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MOLECULAR BIOLOGY

Principles of CRISPR-Cas13 mismatch intolerance enable selective silencing of point-mutated oncogenic RNA with single-base precision

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Single-nucleotide variants (SNVs) are extremely prevalent in human cancers, although most of these remain clinically unactionable. The programmable RNA nuclease CRISPR-Cas13 has been deployed to specifically target oncogenic RNAs. However, silencing oncogenic SNVs with single-base precision remains extremely challenging due to the intrinsic mismatch tolerance of Cas13. Here, we show that introducing synthetic mismatches at precise positions of the spacer sequence enables de novo design of guide RNAs [CRISPR RNAs (crRNAs)] with strong preferential silencing of point-mutated transcripts. We applied these design principles to effectively silence the oncogenic *KRAS* G12 hotspot, *NRAS* G12D and *BRAF* V600E transcripts with minimal off-target silencing of the wild-type transcripts, underscoring the adaptability of this platform to silence various SNVs. Unexpectedly, the SNV-selective crRNAs harboring mismatched nucleotides reduce the promiscuous collateral activity of the *RfxCas13d* ortholog. These findings demonstrate that the CRISPR-Cas13 system can be reprogrammed to target mutant transcripts with single-base precision, showcasing the tremendous potential of this tool in personalized transcriptome editing.

INTRODUCTION

Comprehensive molecular profiling initiatives have revealed the vast landscape of single-nucleotide variants (SNVs) in adult (1) and pediatric cancers (2). On average, adult tumors harbor 2.6 driver SNVs (1), which are often enriched in proliferative and pro-survival pathways such as the mitogen-activated protein kinase (MAPK) pathway. Somatic MAPK aberrations most commonly occur in the proto-oncogenes *KRAS* (Kirsten rat sarcoma viral oncogene homolog) or *BRAF* (v-raf murine sarcoma viral oncogene homolog B1), which collectively drive malignancy in approximately one-third of all human cancers (1). *KRAS* mutations are extremely pervasive, present in over 90% of pancreatic cancers, ~40% of colorectal cancers, and ~30% of lung cancers (3). Virtually, all (>99%) *KRAS* mutations are missense substitutions (4), with the most frequent *KRAS* SNVs occurring at the codon 12-encoded glycine residue (4).

Tumor-associated SNVs are considered an attractive therapeutic target due to their high prevalence and restricted expression in transformed cells. Several small molecule inhibitors targeting oncogenic SNVs have been clinically approved, including a *KRAS* G12C inhibitor (sotorasib). This agent exploits a unique binding pocket generated in the mutated protein, enabling its G12C selectivity (5, 6). However, exclusive targeting of other SNVs remains a pharmacological challenge as these mutated proteins differ from their wild-type (WT) counterparts by only one amino acid, rarely conferring

sufficient structural dissimilarity for SNV-selective targeting. Most *KRAS* aberrations (such as -G12R, -G13D, -Q61H, etc.) lack any accessible binding pockets and are therefore considered clinically unactionable (5). As only ~10% of *KRAS* mutations are of the G12C genotype (7), therapeutic targeting of non-G12C variants remains an unmet clinical challenge. Although a recent landmark study has developed a preclinical, pan-*KRAS* inhibitor, which suppresses multiple *KRAS* variants by nonselectively trapping the protein in its inactive state (8), this drug also suppresses WT *KRAS* and may therefore exhibit toxicity in normal tissues.

Unfortunately, the reported number of deleterious, tumor-associated SNVs far outweighs the number of clinically approved inhibitors. The development of traditional small molecule inhibitors that target proteins requires structure-based design and high-throughput screens, a process that is expensive, labor-intensive, and of low yield. In contrast, innovative sequence-based therapeutics that target oncogenic drivers at their DNA or RNA level can be developed and personalized for each patient's specific mutational profile with rapid manufacturing and minimum cost. In addition, in bypassing any three-dimensional structural constraints, sequence-based therapeutics targeting aberrant nucleic acid molecules might provide an alternative modality for targeting SNVs that generate proteins that lack pharmacophoric vulnerabilities.

CRISPR-Cas9 molecular tools have revolutionized our ability to perform targeted genome editing. Elegant studies have exploited the observation that certain SNVs, such as *EGFR*-L858R, generate nascent 5'-NGG-3' protospacer adjacent motif (PAM) sequences recognizable by SpCas9, thus enabling the selective cleavage of the mutated oncogene while leaving the PAM-free WT sequence intact (9). However, several reports have linked the on-target and off-target nuclease activity of CRISPR-Cas9 to megabase-scale chromosomal truncations, rearrangements, and even chromothripsis at both the target and distal sites (10–13), as well as significantly increased rates of off-target Insertion/Deletion (INDEL) generation (14). Cas9 genome editing has also been shown to positively select for cells with

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defective p53-mediated DNA damage responses and may thus lead to the expansion of *TP53*-mutated clones from a heterogeneous cancer cell population (15, 16). Thus, the risks associated with such permanent, off-target genomic alterations may limit the therapeutic use of current CRISPR-Cas9 technologies in clinical settings.

Conversely, CRISPR-Cas13 is an RNA-guided RNA-targeting nuclease that enables precise and efficient cleavage of single-stranded RNA without altering genomic DNA (17). Previous studies have demonstrated that CRISPR-Cas13 can be successfully reprogrammed to silence various pathogenic RNAs including SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) variants of concern (18, 19) and to alleviate neurodegenerative symptoms in mouse models of Huntington's disease (20). Cas13 enzymes typically require base pairing with a 23- to 30-nucleotide (nt)-long recognition motif to activate its higher eukaryotes and prokaryotes nucleotide (HEPN) domains and cleave the RNA target. This highlights the high specificity of Cas13 enzymes compared to classical eukaryotic RNA interference (RNAi), where only a 7- to 8-nt-long seed region dictates target recognition and degradation (21). As such, traditional RNAi is highly permissive to off-target binding, with some estimates indicating that over one-third of all human transcripts could harbor one or several small interfering RNA binding sites (22). Conversely, the greater specificity of Cas13 renders it a promising tool for targeting oncogenic transcripts. We and others have reprogrammed Cas13 to silence oncogenic fusion transcripts by targeting their tumor-specific breakpoint sequence (23, 24). However, single-base precision silencing of SNVs with Cas13 remains highly challenging due to the well-known mismatch tolerance capability of various Cas13 enzymes (18, 25–27). Thus, systematic and precise silencing of SNVs may only be achieved once we understand the molecular principles behind Cas13 mismatch tolerance and intolerance at the single-base level.

Here, we present an experimental pipeline to design and validate Cas13-compatible CRISPR RNAs (crRNAs) capable of SNV-specific transcript obliteration. By using an extensive analysis of target-spacer interactions at the level of individual nucleotides, we developed a suite of crRNAs with a remarkable ability to selectively silence point-mutant *KRAS* transcripts. Our most effective crRNAs demonstrated marked preferential silencing of various oncogenic *KRAS* mutant transcripts, displaying dose-dependent and SNV-selective silencing. Furthermore, we successfully applied these design principles to silence oncogenic *NRAS* *G12D* and *BRAF* *V600E* transcripts, demonstrating the adaptability of this approach for a diverse range of SNV targets. These findings highlight the versatility of our Cas13 design principles to precisely target mutant transcripts with single-base precision.

RESULTS

Selective cleavage of *KRAS* *G12C* and *G12D* mutant transcripts via precise spacer mutagenesis

We questioned whether Cas13 can efficiently silence mutant *KRAS* transcripts while sparing the WT variant. Among various Cas13 orthologs, we focused on *Ruminococcus flavefaciens* Cas13d (*RfxCas13d*) that has been shown to have high silencing activity (28). To assess the RNA cleavage activity and specificity of *RfxCas13d* and its crRNAs against mutants and WT *KRAS*, we used a reporter assay that enables accurate monitoring of Cas13-mediated mRNA cleavage via loss of fluorescence signal in human embryonic kidney (HEK) 293T cells (Fig. 1A; Materials and Methods) (18).

As *G12D* is the most common *KRAS* SNV (4), we first designed a parental crRNA (cr*KRAS*), which base pairs with the *G12D* transcript with 100% sequence complementarity (termed a “perfect-match” crRNA). Here, the spacer’s “U” nucleotide that base pairs with the “A” nucleotide substitution found in *G12D*-mutated *KRAS* mRNA is located at crRNA position 10 (Fig. 1B, top). Although cr*KRAS* exhibited incredibly potent mRNA cleavage relative to the nontargeting control (crNT), it efficiently silenced both the WT *KRAS* and *G12D* transcripts with equal efficiency (Fig. 1B, bottom), indicating that *RfxCas13d* has an intrinsic single-nucleotide mismatch tolerance that disables the discrimination between WT and SNV tumor transcripts.

Given that the position and number of nucleotide mismatches in crRNA spacer sequences can modulate silencing efficiency (23, 29), we sought to determine the optimal mismatch tolerance threshold that would confer preferential silencing of the *G12* mutant transcript. As any additional perturbations to the crRNA sequence would be made in addition to the original SNV substitution, the number of mismatches in the WT sequence is always $n + 1$, where n is the number of mismatches in the spacer sequence when using the SNV target as a template (Fig. 1A). We first screened a series of *RfxCas13d*-compatible single-mismatch guides for selective silencing of *KRAS* *G12D* relative to *KRAS* WT. Here, the position of the mismatch in each guide was shifted by one nucleotide, allowing the assessment of how each position of the 23-nt spacer sequence contributes to selective silencing activity. However, 22/23 mutagenesis derivatives achieved equipotent, efficient silencing of both variants (fig. S1A), indicating that *RfxCas13d* can intrinsically tolerate single-nucleotide mismatches (MM). Although the crRNA with a mismatch at position 23 showed strongly selective silencing of *KRAS* *G12D*, indicating that this position may be important for achieving SNV selectivity, the off-target silencing of *KRAS* WT was ~50% (fig. S1A).

Thus, to maximize our ability to discriminate between WT and mutant *KRAS* transcripts, we performed systematic mutagenesis of this crRNA by introducing two to three additional synthetic mismatches along the spacer sequence, prioritizing mismatches at nucleotide position 23 (Fig. 1C). When choosing the position of the other synthetic mismatches in this crRNA series, we strategically noted that the *KRAS* *G12D* and *G12C* variants occur at adjacent nucleotides in the *KRAS* mRNA sequence (at positions c.34 and c.35, respectively; fig. S1B). We leveraged this observation by ensuring that all mutagenesis derivatives retained the “U” nucleotide (corresponding to the *G12D* mutation) at crRNA position 10 but additionally incorporated an “A” nucleotide (matching *G12C* SNV) at crRNA position 11. Thus, by conserving this “D₁₀C₁₁” motif across all guides, we anticipated that our engineered crRNAs would now be able to silence both *G12D* and *G12C*.

When screened against the *G12D* variant, all triple-mismatch guides inefficiently silenced both WT and *G12D* (Fig. 1C, top), indicating that three mismatches in the crRNA may be the upper limit for efficient *RfxCas13d*-mediated *KRAS* silencing. Conversely, certain double-mismatch configurations conferred preferential silencing activity against the *G12D* variant (Fig. 1C, top). These SNV-selective guides contained synthetic mismatches at crRNA positions 9 (i.e., crD₁₀C₁₁-MM₉) and 12 (i.e., crD₁₀C₁₁-MM₁₂). Consistent with our *G12D* results, most triple-mismatch guides also showed poor on-target silencing against the *G12C* variant (Fig. 1C, bottom). Unlike *G12D*, crD₁₀C₁₁-MM₂₃ exhibited SNV-selective silencing of *G12C*,

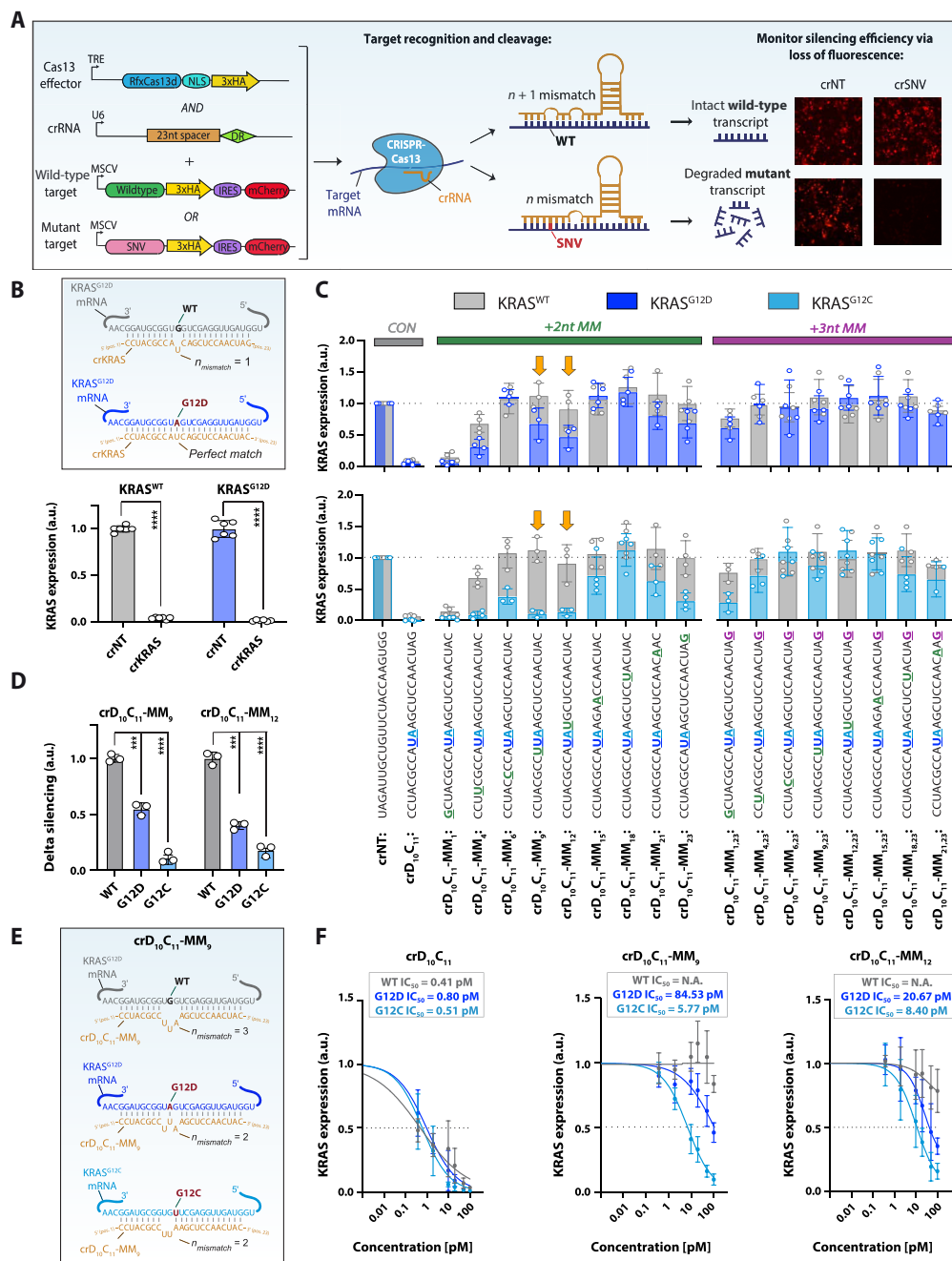


Fig. 1. Selective cleavage of KRAS G12C and G12D mutant transcripts is achieved via precise crRNA mutagenesis. (A) Schematic of the RfxCas13d reporter assay used to assess silencing efficiency of WT and mutant tumor transcripts. (B) Schematic depicting the sequence and base-pairing configuration of perfect-match crKRAS with WT KRAS versus G12D-mutant mRNA targets (top). The silencing efficiency of crKRAS at 48 hours after knock-in of WT (gray) or G12D-mutant (blue) KRAS, normalized against a nontargeting control crRNA (crNT; bottom). a.u., arbitrary units. (C) Systematic mutagenesis of nonselective cr_{10D11} was used to engineer G12D-selective and G12C-selective crRNAs. Spacer sequences are shown below the bar plot: The "U" nucleotide (dark blue) indicates the uracil in the spacer sequence that base pairs with the G12D target, and the "A" nucleotide (light blue) indicates the adenine in the spacer sequence that base pairs with the G12C target (neither of which will base pair with WT target); the colored nucleotides show the position of additional mismatches introduced at various spacer locations through mutagenesis. Bar plot shows the silencing efficiency of cr_{10D11} and its derivatives against KRAS-WT (gray) versus KRAS-G12D (dark blue; top) or KRAS-G12C (light blue; bottom), normalized against a nontargeting control (crNT), at 48 hours after transfection. (D) Delta silencing efficiencies between WT (gray), G12D (dark blue), and G12C (light blue) KRAS variants for the top-performing crRNAs at 48 hours after knock-in. (E) Schematic depicting the sequence and base-pairing configuration of SNV-selective cr_{10D11}-MM₉ with WT versus G12D-mutant and G12C-mutant KRAS mRNA targets. (F) Dose-response curves of parental cr_{10D11}, cr_{10D11}-MM₉, and cr_{10D11}-MM₁₂ against KRAS WT (gray), KRAS-G12D (dark blue), and KRAS-G12C (light blue). All experiments were performed in HEK293T overexpression models. Individual data points show normalized fluorescence intensity averaged from four representative fields of view ($n = 3$ independent experiments), and error bars show means $\pm$ SD. Statistical significance was determined using unpaired t tests, where *** $P < 0.001$ and **** $P < 0.0001$. N.A., not applicable.

aligning with the results of our single-mismatch screen (Fig. 1C and fig. S1A). Unexpectedly, the SNV selectivity of crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂ was strongly enhanced when targeting *G12C* (Fig. 1C, bottom, and fig. S1C), indicating that the specific nucleotide that is mutated in each *G12* variant (i.e., G>A for *G12D* versus G>T for *G12C*) influences the silencing efficiency of individual crRNAs. When normalized against the *WT* variant, these two top-performing guides showed significantly selective silencing of *G12D* (mean silencing: 54.8 and 39.9%, respectively) and *G12C* (mean silencing: 11.0 and 17.9%, respectively) (Fig. 1D). This differential silencing activity is likely attributable to three mismatches with *WT KRAS*—exceeding the upper limit of mismatches that allow for efficient silencing—but only two mismatches with the *G12D* and *G12C* variants (Fig. 1E and fig. S1D). Titration of these engineered crRNAs confirmed that this preferential silencing of *G12C* and *G12D* mutant transcripts was crRNA dose-dependent, with the median inhibitory concentration (IC₅₀) estimated between 5 to 84 and 8 to 21 pM for crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂, respectively. These SNV-selective crRNAs exhibited limited activity against *WT KRAS* at even the highest concentration of crRNA tested (IC₅₀'s nonconverged; Fig. 1F). This highlights the tunability of this targeting strategy as the optimal concentration of crRNA required to maximize silencing of the SNV transcript while also retaining minimal off-target silencing of the *WT* transcript can be easily identified via titration. Collectively, these results indicated that directed mutagenesis of crRNA spacer sequences can unlock SNV selectivity.

Exploiting mismatch tolerance enables selective targeting of all *KRAS G12* hotspot mutations

Having confirmed the *G12* selectivity of the crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂ guides, we next questioned whether they would display cross-reactive silencing activity against the other c.34 and c.35 mutant *KRAS* variants (Fig. 2A). Beyond clinical relevance of Pan-RAS inhibition, targeting the full suite of *G12* mutants also offers a unique opportunity to validate the relative importance of (i) the type of nucleotide substitution (i.e., G>A versus G>T) and (ii) the position of the SNV in the mRNA sequence (i.e., c.34 versus c.35) for efficient RfxCas13d-mediated silencing. We therefore generated a library of *KRAS G12* reporter plasmids encoding each of the remaining *G12* hotspot mutations (fig. S2A) and assessed their sensitivity to crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂. In contrast with the high on-target activity observed against *G12C* and *G12D* mutant transcripts, these crRNAs did not show efficient silencing of the other c.34 (i.e., *G12R* and *G12S*) and c.35 (i.e., *G12A* and *G12V*) variants (Fig. 2B and fig. S2B).

We hypothesized that specificity of our crRNAs could be “switched” from one variant to another by substituting the appropriate nucleotide at the c.34 or c.35 positions in the crRNA spacer (Fig. 2C). For example, a perfect-match *G12C*-targeting guide may contain an “A” nucleotide in spacer position 11 (i.e., crC₁₁), which will hybridize with the “U” nucleotide substitution found in *G12C*-mutated *KRAS* mRNA (i.e., c.34G>U). We reasoned that if we exchange the spacer nucleotide “A” for “G” at position 11 (i.e., crR₁₁), we would promote base pairing with the “C” substitution present in *G12R*-mutated *KRAS* (i.e., c.34G>C), silencing activity might correspondingly “switch” from *G12C* to *G12R* (Fig. 2C). Using this strategy, we designed a series of crRNAs that conserved the structure of crD₁₀C₁₁-MM₉ or crD₁₀C₁₁-MM₁₂ but altered the nucleotide identity at the spacer positions involved in c.34 and c.35 base pairing (e.g., crD₁₀R₁₁-MM₉, crA₁₀C₁₁-MM₁₂, and so on). As predicted, directed mutagenesis of these crRNAs resulted in potent, SNV-selective

silencing of *KRAS G12R*, *G12S*, and *G12A* relative to *KRAS WT* (Fig. 2D).

Unlike the other variants, *G12V* did not follow the expected silencing pattern, with crV₁₀C₁₁-MM₉ and crV₁₀C₁₁-MM₁₂ showing similarly inefficient silencing activity against both the *WT* and *G12V* variants (Fig. 2D). This equipotent silencing trend was confirmed in a further panel of putative *G12V*-selective guides (fig. S2C), where all guides silenced both the *WT* and *G12V* variants with outstanding efficiency. Unexpectedly, one crRNA (crD₁₁C₁₂-MM₁₀) demonstrated selective silencing of *KRAS G12V* relative to *WT KRAS* (Fig. 2D), despite having three mismatches with both *G12V* and *WT* and its intended targets being *G12C* and *G12D*. As the magnitude of *G12V* variant-selective silencing was minor compared to the best candidate crRNAs for the other *G12* variants, we did not proceed with further validation of the *G12V* target. However, this unexpected result caused us to question whether any of our newly engineered guides may outperform the parental crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂ in selectively silencing *G12C* or *G12D*. Screening the entire panel of guides against each *G12* mutant (fig. S3) uncovered several additional guides with improved *G12* selectivity as compared with the parental guides (Fig. 2E), most notably for the *G12D* variant.

Mismatch number and position are key determinants of SNV selectivity

The broad variance in silencing efficiency of our crRNAs observed across a single *G12* target (i.e., some *G12C*-targeting crRNAs are extremely efficient, and others less so) prompted us to investigate whether there are any generalizable features that could differentiate efficient SNV-selective versus inefficient or nonselective crRNAs. To that end, we pooled the silencing data from all crRNAs that targeted any *KRAS* variant and plotted their silencing efficiency relative to the number of mismatches (with a single *G12* target) in their spacer sequences (Fig. 3A). As expected, the perfect-match crRNA for *KRAS G12D* (i.e., no mismatches with *G12D* and one mismatch with *KRAS-WT*) efficiently silenced both *WT* and SNV with equipotency (Fig. 3A, yellow header). Similarly, when there is an equal number of mismatches in the spacer sequence for both the *WT* and the SNV transcript (e.g., two mismatches with *WT* and two mismatches with the SNV variant), crRNAs generally exhibit no selectivity and typically silence both *WT* or SNV with equivalent efficiency (bottom-left quadrant) or inefficiency (top-right quadrant) (Fig. 3A, light green header). Predictably, when crRNAs are engineered using *WT KRAS* as a template (instead of the SNV), thus meaning that the guide will have one fewer mismatch with the *WT* relative to the SNV (i.e., $n - 1$, instead of $n + 1$ described previously), silencing efficiency is skewed in favor of the *WT* variant (Fig. 3A, dark green header).

Guides that contain one mismatch with the SNV (and two with the *WT*) are comparably efficient at silencing both the *WT* and *G12* variants (Fig. 3A, teal header), just as those with three mismatches with the SNV (and four with the *WT*) are comparably inefficient (Fig. 3A, purple header). Over half of all crRNAs containing two mismatches with the SNV (and three with the *WT*) exhibit SNV-selective silencing (Fig. 3A, blue header), suggesting that this number of mismatches might be exquisitely important in the context of targeting mutant *G12* sequences. However, the observation that certain guides with two nucleotide mismatches (relative to the SNV template) remain nonselectively efficient or inefficient suggests that additional factors may also influence silencing efficiency.

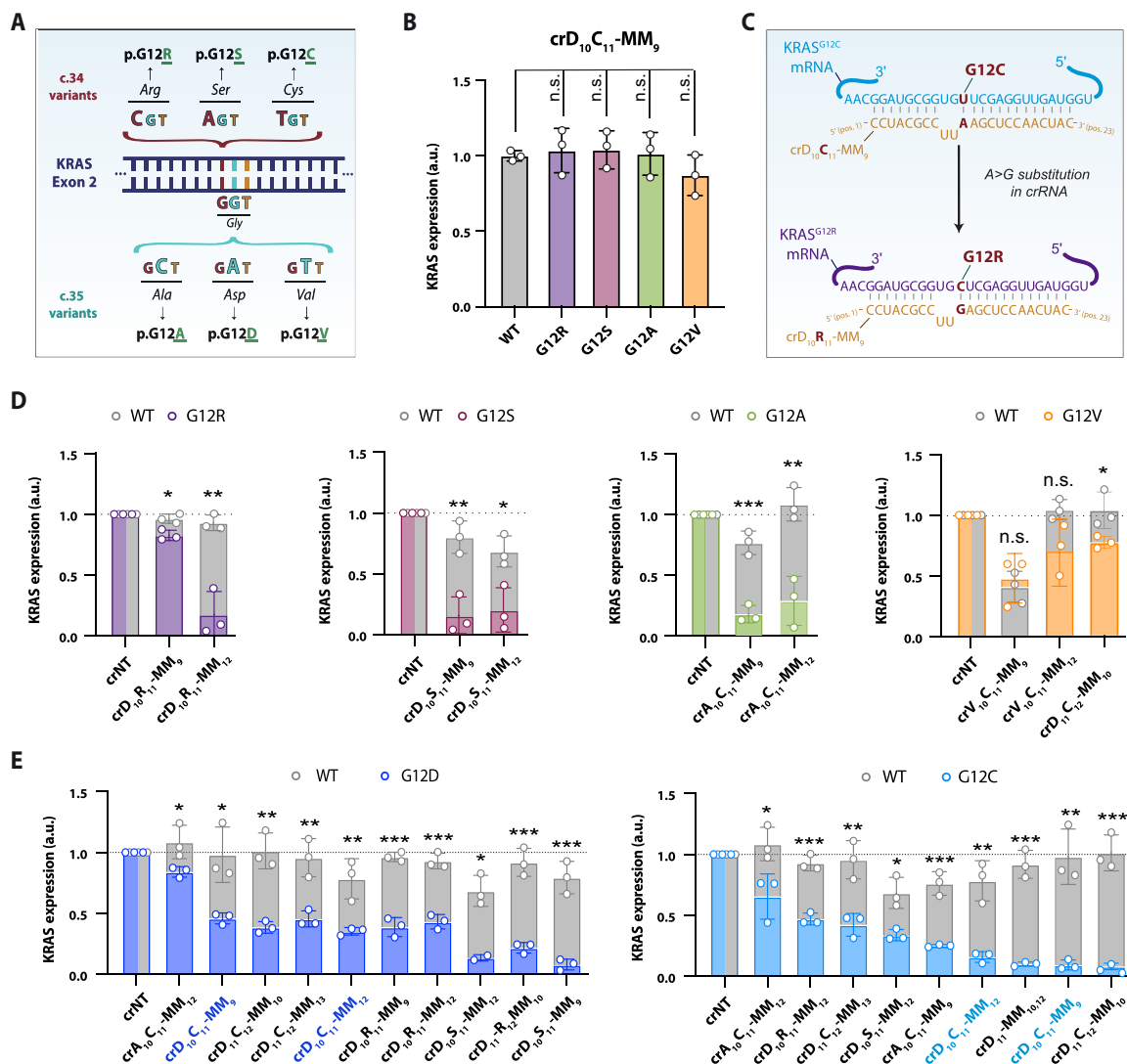


Fig. 2. Exploiting the mismatch tolerance of *RfxCas13d* enables selective targeting of *KRAS* G12 hotspot mutations. (A) The G12 mutation hotspot is found in exon 2 of the *KRAS* gene (codon 12, nucleotides 34 to 36). For WT *KRAS*, the consensus coding sequence "GGT" at codon 12 encodes a glycine (i.e., G12). Missense mutations that affect the "G" nucleotide at position 34, collectively referred to as "c.34 variants," change the amino acid sequence such that arginine (G12R, from c.34G>C substitution), serine (G12S, c.34G>A), or cysteine (G12C, c.34C>T) is encoded instead of glycine. "c.35 variants" arise from missense substitutions at nucleotide 35, causing glycine to be replaced by alanine (G12A, c.35G>C), aspartate (G12D, c.35G>A), or valine (G12V, c.35G>T). (B) Bar plot showing minimal cross-reactive silencing by cr₁₀D₁₁-MM₉ against other c.34 and c.35 *KRAS* variants (G12R, purple; G12S, pink; G12A, green; and G12V, gold), normalized against the silencing of WT *KRAS*, at 48 hours after transfection. (C) Schematic depicting the mutagenesis strategy used to switch the silencing selectivity of our G12C-selective or G12D-selective crRNAs onto other G12 variants (G12R, G12S, G12A, and G12V). In the example, the "A" nucleotide of the cr₁₀D₁₁-MM₉ spacer, which base pairs with the "T" nucleotide present in G12C-mutated *KRAS* mRNA, can be switched to a "G" nucleotide, thus promoting base pairing with the "C" nucleotide present in G12R-mutated *KRAS* mRNA. (D and E) Bar plots showing the silencing efficiency of various cr₁₀D₁₁-MM₉ and cr₁₀D₁₁-MM₁₂ mutagenesis derivatives against all *KRAS* G12 variants, normalized against crNT. Statistics indicate differential silencing efficiency between WT versus G12-mutants. In (E), the parental cr₁₀D₁₁-MM₉ and cr₁₀D₁₁-MM₁₂ are labeled in bold. All experiments were performed in HEK293T overexpression models. For all bar graphs, error bars represent means $\pm$ SD from three independent experiments. Statistical significance was determined using unpaired *t* tests, where **P* < 0.05, ***P* < 0.01, ****P* < 0.001. n.s., not significant.

Although most studies using *RfxCas13d* enzymes use a spacer length of 23 nt, recognition motifs of up to 32 nt have been reported (30). As a recent report has demonstrated that modulating the spacer length alters the mismatch sensitivity of *LbuCas13a* (31), we speculated that mismatch tolerance threshold is dependent on the proportion rather than the absolute number of mismatched nucleotides. In other words, if the crRNA spacer length was altered, would the number of mismatched nucleotides required for SNV-selective silencing

remain two, or would the 2-nt mismatch threshold be unique to 23-nt-long spacer sequences?

To address this question, we generated crRNA variants of a double-mismatched guide targeting *KRAS* G12C with truncated (19 and 21 nt) or extended (25, 27, and 29 nt) spacer sequences and evaluated the effect on silencing efficiency and selectivity. We confirmed that truncating the spacer leads to a drastic reduction in the silencing efficiency against both the SNV-containing target and its WT counterpart (Fig.

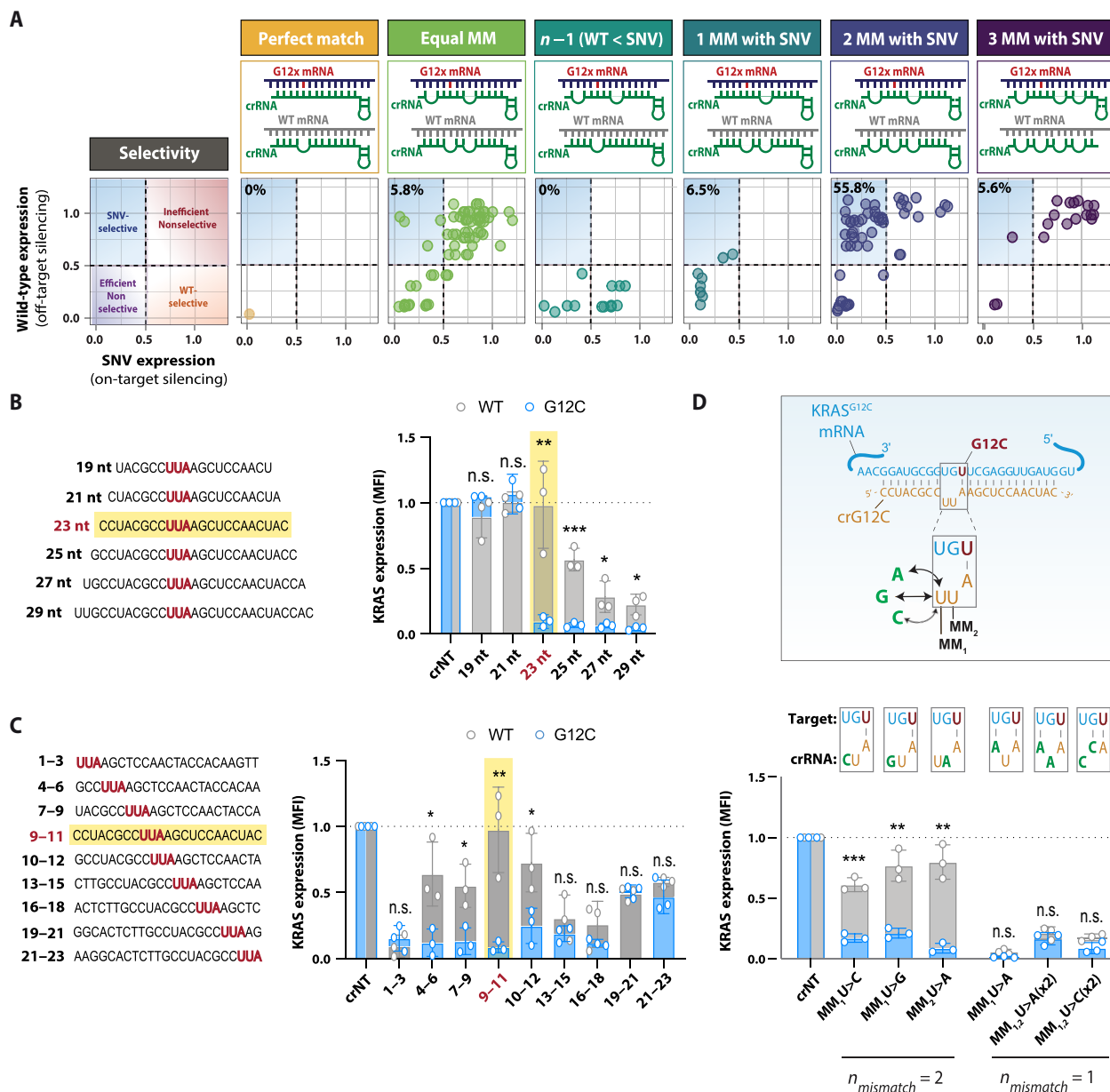


Fig. 3. Synthetic mismatch number, proportion, and position are key determinants of SNV selectivity. (A) Biplots showing the SNV selectivity profile of all G12-targeting crRNAs in this study. Each point represents the mean silencing efficiency of a single guide against *KRAS* WT (y axis) versus G12-mutant (x axis) targets (from $n = 3$ independent experiments). crRNAs that fall within the upper-left quadrant (light blue; indicating $<50\%$ expression of the G12 variant while maintaining $>50\%$ expression of the WT) are considered SNV-selective silencers. (B) Bar plots showing the effect of crRNA length and (C) position of the trinucleotide mismatch motif on SNV selectivity against *KRAS* WT (gray) and *KRAS* G12C (light blue), normalized against crNT at 48 hours after transfection. Sequences for each crRNA are shown adjacent to the bar plot: The “UUA” nucleotides (in red) indicates the trinucleotide mismatch motif that base pairs with the G12 codon. In (B) to (C), the parental, SNV-selective crRNA data (positive control; highlighted in yellow) is duplicated as the experiments were performed in parallel. MFI, mean fluorescence intensity. (D) Bar plot showing the effect of substituting individual nucleotides in the trinucleotide mismatch motif on SNV selectivity against *KRAS* WT (gray) and *KRAS* G12C (light blue), normalized against crNT at 48 hours after transfection. Schematic of the overall substitution strategy is shown in the top panel, with individual base-pairing motifs between the target mRNA (*KRAS* G12C) and the crRNA shown in boxes above each bar. All experiments were performed in HEK293T overexpression models. For all bar graphs, error bars represent means $\pm$ SD from three independent experiments. Statistical significance was determined using unpaired t tests, where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ns, nonsignificant.

3B). We reason that these shorter crRNAs are more sensitive to mismatches as the per-base energetic penalty for a mismatch relative to the free energy of the whole hybridization interaction is proportionally higher. Conversely, extending the spacer sequence leads to a step-wise increase in silencing efficiency for the WT variant, thereby decreasing the SNV selectivity of longer crRNAs, with near-complete

silencing of both the WT and mutant achieved with the 29-nt-long spacer (Fig. 3B). Collectively, these data indicate that mismatch tolerance is a function of the crRNA spacer length and that our mismatch strategy is optimized for crRNAs with a length of 23 nt.

Throughout our study, the mismatches in each spacer sequence were generated using the same nucleotide as in the target sequence

(e.g., “U” in the target forms a mismatch with a “U” in the spacer). We therefore questioned whether the identity of the mismatched nucleotide would influence silencing efficiency or selectivity. We designed a small panel of guide RNAs where we substituted the nucleotide identity of the mismatched base with other nucleotides (e.g., “U” > “C” or “U” > “G”). Broadly, we found that the exact nucleotide substitution in the spacer sequence does not strongly influence SNV selectivity so long as the overall number of mismatches is preserved (Fig. 3C). Conversely, if the nucleotide substitution creates a new base pair and thus allows an additional target:spacer interaction, meaning that the overall number of mismatches dropped from 2 nt to only 1 nt, we saw an improvement in silencing efficacy for both the WT and mutant variants (Fig. 3C). Together, these data indicate that the overall number and position of mismatches, rather than the nucleotide identity of the mismatch, carry more weight in determining SNV selectivity.

Last, we were interested in determining whether the position of the entire mismatch motif, being the trinucleotide sequence at the G12 codon, would affect silencing efficiency and/or selectivity. To do so, we tiled the mismatch motif (UUA) of our *G12C*-targeting crRNA from the 5′ end (spacer positions 1 to 3) to the 3′ end of the spacer sequence and assessed the effect on overall crRNA efficiency and SNV selectivity. Mismatches toward the 5′ end of the crRNA spacer (nucleotides 4 to 12) generally maintained some degree of SNV selectivity (Fig. 3D) although with less discrimination than our optimized cr*G12C* guide (with mismatch motif at positions 9 to 11). However, extreme 5′ mismatches (positions 1 to 3) indiscriminately increased the silencing efficiency against both the WT and the mutant transcript (Fig. 3D). Conversely, mismatches toward the extreme 3′ end of the spacer (positions 19 to 23) appear to indiscriminately reduce silencing efficiency (Fig. 3D).

Collectively, these data suggest that intrinsic mismatch tolerance “rules,” primarily related to the number and position of spacer mismatches, regulate the capacity of a crRNA to achieve SNV-selective silencing. Overall, guide RNAs that harbor two mismatches with the SNV and three mismatches with WT are the most likely to achieve selective silencing of *KRAS* G12 SNVs when using 23-nt-long spacer.

Reprogrammed Cas13 represses “undruggable” RAS mutants

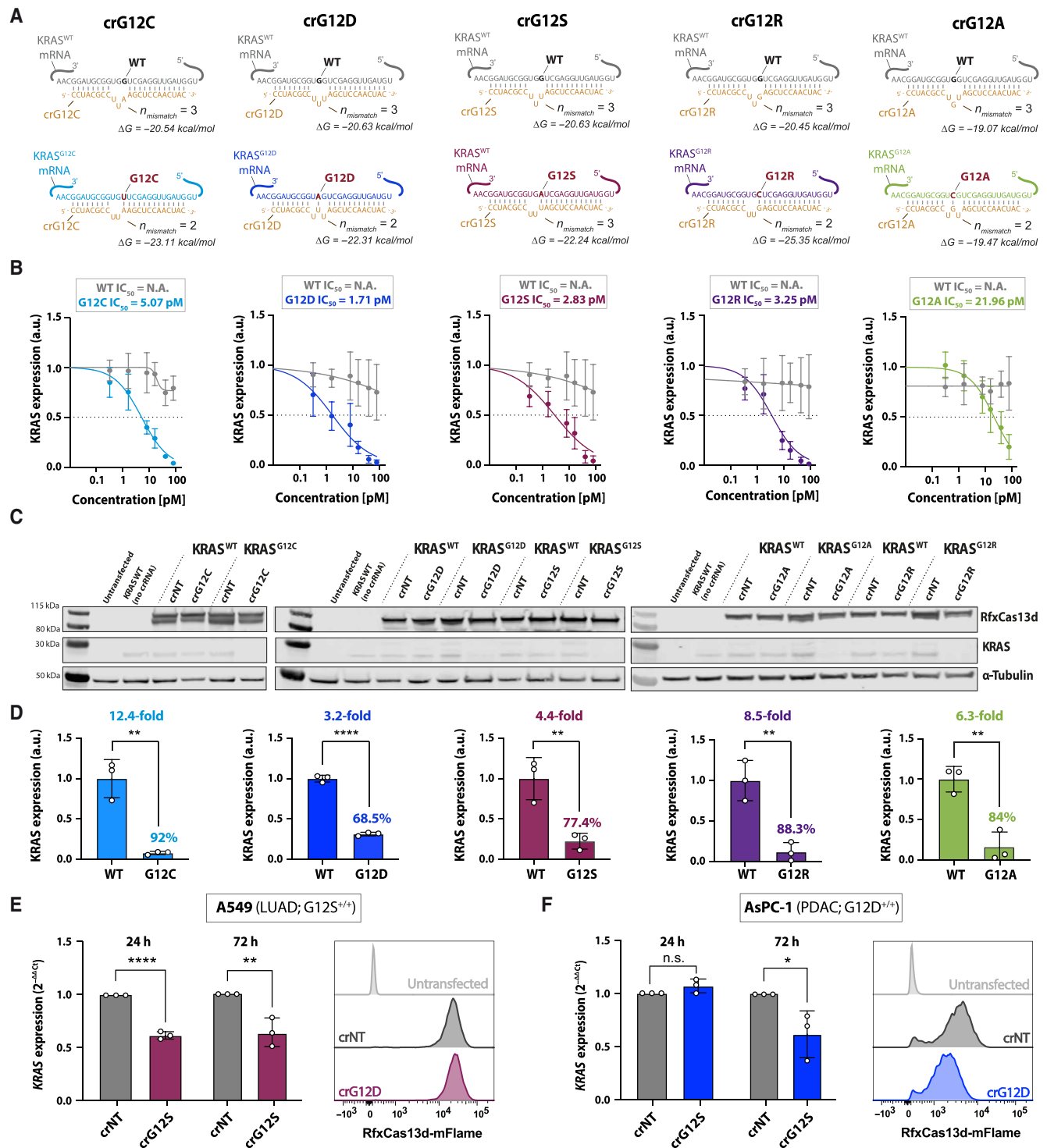
Through our various *KRAS* targeting screens, we identified several crRNAs that exhibited strong preferential silencing of each G12 variant. For further validation, we selected the most potent selective silencers of *G12A* (cr*G12A*), *G12C* (cr*G12C*), *G12R* (cr*G12R*), *G12D* (cr*G12D*), and *G12S* (cr*G12S*; Fig. 4A). For each guide, heteroduplex crRNA:mRNA formation was favored under the mutant-targeting condition [lower change in Gibbs free energy (ΔG)], consistent with the idea that increasing the number of spacer mismatches reduces binding affinity (Fig. 4A). Titration of these top-performing crRNAs confirmed the notably SNV-selective, dose-dependent silencing activity of these crRNAs against all *G12* variants tested (Fig. 4B). These guides were extremely potent, with IC_{50} ’s for each guide ranging from 1 to 22 pM plasmid. Conversely, the IC_{50} ’s for the WT could not be accurately calculated due to the failure of these guides to silence this variant with at least 50% efficiency. Moreover, *G12* selectivity was maintained at the protein level for all variants, with silencing efficiency against the mutant protein enhanced by ~3- to 12-fold versus the WT protein (Fig. 4, C and D). Next, we aimed to validate silencing of endogenous *KRAS* in G12-mutant cell lines. As these

cell lines are notoriously difficult to transfect, we optimized a method to deliver *RfxCas13d* and its crRNA via nucleofection. We nucleofected *RfxCas13d* mRNA and plasmid-encoded crRNA into AsPC-1 (pancreatic adenocarcinoma; G12D homozygote) and A549 (lung adenocarcinoma; G12S homozygote) and monitored *RfxCas13d*-mFlame expression, along with the cleavage of the SNV target transcript at 24 and 72 hours. A549 showed robust expression of *RfxCas13d* protein (Fig. 4E, histograms), leading to 39% reduction in *KRAS* G12S transcript expression as early as 24 hours, which was sustained until 72 hours (mean silencing of 37%; Fig. 4E). Although AsPC-1 exhibited more modest expression of *RfxCas13d* upon mRNA nucleofection (Fig. 4F, histograms) and slower silencing kinetics (i.e., no measurable silencing at 24 hours), a similar level of *KRAS* suppression was achieved by 72 hours (mean silencing of 38%; Fig. 4F). Collectively, these data indicate that reprogramming *RfxCas13d* via directed crRNA mutagenesis allows for selective inhibition of *KRAS* G12 hotspot mutants in both isogenic overexpression models and in endogenously G12-mutated cancer cell lines.

We next interrogated whether our strategy for targeting SNV could be extended to silence other prevalent oncogenic point mutations. To demonstrate this concept, we focused on another member of the RAS family, *NRAS*, for which there are no targeted therapies. Single-base substitutions in *NRAS* are present in ~10 to 15% of acute myeloid leukemia (AML), ~40% of which are *G12D* SNVs (c.35 T>A) (32). The binding site of *RfxCas13d* surrounding the *KRAS* *G12D* motif differs by four nucleotides from the corresponding region in *NRAS* *G12D* (Fig. 5A and fig. S4A). Consequently, the specific crRNAs designed for *KRAS* *G12D* are not capable of silencing the *NRAS* *G12D* variant due to the presence of these four nucleotide differences, exceeding the mismatch tolerance threshold of *RfxCas13d*. We hypothesized that the *KRAS* *G12D*-specific crRNA could be modified into an *NRAS* *G12D*-specific crRNA by introducing targeted substitutions for these four differing nucleotides within the spacer sequence.

As expected, a crRNA that fully base pairs with the *NRAS* *G12D* binding site achieved extremely high silencing efficiency against both *G12D* and WT *NRAS* mRNA without selectivity. However, the two crRNAs we engineered to silence *NRAS* *G12D* (cr*NRAS*-G12Dv1 and cr*NRAS*-G12Dv2, which adopt the G12-targeting motifs from *KRAS*-targeting crD₁₀C₁₁-MM₉ and crD₁₀C₁₁-MM₁₂, respectively; Fig. 5A and fig. S4A), showed clear *G12D* selectivity (fig. S4, B and C). Consistent with our previous observations, this preferential *NRAS* *G12D* silencing effect was dose-dependent (*G12D* relative IC_{50} = 11.83 pM; Fig. 5, B and C). As there are currently no existing small molecule inhibitors that specifically inhibit *NRAS* *G12D*, we aimed to determine whether our Cas13-based approach could effectively inhibit the growth of the THP-1 AML cell line (*NRAS* *G12D* heterozygote). Given that an antiproliferative phenotype would take time to manifest (i.e., several cell divisions), we reasoned that sustained *RfxCas13d* and crRNA expression would be necessary for long-term silencing. To ensure this, we switched our delivery approach from nucleofection to lentiviral transduction. As expected, both the parental cr*NRAS* (nonselective) and cr*G12D*-v2 (SNV-selective) crRNAs showed a moderate reduction in the proliferation of THP-1 cells relative to the crNT control (Fig. 5D).

When applying this lentiviral approach to *KRAS*-mutant A549 cells, we could detect moderate suppression of the MAPK signaling via phospho-Extracellular signal-Regulated Kinase (pERK) (fig. S5A). However, despite a slight reduction in colony size, this did not result



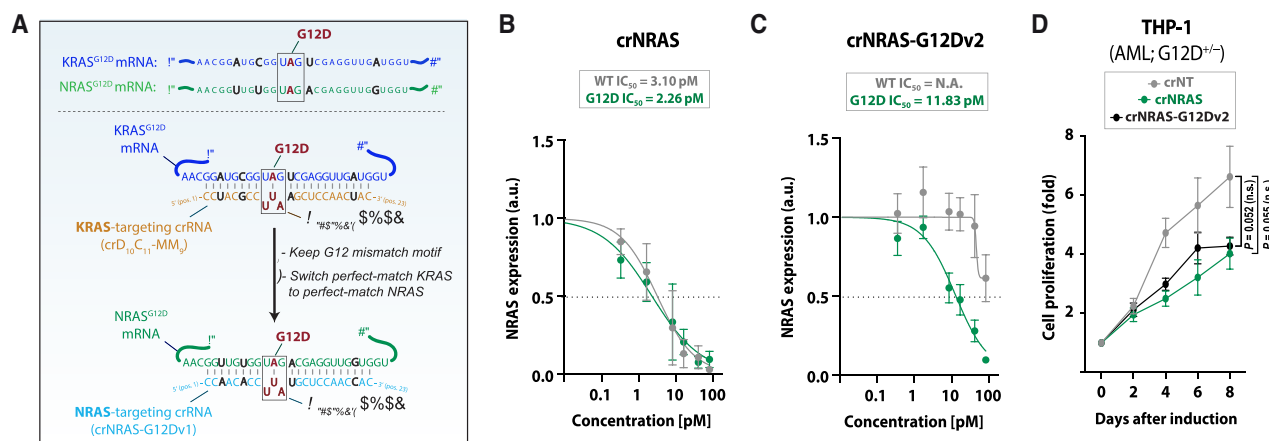


Fig. 5. Selective silencing of the undruggable NRAS G12D variant. (A) Schematic of the guide design strategy used to adapt *KRAS*-G12D targeting crRNAs to *NRAS*-G12D targeting crRNAs, showing the sequence homology between the two mRNAs (top) the sequence of each crRNA (bottom). The box indicates the nucleotides surrounding the codon 12 hotspot, and nucleotides in black indicate bases that are not conserved between *KRAS* and *NRAS* WT sequences. (B and C) Dose-response curves derived from titration of parental crNRAS and crNRAS-G12Dv2, against *NRAS* WT (gray) versus G12D (green) in HEK293T cells. Error bars represent the mean fluorescence intensity $\pm$ SEM from eight fields of view. (D) Proliferation curves showing growth rate of THP-1 AML cells transduced with *RfxCas13d* and either crNT (gray), nonselective crNRAS (black), or SNV-selective crNRAS-G12Dv2 (green), assessed by resazurin reduction assay at 48-hour intervals. Where statistics are shown, error bars indicate means $\pm$ SD [$\pm$ SEM in (D)] from at least $n = 3$ independent experiments. Statistical significance was determined using unpaired t tests.

in a significant antiproliferative effect when assessed by colony-forming assay (fig. S5B). As *RfxCas13d* protein expression was clearly detectable via immunoblotting (fig. S5A), we hypothesized that silencing inefficiency could be due to limited expression of crRNA when delivered via lentiviral transduction. To test this hypothesis, we compared crRNA abundance in transient transfection versus transduction settings in HEK293T cells. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) quantification revealed that the expression of crRNA under the transduction condition was several hundred-fold less than under the Lipofectamine transfection condition (fig. S5C). Consistent with these data, crRNA transfection in HEK293T cells achieved potent silencing, whereas crRNA transduction did not show any silencing activity (fig. S5, D and E). These findings show that lentiviral transduction leads to a limited expression of crRNAs, likely impeding RNA silencing efficiency.

Collectively, these data further underscore the effectiveness and adaptability of our strategy for targeting a broad range of oncogenic SNVs. They also suggest that the cellular abundance of *RfxCas13d* and crRNA is critical for efficient silencing, highlighting the need to optimize delivery methods for individual cell lines or transcripts.

SNV-selective crRNAs with mismatched nucleotides lack collateral activity

Previous reports have shown that *RfxCas13d* is prone to promiscuous collateral activity that can degrade nearby nontarget RNA molecules in mammalian cells (33–35). To assess the potential contribution of collateral activity to our *KRAS*-targeting strategy, we performed cotransfection assays where our target transcript was cotransfected alongside a nontarget fluorescent reporter [enhanced green fluorescent protein (eGFP)]. If collateral activity is present, then the fluorescent signal of both the target transcript (linked to mCherry) and the nontarget eGFP should be diminished. However, degradation of the target-mCherry reporter alone would indicate on-target nuclease activity without collateral cleavage of proximal RNA molecules.

Consistent with the literature, when using the *RfxCas13d* ortholog and perfect-match crRNAs (with zero nucleotide mismatch, “0MM”), we observed substantial collateral cleavage activity against the eGFP nontarget fluorescent reporter (~40 to 50% for G12C and G12D perfect-match crRNAs) (Fig. 6, A and B). Our data revealed that introducing synthetic mismatches into the crRNA sequence drastically impaired collateral activity against the nontarget eGFP reporter, despite retaining relatively high on-target activity (Fig. 6, A to D). We verified this finding against a second transcript by targeting mCherry directly, observing both lack of eGFP cleavage (Fig. 6E) and reduced collateral induced *RfxCas13d* self-cleavage (Fig. 6F) in the context of synthetic nucleotide mismatches. Collectively, these results indicate that targeting SNVs with mismatched crRNAs drastically reduces the inherent collateral activity of *RfxCas13d*.

SNV-selective silencing is achievable using orthogonal *PspCas13b*

To gain a deeper understanding of mismatch tolerance and SNV-specific silencing rules, we expanded our investigation to additional point-mutated transcripts and Cas13 orthologs. Specifically, we questioned whether SNV-selective silencing is unique to *RfxCas13d* or whether this property is instead a conserved feature across Cas13 family members. For this, we investigated whether another Cas13 ortholog, *PspCas13b*, could also achieve SNV-selective silencing. Of note, *PspCas13b* has a 30-nt-long spacer sequence (versus 23 nt for *RfxCas13d*), which may confer not only enhanced target specificity but also a higher mismatch tolerance.

To show that single-base resolution targeting can silence other onco-transcripts (which have limited sequence homology with *KRAS*), we focused our *PspCas13b* designs on targeting the *BRAF*^{V600E} mutation, in which a single-nucleotide substitution (c.1799 T>A) results in the replacement of valine by glutamate at amino acid position 600. This SNV is the most common *BRAF* aberration and is found in ~7% of all human cancers and up to 60% of melanomas (36). We first designed four *PspCas13b* perfect-match crRNAs (crBRAF1-4), which tile across

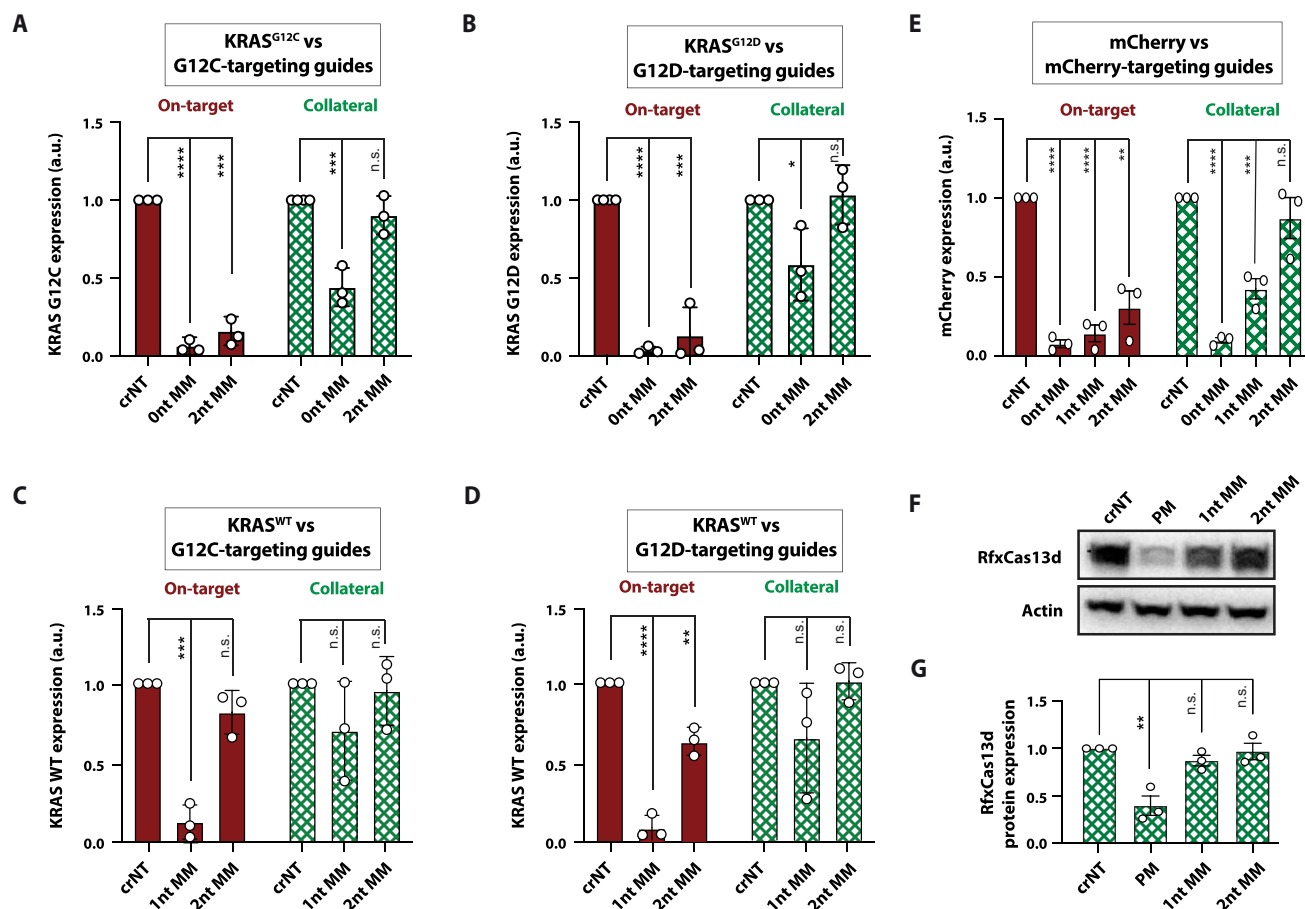


Fig. 6. SNV-selective crRNAs with mismatched nucleotides lack collateral activity. (A) Bar plots showing on-target (red bars) and collateral cleavage (green bars; cotransfected eGFP reporter) activity of perfect-match (“0nt MM”) or double-mismatched (“2nt MM”) crRNAs targeting *KRAS* G12C or (B) *KRAS* G12D or the same guides tested against (C and D) *KRAS* WT (therefore having $n + 1$ mismatches: “1nt MM” and “2nt MM”, respectively), normalized against crNT at 48 hours after transfection. (E) Bar plots showing on-target (red bars) and collateral cleavage (green bars; cotransfected eGFP reporter) activity of perfect-match (0nt MM), single-mismatched (1nt MM), or double-mismatched (2nt MM) crRNAs targeting mCherry, normalized against crNT at 48 hours after transfection. (F) Western blot depicting RfxCas13d self-cleavage activity for the transfection conditions shown in (E), compared with a perfect-match (PM) crRNA. (G) Densitometry of $n = 3$ independent experiments is shown in the bar plot below, where RfxCas13d expression is normalized to the actin loading control, shown relative to crNT. All experiments were performed in HEK293T overexpression models. For all bar graphs, error bars represent means $\pm$ SD from three independent experiments. Statistical significance was determined using unpaired t tests, where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

the V600E point mutation. These guides incorporated “GG” in spacer positions 1 to 2, in line with our recent report that this motif leads to enhanced PspCas13b-mediated silencing (23). Using *BRAF* reporter assays, all perfect-match guides enabled potent *BRAF* mRNA cleavage relative to the non-targeting control, among which the cr*BRAF*-1 guide, which base pairs with V600E at spacer position 5, showed the highest silencing activity (Fig. 7A and fig. S6A). However, none of these guides were able to discriminate between WT and SNV-containing targets.

To achieve SNV selectivity, we serially mutated cr*BRAF*-1 by introducing either contiguous blocks of 2 to 4 mismatched nucleotides flanking the SNV site or a series of two, three, or four single-nucleotide mismatches that were distributed along the spacer sequence (Fig. 7B). Although many crRNAs retained their ability to silence the mutant transcript, often with efficiencies comparable with the parental perfect-match cr*BRAF*-1 (e.g., crE5-MM_{4,15}), certain crRNAs completely lost their silencing activity (e.g., crE5-MM_{4,11,18,25}). As expected, silencing activity against the *BRAF* WT target was more severely impaired as these transcripts had one additional mismatch ($n + 1$) for each of the spacer sequence screened.

The crRNAs that were least efficient at silencing WT *BRAF* transcripts were overrepresented in the mutagenesis groups where spacers had two or three mismatches in the V600E spacer (corresponding to three and four mismatches with the WT, respectively), suggesting that this may represent an optimal mismatch tolerance threshold for the PspCas13b ortholog when targeting *BRAF* V600E sequence. Of these, two crRNAs, crE5-MM_{8,15} and crE5-MM_{11,15}, showed significant differential (Fig. 7C and fig. S6B) and dose-dependent (Fig. 7D) silencing activity in favor of the V600E transcript.

Unlike RfxCas13d, and consistent with recent reports (35, 37), cotransfection assays revealed that target-activated PspCas13b lacks any significant collateral activity against the eGFP (nontarget) transcript, both in the context of perfect-match and V600E-selective crRNAs (fig. S6C).

As the *BRAF* construct used in our reporter assay encoded a truncated, V600E-spanning *BRAF* gene (~500 nt), we next sought to investigate whether the SNV-specificity of crV600E-13 and crV600E-14 would be retained when targeting the full-length *BRAF* transcript. HEK293T cells transfected with constructs encoding full-length

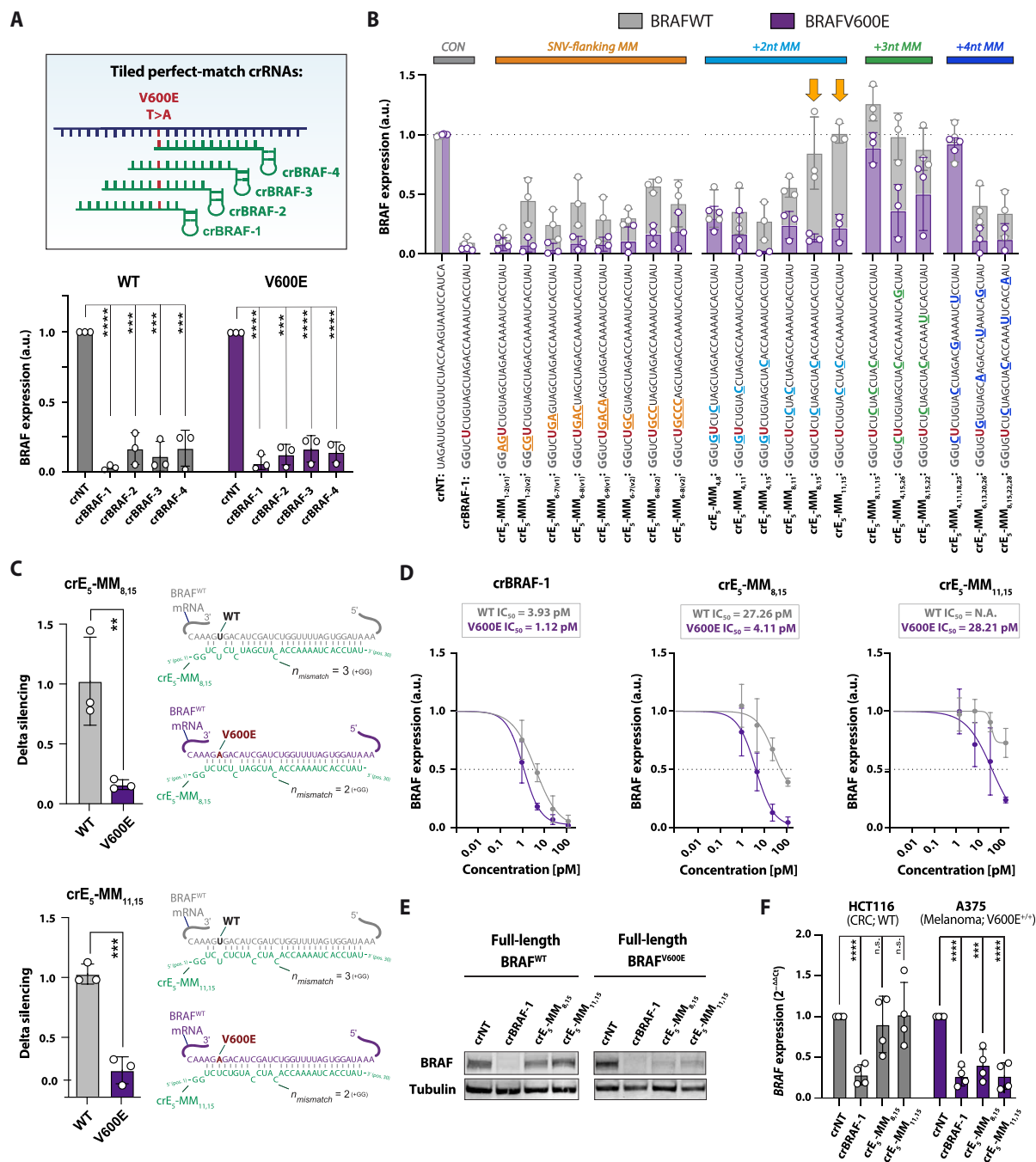


Fig. 7. SNV-selective silencing of *BRAF* V600E using orthogonal *PspCas13b*. (A) Silencing efficiency of four perfect-match, *BRAF*-targeting crRNAs at 48 hours after knock-in of WT (gray) or V600E (purple) *BRAF* variants, normalized against crNT. (B) Systematic mutagenesis of nonselective crBRAF-1 was used to engineer V600E-selective crRNAs. Sequences for each crRNA are shown below the bar plot; the “U” nucleotide (in red) indicates the uracil in the spacer sequence that base pairs with the target V600E but not with the WT *BRAF* RNA, and the colored nucleotides show the position of an additional mismatch introduced at various spacer locations through mutagenesis. Bar plot shows the silencing efficiency of crBRAF-1 and its mutagenesis derivatives against *BRAF*-WT (gray) versus *BRAF*-V600E (purple), normalized against crNT at 48 hours after transfection. crE₅-MM_{8,15} and crE₅-MM_{11,15} (orange arrows) show the highest selectivity against *BRAF* V600E. (C) Delta silencing efficiencies between WT (gray) and V600E (purple) variants for the top-performing crRNAs, indicating the degree of SNV-specificity. Schematics depicting the sequence and base-pairing configuration of crE₅-MM_{8,15} and crE₅-MM_{11,15} with WT versus V600E-mutant *BRAF* mRNA targets are shown adjacent. (D) Dose-response curves derived from titration of parental crBRAF-1, crE₅-MM_{8,15}, and crE₅-MM_{11,15} against WT (gray) or V600E (purple) *BRAF* at 48 hours after transfection. For all graphs in (A) to (C), individual data points show averaged fluorescence intensity from eight representative fields of view ($n = 3$ independent experiments), and error bars show means $\pm$ SD. (E) Silencing efficiency assessed by Western blotting in HEK293T cells transfected with *PspCas13b* and full-length WT or SNV constructs. (F) Silencing efficiency assessed by RT-qPCR in cancer cell lines (A375 melanoma and HCT116 colorectal cancer), which endogenously express the SNV-containing oncogene (means $\pm$ SD from $n = 3$ independent experiments). Statistical significance was determined using unpaired t tests, where $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

BRAF-WT or *BRAF*-V600E also exhibited the expected pattern of silencing, with both guides preferentially knocking down *BRAF*-V600E at the protein level (Fig. 7E). This indicates that the silencing efficiency of these prevalidated crRNAs was not disrupted by any potential secondary structures present in the full-length transcripts expressed in HEK293T cells.

Next, we tested the silencing activity of the engineered crRNAs in cancer cell lines with different *BRAF* genotypes, HCT116 (colorectal cancer, WT) and A375 (melanoma, homozygous V600E). Corroborating the results seen in HEK293T cells (fig. S6B), these data confirmed that the efficiency and SNV selectivity of these crRNAs are retained in cancer cell lines expressing endogenous *BRAF* variants (Fig. 7F). Collectively, these data indicate that the *Psp*Cas13b system can be reprogrammed to target V600E-mutated transcripts in both overexpression and endogenous contexts with high specificity. Moreover, our demonstration of SNV-selective silencing in two orthogonal Cas13 systems reinforces both the sensitivity and versatility of our screening platform to identify SNV-selective crRNAs and suggests that it may be applicable to other SNVs beyond *KRAS* G12 or *BRAF* V600E.

DISCUSSION

In this study, we developed single-nucleotide precision Cas13 tools capable of selectively degrading various oncogenic SNV transcripts with high efficiency and very limited off-targeting of WT mRNA (Fig. 8). The engineering required for this single-base precision RNA targeting was challenging due to the intrinsic mismatch tolerance of various CRISPR-Cas13 enzymes (18, 23). Directed perturbation of spacer-target interactions at precise spacer nucleotide positions through mutagenesis enabled us to identify key positions that are highly sensitive to mismatches, facilitating de novo crRNA design that can discriminate between point-mutated transcripts and their WT variants. Beyond the oncogenic *KRAS*, *NRAS*, and *BRAF* mutant transcripts that we silenced in this study, we aimed to identify the molecular principles of crRNA design, which confer SNV selectivity, allowing us to develop a systematic approach to predict personalized, single-base precision crRNAs capable of targeting other pathogenic SNVs. This strategy was previously unattainable due to the limited comprehension of the

molecular basis of mismatch tolerance and intolerance at single-nucleotide resolution.

Consistent with a previous report (26), different regions of the *Rfx*Cas13d spacer showed marked variation in mismatch intolerance, with the highest mismatch sensitivity exhibited in the central region. When we interrogated generalizable *Rfx*Cas13d design principles to enable selective silencing of *KRAS* SNVs, we found that the transition from mismatch tolerance (active nuclease) to intolerance (inactive nuclease) often occurs when three nucleotide mismatches are introduced at the central region of *Rfx*Cas13d's spacer-target duplex. Two nucleotide mismatches with point-mutated transcripts were well tolerated and resulted in potent silencing of SNVs, whereas the additional third mismatch with the WT transcript led to strong or complete loss of silencing. We speculate that this mismatch intolerance threshold (three nucleotides) may vary when targeting other sequences due to the thermodynamic stability of the A-form RNA helix in the enzyme's central cleft and the potential for subtle, target-specific interactions between the duplex RNA and the surrounding amino-acid residues of *Rfx*Cas13d that are intrinsic to the target sequence. Other high-throughput studies using different spacer-target sequences have shown that the sensitivity to a single, double, or triple nucleotide mismatches may be dependent on the target sequence (25).

Although crRNAs with two nucleotide mismatches most often achieved SNV selectivity, not all double-mismatched guides showed differential silencing of the mutant. Our data suggest that, beyond having a mismatch number of two, the position of these two nucleotide mismatches, both in the context of the entire crRNA spacer and relative to the point mutation, is a key determinant of SNV selectivity. For example, certain double-mismatch crRNAs that were designed to target *KRAS* G12D showed no silencing of either *KRAS* G12D or WT *KRAS*. Upon further analysis, we noted that several of these guides shared a mismatch configuration, containing one mismatch adjacent to the G12D mutation and another at a distal location (e.g., nucleotide position 6, 10, or 15). This is consistent with previous reports that certain spacer positions may be more intolerant to mismatches than others (26, 31).

Our results indicated that a single additional mismatch often confers a sudden transition of Cas13 from an active to inactive

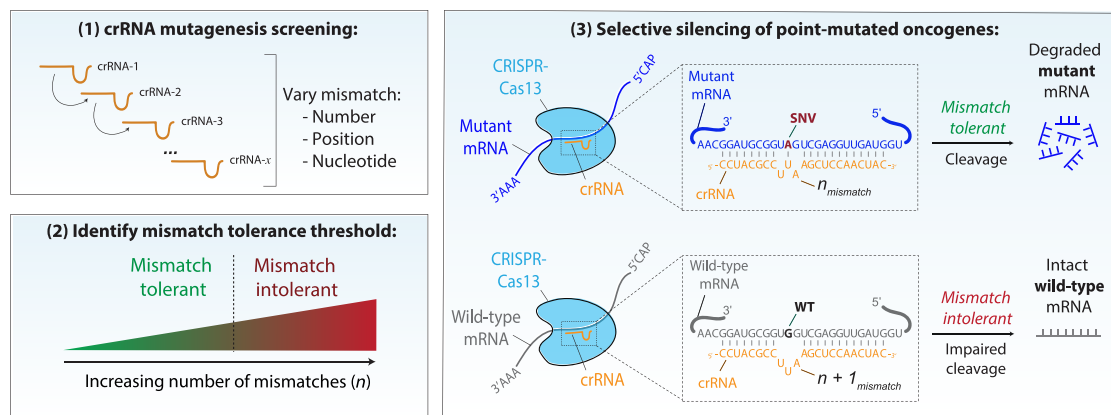


Fig. 8. Molecular principles of CRISPR-Cas13 mismatch intolerance enable selective silencing of point-mutated oncogenic RNA with single-base precision. In this study, we show that comprehensive mutagenesis analysis of target-spacer interactions at single-nucleotide resolution reveals key spacer nucleotide positions intolerant to mismatches and the overall mismatch tolerance threshold of the target transcript. By designing crRNAs that follow these molecular principles, CRISPR-Cas13 can be reprogrammed for strong preferential silencing of SNV transcripts with single-base precision.

state. Although the predicted ΔG favors the hybridization of a given crRNA with the mutant mRNA sequence (i.e., lower ΔG for crRNA:mutant mRNAs heterodimers versus crRNA:WT mRNAs; Fig. 4), it is unlikely that this modest reduction in binding affinity alone can explain the profound shift from mismatch tolerance to intolerance. Rather, the mismatch intolerance threshold may be crossed due to steric hindrance created by bulky unpaired nucleotides at the central cleft of the enzyme, which may constrain the molecular rearrangement required for HEPN1 and HEPN2 nuclease domain activation (38). Structural studies of the Cas13d-crRNA binary complex demonstrated that various regions of the spacer positioned within the open channel of the enzyme are exposed to the solvent (38), suggesting that the Cas13d spacer is unlikely to have a unique mismatch-sensitive seed region responsible for initiating base pairing with the target. Instead, the sensitivity to mismatches in the central region we observed could be due to steric hindrance and the impediment of domain rearrangement. Future structural and dynamic studies may further illuminate the molecular components of this transition from mismatch tolerance to intolerance when various mismatches are located at the central region of the *RfxCas13d* spacer.

Our study also revealed that synthetic mismatches at key spacer positions can impair *RfxCas13d*'s collateral cleavage activity (Fig. 4). This finding may have important implications for future Cas13-based therapeutics as reduced self-cleavage of *RfxCas13d* RNA activity may increase the half-life of the *RfxCas13d* protein. The mechanism by which mismatches negatively affects collateral cleavage is yet to be elucidated. As individual mismatches are predicted to have limited effect on Cas13 binding or dissociation kinetics (due to extensive spacer-target base pairing and the limited change in the stability of the RNA-RNA duplex), we speculate that minor mismatches at sensitive spacer regions may have compromised collateral activity through the creation of steric hindrance that may prevent conformational rearrangement. This hypothesis remains to be tested in future work using sensitive biophysical binding and cleavage assays that can decouple binding and cleavage steps.

When we interrogated the single-base precision capability of the *PspCas13b* using a different target sequence (*BRAF-V600E*), we also found that the mismatch number and position are critical for the activity of this Cas13 ortholog, although their number and position vary from those of *RfxCas13d*. crRNAs harboring two nonconsecutive mismatches with the mutant template (at position 15, combined with position 8 or 11, with an additional mismatch for the WT template arising from the SNV located at position 5) exhibited the most noticeable discrimination between WT and *BRAF-V600E* transcripts. This precise balance between the number and position of mismatches suggested the existence of asymmetry within the spacer with some regions highly sensitive to mismatches while others are resilient. We found that this mutagenesis strategy generated at least one crRNA capable of selective and potent silencing of each of the six possible *KRAS G12* mutants (Fig. 4), albeit with a lower silencing potency for *G12V* compared to the other variants. Further development of these Cas13-based *KRAS* suppressors as RNA therapeutics could be of high clinical significance as 90% of *KRAS*-driven cancers remain clinically unactionable (7).

In this proof-of-concept study, we have demonstrated a highly precise RNA targeting strategy that effectively suppresses SNVs while sparing WT transcripts. This tool may be of great value for elucidating the function of point-mutated variants of unknown significance

or, with further development, to therapeutically suppress known oncogenic drivers with single-nucleotide precision.

However, despite its potential, the major hurdle for these RNA-targeting tools lies in the absence of efficient and safe strategies for delivering large Cas13 enzymes and their crRNAs to hard-to-transfect patient-derived cancer cell lines and animal models. Although a key benefit of CRISPR-Cas13 technology is that it does not mutilate genomic DNA, this means that Cas13-based therapeutics will likely require repeated dosing for sustained RNA cleavage activity, a constraint applicable to all RNAi technologies. This is additionally complicated by the rapid degradation of crRNAs in cells, yielding only transient knockdown. Although a single dose of transiently delivered Cas13 and crRNA resulted in ~40% reduction in endogenous *KRAS G12* expression and ~70% reduction of endogenous *BRAF V600E*, this was insufficient to produce sustained antiproliferative phenotypes in many of the cancer cell lines tested.

Future work should focus on optimizing delivery methods that allow for efficient and sustained expression of Cas13 and crRNA in vitro and in vivo. To this end, important milestones have been met in the delivery of various CRISPR systems using Adeno-Associated Viruses (AAV) (39, 40) or through the delivery of Cas endonuclease mRNA encapsulated into lipid nanoparticles (41–44). Similarly, meaningful advances have been made to enhance the stability of crRNAs, with various chemical modifications improving their half-life in human cells (45–47). These delivery modalities lay a strong foundation for future studies in patient-derived cancer cell lines and animal models, which are needed for the further advancement of these SNV-specific Cas13 drugs.

MATERIALS AND METHODS

Generation of SNV-selective crRNAs

crRNA design and cloning

RfxCas13d crRNA spacers were designed as 27-nt single-stranded forward and reverse oligos containing AAAC and AAAA overhangs, respectively, allowing for ligation into Bsm BI-digested plasmid that encodes *RfxCas13d* direct repeat (Addgene #138150). *PspCas13b* crRNA spacers were designed as 34-nt single-stranded forward and reverse oligos containing CACC and CAAC overhangs, respectively, allowing for ligation into Bbs I-digested plasmid that encodes *PspCas13b* direct repeat (Addgene #103854). Oligos were ordered as single-stranded DNA (IDT) at a concentration of 100 μ M. All crRNA spacer sequences used in this study are available in table S1. To produce double-stranded DNA oligos suitable for plasmid ligation, each forward and reverse DNA oligos (3 μ M) were annealed in a T4 DNA ligase buffer (NEB) by heating to 95°C for 5 min then cooling at a rate of 4°C every 2 min until ambient temperature was reached. The *PspCas13b* crRNA backbone plasmid (Addgene #103854), a gift from F. Zhang (48), was digested using Bbs I (NEB) in 1x NEBuffer r2.1 at 37°C for 2 hours. The *RfxCas13d* crRNA backbone plasmid (Addgene #138150), a gift from N. Sanjana (26), was digested using Bsm BI-v2 (NEB) in 1x NEBuffer r3.1 at 55°C for 2 hours. Digested plasmids were separated on a 1% (w/v) agarose gel in 1x TAE (tris-acetate-EDTA) buffer at 100 V for 1 hour. The linearized fragment was then excised and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The annealed oligos (400 nM) were ligated with 1 ng of the digested crRNA backbone using T4 ligase (Promega) in 1x T4 ligase buffer for 3 hours at room temperature (RT).

Nomenclature of SNV-selective crRNA

To ensure clarity, we have devised a consistent nomenclature system for crRNAs that effectively conveys information about the target SNV and the position of any introduced mismatches. Uppercase characters within the nomenclature signify the specific SNV being targeted by the respective crRNA. In addition, the designation “MM” denotes the presence of synthetic mismatches that have been introduced through spacer mutagenesis. The subscript numbers within the nomenclature correspond to the positions within the crRNA spacer, with position 1 situated at the 5′ end. For instance, consider the crRNA “crD₁₀C₁₁-MM₉,” which is designed to base pair with the *KRAS G12D* transcript. Here, the SNV interacts with spacer nucleotide 10. The same crRNA can also base pair with the *KRAS G12C* transcript, wherein the SNV interacts with spacer nucleotide 11. This crRNA forms an additional synthetic mismatch at spacer position 9, relevant to both *KRAS G12D* and *KRAS G12C* targets. A visual representation of this concept can be found in the schematic in Fig. 1E.

Prediction of crRNA:mRNA heterodimer affinities

Δ*G* analyses were performed using the RNAfold module of the ViennaRNA web server (49), available at <http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>.

Cloning of oncogene expression constructs

The *BRAF* and *KRAS* coding sequences were obtained from NCBI and all fragments for cloning were ordered as dsDNA gBlocks (IDT). Truncated *BRAF* inserts for cloning were designed to contain 250 base pairs upstream and downstream of codon V600, adding in-frame reporter tags to the 3′ end (3xFLAG for *BRAF-WT* and 3xHA for *BRAF-V600E*), and flanking the excerpt with Eco RI/Xho I restriction cut sites. Full-length *KRAS-WT* was engineered in-frame with 3xHA and Bam HI/Xho I cut sites. Cloning into MSCV-IRES reporter backbones encoding GFP (for *BRAF-WT*) or mCherry (for *BRAF V600E* and *KRAS*) was achieved via plasmid digestion, gel purification, and T4 ligation as previously described (18). Plasmids encoding full-length *BRAF WT* (p3xFLAG-CMV-*BRAF*, Addgene #131710) and *V600E* mutant (p3xFLAG-CMV-*BRAF-V600E*, Addgene #131723) were gifts from J. De Grève (50).

Generation of point-mutated plasmids via site-directed mutagenesis

SNV-containing plasmid constructs for *KRAS G12* (-A, -C, -D, -R, -S, and -V) mutants were generated using site-directed mutagenesis. Fifty nanograms of the parental plasmid was amplified with complementary 35-nt-long forward and reverse primer pairs (0.25 μM each) containing the desired nucleotide substitution (at nucleotide position 17) in the presence of 1.25 U *Pfu* DNA polymerase (Promega) and 200 μM deoxynucleotide triphosphates (dNTPs) (each) in 1x MgSO₄ buffer. After initial denaturation at 95°C, the reaction mix underwent 15 cycles of denaturation (95°C, 30 s), annealing (58° to 60°C, 30 s), and extension (72°C, 2 min/kb) followed by final extension (72°C, 5 min). Parental plasmids were selectively linearized from the PCR product via digestion with Dpn I (Promega) in 1x MULTI-CORE buffer for 1 hour at 37°C, and the digestion product was immediately transformed into Stbl3 competent bacteria.

Plasmid amplification and purification

Five microliters of the ligation product or 100 ng of the circularized plasmid was transformed into 25 μl of Stbl3 competent bacteria by

heat shock at 42°C for 90 s, followed by 2 min on ice. The transformed bacteria were recovered in 250 μl of LB media for 1 hour at 37°C in a shaking incubator (200 rpm). The bacteria were pelleted by centrifugation at 8000 rpm for 1 min, resuspended in 30 μl of LB broth, and plated onto prewarmed LB agar plates containing ampicillin (75 μg/ml). Following overnight incubation at 37°C, single colonies were picked and transferred into 3-ml bacterial starter cultures and incubated for ~16 hours prior to mini- or maxi-prep DNA purification using the NucleoSpin Plasmid kit (Machery-Nagel). Successful cloning of all plasmids was verified by Sanger sequencing (Australian Genome Research Facility).

Cell culture

HEK293T (American Type Culture Collection, CRL-3216) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose, Gibco). A375, HCT-116, THP-1, AsPC-1, and A549 cells were cultured in RPMI. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), 2 mM GlutaMAX (Gibco), and 1% penicillin/streptomycin (Gibco). HEK293T cells were incubated at 37°C and 10% CO₂. All other cell lines were incubated at 37°C and 5% CO₂. Cells were passaged twice a week and routinely tested for mycoplasma.

RNA silencing and collateral cleavage assays via transient transfection

This approach requires cloning the coding sequence of the *KRAS* SNV of interest into an Internal Ribosome Entry Site (IRES)–fluorescent protein (FP) reporter construct (as described above). When transfected, this construct generates a chimeric SNV-IRES-FP transcript that is subsequently translated into two separate proteins due to the presence of the IRES sequence. Cas13 cleaves the SNV-IRES-FP chimeric RNA at the SNV site, resulting in RNA fragments lacking key features such as 5′ cap, 5′ and 3′ untranslated regions, and polyadenylate tail, which leads to reduced intracellular RNA stability and/or compromised ribosomal translation. Consequently, the cleaved SNV fragment and downstream reporter gene undergo degradation and/or translational repression, resulting in the loss of fluorescence signal. Therefore, efficient cleavage of the SNV transcript by Cas13 is accompanied by the loss of fluorescence signal. Transfection experiments to determine the silencing efficiency of each crRNA were carried out using an optimized version of the manufacturer's Lipofectamine 3000 protocol (Invitrogen). Approximately 18 hours prior to transfection, HEK293T cells were seeded at densities of 25,000 cells/100 μl or 150,000 cells/500 μl in 96-well or 24-well densities in tissue culture-treated flat-bottom plates (Corning), respectively. For each transfection condition, performed in duplicate, two master mixes were prepared. For 96-well plates, master mix A contained 25 ng of the Cas13 effector plasmid [*Psp*Cas13b-NES-3xFLAG-T2A-BFP (Addgene #173029) or pLentiRNACRISPR-007-RfxCas13d (Addgene #138149)], target plasmid (50 ng of *BRAF* or 100 ng of *KRAS* expression plasmids), crRNA plasmid (0.1 to 100 ng), and 4% (v/v) P3000 enhancer reagent (Invitrogen) in Opti-MEM serum-free medium (Gibco), making up 5-μl total volume for 96-well plates. Master mix B contained 6% (v/v) Lipofectamine 3000 reagent (Invitrogen) in Opti-MEM serum-free medium, making up 5-μl total volume. Master mixes A and B were combined at a 1:1 ratio and incubated at RT for 20 min, after which 10 μl of A+B master mix was added to each well. For larger plates (24 and 96 wells), seeding density and all volumes were upscaled by surface area. For Western blotting experiments shown in Fig. 3C, the

crRNA concentration was increased to 300 ng per 24 wells. For colateral cleavage experiments, the empty vector MSCV-IRES-EGFP plasmid (1:1 ratio with the target plasmid) was also cotransfected. As the *RfxCas13d* effector plasmid is inducible, doxycycline at a final concentration of 1 $\mu\text{g}/\mu\text{l}$ was added to each well at the time of transfection for this ortholog. Transfected cells were incubated at 37°C in 10% CO₂ for 48 hours and then imaged using the EVOS M5000 FL Cell Imaging System (Thermo Fisher Scientific). The mean fluorescence intensity of each image was quantified using an in-house macro written for the ImageJ software in batch mode. Briefly, images were converted to 8 bit, threshold adjusted based on the fluorescence intensity of positive (crNT) and negative (untransfected) controls, converted to a binary mask, and the fluorescence intensity per pixel measured using the Analyze Particles function. Mean fluorescence intensities were obtained from four fields of view per well for each crRNA and subsequently normalized to the crNT control. Plasmid relative IC₅₀'s, where shown, were obtained by first calculating the molecular weight (MW) of the plasmid (DNA length in base pair $\times$ 650, which is the average MW of a single DNA base pair) and then calculating the molarity (i.e., mass transfected per 110- μl total volume per well of a 96-well plate). Molarity calculations and ratios between the Cas13, crRNA, and target plasmid are shown in table S4. [Note: We found that transfection of equivalent mass of the two full-length *BRAF* plasmids led to marked difference in *BRAF* protein expression, where *V600E* expression was several-fold higher than for *BRAF-WT*. Thus, for transfection experiments using these plasmids, we titrated the amount of each plasmid such that they produced equivalent protein expression measured by Western blot. We balanced the overall total amount of DNA transfected under each condition by cotransfecting the residual amount of empty vector plasmid (of equivalent size).]

RNA silencing via nucleofection

For endogenous KRAS silencing, 1×10^6 cells (A549 or AsPC-1) per condition were harvested and premixed with 2 μg of *RfxCas13d* mFlame mRNA (Messenger Bio) and 4 μg of crNT or crG12D/S (Integrated DNA Technologies). The reaction mixture was topped up to 20 μl using the appropriate buffer solution for each cell line and nucleofected using the Lonza 4D-Nucleofector according to the manufacturer's instructions (A549: buffer SF, pulse code CM-130; AsPC-1: buffer SE, pulse code DN-100). Twenty-four hours after nucleofection, *RfxCas13d*-mFlame expression was quantified using via flow cytometry using an LSRFortessa (sample preparation details below). After nucleofection, cells were recovered in 12-well plates in prewarmed RPMI and harvested at 24- and 72-hour time points for RNA extraction and RT-qPCR analysis.

Lentivirus production and transduction

To generate lentiviral particles, $\sim 12 \times 10^6$ HEK293T packaging cells were seeded in complete DMEM in T175 flasks and incubated overnight. A transfection cocktail composed of packaging vectors [pMDLg/pRRE (3 μg ; Addgene #12251), pRSV-Rev (1.5 μg ; Addgene #12253), and pMD2.G/VSVg (1.8 μg ; Addgene #12259)] and 14 μg of the target plasmid [*RfxCas13d* (Addgene #138149), or guide plasmid (cloned into Addgene #138150)] was combined with 150 μl of polyethylenimine (1 mg/ml) and topped up to 1.2-ml total volume with Opti-MEM. The solution was allowed to incubate for 20 min at RT, mixed well, and then added gently to packaging cells and incubated overnight at 37°C and 5% CO₂. The following day, spent media

were refreshed, and for the next 48 hours, lentivirus-containing supernatants were harvested once daily. The harvested media were passed through a 0.4-mm filter to remove cells and cellular debris and then concentrated via centrifugation for 15 min at 2000g in Amicon Ultra-15 (Merck) filter units. For target cell transduction, A549 cells were plated in 500 μl of complete RPMI in a 24-well plate at a density of 100,000 cells per well. After overnight incubation, polybrene (1 mg/ml) and the lentiviral supernatant diluted 1:10 (i.e., 50 μl per 500 ml) were added to the target cells. For THP-1 suspension cells, 5×10^5 cells were incubated with 50 μl of the viral supernatant and polybrene (1 mg/ml) in 6-well plates (3 ml of RPMI) and then spinoculated for 30 min at 600g. For all cell lines, after overnight incubation at 37°C and 5% CO₂, complete RPMI media were refreshed. Transduced cells were selected using puromycin (2 $\mu\text{g}/\text{ml}$; guide RNA positive cells) and blasticidin (10 $\mu\text{g}/\text{ml}$; *RfxCas13d* positive cells).

Cell sorting, RNA extraction, cDNA synthesis, and RT-qPCR

A375 and HCT116 cells were sorted 48 hours after transfection (for Cas13b-BFP+ cells) using a BD FACSaria Fusion 5 or Fusion 3 instrument. For all flow cytometry experiments, single-cell suspensions were resuspended in a fluorescence-activated cell sorting (FACS) buffer [2% FBS and 0.2% EDTA in phosphate-buffered saline (PBS)] and passed through a 35- μm nylon mesh cell strainer to exclude large aggregates. Single cells were gated based on morphology, and the threshold for Blue Fluorescent Protein (BFP) positivity was selected using an untransfected cell suspension as a nonfluorescent control. Total RNA was isolated from the recovered cells ($\sim 5 \times 10^5$ cells) using NucleoZOL one-phase purification (Machery-Nagel) according to the manufacturer's instructions. One microgram of total RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) following the manufacturer's instructions. RT-qPCR was performed in duplicate in a StepOne Real-Time PCR system (Thermo Fisher Scientific) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The total reaction mixture contains 0.2 μl of cDNA, 0.6 μM forward primer, and 0.6 μM reverse primer (sequences available in table S2).

Western blotting

Cells were washed with ice-cold PBS and lysed on ice in a radioimmunoprecipitation assay lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP-40, and 2% SDS (pH 7.5)] containing a protease inhibitor cocktail (Roche, 04693159001) and phosphatase inhibitor cocktail (Roche, 4906845001). Samples were incubated for 30 min at 4°C with rotation (25 rpm) and centrifuged at 16,000g for 10 min at 4°C, after which the supernatants were recovered. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples for polyacrylamide gel electrophoresis were prepared by combining 25 μg of the protein lysate with 4x Bolt LDS sample buffer (Invitrogen) and 10x Bolt Sample Reducing Agent (Invitrogen) in 30- μl total volume and denatured at 95°C for 5 min. Samples were loaded adjacent to a PageRuler prestained protein ladder (Thermo Fisher Scientific) onto Bolt Bis-Tris 4 to 12% gels Mini Protein Gels (Invitrogen) in 1x MES SDS running buffer (Invitrogen). Polyacrylamide gels were electrophoresed at 100 V for 1.5 hours. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes {activated in 100% methanol for 5 s, rinsed with Milli-Q H₂O, and then soaked in a 1x transfer buffer [25 mM tris-HCl

(pH 7.3), 192 mM glycine, and 20% v/v methanol]] using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA, United States) apparatus (20 V, 30 min). After protein transfer, membranes were blocked with 5% skim milk [w/v in PBS/0.5% Tween (PBS-T)] at RT for 1 hour and then incubated with a primary antibody (table S3) diluted in 5% (w/v) skim milk and incubated on a roller at 4°C overnight. The membrane was washed with PBS-T (3 x 5 min) and incubated in a secondary antibody (table S3) diluted in Intercept Blocking Buffer (LI-COR) for 1 hour at RT. After washing (3 x 5 min), the membrane was imaged using an Odyssey DLx Imager (LI-COR). Densitometry quantification was performed using the Image Studio software (LI-COR).

Resazurin reduction assay

A resazurin sodium salt working solution (Sigma-Aldrich, R7017-1G) was prepared by resuspension in PBS (pH 7.4) to a final concentration of 0.15 mg/ml. Working solutions were filter sterilized and stored at 4°C in light-protected tubes. On day 0 of the time course (baseline), 1×10^6 THP-1 cells (lentivirally transduced with doxycycline-inducible *RfxCas13d* and either crNT, crNRAS, or crNRAS-G12Dv2) were seeded in 6-well plates in 3 ml of RPMI containing doxycycline (2 µg/ml). For proliferation assays, at 48-hour intervals, the cell suspension (3 x 200 µl) was aspirated from each well and transferred into a 96-well assay plate, and an equivalent volume was replaced in the THP-1-containing 6-well plates to maintain constant volume. Forty microliters of the resazurin working solution was added to the triplicate wells in the 96-well plate and incubated at 37°C for 4 hours. Resazurin reduction was calculated by subtracting the background absorbance at 600 nm from the absorbance at 570 nm using the Cytation 5 Multi-mode Plate Reader (Agilent), with data normalized to baseline values.

Colony-forming assay

A549 cells (transduced with doxycycline-inducible *RfxCas13d* and either crNT or crG12S) were harvested, counted, and seeded at low density (500 cells per 6-well plate) in 3 ml of complete RPMI containing doxycycline (2 µg/ml). After 10 days of incubation at 37°C and 5% CO₂, cells were washed once with 1x PBS, fix/stained with 0.5% crystal violet staining solution containing 50% v/v methanol, and incubated for 30 min at RT. Each well was then washed three times with 1x PBS, and then the plates were dried for 24 hours and imaged.

Data and statistical analyses

All experiments were performed in at least three biological replicates unless otherwise indicated, and data are expressed as means $\pm$ SD. For data presented in Fig. 2F, silencing efficiencies against WT versus SNV were averaged from three independent experiments and plotted in R (version 4.1.0) using the ggplot2 (51) package (script available in data S1). All statistical analyses were performed with the GraphPad Prism 9 Software (v9.4.1, GraphPad, CA, United States), and *t* tests were used to compare pairs of data. $P < 0.05$ was used as the threshold of rejecting the null hypothesis, and statistical significance is shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Supplementary Materials

The PDF file includes:

Figs. S1 to S6
Tables S1 to S4
Raw Western blots
Legend for data S1

Other Supplementary Material for this manuscript includes the following:

Data S1

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