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ATP-Citrate Lyase regulates DNA double-strand break repair through binding of 53BP1

조선대학교 대학원

의과학과

양 지 예

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이 논문을 석사학위신청 논문으로 제출함

2014 년 11 월

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ACL에 의한 DNA손상 복구 조절 기전연구

양 지 예

지도교수 : 유 호 진

조선대학교 일반대학원

의과학과

ATP-Citrate Lyase(ACL) 단백질은 지질대사 과정 중 지방산 생합성에 관여하는 효소로써, 최근엔 성장인자 자극에 대한 반응과 분화과정 동안에 Acetyl-coA를 생산함으로써 히스톤을 아세틸화 한다고 보고되었다. 여기서 우리는 Yeast two-hybrid screening을 통해 이 ACL단백질과 결합하는 단백질들을 동정하였고 그 중에서 DNA손상반응에 중요한 역할을 하는 53BP1이 결합함을 확인하였다.

53BP1은 DNA손상시 손상인식, 복구 및 세포주기 조절에 관여하는 단백질로 알려져 있다. 본 연구에서는 ACL이 53BP1과의 결합을 통해 DNA손상반응에 관여하는 기전을 밝히고자 한다. 먼저 세포 내(in vivo)와 세포 밖(in vitro)에서 ACL과 53BP1이 결합함을 보았고, ACL이 세포질 뿐 아니라 핵에도 위치함을 관찰하였다. 이는 53BP1이 매개된 DNA손상반응에 ACL이 기능적으로 연관됨을 시사한다. ACL이 53BP1 활성조절에 미치는 영향을 조사하기 위해 먼저 ACL이 결핍된 세포에서 53BP1의 기능을 조사한 결과 53BP1의 DNA손상 foci와 인산화가 감소됨을 관찰하였다. 그러나 다른 DNA손상 반응관련 단백질 BRCA1, MDC1, γ-H2AX의 foci 형성에는 영향이 없음을 보았다. 또한, ACL이 결핍된 세포에서 DNA손상 복구 활성이 떨어짐을 clonal survival assay, comet assay, late γ-H2AX 염색법 등을 통해 관찰하였다. 특히 이는 ACL결손에 의한 53BP1의 기능저하에 따른 비 상동 말단 결합 활성의 저하에 관여함을 확인하였다. 따라서, 본 연구결과는 지질대사 조절효소 ACL이 53BP1과 상호작용을 통해 DNA손상반응, 특히 비 상동 말단 결합 활성을 조절함을 제안한다.

INTRODUCTION

A DNA double-strand break (DSB) has long been recognized as a severe cellular lesion, potentially representing an initiating event for carcinogenesis or cell death. The evolution of DSB repair pathways as well as additional processes, such as cell cycle checkpoint arrest, to minimize the cellular impact of DSB formation was, therefore, not surprising. However, the depth and complexity of the DNA damage responses being revealed by current studies were unexpected. Perhaps the most surprising finding to emerge is the dramatic changes to chromatin architecture that arise in the DSB vicinity[1]. DNA double-strand breaks (DSB) are considered the most lethal form of DNA damage which can be induced by a number of different endogenous and exogenous factors. Since unrepaired or misrepaired DSB can initiate processes leading to mutagenesis, tumorigenesis and cell death, repair of DSB is essential to maintain genome stability and cell viability.

Cells have evolved two major pathways for the repair of DSB, homologous recombination (HR) and non-homologous end-joining (NHEJ). HR utilizes DNA molecules with a significant length of sequence homology to prime DNA synthesis, allowing for accurate repair. For many years, HR had been considered to be a minor pathway that acts on DSB in higher eukaryotes. It was therefore believed that DSB repair in these organisms is almost exclusively undertaken by NHEJ. Later, however, it was established that HR also contributes considerably to DSB repair in higher eukaryotes. In Saccharomyces cerevisiae, HR is the favored DSB repair pathway, whereas NHEJ is only of minor importance. Basically, in S. cerevisiae NHEJ activity can be demonstrated only in the absence of HR and it may thus serve only as a backup system. The HR components in this organism belong to the RAD52 epistasis group, which includes RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2 genes. Cells mutated in these genes are in general sensitive

to ionizing radiation, unable to repair DSB, and defective in mitotic and/or meiotic recombination. In contrast, NHEJ rejoins DNA ends with little or no sequence homology, potentially leading to inaccurate joining. In higher animal cells, NHEJ is believed to play a predominant role in DSB repair. NHEJ process was initially discovered in mammalian cells, where it represents the main DSB repair pathway, and where the core NHEJ factors are the DNA-dependent protein kinase (DNA-PK), consisting of the catalytic subunit (DNA-PKcs) and the two DNA end-binding components (KU70 and KU80), the DNA ligase IV-XRCC4 complex associating with recently identified CERNUNNOS/XLF protein, and the ARTEMIS protein. NHEJ has also been discovered in other organisms including the budding yeast S. cerevisiae, where the KU70, KU80, DNA ligase IV and XRCC4 homologues have been identified (YKu70, YKu80, Dnl4 and Lif1, respectively). Although S. cerevisiae lacks clear homologues of DNA-PKcs and ARTEMIS, it is endowed with an additional regulatory NHEJ factor, Nej1,

which appears to be genuine homologue of CERNUNNOS/XLF[2]. Thus, cells have mechanisms responsible for recognition of DNA damage and activation of cell cycle checkpoints leading to DNA repair.

The generation of DSBs triggers the relocalization of many DNA damage response (DDR) proteins such as MRE11/NBS1/RAD50, MDC1, 53BP1, and BRCA1 to nuclear foci where these proteins colocalize and interact with γ-H2AX[3]. The DNA damage response factor 53BP1 is a key regulator of the processing and repair of double-strand breaks (DSBs). The p53-binding protar structures at the site of DNA damage and forms readily visualized ion-izing radiation (IR) induced foci. Depletion of 53BP1 results in cell cycle arrest in G2/M phase as well as genomic instability in human as well as mouse cells[4]. Moreover, The DNA damage response factor 53BP1 functions at the intersection of two major double strand break (DSB) repair pathways promot-ing nonhomologous end-joining (NHEJ) and homologous recombination repair

(HRR)[5]. Here we show that 53bp1 promote their repair by nonhomologous end joining (NHEJ).

In this study, we found that 53BP1 interacts with ACL by yeast-two hybrid screening. we identified 53BP1 binds ACL, which bind N-terminal of the 53BP1. ATP citrate lyase(ACL) is an enzyme that represents an important step in fatty acid biosynthesis. This step in fatty acid biosynthesis occurs because ATP citrate lyase is the link between the metabolism of carbohydrates (which causes energy) and the production of fatty acid[6]. Moreover, ATP citrate lyase (ACL), which catalyzes the conversion of citrate to cytosolic acetyl-CoA. ACL inhibition by RNAi limits in vitro proliferation and survival of tumor cells[7].

We found that ACL deficient cells were hypersensitive to IR-induced colony formation. Moreover, ACL depletion diminished the recruitment of 53BP1 to site of DNA damage after DNA damage but not MDC1 and BRCA1.

ACL deficient cells showed impaired Non-homologous repair activity after DSB. Based on the results, we propose a vital role of ACL in cellular DNA damage response through association with 53BP1.

MATERIALS AND METHODS

1. Cell culture

293T, Hela cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and streptomycin (0.1 mg/ml), penicillin (100units/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 % CO_2 at saturated humidity. Cells in exponential growth were harvested for subsequent experiments.

2. Immunoprecipitation assay

For the Immunoprecipitation assay, aliquots of soluble cell lysates were precpeared with protein A/G plus-agarose beads(Santa Cruz Biotechnology), G-sepharose and A sepharose(GE Healthcare) as indicated and then incubated at 4° C for 12h. After the addition of fresh protein A/G plus agarose bead, G sepharose and A sepharose, the reaction was incubated overnight at 4° C with

rotation. The beads were washed five times in RIPA buffer without protease inhibitors, resuspended in SDS sample buffer and boiled for 5 min. The samples were then analyzed by western blotting using the appropriate antibodies.

3. Western blot

Cells were lysed in RIPA buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) 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 1 hr with TBS-t (10mM Tris-HCl (pH 7.4), 150mM 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 four times for 15 min with 0.1% Tween 20 containing TBS-t and then incubated for 2 hr with peroxidase-conjugated secondary antibodies (1:4000). The membranes were washed four more times and

developed using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

4. Non-homologous end joining assay

The NHEJ assay was measured in HeLa EJ5-GFP cells. EJ5-GFP contains a promoter that is separated from a GFP coding region by puromycin resistance gene, which is flanked by two I-SecI sites that are in the same orientation. When the I-SecI-induced DSBs is repaired by NHEJ in HeLa EJ5-GFP cells, the puro gene is removed, and the promoter is rejoined to the rest of the GFP expression cassette, leading GFP expression. HeLa EJ5-GFP cells were kindly provided by Dr. Kee at the University of South Florida. After 3days, the percentage of GFP-positive cells which had repaired the DSBs generated by I-SecI was determined by flow cytometry. For each analysis, 10,000 cells were processed and each ex-periment was repeated three times.

5. Comet assay

For the comet assay, HeLa cells were treated with 10 Gy of γ-ray by followed by incubation in culture medium at 37° C. The alkaline version of the comet assay (single cell gel electrophoresis) was performed as described by Singh et al. Briefly, 100 μL of cells were taken, homogenized with low-melting point agarose, spread on a microscope slide pre-coated with normal-melting-point agarose and covered with a coverslip. After 30 min at 4° C, the coverslip was removed from the slides. and they were immersed in cold lysing solution (2.5 M NaCl; 100 mM ethylenediaminetetraacetic acid (EDTA); 10mM Tris, 10% dimethylsulfoxide and 1% Triton-X, pH 10) for 24 h. After lysis, the slides were placed in an electrophoresis chamber, covered with electrophoresis buffer (300 mM NaOH, 200mM EDTA, pH >13) and left for 20 min for the DNA to unwind. The electrophoresis ran for 30 min (1 V/cm tank length), after which the slides were and fixed in 70% ethanol for 5 min. 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).

6. Immunofluorescence microscopy

To visualize nuclear foci, cells were grown on glass coverslips and were ir-radiated with 10 Gy of ionizing radiation. cells were then washed twice with PBS, fixed with 4% paraformaldehyde for 10 min and ice-cold 98% methanol for 5 min, followed by permeabilization with 0.3% Triton X-100 for 15 min at room tem-perature. 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) con-

jugated secondary antibodies, as appropriate. 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 a Zeiss Axioplan 2 imagingepifluorescent microscope equipped with a charge-coupled device camera and ISIS software (MetaSystems, Altlussheim, Germany).

7. GST pull down assay

GST pull down experiments were performed by incubating a various GST-labelled 53BP1 fragments. Bound GST proteins were isolated by incubating the mixture for 1 h at 4ºC in 200ml NETN buffer (300 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 7.4], 0.5% Nonidet P-40), washing 5x with NETN, eluting the proteins with 2x Sample buffer, separating them on SDS PAGE and immunoblottig.

8. Cell survival assay

After treatment with IR, 5×10^2 cells were immediately seeded onto a 60-mm dish in duplicate and grown for 2-3 weeks at 37℃ to allow colony formation. Colonies were stained with 2% methyleneblue in 50%ethanol and counted. The fraction of surviving cells was calculated as the ratio of the plating efficiencies of treated cells to untreated cells. Cell survival results are reported as the mean value ± standard deviation for three independent experiments.

RESULT

1. 53BP1 interacts with ACL

To identify unknown 53BP1 interacting protein, yeast two-hybrid screening was performed. One of the positive clones isolated from the transformants was identified as human ACL. To determine whether 53BP1 interacts with ACL in human cells endogenously expressing both proteins, we utilized co-Immunoprecipitation assay. 293T cells were co-transfected with HA-53BP1 and GFP-ACL. HA-vector and GFP-vector was used for negative control immunoprecipitations. The cells were lysed, and exogenous HA-tagged 53BP1 was immunoprecipitated with a HA specific antibody. Immunoprecipiates were subjected to western blotting with an anti-GFP antibody. Immunoprecipitation results through western blotting showed us the 53BP1 binding to ACL (Fig 1A). To identify which regions of 53BP1 interact with ACL, we subjected HA-

tagged 53BP1, including wild type and serial deletion mutants, N1-N7. 293T cells were transfected with HA-53BP1 mutants, N1-N6 constructs binds GFP-ACL and N7 not bind GFP-ACL (Fig 1B). Co-immunoprecipitation analyses revealed that the 53BP1 segment comprising amino acids 1-100 and 301-500 is critical for 53BP1-ACL interaction. In addition to, to confirm ACL binds to 53BP1, GST-pull down assay were performed (Fig 1C). As a result of the above, Our results from co-immunoprecipitation and GST-pull down assay were revealed that ACL interacts with 53BP1. To investigate 53BP1 interact with ACL upon DNA damage from intact cells, 293T cells was treated with IR to make DSB and harvested after 3hrs, untreated cells was used as control. The cells were lysed, and endogenous 53BP1 was immunoprecipitated with a 53BP1 specific antibody. Immunoprecipitates were subjected to western blotting with an anti-ACL antibody (Fig 2A, upper panel). In this reciprocal experiments, ACL antibody was able to co-immunoprecipitate 53BP1 (Fig 2A,

lower panel). The mouse IgG was used for negative control immunoprecipitations. These results suggest that 53BP1 and ACL proteins interact with each other directly or indirectly.

2. ACL regulates activity of 53BP1

The DNA damage induced by IR or other damaging agent's localize/recruit/interact many DNA repair proteins in the nucleus and form distinct structures called as foci. It was previously shown that 53BP1, a protein proposed to be involved in the repair of DSB, has also been shown to localise to sites of radiation induced DNA DSBs[8]. To identify the physiological relevance of the these interactions, we first investigated localization of ACL and 53BP1. In untreated HeLa cells, 53BP1 forms diffuse nuclear staining and forms discrete 53BP1 nuclear foci in treated. Moreover 53BP1 colocalized with ACL after IR treatment (Fig 2B). Many DNA damage signalling proteins local-

ized nucleus. Therfore, we examined the subcellular localization of ACL after DNA damage. The untreated and IR-treated cells were separated the cytosol and nuclear. As the experimental results found that 53BP1 and ACL exists in both the cytoplasm and the nucleus (Fig 2C). We further investigated 53BP1 foci formation after DSB in control and ACL-depleted HeLa cells. Control and ACL-depleted cells were treated with IR harvested in different time intervals. Cells were fixed with 4%paraformaldehyde and immunofluorescence assay performed. Our observation showed that ACL knockdown impaired DSB-induced 53BP1 foci formation (Fig 3A). After observing our results, 53BP1 foci formation was approximately 87.4% at early time points(0.5hr) but it has increased to 95.7% and then reduced to 70.1% in 6hrs in case of control cells. In contrast to, the foci of 53BP1 was reduced significantly in ACL-depleted cells as compared with control cells (Fig 3B). We next performed whether ACL would affect 53BP1 phosphorylation after DNA damage. Consistent with the

immunofluorescence data, ACL-depleted cells exhibited impaired 53BP1 phosphorylation after IR treatment. Together, these finding suggest that ACL localized with 53BP1 in nucleus, and regulates activity of 53BP1.

3. The foci formation of BRCA1, MDC1, and γ-H2AX is not reduced in ACL-depleted cells

DNA repair-related protein, BRCA1 plays essential roles in homologous recombination and nucleotide excision repair. BRCA1 mediates these functions by interaction with components of the DNA repair machinery and by regulating expression of genes that are involved in these DNA damage repair path-ways[9]. MDC1 also mediated Homologous recombination. We further investi-gated MDC1 and BRCA1 foci formation after DSB in control and ACL-depleted Hela cells. Control and ACL-depleted cells were treated with IR harvested in different time intervals. Cells were fixed with 4% paraformaldehyde and im-munofluorescence assay performed. MDC1, upstream of 53BP1, did not differ

significantly when compared with the ACL-deficient cells and control cells (Fig 4A). Likewise, BRCA1 foci formation did not differ significantly when compared with the ACL-deficient cells and control cells (Fig 4B). Western blotting confirmed that control and ACL knockdown siRNA HeLa cells were treated IR to make DSB and harvested at the indicated time points. As a results, phosphorylation of BRCA1 was not decreased in ACL-deficient cells compared to control cells (Fig 4C).

When cells are exposed to ionizing radiation or DNA-damaging chemo-therapeutic agents, double-stranded breaks (DSBs) are generated that rapidly result in the phosphorylation of histone H2A variant H2AX. Because phos-phorylation of H2AX at Ser 139 (γ-H2AX) is abundant, fast, and correlates well with each DSB, it is the most sensitive marker that can be used to exam-ine the DNA damage produced and the subsequent repair of the DNA lesion.[10] We further investigated γ-H2AX formation after DSB in control and ACL-

depleted Hela cells (Fig 5A). Control and ACL-depleted cells were treated with IR, and harvested in different time intervals. Cells were fixed with 4% paraformaldehyde and immunofluorescence assay performed. γ-H2AX foci formation was approximately 79% at early time points(0.5hr) but it has increased the mount(approximately 100%) in case of control HeLa cells. In contrast to control cells, ACL-deficient HeLa cells showed that γ-H2AX foci formation did not differ significantly (Fig 5B). Western blotting confirmed that control and ACL knockdown siRNA HeLa cells were treated with IR and harvested at the indicated time points. As a results, γ-H2AX reached peak at 3hrs then reduced in 6hrs in case of control HeLa cells. In contrast to control cells, ACL knockdown cells did not differ significantly(Fig 5C). Thus, ACL is regulated with 53BP1 but not BRCA1 and 53BP1.

4. ACL knockdown cells show increased sensitivity to IR and defective NHEJ

To address the overall consequences of the diminished 53BP1 foci formation that occurs in ACL-depleted cells, we analyzed the sensitivity of cells to IR and DSB repair. Control and ACL knockdown HeLa cells were treated with IR, and a clonogenic survival assay was performed. ACL depleted cells were significantly more sensitive to IR than the control cells (Fig 6). These results indicated that ACL supports cell survival in response to IR.

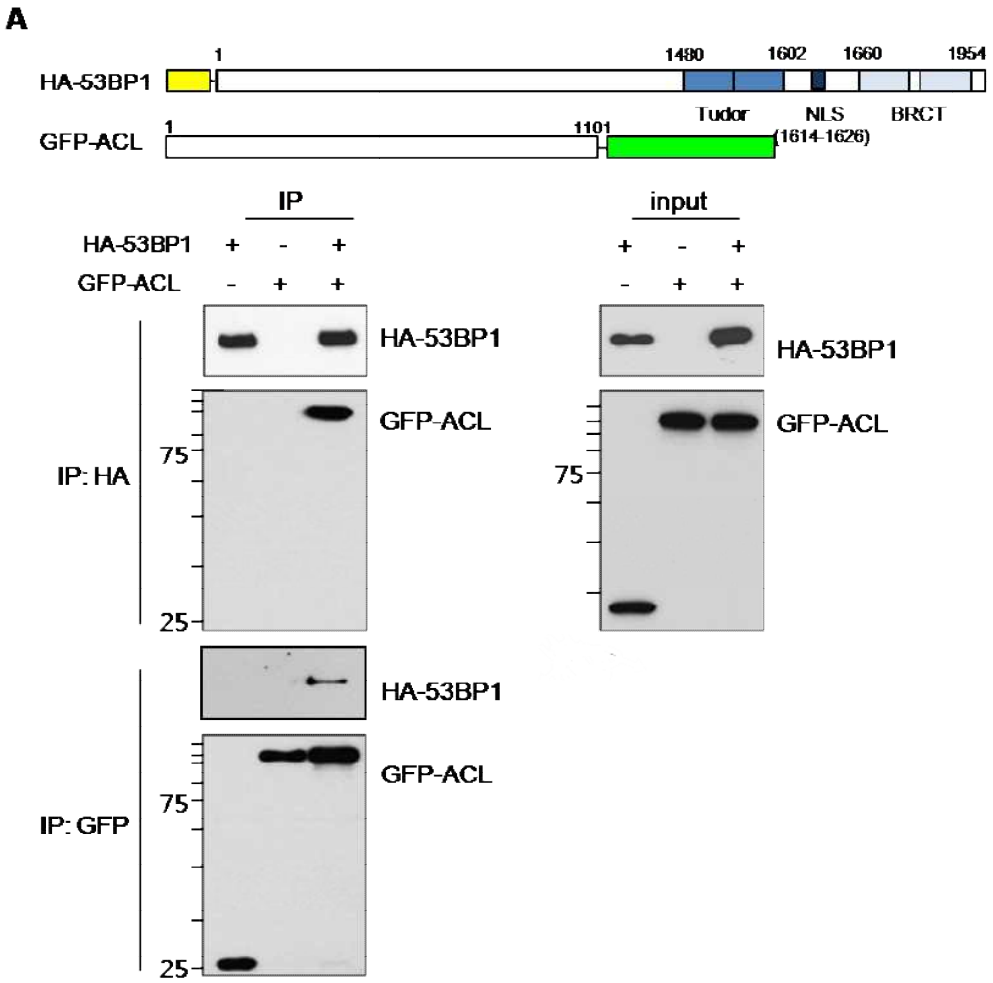
Amongst the different markers of DNA DSBs, one of the most well characterized is the phosphorylation of the histone H2AX (γ-H2AX). Although it is commonly accepted that a γ-H2AX focus indicates the presence of a double strand break (DSB), while foci disappearance is associated with the repair of the DNA damage, the exact relationship between the number of foci and the number of DSBs is still a matter of debate [11]. We further investigated γ-

H2AX formation after DSB in control and ACL-depleted HeLa cells. Control and ACL-depleted cells were treated with IR harvested in different time intervals. Cells were fixed with 4% paraformaldehyde and immunofluorescence assay performed. As shown in Figure 7A, the depletion of ACL leds to increased level of unrepaired DSBs 16hrs after treatment with IR, as evidenced by the number of γ-H2AX foci remaining. This indicates that DSB repair is severely impaired in the absence of ACL. Further evidence of an overt repair defect was shown using the comet assay, which monitors the presence of DSBs in single cells. Based on DNA mobility or comet tail movement DSB repair will be measured. In the initial time point's tail movement will be similar between control and ACL knockdown cells. The comet assay demonstrated that suppression of ACL resulted in extensively longer comet tails after IR treatment than control cells (Fig 7B). The observation of long comet tails in ACL deficient cells indicates impaired DNA DSB repair. These results proved that ACL

knockdown cells unable to repair IR induced DSBs.

Because 53BP1 directly mediates DSB repair through NHEJ, we examined whether an ACL depletion would lead to functional changes in NHEJ of DSBs. Using EJ5-GFP-HeLa cells, we examined the levels of NHEJ when ACL or 53BP1 was depleted and found that the percentage of GFP-positive cells was decreased than in control cells (Fig 8), suggesting that ACL impacts NHEJ. This results indicated ACL and 53BP1 playing an effective role in NHEJ regu-lation and its involvement in DNA damage repair.

Figure 1



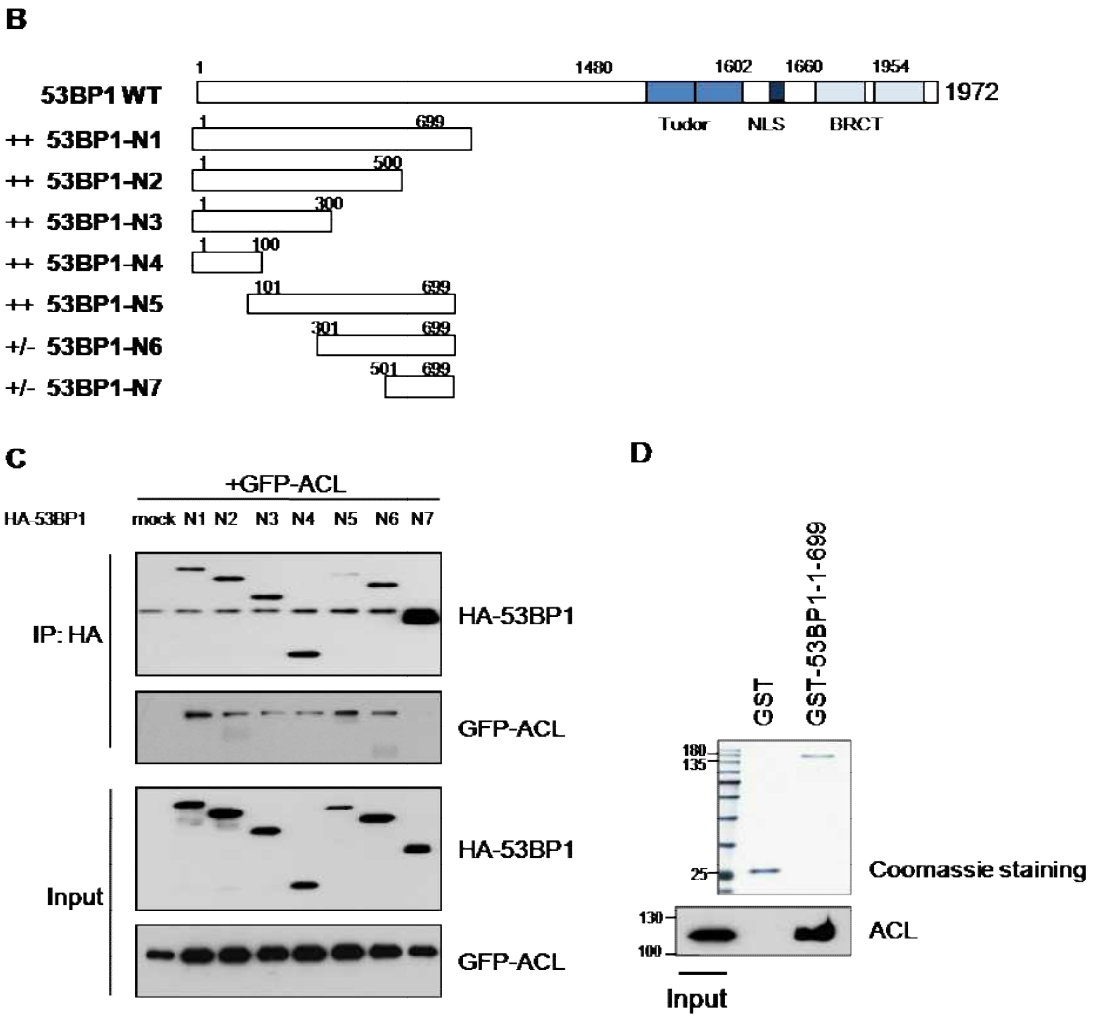


Figure 1. Interaction between ACL and 53BP1.

(A) 293T cells were co-transfected with HA-tagged 53BP1, GFP-tagged ACL. Proteins were immunoprecipitated (IP) from the lysates using an anti-HA anti-body. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for HA or GFP. The input lane contain 5% input for ACL and 20% input for 53BP1. HA-vector and GFP-vector was used for negative control immunoprecipitations. (B) Schematic representation of HA-tagged 53BP1(WT) and the various serial deletion mutants(N1-N7). Relative affinity of ACL binding by each of the 53BP1 deletion mutants is indicated on the left based on the in vi-vo binding assay in Fig 1C. (C) 293T cells were co-transfected with ACL-GFP and various HA-53BP1 constructs named N1-N7 and mock as control HA ex-pression vectors. Immunoprecipitation was done with anti-HA antibody and western blotting with indicated antibodies (D) Confirmation for novel ACL-binding proteins by pull-down assay with GST-53BP1(1-699 a.a) GST-tagged

fragment of 53BP1(1-699 a.a) or GST bead was incubated with ACL protein. GST pull-downs were immunoblotted with ACL antibody. GST-pull downs were confirmed by coomassie staining

Figure 2

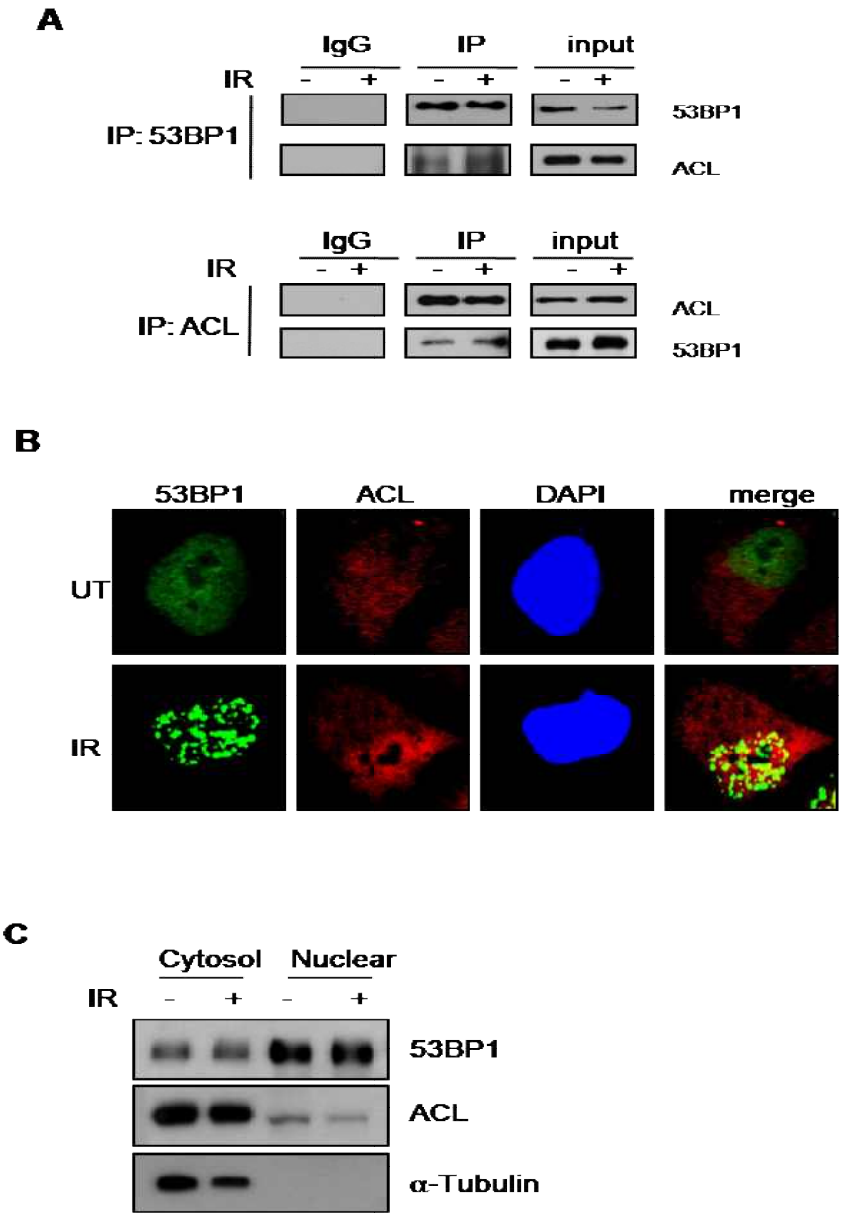


Figure 2. 53BP1 interacts with ACL.

(A) 293T cells were untreated(UT) or 10Gy ionizing radiation(IR) for 3hrs. Proteins were immunoprecipitated from the lysates using an anti-53BP1 or anti-ACL antibodies. Immunoprecipitates were then subjected to western-blot analysis using antibodies specific for ACL or 53BP1. The input lane contain 5% input for ACL and 20% input for 53BP1. Normal rabbit IgG was used for negative control immunoprecipitations. (B) HeLa cells were untreated or treated with IR. After 3hrs cells were fixed and immunostained using anti-53BP1 and anti-ACL antibodies. DAPI staining was performed to indicate the positions of nuclei. (C) HeLa cells were untreated or treated with IR of 10Gy. After 3h, fractionated cytosol and nuclear extracts were subjected western blotting with antibodies against 53BP1, ACL and α-tubulin.

Figure 3

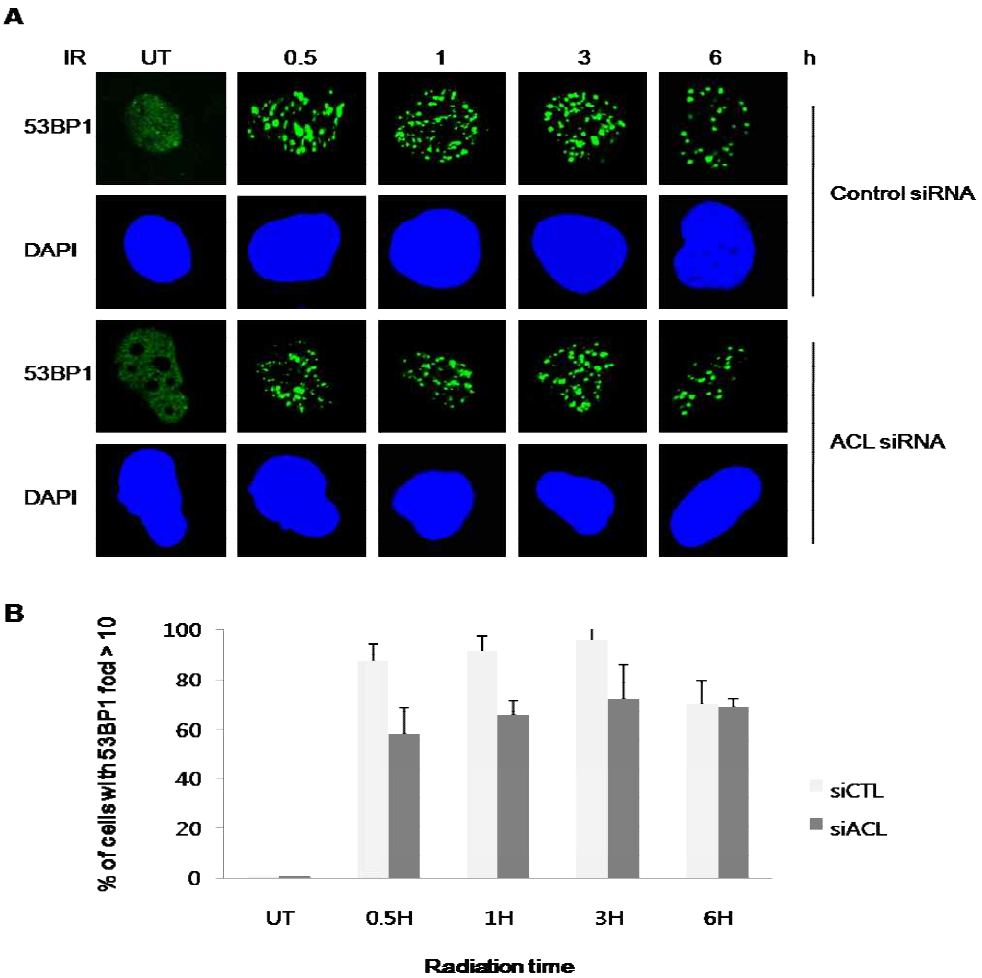
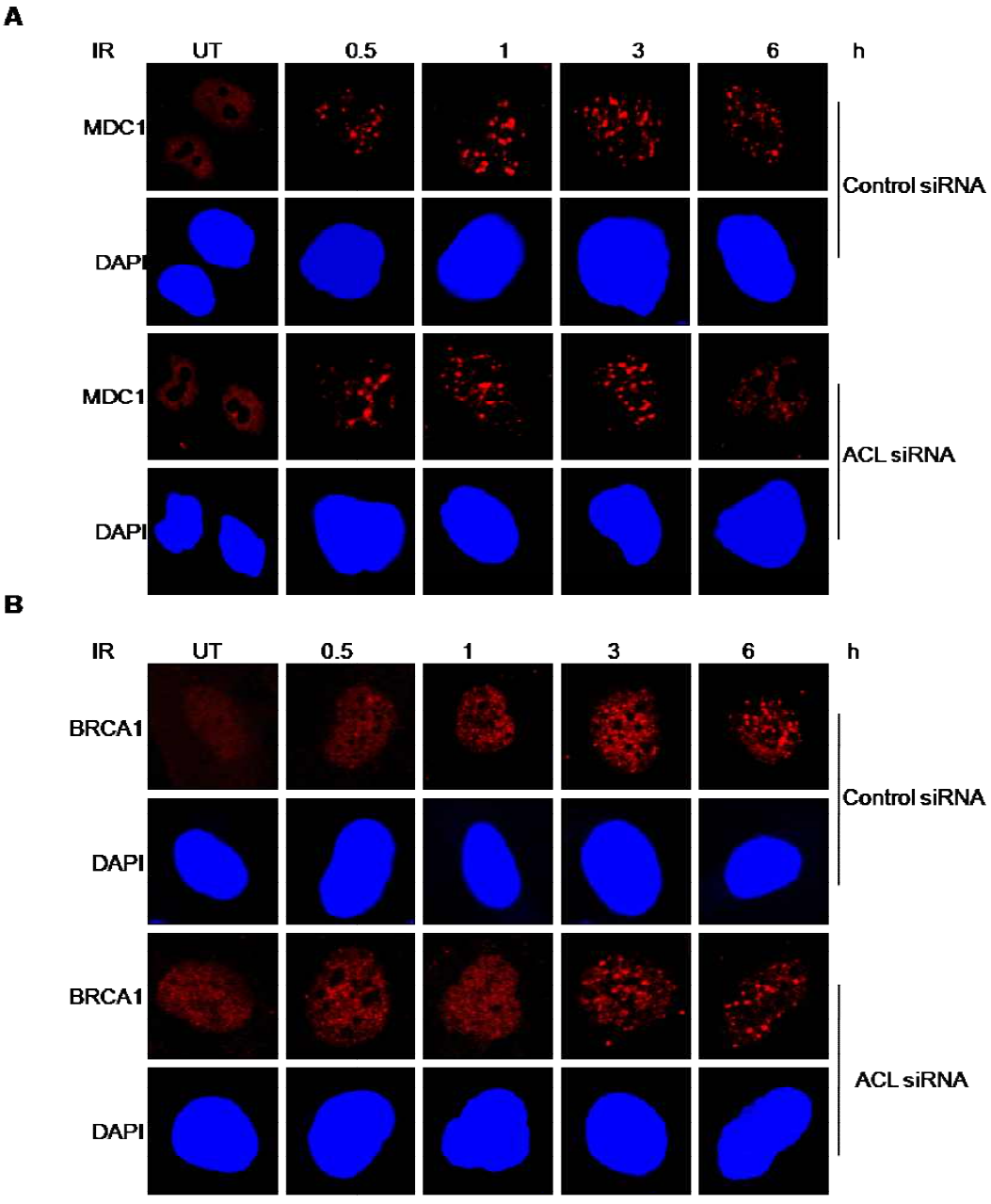


Figure 3. ACL konockdown impairs the recruitment of 53BP1 to DNA damage site.

(A) Control and ACL-depleted HeLa cells were untreated or treated with IR and were then fixed at the indicated times. Cells were stained with an anti-53BP1 antibody and the DNA was counterstained using DAPI. (B) Quantification of cells with 53BP1 damage foci in control or ACL depleted cells with or without IR. The histogram shows the percentage of cells containing > ten distinct 53BP1 foci per cells. At least 100 cells were analyzed for each treatment. Results are shown as means ±SD (n=3). (C) Control cells and ACL-depleted cells were treated with IR and harvested at the indicated time points. Western blot analysis was carried out using specific antibodies against ACL, 53BP1, phospho-53BP1 and β-actin.

Figure 4



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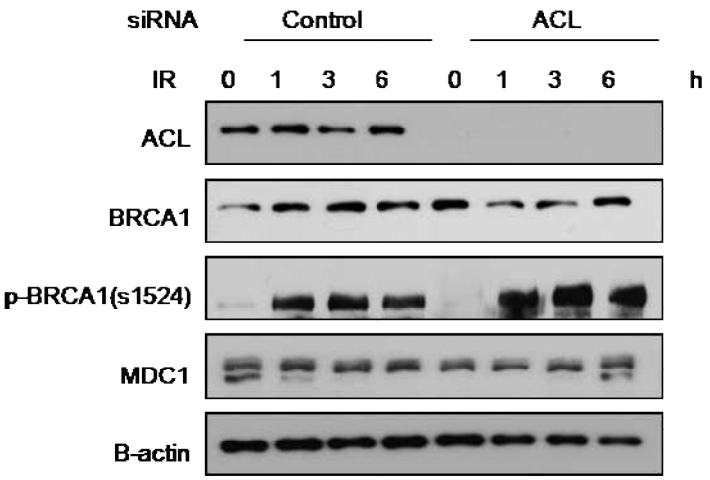

Figure 4. ACL is not effected the recruitment of MDC1 and BRCA1 foci.

(A) Control and ACL-depleted HeLa cells were untreated or treated with IR and were then fixed at the indicated times. Cells were stained with an anti-MDC1 antibody and the DNA was counterstained using DAPI. (B) Control and ACL-depleted HeLa cells were untreated or treated with IR and were then fixed at the indicated times. Cells were stained with an anti-BRCA1 antibody and the DNA was counterstained using DAPI. (C) Control cells and ACL-depleted cells were treated with IR and harvested at the indicated time points. Western blot analysis was carried out using specific antibodies against ACL, 53BP1, BRCA1, p-BRCA1, MDC1 and β-actin.

Figure 5

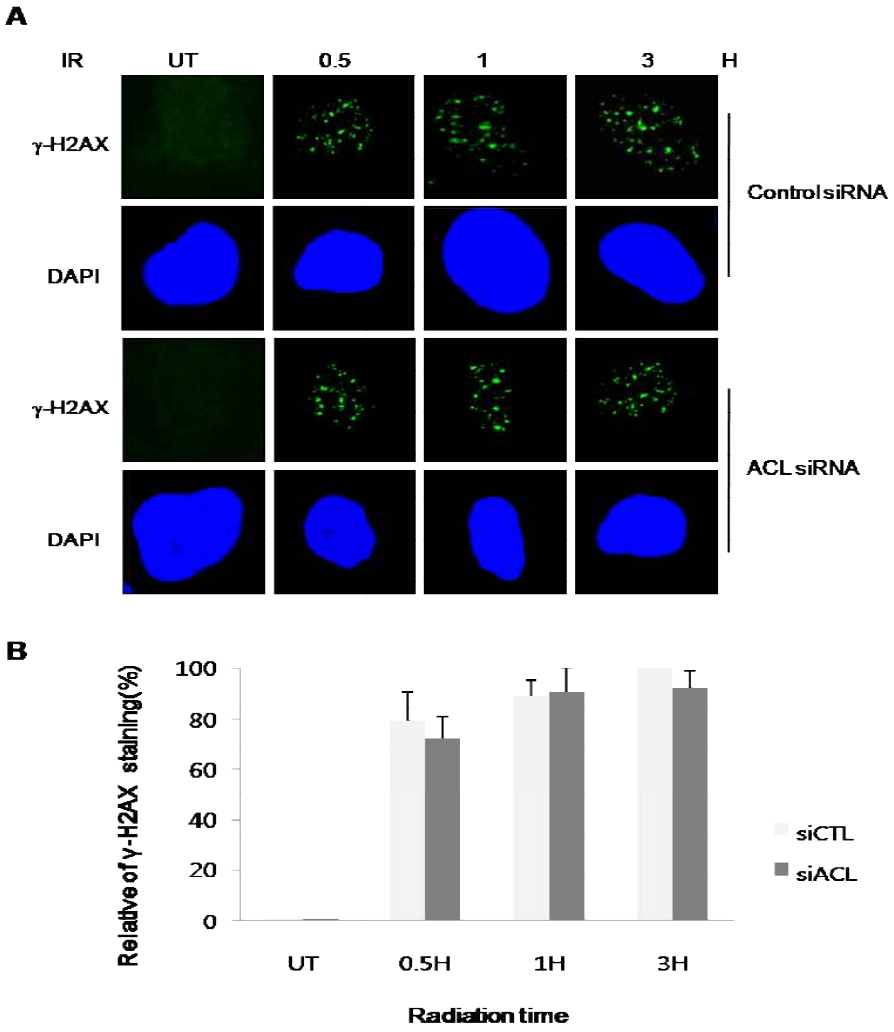

Figure 5. ACL knockdown is not involved in γ-H2AX signals in response to DNA damage.

(A) Control and ACL-depleted HeLa cells were untreated or treated with IR and were then fixed at the indicated times. Cells were stained with an anti-γH2AX antibody and the DNA was counterstained using DAPI. (B) The percentage of cells containing > five γ-H2AX foci are indicated. At least 100 cells were analyzed for each treatment. Data are presented as mean ±SD. (C) Control cells and ACL-depleted cells were treated with IR whole-cell lysates were prepared at the indicated time points, and western blot analysis was carried out using specific antibodies against ACL, 53BP1, γ-H2AX and β-actin.

Figure 6

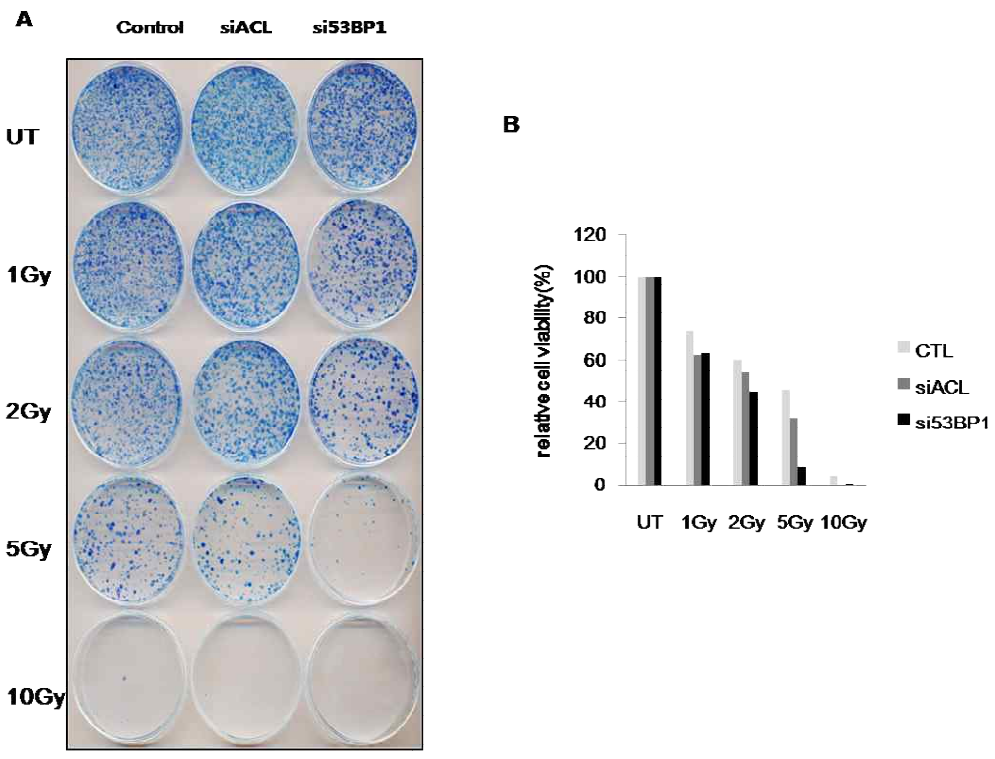

Figure 6. Knockdown of ACL sensitized cells to IR treatment.

Control, ACL and 53BP1 knockdown HeLa cells were untreated or treated with indicated does of IR. After 2 week, (A) cell were stained with methylene blue, and (B) the number of surviving colonies was counted. Data are presented as mean ±SD.

Figure 7

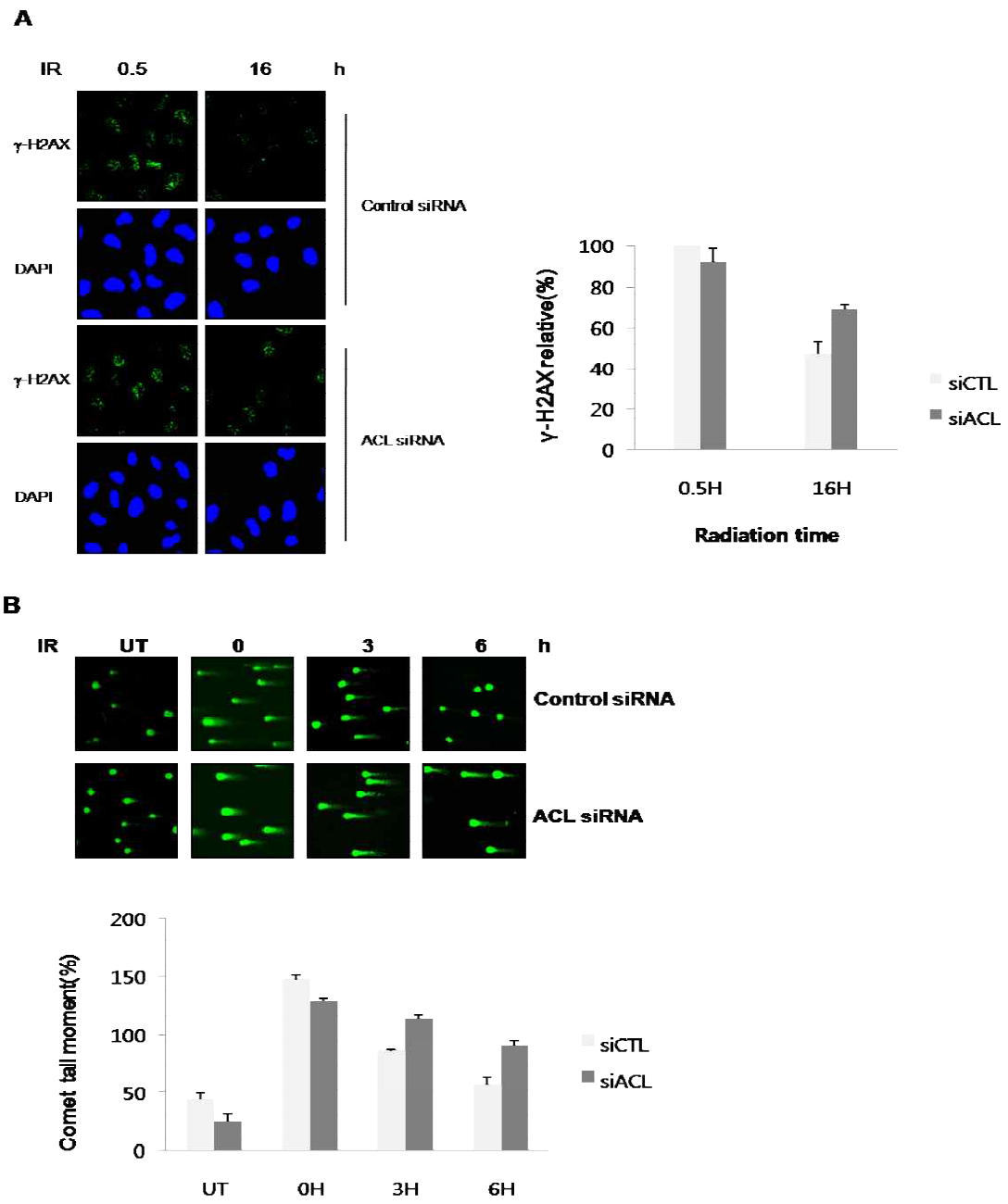

Figure 7. ACL knockdown results in decreased DSB repair.

(A) Counts of γ-H2AX foci at indicated time points after IR in control and ACL-depleted HeLa cells. Representative image(left panel) and quantification(right panel) of unrepaired DSBs are shown. Results are shown as means ±SD. (B) Influence of ACL depletion on single cell electrophoresis(comet) assay of HeLa cells treated with IR. The tail moments of cells for untreated, 0, 3 and 6 hours after IR treatment were measured. The length and intensity of DNA tails relative to heads is shown as % of the tail moment(n=100) in the lower panel. Results are shown as means ±SD.

Figure 8

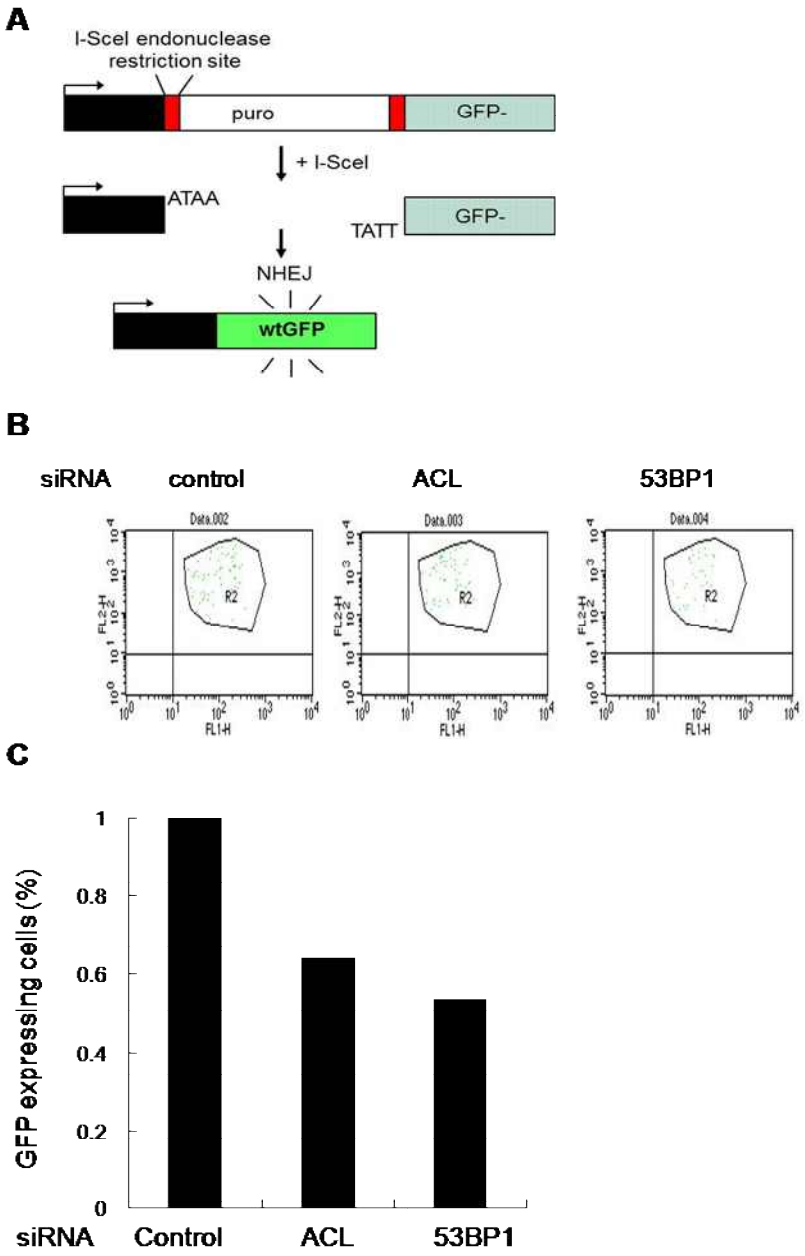

Figure 8. Decreased NHEJ activity in ACL depleted cells.

(A) A diagram for the end-use assay based on the EJ5-GFP reporter, which contains two tandem recognition sites for the I-Sce I endonuclease. Once DSBs are generated by I-Sce I, the reporter construct will then express GFP that can be measured and quantitated. (B) EJ5-GFP-HeLa cells were transfected with the control or ACL siRNA for 24 hours, and then infected with the I-Sce I expressing adenovirus. After 3 days, the population of cells for GFP expression was measured by flow cytometry. (C) Quantification of Fig 8B.

DISCUSSION

Here, we report that ACL is a novel 53BP1-interacting protein identified by yeast two-hybrid screening. We showed ACL interacts with 53BP1 in endoge-nous and exogenous co-immunoprecipitation assay. Different 53BP1 constructs co-immunoprecipitation assay showed us binding site of ACL is N-terminal re-gion 1-100 and 301-500 amino acid of 53BP1. 53BP1 and ACL forms a discrete IR induced nuclear foci and colocalize at DSB site. We report that ACL knock-down cell shows depletion of 53BP1 foci but no change in MDC1 or BRCA1 foci after γ-irradiation. Moreover, ACL deficient cells also showed impaired NHEJ after DSB. ACL depleted cells shown hypersensitive to IR induced less colony formation which is proved by a clonal survival assay. We showed depletion of endogenous ACL accumulates increased amount of unrelieved DSBs, as obser-vations confirmed us ACL is novel interactor of 53BP1 and having critical role in Non-homologous recombination.

It was previously reported, The ATP-driven conversion of citrate and CoA into oxaloacetate and acetyl-CoA by ATP citrate-lyase provides a major source of acetyl-CoA units within the cytosolic compartment of mammalian cells. Because acetyl-CoA is the basic precursor for the synthesis of both sterols and fatty acids, inhibition of ATP citrate-lyase should decrease both cholesterol and fatty acid synthesis.[12; 13; 14; 15] Recent reports showed us ACLY-knockdown in primary human cells triggers cellular senescence and the activation of tumor suppressor p53. In cancer cells, ACLY-silencing-induced p53 activation facilitated DNA-damage-induced cell death. Here we have identified ACL as a novel interacting partner of 53BP1 and involved in functional regulation of NHEJ. In conclusion, we discovered that ACL deficient cells impaired Non-homologous repair activity after DSB [16]. Our data estabilish a mechanistic basis for the ACL-53BP1 cooperation and suggest that, in response to IR, ACL binds directly to 53BP1, promoting the accumulation of 53BP1 at DSB

sites and phosphorylation of 53BP1, leading to NHEJ. Thus, our results illumi-nate a novel interaction between ACL and 53BP1 that is crucial for DSB repair. Furthermore, we should expect to have a effective for the plays an important role in DNA repair.

ABSTRACT

ATP-Citrate Lyase regulates DNA double-strand break repair through binding of 53BP1

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ATP Citrate-Lyase(ACL) is a key enzyme linking glucose metabolism to li-pid synthesis and required for increase in histone acetylation in response to growth factor stimulation and during differentiation. Here, we report that ACL is a novel 53BP1-interacting protein identified by yeast two-hybrid screening. 53BP1 is key regulator of DNA damage response, which plays a critical role in repair of DNA double strand breaks through the non-homologous end joining

pathway. We found that association of ACL and 53BP1 showed in nucleus as well as cytoplasm, suggesting that ACL is functionally related 53BP1-mediated DNA damage response. ACL depletion decreases the recruitment of 53BP1 foci and phosphorylation of 53BP1 after DNA damage. Moreover ACL-deficient cells were hypersensitive to IR-induced cell death and defective to DNA repair, as revealed by a clonal survival assay and comet assay, respectively. ACL-deficient cells were also shown to exhibit impaired non-homologous end-joining. Taken together, these finding suggest that a potential role for ACL in DNA dou-ble strand break repair through interaction with 53BP1.

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