RNA isoform screens uncover the essentiality and tumor-suppressor activity of ultraconserved poison exons

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While RNA-seq has enabled comprehensive quantification of alternative splicing, no correspondingly high-throughput assay exists for functionally interrogating individual isoforms. We describe pgFARM (paired guide RNAs for alternative exon removal), a CRISPR–Cas9-based method to manipulate isoforms independent of gene inactivation. This approach enabled rapid suppression of exon recognition in polyclonal settings to identify functional roles for individual exons, such as an SMNDC1 cassette exon that regulates pan-cancer intron retention. We generalized this method to a pooled screen to measure the functional relevance of ‘poison’ cassette exons, which disrupt their host genes’ reading frames yet are frequently ultraconserved. Many poison exons were essential for the growth of both cultured cells and lung adenocarcinoma xenografts, while a subset had clinically relevant tumor-suppressor activity. The essentiality and cancer relevance of poison exons are likely to contribute to their unusually high conservation and contrast with the dispensability of other ultraconserved elements for viability.

Most biological processes are characterized by alternative splicing1–3, which is correspondingly dysregulated in many diseases4–6. Mapping individual mis-spliced isoforms to specific molecular pathologies can enable the rational design of splicing-targeted therapeutics7–9. However, the vast majority of disease-associated RNA isoforms have not been functionally studied, hindering such therapeutic development.

This disparity between identification and functional characterization of isoforms arises from technological limitations. Antisense oligonucleotides are low throughput8,9, while RNA-mediated interference does not alter alternative splicing. CRISPR–Cas9 has been used to knock out DMD isoforms or long non-coding RNAs by targeting splice sites10,11, but has not been applied in a multiplexed fashion for studying alternative isoforms.

‘Poison exons’ provide a striking example of alternative splicing that is likely critical for organismal function, yet challenging to study. The human genome contains 481 ‘ultraconserved elements’ that are perfectly conserved in the mouse and rat genomes12. Many ultraconserved and highly conserved elements overlap poison exons, defined as alternative exons that interrupt their host genes’ reading frames13,14 and trigger nonsense-mediated RNA decay (NMD)15. Although poison exons do not contribute to the protein-coding capacity of their host genes, a subset are known to play critical cellular roles. For example, poison exons within splicing factors can mediate gene expression autoregulation13,14. However, the vast majority of poison exons have not been functionally interrogated, and their hypothesized essentiality has never been tested.

Results

pgFARM enforces the production of exon exclusion isoforms. Simultaneously delivering two guide RNAs (paired guide RNA, or pgRNA) into cells can induce deletion of the intervening DNA sequence16–19. We therefore hypothesized that pgRNA delivery could manipulate isoform expression by deleting exons, splice sites and/or other cis-regulatory splicing elements. We termed this approach pgFARM (paired guide RNAs for alternative exon removal).

As a proof of principle, we designed pgRNAs that used distinct targeting strategies to remove a constitutive coding exon (exon two) of HPRT1, a non-essential gene whose inactivation permits resistance to 6-thioguanine (6TG; Fig. 1a). We cloned each pgRNA into the lentiGuide-Puro backbone20 and introduced each construct into HeLa cells with doxycycline-inducible Cas9 (HeLa/iCas9 20; Fig. 1e, Extended Data Fig. 1a and Supplementary Table 1). We confirmed that exon skipping arose from on-target genomic DNA (gDNA) editing by sequencing individual HPRT1 alleles. We detected pgRNA/Cas9-dependent edits at 91% of alleles. Complete gDNA excision was the most common editing event (40% of edited alleles), followed by diverse short insertions/deletions (indels; Fig. 1e, Extended Data Fig. 1a and Supplementary Table 1). Although pgRNAs can cause gDNA inversion in addition to excision21, we detected no inversion events.

A recent study reported that Cas9-induced DNA breaks can result in rare large deletions22, which could potentially cause unwanted gene disruptions. Although we did not observe any excision events > 350 base pairs (bp) by Sanger sequencing—far shorter than most
We next used pgFARM to identify cellular roles for a highly conserved but less well-studied poison exon in SMNDC1, which is included at high levels in HeLa and lung adenocarcinoma (PC9) cells (Fig. 2a,b). As SMNDC1 is required for splicing catalysis in vitro\textsuperscript{26}, we hypothesized that its poison exon might influence the widespread intron retention that characterizes most cancers\textsuperscript{27,28}.

The SMNDC1 poison exon enables splicing-dependent autoregulation via NMD in cell culture\textsuperscript{29}. We therefore tested whether the same occurred in primary cancers profiled by The Cancer Genome Atlas (TCGA). Cancer samples exhibiting high SMNDC1 poison exon inclusion relative to patient-matched peritumoral normal samples exhibited low SMNDC1 gene expression, and vice versa (Fig. 2c and Extended Data Fig. 3a). SMNDC1 poison exon inclusion was significantly dysregulated in cancer relative to patient-matched normal samples in 9 of the 14 cohorts with sufficient data for analysis, with reduced poison exon inclusion in most cancer types (Fig. 2d). Low SMNDC1 poison exon inclusion and high gene expression were both associated with significantly poorer survival (Extended Data Fig. 3b,c).

We modeled cancer-associated SMNDC1 poison exon skipping by delivering a pgRNA targeting the poison exon’s 3’ splice site. We targeted the 3’ splice site to maximize the chance of exon skipping even if only one guide RNA (gRNA) induced cutting\textsuperscript{27}. This strategy also allowed us to minimize the deleted region to reduce the chance of inadvertently affecting other functional elements. pgRNA delivery resulted in editing at 82% of sequenced SMNDC1 alleles, with complete gDNA excision being the most common editing event (33%; Extended Data Fig. 4a and Supplementary Table 1). All almost edited alleles exhibited dramatically reduced 3’ splice site strengths\textsuperscript{26}, even when only one cut occurred (Fig. 2e).

We next modeled that individual editing events resulted in poison exon skipping. We generated Cas9-expressing PC9 lung adenocarcinoma cells (Extended Data Fig. 4b,c), delivered SMNDC1-targeting or control pgRNAs, and isolated monoclonal cell lines. Ninety percent of the SMNDC1-targeted clones carried 3’ splice site-dismuting edits (Extended Data Fig. 4d,e). We analyzed ten clones to find that all poison exon-targeted clones exhibited complete loss of SMNDC1 poison exon inclusion, while no control clones did (Fig. 2f).

We functionally characterized the SMNDC1 poison exon by delivering SMNDC1-targeting or control pgRNAs to HeLa/iCas9 cells and quantifying splicing with RNA sequencing (RNA-seq). SMNDC1 poison exon-targeting pgRNA delivery eliminated poison exon inclusion without detectable induction of any cryptic splicing (Fig. 2g). Consistent with our hypothesis that SMNDC1 regulates splicing efficiency, 221 genes exhibited significantly decreased splicing of the targeted exon, while no control clones did (Fig. 2f). We grouped genes into terciles based on SMNDC1 expression and quantified intron retention across each tercile\textsuperscript{27}. Low SMNDC1 poison exon inclusion was associated with notably widespread reductions in intron retention: 59% of constitutive introns exhibiting any retention.

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**Fig. 1** pgFARM facilitates rapid, programmable exon skipping. **a**. Top, RNA-seq read coverage and sequence conservation across HPRT1 in HeLa/iCas9 cells. Bottom, pgRNAs targeting HPRT1 exon two. **b**. A schematic of the pgRNA-expressing vector. **c**. A schematic of the pgRNA delivery strategy. **d**. A phase-contrast image of HeLa/iCas9 cells expressing a non-targeting control (pgNTC) or HPRT1 exon two-targeting pgRNA after selection with 6-thioguanine. Representative images from a gDNA excision events that are too small to resolve. **e**. A schematic of the pgRNA delivery strategy. **f**. Almost all edited alleles exhibited dramatically reduced 3’ splice site strengths, even when only one cut occurred. **g**. We next modeled that individual editing events resulted in poison exon skipping. We generated Cas9-expressing PC9 lung adenocarcinoma cells (Extended Data Fig. 4b,c), delivered SMNDC1-targeting or control pgRNAs, and isolated monoclonal cell lines. Ninety percent of the SMNDC1-targeted clones carried 3’ splice site-dismuting edits (Extended Data Fig. 4d,e). We analyzed ten clones to find that all poison exon-targeted clones exhibited complete loss of SMNDC1 poison exon inclusion, while no control clones did (Fig. 2f).

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**Fig. 2** Sanger sequencing of pgFARM-edited HPRT1 exon two (gray box). Bottom, PCR analysis of the HPRT1 exon two genomic locus. pgHPRT1.a–c create gDNA excision events that are too small to resolve. **f**. A phase-contrast image of HeLa/iCas9 cells expressing a non-targeting control (pgNTC) or HPRT1 exon two-targeting pgRNA after selection with 6-thioguanine. Representative images from a gDNA excision events that are too small to resolve. **g**. We next modeled that individual editing events resulted in poison exon skipping. We generated Cas9-expressing PC9 lung adenocarcinoma cells (Extended Data Fig. 4b,c), delivered SMNDC1-targeting or control pgRNAs, and isolated monoclonal cell lines. Ninety percent of the SMNDC1-targeted clones carried 3’ splice site-dismuting edits (Extended Data Fig. 4d,e). We analyzed ten clones to find that all poison exon-targeted clones exhibited complete loss of SMNDC1 poison exon inclusion, while no control clones did (Fig. 2f).

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were spliced significantly more efficiently in samples with low poison exon inclusion (Fig. 2i,j). This signal persisted after restricting the analysis to cases where intron retention is not predicted to induce NMD (Extended Data Fig. 4f), and was equally strong but opposite on stratifying by SMNDC1 gene expression (Fig. 2k and Extended Data Fig. 4g). We extended this analysis to find that almost all profiled cancer types exhibited significantly reduced intron retention in samples with low SMNDC1 poison exon inclusion.
pgRNA library targeting highly conserved poison exons. We designed a pgRNA library targeting poison exons to perform a highly multiplexed screen (Fig. 3a). We identified 12,653 human poison exons that are predicted to induce NMD and computed each exon’s sequence conservation across 46 species, yielding 520 poison exons with high conservation at their 5’ and 3’ splice sites (Extended Data Fig. 5a–e). In contrast to frame-preserving cassette exons, highly conserved poison exons were uniquely enriched in genes encoding RNA-binding proteins (Fig. 3b,c and Extended Data Fig. 5f), in agreement with previous studies.

We selected 465 and 91 poison exons exhibiting high and low conservation to target with our library, with a preference for highly conserved poison exons given their presumed functional importance.
We analyzed a published dataset\textsuperscript{36} to find that the inclusion of those selected poison exons increased dramatically following SMG6 and SMG7 knockdown in HeLa cells, confirming that they induce NMD (Fig. 3d). Seventy-eight percent of targeted poison exons exhibited inclusion $\geq$5% in NMD-inhibited HeLa cells. We confirmed that representative poison exons were included at high levels and induced NMD in both HeLa/iCas9 and PC9-Cas9 cells (Fig. 3e).

We designed pgRNAs targeting the 3′ splice sites of each poison exon and the corresponding upstream constitutive coding exon (Fig. 3f). This design permitted us to compare the relative consequences of constitutive exon loss, which is typically equivalent to gene knockout, to poison exon loss. Our library targeted 556 poison and 407 upstream constitutive exons with an average of 9 pgRNAs per exon, and additionally included 1,000 non-targeting pgRNAs (Extended Data Fig. 3g–i and Supplementary Table 2).

We synthesized the pgRNA library with an oligonucleotide array and cloned the library at $\geq$1,000-fold coverage using a cloning strategy similar to those from previous pgRNA studies\textsuperscript{17,18} (Fig. 3g). Sanger sequencing of individual bacterial colonies showed that $\geq$98% of sequenced pgRNAs were properly paired after library construction, consistent with low (~7.5%) mis-pairing rates reported in other studies\textsuperscript{17}.

pgEARM enables isofrom-resolution functional screens. We first performed a pilot cell viability screen in HeLa/iCas9 cells (Fig. 4a). We delivered the pgRNA library at a low multiplicity of infection of 0.2, collected gDNA 0, 8 and 14 d after Cas9 induction, and profiled pgRNA abundance by sequencing both gRNAs (Extended Data Fig. 4a). We sequenced each time point to $\geq$400 coverage per pgRNA and computed the numbers of properly paired reads supporting each pgRNA. Non-targeting control pgRNAs were progressively enriched relative to targeting pgRNAs throughout the time course, as expected (Extended Data Fig. 4b).

We confirmed that the pgRNA library functioned in the context of a dropout screen with two metrics. First, we estimated gene expression in HeLa/iCas9 cells with RNA-seq to find that pgRNAs
targeting unexpressed and expressed genes were respectively enriched and depleted, as expected (Fig. 4b). Second, we confirmed that pgRNAs targeting a published set of 'core essential' genes were depleted relative to pgRNAs targeting 'core non-essential' genes (Fig. 4c–e). We validated the on-target activity of a pgRNA targeting a constitutive exon within the essential gene U2AF1 to find that it induced exon skipping and cell death (Fig. 4f–h), as well as differential requirements for the SMNDC1 poison versus constitutive exons for cell growth (Extended Data Fig. 6c).

CRISPR–Cas9-induced DNA breaks can reduce cell fitness in a gene copy number-dependent manner. We computed the copy number of each targeted unexpressed gene in the HeLa genome and compared fold changes between different loci. While this analysis showed no correlation between the copy number and pgRNA depletion, we observed a modest depletion of exon-targeting pgRNAs relative to non-targeting pgRNAs (Extended Data Fig. 6d). We concluded that decreased cell viability caused by DNA breaks contributed to pgRNA depletion, although not in a copy number-dependent manner. We therefore normalized all fold changes relative to the median fold change for pgRNAs targeting unexpressed genes (Supplementary Table 3).

We next functionally validated additional constitutive exons that were identified as essential in our dropout screen. We ranked each exon according to the geometric mean of fold changes for all targeted exons in unexpressed (left) or expressed (right) genes. Median unexpressed genes were identified as essential in our dropout screen. We ranked each exon according to the geometric mean of fold changes for all targeted exons in unexpressed genes (Supplementary Table 3).
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site recognition, induction of exon skipping. Consistent with SNRNP70’s key role in unwanted cryptic isoforms.

We next performed RNA-seq to validate on-target exon skipping, which introduces a frameshift. Consistent with efficient NMD, we observed low levels of the exon exclusion isoform (versus none in control pgRNA-treated cells) with concomitant downregulation (greater than fourfold) of SNRNP70 mRNA levels and reduced inclusion of SNRNP70’s poison exon (approximately fivefold; Extended Data Fig. 6h,i), consistent with the autoregulatory role of this poison exon. We observed no RNA-seq reads indicative of unwanted cryptic isoforms.

We then tested the functional consequences of pgRNA-induced exon skipping. Consistent with SNRNP70’s key role in 5’ splice site recognition, induction of SNRNP70 constitutive exon skipping caused transcriptome-wide exon skipping and a shift towards intron-proximal 5’ splice site usage (Fig. 4i,m and Extended Data Fig. 6j,k). We extended these functional assays to SRSF3, which encodes a sequence-specific splicing factor. We delivered a pgRNA targeting an SRSF3 constitutive exon, confirmed on-target gDNA editing and performed RNA-seq (Fig. 4i, Extended Data Fig. 7a and Supplementary Table 1). pgRNA delivery caused SRSF3 constitutive exon skipping and reduced inclusion of SRSF3’s poison exon (Extended Data Fig. 7b,c), consistent with its autoregulatory role. Cassette exons that were repressed following SRSF3-targeting pgRNA delivery were enriched for SRSF3’s RNA-binding motif (Fig. 4n and Extended Data Fig. 7d). In contrast to SNRNP70 and SRSF3 pgRNA-expressing cells, treatment with an AAVS1-targeting pgRNA resulted in little differential splicing relative to treatment SRSF3 (Fig. 4n and Extended Data Fig. 7d). In contrast to SNRNP70 and SRSF3 pgRNA-expressing cells, treatment with an AAVS1-targeting pgRNA resulted in little differential splicing relative to treatment with a non-targeting pgRNA (Extended Data Fig. 7e). No unwanted cryptic SNRNP70 or SRSF3 isoforms were detectable in any condition (Extended Data Fig. 7f,g). We conclude that pgFARM enables on-target induction of exon skipping in a high-content screen.

Many conserved poison exons are essential for cell growth. Having established the robustness of our method, we next tested the hypothesis that poison exons are important for viability. We performed a second dropout screen in HeLa/iCas9 and PC9-Cas9 cells with a re-cloned pgRNA library in biological quadruplicate (Extended Data Fig. 8a). Biological replicates segregated according to the day of collection and cell line following unsupervised hierarchical clustering (Fig. 5a). Per-pgRNA fold changes estimated for HeLa/iCas9 cells in our pilot and second screens had Pearson correlations of 0.88–0.93 (Extended Data Fig. 8b), highlighting our method’s reproducibility. We therefore pooled data across biological replicates for subsequent analyses to maximize statistical power (Supplementary Table 5). pgRNAs targeting expressed versus unexpressed genes and essential versus non-essential genes were consistently depleted in both cell lines (Fig. 5b and Extended Data Fig. 8c).

As for our pilot screen, we normalized fold changes such that the median fold change for pgRNAs targeting unexpressed genes was equal to 1 for each cell line, replicate and time point. We computed a P value and empirical false discovery rate (FDR) for each exon by comparing the distribution of fold changes for all pgRNAs targeting that exon relative to the fold changes for all pgRNAs targeting unexpressed genes (Supplementary Tables 4 and 5). Gene copy number effects were not a confounding factor (Extended Data Fig. 8d).

We next tested whether poison exons are important for cell fitness. We enumerated exons that exhibited a significant depletion or enrichment (absolute fold change ≥25% with FDR ≤0.01 at day 14). Forty-three percent (169) and 10% (38) of targeted poison exons in expressed genes were depleted and enriched in HeLa/iCas9 cells, versus 58% (170) and 11% (32) of upstream constitutive exons—only a modest increase relative to poison exons. Poison exons that were frequently included in mRNA were preferentially depleted relative to exons that were typically excluded (Fig. 5c; P = 0.004). In PC9-Cas9 cells, 13% (51) and 6% (23) of targeted poison exons in expressed genes exhibited depletion and enrichment, versus 35% (101) and 5% (13) of upstream constitutive exons. Although constitutive Cas9 expression reduced the dynamic range of the PC9-Cas9 screen, skipping of both poison and upstream constitutive exons resulted in highly concordant fitness costs in the two cell lines (Fig. 5d and Extended Data Fig. 8e,f).

We validated our screens’ estimates of cell viability by delivering individual pgRNAs targeting poison exons in CPSF4 and SMG1 and confirming that these exons are important for cell growth (Fig. 5e,f). We sequenced individual CPSF4 and SMG1 alleles to find that 96% of CPSF4 alleles were subject to 3’ splice site-disrupting editing, including 58% with complete gDNA excision, while 75% of SMG1 alleles contained indels that likely compromised exon recognition (Extended Data Fig. 9a and Supplementary Table 1). In neither case did targeting pgRNA delivery induce unwanted cryptic isoforms (Extended Data Fig. 9b,c).

Poison exon skipping leaves a gene's protein-coding capacity intact, while constitutive exon skipping typically does not. Nonetheless, pgRNA-induced skipping of many highly conserved and even some poorly conserved poison exons was associated with only modestly lower fitness costs than was loss of many constitutive exons (Fig. 5g and Extended Data Fig. 9d). These results support the intuitive, but untested, hypothesis that the high conservation of many poison exons is explained by purifying selection arising from those exons’ contributions to cell fitness.

A subset of poison exons exhibit tumor-suppressor activity. We extended our approach to the context of lung adenocarcinoma xenografts to test two distinct hypotheses. First, we hypothesized that many poison exons would prove essential in vivo, just as in cell culture. Second, because of the difficulty of identifying positive selection in cultured transformed cells, we hypothesized that the stringency of growth in vivo might identify poison exons whose loss promoted tumor growth. We utilized PC9 cells, a common preclinical model of lung adenocarcinoma.

We transduced PC9-Cas9 cells with the poison exon pgRNA library using the same conditions as for our previous screens. After selection in cell culture for 4d, we subcutaneously injected 3 × 10⁷ cells (~3,000-fold pgRNA representation) into the flanks of immunocompromised (NU/Albino) mice (Fig. 6a and Supplementary Table 6). We observed similar growth rates for pgRNA library-transduced PC9-Cas9 xenografts and control parental PC9 (lacking Cas9) xenografts (Extended Data Fig. 10a,b). We collected gDNA from four and ten xenografts at early (~3 weeks) and late (~6 weeks) time points and measured the pgRNA abundance in the input plasmid pool, pre-injected cells, early tumors and late tumors with ~2,500-fold pgRNA coverage (Extended Data Fig. 10c).

All samples grouped according to biological condition and time of collection following unsupervised hierarchical clustering (Extended Data Fig. 10d). Late xenografts exhibited lower inter-tumor correlations than did early xenografts, consistent with prior reports. We therefore used data from all replicates for statistical analyses to ensure that our results were robust with respect to high biological variability during tumorigenesis (Supplementary Table 5).

Few pgRNAs had no representation in early xenografts, while thousands were absent from late xenografts (Fig. 6b). Exon-targeting pgRNAs were preferentially lost relative to non-targeting pgRNAs. Therefore, almost all pgRNAs were compatible with engraftment, but negative selection led to subsequent loss of many exon-targeting pgRNAs.
We quantified exon essentiality by computing fold changes in pgRNA abundance in each tumor versus pre-injected cells and normalized the data as described above. One hundred and twelve upstream constitutive and 77 poison exons were significantly depleted in late xenografts. Consistent with our results, parent genes of these 112 constitutive exons were all previously reported as essential for lung cancer xenograft growth. Most upstream constitutive and poison exons that exhibited significant depletion in the late xenografts were also depleted in our PC9-based cell culture screens, although a subset exhibited divergent behavior (Fig. 6c).

Although many poison exons are essential for cell growth, we hypothesized that a subset might have anti-tumorigenic effects. Splicing factors are frequently overexpressed in cancers, although many conserved poison exons are essential for cell fitness. We validated this idea by testing whether modulating exon inclusion within genes encoding splicing factors influenced tumorigenesis. Skipping of constitutive exons within SR and hnRNP genes, many of which are essential, was strongly selected against (Fig. 6d). These data suggest that many RNA splicing factors are proto-oncoproteins whose pro-tumorigenic effects are constrained by poison exons.

The anti-tumorigenic effects of poison exons extend beyond splicing factors, with 61 poison exons enriched in late xenografts. Poison exon loss was more frequently associated with pro-relative pro-tumorigenic roles have been demonstrated only for a few factors. We therefore tested whether modulating exon inclusion within genes encoding splicing factors influenced tumorigenesis. Skipping of constitutive exons within SR and hnRNP genes, many of which are essential, was strongly selected against (Fig. 6d and Extended Data Fig. 10e). In contrast, most targeted poison exons within SR and hnRNP genes exhibited enrichment in late xenografts (Fig. 6d). These data suggest that many RNA splicing factors are proto-oncoproteins whose pro-tumorigenic effects are constrained by poison exons.

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**Fig. 6** | pgFARM uncovers modifiers of in vivo tumorigenesis. a, A schematic of the screens. b, The numbers of pgRNAs with zero counts. c, Normalized fold changes for exons measured in vivo and in vitro. d, Normalized fold changes for exons in SR and hnRNP genes. HNRNPH1 and SRSF7 contain multiple poison exons; SRSF7 has a poison exon with competing 3' splice sites. e, The numbers of significantly depleted (blue) and enriched (red) targets. f, SF3B3 (left) or CLK4 (right) poison exon inclusion in PC9-Cas9 cells expressing the indicated pgRNAs. g, Poison exon inclusion in the indicated genes in PC9-Cas9 cells expressing the indicated pgRNAs. h, Poison exon inclusion in PC9-Cas9 clones expressing the indicated pgRNAs. The data are presented as mean ± s.e.m. i, Normalized fold changes for the EPC1 poison exon. j, EPC1 poison exon inclusion in PC9-Cas9 clones expressing the indicated pgRNAs. The P value was computed with a two-sided Student’s t-test. k, The tumor weights at the endpoint. The P value was computed with a two-sided Mann–Whitney U test. l, The survival of lung adenocarcinoma patients stratified by inclusion of tumor-suppressive poison exons. The P values were computed with a two-sided logrank test. The sample sizes and box plot elements are defined in the Methods.
to anti-tumorigenic effects compared to constitutive exon loss ($P = 0.017$ by the one-sided binomial proportion test; Fig. 6c) We confirmed that enrichment was due to on-target activity by validating poison exon skipping for several pgRNAs (Fig. 6f,g).

We selected a poison exon within EPC1 for further study due to its notable enrichment, previous reports of tumorigenic roles for EPC1, and inclusion at high rates (>40%) in NMD-inhibited cells (Fig. 6h,i). We confirmed on-target induction of exon skipping following pgRNA delivery in monoclonal cell lines (Fig. 6i) as well as a modest fitness advantage in cell culture (Extended Data Fig. 10f). We therefore extended these studies to in vivo tumorigenesis. Tumors derived from engraftment of polyclonal EPC1-targeted PC9-Cas9 cells were significantly larger and exhibited increased Ki-67 staining relative to control tumors (Fig. 6j–l).

We next tested whether poison exons with tumor-suppressor capacity in xenografts were clinically relevant. We stratified lung adenocarcinoma patients according to their inclusion of essential (depleted) and tumor-suppressive (enriched) poison exons. Low inclusion of tumor-suppressive poison exons was associated with significantly worse progression-free and overall survival relative to high inclusion (Extended Data Fig. 10g,h; $P = 0.012$ and 0.0187). Further restricting our analysis to tumor-suppressive poison exons that exhibited high splicing variability across tumors yielded even more significant effects (Fig. 6m; $P = 0.013$ and 0.00072). Inclusion of essential poison exons was associated with no significant survival difference (Extended Data Fig. 10j,i), as expected. We conclude that many poison exons act as clinically relevant tumor suppressors.

**Discussion**

The ongoing discovery of new DNA- and RNA-targeting CRISPR–Cas systems will enable the development of diverse tool kits for manipulating isoform expression. Single gRNA delivery and base editing can alter exon recognition, while RNA-targeting CRISPR–Cas systems can enable direct manipulation of alternative splicing.

Each of these techniques is potentially amenable to a screening format. As a result of their extraordinary sequence conservation, ultrasupervised elements were initially assumed to be essential for life. However, deletion of many ultrasupervised enhancers has no effects on mouse organismal or cell viability.

Although poison exons are similar to enhancers with respect to their gene regulatory activities, we found that many poison exons exert robust effects on cell viability. Most unexpectedly, some poison exons have clinically relevant tumor-suppressive effects.

We focused on cassette exons to address the outstanding mystery of poison exons' high conservation. However, pgFARM can potentially be applied to many other kinds of alternative RNA processing. We expect pgFARM to enable rapid and unbiased functional interrogation of specific RNA isoforms associated with diverse biological processes or disease states.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41588-019-0555-z.

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Methods

pgRNA design, plasmids and cloning. For pgRNA optimization (Fig. 1), candidate gRNAs located near the targeted exon were identified and then paired on the basis of being located within the coding sequence or proximal/distal to splice sites. Both NAG and TAD protease and all motifs were utilized. pgRNAs were cloned following published methods (Fig. 3g). Oligonucleotides containing both pgRNA spacer sequences were synthesized as DNA ultramers, amplified (primers RKB1169 and RKB1170; Supplementary Table 7) using NEBNext High Fidelity 2x Ready Mix (New England Biolabs) and purified with a 1.8x Ampure XP SPRI bead (Beckman Coulter) clean-up. This insert was cloned into BsmBI (FastDigestExp3i, Thermo Fisher Scientific)-linearized lentivideGuide-Puro (Addgene no. 52963) backbone using the NEBuilder HiFi (New England Biolabs) assembly system and transformed into NEB Stable competent Escherichia coli (New England Biolabs) to generate the pLGP-2xSpacer vector. Propagated plasmid was purified using the ZymoPURE Plasmid Miniprep Kit (Zymogen) and linearized with BsmBI. An E1 coding g-block (Integrated DNA Technologies) containing the second Pol III promoter and gRNA backbone was digested with BsmBI, purified using a 1.8x SPRI bead clean-up and ligated into the linearized pLGP-2xSpacer backbone using BsmI Quick Ligate (New England Biolabs). This reaction was transformed into NEB Stable cells to propagate the plasmid and generate final plasmid DNA. All plasmids with verified sgRNA sequence were sequenced using Sanger sequencing (RKB1148 primer). pgRNAs used for validation studies are listed in Supplementary Table 8.

Cas9-expressing cell generation. PC9-Cas9 cells were generated by transducing PC9 cells (M. Meyerson) with pSPX111 lentivirus and selecting with blasticidin for 5–7 d. Cas9 protein was detected with an anti-Cas9 antibody (Cell Signaling no. 14697) and anti-ACTB antibody (Cell Signaling no. 4970). Cas9-expressing B16-F10 (ATCC CRL-6475), Melan-a (D. Bennett) and HEK293T cells were generated by transducing cells with lentiCas9-Blast (Addgene 52962) lentivirus and selecting with blasticidin following by puromycin selection. Cells in all treatment groups constitutively express Cas9, day 0 was defined as the time when all cells in a no-treatment control plate died after puromycin selection. Cells in all treatment groups were periodically tested for mycoplasma contamination. For 6TG resistance assays, we used to confirm proper gRNA pairing throughout the cloning procedure. The presence of the library diversity. Sanger sequencing of individual bacterial colonies was performed by Direct Colony Sanger Sequencing (RKB1148 primer). pgRNAs used for validation studies are listed in Supplementary Table 8.

LentiVirus production and titration. For large-scale production, HEK293T cells were seeded in T25 flasks such that each flask would be ~80% confluent at the time of transfection. After overnight incubation, pCMV-VSV-G (Addgene no. 8454), pSPAX2 (Addgene no. 12260) and pLGP-pgRNA transfer vectors were introduced into cells using PEI Max (Polysciences) transfection. LentiVirus-containing medium was collected 48 h later, filtered and stored at –80 °C until use. For small-scale production, HEK293T cells were seeded into individual wells of a 12-well plate in medium supplemented with 8 µg ml⁻¹ polybrene (EMD Millipore) and incubated at 37 °C for 2 h. Next, dilution of the lentiviral preparation was added to individual wells and incubated for 24 h at 37 °C. The next day, cells from individual wells of the 12-well plate were re-seeded into 8 wells of a 96-well plate. Cells in four of these wells were grown in culture medium supplemented with 1 µg ml⁻¹ puromycin and the other four contained no puromycin. After all cells in the no-infection control wells were dead (typically 2–3 d), cell viability was quantified using a CellTiter-Glo (Promega) assay according to the manufacturer’s instructions. Multiplicity of infection was determined by calculating the ratio of cells in the puromycin-treated compared to no puromycin treatment groups.

pgRNA vector delivery and sample collection. For testing individual pgRNA constructs, HEK293T or PC9-Cas9 cells were seeded in individual wells of a multi-well plate and treated with viral supernatant to deliver pgRNA vectors. The next day, virus-containing medium was exchanged for standard growth medium supplemented with 1 µg ml⁻¹ puromycin to select for stable integration. After selection, 1 µg ml⁻¹ of doxycycline was added to HEK293T or PC9-Cas9 cells to induce Cas9 expression. This was defined as day 0 for each experiment. As the PC9-Cas9 cells constitutively express Cas9, day 0 was defined as the time when all cells in a no-infection control plate died after puromycin selection. Cells in all treatment groups were passaged for 2–3 weeks. During this time, cell confluency and morphology were routinely analyzed using a Cytation 5 Imaging Reader (BioTek), cell number was measured using a CellTiter-Glo assay, and aliquots of cells were collected for molecular analyses.

gDNA PCR, TOPO cloning and Sanger sequencing. gDNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer’s protocol. Regions of interest were amplified by PCR using gene-specific primers (Supplementary Table 7) and analyzed using a 4200 TapStation System (Agilent Genomics). For TOPO cloning and Sanger sequencing, purified amplicons were ligated into vectors for sequencing using the ZeroBlunt TOPO PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer’s protocol. Ligation reactions were transformed into One Shot TOP10 Chemically Competent E. coli (Thermo Fisher Scientific) using the manufacturer’s protocol, plated onto LB agar supplemented with 50 µg ml⁻¹ kanamycin and grown overnight at 37 °C. Sequences corresponding to each region of interest were generated by Direct Colony Sanger Sequencing (GENEWIZ). Sequence alignments were performed using MAFFT. RT–PCR. Total RNA was extracted using the Direct-zol RNA MiniPrep (Zymo Research). cDNA was synthesized using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer’s protocol. RT–PCR was performed using gene-specific primers (Supplementary Table 7) and High Fidelity DNA Polymerase (New England Biolabs) and amplicons were analyzed and quantified using either a 4200 TapStation System (Agilent Genomics) or agarose gel electrophoresis followed by quantification of band intensity using Fiji/ImageJ. To detect poison exon-containing RNA isoforms, cells were treated with 50 µg ml⁻¹ cycloheximide for up to 6 h to inhibit NMD.

Immunofluorescence. Cells grown on glass coverslips were washed with PBS, followed by fixation in 10% phosphate-buffered formalin (Fisher Scientific) for 10 min at room temperature and permeabilization with PBST (PBS, 0.2% Triton X-100) for 10 min at room temperature. Non-specific binding was blocked by incubating cells in PBS + 1% BSA (Fisher Scientific) for 1 h at room temperature followed by overnight incubation with primary antibody (MBL, DSHB, 1:1000) for 1 h at room temperature. Cells were washed three times with PBST for 10 min at room temperature and then incubated with secondary antibodies (goat anti-mouse DyLight 594, Thermo Fisher Scientific) for 1 h at room temperature. Cells were then washed three times with PBST for 10 min at room temperature and mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs). Images were captured using an Aperio ScanScope FL (Leica Biosystems) and quantified using the HALO image analysis software (Indica Labs).

Immunohistochemistry. Xenograft tissue processing, embedding and staining was performed by the Fred Hutchinson Experimental Histopathology core. Human Ki-67 was detected using a mouse monoclonal antibody (Dako MIB-1). To mitigate background staining, mouse-on-mouse blocking was performed as previously described 19. Staining was performed using a BOND RX autostainer (Leica Biosystems) and images were acquired using an Aperio ImageScope (Leica Biosystems).

Western blotting. Total protein lysates were prepared in 1x RIPA buffer (Cell Signaling) and quantified using the Pierce 660 nm Protein Assay Reagent. Total protein lysates were electrophoretically separated and transferred to nitrocellulose membranes using the NuPAGE system (Thermo Fisher Scientific). Membranes were blocked and incubated with Odyssey Blot Blocker (LI-COR Biosciences) at room temperature followed by overnight incubation at 4 °C with primary antibodies diluted in blocking buffer. HPR1 (Abcam ab10479, 1:1,000) and GAPDH (Bethyl a300-639a, 1:5,000) were used as primary antibodies. IRDye (LI-COR Biosciences) secondary antibodies were used for detection and imaged using the Odyssey CLx Imager (LI-COR Biosciences).

pgRNA library design and construction. Poison exons were identified using transcript annotations from MISO v2.0 and pgRNAs targeting the 3′ splice sites of poison exons were designed using the methodology described in Fig. 3. The library cloning method followed previously published strategies 17,18 and was similar to cloning individual pgRNA vectors except for two adaptations. First, pgRNA oligonucleotides were synthesized using a DNA oligonucleotide array (Twist Bioscience) and used as input for the first PCR step. Second, for each step, multiple molecular reactions and bacterial transformations were performed such that each pgRNA was maintained at ≥1,000-fold coverage to prevent bottlenecking of the library diversity. Sanger sequencing of individual bacterial colonies was used to confirm proper gRNA pairing throughout the cloning procedure. The pgRNA library is available to the academic community (https://www.addgene.org/Robert_Bradley).

Cell viability screens. HEK293T or PC9-Cas9 cells were seeded in 15 cm plates at ~10,000 cells per plate to obtain a complete medium supplemented with 1 µg ml⁻¹ puromycin. After no cells remained in uninfected control plates, we collected the day 0 cell pellets and then added 1 µg ml⁻¹ doxycycline to HEK293T cells. At this point, cells were passaged every 2 to 3 d at a sufficient seeding density to maintain library diversity and cell pellets were collected on days 8 and 14 for gDNA extraction.
pgRNA deep sequencing library preparation and sequencing. Cell pellets were digested in lysis buffer (50 mM Tris, 50 mM EDTA, 1% SDS, 100 μg·mL⁻¹ protease K) overnight at 55 °C and gDNA was isolated using isopropanol precipitation. To build sequencing libraries, three PCR steps were performed as outlined in Extended Data Fig. 6a. First, 1 μg gDNA was used as input for amplification with NEBNext High Fidelity 2× Ready Mix using primers RRK2713/RRK2714 followed by Ampure XP SPRI bead clean-up. Second, 10 ng of amplicon from PCR no. 1 was used as input for amplification with primers RRK2715/RRK2716 followed by Ampure XP SPRI bead clean-up. Third, 1 μg of amplicon from PCR no. 2 was used as input for amplification with a common forward primer, RRK2717, and a sample-specific barcoding primer to accommodate multiplexing. For each PCR, multiple reactions were performed for each sample to maintain >1,000-fold coverage of each pgRNA in the library. Finally, purified libraries were combined in equimolar proportions and sequenced using an Illumina sequencer.

Animal use. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees at Fred Hutchinson Cancer Research Center. NU/J (stock no. 002019) mice were obtained from the Jackson Laboratory.

Xenograft screen. PC9-Cas9 cells were grown in multiple 15-cm plates and treated with pgRNA lentiviral libraries at a multiplicity of infection of ~0.3. Infected cells were propagated in cell culture for ~4 d to select (1 with pgRNA lentiviral libraries at a multiplicity of infection of ~0.3. Infected cells were propagated in cell culture for ~4 d to select (1 with pgRNA deep sequencing library preparation and sequencing. PC9-Cas9 cells were grown using standard conditions, supplemented with lentinivirus containing pgRNA expression vectors, and selected with 1 μg·mL⁻¹ puromycin. Before implantation, cells were grown for at least one week post-selection. For injections, adult NU/J mice were anesthetized with isoflurane and 3 × 10⁴ cells were injected subcutaneously into both flanks. Cohorts of mice were euthanized ~3 –6 weeks post injection, corresponding to the early and late time points, respectively (Supplementary Table 6), and tumors were dissected and stored at −80 °C. For gDNA extraction, 100 μg of tissue from each tumor was digested in lysis buffer (50 mM Tris, 50 mM EDTA, 1% SDS, 100 μg·mL⁻¹ protease K) overnight at 55 °C and gDNA was isolated using isopropanol precipitation. pgRNA libraries were constructed using the same methods as for the in vitro screens.

Validation xenograft studies. PC9-Cas9 cells were grown using standard conditions, supplemented with lentinivirus containing pgRNA expression vectors, and selected with 1 μg·mL⁻¹ puromycin. Before implantation, cells were grown for at least one week post-selection. For injections, adult NU/J mice were anesthetized with isoflurane and 3 × 10⁴ cells were injected subcutaneously into both flanks. Tumor dimensions were measured using calipers throughout the time course. For histology, dissected tumors were fixed in 10% formalin solution at room temperature for 3 d before processing and paraffin embedding.

pgRNA deep sequencing data analysis. The first and second reads were separately mapped to a database of pgRNA sequences using Bowtie1. Correct pairings, for which both the first and second reads mapped to a given pgRNA, were kept; incorrect pairings were discarded. If a given first and second read had more than one possible correct pairing, then all correct pairings were kept but the degenerate pairings were down-weighted by 1/the number of possible pairings when counts of reads supporting each pgRNA were computed. A per-pgRNA pseudocount was computed as follows. For each pgRNA, ‘reference’ and ‘comparison’ pseudocounts were computed as max (5, 0.05 × (counts in the reference time point)) and max (5, (reference pseudocount) × (total counts for all pgRNAs in the comparison sample/total counts for all pgRNAs in the reference sample)). The reference and comparison pseudocounts were added to the actual counts for the reference and comparison time points when computing fold changes for each pgRNA. This procedure regularized fold-change computations in a manner proportional to the relative representation of each pgRNA within the library.

Fold changes were then normalized to account for the effects of DNA damage as described in the main text. The median fold change for all pgRNAs targeting unexpressed genes was computed for each time point relative to day 0 and each fold change was then divided by this number. After applying this normalization procedure, the median fold change for pgRNAs targeting unexpressed genes for a given cell type was equal to 1.

Statistical analyses of normalized fold changes were performed as follows at a per-target level. For a given targeted exon at a given time point, a P value for differential enrichment relative to day 0 was computed by performing a two-sided Mann–Whitney test between the fold changes for all pgRNAs targeting that exon relative to the fold changes for all pgRNAs targeting unexpressed genes for a given cell type was equal to 1.

All statistical analyses were performed in the R programming environment with Bioconductor. All plots and figures were generated with the dpyplot and pgplot² packages.

RNA-seq library preparation. RNA was extracted from cell pellets using the Direct-zol RNA MiniPrep (Zymo Research) kit. Poly(A)-selected, stranded Illumina libraries were prepared using the TruSeq protocol per the manufacturer’s instructions. Libraries were analyzed using a 4200 TapeStation System to confirm proper size distribution before sequencing on an Illumina HiSeq. Libraries were sequenced as 2 × 50 bp to obtain ~40 million reads per sample.

RNA-seq data analysis. RNA-seq data were analyzed as previously described. Briefly, reads were mapped to a transcriptome annotation created by merging the Ensemble71, UCSC knownGene and MISO v2.0 annotations using RSEM version v1.2.12 (modified to call Bowtie* with option ‘–v 2’). Unaligned reads were mapped to the genome (hg19/GRCh37 assembly) and a database consisting of all possible pairings between 5′ and 3′ splice sites for a given gene present in our merged transcriptome annotation with TopHat version 2.0.8h. Mapped reads were merged and used as input to MISO v2.0. For TCGA studies, we analyzed the 5,718 available samples from the 14 cancer types with at least 10 patient-matched cancer and normal samples.

Survival analyses. Survival analyses and corresponding statistical tests were performed with the Kaplan–Meier estimator and logrank test (R package surv 曅). Patients were stratified as follows for Fig. 3a, for each sample, we computed the following statistic: (number of tumor-suppressive poison exons for which exon inclusion ≤ 25% of percent of exon inclusion over the entire cohort)/(number of tumor-suppressive poison exons for which exon inclusion ≥ 75% of percent of exon inclusion over the entire cohort). The statistic was computed using the set of tumor-suppressive poison exons with defined exon inclusion for ≥90% of patients and high splicing variability (median exon inclusion level ≥ 10% with a standard deviation of inclusion across patients ≥ 25% of the median inclusion). Sixteen depleted and sixteen enriched poison exons met those criteria. Patients were stratified identically for Extended Data Fig. 10g–j using the sets of essential or tumor-suppressive poison exons described in the main text (as for Fig. 6m, but without filtering based on splicing variability, yielding a total of 62 depleted and 47 enriched poison exons).

Statistics and reproducibility. For Fig. 2d, sample sizes are n = 19; 111; 38; 12; 40; 25; 71; 36; 48; 57; 50; 52; 30; 59 (left to right). For Fig. 2l, sample sizes are n = 105; 121; 326/484; 112/210; 54/66; 14/26; 17/22; 136/201; 68/104; 87/142; 132/237; 120/179; 135/171; 9/14; 88/151 (left to right, formatted as low/high/telcres). Cancer type abbreviations follow TCGA standards (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). For Fig. 6a, sample sizes are n = 4/10 (top/bottom) biologically independent experiments. For Fig. 6b, sample sizes are n = 3 (pgTCG2/pgS3B3) and 1 (pgCL4A/pgDPFP9/ pgKTN1) technically independent experiments. For Fig. 6h, sample sizes are n = 105/121; 326/484; 112/210; 54/66; 14/26; 17/22; 136/201; 68/104; 87/142; 132/237; 120/179; 135/171; 9/14; 88/151 (left to right, formatted as low/high/telcres). Cancer type abbreviations follow TCGA standards (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). For Fig. 6m, sample sizes are n = 171/170 samples for low/high/categorical. For all box plots, the middle line, hinges, notches and whiskers indicate the median, 25th/75th percentiles, 95% confidence interval and most extreme data points within 1.5× the interquartile range from the hinge.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. RNA-seq data generated as part of this study have been deposited in the Gene Expression Omnibus (accession number GSE120703). RNA-seq data generated by TCGA were downloaded from the Cancer Genomics Hub (CGHub) and Genomic Data Commons (GDC). Other data that support this study’s findings are available from the authors upon reasonable request. Source data for Figs. 1–4 and Extended Data Figs. 1, 2, 4, 6 and 10 are presented with the paper.

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**Author contributions**

J.D.T., Q.F. and R.K.B. designed the study. J.D.T., J.T.P., Q.F., E.J.D.N., E.R.H., M.V.M., J.P., A.M.G., A.E.B., J.W., N.T.N. and A.H.B. performed experiments. J.D.T., J.T.P. and R.K.B. analysed data. J.D.T. and R.K.B. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | pgFARM-induced exclusion of HPRT1 exon two and MET exon 14. a, Sanger sequencing of pgFARM-edited HPRT1 exon two in HeLa/iCas9 cells. b, Long range RT-PCR analysis of HPRT1 exon two skipping. c, RT-PCR analysis of HPRT1 exon two (e2) inclusion before/after Cas9 induction (day 0/day 10) and one week treatment with 6-thioguanine (+6TG). d, HPRT1 western blot analysis (n = 1 independent experiments) before (-) and after (+) one week treatment with 6TG. e, Cas9-expressing HEK293T cells (n = 3 biological replicates) that were untreated (wild-type) or expressing the indicated pgRNAs followed by one week treatment with 6TG. f, RT-PCR analysis of HPRT1 exon two (e2) inclusion in Cas9-expressing HEK293T cells (n = 3 biological replicates). g, Top, RT-PCR analysis of MET exon 14 (e14) inclusion with (+) or without (-) Cas9 expression. Bottom, quantification. (n = 1 independent experiments). h, As for (b), but for MET exon 14. Gray, non-targeting pgRNA; green, pgRNA targeting MET exon 14. See Source Data for uncropped gels.
Extended Data Fig. 2 | pgFARM-induced exclusion of MBNL1 exon five in multiple cell lines. a, Sanger sequencing of pgFARM-edited MBNL1 exon two in HeLa/iCas9 cells. b, Long range RT-PCR analysis of MBNL1 exon two skipping (n=1 independent experiments). c, Left, RT-PCR analysis (n=3 biological replicates per group) of MBNL1 exon five (e5) inclusion in Cas9-expressing IMR90 cells expressing a non-targeting pgRNA (pgNTC) or pgMBNL1.a. Right, quantification of MBNL1 exon 5 inclusion. d, Left and center, RT-PCR analysis and associated quantification of Mbnl1 exon five (e5) inclusion in Cas9-expressing B16-F10 cells expressing the indicated pgRNA. Right, RT-PCR analysis (n=3 biological replicates per group) and associated quantification of Mbnl1 exon (e5) inclusion in Cas9-expressing Melan-A cells expressing the indicated pgRNA. e, Individual Mbnl1 alleles that were cloned from gDNA of Cas9-expressing B16-F10 cells following delivery of an Mbnl1 exon five-targeting pgRNA and subjected to Sanger sequencing. f, Scatter plot comparing pgRNA-mediated exclusion of MBNL1 exon five (e5) and inclusion of MBNL2 exon five (e5), a paralogous exon that is regulated by nuclear MBNL1. Datapoints (n=24) are from HeLa/iCas9 cells treated with pgMBNL1.a, pgMBNL1.d, or pgMBNL1.e pgRNAs for two weeks. r, Pearson correlation; p, associated p-value computed using a two-sided Student’s t-test; shaded region, 95% confidence interval. See Source Data for uncropped gels.
Extended Data Fig. 3 | SMNDC1 poison exon inclusion in cancer. a, As Fig. 2c, but for all TCGA cohorts analyzed in Fig. 2d. p computed with two-sided Mann-Whitney U test. Hinges, notches, and whiskers indicate 25th/75th percentiles, 95% confidence interval, and most extreme datapoints within 1.5X interquartile range from hinge. Sample sizes are BLCA: n = 338; BRCA: n = 1089; COAD: n = 451; ESCA: n = 180; HNSC: n = 40; KICH: n = 62; KIRC: n = 430; KIRP: n = 262; LIHC: n = 350; LUAD: n = 502; LUSC: n = 447; PRAD: n = 481; STAD: n = 30; THCA: n = 362. b, Overall survival of lung adenocarcinoma (LUAD) patients, where patients were stratified according to the relative inclusion of the SMNDC1 poison exon. High poison exon, top tercile of samples; low poison exon, bottom tercile of samples. p computed with a two-sided logrank test. n = 237 (low) and 132 (high) samples. The uneven sample allocation arises from edge effects at the boundaries of terciles (MISO only estimates exon inclusion to two significant digits). c, As (b), but for SMNDC1 gene expression. High expression, top tercile of samples; low expression, bottom tercile of samples. p computed with a two-sided logrank test. n = 169 (low) and 174 (high) samples.
Extended Data Fig. 4 | pgFARM-induced exclusion of SMNDC1’s poison exon. 

a, Sanger sequencing of pgFARM-edited SMNDC1 poison exon in HeLa/iCas9 cells. Annotations of eliminated (X) or disrupted (↓) sequence elements are indicated.

b, Western blot for Cas9 and ACTB in parental PC9 and PC9-Cas9 (n = 3 biological replicates) transgenic cell lines.

c, Left, PC9-Cas9 cells expressing the indicated pgRNAs following treatment with 6TG for one week. Right, quantification of cell survival.

d, Representative SMNDC1 allele (n = 25 total sequenced alleles) from individual PC9-Cas9 clones. “small” and “medium” indicate alleles containing indels of length ~1–10 bp and >10 bp without intervening gDNA excision; “gDNA excision” indicates alleles with complete excision of intervening gDNA. Each class of editing event can effectively reduce 3′ splice site strength.

e, MaxEnt 3′ splice site scores for unedited (wild-type) or edited SMNDC1 alleles from individual PC9-Cas9 clones. See Source Data for uncropped gels.

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Extended Data Fig. 4 | pgFARM-induced exclusion of SMNDC1’s poison exon.
Extended Data Fig. 5 | pgRNA library design. a, Regions used to classify each poison exon (n = 12,653) according to its sequence conservation. b, Median conservation scores for each indicated region (violin plot width represents probability density of data distribution). c, Median per-nucleotide sequence conservation for exon groups described in the text. d, Per-nucleotide sequence conservation for an SRSF3 ultraconserved poison exon. e, As (d), but for an MTX2 poorly conserved poison exon. f, The most significant biological processes associated with genes containing unconserved poison exons (n = 2,363), conserved poison exons (n = 352), or conserved non-poison exons (n = 888) (related to Fig. 3c). FDR computed using the Wallenius method and corrected using the Benjamini-Hochberg method. g, pgRNA library summary. h, On-target scores (MIT score) for all gRNAs targeting 3′ splice sites analyzed in our study (“false”) and those included in the final library (“true”). i, As (h), but for off-target scores identified using Cas-OFFinder.
Extended Data Fig. 6 | Analysis of pilot pgFARM screen. a, pgRNA library generation for Illumina sequencing. b, pgRNA counts throughout the time course (n=1,000; 3,604; 4,099; 805 for groups, left to right). c, Relative proliferation of HeLa/iCas9 cells expressing an SMNDC1 upstream constitutive exon-targeting pgRNA relative to control pgRNA (non-essential gene CSPG4; n=2 independent experiments). d, Unnormalized fold-changes for non-targeting pgRNAs (n=1,000) and pgRNAs targeting unexpressed (<1 transcripts per million, TPM) genes, located in genomic regions with the indicated copy numbers (n=2, 38, 45, and 11, left to right). e, Normalized fold-changes for all non-targeting pgRNAs (NTC; n=1,000) and pgRNAs targeting the indicated exons (n=9 pgRNA per exon) in SNRNP70. f, Relative proliferation of HeLa/iCas9 cells expressing a SNRNP70 upstream constitutive exon-targeting pgRNA without (-) or with (+) simultaneous overexpression of a SNRNP70-encoding cDNA (n=6 replicates per condition). g, Representative Sanger sequencing of a pgFARM-edited SNRNP70 upstream exon in HeLa/iCas9 cells (n=19 total sequenced alleles). h, RNA-seq read coverage across the SNRNP70 locus containing the targeted upstream constitutive exon (gray box) from HeLa/iCas9 cells expressing the indicated pgRNA (n=1 per pgRNA). Ψ, percent spliced in. i, SNRNP70 poison exon inclusion for HeLa/iCas9 cells expressing the indicated pgRNA relative to a non-targeting pgRNA (n=1 per pgRNA). j, Scatter plot comparing cassette exon inclusion in HeLa/iCas9 cells treated with a non-targeting control pgRNA (pgNTC) or SNRNP70 upstream constitutive exon-targeting pgRNA (pgSNRNP70). Points are shaded by statistical significance (two-sided Mann-Whitney test). k, As (j), but comparing alternative 5′ splice site usage. For box plots, the line, hinges, and whiskers represent median, 25th and 75th percentiles, and most extreme datapoints within 1.5X interquartile range from hinge. See Source Data for uncropped gels.
Extended Data Fig. 7 | Analysis of pilot pgFARM screen, continued. a, Normalized pgRNA fold-changes (n = 1,000 and 9 for non- and exon-targeting pgRNAs, respectively). The center line, hinges, and whiskers represent median, 25th and 75th percentiles, and most extreme datapoints within 1.5X interquartile range from hinge. b, RNA-seq read coverage across the SRSF3 locus containing the targeted upstream constitutive exon (gray box) from HeLa/iCas9 cells expressing the indicated pgRNA (n = 1 per pgRNA). Ψ, percent spliced in. c, SRSF3 exon inclusion for HeLa/iCas9 cells expressing the indicated pgRNA relative to a non-targeting pgRNA (n = 1 per pgRNA). d, SRSF3 RNA binding motif enrichment in differentially spliced exons (n = 2,046 left; 727 right) in HeLa/iCas9 cells expressing the indicated pgRNA. Data presented as mean ± 95% confidence interval computed by bootstrapping. e, Scatter plot comparing cassette exon inclusion in HeLa/iCas9 cells treated with a non-targeting control pgRNA (pgNTC) or AAVS1-targeting control pgRNA (pgAAVS1). Points are shaded by statistical significance (two-sided Mann-Whitney U test). f, RNA-seq read coverage across the entire SNRNP70 locus in HeLa/iCas9 cells expressing the indicated pgRNA (n = 1 per pgRNA). g, As (f), but for SRSF3 (n = 1 per pgRNA).
Extended Data Fig. 8 | Analysis of large-scale pgFARM screens. **a,** HeLa/iCas9 cells (n = 4 biological replicates) treated with the poison exon pgRNA library and grown in the presence (+ dox) or absence (- dox) of active Cas9. **b,** Scatter plots comparing normalized fold-changes (day 14 vs. day 0; n = 963 targeted exons) estimated with each replicate of the cell viability screen in HeLa/iCas9 cells. Pearson correlations for individual replicate comparisons are indicated. **c,** Normalized fold-changes for pgRNAs targeting exons in unexpressed (TPM ≤ 1; n = 96 for HeLa/iCas9 and 128 for PC9-Cas9) or highly expressed (TPM ≥ 10; n = 681 for HeLa/iCas9 and 661 for PC9-Cas9) genes. Each dot represents the median fold-change computed over all pgRNAs targeting exons in the indicated groups for a representative replicate from the screens in HeLa/iCas9 (left; n = 5) and PC9-Cas9 (right; n = 4) cells. TPM, transcripts per million. **d,** Normalized fold-changes for pgRNAs targeting lowly expressed genes (TPM < 5) located in genomic regions with the indicated copy numbers (n = 6, 165, and 14 per group, left to right, for HeLa/iCas9; n = 60, 107, and 45 per group, left to right, for PC9-Cas9). **e,** Rank plot of mean normalized fold-changes for conserved poison (orange) or upstream constitutive exons (purple) based on all replicates of the HeLa/iCas9 viability screen. **f,** As (**e**), but for all replicates of the PC9-Cas9 viability screen. For box plots, the center line, hinges, and whiskers represent median, 25th and 75th percentiles, and most extreme datapoints within 1.5X interquartile range from hinges, respectively.
Extended Data Fig. 9 | pgFARM-induced exclusion of CPSF4 and SMG1 poison exons. a, Sanger sequencing of pgFARM-edited CPSF4 poison exon in HeLa/iCas9 cells. Annotations of eliminated (X) or disrupted (↓) sequence elements are indicated. b, RNA-seq read coverage across the entire CPSF4 locus in HeLa/iCas9 cells expressing a CPSF4 poison exon-targeting pgRNA (pgCPSF4; n = 1). We observed no read coverage indicative of cryptic splicing in pgCPSF4-treated cells. The two sets of splice junction reads downstream of the CPSF4 poison exon correspond to usage of endogenous (naturally occurring in unedited cells) competing 3′ splice sites. c, As (b), but for an SMG1 poison exon-targeting pgRNA (pgSMG1; n = 1). d, Scatter plot comparing normalized fold-changes for pgRNAs targeting a poison exon compared to matched upstream coding exon within the same gene.
Extended Data Fig. 10 | Analysis of xenograft screens. a, Tumors derived from parental PC9 or PC9-Cas9 cells (n = 4 per group). b, Mice from early and late tumor time points (n = 4 and 10 tumors, respectively). c, pgRNA Illumina libraries. d, Pearson correlation (r) matrix for xenograft screen samples. Unsupervised clustering of library depth-normalized pgRNA counts by the complete-linkage method. e, Normalized counts (mean ± S.D.) for gRNAs targeting coding exons in the indicated genes. Data from Chen et al, 2015 (n = 1, 6, 3, and 9 for groups, left to right). f, Relative cell number (mean ± S.D.) for PC9-Cas9 cells expressing a pgRNA targeting the indicating exons (n = 3 per group). g, Progression-free survival of lung adenocarcinoma patients (n = 167/171 for low/high categories), where patients were stratified by inclusion of tumor-suppressive poison exons. h, As (g), but for overall survival. i, As (g), but for essential poison exons (n = 166/169 for low/high categories). j, As (f), but for overall survival. See Source Data for uncropped gels.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☐  | ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
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| ☐  | ☒ The statistical test(s) used AND whether they are one- or two-sided |
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| ☐  | ☒ A description of all covariates tested |
| ☐  | ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐  | ☒ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐  | ☒ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted |
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| ☐  | ☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☐  | ☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐  | ☒ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |
| ☐  | ☒ Clearly defined error bars |
| ☒  | ☒ State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection | No software was used.

Data analysis | Publicly available software was used in this study. Specific programs are RSEM (v1.2.4), Bowtie (v1.0.0), TopHat (v2.0.8b), MISO (v2.0), and Bioconductor (v3.7) within the R (v3.5.1) programming environment. Fiji/ImageJ (v2.0.0) and HALO (v2.0, Indica Labs) were used for image analysis. MAFFT (v7.0) was used for Sanger sequence alignments.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNA-seq data generated as part of this study has been deposited in the Gene Expression Omnibus (accession number GSE120703). RNA-seq data generated by The Cancer Genome Atlas (TCGA) was downloaded from the Cancer Genomics Hub (CGHub) and Genomic Data Commons (GDC).

Field-specific reporting
Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.
☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences
For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

Sample size
Samples sizes for pooled screens were based on results from our pilot screen as well as information garnered from published literature (Doench 2018, Chen et al 2015).

Data exclusions
No data were excluded.

Replication
All attempts at replication with both technical and biological replicates were successful.

Randomization
This is not relevant to our study, which relied on pooled screening. For experiments involving specific targets, all experiments involved control treatments (non-targeting, AAVS1-targeting, or CSPG4-targeting pgRNAs) or perturbation treatments (pgRNAs targeting exons).

Blinding
Blinding was not relevant to our study because of the pooled nature of the screen.

Reporting for specific materials, systems and methods

Materials & experimental systems
n/a Involved in the study
☐ ☑ Unique biological materials
☐ ☑ Antibodies
☐ ☑ Eukaryotic cell lines
☐ ☑ Palaeontology
☐ ☑ Animals and other organisms
☐ ☑ Human research participants

Methods
n/a Involved in the study
☐ ☑ ChIP-seq
☐ ☑ Flow cytometry
☐ ☑ MRI-based neuroimaging

Unique biological materials
Policy information about availability of materials

Obtaining unique materials
All unique materials are readily available from the authors.

Antibodies
Antibodies used
Antibodies against Cas9 (Cell Signaling #14697, 1:1000), ACTB (Cell Signaling (13E5) Rabbit mAb #4970, 1:5000), HPRT1 (Abcam ab10479, 1:1000), and GAPDH (Bethyl a300-639a, 1:5000) were used as primary antibodies for western blots. For immunofluorescence, MBNL1 (DSHB MB1a(4A8)) was used at a dilution of 1:500.
Validation

Cas9 antibody specificity was confirmed using 293T cells mock transfected or transfected with a construct expressing Cas9 (https://www.cellsignal.com/products/primary-antibodies/cas9-7a9-3a3-mouse-mab/14697).

ACTB antibody specificity was confirmed by western blot analysis which revealed a band of the expected size (https://www.cellsignal.com/products/primary-antibodies/b-actin-13e5-rabbit-mab/4970).

GAPDH antibody specificity was confirmed by western blot analysis which revealed a band of the expected size (https://www.bethyl.com/product/A300-639A/GAPDH+Antibody#).

MBNL1 antibody specificity has been previously described (e.g., PMID: 19095965) and confirmed in our CRISPR-edited cell lines (e.g., Fig. 31).

### Eukaryotic cell lines

| Policy information about | cell lines |
|--------------------------|------------|
| **Cell line source(s)**  | HeLa/iCas9 cells were obtained from Qin Yan (Yale School of Medicine). PC9 cells were obtained from M. Meyerson (Broad Institute/Dana-Farber Cancer Institute). Cas9-expressing IMR90 cells were obtained from Adam Geballe (FHCRC). Melan-a cells were obtained from Dorothy Bennett (St George's University of London). B16-F10 cells were obtained from ATCC (ATCC CRL-6475). HEK293T cells (PMID: 28335006) were obtained from Douglas Fowler (University of Washington). |

| **Authentication**       | Cell lines were authenticated via RNA-seq expression profiling. |
| **Mycoplasma contamination** | Cell lines tested negative for mycoplasma contamination. |
| **Commonly misidentified lines** (See ICLAC register) | None of the cell lines are commonly misidentified cell lines. |

### Animals and other organisms

| Policy information about | studies involving animals; ARRIVE guidelines recommended for reporting animal research |
|--------------------------|---------------------------------------------------------------|
| **Laboratory animals**   | Species: Mus musculus. Strain: NU/J. Sex: male. Age: 8 to 12 weeks. |
| **Wild animals**         | The study did not involve wild animals. |
| **Field-collected samples** | The study did not involve samples collected from the field. |