Therapeutic Genome Editing

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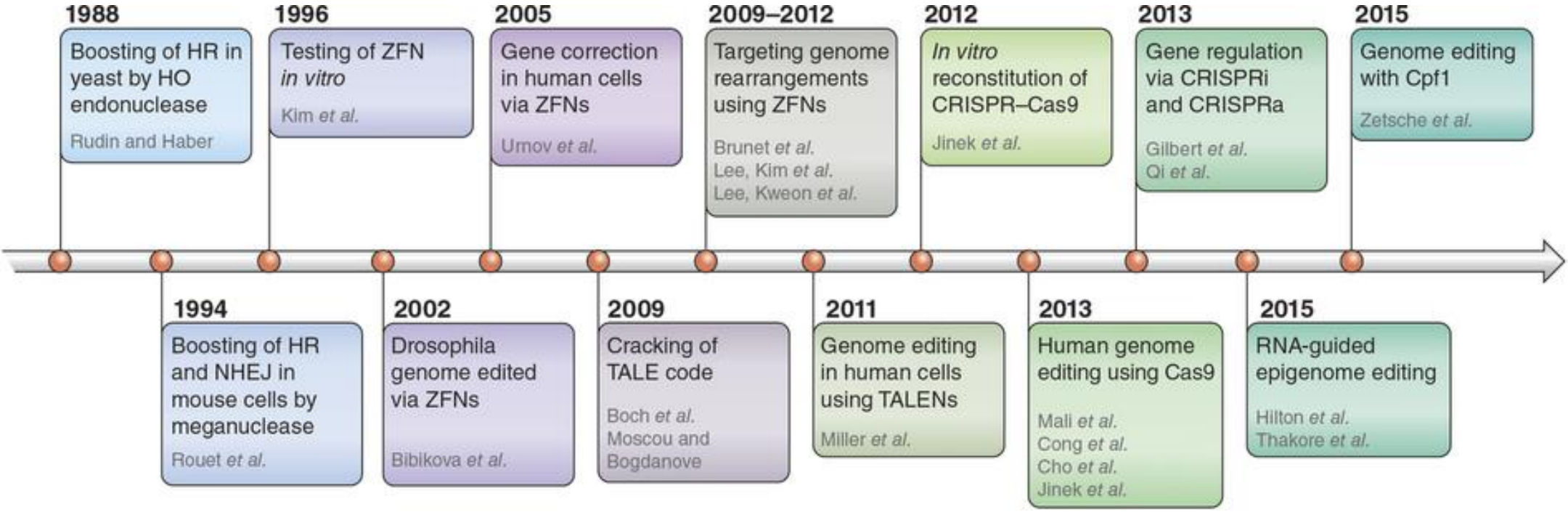
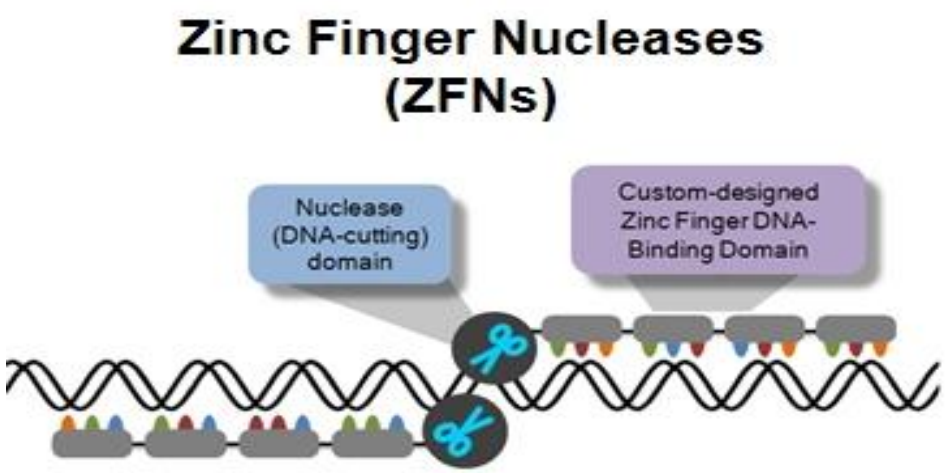
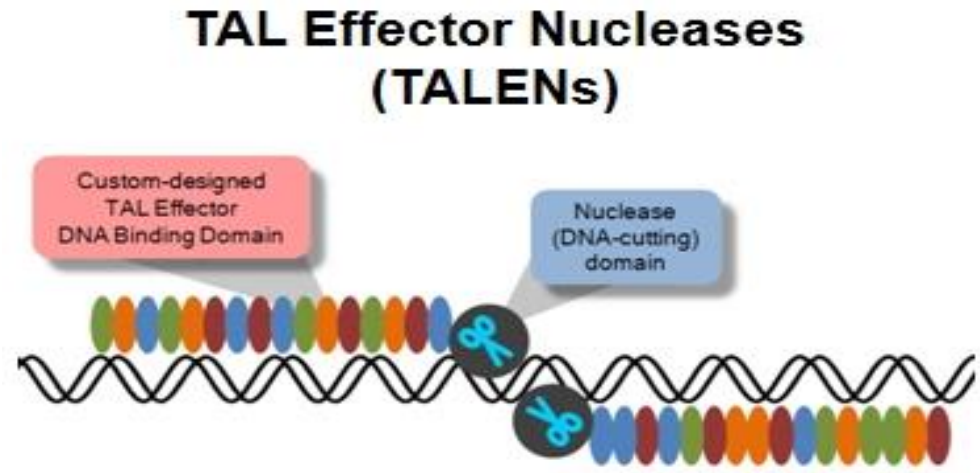
Genome Editing Timeline

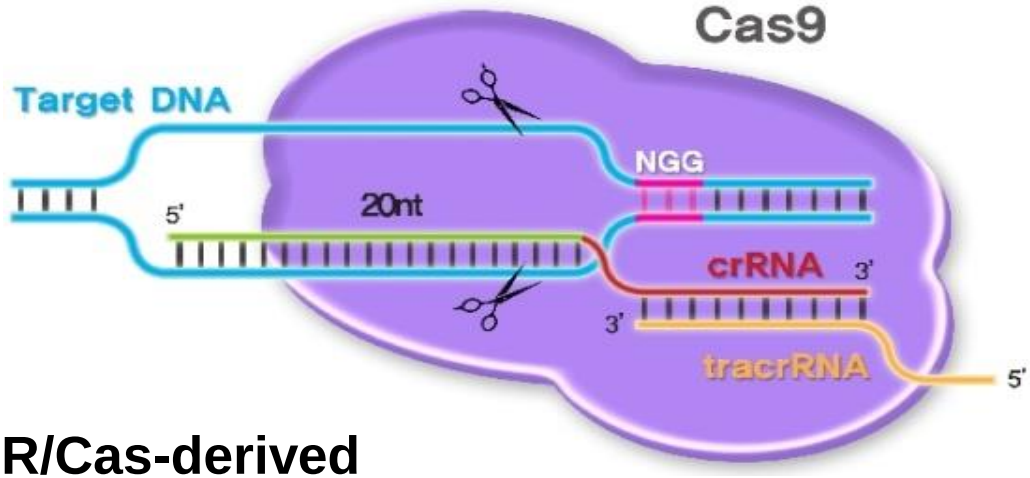


Kim JS. Nature Protocols (2015)

Programmable Nucleases







CRISPR/Cas-derived
RNA-guided endonuclease
(RGEN)

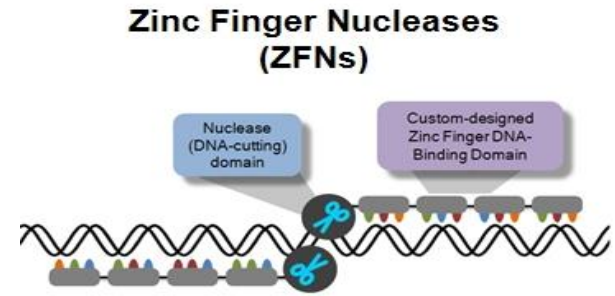
Comparison of Programmable Nucleases

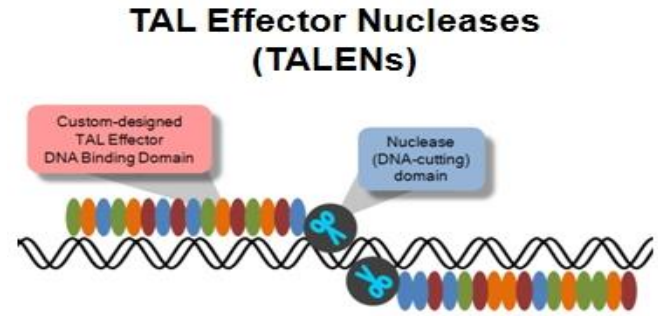
	ZFN	TALEN	CRISPR RGEN
Success rate	~24%	>99%	~90%
Average mutation rate	<10%	~20%	~20%
Length of target site	20 to 36 bp	30 to 40 bp	23 bp
Restriction in target site	Guanine-rich	Start with T and end with A	End with GG (PAM)
Design density	One per ~100 bp	One per every bp	One per 8 bp
Off-target effects	High	Low	Variable
Size	2 x ~2 kbp	2 x ~3 kbp	4.2 kbp + gRNA

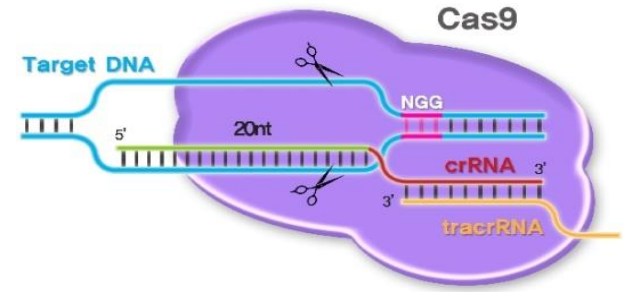
Kim H & Kim JS, Nat. Rev. Genet. (2014)

Challenges in Therapeutic Genome Editing

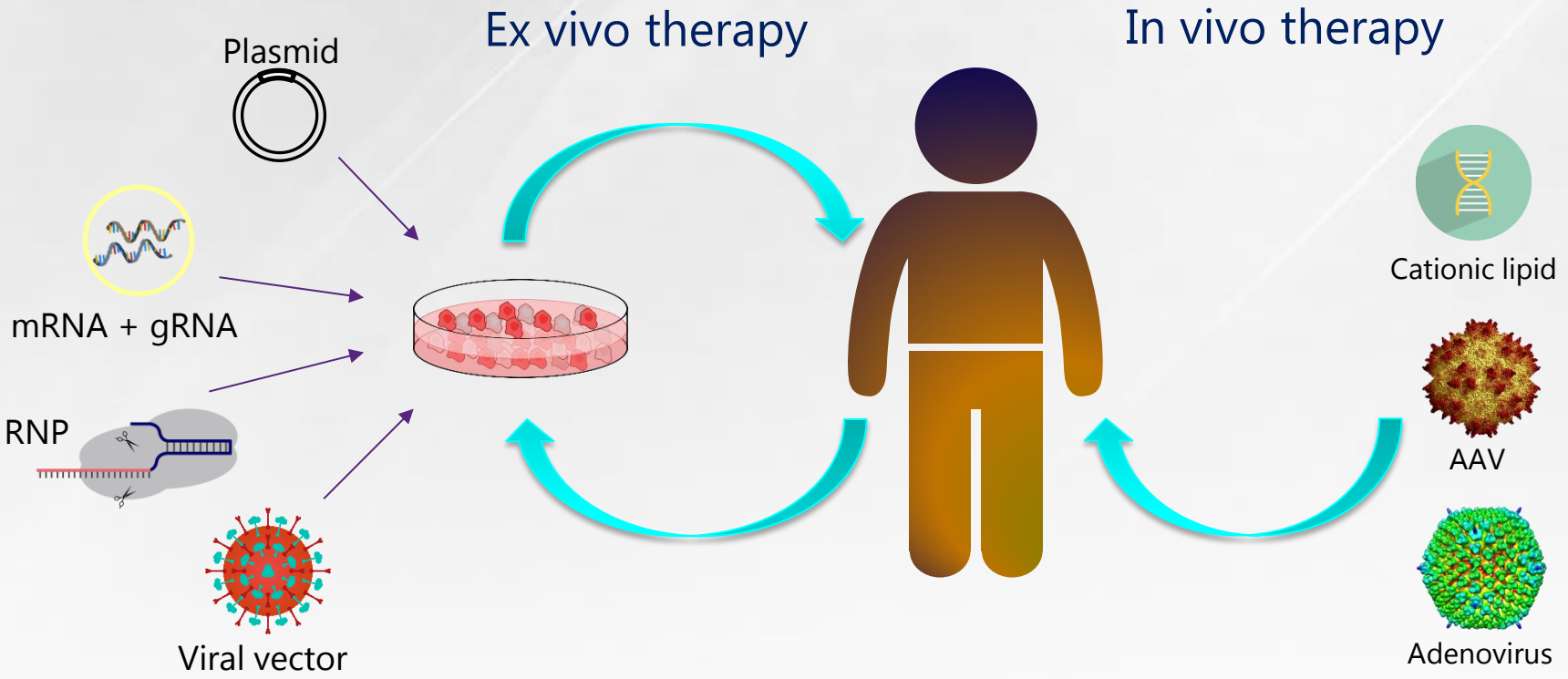
- Delivery
- Immunogenicity
- Mosaicism
- HDR vs. NHEJ
- Specificity: Off-target mutations



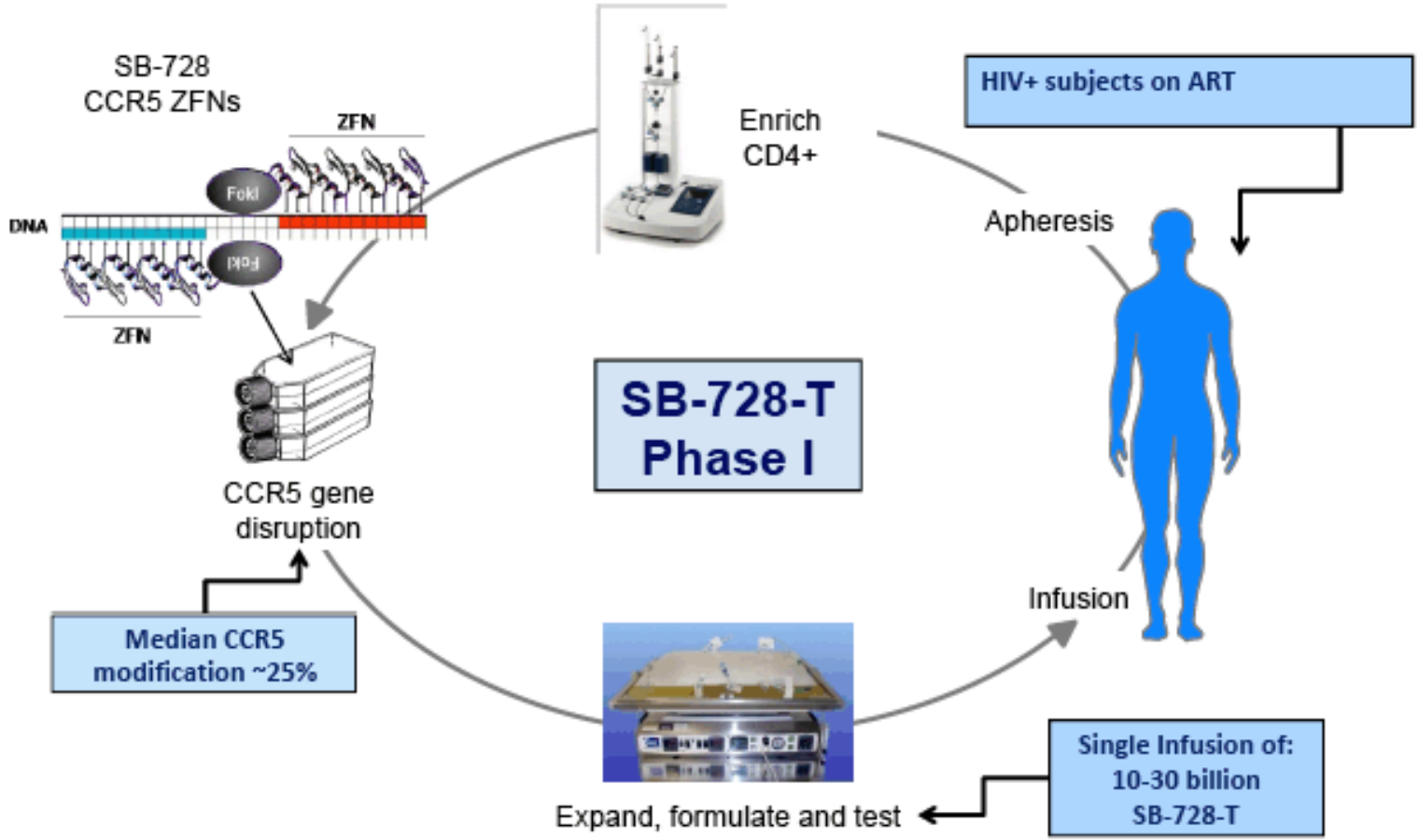




Genome Surgery

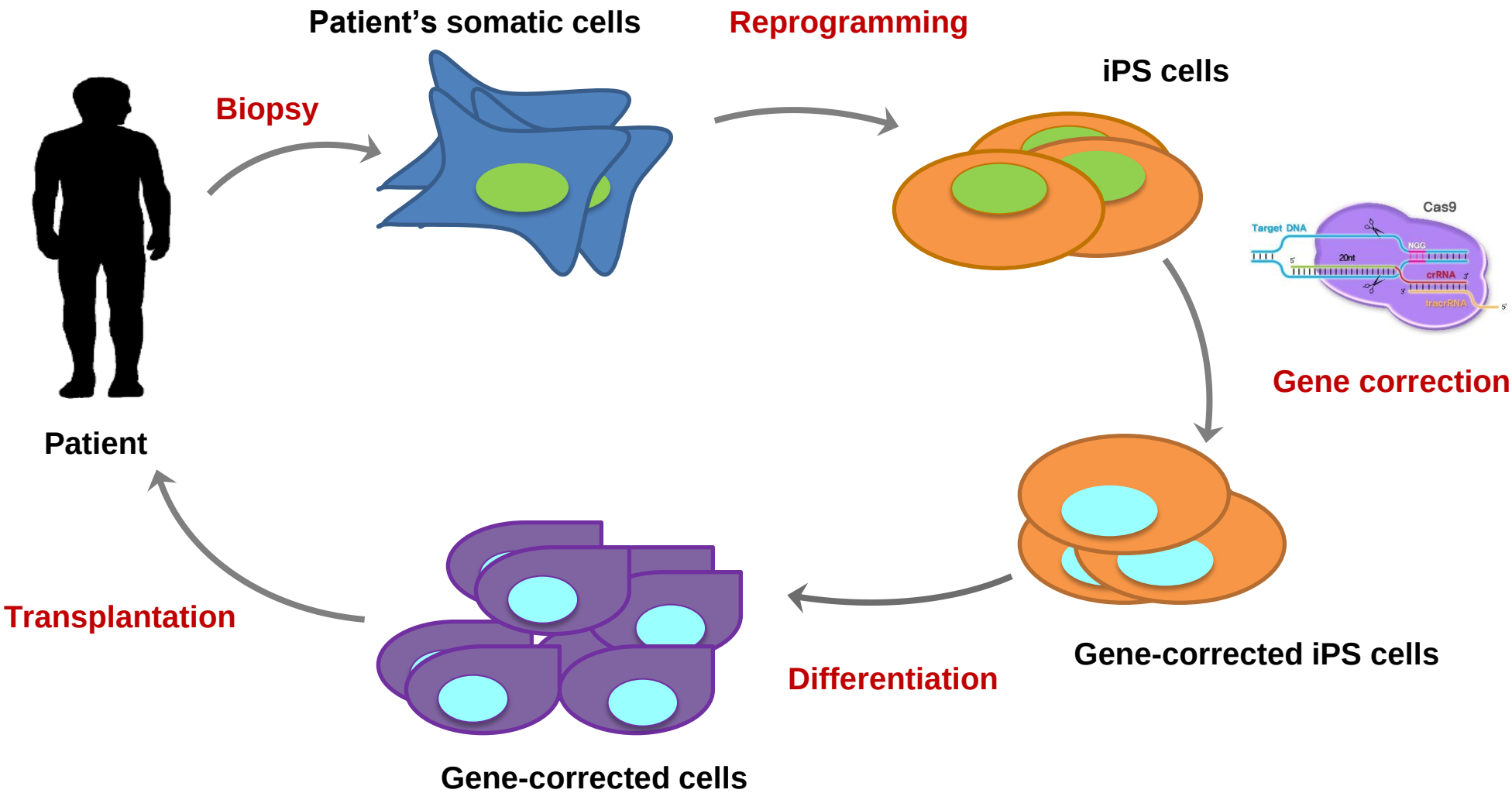


SB-728-T: Zinc Finger Nuclease Driven CCR5 Modified Autologous CD4+ T-cells



- T cells from HIV+ patients are treated with a programmable nuclease.
- CCR5-inactive T cells are delivered back to patients

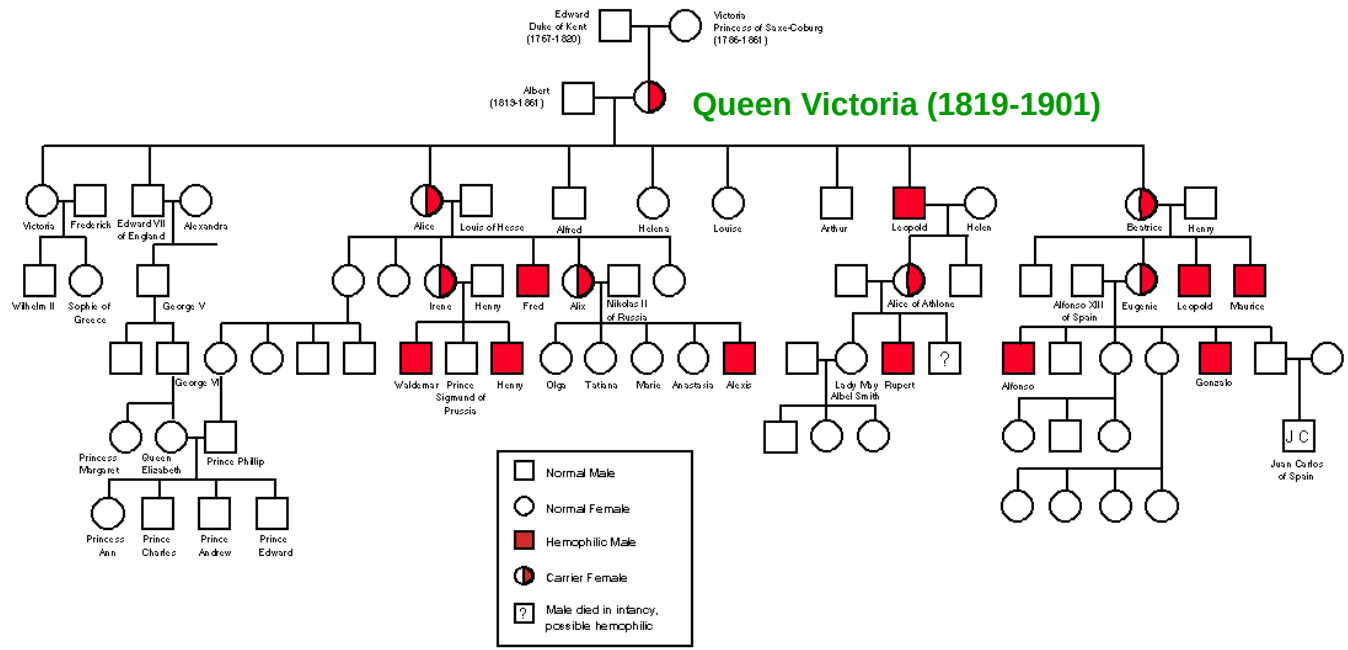
Stem Cell Therapy: Gene Correction in iPS Cells



Park et al. Cell Stem Cell (2015)

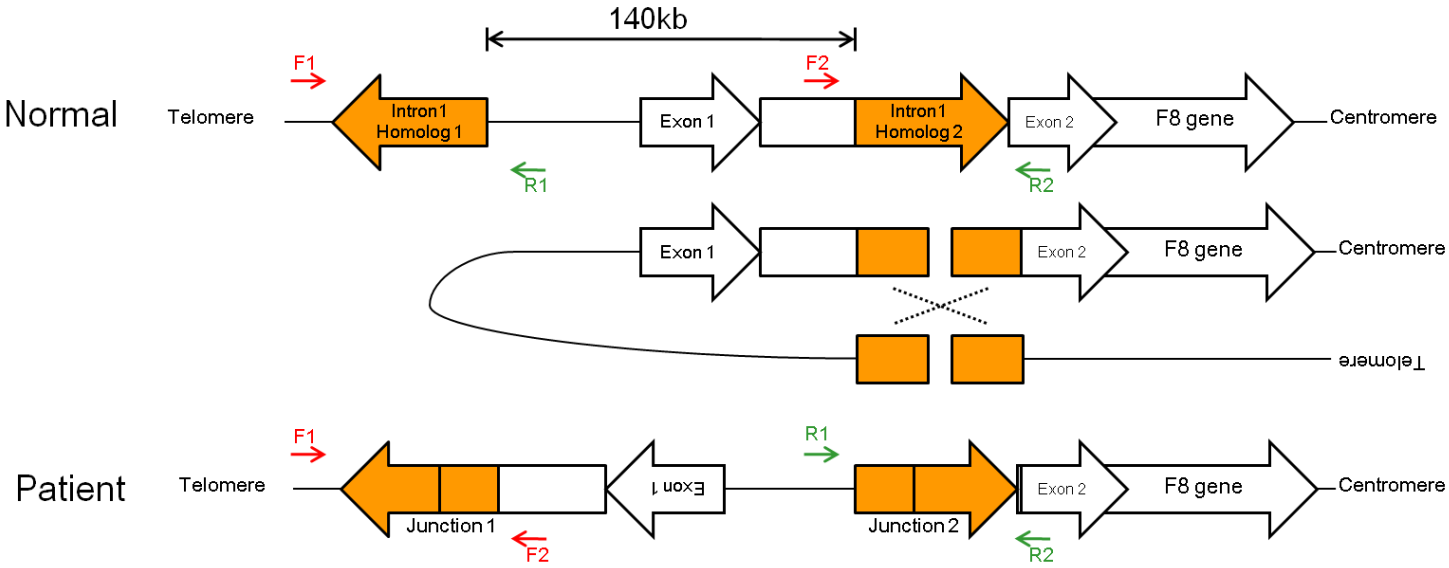
Hemophilia: The Royal Disease

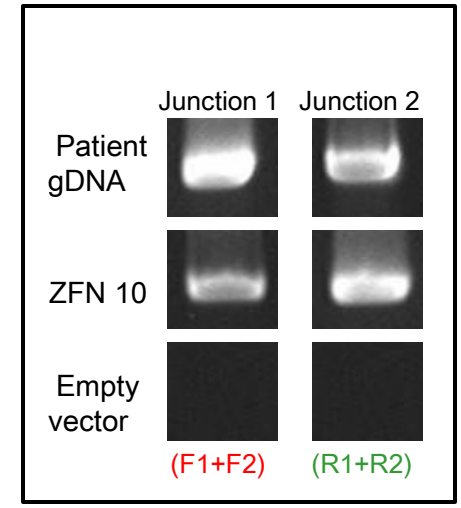
Queen Victoria and her royal family



- British Queen Victoria was a carrier of the hemophilia gene.
- Almost half of the severe form of hemophilia A is caused by DNA inversion.

Inversion of the Hemophilia Gene





Z10 target site(Homology 1) Z10 target site(Homology 2)

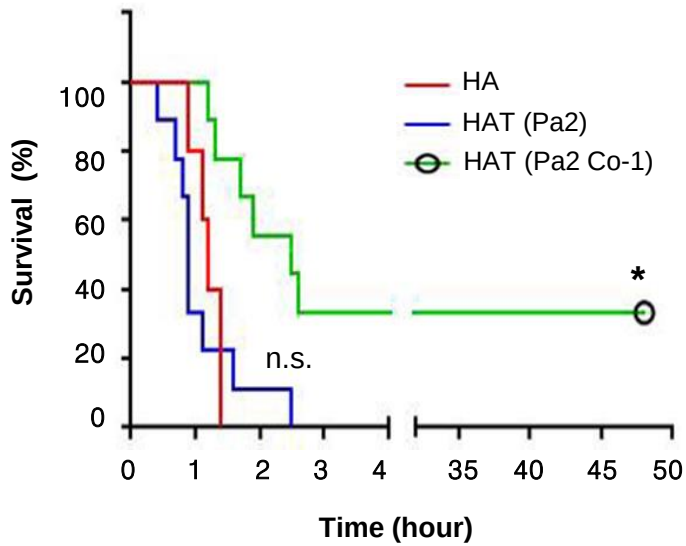
```
WT            ..caaggagacccactgagttgggcaaaggtggggccgac..140kbp..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
              ..gttcctctgggtgactcaacccgtttccaccccggctg..140kbp..CAGCCGGGGTGGAAACGGGTTGAGTCACCCAGAGGAAC ..

Cleaved   ..caaggagacccactga     gttgggcaaaggtggggccgac..140kbp..GTCGGCCCCACCTTTGCC     CAACTCAGTGGGTCTCCTTG..
          ..gttcctctgggtgactcaac     ccgtttccaccccggctg..140kbp..CAGCCGGGGTGGAAACGGGTTG     AGTCACCCAGAGGAAC ..

Flipped   ..caaggagacccactga     GTTGGGCAAAGGTGGGGCCGAC..140kbp..gtcggccccacctttgcc     CAACTCAGTGGGTCTCCTTG..
          ..gttcctctgggtgactcaac     CCGTTTCCACCCCGGCTG..140kbp..cagccggggtggaaacggttg     AGTCACCCAGAGGAAC ..
```

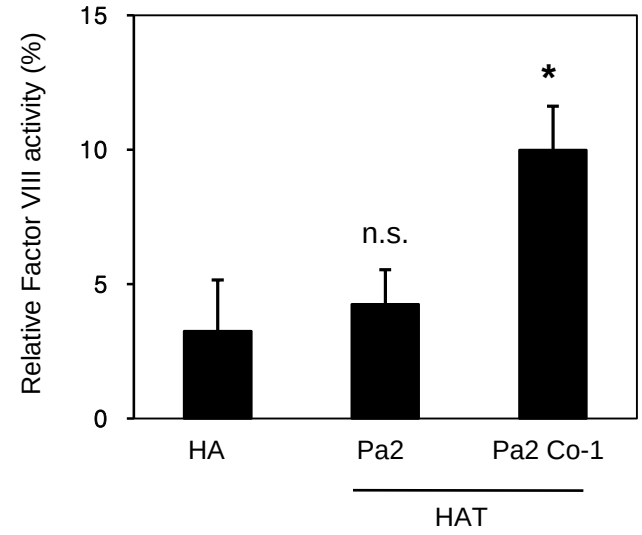
Breakpoint junction 1 Breakpoint junction 2

```
..agtcggccccacctttgcccaa----ctcagtgggtctccttg..   (X6)
..agtcggc----------ccaa----ctcagtgggtctccttg..   (X1)
..agtcggccccacctttgcccaa--------------------..   (x1)
..agtcggccccacctttgcccaaccaactcagtgggtctccttg..   (X2)
```

Lee et al. Genome Res. 22, 539 (2012)

Hemophilia Mice Treated w/ Gene-Corrected Cells

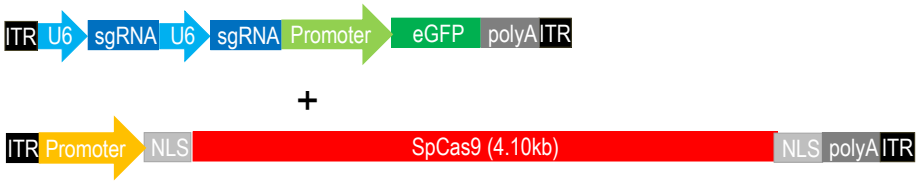




Park et al. Cell Stem Cell (2015)

- CRISPR-Cas9 can revert large inversions in hemophilia iPSCs.
- Endothelial cells derived from corrected iPSCs rescue F8 deficiency in mice.

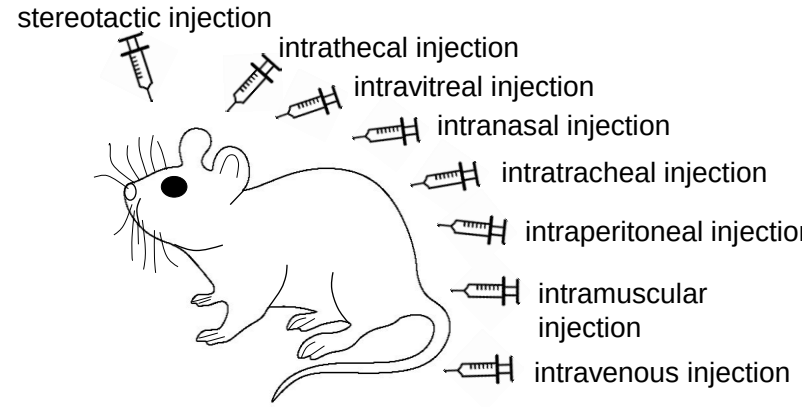
CjCas9: mini-Cas9 for AAV package



AAV-SpCas9 and AAV-sgRNAs-eGFP dual vectors system

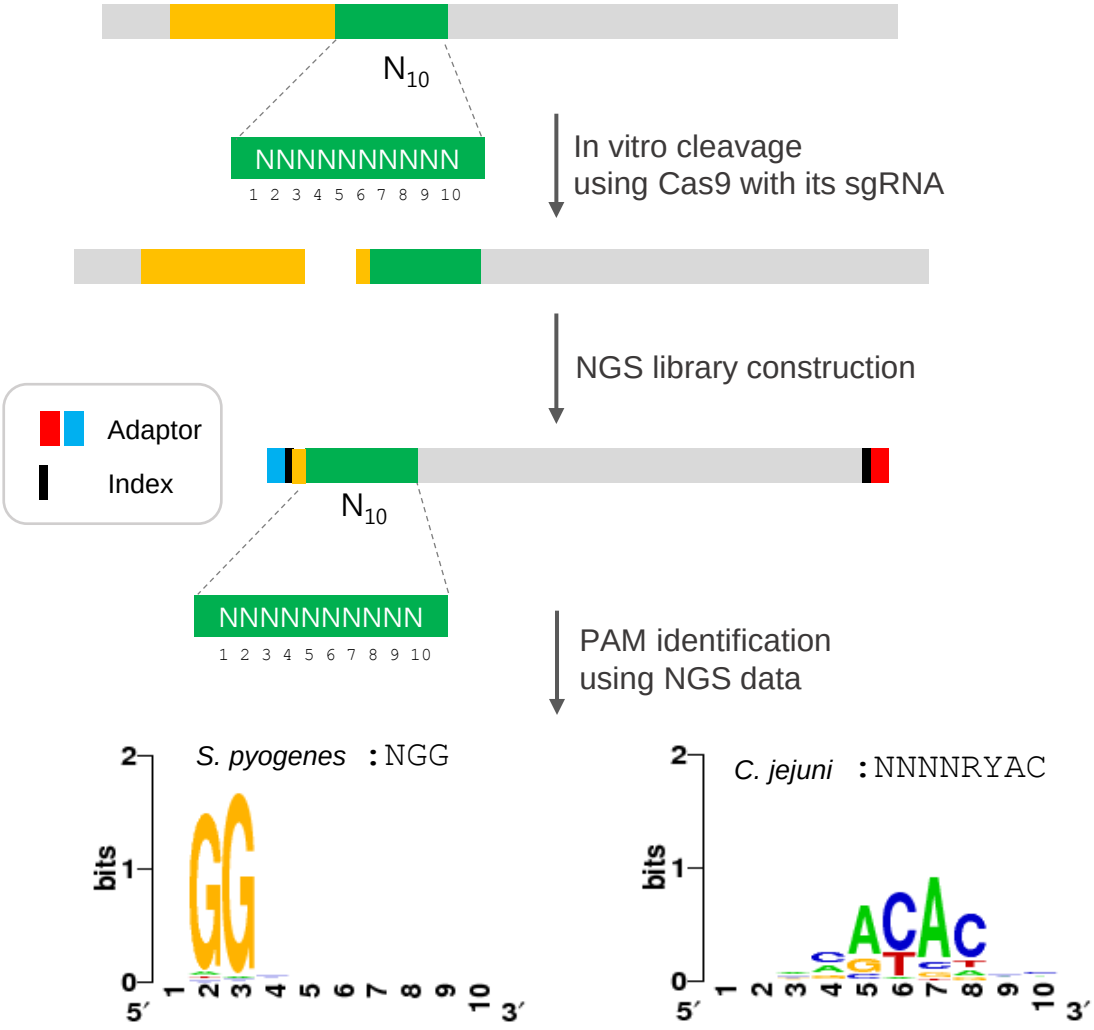
AAV-SaCas9-sgRNAs single vector system

AAV-CjCas9-sgRNAs-eGFP all-in-one vector system

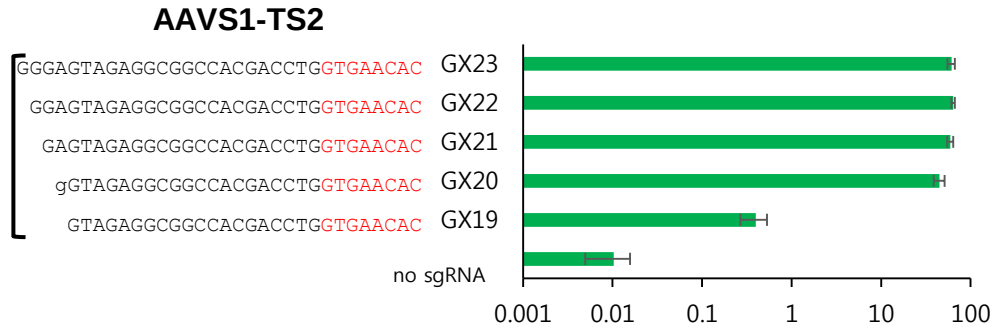


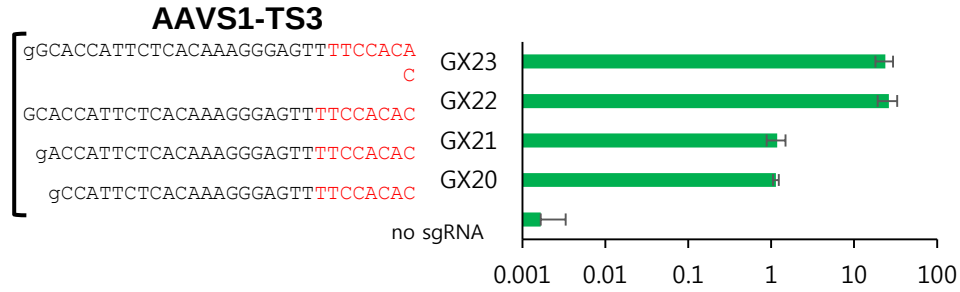
- CjCas9 is the smallest Cas9 ortholog reported to date
- Digenome-seq reveals that CjCas9 is more specific than SpCas9 or SaCas9

CjCas9 PAM characterization



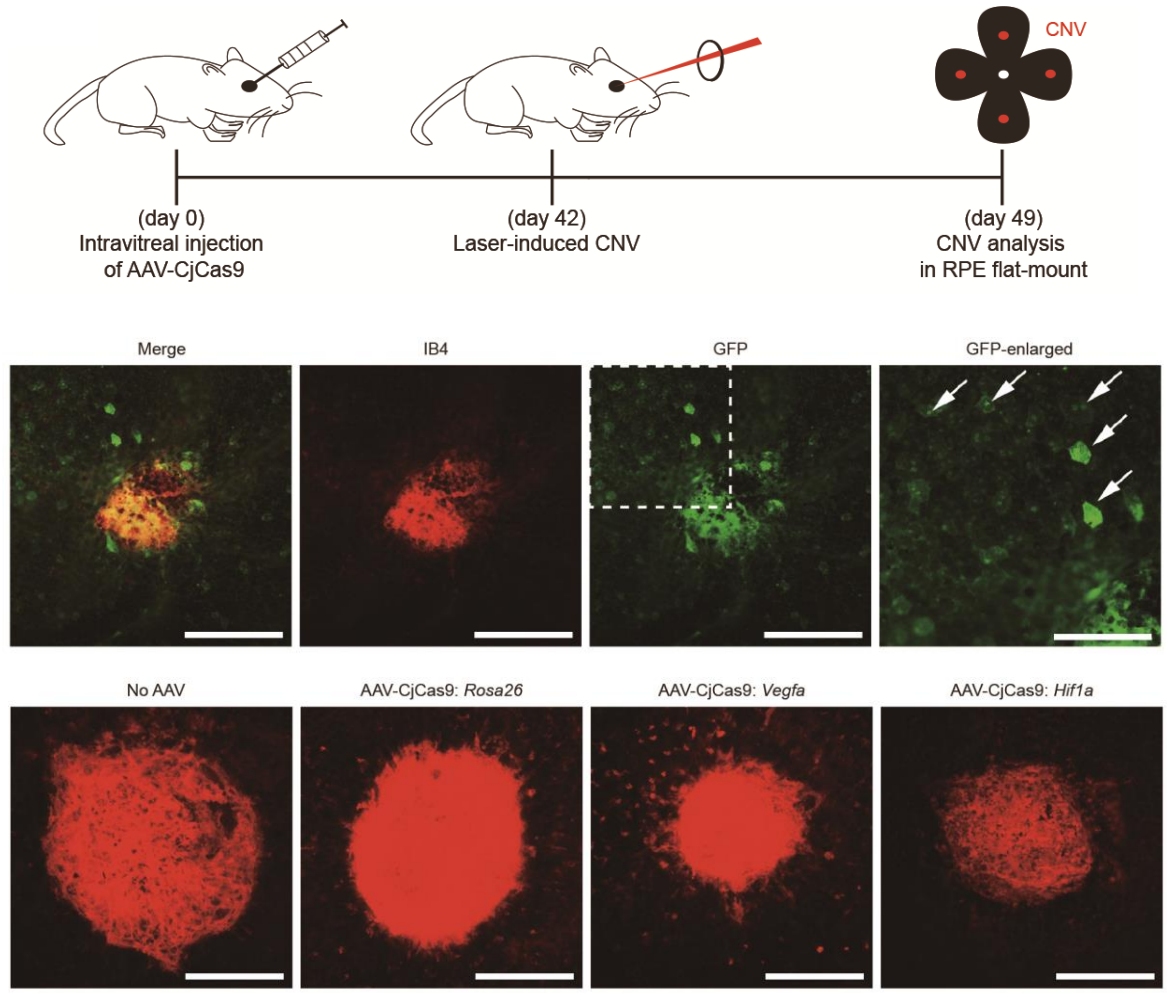
CjCas9 sgRNA optimization





Kim et al. Nat. Commun. (2017)

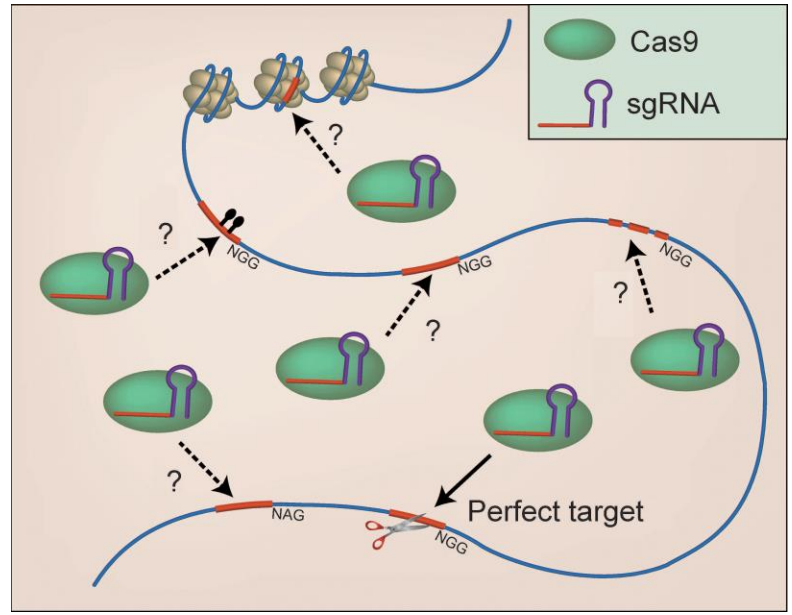
In vivo Genome Editing with CjCas9



- CjCas9 targeted to Hif1a or Vegfa reduced the size of choroidal neovascularization
- Demonstrating the potential for treatment of age-related macular degeneration

Nuclease Off-target Effects

- ZFNs, TALENs, and CRISPR-Cas9 can cleave off-target sites
- Off-target mutations can
  - Inactivate essential genes
  - Activate oncogenes
  - Cause chromosomal rearrangements



Wu et al. Quant. Biol. (2014)

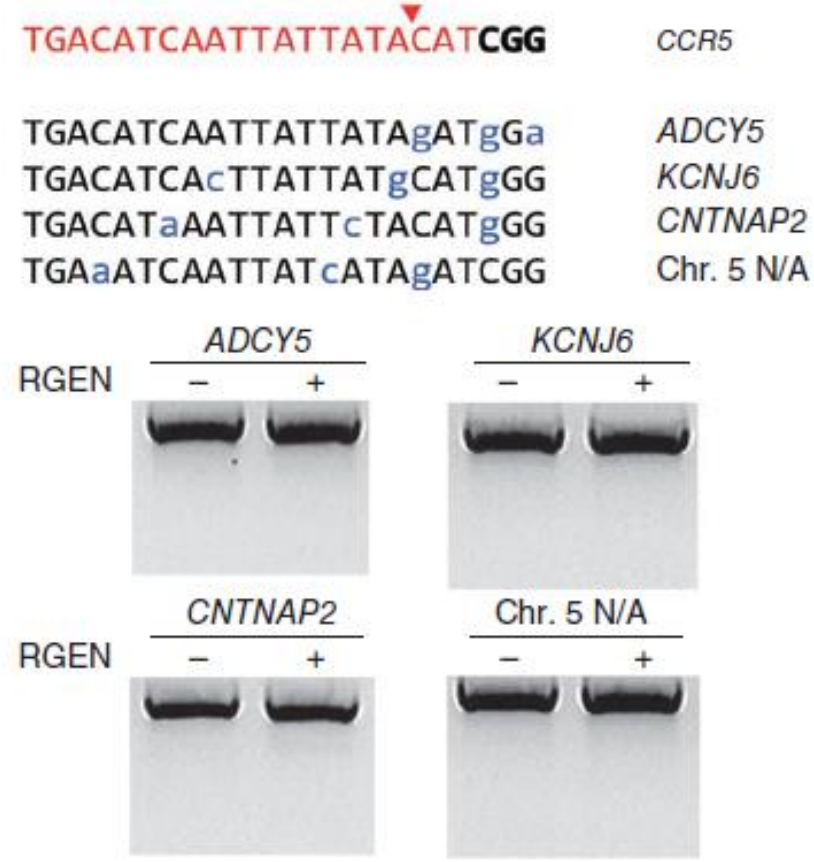
Undetectable CRISPR Off-target Mutations

nature biotechnology

Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease

Seung Woo Cho[1–3], Sojung Kim[1–3], Jong Min Kim[1,2] & Jin-Soo Kim[1,2]

published online 29 January 2013; doi:10.1038/nbt.2507

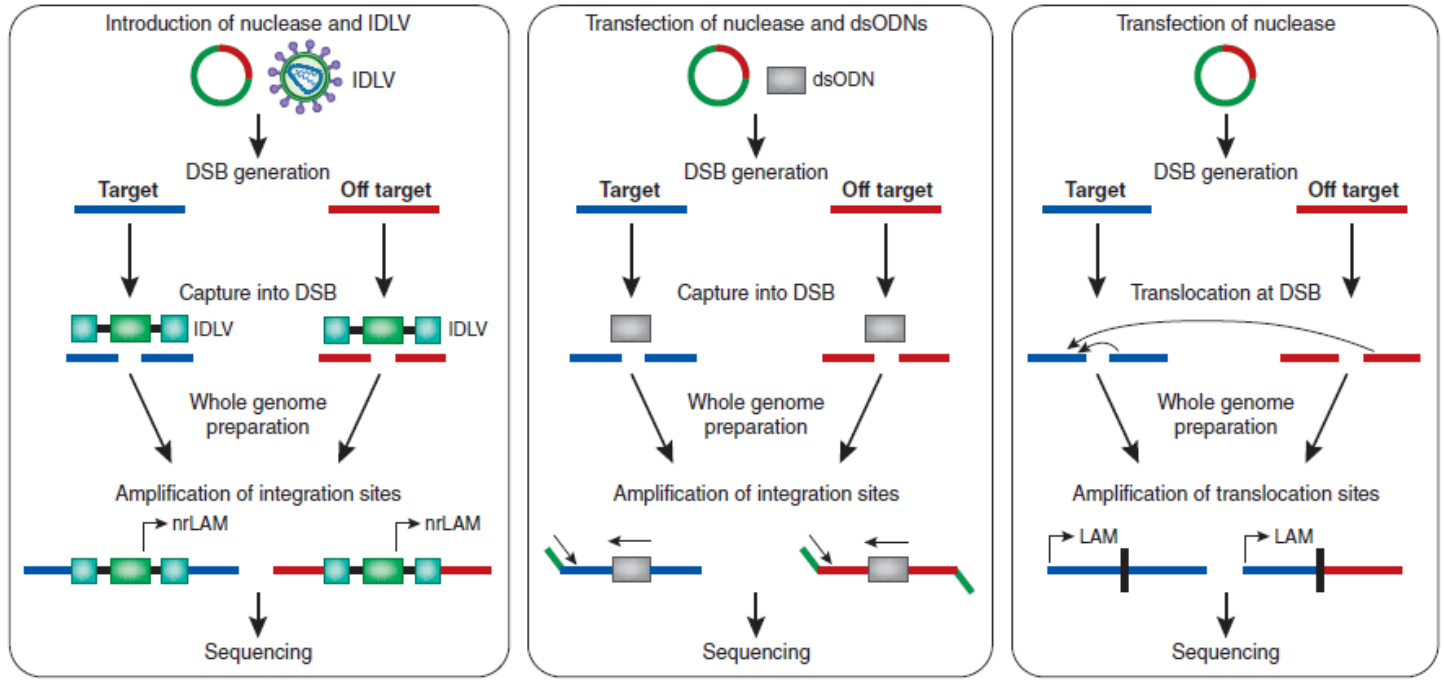


- No detectable off-target mutations at sites with 2- or 3-nucleotide mismatches
- Confirmed by targeted deep sequencing in Cho et al. Genome Res. 2014

How to Assess Genome-wide Off-target Effects

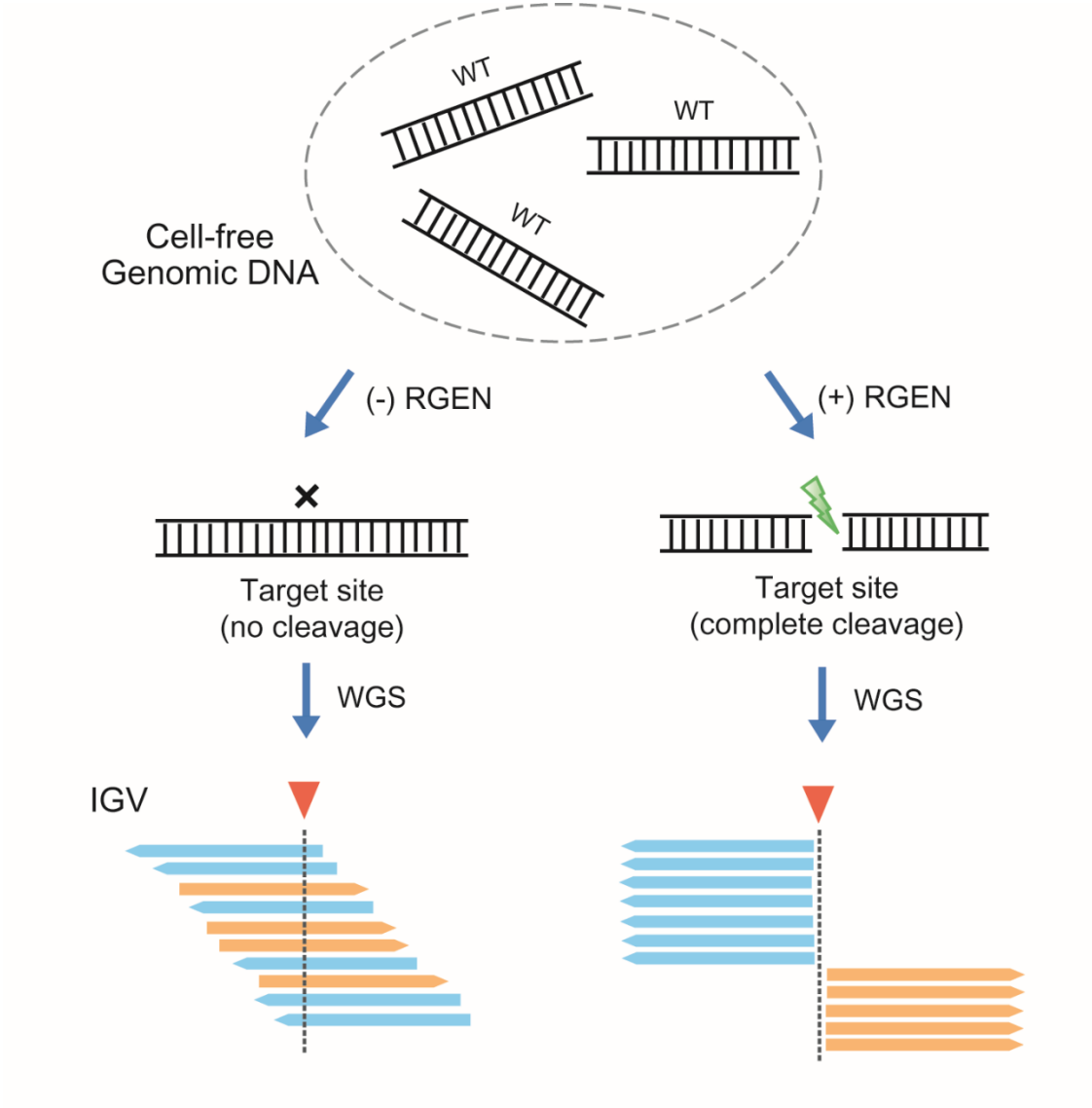
- Whole genome sequencing: Limited by sequencing depth
- Digenome-seq: Nuclease-digested whole genome seq.
- Cell-based methods: GUIDE-seq, Translocation seq., BLESS

Kim et al. Nat. Methods (2015)



Gabriel et al. Nat. Biotechnol. (2015)

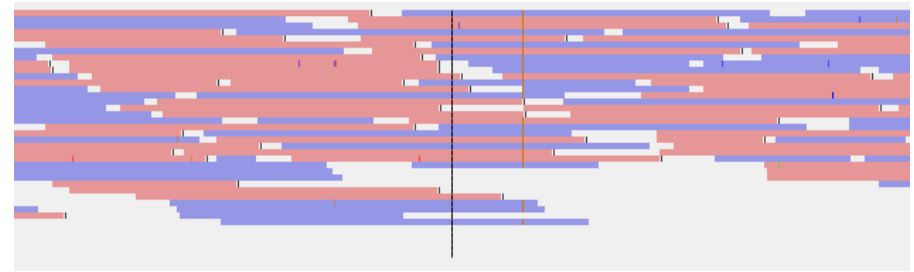
Overview of Digenome-seq



Kim et al. Nat. Methods (2015)

Staggered Alignment vs Straight Alignment

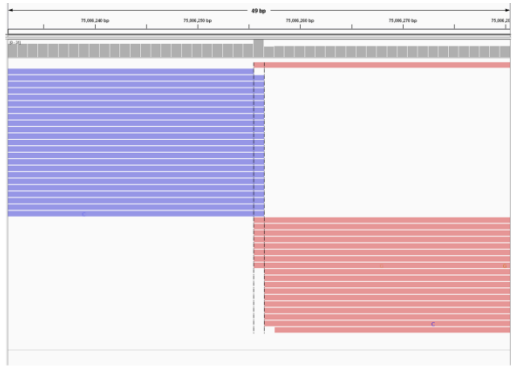
(-) RGEN



(+) RGEN

- After WGS, sequence reads were mapped to the reference human genome.
- Straight alignments of sequence reads are observed at the on-target site.

DNA Cleavage Score



		75006253	75006254	75006255	75006256	75006257	75006258	75006259
Count	Reverse	0	0	1	22	0	0	0
	Forward	0	0	0	9	9	1	0
Depth		23	23	23	31	18	19	19

Score at position i =

$$\sum_{a=1}^{5} \frac{100(F_i - 1)}{D_i} X \frac{100(R_{i-4+a} - 1)}{D_{i-4+a}} \times (F_i + R_{i-4+a} - 2)$$
$$+ \sum_{a=1}^{5} \frac{100(R_{i-1} - 1)}{D_{i-1}} X \frac{100(F_{i-3+a} - 1)}{D_{i-3+a}} \times (R_{i-1} + F_{i-3+a} - 2)$$

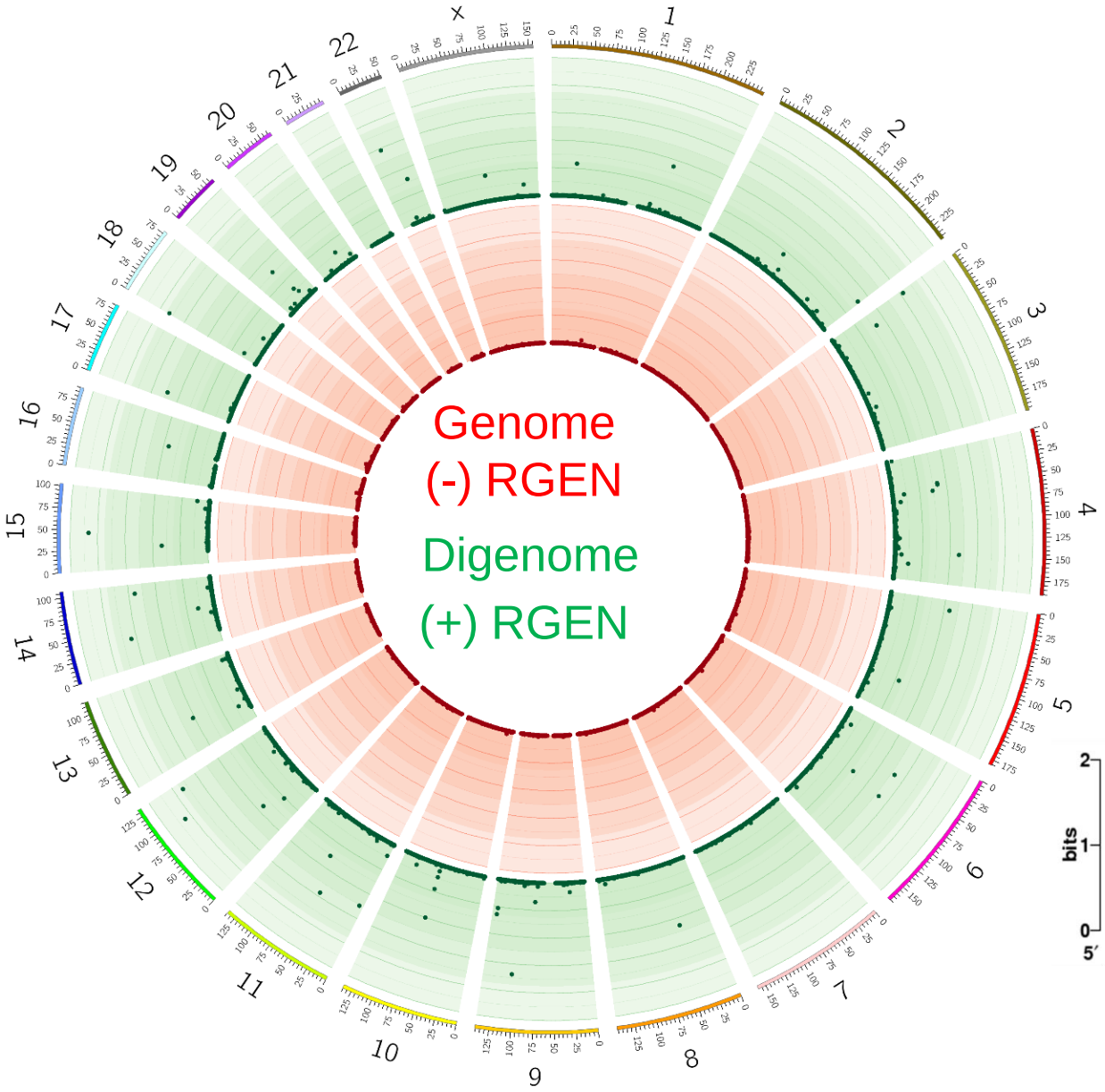
F_i : Number of forward sequence reads starting at position i

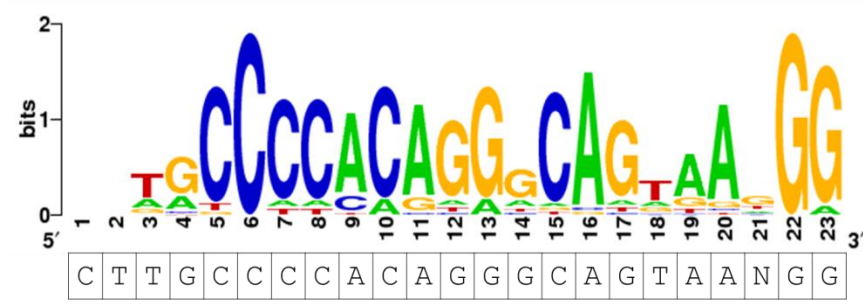
R_i : Number of reverse sequence reads starting at position i

D_i : Sequencing depth at position i

- Sequence reads with the same 5’ end are counted across the genome.
- DNA cleavage scores are assigned to each nucleotide position.

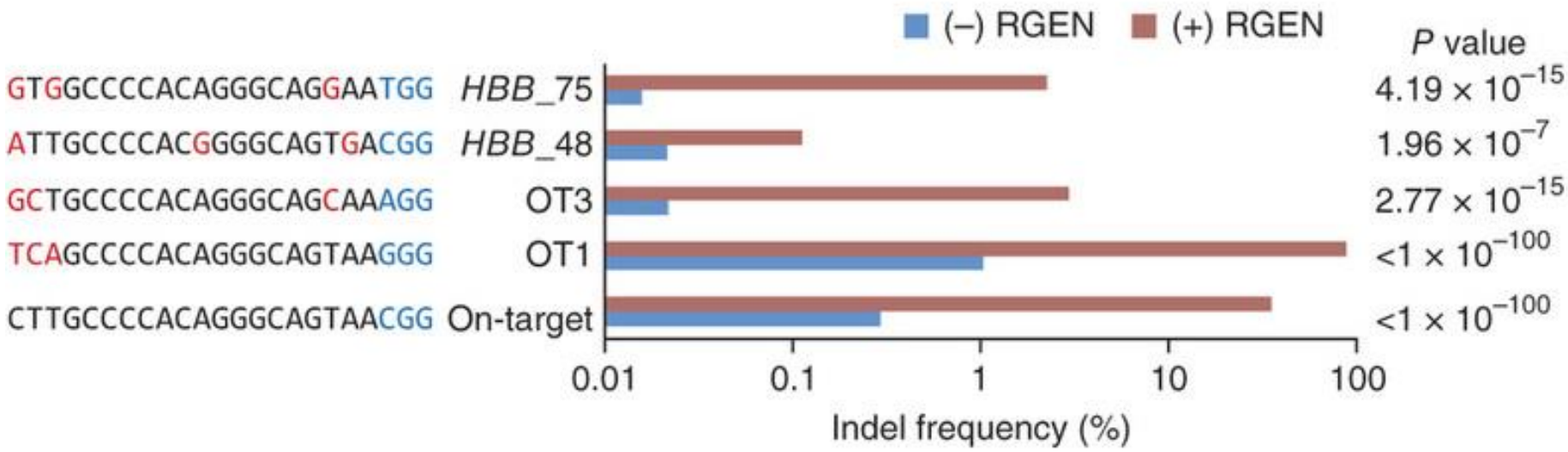
Genome-wide Cleavage Scores





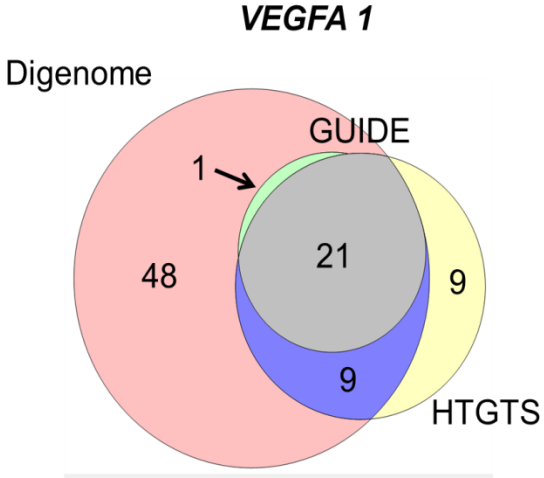
Kim et al. Genome Res. (2016)

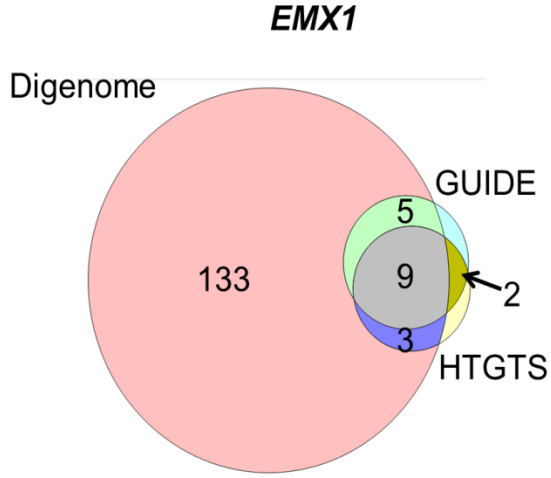
Off-target Sites Validated by Deep Sequencing



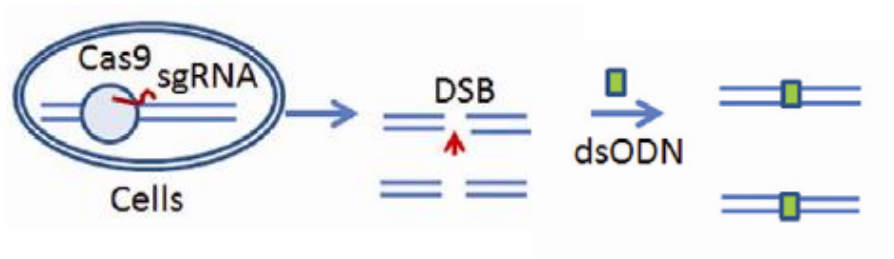
- 74 Digenome-captured sites were validated by targeted deep sequencing.
- Only five sites were mutated at frequencies ranging from 0.1 to 87%.

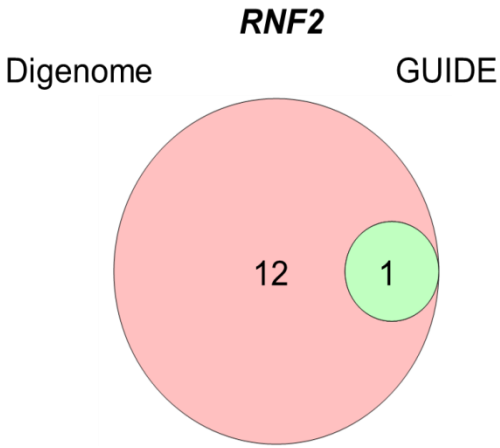
Digenome-seq vs Other Methods

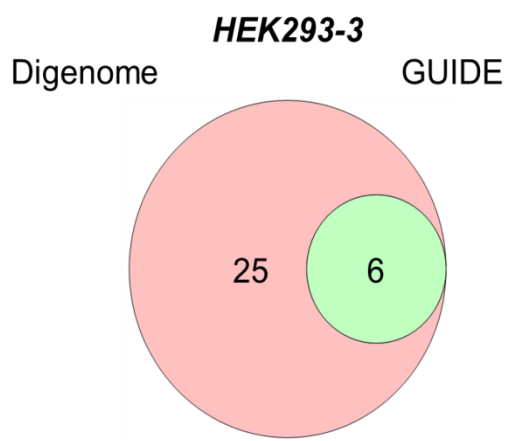




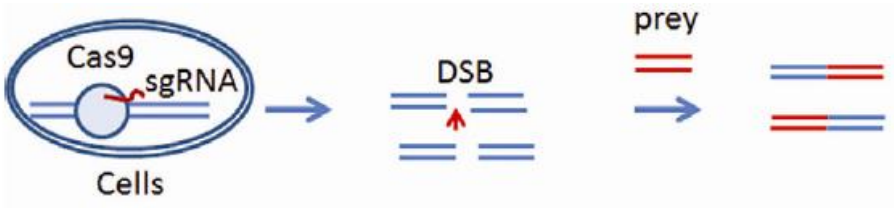
GUIDE-seq





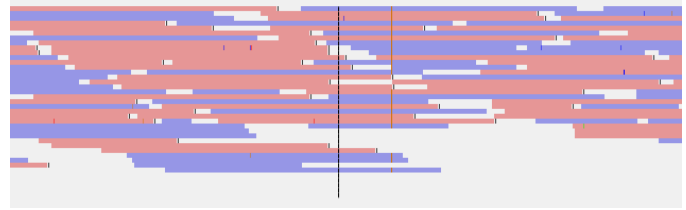


HTGTS

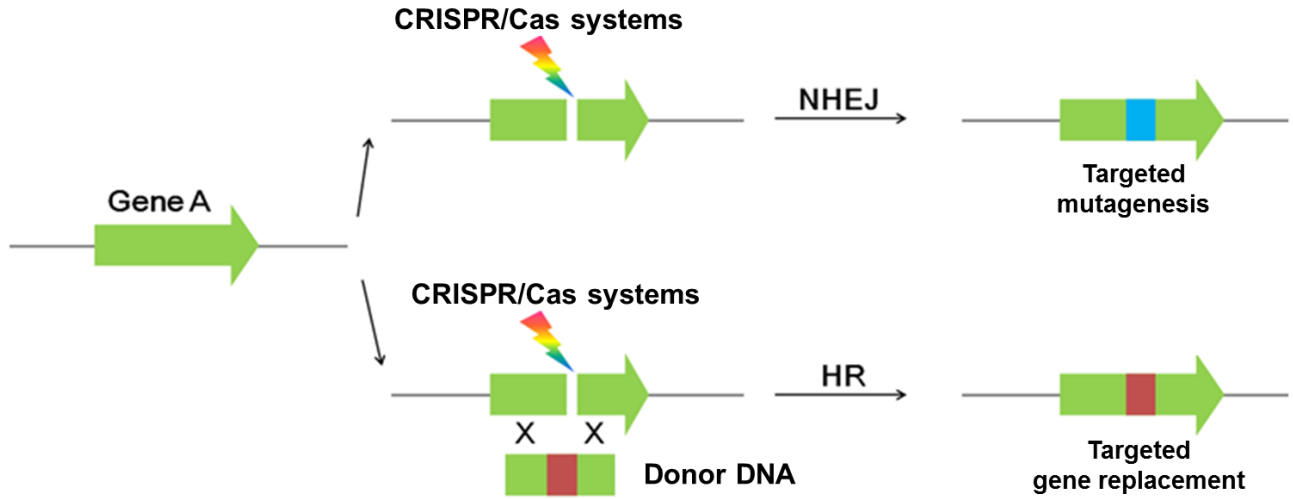


Digenome-seq Advantages

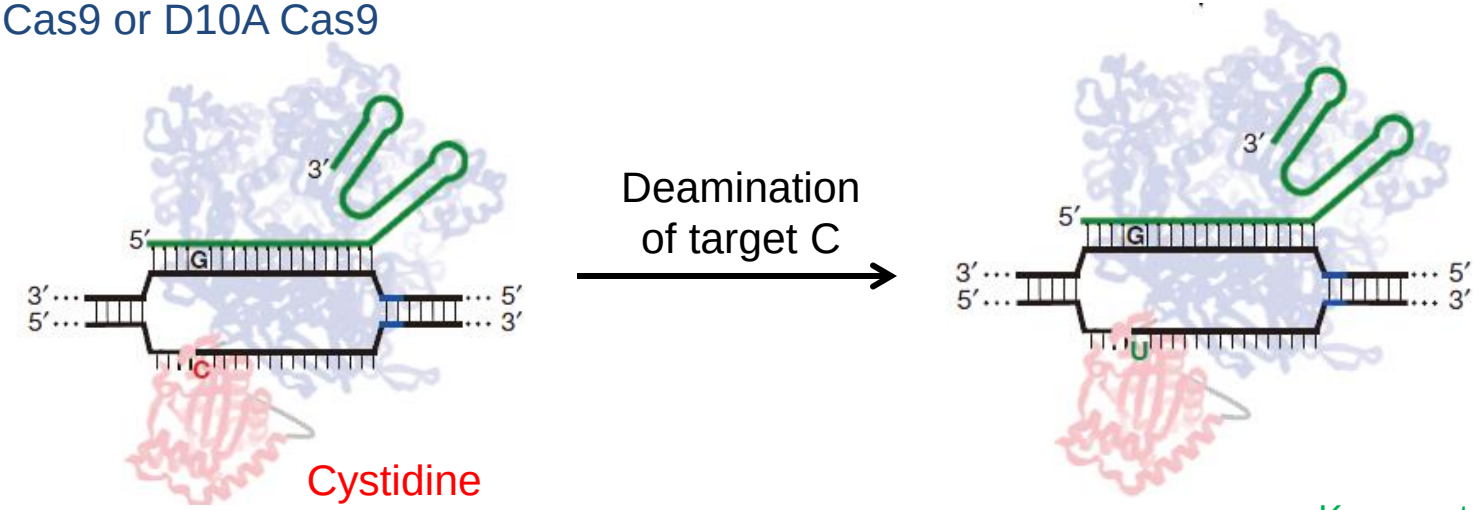
- Highly sensitive; Captures off-target sites w/ <0.1% indel freq.
- Not limited by chromatin accessibility
- Pinpoint off-target sites; no NHEJ-mediated indels in vitro
- Easy to carry out; no PCR steps prior to WGS
- Free form naturally-occurring DSBs in cells and PCR artifacts
- Compatible with RNA-guided programmable deaminases



Programmable Nuclease vs. Deaminase



dCas9 or D10A Cas9



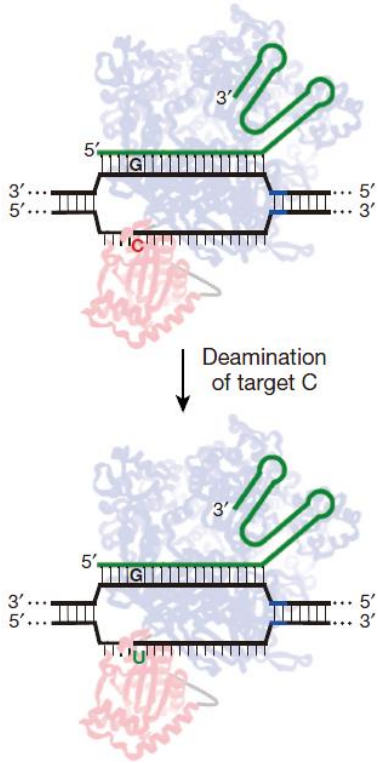
Cystidine
deaminase

Komor et al. Nature (2016)

Nishida et al. Science (2016)

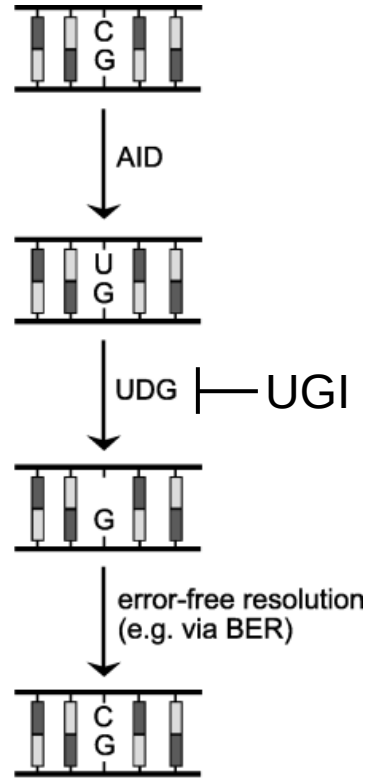
Programmable Deaminases

First-generation base editor (BE1)



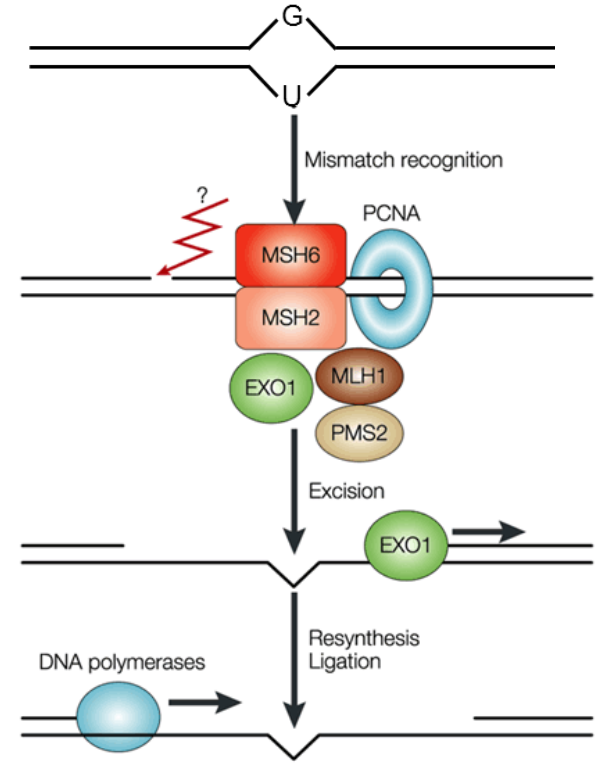
APOBEC1–dCas9

Second-generation base editor (BE2)



APOBEC–dCas9–UGI

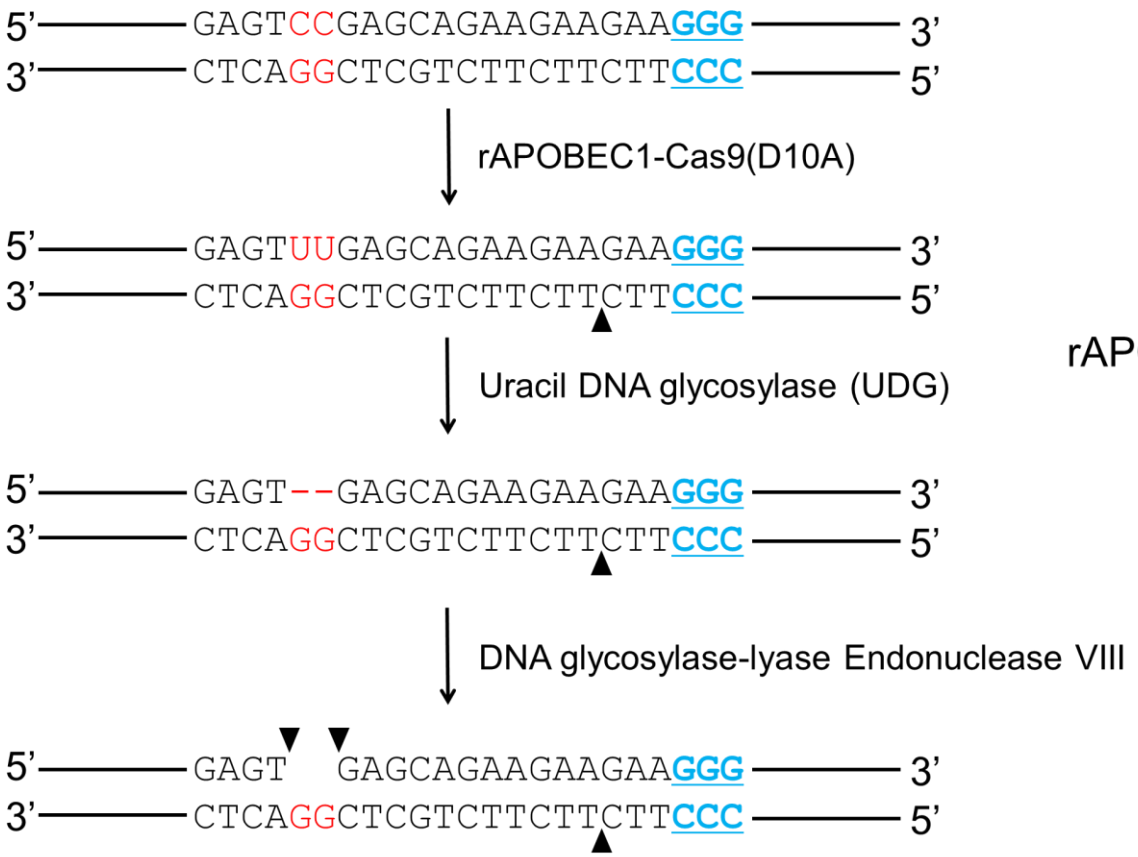
Third-generation base editor (BE3)

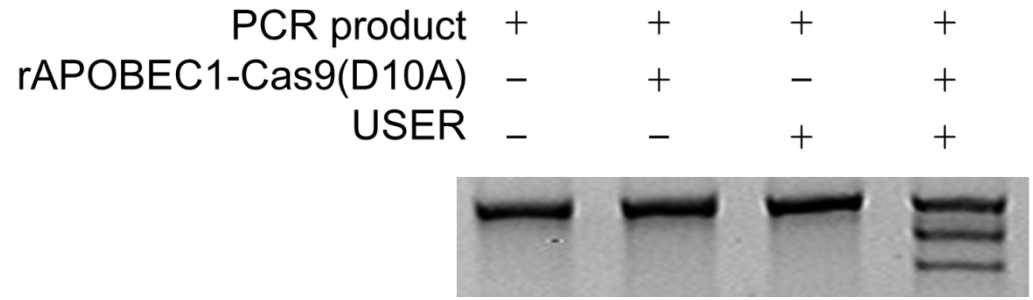


APOBEC–nCas9–UGI

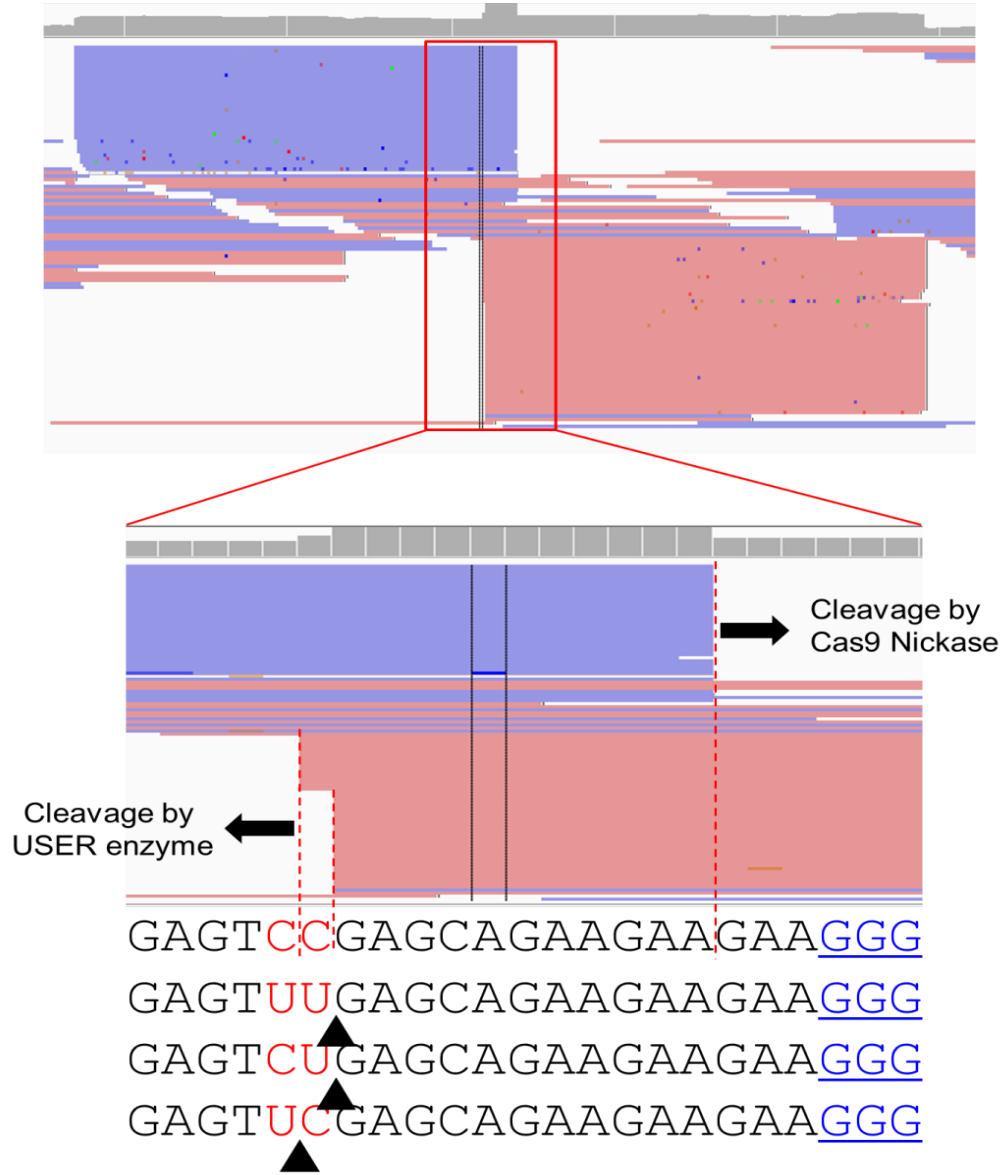
Komor et al. Nature (2016)

DNA cleavage at uracil-containing sites

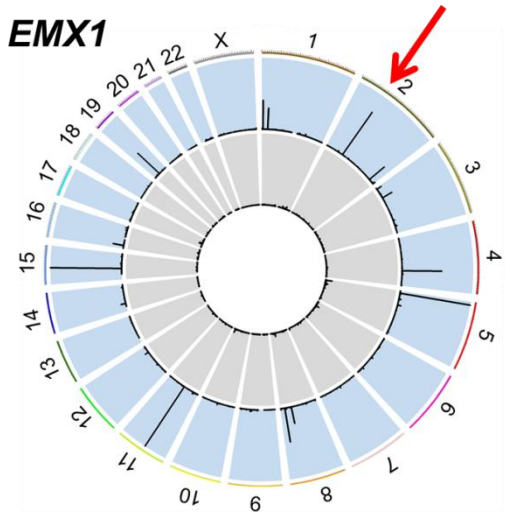




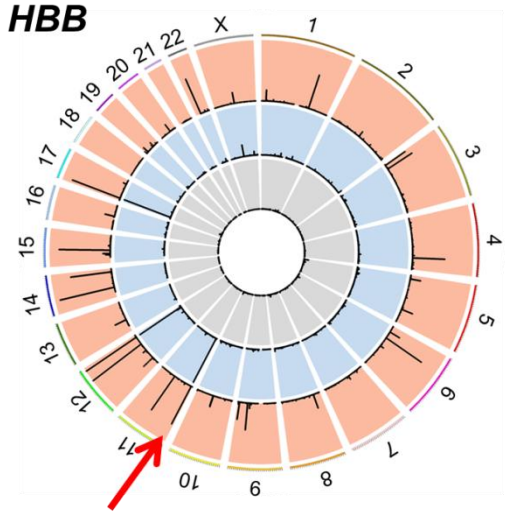
Digenome-seq of BE3-treated genomic DNA



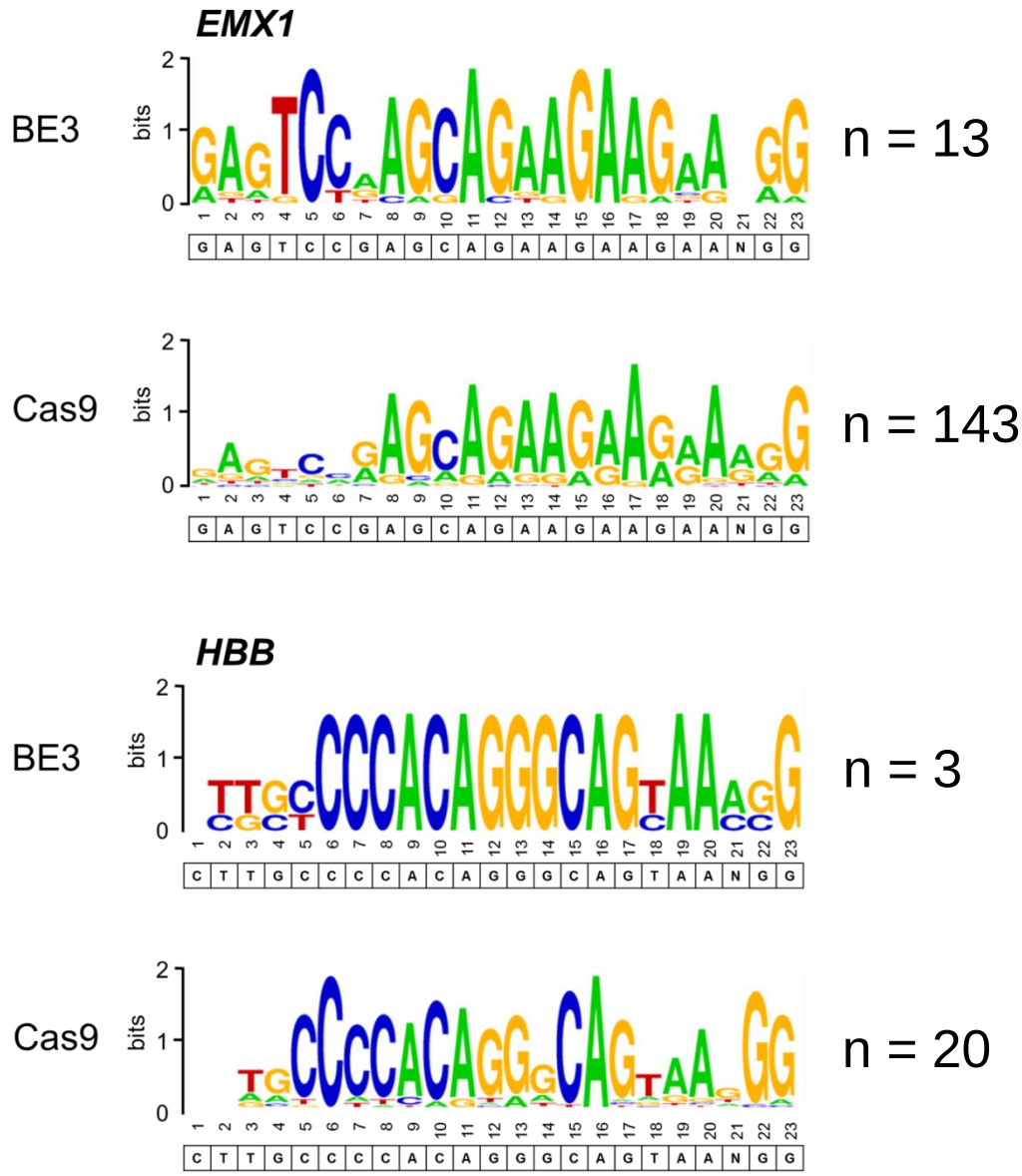
Genome-wide BE3 off-target sites



Untreated (+) Base Editor

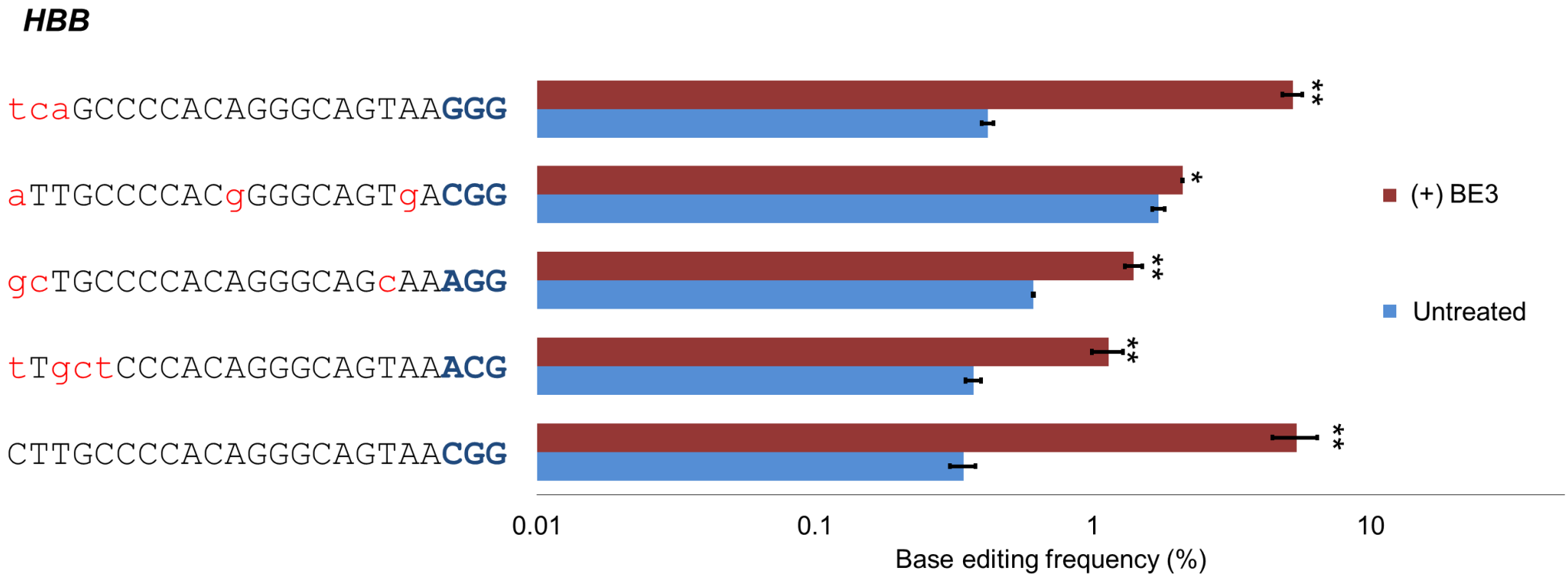


Untreated (+) Base Editor
(+) Cas9 nuclease



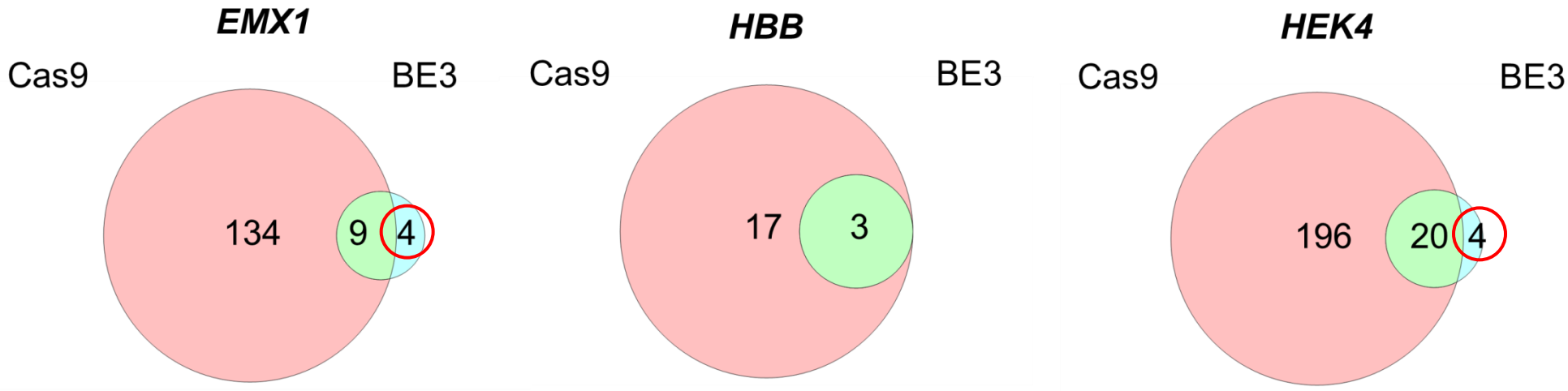
Kim et al. NBT (2017)

Off-target sites validated in human cells



Kim et al. NBT (2017)

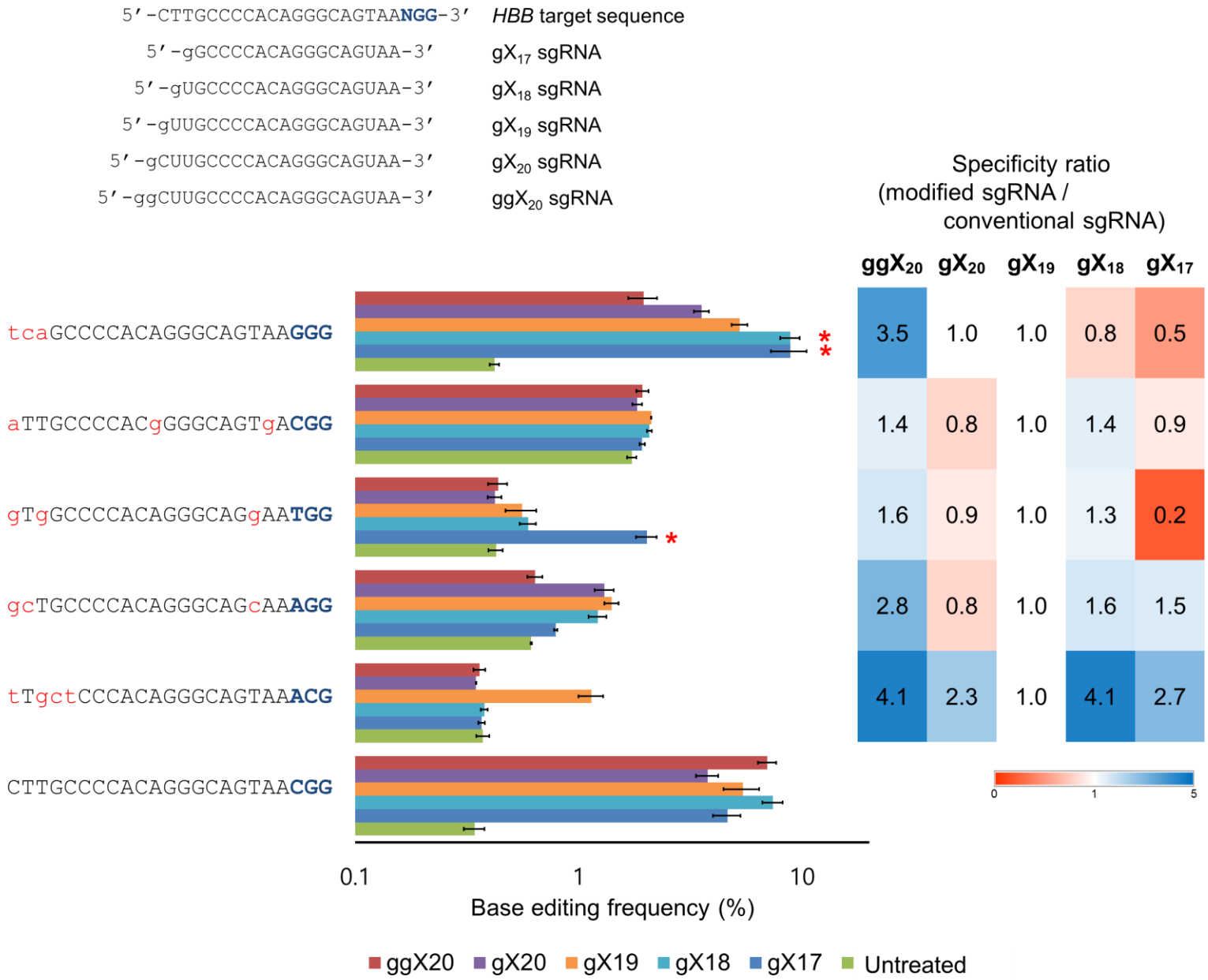
Cas9 and BE3 Off-target Sites



EMX1		
	DNA seq at a cleavage sites	Bulge
EMX1_5	GAaTCCaAG-AGAAGAAGAA**TGG**	RNA bulge
EMX1_8	GtGTCCtAG-AGAAGAAGAA**GGG**	RNA bulge
EMX1_12	GAGTCCacaCAGAAGAAGAA**AGA**	x
EMX1_13	GAGTCCaAG-AGAAGAAGtg**AGG**	RNA bulge

HEK293-4		
	DNA seq at a cleavage sites	Bulge
HEK4_3	GGCACTGCa-CTGGAGGTtG**TGG**	RNA bulge
HEK4_8	GGCACT-gGGCTGaAGGTaG**AGG**	RNA bulge
HEK4_19	GGCACTG-GGCTGGAGGcGG**GGG**	RNA bulge
HEK4_24	GGCACTG-GGCTGGAGaTGG**AGG**	RNA bulge

Reducing BE3 off-target effects via modified sgRNAs



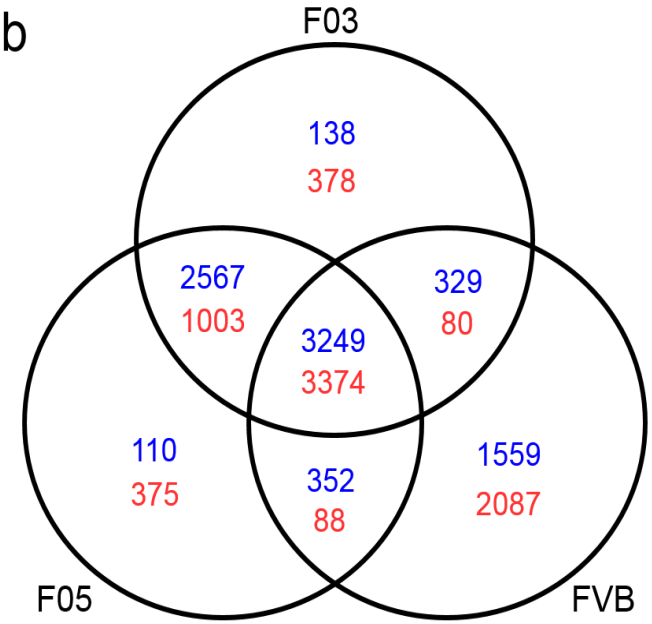
Unexpected CRISPR Off-target Mutations In Mice?

a

	F05 vs F03	F03 vs F05	FVB vs F05	F05 vs FVB	FVB vs F03	F03 vs FVB
Strelka	1482	745	2759	2078	3251	2330
Mutect	2749	1957	5817	4026	6375	4348

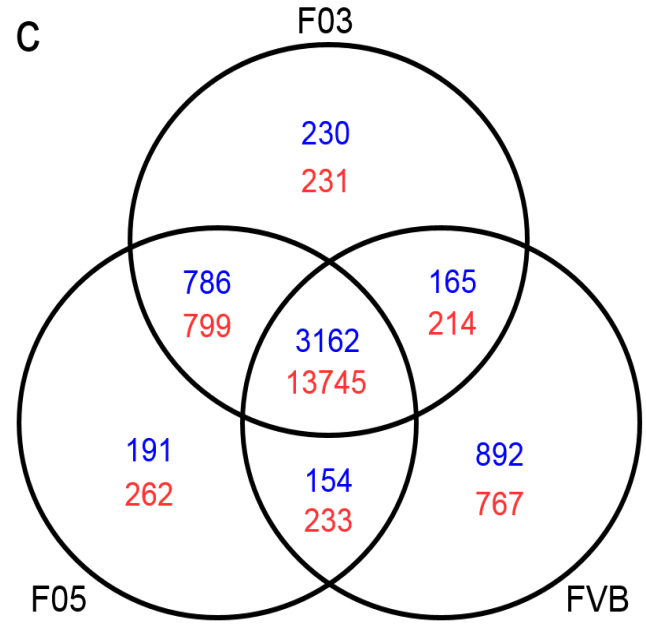
SNVs

b



SNVs

c



Indels

Kim et al. bioRxiv (2017)

- Schaefer et al. (Nature Methods, 2017) did not validate off-target effects
- Neglected SNVs and indels unique to the “co-housed control” mouse

How to Avoid Off-target Effects

- Choose a unique target site
- Use purified Cas9/Cpf1/BE proteins rather than plasmids
- Use modified guide RNAs
- Attenuated Cas9 proteins: eCas9 or Cas9 HF

Koo et al. Molecules and Cells (2015)

Do Off-target Effects Matter?

- No drugs are free from off-target effects, often leading to repositioning
- Etoposide, an anti-cancer drug, cleaves DNA randomly, inducing mutations
- CCR5-targeted ZFN has been proven safe in a clinical test (thus far)
- Biological consequences rather than mutations per se are more relevant

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