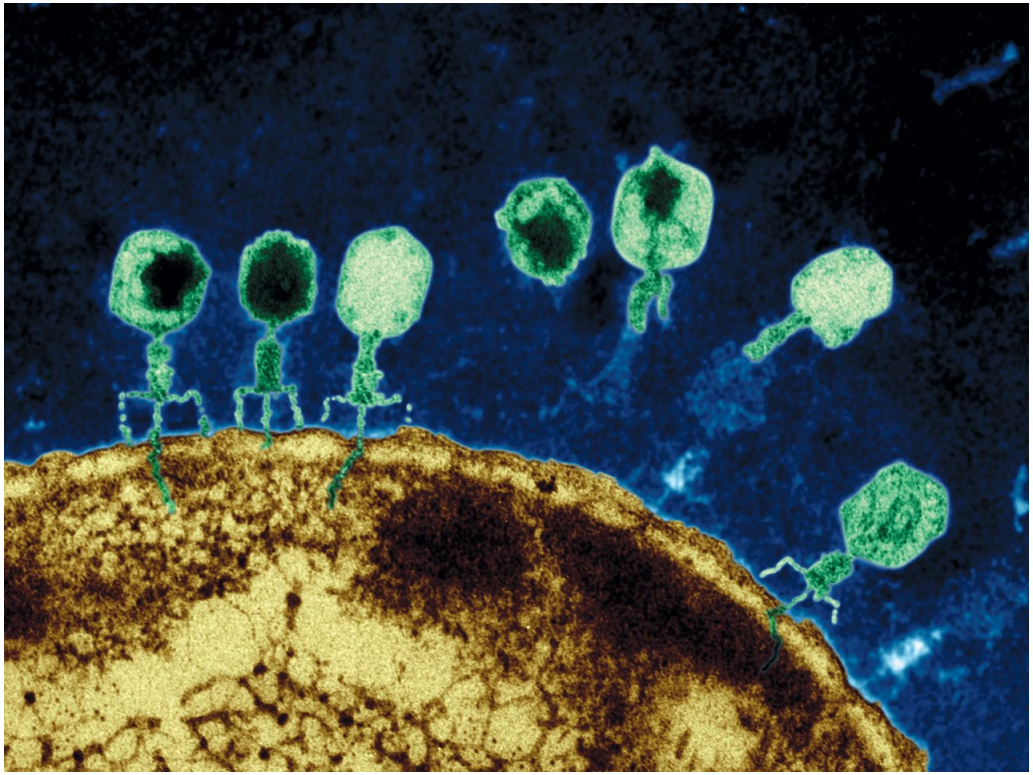
Edição gênica: CRISPR/Cas9

O CRISPR/Cas9 é o nome de uma técnica de biologia molecular capaz de editar (remover, adicionar, trocar) sequências de DNA localizadas em qualquer região do genoma. Essa técnica é baseada em um sistema de memória imunológica presente nas bactérias, usado para protegê-las de invasões por bacteriófagos.

O sistema CRISPR/Cas9 consiste em duas moléculas:

-Cas9: uma enzima (nuclease) que atua como um par de “tesouras” que pode cortar as duas fitas do DNA em um local específico no genoma.

-RNA guia (gRNA): um pequeno pedaço de sequência de RNA (com cerca de 20 bases de comprimento) localizada dentro de uma estrutura de RNA mais longa. Essa molécula de RNA “guia” a Cas9 para a parte do genoma que deverá ser cortada.

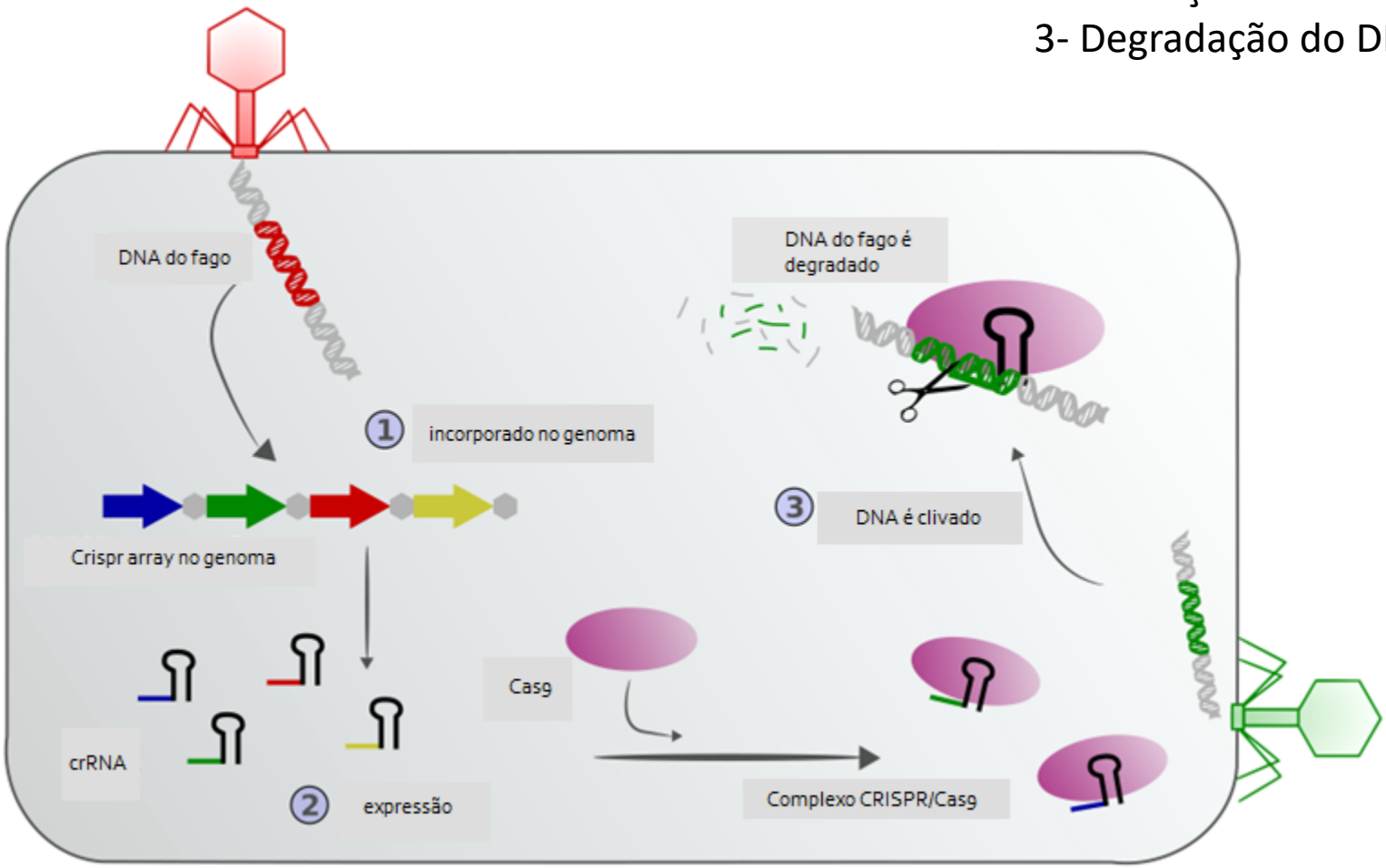


The CRISPR Craze
SCIENCE
23 Aug 2013
Vol 341, Issue 6148
pp. 833-836

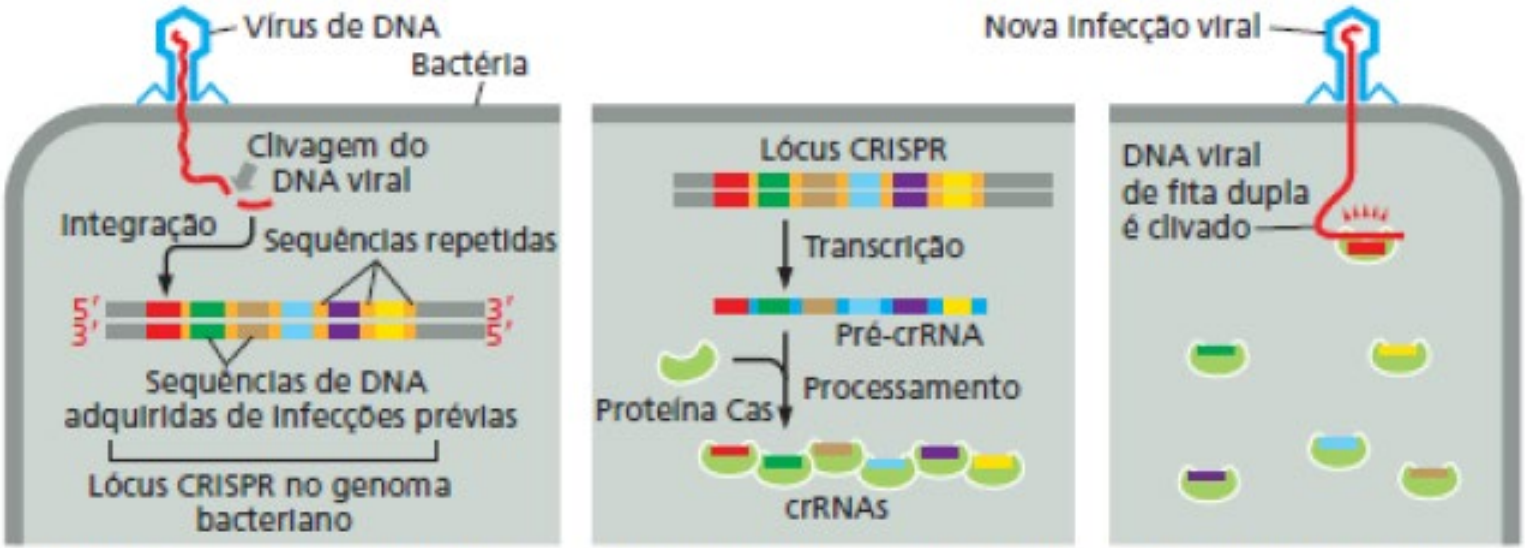
Bactérias como a ***Streptococcus thermophilus*** são utilizadas para para fazer iogurtes e queijos. Mas certos vírus – bacteriófagos podem debilitar a bactéria, causando estragos na qualidade ou quantidade dos alimentos que ela ajuda a produzir. Em 2007, cientistas da Danisco, uma empresa de ingredientes alimentares sediada em Copenhagen, agora propriedade da DuPont, descobriram uma forma de aumentar as defesas destas bactérias a fagos. Expuseram a bactéria a um fago e mostraram que isso essencialmente a vacinava contra esse vírus (Science, 23 de março de 2007, p. 1650). O truque permitiu à DuPont criar cepas de bactérias mais resistentes para a produção de alimentos. Também revelou algo fundamental: as bactérias têm uma espécie de sistema imune adaptativo, que lhes permite lutar contra ataques repetidos de fagos específicos.

Imunidade mediada por CRISPR/Cas em procariotos

1- Aquisição do DNA viral
2- Produção dos RNAs guias
3- Degradação do DNA invasor



Imunidade mediada por CRISPR/Cas em procariotos



ETAPA 1: pequenas sequências de DNA viral são integradas no lócus CRISPR

ETAPA 2: RNA é transcrito a partir do lócus CRISPR, processado e ligado à proteína Cas

ETAPA 3: crRNA pequeno complexado com Cas procura e destrói sequências virais

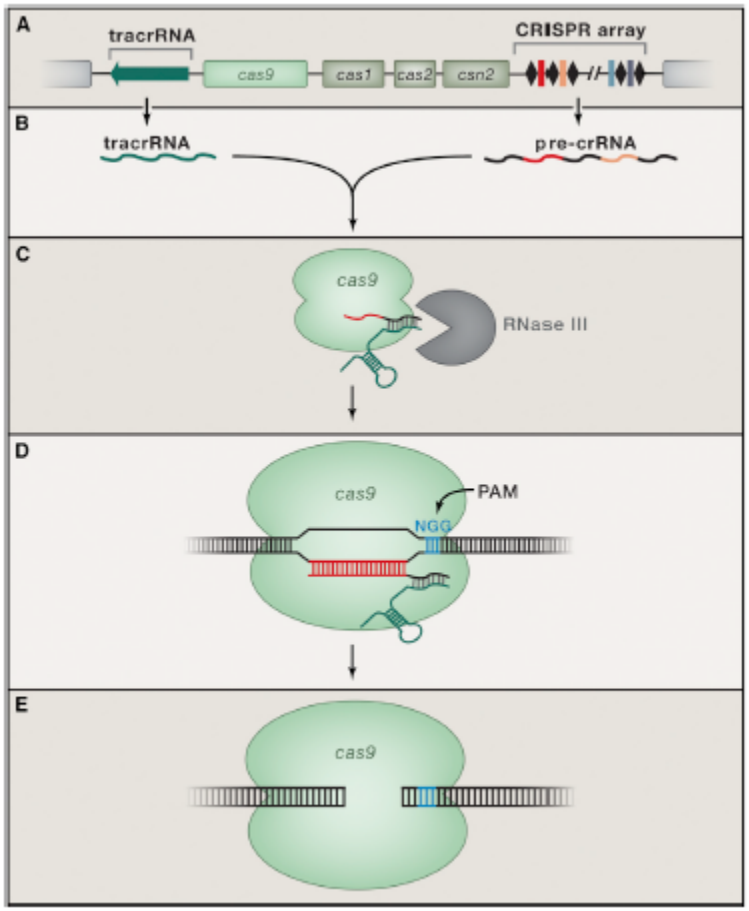


Figure 1. Class 2, Type II CRISPR-Cas9 System from *Streptococcus thermophilus*

Type II systems are the simplest of the three types of CRISPR systems and have been the basis for genome editing technology.

(A) The locus contains a CRISPR array, four protein-coding genes (*cas9*, *cas1*, *cas2*, and *cns2*) and the tracrRNA. The CRISPR array contains repeat regions (black diamonds) separated by spacer regions (colored rectangles) derived from phage and other invading genetic elements. The *cas9* gene encodes a nuclease that confers immunity by cutting invading DNA that matches existing spacers, while the *cas1*, *cas2*, and *cns2* genes encode proteins that function in the acquisition of new spacers from invading DNA.

(B) The CRISPR array and the tracrRNA are transcribed, giving rise to a long pre-crRNA and a tracrRNA.

(C) These two RNAs hybridize via complementary sequences and are processed to shorter forms by Cas9 and RNase III.

(D) The resulting complex (Cas9 + tracrRNA + crRNA) then begins searching for the DNA sequences that match the spacer sequence (shown in red). Binding to the target site also requires the presence of the protospacer adjacent motif (PAM), which functions as a molecular handle for Cas9 to grab on to.

(E) Once Cas9 binds to a target site with a match between the crRNA and the target DNA, it cleaves the DNA three bases upstream of the PAM site. Cas9 contains two endonuclease domains, HNH and RuvC, which cleave, respectively, the complementary and non-complementary strands of the target DNA, creating blunt ends.

tracrRNA= transactivator crispr RNA
PAM= protospacer adjacente motif
cRNA= crispr RNA

Eric S. Lander, The heroes of CRISPR, Cell:164 (2016)

CRISPR-Cas 9

(Conjunto de Repetições Palindrômicas Regularmente Espaçadas em associação com a nuclease Cas9)

Francisco Mojica
Microbiologist who discovered and named CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats

Credit: Alexander Heinel/Picture Alliance/DPA

Jennifer Doudna and Emmanuelle Charpentier showed that the system could be programmed to cut specific sites in isolated DNA. They shared the 2020 Nobel Chemistry Prize.

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]† Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

Bacteria and archaea have evolved RNA-mediated adaptive defense systems called clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) that protect organisms from invading viruses and plasmids (*1*–*3*). These defense systems rely on small RNAs for sequence-specific detection and silencing of foreign nucleic acids. CRISPR/Cas systems are composed of *cas* genes organized in operon(s) and CRISPR array(s) consisting of genome-targeting sequences (called spacers) interspersed with identical repeats (*1*–*3*). CRISPR/Cas-mediated immunity occurs in three steps. In the adaptive phase, bacteria and archaea harboring one or more CRISPR loci respond to viral or plasmid challenge by integrating short fragments of foreign sequence (protospacers) into the host chromosome at the proximal end of the CRISPR array (*1*–*3*). In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets (*4*–*11*). Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs (*10*, *12*–*20*).

There are three types of CRISPR/Cas systems (*21*–*23*). The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process pre-crRNAs by a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA-specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein (fig. S1) (*4*, *24*). Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA (*25*–*27*).

We show here that in type II systems, Cas9 proteins constitute a family of enzymes that require a base-paired structure formed between the activating tracrRNA and the targeting crRNA to cleave target dsDNA. Site-specific cleavage occurs at locations determined by both base-pairing complementarity between the crRNA and the target protospacer DNA and a short motif [referred to as the protospacer adjacent motif (PAM)] juxtaposed to the complementary region in the target DNA. Our study further demonstrates that the Cas9 endonuclease family can be programmed with single RNA molecules to cleave specific DNA sites, thereby raising the exciting possibility of developing a simple and versatile RNA-directed system to generate dsDNA breaks for genome targeting and editing.

Cas9 is a DNA endonuclease guided by two RNAs. Cas9, the hallmark protein of type II systems, has been hypothesized to be involved in both crRNA maturation and crRNA-guided DNA interference (fig. S1) (*4*, *25*–*27*). Cas9 is involved in crRNA maturation (*4*), but its direct participation in target DNA destruction has not been investigated. To test whether and how Cas9 might be capable of target DNA cleavage, we used an overexpression system to purify Cas9 protein derived from the pathogen *Streptococcus pyogenes* (fig. S2, see supplementary materials and methods) and tested its ability to cleave a plasmid DNA or an oligonucleotide duplex bearing a protospacer sequence complementary to a mature crRNA, and a bona fide PAM. We found that mature crRNA alone was incapable of directing Cas9-catalyzed plasmid DNA cleavage (Fig. 1A and fig. S3A). However, addition of tracrRNA, which can pair with the repeat sequence of crRNA and is essential to crRNA maturation in this system, triggered Cas9 to cleave plasmid DNA (Fig. 1A and fig. S3A). The cleavage reaction required both magnesium and the presence of a crRNA sequence complementary to the DNA; a crRNA capable of tracrRNA base pairing but containing a noncognate target DNA-binding sequence did not support Cas9-catalyzed plasmid cleavage (Fig. 1A; fig. S3A, compare crRNA-sp2 to crRNA-sp1; and fig. S4A). We obtained similar results with a short linear dsDNA substrate (Fig. 1B and fig. S3, B and C). Thus, the trans-activating tracrRNA is a small noncoding RNA with two critical functions: triggering pre-crRNA processing by the enzyme RNase III (*4*) and subsequently activating crRNA-guided DNA cleavage by Cas9.

Cleavage of both plasmid and short linear dsDNA by tracrRNA:crRNA-guided Cas9 is site-specific (Fig. 1, C to E, and fig. S5, A and B). Plasmid DNA cleavage produced blunt ends at a position three base pairs upstream of the PAM sequence (Fig. 1, C and E, and fig. S5, A and C) (*26*). Similarly, within short dsDNA duplexes, the DNA strand that is complementary to the target-binding sequence in the crRNA (the complementary strand) is cleaved at a site three base pairs upstream of the PAM (Fig. 1, D and E, and fig. S5, B and C). The noncomplementary DNA strand is cleaved at one or more sites within three to eight base pairs upstream of the PAM. Further investigation revealed that the noncomplementary strand is first cleaved endonucleolytically and subsequently trimmed by a 3′-5′ exonuclease activity (fig. S4B). The cleavage rates by Cas9 under single-turnover conditions ranged from 0.3 to 1 min^{-1}, comparable to those of restriction endonucleases (fig. S6A), whereas incubation of wild-type (WT) Cas9-tracrRNA:crRNA complex with a fivefold molar excess of substrate DNA provided evidence that the dual-RNA–guided Cas9 is a multiple-turnover enzyme (fig. S6B). In

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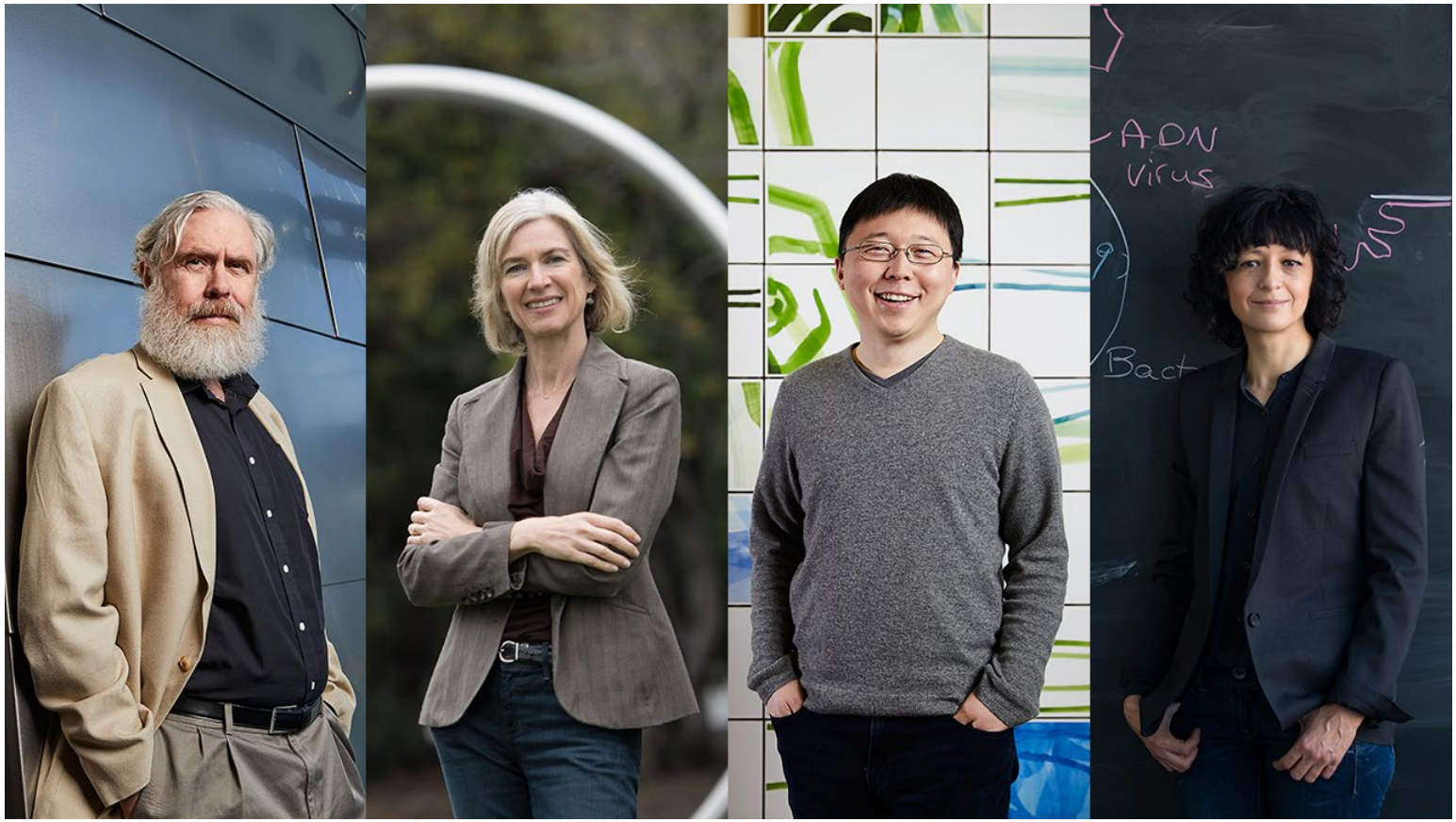
Guerra de patentes

Pesquisadores duelam por direitos de explorar a ferramenta de edição de genes CRISPR-Cas9 na Justiça norte-americana

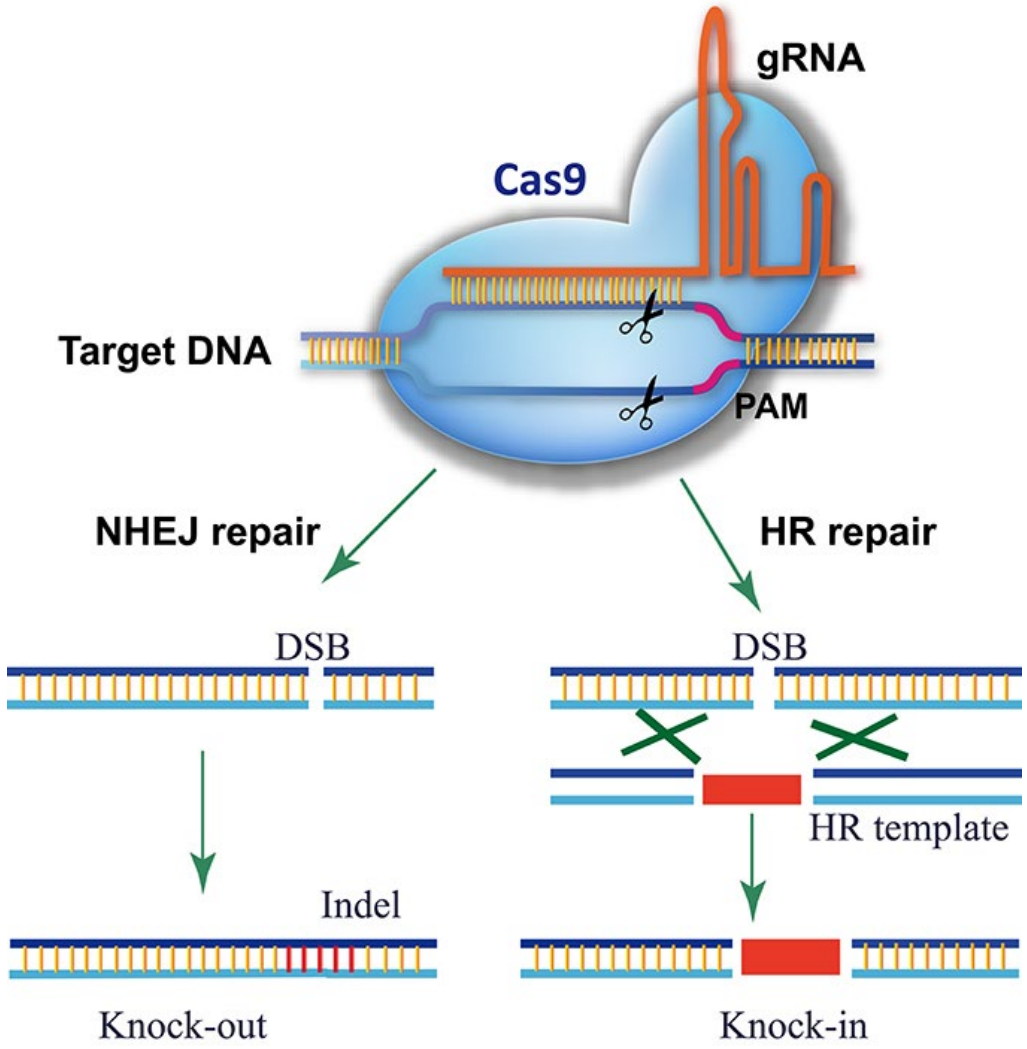


George Church Jennifer Doudna Feng Zhang Emmanuelle Charpentier

https://www.science.org/content/article/how-battle-lines-over-crispr-were-drawn

https://revistapesquisa.fapesp.br/guerra-de-patentes/

Edição de genoma utilizando CRISPR/Cas



gRNA= RNA guia

DBS= double strand break

Edição com Crispr/Cas9 desencadeia reparo endógeno de quebra de DNA dupla fita.

Limitações:

-muitas pesquisas ainda são necessárias para avaliar a eficiência e, principalmente, a segurança de utilizar essa técnica em humanos.

-“off-targets” (fora do alvo):geração de mutações indesejadas.

- edição do DNA levanta muitas questões éticas.

He Jiankui alegou ter modificado um gene (o CCR5) de embriões que foram implantados em uma mulher e resultaram no nascimento de duas crianças com uma alteração que as torna resistentes à infecção por HIV.

https://revistapesquisa.fapesp.br/chines-e-suspenso-por-ter-criado-bebes-com-gene-alterado/

Vantagens:

-Simplicidade e facilidade em desenhar o alvo (complexo Cas com sequência de RNA);

-Edições podem ser realizadas em mais de um gene ao mesmo tempo;

- Mais barato que métodos anteriores de edição de genoma.

https://www.youtube.com/watch?v=4YKFw2KZA5o

https://www.youtube.com/watch?v=2pp17E4E-O8

CRISPR/Cas e tratamento de doenças genéticas

Ex: anemia falciforme

News & analysis

News

https://doi.org/10.1038/d41573-023-00050-8

First CRISPR therapy seeks landmark approval

By Katie Kingwell

Vertex and CRISPR Therapeutics's first-in-modality genome-editor exa-cel, for the treatment of two haemoglobinopathies, has entered the regulatory spotlight.

Vertex and CRISPR Therapeutics have submitted their CRISPR-based ex vivo cell therapy exagamglogene autotemcel (exa-cel) for FDA approval, for sickle cell disease (SCD) and β-thalassaemia. A regulatory decision on the gene-editing candidate is expected within 8 to 12 months. The companies have also filed for approval in Europe and the UK.

"This is an incredible validation for a technology that 10 years ago was a paper," says Santino Sanna, chief of cell and genetic therapies at Vertex, which partnered with CRISPR Therapeutics in 2015 to develop exa-cel, formerly called CTX-001. "It was like opening a bottle: when the right tool came, suddenly we could use all the science that the [haemoglobinopathy] field had stored up for so many years," says Sanna.

"It's astounding," agrees Jennifer Doudna, one of the scientists who showed in 2012 that the CRISPR–Cas system could be used to edit genomes. "It was clear that having the ability to edit genomes was a powerful tool, but I don't think any of us could have imagined how fast the field would move." Doudna co-founded the genome-editing companies Editas and Intellia, while Emmanuelle Charpentier, a co-author of the 2012 paper, co-founded CRISPR Therapeutics. Doudna and Charpentier shared a Nobel Prize in 2020 for their ground-breaking work.

β-haemoglobinopathies are a popular testing ground for gene-targeted approaches, because the underlying genetic drivers are well understood and tractable. The target cells can also be modified ex vivo and returned to patients, sidestepping some delivery hurdles. Bluebird bio recently secured FDA approval of its lentiviral gene therapy betibeglogene autotemcel (beti-cel) in this setting. But with the competitive landscape maturing, some contenders are now retreating (Table 1).

Exa-cel data, from 75 patients in ongoing open-label studies, helped set the bar.

"It's been spectacular, and beyond anybody's expectations," says Martin Steinberg, a haematologist at Boston University and a member of Vertex's steering committee for exa-cel's development.

Vertex's gene-editing therapy stopped painful vaso-occlusive crises in SCD, and led to transfusion-independence in 90% of patients with β-thalassaemia over 1.2 to 37.2 months, the company has reported.

This is a step change over pharmacological therapies for SCD, such as the standard-of-care haemoglobin-boosting agent hydroxyurea, and more recently approved options such as GBT/Pfizer's anti-sickling agent voxelotor and Novartis's P-selectin-blocking antibody crizanlizumab. "Hydroxyurea is extremely useful, but it never cured anybody," says Steinberg. "The idea behind exa-cel is a curative intent."

So far, exa-cel's efficacy looks similar to that of lentiviral approaches, including Bluebird's investigational lentiviral gene therapy for SCD, Steinberg adds.

A 15-year follow-up trial of exa-cel is ongoing to assess its durability and longer-term safety. Concerns linger about the theoretical and practical possibilities of both on-target and off-target editing with CRISPR–Cas-based drugs.

The treatment regimen is also onerous, and is likely to be expensive. Because the majority of haemoglobinopathy patients live in under-resourced countries in Africa and elsewhere, access and affordability will remain huge hurdles. "I see it as a biological breakthrough. But, in the context of things, it's a small first step to making these types of gene therapies applicable to most people who need them," says Steinberg.

While an approval would buoy the rapidly evolving field of CRISPR–Cas therapeutics, more work is needed to improve the delivery and expand the therapeutic potential of the modality. "The two axes along which people are pushing forward are to improve delivery across organ systems, and to develop the technology so you can make gene corrections at higher efficiency," says Samarth Kulkarni, CEO of CRISPR Therapeutics.

Getting to the point

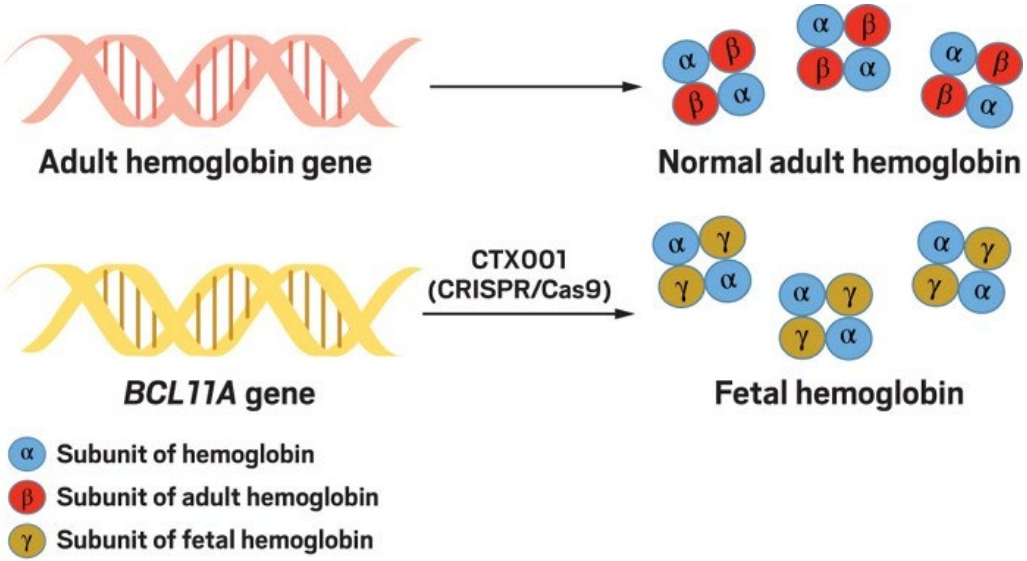
β-haemoglobinopathies are caused by mutations in the β-subunit of haemoglobin, the

nature reviews drug discovery

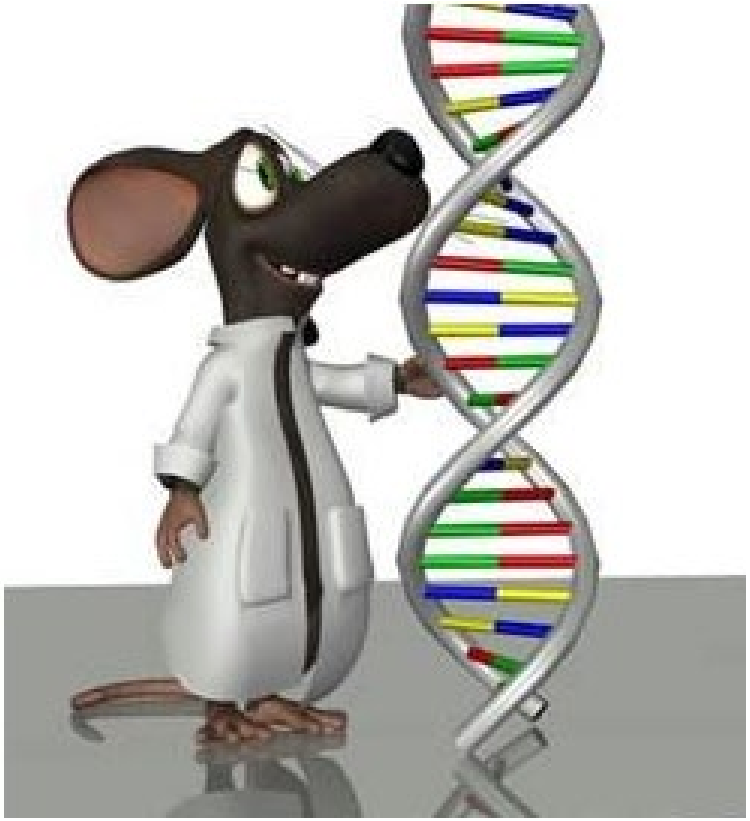
Volume 22 | May 2023 | 339–340 | 339

https://www.nature.com/articles/d41573-023-00050-8

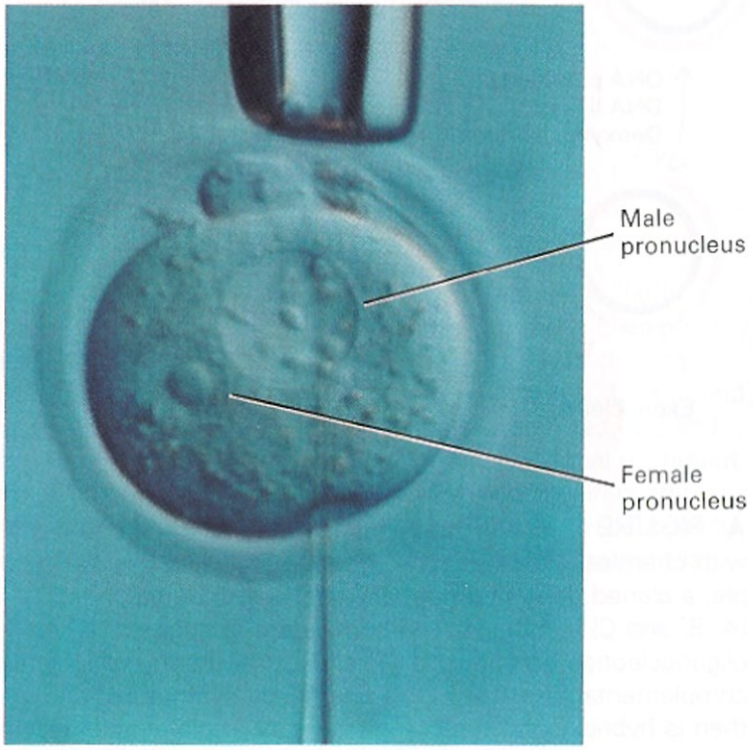
Silenciar o BCL11A, um repressor do gene da hemoglobina fetal, em células progenitoras hematopoiéticas (HSPCs) que foram colhidas de doentes. A hemoglobina fetal é normalmente silenciada nos meses que se seguem ao nascimento, mas a reativação do gene produz proteínas que podem compensar a beta-globina defeituosa em beta-hemoglobinopatias.



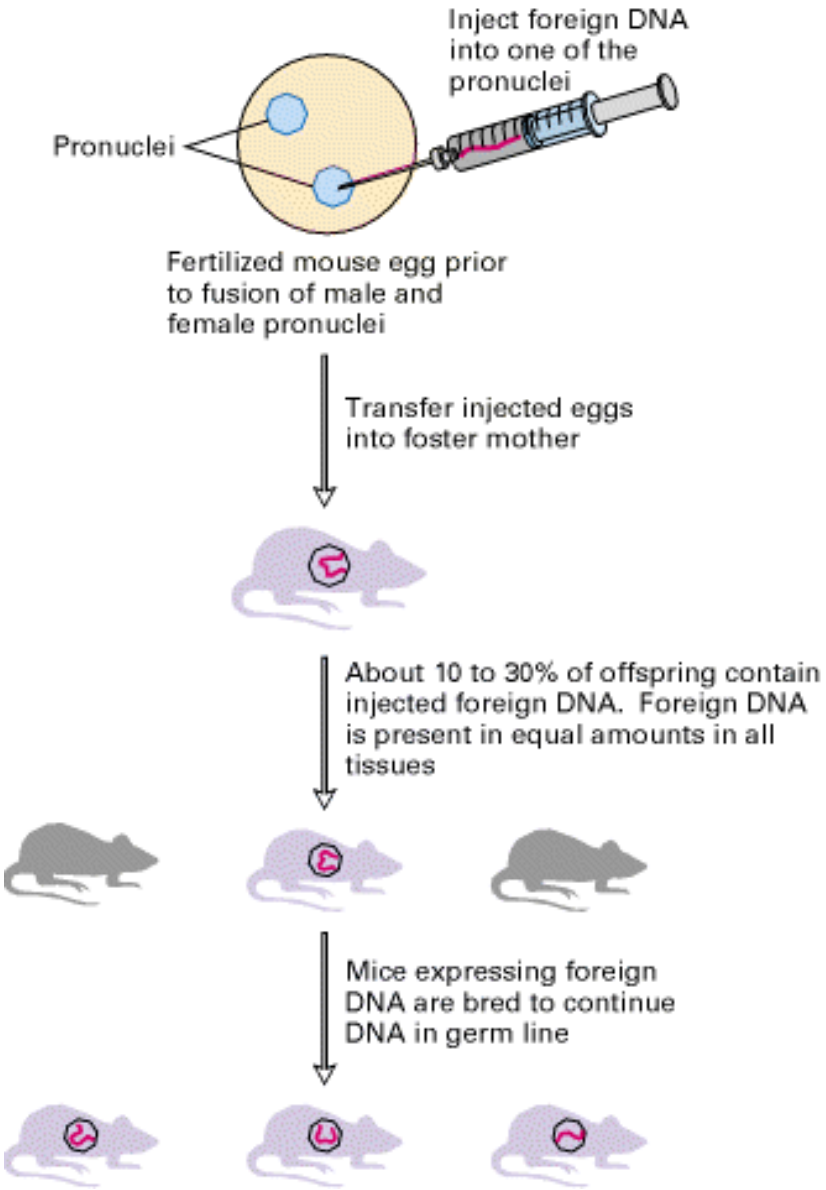
Camundongos transgênicos e *knock out.*



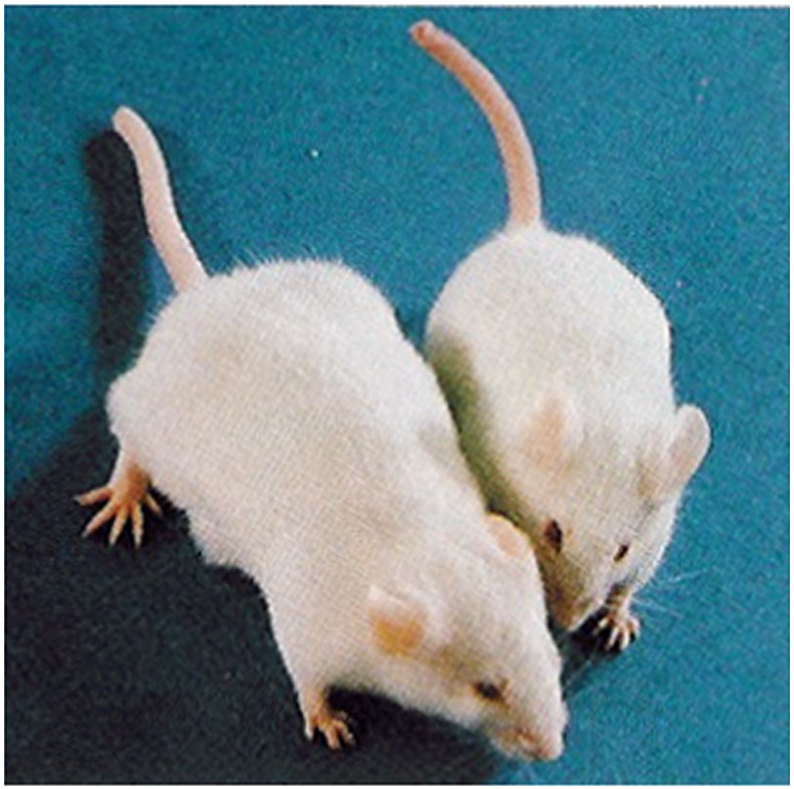
CAMUNDONGOS TRANSGÊNICOS (adição de genes)



Injeção do DNA contendo o transgene no ovócito fecundado de camundongo



Camundongo transgênico que apresenta múltiplas cópias do hormônio de crescimento de ratos, inseridas *in tandem* em um único sítio randômico em um de seus cromossomos.



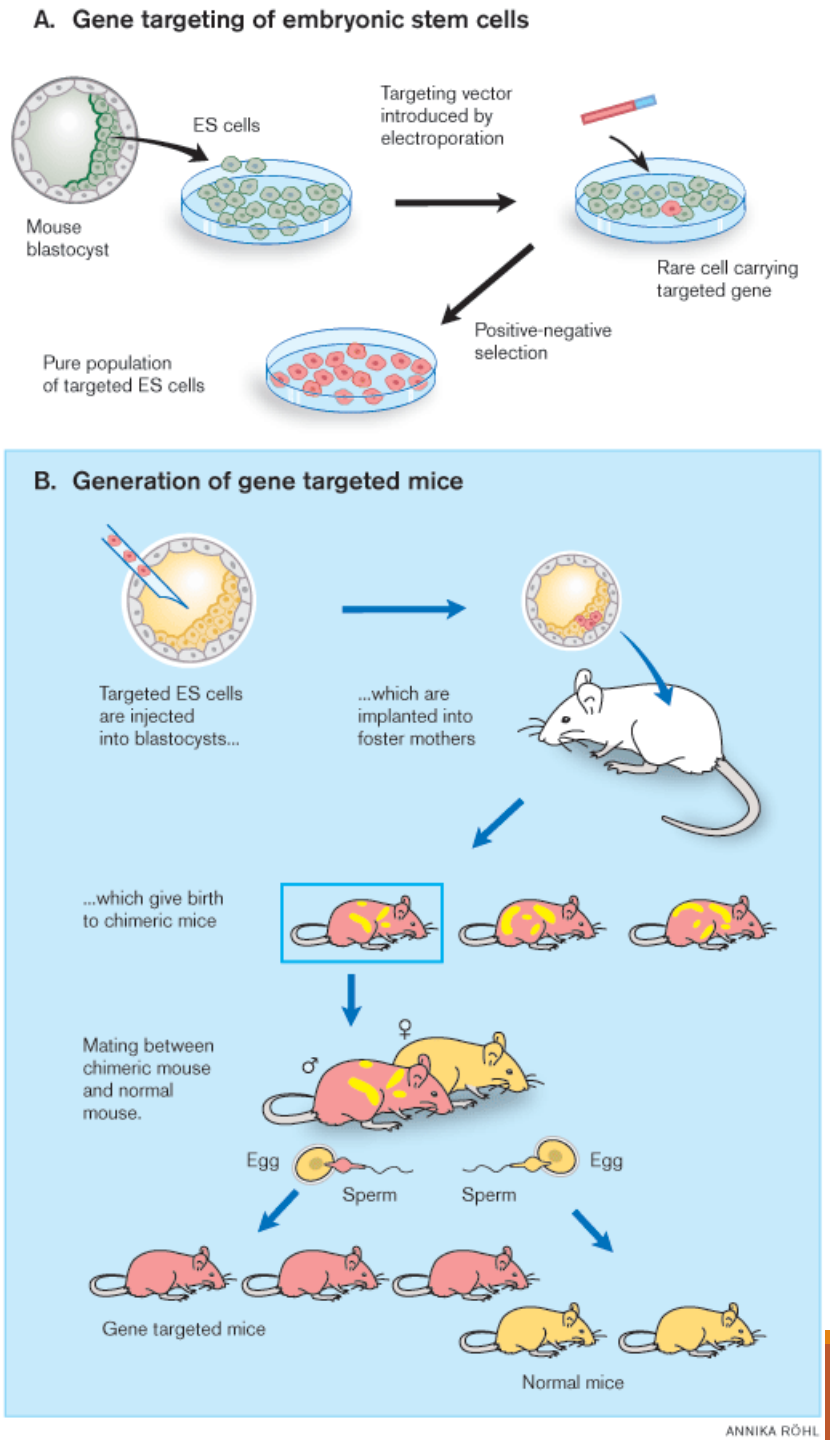
Camundongos *knock out*

KNOCK OUT DE GENES

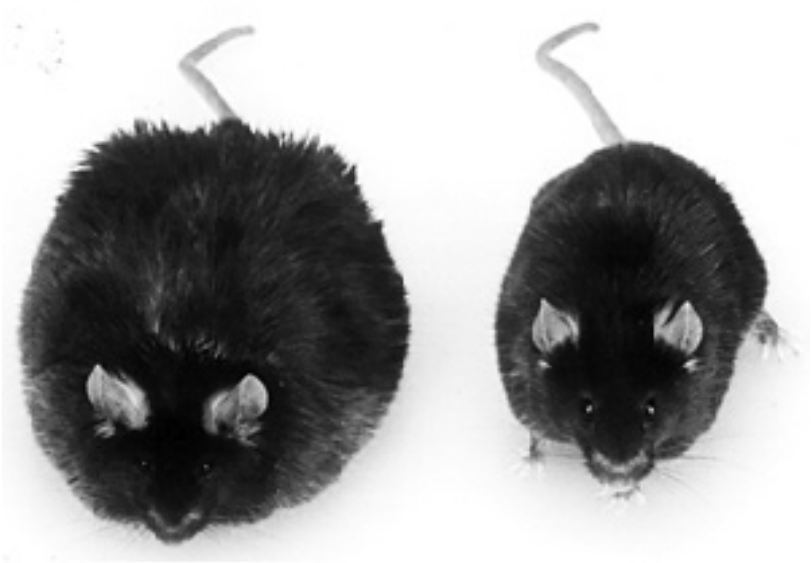
Inativação de um gene.

Através da substituição de um gene por um DNA artificial (*targeting vector*) através da recombinação homóloga

A recombinação homóloga: recombinação genética em que sequências são trocadas entre duas moléculas de DNA semelhantes ou idênticas.

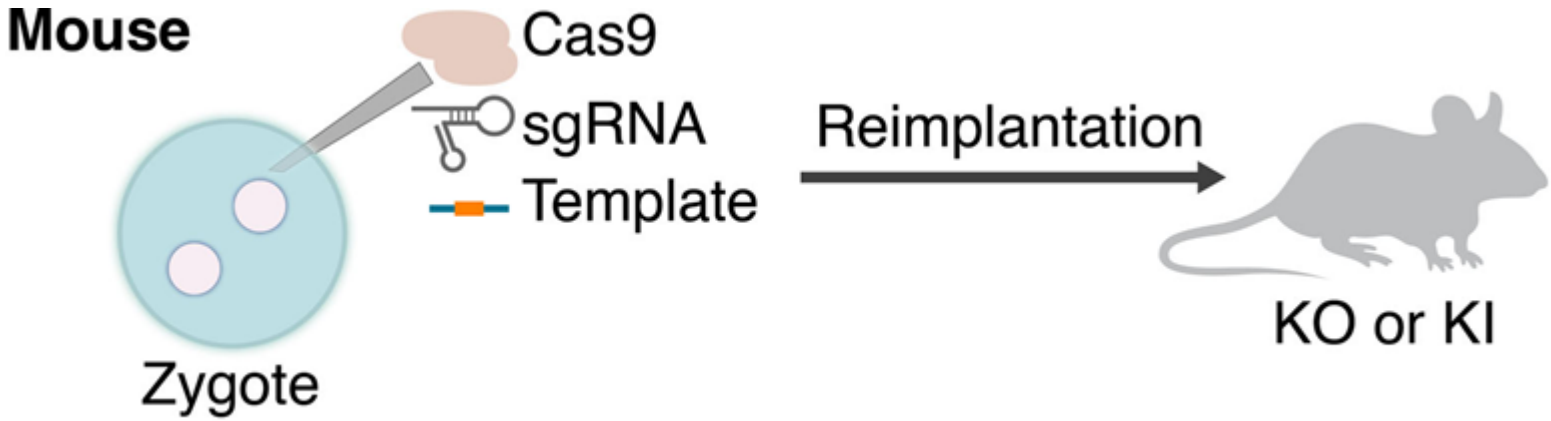


Camundongo quimérico

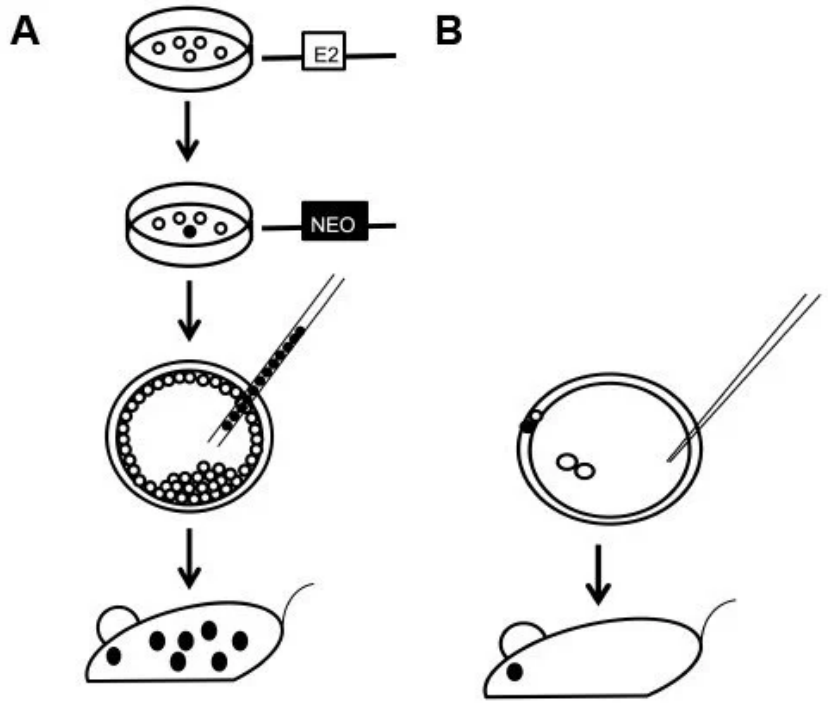


Camundongo
knock out
para o gene
da leptina

Camundongo
tipo *selvagem*



inject the mutated ES cells into a mouse embryo to create chimeric mice



inject CRISPR components directly into a zygote and obtain a knockout mouse