

# **Zinc Finger Nucleases (ZFNs), TAL Effector Nucleases (TALENs), Homing Endonucleases (Meganucleases) and RNA Guided Endonucleases (RGENs)**

Matthew Porteus MD, PhD  
Department of Pediatrics  
Stanford University  
mporteus@stanford.edu

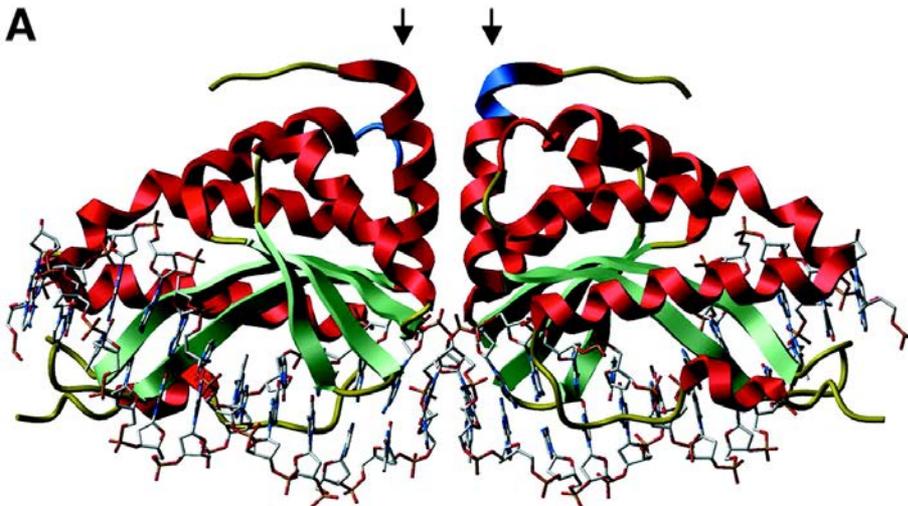
# Conflicts of Interest

CRISPR Therapeutics: Equity and Consultancy

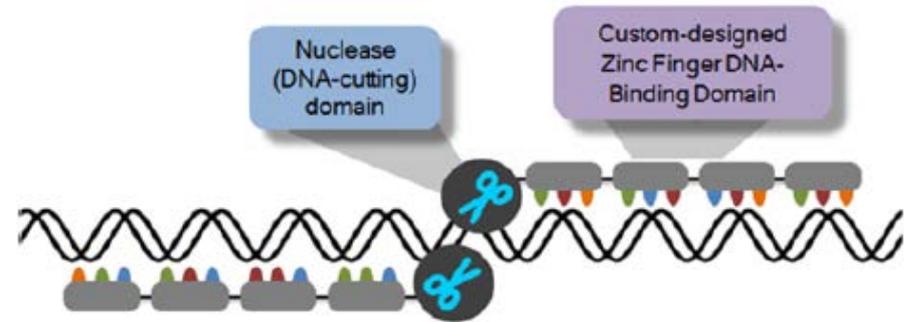
Managed through Stanford in accordance with their  
conflict of interests policy.

# Review of Four Major Engineered Nuclease Platforms

A

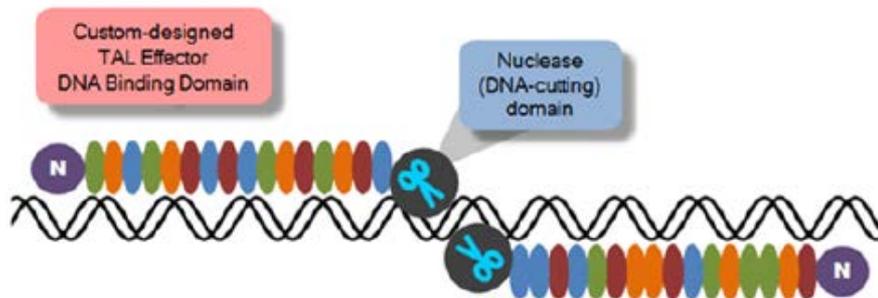


**Homing Endonuclease/Meganuclease**  
**>18 bp specificity**



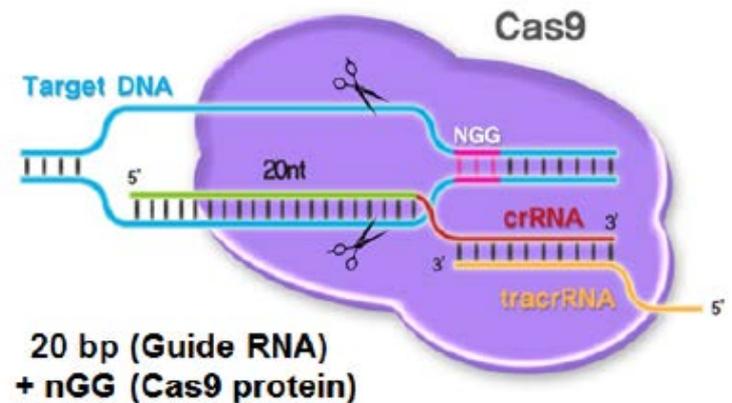
**Zinc Finger Nuclease (ZFN)**  
**Dimer**

**Recognition Sites: 9-18 bp x 2**  
**Spacer: 5-7 bp**



**TAL Effector Nuclease (TALEN)**  
**Dimer**

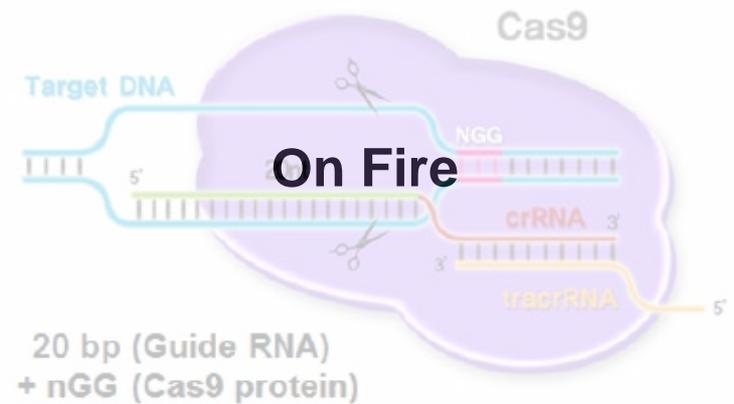
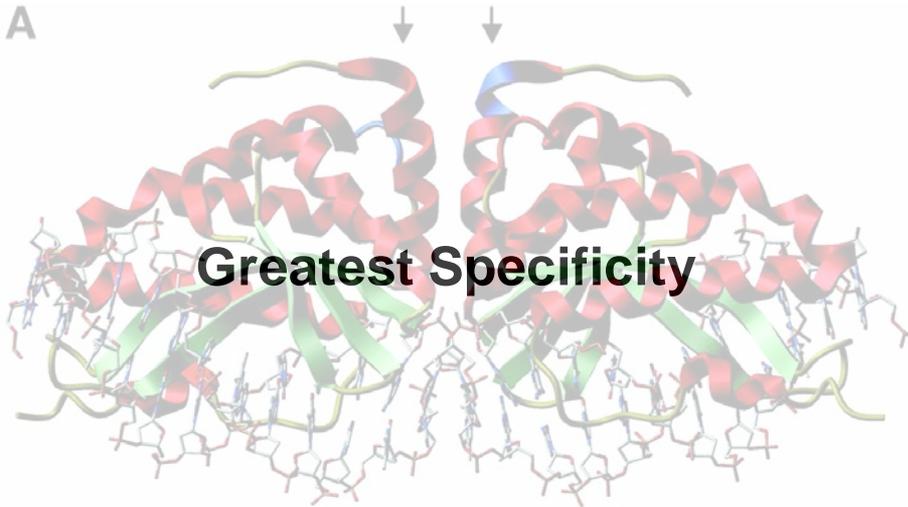
**Recognition Sites: 12-20 bp x 2**  
**Spacer: 12-20 bp**



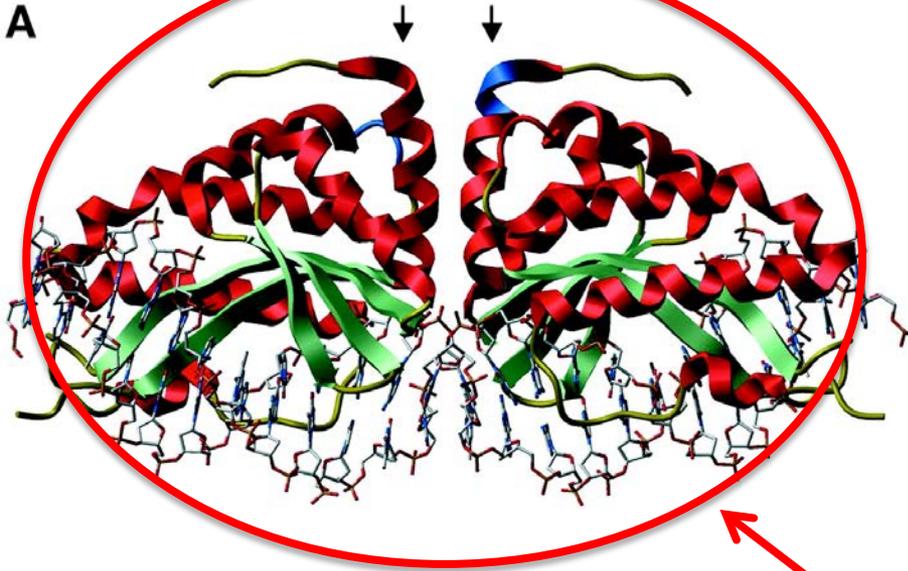
**RNA-Guided Endonuclease (RGEN)**  
**CRISPR/Cas9**  
**Recognition Site: 19-22 bp x 1**

# Alternative Engineered Nuclease Platforms

A



# Alternative Engineered Nuclease Platforms



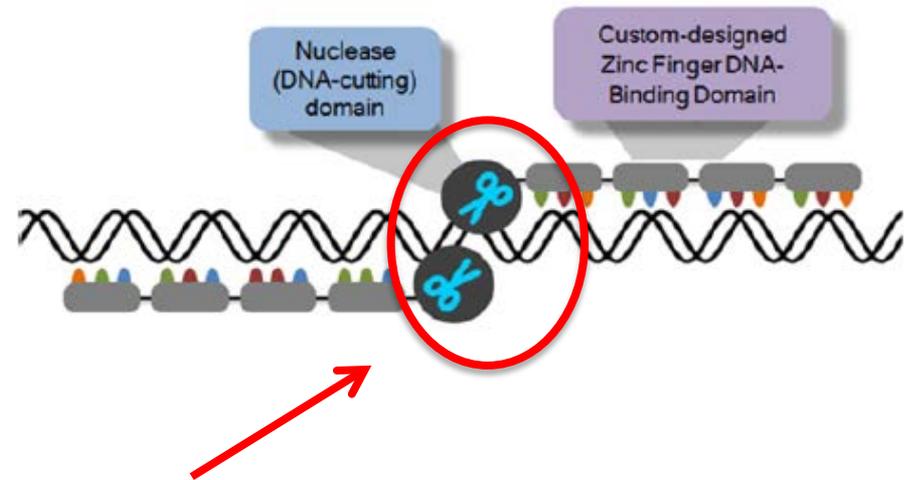
**Clinically Most Advanced**

**Genetic Parasite**

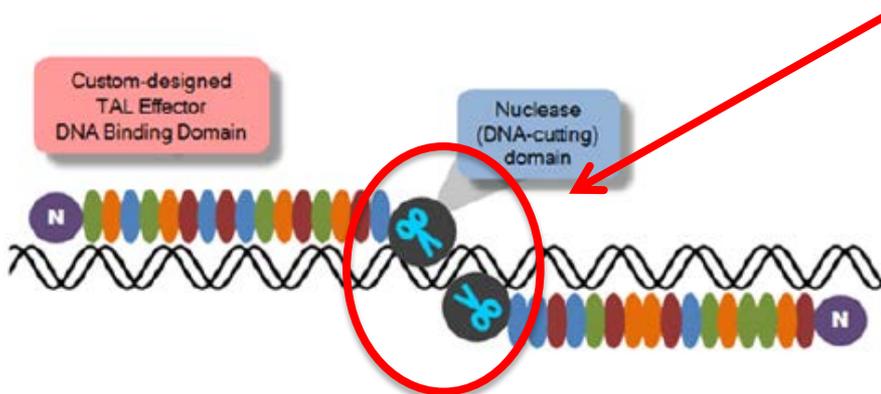
**Renaissance Platform  
(Little Bit of Everything)**

**On Fire**

# Alternative Engineered Nuclease Platforms

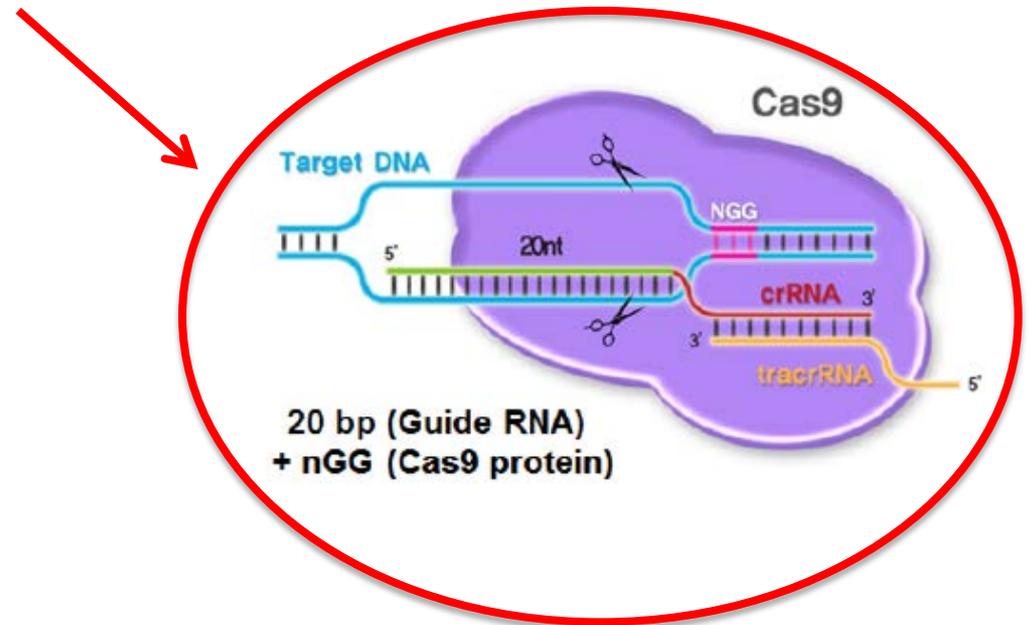


**Bacterial Innate Immune System**



# Alternative Engineered Nuclease Platforms

## Bacterial Adaptive Immune System



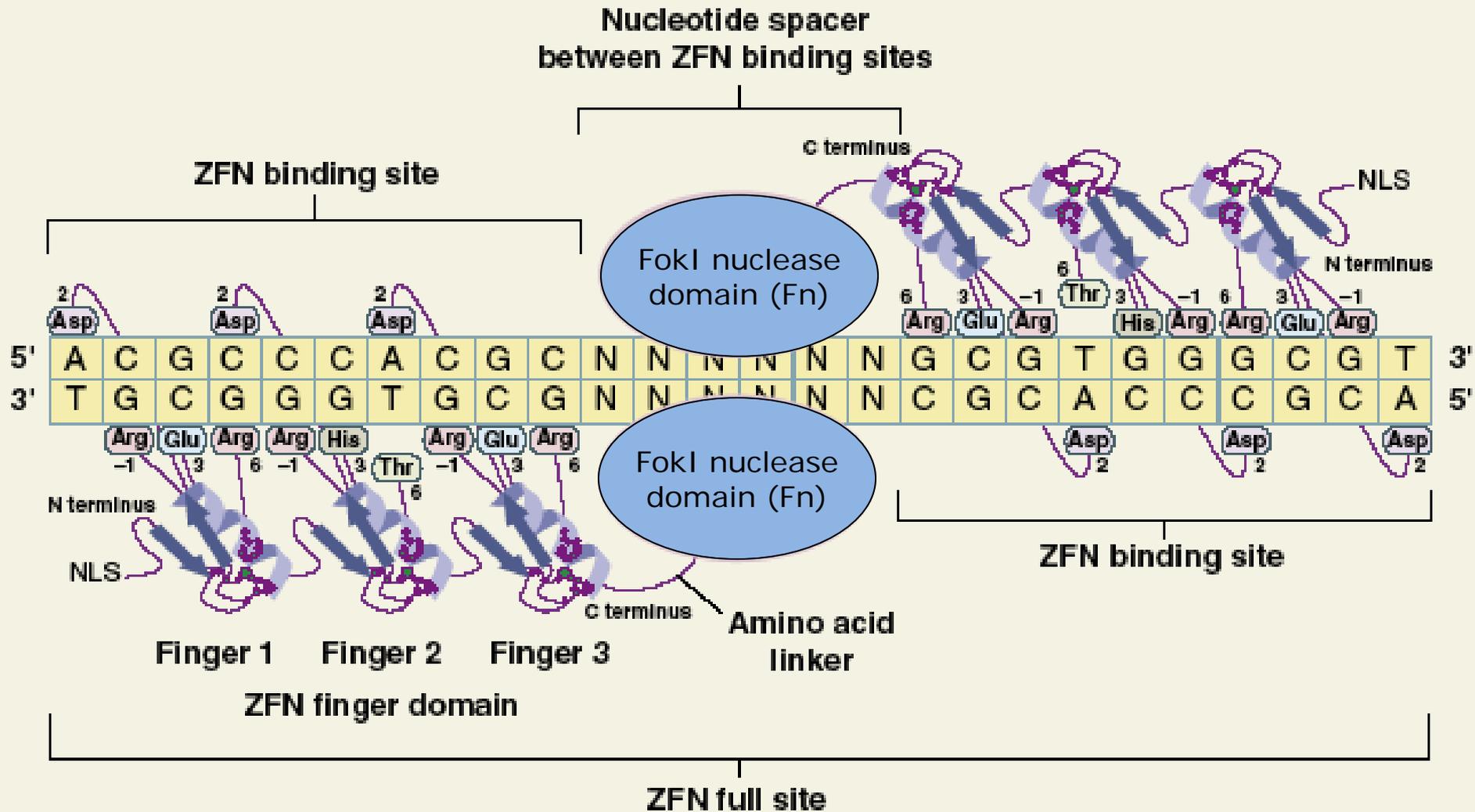
# Homing Endonucleases

- Natural Proteins derived from a large (hundreds) family of genetic elements and proteins that catalyze their own horizontal transfer across cell types.
  - Genetic parasites
- Large recognition sites (>18 basepairs) with high but not absolute degree of specificity.
  - Cryptic off-target sites identified by AAV capture (Miller and Russell)
- Recognition activity and enzymatic activity are intertwined within the protein.
- Small
- Usually result in a DSB with a 4 basepair 3' overhang which has been proposed as being a natural substrate for the homologous recombination machinery.
- Nearly all of the fundamental genome editing manipulations were first done using I-SceI, a founding member of the family.
  - Mutagenic NHEJ (Waldman and colleagues)
  - Gene Targeting by Homologous Recombination (Groups of Jasin, Nickoloff, Wilson, Choulika...)
  - Creation of small deletions (Groups of Porteus and Lopez)
  - Catalyzing the insertion of random DNA into a site of a break (Waldman and colleagues)

# Engineering Homing Endonucleases

- Method of Engineering (Difficult)
  - Detailed structure/function analysis of a small number of homing endonucleases that are structural dimers to determine key residues that need to modify to change target site specificity.
  - Bio-informatically identify which scaffold is closest to new desired target site.
  - Iteratively screen for variants that have high binding and cutting activity at desired target site.
  - (Precision Biosciences has proposed that there is a code to re-engineering but this has not been published nor is available to academics nor have any clear successes been reported.
- Potential Advantages
  - Specificity
  - Small size
- Potential Disadvantages
  - Do re-engineered HE's retain their specificity?
  - Do re-engineered HE's have sufficient activity?
    - Mutagenic NHEJ activity can be clearly enhanced by co-expression of TREX, a 3' single-stranded exonuclease.

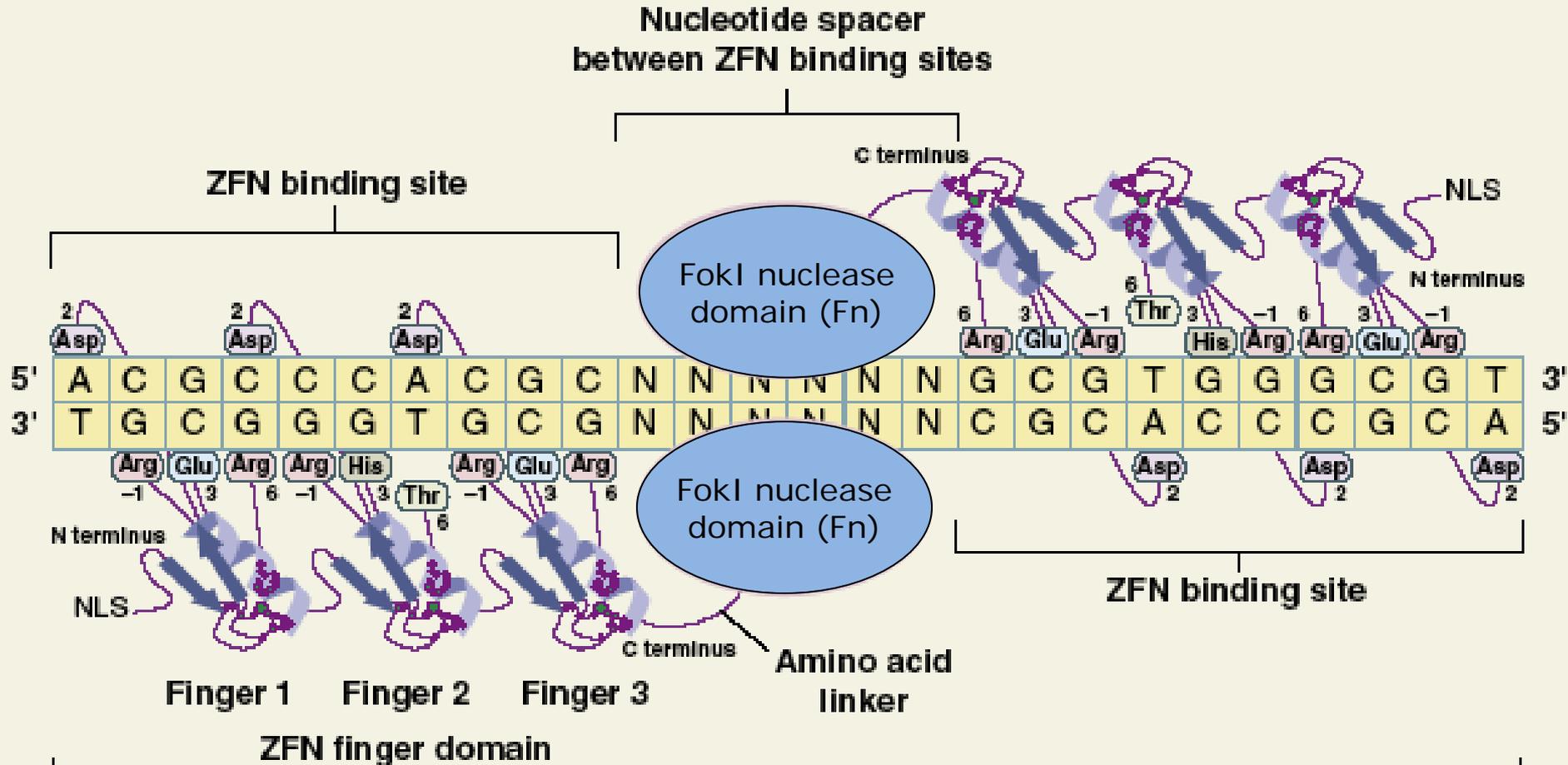
# Zinc Finger Nucleases (ZFNs)



Initially developed by labs of Srinivasan Chandrasegaran (Johns Hopkins) and Dana Carroll (Univ. Utah)

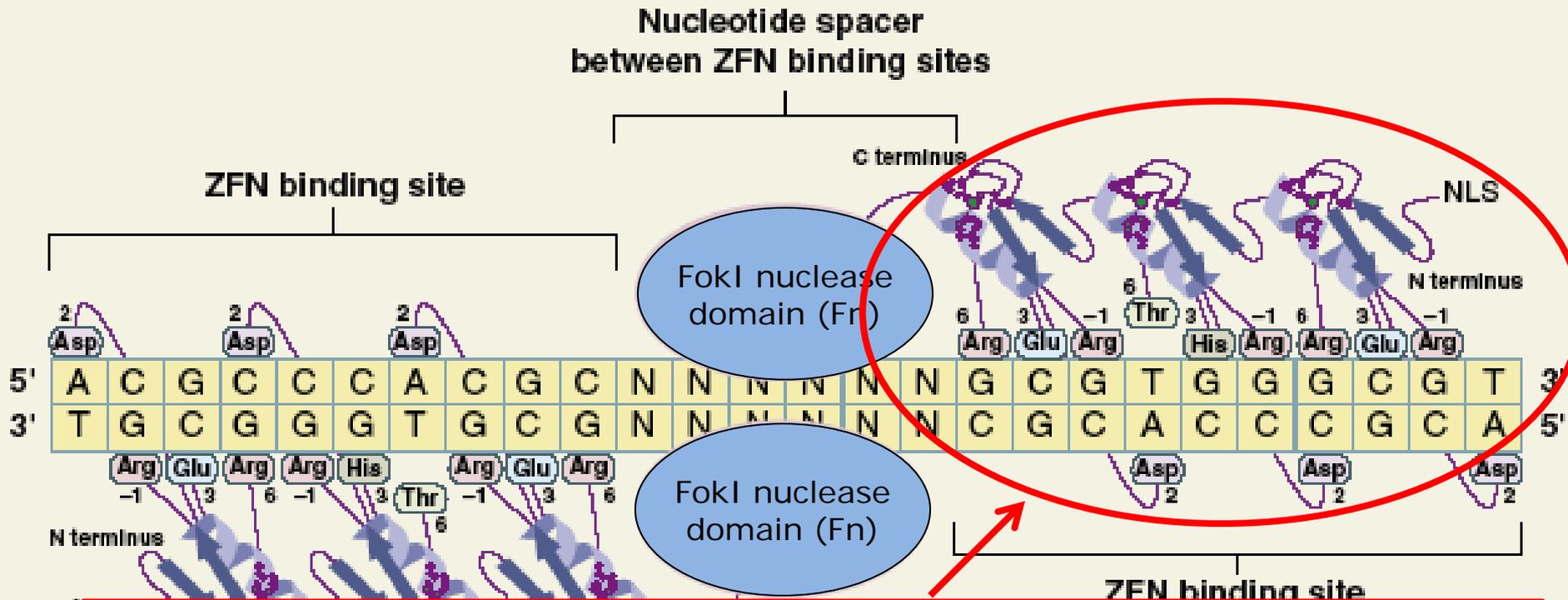
First used to engineer mammalian cells Porteus and Baltimore (2003)

# Zinc Finger Nucleases (ZFNs)



1. Most advanced clinically
2. Demonstrated efficacy in a variety of therapeutically relevant cell types including T-cells, keratinocytes, primary fibroblasts hematopoietic stem/progenitor cells.

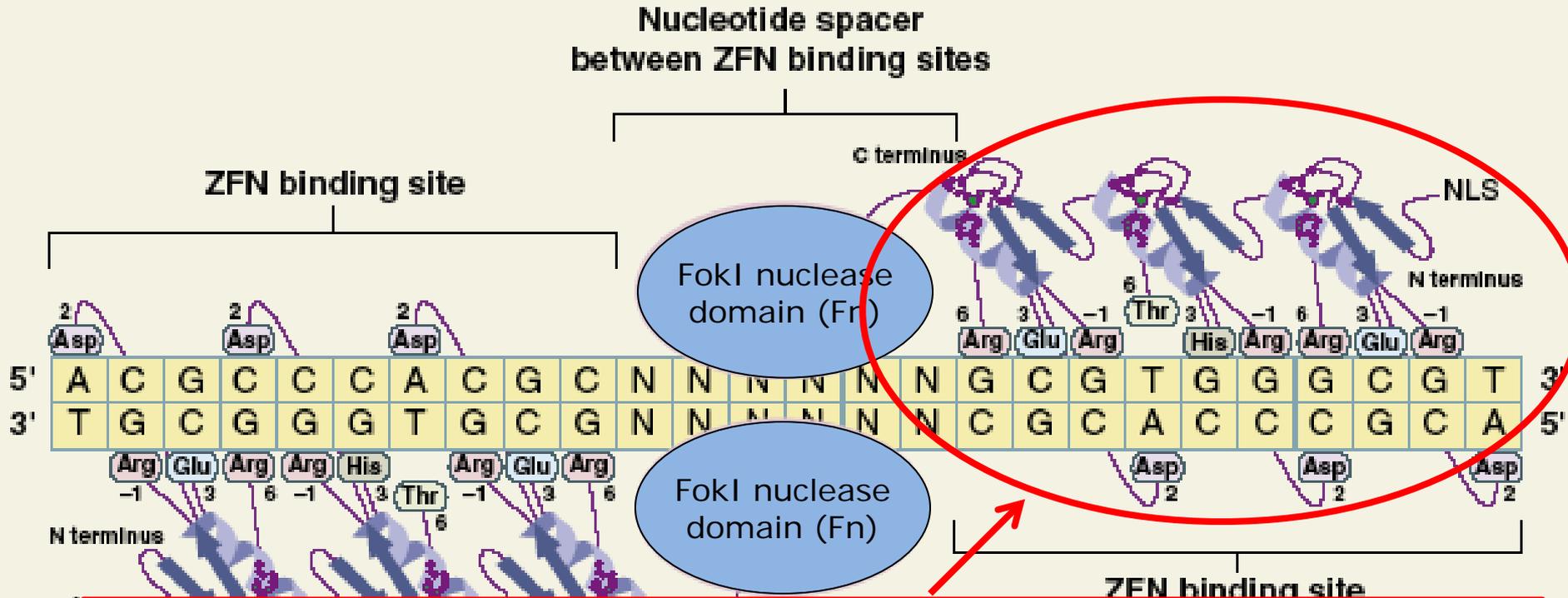
# Zinc Finger Nucleases (ZFNs)



## DNA Binding Domain:

- Modular-Assembly
- Phage Display
- Bacterial 1-Hybrid or Bacterial 2-Hybrid Systems.
- Combination: Phage Display to Generate Two-Finger Modules, Modular Assembly, Iterative Rational and Empiric Design (Sangamo)

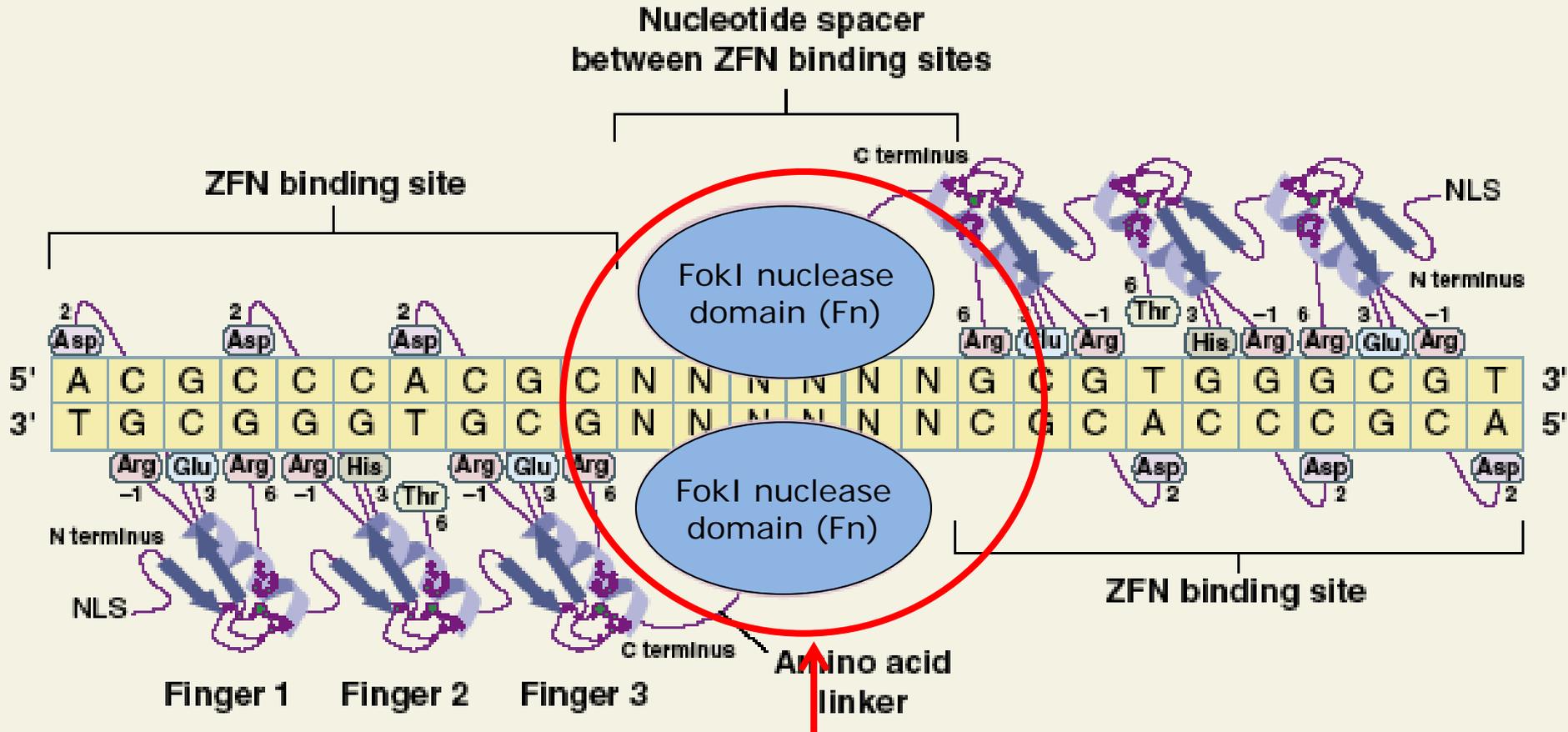
# Zinc Finger Nucleases (ZFNs)



## Engineering DNA Binding Domain:

- Array of Individual Zinc Fingers (3-6)
- Zinc Finger is the most common motif in mammalian proteins.
- ~30 amino acids long
- Each finger mediates binding to a 3-4 bp sequence through the alpha-helix integrating into the major groove of double-stranded DNA
- Re-engineering specificity by changing both the amino acids that make major contacts with the DNA and the surrounding amino acids.
- Sites tend to be rich in "GNN" triplets

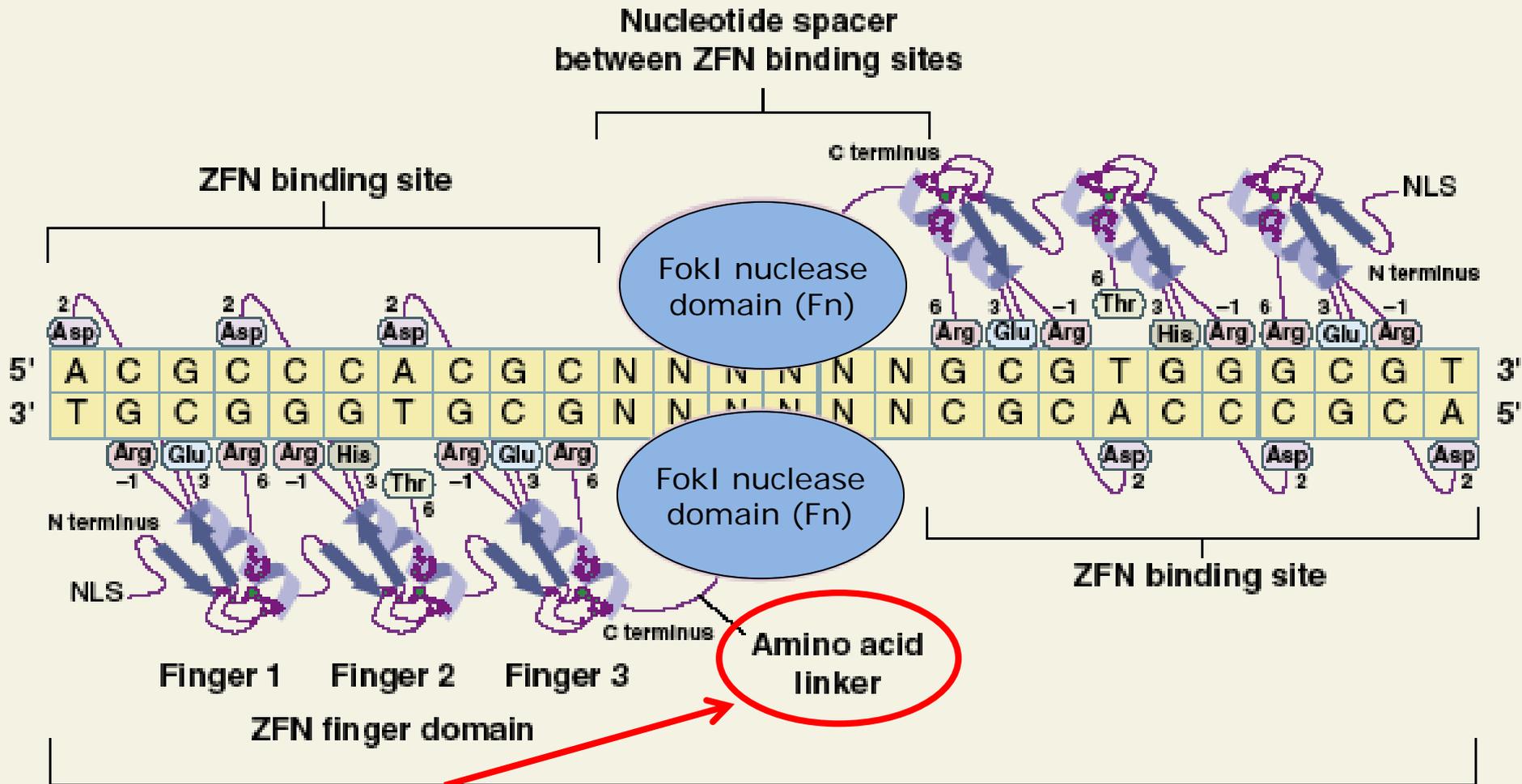
# Zinc Finger Nucleases (ZFNs)



## Nuclease Activity:

- From the N-terminus of the Type IIS Restriction Endonuclease FokI
- Requires dimerization
- Generates a 5' overhang
- Can be engineered to be catalytically inactive ("nickase")
- Can be engineered to only dimerize as a heterodimer to improve specificity

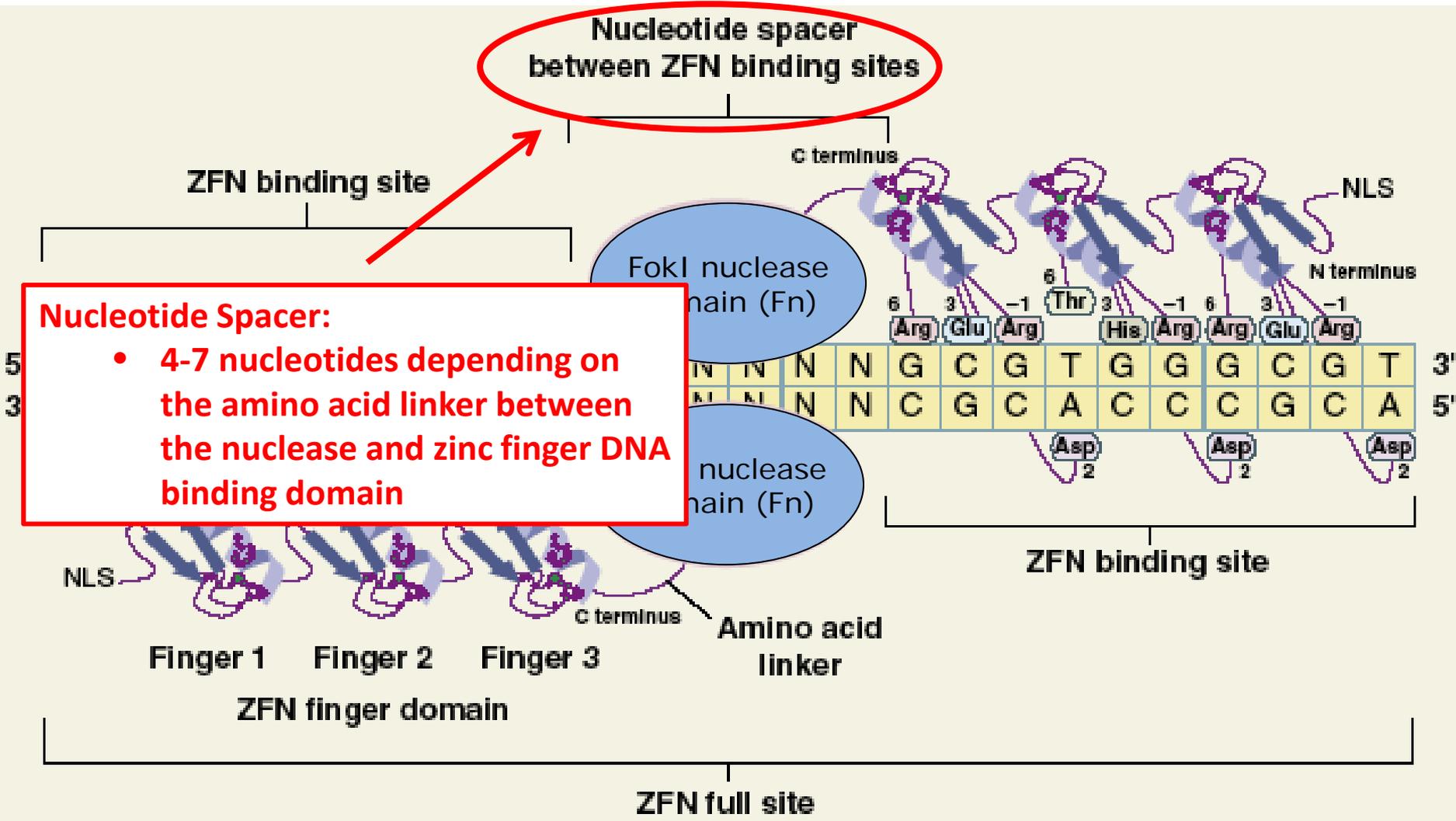
# Zinc Finger Nucleases (ZFNs)



## Amino Acid Linker:

- Usually 2-6 amino acids long and regulates how far the two binding sites can be from each other.

# Zinc Finger Nucleases (ZFNs)

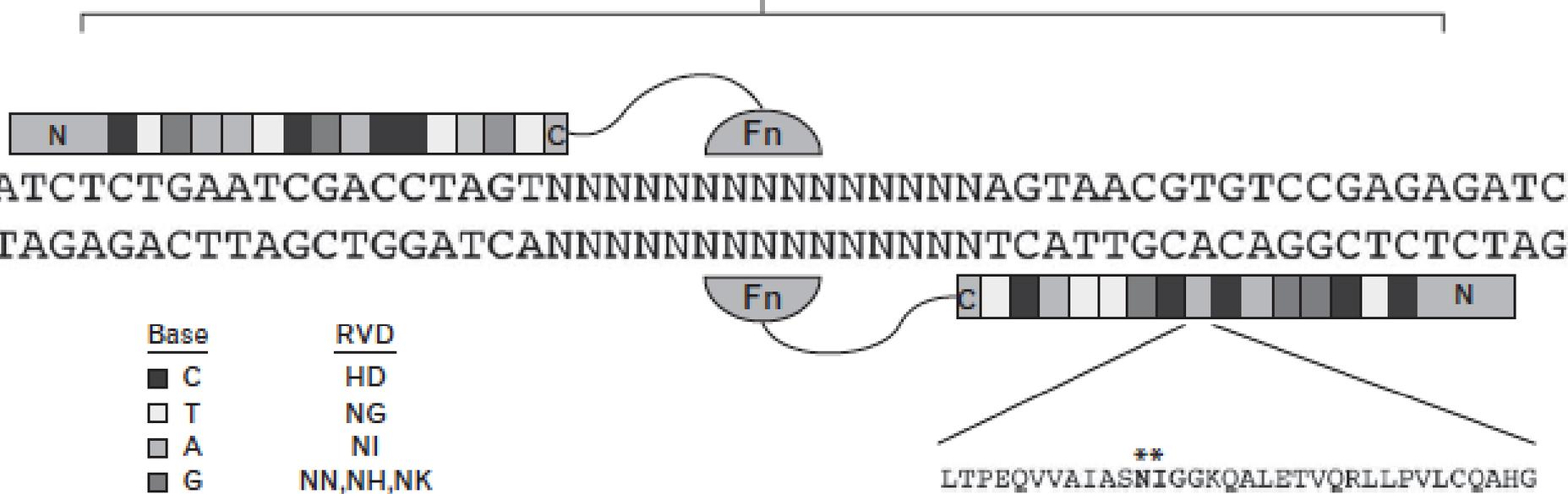


**Nucleotide Spacer:**

- 4-7 nucleotides depending on the amino acid linker between the nuclease and zinc finger DNA binding domain

# TAL Effector Nucleases (TALENs)

TALEN pair target site

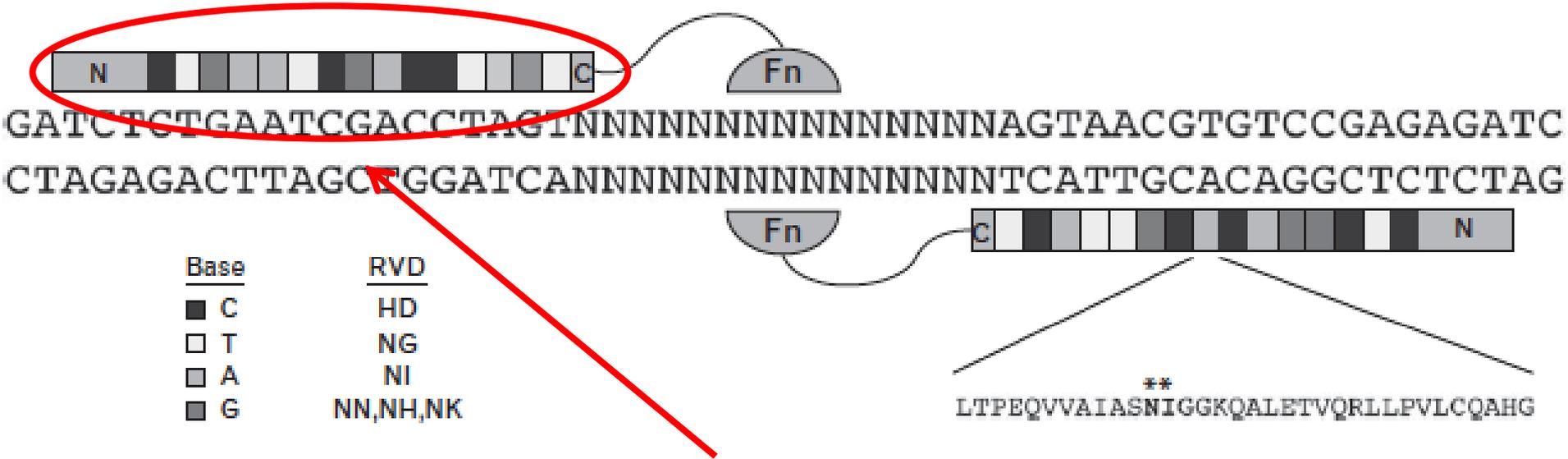


Christian et al (2010)

Miller et al (2010)

# TAL Effector Nucleases (TALENs)

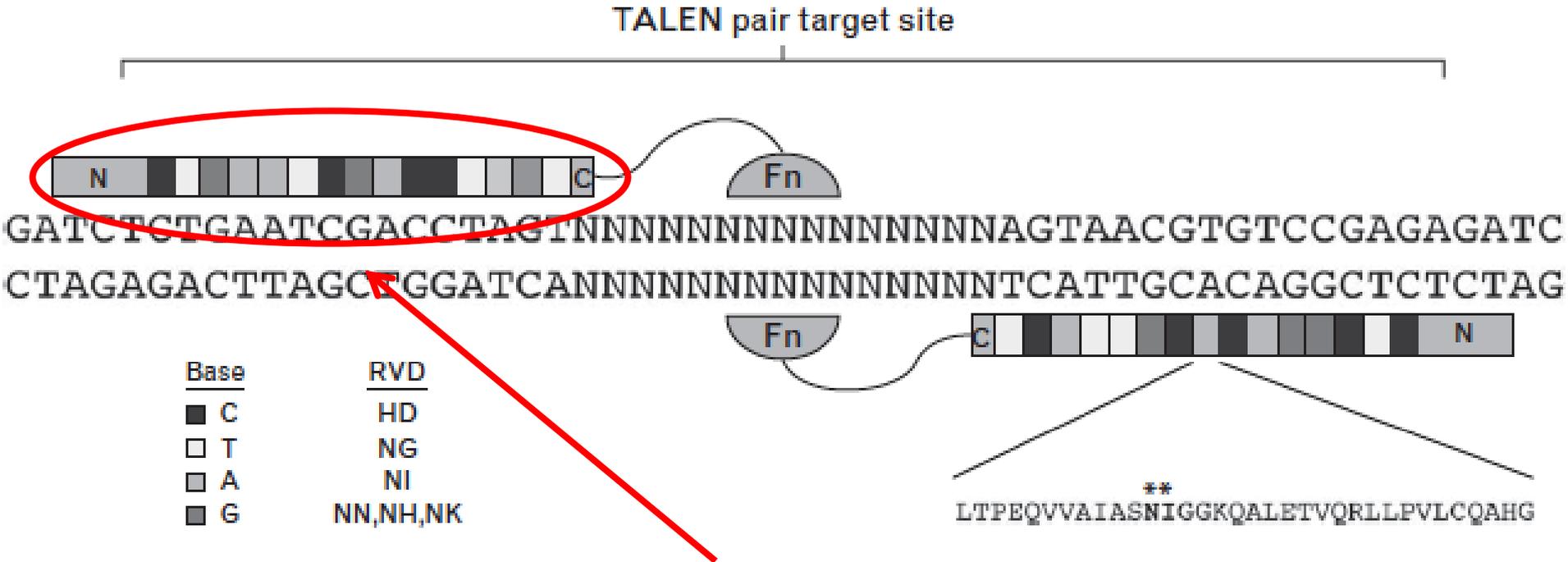
TALEN pair target site



## DNA Binding Domain:

- 34 amino acid domain with RVD at amino acids 12-13.
- Single repeat binds to single nucleotide
- RVD code simultaneously reported by Moscou et al (2009) and Boch et al (2009)
  - Alternative RVDs have been described but original four seem to work best
- Derived from a plant bacterial pathogen (Xanthomonas)

# TAL Effector Nucleases (TALENs)

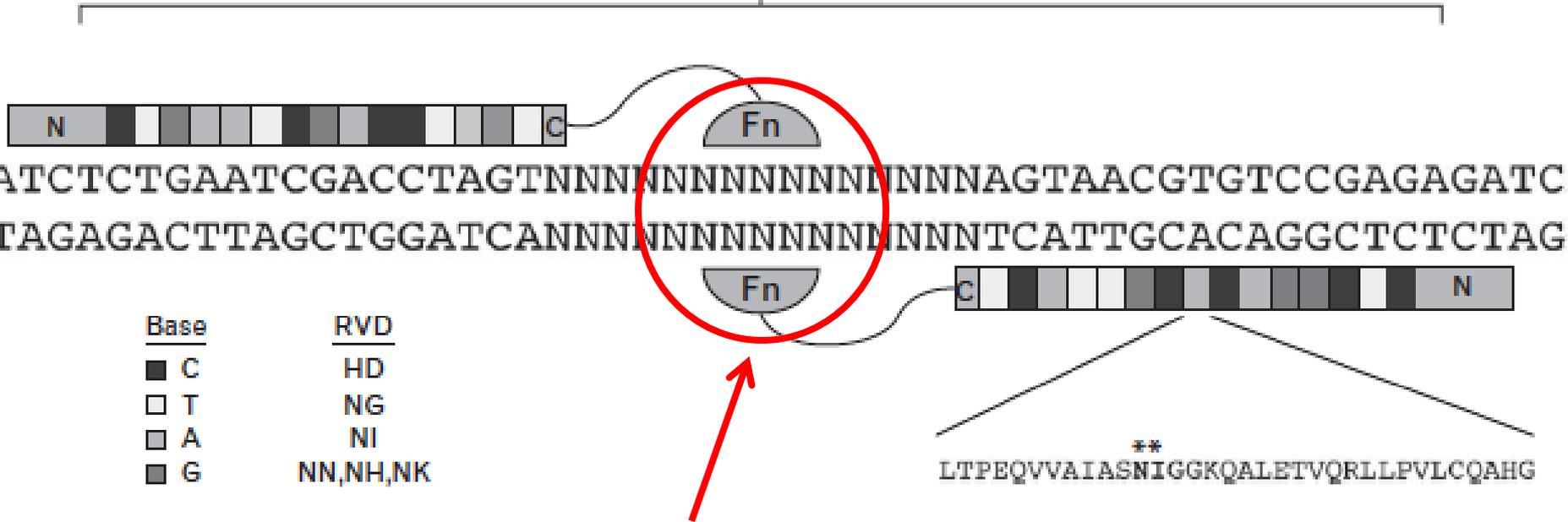


## Engineering DNA Binding Domain:

- Assemble individual TAL repeats into an array using RVD:Nucleotide code either by gene synthesis, Golden Gate cloning, or FLASH  
(Handful of TALENs can be made by 1<sup>st</sup> year graduate student using Golden Gate assembly in 1-3 weeks time)
- Repeat length 13-20 long (so each TALEN recognizes a 13-20 bp sequence)
- Multiple on-line tools.

# TAL Effector Nucleases (TALENs)

TALEN pair target site

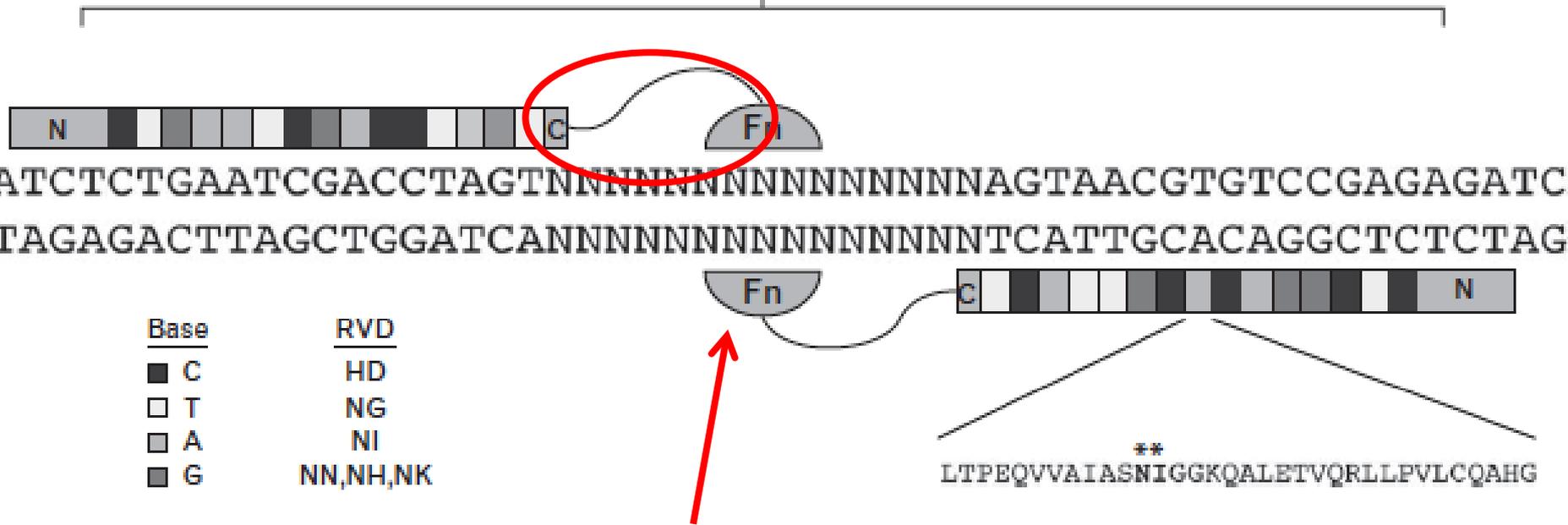


## Nuclease Domain derived from FokI:

- Same as for zinc finger nucleases
- When make one nuclease domain catalytically inactive still get DSBs instead of nicks (in contrast to ZFNs).

# TAL Effector Nucleases (TALENs)

TALEN pair target site

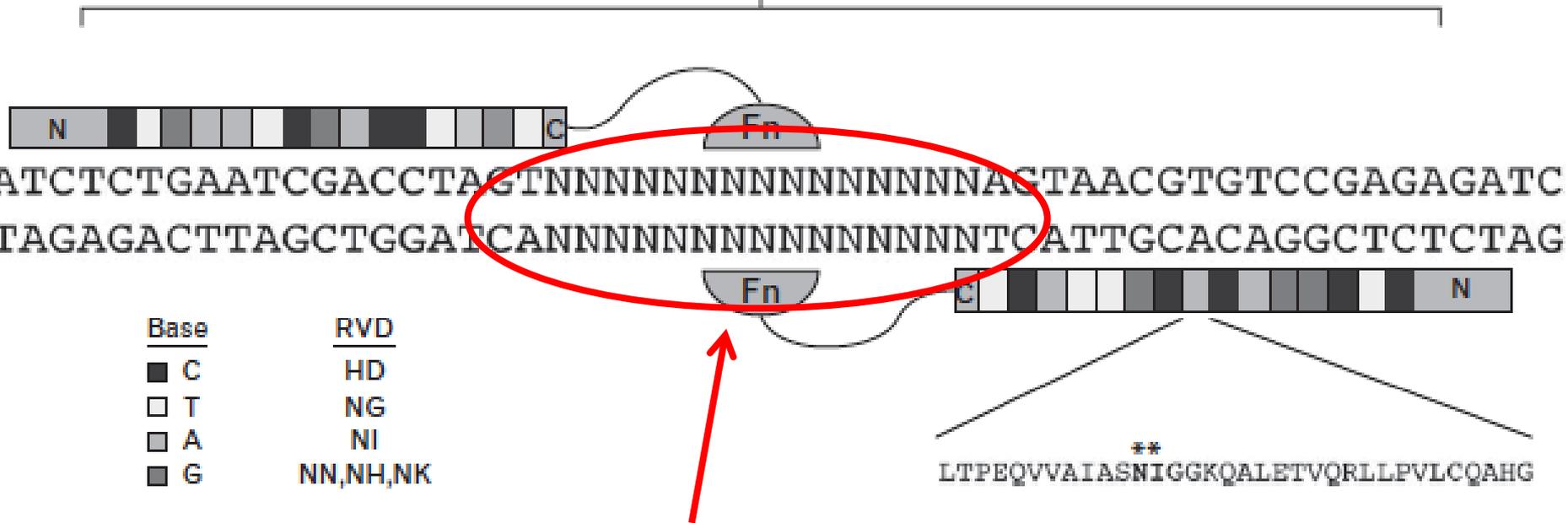


## C-Terminal Amino Acid Linker:

- Essential for Activity
- Various different lengths used including C-18, C-28 and C-63
- Different length linkers do affect on-target activity and specificity

# TAL Effector Nucleases (TALENs)

TALEN pair target site

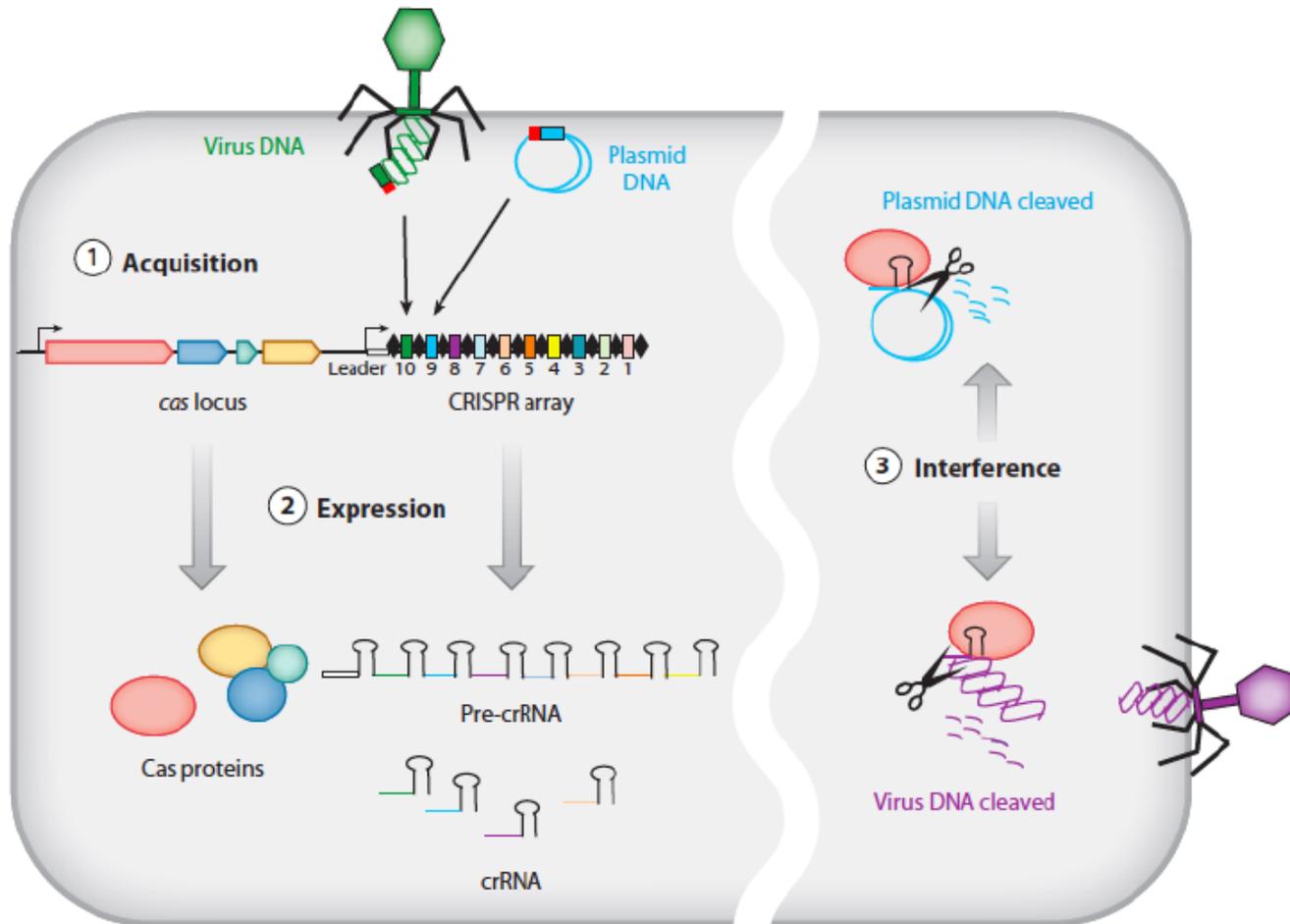


## Spacer:

- 12-20 basepairs long for optimal activity
- Dependent on length of C-terminal linker

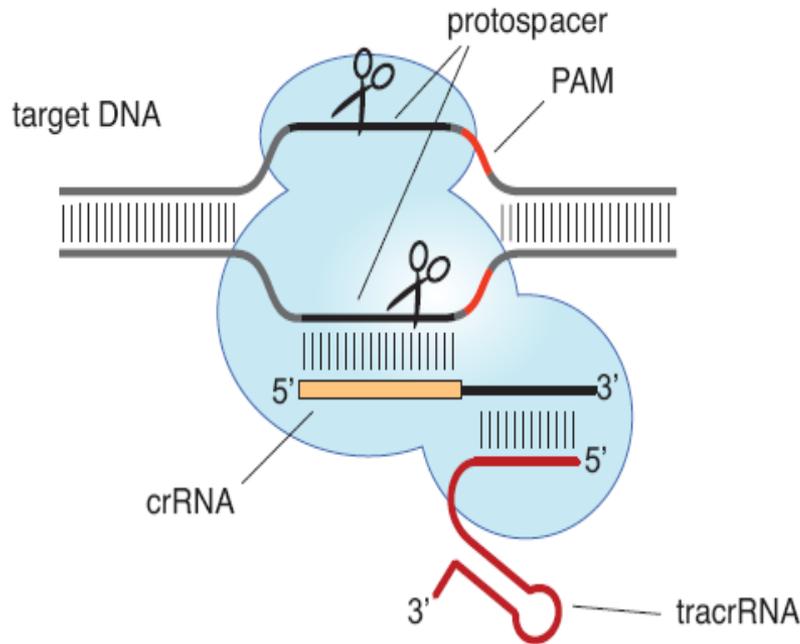
# **RNA Guided Endonucleases (RGENs) from the CRISPR/Cas9 System**

# CRISPR/Cas9: bacterial adaptive immunity

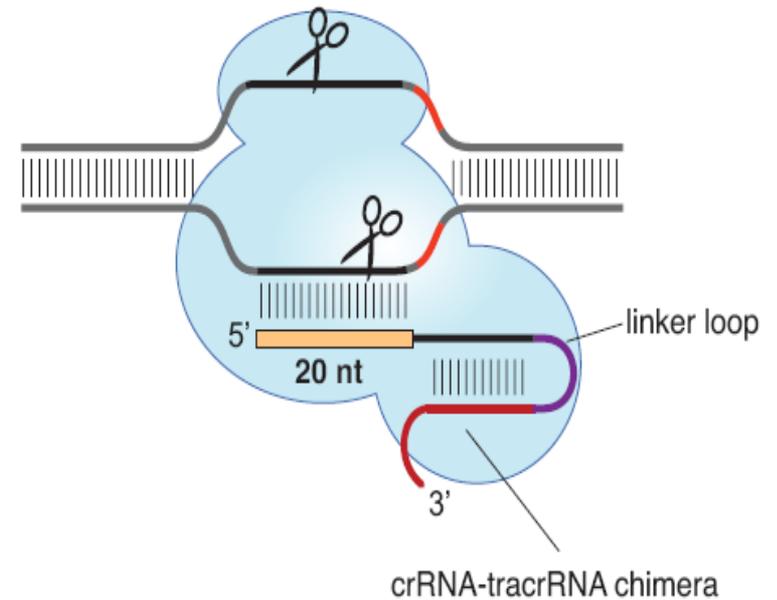


# Nuclease Activity is Programmed by Specific short guide RNAs (sgRNAs)

Cas9 programmed with tracrRNA:crRNA duplex

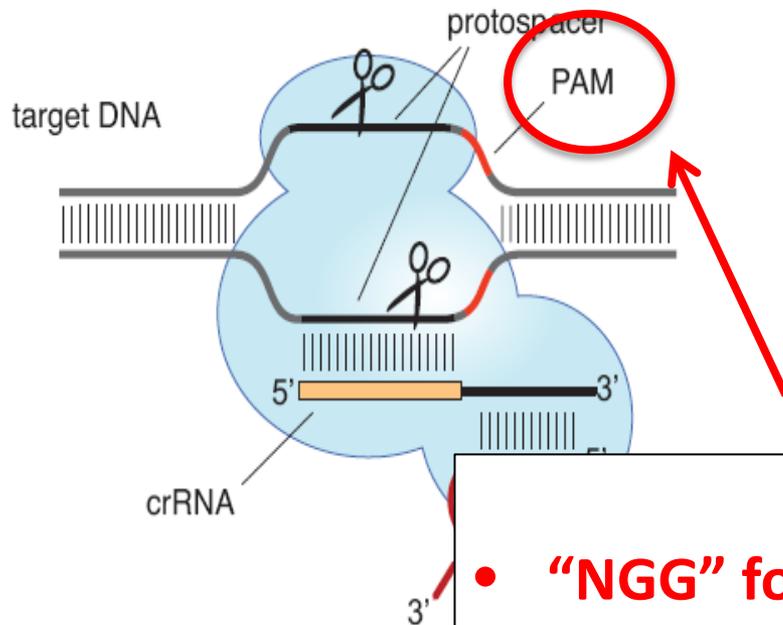


Cas9 programmed with single chimeric RNAs

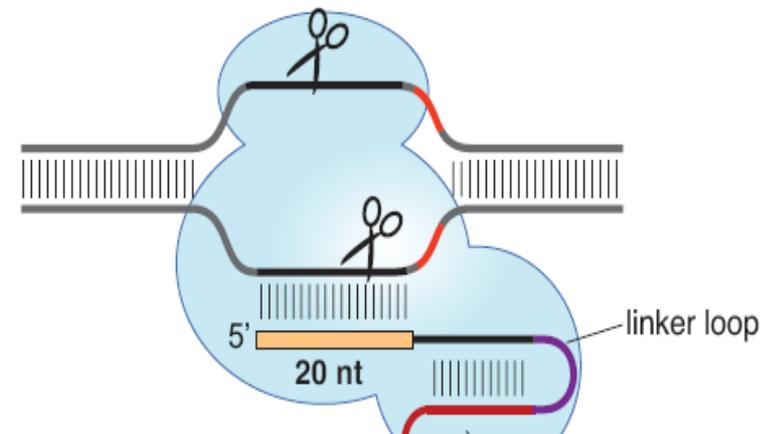


# Nuclease Activity is Programmed by Specific short guide RNAs (sgRNAs)

Cas9 programmed with tracrRNA:crRNA duplex



Cas9 programmed with single chimeric RNAs



## PAM

- “NGG” for Cas9 from *Strep. pyogenes*
- Different PAM sequences for Cas9 from different species.
- Essential for activity.

# Nuclease Activity is Programmed by Specific short guide RNAs (sgRNAs)

Cas9 programmed with tracrRNA:crRNA duplex

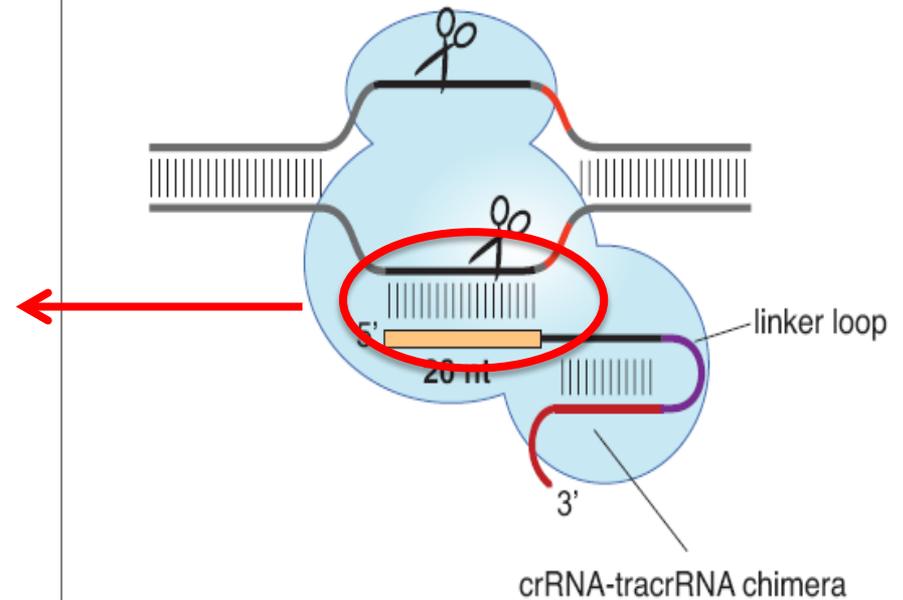
protospacer

## Guide Sequence

- Usually 20 bp long
- Tolerates up to 3-4 mismatches
- Can sometimes be shortened to 17 bp with improved specificity

3' tracrRNA

Cas9 programmed with single chimeric RNAs



# Nuclease Activity is Programmed by Specific short guide RNAs (sgRNAs)

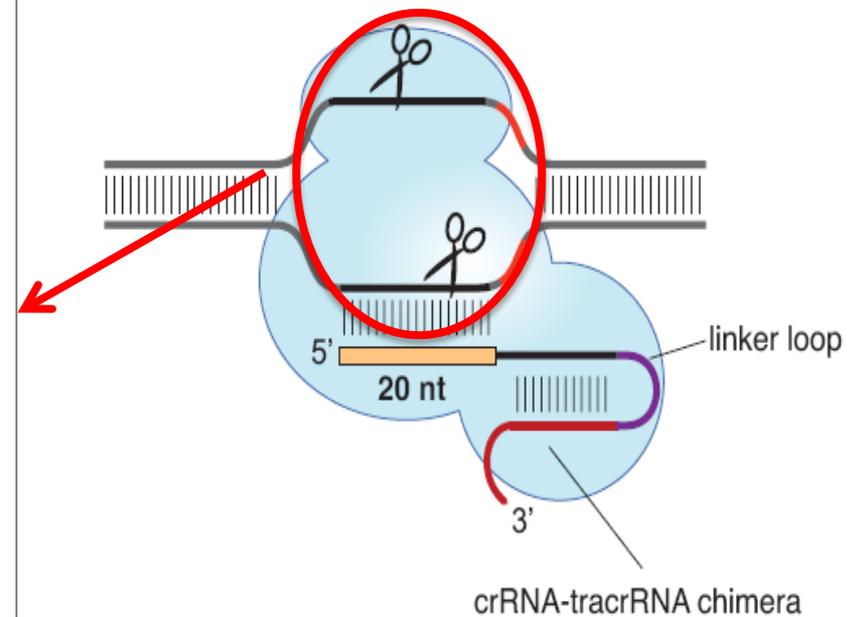
Cas9 programmed with tracrRNA:crRNA duplex



## Nuclease Activity

- Intrinsic to Cas9 protein
- Leaves a blunt end (in contrast to other nuclease platforms)
- Cas9 can be engineered to be a “nickase”

Cas9 programmed with single chimeric RNAs



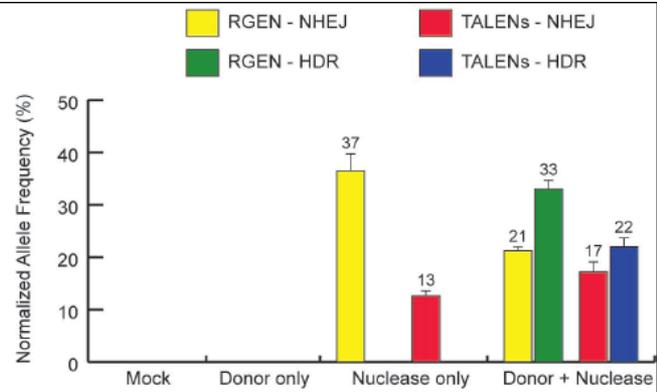
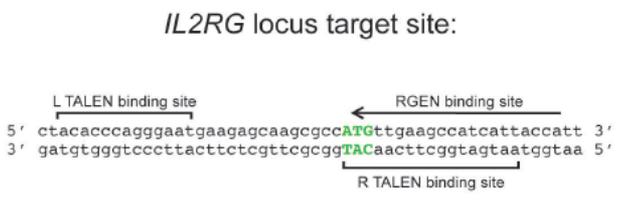


# Quantification of Genome Editing Outcomes at Endogenous Loci in Cell Lines using SMRT Sequencing and Different Nuclease Platforms (Hendel et al 2014)

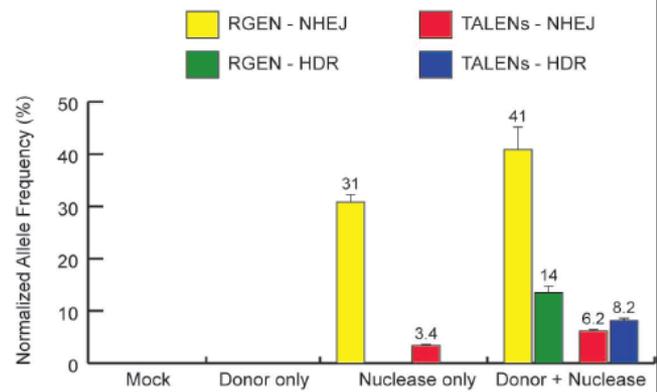
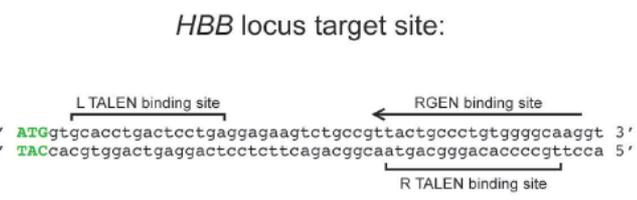
1. They all work.
2. There may be subtle differences.



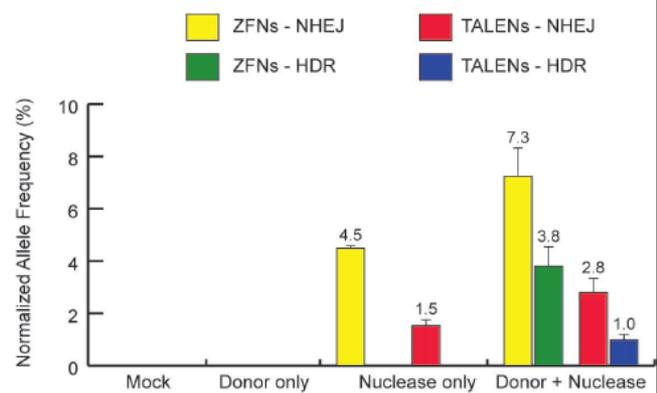
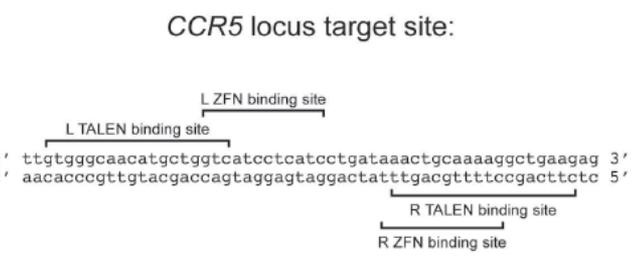
A



B



C



# Summary of Different Platforms

	<b>Meganucleases</b>	<b>Zinc Finger Nucleases</b>	<b>TAL Effector Nucleases</b>	<b>RNA-Guided Endonucleases (CRISPR/Cas9)</b>
<b>Type of Break</b>	Clean 3' Overhang	Clean 5' Overhang	Ragged 5' Overhang	Clean Blunt
<b>Specificity</b>	+++++/?	+++	++++	+++/++++
<b>Ease of Engineering</b>	Difficult	Hard	Easy	Simple
<b>Vectorization</b>	Best	Easy	Difficult	Mod Difficult
<b>Immunogenicity</b>	Yes	Yes	Yes	Yes

# **All Four Platforms Have Been Used for a Variety of Genome Editing Purposes**

- 1. Creation of small insertions/deletions at site of break**
  - 2. Deletion of small and large genetic elements**
  - 3. Single basepair changes by homologous recombination**
  - 4. Targeted transgene addition to specific genomic locations**
- Etc...**

# Thank You

## Porteus Lab

Jen Barker MD, PhD

Erin Breese MD PhD

Joe Clark

Mara Damian

Ayal Hendel PhD

Jennifer Johnston PhD

Eric Kildebeck

Shaina Porter PhD

Kenric Tam

Richard Voit MD, PhD

Will Wagstaff

Gabriel Washington

## Nanomedicine

### Development Center

Gang Bao, Bill Dynan, David Roth

David Spector, Wilbur Lam, Mark

Prausnitz

Leslie Kean, Amy Wagers

**Funding: NIH/NHLBI/NIAID,  
NIH/NDC, Amon Carter  
Foundation, amfAR, Laurie  
Kraus Lacob Faculty Scholar  
Fund**

## RAC Workshop

**Dr. Kathy High**

**Dr. Jacqueline Corrigan-Curay**

**Dr. Paula Cannon**

**Dr. Don Kohn**

**Dr. Saraswati Chatterjee**

**Dr. Marina O'Reilly**