Background & Definition:
What is gene editing/genome engineering?
**Genome Editing**

**Definition**

Wikipedia:

*Genome editing,* or *genome editing with engineered nucleases (GEEN)* is a type of genetic engineering in which DNA is **inserted,** **deleted** or **replaced** in the genome of a living organism using engineered nucleases, or "molecular scissors." These nucleases create **site-specific** double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through **non-homologous end-joining (NHEJ)** or **homologous recombination (HR),** resulting in targeted mutations ('edits').

http://www.greenes.org.uk and http://www.nclack.k12.or.us

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**Genome Editing**

**Available Tools**

**Zinc Finger Technology (ZFN)**

- **FokI** nuclease as the DNA-cleavage domain and binds DNA by engineered Cys2His2 zinc fingers. Specific zinc fingers recognize different nucleotide triplets and dimerize the FokI nuclease (induce DSB).

**TALEN Technology**

- **Transcription Activator-Like Effector Nuclease (TALEN)** systems are a fusion of TALEs derived from the Xanthomonas spp. to a restriction endonuclease FokI. By modifying the amino acid repeats in the TALEs, users can customize TALEN systems to specifically bind target DNA and induce DNA breaks.

**CRISPR-Cas Technology**

- RNA-guided endonucleases (RGEN) utilize a short guide RNA (gRNA) to recognize DNA, bind an endonuclease, and induce site specific cleavage. Derived from the **Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)** found in bacteria that serve to identify/destroy foreign DNA.
**Genome Editing**

**CRISPR: Bacteria Defense Mechanism**

Adaptive bacteria immunity to protect against viral infection:

![CRISPR Defense Mechanism Diagram](http://rna.berkeley.edu)

Different bacterial systems = different specificities:

<table>
<thead>
<tr>
<th>Species/Variant of Cas9</th>
<th>PAM Sequence</th>
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<tr>
<td>Streptococcus pyogenes (SP); SpCas9</td>
<td>NGG</td>
</tr>
<tr>
<td>Staphylococcus aureus (SA); SaCas9</td>
<td>NNGRRT or NNGRR(N)</td>
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<tr>
<td>Neisseria meningitidis (NM)</td>
<td>NNNNGATT</td>
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**Genome Editing**

**Background**

Key dates:

![Genome Editing Timeline](image)
Genome Editing
CRISPR Publications

Exponentially Growing Interest

<table>
<thead>
<tr>
<th>Year</th>
<th># publications on 'CRISPR'</th>
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<tr>
<td>2005</td>
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<td>2015</td>
<td>1262</td>
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<td>2016</td>
<td>1713</td>
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Source: CRISPR Screening Workshop SDCSB 2016

CRISPR-Cas9:
How does it work?

PART II
CRISPR-Cas9

Glossary

CRISPR: Clustered Regularly Interspaced Short Palendromic Repeat, a region in bacterial genomes used in pathogen defense.
Cas: CRISPR Associated Protein, the Cas9 nuclease is the active enzyme for the Type II CRISPR system.

Target sequence: the 20 nucleotides that are incorporated into the gRNA plus the PAM sequence, target sequence is in the genomic DNA.
PAM: Protospacer Adjacent Motif, required sequence that must immediately follow the gRNA recognition sequence but is NOT in the gRNA.
gRNA: guide RNA, a fusion of the crRNA and tracrRNA, provides both targeting specificity and scaffolding/binding ability for Cas9 nuclease, does not exist in nature.
crRNA: the endogenous bacterial RNA that confers target specificity, requires tracrRNA to bind to Cas9.
tracrRNA: the endogenous bacterial RNA that links the crRNA to the Cas9 nuclease, can bind any crRNA.

CRISPR-Cas9

Applications Overview

Gene Knock-out + Gene Knock-in + Mutagenesis

Gene Repression
Gene Activation

Gene of interest

Genome-wide Screening
Imaging genomic loci
Purification of genomic loci

www.addgene.org/crispr/guide/
CRISPR-Cas9 Principle

DSB: Double Strand Break, a break in both strands of DNA, Cut, 2 proximal, opposite strand nicks can be treated like a DSB.
NHEJ: Non-Homologous End-Joining, a DNA repair mechanism that often introduces InDels.
HDR: Homology Directed Repair, a DNA repair mechanism that uses a template to repair nicks or DSBs.
CRISPR-Cas9: Gene KO

- When both Cas9 catalytic domains (RuvC and HNH) are functional, binding of Cas9 through a gRNA will induce a DSB.
- If no repair template is present, then the NHEJ repair pathway will be used to fix the DNA break.
- Errors induced by the NHEJ will disrupt the ORF and disrupt gene expression.

www.addgene.org/crispr/guide/
**CRISPR-Cas9**
Fluo readout of delivery/KO efficiency?

1- For controlled expression level of SpCas9 and enrichment of edited cells

- All-in-one Cas9-GFP-vector (Addgene)
- FACS sorting of GFP cells
- WB analysis of optimal GFP (i.e. Cas9) expression level for max KD

2- Removes the need for antibiotic selection (often unavailable) and allows for transient/controlled expression time (GFP negative cells can be sorted out)

3- Useful for low-throughput multiplexing (2 to 6 targets)

4- 6 colors available: TagBFP2, CFP, GFP, TagRFP-T, iRFP, iRFP670 for SpCas9 WT/D10A and eSpCas9 (1.1) to accommodate with already fluorescently modified cells

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**Example of optimized CRISPR KO: Arf6**

- gRNA cloning in Cas9-GFP-vector
- Transfection + FACS sorting of GFP cells
- WB analysis to evaluate overall protein KD
- Sub-cloning
- Internalization assay by flow cytometry on several clones - 60% endocytosis inhibition

**Further characterization of KO clones:**
- Response to stimulation (degranulation assay)
- Imaging: confocal + electron microscopy
- Additional KO (clathrin, caveolin, ...)

- Screening for KO clones by WB
Integration into your lab workflow

Transcriptomics
- Expression levels of key genes in model cell line
- Guide for siRNA screen

Targeted siRNA HTS screening
- Individual siRNA
- Pooled siRNA (biologically-related)
- “Hit” target ID

Validation using CRISPR/Cas9 KO
- Receptors surface expression
- Response to stimulation
- Internalization rate

Table 3-1. Results of high throughput flow assay in cells treated with siRNA pools
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Resting Cell stimulation for 30 min</th>
<th>Cell stimulation for 4 hours</th>
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<td>3907</td>
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<td>Arf2</td>
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<td>Arf5</td>
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<td>309</td>
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<tr>
<td>Arf6</td>
<td>2298</td>
<td>2153</td>
</tr>
</tbody>
</table>

Double CRISPR KO in RBL cells

Eps15-Eps15R KO

c-Cbl-Nedd4a KO

Sequential CRISPR KO to generate single and double KO cell lines
Double CRISPR KO in RBL cells

Eps15-Eps15R KO  c-Cbl-Nedd4a KO

CRISPR-Cas9: Gene KO & Replacement

PART III
CRISPR-Cas9
Fluo readout of delivery/KO efficiency?

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- WB analysis of optimal GFP (i.e. Cas9) expression level for max KD

Visualizing Syk Recruitment After FceRI Activation

- Syk Aggregates the Result of Dynamic Binding Events

Slide courtesy from Dr. Sam Schwartz
Syk binding lifetime increases with FcεRI Activation

Slide courtesy from Dr. Sam Schwartz

CRISPR-Cas9:
Gene KI: Insertion of tags

PART IV-A
**CRISPR-Cas9**

Generating Gene Ki: Addition of a tag

- Instead of using the NHEJ repair system, a template containing the desired sequence to be inserted is provided for repair of the DSB.
- Need homology arms (size of homologous sequence depends on size of sequence to be inserted/modified).

www.addgene.org/crispr/guide/

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**CRISPR Ki: clathrin light chain a-meGFP**

- In control cells, clta-GFP is found as expected. **Endogenous** & **physiologic** expression of clta-GFP allows for accurate measurement of clathrin dynamics at the plasma membrane.
- As seen by electron microscopy, large clathrin structures are also observed using laser scanning confocal imaging of live cells.

With Dr. Keith Lidke & Farzin Farzam
Double CRISPR KI: clta-meGFP & Arf6-mRuby3

- **Goal**: simultaneous live cell imaging of cross-linked FceRI, clathrin and Arf6 in order to study dynamics of internalization via both endocytic pathways.
- Preliminary results seem to indicate that Arf6 maybe a shared component.

Double CRISPR KI: clta-meGFP & Arf6-RFP

- **Goal**: simultaneous live cell imaging of cross-linked FceRI, clathrin and Arf6 in order to study dynamics of internalization via both endocytic pathways.
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*With Dr. Sam Schwartz*
CRISPR-Cas9: Gene KI: Mutagenesis

- Instead of using the NHEJ repair system, a template containing the desired sequence to be inserted is provided for repair of the DSB.
- Need homology arms (size of homologous sequence depends on size of sequence to be inserted/modified).

www.addgene.org/crispr/guide/
Gene Editing in CAMT

What is CAMT?

- CAMT: Congenital AMegakaryocytic Thrombocytopenia is a rare disorder characterized by isolated thrombocytopenia and megakaryocytopenia in infancy with no associated physical abnormalities. Can evolve into aplastic anemia and leukemia later in life.

- Typically caused by autosomal recessive, or compound heterozygous, germline mutations of the thrombopoietin receptor (Mpl).

Gene Editing in CAMT

New Mpl mutation in CAMT

- Familial case of CAMT:

- Clinical presentation: highly elevated Tpo levels but severe thrombocytopenia

- Goal: Understand what causes the disease and maybe suggest a therapeutic solution
**Gene Editing in CAMT**

Double mutant Mpl do not respond to Tpo

- Characterization of mutants Mpl expressed in Ba/F3 cells:

- Explain why patients have no platelets/MK despite highly elevated Tpo levels

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**Gene Editing in CAMT**

Intracellular trafficking defect

- Expression of mutant Mpl in human UT-7 megakaryoblastic cells

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![](chart1.png)

UT-7 at [Tpo]=10 ng/mL

- Mpl WT
- Mpl K39N
- Mpl W272R
- Mpl K39N/W272R
- UT-7 parental

![](chart2.png)
**Gene Editing in CAMT**

**Mpl Functional Rescue: Approach #2**

- 1 gRNA + WT SpCas9 in Ba/F3 cells:

  - gRNA
  - ssODN Repair Template

- 2 gRNA + SpCas9-D10A in UT-7 cells:

  - gRNA #1
  - ssODN Repair Template

- FACS enrichment of RFP cells
- Switch from IL-3 to Tpo for selection

- FACS enrichment of CFP/RFP cells
- Switch from GM-CSF to Tpo for selection

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**Gene Editing in CAMT**

**Summary & Perspectives**

- New Mpl mutation (W272R) in cis with hereditary thrombocytosis-causing K39N mutation in the context of CAMT Type I.
- Aberrant Mpl trafficking prevents Tpo signaling
- Mpl functional rescue:
  - FACS enrichment
  - Switch from IL-3 to Tpo

- Next steps:
  1. Evaluate precisely off-target in UT-7 cells
  2. Test gRNAs in primary CD34+ cells using RNPs
  3. Identify a suitable patient to establish a xenograft
  4. Perform gene editing on patient’s CD34+

- **Goal:** to edit enough HSC to ensure a polyclonal hematopoiesis!
CRISPR-Cas9: Major issues & How to address them

This is the major issue regarding the use of CRISPR-Cas9 (and other gene editing tools):
- Lack of gRNA specificity, or genomic sequence redundancy, can lead to modifications at additional genomic loci (in coding or non-coding sequences).
- It is critical to address this issue before any biomedical application can be setup.
**CRISPR-Cas9**

How to limit off-target effect?

**Double nickase approach:**
- Mutations within each nuclease domain that are critical for endonuclease activity (D10A for HNH and H840A for RuvC in spCas9) lead to modified versions of the Cas9 enzyme containing only one active catalytic domain (called “Cas9 nickase”).
- Cas9 nickases still bind DNA but are only capable of cutting one of the DNA strands, resulting in a “nick”, or single strand break.
- DNA nicks are rapidly repaired by HDR using the intact complementary DNA strand.
- 2 nickases targeting opposite strands are required to generate a DSB. It increases target specificity, since it is unlikely that two off-target nicks will be generated within close enough proximity to cause a DSB.

Gene Editing in CAMT

What system for less off-target?

1. Single nick-induced HDR using SpCas9-D10A:
   - gRNA #1 + gRNA #2:
     - 30% 2% 95%

2. 1 gRNA + SpCas9-D10A for Mpl editing:
   - SpCas9 D10A
   - ~2% editing

www.addgene.org/crispr/guide/
**CRISPR-Cas9**

**Other engineered Cas9**

eCas9 (1.1) and Cas9-HF1:
- Developed by the Feng Zeng lab and the Kleinstiver labs, respectively.
- Additional mutations in Cas9 protein destabilize its complex formation ability with gRNA and genomic DNA. Hence, if the gRNA hybridization is not perfect, the complex Cas9/gRNA/DNA will not be stable enough to generate a DSB.
- Dramatic positive effect on off-target but also strongly reduces Cas9 on-target activity.

http://documents.tips/documents/ecas9.html#

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**CRISPR-Cas9**

**Other delivery methods**

**Plasmid expression:**
- >12 hours to see a peak in Cas9 production.
- Plasmid expression is infinite (for viruses) or last until plasmid is lost (cell division) (days).

**RNP delivery:**
- Cas9/gRNA complex is ready and functional right away.
- Proteins are being cleared within hours using the cell protein degradation machinery.
- Limit off-target effect due to short lifetime and enhanced activity of preformed complex.
- Can be used for primary cells or zygotes injection (not possible with plasmids/virus).

www.addgene.org/crispr/guide/
CRISPR-Cas9: Conclusion

Applications & Advantages

Applications:
- Gene KO
- Gene replacement (chimeric DNA sequence)
- Knock-in of large DNA fragments (useful for tagging POI with fluorescent tags for example)
- Directed mutagenesis to reproduce point mutations (or to fix them!)
- Activation or repression of gene transcription
- Can replace siRNA screening (GeCKO lentiviral library of human gRNA). Etc ...

Advantages:
- Endogenously regulated expression of POI (no over-expression)
- Gene KO fairly easy (about 80% efficiency) + no residual expression! (need gRNA + Cas9)
- Knock-in of large DNA fragments more difficult (requires a good readout) (need gRNA + Cas9 + Donor vector)
- No need to stabilize the new cell line: the genome itself is modified
CRISPR-Cas9

Resources

Addgene website:

- http://crispr.mit.edu/
  gRNA design

http://arep.med.harvard.edu/
George Church lab

http://www.genome-engineering.org/crispr/?page_id=23
Feng Zhang lab

University of New Mexico
Dr Bridget S. Wilson
Dr Diane S. Lidke
Dr Angela Wandinger-Ness
Dr Samantha Schwartz
Dr Keith A. Lidke
Dr Michael L. Paffett
Farzin Farzam
Shayna R. Lucero
Rachel Grattan
Eun Ho Choi

CHU Nîmes, France
Serge Carillo, PhD
Thierry Lavabre-Bertrand, MD
Éric Jeziorski, MD

INSERM, Nantes, France
Sylvie Hermouet, MD, PhD

Questions?