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

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Abstract

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 likely contribute to their unusually high conservation and contrast with the dispensability of other ultraconserved elements for viability.

Keywords

CRISPR/Cas9; alternative splicing; ultraconserved elements; nonsense-mediated decay; cancer; lung adenocarcinoma; intron retention

INTRODUCTION

Most biological processes are characterized by alternative splicing[1–3], which is correspondingly dysregulated in many diseases[4,5]. Mapping individual mis-spliced isoforms to specific molecular pathologies can enable the rational design of splicing-targeted therapeutics[6,7]. 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-throughput[8,9], while RNAi does not alter alternative splicing. CRISPR/Cas9 has been used to knock out *DMD* isoforms or long non-coding RNAs by targeting splice sites[10,11], 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 genomes[12]. Many ultraconserved and highly conserved elements overlap poison exons, defined as alternative exons which interrupt their host genes' reading frames[13,14] 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 autoregulation[13,14]. 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 sequence[16–19]. 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 backbone[18] and introduced each construct into HeLa cells with doxycycline-inducible Cas9 (HeLa/iCas9[20]; Fig. 1b,c). pgRNA delivery induced rapid and effective skipping of *HPRT1* exon two (Fig. 1d).

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, Supplementary Table 1). Although pgRNAs can cause gDNA inversion in addition to excision[21], we detected no inversion events.

A recent study reported that Cas9-induced DNA breaks can result in rare large deletions[22], which could potentially cause unwanted gene disruptions. Although we did not observe any excision events >350 bp by Sanger sequencing—far shorter than most introns—this assay might not detect extremely large deletions. We therefore used long-range gDNA PCR to test whether pgRNA delivery caused large deletions. Consistent with the reported rarity of large deletions (3–7% of events[22]), we readily detected our positive control (deletion of ~600 bp) but no other large deletions (Fig. 1e). Large deletions therefore occur at sufficiently low rates to not significantly influence phenotypes in our polyclonal assays.

As gDNA excision disrupts gene structures, pgRNA delivery could potentially result in abnormal mis-splicing in addition to targeted exon skipping. We therefore used long-range RT-PCR to confirm that all pgRNAs caused skipping of the targeted *HPRT1* exon, but not production of unwanted additional isoforms (Extended Data Fig. 1b).

Inducing *HPRT1* exon skipping drove the expected 6TG resistance. Both HeLa/iCas9 and Cas9-expressing 293T cells treated with *HPRT1* exon two-targeting, but not non-targeting, pgRNAs formed 6TG-resistant outgrowths that exhibited *HPRT1* exon two skipping and loss of HPRT1 protein (Fig. 1f, Extended Data Fig. 1c–f). We confirmed pgFARM's generalizability by targeting another constitutively included exon. pgRNA delivery drove rapid skipping of *MET* exon 14 without inducing detectable cryptic splicing (Extended Data Fig. 1g–h).

We next used pgFARM to manipulate alternative splicing by targeting an *MBNL1* ultraconserved coding exon (exon five; Fig. 1g). We detected exon skipping two days after pgRNA delivery, with near-complete exon skipping for some pgRNAs after seven days (Fig. 1h). Complete gDNA excision was the most common editing event (91%). We observed no unexpectedly large gDNA deletions, gDNA inversion, or unwanted cryptic isoforms (Fig. 1i, Extended Data Fig. 2a–b, Supplementary Table 1). pgRNA delivery similarly induced *MNBL1* or *Mbnl1* exon skipping in Cas9-expressing untransformed human fibroblasts (IMR90), untransformed mouse melanocytes (Melan-a), and mouse melanoma cells (B16-F10), as well as on-target gDNA editing and splice site disruption (Extended Data Fig. 2c–e, Supplementary Table 1).

Induction of *MBNL1* exon skipping drove expected functional consequences. Nuclear levels of total MBNL1 were quantitatively lower following delivery of each pgRNA that induced appreciable exon skipping (Fig. 1j,k), as expected[12,23,24]. MBNL1 protein encoded by the exon five-containing mRNA was ablated in pgRNA-edited cell lines, while MBNL1 protein encoded by the exclusion isoform remained (Fig. 1l, Extended Data Fig. 2f). Induction of *MBNL1* exon five skipping caused quantitatively correlated differential splicing of *MBNL2*,

whose own exon five is regulated by nuclear MBNL1[24,25] (Extended Data Fig. 2g). Together, these data demonstrate that pgFARM can suppress a specific RNA isoform independent of total gene disruption or induction of unwanted cryptic isoforms.

An *SMNDC1* poison exon regulates intron retention in cancer

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*[26], we hypothesized that its poison exon might influence the widespread intron retention that characterizes most cancers[27,28].

The *SMNDC1* poison exon enables splicing-dependent autoregulation via NMD in cell culture[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, Extended Data Fig. 3a). *SMNDC1* poison exon inclusion was significantly dysregulated in cancer relative to patient-matched normal samples in nine 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 gRNA induced cutting[30]. 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, Supplementary Table 1). Almost all edited alleles exhibited dramatically reduced 3' splice site strengths[31], even when only one cut occurred (Fig. 2e).

We next confirmed 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. 90% of the *SMNDC1*-targeted clones carried 3' splice site-disrupting 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-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 intron retention following delivery of the poison exon-targeting pgRNA relative to an AAVS1-targeting control pgRNA, such as introns in *STK36* and *CENPT* (Fig. 2h).

We tested whether variable *SMNDC1* poison exon inclusion contributed to frequent intron retention in cancers[27,28,32]. We grouped the 512 lung adenocarcinoma samples with RNA-seq data[33] into terciles based on *SMNDC1* poison exon inclusion and quantified intron retention across each tercile[27]. Low *SMNDC1* poison exon inclusion was associated with notably widespread reductions in intron retention: 59% of constitutive introns exhibiting any retention were spliced significantly more efficiently in samples with low poison exon inclusion (Fig. 2i,j). This signal persisted after restricting to cases where intron retention is not predicted to induce NMD (Extended Data Fig. 4f), and was equally strong but opposite upon stratifying by *SMNDC1* gene expression (Fig. 2k, 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 (Fig. 2l). Experimentally targeting the *SMNDC1* poison exon in HeLa/iCas9 cells similarly resulted in significantly decreased intron retention, while targeting the *SMNDC1* upstream exon resulted in significantly increased intron retention affecting 240 genes (Fig. 2l). These data suggest that the *SMNDC1* poison exon controls *SMNDC1* expression to modulate intron retention.

pgRNA library targeting highly conserved poison exons

We designed a pgRNA library targeting poison exons in order to perform a highly multiplexed screen (Fig. 3a). We identified 12,653 human poison exons that are predicted to induce NMD[15] and computed each exon's sequence conservation across 46 species[34], 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, Extended Data Fig. 5f), in agreement with previous studies[13,14,35].

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[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). 78% of targeted poison exons exhibited inclusion   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 coding 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 nine pgRNAs per exon, and additionally included 1,000 non-targeting pgRNAs (Extended Data Fig. 5g–i, Supplementary Table 2).

We synthesized the pgRNA library with an oligonucleotide array and cloned the library at >1,000-fold coverage using a cloning strategy similar to those from previous pgRNA studies[17,18] (Fig. 3g). Sanger sequencing of individual bacterial colonies showed that ~98%

of sequenced pgRNAs were properly paired after library construction, consistent with low (~7.5%) mis-pairing rates reported in other studies[17].

pgFARM enables isoform-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 days after Cas9 induction, and profiled pgRNA abundance by sequencing both gRNAs (Extended Data Fig. 6a). We sequenced each time point to ~400X 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. 6b).

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[37] 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[38–41]. We computed the copy number of each targeted unexpressed gene in the HeLa genome[42] and compared fold-changes between different loci. While this analysis showed no correlation between 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 targeting pgRNAs (Fig. 4i, Supplementary Table 4) and selected a constitutive exon in *SNRNP70*, which encodes a core splicing factor[43], for detailed study. Treating cells with a *SNRNP70* constitutive exon-targeting pgRNA caused dramatic fitness defects that were rescued by overexpressing a SNRNP70-encoding cDNA (Fig. 4j,k, Extended Data Fig. 6e,f). We sequenced individual *SNRNP70* alleles four days after Cas9 induction to find that 79% of alleles exhibited 3' splice site-disrupting edits, with ~40% exhibiting complete gDNA excision (Extended Data Fig. 6g, Supplementary Table 1).

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 down-regulation (>4-fold) of SNRNP70 mRNA levels and inclusion of *SNRNP70*'s poison exon (~5-fold;

Extended Data Fig. 6h,i), consistent with the autoregulatory role of this poison exon[29]. 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[43], induction of *SNRNP70* constitutive exon skipping caused transcriptome-wide exon skipping and a shift towards intron-proximal 5' splice site usage (Fig. 4l,m, Extended Data Fig. 6j,k). We extended these functional assays to *SRSF3*, which encodes a sequence-specific splicing factor[44]. We delivered a pgRNA targeting an *SRSF3* constitutive exon, confirmed on-target gDNA editing, and performed RNA-seq (Fig. 4i, Extended Data Fig. 7a, 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[45]. Cassette exons that were repressed following *SRSF3*-targeting pgRNA delivery were enriched for SRSF3's RNA-binding motif (Fig. 4n, 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 based on 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, 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 4,5). 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 significant depletion or enrichment (absolute fold-change 25% with FDR 0.01 at day 14). 43% (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, 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, 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, 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[46], 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[47–49].

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 four days, we subcutaneously injected 3×10^7 cells (~3,000-fold pgRNA representation) into the flanks of immunocompromised (NU/J) mice (Fig. 6a, 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 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[50]. We therefore used data from all replicates for statistical analyses in order 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 data as described above. 112 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[50]. 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[51], although pro-tumorigenic roles have only been demonstrated for a few factors[52–54]. 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[37,50], was strongly selected against (Fig. 6d, 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.

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 to anti-tumorigenic effects compared to constitutive exon loss ($p = 0.017$ by the one-sided binomial proportion test; Fig. 6e) 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*[55,56], 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* poison exon-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[33] based on 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. 10i,j), 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 toolkits for manipulating isoform expression. Single guide RNA (gRNA) delivery[10,57] and base editing[58,59] can alter exon recognition, while RNA-targeting CRISPR/Cas systems can enable direct manipulation of alternative splicing[60,61]. Each of these techniques is potentially amenable to a screening format.

Because of their extraordinary sequence conservation, ultraconserved elements were initially assumed to be essential for life[12]. However, deletion of many ultraconserved enhancers has no effects on mouse organismal or cell viability[62–65]. 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 in order to address the outstanding mystery of poison exons' high conservation. However, pgFARM can potentially be applied to many other kinds of alternative RNA processing[66–68]. We expect pgFARM to enable rapid and unbiased functional interrogation of specific RNA isoforms associated with diverse biological processes or disease states.

ONLINE METHODS

pgRNA design, plasmids, and cloning

For pgRNA optimization (Fig. 1), candidate gRNAs located near the targeted exon were identified and then paired based on being located within the coding sequence or proximal/distal to splice sites. Both NAG and NGG PAMs were utilized. pgRNAs were cloned following published methods[18] (Fig. 3g). Oligos 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 (FastDigestEsp3I, Thermo Fisher Scientific)-linearized lentiGuide-Puro (Addgene #52963) backbone using the NEBuilder HiFi (New England Biolabs) assembly system and transformed into NEB Stable competent *E. coli* cells (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 H1 drop-in gBlock (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 NEB Quick Ligase (New England Biolabs). This reaction was transformed into NEB Stable cells to propagate the

plasmid and generate final pLGP-pgRNA vectors. All plasmids were sequence verified 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 (Matthew Meyerson) with pXPR_111 lentivirus and selecting with blasticidin for 5–7 days. Cas9 protein was detected with an anti-Cas9 antibody (Cell Signaling #14697) and anti-ACTB antibody (Cell Signaling #4970). Cas9-expressing B16-F10 (ATCC CRL-6475), Melan-a (Dr. Dorothy Bennett), and HEK293T cells were generated by transducing cells with lentiCas9-Blast (Addgene 52962) lentivirus followed by blasticidin selection.

Cell culture

HeLa/iCas9 and Cas9-expressing HEK293T, IMR90, and B16-F10 cells were grown at 37°C and 5% atmospheric CO_2 in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). The same conditions were used for PC9-Cas9 and Cas9-expressing Melan-a cells except that Roswell Park Memorial Institute (RPMI) 1640 media was instead of DMEM. Cas9-expressing Melan-A cell media was supplemented with 200 nM TPA (Sigma-Aldrich). All cell lines were periodically tested for mycoplasma contamination. For 6TG resistance assays, we treated cells with 15 μM 6-thioguanine (Sigma-Aldrich) for one week.

Lentivirus production and titration

For large-scale production, HEK293T cells were seeded in T225 flasks such that each flask would be ~80% confluent at the time of transfection. After overnight incubation, pCMV-VSV-G (Addgene #8454), psPAX2 (Addgene #12260), and pLGP-pgRNA transfer vectors were introduced into cells using PEI Max (Polysciences, Inc.) transfection. Lentivirus-containing media was harvested 48 hours later, filtered, and stored as 1 mL aliquots at −80°C until use. For small-scale production, HEK293T cells were seeded into individual wells of a 6-well plate and all reagents were proportionally scaled. To determine lentiviral titers, HeLa/iCas9 or PC9-Cas9 cells were seeded in individual wells of a 12-well plate in media supplemented with 8 μg/mL polybrene (EMD Millipore) and incubated at 37°C for 2 hours. Next, serial dilution of the lentivirus preparation was added to individual wells and incubated for 24 hours at 37°C. The next day, cells from individual wells of the 12-well plate were re-seeded into eight wells of a 96-well plate. Cells in four of these wells were grown in culture media supplemented with 1 μg/mL puromycin and the other four contained no puromycin. After all cells in the no-infection control wells were dead (typically 2–3 days), 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, HeLa/iCas9 or PC9-Cas9 cells were seeded into individual wells of a multi-well plate and treated with viral supernatant to deliver pgRNA

vectors. The next day, virus-containing media was exchanged for standard growth media supplemented with 1 μg/mL puromycin to select for stable integration. After selection, 1 μg/mL of doxycycline was added to HeLa/Cas9 cells to induce Cas9 expression. This was defined as day 0 for each experiment. Because 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 was 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 assays.

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 TapeStation System (Agilent Genomics). For TOPO cloning and Sanger sequencing, purified amplicons were ligated into vectors for sequencing using the Zero Blunt 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 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[69].

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) using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and amplicons were analyzed and quantified using either an 4200 TapeStation 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 cycloheximide for up to 6 hours 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 minutes at room temperature and permeabilization with PBST (PBS, 0.2 % Triton X-100) for 10 minutes at room temperature. Non-specific binding was blocked by incubating cells in PBS + 1% BSA (Fisher Scientific) for 1 hour at room temperature followed by overnight incubation with primary antibody (Mb1a DSHB, 1:1000) for 1 hour at room temperature. Cells were washed three times with PBST for 10 minutes at room temperature and then incubated with secondary antibodies (Goat Anti-Mouse DyLight 594, Thermo Fisher Scientific) for 1 hour at room temperature. Cells were then washed three times with PBST for 10 minutes 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[70]. 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 660nm Protein Assay Reagent. Total protein lysates were electrophoretically separated and transferred to nitrocellulose membranes using the NuPAGE system (Thermo Fisher Scientific). Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 hour at room temperature followed by overnight incubation at 4°C with primary antibodies diluted in blocking buffer. HPRT1 (Abcam ab10479, 1:1000) and GAPDH (Bethyl a300-639a, 1:5000) 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[71] 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

HeLa/iCas9 or PC9-Cas9 cells were seeded in 15 cm plates at a density of 5×10^6 cells per plate in complete media supplemented with 8 μg/mL polybrene. A volume of the pgRNA library virus was added such that only 20–30% of cells were predicted to survive after selection with puromycin. Media was changed 24 hours later and replaced with complete media supplemented with 1 μg/mL puromycin. After no cells remained in uninfected control plates, we collected the day 0 cell pellets and then added 1 μg/mL doxycycline to HeLa/iCas9 cells. At this point, cells were passaged every 2 to 3 days 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 proteinase 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 2X Ready Mix using primers RKB2713/RKB2714 followed by Ampure XP SPRI bead clean-up. Second, 10 ng of amplicon from PCR #1 was used as input for amplification with primers RKB2715/RKB2716 followed by Ampure XP SPRI bead clean-up. Third, 10 ng of amplicon from PCR #2 was used as input for amplification with a common forward primer, RKB2717, and a sample specific barcoding primers 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. Final, 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 #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 an M.O.I. of ~0.3. Infected cells were propagated in cell culture for ~4 days to select (1 μg/mL puromycin) stable cell lines and grow enough cells for transplantation. For injections, adult NU/J mice were anesthetized with isoflurane and 3×10^7 cells were injected subcutaneously into both flanks. Cohorts of mice were sacrificed ~3 and ~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 mg of tissue from each tumor was digested in lysis buffer (50 mM Tris, 50 mM EDTA, 1% SDS, 100 μg/mL proteinase 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 were grown using standard conditions, transduced with lentivirus containing pgRNA expression vectors, and selected with 1 μg/mL puromycin. Prior to implantation, cells were grown for at least 1 week post-selection. For injections, adult NU/J mice were anesthetized with isoflurane and 2×10^6 cells were subcutaneously injected 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 three days prior to processing and paraffin embedding.

pgRNA deep sequencing data analysis

The first and second reads were separately mapped to a database of pgRNA sequences using Bowtie[72]. 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) x (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. False discovery rates (FDRs) were computed by estimating a distribution of p-values associated with the above procedure for fake targets derived by sub-sampling groups of 9 pgRNAs from all pgRNAs targeting unexpressed genes. A p-value was computed for each group. We performed this procedure 10,000 times in order to estimate an empirical distribution of p-values derived from fake targets and then estimated FDRs for real targets via the cumulative distribution function of the fake p-value distribution. Unless otherwise specified, normalized fold-changes associated with a given target exon were computed as the geometric mean over all targeting pgRNAs. These statistical procedures ensured that fold-changes < 1 corresponded to decreased viability due to on-target effects, independent of DNA breaks, and permitted us to assess the statistical significance of depletion or enrichment of each targeted exon.

All statistical analyses were performed in the R programming environment with Bioconductor[73]. All plots and figures were generated with the dplyr[74] and ggplot2[75] packages.

RNA-seq library preparation

RNA was extracted from cell pellets using the Direct-zol RNA MiniPrep (Zymo Research) kit. Poly(A)-selected, unstranded 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 prior to 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 was analyzed as previously described[76]. Briefly, reads were mapped to a transcriptome annotation created by merging the Ensembl 71[77], UCSC knownGene[78], and MISO v2.0[71] annotations using RSEM version 1.2.4[79] (modified to call Bowtie[72] 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 2.0.8b[80]. 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 survival[81]). Patients were stratified as follows for Fig. 6m. For each cancer sample, we computed the following statistic: (# of tumor-suppressive poison exons for which exon inclusion ⩽ 25th percentile of exon inclusion over the entire cohort) / (# of tumor-suppressive poison exons for which exon inclusion ⩾ 75th percentile 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). 16 depleted and 16 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;30;46;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 terciles). Cancer type abbreviations follow TCGA standards (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). For Fig. 6e, sample sizes are n=4/10 (top/bottom) biologically independent experiments. For Fig. 6f, sample sizes are n=3 (pgNTC/pgSF3B3) and 1 (pgCLK4/pgDPP9/pgKTN1) technically independent experiments. For Fig. 6g, sample sizes are n=1 (*CLK4/DPP9/KTN1*) and 3 (*SF3B3/SRSF2/SRSF5*) technically independent experiments. For Fig. 6h, sample sizes are n=4 (*in vitro*/early tumor) and 10 (late tumor) biologically independent experiments. For Fig. 6i, sample sizes are n=4 (pgNTC) and 8 (pgEPC1) biologically independent clones. For Fig. 6j,k, sample sizes are n=10 tumors per group. For Fig. 6l, sample sizes are n=17 histological analyses. For Fig. 6m, sample sizes are n=171/170 samples for low/high categories.

For all box plots, middle line, hinges, notches, and whiskers indicate median, 25th/75th percentiles, 95% confidence interval, and most extreme datapoint within 1.5X interquartile range from hinge.

Reporting Summary

Additional information on research design is available in the Life Sciences Reporting Summary linked to this article.

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). Other data that support this study's findings are available from the authors upon reasonable request.

Extended Data

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 a *Mbnl1* exon five-targeting pgRNA and subjected to Sanger sequencing. (**f**) Quantification of total MBNL1 protein levels (top) and MBNL1 protein encoded by the exon five-including isoform (bottom) before (day 0) and after (day 14) Cas9 induction in HeLa/iCas9 cells expressing the indicated pgRNA, measured by immunoblot in Fig. 1l. *, pgRNAs that induced the greatest *MBNL1* exon five exclusion. Data are representative of n=2 independent experiments. (**g**) 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.
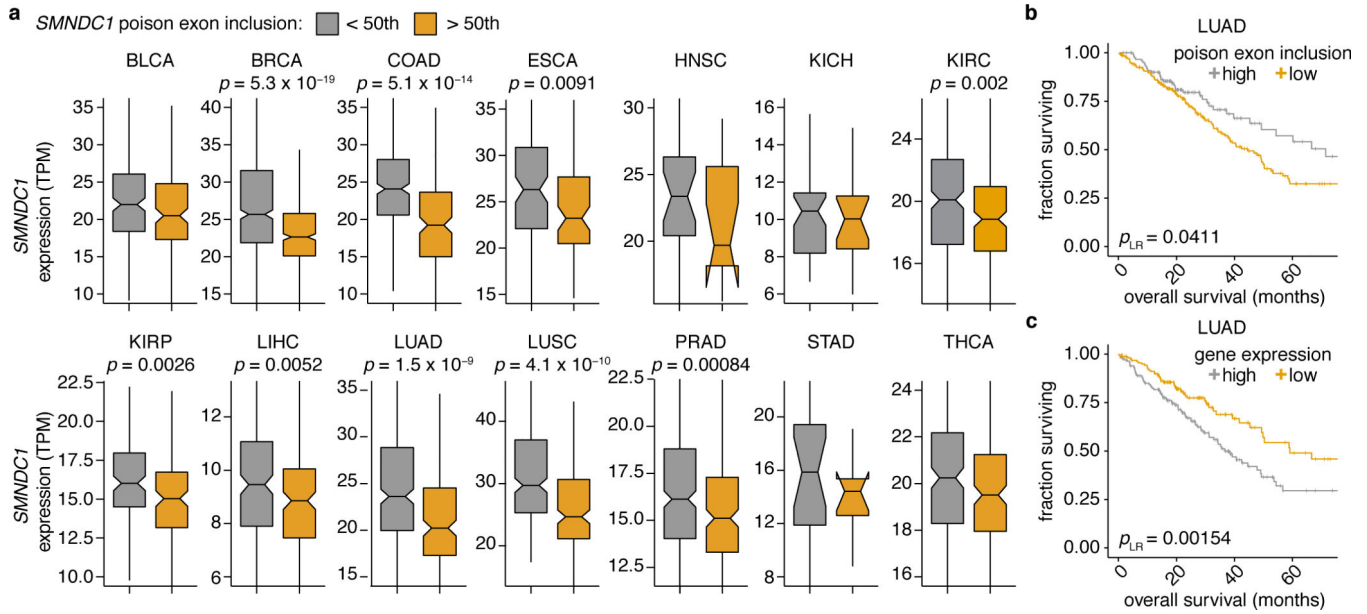
Extended Data Fig. 3. *SMNDC1* poison exon inclusion in cancer.

(**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.
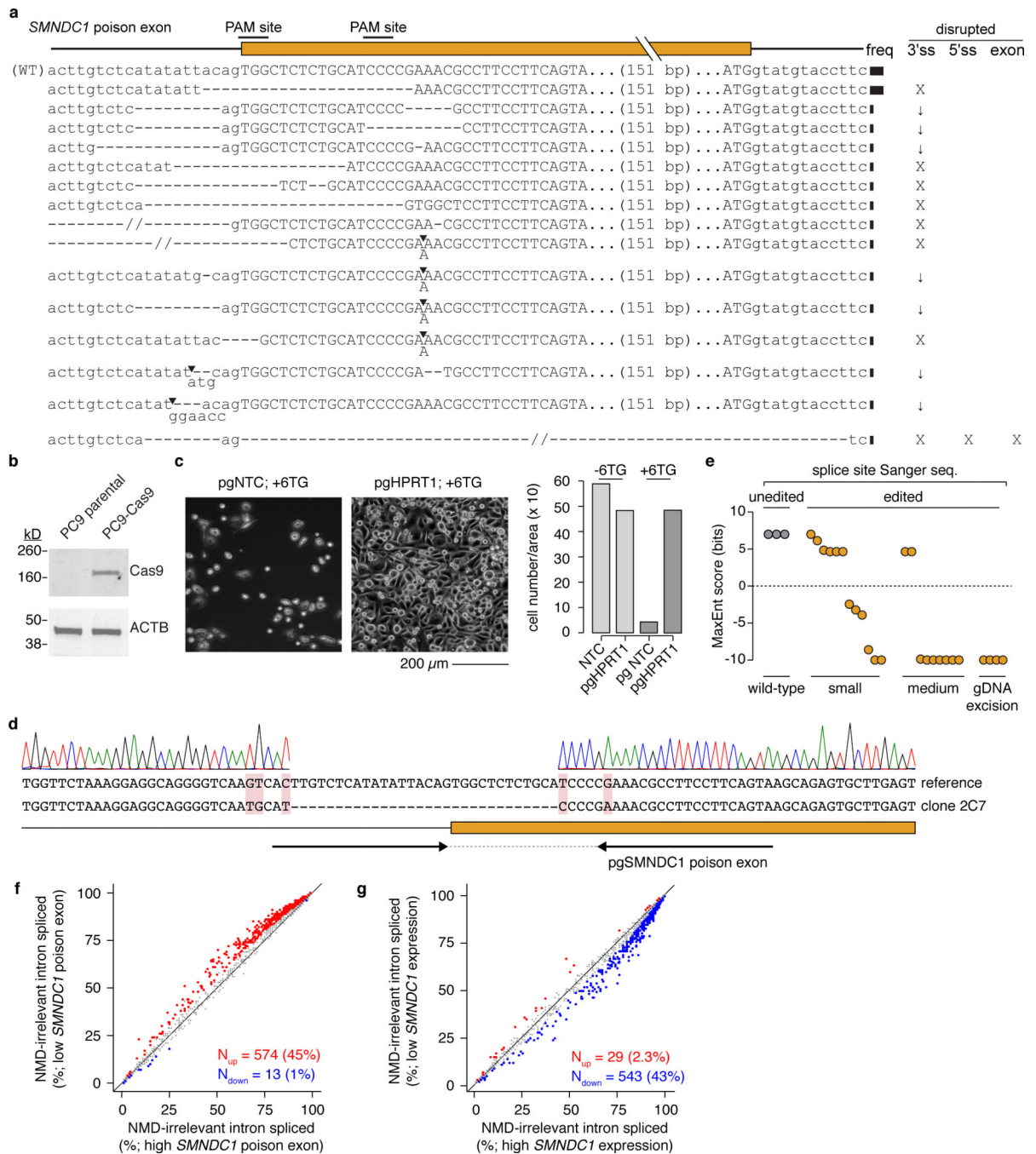
Extended Data Fig. 4. pgFARM-induced exclusion of *SMNDC1*'s poison exon.
(**a**) Sanger sequencing of pgFARM-edited *SMNDC1* poison exon in HeLa/iCas9 cells.
Annotations of eliminated (X) or disrupted (↓) sequence elements are indicated. (**b**) Western
blot for Cas9 and ACTB in parental PC9 and PC9-Cas9 (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) of a PC9-Cas9 clonal cell line isolated
following delivery of an *SMNDC1* poison exon-targeting pgRNA. (**e**) MaxEnt 3' splice site

scores for unedited (wild-type) or edited *SMNDC1* 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. (**f**) As Fig. 2j, but restricted to introns that are not NMD-targets (NMD-irrelevant). (**g**) As Fig. 2k, but restricted to introns that are not NMD-targets (NMD-irrelevant). See Source Data for uncropped gels.
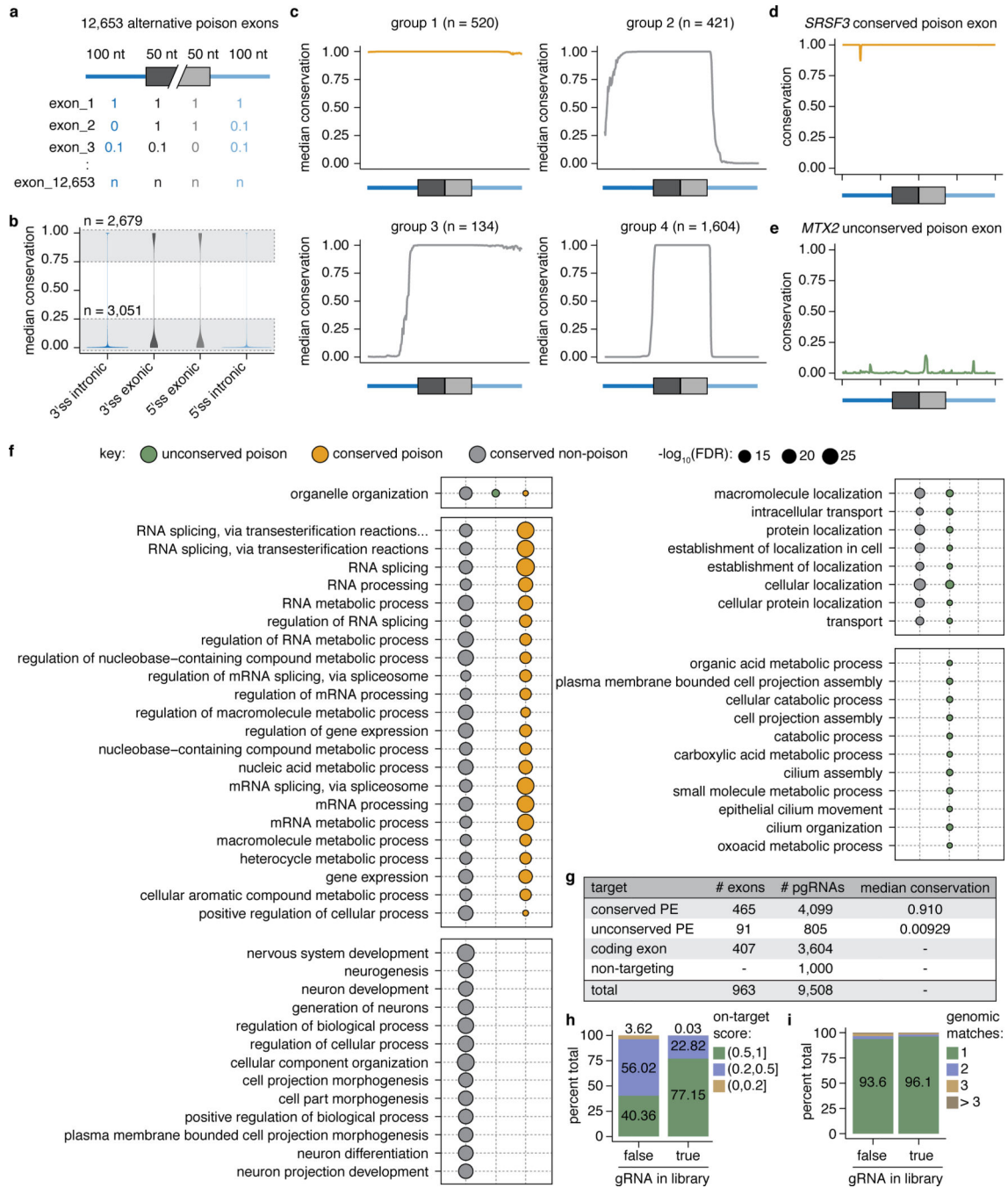
Extended Data Fig. 5. pgRNA library design.
(**a**) Regions used to classify each poison exon (n=12,653) according to its sequence conservation. (**b**) Median conservation scores for each indicated region (violin plot width represents probability density of data distribution). (**c**) Median per-nucleotide 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.
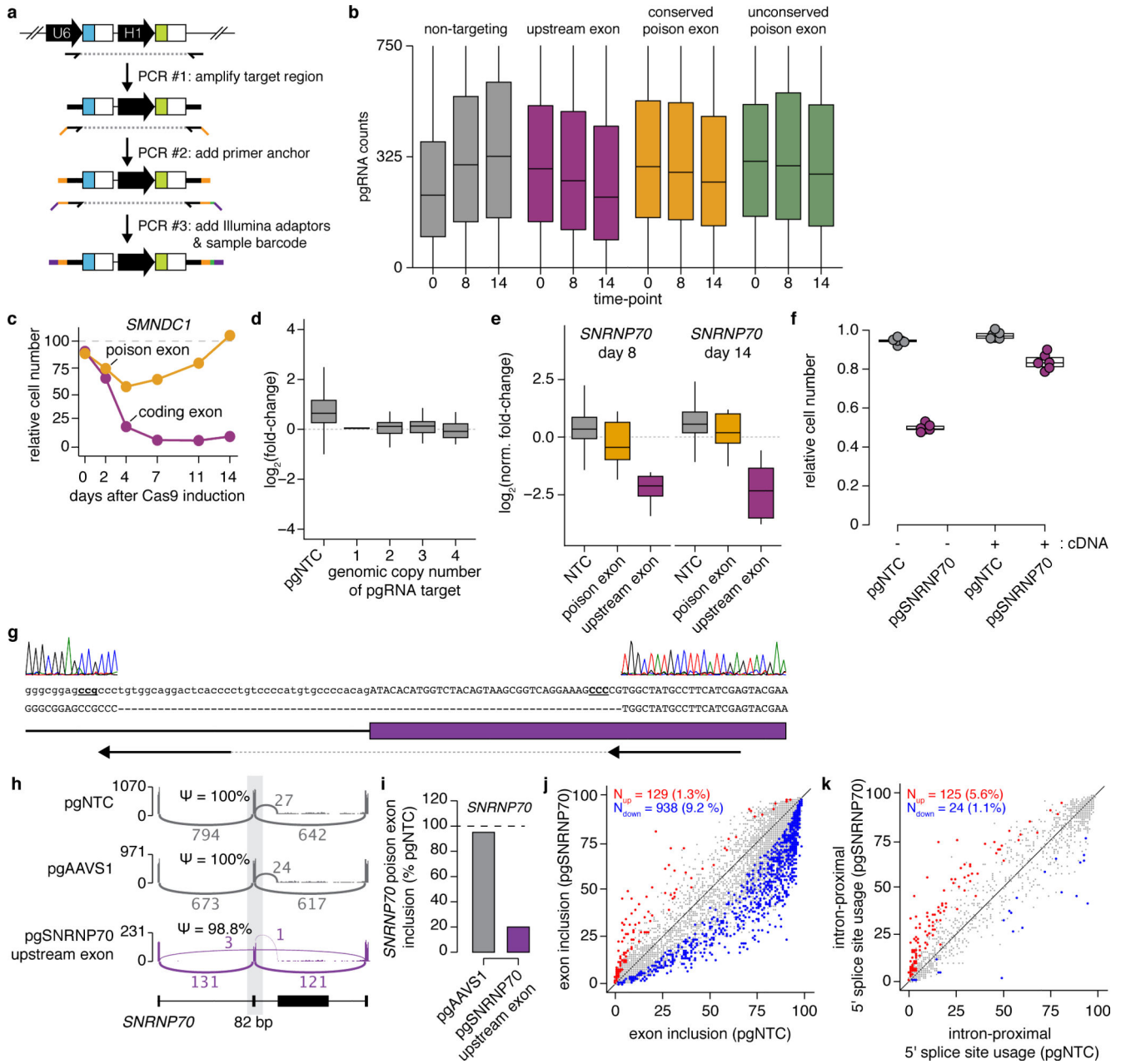
Extended Data Fig. 6. Analysis of pilot pgFARM screen.
(**a**) pgRNA library generation for Illumina sequencing. (**b**) pgRNA counts throughout the time course (n=1,000; 3,604; 4,099; 805 for groups, left to right). (**c**) Relative proliferation of HeLa/iCas9 cells expressing an *SMNDC1* upstream constitutive 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.
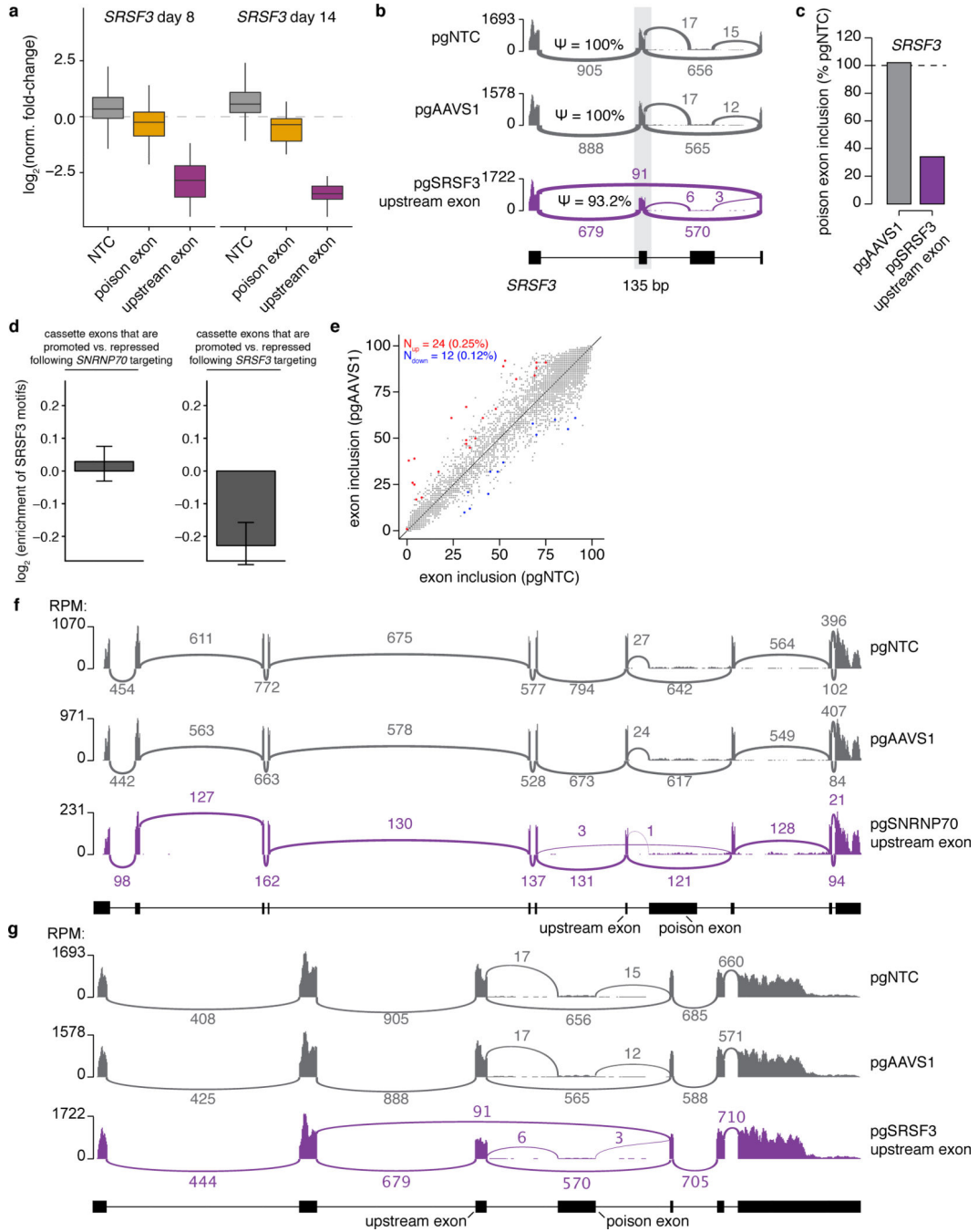
Extended Data Fig. 7. Analysis of pilot pgFARM screen, continued.
(**a**) Normalized pgRNA fold-changes (n=1,000 and 9 for non- and exon-targeting pgRNAs, respectively). The center line, hinges, and whiskers represent median, 25th and 75th percentiles, and most extreme datapoints within 1.5X interquartile 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* poison 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).
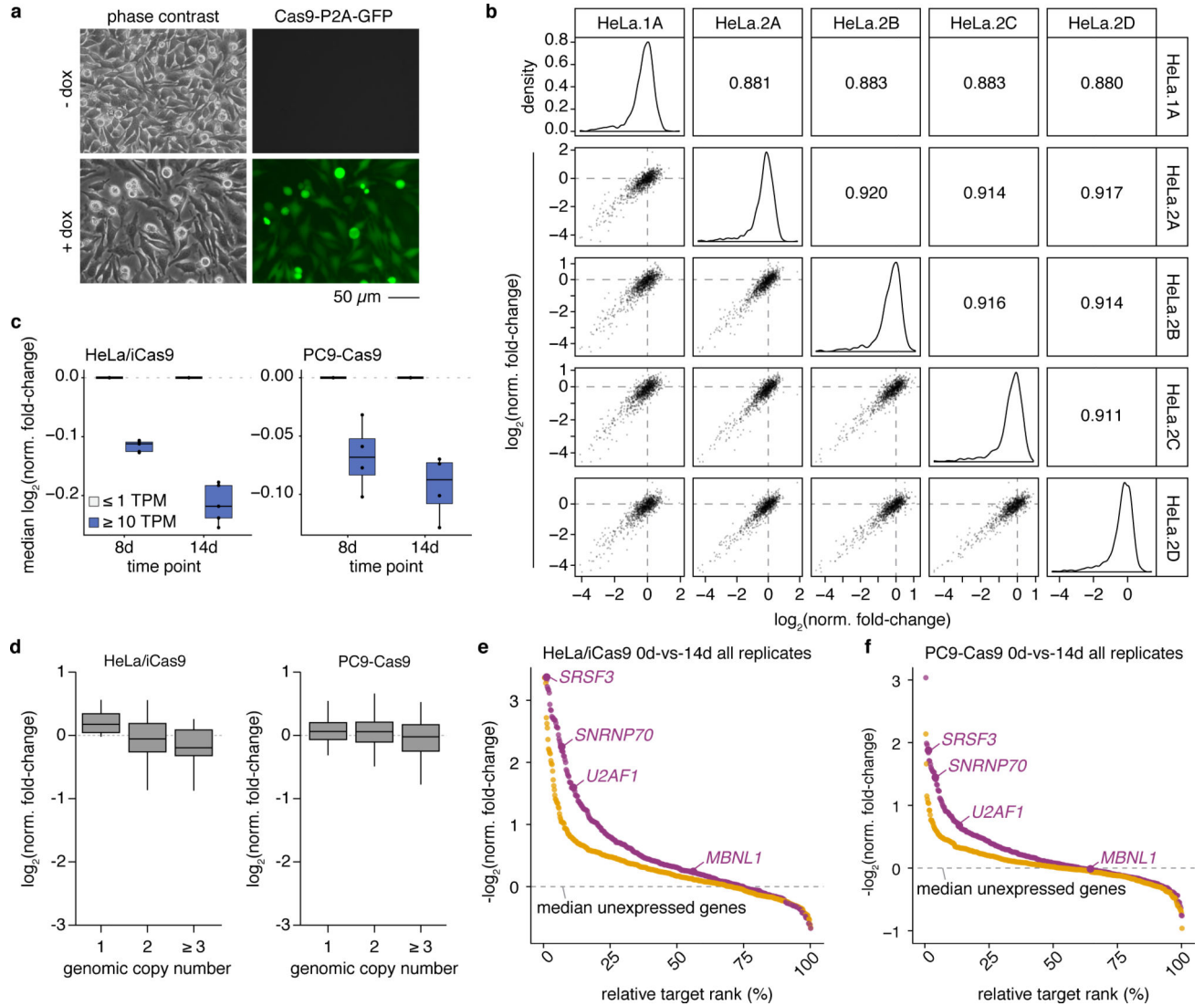
Extended Data Fig. 8. Analysis of large-scale pgFARM screens.
(**a**) HeLa/iCas9 cells (n=4 biological replicates) treated with the poison exon pgRNA library and grown in the presence (+ dox) or absence (- dox) of active Cas9. (**b**) Scatter plots comparing normalized fold-changes (day 14 vs. day 0; 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.

a

b

pgCPSF4
poison exon

c

pgSMG1
poison exon

d
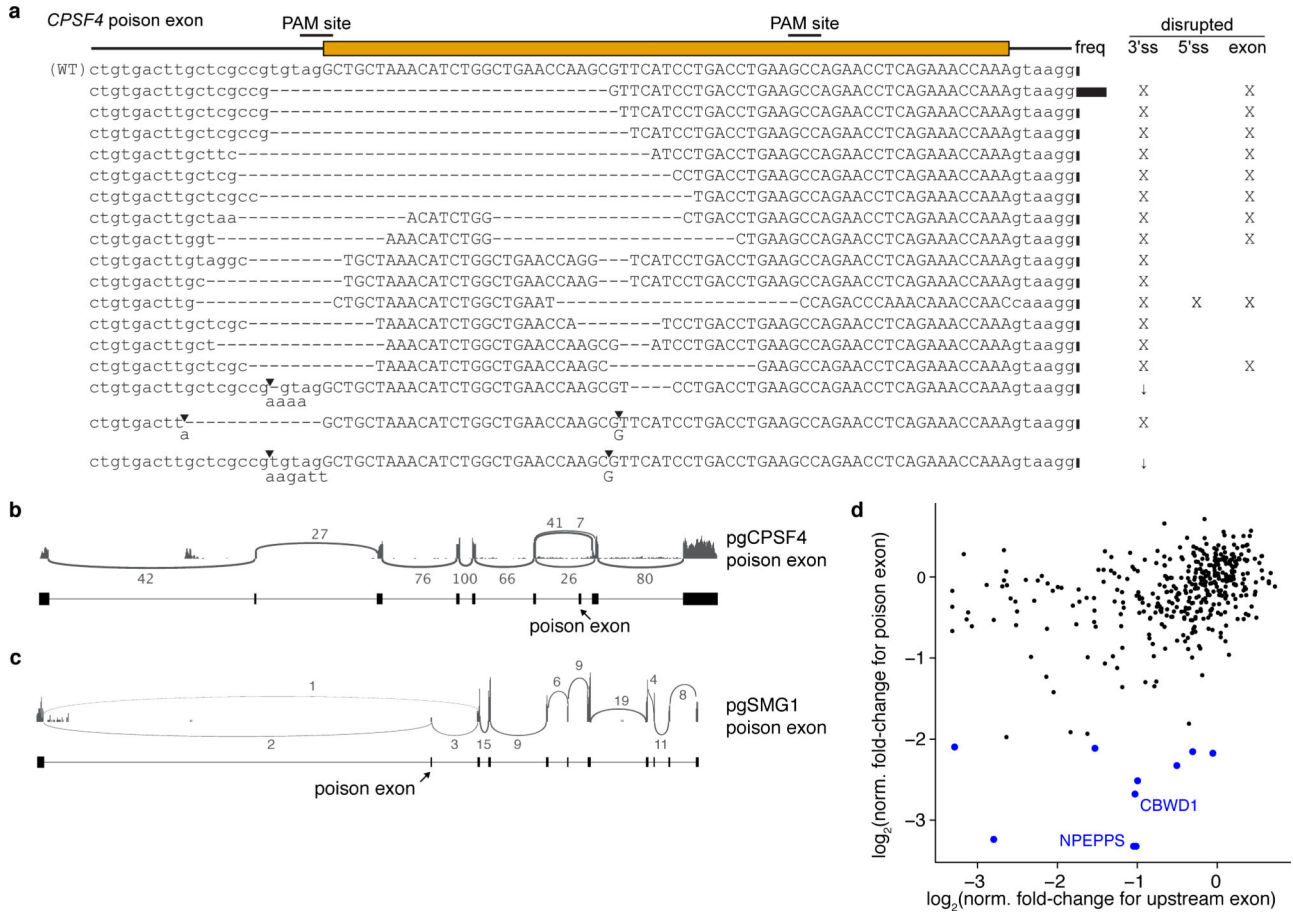
Extended Data Fig. 9. pgFARM-induced exclusion of *CPSF4* and *SMG1* poison exons.
(**a**) Sanger sequencing of pgFARM-edited *CPSF4* poison exon in HeLa/iCas9 cells. Annotations of eliminated (X) or disrupted (↓) sequence elements are indicated. (**b**) RNA-seq read coverage across the entire *CPSF4* 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.
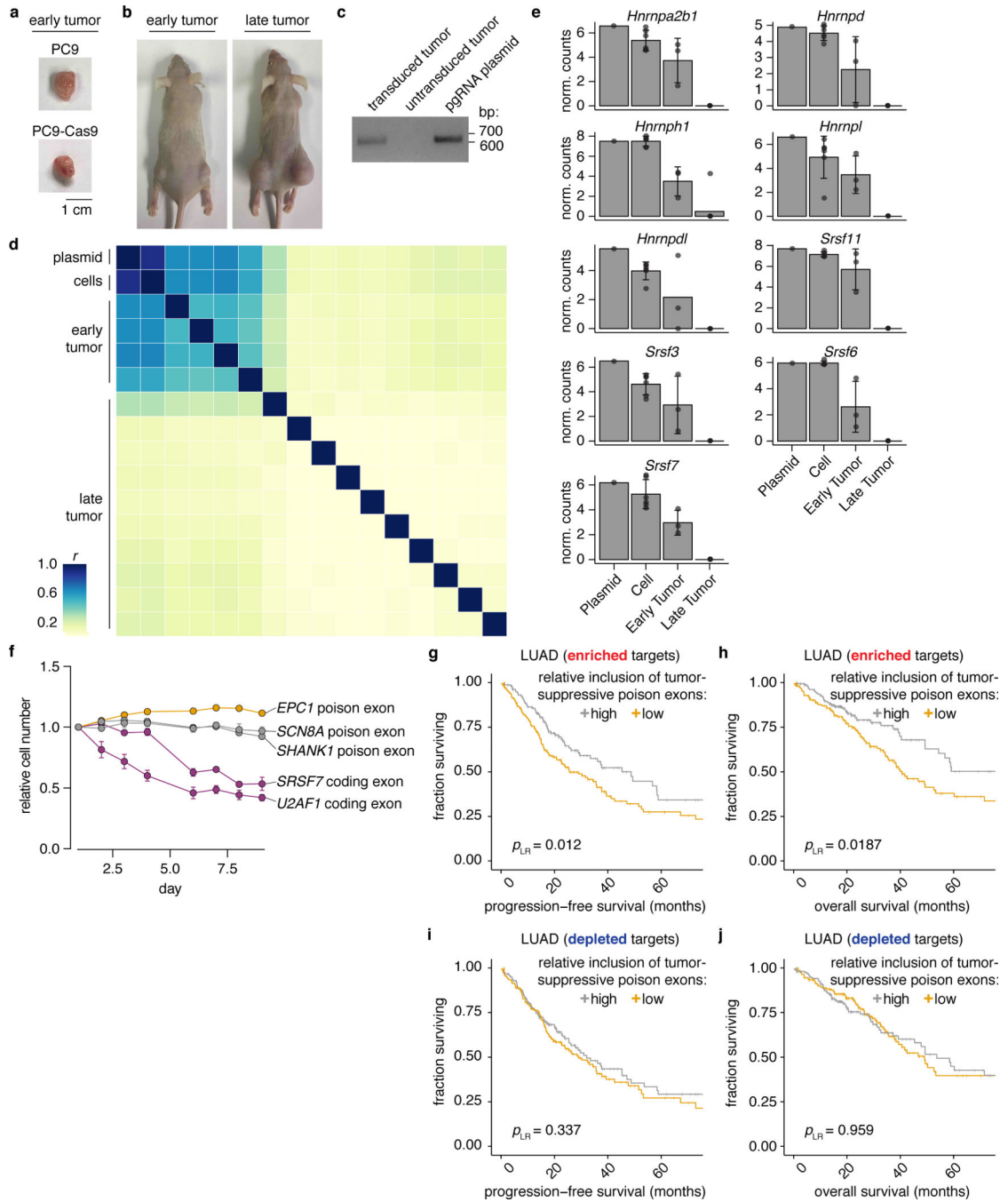
Extended Data Fig. 10. Analysis of xenograft screens.
(**a**) Tumors derived from parental PC9 or PC9-Cas9 cells (n=4 per group). (**b**) Mice from early and late tumor time points (n=4 and 10 tumors, respectively). (**c**) pgRNA Illumina libraries. (**d**) Pearson correlation (*r*) matrix for xenograft 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 (i), but for overall survival. See Source Data for uncropped gels.

Supplementary Material

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ACKNOWLEDGEMENTS

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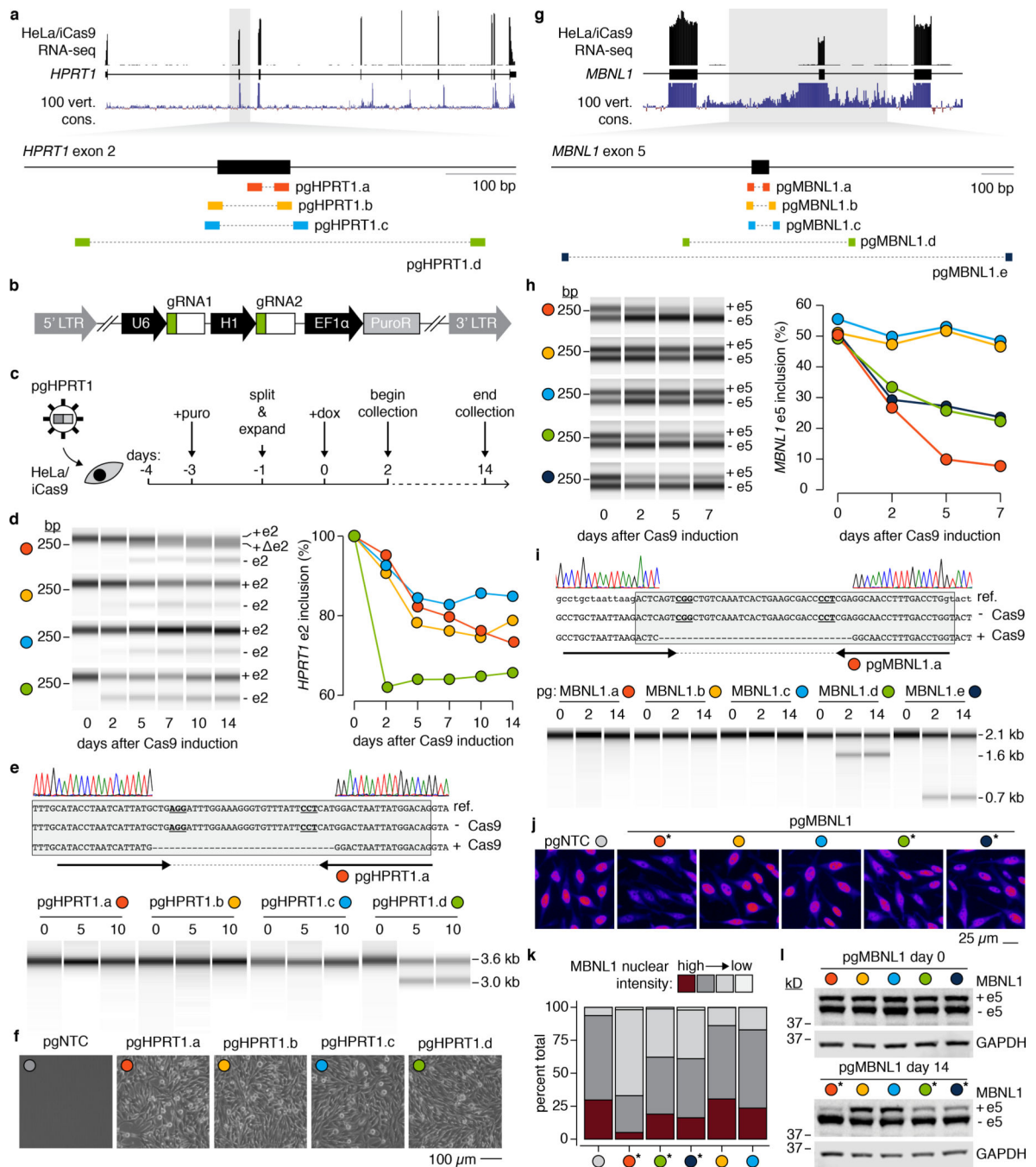
Figure 1. pgFARM facilitates rapid, programmable exon skipping.
a, Top, RNA-seq read coverage and sequence conservation across *HPRT1* in HeLa/iCas9 cells. Bottom, pgRNAs targeting *HPRT1* exon two. **b**, Schematic of pgRNA-expressing vector. **c**, Schematic of pgRNA delivery strategy. **d**, Left, RT-PCR analysis of *HPRT1* exon two (e2) inclusion. Right, RT-PCR quantification. **e**, Top, representative 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**, 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 n=3 independent experiments. **g**, As (a), but for *MBNL1* exon five. **h**, As (d), but for *MBNL1* exon five (e5) inclusion. **i**, As (e), but for *MBNL1* exon five. **j**, Immunofluorescence images comparing nuclear MBNL1 abundance (orange, high intensity; blue, low intensity) in HeLa/iCas9 cells expressing non-targeting or *MBNL1* exon five-targeting pgRNAs. * indicates pgRNAs that induced the greatest exon exclusion. **k**, Quantification of data in (j). **l**, Western blot for MBNL1 and GAPDH from HeLa/iCas9 cells expressing the indicated pgRNAs before (top) and after (bottom) Cas9 induction. Colors as in (j). Unless otherwise indicated, all data are representative results from n=2 independent experiments. See Source Data for uncropped gels.
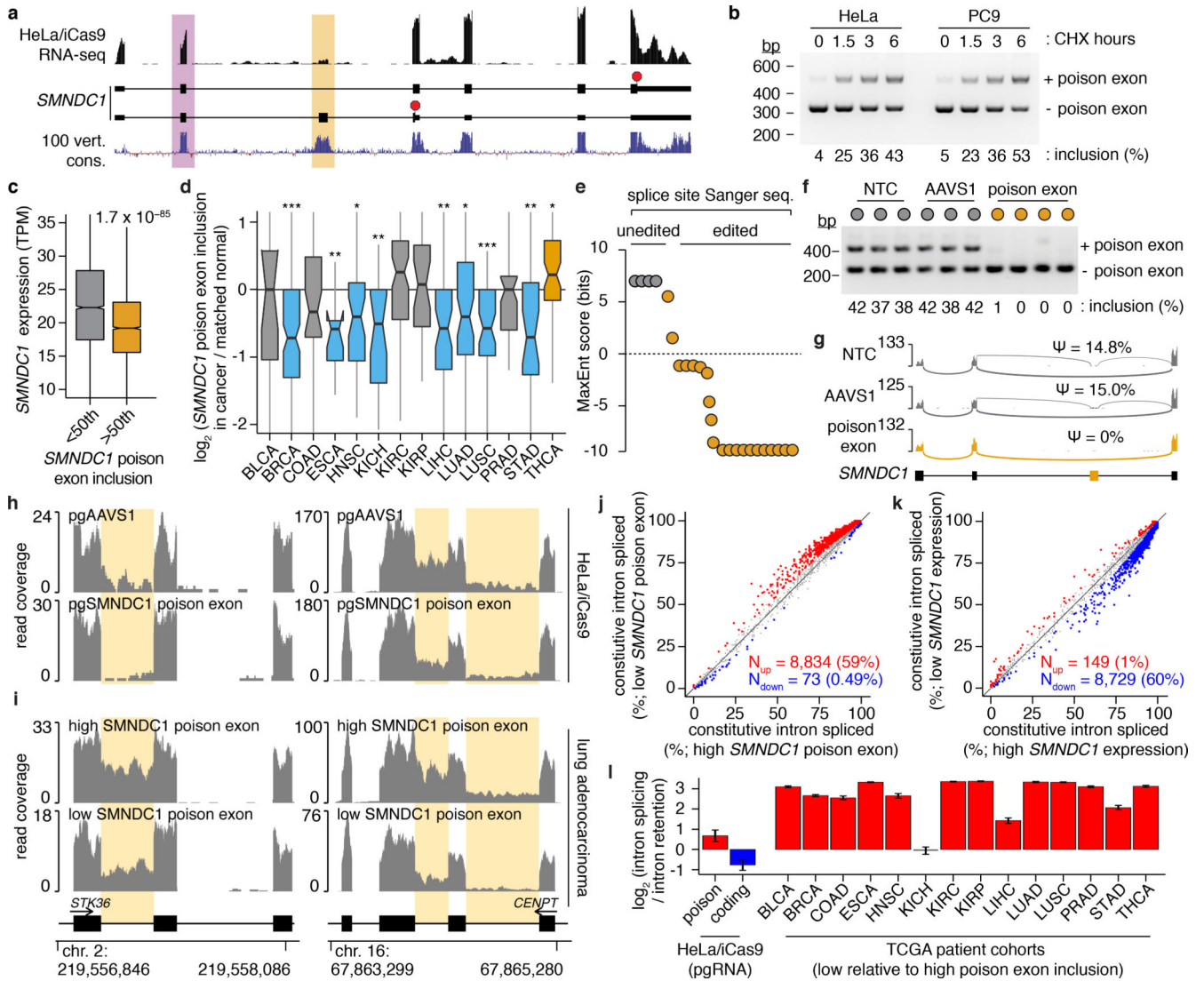
Figure 2. An *SMNDC1* poison exon modulates intron retention.

a, pgRNAs were designed to disrupt inclusion of an *SMNDC1* constitutive coding (purple) or poison exon (yellow). **b**, *SMNDC1* poison exon inclusion following cycloheximide (CHX) treatment to inhibit NMD. n=4 biologically independent time points. **c**, *SMNDC1* expression in cancers with *SMNDC1* poison exon inclusion greater (>50th) or lower (<50th) than the median. TPM, transcripts per million. *p* computed with two-sided Mann-Whitney *U* test. n=8,361 cancers. **d**, Relative *SMNDC1* poison exon inclusion in cancers versus patient-matched peritumoral normal samples. *p* computed with two-sided Mann-Whitney *U* test. *, p ≤ 5×10⁻²; **, p ≤ 5×10⁻³; ***, p ≤ 5×10⁻⁵. **e**, MaxEnt[31] 3' splice site scores for pgFARM-edited *SMNDC1* alleles. **f**, *SMNDC1* poison exon inclusion in CHX-treated PC9-Cas9 clones expressing control (NTC, AAVS1) or *SMNDC1* poison exon-targeting pgRNAs. n=10 biologically independent clones. **g**, RNA-seq coverage across the *SMNDC1* poison exon locus in HeLa/iCas9 cells treated with the indicated pgRNAs. n=1 per pgRNA. ψ, poison exon inclusion. **h**, RNA-seq coverage across representative differentially retained introns in HeLa/iCas9 cells treated with the indicated pgRNAs. n=1 per pgRNA. **i**, As (h),

but for lung adenocarcinoma samples with the highest or lowest *SMNDC1* poison exon inclusion. n=5 per group. **j**, Constitutive intron splicing in lung adenocarcinomas with low (bottom tercile) or high (top tercile) *SMNDC1* poison exon inclusion. Red/blue, significantly increased/decreased splicing. **k**, As (j), but samples stratified by *SMNDC1* expression. **l**, Constitutive intron splicing efficiency. Error bars, 5th/95th percentiles estimated by bootstrapping. Abbreviations, sample sizes, and box plot elements defined in Methods. See Source Data for uncropped gels.
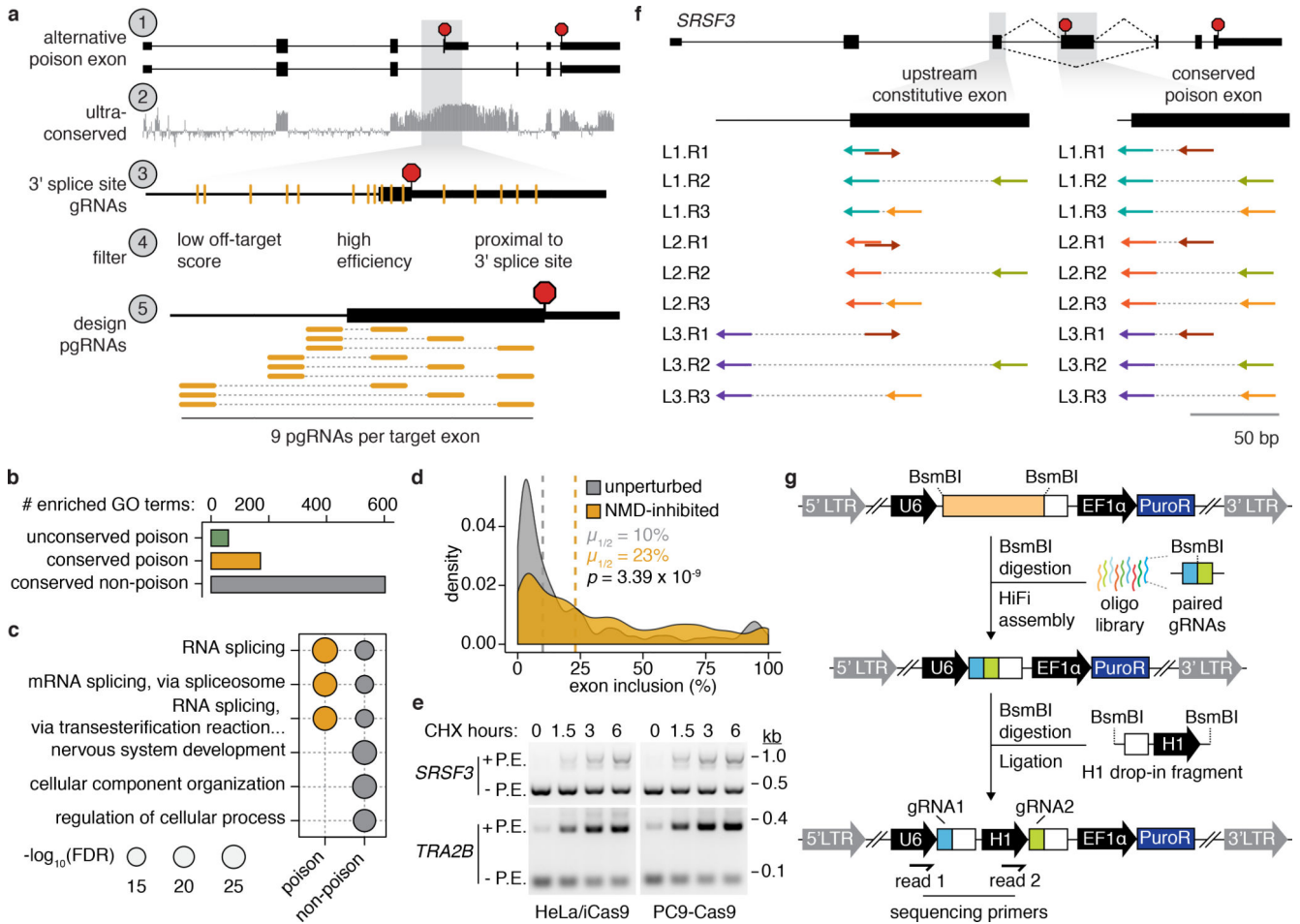
Figure 3. Design and construction of a poison exon loss-of-function library.
a, Schematic of selection criteria for poison exons targeted in this study as well as gRNA filtering criteria. **b**, Bar graph illustrating the numbers of significantly enriched (false discovery rate, FDR 0.01) biological processes associated with the genes containing each of indicated classes of alternative exons (n=2,363, 352, and 888 for unconserved poison, conserved poison, and conserved non-poison, respectively). Non-poison exons do not introduce premature termination codons. **c**, Bubble chart of FDRs for the three most-enriched biological processes that were associated with the sets of genes containing either highly conserved poison exons (left; n=352) or highly conserved non-poison exons (right; n=888). For (b) and (c), FDR computed using the Wallenius method and corrected using the Benjamini-Hochberg method. **d**, Histogram illustrating exon inclusion levels in unperturbed and NMD-inhibited HeLa cells[36] for conserved poison exons (n=337) targeted in our pgRNA library. p computed by the two-sided Mann-Whitney U test. **e**, Inclusion of representative poison exons (P.E.) from (d) following NMD-inhibition (Methods). Representative image from n=2 independent experiments. **f**, Illustration of pgRNA targeting strategy for exemplary 3' splice sites of an ultraconserved poison exon and corresponding upstream constitutive exon in *SRSF3*. **g**, Schematic of the pgRNA library cloning strategy. See Source Data for uncropped gels.
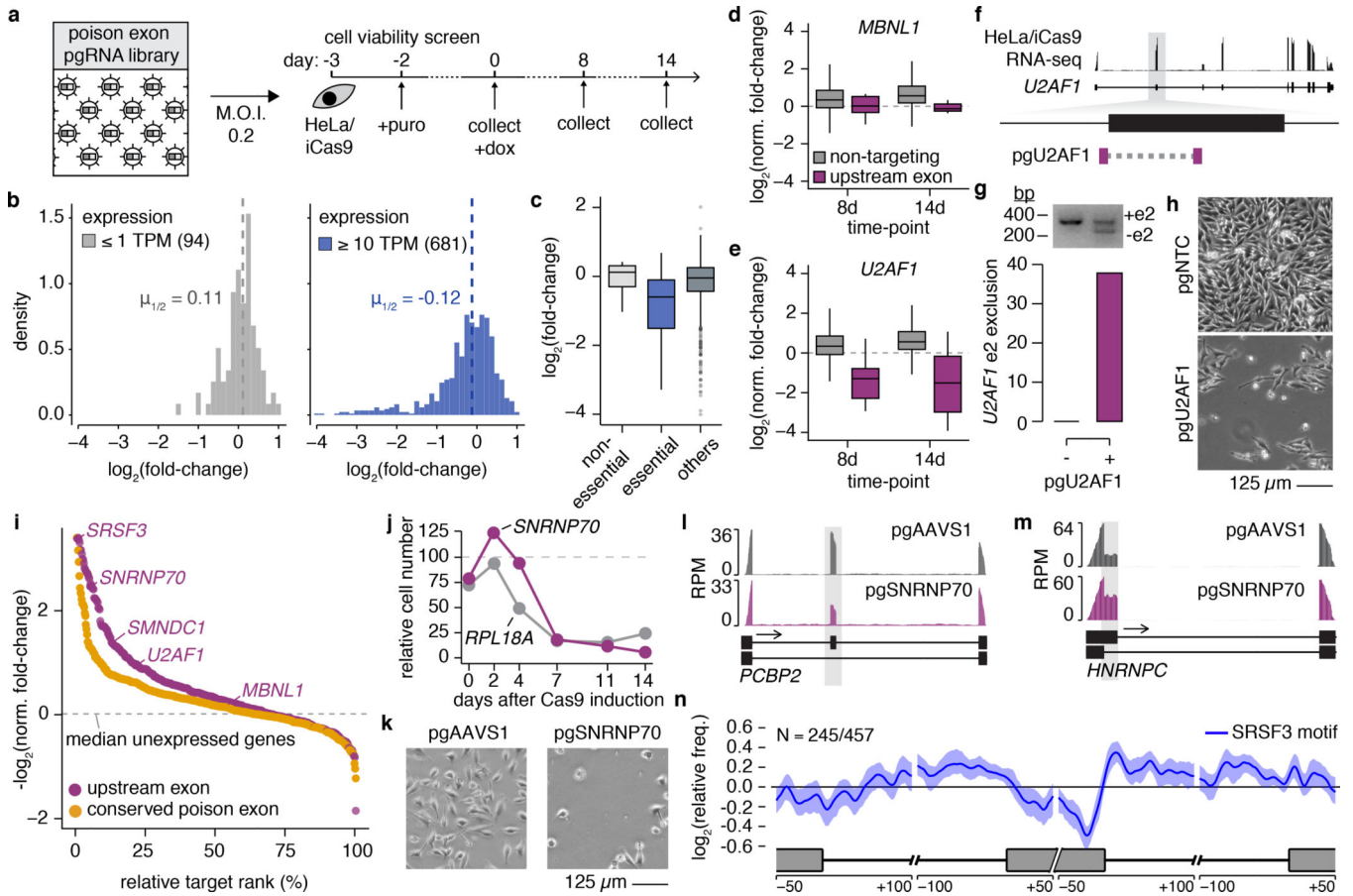
Figure 4. Unbiased detection of essential exons with pgFARM.
a, Schematic of dropout screen. **b**, Histogram illustrating unnormalized fold-changes associated with each targeted exon in unexpressed (left) or expressed (right) genes. TPM, transcripts per million. **c**, Unnormalized fold-changes associated with targeted exons in "core essential" (n=51), "core non-essential" (n=12)[37], or other genes (n=900). **d**, Normalized fold-changes for non-targeting (gray; n=1,000) and *MBNL1* constitutive upstream exon-targeting (purple; n=9) pgRNAs. **e**, As (d), but for a *U2AF1* constitutive exon (purple; n=9 pgRNAs). **f**, Schematic of *U2AF1* exon two-targeting pgRNA. **g**, *U2AF1* exon two (e2) exclusion in cells treated with pgRNA from (f). n=1 independent experiment. **h**, Representative phase contrast images of HeLa/iCas9 cells expressing the indicated pgRNAs. n=3 independent experiments. **i**, Rank plot of normalized fold-changes for conserved poison and upstream constitutive exons. *SRSF3*, *SNRNP70*, *SMNDC1*, and *U2AF1* are essential genes; *MBNL1* is not. **j**, Viability of HeLa/iCas9 cells expressing the indicated pgRNAs relative to an AAVS1-targeting pgRNA. *RPL18A* is an essential gene. n=3 biologically independent experiments. **k**, Representative phase contrast images from (j). **l**, RNA-seq coverage illustrating differential cassette exon inclusion following treatment with an *SNRNP70* constitutive exon-targeting pgRNA. RPM, reads per million. **m**, As (l), but illustrating differential 5' splice site usage. **n**, Metagene plot illustrating relative *SRSF3* binding motif[44] occurrence in cassette exons exhibiting increased (n=245) versus decreased (n=457) inclusion following treatment with an *SRSF3* constitutive exon-targeting pgRNA.

Exons exhibiting increased/decreased inclusion were depleted/enriched for the motif. Shading, 95% confidence interval. Box plot elements defined in Methods. See Source Data for uncropped gels.
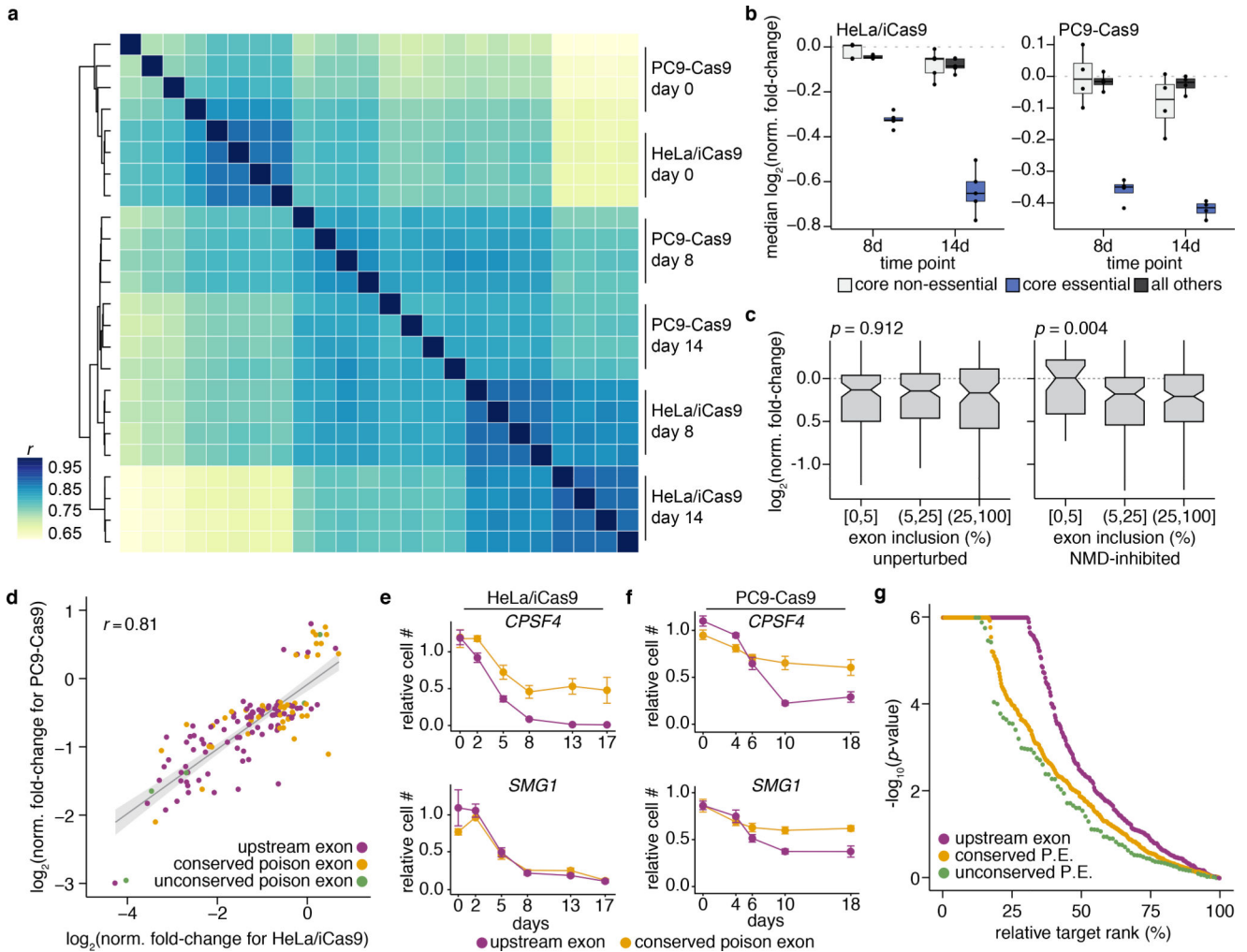
Figure 5. Many conserved poison exons are essential for cell fitness.
a, Heat map illustrating Pearson correlations between raw counts supporting each pgRNA for all samples. Dendrogram, unsupervised clustering of raw counts by complete-linkage method. n=9,508 pgRNAs per sample. **b**, Normalized fold-changes for targeted exons within "core essential", "core non-essential"[37], or all other genes. Each point illustrates median over targeted exons within indicated gene sets for a single screen replicate of the screen. n=5 and 4 screens for HeLa/iCas9 and PC9-Cas9 cells. **c**, Normalized fold-changes for targeted poison exons, stratified based on their inclusion in unperturbed or NMD-inhibited HeLa cells[36]. NMD inhibition decouples splicing and transcript degradation. *p* computed by two-sided Mann-Whitney *U* test. n=154/91/31 (left) and 44/103/129 (right). **d**, Scatter plot comparing normalized fold-changes for exons in HeLa/iCas9 versus PC9-Cas9 cells. Because of the reduced dynamic range of the PC9 screen, plot restricted to exons with absolute log fold-change   1.25 and FDR   0.01 in PC9 cells and within genes with expression   10 TPM in both cell lines. *r*, Pearson correlation. n=86, 46, and 5 for upstream, conserved poison, and unconserved poison exons, respectively. **e**, Relative proliferation of HeLa/iCas9 cells treated with the indicated pgRNAs relative to cells treated with control (non-essential gene *CSPG4*-targeting) pgRNAs. Data presented as mean ± S.D. n=3

biologically independent experiments. **f**, As (e), but for PC9-Cas9 cells. **g**, Rank plot of p-values for each targeted exon in HeLa/iCas9 screen. P.E., poison exon. Box plot elements defined in Methods.
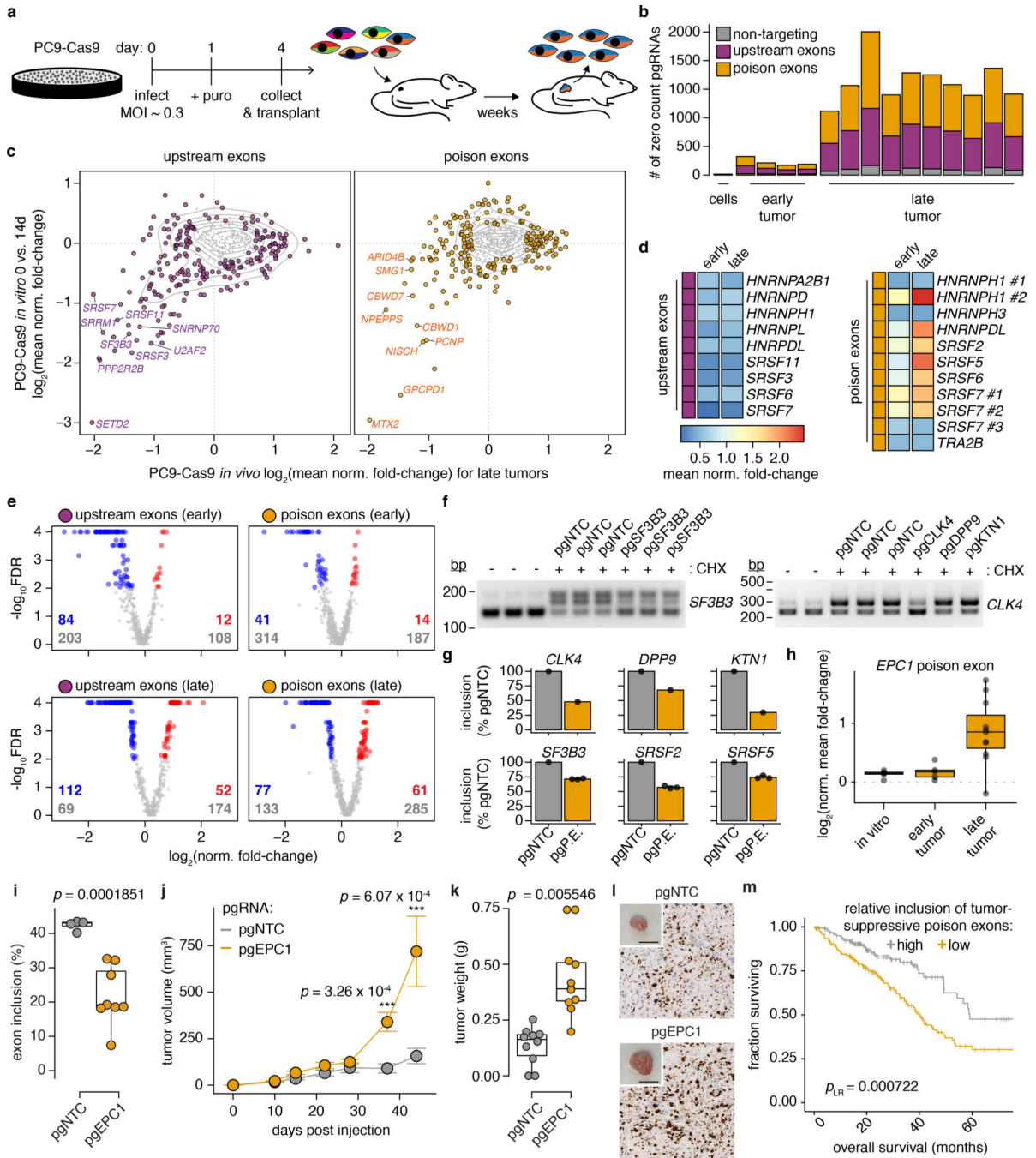
Figure 6. pgFARM uncovers modifiers of *in vivo* tumorigenesis.
a, Schematic of screens. **b**, Numbers of pgRNAs with zero counts. **c**, Normalized fold-changes for exons measured *in vivo* and *in vitro*. **d**, Normalized fold-changes for exons in SR and hnRNP genes. *HNRNPH1* and *SRSF7* contain multiple poison exons; *SRSF7* has a poison exon with competing 3' splice sites. **e**, 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. pgP.E., pgRNA targeting the indicated

poison exon. Data presented as mean ± S.D. **h**, Normalized fold-changes for the *EPC1* poison exon. **i**, *EPC1* poison exon inclusion in PC9-Cas9 clones expressing the indicated pgRNAs. *p* computed with two-sided Student's *t*-test. **j**, Tumor volumes for xenografts established from PC9-Cas9 cells expressing the indicated pgRNAs (n=10 per group). Data presented as mean ± S.E. *p* computed with two-sided Mann-Whitney *U* test. **k**, Tumor weights at endpoint. *p* computed with two-sided Student's *t*-test. **l**, Representative Ki-67 immunohistochemistry images (n=17 total histological analyses; for dissected tumor images, scale bar = 1 cm). **m**, Survival of lung adenocarcinoma patients stratified by inclusion of tumor-suppressive poison exons. *p* computed with two-sided logrank test. Sample sizes and box plot elements defined in Methods.