EDIÇÃO GÊNICA MEDIADA POR CRISPR-CAS9 EM *Cryptococcus neoformans*

Profa. Dra. Renata Castiglioni Pascon
Prof. Dr. Marcelo A. Vallim

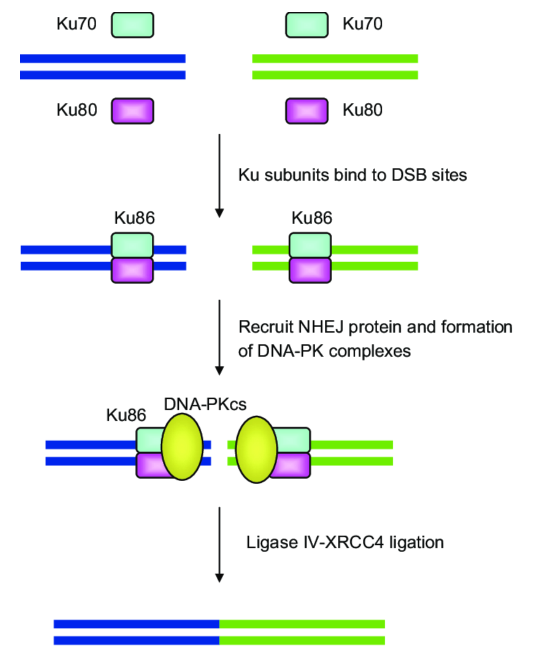
CRISPR-CAS9 EM *Cryptococcus neoformans*

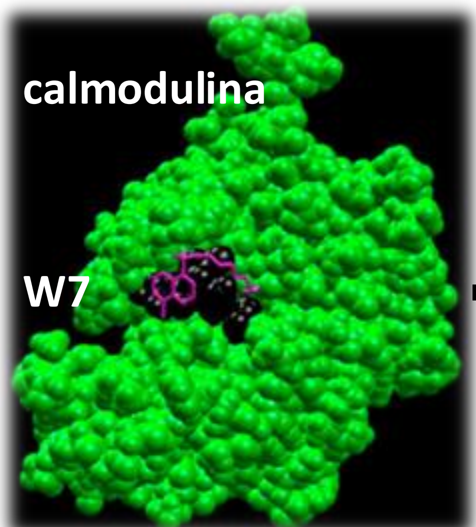
- Baixa frequência de integração homóloga
- Biolística
- Edição de múltiplos genes impossível
- Dificuldades de construção de deleção

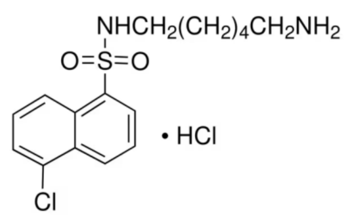
CRISPR-CAS9 EM *Cryptococcus neoformans*

- Knockout do NHEJ
  - ku80Δ (afeta a virulência)
  - W7*

* N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide







Inositol fosfato ↓

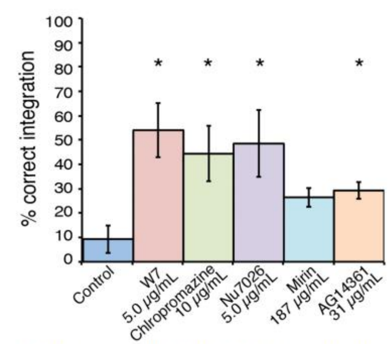


Fig 3. Inhibitors of mammalian NHEJ repair result in increased homologous integration in C. *neoformans* at the *LAC1* locus. Drug treatment resulted in a significant increase in the frequency of homologous integration events for four of the five inhibitors tested. All values show mean, error bars show S. E.M. * = $P<0.05$.

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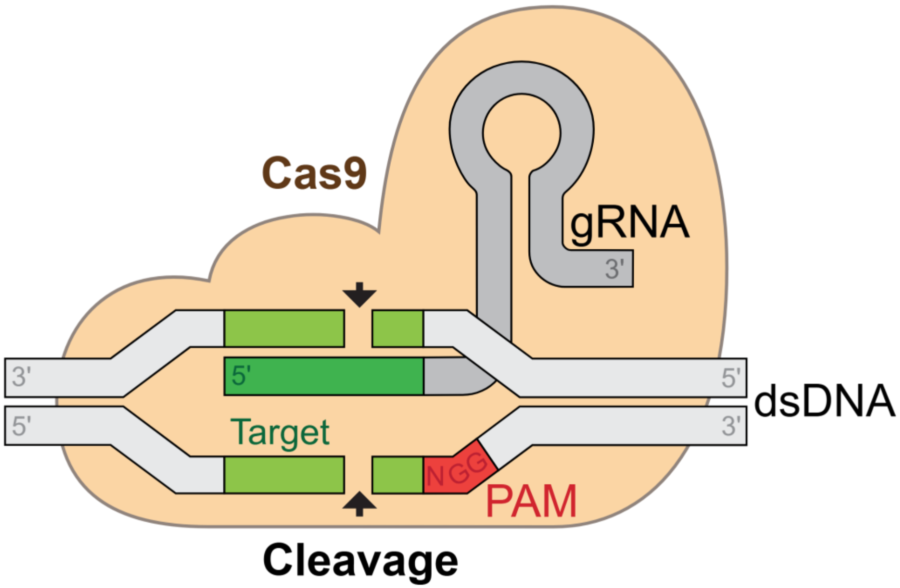
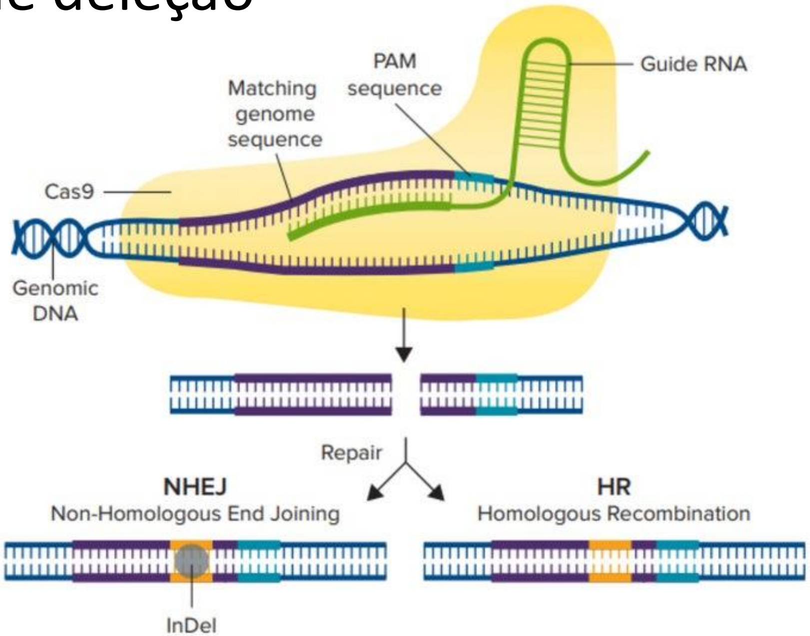
Arras SDM, Fraser JA (2016) Chemical Inhibitors of Non-Homologous End Joining Increase Targeted Construct Integration in *Cryptococcus neoformans*. PLoS ONE 11(9): e0163049. https://doi.org/10.1371/journal.pone.0163049

CRISPR-CAS9 EM *Cryptococcus neoformans*

- Por que CRISPR em *C. neoformans*?
  - Baixa frequência de integração homóloga
  - Biolística → eletroporação
  - Edição de múltiplos genes
  - Facilidade de construção de deleção





CRISPR-CAS9 EM *Cryptococcus neoformans*

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  - Baixa frequência de integração homóloga
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  - Facilidade de construção de deleção

PLOS | ONE

RESEARCH ARTICLE

Targeted Genome Editing *via* CRISPR in the Pathogen *Cryptococcus neoformans*

Samantha D. M. Arras[1,2], Sheena M. H. Chua[1,2], Maha S. I. Wizrah[1,2], Joshua A. Faint[1,2], Amy S. Yap[1,2], James A. Fraser[1,2]*

1 Australian Infectious Diseases Research Centre, St Lucia, Queensland, Australia, **2** School of Chemistry & Molecular Biosciences, The University of Queensland, St Lucia, Queensland, Australia

* jafraser@uq.edu.au

Arras SDM, Chua SMH, Wizrah MSI, Faint JA, Yap AS, Fraser JA (2016) Targeted Genome Editing via CRISPR in the Pathogen Cryptococcus neoformans. PLoS ONE 11(10): e0164322. doi:10.1371/journal.pone.0164322

CRISPR-CAS9 EM *Cryptococcus neoformans*

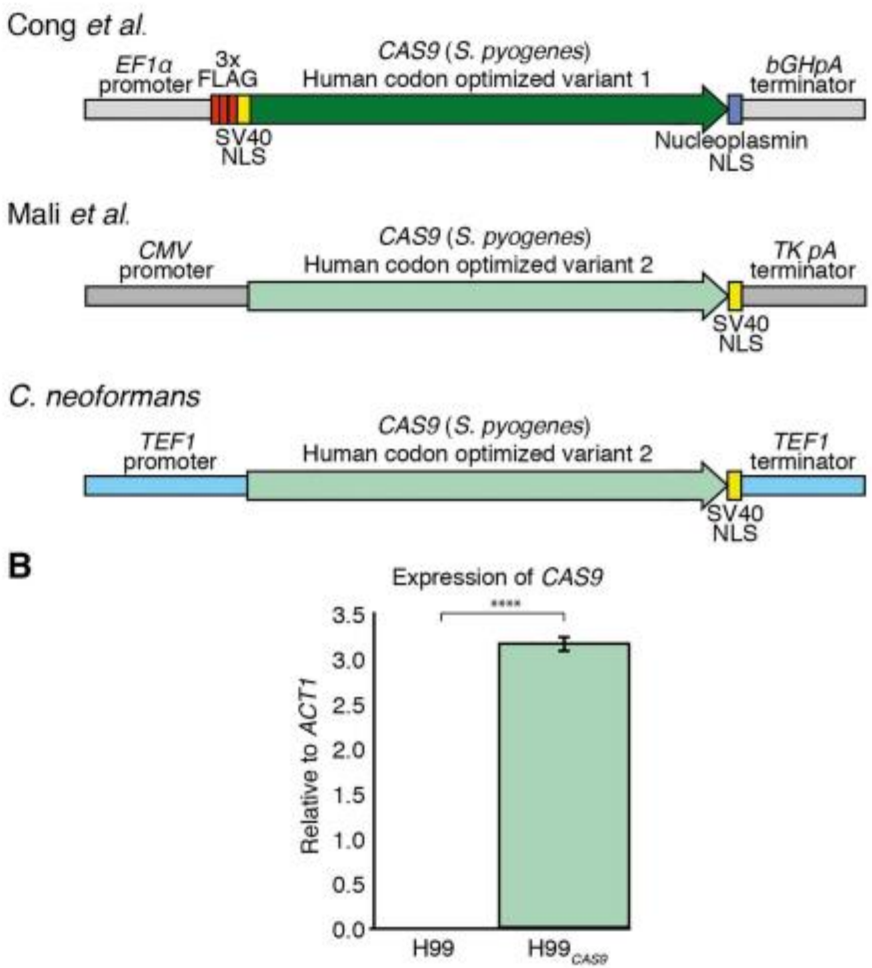
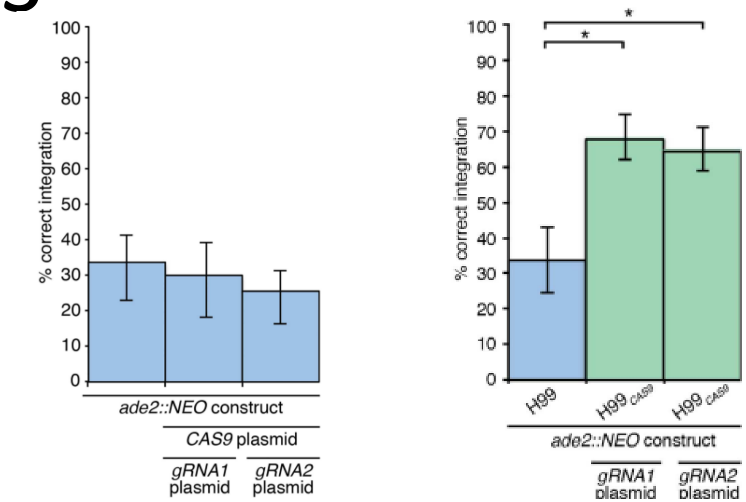
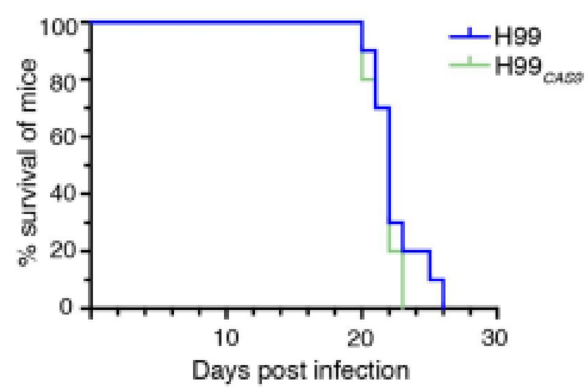


Fig 1. Expressing Cas9 in *C. neoformans*. A. Comparison of the *CAS9* expression constructs from human [37, 47] and *C. neoformans*. **B.** Transcript abundance of *CAS9* in H99 and $H99_{CAS9}$ relative to *ACT1*. Values show mean, error bars show S.E.M.



BIOLÍSTICA



Arras SDM, Chua SMH, Wizrah MSI, Faint JA, Yap AS, Fraser JA (2016) Targeted Genome Editing via CRISPR in the Pathogen Cryptococcus neoformans. PLoS ONE 11(10): e0164322. doi:10.1371/journal.pone.0164322

CRISPR-CAS9 EM *Cryptococcus neoformans*

- Sem biolística
- > frequência de RH
- Linhagem hospedeira H99 selvagem

Multiple Applications of a Transient CRISPR-Cas9 Coupled with Electroporation (TRACE) System in the *Cryptococcus neoformans* Species Complex

Yumeng Fan and Xiaorong Lin[1]
Department of Microbiology, University of Georgia, Athens, Georgia 30602
ORCID IDs: 0000-0003-4975-3751 (Y.F.); 0000-0002-3390-8387 (X.L.)

ABSTRACT *Cryptococcus neoformans* is a fungal pathogen that claims hundreds of thousands of lives annually. Targeted genetic manipulation through biolistic transformation in *C. neoformans* drove the investigation of this clinically important pathogen at the molecular level. Although costly and inefficient, biolistic transformation remains the major method for editing the *Cryptococcus* genome as foreign DNAs introduced by other methods such as electroporation are predominantly not integrated into the genome. Although the majority of DNAs introduced by biolistic transformation are stably inherited, the transformation efficiency and the homologous integration rate (~1–10%) are low. Here, we developed a Transient CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 coupled with Electroporation (TRACE) system for targeted genetic manipulations in the *C. neoformans* species complex. This method took advantages of efficient genome integration due to double-strand breaks created at specific sites by the transient CRISPR-Cas9 system and the high transformation efficiency of electroporation. We demonstrated that TRACE can

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CRISPR-CAS9 EM *Cryptococcus neoformans*

- Prova de conceito *ade*2Δ

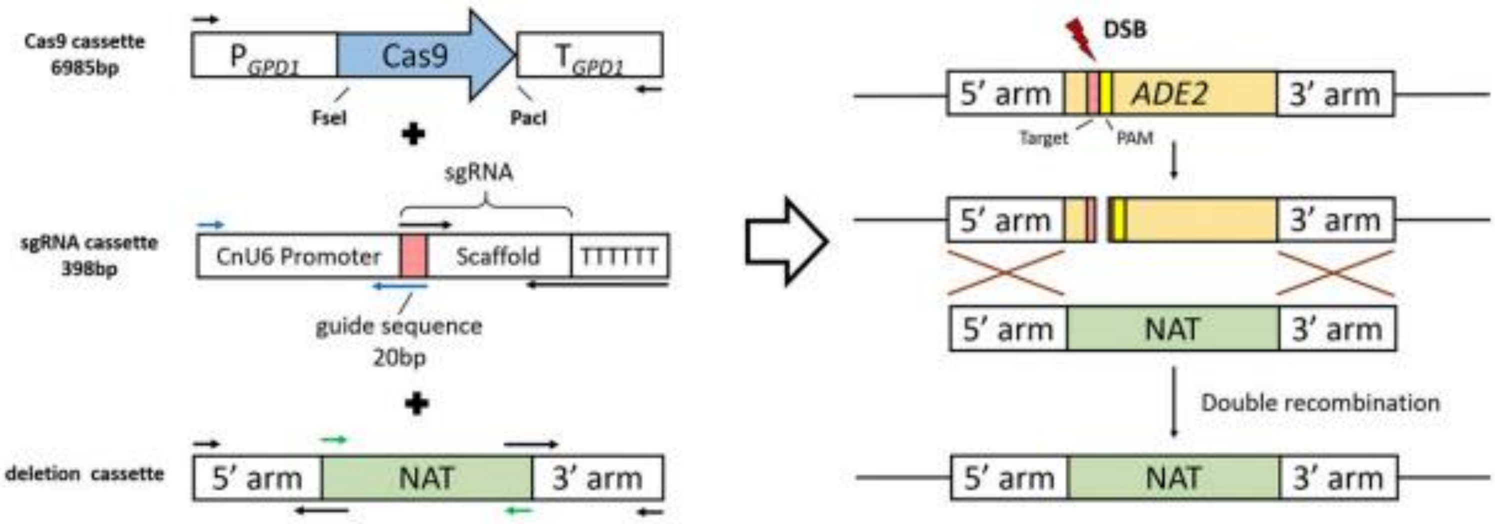

Figure 1 Construction of the Transient CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 coupled with Electroporation (TRACE) system. A diagram of the three elements of the TRACE system generated via PCR (left panel). Arrows represent the position and the direction of the primers. A diagram for the working concept of the TRACE system (right panel). When the three elements are all expressed in *Cryptococcus* cells, single-guide RNA (sgRNA) will guide Cas9 to the specific site that matches the target sequence and then Cas9 will generate a double-strand break (DSB). The deletion construct will serve as the template during DSB repair and eventually replace the gene of interest with the drug-resistant marker by homologous recombination. NAT: nourseothricin resistance cassette

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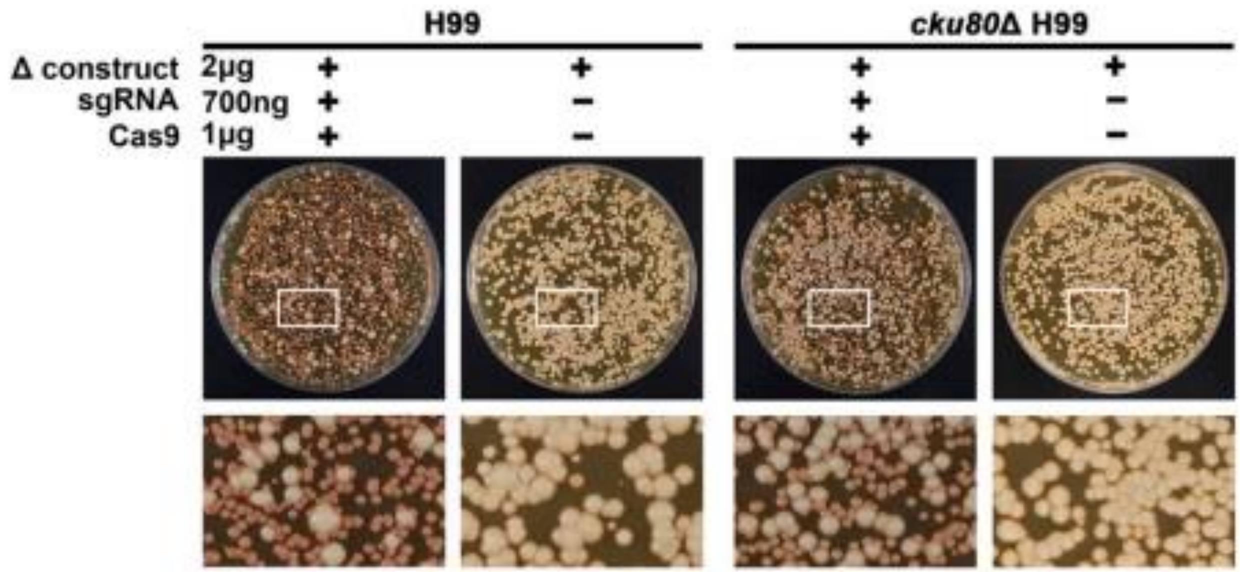

Table 1 Deletion of *ADE2* with a 1-kb arm deletion construct

Strain	Deletion construct	Cas9	sgRNA	Red colonies	Total colonies	*ADE2* disruption frequency (%)
H99	+	+	+	4392	4728	92.90
H99	+	+	+	2648	2864	92.40
H99	+	−	−	20	1120	1.78
H99	+	−	−	16	656	2.44
*cku80*Δ H99	+	+	+	2080	2528	82.30
*cku80*Δ H99	+	+	+	2504	3184	78.60
*cku80*Δ H99	+	−	−	26	1456	1.78
*cku80*Δ H99	+	−	−	23	1888	1.22

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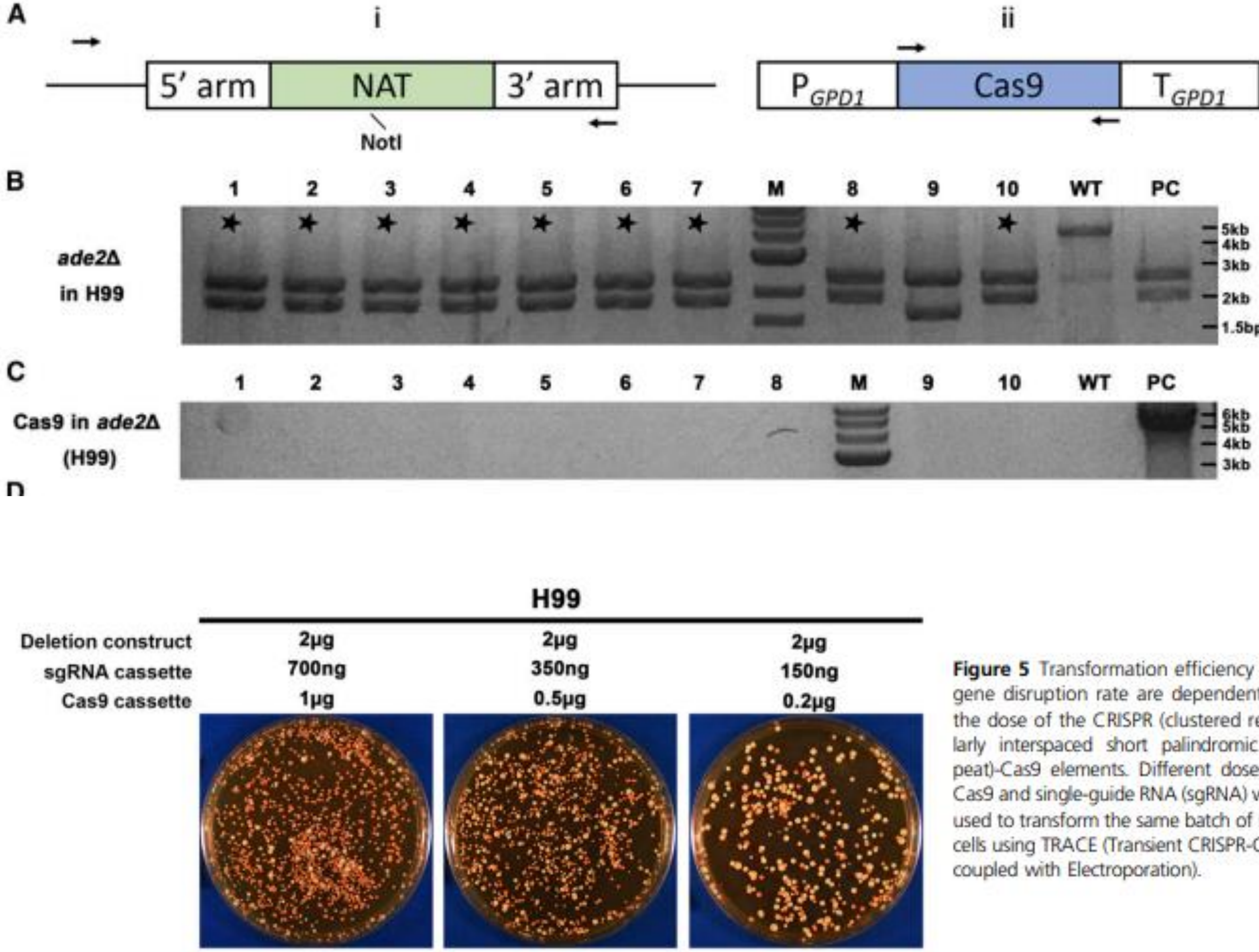

Figure 5 Transformation efficiency and gene disruption rate are dependent on the dose of the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 elements. Different doses of Cas9 and single-guide RNA (sgRNA) were used to transform the same batch of H99 cells using TRACE (Transient CRISPR-Cas9 coupled with Electroporation).

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CRISPR-CAS9 EM *Cryptococcus neoformans*

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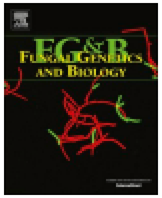

Resource

Transformation of *Cryptococcus neoformans* by electroporation using a transient CRISPR-Cas9 expression (TRACE) system

Jianfeng lin[1,2], Yumeng Fan[1], Xiaorong Lin[*]

Department of Microbiology, University of Georgia, Athens, GA 30602, USA

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TRACE
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Allele Swap

ABSTRACT

The basidiomycete *Cryptococcus neoformans* is not only a clinically important pathogen, but also a model organism for studying microbial pathogenesis and eukaryotic biology. One key factor behind its rise as a model organism is its genetic amenability. The widely used methods for transforming the *C. neoformans* species complex are *Agrobacterium*-mediated transformation (AMT) for random insertional mutagenesis and biolistic transformation for targeted mutagenesis. Electroporation was introduced to *C. neoformans* in early 1990s. Although electroporation is economic and yields a large number of transformants, introduced DNA rarely integrates into cryptococcal genome, which limits its use. Biolistic transformation, although costly and inefficient, has been the only method used in targeted mutagenesis in the past two decades. Several modifications, including the use of a donor DNA with split markers, a drug-resistant selection marker, and a recipient strain deficient in non-homologous end joining (NHEJ), have since modestly increased the frequency of genome integration and the rate of homologous replacement of the DNA introduced by electroporation. However, electroporation was not the method of choice for transformation until the recent adoption of CRISPR-Cas9 systems. We have developed a Transient CRISPR-Cas9 coupled with Electroporation System (TRACE), which dramatically facilitates targeted mutagenesis in the *Cryptococcus* species complex. TRACE combines the high transformation efficiency of

CRISPR-CAS9 EM *Cryptococcus neoformans*

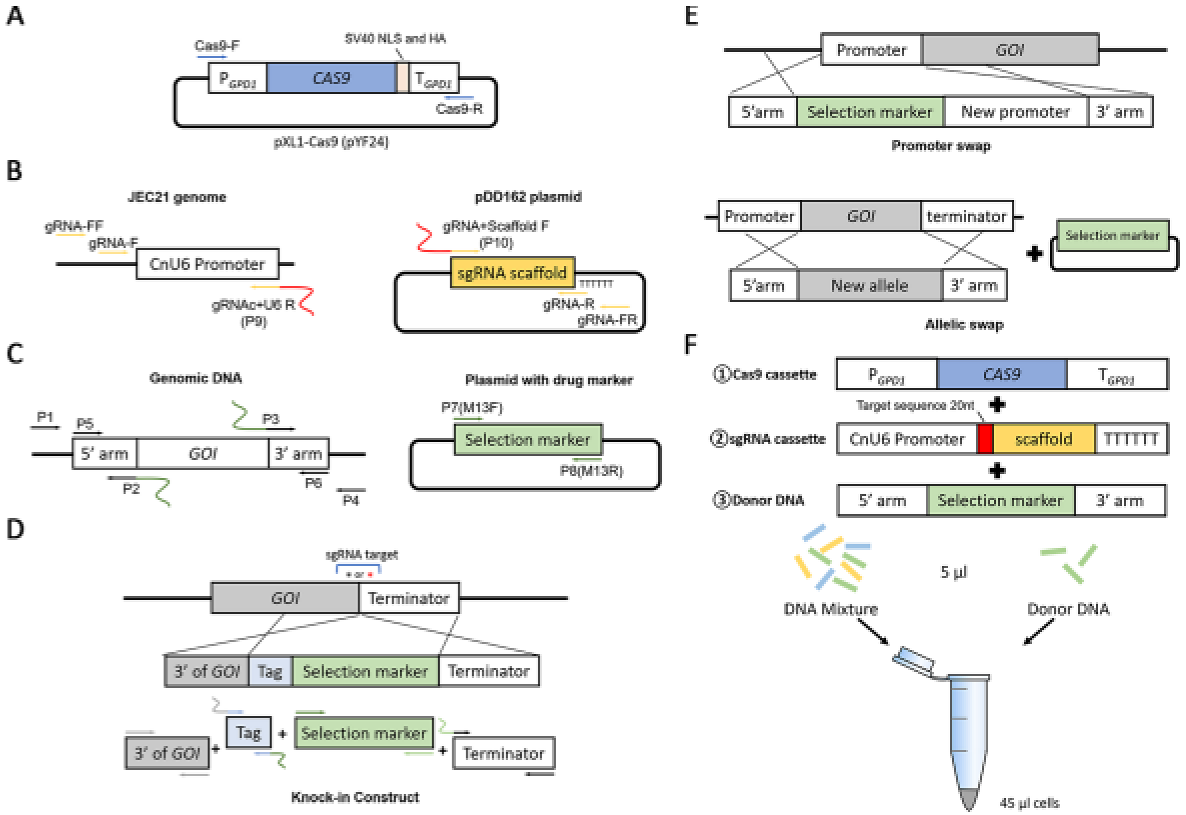

Fig. 1. Diagrams of the cassettes used in a TRACE system. (A) The simplified layout of the pXL1-Cas9 (pYF24) plasmid. The Cas9 construct (~7kb) for TRACE is amplified from this vector with the Cas9-F/Cas9-R primer set. (B) The gRNA construct is amplified by fusing the U6 promoter and the gRNA scaffold in an overlap extension PCR with primers gRNA-F/gRNA-R. The U6 promoter and the gRNA scaffold are amplified from the genomic DNA of strain JEC21 and the vector pDD162 respectively. (C) The gene deletion construct is amplified with the primer set P5/P6 by fusing the 5′ arm, the selection marker, and the 3′ arm in a double-joint PCR. The homologous arms are amplified from the genomic DNA of the parent strain and the drug selection marker is amplified from a vector with the M13F/M13R primer set. (D) The native-tagging knock-in construct is generated by fusing 4 fragments: the 3′ end of *GOI*, the tag cassette, the selection marker, and the downstream of *GOI* (terminator). Asterisks indicate the locations of the gRNA target sequences. (E) Both promoter swap and ORF allelic swap constructs contain two homologous arms in the donor construct. However, in allelic swap, the selection marker needs to be co-transformed into cells separately. (F) The linear Cas9, gRNA, and donor DNA are co-transformed into *Cryptococcus* cells in the TRACE system.

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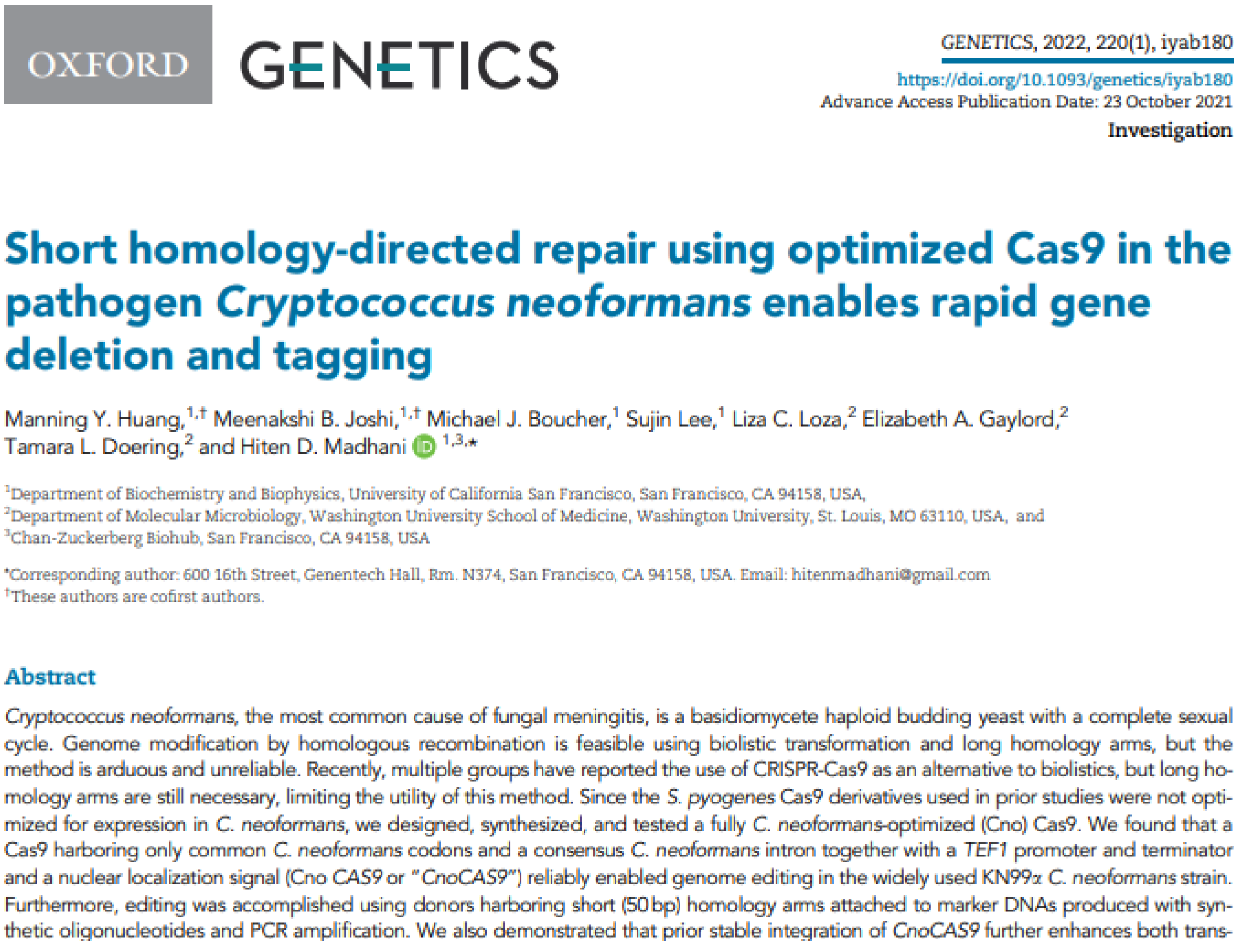
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Investigation

Short homology-directed repair using optimized Cas9 in the pathogen *Cryptococcus neoformans* enables rapid gene deletion and tagging

Manning Y. Huang,[1,†] Meenakshi B. Joshi,[1,†] Michael J. Boucher,[1] Sujin Lee,[1] Liza C. Loza,[2] Elizabeth A. Gaylord,[2] Tamara L. Doering,[2] and Hiten D. Madhani [1,3,*]

[1]Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA 94158, USA,
[2]Department of Molecular Microbiology, Washington University School of Medicine, Washington University, St. Louis, MO 63110, USA, and
[3]Chan-Zuckerberg Biohub, San Francisco, CA 94158, USA

*Corresponding author: 600 16th Street, Genentech Hall, Rm. N374, San Francisco, CA 94158, USA. Email: hitenmadhani@gmail.com
†These authors are cofirst authors.

Abstract

Cryptococcus neoformans, the most common cause of fungal meningitis, is a basidiomycete haploid budding yeast with a complete sexual cycle. Genome modification by homologous recombination is feasible using biolistic transformation and long homology arms, but the method is arduous and unreliable. Recently, multiple groups have reported the use of CRISPR-Cas9 as an alternative to biolistics, but long homology arms are still necessary, limiting the utility of this method. Since the *S. pyogenes* Cas9 derivatives used in prior studies were not optimized for expression in *C. neoformans*, we designed, synthesized, and tested a fully *C. neoformans*-optimized (Cno) Cas9. We found that a Cas9 harboring only common *C. neoformans* codons and a consensus *C. neoformans* intron together with a *TEF1* promoter and terminator and a nuclear localization signal (Cno *CAS9* or "*CnoCAS9*") reliably enabled genome editing in the widely used KN99α *C. neoformans* strain. Furthermore, editing was accomplished using donors harboring short (50 bp) homology arms attached to marker DNAs produced with synthetic oligonucleotides and PCR amplification. We also demonstrated that prior stable integration of *CnoCAS9* further enhances both trans-

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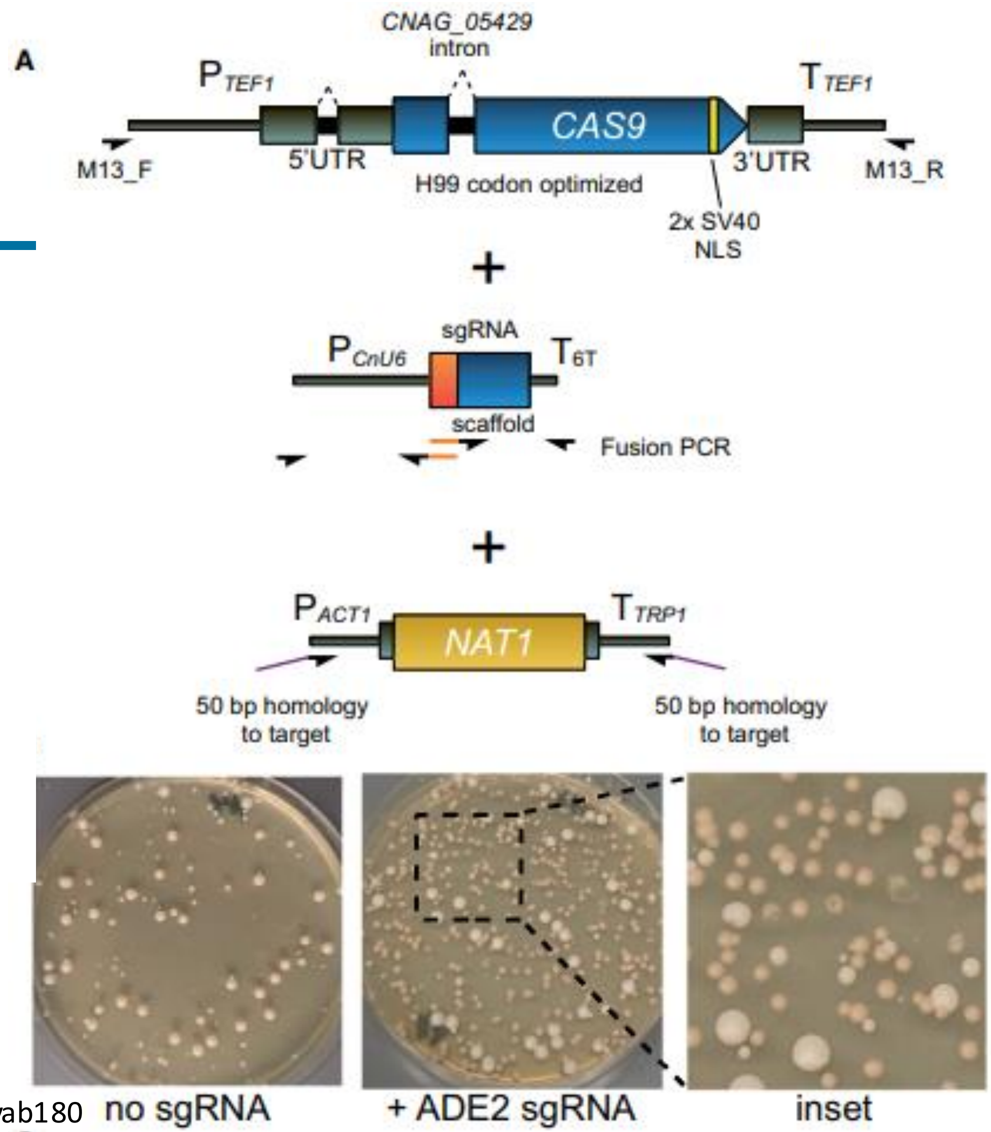
CRISPR-CAS9 EM *Cryptococcus neoformans*

6 | GENETICS, 2022, Vol. 220, No. 1

Table 1 Colony counts of Ade- and Ura- transformants

Experiment	Cas9 state	# aux −	# aux +	% aux −
ade2Δ	Transient	174	76	69.6
		158	69	69.6
		129	58	69.0
ura5Δ	Transient	11	131	7.7
		11	101	9.8
		24	108	18.2
ade2Δ	Integrated	1730[a]	4	99.8
		1950[a]	8	99.6
		1520[a]	7	99.5
ura5Δ	Integrated	82	53	60.7
		106	17	86.2
		69	22	75.8

[a] Values extrapolated from colony counts of a 1:100 dilution of transformed cells.



Prós: 50 bp de braço
Contras: Cas9 integrado ao genoma

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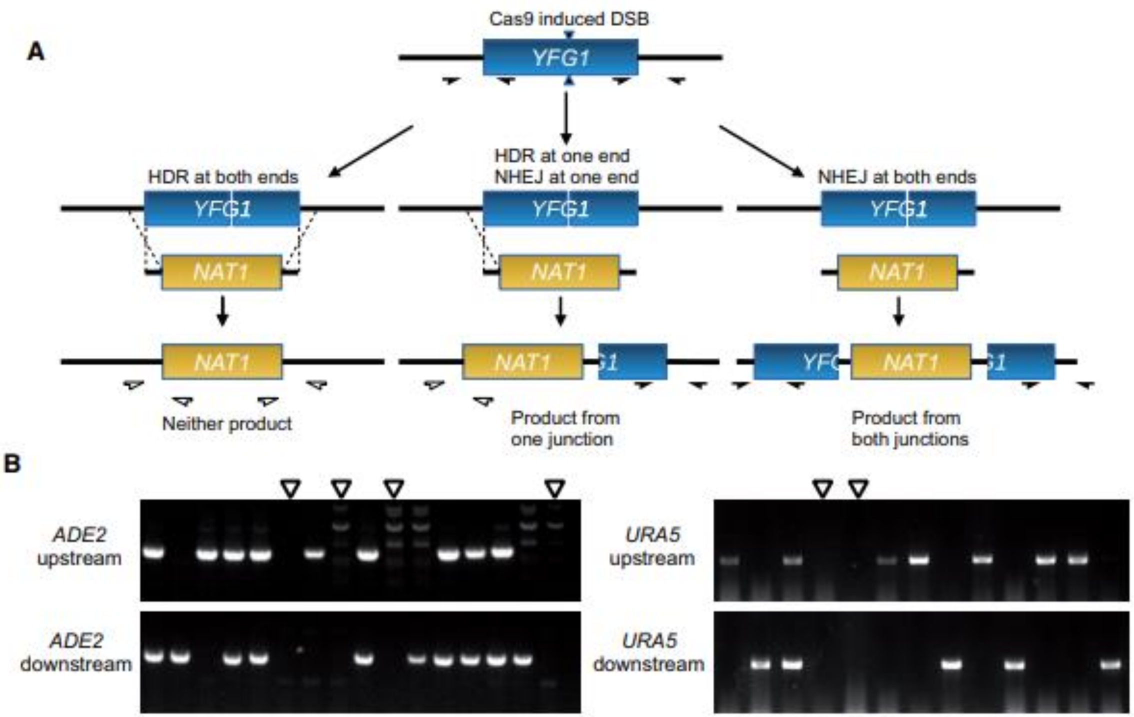

Table 2 Colony PCR results by experiment

Experiment	Cas9 state	Total colonies tested	5' HDR only	3' HDR only	Both
*ade2*Δ	Transient	48	7	6	11
*ade2*Δ	Integrated	48	14	5	6
*ade2*Δ + W7	Transient	48	4	0	21
*ura5*Δ	Transient	26	1	5	10
*ura5*Δ	Integrated	31	4	3	5
*ura5*Δ + W7	Transient	22	3	3	2

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CRISPR-CAS9 EM *Cryptococcus neoformans*

- TRACE
- Eliminou a biolística
- > frequência de transformação e de integração homóloga
- Simplificação da construção de deleção para 50 pb de homologia nas pontas
- Múltiplas modificações em um só passo de transformação
- **Vale a pena tentar!**