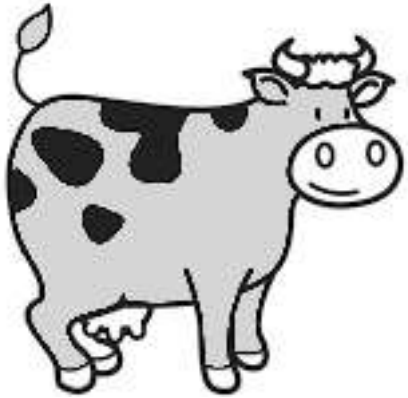
Las herramientas CRISPR: nuevas soluciones para la mejora genética animal

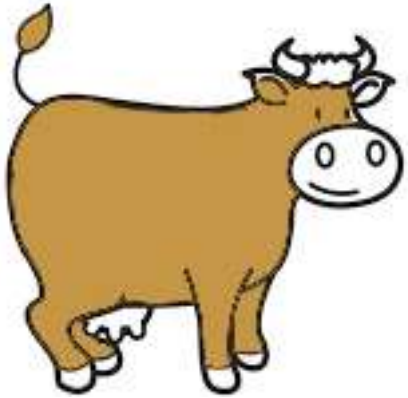
Lluís Montoliu
CNB-CSIC, Madrid, Spain

The paradigm of genetic selection/improvement

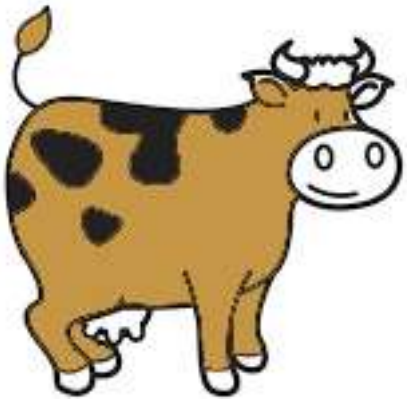


High milk production
Sensitive to disease X

X



Low milk production
Resistance to disease X



High milk production
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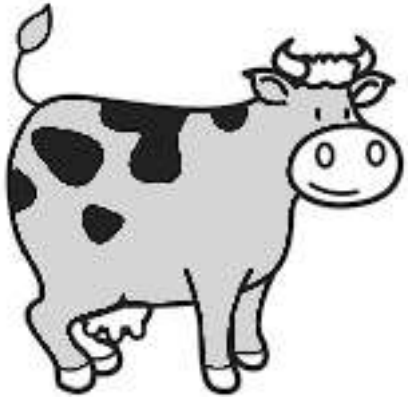
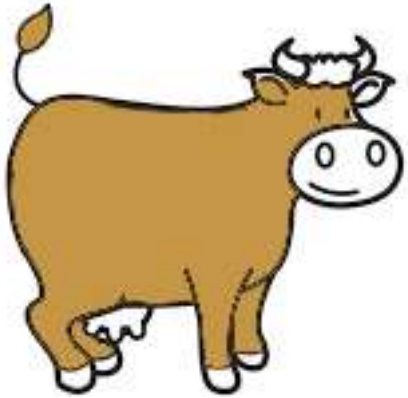
The paradigm of genetic selection/improvement

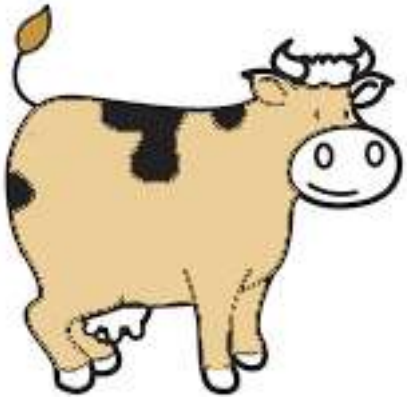


X



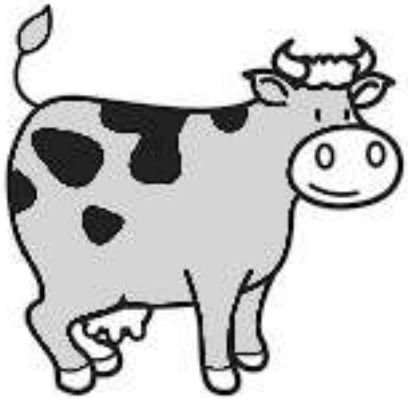
High milk production
Sensitive to disease X

Low milk production
Resistance to disease X

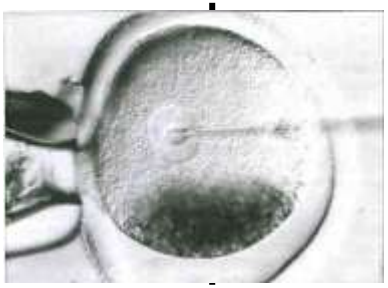


Intermediate milk production
Intermediate susceptibility to disease X

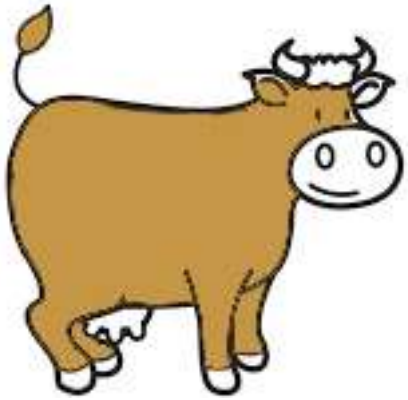
The paradigm of genetic selection/improvement



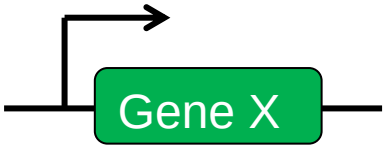
High milk production
Sensitive to disease X

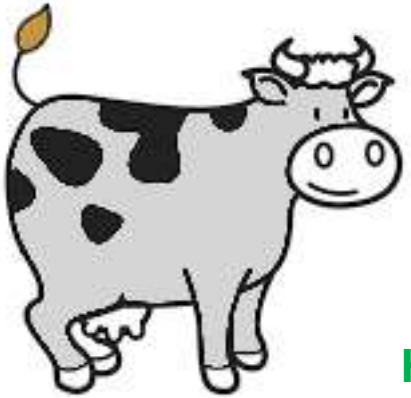


Transgenesis



Low milk production
Resistance to disease X

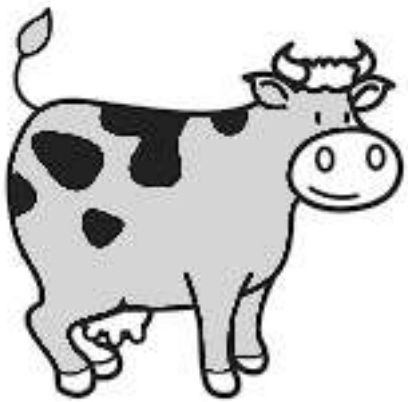


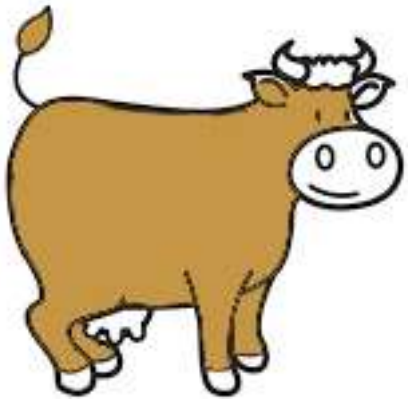


High milk production
~Sensitive to disease X

Low expression of transgene
Chromosomal position effects

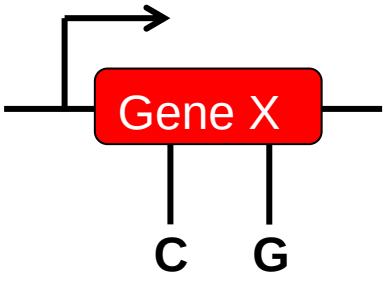
The paradigm of genetic selection/improvement



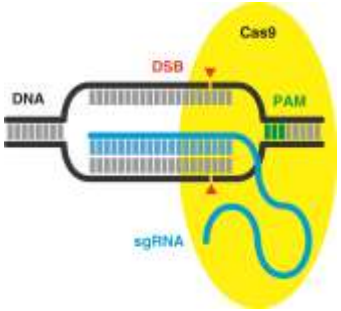


High milk production
Sensitive to disease X

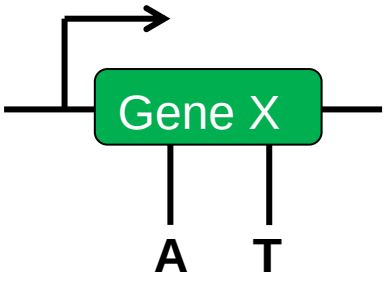
Low milk production
Resistance to disease X



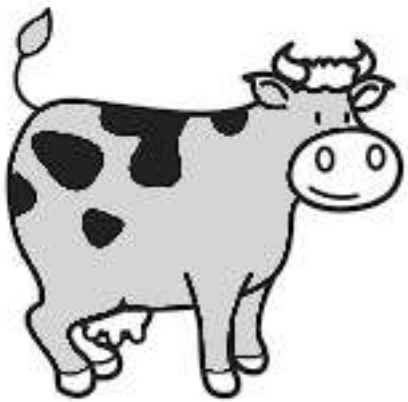
Gene Editing

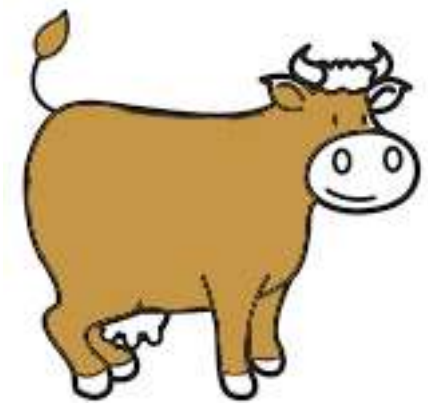


CRISPR-Cas9



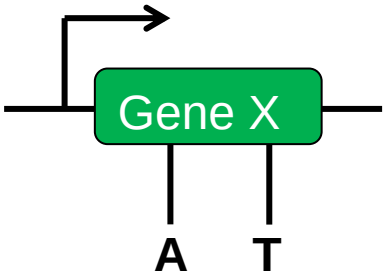
The paradigm of genetic selection/improvement

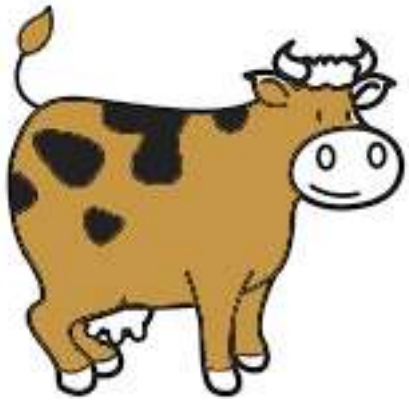


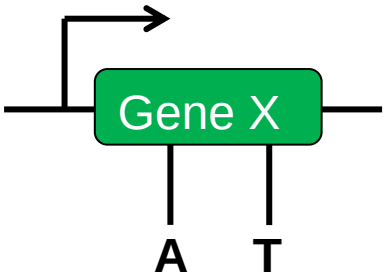


High milk production
Sensitive to disease X

Low milk production
Resistance to disease X







High milk production
Resistance to disease X

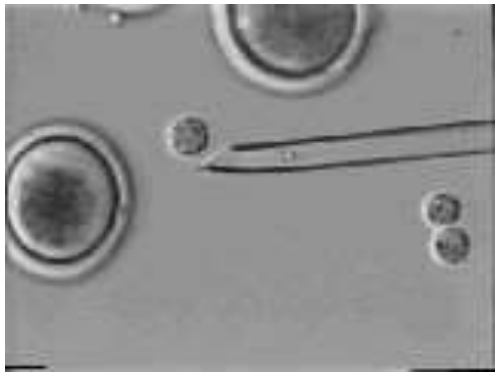
CRISPR

Modifying the Mammalian Genome

ISTT

ISTT

ISTT



Pronuclear microinjection

1980 → …

ES / iPS cells

1986 → …

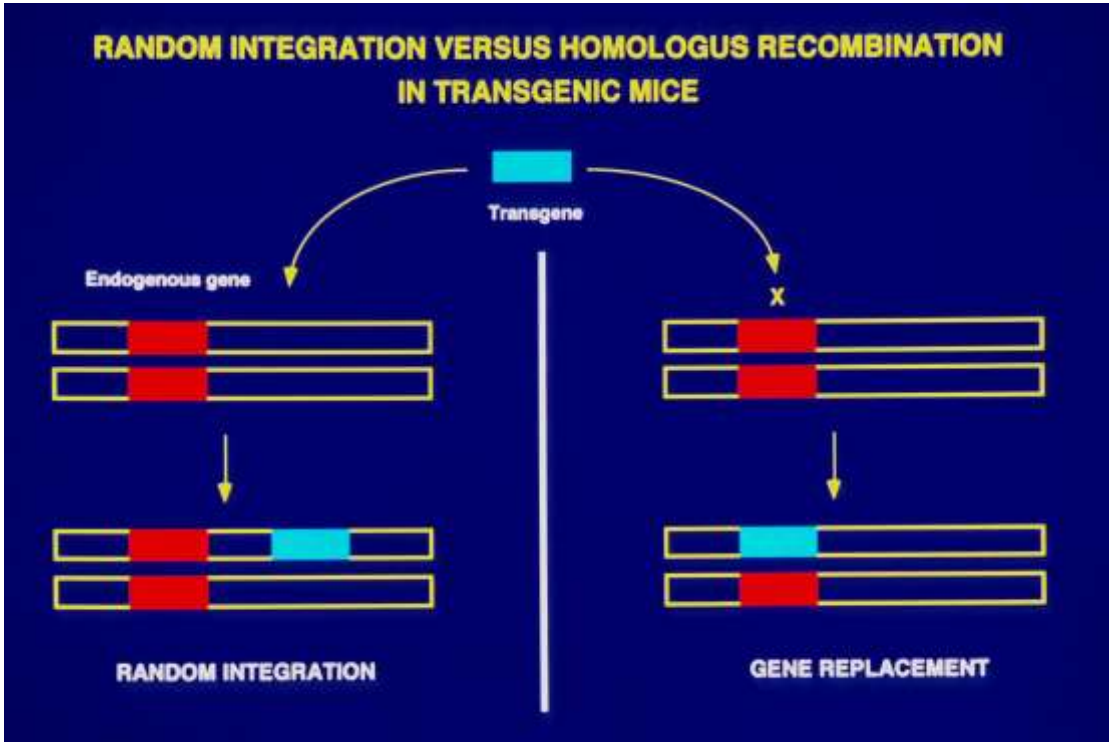
SCNT

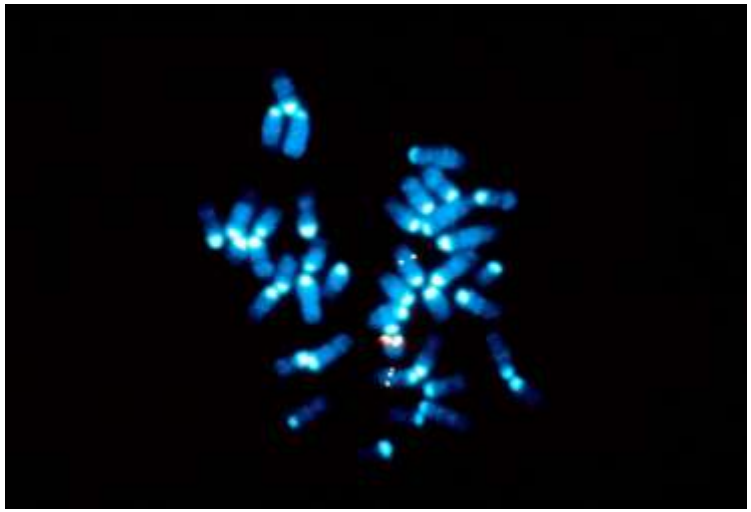
1996 → …

ADDING A GENE

DELETING A GENE

The most relevant and distinctive feature among all gene modifying methods is

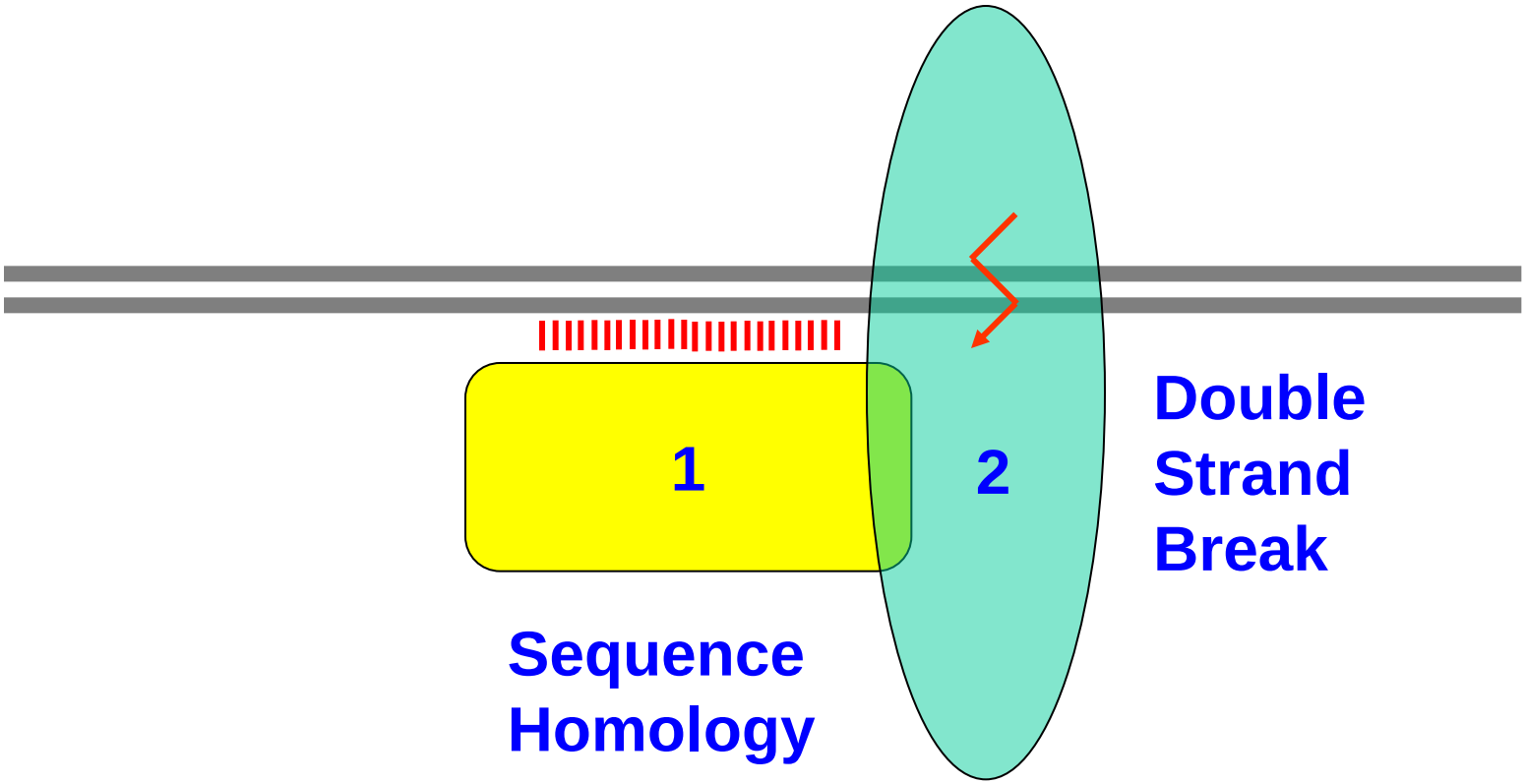




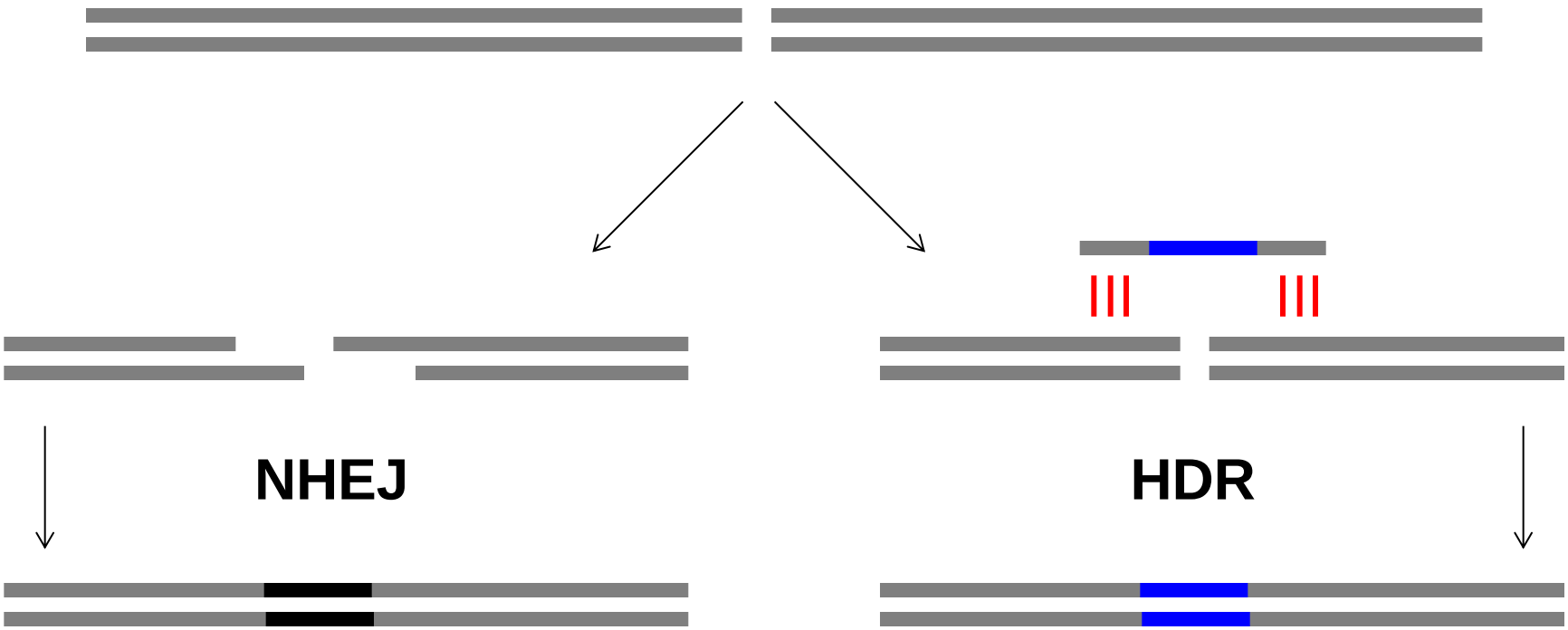
Slides from 1991

Random versus Targeted genetic modification

Genomic Editing Tools: 2 elements to precisely target a gene modification



Genomic Editing Tools: 2 pathways to fix the Double Strand Break (DSB)



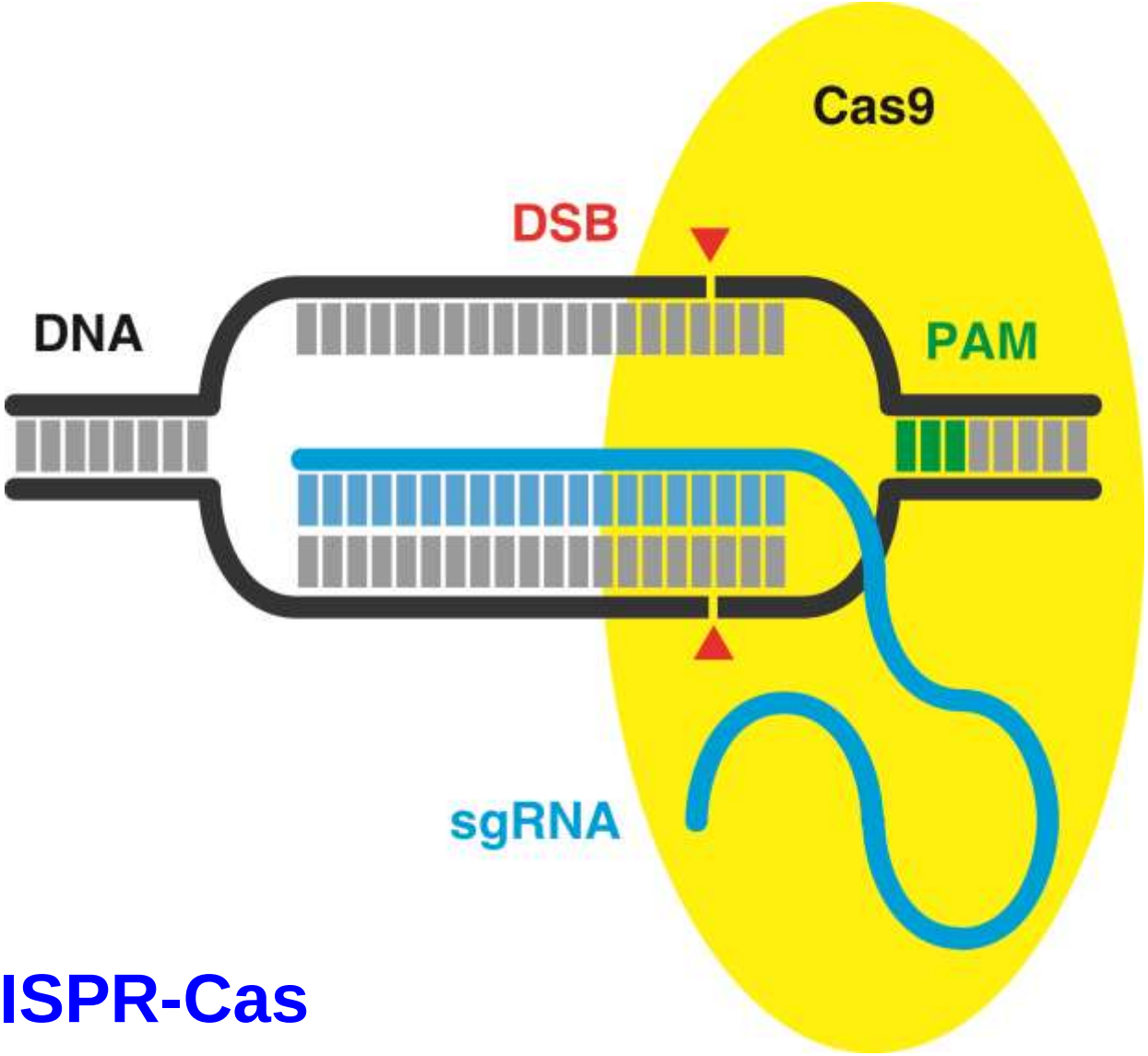
INDELs – Gene disruption

Gene repair / edit

Genomic Editing Tools: 3 flavours

Type	Sequence Homology	Double Strand Break
Zinc-Finger Nuclease (ZFN)	**PROTEIN** 1 Zinc finger (3 AA) → 3 bp	**PROTEIN** FokI
TALEN	**PROTEIN** 2 AA → 1 bp	**PROTEIN** FokI
CRISPR-Cas9	**RNA** 1 ribonucleotide → 1 bp	**PROTEIN** Cas9

The Watson&Crick base pairing champions CRISPRs



The CRISPR-Cas system

1993

Transcription at different salinities of *Haloferax mediterranei* sequences adjacent to partially modified *Pst*I sites

F. J. M. Mojica, G. Juez and F. Rodríguez-Valera*
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Summary

Two genomic sequences from the halophilic archaeon *Haloferax mediterranei*, where we had found *Pst*I restriction-pattern modifications depending on the salinity of the growth medium, have been studied. A markedly salt-dependent differential expression has been detected in the nearby regions. Two of the open reading frames characterized correspond to two of the differentially expressed transcripts. In both cases the *Pst*I sites were included in purine–pyrimidine alternancies suggestive of Z-DNA structures and located in non-coding regions with frequent repetitive motifs. A long alternating adenine–thymine tract also appears in the upstream regions of one of these open reading frames. A possible role of local DNA configuration in osmoregulation in this organism is discussed.

involved in the high-affinity K^+ transport, whose regulation is effected at transcriptional level and is being extensively studied (Csonka, 1989; May *et al.*, 1989; Mizuno and Mizushima, 1990; Sugiura *et al.*, 1992). A role for the topology of DNA and intracellular K^+ concentrations in osmoregulation has been suggested (Sutherland *et al.*, 1986; Higgins *et al.*, 1987; 1988; Graeme-Cook *et al.*, 1989; Ramirez and Villarejo, 1991). In the case of halobacteria there is little evidence of the effect of salinity on gene expression. To our knowledge, the only reference to the subject concerns a markedly different expression of the *mc-vac* gene encoding the major gas vesicles protein of *Haloferax mediterranei* at different salinities (Englert *et al.*, 1990).

We previously described the existence of certain *Pst*I sites in the *H. mediterranei* genome which appeared to be more susceptible to cleavage, or less, depending on the salt concentration at which the cells were grown (Juez *et al.*, 1990). At least 5% of the clones from a genomic library of the organism used as probes revealed restriction-pattern modifications which appeared to be consistently associated with the salinity of the growth medium. To clarify whether this phenomenon could have any biological significance implicated in the adaptation of the

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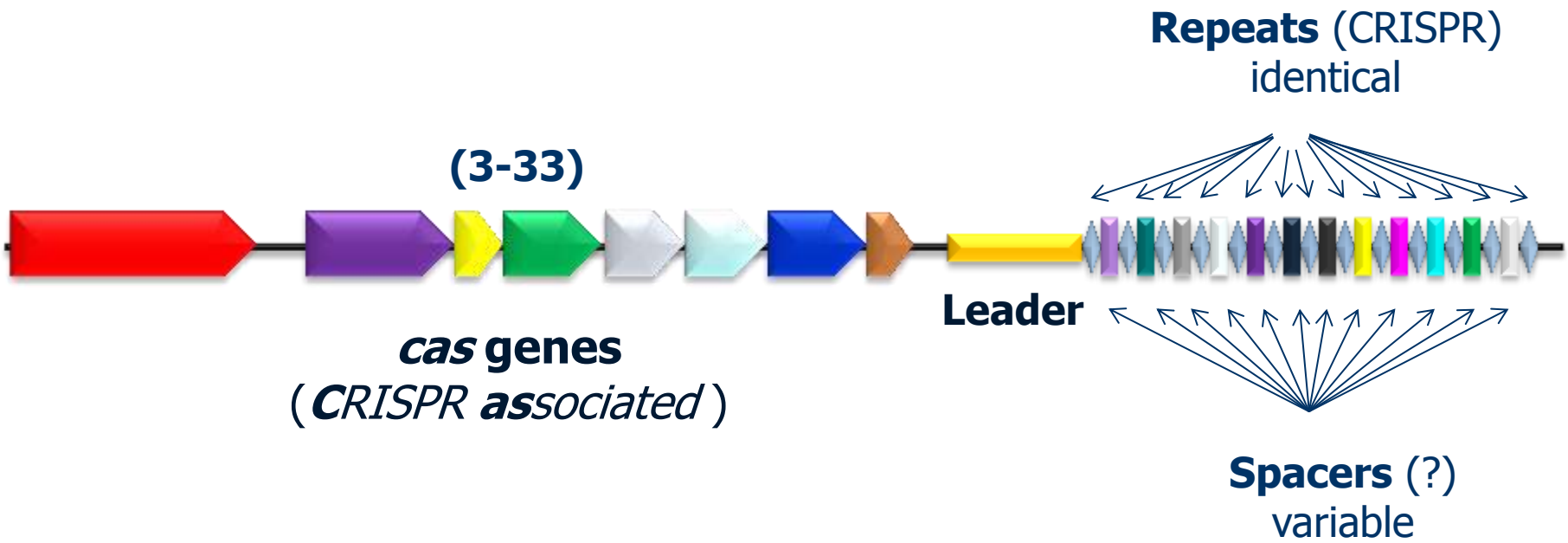
Spain

CRISPR arrays discovered by Mojica et al. (1993) Molecular Microbiology, in *archeas*

The CRISPR-Cas system in prokaryotes



Scheme by Francis Mojica

Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements

Francisco J.M. Mojica, César Díez-Villaseñor, Jesús García-Martínez, Elena Soria

División de Microbiología, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Campus de San Vicente, E-03080, Spain

Table 2. Distribution of CRISPR-spacer homologs

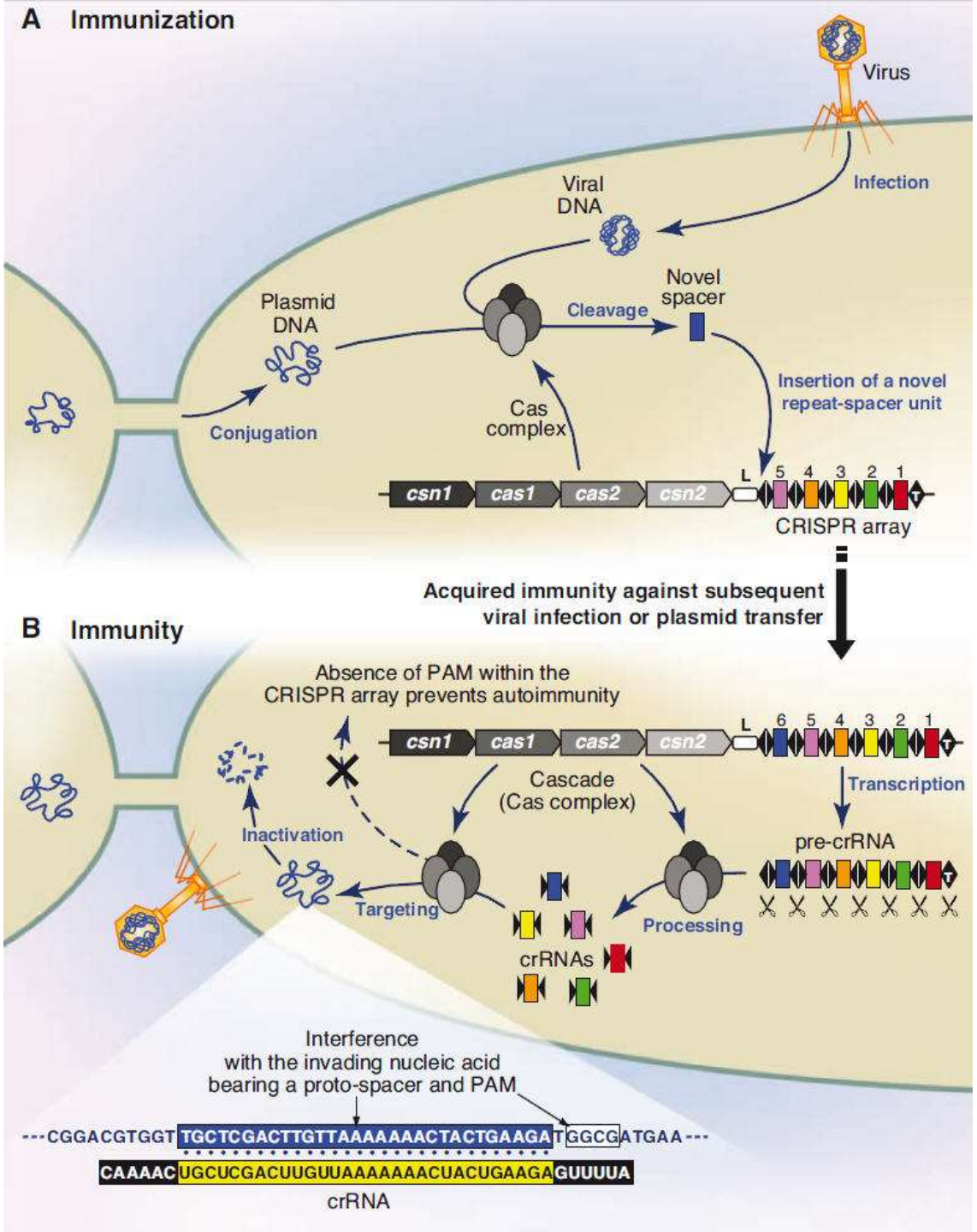
Strain	No. of spacers analyzed	No. of spacers with homologs in		
		Phages[a]	Plasmids	NF[b]
Chlorobium tepidum TLS	62		1	
Clostridium tetani Massachusetts E88	62	1		6
Corynebacterium efficiens YS-314T	22		1	2
Escherichia coli ECOR42	14		1	
Escherichia coli ECOR44	10	1		
Escherichia coli ECOR47	17	1		
Escherichia coli ECOR49	11		1	
Listeria innocua Clip11262	9	3		
Listeria monocytogenes EGD-e	4	1		
Methanothermobacter thermoautotrophicum ΔH	169	9		
Mycoplasma gallisepticum R	71			1
Neisseria meningitidis Z2491 (serogroup A)	16			4
Photorhabdus luminescens laumondii TT01	65	7		3
Porphyromonas gingivalis W83	44			4
Pyrobaculum aerophilum IM2	129			1
Salmonella typhimurium LT2 SGSC1412	57	1		
Shigella sonnei 53G	3			1
Streptococcus agalactiae NEM316	13	1		1
Streptococcus agalactiae 2603V/R	25	1	1	3
Streptococcus pyogenes M1 GAS SF370	9	8		
Sulfolobus solfataricus P2	424	6	3	
Sulfolobus tokodaii 7	471	2	2	
Thermoanaerobacter tengcongensis MB4T	306			5
Yersinia pestis CO-92 (Biovar Orientalis)	16	4		
Yersinia pestis KIM5P12 (Biovar Mediaevalis)	10	1		

[a]Prophages are included.
[b]Number of spacers with homology to chromosomal sequences not directly related to foreign DNA (prophages are excluded).

(Kawarabayasi et al. 2001). Indeed, the preferential occurrence of CRISPR spacers derived from genetic elements that fail to infect the corresponding spacer-carrier strain, but not from those successfully propagated in the population, strongly suggests a relationship between CRISPR and such immunity. Most

2005

CRISPR-Cas system first suggested by Mojica et al. as immunity system (2005) J. Mol. Evol.



CRISPR-Cas System

(adaptive immunological system in archaeas and bacterias)

Clustered
Regularly
Interspaced
Short
Palindromic
Repeats

CRISPR
associated
proteins

Horvath & Barrangou (Science, 2010)

The CRISPR-Cas System: targeting nucleases to specific DNA sequences
a binary system: Cas9 protein and sgRNA

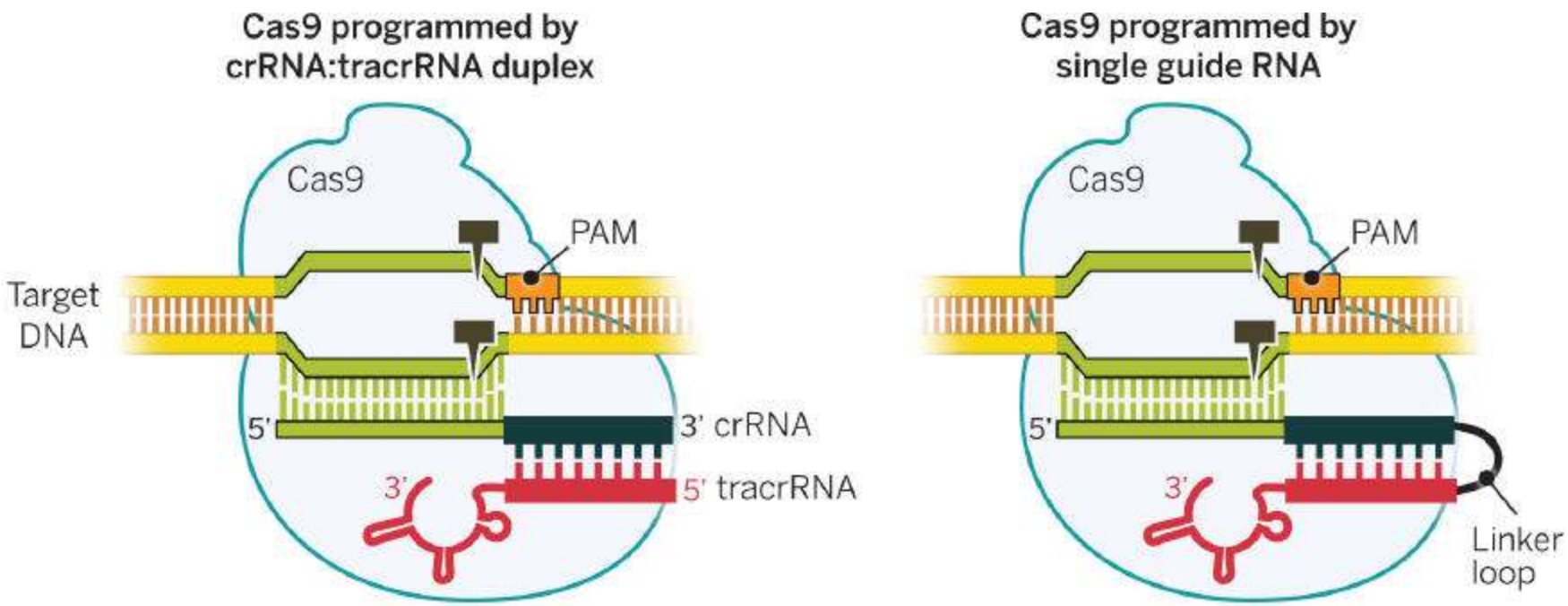
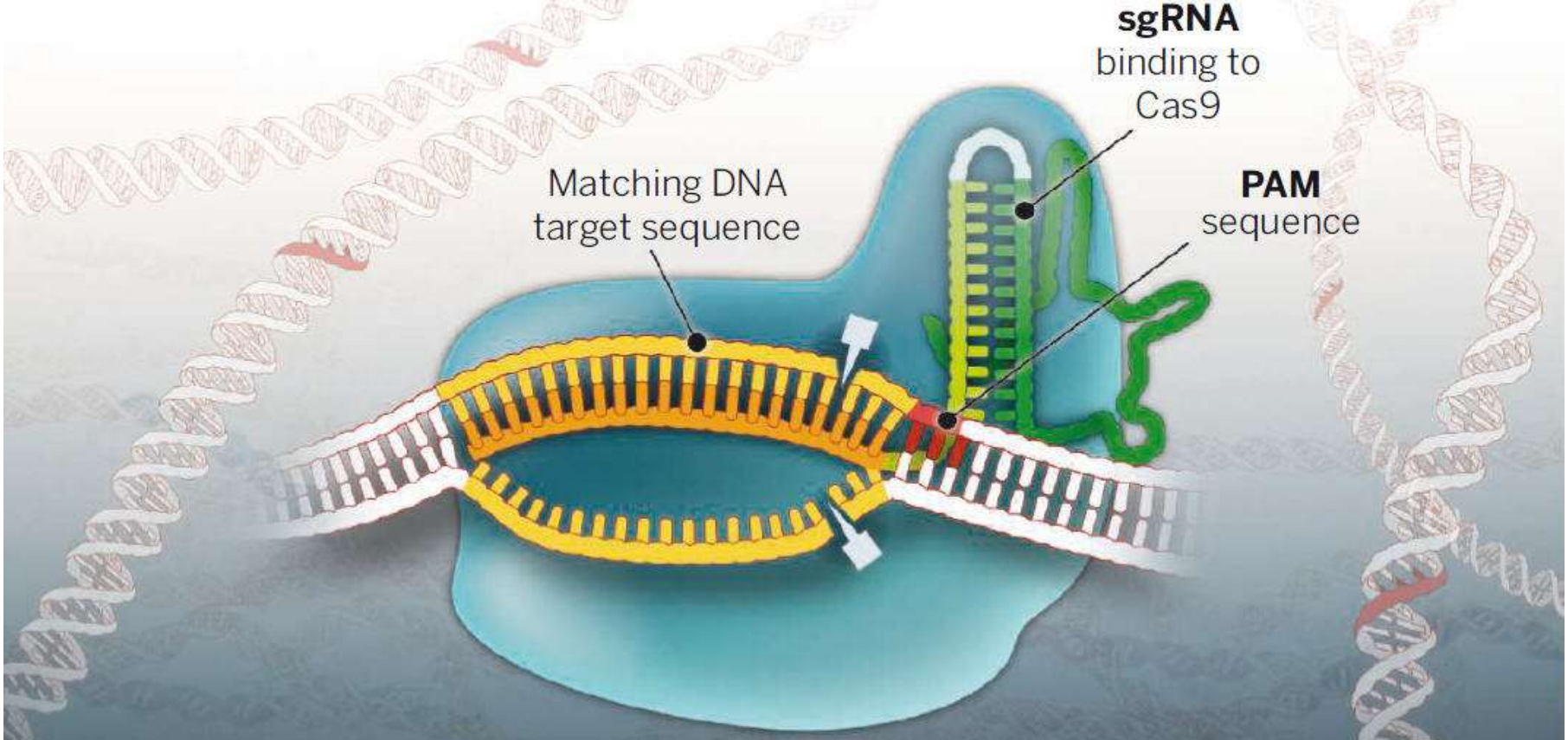


Fig. 3. Evolution and structure of Cas9. The structure of *S. pyogenes* Cas9 in the unliganded and RNA-DNA–bound forms [from (*77*, *81*)].

Jennifer Doudna
Emmanuelle Charpentier

Jinek et al. (2012) Science
Doudna & Charpentier (2014) Science



CRISPR-Cas9 development

- DNA deletion
- DNA insertion
- DNA replacement
- DNA modification
- DNA labeling
- Transcription modulation
- RNA targeting
- ...

CRISPR-Cas9 applications

- Biological research
- Research and development
- Human medicine
- Biotechnology
- Agriculture
- ...

CRISPR-Cas9 tools & Animal Models

Deletions, Inversions, Disruptions, Editions, Knockins

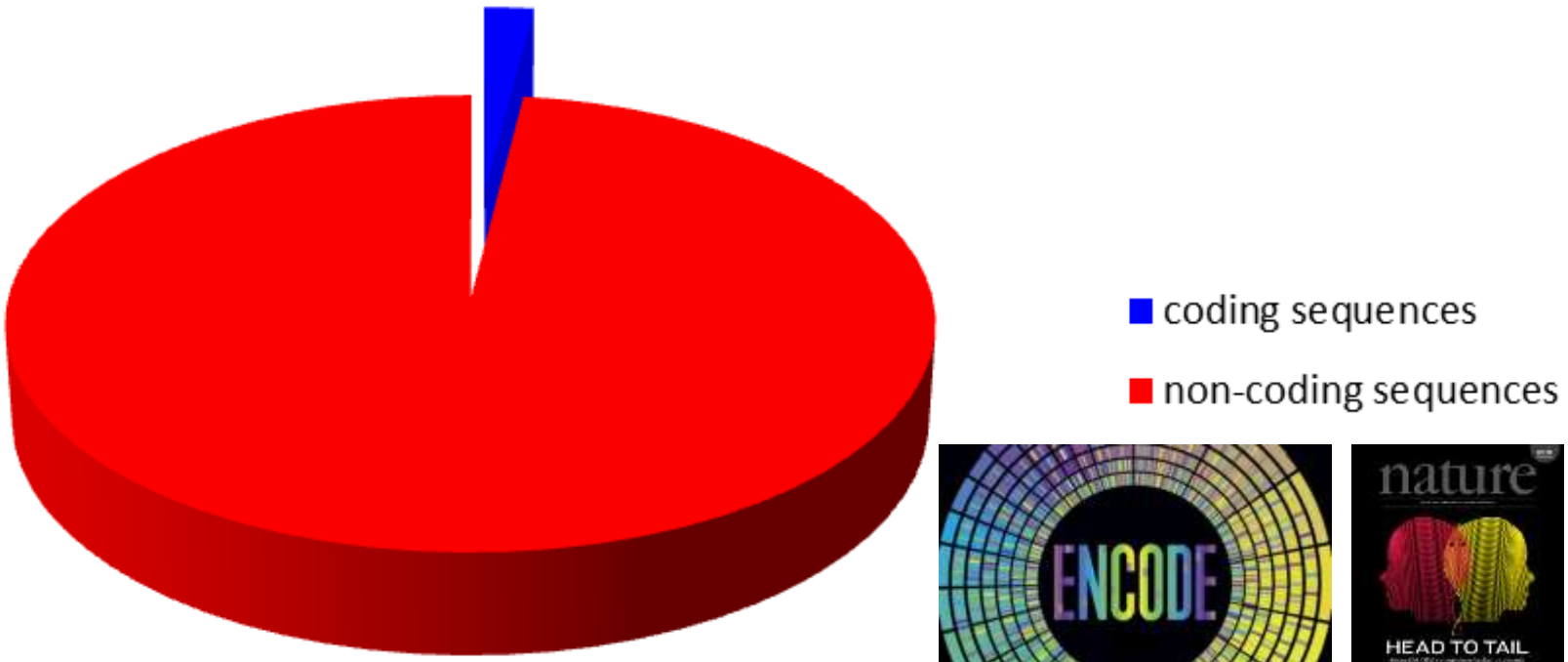
We use the Tyrosinase gene (*Tyr*) in mice as experimental model to study mammalian gene regulation and …

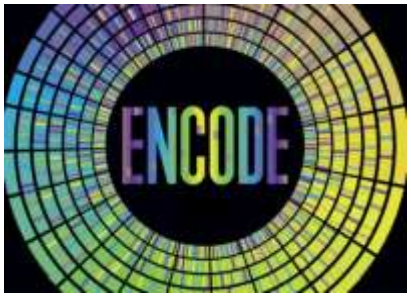
…as animal models of albinism, a human rare disease

The non-coding genome

DNA coding sequences represent 2% genome

DNA non-coding sequences represent 98% genome



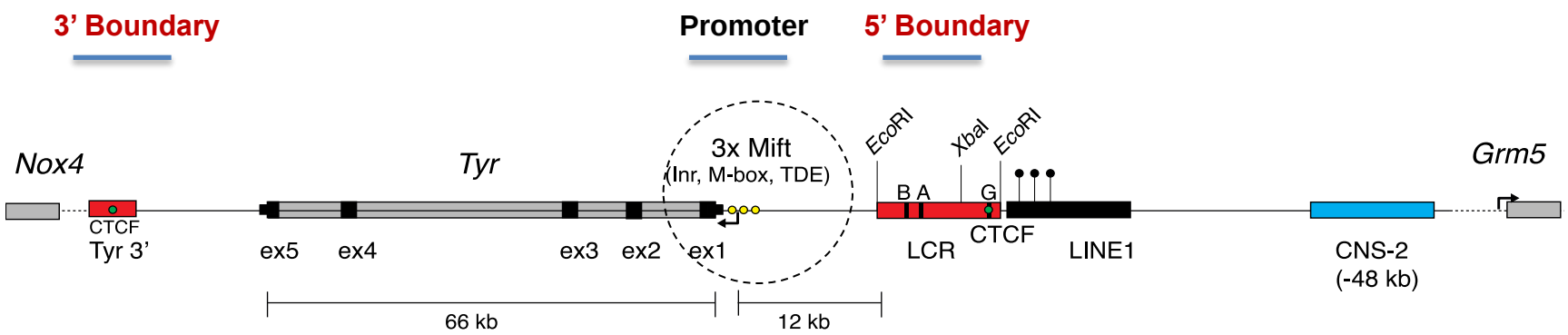


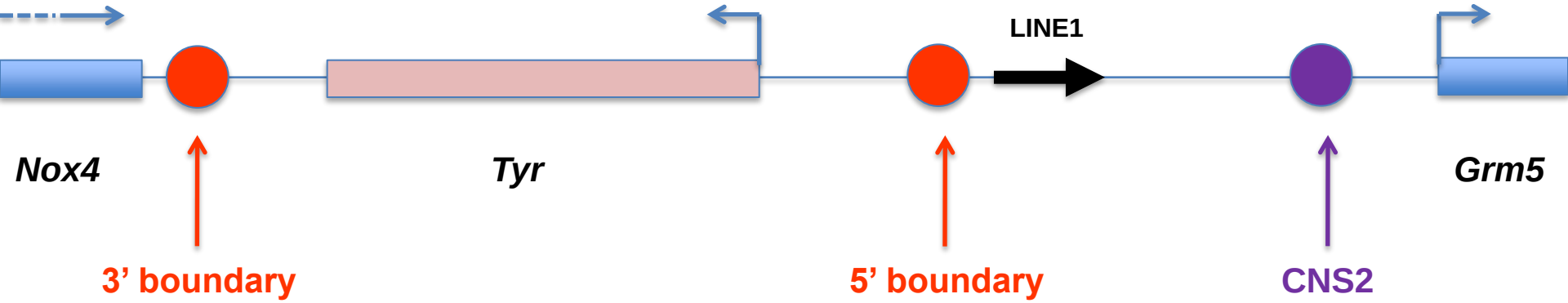
DNA non-coding sequences contain mainly:

DNA repetitive elements, mobile elements and

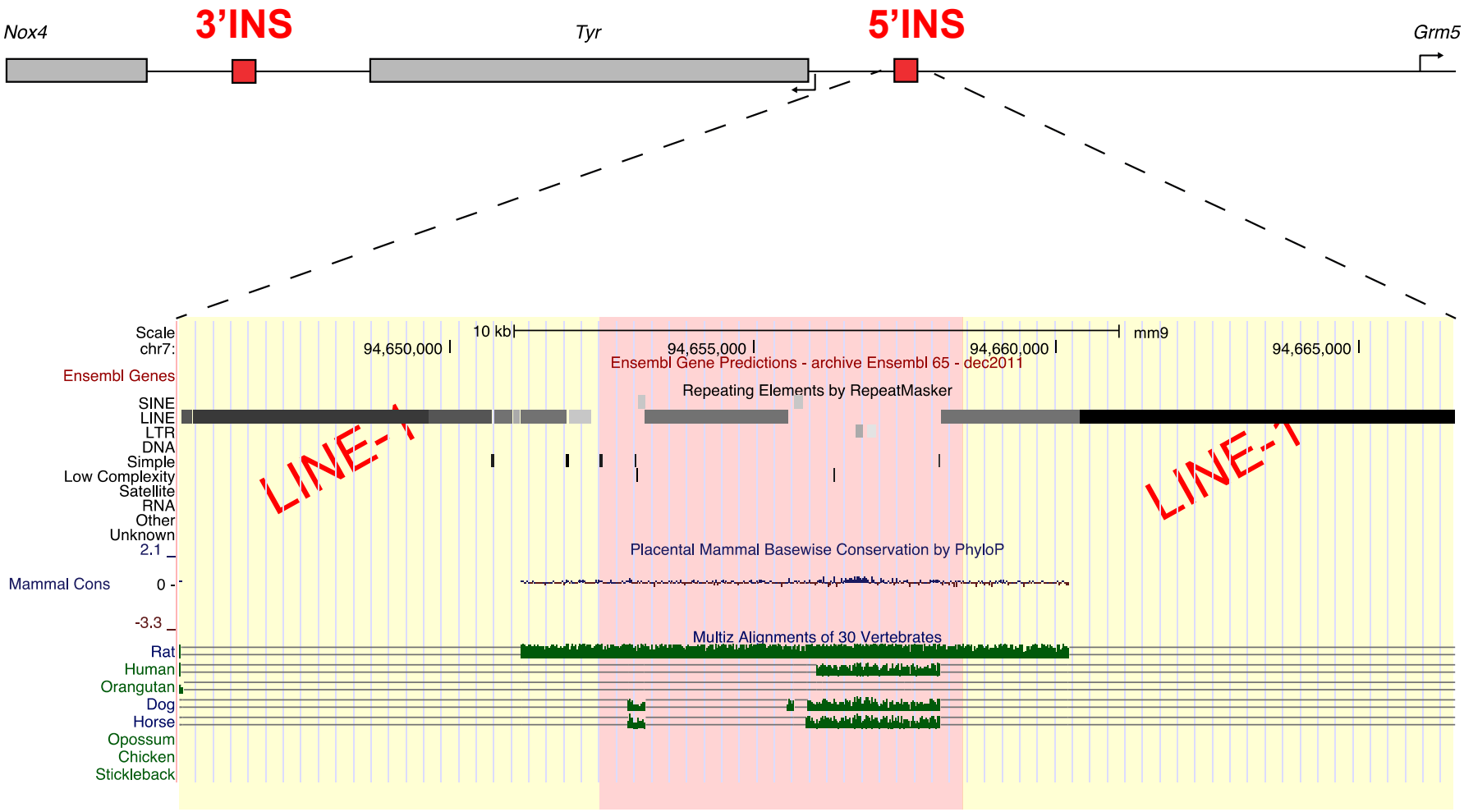
<u>DNA regulatory elements</u>

The known DNA regulatory elements at the mouse *Tyr* locus

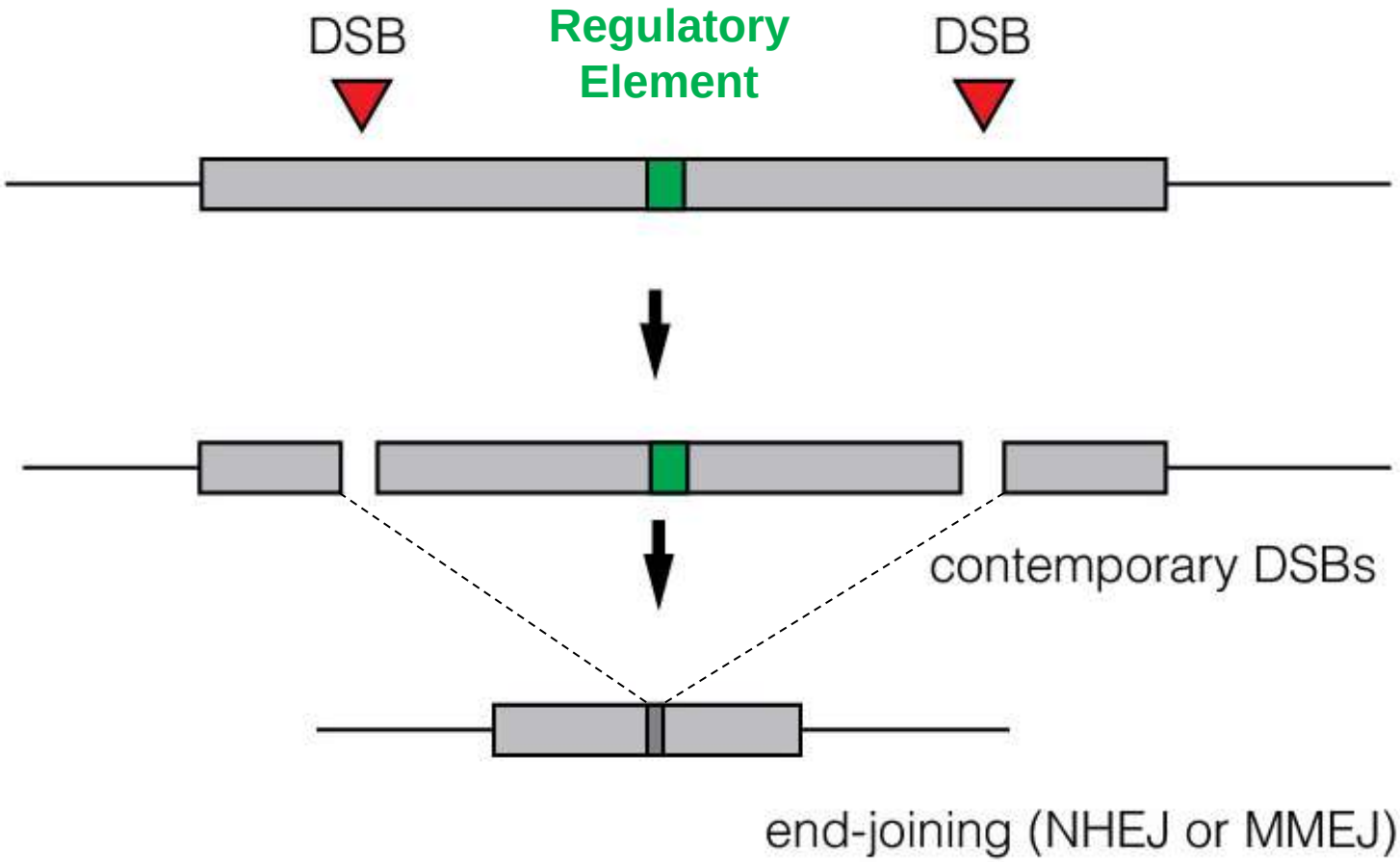




Deleting mouse *Tyr* intergenic regions is challenging due to the presence of repetitive DNA elements

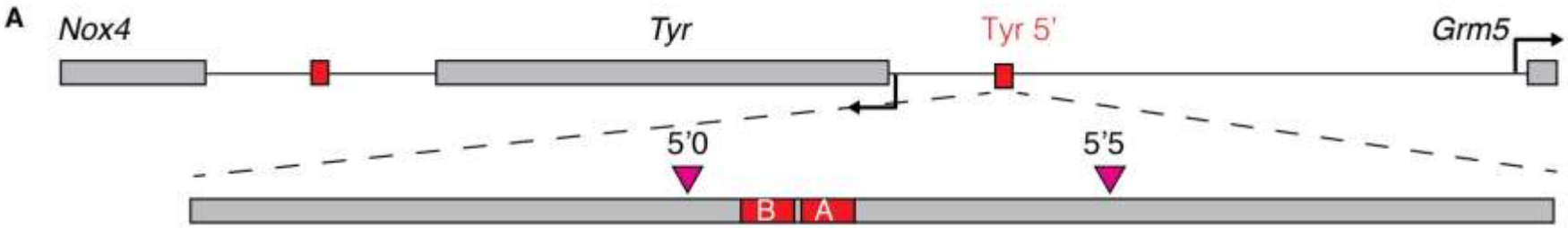


Alternative: using CRISPR-Cas9 genome editing to target *Tyr* regulatory elements

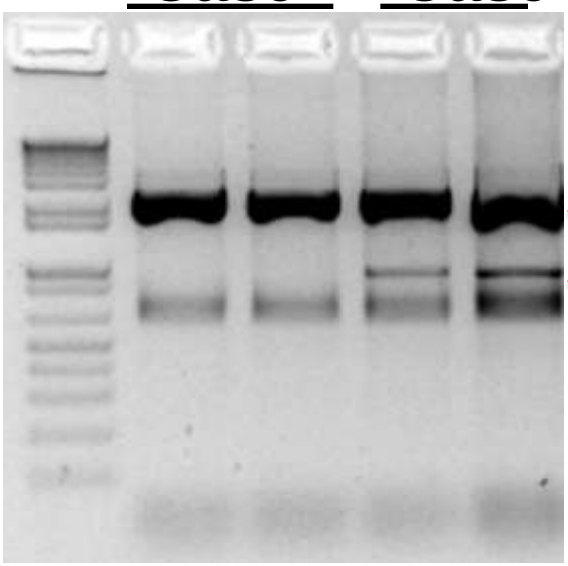


CRISPR-Cas9 genome editing

Deletion of the *Tyr* 5' insulator with CRISPR *in vitro*



Cas9 Cas9 + sgRNAs



wt

deletion

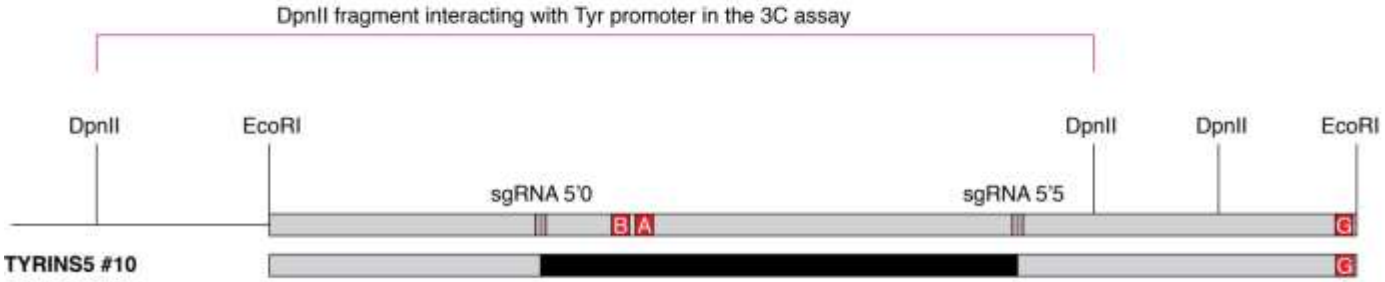
Davide Seruggia

Mouse N2a cells, unsorted
72 hours post transfection

CRISPR-Cas9 genome editing: First always testing *in vitro*, then *in vivo*

Deleting *Tyr* 5' boundary with CRISPRs

Founder mosaic mice with clear coat colour pigmentation phenotype carrying BIALLELIC deletion of *Tyr* 5' boundary




```
reference:     CAAGAATTAAGTGTGACAGTGCAAGATAACAGGAAAATAA......1170bp........CTCTAGGCCAAAATTGCCTAGTTTTATCACTACAAAACTT
TYRINS5#18_1: CAAGAATTAAGTGTGACAGTGCAAGAT-------------......1170bp........-------------TTGCCTAGTTTTATCACTACAAAACTT
TYRINS5#18_1: CAAGAATTAAGTGTGACAGTGCAAGAT-------------......1170bp........-------------TTGCCTAGTTTTATCACTACAAAACTT
```

Many mutant alleles generated upon targeting the 5' *Tyr* Boundary by CRISPR-Cas9

Table 1. CRISPR-Cas9 RNA microinjection into B6CBAF2 fertilized eggs

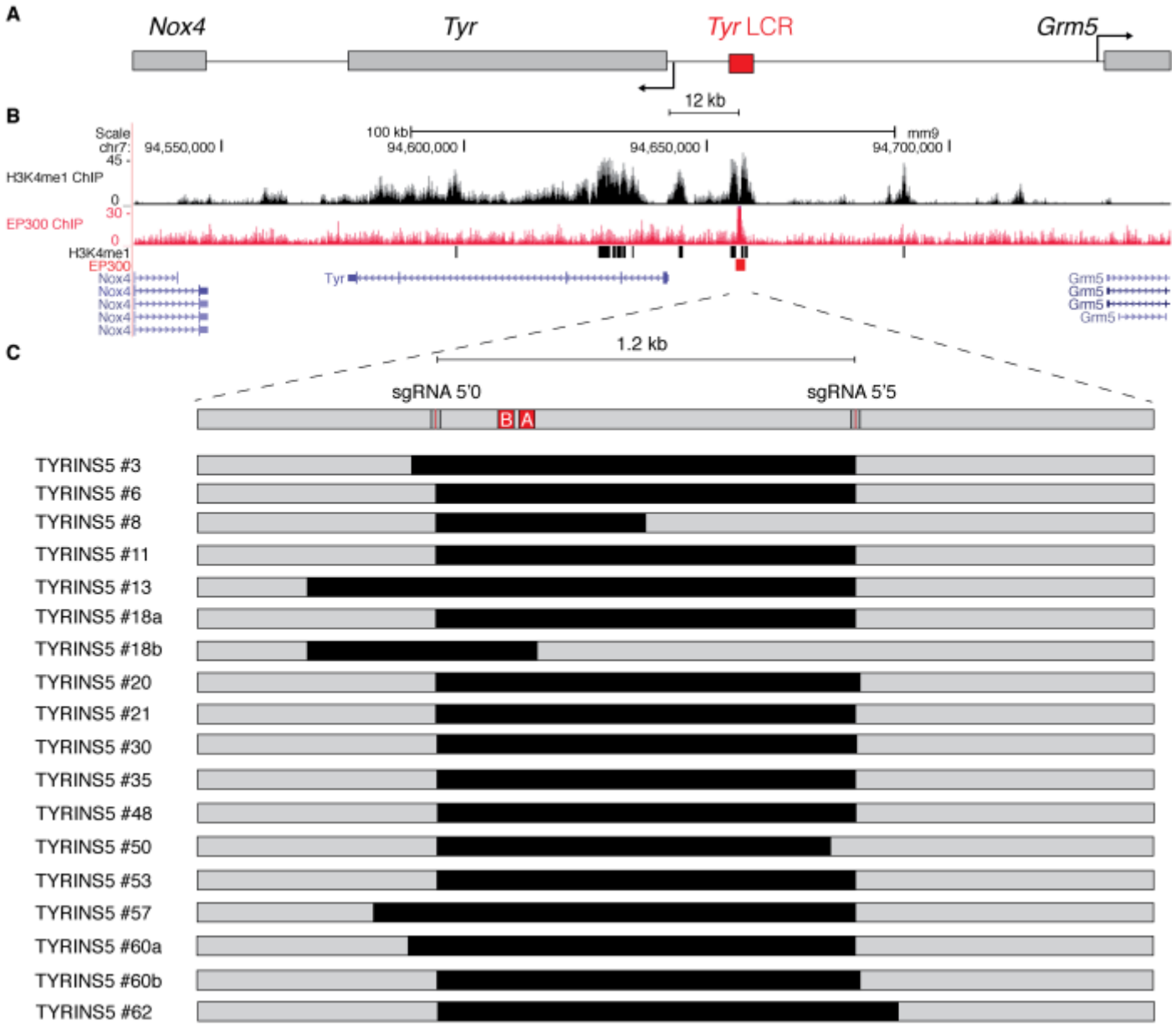
RNA concentration	Microinjected mouse fertilized eggs	Transferred mouse embryos (% microinjected)	Number of fosters used	Live pups obtained (% transferred)	Positive mouse lines (deletions) (% live pups)
35 ng/µl Cas9 60 ng/µl sgRNA	163	86 (52%)	5	31 (36%)	7 (23%)
50 ng/µl Cas9 90 ng/µl sgRNA	225	99 (44%)	7	33 (33%)	12 (36%)
TOTAL	**388**	**185 (47%)**	**12**	**64 (35%)**	**19 (30%)**

Out of **64 pups produced** in various injection sessions:
54 founder mice (84%) carry indels in at least one of the two sites
19 founder mice (30%) carry indels in both target sites
19 founder mice (30%) carry the expected deleted alleles
7 founder mice (11%) carry inverted-region alleles
4 founder mice (6%) carry biallelic deletions and show a phenotype

One CRISPR-Cas9 experiment: far too many lines to analyze!

RNA injections done by Belén Pintado (CNB-CSIC)

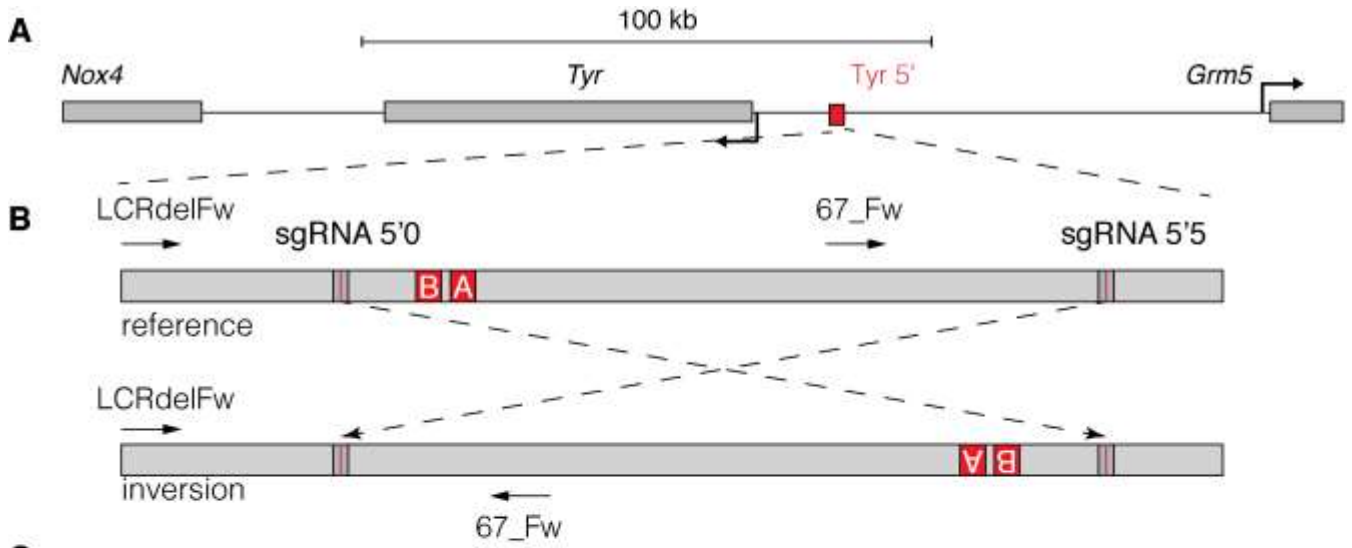
Many mutant deletion alleles generated upon targeting the 5' *Tyr* Boundary by CRISPR-Cas9



Seruggia et al. 2015 NAR

Inversion deletion alleles generated upon targeting the 5' *Tyr* Boundary by CRISPR-Cas9

Inversions are often found in humans and associated with disease

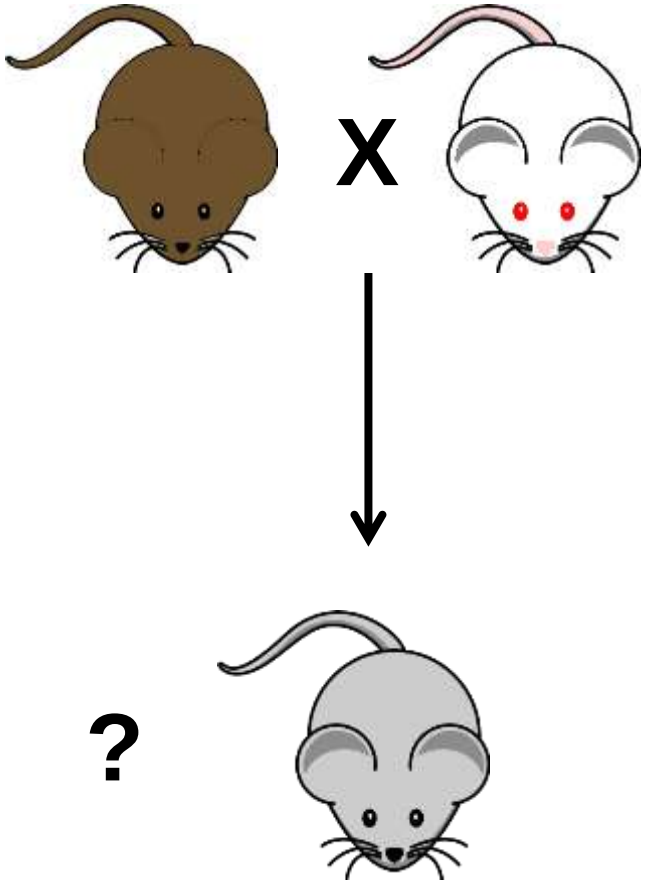


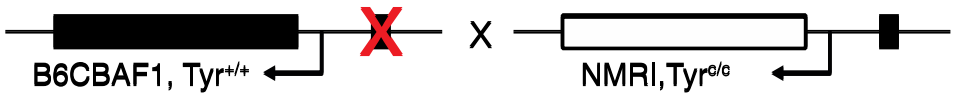
C

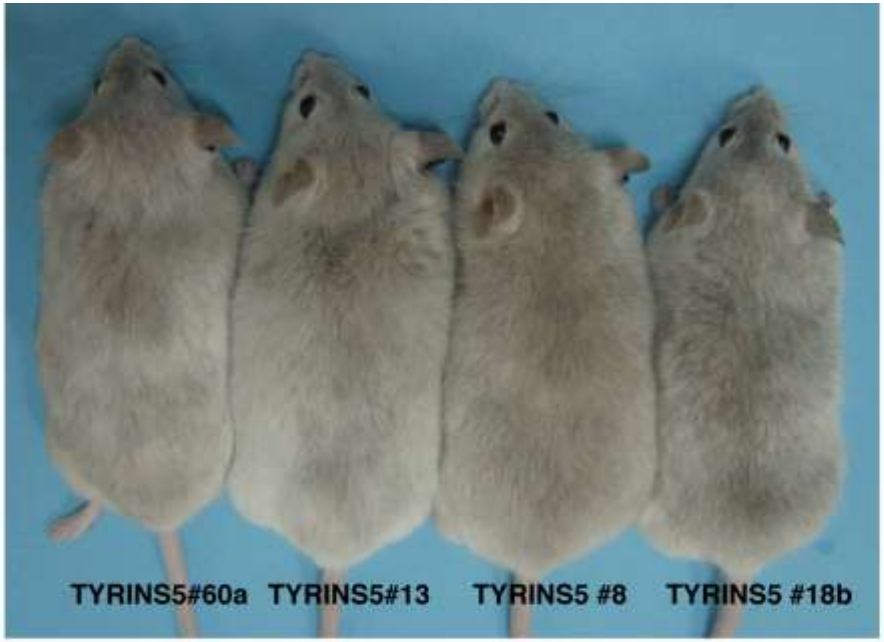
```
                                   sgRNA 5'0          sgRNA 5'5
reference:      TCCCAAGAATTAAGTGTGACAGTGCAAGAT****TTTTGGCCTAGAGCTTTGCAGTGATGATTA

TYRINS5#19_1:   TCCCAAGAATTAAGTGTGACAG-----(+120-8)--TGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#19_2:   TCCCAAGAATTAAGTGTGACAG-----(+120-8)--TGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#23_1:   TCCCAAGAATTAAGTGTGACAGTGCAAGA(+6-2)TTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#23_2    TCCCAAGAATTAAGTGTGACAGTGCAAGA(+6-2)TTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#27_1:   TCCCAAGAATTAAGTGTGACAGTGCAAGAT****TTTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#27_2:   TCCCAAGAATTAAGTGTGACAGTGCAAGAT****TTTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#30_1:   TCCCAAGAATTAAGTGTGACAGTGCAAGAT(+5)TTTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#30_2:   TCCCAAGAATTAAGTGTGACAGTGCAAGAT(+5)TTTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#31_3:   TCCCAAGAATTAAGTGTGACAGTGCAAGA-****------------GCTTTGCAGTGATGATTA
TYRINS5#41_1:   TCCCAAGAATTAAGTGTGATA---------****TTTTGGCCTAGAGCTTTGCAGTGATGATTA
TYRINS5#41_1:   TCCCAAGAATTAAGTGTGATA---------****TTTTGGCCTAGAGCTTTGCAGTGATGATTA
```

Seruggia et al. 2015 NAR

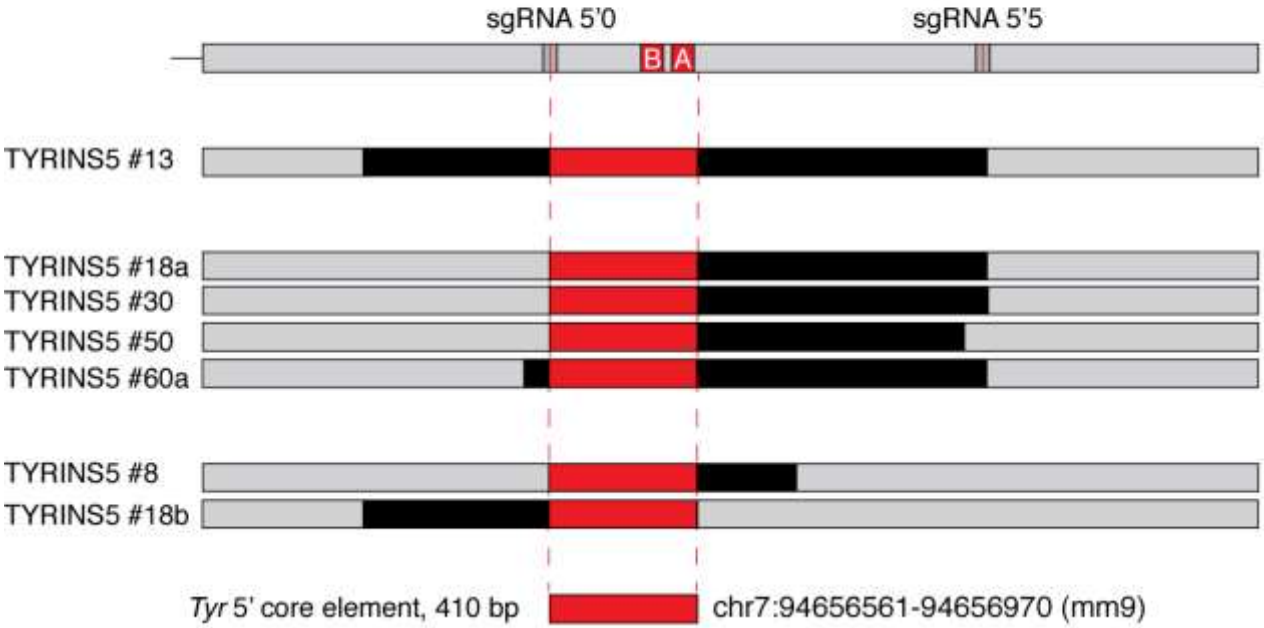
Phenotype of *Tyr* 5' Boundary CRISPR genome-edited mice crossing F0 founder mice with albino mice







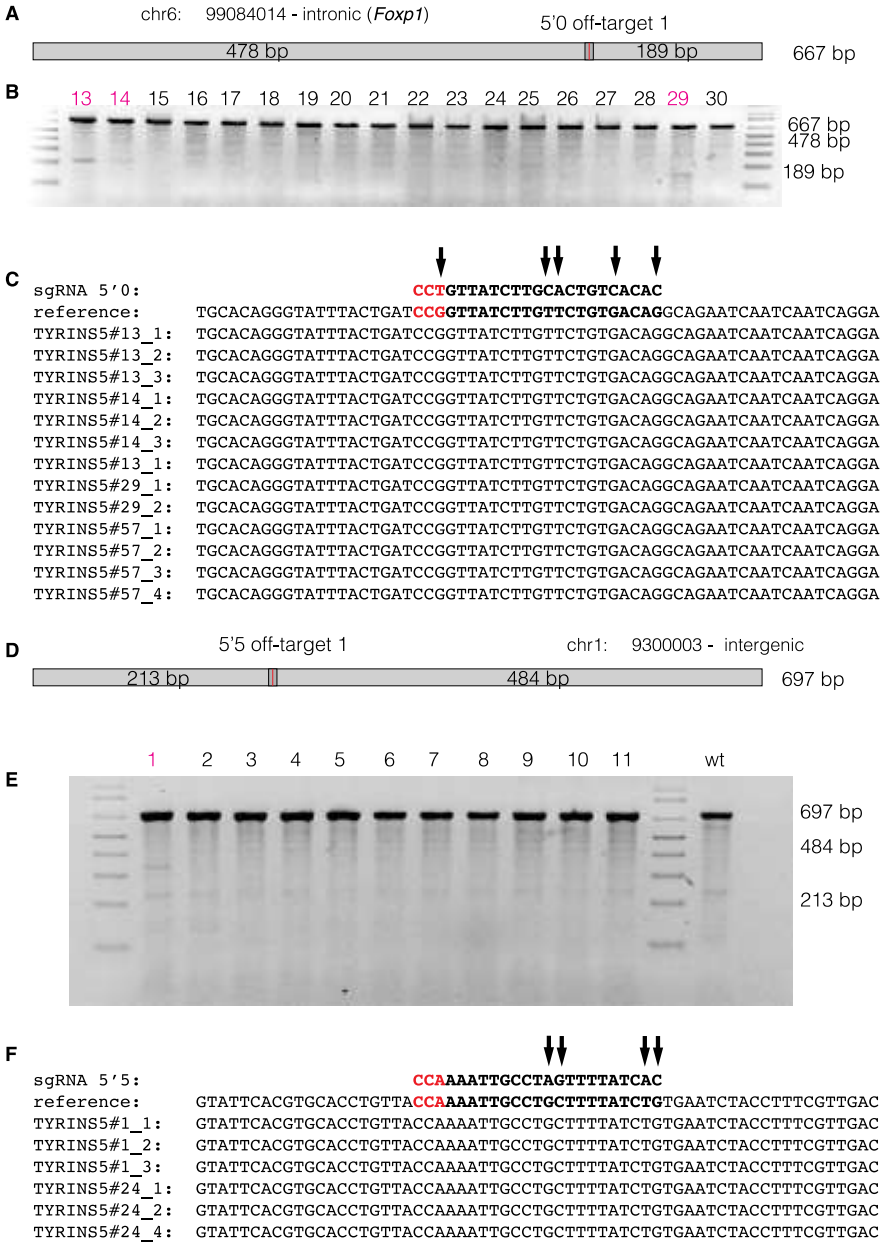
Comparing different *Tyr* 5' Boundary targeted alleles with similar phenotypes reveals the location of the functionally relevant endogenous regulatory DNA sequences



Genetic analysis now possible in mice!

Seruggia et al. 2015 NAR

We have not found off-target sites with altered sequences



We have found some (very few) animals positive to the T7 assay (very sensitive to detect mismatches), that are probably mosaic or polymorphisms between the various mouse strains involved

Off-target mutations are rare in Cas9-modified mice

Vivek Iyer, Bin Shen, Wensheng Zhang, Alex Hodgkins, Thomas Keane, Xingxu Huang & William C Skarnes

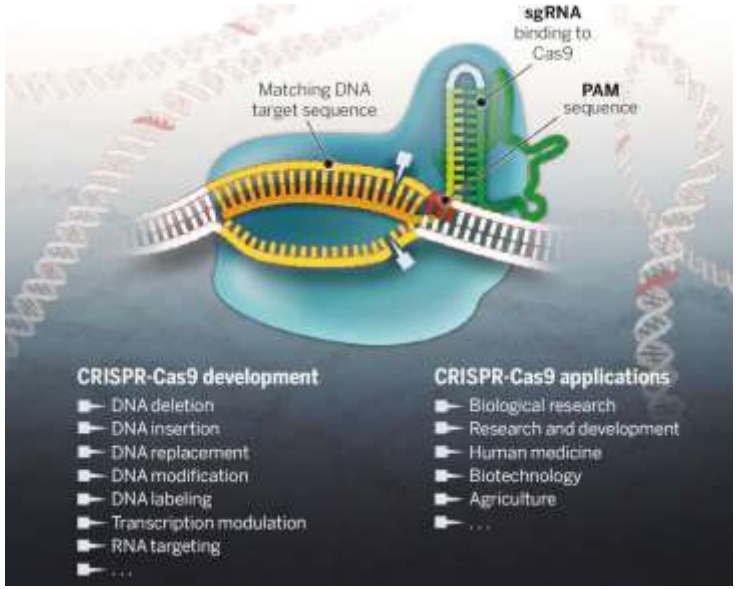
Affiliations | Corresponding authors

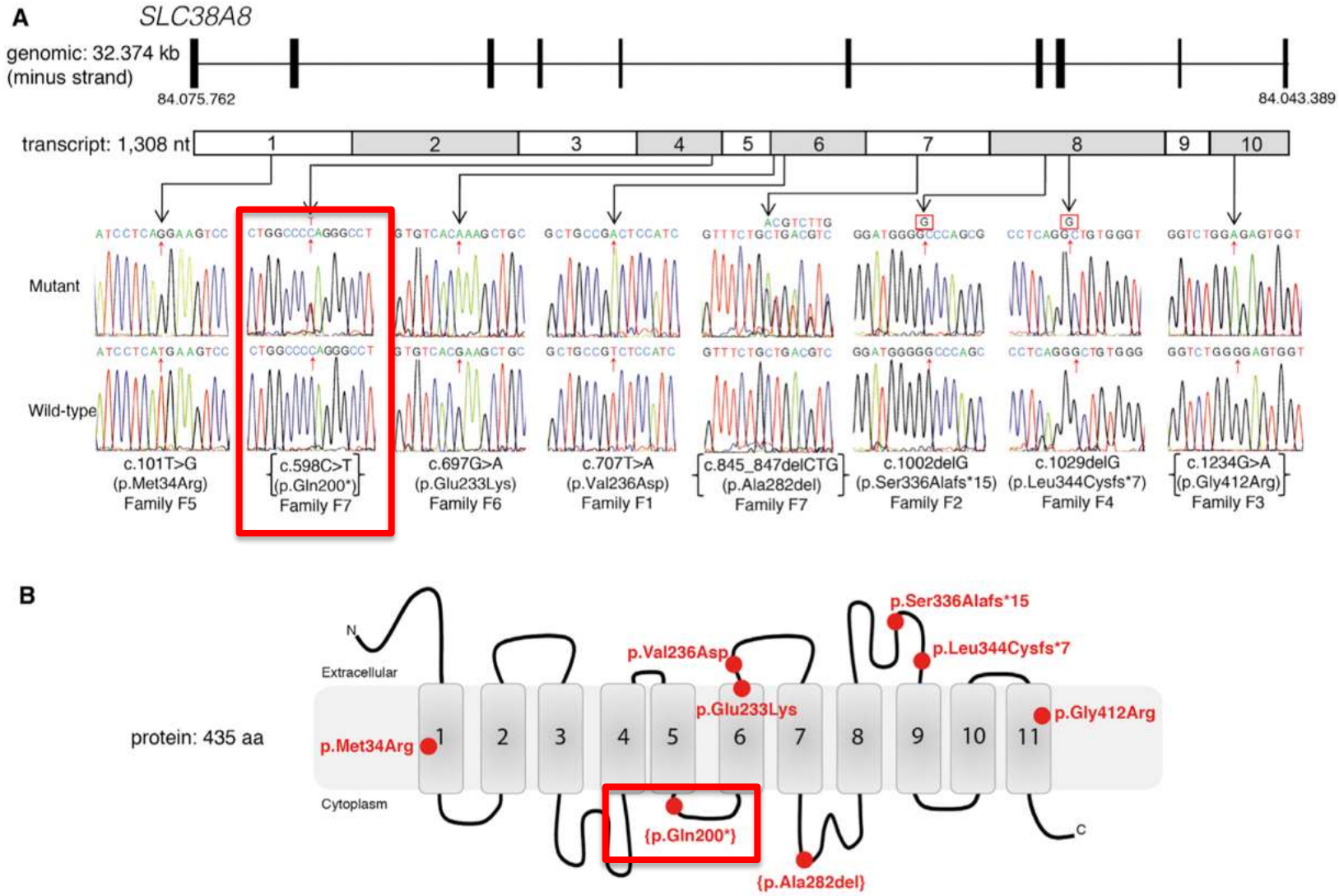
Nature Methods **12**, 479 (2015) | doi:10.1038/nmeth.3408
Published online 28 May 2015

Confirmed by NGS

AVATAR CRISPR mice

- Easier approach to reproduce human mutations in animal models
- Easier way to functionally assess the relevance of coding and non-coding DNA sequences



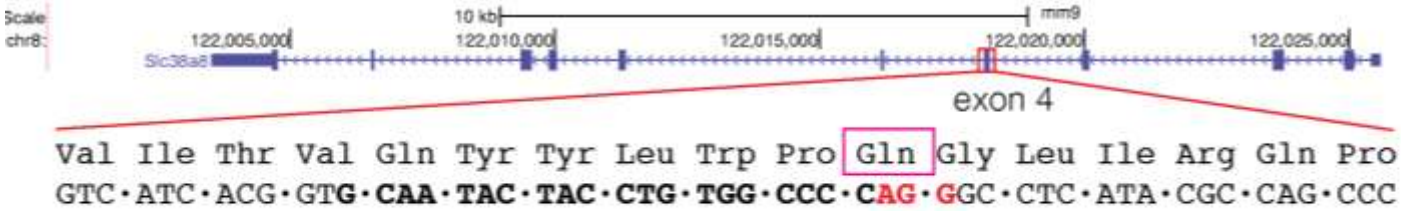


Mouse model of FHONDA syndrome

We set to reproduce the Gln200* in mice using CRISPR-Cas9

Mouse model of FHONDA (*Slc38a8*)

Prediction of protein sequence after mutagenesis: 16 different proteins

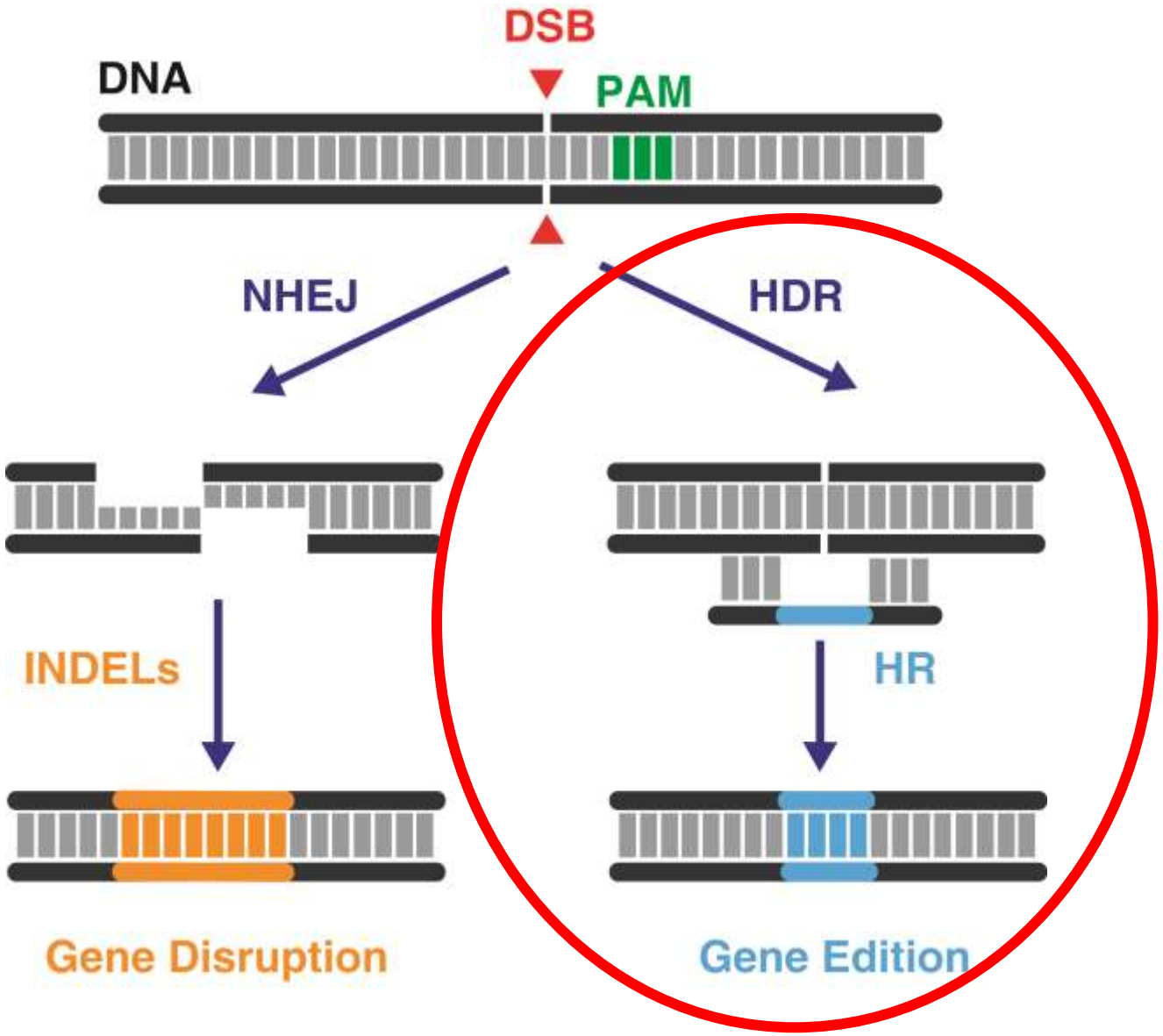


Val Ile Thr Val Gln Tyr Tyr Leu Trp Pro Gln Gly Leu Ile Arg Gln Pro
GTC·ATC·ACG·GTG·CAA·TAC·TAC·CTG·TGG·CCC·CAG·GGC·CTC·ATA·CGC·CAG·CCC

exon 3 | exon 4 | exon 5 | exon 6

	alleles in founder#
ALQKYTSILGTLAACYLALVITVQYYLWPQGLIRQPGPLLSPSPWTSVFSVFPTICFGFQCHEAAVSI	
ALQKYTSILGTLAACYLALVITVQYY---QGLIRQPGPLLSPSPWTSVFSVFPTICFGFQCHEAAVSI	69; 70; 80; 83; 86; 89
ALQKYTSILGTLAACYLALVITVQYYLWPGPHTPARSFAEPVSLDLCV*	69; 77; 91; 94
ALQKYTSILGTLAACYLALVITVQYYLWHRLGATFSS*	70
ALQKYTSNPRYFAEPVSLDLCV*	70
ALQKYTSILGTLAACYLALVITVQYYLWPHTPARSFAEPVSLDLCV*	71; 73; 74; 81; 82; 88; 89; 95
ALQKYTSILGTLAACYLALVITVQYYLPHTPARSFAEPVSLDLCV*	72
ALQKYTSILGTLAACYLALVITVQYYLWPRASYASPVLC*	72; 89; 95
ALQKYTSILGTLAACYLALVITVQYYLWPPGPHTPARSFAEPVSLDLCV*	73
ALQKYTSIPGPHTPARSFAEPVSLDLCV*	73
ALQKYTSILGTLAACYLALVITVQYYL-----IRQPGPLLSPSPWTSVFSVFPTICFGFQCHEAAVSI	74
ALQKYTSILGTLAACYLALVITVQYYLWPRASYASPVLC*	75; 80; 83; 87; 88
[illegible]	76; 79; 92; 93; 94
ALQKYTSILGTLAACYLALVITVQYYLW*	78
[illegible]	79
ALQKYTSILGTLAACYLALVITVQYYLRPHTPARSFAEPVSLDLCV*	87
ALQKYTSILGTLAACYLVLVISVQYYLWPTPCRFFELITDYPYFNLLFLALKLGPQGLIHQPGPLLSP	91

NHEJ vs HDR (towards genome edition)



Aim: to introduce a single base pair substitution resulting in AA change

-Identify a sgRNA within 50 bp from the desired mutation
-Design a donor DNA including the desired mutation
-study a silent mutation to introduce a convenient novel restriction site

target sequence

```
 D  T  F  L  E  H  M  C  R  L  D  I  D  S  A  P  I  T  A  R  N  T  G  I  I  C  T  I  G  E
GACACCTTCCTGGAACACATGTGCCGCCTGGACATTGACTCTGCCCCCATCACGGCCCGCAACACTGGCATCATTTGTACCATTGGTGAG
```

```
GACACCTTCCTGGAACACATGTGCCGCCTGGACATTGACTCTCCCCCGATCACGGCCCGCAACACTGGCATCATTTGTACCATTGGTGAG
 D  T  F  L  E  H  M  C  R  L  D  I  D  S  P  P  I  T  A  R  N  T  G  I  I  C  T  I  G  E
```

donor DNA
(90 bp oligo)

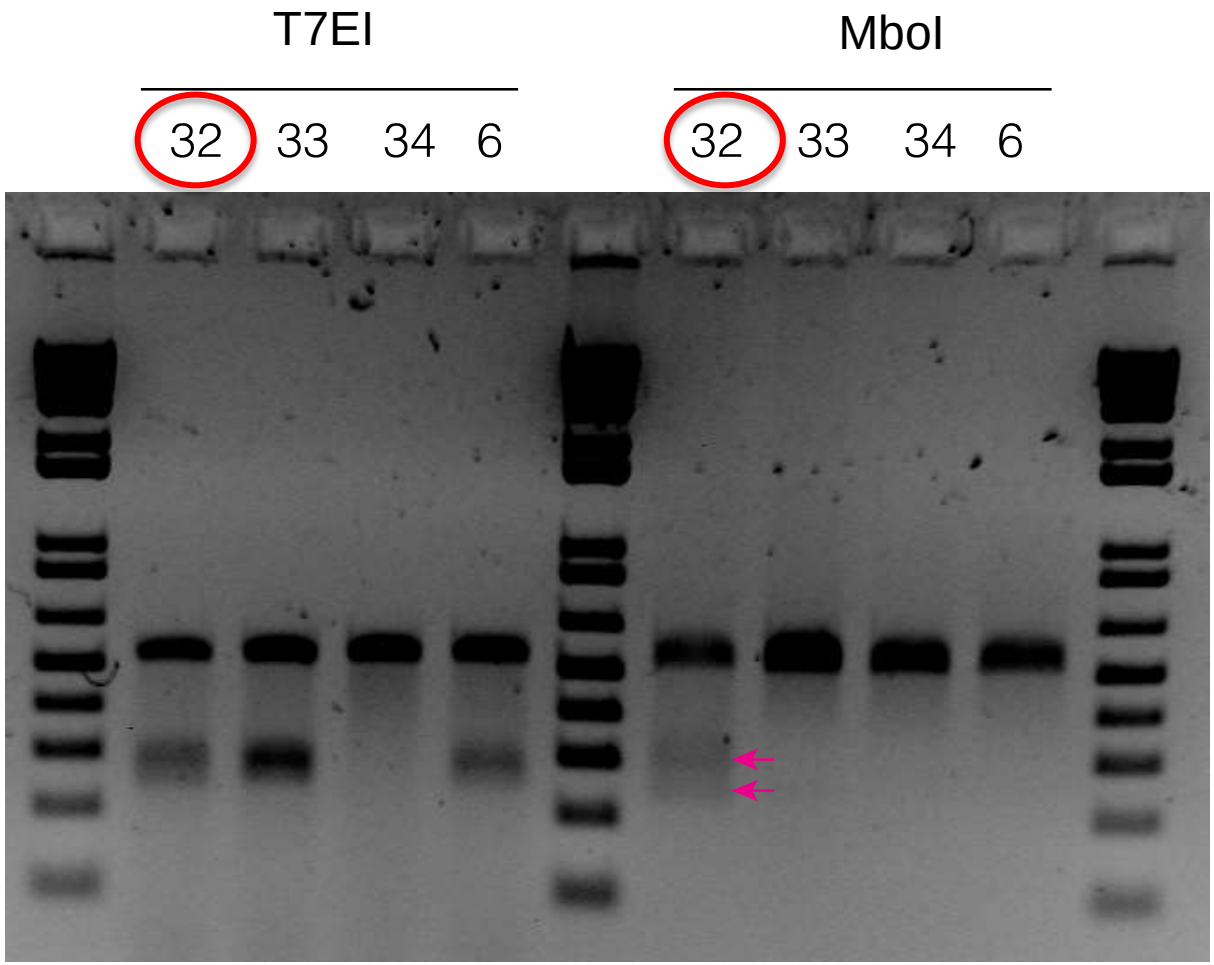
point mutation
(Ala to Pro)

silent mutation
(MboI site)

Collaboration with Guadalupe Sabio (CNIC)

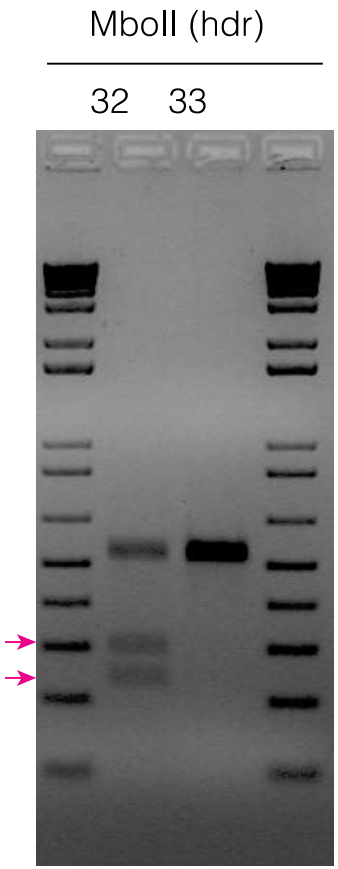
unpublished

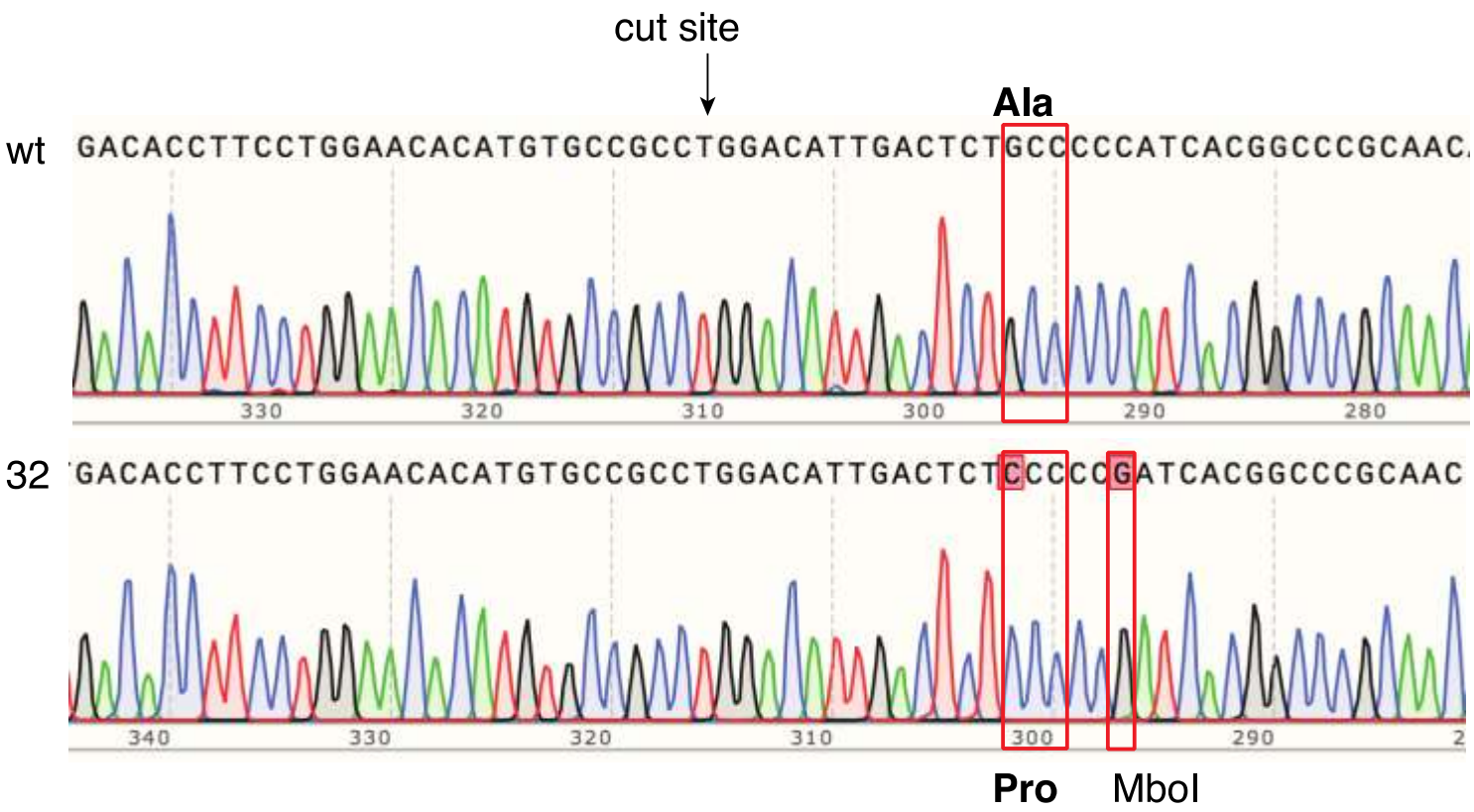
Silent mutation associated with a new restriction site useful to identify genome-edited mice



Founder 32 is pos
At the T7EI and by
MboI digestion

Sequencing of founder #32: the two mutations are introduced





Founder 32 has been genome edited as we planned

CRISPR-Cas is the beginning of the future

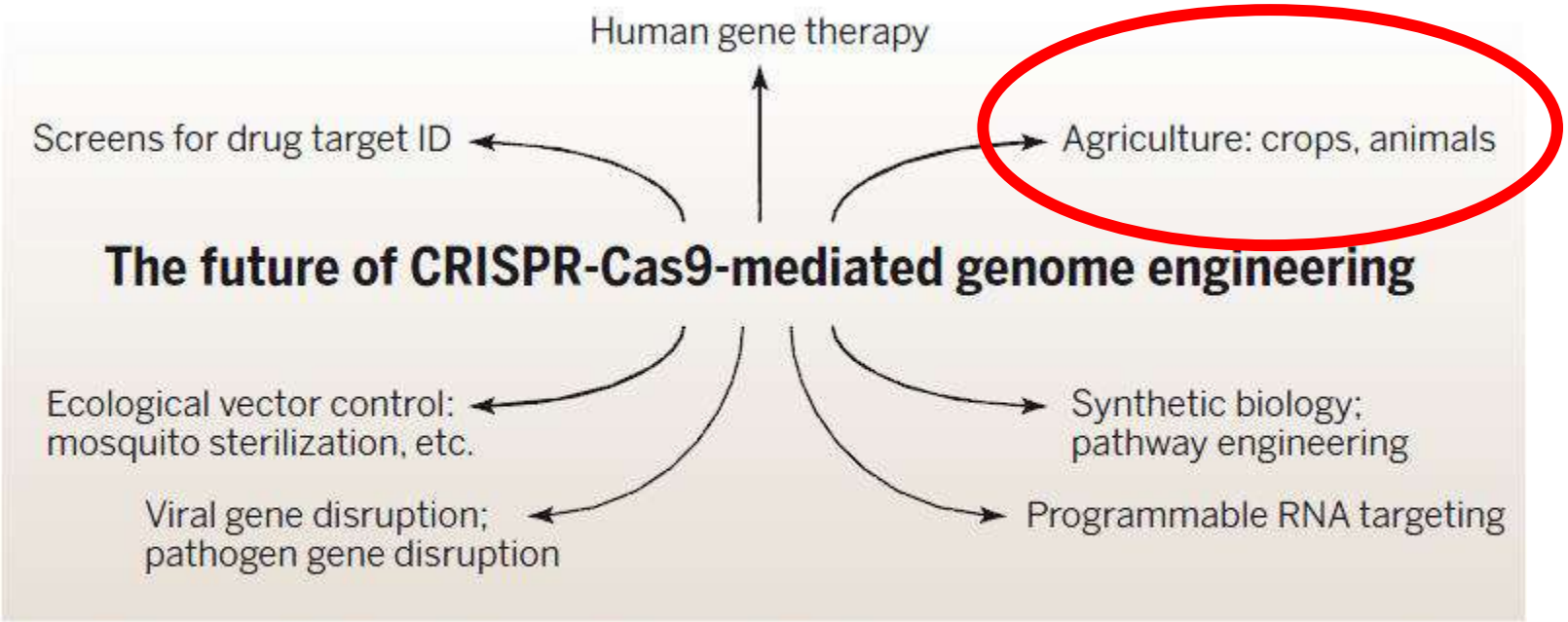


Fig. 6. Future applications in biomedicine and biotechnology. Potential developments include establishment of screens for target identification, human gene therapy by gene repair and gene disruption, gene disruption of viral sequences, and programmable RNA targeting.

Doudna & Charpentier (2014) Science

Large Animals

CRISPR-Cas and animal biotechnology

Xenotransplantation

PERV

Xenotransplantation – down and up

1997

2016

nature biotechnology

EDITORIAL

In the 1990s, concerns about potential transfer of porcine retroviruses to xenotransplant recipients shut down commercial programs, even though **transmission had not (and still has not) been demonstrated *in vivo***. Given the continuing shortage of human organs for transplant, **return of commercial funding to xenotransplantation is encouraging**.

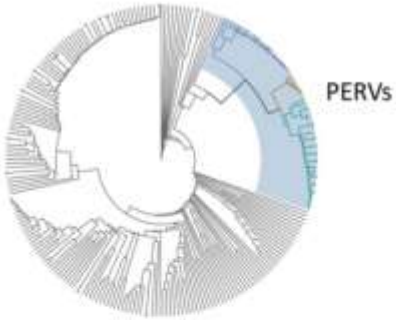
Government funders should take note. Yes, stem cell–derived therapies offer great long-term promise for degenerative diseases. But **xenotransplants represent an additional intriguing option - one with potentially shorter horizons to the clinic.**

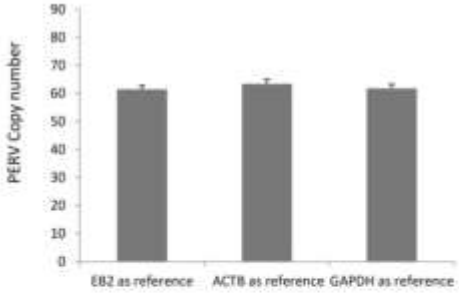
NATURE BIOTECHNOLOGY
VOLUME 34 NUMBER 1 JANUARY 2016

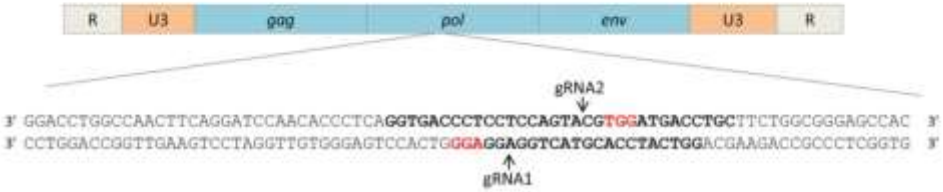
Sciencexpress Reports

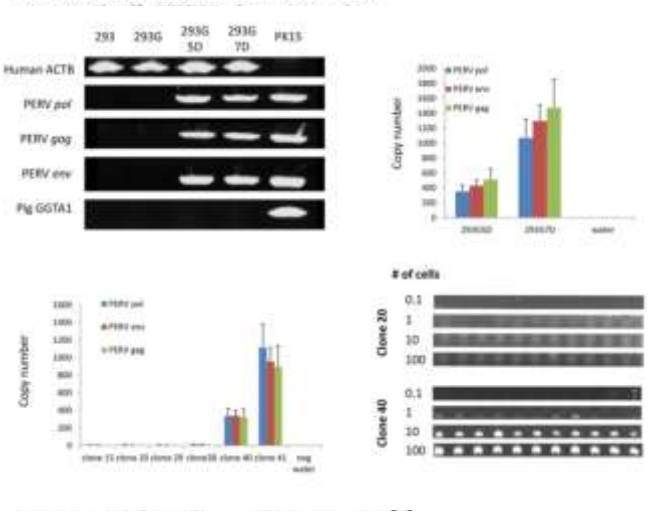
Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

Luhan Yang,[1,2,3]*† Marc Güell,[1,2,3]† Dong Niu,[1,4]† Haydy George,[1]† Emal Lesha,[1] Dennis Grishin,[1] John Aach,[1] Ellen Shrock,[1] Weihong Xu,[6] Jürgen Poci,[1] Rebeca Cortazio,[1] Robert A Wilkinson,[5] Jay A. Fishman,[5] George Church[1,2,3]*





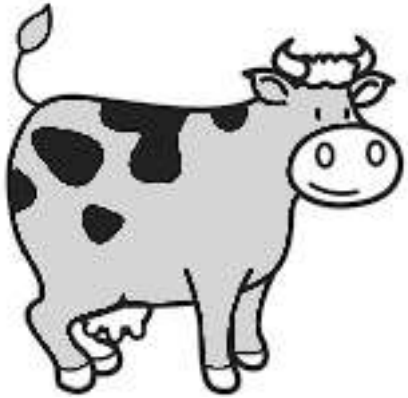




Inactivation of 62x PERV integrated in the pig genome via CRISPR-Cas9

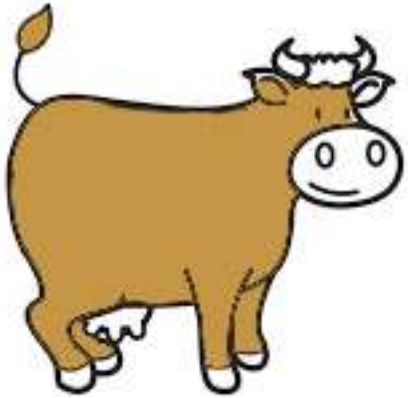
Yang et al. 2015 (Science)

The paradigm of genetic selection/improvement

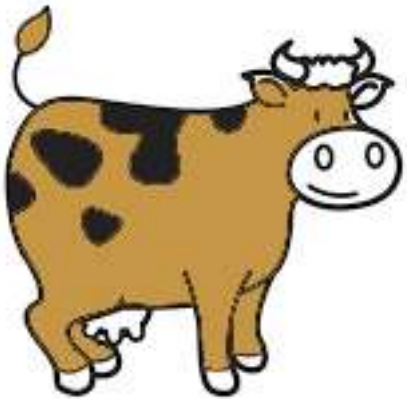


High milk production
Sensitive to disease X

X



Low milk production
Resistance to disease X



High milk production
Resistance to disease X

CRISPR-Cas and animal biotechnology

Texel

↑ Great meat

↓ Poor wool

Merina

↓ Poor meat

↑ Great wool

MSTN (myostatin) natural mutation

CRISPR-Cas and animal biotechnology

Texel

↑ Great meat

↓ Poor wool

Merina

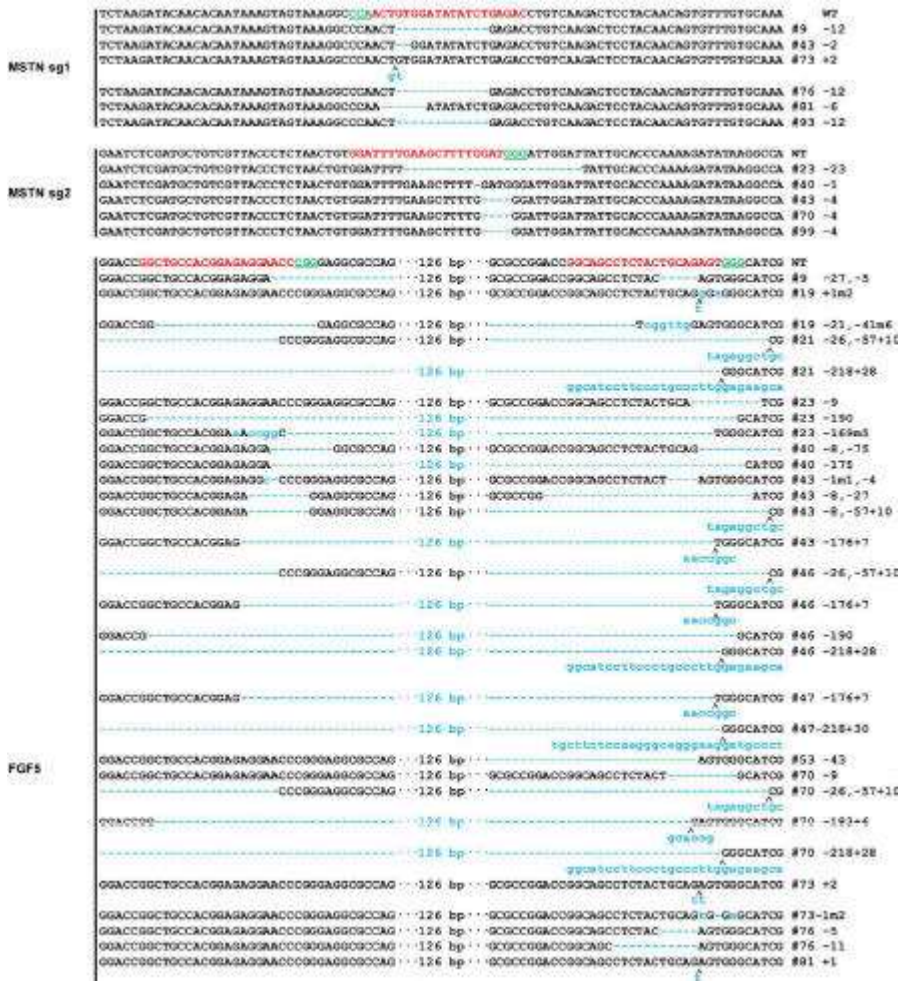
↓ Poor meat

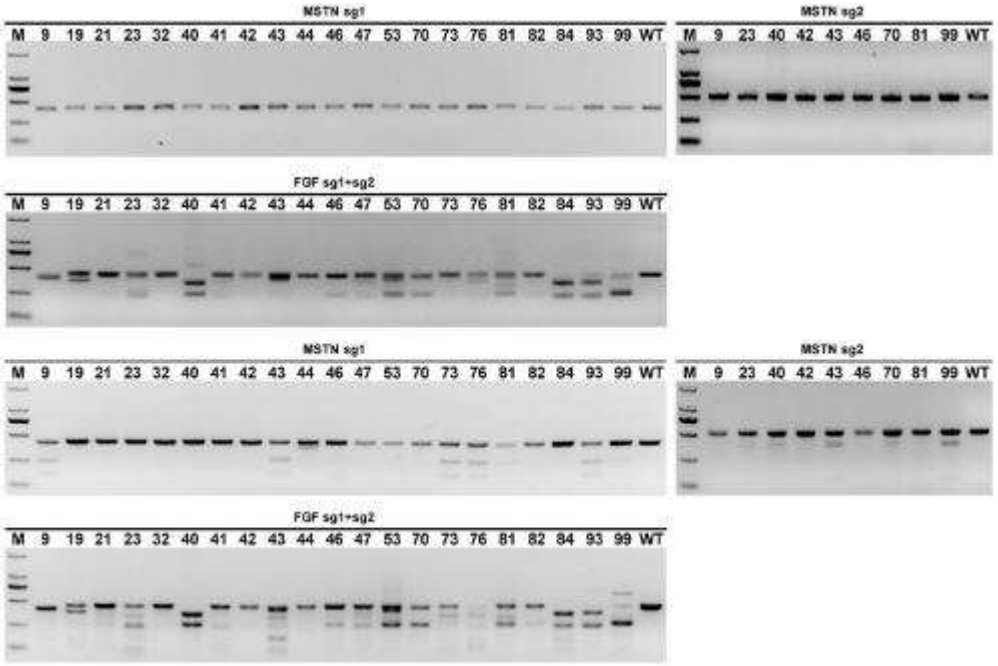
↑ Great wool

Targeting *MSTN* by CRISPR-Cas9

Crispo et al. PLoS ONE 2015

CRISPR-Cas and animal biotechnology



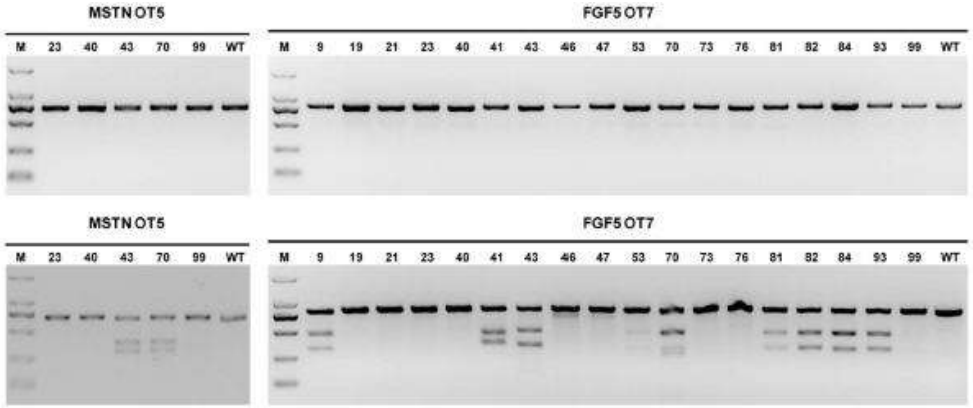


Founder animals
are complex mosaic

Targeting *MSTN* and *FGF5* by CRISPR-Cas9 in goat embryos

Wang et al. Sci Rep. 2015

CRISPR-Cas and animal biotechnology



Targeting *MSTN* and *FGF5* by CRISPR-Cas9 in goat embryos

	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	N	G	G
MSTN sg2	G	G	A	T	T	T	T	G	A	A	G	C	T	T	T	T	G	G	A	T	G	G	G
OT5	A	G	A	T	T	A	A	G	A	A	G	C	T	T	T	T	G	G	A	T	G	G	G

```
GATTTACTGAGCATGGCCCCACCCATCCAAAAGCTTCTTAATCTTCTCCATCAGAGGGCAGACA OT5 WT
GATTTACTGAGCATGGCCCCACCCA----AAAGCTTCTTAATCTTCTCCATCAGAGGGCAGACA #43 -4
GATTTACTGAGCATGGCCCCACCCA----AAAGCTTCTTAATCTTCTCCATCAGAGGGCAGACA #70 -4
```

	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	N	G	G
FGF5 sg1	G	G	C	T	G	C	C	A	C	G	G	A	G	A	G	G	A	A	C	C	C	G	G
OT7	G	G	T	T	G	C	C	A	A	G	A	A	G	A	G	G	A	A	C	C	T	G	G

```
TGAGGGAGGAGCATTTGTCTTCCAGGTTCCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC OT7 WT
TGAGGGAGGAGCATTTG-------------TCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #9  -13
TGAGGGAGGAGCATTTGTCTTCCAtG--CCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #43 -2m1
TGAGGGAGGAGCATTTGTCT-------TCCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #70 -7
TGAGGGAGGAGCATTTGTCTTCCtG---CCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #70 -3m1
TGAGGGAGGAGCATTTGTCT-------TCCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #82 -7
TGAGGGAGGAGCATTTGTCTTCCAGGTTCCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #84 +5
                          ^
                          gtctt
TGAGGGAGGAGCATTTGTCTTCCAGG-------------CAACCAGTTCAGAATTCATCTCCTC #84 -13
TGAGGGAGGAGCATTTGTCT-------TCCTCTTCTTGGCAACCAGTTCAGAATTCATCTCCTC #93 -7
```

Founder animals show off-target gene editions

Wang et al. Sci Rep. 2015

CRISPR-Cas and animal biotechnology

Targeting *MSTN* by CRISPR-Cas9 in pig fibroblasts
Obtaining MSTN mutant pigs by SCNT

Wang et al. Sci Rep. 2015

Editing pig genome with ZFNs

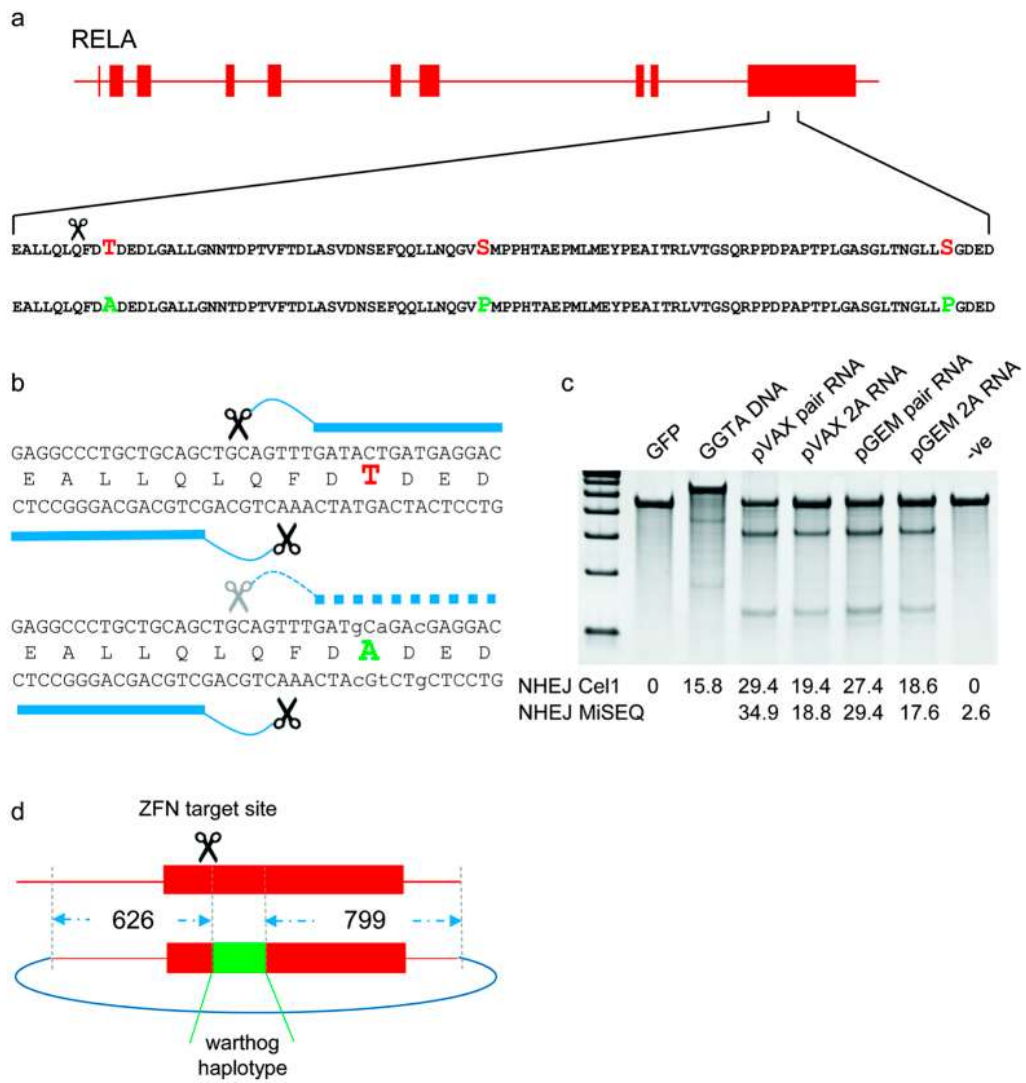
Targeting *MSTN* by ZFNs in pig fibroblasts
Obtaining MSTN mutant pigs by SCNT

Qian et al. 2015 *Sci. Rep.*

Editing pig genome with ZFNs

Warthog (wild pig)
resilient to African Swine Fever

Domestic pig
vulnerable to African Swine Fever



Lillico et al. 2016 *Sci. Rep.*

Editing pig genome with ZFNs

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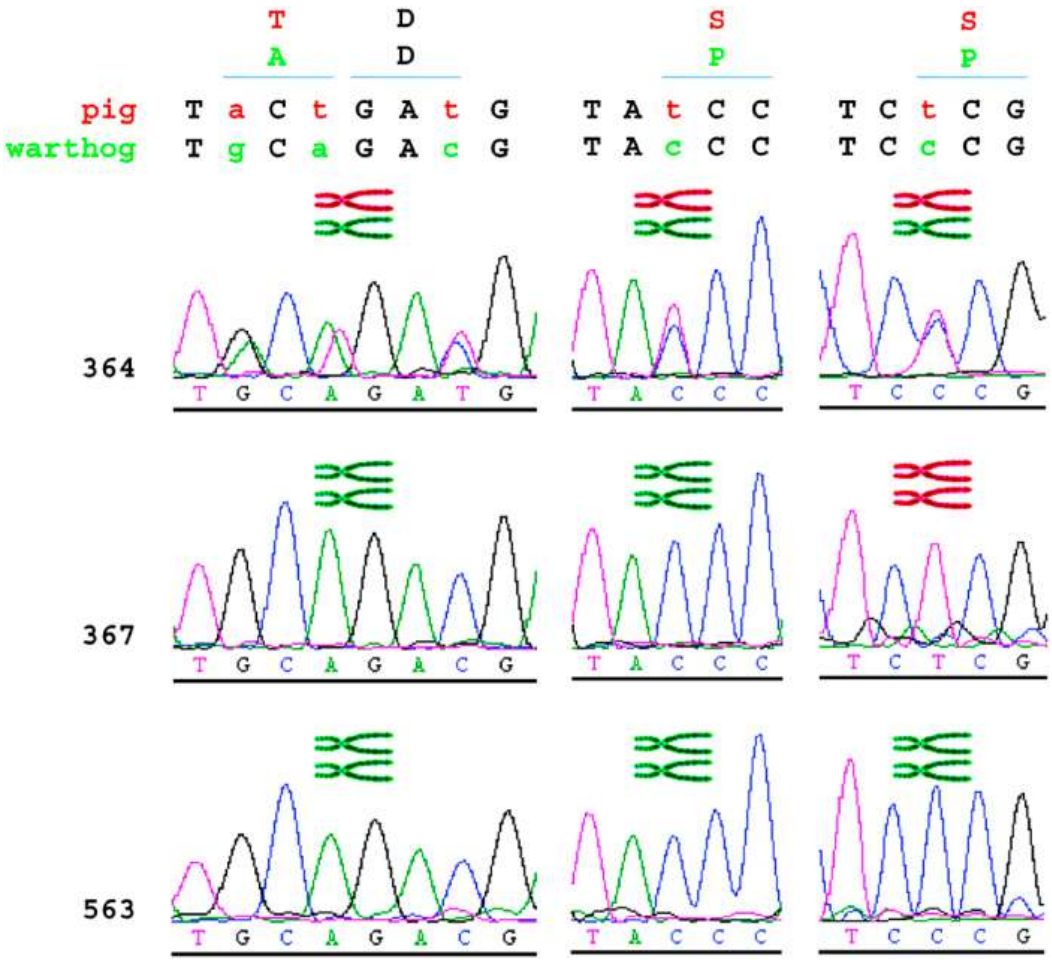


Lillico et al. 2016 *Sci. Rep.*

Production of hornless dairy cattle from genome-edited cell lines

HORNS

Holsteins dairy cattle (80%)
Angus beef cattle (20%)

Dehorning…

recombinetics

Scott Fahrenkrug

Tan et al. 2013 PNAS
Carlsson et al. 2016 Nature Biotech

Production of hornless dairy cattle from genome-edited cell lines

Introgression of the Pc (*POLLED*) allele through TALENs in Holstein bovine fibroblasts and using homozygous clones to obtain hornless cattle by SCNT

Table 1 Animal production statistics

Cell line	Parental cells	Genotype	SCNT rep	Blast rate (%)	Embryos/ recipients	Pregnant at day 40	Pregnant at day 90	Liveborn	Alive at 60 d
HP14-B6	2122	Homozygous *POLLED*	1	27/64 (42)	9/9	6	0	0	0
HP14-B4	2122	Homozygous *POLLED*	1	3/15 (20)	1/1	1	1	1[a]	0
HP7-P4-A1	2120	Homozygous *POLLED*	2	25/82 (30)	9/9	2	2	2	2[b]
HP-24.8	2120	Heterozygous *POLLED*	3	35/151 (23)	7/7	5	2	2[a]	0
Summary				70/295 (24%)	26/26	14/26 (54%)	5/26 (19%)	5/26 (19%)	2/26 (7%)

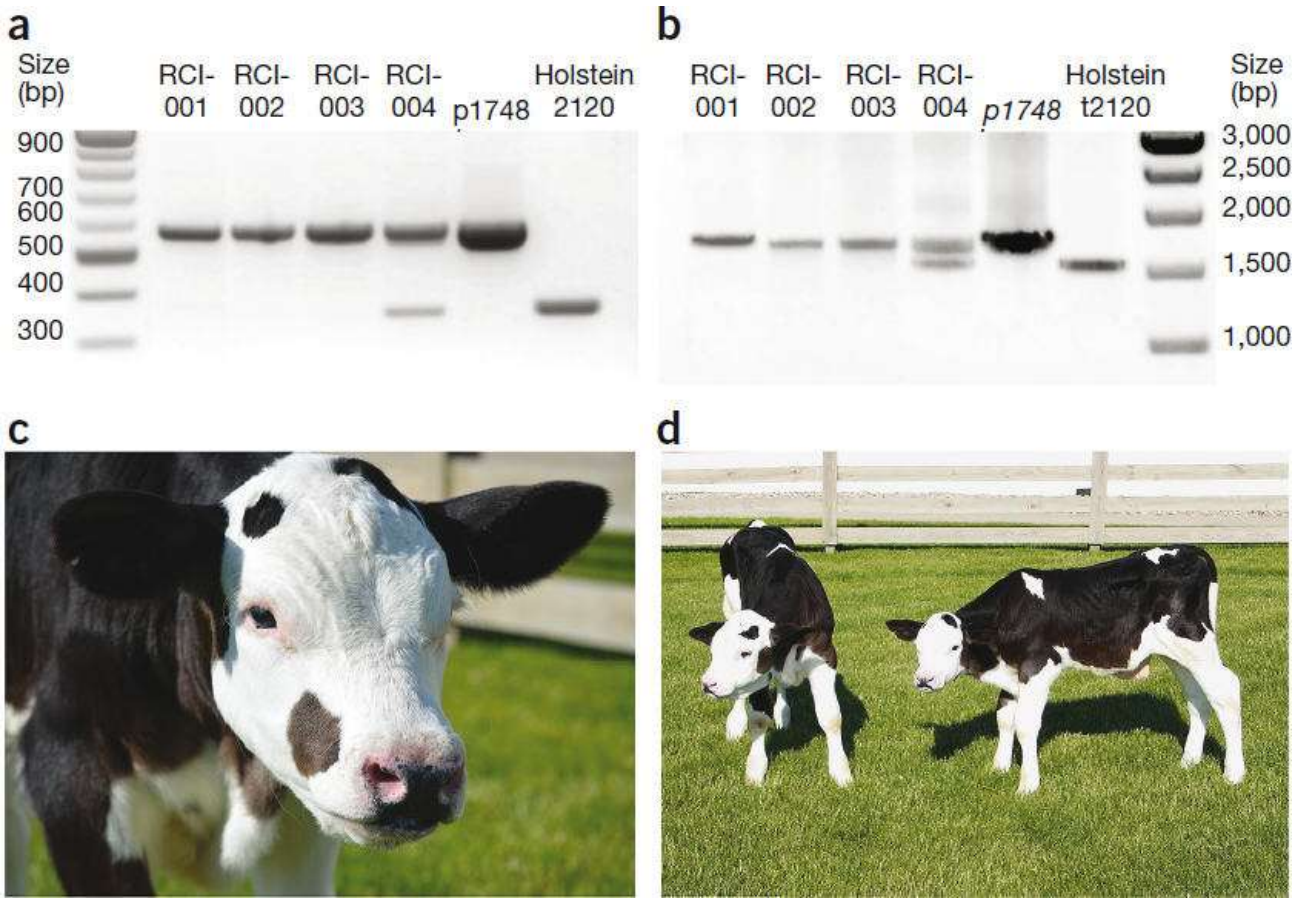
[a]RCI-001 (HP14-B6), RCI-004 and RCI-005 (HP-24.8). Consistent with known cloning inefficiencies, these animals were not viable and were humanely euthanized within 24 h of birth[11].
[b]Spotigy (RCI-002) and Buri (RCI-003). SCNT rep, somatic cell nuclear transfer replicates.

recombinetics

Scott Fahrenkrug

Tan et al. 2013 PNAS
Carlsson et al. 2016 Nature Biotech

Production of hornless dairy cattle from genome-edited cell lines

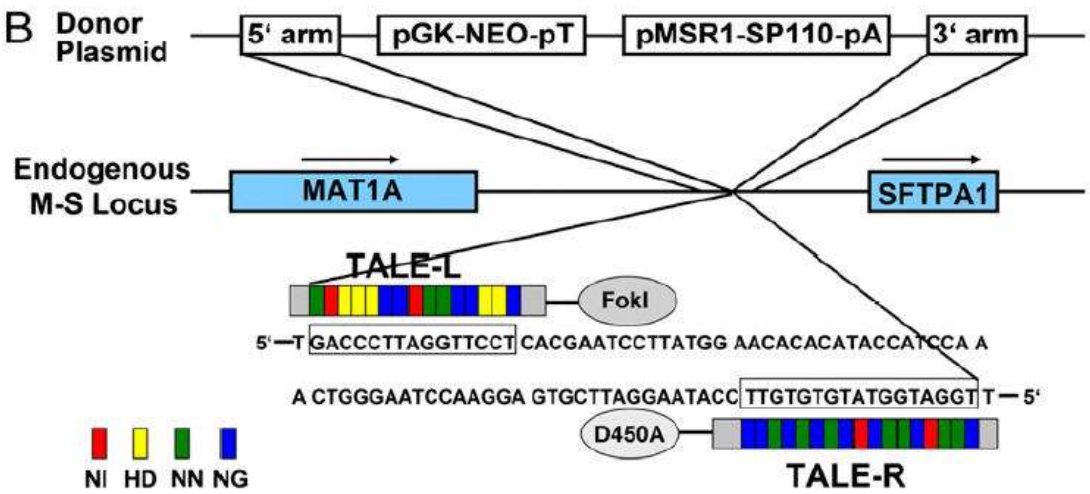


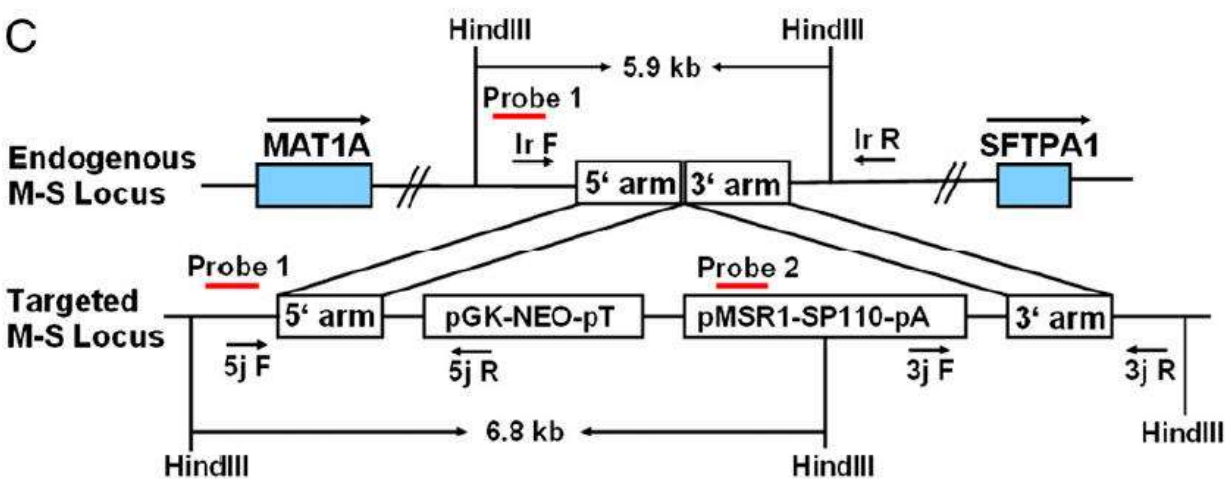
recombinetics

Scott Fahrenkrug

Tan et al. 2013 PNAS
Carlsson et al. 2016 Nature Biotech

Production of transgenic cattle with increased resistance to tuberculosis via TALENs and SCNT





-Knockin of a SP110-expressing transgene in an intergenic area in cells

-SCNT from selected clones to derive transgenic cattle

Wu et al. 2015 PNAS

Production of transgenic cattle with increased resistance to tuberculosis via TALENs and SCNT

A

Table 2. Gross pathology of transgenic cattle challenged with *M. bovis* by endobronchial instillation

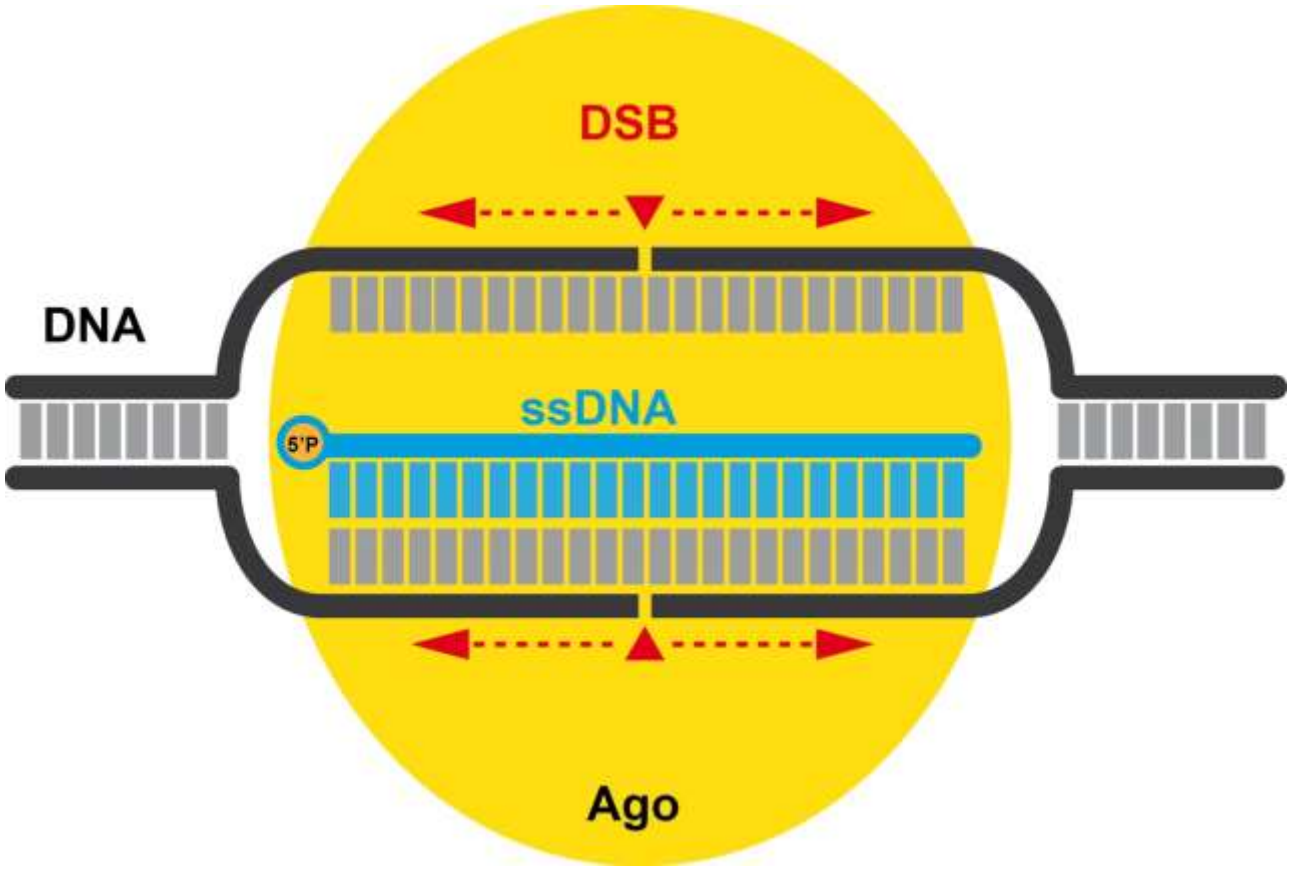
Animal	No. of lobes infected*	Lung score	No. of lymph nodes infected†	Lymph node score	Total pathology score	Mean‡
Transgenic 1	2	4	3	4	8	6.5
Transgenic 2	1	2	2	3	5	
Transgenic 3	0	0	0	0	0	
Control 1	5	21	6	14	35	32.0
Control 2	4	15	8	18	33	
Control 3	4	14	6	14	28	

Wu et al. 2015 PNAS

Genomic Editing Tools: 4 flavours!

Type	Sequence Homology	Double Strand Break
Zinc-Finger Nuclease (**ZFN**)	**PROTEIN** 1 Zinc finger (3 AA) → 3 bp	**PROTEIN** FokI
TALEN	**PROTEIN** 2 AA → 1 bp	**PROTEIN** FokI
CRISPR-Cas9	**RNA** 1 ribonucleotide → 1 bp	**PROTEIN** Cas9
Argonaute	**DNA** 1 nucleotide → 1 bp	**PROTEIN** Ago

Argonaute



5' Phosphorylated single-strand DNA guides (24 nucleotides)
PAM sequences is not required
Less tolerance to mismatches (lower risk for off-targets)

Swarts et al. 2014 Nature (Pepe Berenguer, CBMSO)

Gao et al. 2016 Nature Biotech

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The CRISPR page at CNB

by Lluís Montoliu (CNB-CSIC)

with help and comments from Davide Seruggia (Boston Children's Hospital) and Francisco Mojica (Universidad de Alicante)

Lluís Montoliu's Lab Web Page

LAST UPDATED: 26 April 2016

www.cnb.csic.es/~montoliu/CRISPR/

Google for "CRISPR CNB"

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Br[35]eaking Ca[20]s

Breaking-Cas

Oligo guide design tool for CRISPR based genome editing. Any eukaryote genomic sequence available in ENSEMBL (release 84) or ENSEMBLGENOMES (release 31) can be used as reference.

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Tutorial

1 Choose organism: (alphabetic list) [ ] *Write 3 letters or more and select it.*

2 Paste one or several query DNA sequences in FASTA format (up to 20.000 nucleotides in total):

Or upload FASTA file (DNA): Seleccionar archivo Ningún archivo seleccionado

3 Select nuclease settings: Cas9: Streptococcus pyogenes Cas9 (SpCas9) ("NGG" in 3')

Or set your own parameters:

PAM sequence: NGG
PAM position: 5' 3'
Guide length: 20 nts.
Mismatches: Up to 4

Use predefined settings for Cas9 or Cpf1, or set custom parameters for other nucleases. If necessary, write a different PAM sequence (in IUPAC notation). For Cas9, positional weights based on Hsu et al. (2013) are used by default. See tutorial pages for details on off-targets score's calculation.

Position-dependant weights Edit

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	(PAM)	
5'-	0	0	0.014	0	0	0.395	0.317	0	0.389	0.079	0.445	0.508	0.613	0.851	0.732	0.828	0.615	0.804	0.685	0.583	NGG	-3'

Confirmation email (optional): [ ] *To receive a message as soon the job finishes. **Write it carefully** (it will not be checked).*

Fill with example Submit Clear fields

Oliveros *et al.* Nucleic Acids Res. 2016

The CRISPR web page at CNB

www.cnb.csic.es/~montoliu/CRISPR/

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