MOD1 – DNA ENGINEERING

Fall 2010

Day 2

Mod1:

In this module, you will create a plasmid that will be used in an assay to measure homologous recombination activity in mammalian cells.

Background & Significance:

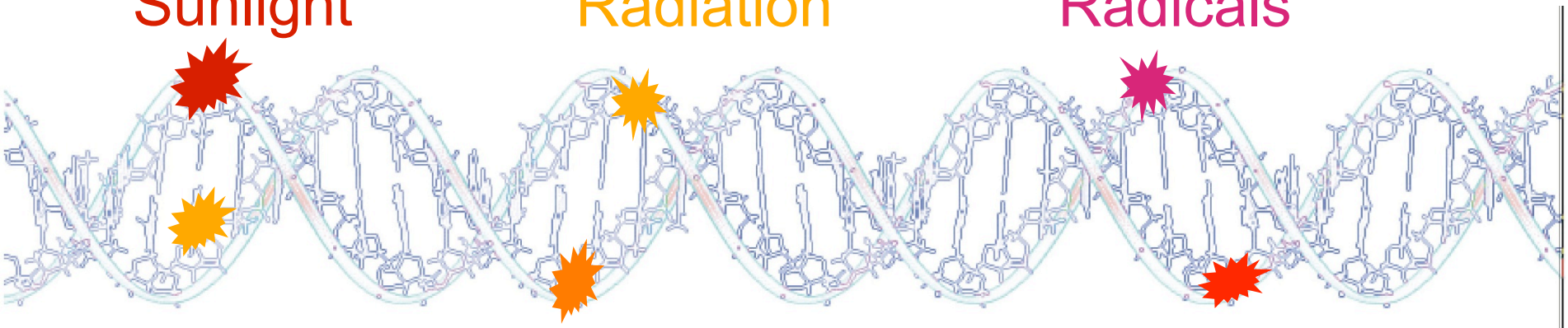
“Homology-Directed Repair” for double strand breaks

You will need to understand this material in order to analyze your data.

Sunlight

Radiation

Oxidative Radicals



Cigarette Smoke

Pollution

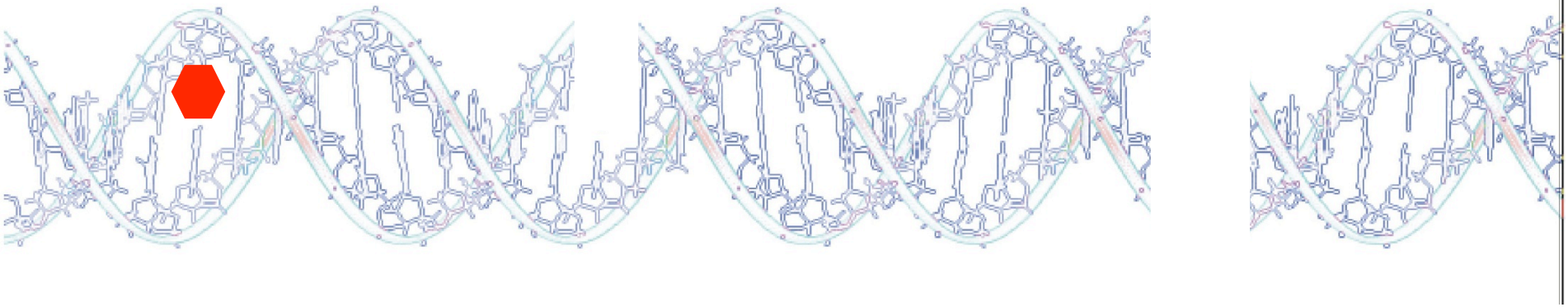
Nitric Oxide

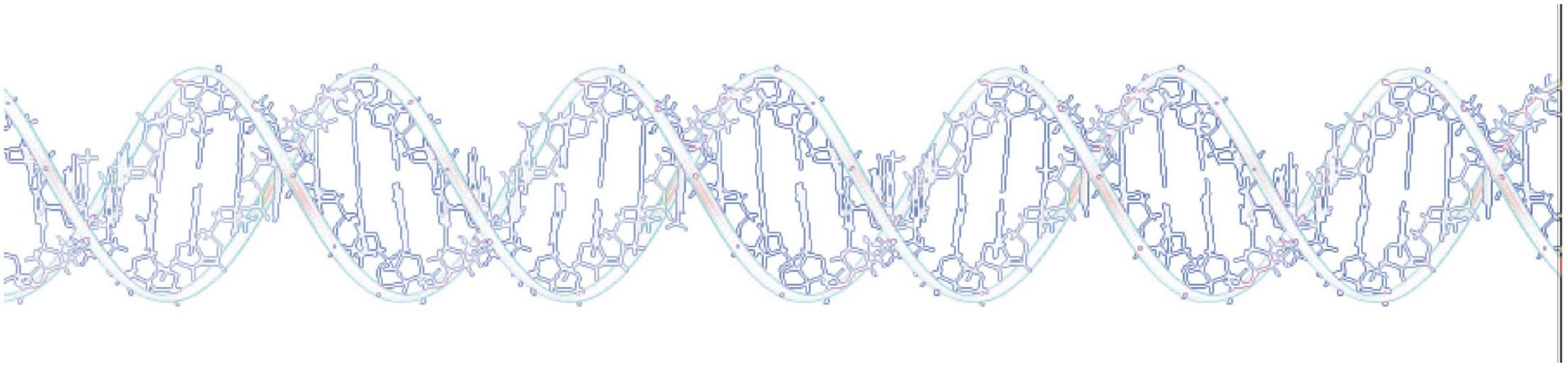
After damage, what might happen?

Base Lesions

Single Strand Breaks

Double Strand Breaks





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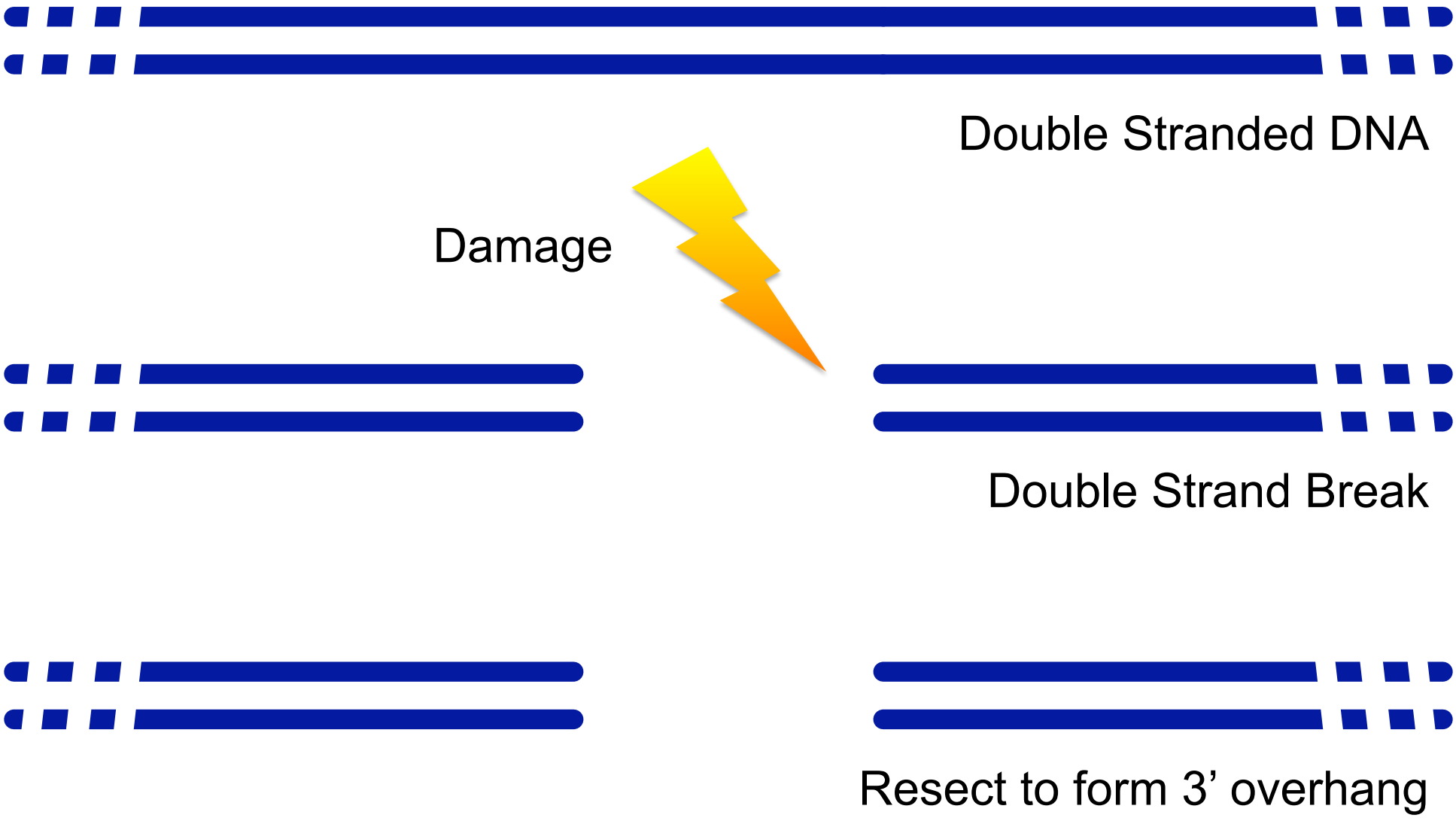
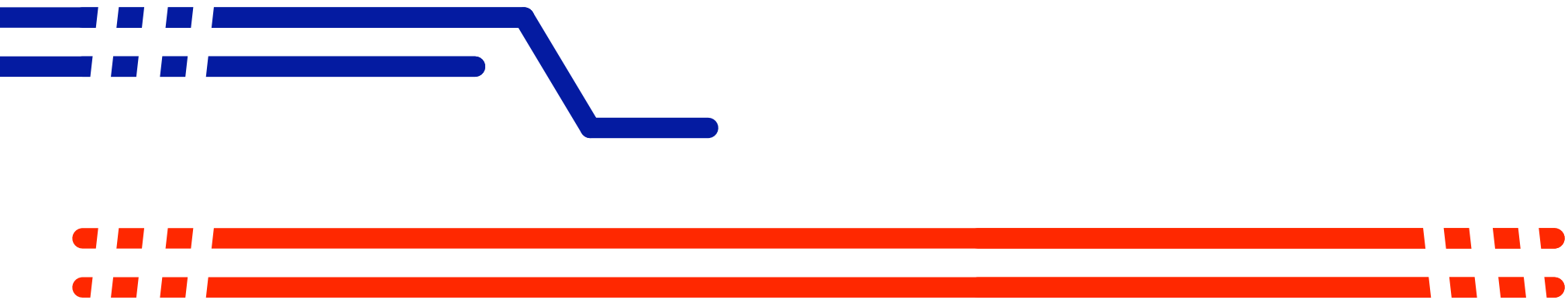
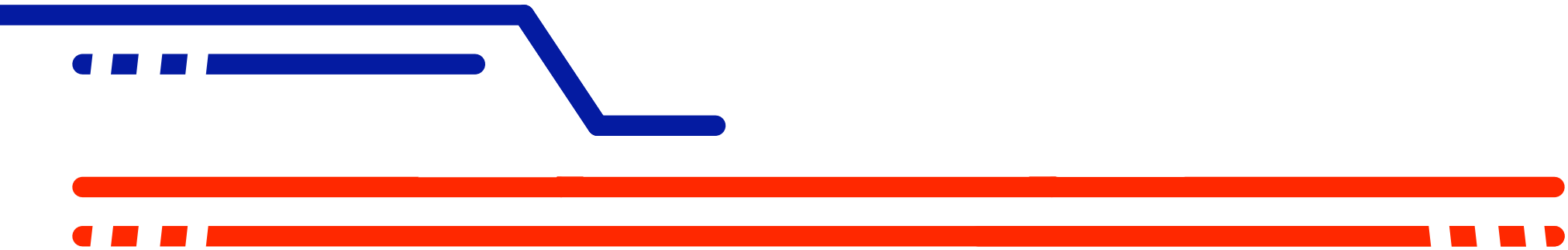
Double Stranded DNA

Sister Chromatid

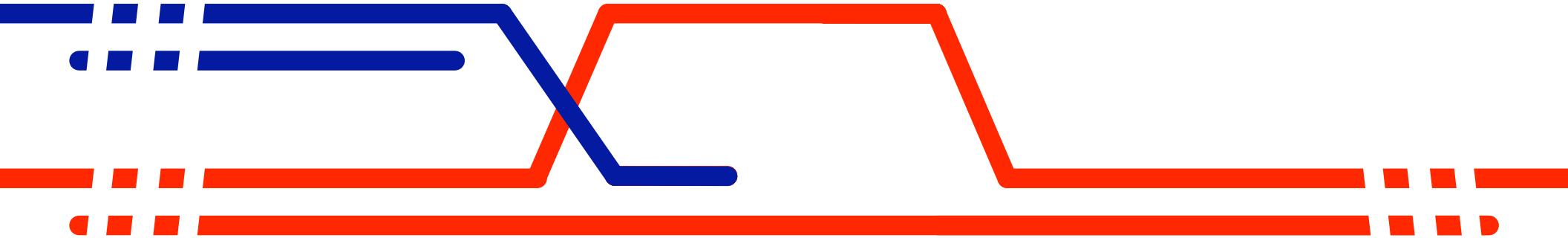

Double Stranded DNA
Damage
Double Strand Break
Resect to form 3’ overhang



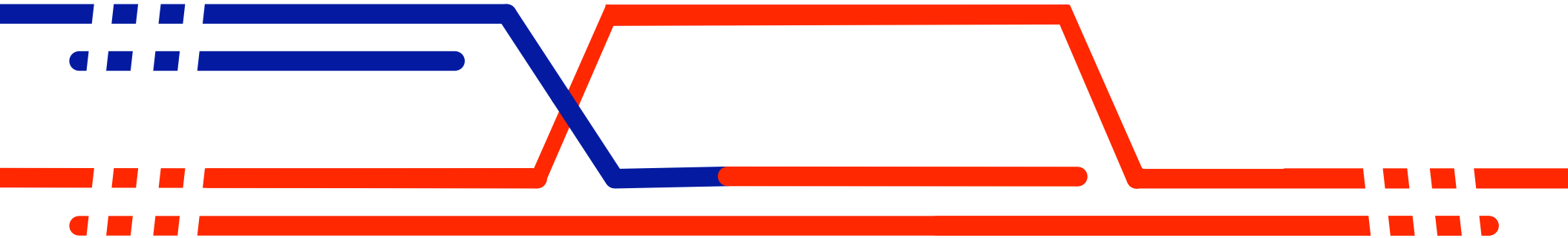
Find Homology



Form D-loop



Polymerize DNA using invading strand with 3’OH as a primer and the homologous donor DNA as a template

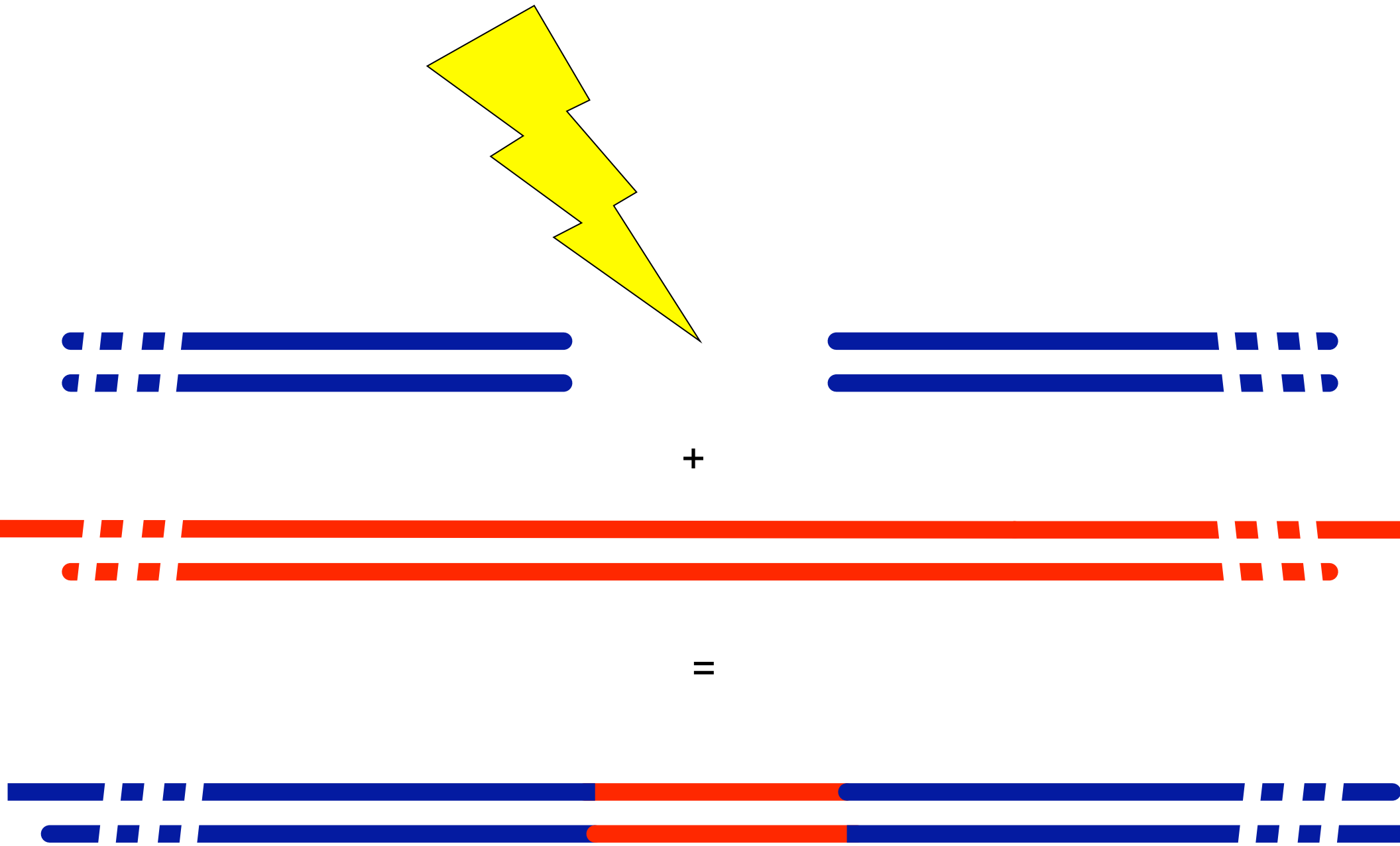


Branch Migration

Bring extended ends together

Single Strand Annealing

Fill in gaps



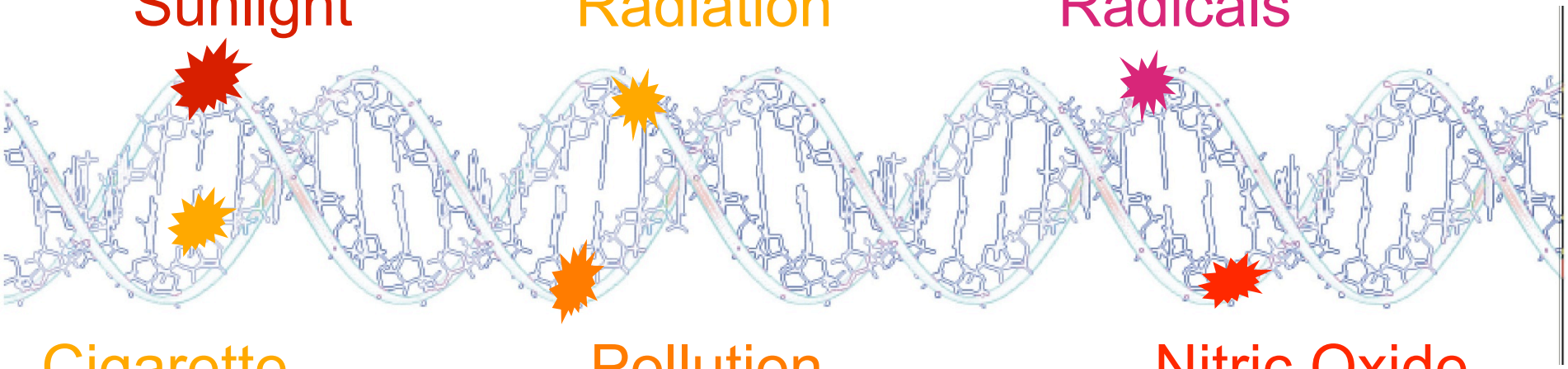
See SDSA
Prototypic Model
& Replication Fork

Animations by Justin Lo

Sunlight

Radiation

Oxidative Radicals



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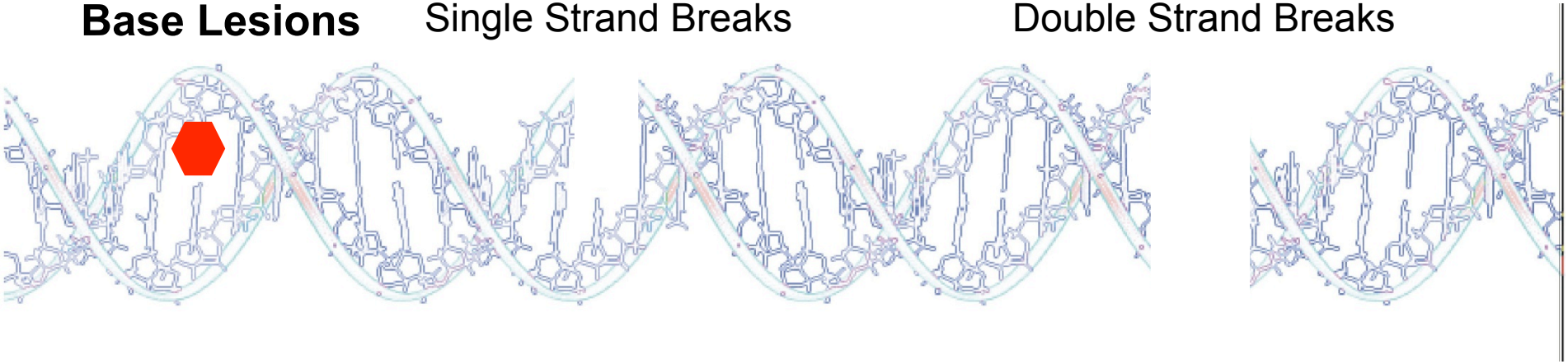
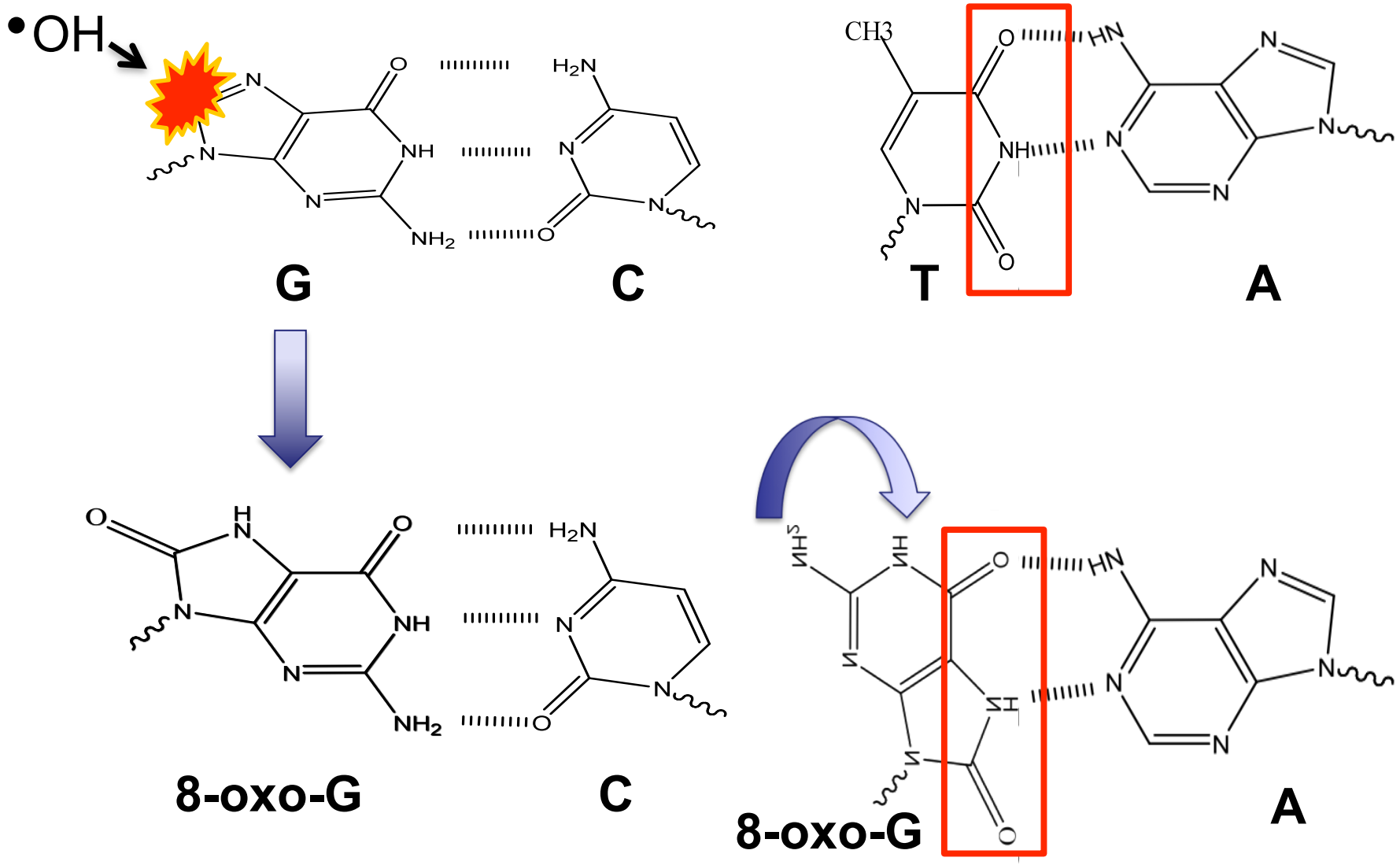



•OH
G
C
T
A
8-oxo-G
C
8-oxo-G
A
GT -> AT

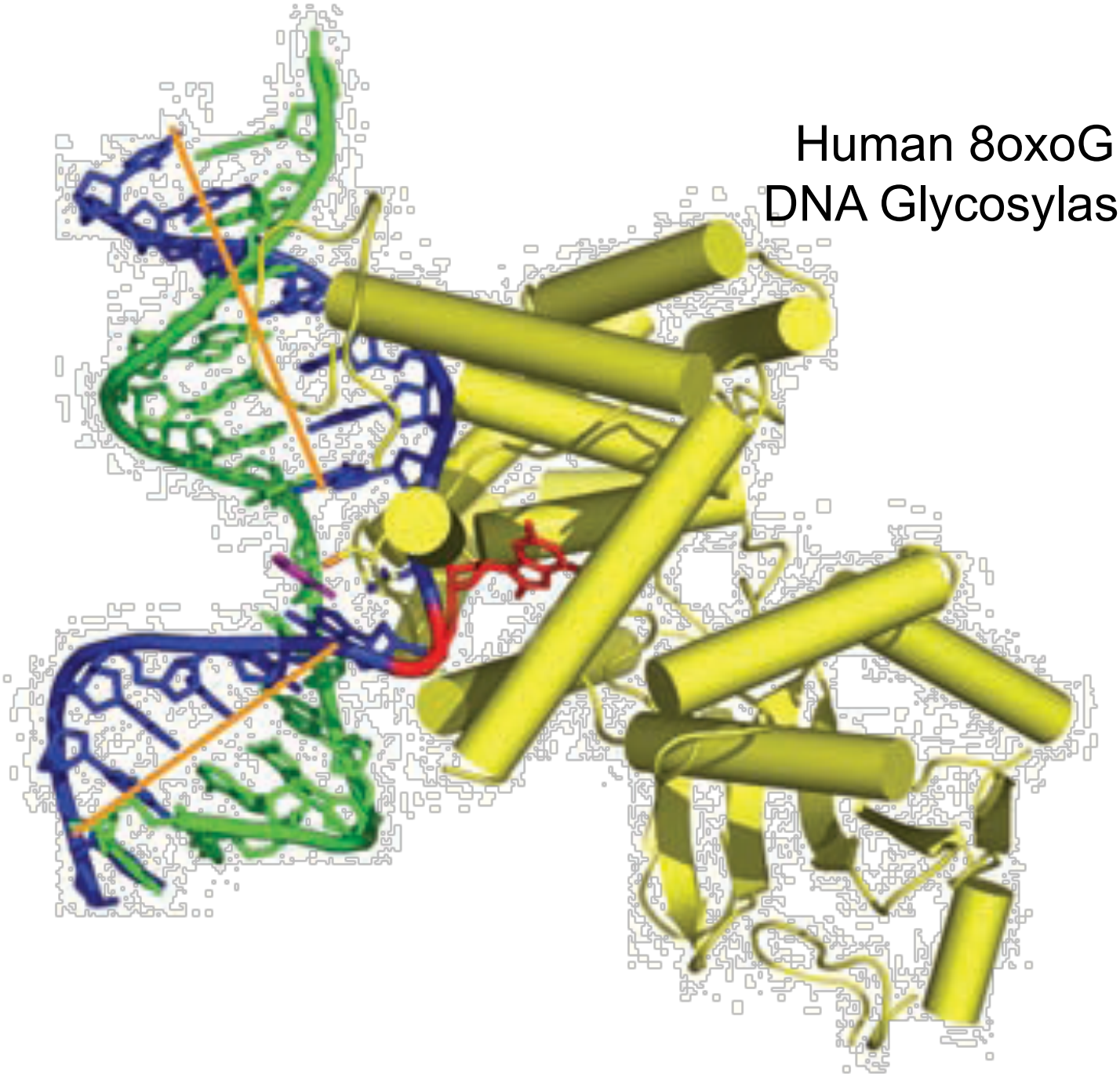
Does this damage matter?

- What can the cell do?

  Apoptose Necrose Nothing **Fix it**

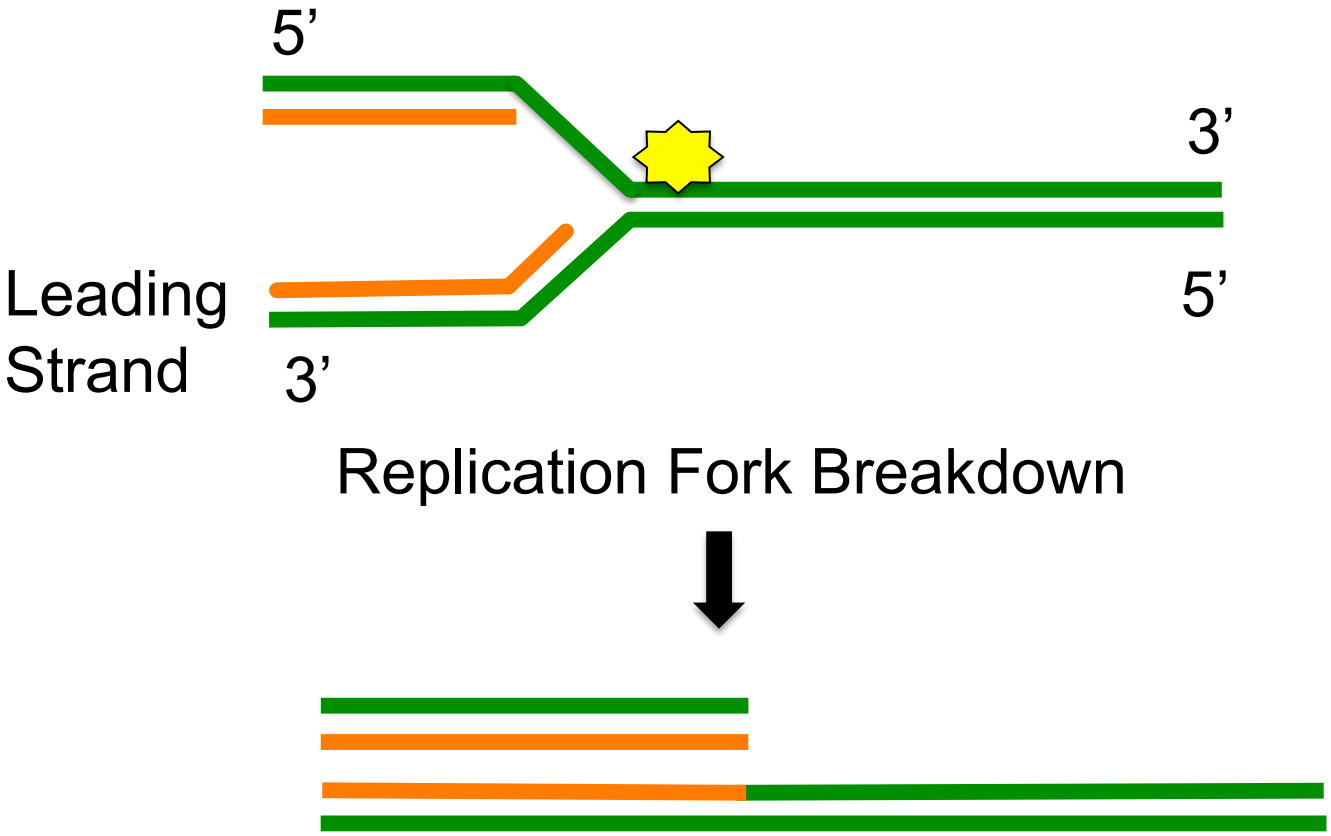
- If the cell is not dividing?

- If the cell is dividing?


Human 8oxoG
DNA Glycosylase

Dangers of Bulky Damage

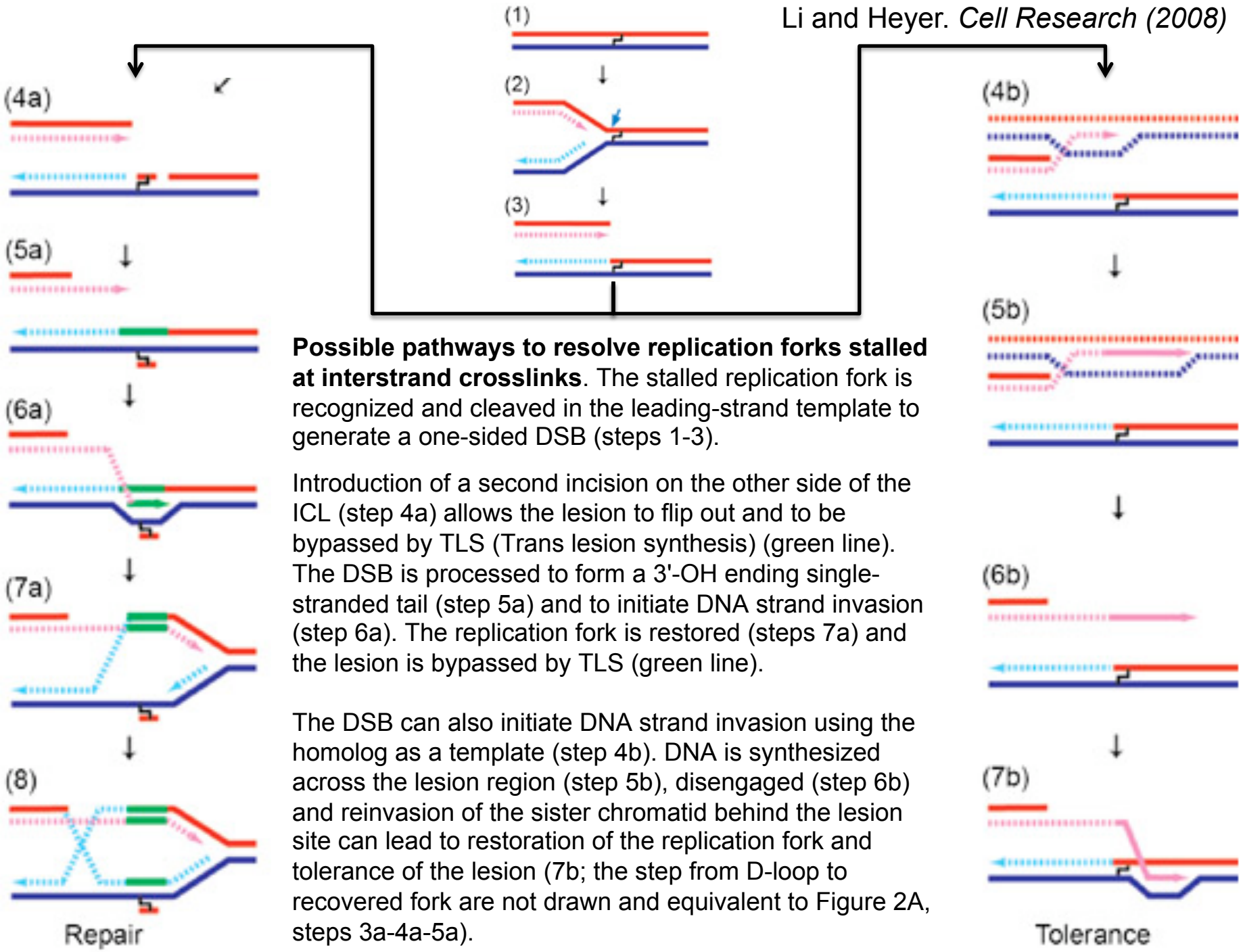
- Polymerase is unable to bypass lesions
- This leads to replication fork breakdown



Homologous Recombination Videos

- http://web.mit.edu/engelward-lab/animations/forkHR.html

Li and Heyer. *Cell Research (2008)*



Possible pathways to resolve replication forks stalled at interstrand crosslinks. The stalled replication fork is recognized and cleaved in the leading-strand template to generate a one-sided DSB (steps 1-3).

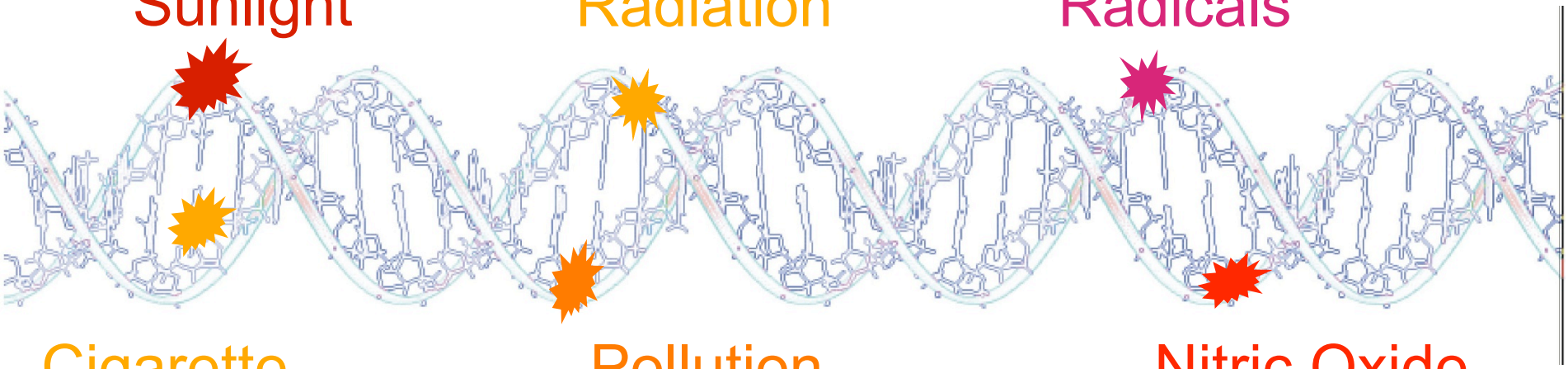
Introduction of a second incision on the other side of the ICL (step 4a) allows the lesion to flip out and to be bypassed by TLS (Trans lesion synthesis) (green line). The DSB is processed to form a 3'-OH ending single-stranded tail (step 5a) and to initiate DNA strand invasion (step 6a). The replication fork is restored (steps 7a) and the lesion is bypassed by TLS (green line).

The DSB can also initiate DNA strand invasion using the homolog as a template (step 4b). DNA is synthesized across the lesion region (step 5b), disengaged (step 6b) and reinvasion of the sister chromatid behind the lesion site can lead to restoration of the replication fork and tolerance of the lesion (7b; the step from D-loop to recovered fork are not drawn and equivalent to Figure 2A, steps 3a-4a-5a).

Sunlight

Radiation

Oxidative Radicals



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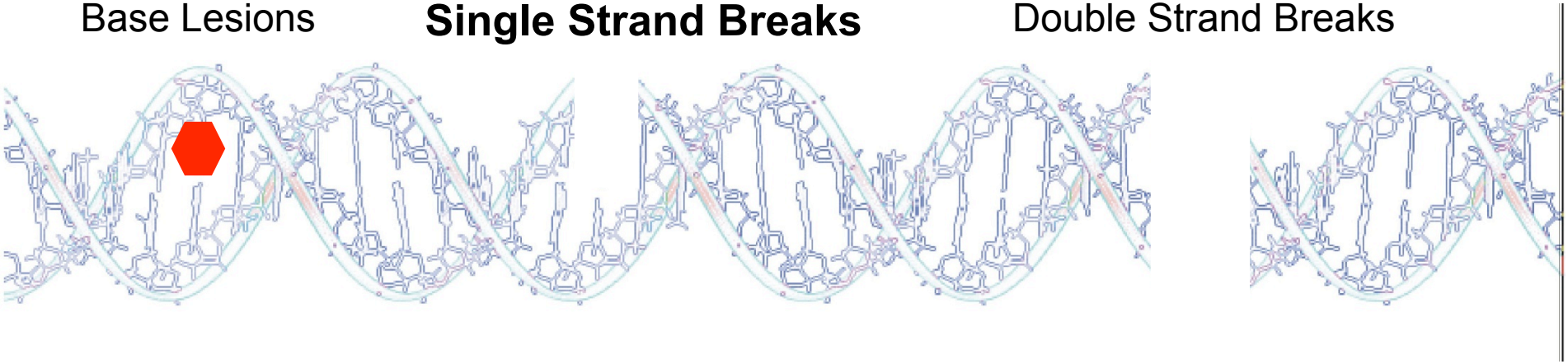
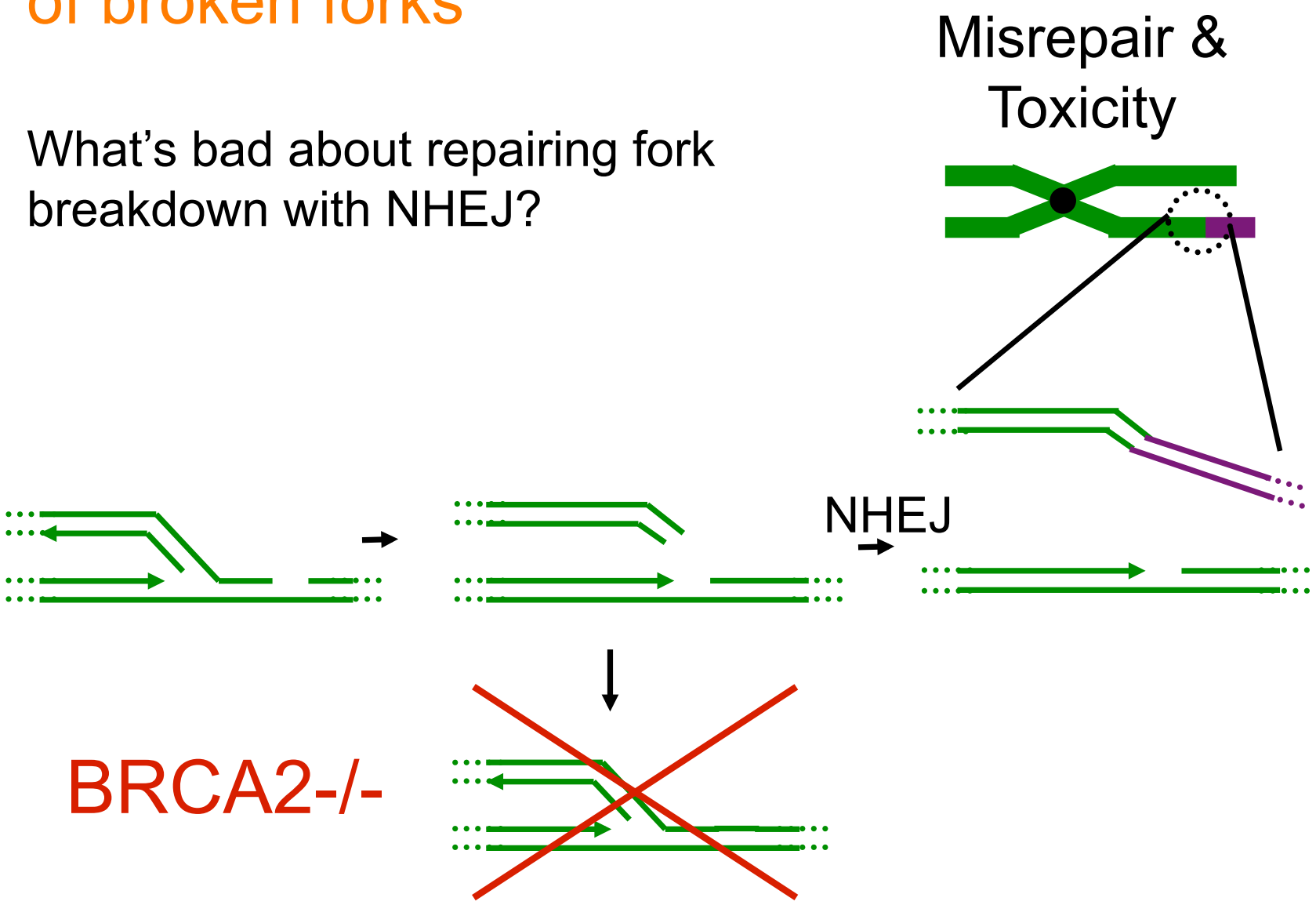
Double Strand Breaks



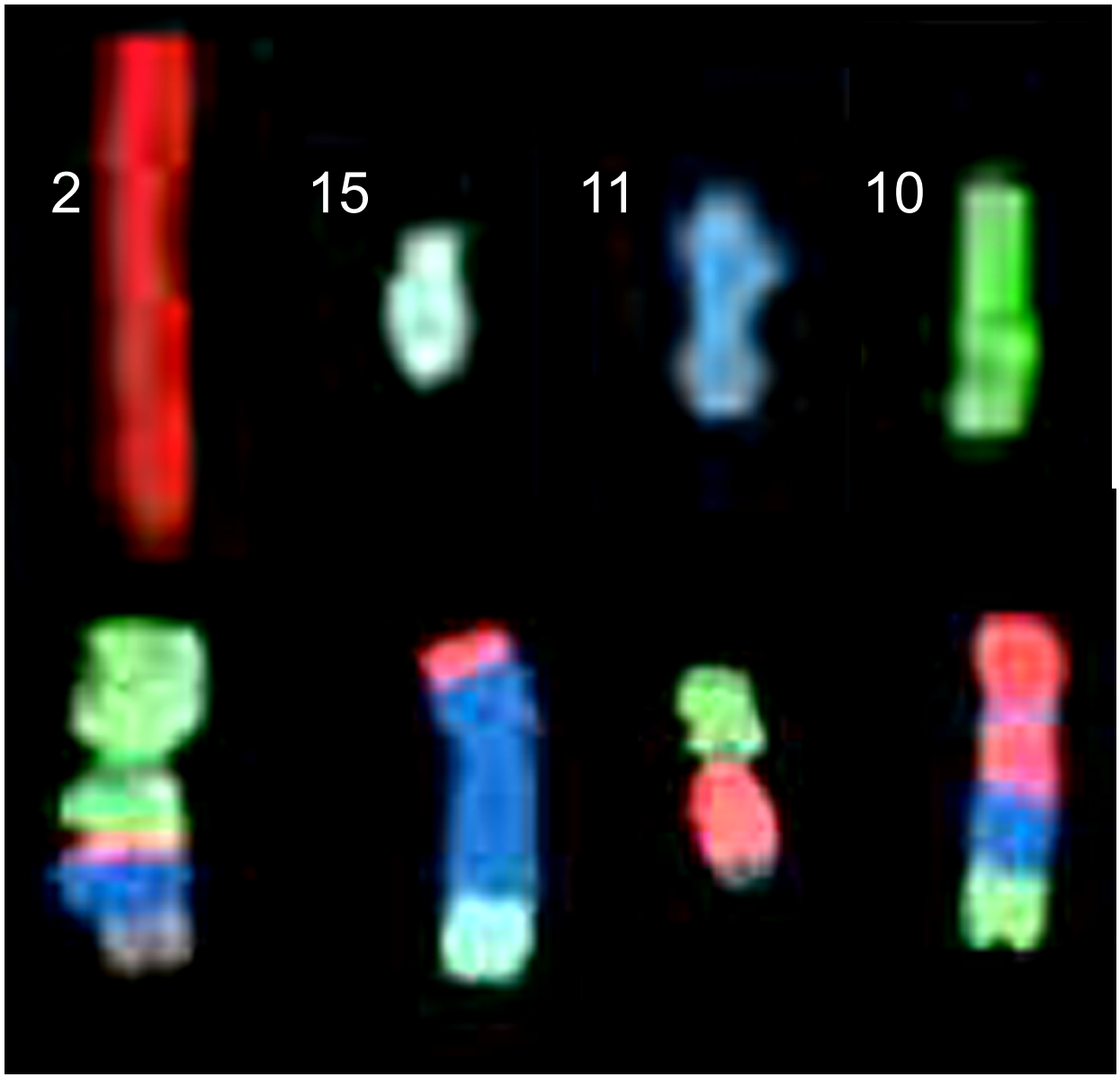
BRCA2 is critical for repair of broken forks

What's bad about repairing fork breakdown with NHEJ?

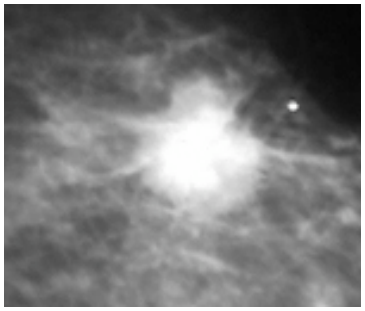


Misrepair

Normal Human Chromosomes



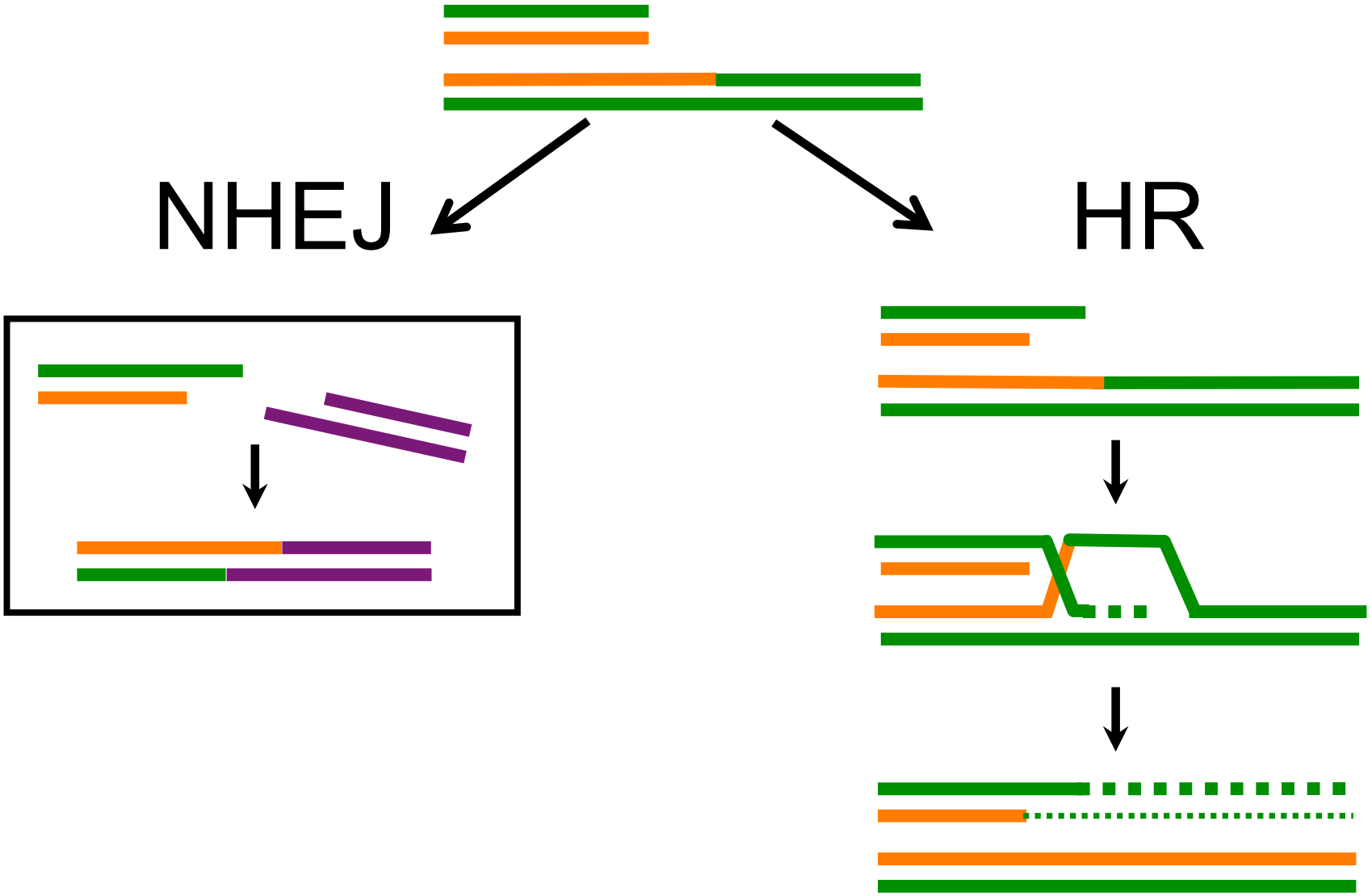
BRCA2 -/- Chromosomes



www.rctradiology.com

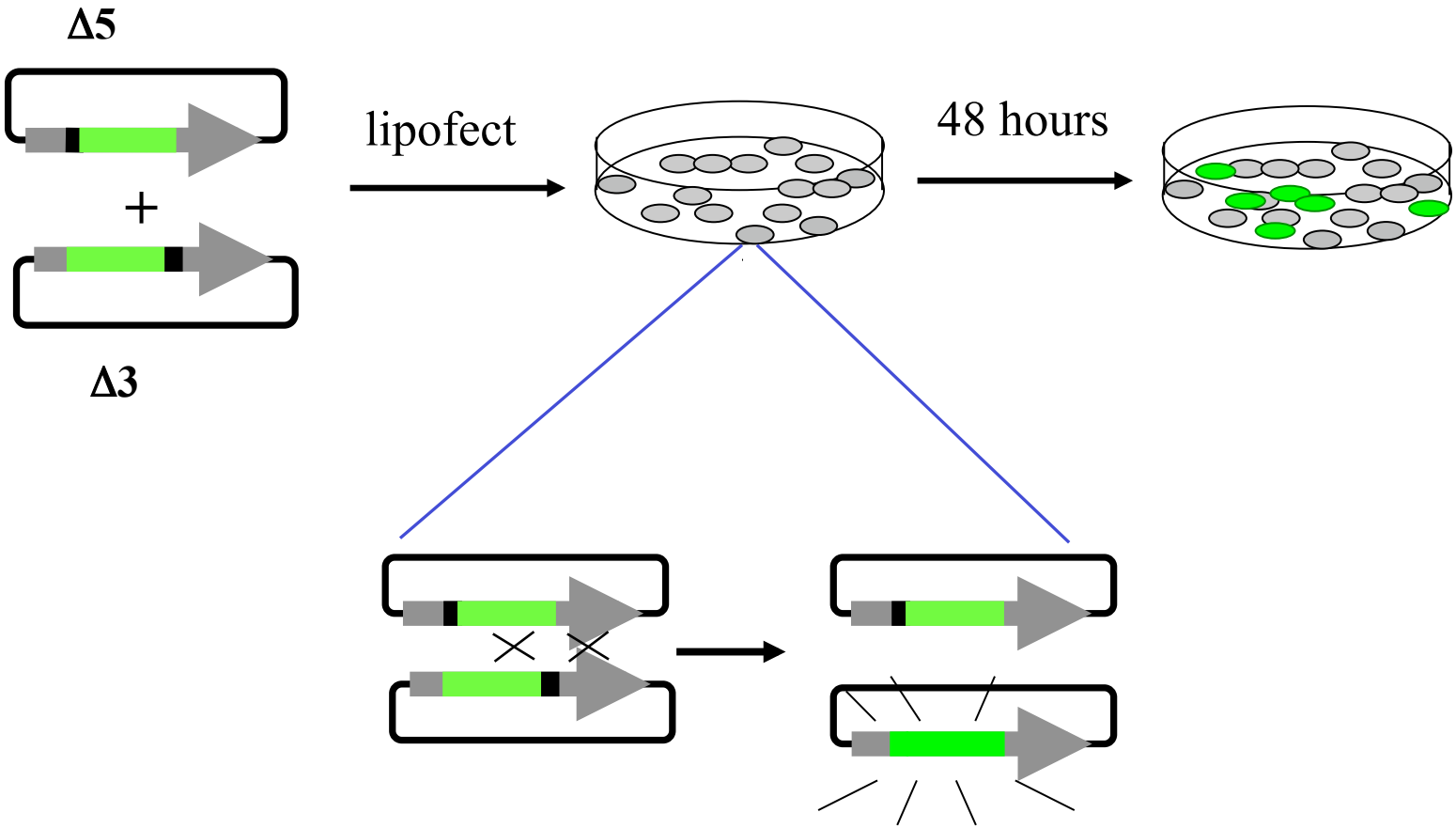
Raw data from Grigorova *et al*., Cytogen. and Gen. Res. 104:333-340 (2004)

Broken Fork Repair



Your Assay for Homologous Recombination

A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells



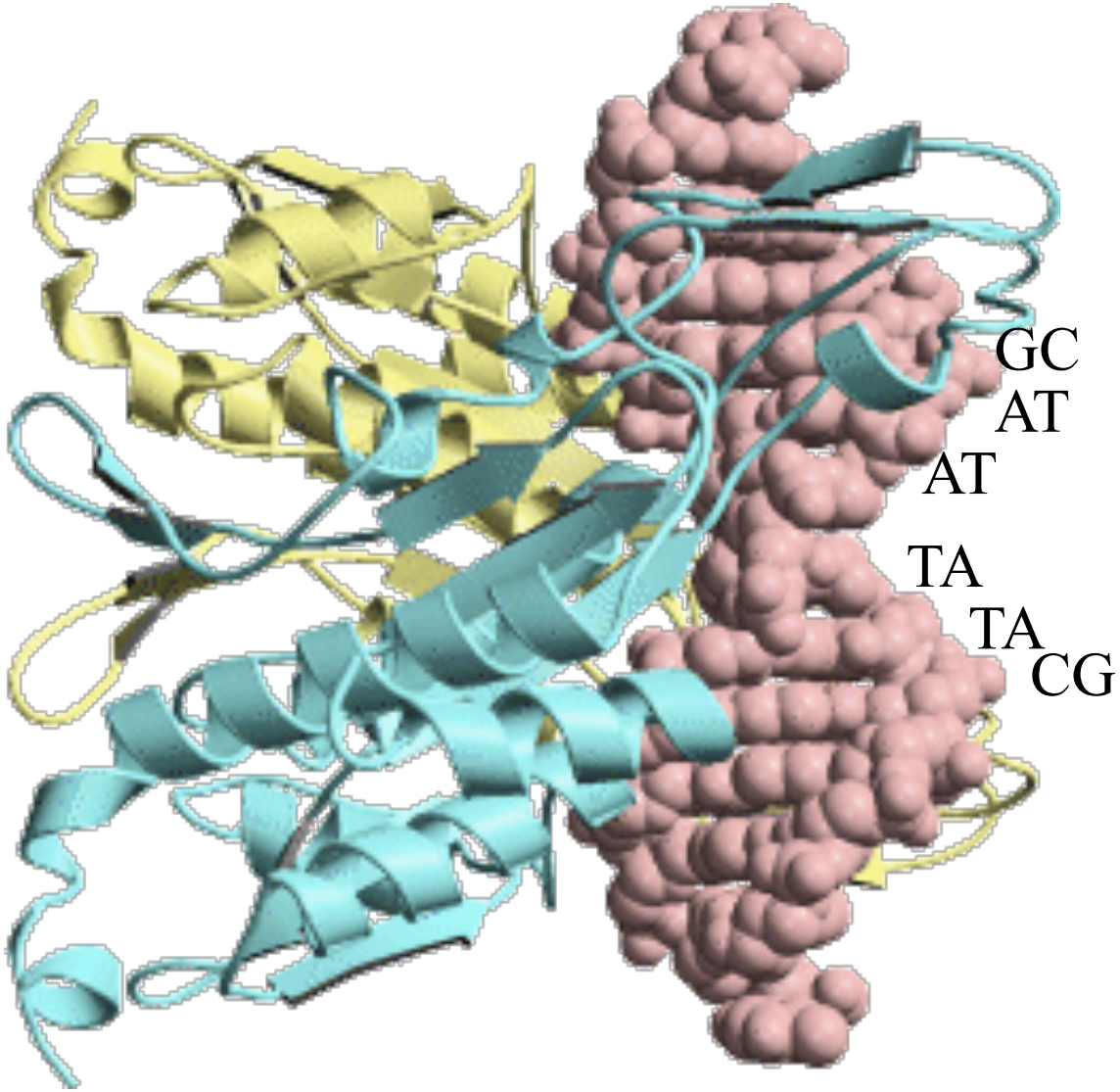
- **About the experiments in Mod1**
  - How does DNA damage cause mutations?
  - How is recombination used to fix double strand breaks?
  - Overview of the experiments you will be doing
- **Restriction Enzymes**
  - Basics restriction enzymes
- **Anticipating Potential Problems & Pitfalls**
  - What controls are needed and why?

Overview of the Experiments in Mod1

Where you are,
and where you are going

Restriction Enzymes

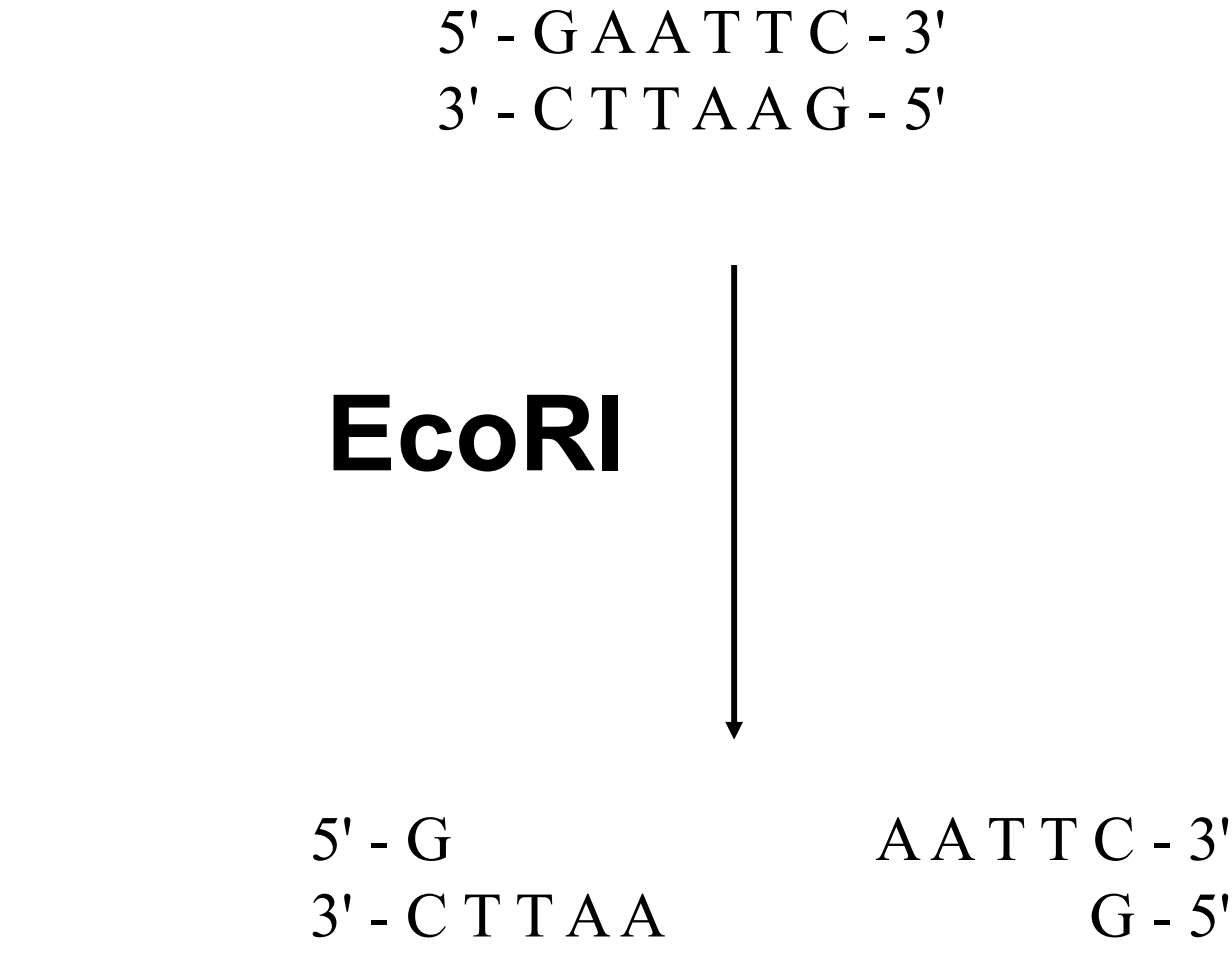
- Where they come from?
  - Bacteria
- What do they do?
  - Chop up the DNA of infecting phages
- How do cells protect themselves?
- How can we use them?



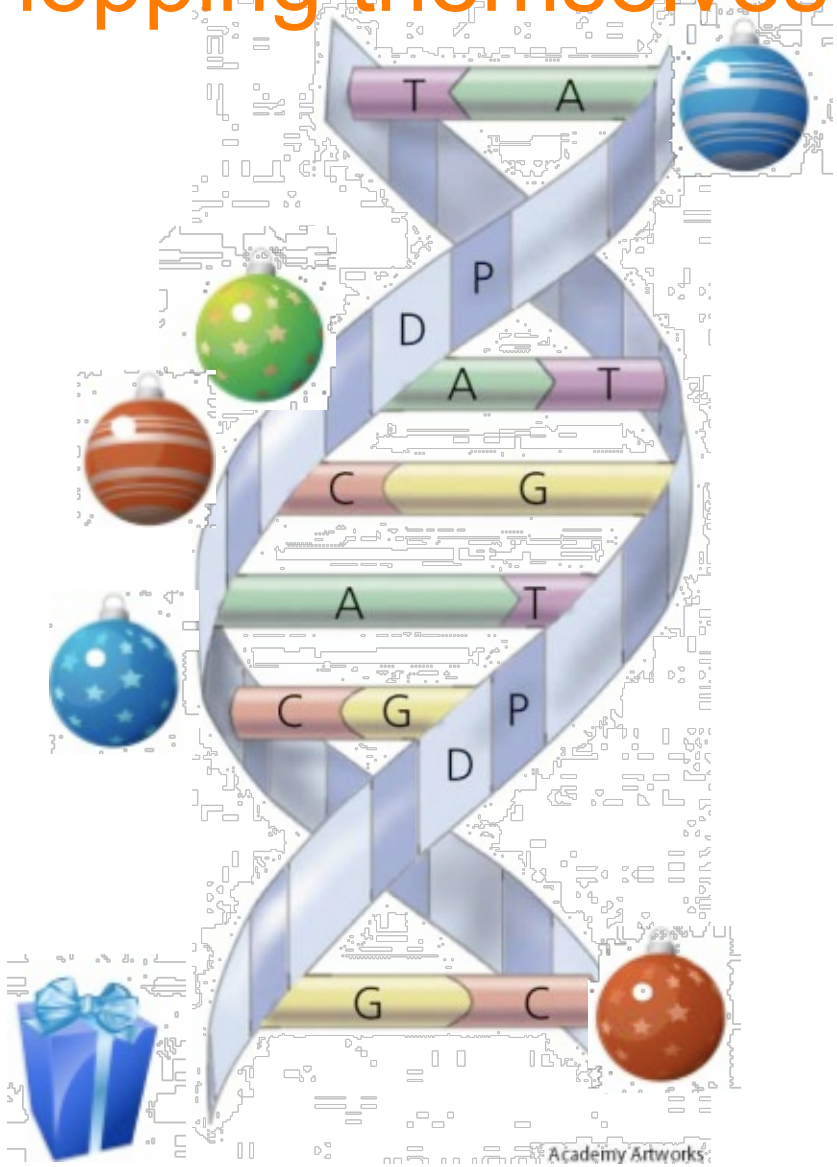
5' - G A A T T C - 3'
3' - C T T A A G - 5'

EcoRI

Image from: Rosenberg, J. M. Curr. Opin. Struct. Biol. 1: 104-110 (1991)


5' - G A A T T C - 3'
3' - C T T A A G - 5'
EcoRI
5' - G
3' - C T T A A
A A T T C - 3'
G - 5'

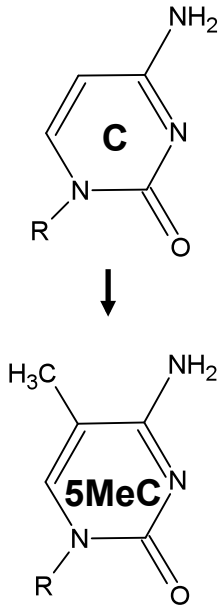
How do bugs keep from chopping themselves up?



“Cognate Methyltransferases”

M.HaeIII

5′–GG**C**C–3′
3′–C**C**GG–5′



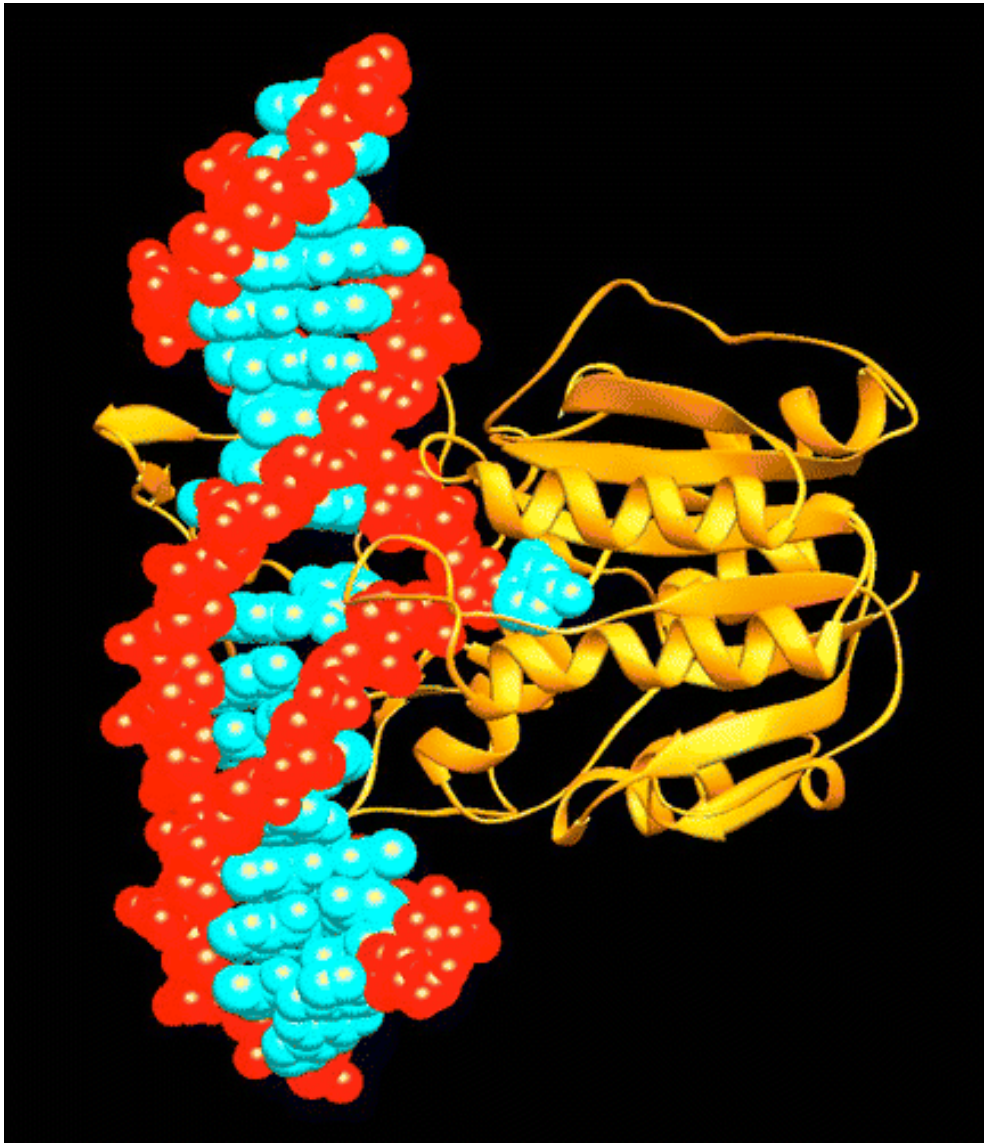


Figure from R. Roberts, Annual Review of Biochemistry (1998) 67: 181-198.

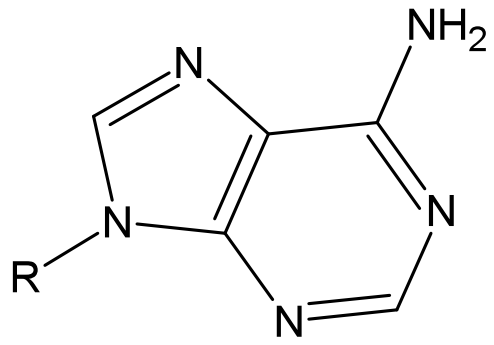
Who's Who?

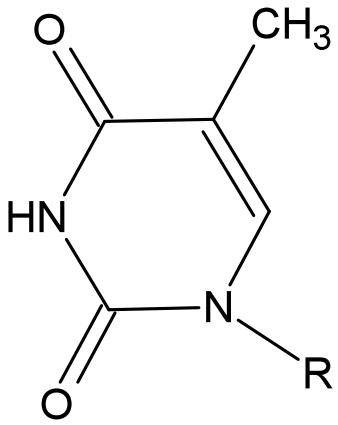
Adenine

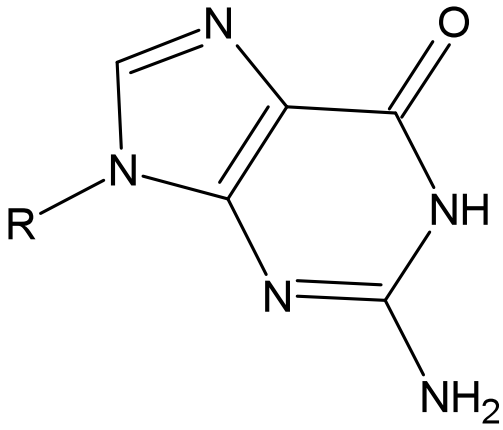
Guanine

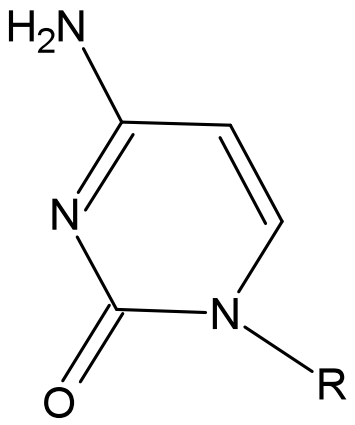
Thymine

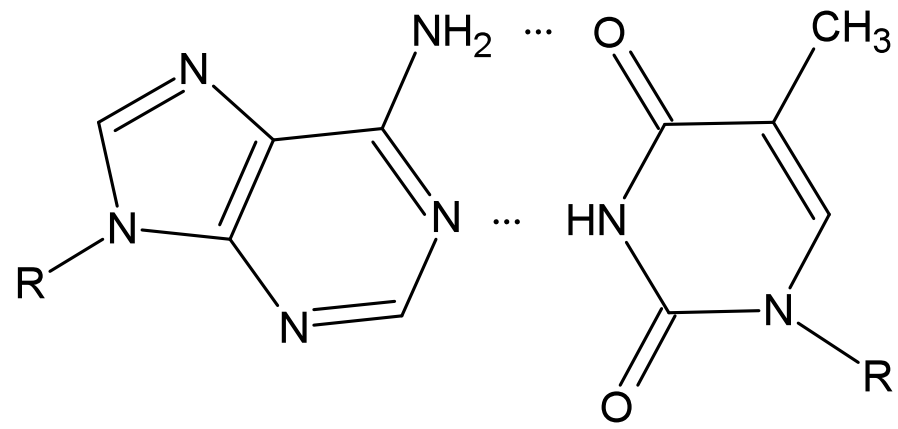
Cytosine



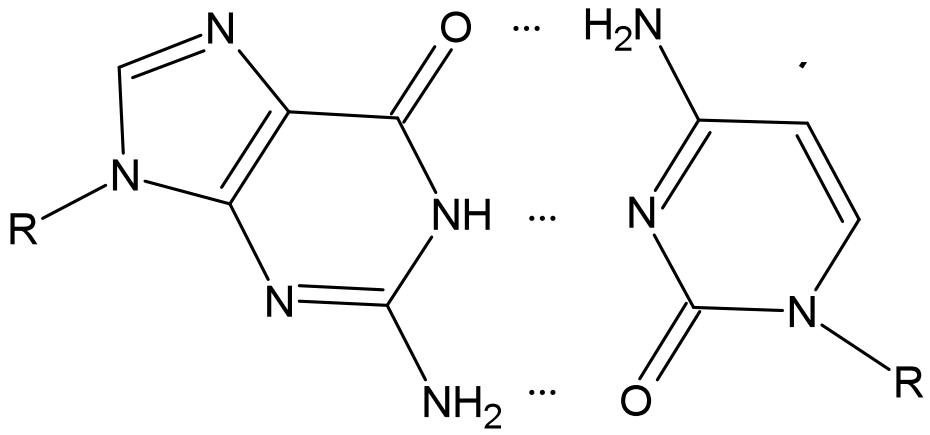






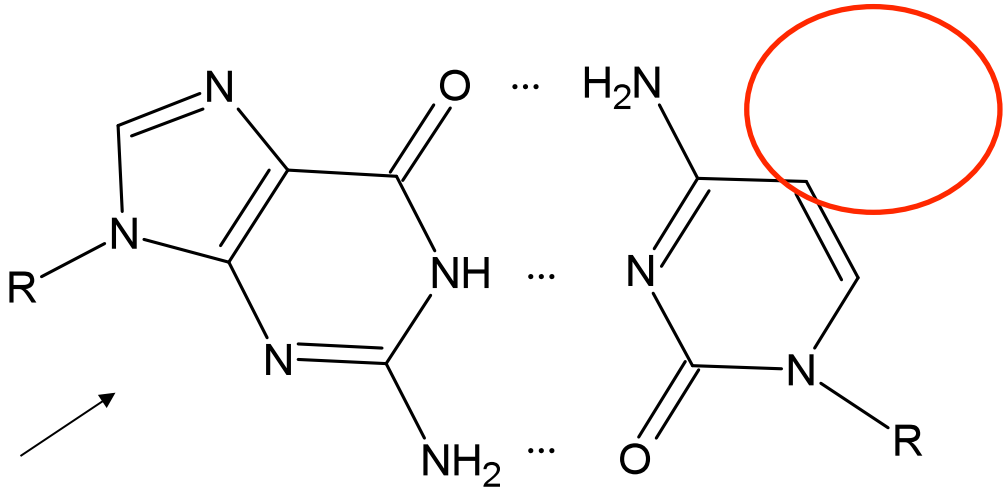


A:T

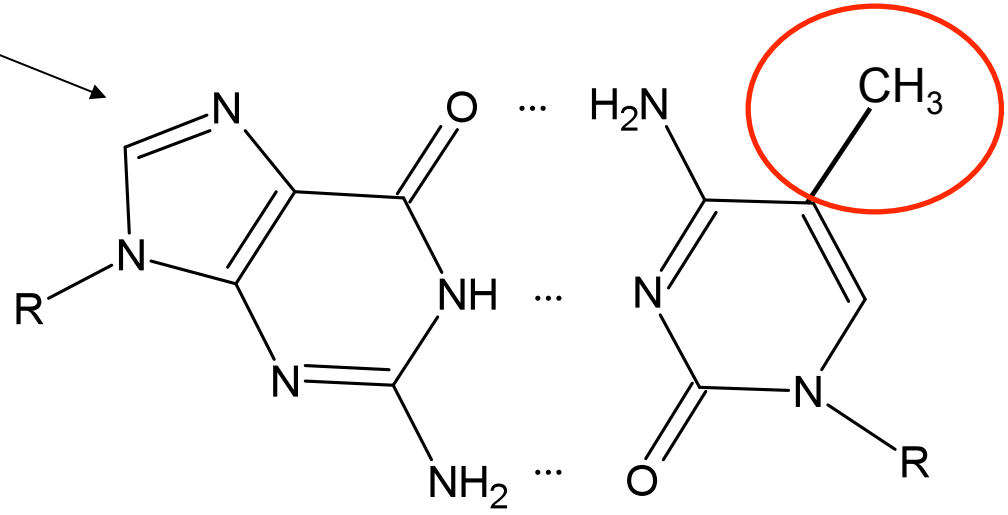


G:C

Proper G:C Base Pairs

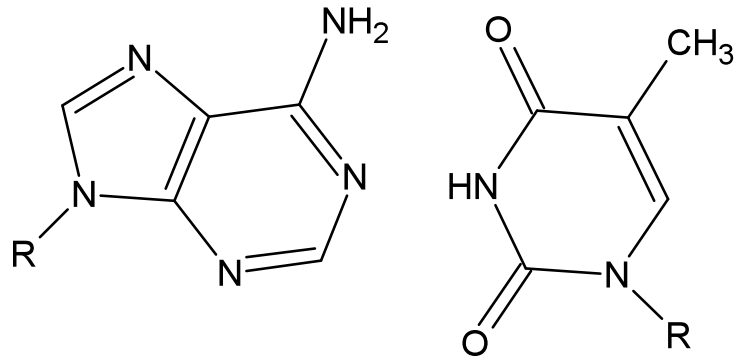


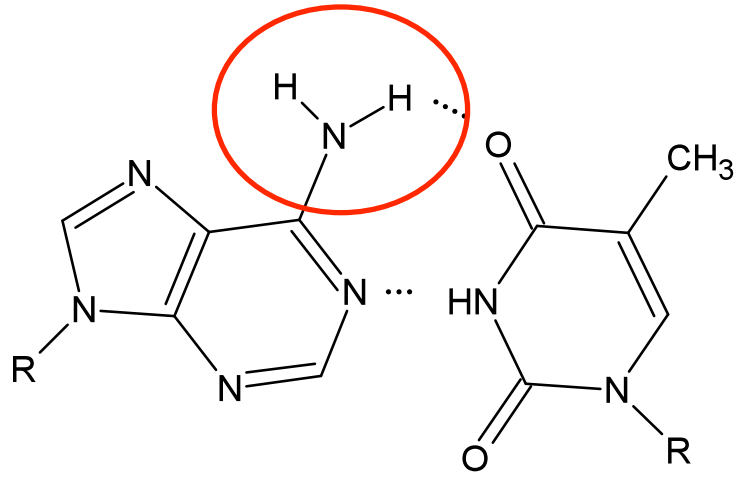
G:C



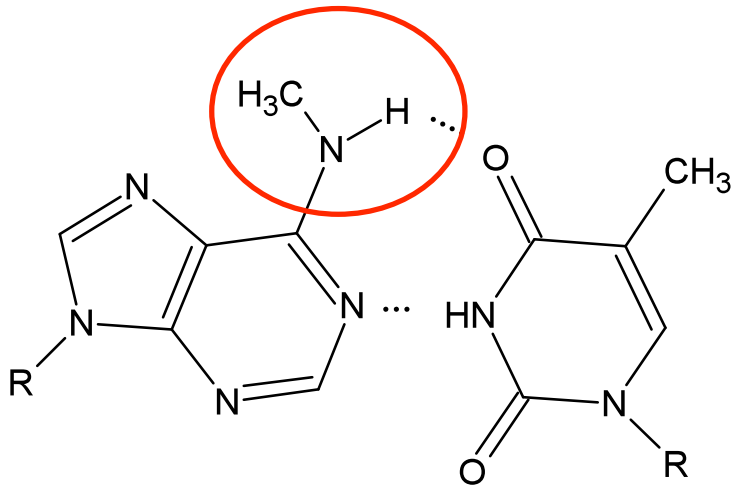
G:5MeC

Proper
A:T Base
Pairs





6MeA



On a practical level…
Using Restriction Enzymes

-Different kinds of restriction enzymes (blunt/distal)
-Shared recognition sequences
-Shared overhangs

-Buffer conditions
-Storing and diluting your restriction enzymes
-Specificity (potential pitfalls!)
-Lack of activity (host cells & potential pitfalls)

Double check you're using the enzyme you think you are using!

Construction of the Δ5 Plasmid

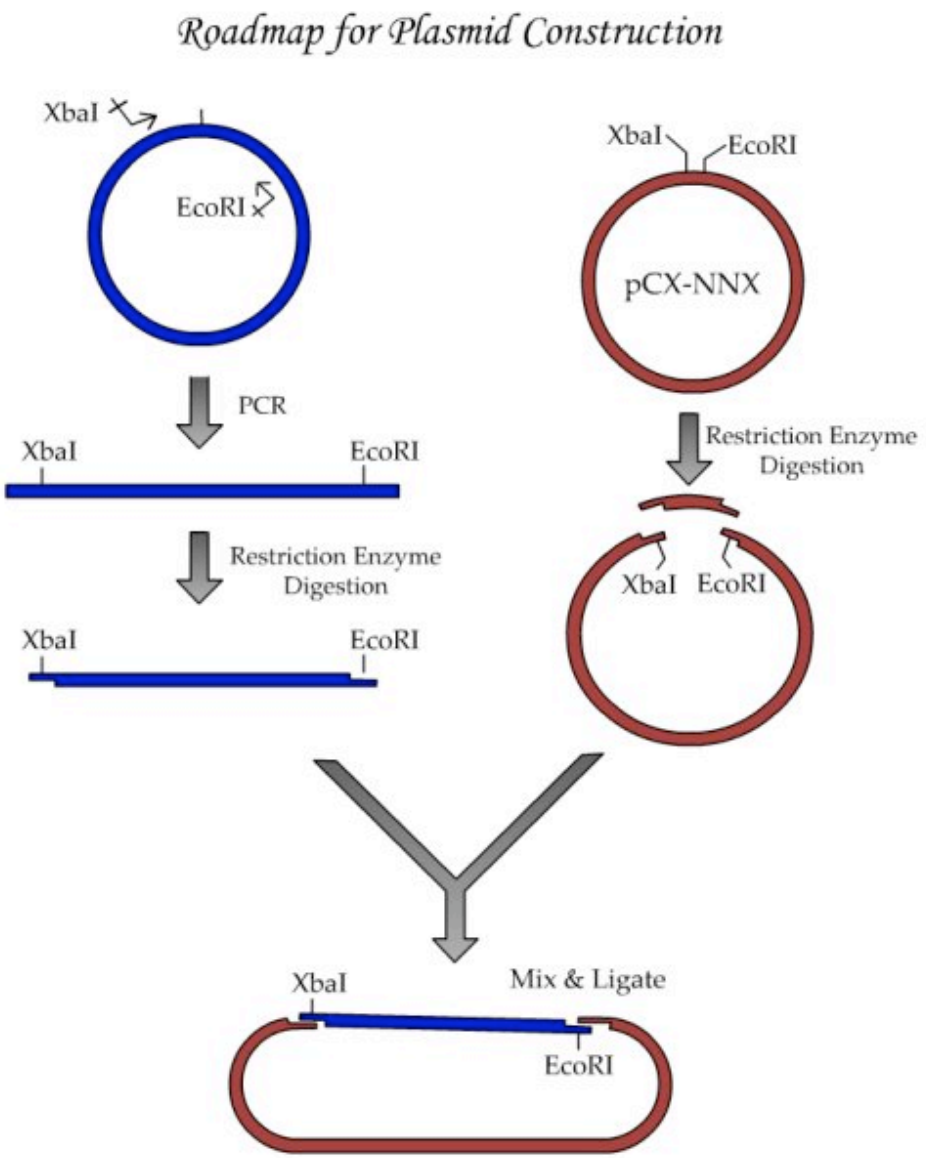


Figure by Justin Lo

Anticipating Problems & Pitfalls:

What might go wrong in your experiment?

Incomplete Reactions

Controls: How can you tell if your DNA has actually been cut?

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