# Gene targeting of the HBB locus by Crispr/Cas9 to investigate repair pathway choice in response to different types of DNA lesions

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## Introduction

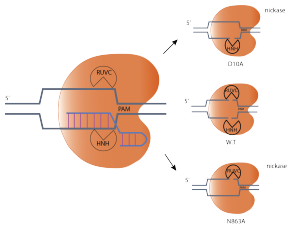
The CRISPR/Cas9 system has recently been propelled to the forefront of the genome editing field as a fast and reliable method for introducing targeted DNA double-strand breaks into the genome. Derived from a bacterial adaptive immune system, this technology uses short guide RNAs (gRNAs) to direct the cleavage activity of the Cas9 protein in a site-specific manner. Inactivating point mutations engineered into either the HNH or RuvC catalytic domains enable conversion of Cas9 from a nuclease to a single-strand nickase.

Here we investigate the use of this powerful genome editing technology to target the human *HBB* gene in the region of the sickle cell anemia-causing mutation. Sickle Cell Anemia is a recessive disorder caused by a single point mutation in the human beta globin gene.

We demonstrate the use of the CRISPR/Cas9 system to target the human *HBB* gene and examine how the nature of the targeted break affects the frequency of different DNA repair outcomes.

Utilizing the wild type Cas9 nuclease, as well as two different Cas9 nickases we are able to introduce blunt double-strand breaks, single-strand nicks, and dual-nicks in which the nicks are placed on opposite strands and leave either 3' or 5' overhangs of varying lengths. Using either single-strand oligonucleotide (ssODN) or plasmid DNA donors, we characterize several different DNA repair outcomes including indel mutations resulting from non-homologous end-joining, homology-dependent repair (HDR) using the donor as a template, and HDR using the closely related *HBD* gene as an endogenous template. The frequency with which we observe these various repair outcomes under different conditions offer insight into the mechanisms of DNA repair and how it is impacted by the nature of the DNA break. The data also suggests a potential therapeutic approach in which correction of the sickle-cell mutation is efficiently mediated through HDR with either a donor template or with the *HBD* gene.

**Fig 1: Schematic of Cas9 variants used in this study**



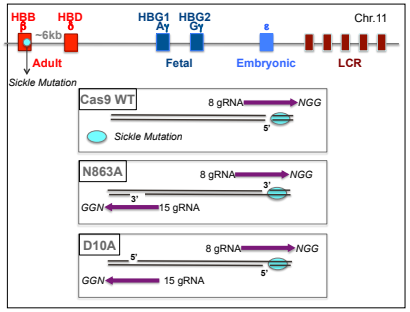




**Fig.2**
Schematic of the HBB locus indicating the position of the different gRNAs used in the study.

### Different Cas9 variants cut with a comparable aggregate efficiency

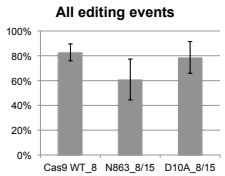
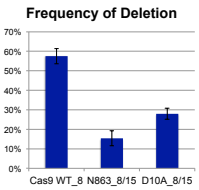



**Fig.3**
Total editing observed with three Cas9 variants. Results are compiled from at least 3 independent experiments for each condition.

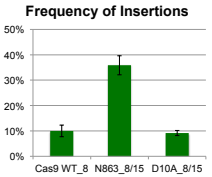
### WT _Cas9 leads to higher levels of deletions




- With Cas9 WT deletions are mostly less then 5 bp
- With Cas9 Nickase deletions are mostly around 40-50 bp

**Fig.4**
Frequency of deletion observed with three Cas9 variants. Results are compiled from 3 independent experiments for each condition.

### Nickase N863A_Cas9 leads to higher levels of insertions




N863A Cas9 insertions are mostly duplication of the 3' protruding arm

**Fig.5**
Frequency of insertion observed. Results are compiled from 3 independent experiments for each condition tested.

### Examples of the Insertion after nicking with N863A

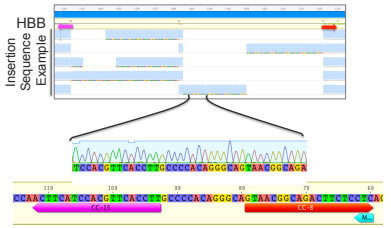



**Fig.6**
Example of common Sanger reads observed in U2OS cells electroporated with Cas9 N863A and gRNA 8/15. The HBB reference is indicated on the top.

### Nickase D10A_Cas9 leads to higher levels of Gene Conversion from HBD

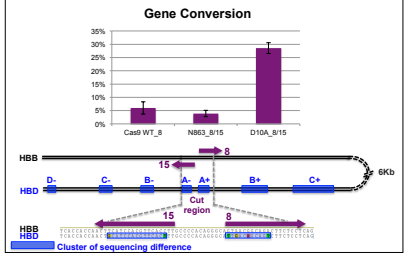



**Fig.7**
Frequency of gene conversion. A representation of at least 3 independent experiments for each condition is shown. Schematic representing the genomic organization and the region of similarity between HBB and HBD is shown on the bottom.

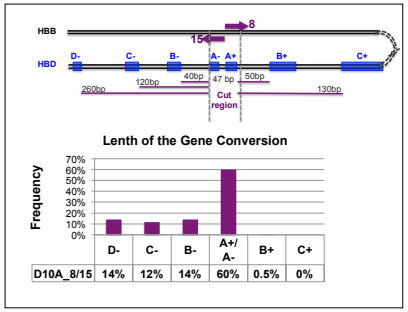



**Fig.8**
Frequency with which different lengths of HBD sequence are incorporated into HBB.

### D10A Cas9 induces higher rate of HDR with lower strand ssDNA as donor template

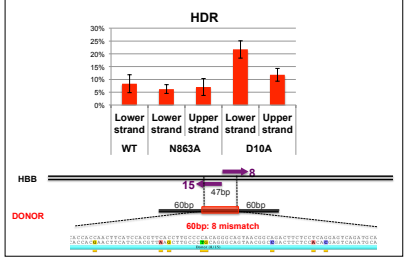



**Fig.9**
Frequency of HDR in response to different donors. Schematic illustrating the donor template is shown on the bottom.

## Methods:

U20S cells were electroporated with gRNA and Cas Wt or mutant plasmid. Cells were collected 6 days after electroporation. gDNA was extracted, PCR amplification of HBB locus was performed and sub cloned into a Topo Blunt Vector. For each condition in each experiment 96 colonies were sequenced by Sanger sequencing.

## Conclusion:

- Cas9 wt, N863A and D10A cut with a comparable efficiency
- Cas9 N863A Nickase induces a higher rate of insertions
- Cas9 D10A Nickase induces modification of the HBB locus using the HBD gene as a donor template
  - Predicted to repair the sickle mutation
- Cas9 D10A Nickase achieves a higher rate of HDR