

1 **ADAR1 editing dependency in triple-negative breast cancer**

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14 **Summary**

15 Triple-negative breast cancer (TNBC) is the deadliest form of breast cancer. Unlike other  
16 types of breast cancer that can be effectively treated by targeted therapies, no such targeted  
17 therapy exists for all TNBC patients. The ADAR1 enzyme carries out A-to-I editing of RNA to  
18 prevent sensing of cellular double-stranded RNAs (dsRNA). ADAR1 is highly expressed in  
19 breast cancer including TNBC. Here, we demonstrate that ADAR1 expression and editing  
20 activity is required in TNBC cell lines but not in ER+ and/or Her2+ cells. In TNBC cells,  
21 knockdown of ADAR1 attenuates proliferation and tumorigenesis. PKR expression is elevated in  
22 TNBC and its activity is induced upon ADAR1-knockdown, which correlates with a decrease in  
23 translation. ADAR1-dependent TNBC cell lines also exhibit elevated IFN stimulated gene  
24 expression. IFNAR1 reduction significantly rescues the proliferative defects of ADAR1 loss.  
25 These findings establish ADAR1 as a novel therapeutic target for TNBC tumors.

26

27 **Keywords:** ADAR1, triple-negative breast cancer, RNA editing, Type I IFN, PKR

28

## 29 **Introduction**

30 Generally defined by the lack of estrogen receptor (ER), progesterone receptor (PR) and  
31 HER2 expression, triple-negative breast cancer (TNBC) accounts for 15 to 20 percent of all  
32 breast cancer diagnoses in the United States each year(Ademuyiwa et al., 2017). Unlike ER-  
33 positive (tamoxifen, fulvestrant, and other ER modulators) and HER2-positive (Herceptin and  
34 other HER2 inhibitors) breast cancers, there are no targeted therapies for all TNBC  
35 patients(Waks and Winer, 2019). The lack of targeted therapies for TNBC leaves chemotherapy  
36 as the main treatment option that carries a generally worse prognosis(Garrido-Castro et al.,  
37 2019). Efforts to develop effective targeted therapies against TNBC have focused on further sub-  
38 categorizing TNBC based on gene expression signatures, as well as looking to exploit common  
39 genetic vulnerabilities(Perou, 2011, Anders et al., 2016).

40 A potential therapeutic target for TNBC is Adenosine Deaminase Acting on RNA  
41 (ADAR1, encoded by *ADAR*). ADAR1 carries out the enzymatic reaction of deaminating  
42 adenosine to inosine within cellular dsRNA, in a process known as A-to-I editing. Induction of  
43 ADAR1 expression is prevalent in breast cancer(Fumagalli et al., 2015, Han et al., 2015, Paz-  
44 Yaacov et al., 2015, Peng et al., 2018, Anantharaman et al., 2017) and ADAR1-mediated A-to-I  
45 editing has been found to influence the levels of its targets in breast cancer(Gumireddy et al.,  
46 2016, Binothman et al., 2017, Dave et al., 2017, Nakano et al., 2017). Recent studies indicate  
47 that ADAR1 is over-represented in TNBC and may be correlated with poor prognosis when  
48 RNA editing is increased(Song et al., 2017, Sagredo et al., 2018).

49 ADAR1 acts in a negative feedback loop to inhibit the type-I IFN pathway triggered by  
50 endogenous dsRNAs or dsRNAs introduced upon viral infections(Mannion et al., 2014,  
51 Liddicoat et al., 2015). ADAR1 has been shown to suppress type-I IFN pathway through

52 multiple mechanisms, including destabilization of the dsRNA structure, reduced expression, and  
53 activation of the dsRNA sensors MDA5 and RIG-I, and inhibition of IFN expression(Mannion et  
54 al., 2014, Liddicoat et al., 2015, Pestal et al., 2015, George et al., 2016, Li et al., 2012, Pujantell  
55 et al., 2017). ADAR1-mediated A-to-I RNA editing by the IFN-inducible p150 isoform (not the  
56 constitutive p110 isoform) is essential for its ability to modulate dsRNA-induced IFN  
57 signaling(Liddicoat et al., 2015, Pestal et al., 2015, George et al., 2016). ADAR1's ability to  
58 regulate this response was recently linked to the development of ADAR1 dependency in some  
59 cancer cell lines; two groups showed that by removing ADAR1 from cancer cells with elevated  
60 innate immune signaling, cells became susceptible to inflammation-induced cell death(Gannon et  
61 al., 2018, Liu et al., 2019). This is consistent with previous findings that ADAR1 prevents  
62 immune and translational catastrophes by blocking dsRNA-activated IFN pathway(Mannion et  
63 al., 2014, Chung et al., 2018).

64 Here we demonstrate that TNBC cell lines are dependent on ADAR1 expression and  
65 activity; loss of ADAR1 in these cell lines inhibits cellular growth and tumorigenesis,  
66 highlighting the therapeutic potential of ADAR1 inhibitors for the treatment of TNBC.

## 67 **Results**

### 68 **ADAR1 is highly expressed in all breast cancer subtypes**

69 Using publicly available data from TCGA (The Cancer Genome Atlas)(Han et al., 2015,  
70 Fumagalli et al., 2015), we found that high expression of ADAR1 correlated with poor prognosis  
71 of breast cancers (Figure 1A). Recent studies indicated that ADAR1 promotes tumorigenesis of  
72 metaplastic breast cancers, and that high expression of ADAR1 correlates with poor prognosis in  
73 basal-like breast cancers(Sagredo et al., 2018, Dave et al., 2017). Since both basal-like and

74 metaplastic breast cancers share similar characteristics with TNBC, we sought to determine the  
75 importance of ADAR1 in the tumorigenesis of TNBC. By evaluating the TCGA database, we  
76 found that while mRNA expression of ADAR1 was higher in TNBC compared to normal, it was  
77 not significantly different between TNBC and non-TNBC tumors (Figure 1B). Additionally,  
78 ADAR1 expression was not significantly higher in any one subtype of breast cancer based on  
79 PAM50 classification(Lehmann et al., 2016) (Supplemental Figure 1A). This observation is  
80 consistent with data from the Cancer Cell Line Encyclopedia (CCLE), which uses both RNA-seq  
81 and Reverse Phase Protein Array (RPPA) to determine RNA and protein expression levels in  
82 numerous cancer cell lines (Supplemental Figure 1B-C). Data from both the TCGA and CCLE  
83 datasets also revealed that both p150 and p110 isoforms of ADAR1 were expressed at similar  
84 levels between TNBC and non-TNBC specimen (Supplemental Figure 1D-H), with p110  
85 expression being consistently higher than p150 in all samples. Additionally, we assessed p150  
86 isoform expression by immunohistochemistry in TNBC and non-TNBC patient tumors, Figure  
87 1D. We sought to determine the protein expression level of the ADAR1-p150 isoform in a panel  
88 of established breast cancer cell lines representing TNBC and non-TNBC. Immunoblot analysis  
89 showed that ADAR1 (p150 isoform) is overexpressed, compared to normal human mammary  
90 epithelial cells (HMECs), in over half of all TNBC (6/8) and non-TNBC (5/8) cell lines assayed  
91 (Supplemental Figure 1I-K). These results indicate that ADAR1-p150 is overexpressed in many  
92 breast cancer cell lines regardless of subtype.

### 93 **ADAR1 is required for TNBC proliferation**

94 Several recent studies have suggested that some established cancer cell lines display  
95 strong dependencies on ADAR1 expression(Liu et al., 2019, Ishizuka et al., 2019, Gannon et al.,  
96 2018). Given the high expression of ADAR1-p150 in most breast cancer cell lines, we sought to

97 determine whether these breast cancer cell lines exhibited ADAR1-dependency. We analyzed  
98 publicly available RNAi and CRISPR-Cas9 datasets to determine if ADAR1 was required for the  
99 survival of breast cancer cell lines representing various subtypes(McFarland et al., 2018, Meyers  
100 et al., 2017). TNBC and basal-like cell lines made up the majority of breast cancer cells  
101 exhibiting high ADAR1 sensitivity scores (DEMETER2 Score < -0.5) (Figure 1C, Supplemental  
102 Figure 2A-C). Importantly, we did not observe a correlation between ADAR1 expression and  
103 ADAR1-dependency across these breast cancer cell lines (Supplementary Figure 2D). To  
104 experimentally validate ADAR1-dependency among breast cancer cell lines, we knocked-down  
105 ADAR1 expression in eight cell lines (Four TNBC: MDA-MB231, MDA-MB468, BT549,  
106 HCC1806; Four non-TNBC: SKBR3, CAMA1, MCF7, T47D); all of these cell lines showed  
107 noticeable ADAR1-p150 isoform overexpression over HMEC controls in our immunoblot  
108 analysis (Supplemental Figure 1I). Long-term (7-28 days) and short-term (4 days) cell  
109 proliferation was evaluated for each cell line following ADAR1 knockdown. Notably, similar  
110 levels of ADAR1 knockdown were achieved for each cell line (Figure 1E). All four TNBC cell  
111 lines displayed significant attenuation in both long- and short-term proliferation following  
112 ADAR1 knockdown (Figure 1F-G, Supplemental Figure 2E). Conversely, ADAR1 expression  
113 proved dispensable for proliferation in all four non-TNBC cell lines.

#### 114 **ADAR1-p150 editing activity rescues TNBC proliferation**

115 While both isoforms of ADAR1 are expressed in TNBC, our knockdown experiment  
116 does not distinguish between p150 or p110 dependence. To address this, we set up a knockdown-  
117 rescue system. We overexpressed either the p110 or p150 isoform following ADAR1  
118 knockdown in MDA-MB231 cells and evaluated their ability to rescue cell proliferation (Figure  
119 1H-J). Overexpression of ADAR1-p150, but not p110, resulted in significant rescue of cell

120 proliferation in MDA-MB231 TNBC cells. Having established a rescue system for ADAR1  
121 dependent proliferation, we next aimed to determine whether the editing activity of ADAR1-  
122 p150 was required for this rescue. An editing-defective mutant (E912A) of the p150 isoform was  
123 incapable of rescuing the ADAR1 knockdown phenotype, indicating that the A-to-I editing  
124 function of ADAR1 is absolutely required for cellular proliferation in TNBC cells (Figure 1I-J).

### 125 **ADAR1 is required for TNBC transformation and tumorigenesis**

126 To assess the functional relevance of our findings, we investigated the requirement of  
127 ADAR1 for the transformation of breast cancer cell lines. We utilized anchorage independent  
128 growth in soft agar as a measure of cellular transformation. Knockdown of ADAR1 dramatically  
129 reduced soft agar colonies of MDA-MB231 and HCC1806 TNBC cells while not significantly  
130 affecting the numbers of colonies formed by SKBR3 and T47D non-TNBC cells (Figure 2A-D).

131 To extend these *in vitro* findings, we next determined whether ADAR1 was required for  
132 TNBC cell lines to form tumors *in vivo*. We performed mammary gland orthotopic  
133 transplantations using TNBC and non-TNBC cells following ADAR1 knockdown. Parental  
134 (shRNA-NonTargeting, shNT) MDA-MB231 and MDA-MB468 TNBC cells and SKBR3 non-  
135 TNBC cells were all able to form visible tumors in the mammary glands of four-five  
136 independently transplanted female immune compromised mice (Figure 2E-H). Knockdown of  
137 ADAR1 in MDA-MB231 and MDA-MB468 TNBC cells completely abrogated their ability to  
138 form tumors in transplanted mice. In contrast, ADAR1 knockdown in SKBR3 cells did not  
139 significantly affect tumor formation in transplanted mammary glands. Collectively, these results  
140 demonstrate that ADAR1 expression is required for *in vitro* transformation and *in vivo* tumor  
141 formation of TNBC cells, but is completely dispensable for these properties in non-TNBC cells.

### 142 **PKR is overexpressed in TNBC and activated upon ADAR loss**

143 Previous reports have shown that ADAR1 dependency in human cancer cells could be  
144 mediated through several downstream pathways, including translational inhibition triggered by  
145 activated PKR and ribonuclease L (RNASEL), as well as type-I IFN signaling(Li et al., 2017,  
146 Gannon et al., 2018, Liu et al., 2019). To investigate if these pathways contribute to the ADAR1  
147 dependency observed in TNBC cells, we first analyzed the TCGA and CCLE datasets to  
148 determine if these pathways are intrinsically elevated in TNBC. Across TCGA breast cancer  
149 samples, RNA expression of PKR is significantly higher in TNBC samples compared to non-  
150 TNBC (Figure 3A). This is consistent with RNA-seq data for breast cancer cell lines within the  
151 CCLE (Figure 3B). Moreover, elevated PKR expression positively correlates with the ADAR1  
152 sensitivity scores, suggesting a strong relationship between PKR and TNBC-associated ADAR1  
153 dependency (Figure 3C, Supplemental Figure 3A-C). We further confirmed this observation by  
154 immunoblot analysis among our panel of sixteen breast cancer cell lines to show a general  
155 elevation of PKR expression across all TNBC cell lines (Figure 3D). We also detected  
156 heightened levels of PKR phosphorylation as well as its downstream substrate eIF2 $\alpha$  in TNBC  
157 cells compared to non-TNBC cells. Upon ADAR1 knockdown, phosphorylation of PKR and  
158 eIF2 $\alpha$  was markedly induced in all TNBC cell lines but remained unchanged in the non-TNBC  
159 cell lines (Figure 3E). These observations suggest that TNBC-associated ADAR1 dependency  
160 might be attributed to PKR-mediated translational inhibition. To investigate this, we performed  
161 polysome profiling. ADAR1 knockdown in MDA-MB231 and HCC1806 TNBC cells led to  
162 inhibition of translation, demonstrated by the substantial reduction of polysome peaks (Figure  
163 3F-G). These results suggest that translational repression contribute to TNBC-associated  
164 ADAR1 dependency. While attempting to rescue the ADAR-knockdown phenotype in MDA-  
165 MB231 and HCC1806 cells by knockdown of PKR, we observed that knockdown of PKR alone



166 greatly reduced foci formation (Supplemental Figure 3D-E). This suggests basal PKR expression  
167 is required for the proliferation of these cell lines and precluding us from determining if  
168 expression of PKR is required for the ADAR-knockdown phenotype.

### 169 **RNASEL is not activated following loss of ADAR1 in TNBC**

170       Activation of RNASEL and subsequent translational inhibition has also been shown to  
171 result in cell lethality in the absence of ADAR1(Li et al., 2017). The CCLE dataset indicated that  
172 RNASEL activators OAS1, OAS2 and OAS3 were highly expressed in ADAR1 dependent cell  
173 lines, while the expression of *RNASEL* showed modest correlation with ADAR1 dependency  
174 (Supplemental Figure 3F-G). A hallmark of RNASEL activation is degradation of  
175 rRNA(Silverman et al., 1983). However, we did not observe rRNA degradation in ADAR1-  
176 dependent TNBC cells after ADAR1 knockdown (Supplemental Figure 3H), further suggesting  
177 that the RNASEL pathway does not significantly contribute to TNBC-associated ADAR1  
178 dependency and the induction of OAS genes likely reflects the fact that OAS genes are also  
179 known ISGs (see below).

### 180 **ADAR1-dependent TNBCs exhibit elevated ISG expression**

181       Another factor contributing to ADAR1 dependency in cancer cell lines is the type-I IFN  
182 pathway(Liu et al., 2019). It has been shown previously that this connection is mediated through  
183 either altering the expression of type I IFN regulators or activating the feed-forward loop of IFN  
184 signaling(Gannon et al., 2018, Liu et al., 2019). RNA expression data from the TCGA and CCLE  
185 datasets showed that TNBC have higher ISG expression (Core ISG Score(Liu et al., 2019))  
186 compared to non-TNBC (Figure 4A-B). This is consistent with the elevated expression of PKR  
187 and ISG15 in our immunoblot analysis among breast cancer cell lines (Figure 3D, Supplemental

188 Figure 4A). Like PKR expression, the Core ISG Score positively correlated with ADAR1  
189 sensitivity among TNBC cell lines (Figure 4C and Supplemental Figure 4B-C).

### 190 **INFAR1 loss rescues ADAR1 knockdown phenotype**

191 To establish whether the type-I IFN pathway accounts for the significant differences of  
192 ADAR1-dependency between TNBC and non-TNBC cell lines, non-TNBC cell lines (SKBR3  
193 and MCF7) were treated with IFN $\beta$  in ADAR1-intact and ADAR1-deficient cells (Supplemental  
194 Figure 4D-G). Expression of ADAR1 and ISG15 were induced upon IFN $\beta$  treatment, as well as  
195 phosphorylation of STAT1. However, while the treatment of IFN $\beta$  generally reduced cell  
196 proliferation, it did not sensitize non-TNBC cells to ADAR1 deficiency (Supplemental Figure 4E  
197 and G), implying that IFN $\beta$  alone is not capable of switching ADAR1-resistant cells to ADAR1-  
198 dependent cells.

199 To determine if the type-I IFN pathway functionally contributes to ADAR1 dependency  
200 in TNBC, we knocked-down ADAR1 and the IFN alpha-receptor subunit 1 (IFNAR1)  
201 simultaneously in both MDA-MB231 and MDA-MB468 cells (Figure 4D and Supplemental  
202 Figure 4H). The knockdown of IFNAR1 partially rescued the proliferation of both cell lines,  
203 suggesting that TNBC-associated ADAR1 dependency can be partially attributed to type I IFN  
204 pathway (Figure 4E-F, Supplemental Figure 4I). However, knockdown of IFNAR1 in TNBC  
205 cells did not alter the levels of phosphorylated PKR (Figure 4D, Supplemental Figure 4H),  
206 suggesting that in these TNBC cells, either type-I IFN and PKR pathways independently  
207 contribute to ADAR1 dependency or IFNAR1 resides downstream of PKR.

### 208 **Discussion**

209 Recent studies have highlighted the dependence of some cancer cell lines on ADAR1  
210 expression(Gannon et al., 2018, Liu et al., 2019). Here, we characterized the requirement for

211 ADAR1 in a panel of established breast cancer cell lines. ADAR1-dependent cell lines shared an  
212 elevated ISG-expression signature. Loss of ADAR1 in these cell lines led to activation of the  
213 translational regulator PKR and translational repression. The ADAR1-dependence phenotype  
214 could be partially abrogated by knockdown of IFNAR1. It is not currently understood what  
215 makes select cancer cell lines ADAR1-dependent, or conversely why others are refractory to  
216 ADAR1-loss. It has been proposed that the higher ISG expression might potentiate these cells  
217 towards ADAR1-dependency – loss of ADAR1 would further elevate ISG expression leading to  
218 the growth inhibition phenotype(Liu et al., 2019, Gannon et al., 2018). However, we have  
219 demonstrated that for cell lines refractory to ADAR1 loss, treatment with IFN- $\beta$  did not render  
220 them sensitive to ADAR1 knockdown. Furthermore, we observed no activation of PKR in the  
221 ADAR1 refractory cell lines following ADAR1 loss. These findings suggest that the link  
222 between ADAR1 loss and the IFN pathway or PKR activation in ADAR1-refractory cell lines is  
223 missing. Loss of ADAR1 is thought to activate the IFN pathway and PKR by causing an increase  
224 in dsRNA – stemming from a reduction in A-to-I editing(Mannion et al., 2014, Liddicoat et al.,  
225 2015). It is possible that ADAR1-refractory cell lines either do not accumulate dsRNA following  
226 ADAR1 loss or there exists a system that prevents dsRNAs from activating the IFN pathway or  
227 PKR. Understanding the molecular basis of this process would help to predict which cell lines –  
228 or more importantly which tumors – should be sensitive to ADAR1 loss.

229       Important clinical implications can be drawn from these observations. Our data suggest  
230 that ADAR1 is a legitimate candidate for targeted therapies in TNBC. We found that TNBC cell  
231 lines and patient samples exhibit elevated ISG and PKR expression, which is consistent with  
232 ADAR1-dependent cell lines. With increased understanding of ADAR1 functions, novel  
233 therapeutic strategies against ADAR1 could benefit ADAR1-dependent cancers, including

234 TNBC(Kung et al., 2018). Secondly, the relationship between ADAR1 dependency and type-I  
235 IFN pathway could point to new directions for TNBC interventions. Recent studies revealed that  
236 the increased IFN $\beta$  target gene signature correlates with improved recurrence-free survival in  
237 TNBC, and IFN $\beta$  treatment inhibits tumor progression in TNBC by reducing cancer stem cell  
238 (CSC) plasticity(Doherty et al., 2017, Doherty et al., 2019). In addition to cell-intrinsic effects of  
239 ADAR1-loss in cancer cells, removal of ADAR1 has been shown to sensitize tumors to  
240 immunotherapy by overcoming resistance to checkpoint blockade(Ishizuka et al., 2019).

241 It was recently demonstrated that chemotherapies elicit a state of immunological  
242 dormancy in ER-negative breast cancers, marked by sustained type-I IFN signaling, reduced cell  
243 growth, and longer progression-free survival(Lan et al., 2019). This indicates a possible shared  
244 mechanism between chemotherapy-induced immunological dormancy and ADAR1-dependency  
245 in TNBC. It is important to note that careful considerations need to be given when applying the  
246 concepts of ADAR1 inhibition and type-I IFN application in the treatment of TNBC. It is  
247 recognized that type-I IFN can elicit paradoxical effects on cancer development(Snell et al.,  
248 2017). For example, it has been suggested that type-I IFN pathway, potentially through ISG15-  
249 mediated ISGylation, can promote the aggressiveness of TNBC(Forys et al., 2014, Lo et al.,  
250 2018). Therefore, further understanding of the relationship between ADAR1 functions and  
251 TNBC tumorigenesis should better inform the context in which this strategy can provide the  
252 maximum benefit.

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### 259 **Author Contributions**

260 Conceptualization, C-PK, KAC, and JDW; Methodology, C-PK, KAC, and JDW; Software,  
261 KAC; Investigation, C-PK, KAC, SR, ERB, RK, EAB, LM, and JDW; Writing – Original Draft,  
262 C-PK and KAC; Writing – Review & Editing, C-PK, KAC, SR, ERB, RK, LM, and JDW;  
263 Funding Acquisition, JDW; Supervision, JDW

### 264 **Declaration of Interests**

265 The authors declare no competing interests.

266 **Figure 1: ADAR1 is highly expressed in all breast cancer subtypes and required for TNBC**  
267 **proliferation**

268 **A)** Kaplan-Meier survival curves of breast cancer patients. Patients were stratified by ADAR1  
269 expression, above or below z-score = 2.34. **B)** Relative mRNA expression of ADAR1 in normal,  
270 TNBC and Non-TNBC breast cancer. Data were extracted from TCGA database. **C)** ADAR1-  
271 dependency scores in breast cancer cell lines. Lower DEMETER2 scores indicate stronger  
272 ADAR1-dependency. ERBB2 = HER2-positive. **D)** Representative images of IHC staining of  
273 ADAR1 in TNBC and Non-TNBC breast cancer tissues (scale: 100  $\mu$ M). Numbers below the  
274 image indicate the ratio of samples identified as high p150-ADAR based on IHC scoring. **E)**  
275 Immunoblots showing protein levels of ADAR1 p150 isoform and GAPDH (loading control)  
276 with or without ADAR1-knockdown in breast cancer cell lines. Fold change of ADAR1  
277 (ShADAR1/ShNT) is indicated, normalized to GAPDH. Focus formation (FF) assay showed that  
278 ADAR1-knockdown reduced proliferation of TNBC but not Non-TNBC cells. Images are  
279 representative, N=3. **F)** Quantification of FF in **E)**. Relative plate occupancy was determined  
280 using ImageJ software and normalized to ShNT samples for each cell line. Data are represented  
281 as mean  $\pm$  SD, N=3. **G)** Cell proliferation assay showing that ADAR1-knockdown reduced  
282 proliferation of TNBC but not Non-TNBC cells. Data are represented as mean  $\pm$  SD, N=2.  
283 (\*\*\*\*)  $p < 0.0001$ . ns, not significant. **H)** Immunoblots showing protein levels of ADAR1 and  
284 GAPDH (loading control) with overexpression of p150, p110 or editing-defective p150<sup>E912A</sup> in  
285 ShADAR1-treated MDA-MB231 cells. Images are representative, N=3. EV, empty virus. **I)** FF  
286 assay showing that p150, but not p110 or editing-defective p150<sup>E912A</sup>, partially rescued  
287 proliferation of ShADAR1-treated MDA-MB231. Images are representative, N=3. **J)**  
288 Quantification of FF in **I)**. Relative plate occupancy was determined using ImageJ software and  
289 normalized to ShNT-EV. Data are represented as mean  $\pm$  SD, N>3. (\*\*)  $p < 0.01$ . n.s., not  
290 significant. See also Figure S1 and S2.

291 **Figure 2: ADAR1 is required for TNBC transformation and tumorigenesis**

292 **A)** Soft agar assay (SAA) showing that ADAR1-knockdown reduced anchorage-independent  
293 growth of TNBC cells (HCC1806 and MDA-MB231). Images are representative, N=3. Scale-  
294 bar, 100  $\mu$ M. **B)** Quantification of SAA in **A)**. Colonies bigger than 100  $\mu$ M in diameter were  
295 counted. Data are represented as mean  $\pm$  SD, N=3. (\*\*\*\*)  $p < 0.0001$ . (\*\*)  $p < 0.01$ . **C)** SAA  
296 showing that ADAR1-knockdown did not affect anchorage-independent growth of Non-TNBC  
297 cells (SKBR3 and T47D). Images are representative, N=3. Scale-bar, 100  $\mu$ M. **D)** Quantification  
298 of SAA in **C)**. Colonies bigger than 100  $\mu$ M in diameter were counted. Data are represented as  
299 mean  $\pm$  SD, N=3. ns, not significant. **E)** Orthotopic implantation of MDA-MB231 cells into  
300 abdominal mammary fat pad. Tumors were removed from the mice ~4 weeks post injection and  
301 weighed (ShNT, N=4; ShADAR1, N=5). Red arrows indicate the location of mammary fat pad.  
302 **F)** Orthotopic implantation of MDA-MB468 cells into abdominal mammary fat pad. Tumors  
303 were removed from the mice ~12 weeks post injection and weighed (N=5). Red arrows indicate  
304 the location of mammary fat pad. **G)** Orthotopic implantation of SKBR3 cells into abdominal  
305 mammary fat pad. Tumors were removed from the mice ~4 weeks post injection and weighed  
306 (N=5). Red arrows indicate the location of mammary fat pad. **H)** Quantification of the result

307 shown in **E)–G**). Data are represented as mean  $\pm$  SD. (\*\*\*\*)  $p < 0.0001$ . (\*)  $p < 0.05$ . ns, not  
308 significant.

309 **Figure 3: PKR is overexpressed in TNBC and activated upon ADAR loss**

310 **A)** Relative mRNA expression of PKR in TNBC and Non-TNBC. Data were extracted from  
311 TCGA database. **B)** Relative mRNA expression of PKR in ER-positive, ERBB2(HER2)-positive  
312 and TNBC cell lines. Data were extracted from CCLE database. **C)** ADAR1-dependency scores  
313 positively correlate with PKR expression. Upper panel: PKR expression z-score in breast cancer  
314 cell lines. Lower panel: ADAR1-dependency scores. Lower DEMETER2 scores indicate  
315 stronger ADAR1-dependency. **D)** Immunoblots showing protein levels of PKR, p-PKR (T446),  
316 p-eIF2 $\alpha$  (S51) and GAPDH (loading control) in breast cancer cell lines. Densitometry  
317 quantification of gel images was normalized to GAPDH and set relative to HMEC signal. Data  
318 shown are representative, N=3. **E)** Immunoblots showing protein levels of PKR, p-PKR (T446),  
319 p-eIF2 $\alpha$  (S51) and  $\beta$ -tubulin (loading control) in TNBC and non-TNBC breast cancer cell lines  
320 with or without ADAR1-knockdown. Densitometry quantification of gel images was normalized  
321 to GAPDH and compared to HMEC signal set as 1-fold. Data shown are representative, N=3. **F)**  
322 Polysome profiling of MDA-MB231 cells with or without ADAR1-knockdown. Data shown are  
323 representative of three replicates. **G)** Ribosomal profiling of HCC1806 cells with or without  
324 ADAR1-knockdown. See also Figure S3.

325 **Figure 4: ADAR1-dependent TNBCs exhibit elevated ISG expression and IFNAR1 loss**  
326 **rescues ADAR1 knockdown phenotype**

327 **A)** Relative ISG Core Scores in TNBC and Non-TNBC breast cancer samples. Data were  
328 extracted from TCGA database. **B)** Relative ISG Core Scores in ER-positive, ERBB2(HER2)-  
329 positive and TNBC cell lines. Data were extracted from CCLE database. **C)** ADAR1-  
330 dependency scores positively correlate with ISG Core Scores in breast cancer cell lines. Upper  
331 panel: ISG Core Scores in breast cancer cell lines. Lower panel: ADAR1-dependency scores.  
332 Lower DEMETER2 scores indicate stronger ADAR1-dependency. **D)** Immunoblots showing  
333 protein levels of IFNAR1, PKR, p-PKR (T446), p-eIF2 $\alpha$  (S51) and GAPDH (loading control) in  
334 MDA-MB231 cells. IFNAR1 was knocked down in ShADAR1-treated MDA-MB231 cells to  
335 determine if IFNAR1 loss reverses ADAR1-knockdown phenotype. Images are representative,  
336 N=3. **F)** FF assay showing that IFNAR1 loss partially rescued ADAR1-knockdown phenotype in  
337 MDA-MB231 cells. Images are representative, N=3. **G)** Quantification of FF in **F**). Relative  
338 plate occupancy was determined using ImageJ software and normalized to ShNT-ShNT. Data are  
339 represented as mean  $\pm$  SD. N=3. (\*\*\*\*)  $p < 0.0001$ . (\*\*\*)  $p < 0.001$ . See also Figure S4.

340

## 341 **Experimental Procedures**

### 342 **Cell lines and reagents**

343 Human mammary epithelial cells (HMEC) and breast cancer cells lines were obtained  
344 from American Tissue Cells Consortium (ATCC). HMECs were cultured in MammaryLife Basal  
345 Medium (Lifeline Cell Technology) and passaged by using 0.05% trypsin-EDTA (Gibco) and  
346 Defined Trypsin Inhibitor (DTI, Gibco). All breast cancer cell lines were maintained in  
347 Dulbecco's Modification of Eagle's Medium (DMEM, GE Life Sciences) supplemented with  
348 10% fetal bovine serum (Gibco, 10091-148), Sodium Pyruvate (Cellgro, 30-002-CI), Non-  
349 Essential Amino Acids (NEA, Cellgro, 25-030-CI), and L-glutamine (Cellgro, 25-005-CI).  
350 Lipofectamine 2000 (Invitrogen) was used for transfection to generate lentivirus. Fugene 6  
351 transfection reagent (Roche) was used for all other transfection experiments.

### 352 **Immunoblot analysis**

353 Cell lysates were extracted from cells at ~90% confluence. Cell were washed with  
354 phosphate-buffered saline (PBS, GE Life Sciences), scrape harvested, centrifuged at 1000 × g for  
355 5 min, and lysed with RIPA buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1% NP-  
356 40, 0.1% SDS, 0.1% Deoxycholate) supplemented with 10mM PMSF and HALT protease  
357 inhibitor cocktail (Thermo Fisher Scientific). Lysates were clarified by centrifugation and the  
358 protein concentration was determined using DC protein assay system (Bio-Rad Laboratories).  
359 Equal amount of protein was resolved by sodium dodecyl sulfate-polyacrylamide gel  
360 electrophoresis (SDS-PAGE) using Criterion TGX Stain-Free Precast Gels (Bio-Rad) and  
361 transferred onto Immobilon-P membranes (MilliporeSigma). Primary antibodies used in this  
362 study include ADAR1 (Santa Cruz, sc-73408), MDA5 (Cell Signaling, #5321), RIG-I (Cell  
363 Signaling, #3743), PKR (Cell Signaling, #3072), PKR Thr-446-P (Abcam, ab32036), STING  
364 (Cell Signaling, #13647), IFNAR1 (Bethyl Laboratories, A304-290A), ISG15 (Santa Cruz, sc-



365 166755), GAPDH (Bethyl Laboratories, A300-641A),  $\beta$ -Tubulin (Abcam, ab6046),  
366 EIF2S1/eIF2 $\alpha$  Ser-51-P (Abcam, 32157), EIF2S1 (Abcam, ab5369). Secondary antibodies  
367 conjugated to Horseradish peroxidase were used at a dilution of 1:5-10,000 (Jackson  
368 Immunochemicals). Clarity Western ECL Substrate (Bio-Rad) was then applied to blots and  
369 protein levels were detected using autoradiography with ChemiDoc XRS+ Imager (Bio-Rad).  
370 Densitometry quantification of protein signals was performed using ImageJ software (NIH,  
371 Bethesda, MD).

### 372 **Quantitative reverse-transcription polymerase chain reaction**

373 Total RNA was isolated from cells using RNeasy Mini Plus kit (Qiagen, Hilden,  
374 Germany) including on-column DNase digestion following the manufacturer's protocol. High  
375 Capacity cDNA Reverse Transcription Kit (Life technologies, CA, USA) was used to transcribe  
376 RNA to cDNA. Quantitative PCR (qPCR) was performed using iTaq Universal SYBR Green  
377 Supermix (Bio-Rad, #1725121) on the C1000 Thermal Cycler (CFX96 Real-Time System, Bio-  
378 Rad), and data analysis was performed using the  $2(-\Delta\Delta C_T)$  method. Messenger RNA expression  
379 levels were normalized to GAPDH. Primers used in this study are listed in Supplemental Table  
380 1.

### 381 **Lentiviral production and transduction**

382 To generate lentivirus, transformed human embryonic kidney HEK293T cells were  
383 transfected using Lipofectamine 2000 (Invitrogen) with pCMV-VSV-G, pCMV- $\Delta$ R8.2, and  
384 expression constructs (with pLKO.1-puromycin or pLKO.1-hygromycin backbone for short-  
385 hairpin RNAs and with pLVX-hygromycin backbone for overexpression constructs). Growth  
386 medium was replaced with fresh medium 24hr after transfection, and supernatants containing  
387 lentivirus were harvested 24hr later. For transduction, one million cells were infected with

388 lentivirus for 24hr in the presence of 10 µg/ml protamine sulfate to facilitate viral entry.  
389 Sequences of shRNAs are listed in Supplemental Table 1. ADAR1 overexpression constructs  
390 (p150; p110; p150-E912A mutant) were subcloned from pBac-ADAR1 constructs (generous  
391 gifts from Kazuko Nishikura at The Wistar Institute, Philadelphia) into pLVX-hygromycin  
392 vectors (Cho et al., 2003).

### 393 **Cell proliferation and Focus Formation Assays**

394 For cell proliferation assays,  $2-5 \times 10^4$  cells were plated in triplicate in six-well plates.  
395 Cells were trypsinized, harvested and counted using a hemocytometer or the Cello Cell counter  
396 every 24 hours for 4 days post-plating. For the focus formation assay,  $3-5 \times 10^3$  cells were plated  
397 in triplicate in 10-cm cell culture dishes 7-28 days (depending on the cell line: MDA-MB231 and  
398 BT549, ~7 days; HCC1806, ~14 days; SKBR3, ~14-21 days; T47D, ~21 days; MDA-MB468,  
399 CAMA1 and MCF7, ~28 days) post-plating until foci became visible. Cells were washed with  
400 PBS twice, fixed with 100% methanol, dried, and stained with Giemsa staining reagent (Sigma  
401 Aldrich). Stained plates were scanned and surface areas occupied by cell foci were measured  
402 using ImageJ software (NIH, Bethesda, MD).

### 403 **Soft agar transformation assay**

404 Equal volumes of 2X concentrated DMEM culture media and 1% noble-agar solution  
405 (made with sterile cell-culture-grade water) were mixed to make 0.5% agar-media solution and  
406 plated in the bottom of six-well plates. Equal volumes of 2X concentrated DMEM culture media  
407 and 0.6% noble-agar solution were mixed to make 0.3% agar-media solution for cell suspension.  
408  $2-5 \times 10^4$  cells were suspended in 0.3% agar-media solution and layered, in triplicate, onto the  
409 bottom layer. Cells were fed with fresh media every 7 days and incubated in 37°C for 21-30

410 days, before being stained with 0.005% crystal violet and examined under a microscope.  
411 Colonies bigger than 100  $\mu$ M in diameter were manually counted.

## 412 **Mammary gland orthopedic implantation**

413 The abilities of human breast cancer cell lines to form tumors *in vivo* were evaluated by  
414 performing mammary gland orthopedic implantation as described previously (Brenot et al.,  
415 2018). Immuno-deficient NOD scid gamma (NSG) female mice at 6-8 week-old were purchased  
416 from Jackson Laboratory (Bar Harbor, ME) and used for this experiment.  $1-3 \times 10^6$  cells were  
417 harvested and resuspended in PBS, mixed with standard base-membrane Matrigel Matrix  
418 (Corning, MA, USA) at 1:1 volume ratio, and kept at 4°C until implantation. In total 100 $\mu$ l of  
419 cells-Matrigel solutions were injected into the right inguinal mammary glands of NSG mice,  
420 which were monitored closely to observe tumor formation. Mice were euthanized before tumors  
421 in control groups reached 2 cubic cm in size, and palpable tumors were dissected from the mice  
422 for weight measurement. All animal-related experimental procedures were performed in  
423 compliance with the guidelines given by the American Association for Accreditation for  
424 Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of  
425 Laboratory Animals. All animal studies were approved by the Washington University  
426 Institutional Animal Care and Use Committee (IACUC) in accordance with the Animal Welfare  
427 Act and NIH guidelines (Protocol 20160916)

## 428 **Statistical analysis**

429 Unless otherwise stated, the two-tailed unpaired Student t test was performed for  
430 statistical analysis. All *in vitro* and *in vivo* data are reported as the mean  $\pm$  SD unless stated  
431 otherwise, Statistical analyses were performed using GraphPad Prism. P values are as indicated:  
432 \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ; n.s., not significant.

### 433 **Polysome profiling**

434           Either MDA-MB231 or HCC1806 cells were treated with 100 µg/mL cycloheximide in  
435 growth media for 5 minutes at 37 °C. The cells were washed with ice-cold 1x PBS containing  
436 100 µg/mL cycloheximide prior to harvesting by scraping. The cells were lysed in polysome  
437 lysis buffer (20 mM Tris pH 7.26, 130 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5% NP-40, 0.2 mg/mL  
438 heparin, 200 U/mL RNasin, 2.5 mM DTT, 1x HALT, 100 µg/mL cycloheximide, 0.5% sodium  
439 deoxycholate) for 20 minutes on ice prior to clarification at 8000 g for 10 minutes at 4 °C. The  
440 absorbance at 260 nm was determined for each lysate. An equal number of A260 units for each  
441 lysate was overlaid on a 10-50% sucrose gradient (10 mM Tris pH 7.26, 60 mM KCl, 10 mM  
442 MgCl<sub>2</sub>, 2.5 mM DTT, 0.2 mg/mL heparin, 10 µg/mL cycloheximide). The gradients were  
443 subjected to centrifugation at 30,000 RPM for 3 hours at 4 °C. The absorbance at 254 nm was  
444 measured along the gradient using a fractionation system (Teledyne ISCO).

### 445 **Analysis of rRNA integrity**

446           For analysis of rRNA integrity, total RNA isolated from cells of interest as described  
447 above was denatured in 1x RNA Loading Dye (NEB) containing 100 ng/µL ethidium bromide  
448 by incubation at 65 °C for 10 minutes. The denatured RNA was resolved on a 1.5% denaturing  
449 formaldehyde agarose gel as described previously (Rio, 2015).

### 450 **Analysis of CCLE RNAseq Data and ADAR1 Dependency**

451           Raw CCLE RNaseq count data from breast cancer cell lines were normalized by the  
452 ‘cpm’ function of ‘edgeR’(Robinson et al., 2010). From the cpm values z-scores were  
453 determined for each gene across all cell lines. To determine ‘ISG Core Score’ or ‘ISG Score’ we  
454 calculated the median z-score of previously identified ‘Core ISGs’ (Liu et al., 2019) or all ISGs  
455 defined by the GSEA/mSigDB hallmark gene set collection

456 (HALLMARK\_INTERFERON\_ALPHA\_RESPONSE and  
457 HALLMARK\_INTERFERON\_GAMMA\_RESPONSE) (Liberzon et al., 2015) . Molecular  
458 subtypes of breast cancer cell lines were defined previously (Marcotte et al., 2016).

#### 459 **Analysis of TCGA RNAseq Data**

460 Unnormalized RSEM values were normalized by the ‘cpm’ function of edgeR(Robinson  
461 et al., 2010). From the cpm values modified z-scores were determined using the following  
462 formula.

$$z = \frac{[(cpm \text{ gene } X \text{ in breast cancer sample}) - (mean \text{ gene } X \text{ in normal})]}{(standard \text{ deviation } X \text{ in normal})}$$

463 We calculated ‘ISG Core Score’ and ‘ISG Score’ as described above. Molecular subtypes of  
464 TCGA samples were defined previously (Lehmann et al., 2016).

#### 465 **Data and Code Availability**

466 CCLE RNAseq count data (CCLE\_RNAseq\_genes\_counts\_20180929.gct.gz,  
467 CCLE\_RNAseq\_rsem\_transcripts\_tpm\_20180929.txt.gz) were obtained from the Broad Institute  
468 Cancer Cell Line Encyclopedia and is available online at  
469 <https://portals.broadinstitute.org/ccle/data>. Dependency data  
470 (D2\_combined\_gene\_dep\_scores.csv, Achilles\_gene\_effect.csv) were obtained from Broad  
471 Institute DepMap Portal and is available on at <https://depmap.org/portal/download/>. TCGA  
472 breast cancer RNAseq (illuminahisec\_rnaseqv2-RSEM\_genes, illuminahisec\_rnaseqv2-  
473 RSEM\_isoforms\_normalized) and clinical data (Merge\_Clinical) were obtained from the Broad  
474 Institute FireBrowse and is available online at <http://firebrowse.org/>.

475 All custom R scripts used in this are available upon request. All other data are available  
476 in the main text and figures or supplemental information.

#### 477 **Immunohistochemistry**

478 Human breast formalin fixed paraffin embedded tissue array sections (5 $\mu$ m) on positively  
479 charged slides were obtained from US Biomax Inc. (BC081116d). For immunohistochemistry,  
480 sections were stained using a Bond RXm autostainer (Leica). Briefly, slides were baked at 65°C  
481 for 15min and automated software performed dewaxing, rehydration, antigen retrieval, blocking,  
482 primary antibody incubation, post primary antibody incubation, detection (DAB), and  
483 counterstaining using Bond reagents (Leica). Samples were then removed from the machine,  
484 dehydrated through ethanols and xylenes, mounted and cover-slipped. An antibody for ADAR1-  
485 p150 (Abcam ab126745) was diluted 1:100 in Antibody Diluent (Leica).

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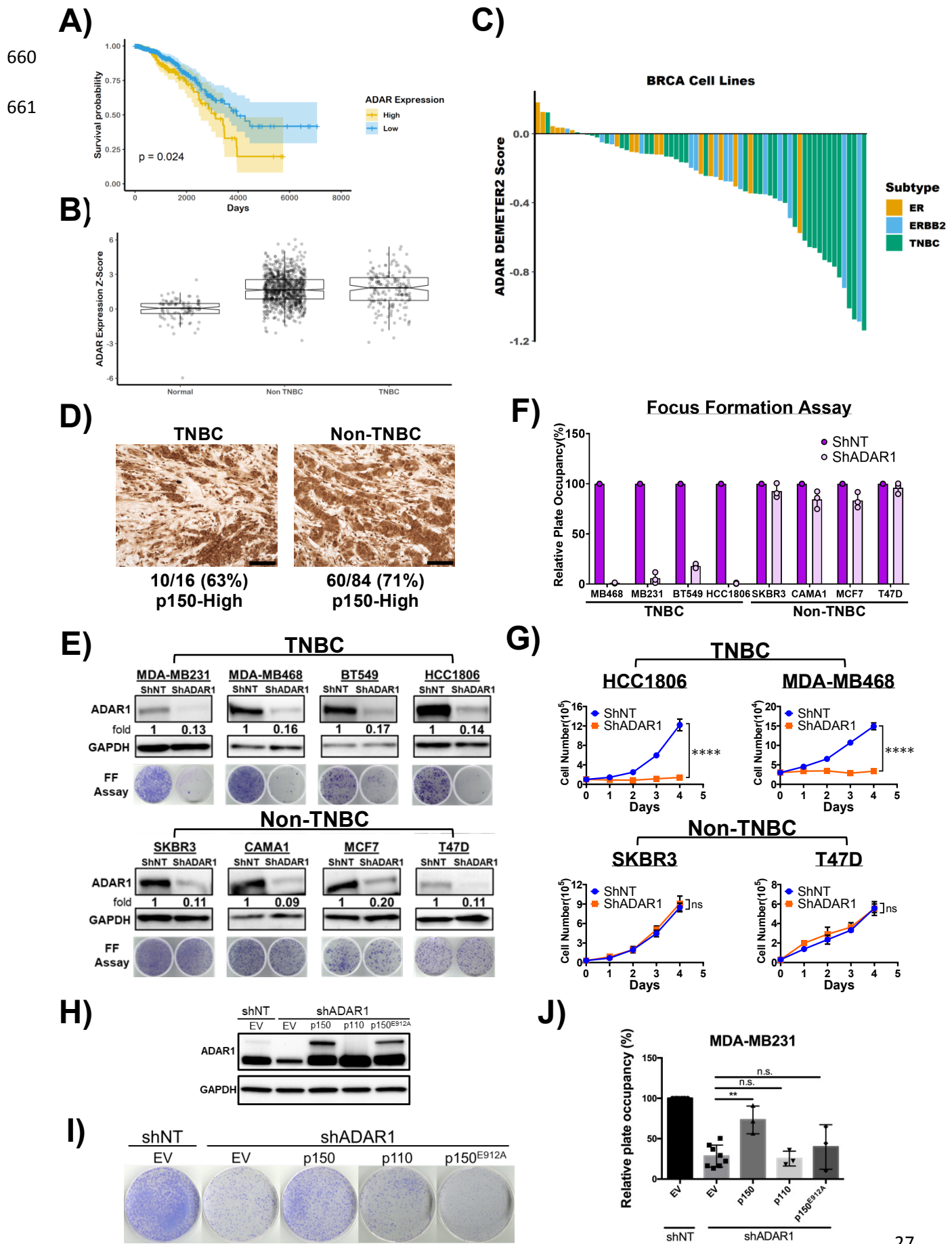


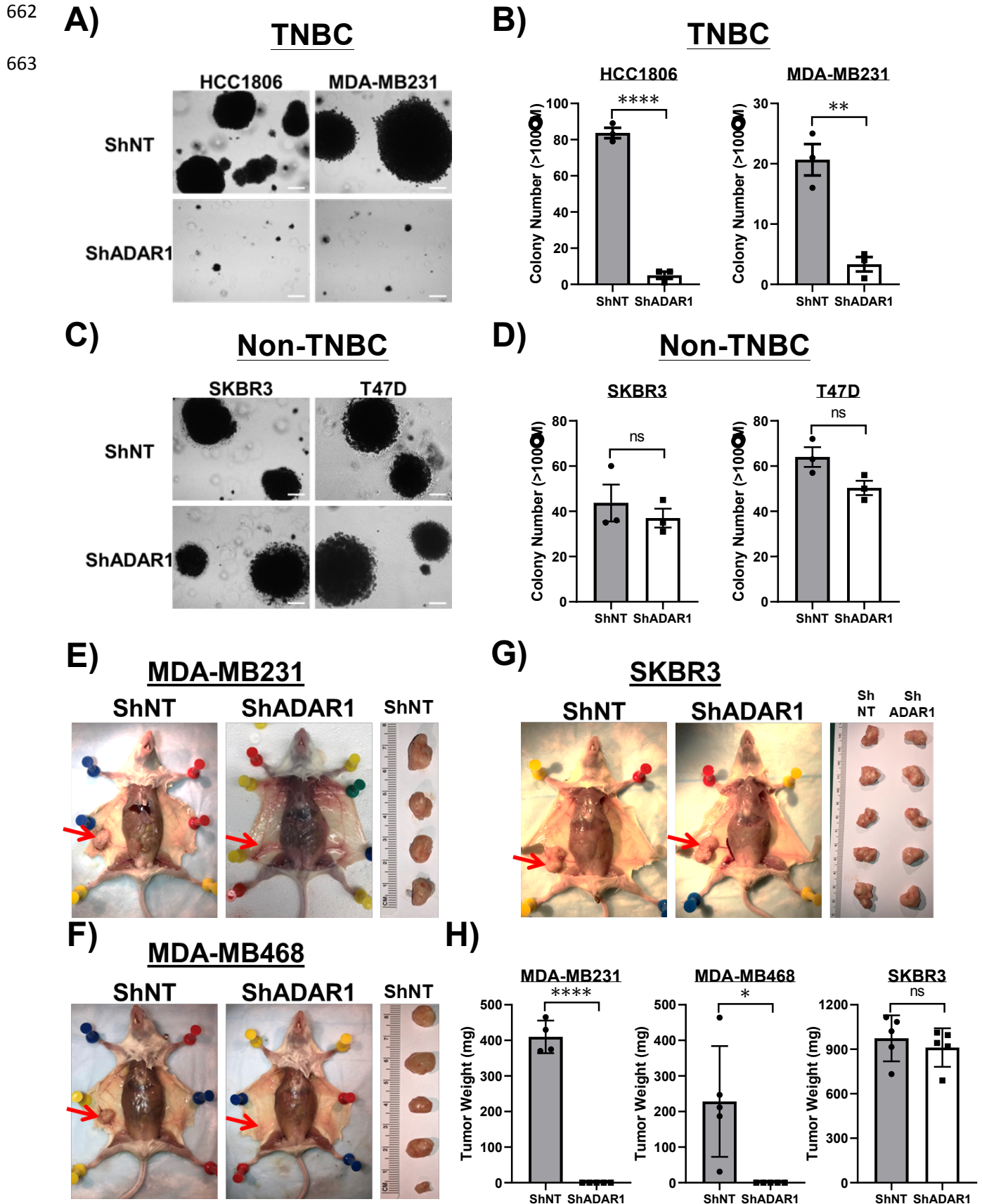
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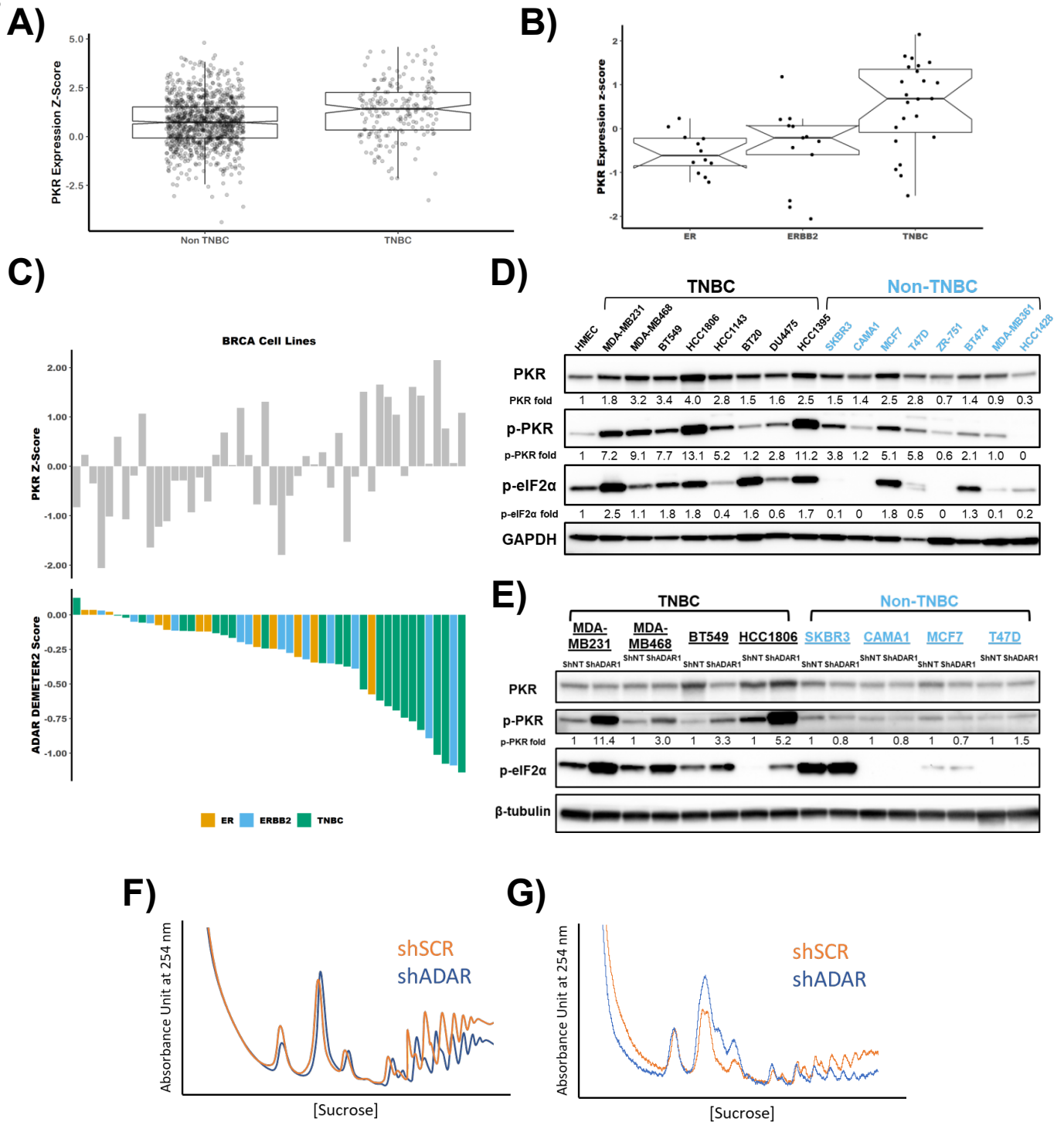
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