

Edição genética via CRISPR-Cas9

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GS Treinamentos e Consultoria

CRISPR - Origem

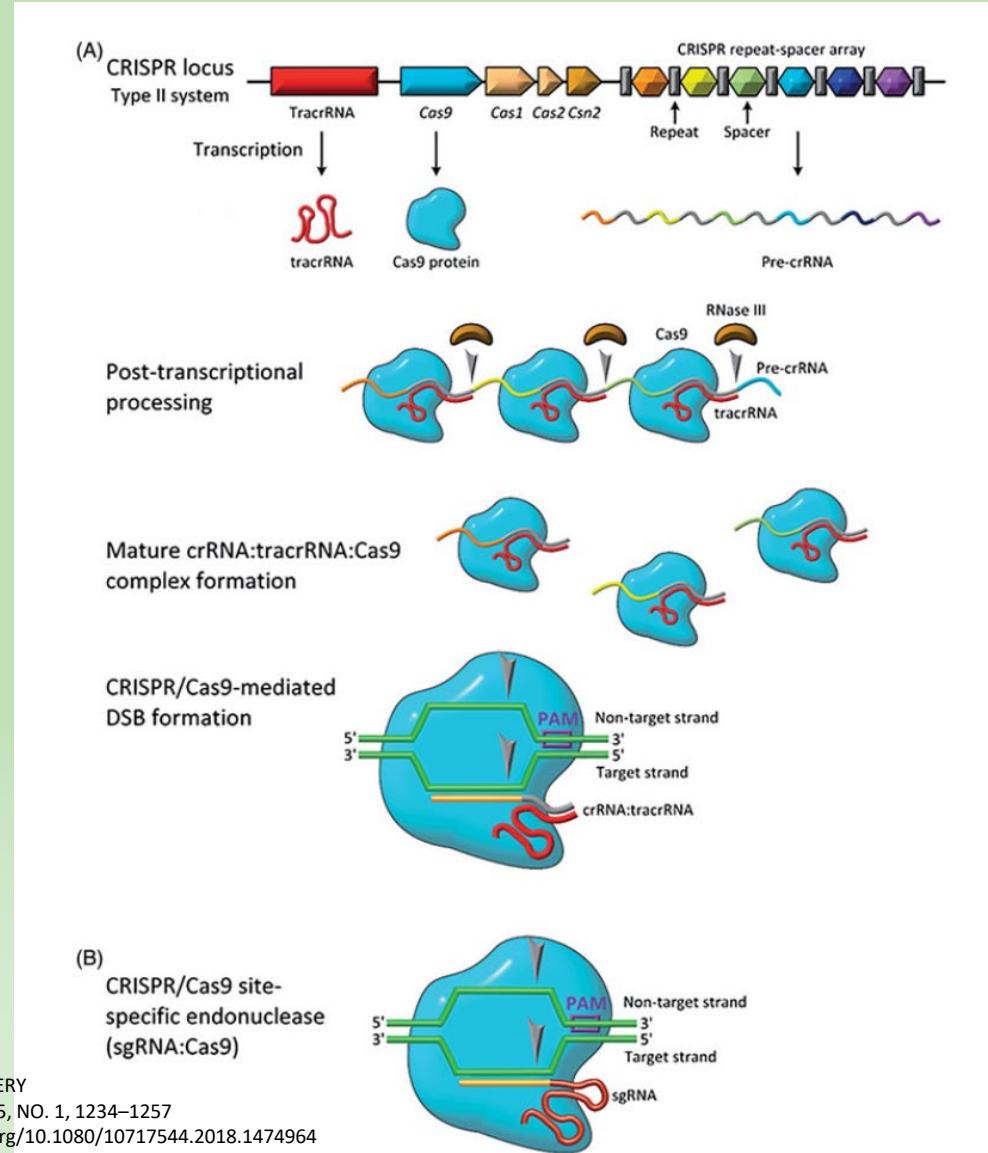
1. CRISPR – clustered regularly interspaced short palindromic repeats
2. descoberto pela primeira vez nas sequências de DNA de *Escherichia coli*
3. descrito em 1987 por Ishino et al. da Universidade de Osaka (Japão) – sem entender o seu significado
4. Aplicação médica: identificação de cepas bacterianas em condições clínicas
 - a) loci CRISPR tem um alto grau de polimorfismo em diferentes cepas da mesma espécie de bactéria patogênica

1. 1995: Francisco Mojica da Universidade de Alicante (Espanha) - encontrou estruturas semelhantes no genoma da archaea *Haloferax mediterranei*
2. Hipótese: esses loci incomuns incluem fragmentos de DNA estranho e são, de fato, uma parte do sistema imunológico de bactérias e archaea
3. Repetições CRISPR: encontradas em
 - a) maioria dos genomas de archaeas
 - b) quase metade dos genomas bacterianos estudados
 - c) não foram encontradas em sequências de DNA eucarióticas ou virais

Sistema CRISPR – Cas

1. Bactérias e archaea desenvolveram sistemas de defesa adaptativos mediados por RNA, chamados de repetições palindrômicas curtas regularmente intercaladas (CRISPR) que protegem os organismos de vírus e plasmídeos invasores
2. Esses sistemas de defesa dependem de pequenos RNAs para detecção específica de sequência e silenciamento de ácidos nucléicos exógenos
3. Os sistemas CRISPR / Cas são compostos por:
 - a) genes cas organizados em operon (s)
 - b) CRISPR que consiste em sequências de direcionamento de genoma (chamadas espaçadores) intercaladas com repetições idênticas

Biologia do sistema CRISPR / Cas tipo II

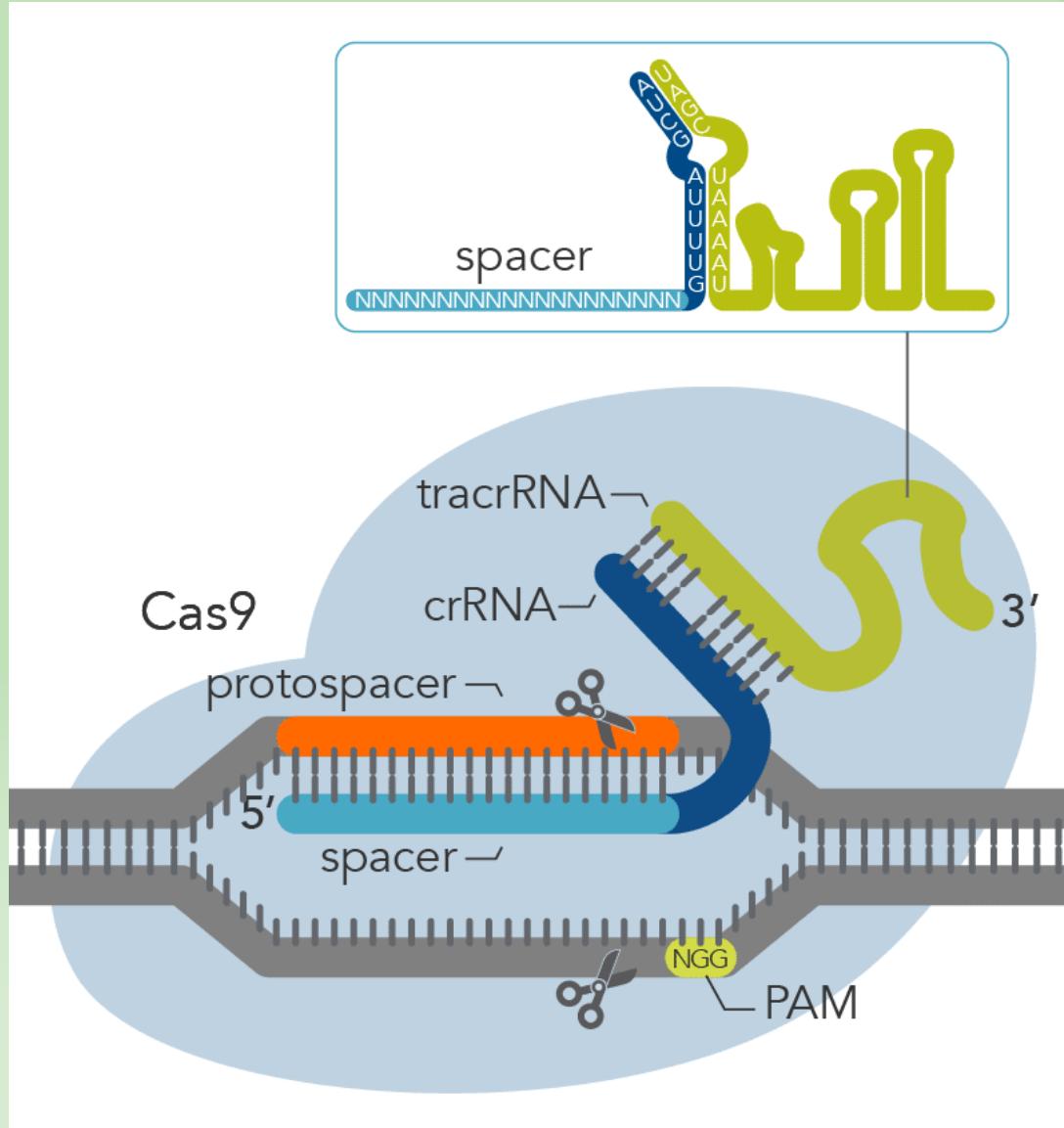


- (A) Representação genômica de CRISPR / Cas9 juntamente com produtos de transcrição / tradução relevantes.
- (B) CRISPR / Cas9 projetado para edição de genes específicos do local (sgRNA: Cas9).
- Setas cinza indicam locais de quebras de nucleotídeo de fita simples.

Reconhecimento pelas células procarióticas

- PAMs (protospacer adjacent motifs) - Motivos curtos adjacentes aos protoespaçadores
- Protoespaçadores - fragmentos de DNA que são atacados pelo sistema imunológico dos procariotos e são idênticos aos espaçadores correspondentes no locus CRISPR, exceto pelo motivo PAM
- Importantes na fase de reconhecimento de informações genéticas potencialmente perigosas
 - Sua presença no final da sequência sinaliza que o fragmento de DNA é estranho e precisa ser destruído
 - As sequências de DNA armazenadas no locus CRISPR como espaçadores e não contendo motivos PAM não são atacadas pelo sistema imune procariótico

Componentes do Sistema CRISPR-Cas9

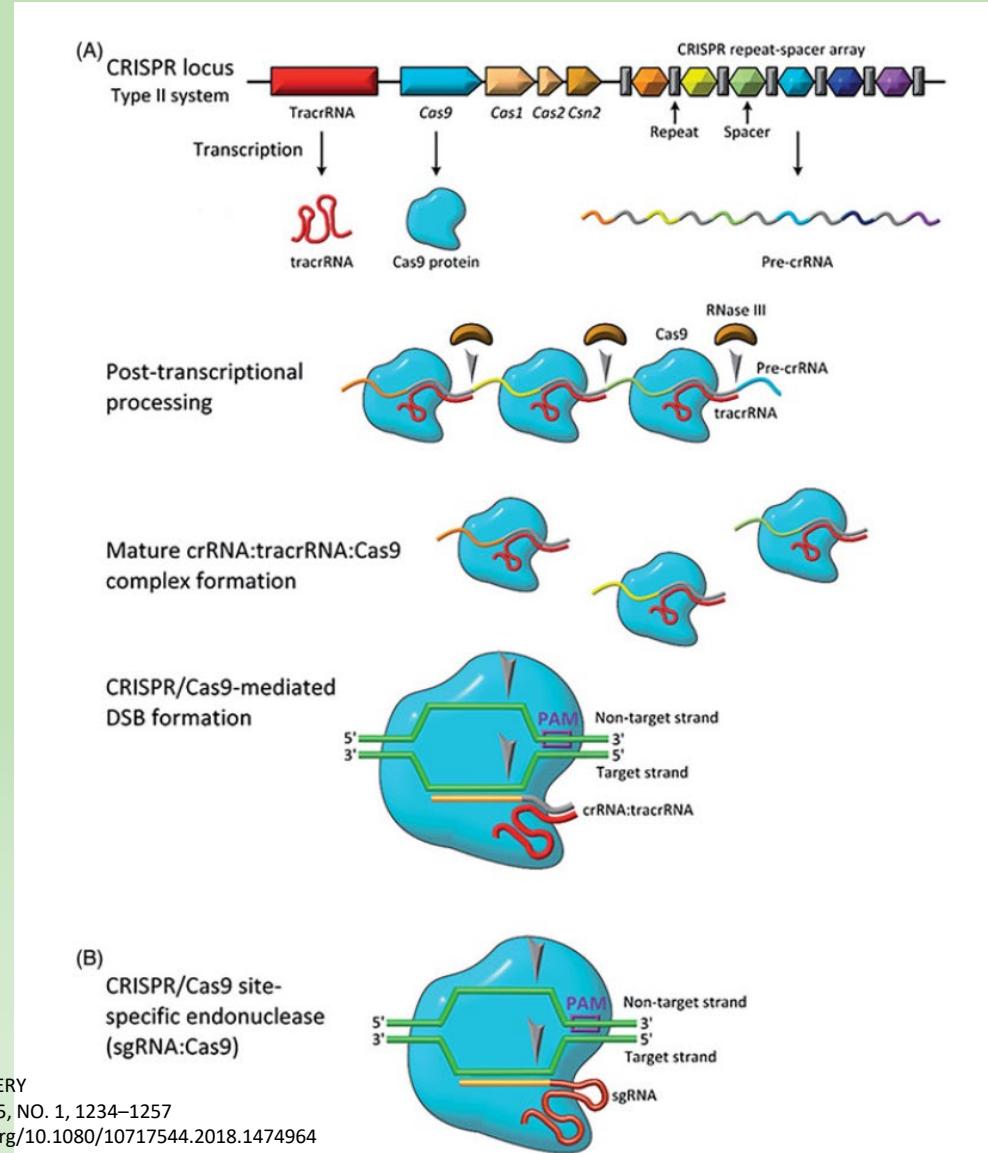


- RNA guia:
 - Spacer (região que tem complementariedade com o DNA)
 - crRNA (Complementariedade com o tracrRNA - trans-activating crRNA)
- PAM (protospacer adjacent motif):
 - NGG para a Cas9 nativa
 - Sequencia de reconhecimento da Cas9 (fita complementar)
 - Abertura da molécula

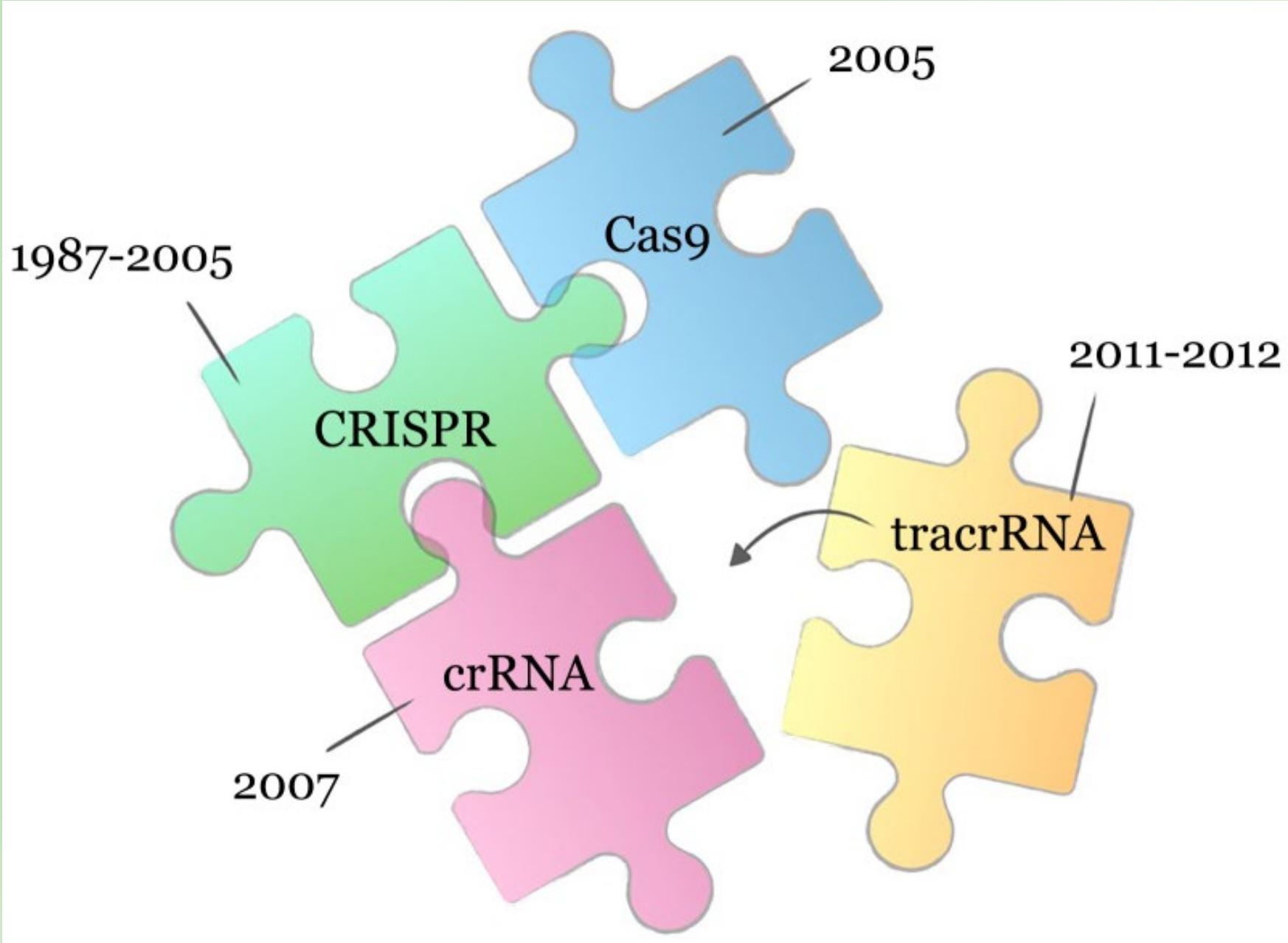
- crRNA (RNA associado a CRISPR) – pequenas moléculas de RNA (RNA guia)
- A transcrição do locus CRISPR é uma molécula precursora de pré-crRNA consistindo em vários espaçadores e repetições, que posteriormente é clivada em fragmentos individuais
- Virginijus Siksnys (Universidade de Vilnius, Lituânia) - o comprimento da sequência real de crRNA “guia” de **20 pares de bases**, complementar ao DNA alvo, é necessário e suficiente para a atividade da nuclease do complexo CRISPR-Cas, mesmo que o espaçador no locus CRISPR seja representado por uma sequência mais longa de nucleotídeos

- Emmanuelle Charpentier e colaboradores em 2011
 - Descobriu uma molécula de RNA essencial para a atividade da nuclease
 - Foi batizada de tracrRNA (trans-activating CRISPR RNA)
 - <https://www.youtube.com/watch?v=4YKFw2KZA5o&t=2s>

Biologia do sistema CRISPR / Cas tipo II



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Premio Nobel em química em 2020

2012: Todos os componentes foram montados in vitro e duas moléculas de RNA combinadas em uma cadeia para facilitar o uso do sistema



Jennifer Doudna ([University of California Berkeley](#)) e Emmanuelle Charpentier ([Max Planck Unit for the Science of Pathogens](#), Berlin)

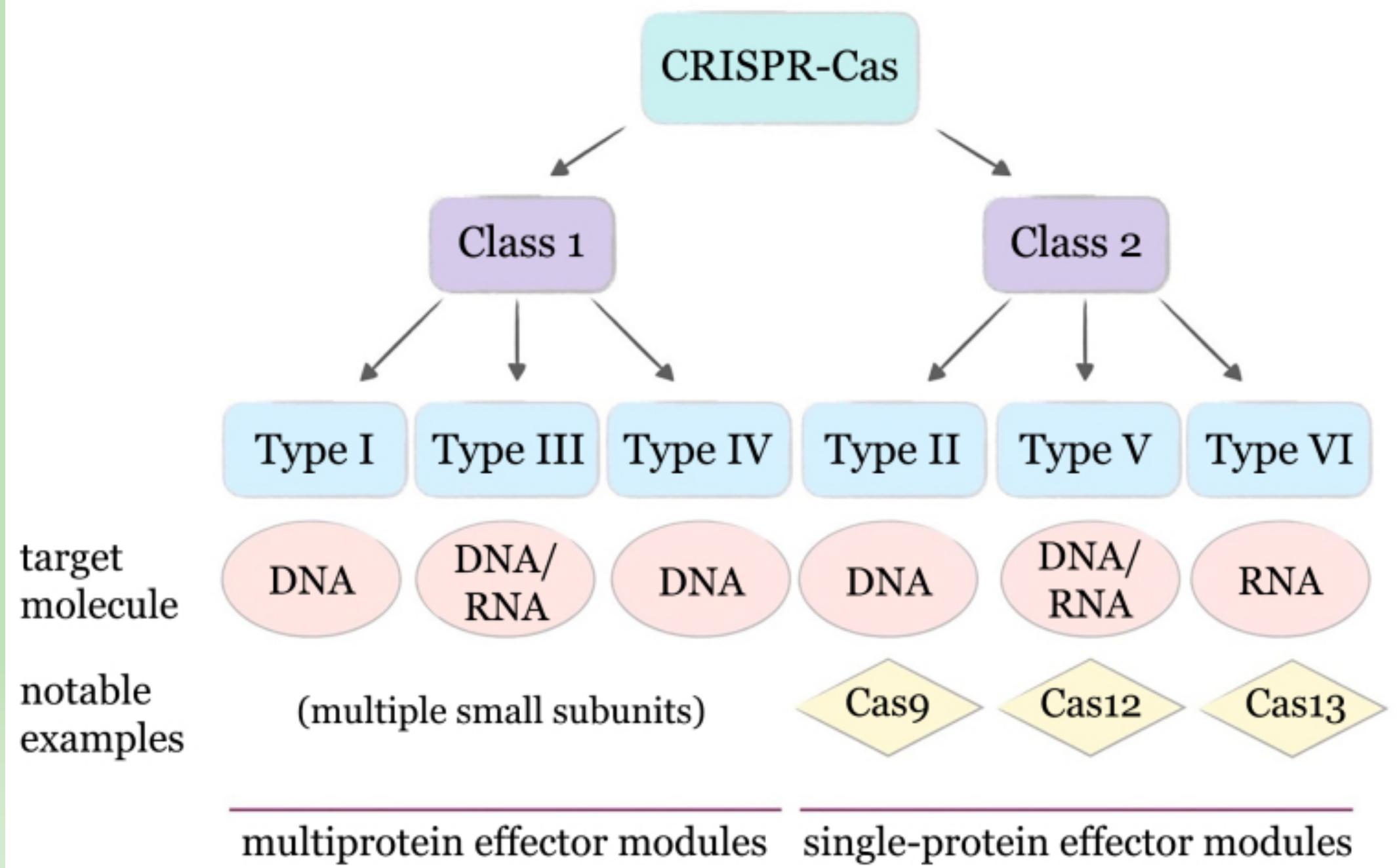
1. Janeiro de 2013: cinco publicações
2. Science – duas publicações com células humanas:
 - a) George Church (Harvard University, EUA) e Feng Zhang (Broad Institute, EUA)
3. Etapas para uma edição de DNA bem-sucedida:
 - a) Otimização de códons
 - b) Adição de um sinal de localização nuclear ao gene cas9
 - c) **Alongamento da molécula de sgRNA** (para melhorar a eficiência do sistema)
 - d) Adição de um molde de DNA para recombinação homóloga
com o qual as células podem reparar a dupla quebra do DNA

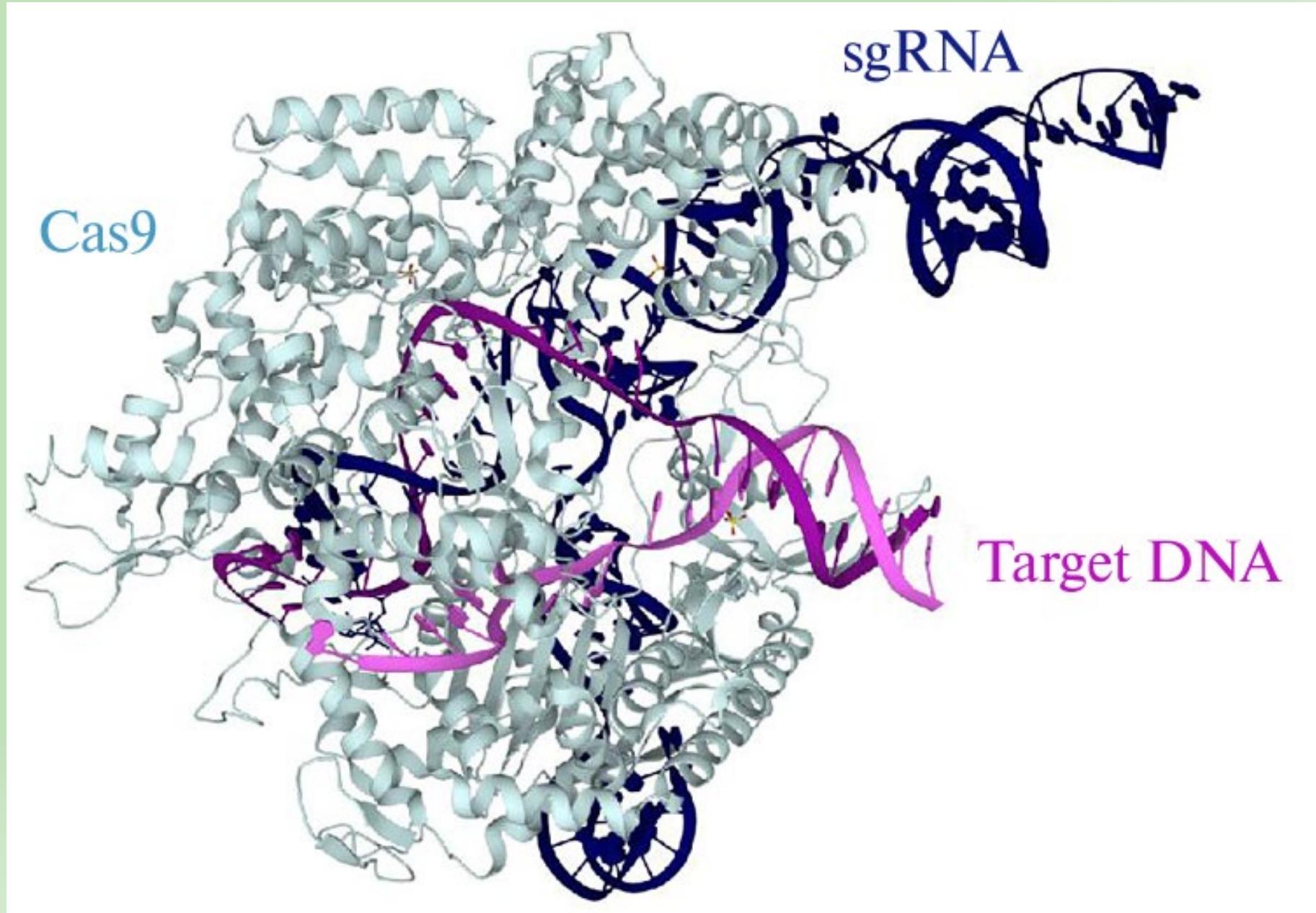
Edição genética via CRISPR / Cas9

- Dois métodos tradicionais
 - transformação mediada por protoplasto (PMT)
 - **transformação mediada por Agrobacterium (AMT)**
 - Maior eficiência
- CRISPR / Cas9: repetição palindrômica curta regularmente interespaciada agrupada (em tandem) / proteína associada (CRISPR / Cas9)
- clustered regularly interspaced short palindromic repeat/associated protein system (CRISPR/Cas9)

CRISPR

- Ferramenta de edição de genomas descrita para:
 - Nocaute
 - inserção
 - substituição de genes
- CRISPR tipo II mais populares têm dois componentes:
 - (i) uma endonuclease Cas9 associada a CRISPR de *Streptococcus pyogenes* e
 - (ii) um RNA guia único (sgRNA), que é a fusão de um RNA CRISPR precursor (precrRNA) e um RNA CRISPR transativador (tracrRNA) 19





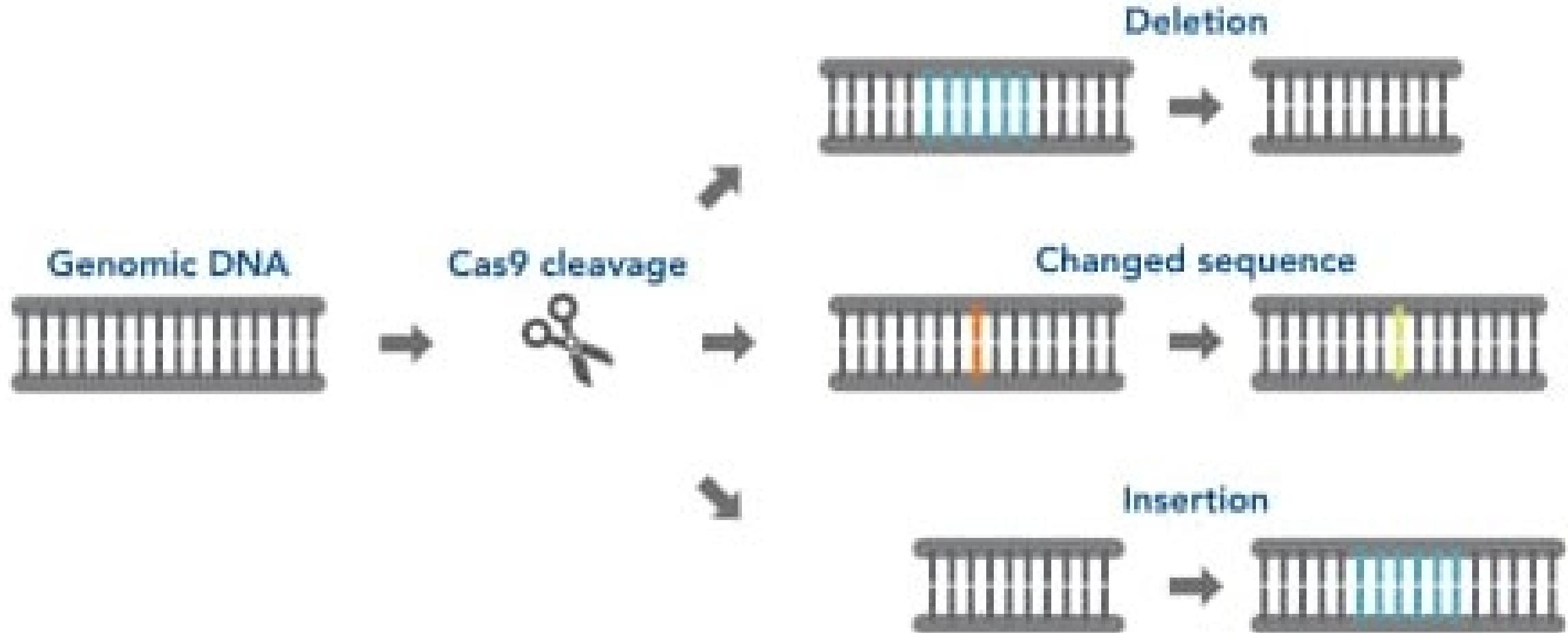
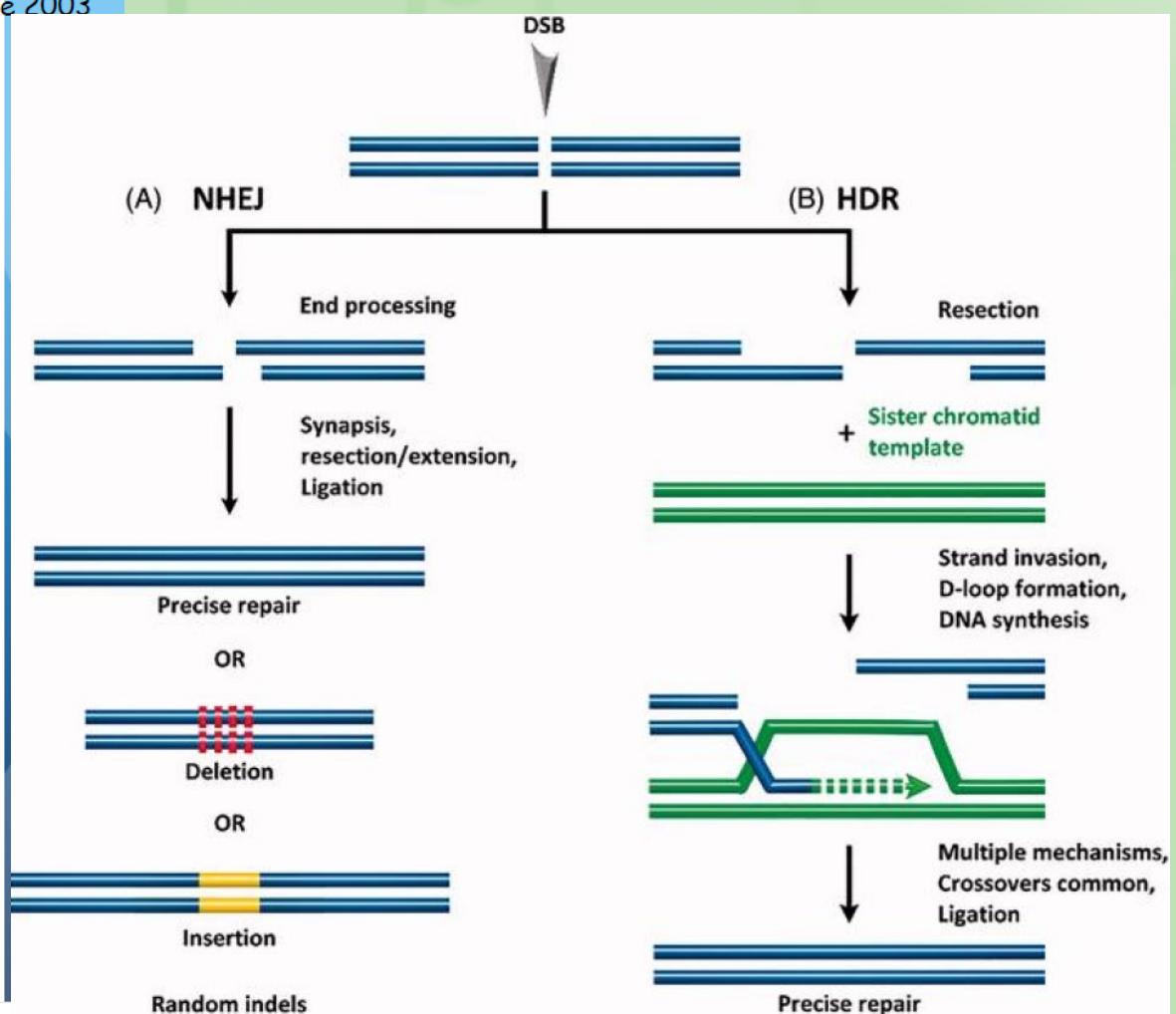
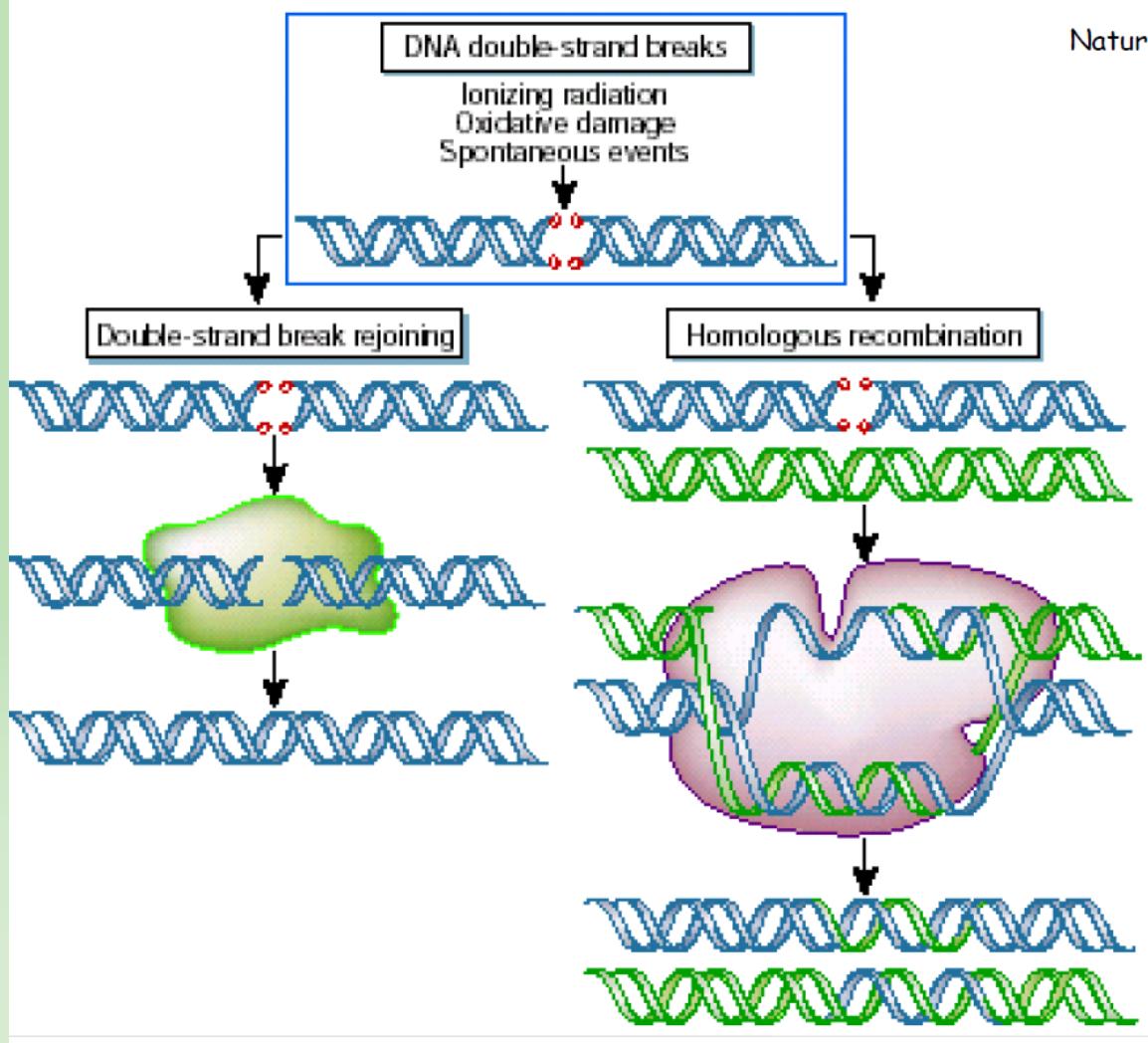
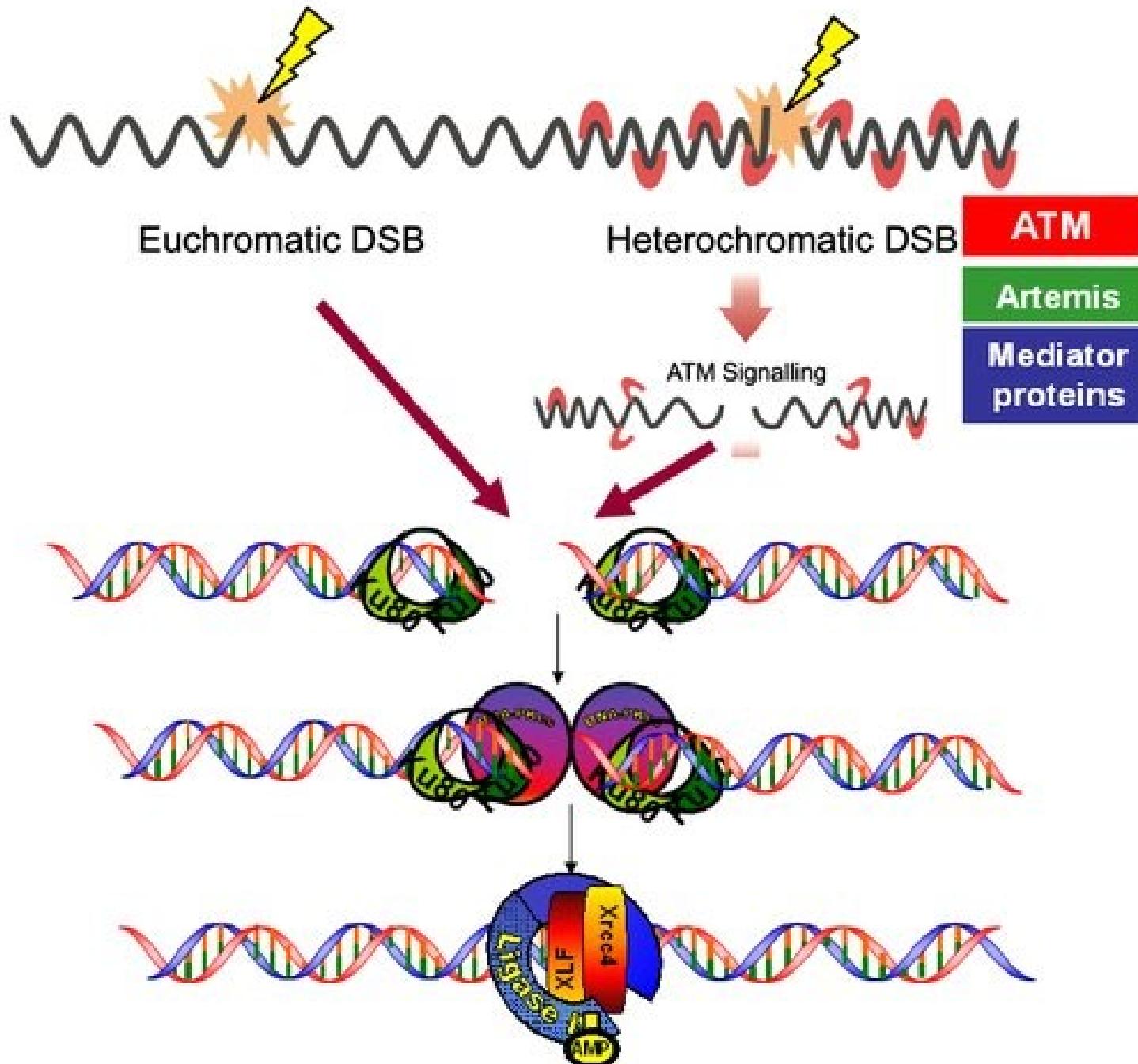
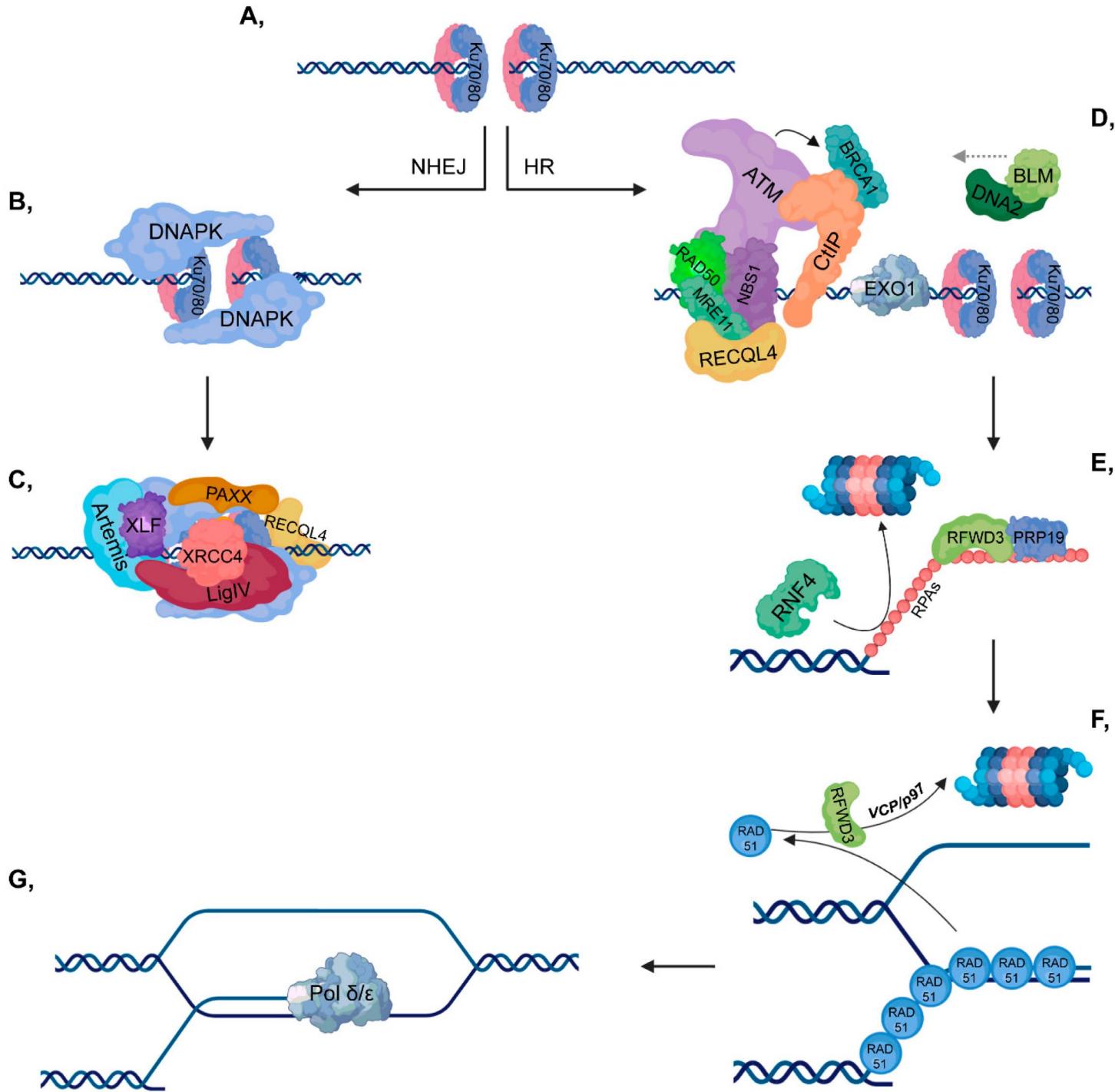


Figure 3. Possible outcomes after genomic DNA cleavage. Cellular DNA repair pathways such as NHEJ can lead to deletions, changed sequences, and small insertions.

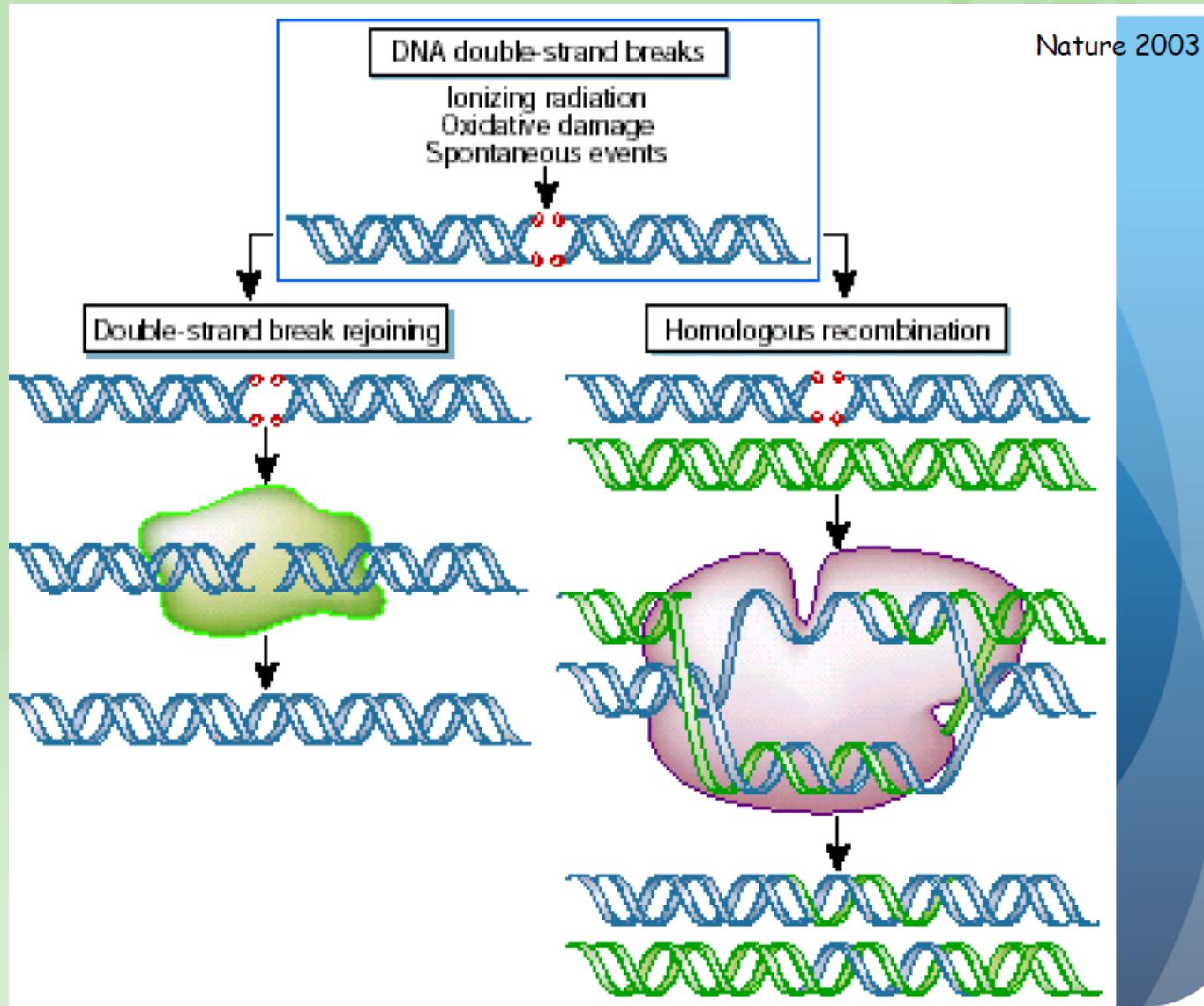
Mecanismos de reparo de dupla quebra de DNA



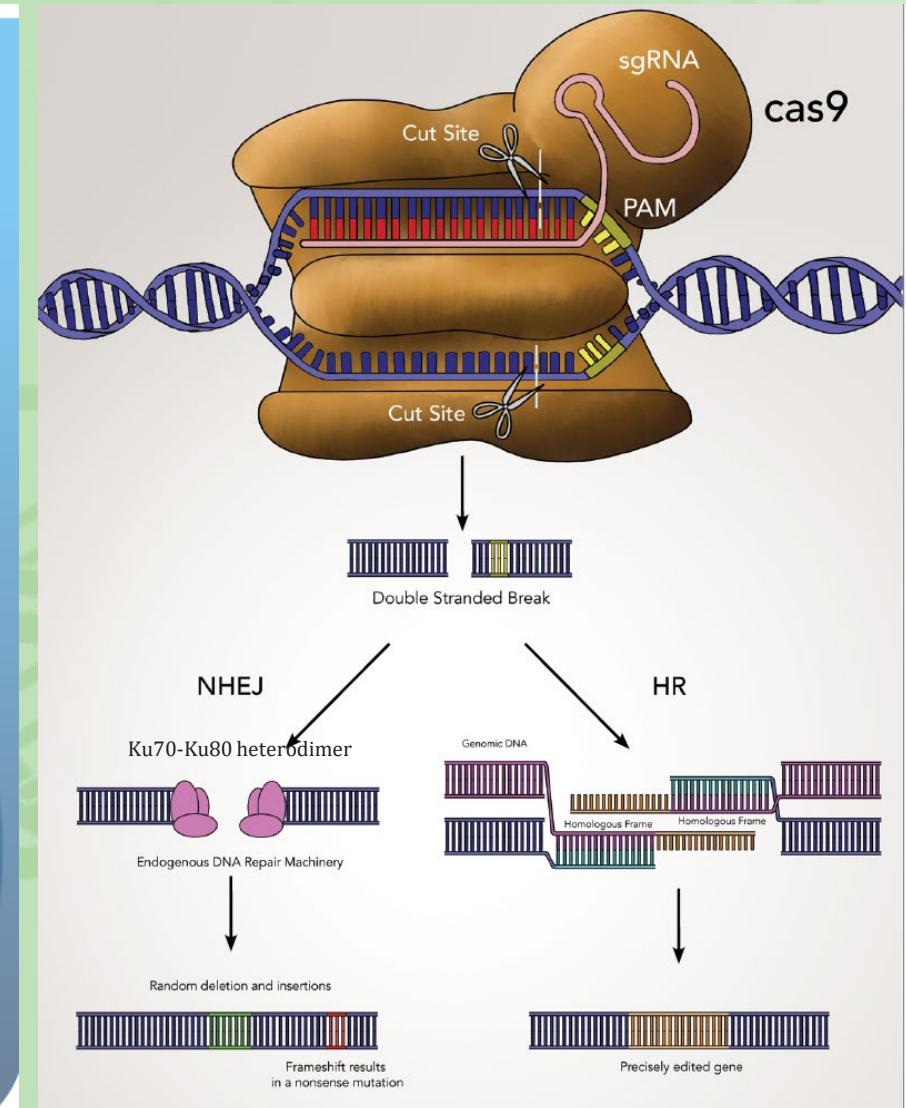




Reparo de dupla quebra de DNA e CRISPR Cas9



Nature 2003



DOI: 10.23861/EJBM201631754



HDR for gene correction or modification

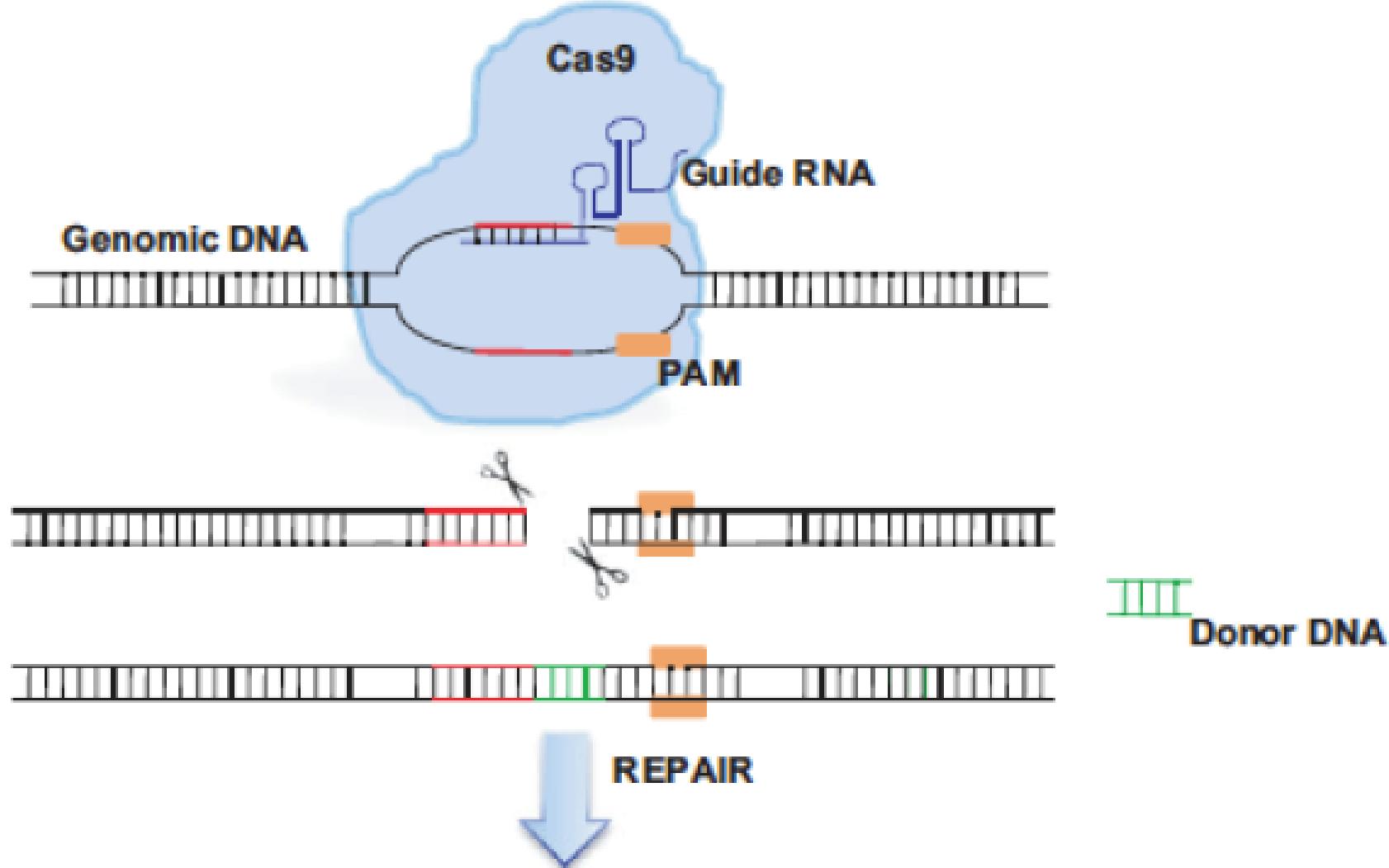


DNA modelo com sequências homólogas (normalmente > 100 bp)

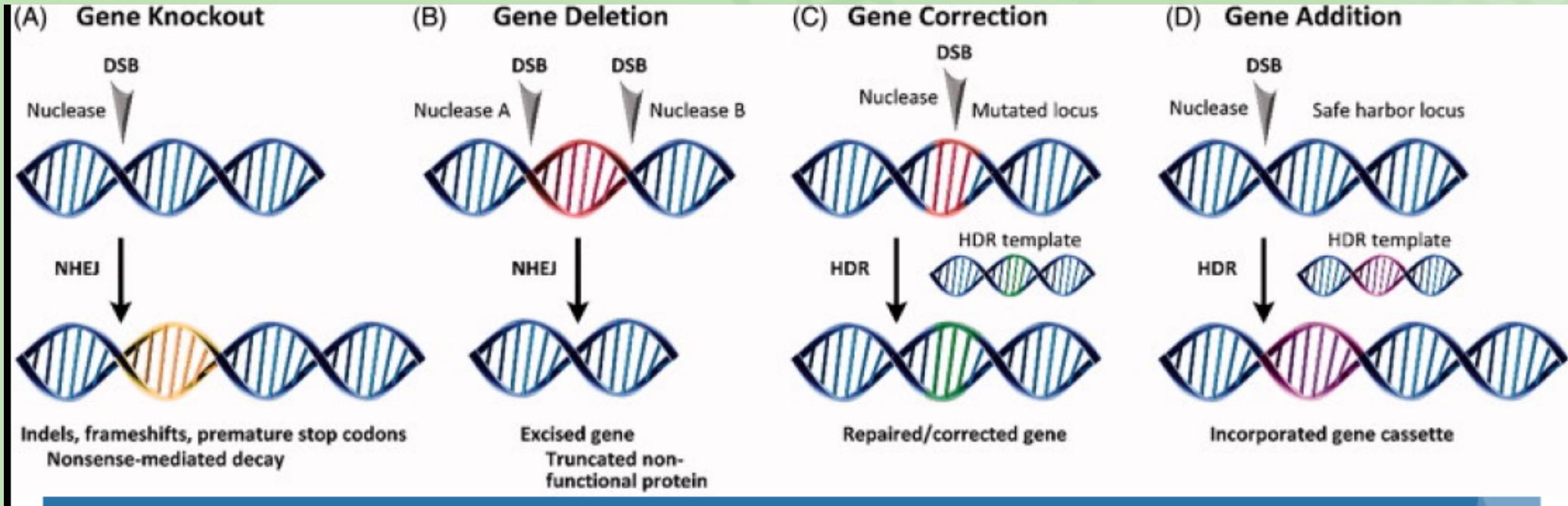
HDR for gene addition



CRISPR-Cas9



Edição de DNA - CRISPR Cas9



Drug Deliv. 2018; 25(1): 1234-1257.

Published online 2018 May 25. doi: 10.1080/10717544.2018.1474964

PMCID: PMC6058482

PMID: 29801422

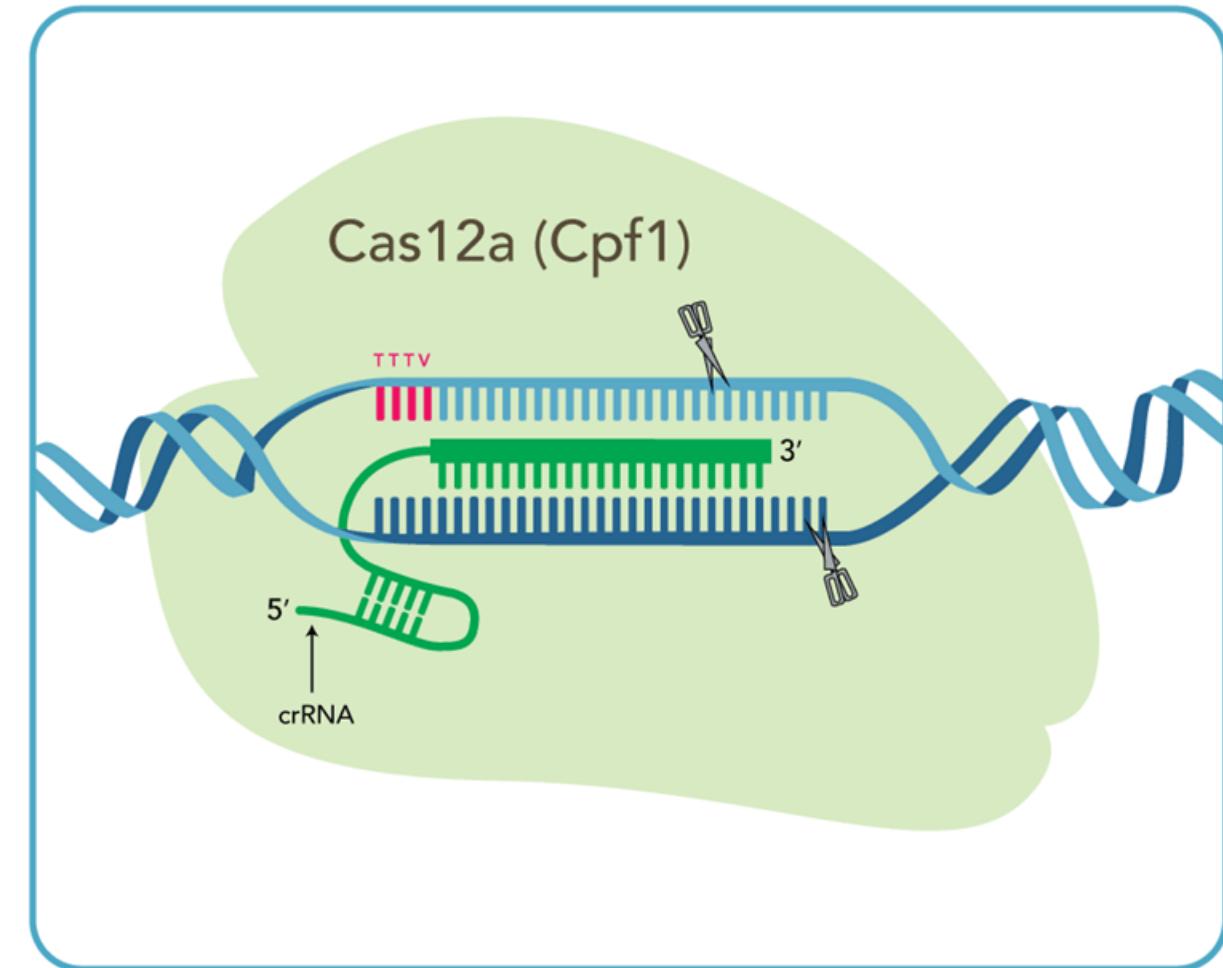
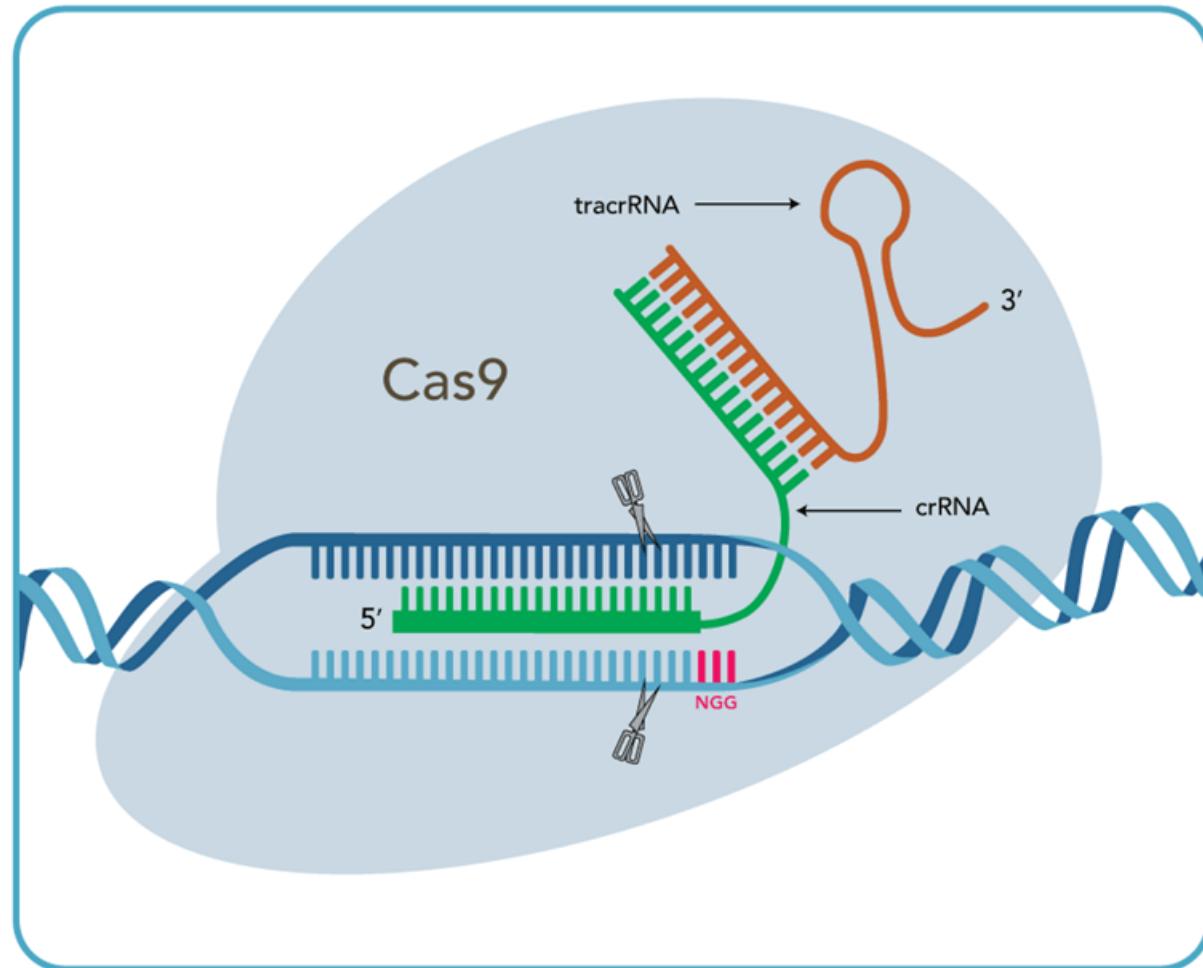
Delivering CRISPR: a review of the challenges and approaches

Christopher A. Lino, Jason C. Harper, James P. Carney, and Jerilyn A. Timlin

Reparo de DSB em fungos

1. HDR (homology directed repair) sendo altamente ativo na levedura *S. cerevisiae*
2. C-NHEJ (canonical non-homologous end joining) predomina na maioria dos fungos filamentosos
3. Mutantes deletados Ku70 e Ku80

Endonucleases Cas9 e Cas12a (Cpf1)



https://www.idtdna.com/pages/products/crispr-genome-editing?utm_source=google&utm_medium=cpc&utm_campaign=ga_crispr_cas&utm_content=ad_group_cas9&gclid=CjwKCAjwtJ2FBhAuEiwAIKu19m1SCZSmhNJ8yk0lMbomT3zKjsJOzEYHKb3yhk4ePHNbtBciTPXpzzoCZpkQAvD_BwE

Endonucleases Cas9 e Cas12a (Cpf1)

	Cas9 system	Cas12a system
Applications	General genome editing	<ul style="list-style-type: none">•For species with AT-rich genomes•For regions with limiting design space for use of the CRISPR-Cas9 system
Ribonucleoprotein components	<ul style="list-style-type: none">•gRNA options:<ul style="list-style-type: none">• crRNA and tracrRNA• sgRNA•Cas9 endonuclease	<ul style="list-style-type: none">•crRNA•Cas12a endonuclease
PAM sequence [†]	NGG	<ul style="list-style-type: none">•TTTV for Cas12a V3•TTTN for Cas12a Ultra

https://www.idtdna.com/pages/products/crispr-genome-editing?utm_source=google&utm_medium=cpc&utm_campaign=ga_crispr_cas&utm_content=ad_group_cas9&gclid=CjwKCAjwtJ2FBhAuEiwAIKu19m1SCZSmhNJ8yk0lMbomT3zKjsJOzEYHKb3yhk4ePHNbtBciTPXpzxoCZpkQAvD_BwE

Esquema de ribonucleoproteínas ativas contendo enzimas Cas ligadas a RNAs guia

Cas9

protospacer

spacer

5'

spacer

3'

spacer

NGG

PAM

crRNA

tracrRNA

Cas9

protospacer

spacer

5'

spacer

3'

spacer

NGG

PAM

linker loop

crRNA

tracrRNA

Cas12a
(Cpf1)

crRNA

5'

TTV

PAM

3'

TTV

PAM

A. Cas9 and 2-part gRNA

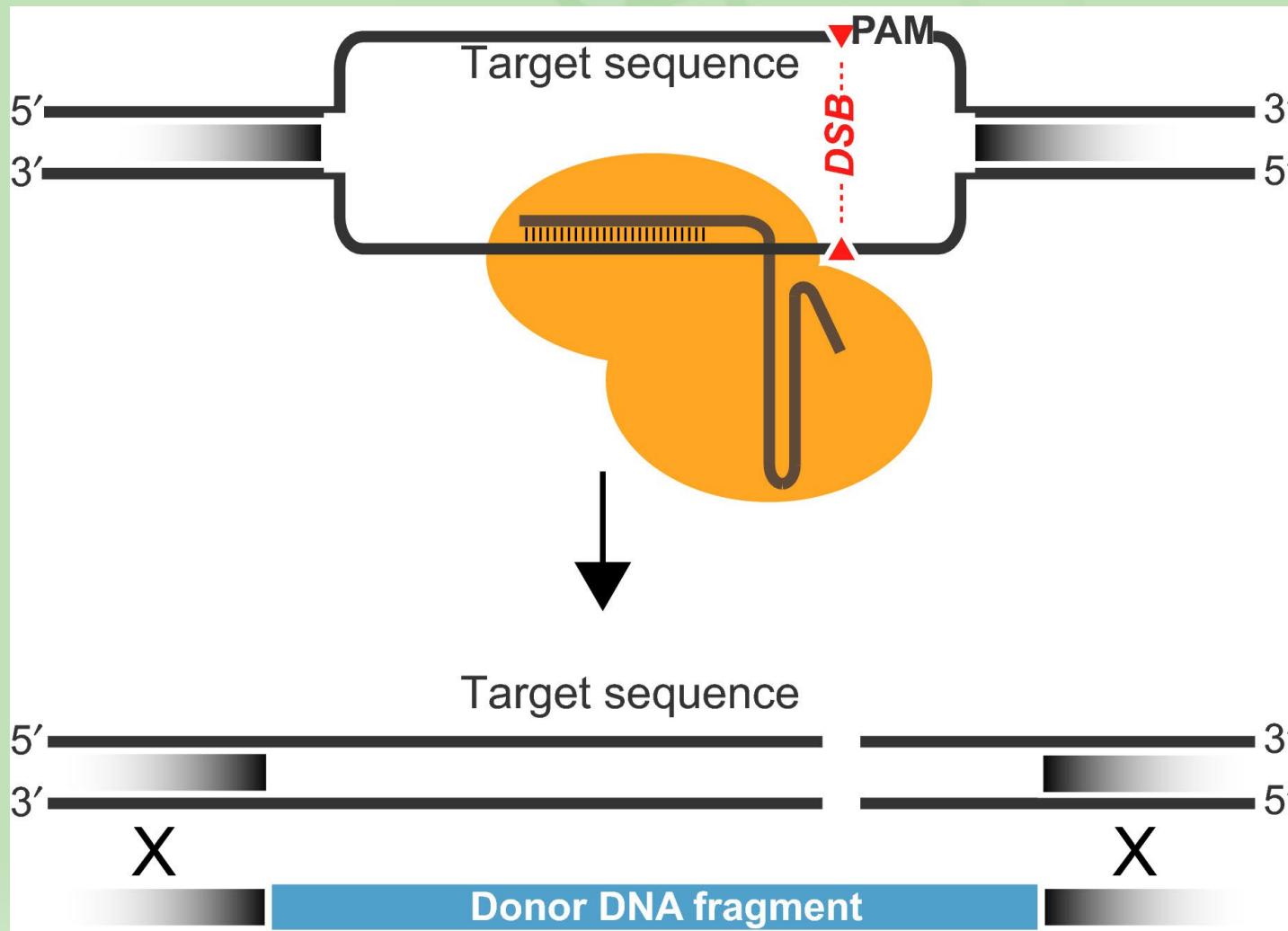
B. Cas9 and sgRNA

C. Cas12a and crRNA

(A) Cas9 with 2-part guide RNA, comprising crRNA and tracrRNA. (B) Cas9 with single-guide RNA. (C) Cas12a with crRNA (gRNA).

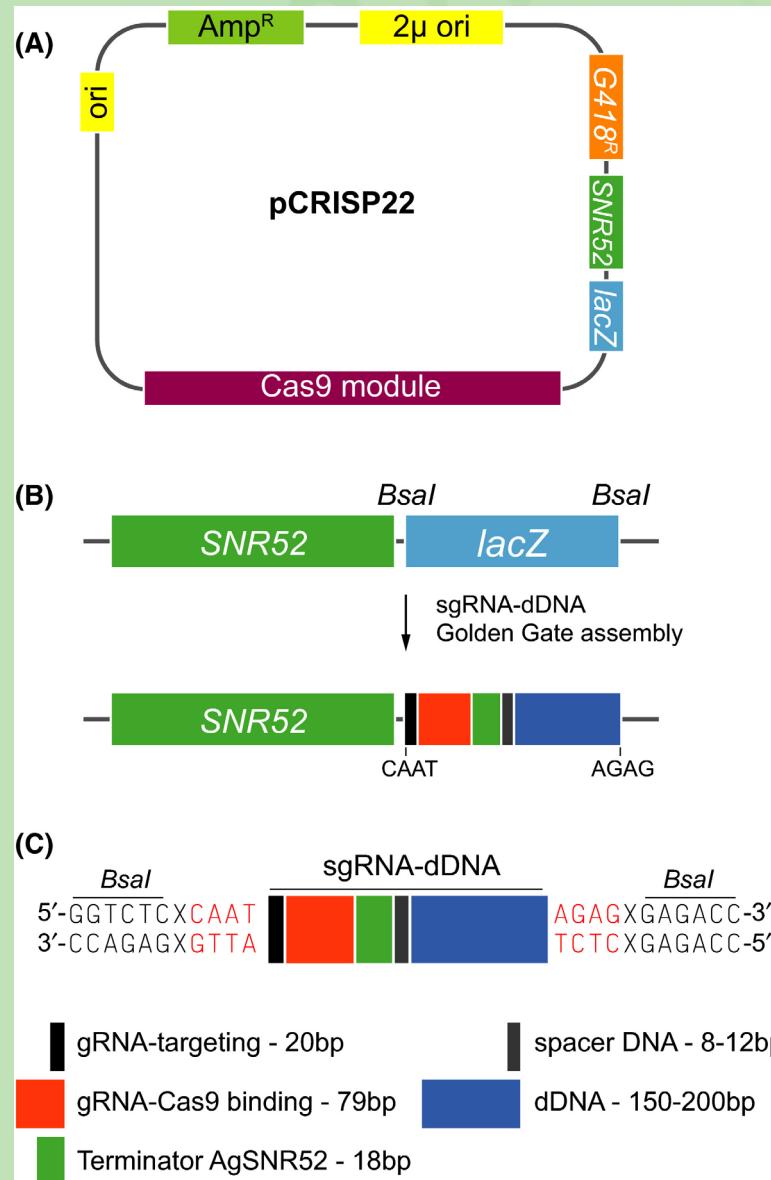


One-vector CRISPR/Cas9 - edição genética do fungo *Ashbya gossypii*





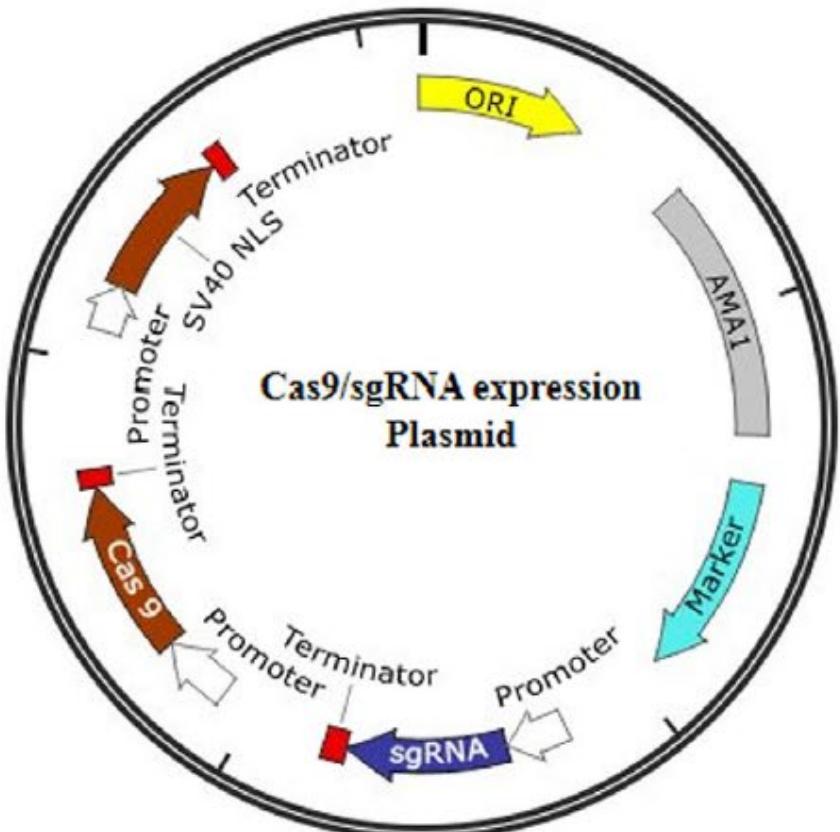
One-vector CRISPR/Cas9 - edição genética do fungo *Ashbya gossypii*



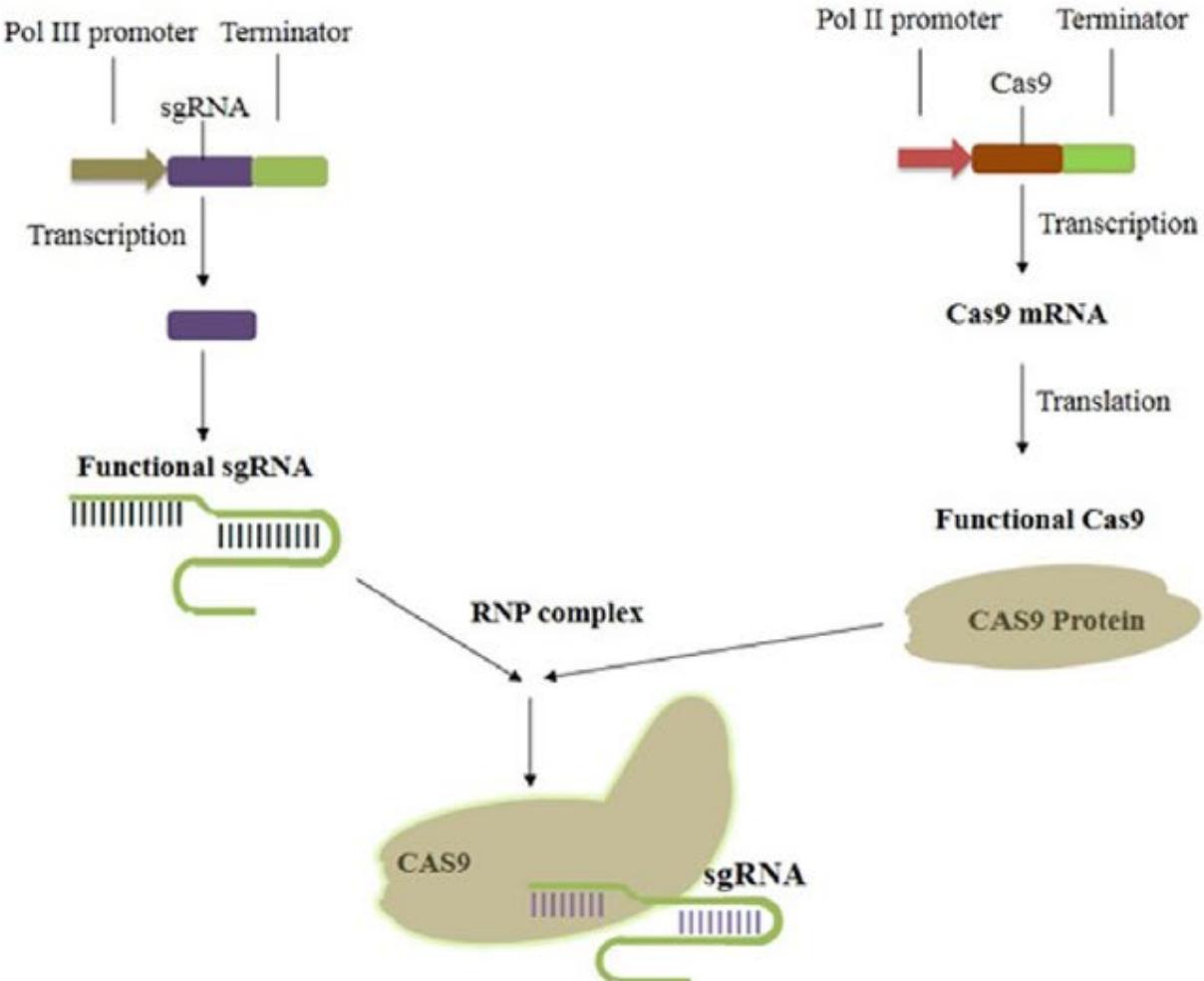
Genetic Modification	Application	Nuclease Activity	gRNA
Knock-out	Permanently remove gene function	Cas or Cas9n	gRNA targeting 5' exon or essential protein domains
Knock-in	Generate a specific sequence change	Cas or Cas9n	gRNA targeting region of interest
Interference	Reduce gene expression	dCas-repressor	gRNA targeting gene promoter elements
Activation	Increase gene expression	dCas-activator	gRNA targeting gene promoter elements

Edição de DNA - CRISPR Cas9

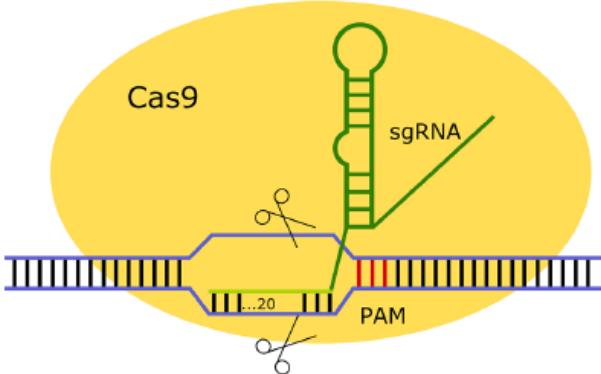
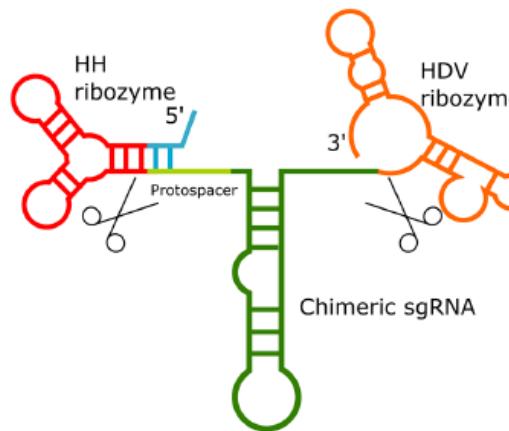
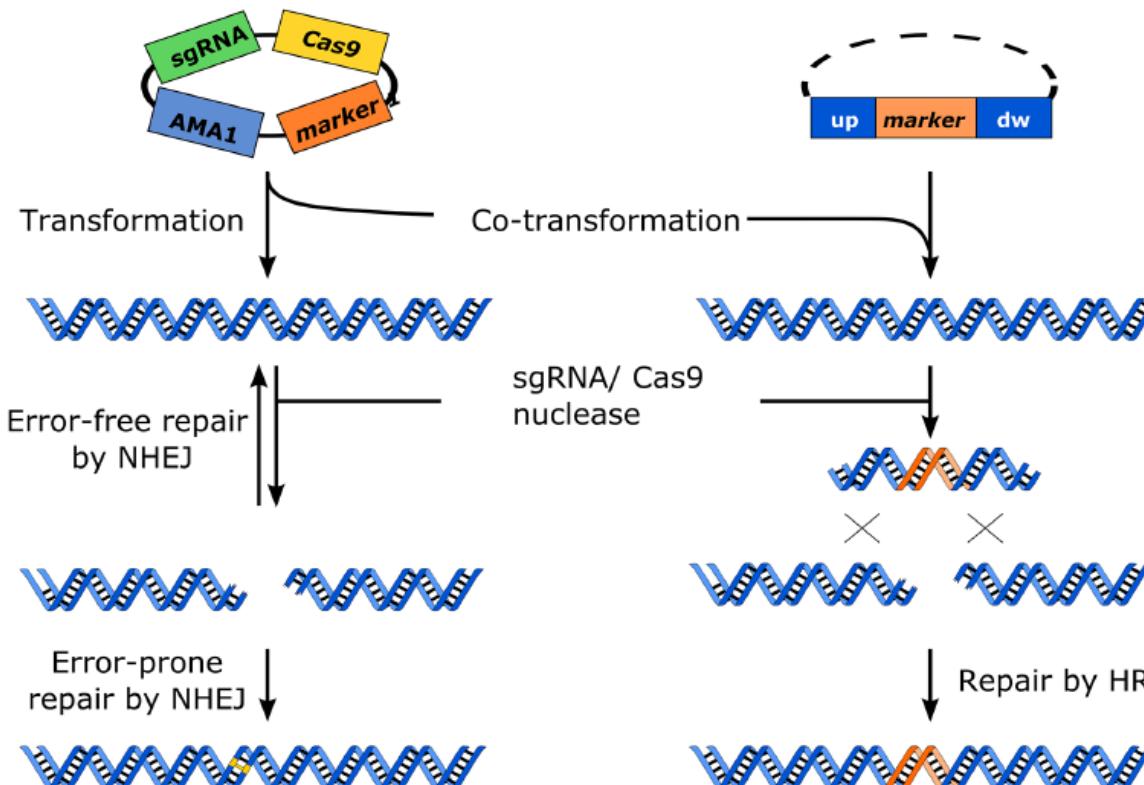
A



B

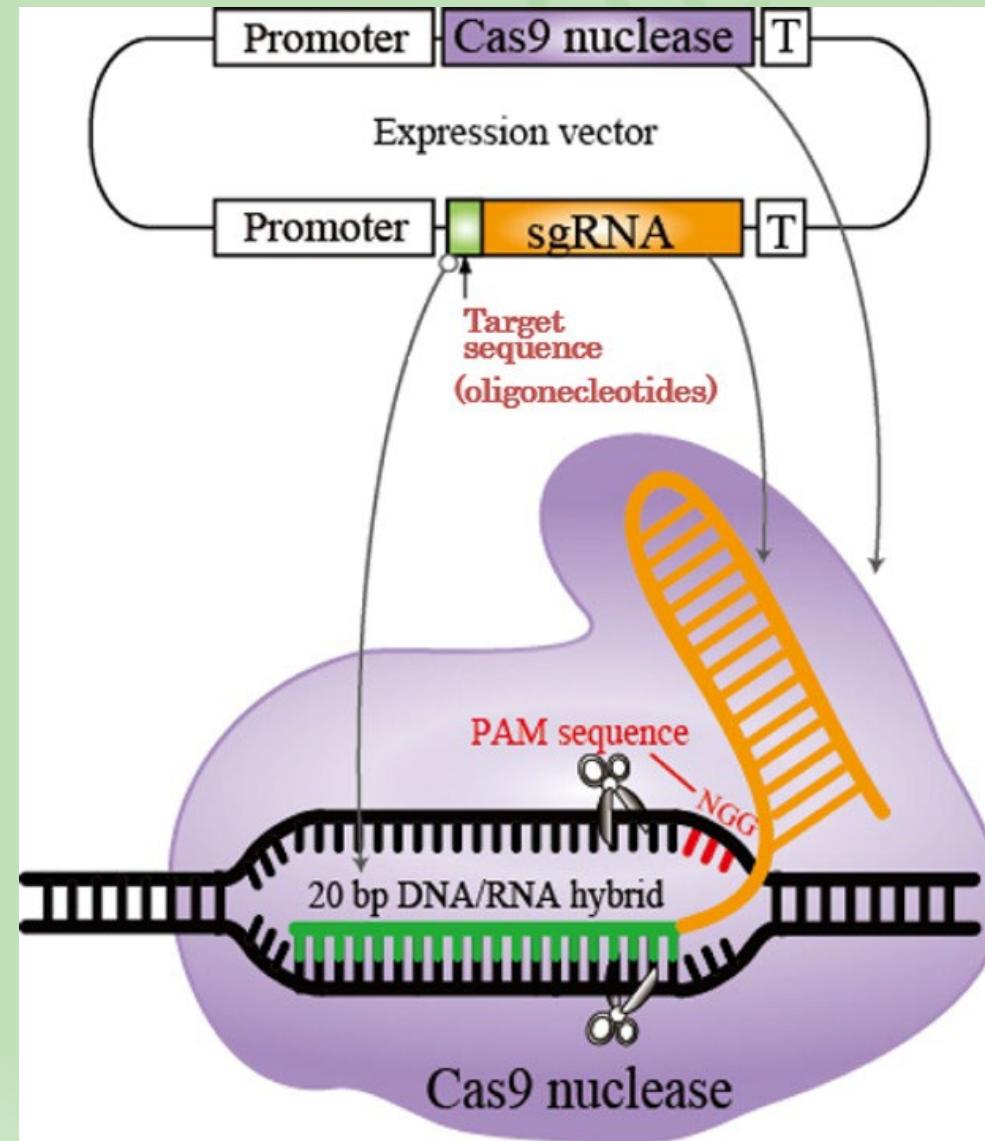


- DOI: 10.1002/bab.2077
- Published online 29 November 2020

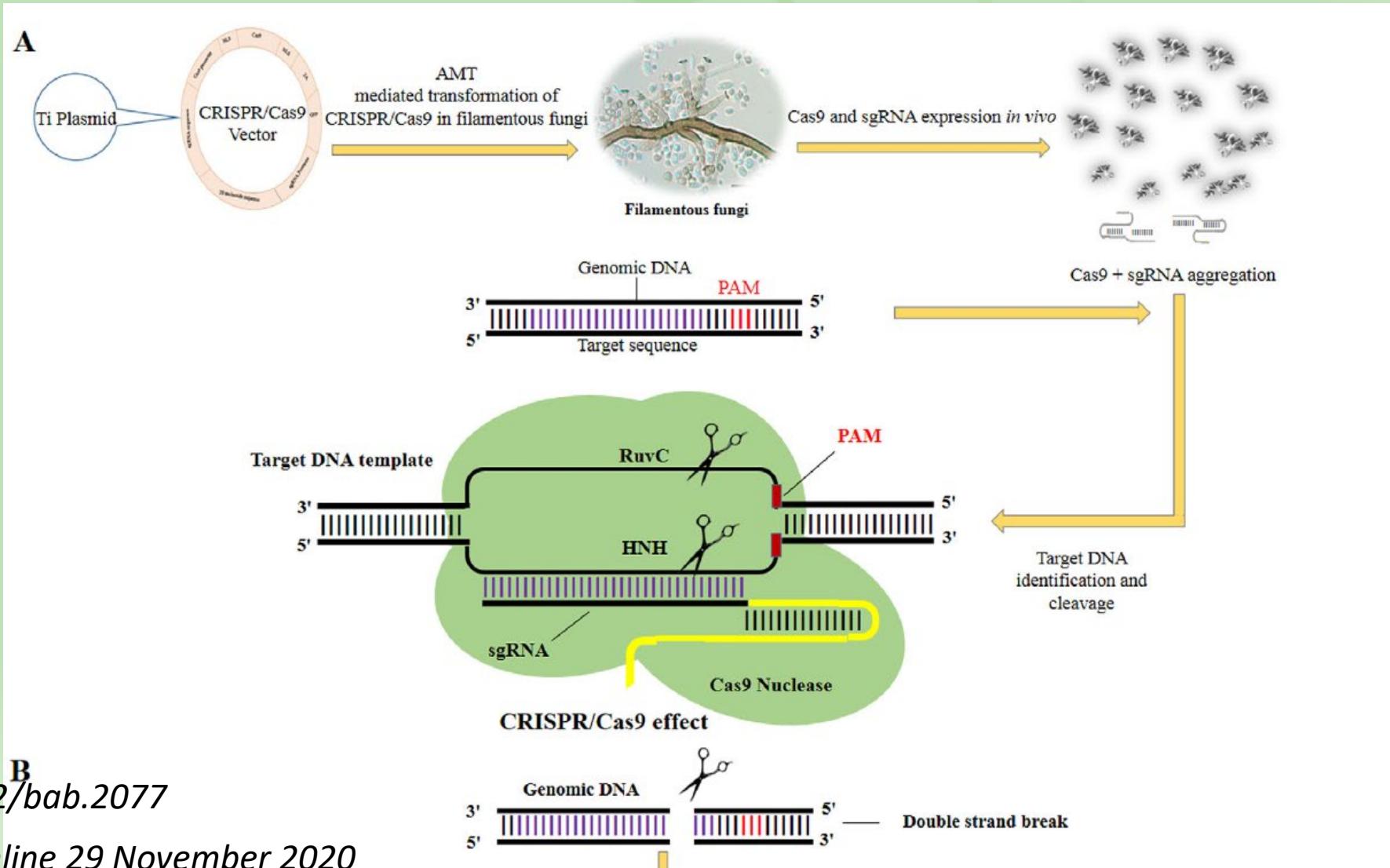
A**B****C**



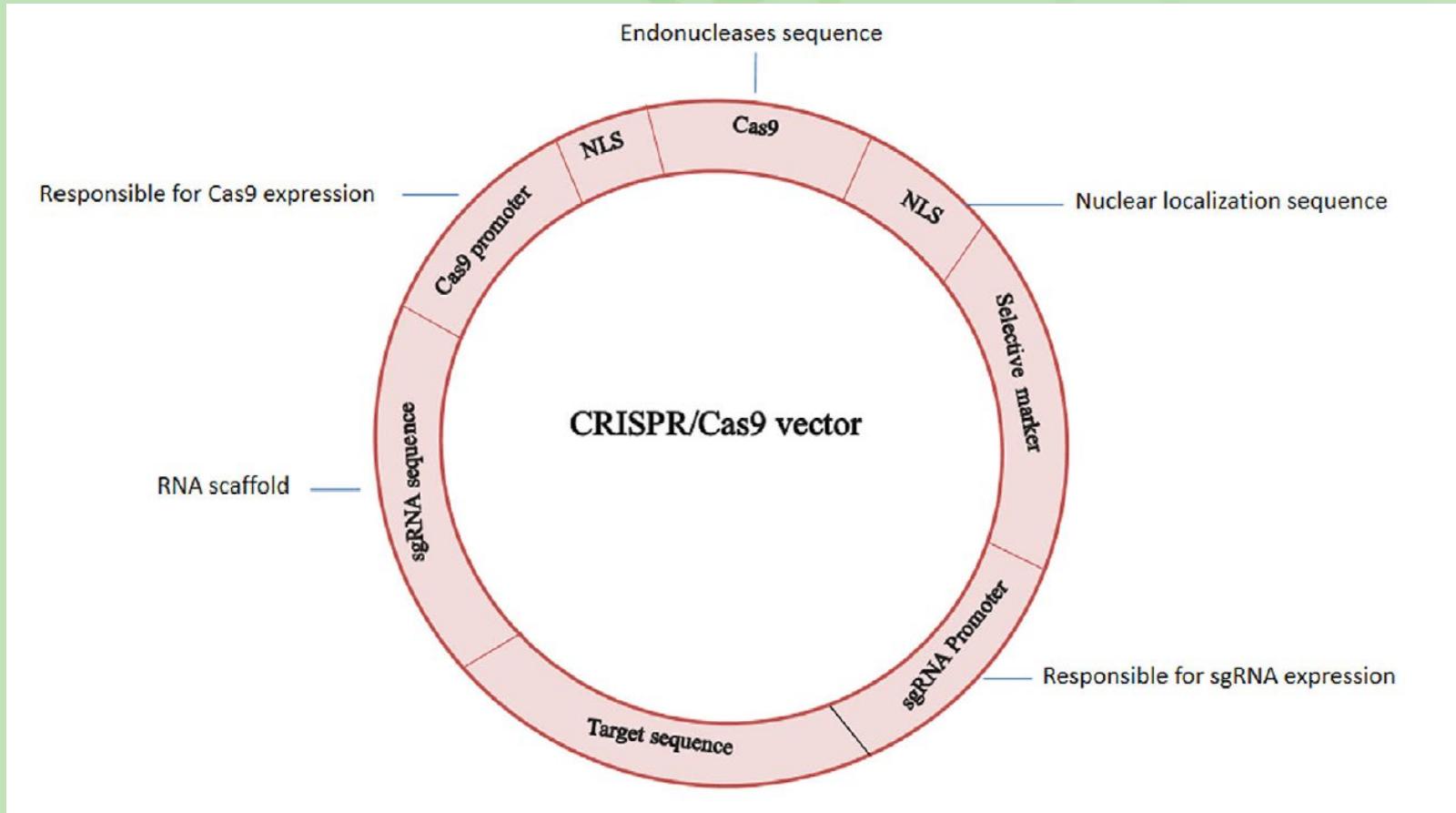
Sistema CRISPR / Cas feito sob medida para substituição dirigida de genes altamente eficiente no fungo brusone do arroz



Edição de DNA - CRISPR Cas9



Edição de DNA - CRISPR Cas9



- DOI: 10.1002/bab.2077
- Published online 29 November 2020

Edição genética via CRISPR Cas9 e Cas12a (Cpf1) (nucleases)

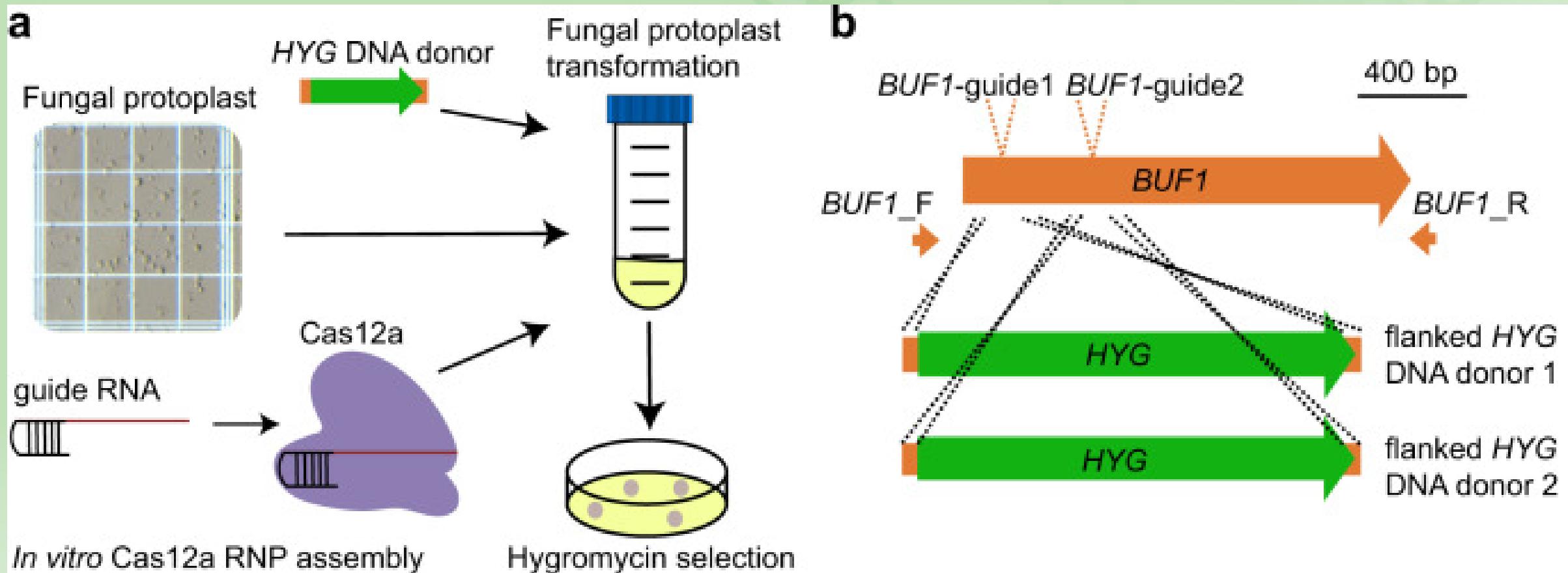
- Nuclease Cas9 de *Streptococcus piogenes* (SpCas9)
- Nucleases Cas12a (antes chamadas de Cpf1) de *Francisella novicida* (FnCpf1), *Acidaminococcus* sp. (AsCpf1) e *Lachnospiraceae bacterium*
- Em comparação com Cas9, Cas12 (Cpf1) reconhece sequências PAM ricas em T e não precisa de um crRNA de ação trans (tracrRNA) devido à atividade única de nuclease dupla que cliva não apenas o DNA alvo, mas também seu próprio CRISPR-RNA (crRNA)
- A sequência de reconhecimento:
 - Cas9 é 5' - NGG - 3' (cria extremidades blunt)
 - Cas12a: 5' - TTN - 3' ou 5' - TTTN - 3' (criam extremidades 5' overhangs)

Linhagens modelo de fungos filamentosos para as quais as ferramentas de edição de genes CRISPR já foram estabelecidas

Species	CRISPR protein	Nuclease delivery	References
<i>Aspergillus fumigatus</i>	Cas9	Plasmid-based and RNP-based	[36, 37]
<i>A. nidulans</i>	Cas9, Cpf1	Plasmid-based	[9]
<i>A. niger</i>	Cas9, Cpf1	Plasmid-based and RNP-based	[5, 9]
<i>A. oryzae</i>	Cas9	Plasmid-based	[38]
<i>Neurospora crassa</i>	Cas9	Plasmid-based	[39]
<i>Penicillium chrysogenum</i>	Cas9	Plasmid- based and RNP-based	[33]
<i>Thermothelomyces thermophilus</i>	Cas9	Plasmid-based	[12]
<i>Trichoderma reesei</i>	Cas9	Plasmid-based	[40]

Note that the nuclease can be delivered to the cell either plasmid encoded or as a purified protein, which has to be preassembled with in vitro transcribed gRNA to form ribonucleoproteins (RNPs)

Transformação com a Cas12a + gRNA + DNA molde



Huang J, Rowe D, Subedi P, Zhang W, Suelter T, Valent B, Cook DE. CRISPR-Cas12a induced DNA double-strand breaks are repaired by multiple pathways with different mutation profiles in *Magnaporthe oryzae*. Nat Commun. 2022 Nov 22;13(1):7168. doi: 10.1038/s41467-022-34736-1.

Reduzindo a escala do procedimento de transformação

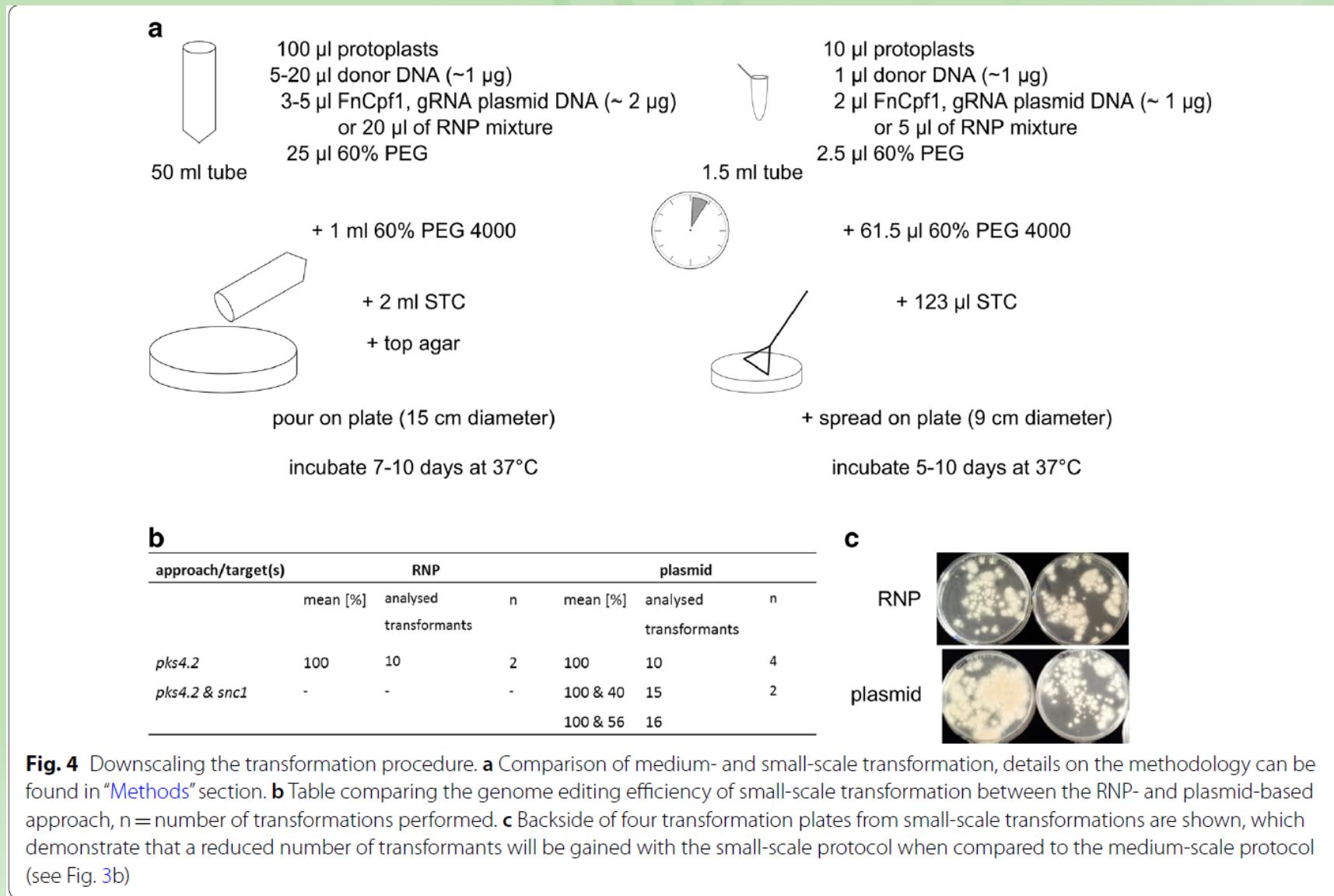


Fig. 4 Downscaling the transformation procedure. **a** Comparison of medium- and small-scale transformation, details on the methodology can be found in “Methods” section. **b** Table comparing the genome editing efficiency of small-scale transformation between the RNP- and plasmid-based approach, n = number of transformations performed. **c** Backside of four transformation plates from small-scale transformations are shown, which demonstrate that a reduced number of transformants will be gained with the small-scale protocol when compared to the medium-scale protocol (see Fig. 3b)

Sistema CRISPR-Cas9 para edição genética do fungo *Fusarium proliferatum*

ID	Protospacer sequence 5'→3'	PAM site
sgRNA1175	TCACCCCCGAGTACCGCTGT	AGG
sgRNA9269	TGATGCGTATCTGGAAATGA	AGG

Table 1. sgRNAs used in this study

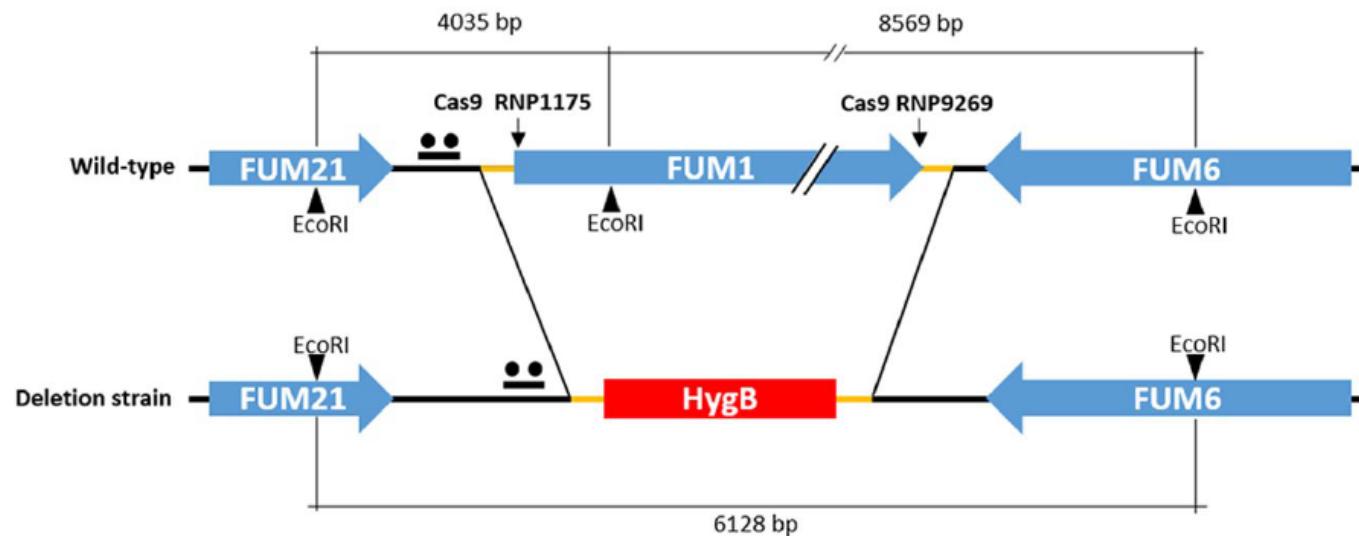


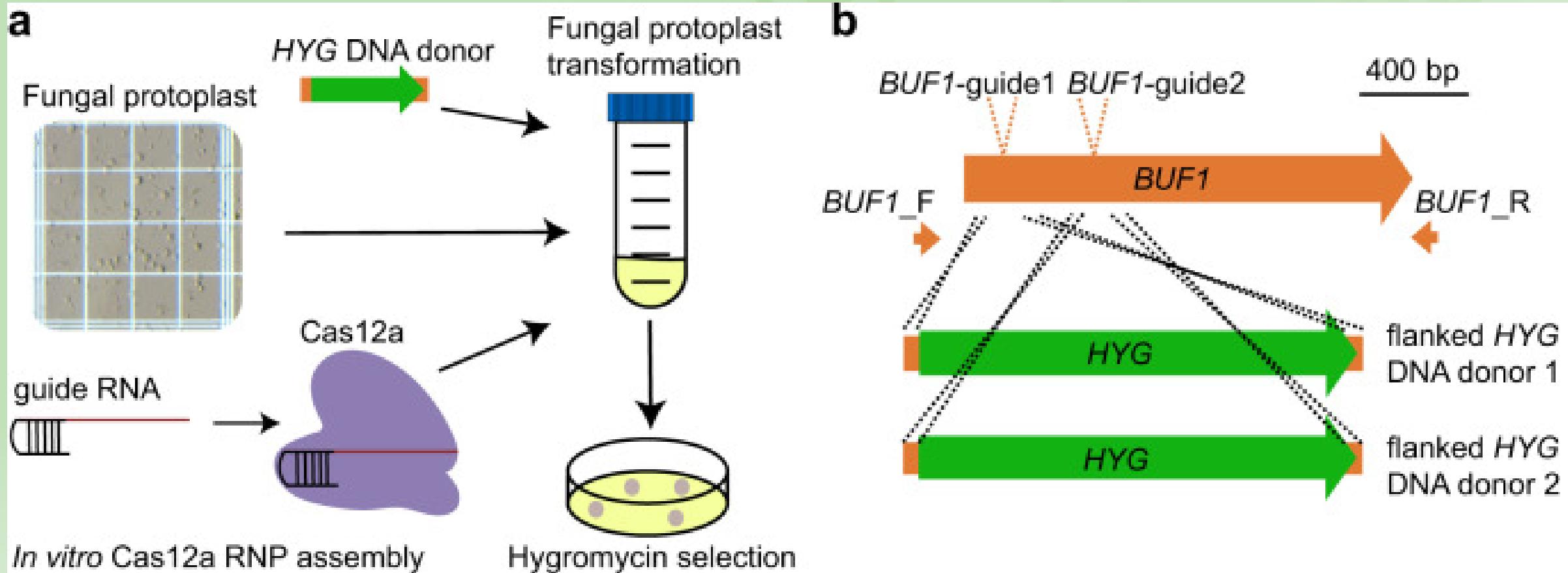
Figure 1. Schematic representation of the *FUM1* gene deletion by *in vitro*-assembled dual Cas9 ribonucleoproteins coupled with homology directed repair (HDR). The cleavage sites of the *in vitro*-assembled Cas9 RNP1175 and RNP9269 (↓), the 50 bp microhomology regions for HDR (orange segment), the EcoRI cut sites, and the pks*FUM1*-specific probe (●●) are represented for the genomic locus of the wild-type ITEM 7595 and Δ *FUM1* deletion strain.

1. Deleção de gene clusler (8000bp) em um único evento de transformação

2. Sistema CRISPR-Cas9 acoplando um sitio específico de quebra de DNA de fita dupla mediada por duas ribonucleoproteínas Cas9 (recombinação em microhomologia requerendo apenas regiões de 50 pb que flanqueiam o gene alvo)

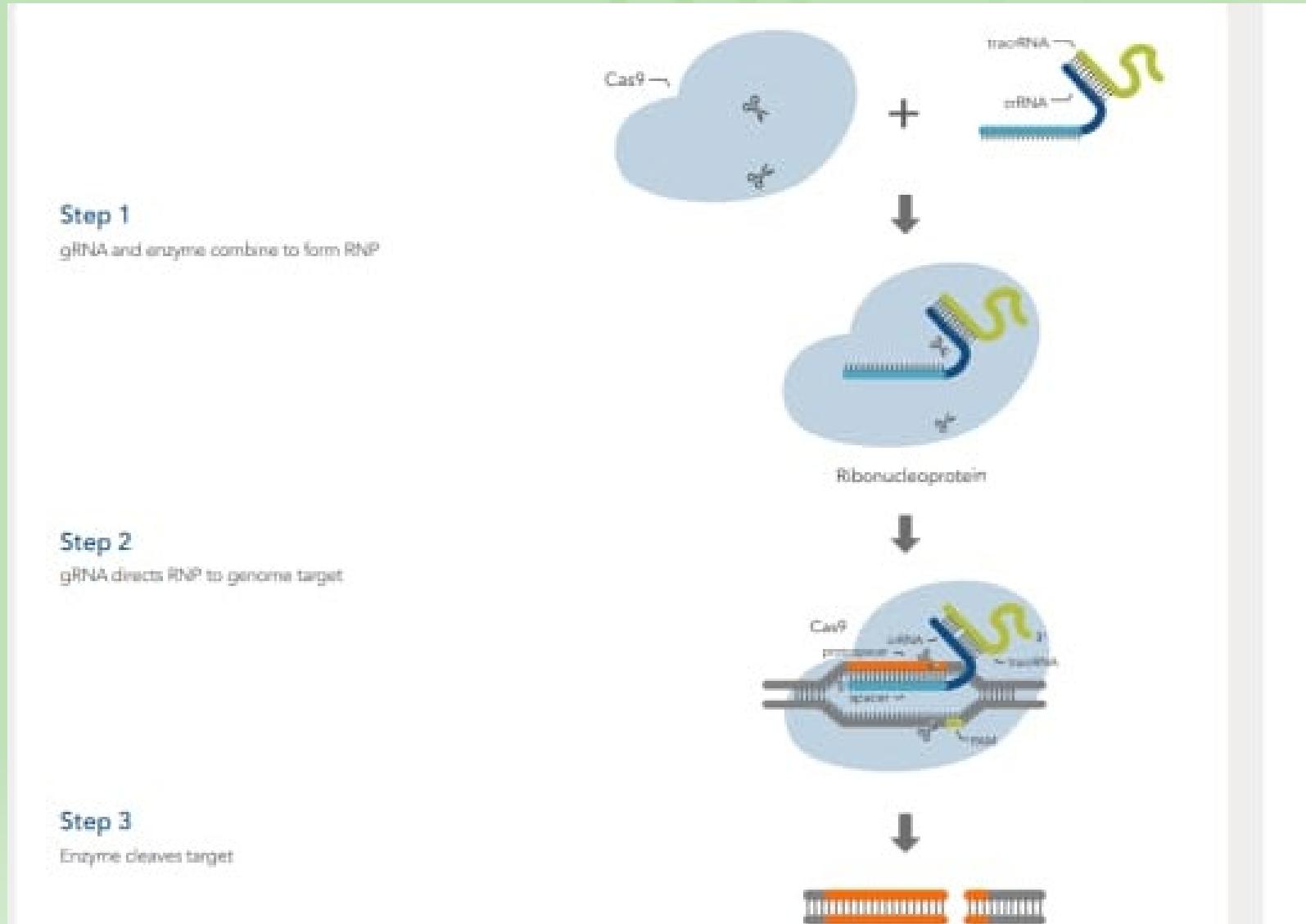
3. Este sistema reduz o risco de mutações fora do alvo e minimiza o risco de alterar qualquer gene adjacente à região alvo

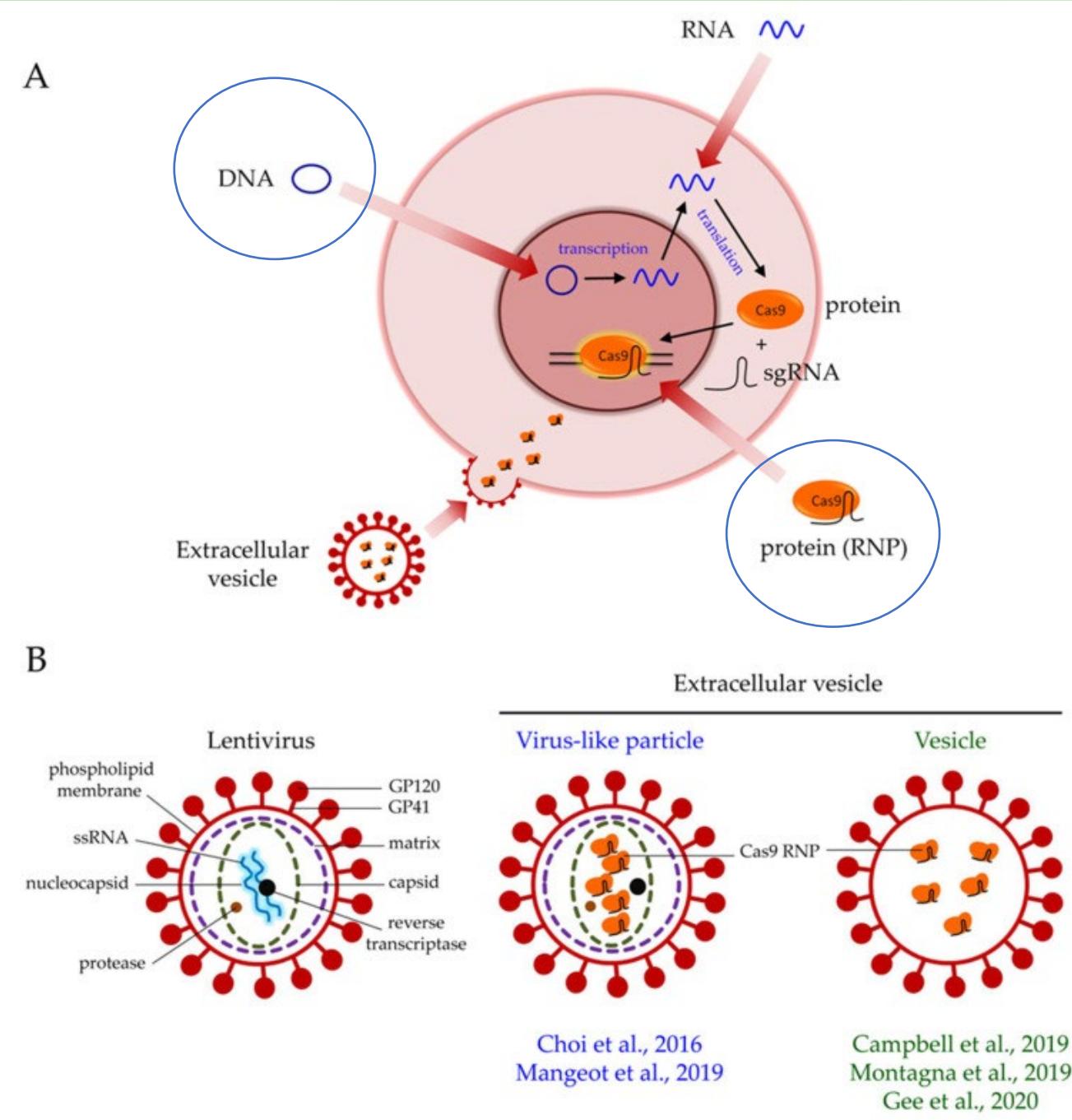
Uso da Cas12a - aumento de eventos de inserção



Huang J, Rowe D, Subedi P, Zhang W, Suelter T, Valent B, Cook DE. CRISPR-Cas12a induced DNA double-strand breaks are repaired by multiple pathways with different mutation profiles in *Magnaporthe oryzae*. Nat Commun. 2022 Nov 22;13(1):7168. doi: 10.1038/s41467-022-34736-1.

Passo a passo da Edição via CRISPR Cas9



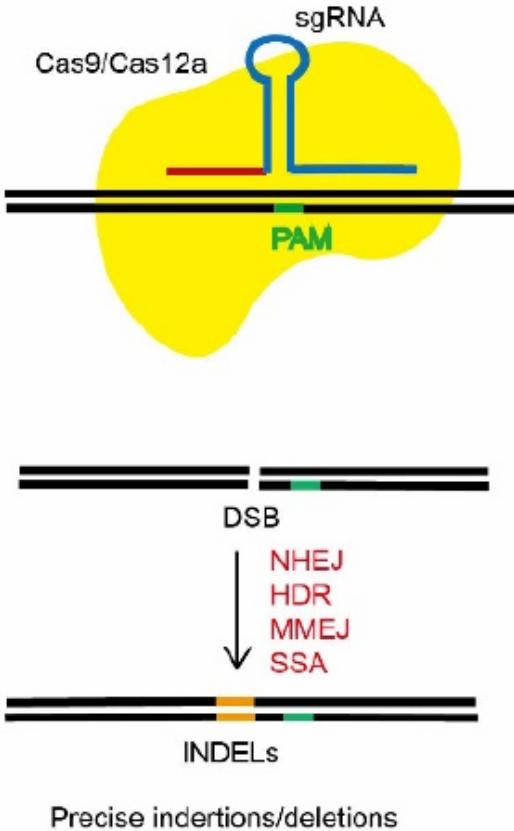


Orientação prática para a implementação da tecnologia CRISPR em fungos filamentosos com base nos dados obtidos para *Thermothelomyces thermophilus*

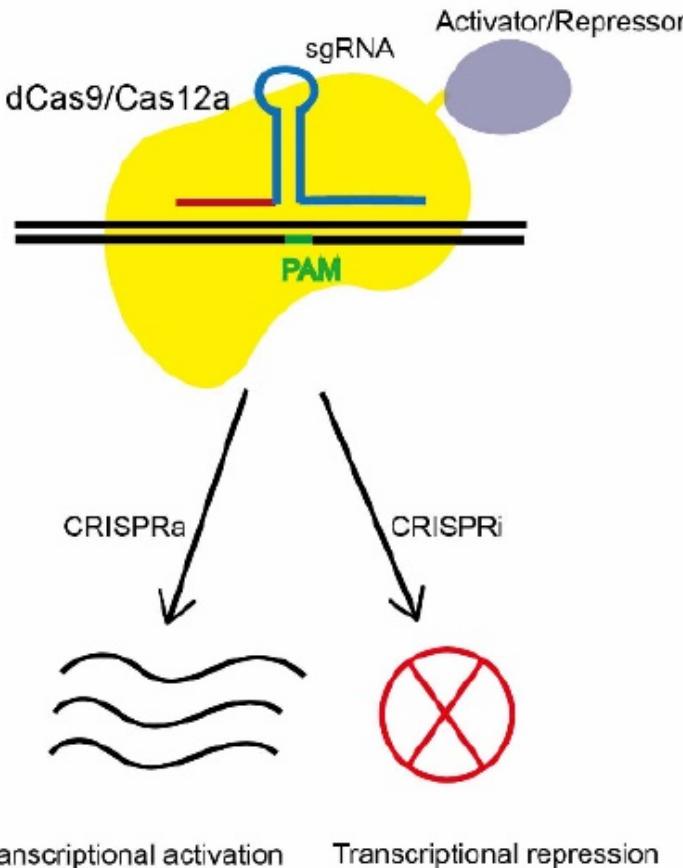
	Plasmid-based approach	RNP-based approach
Preparation of nuclease	Cloning of the nuclease into a plasmid prior transformation is mandatory. When constitutively expressed, risk of off-targets should be considered. When present on AMA-plasmid, the risk should be lower but still present	Cloning of the nuclease into a plasmid allowing heterologous expression, e.g. in <i>E. coli</i> , is a prerequisite. Once established and purified, the nuclease can be aliquoted and stored prior to use. As the protein does not become expressed in the targeted fungus, the risk of off-targets should be very small
Preparation of guide RNA	Plasmid-based, thus more stable during handling and storage	Involves in vitro transcription, hence potentially sensitive to handling errors
Transformation procedure	Easy	Easy but requires preassembly of RNPs
Transformation rate	Very high also with four targets	Very high for single and double targets Low for three and four targets
Single-targeting efficiency of FnCpf1, AsCpf1, SpCas9	Locus-dependent	Locus-dependent
Multiplex-targeting efficiency of FnCpf1	High ($34\% \pm 6\%$ in this study)	Low ($13\% \pm 2\%$ in this study)
MTP-based down-scaling for FnCpf1	Possible with no loss in efficiency with respect to single and double targeting	Possible with no loss in efficiency with respect to single targeting*

Aplicações

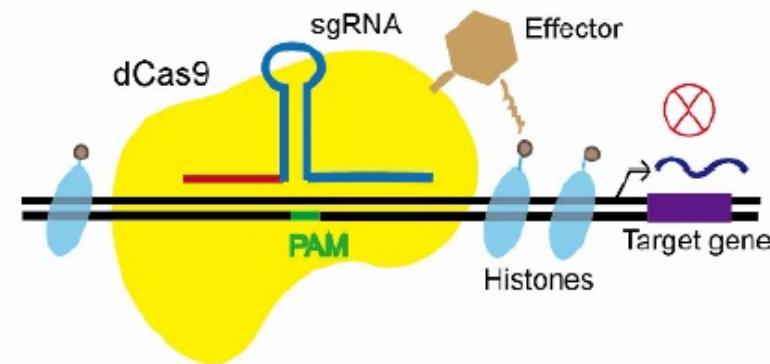
(a) CRISPR-mediated gene editing



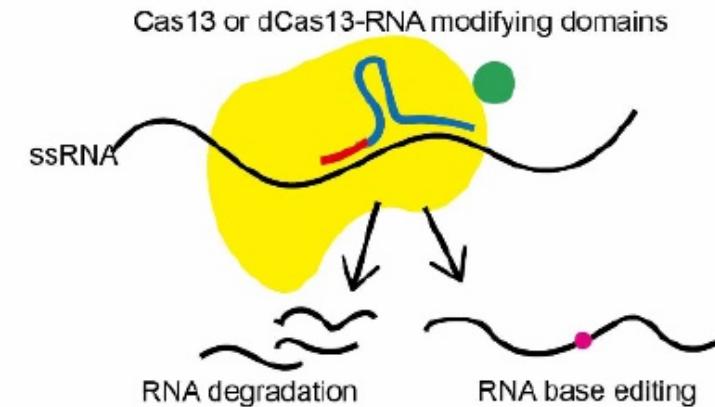
(b) CRISPR-mediated transcriptional regulation



(c) CRISPR-mediated epigenetic editing



(d) CRISPR-mediated RNA editing





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

Dois lados da moeda

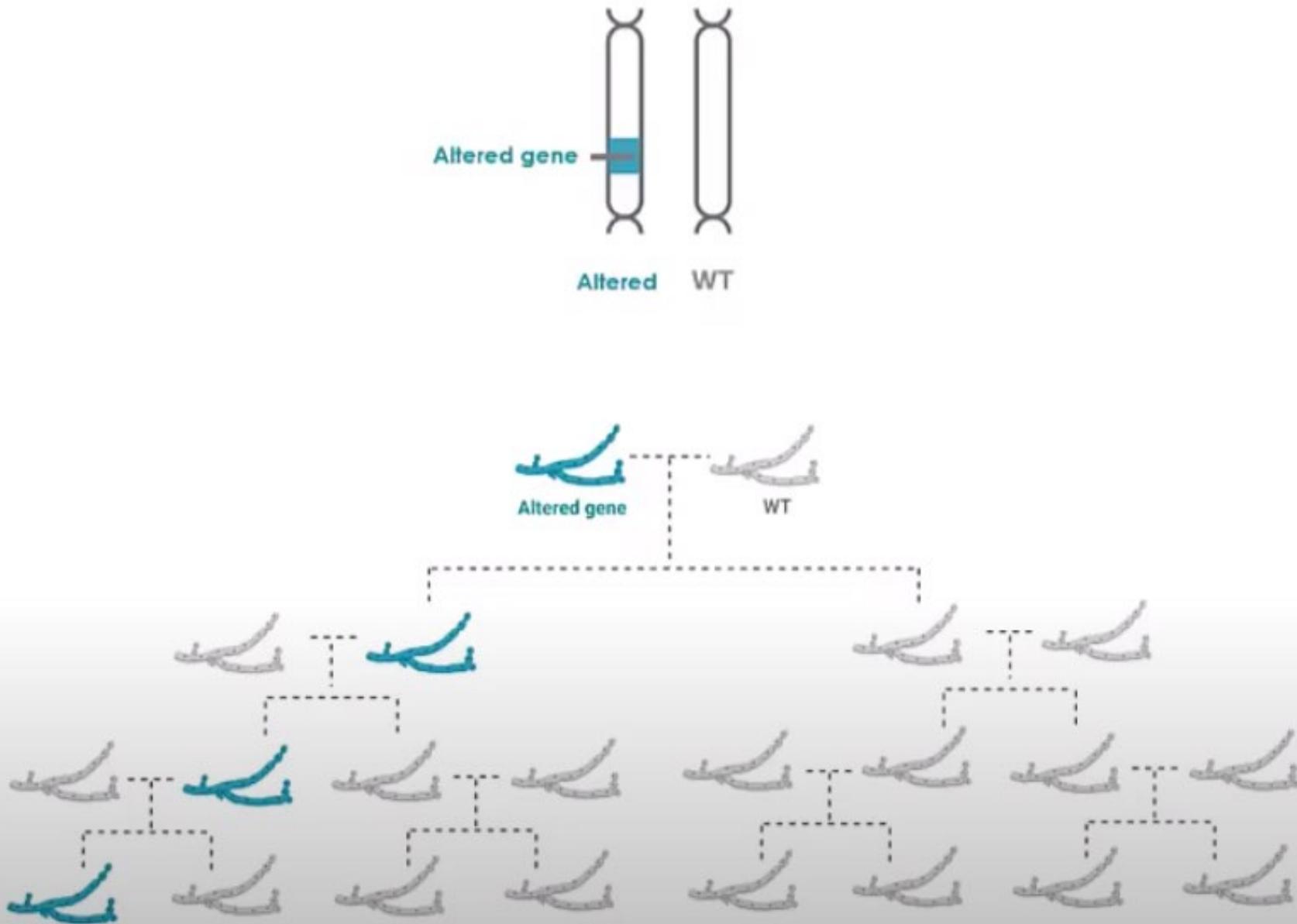
Austin Burt sugeriu em 2003 que as homing endonucleases poderiam formar a base de genes sintéticos que poderiam alterar populações selvagens de organismos que se reproduzem sexualmente

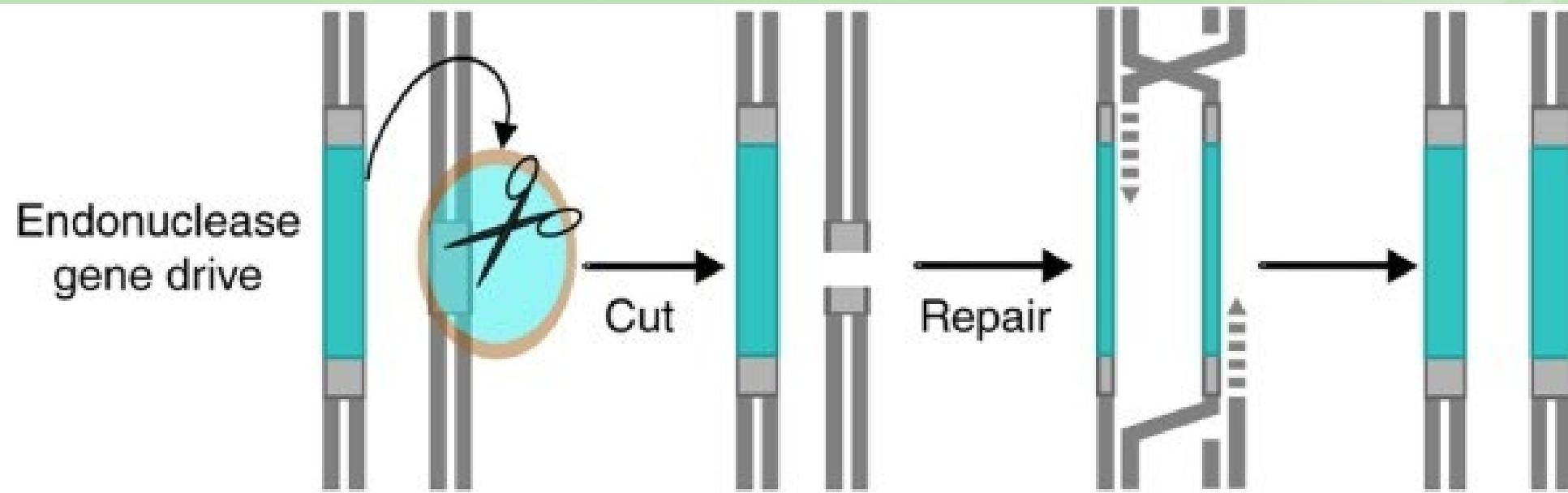
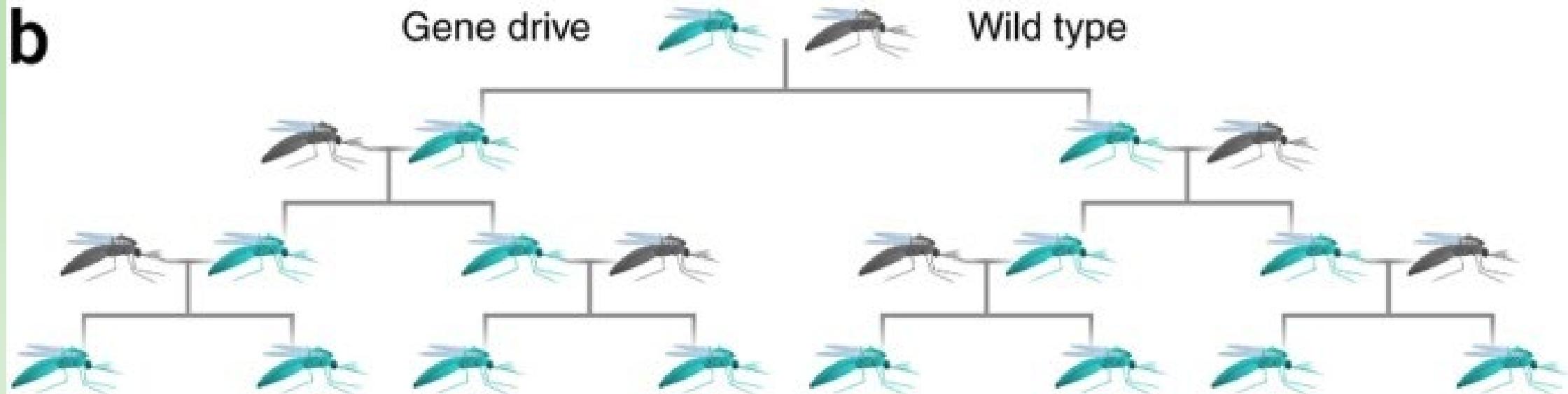
- 1) Eliminação de vetores de doenças e patógenos
- 2) Disseminação de uma população natural por escapes em laboratórios

<https://www.youtube.com/watch?v=7X715cD02sA>

O risc

Altered gene spread by **normal inheritance**



a**b**

Exemplo de aplicação

Developing safeguarding CRISPR-Cas9 gene drive in the plant pathogenic fungus, *Sphaerulina musiva*, to mitigate *Populus* diseases

Joanna Tannous

Oak Ridge National Laboratory

April 26, 2023



ORNL is managed by UT-Battelle, LLC for the US Department of Energy





Fungal Genetic Background and Experiences

Genetic engineering of **model** and **non-model** filamentous fungi

Fungal metabolism

- Characterization of novel fungal metabolites
- Elucidation of biosynthetic pathways

Microbiome dynamics

- Uncover complex interaction of fungi with their hosts and other surrounding microbes

Fungal pathogenicity

- Dissection and elucidation of the mechanisms regulating pathogenicity
- Characterize virulence factors

Overall approach



Development of a CRISPR-Cas9 transformation system in *S. musiva*



Identification of genetic targets for establishment and pathogenesis of *S. musiva*



Evaluation of candidate gene drive targets through knockout and plant phenotyping assays

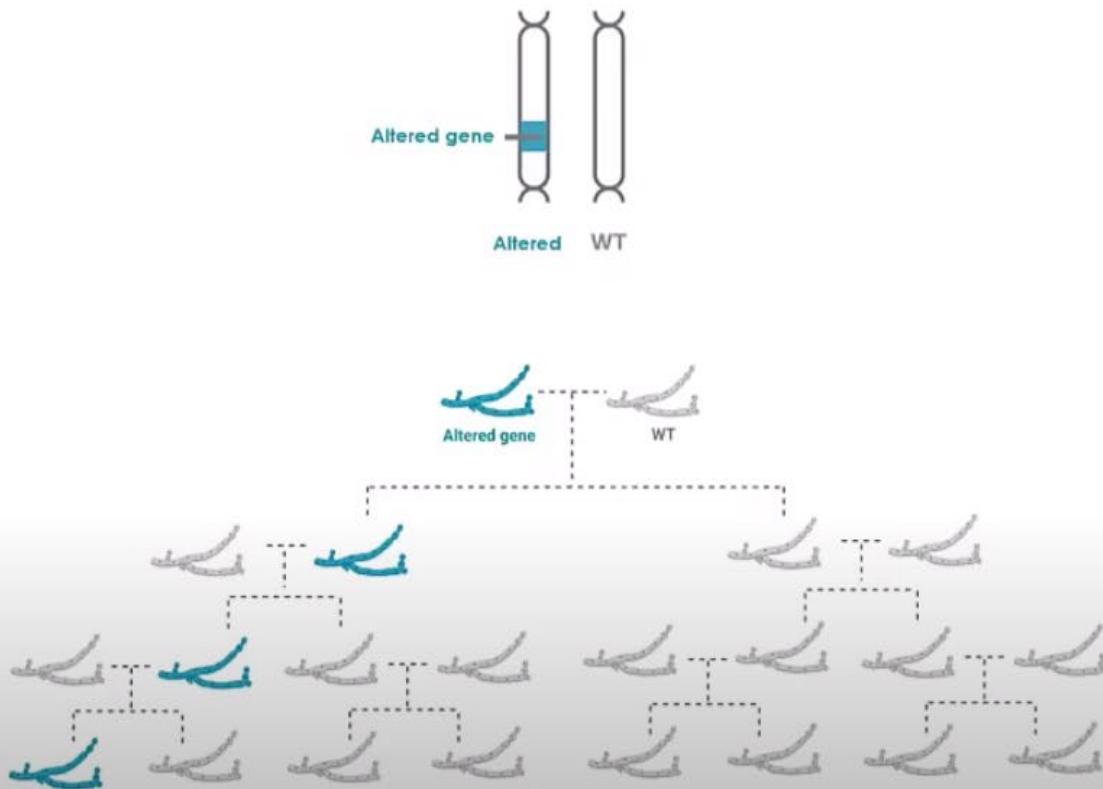


Working towards the establishment of a safeguarding (or split) CRISPR-Cas9 gene drive

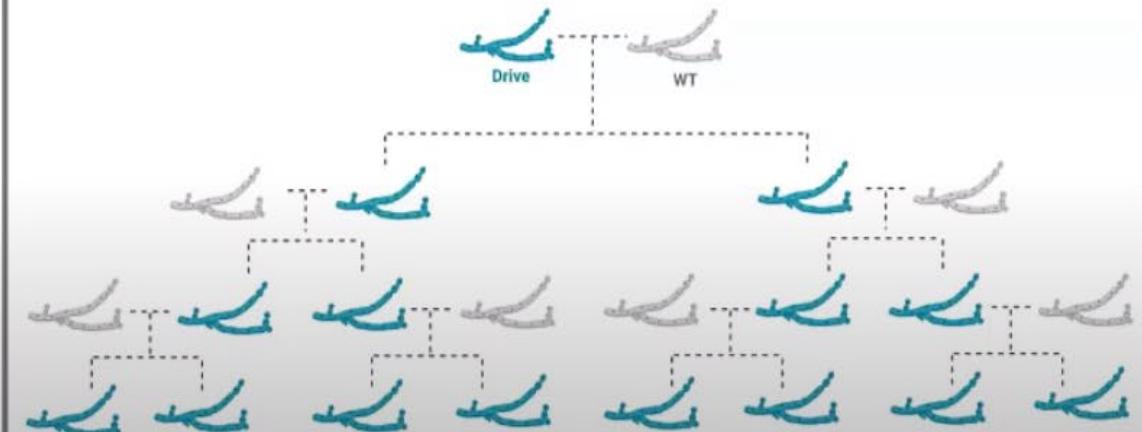
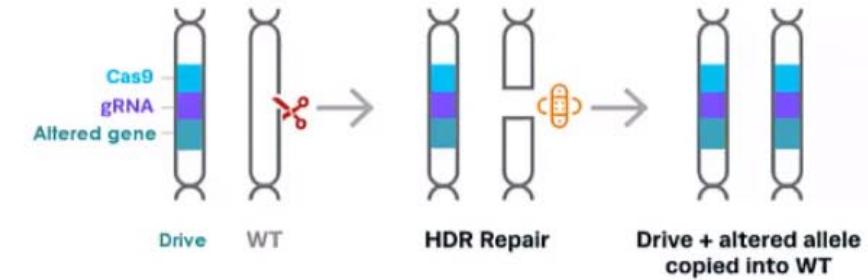
O risco de Synthetic gene drive systems

Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases

Altered gene spread by **normal inheritance**

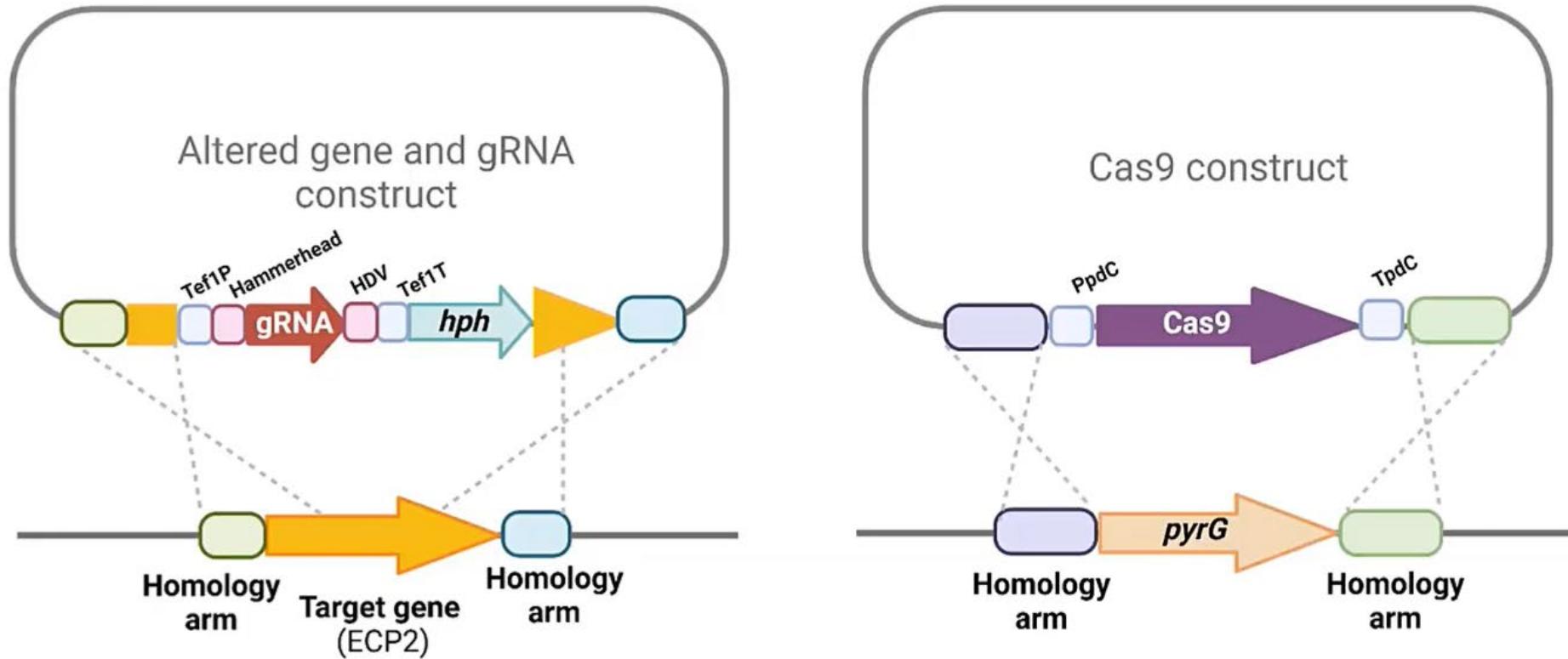


Altered gene spread by **gene drive**

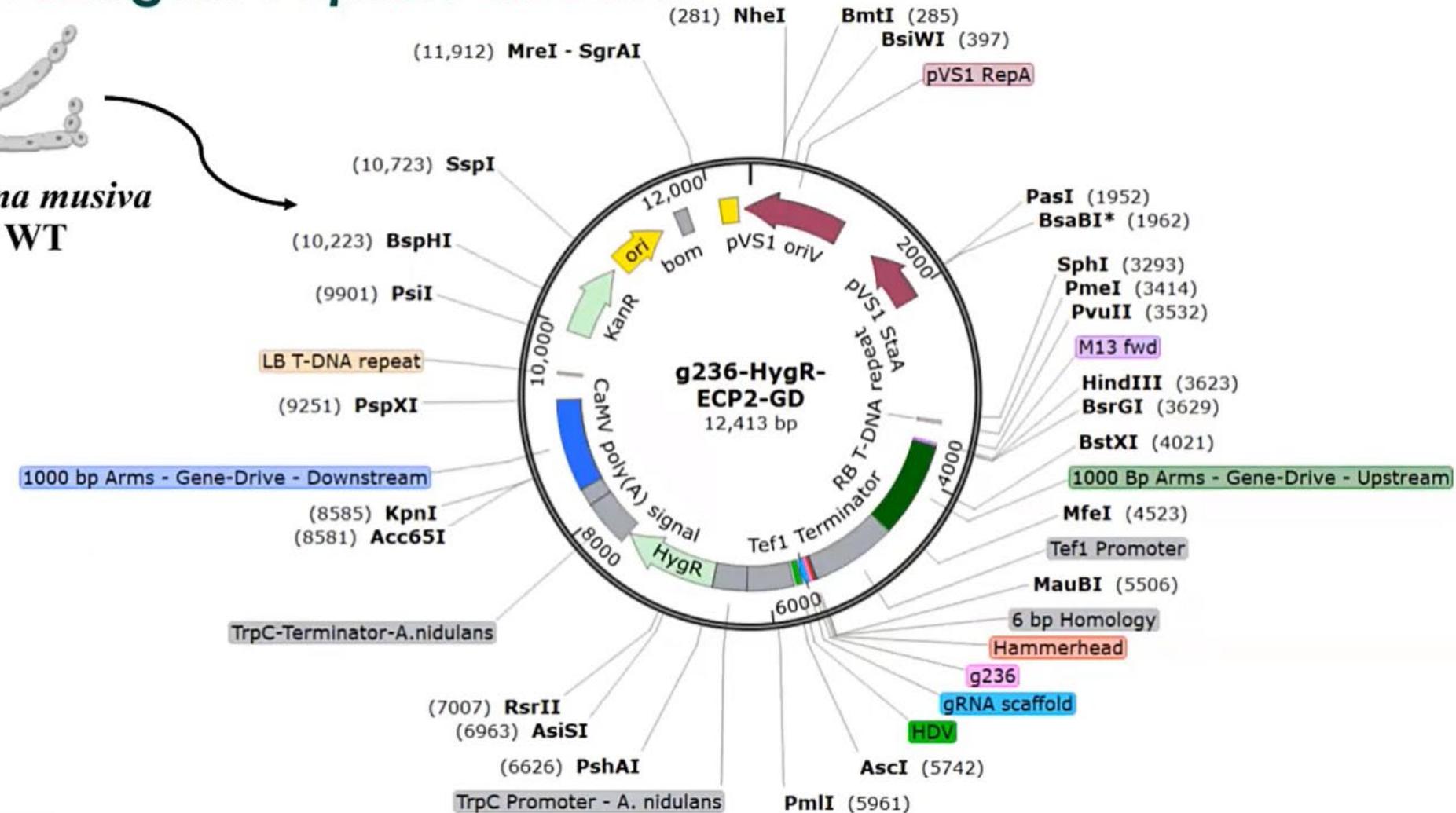


Safeguarding (split)

Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases



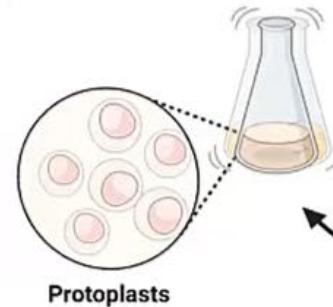
Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases



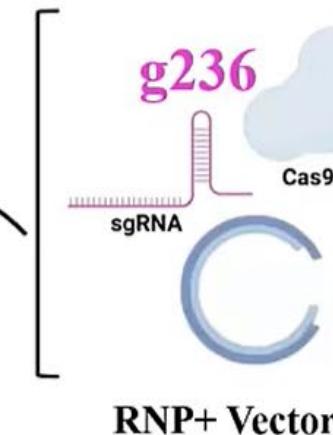
Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases



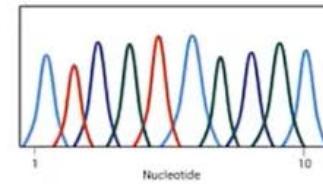
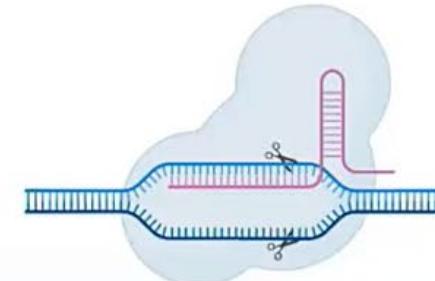
Sphaerulina musiva
strain Mn5



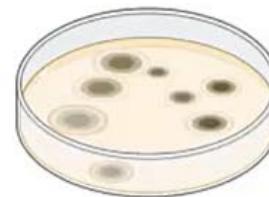
Protoplasts



Target gene:
ECP2



PCR screening for
insertion at the locus and
sequencing at the cut site

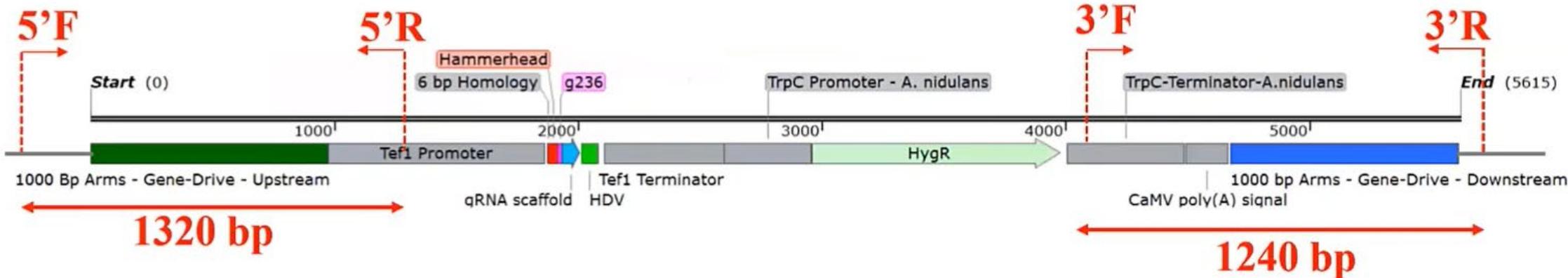


Selection on SMM supplemented
with hygromycin (150 µg/ml)

Seleção dos mutantes via PCR – dos que cresceram em hygromicina

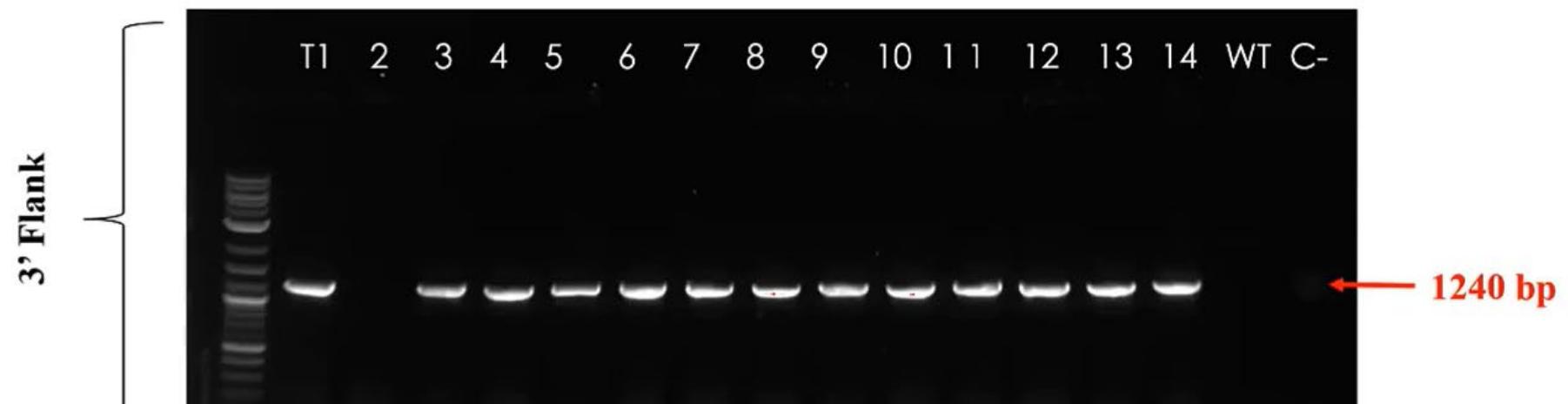
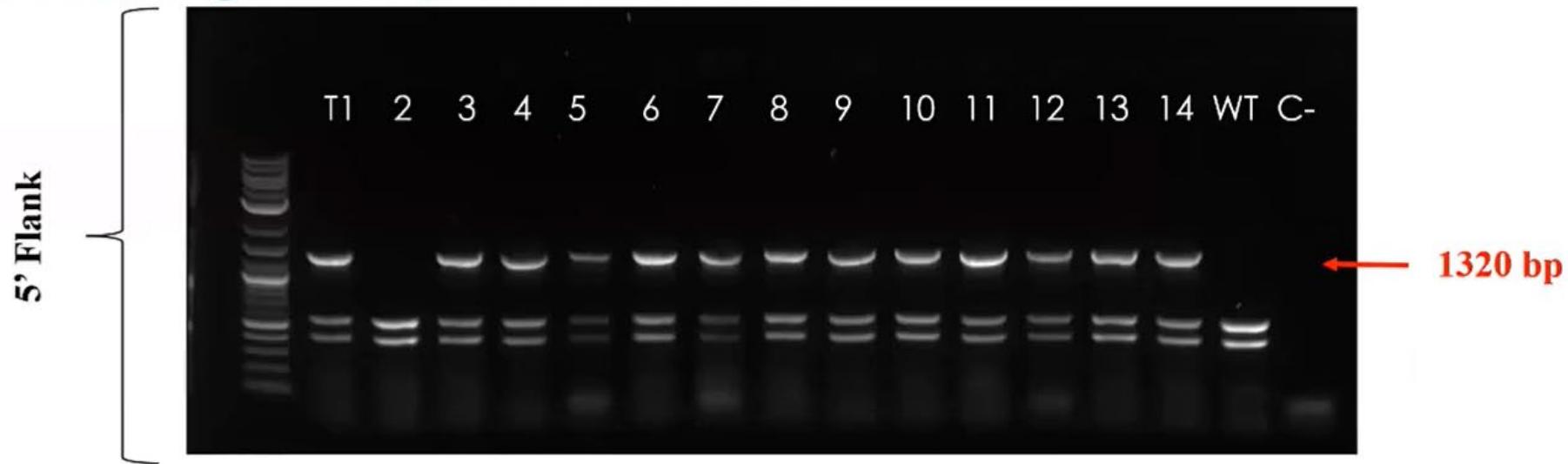
Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases

PCR validation of the knockout strains



Seleção dos mutantes via PCR – apenas 1 não inseriu no local correto

Implementation of a Safeguarding CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases

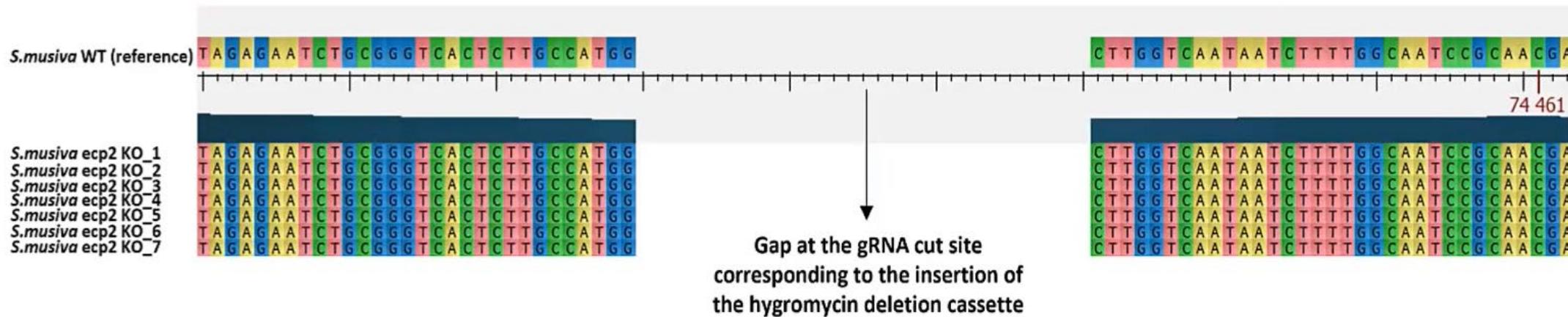


Sequenciamento do Genoma de um dos mutantes

Implementation of a Safeguarding (split) CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases



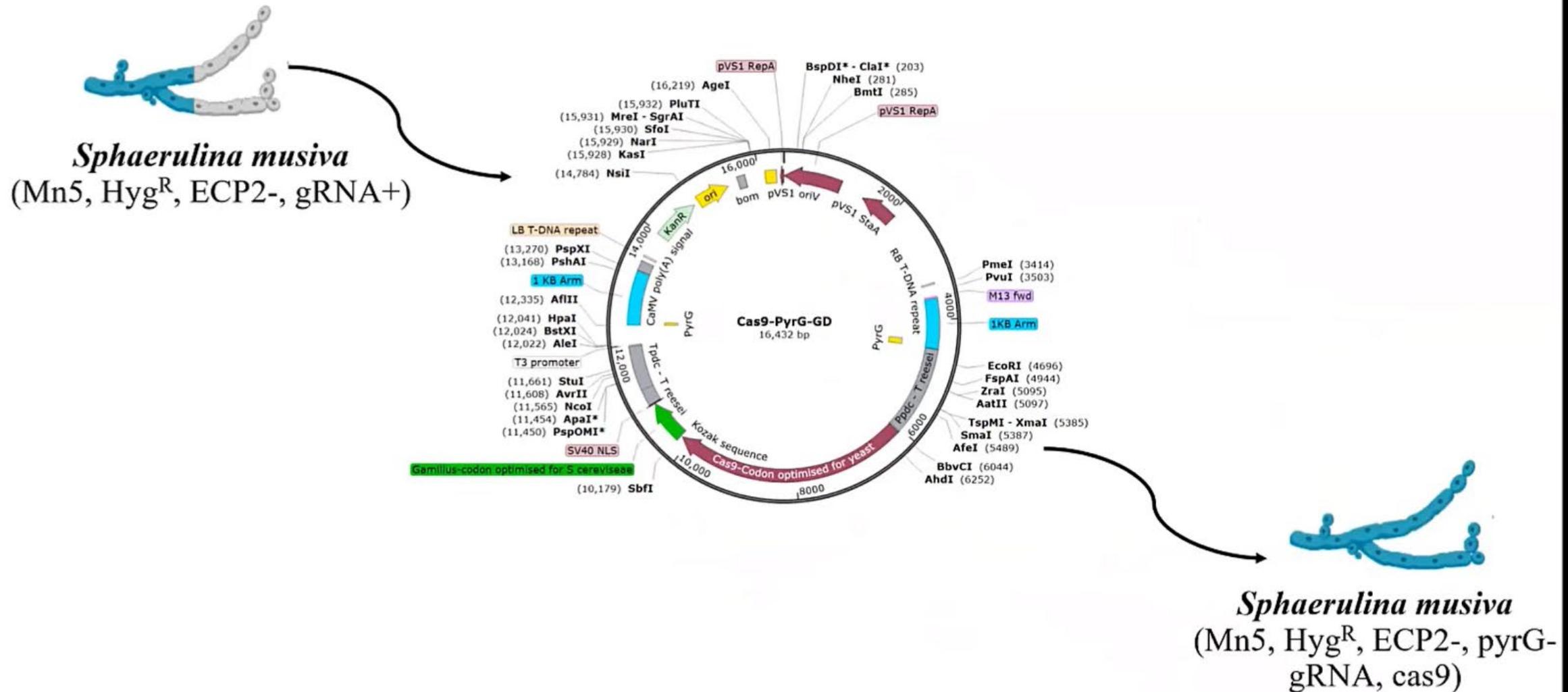
Whole genome nanopore sequencing



- Single insertion of the gRNA construct at the targeted locus
- No SNPs detected due to Cas9-off targets

Segundo evento de transformação

Implementation of a Safeguarding (split) CRISPR-Cas9 gene drive
in *S. musiva* to mitigate *Populus* diseases



Avaliação do sistema de cruzamento

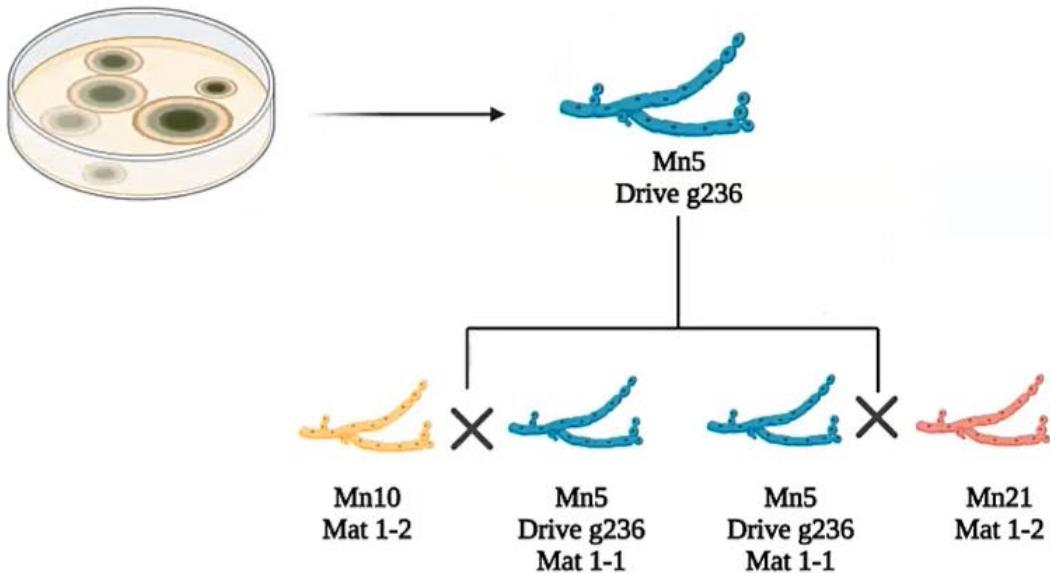
Implementation of a Safeguarding (split) CRISPR-Cas9 gene drive in *S. musiva* to mitigate *Populus* diseases



Strains that can be used to confirm the gene drive system:

Mn-10 – Can be cut by g236, different mating type than Mn5

Mn-21 – Cannot be cut by g236, different mating type than Mn5



Condições para desenho do gRNA

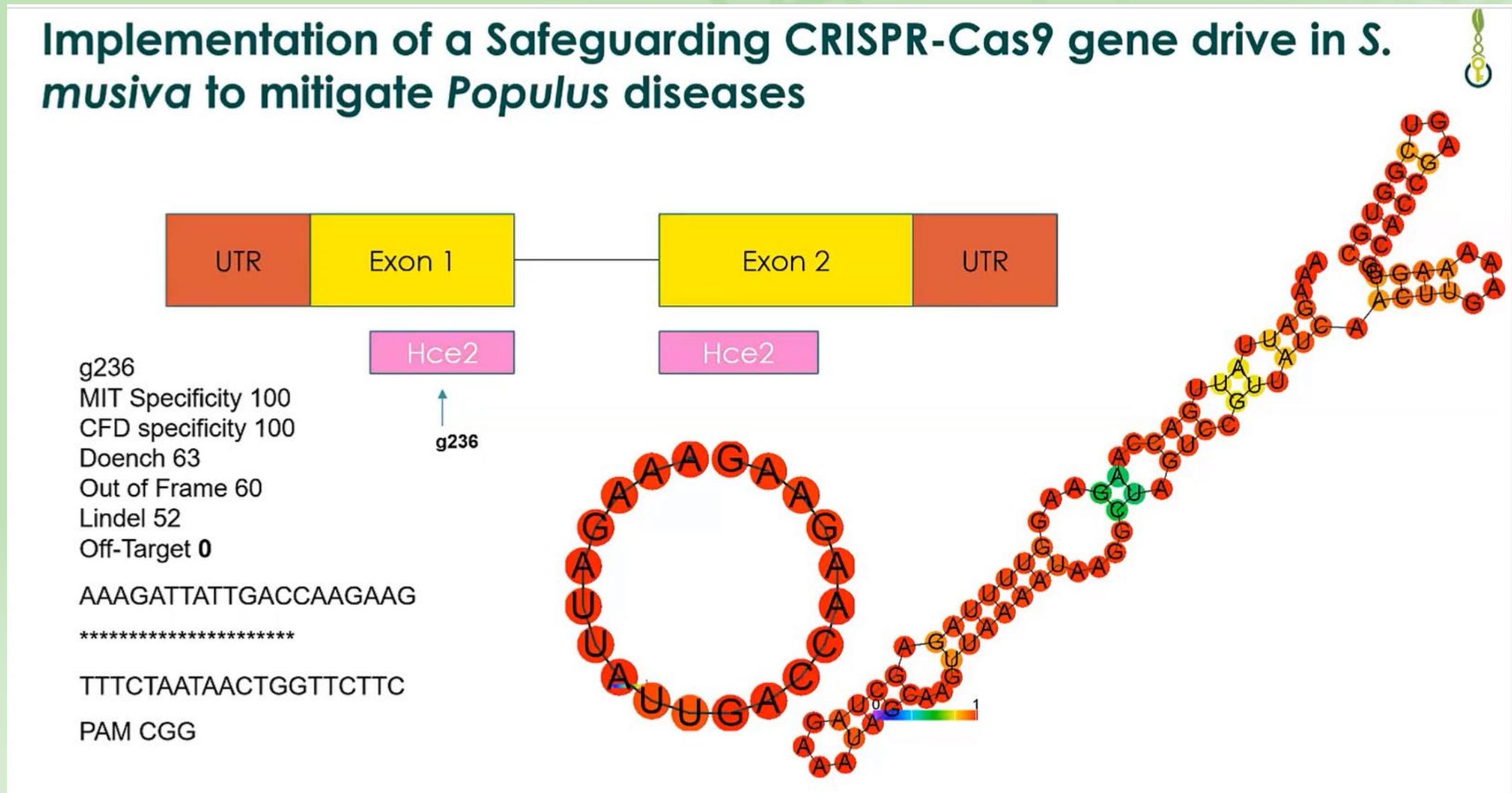
1. Para perda de função:

- a) Região de desenho deve ser de domínio funcional e conservado - gerando produtos não funcionais após edição

2. Avaliação de off-target

- a) Genoma disponível

Condições para desenho do gRNA



Alterações genéticas e epigenéticas

<https://www.youtube.com/watch?v=4YKFw2KZA5o&t=2s>