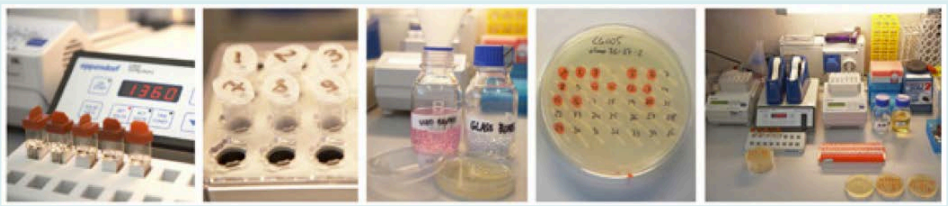
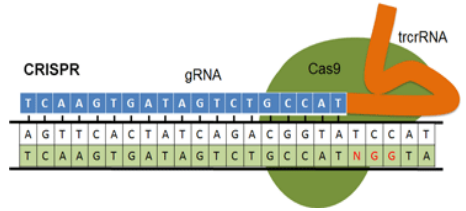
Engineering Genomes:

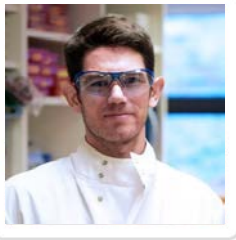
An Introduction to CRISPR/Cas9 Technology

Weatherall Institute of Molecular Medicine,
Virtual D.Phil. course, Oxford
November 12th, 2020

Philip Hublitz, PhD
University of Oxford, MRC WIMM, Genome Engineering Services





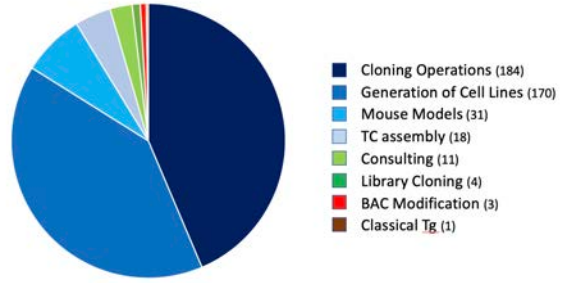


The WIMM Genome Engineering Core

We assist PIs in the design and execution of Genome Engineering needs.
We assemble custom DNA-based targeting constructs.
→ **Regular cloning, RedET Recombineering, ELAN, Gibson & Golden Gate assembly** and **Directed Synthesis**.

On average we handle about 80 projects per year.

- 8% **Mouse production** by ESC targeting.
- 42% **KO/KI** cell line-generation.
- 6% **CRISPR screens**, **CRISPRa** & **CRISPRi** approaches.
- 44% **Complex cloning** operations, other model animals.



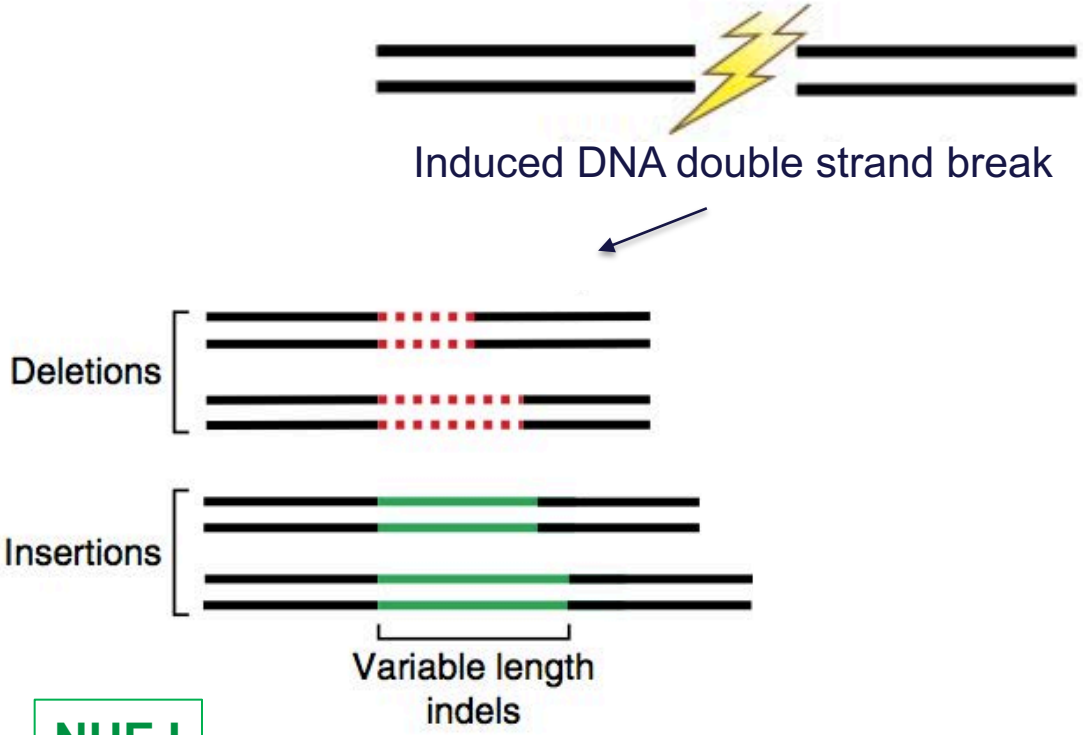
Ample experience in the use of **CRISPR/Cas9** for gene editing.
Development of **custom targeting strategies**.
Execution of **screening strategies and QC** to verify new model systems.

Any Genome Engineering Starts with a DSB

Induced DNA double strand break

Sander and Joung, NBT, 2014

Cellular DNA Repair Pathways



NHEJ

Repair w/o template.

Sander and Joung, NBT, 2014

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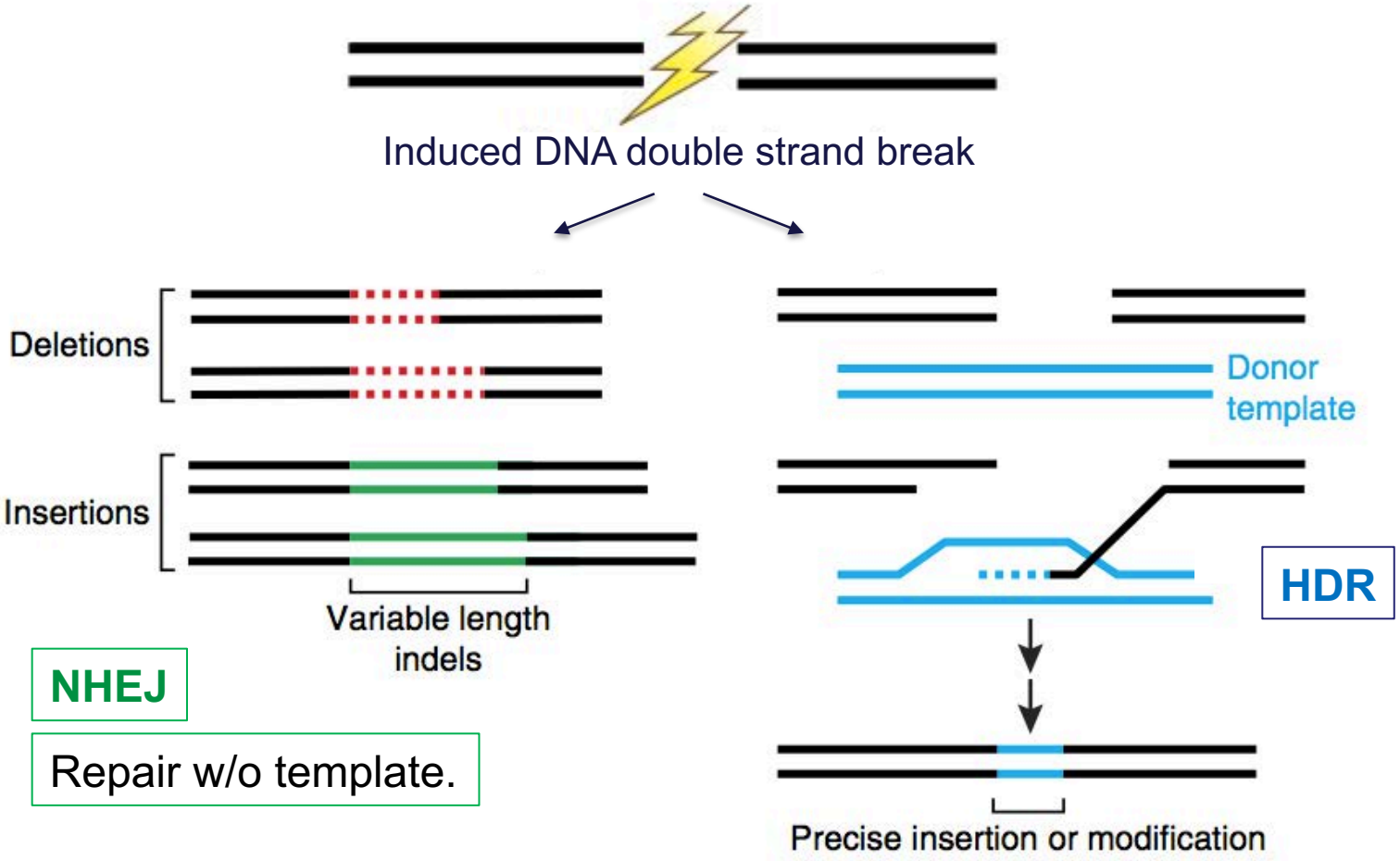


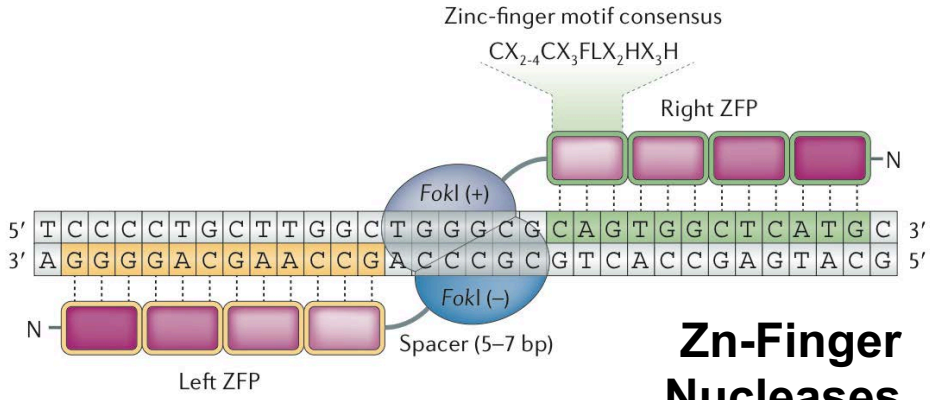
NHEJ

Repair w/o template.

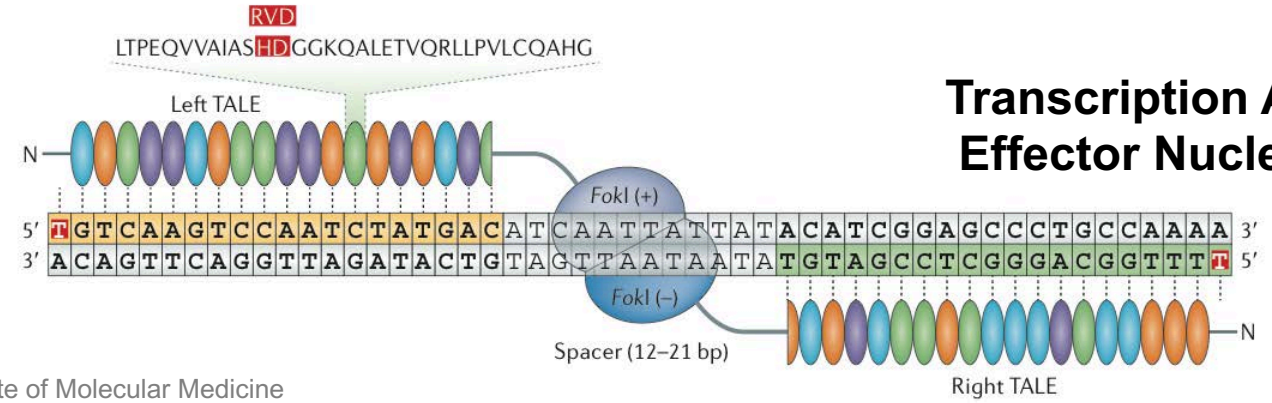
Sander and Joung, NBT, 2014

Pre-CRISPR Times

	ZFNs	TALENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors
Nuclease	*FokI*	*FokI*
Success rate‡	Low (~24%)	High (>99%)
Average mutation rate§	Low or variable (~10%)	High (~20%)
Specificity-determining length of target site	18–36 bp	30–40 bp
Restriction in target site	G-rich	Start with T and end with A (owing to the heterodimer structure)
Design density	One per ~100 bp	At least one per base pair
Off-target effects	High	Low
Cytotoxicity	Variable to high	Low
Size	~1 kb×2	~3 kb×2

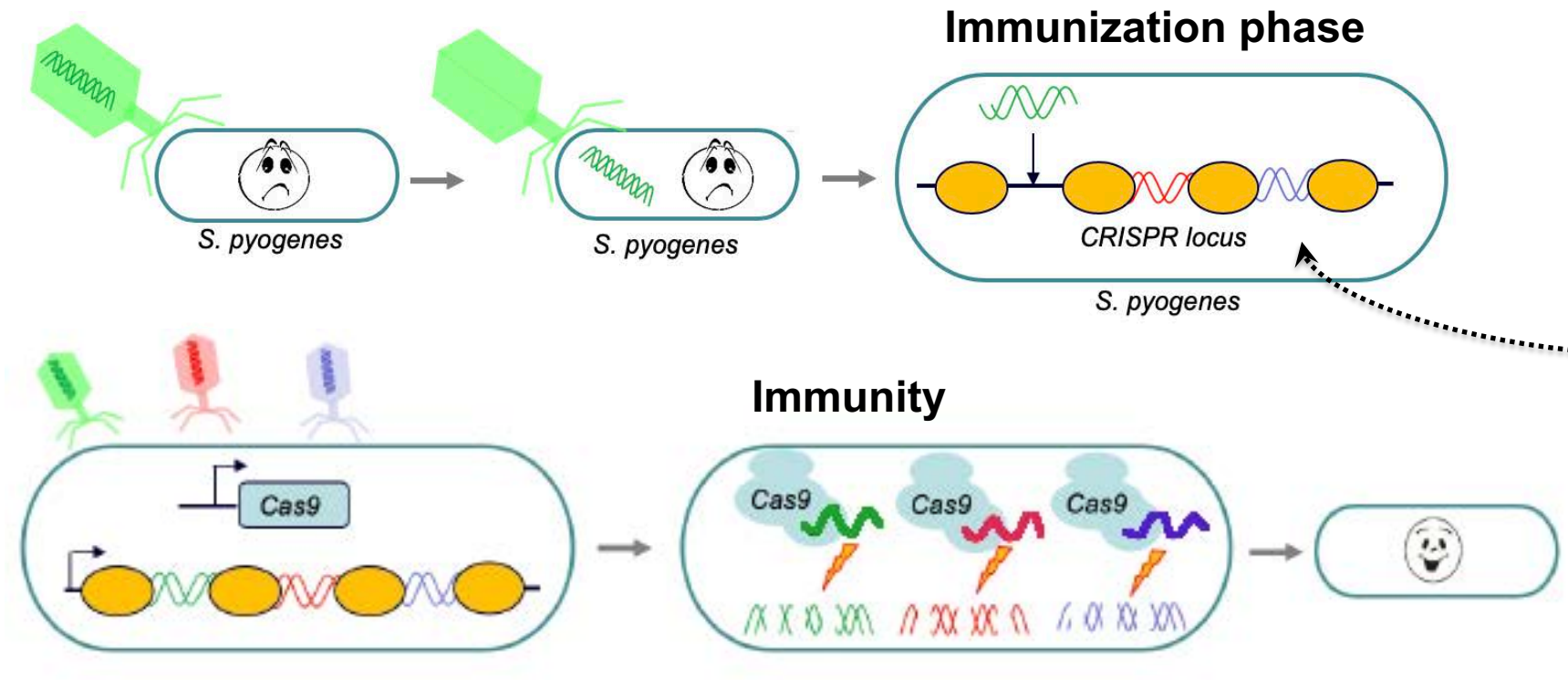


Zn-Finger Nucleases



Transcription Activator-Like Effector Nucleases

CRISPR

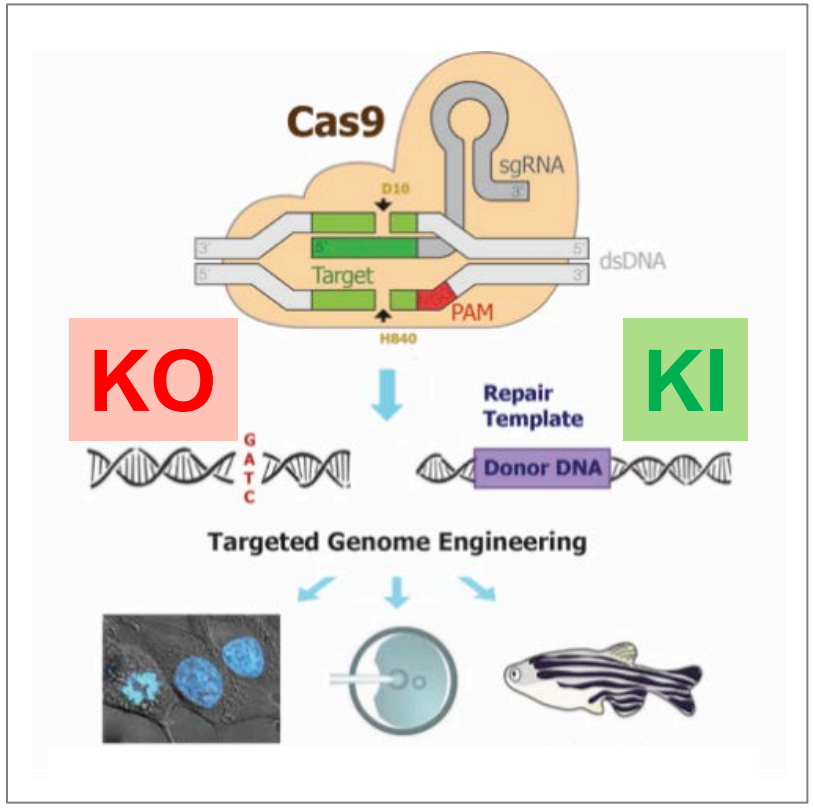


Clustered
Regularly
Interspersed
Short
Palindromic
Repeats

Ben Davis, Addgene,
Mali et al., Nature Methods, 2013

CRISPR/Cas9

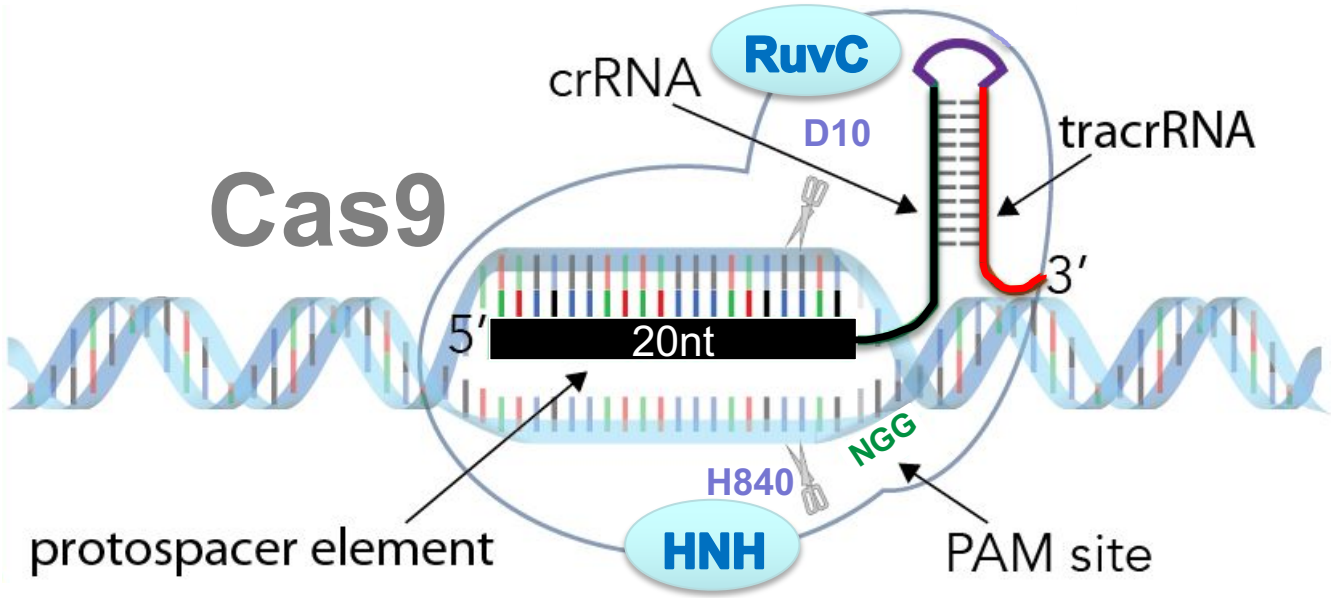
Nature, March 6th, 2014



WIMM Genome Engineering, Website, 2018

Stockholm, Nobel Prize for Chemistry 2020
The Guardian, 07.10.2020

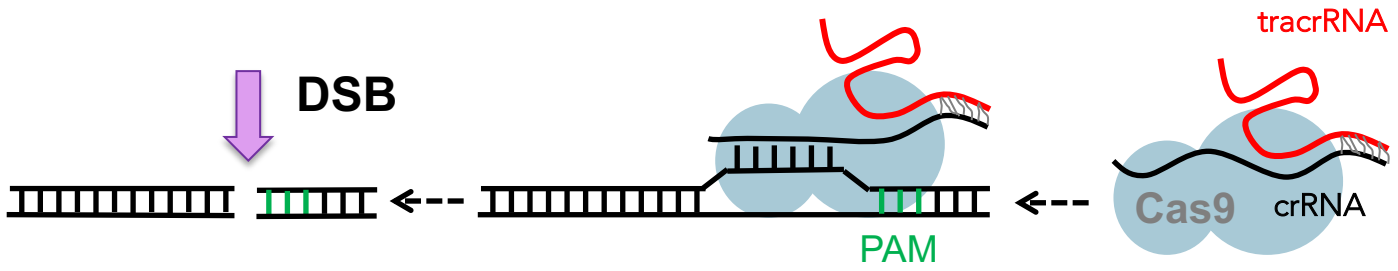
Cas9: CRISPR Associated Protein 9



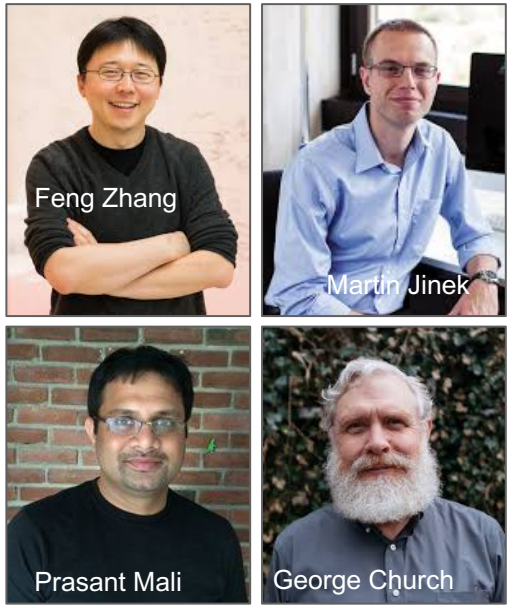
Nobel Committee
Illustration for the 2020 Nobel Prize for Chemistry

Cas9: Nuclease, DNA cutting tool
crRNA: Specific localization by a 20nt RNA
PAM: Specificity, only when present cut takes place

Cas9 Targeting

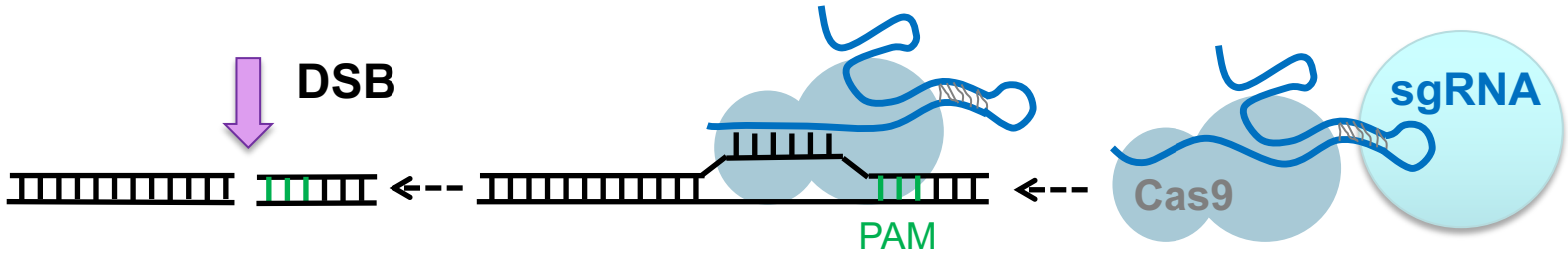


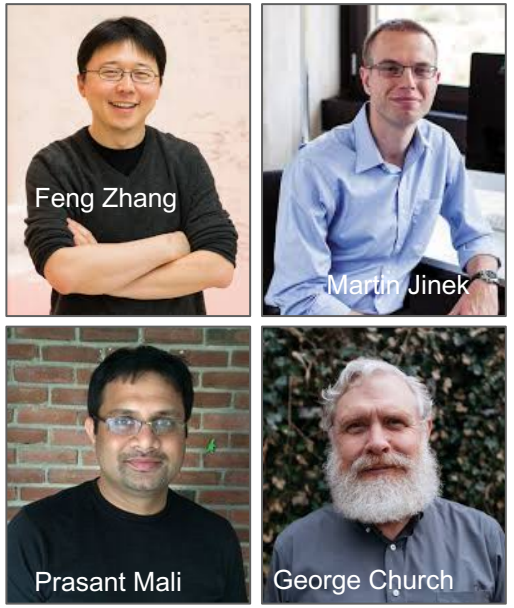
Only if a **PAM** is present, a blunt cut 3 bp upstream of the PAM can be made (conformational rearrangement).



Jinek *et al.*, Science, 2012
Mali *et al.*, Nature Methods, 2013

Cas9 Targeting





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Fusion of crRNA and tracrRNA is possible. Easy-to-use 2-component system of Cas9 and a **single guide RNA**.

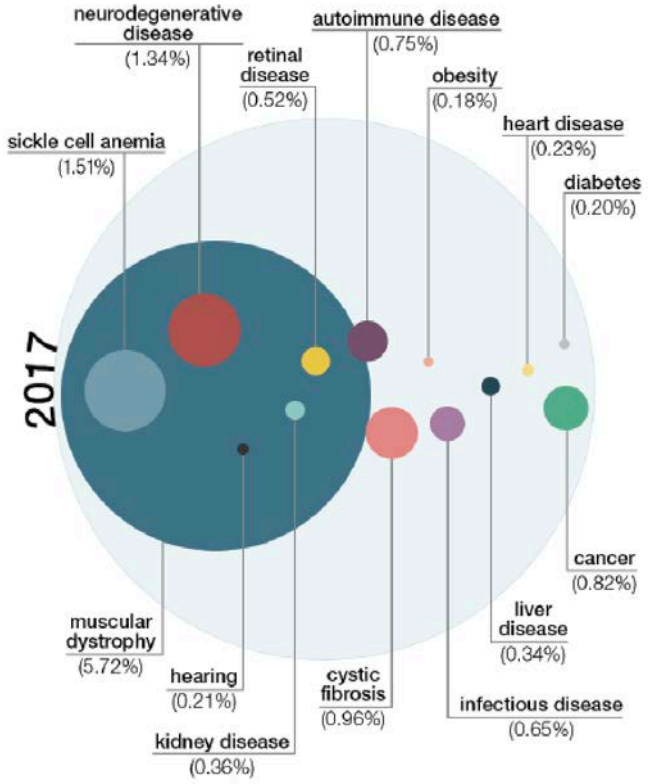
Jinek *et al.*, Science, 2012
Mali *et al.*, Nature Methods, 2013

CRISPR/Cas9: What is it good for?

Generation of **LOF/GOF models**: Engineering cells and organisms, attempting to cure diseases.

Nature, 2015

Nature, 2016



Research Arc Cell Press, 2018

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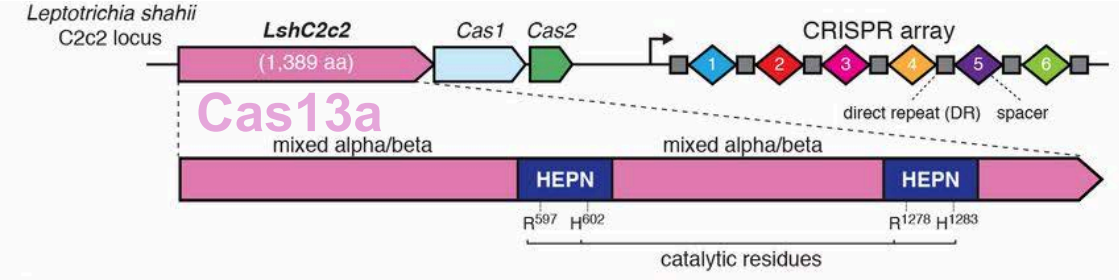
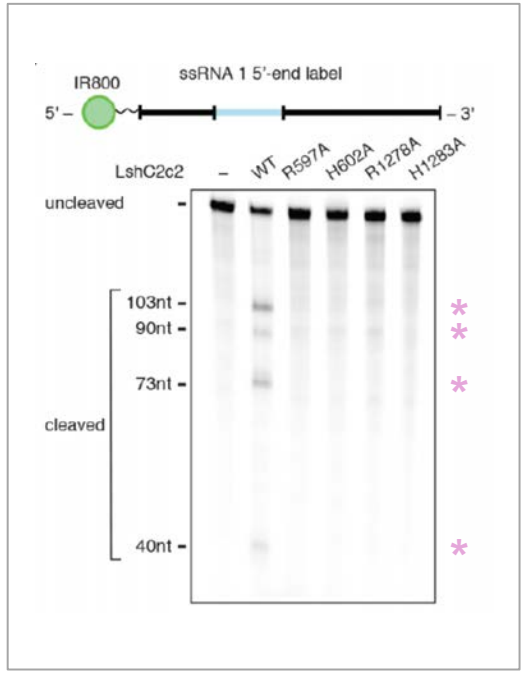
Generation of **LOF/GOF models**.

mRNA editing/detection



Abbudayyeh, Sherlock BioSciences



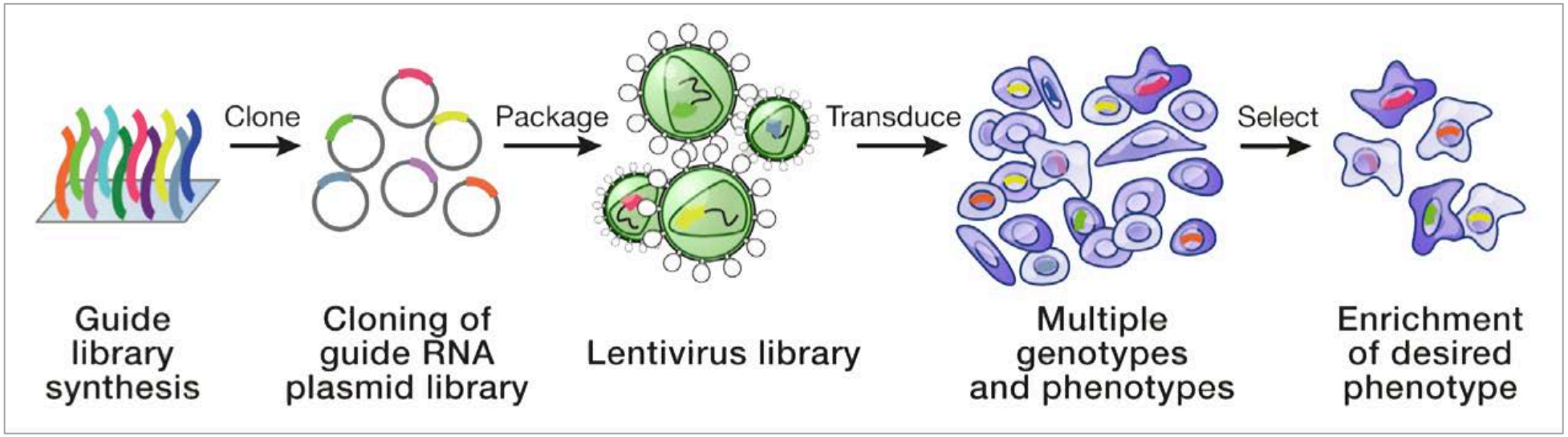
Abbudayyeh *et al.*, Science, 2016

CRISPR/Cas9: What is it good for?

Generation of **LOF/GOF models**.

mRNA editing/detection

High-throughput screens: KO, CRISPRi, CRISPRa



Hsu *et al.*, Cell, 2014
Bak *et al.*, NBT, 2015

CRISPR/Cas9: What is it good for?

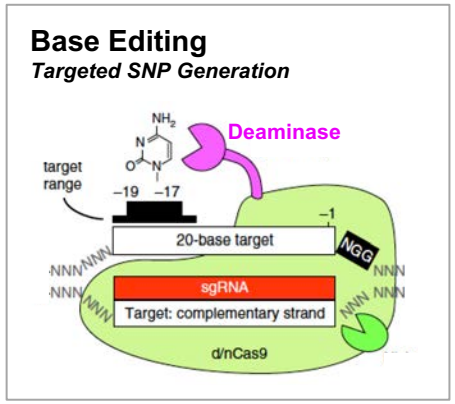
Generation of **LOF/GOF models**.

mRNA editing/detection

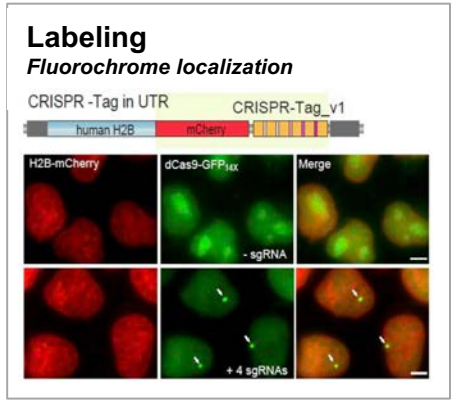
High-throughput screens: KO, CRISPRi, CRISPRa

Targeting fusion proteins:

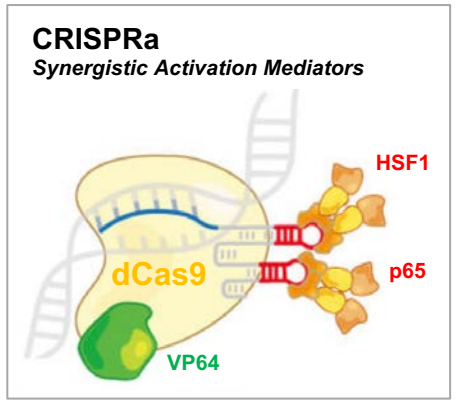
- Labeling
- Gene activation
- Gene repression
- Epigenetic remodeling
- Base editing



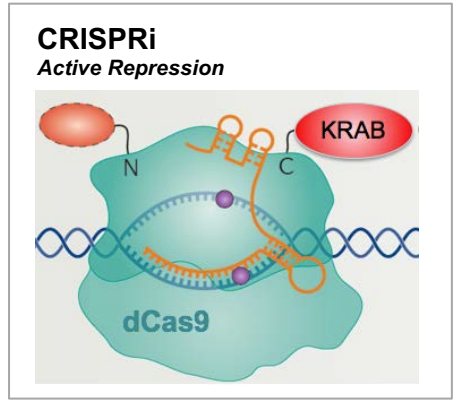
Komor *et al.* Nature, 2016



Ma *et al.*, NBT, 2016



Konermann *et al.*, Science, 2015



Gilbert *et al.*, Cell, 2014

CRISPR Pipeline

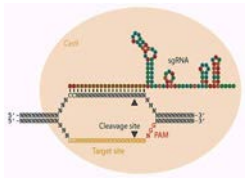
Pros and Cons

Depends on the experiment.

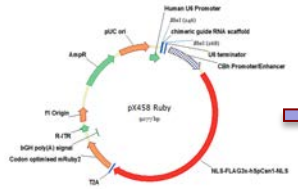
Cell Line and Reagent-specific

To be empirically determined.

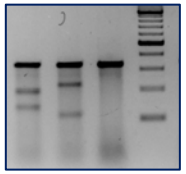
sgRNA design



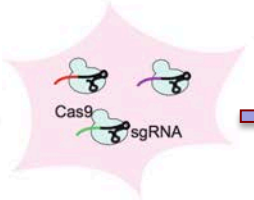
Vector or RNP



ON-target validation

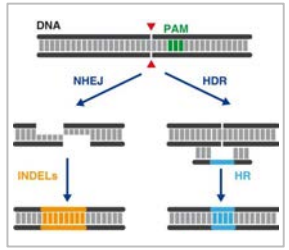


Delivery



Enrichment

Target Cleavage and Repair



Screen

Algorithms & sgRNA choice

Avoidance and Requirements.

Surveyor assay

I am a believer.
Especially when using 2 sgRNAs…

Plasmid allows FP sorting and/or antibiotic selection.
HDR allows selection.

No enrichment with RNPs.
Is it necessary?

Custom designed

Tips and Tricks

Plasmid based sgRNAs: Key Requirements

Thoru Pederson: **U6, H1** or **7SK** need a G to start (3 in WT).
Feng Zhang: One **G at 0** won't disturb. Replacing 1 + 2 with G's might affect Cas9 specificity.
Keith Joung: **18bp** guides are less prone for OFF-targeting due to binding thermodynamics.
Jin-Soo Kim: A **double G** at start is perfect (WGS based).

Ran *et al.*, Nature Protocols, 2014

CACC G GNN NNN NNN NNN NNN NNN NN
C CNN NNN NNN NNN NNN NNN NN **CAAA**
19nt

"Standard design" for guides to be used by plasmid delivery.

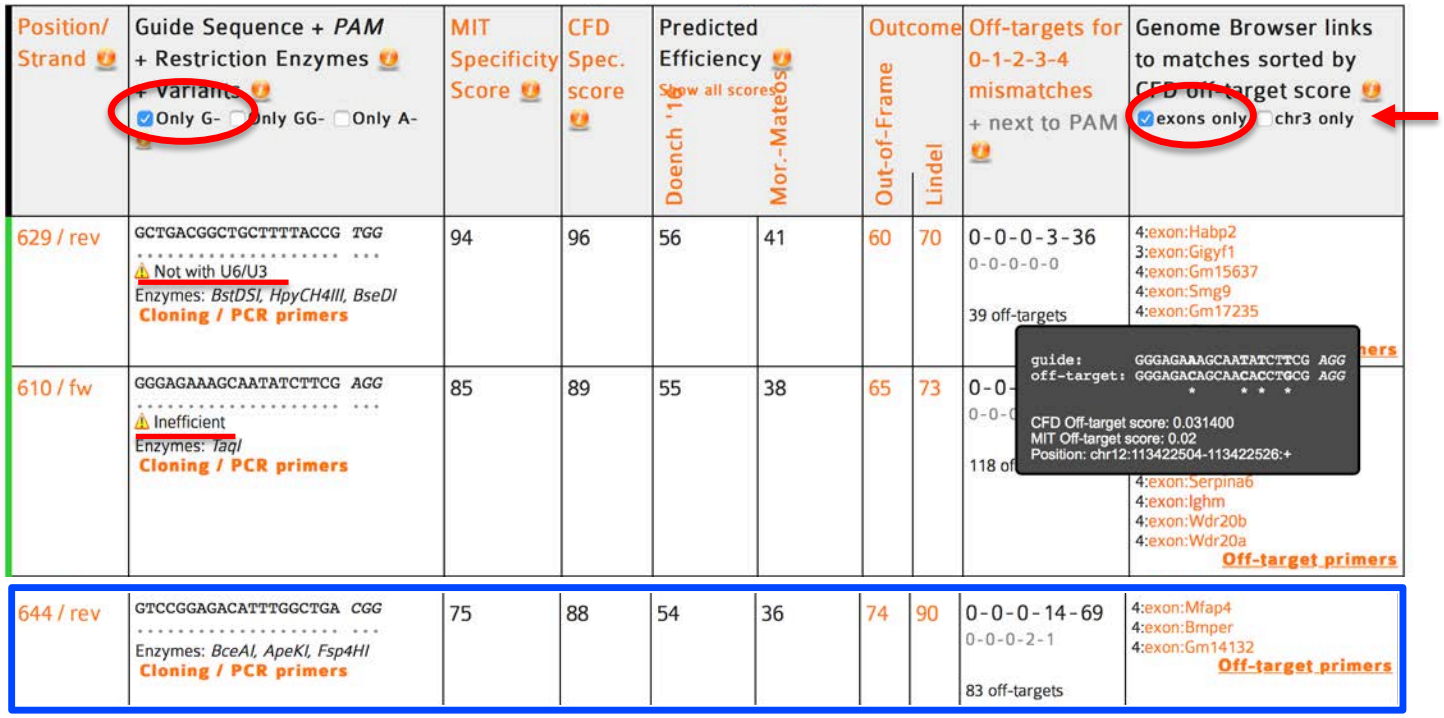
Hublitz and many others, unpublished

sgRNA Predicting Websites

Many available, all current online versions use very similar algorithms and exclusion criteria.

→ Personal choice: whichever graphic display you like best.

→ I use **CRISPOR** and BreakingCas.



Custom selection of best-possible guides:

(a) Least general OFFs
(b) No genic OFF < 4MM
(c) No OFF with < 3MM
(d) Scrutinize where MMs are
(e) Relaxed on intergenic and intronic OFFs
(f) Avoid “inefficient” guides?

Hublitz and many others, unpublished
Graf *et al.*, Cell Reports, 2019
Haeusler *et al.*, Genome Biology, 2016

CRISPR/Cas9 in Cell Lines

Good idea to test **3 sgRNAs** per target site: genome and epigenome.

Housekeeping on cell lines: (A) determine cell **ploidy** → you need to know what to expect, (B) screen and test for eventual **SNPs**, (C) find out if they can be **cloned** (crucial for establishment of cell lines), (D) are they mycoplasma free.

Establish **optimum delivery conditions**: efficiency vs. viability, delivery method (LPF, Amaxa, Neon), recovery for 48 to **72h** (test for maximum clonal survival rate).

Selection by Cas9-2A-**eGFP**/**BFP**/**Ruby**/etc. by FACS or use of Cas9-2A-**Puro**/**Bla**/**Gen** for enrichment culture. Isolation of single cell clones.

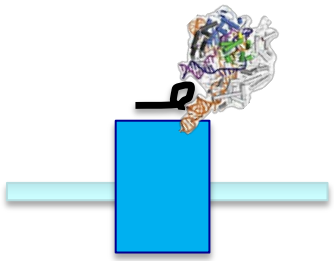
Screen: Analyze experimental clones by method of choice.

N.B.: **All off-target modifications are contained in the cell.**
Establish ≥ 3 independent lines for phenotype comparison.

Hublitz, personal summary

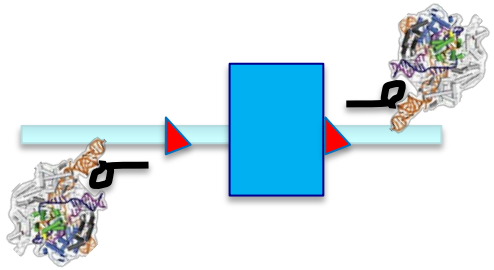
Which sgRNA to Choose

KO by NHEJ



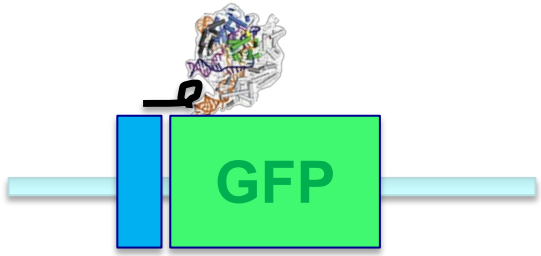
- Not w/in 1st exon.
- **Scan for downstream in-frame ATGs.**
- Do target all isoforms.
- Out-of-frame score indicates guides that are ≥ 2/3 useful...

KO by exon deletion



- Targeting the **critical exon** delivers **defined outcome**.
- Must delete the splice branch point.
- Requires roughly equal ON-target activity.

Knock In



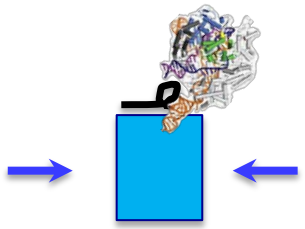
- Best placed **at insertion site**: the closer the higher is HDR-efficiency, more important for ssODN donors.
- Insert REN with ssODN donors.
- Inactivation of PAM/protospacer?

Lonowski *et al.*, Nature Protocols, 2017
Blayney *et al.*, Methods and Protocols, 2020

Best Practice Screening Approaches

Standard applications:

KO by single sgRNA

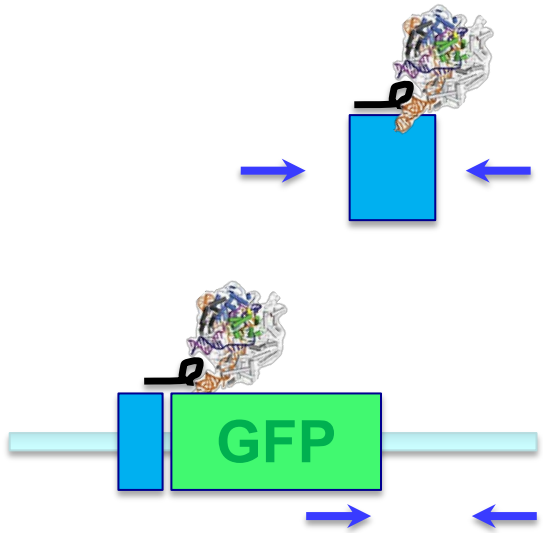


PCR the region of interest and Sanger.
HT-MiSeq screen.
Assays based on predicted gene function.
Protein based: WB or IIF.

Best Practice Screening Approaches

Standard applications:

KO by single sgRNA
<u>KI</u>



PCR the region of interest and Sanger.
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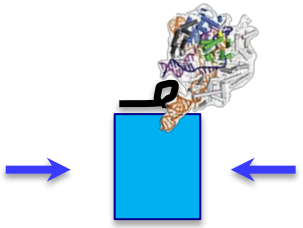
Assays based on predicted gene function.
Protein based: WB or IIF.

Inside out PCR.
Fluorescence microscopy if target is expressed.
Protein based: WB.

Best Practice Screening Approaches

Standard applications:

KO by single sgRNA
KI
SNPs



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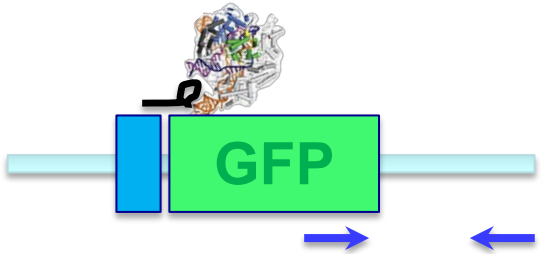

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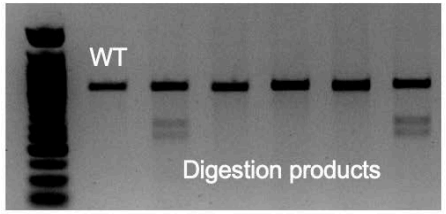
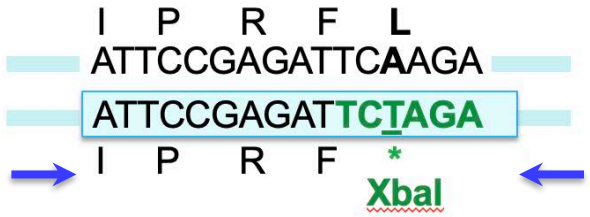
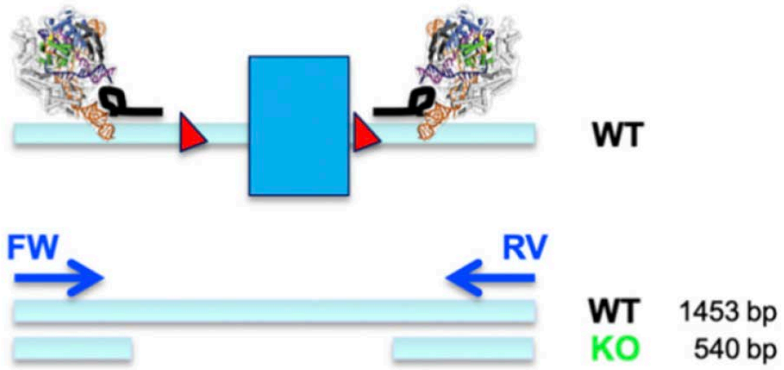
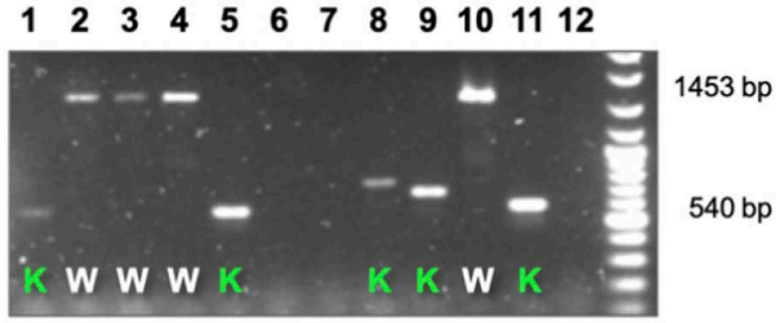




Same as for KO.
If possible, co-insert *de novo* REN with SNP:
PCR and digest.

Exon Deletion: Screen



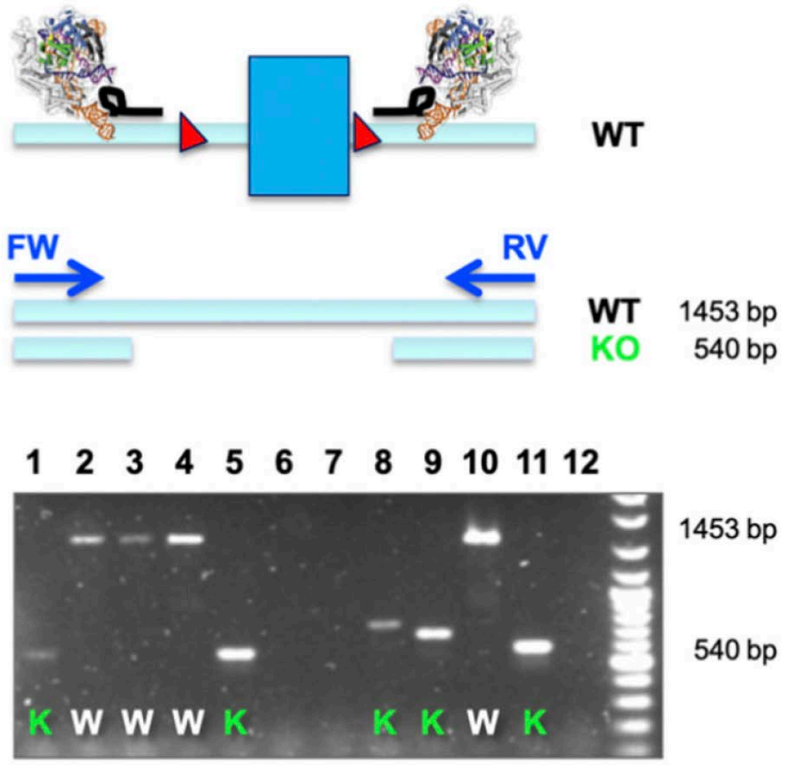


Irrespective of ploidy

One PCR gives information about the **full allelic status**

Blayney *et al.*, Methods and Protocols, 2020

Exon Deletion: Screen and Surprises Worth Considering



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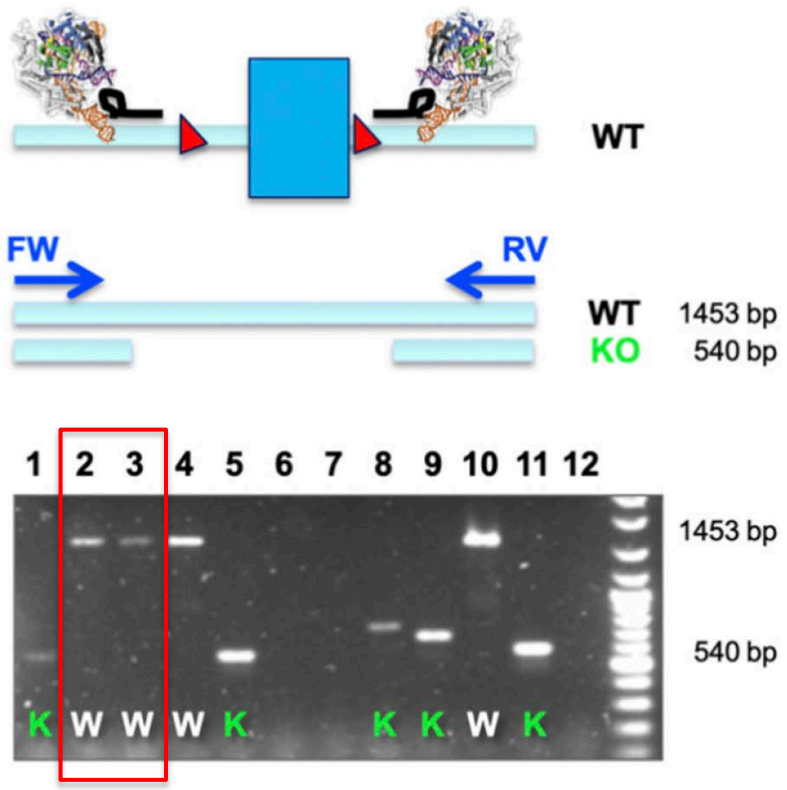
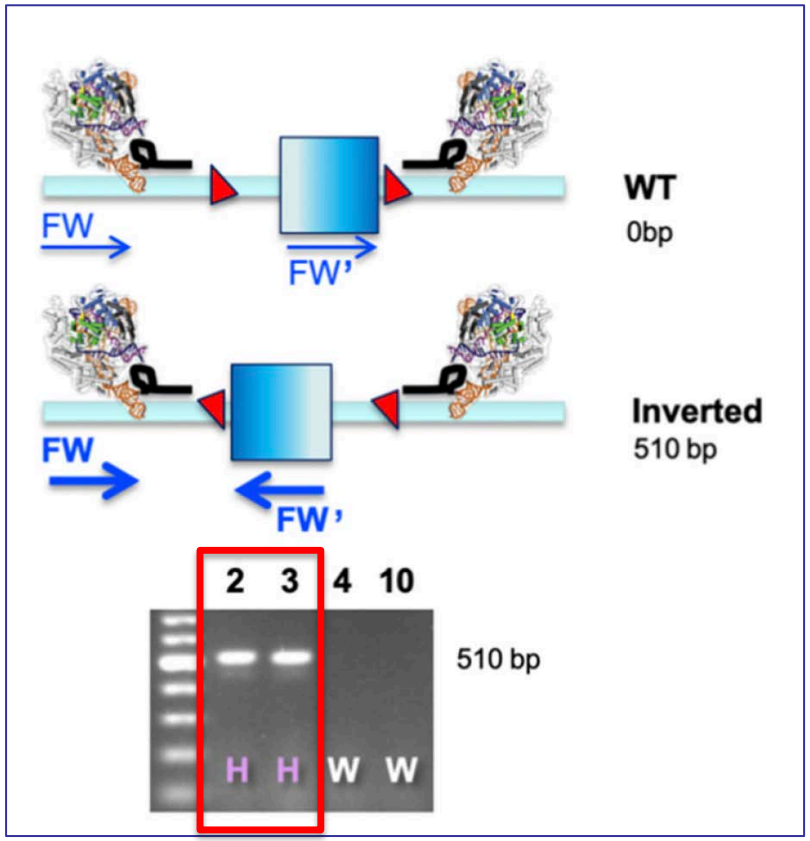
Exon Deletion: Screen and Surprises Worth Considering





Irrespective of ploidy

One PCR gives information about the **full allelic status**

Inverted re-insertions occur frequently after excision.
→ Screen for it.

Hidden **WT to HET** and **HET to HOM !**

Blayney *et al.*, Methods and Protocols, 2020

Exon Deletion: Surprises That Work Your Way

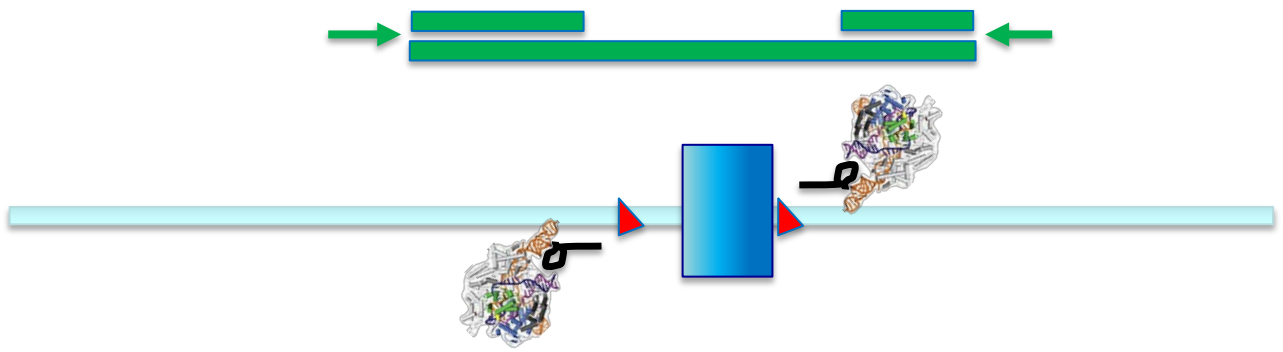
Cell line	Target	Total	WT/HET	Δ	%Δ	INV	%INV	
293	Exon PEX5	5	4	1	20	1	20	
293 T-Rex	Exon PEX5	26	18	8	31	5	19	
293	Exon PEX14	2	1	1	50	1	50	*
293 T-Rex	Exon PEX14	34	28	6	18	1	3	
4T1	Exon Car9	6	3	3	50	3	50	**
E14 mESC	Enhancer Hba R3	28	16	12	43	3	11	
E14 mESC	Enhancer Hba R4	62	53	9	15	2	3	
E14 mESC	Enhancer Hba Rm	27	17	10	26	5	19	
HeLa	Exon SCL38A2	23	17	6	26	2	9	
HeLa	Exon SMPD1	150	147	3	2	4	3	
HT29-mTX-E12	Exon WFDC2	20	18	2	10	2	10	
CTR M3 36S hiPSC	Exon CLU	80	80	0	0	0	0	***

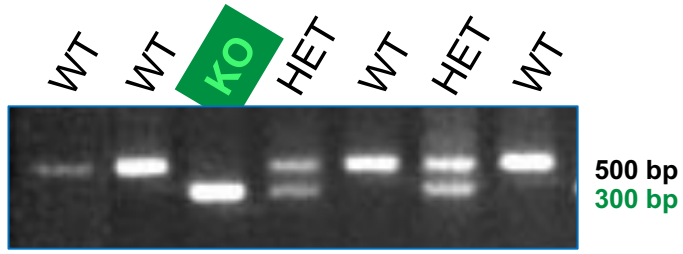
Dual sgRNA approach is efficient. But, as always, is cell line and context dependent.

In average, **5-20% inverted re-insertions** help finding the proper genotype faster.

Blayney *et al.*, Methods and Protocols, 2020

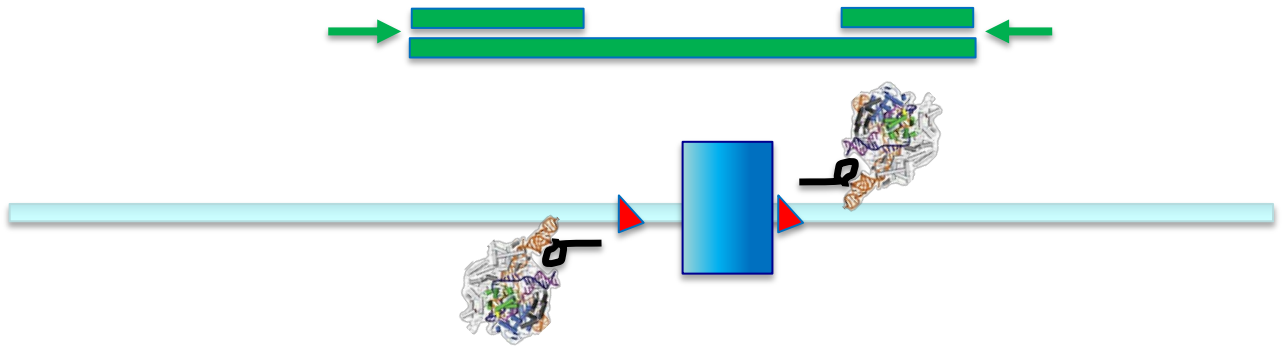
Screening: Surprises Worth Considering

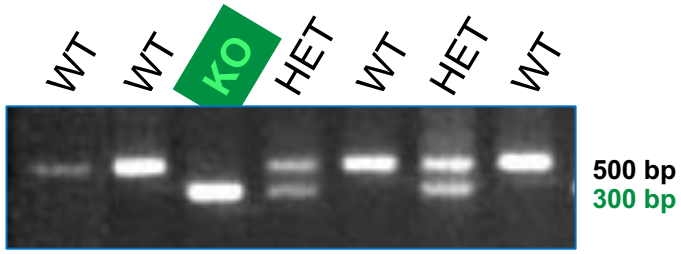


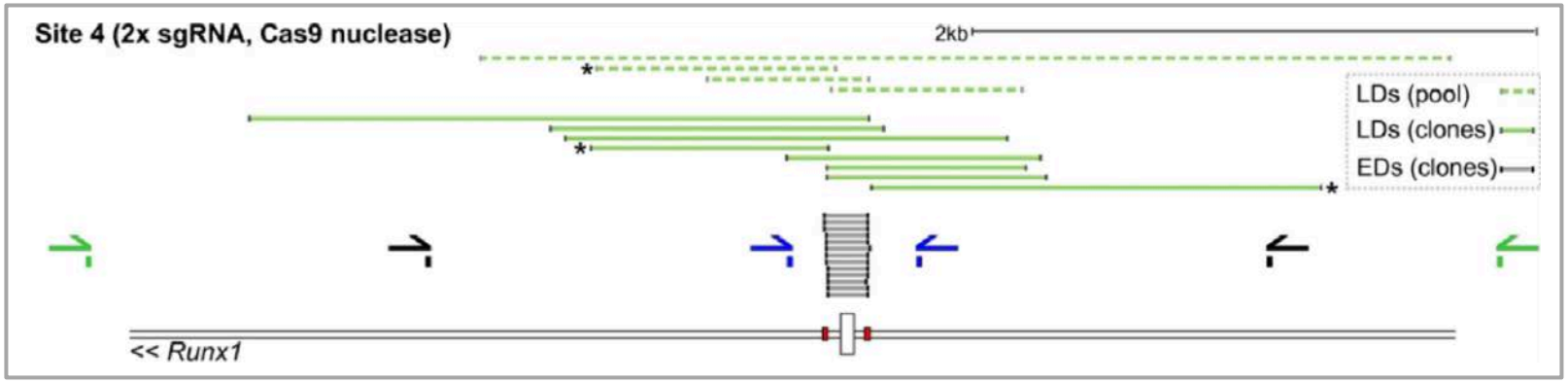


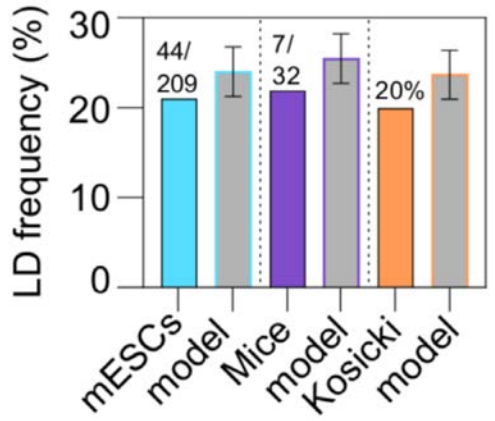
Owens *et al.*, Nucleic Acids Research, 2019

Screening: Surprises Worth Considering





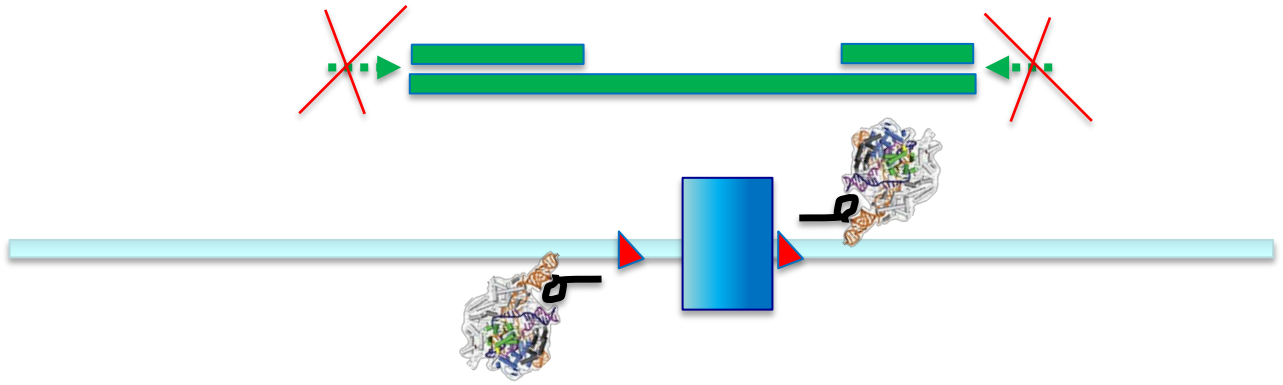


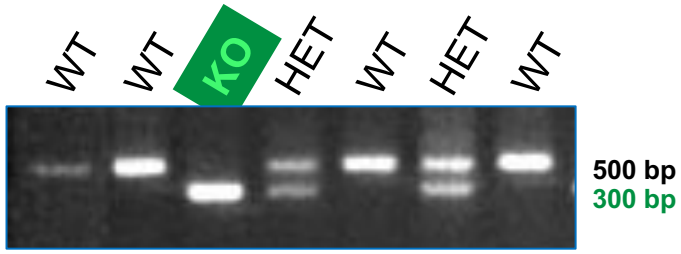


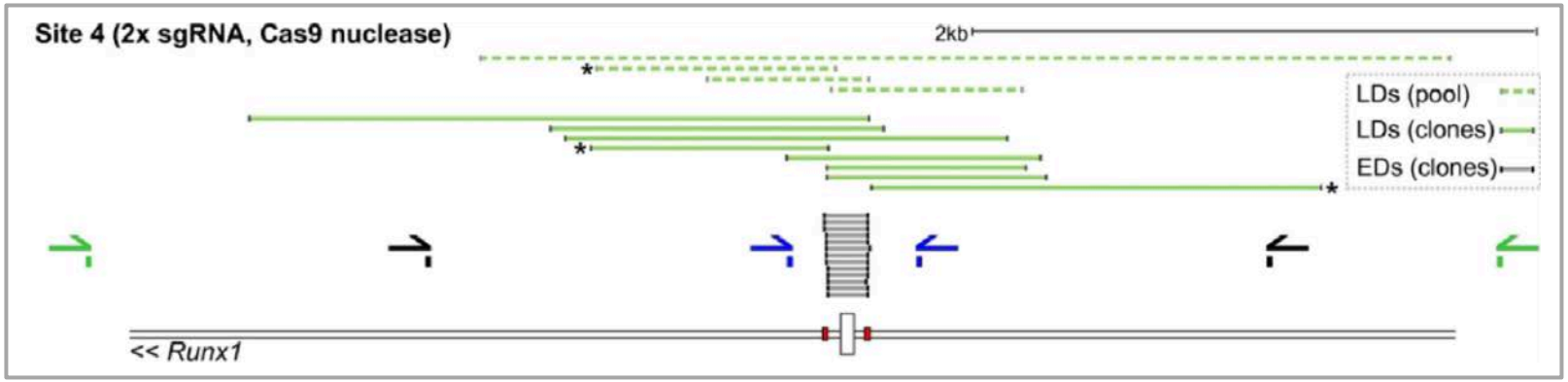
LD: Large Deletions. **ED: Expected Deletions.**

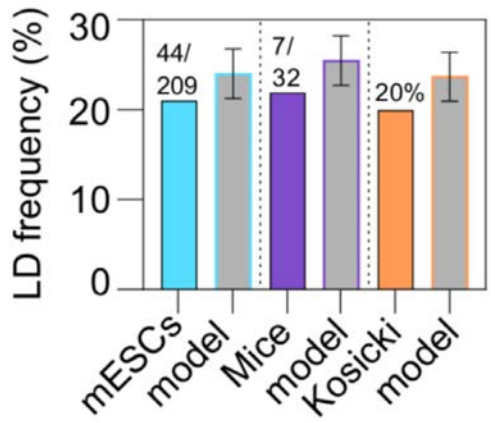
Owens *et al.*, Nucleic Acids Research, 2019

Screening: Surprises Worth Considering





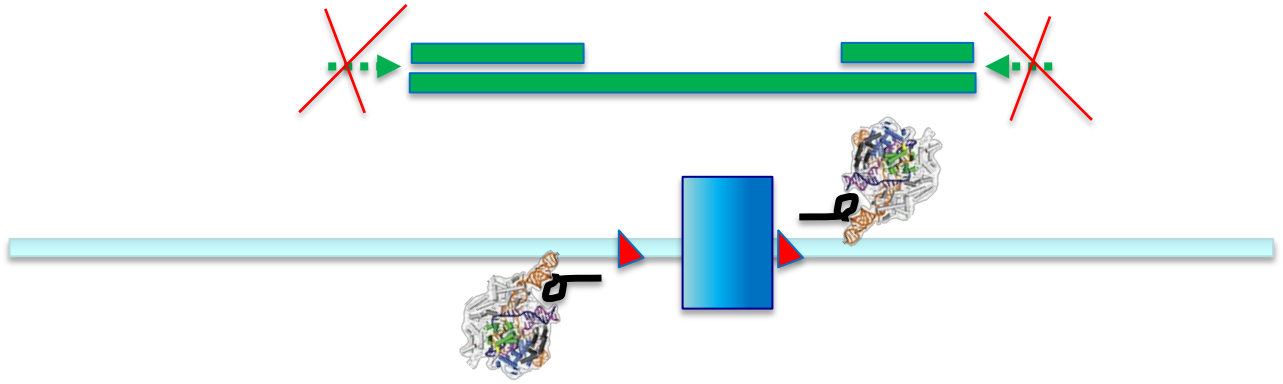


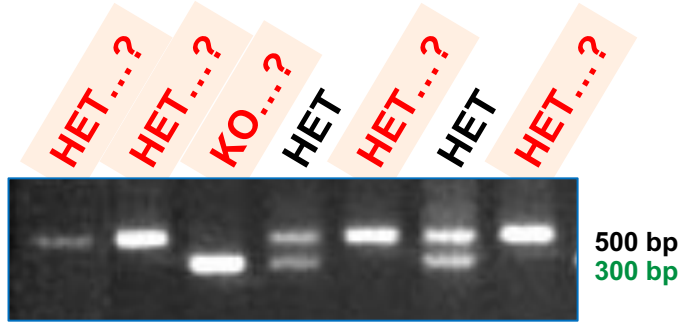


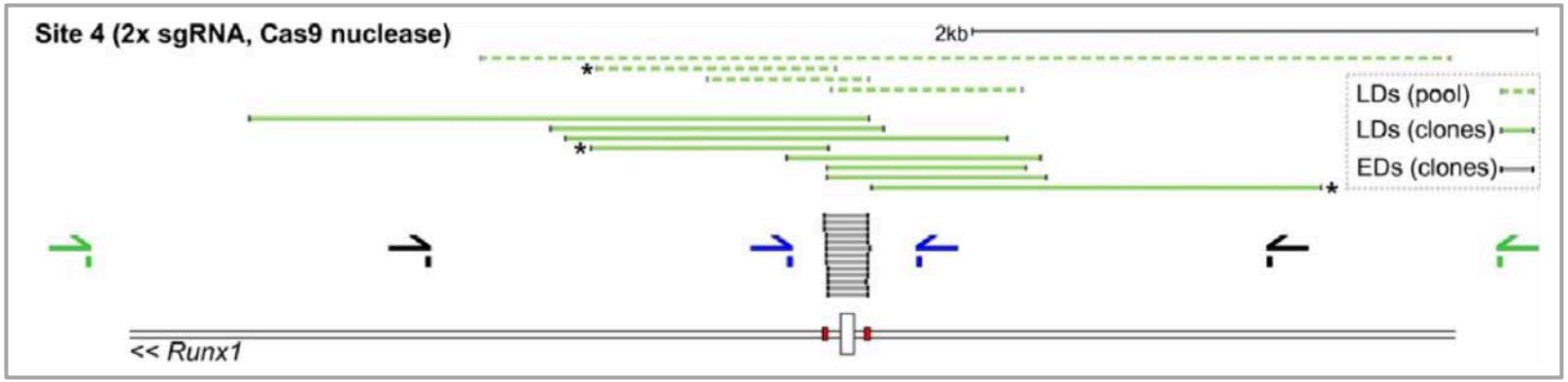
LD: Large Deletions. **ED: Expected Deletions.**

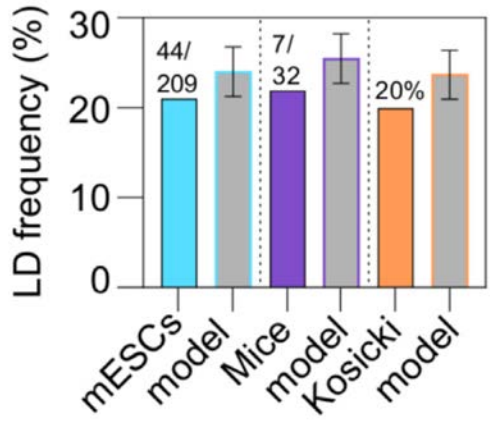
Owens *et al.*, Nucleic Acids Research, 2019

Screening: Surprises Worth Considering









LD: Large Deletions. **ED: Expected Deletions.**

Owens *et al.*, Nucleic Acids Research, 2019

Adjusting the Right level of QC

Mouse: Breeding removes SNPs.

Cell lines: Test several independent clones.

Clinical Applications: All that is possible.

Table 1 | Summary of variants detected by whole-genome sequencing

sgRNA	No. of mismatches	No. of NGG genomic sites	No. of NAG genomic sites	No. of off-target sites
Ar-a	0	1	0	N/A
	1	2	2	0
	2	3	6	1[a]
	3	26	38	0
	4	358	446	0
	5	2,432	3,130	0
Ar-b	0	1	0	N/A
	1	0	0	N/A
	2	0	0	N/A
	3	9	3	0
	4	98	82	0
	5	920	884	0
Total	–	**3,850**	**4,591**	**1**

[a]OTS3 (chromosome 8: 121017178), a 2-base-pair deletion experimentally validated in F_1 animal F18-9.

Iyer et al., 2015, Nature Methods

BLISS, BLESS, GUIDE-Seq, Digenome-Seq, Circle-Seq, TLA, WGS...

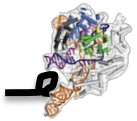
Bolukbasi *et al.*, 2015, Nature Methods
Tsai *et al.*, 2017, Nature Methods
deVree *et al.*, NBT, 2014

Refining the Technology: Key Steps Over the Years

Weatherall Institute of Molecular Medicine

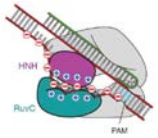
1 Use of RNPs instead of Cas9 mRNA.

Empirically determined.



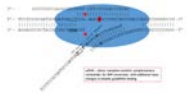
2 HiFi Cas9 with less OFF-targets.

Slaymaker *et al.*, Science, 2015
Kleinstiver *et al.*, Nature, 2016
Chen *et al.*, Nature, 2017



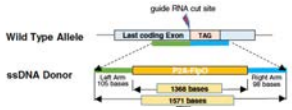
3 Asymmetric ssODN donors.

Richardson *et al.*, NBT, 2016



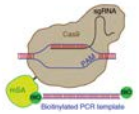
4 Long ssDNA donors.

Quadros *et al.*, Genome Biology, 2017



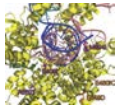
5 2C-HR-CRISPR and Biotin-tagging

Gu *et al.*, NBT, 2018



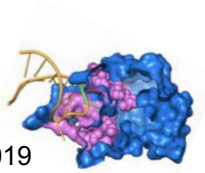
6 xCas9 with lower PAM stringency

Hu *et al.*, Nature, 2018



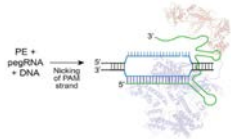
7 Base editors: **No DSB required**.

Komor *et al.*, Nature, 2016
Kim *et al.*, NBT, 2017
Koblan *et al.*, NBT, 2018
Gruenewald *et al.*, NBT, 2019
Thuronyi *et al.*, NBT, 2019
Cheng *et al.*, Nature Communications, 2019



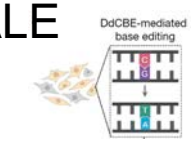
8 Prime editing and pegRNA

Anzalone *et al.*, Nature, 2019



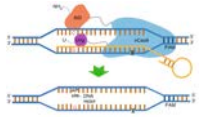
9 RNA-less and cut-free deamination: DddA-TALE

Mok *et al.*, Nature, 2020



10 C to G and C to A base editors

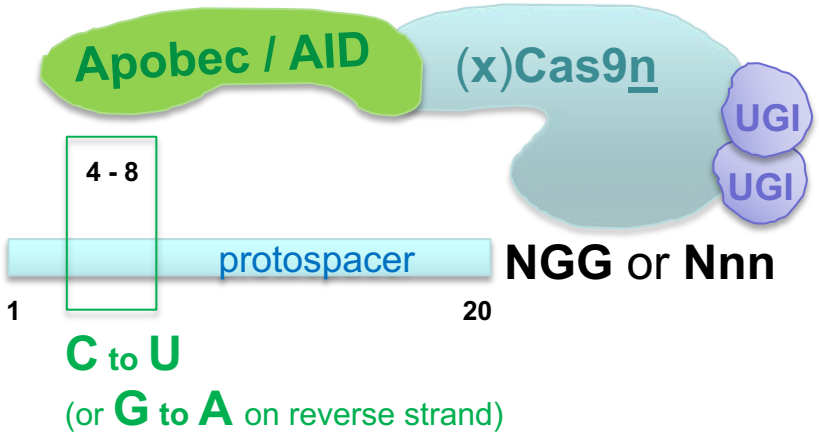
Kurt *et al.*, NBT, 2020
Zhao *et al.*, NBT, 2020

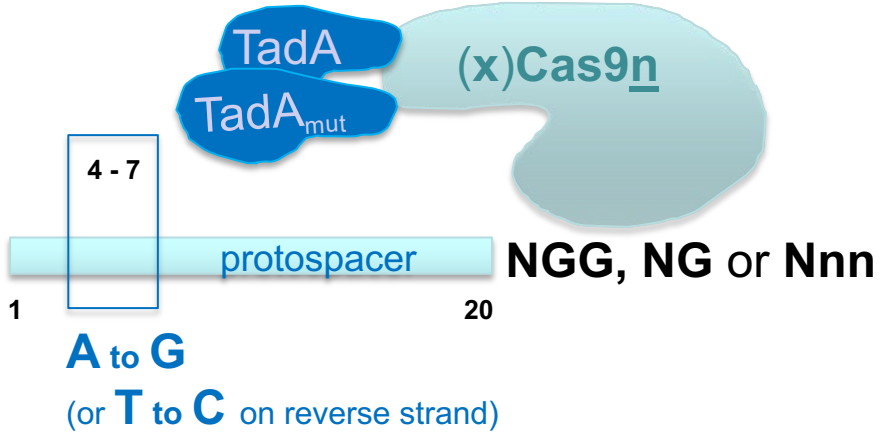


11 CRISPR-CasΦ

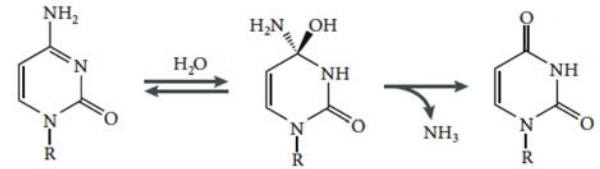
Pausch *et al.*, Science, 2020

Base Editing

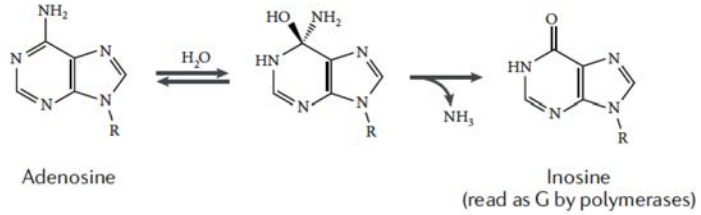




CBE4 max

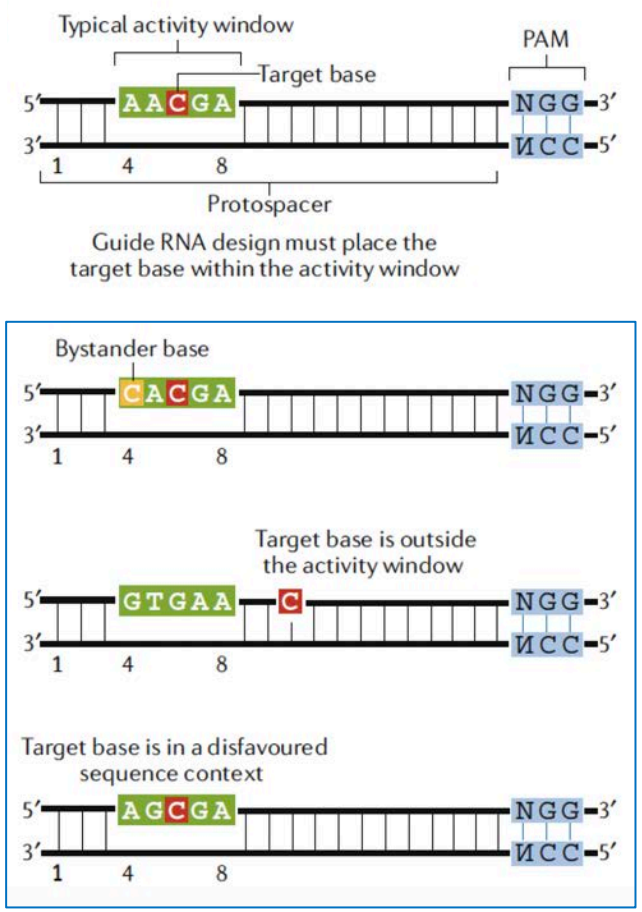


ABE7.10
ABE8



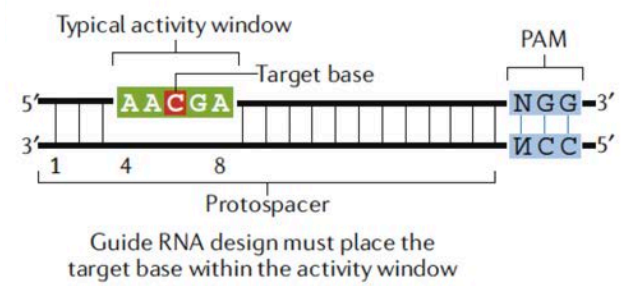
Modified after Rees and Liu, NRG, 2018

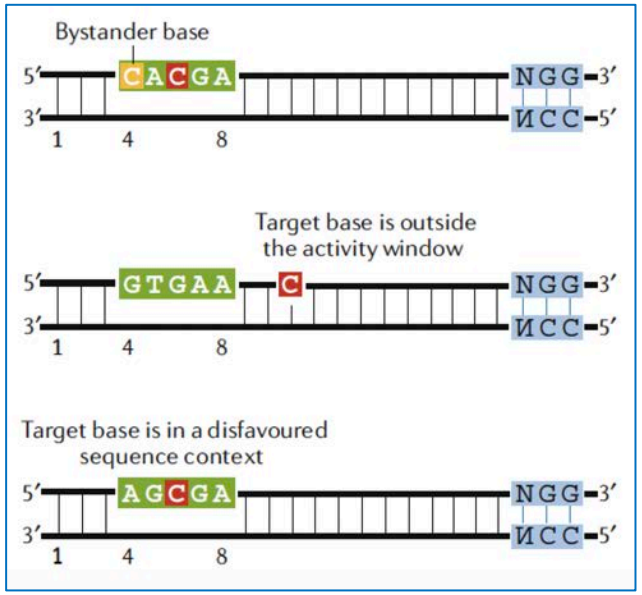
Base Editing

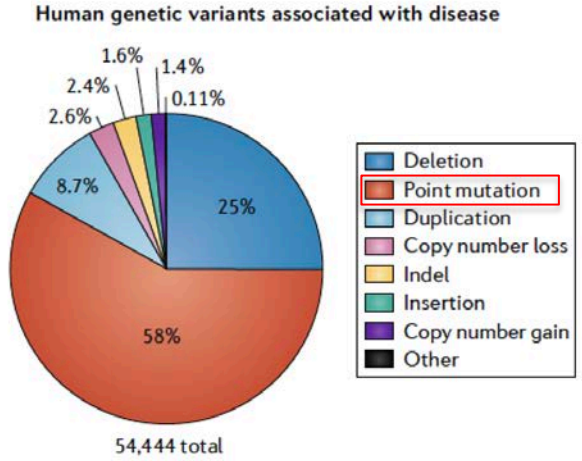


Rees and Liu, NRG, 2018

Base Editing

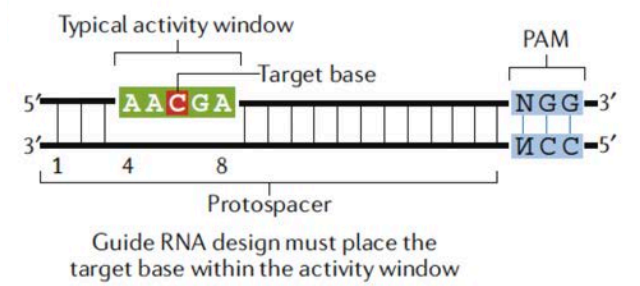


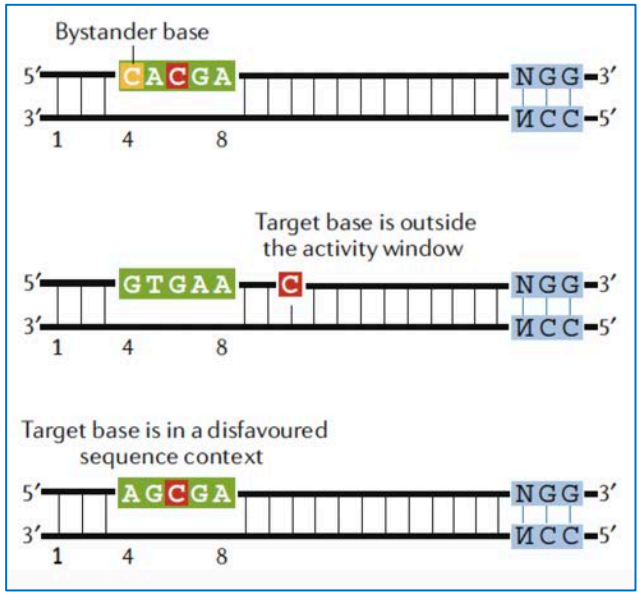


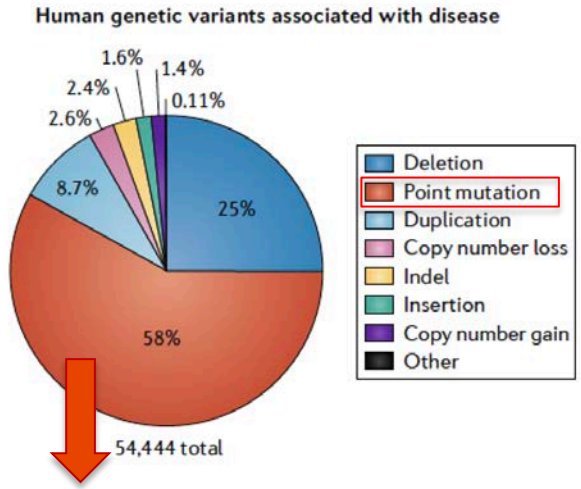


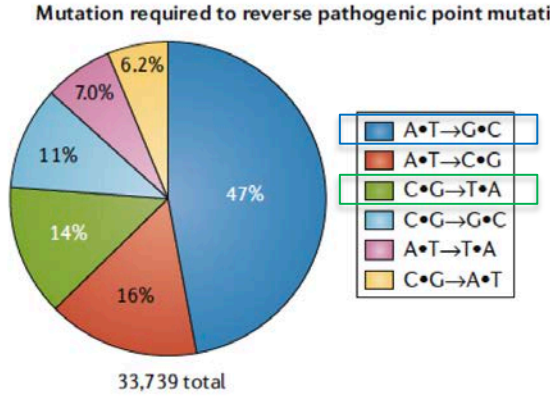
Rees and Liu, NRG, 2018

Base Editing









58% approachable.
61% thereof treatable.
→ **40% of total SNPs**.

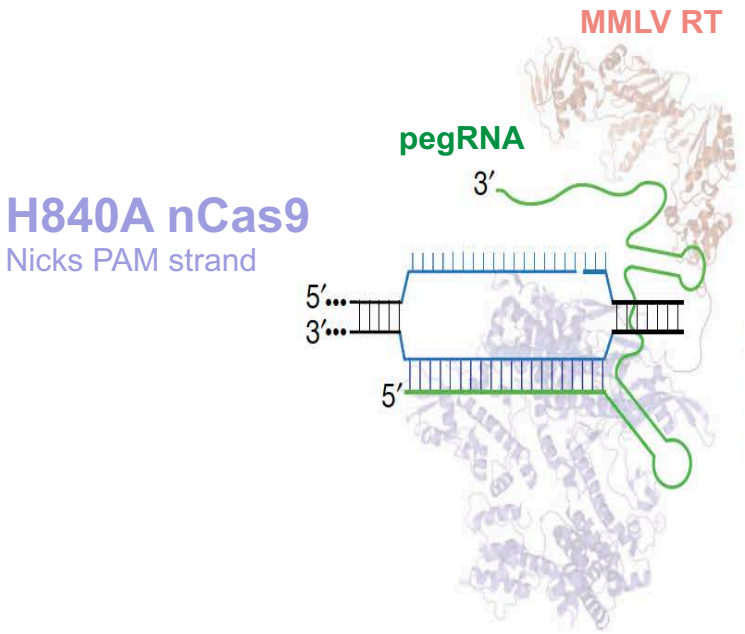
26% of that available to WT Cas9, but **95%** for xCas9…

ABE7.10/ABE8

CBE4 max

Rees and Liu, NRG, 2018

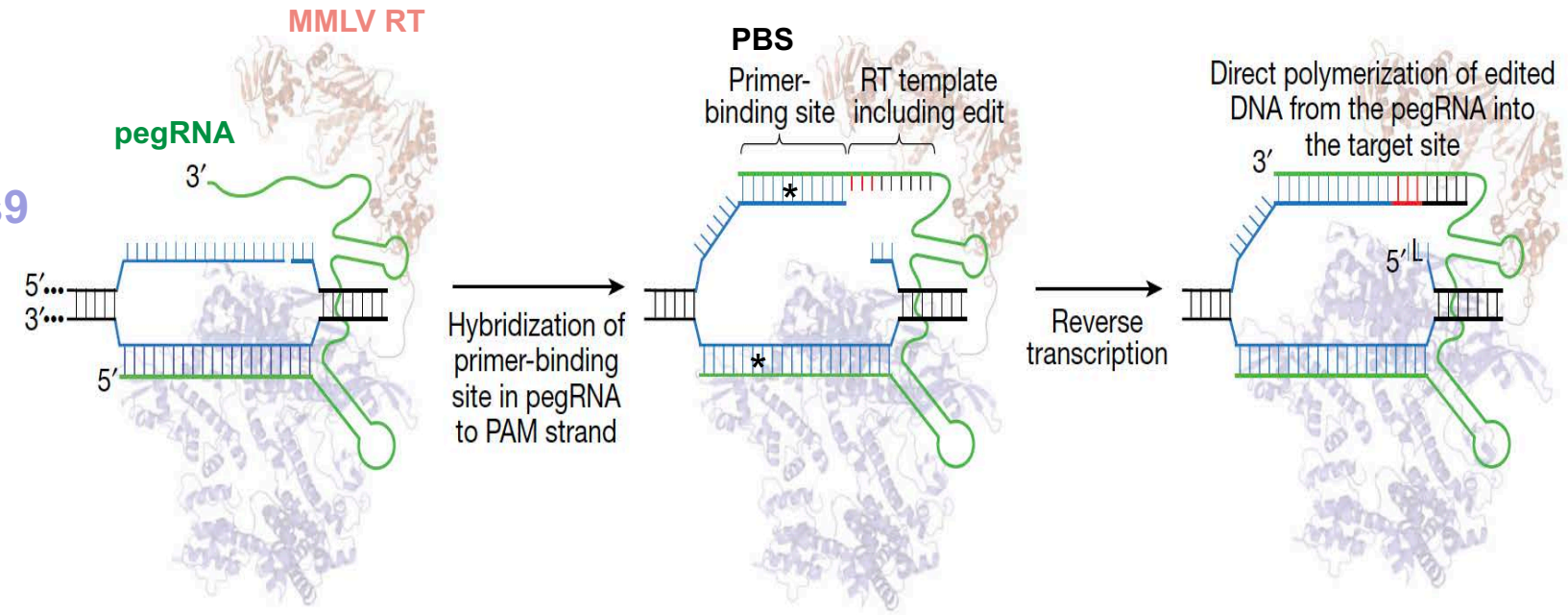
Prime Editing



Anzalone *et al.*, Nature, 2019

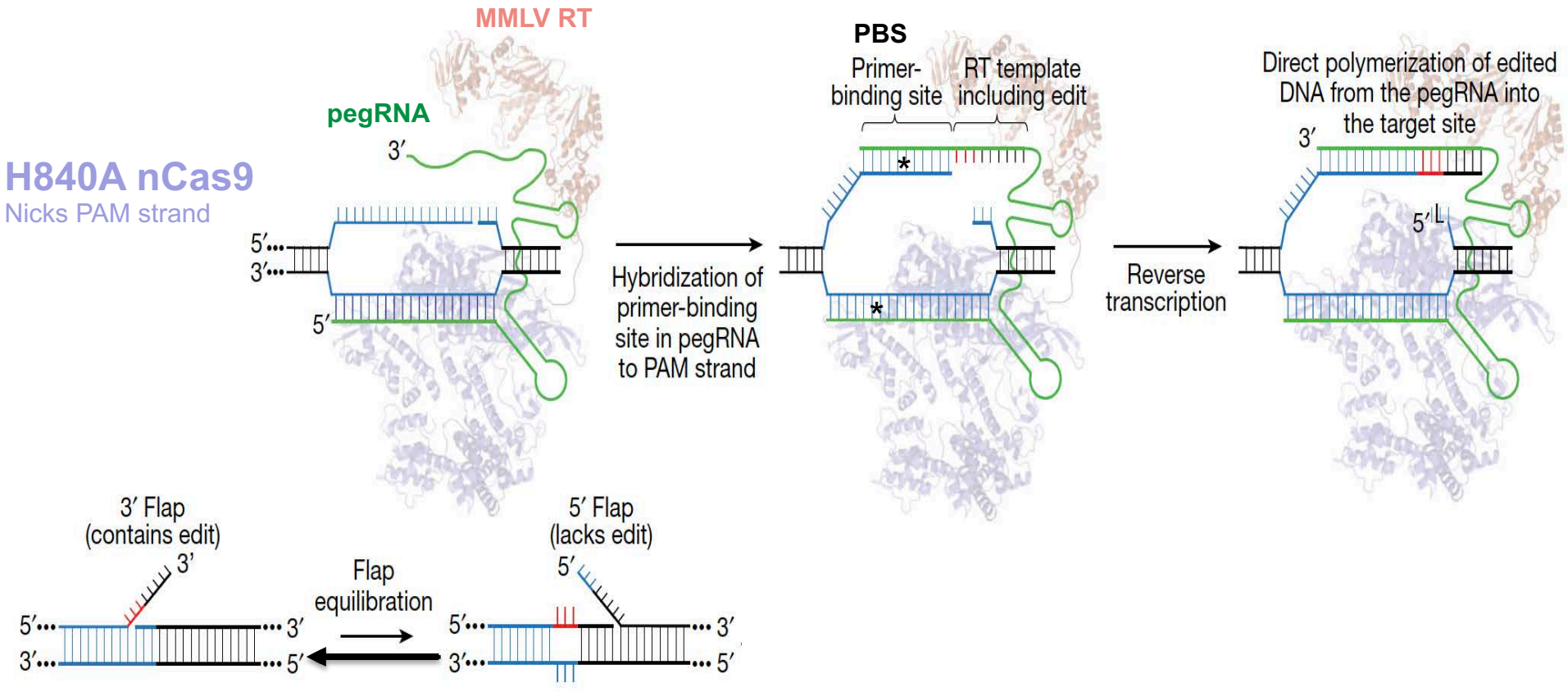
Prime Editing

H840A nCas9
Nicks PAM strand



Anzalone *et al.*, Nature, 2019

Prime Editing



5’ flap degradation by endogenous enzymes favors edited strand preference.

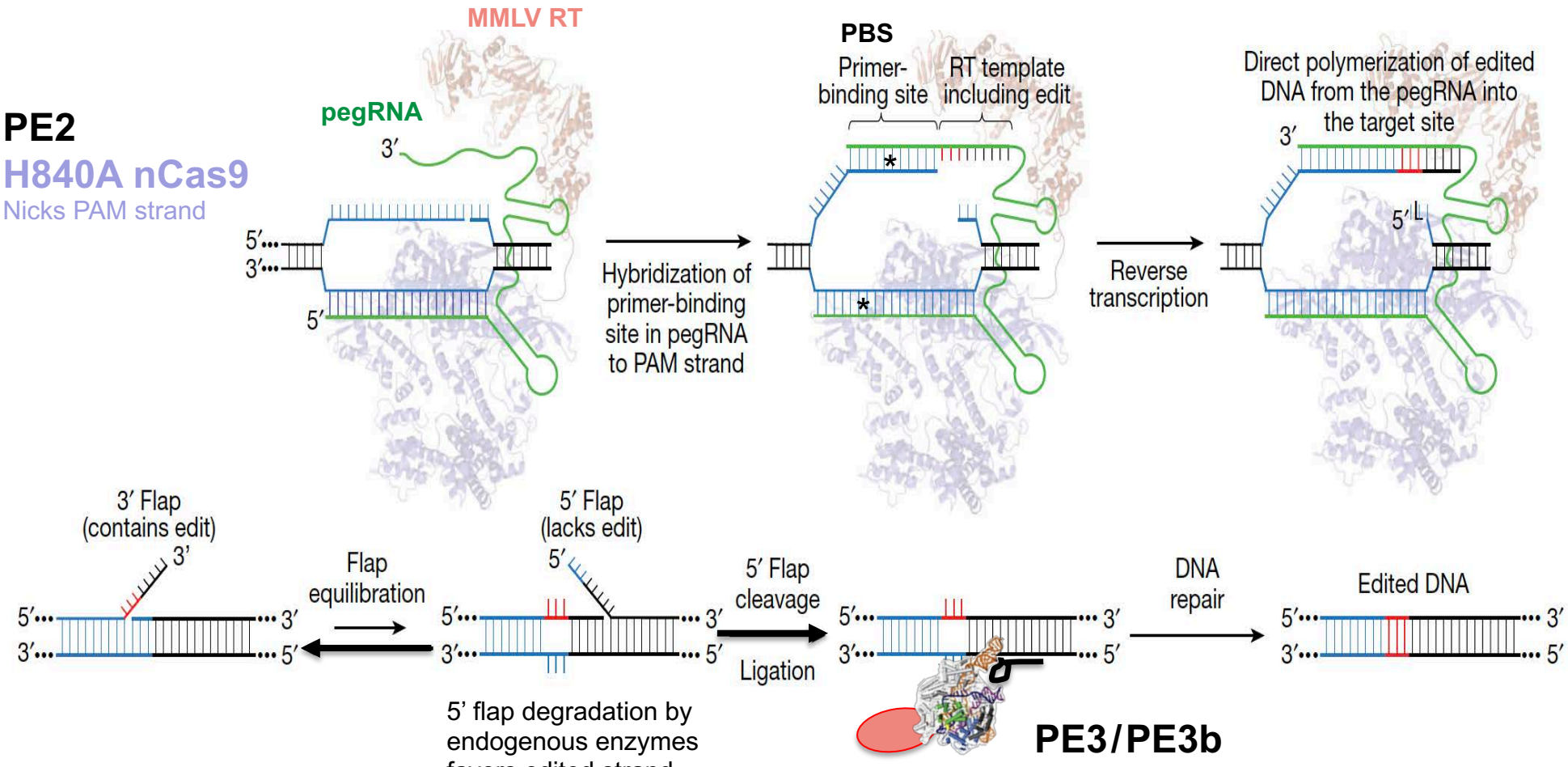
Anzalone *et al.*, Nature, 2019

Prime Editing

PE2

H840A nCas9

Nicks PAM strand



5’ flap degradation by endogenous enzymes favors edited strand preference.

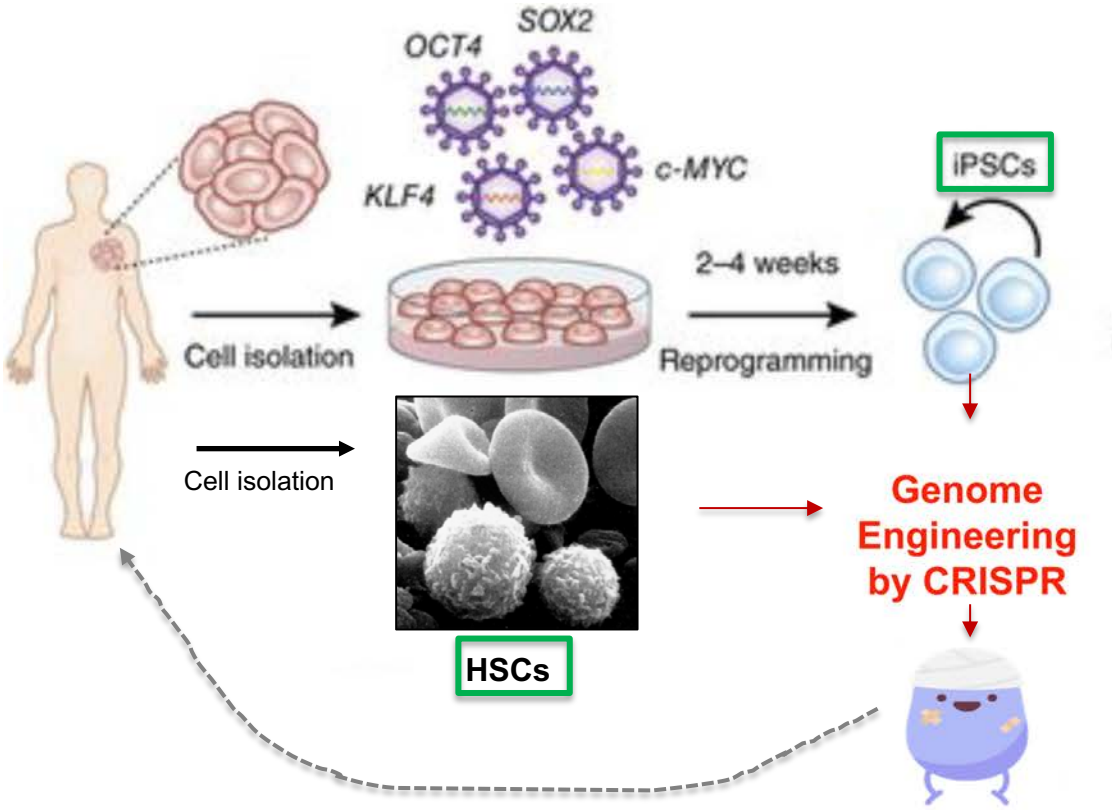
PE3/PE3b

2nd WT sgRNA to target non-edited strand.

Anzalone *et al.*, Nature, 2019

Clinical Applications

Ex vivo approaches



Several 100's of publications 2013 to 2020

In vivo

First in vivo CRISPR candidate enters the clinic

Editas Medicine and its partner Allergan have advanced AGN-151587 into a phase I/II trial for patients with Leber congenital amaurosis type 10, a rare and inherited form of blindness.

AGN-151587, previously called Edit-101, is the first CRISPR–Cas9 genome-editing medicine that is administered directly to patients. Doctors inject the adeno-associated virus-based candidate subretinally, so that it can cut out a mutation in the *CEP290* gene in photoreceptor cells in the eye. Spark Therapeutics and Novartis's voretigene neparvovec, the first gene therapy to gain approval in the US, corrects a different form of the inherited eye disease, by introducing a normal copy of the *RPE65* gene to patients with Leber congenital amaurosis type 2.

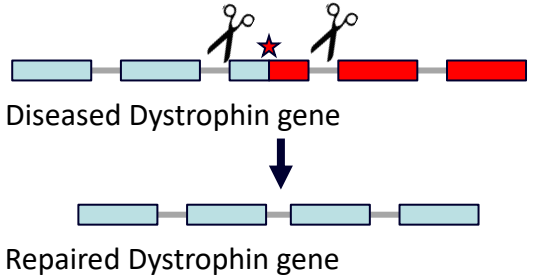
Mullard, Nature Reviews Drug Discovery, September 2019

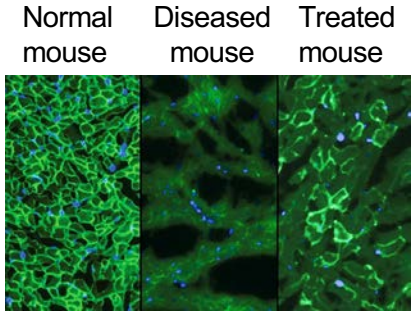
Clinical Applications: DMM

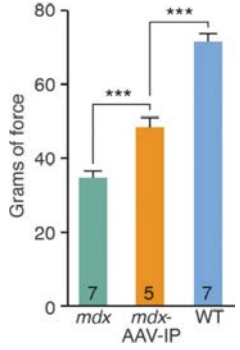
Dystrophin is a key muscle protein.

Damaged Dystrophin leads to severe muscle wasting, known as **Muscular Dystrophy**.

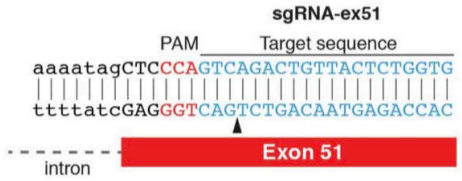
Genome editing by CRISPR-Cas9 is therapeutically used for a cure.

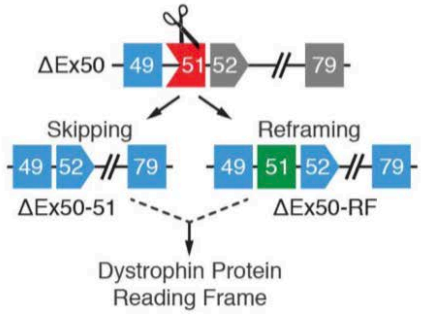


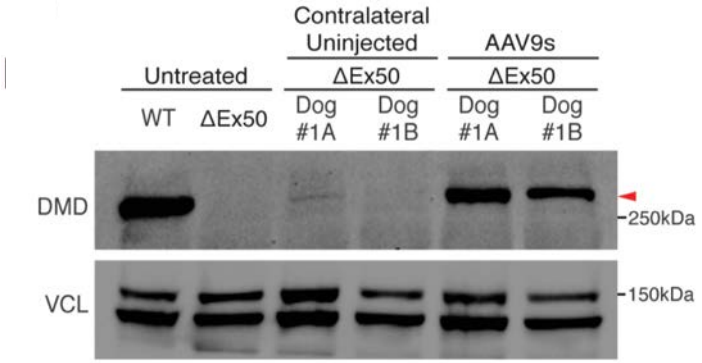




Long *et al.*, Science, 2015
Nelson *et al.*, Science, 2015



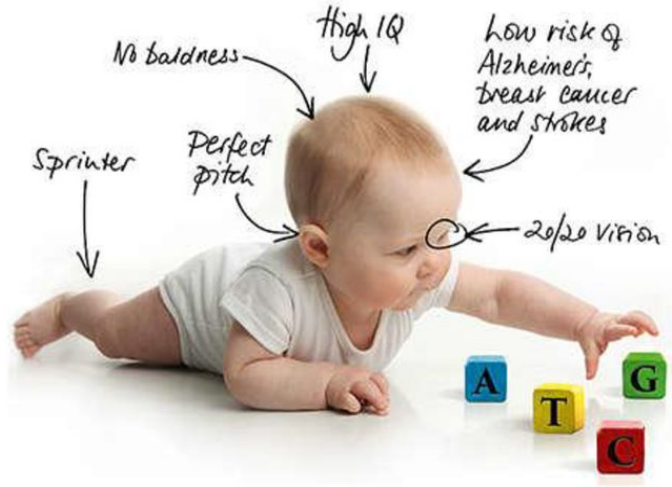




Amoasii *et al.*, Science, 2018

1 sgRNA.
No detected OFF target events.
In vivo, dog.

Ethical Considerations: Germline Editing



The Economist, 2015

NEWS · 18 OCTOBER 2019 · CORRECTION 18 OCTOBER 2019

Russian 'CRISPR-baby' scientist has started editing genes in human eggs with goal of altering deaf gene

Denis Rebrikov also told *Nature* that he does not plan to implant gene-edited embryos until he gets regulatory approval.

CRISPR-CAS9 GENOME EDITING · 29 OCTOBER 2019

A crop that feeds billions freed from blight by CRISPR

Nature, 29.10.2019

NEWS · 24 SEPTEMBER 2019

CRISPR might be the banana's only hope against a deadly fungus

Nature, 24.09.2019

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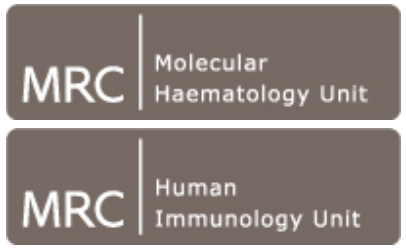

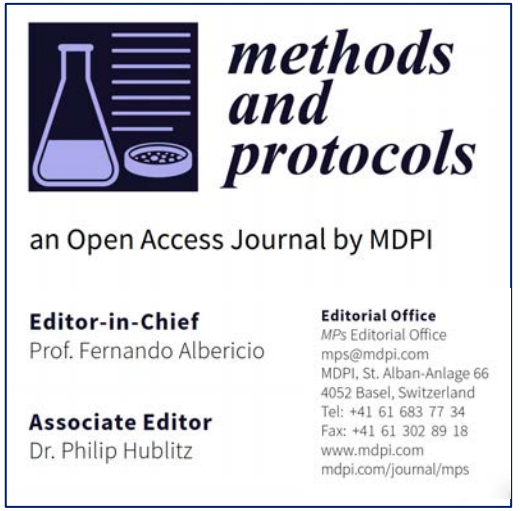

https://www.imm.ox.ac.uk/research/facilities/**genome-engineering-facility**
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