ARTICLE

Evaluating the therapeutic potential of ADAR1 inhibition for triple-negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is the deadliest form of breast cancer. Unlike other types of breast cancer that can be effectively treated by targeted therapies, no such targeted therapy exists for all TNBC patients. The ADAR1 enzyme carries out A-to-I editing of RNA to prevent sensing of endogenous double-stranded RNAs. ADAR1 is highly expressed in breast cancer including TNBC. Here, we demonstrate that expression of ADAR1, specifically its p150 isoform, is required for the survival of TNBC cell lines. In TNBC cells, knockdown of ADAR1 attenuates proliferation and tumorigenesis. Moreover, ADAR1 knockdown leads to robust translational repression. ADAR1-dependent TNBC cell lines also exhibit elevated IFN stimulated gene expression. IFNAR1 reduction significantly rescued the proliferative defects of ADAR1 loss. These findings establish ADAR1 as a novel therapeutic target for TNBC tumors.

Introduction

Generally defined by the lack of estrogen receptor (ER), progesterone receptor, and HER2 expression, triple-negative breast cancer (TNBC) accounts for 15–20% of all breast cancer diagnoses in the United States each year [1]. Unlike ER-positive and HER2-positive breast cancers, there are no targeted therapies for all TNBC patients [2]. The lack of targeted therapies for TNBC leaves chemotherapy as the main treatment option, which carries a generally worse prognosis [3]. Efforts to develop effective targeted therapies against TNBC have focused on further

subcategorizing TNBC based on gene expression, as well as looking to exploit common genetic vulnerabilities [4, 5].

A potential therapeutic target for TNBC is adenosine deaminase acting on RNA (ADAR1, encoded by *ADAR*). ADAR1 carries out the enzymatic reaction of deaminating adenosine to inosine within cellular dsRNA, in a process known as A-to-I editing. Induction of ADAR1 expression is prevalent in breast cancer [6–10] and ADAR1-mediated A-to-I editing has been found to influence the levels of its targets in breast cancer [11–14]. Recent studies have indicated that expression of ADAR1 is elevated in TNBC and may be correlated with poor prognosis when RNA editing is increased [15, 16].

ADAR1 acts in a negative feedback loop to inhibit activation of the type I interferon (IFN) pathway triggered by endogenous dsRNAs or dsRNAs introduced upon viral infection [17, 18]. ADAR1 has been shown to suppress type I IFN pathway through multiple mechanisms, including destabilization of the dsRNA structure, reduced expression, and activation of the dsRNA sensors MDA5 and RIG-I, and inhibition of IFN expression [17–22]. ADAR1-mediated A-to-I RNA editing by the IFN-inducible p150 isoform (not the constitutive p110 isoform) is essential for its ability to modulate dsRNA-induced IFN signaling [18–20]. ADAR1's ability to regulate this response was recently linked to the development of ADAR1 dependency in some cancer cell lines; two groups showed that by removing

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ADAR1 from cancer cells with elevated IFN signaling, cells became susceptible to inflammation-induced cell death [23, 24]. This is consistent with previous findings that ADAR1 prevents immune and translational catastrophes by blocking dsRNA-activated pathways [17, 25].

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

Results

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

Using publicly available data from The Cancer Genome Atlas (TCGA) [6, 7], we found that high expression of ADAR1 correlated with poor prognosis of breast cancers (Fig. 1A). Recent studies indicated that ADAR1 promotes tumorigenesis of metaplastic breast cancers, and that high expression of ADAR1 correlates with poor prognosis in basal-like breast cancers [13, 16]. Since both basal-like and metaplastic breast cancers share similar characteristics with TNBC, we sought to determine the importance of ADAR1 in the tumorigenesis of TNBC. By evaluating the TCGA database, we found that while mRNA expression of ADAR1 was higher in TNBC compared to normal, it was not significantly different between TNBC and non-TNBC tumors (Fig. 1B). In addition, ADAR1 expression was not significantly higher in any one subtype of breast cancer based on PAM50 classification [26] (Fig. 1C). This observation is consistent with data from the Cancer Cell Line Encyclopedia (CCLE), which uses both RNA-seq and reverse phase protein array to determine RNA and protein expression levels in numerous cancer cell lines (Fig. 1D and Supplementary Fig. S1a). Data from both the TCGA and CCLE datasets also revealed that both p150 and p110 isoforms of ADAR1 were expressed at similar levels between TNBC and non-TNBC specimen (Fig. 1E and Supplementary Fig. S1b–e), with p110 expression being consistently higher than p150 in all samples. The lack of distinctive expression patterns for p150 and p110 between TNBC and non-TNBC was also confirmed by performing quantitative RT-PCR in a panel of breast cancer cell lines (Supplementary Fig. S1f, g). To independently investigate ADAR1 expression in BRCA tumor samples, we assessed p150 isoform expression by immunohistochemistry in TNBC and non-TNBC patient tumors using an antibody that specifically recognizes only the p150 isoform (Fig. 1F and Supplementary Fig. S1h). Consistent with RNA-seq data from TCGA, p150 was upregulated in the majority of

TNBC and non-TNBC tumors compared to normal tissues. Next, we sought to determine the protein expression level of the ADAR1-p150 isoform in a panel of established breast cancer cell lines representing TNBC and non-TNBC. Immunoblot analysis showed that ADAR1 (p150 isoform) is overexpressed, compared to normal human mammary epithelial cells (HMECs), in over half of all TNBC (6/8) and non-TNBC (5/8) cell lines assayed (Fig. 1G). These results indicate that ADAR1-p150 is overexpressed in many breast cancer cell lines regardless of subtype.

ADAR1 is required for TNBC proliferation

Several recent studies have suggested that some established cancer cell lines display strong dependencies on ADAR1 expression [23, 24, 27]. Given the high expression of ADAR1-p150 in most breast cancer cell lines, we sought to determine whether these breast cancer cell lines exhibit ADAR1 dependency. We analyzed publicly available RNAi and CRISPR-Cas9 datasets to determine if ADAR1 was required for the survival of breast cancer cell lines representing various subtypes [28, 29]. TNBC and basal-like cell lines made up the majority of breast cancer cells exhibiting high ADAR1 sensitivity scores (DEMETER2 score < −0.5) (Fig. 2A and Supplementary Fig. S2a–c). Importantly, we did not observe a correlation between ADAR1 expression and ADAR1 dependency across these breast cancer cell lines (Supplementary Fig. S2d). This lack of correlation was noted for both p110 and p150 isoforms of ADAR1 (Supplementary Fig. S2e, f). To independently validate ADAR1 dependency among breast cancer cell lines, we knocked down ADAR1 expression in eight cell lines (four TNBC: MDA-MB231, MDA-MB468, BT549, and HCC1806; four non-TNBC: SKBR3, CAMA1, MCF7, and T47D); all of these cell lines showed noticeable ADAR1-p150 isoform overexpression over HMEC controls in our immunoblot analysis (Fig. 1G). Long-term (7–28 days, foci formation) and short-term (4 days, growth curve) cell proliferation was evaluated for each cell line following ADAR1 knockdown. Notably, similar levels of ADAR1 knockdown were achieved for each cell line (Fig. 2B). All four TNBC cell lines displayed significant attenuation in both long- and short-term proliferation following ADAR1 knockdown (Fig. 2C, D and Supplementary Fig. S2g). Conversely, ADAR1 expression proved dispensable for proliferation in all four non-TNBC cell lines.

Previous work has shown that loss of ADAR1 leads to cell death via apoptosis [30, 31]. Knockdown of ADAR1 in two TNBC cell lines (HCC1806 and MB231) caused apoptosis (Fig. 2E–G and Supplementary Fig. S3a–d). Apoptosis induction was detected by co-staining of Annexin V and propidium iodide, as well as immunoblot analysis showing increased levels of cleaved PARP. Taken
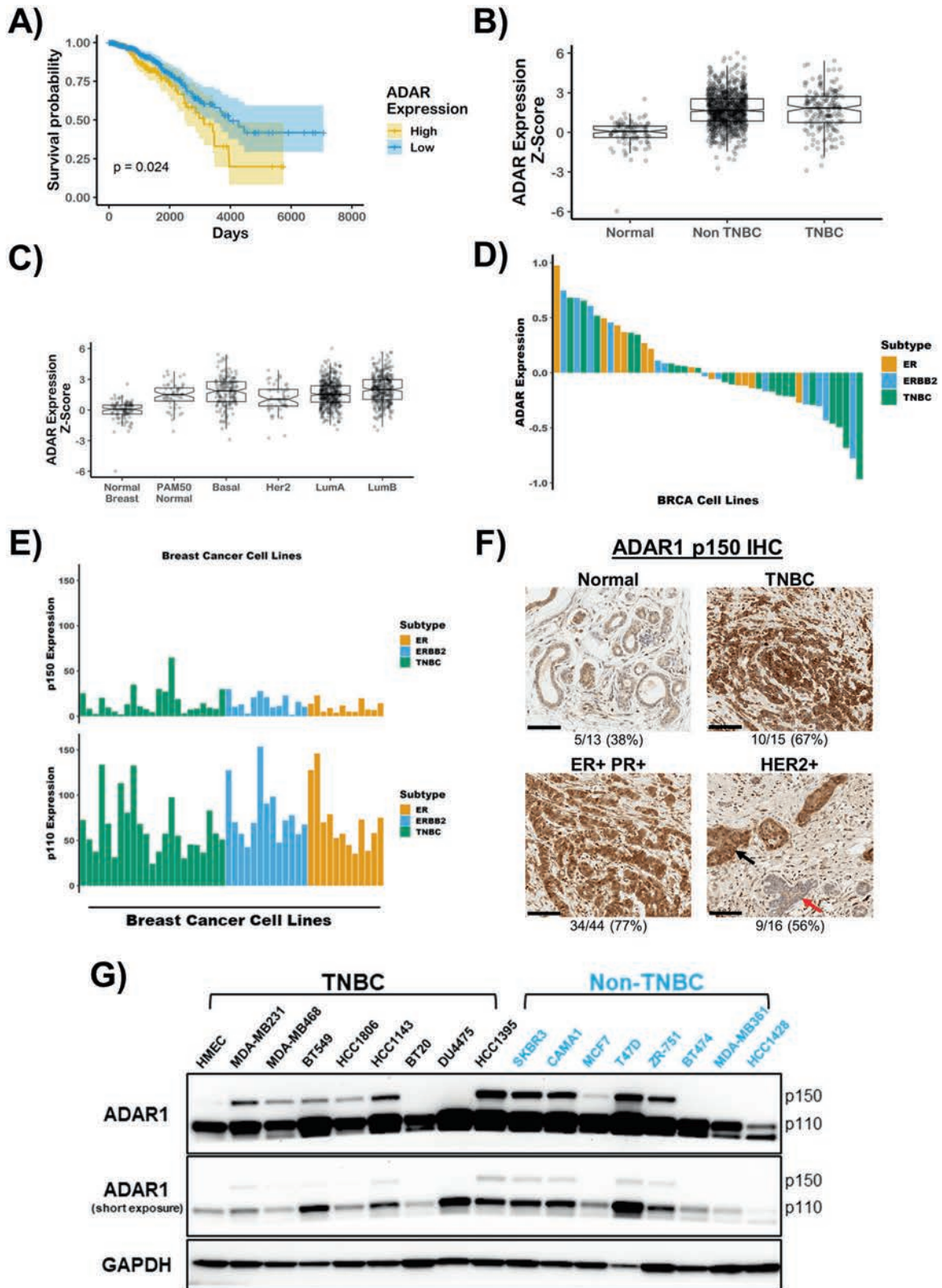
together these data show that ADAR1 is essential for the survival and growth of many TNBC cells.

As expected, knockdown of ADAR1 reduced RNA editing level of its targets, such as antizyme inhibitor 1 (AZIN1) (Supplementary Fig. S3e, f). Edited AZIN1 (S367G) has been shown to block antizyme-mediated degradation of ornithine decarboxylase and cyclin D1, leading to increased cell proliferation, tumor initiation, and

◀ **Fig. 1 ADAR1 is highly expressed in all breast cancer subtypes.**
A Kaplan–Meier survival curves of breast cancer patients. Patients
were stratified by ADAR1 expression, above or below z-score = 2.34.
B, C Z-score modified mRNA expression of ADAR1 in normal,
TNBC and non-TNBC breast cancer, or by PAM50 classification.
LumA luminal A, LumB luminal B. Data were extracted from TCGA
database. Tumor classification described previously [26]. **D** Protein
expression of ADAR1 in breast cancer cell lines. ERBB2 = HER2.
Data were extracted from CCLE database. **E** mRNA expression of
ADAR1-p150 and -p110 isoforms in breast cancer cell lines. Data
were extracted from CCLE database. **F** Representative images of IHC
staining of ADAR1 (p150 isoform) in normal, TNBC and non-TNBC
(ER+PR+ and HER2+) breast cancer tissues (scale bar: 100 μM).
Numbers below the image indicate the ratio of samples identified as
high p150-ADAR1 based on IHC scoring. Black and red arrows in the
HER2+ image point to adjacent p150-high cancerous and p150-low
noncancerous ductal epithelial cells, respectively. **G** Immunoblots
showing protein levels of ADAR1 and GAPDH (loading control) in
breast cancer cell lines. Images are representative of three replicates.

metastasis [32, 33]. To determine if *AZIN1* editing con-
tributes to TNBC-associated ADAR1 dependency, we per-
formed RNA editing site-specific quantitative PCR to assess
the editing level of *AZIN1* with or without ADAR1
knockdown [34]. The editing level of AZIN1 is generally
lower in TNBC compared to non-TNBC cell lines, and
ADAR1 knockdown did not result in more significant
reduction of AZIN1 editing in TNBC cell lines (Supple-
mentary Fig. S3f, g). These data suggest that it is unlikely
that ADAR1-mediated *AZIN1* editing contributes to the
TNBC-associated ADAR1 dependency.

ADAR1-p150 is required for TNBC proliferation

While both isoforms of ADAR1 are expressed in TNBC,
our knockdown experiment does not distinguish between
p150 or p110 dependence. To address this, we set up a
knockdown-rescue system. We overexpressed either the
p110 or p150 isoform following ADAR1 knockdown in
HCC1806 and MDA-MB231 cells and evaluated their
ability to rescue cell proliferation. Overexpression of p110
and p150 caused increased editing of MDM2 mRNA as
determined by Sanger sequencing of cDNA (Supplementary
Fig. S3h, i). Overexpression of ADAR1-p150, but not p110,
resulted in significant rescue of cell proliferation in
HCC1806 and MDA-MB231 TNBC cells (Fig. 2H–J and
Supplementary Fig. S3j–l). Having established a rescue
system for ADAR1-dependent proliferation, we next aimed
to determine whether the editing activity of ADAR1-p150
was required for this rescue. Overexpression of the editing-
defective mutant (E912A) p150 had no effect on editing of
MDM2 mRNA (Supplementary Fig. S3h, i). Over-
expression of p150-E912A was capable of rescuing the
ADAR1 knockdown phenotype in HCC1806 to the same
extent as WT p150 (Fig. 2H–J). For the MDA-MB231 cell
line, p150-E912A rescued to a lesser extent than WT p150

(Supplementary Fig. S3j–l). These data indicate that
dependency on A-to-I editing by ADAR1-p150 isoform
varies across TNBC cell lines.

ADAR1 is required for TNBC transformation and tumorigenesis

To assess the functional relevance of our findings, we
investigated the requirement of ADAR1 for the transfor-
mation of breast cancer cell lines. We utilized anchorage-
independent growth in soft agar as a measure of cellular
transformation. Knockdown of ADAR1 dramatically
reduced soft agar colonies of MDA-MB231 and HCC1806
TNBC cells while not significantly affecting the numbers of
colonies formed by SKBR3 and T47D non-TNBC cells
(Fig. 3A–D). The effect of ADAR1 knockdown on
anchorage-independent growth could be rescued by WT and
editing defective ADAR1-p150 in HCC1806 cells (Fig. 3E,
F), implying that ADAR1 does possess critical non-editing
functions.

To extend these in vitro findings, we next determined
whether ADAR1 was required for TNBC cell lines to form
tumors in vivo. We performed mammary gland orthotopic
transplantations using TNBC and non-TNBC cells follow-
ing ADAR1 knockdown. MDA-MB231, MDA-MB468,
and SKBR3 cells were all able to form visible tumors in the
mammary glands of independently transplanted female
immune compromised mice (Fig. 3G–J). Knockdown of
ADAR1 in MDA-MB231 and MDA-MB468 TNBC cells
completely abrogated their ability to form tumors in trans-
planted mice. In contrast, ADAR1 knockdown in SKBR3
cells did not significantly affect tumor formation in trans-
planted mammary glands. Collectively, these results
demonstrate that ADAR1 expression is required for in vitro
transformation and in vivo tumor formation of TNBC cells
but is completely dispensable for these properties in non-
TNBC cells.

PKR is overexpressed in TNBC and activated upon ADAR1 loss

Previous reports have shown that ADAR1 dependency in
human cancer cells could be mediated through several
downstream pathways, including translational inhibition
triggered by activated PKR or ribonuclease L (RNASEL),
as well as type I IFN signaling [23, 24, 35]. To investigate if
these pathways contribute to the ADAR1 dependency
observed in TNBC cells, we first analyzed the TCGA and
CCLE datasets to determine if these pathways are intrinsi-
cally elevated in TNBC. Across TCGA breast cancer sam-
ples, RNA expression of PKR is significantly higher in
TNBC samples compared to non-TNBC (Fig. 4A). This is
consistent with RNA-seq data for breast cancer cell lines
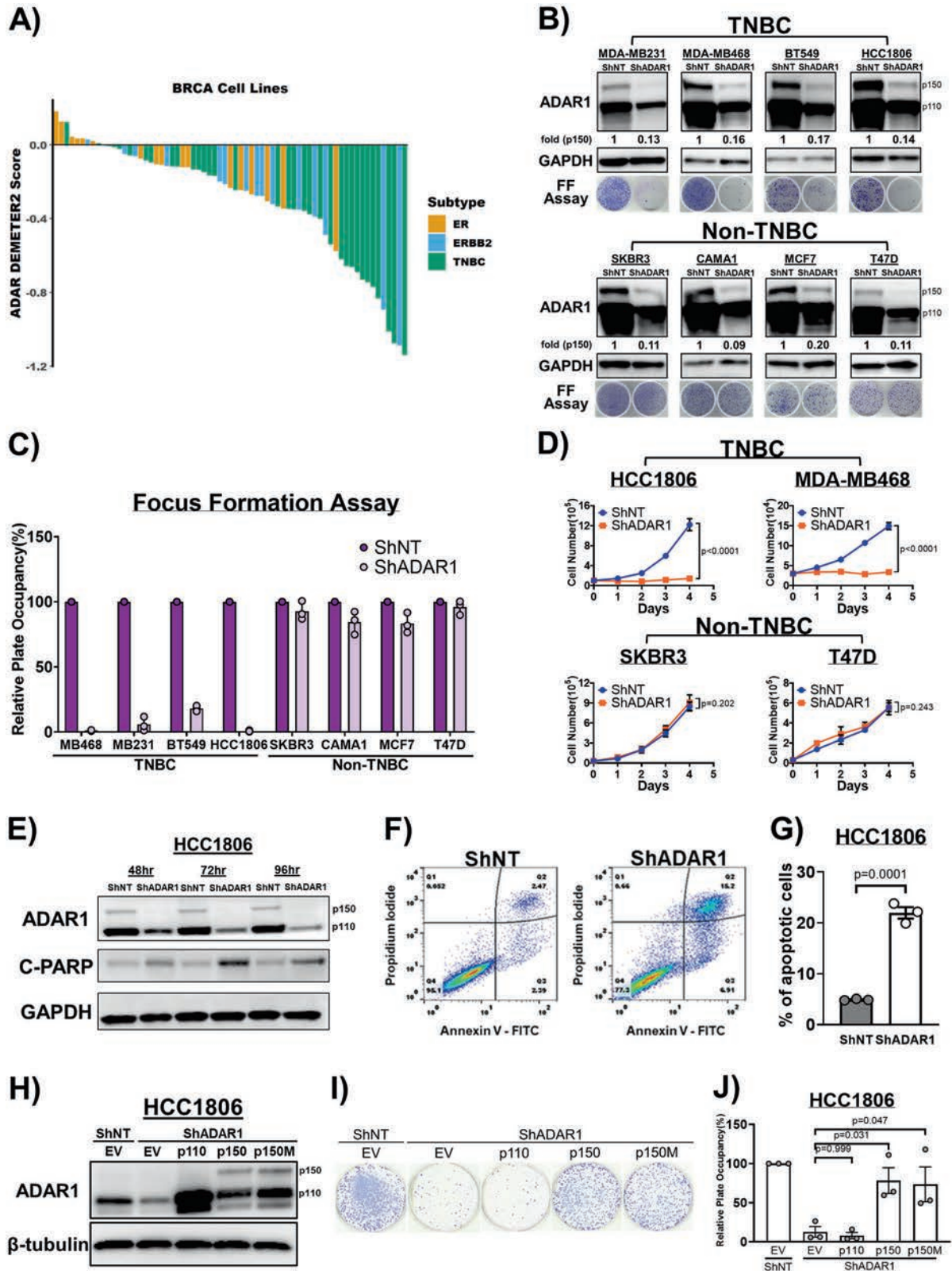
within the CCLE (Fig. 4B). Moreover, elevated PKR expression positively correlates with the ADAR1 sensitivity scores, suggesting a strong relationship between PKR and TNBC-associated ADAR1 dependency (Fig. 4C and Supplementary Fig. S4a–c). We further confirmed this observation by immunoblot analysis among our panel of sixteen

◀ **Fig. 2 ADAR1 is required for TNBC survival and proliferation.**
A ADAR1 dependency scores in breast cancer cell lines. Lower
DEMETER2 scores indicate stronger ADAR1 dependency. ERBB2 =
HER2. **B** Immunoblots showing protein levels of ADAR1 and
GAPDH (loading control) with or without ADAR1 knockdown in
breast cancer cell lines. Fold change of ADAR1-p150 isoform (ShA-
DAR1/ShNT) is indicated, normalized to GAPDH. Focus formation
(FF) assay showed that ADAR1 knockdown reduced proliferation of
TNBC but not non-TNBC cells. Images are representative, $N = 3$.
C Quantification of FF in **B**. Relative plate occupancy was determined
using ImageJ software and normalized to ShNT samples for each cell
line. Data are represented as mean ± SD, $N = 3$. **D** Cell proliferation
assay showing that ADAR1 knockdown reduced proliferation of
TNBC but not non-TNBC cells. Data are represented as mean ± SD,
$N = 2$. **E** Immunoblots showing protein levels of ADAR1, GAPDH
(loading control), and cleaved PARP (C-PARP; apoptosis marker) in
ShADAR1-treated HCC1806 cells. **F** Flow cytometry analysis with
propidium iodide (PI) and Annexin V-FITC (AV) staining to
detect portions of apoptotic cells in ShNT- or ShADAR1-treated
HCC1806 cells. $PI^{low}AV^{low}$: live cells. $PI^{low}AV^{high}$: early apoptotic
cells. $PI^{high}AV^{high}$: late apoptotic cells. **G** Quantification of relative
level of apoptosis (early + late) in ShNT- or ShADAR1-treated
HCC1806 cells in **F**. Data are represented as mean ±±±± SD, $N = 3$.
H Immunoblots showing protein levels of ADAR1 and β-tubulin
(loading control) with overexpression of p150, p110, or editing-
defective $p150^{E912A}$ (p150M) in ShADAR1-treated HCC1806 cells.
Images are representative, $N = 3$. EV empty virus. **I** FF assay showing
that p150 and p150M, but not p110, partially rescued proliferation of
ShADAR1-treated HCC1806 cells. Images are representative, $N = 3$. **J**
Quantification of FF in **I**. Relative plate occupancy was determined
using ImageJ software and normalized to ShNT-EV. Data are repre-
sented as mean ± SD, $N > 3$.

breast cancer cell lines which showed a general elevation of
PKR expression across all TNBC cell lines (Fig. 4D). We
also detected heightened levels of PKR phosphorylation as
well as its substrate alpha subunit of eukaryotic translation
initiation factor 2 in TNBC cells compared to non-TNBC
cells. Upon ADAR1 knockdown, phosphorylation of PKR
and eIF2α was markedly induced in all TNBC cell lines but
remained unchanged in the non-TNBC cell lines (Fig. 4E).
These observations suggest that TNBC-associated ADAR1
dependency might be facilitated by PKR activation. We
observed another potential connection between PKR-eIF2α
signaling and ADAR1 dependency by comparing levels of
PKR and eIF2α phosphorylation in aforementioned rescue
experiments using different isoforms of ADAR1 (Fig. 2H–J
and Supplementary Fig. S3j–l). Overexpression of WT
p150 and p150-E912A, both capable of rescuing ADAR1-
dependent phenotypes in TNBC cells, resulted in decreased
phosphorylation of PKR and eIF2α (Fig. 4F).

Activation of PKR causes global translational repression
through phosphorylation of eIF2α [36]. To investigate if
translational repression occurs following ADAR1 knock-
down, we performed polysome profiling. ADAR1 knock-
down in MDA-MB231 and HCC1806 TNBC cells leds to
inhibition of translation, demonstrated by the substantial
reduction of polysome peaks (Fig. 4G and Supplementary
Fig. S4d). These results suggest that translational repression

may contribute to TNBC-associated ADAR1 dependency,
however it is not clear if the observed translational repres-
sion is caused by PKR activation. To address this, we
attempted a rescue experiment by knocking down PKR in
conjunction with ADAR1 knockdown. While attempting
this experiment in MDA-MB231 and HCC1806, we
observed that knockdown of PKR alone greatly reduced
foci formation (Supplementary Fig. S4e, f). Treatment of
MDA-MB231 with a PKR inhibitor also caused reduced
foci formation (Supplementary Fig. S4g). These data sug-
gest that basal PKR expression and activity is required for
the proliferation of these cell lines, thus precluding us from
directly determining if expression of PKR is required for the
ADAR-knockdown phenotype, or if increased PKR activity
drives translational repression following ADAR
knockdown.

Due to the essentiality of PKR in the cell lines used in
this study, we used a pharmacological approach to blunt the
effects of eIF2α phosphorylation by PKR. We used the
small molecule ISRIB, which inhibits the translational
repressive function of p-eIF2α [37]. ISRIB was only cap-
able of modestly rescuing the proliferation defect of
ADAR1 knockdown in HCC1806 (Fig. 4H and Supple-
mentary Fig. S4h) but not MDA-MB231 cells (data not
shown). These data suggest that phosphorylation of eIF2α
by PKR, following ADAR knockdown, contributes only
modestly to reduced proliferation and is cell-line-dependent.

An important downstream effector of PKR activation is
the pro-survival gene ATF4. Unlike most cellular mRNAs,
the ATF4 mRNA is translationally upregulated following
activation of PKR and phosphorylation of eIF2α [38].
Because of the pro-survival role of ATF4, it is possible that
it may play a role in protecting cells from cellular death
following ADAR1 knockdown and PKR activation.
Assessment of ATF4 expression across breast cancer cell
lines revealed no correlation between ATF4 expression and
ADAR1 dependency (Supplementary Fig. S5a, b). There
was no clear pattern in ATF4 protein expression between
TNBC and non-TNBC following ADAR1 knockdown,
suggesting that ATF4 expression alone cannot be used to
determine ADAR1 dependency in TNBC cells (Supple-
mentary Fig. S5c). Together these data suggest that ATF4
expression does not contribute to ADAR dependency or
growth inhibition following ADAR1 knockdown.

RNASEL is not activated following loss of ADAR1 in TNBC

Activation of RNASEL and subsequent translational inhi-
bition has also been shown to result in cell lethality upon
ADAR1 loss [35]. The CCLE dataset indicated that RNA-
SEL activators OAS1, OAS2, and OAS3 were highly
expressed in ADAR1-dependent cell lines, while the
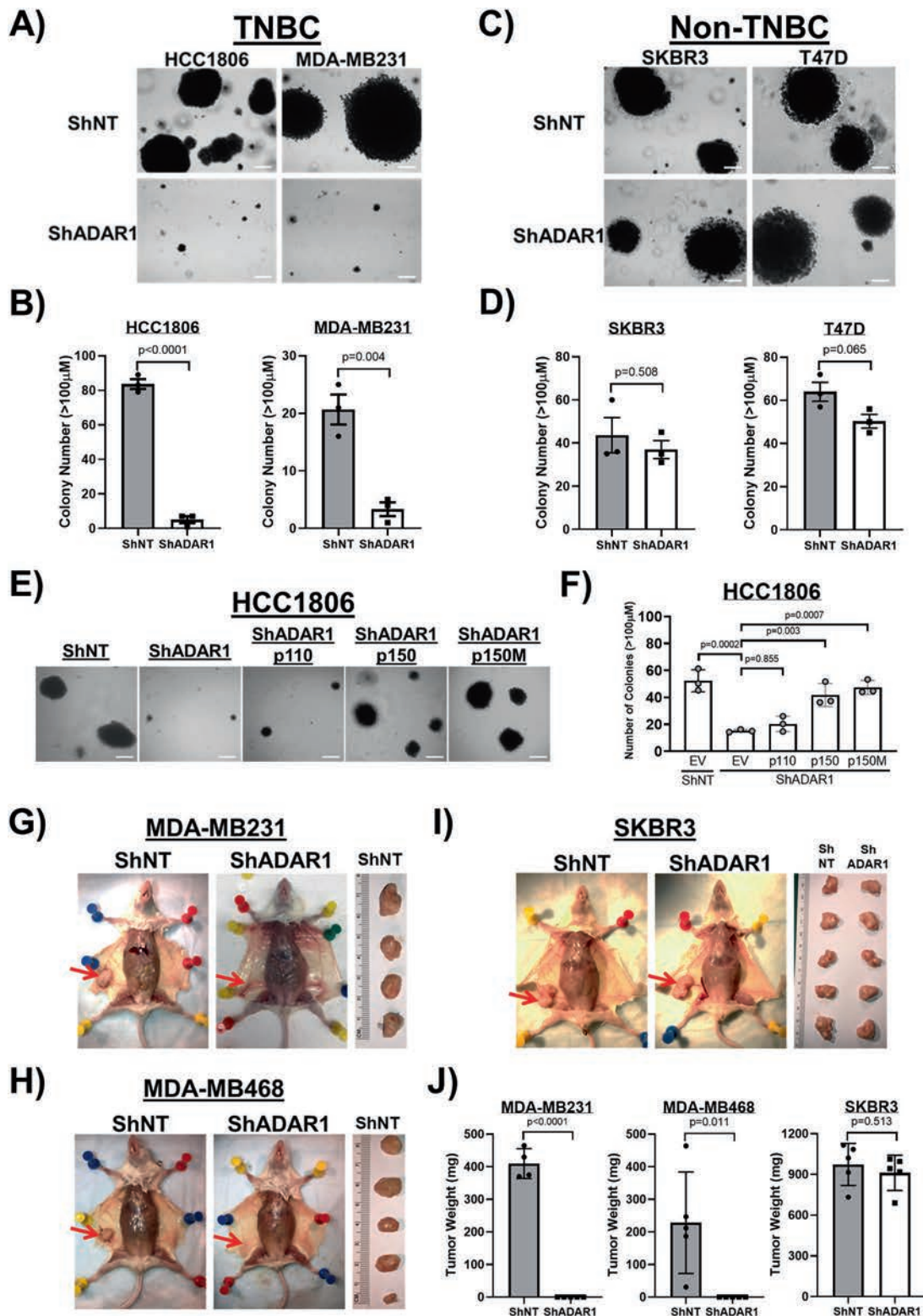
expression of *RNASEL* showed modest correlation with ADAR1 dependency (Supplementary Fig. S5d, e). A hallmark of RNASEL activation is degradation of rRNA [39]. However, we did not observe rRNA degradation in ADAR1-dependent TNBC cells after ADAR1 knockdown (Supplementary Fig. 5f), further suggesting that the RNASEL pathway does not significantly contribute to TNBC-associated ADAR1 dependency and the induction of OAS genes likely reflects the fact that OAS genes are known ISGs (see below).

◀ **Fig. 3 ADAR1 is required for TNBC transformation and tumorigenesis. A** Soft agar assay (SAA) showing that ADAR1 knockdown reduced anchorage-independent growth of TNBC cells (HCC1806 and MDA-MB231). Images are representative, $N = 3$. Scale bar, 100 μM. **B** Quantification of SAA in **A**. Colonies bigger than 100 μM in diameter were counted. Data are represented as mean ± SD, $N = 3$. **C** SAA showing that ADAR1 knockdown did not affect anchorage-independent growth of non-TNBC cells (SKBR3 and T47D). Images are representative, $N = 3$. Scale bar, 100 μM. **D** Quantification of SAA in **C**. Colonies bigger than 100 μM in diameter were counted. Data are represented as mean ± SD, $N = 3$. **E** SAA showing that overexpression of p150 and p150M, but not p110, partially rescued reduced anchorage-independent growth of HCC1806 cells due to ADAR1 knockdown. Images are representative, $N = 3$. Scale bar, 100 μM. **F** Quantification of SAA in **E**. Colonies bigger than 100 μM in diameter were counted. Data are represented as mean ± SD, $N = 3$. **G** Orthotopic implantation of MDA-MB231 cells into abdominal mammary fat pad. Tumors were removed from the mice ~4 weeks post injection and weighed (ShNT, $N = 4$; ShADAR1, $N = 5$). Red arrows indicate the location of mammary fat pad. **H** Orthotopic implantation of MDA-MB468 cells into abdominal mammary fat pad. Tumors were removed from the mice ~12 weeks post injection and weighed ($N = 5$). Red arrows indicate the location of mammary fat pad. **I** Orthotopic implantation of SKBR3 cells into abdominal mammary fat pad. Tumors were removed from the mice ~4 weeks post injection and weighed ($N = 5$). Red arrows indicate the location of mammary fat pad. **J** Quantification of the result shown in **G–I**. Data are represented as mean ± SD.

ADAR1-dependent TNBCs exhibit elevated ISG expression

Another factor contributing to ADAR1 dependency in cancer cell lines is the type I IFN pathway [24]. It has been shown previously that this connection is mediated through either altering the expression of type I IFN regulators or activating the feed-forward loop of IFN signaling [23, 24]. RNA expression data from the TCGA and CCLE datasets showed that TNBC has higher ISG expression (core ISG score [24]) compared to non-TNBC (Fig. 5A, B). This is consistent with the elevated expression of PKR and ISG15 in our immunoblot analysis among breast cancer cell lines (Fig. 4D and Supplementary Fig. S6a). Like PKR expression, the core ISG score positively correlated with ADAR1 sensitivity among TNBC cell lines (Fig. 5C and Supplementary Fig. S6b, c).

INFAR1 loss rescues ADAR1-knockdown phenotype

To establish whether the type I IFN pathway accounts for the significant differences of ADAR1 dependency between TNBC and non-TNBC cell lines, non-TNBC cell lines (SKBR3 and MCF7) were treated with IFNβ in ADAR1-intact and ADAR1-deficient cells (Supplementary Fig. S6d–g). Expression of ADAR1 and ISG15 was induced upon IFNβ treatment, as well as phosphorylation of STAT1. However, while the treatment of IFNβ generally reduced

cell proliferation, it did not sensitize non-TNBC cells to ADAR1 deficiency (Supplementary Fig. S6e, g), implying that IFNβ alone is not capable of switching ADAR1-resistant cells to ADAR1-dependent cells.

To determine if the type I IFN pathway functionally contributes to ADAR1 dependency in TNBC, we knocked down ADAR1 and the IFN alpha-receptor subunit 1 (IFNAR1) simultaneously in both MDA-MB231 and MDA-MB468 cells (Fig. 5D and Supplementary Fig. S6h). The knockdown of IFNAR1 partially rescued the proliferation of both cell lines, suggesting that TNBC-associated ADAR1 dependency can be partially attributed to type I IFN pathway activation (Fig. 5E, F and Supplementary Fig. S6i). However, knockdown of IFNAR1 in TNBC cells did not alter the levels of phosphorylated PKR (Fig. 5D and Supplementary Fig. S6h), suggesting that in these TNBC cells, type I IFN and PKR pathways might independently contribute to ADAR1 dependency.

Discussion

Recent studies have highlighted the dependence of some cancer cell lines on ADAR1 expression [23, 24]. Here we characterized the requirement for ADAR1 in a panel of established breast cancer cell lines. ADAR1-dependent cell lines shared an elevated ISG-expression signature. Loss of ADAR1 in these cell lines leds to activation of the translational repressors PKR and eIF2α, as well as translational repression. The ADAR1-dependence phenotype could be partially abrogated by knockdown of IFNAR1.

It is not currently understood what makes select cancer cell lines ADAR1-dependent, or conversely why others are refractory to ADAR1 loss. It has been proposed that the higher ISG expression might potentiate these cells toward ADAR1 dependency—loss of ADAR1 would further elevate ISG expression leading to the growth inhibition phenotype [23, 24]. However, we have demonstrated that for cell lines refractory to ADAR1 loss, treatment with IFN-β did not render them sensitive to ADAR1 knockdown. Furthermore, we observed no activation of PKR in the ADAR1-refractory cell lines following ADAR1 loss. These findings suggest that the link between ADAR1 loss and the IFN pathway or PKR activation in ADAR1-refractory cell lines is missing. Loss of ADAR1 is thought to activate the IFN pathway and PKR by causing an increase in dsRNA—stemming from a reduction in A-to-I editing [17, 18]. It is possible that ADAR1-refractory cell lines either do not accumulate dsRNA following ADAR1 loss, fail to accumulate a specific subset of dsRNA responsible for PKR or type I IFN pathway activation, or there exists a system that prevents dsRNAs from activating the IFN pathway or PKR. Understanding the molecular basis of this process would
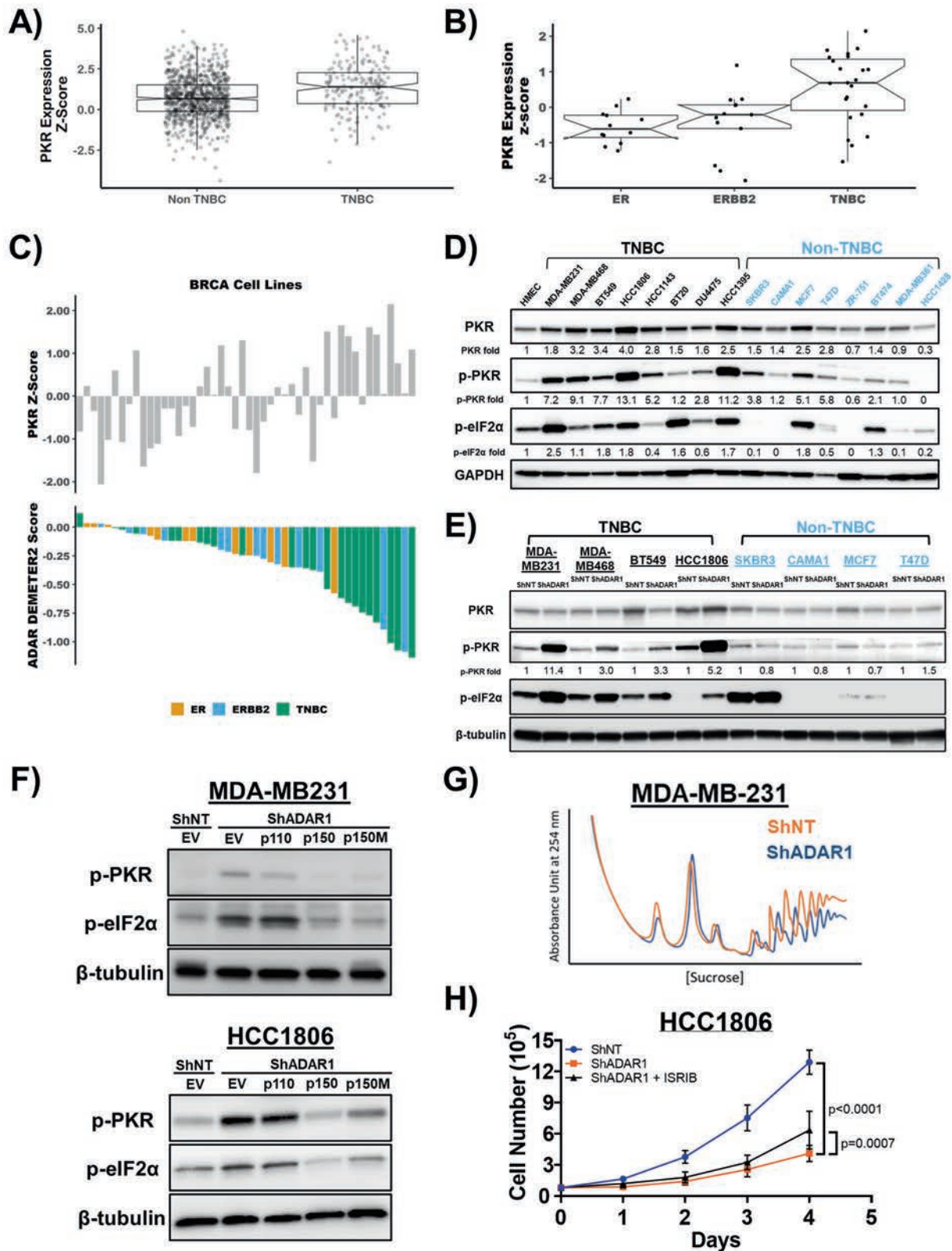
help to predict which cell lines— or more importantly which tumors—should be sensitive to ADAR1 loss or inhibition.

Translational repression mediated through PKR activation and phosphorylation of eIF2α has been shown to contribute to ADAR1 dependency in other cancers and

◀ **Fig. 4 PKR is overexpressed in TNBC and activated upon ADAR loss. A** mRNA expression of PKR in TNBC and non-TNBC. Data were extracted from TCGA database. **B** mRNA expression of PKR in ER-positive, ERBB2(HER2)-positive, and TNBC cell lines. Data were extracted from CCLE database. **C** ADAR1-dependency score positively correlates with PKR expression. Upper panel: PKR expression *z*-score in breast cancer cell lines. Lower panel: ADAR1-dependency scores. Lower DEMETER2 scores indicate stronger ADAR1 dependency. **D** Immunoblots showing protein levels of PKR, p-PKR (T446), p-eIF2α (S51), and GAPDH (loading control) in breast cancer cell lines. Densitometry quantification of gel images was normalized to GAPDH and set relative to HMEC signal. Data shown are representative, $N = 3$. **E** Immunoblots showing protein levels of PKR, p-PKR (T446), p-eIF2α (S51), and β-tubulin (loading control) in TNBC and non-TNBC breast cancer cell lines with or without ADAR1 knockdown. Densitometry quantification of gel images was normalized to GAPDH and compared to HMEC signal set as onefold. Data shown are representative, $N = 3$. **F** Immunoblots showing protein levels of p-PKR (T446), p-eIF2α (S51), and β-tubulin (loading control) with overexpression of p150, p110, or editing-defective p150^{E912A} (p150M) in ShADAR1-treated MDA-MB231 and HCC1806 cells. Data shown are representative, $N = 3$. **G** Polysome profiling of MDA-MB231 cells with or without ADAR1 knockdown. Data shown are representative of three replicates. **H** Cell proliferation assay showing that treatment of ISRIB (5nM) resulted in a modest rescue of ADAR1-knockdown phenotype in HCC1806 cells. Data are represented as mean ± SD. $N = 3$.

remains a possible explanation for TNBC-associated ADAR1 dependency [23]. We observed elevated PKR expression in TNBC tumors and cell lines, induced phosphorylation of PKR and eIF2α in TNBC cell lines upon ADAR1 loss, and decreased overall translation in ADAR1-deficient TNBC cells. The lack of significant reversal of ADAR1 dependency by mitigating the effects of eIF2α phosphorylation, however, suggests that this phenotype is (1) only modestly influenced by PKR-eIF2α-translation pathway; (2) attributed to a specific subset of targets downstream of PKR other than eIF2α [40]. We investigated the functional relevance of one such target, ATF4, in the context of ADAR1 dependency. The lack of association between ATF4 expression and ADAR1 dependency in breast cancer cells suggests that ATF4 does not play a significant role in this TNBC-specific phenotype.

It is not well understood how the RNA editing activity of ADAR1 contributes to its essentiality in certain cancer cells. Consistent with previous reports, we found that both catalytic and nonenzymatic functions of ADAR1 contribute to the ADAR1-dependent phenotype in a cell-line-specific manner. ADAR1-mediated editing of individual targets, including *AZIN1*, has been shown to regulate different aspects of tumorigenesis, including initiation, metastasis, and drug response [41]. However, we have shown that editing of *AZIN1* does not contribute to TNBC proliferation, opening up future investigations into other ADAR1-edited targets in TNBC. Moreover, rescue with either wild-type ADAR1 or editing-defective p150 in ADAR1 knockdown

TNBC cells resulted in reduced phosphorylation of PKR and eIF2α. Thus, the potential connection between ADAR1 and PKR/eIF2α in TNBC-associated ADAR1 dependency likely involves ADAR1 editing-independent functions and warrants further investigation.

Important clinical implications can be drawn from these observations. Our data suggest that ADAR1 is a legitimate candidate for targeted therapies in TNBC. First, we found that TNBC cell lines and patient samples exhibit elevated ISG and PKR expression, which is consistent with ADAR1-dependent cell lines. With increased understanding of ADAR1 functions, novel therapeutic strategies against ADAR1 could benefit ADAR1-dependent cancers, including TNBC [41]. Second, the relationship between ADAR1 dependency and type I IFN pathway activity could point to new directions for TNBC interventions. Recent studies revealed that the increased IFNβ target gene signature correlates with improved recurrence-free survival in TNBC, and IFNβ treatment inhibits tumor progression in TNBC by reducing cancer stem cell plasticity [42, 43]. In addition to cell-intrinsic effects of ADAR1 loss in cancer cells, removal of ADAR1 has been shown to sensitize tumors to immunotherapy by overcoming resistance to checkpoint blockade [27].

It was recently demonstrated that chemotherapies elicit a state of immunological dormancy in ER-negative breast cancers, marked by sustained type I IFN signaling, reduced cell growth, and longer progression-free survival [44]. This indicates a possible shared mechanism between chemotherapy-induced immunological dormancy and ADAR1 dependency in TNBC. It is important to note that careful considerations need to be given when applying the concepts of ADAR1 inhibition and type I IFN application in the treatment of TNBC. It is recognized that type I IFN can elicit paradoxical effects on cancer development [45]. For example, it has been suggested that type I IFN pathway, potentially through ISG15-mediated ISGylation, can promote the aggressiveness of TNBC [46, 47]. Therefore, further understanding of the relationship between ADAR1 functions and TNBC tumorigenesis should better inform the context in which this strategy can provide the maximum benefit.

Materials and methods

Cell lines and reagents

HMECs and breast cancer cell lines were obtained from American Tissue Cells Consortium. HMECs were cultured in MammaryLife Basal Medium (Lifeline Cell Technology) and passaged by using 0.05% trypsin-EDTA (Gibco) and Defined Trypsin Inhibitor (Gibco). Other cell lines were maintained in Dulbecco's Modification of Eagle's Medium (GE Life Sciences) supplemented with 10% fetal bovine
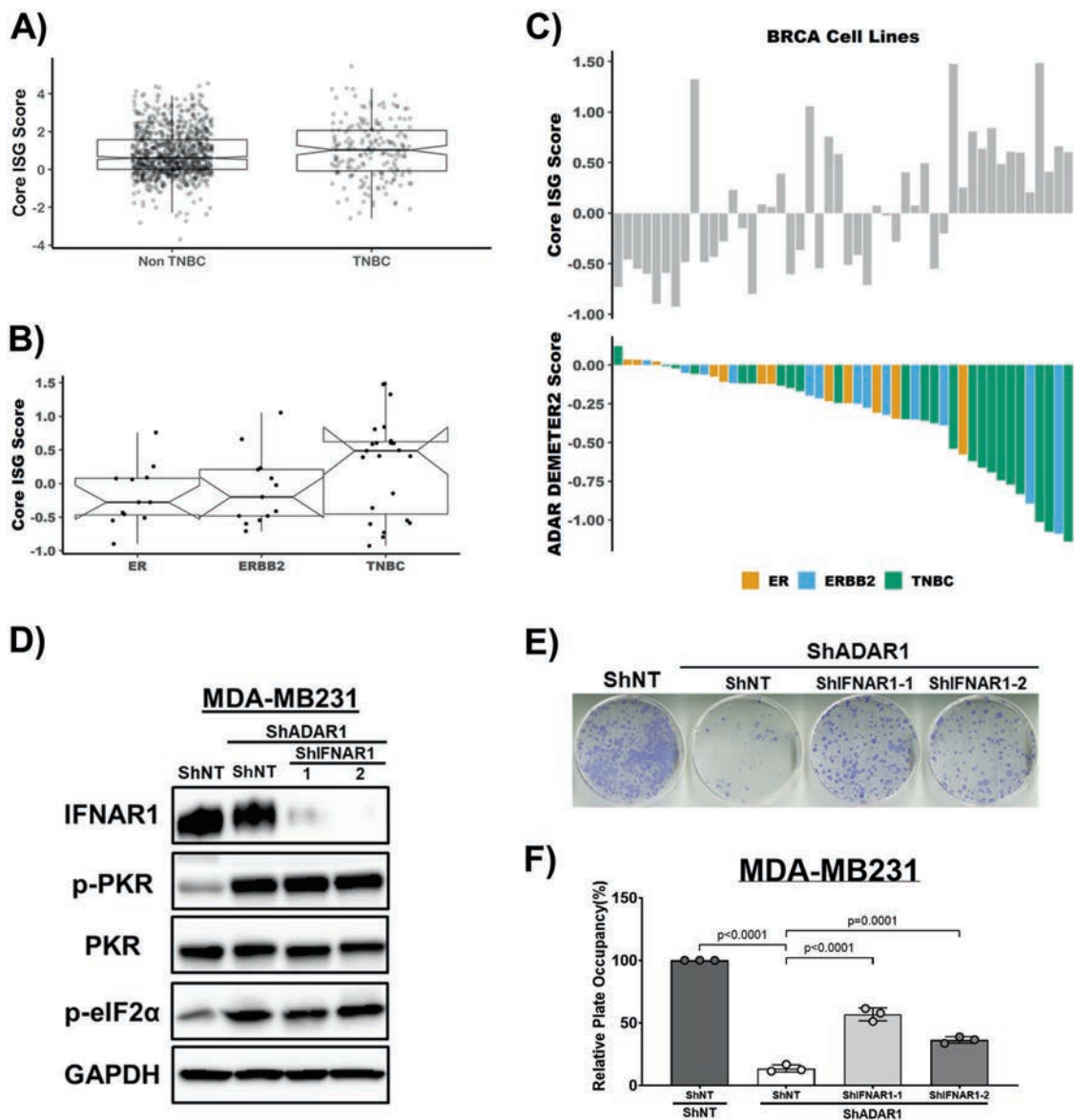
Fig. 5 ADAR1-dependent TNBCs exhibit elevated ISG expression and INFAR1 loss rescues ADAR1-knockdown phenotype. A Core ISG score in TNBC and non-TNBC breast cancer samples. Data were extracted from TCGA database. **B** Core ISG score in ER-positive, ERBB2(HER2)-positive, and TNBC cell lines. Data were extracted from CCLE database. **C** ADAR1-dependency score positively correlates with core ISG score in breast cancer cell lines. Upper panel: core ISG score in breast cancer cell lines. Lower panel: ADAR1-dependency scores. **D** Immunoblots showing protein levels of IFNAR1, PKR, p-PKR (T446), p-eIF2α (S51), and GAPDH (loading control) in MDA-MB231 cells. IFNAR1 was knocked down in ShADAR1-treated MDA-MB231 cells to determine if IFNAR1 loss reverses ADAR1-knockdown phenotype. Images are representative, $N = 3$. **E** FF assay showing that IFNAR1 loss partially rescued ADAR1-knockdown phenotype in MDA-MB231 cells. Images are representative, $N = 3$. **G** Quantification of FF in **F**. Relative plate occupancy was determined using ImageJ software and normalized to ShNT-ShNT. Data are represented as mean ± SD. $N = 3$.

serum (Gibco, 10091-148), sodium pyruvate (Cellgro, 30-002-CI), non-essential amino acids (Cellgro, 25-030-CI), and L-glutamine (Cellgro, 25-005-CI). Lipofectamine 2000 (Invitrogen) was used for transfection to generate lentivirus. Fugene 6 transfection reagent (Promega) was used for all other transfection experiments. PKR inhibitor (MilliporeSigma, CAS-608512-97) and eIF2α inhibitor ISRIB (MilliporeSigma, SML0843) were used for rescue experiments.

Immunoblot analysis

Immunoblot analysis was performed as described previously, further details can be found in the Supplementary Information [46]. Primary antibodies: ADAR1 (Santa Cruz, sc-73408; Bethyl Laboratories, A303-883A; Abcam, ab126745), cleaved PARP (Cell Signaling, #9541), PKR (Cell Signaling, #3072), PKR Thr-446-P (Abcam, ab32036), IFNAR1 (Bethyl, A304-290A), ISG15 (Santa Cruz, sc-166755), GAPDH (Bethyl,

A300-641A), β-Tubulin (Abcam, ab6046), EIF2S1/eIF2α Ser-51-P (Abcam, 32157), EIF2S1 (Abcam, ab5369), and ATF4 (Cell Signaling, #11815).

Mammary gland orthopedic implantation

The abilities of human breast cancer cell lines to form tumors in vivo were evaluated by performing mammary gland orthopedic implantation as described previously, further details can be found in the Supplementary Information [48]. Five mice were used in each experiment group to achieve type I error rate at 0.05 with 90% power based on results from in vitro cell proliferation and transformation assays. One mouse from MDA-MB231-ShNT experiment group was excluded due to premature death (Fig. 3G). All animal-related experimental procedures were performed in compliance with the guidelines given by the American Association for Accreditation for Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All animal studies were approved by the Washington University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act and NIH guidelines (Protocol 20160916)

Statistical analysis

All experiments were performed with biological replicates, with the exact sample size stated in figure legends. All in vitro experiments were performed in triplicate, unless otherwise stated. Nonlinear regression test for Malthusian growth was used for statistical analysis for cell proliferation assay. One-way ANOVA with Tukey's multiple comparisons was used for statistical analysis in ADAR1-overexpressing rescue experiments. The two-tailed unpaired Student t test was performed for statistical analysis for other experiments. All in vitro and in vivo data are reported as the mean ± SD unless stated otherwise, statistical analyses were performed using GraphPad Prism.

Analysis of CCLE RNA-seq data and ADAR1 dependency

CCLE RNa-seq count data from breast cancer cell lines were normalized by the "cpm" function of "edgeR" [49]. From the cpm values, z-scores were determined for each gene across all cell lines. To determine "core ISG score," we calculated the median z-score of previously identified "core ISGs" [24]. Molecular subtypes of breast cancer cell lines were defined previously [50].

Analysis of TCGA RNA-seq data

Unnormalized RSEM values were normalized by the "cpm" function of edgeR [49]. From the cpm values, modified

z-scores were determined using the following formula:

$$z = \frac{[(\text{cpm gene X in breast cancer sample}) - (\text{Mean gene X in norma}}{(\text{Standard deviation X in normal})}$$

We calculated "core ISG score" as described above. Molecular subtypes of TCGA samples were defined previously [26].

Data and code availability

CCLE RNA-seq count data (CCLE_RNAseq_genes_counts_20180929.gct.gz, CCLE_RNAseq_rsem_transcripts_tpm_20180929.txt.gz) were obtained from the Broad Institute Cancer Cell Line Encyclopedia and is available at https://portals.broadinstitute.org/ccle/data. Dependency data (D2_combined_gene_dep_scores.csv, Achilles_gene_effect.csv) were obtained from Broad Institute DepMap Portal and is available at https://depmap.org/portal/download/. TCGA breast cancer RNA-seq (illuminahiseq_rnaseqv2-RSEM_genes, illuminahiseq_rnaseqv2-RSEM_isoforms_normalized) and clinical data (Merge_Clinical) were obtained from the Broad Institute FireBrowse and are available at http://firebrowse.org/. All custom R scripts used in this study are available on GitHub (https://github.com/cottrellka/ADAR_TNBC). Lentiviral production and transduction; flow cytometric analysis of apoptosis; cell proliferation and focus formation assays; soft agar transformation assay; polysome profiling; immunohistochemistry. These experiments were performed as previously described, and further details can be found in the Supplementary Information [46, 51, 52].

Author contributions Conceptualization: C-PK, KAC, and JDW; methodology: C-PK, KAC, and JDW; software: KAC; investigation: C-PK, KAC, SR, ERB, RDK, EAB, ECF, TS, LM, and JDW; writing—original draft: C-PK and KAC; writing—review and editing: C-PK, KAC, SR, ERB, RDK, LM, and JDW; funding acquisition: JDW; supervision: JDW.

Compliance with ethical standards

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