1 **Induction of viral mimicry upon loss of DHX9 and ADAR1 in breast cancer cells**

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Abstract

Detection of viral double-stranded RNA (dsRNA) is an important component of innate immunity. However, many endogenous RNAs containing double-stranded regions can be misrecognized and activate innate immunity. The interferon inducible ADAR1-p150 suppresses dsRNA sensing, an essential function for ADAR1 in many cancers, including breast. Although ADAR1-p150 has been well established in this role, the functions of the constitutively expressed ADAR1-p110 isoform are less understood. We used proximity labeling to identify putative ADAR1-p110 interacting proteins in breast cancer cell lines. Of the proteins identified, the RNA helicase DHX9 was of particular interest. Knockdown of DHX9 in ADAR1-dependent cell lines caused cell death and activation of the dsRNA sensor PKR. In ADAR1-independent cell lines, combined knockdown of DHX9 and ADAR1, but neither alone, caused activation of multiple dsRNA sensing pathways leading to a viral mimicry phenotype. Together, these results reveal an important role for DHX9 in suppressing dsRNA sensing by multiple pathways.

Introduction

RNA editing enhances protein diversity and modulates multiple aspects of RNA metabolism[1–3]. A-to-I editing is carried out by adenosine deaminase acting on RNA 1 (ADAR1), an RNA editase that binds double-stranded RNA (dsRNA) and converts adenosines to inosines[4,5]. The main domains of ADAR1 include the Z-DNA binding domains (ZBD), the double-stranded RNA (dsRNA) binding domains (dsRBD), and the deaminase domain[6,7]. There are two isoforms, p110 and p150, produced by alternative transcriptional start sites[8]. They share the same deaminase domain, dsRBDs, and a ZBD, but exhibit a distinct subcellular localization[9,10]. ADAR1-p150 is predominantly cytoplasmic, whereas ADAR1-p110 is nuclear-localized[9,11].

An important role for ADAR1 is to suppress dsRNA sensing[12–15]. Many endogenously encoded RNAs can form large double-stranded regions, often through base-pairing between inverted Alu elements[16]. ADAR1 edits the majority of human genes, with most editing occurring within inverted Alu repeats[3,4]. By binding, editing, and thus altering the structure of dsRNA, ADAR1 suppresses the detection of dsRNA by various cytoplasmic sensors such as MDA5, RIG-I, and PKR[12,13,15,17]. These RNA sensors are part of innate immunity against viral infections[16,18–20]. Thus, ADAR1 prevents the activation of innate immunity pathways by endogenous immunogenic RNAs[16,19,21].

The cell-intrinsic antiviral response against foreign dsRNA - or misrecognized endogenous dsRNA - involves multiple pathways: (1) Recognition of dsRNA by MDA5 or RIG-I results in the activation of type I interferon (IFN-I) signaling[16,19,21]. (2) Activation of the dsRNA-binding kinase PKR triggers translational shutdown by phosphorylation of the translation initiation factor eIF2a[12]. (3) Detection of dsRNA by OAS proteins activates RNase L, which carries out non-specific cleavage of RNA and triggers cell death[22]. There is significant crosstalk between the three pathways, and the aforementioned dsRNA sensors are transcriptionally

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78   controlled by IFN-I, known as interferon-stimulated genes (ISGs)[23,24]. The ADAR1-p150 isoform

79   is itself an ISG and is the isoform responsible for suppressing the activation of dsRNA

80   sensors[8,10,14,25]. ADAR1-p110, however, is constitutively expressed, though its functions are less

81   established[26,27].

82   Because IFN-I signaling is often cytotoxic and antiproliferative, ADAR1's ability to

83   suppress IFN-I signaling was shown to exert pro-tumor effects[21,28,29]. As such, ADAR1 has been

84   proposed as a potential therapeutic target for various cancers, including breast cancer[28,30–32].

85   ADAR1 mRNA expression is elevated in breast cancer and is correlated with a poor

86   prognosis[28,33]. Furthermore, ADAR1 is essential for a subset of breast cancer cells

87   overrepresented by triple-negative breast cancer (TNBC)[33]. Known as 'ADAR1-dependency,'

88   depletion of ADAR1-p150 leads to IFN-I signaling and global translational repression in cells

89   that are sensitive to depletion of ADAR1[32,33]. What makes cancer cells sensitive or refractory to

90   ADAR1 loss is not yet determined[33].

91   ADAR1 interacts with numerous RNA binding proteins including RNA helicases,

92   transcription machinery, and DNA repair proteins [27,34–37]. The influence of ADAR1-interacting

93   proteins on A-to-I editing has previously been reported[35,38–41]. In this study, we evaluated

94   components of the ADAR1 interactome in breast cancer cells and identified the RNA helicase

95   DHX9 as a redundant suppressor of immunogenic dsRNA in ADAR1-independent breast cancer

96   cells. We demonstrate that co-depletion of ADAR1 and DHX9 is sufficient to trigger a viral

97   mimicry phenotype in ADAR1-independent cells.

98   **Results**

99   **Proximity Labeling by APEX2 Reveals ADAR1 Interacting Proteins**

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100   To better understand the role of ADAR1-p110 in breast cancer, we turned to a proximity

101   labeling approach using APEX2 to identify putative ADAR1-p110-interacting proteins. Proximity

102   labeling by APEX2 allows for the identification of proteins within 20 nm of an APEX2 fusion

103   protein via biotin-mediated pulldown[42]. An APEX2-ADAR1-p110 fusion construct or APEX2

104   alone was expressed in MDA-MB-231, MCF-7, and SK-BR-3 (Fig. 1a). Following proximity

105   labeling, biotinylated proteins were purified by streptavidin pulldown and subsequently identified

106   by mass spectrometry (Fig 1b-c, Extended Data Fig. 1a, Supplementary Table 2). In total, we

107   identified over one hundred enriched proteins across the three cell lines, with many identified in

108   all three lines (Fig. 1d). Over-representation analysis of gene ontology (GO) terms revealed that

109   many of the proteins identified by proximity labeling have roles in multiple aspects of RNA

110   metabolism and localize to the nucleus and nucleolus (Fig. 1e, Supplementary Table 3). This

111   finding was supported by a comparison to proteins previously observed to localize to the

112   nucleus and nucleolus within MCF-7 in the SubCellBarcode dataset[43] (Fig.1f and 1g). These

113   findings are largely consistent with the localization of ADAR1 within the cell lines studied.

114   Immunofluorescence for ADAR1, which largely reflects the localization of ADAR1-p110 as the

115   predominant isoform, showed that ADAR1 was localized in both the nucleus and nucleolus (Fig.

116   1h).

Validation of Protein Interactions Identified by Proximity Labeling

118   Although proximity labeling by APEX2 is a powerful technique for identifying putative

119   protein-protein interactions, it does not distinguish between interactions and close

120   associations[42]. To validate the proximity labeling findings and provide supporting evidence for

121   direct protein-protein interactions, we performed co-immunoprecipitation followed by

122   immunoblotting for five proteins identified by proximity labeling. ADAR1 could be

123   immunoprecipitated by antibodies against the helicases DHX9, DDX17, and DDX54 (Fig. 2a-g).

124   ADAR1 could also be immunoprecipitated with antibodies against XRN2 and PARP (Extended

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125   Data Fig. 1b-c). To assess whether these potential interactions depended on RNA, we treated

126   the lysates with RNase A to degrade RNA prior to immunoprecipitation (Extended Data Fig. 1d).

127   Immunoprecipitation of ADAR1 by antibodies against DHX9, DDX17, and DDX54 was possible

128   even in the presence of RNase A and improved in some cases (Fig. 2a-g). For DDX17 and

129   DDX54, RNase A treatment did not change the co-immunoprecipitation results. However, DHX9

130   immunoprecipitated with both isoforms of ADAR1 in the absence of RNase A, but only with

131   ADAR1-p110 when treated with RNase A. These findings suggest that DHX9, DDX17, and

132   DDX54 directly interact with ADAR1-p110, and DHX9 interacts with ADAR1-p150 through an

133   RNA bridge/scaffold. The co-immunoprecipitation findings are also consistent with the

134   localization of the proteins studied. Much like ADAR1, the helicases DHX9, DDX17, and DDX54

135   localized to the nucleus or nucleolus (Fig. 2h-j). Together, these findings validate the results of

136   the proximity labeling described in Fig. 1 and provide evidence for direct interactions between

137   ADAR1 and multiple helicases.

138   **DHX9 is overexpressed in breast cancer**

139   Of the identified helicases, DHX9 was of particular interest for several reasons. First,

140   DHX9 is the only helicase in humans that has a dsRBD. The dsRBD that is present in DHX9 is

141   of the same class present in ADAR1 and PKR (Fig. 3a). Analysis of publicly available RNA-seq

142   data for human cell lines and tumors revealed that DHX9 expression closely correlates with

143   ADAR1 expression (Fig. 3b-d, Extended Data Fig. 2a-c). The correlation is stronger between

144   DHX9 and the transcript encoding ADAR1-p110, than between DHX9 and ADAR1-p150 (Fig.

145   3c-d, Extended Data Fig. 2d-e). Furthermore, the expression of DHX9 and ADAR1 correlates

146   better than any other helicase identified by proximity labeling (Fig. 3b, Extended Data Fig. 2b).

147   Consistent with this correlated expression, and much like ADAR1, DHX9 is highly expressed in

148   breast cancer and is correlated with a poor prognosis[33] (Fig. 3c, Extended Data Fig. 2f-h).

149 **DHX9 is essential in TNBC cell lines and suppresses PKR activation**

150   The similarities between ADAR1 and DHX9 led us to further study the role of DHX9 in

151 breast cancer. Analysis of publicly available data from DepMap

152 (https://depmap.org/portal/download/custom/) revealed that DHX9 is commonly essential in

153 breast cancer cell lines (Fig. 3f). We validated this finding by knocking down DHX9 in four TNBC

154 cell lines previously shown to be ADAR1-dependent[33]. In all four lines, knockdown of DHX9

155 caused cell death, likely through caspase-mediated apoptosis as indicated by cleaved PARP

156 (Fig. 3g and h). Given the presence of the common dsRBD in DHX9 and PKR, and the role of

157 DHX9 in regulating the abundance of dsRNA[44], we asked whether DHX9 could influence

158 activation of PKR. To our surprise, we found that in three of the TNBC cell lines studied,

159 knockdown of DHX9 caused activation of PKR (Fig. 3g and 3i). Together these findings show

160 that DHX9 is essential in breast cancer cell lines and that in some ADAR1-dependent cell lines,

161 DHX9 suppresses PKR activation.

162 **DHX9 and ADAR1 redundantly suppress PKR activation**

163   The experiments above were performed in ADAR1-dependent TNBC cell lines - cell

164 lines that activate PKR following ADAR1 knockdown[33]. We were curious if DHX9 knockdown

165 would also cause PKR activation in ADAR1-independent cell lines - cell lines that do not

166 activate PKR following ADAR1 knockdown. Using shRNAs, we knocked down DHX9 in two

167 ADAR1-independent breast cancer lines, MCF-7 and SK-BR-3 (Fig. 4a-b and 4f-g, Extended

168 Data Fig. 4a-b and 4g-h). Unlike in ADAR1-dependent cell lines, knockdown of DHX9 did not

169 cause activation of PKR in ADAR1-independent cell lines (Fig. 4a, 4d, 4f, 4i). Next, we asked

170 whether combined knockdown of ADAR1 and DHX9 in these cell lines would lead to activation

171 of PKR. As we had previously observed, knockdown of ADAR1 in SK-BR-3 and MCF-7 did not

172 cause PKR activation[33]. However, combined knockdown of DHX9 and ADAR1 caused robust

173   activation of PKR in both cell lines (Fig. 4a, 4d, 4f, 4i). Consistent with PKR activation, we

174   observed increased phosphorylation of the PKR substrate eIF2α following combined knockdown

175   of ADAR1 and DHX9 (Fig. 4a and 4d, Extended Data Fig. 4c and 4i). Like in ADAR1-dependent

176   TNBC cell lines, knockdown of DHX9 caused reduced proliferation of MCF-7 and SK-BR-3 (Fig.

177   4c, 4d, 4h, 4j), likely through caspase-dependent apoptosis, as indicated by elevated cleaved

178   PARP (Fig. 4a, 4d, Extended Data Fig. 4e, 4k). Although PARP cleavage was increased upon

179   combined knockdown of DHX9 and ADAR1, this did not statistically reduce the proliferation of

180   the cells compared to single knockdown of ADAR1 and DHX9 as measured by foci formation.

181   Together, these results reveal that ADAR1 and DHX9 redundantly suppress PKR activation in

182   ADAR1-independent breast cancer cell lines.

DHX9 and ADAR1 redundantly suppress RNase L activation

184   Next, we wanted to evaluate the activation of other dsRNA sensing pathways in ADAR1-

185   independent cell lines after the combined knockdown of ADAR1 and DHX9. To assess whether

186   the IFN-I pathway, or other pathways, is activated after combined knockdown of ADAR1 and

187   DHX9 we turned to analysis of differential gene expression by RNA-seq. In the process of

188   preparing RNA for sequencing, we were surprised to find specific degradation of rRNA in MCF-7

189   cells following combined knockdown of ADAR1 and DHX9 (Fig. 4k). Single knockdown of either

190   DHX9 or ADAR1 did not cause rRNA degradation. The observed rRNA degradation in the

191   combined knockdown cells was reminiscent of the degradation products caused by RNase L[45].

192   Transfection with poly(I:C), which activates the IFN-I pathway and RNase L[46], created an

193   identical band pattern to that of combined knockdown of DHX9 and ADAR1, indicating that the

194   degradation of rRNA observed in these cells is likely caused by RNase L activity (Fig. 4k). We

195   performed the same experiment with the ADAR1-dependent TNBC cell lines described above.

196   In these cells, we did not see activation of RNase L after the knockdown of DHX9 alone

197   (Extended Data 3h).

198 **DHX9 and ADAR1 redundantly suppress multiple innate immunity pathways**

199   RNA sequencing revealed that many more RNAs were differentially expressed after

200 combined knockdown of DHX9 and ADAR1, compared to single knockdown of ADAR1 or DHX9

201 (Fig. 5a, Extended Data Fig. 5a-f). Analysis of differential gene expression by gene set

202 enrichment after combined knockdown of ADAR1 and DHX9 in MCF-7 revealed activation of

203 multiple pathways involved in the innate response to viral infection and repression of several

204 pathways involved in translation (Fig. 5b and Supplemental Table 14). An enrichment map

205 showed that the activated pathways associated with innate immunity formed one cluster and the

206 depressed pathways formed a separate cluster (Extended Data Fig. 5g). Of the upregulated

207 pathways, several were associated with activation of IFN signaling (Fig. 5b, and Supplementary

208 Table 14). Analysis of core ISG expression revealed significant upregulation of ISGs in MCF-7

209 after combined knockdown of ADAR1 and DHX9 (Fig. 5c and Extended Data 5c). On the

210 contrary, knockdown of DHX9 or ADAR1 alone did not increase ISG expression. Activation of

211 RNase L described above also indicated that the type I IFN pathway was active in MCF-7 after

212 double knockdown of DHX9 and ADAR1, because the activators of RNase L, the OAS proteins,

213 are ISGs[22]. In fact, a GO term associated with OAS activity was upregulated in MCF-7 after

214 combined knockdown of ADAR1 and DHX9 (Supplementary Table 14). Consistent with

215 activation of PKR, we also observed increased expression of ATF4 targets (Fig. 5d) and NF-KB

216 targets (Fig. 5e). Interestingly, we did not observe activation of any of these pathways or RNase

217 L in SK-BR-3 following combined ADAR1 and DHX9 knockdown.

218   The finding that combined knockdown of ADAR1 and DHX9 did not induce ISG

219 expression in SK-BR-3 is consistent with our findings in TNBC cell lines. While knockdown of

220 DHX9 alone caused activation of PKR in several TNBC cell lines, we observed no activation of

221 the type I IFN pathway, as indicated by no change in ISG15 expression (Extended Data Fig. 3f-

222 g). ISG15 was found to be highly upregulated at the RNA and protein level in MCF-7 after

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223   double knockdown of ADAR1 and DHX9 (Extended Data Fig. 4f, 4m, Supplementary Table 8).

224   Like ISG expression overall, ISG15 expression in SK-BR-3 was not changed by knockdown of

225   DHX9 and/or ADAR1 (Extended Data Fig. 4l, 4n).

226   Given the previously described role of DHX9 in the control of Alu containing RNAs, we

227   next sought to assess if increased expression of transposable elements, especially Alus, could

228   explain the activation of PKR or the IFN pathway upon combined knockdown of DHX9 and

229   ADAR1. Analysis of our RNA-seq data revealed that transposable element expression was

230   generally unchanged upon either single knockdown of ADAR1 or DHX9, or combined

231   knockdown of both proteins (Extended Data Fig. 6a-f).

232   **The dsRBDs of DHX9 are sufficient to suppress PKR activation in the absence of ADAR1**

233   Having shown through knockdown studies that ADAR1 and DHX9 function redundantly

234   to suppress dsRNA sensing, we next wanted to assess which functions of DHX9 and which

235   isoforms of ADAR1 are important for this role. To determine which functions of DHX9 are

236   sufficient to suppress PKR activation, we performed a rescue experiment with wild-type DHX9,

237   a helicase deficient mutant DHX9, and a truncated DHX9 that possess the N-terminal dsRBDs

238   fused to EGFP (Fig. 6a). Overexpression of wild-type DHX9 rescued the PKR activation

239   phenotype caused by double knockdown of ADAR1 and DHX9, confirming that the observed

240   phenotypes are not an off-target effect of the shRNAs used for knockdown (Fig. 6b and 6d).

241   Interestingly, the DHX9^{K417R} mutant, which lacks helicase activity due to its inability to bind

242   ATP[47], was also capable of suppressing PKR activation. The same was true for a construct

243   which contained the dsRBDs of DHX9 fused to EGFP (dsRBD-EGFP). These findings indicate

244   that the DHX9 dsRBDs are likely sufficient to suppress PKR activation in the absence of ADAR1

245   and DHX9. However, only wild-type DHX9 could rescue the reduced foci formation observed

246   after DHX9 knockdown (Fig. 6c and 6e). This finding indicates that the PKR activation

247   phenotype and the reduced proliferation phenotype are uncoupled.

248   Consistent with the observation that the DHX9 dsRBDs are sufficient to suppress PKR

249   activation, knockdown of DDX17, which lacks dsRBDs, did not cause substantial PKR activation

250   in SK-BR-3 (Extended Data Fig. 9). Unlike DHX9, knockdown of DDX17 had no effect on cell

251   proliferation as measured by the foci formation assay (Extended Data Fig. 9b and 9d). While

252   combined knockdown of DHX9 and ADAR1 in SK-BR-3 caused a 5-10 fold increase in PKR

253   phosphorylation, the combined knockdown of DDX17 and ADAR1 caused only a modest 1.5-

254   fold increase in PKR phosphorylation (Extended Data Fig. 9a and 9c). This finding underscores

255   the novel role of the DHX9 helicase and its dsRBD, a unique domain among this large family of

256   RNA helicases.

257   **The p110 and p150 isoforms of ADAR1 suppress PKR activation in the absence of DHX9**

258   Next, we turned to ADAR1, and asked which isoform of ADAR1 is sufficient to suppress

259   PKR activation in the absence of DHX9. We used the same approach as above, a rescue

260   experiment with overexpression of ADAR1-p110 or ADAR1-p150. Interestingly, we found that

261   both ADAR1 isoforms were sufficient to suppress PKR activation upon loss of DHX9 (Fig. 6f and

262   6h, Extended Data Fig. 8a-b). However, overexpression of neither ADAR1 isoform was able to

263   rescue the foci formation phenotype, again indicating that the PKR activation and cell

264   proliferation phenotypes are uncoupled (Fig. 6g and 6i).

265   **Discussion**

266   In recent years, ADAR1 has become an important therapeutic target for breast and other

267   cancers. It is clear from the literature that depletion of ADAR1 in ADAR1-dependent cell lines

268   leads to activation of dsRNA sensors and innate immunity programs that lead to cell

269   death[21,32,33]. Yet unclear is what distinguishes ADAR1-dependent cell lines from ADAR1-

270   independent cell lines - those that are insensitive to ADAR1 depletion. Elevated ISG expression

271   has been proposed as a potential prerequisite for ADAR1-dependency, but some ADAR1-

272   independent cell lines exhibit elevated ISG expression[21,33]. As such, more information is needed

273   to identify the factors that establish ADAR1-dependency or ADAR1-independency. To begin to

274   fill in some of the knowledge gaps surrounding ADAR1, we utilized proximity labeling to identify

275   putative ADAR1 interacting proteins, specifically focusing on the less studied ADAR1-p110

276   isoform.

277   Of the proteins identified by proximity labeling, the DEAH box helicase DHX9 was of

278   particular interest. Like ADAR1 and PKR, DHX9 possesses dsRBDs, a singularly unique trait

279   among the DEAD/DEAH box RNA helicase family. DHX9 expression is strongly correlated with

280   ADAR1-p110 expression in breast cancer. Interestingly, both genes are located on the q-arm of

281   chromosome 1, though they are separated by 28 mb and are thus unlikely to be physically co-

282   regulated. Consistent with other reports in the literature, we show here that ADAR1 and DHX9

283   likely interact directly[38]. We observed that ADAR1-p110 and DHX9 interact in an RNA-

284   independent manner, while the interaction between ADAR1-p150 and DHX9 was disrupted by

285   RNase treatment. This result contradicts previous findings, in which ADAR1-p150 and DHX9 co-

286   immunoprecipitated after RNase A treatment[44]. This discrepancy may be due to different cell

287   lines used, HEK293T was used for the previous study, or different accessibility of DHX9

288   epitopes during immunoprecipitation. The importance of the ADAR1-DHX9 interaction is unclear

289   from this work. Further studies are needed to structurally assess the interaction and directly

290   perturb the interaction to understand what function it may have.

291   Here we report that in addition to being a commonly essential gene in breast cancer,

292   DHX9 suppresses dsRNA sensing. In ADAR1-dependent cell lines, knockdown of DHX9 alone -

293   much like knockdown of ADAR1 alone - caused activation of the dsRNA sensor PKR[33]. Like

294   ADAR1 knockdown, DHX9 knockdown had no effect on PKR activation in ADAR1-independent

295   cell lines. However, combined knockdown of DHX9 and ADAR1 caused robust activation of

296   PKR in these cells. This finding indicates that ADAR1 and DHX9 function redundantly to

297   suppress PKR activation in ADAR1-independent cell lines and provides an explanation for why

298   PKR is not activated in ADAR1-independent cells upon ADAR1 knockdown. Interestingly, like

299   ADAR1, DHX9 has been shown to interact with PKR and is phosphorylated by PKR[48]. More

300   research is needed to understand the importance of PKR phosphorylation of DHX9 and whether

301   or not it may serve as a feedback mechanism.

302   Rescue experiments revealed that the helicase activity of DHX9 was dispensable for

303   suppression of PKR activation. In fact, the N-terminal dsRBDs of DHX9 were sufficient to

304   suppress PKR activation in the absence of ADAR1 in ADAR1-independent cell lines. This

305   finding provides evidence for a model in which DHX9 competes with PKR for dsRNA binding

306   through its dsRBDs (Fig. 7). This competition is likely to be indirect, as DHX9 is nuclear

307   localized while PKR is generally localized in the cytosol[49–52]. As such, DHX9 may function to

308   suppress PKR activation by sequestering dsRNAs in the nucleus.

309   Previously, the ADAR1 p150 isoform, and not the p110 isoform, was shown to be

310   responsible for suppression of PKR activation. Through rescue experiments, we show here that

311   both isoforms are sufficient to suppress PKR activation in the absence of DHX9 in ADAR1-

312   independent cell lines. A preprint that was published during the preparation of this manuscript

313   showed that the dsRBDs of ADAR1, ADAR2, and STAU1 were sufficient to suppress PKR

314   activation[53]. Based on this finding and our rescue experiment with the DHX9 dsRBDs, it is likely

315   that ADAR1-p150 and ADAR1-p110 suppress dsRNA sensing in the absence of DHX9 by

316   competing with PKR for dsRNA binding. Although this may be direct competition in the case of

317   ADAR1-p150 localized cytoplasmically, ADAR1-p110 may function like DHX9 to sequester

318   dsRNAs in the nucleus.

319   In addition to suppression of PKR activation, we also observed that DHX9 and ADAR1

320   redundantly suppress activation of several other dsRNA sensing pathways in MCF-7.

321   Knockdown of both proteins caused activation of IFN-I signaling, likely via MDA5 activation, as

322   previously shown for ADAR1[13,15]. We also observed activation of OAS-RNaseL and increased

323   expression of the ATF4 and NF-KB targets, likely downstream of PKR activation[54–56]. Taken

324   together, combined knockdown of DHX9 and ADAR1 in MCF-7 creates a viral mimicry

325   phenotype, where multiple innate immune pathways against RNA viruses have been activated

326   (Fig. 7). Interestingly, we only observed activation of PKR in other cell lines after either DHX9

327   knockdown alone or combined knockdown with ADAR1. Two possible explanations for this

328   discrepancy could be 1) Another factor may be present in some cells that suppresses dsRNA

329   sensing in the absence of DHX9 and/or ADAR1. 2) The expression of endogenous dsRNAs that

330   cause activation of sensors other than PKR may vary, leading to the differential effects of

331   ADAR1 and/or DHX9 knockdown. Further studies are needed to identify the immunogenic

332   RNAs that activate the various sensors.

333   Induction of viral mimicry has great potential as a therapeutic approach for multiple

334   cancers, including TNBC[57–60]. In addition to the cell intrinsic effects of activating innate immune

335   pathways within the tumor, the signaling that occurs after activation of those pathways can

336   promote anti-tumor immunity, especially when combined with checkpoint inhibitors[61–63].

337   Combined therapies targeting ADAR1 and DHX9 may serve as an effective means of treating

338   breast and other cancers by inducing viral mimicry.

339   **Materials and Methods**

340   **Cell culture**

341   Breast cancer cell lines (MCF-7 (RRID:CVCL_0031), SK-BR-3 (RRID:CVCL_0033), BT-

342   549 (RRID: CVCL_1092), MDA-MB-231 (RRID: CVCL_0062), HCC1806 (RRID: CVCL_1258),

343   MDA-MB-468 (RRID: CVCL_0063) and 293T (RRID: CVCL_0063) were obtained from

344   American Type Culture Collection. All cell lines were cultured in Dulbecco's modified Eagle's

345   medium (DMEM) (Hyclone) with 10% fetal bovine serum (Invitrogen), 2 mM glutamine

346   (Hyclone), 0.1 mM nonessential amino acids (Hyclone), and 1 mM sodium pyruvate (Hyclone).

Viral Production and Transduction

348   Lentivirus was produced by Turbo DNAfection 3000 (Lamda Biotech) transfection of

349   293T cells with pCMV-VSV-G, pCMV-ΔR8.2, and the appropriate plasmid for expression of

350   genes of interest or shRNAs. Virus was harvested 48 hours post-transfection. Cells were

351   transduced with lentivirus for 16 hours in the presence of 10 µg/mL protamine sulfate (Sigma-

352   Aldrich). The cells were selected with puromycin at 2 µg/mL (Sigma-Aldrich), 150 µg/mL

353   hygromycin (Invitrogen) or 10 µg/mL Blasticidin (Invitrogen). The sequences for the shRNA-

354   scramble (shSCR) and shRNA-ADAR1 (shADAR1) were described and validated previously[33].

355   The sequences for shRNAs against DHX9 and DDX17 are in Supplementary Table 1.

Plasmids

357   APEX2 was PCR amplified from pcDNA3-APEX2-NES, a kind gift from the laboratory of

358   Kendall Blumer at Washington University in St. Louis. ADAR1-p110 was PCR amplified from

359   pLVX-p110-ADAR1 described previously[33]. APEX2 and p110 were cloned into pLVX-IRES-puro

360   (Takara, 632183) via a series of restriction enzyme digests and ligations. The final plasmids

361   pLVX-3xFLAG-APEX2 and pLVX-3xFLAG-APEX2-linker-p110 were confirmed by digestion and

362   sequencing. The linker consists of three repeats of Gly-Gly-Gly-Gly-Ser. Lentiviral shRNA

363   constructs in the pLKO.1-puro vector were purchased as glycerol stocks from Millipore Sigma

364   (Supplemental Table 1). For shADAR1, the shRNA was subcloned into pLKO.1-hygro

365   (Addgene, #24150). Overexpression constructs for DHX9 and ADAR were generated by PCR

366   amplification and ligation into pLV-EF1a-IRES-Blast vector (Addgene, #85133). For DHX9

367   overexpression, wobble mutants were made to reduce shRNA targeting. Mutagenesis primers

368   for DHX9 K417R and shRNA-resistant codons (designed using the Synonymous Mutation

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369   Generator[64]) are included in Supplemental Table 1. The DHX9-dsRBD-EGFP construct was

370   generated by digestion of pLV-EF1-DHX9 with SpeI and EcoRI and ligation of EGFP in place of

371   the 3' portion of DHX9. The resulting construct codes for the first 344 amino acids of DHX9

372   fused to EGFP.

373   **Immunoblot**

374   Cell pellets were lysed and sonicated in RIPA Buffer (50 mM Tris pH 7.4 (Ambion), 150

375   mM NaCl (Ambion), 1% Triton X-100 (Sigma-Aldrich), 0.1% sodium dodecyl sulfate (Sigma-

376   Aldrich) and 0.5% sodium deoxycholate (Promega) with 1x HALT Protease Inhibitor (Pierce).

377   Protein was quantified using the DC Assay kit (Bio-Rad) and diluted in SDS Sample Buffer (125

378   mM Tris pH 6.8, 30% glycerol, 10% sodium dodecyl sulfate, 0.012% bromophenol blue) prior to

379   denaturation by heating to 95 °C for 7 minutes. Thirty micrograms of protein lysate were

380   resolved on 4-12% TGX Acrylamide Stain-Free gels (Bio-Rad). Stain-Free gels were imaged

381   prior to transfer to PVDF membrane (Millipore or Bio-Rad) by TransBlot Turbo (Bio-Rad). The

382   blots were then probed with the appropriate primary antibodies: ADAR1 (Santa Cruz, sc-73408;

383   Bethyl Laboratories, A303-883A), DDX17 (Thermo Scientific, PA5-84585), DHX9 (Bethyl, A300-

384   855A), DDX54 (Novus Biologicals, NB100-60678), eIF2a (Abcam, ab5369), eIF2a-Ser-51-P

385   (Abcam, ab32157), Fibrillarin (Santa Cruz, sc-25397), beta-tubulin (Abcam, ab6046), ISG15

386   (Santa Cruz, sc-166755), cleaved PARP (Cell Signaling, #9541), PKR (Cell Signaling, #3072),

387   PKR Thr-446-P (Abcam, ab32036). Primary antibodies were detected with horseradish-

388   peroxidase conjugated secondary antibodies (Jackson ImmunoResearch) and detection was

389   carried out with Clarity Western ECL Substrate (Bio-Rad). Densitometry was performed using

390   Image Lab (Bio-Rad). Band intensity was normalized to total protein measured by imaging of

391   the Stain-Free gel.

392   **Proximity Labeling by APEX2**

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393 SK-BR-3, MCF-7, or MDA-MB-231 cells expressing pLVX-puro-FLAG-APEX2 or pLVX-

394 puro-FLAG-APEX2-ADAR1p110 were grown to ~80% confluency in a 15 cm dish. Quencher

395 solution (10 mM sodium azide, 10 mM sodium ascorbate, and 5 mM Trolox (Sigma-Aldrich,

396 238813-1G) in 1X PBS) was prepared at 1X and 2X concentrations. Prior to labeling, cells were

397 incubated in 10 mL of culture media containing 500 µM biotin phenol (Toronto Research

398 Chemicals, B397770) for 30 min at 37 °C. Next, hydrogen peroxide was added to the cells at 1

399 mM and incubated at room temperature for 1 min. Immediately one volume of 2X quencher

400 solution was added to the cells to stop labeling. The cells were washed twice with 1X quencher

401 solution. Cells were harvested by scraping in 1X quencher solution and lysed in RIPA buffer

402 containing 10 mM sodium azide, 10 mM sodium ascorbate, 5 mM Trolox and 1x HALT. Biotin

403 labeling was verified by immunoblotting with HRP-streptavidin (Abcam, ab7403). Biotinylated

404 proteins were purified using streptavidin magnetic beads (Thermo Fisher Scientific, 88816).

405 Streptavidin magnetic beads were washed twice with RIPA containing HALT and quenching

406 agents. The lysate from above was incubated with the beads for 1 hour at room temperature.

407 The beads were washed in the following order: once with RIPA containing HALT and quenching

408 agents, once with RIPA, once with 1 M KCl, once with 2 M urea pH 8.0, twice in RIPA and once

409 with water. Elution was performed in 1X SDS-Sample buffer by heating at 95 °C for 10 minutes.

410 The eluate was analyzed by LC-MS-MS, see below.

411 **Mass Spectrometry**

412 Liquid-chromatography and tandem mass spectrometry was performed by MSBioWorks

413 (Ann Arbor, MI). The eluates from the streptavidin pulldown above were processed by SDS-

414 PAGE using 10% Bis-Tris NuPage Mini-gel (Invitrogen) with the MES buffer system. The gel

415 was run 2cm. The mobility region was excised and processed by in-gel digestion with trypsin

416 using a robot (ProGest, DigiLab). For the trypsin digestion, the gel slices were washed with

417 25mM ammonium bicarbonate followed by acetonitrile. The samples were reduced with 10mM

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418   dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at room temperature.

419   Subsequently proteins were digested with trypsin (Promega) at 37°C for 4 h. The trypsin

420   digestion was quenched with formic acid and the supernatant was analyzed directly without

421   further processing. The digested sample was analyzed by nano LC-MS/MS with a Waters M-

422   Class HPLC system interfaced to a ThermoFisher Fusion Lumos mass spectrometer. Peptides

423   were loaded on a trapping column and eluted over a 75 µm analytical column at 350 nL/min;

424   both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was

425   operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 15,000

426   FWHM for MS and MS/MS respectively. APD was enabled and the instrument was run with a 3

427   s cycle for MS and MS/MS. Five hours of instrument time was used for the analysis of each

428   sample.

429   **Analysis of Mass Spectrometry Data**

430   Data were searched using a local copy of Mascot (Matrix Science) with the following

431   parameters: Enzyme - Trypsin/P; Database - SwissProt Human (concatenated forward and

432   reverse plus common contaminants); Fixed modification – Carbamidomethyl; Variable

433   modifications - Oxidation; Acetyl; Pyro-Glu; Deamidation; Mass values – Monoisotopic; Peptide

434   Mass Tolerance - 10 ppm; Fragment Mass Tolerance - 0.02 Da; Max Missed Cleavages - 2.

435   Mascot DAT files were parsed into Scaffold (Proteome Software) for validation, filtering and to

436   create a non-redundant list per sample. Data were filtered using a 1% protein and peptide FDR

437   and requiring at least two unique peptides per protein. Fold change of protein abundance was

438   determined by DESeq2 using spectral counts (see Data Availability below for scripts).

439   Overrepresentation analysis was performed using 'enrichR' in R[65]. The cutoff used for

440   enrichment for the overrepresentation analysis was an FDR < 0.05 and a log2 fold change of >

441   0.5.

Immunoprecipitation

442

443   Cell lysates prepared in RIPA with 1X HALT. RNaseOUT (Thermo Fisher) RNase

444 inhibitor was added to the lysis buffer at 0.5 U/µL when RNase A was not used. One milligram

445 of protein lysate was mixed with 2-10 µg of IgG or specific antibody overnight at 4 °C with

446 rotation. For samples treated with RNase A, 20 µg of RNase A (Invitrogen) was added to the

447 lysate during overnight mixing with the antibody. Protein G Dynabeads (Thermo Fisher, 25 µL

448 per sample) were prepared by washing twice in the lysis buffer. Prepared beads were mixed

449 with lysates for 30 min at 4 °C with rotation. Supernatants were collected and beads were

450 washed three times in the lysis buffer, and eluted by mixing the beads in SDS sample buffer and

451 incubating at 95 °C for 7 min. Antibodies: Rabbit IgG (Jackson ImmunoResearch, 011-000-003),

452 Mouse IgG (Jackson ImmunoResearch, 015-000-003), DHX9 (Bethyl, A300-855A), PARP (Cell

453 Signaling, 9532S), XRN2 (Novus, NB100-57541), DDX54 (Novus, NB100-60678), DDX17

454 (Thermo Scientific, PA5-84585).

Immunofluorescence

455

456   Cells were plated on glass coverslips (Corning) two days prior to fixation for

457 immunofluorescence. Cells were washed in PBS prior to fixation with 4% paraformaldehyde

458 (Thermo Scientific) and permeabilization with 0.15% Triton-X100 in PBS. Following

459 permeabilization the cells were washed three times with PBS then blocked with Protein Block

460 (Aligent/Dako, X090930-2). Primary antibodies (ADAR1 (Santa Cruz, sc-73408), Fibrillarin

461 (Santa Cruz, sc-25397), DDX54 (Novus Biologicals, NB100-60678) or DDX17 (Thermo

462 Scientific, PA5-84585), DHX9 (Bethyl, A300-855A)) and secondary antibodies (Thermo

463 Scientific, A21207, A21203, A21202, A21206) were diluted in Antibody Diluent (Agilent/Dako,

464 S302283-2). Antibody binding was performed in a humidity chamber for 1.5 hours for primaries

465 and 30 minutes for secondaries. Between primary and secondary antibodies, and after

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466   secondary antibody binding the coverslips were washed in PBS. The coverslips were washed

467   once in water before mounting on glass slides with Vectashield Antifade Mounting Media with

468   DAPI (Vector Laboratories, H-1200-10). Fluorescence microscopy images were obtained with

469   an Eclipse 90i microscope (Nikon) using a Plan Apochromatic 20x/NA 0.75 objective (Nikon)

470   and a CoolSNAP ES2 monochrome digital camera cooled to 0°C (Photometrics). Fluorescence

471   images were captured with MetaMorph version 7.8.0.0 software (Molecular Devices) and

472   resized and formatted with Fiji.

Transfection of poly(I:C)

473

474   The cell line indicated was transfected with high-molecular weight poly(I:C) (Invivogen)

475   with Lipofectamine LTX. Three microliters of Lipofectamine LTX was used per microgram of

476   poly(I:C). Sixteen hours after transfection, cells were harvested in RIPA with 1X HALT or the

477   RNA lysis buffer from the Nucleospin RNA kit (Macherey-Nagel).

RNA Purification and RNA sequencing

478

479   RNA-sequencing was performed for two replicates of ADAR1 and/or DHX9 knockdown

480   in MCF-7 and SK-BR-3. RNA was purified using the Nucleospin RNA kit (Macherey-Nagel).

481   Assessment of rRNA integrity and RNA-sequencing was performed by the Genome Technology

482   Access Center at Washington University in St. Louis. Total RNA integrity was determined using

483   Agilent TapeStation 4200. Library preparation was performed with 500 ng to 1 ug of total RNA.

484   Ribosomal RNA was removed by an RNase-H method using RiboErase kit (Kapa Biosystems).

485   After rRNA depletion, the remaining RNA was then fragmented in reverse transcriptase buffer

486   (Life Technologies) by heating to 94 degrees for 8 minutes. The RNA was reverse transcribed to

487   yield cDNA using SuperScript III RT and random hexamers (Life Technologies) per

488   manufacturer's instructions. A second strand reaction was performed with DNA Polymerase I

489   and RNase H (Qiagen) to yield ds-cDNA. The cDNA was then blunted with T4 DNA

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490   Polymerase, Polynucleotide Kinase and Klenow DNA Polymerase (Qiagen). An A base was

491   added to the 3' ends with Klenow (3'-4' exo-) (Qiagen). The processed ds-cDNA was then

492   ligated to Illumina sequencing adapters with T4 DNA Ligase (Qiagen). Ligated fragments were

493   then amplified for 12-15 cycles using primers incorporating unique dual index tags with VeraSeq

494   polymerase (Qiagen). Fragments were sequenced on an Illumina NovaSeq-6000 using paired

495   end reads extending 150 bases.

496   **RNA Sequencing Analysis**

497   The Illumina bcl2fastq software was used for base calling and demultiplexing, allowing

498   for one mismatch in the indexing read. STAR version 2.7.9a1 was used for read alignment to

499   RNA-seq to the Ensembl GRCh38.101 primary assembly. Gene counts were determined using

500   Subread:featureCount version 2.0.32, only uniquely aligned unambiguous reads were counted.

501   Differential gene expression was determined using DESeq2 (see Data Availability below for

502   scripts)[66]. The experimental design for DESeq2 analysis included an interaction term between

503   the shRNAs used for knockdown ('shrna1 + shrna2 + shrna1:shrna2'; where 'shrna1' was either

504   shSCR or shADAR and 'shrna2' was either shSCR or shDHX9-3). Contrasts were used to

505   assess differential expression after singular knockdown of either ADAR1 or DHX9. Fold

506   changes were shrunken using the 'apeglm' approach from DESeq2[67]. Gene set enrichment

507   analysis was performed using 'clusterProfiler'[68]. For analysis of transposable element

508   expression, 'TEcount' from TEtranscripts[69] was used to determine family level counts for

509   transposable elements using a GTF file containing transposable element information from

510   RepeatMasker (http://www.repeatmasker.org). (see Data Availability section for more

511   information about the GTF file).

512   **Foci Formation Assay**

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513   Five thousand cells were plated for each condition in a 10 cm culture dish. After 10 (BT-

514   549, MDA-MB-MB231, HCC1806, MDA-MB-468 and SK-BR-3) to 20 (MCF-7) days the cells

515   were washed briefly with 1x PBS prior to fixation in 100% methanol for 5 min. After drying, the

516   cells were stained with 0.005% Crystal Violet solution containing 25% methanol (Sigma-Aldrich)

517   prior to washing excess stain away with deionized water. The plates were scanned using an

518   ImageScanner III (General Electric). Foci area was calculated using ImageJ.

519   **Analysis of CCLE and TCGA data**

520   RNA-seq normalization and calculation of z-scores was performed as described

521   previously[33]. Molecular subtypes of breast cancer cell lines and TCGA samples were defined

522   previously[70]. Breast cancer survival analysis was performed using the R packages RTCGA and

523   survminer[71,72]. The DHX9 expression level used for stratification in survival analysis was

524   determine by the surv_cutpoint function of survminer.

525   **Data and Code Availability**

526   Scripts used for analysis of mass spectrometry, RNA-seq and generation of all plots are

527   available at (https://github.com/cottrellka/Cottrell-Ryu-et-al-2023), raw sequencing data and

528   count files were deposited at the Gene Expression Omnibus (GEO) under accession

529   GSE224677. RNA-seq data for cancer cell lines (CCLE_expression_full.csv,

530   CCLE_RNAseq_rsem_transcripts_tpm_20180929.txt) were obtained from the DepMap portal

531   (https://depmap.org/portal/download/custom/)[73]. RNAi based dependency data for DHX9

532   (D2_combined_gene_dep_scores) was obtained from DepMap Portal

533   (https://depmap.org/portal/download/custom/)[74]. RNA-seq data for TCGA BRCA samples

534   (illuminahiseq_rnaseqv2-RSEM_genes, illuminahiseq_rnaseqv2-RSEM_isoforms_normalized)

535   and clinical data (Merge_Clinical) were obtained from the Broad Institute FireBrowse and are

536   available at http://firebrowse.org/. The GTF file used for TEcount

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537   (GRCh38_Ensembl_rmsk_TE.gtf) is available at

538   https://www.dropbox.com/sh/1ppg2e0fbc64bqw/AACUXf-TA1rnBIjvykMH2Lcia?dl=0.

539

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552

Author Contributions

554   K.A.C, S.R. and J.D.W. conceived the project. K.A.C., S.R., L.S.T., and A.M.S.

555   performed the experiments. K.A.C., S.R., and L.S.T. performed the data analysis. K.A.C. and

556   S.R. wrote the manuscript. All authors edited the manuscript.

557

Figure 1: Identification of putative ADAR1 interacting proteins by APEX2 proximity labeling

a Immunoblot showing expression of the constructs used for proximity labeling in MCF-7, MDA-MB-231 and SK-BR-3. **b** Representative fluorescently stained gel image showing proteins purified by streptavidin-biotin pulldown following proximity labeling in MCF-7. **c** Volcano plot summarizing the proteins identified by mass spectrometry following streptavidin pulldown subsequent to proximity labeling. Differential abundance of all proteins in each cell line can be found in Supplementary Table 2. **d** Venn diagram showing overlap between enriched proteins from all three cell lines. The cut-off for enriched proteins was an FDR adjusted p-value of less than 0.05 and a Log2 fold change of greater than 0.5. **e** GO terms found to be overrepresented in the list of the enriched proteins. Only the top ten GO terms, by FDR, are shown for each category. All other significant GO terms are available in the Supplementary Table 3-4. **f** and **g** Venn diagrams showing overlap between the enriched proteins identified and those proteins found previously to localize to the nucleus or nucleolus in MCF-7[43]. **h** Representative indirect immunofluorescence micrographs showing localization of ADAR1, fibrillarin (a nucleolar marker) and DAPI.

574 **Figure 2: Validation of putative protein-protein interactions identified by proximity**

575 **labeling**

576 Immunoprecipitation of DHX9 **a-c**, DDX54 **d-e** or DDX17 **f-g** followed by immunoblot for ADAR

577 in breast cancer cell lines**.** Immunoblot of immunoprecipitation eluates and inputs from SK-BR-3

578 **a**, **d** and **f**, MCF-7 **b**, **e** and **g**, and MDA-MB-231 **c**. Input represents 5% of the lysate used for

579 immunoprecipitation. The IgG lanes represent immunoprecipitation eluates from pulldown with

580 anti-rabbit IgG antibody. The lanes labeled DHX9, DDX54 and DDX17 indicate the eluates from

581 immunoprecipitation with antibodies against those proteins respectively. The IgG^{HC} label

582 indicates the band corresponding to the IgG heavy chain from the antibody used for

583 immunoprecipitation. Uncropped immunoblots for panels **a-g** can be found in Source Data

584 Figures. Immunofluorescence for ADAR1 and DHX9 **h**, DDX54 **i**, or DDX17 **j** in SKBR3 or MCF-

585 7.

586

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587   **Figure 3: DHX9 is overexpressed in breast cancer and suppresses PKR activation**

588   **a** Schematic showing the domain structure of PKR, ADAR1, DHX9 and other helicases

589   identified by proximity labeling in Fig. 1, dsRBD refers to the dsRNA Binding Domain. **b**

590   Pearson and Spearman correlation coefficients for the correlation between ADAR1 expression

591   at the RNA level and the expression of each indicated helicase at the RNA level, data from

592   breast tumors within TCGA. Scatterplots showing the correlation between ADAR1-p110 **c**, or

593   ADAR1-p150 **d**, and DHX9 expression in normal breast or breast tumors. **e** Expression of DHX9

594   at the RNA level in normal breast, non-TNBC or TNBC tumors. **f** Waterfall plot showing DHX9

595   dependency of breast cancer cell lines using data from DepMap, ER = estrogen receptor

596   positive cell lines, ERRB2 = HER2 positive cell lines. **g** Representative immunoblot following

597   knockdown of DHX9 with two different shRNAs in four TNBC cell lines. Immunoblots for other

598   replicates and uncropped blots can be found in Source Data Figures. **h** Foci formation assay for

599   the same cells used in **g** for immunoblot. **i** Quantification of PKR phosphorylation as determined

600   by the immunoblot in **g**. Quantification of protein expression for other proteins of interest can be

601   found in Extended Data Fig. 3a-e. Bars represent the average of at least three replicates, error

602   bars are +/- SD. * p <0.05, ** p <0.01, *** p < 0.001. P-values determined by Dunnett's test.

603

604 **Figure 4: DHX9 and ADAR1 redundantly suppress dsRNA sensing in ADAR1-independent**

605 **cell lines**

606 Representative immunoblot showing the phenotype of ADAR1 and/or DHX9 knockdown in

607 MCF-7 **a**, or SK-BR-3 **f**. Immunoblots for other replicates and uncropped blots can be found in

608 Source Data Figures. Protein abundance from the immunoblot in **a** and **f** was normalized by

609 total protein abundance by quantification of the Stain Free gel in **b** and **g** respectively. Fold

610 change of PKR phosphorylation at Thr-446 in MCF-7 **d** or SK-BR-3 **i** as determined by the

611 immunoblots in **a** or **f** respectively. Quantification of protein expression for other proteins of

612 interest can be found in Extended Data Fig. 4a-l. Representative foci formation phenotype of

613 ADAR1 and/or DHX9 knockdown in MCF-7 **c** or SK-BR-3 **h**, quantification of relative foci area is

614 shown in **e** or **j** respectively. **k** Analysis of rRNA integrity upon knockdown of ADAR1 and/or

615 DHX9 in MCF-7 or SK-BR-3. Additionally, panel **k** shows the effect of poly(I:C) (p(I:C))

616 transfection on rRNA integrity in MCF-7. Bars represent the average of at least three replicates,

617 error bars are +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P-values determined by one-way

618 ANOVA with post-hoc Tukey. Comparisons between the two different shRNAs targeting DHX9

619 (shDHX9-3 and shDHX9-5) were not included for clarity.

620

621 **Figure 5: Induction of a viral mimicry phenotype upon knockdown of DHX9 and ADAR1 in**

622 **MCF-7**

623 **A** Volcano plot showing changes in RNA expression upon knockdown of DHX9 and ADAR1 in

624 MCF-7, a volcano plot for SK-BR-3 is in Extended Data Fig. 5f. Fold-change of RNA expression

625 shown in **a** was determined using an interaction term between ADAR1 and DHX9 knockdown,

626 volcano plots for fold change of RNA expression for single knockdown of ADAR1 or DHX9 is in

627 Extended Data Fig. 5a-b and 5d-e. **b** GO terms identified by gene set enrichment analysis of the

628 RNA-seq data in **a**. **c-e** Heatmaps and summary box plots showing RNA expression changes in

629 MCF-7 and SK-BR-3 upon knockdown of ADAR1 and/or DHX9. Panel **c** shows RNA expression

630 for core ISGs[21,33], panel **d** shows ATF4 targets and panel **e** shows NF-KB targets with ISGs

631 removed.

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632 **Figure 6: Rescue of PKR activation by ADAR1-p110, ADAR-p150, DHX9 and DHX9**

633 **mutants**

634 **a** Schematic showing the domain structure of ADAR1 isoforms, DHX9 and mutants of DHX9,

635 dsRBD refers to the dsRNA Binding Domain. Representative immunoblot showing the

636 phenotype of ADAR1 and DHX9 knockdown with DHX9, DHX9^{K417R} or dsRBD-EGFP

637 overexpression **b**, or ADAR1 isoform overexpression **f**. Immunoblots for other replicates and

638 uncropped blots can be found in Source Data Figures. Fold change of PKR phosphorylation at

639 Thr-446 upon ADAR1 and DHX9 knockdown with DHX9, DHX9^{K417R} or dsRBD-EGFP

640 overexpression **d**, or ADAR1 isoform overexpression **h**, quantified from immunoblots in **b** and **f**

641 respectively. Quantification of protein expression for other proteins of interest can be found in

642 Extended Data Fig. 7b-f and 8a-f. Representative foci formation phenotype of ADAR1 and

643 DHX9 knockdown with DHX9, DHX9^{K417R} or dsRBD-EGFP overexpression **c**, or ADAR1 isoform

644 overexpression **g**. Quantification of relative foci area is shown in **e** or **i**, respectively. Bars

645 represent the average of at least three replicates, error bars are +/- SD. * $p < 0.05$, ** $p < 0.01$,

646 *** $p < 0.001$. P-values determined by Dunnett's test.

647

648 **Figure 7: Model of ADAR1 and DHX9s roles in suppression of dsRNA sensing.**

649 A hypothetical model based on published data and the findings presented here. Dashed lines

650 indicate multiple steps. Lines marked with question marks are proposed and require further

651 investigation. Created with BioRender.com

652

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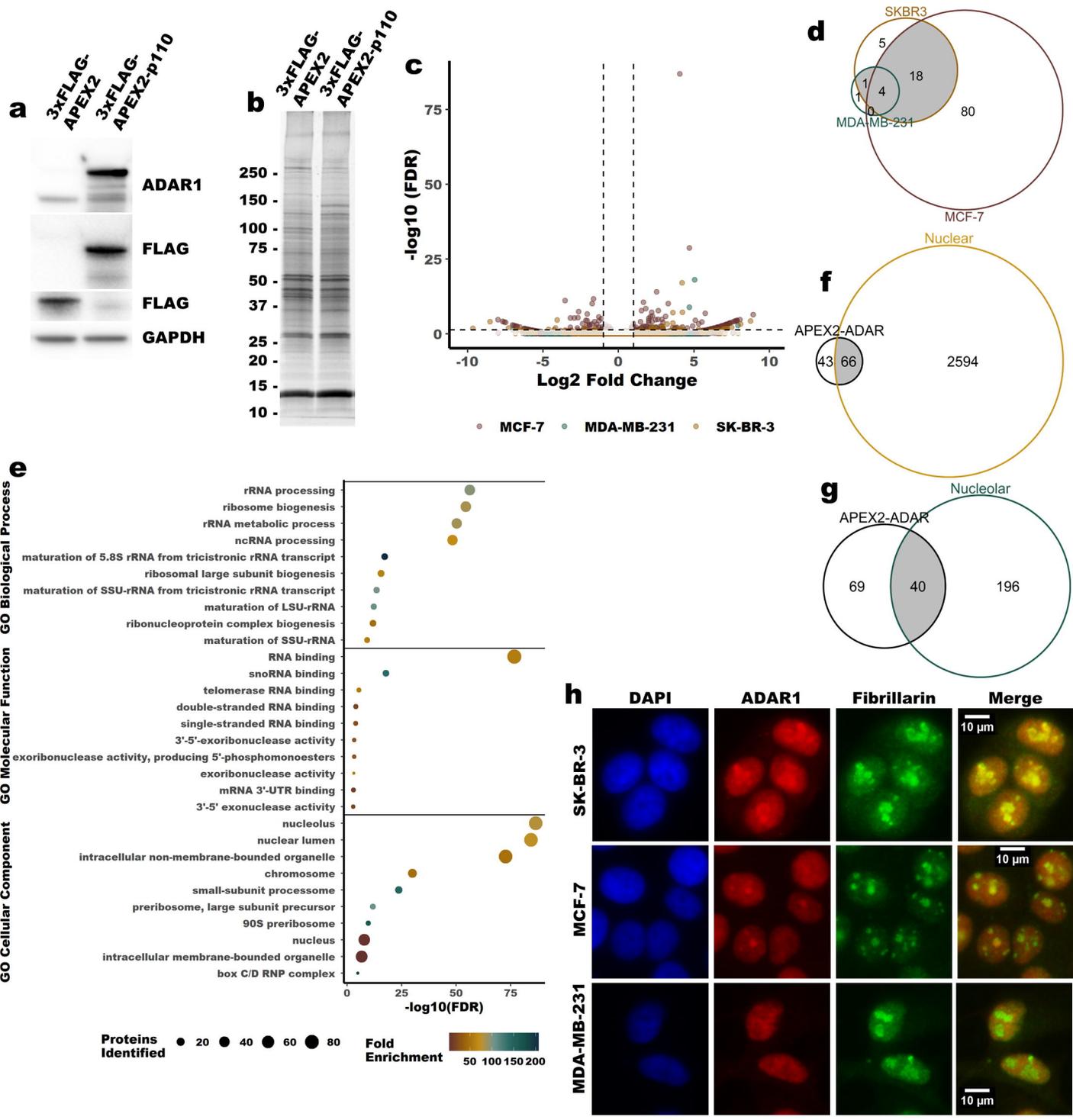820   74. DepMap 22Q4 Public. (2022) doi:10.6084/m9.figshare.21637199.v2.

821

Figure 1
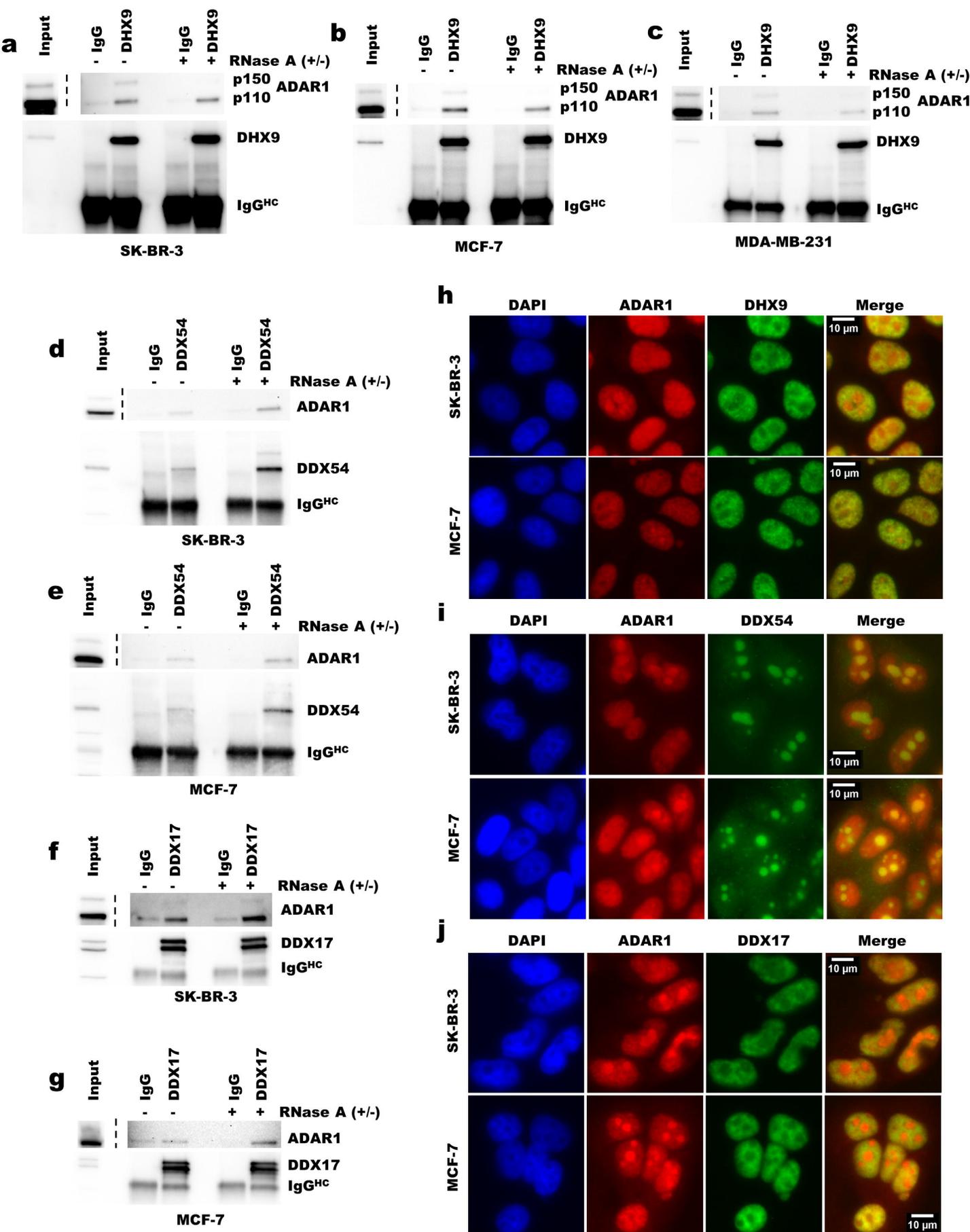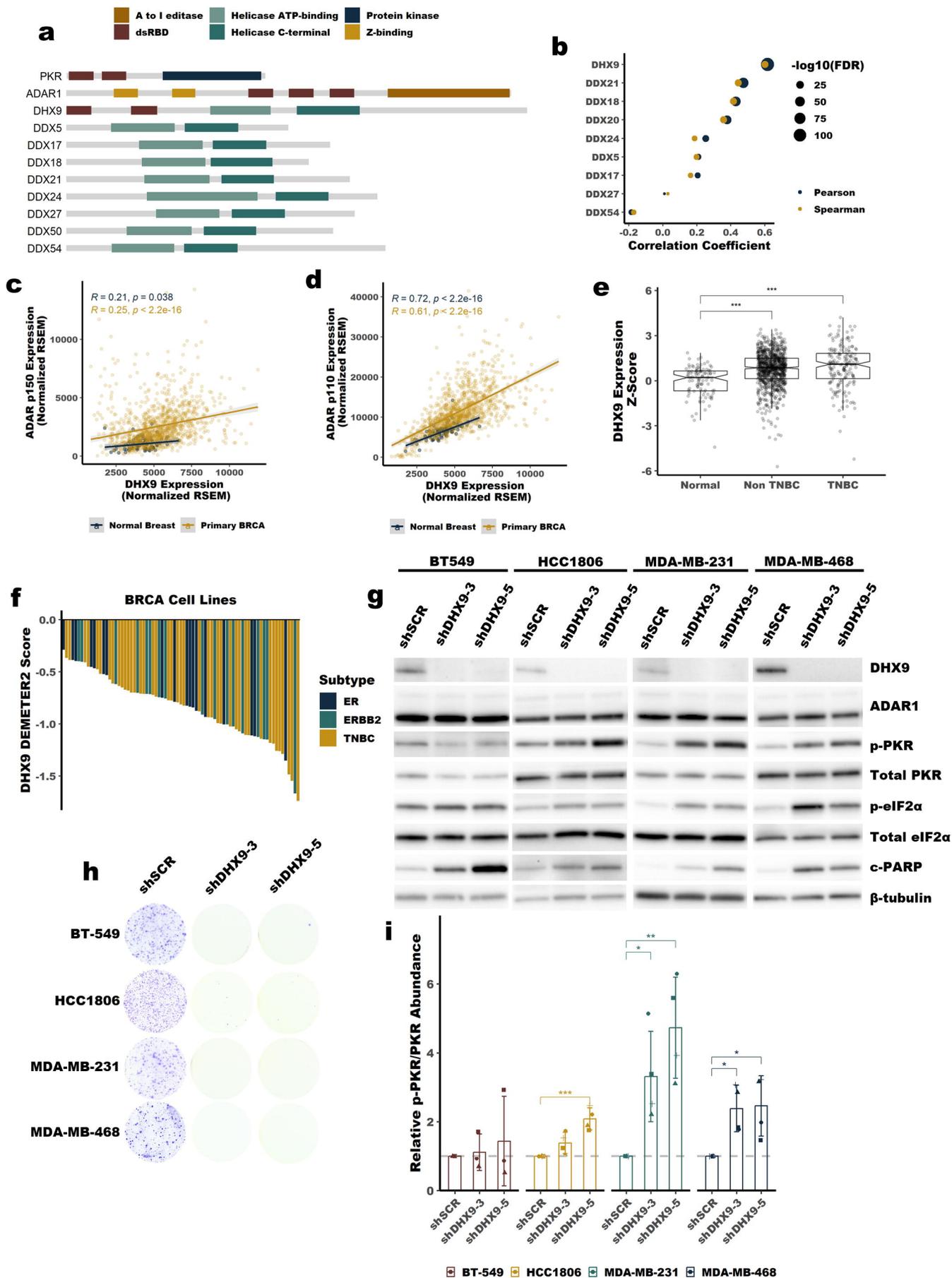

a

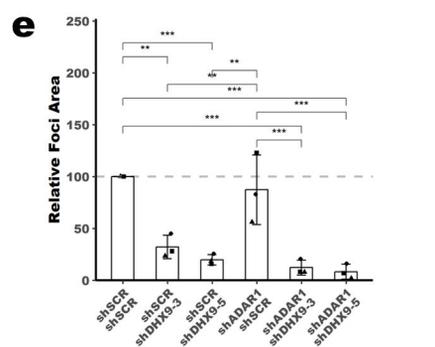3xFLAG-APEX2 | 3xFLAG-APEX2-p110

ADAR1
FLAG
FLAG
GAPDH

b

3xFLAG-APEX2 | 3xFLAG-APEX2-p110

250 —
150 —
100 —
75 —
50 —
37 —
25 —
20 —
15 —
10 —

c

-log10 (FDR)

75

50

25

0

-10   -5   0   5   10

Log2 Fold Change

● MCF-7   ● MDA-MB-231   ● SK-BR-3

d

SKBR3
5
18
1
1 4
0
MDA-MB-231
80
MCF-7

f

Nuclear
APEX2-ADAR
43 66
2594

g

Nucleolar
APEX2-ADAR
69 40 196

e

GO Biological Process
rRNA processing
ribosome biogenesis
rRNA metabolic process
ncRNA processing
maturation of 5.8S rRNA from tricistronic rRNA transcript
ribosomal large subunit biogenesis
maturation of SSU-rRNA from tricistronic rRNA transcript
maturation of LSU-rRNA
ribonucleoprotein complex biogenesis
maturation of SSU-rRNA

GO Molecular Function
RNA binding
snoRNA binding
telomerase RNA binding
double-stranded RNA binding
single-stranded RNA binding
3'-5'-exoribonuclease activity
exoribonuclease activity, producing 5'-phosphomonoesters
exoribonuclease activity
mRNA 3'-UTR binding
3'-5' exonuclease activity

GO Cellular Component
nucleolus
nuclear lumen
intracellular non-membrane-bounded organelle
chromosome
small-subunit processome
preribosome, large subunit precursor
90S preribosome
nucleus
intracellular membrane-bounded organelle
box C/D RNP complex

0   25   50   75

-log10(FDR)

Proteins Identified   ● 20 ● 40 ● 60 ● 80

Fold Enrichment   50 100 150 200

h

DAPI | ADAR1 | Fibrillarin | Merge

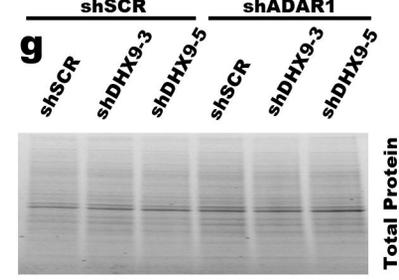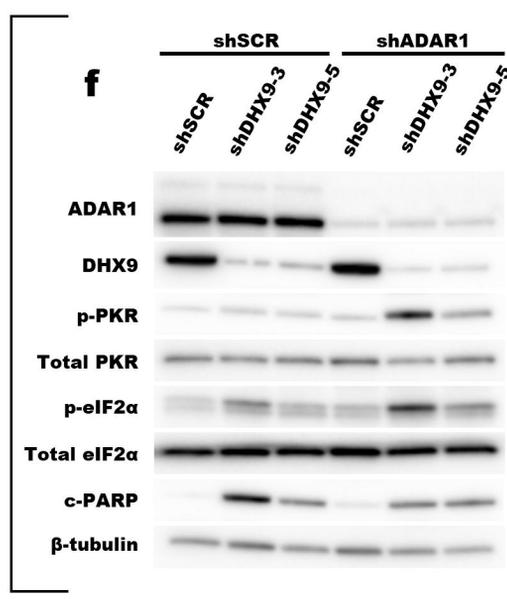SK-BR-3   10 µm
MCF-7   10 µm
MDA-MB-231   10 µm

Figure 2

Figure 3

Figure 4
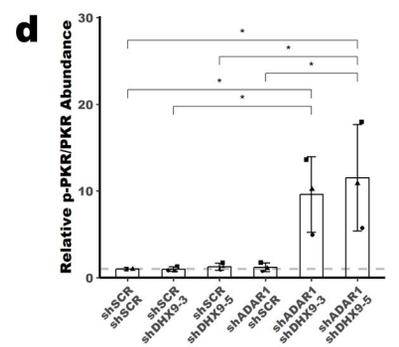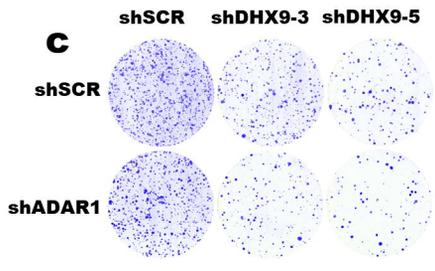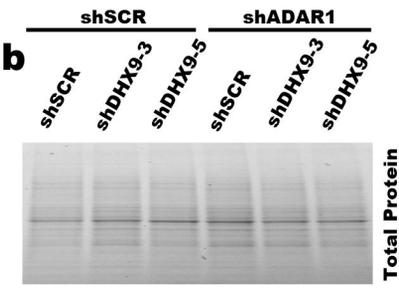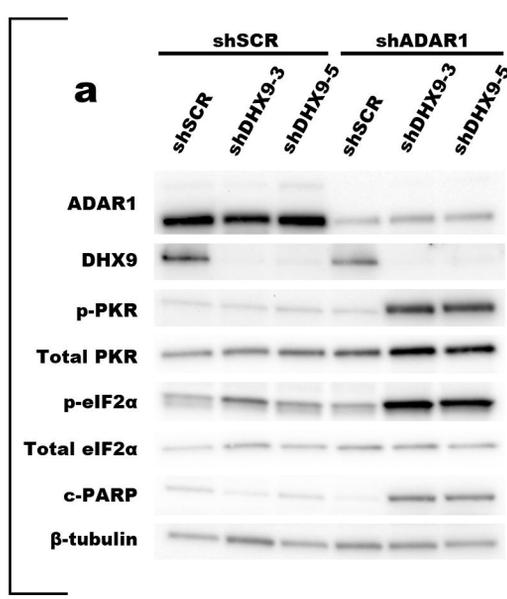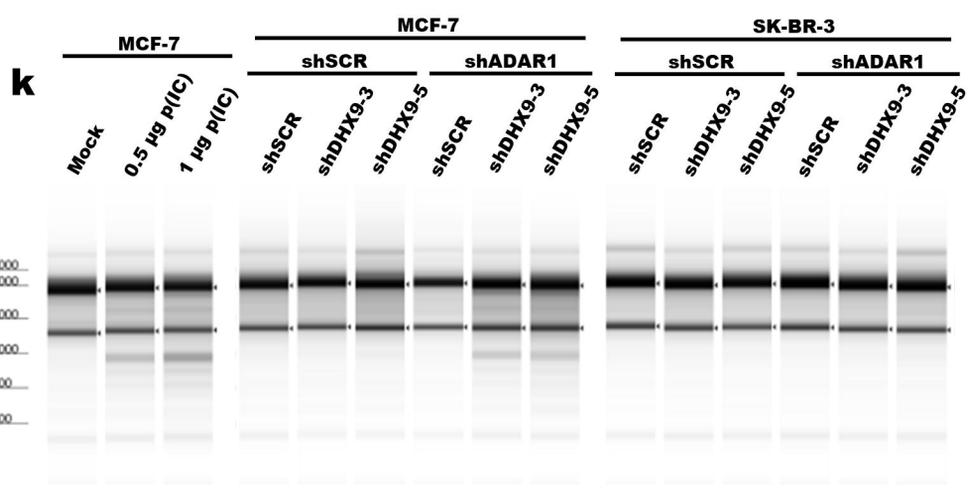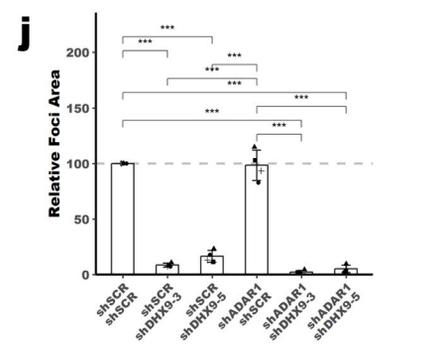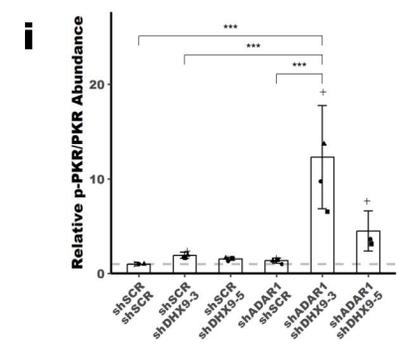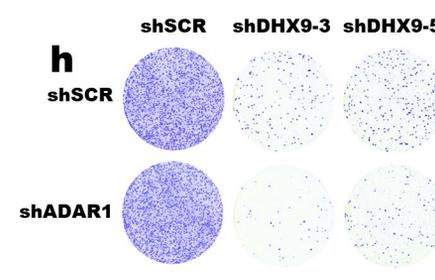
Figure 5

Figure 6

Figure 7