Epigenetic therapy induces transcription of Inverted SINEs and ADAR1 dependency

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SUMMARY:

Cancer therapies that target epigenetic repressors can mediate their effects by activating retroelements within the human genome. Retroelement transcripts can form double-stranded RNA (dsRNA) that activates the MDA5 pattern recognition receptor[1–6]. This state of viral mimicry leads to loss of cancer cell fitness and stimulates innate and adaptive immune responses[7,8]. However, the clinical efficacy of epigenetic therapies has been limited. To find targets that would synergize with the viral mimicry response, we sought to identify the immunogenic retroelements that are activated by epigenetic therapies. Here we show that intronic and intergenic SINE elements, specifically inverted-repeat Alus, are the major source of drug-induced immunogenic dsRNA. These inverted-repeat Alus are frequently located downstream of 'orphan' CpG islands[9]. In mammals, the ADAR1 enzyme targets and destabilizes inverted repeat Alu dsRNA[10], which prevents activation of the MDA5 receptor[11]. We found that ADAR1 establishes a negative-feedback loop, restricting the viral mimicry response to epigenetic therapy. Depletion of ADAR1 in patient-derived cancer cells potentiates the efficacy of epigenetic therapy, restraining tumour growth and reducing cancer initiation. Therefore, epigenetic therapies trigger viral mimicry by inducing a subset of inverted-repeats Alus, leading to an ADAR1 dependency. Our findings suggest that combining epigenetic therapies with ADAR1 inhibitors represents a promising strategy for cancer treatment.

Main

Therapies that target repressive epigenetic machinery can mediate their anti-tumour effects by activating retroelements[1–6]. Active retroelements generate dsRNA, which in turn stimulate the pattern recognition receptor MDA5[1–6]. While the viral mimicry state has been well characterized by a type I/III interferon response, loss of cancer cell fitness and activation of innate and adaptive immune responses[7,8], the identity of epigenetically induced immunogenic retroelements remain unknown.

To identify drug-induced immunogenic retroelements on a global scale, we examined the formation of endogenous dsRNA in patient-derived colorectal cancer (CRC) cells treated with low doses of 5-AZA-CdR, an FDA-approved DNA methyltransferase inhibitor (DNMTi). Total-stranded RNA sequencing (RNA-seq) analysis revealed an increase in sense and/or antisense transcription of long interspersed nuclear elements (LINEs) and endogenous retroviruses (ERVs), but not short interspersed nuclear elements (SINEs), after DNMTi treatment **(Extended Data Fig. 1a)**, consistent with previous studies showing that disruption of repressive epigenetic marks lead to formation of dsRNA from LINEs and ERVs through bidirectional transcription[1–5,12,13].

The viral mimicry response to epigenetic therapy is dependent on activation of MDA5 by endogenous dsRNAs[1,4,5]. To directly identify drug-induced immunogenic retroelements, we performed an MDA5-protection assay[14], followed by RNA-seq analysis **(Extended Data Fig. 2a).** SINEs, specifically Alu retroelements, were remarkably enriched in MDA5-protected RNA compared to total cytosolic RNA **(Fig. 1a–c)**. Notably, the enrichment of Alu increased from 53.03% in mock-treated samples **(Fig. 1a)** to 72.33% in DNMTi-treated samples **(Fig. 1b).** By contrast, MDA5-protected RNA contained only 1.71% long-terminal repeats (LTRs) and 6.40% LINEs in mock-treated samples **(Fig. 1a)**, and only 1.81% LTRs and 7.56% LINEs in DNMTi-treated samples **(Fig. 1b).**

To identify immunogenic retroelements induced only by DNMTi treatment, we excluded those that were expressed in both the mock and DNMTi-treated samples **(Extended Data Fig. 2b).** Alu retroelements represented 88.72% of treatment-induced immunogenic RNA **(Fig. 1c)**, with the remaining RNA originating from non-repeats (5.15%), LINEs (3.86%) and LTR elements (1.37%) **(Fig. 1c)**. Collectively, these results demonstrate that although many dsRNA species are induced after DNMTi treatment, Alu elements, but not LTRs or ERVs[1,2,4], are the main source of immunogenic dsRNA induced upon epigenetic therapy.

To uncover how repetitive elements generate immunogenic dsRNA, we examined all repeats in MDA5-protected transcripts and searched for repeat pairs within a 3-kb window. Notably, we found that most aligned pairs in both baseline and treatment-induced RNA were inverted-repeat Alus (IR-Alus) **(Extended Data Fig. 2c, d)**. MDA5-

protected RNA also contained higher expression levels of IR-Alus compared with non-IR Alus **(Extended Data Fig. 1b, c)**. Although the expression of baseline IR-Alus was maintained after 5-AZA-CdR treatment **(Extended Data Fig. 2e)**, treatment-induced IR-Alu hybrids became highly expressed in MDA5-protected RNA only after treatment with 5-AZA-CdR **(Extended Data Fig. 2f)**. Similarly, we found that both repeats within baseline immunogenic IR-Alu pairs were concordantly enriched in MDA5-protected RNA before and after treatment with DNMTi **(Extended Data Fig. 1d)**, whereas both repeats in treatment-induced immunogenic IR-Alu pairs became concordantly enriched only after treatment with DNMTi **(Extended Data Fig. 1e)**. Moreover, most IR-Alu pairs were unidirectionally transcribed either in the sense (+/+) or antisense (−/−) direction in both baseline **(Fig. 1d)** and treatment-induced **(Fig. 1e)** dsRNA. Together, these observations suggest that DNMTi treatment gives rise to immunogenic dsRNA via intramolecular pairing of IR-Alus, forming RNA stem–loops, rather than the previously proposed model of sense or antisense transcription[4] **(Extended Data Fig. 2g).**

Next, we investigated how DNMTi treatment induces the expression of IR-Alu elements. We detected 746,470 IR-Alu pairs in the human genome, with about 55% originating from introns and 43% from intergenic regions **(Fig. 2a)**. Consistent with previous studies[14,15], most baseline immunogenic IR-Alu pairs were within 3′ untranslated regions (UTRs) (35%) and introns (42%) **(Fig. 2a)**. Unexpectedly, the vast majority of treatment-induced immunogenic IR-Alu pairs were within introns (70%), followed by intergenic regions (16%) **(Fig. 2a)**. These distributions were broadly independent of genomic orientation of each repeat within the pair **(Extended Data Fig. 3a, b).** We also investigated the distribution of Alu subfamilies of the immunogenic IR-Alus. Although the evolutionary younger AluY subfamilies[16] were observed at the same rate as IR-Alu pairs in the human genome, we observed an enrichment of the more active AluS subfamilies and a depletion of the more ancient AluJ subfamilies[16] **(Extended Data Fig. 3c).**

In addition, we mapped the average CpG density flanking IR-Alu pairs. Notably, CpG density was high directly upstream of IR-Alu pairs induced by DNMTi treatment **(Fig. 2b)**, but not upstream of baseline IR-Alus **(Extended Data Fig. 3d).** Indeed, we detected a high density of bona-fide CpG islands (CGIs) upstream of treatment-induced IR-Alu pairs compared to baseline and all other IR-Alu pairs in the human genome **(Extended Data Fig. 3e, f)**. Of the roughly 25,000 CGIs present in the human genome, nearly half reside outside of promoters of annotated genes, and are generally found in intergenic and intronic regions[9]. These ‘orphan’ CGIs are more frequently methylated than CGIs at the promoters of annotated genes[9]. We therefore assessed the methylation status of CGIs directly upstream of treatment-induced immunogenic IR-Alu repeats. Analysis of a panel of 51 CRC cancer cell lines[17] revealed that most (57%) CGIs adjacent to immunogenic IR-Alus are either partially or fully methylated in untreated cells **(Extended Data Fig. 4a)**. These results were validated across a larger panel of 988 cancer cell lines from 33 different cancer types[17] **(Extended Data Fig. 4b)**.

Active promoters and enhancers are often marked by histone 3 lysine 4 trimethylation (H3K4me3) and H3K27ac, respectively. We performed genome-wide CUT&RUN (cleavage under targets and release using nuclease)[18] analysis and found that DNMTi treatment led to increased levels of H3K4me3, but not H3K27ac, directly upstream of treatment-induced immunogenic IR-Alu repeats **(Fig. 2c)**. Notably, most of these specific regulatory regions were also found to be frequently methylated before treatment **(Extended Data Fig. 4c, d)** and their methylation status was inversely correlated with expression of a panel of interferon-stimulated genes (ISGs) (n = 22) from our viral mimicry signature[1] across the 988 pan-cancer cell lines **(Extended Data Fig. 4e)**. Overall, our data suggest that orphan CGIs located upstream of treatment-induced IR-Alus in intronic and intergenic regions are frequently methylated and act as sites of cryptic transcription initiation after treatment with DNMTi **(Fig. 2d, Extended Data Fig. 3g, h).** By contrast, IR-Alus transcribed basally are mainly located at 3′ UTRs **(Extended Data Fig. 3i)** and probably originate from annotated transcriptional start sites as previously suggested[14,15]. DNMTi therefore preferentially activate cryptic transcription of intronic and intergenic IR-Alus, leading to RNA stem–loops that stimulate MDA5 **(Extended Data Fig. 3j).**

To further characterize how immunogenic IR-Alus can reach the cytoplasm, where they can be recognized by MDA5, we assessed the presence of poly(A) signals (PASs) within immunogenic IR-Alu pairs. We searched for known PAS motifs[19] in the second half (3′ end) of the distal Alu of each pair using the MDA5-protected RNA-seq data and observed that most IR-Alu pairs have at least one PAS **(Extended Data Fig. 5a, b).** Notably, the MDA5-protected RNA-seq signal peaked immediately upstream of PAS motifs and finished abruptly at the 3′ end of these PAS **(Extended Data Fig. 5c, d),** which suggests that cleavage and polyadenylation occur at the 3′ end of the dsRNA stem, mediating the export of immunogenic dsRNA to the cytoplasm. Consistent with these findings, analysis of cytoplasmic RNA-seq revealed a significant enrichment of SINEs compared to total RNA-seq (nuclear and cytoplasmic), among which Alus were particularly enriched in 5-AZA-CdR-treated cells **(Extended Data Fig. 5e**). Together, these results help to explain the accumulation of cytoplasmic dsRNA observed after epigenetic therapy[1,4,5] **(Extended Data Fig. 5f).**

RNA stem–loops formed by IR-Alu elements are a main substrate for the RNA-editing enzyme ADAR1[10]. ADAR1 regulates dsRNA through adenosine-to-inosine (A-to-I) editing and subsequent destabilization of RNA duplexes[11,20]. To determine whether depletion of ADAR1 affects dsRNA formation by IR-Alus, we first generated ADAR1-knockdown CRC cells using two different short hairpin RNAs ($ADAR1^{KDA/B}$) and confirmed depletion of both the constitutively expressed (p110) and the interferon-inducible (p150) ADAR1 isoforms (**Fig. 3a, Supplementary Table 1**). Immunostaining with a monoclonal antibody against dsRNA greater than 40 bp revealed a significant increase in cytoplasmic dsRNA in ADAR1-depleted cells compared to wild-type cells, as expected (**Fig. 3b, Extended Data Fig. 6a**). Notably, cells treated with 5-AZA-CdR upregulated ADAR1 expression, which was sustained for about 14 days after drug withdrawal **(Extended Data Fig. 6b)**.

To determine whether MDA5 ligands also increase after ADAR1 depletion, we performed the MDA5-protection assay followed by RNA-seq in ADAR1-depleted cells and found SINE elements were markedly enriched in MDA5-protected RNA (63.9%) compared to total CytoRNA (8.6%) **(Fig. 3c, d)**. We then examined MDA5 ligands that were specifically induced by ADAR1 depletion by excluding those that were present in both wild-type and ADAR-depleted cells **(Extended Data Fig. 6c)**. We found 76.76% of these immunogenic dsRNA to be Alus **(Fig. 3e)**, consisting primarily of IR-Alu pairs unidirectionally transcribed in either the sense (+/+) or antisense (−/−) direction **(Extended Data Fig. 6d–k)**. Next, we tested whether epigenetic therapy can activate ADAR1-editing activity. As a control, we verified that cells transiently transfected with synthetic dsRNA (poly(I:C)) upregulated the interferon-inducible isoform of ADAR1 (p150) (**Extended Data Fig. 7b**). We found that treatment with 5-AZA-CdR resulted in increased genome-wide A-to-I editing of MDA5-protected Alus, and depletion of ADAR1 abrogated this effect **(Fig. 3f)**. Together, these results suggest that DNMTi treatment stimulates ADAR1 transcription and A-to-I editing resulting in destabilized immunogenic IR-Alu dsRNA.

To assess how ADAR1 depletion affects 5-AZA-CdR-treated samples, we performed RNA-seq analyses. Gene set enrichment analysis (GSEA) revealed that the type I interferon response was the most enriched pathway between wild-type and ADAR1-depleted samples treated with 5-AZA-CdR **(Fig. 3g)**. Expression of ISGs was also enhanced upon ADAR1 depletion in two other CRC cell lines treated with 5-AZA-CdR **(Extended Data Fig. 8a–d, Supplementary Table 2)**. Notably, these effects persisted for as long as 24 days after initial treatment **(Fig. 3h, Extended Data Fig. 7c)**. By contrast, transfection of poly(I:C) in both wild-type and ADAR1-depleted cells led to comparable activation of ISGs across time points **(Extended Data Fig. 7d, e)**. As a molecular read-out of dsRNA pattern recognition activation, we measured aggregation of the mitochondrial protein MAVS in the mitochondrial fraction of cell lysates[21]. Depletion of ADAR1 in 5-AZA-CdR-treated cells resulted in enhanced MAVS activation **(Extended Data Fig. 7a)**. In addition, we generated cells that overexpress the interferon-inducible isoform of ADAR1 (ADAR1-p150) **(Extended Data Fig. 7f)** and observed a rescue in the ability of 5-AZA-CdR to induce expression of ISGs and dsRNA formation **(Extended Data Fig. 7g–i).** Together, ADAR1 restricts the viral mimicry response induced by DNMTi treatment in cancer cells, acting as a negative-feedback loop.

Finally, we sought to investigate whether epigenetic therapy would create an ADAR1 synthetic dependency. ADAR1 knockdown led to a four- to sixfold reduction of the 5-AZA-CdR half-maximal effective concentration (EC50) in vitro **(Fig. 4a)**. Next, we injected ADAR1$^{KDA/B}$ or wild-type patient-derived xenograft CRC cells subcutaneously into immunodeficient NSG mice and treated them with 5-AZA-CdR **(Fig. 4b).** Low-dose 5-AZA-CdR treatment had limited anti-tumour activity in mice, similar to observations in clinical trials of human solid tumours[22] **(Fig. 4c, d)**. However, low-dose 5-AZA-CdR treatments showed remarkable anti-tumour activity in

mice injected with ADAR1$^{KDA/B}$ cells **(Fig. 4c, d, Extended Data Fig. 9a)**. Of note, ADAR1$^{KDA/B}$ and wild-type cells grew equivalently in mock-treated mice **(Fig. 4c, d, Extended Data Fig. 9a, b)**, consistent with recent reports showing that ADAR1 depletion alone does not have considerable anti-tumour effects in most cancer cells[23,24]. Thus, ADAR1 depletion in human cancer cells profoundly sensitizes tumours to 5-AZA-CdR treatment *in vivo*.

One of the major challenges facing conventional chemotherapy is the persistence of cancer-initiating cells, often resulting in relapse[25]. DNMTi can target colorectal cancer-initiating cells by inducing the viral mimicry response[1,26]. To test whether ADAR1 knockdown by itself or in combination with 5-AZA-CdR can decrease tumour initiation, we first used an in vitro sphere-initiation assay followed by limiting dilution analysis (LDA) using patient-derived CRC cells **(Extended Data Fig. 9c)**. Relative to wild-type, ADAR1$^{KDA/B}$ cells treated with 5-AZA-CdR showed a reduced spheroid-forming potential **(Extended Data Fig. 9d)**. Next, we performed in vivo tumour initiation assays by injecting serially diluted patient-derived CRC cells into NSG immunodeficient mice **(Fig. 4e)**. Depletion of ADAR1 alone had minimal effects, but substantially reduced the tumour engraftment potential of CRC cells treated with 5-AZA-CdR **(Fig. 4f, Extended Data Fig. 9e)**.

Finally, to investigate the potential of ADAR1 depletion to synergize with other anti-cancer therapies, we combined ADAR1 knockdown with inhibition of CDK4/6. CDK4/6 inhibitors were recently shown to upregulate dsRNA by reducing levels of DNMT1, leading to a viral mimicry response[3]. Indeed, we observed a small accumulation of dsRNA in wild-type CRC cells treated with the CDK4/6 inhibitor Palbociclib **(Extended Data Fig. 9f, g)**. However, depletion of ADAR1 in combination with palbociclib treatment resulted in stronger accumulation of dsRNA in the cytosol **(Extended Data Fig. 9f, g)** and a synergistic upregulation of ISGs, indicative of an enhanced viral mimicry response **(Extended Data Fig. 9h)**.

In summary, our study identifies IR-Alus, rather than bi-directional ERV transcripts, as the main immunogenic dsRNA induced by DNA-hypomethylating agents. Molecularly, treatment-induced inverted-repeat pairs are transcribed from cryptic promoters within intronic or intergenic regions, which are enriched for orphan CpG islands and acquire active epigenetic marks after DNMTi treatment. We also find that ADAR1 induction establishes a negative-feedback loop that limits the ability of epigenetic therapy to induce a robust and sustained immunogenic dsRNA response. Depletion of ADAR1 in combination with DNMTi treatment profoundly restrained the growth of colorectal cancer tumours. Our findings suggest that the combination of ADAR1 inhibition with epigenetic therapy represents a potent therapeutic strategy.

Figures

Fig. 1. IR-Alus are the major source of immunogenic RNAs induced by 5-AZA-CdR treatment.

a–c, Percentage of non-repeats and repetitive elements in the total cytoplasmic (total CytoRNA) and MDA5-protected fractions after mock treatment (**a**), treatment with 5-AZA-CdR (P<2.2e-16) (**b**), and in 5-AZA-CdR specific transcripts (P<2.2e-16) (**c**). Each plot is the average percentage of two independent replicates. P values represent two-sided Fisher exact test for enrichment of SINEs and depletion of non-repeats in immunogenic RNAs (5-AZA-CdR) and treatment-induced immunogenic RNA compared to the baseline immunogenic RNA. **d, e,** Transcriptional orientation of each IR-Alu pair identified as immunogenic RNA at baseline (n = 1,040) (**d**) and treatment-induced immunogenic RNA (n = 3,687) (**e**). Each bar shows the count of inverted-repeat (IR)-pairs where both repeats are in the sense strand (+/+, blue), antisense strand (−/−, red), or discordant strands (+/− or −/+). Shown are the counts in the RNA-seq data from mock-treated (left) and 5-AZA-CdR-treated (right) cells.

Fig. 2. Genomic distribution and regulatory features IR-Alus induced by 5-AZA-CdR treatment.

a, Genomic distribution of the baseline immunogenic IR-Alu pairs (n = 1,040), treatment-induced immunogenic IR-Alu pairs (n = 3,687), and all IR-Alu pairs present in the human genome (n = 746,470). Odds ratio (OR) shows depletion or enrichment for each genomic category of baseline immunogenic IR-Alu pairs or treatment-induced immunogenic IR-Alu pairs compared to the distribution of all IR-Alu pairs present in the human genome. ****P < 0.0001, two-sided Fisher exact test. **b**, Average CpG density flanking (−50 kb/+50 kb; upstream/downstream) the treatment-induced IR-Alu pairs (n = 3,687) and all existing IR-Alu pairs in the human genome (n = 746,470). For treatment-induced IR-Alu pairs, orientation was based on transcriptional orientation from the MDA5-protected RNA-seq data. **c**, CUT&RUN analysis depicting heat maps and average profiles of H3K4me3 (left) and H3K27ac (right) signal at ±50 kb (upstream/downstream) of the treatment-induced IR-Alu pairs from two independent experiments (n = 3,687). Orientation was based on the transcriptional orientation from the MDA5-protected RNA-seq data. **d**, Representative genomic track of a treatment-induced IR-Alu pair located at an intronic region. The top four tracks represent antisense transcription (in red) and sense transcription (in blue). The bottom track represents CpG density (green).

Fig. 3. ADAR1 suppresses immunogenic Alus and limits viral mimicry response to 5-AZA-CdR treatment.

a, Quantitative PCR (qPCR) (top) and immunoblot (bottom) analysis of ADAR1 knockdown efficiency of two distinct short hairpin RNAs (shRNAs; KDA and KDB) in patient-derived CRC cells. qPCR data are shown as mean ±s.e.m (n = 3 from three independent experiments). ****P < 0.0001, Tukey-corrected one-way ANOVA. Representative immunoblot from two independent experiments depicts two ADAR1 isoforms marked by arrowheads. Anti-tubulin served as a loading control. WT, wild type. **b**, Representative confocal microscopy images from two independent experiments of wild-type ADAR1 and two knockdown ADAR1 cells. DNA was stained with DAPI (blue) and dsRNA was stained using the J2 antibody (red). Scale bars, 50 μm. **c–e**, Percentage of non-repeats and repetitive elements in total cytoplasmic and MDA5-protected fractions at baseline (**c**), after ADAR1 knockdown (P<2.2e-16) (**d**), and in ADAR1-knockdown-induced immunogenic RNA (P<2.2e-16) (**e**). Each plot is the average percentage of two independent replicates. P values represent

two-sided Fisher exact test for enrichment of SINEs and depletion of non-repeats in $ADAR1^{KD}$-baseline immunogenic RNA and $ADAR1^{KD}$-induced immunogenic RNAs compared to MDA5-protected $ADAR1^{WT}$-baseline immunogenic RNA. Baseline plots (**c**) are as in **Fig. 1a** and are plotted for comparison. **f**, Dot plot depicting genome-wide A-to-I editing loci counts of Alus in RNA-seq data performed on total cytoplasmic fraction MDA5-protected fraction at mock-treated (MT) and 5-AZA-CdR-treated wild-type ADAR1 and ADAR1-knockdown conditions. **g**, GSEA showing the top gene signature in 5-AZA-CdR-treated wild-type and ADAR1-knockdown cells (P<2.2e-16), Kolmogorov–Smirnov test. ES, enrichment score. **h**, Interferon (IFN)-responsive single-sample GSEA (ssGSEA) score in mock-treated and 5-AZA-CdR-treated wild-type and ADAR1-knockdown conditions. Samples were collected 5, 14 and 24 days after treatment. For gel source data, see **Supplementary Fig. 1**.

Fig. 4. ADAR1 depletion synergizes with anti-tumour effects of 5-AZA-CdR.

a, Survival of wild-type ADAR1 (black) and ADAR1-knockdown (red) patient-derived CRC cells after treatment with 5-AZA-CdR. Luminescence signal was normalized, and dose–response curves and EC50 values were calculated using a nonlinear regression curve fit. **b**, Schematic representation of in vivo experimental scheme of non-infected, $ADAR1^{WT}$, $ADAR1^{KDA}$ and $ADAR1^{KDB}$ patient-derived xenograft CRC cells injected subcutaneously into flanks of NSG mice (n = 10 mice per group; 2 tumours per mouse). Arrows represent days of treatment (0.5 mg/kg 5-AZA-CdR by intraperitoneal injection or vehicle). **c**, Tumour volume measured at the indicated time points. Arrows represent days of treatment. $ADAR1^{KDA}$ 5-AZA-CdR (n = 20 tumours) and $ADAR1^{KDB}$ 5-AZA-CdR (n = 18 tumours at day 12 and n = 14 tumours at day15) compared with non-infected (NI) 5-AZA-CdR (n = 19 tumours) cohort. Data are mean ± s.e.m. ****P < 0.0001, Tukey-corrected two-way ANOVA. **d**, Tumour weight for each group at the time of dissection (day 14 after treatment). Data are mean + s.d. ****P < 0.0001, Tukey-corrected ordinary-one-way ANOVA. **e**, Schematic representation of in vivo LDA measuring tumour-initiation potential. NSG mice were injected with 10,000 cells (n = 8 tumours), 1,000 cells (n = 8 tumours), 100 cells (n = 8 tumours) and 10 cells (n = 16 tumours). **f,** Tumour-initiating cell frequency from the in vivo LDA of patient-derived CRC cells for each condition. $ADAR1^{WT}$ 5-AZA-CdR vs $ADAR1^{KDA}$ 5-AZA-CdR P = 0.00862; $ADAR1^{WT}$ 5-AZA-CdR vs $ADAR1^{KDB}$ 5-AZA-CdR P = 0.009; chi-square test.

Extended Data Fig. 1. Detecting dsRNA forming repeat element transcription after 5-AZA-CdR treatment.

a, Effect of 5-AZA-CdR on expression of repeat elements in patient-derived xenograft CRC cells after 5 days of treatment. Plots show log-transformed fold change for 5-AZA-CdR treated cells compared with mock-treated cells for antisense (y axis) and sense (x axis) transcripts of each SINE, LINE and ERV repeat element (CPM). Green dots in the top-right quadrant represent repeats that are significantly upregulated in 5-AZA-CdR-treated condition at both the antisense and sense strands as compared to the mock condition. Blue dots in the top-right quadrant represent repeats that have baseline expression (CPM ≥ 1) at the sense strand and are significantly upregulated at the antisense strand after 5-AZA-CdR treatment. Red dots in the top-right quadrant represent the repeats that have baseline expression (CPM ≥ 1) at the antisense strand and are significantly upregulated at the sense strand after 5-AZA-CdR treatment. Significance was determined as P < 0.05 and |logFC| ≥ 1 at each strand, BH (Benjamini– Hochberg)-corrected for multiple testing. **b, c,** Counts of expressed Alus in terms of the log10-transformed fold change of their MDA5-protected expression compared with total CytoRNA expression for baseline immunogenic RNA (n = 187 non-IR-Alus and 1,602 IR-Alus) (**b**), and the treatment-induced immunogenic RNA (n = 992

non-IR-Alus and 5,199 IR-Alus) (**c)**. Histograms on the left and right show the count of Alus from mock- and 5-AZA-CdR-treated cells respectively. The colour code represents Alus making IR-Alus (red) and non-IR Alus (blue). IR-Alus that are MDA5 dsRNA agonists have positive values on the x axis. **d, e**, Scatterplots showing the log10-transformed fold change of MDA5-protected compared with total CytoRNA for each repeat in the pair of Alus (y axis represents the fold change in the first repeat, x axis represents the fold change in the second repeat in the pair) identified for baseline immunogenic RNA (n = 1,040 IR-Alu pairs) (**d**) and treatment-induced immunogenic RNA (n = 3,687 IR-Alu pairs) (**e**).

Extended Data Fig. 2. Detecting immunogenic RNAs that are induced after treatment with 5-AZA-CdR using MDA5/RNase-protection assay.

a, MDA5/ RNase-protection assay experimental scheme created with BioRender.com. To identify the primary ligand for MDA5, total cytosolic RNA (5 ng/μl) was purified from mock or 5-AZA-CdR treated (300 nM for 5 days) patient-derived CRC cells and pre-incubated with MDA5-Δ2CARD protein, followed by either RNase A (+RNase A) digestion or without RNase A digestion (−RNase A). The remaining RNA was then purified and sequenced. **b**, Venn diagram showing the number of IR-Alu pairs at the baseline (n = 1,040 IR-Alu pairs), immunogenic (5-AZA-CdR) (n = 4,482 IR-Alu pairs) and treatment-induced (n = 3,687 IR-Alu pairs) conditions. **c**, Bar plot showing the number of baseline immunogenic RNA (IR-Alu pairs = 1,040, non-IR Alu pairs = 97). **d**, Bar plot showing the number of treatment-induced immunogenic RNA (IR-Alu pairs = 3,687, non-IR Alu pairs = 547). **e, f**, Average reads per million (RPM) profile of baseline MDA5- protected inverted-repeat transcripts (n = 1,176 transcript regions) (**e**) and treatment-induced MDA5-protected inverted-repeat transcripts (n = 3,895 transcript regions) (**f**). The box plots include the RPM scores of these transcripts. P value was calculated using a two-sided Wilcoxon signed-rank test. Box plot statistics containing the minimum, first quantile, median, third quantile, and maximum are 4.76, 10.21, 15.15, 27.44, and 52.53, respectively, for the mock-treated sample, and 0.0, 7.69, 15.24, 29.96, and 63.35 for the 5-AZACdR- treated sample in **e**, and 0.00, 0.79, 2.976, 6.185, and 14.26 for the mock-treated sample, and 2.41, 5.47, 7.47, 10.98, and 19.22 for the 5-AZA-CdR-treated sample in **f. g**, Schematic representation of inverted-repeats intramolecular pairing versus sense and antisense transcription created with BioRender.com. Inverted repeats can form intramolecular stem–loops when one strand is transcribed, as well as intermolecular duplexes when both strands are transcribed.

Extended Data Fig. 3. Genomic distribution and regulatory features of baseline and treatment-induced IR-Alus.

a, b, Distribution of the IR-Alu pairs based on their genomic orientation for the baseline immunogenic RNA (**a**) and treatment-induced immunogenic RNA (**b**). Arrows represent the genomic orientation (5′ to 3′) and arrow colour represent the transcriptional orientation (blue for sense and red for antisense). The number of IR-Alu pairs is represented by n. **c**, Distribution of Alu subfamilies from baseline immunogenic IR-Alus (n = 1,602 IR Alus), treatment-induced immunogenic IR-Alus (n = 5,199 IR Alus), and IR-Alus present in the human genome (n = 641,262 IR Alus). Odds ratio shows depletion or enrichment for each Alu subfamily (AluS, AluJ and AluY) at baseline immunogenic IR-Alus or treatment-induced immunogenic IR-Alus compared to Alu subfamily distribution of all IR-Alus present in the human genome. ****$P < 0.0001$, two-sided Fisher exact test. **d**, Profile of average CpG density 50 kb upstream and 50 kb downstream of the baseline IR-

Alu pairs and all existing IR-Alu pairs in the human genome. **e,** Profile of average bona-fide CpG island (CGI) intersection density flanking (−50 kb/+50 kb; upstream/ downstream) the treatment-induced IR-Alu pairs (n = 3,687 IR-Alu pairs, red line) and all existing IR-Alu pairs in the human genome (n = 746,470 IR-Alu pairs, blue line). For treatment-induced IR-Alu pairs, orientation was based on the transcriptional orientation in the MDA5-protected RNA-seq data. **f**, Profile of average CpG island intersection density flanking (−50 kb/+50 kb; upstream/ downstream) the baseline IR-Alu pairs (n = 1,040 IR-Alu pairs, red line) and all existing IR-Alu pairs in the human genome (n = 746,470 IR-Alu pairs, blue line). **g–i**, Representative genomic tracks of treatment-induced IR-Alu pairs located at intergenic regions (g, h) and baseline IR-Alu pair located at a 3′ UTR region (**i**). The top four tracks represent antisense transcription (in red) and sense transcription (in blue). The bottom track represents CpG density (green). **j**, Schematic representation of baseline and treatment-induced immunogenic IR-Alu pairs.

Extended Data Fig. 4. DNA methylation status of CGI adjacent to immunogenic IR-Alus and correlation of methylation score of immunogenic IR-Alus regulatory regions with viral mimicry signature.

a, b, Percentage of CGIs (n = 1338) directly upstream of 5-AZA-CdR induced immunogenic IR-Alus with at least one fully methylated (green), partially methylated (blue), or fully unmethylated (red) CpG site in CRC cells (n = 51) (**a**) or pan-cancer cell lines (n = 988) (**b**) from the GDSC project. **c, d**, Regions that after epigenetic therapy become H3K4me3 marked directly upstream of induced immunogenic IR-Alus (n = 991 peaks) with at least one fully methylated (green), partially methylated (blue), or fully unmethylated (red) CpG site in CRC cells (**c**) or pan-cancer cell lines (**d**) from the GDSC project. **e**, Scatter plot showing inverse correlation ($r=-0.23$, $p=7.58e^{-13}$) between the DNA methylation score of regulatory regions that after epigenetic therapy become H3K4me3 marked directly upstream to the induced immunogenic IR-Alus (n = 991 H3K4me3 peaks) and viral mimicry ISG signature ssGSEA score (n = 22 ISGs). Each dot is one pan-cancer cell line (n = 988) from the GDSC project. The grey area represents the 95% confidence interval around the linear model fit represented by the black line. The test used was a two-sided Pearson's correlation test, uncorrected for multiple testing.

Extended Data Fig. 5. Characterization of the cytoplasmic immunogenic IR-Alus.

a, b, Distribution of each known human polyA signal (PAS) motif with respect to the distance from the end of the downstream Alu of each IR-Alu pair in the set of baseline immunogenic IR-Alus (n = 1,040 IR-Alu pairs) (**a**) and the set of treatment-induced immunogenic IR-Alus (n = 3,687 IR-Alu pairs) (**b**). The y axis is the counts of the IR-Alu pairs that include the motif in the MDA5- protected RNA-seq, and the x axis is the distance in bp from the end of the downstream Alu in an IR-Alu pair. **c, d,** Heatmap and average profile of the MDA5-protected RNA-seq signal centred at the PAS locations detected in **a** and **b**. Each row represents the downstream Alu for each IR-Alu pair. The orientation and the strand are based on the MDA5-protected transcriptional orientation. **e**, Percentage of non-repeats and several families of repetitive elements in total RNA-seq (nuclear and cytoplasmic RNA) and in RNA-seq from the total cytoplasmic fraction after 5-AZA-CdR treatment at 300 nM for 5 days. The total Cyto RNA-Seq donut plot (on right) is as in **Fig. 1b** and plotted here for reference. The odds ratio for SINEs is 1.68 ($P<2.2e-16$) and 2.21 ($P<2.2e-16$) for Alus. Odds ratio was calculated between total cytoplasmic RNA-seq and total RNA-seq. ****$P < 0.0001$, two-sided Fisher exact test. **f**, Representative confocal microscopy images from two independent experiments of mock treated and 5-AZA-CdR-treated $ADAR1^{WT}$

patient-derived xenograft CRC cells. DNA was stained with DAPI (blue) and dsRNA was stained using the J2 antibody (red). Scale bar, 50 μm.

Extended Data Fig. 6. ADAR1 induces immunogenic inverted Alus.

a, dsRNA quantification based on J2/DAPI staining measured by ImageJ. Data are mean ± s.d. from n = 20 randomly sampled regions of two independent experiments. ***P < 0.001, ****P < 0.0001, Dunnett-corrected ordinary one-way ANOVA. **b**, qPCR analysis of total ADAR1 mRNA level after treatment with 5-AZA-CdR (300 nM, for 5 days) or transfection with 100 ng/ml poly(I:C) over a course of 24 days. Cells were washed out at day 5 and seeded in drug-free medium. Data are mean ± s.e.m. (n = 3 from three independent experiments). ****P < 0.0001, Sidak-corrected two-way ANOVA. **c**, Venn-diagram showing the number of IR-Alu pairs at baseline (n = 1,040 IR-Alu pairs), $ADAR1^{KD}$-baseline immunogenic (n = 9,030 IR-Alu pairs) and $ADAR1^{KD}$-induced immunogenic conditions. **d, e**, log10-transformed fold change of MDA5-protected total CytoRNA enriched IR-Alus and non-IR Alus for baseline immunogenic RNA (**d**) and $ADAR1^{KD}$-induced immunogenic RNA (**e**). The histogram on the left shows the count of IR-Alus from the $ADAR1^{WT}$ cells, and the histogram on the right shows the counts from the $ADAR1^{KD}$-treated cells. The colour code represents Alus making inverted-repeats (red) and non-IR-Alus (blue) for baseline immunogenic RNAs (n = 187 non-IR-Alus and 1,602 IR-Alus) (**d**; $ADAR1^{WT}$ data are the same as mock-treated data in **Extended Data Fig. 1b** and plotted here for reference) and for the $ADAR1^{KD}$-induced immunogenic RNA (n = 1,684 non-IRAlus and 11,085 IR-Alus) (**e**). **f, g,** Scatterplots showing the log10-transformed fold change of MDA5-protected over total CytoRNA for each repeat in the pair of Alus (y axis represents the fold change in the first repeat, x axis represents the fold change in the second repeat in the pair) identified for baseline immunogenic RNA (n = 1,040 IR-Alu pairs) (**f**; $ADAR1^{WT}$ data are the same as mock-treated data in **Extended Data Fig. 1d**) $ADAR1^{KD}$ patient-derived CRCs (n = 8,148 IR-Alu pairs) (**g**). **h, i,** Transcriptional orientation of each IR-Alu pair identified as immunogenic RNA at baseline (n = 1,040 IR-Alu pairs) (**h**) and $ADAR1^{KD}$-induced immunogenic RNA (n = 8,148 IR-Alu pairs) (**i**). Each bar shows the count of IR-pairs where both repeats are in the sense strand (+/+, blue), antisense strand (−/−, red), or discordant strands (+/− or −/+). The plot on the left shows the counts in the RNA-seq data from the $ADAR1^{WT}$ cells and the plot on the right shows the counts in the RNA-seq data from the $ADAR1^{KD}$ cells.
j, k, Average RPM profile of baseline MDA5-protected RNA transcripts (n = 1,176 transcript regions) that include baseline IR-Alus (**j**; $ADAR1^{WT}$ data are the same as mock-treated data in **Extended Data Fig. 2e**), and $ADAR1^{KD}$-induced MDA5-protected transcripts (n = 8,432 transcript regions) that include ADAR1KD induced IR-Alus (**k**). The box plots indicate the RPM scores of these transcripts. P values determined by two-sided Wilcoxon signed-rank test. Box plot statistics that contain the minimum, first quantile, median, third quantile, and maximum are 4.76, 10.21, 15.15, 27.44, and 52.53, respectively, for $ADAR1^{WT}$, and 0, 7.52, 14.71, 28.38, and 59.37 for the $ADAR1^{KD}$ box plots in **j**, and 0, 0, 2.55, 5.06, and 12.65 for the $ADAR1^{WT}$ and 1.21, 3.17, 4.62, 7.23, and 13.32 for the $ADAR1^{KD}$ box plots in **k**.

Extended Data Fig. 7. ADAR1 depletion synergizes with anti-tumour effects of DNMTi through loss of its catalytic activity.

a, Representative immunoblot of two independent experiments showing MAVS aggregation analysed by SDS-AGE and MAVS protein level analysed by SDS–PAGE. VDAC served as a loading control in SDS–PAGE. **b**, ADAR1 isoforms (p110 and p150) relative expression analysed by qPCR. LACZ denotes $ADAR1^{WT}$; PIC denotes 100 ng/ml poly (I:C) for 5 days; AZA denotes 300 nM 5-AZA-CdR for 5 days). Data are mean ± s.e.m. (n = 3 from three independent experiments). ***P < 0.001, ****P < 0.0001, Tukey-corrected two-way ANOVA. **c**, Kinetics of ISGs (ISG15, DDX58 and IRF7) relative expression in $ADAR1^{WT}$ (grey) and $ADAR1^{KD}$ (salmon) patient-derived CRCs treated with 5-AZA-CdR analysed by qPCR. Data are mean ± s.e.m. (n = 3 from three independent experiments). *P < 0.05, **P < 0.01, ****P < 0.0001, Sidak-corrected two-way ANOVA. **d**, Interferon-responsive ssGSEA score in $ADAR1^{WT}$ and $ADAR1^{KD}$ mock-treated and poly(I:C)-transfected samples collected 5 days, 14 days and 24 days after treatment. **e**, Kinetics of ISGs (ISG15, DDX58 and IRF7) relative expression in $ADAR1^{WT}$ (black) and $ADAR1^{KD}$ (pink) patient-derived CRCs transfected with poly(I:C), analysed by qPCR. Data are mean ± s.e.m. (n = 3 from three independent experiments). **P < 0.01, ****P < 0.0001, Sidak-corrected two-way ANOVA. **f**, qPCR (left) and immunoblot (right) analysis of ADAR1-p150 overexpression efficiency (ADAR1p150-OE) in patient-derived xenograft CRCs. Data are mean ± s.e.m. (n = 3 from three independent experiments). P = 0.0144, Wilcoxon test. Representative immunoblot of two independent experiments depicts two ADAR1 isoforms (p110 and p150). α-Tubulin served as a loading control. **g**, ISGs (ISG15, DDX58 and IRF7) relative expression in control (dark blue) and ADAR1p150-OE (light blue) patient-derived CRCs treated with 5-AZACdR, analysed by qPCR. Data are mean ± s.e.m. (n = 3 from three independent experiments). **P < 0.01, ***P < 0.001, Sidak-corrected two-way ANOVA. **h**, IFN responsive ssGSEA score in mock-treated or 5-AZA-CdR-treated control and $ADAR1^{p150\text{-}OE}$ samples collected 5 days after treatment and measured by RNAseq. Box plot statistics that contain the minimum, first quantile, median, third quantile, and maximum are 0.67, 0.73, 0.78, 0.84, and 0.90 respectively for 5-AZA-CdR control and 0.12, 0.23,0.35,0.46,0.58 for the ADAR1-p150- overexpressing 5-AZA-CdR samples. **i**, dsRNA quantification by ImageJ in control and $ADAR1^{p150\text{-}OE}$ cells mock-treated or treated with 5-AZA-CdR. Data are mean ± s.d. from n = 20 randomly sampled regions of two independent experiments. ****P < 0.0001, Tukey-corrected ordinary one-way ANOVA. ns, not significant. For gel source data, see **Supplementary Fig. 1.**

Extended Data Fig. 8. ADAR1 depletion synergizes with DNMTi in induction of ISGs in CRCs.

a, qPCR analysis of ADAR1 p150 and p110 isoforms relative expression in LIM1215 CRC cells. **b**, qPCR analysis of ISGs (MDA5, IRF7 and DDX58) relative expression in $ADAR1^{WT}$ and $ADAR1^{KD}$ LIM1215 CRCs mock-treated or treated with 300 nM 5-AZA-CdR for 5 days. **c**, qPCR analysis of ADAR1 p150 and p110 isoforms relative expression in HT29 CRCs. **d**, qPCR analysis of ISGs (MDA5, ISG15 and DDX58) relative expression in $ADAR1^{WT}$ and $ADAR1^{KD}$ HT29 CRCs mock-treated or treated with 300 nM 5-AZA-CdR for 5 days. Data are mean ± s.e.m. (n = 3 from three independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Tukey-corrected two-way ANOVA.

Extended Data Fig. 9. Epigenetic therapies which induce dsRNA can synergize with ADAR1 inhibition.

a, Colorectal cancer tumours dissected from vehicle and 5-AZA-CdR cohorts at day 14 after treatment are depicted for each condition (n = 5). **b**, Change in mean body weight of NSG mice transplanted with non-infected, $ADAR1^{WT}$ and $ADAR1^{KDA/B}$ patient-derived CRCs treated with vehicle or 5-AZA-CdR (0.5 mg/kg through intraperitoneal injection, for two cycles of 4 days with a 3 day break). **c**, Schematic representation of in vitro LDA in patient-derived xenograft CRCs mock-treated or treated with 5-AZA-CdR, created with BioRender.com. For each condition, cells were seeded as 1,000 cells per well, 100 cells per well, 10 cells per well and 1 cell per well. Spheroids were scored (absence or presence) 4 weeks after plating the cells. **d**, Normalized percentage of reduction in sphere forming ability calculated in each condition compared to mock- treated. **e**, Number of engrafted tumours compared to the number of injected tumours in each cohort for in vivo LDA. **f**, Representative confocal microscopy images from two independent experiments of $ADAR1^{WT}$ and $ADAR1^{KDA}$ cells treated with CDK4/6 inhibitor (250 nM palbociclib) for 7 days. DNA was stained with DAPI (blue) and dsRNA was stained using the J2 antibody (red). Scale bar, 50 μm. **g**, dsRNA quantification using ImageJ. Data are mean ± s.d. from n = 15 randomly sampled regions of two independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey-corrected ordinary one-way ANOVA. **h**, qPCR analysis of relative expression of ISGs (ISG15, DDX58 and IRF7) in cells treated with 250 nM palbociclib. Data are mean ± s.e.m., n = 3 from three independent experiments. $^{***}P < 0.001$, $^{****}P < 0.0001$, Tukey-corrected two-way ANOVA.

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Data availability:

RNA-sequencing and CUT&RUN data have been deposited at the GEO (https://www.ncbi.nlm.nih.gov/geo) under the accession number GSE145639.

Code availability:

Code is available at https://github.com/smarhon/IR

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D.D.D.C. received research funds from Pfizer and Nektar therapeutics. D.D.D.C. is co-founder and shareholder of DNAMx, Inc. All the other authors declare no conflict of interest.

Author contributions:

P.M., S.A.M., I.E. and D.D.D.C. contributed to the study design. P.M. and I.E. performed the in vitro experiments with technical support from A.H., F.A.d.C., H.L.Y., C.I. and S.A. P.M. and Y.W. performed the in vivo experiments, C.A.O. contributed to designing the in vivo experiments. S.A.M. performed the bioinformatics data analysis and developed the RNA-editing and the inverted-repeat pipelines. A.C. performed public DNA methylation data analysis. P.M., S.A.M., I.E. and D.D.D.C. wrote the manuscript.