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Controlling the Activity of Gene Editing Tools

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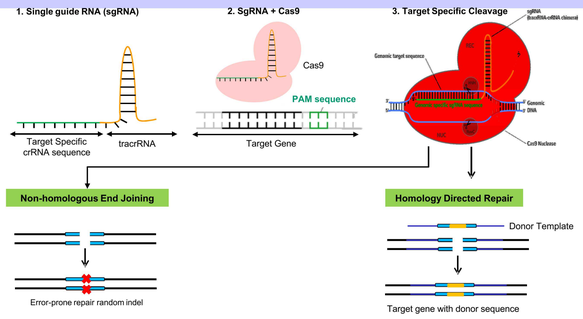
Introduction

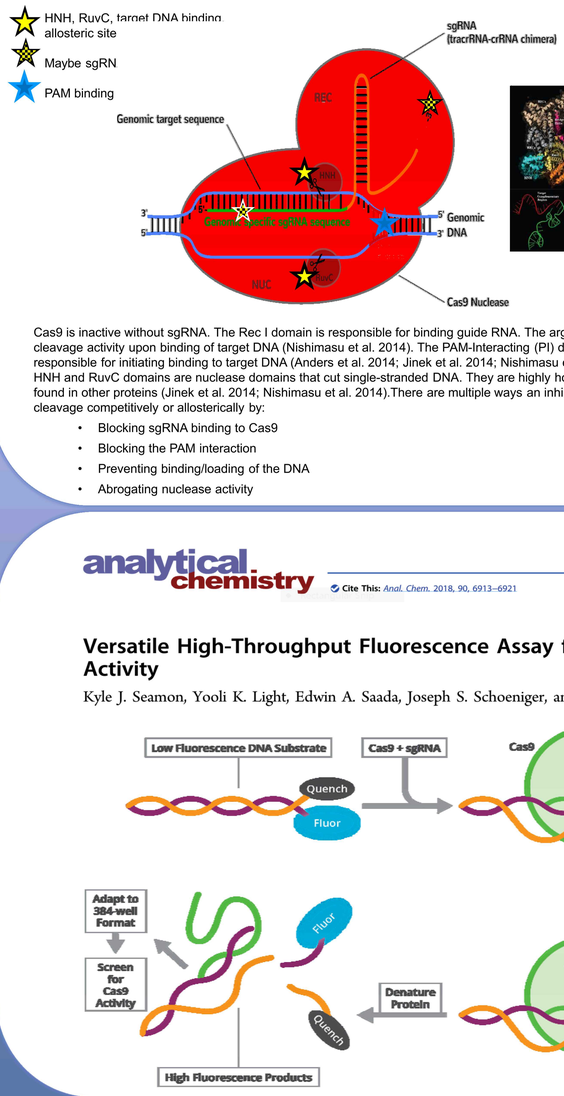
The revolution in biotechnology holds immense promise for addressing human disease, creating biotechnology solutions for bioenergy, understanding basic biology and advancing the drug-discovery field. Unfortunately, explosively growing use of gene editing tools also raises profound dual use concerns. Therefore, a flexible toolbox of inhibitors will be needed to combat any hazards that result from the use of gene editing tools. Inhibitors that block Cas9 activity in vivo could ensure the safety of CRISPR-based gene therapies and provide an antidote in the event of unwanted exposure. At Sandia National Laboratories (SNL) we are developing a pipeline to discover clinically-useful inhibitors that block CRISPR-Cas9 activity for use in treatment or prophylaxis when Cas9 containing agents are encountered. To discover inhibitors, SNL is developing computational and experimental assays to rapidly screen for inhibitors (small molecules, peptides, or antibodies) of Cas9 activity, including a high-throughput fluorescence-based assay using a dual-quencher substrate with low background and high sensitivity. A number of peptide and small molecules have been identified and SNL is currently validating and optimizing these potential inhibitors.

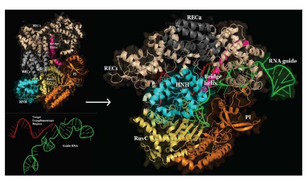
Targeted Genome Editing



1. Single guide RNA (sgRNA) is a chimera of mature crRNA (sequence homology to target gene) and tracrRNA (binds Cas9).
2. Cas9 binds to a sgRNA and undergoes RNA-induced conformational changes to induce DNA binding. PAM recognition is the initial step in DNA binding and is required for strand separation and sgRNA-DNA heteroduplex formation.
3. Two endonuclease domains within Cas9 protein cleave double stranded DNA 3bp upstream of the PAM. These DNA double strand breaks (DSBs) can facilitate genome editing through the error-prone non-homologous end-joining (NHEJ) repair pathway or homology-directed repair (HDR) with donor DNA.

Multiple Target Sites for Inhibition





Cas9 is inactive without sgRNA. The Rec I domain is responsible for binding guide RNA. The arginine-rich bridge helix is crucial for initiating cleavage activity upon binding of target DNA (Nishimasu et al. 2014). The PAM-Interacting (PI) domain confers PAM specificity and is responsible for initiating binding to target DNA (Anders et al. 2014; Jinek et al. 2014; Nishimasu et al. 2014; Sternberg et al. 2014). The HNH and RuvC domains are nuclease domains that cut single-stranded DNA. They are highly homologous to HNH and RuvC domains found in other proteins (Jinek et al. 2014; Nishimasu et al. 2014).There are multiple ways an inhibitor could block Cas9-catalyzed DNA cleavage competitively or allosterically by:

- Blocking sgRNA binding to Cas9
- Blocking the PAM interaction
- Preventing binding/loading of the DNA
- Abrogating nuclease activity

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Versatile High-Throughput Fluorescence Assay for Monitoring Cas9 Activity

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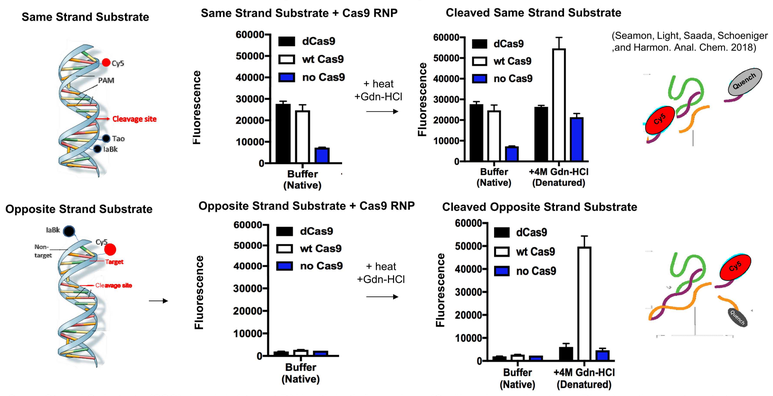
Quenching of Same Strand Substrate Sensitive to Disruption in Duplex Helix

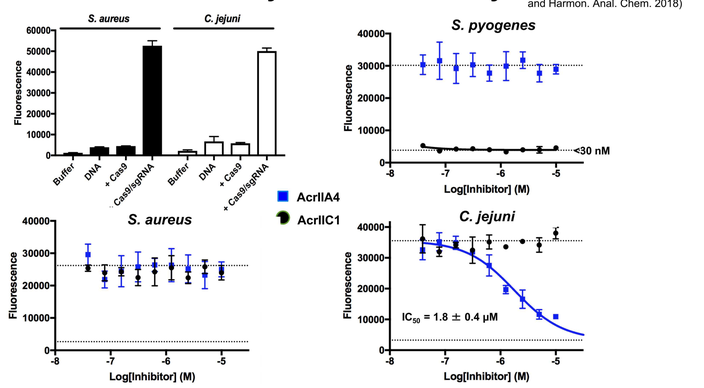


Same Strand Substrate (SSS): Lambda1 target DNA with both fluorophore (Cy-5) and quencher (Iowa-Black) on the non-target strand 11 bp 42.9 Å apart; Cy5 intensifies with binding alone and Cy5 intensities increased after cleavage and heat-induced or chemical denaturation of Cas9. The location of the fluorophore/quenchers on the same strand along the length of the helix makes the quenching efficiency highly sensitive to any changes in the helical properties of the duplex. For the SSS substrate binding of Cas9 and insertion of guide RNA into the substrate is sufficient to disrupt the DNA helix, change the relative orientation of the fluorophore/quenchers, and decrease quenching efficiency without any cleavage of the DNA.

Opposite Strand Substrate: Lambda1 target DNA with end-labeled opposite-strand flurophore/quencher. Distance between fluorophore and quencher is minimal (<15 Å) . Cy5 intensity is unaffected by Cas9 binding alone, Cy5 intensity is substantially increased after cleavage and chemical denaturation of Cas9.

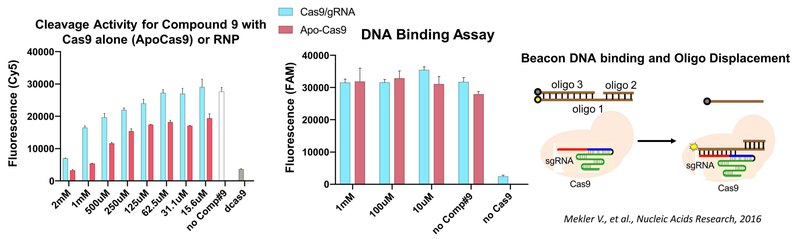
Cas9 Activity Assay is Adaptable to Other Species and Quantitatively Measures Activity

(Seamon, Light, Saada, Schoeniger, and Harmon. Anal. Chem. 2018)



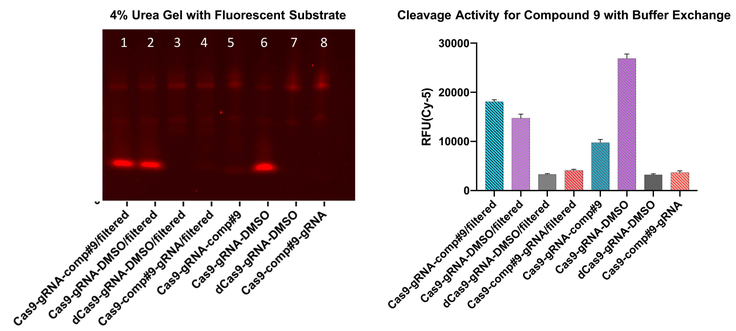
Additional Species of Cas9: Fluorescence of Sau Cas9 and Cje Cas9 substrates when treated with an excess of Cas9 and sgRNA and quenched with 4M Gdn-HCl and 1 hour incubation at 55 °C. Data are the average and standard deviation of three replicate wells. **Profiling the Species Specificity of Anti-CRISPRs.** Fluorescence values of Spy Cas9 (A), Sau Cas9 (B), or Cje Cas9 (C) with the indicated concentration of AcrIIA4 or AcrIIC1. The average fluorescence of controls containing either no Cas9 (lower dashed line) or no inhibitor (upper dashed line) are shown.

Electrophile Compound 9 Inhibits Cas9 Cleavage Activity Without Blocking DNA Binding



Cas9 Cleavage Assay with Different Incubation Steps: Compound 9 incubated with RNP (Cas9/sgRNA, blue bars), or Cas9 alone (ApoCas9, red bars) for 30 minutes prior to addition of substrate and sgRNA (for Apo condition) and additional 30 minute incubation before quench. Data are the average and standard deviation of three replicate wells.
Beacon DNA binding assay (adapted from Mekler V. et al., 2016): both beacons mimic native target DNA and bind Cas9/sgRNA. Cas9 binding to beacon 1 and formation of a DNA–RNA heteroduplex should result in dissociation of oligo 3, leading to increase in fluorescence intensity due to disappearance of the quenching effect. This fluorescence is observed upon addition of Cas9 complexes and is unaltered by incubation with Compound 9.

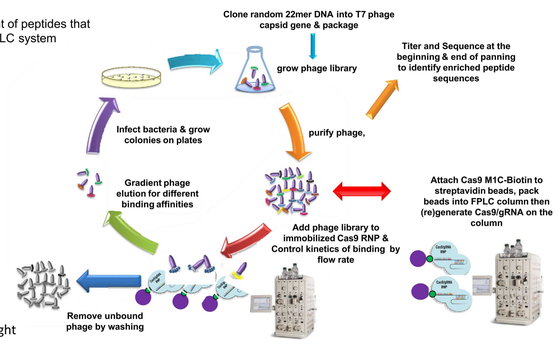
Compound #9 Covalently binds to Apo-Cas9 but Binds Reversibly to Cas9/gRNA Complex



Compound 9 incubated with RNP (Cas9-gRNA-comp #9), or Apo-Cas9 (Cas9-comp#9-gRNA) for 30 minutes, the unbound compound is removed by filtration (filtered) prior to addition of substrate and sgRNA (for Apo condition), or is left in the reaction for an additional 30 minutes prior to quench. This experiment was repeated twice with similar data. Data are the average and standard deviation of three replicate wells. Comp#9 did not covalently inhibit RNP (Cas9 complexed with gRNA) but it seems to covalently inhibit when incubated with Cas9 in the absence of gRNA (apo Cas9).

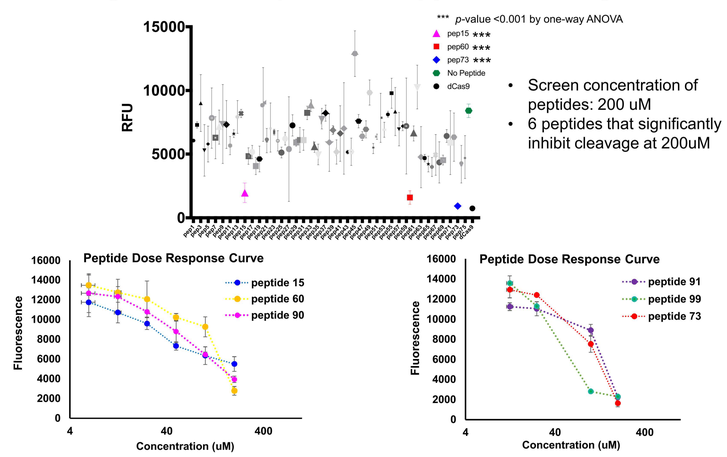
Screening Peptide Inhibitors of Cas9 Using T7 Phage Display System

Phage Panning:
3 rounds of enrichment of peptides that bind to Cas9 using FPLC system



- biotinylated Cas9 was prepared by conjugation via single cystidine on Cas9 protein and immobilized as Cas9/sgRNA complex on Neutravidin or Monoavidin bead columns. Phage library was generated to display ~10^8 possible peptides on phage surface
- Phage peptides bound to Cas9 RNP are eluted with different conditions. The eluted phages are amplified and subjected to additional rounds of enrichment.
- Peptide sequences obtained by high throughput sequencing and bioinformatics pipeline is built to identify most enriched and abundant peptide sequences after the final panning

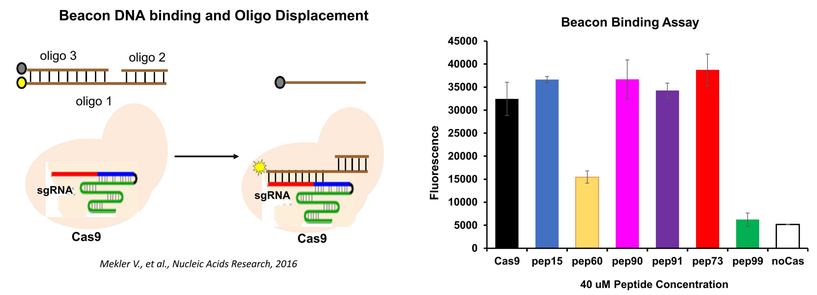
Screening Enriched Peptides for SpyCas9 Cleavage Inhibition



- Screen concentration of peptides: 200 uM
- 6 peptides that significantly inhibit cleavage at 200uM

192 peptides that are abundant and highly enriched in 3rd panning from both NeutrAvidin and MonoAvidin columns, were synthesized and tested. The first round of screening was done at high concentration of peptides (200uM) to quickly identify possible candidates for Cas9 cleavage inhibition. Urea PAGE and 4% agarose gel with Cy5/BHQ was run with top peptides to make sure that they are really blocking DNA cleavage. Peptide that demonstrated inhibition, with uncleaved substrate in the Cas9 cleavage reaction (visualized by Gel), were further analyzed for dose responses.

Peptides 60 and 99 inhibit Cas9 binding to DNA substrate



Mekler V., et al., Nucleic Acids Research, 2016

Beacon DNA binding assay (adapted from Mekler V. et al., 2016): both beacons mimic native target DNA and bind Cas9/sgRNA. The fluorescent signal is increased when Cas9 binds the DNA substrate and a helix forms with the target substrate strand and gRNA resulting in dissociation of oligo 3, leading to increase in fluorescence intensity due to disappearance of the quenching effect. This fluorescence observed upon addition of Cas9 complexes is unaltered by incubation with peptides 15, 90, 91, and 73 but is significantly reduced by peptide 60 and peptide 99.

Summary and Future Directions

A. Six peptides were identified by Phage display and panning by Cas9 affinity chromatography
- a. validated by Gel electrophoresis and the FRET-based cleavage activity assay.
- b. 2 peptides block target DNA binding

B. Use error-prone PCR to optimize the peptides from the screen and re-run through phage display assay.
- **b.** Peptide hits have been mutated, the pool of mutated peptides will be expressed in phage and phage panning with immobilized Cas9 will be performed. More stringent elution conditions will be used to pick up stronger binders (the original 6 hits)

C. Deliver and validate peptide inhibitors in mammalian cells
- **c.** similar amino acid profile as established cell penetrating peptides.

D. One Compound was identified from a screen of 1300 Electrophile compounds for inhibition of Cas9 Cleavage activity
- **d.** We have purchased compounds that are chemically similar to compound 9 that will be screened to find a stronger hit and get information on the mechanism of action.

E. Lead candidates identified with these methods will be tested for activity against Cas9 variants, and for toxicity and efficacy in tissue culture and *in vivo*

Funding Statement

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