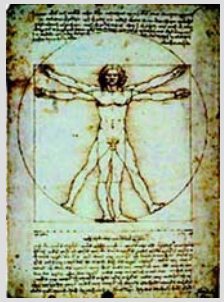
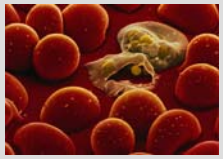
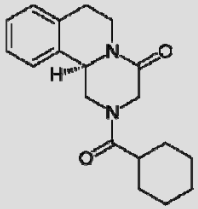
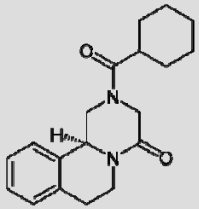








University of São Paulo
Institute of Biomedical Sciences
Department of Parasitology
Unit for Drug Discovery
Carsten Wrenger

Drug Target Validation

Genome of *Plasmodium*

***Plasmodium* Genome :**

•. 20-25 Megabases
•. 14 Chromosomes
•. Haploïd

•. ~5500 Genes
•. 60% with no orthologue in databases (hypothetical genes)

•. Most genes 1-2 kb
•. 50% genes have more than one exon
•. Genomes of rodent malaria parasites are homologous to those of human malaria parasites (except from subtelomeric regions).

Manipulating the *Plasmodium* Genome

Reverse Genetics : Site-directed Mutagenesis
(From Genotype to Phenotype, Gene Characterization)

Homologous Recombination
‘Constant’ Mutagenesis
‘Conditional’ Mutagenesis
~~RNA Interference~~

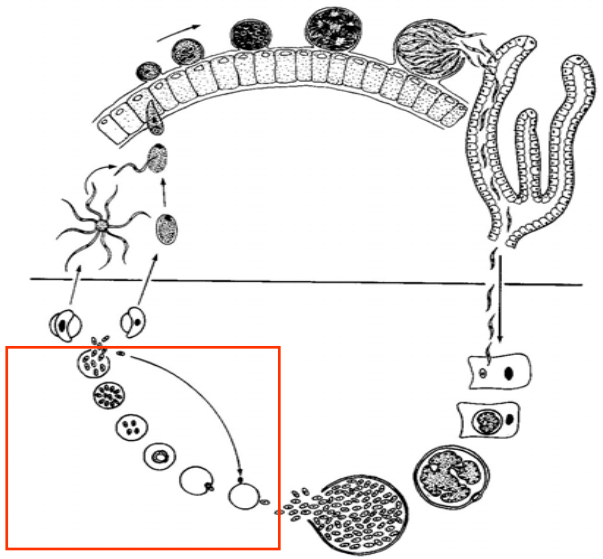
~~**Forward Genetics: Random Mutagenesis**~~
~~(From Phenotype to Genotype, Gene Identification)~~

~~Transposition~~
~~Non Homologous Recombination~~

Systematic Mutagenesis: Pool, Gene-by-Gene Mutagenesis

Homologous Recombination or RNAi…

Transfection of *Plasmodium*



TRANSFECTION

- *P. falciparum* : in vitro selection
- *P. berghei* : in vivo selection

	Stage	Linear DNA	SCO	DCO	Selection
P. berghei	**Extra**	+	+	+	7-10 days
P. falciparum	**Intra**	-	+	+	Months

P. yoelii, P. knowlesi

History of genetic manipulations in *Plasmodium*

1995:

Episomal transfection of *P. berghei* RBC stages (van Dijk et al, 1995, Science 268, 1358-62)

P. falciparum RBC stages (Wu et al, 1995, PNAS 92, 973-7)

1996:

Integrative transfection of *P. berghei* RBC stages (van Dijk et al, 1996, Science 271, 662-5)

P. falciparum RBC stages (Wu et al, 1996, PNAS 93, 1130-4)

100% homologous recombination (HR)

1997:

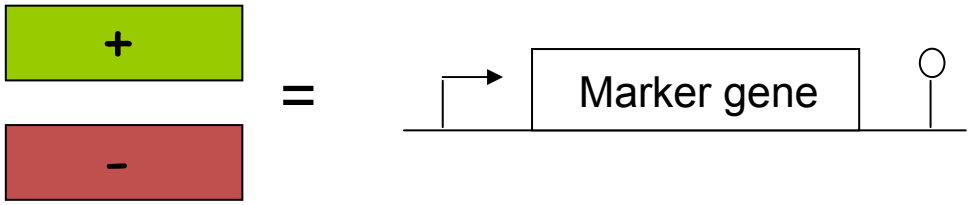
Gene Inactivation in *P. berghei* mosquito stages (Menard et al, 1997, Nature 385, 336-40)

P. falciparum RBC stages (Crabb et al, 1997, Cell 89, 287-296)

Homologous recombination

For genome modifications

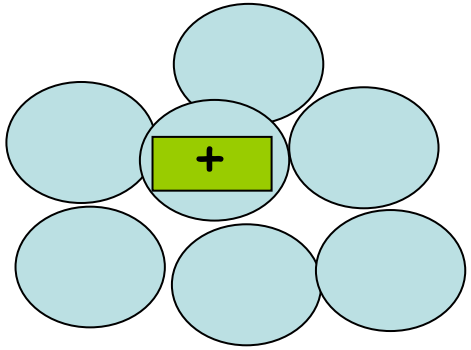
Need markers



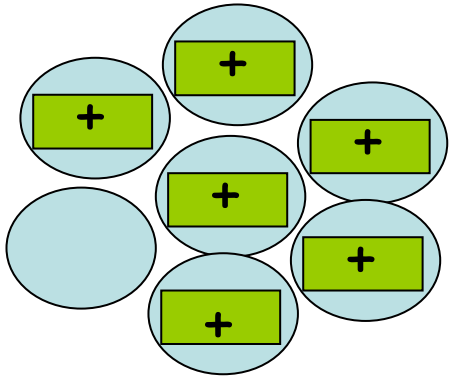
Manipulating the *Plasmodium* Genome

Positive Selectable Markers

Example: hDHFR (human Dihydrofolate-reductase)

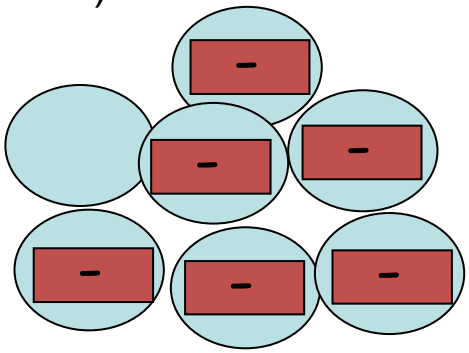


pyrimethamine

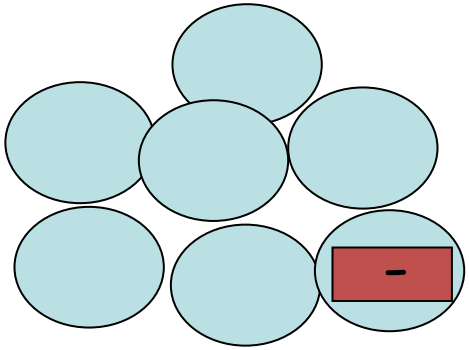


Negative Selectable Markers

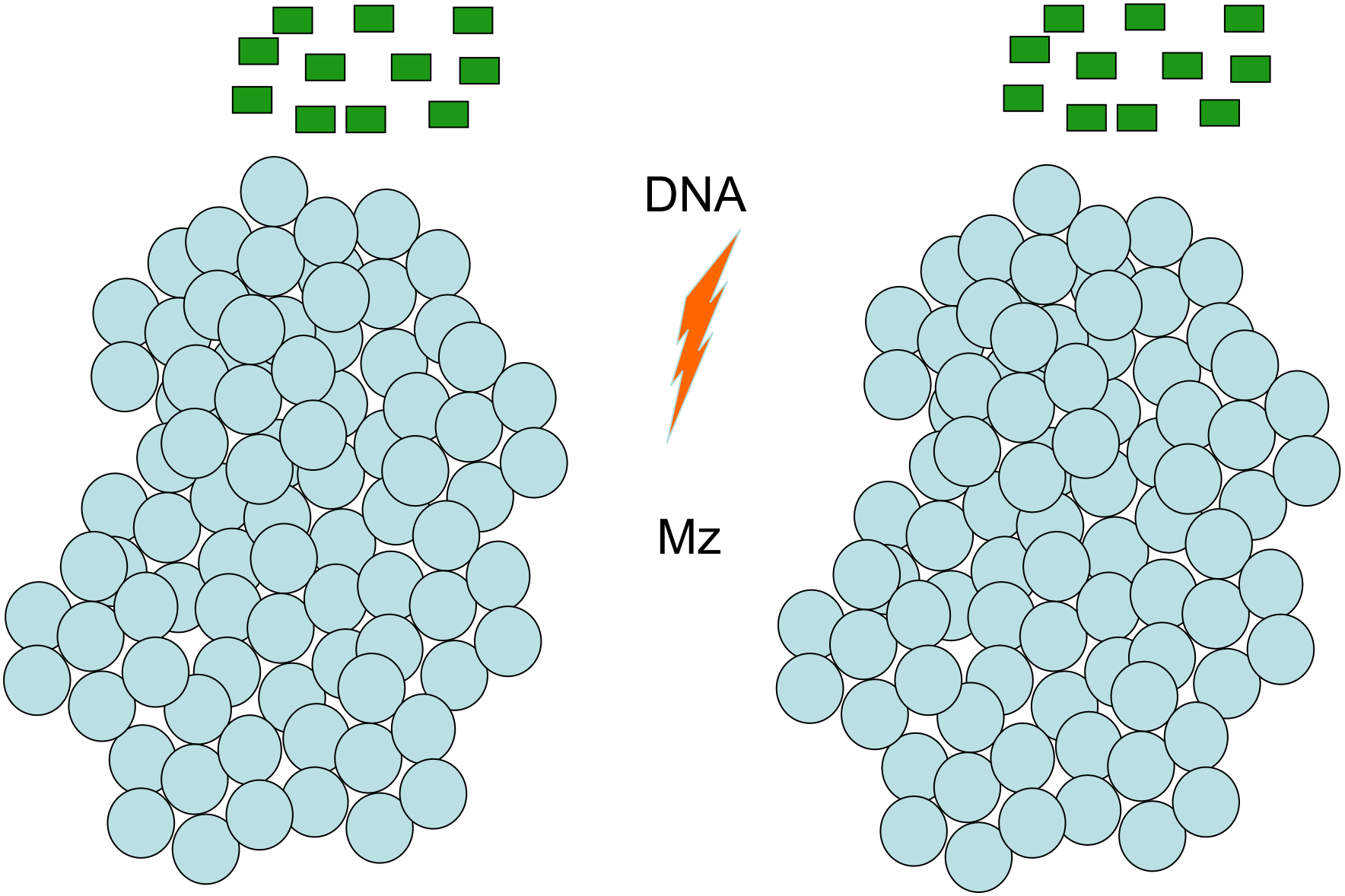
Example: *yFCU* (yeast cytosine deaminase-uridyl phosphoribosyl transferase): 5-FC^S



5-FC(ytosine) $\xrightarrow{CD}$ 5-FU(racil) : TOXIC !!



Transfection efficiency

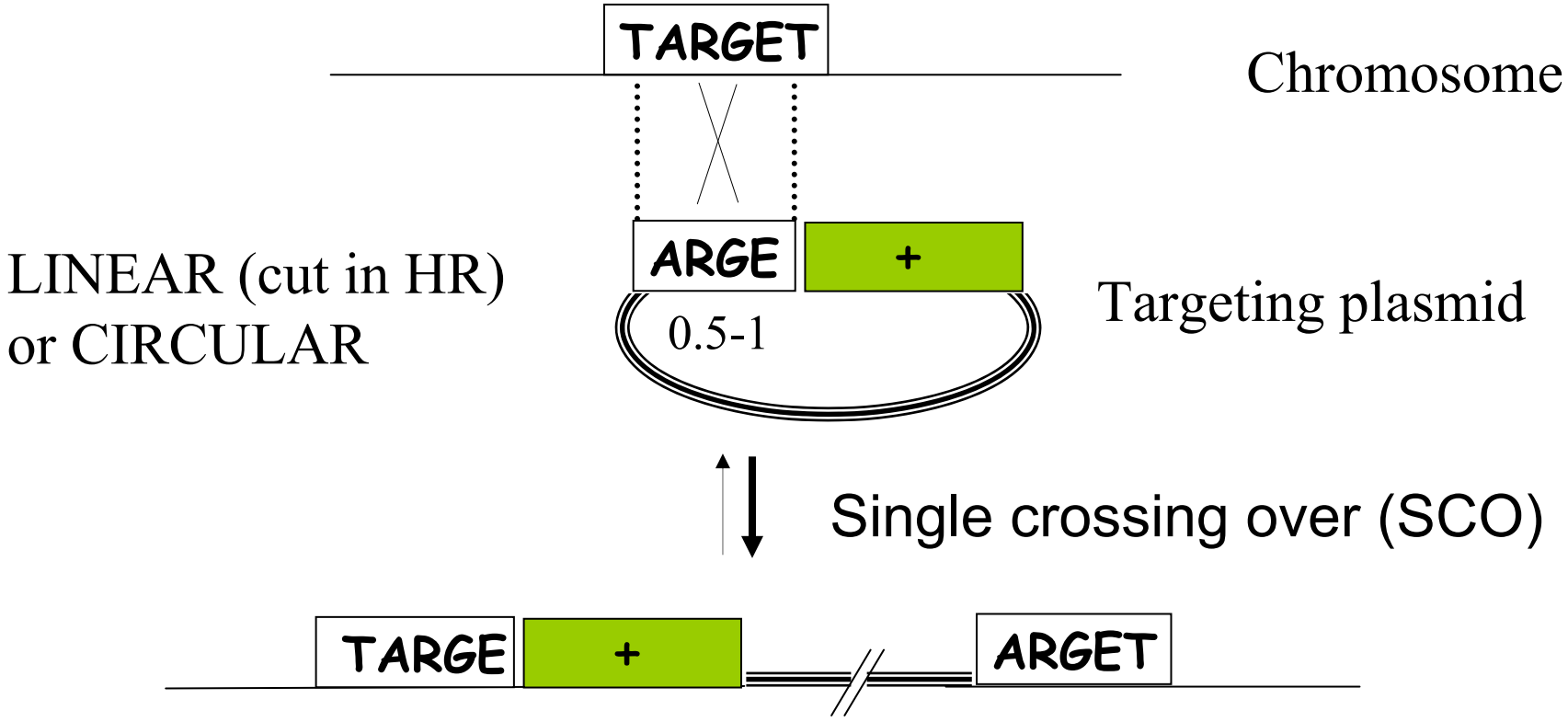


Gene Inactivation

Gene Knock Out

Manipulating the *Plasmodium* Genome

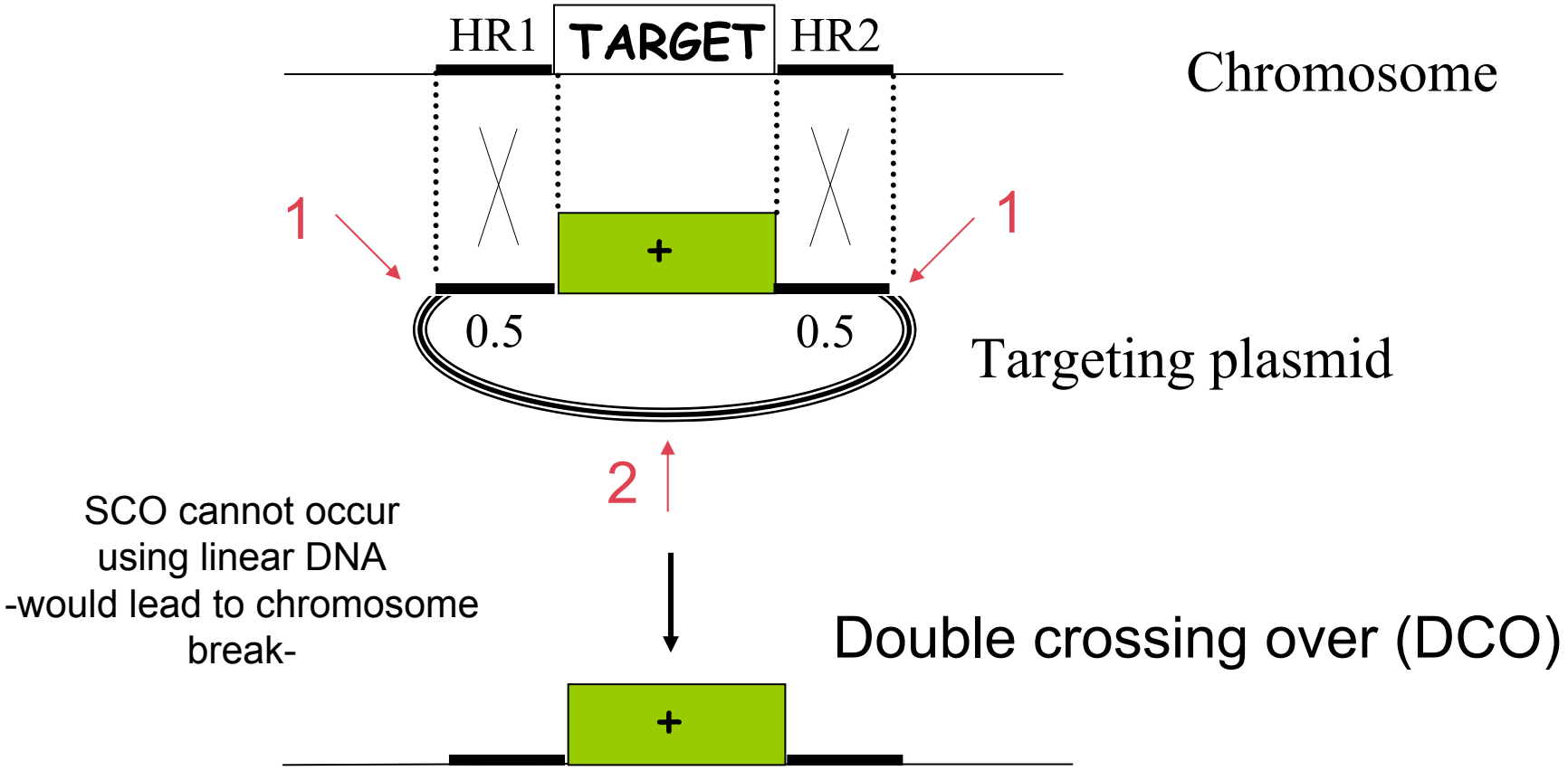
Inactivation by Insertion (Pb & Pf)



CDS interrupted, mutation unstable

Manipulating the *Plasmodium* Genome

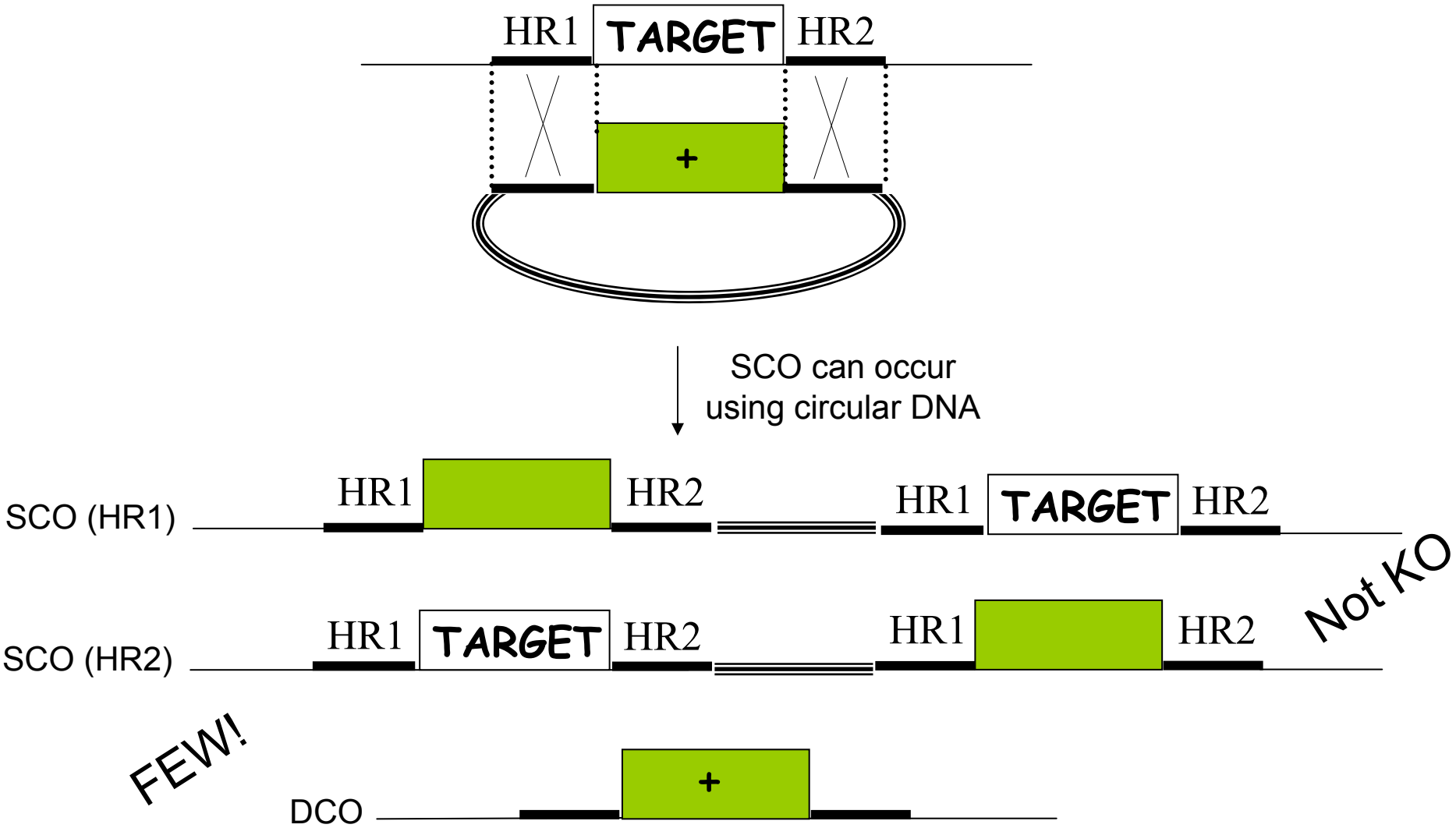
Inactivation by Replacement (Linear DNA, Pb)



CDS deleted, mutation stable

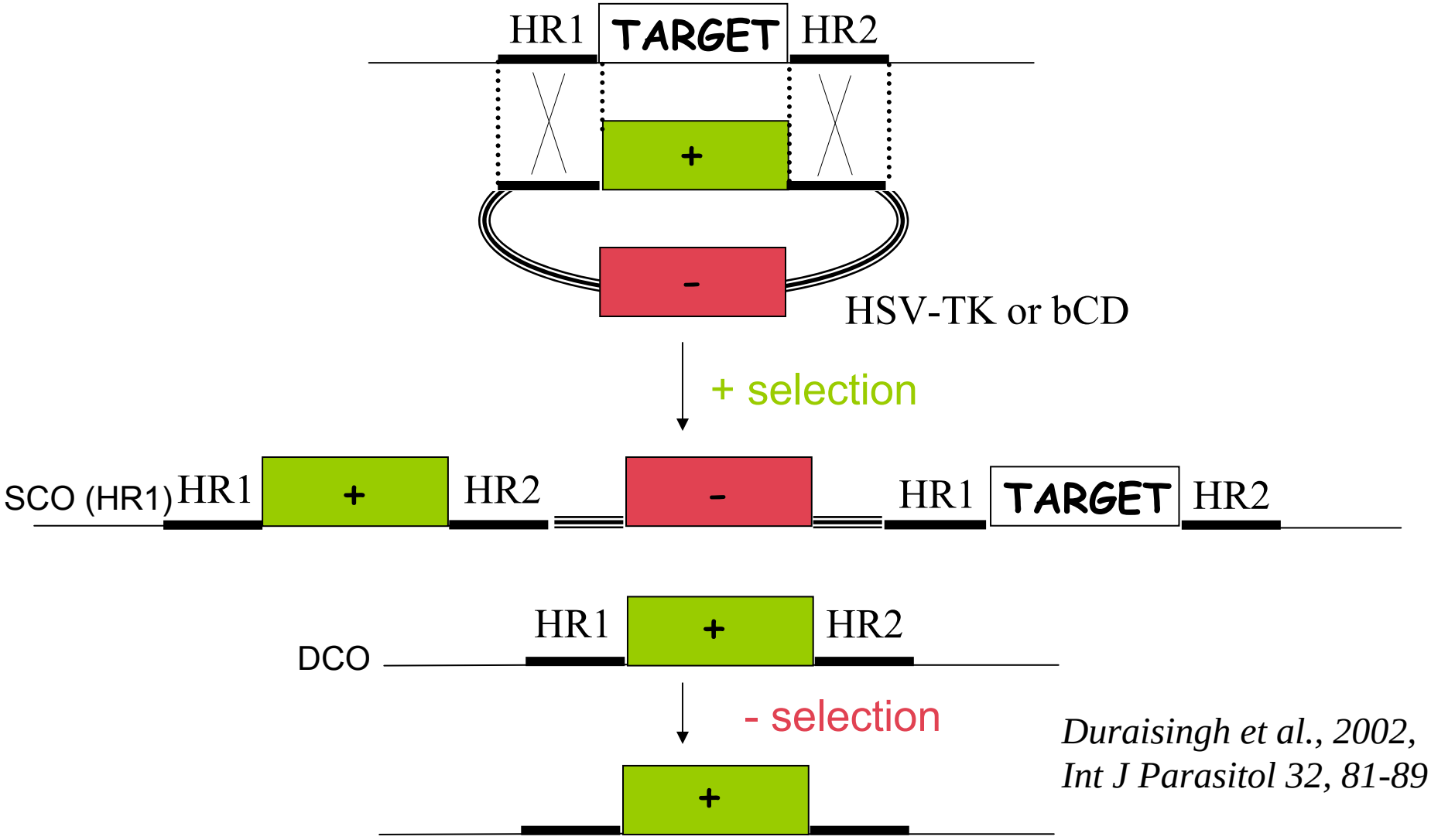
Manipulating the *Plasmodium* Genome

Inactivation by Replacement (Pf, No linear DNA)



Manipulating the *Plasmodium* Genome

Inactivation by Replacement (Pf, No linear DNA)



Duraisingh et al., 2002,
Int J Parasitol 32, 81-89

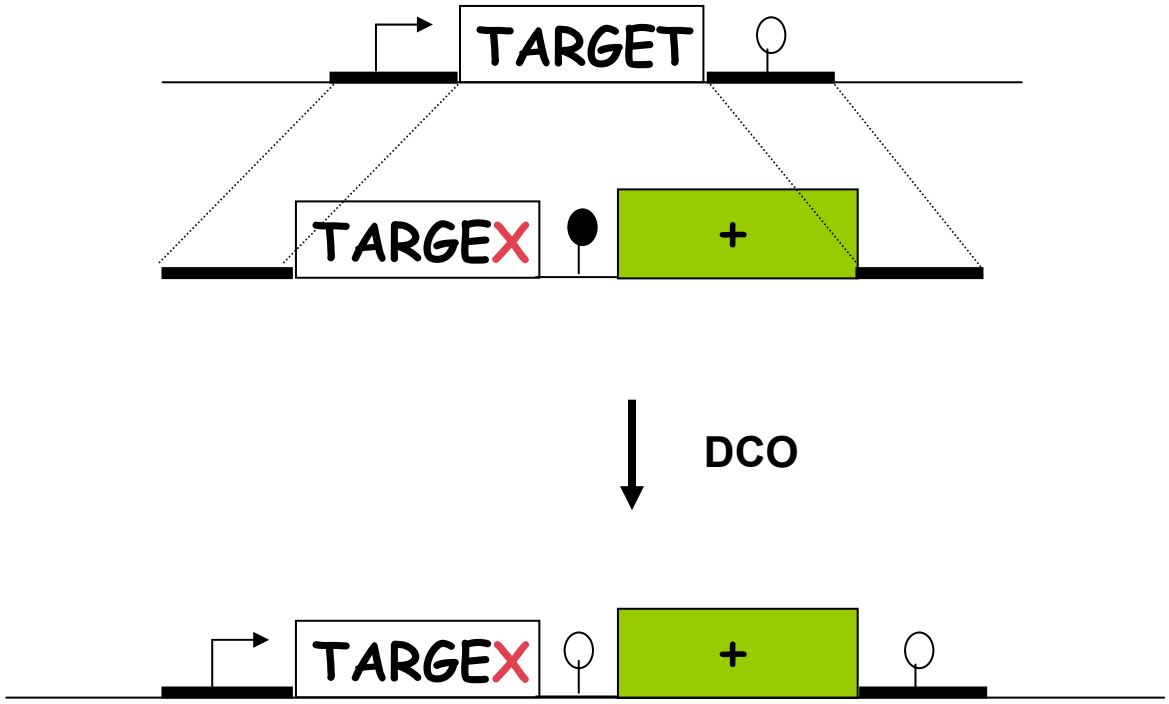
Gene Modification

Structure-Function Analysis

Function of individual domains of a protein

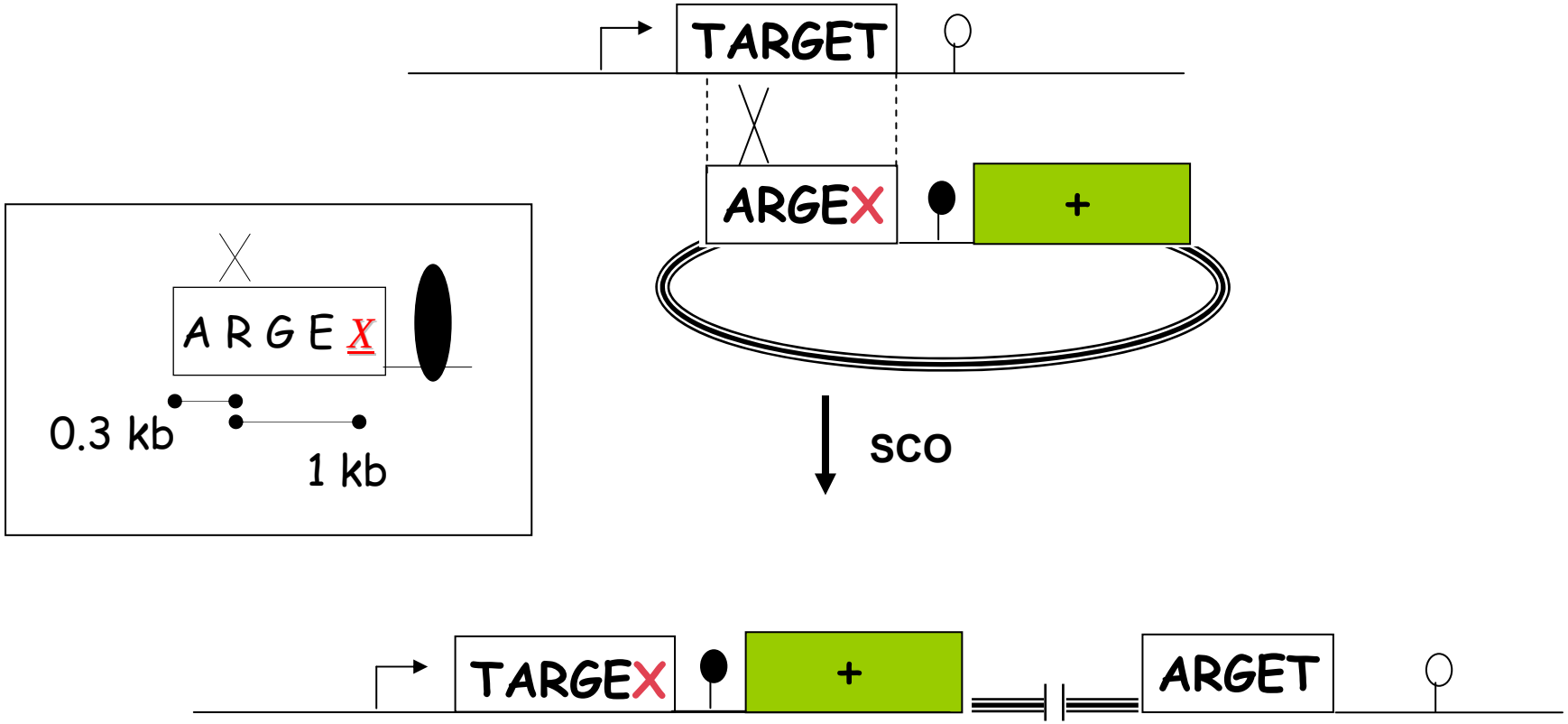
Manipulating the *Plasmodium* Genome

Gene Modification by Replacement (Pb)



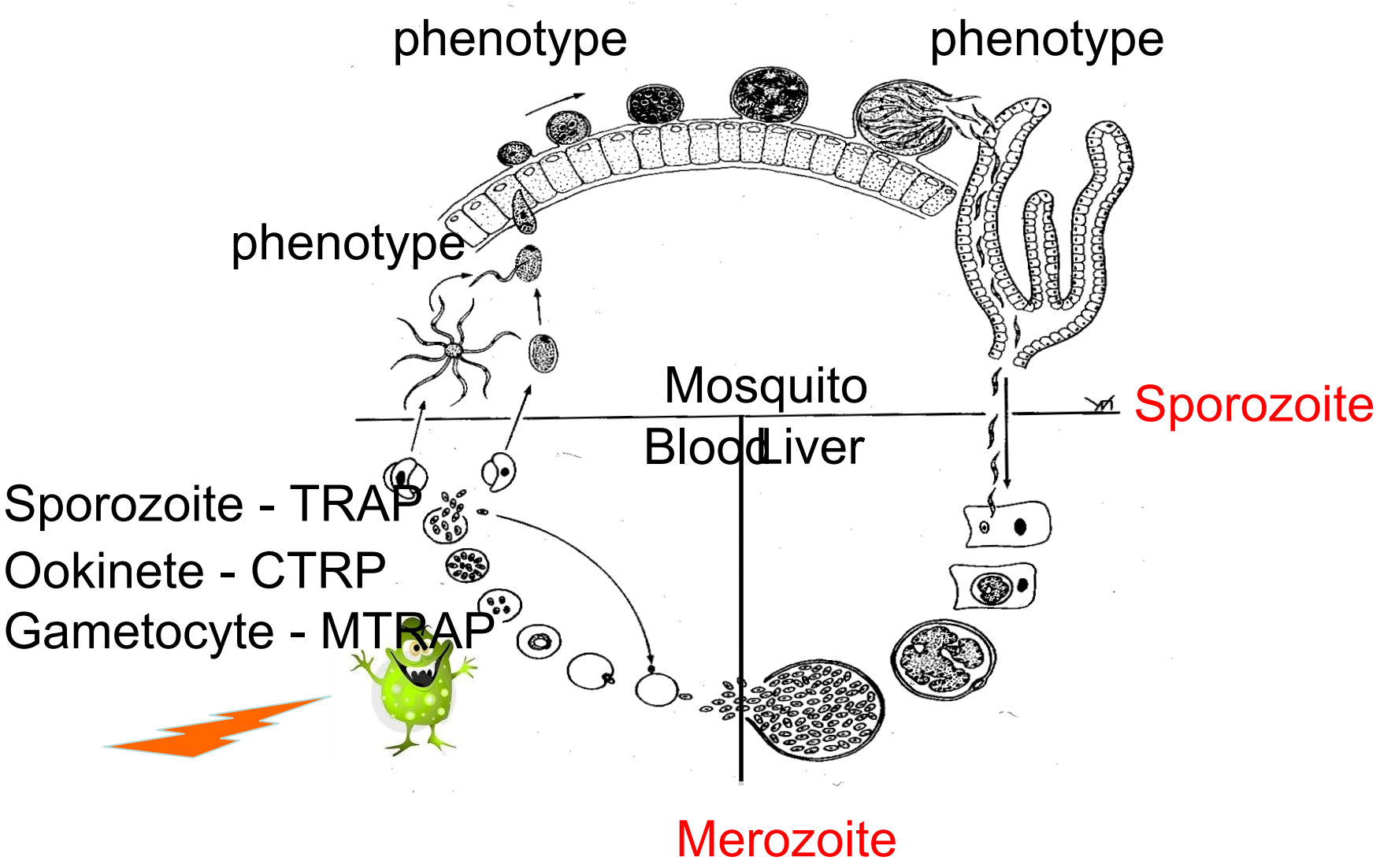
Manipulating the *Plasmodium* Genome

Gene Modification by Insertion (Pb & Pf)



Nunes et al., 1999, Mol Cell Biol 19, 2895-902

Transfection and phenotyping



Transfection and phenotyping

Phenotype: *what to conclude?*

Phenotype can be due to mutation in locus Y unrelated to gene X KO

Characterize 2 (independent) clones

Phenotype can be due to distance/polar effect on another gene than gene X

All clones have the same defective phenotype

Phenotype is indeed due to the lack of protein X

Transfection and phenotyping

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Characterize 2 (independent) clones

Phenotype can be due to distance/polar effect on another gene than gene X
All clones have the same defective phenotype

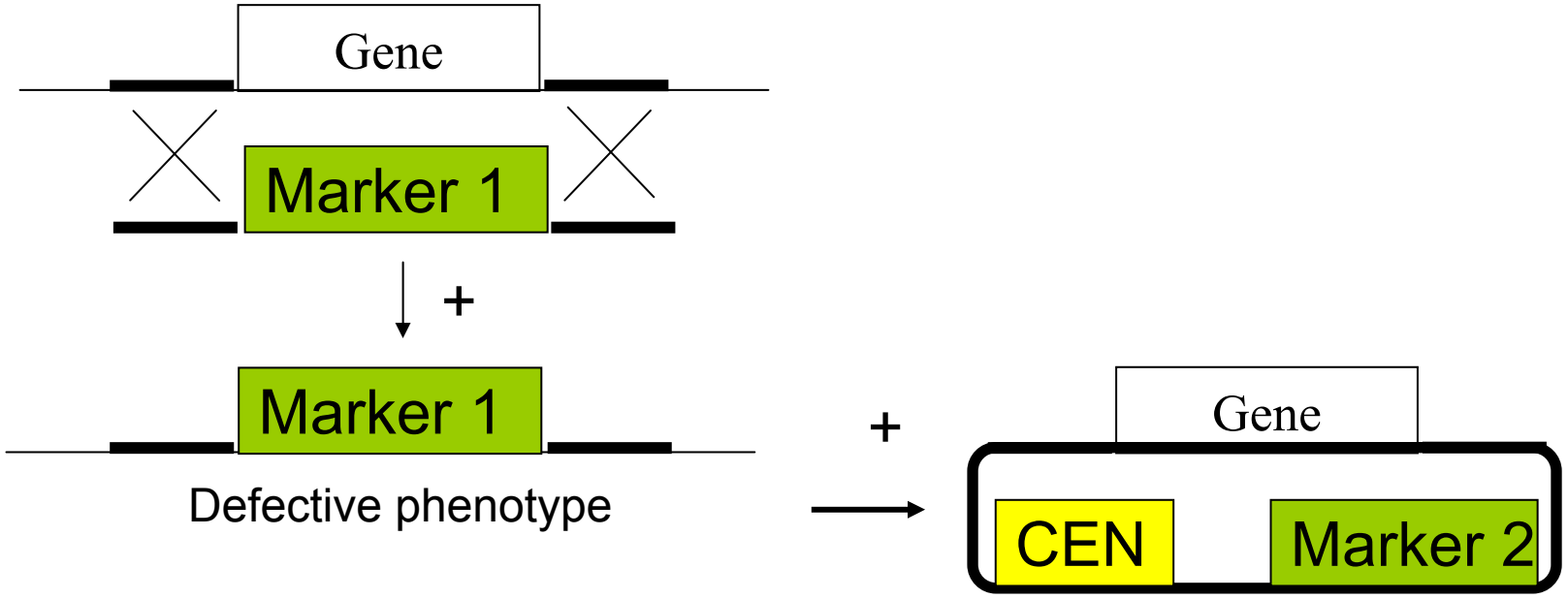
Phenotype is indeed due to the lack of protein X

KO Complementation

Complementation (restoration of WT phenotype upon reintroduction of the targeted gene**) is the only way to demonstrate that the phenotype of the KO is due to the inactivation of the targeted gene**

How to complement a KO Clone (Pb) ?

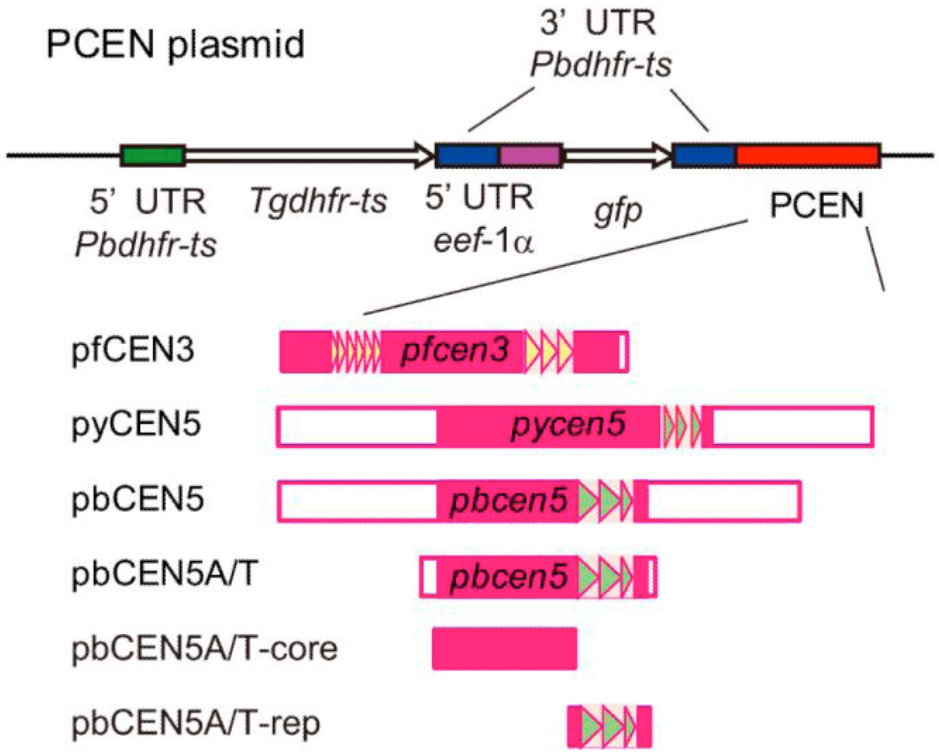
Gene deletion (KO) using *DHFR-TS* (Pyr) and reintroduction of the gene (episomal or inserted) using *hDHFR* (WR).

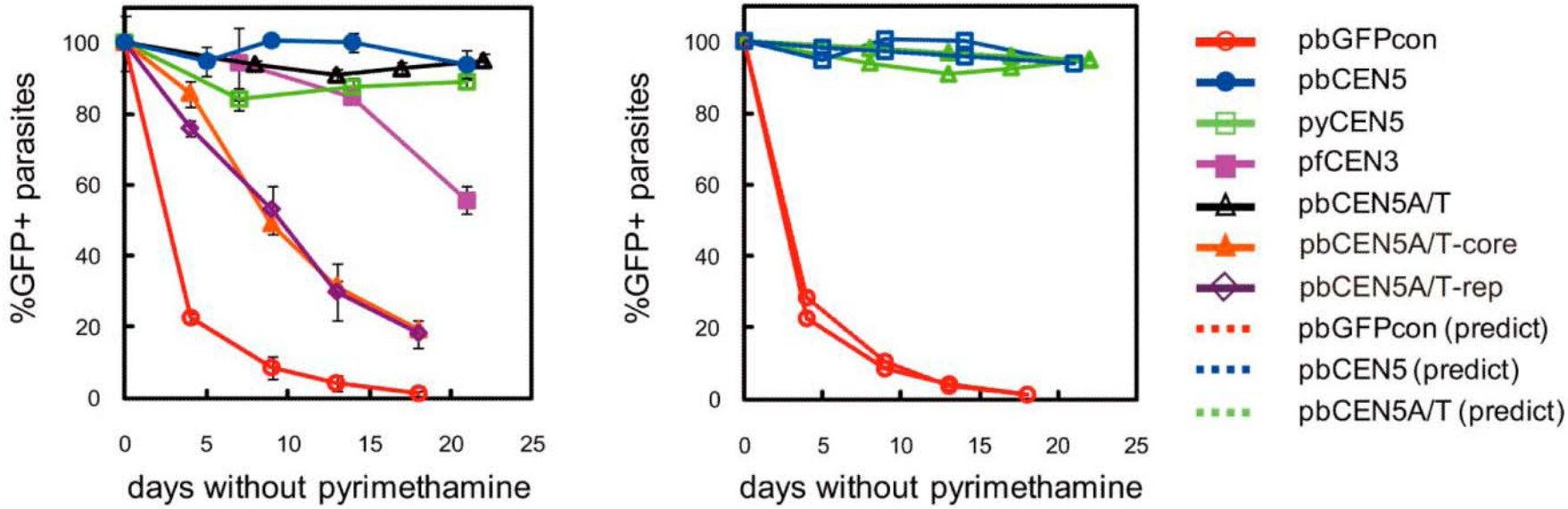


WT phenotype?

Episomes

Iwanaga et al., 2005,
Cell Host & Microbe 7, 245-255





Manipulating the *Plasmodium* Genome

Reverse Genetics : Site-directed Mutagenesis
(From Genotype to Phenotype, Gene Characterization)

Homologous Recombination
'Constant' Mutagenesis
→ 'Conditional' Mutagenesis
~~RNA Interference~~

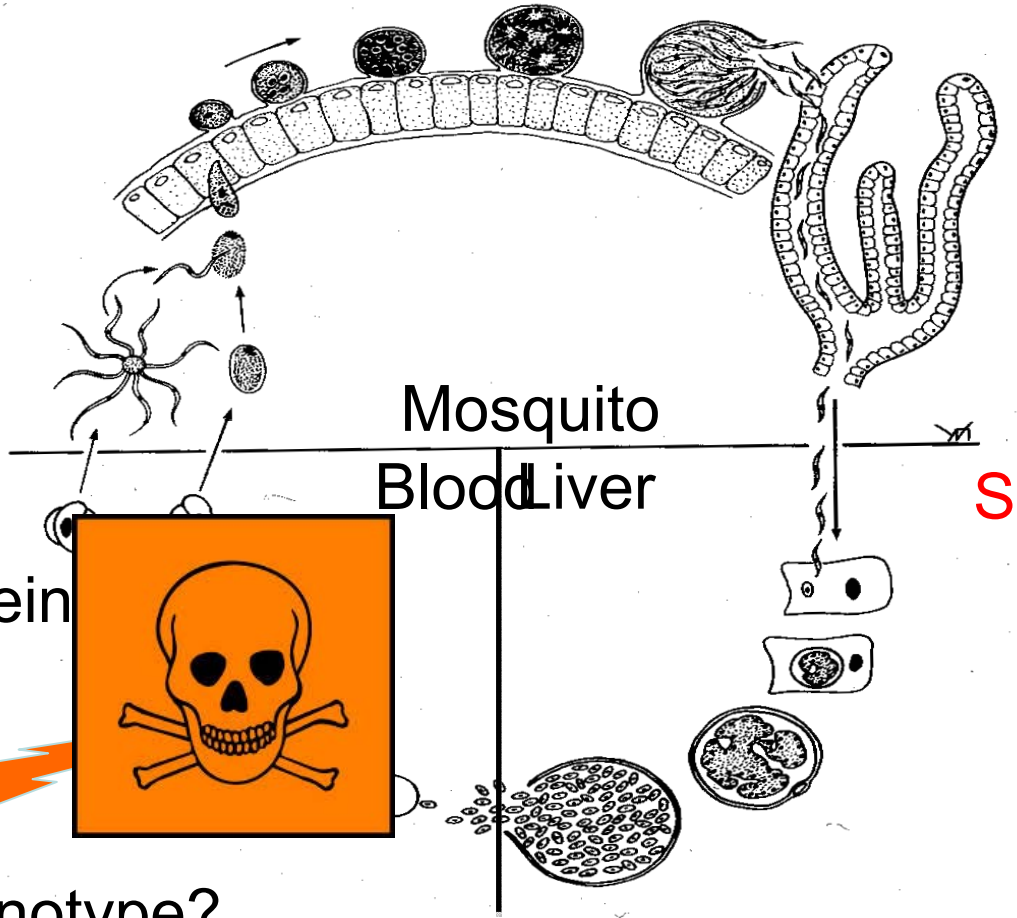
~~**Forward Genetics: Random Mutagenesis**~~
~~(From Phenotype to Genotype, Gene Identification)~~

~~Transposition~~
~~Non Homologous Recombination~~

Systematic Mutagenesis: Pool, Gene-by-Gene Mutagenesis

Homologous Recombination

Targeting genes essential for blood stage



Blood stage important protein

-MSPs
-AMA1
-RONs
-Exports
-Etc…

phenotype?

Targeting genes essential for blood stage

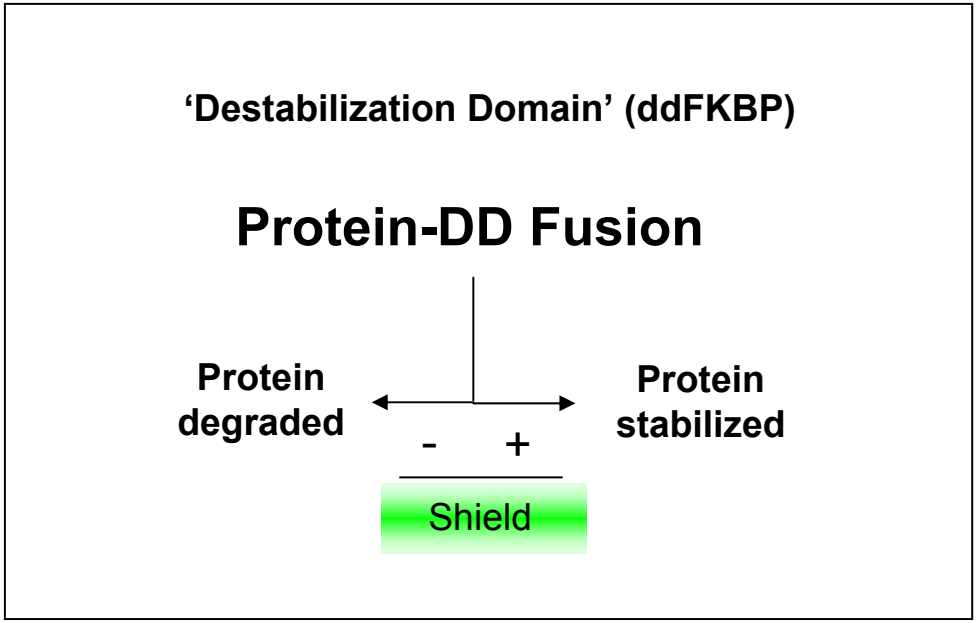
Three ways:

Level of Protein: Protein Destabilization

Level of RNA: Transcriptional Shut-down

Level of DNA: Site-specific Excision

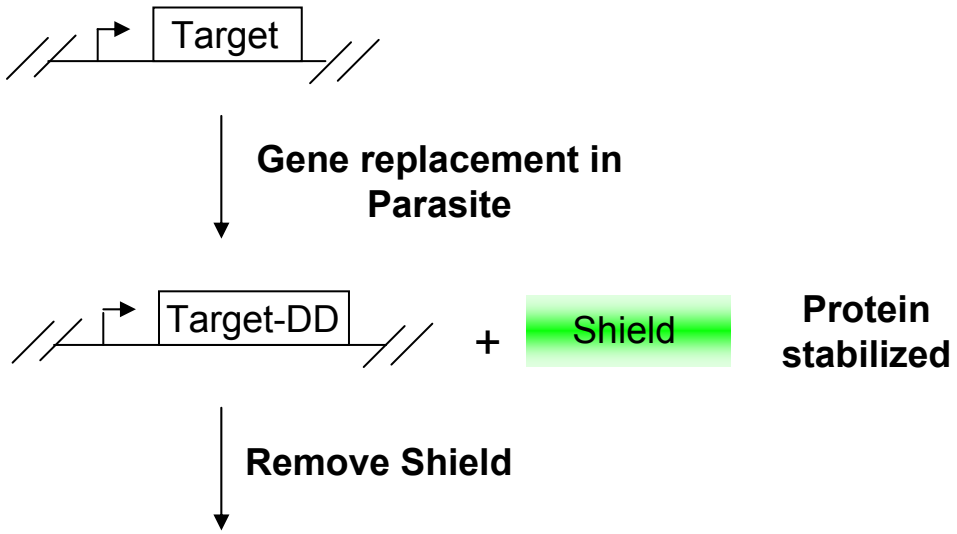
Conditional Protein Inactivation



Amstrong and Golberg., 2007, Nat Methods
Herm-Götz et al., 2007, Nat Methods

Conditional Protein Inactivation

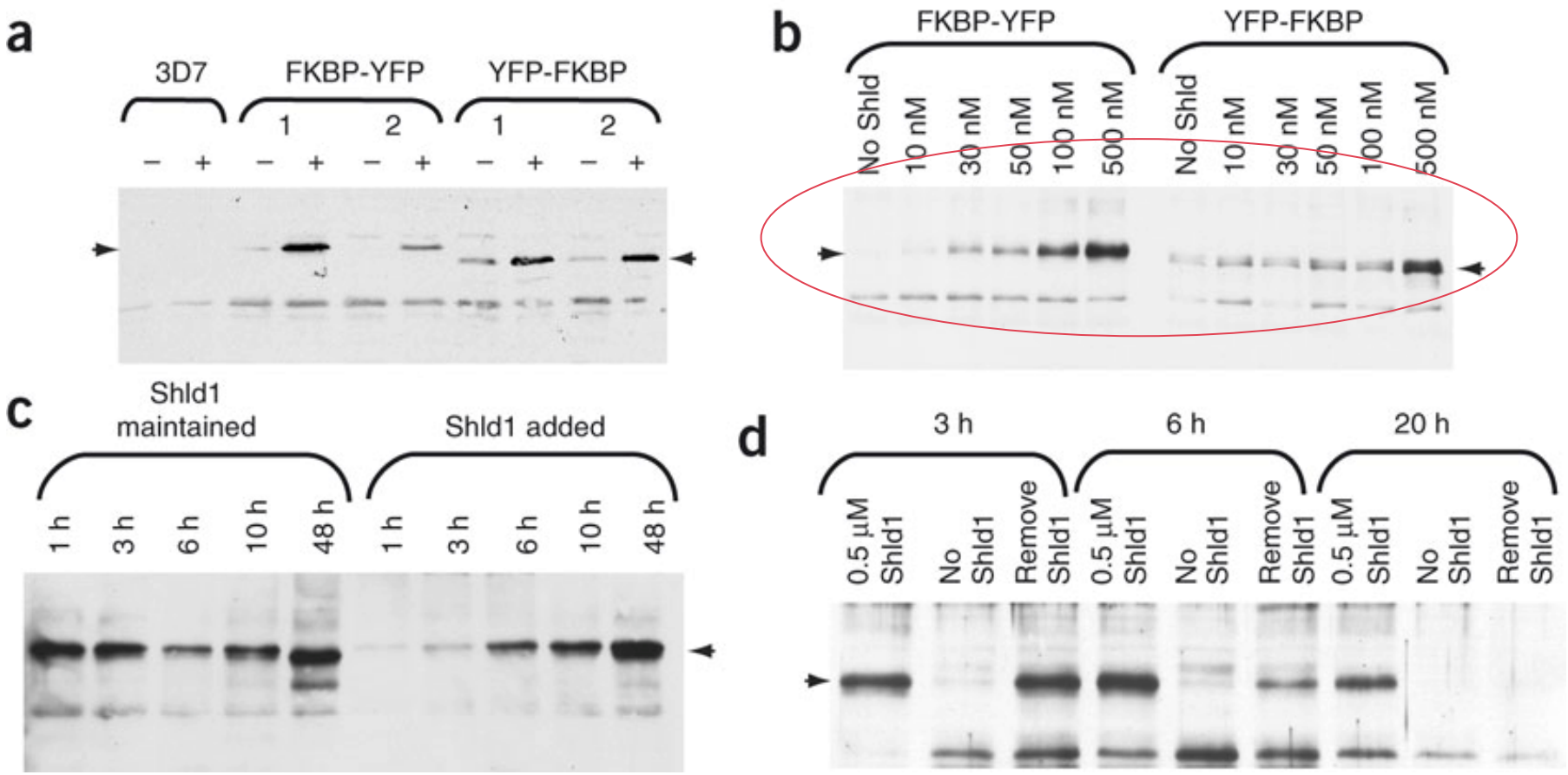
Conditional Protein Inactivation using ‘dd’ System



If only Episomal Expression : Conditional expression of dominant-negative (DN) constructs (- then +)

If replacement of WT copy possible : **Conditional protein depletion** (+ then -) !!

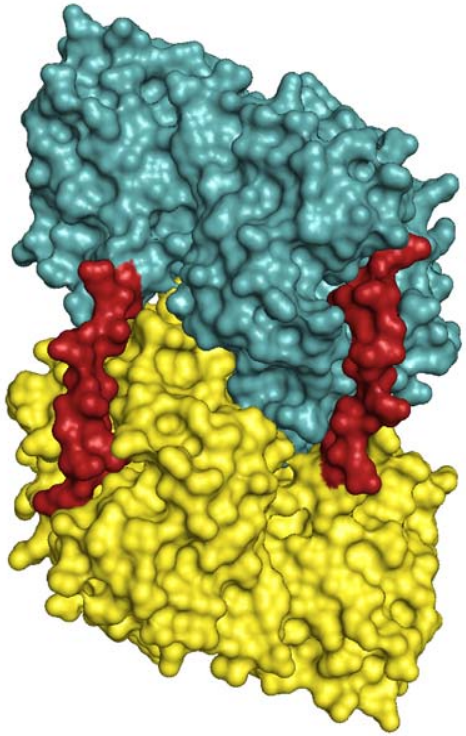
Episomal Expression of ddFKBP fusions to YFP in Pf



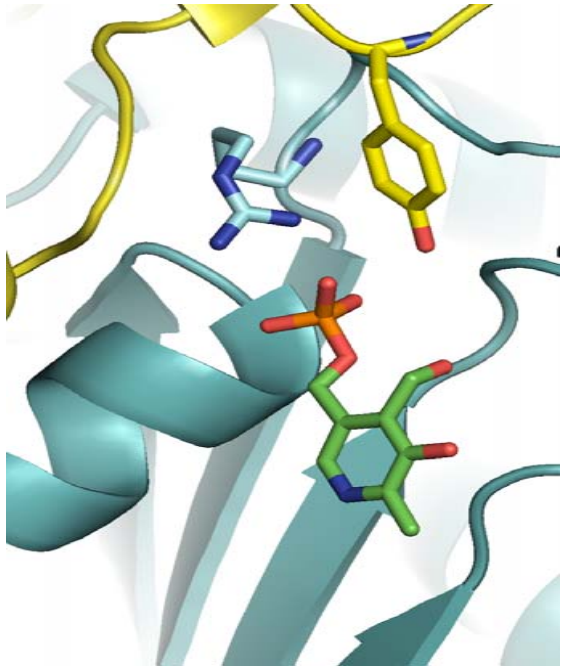
Amstrong and Golberg., 2007, Nat Methods 4, 1007-1009

PIA – Protein Interference Assays

A



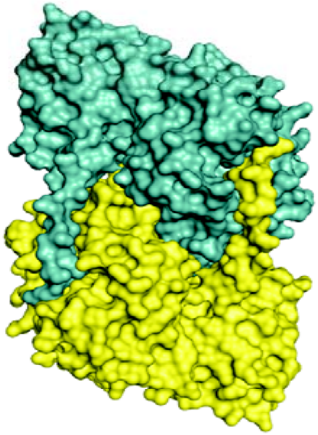
B

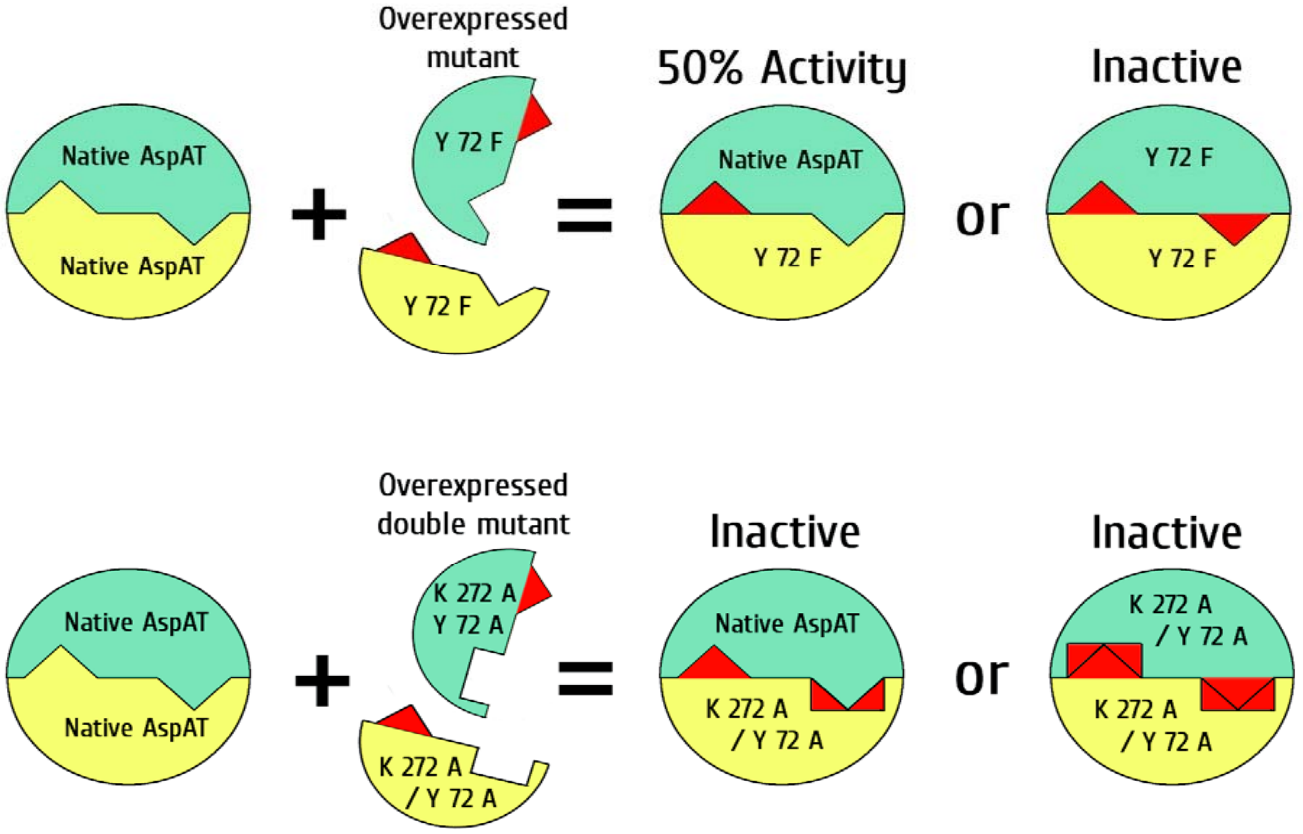


A) *Pf*AspAT is a dimer (individual monomers shown in yellow and blue), with the dimeric interface stabilize by a 13-residue “Arm” (red). B) The two active sites of *Pf*AspAT are formed by contributions from both of the monomers (Y72) from one monomer (yellow) and R257 (blue) from the other.

Meissner et al., 2017, Curr Drug Targets

PIA – Protein Interference Assays





Meissner et al., 2017, Curr Drug Targets

Conditional Protein Inactivation

Three ways:

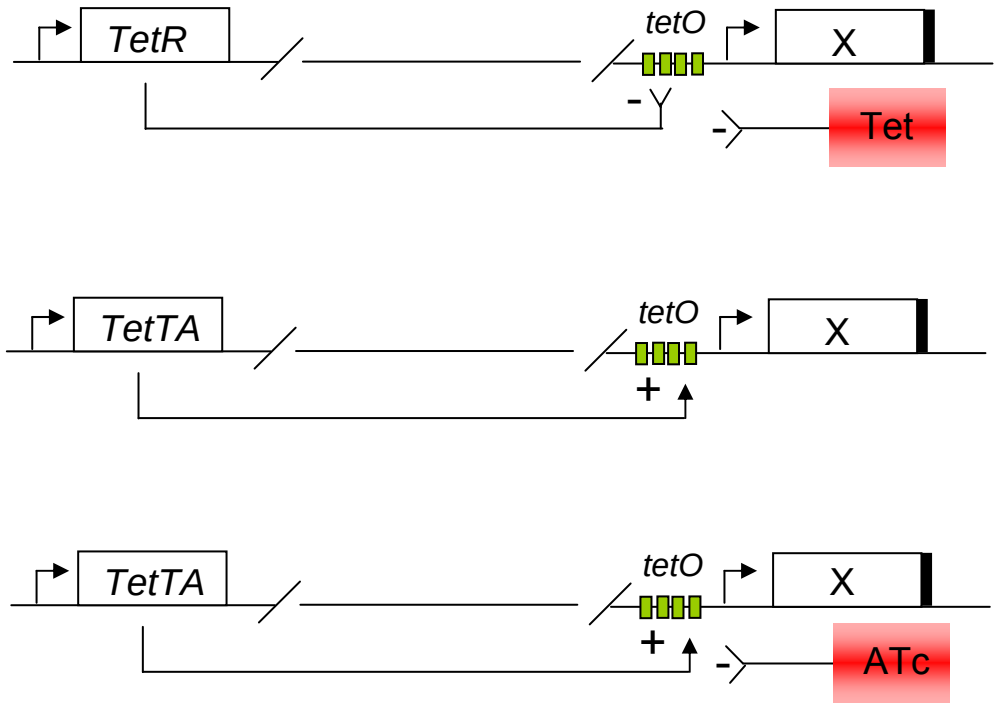
Level of Protein: Protein Destabilization

Level of RNA: Transcriptional Shut-down

Level of DNA: Site-specific Excision

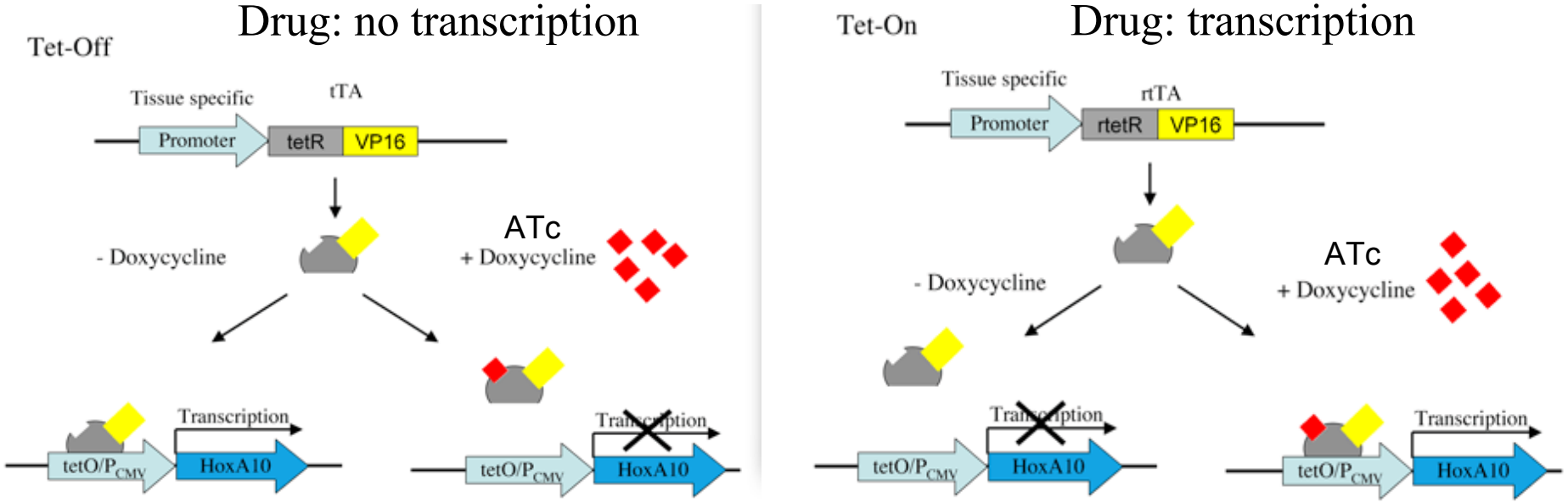
Conditional Protein Inactivation

Transcriptional Regulators (RNA)



Conditional Protein Inactivation

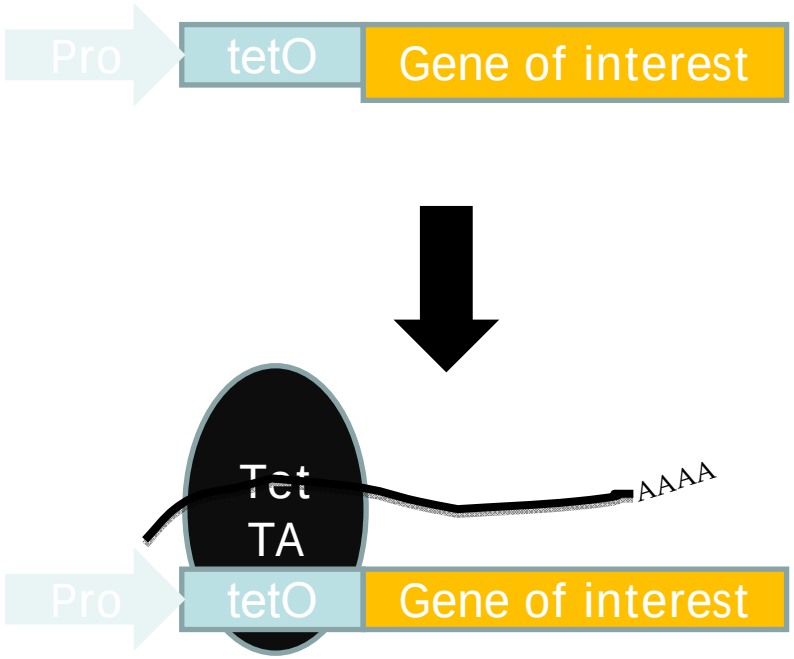
Tetracycline Transactivators



'*Toxoplasma*' tTA (TATi)

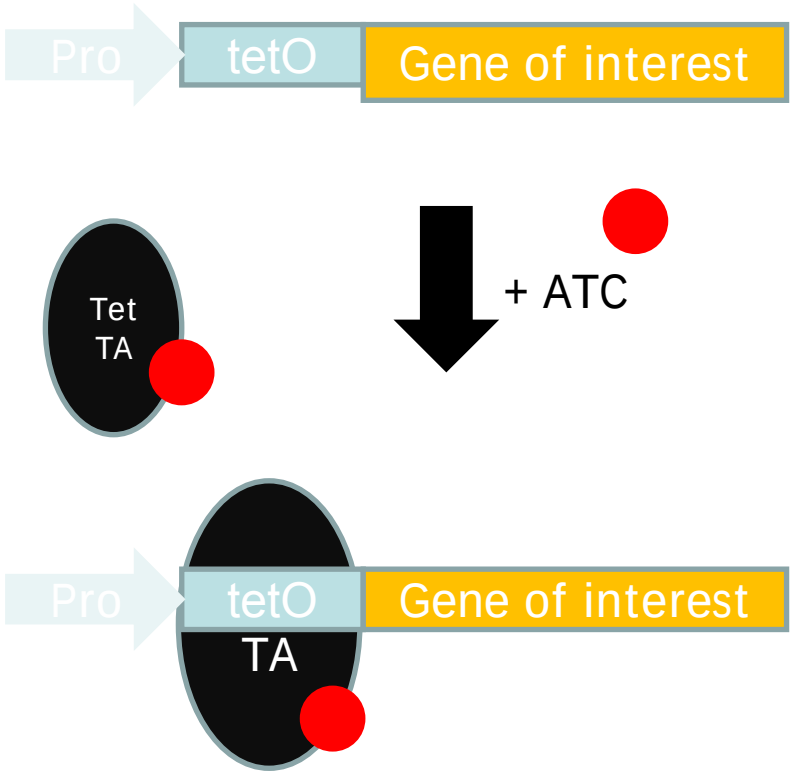
Meissner et al., 2002, Science 298, 837-840
Mital et al, 2005, Mol Biol Cell 16, 4341-4349

Tet- System in *P. falciparum*

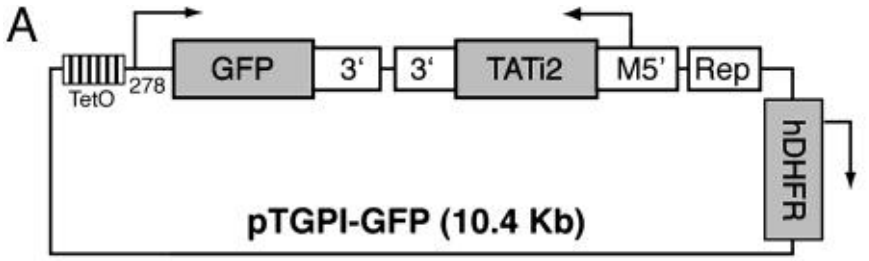


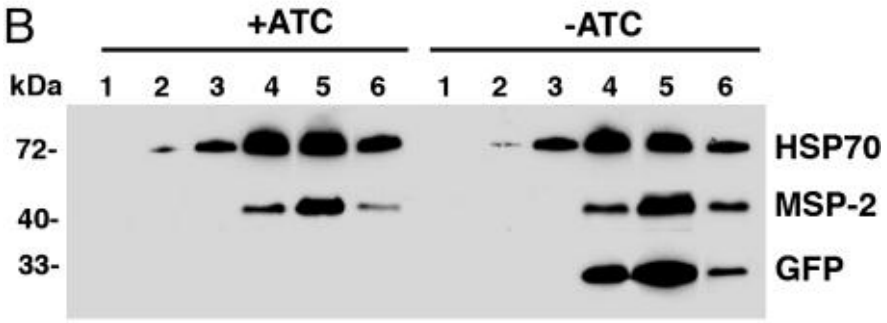
(Meissner et al. 2005, PNAS)

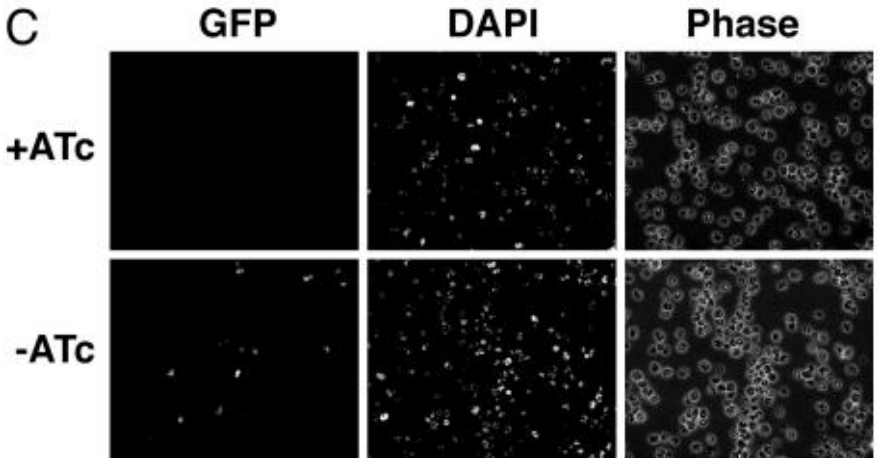
Tet- System in *P. falciparum*



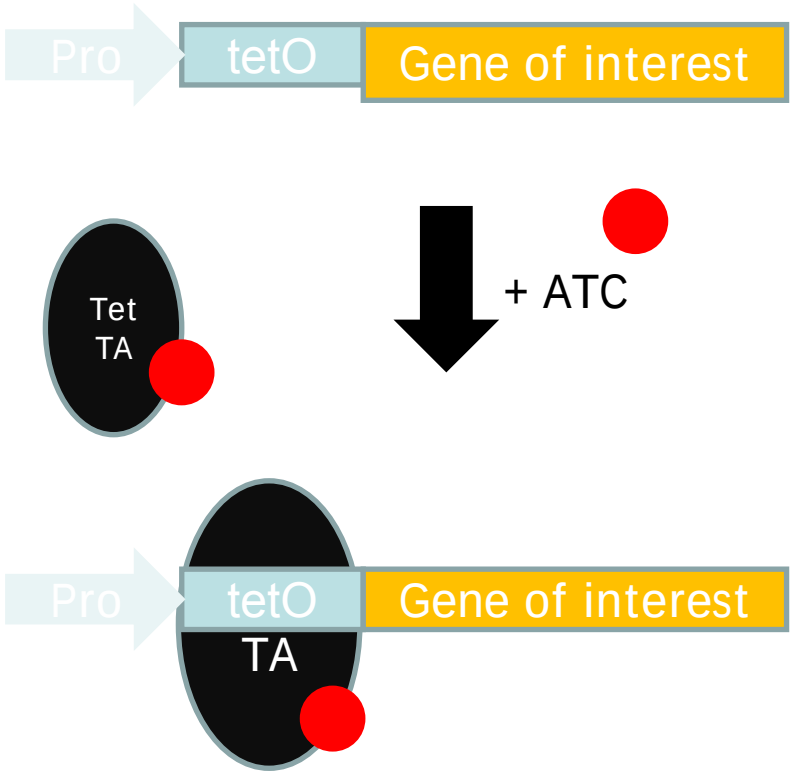
(Meissner et al. 2005, PNAS)



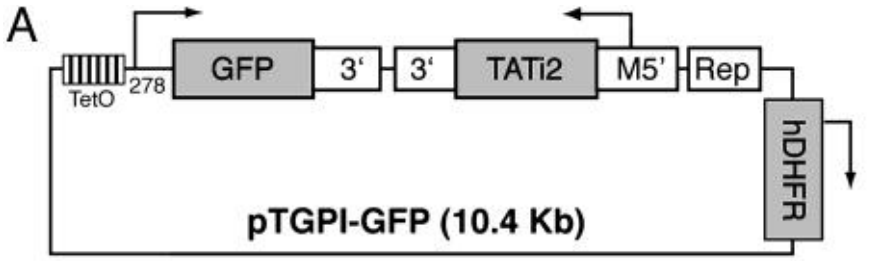


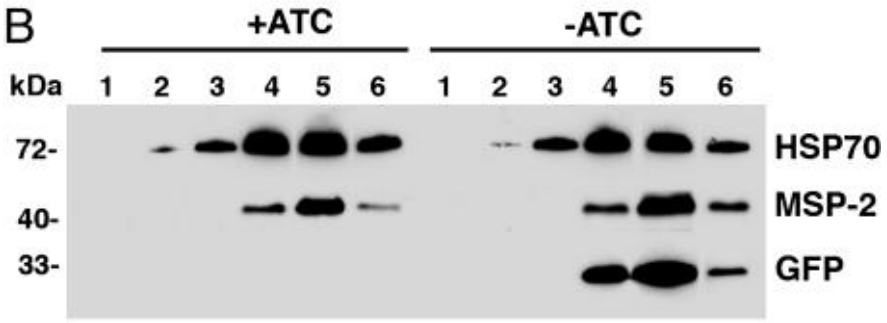


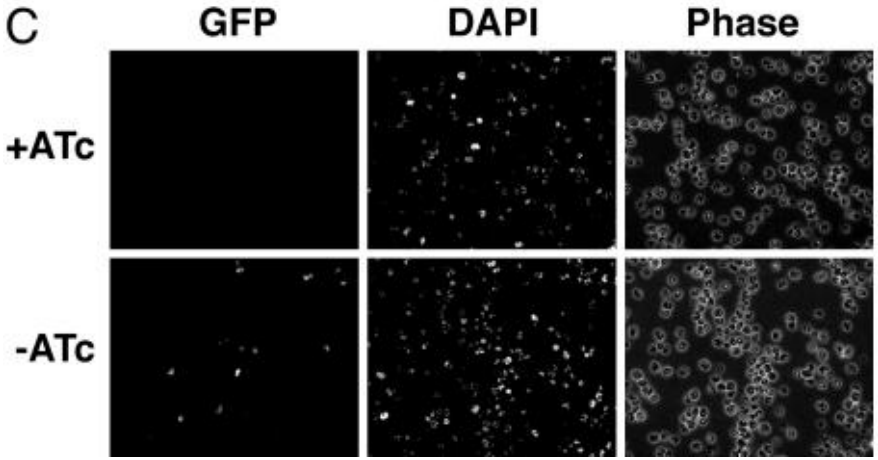
Tet-OFF System in *P. falciparum*



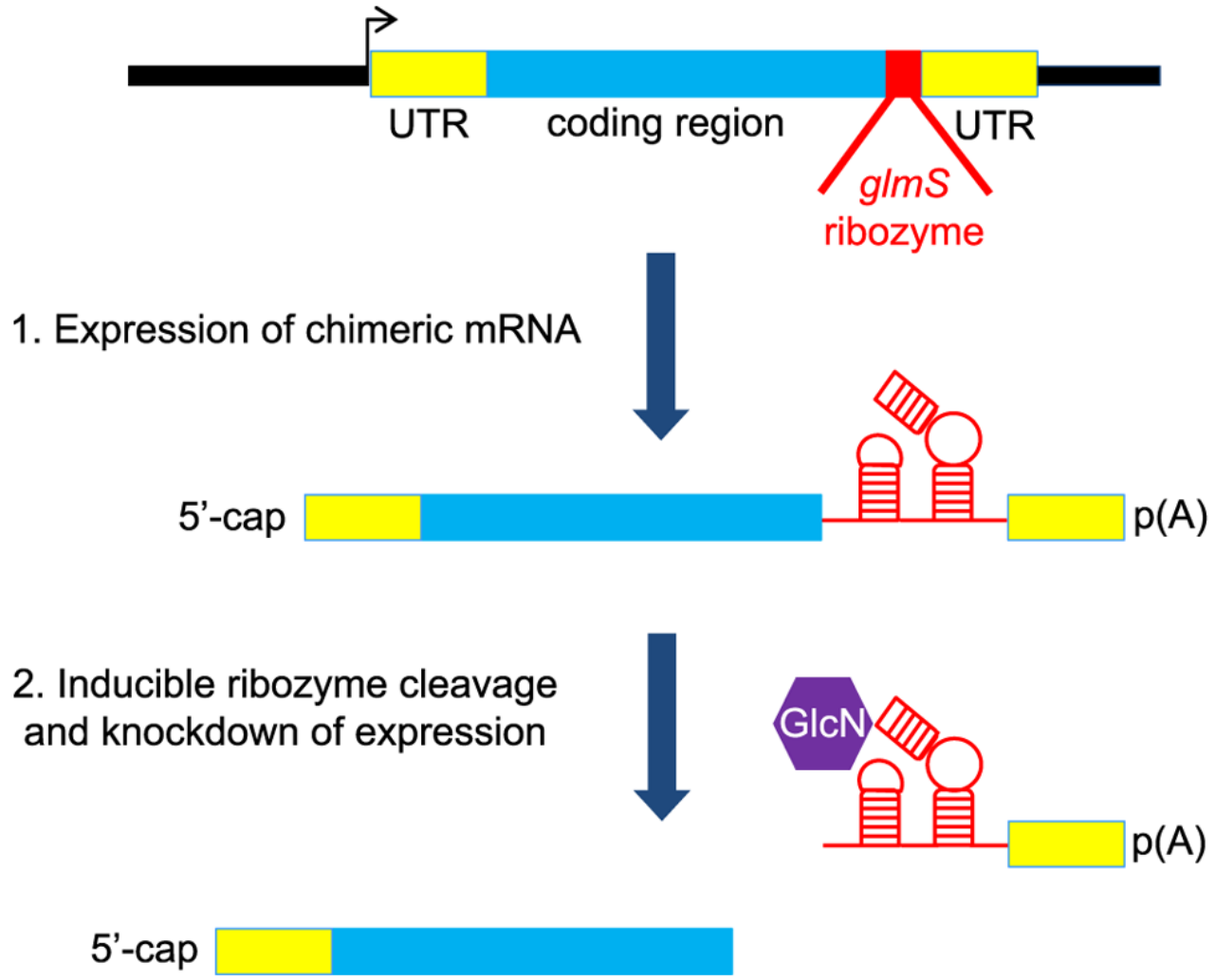
(Meissner et al. 2005, PNAS)





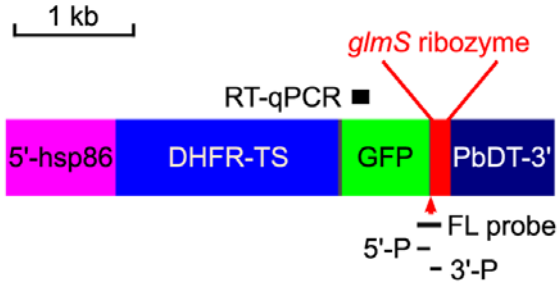


glmS system – ribozyme RNA degradation

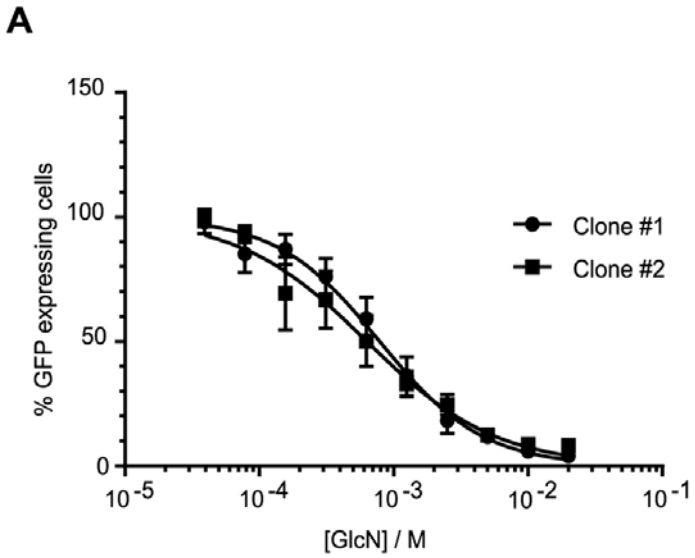


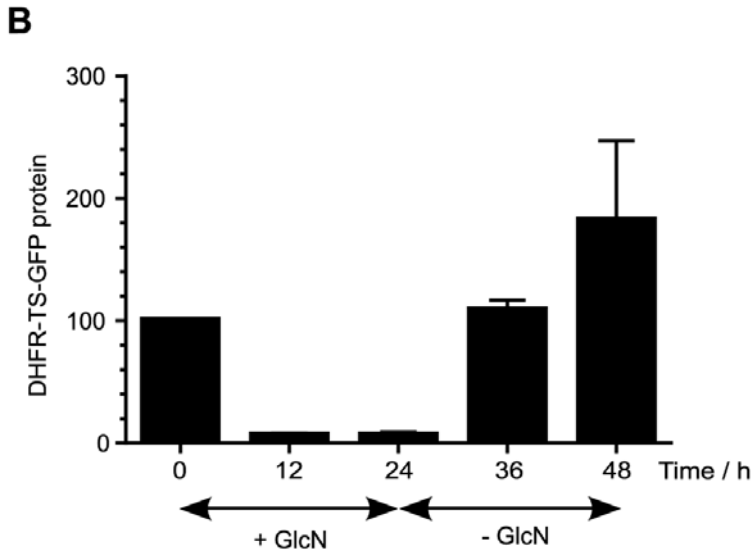
(Prommana et al. 2013)

glmS system – ribozyme RNA degradation



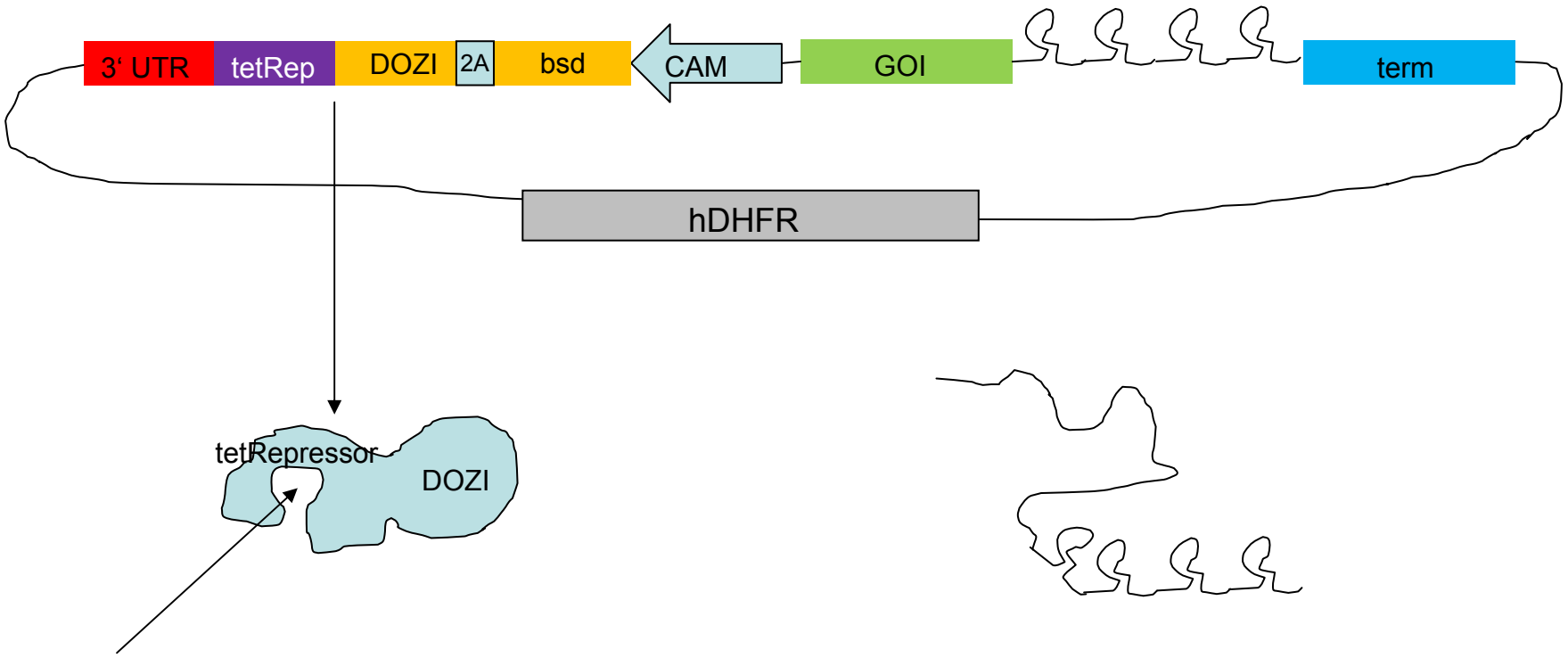
The ribozyme efficiently cleaves the 3’ end of the transcript when exposed to glucosamine





New kid on the block: inserting aptamer

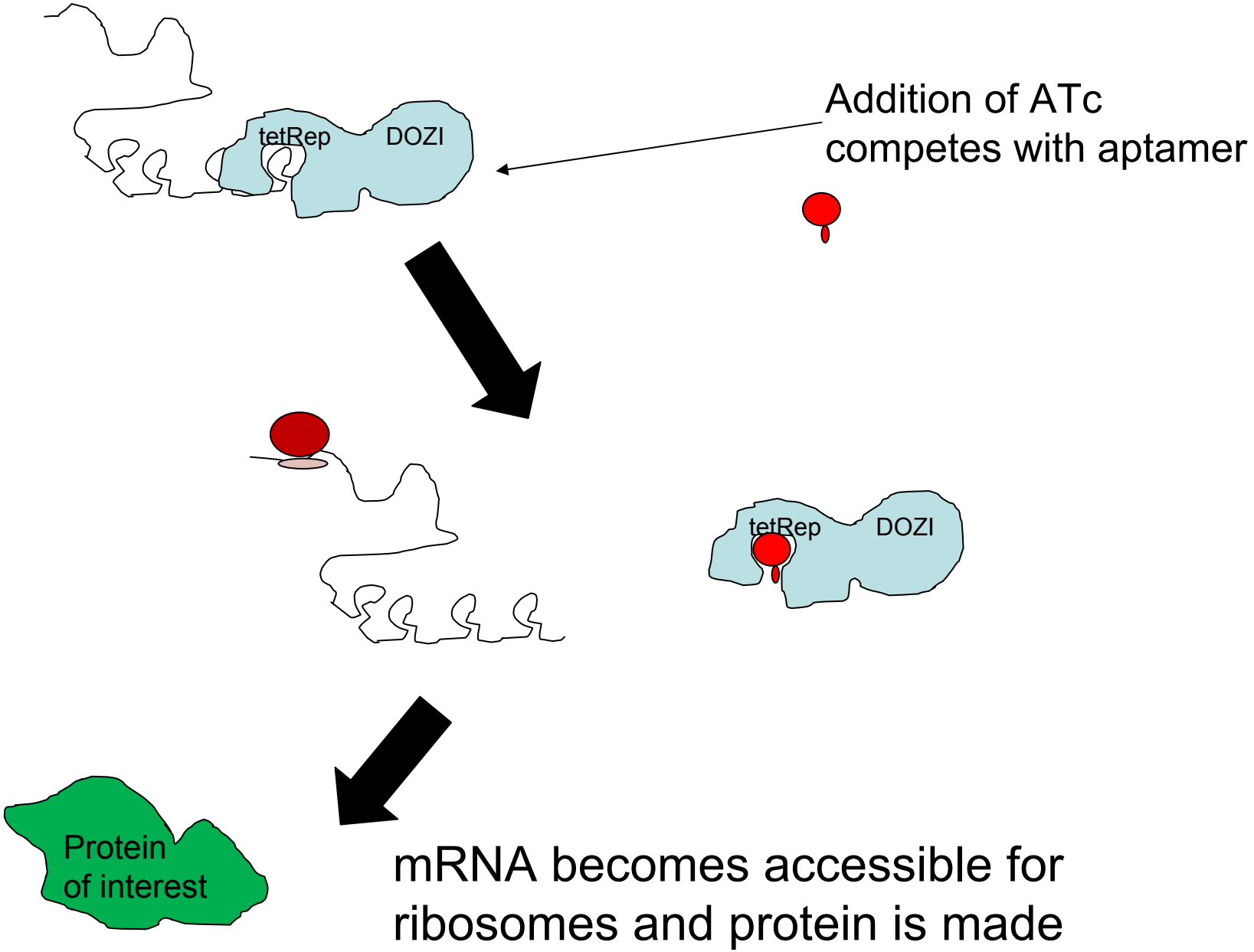
Inserting aptamer sequences in 3‘ UTR which lead to the sequestration of the transcript (Goldfless et al. 2016, Nat Comm.)



Binding site of aptamer *and* tetracyclin

RNA cannot be translated

New kid on the block: inserting aptamer



Conditional Protein Inactivation

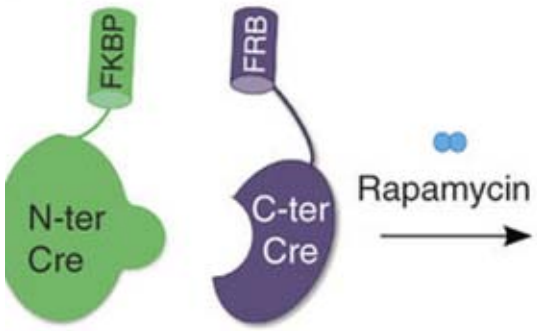
Three ways:

Level of Protein: Protein Destabilization

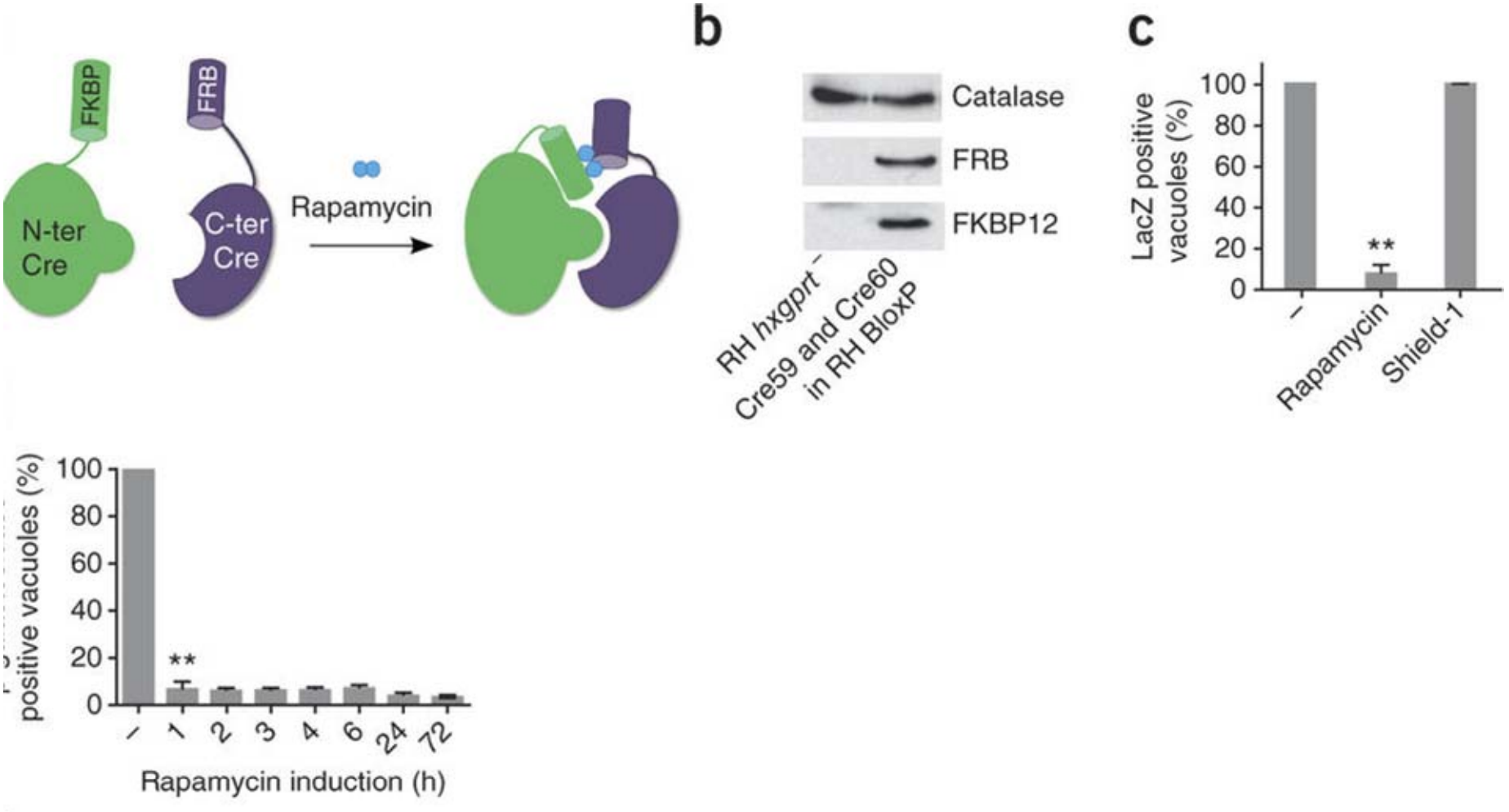
Level of RNA: Transcriptional Shut-down

Level of DNA: Site-specific Excision

Conditional DiCre recombinase system in *T. gondii*

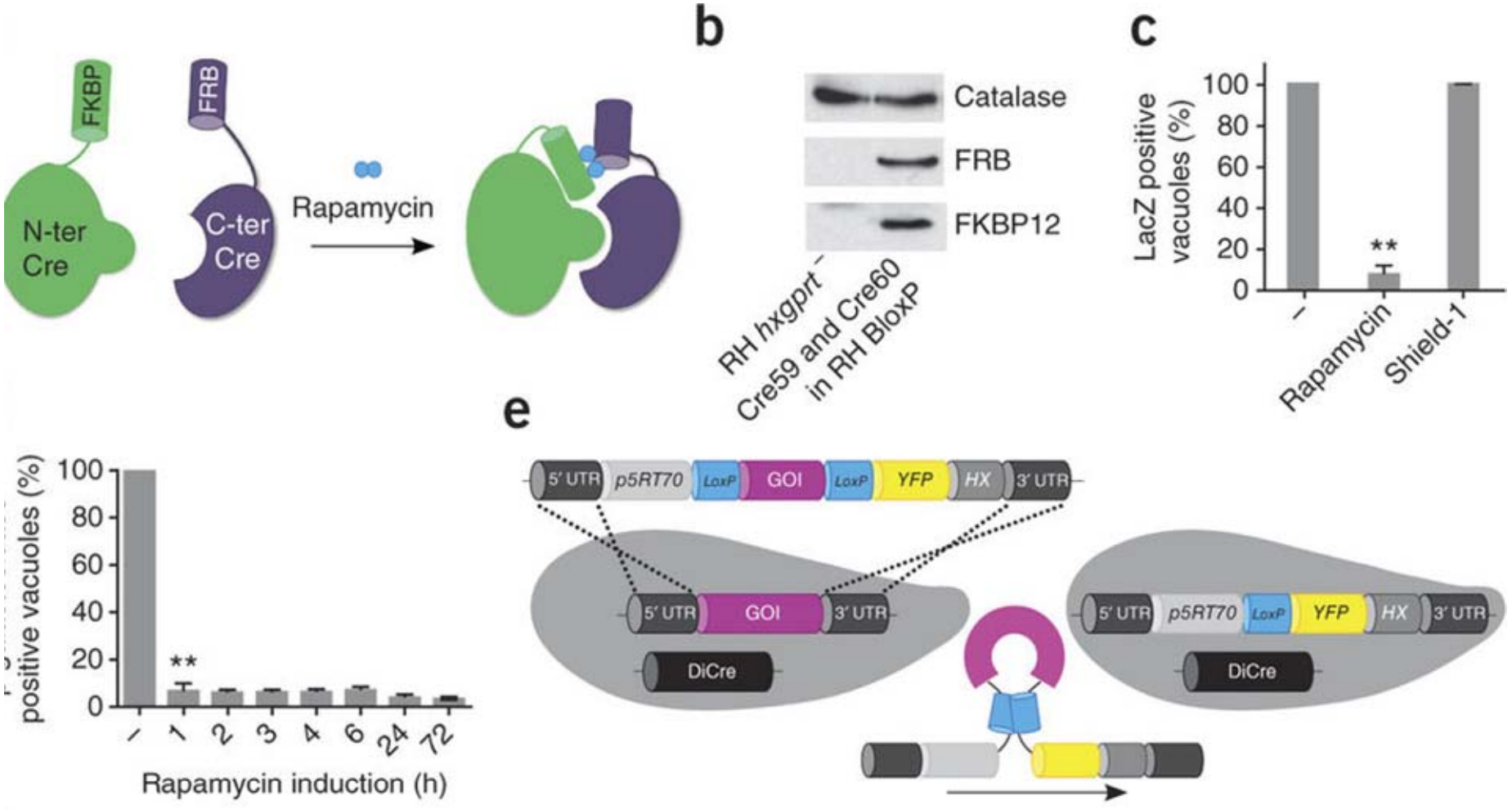


Conditional DiCre recombinase system in *T. gondii*



Andenmatten et al., 2013, Nat Methods

Conditional DiCre recombinase system in *T. gondii*



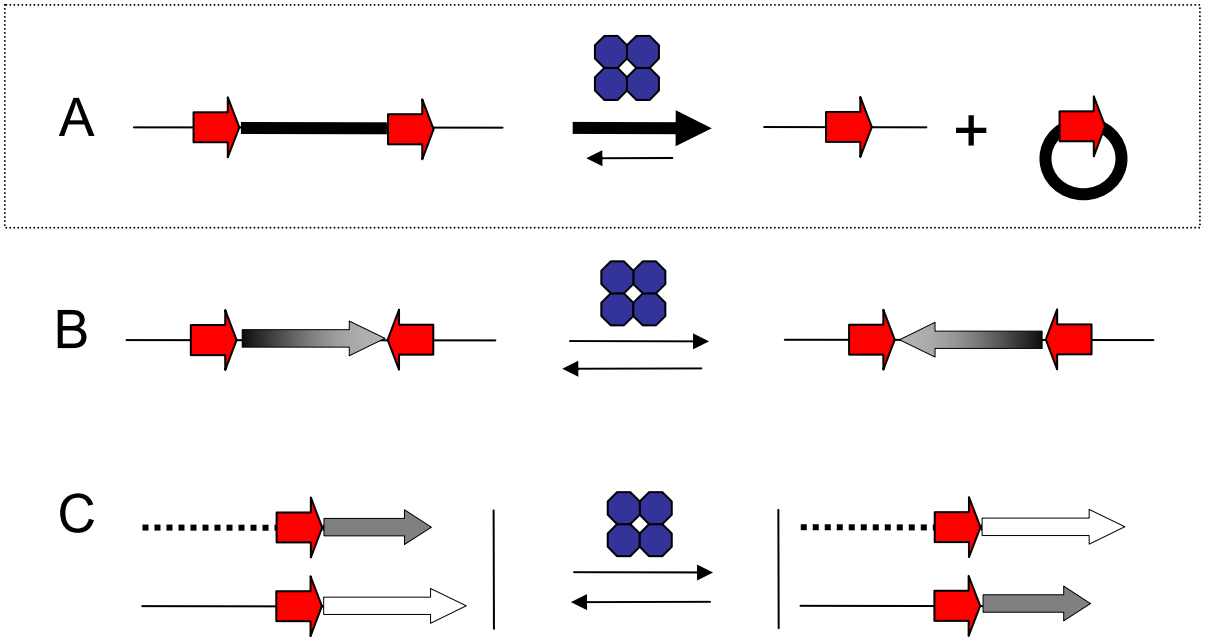
Andenmatten et al., 2013, Nat Methods

Site-specific Recombination: FLP/*FRT* system

-10 +1 +10

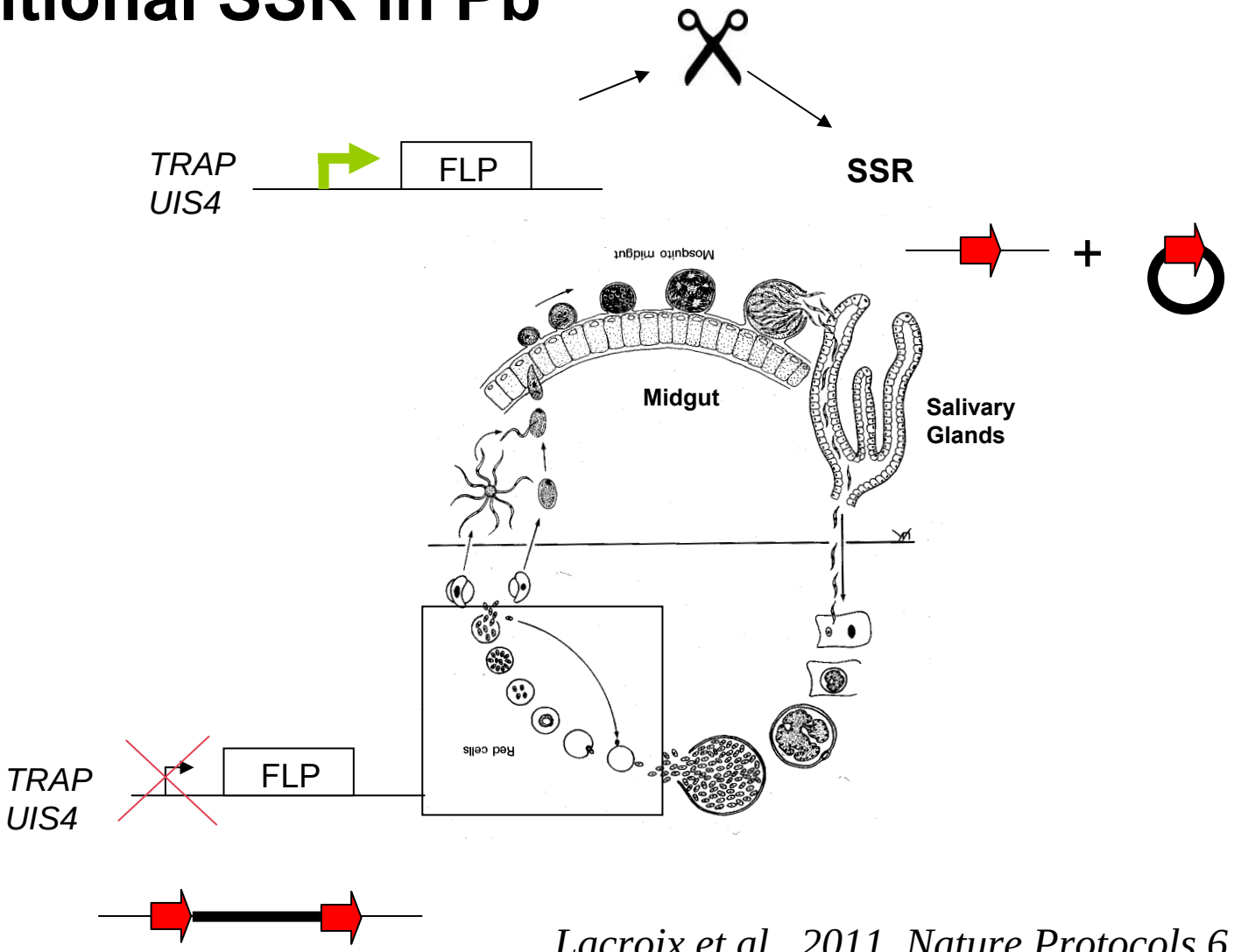
= 5' *GAAGTTCCTATTC***TCTAGAAA***GTATAGGAACTTC* 3' = FRT sequence (min 34bp)

= Rekombinase *Flippase* (*Flp*)



Site-specific Recombination: FLP/*FRT* system

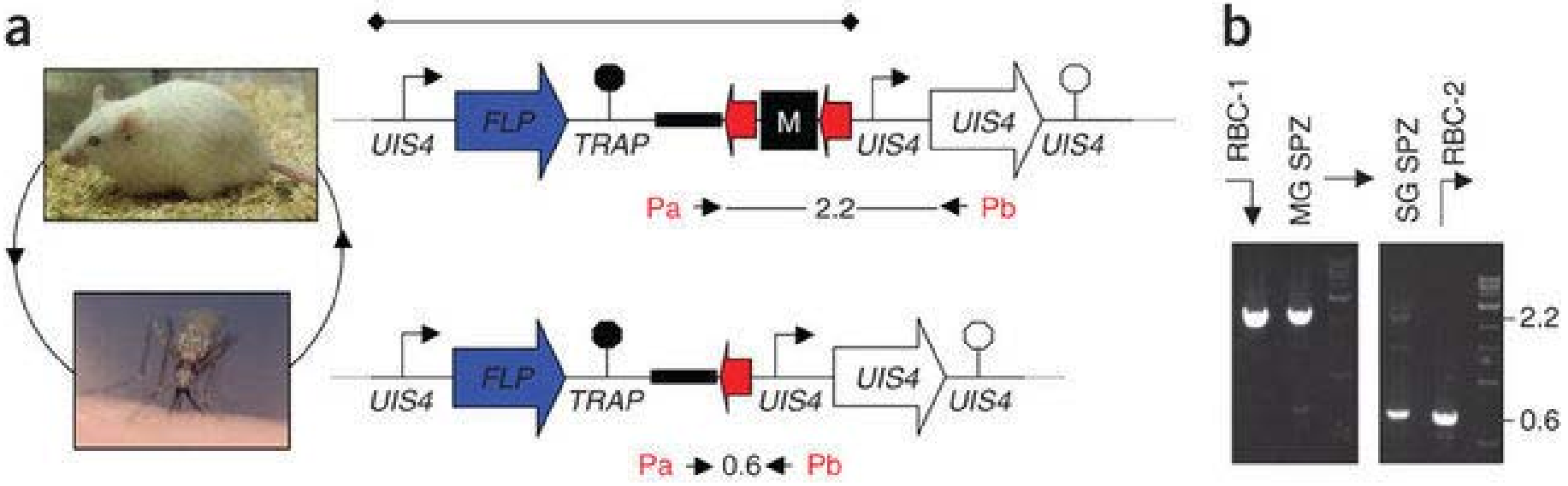
Conditional SSR in Pb



Lacroix et al., 2011, Nature Protocols 6, 1412-1428

Site-specific Recombination: FLP/*FRT* system

Conditional SSR in Pb



Lacroix et al., 2011, Nature Protocols 6, 1412-1428

The CRISPR/Cas9 system

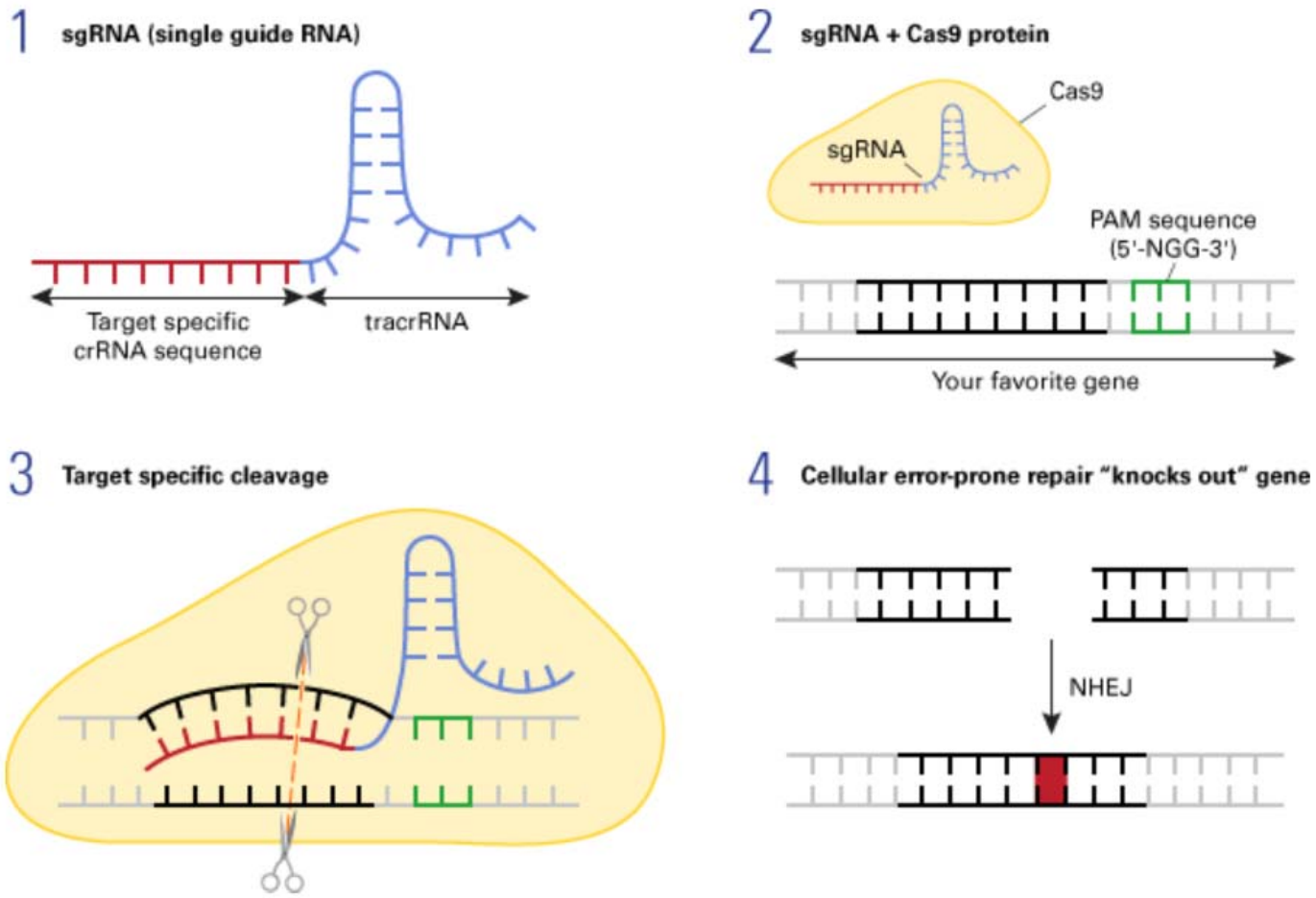
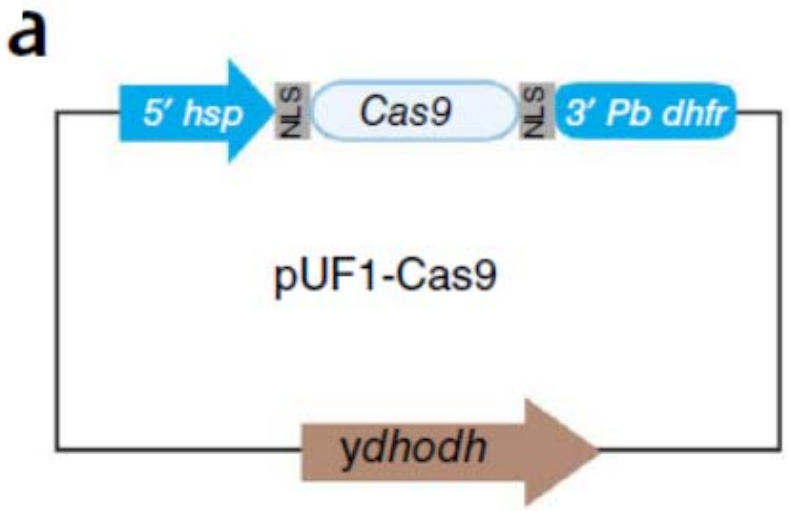
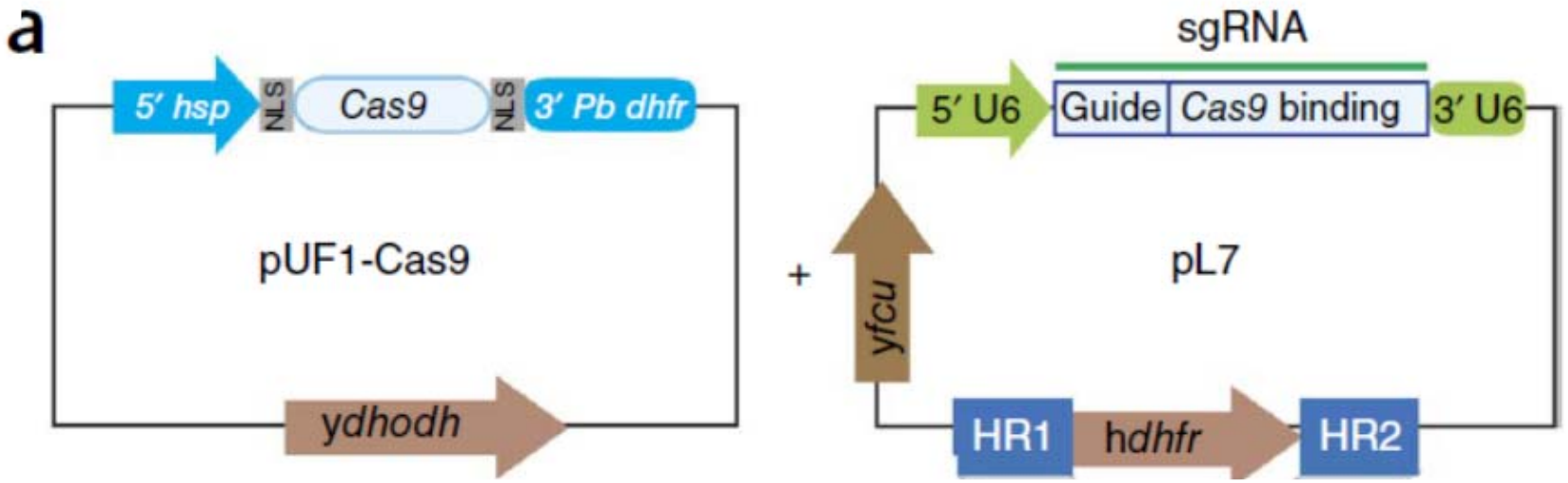


Figure 1. The principle of CRISPR/Cas9-mediated gene disruption. A single guide RNA (sgRNA), consisting of a crRNA sequence that is specific to the DNA target, and a tracrRNA sequence that interacts with the Cas9 protein (1), binds to a recombinant form of Cas9 protein that has DNA endonuclease activity (2). The resulting complex will cause target-specific double-stranded DNA cleavage (3). The cleavage site will be repaired by the non-homologous end joining (NHEJ) DNA repair pathway, an error-prone process that may result in insertions/deletions (INDELs) that may disrupt gene function (4).

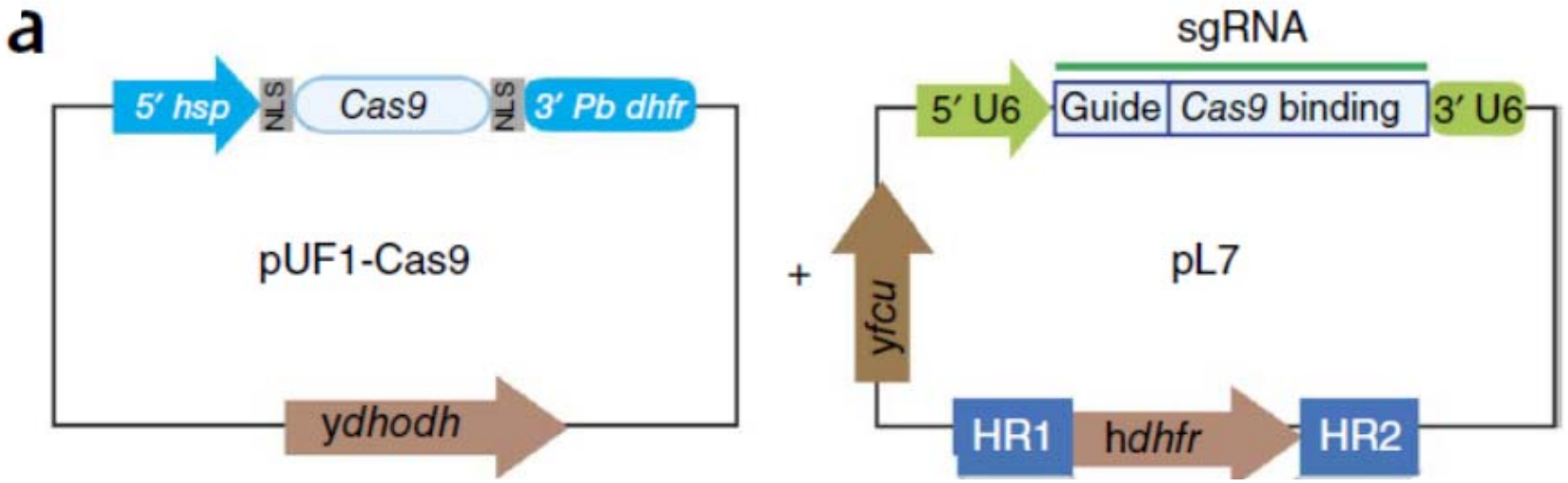
The CRISPR/Cas9 system



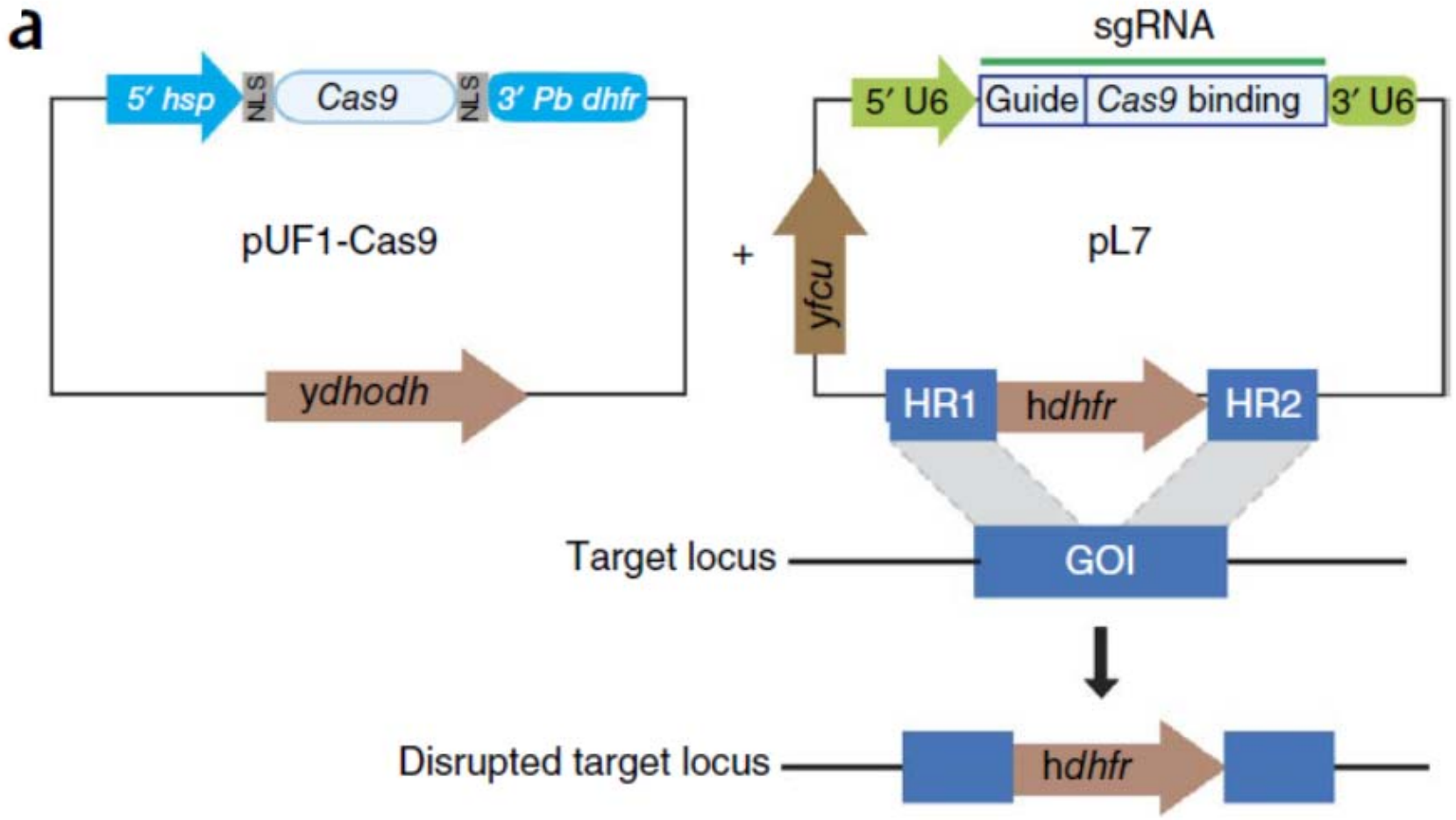
The CRISPR/Cas9 system



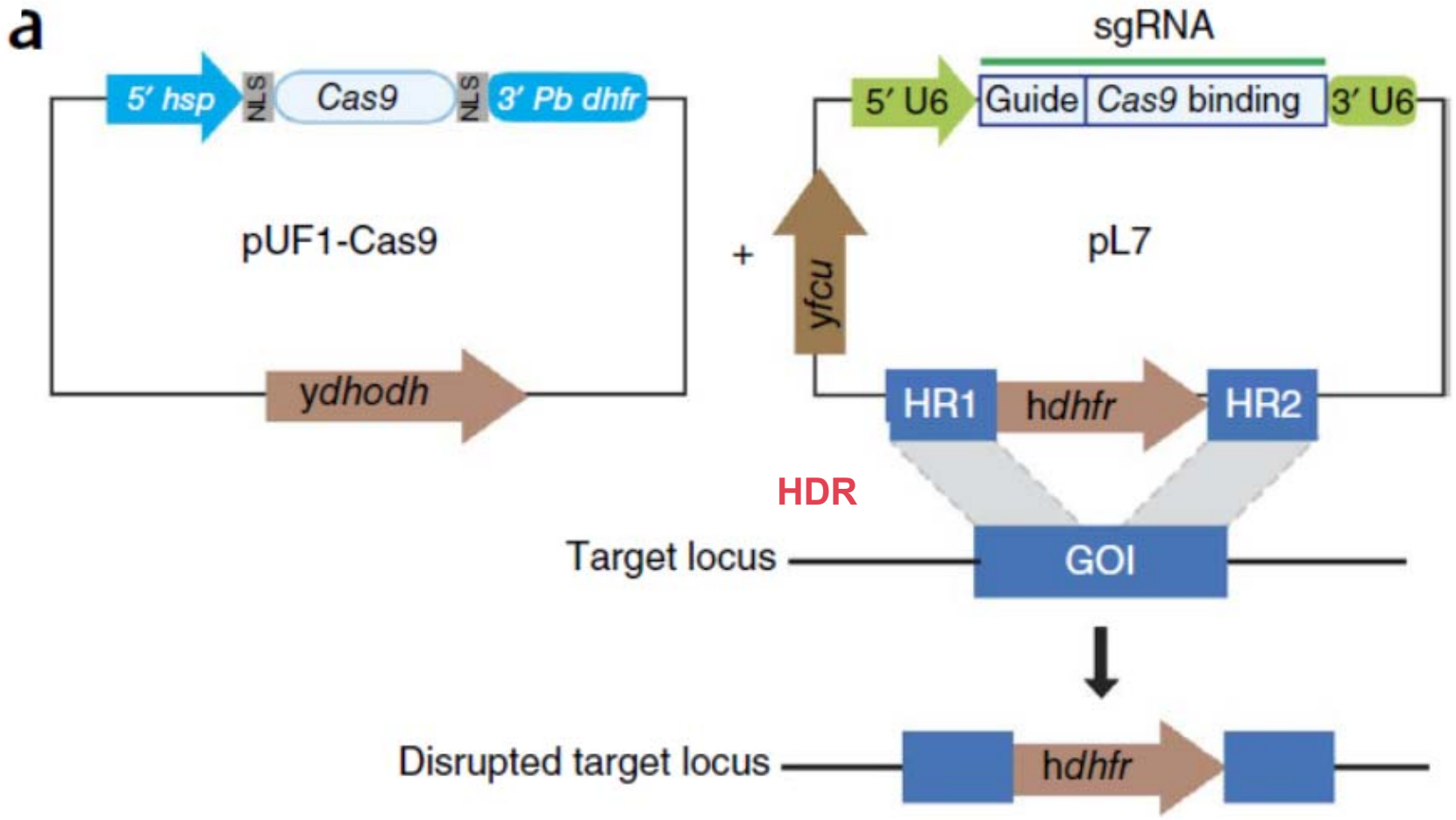
The CRISPR/Cas9 system



The CRISPR/Cas9 system



The CRISPR/Cas9 system



Cellular Repair System:

Non-homologous end joining (**NHEJ**) is a pathway that repairs double-strand breaks in DNA. NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template. **GENE DELETION**

Homology directed repair (**HDR**) is a mechanism in cells to repair double strand DNA lesions. The most common form of HDR is homologous recombination. The HDR repair mechanism can only be used by the cell when there is a homologue piece of DNA present in the cell. **GENE INSERTION**