Lecture 5
Immunology, Enzyme Inhibitors, Gene Editing

To do:
- Presentation topic for approval (ASAP)
- Draft slides by Sept 17th for feedback (extended deadline).

B-Cell Biology - From Stem Cells to Pathogen Killing.

1. Generation of high diversity of chains during development of stem cells to B-cells in bone marrow.

- DNA rearrangements to generate functional exons for variable segments of both light and heavy chain.

2. Molecular & cellular biology of membrane bound antibodies on cell surface = B-cell receptor (BCR)

- Transcriptional enhancers, mRNA splicing
- Light chain and heavy chain exported to surface of B-cells.

3. Self tolerance test to prevent autoimmune diseases, autoreactive B-cells eliminated.

4. Encounter and capture of antigen in lymph nodes

5. Activation of B-cells by T_H cells

- Peptides from pathogen presented on major histocompatibility proteins (MHC II).
- T-cell activation by tyrosine kinase receptors (T-cell Receptor, TCR), secretion of signaling molecules.

6. Development of

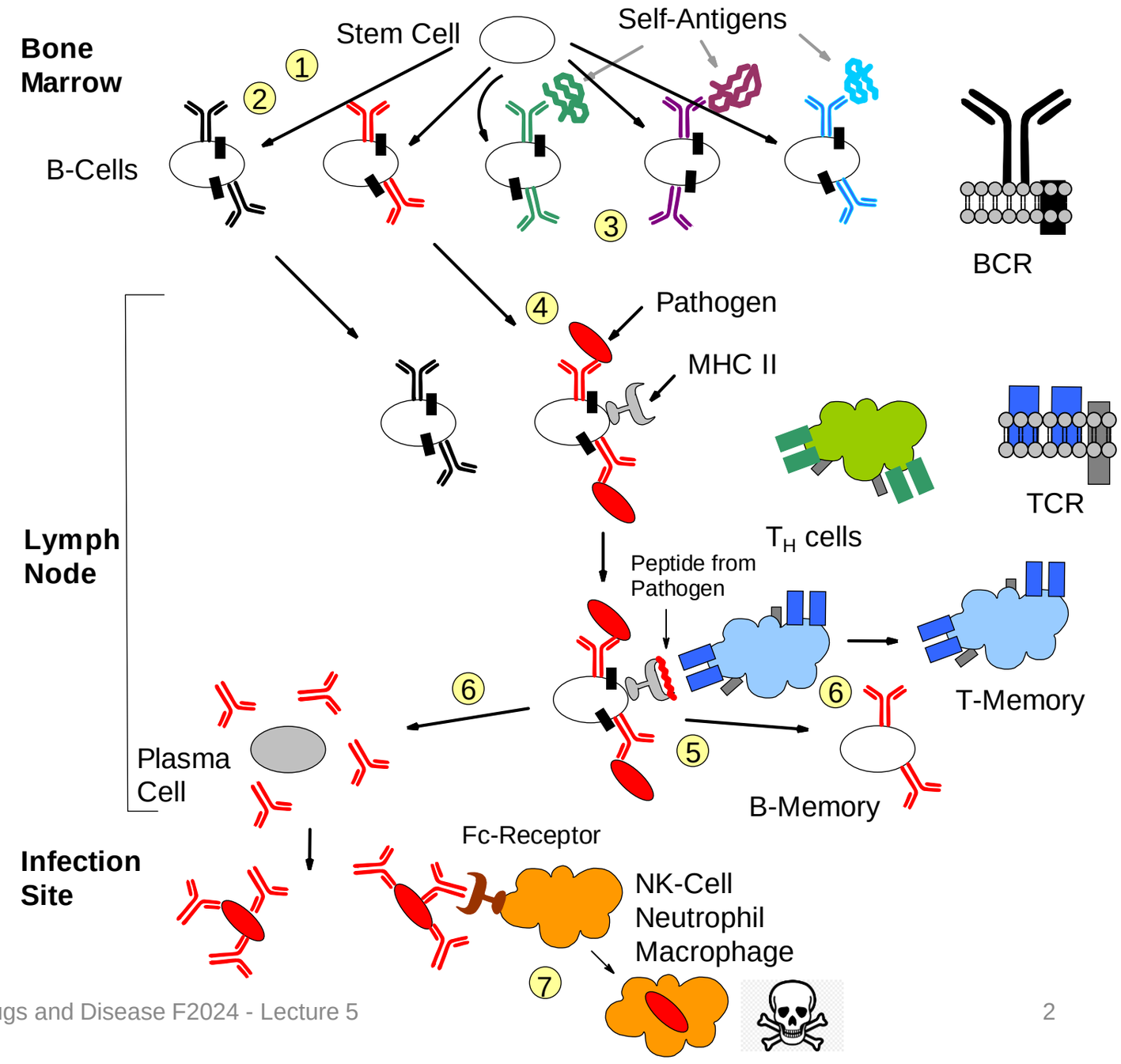
- Plasma cells - Production of soluble antibodies of the same specificity as the parent B-cell.
- B-memory cells (basis of immunity)
- T-memory cells (basis of immunity)

7. Destruction of Pathogens

- Fc region of antibody binds to Fc Receptor on NK cells, neutrophiles, macrophages
- Pathogen internalized and destroyed.

BCR – B-cell receptor = antibody + signaling chains.

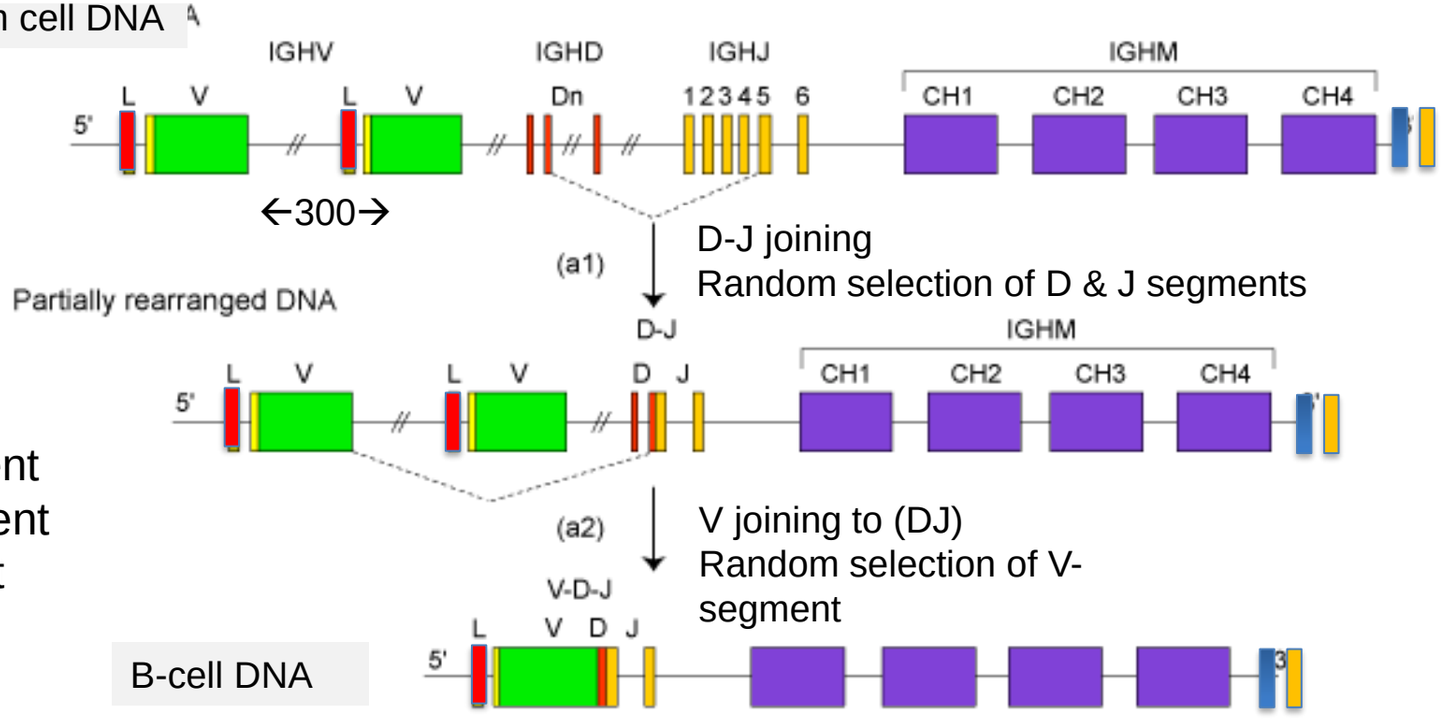
TCR – T cell receptor = MHC-peptide recognition + signaling.



Antibody Genes are Assembled From DNA Segments: Giving many different sequences.

Production of Heavy Chain Gene:

V_H exon = V+D+J segment (selected at random)

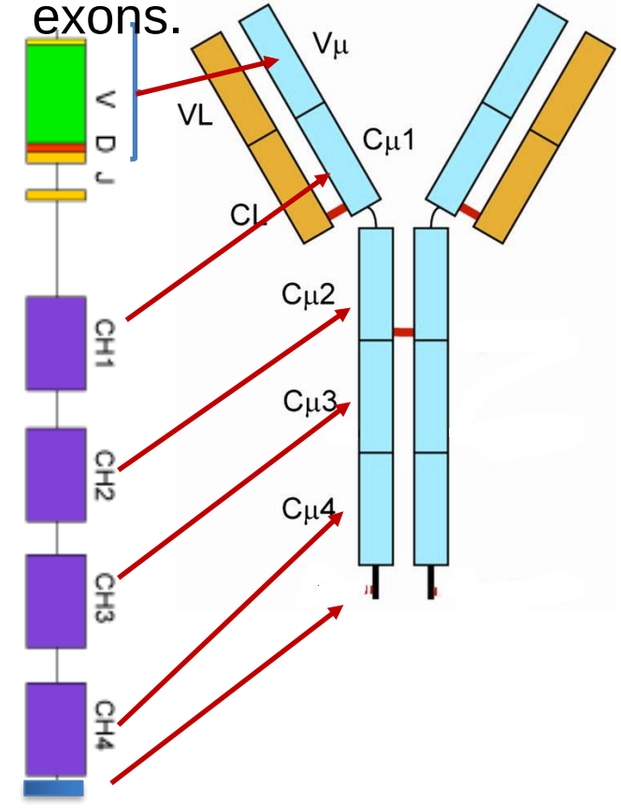


L = leader (signal)
V = variable segment
D = diversity segment
J = joining segment

V_H exon differs from one cell to the next.

Heavy chains have the same constant sequences in all B-cells.

The mRNA coding for antibodies contains 5 exons.



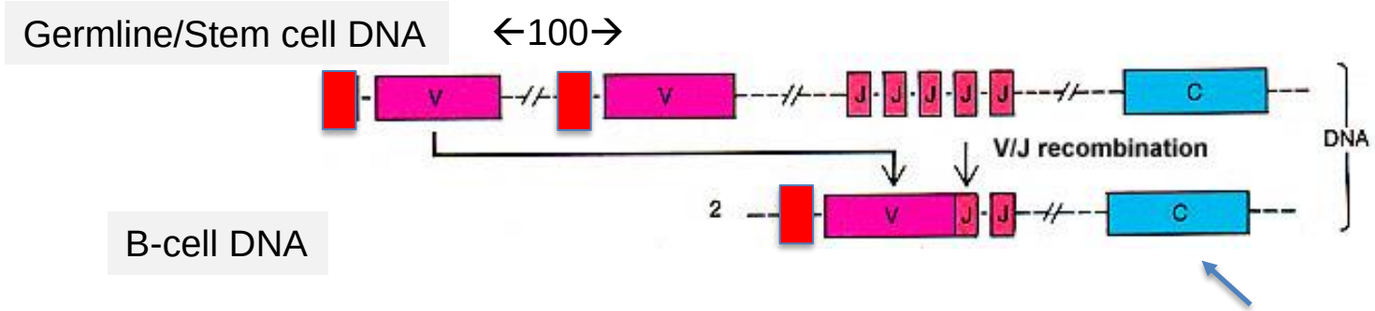
Stem cells -> B-cells

- The exon that codes for the variable region of the heavy chain is generated by the random joining of a V, D, and J DNA segments.
- Each B-cell will generate a unique sequence for its heavy and light chain DNA.
- This is a permanent change to the DNA (***genome)*** of the B-cell.

1. If there are 300 possible V-heavy segments, 10 possible D segments, and 6 possible J segments, how many different heavy chains can be made?

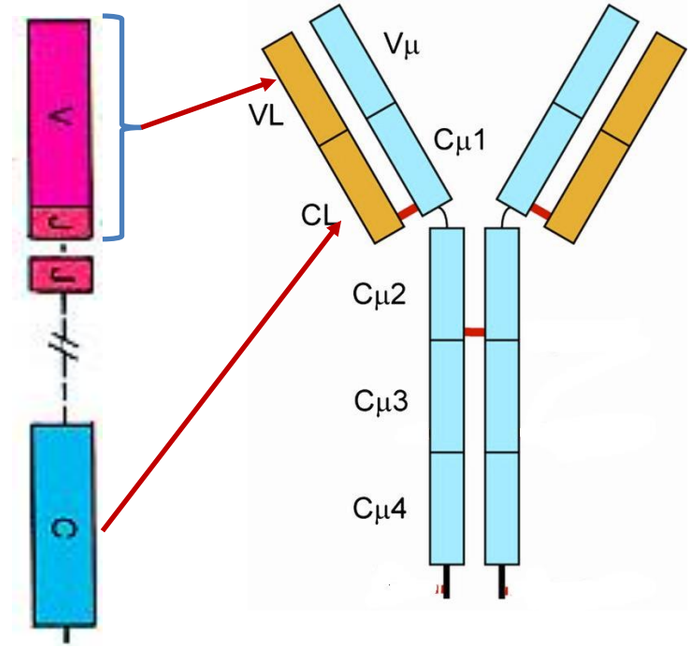
Light-chain Genes are Assembled From DNA Segments: Giving many different sequences.

Production of Light Chain Gene



Light chains have different V-sequences in different B-cells.

Light chains have the same constant sequences in all B-cells.



Antibody Diversity

1. If there are 100 possible V-heavy segments and 5 possible J segments, how many different light chains can be made?

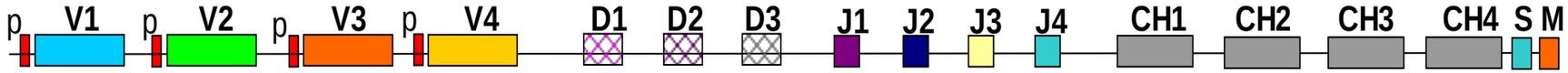
2. If any possible heavy chain can pair with any possible light chain, how many different antibodies can be generated, assuming there are 10,000 possible heavy chains and 500 different light chains?

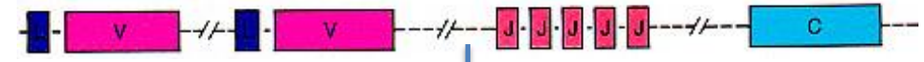
Stem cells -> B-cells

- In the case of the light chain, the variable region is generated by VJ joining.
- Each B-cell will generate a unique sequence for its heavy and light chain DNA.
- This is a permanent change to the DNA (***genome)*** of the B-cell.

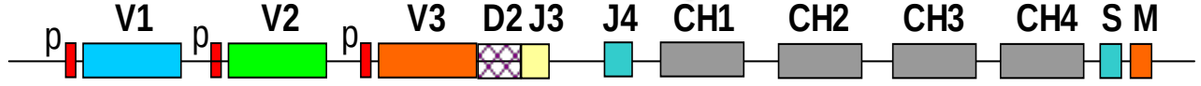
Antibody Production – From Stem Cells to B-Cells

Stem Cell





B-Cell



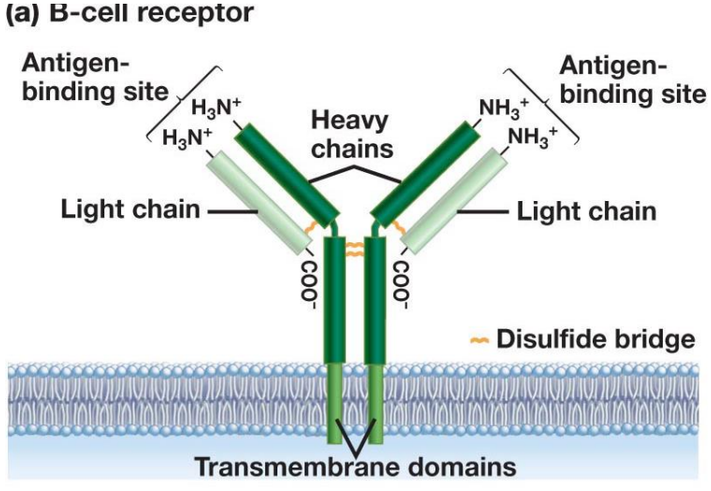
Rearranged heavy chain gene

Rearranged light chain gene

Transcription

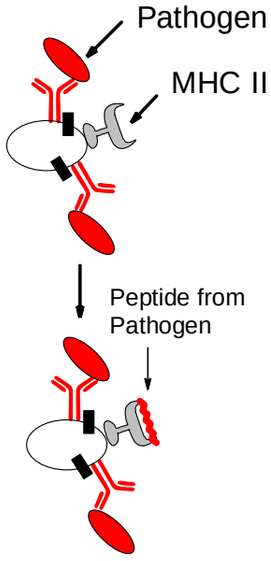
mRNA Splicing

Translation

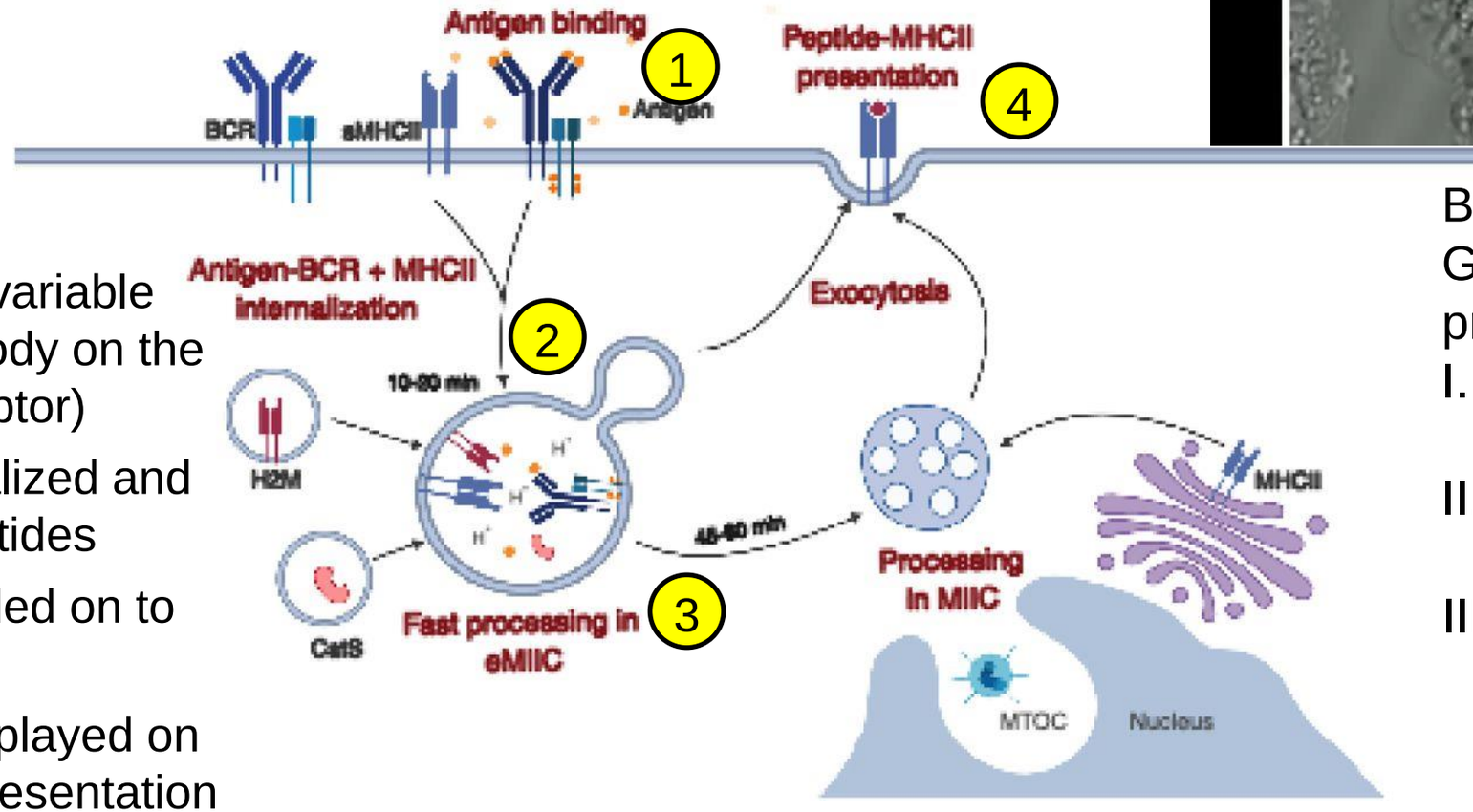


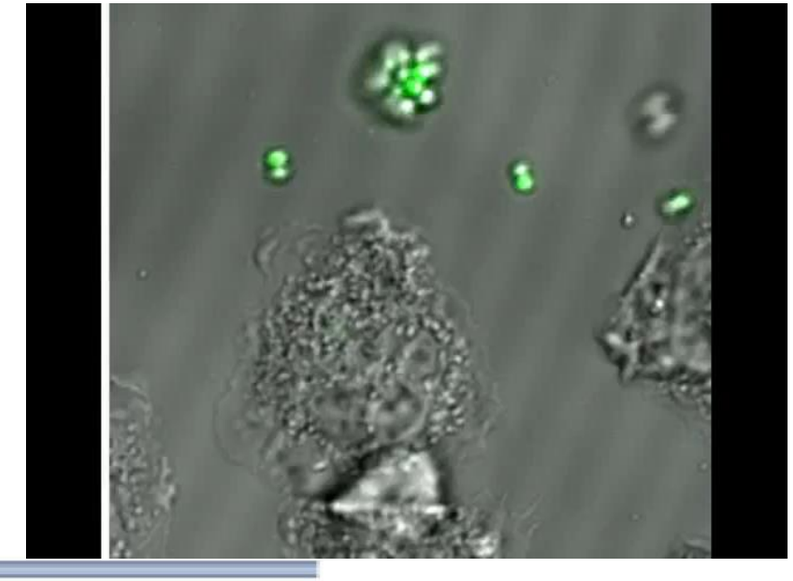
Export to cell surface (ER -> golgi -> Membrane)

Antigen Capture by B-Cells - Endocytic Pathways



Endocytosis of bacteria by a B-cell





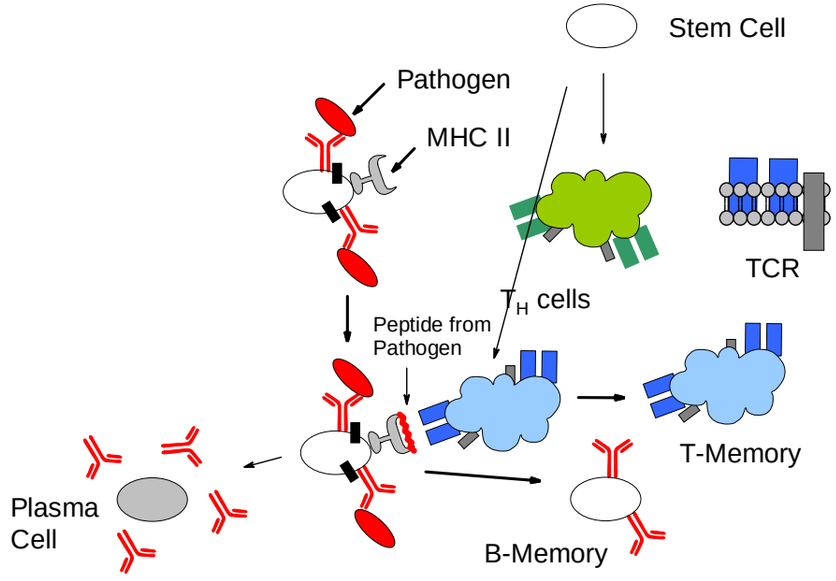
Bacteria labeled with Green fluorescent protein.

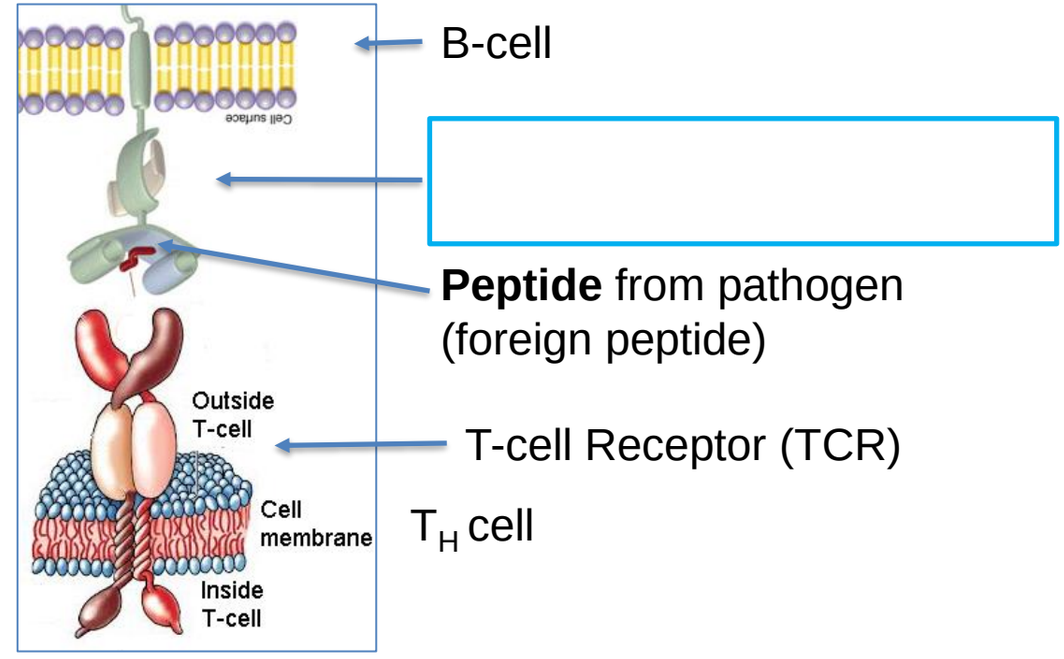
I. Capture of the bacteria
II. Internalization (endocytosis)
III. Degradation of the bacterial proteins, producing peptides.

1. Antigen binds to variable domains of antibody on the BCR (B-cell receptor)
2. Antigen is internalized and digested into peptides
3. Peptides are loaded on to class II MHC
4. Peptide-MHC displayed on membrane for presentation to T-cells

Journal of Cell Science doi: 10.1242/jcs.235192

Activation of B cells by Antigen - Lymph Node





T_H Cells

- Mature in thymus
- High diversity of TCR (10^{10})
- Homogenous on one T_H-cell
- Recognize **foreign** peptide on class II MHC

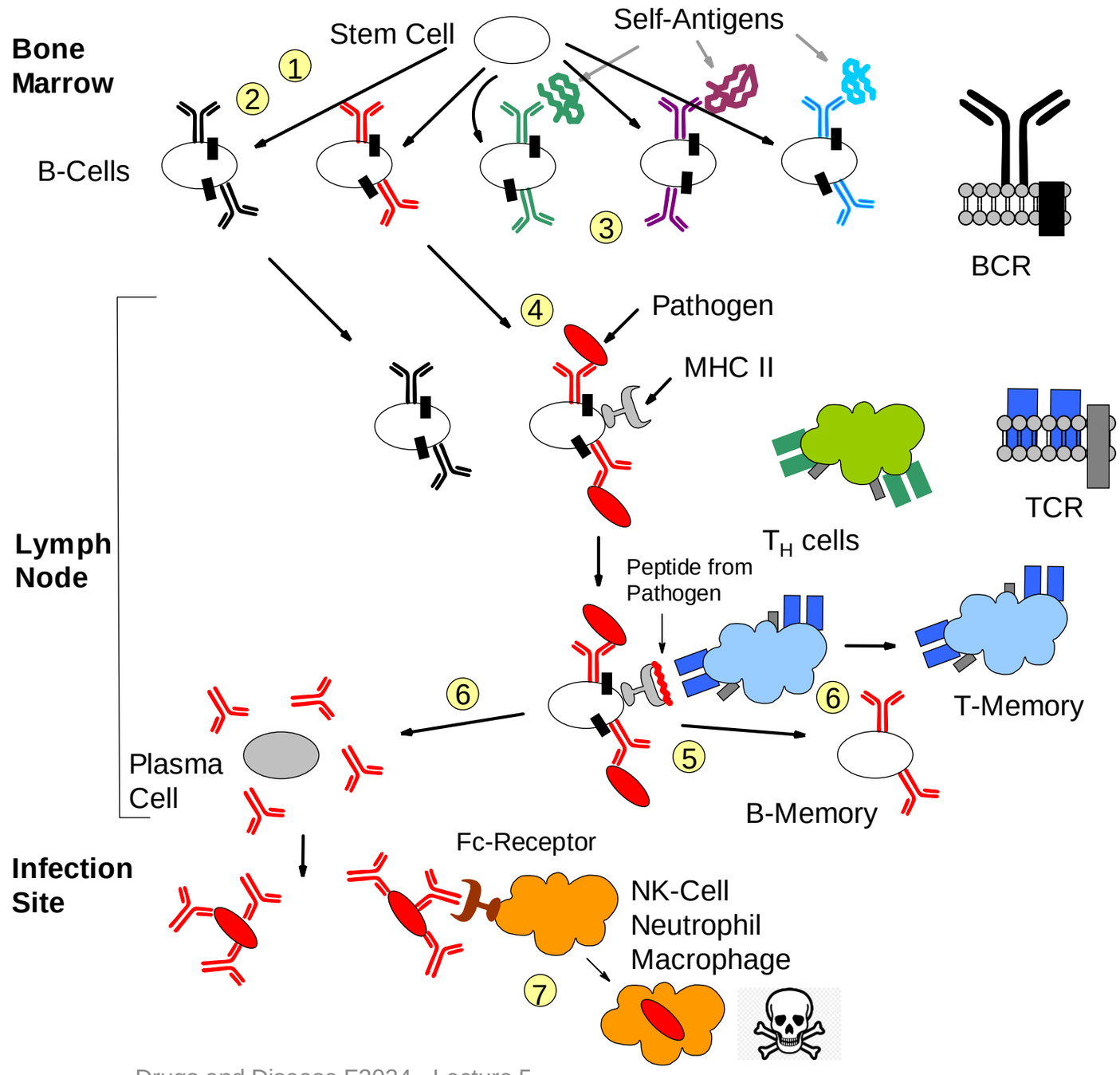
Events:

1. Recognition of MHC II-peptide by TCR
2. Tyrosine kinase signaling in T_H cell
3. Cytokines (protein messengers) produced.
4. Cytokines activate B-cells.

- B-cells develop into antibody secreting *plasma cells*.
- B and T-helper cells develop into **memory** cells, that are long-lived and are quickly activated by the same pathogen. ***This is the basis of vaccination.***
- Soluble antibody from plasma cells has the same light and heavy chains as the original B-cell.
- Membrane anchors are missing, so antibody is secreted outside the cell.

Can you:

- Describe how the genes for the heavy and light chain are generated, and how this gives rise to many different antibodies?
- Do you understand the process of B-cell activation, including presentation of foreign peptides on MHC II and the role of the T-helper cell.
- Describe how antibodies inactivate pathogens?



Cell Based Immunology

Key Questions:

1. How does your immune system fight viruses?
2. How does your immune system detect and destroy cancer cells?
3. How can the immune response be engineered to fight cancer?

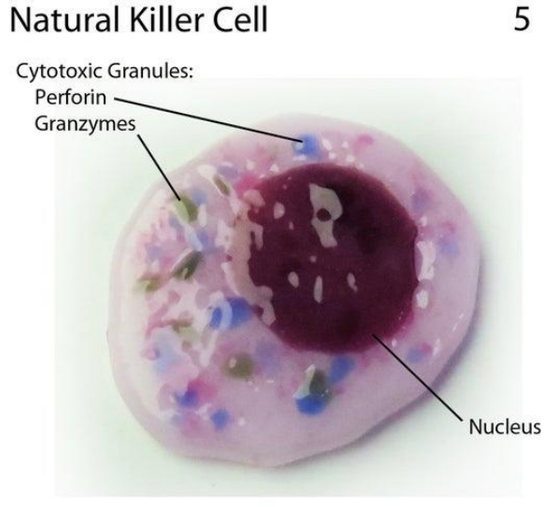
Cell Types:

Innate

- Natural Killer (NK) cell

Acquired

- T_H
- T_C, T_{CTL}

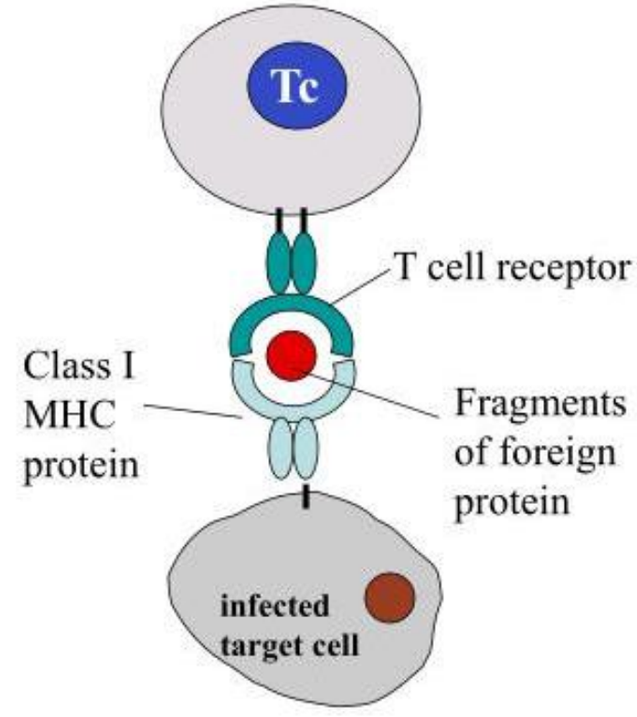


NK: Innate

- Kill virally infected cells
- Kill cancer cells

T_C → T_{CTL}

T_C Memory



Activation of Tc cells requires:

1. Recognition of ***foreign*** peptide on class I MHC.
2. Assistance from T-helper cells.

Activated Tc cell becomes a cytotoxic T-lymphocyte T_{CTL}

T_{CTL}

- Kill virally infected cells
- Kill cancer cells

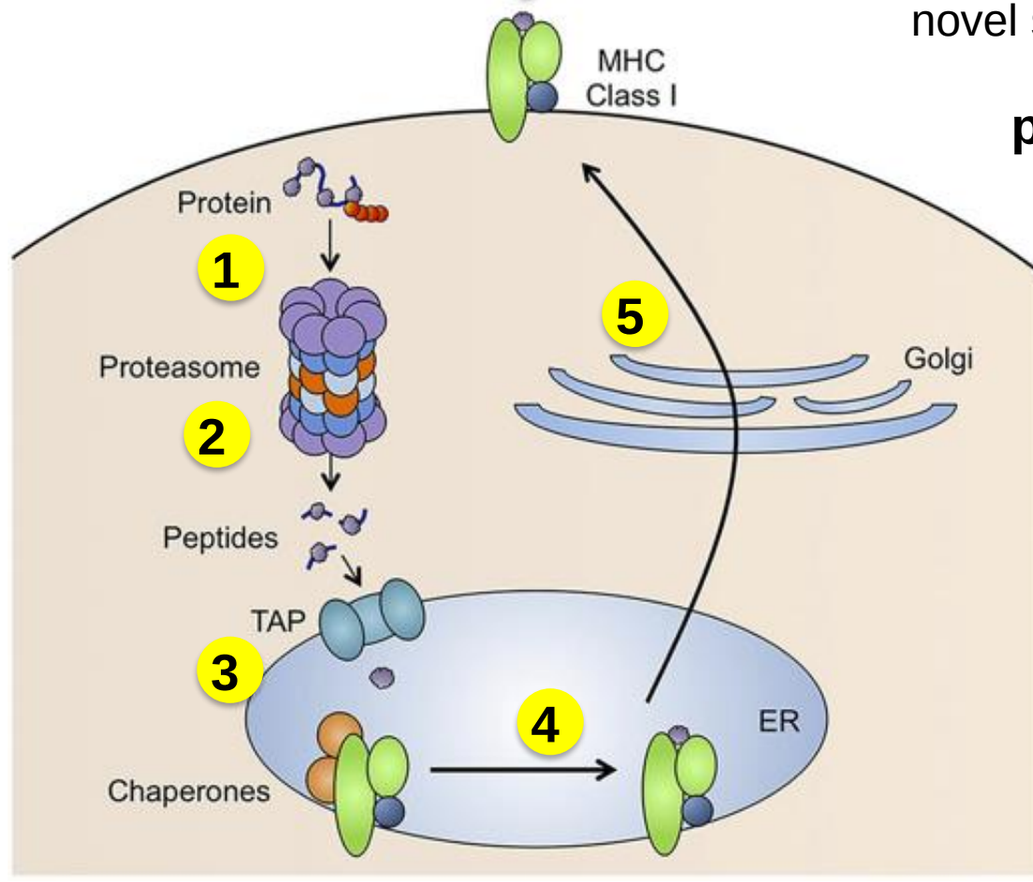
Tc memory cells are produced after activation.

T_c Detection of Diseased/Cancer Cells - Role of MHC I

- MHC I present peptides
- Peptides are generated from of ***all*** of the proteins that are made in the cell.

- Steps:
  1. protein targeted for degradation by ubiquitin
  2. Protein digested by proteasome
  3. Peptides transported into ER
  4. Peptides loaded on to MHC I
  5. Peptide/MHC complex transported to cell membrane.

Only foreign peptides activate T-cells



Possible Sources of Foreign Peptides:

1. From replication of viruses in the cell
2. From replication of intracellular bacteria (e.g. TB)
3. New coding sequences in cancer cells due to genetic changes (e.g. mutations in p53 lead to novel sequences).

p53 Protein Sequence

Zn Fingers (DNA binding)

```
        10         20         30         40         50
MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI
        60         70         80         90        100
EQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ
       110        120        130        140        150
KTYQGSYGFR LGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST
       160        170        180        190        200
PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
       210        220        230        240        250
LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS SCMGGMNRRP
       260        270        280        290        300
ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP
       310        320        330        340        350
PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL
       360        370        380        390
KDAQAGKEPG GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD
```

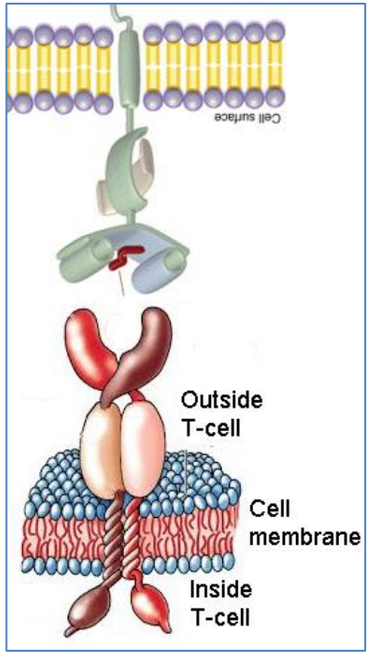
EVVRRCPHHE — Normal seq., ***ignored*** by TCR

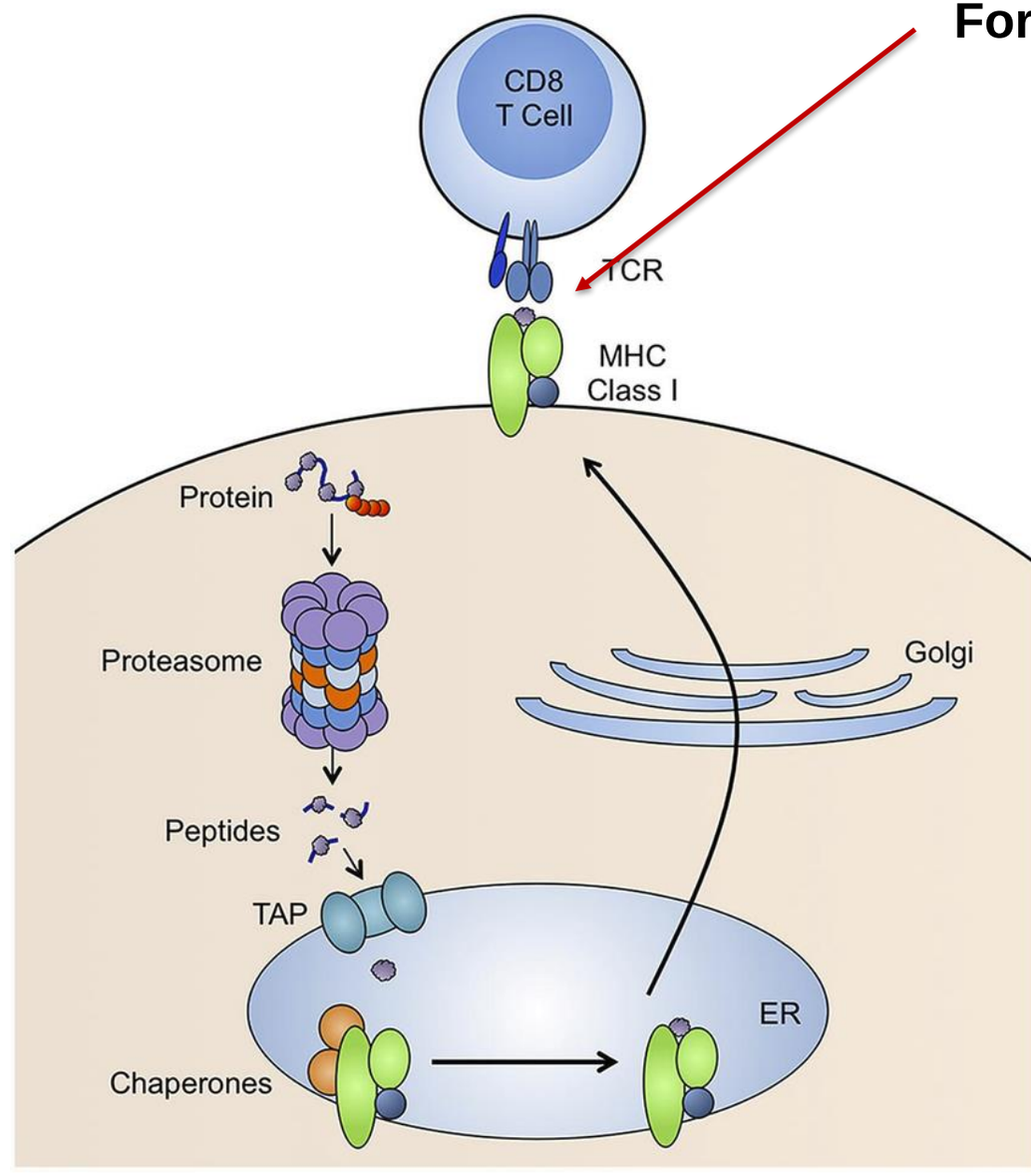
EVV**GG**CPHHE — Mutant seq. in cancer, ***detected*** by TCR

T_c Detection of Diseased/Cancer Cells - Activation

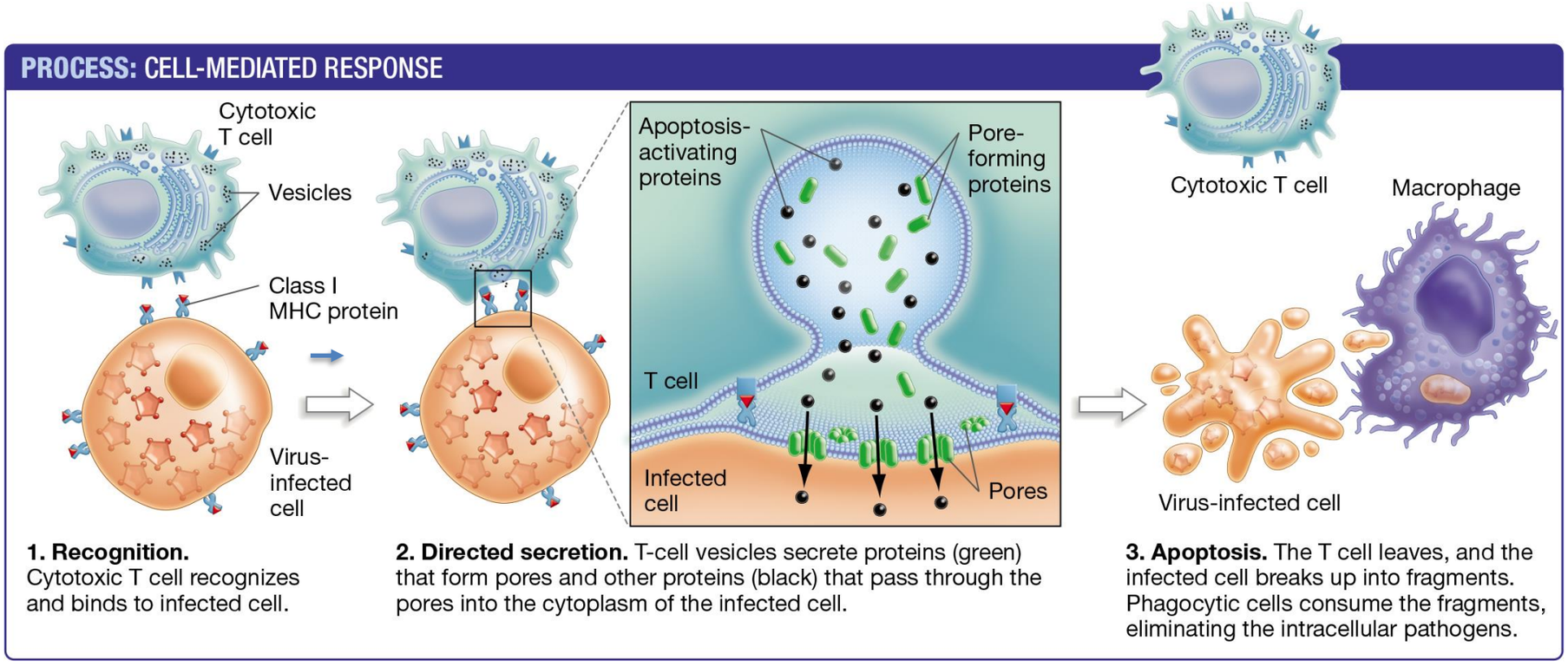
Activation of T_C cells requires stimulation from activated T_H cells via MHC II pathway.

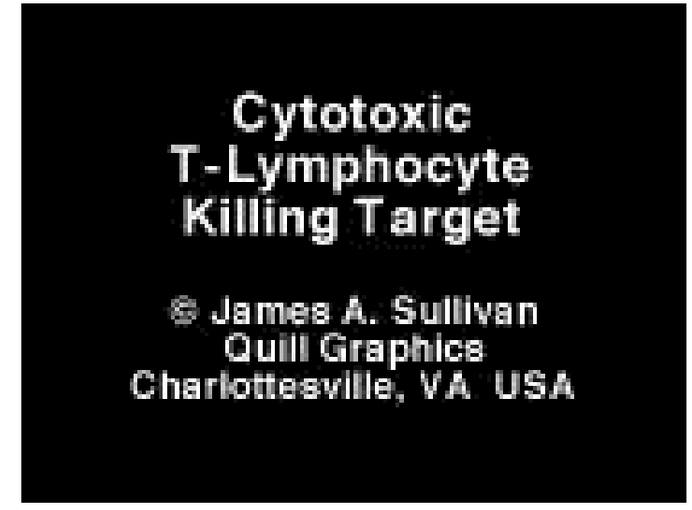
- Antigen captured by B-cells and other phagocytotic cells (macrophages, dendritic cells.
- Peptides presented on class II – T_H activated





T_C Cells: Detection and Killing of Virally Infected or Cancer Cells



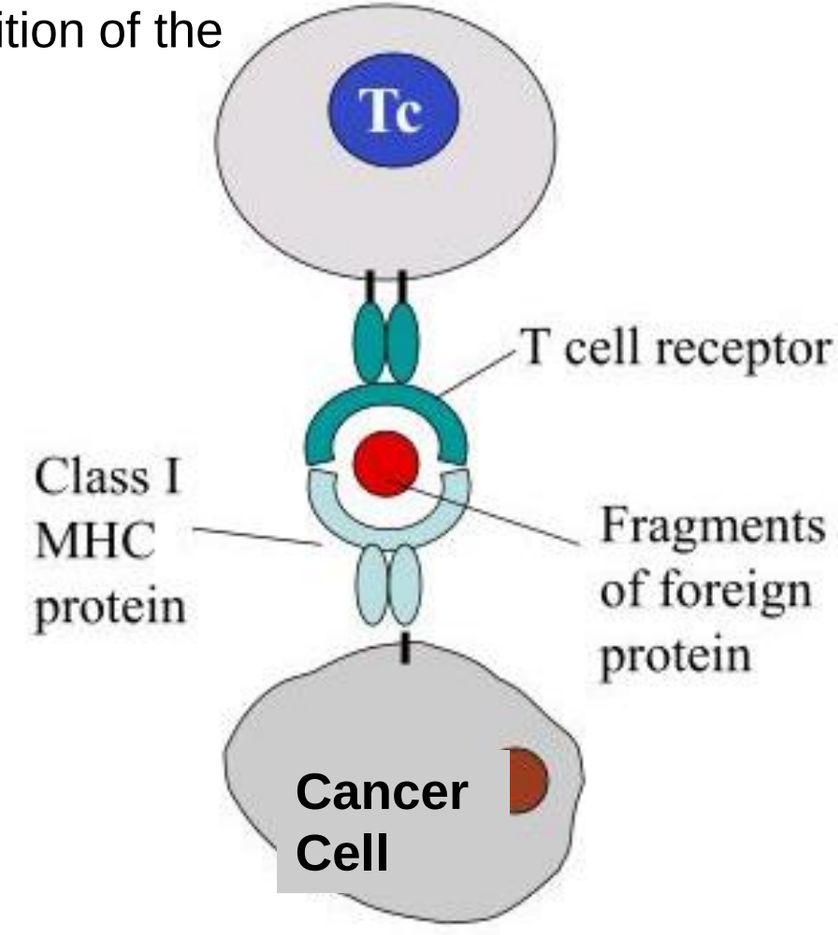


Cancer cell or Infected cell

- Granzymes (apoptosis activating proteins) enter through perforin pores and cause cell undergo programmed cell death (apoptosis)

Cancer Evasion Mechanism - Loss of MHC I on Tumor Cell

Loss of MHC I expression means that T_{CTL} cells can no longer recognize and kill cancer cells because T-cell activation requires recognition of the MHC-peptide complex.

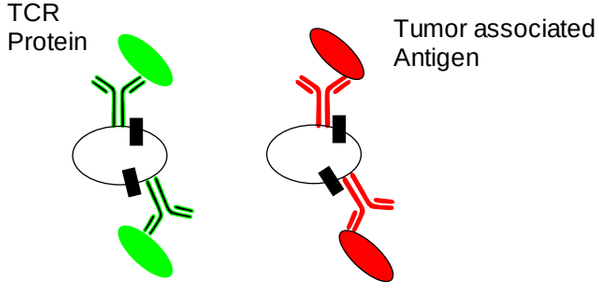


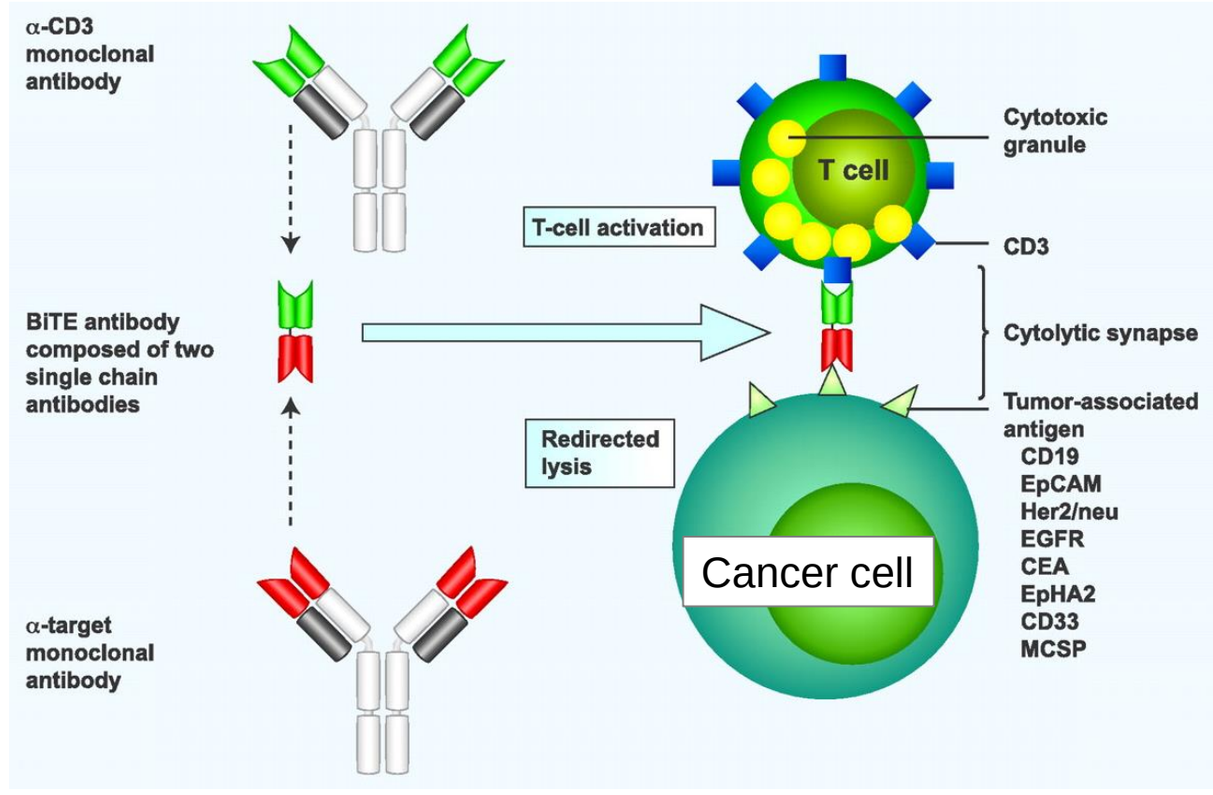
How to re-establish T_C contact with tumor cell and activation of the T-cell so that the cancer cell is killed?

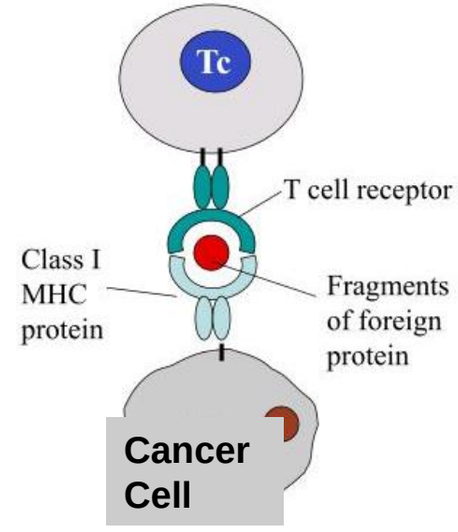
Cancer Treatment with Antibodies - Cancer Evasion - Loss of MHC I on Tumor Cell

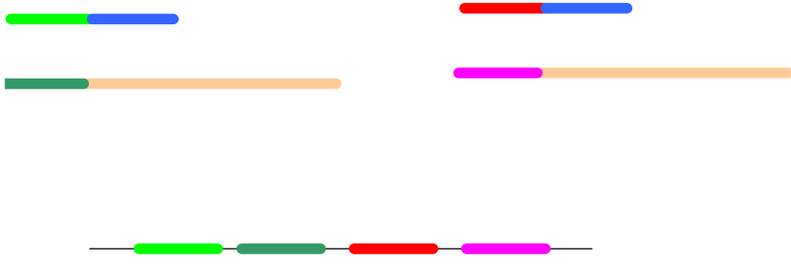
Tumor-associated antigen: An antigen that is found only on tumor cells:

- Mis-regulation
- Mutation





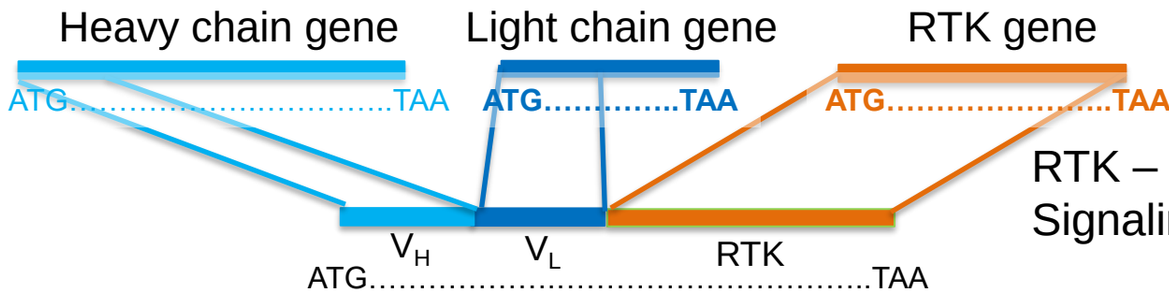




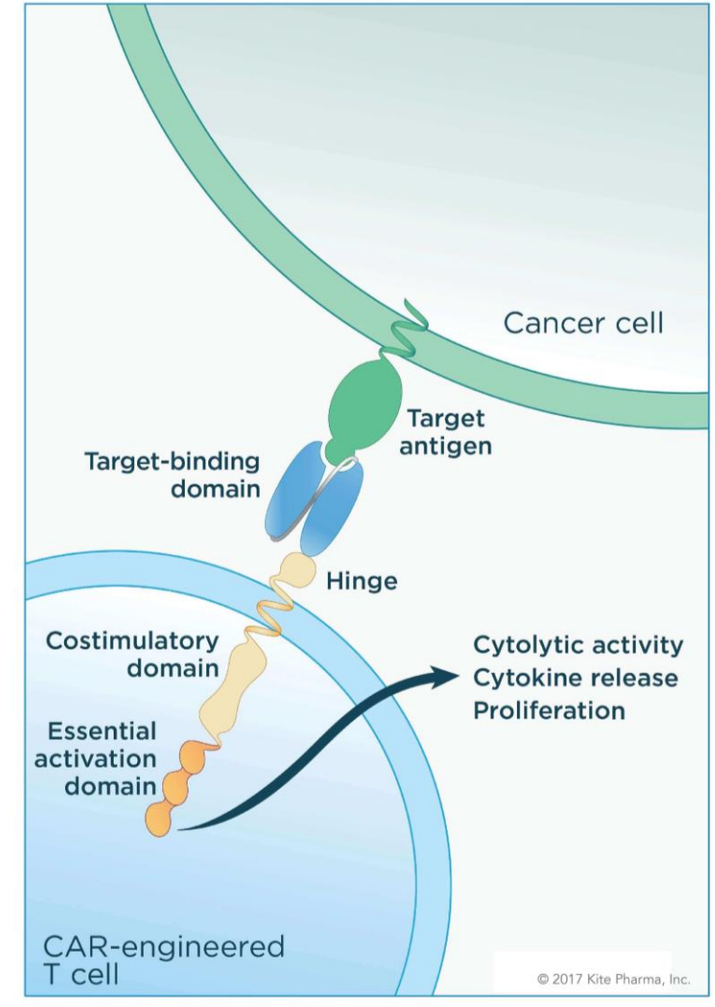
- Bispecific antibodies are generated from two separate antibodies:
  - One recognizes CD3, which is part of the T-cell receptor (TCR)
  - Other recognizes a tumor antigen.
- The two variable regions are linked into a single polypeptide chain by construction of a synthetic DNA molecule.
- The dual binding event mimics the original MHC-I TCR interaction.

Chimeric Antigen Receptor T-cells = CAR T-Cells

A. Obtain antibodies against cancer antigen, isolate genes that code for light and heavy chains for those antibodies.
B. Fuse coding region for variable light and heavy domains to coding region for RTK on T-cells = single CAR-T gene.

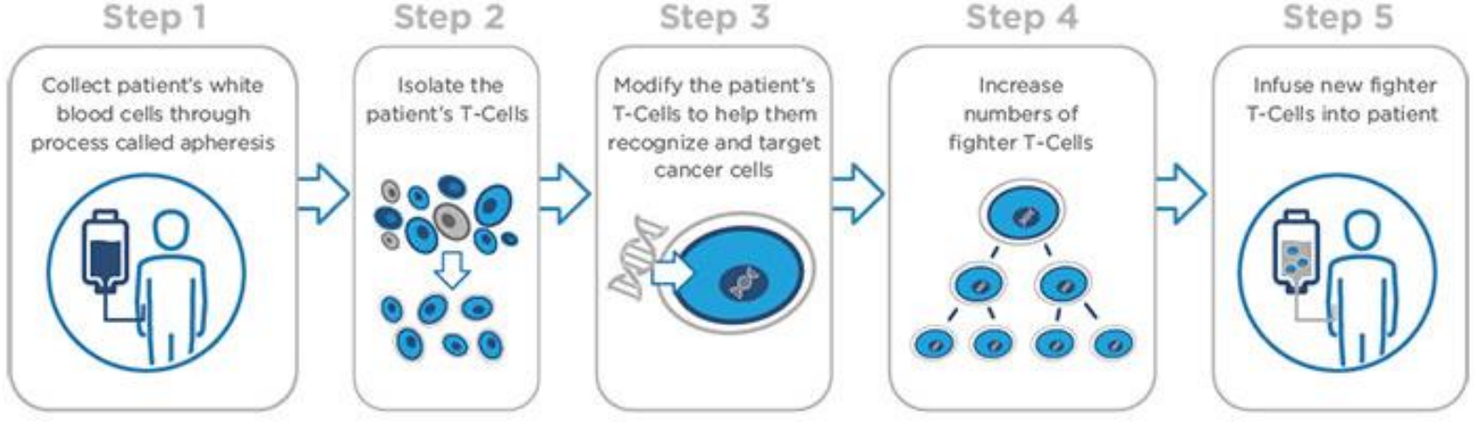


RTK – receptor tyrosine kinase
Signaling domain on T-cells



C. Introduce gene for CAR-T cell into Patient
1. Obtain white blood cells from patient
2. Isolate T-cells
3. Introduce DNA into T-cells
4. Obtain large amounts of T-cells by cell culture
5. Inject CAR-T cells into cancer patient.

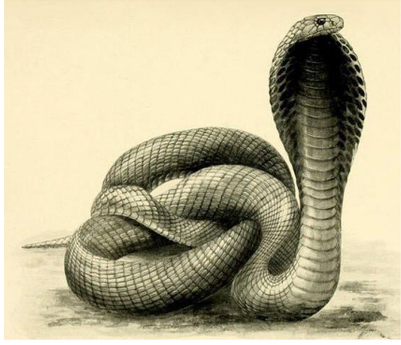
D. What happens when cancer cell is encountered by CarT cell?

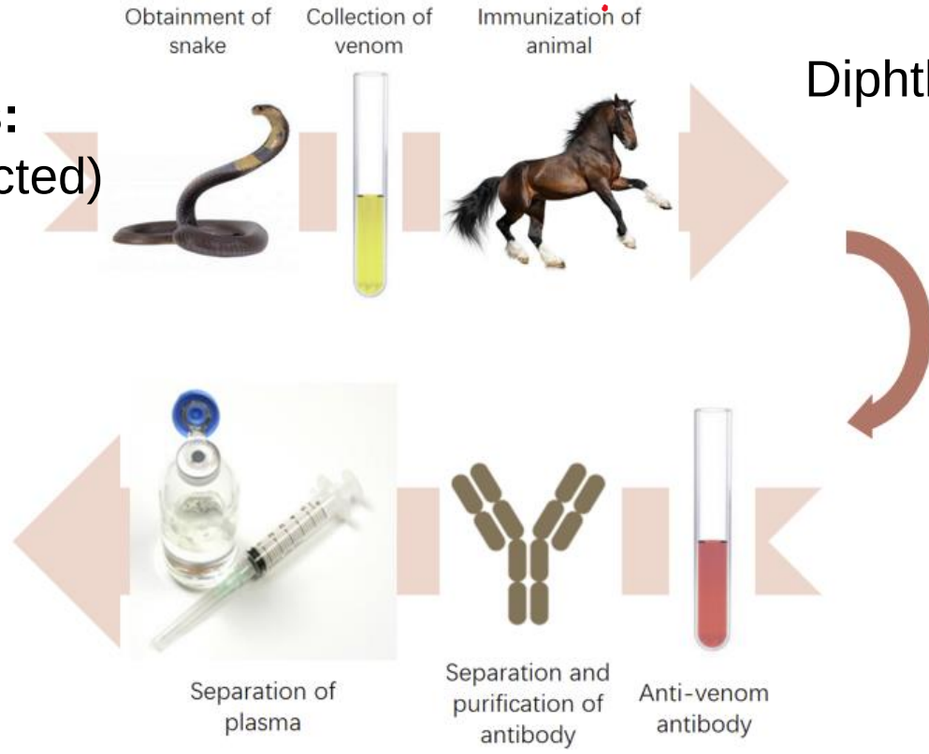


Vaccination

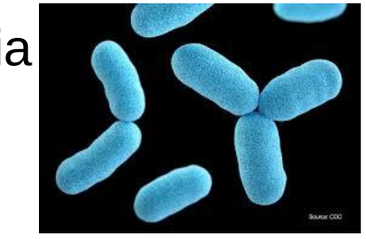
Types of vaccines:

1. Passive (Ab injected)

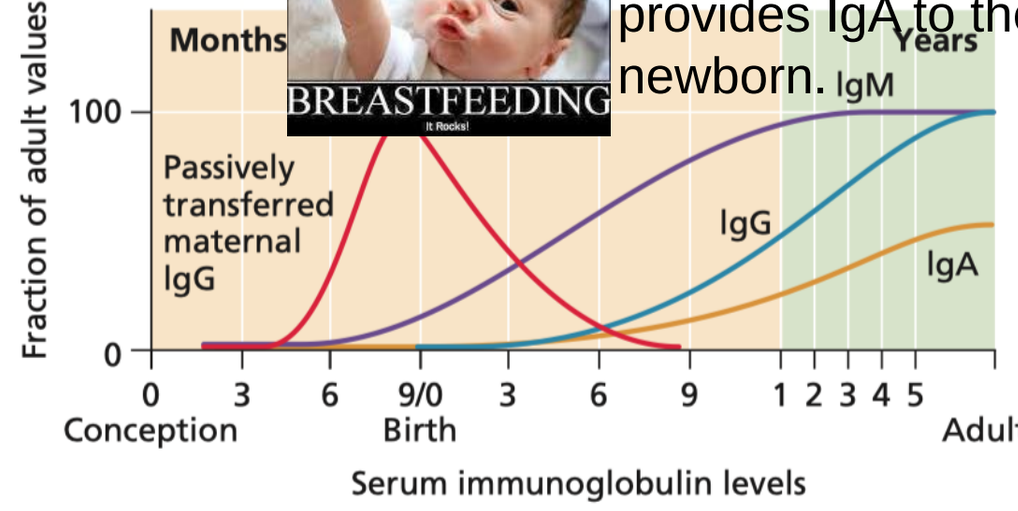




Diphtheria

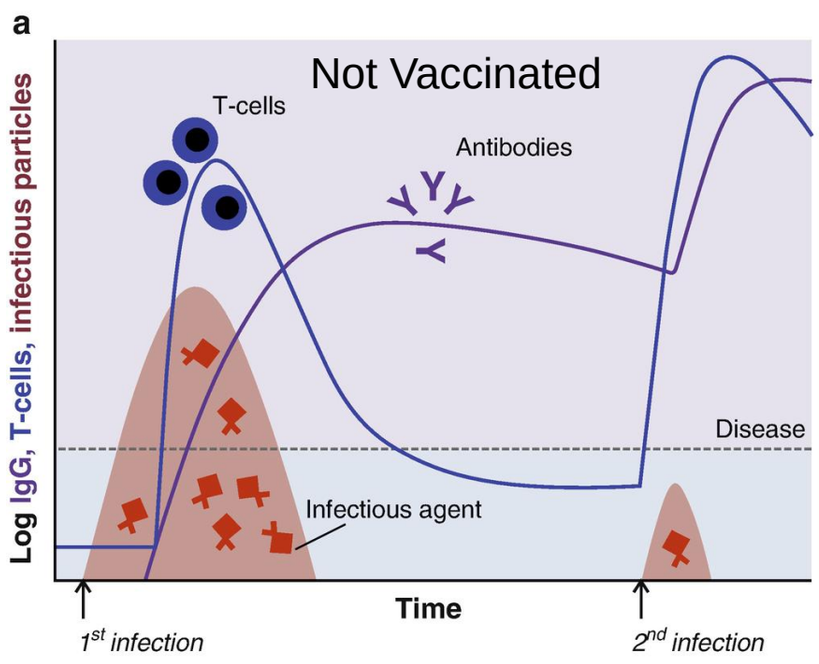


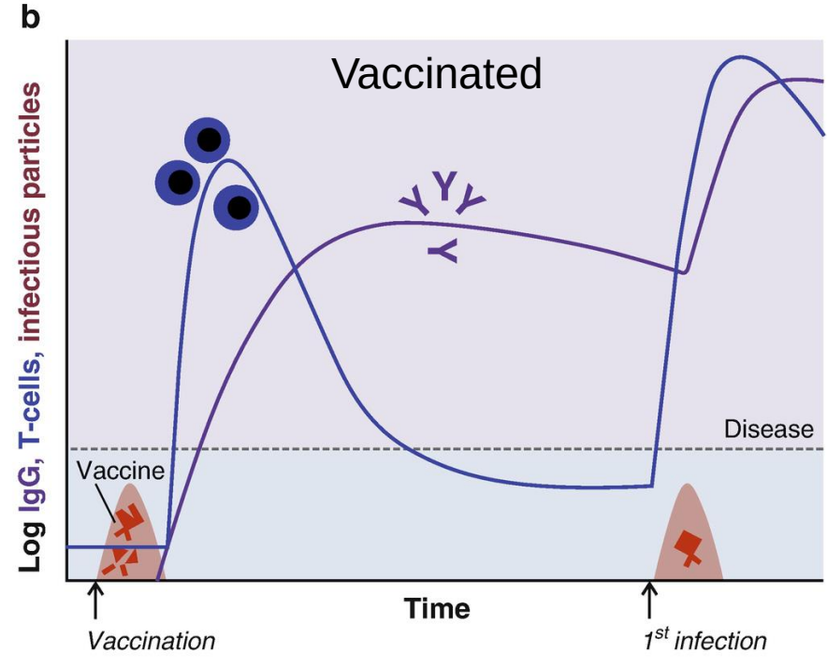
Breast feeding provides IgA to the newborn.

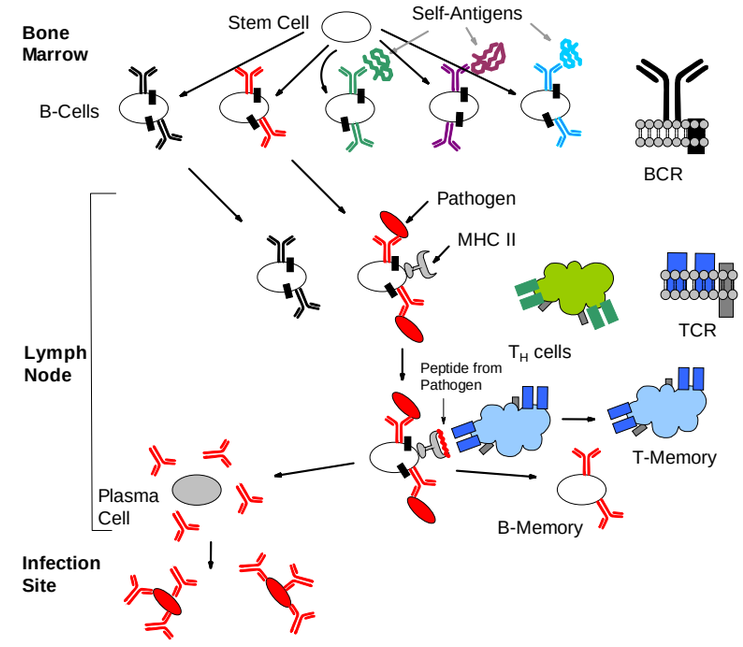


2. Active (Antigen Provided)

Primary and Secondary Response & Protection by Vaccines







Large number of pathogens during first (primary) infection causes disease symptoms

- Antigen from pathogen prompts acquired immune response.

More rapid & intense secondary response prevents extensive pathogen growth – no symptoms.

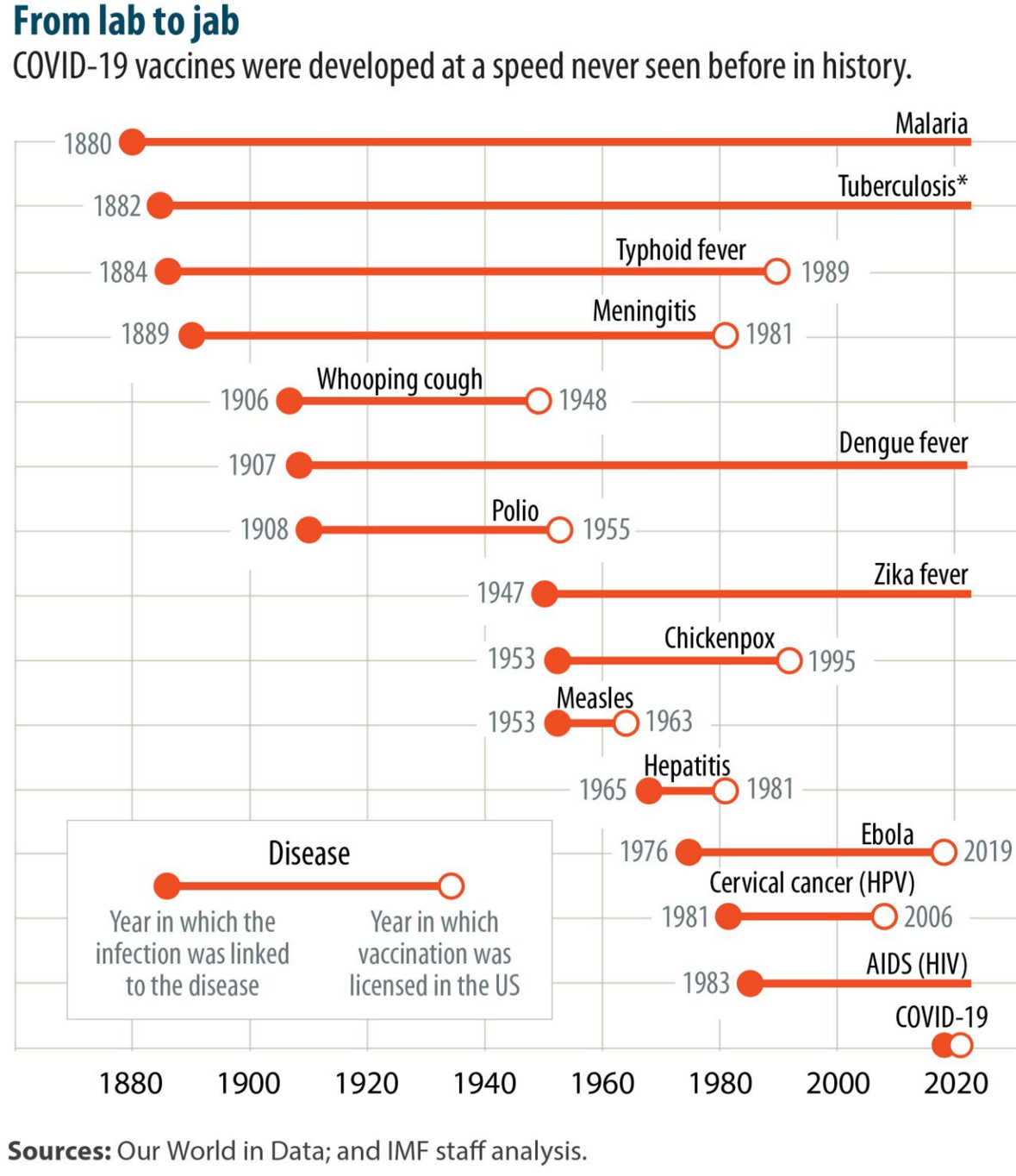
Vaccine: **antigen** induces primary response = memory B and T (T_H and T_C) cells specific for that antigen.

More rapid & intense secondary response prevents extensive pathogen growth – no symptoms.

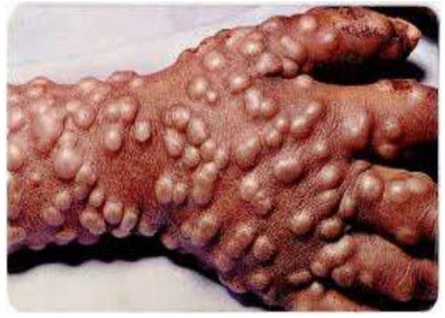
Vaccine History

- Some diseases still do not have vaccines (Malaria, HIV)
- Many diseases are controlled by vaccination (Typhoid, Meningitis, Whooping cough, polio, chickenpox, measles,…)
- A few diseases have been completely eliminated by vaccination (Smallpox)

https://www.imf.org/en/Publications/fandd/issues/2021/12/Journey-covid-19-vaccine-Stanley



Smallpox - A Success Story for Vaccination

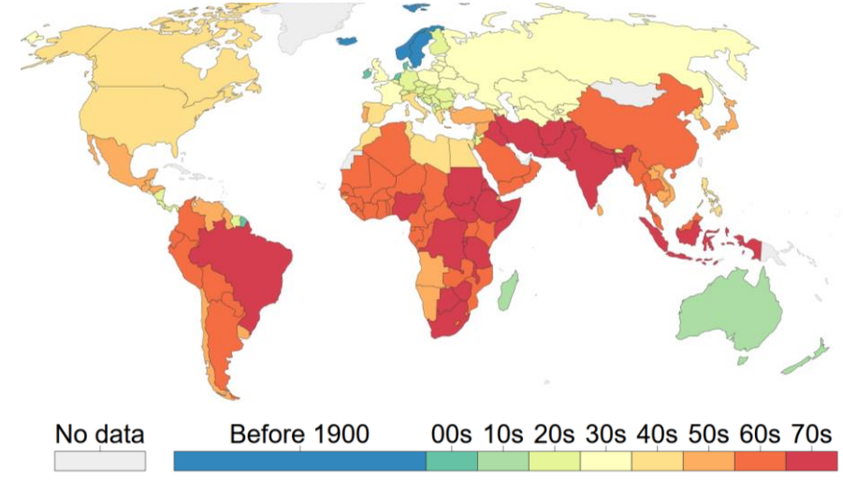


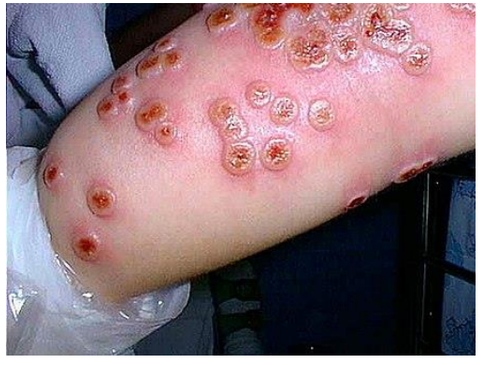
10,000 BC Smallpox – 20-90% lethality

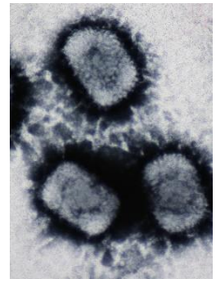
Variolation (1670) provided protection by exposing people to small amounts of smallpox virus (obtained from blisters on infected people). Practice spread from Istanbul to Europe.

Risky because smallpox was used to vaccinate (2% risk of death)

Decade in which smallpox ceased to be endemic

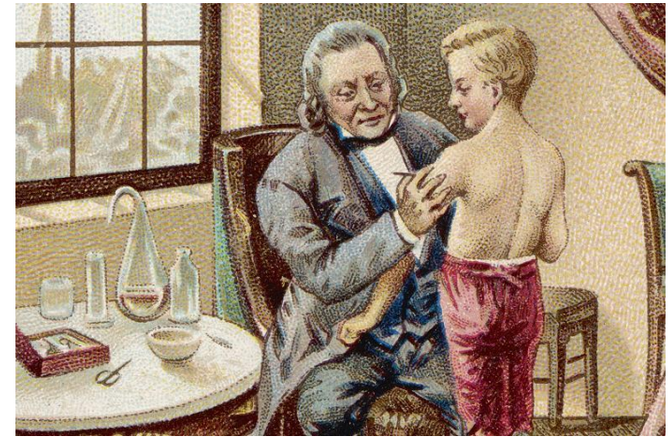






Cowpox virus:

- Not lethal
- Similar to smallpox virus
- Causes production of ***cross-reactive*** antibodies that can bind to smallpox



Jenner was the first to use cowpox to vaccinate against smallpox (1796)

- Vaccinated with cowpox (ill for 9 days)
- Infected with smallpox (2 months later)
- Subject did not develop smallpox

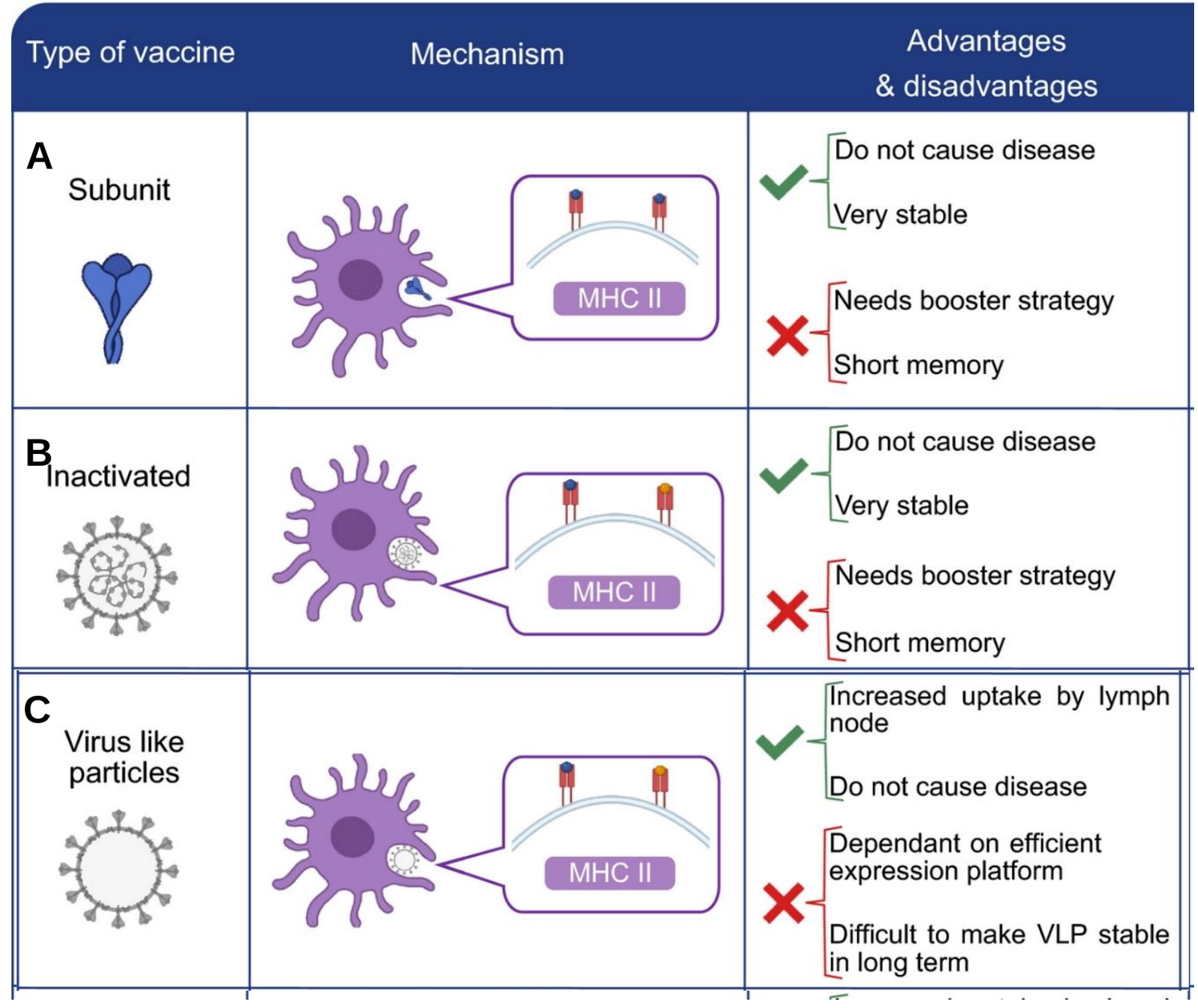
Vaccinia virus (similar to smallpox) is one form of the current vaccine.

Types of Vaccines

A. Subunit Vaccine:
A protein from the pathogen is used to induce memory cells, e.g. spike protein from the virus. The protein can be produced by recombinant DNA technology.

B. Inactivated Virus
The virus is chemically inactivated before administration. Peptides from virus activate B and T_H cells.

C. Virus Like Particles:
Proteins isolated from the virus form virus-like-particles, *without* the genetic material of the virus



D. Live Attenuated

The virus is grown under conditions that select for mutant viruses that:

i) Induce memory cells in humans

ii) Do not cause disease symptoms

E. Recombinant Virus:

A “safe virus” is used (e.g. cold virus)

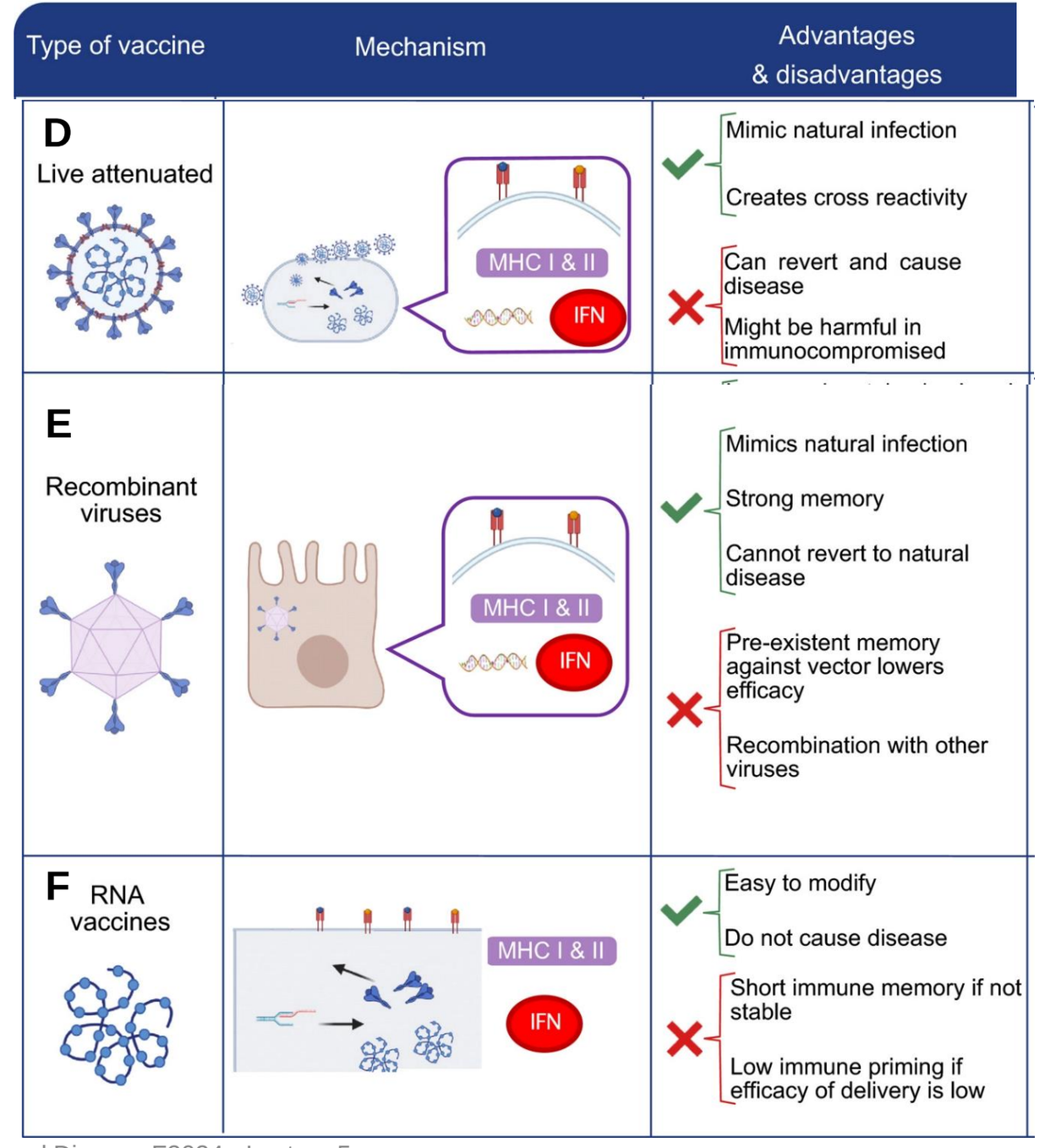
Gene for a protein from a pathogen is inserted into the DNA of the virus.

- When virus grows it produces the protein from the pathogen generating immunity.

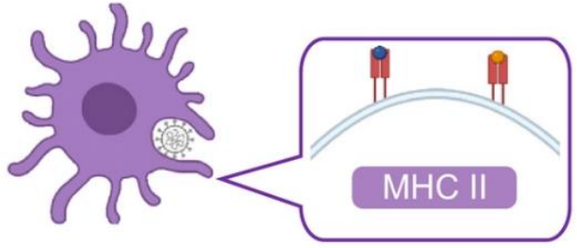
Also includes vaccines that are a mixture of genetic material from human and animal viruses (reassortment viruses)

F. RNA Vaccines (Pfizer Covid Vaccines)

RNA coding for a viral protein is introduced into cells. The RNA is used by the cell to make viral proteins, inducing an immune response.



B. Inactivated – Salk Polio Vaccine

Inactivated 		✓ Do not cause disease Very stable ✗ Needs booster strategy Short memory

THE TAMPA DAILY TIMES NIGHT FINAL

SIXTY-THIRD YEAR—No. 56 TAMPA, FLORIDA, TUESDAY, APRIL 12, 1955 PRICE FIVE CENTS

POLIO VACCINE 'SUCCESSFUL'

End of Quick Divorce Law Gains Favor

Senate Committee Clears Bill for 6 Months' Residence

Top Prize Story In Today's News

Oakland, Cal., April 12 (AP). John Cox's headache turned out to be one for his whole fire station here last night.

Cox, a tillerman on a hook and ladder rig, obtained permission from his captain to step across the street to get some aspirin.

While he was in the drug store, an alarm rang.

And away went the hook and ladder truck—without a tillerman.

At the first corner, the wild-

Auditorium, Bridge Bonds Get Okay

Consultants Give Go Ahead on Tampa Improvement Projects

By BOB TURNER Times Staff Writer

...e Hixon said today the bond consultants in ...ork have given the go-for floating a $5,000,-...id issue to finance con-...n of a new auditorium, ...dges and several build-...provements here.

Paralysis Prevention Proved in Mass Tests

'Effective, Safe' Verdict Reported After Long Study of Innoculations

By ALTON L. BLAKESLEE Associated Press Science Reporter

Ann Arbor, Mich., April 12 (AP). The Salk polio vaccine works—safely, potently—and can virtually end the icy fear that long has gripped the hearts of parents.

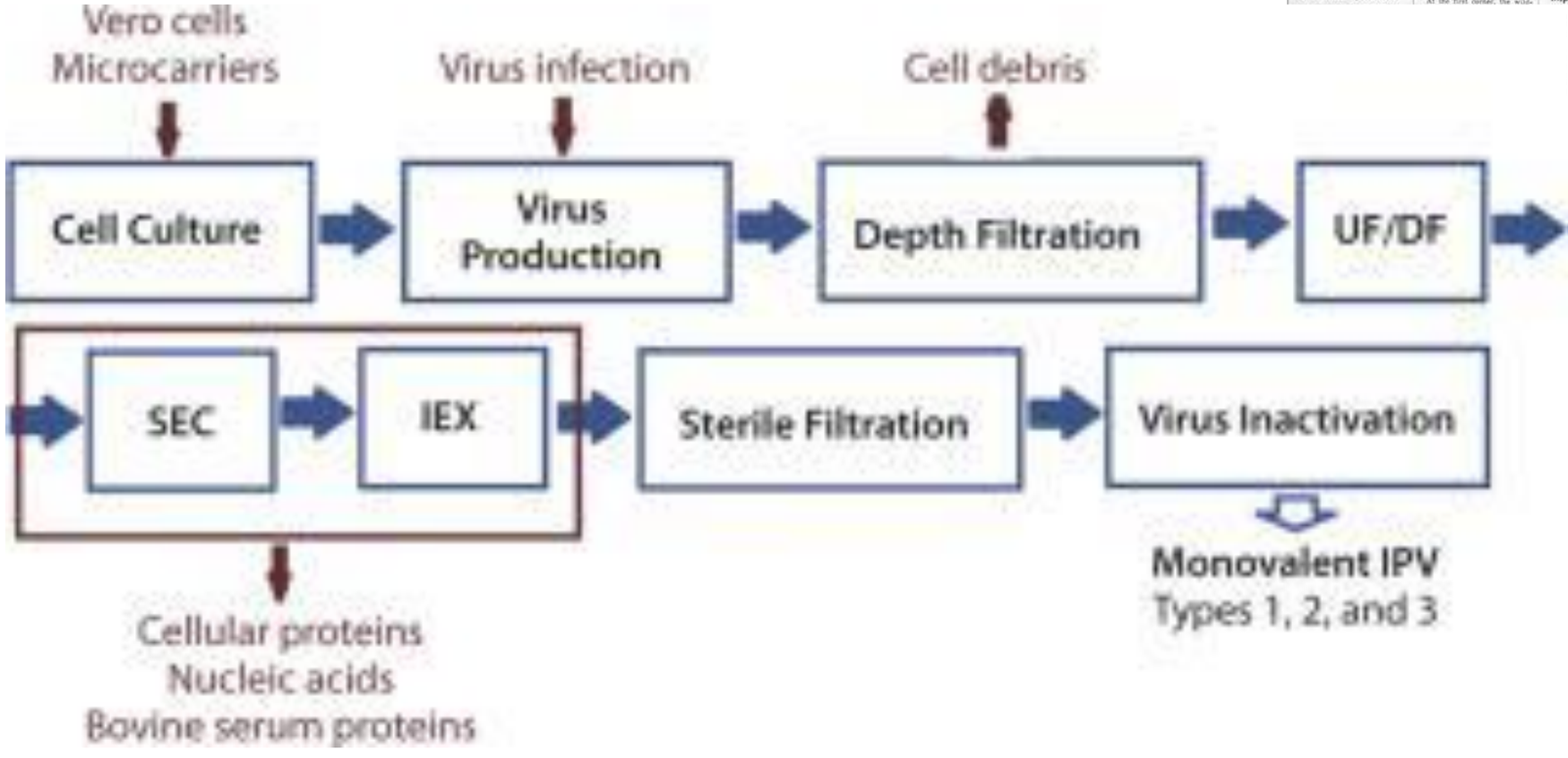
The vaccine was officially declared today to have proved to be 80 to 90 percent effective in preventing paralytic polio.

And young Dr. Jonas E. Salk, who developed it, declared he is certain it can be potentially made almost 100 percent potent to smash polio's terror and tragedies.

Supercharged Atmosphere

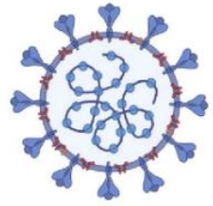
The triumph of the vaccine was announced in an atmosphere of

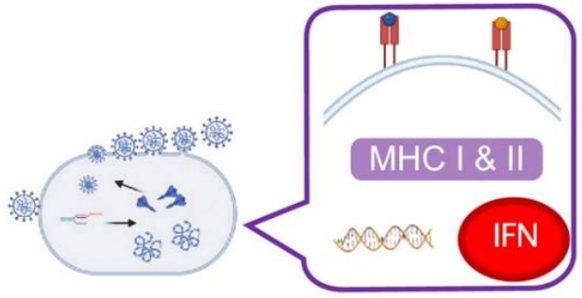
Dr. Salk Says 'Too Busy Now For Comment'



D. Attenuated – Sabin Polio Vaccine

Live attenuated





✓ Mimic natural infection

✓ Creates cross reactivity

✗ Can revert and cause disease

✗ Might be harmful in immunocompromised

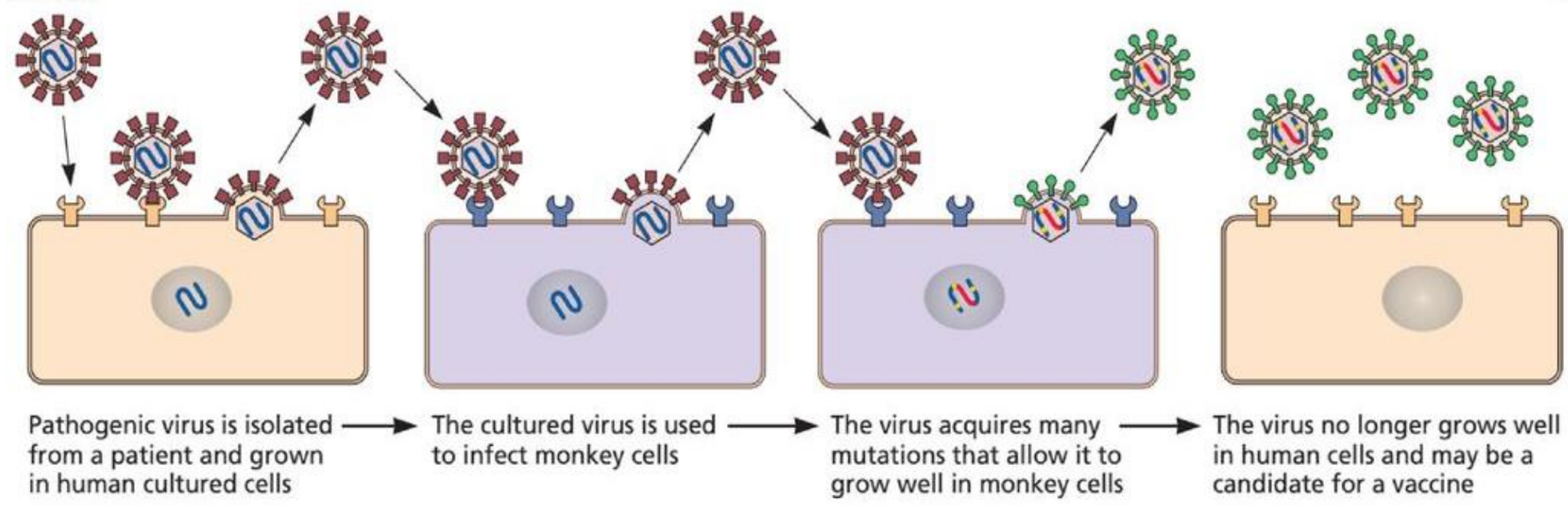
THE CINCINNATI ENQUIRER

122nd YEAR NO. 76—DAILY SUNDAY MORNING, JUNE 24, 1962 FINAL EDITION—PRICE 25 CENTS

'Sabin Sundaes' Ready For Area Polio Drive Beginning At 11 A. M.

'First' Family Gets Sabin Vaccine On Sugar

Attenuation Process Requires Mutations → Change growth characteristics on human cells.



C. Attenuated Viruses – Return to Virulence by Reversion

World Health Organization

Home / Disease Outbreak News / Item / Circulating vaccine-derived poliovirus type 2 - Indonesia

Disease Outbreak News

Circulating vaccine-derived poliovirus type 2 (cVDPV2) - Indonesia

11 January 2024

Cell Host & Microbe

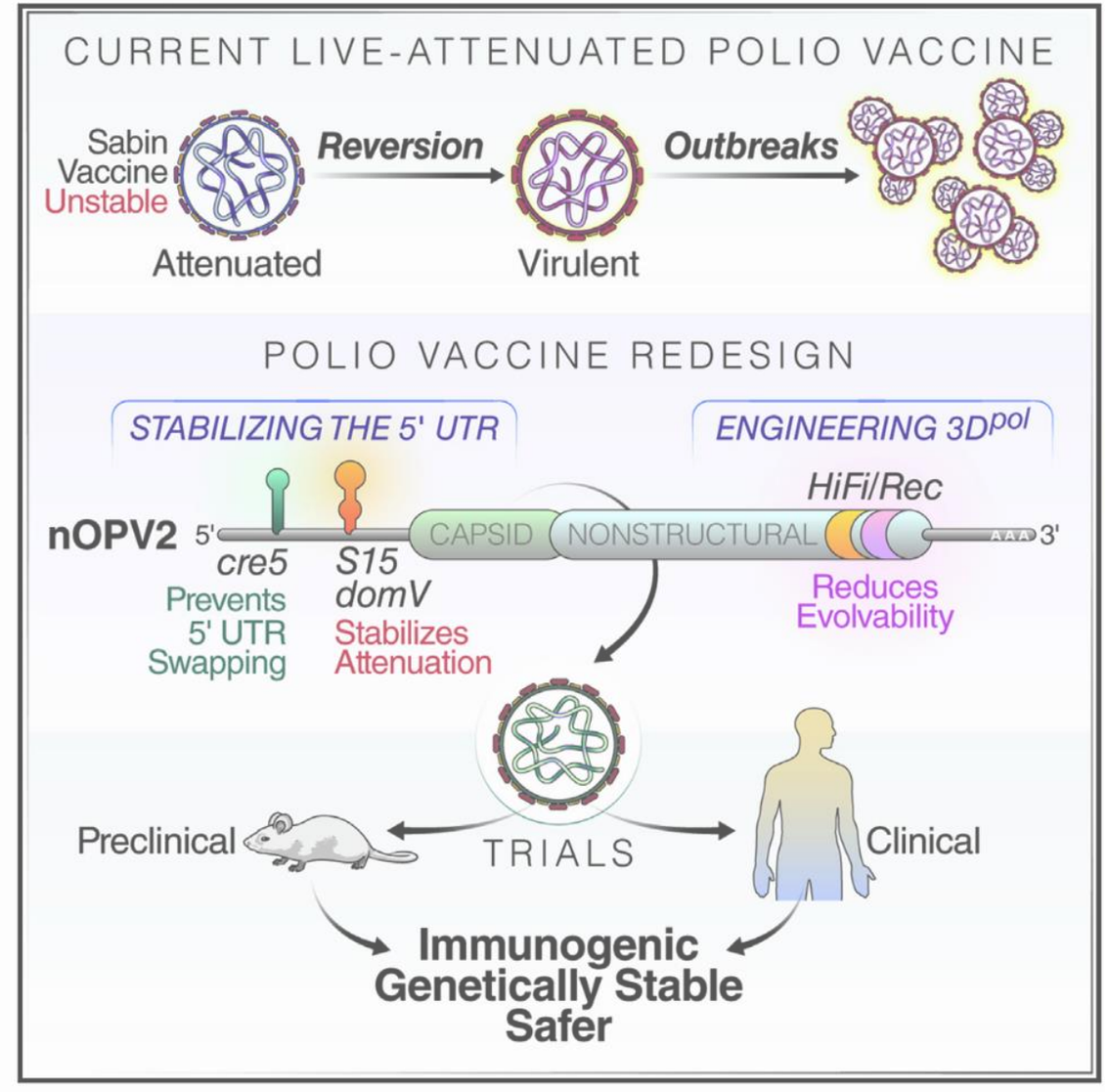
CellPress

Volume 27, Issue 5, 13 May 2020, Pages 736-751.e8

Article

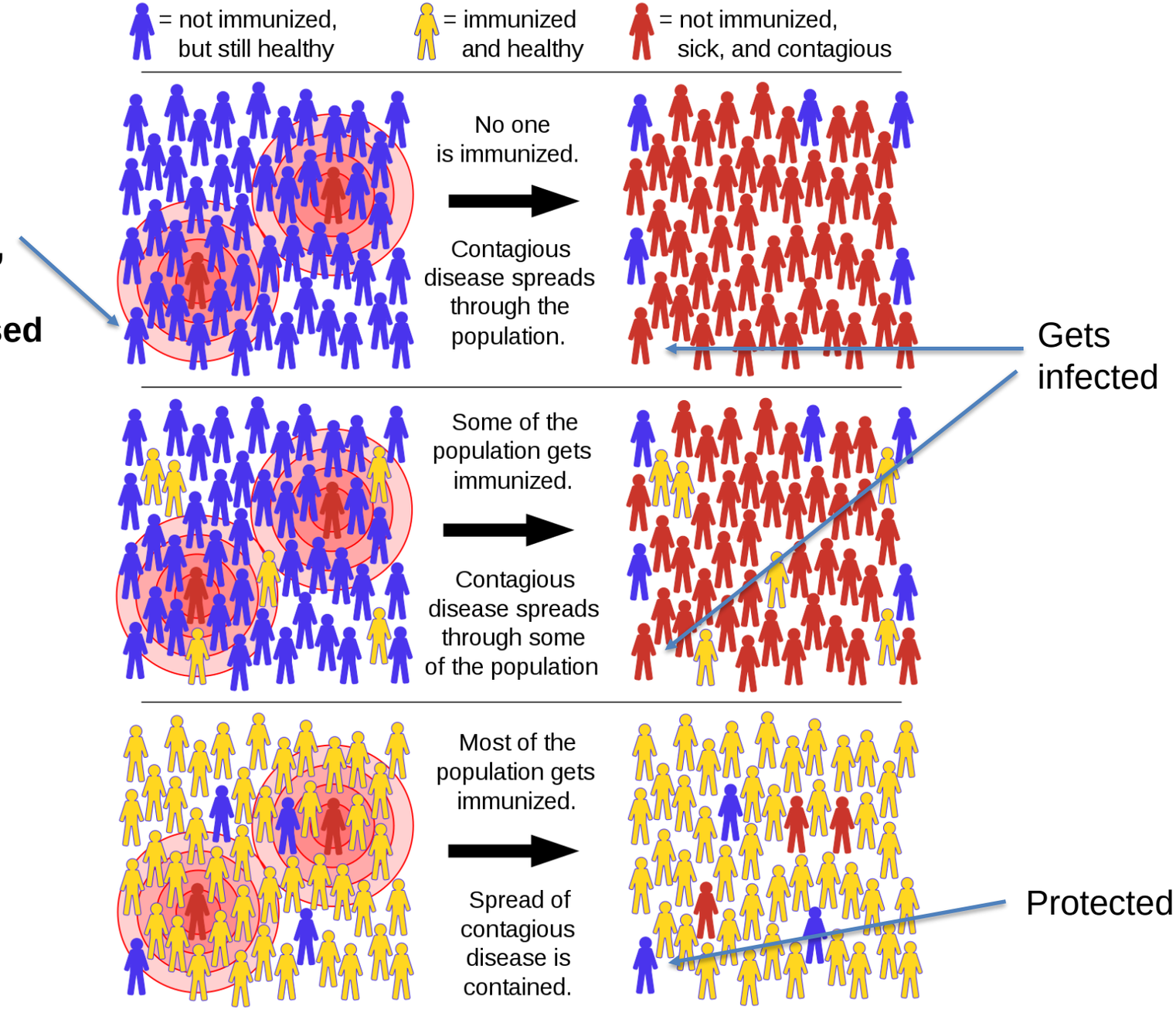
Engineering the Live-Attenuated Polio Vaccine to Prevent Reversion to Virulence

Ming Te Yeh [1], Erika Bujaki [2], Patrick T. Dolan [1], Matthew Smith [2], Rahnuma Wahid [3], John Konz [3], Amy J. Weiner [4], Ananda S. Bandyopadhyay [4], Pierre Van Damme [5], Ilse De Coster [5], Hilde Revets [5], Andrew Macadam [2], Raul Andino [1 6]



Herd Immunity:

- Vaccinated individuals prevent disease from spreading from sick to unvaccinated.
- At sufficient levels, the “herd” is immune because the virus cannot spread, eventhough some people get sick.



Herd Immunity

How Many People need to be vaccinated to achieve herd immunity?

10% ?

20% ?

50% ?

90% ?

100% ?

It depends on the how infectious the virus is

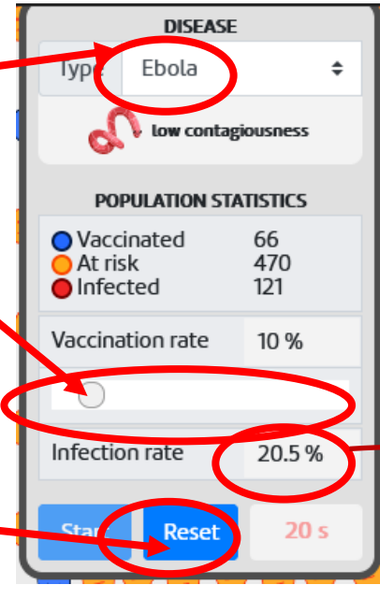
Our Experimental Viruses:

Ebola: Low infectivity
Polio: Moderate infectivity
Measles: High infectivity

Simulation to Determine Infectivity Versus Vaccination Level (Pset)

1. Go to the following web site and open **both** links: http://www.andrew.cmu.edu/~rule/stayin-alive
2. Copy the googlesheet.

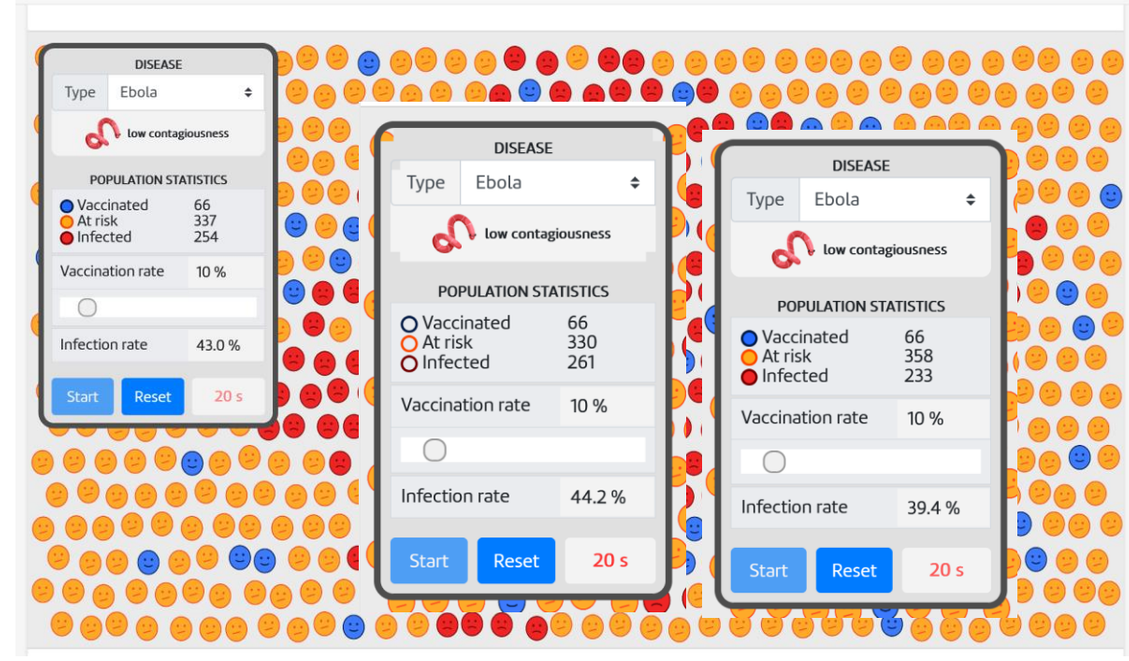
3. On the **Infection Simulator link**, scroll down (2/3 page) to the image of the plane, and click on it.



A. Select the virus.

B. Use the slider to select the different vaccination levels. Use 10, 20, 40, 50, 70, 80, 90 %. For each of the vaccination levels do ***three*** simulations.

C. Enter the value for the ***% Infection rate at 20s*** into the appropriate cell of the google sheet. Your data will be automatically averaged and plotted.



3 Simulation Runs at 10%

	A	B	C	D	E	F	G	H	I	J	K	L	M
		Ebola (Ave)	Polio (Ave)	Measles (Ave)		Ebola			Polio			Measles	
	%Vaccinated				#1	#2	#3	#1	#2	#3	#1	#2	#3
10		42.2%	#DIV/0!	#DIV/0!	43.0%	44.2%	39.4%						
20		#DIV/0!	#DIV/0!	#DIV/0!									
40		#DIV/0!	#DIV/0!	#DIV/0!									
50		#DIV/0!	#DIV/0!	#DIV/0!									
70		#DIV/0!	#DIV/0!	#DIV/0!									
80		#DIV/0!	#DIV/0!	#DIV/0!									
90		#DIV/0!	#DIV/0!	#DIV/0!									

Summary Questions for Immunology:

1. What are the two major branches of the immune system? Why are both important?
2. What are the roles of different cell types in each system, e.g. what would happen if T_H-cells disappeared?
3. What is the quaternary structure of an antibody? Can you sketch an antibody and indicate where the antigen binds?
4. What part of the antibody defines the specificity?
5. What are the steps in the production of antibody genes, at the molecular level:
    a) How do DNA rearrangements produce functional heavy and light chain genes
    b) What is the difference between the heavy chain for B-cells versus plasma cells.
6. Can you describe how antibodies kill/inactivate pathogens
7. How are virally infected cells and tumor cells recognized by Tc cells?
8. How does the Tc cell kill those cells?
9. What evasion mechanisms are used by cancer cells and how have these been addressed by antibody therapy?

10. What was the origin of the idea for vaccination?
11. What was one of the first “safe” vaccines? What disease has now been eradicated due to this vaccine?
12. Can you describe one way to generate a vaccine for a pathogen? Do you know the pros and cons for that method?

Enzyme Inhibitors as Drugs

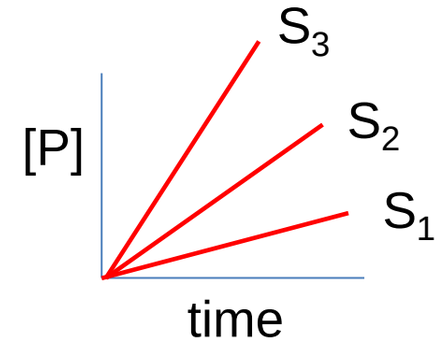
- Types of inhibitors
  - Covalent
  - Competitive
  - Allosteric
- HIV drug therapy
- Antibiotics – inhibitors of RNA and protein synthesis

Genome Editing – Cas9

- Discovery & Engineering of CRISPR systems
- Off-target effects

Key Points:

$$(E) + (S) \rightleftarrows (ES) \xrightarrow{k_{CAT}} (EP) \longrightarrow (E) + (P)$$



Kinetics

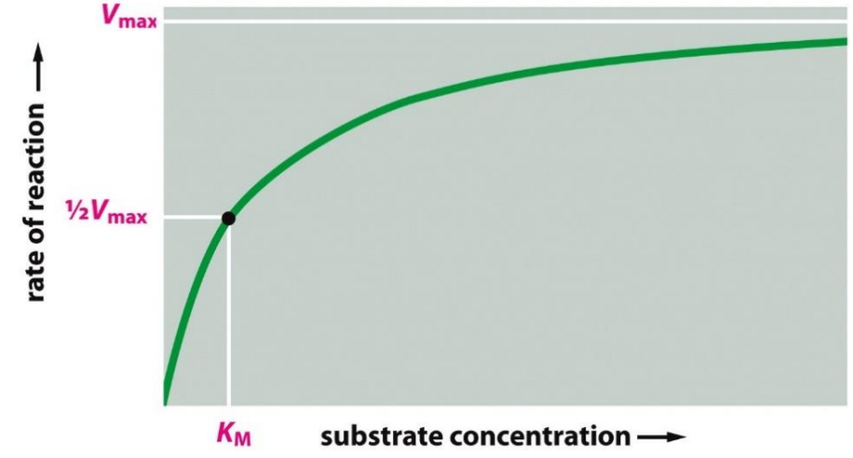
Rate = dP/dt, proportional to [ES].

V_{max} = measured velocity at saturating substrate:

$V_{max} = k_{CAT} \times E_{total}$

K_M:

- Substrate concentration to ½ saturate the enzyme, v = Vmax/2
- Measure of substrate affinity, lower K_M, better binding.



Enzyme Inhibitors

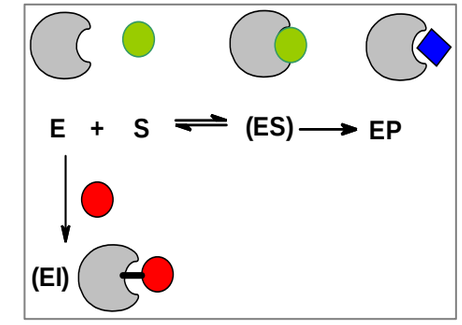
Studies on Inhibitors are useful for:

1. Mechanistic studies to learn about how enzymes interact with their substrates.
2. Understanding the role of inhibitors in enzyme regulation.
3. Drugs if they inhibit aberrant biochemical reactions:
   - penicillin, ampicillin, etc. interfere with the synthesis of bacterial cell walls, acting as suicide inhibitors.
4. Understanding the role of biological toxins.
   - Amino acid analogs - useful herbicides (i.e. roundup)
   - Insecticides - chemicals targeted for insect nervous system.

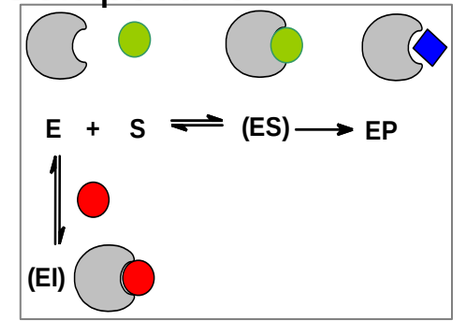
Types of Inhibitors:

1. Covalent – inhibitor *covalently* modifies enzyme, usually in active site, these are generally *irreversible* – the enzyme is dead! *Example – Sarin gas (Tokyo subway 1995)*
2. Competitive – inhibitor blocks substrate, binds *reversibly to active site with a* $K_D = K_I$. Enzyme activity returns when drug is removed.
3. Allosteric (mixed type) – inhibitor causes allosteric change. Binds *reversibly to a different location, with two different* K_D*s:* K_I *and* K_I*'. Enzyme activity returns when drug is removed.*

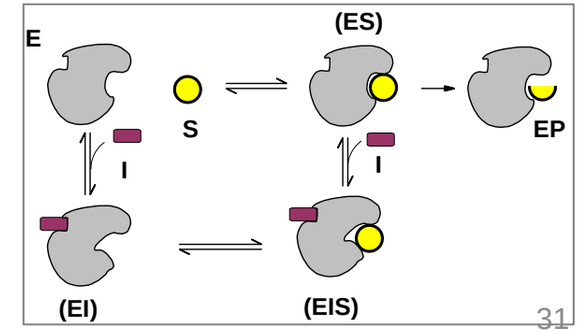
Covalent



Competitive

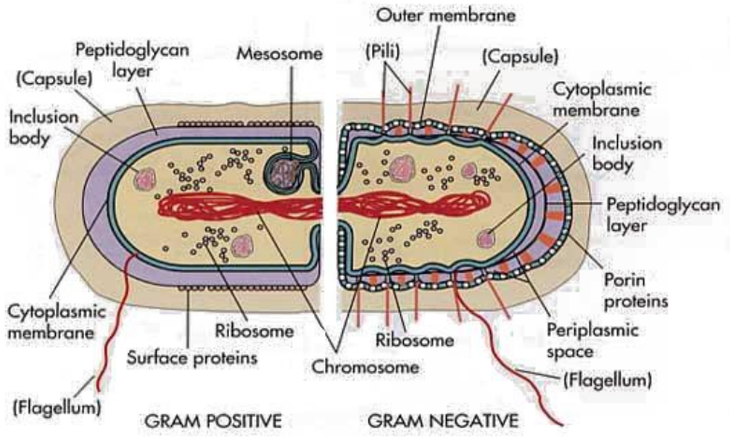


Allosteric (Mixed type)



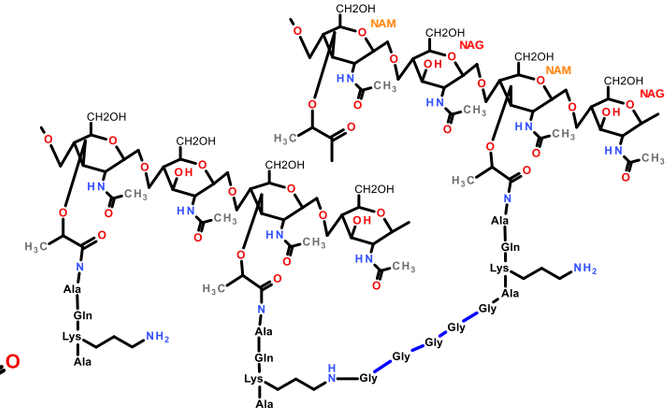
Bacterial Cell Wall

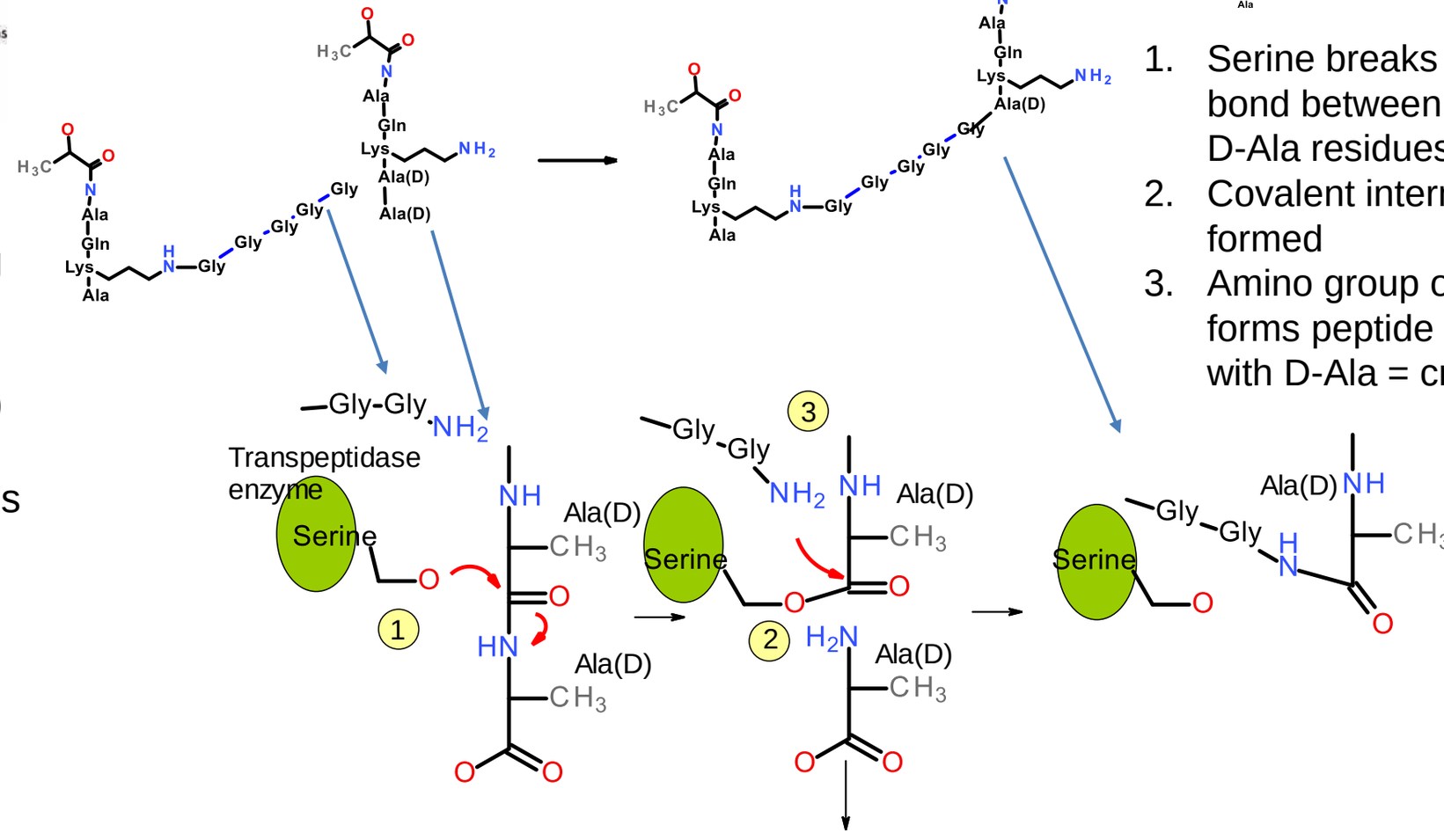
Mechanism of Penicillin – A Suicide Inhibitor



Bacterial cell wall:

- Linear polymers of alternating NAM (N-acetylmuramic acid) and NAG (N-acetylglucosamine), beta(1-4) linkage
- NAM units on adjacent strands are linked via a peptide linker.
- Crosslinking catalyzed by serine-containing ***transpeptidase.***



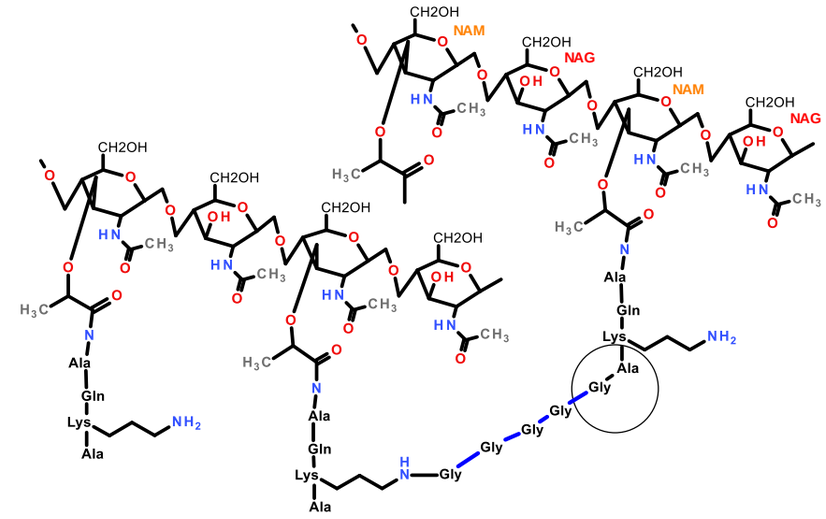


1. Serine breaks peptide bond between terminal D-Ala residues
2. Covalent intermediate formed
3. Amino group of glycine forms peptide linkage with D-Ala = crosslink

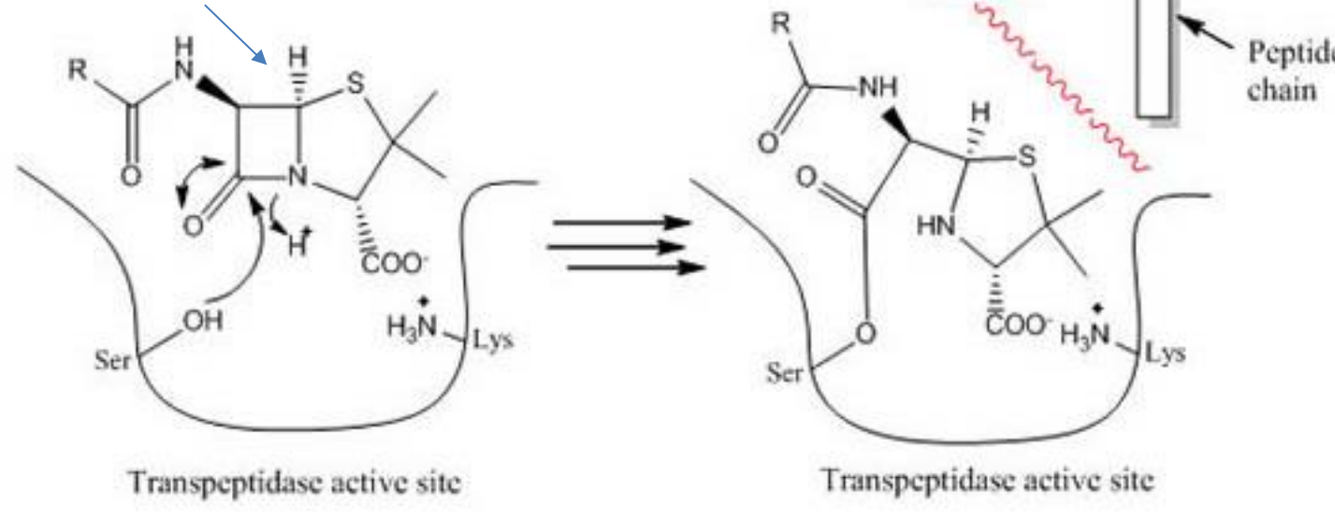
Mechanism of Penicillin

Mechanism of Action of Penicillin:

- Penicillin inhibits the transpeptidase enzyme that is responsible for crosslinking the Gly_5 chain to alanine (circled on diagram).
- The crosslinking of the cell wall is broken, making the bacteria fragile to breakage.
- Inhibition is by formation of a chemical bond between penicillin and the enzyme (covalent inhibitor).



Penicillin

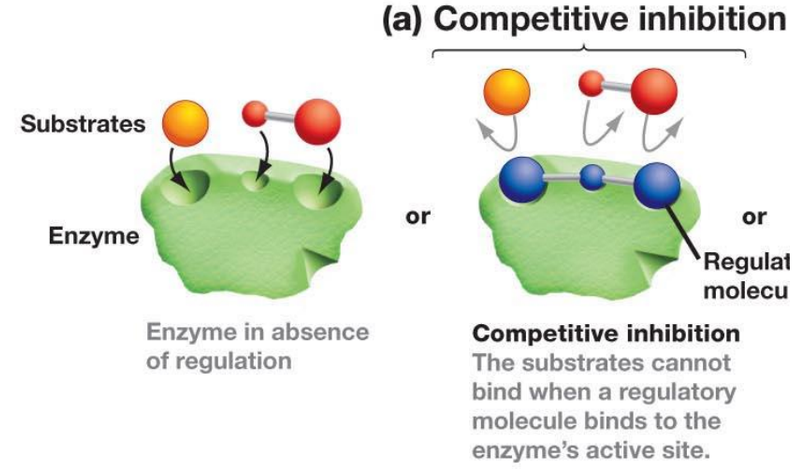


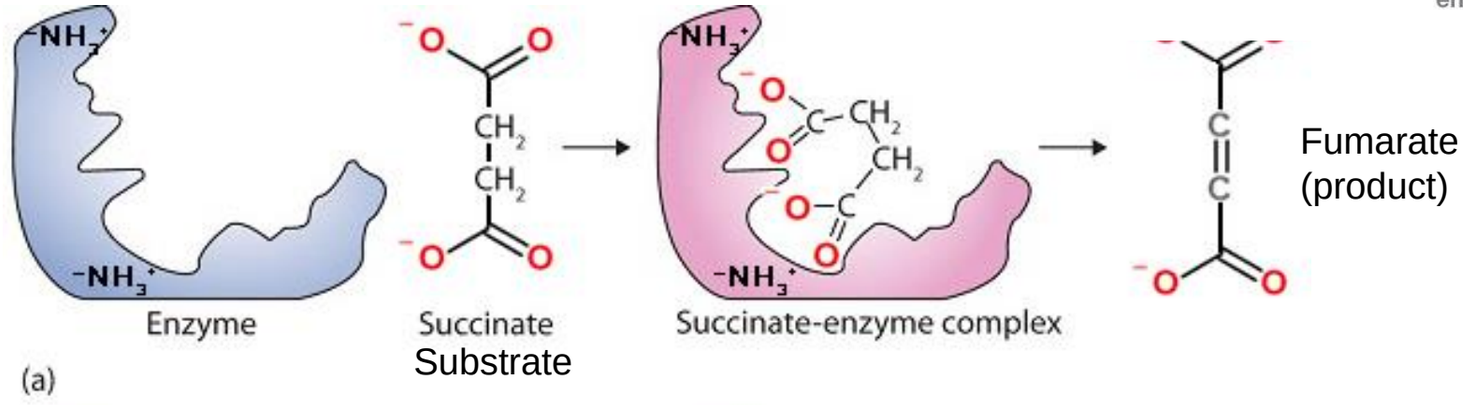
Competitive Inhibitors

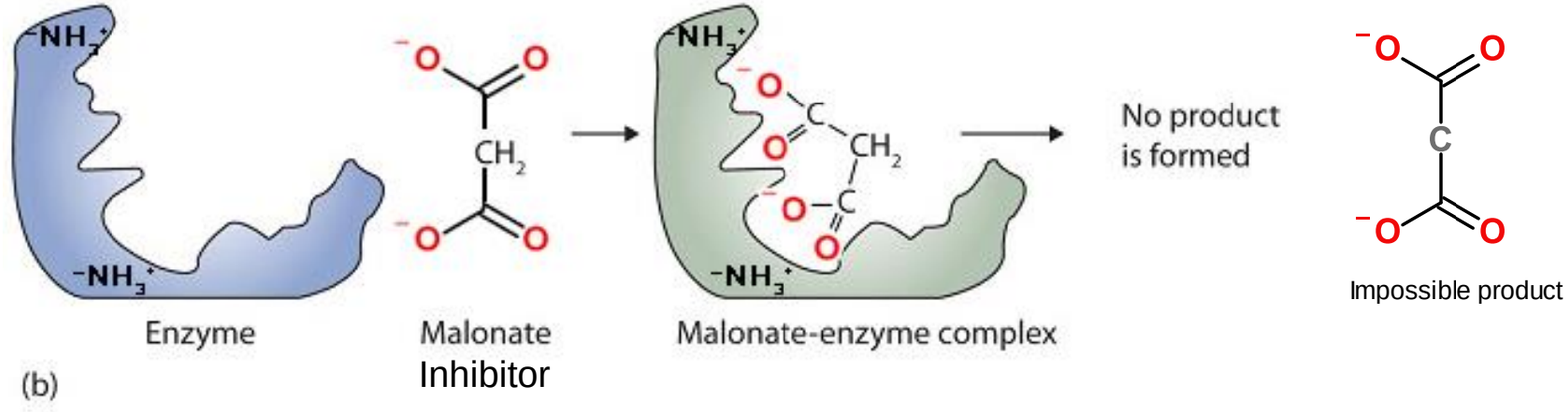
Succinate dehydrogenase converts succinate to fumarate by removal of two hydrogens.

Malonate is a **competitive inhibitor**, because:

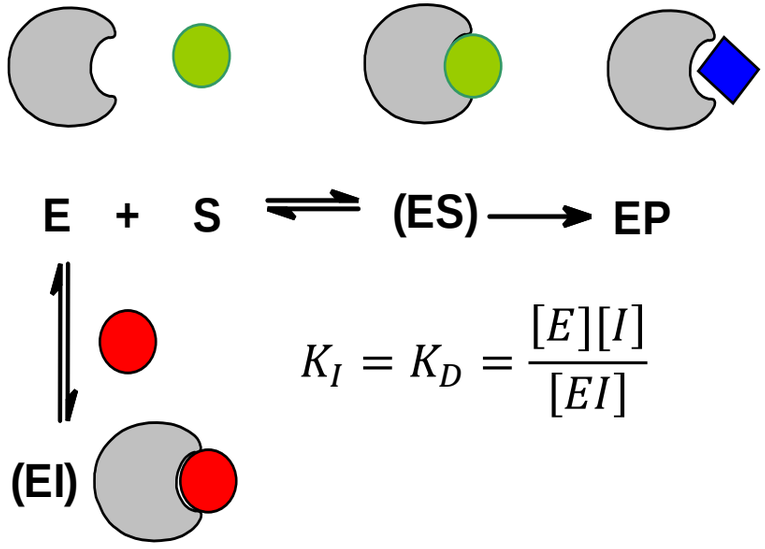
- It is similar in structure to the substrate – so it binds in active site – substrate cannot bind at the same time.
- Malonate **cannot** undergo the chemical reaction – it is not possible to remove two hydrogens without leaving carbon with too few bonds.





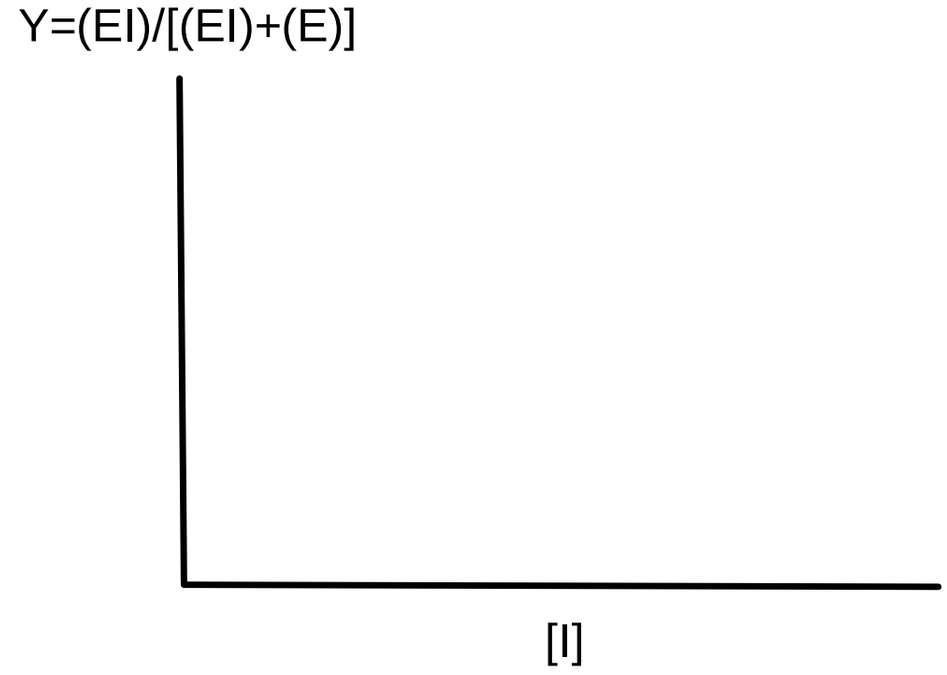


Quantification of Inhibitor Binding



$$K_I = K_D = \frac{[E][I]}{[EI]}$$

Fractional Saturation of Enzyme by Inhibitor



K_I = equilibrium constant for dissociation of inhibitor from enzyme

Low K_I = higher affinity (same principle as K_D)

K_I can be found from ½ point in binding curve

K_I can be determined by measuring the effect of inhibitor on the enzyme kinetics.

Effect of Competitive Inhibitor on Steady-State Kinetics:

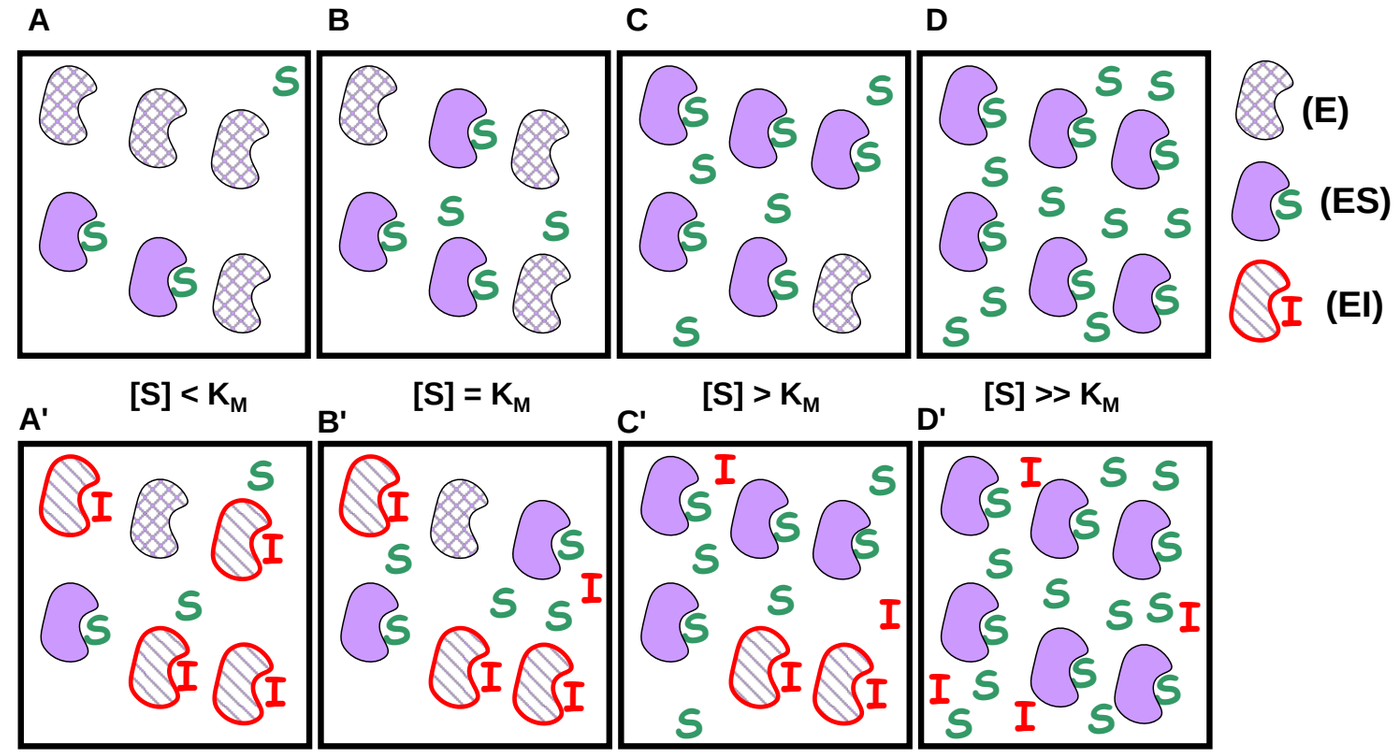
- A competitive inhibitor reduces the amount of [E] by the formation of [EI] complex.
- The inhibitor cannot affect the [ES] complex since the inhibitor can no longer bind.

There are two consequences of a competitive inhibitor binding on the kinetics of the enzyme:

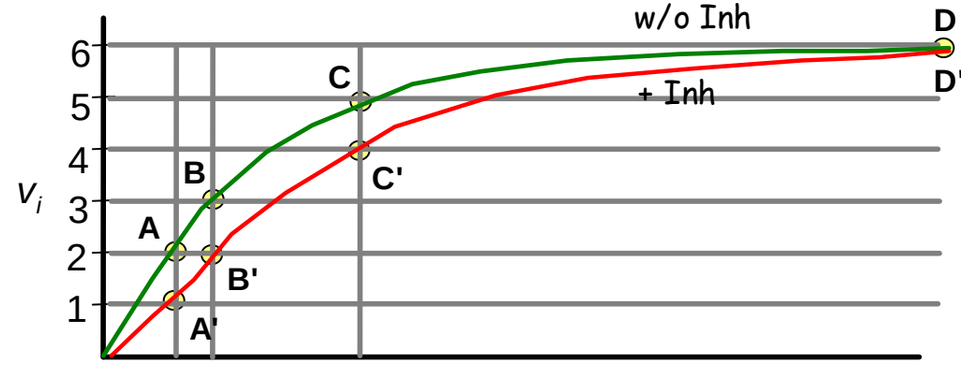
1. **V_{MAX} is unchanged**: At high levels of substrate all of the inhibitor is displaced by substrate, so [ES]=E_{TOTAL}, and v_{MAX} = $k_{CAT}[E_{TOT}]$.
2. **The *observed* K_M is increased:** It requires more substrate to reach 1/2 maximal velocity because some of the enzyme is complexed with inhibitor.

$$K_M^{OBS} = \alpha K_M$$

The change in K_M can be used to determine how well the inhibitor binds to the free enzyme, *if we know how α is related to K_I.*



v = k_{cat} [ES], k_{cat} =1



No inhibitor

$$v = V_{MAX}\frac{[S]}{K_M + [S]}$$

Comp inhibitor

$$v = V_{MAX}\frac{[S]}{\alpha K_M + [S]}$$

$$\alpha = 1 + \frac{[I]}{K_I}$$

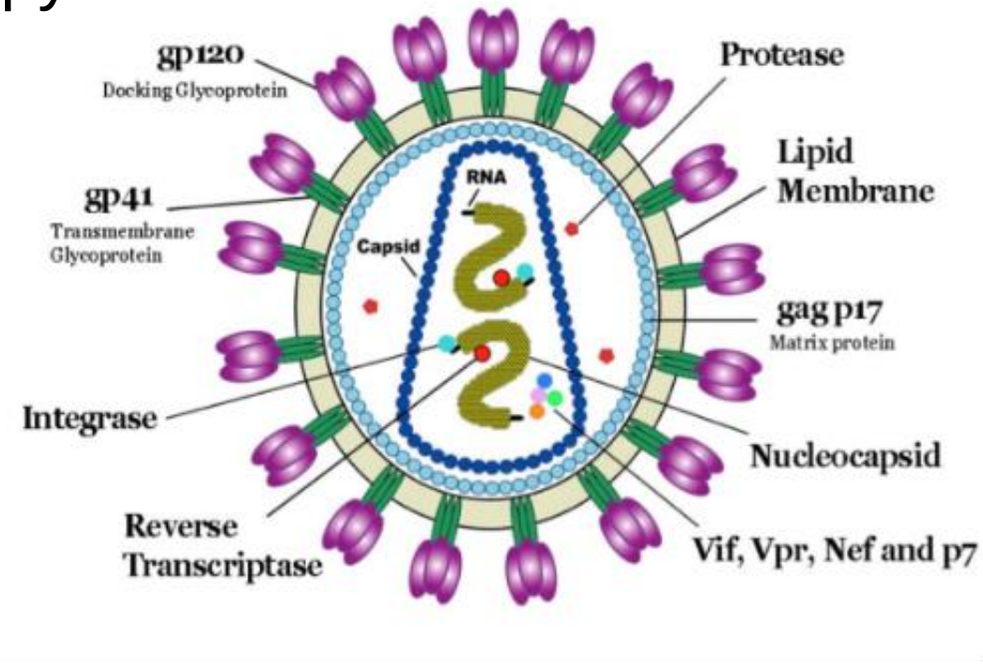
HIV Drug Therapy

Retroviruses & Inhibitors - HIV Protease.

- Identify potential drug targets, based on viral life cycle.
- Measure inhibitor binding to characterize drug efficiency.
- Rational drug design in response to mutations.

Human Immunodeficiency Virus (HIV)

- Infects specialized cells in the immune system – *T-helper cells* (T_H) cells, killing them.
- T_H cells are required for activation of the immune response to all pathogens (bacteria, virus)
- Killing of T_H cells by the HIV virus causes AIDS (acquired immunodeficiency), making the individual susceptible to serious infection by many otherwise harmless bacteria as well as developing rare cancers.



Viral particle contains enzymes required for the replication of the virus:

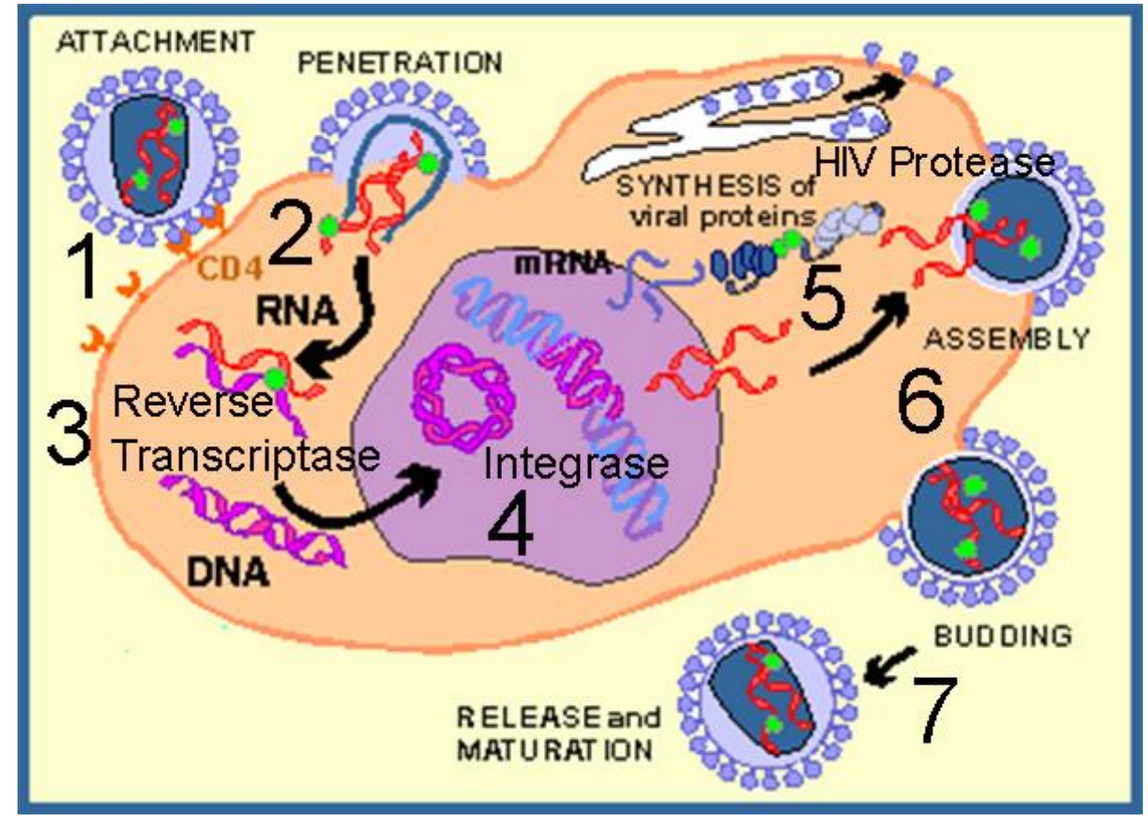
- ***Reverse Transcriptase:*** Copies viral RNA to DNA
- ***Integrase:*** Integrates viral DNA into host chromosome.
- ***HIV Protease:*** Cleaves immature viral protein to produce smaller mature proteins.

The HIV virus is a ***retrovirus***:

The genetic information is stored in RNA (viral RNA, vRNA) which must be first be copied into DNA: vRNA → DNA → mRNA → viral protein

HIV Viral Infection of T-Helper Cells:

1. Viruses bind to molecules displayed on the T_H cell surface.
2. The virus then fuses with the cell membrane and releases its RNA genome from its lipid envelope.
3. The HIV enzyme **reverse transcriptase** first makes a double-stranded DNA copy of the viral RNA molecule. This process is error prone, leading to mutations in the virus. ***These mutations cause drug resistant strains of the virus to arise.***
4. The DNA is integrated into the host cell's DNA by an enzyme called **integrase, also from the HIV virus.**
5. Integrated DNA produces vRNA, the genetic material for new virus particles. mRNA is also made from this DNA, to produce proteins for new particles.
6. **HIV protease** required for maturation of viral proteins, by cleaving them into smaller proteins that form the mature virus.
7. Mature virus buds out of cell.



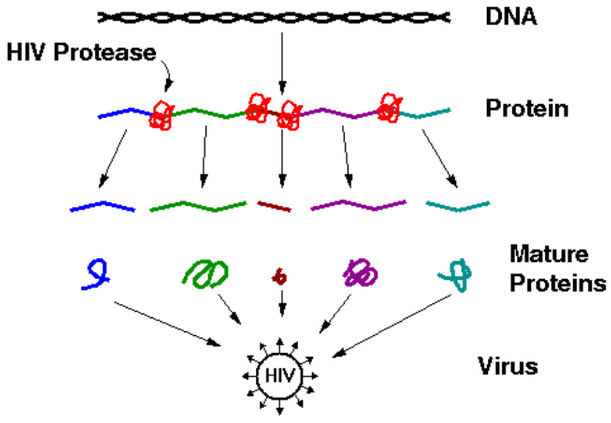
Drug Targets to Combat the HIV Virus –

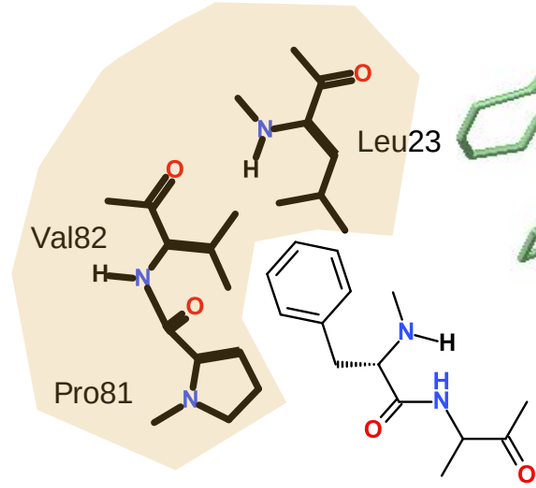
a) Viral fusion
b) Reverse transcriptase
c) Integrase
d) HIV Protease

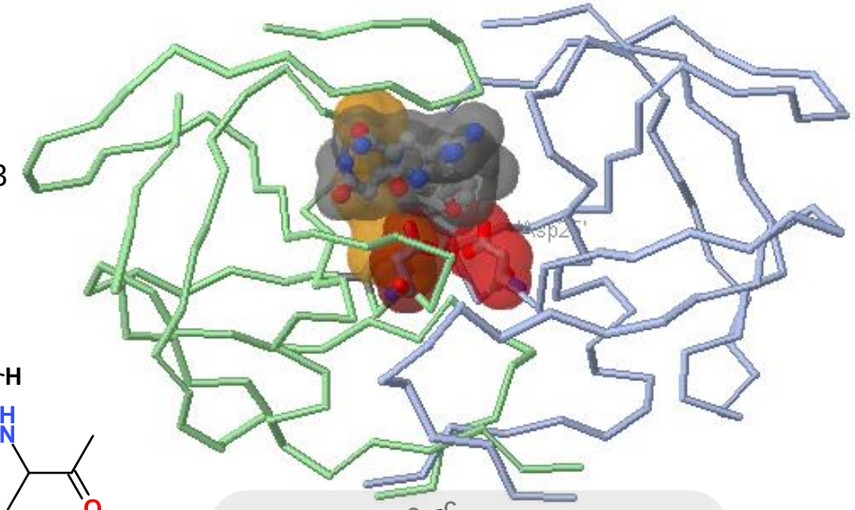
These are good drug targets because:

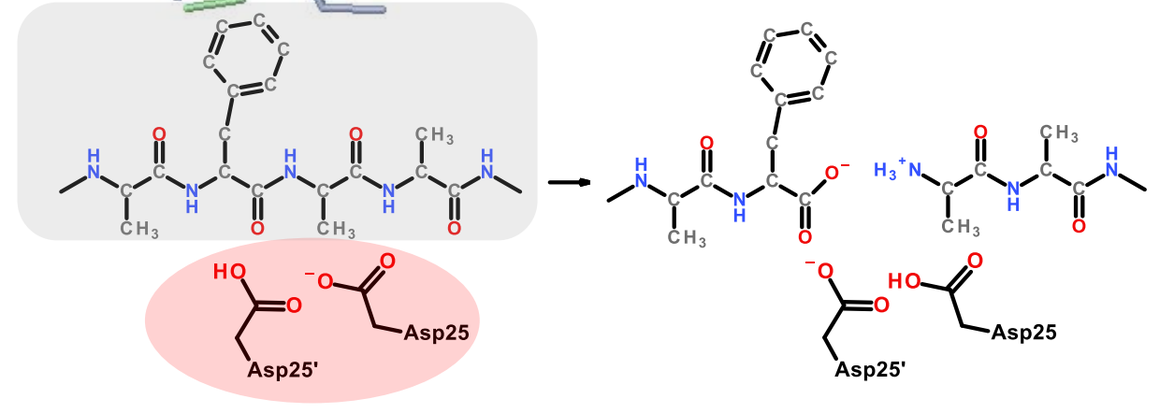
- Required for viral replication
- Activities are not found in humans

HIV Protease (Aspartyl protease)









- The original viral protein is a long pre-protein containing many smaller mature proteins.
- HIV Protease cleaves the pre-protein, releasing the smaller mature proteins.

HIV Protease:

1. An essential enzyme in the maturation of the HIV virus. If inhibited, the virus cannot replicate.
2. Prefers hydrophobic substrates (e.g. Phe) due to Val82 plus other non-polar residues in its active site (Pro81, Leu23).

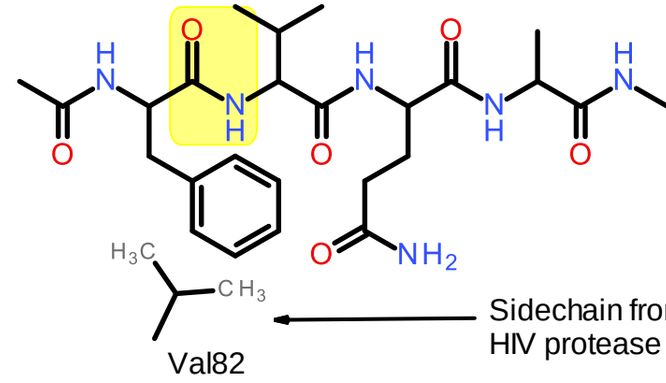
Inhibition of HIV Protease (HIV Drugs):

- Most drugs are small peptide-like analogs with non-cleavable bonds that resemble peptide bonds.

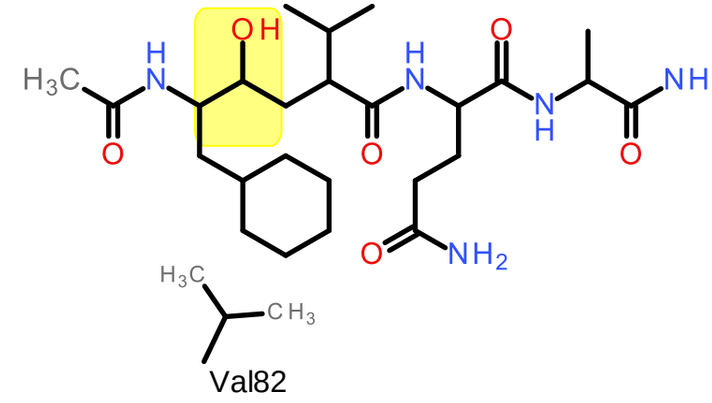
Where will they bind on the enzyme?

What will happen to them after they bind?

Substrate

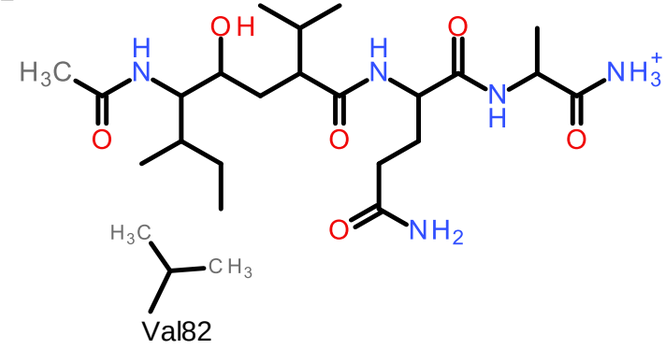


Inhibitor

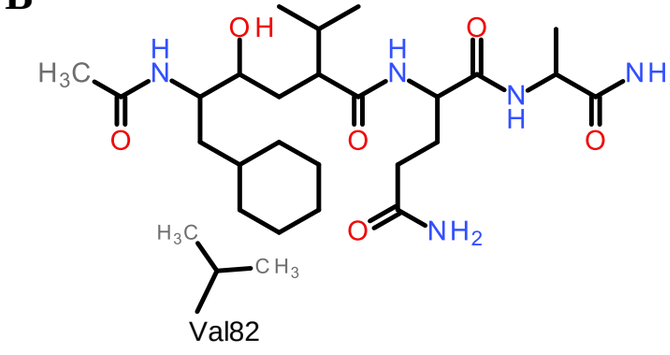


Drug Design: Compounds A (Isobutyl) and B (cyclohexane) are candidates for HIV protease inhibitors. Which of the two drugs will be more effective at inhibiting the wild-type protease?

A



B



Answer: We will assume that these are competitive inhibitors. Therefore**,** we need to compare the K_I values for each inhibitor binding to the protease.

Measuring K_I for both Drugs:

a) Acquire velocity versus substrate, no inhibitor.

b) Acquire velocity versus substrate, fixed inhibitor.

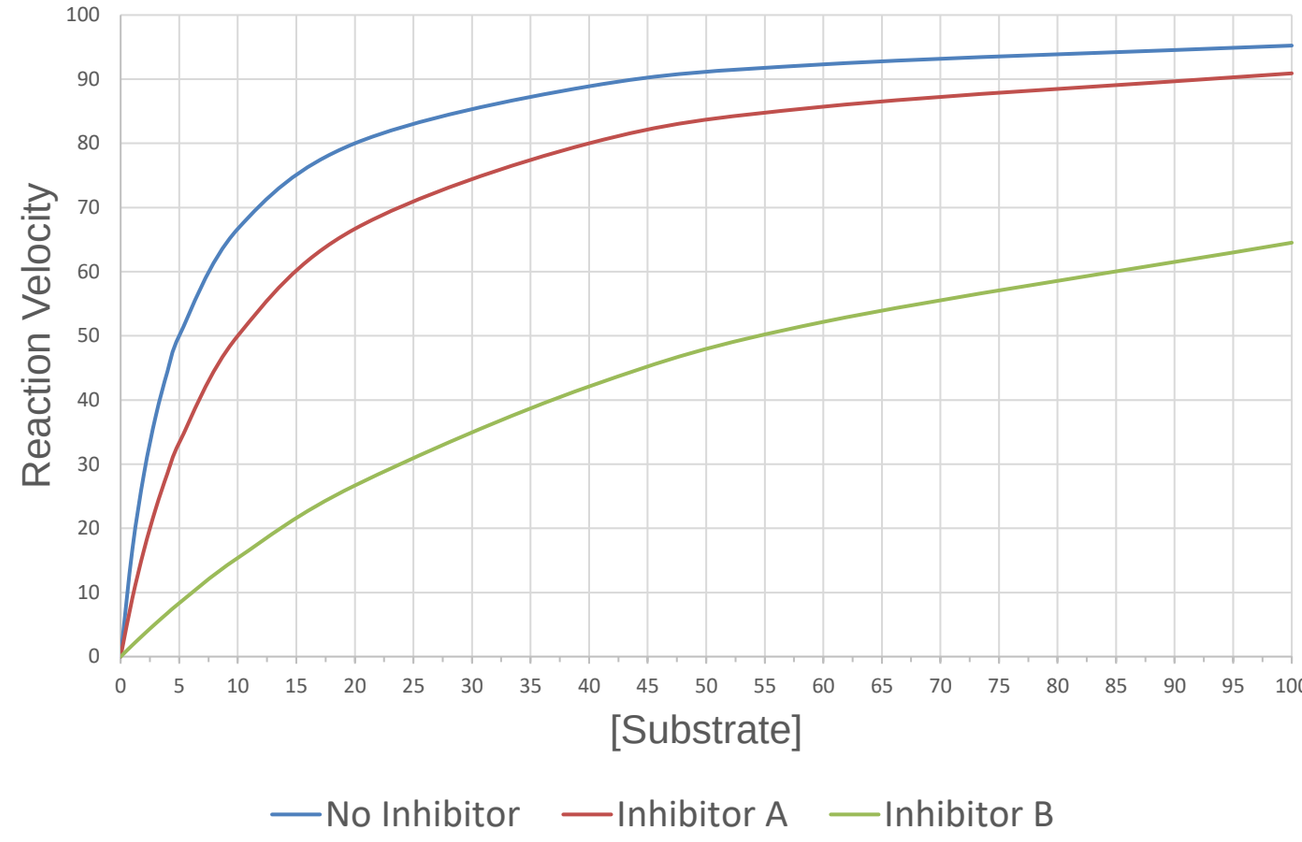
Analysis:

i) Plot velocity versus [S]

ii) Obtain α from the observed Km values

[S]	no inh	A	B
0	0	0	0
1	17	9	2
2	29	17	4
3	38	23	5
4	44	29	7
5	50	33	8
10	67	50	15
20	80	67	27
40	89	80	42
60	92	86	52
100	95	91	65

The units of velocity are μmoles product/sec.

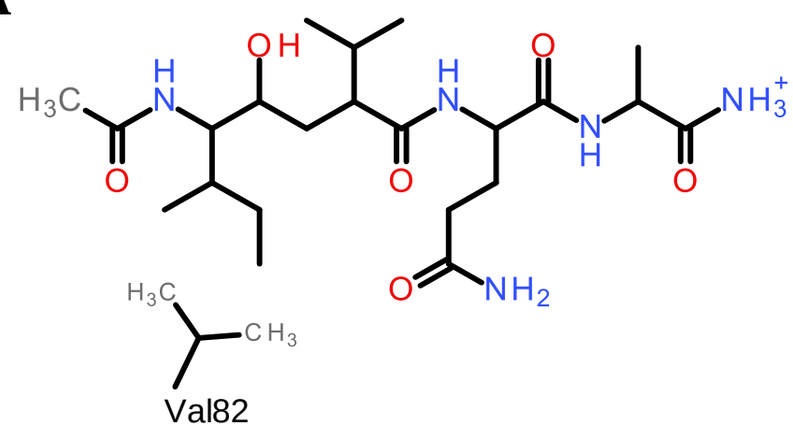


Once the α values are found, we can calculate the K_I for each inhibitor using the formula: **K_I=[I]/(α-1).**

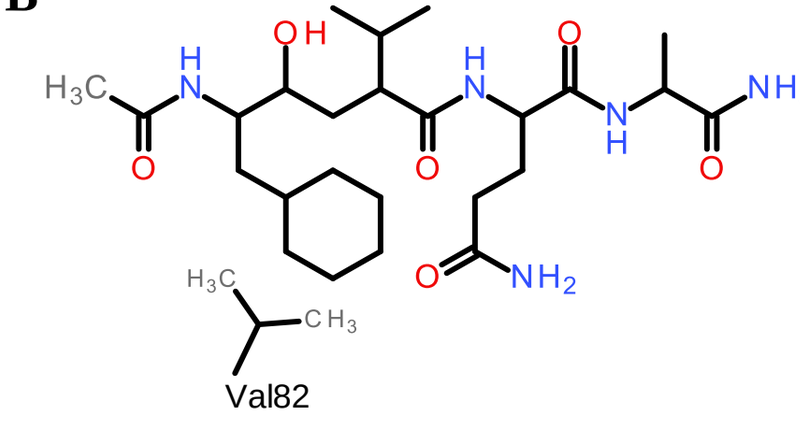
Data	Km	Alpha (K_M^{obs}/K_M)	K_I =[I]/(α-1) ([I]= 10 nM)
No Inh	5		
Inh A	10	2	K_I = 10/(2-1) = 10 nM
Inh B	54	10.8	K_I = 10/(10.8-1)=1.1 nM

Explain the difference in K_I based on the molecular interactions between each inhibitor

A



B

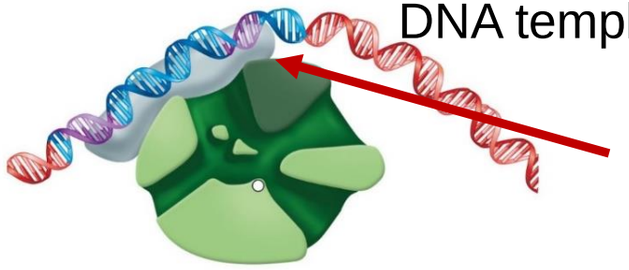


Potential Interaction	Drug A (K_I = 10 nM)	Drug B (K_I = 1.1 nM)
Van der Waals		
Hydrophobic effect		

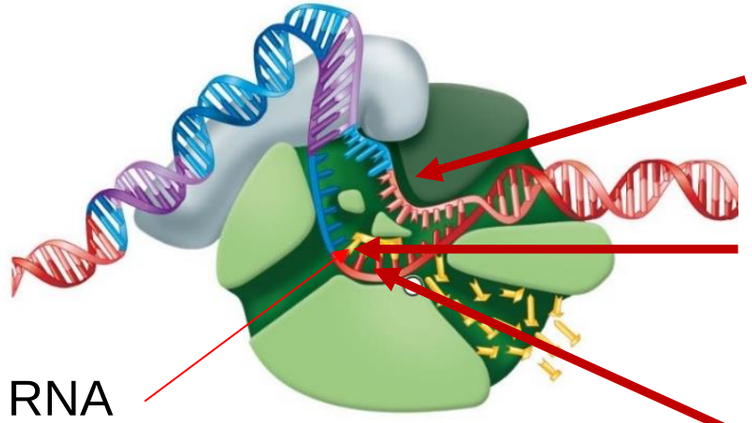
Drugs that inhibit Transcription and Translation

Drugs as inhibitors of Transcription:

Initiation

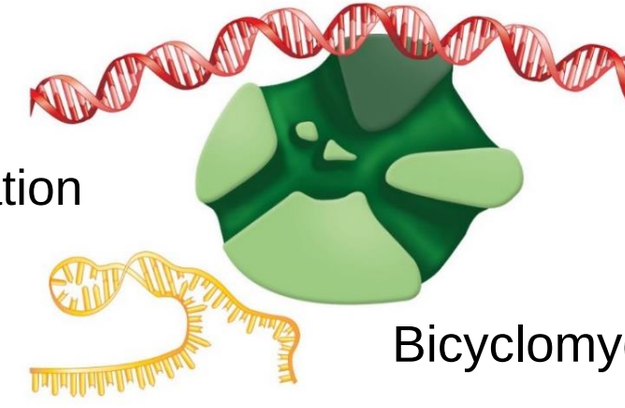


Elongation



- Transcription is the process of copying DNA to RNA
- RNA polymerase binds to a specific DNA sequence called the promoter
- RNAP generates a complementary copy of one strand, T is replaced by U.
- Termination occurs due to specific signals in the RNA sequence.

Termination



Protein Synthesis – tRNA & Ribosomes

Role of different Ribosomal subunits

30S (Small) – RBS & mRNA codon/anticodon
50S (Large) – Peptide bond synthesis
Exit tunnel – new protein emerges

tRNA sites:

A – aminoacyl – next tRNA-AA binds
P – 1st tRNA-Met & growing peptide
E – empty tRNA leave from here

Initiation:

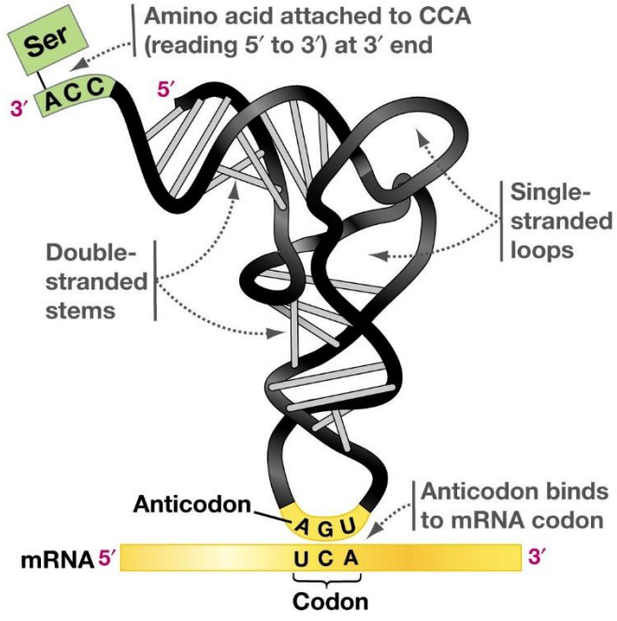
1. Ribosome binding site & rRNA interaction (Proks)/AUG scanning (Euks).
2. fMet-tRNA (Proks) or Met (Euks) in P site

Elongation:

1. New AA-tRNA in A site
2. Peptide bond formation (amino acid in A site added to C-term of peptide in P site)
3. Translocation (tRNA-peptide moves to P site)
4. tRNA exits

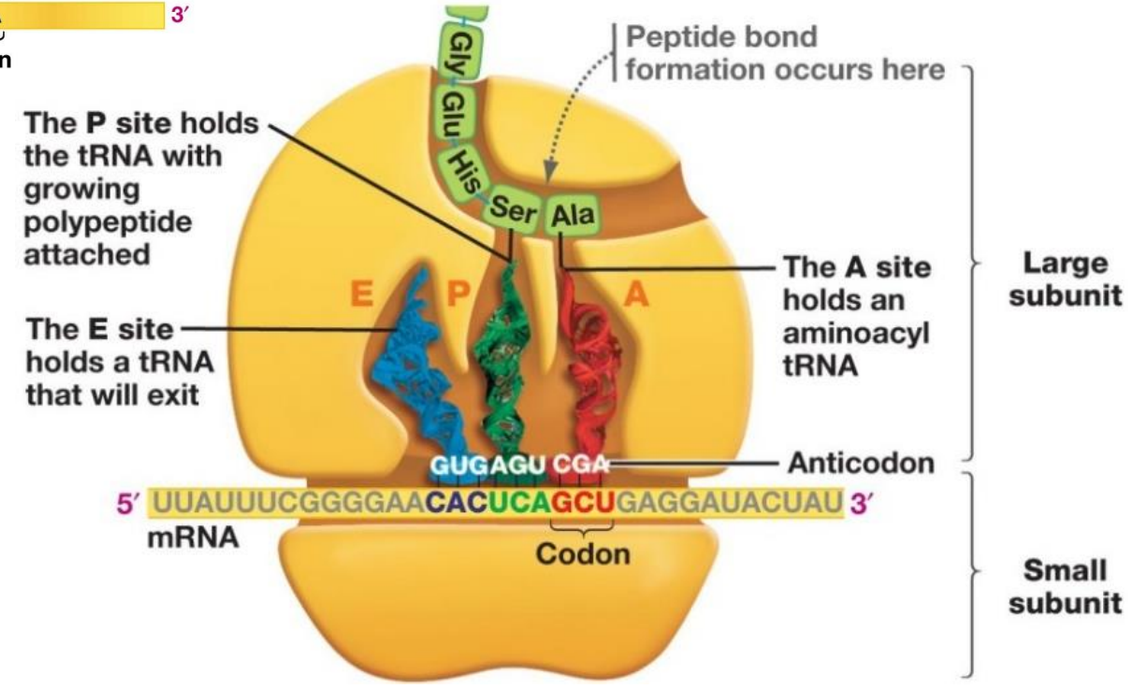
Termination:

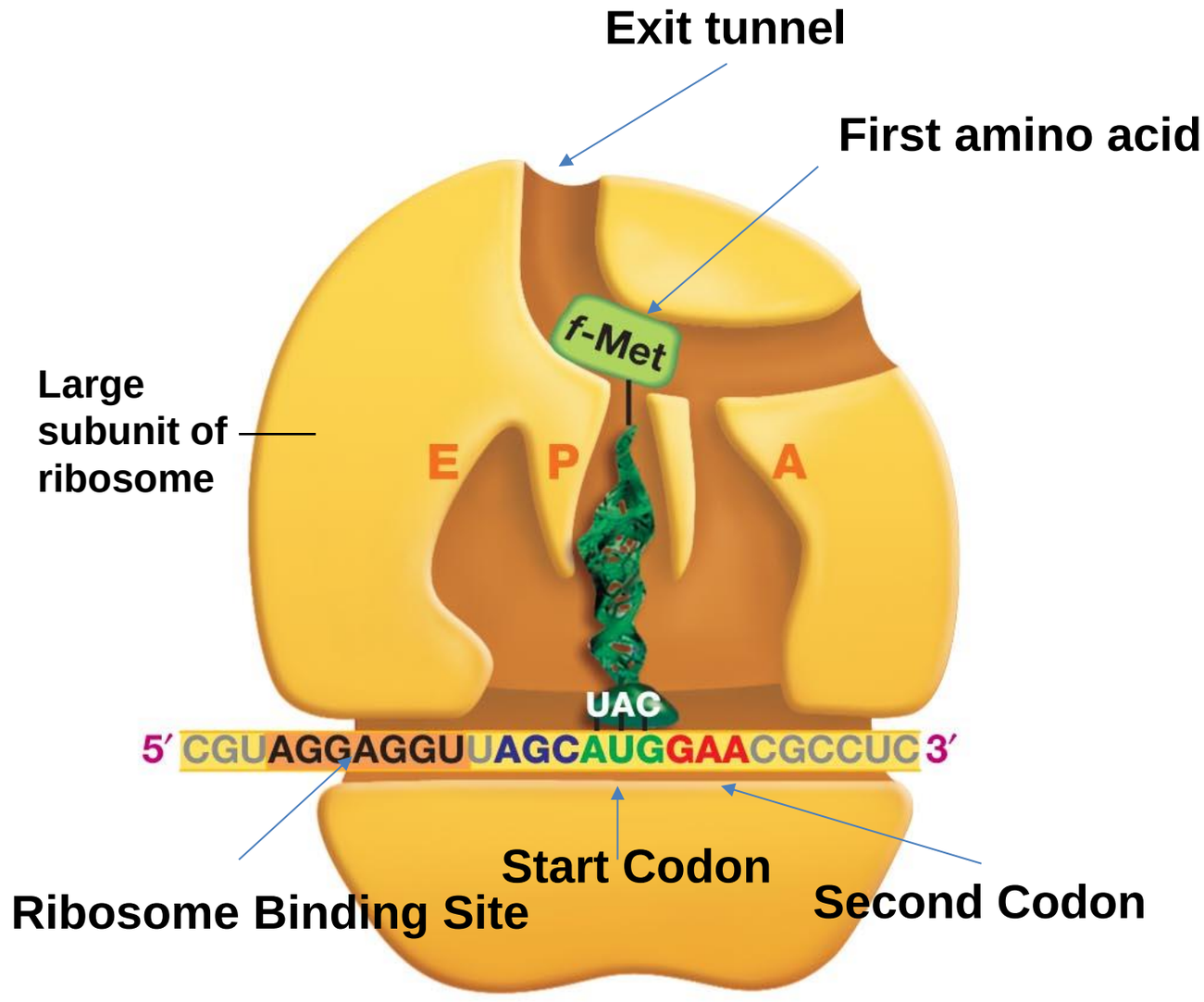
1. Stop codon at A site
2. Termination factor (protein) adds water to cleave peptide from last tRNA



tRNAs

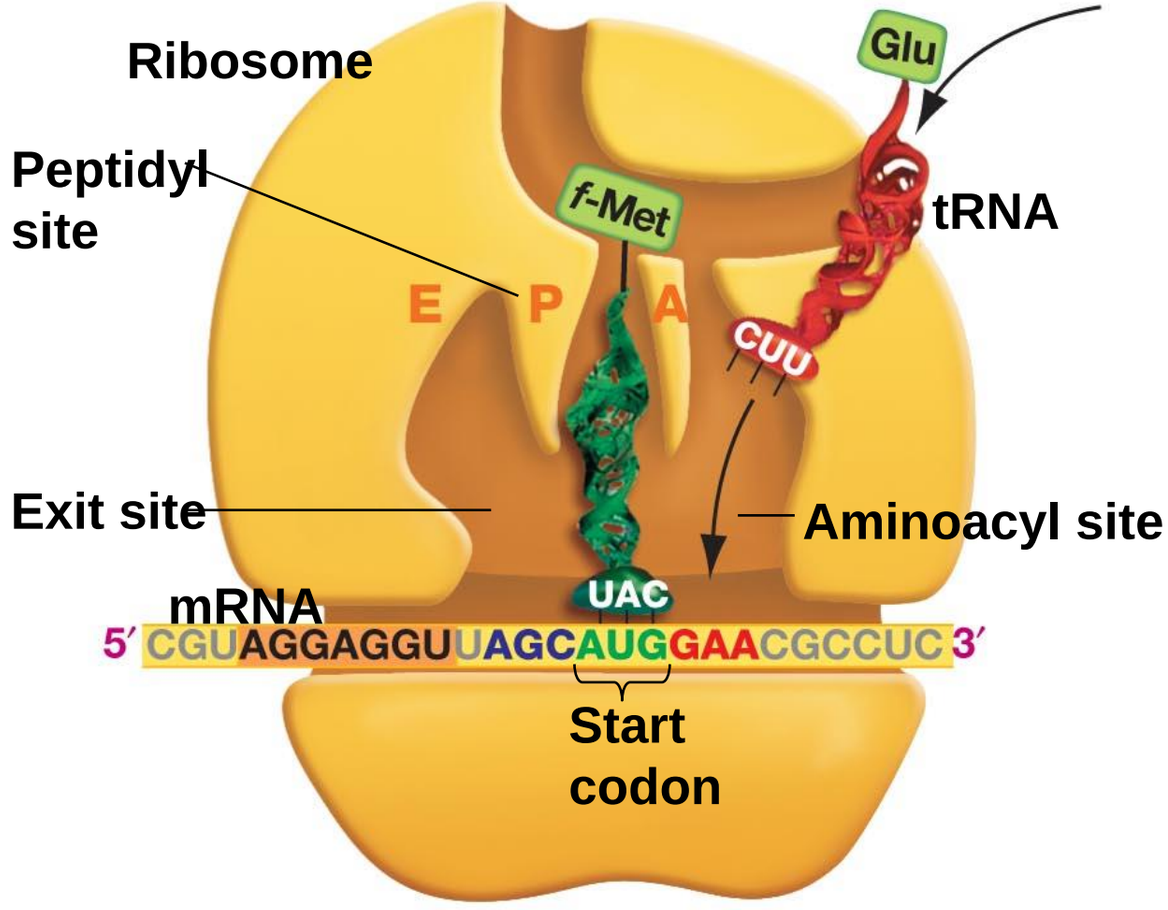
- The adapter molecules are called transfer RNAs or tRNAs.
- Contain a CCA sequence at 3’ end where the amino acid is attached
- a triplet anticodon to form base pairs with the appropriate mRNA codon



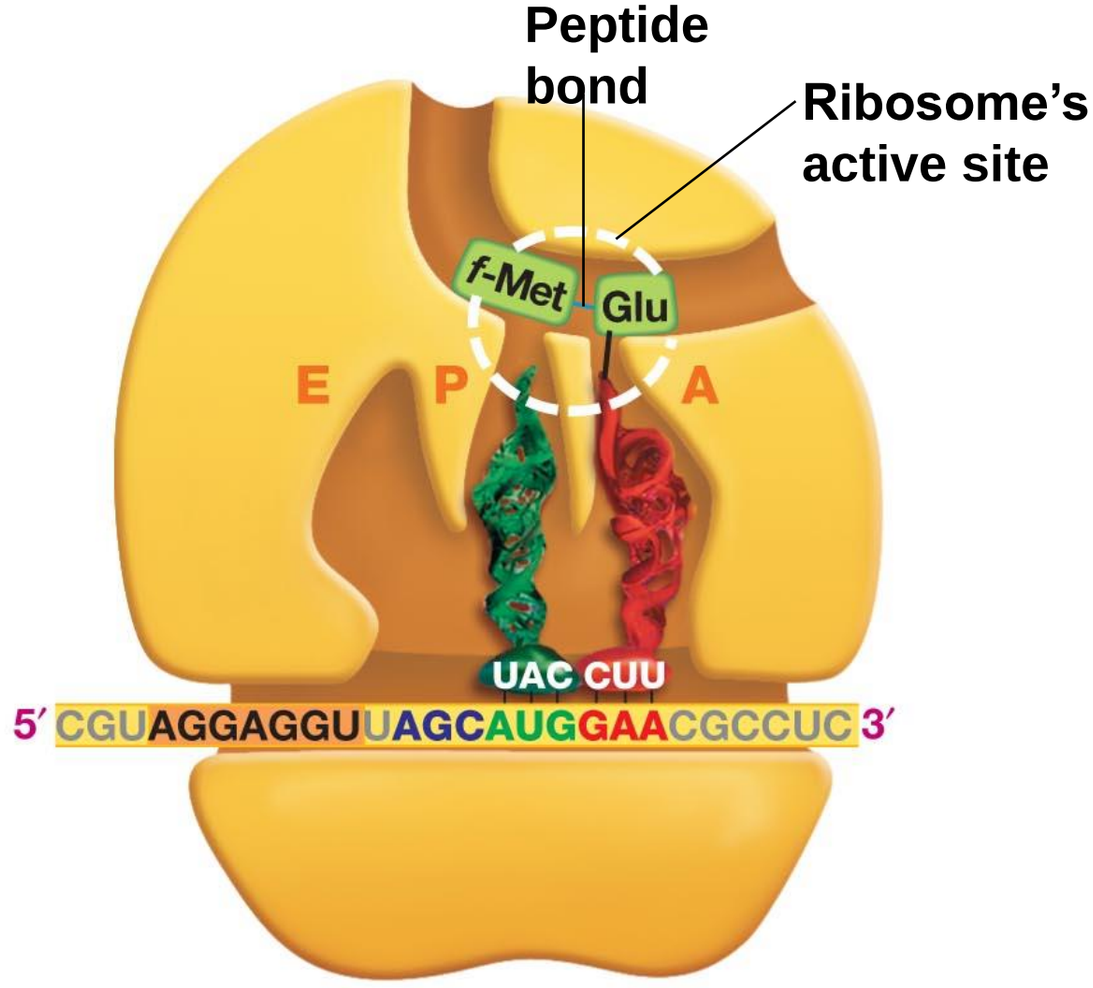


Initiation 3. Large subunit binds completing the complex

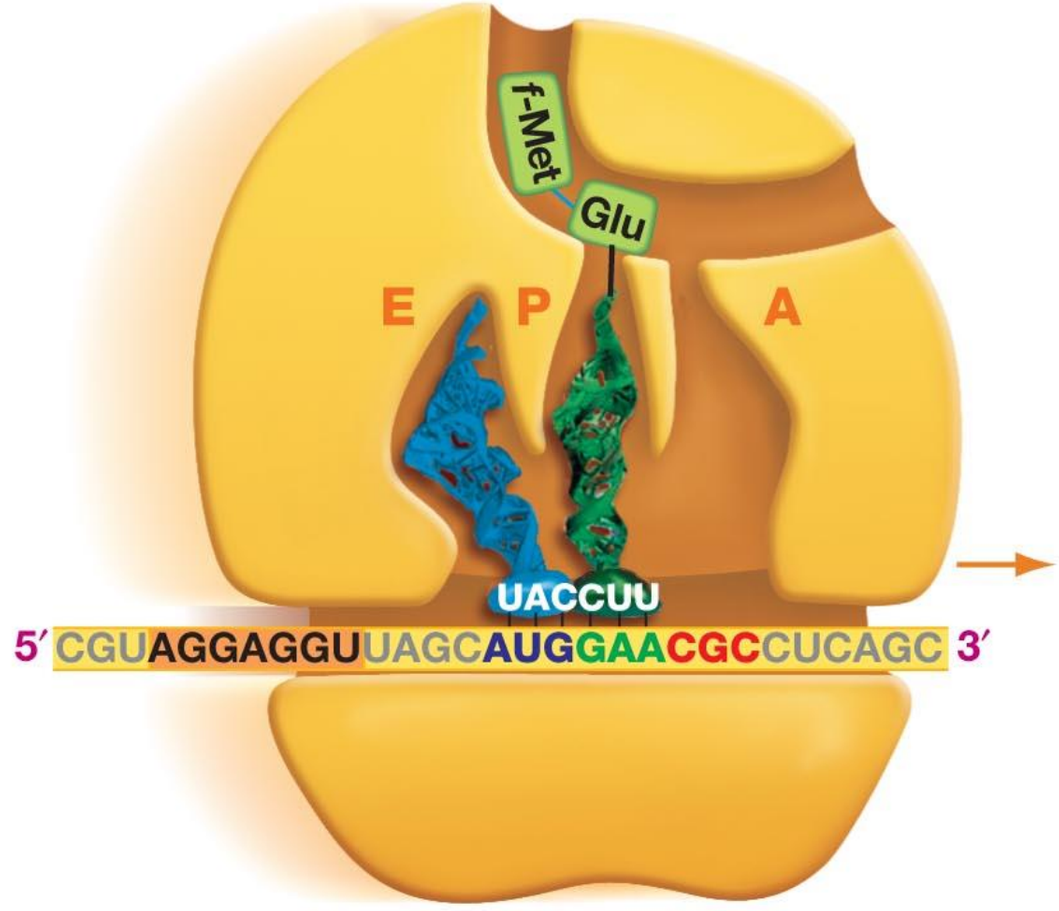
Step 2 - Elongation



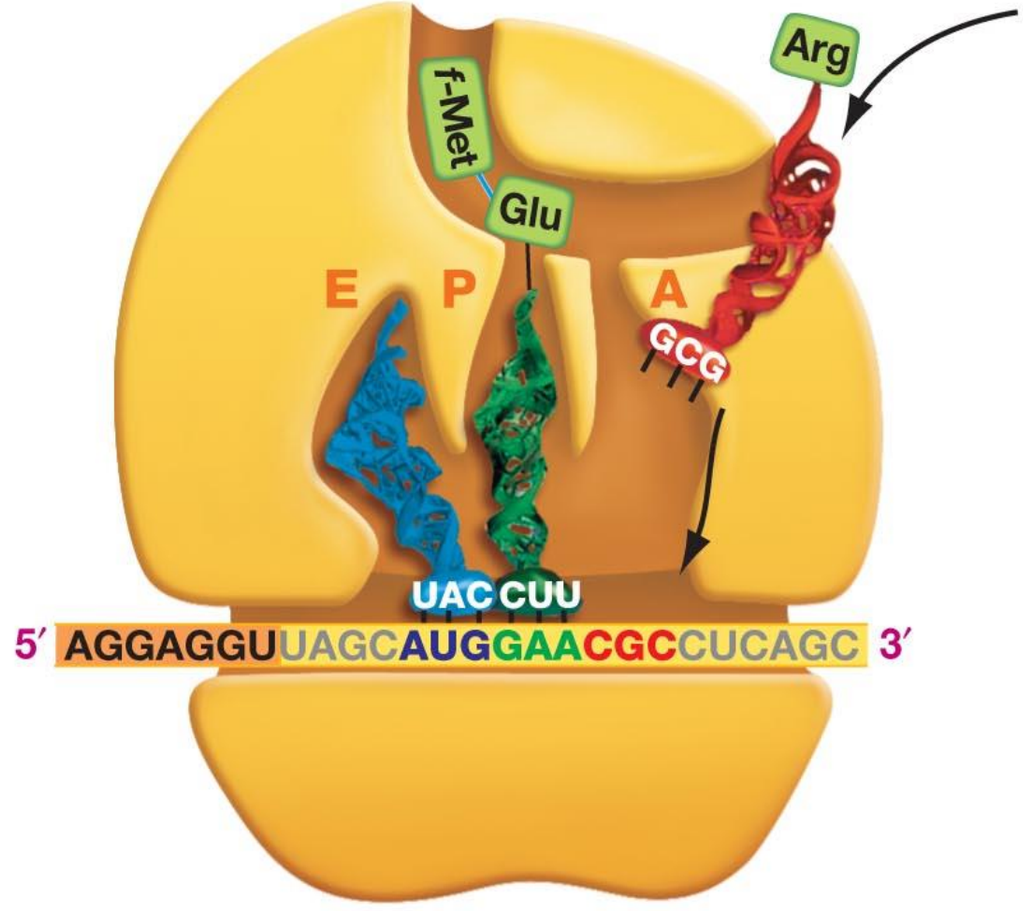
4. Incoming aminoacyl tRNA arrives and binds in A site



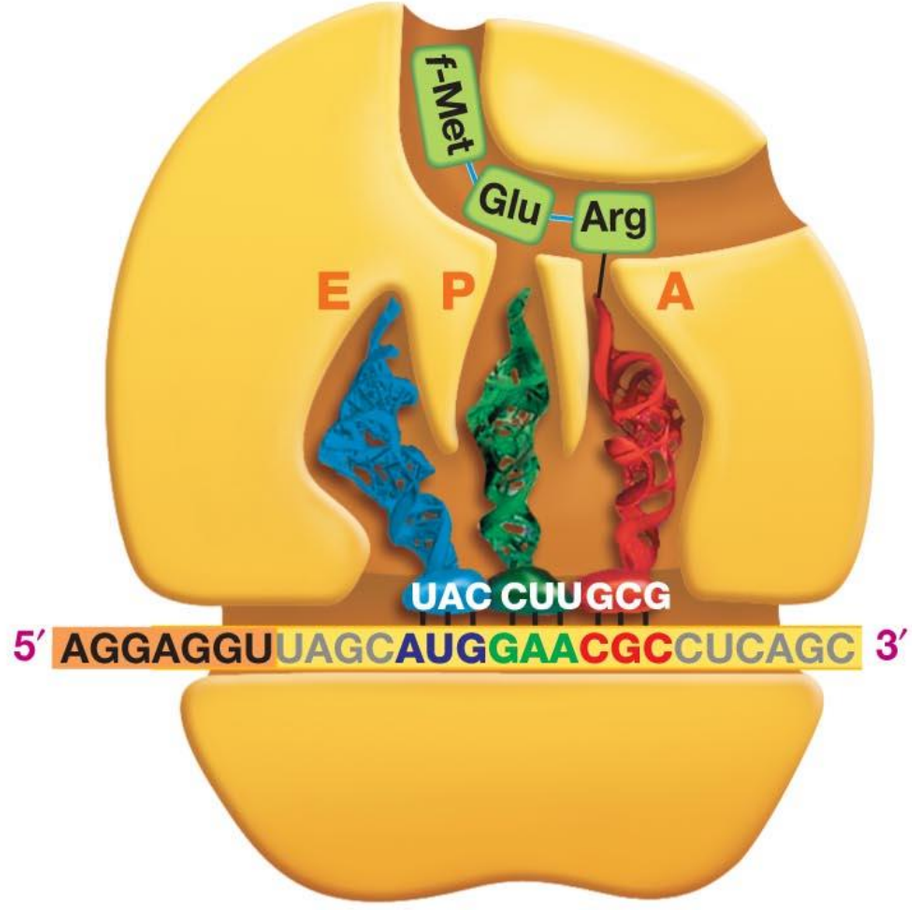
5. Peptide bond formation



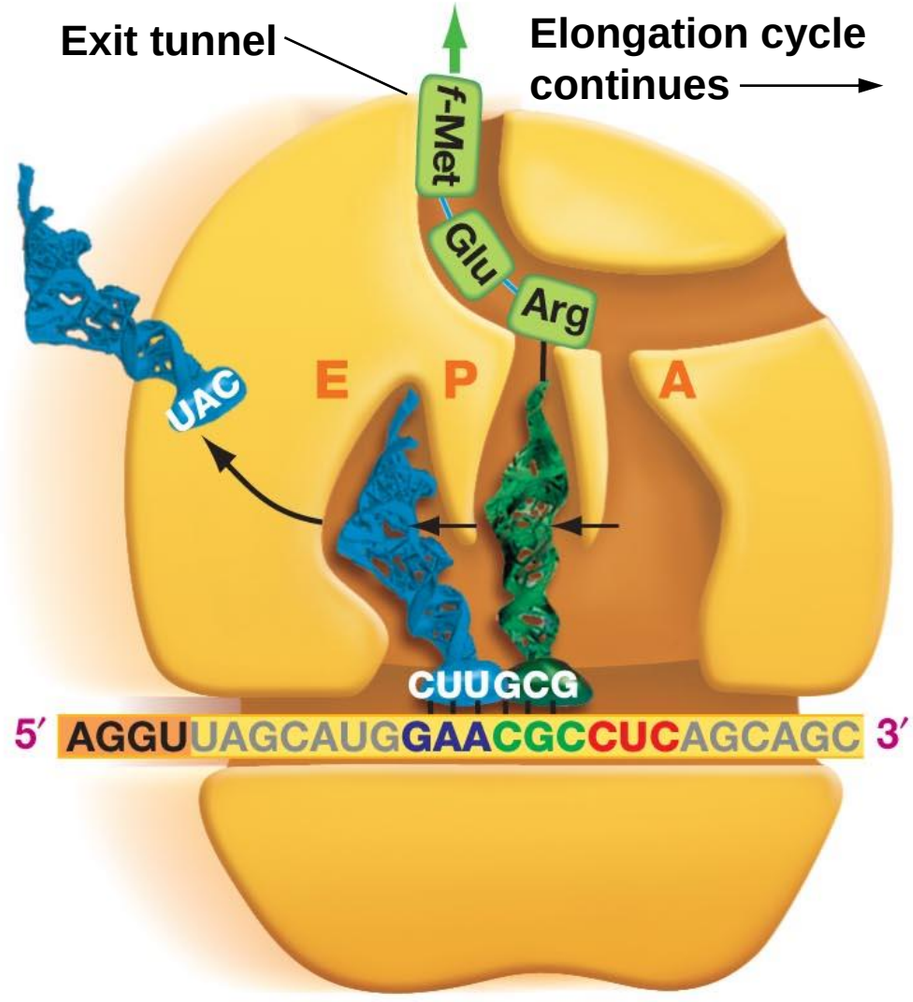
6. Translocation



7. Incoming aminoacyl tRNA arrives and binds in A site

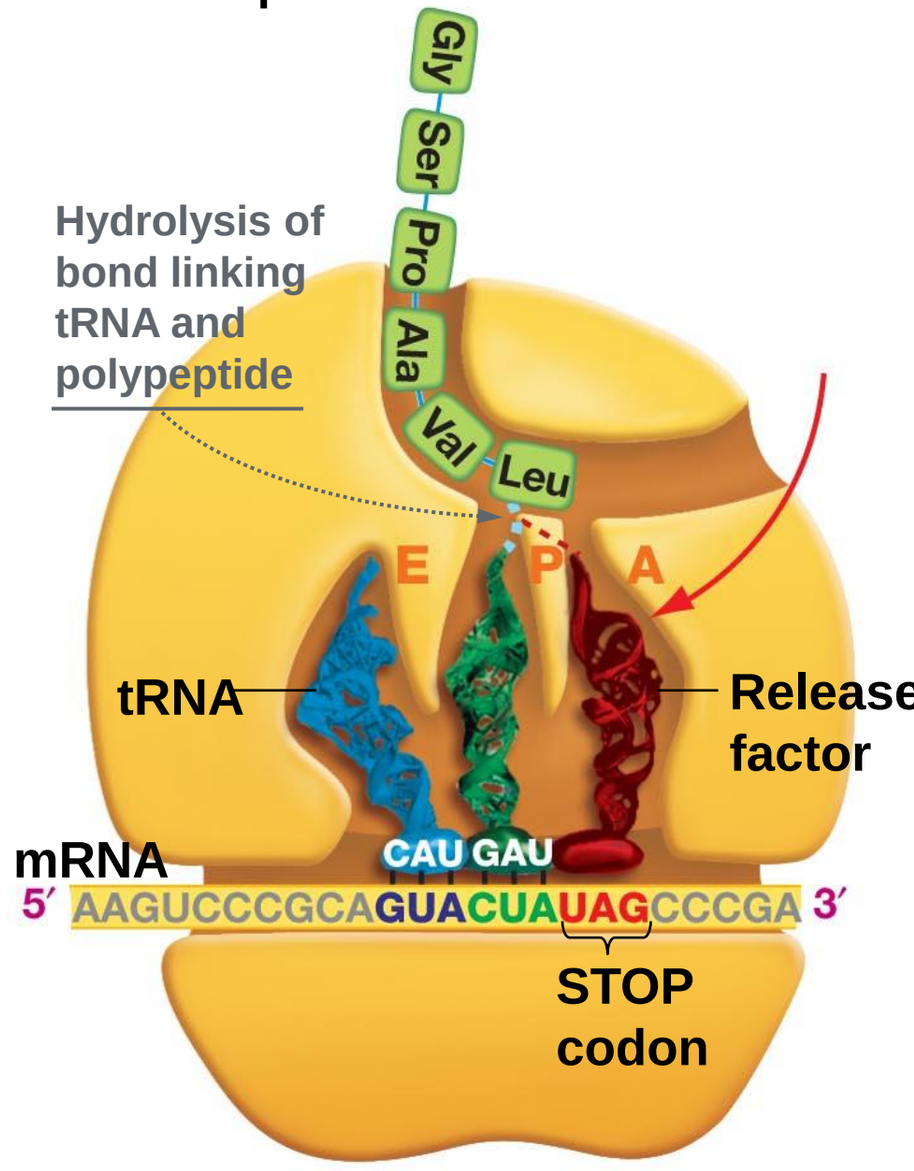


8. Peptide bond formation



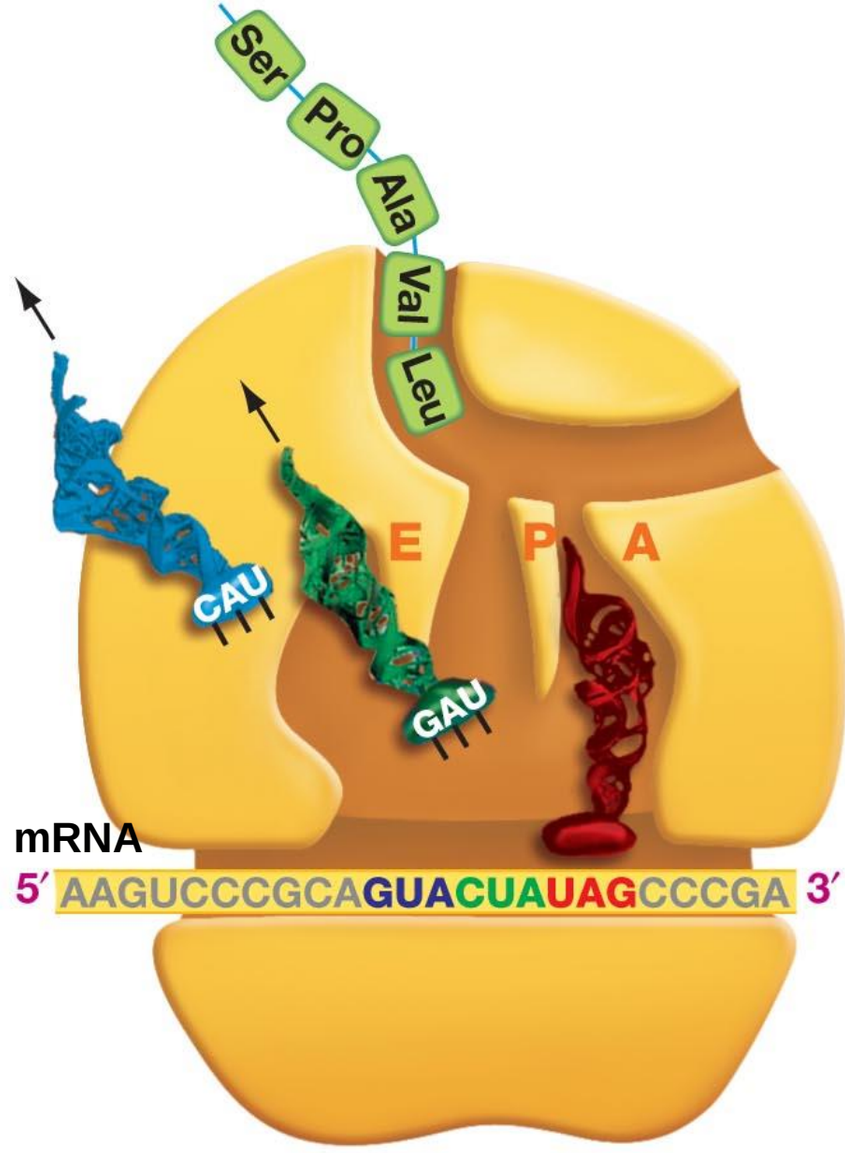
9. Translocation

Step 3 - Termination



10. Release factor binds to stop codon

When translocation opens the A site and exposes a stop codons, a protein called release factor fills the A site. This hydrolyzes the bond linking the tRNA in the P site to the polypeptide chain, releasing the protein.

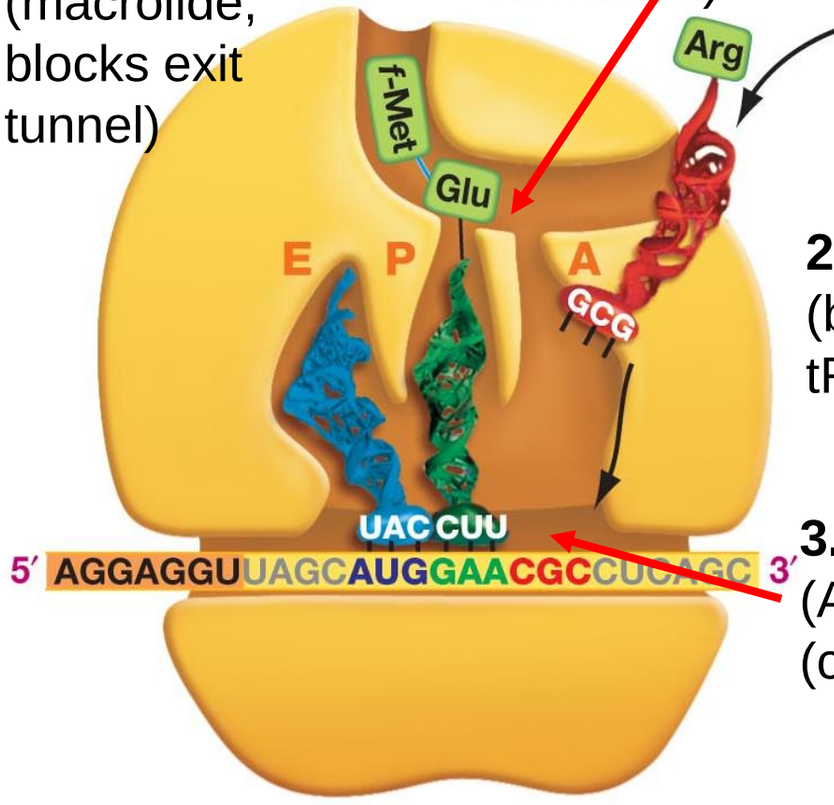


11. Polypeptide is released

Antibiotics that Inhibit Protein Synthesis

6. **E**rythromycin (macrolide, blocks exit tunnel)

4. **C**hloramphenicol (peptide bond formation)



5. **V**iomycin (blocks translocation)

2. **T**etracycline (binding of charged tRNA)

1. **L**inezolid (blocks 70s formation from two subunits)

3. **K**anamycin (Aminoglycoside) (codon/anticodon pairing)

Genome Editing

Genome Editing – CRISPR Cas9

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]†
Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

17 AUGUST 2012 VOL 337 **SCIENCE** www.sciencemag.org

The Nobel Prize in Chemistry 2020

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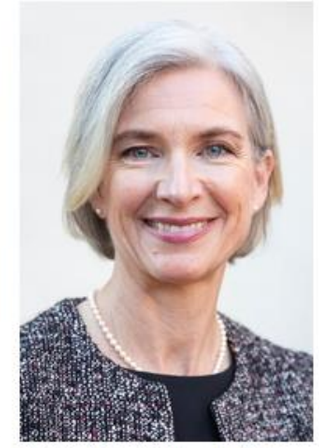
Emmanuelle Charpentier
Prize share: 1/2



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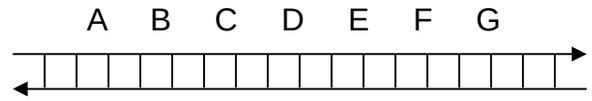
Jennifer A. Doudna
Prize share: 1/2

The Nobel Prize in Chemistry 2020 was awarded jointly to Emmanuelle Charpentier and Jennifer A. Doudna "for the development of a method for genome editing"

Key Concepts in Genome Editing

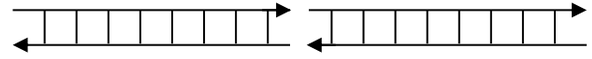
Repair of a targeted double strand break = modification of the genome at a defined location.



Genomic DNA (A, B, C, D, E, F, G represent short sequences)

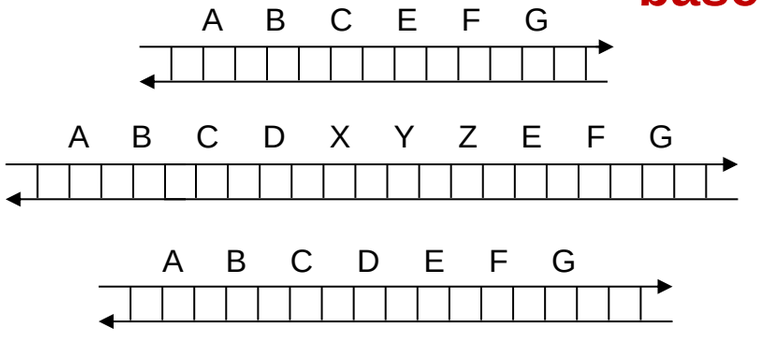
Nuclease (sequence specific)

Genomic DNA with double stranded break



Non-Homologous End Joining (NHEJ)

Repair Method I: Addition or deletion of bases



Deletion

Insertion

together called "***Indels***"

Restoration (undetected)

Original Sequence

```
--ATG......GGGTGGCCGATT...CGATAA—
--Met......GlyTrpProIle...Arg
```

Deletion of one base

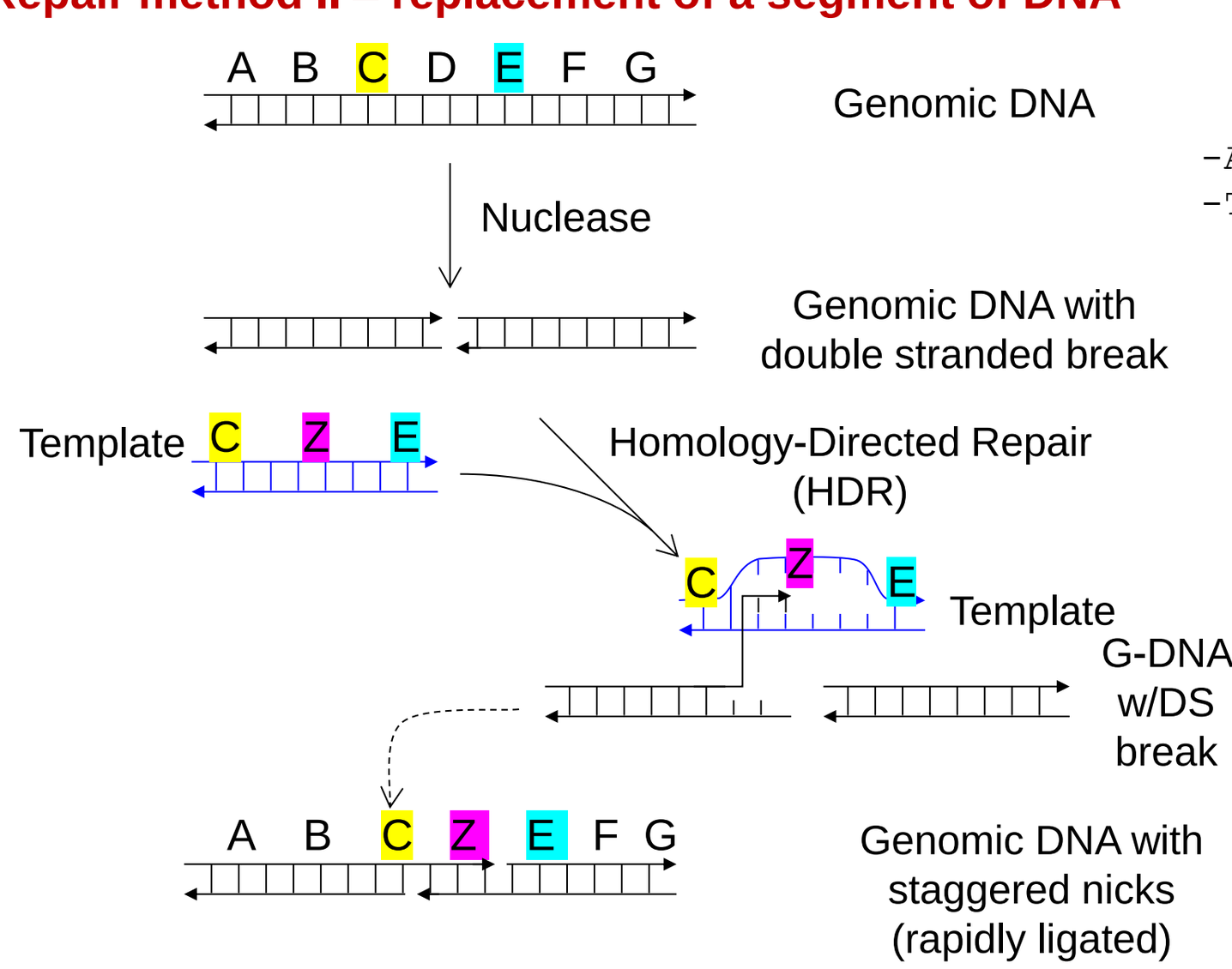
```
--ATG......GGGTGCCGATT...CGATAA—
--Met......GlyCysArgLeu.ArgIle...
```

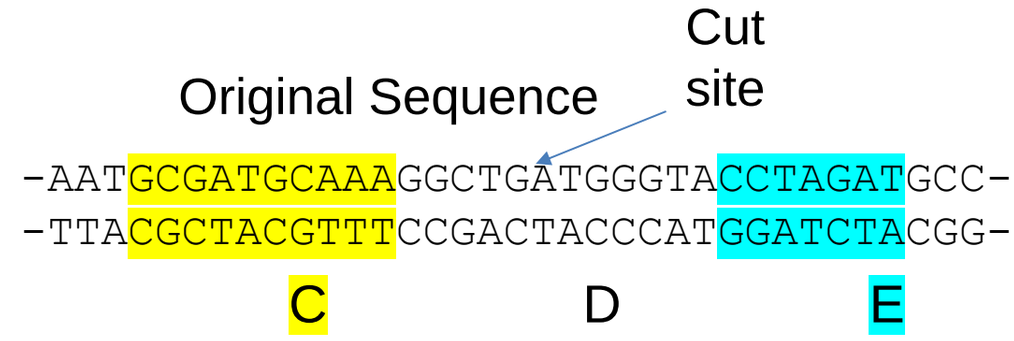
Indels:

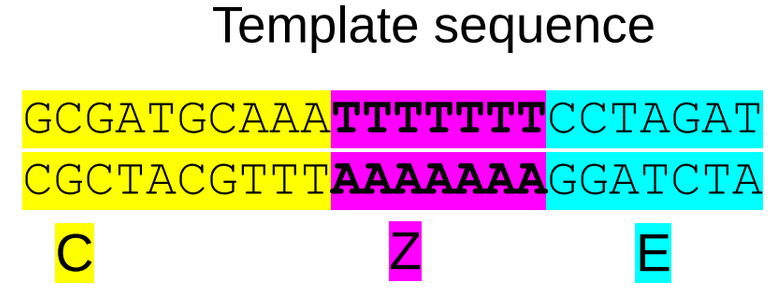
- +/- 3*n* = addition or loss of amino acids
- +/- 1 or 2 bases = Frame shift. changes the amino acid sequence after the indel. The ribosome considers three bases (codon) relative to the start codon.

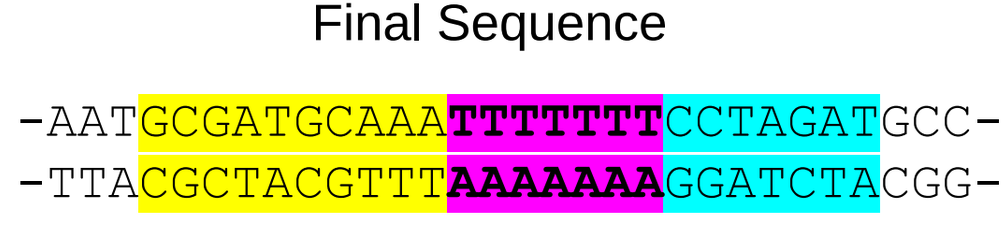
Key Concepts in Genome Editing

Repair method II – replacement of a segment of DNA







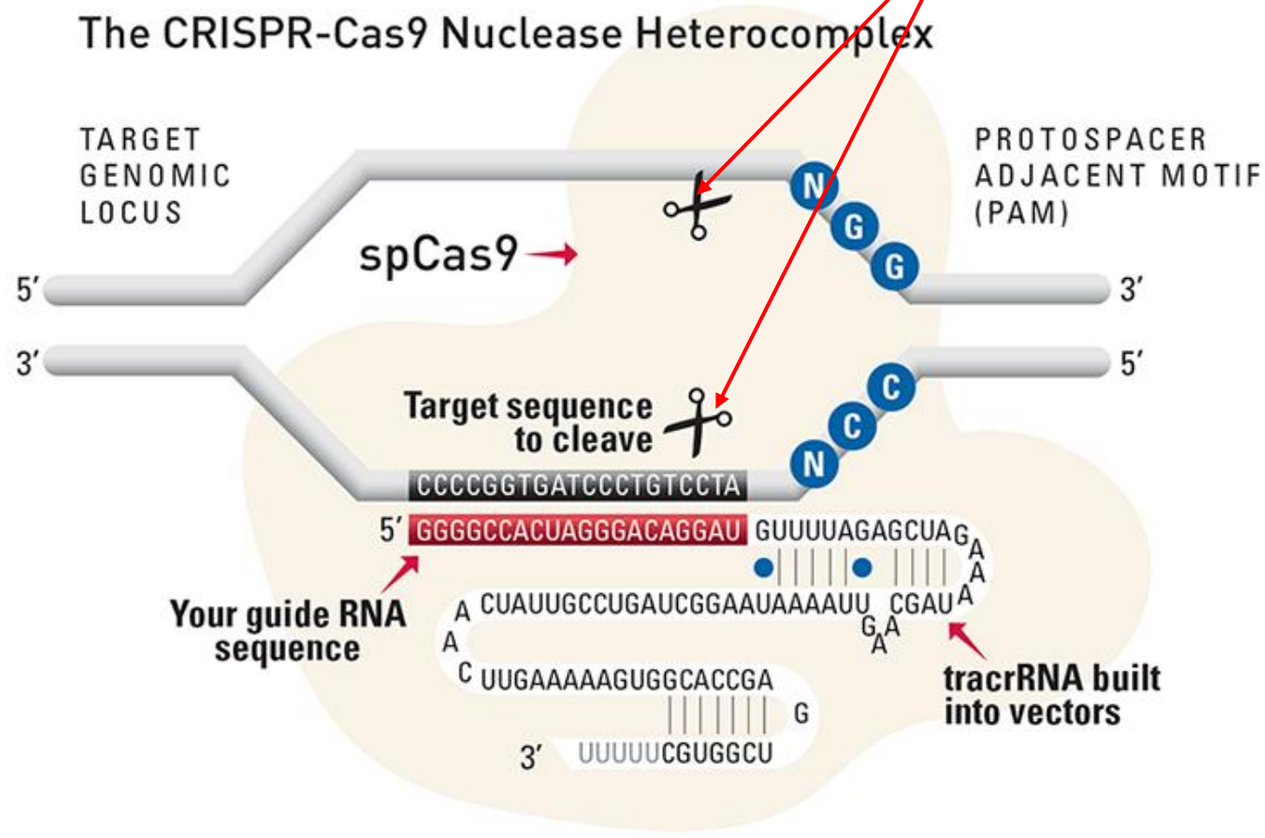


How to Cut at a Defined Location - Cas9 + Guide RNA

Cas9-RNA complex

- Cas9 – nuclease that cuts DNA after activation
- Guide RNA:
  - 5’ end complementary to target sequence
  - 3’ end required for Cas9 activity (tracrRNA)

Double stranded break
(3 bases from PAM)

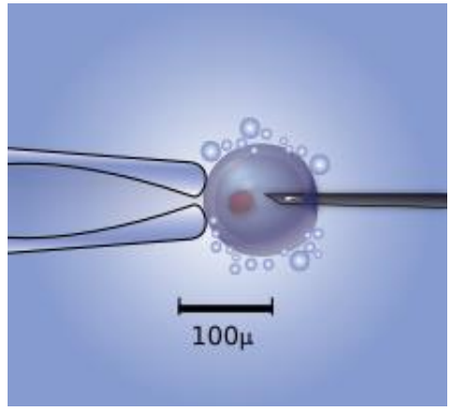


1. Cas9 Binds to PAM, then checks if RNA is complimentary to DNA sequence 5’ to PAM.

2. After PAM recognition by Cas9, guide RNA unwinds DNA, by pairing with one DNA strand.
3. Cas9 cleaves both strands near site, generating a double strand break.
4. Double stranded break triggers DNA repair, using injected replacement DNA for homologous repair

Altering the Genome Sequence with Cas9-CRISPR

Components to microinject:
1. Cas9 enzyme (nuclease)
2. Guide RNA, specific for site of cleavage, bound to the Cas9 protein
3. Copy of replacement DNA sequence (dsDNA)



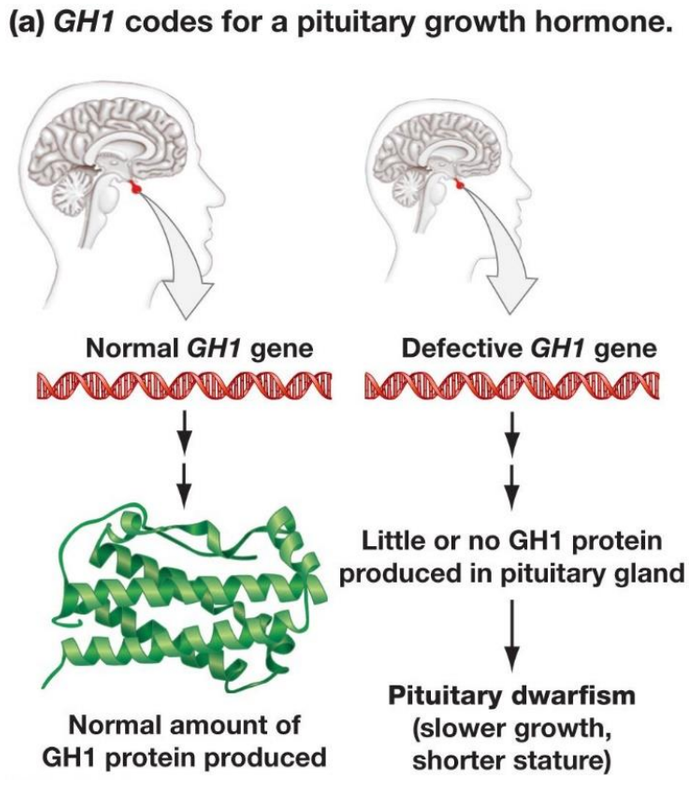
1. Guide RNA directs Cas9 to desired site, by pairing with one DNA strand.
2. CRISPR cleaves both strands near site, generating a double strand break.
3. Double stranded break triggers DNA repair, using injected replacement DNA for homologous repair

(Video originally from Nature)

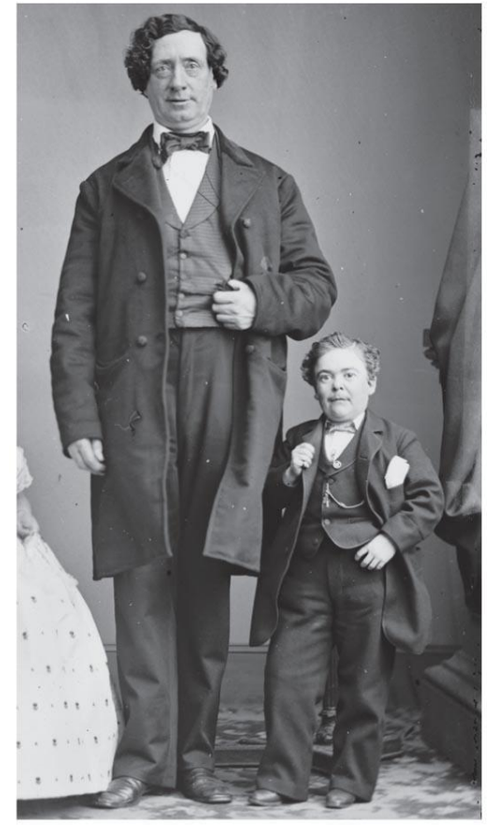
Also view:
https://wyss.harvard.edu/media-post/gene-editing-mechanism-of-crispr-cas9/

Using CRISPR-Cas9 to Correct Genetic Diseases
Human growth hormone (hGH)

Pituitary Dwarfism



(b) Normal versus GH1-deficient

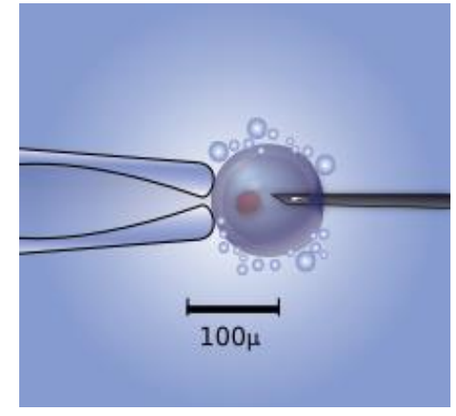


1860 William Harrison and Charles Stratton - comedians and performers.

Between one in 14,000 and one in 27,000 babies born each year have some form of dwarfism.

Components to microinject:

1. Cas9 enzyme (nuclease)
2. Guide RNA, specific for site of cleavage, bound to the Cas9 protein
3. Copy of replacement DNA sequence (dsDNA)



CRISPR Repair of Grown Hormone Gene

Human growth hormone gene

```
>M13438.1:497-2129 Human growth hormone gene (HGH-N), complete cds
AGGATCCCAAGGCCCAACTCCCCGAACCACTCAGGGTCCTGTGGACAGCTCACCTAGCTGCAATGGCTAC  70
AGGTAAGCGCCCCTAAAATCCCTTTGGCACAATGTGTCCTGAGGGGAGAGGCAGCGACCTGTAGATGGGA 140
CGGGGGCACTAACCCTCAGGGTTTGGGGTTCTGAATGTGAGTATCGCCATCTAAGCCCAGTATTTGGCCA 210
ATCTCAGAAAGCTCCTGGCTCCCTGGAGGATGGAGAGAGAAAAACAAACAGCTCCTGGAGCAGGGAGAGT 280
GTTGGCCTCTTGCTCTCCGGCTCCCTCTGTTGCCCTCTGGTTTCTCCCCAGGCTCCCGGACGTCCCTGCT 350
CCTGGCTTTTGGCCTGCTCTGCCTGCCCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATTCCCTTATCC 420
AGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTGGCCTTTGACACCTACCAGGAGT 490
TTGTAAGCTCTTGGGGAATGGGTGCGCATCAGGGGTGGCAGGAAGGGGTGACTTTCCCCCGCTGGAAATA 560
AGAGGAGGAGACTAAGGAGCTCAGGGTTTTTCCCGACCGCGAAAATGCAGGCAGATGAGCACACGCTGAG 630
CTAGGTTCCCAGAAAAGTAAAATGGGAGCAGGTCTCAGCTCAGACCTTGGTGGGCGGTCCTTCTCCTAGG 700
AAGAAGCCTATATCCCAAAGGAACAGAAGTATTCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTC 770
AGAGTCTATTCCGACACCCTCCAACAGGGAGGAAACACAACAGAAATCCGTGAGTGGATGCCTTCTCCCC 840
AGGCGGGGATGGGGGAGACCTGTAGTCAGAGCCCCCGGGCAGCACAGCCAATGCCCGTCCTTGCCCCTGC 910
AGAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAG 980
GAGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTA 1050
GAGGAAGGCATCCAAACGCTGATGGGGGTGAGGGTGGCGCCAGGGGTCCCCAATCCTGGAGCCCCACTGA 1120
CTTTGAGAGACTGTGTTAGAGAAACACTGGCTGCCCTCTTTTTAGCAGTCAGGCCCTGACCCAAGAGAAC 1190
TCACCTTATTCTTCATTTCCCCTCGTGAATCCTCCAGGCCTTTCTCTACACTGAAGGGGAGGGAGGAAAA 1260
TGAATGAATGAGAAAGGGAGGGAACAGTACCCAAGCGCTTGGCCTCTCCTTCTCTTCCTTCACTTTGCAG 1340
AGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAAACT 1410
CACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGT 1480
CGAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCTAGCTGCCCGGGTGG 1550
CATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTG 1620
TCCTAATAAAATTAAGTTGCATCATT
```

Possible PAM Sites

Location of mutation
Isoleucine (I) to Asparagine (N)

Wild type (normal)

```
          R  L  E  D  G  S  P  R  T  G  Q  I  F  K  Q  T  Y  S
-CTTTGCAGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAA-
-GAAACGTCTCCGACCTTCTACCGTCGGGGGCCTGACCCGTCTAGAAGTTCGTCTGGATGTCGTT-
```

Mutant (growth hormone non-functional)

```
          R  L  E  D  G  S  P  R  T  G  Q  N  F  K  Q  T  Y  S
-CTTTGCAGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGAACTTCAAGCAGACCTACAGCAA-
-GAAACGTCTCCGACCTTCTACCGTCGGGGGCCTGACCCGTCTTGAAGTTCGTCTGGATGTCGTT-
```

- The cut site needs to be close to site of mutation so that the injected dsDNA repair template can be as short as possible.
- A NGG (PAM site) is needed for Cas9 to bind & then test whether the RNA is complementary to the DNA.
- There are four possible PAM sites in the DNA sequence on the bottom left. The PAM site closest to the mutation was selected so that the cut site is close to mutation site.

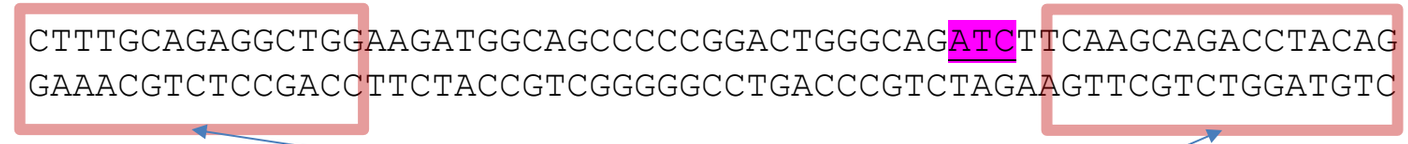
CRISPR Repair of Grown Hormone Gene

- The PAM site closest to the mutation was selected so that the cut site is close to mutation site.
- The targeting section of the guide RNA should have the same sequence as 5' to the XGG, 18 bases are required:

  5'AGAUGGCAGCCCCCGGAC---------- plus additional RNA needed for Cas9 function

- This RNA would cause cleavage of both the wild-type or mutant sequence since they are identical in this region. This is OK since the repair DNA will contain the wild-type sequence.
- The site of Cas9 cleavage is between the PAM and the guide RNA sequence.
- The injected DNA contains sequences on both sides of the ds break, causing the replacement of the sequences at the double stranded break due to repair.

Injected dsDNA for Homologous Repair

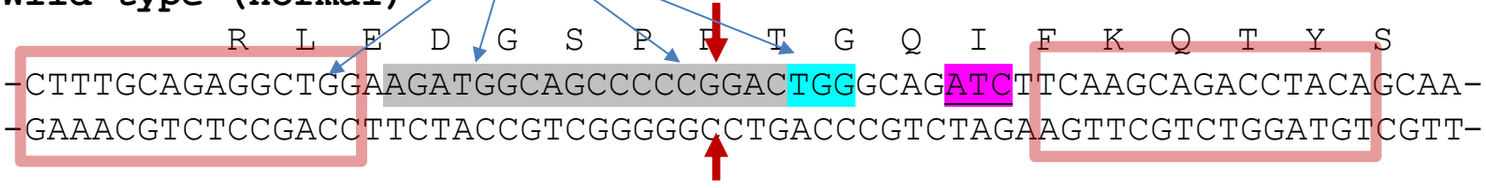


homology regions required for repair

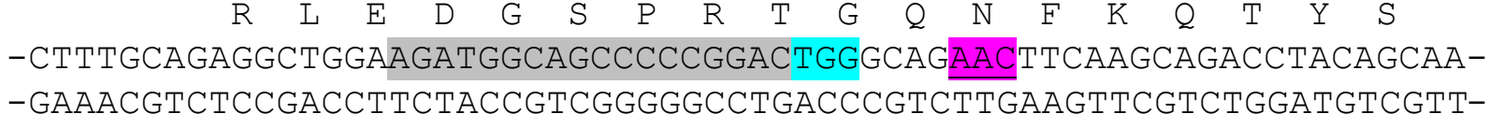
Possible PAM Sites

DNA cuts by cas9

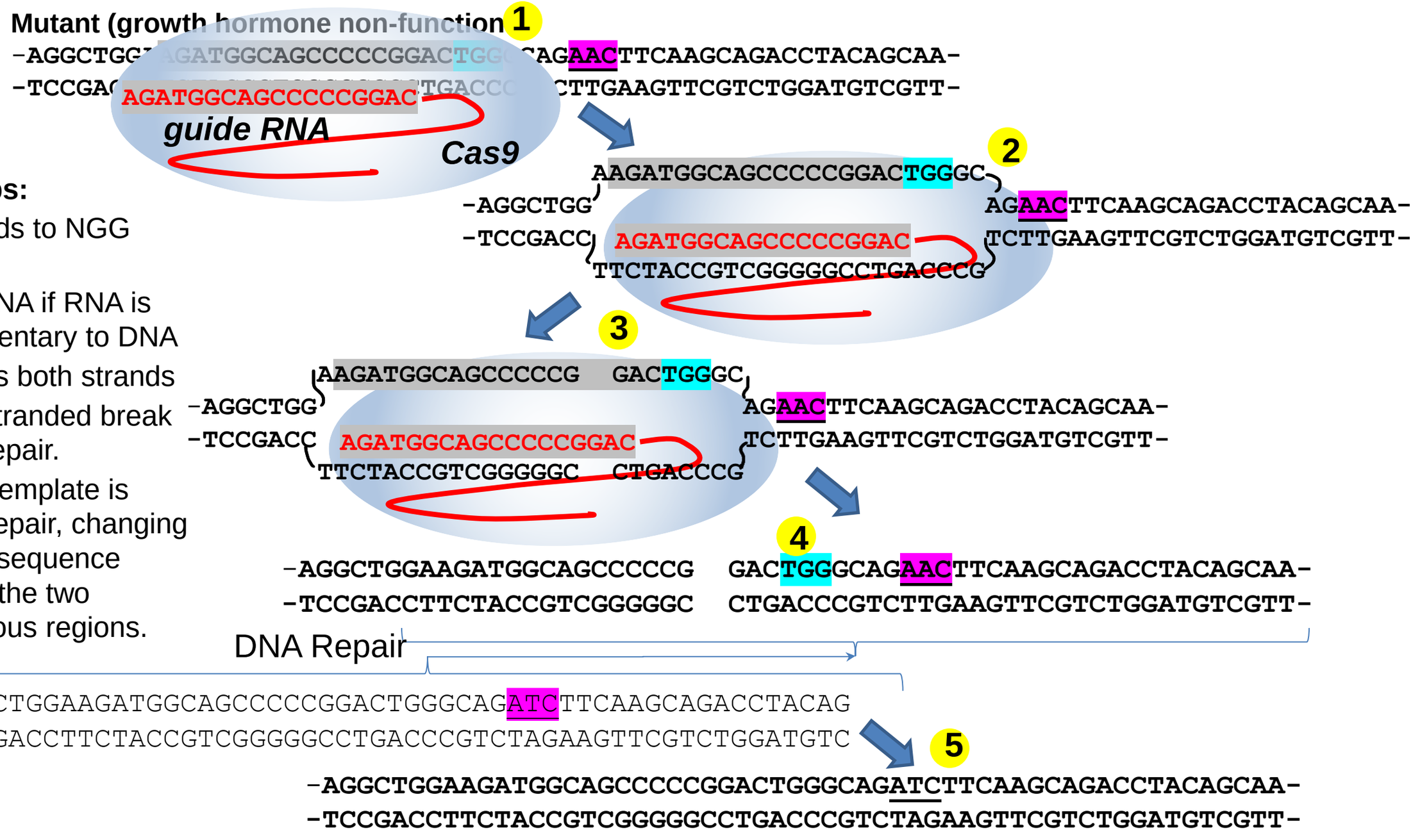
Wild type (normal)



Mutant (growth hormone non-functional)



Note that Cas9 will cut both the wild-type and the mutant, but repair will insert the wild-type sequence.



Editing Steps:

1. Cas9 binds to NGG (PAM)
2. Opens DNA if RNA is complementary to DNA
3. Cas9 cuts both strands
4. Double stranded break causes repair.
5. Injected template is used to repair, changing the DNA sequence between the two homologous regions.