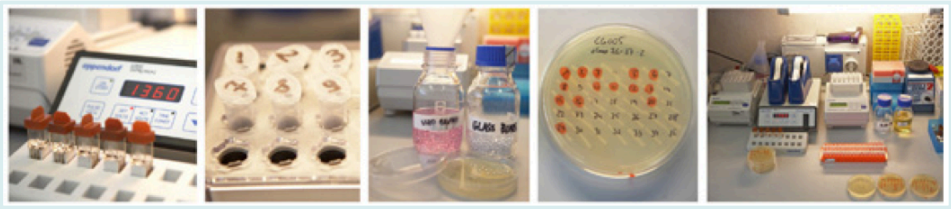
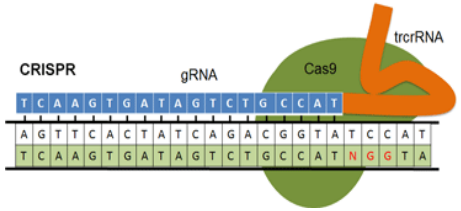
Engineering Genomes

An Introduction to CRISPR/Cas9 Technology

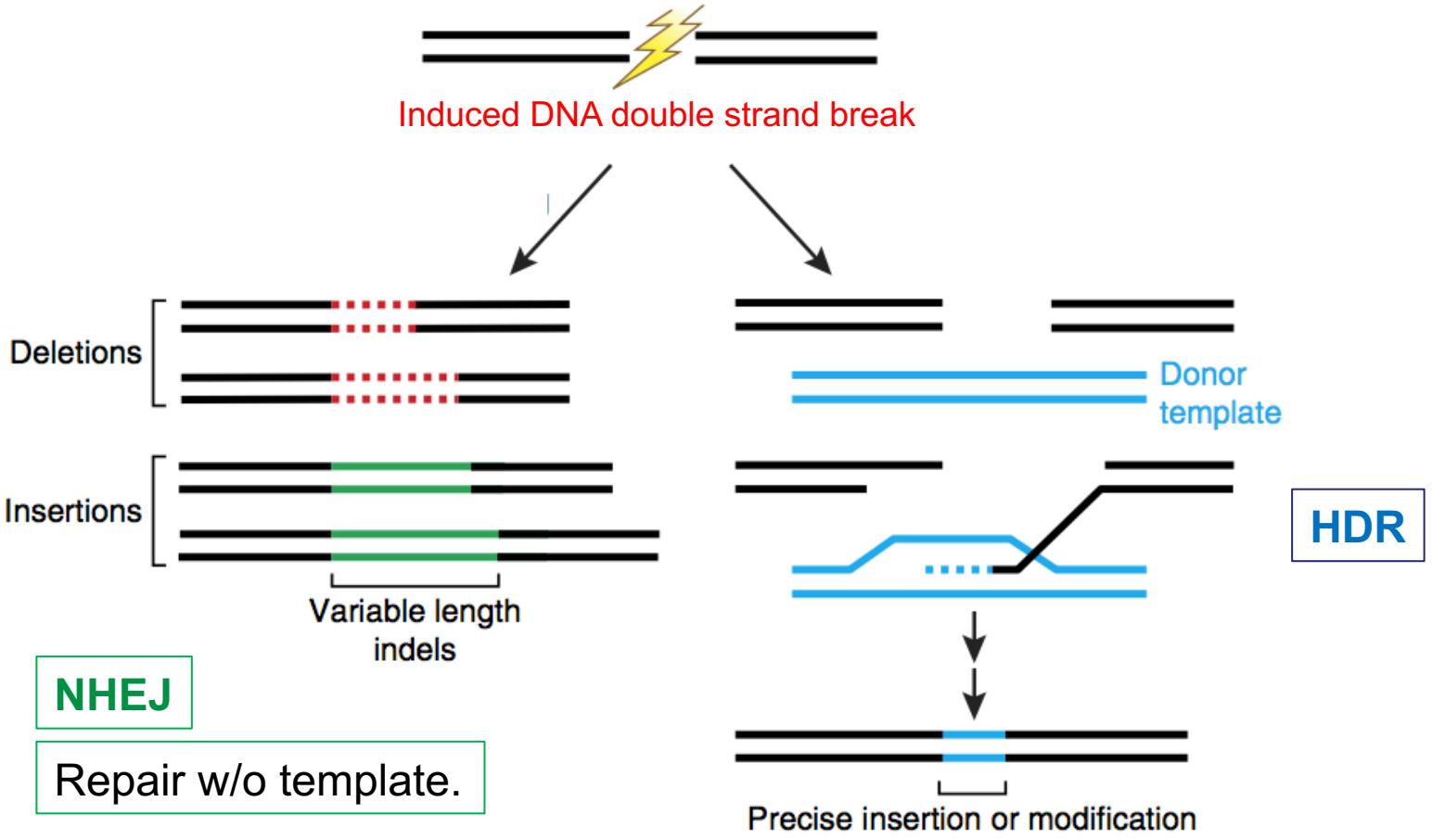
Weatherall Institute of Molecular Medicine,
D.Phil. Course Oxford
November 14th, 2019

Philip Hublitz, PhD
WIMM, Genome Engineering Services





Cellular DNA Repair Pathways



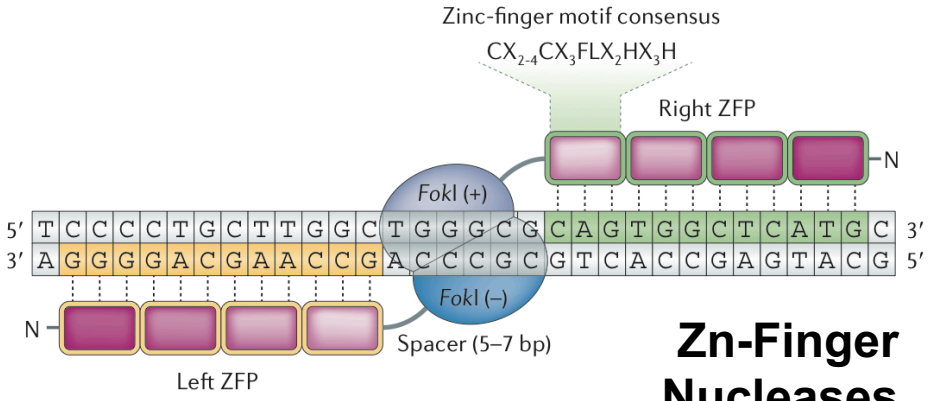
NHEJ

Repair w/o template.

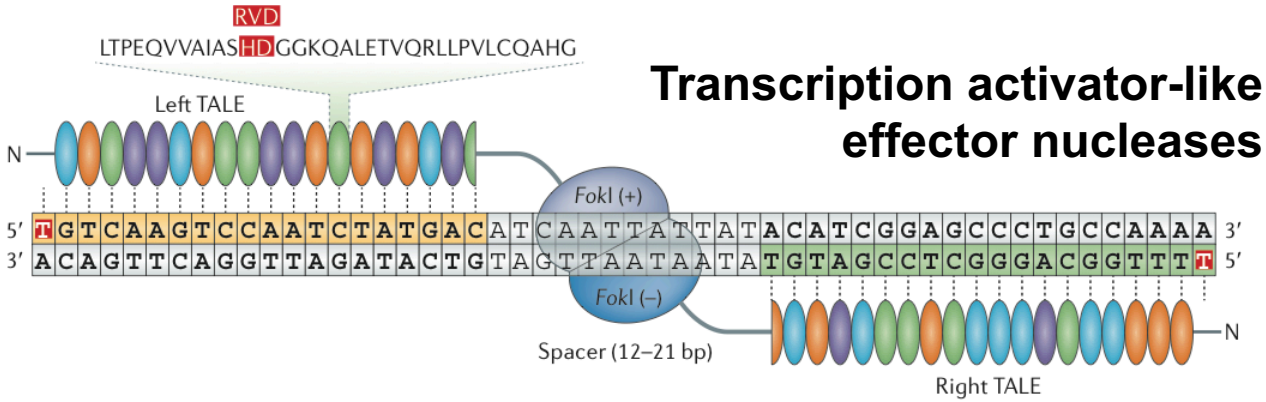
Sander and Joung, NBT, 2014

Pre-CRISPR Times

	ZFNs	TALENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors
Nuclease	*Fok*I	*Fok*I
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



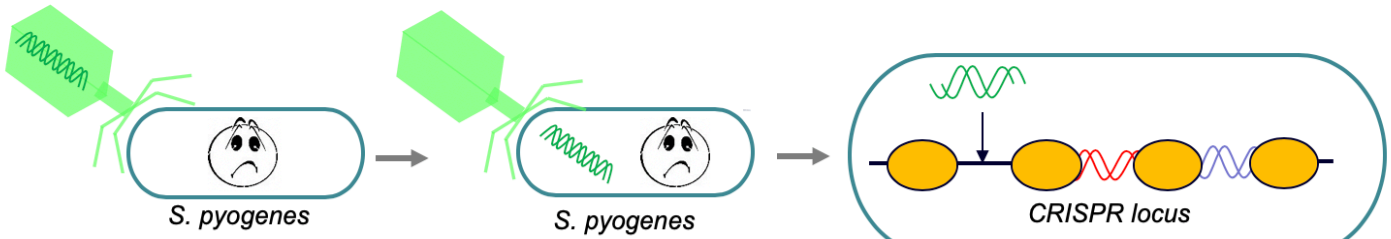
Zn-Finger Nucleases



Transcription activator-like effector nucleases

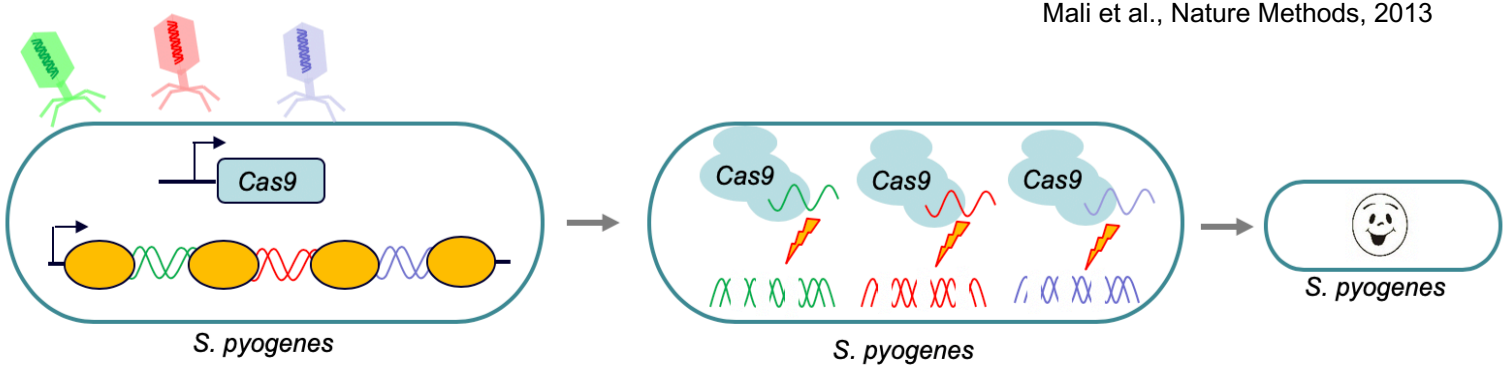
Sander and Joung, NBT, 2014

CRISPR: an Innate Immune System



Immunization phase

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

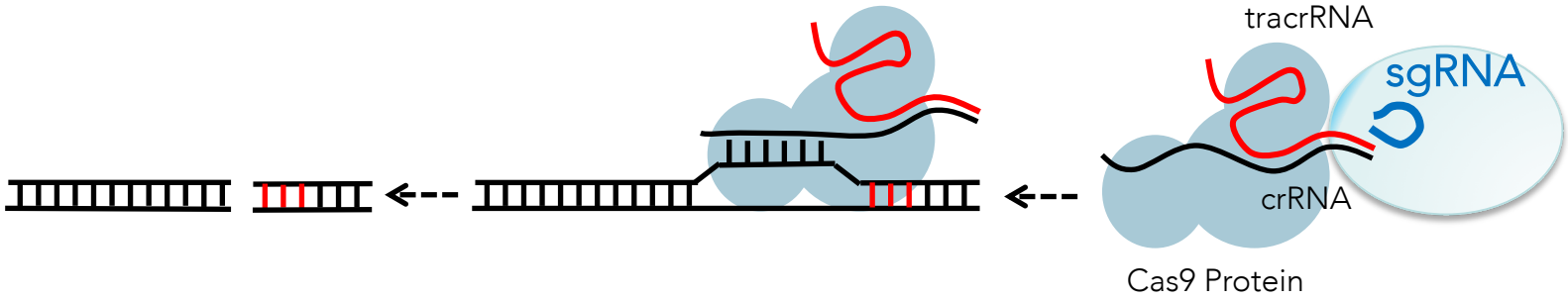


Immunity

Clustered **R**egularly **I**nterspersed **S**hort **P**alindromic **R**epeats

Cas9: a Site-Specific Nuclease

Cas9: CRISPR **as**sociated protein **9**



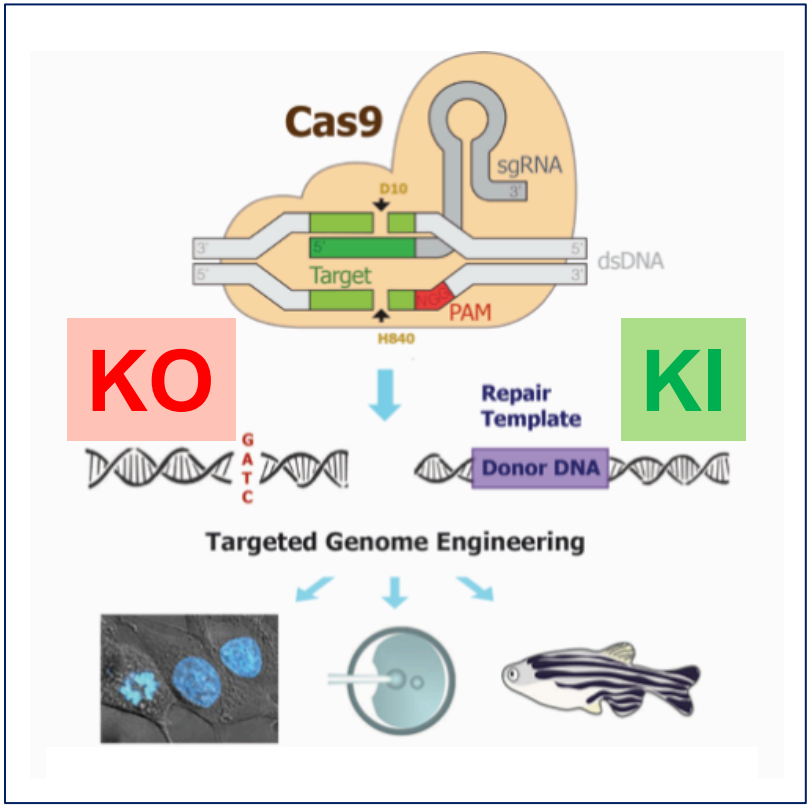
DNA targeting and cleavage only if **PAM** is present.
crRNA complimentary enables specific targeting.
Blunt cut 3 bp upstream of PAM.

Fusion of crRNA and tracrRNA possible, generates an easy to use 2-component system of Cas9-protein and a single guide RNA: **sgRNA**.

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

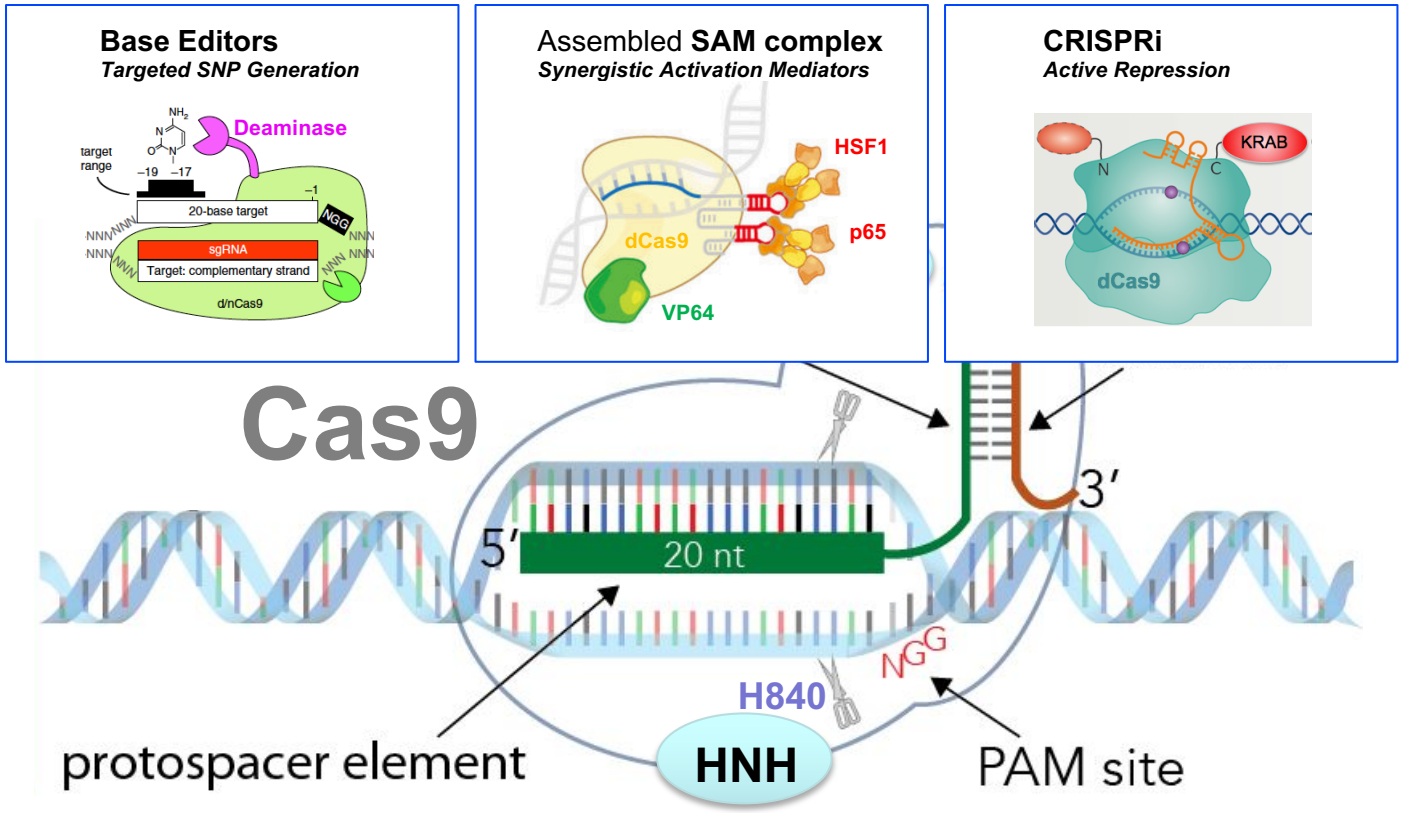
CRISPR/Cas9

Nature, March 6th, 2014



WIMM Genome Engineering, Website, 2018

CRISPR/Cas: What is it good for?



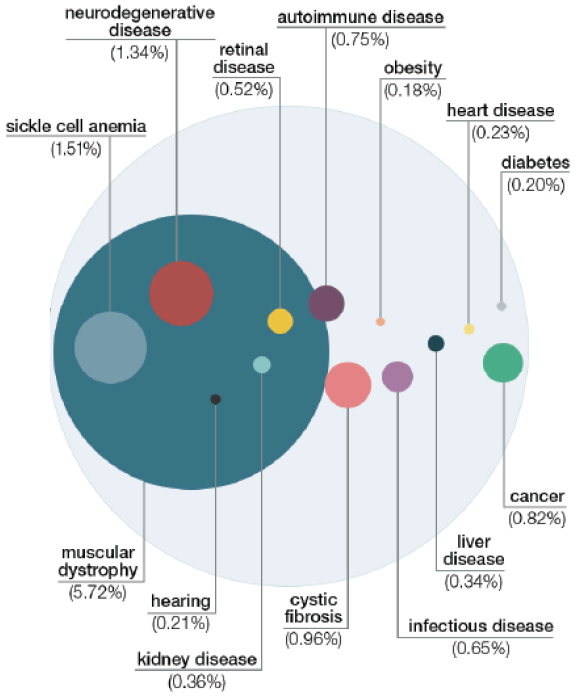
CRISPR/Cas: What is it good for?

Weatherall Institute of Molecular Medicine

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

Nature, 2015

Nature, 2016

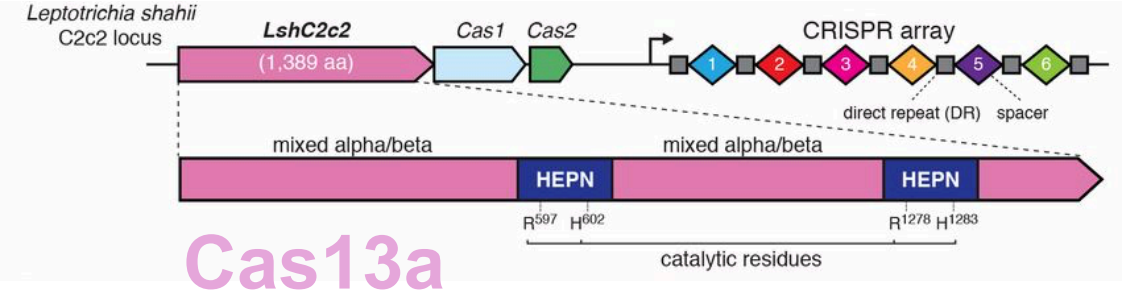


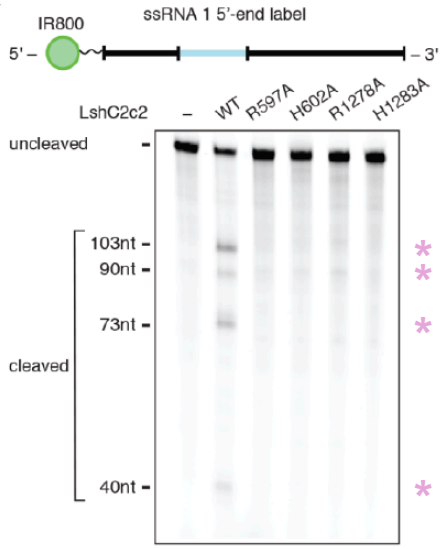
Research Arc Cell Press, 2018

CRISPR/Cas: What is it good for?

Generation of LOF/GOF models.

mRNA editing:





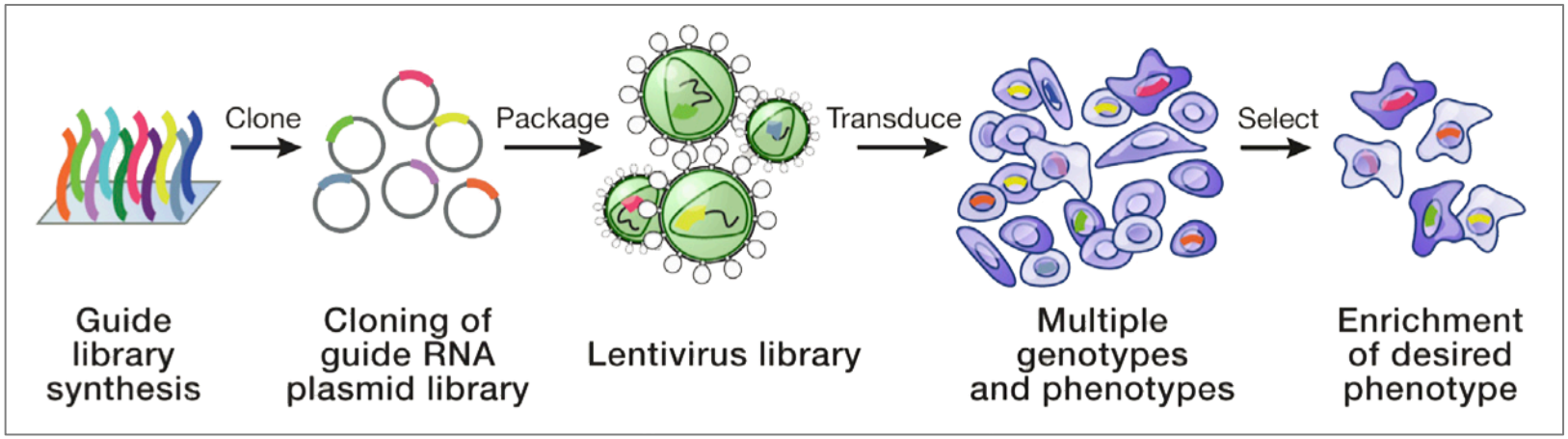
Abbudayyeh *et al.*, Science, 2016

CRISPR/Cas: What is it good for?

Generation of LOF/GOF models.

mRNA editing.

High-throughput screens: KO, CRISPRi, CRISPRa.



Hsu, Cell, 2014

CRISPR/Cas: What is it good for?

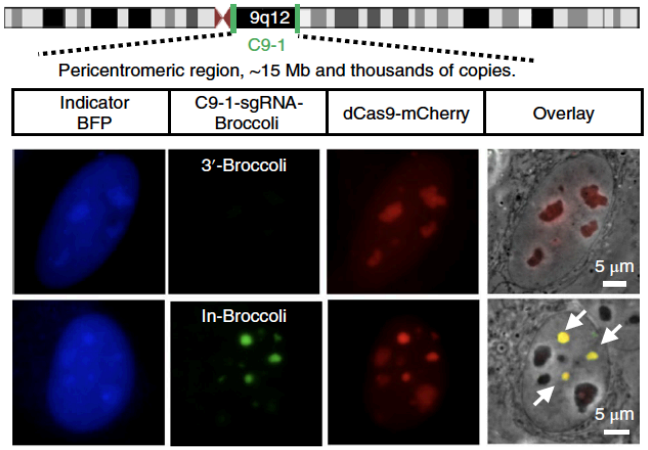
Generation of LOF/GOF models.

mRNA editing.

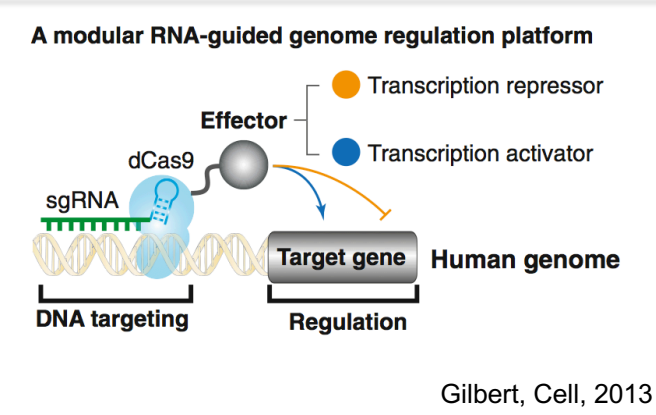
High-throughput screens.

Targeting fusion proteins:

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



Ma *et al.*, Nature Methods, 2018

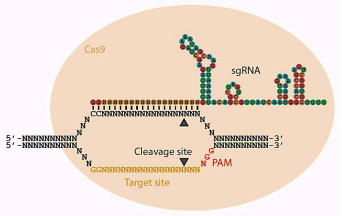


Gilbert, Cell, 2013

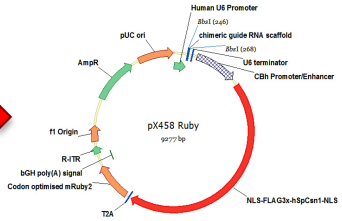
Pipeline

Weatherall Institute of Molecular Medicine

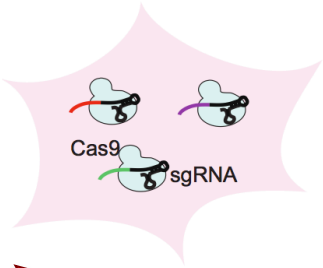
sgRNA design



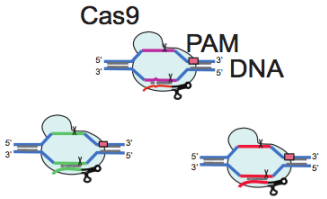
Vector preparation
RNP assembly



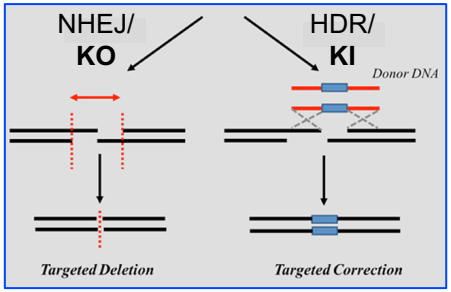
Delivery



Target Cleavage

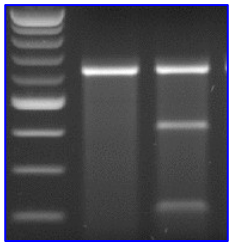


Repair



SCREEN

Guide validation



HELP and ADVICE: come and see me… ;-)

sgRNA design

Thoru Pederson: **U6, H1** & **7SK** need a G at start (3 in WT).

Feng Zhang: add a G at 0, it won't disturb. Replacing 20 and 19 with G's (might) affect Cas9 specificity. Keith Joung: shorter guides (18bp) are better and have less off-target cleavage. Jin-Soo Kim: double G at start is perfect.

Fei-Ann Ran, 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 in transient transfections into cells.

Hublitz and many others, unpublished

Consideration: Cas9 Source

mRNA as plasmid	**mRNA** as mRNA	**Protein**
Txn/Tln takes time. **No go in oocyte**. **Good for cell lines**.	Tln takes time. Risk of mosaicism in PNI.	**Immediate action**. Good for NHEJ and HDR in oocytes.
Gradual action. **Cheap and easy**.	Gradual action, double modification? (e.g. double LoxP).	Multiplexing difficult: deletion vs. double repair.
Stable for several cell divisions: **OFF-targets**.	**Rapidly degraded**.	**Rapidly degraded**.
Pretty efficient.	Variable efficiency?	**Highly efficient**.

Hublitz, personal summary

Consideration: sgRNA Source

Weatherall Institute of Molecular Medicine

Plasmid	**SygRNA (RNP)**
Cheap.	Slightly more expensive. 1 or 2 components.
Persists for several cell divisions: **OFF-target issues**.	**Rapidly degraded, Immediate action. High efficiency.**
Selection possible by 2A-GFP *et al.* coupling.	Labeled tracrRNA not functional → labeled Cas9 might work sooner or later...
Good efficiency.	**Good efficiency**.

SygRNA /Cas9 protein **RNPs** as the reagent of choice.

It all depends on the experimental approach, though.

Hublitz, personal summary

CRISPR/Cas9 in Cell Lines

Weatherall Institute of Molecular Medicine

Test **3 sgRNAs** per target site (genome and epigenome).

Test cells thoroughly for **ploidy** and eventual **SNPs**. Find out if they can be **cloned** (crucial for establishment of cell lines).

Establish **optimum transfection conditions**: efficiency vs. viability, delivery method (LPF, Amaxa, Neon), allow 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