의학석사 학위논문

암세포에서의 DNA 손상 반응에 관한 CIP2A의 역할에 대한 고찰

Oncogenic role of CIP2A on DNA damage response

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고 진 영

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이 논문을 의학석사 학위 논문으로 제출함

2021년 2월

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Abstract

CIP2A (Cancerous inhibitor of PP2A) is widely known for its aberrant overexpression in various tumors and correlation with poor prognosis. Its functions related to inhibition of PP2A have been deeply investigated and MYC, AKT, and mTOR were reported to be affected by PP2A – CIP2A interaction. However, functions of CIP2A independent of PP2A are relatively unrecognized. So, we tried to elucidate the role of CIP2A in cancer irrelevant of PP2A. Here, we show that CIP2A has distinct intracellular dynamics with stress granule, and is degraded on $NaAsO_2$, stress granule inducer. Also, we reveal that CIP2A is deeply connected with DNA repair and DNA damage response network by analyzing high-throughput gene dependency screening data. Further analysis also suggest proliferative effect of CIP2A is mainly related to ATR activity. Additional experiments show that CIP2A influences activation of ATR, possibly interacting with TOPBP1. this study identifies participation in DNA damage response as new mechanism of proliferative effect of CIP2A and provides the possibility that CIP2A might be available target for suppressing ATR as inhibition of ATR activity has been investigated for new chemotherapeutic strategy.

Keywords: CIP2A, PP2A, stress granule, DNA damage, ATR, TOPBP1

Contents

List of figures

Introduction

CIP2A was first reported as p90 autoantigen overexpressed in gastric cancer[1]. This autoantigen was shown to bind and inhibit PP2A, protein phosphatase known as a tumor suppressor, and lead to stabilization and activation of c-Myc[2]. After this study, various reports showed that overexpression of CIP2A was common phenomenon and could be a prognostic factor in different types of cancers[3–5]. Thus far, CIP2A is known to affect the level and activity of diverse proteins related with cell growth and proliferation such as mTOR, KRAS, and AKT through inhibiting dephosphorylation by PP2A[6–8].

Although the name CIP2A was coined from ″cancerous inhibitor of PP2A″, there were researches that described function of CIP2A independent with PP2A. For example, CIP2A was shown to localize to centrosome, a microtubule organizing center of mammalian cells and regulate organization of centrosome via interaction with CEP192 and PLK1[9]. Also, CIP2A was found to be involved Th17 differentiation by modulating interaction of AGK and STAT3 [10]. Furthermore, a biochemical study about binding motifs of PP2A interacting proteins[11] revealed that binding affinity of CIP2A with PP2A-B′γ1, well-established target of CIP2A among 13 PP2A regulatory subunit, was over 10-fold lower than domains of other assayed

proteins. These results imply CIP2A might not be a good inhibitor of PP2A, and the function of CIP2A apart from PP2A could be crucial as well for tumor.

In this regard, we attempted to uncover oncogenic role of CIP2A aside from inhibition of PP2A. two reports gave us cues about the pathways CIP2A is involved. The study about proteome of stress granule reported that CIP2A might be one of components of stress granule [12]. Stress granule is membraneless organelle which forms in response to various stress such as reactive oxygen species, ER stress or osmotic stress. Stress granule is mainly assembled with mRNA and mRNA binding proteins, and consequently its formation induces translational arrest. This temporary hold of protein synthesis helps a cell to prevent overload of damage and to save time for recovery. Tumors grow in a hostile microenvironment. Lack of vascularization compared to its proliferation inevitably causes hypoxic condition, and over-synthesis of protein for growth provokes ER stress[13]. The importance of stress granule to progression of cancer is only to be expected in the light of these unfavorable circumstances. Furthermore, formation of stress granule could provide resistance to chemotherapeutic drugs such as bortezomib[14].

The other study performed a CRISPR-knockout gene library screening to investigate dependency of each gene to hundreds of cancer cell lines[15]. This work made possible to

measure the effect of knockout of specific gene to proliferation of specific cell line. In this study, CIP2A was shown to have similar dependency profile with well-known DNA damage response (DDR) genes such as H2AFX, MDC1, and TOPBP1 rather than subunits of PP2A. This suggests CIP2A might be related in DDR pathway on its own without involvement of PP2A. Furthermore, CIP2A was reported to invoke resistance to doxorubicin and cisplatin, chemotherapeutic agents acting by inducing DNA damage[16,17]. Phosphorylation of AKT by inhibiting PP2A was proposed as a mechanism of resistance. However, participation of CIP2A itself in DDR could be another mechanism of resistance against these reagents.

Therefore, we investigated whether CIP2A were associated with stress granule or DDR pathways. Our results show that CIP2A is not colocalized components of stress granule and does not affect assembly or disassembly of stress granule. In addition, we demonstrate that CIP2A is deeply connected with DDR network proteins by analyzing gene dependency data. Additional experiments show that CIP2A influences activation of ATR on replication stress and provide the possibility that CIP2A interacts with TOPBP1, the ATR activator.

Materials and methods

2.1. Reagents

Sodium arsenite, hydrogen peroxide, and hydroxyurea were purchased from Sigma-Aldrich. Camptothecin was purchased from Selleck Chemicals.

2.2. Cell lines

A549 and U2-OS cell lines were obtained from Korean Cell Line Bank (KCLB). HEK293T Lenti-X cell lines were obtained from Takara Bio. A549 cells were maintained in RPMI 1640. U2-OS cells were maintained in McCoy's 5A media. HEK293T Lenti-X cell lines were maintained in DMEM. All media were supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

2.3. Immunoblotting

Cells were lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl pH 8.0) containing protease and phosphatase inhibitors. After centrifugation at 12,000 g for 20 min, supernatants were collected and boiled with Laemmli sample buffer for 5 min. SDS-PAGE were performed to discriminate by molecular weight of proteins and transferred to nitrocellulose membrane. Chemiluminescence emitting from Horseradish peroxidase (HRP) conjugated antibodies was detected by ChemiDoc XRS+ (Bio-rad). Antibodies used in immunoblotting included: CIP2A (sc-80659, Santa Cruz, 1 : 10,000 dilution), G3BP1 (sc-365338, Santa Cruz, 1 : 10,000 dilution), Actin (A2228, Sigma Aldrich, 1: 10,000 dilution), pATM S1981 (5883, Cell Signaling Technology, 1: 10,000 dilution), pCHK1 S345 (2348, Cell Signaling Technology, 1: 10,000 dilution), pCHK2 T68 (2197, Cell Signaling Technology, 1: 10,000 dilution), FLAG (14973, Cell Signaling Technology, 1: 10,000 dilution). All immunoblotting experiments were performed at least 3 times and representative result is shown.

2.4. Immunofluorescence

Cells were seeded on cell culture chamber slide. After drug treatment, cells were fixed by 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were

incubated in 5% normal goat serum with 0.3% Triton X-100 for 1 h. cells were then incubated with PBS containing antibody diluted as manufacturer's instruction for overnight at 4°C. After washing off unbound antibody, fluorescent dye conjugated antibodies were added to chamber and incubated for 2 h at room temperature. Slides were mounted using Vectashield antifade mounting media with DAPI (4′,6-diamidino-2-phenylindole) and cured overnight before imaging. Images were acquired with Carl Zeiss Axio Observer.Z1 microscope equipped with AxioCamHR3. C-Apochromat 63x/1.20 objective was used. Antibody used in immunofluorescence included: G3BP1(61559, Cell Signaling Technology, 1:400 dilution).

2.5. Immunoprecipitation

HEK293T cells were lysed in IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol). After centrifugation at 12,000 g for 20 min, supernatants were collected. 2 μg of antibody against HA (M180-3, MBL) were added to lysate. Lysate were incubated at 4°C with rotation for overnight. Protein A agarose beads (20333, Thermo scientific) were added to lysate and incubated for 2 h for antibody binding. After several washing, immunoprecipitates were eluted by boiling 5 min with 2X Laemmli sample buffer. Eluted sample were immunoblotted as described above.

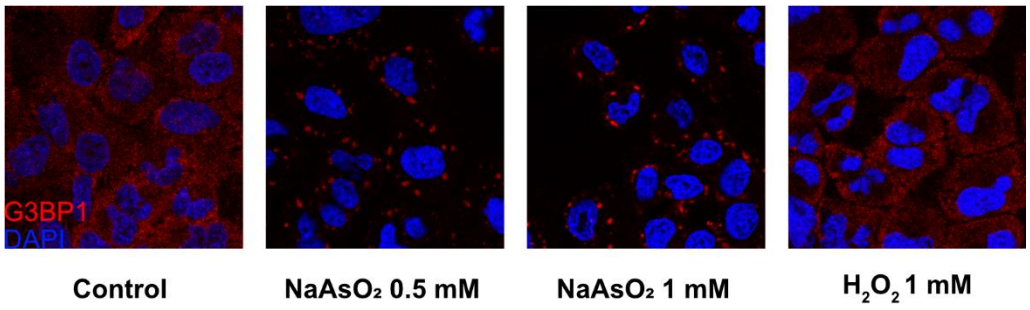
2.6. Gene dependency score analysis

The CRISPR-knockout gene library screening data we used in this study is: DepMap 20Q2 Public. figshare. Dataset. (https://doi.org/10.6084/m9.figshare.12280541.v4.). R version 4.0.0 was used for calculation of Pearson's correlation coefficient. R package pheatmap (v1.0.12) was used to generate heatmap of gene dependency value. Protein interaction network analysis and gene ontology analysis were performed at STRING (https://string-db.org/).

Results

3.1. Formation of stress granules upon treatment of sodium arsenite

First, we chose to confirm the formation of stress granules upon treatment of drugs including sodium arsenite ($NaAsO_2$) in A549 lung cancer cells. G3BP1 was used as marker for stress granule assembly. A549 cells were stained with antibody against G3BP1 for immunofluorescence (IF), after $NaAsO_2$ or hydrogen peroxide (H_2O_2) treatment. G3BP1 was dispersed across cytoplasm evenly in untreated cells (Figure 1A). With $NaAsO_2$, cytoplasmic foci of G3BP1 appeared at 1 hour after treatment, indicating formation of stress granule. There was no prominent variance of size, number, or shape of stress granule with different concentration of $NaAsO_2$. H_2O_2 that were reported to induce stress granule in some cell lines[18] did not induce foci of G3BP1, implying formation of stress granule to specific stress could be affected by cell type.



[Fig. 1] Formation of stress granules with treatment of $NaAsO_2$

A549 cells were treated with $NaAsO_2$ or H_2O_2 for 1 hour and stained for G3BP1.

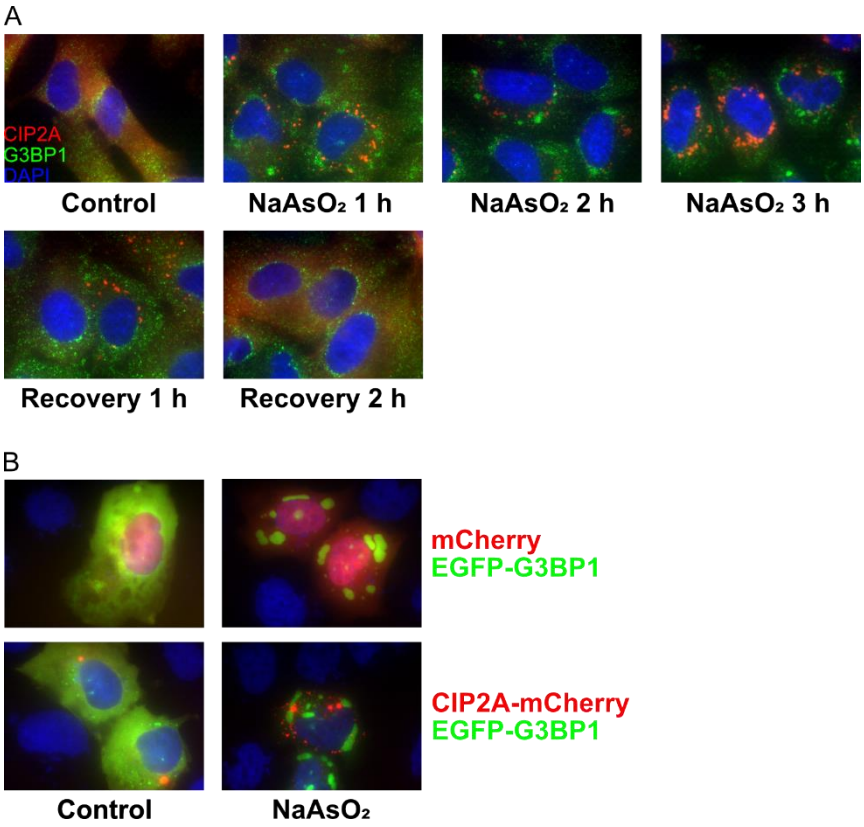
3.2 CIP2A does not participate in the formation of stress granules

Next, we tried to determine whether CIP2A was indeed a part of stress granule by detecting intracellular localization of CIP2A when stress granules were assembled. As commercially available antibodies of CIP2A we had failed to stain CIP2A for IF, we decided to ectopically express mRFP1 tagged CIP2A to A549 cells. Lentivirus was used to generate stable cell line expressing CIP2A tagged with mRFP1 to its N-terminus. Without treatment of $NaAsO_2$, Both CIP2A and G3BP1 were distributed throughout cytoplasm. (Figure 2A). After $NaAsO_2$ treatment, against our expectations, CIP2A did not colocalize to G3BP1 at all, while G3BP1 foci were formed as usual. Instead, CIP2A formed distinct foci that does not overlap with G3BP1. These CIP2A foci was smaller than G3BP1 foci and tended to be located to perinuclear region. Dynamics of these foci were also different to that of stress granule. Stress granule disappeared with recovery in normal media for 1 hour. However, CIP2A foci remained at that time, although the number of foci decreased. At the point of recovery for 2 hours, the intracellular distribution of CIP2A were restored to that of untreated cells.

As the result against our anticipation was acquired, we sought to improve experiment conditions that could affect intracellular localization of CIP2A. A549 cells were replaced with U-2 OS osteosarcoma cell line since it was the cell line the proteomics analysis of stress

granule was performed. mCherry was used for fluorescent tagging instead of mRFP1 due to its superior photostability. Tagging of fluorescent protein into either N or C terminus is known to be able to alter distribution of a protein inside a cell. In general, C terminus tagging shows more consistent correlation with IF than N terminus tagging[19]. So, we investigated intracellular positioning of C terminus tagged CIP2A. Also, it is reported that sometimes fluorescent protein itself can induces protein aggregation[20]. Therefore, transfection of mCherry expression plasmid was used to determine whether formation of CIP2A foci were due to fluorescent protein.

In nontreated cell, mCherry were evenly dispersed throughout nucleus and cytoplasm, and it showed brighter signals in nucleus than cytoplasm (Figure 2B). In contrast, CIP2A localized predominantly in cytoplasm, and formed one perinuclear focus. Given its position and that CIP2A were revealed to be localized to centrosome[9], it might correspond to centrosomal CIP2A. this focus was not observed for CIP2A with N terminus tag, implying different location of tag can impact the movement of CIP2A. After $NaAsO_2$ treatment, distribution of mCherry was not changed even though formation of stress granule was occurred normally. CIP2A formed separate foci from stress granule like before. these results demonstrate that CIP2A was not the component of stress granule, at least formed by $NaAsO_2$.



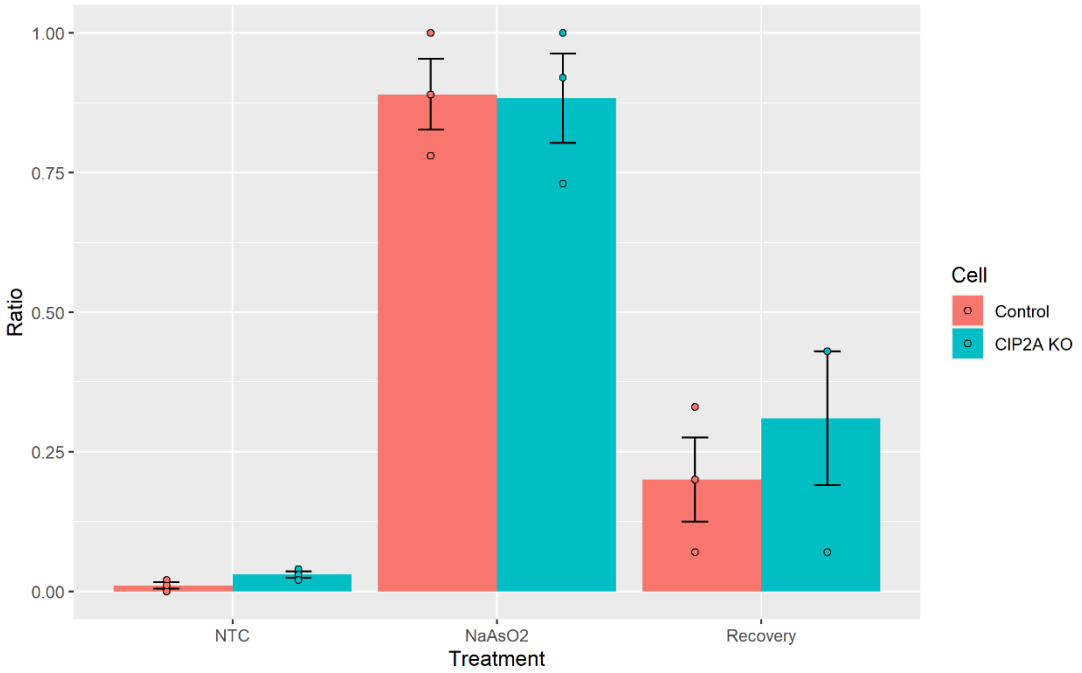
[Fig. 2] intracellular localization of CIP2A with formation of stress granules

(A) A549 cells transduced with lentivirus for expression of mRFP1 tagged CIP2A were treated with $NaAsO_2$ 0.5 mM for indicated time and stained for G3BP1. For recovery, cells were washed with PBS for 3 times, and replenished with fresh media.

(B) U-2 OS cells were transfected with EGFP tagged G3BP1 and either mCherry or CIP2A fused with mCherry at its C terminus. Cells were treated with $NaAsO_2$ 0.5mM for 1 hour and imaged with fluorescent microscopy.

3.3. Loss of CIP2A does not induce changes the dynamics of stress granules

Although CIP2A itself was not the part of the proteome of stress granule, there was still a chance that CIP2A influences stress granule by interacting proteins belong to proteome of stress granule. Indeed, In mass spectrometry analysis of CIP2A interacting proteins in helper T 17 cells[21], multiple proteins known as component of stress granule were detected such as G3BP1, PABP1, and CAPRIN1. So, we investigated whether existence of CIP2A could change the dynamics of stress granule by estimating the ratio of cells with stress granule after treatment or removal of $NaAsO_2$. CRISPR/Cas9 gene editing was employed to generated CIP2A knockout A549 cell lines (CIP2A-KO). Knockout of CIP2A itself did not induce any stress granules (Figure 3). With $NaAsO_2$, stress granules were assembled in most wildtype and CIP2A-KO cells. The ratio of cells with stress granule was not different depending on the expression of CIP2A. After 1 h recovery with fresh media, stress granules were largely disappeared. The percentage of cells with remaining stress granules were slightly higher in CIP2A-KO cells, but the difference was marginal. Therefore, we concluded that CIP2A was not associated with the dynamics of stress granule.

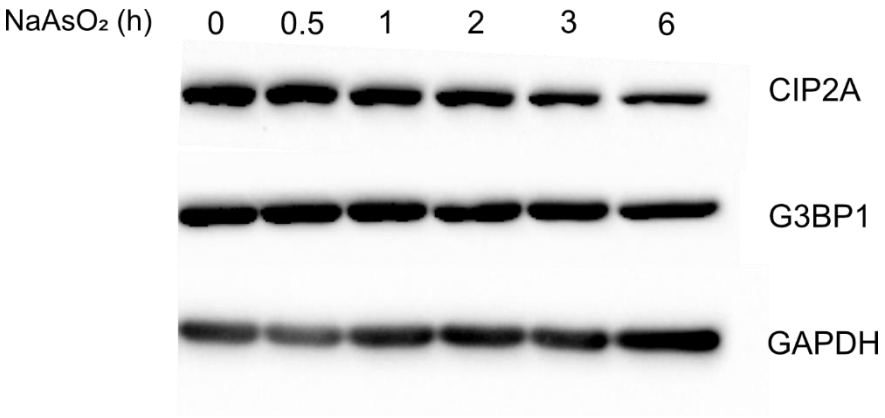


[Fig. 3] Assembly and disassembly of stress granules with loss of CIP2A

A549 cells transduced with lentivirus for expression of Cas9 with non-targeting sgRNA or CIP2A targeting sgRNA were treated with $NaAsO_2$ 0.5 mM for 1 h. For recovery, cells were washed with PBS for 3 times, and replenished with fresh media for 1 h.

3.4. CIP2A is degraded with treatment of $NaAsO_2$

Although CIP2A was not colocalized with stress granules, the formation of distinct foci after $NaAsO_2$ suggested that CIP2A was one of the proteins related to intracellular response to cellular stress. To ascertain the fate of CIP2A with $NaAsO_2$ treatment, we performed western blot for CIP2A and G3BP1 with elongation of treatment time by 6 hours, which was not commonly used condition due to rapid assembly of stress granule within 1 hour. CIP2A was degraded with over time unlike G3BP1, which maintained constant level (Figure 4). this result suggests that CIP2A foci observed earlier were CIP2A in degradation process, possibly protein aggregates.



[Fig. 4] Degradation of CIP2A with $NaAsO_2$

A549 cells were treated with $NaAsO_2$ 0.5 mM for indicated time. Cell lysates were subjected to western blot analysis against CIP2A, G3BP1, and GAPDH.

3.5. Gene dependency score analysis shows strong connection of CIP2A with DNA damage repair and response pathway

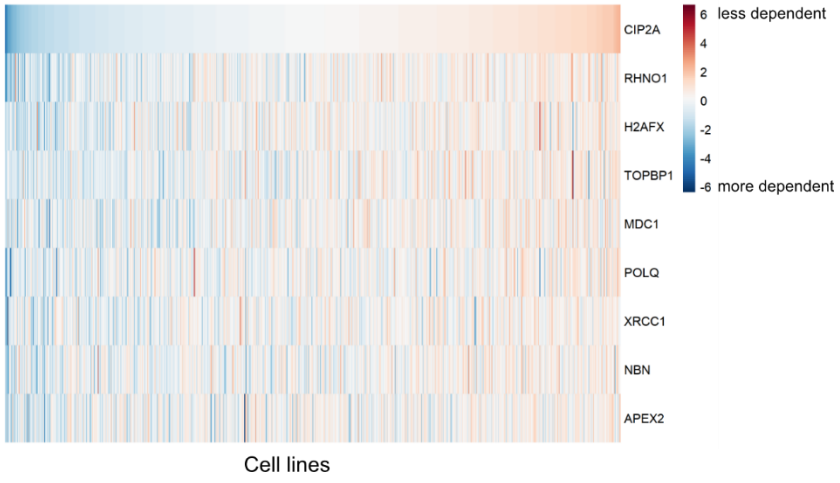
After CIP2A was revealed that it was not associated with stress granule, we explored gene dependency data of cancer cell lines created by DepMap project to gain insights about function and pathways related to CIP2A. CIP2A was categorized as dependent for 102 out of 789 cell lines. As mentioned above, correlation analysis of gene dependency score with that of CIP2A unveiled that genes encoding proteins of DDR such as H2AFX, TOPBP1, MDC1, and NBN were top co-dependent genes (Figure 5A). High correlation of dependency score means that knockout of specific gene had similar effect with another gene, implying the products of two genes have similar role or belong to same biological pathway. On the other hand, correlation coefficient of the subunits of PP2A that were already shown to be interact with CIP2A such as PPP2R5A and PPP2R5C [22] is 0.075, and 0.037, respectively. This result suggests that the function of CIP2A might be more related to DNA damage than suppression of PP2A in the perspective of viability and proliferation of tumor cells. Heatmap of dependency score also showed tight correlations among these genes (Figure 5B). These results show that proliferative effect of CIP2A on cancer cell lines is mainly related with DNA damage response.

To investigate the common function of proteins encoded by highly correlated genes, we analyzed previously revealed interaction among these proteins using STRING database. Network map drawn by STRING clearly showed that almost all these proteins interacted with one another (Figure 6A). Gene ontology analysis of these genes also showed enrichment of DNA damage related process such as DNA repair, DNA recombination, and double-strand break repair (Figure 6B). In summary, these results suggest that CIP2A might have unrecognized interactions with DDR network proteins.

A

Gene	Correlation
RHNO1	0.487
H2AFX	0.397
TOPBP1	0.38
MDC1	0.342
POLQ	0.334
XRCC1	0.308
NBN	0.307
APEX2	0.301
PPP2R5A	0.075
PPP2R5C	0.037

B

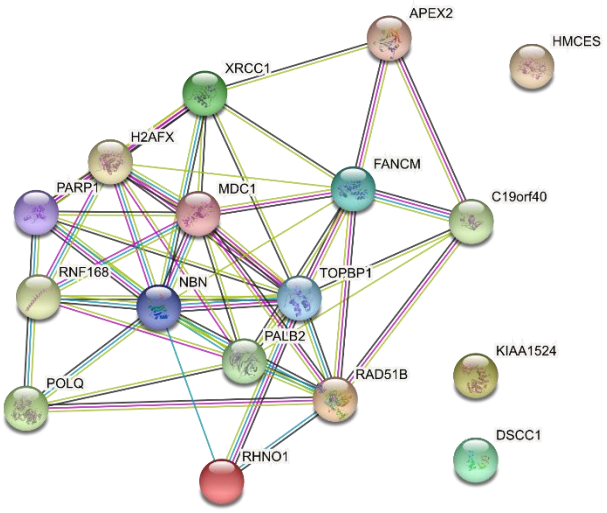


[Fig. 5] Correlations among gene dependency scores with CIP2A

(A) Pearson's correlation score of gene knockout effect with CIP2A was calculated for each gene. Genes with correlation score over 0.3 are shown. The cutoff value of 0.3 was arbitrarily chosen due to length of table. Correlation coefficients of PPP2R5A and PPP2R5C are added for comparison.

(B) Heatmap analysis of highly correlated genes listed in figure 5A. Each cell line was lined up on x axis and arranged with score of CIP2A in ascending order. Normalized Z score of gene knockout effect was used to fill heatmap.

A



B

#term ID	term description	observed gene count	background gene count	strength	false discovery rate
GO:0006281	DNA repair	14	491	1.53	2.07E-17
GO:0006259	DNA metabolic process	15	773	1.31	4.41E-17
GO:0006310	DNA recombination	11	202	1.88	5.13E-16
GO:0000725	recombinational repair	8	89	2.06	8.56E-13
GO:0006302	double-strand break repair	9	178	1.64	1.40E-12

[Fig. 6] Connection among highly co-dependent genes with CIP2A

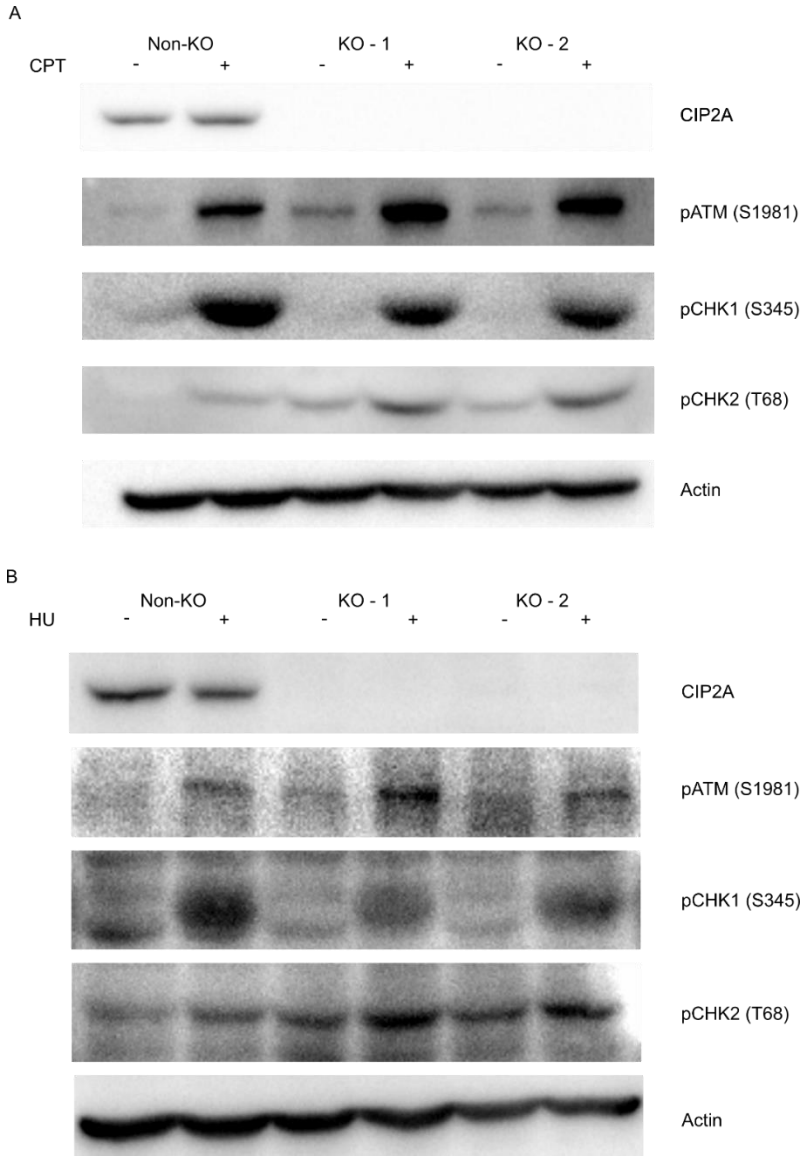
(C) Genes with correlation score above 0.25 were analyzed for interaction mapping using STRING database. KIAA1524 is gene name of CIP2A.

(D) Gene ontology analysis of genes with correlation score above 0.25. Top 5 biological processes with the lowest false discovery rate are shown.

3.6. Loss of CIP2A induces impairment of ATR activation

As the list of figure 5A included genes encoding proteins related to ATR activation such as RHNO1, TOPBP1, and NBN, we investigated whether knockout of CIP2A could induce impairment of ATR activation. ATR is one of a family of phosphoinositide 3-kinase related kinases (PIKKs). unlike ATM and DNA-PKcs, two other PIKK, ATR is mainly activated by single strand DNA (ssDNA) lesion, commonly occurring in DNA replication and transcription. So, we treated camptothecin (CPT), topoisomerase I inhibitor to activate ATR pathway to A549 cells and examined the activation of DDR proteins by western blot. As expected, the phosphorylation of checkpoint kinase 1 (CHK1) on serine 345 (S345) residue was decreased in CIP2A-KO cells of two different sgRNA (KO - 1, and KO - 2) (Figure 7A). CHK1 is the main target of ATR, and phosphorylation of S345 by ATR induces activation of CHK1. Reduced CHK1 phosphorylation upon same treatment implied that activation of ATR might be impaired in cells without CIP2A. There could be alternative explanation of this result such as less damage might be induced by CPT in CIP2A-KO cells, and hence ATR did not need to be activated. However, this possibility was hardly likely as the phosphorylation of checkpoint kinase 2 (CHK2) on threonine 68 (T68) was increased in CIP2A-KO cells. This site is phosphorylated by ATM. Phosphorylation on serine 1981 (S1981) of ATM,

autophosphorylation site which indicates activation of ATM was also increased more with CPT in CIP2A-KO cells. This implied that more DNA double strand breaks (DSBs) were generated by CPT, and DSBs induced by CPT are known to be increased when activity of ATR is impaired[23]. We also treated hydroxyurea (HU) to A549 cells. Hydroxyurea induces replication fork collapse by deoxyribonucleotide pool depletion, thus activates ATR signaling pathway in a different way to CPT. Like CPT, hydroxyurea treatment also induced less CHK1 phosphorylation, and more CHK2 phosphorylation in CIP2A-KO cells (Figure 4B). In summary, these results show that activation of ATR related to replication stress is hampered by loss of CIP2A.



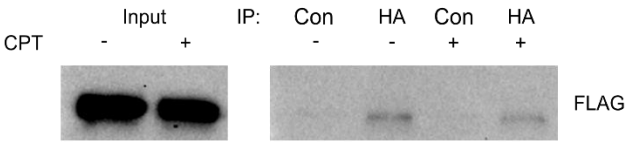
[Fig. 7] Activation of ATR and ATR pathways on replication stress-inducing drugs

(A) A549 cells were transduced as described above and treated with 1μM CPT for 2 h. Cell lysates were subjected to western blot analysis.

(B) A549 cells prepared as described above were treated with 2mM HU for 3 h. Cell lysates were subjected to western blot analysis.

3.7. CIP2A interacts with TOPBP1, activator of ATR

Next, we tried to examine whether CIP2A exerts influence on ATR activation by interacting proteins of ATR activation complex. Over 10 proteins are reported to be localized with ATR to collapsed replication fork and participate ATR activation. We selected binding candidates by co-dependency score (RHNO1, TOPBP1) and previous high-throughput experiments that investigated interaction partner of CIP2A[10] (RAD50). With coimmunoprecipitations experiments using HEK293T cells overexpressing HA tagged CIP2A and FLAG tagged TOPBP1, we observed binding of CIP2A with TOPBP1 in untreated or CPT treated cells (Figure 8). this result implies that CIP2A might participate in ATR activation by interacting TOPBP1.



[Fig. 8] Coimmunoprecipitation assay of CIP2A and TOPBP1

HEK293T cells were co-transfected with HA tagged CIP2A and FLAG tagged TOPBP1 expressing plasmids. After 24 h of transfection, Whole cell lysates were immunoprecipitated with anti-CIP2A antibody. Eluates were subjected to western blot analysis against FLAG.

Discussion

Here, we tried to elucidate the functions of CIP2A other than inhibitor of PP2A, mainly related to stress granules and DNA damage. Although high-throughput assay designated CIP2A as one of components of stress granules, we could not observe the colocalization of CIP2A and G3BP1 with treatment of $NaAsO_2$ (Figure 2). We did not exclude the case that CIP2A interacted with other proteins of stress granule like FUS or HuR except G3BP1. However, we considered it hardly possible as the proteomics assay used G3BP1 for purification of proteome of stress granule. This disagreement between high-throughput assay and validation assay was not surprising as authors themselves reported that only 9 out 15 proteins they found by assay actually colocalized with G3BP1, showing mediocre performance in the perspective of specificity. Also, there are other several researches that studied proteomics of stress granule [24,25] and none of articles reported CIP2A as components of stress granule, suggesting low probability of involvement of CIP2A to stress granule.

Although CIP2A did not colocalize with stress granule, $NaAsO_2$ clearly made changes of subcellular localization of CIP2A (Figure 2). Considering western blot results showing degradation of CIP2A with prolonged exposure to $NaAsO_2$ (Figure 4), the foci could be protein aggregates of misfolded CIP2A. CIP2A is mainly located in cytoplasm but has amino acid

sequence of high hydrophobicity that is predicted as transmembrane region. Exposure of transmembranous hydrophobic region to cytoplasm is known to induce protein aggregates, leading to the formation of aggresome[26]. Also, CIP2A has been reported to form aggresome with natural compound celastrol[27]. Direct observation of aggresome formed by prolonged $NaAsO_2$ exposure in A549 cells was failed due to impaired cell attachment to slide by increased cell toxicity. Additional experiments with different exposure time and concentration of $NaAsO_2$ are required.

Additionally, we discovered CIP2A had a role in DDR. The assumption that CIP2A is related to DDR came from high throughput gene knockout effect screening data from DepMap project. In the paper reporting the results of DepMap project[28], the authors showed high correlation among genes in MTOR-AKT pathway which was already established to interact with each other. So, we tried to discover new interacting proteins of CIP2A with similar approach. It was surprising that DNA repair and DDR related genes were top hit considering CIP2A was barely reported to be in nucleus.

We also revealed that activation of ATR to replication stress were impaired by loss of CIP2A (Figure 7). ATR, and its downstream kinase CHK1 is indispensable for cellular viability and complete loss of these proteins induces cell death. Unlike ATM, other DNA damage related

PIKK that is well-known tumor suppressors, ATR helps tumor cells to proliferate by preserving genome integrity against increased replication stress[29]. Direct inhibition of ATR activity has been tested in clinical trials of multiple types of cancers. Currently, there are over 30 ongoing clinical trials of ATR inhibitors such as M6620 or AZD6738 registered in clinicaltrials.gov. Given its low expression in normal tissues[2], CIP2A might be a legitimate target to suppress activity of ATR specifically in tumors.

Mechanistically, we showed that CIP2A interacted with TOPBP1 by co-immunoprecipitation assay (Figure 8). TOPBP1 is well-known ATR activator, binding ATR with its ATR-Activation-Domain (AAD)[30], and has multiple BRCA1 C-terminal (BRCT) domain. Therefore, we have assumed that CIP2A helps activation of ATR by cooperating with TOPBP1. Recently, an article was published as preprint at Biorxiv that argued CIP2A disturbed the function of TOPBP1 in G2/M checkpoint by inhibiting formation of TOPBP1 complex[31]. The discrepancy between results could be derived from the difference of experimental settings such as cell lines or type of DNA damage. The authors used ionizing radiation (IR) to induce DSBs that activates mainly ATM pathway, while we used CPT or HU to induce replication stress to activate ATR. Also, they focused on G2/M checkpoint, but CPT or HU-induced replication stress primarily

occurs in S phase. Hence, it could be interesting to investigate whether CIP2A – TOPBP1 relationship changes based on activated pathway or cell cycle.

We could not determine the precise mechanism about how CIP2A interacts with TOPBP1 and affects activation of ATR. Unlike CIP2A, TOPBP1 is known to be mainly located in nucleus and form foci at stalled replication forks[32]. There was no the change of subcellular localization of CIP2A with CPT or HU (data not shown), suggesting the way CIP2A influences DDR might not be associated with nuclear import of CIP2A. we speculate that cytoplasmic TOPBP1 could interact with CIP2A as there were reports that TOPBP1 was aberrantly expressed in cytoplasm, especially in tumors[33,34]. Also, both protein was shown to be located in centrosome,[9,35] suggesting CIP2A and TOPBP1 could interact besides at the replication forks. Although a study of immunoprecipitation-based approach for detecting proteins interacting with CIP2A was recently reported[21], few DDR-related proteins were listed in CIP2A interactome, suggesting experiments with cells in DNA damage might be needed to identify interaction partners of CIP2A in context of DNA damage. Currently, we are trying to determine interactome of CIP2A in cells suffering from DNA damage by proximity labeling which is tagging proximate proteins by exploiting promiscuous biotinylation[36].

Overall, our results indicate CIP2A influences proliferation of cancer cells similarly to DDR proteins, and contribute ATR activation, which is under active research for development of anti-cancer treatment.

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국문요약

CIP2A (Cancerous inhibitor of PP2A)는 여러 암종에서 과발현되는 것으로 알려진 단백질로서, 예후에 부정적인 영향을 미치는 것으로 보고되었다. PP2A 억제와 관련된 CIP2A의 기능은 기존에 많은 연구가 진행되었고 MYC, AKT, mTOR 등의 다양한 단백질에 대해 PP2A를 통하여 영향을 미치는 것으로 알려져 있다. 그러나, PP2A와는 별개로서의 CIP2A의 기능은 상대적으로 연구의 진행이 미진한 상황이다. 본 연구는 PP2A와는 독립적인 CIP2A의 기능을 연구하고자 하였으며, stress granule과 DNA 손상 반응과 관련된 CIP2A의 기능을 중점적으로 확인하였다. 실험 결과, CIP2A가 stress granule이 형성되는 조건에서 stress granule과는 별개의 반응을 통해 분해되는 것을 확인할 수 있었다. 또한, CRISPR 기술을 이용한 유전자 의존성 데이터 분석을 통하여 CIP2A가 DNA 손상 반응 관련 단백질과 깊은 연관이 있음을 확인하였다. 우리는 추가적인 실험을 진행하여 CIP2A가 대표적인 DNA 손상 반응 관련 단백질인 ATR의 활성화에 영향을 미치며, ATR 활성에 필요한 TOPBP1과 상호작용하는 것을 확인하였다. ATR 활성의 억제가 최근 새롭게

연구되는 항암치료전략 중 하나라는 점을 고려할 때, 본 연구의 결과는 CIP2A가 ATR을 표적으로 한 새로운 항암치료 기전 중 하나로 고려될 수 있음을 보여준다.