UNIVERSITY OF CALIFORNIA SAN DIEGO

Cas13d binding specificity mitigates off-target activity

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by

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The Thesis of Seungmin Nam is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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CRISPR-Cas13 has been developed as a tool for genome editing by enabling precise RNA manipulation without permanently altering the genome. While its potential in therapeutics and diagnostics is promising, Cas13d's off-target activities have limited its further use. To enhance our understanding of Cas13d's binding specificity, we performed a massively parallel filter-binding assay probing the impact of mismatches in the target RNA on

binding. Our results revealed that Cas13d binding is greatly affected by the specific position of the mutations in the target sequence, with less dependence on the type of mutation. Mismatches in the 5’ region of the target sequence were well tolerated, while those in the central region abrogated binding. These results emphasize the requirement for a complementary sequence in the central region for specific Cas13d binding. Additionally, this study expands the filter-binding method as a valuable tool for analyzing the binding specificity and off-target effects of Cas13d. Thus, our work lays a solid foundation for future high-throughput profiling of Cas13d binding and applying Cas13d for safe and effective transcriptional perturbation.

INTRODUCTION

CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, has been developed as a tool for genetic engineering following its discovery as an adaptive immune system in bacteria (Abudayyeh et al., 2016). The CRISPR systems are largely divided into two classes based on the composition of the CRISPR-associated (Cas) proteins. Class 1 CRISPR systems are made up of several protein subunits while the Class 2 systems have a single protein (Makarova et al., 2020). Class 2 CRISPR systems are known to be easier to engineer and manipulate for various applications. CRISPR-Cas13 is a Class 2 CRISPR system and the only CRISPR system that exclusively targets RNA instead of DNA (Abudayyeh et al., 2016; Konermann et al., 2018), making Cas13 a powerful tool for a wide range of applications.

With increased efficiency and specificity, Cas13 has emerged as a versatile tool for diagnostic, therapeutics, and research (Zhao et al., 2018). As such, since its discovery, Cas13 has been adapted for use in diagnostic applications, offering a tool for detecting the presence of specific nucleic acid sequences (Kellner et al., 2019). Recently, Cas13 was used to detect SARS-CoV-2 RNA for diagnosis (Casati et al., 2022; Zhou et al., 2022) and has also been studied to diagnose cancer at an early stage (Palaz et al., 2021). Additionally, Cas13 has potential therapeutic applications for treating genetic disease as programming of the CRISPR-Cas13 system successfully targeted and reduced mutant alleles of Amyotrophic Lateral Sclerosis and Huntington's disease (Morelli et al., 2023; Powell et al., 2022). Taken together, Cas13 holds great potential for studying and intervening RNA-related diseases.

Off-target effects remain an impediment to therapeutic and diagnostic applications of Cas13. Cas13 can load a programmable guide RNA (gRNA) that directs its targeting to RNA templates. The full ribonucleoprotein (RNP) complex binds to a target RNA sequence and

catalyzes a ssRNA break (Wessels et al., 2020). Off-target activity occurs when the Cas13 complex binds to a similar sequence of RNA that is not the target site and activates despite one or more bulges or mismatches. As a relatively recent addition to the Type VI Cas13 family, Cas13d has been less studied than the other subtypes of Cas13 but has been shown to be efficient even with its small and compact size (Gupta et al., 2022; W. X. Yan et al., 2018). While there have been many studies confirming the presence of such off-target effects in Cas13d (Ai et al., 2022; F. Yan et al., 2019), there is still a lack of understanding on the binding specificity and off-target effects of Cas13d.

An important step to minimizing the off-target effects of Cas13d is to design an efficient and specific guide RNA since sequence similarity of the target RNA is a cause for off-target binding (Li et al., 2022). Therefore, by designing various sequence perturbations of the target RNA ranging from a perfect match to mismatches and indels, we sought to analyze how Cas13d binding is affected by diverse perturbations to target sequence.

In this work, we measure dCas13d binding using massively parallel filter binding (Boyle et al., 2021) with a custom designed 3D printed part. We design a library of target RNAs containing systematic mismatches, deletions, and insertions. We separated protein-bound RNA, retained on the nitrocellulose membrane, from unbound RNA that pass through nitrocellulose membrane into a collection tube. We quantify dCas13d binding and learn principles of Cas13d activity. Through this study, we demonstrate the power of massively parallel filter binding for characterizing RNA-protein interactions and improve understanding of binding rules for Cas13d.

RESULTS

Measuring dCas13d binding through filter binding assay

We first designed an oligonucleotide pool of 1,328 target sequences for a well characterized guide RNA targeting SNRNP 200. This comprehensive library included not only the wildtype target sequence, perfectly complementary to the guide RNA, but also sequences with a spectrum of sequence perturbations, spanning single mismatches, consecutive and pair mismatches, as well as single, double, and triple deletions and insertions (Figure 1A, 1B). The library of target sequences was incubated with the dCas13d RNP for various time intervals of 0, 1, 2, 3, 4, 5, 8, 11, 15.5, 21.5, 30, 42, and 60 minutes. We then performed a massively parallel filter binding assay to measure dCas13d target RNA binding. The filter binding method used a vacuum to filter out the unbound RNA that passed through the nitrocellulose membrane in the flow-through while the protein-bound RNA remained on the nitrocellulose membrane. The unbound RNA in the flow-through was then barcoded based on the duration of incubation with the dCas13d-gRNA complex and were then sequenced (Figure 1C).

We counted the occurrences of each sequence in the flow-through, generating a count data representing the unbound RNA, which was then utilized to calculate the fraction of bound RNA for each target sequence at each time point (Materials and Methods). This approach allowed us to create a comprehensive data from the sequencing results, providing insight into the binding of different target sequences at each time point. The obtained data demonstrated reproducibility across all sequence variations, as evidenced by consistent results from two biological replicates (Figure 1D).

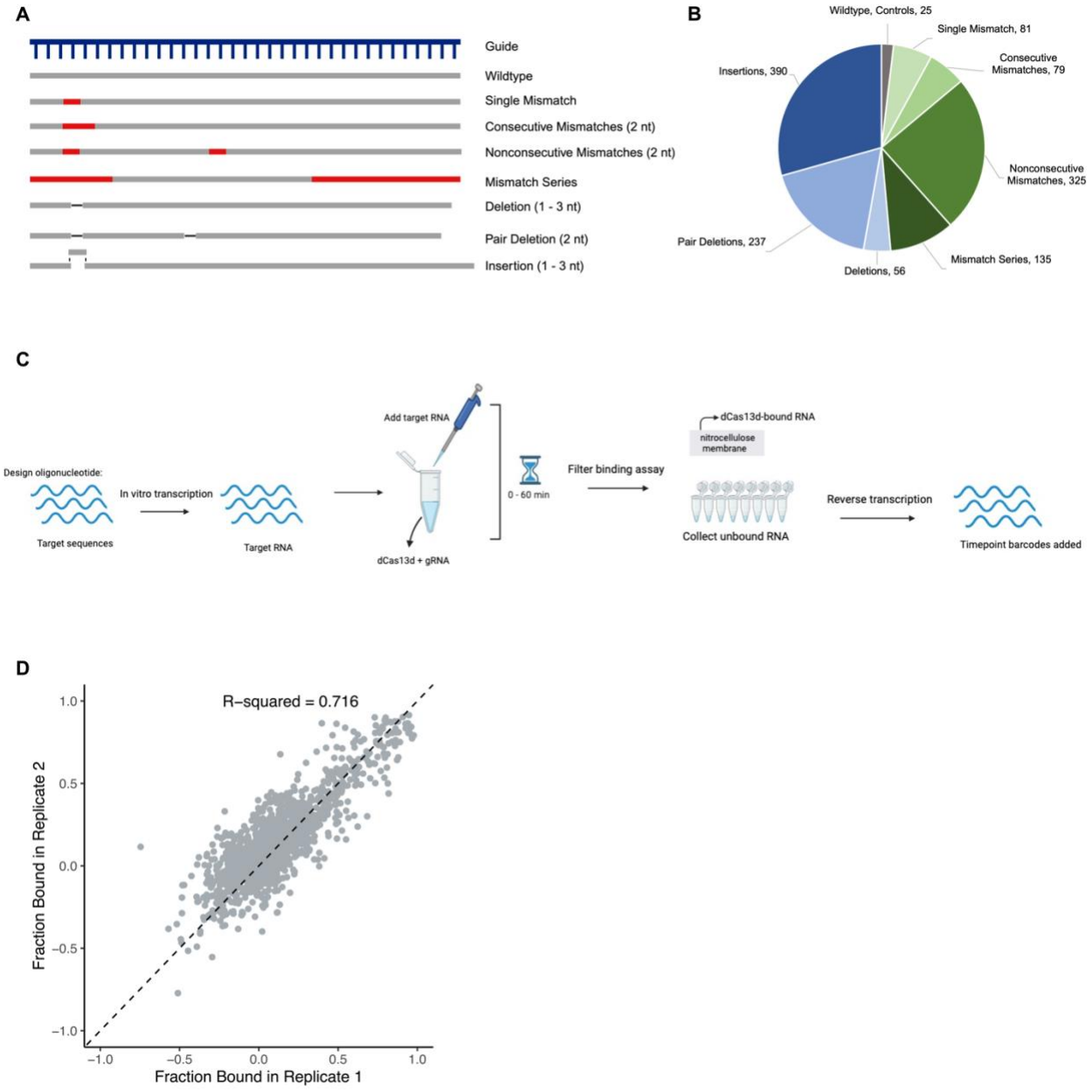

Figure 1. Analysis of Cas13d binding. (A) Design of target sequences in oligonucleotide pool. **(B)** Distribution of sequence perturbations in target library. **(C)** Overall experiment workflow. **(D)** Reproducibility of fraction bound at 60 min for two filter-binding experiments.

Position-dependent binding activity observed for Cas13d

To evaluate how mismatches in the target RNA affect the binding to dCas13d protein, we calculated the fraction bound of each target RNA sequence relative to the fraction bound of the wildtype sequence. The study revealed that the binding was more affected by the position of the mismatch within the target sequence, while being independent of the relative nucleotide at the mismatch site for single mismatches. The binding pattern was similar across all mismatches regardless of the nucleotide (Figure 2A). The 5' region in the target sequence showed little to no decrease in fraction bound compared to the wildtype sequence (Figure 2B). Interestingly, the fraction bound was higher than the fraction bound of the wildtype sequence in some cases. This suggests that a single mismatch in this region has little effect on determining the binding specificity of dCas13d protein. On the other hand, mismatches located near the central region of the target (positions -18 to -11) showed a large reduction in the fraction bound when compared to the wildtype target sequence (Figure 2B). This suggests that any mismatches in this region of the target RNA can have a severe impact on the binding specificity of dCas13d.

Deletions in the target sequence had a more severe impact on binding than mismatches. As expected, a single deletion was better tolerated than double and triple deletions (Figure 2C). However, the position of the mismatch typically determined the fraction bound compared to the number of deletions in the target sequence. A similar binding pattern to single mismatches was observed as the 5' region was better tolerated than other positions in the target sequence. While the positions that were greatly affected by deletions were larger than single mismatches, it was similar in that the central region was still shown to be intolerant to mismatches as it showed a large decrease in binding with the deletions (Figure 2D).

Insertions in the target sequence seemed to be better tolerated than deletions and single mismatches, with single insertions as the most tolerated (Figure 2E). As with other sequence perturbations, the central region seemed to be the sensitive region in the target sequence for insertions as well. Interestingly, insertions were well tolerated in both the 5' and 3' regions of the target sequence (Figure 2F).

To evaluate how mismatches in the target RNA affect the binding of dCas13d protein, each target sequence was visualized based on the type of mismatches present at each position to see how binding changed over time (Figure 3). While the other mismatches showed little binding, position -23 to -20 in the target sequence showed a binding curve similar to the wildtype for the single mismatches, single deletions, and single insertions. The binding curve for these positions confirmed association of dCas13 protein over time.

These findings show that the type of mismatch present in the target sequence did not have as large an effect on the binding as the position of the mismatch. It is true that deletions had the most detrimental effect on the fraction bound than mismatches and insertions. Most single mismatches and insertions showed a modest decrease in fraction bound at the center region of the target sequence. In contrast, deletions showed greater decrease in fraction bound at the central region. Notably, deletions also exhibited a substantially lower fraction bound in the 3' region of the target sequence. However, single mismatches and insertions were less affected in the 3' region, showing little to no difference in fraction bound compared to the wildtype sequence. These results show that while deletions were less tolerated than mismatches and insertions, the mismatch position was more important when determining the binding specificity.

Overall, these findings emphasize the critical role of the central region in determining the binding specificity of dCas13d. It also confirms that mismatched sequence in the 5' region of the

target sequence in some cases does not interfere with binding of dCas13d protein. These results highlight the importance for complementarity to target sequence in the central region for the specific binding of the dCas13d protein.

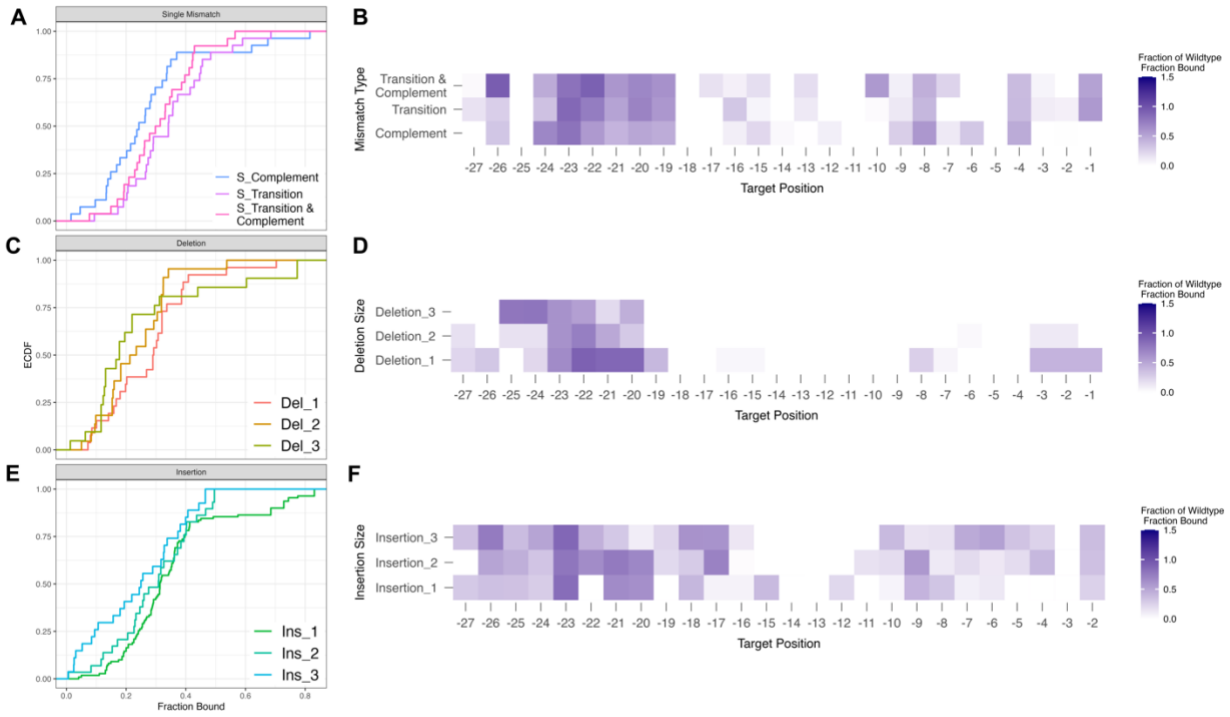

Figure 2. Fraction of target RNA bound to dCas13d protein across mutation positions. Distribution of fraction bound for target sequences with **(A)** single mismatch **(C)** deletions **(E)** insertions. Fraction of bound RNA is compared to the wildtype sequence for mismatch positions in the target sequence for **(B)** single mismatch **(D)** deletions, **(F)** insertions.

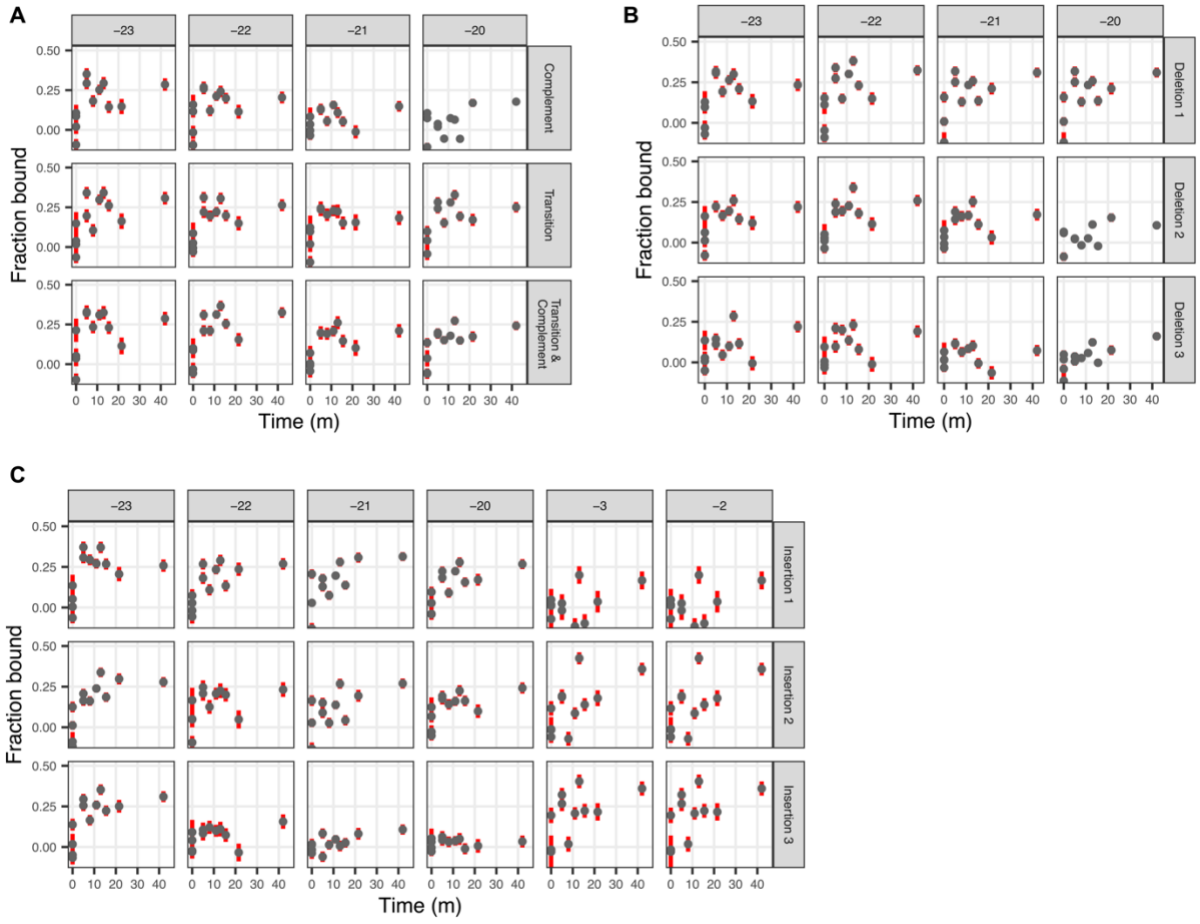

Figure 3. Position dependent binding of dCas13d. Fraction bound over time for **(A)** single mismatch **(B)** deletions **(C)** insertions.

Double and pair mismatches show position importance in determining binding

We expanded our examination to consecutive and nonconsecutive double pair mismatches, aiming to explore how these combinations impact the target RNA binding. Relative binding activity of off-targets was similar to what we observed with single mismatches and deletions. In cases of consecutive mismatches, a similar binding pattern was shown compared to single mismatches at the same positions. Here, the type of mismatch exhibited no detectable impact on binding (Figure 4A). Instead, the pivotal factor remained the position of the mismatches within the sequence (Figure 4B).

For nonconsecutive sequence perturbations, paired mismatches were generally more tolerated than paired deletions, which aligns with our observation with single mismatches and deletions. Again, both the paired deletions and mismatches showed a similar position-dependent binding pattern. When both mismatches occupied the central region, binding substantially decreased (Figure 4C). On the other hand, when mismatch pairs were both present in the 5' region, they were well tolerated (Figure 4C). Interestingly, the central region did not greatly affect the fraction bound when it was paired with a mismatch in the 5' region (Figure 4C). We found this surprising because a single mismatch in the central region showed a large decrease in binding (Figure 2). This suggests that a mismatch in the 5' region might be a factor that increases the binding of dCas13d. This is consistent with our data showing that a single mismatch in the 5' region can increase binding as well (Figure 2B).

The position of any mismatches carries more weight in determining the impact on binding than adding a second mismatch. Our investigation in double and pair mismatches underscores the position-specific nature of their influence on binding. While the double mismatches generally showed a similar binding activity compared to single mismatches and

deletions, the relationship between the 5’ region and central region emerged as key determinants in shaping the binding. This enhances our comprehension of the intricate relationship between mismatch types, their positions, and their collective impact on the binding specificity of dCas13d proteins.

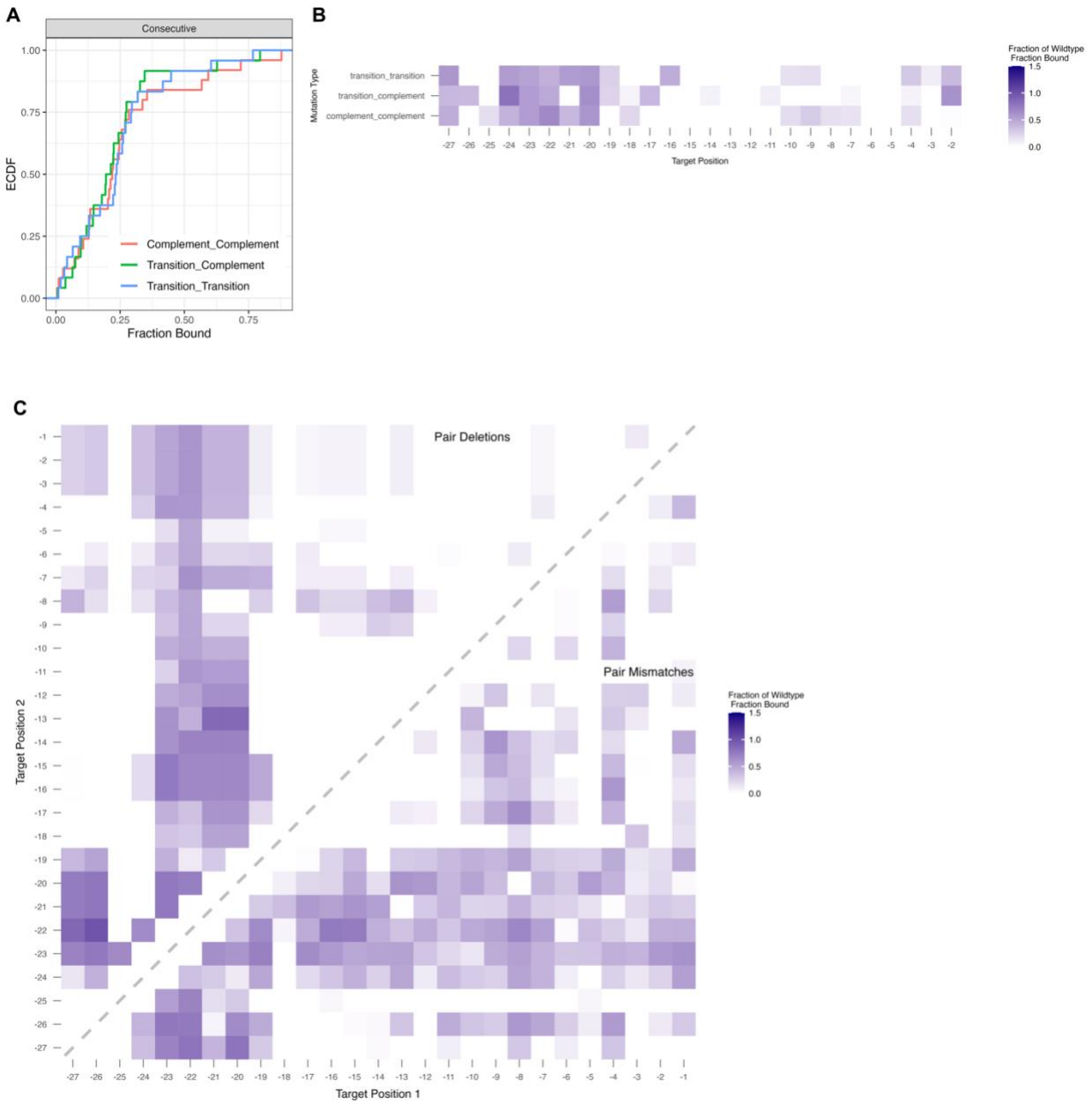

Figure 4. Fraction bound of pair mismatches and deletions. (A) Distribution of fraction bound across consecutive double mismatches. **(B)** Fraction bound across mismatch position of consecutive double mismatches. **(C)** Fraction bound across each pair of mutations for pair mismatches and pair deletions. (Above: pair deletions; Below: pair mismatches)

DISCUSSION

In this study, our goal was to increase the understanding of off-target effects associated with Cas13d proteins using a filter binding assay. We designed target sequence perturbations to explore how mismatches across that target sequence affect Cas13d binding. Our exploration through this method enabled crucial insights into the specificity of the Cas13d protein. Notably, our observations indicate that the binding of Cas13d is heavily influenced by the specific position of mutations within the target RNA sequence, with less dependence on the type of mutation - mismatch, deletion, or insertion. We have identified specific regions within the target RNA that exhibit a higher tolerance for mutations with a distinct region intolerant to such mutations. Thus, we propose the central region as the "seed" region that determines the binding specificity of Cas13d.

Our study shows Cas13d binding greatly depends on the position of the mutation in the target RNA, specifically mismatches in the central region of the target were intolerable to any type of mutations. Previous studies have identified Cas13a and Cas13b to have a sensitive region around the center that significantly affected the binding (Bandaru et al., 2020; Liu et al., 2017; O'Connell, 2019; Tambe et al., 2018). Cas13d has also been shown to have a region intolerable to mismatches (O'Connell, 2019; Wessels et al., 2020). While the exact locations vary, our findings for Cas13d agree with previous findings and propose the central region being a crucial region that determines the specificity of Cas13d activity. This study expands on previous findings by including more types of mutations across each position in the target sequence and clarifies that the loss of activity is mediated by reduced protein occupancy at target sites. On the other hand, the 5' region of the target RNA showed high tolerance for any type of mutations introduced including pair mismatches and deletions. Mutations in these positions did not affect

the fraction bound when compared to wildtype sequence. These results show the importance of the central region having a complementary sequence to the guide RNA for efficient binding of Cas13d.

Interestingly, certain mismatches in the 5' region modestly increased binding and appeared to mitigate the effect of mismatches in the central region. Thus, it is possible that off-targets that are more dissimilar in terms of sequence distance may exhibit superior binding. A possible explanation could be that introducing a mismatch in the 5' region leads to changes in the secondary structures of RNA that favor the bound complex state or disfavor the free RNA state. Further research is needed to explore this behavior across other guide RNAs to see if this is unique to this guide sequence.

This study is limited in that only one guide RNA was screened through the filter binding assay. However, the reliability demonstrated by the filter binding assay proposes its potential as a valuable tool for understanding Cas13d off-target effects across a diverse array of guide RNAs. Varying concentrations of both the protein and guide RNA will give insight into the kinetics of Cas13d binding. The mismatch "seed" region proposed in this study only refers to the binding of Cas13d. It must be noted that the cleavage activity might be affected differently as previous research has shown the case in Cas13a (Tambe et al., 2018).

Altogether, our study opens promising avenues for further research, deepening our understanding of Cas13d off-target effects. Future studies could include an extensive analysis of the binding activity across diverse guide RNA sequences for various concentrations, shedding light on the binding kinetics of Cas13d. Moreover, linking biochemical determinants of Cas13 activity to activity *in vivo* will provide a more complete understanding of how Cas13-based technologies work. Our findings establish a foundational groundwork, paving the way for future

studies in enhancing the specificity of Cas13d, potentially transforming its application in therapeutic contexts and unlocking the full potential of Cas13d for transcriptome engineering.

MATERIALS AND METHODS

Target library design and preparation

An oligonucleotide library of target sequences was designed against the SNRNP200 guide through a custom python script. Each sequence featured a 27-bp target region with 8-bp flanking regions on both the 5' and 3' ends. Additionally, an 18-bp region each end accommodated forward and reverse primers. This target library encompassed various sequence perturbations, including the wildtype, control, mismatches, insertions, and deletions.

The wildtype sequence represented the complementary sequence to the guide. The control sequences included a randomized arrangement of nucleotides, which were later used for calculating the fraction bound. For single mismatches, each position within the 27-bp target region was mutated to include the other three nucleotides. Deletions and insertions ranged from single to double and triple nucleotides, resulting in a different length of the target region. The library also included consecutive mismatches, where the adjacent nucleotides were mutated, along with pair mismatches, where the nonadjacent pair of nucleotides were mutated. Pair deletions were nonadjacent pairs of nucleotides deleted, and series mismatches included a long stretch of nucleotides being mutated. After removing duplicates, the target library comprised 1,328 unique sequences. This comprehensive set included 1 wildtype sequence, 24 control sequences, 81 single mismatches, 69 consecutive mismatches, 325 pair mismatches, 135 insertions, 56 deletions, 237 pair deletions, and 390 mismatch series. Each unique sequence was assigned a number and a unique identifier for further analysis.

To prepare the target RNA, the library was amplified with forward and reverse primers (Table 1). In-vitro transcription was performed using T7 Polymerase. The target sequence was

incubated at 37℃ for 16 hours with T7 RNA Polymerase Mix, NTP, and 10X reaction buffer (Catalog #: E2040S) then treated with DNase I. Finally, the target RNA was diluted to 45nM.

Filter Binding Assay

To prepare dCas13d RNP, the dCas13d protein was diluted to 150nM in dCas13d buffer [250mM KCL, 100mM HEPES pH 6.8, 25mM $MgCl_2$, 25% glycerol]. Subsequently, the sgRNA was incubated at 98℃ for 1 minute, followed by dilution to 175.5nM (5% excess compared to the protein concentration). The sgRNA was loaded to dCas13d protein in a 1:1 ratio, allowing for a minimum 1-minute incubation at room temperature, resulting in a final concentration of 75nM dCas13d and 78.75nM sgRNA. Once all components were prepared, the filter binding apparatus, a custom designed 16 well base and an adapter, was assembled with a vacuum source connected to it. The nitrocellulose membrane was soaked in dCas13d buffer before being placed on top of the adapter.

Then, target RNA was added to dCas13d RNP to reach a final concentration of 1.5nM. The target RNA was incubated with RNP at room temperature for 0, 1, 2, 3, 5, 8, 11, 15.5, 21.5, 30, 42, 59, and 60 minutes. When the incubation reached each time point, each sample was pipetted onto the nitrocellulose of a well on the custom designed filter binding apparatus. The vacuum attached to the apparatus pulled down the unbound RNA at each time point into the flow-through tubes, which was then collected for further analysis.

The flow-through from the filter-binding experiment was then reverse transcribed with Superscript IV Reverse Transcriptase. The primers were unique to each time point to serve as time point barcodes (Table 2). Once these primers were annealed to target RNA, the RT reaction mix was prepared with 5x SSIV buffer, 100mM DTT, RNase Inhibitor, and Superscript IV

Reverse Transcriptase (Catalog #: 18090010). These were incubated at 55℃ for 10 minutes following an inactivation of 10 minutes at 80℃, followed by a PCR amplification.

Sequencing Data Analysis

After sequences were preprocessed for quality control, the sequencing data was analyzed through custom python scripts. The target sequences were counted for each unique occurrence at each timepoint, which were then aggregated into a count table. These counts were used to calculate the fraction bound at each time point (Boyle et al., 2021).

Table 1. Primers for target library amplification.

Name	Sequence
Twist_AmpF2	TTCTAATACGACTCACTATAGCGTAAACCGTG
Twist_AmpR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

Table 2. Primers for Reverse Transcription (Time point barcodes).

Name	Sequence
A1_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATCCTAGTCGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A2_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATTTAGCCTAGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A3_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATACCATCTTGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A4_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATTAACTCCTGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A5_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATAGCCTCTAGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A6_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATCACCATCGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A7_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATGATTGGAAGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
A8_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATCCTCTCAGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B1_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATATGTACGGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B2_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATACCAGGTAGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B3_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATTTGGCATTGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B4_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATGGTTCTAGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B5_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATATTCCAGTGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B6_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATACTATGCTGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B7_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATAGGTGTTAGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
B8_TruSeq_i7	CAAGCAGAAGACGGCATACGAGATGCACATTGGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC

Table 3. Primers for PCR amplification of RT reaction.

Name	Sequence
E1_TruSeq_i5	AATGATACGGCGACCACCGAGATCTACACTTGATAGGACACTCTT TCCCTACACGACGCTCTTCCGATCTGCGTAAACCGTG
E2_TruSeq_i5	AATGATACGGCGACCACCGAGATCTACACCATTCCATACACTCTT TCCCTACACGACGCTCTTCCGATCTGCGTAAACCGTG
i7_PCR_primer	CAAGCAGAAGACG

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