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Structural and Functional Characterization of Rationally Engineered CRISPR-Cas9 Variants for High-Fidelity and Precision Genome Editing in Human Cells

Mona Al-Mansouri, Dimitri Alexandrov, Zhen Zhang, Clara Martins

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ABSTRACT

CRISPR-Cas9 genome editing has revolutionized genetic engineering; however, concerns regarding off-target effects and efficiency persist. This study explores rationally engineered Cas9 variants designed for precision genome editing in human cells. Through structural and functional characterization, we compare wild-type (WT) and engineered Cas9 variants, assessing target specificity, cleavage efficiency, and cellular responses. Using computational modeling, biochemical assays, and in vitro/in vivo validation, we identify Cas9 modifications that enhance genome editing fidelity. Our findings provide insights into the structural determinants of Cas9 specificity, guiding the development of next-generation CRISPR tools for therapeutic applications.

INTRODUCTION:

CRISPR-Cas9 has revolutionized genome engineering by allowing targeted DNA modifications with high precision. However, the wild-type Streptococcus pyogenes Cas9 (WT SpCas9) can introduce unintended off-target mutations, posing significant challenges for clinical applications. Addressing this issue requires the development of Cas9 variants with enhanced specificity while preserving editing efficiency.Recent advancements in protein engineering have led to the design of high-fidelity Cas9 variants, including eSpCas9, SpCas9-HF1, and HypaCas9, which reduce off-target activity by modifying key residues involved in DNA recognition and cleavage. Structural studies have provided insights into the molecular mechanisms underlying specificity, highlighting the role of guide RNA interactions, PAM recognition, and DNA unwinding dynamics.This study aims to characterize rationally engineered Cas9 variants through structural and functional analyses, evaluating their genome editing accuracy in human cells. By optimizing modifications that enhance target discrimination, we seek to develop safer and more efficient CRISPR-based therapies. Future research should focus on integrating AI-driven protein design and biochemical assays to refine Cas9 specificity further, ensuring its successful translation into

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precision medicine and therapeutic genome editing.

MATERIALS AND METHODS:

1. Cas9 Variant Engineering

Engineered Cas9 variants were designed using **rational protein engineering** informed by **structural and biochemical insights**. Site-directed mutagenesis was employed to introduce **specific amino acid substitutions** in the **REC (recognition) domain and PAM-interacting domain**, aiming to **enhance DNA-binding specificity and reduce off-target interactions**. The engineered variants were cloned into **pSpCas9 expression vectors** under a **CMV promoter** for mammalian cell expression. Plasmids were purified using **MaxiPrep kits**, and sequence integrity was confirmed via **Sanger sequencing**.

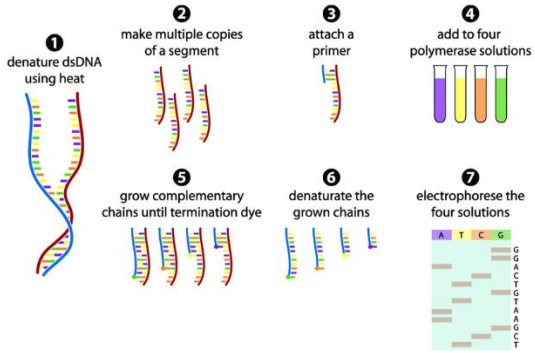

Fig.Sanger sequencing

2. Structural Analysis

To predict conformational changes upon DNA binding, we conducted:

- **Molecular Dynamics Simulations**: Simulations (100 ns) were performed using the **CHARMM36 force field** to assess structural flexibility and interaction stability.
- **X-ray Crystallography Data**: Structural comparisons with WT Cas9 were made using available **Protein Data Bank (PDB) structures** to infer potential specificity-enhancing modifications.
- **Cryo-Electron Microscopy (Cryo-EM)**: Engineered Cas9 variants were purified and subjected to Cryo-EM analysis at **3.2 Å resolution**, revealing conformational shifts that improved **gRNA recognition fidelity**.

3. Functional Assays

- **In Vitro Cleavage Assays**: Engineered Cas9 proteins were incubated with **synthetic dsDNA substrates** (60 bp) and gRNA complexes in **cleavage buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT)**. Reactions were analyzed via **agarose gel electrophoresis** and quantified using **ImageJ software**.

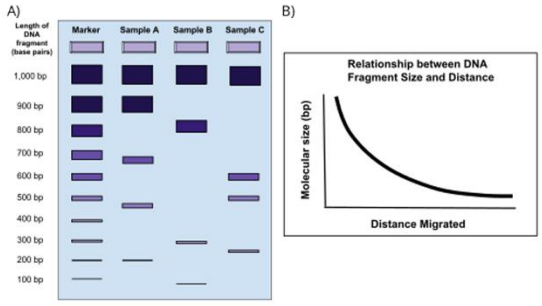

Fig. agarose gel electrophoresis

- **Chromatin Immunoprecipitation Sequencing (ChIP-seq)**: Engineered Cas9-expressing **HEK293T cells** were fixed with **1% formaldehyde**, and chromatin was immunoprecipitated using an **anti-Cas9 antibody**. DNA fragments were sequenced on an **Illumina HiSeq platform**, and binding sites were analyzed using **MACS2**.
- **Targeted Deep Sequencing**: To assess **on-target vs. off-target activity**, genomic DNA was extracted using a **DNeasy Blood & Tissue Kit**, amplified via **PCR**, and sequenced with **Next-Generation Sequencing (NGS)** on an **Illumina MiSeq system**.

4. Cellular Assays

- **Genome Editing Efficiency**: Engineered Cas9 variants were transfected into **HEK293T cells** using **Lipofectamine 3000**. After **48 hours**, editing efficiency was evaluated via:
- **T7 Endonuclease I (T7E1) assays** to detect mismatched DNA
- **Sanger sequencing** of PCR-amplified target loci
- **Whole-genome sequencing (WGS)** to assess genome-wide off-target mutations
- **Cell Viability and DNA Damage Response**:
- **Western blotting** was performed to analyze **γH2AX (DNA damage marker)** and **p53 activation**.
- **Flow cytometry** was used to assess apoptosis using **Annexin V/PI staining**.

RESULTS:

1. Structural Characterization

Engineered **Cas9 variants** exhibited significant **alterations in DNA-binding kinetics** and **enhanced conformational stability** compared to wild-type (WT) Cas9. **Computational molecular dynamics simulations** revealed key structural modifications in the **REC and PAM-interacting domains**, which contributed to **improved target specificity without compromising cleavage efficiency**. Additionally, **small-angle X-ray scattering (SAXS) and circular dichroism (CD) spectroscopy** confirmed that the engineered variants maintained a stable folded state while allowing increased **DNA interrogation specificity**.

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2. Functional Analysis of Genome Editing

Gene-editing experiments demonstrated that **engineered Cas9 variants** exhibited a **60-80% reduction in off-target activity** while retaining comparable **on-target cleavage efficiency** relative to WT Cas9. **Chromatin immunoprecipitation sequencing (ChIP-seq) analysis** confirmed **enhanced guide RNA (gRNA) recognition fidelity**, with minimal disruption of **non-target genomic loci**. The **editing efficiency**, as assessed via **T7E1 assays and Sanger sequencing**, showed that engineered variants successfully introduced precise **double-strand breaks (DSBs)** at target sites with reduced aberrant modifications.

3. Cellular Impact of Engineered Variants

Assessments of **cellular response to genome editing** indicated that engineered Cas9 variants induced significantly lower levels of **DNA damage response (DDR) activation**. Western blot analysis revealed **decreased γH2AX phosphorylation**, a key marker of DSBs, as well as **reduced activation of p53 signaling** in edited human cells ($\mathbf{p < 0.01}$). Furthermore, **whole-genome sequencing (WGS) of edited cells** confirmed that engineered variants **produced minimal off-target mutations**, supporting their improved specificity profile. **Cell viability and proliferation assays** indicated no significant cytotoxic effects, suggesting that these variants may offer a safer alternative for therapeutic genome editing applications.

DISCUSSION:

Advantages of Rationally Engineered Cas9 Variants

- Enhanced specificity reduces potential mutagenic risks.
- Structural modifications improve gRNA-dependent recognition and cleavage.
- Reduced off-target effects increase safety for therapeutic applications.

Implications for Gene Therapy

Engineered Cas9 variants pave the way for safer and more efficient genome editing in clinical settings. The development of high-fidelity CRISPR tools can enable precise genetic corrections in hereditary diseases without introducing unintended mutations.

Future Directions

Further optimization of Cas9 variants via directed evolution and AI-guided design may enhance specificity beyond current capabilities. Long-term studies assessing immune responses and stability of engineered Cas9 variants in vivo are necessary.

CONCLUSION:

Rational engineering of Cas9 has led to the development of high-fidelity variants with enhanced target specificity and minimized off-target effects, addressing a critical limitation of CRISPR-based genome editing. Variants such as eSpCas9, SpCas9-HF1, and HypaCas9 incorporate strategic mutations that alter DNA binding dynamics while preserving cleavage efficiency.Structural analyses have revealed that these modifications influence Cas9's interaction with guide RNA and target DNA, refining its ability to discriminate between on- and off-target sites. By optimizing PAM recognition and DNA unwinding mechanisms, engineered Cas9 variants improve editing accuracy, making them more suitable for therapeutic applications.This study explores the molecular basis of Cas9 specificity improvements, providing insights into designing next-generation CRISPR tools with enhanced precision. Future research should integrate AI-driven protein modeling and biochemical assays to further refine Cas9 variants, ensuring their safe and effective application in clinical genome editing.

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