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# **The Arp2/3 inhibitory protein Arpin inhibits homology-directed DNA repair**

Running title: Arpin in DNA repair

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## **Abstract**

Arpin, an Arp2/3 inhibitory protein, inhibits lamellipodial protrusions and cell migration. Arpin expression is lost in tumor cells of several cancer types. Here we analyzed expression levels of Arpin and various markers using Reverse Phase Protein Array (RPPA) in human mammary carcinomas. We found that Arpin protein levels were correlated with those of several DNA damage response markers. Arpin-null cells display enhanced clustering of double strand breaks (DSBs) when cells are treated with a DNA damaging agent, in line with a previously described role of the Arp2/3 complex in promoting DSB clustering for homologous DNA repair (HDR) in the nucleus. Using a specific HDR assay, we further showed that Arpin depletion increased HDR efficiency two-fold through its ability to inactivate the Arp2/3 complex. Arpin thus regulates both cell migration in the cytosol and HDR in the nucleus, suggesting that loss of Arpin expression coordinates enhanced cell migration with up-regulated DNA repair, which is required when DNA damage is induced by active cell migration.

**Keywords:** Arpin, Arp2/3, DNA repair, double-strand breaks, homology-directed repair.

**Abbreviations:** BFP, blue fluorescent protein; NHEJ, Non-Homologous End Joining; DSB, double-strand break; GFP, green fluorescent protein; HDR, homology-directed repair; MRN, Mre11-Rad50-Nbs1; NCS, Neocarzinostatin; NPF, Nucleation Promoting Factor; RPPA, Reverse Phase Protein Array.

## Introduction

Membrane remodeling driven by branched actin polymerization is crucial for numerous cell processes, including cell migration, shape control and intracellular traffic (Papalazarou & Machesky, 2021). The Arp2/3 complex is the only machinery in the cell capable of polymerizing branched actin and its activity is tightly regulated by specifically localized activators, called Nucleation Promoting Factors (NPFs) (Molinie & Gautreau, 2018; Pollard, 2007). At the leading edge of the migrating cell, Arp2/3 is activated by the WAVE complex to create membrane protrusions called lamellipodia. The stability of lamellipodia is regulated by the Arp2/3 inhibitory protein Arpin (Dang et al., 2013). Specifically, Arpin regulates the lifetime of lamellipodia, which in turn, controls cell migration persistence and allows the cell to change the direction of its movement (Dang et al., 2013; Gorelik & Gautreau, 2015). The interaction of Arpin with Tankyrases 1 and 2 has also been shown to participate in the regulation of migration persistence (Simanov et al., 2021). There are, thus, two independent pathways, whereby Arpin regulates the persistence of cell migration.

In addition to its role in cell migration, new Arpin functions, relying on its inhibitory activity towards the Arp2/3 complex, have been uncovered. For example, Arpin participates in the regulation of cell proliferation (Molinie et al., 2019). In untransformed cells, the network of cortical branched actin generated by the Arp2/3 complex controls cell cycle progression by integrating various extracellular stimuli. Furthermore, because of its Arp2/3 inhibitory role, Arpin is important for the regulation of actin polymerization during phagocytosis in macrophages (Jubrail et al., 2020) and for maintenance of the integrity of the intestinal epithelial barrier (Chávez-Paredes et al., 2021). Recently, Arpin was found to control shape sensing in dendritic cells through the regulation of a perinuclear branched actin network (Alraies et al., 2024).

The Arp2/3 complex has other functions in the nucleus. Arp2/3 was first shown to regulate gene transcription by promoting RNA polymerase II clustering (Wei et al., 2020; Yoo, Wu, & Guan, 2007). More recently, it has been implicated in DNA repair through the ability of branched actin networks to move and cluster double strand breaks (DSBs) for repair (Caridi et al., 2018; Schrank et al., 2018). This role in DNA repair appears important both in S-phase during replicative stress (Lamm et al., 2020; Nieminuszczy et al., 2023; Palumbieri et al., 2023) and in G2 when homology-directed repair (HDR) is enabled in the presence of a newly replicated DNA template (Schrank et al., 2018). Defective branched actin, like DNA damage, activates the p53 tumor suppressor similar to DNA damage and leads to genome instability (Haarer, Theodore, Guo, Frier, & Campellone, 2023; Rodriguez-Pastrana, Birli, & Coutts, 2023).

The inhibition of the Arp2/3 complex is a key cellular function attributed to Arpin. Strikingly, Arp2/3 is frequently upregulated in tumors and associated with poor prognosis in many cancers (Molinie & Gautreau, 2018). In contrast, Arpin is downregulated in breast, gastric and pancreatic

cancers (Y. Li et al., 2017; Liu et al., 2016; Lomakina et al., 2016; Zhang et al., 2018) and its downregulation is associated with poor prognosis in breast cancer (Lomakina et al., 2016). Here we report that Arpin levels in mammary carcinomas correlate with those of several key players in DNA damage and uncover a novel function of Arpin in HDR of DSBs through its ability to inhibit Arp2/3 activity.

## **Materials and methods**

### ***Cell culture and drugs***

The 293T cells were maintained in DMEM medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific). MCF10A cells were maintained in DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 5% horse serum (Sigma, Hongkong, China), 100 ng/mL cholera toxin (Sigma), 20 ng/mL epidermal growth factor (Sigma), 0.01-mg/mL insulin (Sigma), 500 ng/mL hydrocortisone (Sigma), and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific). Cells were incubated at 37°C in 5% CO<sub>2</sub>. All cells and stable clones were routinely tested for mycoplasma. The 293T BFP+ cell line was kindly provided by Dr. Alberto Ciccia, Columbia University Medical Centre, New York, USA. For the experiments with DSB induction, cells were treated with Neocarzinostatin (Sigma-Aldrich) at 200 ng/mL for 1 h. For Arp2/3 inhibition, CK-666 (Merck) was used.

### ***siRNA transfection***

Arpin was targeted by individual siRNAs at the final concentration of 25 nM (ON-TARGETplus, Dharmacon): Arpin #1 GUGGAUGUAUCUCGGCACA and Arpin #2 AUGGCAGGAAGGAGCGCUA. The control siRNA was the non-targeting siRNA designed by Dharmacon. The genes encoding the Arp2/3 activators were knocked down using in vitro generated sets of siRNAs targeting large portions of their ORF called esiRNAs (Sigma). An esiRNA corresponding to firefly luciferase was used as a control. The 293T BFP+ cells were transfected using Lipofectamine RNAiMAX according to the recommended reverse transfection protocol (Thermo Fisher Scientific).

### ***Plasmids and cell lines***

The C-terminal region of 53BP1 isoform 1, encoding amino acids 1224-1716, was amplified from pTRIPSSFFV-EGFP-53BP1 plasmid (a kind gift from Nicolas Manel, Institut Curie, Paris, France) and subcloned into a homemade plasmid, MXS AAVS1L SA2A Puro bGHpA EF1Flag GFP Blue2 SV40pA AAVS1R, between FseI and AscI sites. The obtained plasmid was transfected into parental MCF10A cells and the MCF10A *ARPIN*<sup>-/-</sup> clone together with two TALENs targeting the AAVS1 site as previously described (Molinie et al. 2019) using Lipofectamine3000 (Thermo Fisher Scientific), according to a forward transfection protocol. Stable clones were selected with 1 µg/mL of puromycin (Invivogen), single clones were isolated by cloning rings, expanded, and characterized using Western blot. The sgRNA-Cas9 and GFP-donor plasmids were kind gifts from Dr. Alberto Ciccia, Columbia University Medical Centre, New York, USA.

### ***Reverse Phase Protein Array and tumor samples***

Reverse Phase Protein Array (RPPA), a miniaturized ‘dot-blot’ immunoassay, was performed as previously described (Rondeau et al., 2015). Overall, 233 biopsy samples from patients with unilateral invasive primary breast tumors treated at the Institut Curie-Hôpital René Huguenin (Saint-Cloud, France) from 1978 to 2008 have been analyzed. These samples were previously analyzed by our group and included in a previous report (Lomakina et al., 2016). Since 2007, patients entering this institution have provided their approval by signing in informed consent. This study was approved by the local ethics committee (Breast Group of René Huguenin Hospital). Specific antibodies used in this study are indicated in the Table S1.

### ***HDR assays with the reporter cell line***

To examine the levels of Cas9-induced HDR upon Arpin, Arp2/3 or Arp2/3 activators depletion, 293T BFP+ cells were reverse transfected with non-targeting siRNA/esiRNA or corresponding targeting siRNA/esiRNA; the next day cells were transfected with sgRNA-Cas9 plasmid targeting BFP with or without GFP-donor plasmid using jetPEI according to the manufacturer’s protocol. CK-666 was added to cells 6 h before the transfection of the sgRNA-Cas9-GFP donor. The cells were collected 3 days after transfection and 100 000 cells per condition were analyzed by flow cytometry using a Guava easyCyte system (Millipore).

### ***Western blot and antibodies***

Anti-Arpin home-made rabbit polyclonal antibodies were previously described (Dang et al. 2013). Following commercial antibodies were used: mAb Tubulin (clone DM1A, Sigma-Aldrich), mAb GFP (13.1 and 7.1 clones, Roche), ARPC2 (#07–227, Millipore). To verify the expression of selected markers from the RPPA experiment, MCF10A WT and ARPIN<sup>-/-</sup> cell lysates were probed with the same antibodies as those used in the RPPA, except for Phospho-p44/42 MAPK (#9101S, Cell Signaling). 293T BFP+ and MCF10A cells were lysed in RIPA buffer and analyzed using Western blot. SDS-PAGE was performed using NuPAGE 4–12% Bis-Tris gels (Life Technologies). Nitrocellulose membranes were developed with alkaline phosphatase-coupled antibodies (Promega) and NBT-BCIP as substrates (Promega).

### ***Immunofluorescence***

For immunofluorescence assay, MCF10A cells expressing GFP-53BP1-Cter were seeded on glass coverslips coated with 20 µg/mL bovine fibronectin (Sigma) for 1 h at 37°C in PBS. Then, cells were fixed with 4% paraformaldehyde and incubated with DAPI (Thermo Fischer Scientific). Imaging was performed using an Axio Observer microscope (Zeiss, Jena, Germany) with the 63×/1.4 oil immersion objective.

### ***Image and statistical analyses***

GFP-53BP1-Cter foci were automatically quantified using a custom-made ImageJ macro script: first, a nucleus mask was created using thresholding, then foci masks were generated with thresholding and watershed. Using this binary mask, the number of individual foci was automatically counted in each nucleus. In a given nucleus, the intensity of foci was determined by measuring fluorescence intensity on the GFP-53BP1 channel of each area corresponding to an individual focus on the foci mask. Foci number is represented as the mean  $\pm$  SD. The mean intensity of individual foci is represented as the median. Statistical significance was assessed using two-tailed Mann-Whitney test.

The percentage of GFP+ cells was normalized to that of control cells (non-targeting siRNA) in each independent experiment ( $n = 3$ ) and represented as the mean  $\pm$  SEM, paired  $t$  test was used to assess statistical significance. Relationships between Arpin protein levels and those of 61 other targets from RPPA experiments were identified as previously described (Rondeau et al. 2015), using the Spearman's rank correlation test. A p-value of less than 0.05 was considered statistically significant.



## Results

### *Arpin protein levels correlate with those of DNA damage response markers*

We had previously analyzed the downregulation of Arpin at the mRNA and protein levels in mammary carcinomas in a retrospective cohort of 454 patients (Lomakina et al., 2016). Out of 454 tumors, 233 biopsy samples were available to produce extracts that were automatically spotted on microscopy slides. These slides were incubated with various high-quality antibodies to produce quantitative signals in a miniaturized “dot-blot” assay, referred to as RPPA. Because the same samples were used for various projects, we examined the correlation of Arpin protein levels with signals previously obtained with 61 antibodies targeting major receptors and effectors signaling growth, proliferation, cell cycle, apoptosis, cytoskeleton reorganization, migration/invasion, and DNA repair (Figure 1A). Out of the 61 markers, Arpin levels were well correlated with those of eight, six of which were related to the DNA repair (Spearman’s correlation coefficient  $r$  of 0.189 or above in absolute value; Table S1).

Arpin levels were negatively correlated with those of phosphorylated histone H2AX ( $r=-0.315$ ,  $p=0.0000034$ ; Figure 1B), a marker of the DNA DSBs (Mah, El-Osta, & Karagiannis, 2010). A significant negative correlation was observed with levels of tumor suppressor protein p53, a master inducer of the DNA damage response, and its phosphoforms on Ser15 and Ser392 (p53:  $r=-0.280$ ,  $p=0.000032$ ; p53 phosphoSer15:  $r=-0.263$ ,  $p=0.000086$ ; p53 phosphoSer392:  $r=-0.247$ ,  $p=0.00021$ ). Serine 15 is the primary target of the DNA damage response on the p53 protein and is phosphorylated by both the ATM and ATR protein kinases. Phosphorylation of p53 at Ser392 influences the growth suppressor function, DNA binding, and transcriptional activation of p53.

Markers of the DNA damage response involved in the early steps of the repair process were examined among the 61 markers. Neither the levels of Ku80, belonging to canonical Non-Homologous End Joining (NHEJ) pathway, nor those of NHEJ promoting factor, 53BP1 protein, were correlated with Arpin levels. In contrast, two subunits of the Mre11-Rad50-Nbs1 (MRN) complex, which is recruited to DSBs shortly after DNA damage to initiate DNA end resection (Syed & Tainer, 2018), showed a significant positive correlation with Arpin levels (Rad50:  $r=+0.286$ ,  $p=0.000021$ ; Mre11:  $r=+0.189$ ,  $p=0.0039$ ; Figure 1B). Surprisingly, there was no correlation with the levels of the third subunit of MRN complex, the Nbs1 protein. Similarly, there was no correlation with the levels of the regulator of DNA DSB signaling recruited by Nbs1, ataxia telangiectasia mutated (ATM) kinase (Lee & Paull, 2007). Together, these correlation data suggested that Arpin could be involved in the DNA damage response signaling.

Regarding the other signaling proteins tested, there was a significant positive correlation detected with the amounts of p42/p44 MAP kinases (ERK1/2;  $r=+0.306$ ,  $p=0.000006$ ) and with the product of the *NF2* tumor suppressor gene, Merlin ( $r=+0.315$ ,  $p=0.0000033$ ). Nevertheless, there was

neither a correlation with p42/p44 MAP kinases activated state (phosphorylation on Thr202 and Tyr204), nor with the immediately upstream kinases, MAPKK, MEK1, and MEK2, or their active phosphoforms, which activate p42/p44 MAP kinases by dual phosphorylation.

We then attempted to validate observed correlations using previously established *ARPIN*<sup>-/-</sup> MCF10A clones (Molinie et al., 2019). We assessed protein levels of selected RPPA markers that were correlated with Arpin protein levels in parental MCF10A and *ARPIN*<sup>-/-</sup> cells (Figure S1). Several markers that showed no correlation in the RPPA experiment were also included in the assessment. Strikingly, Arpin depleted cells demonstrated no significant change in protein levels of markers that were correlated with Arpin levels in the RPPA experiment, suggesting that there is co-regulation in tumors, but no direct causal link in the untransformed mammary epithelial cell line MCF10A. Nonetheless, we decided to examine whether the correlation of Arpin levels with those of DNA damage markers indicated a functional link between Arpin and DNA damage in non-transformed cells.

#### ***Arpin depleted cells exhibit increased DSB clustering***

To obtain evidence of Arpin involvement in the DNA repair process, we used a reporter system to visualize DNA DSB foci. We isolated parental MCF10A and *ARPIN*<sup>-/-</sup> cells stably expressing the C-terminal fragment of 53BP1 protein, which is sufficient for its recruitment to the DSB's sites but unable to promote NHEJ, called hereafter 53BP1-Cter, described in (Gentili et al., 2019, Figure 2A). The plasmid expressing GFP-53BP1-Cter fusion was successfully integrated at the AAVS1 site of MCF10A cells and the fusion protein showed nuclear diffused localization with a few bright spots corresponding to the sites of naturally occurring DSBs and DNA repair. To increase DSBs, we used the DNA damaging agent Neocarzinostatin (NCS). Upon NCS treatment for one hour, the number of DSB foci was dramatically increased, while their intensity decreased, indicating that DSBs did not cluster as efficiently upon NCS treatment as before (Figure 2B). *ARPIN*<sup>-/-</sup> cells exhibited a slightly higher number of DSB foci than control cells in control conditions and showed inconsistent variations upon NCS treatment (Figure S2). However, the most important effect was a consistent increase in the intensity of DSB foci in *ARPIN*<sup>-/-</sup> cells upon NCS treatment, suggesting that Arpin depletion might increase DSB clustering in line with the critical role of Arp2/3 in mediating DSB clustering in HDR (Schrank et al., 2018).

#### ***Arpin depletion increases the level of CRISPR-mediated homology-directed repair***

To specifically evaluate the role of Arpin in HDR, we used a fluorescence-based reporter system based CRISPR/Cas9 that was previously described (Nambiar et al., 2019). In brief, we used

293T cells stably expressing blue fluorescent protein (BFP) that could be converted into green fluorescent protein (GFP) by the substitution of one amino acid. This substitution is mediated by CRISPR/Cas9-induced cleavage of the BFP sequence and following its repair via homologous recombination using a donor plasmid containing the GFP template sequence. Then, the percentage of GFP-positive cells is monitored using flow cytometry, which determines the efficiency of HDR-mediated repair of DSBs (Figure 3A).

Using this reporter system, we assessed the HDR efficiency under Arpin depletion. First, using the control condition without the GFP donor plasmid, we established the sensitivity thresholds to eliminate a nonspecific signal. We then depleted Arpin using two different siRNAs in 293T BFP+ cells and transfected them with the guide RNA/Cas9 and GFP-expressing plasmids. We then measured the proportion of GFP-positive cells (Figure 3B). Arpin depletion led to a two-fold increase in HDR level. This effect was reproducible and significant for both siRNAs (Figure 3C). These data indicate that Arpin plays a role in the negative regulation of this repair pathway, possibly through its interaction with Arp2/3. To validate this hypothesis, we first performed the CRISPR-mediated HDR efficiency assay under depletion of the ARPC2 subunit of the Arp2/3 complex. ARPC2 depletion decreased HDR efficiency two-fold (Figure 3D). We then employed CK-666 to obtain chemical inhibition of the Arp2/3 complex activity. CK-666 treatment similarly decreased HDR efficiency and abolished the increased HDR induced by Arpin depletion (Figure 3E), indicating that increased HDR efficiency associated with Arpin depletion was due to Arp2/3 inhibition. We also conducted a miniscreen targeting known Arp2/3 activators such as WAVE and WASH complexes and other NPFs. However, the depletion of none of these activators resulted in a decrease in HDR efficiency as pronounced as the one obtained upon Arp2/3 inactivation. Only WASH complex depletion slightly reduced HDR efficiency, but it was unable to abrogate the enhanced HDR efficiency associated with Arpin depletion (Figure S3).

## Discussion

The Arp2/3 inhibitory protein Arpin was previously shown to inhibit cell migration persistence (Dang et al., 2013; Simanov et al., 2021) and to be downregulated in several cancers (T. Li et al., 2017; Liu et al., 2016; Lomakina et al., 2016; Zhang et al., 2018). Here, we analyzed a previously obtained RPPA dataset from breast tumor samples to examine the correlation of Arpin protein levels with those of several markers of cell growth, proliferation, invasion, cytoskeleton reorganization and DNA repair. Our results identified several markers that were well correlated with Arpin levels. Consistent with the fact that Merlin, like Arpin, was reported to be downregulated in breast cancer (Morrow et al., 2011), Merlin protein levels were positively correlated with those of Arpin. Furthermore, we found a significant correlation between Arpin levels and markers of DNA

damage response. Importantly, Arpin levels were negatively correlated with those of universal DNA damage response markers Phospho-H2AX, p53 and its phosphoforms, suggesting that decreased Arpin levels were associated with the activation of the p53-dependent DNA damage response pathway in tumor cells. In contrast, Arpin levels were positively correlated with those of Rad50 and Mre11. These two proteins are involved in DNA cleavage prior to HDR mediated repair, suggesting a possible defect in HDR in tumor cells displaying reduced Arpin levels.

In Arpin depleted cells, we found increased levels of HDR, in line with the previously identified role of Arp2/3 in this process (Schrank et al., 2018). It is likely that Arpin controls HDR through its inhibition of the Arp2/3, since NCS-induced increase of DSB foci intensity, an indication of DSB clustering, depends on Arp2/3. Moreover, enhanced HDR induced by Arpin depletion also depends on Arp2/3 activity. The specific NPF that Arpin counteracts in its novel role in HDR remains unclear. In cell migration, Arpin was found to counteract the WAVE complex (Krause & Gautreau, 2014). However, multiple NPFs from the four major NPF families, WASP, WAVE WASH and JMY, have previously been implicated in DSB repair (Caridi et al., 2018; Rodriguez-Pastrana et al., 2023; Schrank et al., 2018). We were unable to identify a single NPF that would decrease HDR to the same level as Arp2/3 inhibition. It was reported that WAVE and WASH must be simultaneously inactivated to prevent DSB movement to the nuclear periphery in *Drosophila* cells (Caridi et al., 2018). Arpin is a competitive inhibitory protein that blocks one of the two essential NPF binding sites on the Arp2/3 (Fregoso et al., 2022). Therefore Arpin has the potential to block any NPF-mediated Arp2/3 activation.

The migrating cell can be subjected to the deformation or even disruption of the nuclear envelope, which results in DNA damage (Denais et al., 2016; Irianto et al., 2017; Raab et al., 2016; Sahu et al., 2021). The DNA damage requires efficient repair for the cell to maintain migration and proper functioning. Arpin depletion renders cell migration more persistent (Dang et al., 2013) but also, according to our results, is accompanied by increased HDR-mediated DNA repair. The slightly increased number of DSB foci in Arpin-null cells that we report here is also consistent with the fact that these cells migrate more actively than parental cells. Given the role that branched actin polymerization plays in cell migration and its emerging functions in DNA repair processes, Arpin regulation might be a way through which the cell coordinates the two phenomena.

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## **Author contributions**

GS and AMG conceived and designed the study. GS performed all cell biology experiments, analyzed data, and wrote the original draft of the paper. NR and SR performed image and statistical analysis of the GFP-53BP1-Cter experiments. LK, SV, TD and IB performed the RPPA experiments. AMG coordinated the work, acquired the funding, and wrote the final version of the manuscript. All authors have read and approved the final manuscript.

## **Conflicts of interest**

The authors declare no conflicts of interest.

## **Appendix A. Supplementary materials**

Table S1. RPPA correlation results.

Figure S1. Examination of selected RPPA markers in parental MCF10A and Arpin null cells.

Figure S2. Quantification of DSB foci in parental MCF10A and Arpin null cells.

Figure S3. CRISPR-mediated HDR efficiency in 293T BFP reporter cells under depletion of various Arp2/3 activators.

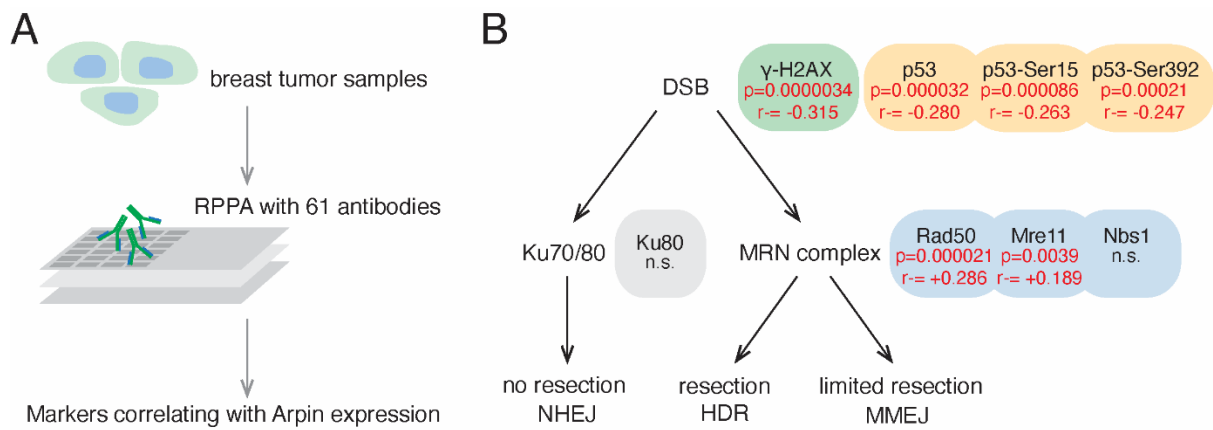
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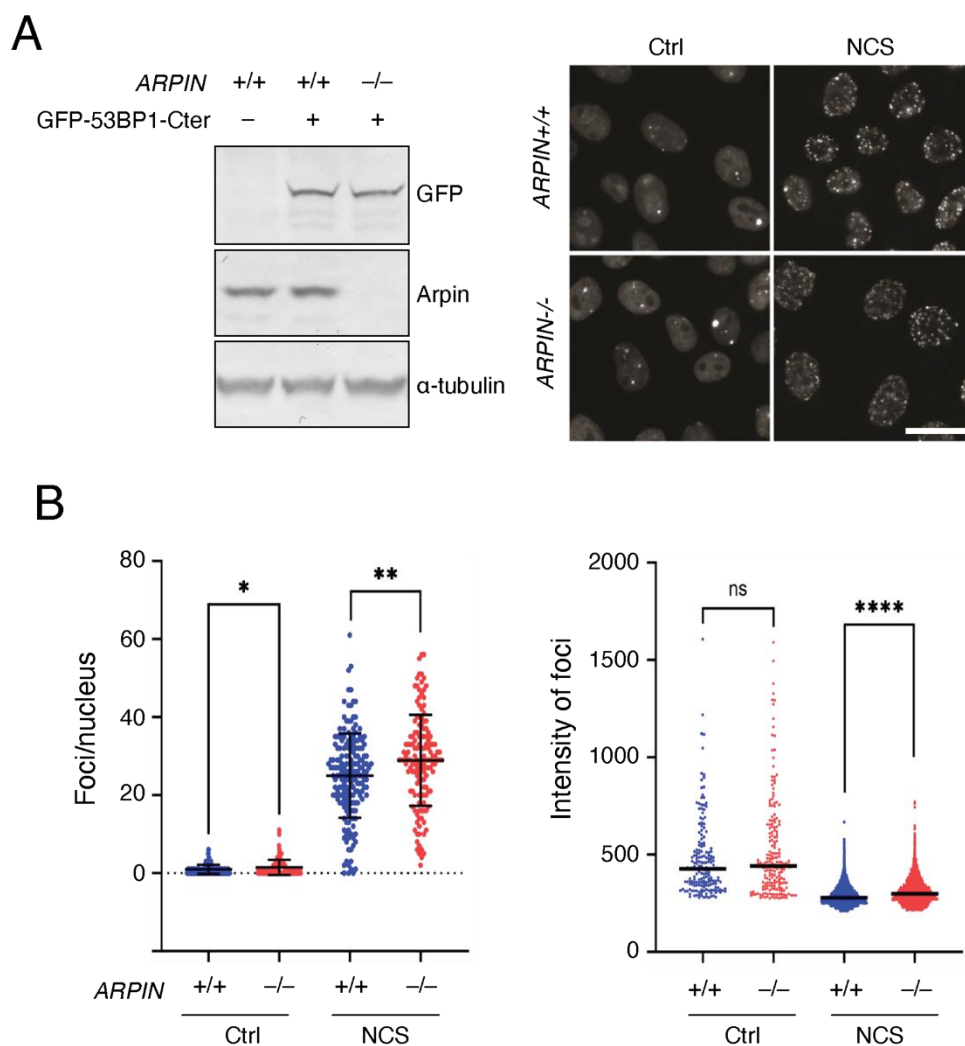
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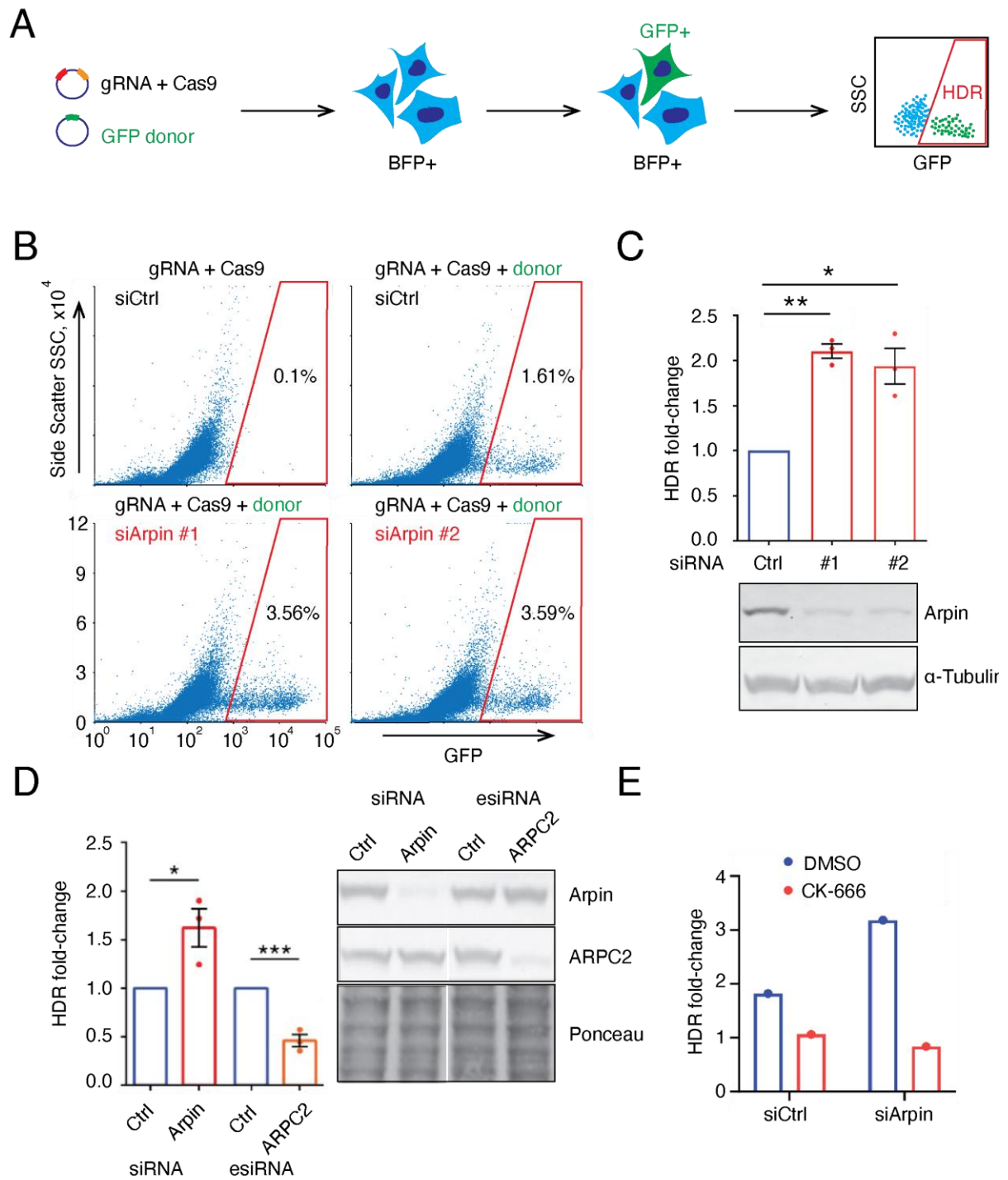
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**Figure 1. Correlation of Arpin levels with those of different markers in breast tumor samples assessed using Reverse Phase Protein Array (RPPA).** (A) Lysates from tumor samples are probed with 61 antibodies. (B) Levels of several actors of the DNA damage response are positively or negatively correlated with Arpin levels (Spearman's coefficient  $r > 0$  or  $< 0$ , respectively). Corresponding p-values are indicated. DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair; MMEJ, microhomology-mediated end joining.



**Figure 2. Visualization and quantification of DSB foci in control and Arpin-null cells.** (A) Parental MCF10A and *ARPIN*<sup>-/-</sup> cells expressing GFP-53BP1-Cter are isolated and GFP and Arpin expression is validated using Western blot. Representative images of GFP-53BP1-Cter expressing cells under normal culture conditions and under DSB-inducing treatment; scale bar: 20  $\mu$ m. (B) GFP-53BP1-Cter foci number and intensity of individual foci are assessed in *ARPIN*<sup>-/-</sup> and parental cells, treated or not with the DNA damage agent Neocarzinostatin (NCS) for 1 h. Values from one representative experiment out of three independent ones are plotted (196, 138, 181, 144 cells analyzed in respective conditions); foci number is represented for each nucleus, with mean  $\pm$  SD, intensity of foci is represented for each focus as median value; statistical significance is calculated using non-parametric Mann-Whitney test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ ).



**Figure 3. CRISPR-mediated HDR in 293T reporter cells under Arpin depletion.** (A) Experimental workflow. 293T BFP reporter cells are transfected with a plasmid expressing Cas9 and a gRNA targeting BFP gene together with a plasmid containing GFP donor sequence and homology arms. The percentage of GFP+ cells is assessed using flow cytometry. (B) Representative flow cytometry plots of reporter cells transfected with a non-targeting siRNA or siRNAs targeting Arpin 24 h prior to transfection of the plasmids that mediate HDR. (C) Quantification of the HDR assay and validation of Arpin depletion using Western blot. (D) Transfected cells are previously depleted of Arpin or of the ARPC2 subunit of the Arp2/3 complex using siRNAs or esiRNAs. (E) Cells are treated or not with 150  $\mu$ M CK-666 for 6 h prior to transfection of the plasmids. Mean  $\pm$  SEM (n = 3 in C, D; n = 1 in E; paired *t* test, \* *p* < 0.05, \*\* *p* < 0.001, \*\*\* *p* < 0.001). gRNA, guide RNA; HDR, homology-directed repair.

