Gene Editing of Livestock

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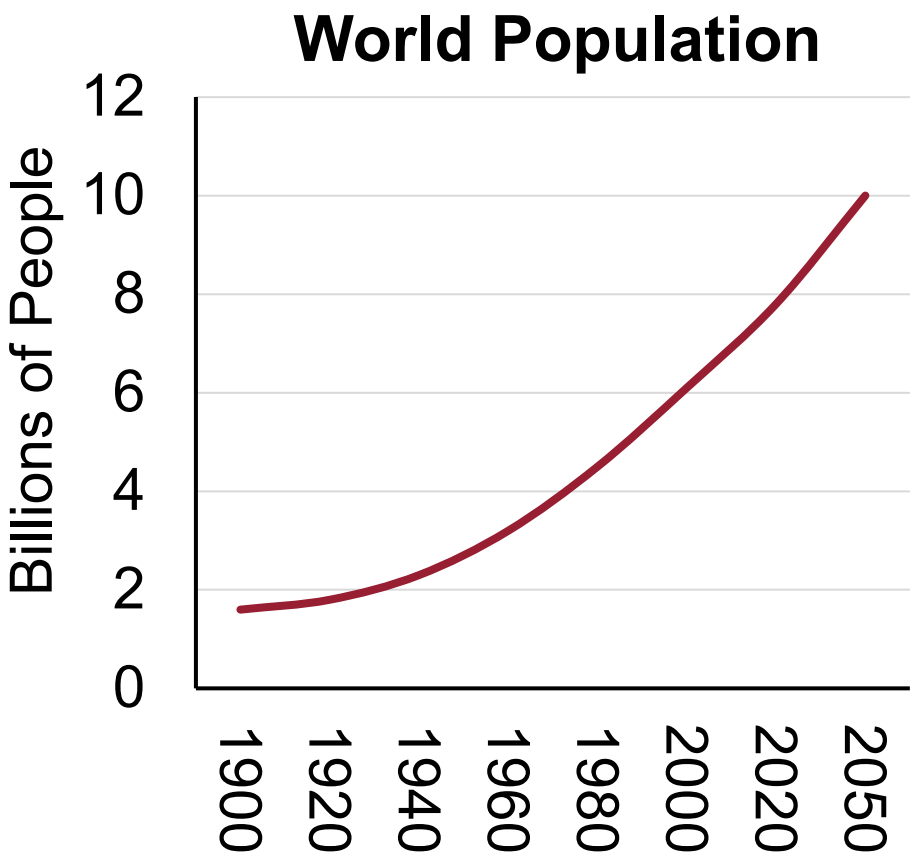
Presentation Primer

1. Why humans work to modify the genome of food animals
2. Strategies to edit genes in livestock cells
3. Strategies to generate gene edited livestock
4. Conscientious considerations

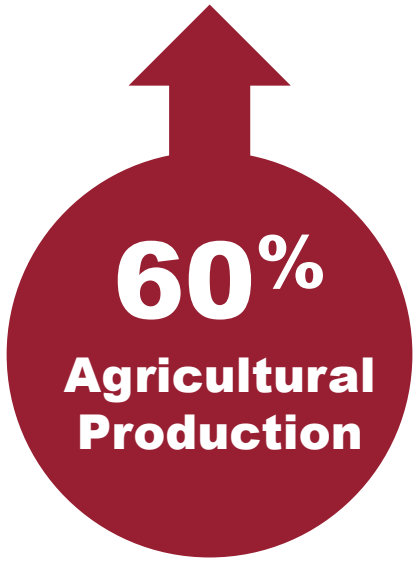
Animal Genetic Engineering

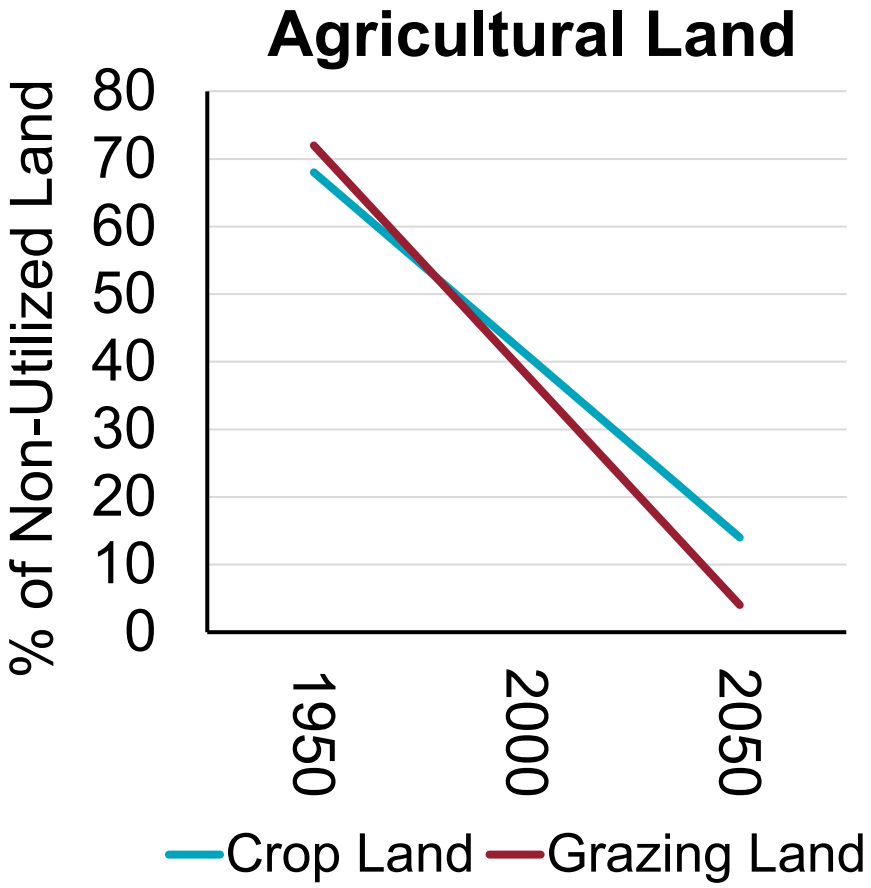
Genome Shaped via Human Intervention

Food (In)Security



Source: US Census Bureau

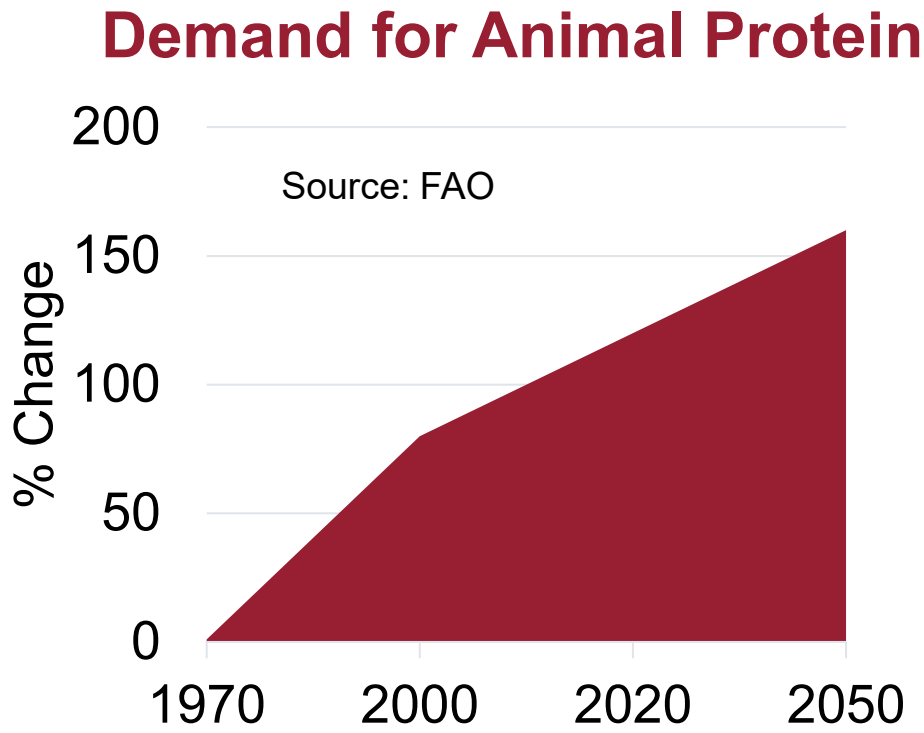




Source: Food and Agricultural Organization of the United Nations

Global Problem

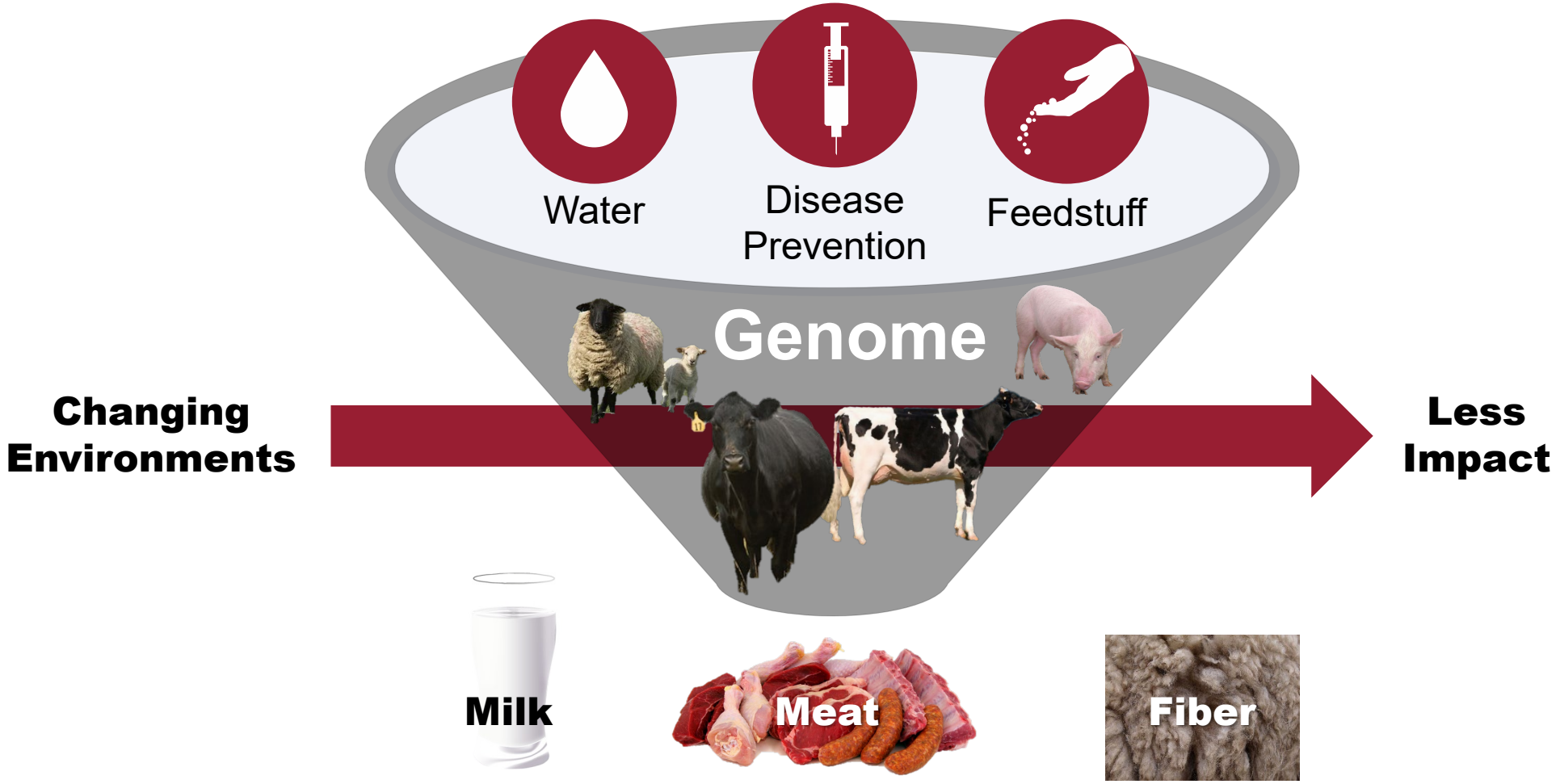
↓ Agricultural Land ↑ Demand for Food



Solution

Enhance livestock production by engineering resiliency & efficiency

Livestock Production Efficiency & Resiliency

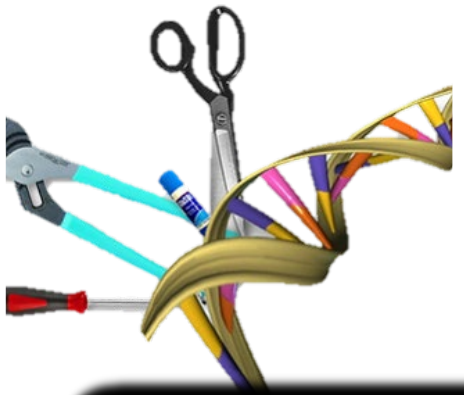


Selective Breeding Shapes the Genome

+ Genetic Gain, but...

- Based Primarily on Observable Phenotype
- 5+ Generations & 10+ Years for Incremental Changes
- Genetic Drift & Inadvertent Negative Phenotypes

Promise of Gene Editing



Molecular Tools

+

DNA Information

Efficient & Precise Genetic Engineering

1-2 Generations

Gene Editing Strategies

CRISPRs

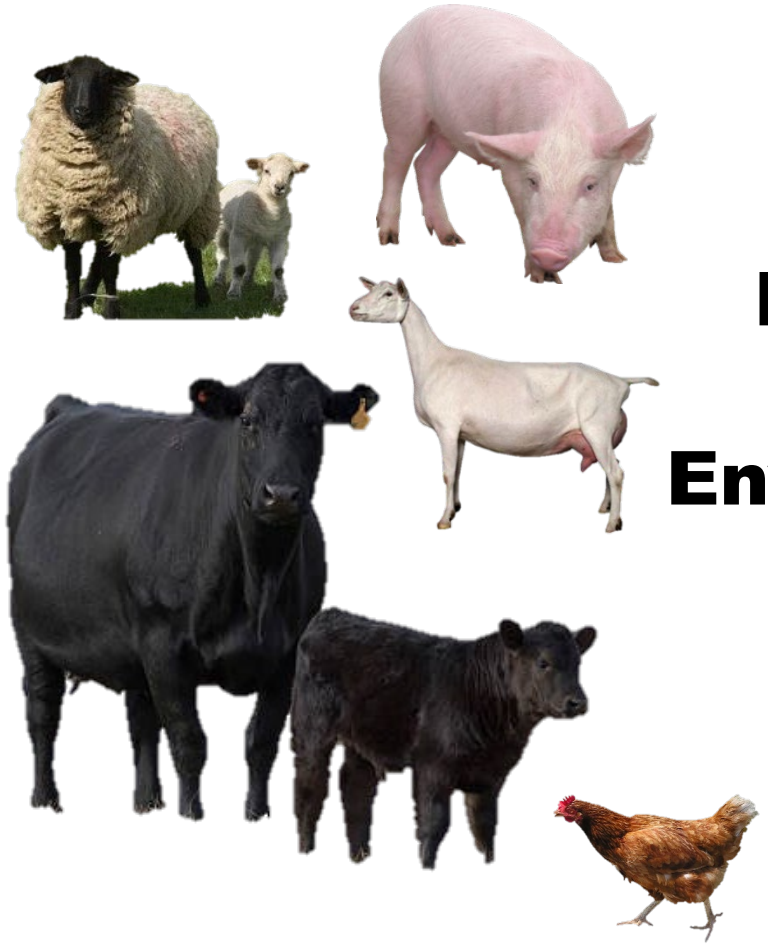
ZFNs

TALENs

Absence of Foreign DNA Sequence

Genomic Changes that Could Arise in Nature

The Leading Edge of Gene Editing for Livestock Resilience



Welfare

Disease Resistance

Environmental Adaption

Growth

Reproduction

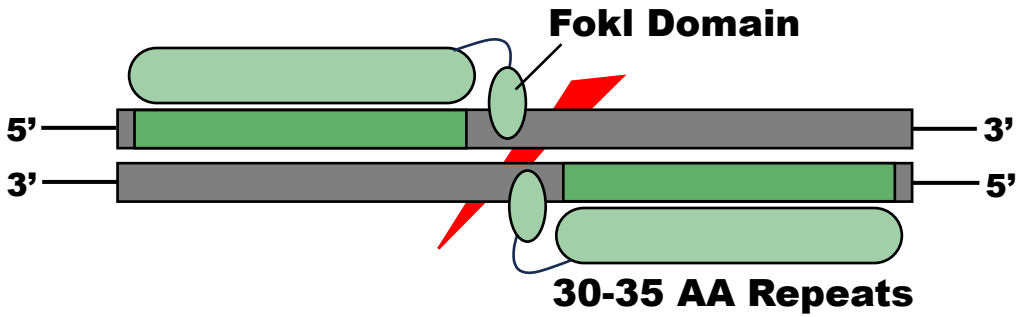
Future of Food Security

Strategies to Edit Livestock Genomes

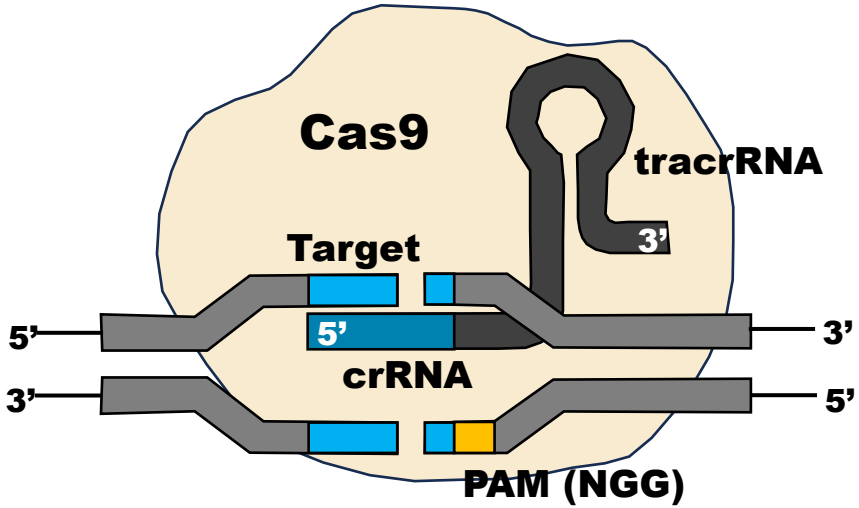
Conventional & Next Generation

Conventional Strategies are the Primary Use with Food Animals

TALEN



CRISPR-Cas9



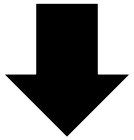
+ Repair Template (ssDNA, long dsDNA, plasmid)

Site-Specific DNA Double Strand Break (DSB)

- **Non-Homologous End Joining (NHEJ) – Random Insertion-Deletion Mutations (INDELs)**
- **Homology Directed Repair (HDR) – Sequence-Specific Insertions or Replacements**

Cas Variants

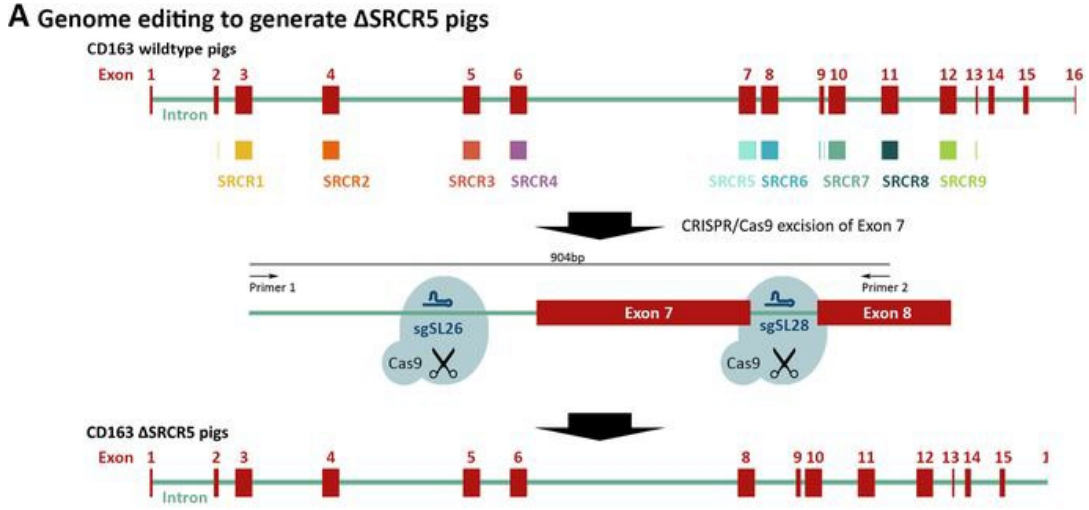
	Cas9	Cas9 Nickase (D10A/H840A)	Cas12
Cas Family	Type II	Type II	Type V
PAM sequence	G-rich	G-Rich	T-rich
Cut type	Blunt double-strand, 3 bp upstream of PAM	Blunt single strand, 3 bp upstream of PAM	Staggered, 18–23 bp downstream of PAM
RNAs needed	crRNA + tracrRNA	crRNA + tracrRNA	crRNA
Major Application	Mammalian gene editing	Mammalian gene editing	Diagnostics



Generate gDNA breaks for INDEL or HDR

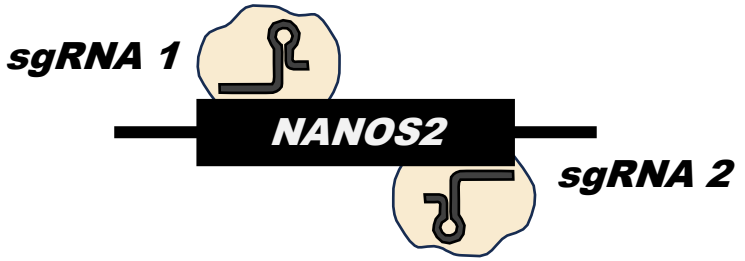
Livestock w/ Engineered INDEL Alleles

PRRS Resistant Pigs – Editing CD163
University of Missouri & Roslin Institute

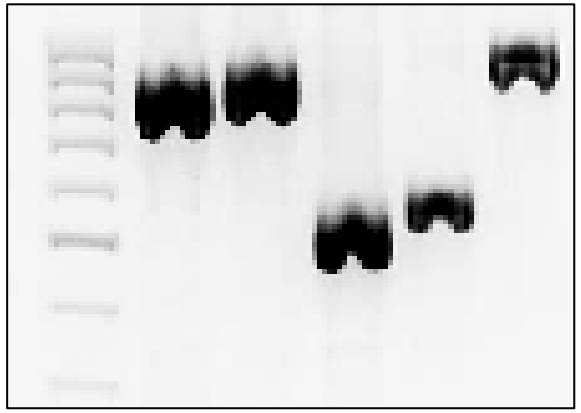


Burkard et al., 2018, J Virol

Surrogate Sires – Editing *NANOS2*
Washington State University



Knockout via Large Exon Deletion



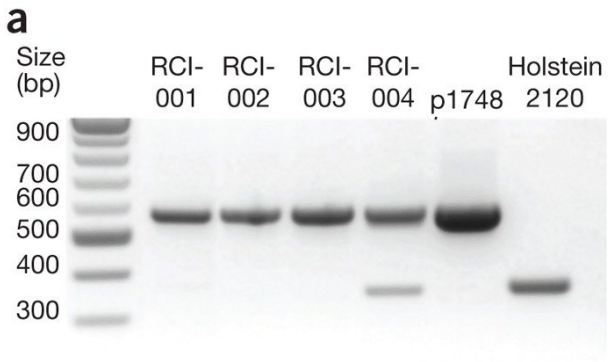
160 bp Δ / 510 bp Δ

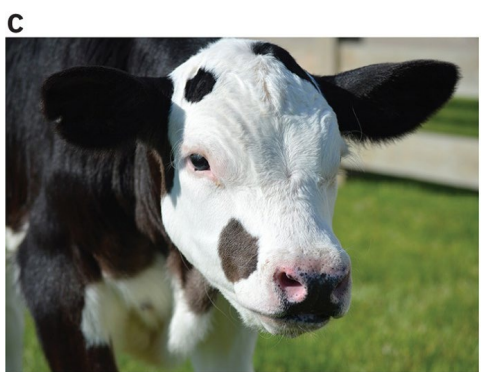
Miao et al., 2019, Biol Repro; Ciccarelli et al., 2020, PNAS

Livestock w/ Engineered HDR Alleles

Hornless Dairy Cattle – Polled Allele
Recombinetics

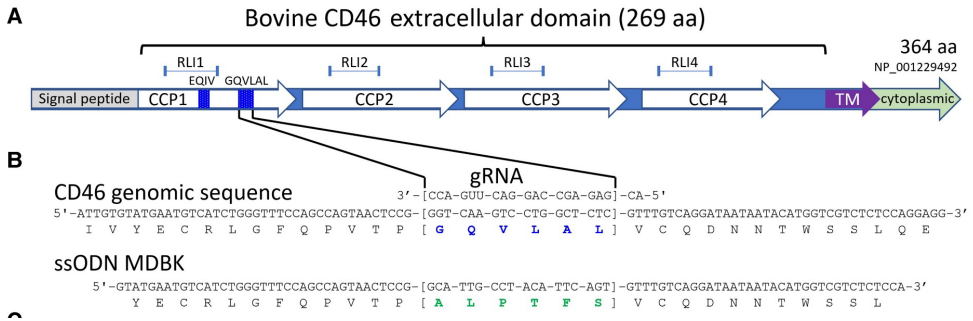
212 bp Introgression to Replicate Celtic Polled Allele



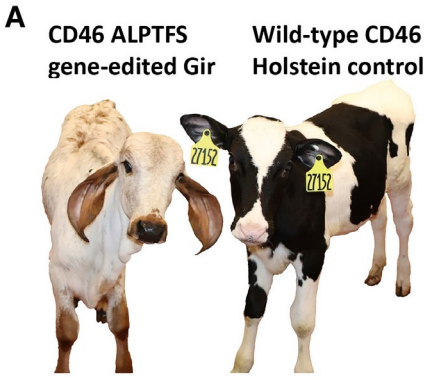


Carlson et al., 2016, Nat Biotech

BVD Resistant Cattle – Editing *CD46*
USDA-MARC & Recombinetics



6AA Substitution – Viral Binding Domain



Workman et al., 2023, PNAS Nexus

Array of Editing Outcomes

Peer-Reviewed Scientific Literature + Washington State University (Unpublished Data)

Mice, Cattle, Pigs, Goats, and Sheep

Allelic Inactivation/Alteration via INDELs

- ❖Range of modifications (1 bp to >1 kb)
- ❖Insertions from other chromosomes
- ❖Retrotransposon insertions (e.g. LINE1)
- ❖Mono-allelic or bi-allelic editing
- ❖Alleles with same edit or each allele edited differently

Site-Specific Insertion/Replacement via HDR

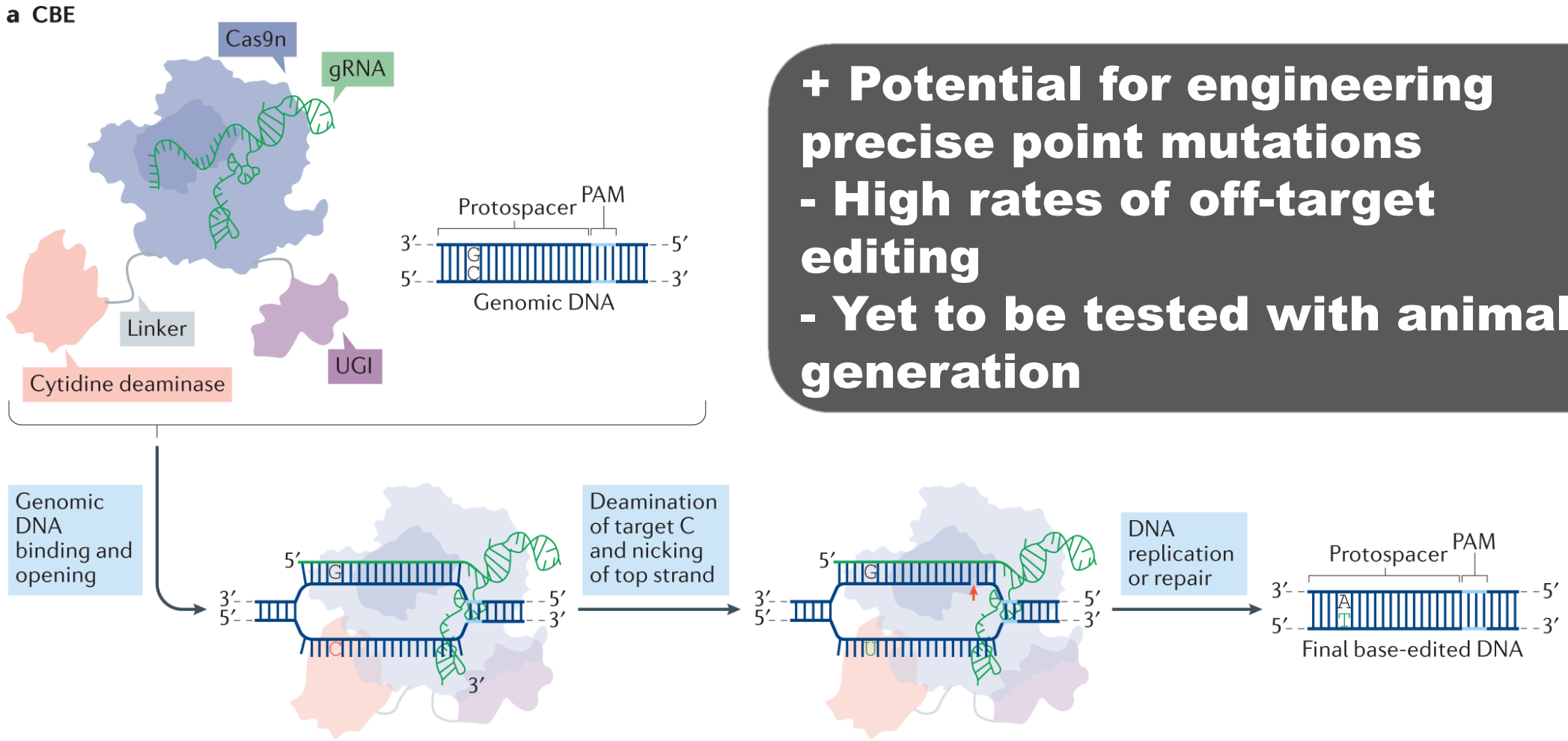
- ❖Designed edit with no unexpected modification
- ❖Designed edit with unexpected INDEL
- ❖Rearranged insert
- ❖Concatemer integration

What transmits through the Germline to the Next Generation is Key

Next Generation Editing

Expand the Toolbox for Specificity & Precision

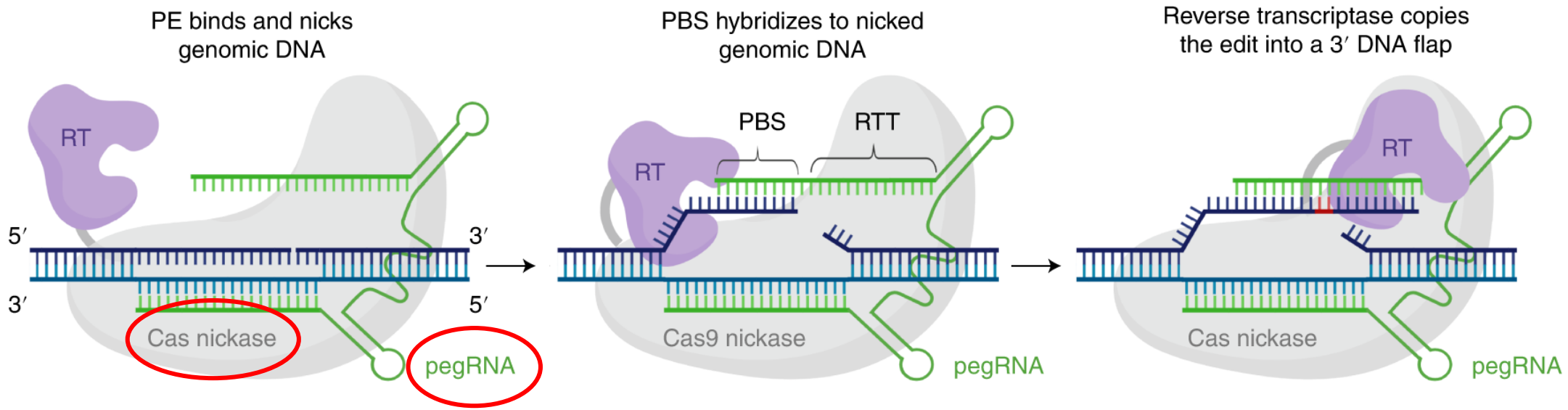
Base Editing



+ Potential for engineering precise point mutations
- High rates of off-target editing
- Yet to be tested with animal generation

Porto et al., 2020, Nat Rev Drug Discov

Prime Editing



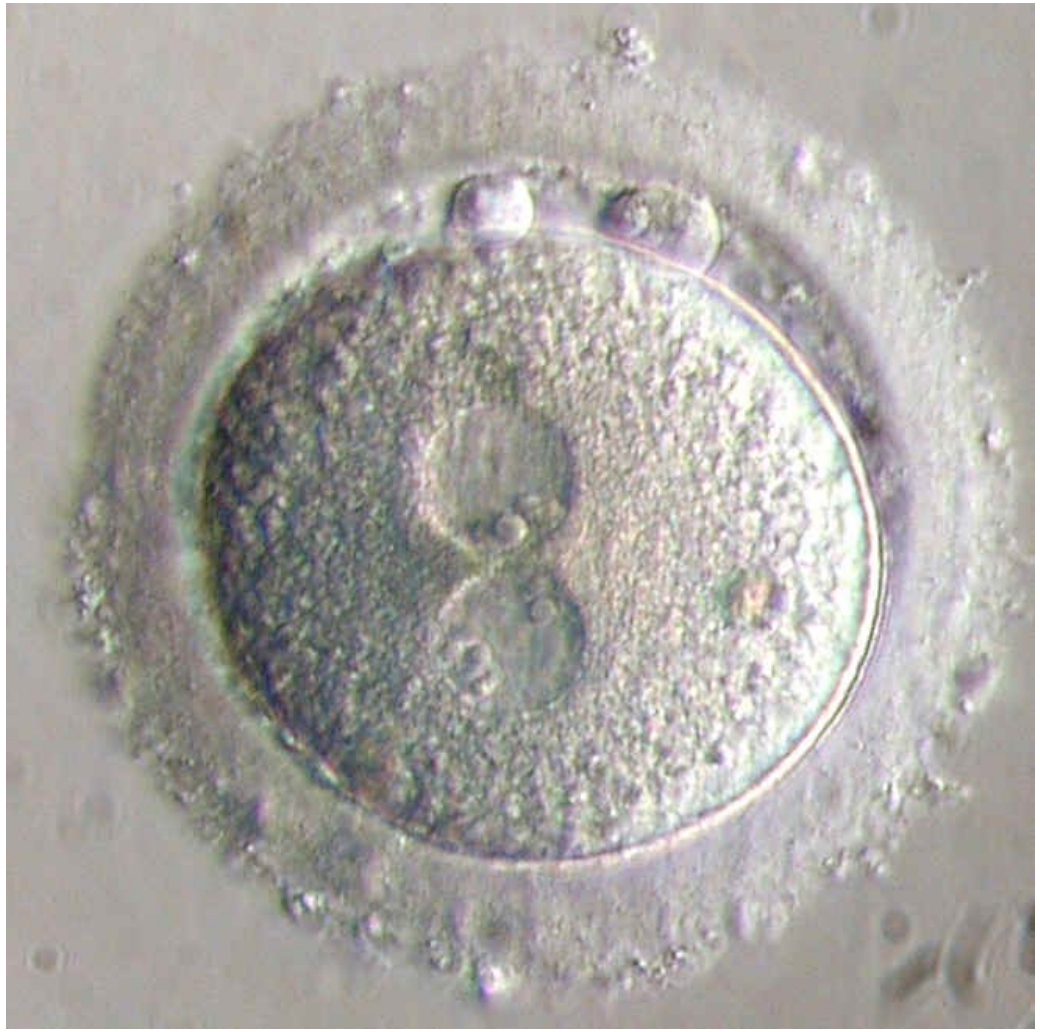
- ❖ **Low efficiency in mammalian embryos**
- ❖ **Limited reports of success in livestock**
  - **Zhou et al., 2023, BMC Genomics: ~1% efficiency with sheep embryos**
- ❖ **Recent report of increased efficiency in mice**
  - **Kim-Yip et al., 2024, Nat Biotech: ~60% efficiency with mouse embryos**

Strategies for Generating Food Animals Possessing DNA Edits

Goal

Germline Transmission = Heritable

Current: Edited Embryos

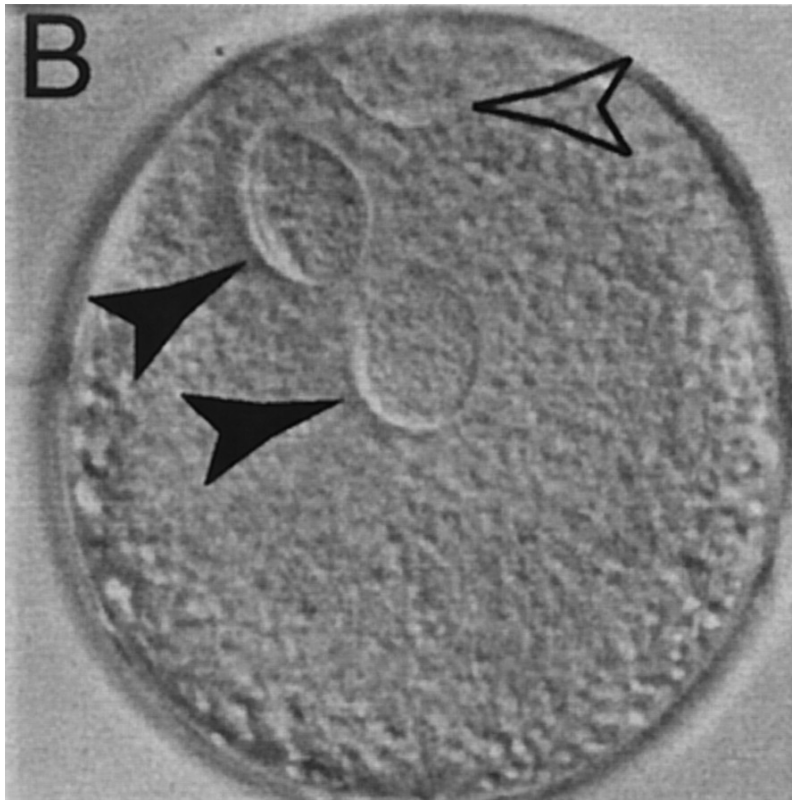


➡ Pregnancy ➡ Offspring (Founder)

Zygote Manipulation

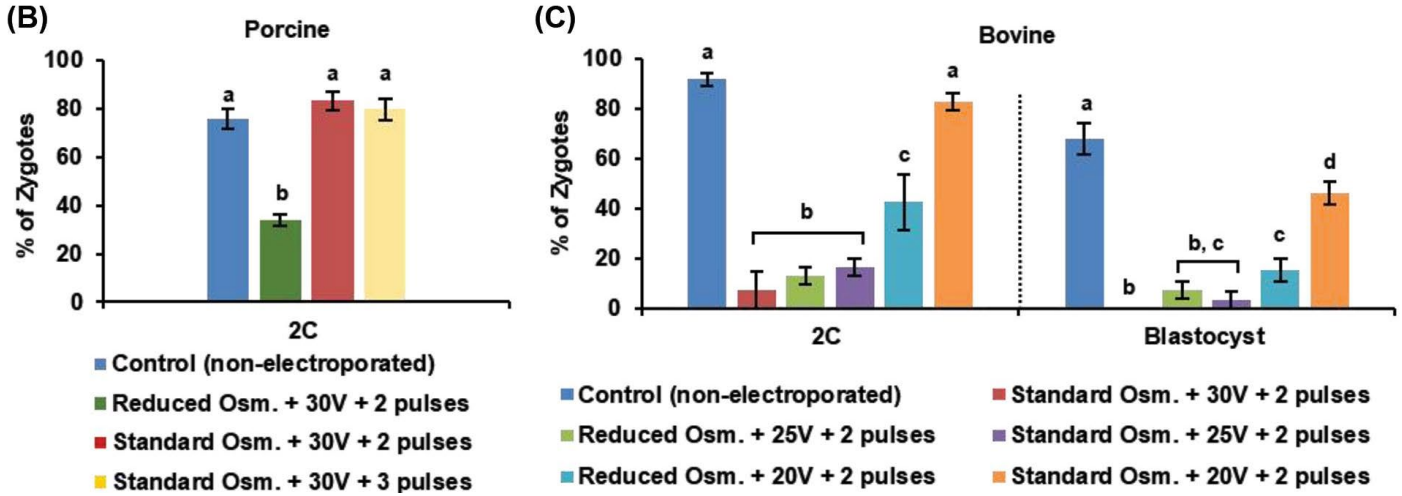
Introduction of Editing Components

Bovine Zygote



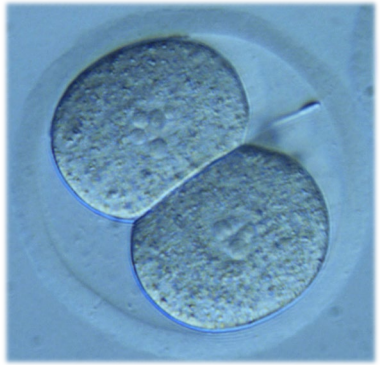
Chan et al., 1998, PNAS

- **Viral Vectors**
  - ❖**Limited utility for livestock**
- **Microinjection**
  - ❖**Cytoplasmic deposit but technical skill needed**
- **Electroporation**
  - ❖**Simple but not standard across species**



Miao et al., 2019, Biol Reprod

2C Microinjection or Electroporation
Introduction of Editing Components



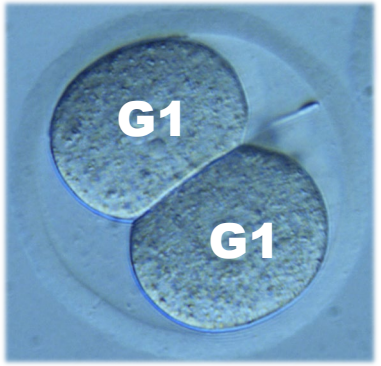
Higher Rate of HDR
vs
Zygote Manipulation

Knock-in	Repair Method	Embryos Transferred	Offspring	+ Founders
Sox2-mCherry	Plasmid	150	32	3 (9%)
Nanog-mCherry	ssDNA	75	25	2 (8%)
Gata6-Halo	ssDNA	60	3	3 (100%)
R26-CAG-H2B-miRFP703	Plasmid	75	22	2 (9%)
Arrdc5-3X FLAG	ssDNA	50	8	4 (50%)
Arrdc5-eGfp	ssDNA	50	12	2 (17%)

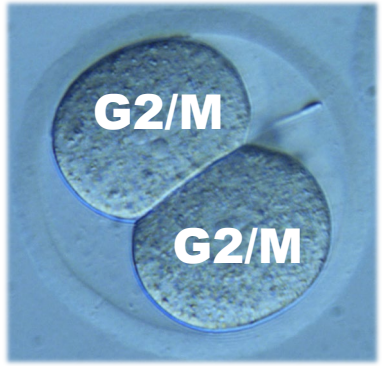
Gu et al., 2018, Nat Biotech
Giassetti et al., 2023, Nat Comm

<u>Caveat/Limitation</u>
Increased Mosaicism

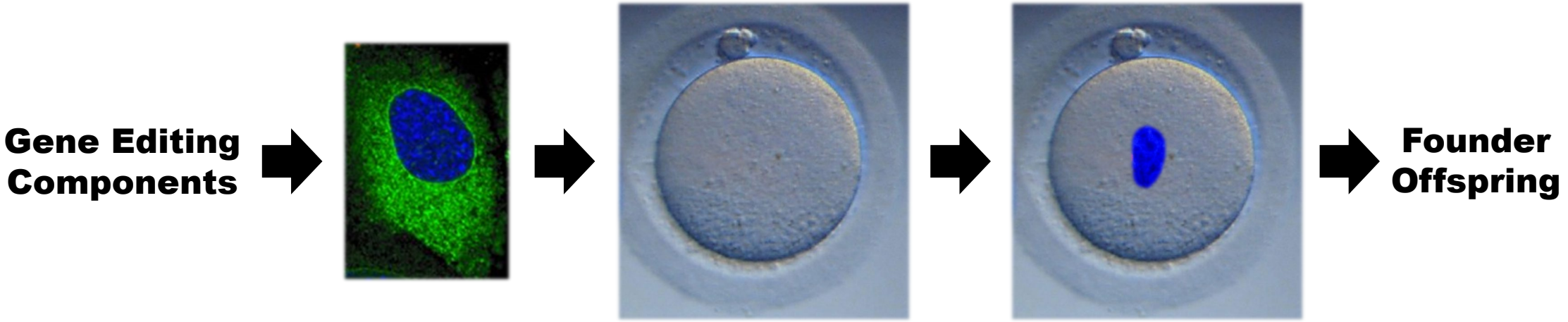
2N = 4 Copies

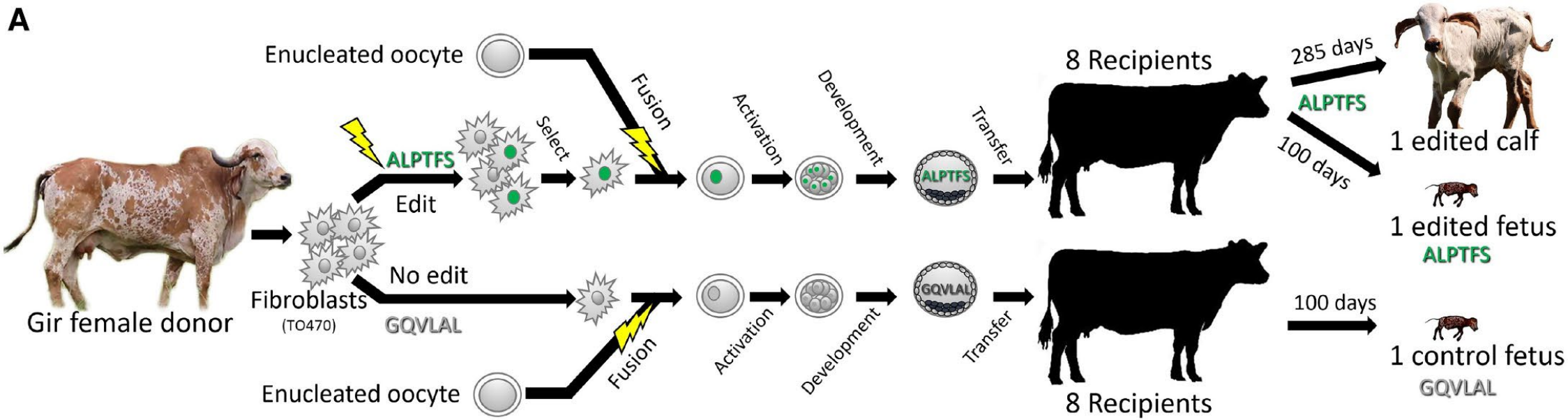


4N = 8 Copies



Editing Somatic Cell DNA + Cloning





Workman et al., 2023, PNAS Nexus

Regardless of strategy…
intended output is founders
&
Key is Edited Germline

Conscientious Considerations

Form of Gene Editing Components Introduced to Embryos & Cells

Editors

- Plasmid Expression
  - Risk of foreign DNA integration
- Synthesized RNA
  - sgRNA
  - Cas9 mRNA
- Ribonucleotide Protein (RNP)
  - sgRNA + Cas9 Protein
  - Standard for many applications

Repair Template

- Plasmid Based
  - Risk of foreign DNA integration
- Synthesized DNA
  - ssDNA (ssODN) – size limits
  - Long dsDNA
- Adeno Associated Virus (AAV)
  - Efficient for HDR but…
  - Potential for foreign sequence integration (Luqman et al., bioRxiv)

Mosaicism

- Genotyping detection of multiple different alleles in Founders
- Soma vs Germline
- If germline mosaic, what will transmit to offspring?
- Only one edited allele will be inherited, but…which one?

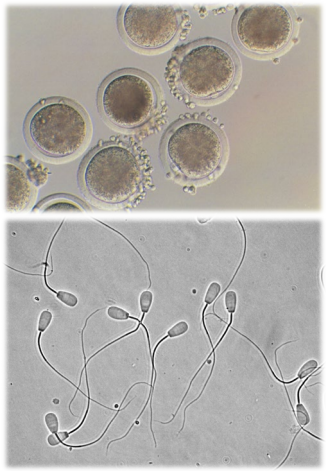
CRISPR-Cas9 Editing *NANOS2* in Pigs

c

Boar #	Ear DNA Sequencing	Sperm DNA Sequencing	Genotype
136	5bpΔ/3bpΔ/3bp (insertion)	5bpΔ/3bpΔ/3bp (insertion)	Mosaic
137	1bp (insertion)/1bpΔ	1bpΔ/3bpΔ	Mosaic
142	3bpΔ/5bpΔ	3bpΔ/200bpΔ	Mosaic
143	3bpΔ/10bpΔ	3bpΔ/10bpΔ/5bpΔ	Mosaic
144	1bp (insertion)/4bpΔ	N/A	Knockout
146	4bpΔ/150bpΔ	N/A	Knockout
147	3bpΔ/5bpΔ	N/A	Heterozygous
148	1bpΔ/3bpΔ	1bpΔ/3bpΔ	Heterozygous
251	1bpΔ/21bpΔ	1bpΔ/3bpΔ	Mosaic

Park et al., 2017, Sci Reports

Germline Transmission is Key!



- Not all founders with edits genotyped in soma transmit through the germline
- Mosaicism in germline also occurs
- Litter bearing vs singleton consideration
- Future is direct germline editing

Knock-in	Method	Embryos Transferred	Offspring	+ Founders	Germline Transmission
Sox2-mCherry	Plasmid	150	32	3 (9%)	8%
Nanog-mCherry	ssDNA	75	25	2 (8%)	47%
Gata6-Halo	ssDNA	60	3	3 (100%)	46%
R26-CAG-H2B-miRFP703	Plasmid	75	22	2 (9%)	41%
Arrdc5-3X FLAG	ssDNA	50	8	4 (50%)	62%
Arrdc5-eGfp	ssDNA	50	12	2 (17%)	100%

On-Target vs Off-Target Edits

- **Stability of on-target edits**
  - **If it does change, how to account for natural evolution of DNA?**
- **Potential for off-target editing to reduce animal welfare and compromise food safety**
  - ❖**Do we need to assess?**
  - ❖**What will the standard practice be?**
  - ❖**Can we really distinguish off-target from random mutations?**
  - ❖**Is this an impossible box to check?**
  - ❖**Should phenotype be the guide?**
- **Enhanced strategies may mitigate concern**
  - **Cas9 Variants like Cas9-HF1 are reported to reduce non-specific contacts, yet to be tested with livestock**

Takeaways

- The future of global food security needs genome engineering of livestock
  - ✓Gene editing offers the means to engineer resiliency and efficiency

- Primary strategies for livestock are conventional TALEN and CRISPR-Cas9 to generate embryos with INDEL and HDR gene edits
  - Expected and unexpected DNA modifications can occur
  - ✓Strategy of choice and research design matter

- Science is the Guide and the Solution!

The Future of Food Security

Minimal Input
+
Maximal Output

Resilient &
Resistant

Precision
Engineered
Food Animal
Genomes

Climate Smart

Matched to
Environment

THANK YOU

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