이학박사 학위논문

The role of ADAR1 in the Mobility and Metabolic adaptation

유방암 세포의 이동성 및 대사 적응에서 ADAR1의 역할

울 산 대 학 교 대 학 원

의 학 과

박 민 지

The role of ADAR1 in the Mobility and Metabolic adaptation in Breast cancer cells

지 도 교 수　장 수 환

이 논문을 이학박사 학위 논문으로 제출함

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울 산 대 학 교 대 학 원
의 학 과
박 민 지

박민지의 이학박사학위 논문을 인준함

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심사위원	이 새 별	인
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심사위원	장 수 환	인

울산대학교 대학원

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ABSTRACT

RNA editing is a well-known post-transcriptional event and deamination is one of the major processes. There are two types of deamination of RNA: cytidine to uridine and adenosine to inosine. The A-to-I transition is edited by ADAR1 (Adenosine Deaminase, RNA Acting on RNA), which recognizes double-stranded RNA. A to I RNA deamination occurs primarily at the Alu repeats and functionally at the 3'UTR region, coding sequence, and microRNA precursors. This can result in recoding of a target gene or alter its expression and function. Recent studies suggested the novel role of ADAR1 in the treatment of human cancers, including epigenetic therapy and immunotherapy. We aimed to find out the role of ADAR1 in human breast cancer cells by combined analysis of RNA seq and cellular analysis after ADAR1 depletion. As a result, we found the depletion of ADAR1 suppresses the expression of ARPIN in an editing-independent manner. As the ARPIN inhibits the branching of actin filaments, the ADAR1 depletion stimulated cell mobility. On the other hand, we revealed the role of ADAR1 in the glucose-limiting condition. Specifically, when ADAR1 was depleted, we found an enhanced autophagic response and increased viability of breast cancer cells. This response seems to be triggered by activated AMPK that subsequently activated Beclin-1 and LC3. Altogether, these results present a new role of ADAR1 in breast cancer.

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INTRODUCTION

RNA editing is a molecular process in which a specific nucleotide sequence within an RNA molecule can be individually changed after a post-transcription process. [1, 2]. RNA editing is catalyzed by ADAR (adenosine deaminase acting on RNA) or APOBEC (apolipoprotein B mRNA editing catalytic polypeptide-like 1) enzymes [3]. The APOBEC family catalyzes Cytidine (C) to Uridine (U) editing as a cytidine deaminase [4]. The characteristic of an RNA editing event is the Adenosine (A) to Inosine (I) conversion mediated by ADAR1 (Adenosine Deaminase Acting on RNA) enzyme. Deamination occurs at the Alu-repeat, an intron or intergenic region and 3'UTR (3'-untranslated regions) and is generally not directly related to the expression of the editing target gene [5-7]. Inosine (I) is recognized as Guanosine (G) by translational or splicing machines. RNA A to I editing causes binding of amino acids, alternative splicing, and acquisition or loss of binding sites to miRNA or RNA binding proteins. This occurrence may result in changes in protein and mRNA expression. [8-11]. There are three families of ADAR1, ADAR2, and ADAR3. The enzymes in the family of ADAR share a common deaminization domain but show differences in structure and enzyme activity. ADAR1 and ADAR2 are known to mediate all known editing events in vertebrates [12]. In addition, ADAR1 may form a heteromolecular body with DICER to facilitate miRNA processing [13]. ADAR1 isoform p110 and p150 are controlled under different promoter control. While p110 isoform is expressed as a whole and is mainly located in the cell nucleus, p150 isoform is expressed in both the nucleus and the cytoplasm. In addition, p150 is induced by IFN and inhibits harmful effects on cells after viral infection [14]. In this regard, recent studies have reported that inhibition of ADAR1 activity improves immunotherapy effectiveness by inducing tumor target immunity in melanoma models. [15]. We previously revealed ADAR1-dependent microRNA regulation and non-canonical interferon signaling on cancerous gastric cancer cells [16, 17]. Other reports have also revealed their function in editing-dependent expression regulation of tumor suppressors, oncogenes, including AZIN1 [18]. On the other hand, it was also reported that ADAR1 causes cancer metastasis in melanoma. [19].

An important mechanism of the actin cytoskeleton is the formation of new filaments. The Arp2/3 (actin-

related protein 2, 3) complex branches into the actin cytoskeleton [20]. Therefore, it is known to play an important role in cell mobility, adhesion, and endoplasmic reticulum transport [21]. This Arp2/3 complex activity is upregulated in several types of metastatic cancer. Binding to GMG, coronin, and ARPIN is known as such Arp2/3 complex inhibitors [20, 22, 23]. As shown in these results, downregulation of ARPIN, an Arp2/3 inhibitor, has a poor prognosis in breast cancer [24]. Therefore, the regulation of Arp2/3 through ARPIN appears to play an important role in cancer cell mobility and metastasis. We indicate in this paper that ADAR1 deficiency in human breast cancer cell lines reduces the expression of ARPIN, resulting in increased cell mobility.

Autophagy is a lysosomal-dependent mechanism that removes unnecessary or non-functional components through this mechanism [25]. Recycling is possible through this decomposition process [26]. Although initially known as an induced degradation pathway to protect against starvation, it is known to play an important role in maintaining cellular homeostasis [27]. In cancer, autophagy plays an important role in preventing cancer as well as potentially contributing to cancer growth. Autophagy may contribute to cancer growth by promoting survival of starved tumor cells or by lowering apoptosis mediators [28]. As cancer cells grow, their autophagy function is affected to relieve metabolic stresses such as hypoxia, undernutrition, and increased proliferation [29]. It has been reported that ADAR1 regulates the expression of HIF1 under cellular stress such as hypoxia [30]. Also, autophagy is activated by glucose [31]. We indicate in this paper that ADAR1 deficiency increases the expression of autophagy markers and cell survivability under the glucose starvation in human breast cancer cells. Through this paper, we suggest a novel function that differs from previously known roles of ADAR1 in cancer to induce cancer mobility and induce high viability in nutrient-deficient conditions.

MATERIALS AND METHODS

Cell Cultures

MDA MB-468, MDA MB-231 and MCF7 human breast cancer cell lines and human embryonic kidney cells of HEK-293T were purchased from Korean Cell Line bank (Seoul Korea). All cells were grown in high glucose DMEM media, free glucose DMEM media with 10% fetal bovine serum and 1% penicillin-streptomycin(P/S) and incubated at 37°C and 5% CO_2. When the performance incubation under Glucose free media

Transfection of human breast cancer cell lines with shRNA of ADAR1

The generated stable knockdown of ADAR1 in MDA MB-231, MDA MB-468 and MCF7 cell lines using the shADAR1 viral vector (TRCN 0000050788, sequence: CCGGGCCCAGTGTTATCTTCACTTTCTCGAGAAAGTGAAGATAACAGTGGGCTTTTTG, sigma, MO, USA). To produce shADAR1 lentivirus, HEK-293T cell was plated at a density of 3 $X10^6$ cells in 100mm plates. The co-transfected with viral plasmid vectors (shcontrol, shADAR1), packaging plasmids (pMD2G, psPAX2) by using the Lipofectamine 3000(Invitrogen) and lentivirus supernatants were harvested every 12hr for 48hr. And then each time of the collected virus were used for infection with MDA MB-231, MDA MB-468 and MCF7. For the selection of stable KD ADAR1 using the puromycin (1ug~2ug/ml).

Protein extraction and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

For protein extraction of cells, EB and RIPA buffers were lysed by protease inhibitor cocktail (Roche) purification and phosphatase inhibitor cocktail (Roche) purification. All sample extractions were incubated on ice for 10 min, then centrifuged for 10 min at 13000 rpm at 4°C and the supernatant was collected. Proteins were quantified using a BCA protein assay kit (BioRad), and normalized protein

extracts were mixed with 6X protein sample buffer and boiled at 95 °C for 10 min. The protein samples were separated in 8%,10%,12% and 15% SDS-PAGE gels, transferred on to nitrocellulose membrane (NC) and each target protein was detected by specific antibodies. The blot was probed with anti-*β*-actin (1:2000 Santa Cruz, CA, USA), anti-ADAR1 (1:2000 Santa Cruz, CA, USA) and anti-ARPIN (1:1000, Abcam, ab235421), E-cadherin (1:1000 Cell signaling Technology), N-cadherin (1:1000 Cell signaling Technology), Slug (1:1000, Cell signaling Technology), Snail (1:10000, Cell signaling Technology). The measured relative densities of bands were analyzed using NIH image J 1.47v software.

cDNA synthesis and Quantitative Real-time PCR analysis

TRIzol (Invitrogen, Carlsbad, CA) was used for total RNA extraction from cells following the instructions of the manufacturer. To synthesis cDNA of mRNA, 1ug of RNA was used for cDNA synthesis by Primer Script 1st strand kit (Takara, Kyoto, Japan), and used for quantitative real-time PCR (qRT-PCR) analysis of ADAR1, METTL7a (Methyltransferase Like 7A), RSL1D1 (Ribosomal L1 Domain Containing 1), ARPIN (Actin Related Protein 2/3 Complex inhibitor), and E-cad, N-cad, Snail, Slug, Twist. As normalized controls were used the human RPL14a, GAPDH. For miRNA analysis, miScriptII RT Kit (Qiagen, Hilden, Germany) was used to synthesize reverse transcriptase RNA. A miScript SYBER Green PCR Kit (Qiagen, Hilden, Germany) was used to detect miRNA-1285-3p, 1285-5p and 619-5p expression in LightCycler 480 Ⅱ (Roche). The PCR condition: initial denaturation at 95°C for 15min followed by 40 cycles with 94°C for 15sec, 55°C for 30sec, 70°C for 30sec. The above kits follow the manufacturer's instructions. As a was normalized to the noncoding, RNU6 (small nuclear RNA molecule U6). The calculation of relative quantification by using 2-(ΔΔCt) method (1), The primer sequences for qRT-PCR are shown in table 3.

Transwell assay and overexpression of ARPIN

Migration and invasion of cells were measured by using transwell chambers with 6.5-mm diameter

(polycarbonate filters 8.0μm pore size). To the migration assay, after coating the upper chamber filters with Poly L lysine and drying it well, filled the resuspended cells with serum free medium. Then the lower chamber was filled with DMEM medium with 10% FBS, 1% PS and incubation for 24hr at 37°C and 5% CO_2. In case of invasion assay, before the upper chamber filled, Matrigel (BD) entered the upper chamber first and dried at 37°C for 40min. The following is the same as the migration process. After incubation 24hr, filters were fixed by MetOH (100%) and stained with eosin and hematoxylin. And then measured by counting cells on the lower side of the filter by used NIH Image J 1.47v software. For overexpression of ARPIN in breast cancer cell lines, $3x10^6$ cells were plated at 100mm, and total of 1ug of CMV ARPIN overexpression vector and CMV empty vector were transfected with Lipofectamine 3000 (Invitrogen) and incubation for 24hr. And then proceeded in the same way as above.

Luciferase reporter assay and miRNA mimic treatment

$3x10^4$ Cells were plated at 24well in DMEM 10% FBS, 1% PS medium. After 24hr of inubation.100ng of pMIR-REPORT^{TM} Luciferase vector (Life technologies, MD, USA), UTR of ARPIN containing pMIR vector and pGL3 enhancer luciferase vector () and 10ng of internal control TK luc plasmid (Renilla luciferase) were transfected per well with Lipofectamine 3000. miRNA 1285-3p, miRNA 1285-5p, miRNA 619-5p mimic or scramble RNA duplexes (Genolution, Korea) were also co-transfected and used for target analysis of ARPIN UTR. The cells were harvested 24hr or 48hr later, and then cells were lysed using 1X PLB (Promega), and luciferase activity was measured by used Dual-Luciferase Reporter Assay system (Promega) and GloMax Luminometer (Promega), following the manufacturer's instructions. Relative luciferase activity was calculated as the ratio of Firefly to Renilla luciferase activity.

Immunocytochemistry (ICC) of ARPIN and Autophagy related genes

Cells were plated in ibidi Dish 35mm, incubated for 24hr, and then washed with 1x PBS. And 1'Fixed

with 5% formaldehyde in DMEM media at 37°C for 10min incubation, and then 2' Fixed with 4% formaldehyde in 1x PBS at 37°C for 10min incubation. Cells were permeabilized with 0.5% triton X100 for 20min. Cells were incubated for 24hr with anti-ARPIN (1:50) (ab150113, Abcam). After 1xPBS washed, and then incubated for 1hr with Alexa 488-conjugated anti-mouse IgG antibodies (ab150113, Abcam). The above referenced the abcam ICC protocol. Fluorescence microscope images were obtained using an Inverted microscope (Carl Zeiss) and Excitation and emission filters were 488 for ARPIN and 555 for Rhodamine phalloidin.

Analysis of Editing events to detection from RNA-seq Data and miRNA sequencing

Analysis of Editing events to detection from RNA-seq Data and miRNA sequencing RNA-sequencing (RNA-seq) data of control clonal cells (shGFP) and shADAR1 clonal cells in MCF7 were generated transcriptome libraries, according to the standard manufacturer's protocol. The libraries were sequenced using the Illumina HiSeq™ 2500 platform (Illumina Inc, San Diego, CA, USA) and a read length of 101 base pair. Transcript quantification and gene annotation of RNA sequencing data were UCSC genome hg19 in STAR software. Then the transform as called from SAMtools. (2) The candidates to be edited were filters by editing regions of Alu and non- Alu, UTR and Intron, Exon. And then compared control clonal cells (MCF7, shGFP-6, shGFP-8) and shADAR1 clonal cells (shADAR1-4, shADAR1-5, shADAR1-7) to sort the site of A to G editing events and frequency of edit changes. Small RNA sequencing was performed at Theragen Etex Bio Institute (Suwon, Korea) using the Illumina platform (NEXTflex Illumina Small RNA Sequencing Kit v3). And 126.4 million sequence reads were obtained from both samples (shGFP-6, shADAR1-4 of MCF7).

Results

Part1. ADAR1 depletion promotes migration and invasion in cancer via the suppression of ARPIN

1.1 The stable knockdown of ADAR1, an A to I (G) Editing enzyme, in human breast cancer cells downregulate ARPIN expression

Using ADAR1 knockdown induced MCF7 cells, the next aim was to identify novel editing targets regulated by ADAR1. RSL1D1 (Ribosomal L1 Domain containing 1), METTL7A (Methyltransferase Like 7A) and ARPIN (Actin Related Protein 2/3 Complex Inhibitor), which are genes with a large difference in editing frequency between control clonal (MCF7, shGFP-1 (6), shGFP-2 (8)) cells and Knockdown clonal (shADAR1-1 (4), shADAR1-2 (5), shADAR1-3 (7)) cells, were selected (P1_Table1). As a result of confirming the mRNA expression of METTL7A and ARPIN, it was confirmed that RNA expression was decreased in three ADAR1 Knockdown clonal cells compared to the three clonal control cells, whereas the RNA expression of RSL1D1 was not significant (P1_Figure 3A, B, C and D). When confirmed at the protein level in three control clone cells and three ADAR1 knockdown clone cells, METTL7A was not significant change in ADAR1 knockdown clone cells, and it was confirmed that the expression of ARPIN was reduced (P1_Figure 3E). As a result of confirming intracellular ARPIN expression through ICC (Immunocytochemistry), it was confirmed that green fluorescence of ARPIN expression was decreased in ADAR1 knockdown cells. (P1_Figure 3F). To confirm that the decrease in ARPIN expression is due to A to I (G) editing of ADAR1, the editing sequence was confirmed using sanger sequencing. Since genomic DNA (gDNA) was not subject to editing, there was no change sequence (A) in editing, and it was confirmed that the G peaks of shADAR1-5 and shADAR1-7 were deceased in cDNA compared to control and shGFP-8 (P1_Figure 3G). From the results in Chapter 1_Figure 3, it was hypothesized that it could cause a change in the expression of ARPIN, a target gene of ADAR1.

1.2 Depletion of ADAR1 in breast cancer cells altered EMT marker expression, increased cancer cell migration and invasion

We focused on the reduction of ARPIN, which functions to inhibit the ARP2/3 complex of actin polymerization, in ADAR1 knockdown cells. Since it is known that the migration in cells was increased due to the decrease in the expression of ARPIN [32], it was confirmed whether the ADAR1-dependent ARPIN down-regulation affects cell migration and invasion.

As a result, it was confirmed that ARPIN expression was reduced in ADAR1 knockdown of MCF7, MDA MB-468 cells (P1_Figure 4A, D). When migration and matrigel invasion assays were performed in MCF7 cells, it was confirmed that shADAR1 increased migration, invasion activity than shGFP (P1_Figure 4B, C). In MDA MB-468 cells, the same results as those of MCF7 were confirmed (P1_Figure 4E, F) Based on the results of previous experiments, it was confirmed as follows whether it affects the expression of EMT (Epithelial- Mesenchymal Transition) marker. As a result of confirming the mRNA expression of EMT markers through Real-Time PCR, there was no significant change in N-cad (neural cadherin, CDH2) and E-cad (epithelial cadherin CDH1), but Snail was decreased, and Slug, Twist was expressed higher in shADAR1 than shGFP. As a result of confirming the protein of the EMT marker using Western blot analysis, it was confirmed that the expression of Twist and Slug was significantly increased in shADAR1 than shGFP (P1 Figure 4G, H). As a result of Chapter 1_Figure 4, it was demonstrated that knockdown of ADAR1 cell regulated cell mobility under the EMT marker regulation.

1.3 Overexpression of ARPIN restores increased mobility of ADAR1 knockdown cancer cell

To determine whether the reduction in ARPIN expression in ADAR1 knockdown cells affects EMT markers and cell mobility, an experiment to restore ARPIN in ADAR1 expression-suppressed cells of MCF7 was performed. This experiment was conducted with reference to the previously published

papers related to ARPIN restoration [33]. Increased migration and invasion activity in ADAR1 knockdown cells with reduced ARPIN in transwell assays. On the other hand, after transfection with ARPIN overexpression vector (0.8ug), restore expression of ARPIN significantly reduced migration and invasion activity in ADAR1 knockdown cells (shADAR1) (P1_Figure 5A). This is the result of counting cells that migrated through transwell analysis (P1_Figure 5B migration, 5C invasion). It was confirmed by Western blot that the decreased protein level of ARPIN in ADAR1 knockdown cells (shADAR1) was increased by overexpression of ARPIN. And the expression of the EMT marker was confirmed. When ARPIN was overexpressed, Snail and Twist did not change. However, Slug protein expression confirmed the decrease (P1_Figure 5D). The slug mRNA confirmed using Real-Time PCR was also confirmed to be the same as the western blot result (P1_Figure 5E). As a results of Figure 5, it was confirmed that cell mobility was regulated by ARPIN.

1.4 Editing independent regulatory role of ADAR1 on the 3'UTR of ARPIN gene

ADAR1 is known to be well edited in 3'UTR. Therefore, to confirm whether ADAR1 is due to the editing of the 3'UTR region of ARPIN, the 3'UTR reporter assay of ARPIN was performed and confirmed by using luciferase assay. In addition, it was confirmed through ARPIN promoter analysis whether a change in transcription factor was another factor in the decrease in ARPIN expression. First, the ARPIN promoter assay result confirmed that ARPIN promoter activity was increased in shADAR1 cells than in shGFP cells (P1_Figure 6A). So, it was confirmed that the decrease in the expression of ARPIN was not affected by the transcription factor. Before proceeding with the luciferase reporter assay, ARPIN 3'UTR reporters 1 and 2 were made by dividing the ARPIN UTR in half because of its large size. As a result of the experiment using the two ARPIN 3'UTR reporters, it was confirmed that the luciferase activity of ARPIN 3'UTR reporter-2 was reduced compared to ARPIN 3'UTR reporter-1 (P1_Figure 6B). Future experiments were mainly conducted with ARPIN 3'UTR-2 reporters. And, as

result of the ARPIN 3'UTR assay, it was confirmed that the luciferase activity was decreased in shADAR1 than in shGFP (P1_Figure 6C). It is known that miRNA binding is a factor that reduced the activity of the 3'UTR reporter assay. Therefore, selected candidate miRNAs that are expected to bind to ARPIN 3'UTR from the predictive database of miRbase (P1_Figure 6D). As a result of Small RNA-seq results for miRNA-1285-3p, miRNA-1285-5p and miRNA-619-5p, that expression was higher in shADAR1 than shGFP in MCF7 cell line (P1_Table 2). Therefore, because of confirming miRNA expression in shGFP and shADAR1 in MCF7 cell line by Real-Time PCR, it was confirmed that the expression of miR-1285-3p, miR-1285-5p, and miR-619-5p was increased in shADAR1 than in shGFP (P1_Figure 6E, F, G). To determine whether luciferase activity is reduced by candidate miRNAs, we performed an experiment to co-transfection of miRNA mimic and ARPIN 3'UTR reporter vector in HEK-293t cell line. As a result, it was confirmed that the activity of miR-1285-3p, miR-1285-5p and miR-618-5p was decreased compared to sicontrol (P1_Figure 6H). And when each miRNA was transfected, it was confirmed that the expression of ARPIN protein level decreased compared to the sicontrol (P1_Figure 6I). And miRNA can reduce the expression of ARPIN by binding to the edited A to I(G) sequence of ARPIN, so the binding sequence of miRNA in ARPIN 3'UTR was confirmed. It was confirmed that miRNA did not bind to the edited A to I sequence of ARPIN (P1_Supplement Figure 1). Therefore, the binding site of miRNA in ARPIN 3'UTR was confirmed. As a result, it was confirmed that the binding site of miR-618-5p was more in ARPIN 3'UTR reporter -2 than in ARPIN 3'UTR reporter-1 (P1 Supplement Figure 2). As a result of Figure 6, it was confirmed that the expression of ARPIN is an in-dependent regulation of editing.

Part 2. ADAR1 suppresses autophagic response in breast cancer cells upon glucose limiting conditions

2.1 In glucose limiting condition, ADAR1 deficient cells had higher cell viability

We previously confirmed proliferation assays using cells that constituted the knockdown of ADAR1 in MCF7, MDA MB-468 cells. As in the previously published papers related to ADAR1, it was confirmed in MCF7 and MDA MB-468 cells that the proliferation of shADAR1 (ADAR1 Knockdown, P2_Figure 1A, B, G, H) cells decreased compared to the shGFP when ADAR1 was depleted (P2_Figure 1C, G). We performed experiments to confirm the action of ADAR1 deficient cells under glucose starvation stress. Therefore, MCF7 and MDA MB-468 cells were cultured under glucose limiting conditions (Low Glucose: LG) for 24, 48 and 72 hours to observe the cell viability of the cells. As a result, there was no difference between MDA MB-468 shADAR1 and shGFP. On the other hand, the survival rate of MCF7 shADAR1 was higher than of shGFP (P2_Figure 1D, H). The experiment was conducted focusing on MCF7, which had a high survival rate in cell viability under glucose limiting conditions (LG). To determine whether the high survival rate of MCF7 shADAR1 is associated with apoptosis in glucose limiting conditions, Annexin V, known as an early marker of apoptosis, and PI (Propidium iodide), known as a marker of late apoptosis, were identified through FACS (Flow cytometry) analysis.

2.2 Downregulation of apoptosis markers in ADAR1 deficient cells under glucose limiting condition

Induction of apoptosis was observed using FACS analysis in glucose limiting conditions (Free glucose: FG) at various incubation times (12hr, 24hr and 36hr, 48hr). As a result of FACS analysis, when comparing shGFP and shADAR1 in PI (Propidium iodide), it was confirmed that the ratio of shADAR1

was significantly lower than that of shGFP at each time point. Annexin V identified a significantly lower percentage of shADAR1 cells at 12 and 24hr of glucose limiting condition media (FG) incubation (P2_Figure 2A, B). We performed experiments to confirm protein expression of apoptosis markers. Protein levels of Cytochrome C and Bim, markers of apoptosis, were confirmed in cells cultured for 12 and 24hr under glucose limiting condition (FG). As a result, it was confirmed that shADAR1 significantly reduced Cytochrome C and Bim levels than shGFP in the 24hr culture condition (P2_Figure 2C). As result, the viability of shADAR1 cells was high when shGFP and shADAR1 were compared under glucose limiting conditions. We wondered the reason for the high survival rate under these glucose limiting conditions (FG). Therefore, the following experiments were conducted focusing on autophagy, which is known to be related to nutrients.

2.3 Autophagy markers LCB and Beclin-1 was increased in ADAR1 deficient cells under glucose limiting condition

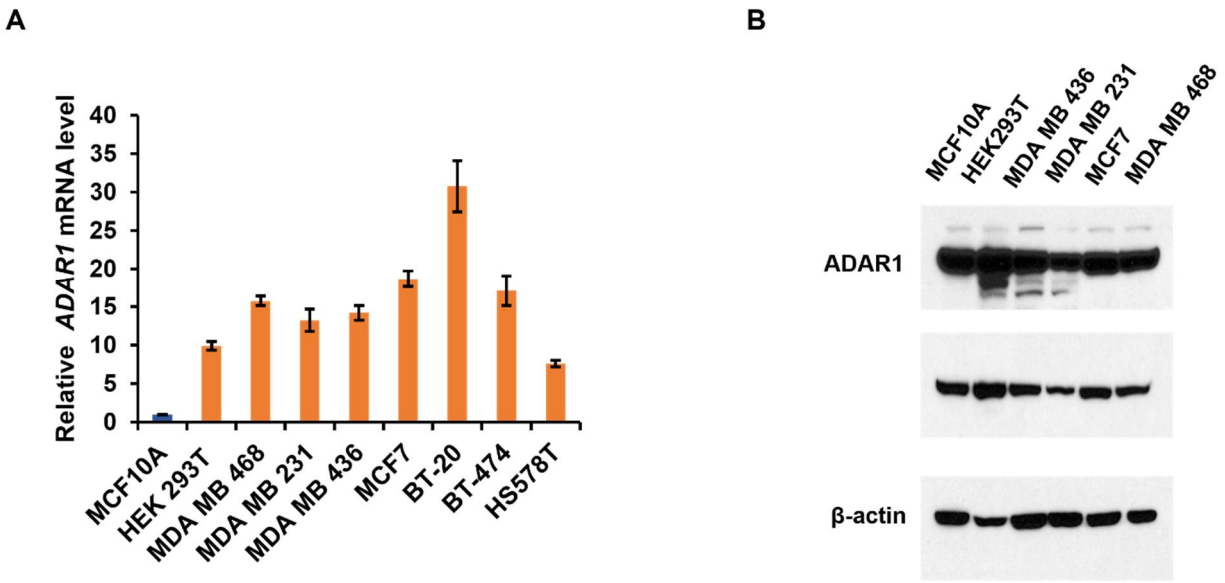
Incubated in glucose limiting conditions (FG) media for 12hr and 24hr. As a result of incubation for 12hr, it was confirmed that the expression of LC3B and Beclin-1, known as autophagy markers, was increased in shADAR1 than in shGFP (P2_Figure 3A). To summarize statistically based on these results, it was confirmed that the autophagy genes LC3b and Beclin1 significantly increased in culture conditions for 12hr, and p-AMPKa was also increased dependent on glucose limiting conditions (FG) incubation times (P2_Figure 3B). And whether there is a change in expression in the cell was confirmed through ICC. In the ICC experiment, CQ (Chloroquine) Was used as a positive control in the experiment as a drug that inhibits autophagosome formation in autophagy and induces the accumulation of autophagy (P2_Figure 3C). As a result of the experiment, it was confirmed that the expression of LC3B in shADAR1 was higher in shADAR1 than in shGFP as the glucose limiting conditions culture time increased to 12hr and 24hr (P2_Figure 3D). Based on the results of previous experiments, we hypothesized that the LC3B of autophagy is increased in ADAR1 deficient cells under glucose limiting conditions (FG), and that this increased autophagy will increase the survival

rate under glucose limiting conditions (FG). In the next experiment, it was confirmed that autophagy was inhibited and survival rate, which was high under glucose limiting conditions (FG), was lowered.

2.4 Autophagy leads high survival of ADAR1-depleted cells in glucose limiting condition.

A 3-mA (3-Methyladenine) drug was used to inhibit the autophagy effect, and this drug is known as an inhibitor of the autophagy initiation step. So before using a 3-mA drug, that needs to be pre-incubated at 24hr. First, it was confirmed whether the cell viability decreased when glucose limiting conditions (FG) and glucose limiting conditions (FG) were treated with 3-mA. As a result, the difference in survival between shGFP and shADAR1 was significant at 12hr, 24hr and 36hr of glucose limiting conditions (FG) media incubation. On the other hand, it was confirmed that the difference in survival between shGFP and shADAR1 decreased at 12hr, 24hr and 36hr of glucose limiting conditions (FG) and 3mA treatment (P2_Figure 4A). A western blot experiment was performed to confirm whether the decrease in the survival difference was an effect of autophagy when glucose limiting conditions (FG) and 3-mA were regulated together. As a result, it was confirmed that Beclin-1 increased from shGFP to shADAR1 for 12hr under glucose limiting conditions (FG) culture conditions. As confirmed by image J quantification, shGFP was 0.3 and shADAR1 0.8, and Beclin-1 was further increased in shADAR1. In the case of 3-mA simultaneous treatment, Beclin-1 of shADAR1 increased more than shGFP at 12hr. As confirmed by Image J quantification, it increased from shGFP 0.6 to shADAR1 1.1. However, because of confirming the increased ratio of shGFP and shADAR1, it was confirmed that the increased amount of shADAR1 decreased compared to the ratio without 3-mA treatment (P2_Figure 4B). It was confirmed that 3-mA treatment reduced the expression of autophagy, and additional experiments were needed to confirm whether cell viability increased. So, in the experiment, FACS was performed to measure apoptosis. As a result of confirming the % of PI and Annexin V in FACS analysis, it was confirmed that the % of PI and Annexin V increased at 24hr and 36hr in shGFP not treated with conventional 3-mA compared to shADAR1. It was confirmed that

when 3-mA was treated, the % of PI and annexin V of shADAR1 increased compared to the of shADAR1 that was not treated. These results suggest that autophagy is a factor in which shADAR1 had a higher survival rate than shADAR1 in the glucose limiting conditions (FG) (P2_Figure 4C, D).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Relative ADAR1 mRNA level
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25
20
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10
5
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HEK 293T
MDA MB 468
MDA MB 231
MDA MB 436
MCF7
BT-20
BT-474
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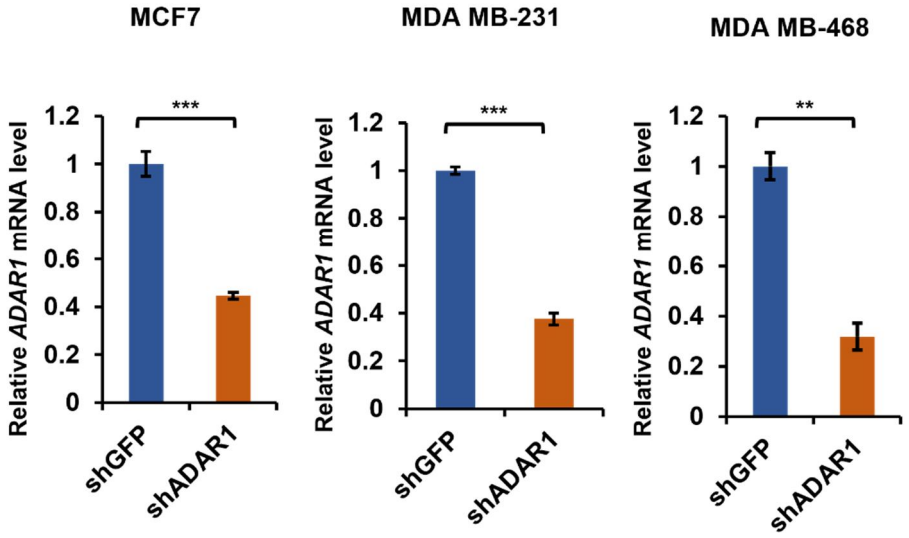
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MDA MB-468
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shADAR1

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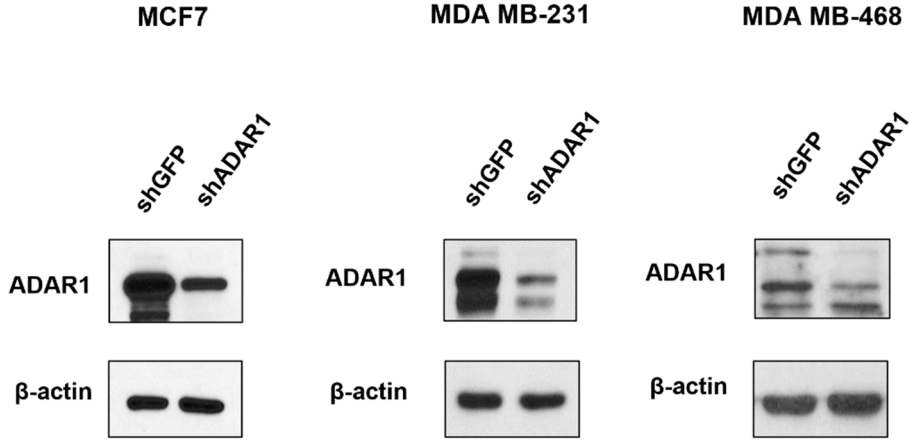
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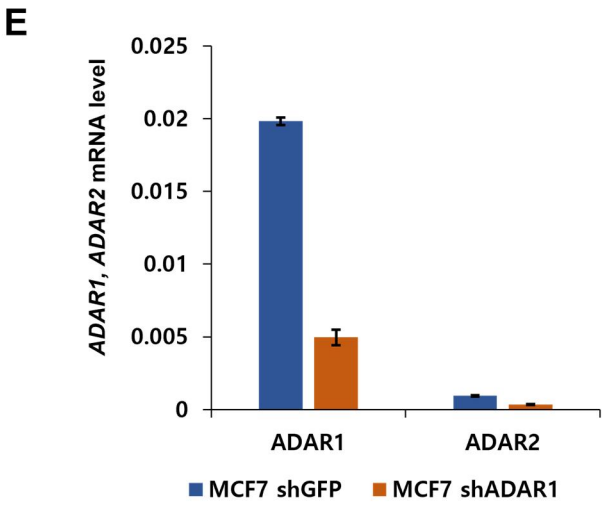
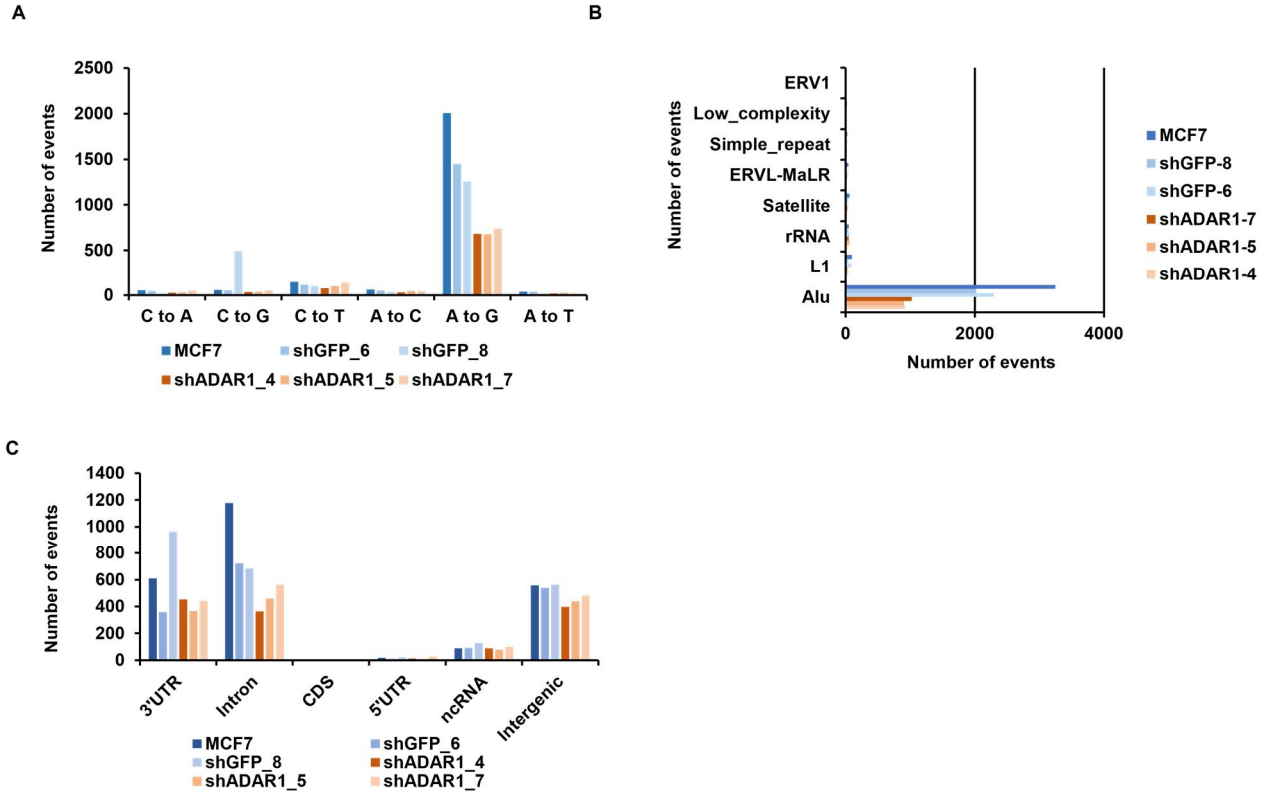


Part 1_Figure 1. Expression of ADAR1 mRNA and protein in human breast cancer cell lines.

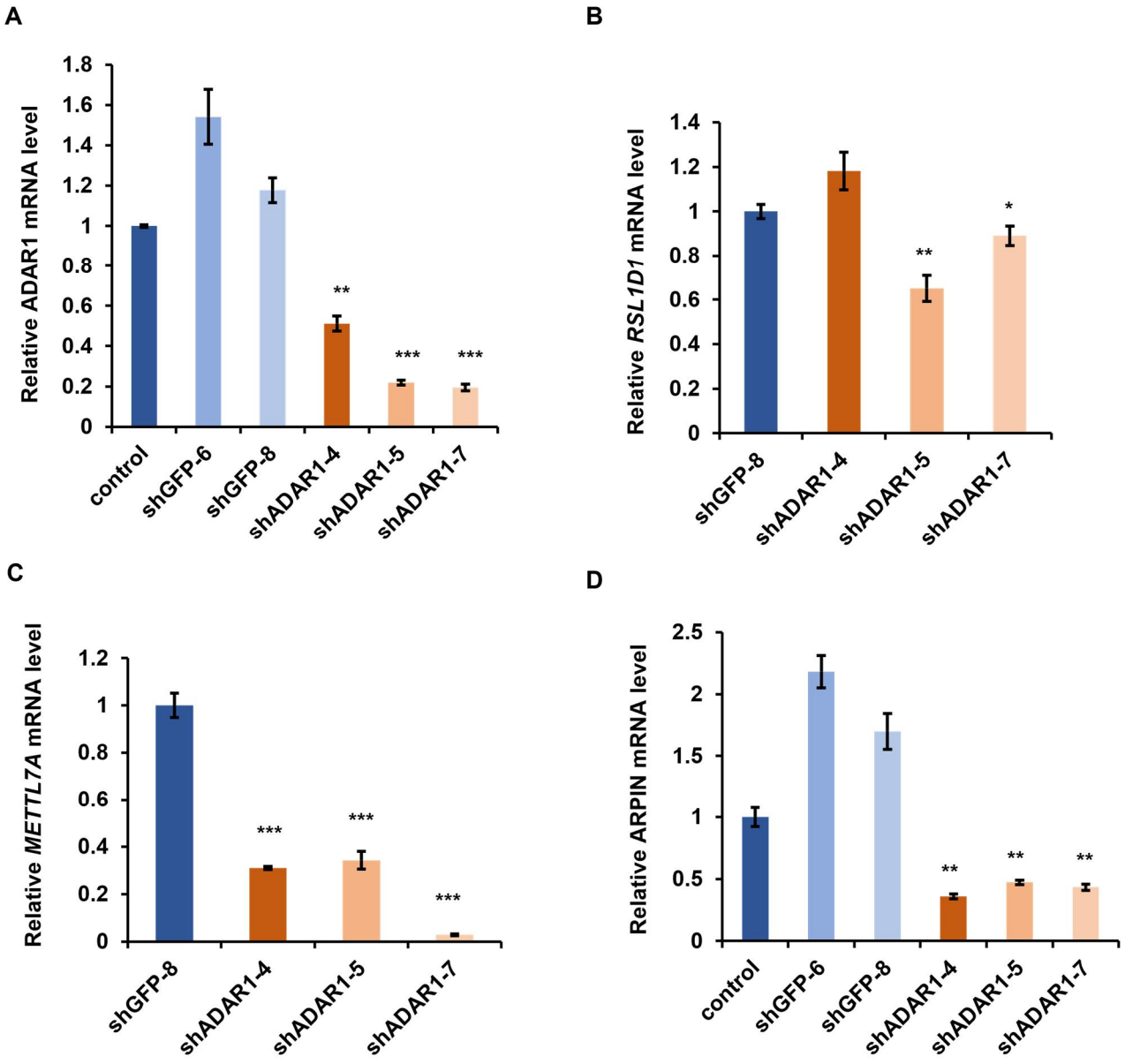
(A) Real-Time PCR (qRT-PCR), (B) Western blot and (C) Real-Time PCR, (D) Western blot of assay of ADAR1 in MCF7, MDA MB-231 and MDA MB-468 cell line infected shADAR1, shGFP lentivirus.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (E) Real time PCR of ADAR1, ADAR2

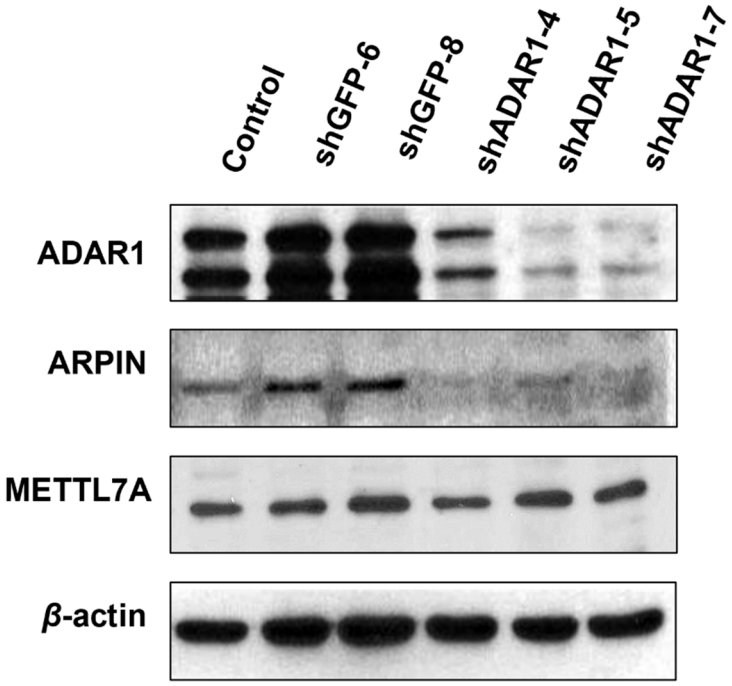


Part 1_ Figure 2. Analysis of RNA-seq data in MCF7

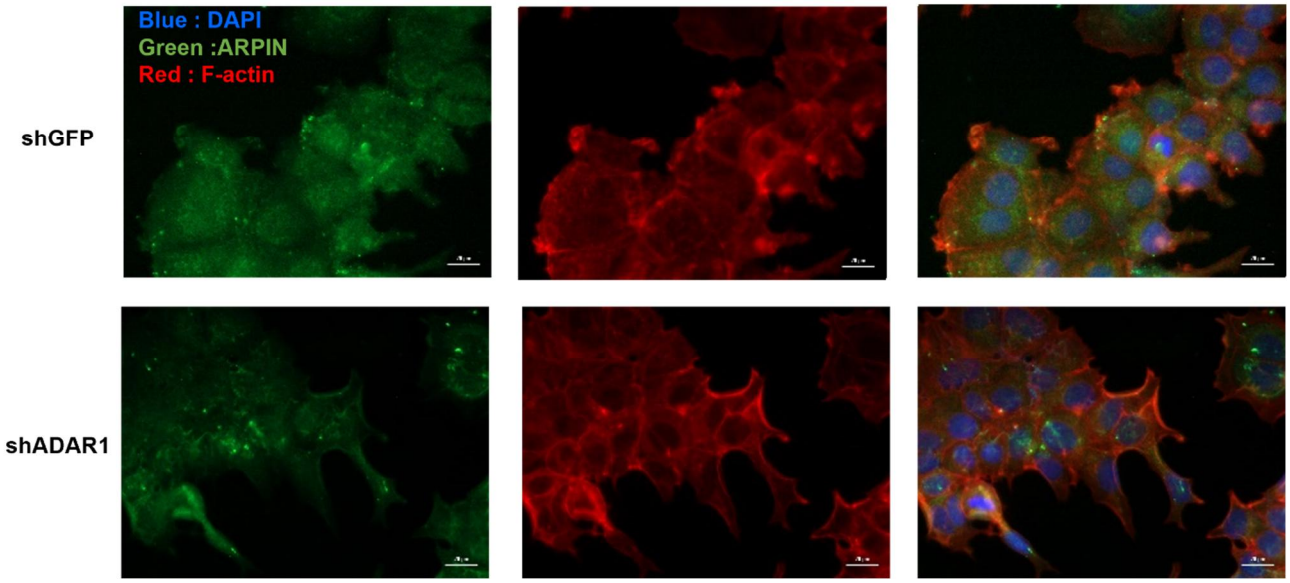
(A) Genomic distribution of edited regions in MCF7 cells form RNA-seq analysis. (B, C) Targets of A to G editing events in the Alu repeat region in RNA-seq analysis of MCF7 cells.


A
Relative ADAR1 mRNA level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
***
***
control
shGFP-6
shGFP-8
shADAR1-4
shADAR1-5
shADAR1-7
B
Relative RSL1D1 mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
shGFP-8
shADAR1-4
shADAR1-5
shADAR1-7
C
Relative METTL7A mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
shGFP-8
shADAR1-4
shADAR1-5
shADAR1-7
D
Relative ARPIN mRNA level
2.5
2
1.5
1
0.5
0
**
**
**
control
shGFP-6
shGFP-8
shADAR1-4
shADAR1-5
shADAR1-7

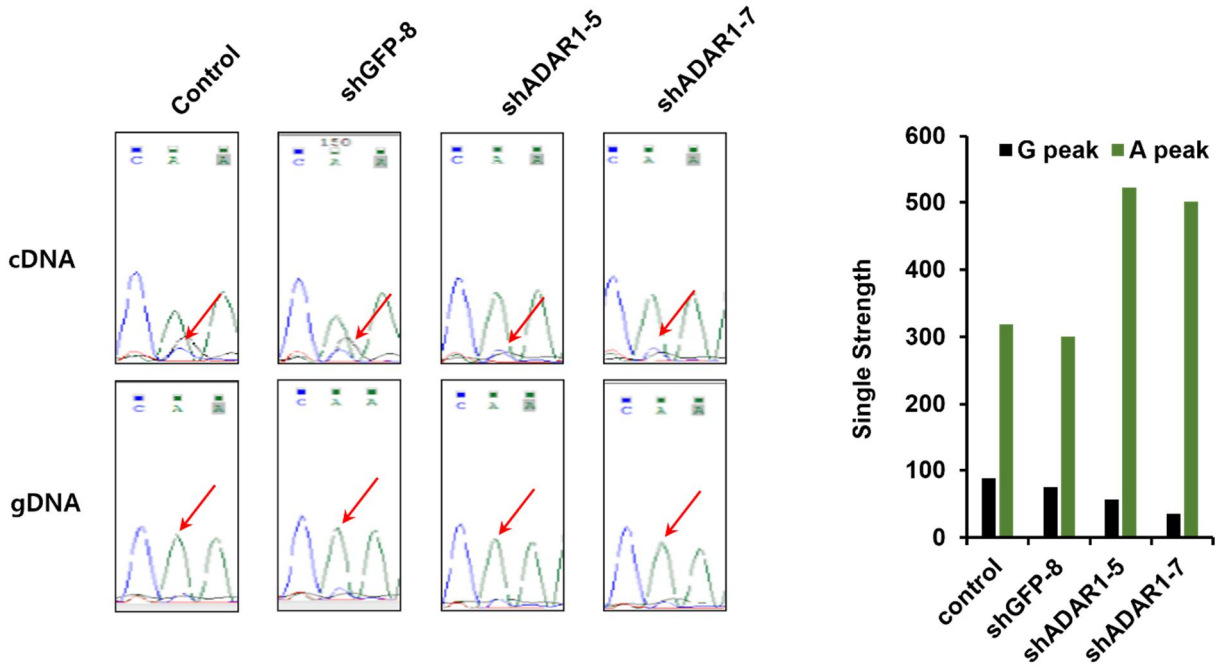
E


Control
shGFP-6
shGFP-8
shADAR1-4
shADAR1-5
shADAR1-7
ADAR1
ARPIN
METTL7A
β-actin

F



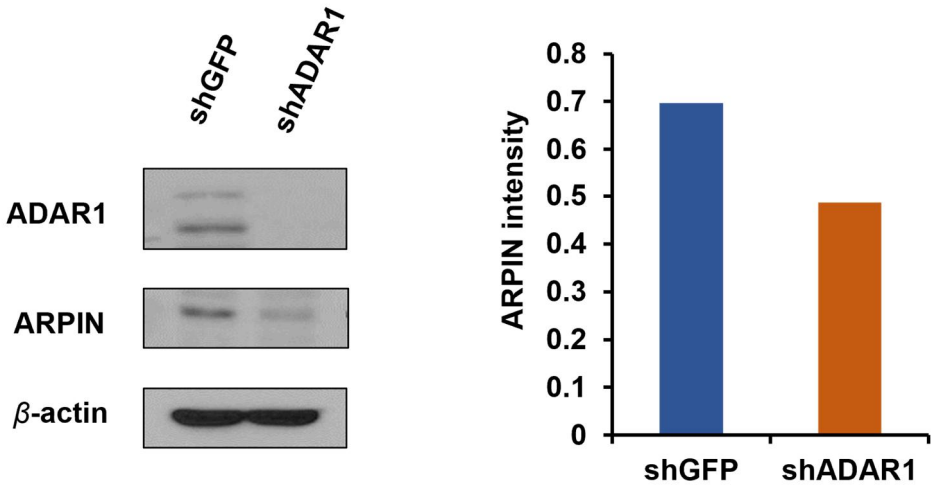
G

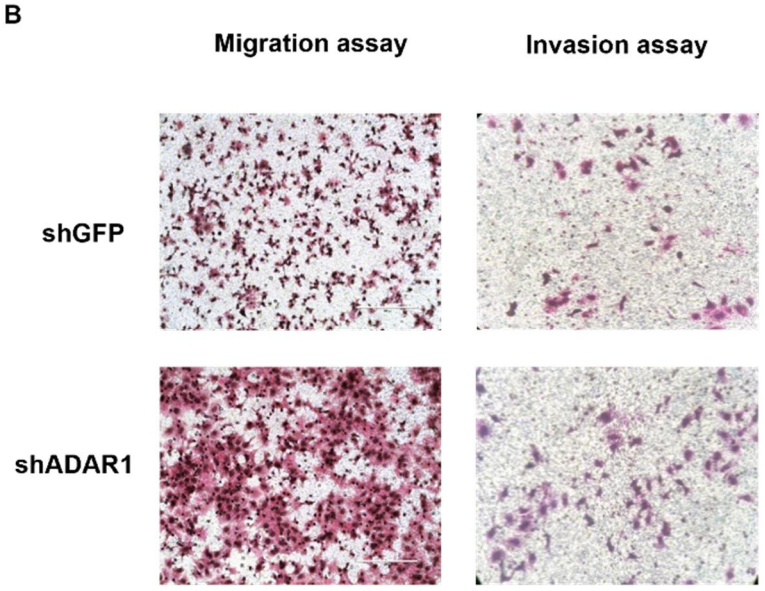


Part 1_Figure 3. Expression of RSL1D1, METTL7A and ARPIN upon ADAR1 depletion cells.

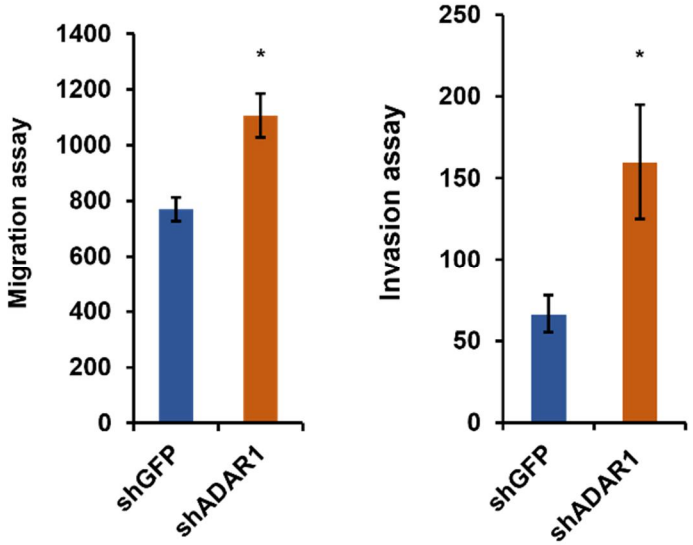
(A, B, C, D) mRNA expression of RSL1D1, METTL7A, ARPIN using Real-Time PCR in MCF7. (E) protein expression of METTL7A, ARPIN using Western Blot in MCF7. (F) Immunofluorescence (ICC) image of ARPIN (Green) in MCF7. (G) Sanger sequencing results of sites edited from A to G within ARPIN 3'UTR in MCF7. * $P < 0.05$, ** $P < 0.01$, *** $P < 0$.

A

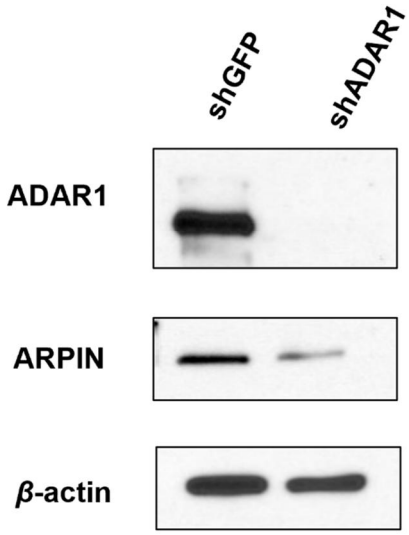

shGFP
shADAR1
ADAR1
ARPIN
β-actin
ARPIN intensity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
shGFP
shADAR1

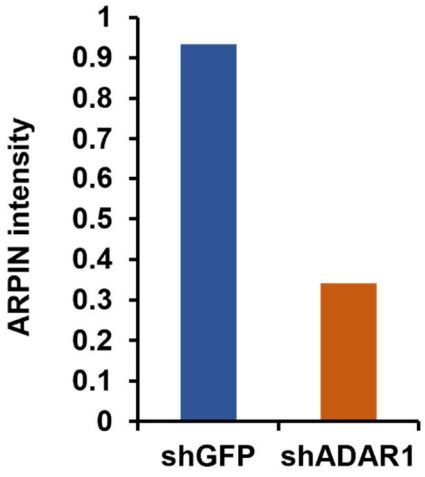

B
Migration assay
Invasion assay
shGFP
shADAR1

C

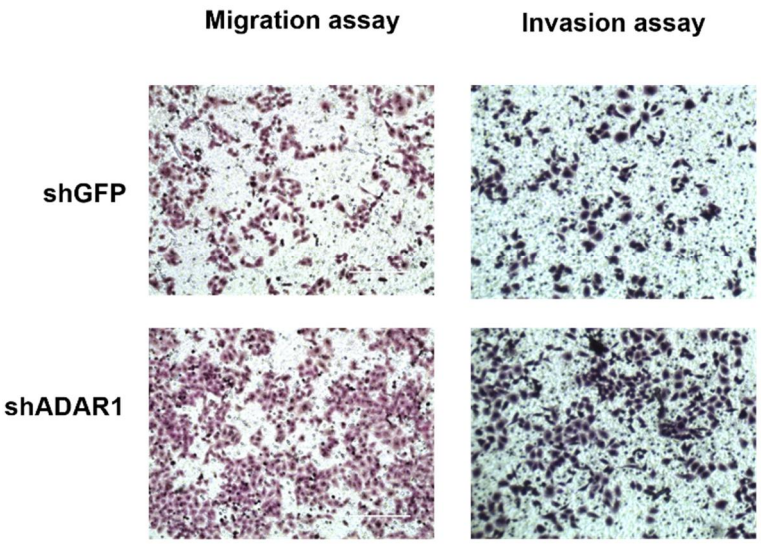

Migration assay
1400
1200
1000
800
600
400
200
0
*
shGFP
shADAR1
Invasion assay
250
200
150
100
50
0
*
shGFP
shADAR1

D

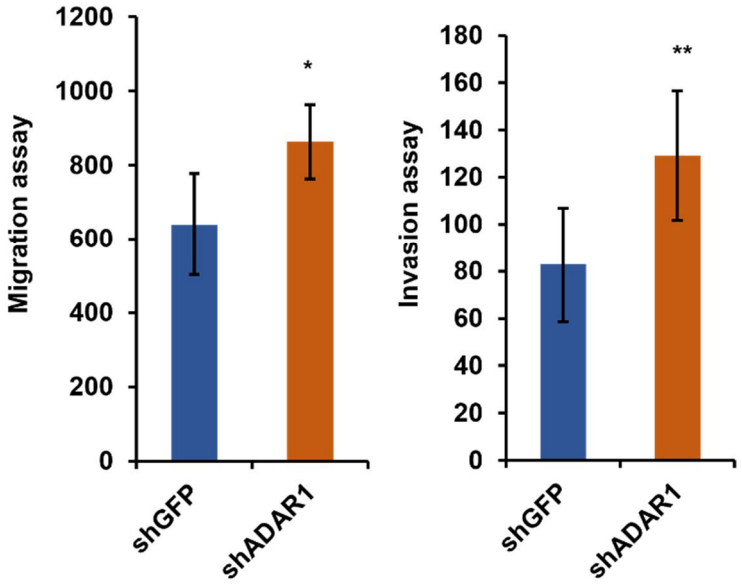

shGFP
shADAR1
ADAR1
ARPIN
β-actin


ARPIN intensity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
shGFP
shADAR1

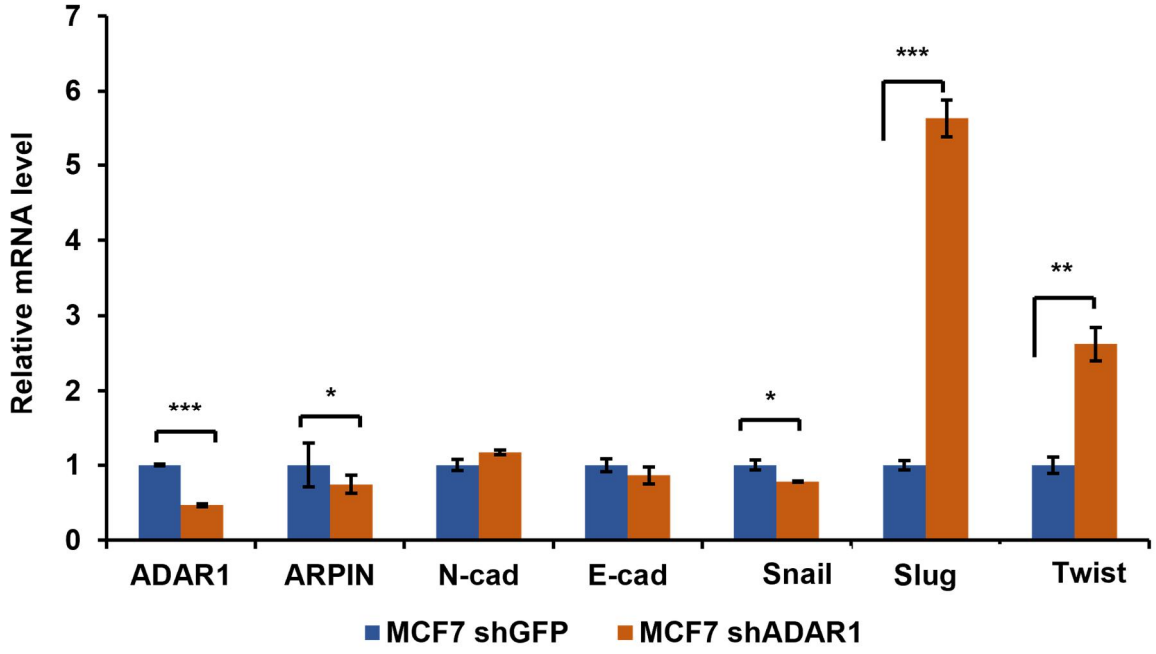
E

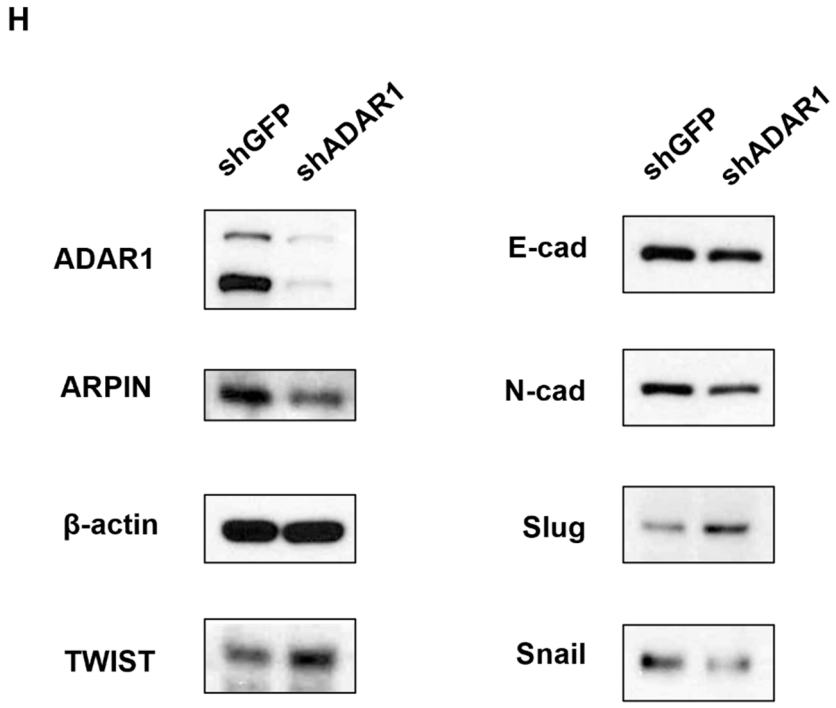


F


Migration assay
1200
1000
800
600
400
200
0
*
shGFP
shADAR1
Invasion assay
180
160
140
120
100
80
60
40
20
0
**
shGFP
shADAR1

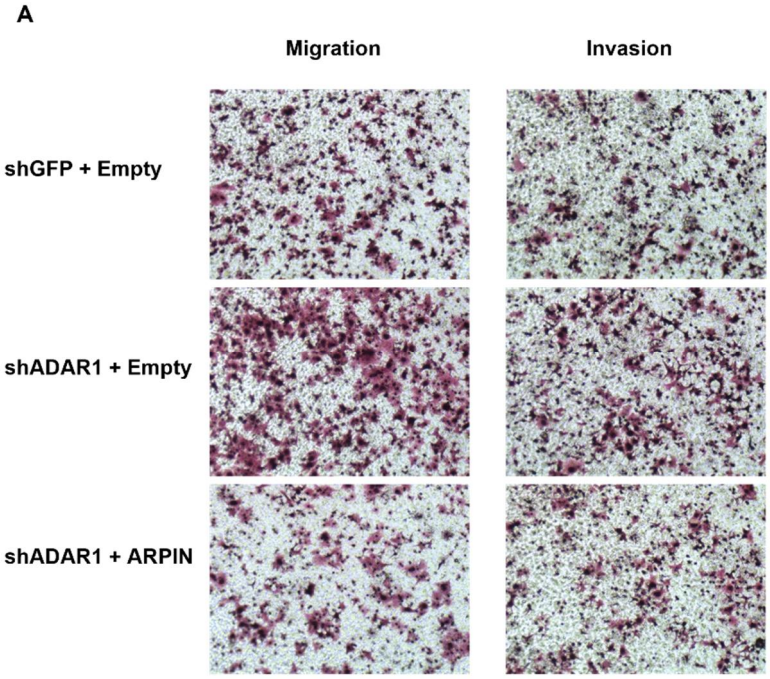
G


Relative mRNA level
7
6
5
4
3
2
1
0
***
*
*
***
**
ADAR1
ARPIN
N-cad
E-cad
Snail
Slug
Twist
MCF7 shGFP
MCF7 shADAR1

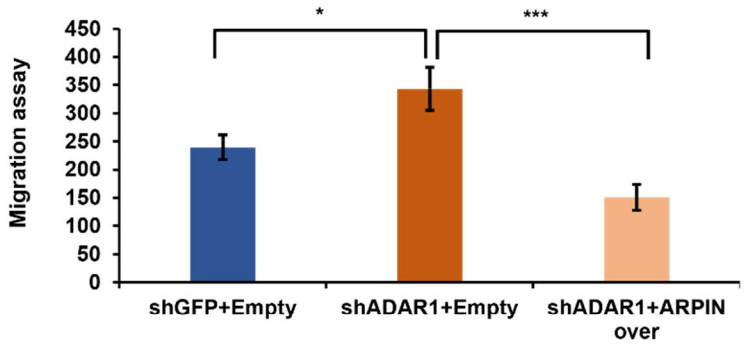


Part1_Figure 4 Increased cell mobility due to ARPIN downregulation of ADAR1 depletion cells in MCF7, MDA MB-468

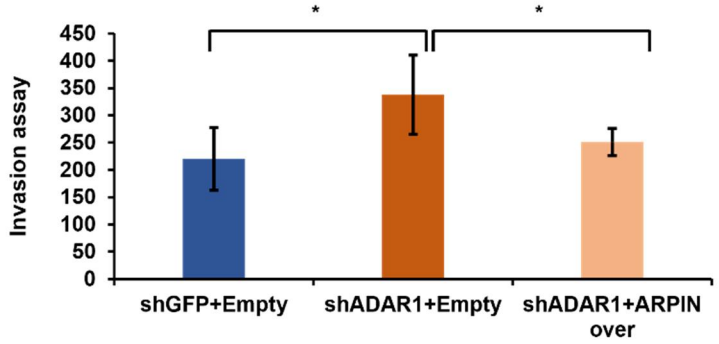
Decreased expression of ARPIN in shADAR1 cells (A), MDA MB-486 (D). Transwell migration and matrigel invasion assay of shGFP, shADAR1 in MCF7 (B), MDA MB-468 (E). Representative microscopic images of cells that migrated through transwell in migration and matrigel invasion assays. Statistical graphical representation of transwell migration, matrigel invasion assay of shGFP, shADAR1 in MCF7 (C), MDA MB-468 (F). (G) Real-Time PCR analysis of EMT marker genes in MCF7, (H) Western blot of EMT marker genes in MCF7. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

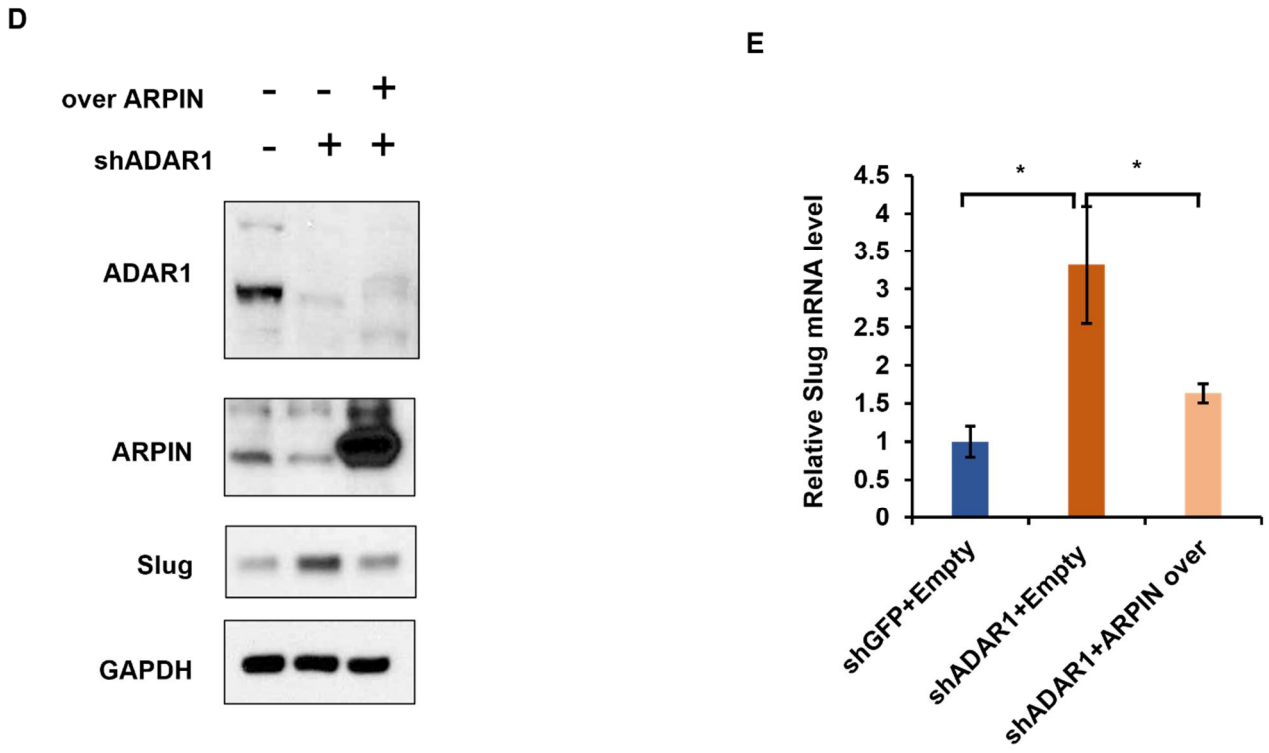

A
Migration
Invasion
shGFP + Empty
shADAR1 + Empty
shADAR1 + ARPIN

B


*
***
Migration assay
450
400
350
300
250
200
150
100
50
0
shGFP+Empty
shADAR1+Empty
shADAR1+ARPIN over

C

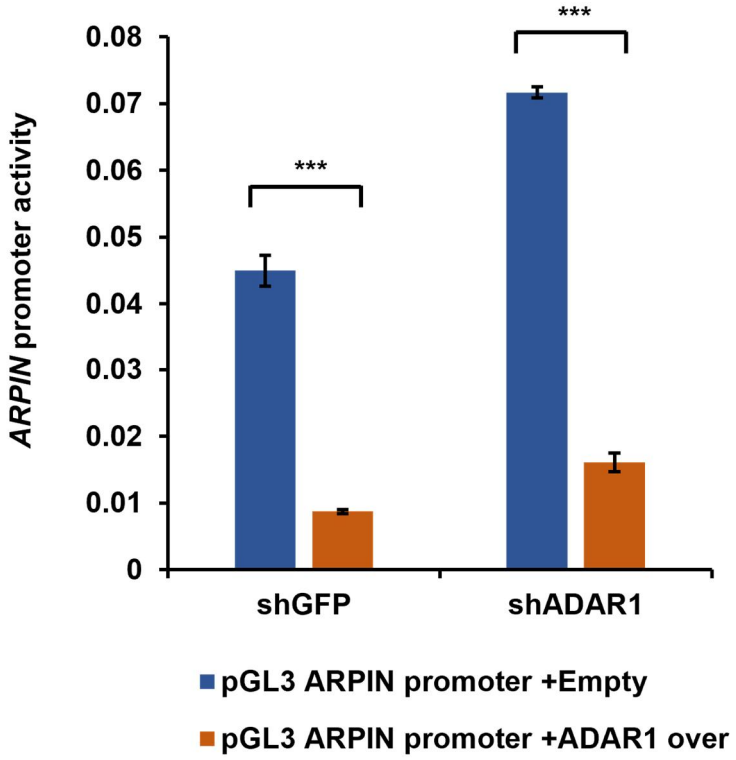

*
*
Invasion assay
450
400
350
300
250
200
150
100
50
0
shGFP+Empty
shADAR1+Empty
shADAR1+ARPIN over

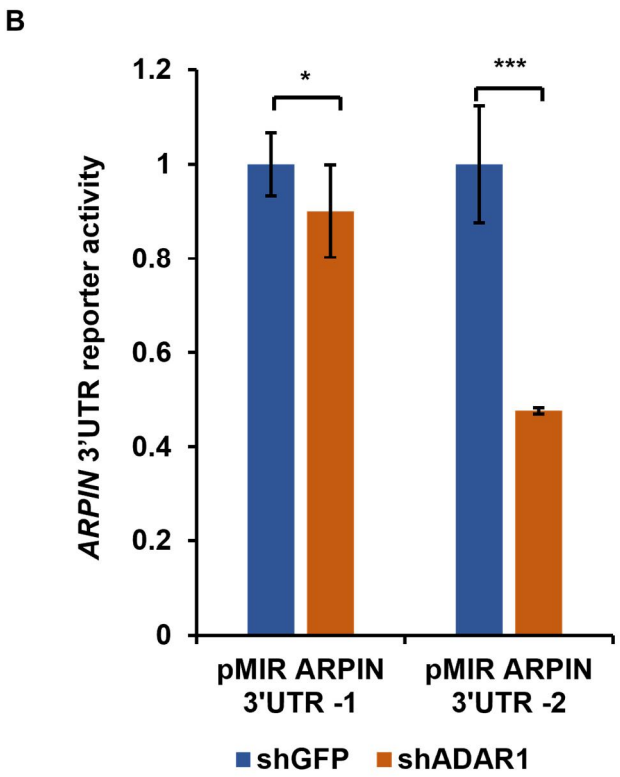


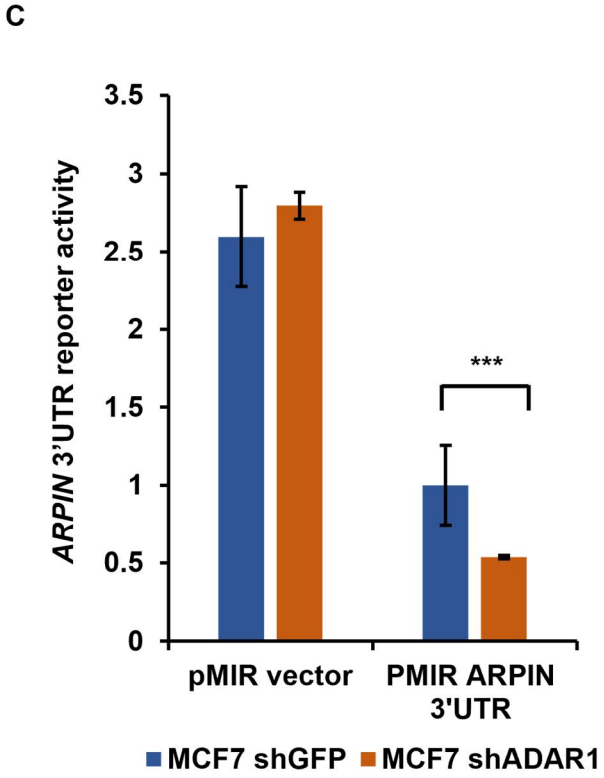
Part 1_Figure 5. Reduced migration and invasion of transwell in overexpression ARPIN of MCF7 ADAR1 knockdown cell

Transwell migration and matrigel invasion assay of ARPIN overexpression using CMV2 ARPIN vector (0.8ug) in MCF7 (A). Statistical graphical representation of transwell migration, matrigel invasion assay of ARPIN overexpression in MCF7 (B). western blot of Slug in MCF7 (D), Real-Time PCR of Slug in MCF7 (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

A


***
***
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
ARPIN promoter activity
shGFP
shADAR1
pGL3 ARPIN promoter +Empty
pGL3 ARPIN promoter +ADAR1 over


B
1.2
1
0.8
0.6
0.4
0.2
0
*
***
ARPIN 3'UTR reporter activity
pMIR ARPIN 3'UTR -1
pMIR ARPIN 3'UTR -2
shGFP
shADAR1


C
3.5
3
2.5
2
1.5
1
0.5
0
***
ARPIN 3'UTR reporter activity
pMIR vector
PMIR ARPIN 3'UTR
MCF7 shGFP
MCF7 shADAR1

D

Alignment of Query to mature miRNAs

Query: 289-309 hsa-miR-1285-3p: 2-22 score: 96 evalue: 0.092

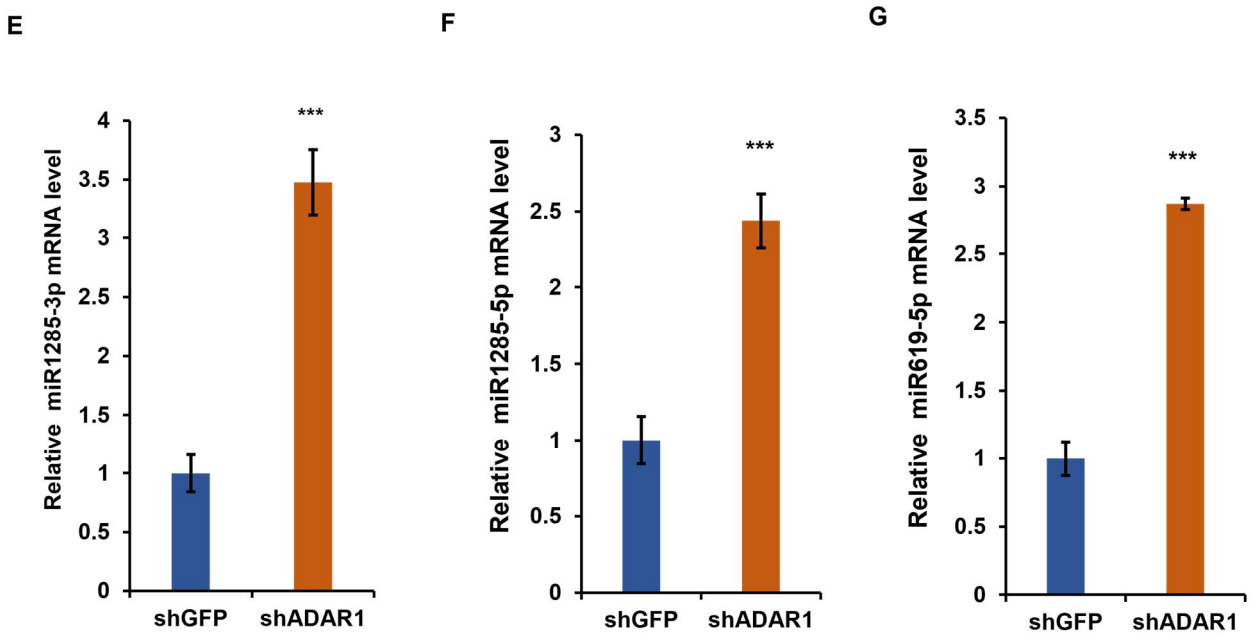
```
UserSeq          289  aggucucacuauguugcccag  309
                      |||||||||| ||||||||||
hsa-miR-1285-3p   22  aggucucacuuuguugcccag  2
```

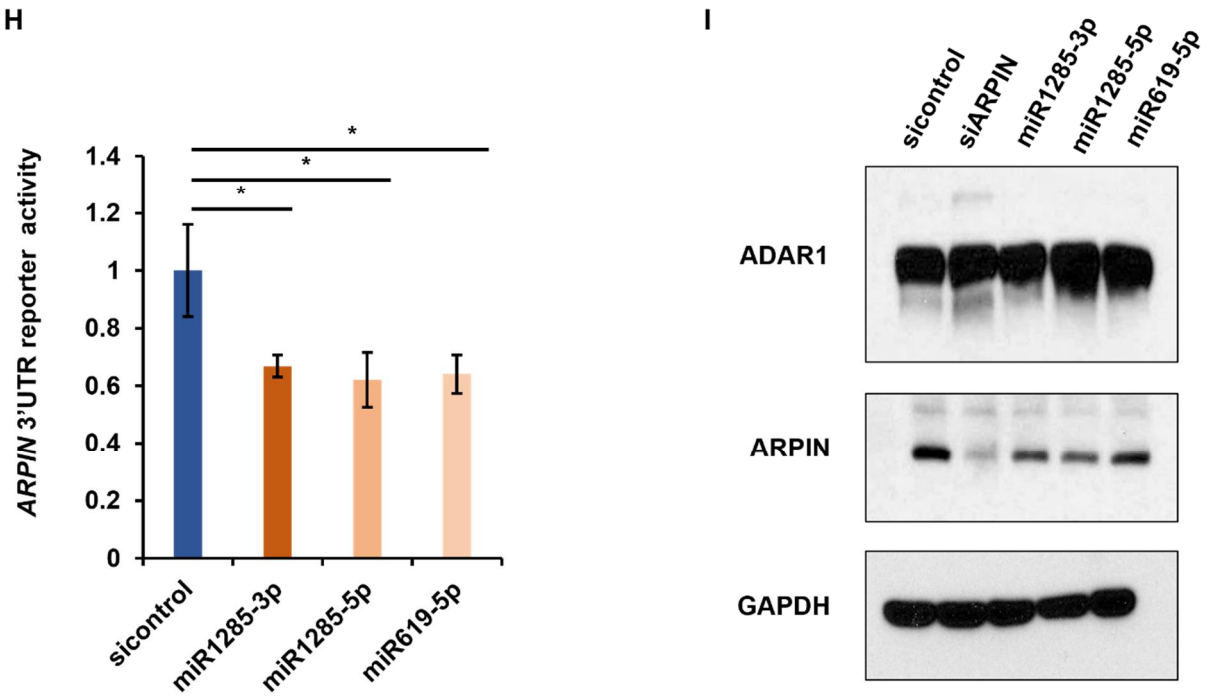
Query: 364-385 hsa-miR-619-5p: 1-22 score: 92 evalue: 0.20

```
UserSeq          364  guugggauuacaggcgugagcc  385
                      | |||||||||||||| ||||||
hsa-miR-619-5p     1  gcugggauuacaggcaugagcc  22
```

Query: 290-310 hsa-miR-1285-5p: 1-21 score: 87 evalue: 0.52

```
UserSeq          290  ggucucacuauguugcccagg  310
                      | ||||||| ||||||||||||
hsa-miR-1285-5p    1  gaucucacuuuguugcccagg  21
```


E
F
G
Relative miR1285-3p mRNA level
Relative miR1285-5p mRNA level
Relative miR619-5p mRNA level
***
shGFP
shADAR1



Part1_Figure 6. Decreased ARPIN expression due to independent regulation of ADAR1

Promoter luciferase assay of ARPIN in MCF7 (A), 3'UTR luciferase assay of ARPIN in MCF7 (B (ARPIN 3'UTR-1,2), C (ARPIN 3'UTR-2)). (D) Predictive miRNA binding database of miRbase in ARPIN 3'UTR. (E, F, G) Expression of miRNA-1285-3p, miRNA-1285-5p, miRNA-619-5p in MCF7 using Real-Time PCR. (H) ARPIN 3'UTR luciferase assay after each miRNA overexpression in HEK-293t cell. (I) Protein expression of ARPIN after each miRNA overexpression in MDA MB-468 cell.

Non- Editing (A) TCTCAAACTC **Editing (G) TCTCGAACTC**

```
Query: 289-309   hsa-miR-1285-3p: 2-22   score: 96   evalue: 0.092
UserSeq          289  aggucuacuaaguugcccag  309
                      ||||||||| ||||||||||
hsa-miR-1285-3p   22  aggucuacuuaguugcccag  2

Query: 364 385   hsa-miR-619-5p: 1-22   score: 92   evalue: 0.20
UserSeq          364  gaugggauuacaggcgugagcc  385
                      | ||||||||||||| ||||||
hsa-miR-619-5p     1  gcuuggauuacaggcaugaccc  22

Query: 290-310   hsa-miR-1285-5p: 1-21   score: 87   evalue: 0.52
UserSeq          290  ggucucacuauguugcccagg  310
                      | ||||||| ||||||||||
hsa-miR-1285-5p    1  gaucucacuuuguugcccagg  21
```

```
Query: 289 309   hsa-miR-1285-3p: 2-22   score: 96   evalue: 0.092
UserSeq          289  aggucuacuaaguugcccag  309
                      ||||||||| ||||||||||
hsa-miR-1285-3p   22  aggucuacuuaguugcccag  2

Query: 364-385   hsa-miR-619-5p: 1-22   score: 92   evalue: 0.20
UserSeq          364  gaugggauuacaggcgugagcc  385
                      | ||||||||||||| ||||||
hsa-miR-619-5p     1  gcuuggauuacaggcaugaccc  22

Query: 290-310   hsa-miR-1285-5p: 1-21   score: 87   evalue: 0.52
UserSeq          290  ggucucacuauguugcccagg  310
                      | ||||||| ||||||||||
hsa-miR-1285-5p    1  gaucucacuuuguugcccagg  21
```

Part1_ Supplement Figure 1. Searching for altered miRNAs in the ARPIN 3'UTR sequence edited by ADAR1

Has-miR-1285-3p AGGTCTCACTATGTGCCCAG

Has-miR-1285-5p GACCCGTGTATCACTCTGGA

Has-miR-619-5p GTTGGGATTACAGGCGTGAGCC

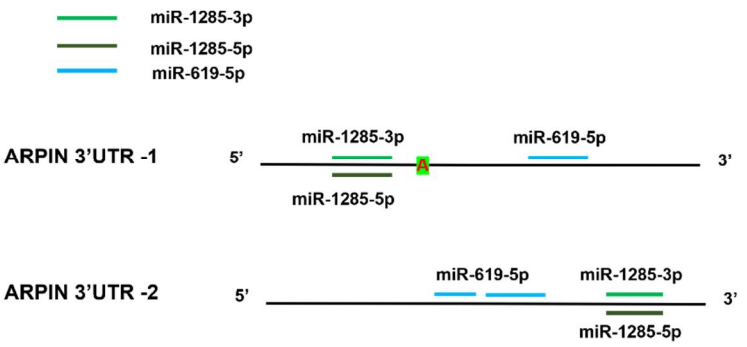
ARPIN 3'UTR 3'UTR-1

GGGACGTGGCTGCCTCTGGGGGCACCAGCACCTTCTGTAGCATTGTGGAACATTTCTTCCATGAACTTCTACTCTGGAGCTGGAGAGAACAGTCTGCCAAGTCTTACCTGGTTCTTCCCCATAGCC
ACCTTGGAATACTCTTTGAACAAGCTCTGCTAAAACCAAACCCATGACCACTGTGTTGGTGGTGGGGCCTCATGCTGTGGCTCAGGGAGGGCCCCTCCCTGCATGTGAGTCTAGCCTTCCTAGACA
TTACATGATGACCCTTTAAAAAAAAATTAGAGATGGAGGTCTCACTATGTTGCCCAGGCTGATCTCAAACTCCTGGACTCAAGCGATCCACCCCCCTCCACCTCCCAAGGTGTTGGGATTACAGGCG
TGAGCCACTGCACCCAGCCTCATGTTGACCCTGGCCCTGGATAGCAGCTTTGTTTGTAGAGGCAGGTGGTGGGAGAGACCCTAGGACAGCCTGAGAGTAACTGGACCTGGTATTGGTGCGGAGT
GGGGAATATCCCTTGTCTAAATGCAGAGCAGCTGAGAGAAGTTAGCGGGGCTCTTCCTCCGTTCTCATTGTTTCCTGCTTTCCTTCCCCACCCACCCCTTCCTCAGCACTCTGTGTTGTAGCCTGTA
CTATCCTGCTTCAAGCCTCCGTGTCCCTTGTACCTTCCCCCTACCTTGAGTTGCCAGTTCTTTACTCCCTCAAATGTTCCCATGCCACCTCTGGGGTTTCTGTTATCTGACATCCCTGGCTGCTTTCC
TGGTAGATGCCATTCCCAGAGAGGCCAACAGCGTTCCCAGGCGTGGCTGGCTGGTCCGCATCTCTCAGACTCACTCTGCCAGGCTACAGTTTTGAGAATACCTTTTTTGTTGTTGTTTGTACCTG
GAGAACTTTTTATGAATGAGTAATCTTCAGAAAGGAATTCCTCCACCCTTACTGGGTTATTGTCAGGTGTAAACGGCCTTTCAGCTGAGCAATGTTCCTATGAGAAGGAAGCCATTTCTCTTGGCTCA
TGAACACTGCCTTTTGGGTAGTATACCCACACCCAGATCTGTGAAGAGACCTGGAATTGAAGTCTCCATGTCGTCAGGACTTGGTGGCATGTGGATCTTGCTTGACTTGGGAAAAGAGAGGGTGGT
GGTGAACTAATAATTATGGAGTCCCCCTAATGTGTCGAACACAATGCTAGATGATTTCCATGATAATAATTACTCATACTTGTGCTAAGCACTTGCGATATCGTACTTCTCA

ARPIN 3'UTR 3'UTR-2

CAACAGTCCATGAGGTACTGATTATTATTCCCATTTTCTCATGAGACTAAAGCTCAGAGCTCTAGGATAGTATACAGTGGCAAGAGGAATTTACATCCAAGCACTCAGATTTCAAAGCCCACATTCTTAACTACCGGACTATTC
ATATTACAGAGCAGGAAAAGGGTCTCAGACAGGTTAAGCAACCTGCCCAGAGTCACACAGCTAATGAGTAGCAGAATCAGGATTCAAACCCAGAGCTGTCAAAAGCAAACCTTATGTTCCTTCCACTGTCCCCTGCTTCCT
TGCCCTTCAGAAAGGTCTTGGAAACAGGGGCCAGAGGATGTGGTAGCCACTTCACTGGTGTTCTGTGTCTGAATGGGACTTTCTCACAGGCAGGCCATGCTGGGACCTCAGCCATGGGGGTGGCCAGGGAGGTGGTTGA
GCAGAAGGATAGCTGGGGACACTGACTGGGGGTAGAGTCACCCAGTGTCTTTAGACATCCTGCTGTCTAAAGCACTGTGTCCAAAGGGTATGGCACACTTATATGGACAGGTCACCATTCCAAACAAGGAGGCCCAGTGT
GTCAATAGGAGAGGTCCTAAAGCAACAAAAAGGGCGGGGTGTCTTGGATGGGCTTCAGGAAGGGAAAGACCATTGTGTCTGGAGCTCAGAGAATGGGGGCTGGTGTTTGAGATGAGGCTGGAGATTGGGCATGGCATC
CTCACGCATGTTAAGGAATTGGGGACTTTATCCTTAACATCCTGGAAGCCAGTGATCCCATGGAGAGTGAGAAGATCTGATCTGTGTTCAAAGGTCACTTGACTGCCGAGCAGCTGGAGGCGAGTTGCTCCTTAGGAGAC
TGCTGCAGTTGCCTGGGTGAGAGGCTAGAGGGGTCTGGTCTATAGCAGTGGCAGGGGAGATGGAAAGAAGAGAATGATTTGAGGGGCATTCGGGGGGCAAAATCAACAGGACCTGGGGGGAGACGAAGGGATCAAG
AATGATGCCCAGATTCTGGGTGAGTGGCAGTGCTGTGGTGCACAAGTAGTGATGTTATGTTTCCGGGTGACCTGCTGTTTAGCAGGAGCCCAGCCTGTGGCTAAGAAGTATAGAGAACTGAAACTAGCTCCCTAGGGATGG
GAAAACCAAGTGGGCTGAGGATAAGAACAGGGCCCCAGGGCTGGGTGCAGTGCTCACGCCTGTAATCTTAGCACTTTGGGAGGCCGAGGTGGGCGGATCACTTGAGGTCAGGAGTTCGAAACCAGCCTGATCAACAC
AGTGAAACCCCGTCTCTACCAAAAAATACAAAAATTACCCGGACATGGTGGCACATGCCTGTAGTTCCAGCGCCTTGGGAGGCTGAGGCATGAGAATTTCTTGAACCCGGGAGGTGGAGGTTGCAGTGAGCTGAGATTGC
CACTGCACTCCAGCCTGGGCAACACAGTGAGACCCTGTCTCAAAAAAAAAAAAAAAAAAAAAGAATAGGGTCCCAGGCTCAGCAGGTAGGGACACTGAGGTAAAATCCTATTCCCTAGGACTAGGGACTAGGAAGGGAG
AAAATAGGAAGCTGCTGTGGCATAGGCATCTGCATGGGCAGATGGTGAGGCCAAAGCCCCTGATTTTGCCATACCTCCTGCCCTGGTCAGGACTGGTTCAGGCATTAGGGCAGGCCCAGCCAGGGCAGAAGCCTTCCTAG
ATACACCTGTGGGGAGTTGAGGAACTACAGCAGGGCTGAAACCCTGCTTTCAGGGGCCCCCATGTGGCTGGTAGGGGCAGCTTTTGGAAGCTAGACTGGATCCTATTACTCCCCAGATGAAAAGTTCAAATTCCTTAATAC
AACTCAGAGCTCATCACAATGTCACAACCCTCCTTTCTCGGC

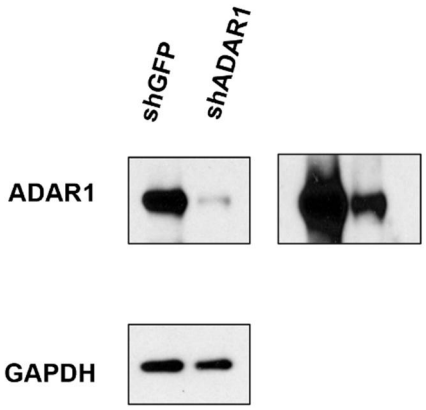
Prediction of miRNA binding in ARPIN 3'UTR



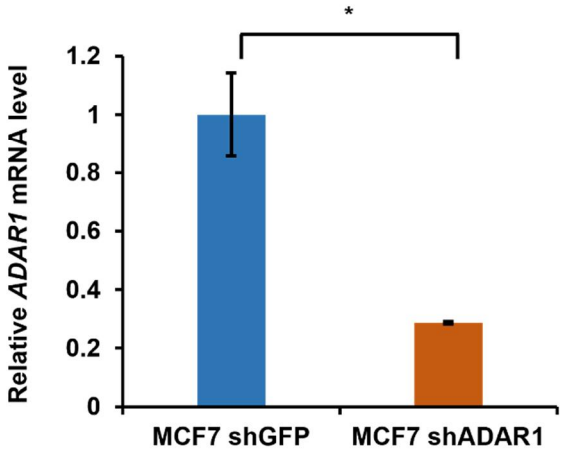
Part1_ Supplement Figure 2. Searching of miRNA in ARPIN 3'UTR sequence

MCF7

A

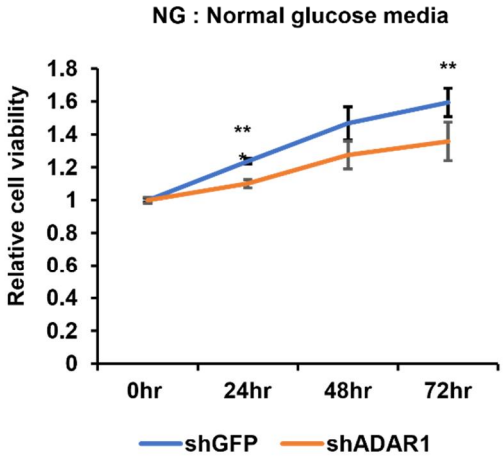

shGFP
shADAR1
ADAR1
GAPDH

B

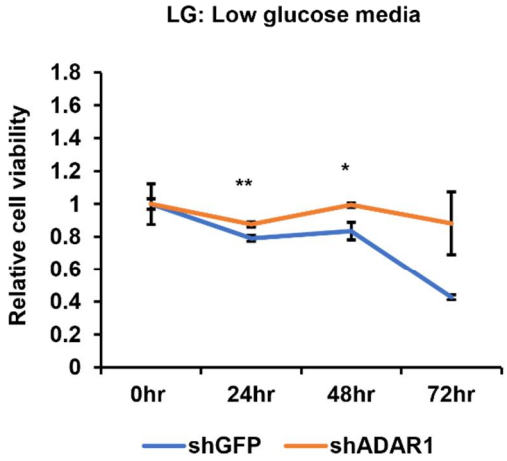

*
Relative ADAR1 mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
MCF7 shGFP
MCF7 shADAR1

MCF7

C

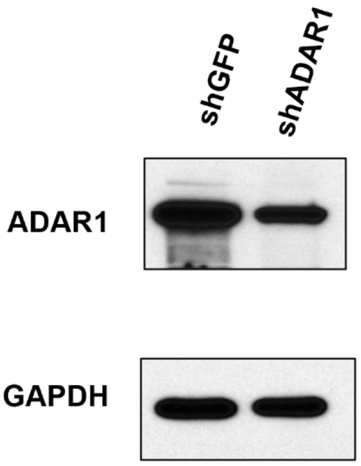

NG : Normal glucose media
Relative cell viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
**
0hr
24hr
48hr
72hr
shGFP
shADAR1

D

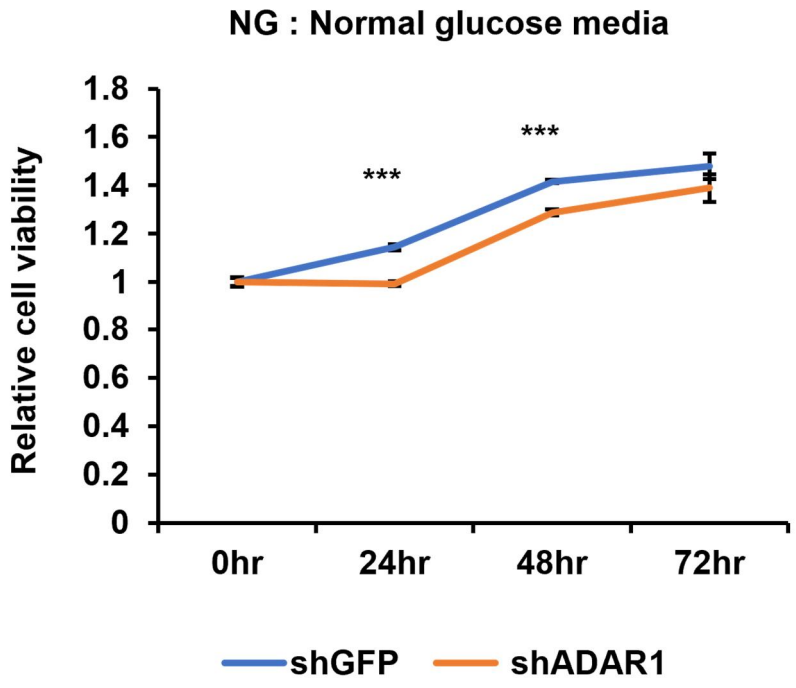

LG: Low glucose media
Relative cell viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
0hr
24hr
48hr
72hr
shGFP
shADAR1

MDA MB-468

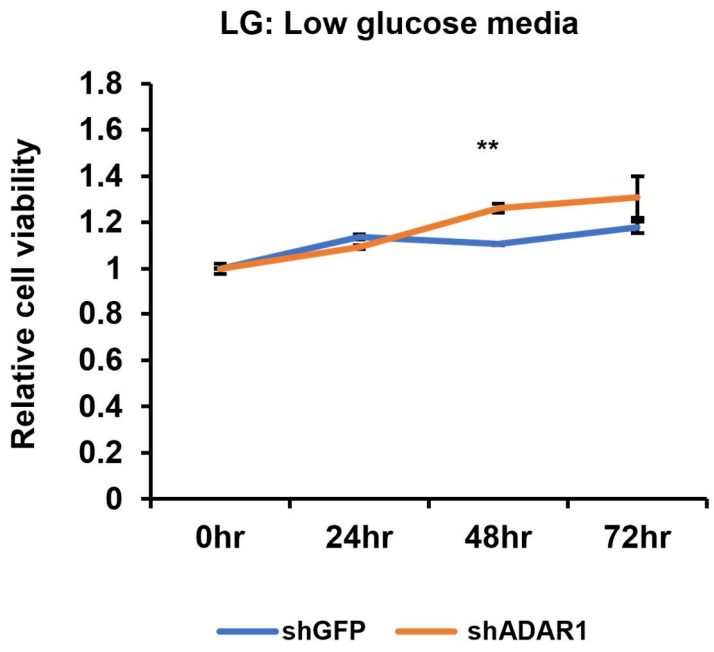
E



F

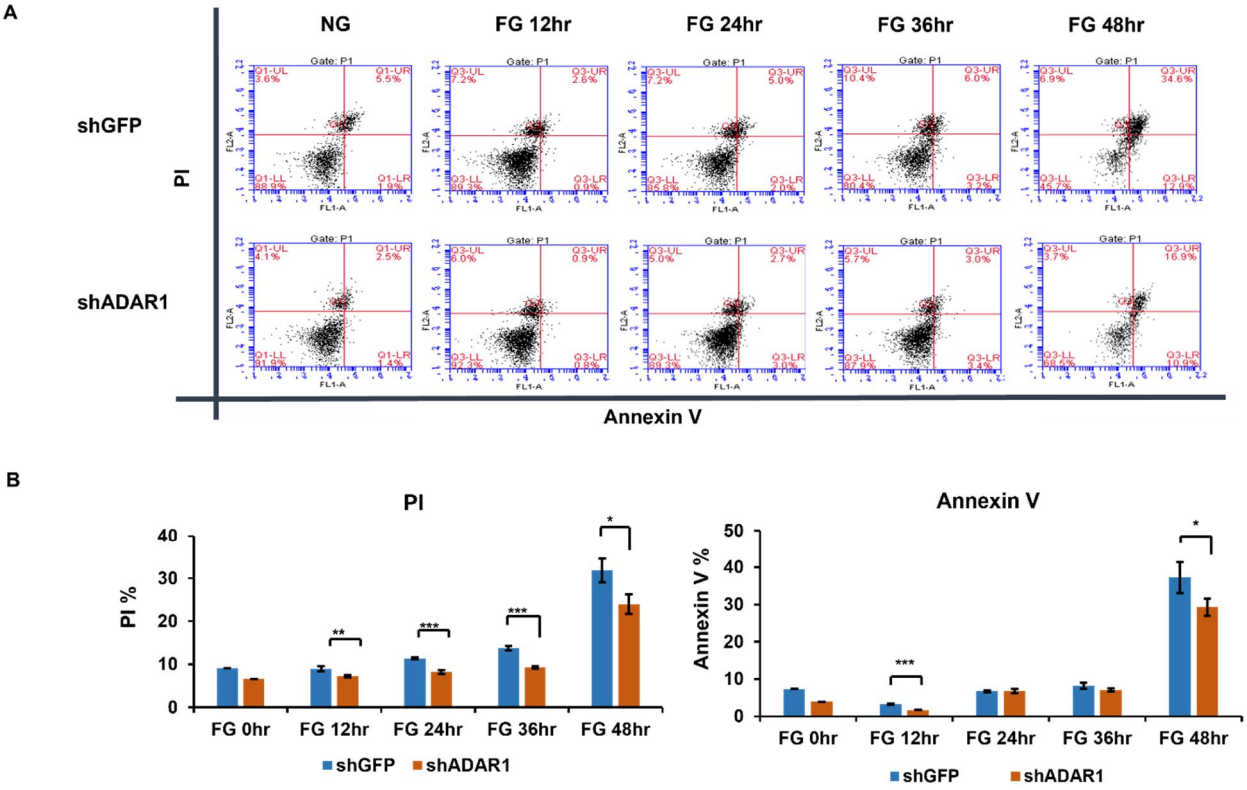

NG : Normal glucose media
Relative cell viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
0hr
24hr
48hr
72hr
shGFP
shADAR1

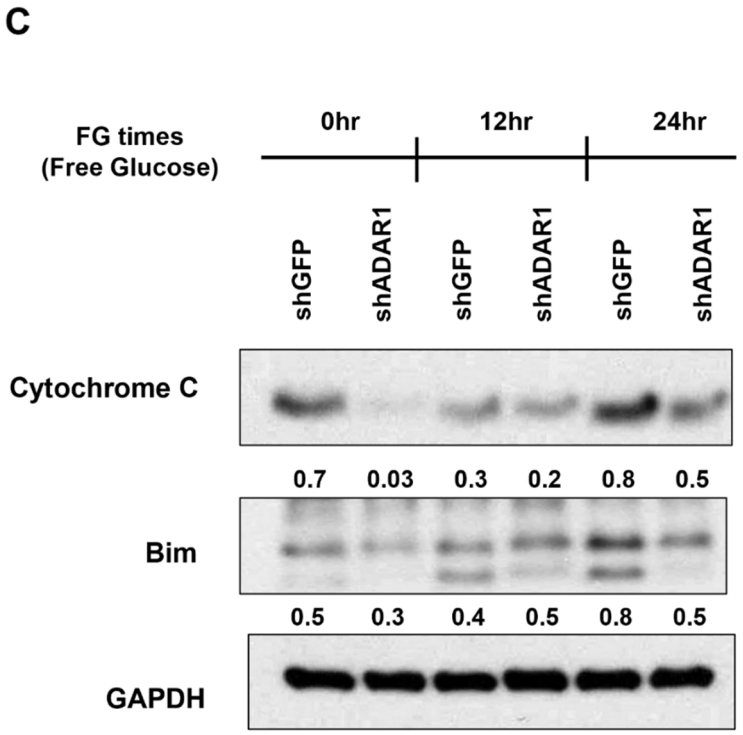
G


LG: Low glucose media
Relative cell viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
0hr
24hr
48hr
72hr
shGFP
shADAR1

Part2_Figure 1. Increased cell viability of ADAR1 depletion cells in glucose starvation condition

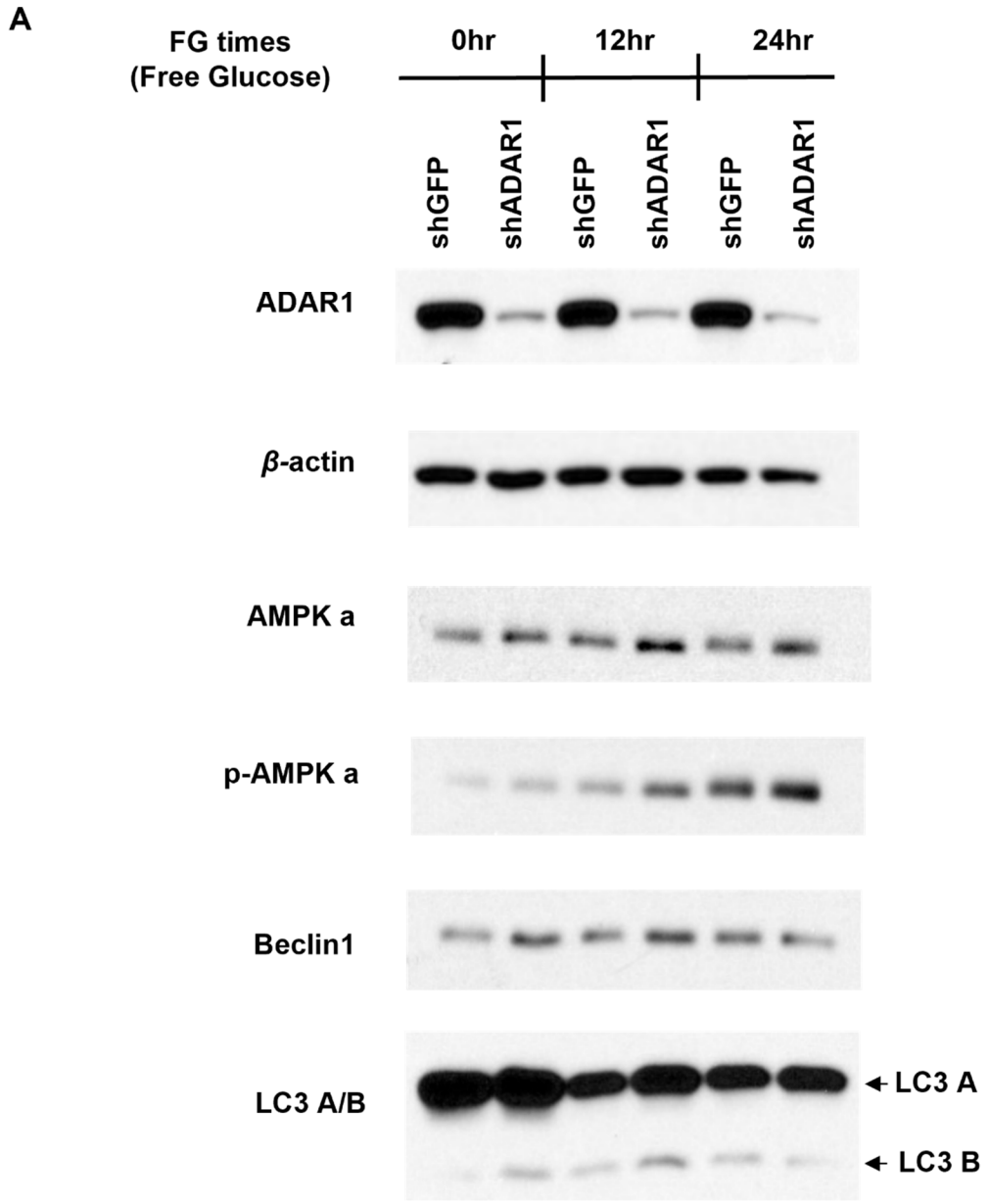
Expression of ADAR1 protein level in MCF7 cell (A), MDA MB-468 cell (E). Expression of ADAR1 mRNA level in MCF7 cell (B), MDA MB-468 cell (F) using Real-Time PCR (qRT-PCR). Cell viability assay under low glucose (7.5mg/ml) media and Normal glucose (4500mg/L) media in MCF7 cell (C, D), MDA MB-468 cell (G, H). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$


A
NG
FG 12hr
FG 24hr
FG 36hr
FG 48hr
shGFP
shADAR1
PI
Annexin V
B
PI
PI %
FG 0hr
FG 12hr
FG 24hr
FG 36hr
FG 48hr
shGFP
shADAR1
Annexin V
Annexin V %

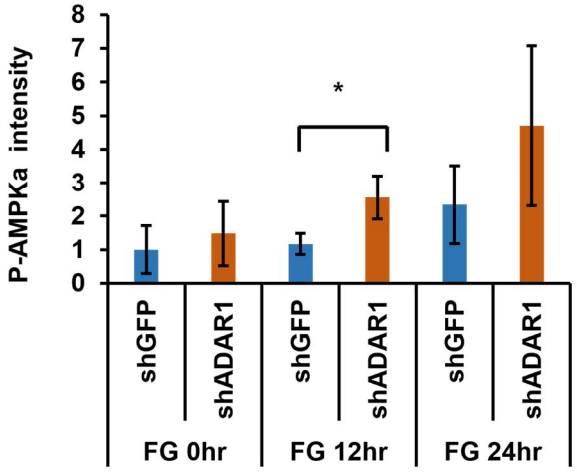


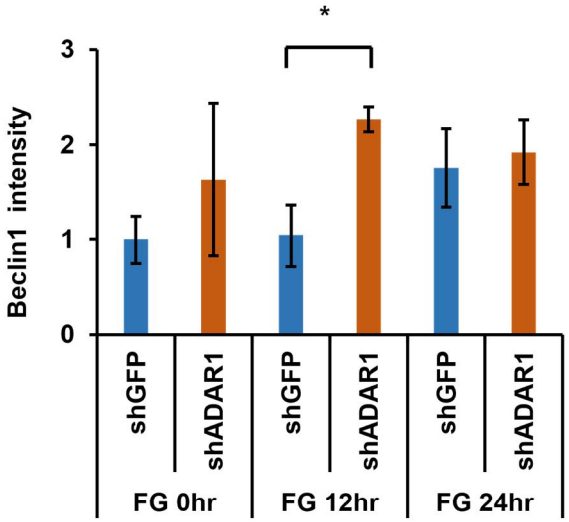
Part2_Figure 2. Reduced apoptosis under glucose starvation conditions in ADAR1 depletion cells

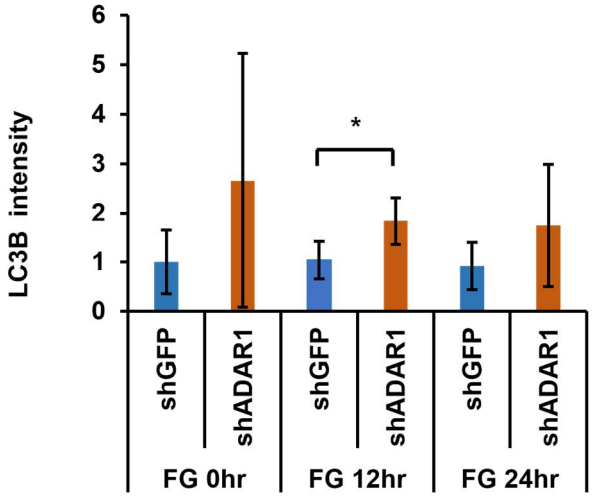
(A) FACS analysis in MCF7 cells. (B) Statistic of Apoptosis FACS analysis. (C) Western blot of cell death markers cytochrome C and Bim. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$


A
FG times
(Free Glucose)
0hr
12hr
24hr
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
ADAR1
β-actin
AMPK a
p-AMPK a
Beclin1
LC3 A/B
LC3 A
LC3 B

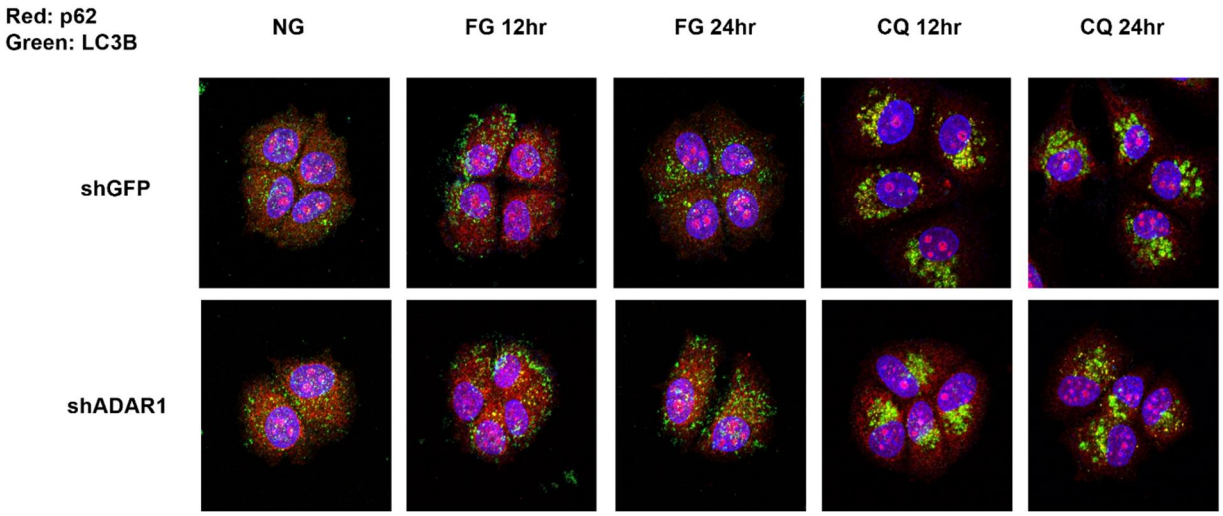
B

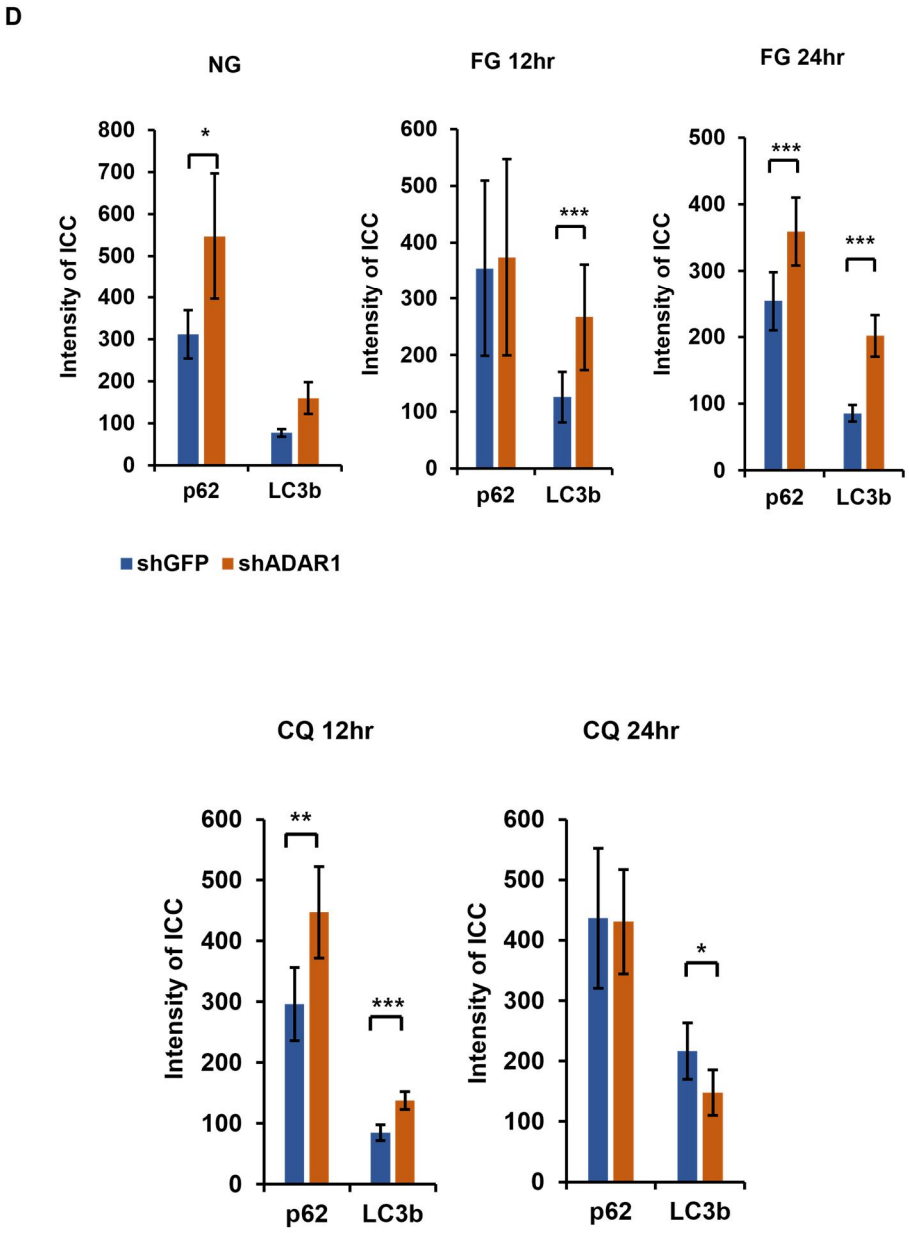

P-AMPKa intensity
8
7
6
5
4
3
2
1
0
*
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
FG 0hr
FG 12hr
FG 24hr


Beclin1 intensity
3
2
1
0
*
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
FG 0hr
FG 12hr
FG 24hr


LC3B intensity
6
5
4
3
2
1
0
*
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
FG 0hr
FG 12hr
FG 24hr

C

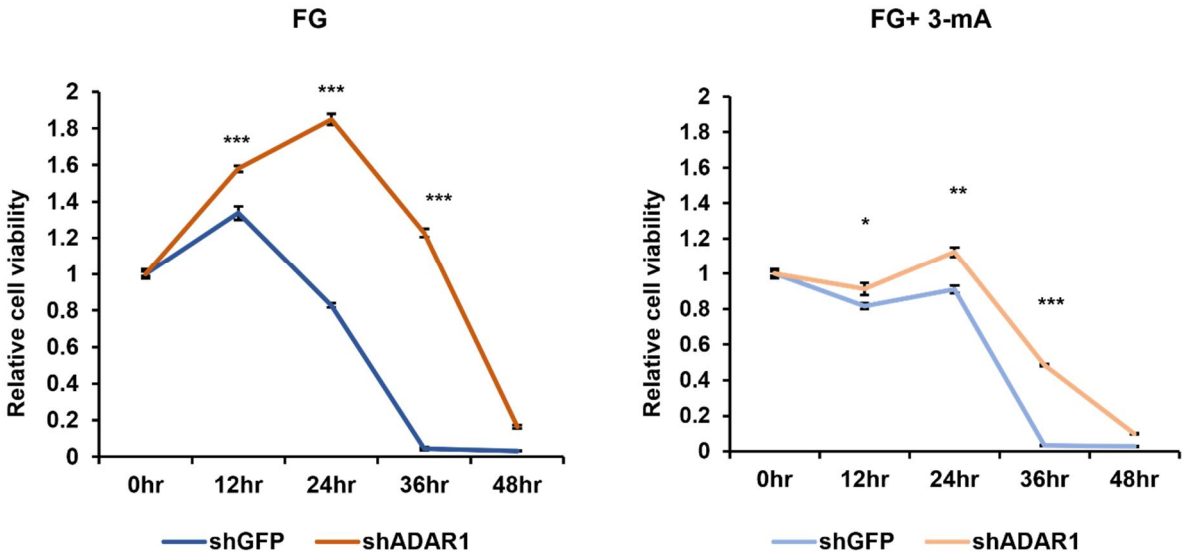

Red: p62
Green: LC3B
NG
FG 12hr
FG 24hr
CQ 12hr
CQ 24hr
shGFP
shADAR1



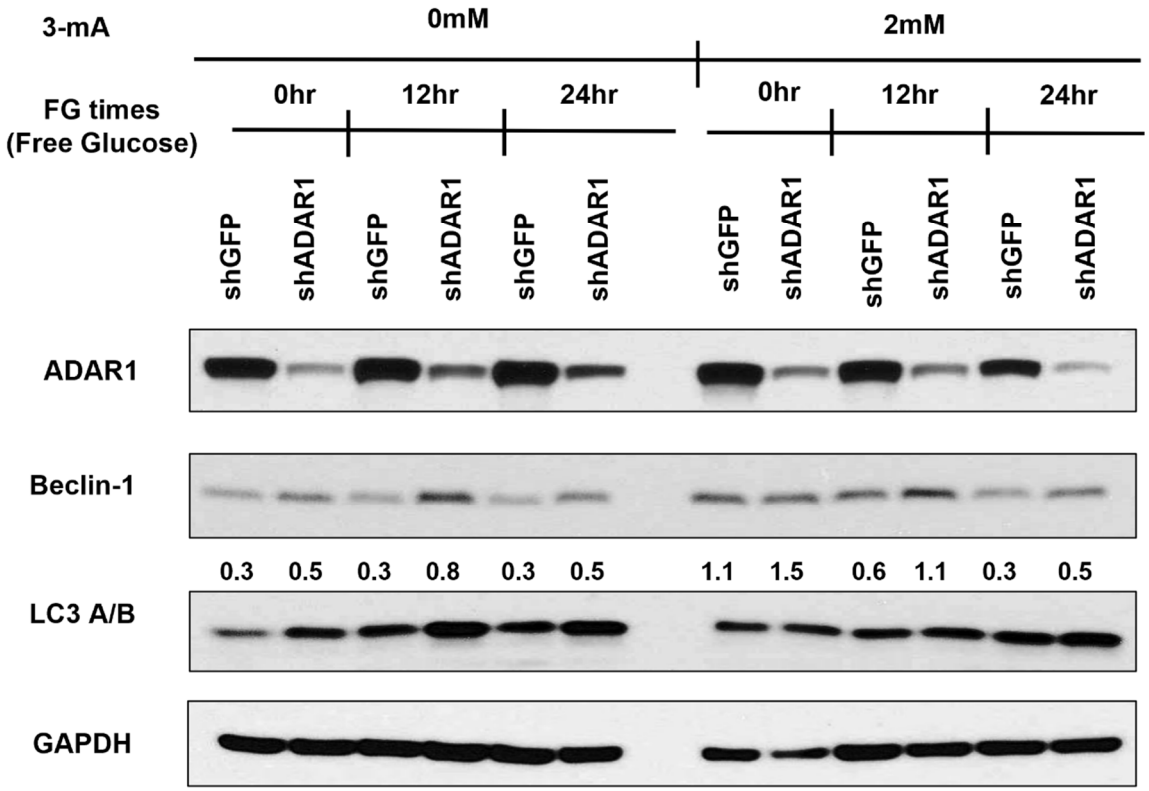
Part2_Figure 3. Increased autophagy under the glucose starvation condition in ADAR1 depletion cell

(A) Expression of Autophagy related genes under glucose limiting conditions in MCF7 cell. (B) Autophagy Western blot quantitative graph using Image J. (C) LC3b (Green), p62(Red) fluorescence expressed in cells using ICC analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

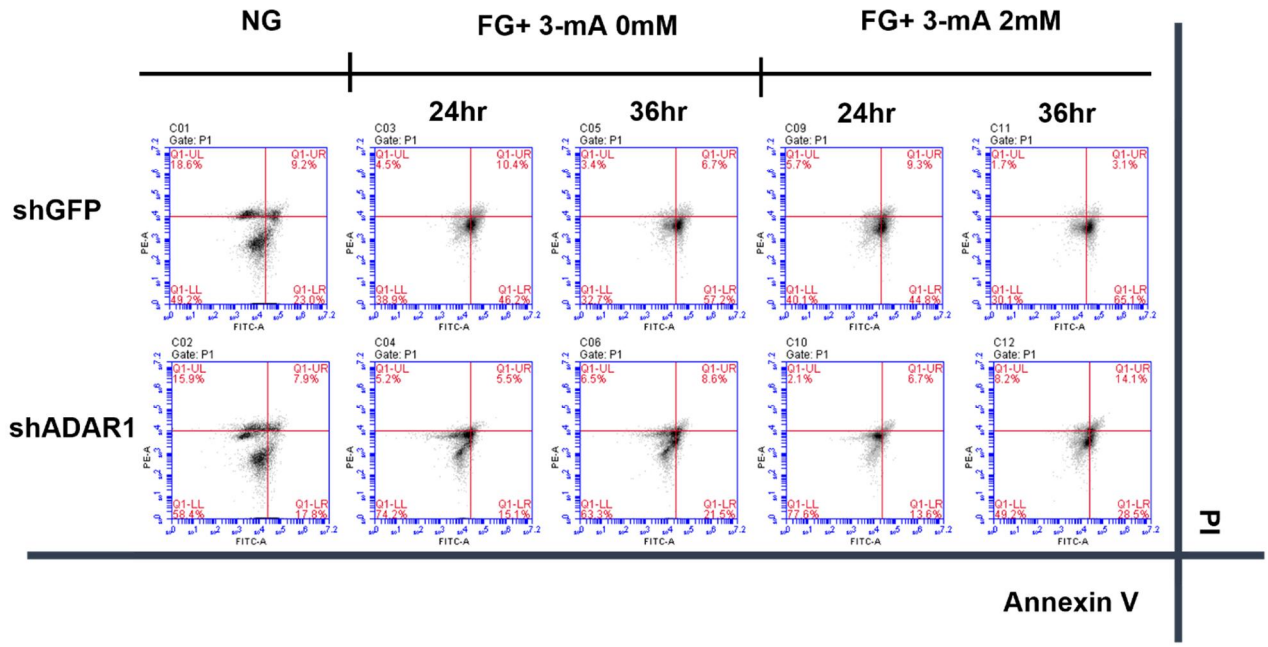
A


FG
FG+ 3-mA
Relative cell viability
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0hr
12hr
24hr
36hr
48hr
***
*
**
shGFP
shADAR1

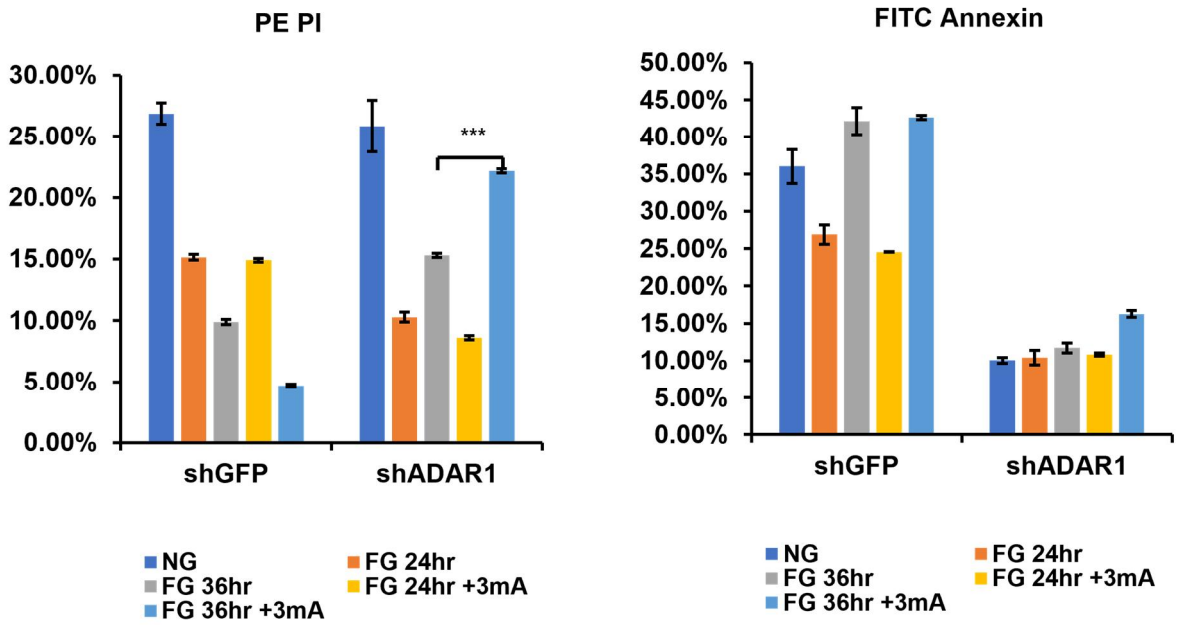
B


3-mA
0mM
2mM
FG times
(Free Glucose)
0hr
12hr
24hr
0hr
12hr
24hr
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
shGFP
shADAR1
ADAR1
Beclin-1
0.3
0.5
0.3
0.8
0.3
0.5
1.1
1.5
0.6
1.1
0.3
0.5
LC3 A/B
GAPDH

C


NG
FG+ 3-mA 0mM
FG+ 3-mA 2mM
24hr
36hr
24hr
36hr
shGFP
shADAR1
C01 Gate: P1 Q1-UL 18.6% Q1-UR 9.2% Q1-LL 49.2% Q1-LR 23.0%
C03 Gate: P1 Q1-UL 4.5% Q1-UR 10.4% Q1-LL 38.9% Q1-LR 46.2%
C05 Gate: P1 Q1-UL 3.4% Q1-UR 6.7% Q1-LL 32.7% Q1-LR 57.2%
C09 Gate: P1 Q1-UL 5.7% Q1-UR 9.3% Q1-LL 40.1% Q1-LR 44.8%
C11 Gate: P1 Q1-UL 1.7% Q1-UR 3.1% Q1-LL 30.1% Q1-LR 65.1%
C02 Gate: P1 Q1-UL 15.9% Q1-UR 7.9% Q1-LL 58.4% Q1-LR 17.8%
C04 Gate: P1 Q1-UL 5.2% Q1-UR 5.5% Q1-LL 74.2% Q1-LR 15.1%
C06 Gate: P1 Q1-UL 6.5% Q1-UR 8.6% Q1-LL 63.3% Q1-LR 21.5%
C10 Gate: P1 Q1-UL 2.1% Q1-UR 6.7% Q1-LL 77.6% Q1-LR 13.6%
C12 Gate: P1 Q1-UL 8.2% Q1-UR 14.1% Q1-LL 49.2% Q1-LR 28.5%
PE-A
FITC-A
PI
Annexin V

D



Part2_Figure 4. Reduced autophagy in glucose starvation condition after with 3-mA treatment in ADAR1- depleted cells

(A) Cell viability assay in MCF7 cells under glucose limiting conditions and with 2mM of 3-mA treatment. (B) Western blot of Beclin-1, p-AMPKa in MCF7 cell. (C) FACS analysis in MCF7 cells with 2mM of 3-mA treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

		Editing			A to G Editing alteration frequency					
Gene	**Chr**	**Site**	**Start**	**End**	**MCF7**	**shGFP-1**	**shGFP-2**	**shADAR1-1**	**shADAR1-2**	**shADAR1-3**
RSL1D1	chr16	3'UTR	11929175	11929176	0.769	0.4	0.583333	0.1875	0	0
METTL7A	chr12	3'UTR	51325236	51325237	0.500	0.5	0.352941	0	0	0
METTL7A	chr12	3'UTR	51324118	51324119	0.600	0.304348	0.444444	0	0	0
RSL1D1	chr16	3'UTR	11928845	11928846	0.500	0.5	0.578947	0	0	0.238095
METTL7A	chr12	3'UTR	51324201	51324202	0.556	0	0.461538	0	0	0
ARPIN	**chr15**	**3'UTR**	**90444707**	**90444708**	**0.476**	**0**	**0.5**	**0**	**0**	**0**

Part1_ Table 1. Genes frequently edited at 3'UTR in RNA-seq data of MCF7 cell

Expression of Small RNA seq		
Mature miRNA	shGFP	shADAR
hsa-miR-1285-3p	113	230
hsa-miR-1285-5p	4	12
hsa-miR-4512	0	1
hsa-miR-619-5p	13	29

Part 1_ Table 2. Expression of miRNAs predicted to bind to ARPIN 3'UTR from Small RNA-seq data of MCF7

hADAR1_RT_F	GCTTGGGAACAGGGAATCG
hADAR1_RT_R	CTGTAGAGAAACCTGATGAAGC
hRPL13a_RT_F	CGAAGATGGCGGAGGTGCAG
hRPL13a_RT_R	GGTTTTGTGGGGCAGCATAC
hARPIN_RT_F2	TGTATCTCGGCACAGCATCTTG
hARPIN_RT_R	CAGCTCGAGTTCCATCACCT
hGAPDH_RT_F	CCCATGTTCGTCATGGGTGT
hGAPDH_RT_R	TGGTCATGAGTCCTTCCACGATAT
siADAR1	GCGACUAUCUCUUCAAUGUUU

Table 3. Real time PCR primer sequence and siADAR1 sequences

Discussion

ADAR1 is widely known as an RNA editing enzyme. Depending on the role of ADAR1, several genes are altered, affecting their expression, which in turn affects the specificity of the cell. It is widely known that ADAR1 expression is high in most solid cancers. However, low expression of ADAR1 is known in various carcinomas. Research into whether reduced ADAR1 directly promotes cancer remains unclear. However, the expression of ADAR1 p110 and p150 in metastatic melanoma tumors was found to be low in common, and this low expression is known to affect the high expression of miRNA-17 and miR-432. [34],

In this paper, mobility was increased in ADAR1 KD stable cells in MCF7 breast cancer cells. It was confirmed that this factor was low in ARPIN expression by direct regulation due to high expression of miR1285-3p,1285-5p,619-5p in ADAR1 KD cells. ARPIN is known to be an ARP2/3 complex inhibitor, and it is known to destabilize membrane protrusions and consequently inhibit cellular migration and invasion in 3D collagen gels. [35] [32]. ARPIN and WAVE are known as regulatory genes of the ARP2/3 complex. These two genes antagonize the ARP2/3 complex. When actin filaments are formed, ARPIN inactivates the ARP2/3 complex. The Arp2/3 regulators ARPIN and WAVE are under the control of the Rac small GTPase (Rac). Rac and ARPIN are important for breast cancer cell proliferation, and low ARPIN expression is known to have a poor prognosis in breast cancer patients. [36] [37]. The relationship between ADAR1 and miRNA is well known. When it is dependent on ADAR1 editing, the miRNA sequence may be edited, or the UTR seed sequence of the target gene may be edited. In addition, it is known that the independent case of RNA editing influences miRNA biogenesis and ADAR1 increases pre-miRNA cleavage at a maximum rate through protein interaction with Dicer [38]. When ADAR1 was inhibited in melanoma cells, the expression of ITGB3 was increased due to increased expression of miRNA22 and PAX2 transcription factors, and the invasion of melanoma cells was increased [19]. Based on Part 1 of this paper, it was suggested that ADAR1 expression could affect cancer progression and metastasis. In this context, low ADAR1 expression

and ARPIN expression in the advanced stage of cancer are associated with metastasis-free survival (MFS).

The results of the PART1 study that miRNA expression is regulated by ADAR1 expression and that ARPIN expression is affected to change cell mobility are thought to play an important role in cancer metastasis. Expression of ADAR1 and ARPIN at the metastatic stage of cancer is thought to play an important role in metastasis.

Autophagy is a physiological cellular process for the breakdown and removal of damaged organelles in cells to function adaptively to starvation, development, apoptosis, and tumor suppression [39, 40]. Autophagy can be nonselective or selective. Non-selective autophagy packages a portion of the cytoplasm of the cytoplasm into autophagosomes and migrates to lysosomes where they are degraded. In contrast, selective autophagy works by recognizing specific targets, such as damaged organelles, intracellular pathogens beneath protein aggregates. An important autophagosome of this autophagy is the delivery of degraded cytoplasmic components to lysosomes for recycling under cellular stressful conditions. These mechanisms are essential for protecting organelles, maintaining metabolic and energy homeostasis, and promoting cell survival [41].

Autophagy is known as a regulator of many oncogenes and tumor suppressor genes. [42, 43], on the other hand, in other studies, autophagy is known to be involved in both the promotion of tumorigenesis and the development and suppression of cancer. [44-47]. In the regulation of autophagy, tumor suppressor factors are negatively regulated by mTOR and AMPK to induce autophagy and inhibit cancer development [48]. This regulation of autophagy is involved in both suppression and promotion of tumor growth. As a role of tumor suppression, Beclin1 plays an important role in the formation of phagophores and is also known to act as a tumor suppressor [49-51]. In other studies, autophagy promotes tumor survival and growth in advanced cancers. As tumors grow, they are exposed to several extreme stresses, including hypoxia and territorial deprivation. One way to overcome this stress is autophagy. During tumor growth, a hypoxic state is maintained in the central region, thereby activating autophagy. Inhibition of autophagy enhances apoptosis [52, 53]. In addition, the high

metabolic and energy requirements for tumor growth are supplied by recycling intracellular components using Autophagy [54, 55].

In this study, part 2, it was confirmed that when ADAR1 expression was inhibited under glucose-limited conditions using breast cancer cells, the cell viability was high, and apoptosis decreased compared to the control. Autophagy increases tumor survival by supplying imaging elements to meet metabolic demands for tumor growth. It is known that this inhibition of autophagy leads to tumor cell death [42, 43, 56]. As above, in this paper, the expression of autophagy was increased in cells that inhibited ADAR1 expression under glucose-limited conditions, resulting in increased cell viability and decreased apoptosis. To suppress this increased autophagy, an autophagy inhibitor drug called 3-MA was used, and as a result, the survival rate was lowered, and apoptosis increased. In this context, ADAR1 is consistent with the idea of regulating autophagy for adaptation to extreme stress in breast cancer cells.

Summarizing the contents of Part 1 and Part 2, when tumors are formed and grown, cell mobility increases, and ADAR1 can be influenced independently or dependently in the process of adaptation to metabolic stress. Through these studies, another novel role of ADAR1 is needed to investigate as a potentially effective treatment strategy in step-by-step anticancer therapy for cancer.

Conclusion

The target gene for RNA editing of ADAR1 acts broadly. We demonstrated a novel role for this ADAR1 in breast cancer.

In Part 1 results, it was confirmed that ARPIN expression was reduced when ADAR1 was deficient using MCF7, a breast cancer cell. In addition, it was confirmed that the expression of miRNA1285-3p, miR1285-5p, and miR619-5p increased due to ADAR1 deficiency. And it was confirmed that the expression of ARPIN was decreased because the increased miRNAs targeted the 3'UTR of ARPIN. Therefore, cell migration and invasion increased due to inhibition of ARPIN expression, and as a result, cell migration ability was improved.

As a result of Part 2, it was confirmed that ADAR1-deficient cells using breast cancer cells had higher cellular adaptability to glucose-limiting conditions than the control group. It was confirmed that apoptosis was decreased in ADAR1-deficient cells than in the control under glucose-restricted conditions, and it was confirmed that this factor plays an important role in increasing the expression of autophagy.

Collectively, we found that ADAR1 expression correlates with metastasis during tumor growth and enhances cancer cell viability by modulating the expression of autophagy under nutrient stress due to glucose restriction, among several stresses encountered during tumor growth.

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국문 요약 (Korean Abstract)

RNA 편집은 잘 알려진 전사 후 사건이며 탈아미노화는 주요 과정 중 하나이다. RNA의 탈아미노화에는 시티딘(C)에서 우리딘(U)으로, 아데노신(A)에서 이노신(I)으로의 두 가지 유형이 있다. A-to-I transition은 이중 가닥 RNA를 인식하는 ADAR1(Adenosine Deaminase, RNA Acting on RNA)에 의해 편집된다. A to I RNA 탈아미노화는 주로 Alu 반복부에서 발생하며 기능적으로는 3'UTR 영역, 코딩 서열 및 microRNA 전구체에서 발생한다. 이것은 표적 유전자의 재암호화를 초래하거나 그의 발현 및 기능을 변경할 수 있다. 최근 연구는 후성 유전 요법 및 면역 요법을 포함한 인간 암 치료에서 ADAR1의 새로운 역할을 제안한다. 우리는 RNA seq의 결합 분석과 ADAR1 고갈 후 세포 분석을 통해 인간 유방암 세포에서 ADAR1의 역할을 찾는 것을 목표로 한다. 결과적으로, 우리는 ADAR1의 고갈이 편집 독립적인 방식으로 ARPIN의 발현을 억제한다는 것을 발견했다. ARPIN이 액틴 필라멘트의 분기를 억제함에 따라 ADAR1 결핍은 세포 이동성을 자극했다. 반면에 우리는 포도당 제한 조건에서 ADAR1의 역할을 밝혔다. 특히, ADAR1이 고갈되었을 때, 우리는 향상된 자가포식 반응과 유방암 세포의 생존력 증가를 발견했다. 이 반응은 이후 Beclin-1 및 LC3를 활성화한 활성화된 AMPK에 의해 유발되는 것으로 보인다. 종합하면, 이러한 결과는 유방암에서 ADAR1의 새로운 역할을 제시한다.

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