



ADAR1 Transcriptome editing promotes breast cancer progression through the regulation of cell cycle and DNA damage response

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ABSTRACT

RNA editing has emerged as a novel mechanism in cancer progression. The double stranded RNA-specific adenosine deaminase (ADAR) modifies the expression of an important proportion of genes involved in cell cycle control, DNA damage response (DDR) and transcriptional processing, suggesting an important role of ADAR in transcriptome regulation. Despite the phenotypic implications of ADAR deregulation in several cancer models, the role of ADAR on DDR and proliferation in breast cancer has not been fully addressed. Here, we show that ADAR expression correlates significantly with clinical outcomes and DDR, cell cycle and proliferation mRNAs of previously reported edited transcripts in breast cancer patients. ADAR's knock-down in a breast cancer cell line produces stability changes of mRNAs involved in DDR and DNA replication. Breast cancer cells with reduced levels of ADAR show a decreased viability and an increase in apoptosis, displaying a significant decrease of their DDR activation, compared to control cells. These results suggest that ADAR plays an important role in breast cancer progression through the regulation of mRNA stability and expression of those genes involved in proliferation and DDR impacting the viability of breast cancer cells.

1. Introduction

Double stranded RNA-specific adenosine deaminase (ADAR) enzymes, catalyze the adenosine to inosine conversion across different RNA families, modifying the canonical base-pairing pattern of adenosine nucleosides [1–3], allowing a plethora of consequences for the

modified transcript [2]. Editing events across the different pre-mRNA structures leads to different consequences [4] for the edited target, including amino acid changes, modification of transcripts variants by altering splicing and/or canonical polyadenylation sites, among others [4–7]. Alterations in the editing process are related with different diseases occurrence, including amyotrophic lateral sclerosis [7], systemic

Abbreviations: ADAR, double stranded RNA-specific adenosine deaminase; DDR, DNA damage response; DFS, disease free survival; HDR, homology directed repair; KM, Kaplan-Meier; METABRIC, The Molecular Taxonomy of Breast Cancer International Consortium; OS, overall survival; SEER, surveillance, epidemiology and end results; TCGA, The Cancer Genome Atlas; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling

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lupus erythematosus [8] and several cancer types [9–13].

Advances on transcriptomic and RNA-protein interaction genome-wide methodologies have shown the fundamental importance of RNA editing mechanisms under physiological and disease conditions [4,13–16] [6,13,17–19], showing that ADAR is an enzyme capable of modifying a large fraction of the transcriptome. Although ADAR generates a small number of editions in coding regions that produce non-synonymous amino acidic changes, several groups established the importance of RNA editing in the protein function that further impacts the phenotype of the cell [17–20]. Recent evidence has revealed the importance of ADAR editing function in cancer progression, including liver [18], lung [13], colorectal [11], melanoma [21,22], and breast cancer [23,24] playing a role in carcinogenesis by either inactivating tumor suppressor genes or promoting the activation of proto-oncogenes [23,25]. Several studies have suggested that ADAR activity regulates several mRNAs of proteins that participate in the DNA Damage response (DDR) and cell cycle regulation [26–29], suggesting a novel role of ADAR activity on tumor development and progression. Recently, it has been shown that ADAR regulates ATM and RAD51 mRNA expression under stress conditions, inhibiting their degradation by an Staufen-mediated mechanism in glioblastoma cells [30]. Also, Zhang et al. [28] described an increased editing on the 3'UTR of MDM2 transcript, affecting the canonical RNA-miRNA interaction and therefore, abolishing the mRNA repression. This could further lead to a down regulation of p53 surveillance in the breast cancer context. Lastly, Guo et al. [27] recently revealed that ADARp150 overexpression leads to a protein overexpression of components of the DDR like PRPF19, XRCC5 and PCNA in HEK293 cells [31–33].

In this work we show that the expression of ADAR and its editing activity correlate positively with breast cancer progression, showing an increased editing on transcripts associated with cellular proliferation, DDR and transcriptional regulation. Moreover, *in vitro* manipulation of ADAR expression produces significant changes on the mRNA stability of edited transcripts involved in DDR and cell cycle progression. In addition, ADAR knock-down cells display a decreased proliferation, viability and increased apoptosis compared to control cells. Finally, ADAR knock-down cells exhibit a significant decrease of their DDR, showing an overall decrease in the activity of this pathway. Taken together, this work provides novel insights on the critical role played by ADAR in the regulation of those edited mRNAs, related to DDR and cell cycle, suggesting that ADAR could be involved in DDR regulation in breast cancer.

2. Methods

2.1. Cell culture

MDA-MB-231, MCF7 and ZR-75-1 breast cancer cell lines were obtained from ATCC and cultured under standard conditions, at 37 °C in a humidified incubator containing 5% CO₂. HeLa ADARp110 Flp-In T-REx and GFP Flp-In T-REx cells were generated at Dr. Alfredo Castello Laboratory, University of Oxford, UK. Cells were routinely tested for mycoplasma contamination using the PCR Mycoplasma Test Kit EZ-PCR (Biological Industries) following the manufacturer's instructions. siRNAs transfections, in MDA-MB-231 were carried out using Lipofectamine 3000 Transfection Reagent following manufacturer's instructions. Briefly, 500.000 cells were plate in a 60 mm dish. The day after, control (Cell Signaling, 6568S) or ADAR1 (Thermo Scientific, 119580) siRNAs were transfected at a final concentration of 20 nM and incubated for 48 h for further experiments.

2.2. Survival analysis

Overall survival data from breast cancer patients was obtained from KMplotter website (<http://kmplot.com/analysis/>) [34]. For overall survival (OS), only SEER like patients were analyzed. ADAR expression

levels were measured using 201786_s_at probe and auto select cut-off option (cut-off value for OS analysis: 5294, cut-off value for DMFS: 6730). KM-plots were created and analyzed using GraphPad Prism 7.0. A log-rank Test p-value < 0.05 is considered significant.

2.3. Adenoviral and lentiviral transduction

MDA-MB-231 cells were transduced (MOI: 200) with commercial pre-package adenoviral particles (VectorBuilder Inc., Shenandoah, TX, USA. VB170930-1035dgj) coding for ADARp110 (based on NM_001025107.2) or transduced (MOI: 200) with eGFP control adenovirus (VB150925-10024 vector). Cells were processed 48 h after transduction. MCF7 and ZR-75-1 cells were transduced (MOI: 10) with commercial pre-package lentiviral particles (GeneTarget, cat. number LTSH-U6-RP) coding shRNAs directed against ADAR mRNA (SHADAR) or a scramble shRNA (SHC) (U6(shRNA-Ctr)-RP) as a control. ADAR shRNA sequences: TRCN0000336832 (SHADAR#1) and TRCN0000336886 (SHADAR#2) were obtained from The RNAi consortium (Broad Institute). Cells were maintained in growth media with 1 µg/mL Puromycin (Thermo Scientific) for selection.

2.4. Gene ontology and pathway enrichment analysis

Gene ontology enrichment was carried out using Cytoscape [35] v3.0.1 software and ClueGO (v3.3.3) plugin [36]. Briefly, a gene list from each analysis was submitted on this software using Reactome pathway enrichment database (v01/03/2017) for further comparisons. Only statistically significant groups were displayed, using a Bonferroni step-down multiple comparison post-hoc test. A corrected p < 0.05 was considered statistically significant. For the breast cancer TCGA RNA-seq enrichment analysis, we used all genes with more than $r \geq 0.2$ (Pearson correlation) obtained from cBioportal website (www.cbioportal.org) [37]. Finally, for breast cancer TCGA array data we performed a K-means clustering (K = 10 with 100 iterations) in Multi Experiment Viewer software (MeV 4.9). The gene cluster which contains the ADAR expression values was extracted and used as input into ClueGO plugin and DAVID 6.7 db (OMIM_Disease).

2.5. ADAR mRNA expression analysis in TCGA and METABRIC cohorts

ADAR expression values were obtained from the TCGA (The Cancer Genomic Atlas) [38] and METABRIC (The Molecular Taxonomy of Breast Cancer International Consortium) [39,40] cohorts. The corresponding files were downloaded from the TCGA data portal and the database cBioPortal, respectively.

2.6. Quantitative RT-PCR

Total RNA was extracted using RNeasy columns following the manufacturer's instructions (QIAGEN). 1 µg of total RNA was reverse-transcribed using an AffinityScript qRT-PCR cDNA Synthesis Kit (Agilent Technologies Inc.) and diluted 5 times. Quantitative expression analysis was performed using specific oligonucleotide primers and Brilliant II SYBR Green qRT-PCR Master Mix (Agilent Technologies Inc.). The reactions were carried out in an Eco Real-Time PCR (Illumina) using the following program: 95 °C for 15 s, 58 °C for 15 s and 72 °C for 15 s at 40 cycles. Expression values were calculated using the $\Delta\Delta C_t$ method and expressed as the fold of change relative to control samples. ACTNB was used as a housekeeping gene. In addition, RESS-qPCR assays for AZIN1 and MDM2 targets were performed according to Crews, et al. [41]. The following primers were used: ADAR: Fw: AAG GCA GAA CGC ATG GGT TTC A; Rv: AGT GTC TTT GGC TGT GCT TCT GG; ACTNB: Fw: AAC GGC TCC GGC ATG TGC AAG; Rv: GCC GTG CTC GAT GGG GTA CTT.

2.7. Western blotting

Protein lysates were processed as described in Sagredo et al. [42]. Briefly, protein lysates were generated using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% v/v TritonX-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS) and protease (Calbiochem) and phosphatase (Roche Life Science) inhibitor cocktails. Protein lysates (30 µg per lane) were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto a nitrocellulose membrane. Membranes were blocked in 5% w/v BSA (Winkler), and then incubated with primary antibodies at 4 °C overnight. Rabbit anti ADAR (Cell Signaling, 14175), rabbit anti phospho-Atr (Ser428) (Cell Signaling, 2853), rabbit anti Atr (Cell Signaling, 279), rabbit anti phospho-Chk2 (Thr68) (Cell Signaling, 2197), mouse anti Chk2 (Cell Signaling, 3440), mouse anti p53 (Cell Signaling 2524) and mouse anti phospho-p53 (Ser15). Mouse anti α -tubulin (Sigma-Aldrich, T5168), mouse anti β -actin (Sigma-Aldrich, A1978) and anti HSP70 (Origene, TA309356) were used as loading controls. All primary antibodies were detected using appropriate HRP-conjugated secondary antibodies (Thermo Scientific, 170–6515 and 170–6516) and a chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). Finally, images were obtained using the ChemiScope3500 Mini chemiluminescence imaging system (Clinx Science Instruments).

2.8. Caspase 3/7 activation assay

Caspase-3/7 activation was measured using the Caspase-Glo 3/7 Assay System (Promega), following the manufacturer's instructions. Briefly, 10,000 cells were seeded in 96 well plates and 24 h after seeding, Caspase activity was measured. Measurements were done in triplicates. As a positive control, cells were exposed to sorbitol 300 mM for 8 h. Luminescence was detected using the Cytation 3 Multi-Mode Reader (BioTek Instruments).

2.9. Indirect immunofluorescence

Cells were fixed (4% w/v formaldehyde, Sigma-Aldrich), permeabilized, blocked and incubated with rabbit anti γ H2AX (Millipore, 5636) (1:1000) followed by anti-mouse coupled with Alexa-Fluor 488 (Invitrogen, 1:1000). Cells were stained and mounted in Prolong-Diamond-DAPI (Invitrogen). Images were recorded in an inverted microscope (IX81 Spinning Disc Confocal, Olympus) and analyzed using NIH ImageJ 1.47 software [43] to measure the relative levels of nuclear γ H2AX fluorescence.

2.10. TUNEL assay

Cells were seeded under standard conditions in Lab-tek® II CC2 Chambers (Thermo Scientific) to further perform TUNEL immunofluorescence assay following the manufacturer's instructions (Promega). Positive foci were counted based on the DAPI nuclei channel signal.

2.11. Viability assays

Cells were seeded in 96-well plates (1×10^3 cells/well) and allowed to attach overnight. Cell proliferation was assessed using the Cell-Titer 96 AQueous-MTS Kit (Promega) at 24, 48 and 72 h, measuring absorbance at 490nm in the Cytation 3 Multi-Mode Reader (BioTek Instruments). Additionally, viability was evaluated by mixing cells with an equal volume of 0.4% trypan blue solution (Logos Biosystems). Cells were counted using a LUNA Automated Cell Counter (Logos Biosystem).

2.12. BrdU incorporation and cell cycle analysis

Cells growing in exponential phase were incubated with 25 µM of

BrdU (Santa Cruz) for 45 min before harvesting. Cells were fixed in 80% methanol and kept overnight at -20 °C. Double staining with 50 µg/mL PI and FITC-anti-BrdU antibody (BD Pharmingen) was performed according to the manufacturer's protocol. Cell cycle profiles and BrdU uptake were determined by FACS (BD Bioscience). Data were analyzed using the BD FACSDiva software.

2.13. Reporter assay and transfections

Reporter assay was performed according to Armisen et al. (2011) [44]. Briefly, MCF7 SHC and MCF7 SHADAR cells were co-transfected with 0.5 µg of Renilla plasmid (pRL-Renilla, Promega) and 1 µg of pGL2-p21 promoter plasmid. Afterwards, cells were processed according to the manufacturer's instructions (Promega) using the Dual-Glo Luciferase system. Luminescence signal was measured using a Multi-Mode Reader (Cytation 3, BioTek Instruments). Renilla luminescence signal was used to normalize signals across samples.

2.14. Triptolide treatment

24 h post seeding, ZR-75-1 SHC, and ZR-75-1 SHADAR cells were treated using 100 nM Triptolide (Invivogen) or DMSO used as a vehicle. After 16 h of treatment, total RNA was extracted as described in the previous section.

2.15. RNA-seq experiments and workflow

RNA quality from each biological replicate ($N = 3$) was analyzed using the Experion (Biorad) system to further generate RNA-seq libraries using the TruSeq Stranded Total RNA kit (Illumina). RNA-seq libraries were sequenced using a HiSeq4000 (BGI, Korea). Fastq files were aligned using STAR and hg19 and transcripts were counted using HT-seq software. Differential expression analysis was performed using DESeq2 software following standard recommendations. To evaluate stability changes, DMSO and triptolide treated (16 h) samples were compared for those SHC and SHADAR cells.

2.16. Gene level A to G(I) editing comparisons between control and ADAR knock-down cells; and between control and ADAR overexpressing cells

Variant files were generated using GATK v4 following GATK best practices for RNA-seq to further proceed to the variant calling using HaplotypeCaller (GATK v4, stand call conf 20 and stand emit conf 20). The resulting variant files were annotated using dbSNP v1.47 and filtered using GATK Variant filtering walker with the following filtering options: QD < 2.0 and FS > 30. Just addressable editing variants were considered. For further analysis, such as variation count for each gene, we used IntersectBed (Bedtools v2.20.1) and Unix command line tools. A to G(I) counts were sum for each gene based on gencode (hg19) coordinates for each position called to further normalize them using the coverage of each gene and sample, the analysis was restricted to those transcripts with non-ambiguous reads. All the data generated was processed in R (3.2.0) or by Unix command line.

2.17. Site level A to G(I) editing comparisons between control and ADAR knock-down cells, and control and ADAR overexpressing cells

Comparisons at shared edited sites were made using a multi-sample variant calling file (.VCF) to further compare control cells, either SHC or GFP cells, against ADAR knock-down or cells overexpressing ADAR, respectively. Combined A and G counts from each group were compared using Fisher's exact test. Confidence level of 0.05 was used as a cut-off for further analysis. All the data generated was processed in R (3.2.0) or by Unix command line.

2.18. Site level A to G(I) editing comparisons between tumoral and matched normal samples from breast cancer TCGA cohort

Briefly, variant calling dataset that contains the editing proportion of each A to G(I) variant called across the breast cancer TCGA samples was obtained from Han et al. [10] For matched comparisons, 105 matched tumoral and normal samples were selected, and paired Wilcoxon test was performed to find sites with an increased editing in tumoral samples compared to control samples. Confidence level of 0.05 was used as the cut-off for further analysis. All the data generated was processed in R (v3.2.0).

2.19. Statistical analysis for qRT-PCR and Western blot

Statistical analysis was performed using GraphPad Prism 7.0. Two-tailed Student's *t*-test was used to establish differences in qRT-PCR and Western Blot. $p < 0.05$ was considered statistically significant. Each analysis was performed at least in 3 independent experiments. Correlation tests were evaluated using the Two-Tailed Spearman correlation test.

2.20. Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The data is publicly available at the Gene Expression Omnibus (GEO) IDs [GSE132287](#) and [GSE132288](#).

2.21. Ethics approval

The Ethics committee of Facultad de Medicina, Universidad de Chile, approved the study, to follow the Helsinki Declaration, the International Ethical Guidelines for Health-related Research Involving Humans CIOMS 2002 and the ICH 1996 Guidelines for Good Clinical Practice. In this study, all the patients had informed consent to participate in scientific research.

3. Results

3.1. ADAR expression correlates with cancer-related pathways in breast cancer

Several authors have described an overexpression of *ADAR* across different cancer types [12,13,24,45,46]. To analyze *ADAR* expression across breast cancer tissues, we explored the well-characterized METABRIC and TCGA breast cancer cohorts. In both databases, *ADAR* was significantly overexpressed in tumors, with a 1.85 and 1.72-fold change relative to normal tissue samples in METABRIC and TCGA cohorts, respectively (Fig. 1A and Supplementary Fig. 1A). In addition, the publicly available Kaplan-Meier plotter database for breast cancer [47] was used to address the clinical significance of *ADAR* expression levels in breast cancer patients. In agreement with previous works [24,48,49], *ADAR* overexpression is significantly associated with lower overall survival (OS) and disease-free survival (DFS) ($p < 0.0042$ and $p < 0.0351$, respectively Fig. 1B), suggesting that *ADAR* expression is a significant factor in breast cancer progression. In addition, the analysis of *ADAR* co-expression on the TCGA dataset, shows that *ADAR* is significantly co-expressed with breast cancer and cancer-related disease genes (OMIM breast cancer $p < 2.24E-04$) (Fig. 1C, upper panel) and with cancer related ontologies, including cell cycle ($p = 3.13E-28$), DNA replication ($p = 2.9E-10$) and DNA repair ($p = 1.95E-4$) (Fig. 1C, lower panel, and Supplementary Fig. 1B). To evaluate the A-to-I RNA editing activity in breast cancer, we re-analyzed the breast cancer TCGA mRNA variant calling data described by Han et al. [10]. 4644 non-redundant transcripts are A-to-I edited across the dataset. These transcripts are involved in different signalling pathways, including Generic

Transcription pathway ($p = 1.3E-27$), Signalling by NGF ($p = 8.3E-28$), Transcript regulation (mRNA splicing pathway ($p = 7.5E-7$), Apoptosis ($p = 2.3E-16$) and DNA repair ($p = 1.5E-3$), among other pathways (Fig. 1D, plotted in red scale). More importantly, we compared the RNA editing for each site shared across the 105-matched normal/tumor samples on the breast cancer TCGA cohort, showing 18,693 variants are significantly more edited in the tumors, compared to the controls included in 1717 non-redundant transcripts (shown in Supplementary Material 1). Further, and to determine if A-to-I RNA editing is differentially enriched in transcripts from specific gene ontologies in breast cancer, we performed a pathway enrichment analysis, showing that transcripts related with Generic transcription pathways ($p = 7.13E-13$), Apoptosis ($p = 2.5E-8$) and Homologous DNA recombination (HDR) through single-strand annealing ($p = 0.004$) show a significant increased edition in the 105 tumor samples compared to controls (Fig. 1D, blue scale colors and Supplementary Material 2). These results indicate that *ADAR* expression and A-to-I RNA editing are closely related with DDR, apoptosis and transcriptional regulation, showing that tumoral samples present an increased editing of transcripts associated with breast cancer progression (shown in Supplementary Material 1 and 2).

To corroborate the participation of *ADAR* in those processes, *ADAR* expression was manipulated in breast cancer cell lines. We transduced ZR-75-1 cells with a shRNA against *ADAR* mRNA (Fig. 2A and Supplementary Fig. 2). These cells were chosen because they express high levels of *ADAR1* [48]. Next, a RNA-seq analysis was performed to further identify differentially expressed mRNAs in control (SHC) and *ADAR* knock-down (SHADAR) cells. In *ADAR* knock-down cells, there is a significant down regulation of transcripts related to DNA replication (described as meiotic synapsis ($p = 4.91E-04$) and proliferative pathways, such as IRS-mediated signalling ($p = 0.003$) and *AKT2* activation ($p = 0.01$) pathways (Fig. 2B, upper panel and Supplementary Material 3); while there is an increased expression of mRNAs related with Generic transcription ($p = 2.23E-05$) and apoptotic related pathways, including *NRAGE* and *JNK* mediated death signalling pathways ($p = 4.12E-04$) (Fig. 2B, lower panel). To confirm the role of *ADAR* on these specific pathways, we compared transcriptome differences in a different breast cancer cell line model. To do that, we used MDA-MB-231 cells overexpressing *ADARp110* (MDA-MB-231 OE) or GFP as a control (MDA-MB-231 Control) (Fig. 2C). *ADARp110* overexpression correlates with a significant increase in transcripts related with cell cycle and proliferative pathways (Signalling to RAS, Signalling by EGFR and Cell cycle, $p = 1.5E-18$), in addition to other pathways such as IL-10 signalling ($p = 8.3E-15$) and ECM proteoglycans ($p = 1.6E-9$) (Fig. 2D and Supplementary Material 3). To begin to understand *ADAR* role on gene expression, next, we compared the mRNA A to G(I) editing differences between SHC and SHADAR ZR-75-1 cells, and between MDA-MB-231 control and overexpressing *ADARp110* (OE), in edited sites present in both conditions. Overall, ZR-75-1 SHADAR cells display a significant decrease in the number of A to G(I) variants compared to SHC cells, while MDA-MB-231 *ADAR* OE display a significant increase in the number of A to G(I) variants compared to control cells (Fig. 3A, upper and lower panel, respectively). From the 8352 edited transcripts identified in ZR-75-1 cells, we found that 4919 show at least a 15% reduction in the number of addressable A-to-G(I) counts. In other hand, *ADAR* overexpression on MDA-MB-231 cells results in an increase in the edition of 1641 mRNAs out of the 1859 addressable edited mRNAs found in MDA-MB-231 cells, showing that *ADAR* targets such as *ATM*, *MDM2*, *MDM4*, *CENPN* and *XPO1* are among the affected transcripts (shown in Supplementary Material 4).

There are 1535 sites with a significant decrease in their editing proportion in ZR-75-1 SHADAR cells, compared to ZR-75-1 SHC cells (Supplementary Material 5). In agreement with the previous results, transcripts related with Generic Transcription ($p = 4.4E-8$), Apoptosis ($p = 4.1E-7$), cell cycle ($p = 1.2E-4$) among other pathways, show a significant decrease in their editing profile in the *ADAR* knockdown

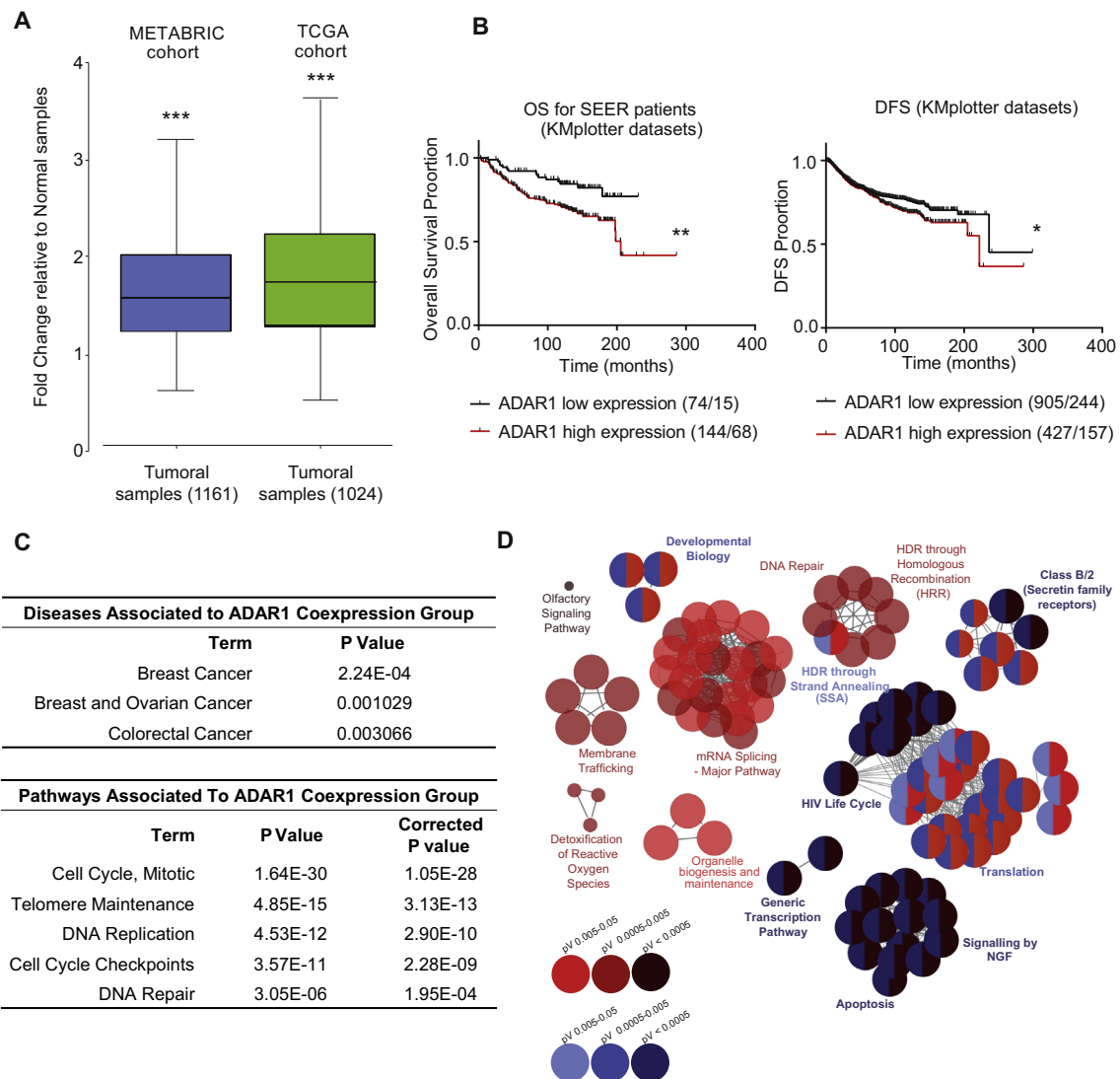


Fig. 1. ADAR displays an increased expression and activity in breast cancer, showing a clinical significance. (A) ADAR mRNA expression values in METABRIC (left panel) and TCGA (right panel) cohorts. ADAR mRNA fold change relative to normal samples, Box and Whiskers plots ***: $p < 0.0001$. (B) Overall survival and Disease-free survival for breast cancer patients segregated by ADAR expression present on KMplot data portal. **: $p = 0.0042$, *: $p = 0.035$. (C) TCGA ADAR mRNA co-expression disease (OMIM, upper) and Biological Process (GO: BP, lower) analysis to ADAR cluster enrichment. (D) Reactome pathway enrichment analysis for those edited transcripts present in breast cancer TCGA patients (in red scale) and those pathways significantly more edited in tumoral samples compared to normal samples, using matched tumoral/normal samples from the breast cancer TCGA cohort after Fisher exact test (in blue scale). Only statistically significant groups are displayed, using a Bonferroni step-down multiple comparison post-hoc test.

cells (Fig. 3B and Supplementary Material 6). On the other hand, 657 sites show significantly increased editing in MDA-MB-231 cells over-expressing ADARp110, compared to control cells (Supplementary Material 5). Consistently, transcripts involved in Generic Transcription ($p = 2.2E-7$), cell cycle ($p = 1.7E-4$), and Apoptosis ($p = 1.93E-3$) showed a significant increase in the editing rates (Fig. 3C). Overall, differential expression and edited targets analysis suggest that ADAR could be affecting the expression of mRNAs related to cell cycle, apoptosis, DDR and generic transcription, among other pathways.

3.2. ADAR regulates the stability of their targets

Previous works suggest that ADAR-mediated mRNA editing may affect the stability and/or the expression of edited targets [30,50,51]. Thus, to evaluate whether ADAR-mediated editing affects the stability on their targets, we treated ZR-75-1 SHC and ZR-75-1 SHADAR cells with triptolide, a robust RNA pol II inhibitor [52], and 16 h later, mRNA levels were analyzed by RNA-seq. Differential expression

analysis between vehicle (DMSO) and triptolide treated cells showed that after triptolide treatment, 1829 transcripts display significant changes in ZR-75-1 SHC cell: 584 transcripts were increased and 1245 were decreased (Fig. 4A and Supplementary Material 7). On the other hand, 930 transcripts increased and 1714 decreased in ZR-75-1 SHADAR cells treated with triptolide compared to DMSO SHADAR cells (Fig. 4A and Supplementary Material 7). Overall, 1565 of the transcripts were affected by triptolide treatment in both SHC and SHADAR cells (Fig. 4B). The affected transcripts are related to several ontologies, including Gene expression ($p = 1.86E-24$), Cell cycle ($p = 9.13E-11$), Transcriptional regulation by P53 and DNA repair ($p = 4.34E-6$) (Fig. 4B and Supplementary Material 8). Interestingly, the effect of triptolide in SHADAR cells was higher, in terms of fold change and number of affected transcripts showing 1079 more transcripts differentially expressed compared to SHC triptolide treated cells (Fig. 4A and B). To identify whether ADAR-mediated editing affected stability, the stability of target transcripts (according to results described in Supplementary Material 4) were evaluated comparing the effect of

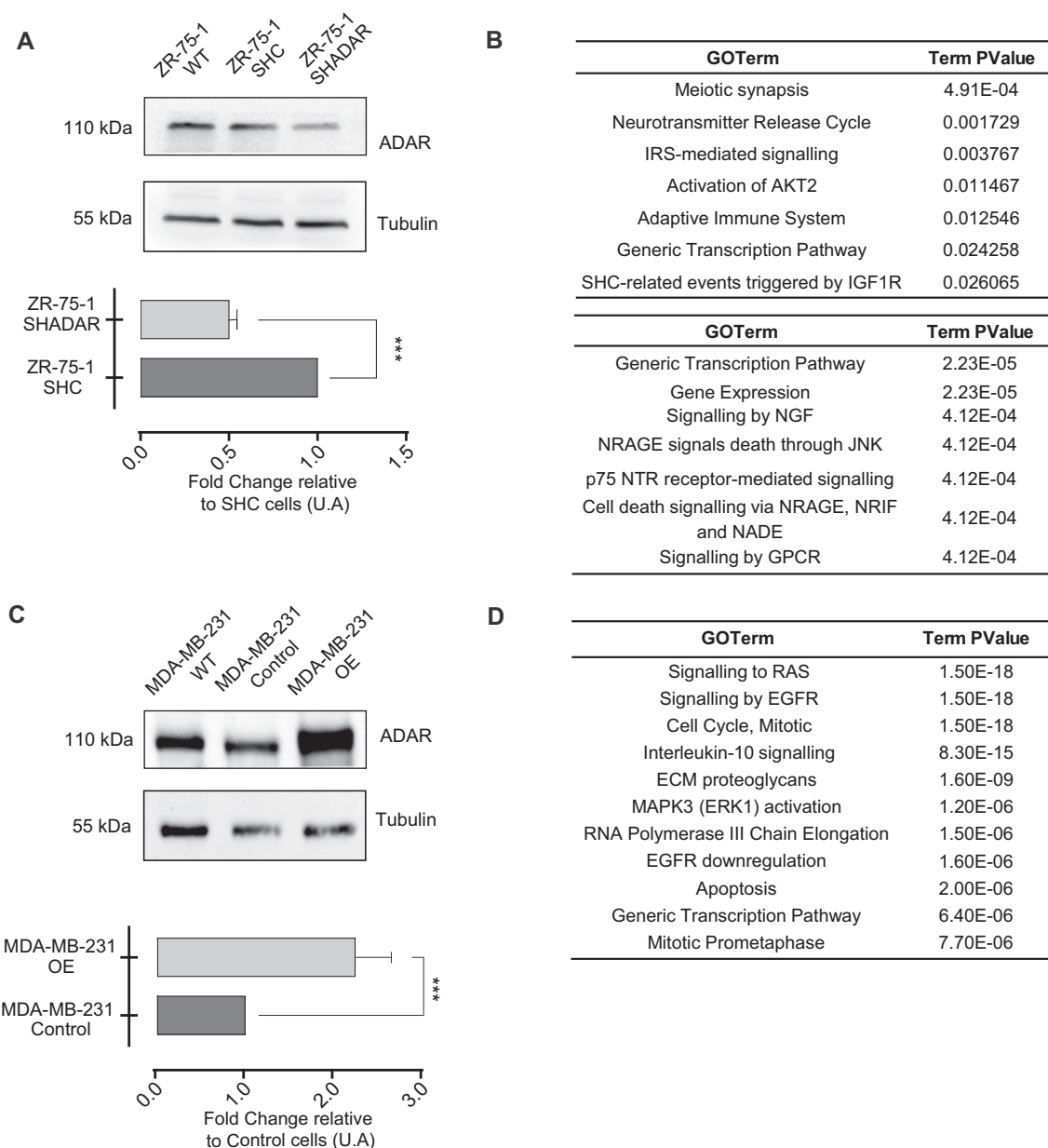


Fig. 2. ADAR is associated with pro-proliferative pathways. (A) ADAR protein expression in ZR-75-1 Wild type (WT), and ZR-75-1 cells expressing an scramble control shRNA (SHC) and an shRNA against ADAR1 (SHADAR). (B) Reactome pathway enrichment associated to the Differential expression analysis between ZR-75-1 SHC and ZR-75-1 SHADAR cells, showing the significant pathways down regulated (upper) and upregulated (lower) in SHADAR cells. (C) ADAR protein expression in MDA-MB-231 WT, Control and overexpressing ADARp110 (OE) cells. At the bottom, quantification of 3 independent assays. Two-way *t*-test, ***: $p < 0.001$. (D) Reactome pathway enrichment associated to the Differential expression analysis comparison between MDA-MB-231 Control and MDA-MB-231 ADAR OE cells.

triptolide in both SHC and SHADAR cells. 837 edited transcripts significantly changed their stability after triptolide treatment in ADAR knock-down cells, while 589 edited transcripts changed their stability in the SHC cells (Fig. 4C and Fig. 4E). Overall, 532 edited transcripts changed their levels in both treated cell types, with 378 of them presented at least 10% greater change on ZR-75-1 SHADAR cells compared to SHC cells (Fig. 4D, right panel). More importantly, for all the transcript shared in both comparisons, not edited and edited transcripts show a systematic, larger and significant reduction in their stability in those ADAR knock-down cells compared to the SHC cells treated during 16 h with triptolide, suggesting an important role of ADAR on mRNA stability (Fig. 4D, upper panel). Where, 309 out 387 edited transcripts presented a larger decrease in their stability on ZR-75-1 SHADAR cells compared to ZR-75-1 SHC cells (Fig. 4D, upper right panel); and 69 edited transcripts present in both comparisons displayed an increased

stability on the SHADAR background cells compared to the SHC cells (Fig. 4D, lower right panel). Moreover, 513 non-edited transcripts decreased at least 10% more in the SHADAR cells compared to control cells, while 209 non-edited transcripts shared in both comparisons displayed an increased stability on the SHADAR background cells compared to the SHC cells (Fig. 4D, upper left panel). Finally, a pathway enrichment analysis for those shared edited transcripts was performed (shown in the Venn diagram Fig. 4E), showing that transcript related to General transcription ($p = 4.308E-17$), Cell cycle ($p = 1.145E-5$), Regulation of p53 activity ($p = 1.1E-4$), among others like DNA Repair by Homologous Recombination change their stability (shown in Supplementary Material 8).

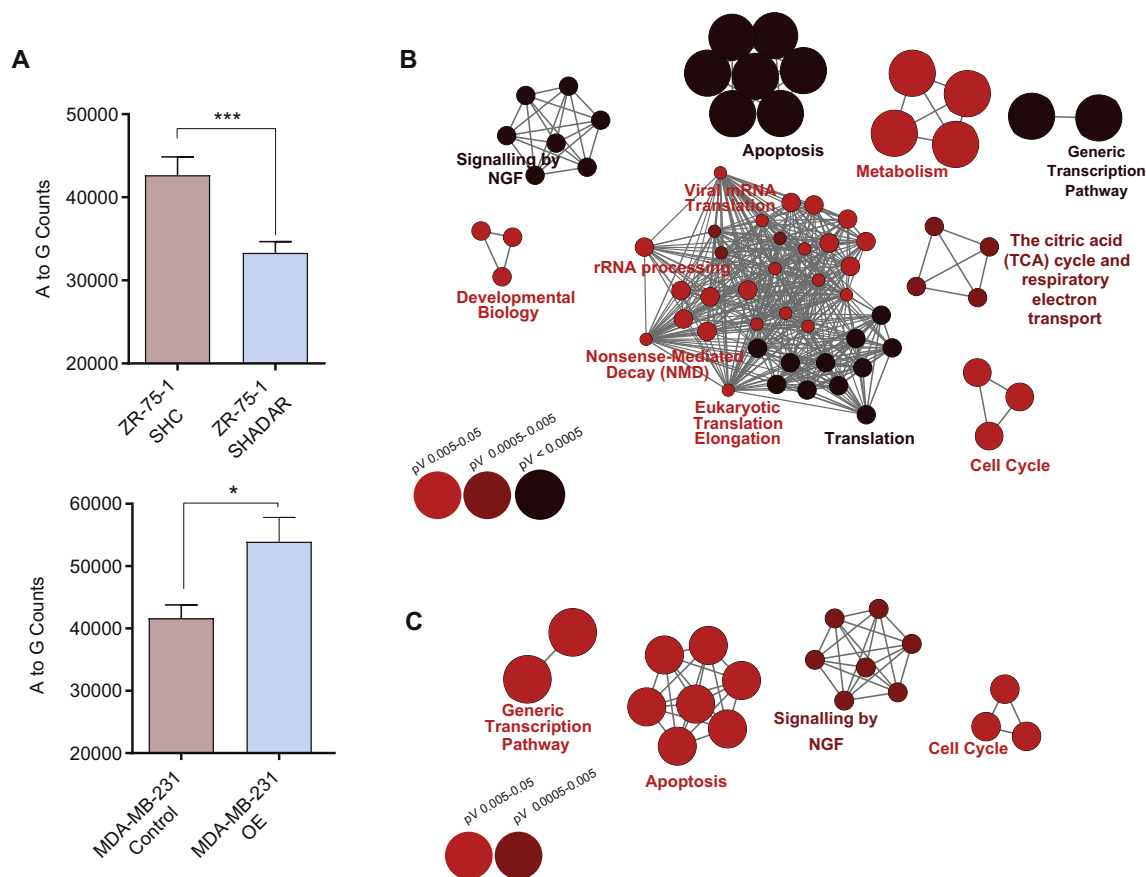


Fig. 3. ADAR activity significantly modifies transcripts related with cell cycle and apoptosis pathways. (A) A to G(I) differential count comparison between ZR-75-1 SHC and ZR-75-1 SHADAR cells (Upper) and MDA-MB-231 ADAR OE and MDA-MB-231 Control cells (Lower). *: $p < 0.05$, ***: $p < 0.001$, One-way *t*-test. (B) Reactome pathway enrichment for those significantly under edited sites in ZR-75-1 SHADAR compared to ZR-75-1 SHC cells. (C) Reactome pathway enrichment for those significantly over edited sites in MDA-MB-231 ADAR OE compared to Control cells. Only statistically different groups are displayed, using a Bonferroni step-down multiple comparison post-hoc test.

3.3. Role of ADAR in viability and apoptosis

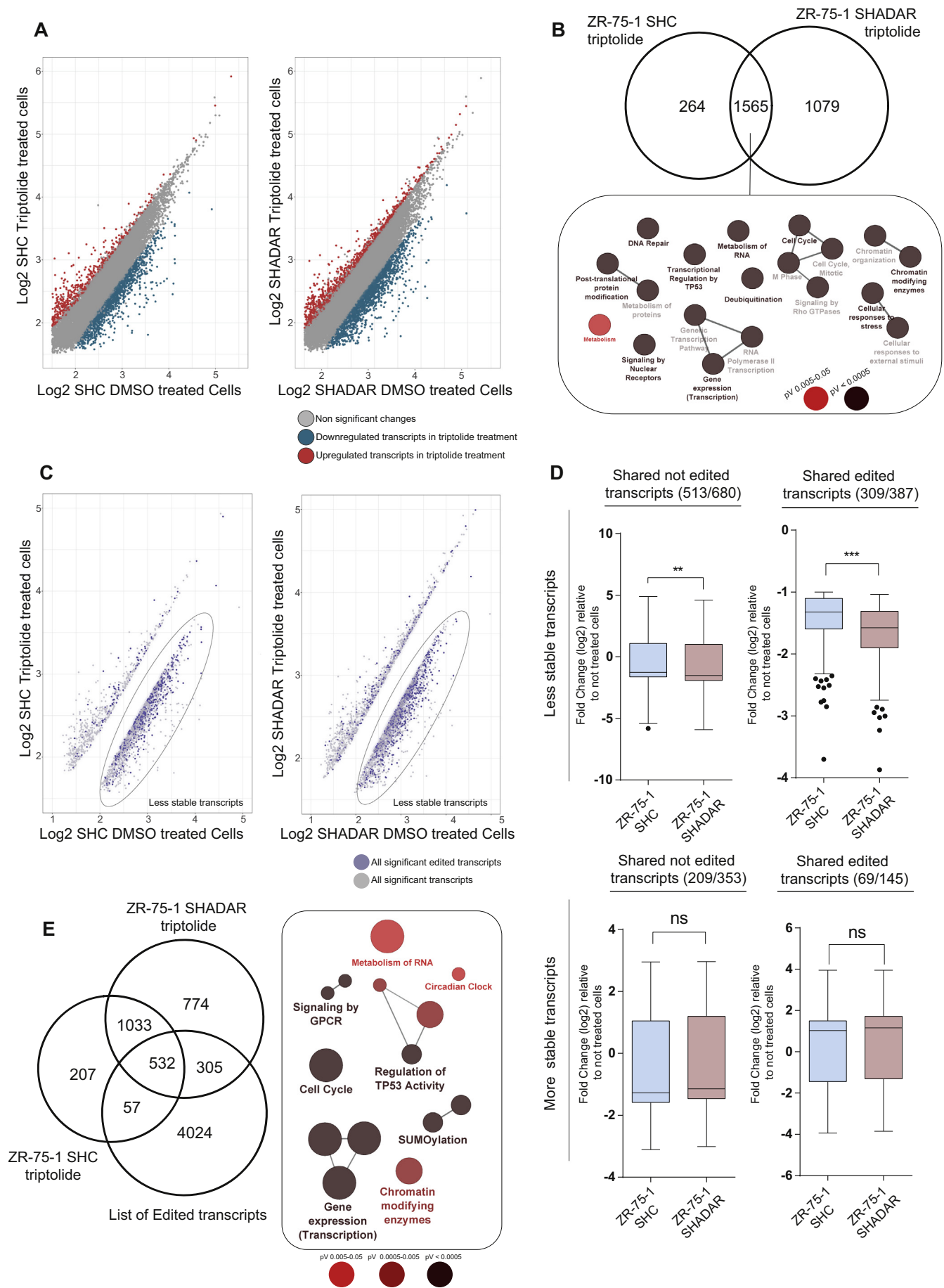
Overall, our results suggest that ADAR activity could impact the viability, DDR and apoptosis of breast cancer cells. Thus, we analyzed the viability of ZR-75-1 SHC and ZR-75-1 SHADAR cells and found that knock-down of ADAR1 decreased the viability and proliferation of the cells (Fig. 5A). Similar results were obtained ZR-75-1 using a different shRNA (Supplementary Fig. 2A-C) and MCF7 SHADAR cells (Supplementary Fig. 2D-F). Conversely, overexpression of ADARp110 in Hela and in MDA-MB-231 cells resulted in an increased proliferation rate (Supplementary Fig. 3). Cell cycle distribution was assessed in ZR-75-1 SHADAR and ZR-75-1 SHC cells, using PI and BrdU incorporation assays and FACS. ZR-75-1 SHADAR cells displayed a significant decrease in the G1 phase, and an increase in S phase (Fig. 5C and D). Considering the decreased proliferation and decrease in the proportion of cells in G1, without an increase in G2/M, the observed increased percentage of cells in the S phase might be reflecting an extended duration of the replication process. Alternatively, this could be partially explained by an increase in cell death. Thus, we analyzed the apoptotic activity of ZR-75-1 SHC and ZR-75-1 SHADAR cells and found that the ZR-75-1 SHADAR cells displayed a significantly increased activity of the intrinsic caspases 3/7 (Fig. 5E), and increased TUNEL staining (Fig. 5F), indicating an increased apoptosis in these cells. Together, these pieces of evidence corroborate previous insights, suggesting that ADAR knock-down would produce a decreased viability through an increase in apoptosis.

3.4. Role of ADAR on DNA damage response in breast cancer cells

Considering that ADAR is likely involved in the modulation of the expression of RNAs associated with the DNA damage response (DDR) (Fig. 4E and Supplementary Material 8), the DDR status of the ZR-75-1 SHADAR and SHC cells under basal conditions was analyzed. ZR-75-1 SHADAR cells displayed a significant decrease in the number of cells positive for γ H2AX staining (a measure of DNA damage) in comparison to SHC cells (Fig. 6A). Consistently, the active form of the DNA damage sensing protein ATR pS428 and Chk2 pT68 were decreased in ZR-75-1 SHADAR compared to control cells, while total levels of the proteins were not different (Figs. 6B-C). In the same way, the active form of P53 (pS15) was decreased in MCF7 SHADAR cells (Fig. 6D). This cell line expresses wild type P53 [53]. Accordingly, activity of P53 on a reporter promoter was decreased in MCF7 SHADAR cells, compared to MCF7 SHC cells (Fig. 6E). Altogether these results indicate that DDR is diminished in the ADAR knock-down breast cancer cells, suggesting a role for ADAR in the regulation of this response.

4. Discussion

In recent years, the participation of ADAR in the cancer progression has been established [17,45,54,55]. Particularly in breast cancer, ADAR expression and activity is significantly enhanced, showing that patients with an increased ADAR expression have a significantly lower overall survival and disease-free survival [19,24,48,49]. ADAR is a critical player in several RNA biology processes, contributing to transcriptome variability [19], mRNA stability [50], alternative splicing [5] and



(caption on next page)

Fig. 4. ADAR preferably regulates the mRNA stability of their targets. (A) Scatterplot comparing transcriptome differences between DMSO (vehicle) or triptolide treated ZR-75-1 SHC (left panel) or ZR-75-1 SHADAR (right panel) cells. Transcripts with p -adj 0.01 and Log2 fold change $< \pm 1$ were considered significant. (B) Venn diagram showing differential gene list intersection between ZR-75-1 SHC and ZR-75-1 SHADAR cells after triptolide treatment and the corresponding Reactome enrichment analysis for those mRNAs present in both lists. (C) Scatterplot displaying those significantly changed transcripts after DMSO (vehicle) and triptolide treated comparison on ZR-75-1 SHC (left panel) or ZR-75-1 SHADAR (right panel) background cells including that of edited transcripts between SHC and SHADAR cells. Transcripts with p -adj 0.01 and Log2fold change $< \pm 1$ were considered as significant. (D) Box and Whiskers plots comparing each pair of transcripts present in both treatment analysis and their stability changes for non-edited and edited transcripts. ***: $p < 0.003$, **: $p = 0.005$ ns: non-significant changes, Two-way t-test with Welch correction. (E) Venn diagram and Reactome enrichment analysis for those differentially edited with significant changes on their mRNA stability after triptolide treatment in ZR-75-1 SHADAR cells.

mRNA localization [55]. As the expression of ADAR positively correlates with the edition of its target transcripts [56], it is reasonable to expect that the increased expression pattern detected in breast cancer and other types of tumors [10,13,46] results in an increased edition of its targets. In agreement, we found that in breast cancer tissues, the mRNA ADAR expression and ADAR activity is significantly correlated with DDR, cell cycle and proliferation-related ontologies, suggesting a further relation of ADAR with those biological processes as is shown in Fig. 1. Indeed, after ADAR knock-down on ZR-75-1 cells we found a significant downregulation for mRNAs related to proliferative pathways and an increase of the expression of mRNAs associated with apoptosis process (Fig. 2). In addition, our results show an important number of those mRNAs edited by ADAR display a significant change in their stability. Our results suggest that after ADAR knock-down and triptolide treatment, DNA repair (regulation of P53), cell cycle, gene expression, among other pathways, suffer a significant decrease in their mRNA stability. On the contrary, mRNAs of genes associated with rRNA processing, mRNAs of genes involved in the surveillance through the nonsense-mediated decay and pro-apoptotic related mRNAs exhibited increased stability after ADAR knock-down (Fig. 4).

Previously, Wang et al. [50] showed that ADAR could modulate the stability and/or expression of their mRNA target through the modification of AU-rich elements at 3'UTRs, modifying the RNA-protein interactions with HuR, an RNA-binding protein that favors mRNA stability through the RNA. Interestingly, this work describes that ADAR is significantly co-expressed with cell cycle related pathways, suggesting that MCM4, MCM8 and ORC2, three critical genes involved in the replication process, are regulated by ADAR. Complementary to our results, Sakurai et al. [30], showed that after ADAR knock-down, apoptosis and DDR related mRNAs suffer expression/stability changes in a Staufen dependent manner, describing that *ATM* and *RAD51* mRNAs are protected from Staufen mediated decay under non-stressed conditions by ADAR.

Additionally to this findings and in agreement with previous reports showing a decreased viability in ADAR knock-down cells [25,29], we found an overall decrease of cell viability with a significant decrease in the viability of MCF7 and ZR-75-1 ADAR knock-down cells and an increased apoptosis for ZR-75-1 ADAR knock-down cells (Fig. 5). In agreement with our results, recently, it was reported that the interferon (INF)-induced double-stranded RNA-activated protein kinase (*PKR*) activates pro-apoptotic signalling after ADAR KO cells. We found deregulation of several transcripts associated with INF response in ADAR knock-down cells, including *PKR*. Thus, in our model, apoptosis could be mediated at least in part by this mechanism. In addition, ADARp110 transduced MDA-231 and HeLa ADAR Flp-In T-Rex induced cells showed a significant increase of cell viability, suggesting that ADAR is involved in those processes.

The emerging role of ADAR in cancer-related pathways such as DDR, was recently suggested by Guo et al. [27]. They noted that HEK293 ADARp150 overexpressing cells showed an increased expression of the proliferation and DDR related proteins, described by the authors as *PCNA* network. Also, Zhang et al. [28] described an increased fraction of editing sites on *MDM2* 3'UTR, a key regulator of p53 abundance, abolish the RNA-RNA interaction between *MDM2* and miR-200b, inhibiting the repression of this mRNA in breast cancer patients, suggesting an expanded model where most oncogenes, that have an

increased in cancer, suffer an unbalance regulation favoring cancer progression.

Interestingly, we found that ZR-75-1 SHADAR cells presented an overall lower DDR activity compared to control cells, with ADAR knock-down cells displaying a significant decrease in the number of γ H2AX foci, which could suggest a general decrease in the response of DNA damage in this model. Moreover, ZR-75-1 SHADAR presented a lower activation of pATR under non-stimulated or basal conditions. Additionally, MCF7 ADAR knock-down cells showed a lower phosphorylation on Ser15, suggesting a lower activity of the p53 (Fig. 6). In that line, Jiang et al. [29] recently described that ADAR expression and activity favor the cell cycle progression by modulating miRNA maturation but also modifying the RNA-RNA interaction between *MDM2* and miR-155, in hematopoietic stem cells and chronic myelogenous leukemia progenitors, respectively. Given the complexity of ADAR function as a RNA binding protein with a dynamic and widely distributed function, as it is the editing function, it is reasonable to expect that the phenotype behind ADAR manipulation would be the result of a cumulative and complex effect on a network of genes and mechanisms under ADAR regulation. Thus, the existence of a significant number of ADAR-edited targets intimately related to cancer pathways such as apoptosis, cell cycle, and DDR opens an intricate and novel role for ADAR in tumor progression. However, further insights are necessary to understand and explain the specific components of these networks behind ADAR's role on cancer progression and how ADAR is involved in apoptosis, cell cycle and DDR.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbamcr.2020.118716>.

CRediT authorship contribution statement

Eduardo A. Sagredo: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Alfredo I. Sagredo:** Investigation, Formal analysis, Data curation, Writing - review & editing. **Alejandro Blanco:** Investigation, Writing - review & editing. **Pamela Rojas De Santiago:** Investigation, Writing - review & editing. **Solange Rivas:** Investigation, Writing - review & editing. **Rodrigo Assar:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - review & editing. **Paola Pérez:** Formal analysis, Data curation, Writing - review & editing. **Katherine Marcelain:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Ricardo Armisén:** Writing - original draft, Writing - review & editing.

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The results shown here are in part based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>, and the Molecular Taxonomy of Breast Cancer International Consortium

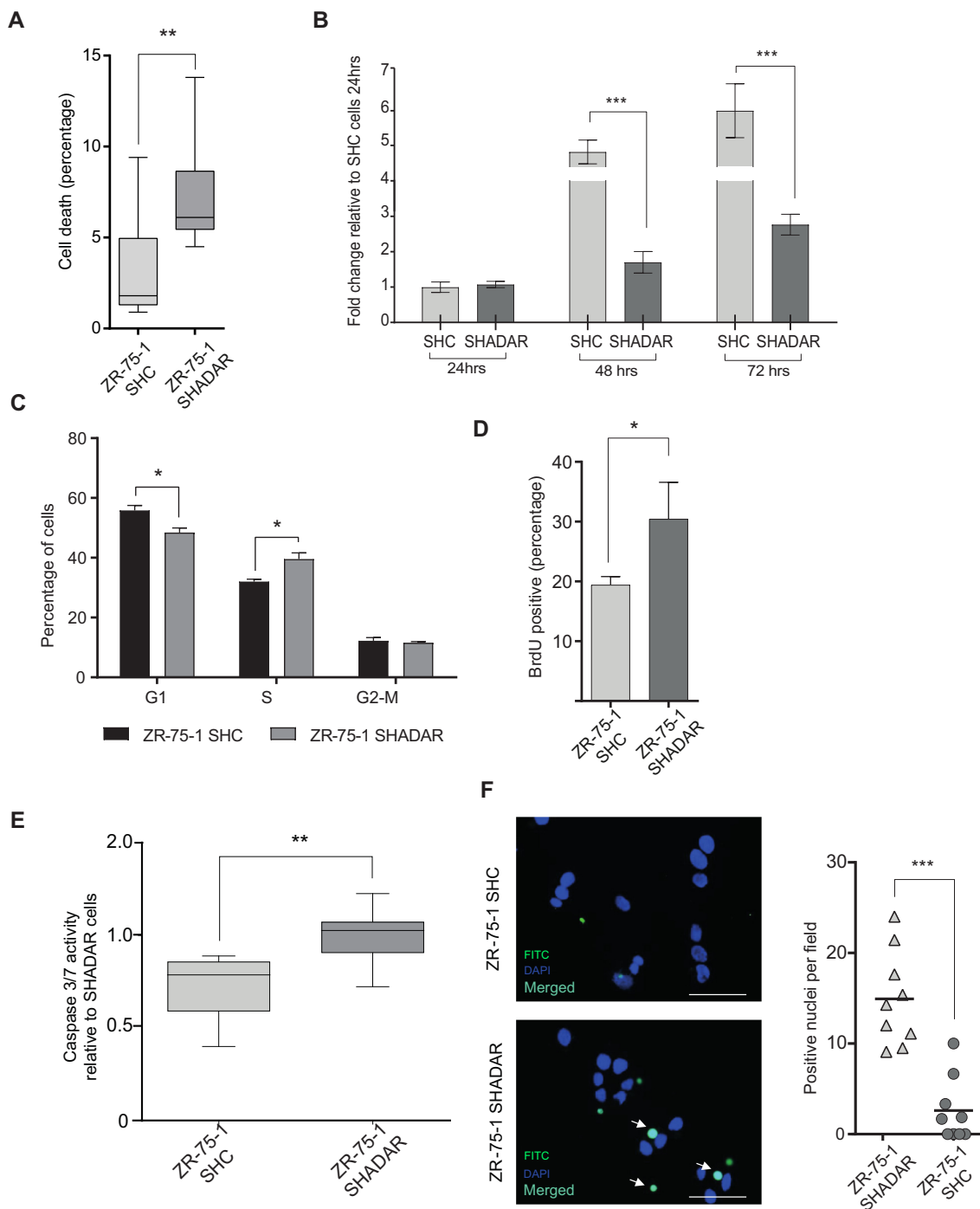


Fig. 5. ADAR is associated with apoptotic and viability phenotype. (A) Cell death was evaluated by trypan blue staining in ZR-75-1 SHC and ZR-75-1 SHADAR cells. Results are expressed as dead cell percentage. (B) Cell proliferation in ZR-75-1 SHC and ZR-75-1 SHADAR cells assessed using MTS assay. Three independent assays were performed in triplicate and proliferation was assessed 24–72 h after culturing. Mean ± SEM are shown. (C) Cell cycle determination on ZR-75-1 SHC and ZR-75-1 SHADAR cells using propidium iodide and flow cytometry. (D) BrdU incorporation assay on ZR-75-1 SHC and SHADAR cells. Positive cells that incorporated BrdU were detected using a FITC-conjugated anti-BrdU antibody. (E) 3/7 intrinsic Caspase activity assay was determined by using a luminescence assay in ZR-75-1 SHC and ZR-75-1 SHADAR. (F) TUNEL fluorometric assay in ZR-75-1 SHC and ZR-75-1 SHADAR cells. Representative images from each condition ZR-75-1 SHC (upper) and ZR-75-1 SHADAR (lower) showing merged images. Scale bar 20 μm. Positive nuclei per field were quantified in at least three independent experiments for each condition. For all assays, at least three independent experiments were performed. Significance was determined by Two-way t-test. *: p < .05, **: p < 0.01, ***: p ≤ 0.001.

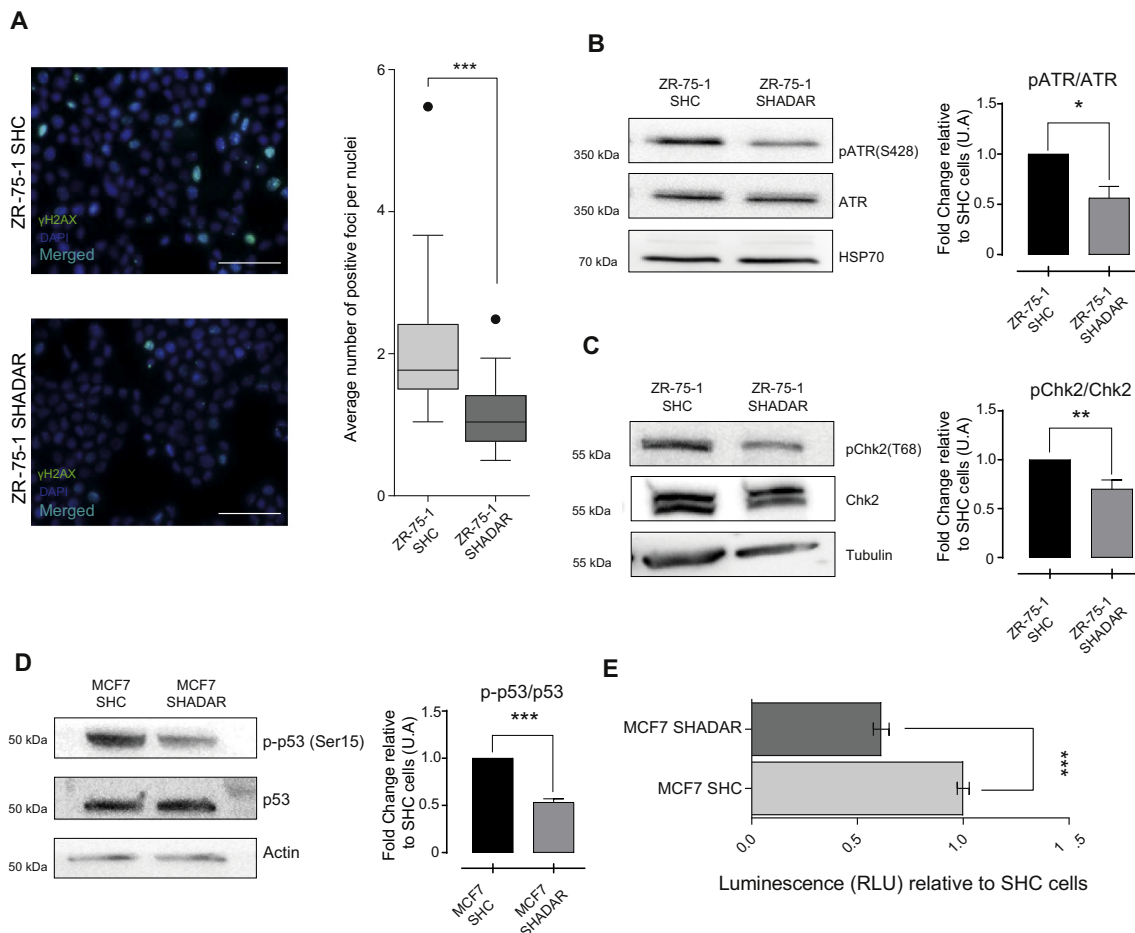


Fig. 6. DNA damage response is decreased in breast cancer cells with lower expression of ADAR1. (A) ADAR knock-down ZR-75-1 cells show a decreased number of γ H2AX positive nuclei. Representative images of nuclear γ H2AX detected by immunofluorescence in ZR-75-1 SHC (upper) and ZR-75-1 SHADAR cells (lower). Graph shows arbitrary units of fluorescence of γ H2AX nuclear ratio quantified in at least three independent experiments. Mean \pm SEM are shown. Scale bar 20 μ m. Two-way t-test $***p \leq .001$. (B) ZR-75-1 SHADAR cells show a lower rate of ATR/ATR pS428, relative to ZR-75-1 SHC cells. No changes in total ATR were detected. (C) ZR-75-1 SHADAR cells show a decreased phosphorylation of Chk2 at Thr68, relative to ZR-75-1 SHC cells. No changes in total Chk2 were detected. (D) MCF7 SHADAR cells show a decreased phosphorylation of p53 at Ser15, relative to ZR-75-1 SHC cells. No changes in total p53 were detected. In B-D, representative western blots and densitometries of three independent experiments. (E) MCF7 SHADAR cells show a significant decrease in the activation of a p53 reporter compared to MCF7 SHC cells. All experiments were performed at least three times. Mean \pm SEM are shown. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Two-way t-test.

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Declaration of competing interest

RA, RAs, PP, EAS, AB, AIS and PR were Pfizer Chile employees.

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