Overexpression of SIAH2 in SIAH2-depleted Hela cells restored normal rate(Figure8B), suggesting that SIAH2 contributes to cell survival during the DNA damage response.

Figure 8

A.



B.

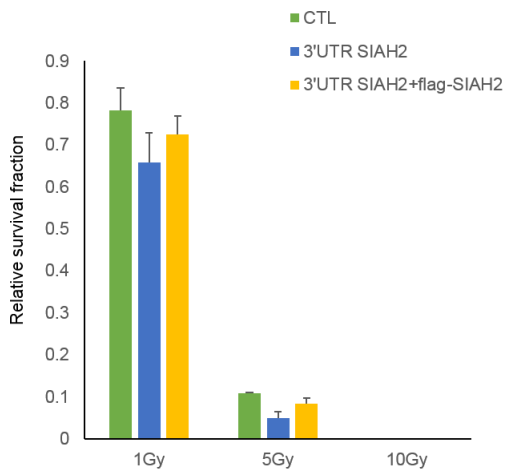


Figure 8. Effect of SIAH2 on the clonal survival of Hela cells after IR

(A) Hela cells were transfected with control and SIAH2 siRNA. (B)SIAH2-depleted Hela cells were transfected with flag-tagged siRNA-resistant SIAH2 construct. (A.B) After48hr, cells treated with indicated dose of IR and after 14days, analyzed for clonal survival ability by crystal violet staining.

7. SIAH2 recruits to DSB sites in S/G2 phase cells

γH2AX is considered as one of the earliest markers and central player of the DSB signaling, leads to the accumulation of other DDR proteins at damage sites[31]. To determine whether SIAH2 is recruited to DNA damage sites, cells were treated with IR-induced damage, performed co-immunostaining assay using anti-SIAH2 and anti-γH2AX antibody. We found that SIAH2 foci formation was not detected in untreated Hela cells. However SIAH2 foci was increased after IR treatment. We also observed that SIAH2 foci co-localized with γH2AX foci, which has been shown to represent sites of DSBs processing. This results suggested that these SIAH2 foci represent actual sites of DNA breaks(Figure9A).

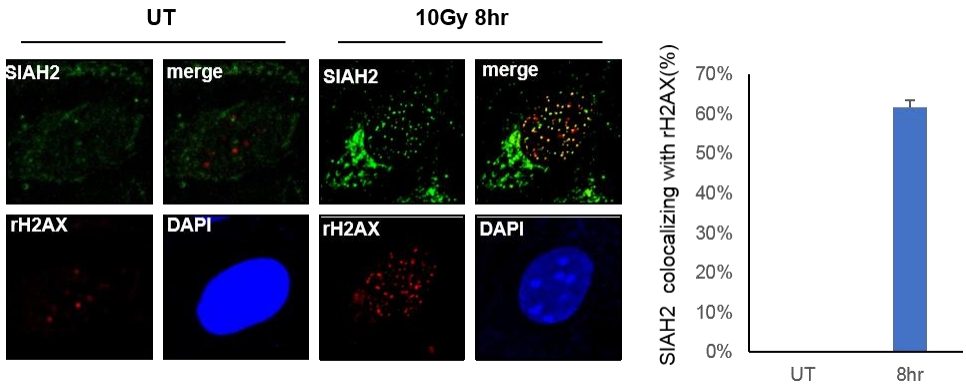
Next, SIAH2 might be involved in HR because it interacts with CtIP. In general HR, which requires a sister chromatid as template and hence only operates in S/G2 phase[32]. We hypothesized that SIAH2 would act on the S/G2 phase cells for regulation of HR. To verify this hypothesis, we showed SIAH2 foci in different cell cycle phase using the S/G2 marker CENPF. CENPF negative cells were G1

phase and CENPF positive cells were S/G2 phase. Most of the SIAH2 foci formation occurred in the S/G2 phase(Figure9B). We have already confirmed that CtIP interacts with SIAH2, and wondered if their interaction depended on the cell cycle because SIAH2 recruit in the S/G2 phase on DNA damage. HEK293T cells were transfected with flag-tagged SIAH2 and then synchronized the cells in G1/M or S/G2 phases using nocodazole or aphidicolin, respectively. Immunoprecipitation was performed using an anti-flag antibody. Interestingly, we showed that CtIP was strongly bound to SIAH2 in the S/G2 phase cells(Figure9C). Previous reported that interaction of CtIP with BRCA1 requires phosphorylation of CtIP at Ser327 by CDK, which contributes to the activation of HR-mediated DSB repair in the S and G2 phases of the cell cycle[24, 33]. Thus, the effect of SIAH2 on interaction with CtIP and BRCA1 was investigated. We transfected with SIAH2 siRNA into HEK293T cells and treated to IR, and then co-immunoprecipitation was performed using anti-CtIP antibody. We observed that the binding of CtIP and BRCA1 was diminished in the absence of SIAH2(Figure10).

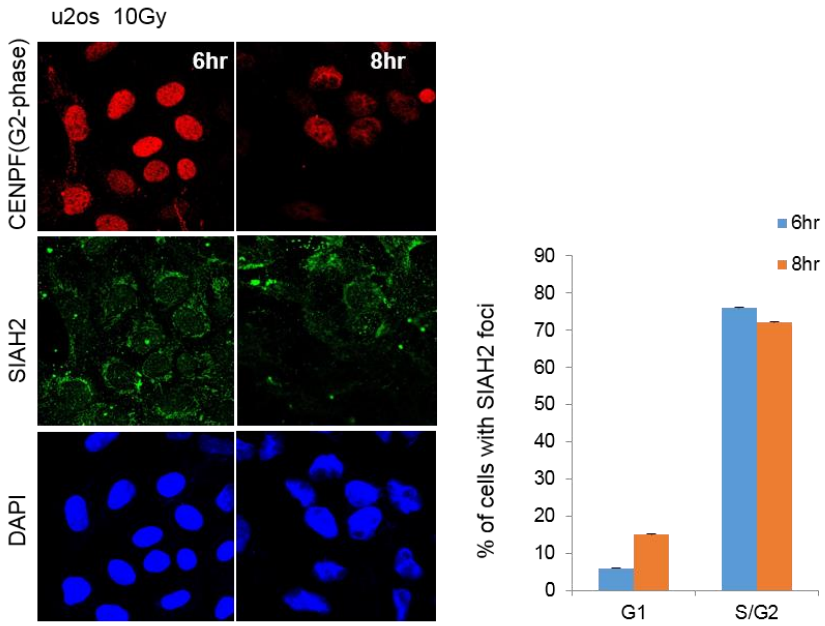
These data suggest that SIAH2 interacts and ubiquitinates CtIP, and leads to HR through stimulation of end resection.

Figure 9

A.



B.



C.

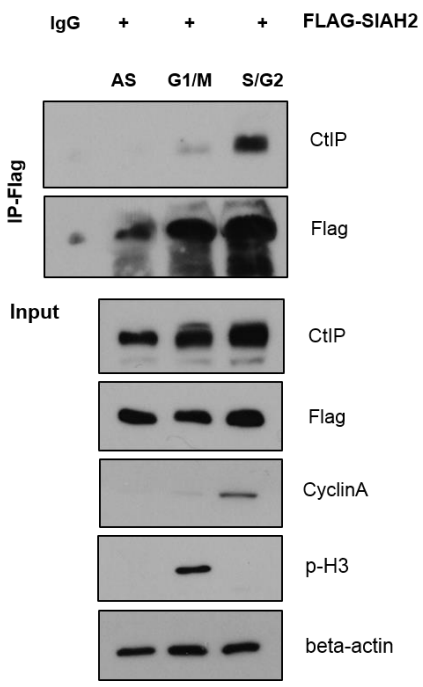


Figure 9. SIAH2 recruit to DSB sites in S/G2 phase

(A) U2OS cells were treated with IR(10Gy) for 8hr. Immunostaining experiment were performed using anti-γH2AX and anti-SIAH2 antibodies. SIAH1 recruitment foci was quantified in rH2AX-positive U2OS cells. (B) After 6hr or 8hr after IR(10Gy), cells were fixed and immunostained with SIAH2 antibody. Nuclei were co-stained with CENPF to distinguish G1 and S/G2 cells. (C)

HEK293T cells were transfected with flag-tagged-SIAH2. After 48hr, cells were untreated (asynchronous, AS) or G1/M phase cells treated with nocodazole for 16 hr, S/G2-phase cells treated with Aphidicolin for 16hr. Cell lysates were co-immunoprecipitated with anti-flag-antibody followed by immunoblotting using anti-CtIP or anti-flag antibodies.

Figure 10

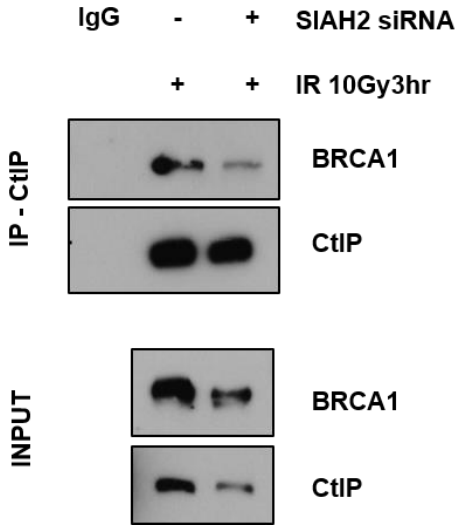


Figure 10. SIAH2 knockdown decreases interaction between CtIP and BRCA1

HEK293T cells were transiently transfected with control and SIAH2 siRNA. After 48hr, cells were treated with IR(10Gy) for 3hr. Cell lysates were co immunoprecipitated with anti-CtIP antibody followed by immunoblotting using anti-BRCA1 or anti-CtIP antibodies.

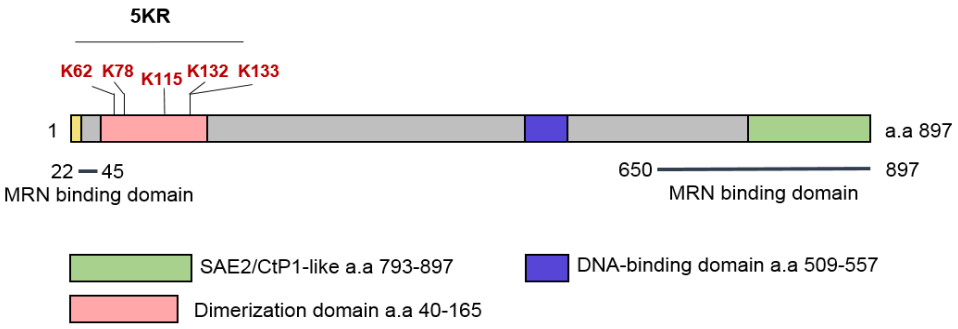
8. Ubiquitination of N-terminal CtIP promotes HR repair in response to DSBs

Previous studies have shown that ubiquitination of CtIP at N-terminal region(5K) is important for CtIP recruitment at DSBs[22]. Based on these reports, we generated CtIP mutants with lysine-to-arginine mutations in the N-terminal CtIP regions(K62/78/115/132/133K) and examined the ability of these sites to be ubiquitinated in vivo by introducing these mutant into HEK293T cells(Figure11A). We transfected with flag-tagged-CtIP WT, flag-tagged-mutant CtIP-5KR, and HA-tagged ubiquitin in to HEK293T cells and treated with IR, and then IP assay was performed. Ubiquitination of 5KR mutant was abolished, suggesting that 5K site is essential for CtIP ubiquitination after DNA damage(Figure11B). We investigated whether the effect of CtIP N-terminal region (5K) on the CtIP-mediated DDR. For this purpose, we depleted SIAH2 with siRNA targeting its 3'UTR and complemented with flag-CtIP-WT or flag-CtIP-5KR, and then chromatin fractionation and immunostaining was performed. As shown in Figure11C, flag-CtIP-WT reconstituted in Hela recruited to DNA

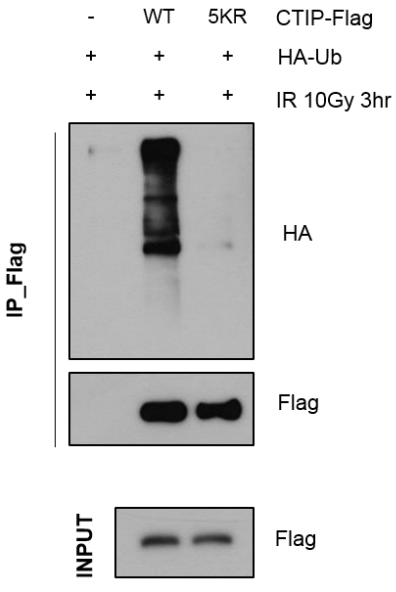
damage site. In contrast, flag-CtIP-5KR showed a significant decrease chromatin recruitment of CtIP, suggest that ubiquitination of CtIP is required for the chromatin recruitment of CtIP to DSBs. Similarly, mutant CtIP-5KR not rescued RPA and Rad51 foci formation to DSBs(Figure11D,E). One of the hallmarks of defective DNA damage responses is increased ionizing radiation sensitivity. Sensitivity to IR was compared in WT and 5KR mutant CtIP expressed cells. As a result, CtIP 5KR-expressing cells were more sensitive to IR-induced DNA damage than CtIP-WT(Figure11F). These results indicate that CtIP ubiquitination at N-terminal region is recruited to CtIP activation in the DDR and plays an important role maintenance of by HR regulation.

Figure 11

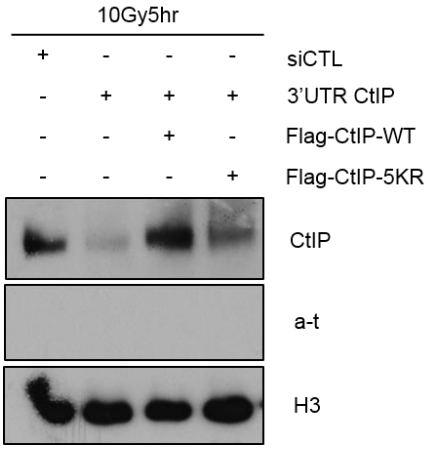
A.



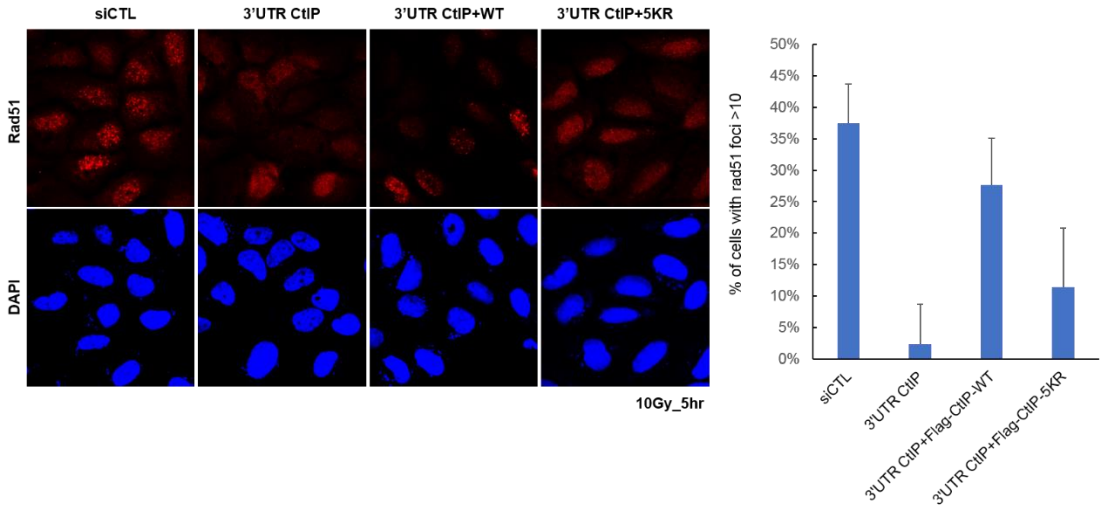
B.



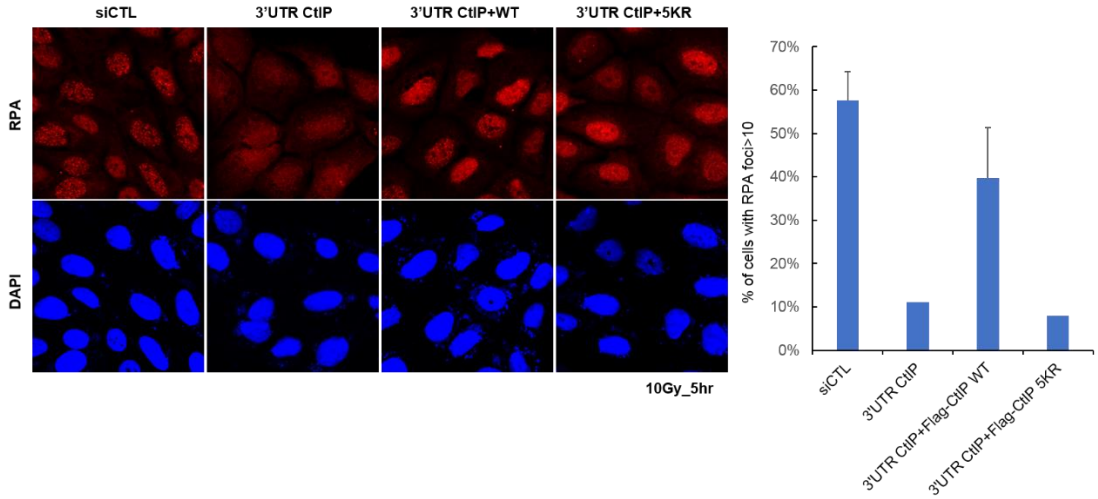
C.



D.



E.



F.

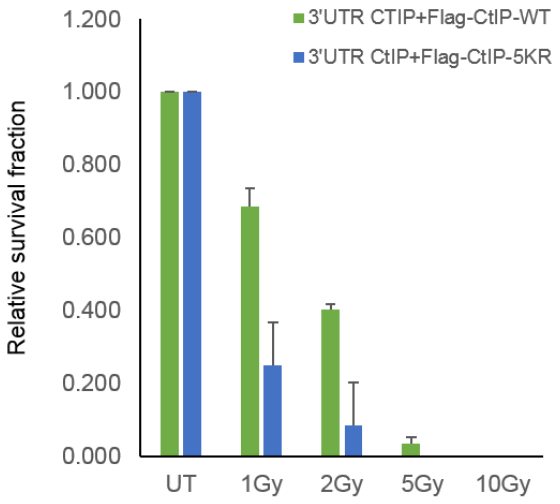


Figure 11. CtIP ubiquitination at N-terminal 5K site is important to CtIP function

(A) Schematic representation of CtIP, five ubiquitination sites are highlighted in red. Red lysines were mutated to arginines(R), as indicated, leading to CtIP mutants 12KR, 5KR and 6KR. (B) HEK293T cells were co-transfected with flag-tagged CtIP-WT and flag-tagged CtIP-5KR, and with HA-Ub. After 48hr, cells were treated with IR(10Gy) for 3hr. Cell lysates were co-immunoprecipitated with anti-flag antibody followed by immunoblotting using anti-HA or anti-flag

antibodies. (C,D,E,F) CtIP-depletion cells were transfected with flag-tagged siRNA-resistant CtIP WT and CtIP 5KR construct (C) CtIP recruitment U2OS cells was detected by chromatin fraction after treatment with IR(10Gy). U2OS cells were treated with IR(10Gy) for 5hr. Immunostaining experiments were performed using anti-Rad51(D) and anti-RPA(E) antibodies. (F) Hela cells were treated with indicated dose of IR and after 14days, analyzed for clonal survival ability by crystal violet staining.

PARTⅡ.

SIAH1 ubiquitinates BRCA2 and regulates homologous recombination

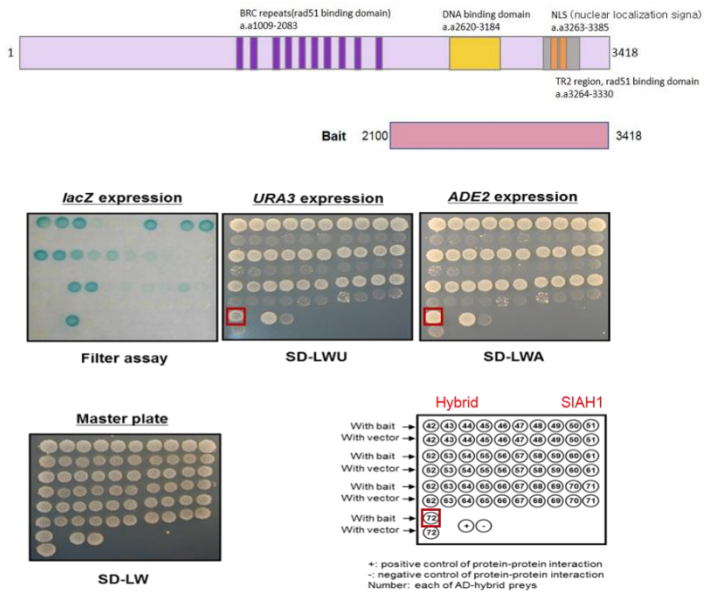
1. BRCA2 colocalizes and interacts with SIAH1

BRCA2 has a major role in DNA repair, to explore the mechanism of BRCA2 function, we conducted by yeast two-hybrid screening to identify proteins that interact with BRCA2. The yeast two-hybrid screening resulted in identification of E3 ubiquitin ligase SIAH1 protein that interacts with BRCA2 (Figure12A). To confirm the interaction between SIAH1 and BRCA2 in vivo, we subjected whole cell extracts from HEK293T cells co-transfecting BRCA2 and HA-SIAH1 expression vector to co-immunoprecipitation analysis. We showed that SIAH1 and BRCA2 interacted each other and these associations increased in response to IR irradiation(Figure12B).

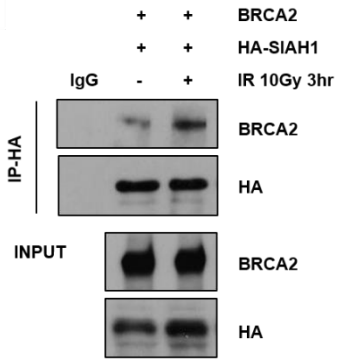
Similarly, to investigate the co-localization of BRCA2 and SIAH1 on response to DNA damage, we performed immunofluorescence assay using anti-SIAH1 and anti-BRCA2 antibodies. The untreated condition, BRCA2 and SIAH1 foci formation did not detected. In IR-treated cells, SIAH1 accumulated in DSB site, and co-localized with BRCA2 foci(Figure12C). Moreover, SIAH1 foci overlapped extensively with those of the DSBs marker γH2AX(Figure13), indicating that SIAH1 is accumulated at the sites of DSBs through interaction with BRCA2.

Figure 12

A.



B.



C.

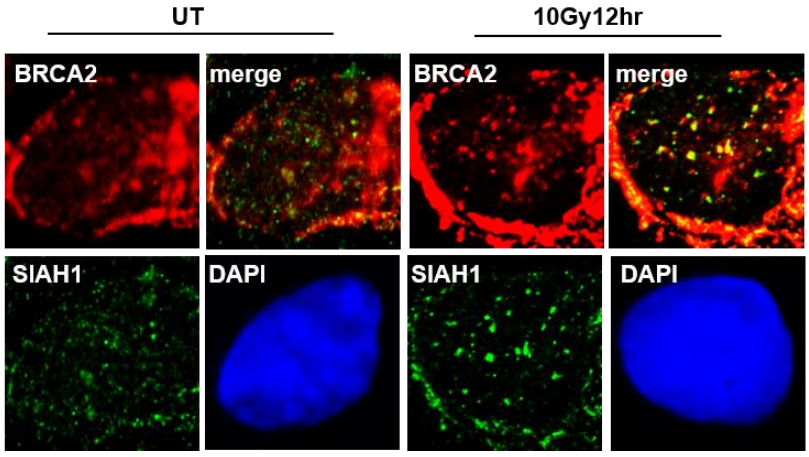


Figure 12. E3 ubiquitin ligase SIAH1 interacts with BRCA2

(A) Yeast two-hybrid assay using BRCA2 (2100-3418a,a) as bait. (B) HEK293T cells were co-transfected with BRCA2 cDNA and with HA-SIAH1 construct. After 48hr, the interaction exogenously expressed BRCA2 and HA-SIAH1 was determined by immunoprecipitation and western blotting with the indicated antibodies. (C) U2OS cells were treated with IR(10Gy) for 12hr. Co-immunostaining experiments were performed using anti-BRCA2 and anti-SIAH1 antibodies.

Figure 13

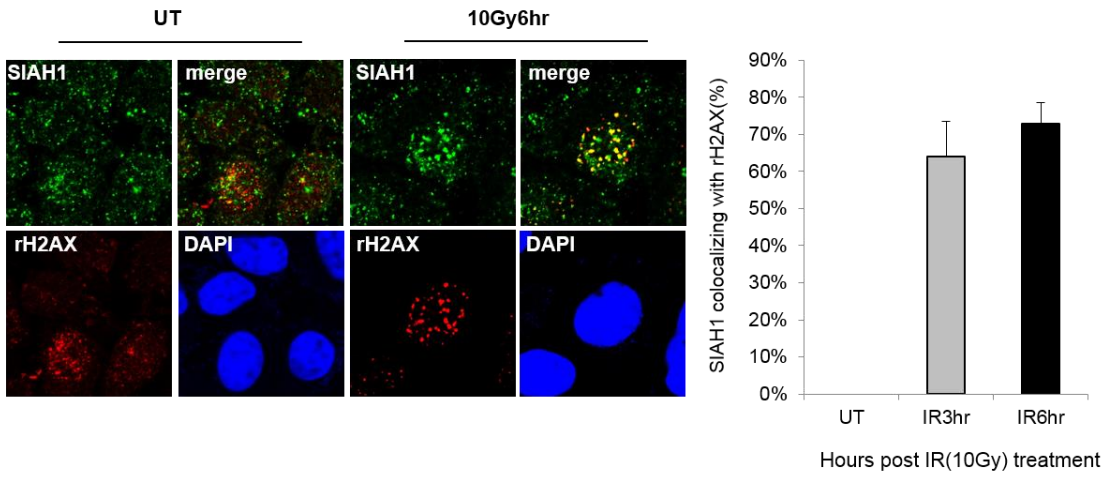


Figure 13. SIAH1 recruit to sites of DNA damage

U2OS cells were treated with IR(10Gy) for 6hr. Immunostaining experiment were performed using anti-γH2AX and anti-SIAH2 antibodies. SIAH1 recruitment foci was quantified in rH2AX-positive U2OS cells(right panel).

2. BRCA2 is ubiquitinated

Previous studies have implicated that BRCA2 participates in multiple functions, including DNA repair, transcription, and cell cycle control. Recently, ubiquitination of BRCA2 in response to MMC-induced DNA damage has been reported[34]. We first investigated whether BRCA2 is ubiquitinated by SIAH1. We co-transfected with BRCA2 cDNA and HA-tagged ubiquitin into HEK293T cells followed by IR exposure. BRCA2 was then immuneprecipitated, separated by SDS-PAGE and immunoblotted with anti-HA antibody. HA-ubiquitinated form of BRCA2 was detected(Figure14).

Figure 14

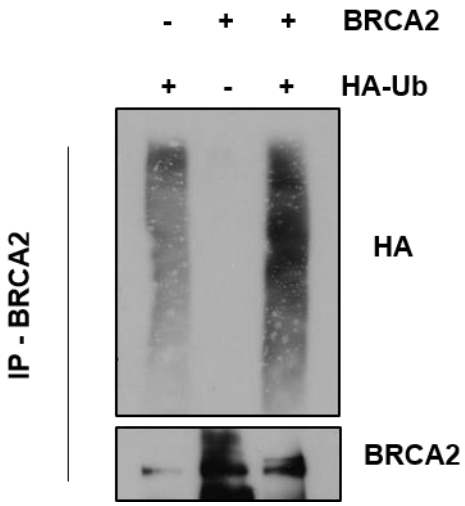


Figure 14. The poly-ubiquitination of BRCA2

HEK293T cells were transiently co-transfected with BRCA2 cDNA and with HA-tagged-Ub. After 48hr, cell lysates were co-immunoprecipitated with anti-BRCA2 antibody followed by immunoblotting using anti-HA or anti-BRCA2 antibodies.

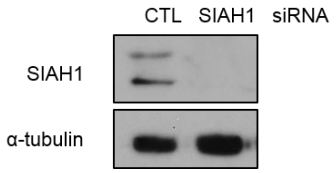
3. E3 ubiquitin ligase SIAH1 promotes ubiquitination of BRCA2

Since SIAH proteins have been implicated in ubiquitination of several interaction protein partners and given also BRCA2 is a ubiquitinated protein, we investigated to BRCA2 ubiquitination by SIAH1 on DNA damage response. First, we designed siRNA, recognizes CDS sequence of SIAH1 mRNA. We named this siRNA to siSIAH1#1(Figure15A). To check of siRNA efficiency, we performed western blotting using anti-SIAH1 antibody following each siRNA treatment(Figure15B). Next, we co-transfected with either SIAH1 siRNA or control siRNA, HA-tagged ubiquitin and BRCA2 cDNA into HEK293T cells, co-IP was performed using HA antibody. In control siRNA cells, BRCA2 ubiquitination was increased by IR-induced damage. But, in SIAH1 depleted cells, BRCA2 ubiquitination was not detected. These results suggest that SIAH1 promote BRCA2 ubiquitination in response to DNA damage(Figure15C).

Figure 15

A.

B.



C.

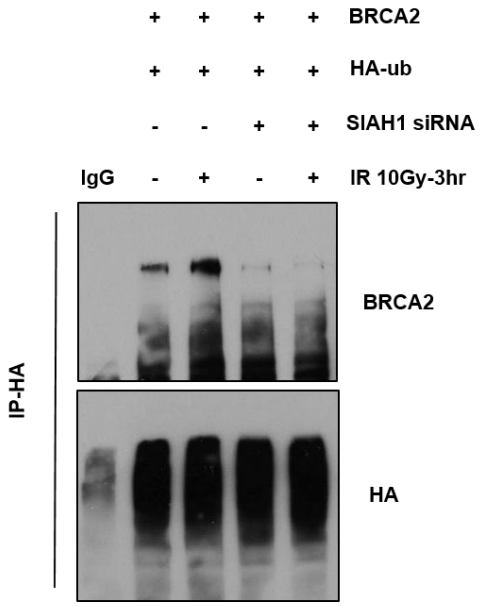


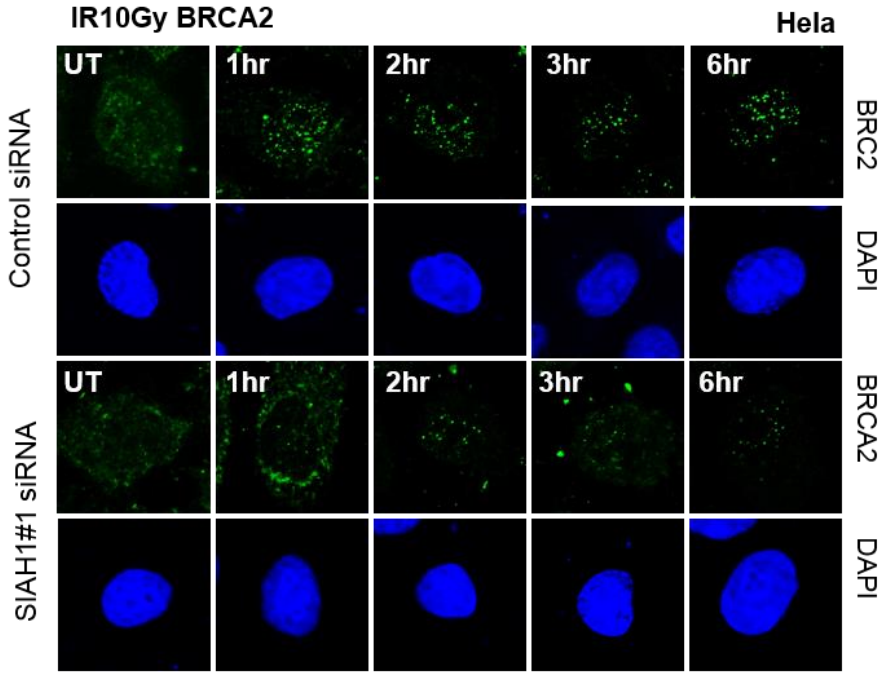
Figure 15. SIAH1 mediates ubiquitination of BRCA2

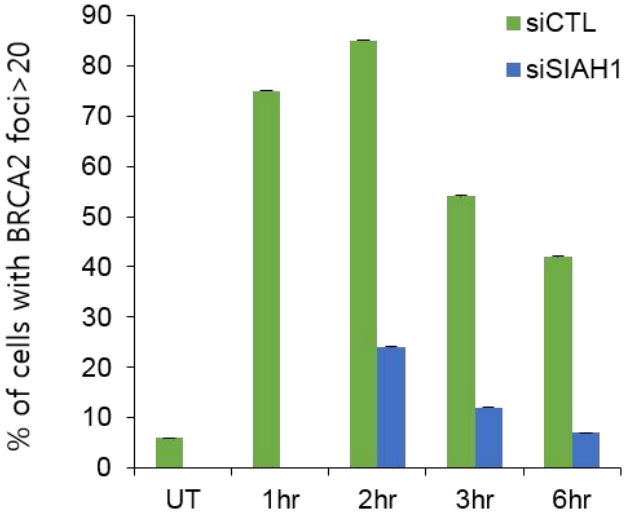
(A) Schematic representation of SIAH1 siRNA sequence. (B)The levels of endogenous SIAH1 in control and SIAH1 siRNA treated cells. (C) HEK293T cells were co-transfected with the indicated siRNA, BRCA2 and HA-Ub. Cells were treated with IR(10Gy) for 3hr, Cell Lysates were co-immunoprecipitated with anti-HA antibody followed immunoblotting using anti-BRCA2 or anti-HA antibodies.

4. SIAH1 is required for the BRCA2, BRCA1, CtIP foci formation

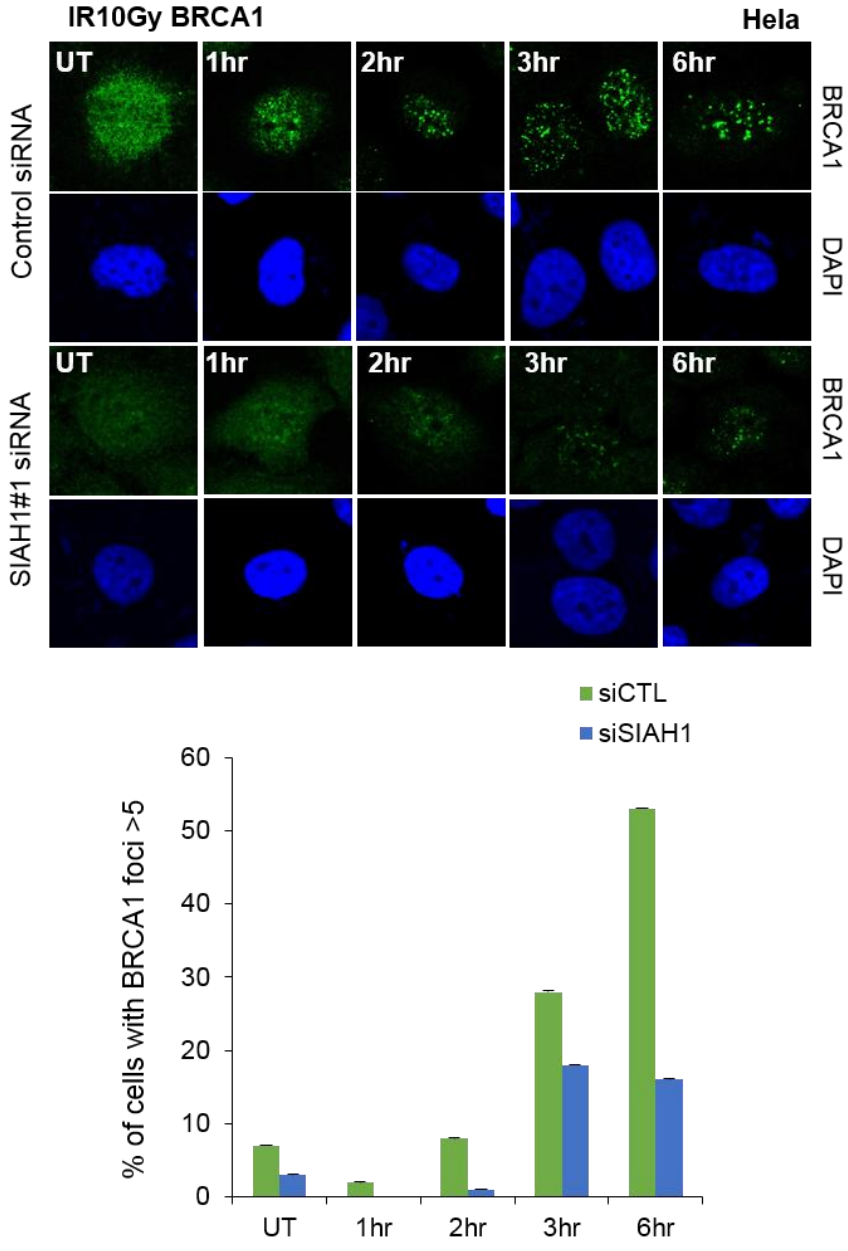
SIAH1 is a key E3 ubiquitin ligase of BRCA2 ubiquitination, we thought that SIAH1 also contributed to DNA damage response through regulation of BRCA2 function. To investigate the role of SIAH1 in DDR, first, we examined whether SIAH1 affects the foci formation of BRCA2, BRCA1, and CtIP. Hela cells were transfected with SIAH1 siRNA and induced IR during indicated time points, and immunostaining was performed using anti-BRCA2, anti-BRCA1 and anti-CtIP antibodies. SIAH1-depleted U2OS cells had significantly fewer BRCA2, BRCA1 and CtIP foci following IR exposure when compared to control cells, and the percentage of cells with more than ten foci remained low throughout the entire time course, indicating that SIAH1 facilitates recruitment of BRCA2, BRCA1, and CtIP to the damage foci(Figure16A,B,C).

Figure 16 A.

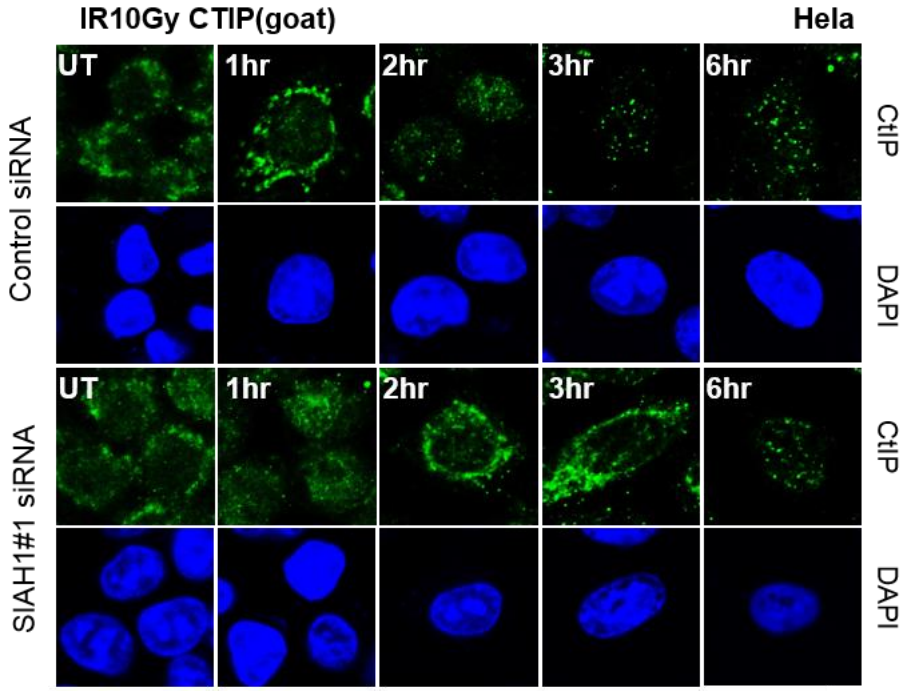




B.



C.



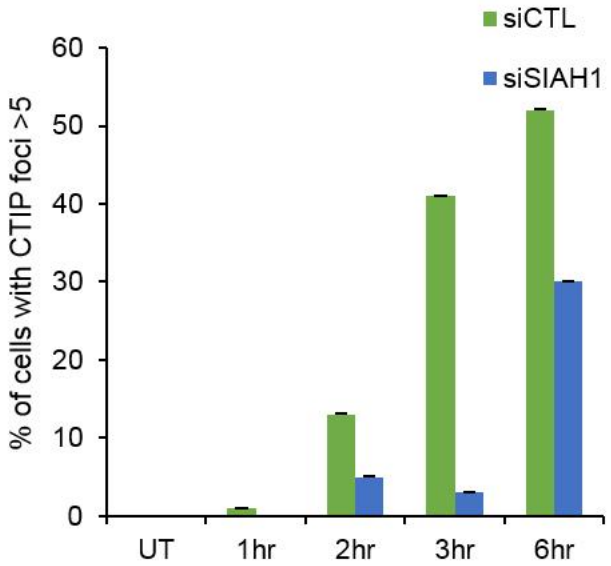


Figure 16. SIAH1 is required for the recruitment of BRCA2, BRCA1 and CtIP foci at DSBs

Control- and SIAH1-depleted Hela cells were untreated or treated with IR(10Gy) and fixed at the indicated time point. Immunostaining experiments were performed using an anti-BRCA2(A), anti-BRCA1(B) or anti-CtIP(C) antibodies. Representative images (upper panel) and quantification(lower panel) of BRCA2, BRCA1 or CtIP foci in control and SIAH1 depleted Hela cells.

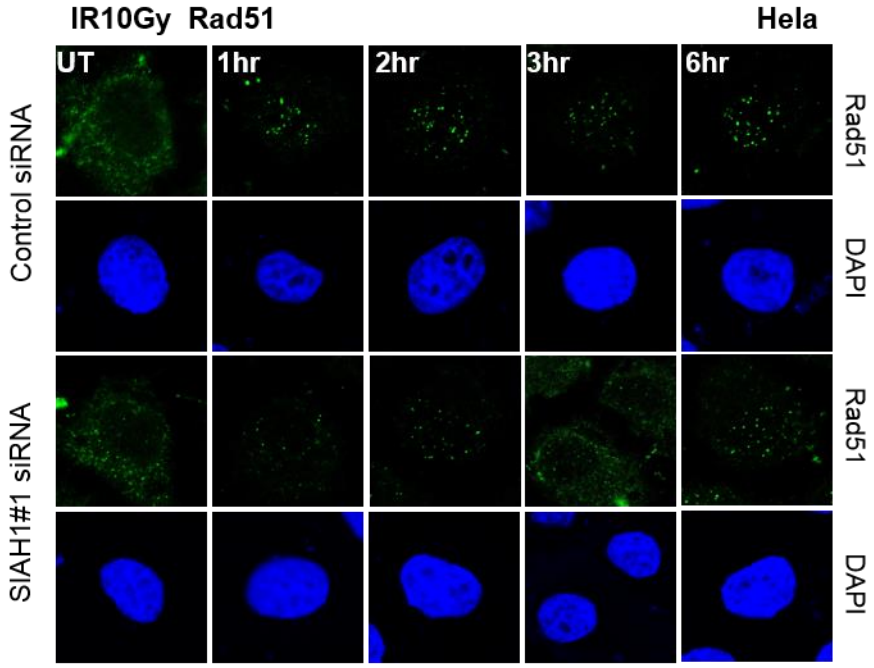
5. SIAH1 promotes HR through interaction with BRCA2

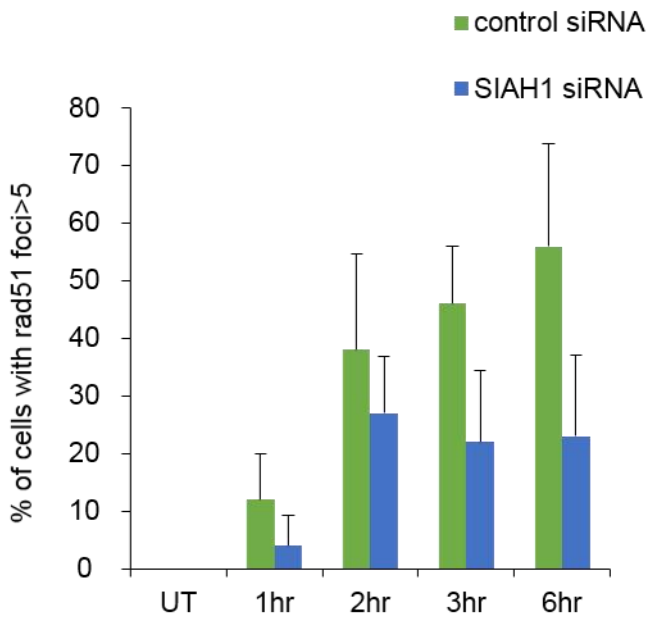
Previous reports have suggested that BRCA2 promotes homologous recombination[24-26]. In the above results, SIAH1 regulated BRCA2, BRCA1, and CtIP foci formation. We hypothesized that SIAH1 would also affect recruitment of Rad51 and DNA repair. To address this, Hela cell transfected with SIAH1 siRNA and induced IR during indicated time points, and Immunostaining was performed using anti-Rad51 antibody. SIAH1-depleted cells were decreased Rad51 foci following IR exposure when compared to control cells(Figure17A). These results suggest that SIAH1 facilitates recruitment of Rad51 to the damage foci.

Next, we investigated whether SIAH1 affect to homologous recombination. We constructed the recombination substrate DR-GFP. Control siRNA or SIAH1 siRNA treated DR-GFP HeLa cells were transiently transfected with an I-SceI expression vector and evaluated for the appearance of GFP-positive cells by flow cytometry, resulted in a 0.6-fold decrease in the number of GFP positive cells in

SIAH1 deficient cell compared with control cells(Figure17B). These results suggest that SIAH1 function is required for HR.

Figure 17 A.





B.

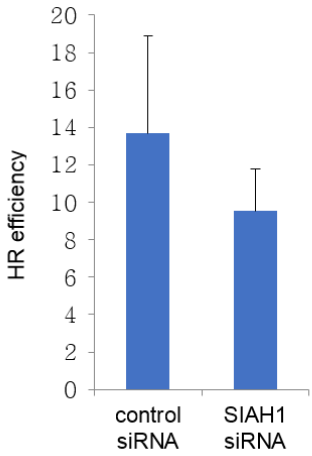


Figure 17. SIAH1 promotes HR by facilitating RAD51 accumulation at DSBs

(A) Control and SIAH1-depleted Hela cells were treated with IR(10Gy) for indicated time and fixed for immunofluorescence staining of Rad51. DAPI was used for nuclear staining. Representative images(upper panel) and quantification(lower panel) of Rad51 foci un control and SIAH1-deplted cells.(B) The efficiency HR repair was measured by FACS analysis in DR-GFP Hela cells transfected with either control or SIAH1 siRNA.

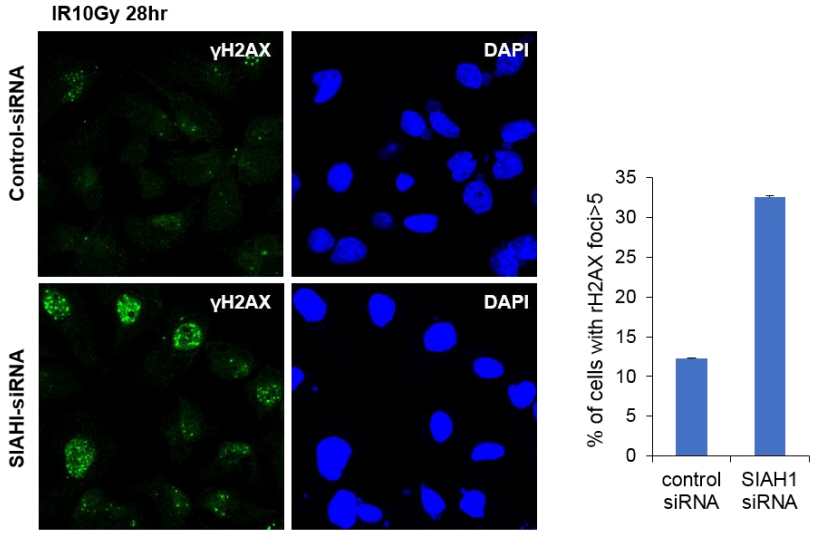
6. Depletion of SIAH1 impairs DSB repair

The accumulation of γ-H2AX at the site of DNA damage and the clearance of γ-H2AX foci reflects successful DNA repair. To test whether SIAH1-deficient cells are defective for repair of DSBs, Hela cells were transfected with control siRNA or SIAH1 siRNA. After irradiation for 28hr, we observed γ-H2AX foci formation using immunostaining assay. SIAH1 depletion cells display a significantly higher number of γ-H2AX foci following IR treatment compared with control cells(Figure18A). Next, we examined whether DSB repair is altered in Hela cells depleted for SIAH1 by analyzing comet assay. Comet assay is based on single cell gel electrophoresis. The cells are permeabilized under alkaline condition, embedded in agarose, electrophoresed at low voltage, and finally fluorescent stained with SYBR green. If DNA breakage occurs, fragmented DNA that resembles a comet's tail can be observed. We found that depletion of SIAH1 resulted in extensively longer comet tails after IR treatment than control cells. Quantitation of these observations is depicted in the graphs(Figure18B),

suggesting that SIAH1 deficient cells have defective or delayed DNA damage repair.

Figure 18

A.



B.

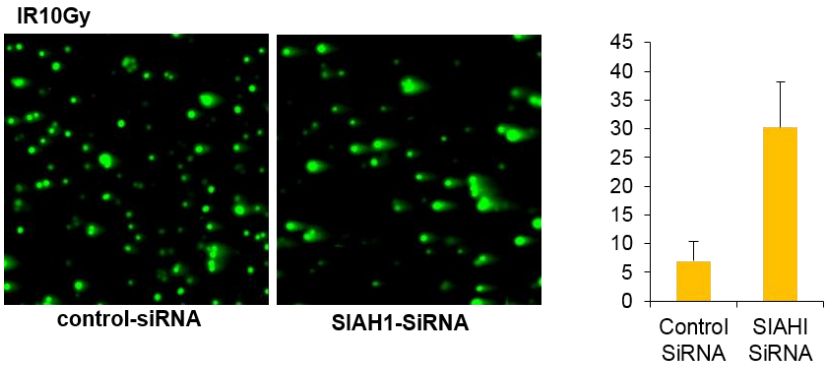


Figure 18. Effect of SIAH1 on DSB repair

(A) Control and SIAH1-depleted Hela cells were treated with IR(10Gy). After 28hr, immunofluorescence staining were performed using anti-γH2AX antibody.

(B) Hela cells were transfected with control and SIAH1 siRNA. Effect of SIAH1 on single cell electrophoresis(comet) assay of Hela cells with treated with IR(10Gy). Representative comet figures for 1hr after IR are shown in the left panel. The comet tail moments of 100 cells were measured, values with standard deviations are shown in the right panel.

7. SIAH1 deficiency reduces cell survival in response to DNA damage

Defects in the DDR usually lead to cellular hypersensitivity to IR-induced DNA damage. In order to investigate the effect of SIAH1 deficient on cell survival to DNA damage, we performed a clonal survival assay. HeLa cells were transfected with SIAH1 siRNAs and control siRNA. The next day cells were reseeded and exposed to IR. SIAH1 depleted cells were more sensitive to IR exposure than the control cells. These findings indicate that SIAH1 contributes to cell survival during the DDR(Figure19). Collectively, these results suggest that SIAH1 mediates ubiquitination of BRCA2 its critical for functions in DDR and DSB repair by homologous recombination.

Figure19

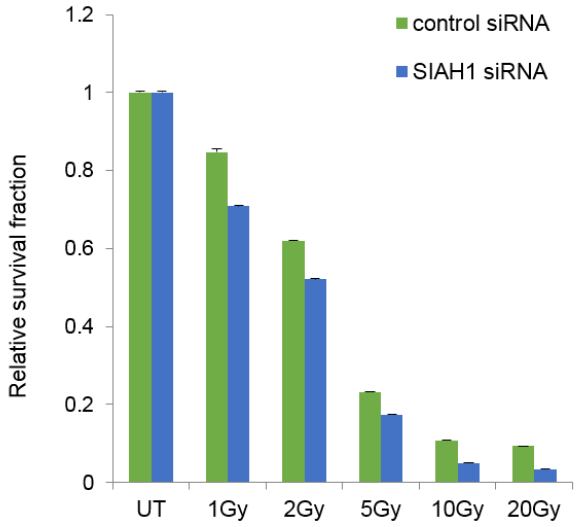


Figure 19. Effect of SIAH1 on the clonal survival of Hela cells after IR

Hela cells were transfected with control and SIAH1 siRNA. After24hr, cells treated with indicated dose of IR and after 14days, analyzed for clonal survival ability by crystal violet staining.

DISCUSSION

Ubiquitination is a post-translational modification that plays variety of cellular roles.[35] Recently, different groups reported the critical role of ubiquitination in the DNA damage response and DSBs repair mechanisms[9]. Our data demonstrate that CtIP and BRCA2 are ubiquitinated during DNA damage. Moreover, the five lysines of CtIP are important sites for ubiquitination. Ubiquitinated WT CtIP but not non-ubiquitinated MT CtIP enhances Rad51 and CtIP foci formation and increases HR. Therefore, based on the above results, we can demonstrate how ubiquitination plays a role in cellular responses to DNA damage. In addition, Yeast two-hybrid system and Co-IP were showed that BRCA2 interacts with SIAH1 and CtIP interacts with SIAH2. SIAH has generally been reported to bind with substrates, through the SBD region. In our study, CtIP also interacts with SIAH2 through SBD domain.

Several RING E3 ubiquitin ligase plays a critical role in the response to DNA damage(Table1)[20, 22, 36-38]. Especially, we found that this ubiquitination was

promoted by the SIAH protein on response to DNA damage.

RING E3s	Target	Ubiquitination type	Function
SIAH1	BRCA2	poly	DSB signaling and HR repair
SIAH2	CtIP	poly	DSB signaling and HR repair
RNF8	H2A/H2AX and other unknown	K63 chains	DSB signaling and HR rep
RNF168	H2A/H2AX	mono	DSB signaling and HR rep
BRCA1	unknown	K6 and other unknown	Promote HR
BMI	H2A	Mono on K119 of H2A	Gene silencing; DSB signaling?
RING1B	H2A	Mono on K119 of H2A	Gene silencing; DSB signaling?
RAD18	PCNA	Mono on K164 of PCNA	PRR
FANCL	FANCD2	Mono on K561 of FACD2	CL repair (FA pathway)

Table1. Ring finger E3s involved in DDR pathway

SIAH protein is members of the RING finger E3 ubiquitin ligases, which exist two genes encode Sina-like proteins, SIAH1 and SIAH2[39][40]. Activity of SIAH1 and SIAH2 are increased in response to various forms of cellular stress, including oxygen deprivation (hypoxia), glucose deprivation, glucose elevation, DNA damage, and apoptosis, supporting their possible roles in normal homeostasis and the stress response[20, 41, 42]. Our result also show that SIAH1/2 are one

of the E3 ubiquitin ligases recruited to DNA damage sites(Figure9A & Figure13). But, It is unclear how the SIAH protein regulates the DNA damage response. In the present study demonstrate that SIAH protein directly participates in the DNA repair process. Our result shows that interestingly, recruitment of CtIP, BRCA2, BRCA1, RPA and Rad51 were decreased in SIAH1/2 depleted cells. And efficiency of homologous recombination decreased in SIAH1/2 depleted cells. SIAH depleted cells were increased ionizing radiation hypersensitivity as determined by the clonal survival assay. Previous study showed that BRCA1, MRN complex, CtIP play a critical role in DSB end resection and homologous recombination-mediated DSB repair[24, 43]. Consistently, we found that DSB end resection is decreased in SIAH2-depleted cells, which proves that SIAH2 is a direct regulator of end resection and DSB repair.

The most well-known function of ubiquitination is a signal to target the protein to the proteasome for degradation. However, several reported that mono-ubiquitination or poly-ubiquitination is involved in the cell signaling pathway as a

non-degradative function[44]. Our data reveals that CtIP poly-ubiquitination and BRCA2 poly-ubiquitination was promoted by SIAH(Figure3A,B & Figure14). SIAH target proteins, CtIP and BRCA2 were non-degrade. Therefore, this poly-ubiquitination, non-proteasomal ubiquitin signal induced by E3 ubiquitin ligase SIAH.

This study aims to understand the mechanism of ubiquitination of CtIP and BRCA2 in response to DNA damage. Taken together, SIAH1/2 is an E3-ligase that promotes ubiquitination and plays an important role in regulating DNA repair through homologous recombination. We must have done to further demonstrate with detailed mechanism of SIAH-related DDR.

ABSTRACT

Effect of ubiquitin E3 ligase SIAH1/2 in the DNA double-strand break repair

Jeong Seo Yeon

Advisor : Prof. Lee Jung Hee, Ph.D.

Co-Advisor : Prof. Yoo Ho Jin, Ph.D.

Department of biomedical sciences

Graduate School of Chosun University

DNA double-strand break(DSB) repair is the most important mechanism to maintain the normal cellular homeostasis. The DSBs were repaired by two main pathway, Homologous recombination(HR) or non-homologous end joining(NHEJ). Ubiquitination is a form of protein stabilization, intracellular localization of protein,

and intracellular signal at DNA damage. Although ubiquitination is commonly known as a critical mark targeting proteins for proteasome-dependent degradation, recent studies have revealed that it also has non-proteolytic functions such as activation and inactivation of target protein. Here, we report non-proteolytic function of ubiquitination by SIAH E3 ubiquitin ligase. We identify that E3 ubiquitin ligase, SIAH1 and SIAH2 are targeting BRCA2 and CtIP, respectively. We demonstrate that the E3 ubiquitin ligase SIAH mediates the target protein ubiquitination. E3 ubiquitin ligases SIAH1/2 is recruited to DNA damage site and recruitment of CtIP, BRCA2, BRCA1 and RAD51 at DSB sites following IR is dramatically decreased in SIAH1/2-deficient cells. The deletion of SIAH results in impaired HR, suppression of DNA end resection and cellular hypersensitivity to IR. Our finding reveal a role for SIAH1/2 in regulation of HR repair and maintaining genome stability, and suggest that ubiquitination of CtIP and BRCA2 by SIAH is critical for DDR.

REFFERENCE

1. Symington, L.S., *End resection at double-strand breaks: mechanism and regulation.* Cold Spring Harb Perspect Biol, 2014. **6**(8).
2. Heyer, W.D., K.T. Ehmsen, and J. Liu, *Regulation of homologous recombination in eukaryotes.* Annu Rev Genet, 2010. **44**: p. 113-39.
3. Lieber, M.R., et al., *The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination.* DNA Repair (Amst), 2004. **3**(8-9): p. 817-26.
4. New, J.H., et al., *Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A.* Nature, 1998. **391**(6665): p. 407-10.
5. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
6. Lans, H., J.A. Marteijn, and W. Vermeulen, *ATP-dependent chromatin remodeling in the DNA-damage response.* Epigenetics Chromatin, 2012. **5**: p. 4.
7. Yoshida, K. and Y. Miki, *Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage.* Cancer Sci, 2004. **95**(11): p. 866-71.
8. Xia, B., et al., *Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2.* Mol Cell, 2006. **22**(6): p. 719-29.
9. Jackson, S.P. and D. Durocher, *Regulation of DNA damage responses by ubiquitin and SUMO.* Mol Cell, 2013. **49**(5): p. 795-807.
10. Ciechanover, A., D. Finley, and A. Varshavsky, *Mammalian cell cycle mutant defective in intracellular protein degradation and ubiquitin-protein conjugation.* Prog Clin Biol Res, 1985. **180**: p. 17-31.
11. Brinkmann, K., et al., *Regulation of the DNA damage response by ubiquitin conjugation.* Front Genet, 2015. **6**: p. 98.
12. Schwertman, P., S. Bekker-Jensen, and N. Mailand, *Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers*. Vol. 17. 2016. 379-94.
13. Chen, S., et al., *The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner.* Nucleic Acids Res, 2005. **33**(18): p. 5878-86.
14. Thrower, J.S., et al., *Recognition of the polyubiquitin proteolytic signal.* EMBO J, 2000. **19**(1): p. 94-102.
15. Carthew, R.W. and G.M. Rubin, *seven in absentia, a gene required for specification of R7 cell fate in the Drosophila eye.* Cell, 1990. **63**(3): p. 561-77.
16. Polekhina, G., et al., *Siah ubiquitin ligase is structurally related to TRAF and*

modulates TNF-alpha signaling. Nat Struct Biol, 2002. **9**(1): p. 68-75.
17. Kramer, O.H., et al., *SIAH proteins: critical roles in leukemogenesis.* Leukemia, 2013. **27**(4): p. 792-802.
18. Fujita, K., et al., *Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence.* Nat Cell Biol, 2010. **12**(12): p. 1205-12.
19. Nakayama, K., J. Qi, and Z. Ronai, *The ubiquitin ligase Siah2 and the hypoxia response.* Mol Cancer Res, 2009. **7**(4): p. 443-51.
20. Winter, M., et al., *Control of HIPK2 stability by ubiquitin ligase Siah-1 and checkpoint kinases ATM and ATR.* Nature Cell Biology, 2008. **10**: p. 812.
21. Zhou, Y., et al., *Quantitation of DNA double-strand break resection intermediates in human cells.* Nucleic Acids Res, 2014. **42**(3): p. e19.
22. Schmidt, C.K., et al., *Systematic E2 screening reveals a UBE2D-RNF138-CtIP axis promoting DNA repair.* Nat Cell Biol, 2015. **17**(11): p. 1458-1470.
23. Ferretti, L.P., et al., *Cullin3-KLHL15 ubiquitin ligase mediates CtIP protein turnover to fine-tune DNA-end resection.* Nat Commun, 2016. **7**: p. 12628.
24. Chen, L., et al., *Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair.* J Biol Chem, 2008. **283**(12): p. 7713-20.
25. Venkitaraman, A.R., *Functions of BRCA1 and BRCA2 in the biological response to DNA damage.* J Cell Sci, 2001. **114**(Pt 20): p. 3591-8.
26. Chen, J.J., et al., *BRCA1, BRCA2, and Rad51 operate in a common DNA damage response pathway.* Cancer Res, 1999. **59**(7 Suppl): p. 1752s-1756s.
27. You, Z., et al., *CtIP links DNA double-strand break sensing to resection.* Mol Cell, 2009. **36**(6): p. 954-69.
28. Marechal, A. and L. Zou, *RPA-coated single-stranded DNA as a platform for post-translational modifications in the DNA damage response.* Cell Res, 2015. **25**(1): p. 9-23.
29. Bunting, S.F., et al., *53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.* Cell, 2010. **141**(2): p. 243-54.
30. Chen, H., M. Lisby, and L.S. Symington, *RPA coordinates DNA end resection and prevents formation of DNA hairpins.* Mol Cell, 2013. **50**(4): p. 589-600.
31. Rogakou, E.P., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.* J Biol Chem, 1998. **273**(10): p. 5858-68.
32. Rothkamm, K., et al., *Pathways of DNA double-strand break repair during the mammalian cell cycle.* Mol Cell Biol, 2003. **23**(16): p. 5706-15.
33. Yun, M.H. and K. Hiom, *CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle.* Nature, 2009. **459**(7245): p. 460-3.
34. Schoenfeld, A.R., et al., *BRCA2 is ubiquitinated in vivo and interacts with USP11, a deubiquitinating enzyme that exhibits prosurvival function in the cellular response to DNA damage.* Mol Cell Biol, 2004. **24**(17): p. 7444-55.

35. Weissman, A.M., *Themes and variations on ubiquitylation.* Nat Rev Mol Cell Biol, 2001. **2**(3): p. 169-78.
36. Bartocci, C. and E.L. Denchi, *Put a RING on it: regulation and inhibition of RNF8 and RNF168 RING finger E3 ligases at DNA damage sites.* Front Genet, 2013. **4**: p. 128.
37. Bohgaki, M., et al., *RNF168 ubiquitylates 53BP1 and controls its response to DNA double-strand breaks.* Proc Natl Acad Sci U S A, 2013. **110**(52): p. 20982-7.
38. Galanty, Y., et al., *RNF4, a SUMO-targeted ubiquitin E3 ligase, promotes DNA double-strand break repair.* Genes Dev, 2012. **26**(11): p. 1179-95.
39. Hu, G., et al., *Characterization of human homologs of the Drosophila seven in absentia (sina) gene.* Genomics, 1997. **46**(1): p. 103-11.
40. Tang, A.H., et al., *PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism.* Cell, 1997. **90**(3): p. 459-67.
41. Nakayama, K., et al., *Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia.* Cell, 2004. **117**(7): p. 941-52.
42. Yego, E.C. and S. Mohr, *siah-1 Protein is necessary for high glucose-induced glyceraldehyde-3-phosphate dehydrogenase nuclear accumulation and cell* death *in Muller cells.* J Biol Chem, 2010. **285**(5): p. 3181-90.
43. Cruz-Garcia, A., A. Lopez-Saavedra, and P. Huertas, *BRCA1 accelerates CtIP-mediated DNA-end resection.* Cell Rep, 2014. **9**(2): p. 451-9.
44. Ulrich, H.D. and H. Walden, *Ubiquitin signalling in DNA replication and repair.* Nat Rev Mol Cell Biol, 2010. **11**(7): p. 479-89.