# Microbial enzymes for DNA manipulation

Dr. Nick Coleman

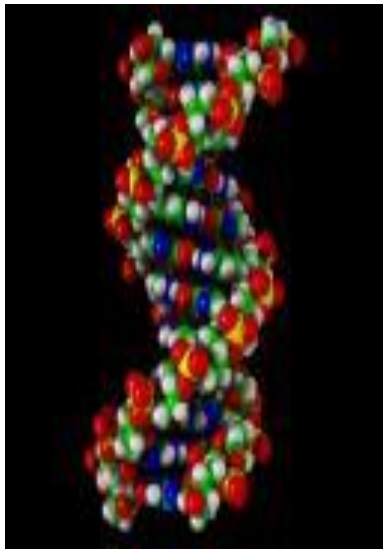
# Enzymes of DNA manipulation

## Learning outcomes. Be able to…

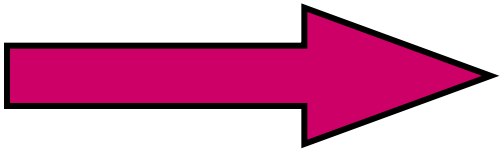
- **Name four different types of DNA-modifying enzymes used in biotechnology, and describe in each case:**
  - **What reaction is catalysed?**
  - **What is the microbial source of the enzyme?**
  - **What is the natural role of the enzyme?**
  - **How is it the enzyme used in biotechnology?**

# Enzymes of DNA manipulation

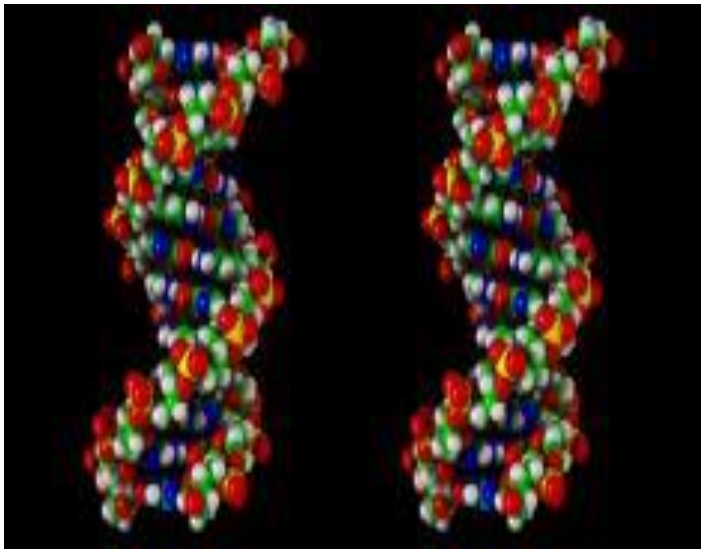
- Enzymes that modify DNA are crucial to biotechnology
- Three* broad categories: cutting, joining, copying - we will focus in this lecture just on cutting and joining.
- Majority of biotech enyzmes are from microbes or associated elements (eg. bacteriophage)
- DNA-modifying enzymes have fundamental significance:



DNA polymerase



Replication of DNA is a core function of all cells



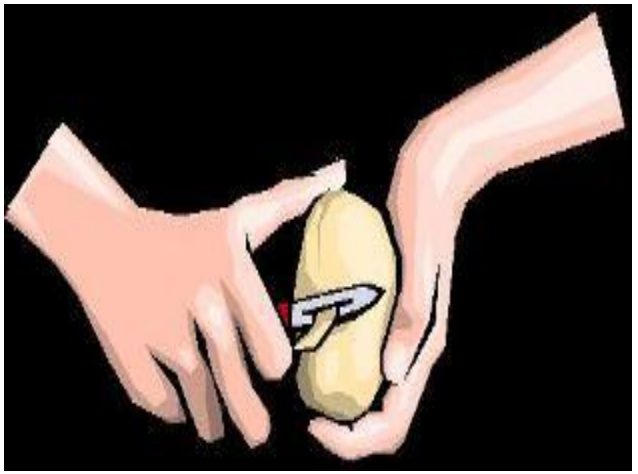
# Enzymes of DNA manipulation

Restriction enzyme

Cre recombinase

Homing endonuclease

T4 ligase



Lambda exonuclease

# Cutting DNA – Nucleases

- **Nucleases: hydrolyze phosphodiester bond in DNA/RNA**
  - **Endonucleases: cut within nucleic acid**
  - **Exonucleases: cut at ends of nucleic acid**

- **Nucleases may be friend or foe to the molecular biologist, depending on their action on the DNA**

→ **Specific nucleases** **added intentionally**

→ **Non-specific nucleases** **present in initial sample**

# Restriction endonucleases (REs)

- Found in many bacteria, >200 available
- Recognise specific sites, usu. 4-8 bp palindromes

eg. recognition site for HindIII:

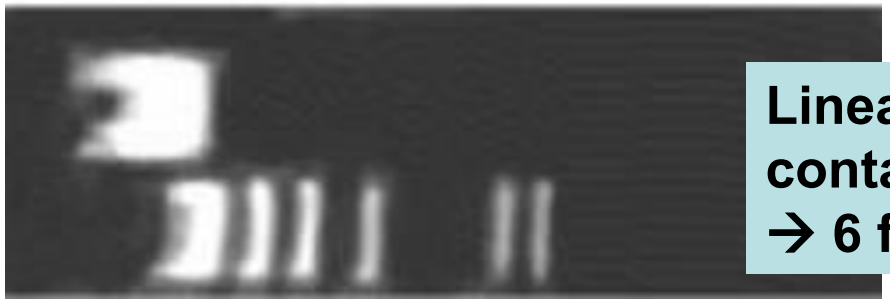
5′–AAGCTT–3′
3′–TTCGAA–5′

(Type I and III RE's not so useful for biotechnology )

- Type II RE's: — cut site within recognition site
  — very useful for biotechnology

Uncut

Cut with HindIII



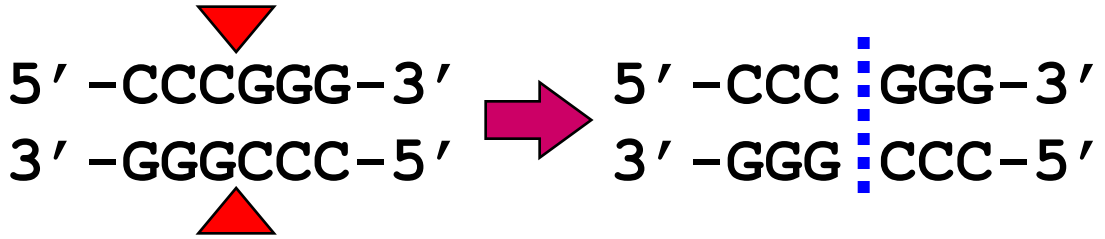
Linear Lambda phage DNA contains 5 HindIII sites,
→ 6 fragments on gel

# Restriction endonucleases

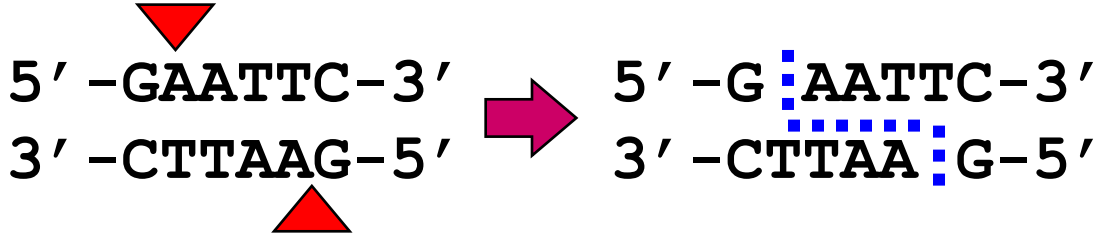
**Role in biotechnology:**

- **DNA cut with REs → blunt or 'sticky' ends**
- **Sticky ends can have either a 5' overhang or 3' overhang**

**eg. enzyme SmaI from *Serratia marcescens* → blunt ends**




**eg. enzyme EcoRI from *Escherichia coli* → sticky 5' overhang**



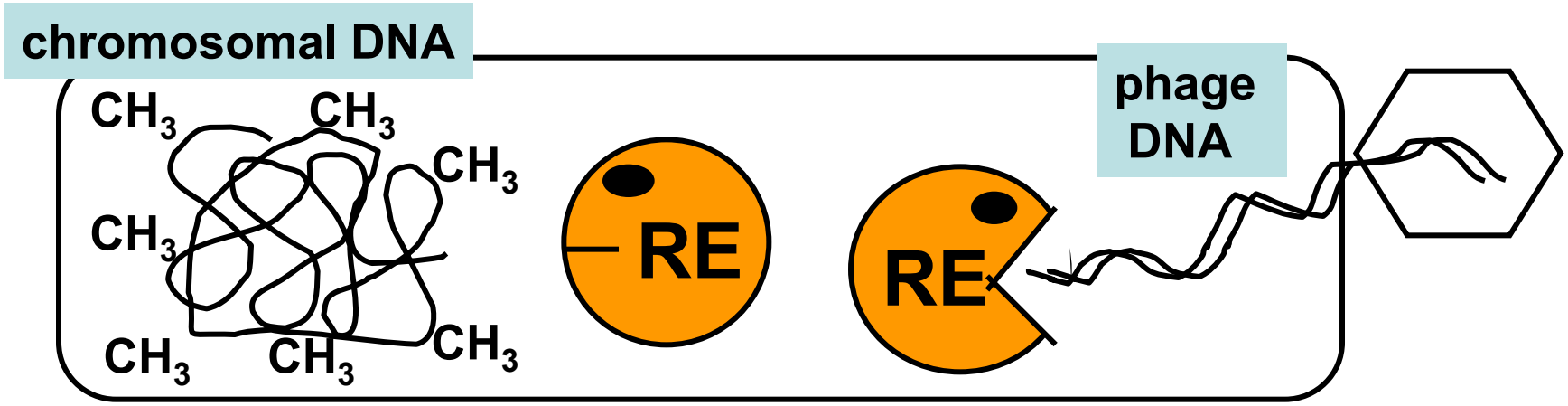

- **Both blunt and sticky ends can be rejoined by DNA ligase**

# Restriction endonucleases

**What is the biological role of REs?**

**1. An immune system for bacteria.**

- **REs are paired with modification enzymes (Mod), which methylate bases in the same recognition site.**
- **Mod acts on newly-replicated DNA (hemimethylated) → protects host DNA, doesn't protect foreign DNA**

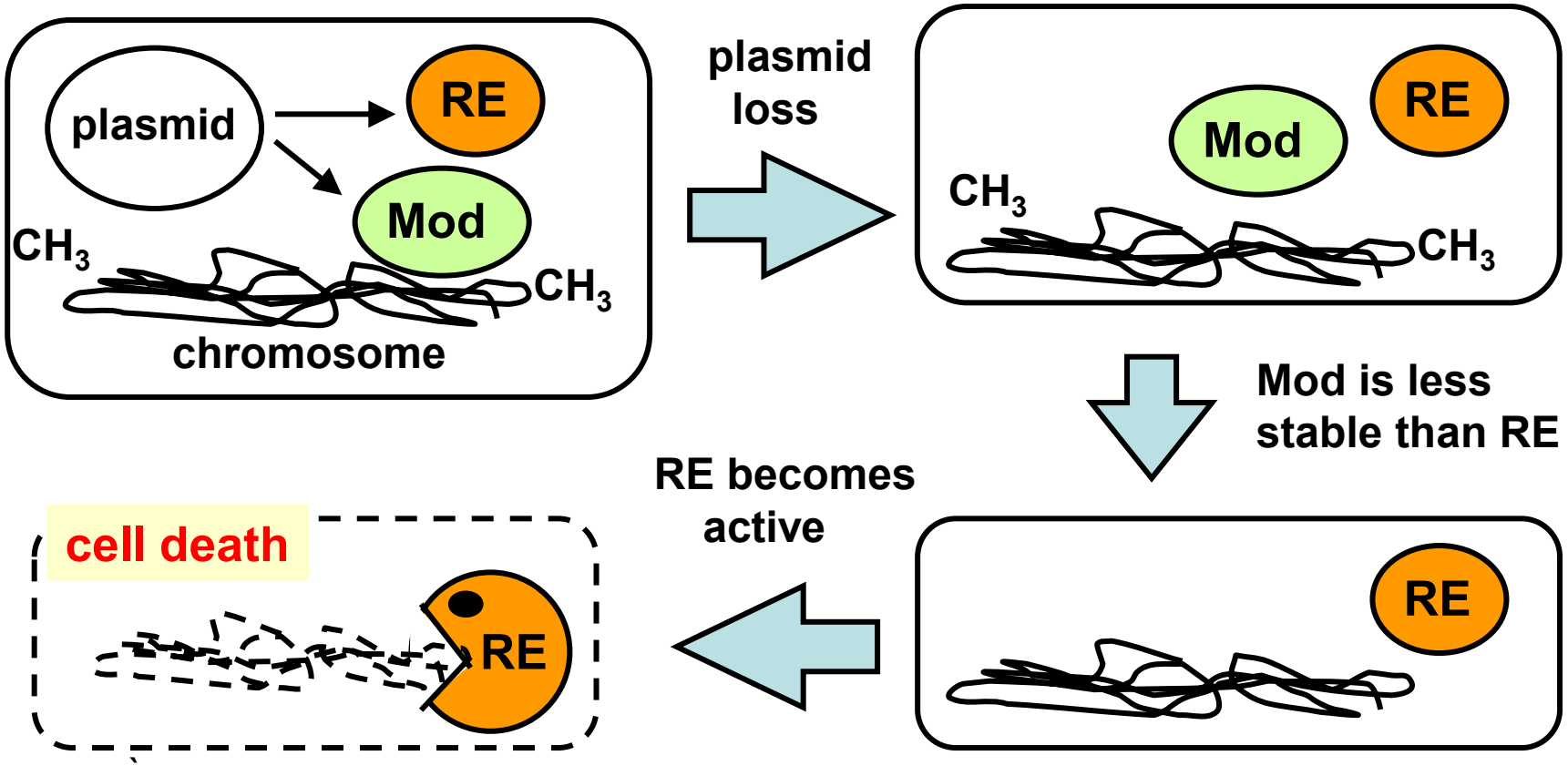


# Restriction endonucleases

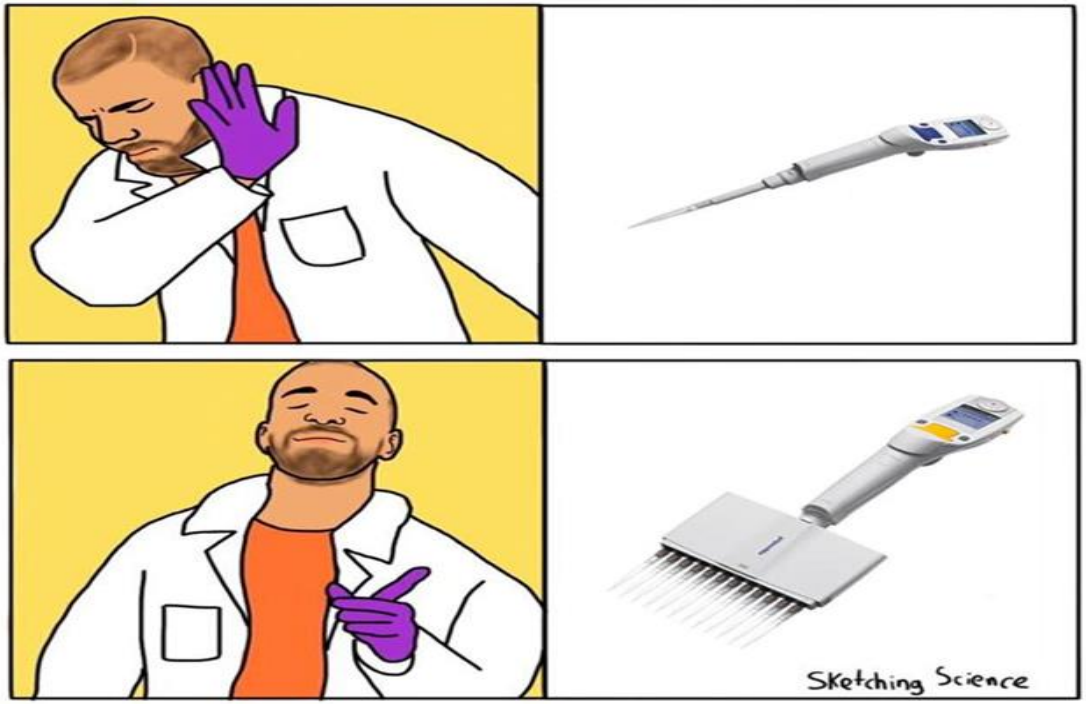
## What is the biological role of REs?

### 2. Act as stabilisers of mobile DNAs

- Presence of RE/Mod gene pairs selects for maintenance of mobile genetic elements in host cell – how ?



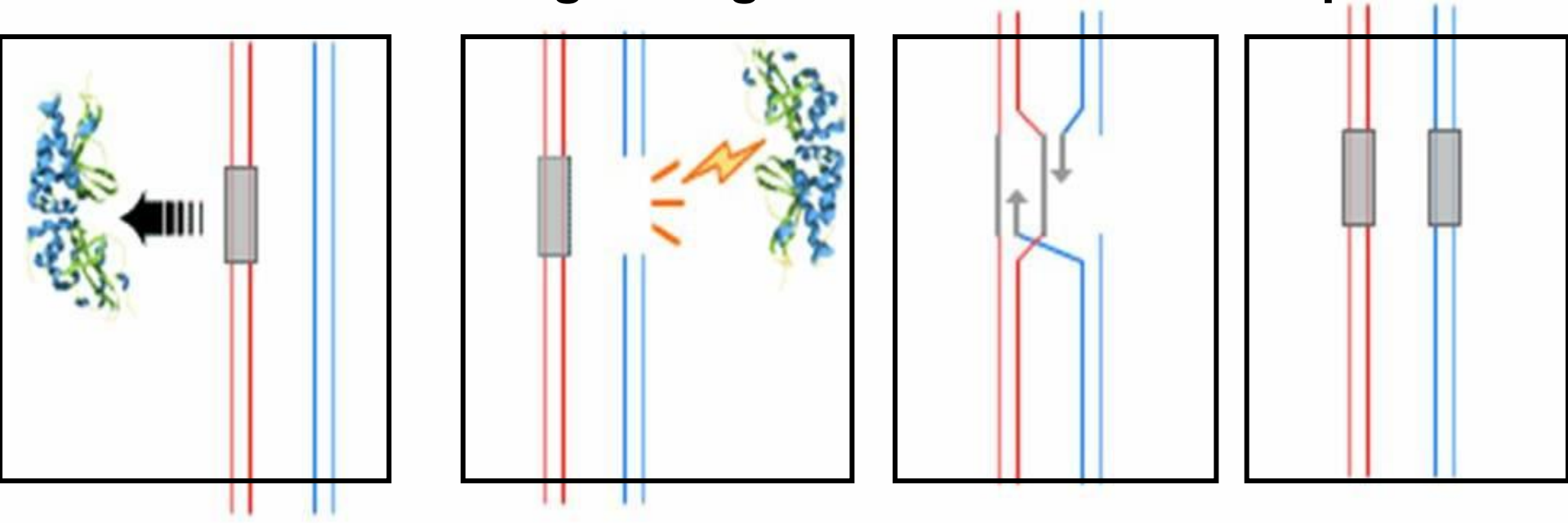
# Restriction endonucleases (REs)



# Homing endonucleases (HEs)

- HEs: special RE from introns
- Natural function is different to other REs: to provide mobility for parent element; homes into specific site

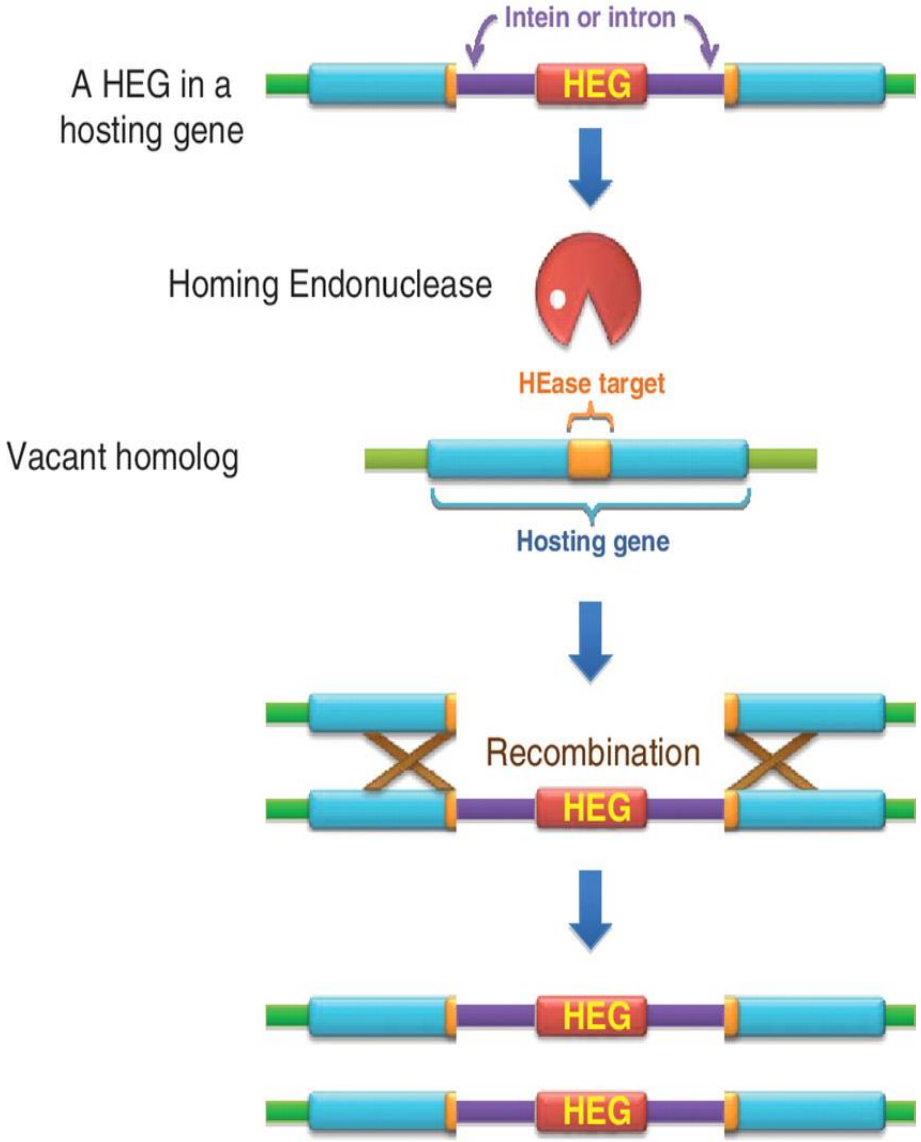
1. Expression of HE
2. Cutting of target site
3. Recombination
4. Duplication



# Homing endonucleases (HEs)

## How do they enable gene movement?

- **Depends on presence of <u>multiple copies</u> of target gene e.g. rRNA in bacteria**
- **HE cuts at location <u>not yet occupied</u> by HE-intron, but which has identical DNA surrounding occupied site**
- **Presence of long identical DNA regions is essential for <u>homologous recombination</u>**

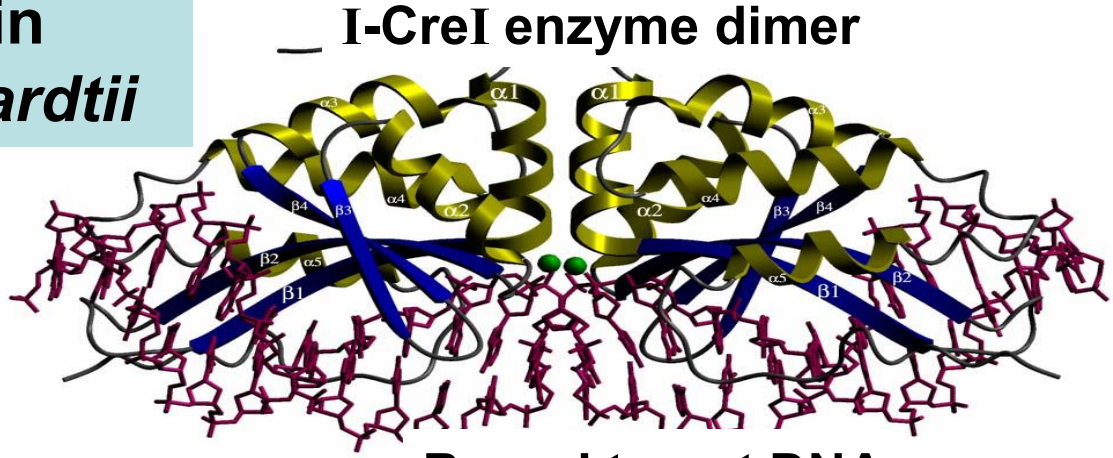


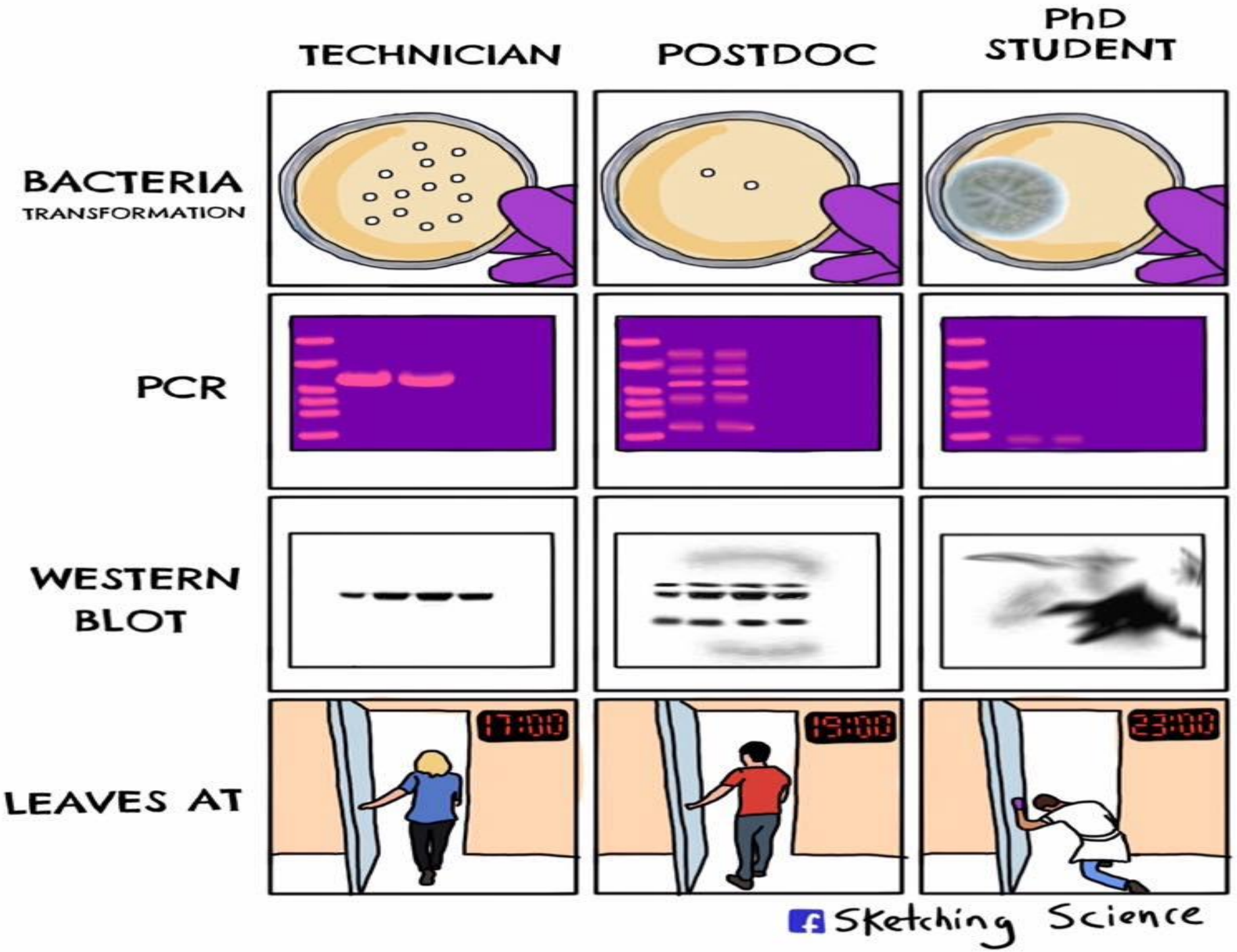
# Homing endonucleases

## Applications in biotechnology

- Target site of HEs (12-40 bp) much longer than typical RE → HE sites are rare in genomes
- Adding HE gene to cell generates a few double-strand breakages; useful for studying DNA repair systems
- HE sites in plasmid vectors allows cloning of large DNAs that would be digested by regular REs

**I-CreI: HE from intron in *Chlamydomonas reinhardtii***

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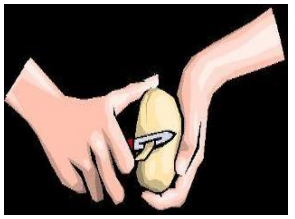
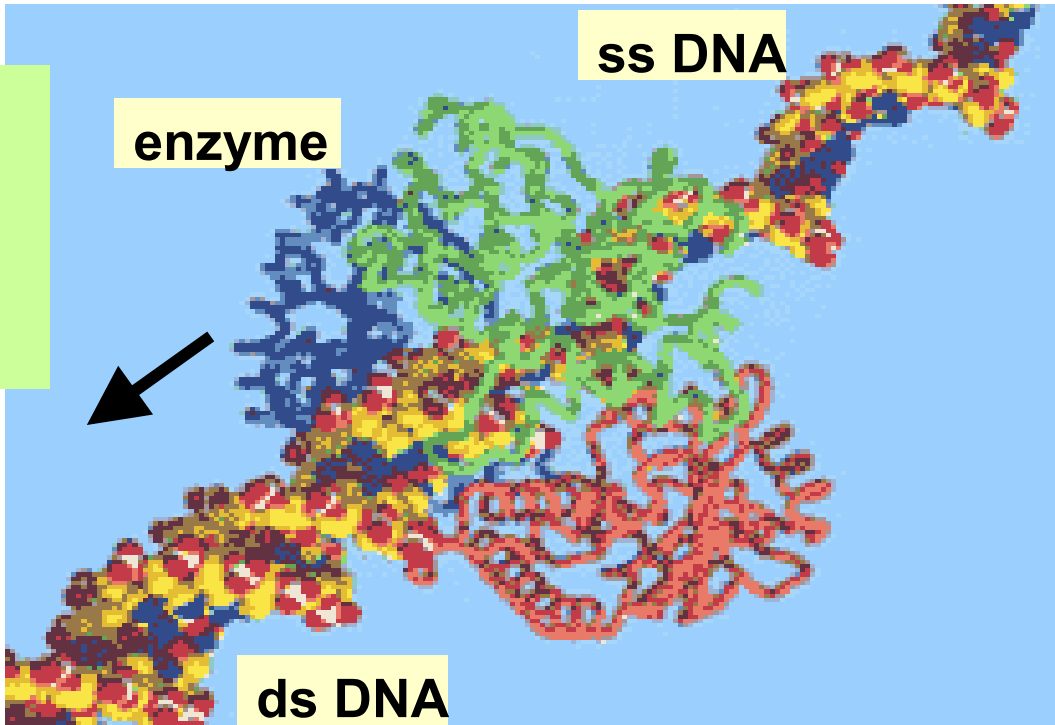
# Lambda exonuclease – Exo



- Exo is derived from bacteriophage lambda it converts ds. DNA to ssDNA*
- Selectively digests dsDNA that has a 5'-phosphate
- Example of a molecular motor: hydrolysis of one DNA strand provides energy for enzyme to move along

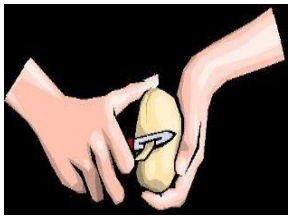
Homotrimer of Exo subunits makes a ring shape.

Central channel allows dsDNA to enter, and ssDNA to exit




*ds: double stranded
ss: single stranded

# Lambda Exo – role in phage life ?



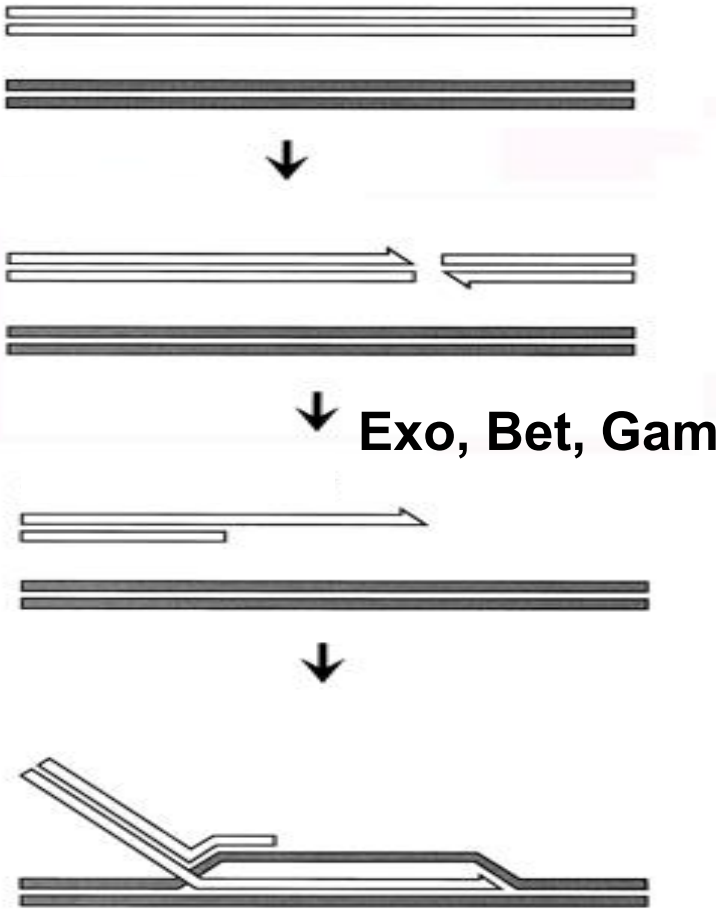
- Exo, Bet, Gam make + stabilise ss DNA,
  → This increases the recombination rate

- Function: DNA repair (?)
  similar to E.coli RecABCD.

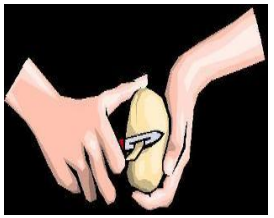
- Exo: makes the ssDNA

- Bet: binds ssDNA

- Gam: inhibits *E. coli*
  recombination (RecBCD)

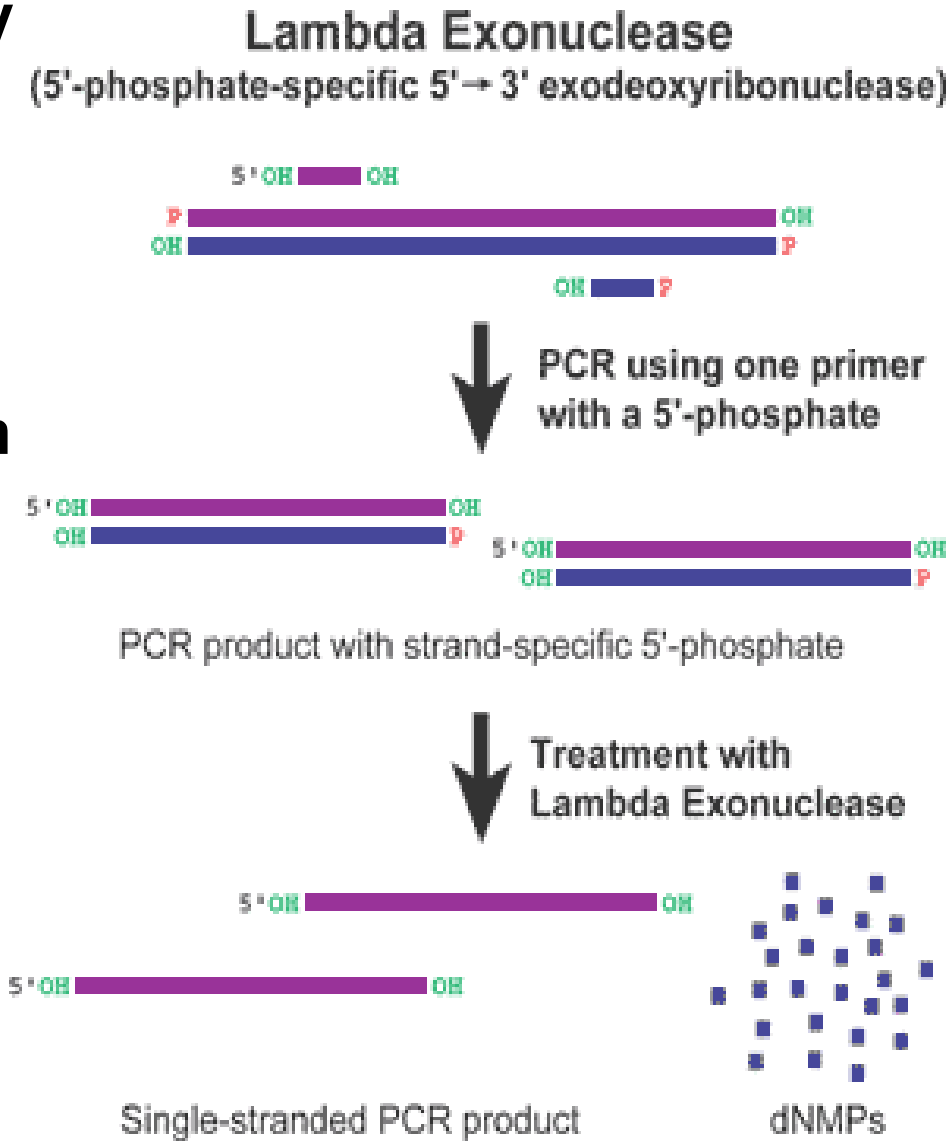


# Lambda Exo – biotech applications

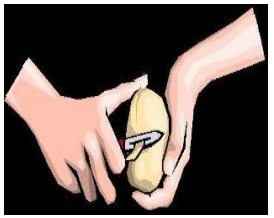


## 1. Making ss DNA for sequencing or SSCP

- ss DNA can also be made by asymmetric PCR, or by denaturation (heat/alkali)
- SSCP: single strand conformation polymorphism
- SSCP can separate DNA seqs that have same size but different *sequence*



# Lambda Exo – biotech applications

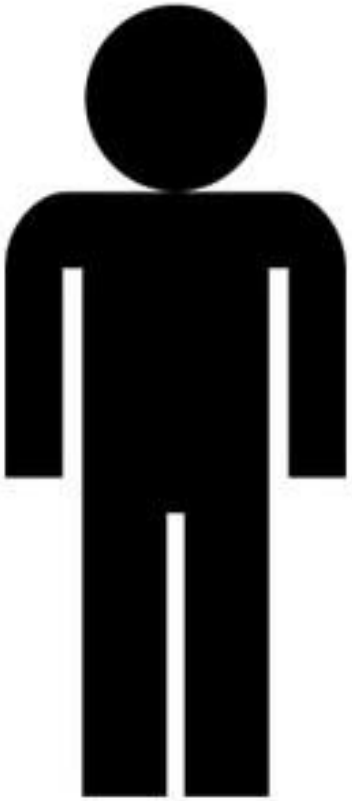


## 2. Recombineering

- Overexpression of Exo + Bet + Gam in E.coli yields strains with very high rates of recombination
- Can be used to make gene knockouts - see later lecture
- Using the lambda recombination enzymes for DNA manipulation is called "recombineering"

RECOMBInation-mediated genetic engiNEERING.

Mutation of galK galactose metabolic gene in E.coli at high frequency by recombineering



# Human v 1.1

## Hotfix patch

- dislodged eyelashes will no longer enter eyeball area and become inaccessible

- random cheek and tongue biting issue during food consumption fixed

- memory leak patched, should fix the "enter room and forget why" and item misplacement issues

- fixed a bug where the motivation module would randomly fail to load

**GENETIC ENGINEERING**

ONE DAY IT WILL MAKE THIS AND OTHER GLITCH FIXES REAL

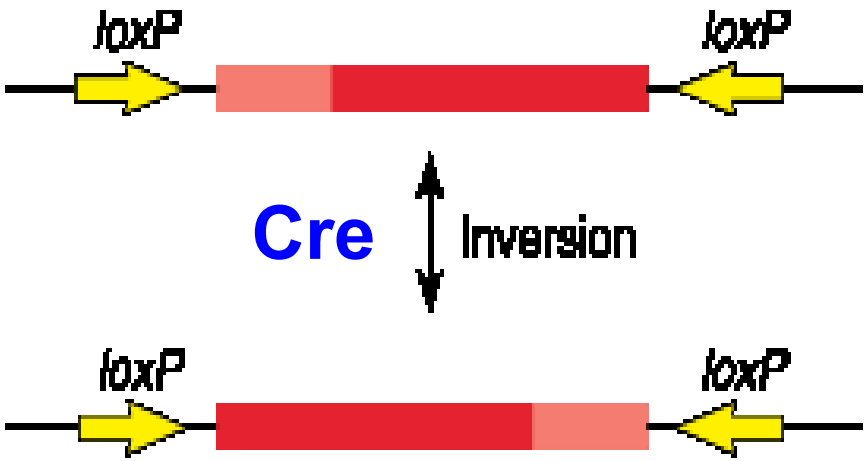
# Cre recombinase

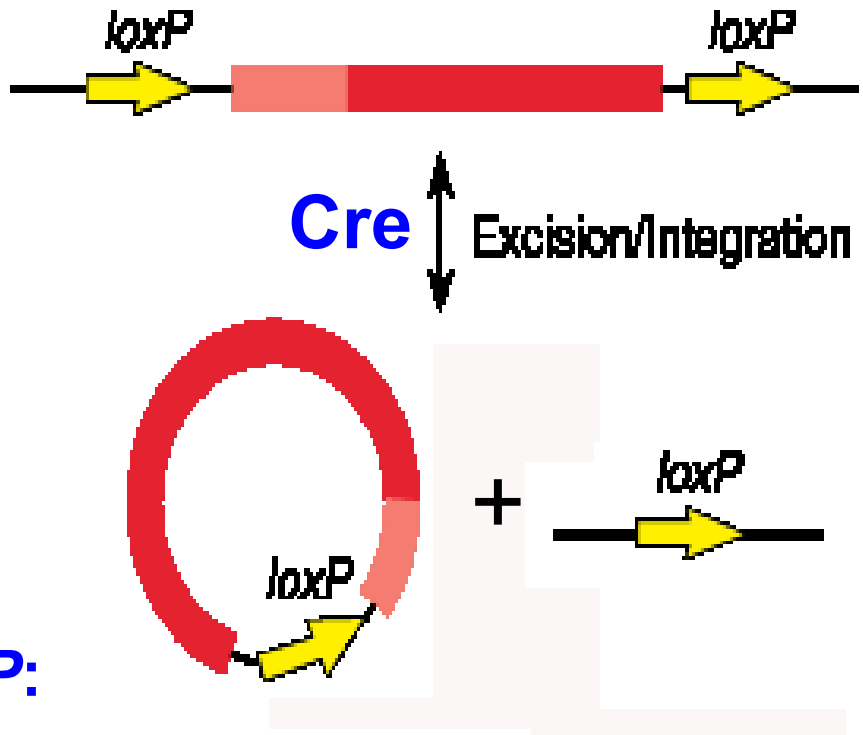
- Site-specific recombinase from phage P1
- Contains tyrosine at active site.
  Other tyrosine recombinases include:
  - lambda integrase: integrates phage into chromosome
  - Integron integrase: integration/excision of gene cassettes
- Cre targets *loxP* sites: 34 base pair inverted repeat
  - much higher specificity than REs: a 34 bp sequence is unique in a genome, unlike a 6 bp RE target site

structure of loxP site

# Cre recombinase

- Causing recombination:
  integration, excision, inversion



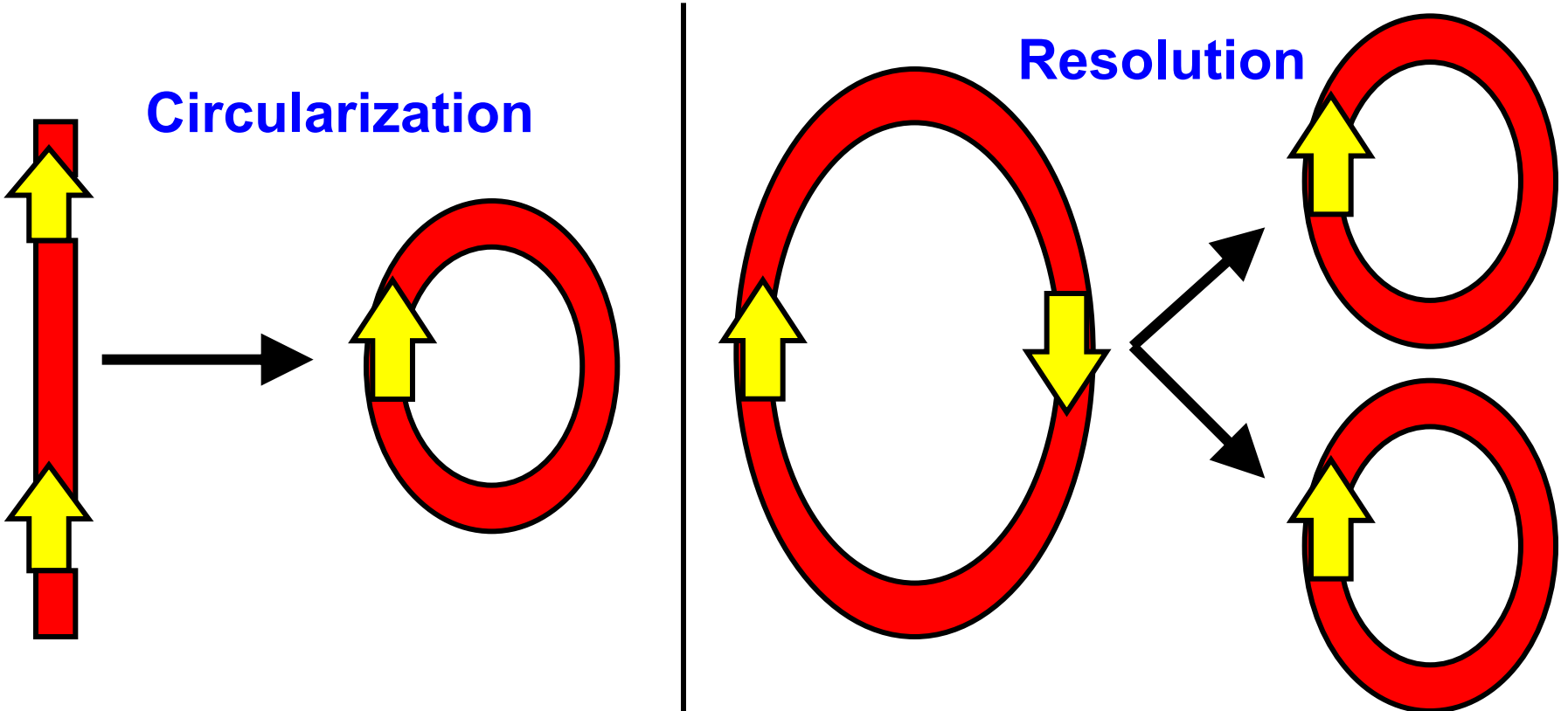

## Diverse reactions of Cre and *loxP*:

- DNA between oppositely-oriented loxP sites is inverted
- DNA between identically-oriented loxP sites is excised
- Two DNAs, each with one loxP site, will be joined

# Cre recombinase

**Role of Cre in phage P1 life cycle:**

1. **Circularization of phage genome after entry into cell yields plasmid form of P1**
2. **Resolution of P1 plasmid dimers ensures accurate partioning of plasmids into daughter cells at cell division**

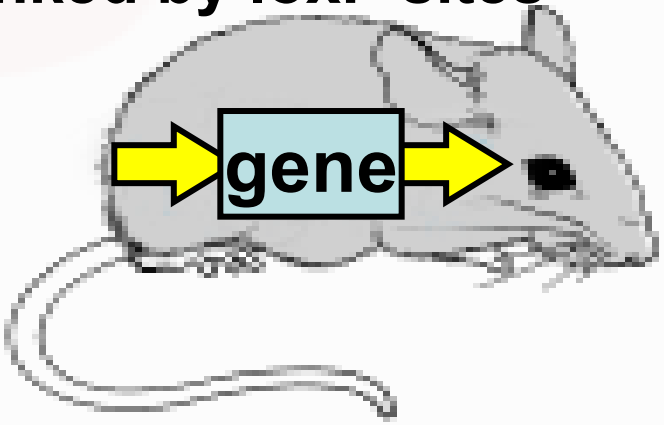


# Cre recombinase

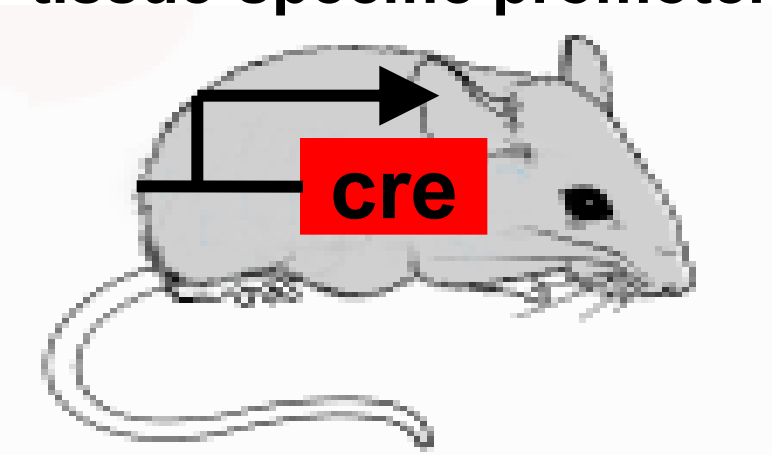
## Uses of Cre/loxP for biotechnology

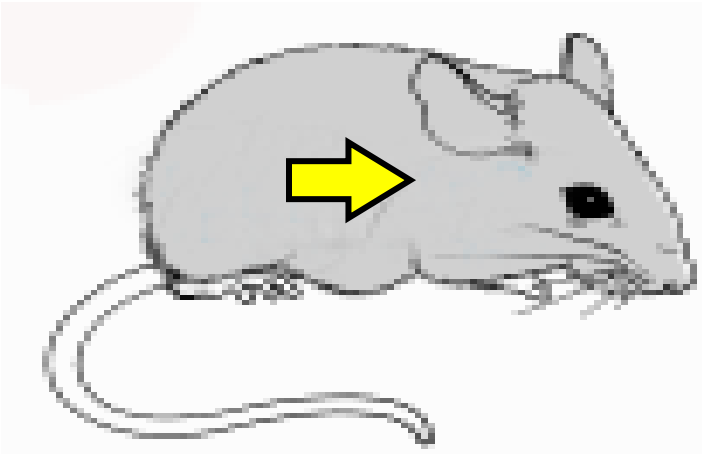
**eg. Tissue-specific knockout mutations in mice:**

**Parent 1: Gene of interest flanked by loxP sites**




**Parent 2: Cre gene controlled by tissue-specific promoter**



**Offspring: Gene of interest deleted in specific tissue**

# Joining DNA – T4 ligase

- Joining DNA from different sources is the key step in all recombinant DNA work
- T4 ligase: joins 5' phosphates and 3' hydroxyls of two double-stranded DNAs, 'reverse' of RE reaction
- T4 ligase can join blunt or sticky ends; sticky end reaction more efficient (assisted by hydrogen bonding)

**Blunt ligation of two DNAs cut with SmaI**

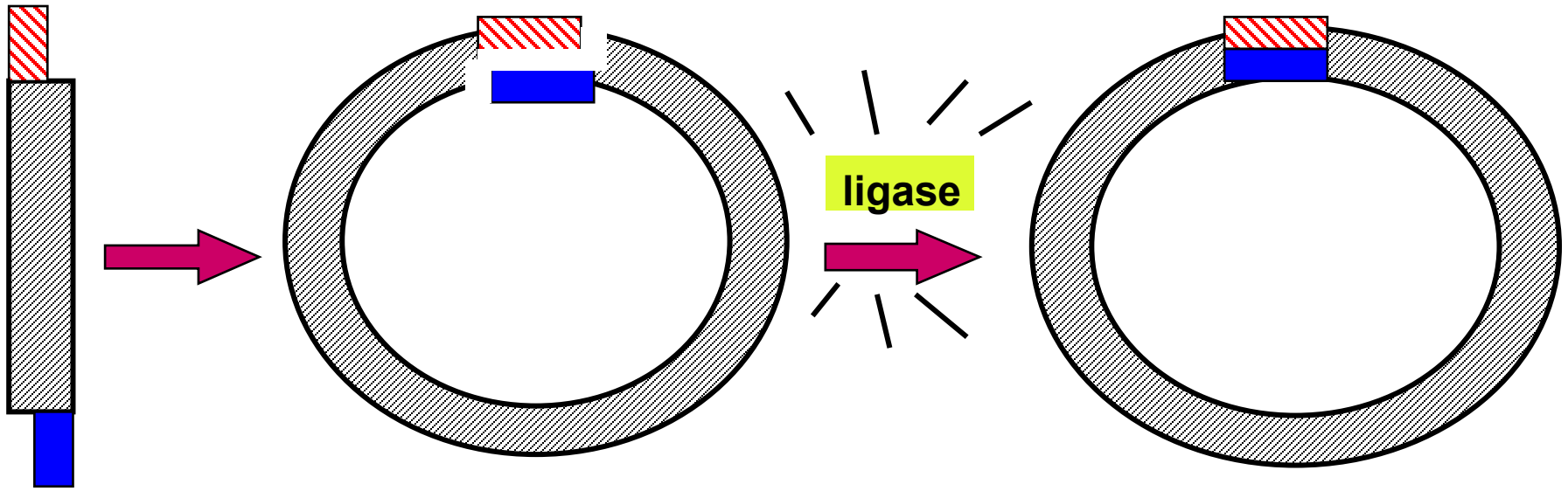
```
5′-CCC   GGG-3′          5′-CCCGGG-3′
3′-GGG   CCC-5′    →     3′-GGGCCC-5′
```

**Sticky ligation of two DNAs cut with EcoRI**

```
5′-G   AATTC-3′          5′-GAATTC-3′
3′-CTTAA   G-5′    →     3′-CTTAAG-5′
```

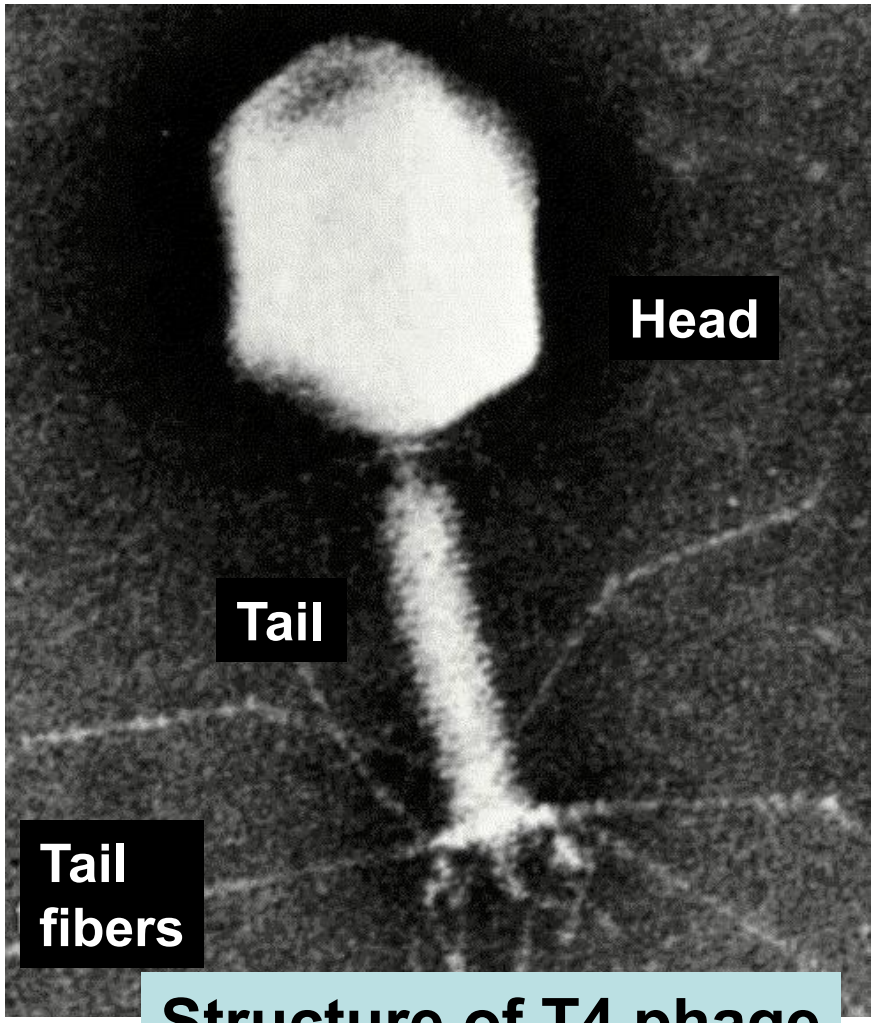
# Joining DNA – T4 ligase

- T4 is a bacteriophage that infects *E.coli*
- DNA packaged in phage as linear molecules
- After infection, T4 ligase joins ends of phage DNA to give circular form, allowing 'rolling circle' DNA replication



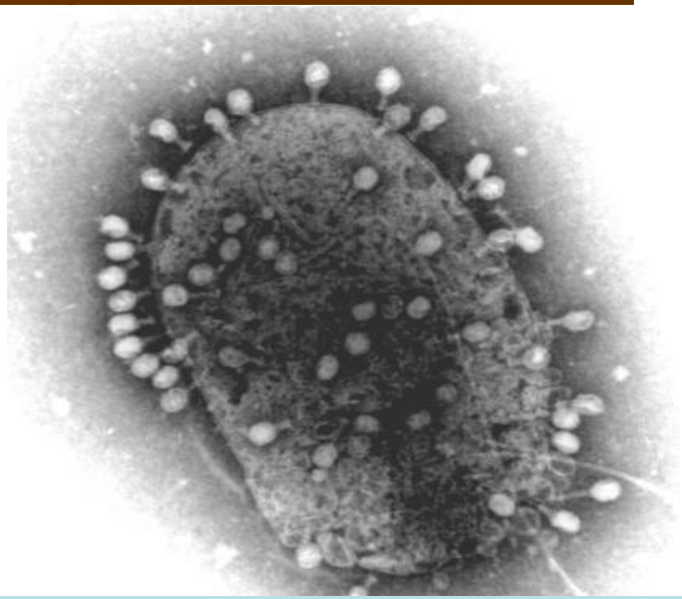
# T4 phage: source of ligase and more

- T4: large genome (170 kb), complex biology
- Source of many useful enzymes for biotech eg.




Structure of T4 phage

- DNA Ligase
- RNA Ligase
- DNA polymerase
- Polynucleotide kinase



T4 phages attacking E.coli

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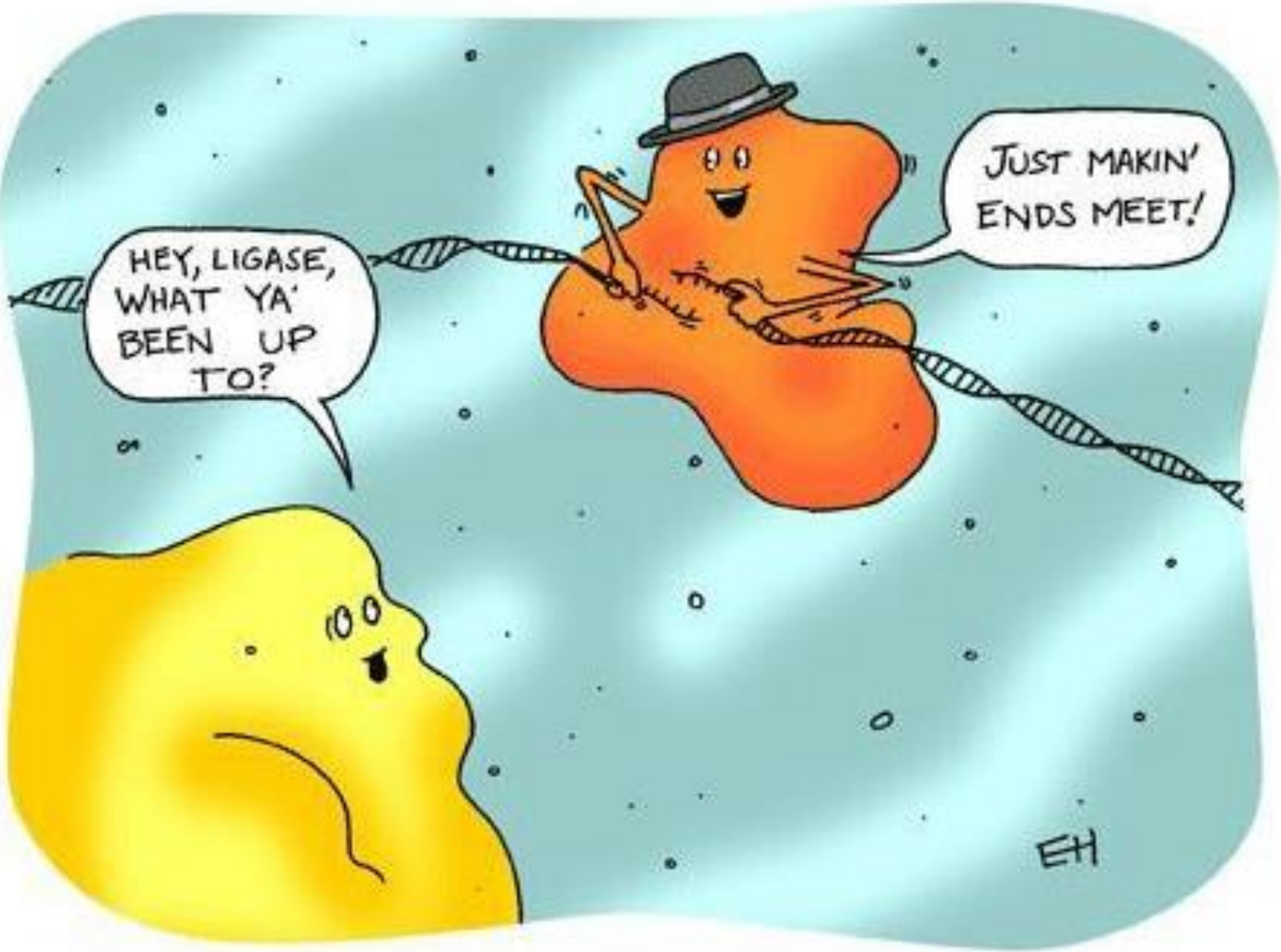

HEY, LIGASE, WHAT YA' BEEN UP TO?
JUST MAKIN' ENDS MEET!
EH

# Putting it all together. Clone army construction

PCR

# Putting it all together. Heterologous expression

Biological sample

DNA extraction

or PCR

DNA polymerase

genomic DNA

PCR product

Plasmid or other vector

Restriction or homing endonuclease

sticky-end vector

sticky-end fragments

DNA ligase

Ligation mixture

Transformation into host

Individual clones (e.g. colonies on agar)

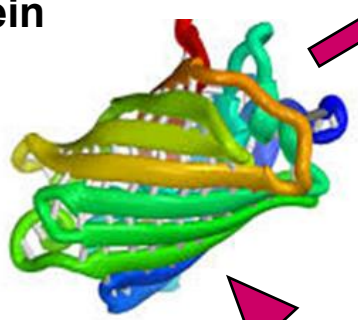
PCR Screening

DNA polymerase

Cloned gene of interest

expression, purification

Recombinant protein



- Phenotypes
- Functions
- Applications

# Putting it all together:

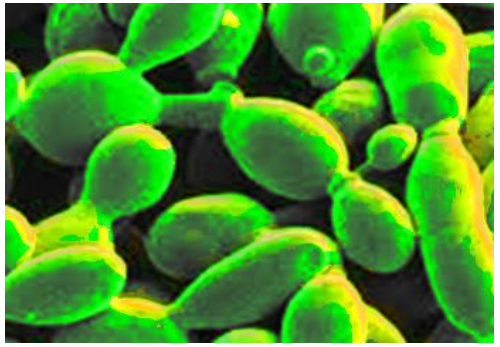
## 2. Knockout / integration

Biological sample

Recombineering

Lambda Exonuclease
+beta + gamma proteins

Marker gene
and flanking DNA

Cre/lox

Cre recombinase
+ loxP sites