Conformational Control of Cas Endonucleases by CRISPR Hybrid RNA-DNA Guides Mitigates Off-Target Activity in T Cell

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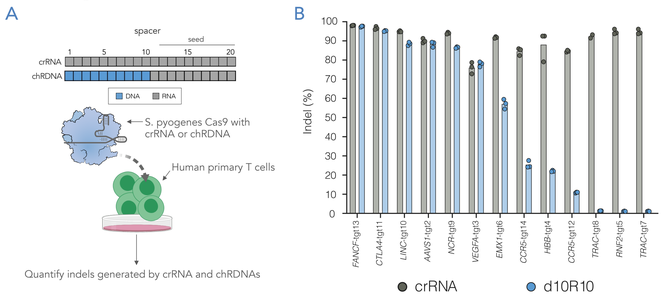
Overview

CRISPR-based genome editing of primary human T cells has the potential to revolutionize immunotherapies; however, unintended editing at off-target sites remains a major concern. To address this, we show that CRISPR hybrid RNA-DNA (chRDNA) guides composed of both RNA and DNA nucleotides are a highly effective approach to minimize off-target editing while preserving on-target editing activity. From the combined understanding of chRDNA design principles and mechanistic data, we designed chRDNA guides for use with the Cas12a CRISPR system. Much like Cas9 chRDNA guides, engineered Cas12a chRDNA guides support robust Cas12a-mediated on-target editing in human T cells without detectable off-target editing. Our results demonstrate the portability of the chRDNA platform across CRISPR systems and its utility for enabling highly efficient and precise genome editing for the generation of cell therapies.

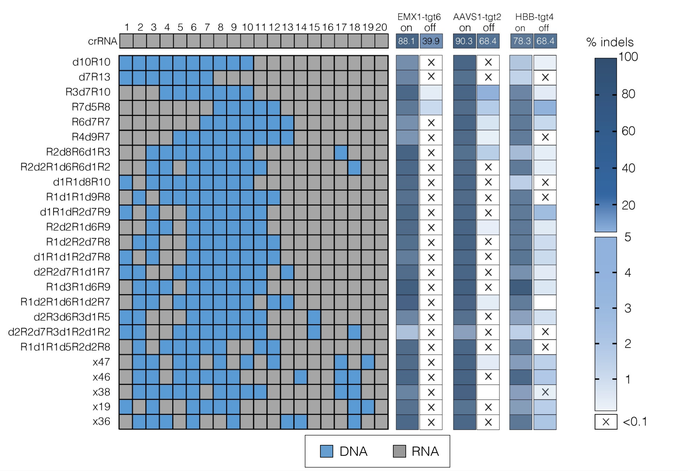
Variable on-target editing across targets with fixed Cas9 chRDNA design

To understand the design principles for chRDNAs, we selected 14 targets and a single chRDNA design comprising 10 nt of DNA at the 5′ end and 10 nt of RNA at the 3′ PAM-proximal end (d10R10). We then programmed recombinant Cas9 with an RNA or a chRDNA guide and transfected the complexes into human primary T cells. 48 hrs later we harvested genomic DNA and quantified the indels via NGS (A). We observed that the d10R10 chRDNA design showed equivalent levels of on-target editing for some targets; however other targets showed large reductions in editing (B). This finding hinted that there might not be a "one-size-fits-all" approach for chRDNA designs across different target sites.



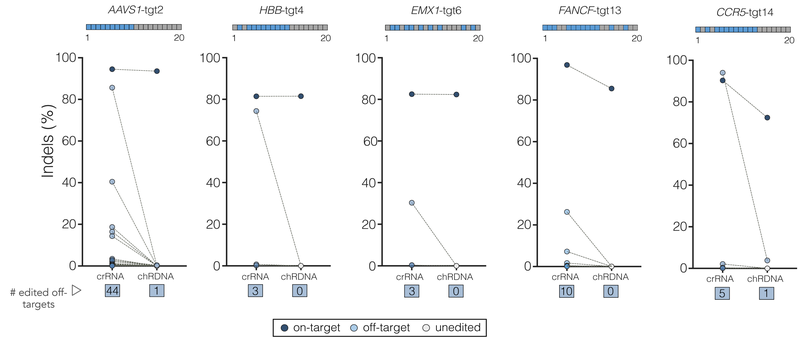
Target specific screening to optimize Cas9 chRDNA designs

To identify chRDNAs in a target-specific manner, we designed a panel of 25 chRDNAs varying the number and positions of DNA bases. We screened this panel against three of the targets from our initial set of targets and evaluated chRDNA specificity at a high activity off-target site (three are shown in the figure below) in T cells. From this panel we identified multiple chRDNA designs for each target with on-target editing comparable to the all-RNA guide control, but with a substantial reduction in off-target editing (to below our limit of detection of 0.1%). We observed that, although multiple top designs were identified, no single design was universally the best across the three targets, reinforcing the idea that optimization of a chRDNA is essential for robust guide performance.



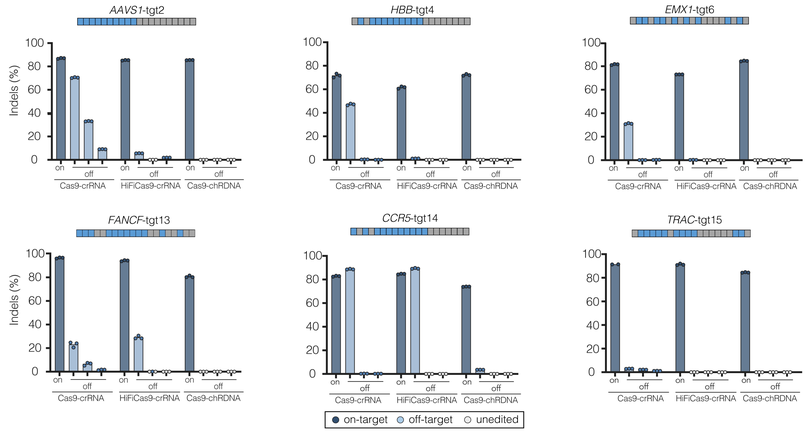
Tunable chRDNA activity across a broad collection of on- and off-target sites

We used our target-based screening approach to identify optimal chRDNA designs for other targets and evaluated chRDNA off-target activity across a broad set of cellular off-targets. Evaluation of optimized chRDNAs in T cells across this broad collection of off-targets showed that optimized chRDNAs resulted in either elimination of off-target editing or a severe reduction across multiple loci.



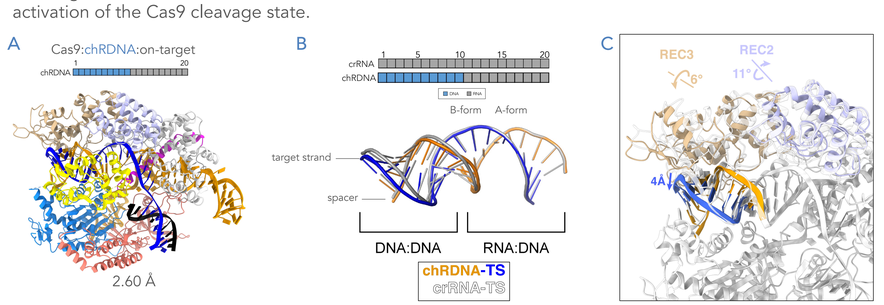
chRDNAs show higher specificity than an engineered high-fidelity Cas9 variant

To understand how our optimized chRDNAs compare to other high-specificity technologies, we performed a head-to-head comparison with a commercially available engineered Cas9 previously reported to exhibit improved specificity[15]. The "high-fidelity" Cas9 showed marked improvement to off-target editing rates compared to wild-type Cas9; however, at some of the targets tested we detected residual off-target editing, or in the case of the CCR5-tgt14 target, no improvement in specificity. In contrast, our Cas9 chRDNAs displayed comparable on-target editing rates to the all-RNA guide and greater specificity than the engineered Cas9 across all targets evaluated.



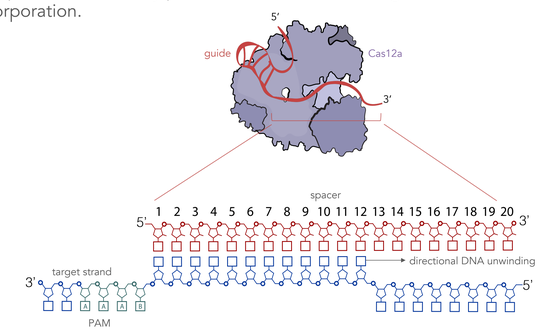
Cas9 chRDNAs improve off-target discrimination through conformation distortion of the guide-target heteroduplex

To understand how chRDNAs improved specificity of the Cas9 system, we co-crystalized Cas9 in the presence of a DNA target with an all-RNA guide or a chRDNA. Comparison of the spacer-target strand heteroduplex showed that the DNA:DNA portion of the chRDNA structure was distorted into a B-form conformation compared to an A-form conformation seen for the entire length of the all-RNA guide heteroduplex. This altered structure results in discordant positioning of the duplex and the rearrangement of the REC3 and REC2 domains, both of which are critical in the conformational activation of the Cas9 cleavage state.



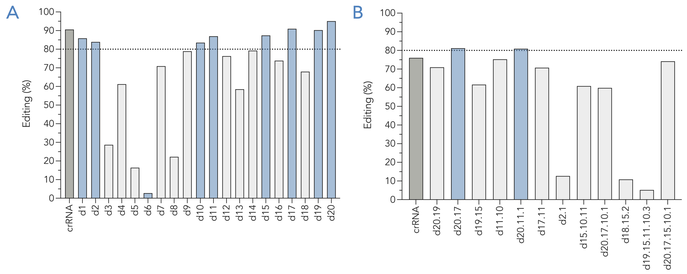
Engineering chRDNA guides for the Cas12a platform

Based on our work with the Cas9 chRDNA platform, we postulated that chRDNA guides could be engineered for other Cas nucleases. To test this, we selected the Type V *Acidaminococcus* Cas12a. Because of the significant difference in protein sequence and guide architecture between Cas9 and Cas12a, we developed a unique screening platform to identify positions within the Cas12a guide that are amenable to DNA incorporation.



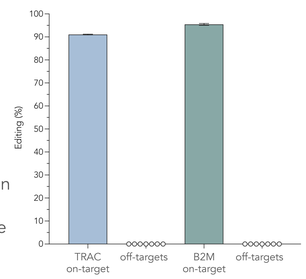
Iterative DNA position 'blending' for optimal Cas12a chRDNAs

For a given target sequence, individual chRDNAs were tested with a single DNA base at each position in the 20-nucleotide spacer and then evaluated for on-target editing activity in T cells (A). Those positions where DNA bases did not reduce editing were combined in subsequent chRDNA guide designs to identify optimal chRDNAs with multiple DNA bases in the optimized sequence (B).



Demonstrated high specific and activity of Cas12a chRDNAs

To confirm the specificity of optimized Cas12a chRDNAs guides, we transfected T cells with Cas12a chRDNA complexes and evaluated editing at the on-target site and a plurality of off-target sites. We observed robust on-target editing consistent with rates observed during chRDNA development and confirmed that the optimized Cas12a chRDNA demonstrated no off-target editing above our limit of detection (0.1%) at any of the off-target sites tested. Taken together, we confirmed that chRDNA guides function with multiple Cas nucleases to improve the specificity of each editing platform while maintaining robust on-target editing rates.



Conclusion

In summary, chRDNA guides provide a highly customizable approach for improving the specificity of CRISPR genome editing superior to that achievable with a high-fidelity Cas protein variant. Through the iterative engineering of DNA positions in chRDNA guides, we can "tune" the activity and specificity of chRDNA guides in a target-specific manner. Our structural analysis with the Cas9 nuclease shows that chRDNA guides adopt distorted helical conformations upon target hybridization, which disfavors engagement of off-target sequences. Additionally, we validated the portability of the chRDNA platform across CRISPR systems by designing high specificity chRDNA guides for the Cas12a nuclease. Together, these results demonstrate that chRDNAs enable highly efficient and precise genome editing, paving the way for their utilization across CRISPR systems for application in therapeutics.

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[15]Vakulskas et al., 2018, Nat Med, 24(8):1216-1224.