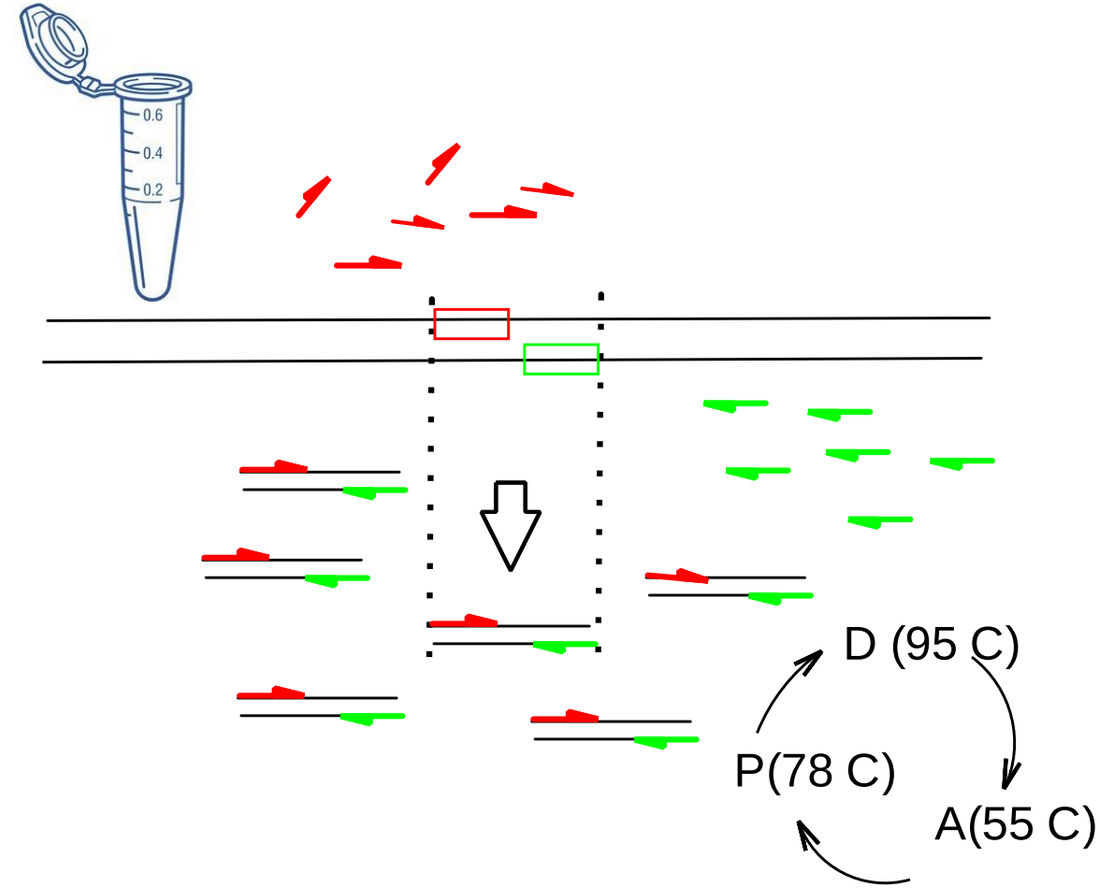
Lecture 5
Immunology & Drugs & Genome Editing (and a little on PCR)

- PCR
- Immunotherapies
- Drugs that inhibit key processes
- How do you edit the genome of an organism

Polymerase Chain Reaction

- PCR is an *in vitro* DNA synthesis reaction in which a specific section of DNA is replicated over and over generating exponentially large amounts of a specific piece of DNA from trace amounts of starting material (template).
- Template can be trace amounts of DNA from a drop of blood, a single hair follicle, or a cheek cell.
- The region of DNA that is copied is specified by the sequence of two primers, which are short ssDNA that initiate polymerase activity. The primers are in vast excess over the DNA.
- The location of the amplified segment is *defined* by two primers (left = upstream, right = downstream):
  - they anneal to their templates according to Watson-Crick pairing rules (A-T, G-C),
  - initiate polymerization from those sites,
  - they are incorporated into the final PCR product.
  - Left primer = sequence of top strand at left boundary
  - Right primer = sequence of bottom strand at right boundary
- The primers are DNA and are synthesized chemically, they can be any desired sequence.
- If there is no homology between the primers and the input DNA, then no PCR product will be formed.

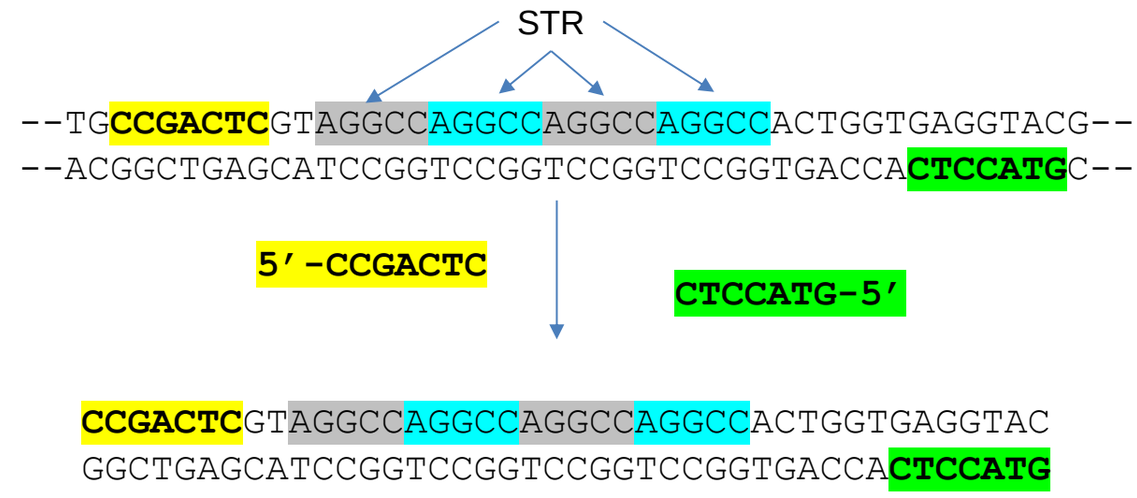


Each PCR cycle consists of three steps:

1. Denaturation of the DNA to make it single stranded (2 min at 98 C)
2. Lowering of temperature to let the primers form double-stranded DNA (1 min at 55 C)
3. Elongation by DNA polymerase (1 min/kb at 78 C)

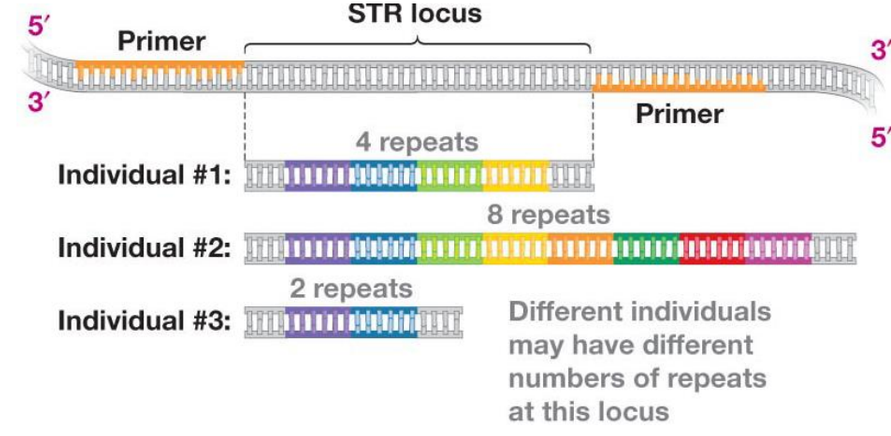
PCR Applications – Identification of Individuals

- Regions of DNA have variable numbers of repeated DNA sequences (Short tandem repeats, STR). The number of STR can differ from one person to the next and can change over time due to replication errors (repeat expansion disease).
- Individuals will inherit one copy of the repeat from each parent. The length of the inherited DNA can be the same or different, depending on the number of repeats in each parent.
- PCR Primers are designed to be outside the repeated region, so that they will anneal to a single location on the chromosome and then amplify the region containing the STR
- PCR Product length = primer lengths + number of tandem repeats (+ any DNA between the primers and the repeats). *Individuals can be differentiated by the length of the PCR product if they have different numbers of STR*



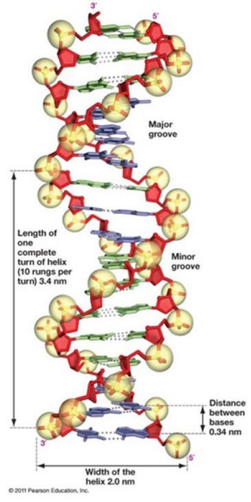
Which individual has the shortest PCR product?

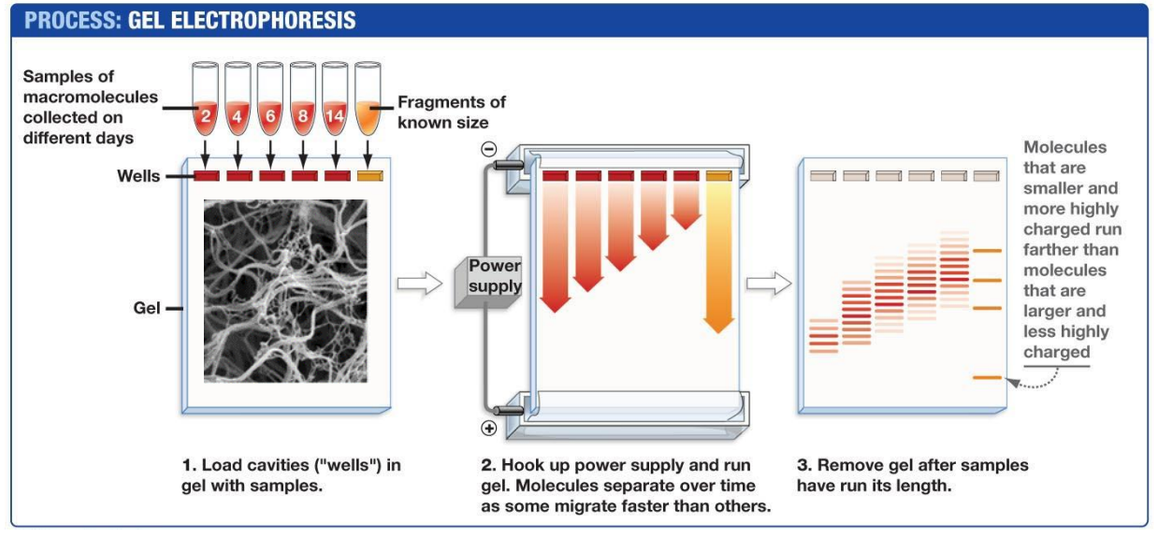
Which has the longest?

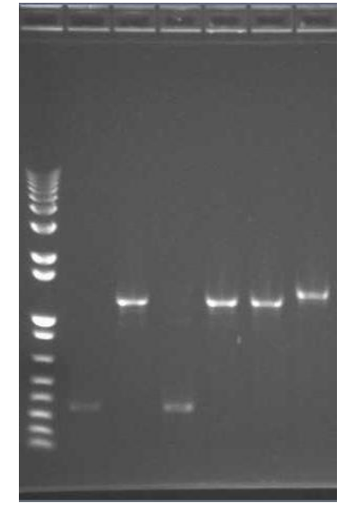


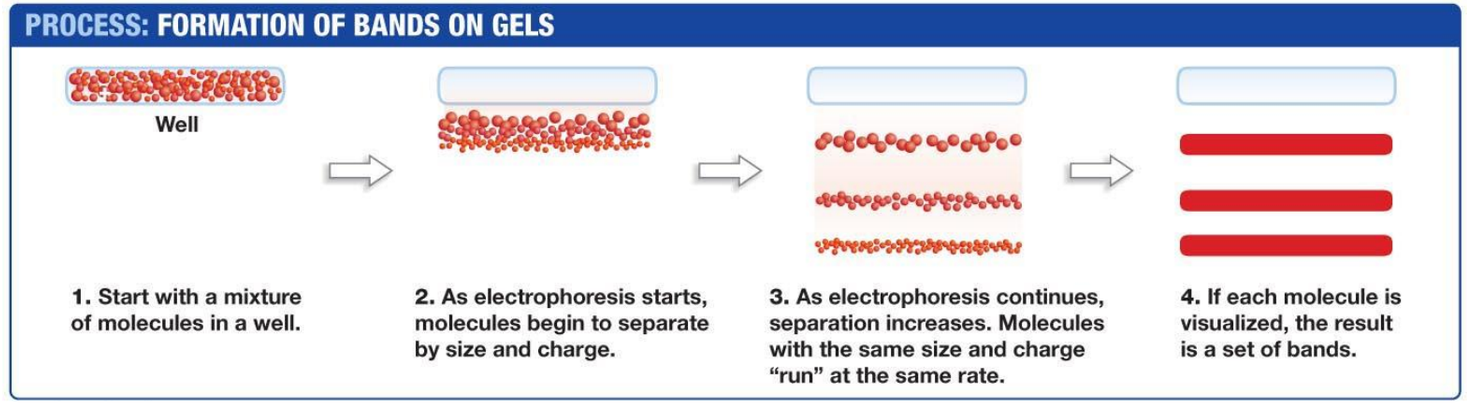
Size Determination of PCR products - Agarose Gel Electrophoresis.

https://dnalc.cshl.edu/resources/animations/gelelectrophoresis.html





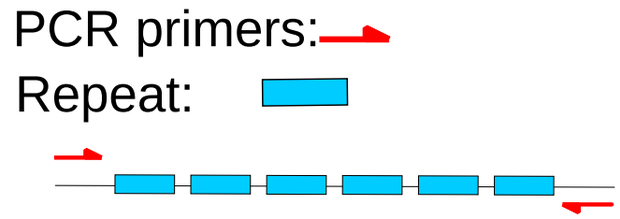




Which are the smallest PCR fragments?

Short Tandem Repeats to Test Paternity

1. DNA samples (blood, cheek cells) would be obtained from:
   - Mother
   - Child
   - Candidate fathers.
2. PCR would be preformed using primers that amplify a segment of the chromosome containing repeats.
3. Each individual would show 2 bands on the gel, corresponding to the PCR product from each chromosome (we have two copies of each chromosome).
4. The child would inherit one copy from the mother and the other from the father:
   - One of the child's PCR product would match one of the mothers.
   - The other PCR product from the child would match one of the PCR products from the father.



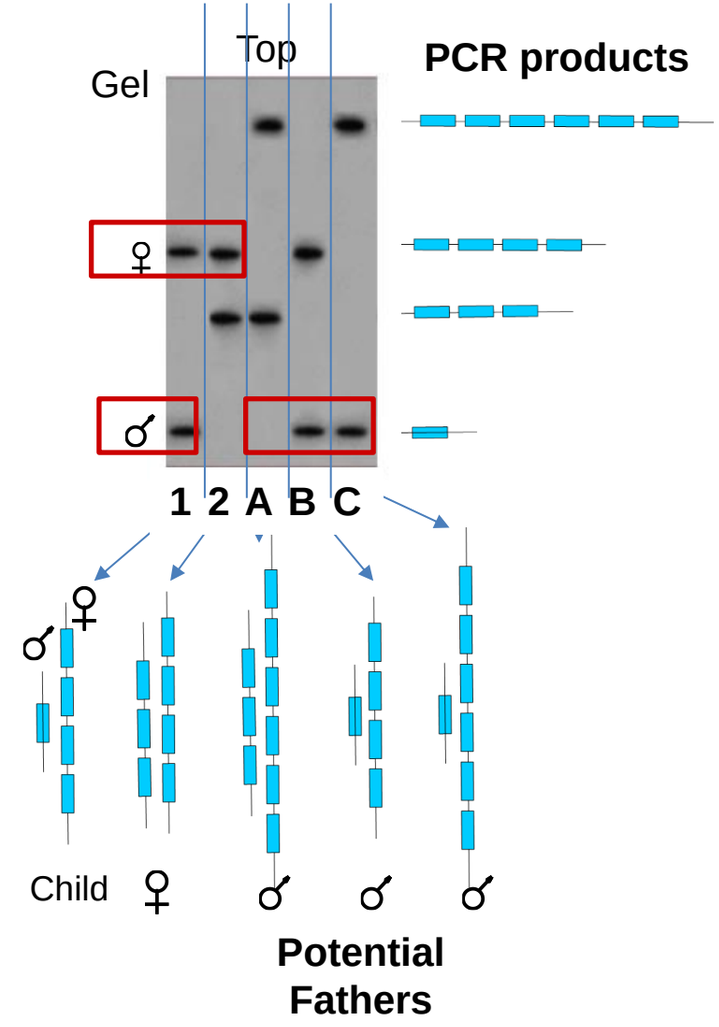
Lane 1: Child
Lane 2: Mother

Lanes A, B, C: Possible Fathers

1. *For the child, which PCR product is from the mother? From the father?*

2. *Who is **not** the father?*

3. *Who **may** be the father?*



Introduction to Immunology

1. Branches of the immune system (Innate and acquired)
2. Properties of antibodies (Quaternary structure, antigen recognition)
3. How diverse antibodies are produced:
    - Genome DNA changes
4. How antibodies eliminate pathogens

Key Questions:

1. Why is the innate system important?
2. What is the origin of diversity in acquired immunity?

The Nobel Prize in Physiology or Medicine 2018

Ill. Niklas Elmehed. © Nobel Media

James P. Allison

Prize share: 1/2

Ill. Niklas Elmehed. © Nobel Media

Tasuku Honjo

Prize share: 1/2

The Nobel Prize in Physiology or Medicine 2018 was awarded jointly to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation."

Some Important Definitions:

Antigen = something that is recognized by the immune system, e.g. bacteria, virus, pollen.

Epitope = the part of the antigen that is contacted by the antibody.

Antibody (Ab) = Y-shaped protein that recognizes antigens, found on the surface of B-cells or secreted by plasma cells. When bound to antigen, it can initiate a process that results in the destruction of the antigen. *Specificity is high due to AA sequence in the variable segments.*

Immunoglobulin (Ig) = antibody.

B-cell = involved in antibody production and recognition of pathogen. Has antibody molecule on its surface (as part of the B-cell receptor). Develops into plasma cells after activation by T_H cells. Called B-cells because they are generated in the organ called the Bursa in birds.

Plasma cell = derived from B-cell after activation of the B-cell, produces secreted antibodies with the *same specificity as the original B-cell.*

T_H cell = T-helper: *Required* to activate both B and T_C cells, as well as other cells in the immune system.

Called T-cells because they mature in the thymus.

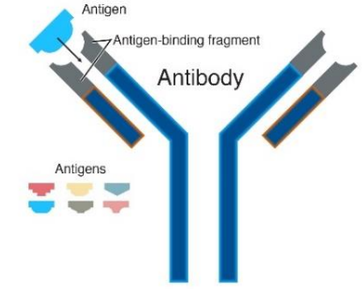
T_c cell = T-cellular: Involved in defense against viruses and cancer.

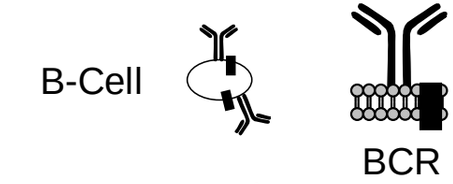
TCR = T-cell receptor – found on the surface of T-cells, recognizes MHC proteins + bound peptide, RTK.

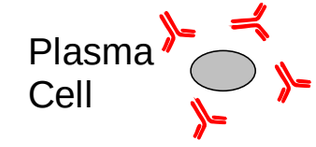
- **T_c cell** = recognizes MHC I + peptide
- **T_H cell** = recognizes MHC II + peptide

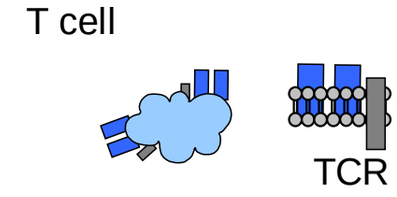
MHC = major histocompatibility complex – required for acquired immunity (basis of transplant rejection)

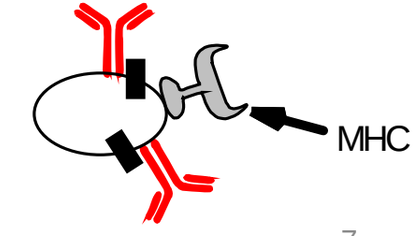
- MHC I = protein found on the surface of ***all*** cells, "presents" peptides derived from the proteins that were made by the cell. The MHC-peptide complex is recognized by T_c cells. ***Only foreign peptides produce a response***.
- MHC II = on the surface of B-cells, macrophages, and dendritic cells. Presents external peptides to T_H cells, leading to activation of the cell by T_H cells. ***Only foreign peptides produce a response***.





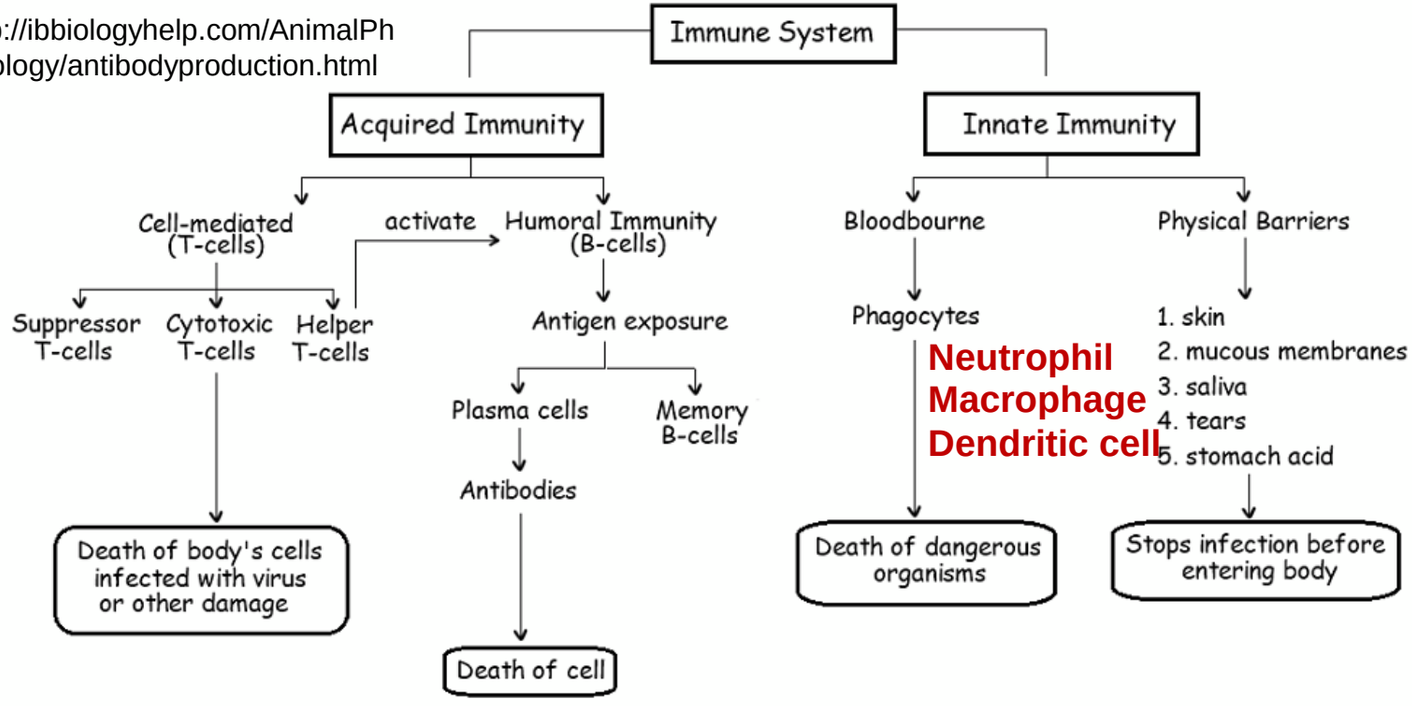


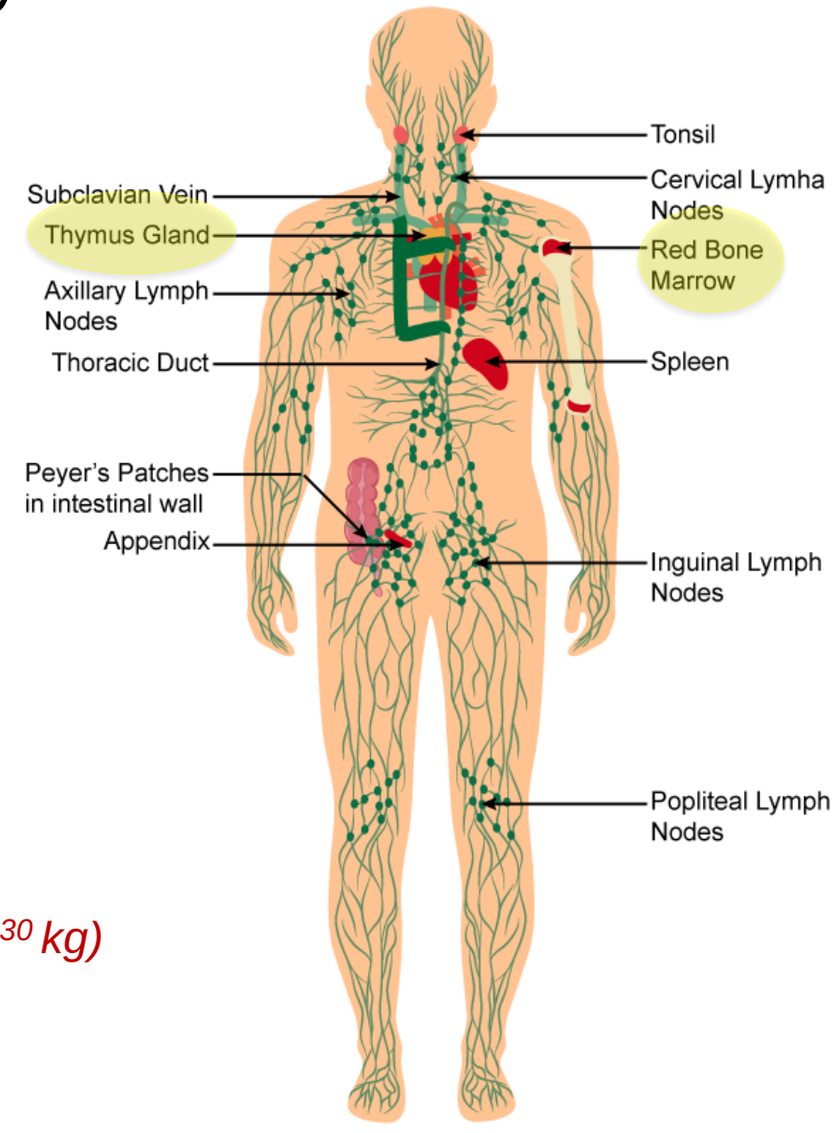




Branches of the Immune System

http://ibbiologyhelp.com/AnimalPhysiology/antibodyproduction.html





https://www.topperlearning.com/

Why is the innate system essential?

- *A pathogen doubles every hour.*
- *It takes 7 days to produce antibody (after 1st exposure)*
- *Uncontrolled growth would produce many bacteria: $2^{24 \times 7} = 3.7 \times 10^{50}$ (~10^{30} kg)*

- Important **primary** lymphatic organs: **bone marrow** (B), **thymus** (T)-Generate all immune cell.
- Important **secondary** lymphatic organs: lymph nodes, spleen, Peyer's patches – Activation of immune cells.

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

6. Development of

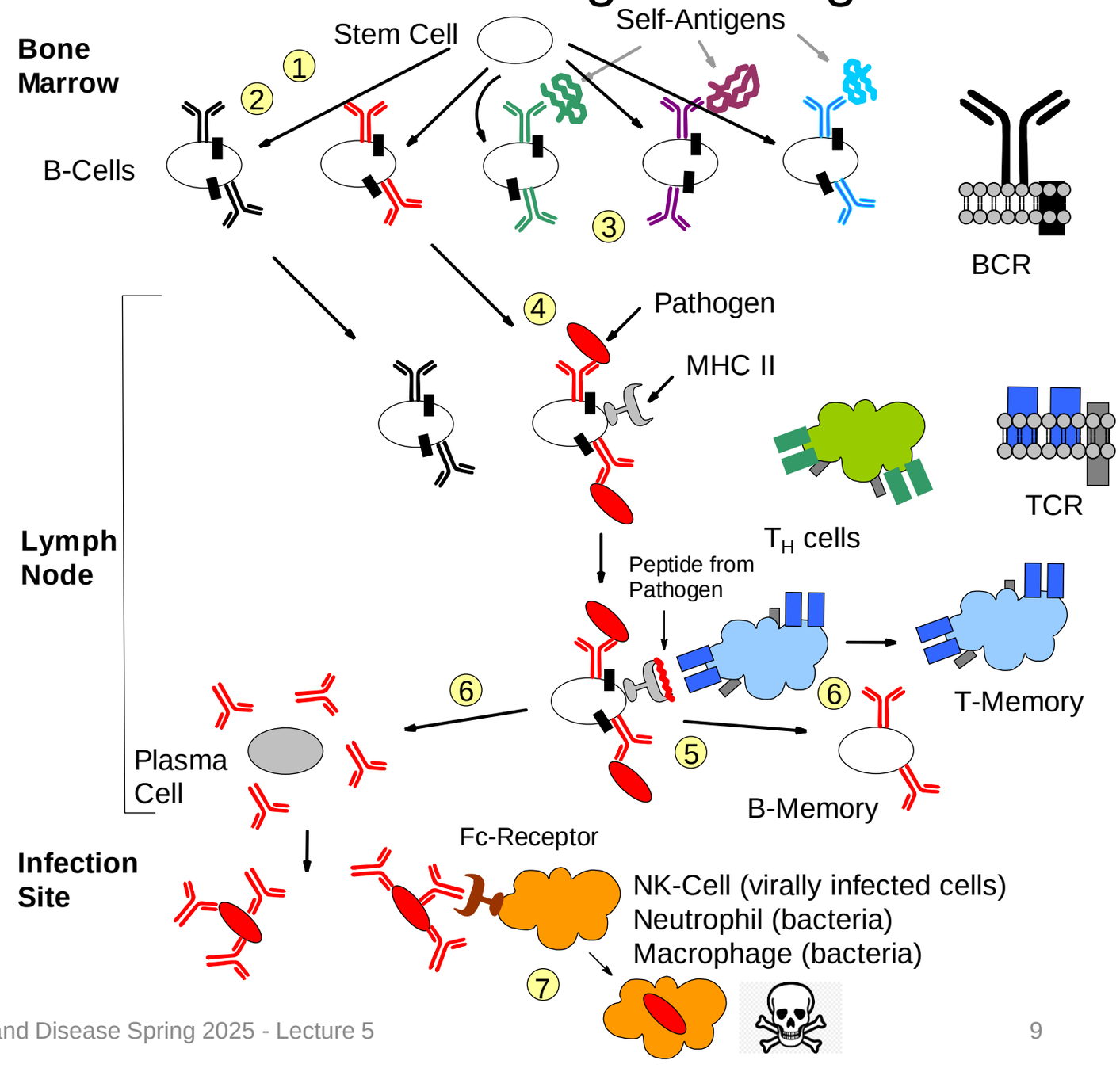
- Plasma cells - Production of soluble antibodies of the same specificity as the parent B-cell.
- B-memory cells (basis of immunity)
- T-memory cells (basis of immunity)

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.

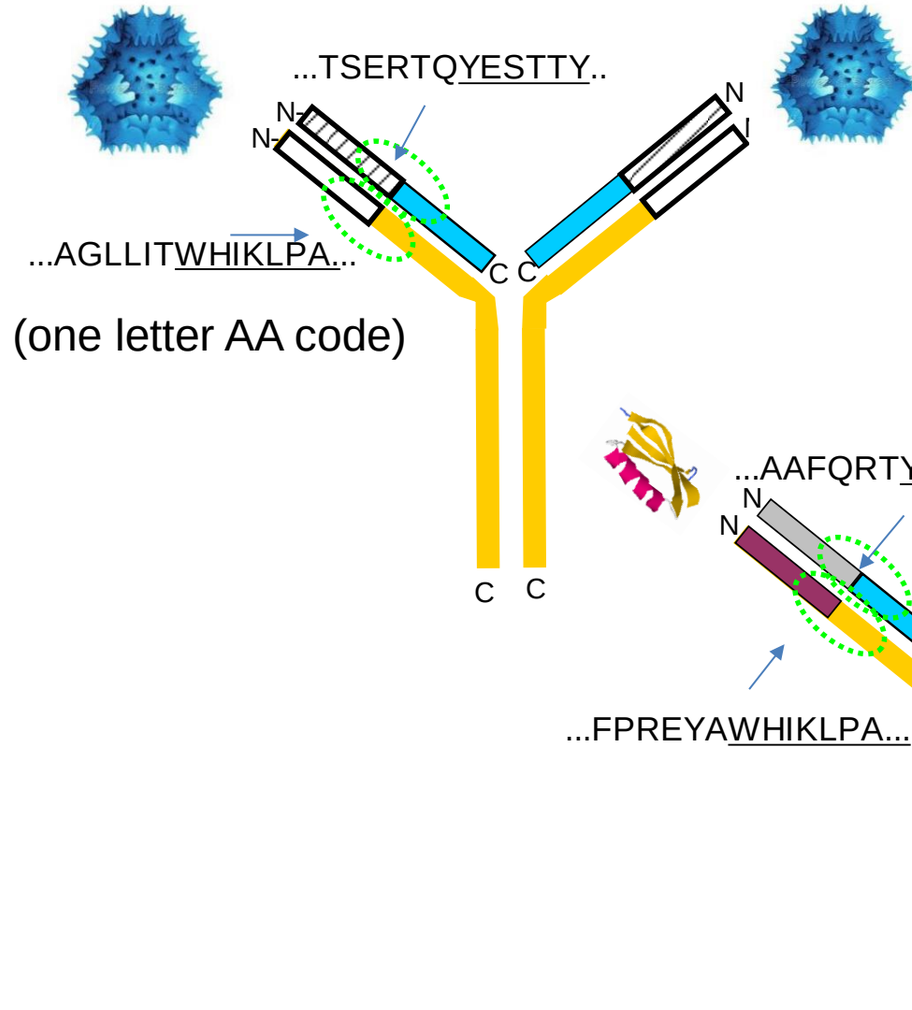
TCR – T cell receptor = MHC-peptide recognition + signaling.



Antibody Structure and Diversity

Each Antibody:

- Two identical light chains
- Two identical heavy chains
- First ~100 Amino acids on each chain are called the variable region and differ from antibody to antibody.
- Unique sequence for variable region of both heavy and light chains ***– defines specificity – different antibodies bind different antigens.***
- Constant regions - same protein sequence for all.



Light chain sequences:

```
M..TSERTQYESTTY..RGEC
M..AAFQRTYESTTY..RGEC
1.....110.........210
```

Coded by different exons

Heavy chain sequences:

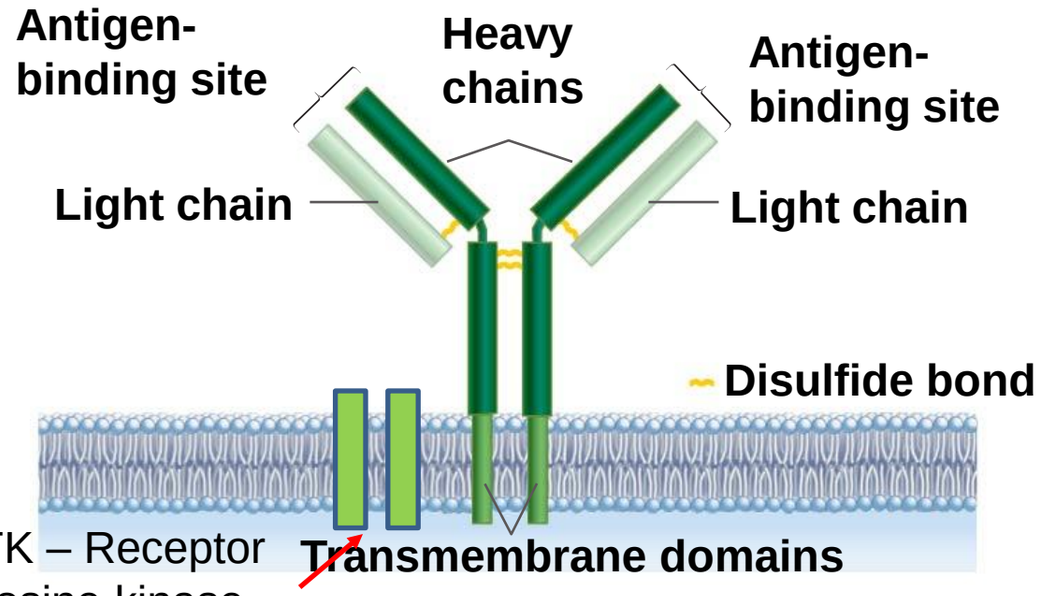
```
M...AGLLITWHIKLPA...SPGK
M...FPREYAWHIKLPA...SPGK
1......120............449
```

Coded by different exons

Production of Antibodies by B-cells & Plasma Cells

B- Cells & B-cell Receptor (BCR)

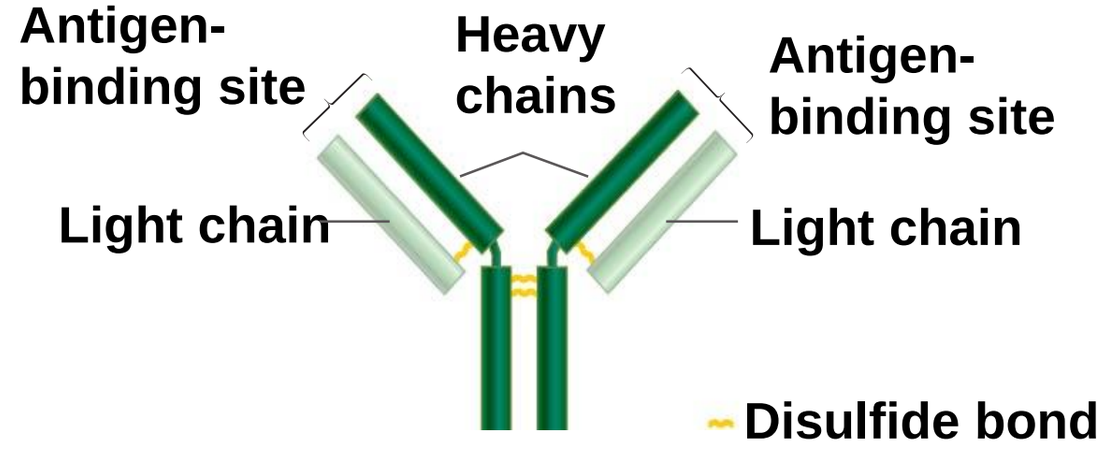
- Each B-cell has only one type of antibody as part of its BCR (B-cell receptor), i.e. the 10^5 BCRs are *homogeneous* on the same cell.
- Approximately 10^8 different specificities at any one time. i.e. *10^8 different B-cells!*



RTK – Receptor tyrosine kinase – signaling domain.

Plasma Cells:

- After activation, a B-cell develops into a plasma cell.
- The antibody is secreted.
- The same light chains are produced.
- The heavy chains differ only in the absence of the transmembrane domains.



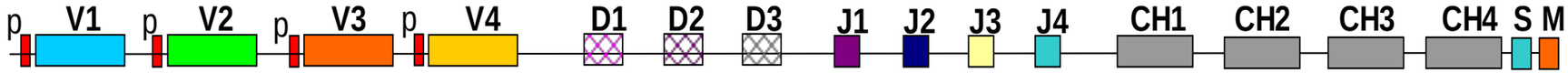
mRNA that codes for antibodies contains two types of sequences:

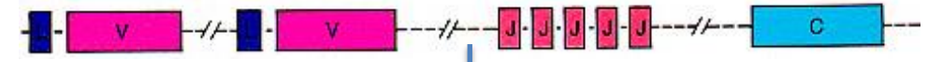
- Exons – contain codons for the amino acids
- Introns – removed before translation

Different exons are used to produce membrane bound or soluble antibodies.

Antibody Production – From Stem Cells to B-Cells

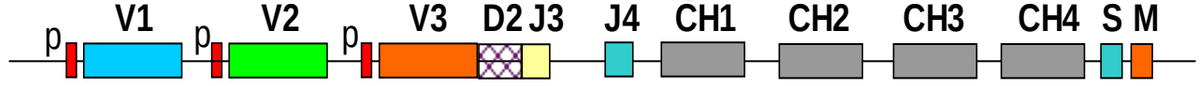
Stem Cell





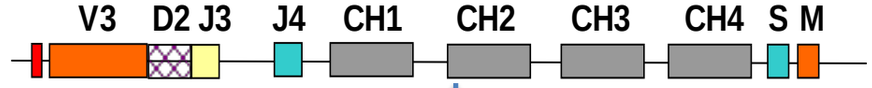
B-Cell

Rearranged heavy chain gene



Rearranged light chain gene

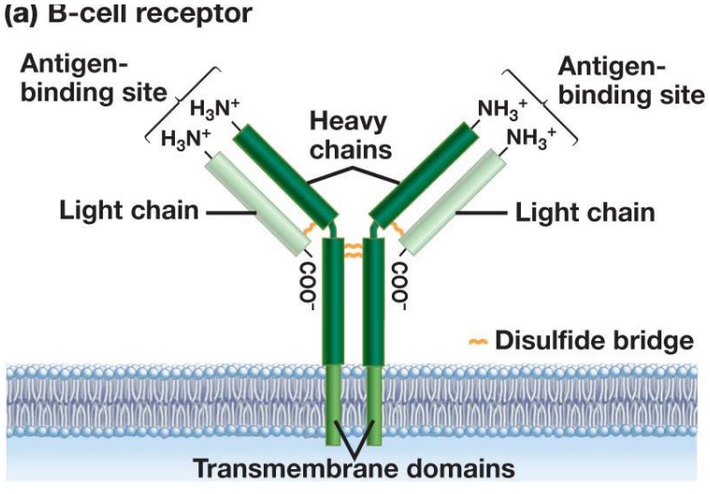
Transcription



mRNA Splicing (introns removed)

Translation

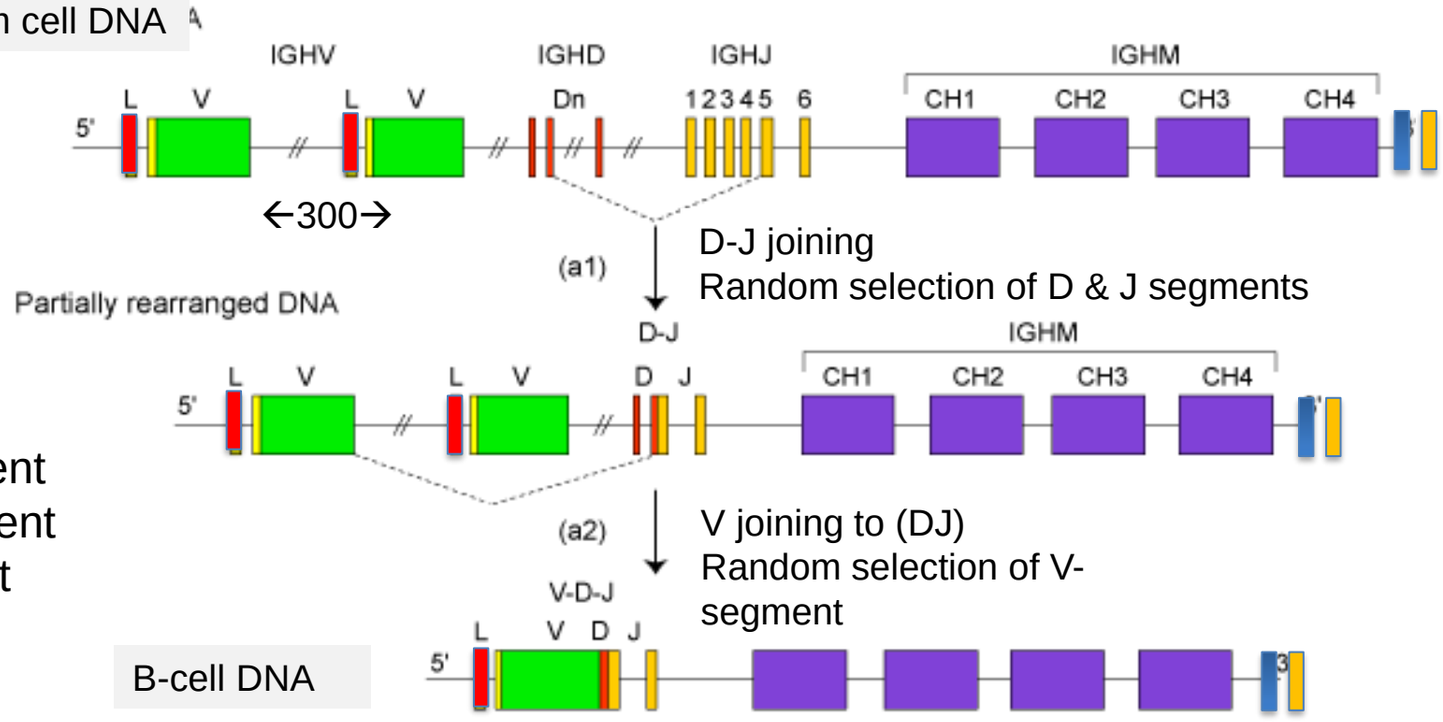
Export to cell surface (ER -> golgi -> Membrane)

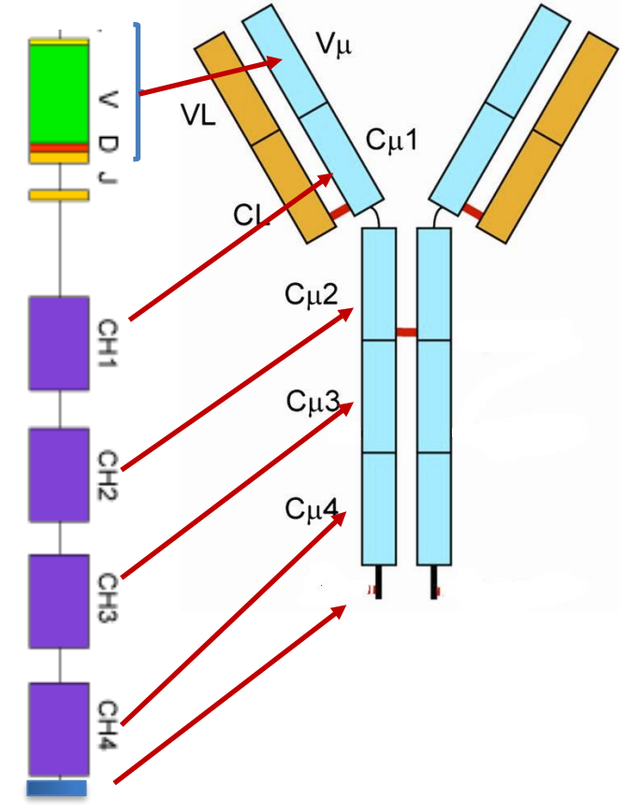


Antibody Genes are Assembled From DNA Segments: Giving Many Different Sequences.

Production of Heavy Chain Gene:
V_H exon = V+D+J segment (selected at random)

The mRNA coding for antibodies contains 5 exons.





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.

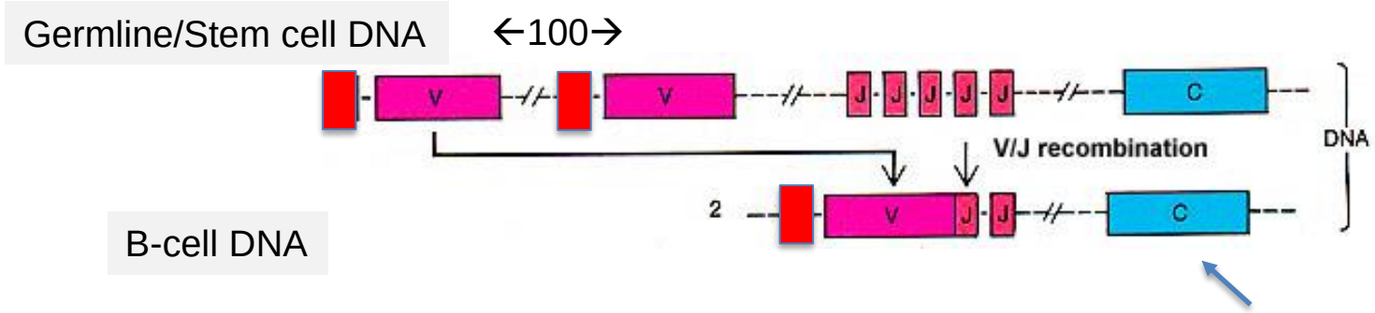
Stem cells -> B-cells

- 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?

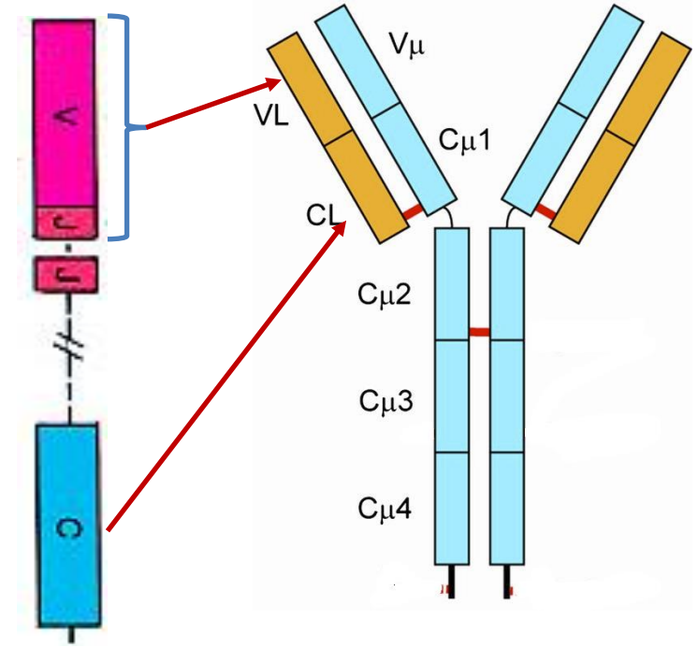
Light-chain Genes are Assembled From DNA Segments: Giving many different sequences.

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.



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 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 heavy chain that is generated can pair with any light chain that is generated, how many different antibodies can be generated (assuming there are 10,000 possible heavy chains and 500 different light chains) ?

Cell Based Acquired 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:

- T_H
- T_C, T_{CTL}

MHC = major histocompatibility complex
Membrane bound protein that “Presents” or displays peptides to T-cells:

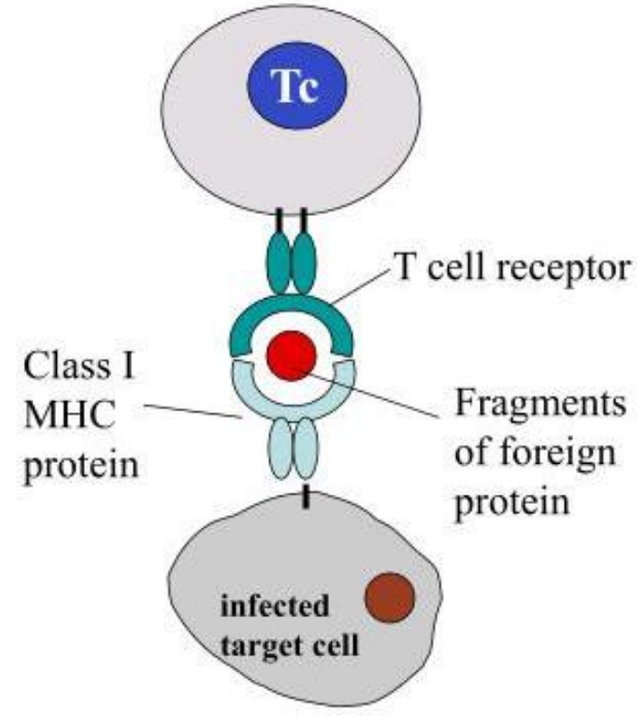
- MHC I – T_C cells
- MHC II – T_H cells

A single MHC can present many different peptides (low specificity)

Peptide + MHC recognized by T-cell (T-cell receptor)

Responsible for transplantation rejection.

T_C → T_{CTL}
T_C Memory



Activation of Tc cells requires:

1. Recognition of ***foreign*** peptide on MHC I.
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

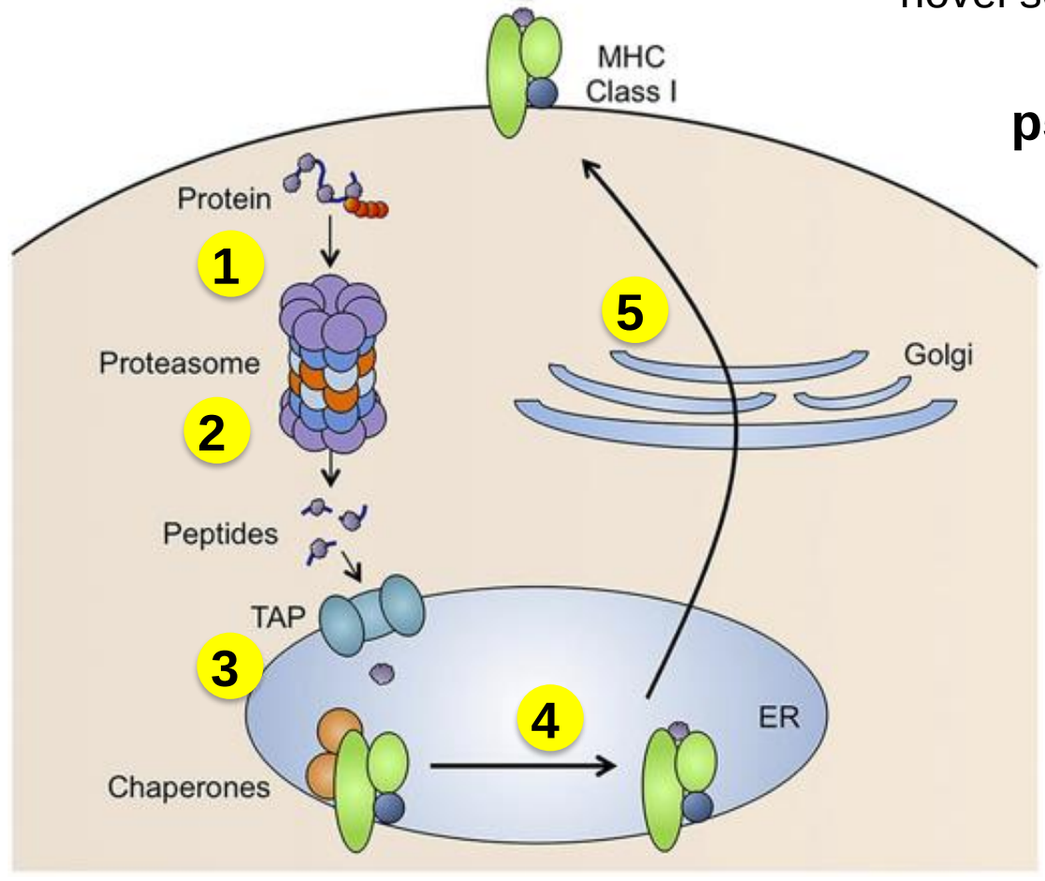
T_c Detection of Diseased/Cancer Cells - Role of MHC I

- MHC I present peptides
- Peptides are generated from of ***all*** of the proteins that are made in the cell, both self and foreign from pathogens.

- Steps for Presentation

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

- ***Only foreign peptides activate T-cells***



Foreign Peptide Source:

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

p53 Protein Sequence

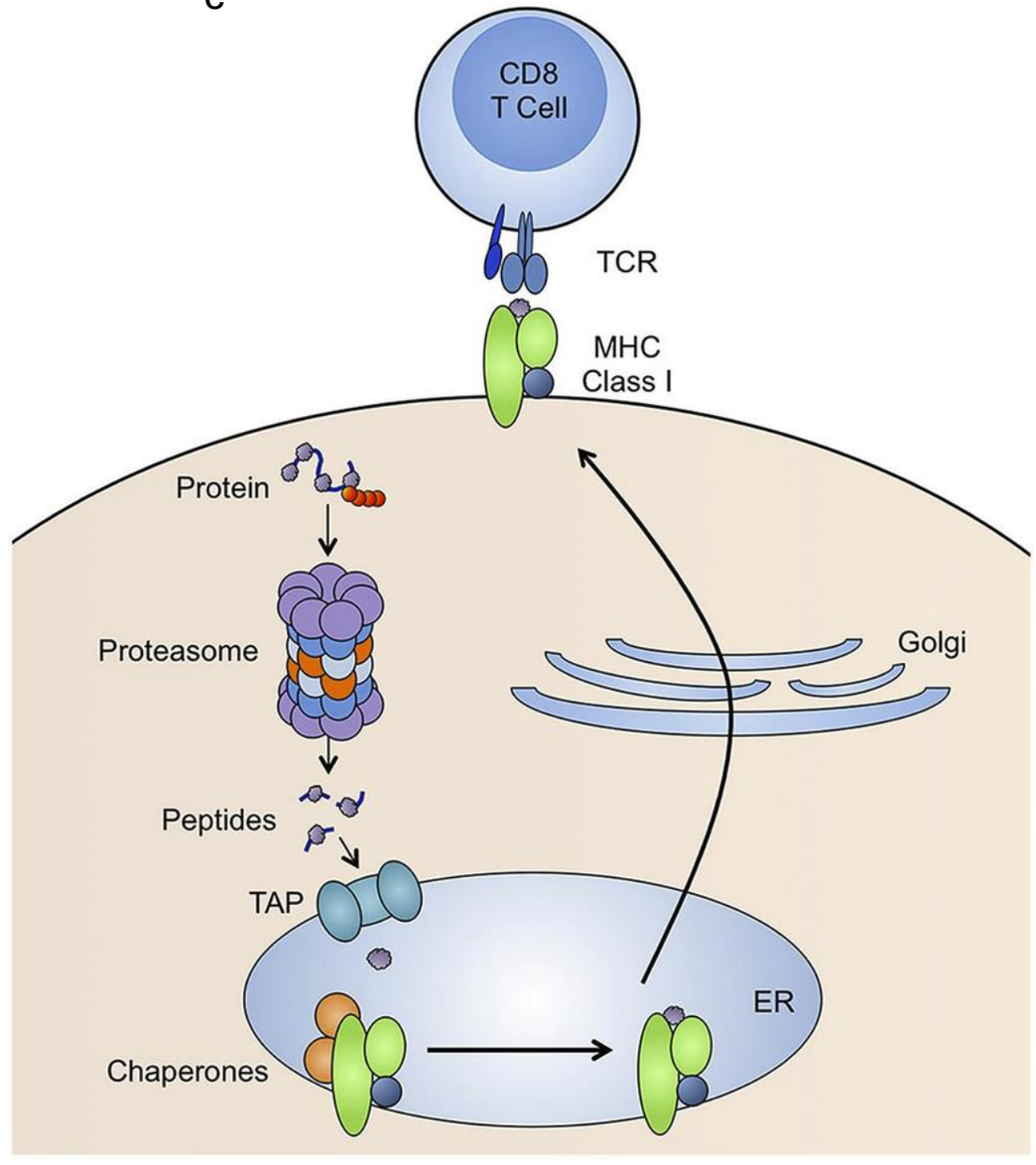
Zn Fingers (DNA binding)

```
        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
```

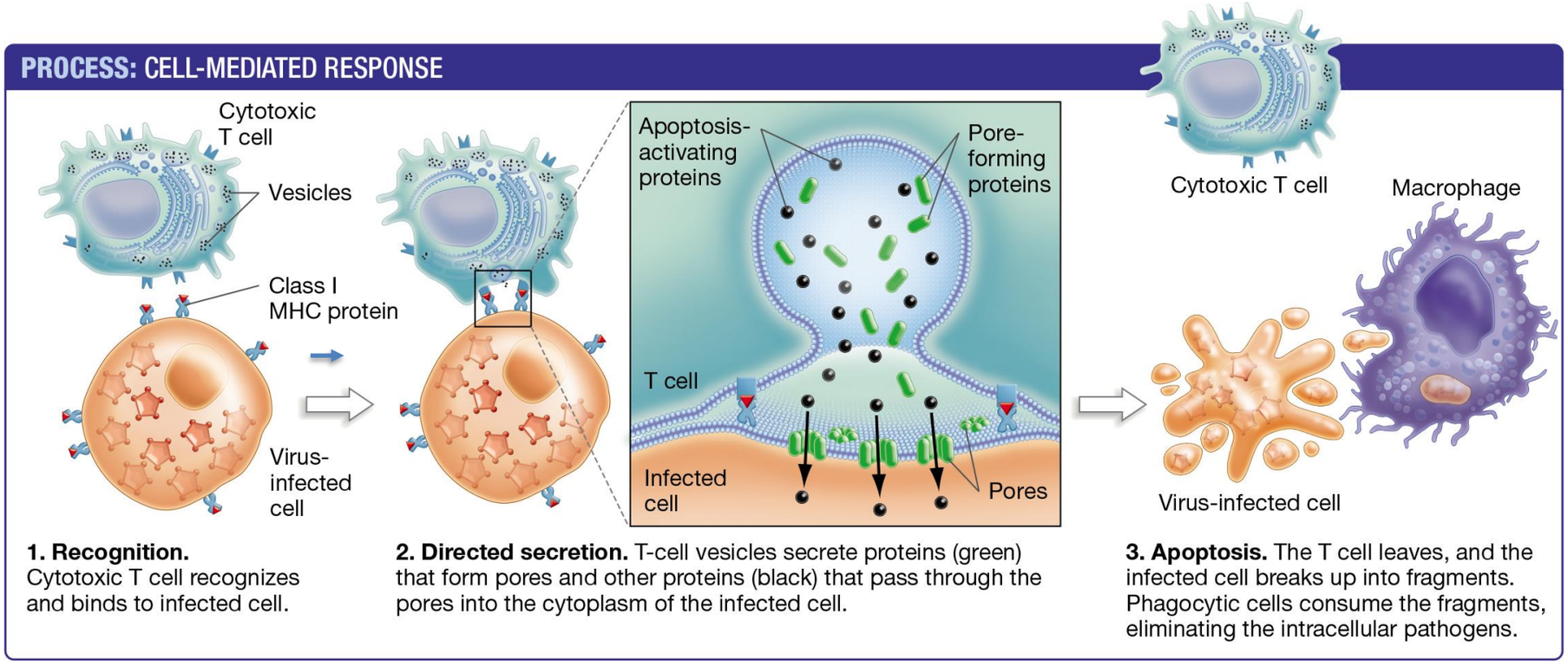
EVVRRCPHHE — Normal seq., ***ignored*** by TCR

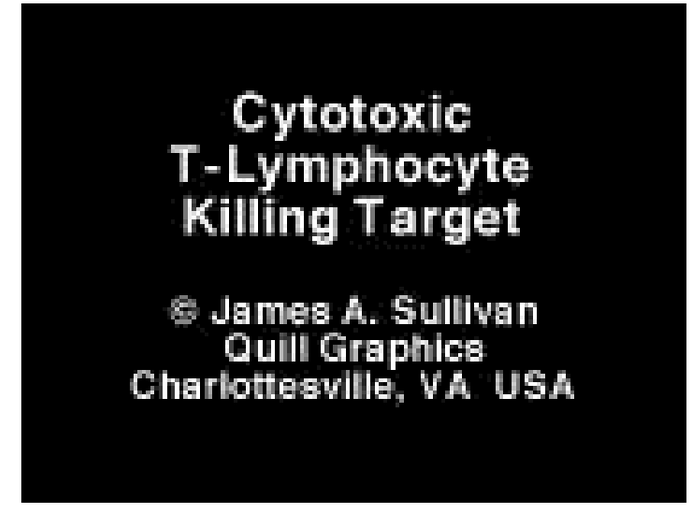
EVV**GG**CPHHE — Mutant seq. in cancer, ***detected*** by TCR

T_c Detection of Diseased/Cancer Cells



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



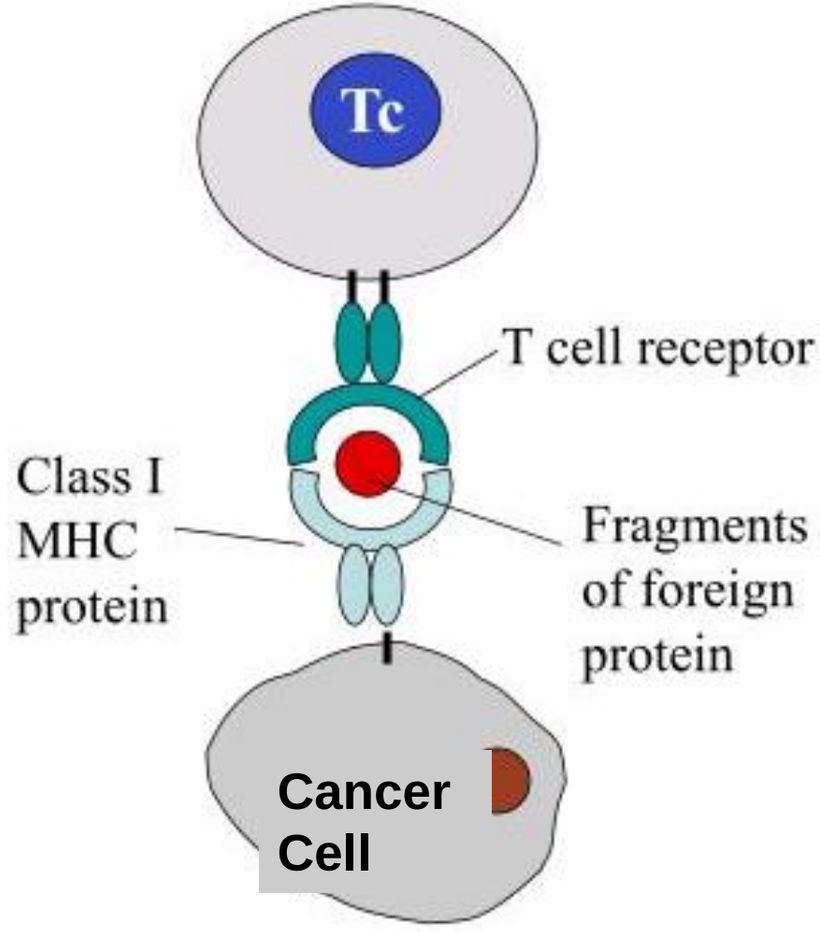


Cancer cell or Infected cell

- Granzymes enter through perforin pore 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.

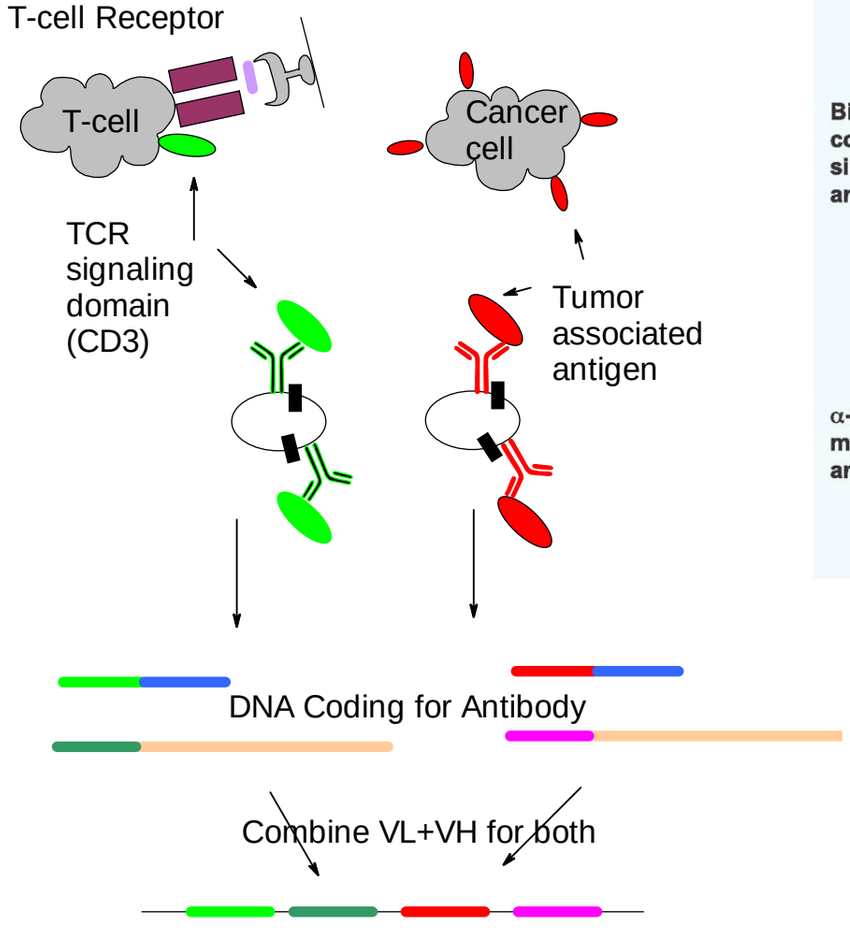


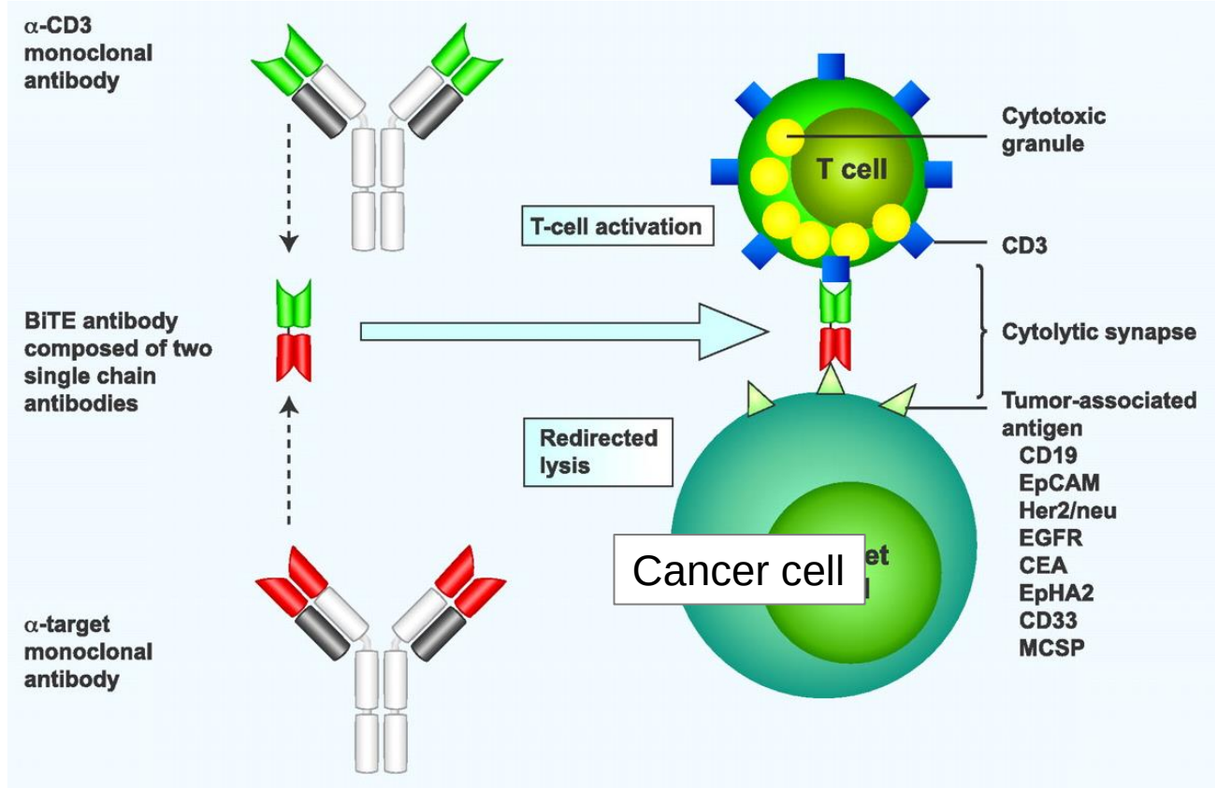
How to re-establish T_C contact with tumor cell and activation of the T-cell so that the cancer cell is killed?

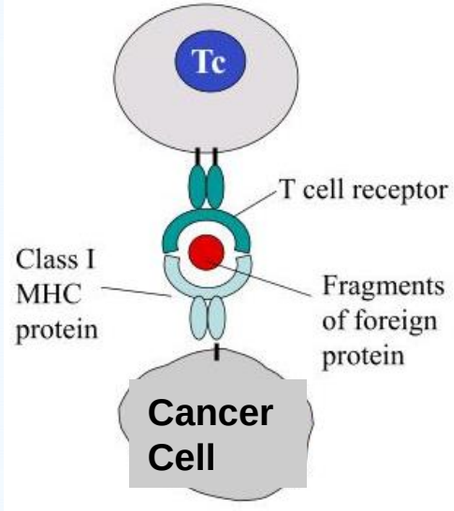
Cancer Treatment with Antibodies - Cancer Evasion - Loss of MHC I on Tumor Cell

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

- Up-regulation
- Mutation



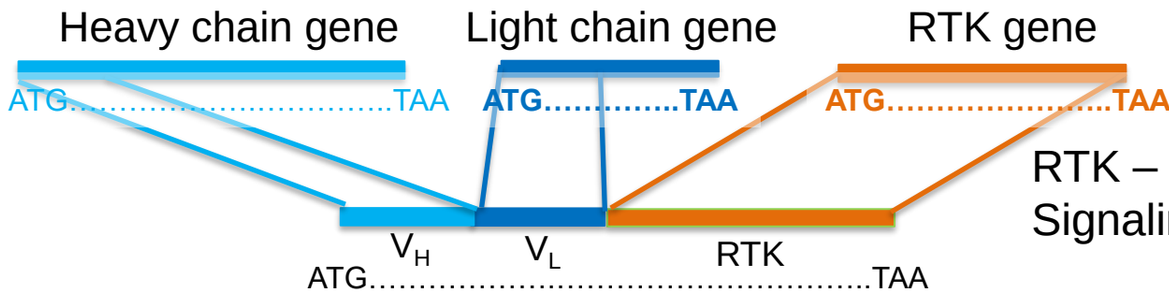




- Bispecific antibodies are generated from two separate antibodies:
  - One recognizes CD3, which is part of the T-cell receptor (TCR)
  - Other recognizes a tumor antigen.
- The two variable regions are linked into a single polypeptide chain by construction of a synthetic DNA molecule.
- The dual binding 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 signaling on T-cells = CAR-T gene.

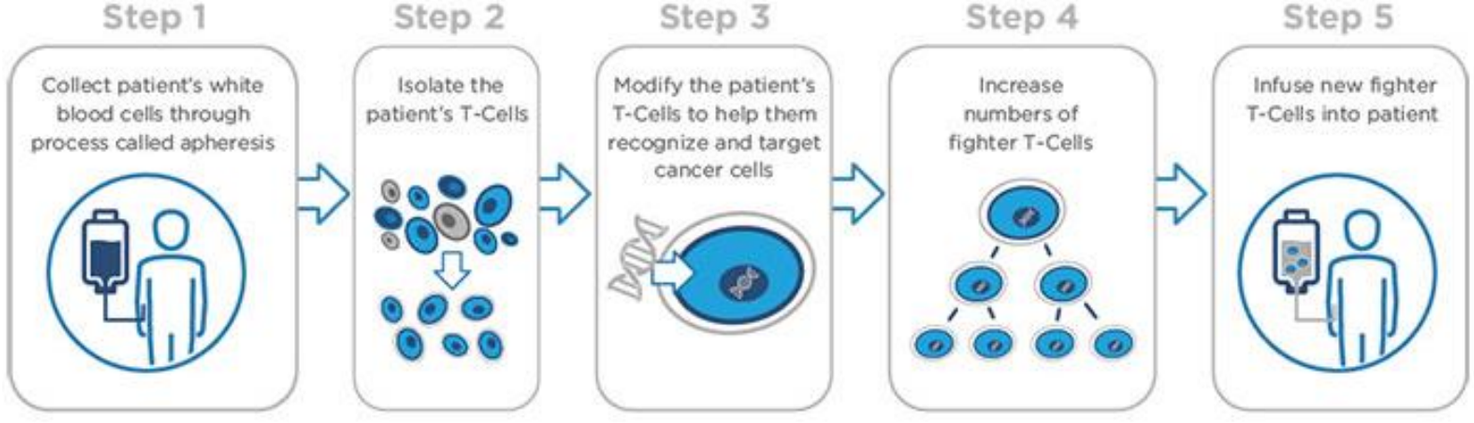


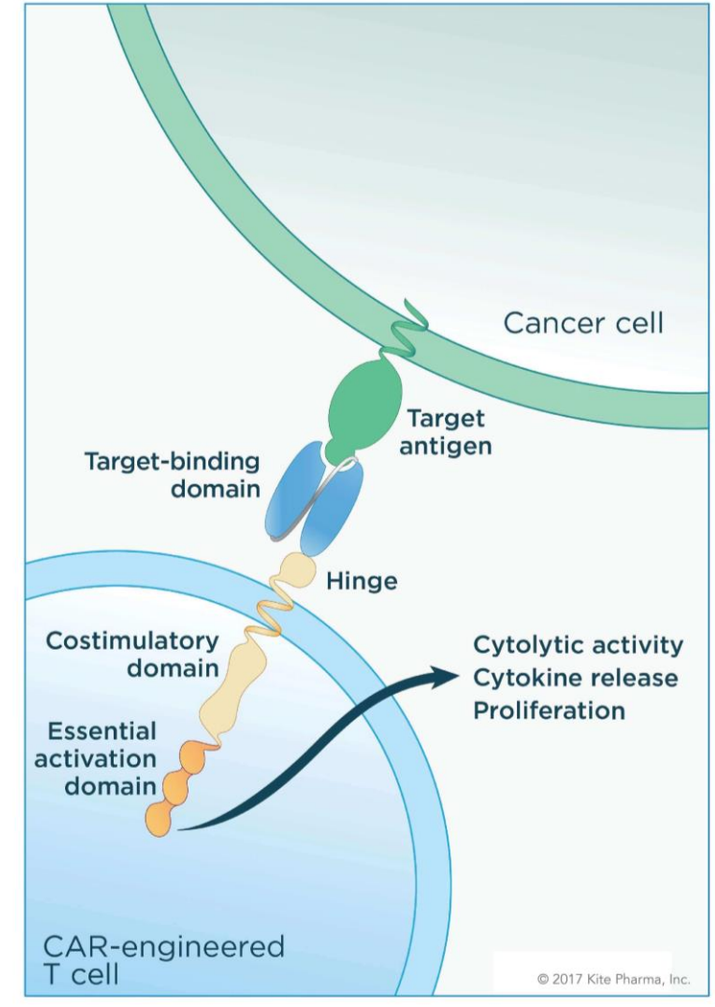
RTK – receptor tyrosine kinase
Signaling domain on T-cells

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?

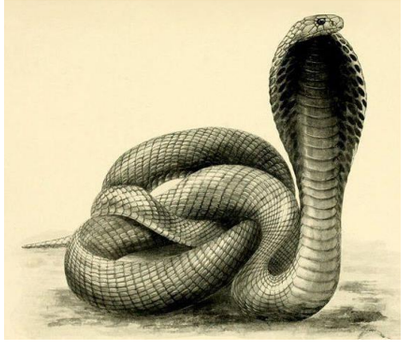


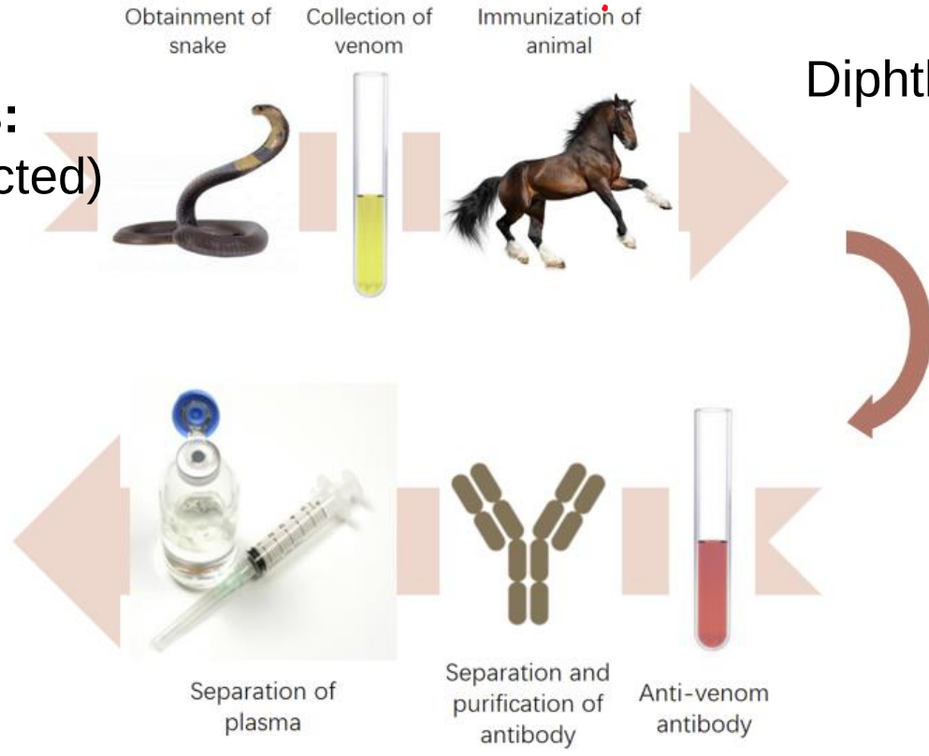


Vaccination

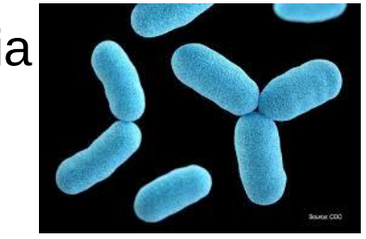
Types of vaccines:

1. Passive (Ab injected)

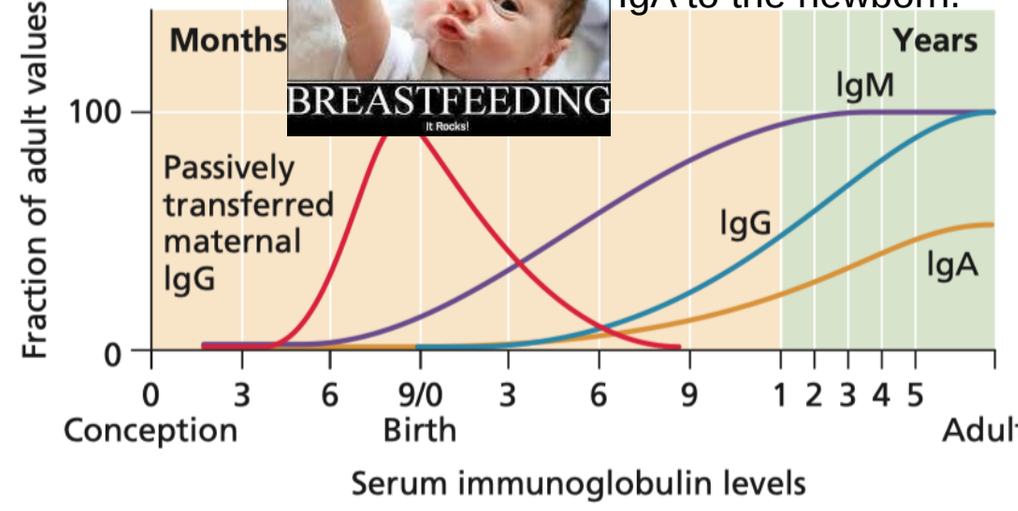




Diphtheria

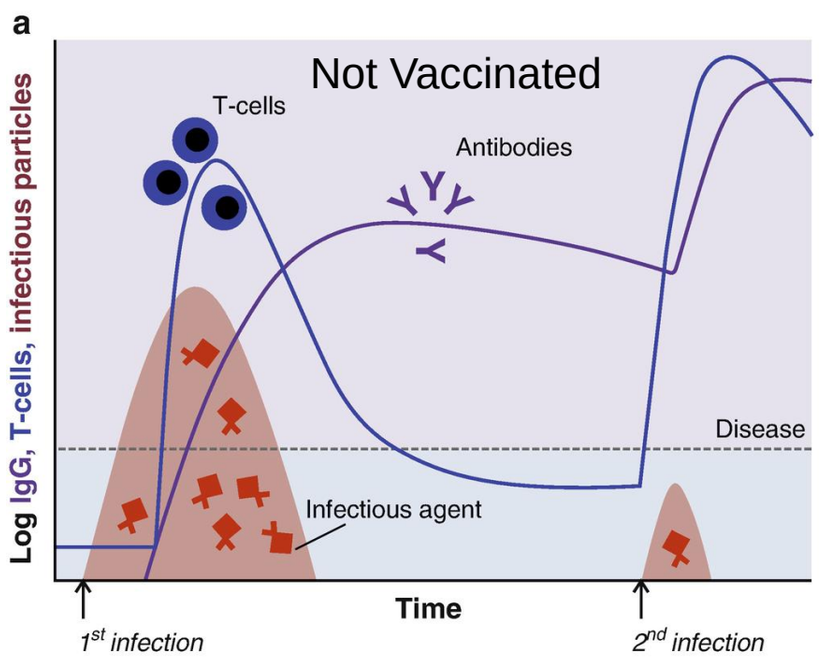


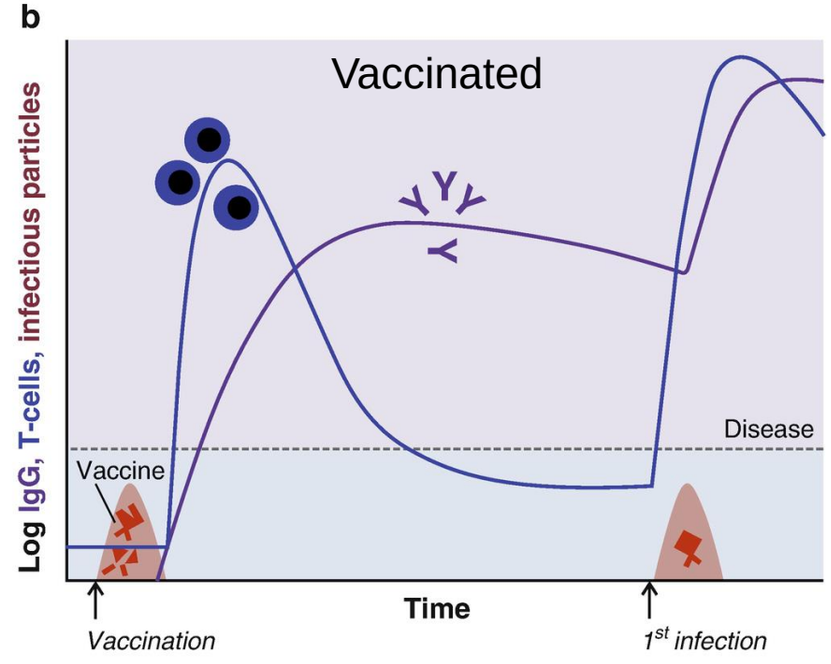
Breast feeding provides IgA to the newborn.

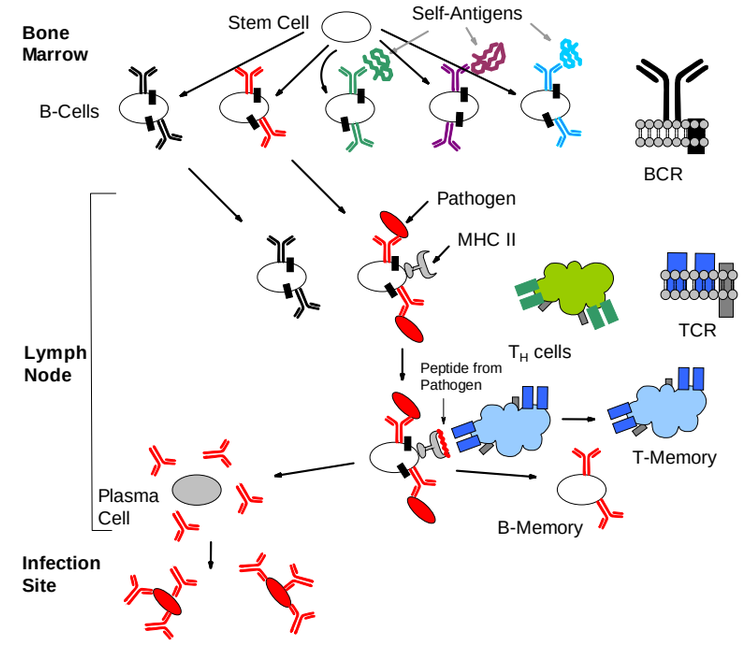


2. Active (Antigen Provided)

Primary and Secondary Response & Protection by Vaccines







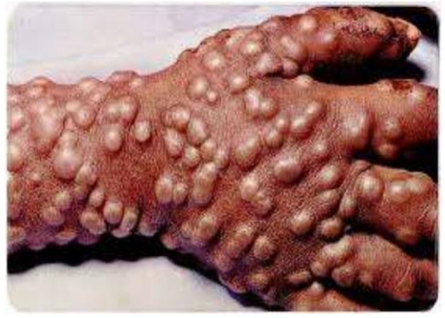
Large number of pathogens during first (primary) infection causes disease symptoms

- Antigen from pathogen prompts acquired immune response.
- Generates long-lived memory cells.
- 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.

Smallpox - A Success Story for Vaccination

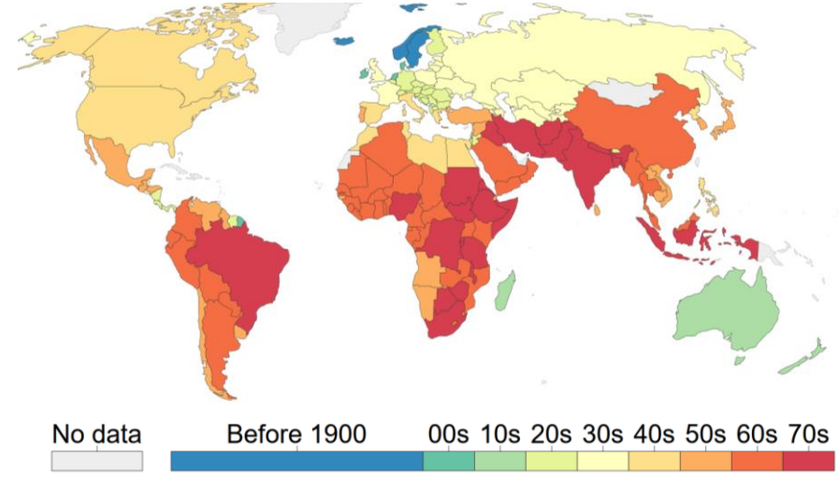


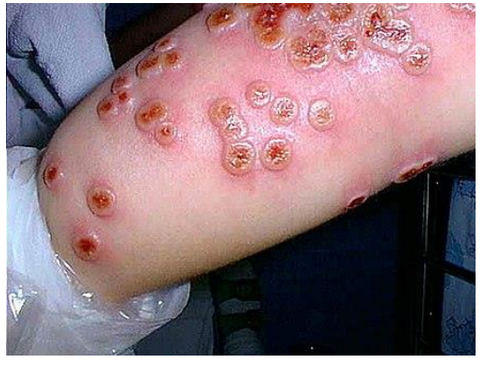
10,000 BC Smallpox – 20-90% lethality

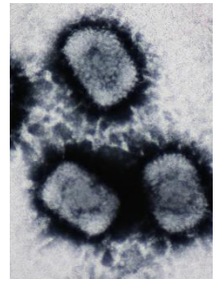
Variolation (1670) provided protection by exposing people to small amounts of smallpox virus (obtained from blisters on infected people). Practice spread from Istanbul to Europe.

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

Decade in which smallpox ceased to be endemic

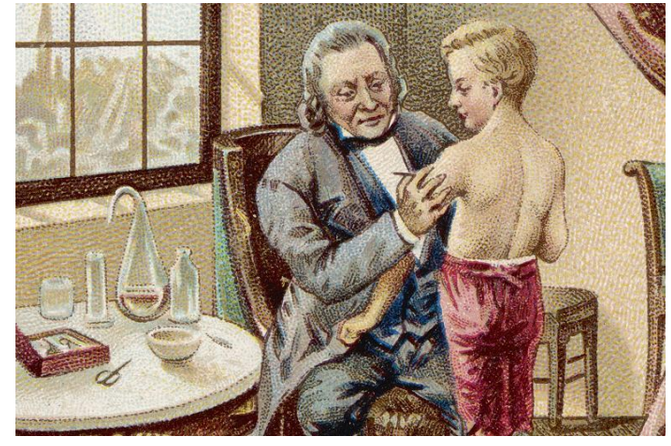






Cowpox virus:

- 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

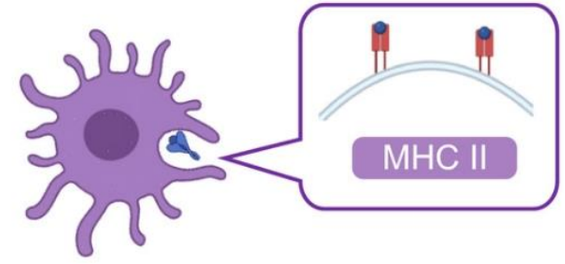
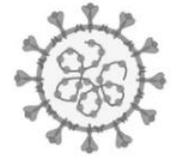
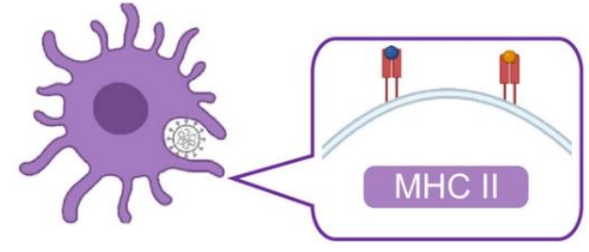
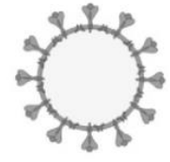
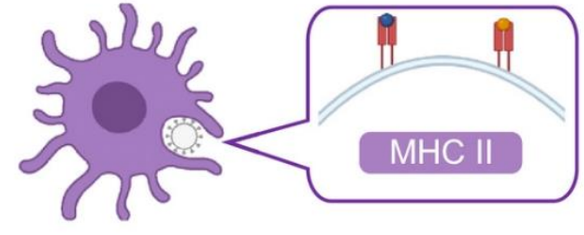
Vaccinia virus (similar to smallpox) is one form of the current vaccine.

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 cells.

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

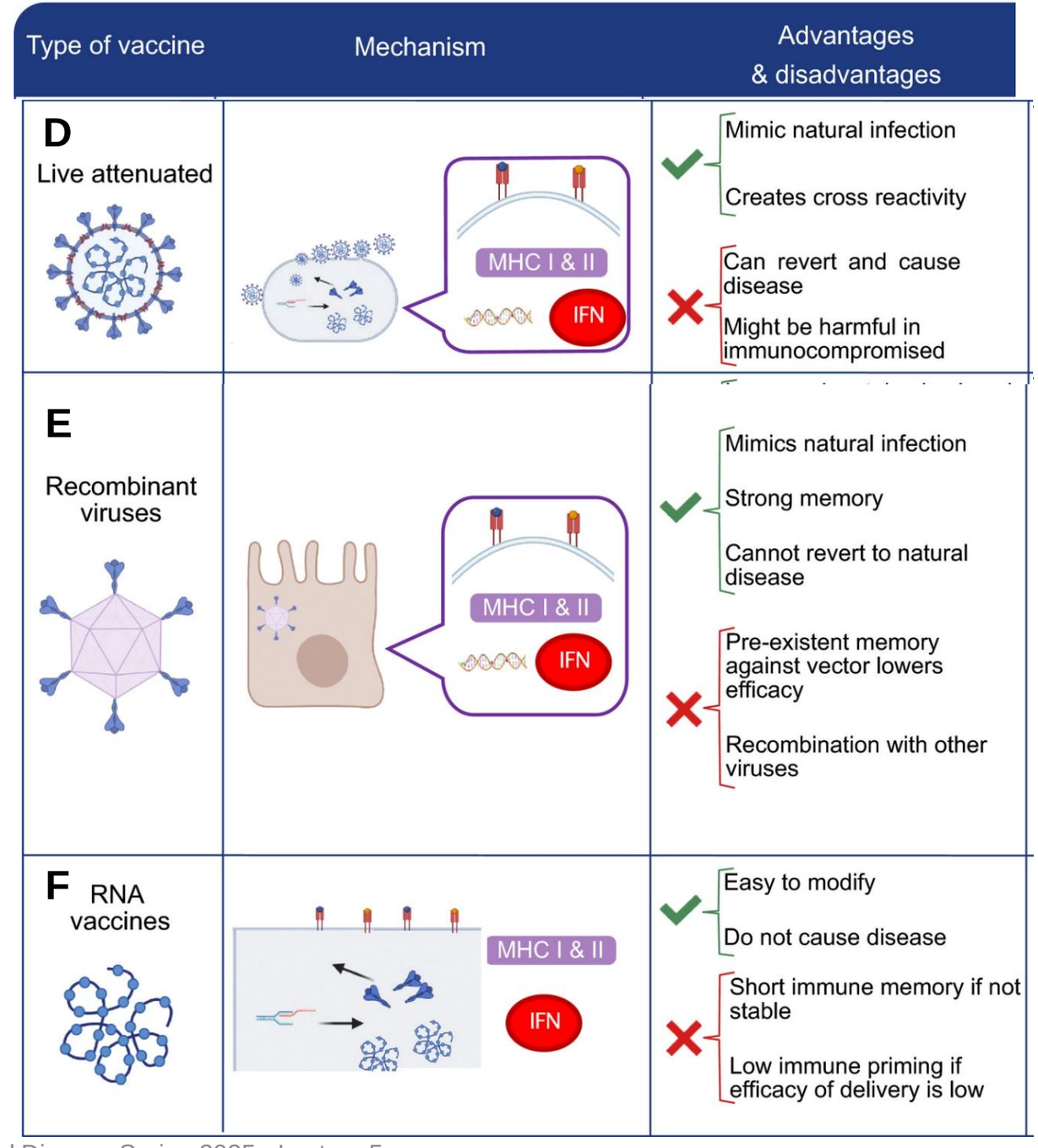
Type of vaccine	Mechanism	Advantages & disadvantages
A Subunit 		✓ Do not cause disease ✓ Very stable ✗ Needs booster strategy ✗ Short memory
B Inactivated 		✓ Do not cause disease ✓ Very stable ✗ Needs booster strategy ✗ Short memory
C Virus like particles 		✓ Increased uptake by lymph node ✓ Do not cause disease ✗ Dependant on efficient expression platform ✗ Difficult to make VLP stable in long term

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

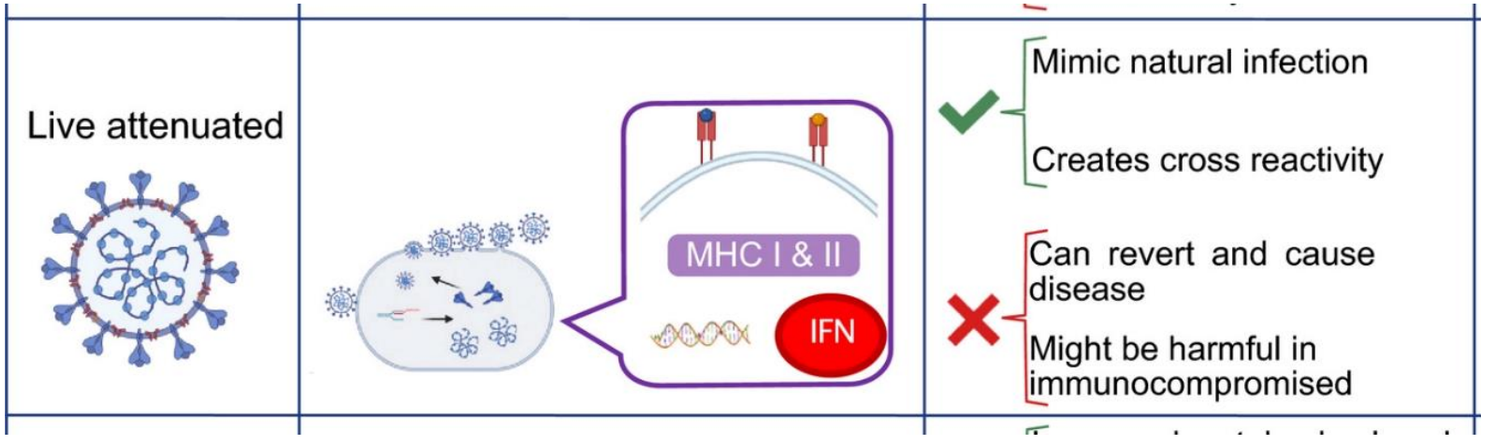
E. Recombinant Virus:
A “safe virus” is used (e.g. cold virus)
Gene for a protein from a pathogen is inserted into the DNA of the virus.
- When virus grows it produces the protein from the pathogen generating immunity.

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.



D. Attenuated – Sabin Polio Vaccine



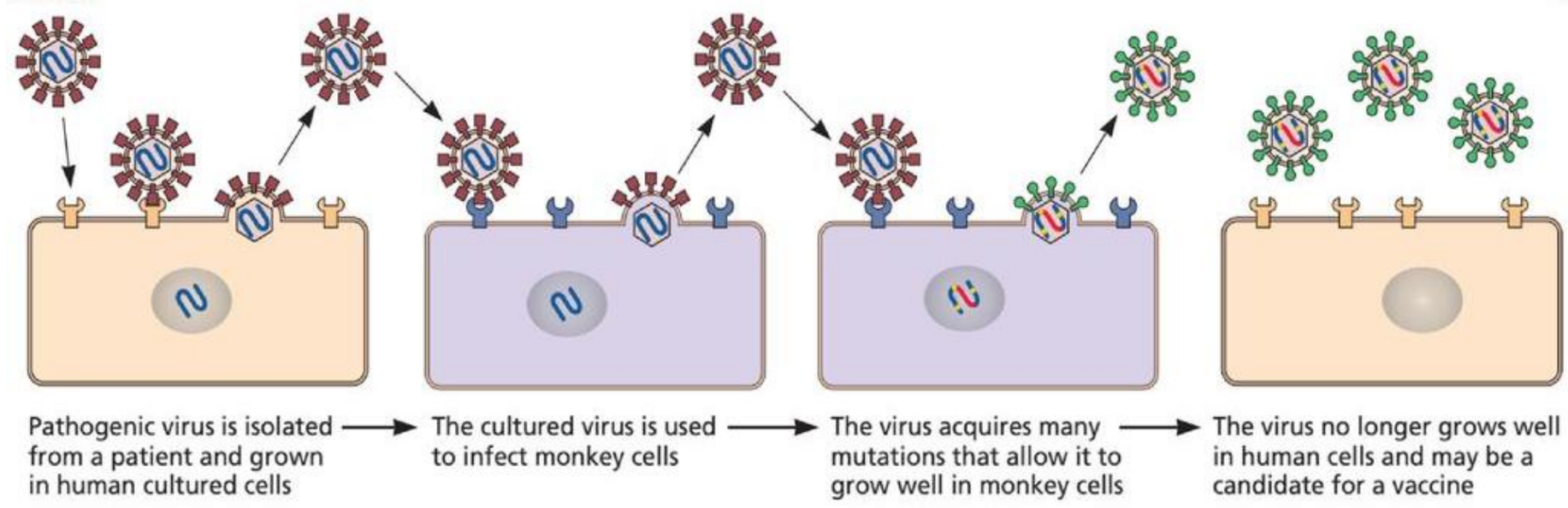
THE CINCINNATI ENQUIRER

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

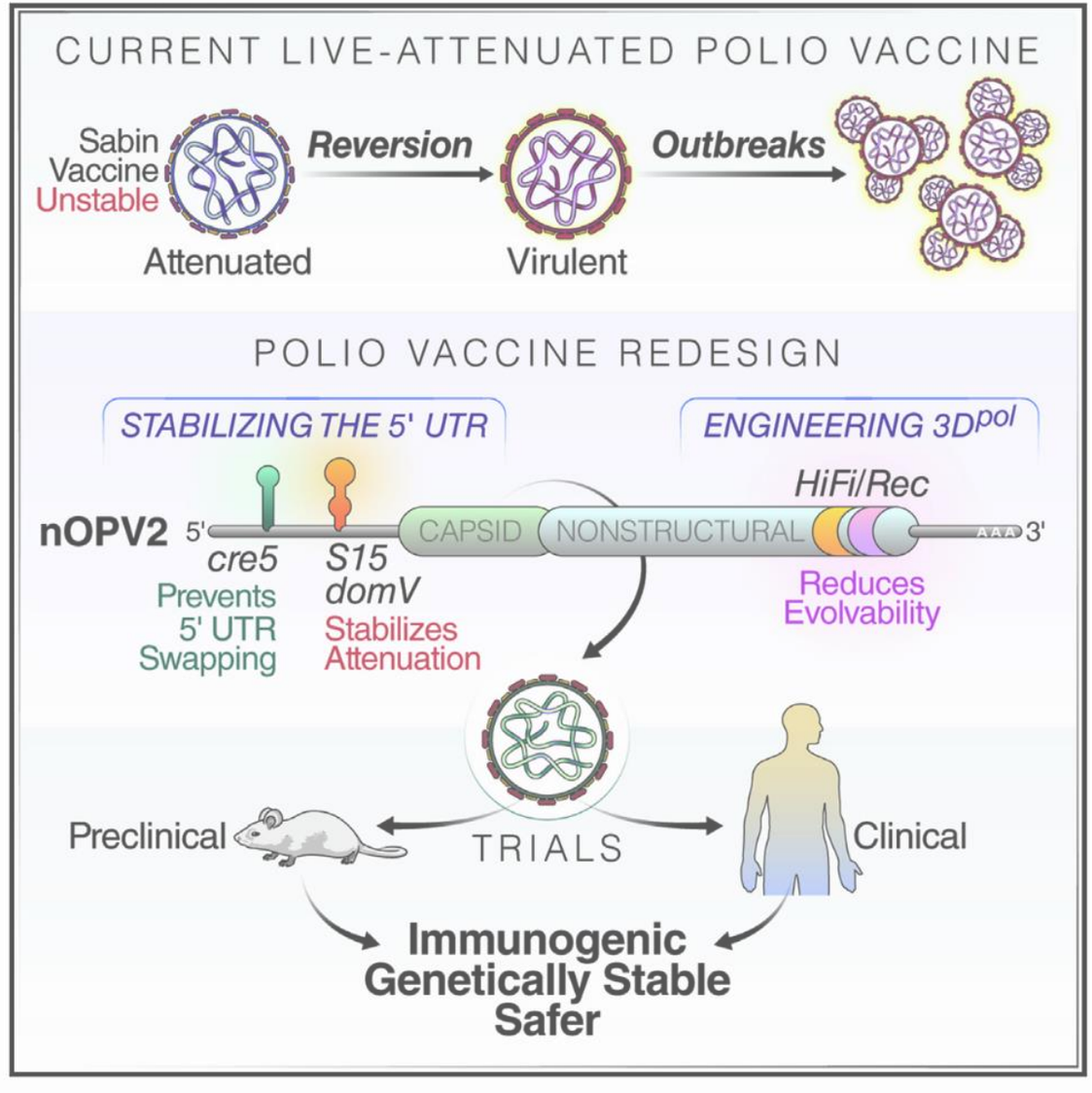
Home / Disease Outbreak News / Item / Circulating vaccine-derived poliovirus type 2 - Indonesia

Disease Outbreak News

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

11 January 2024

- Mutations that attenuated the virus revert to the original sequence during infection.
- This is not surprising because growth of the virus in infected humans will select for viruses that grow better in humans.



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]

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?

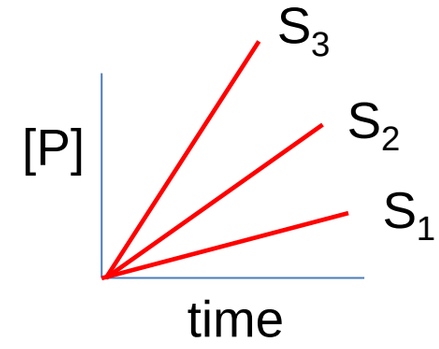
10. What was the origin of the idea for vaccination?
11. What was one of the first “safe” vaccines? What disease has now been eradicated due to this vaccine?
12. Can you describe one way to generate a vaccine for a pathogen? Do you know the pros and cons for that method?

Enzyme Inhibitors as Drugs

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

Key Points:

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



Kinetics

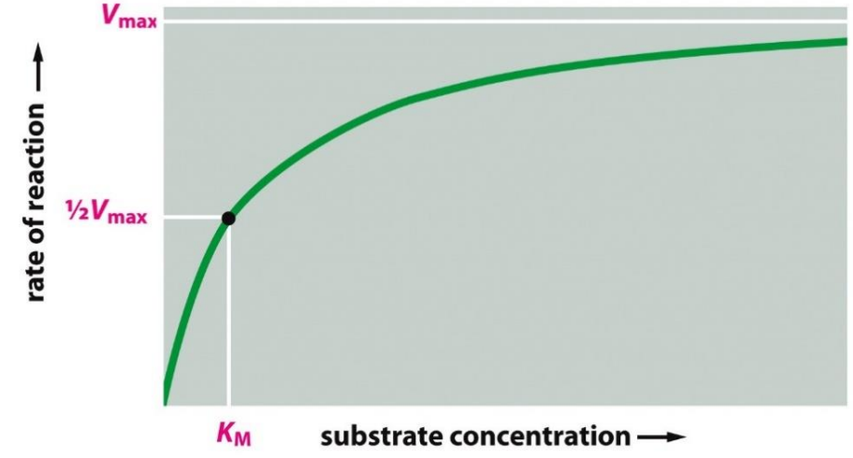
Rate = dP/dt, proportional to [ES].

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 (K_M is very similar to K_D).



Enzyme Inhibitors

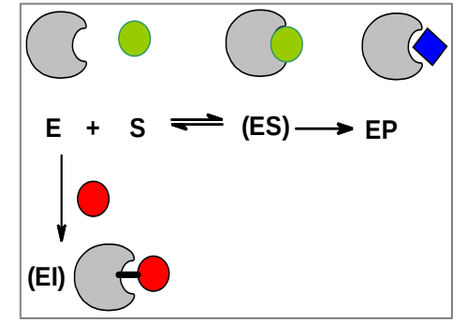
Studies on Inhibitors are useful for:

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.

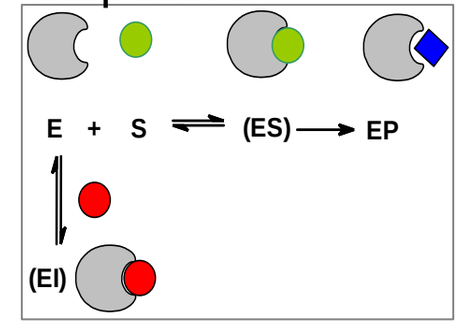
Types of Inhibitors:

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*. Enzyme activity returns when drug is removed.
3. Allosteric (mixed type) – inhibitor causes allosteric (different shape) change, *distorting the active site*. Binds *reversibly to a different location. Enzyme activity returns when drug is removed.*

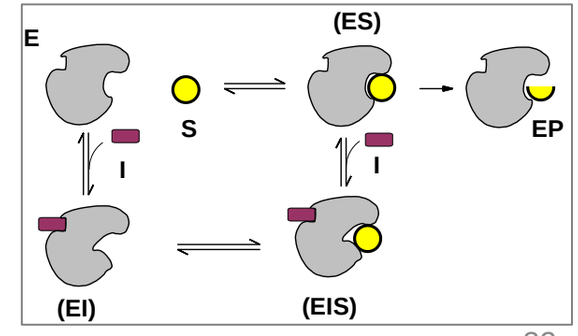
Covalent



Competitive

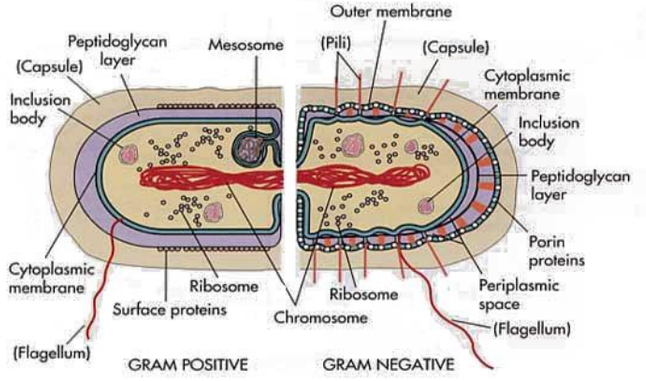


Allosteric (Mixed type)



Mechanism of Penicillin – A Covalent Inhibitor

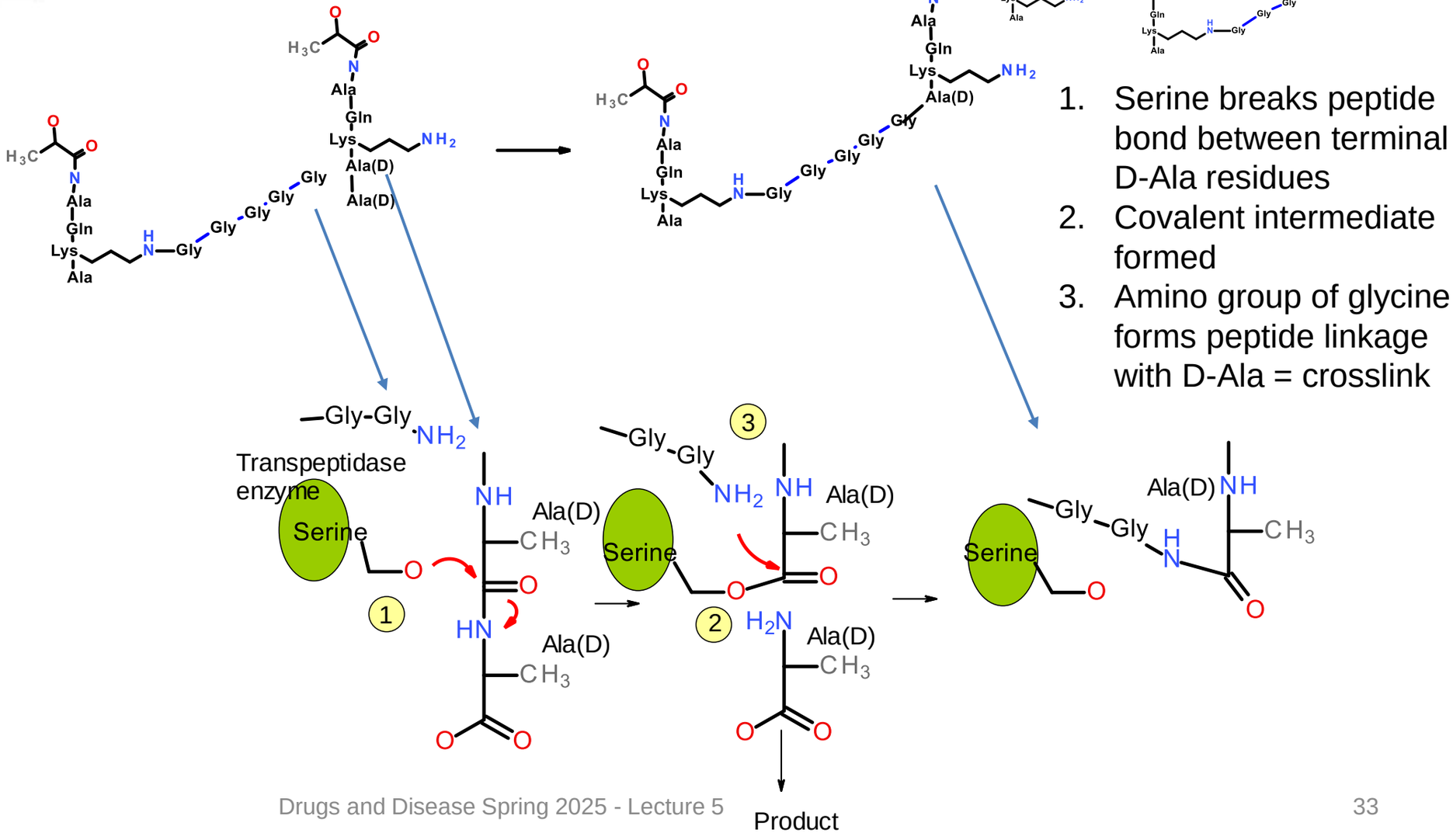
Bacterial Cell Wall



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.***

Synthesis of bacterial cell wall – generation of protein crosslink

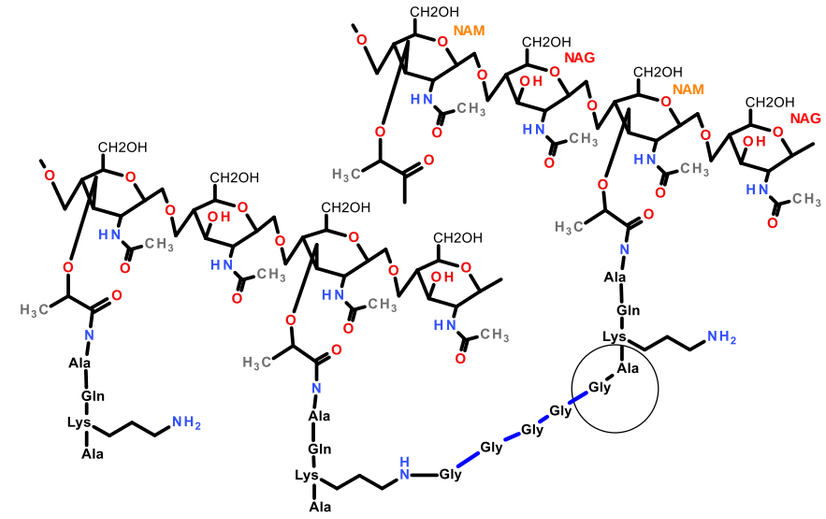


1. Serine breaks peptide bond between terminal D-Ala residues
2. Covalent intermediate formed
3. Amino group of glycine forms peptide linkage with D-Ala = crosslink

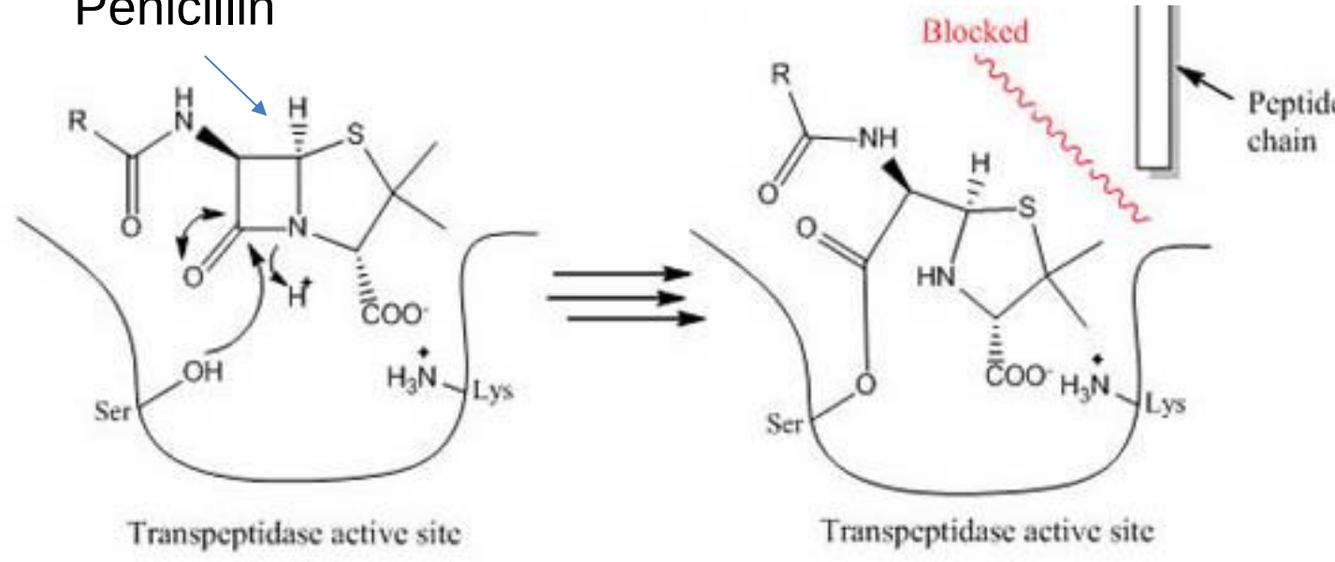
Mechanism of Penicillin

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

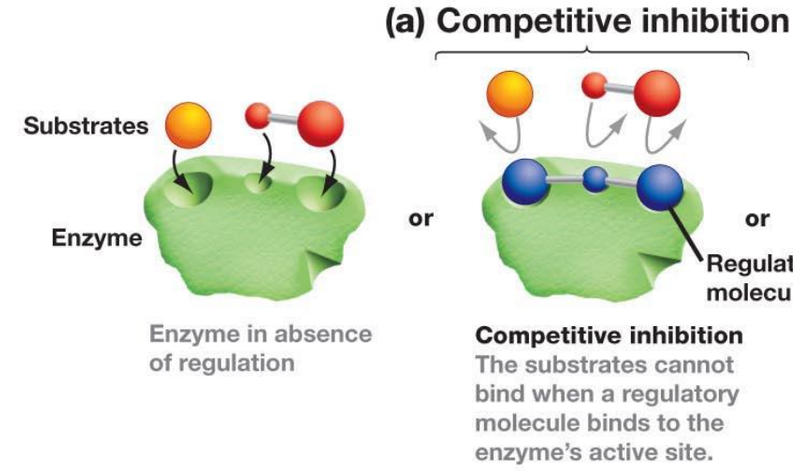


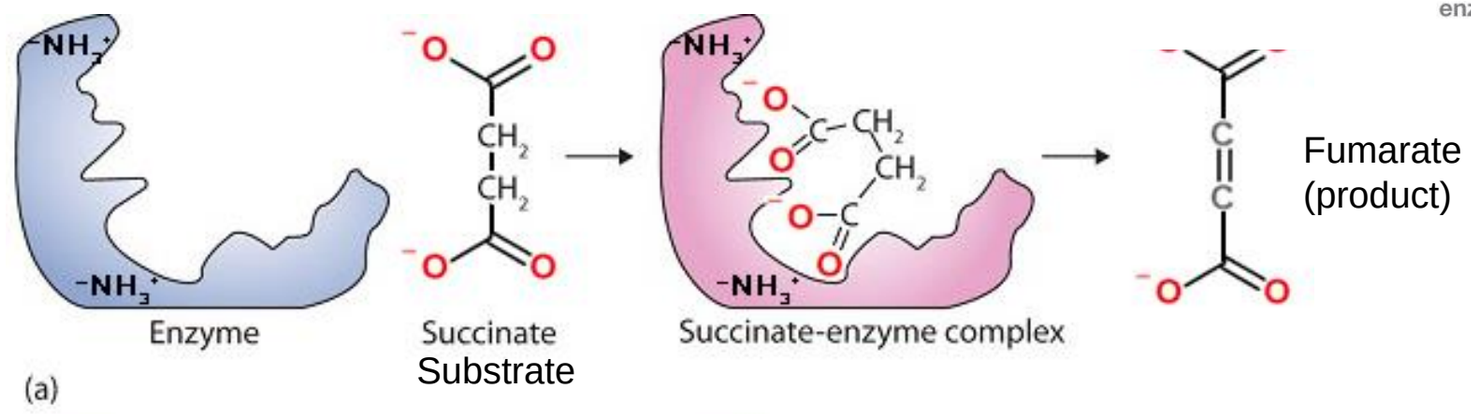
Competitive Inhibitors

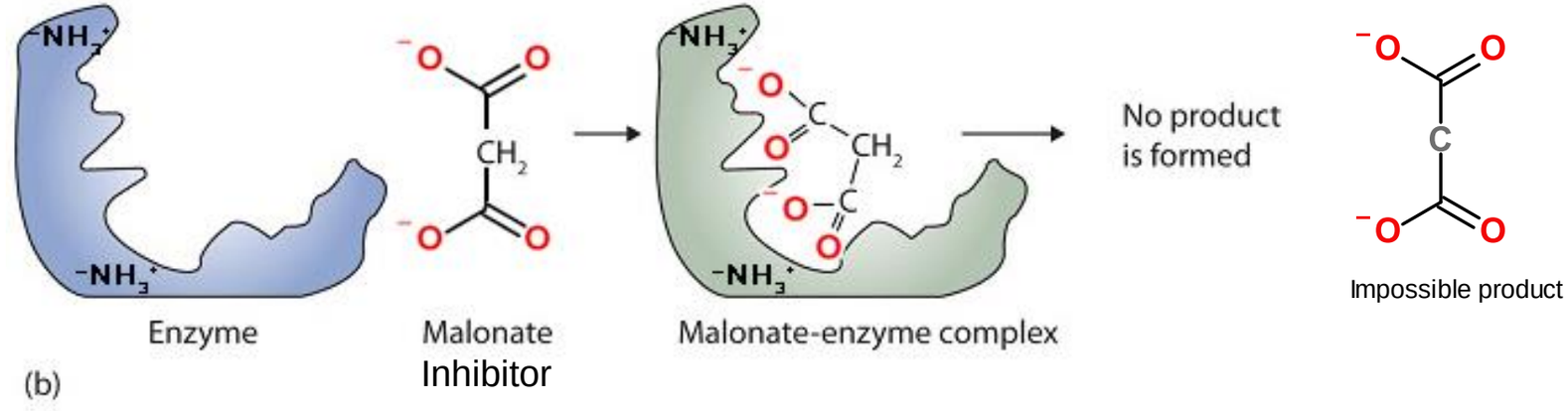
Succinate dehydrogenase converts succinate to fumarate by removal of two hydrogens.

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

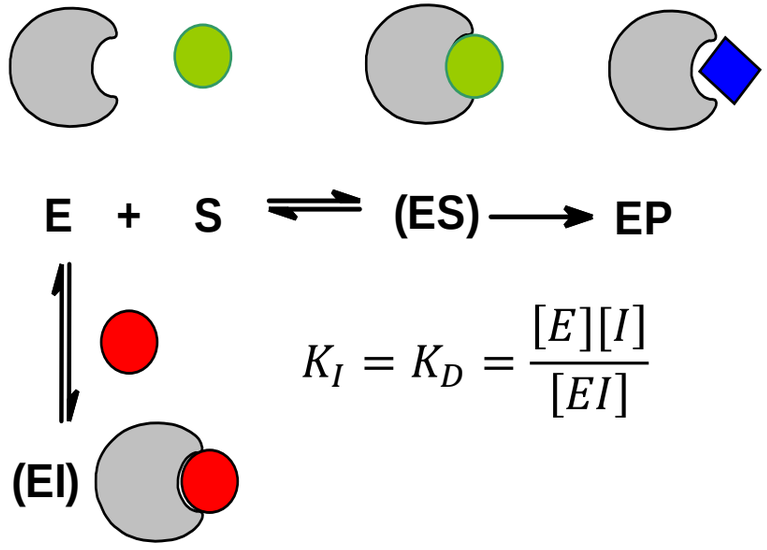
- It is similar in structure to the substrate – so it binds in active site – substrate cannot bind at the same time.
- Malonate **cannot** undergo the chemical reaction – it is not possible to remove two hydrogens without leaving carbon with too few bonds.





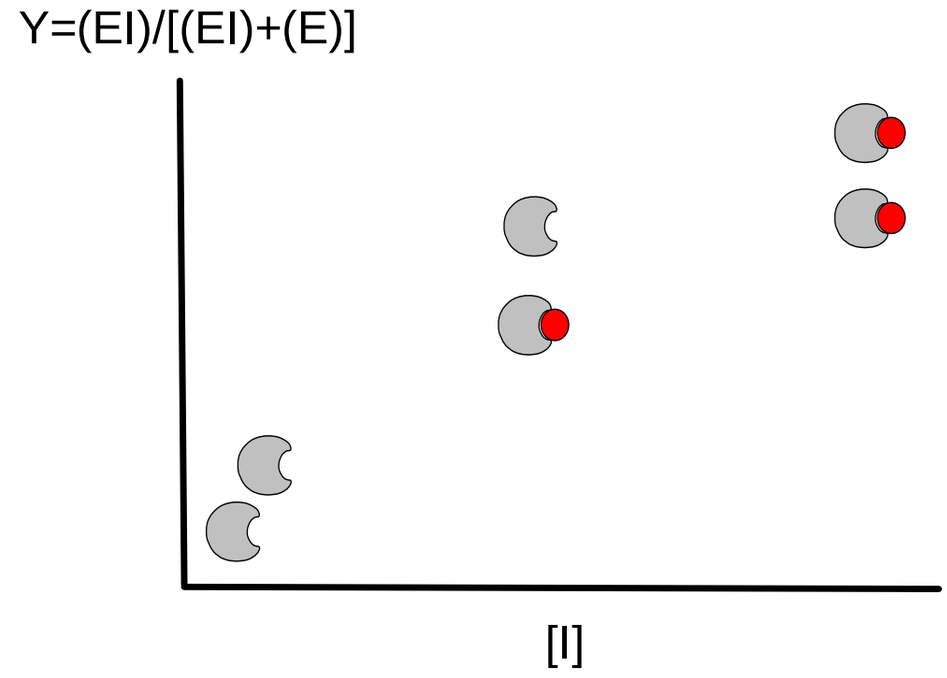


Quantification of Inhibitor Binding



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

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 determined by measuring the effect of inhibitor on the enzyme kinetics.

Effect of Competitive Inhibitor on Steady-State Kinetics:

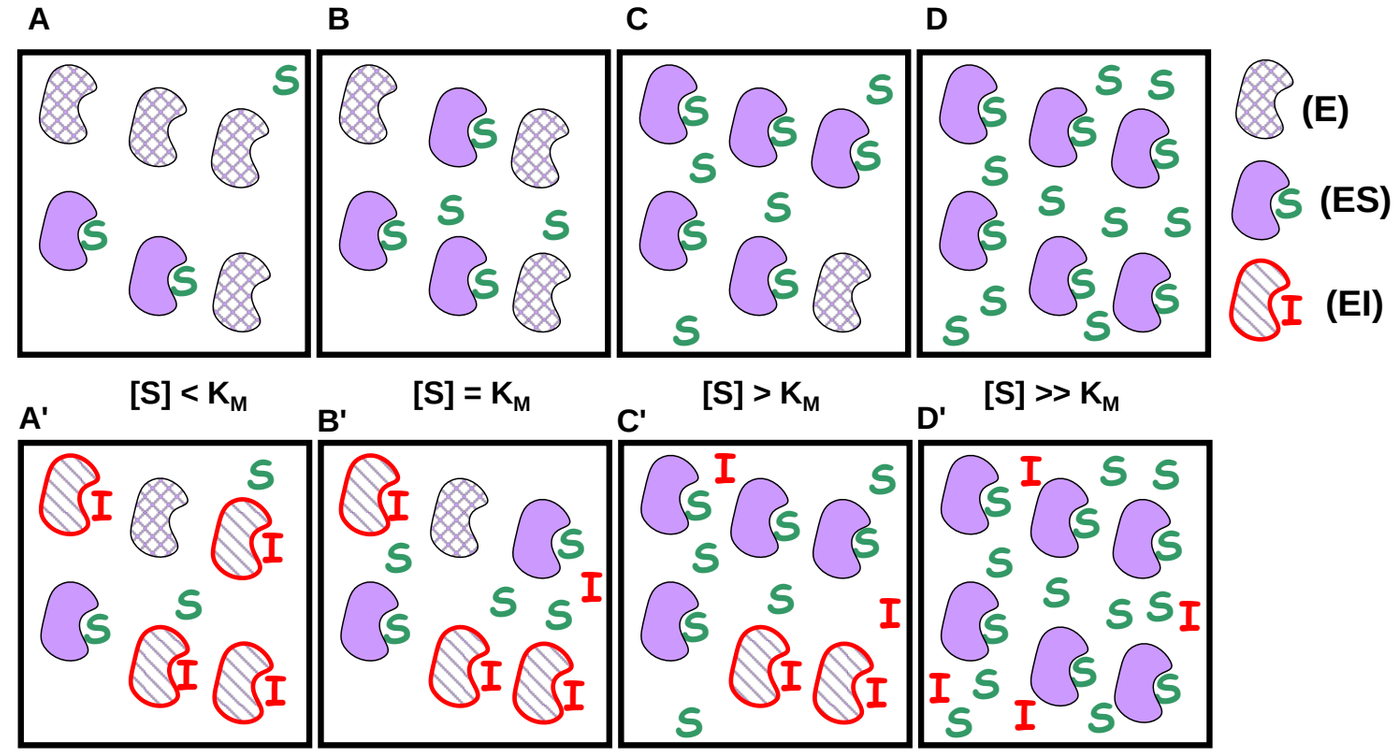
- A competitive inhibitor reduces the amount of [E] by the formation of [EI] complex.
- The inhibitor cannot affect the [ES] complex since the inhibitor can no longer bind.

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

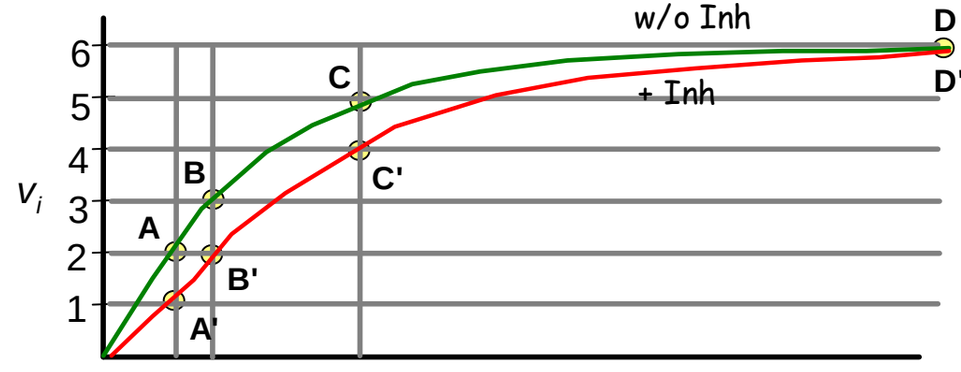
1. **V_{MAX} is unchanged**: At high levels of substrate all of the inhibitor is displaced by substrate, so $[ES]=E_{TOTAL}$, and $v_{MAX} = k_{CAT}[E_{TOT}]$.
2. **The *observed* K_M is increased:** It requires more substrate to reach 1/2 maximal velocity because some of the enzyme is complexed with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



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



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

No inhibitor

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

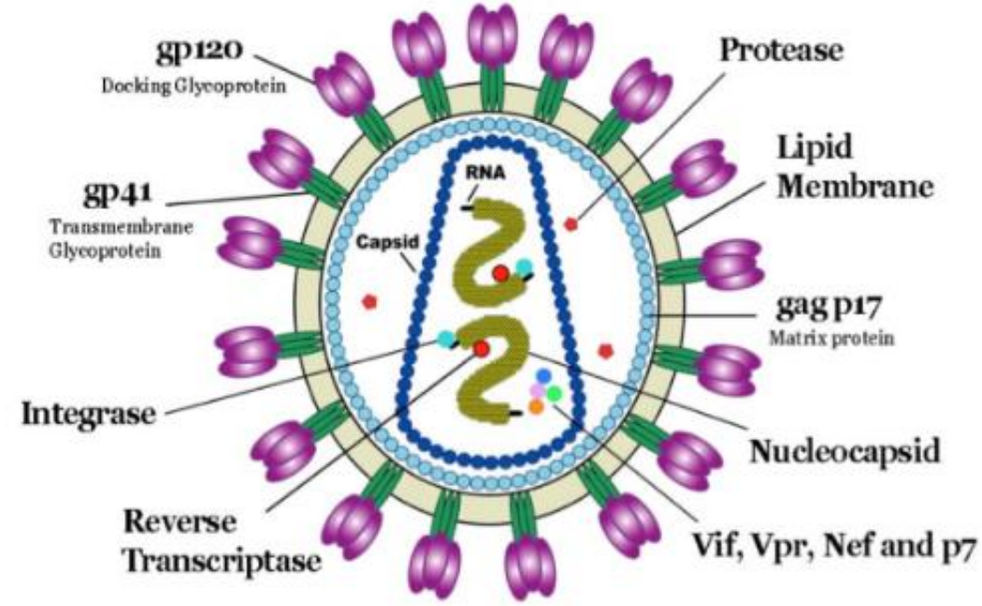
Comp inhibitor

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

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.



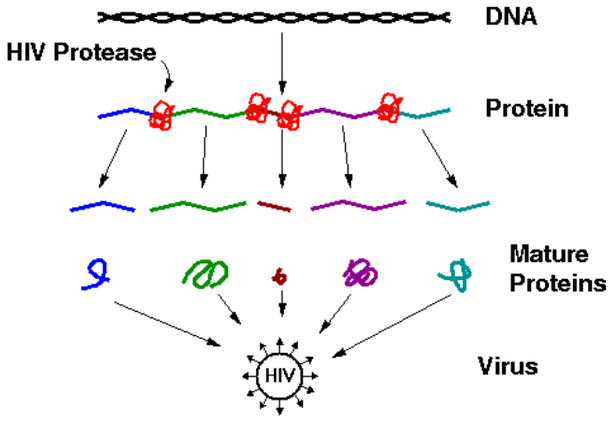
Human Immunodeficiency Virus (HIV)

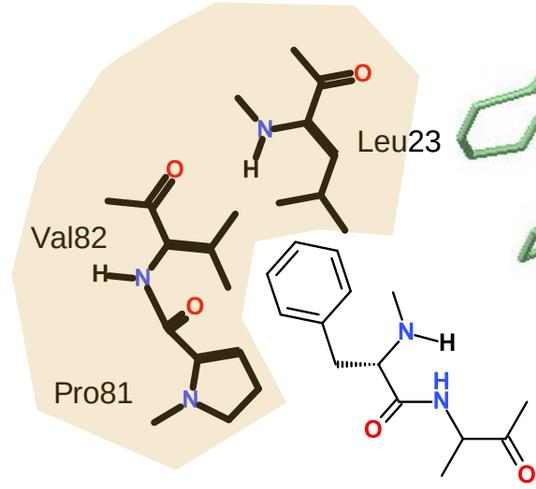
- Infects specialized cells in the immune system – *T-helper cells* (T_H) cells, killing them.
- T_H cells are required for activation of the immune response to all pathogens (bacteria, virus)
- Killing of T_H cells by the HIV virus causes AIDS (acquired immunodeficiency), making the individual susceptible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

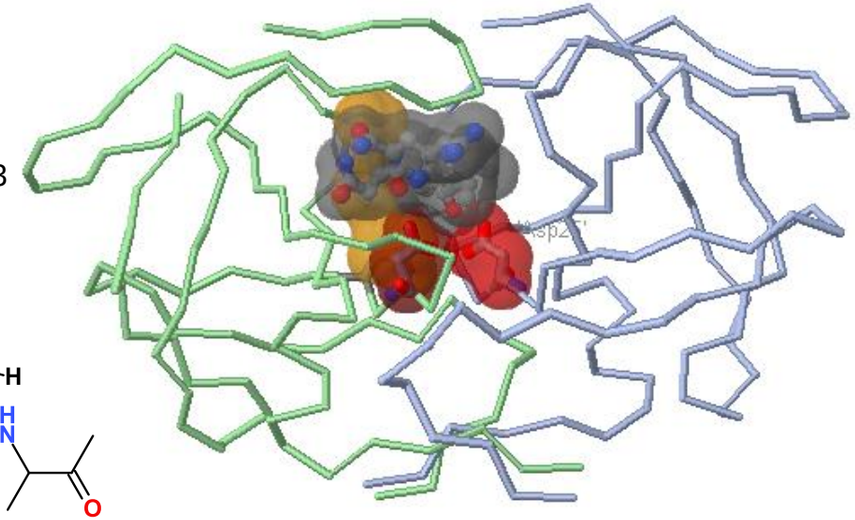
Viral particle contains enzymes required for the replication of the virus:

- ***Reverse Transcriptase:*** Copies viral RNA to DNA
- ***Integrase:*** Integrates viral DNA into host chromosome. This DNA is used to make new copies of the viral RNA as well as mRNA to make viral proteins.
- ***HIV Protease:*** Cleaves immature viral protein to produce smaller mature proteins.

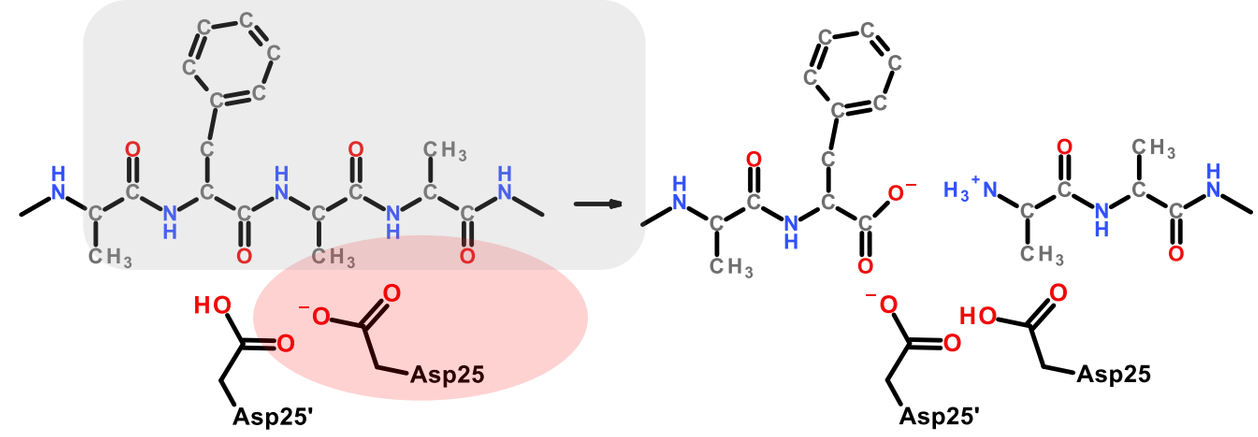
HIV Protease (Aspartyl protease)







- 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.



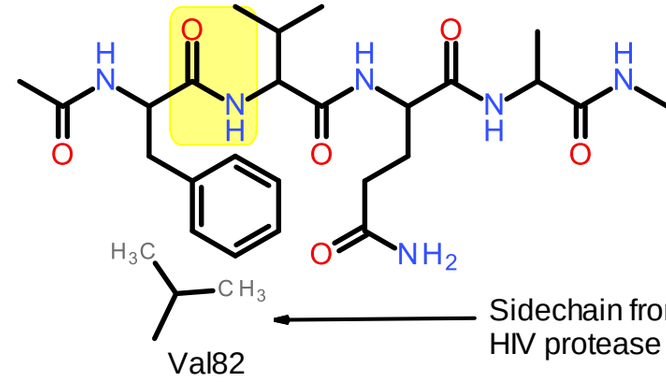
HIV Protease:

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).
3. Cleaves peptide bond after large non-polar residues

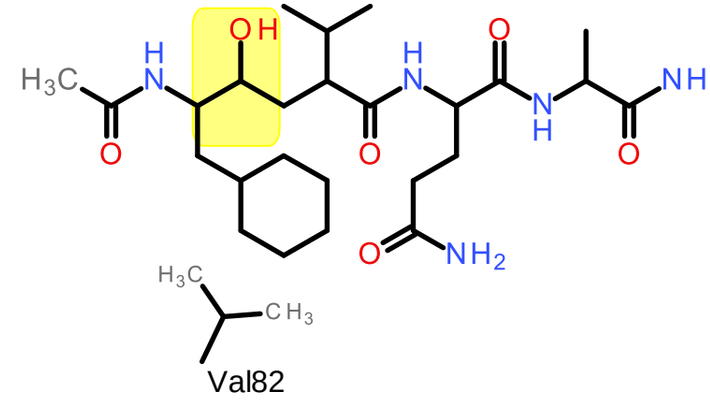
Inhibition of HIV Protease (HIV Drugs):

- Most drugs are small peptide-like analogs with non-cleavable bonds that resemble peptide bonds. These are competitive inhibitors because:
  - ❑ They bind to the active site (similar to substrate)
  - ❑ They are unreactive (no peptide bond)

Substrate

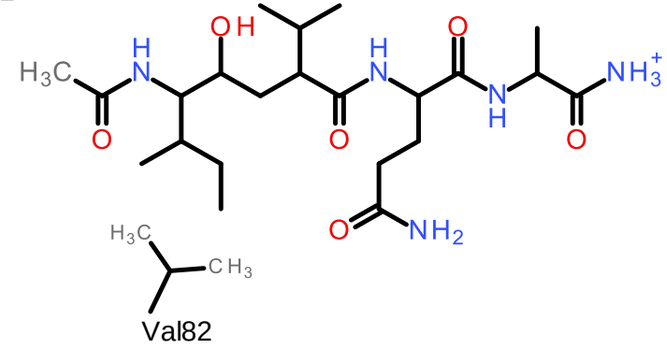


Inhibitor

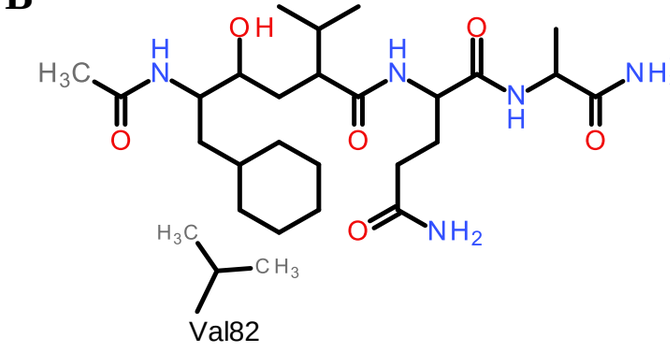


Drug Design: Compounds A (Isobutyl) and B (cyclohexane) are candidates for HIV protease inhibitors. Which of the two drugs will be more effective at inhibiting the wild-type protease?

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:

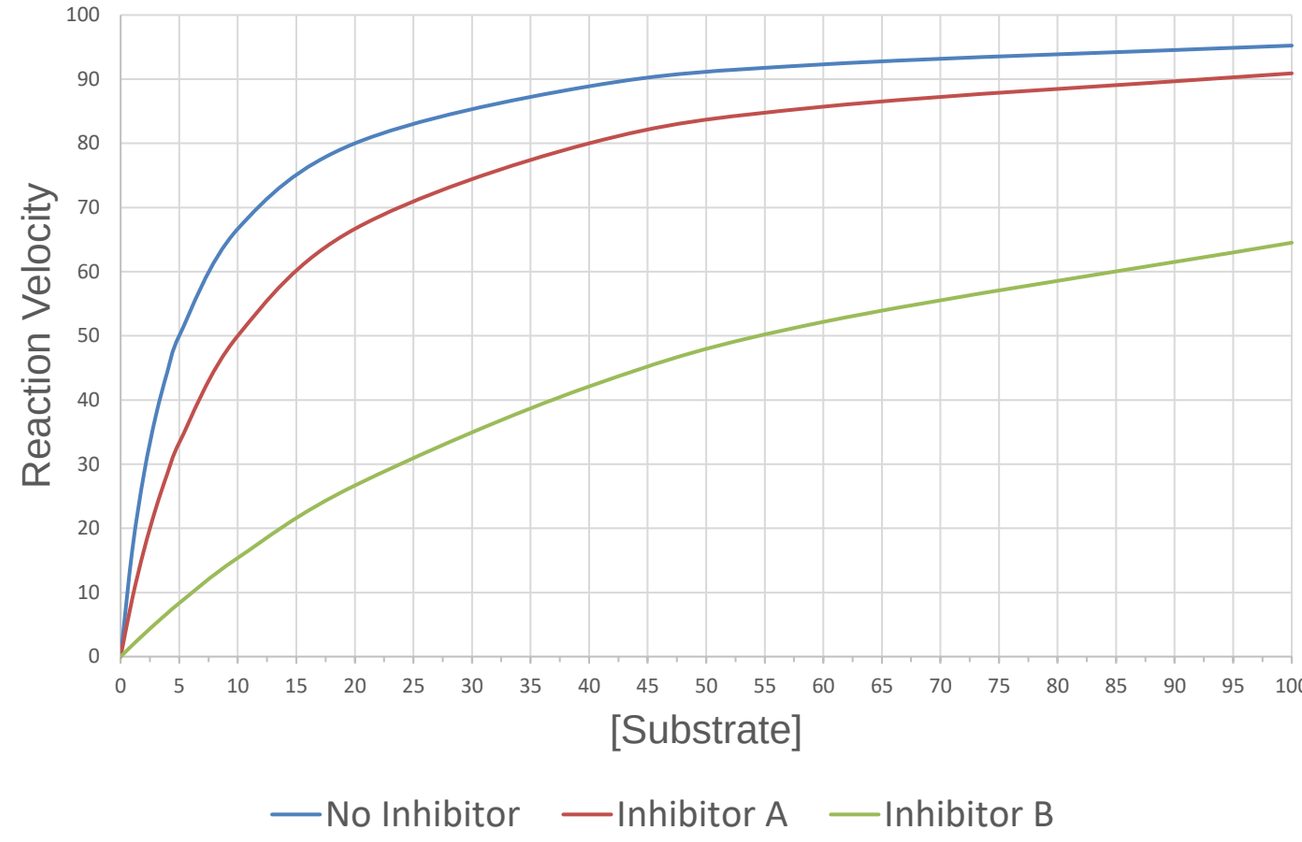
i) Plot velocity versus [S]

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.

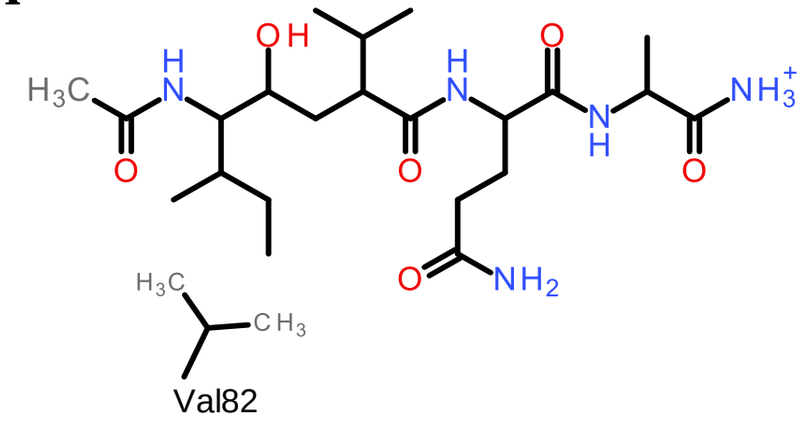
Once the α values are found, we can calculate the K_I for each inhibitor using the formula: **K_I=[I]/(α-1).**



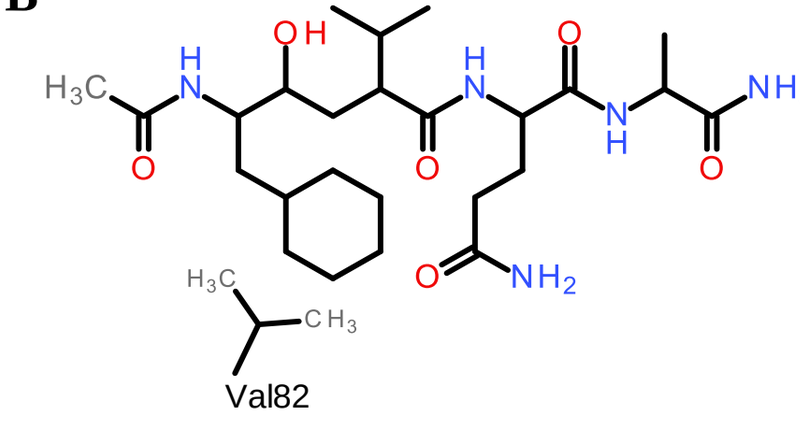
Data	Km	Alpha (K_M^{obs}/K_M)	K_I =[I]/(α-1) ([I]= 10 nM)
No Inh	5		
Inh A	10	2	K_I = 10/(2-1) = 10 nM
Inh B	54	10.8	K_I = 10/(10.8-1)=1.1 nM

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

A



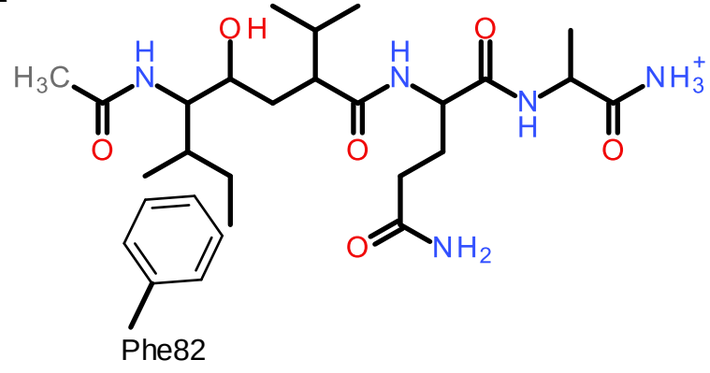
B



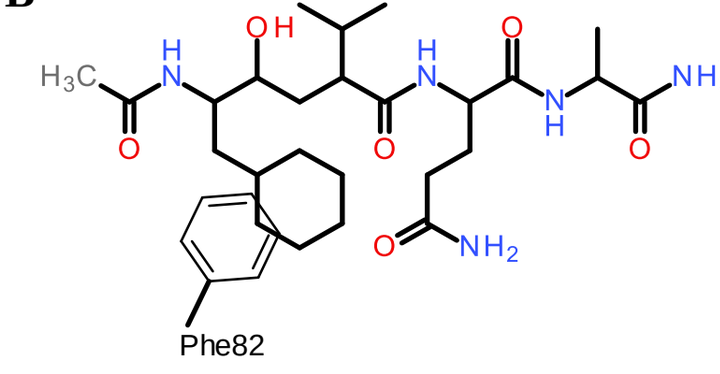
Potential Interaction	Drug A (K_I = 10 nM)	Drug B (K_I = 1.1 nM)
Van der Waals		
Hydrophobic effect		

Rational Drug Design. Which Drug would be more effective if the virus, via errors in replication, replaced Val82 with Phe?

A



B



Antibiotics That Inhibit Prokaryotic Translation

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:

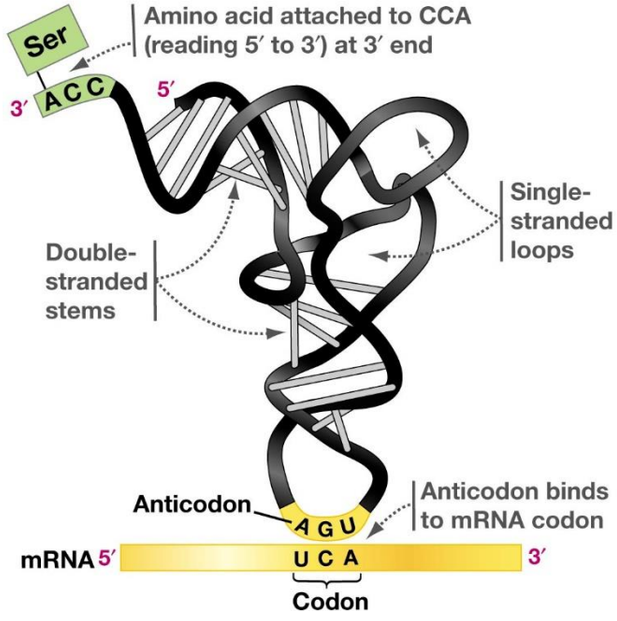
1. mRNA binds to 30S
2. fMet-tRNA binds to P site
3. 50s binds to complete initiation complex

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

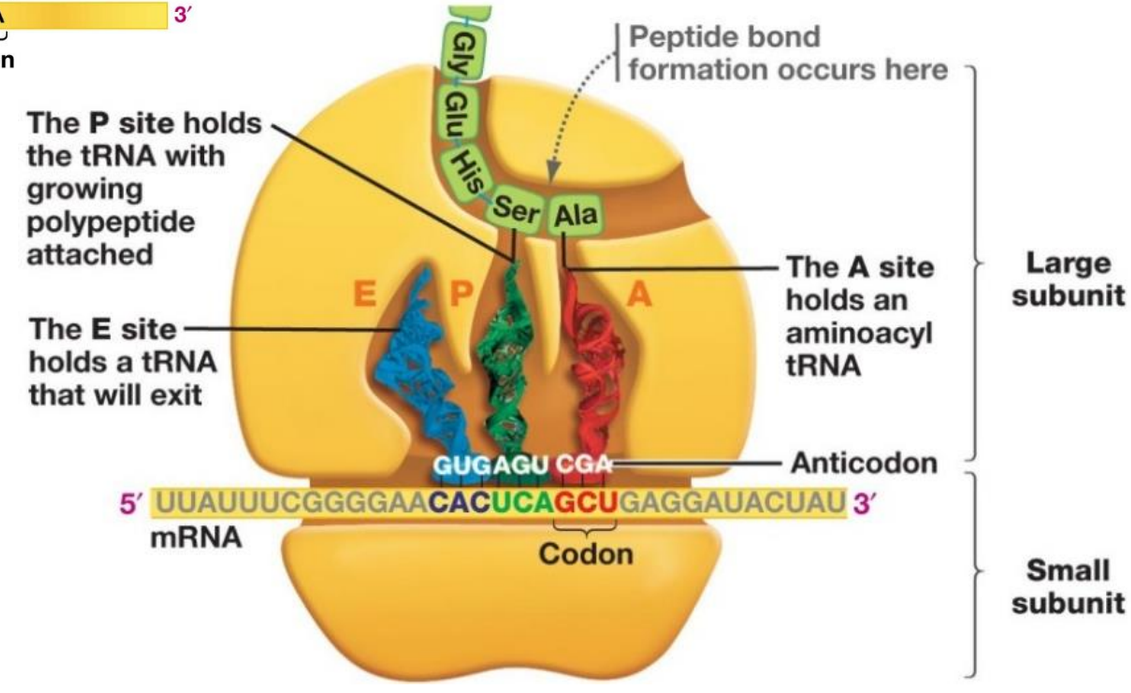
Termination:

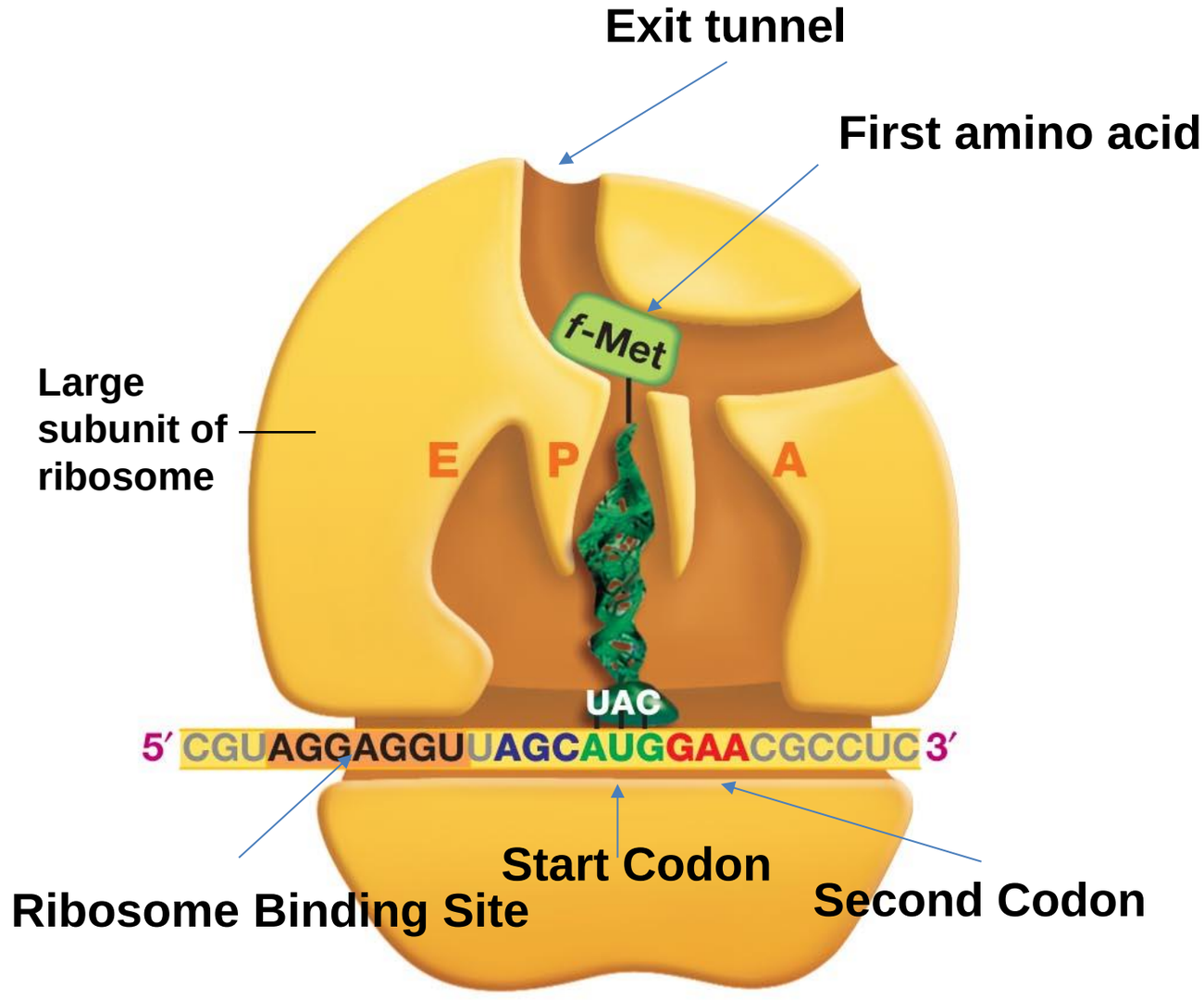
1. Stop codon at A site
2. Termination factor (protein) adds water to cleave peptide from last tRNA



tRNAs

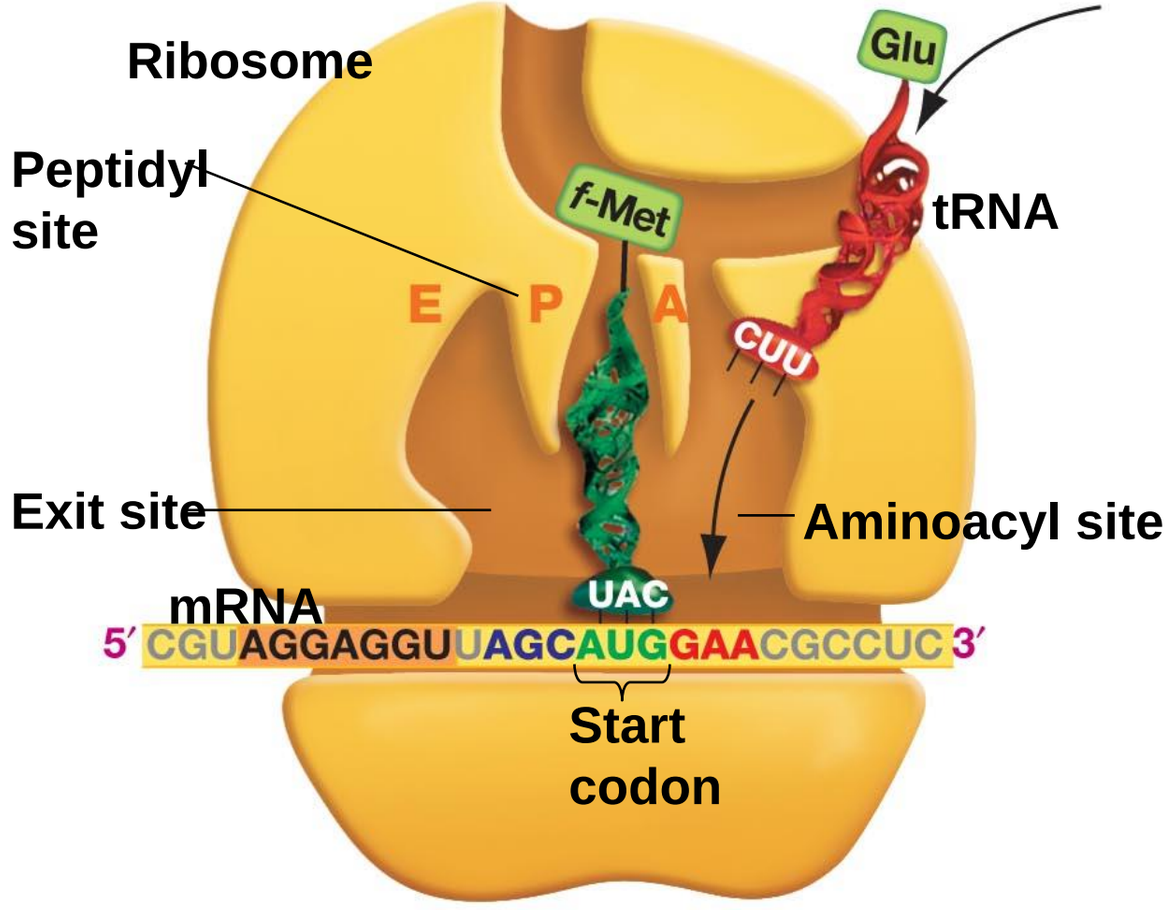
- The adapter molecules are called transfer RNAs or tRNAs.
- Contain a CCA sequence at 3’ end where the amino acid is attached
- a triplet anticodon to form base pairs with the appropriate mRNA codon



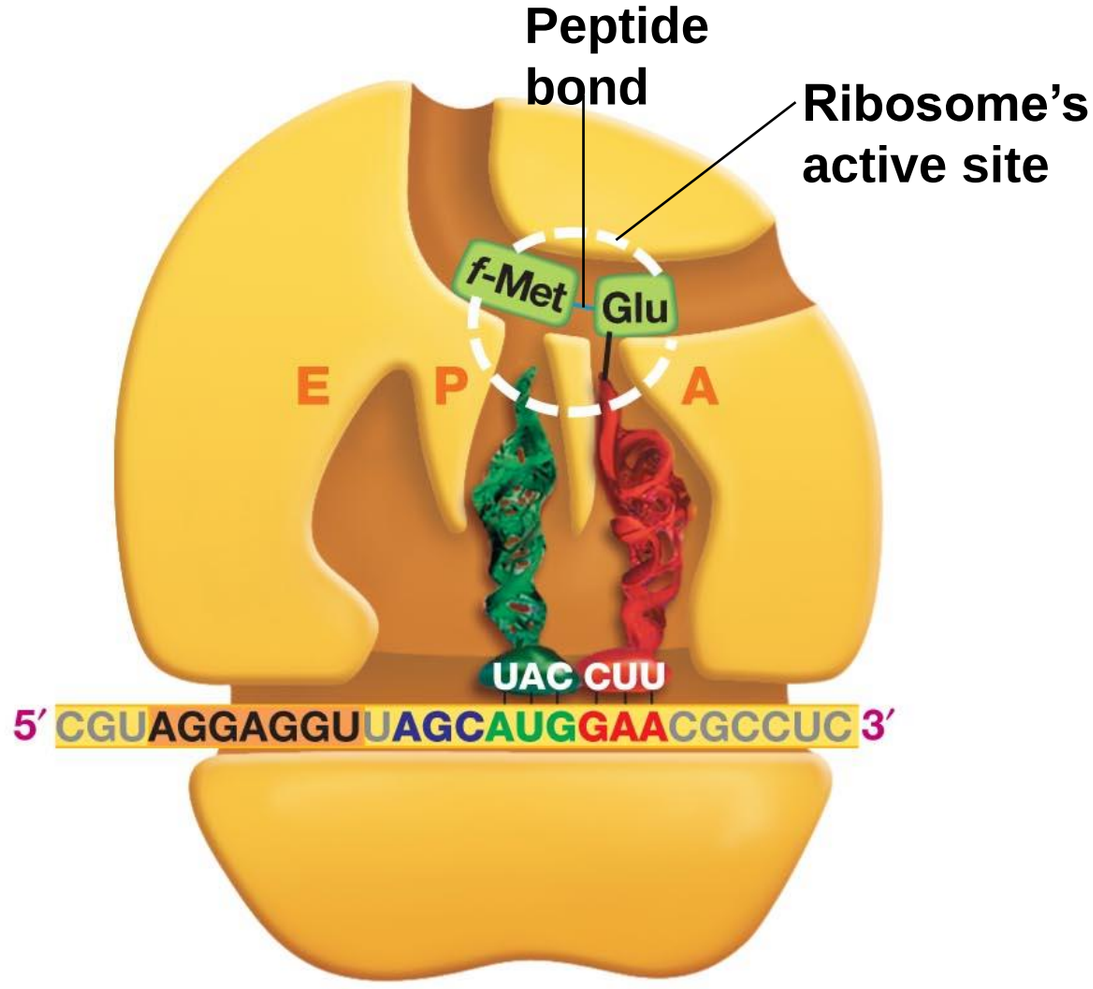


Initiation 3. Large subunit binds completing the complex

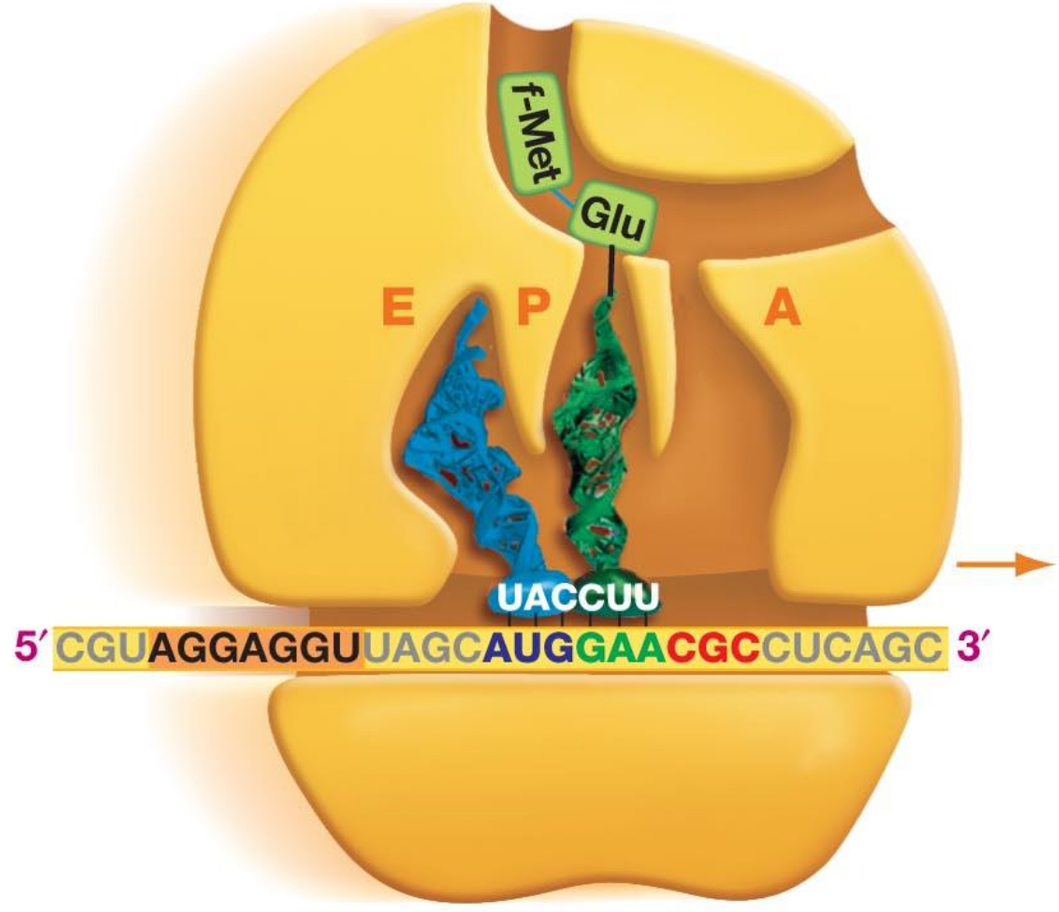
Step 2 - Elongation



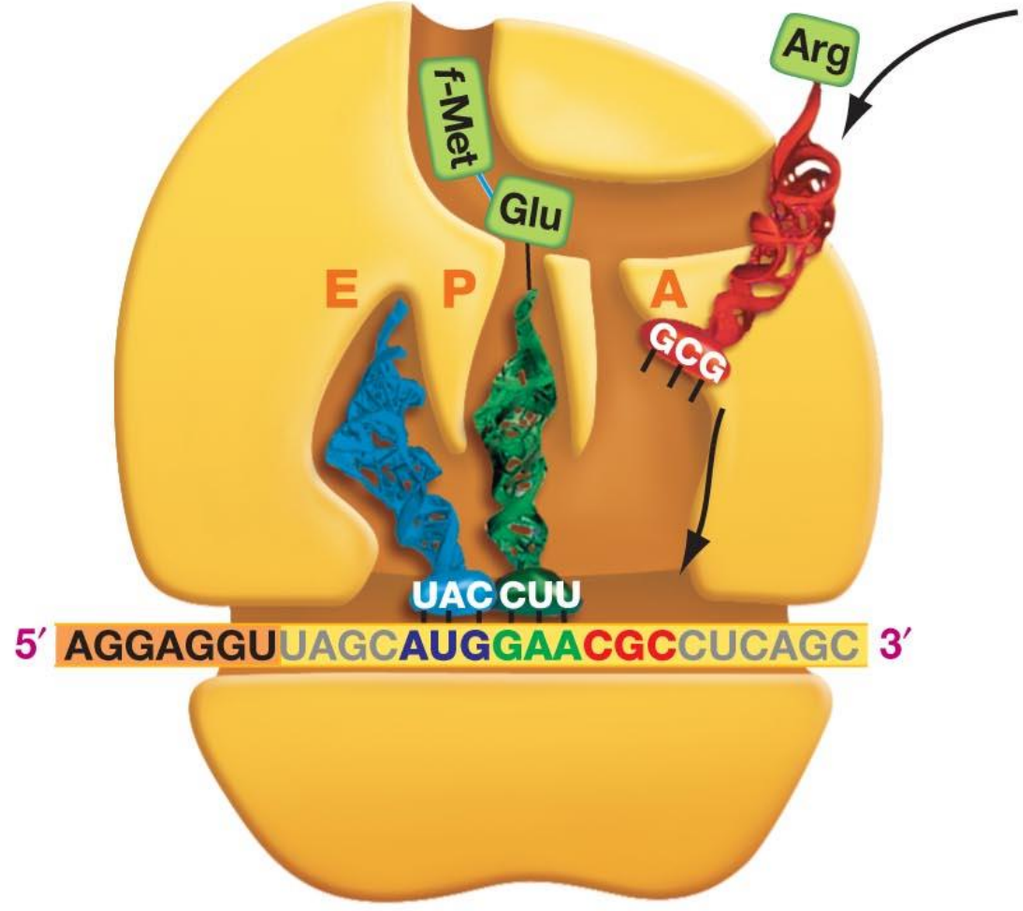
4. Incoming aminoacyl tRNA arrives and binds in A site



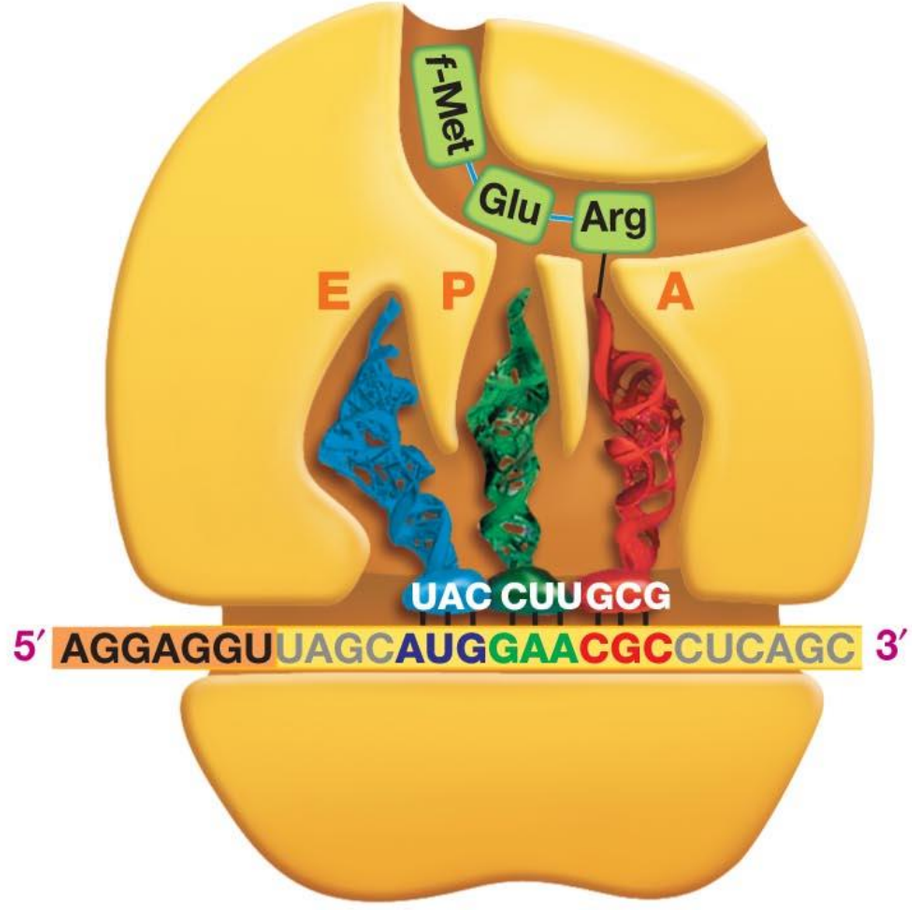
5. Peptide bond formation



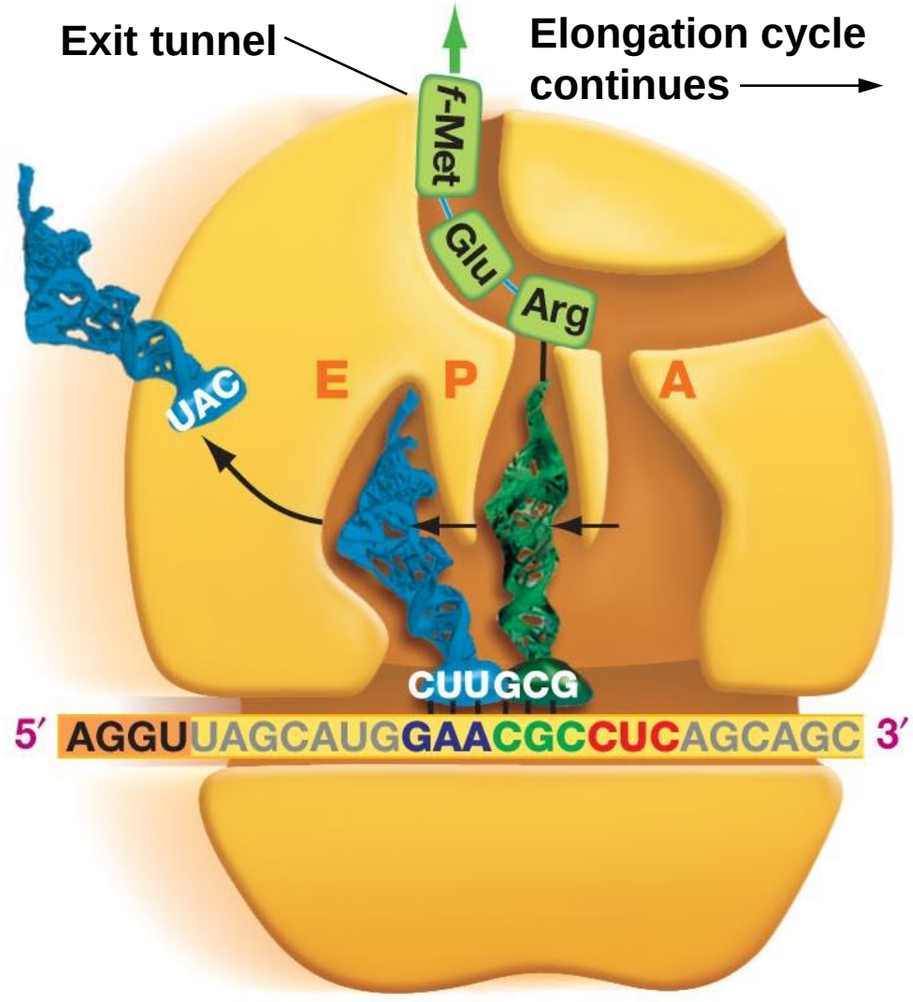
6. Translocation



7. Incoming aminoacyl tRNA arrives and binds in A site

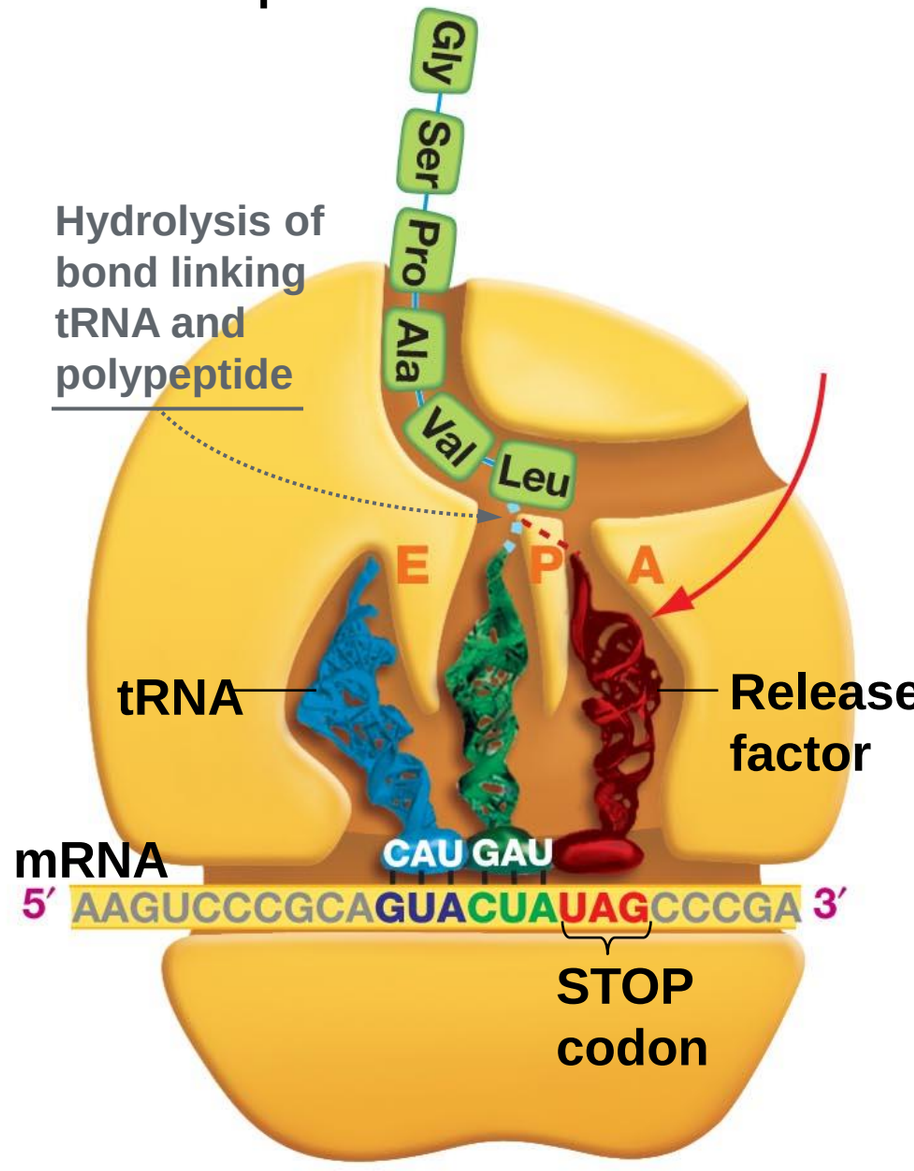


8. Peptide bond formation



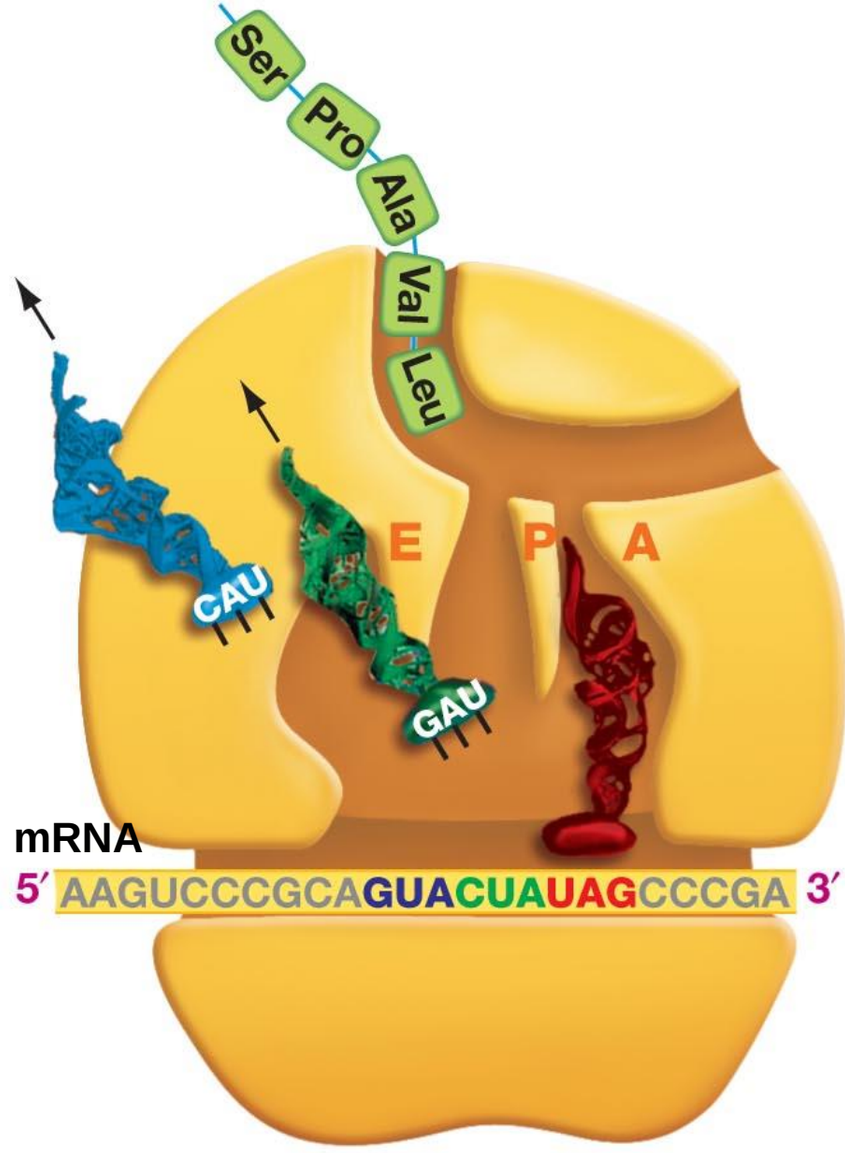
9. Translocation

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.



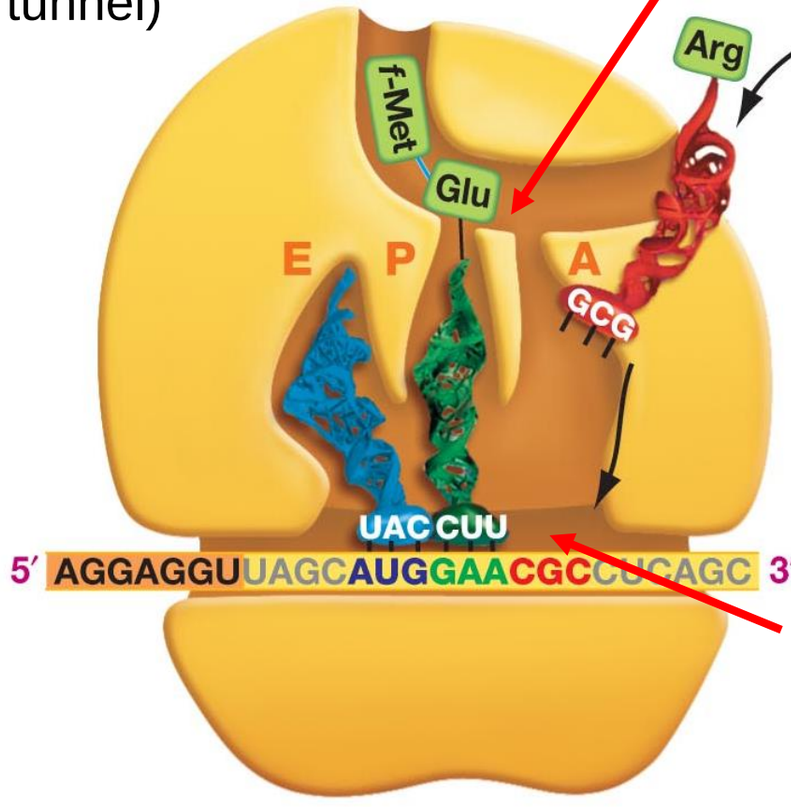
11. Polypeptide is released

Antibiotics that Inhibit Protein Synthesis

6. Erythromycin (macrolide, blocks exit tunnel)

4. Chloramphenicol (peptide bond formation)

2. Tetracycline (binding of charged tRNA)



5. Viomycin (blocks translocation)

1. Linezolid (blocks 70s formation from two subunits)

3. Kanamycin (Aminoglycoside) (codon/anticodon pairing – wrong amino acid gets added)

Genome Editing – CRISPR Cas9

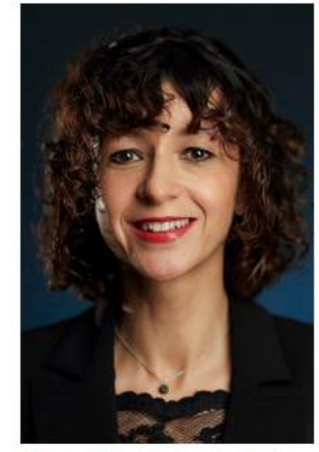
A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

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



Emmanuelle Charpentier

Prize share: 1/2

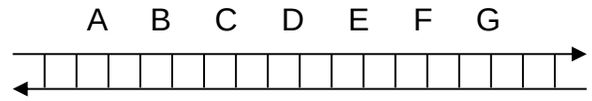
Jennifer A. Doudna

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

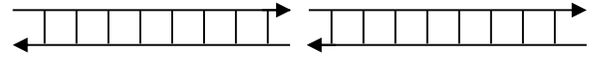
Repair of a ***targeted*** double strand break = modification of the genome at a ***defined location***.



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

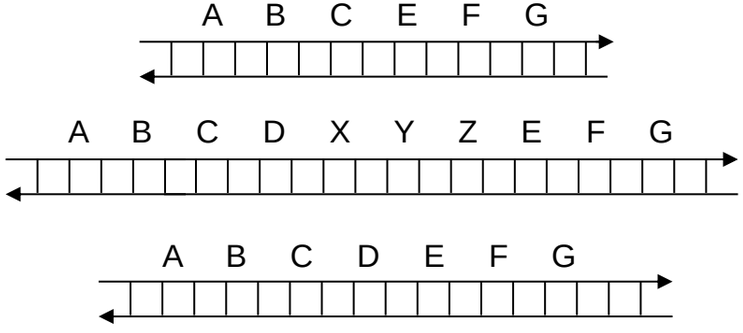
Genomic DNA with double stranded break

Nuclease (sequence specific)



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

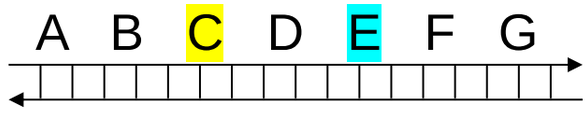
```
--ATG......GGGTGCCGATT...CGATAA—
--Met......GlyCysArgLeu.ArgIle...
```

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



Genomic DNA

Nuclease

Genomic DNA with double stranded break

Template C Z E

Homology-Directed Repair (HDR)

C Z E

Template

G-DNA w/DS break

A B C Z E F G

Genomic DNA with staggered nicks (rapidly ligated)

Cut site

Original Sequence

```
-AATGCGATGCAAAGGCTGATGGGTACCTAGATGCC-
-TTACGCTACGTTTCCGACTACCCATGGATCTACGG-
```

C D E

Template sequence

```
GCGATGCAAATTTTTTTCCTAGAT
CGCTACGTTTAAAAAAAGGATCTA
```

C Z E

Final Sequence

```
-AATGCGATGCAAATTTTTTTCCTAGATGCC-
-TTACGCTACGTTTAAAAAAAGGATCTACGG-
```

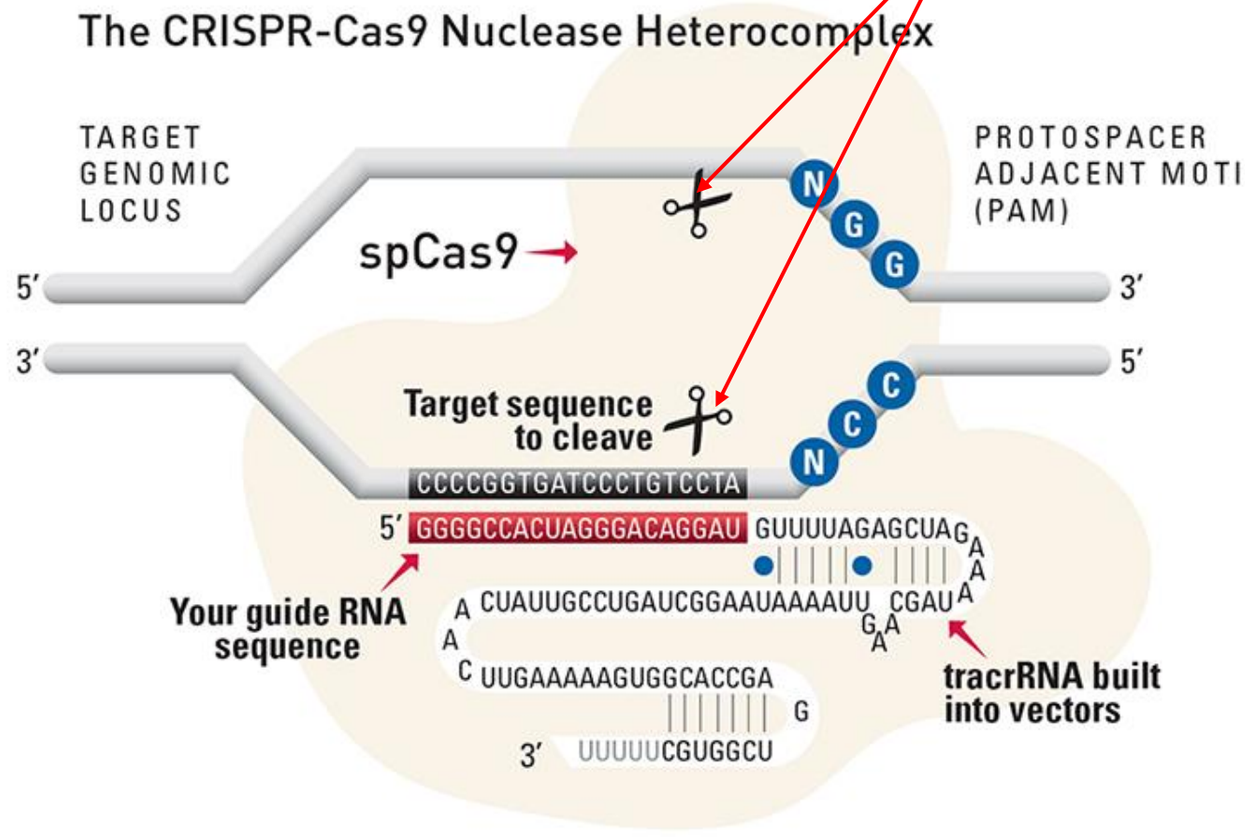
How to Cut at a Defined Location - Cas9 + Guide RNA

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)

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



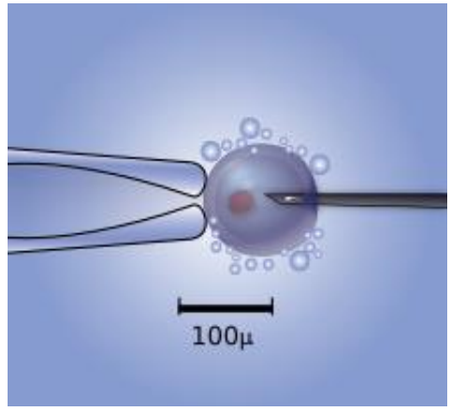
Step 2. After PAM recognition by Cas9, guide RNA unwinds DNA, by pairing with one DNA strand.

Step 3. Cas9 cleaves both strands near site, generating a double strand break.

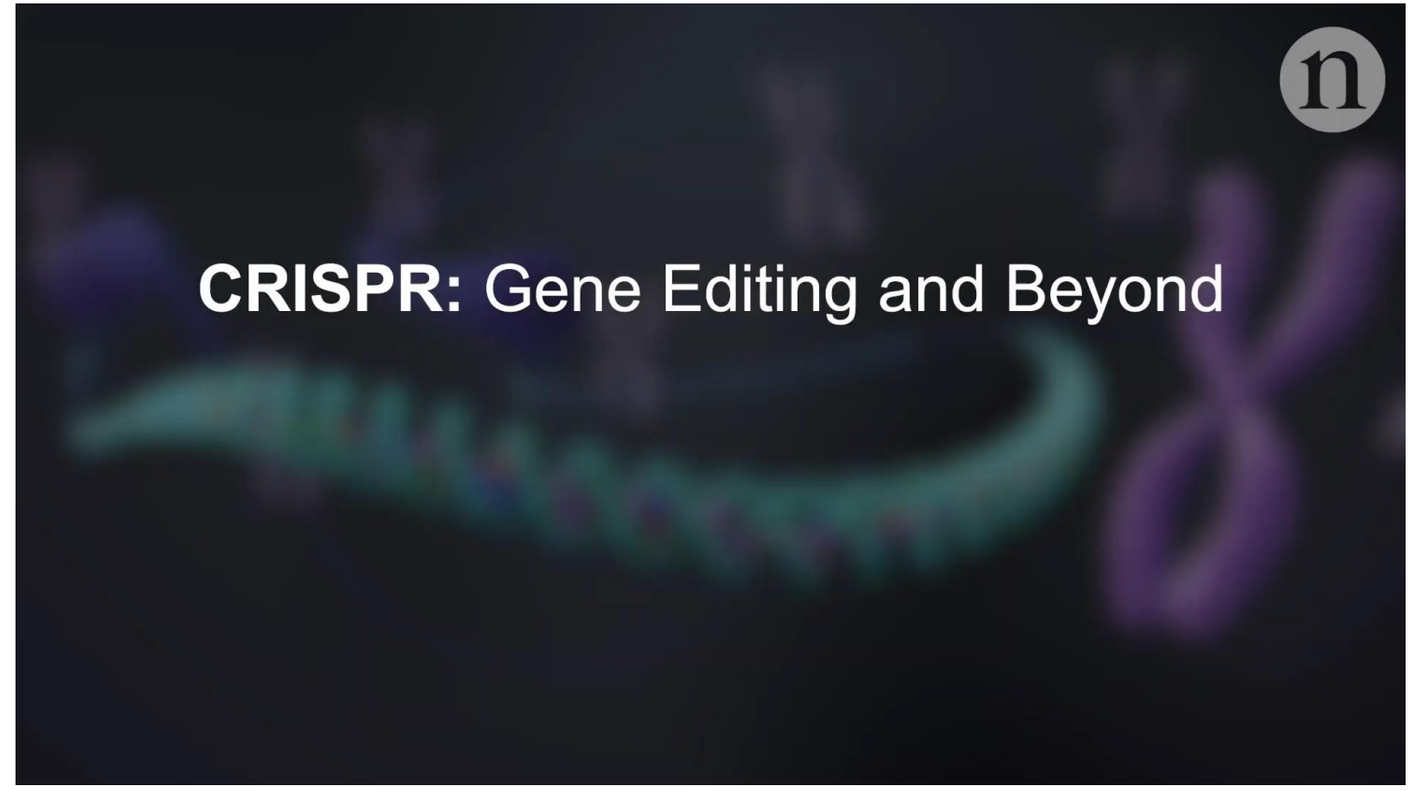
Step 4. Double stranded break triggers DNA repair, using injected replacement DNA for homologous repair

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

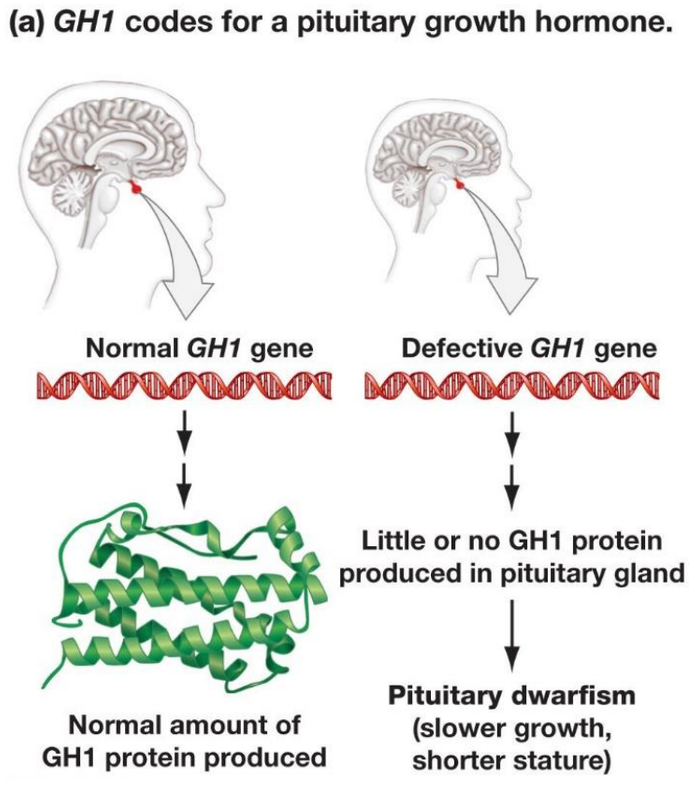


(Video originally from Nature)

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

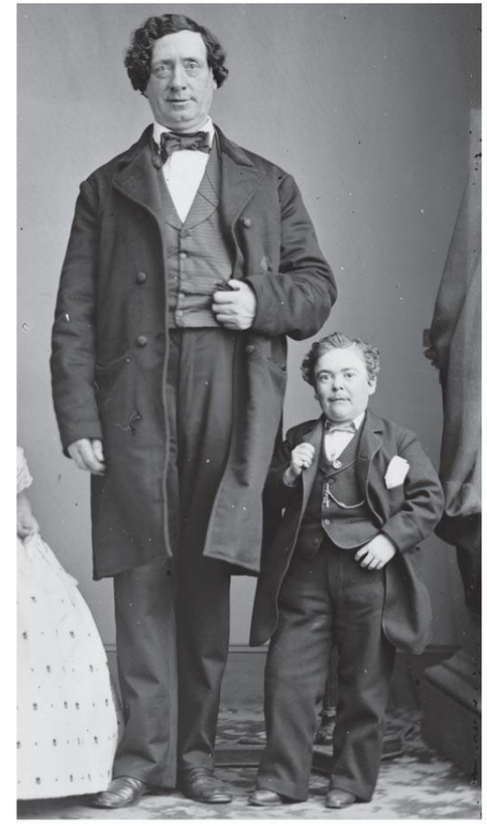
Using CRISPR-Cas9 to Correct Genetic Diseases
Human growth hormone (hGH)

Pituitary Dwarfism



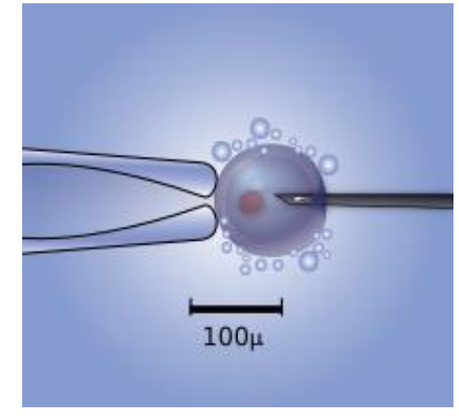
Between one in 14,000 and one in 27,000 babies born each year have some form of dwarfism.

(b) Normal versus GH1-deficient



1860 William Harrison and Charles Stratton - comedians and performers.

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.
- 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.

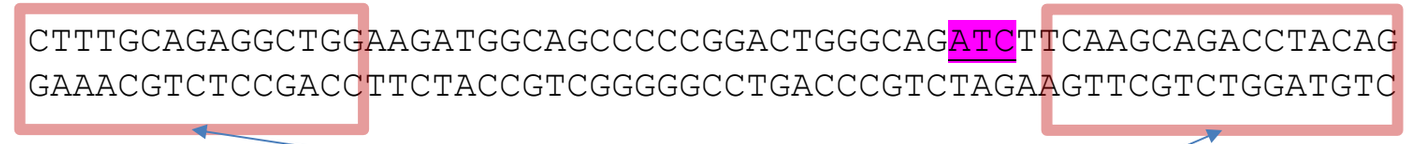
CRISPR Repair of Grown Hormone Gene

- 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.

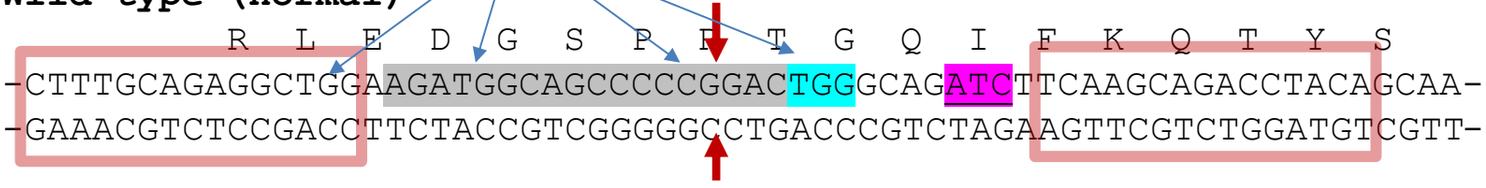
Injected dsDNA for Homologous Repair



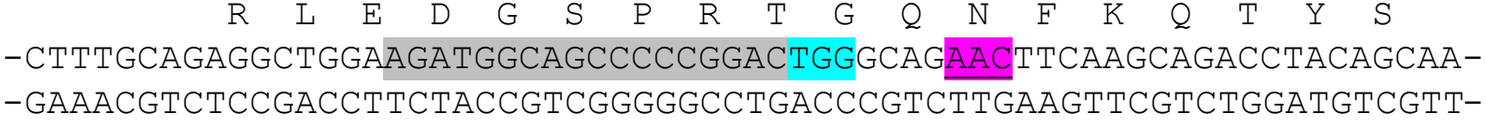
Possible PAM Sites

DNA cuts by cas9

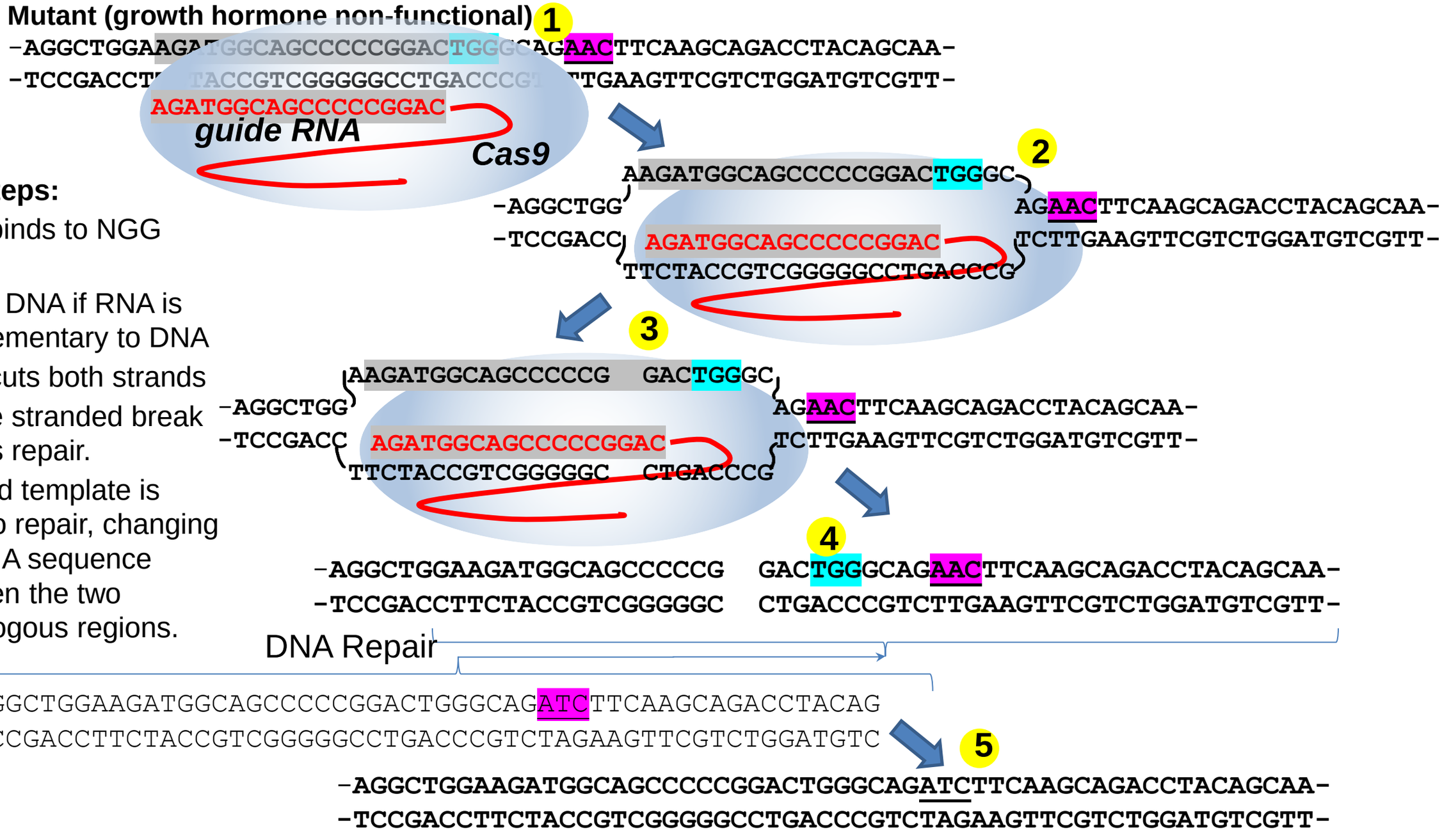
Wild type (normal)



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.