Improved genome editing with Cas12a and Cas9 chRDNA platform in T, NK, B, and iPS cells

Abstract
CRISPR-based genome editing of primary human T cells has the potential to revolutionize cell therapies. However, substantial improvements in CRISPR-Cas9 specificity are needed to significantly reduce its off-target activity in cells. Here we show that Cas12a-Cas9 hybrid RNA-DNA (chRDNA) guides designed with both RNA and DNA nucleotides are a highly effective approach to increase the specificity of Cas9 and Cas12a genome editors while preserving on-target editing activity. Across multiple genome targets in primary human T cells, we show that 2′-deoxyuridine positioning affects guide activity in a sequence-dependent manner, and we leveraged this observation to engineer chRDNA guides with minimal to no detectable off-target activities. To further enhance the capability of the Cas12a chRDNA platform for engineering of cell therapy, we optimized the nuclear trafficking sequence, thereby improving editing efficiency even at low concentrations of Cas12a across multiple cell types.

Cas9 chRNA design demonstrates improved specificity compared to an engineered Cas9 variant
The initial investigation of DNA incorporation into Cas9 RNA guides showed that Cas9 tolerated high levels, as high as 80%, of RNA replacement in the target-complementary spacer region. To identify ideal CRISPR designs, we employed an iterative approach to empirically determine the position and combination of DNA bases through successive rounds of Cas9 minimal to no detectable off-target activity. We then developed a new screening pipeline where we tested chRDNA with a single DNA base at each position in the spacer region (A). We then blinded individual DNA-permissive positions to generate new designs with multiple DNA bases positioned in the spacer without compromising on-target editing (B). Final chRDNA designs were tested for on- and off-target editing efficiencies in human T cells. Despite Cas12a all-RNA guides generally showing low off-target editing (compared to Cas9 all-RNA guides), optimized chRDNA guides further improved specificity resulting in no off-target edits above 0.1% when analyzed by next-generation sequencing. These results demonstrate the utility of the chRDNA platform across different classes of Cas nucleases and that incorporation of DNA bases into the spacer region sensitizes Cas nucleases to stable engagement at off-target sites.

Enhancing Cas12a editing levels through optimization of NLS sequence
Knowing that the manufacturing of single- and multiplex genome-edited cell therapies would benefit from consistent editing efficiencies, we endeavored to enhance the editing capability of our Cas12a platform through optimization of the nuclear localization signal sequence (NLS) appended to the C-terminus of the nuclease. We screened a variety of designs evaluating the type and number of NLSs as well as the linker length and composition used to append the NLS to the Cas12a. We found that multiple designs afforded improved editing levels across numerous targets compared to the standard gly-ser linker and (GGGGS)6 NLS (A). Selecting top targets from (A), we compared the editing in a time course assay and showed that the optimized NLS resulted in a more rapid accumulation of edits in T cells, perhaps indicating more rapid nuclear trafficking (B).

Optimized Cas12a-NLS improves sequential editing rates in iPSCs
To confirm the utility of the optimized Cas12a-NLS variant we utilized it for sequential editing in induced pluripotent stem cells (iPSCs). iPSCs were edited in a step-wise fashion at four genes with either the standard NLS or the optimized NLS, and we observed that all genes containing the optimized NLS yielded a population of cells where 75% of the cells would contain all edits, a 5-fold improvement in efficiency (C).

Cas12a achieves robust editing across multiple immune cell types
To further understand the broad utility of the optimized Cas12a-NLS beyond T and NK cells, we utilized Cas12a editing in human iNK cells, primary B cells, and macrophages achieving ≥80% on-target editing across multiple gene targets.

Summary
Knowing that the manufacturing of single- and multiplex genome-edited cell therapies would benefit from consistent editing efficiencies, we endeavored to enhance the editing capability of our Cas12a platform through optimization of the nuclear localization signal sequence (NLS) appended to the C-terminus of the nuclease. We screened a variety of designs evaluating the type and number of NLSs as well as the linker length and composition used to append the NLS to the Cas12a. We found that multiple designs afforded improved editing levels across numerous targets compared to the standard gly-ser linker and (GGGGS)6 NLS. Selecting top targets from (A), we compared the editing in a time course assay and showed that the optimized NLS resulted in a more rapid accumulation of edits in T cells, perhaps indicating more rapid nuclear trafficking (B).

Comprehensive overview of the Cas12a and Cas9 chRDNA platform
Cas12a and Cas9 chRDNA demonstrate improved specificity compared to all-RNA guides, while maintaining robust on-target editing rates
• Editing efficiency of Cas12a chRDNA is further enhanced through utilization of an optimized NLS, resulting in faster target cleavage and improved yields of multi-edited cell populations
• Cas12a chRDNA demonstrate broad and robust editing across multiple immune cell types
• Caribou utilizes both Cas9 and Cas12a chRDNA across three clinical-stage CAR T cell programs CB-010 (NCT04637836), CB-011 (NCT07522418), and CB-012 (NCT01260394) for the treatment of patients with hematologic malignancies.