Lecture 5:

Enzyme Inhibitors as Drugs

- Suicide inhibitors
- Competitive inhibitors
- Allosteric inhibitors
- HIV drug therapy
- Antibiotics – inhibitors of central dogma

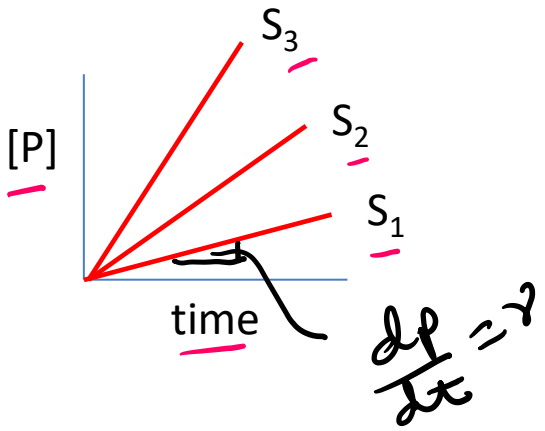
Genome Editing – Cas9

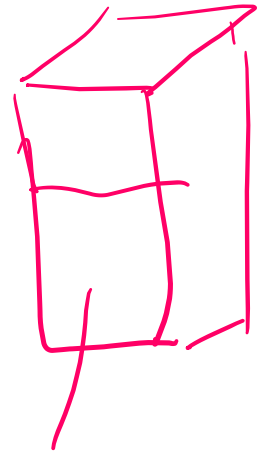
- Discovery of CRISPR systems
- Engineering of Cas9
- Off-target effects

Key Points:

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

$$v = \frac{E_{TOT}\, k_{CAT}}{V_{max}} \cdot \frac{[S]}{[S] + K_M}$$





Kinetics

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

V_{max} = measured velocity at saturating substrate:

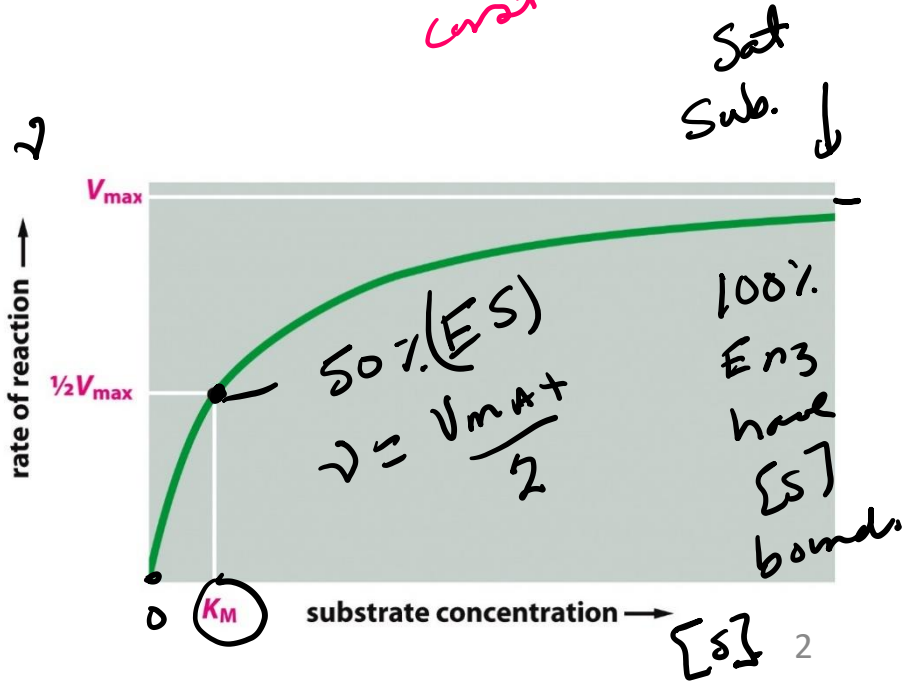
$V_{max} = k_{CAT} \times E_{total}$

K_M:

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

lower K_M = higher affinity

$$K_M = \frac{k_{off} + k_{CAT}}{k_{ON}} \approx K_D$$



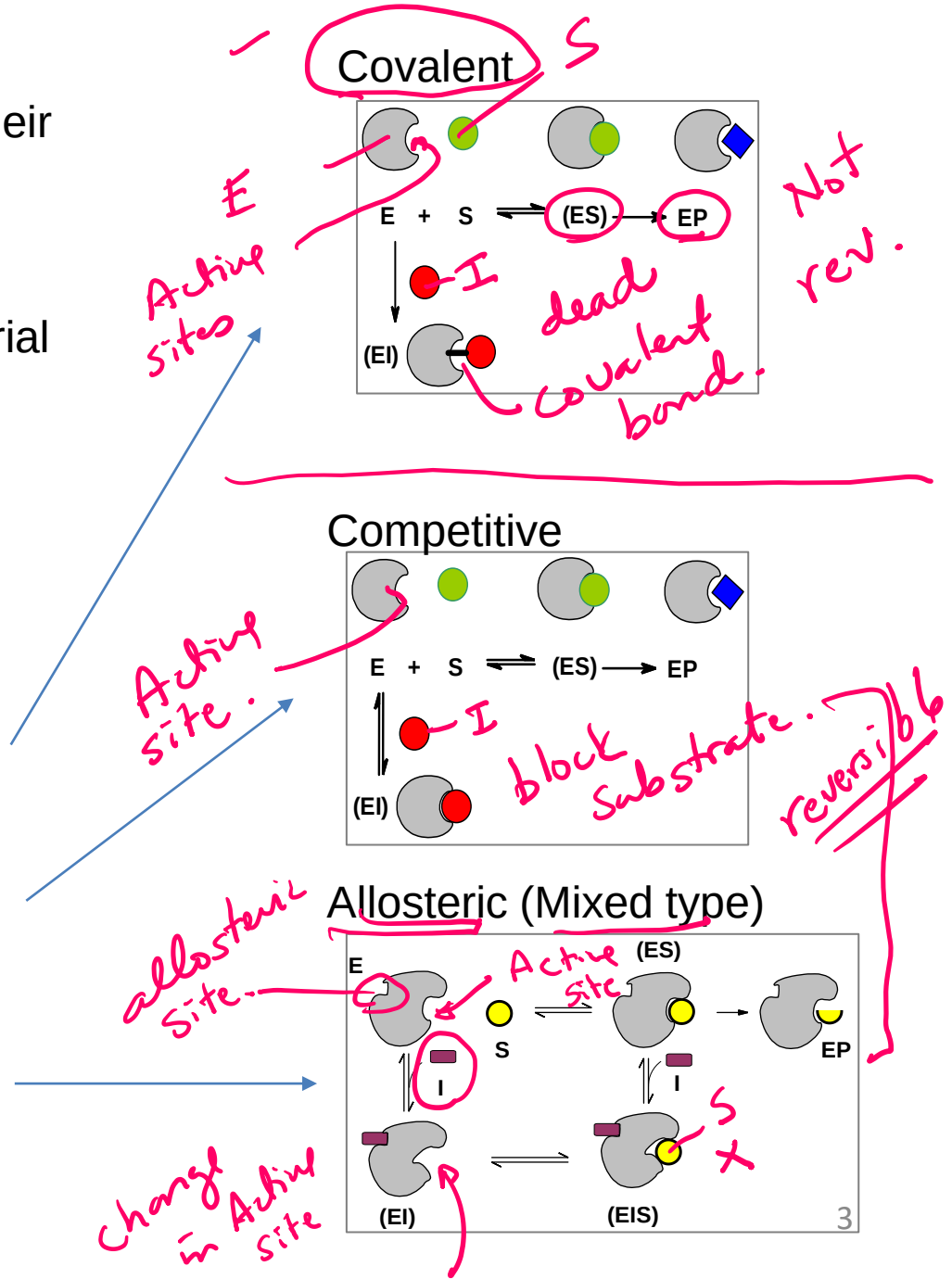
Enzyme Inhibitors

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.

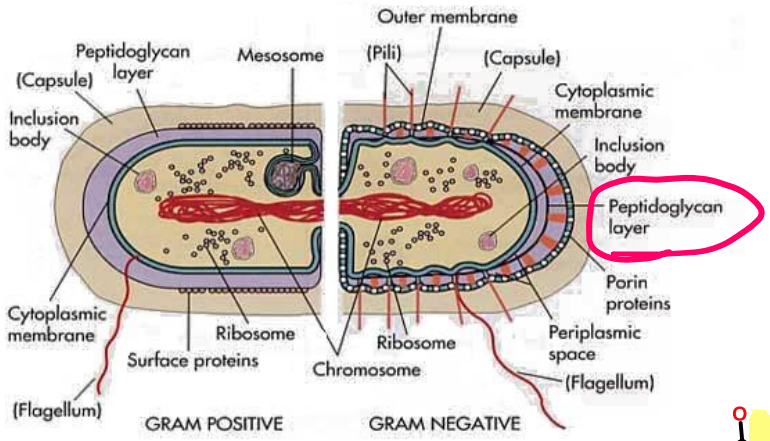
Types of Inhibitors:

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 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.*



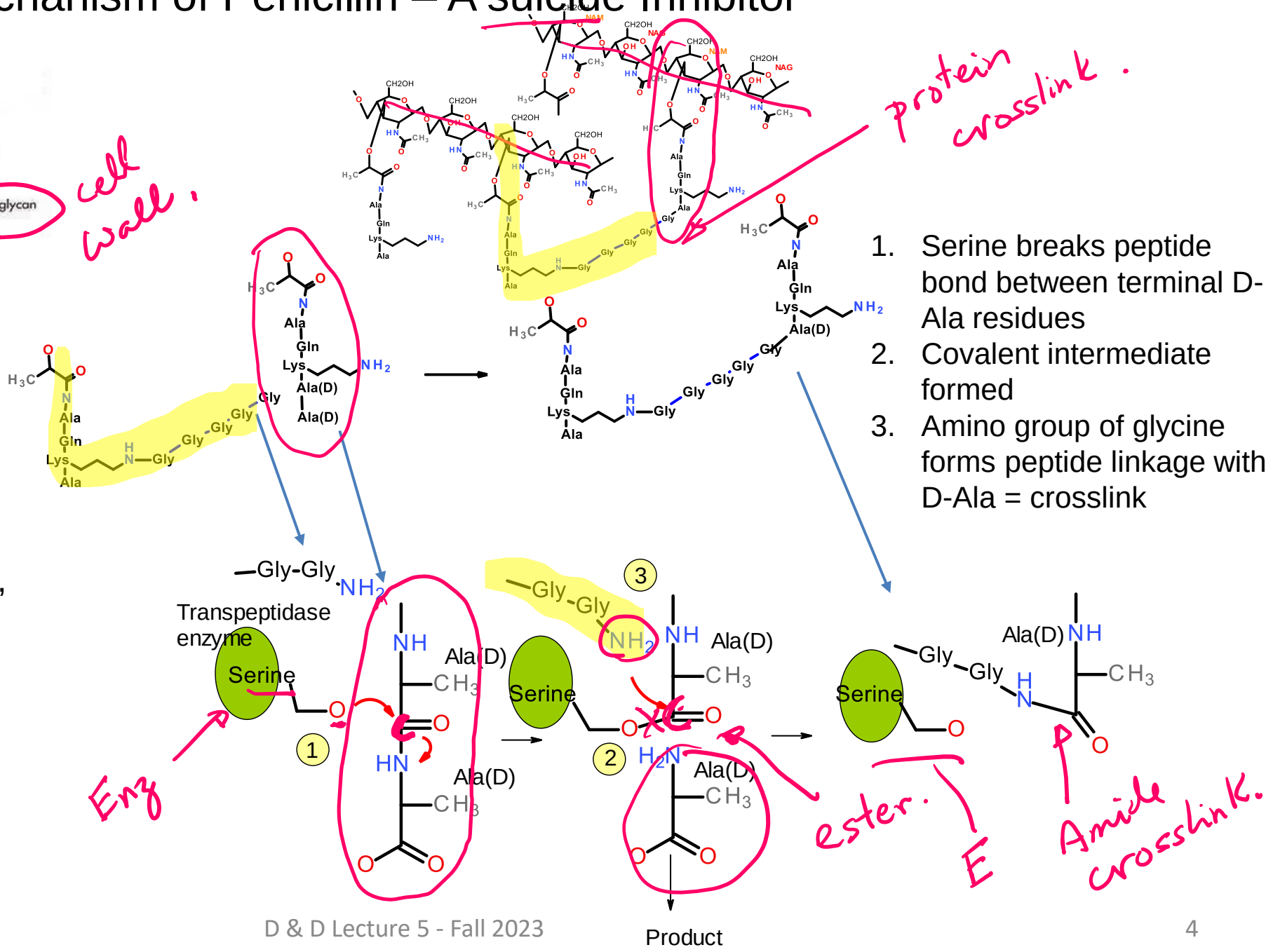
Mechanism of Penicillin – A suicide Inhibitor

Bacterial Cell Wall



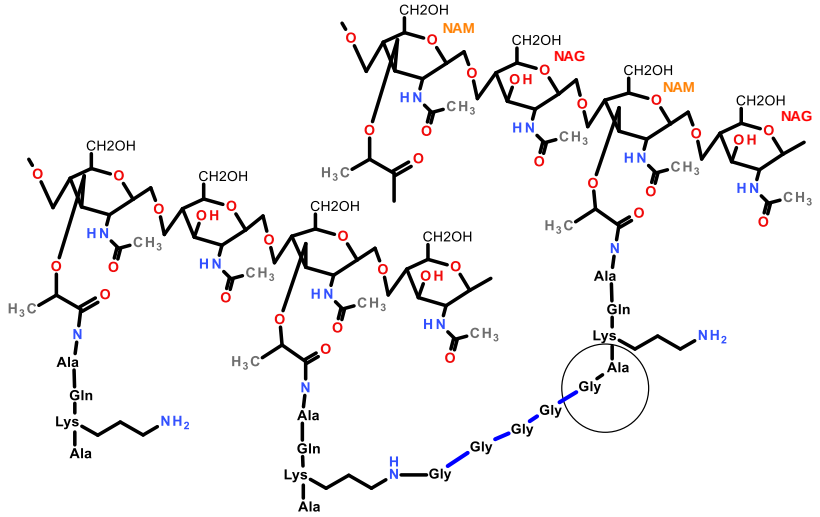
Bacterial cell wall:

- 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.***



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

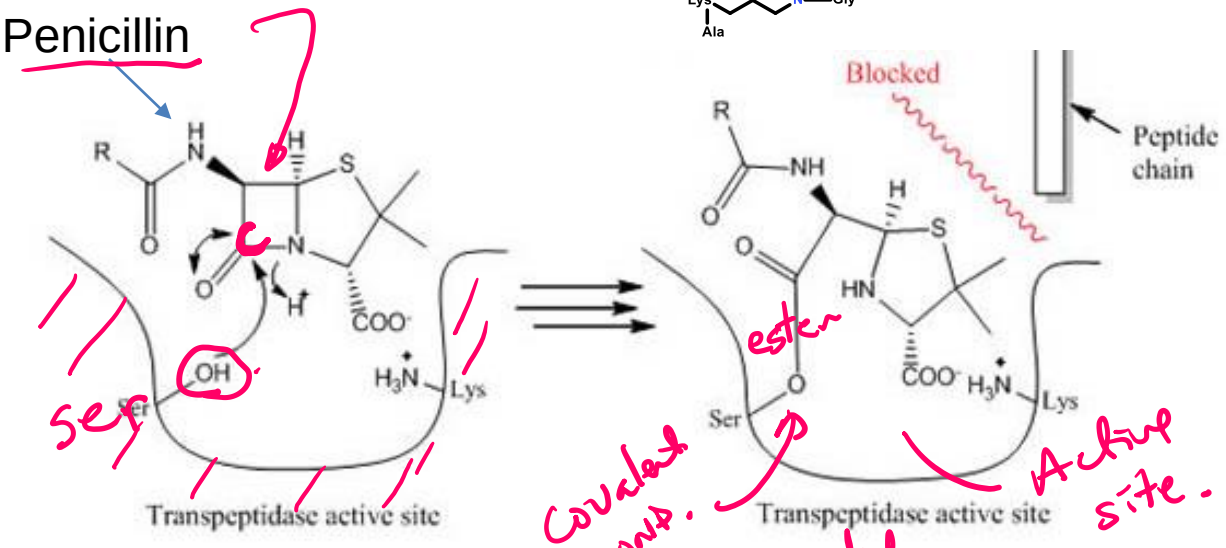
Mechanism of Penicillin

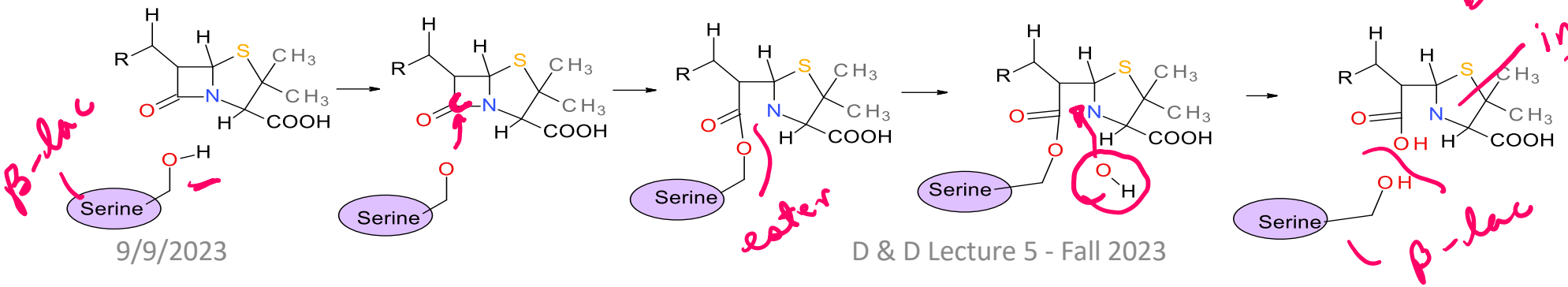


Mechanism of Action of Penicillin:

- 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 Resistance: Bacteria produce a protein that degrades penicillin (β-lactamase). This is a common antibiotic resistance gene that is used on plasmids. The transformed bacterial are resistant to penicillin.





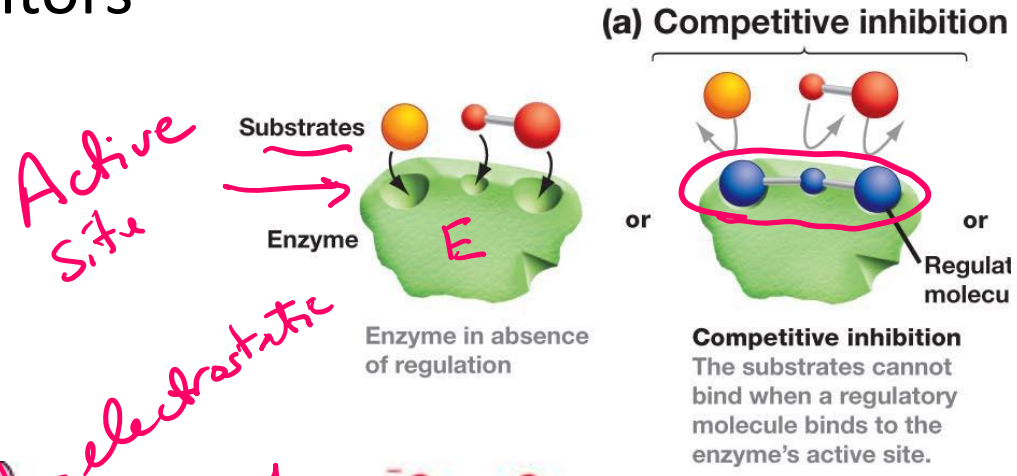
Lactamase can regenerate the serine, regenerating the enzyme.

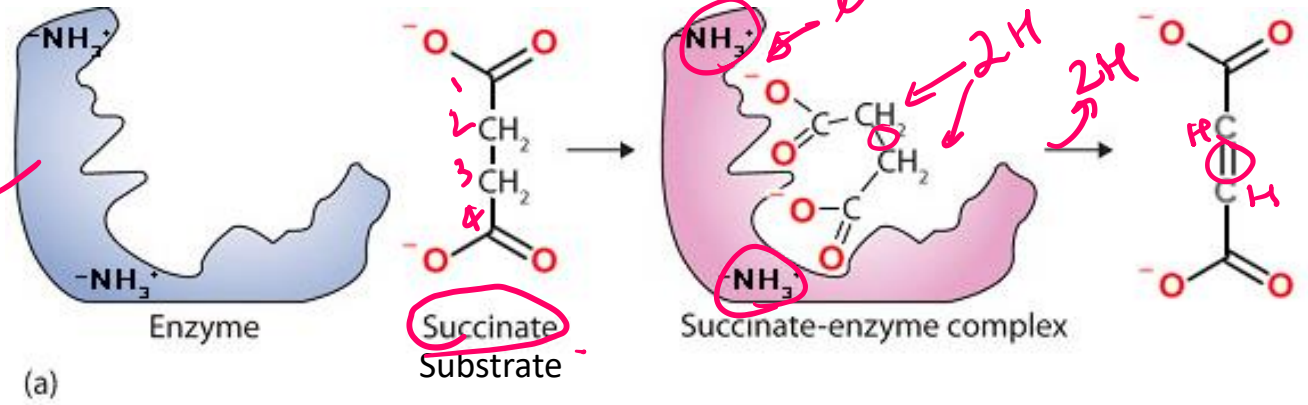
Competitive Inhibitors

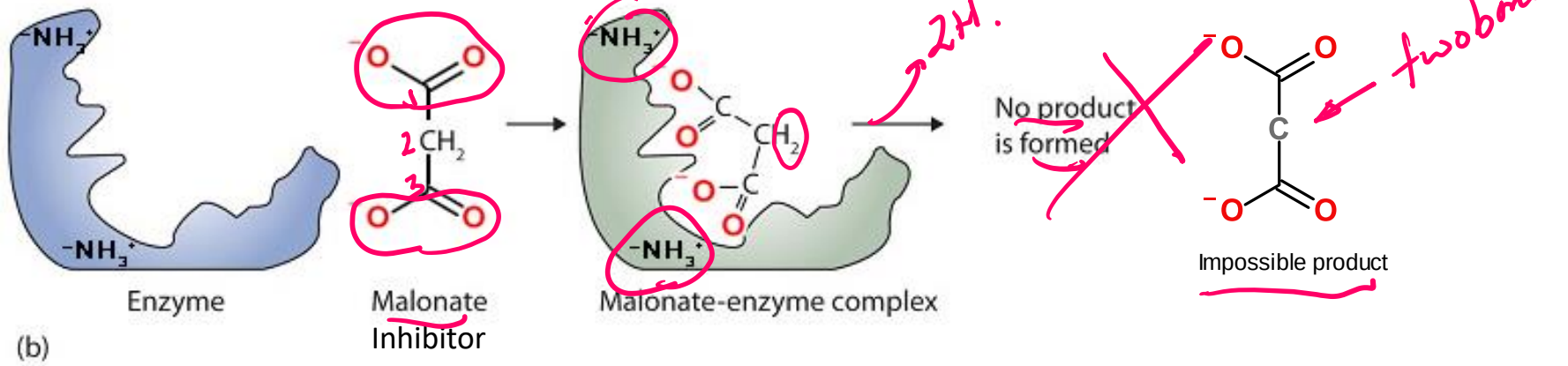
Succinate dehydrogenase converts succinate to fumarate by removal of two hydrogens.

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

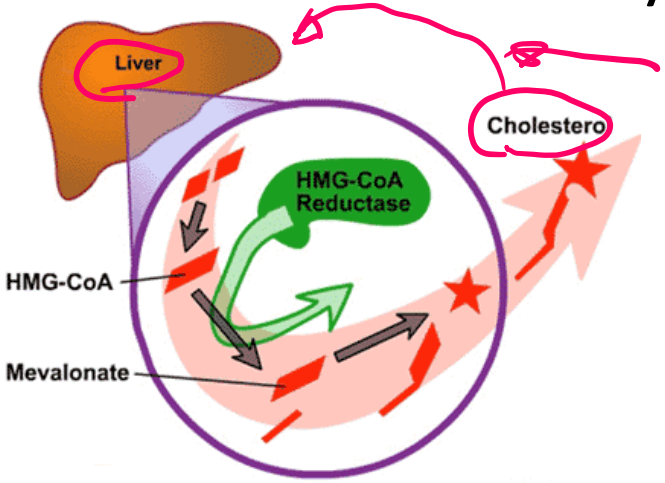
- It is similar in structure to the substrate – so it binds in active site – substrate cannot bind at the same time.
- Malonate **cannot** undergo the chemical reaction – it is not possible to remove two hydrogens without leaving carbon with too few bonds.

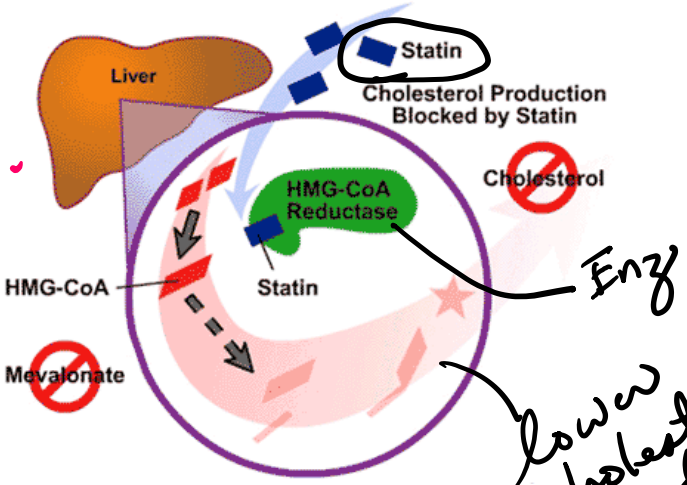


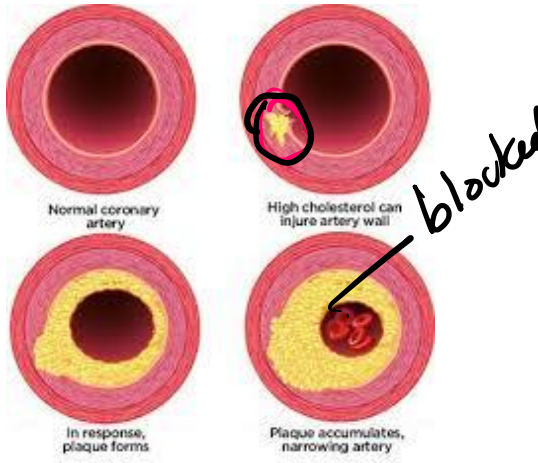


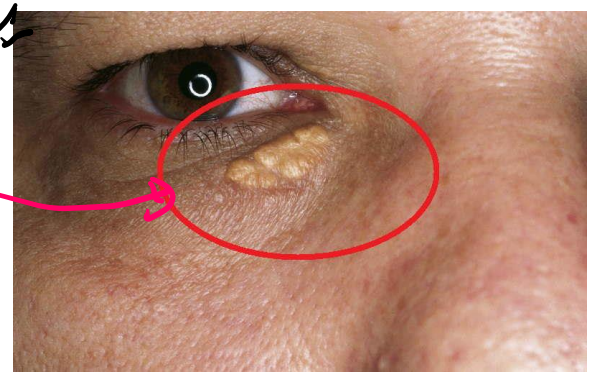


Many drugs are Competitive Inhibitors

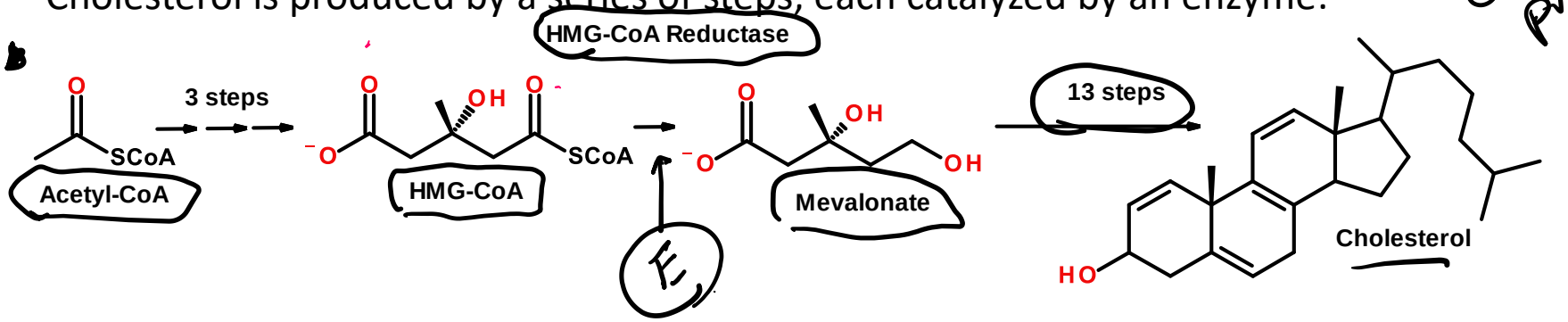


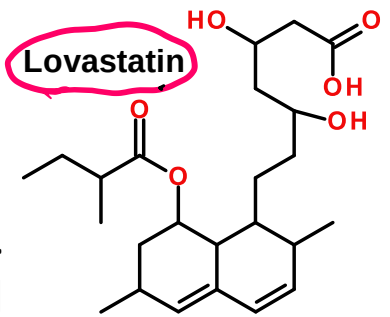






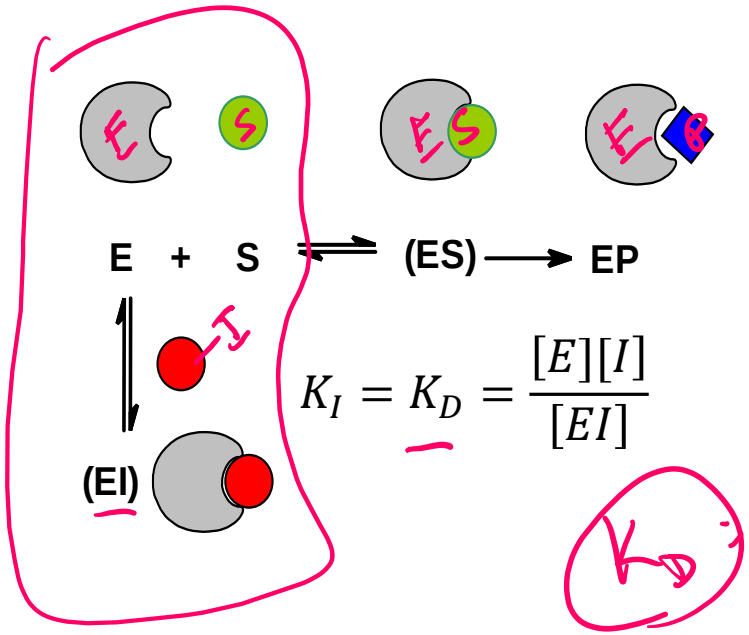
Cholesterol is produced by a series of steps, each catalyzed by an enzyme:





- In normal individuals, cholesterol production in the liver is tightly controlled by cholesterol levels in the blood by a feedback mechanism (the liver actively takes up cholesterol from the blood).
- A genetic disease causes this regulation to be dysfunctional in some individuals, leading to high cholesterol levels, leading to damage of the arterial walls and cholesterol deposits (often near the eyes).
- **Statins** are competitive inhibitors that inhibit one of the enzymes (HMG-CoA Reductase) that is required to make cholesterol

Quantification of Inhibitor Binding



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

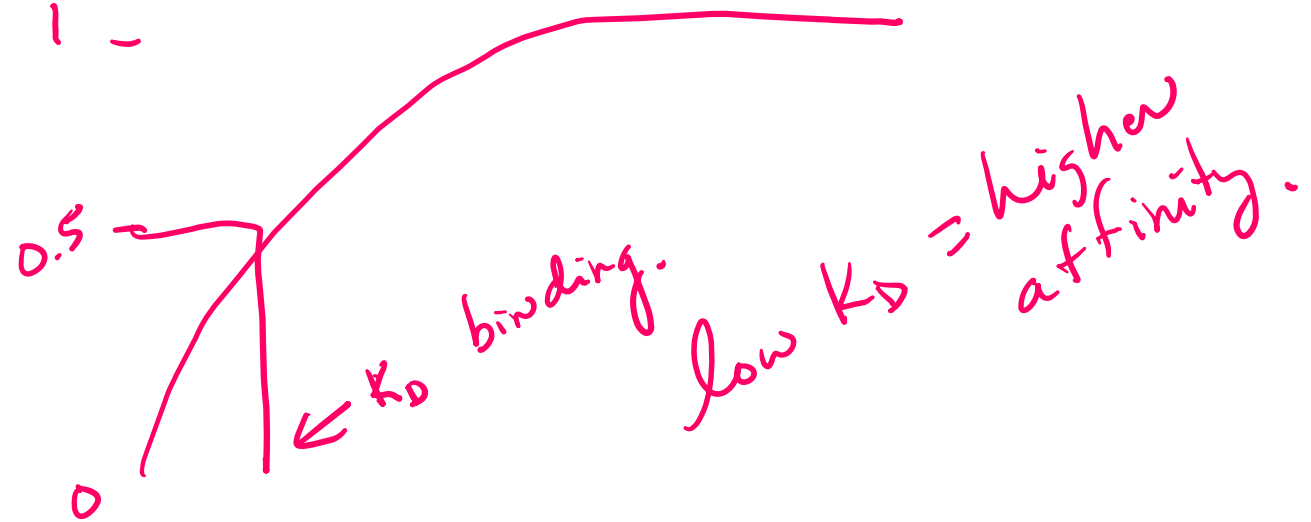
K_D: $\frac{(E)(I)}{(EI)}$

$(EI) \rightleftharpoons (E) + (I)$

$(ML) \rightleftharpoons M + L$

Y=(EI)/[(EI)+(E)]

bound / total



$K_I \simeq K_D$

Effect of Competitive Inhibitor on Steady-State Kinetics:

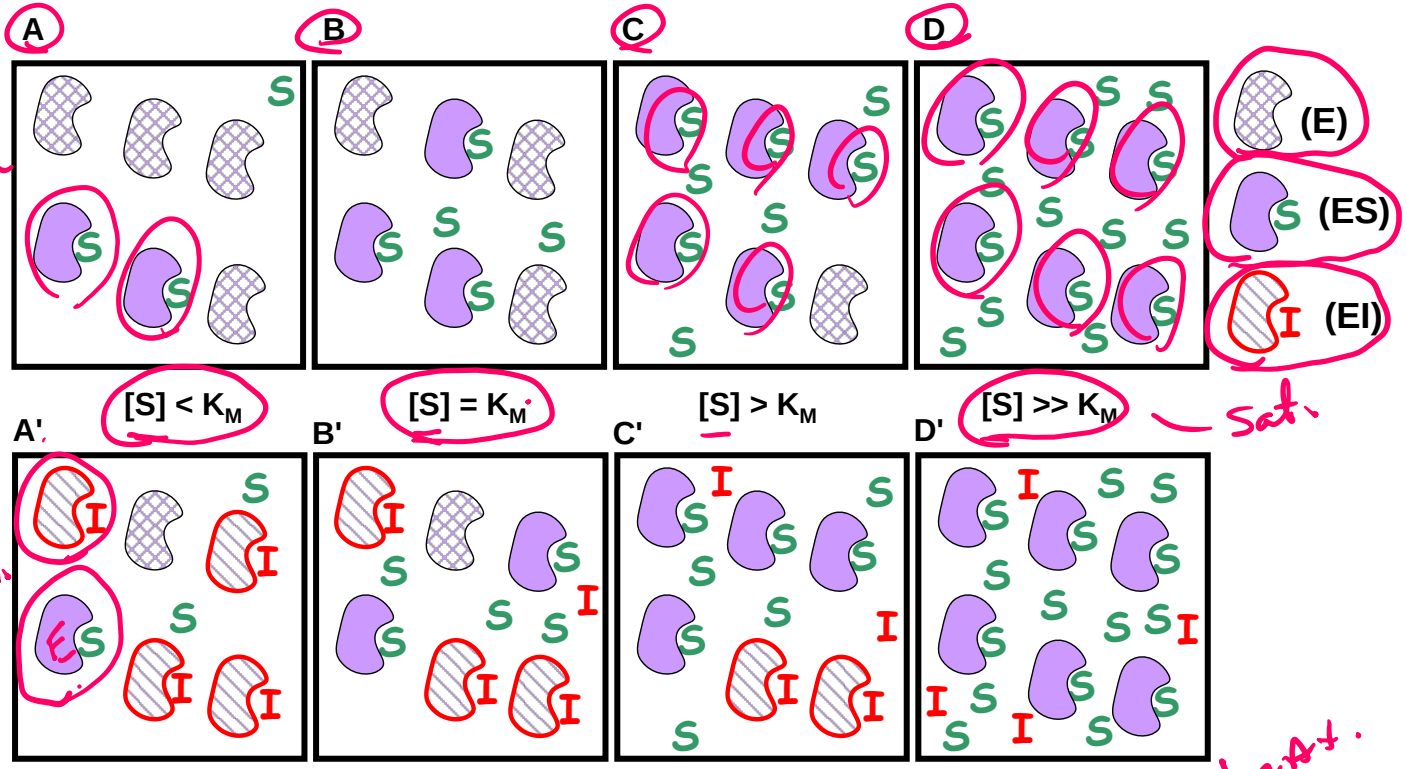
- A competitive inhibitor reduces the amount of [E] by the formation of [EI] complex.
- The inhibitor cannot affect the [ES] complex since the inhibitor can no longer bind.

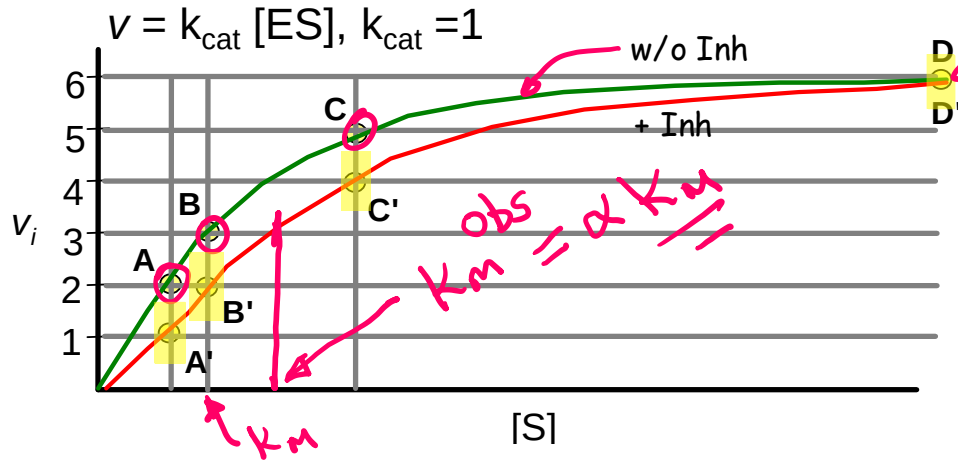
There are two consequences of a competitive inhibitor binding on the kinetics of the enzyme:

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.*





No inhibitor

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

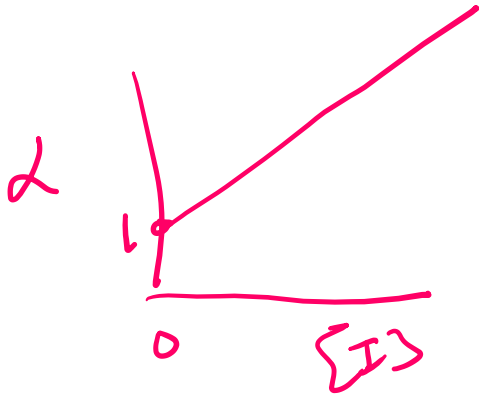
Comp inhibitor

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

Steady-State Analysis of Competitive Inhibitors

α = degree of inhibition

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



A double reciprocal plot can be used to obtain α:

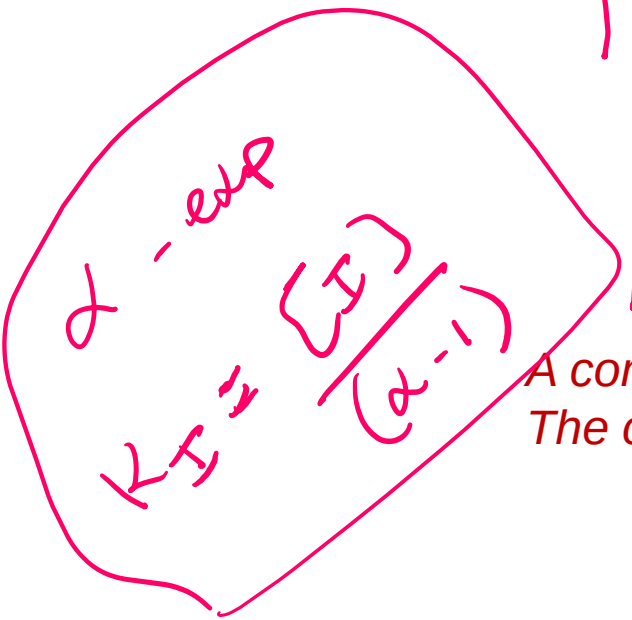
No Inhibitor Present → Double Reciprocal Equation

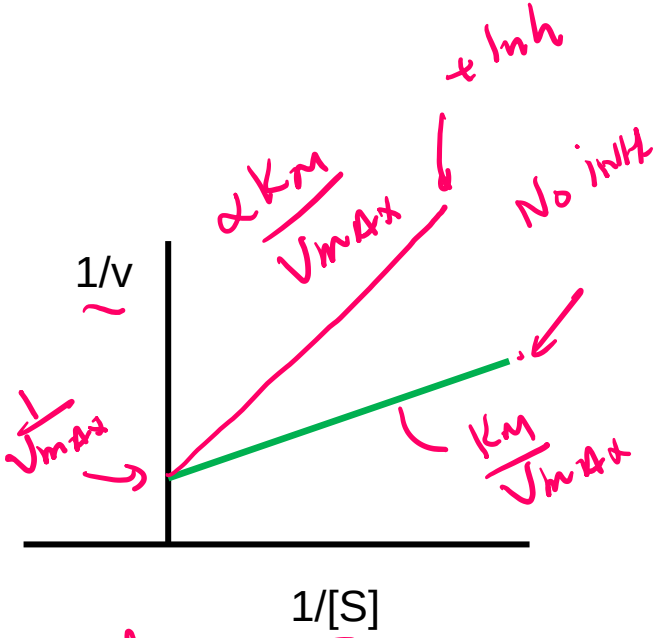
$$v = V_{MAX}\frac{[S]}{K_M + [S]} \qquad \frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$

Inhibitor Present → Double Reciprocal Equation

$$v = V_{MAX}\frac{[S]}{\alpha K_M + [S]} \qquad \frac{1}{v} = \frac{\alpha K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$

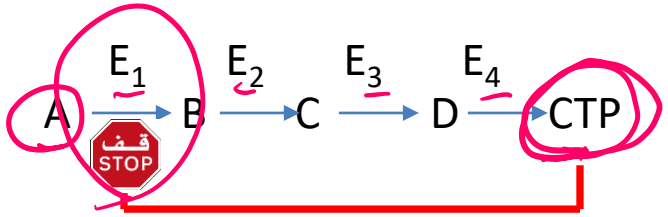
A competitive inhibitor changes the slope of a double recip. plot!
The change in slope can be used to obtain α and then K_I





α : ratio of slopes.

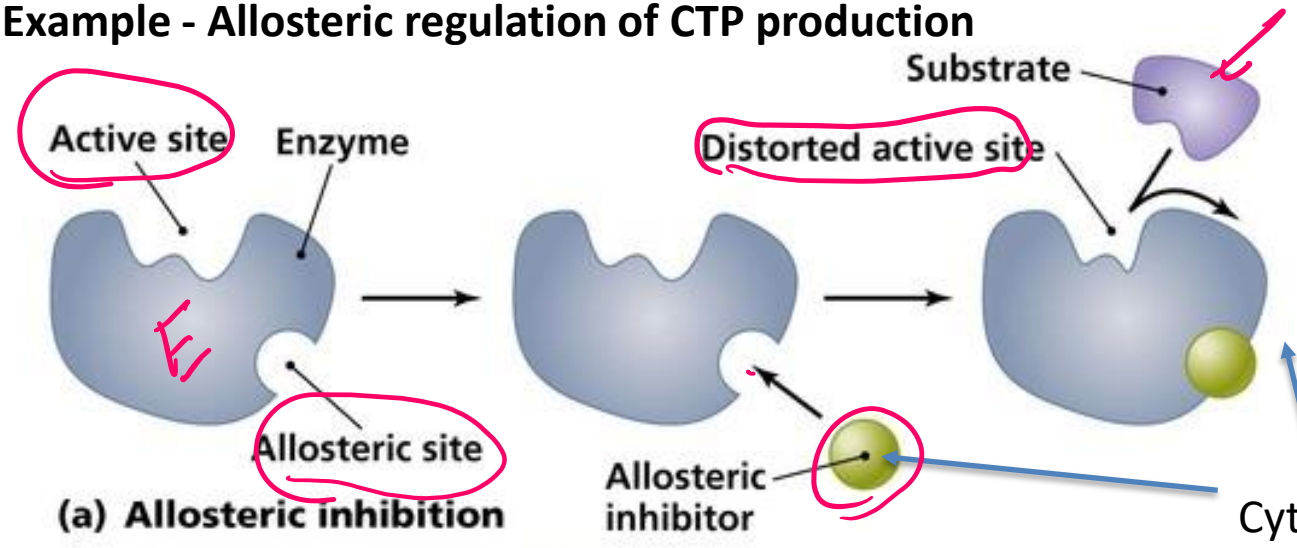
Allosteric Inhibition of Enzymes – Often Used in Control



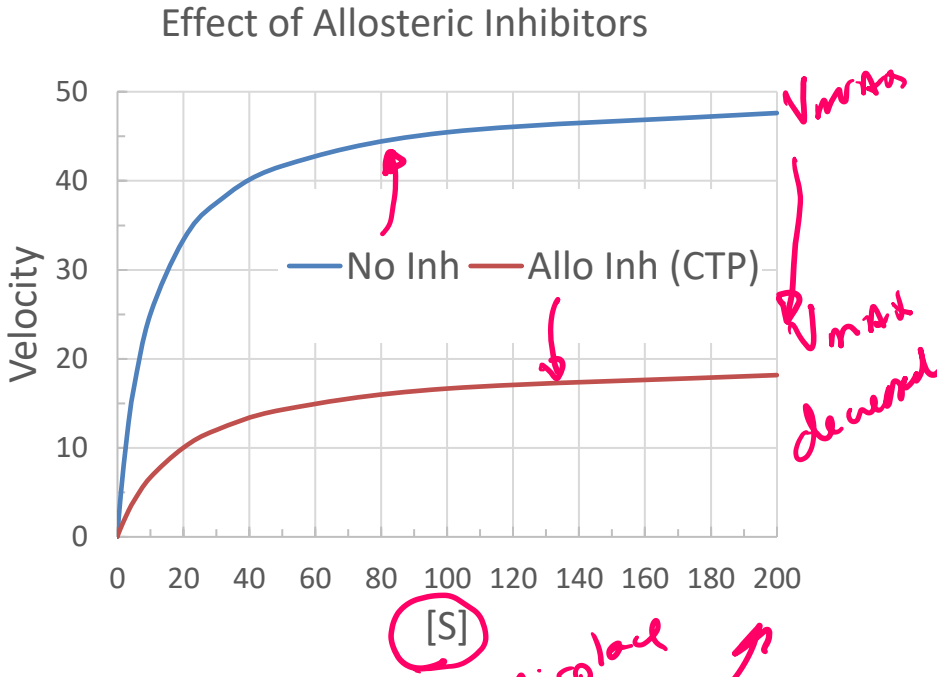
- Inhibitor does not bind in the active site.
- Inhibitor can bind to both the free enzyme (E) and the (ES) complex

Feedback Regulation:
Product of pathway prevents production by inhibition of enzyme earlier in the pathway.

Example - Allosteric regulation of CTP production

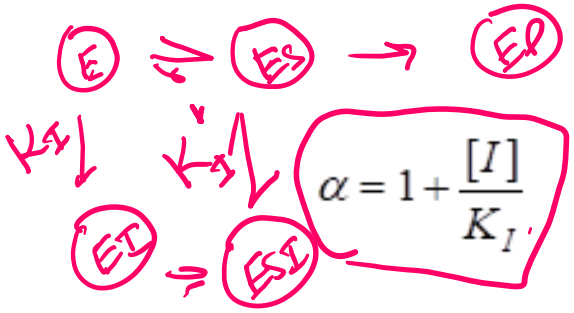


Cytosine triphosphate



- CTP (a component of RNA) is generated by a series of enzymatic steps – a metabolic pathway.
- The first enzyme in the pathway is inhibited by the final product of the pathway, CTP (cytosine triphosphate)
- The CTP binds at an allosteric site, cause a conformational change in the active site, decreasing V_{MAX}, ***shutting off its own production.***

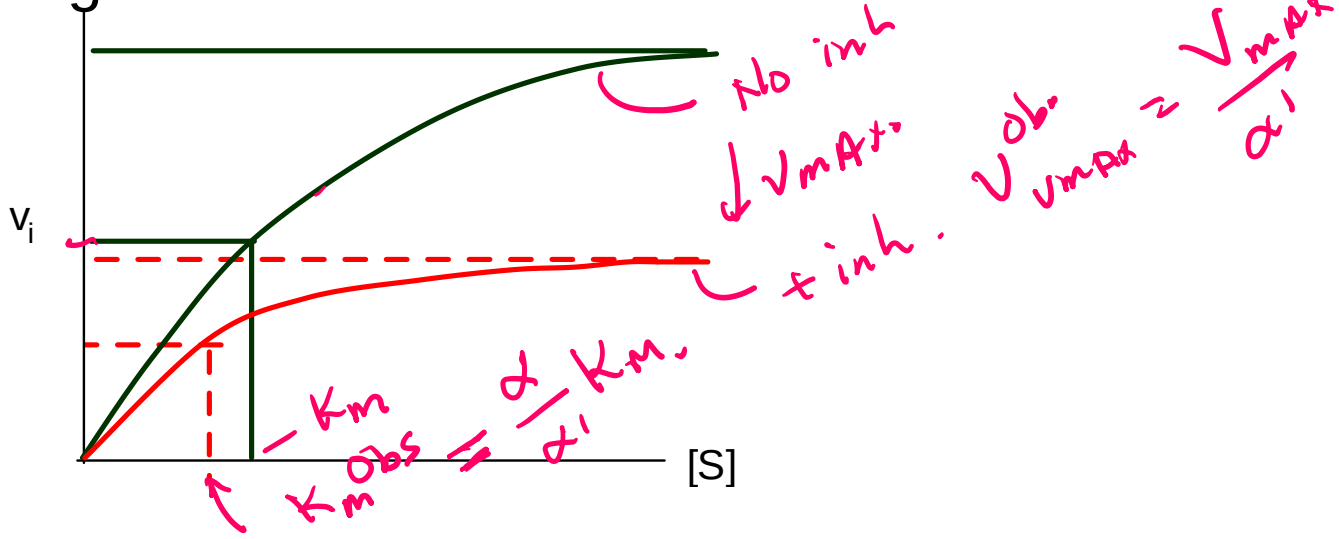
Determining Binding of Allosteric Inhibitors:



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

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

- The change in V_{MAX} can be used to find K_I': $\mathbf{V_{MAX}^{OBS} = V_{MAX}/\alpha'}$
- The change in K_m can be used to find K_I. $\mathbf{K_M^{OBS} = (\alpha/\alpha')\, K_M}$



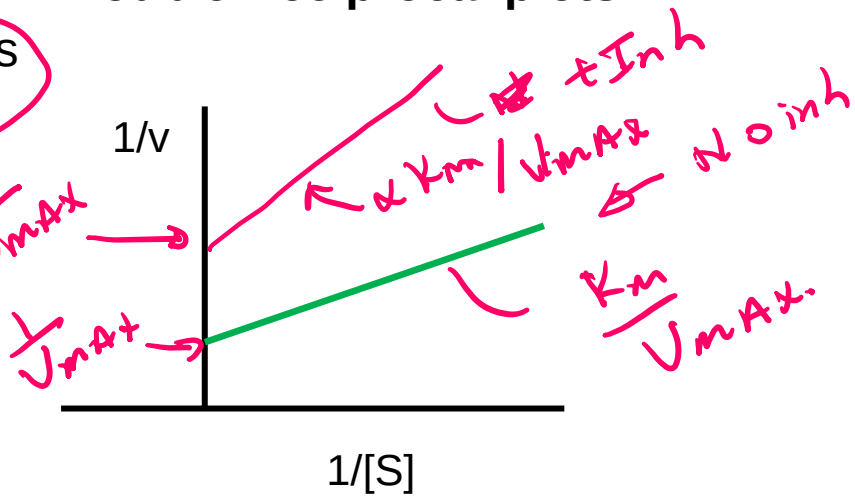
Obtaining K_is from Double Reciprocal plots:

α = ratio of slopes (+Inh/no inh)

$K_I = [I]/(\alpha - 1)$

α' = ratio of y-intercept (+Inh/no inh)

$K_I' = [I]/(\alpha' - 1)$



No Inhibitor

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

$$\frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$

Allosteric Inhibitor

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

$$\frac{1}{v} = \frac{\alpha K_M}{V_{MAX}}\frac{1}{[S]} + \frac{\alpha'}{V_{MAX}}$$

Key Points:

Inhibition/Regulation

Suicide inhibitors inactivate the enzyme.

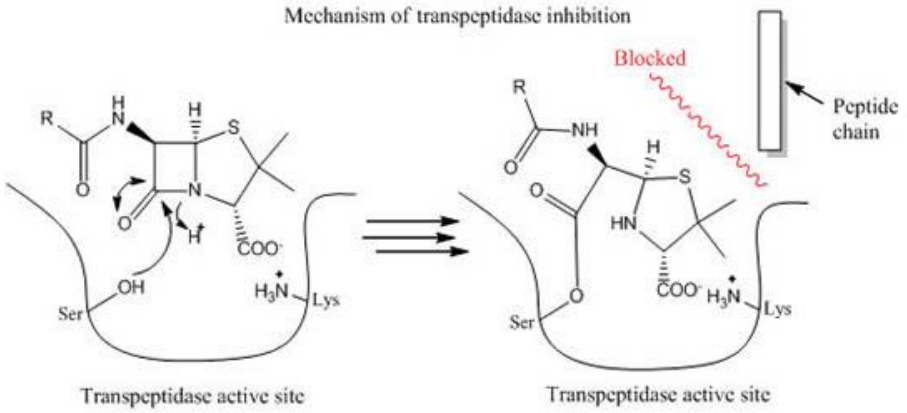
Competitive:

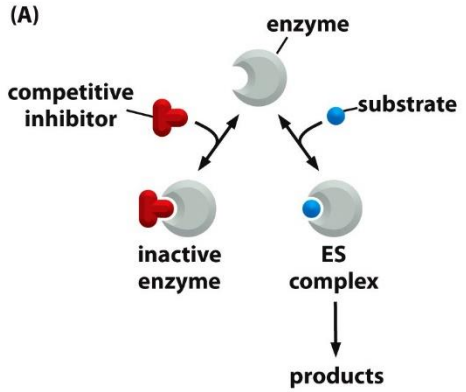
- Similar to substrate, so binds in the active site, blocking substrate binding
- Cannot be converted to product by enzyme
- Only affects K_M, not V_{max} since at high [S] the inhibitor cannot bind.

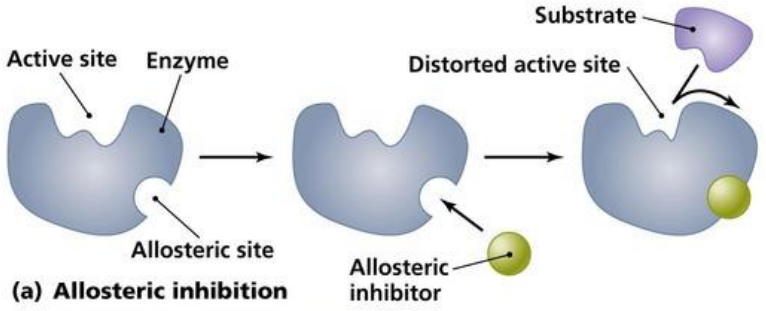
Allosteric Inhibition:

- Does not bind to active site
- Causes change in shape of active site, reducing activity
- V_{max} decreases, K_M can also change.

Competitive inhibitors and Allosteric inhibitors bind **reversibly**.







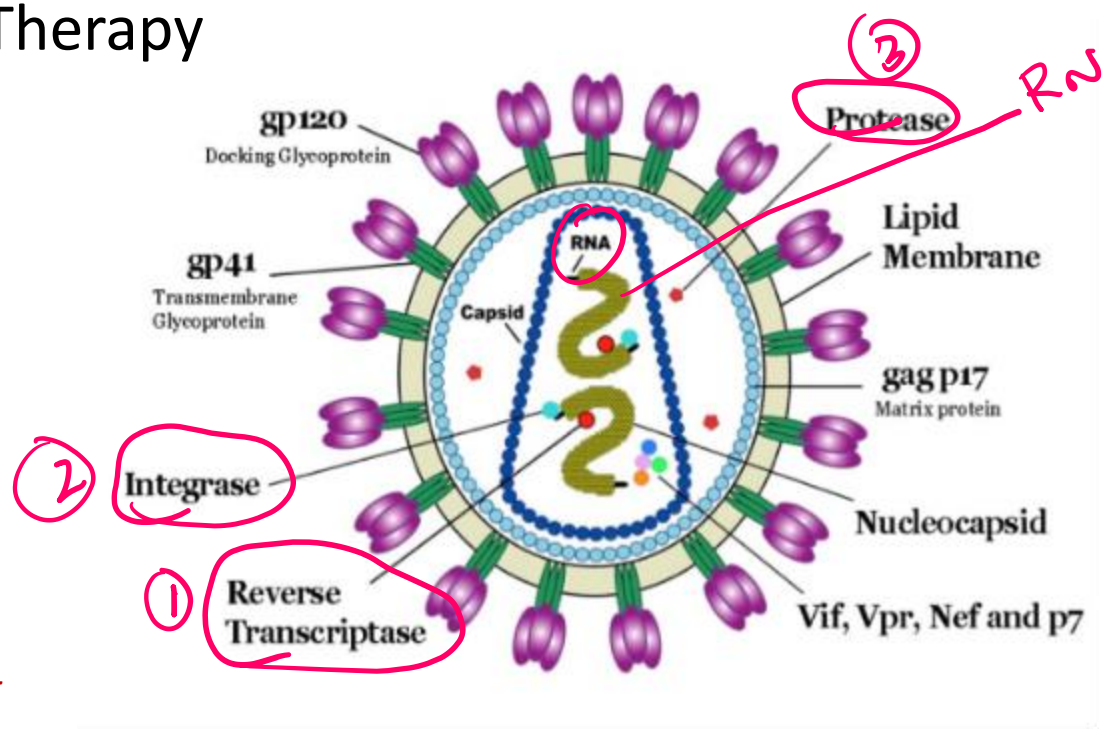
HIV Drug Therapy

Retroviruses & Inhibitors - HIV Protease.

- Identify potential drug targets, based on viral life cycle.
- Compare and contrast serine to aspartyl proteases
- 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.

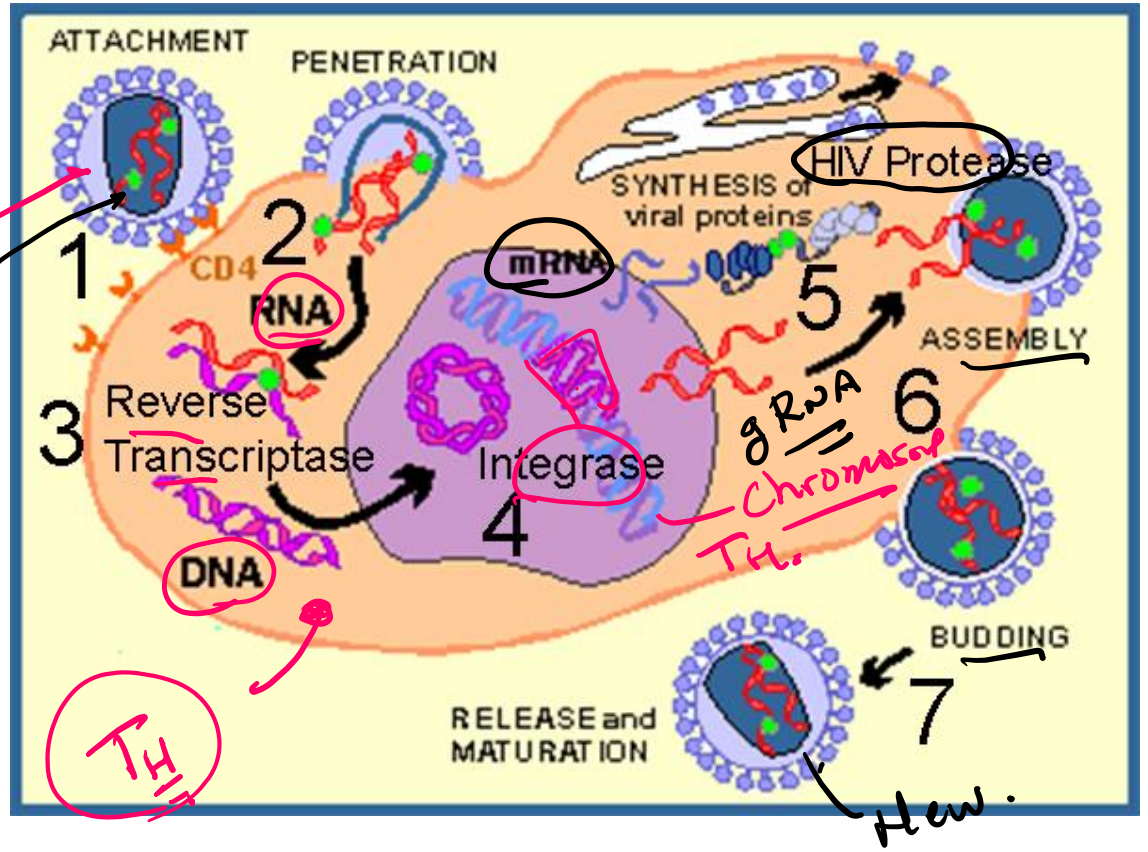
HIV Protease: Cleaves immature viral protein to produce smaller mature proteins.

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.

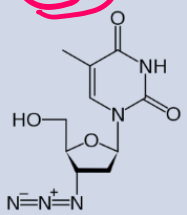
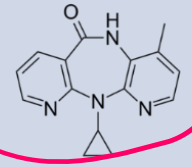


Drug Targets to Combat the HIV Virus –

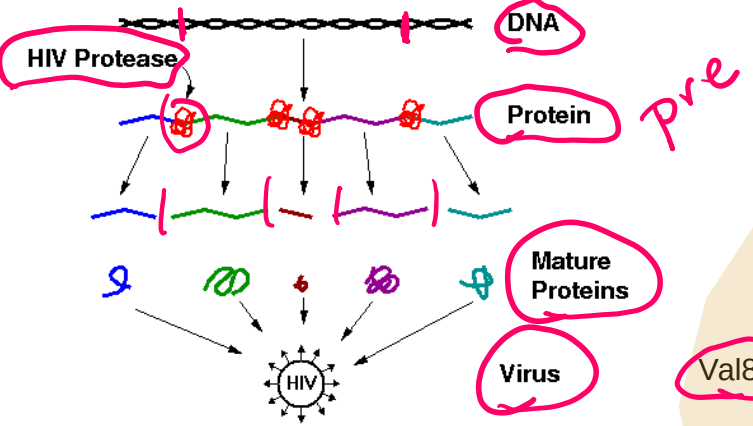
a) Viral fusion ✓
b) Reverse transcriptase ✓
c) Integrase ✓
d) HIV Protease ✓

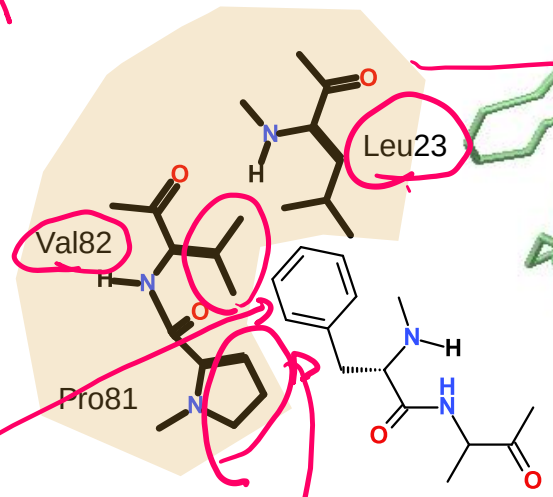
These are good drug targets because:

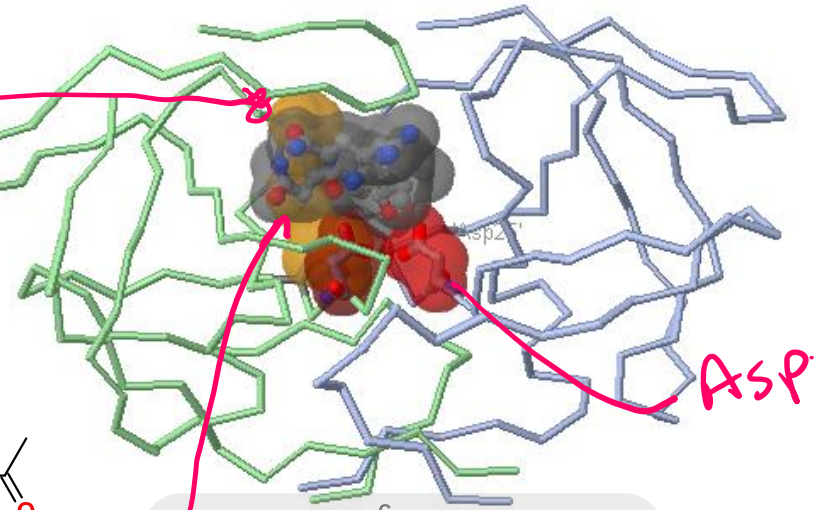
- Required for viral replication ✓
- Activities are not found in humans ✓

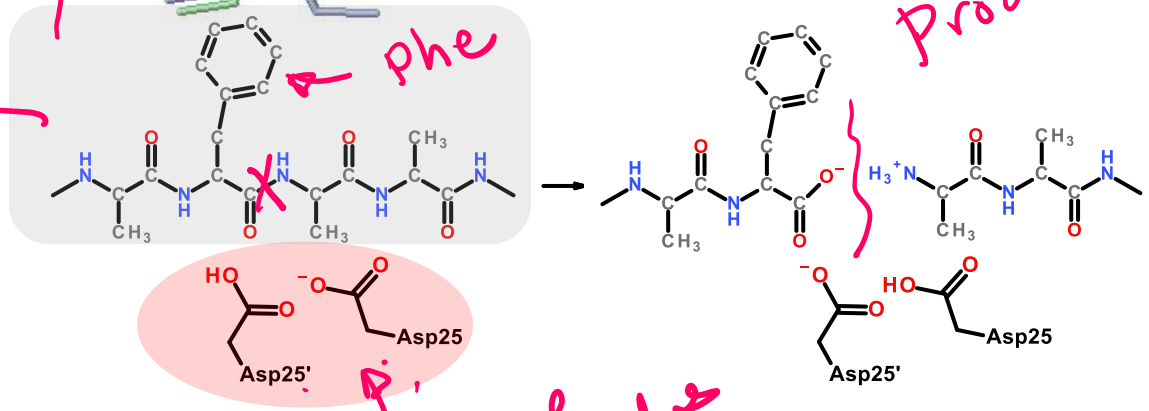
1980-84 HIV virus Characterized	Fusion Inhibitors	RevTrans Inhibitors – NRTI (competitive)	RevTrans Inhibitors – NNRTI (allosteric)	Integrase Inhibitors	Protease Inhibitors (competitive)
1985-89		AZT 			
1990-94		Didanoisine, Zalcitabine, Stavudine			
1995-99		Lavidudine	Nevirapine, Delavirdine, Efavirenz 		Saquinavir, Ritonovair, Indinavir, Nelfinavir
2000-04	Enfuvirtide	Didanosine Emtricitabine			Atazanavir
2005-09	Maraviroc		Etravirine	Raltegravir	Darunavir, Tipranavir
2010-14			Nevirapine XR, Rilpivirine	Dolutegravir, Evitegravir	

HIV Protease (Aspartyl 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):

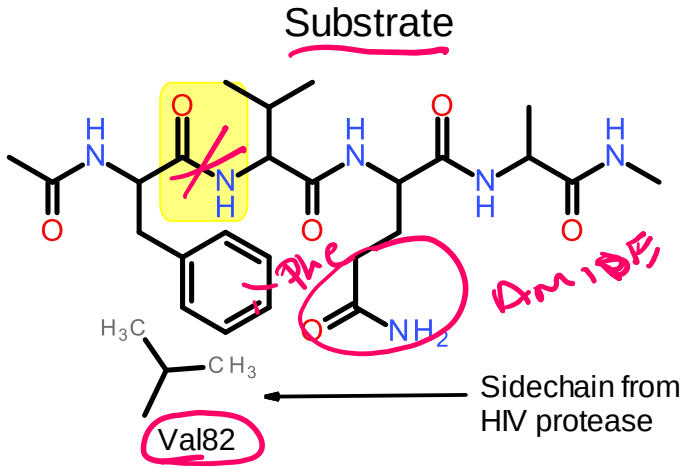
- Most drugs are small peptide-like analogs with non-cleavable bonds that resemble peptide bonds.

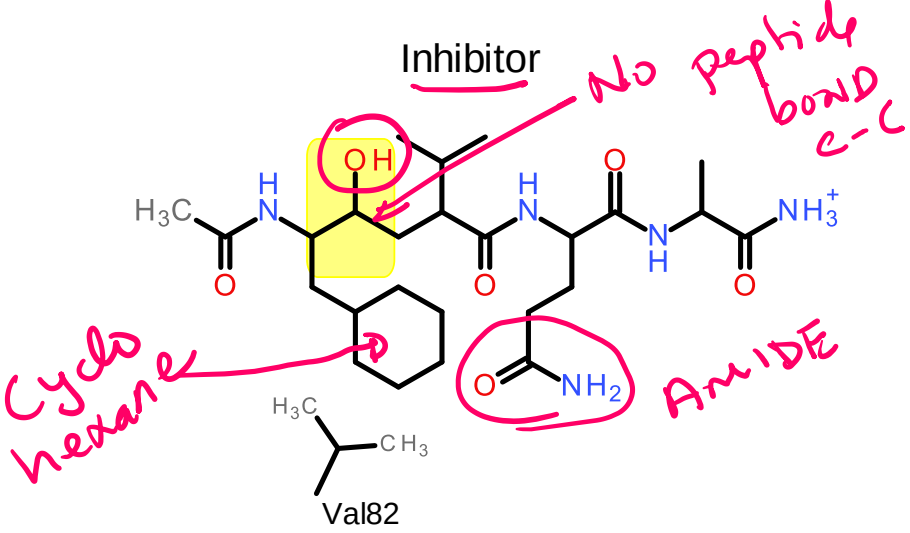
- *Where will they bind on the enzyme?*

Active site.

What will happen to them after they bind?

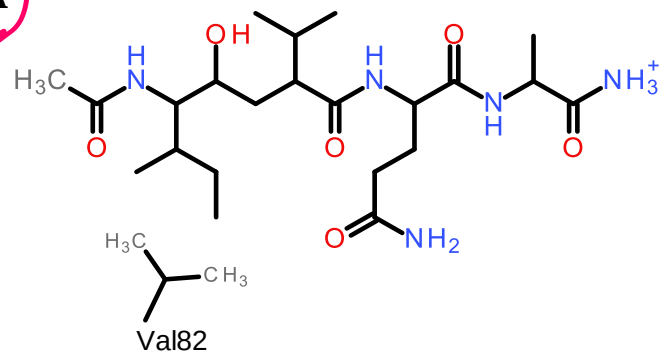
Nothing (no peptide bond).



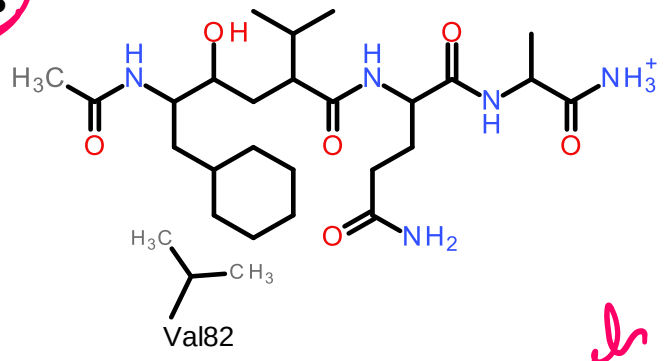


Drug Design: Compounds A (Isobutyl) and B (cyclohexane) are candidates for HIV protease inhibitors. Which of the two drugs will be more effective at inhibiting the wild-type protease?

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.

K_I for each inh. = K_D.

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]

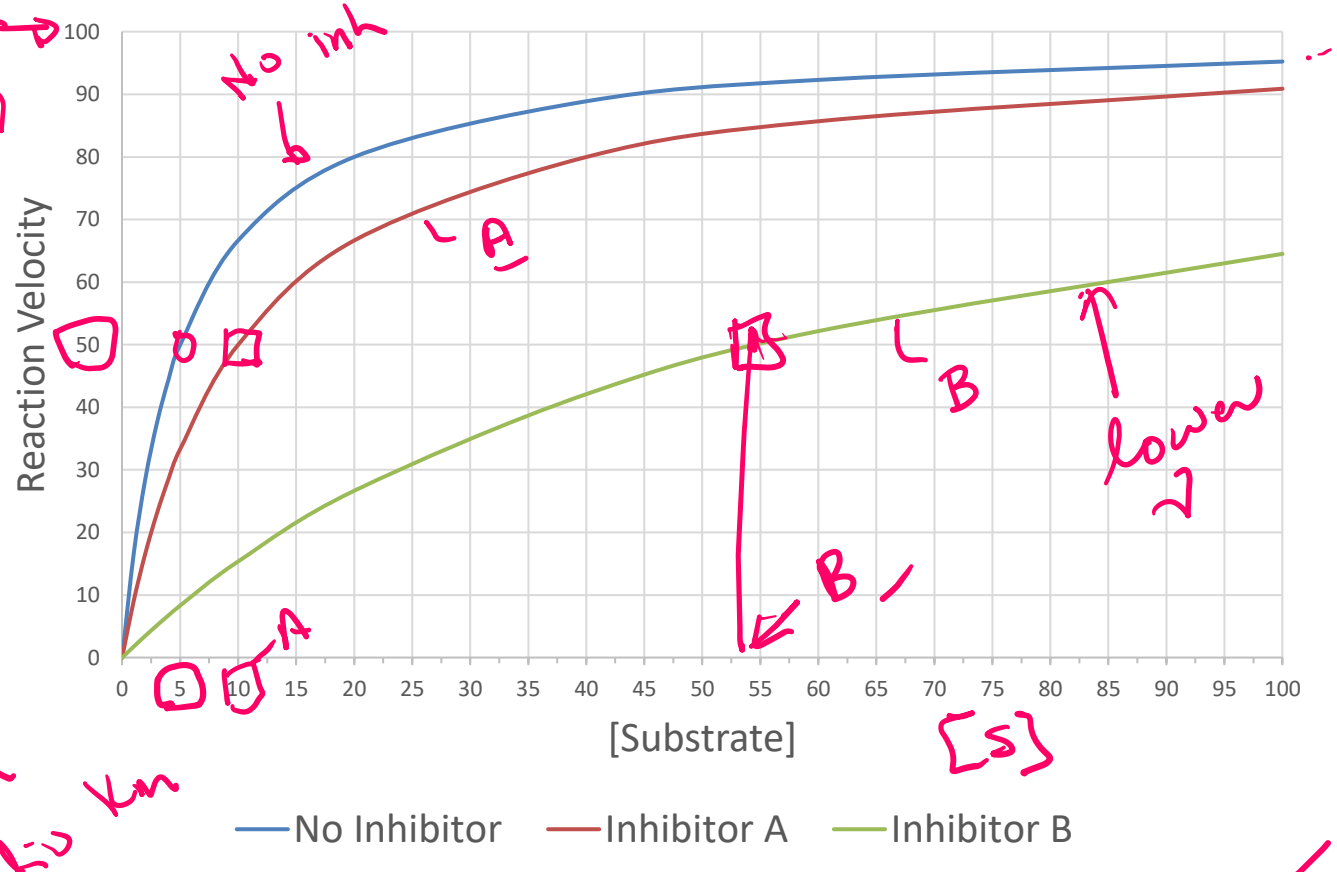
ii) Obtain α from the observed Km values

[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).**

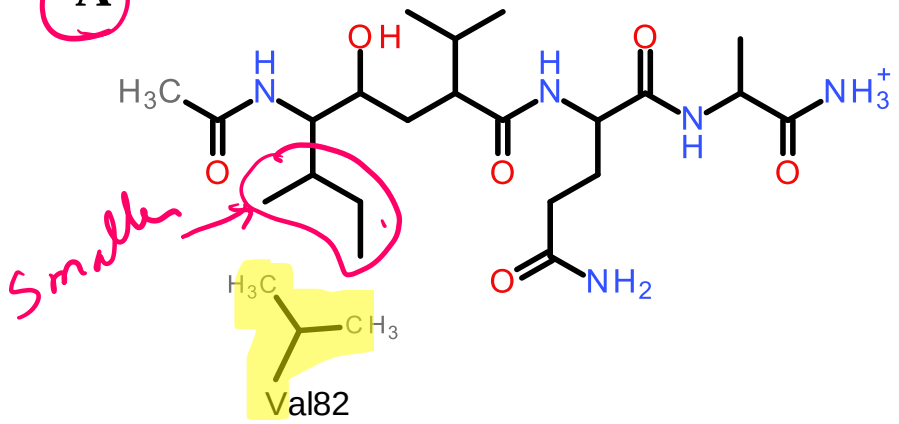
Note: more accurate Km values would be obtained using double reciprocal plots.



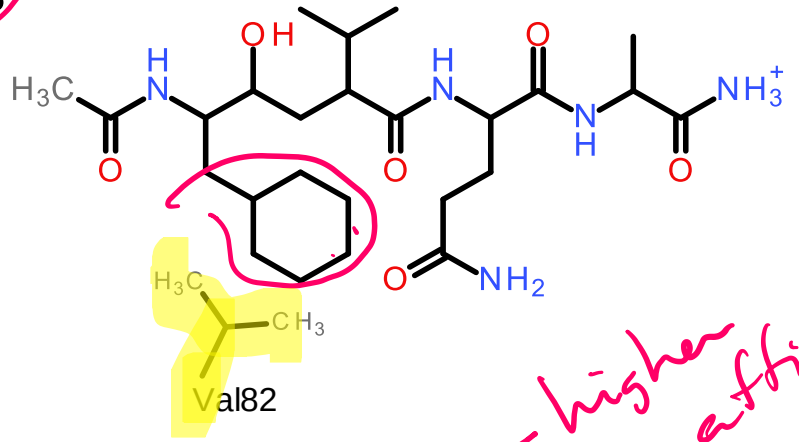
Data	Km	Alpha	K_I ([I]= 10 nM)
No Inh	5		
Inh A	10	2	K_I = 10 nM
Inh B	54	10.8	K_I = 1 nM

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

A



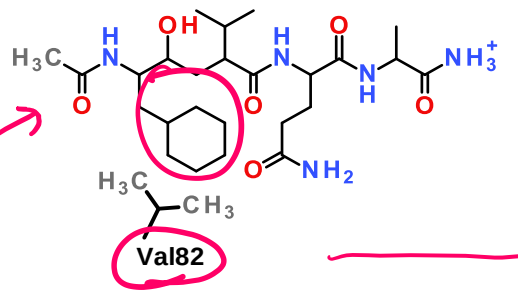
B

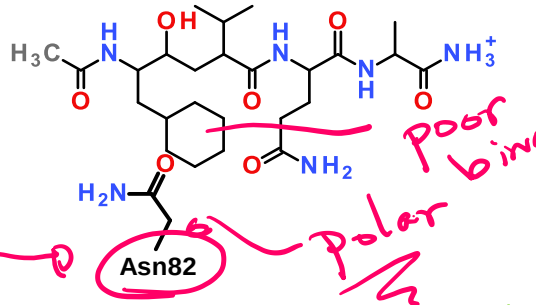


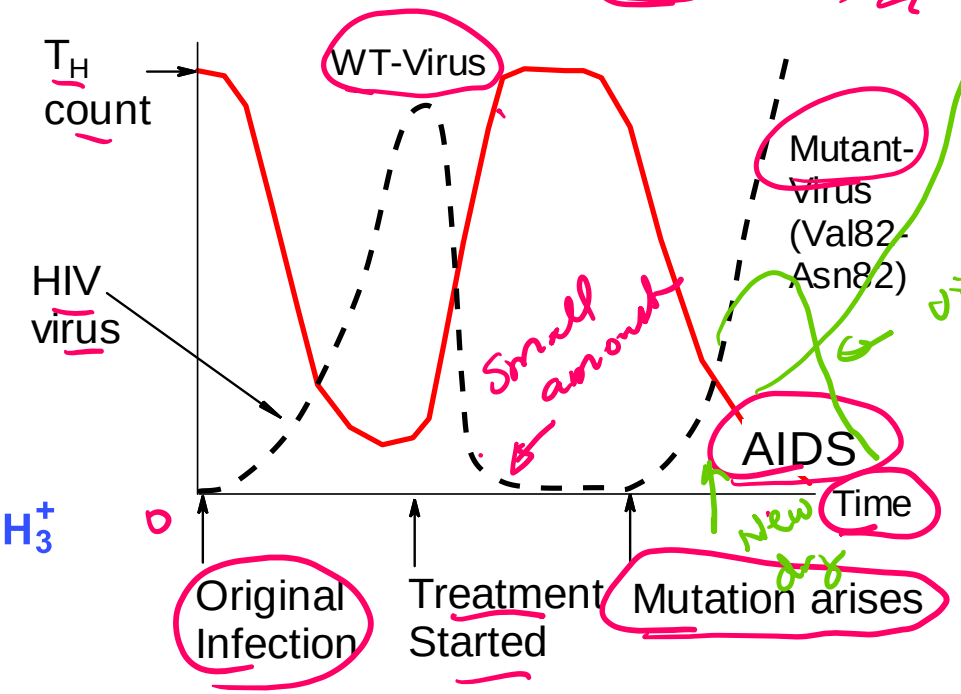
Potential Interaction	Drug A (K_I = 10 nM)	Drug B (K_I = 1 nM)
Van der Waals		
Hydrophobic effect		

Drug resistance & Rational Drug Design:

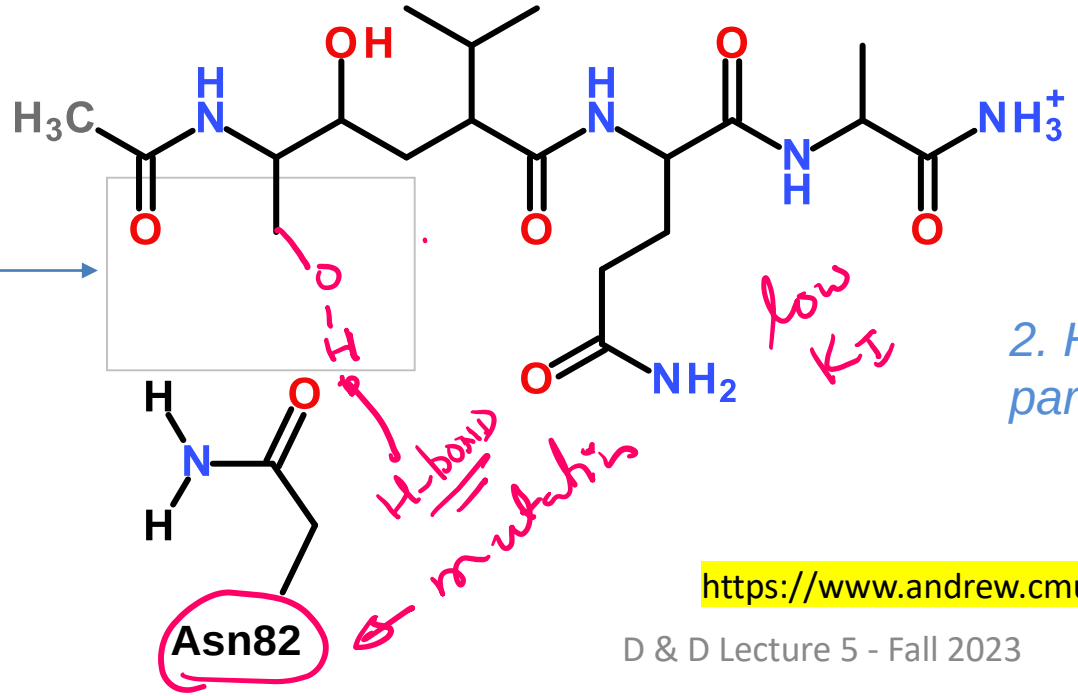
- Error prone copying of vRNA to DNA introduces changes in the sequence of the viral RNA (mutations), leading to altered amino acids in the viral proteins.
- Changes in the residues that are involved in drug binding may reduce binding.
- The mutant virus is no longer inhibited and will quickly overgrow the wild-type virus.
- A common mutation that arises in many HIV patients is changing Val82→Asn82 in HIV protease.
- The altered HIV protease can be inhibited with modified protease inhibitors (personalized medicine).







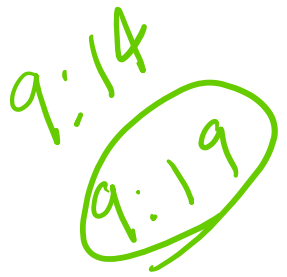
1. How might you alter the existing inhibitor to be effective at binding to HIV protease with the Asn82 mutation?



2. How would you test your new drug? What parameter would you measure?

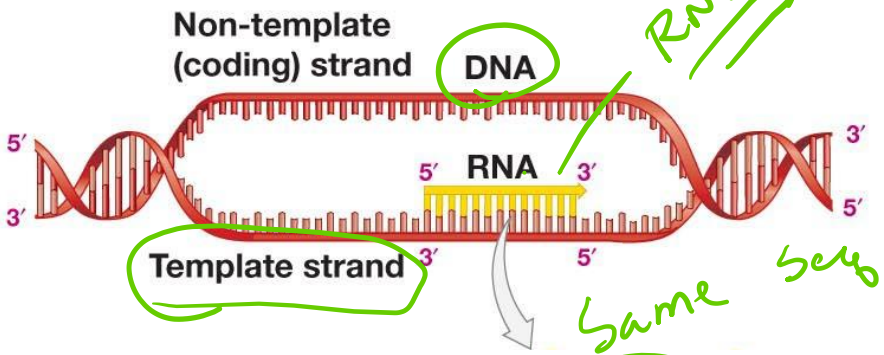
K_M or k_{CAT} or K_I

https://www.andrew.cmu.edu/user/rule/03_131/Pset/PS05/jsmol_hiv.html

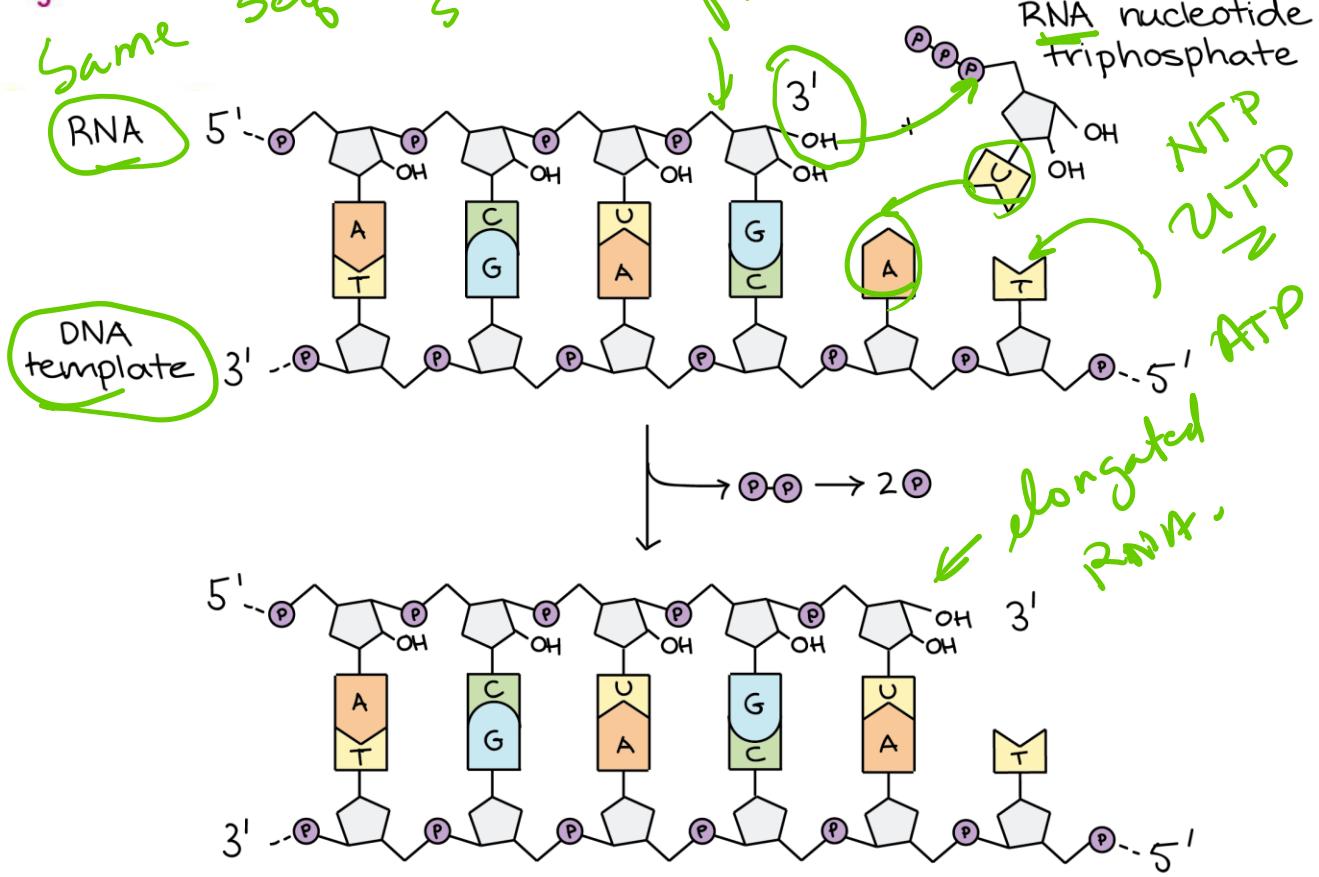


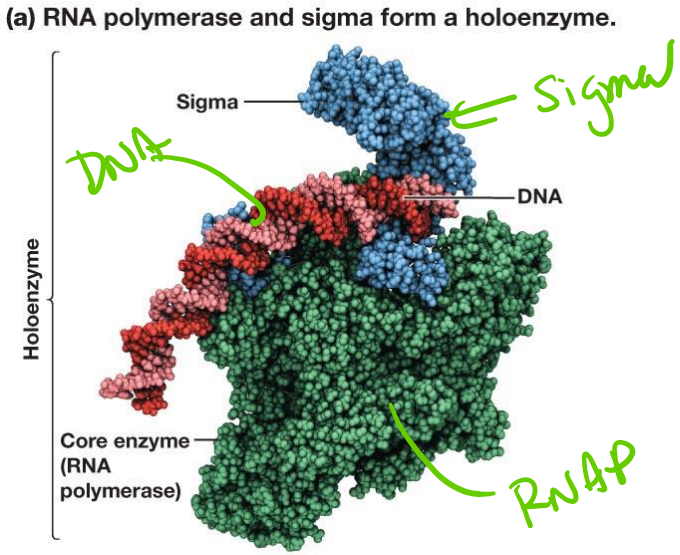
Drugs that inhibit Transcription and Translation

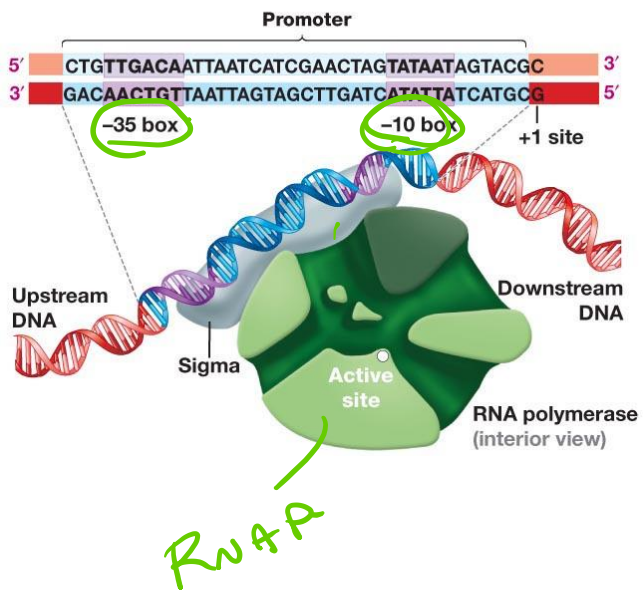
Transcription



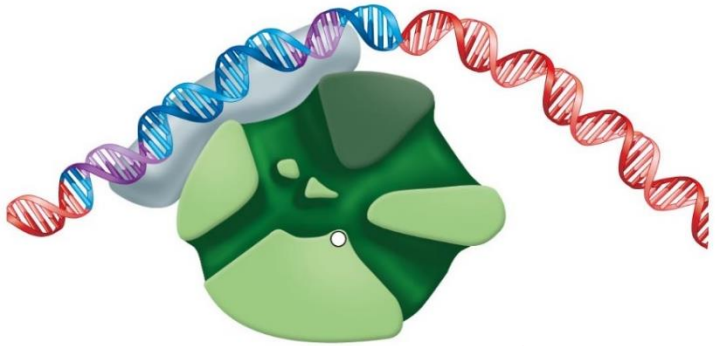
1. No primer (self generated)
2. Template is DNA
3. Product is RNA
4. Bases are added to 3’-OH
5. No error correction
6. NTPs used to elongate - RNA is being made



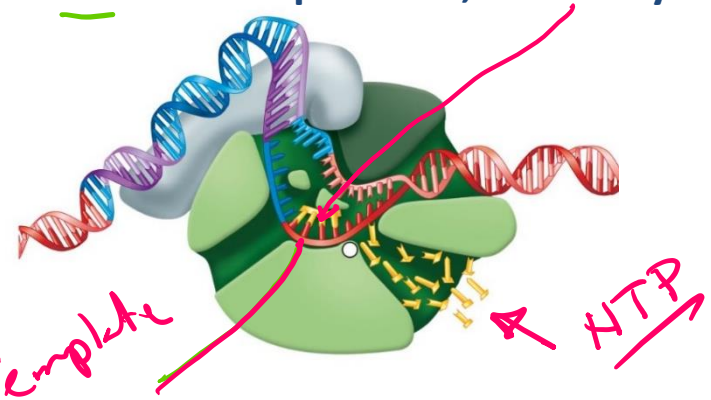




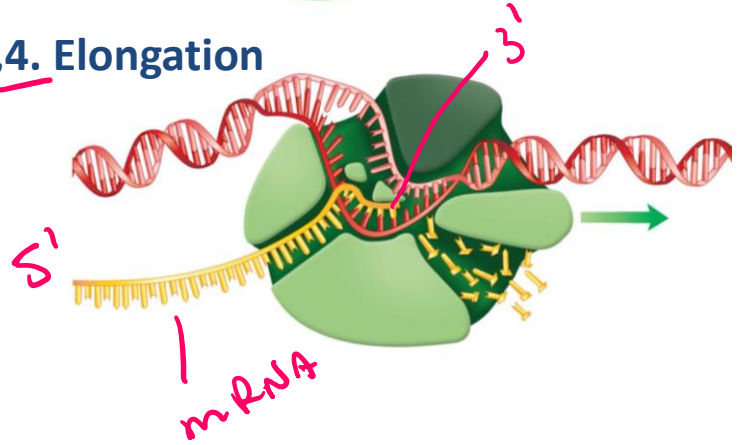
1. Promoter recognition (sigma factor)



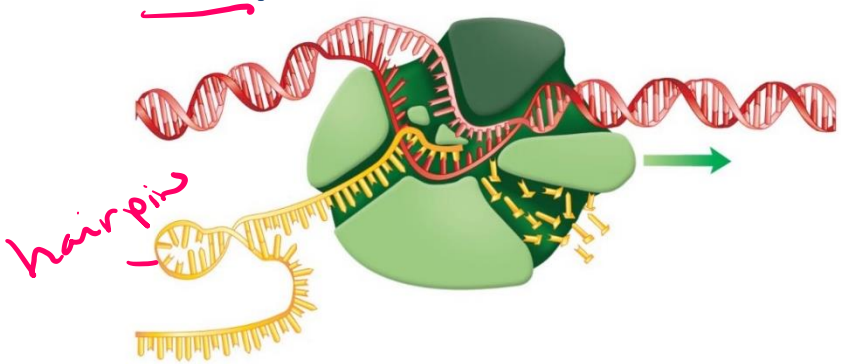
2. Strand separation, Primer synthesis



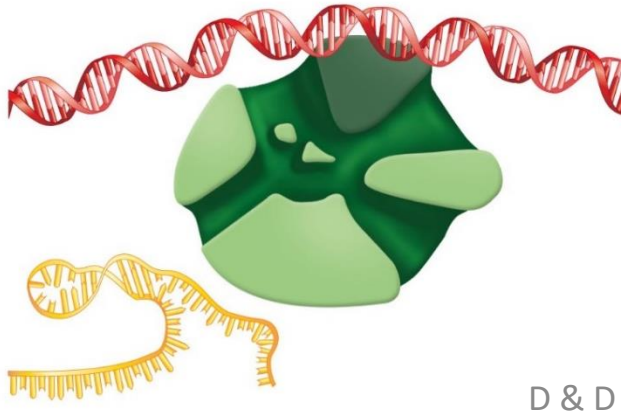
3,4. Elongation



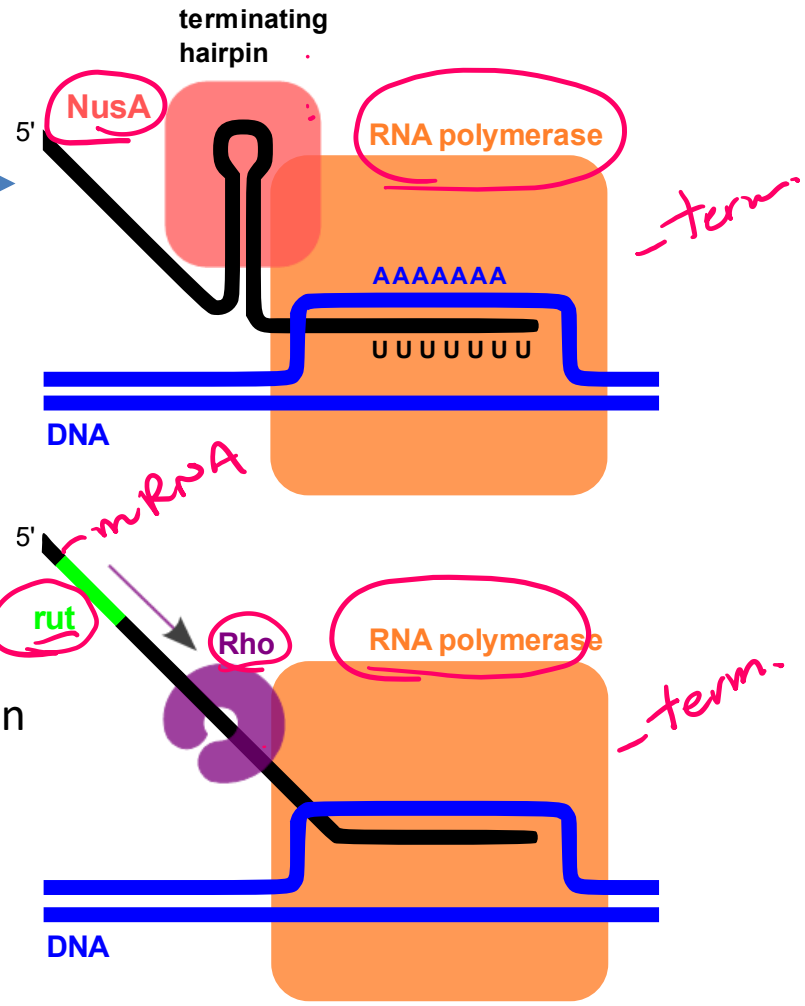
5. Hairpin formation



6. Release of mRNA

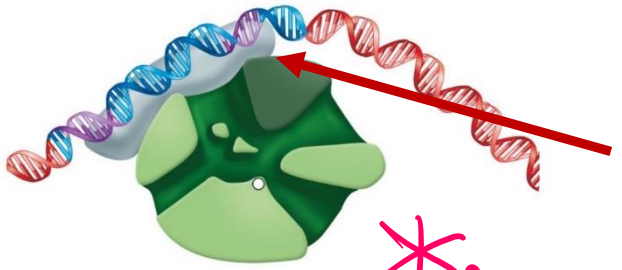


Termination of Transcription

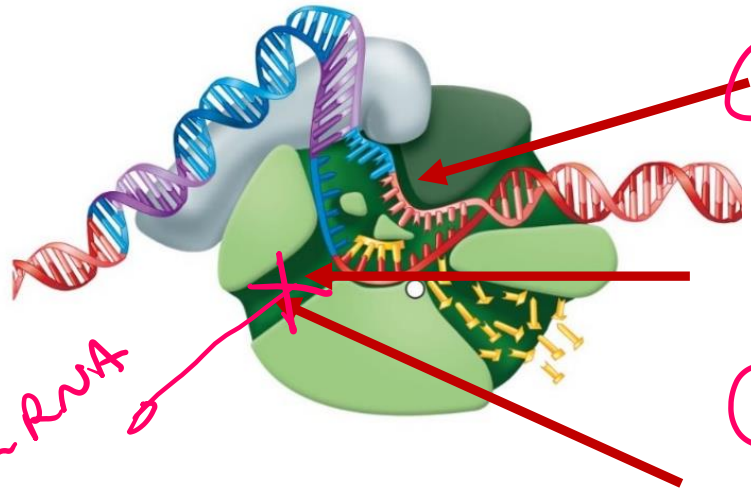


Rho protein can also terminate transcription

Drugs as inhibitors of Transcription:



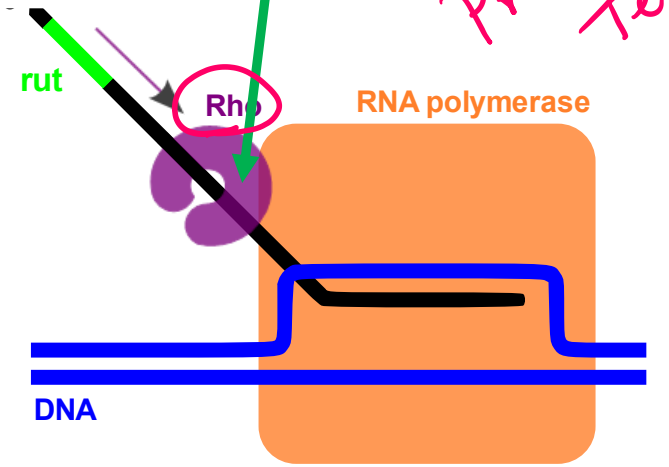
Fidaxomicin, Myxopryronin



Streptolydigin

GE23077

Rifamycin (rifampin, rifapentine, rifabutin, rifamixin)



Bicyclomycin

Prokaryotic Transcription Video

Animation - Transcription in Prokaryotes →

- Video shows an alternate way that bacterial terminate transcription – rho factor.
- It also shows a ribosome attaching to the mRNA, generating protein.

Structure and Function of Ribosomes

- The process of translations occurs inside a ribosome. Ribosomes can be separated into two major substructures called the large (50S) and small subunit (30S).

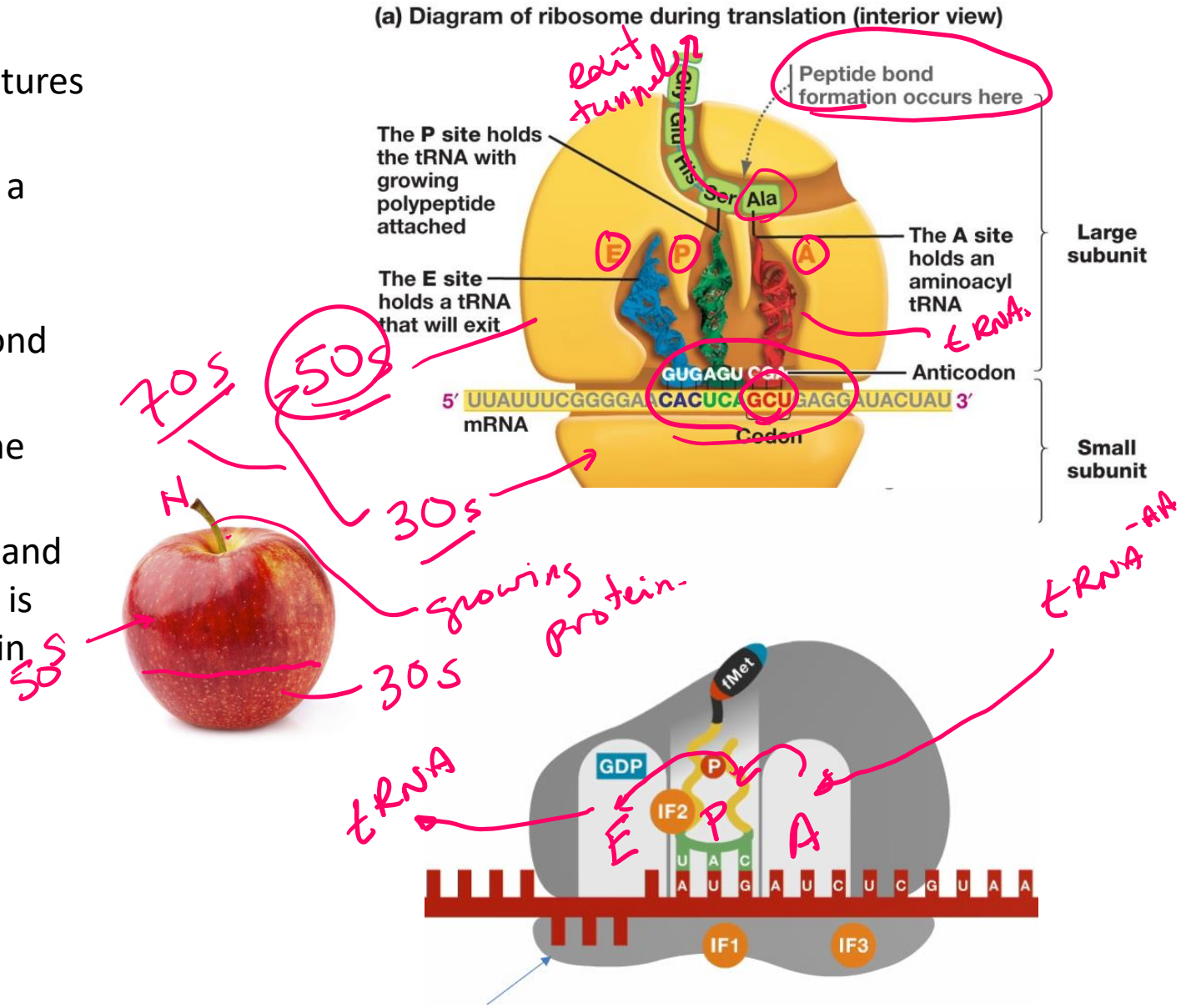
  S is a measure of how fast a particle will sediment in a centrifuge. Larger S, bigger particle.
- The small subunit holds the mRNA in place during translation and the large subunit is where peptide-bond formation takes place.
- The growing peptide (apple stem) leaves the ribosome through the exit tunnel (dimple at top of apple).
- tRNA (transfer RNA) molecules recognize the codons and bring the next amino acid into the ribosome where it is attached to the COOH terminus of the growing protein.

Path of a tRNA during protein synthesis:

A site = tRNA + AA

P site = tRNA + entire protein

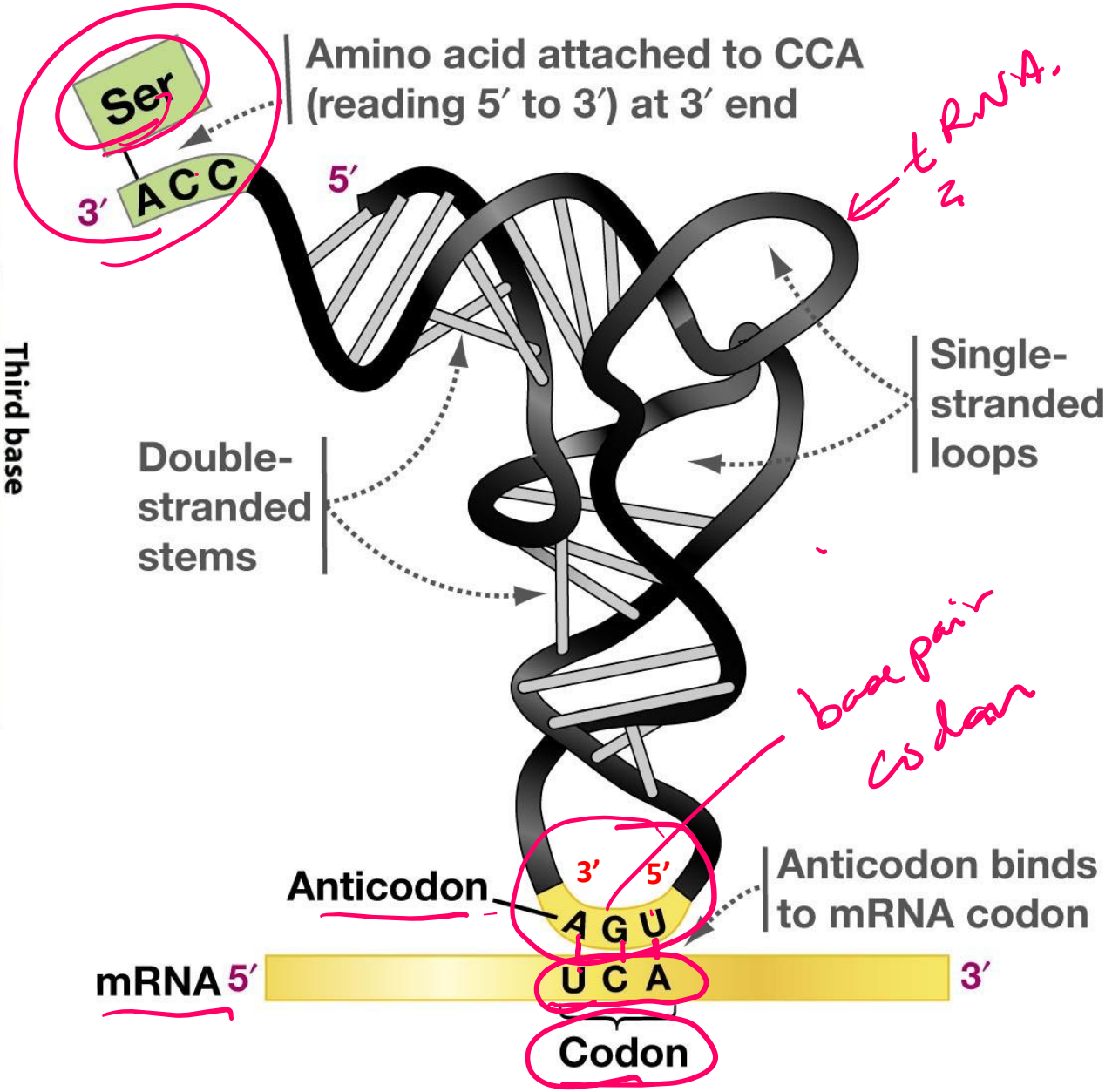
E site = tRNA



Translation- The Adapter Molecule (tRNA)

Second base

First base	U	C	A	G	Third base
U	UUU, UUC Phenylalanine UUA, UUG Leucine	UCU, UCC, UCA, UCG Serine	UAU, UAC Tyrosine UAA Stop codon UAG Stop codon	UGU, UGC Cysteine UGA Stop codon UGG Tryptophan	U C A G
C	CUU, CUC, CUA, CUG Leucine	CCU, CCC, CCA, CCG Proline	CAU, CAC Histidine CAA, CAG Glutamine	CGU, CGC, CGA, CGG Arginine	U C A G
A	AUU, AUC, AUA Isoleucine AUG Methionine (start codon)	ACU, ACC, ACA, ACG Threonine	AAU, AAC Asparagine AAA, AAG Lysine	AGU, AGC Serine AGA, AGG Arginine	U C A G
G	GUU, GUC, GUA, GUG Valine	GCU, GCC, GCA, GCG Alanine	GAU, GAC Aspartic acid GAA, GAG Glutamic acid	GGU, GGC, GGA, GGG Glycine	U C A G



- The adapter molecules are called transfer RNAs or tRNAs.
- Contain a CCA sequence at 3’ end where AA’s are attached
- a triplet anticodon to form base pairs with the mRNA codon – ***anti-parallel as always.***

Protein Synthesis – Prokaryotic Cells

Initiation

1. 30 s subunit binds to RBS
2. tRNA-fMet in P-site
3. 50 s subunit binds

Elongation

1. tRNA-AA binds to A-site
2. Peptide bond forms
3. Ribosome shifts, protein-tRNA returns to P-site

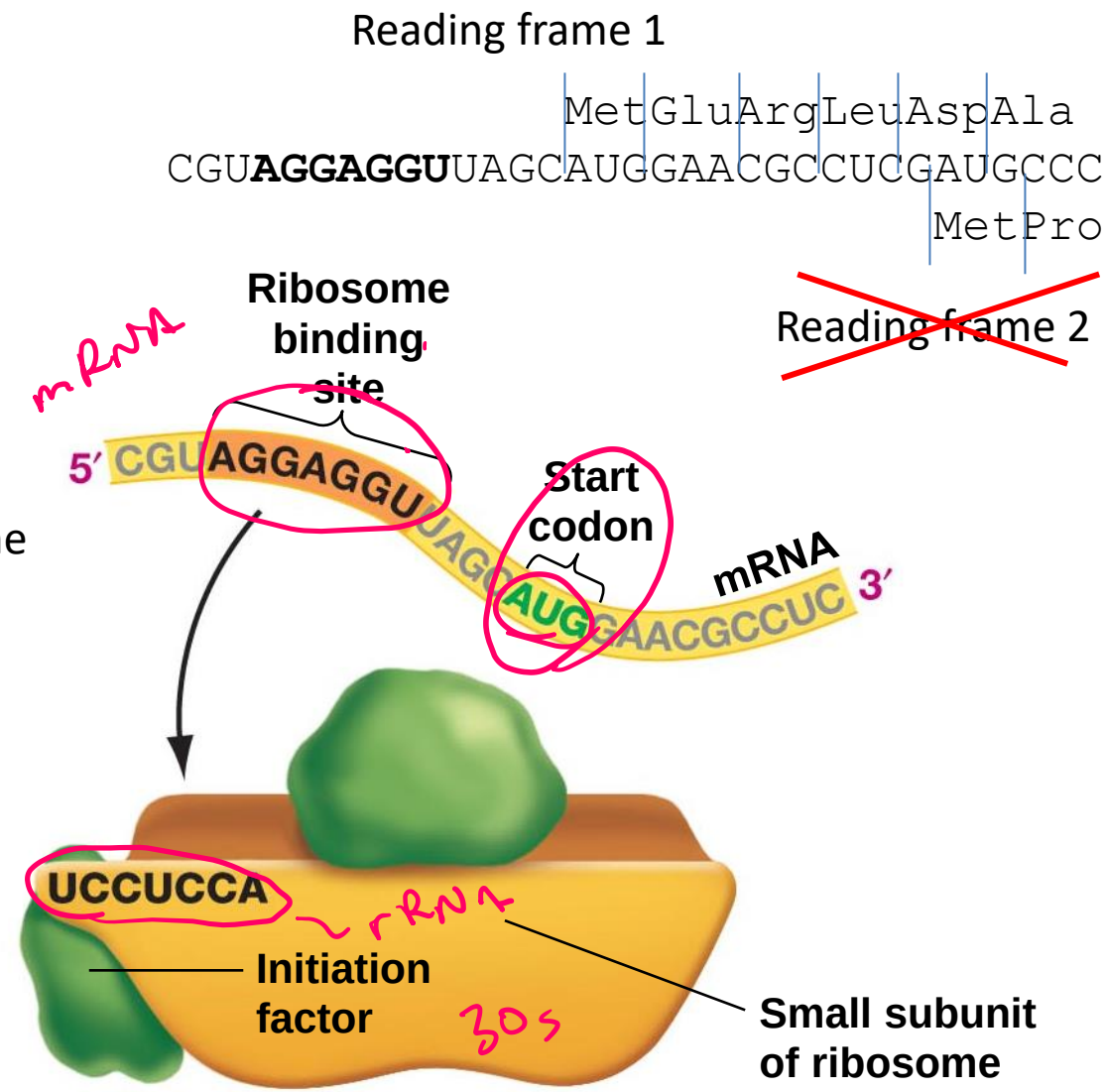
Termination

1. Stop codon interpreted by release factor (protein)
2. Covalent bond between C-term of protein and last tRNA used is hydrolyzed.

Initiation 1. mRNA binds to small subunit of ribosome

Formation of Initiation Complex -

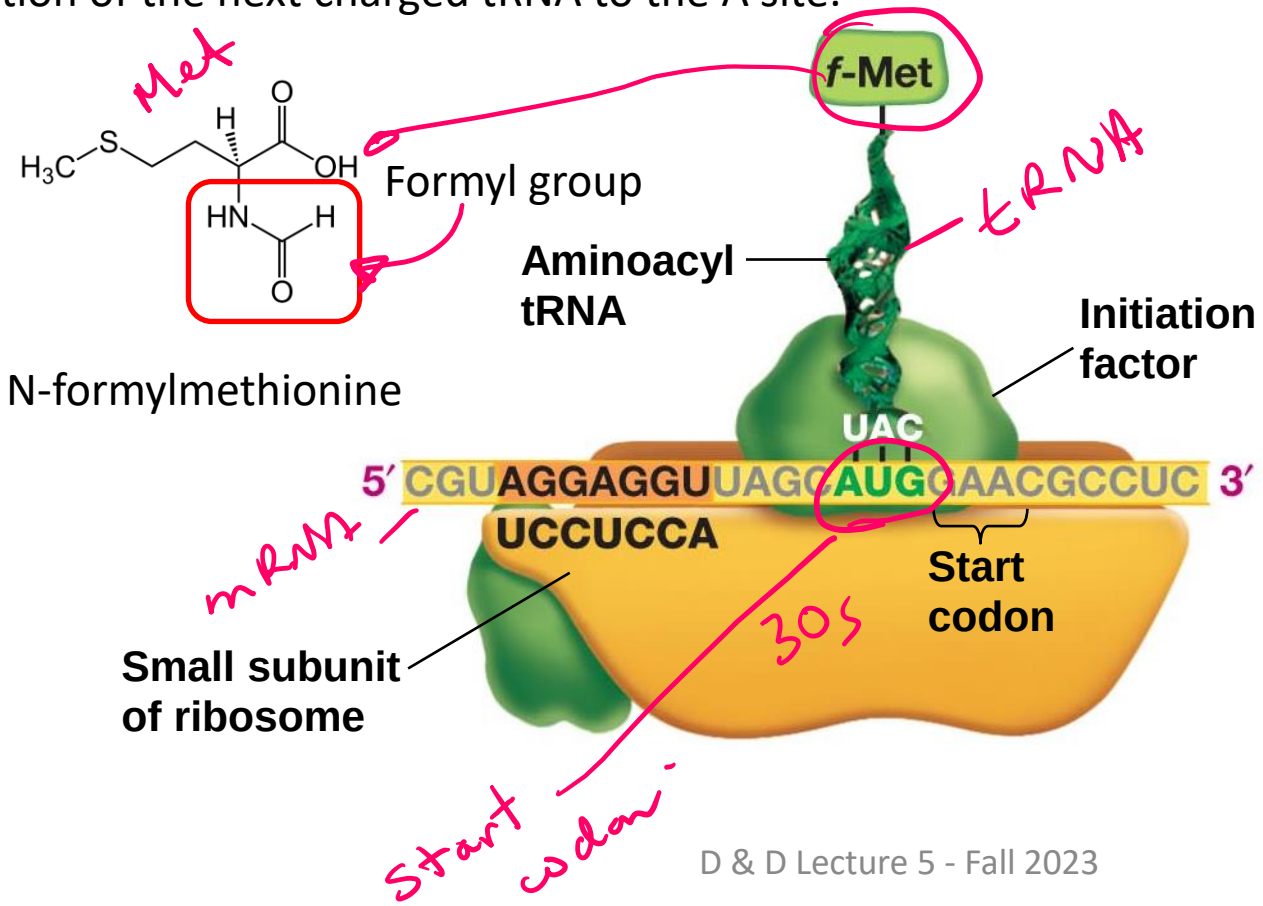
- 30 S + mRNA
- Ribosome binding site binds to rRNA
- AUG start codon is ~4-6 bases from ribosome binding site, and will be in the P-site after initiation is complete.



Initiation 2. Initiator aminoacyl tRNA binds to start codon carrying f-met.

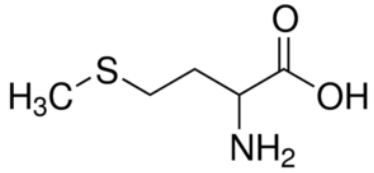
Prokaryotic Cells

- The initiator tRNA is charged with a modified form of methionine called ***N-formylmethionine***.
- Only the initiator tRNA is capable of binding the P site on the small subunit.
- Because it is in the P site, protein synthesis is ready to begin with the addition of the next charged tRNA to the A site.



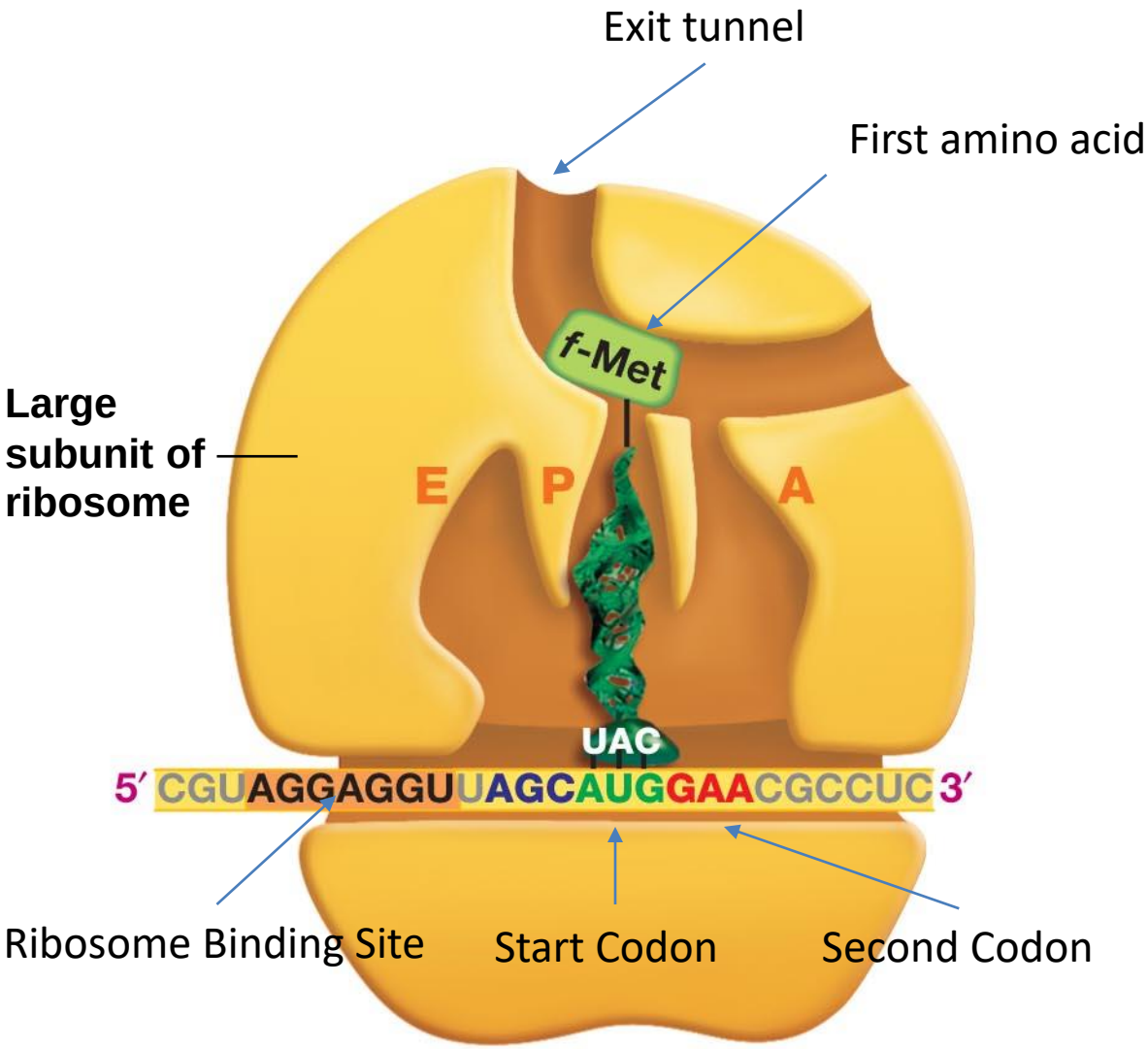
Eukaryotic Cells:

- ***normal methionine*** is used to start the protein
- There is no ribosome binding site on the mRNA, ribosome scans for the first start codon, starting from the 5' end of the mRNA



These, and other, differences allow the selective inhibition of prokaryotic ribosomes by antibiotics.

Initiation 3. Large subunit binds completing the complex



Summary of Protein Synthesis & Ribosomes

Role of different Ribosomal subunits

- 30S – RBS & mRNA codon/anticodon
- 50S – Peptide bond synthesis
- Exit tunnel – new protein emerges

tRNA sites:

- A – aminoacyl – next tRNA-AA binds
- P – initiation & contains the growing peptide
- E – empty tRNA leave from here

Initiation:

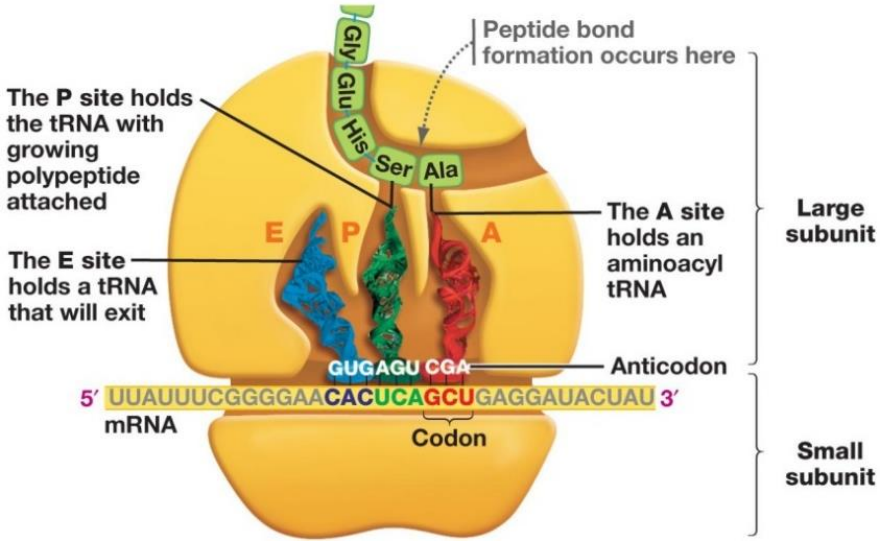
1. Ribosome binding site & rRNA interaction (Proks)/AUG scanning (Euks).
2. fMet-tRNA (Proks) or Met (Euks) in P site

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 bond between C-terminal of peptide & last tRNA



Antibiotics that Inhibit Protein Synthesis

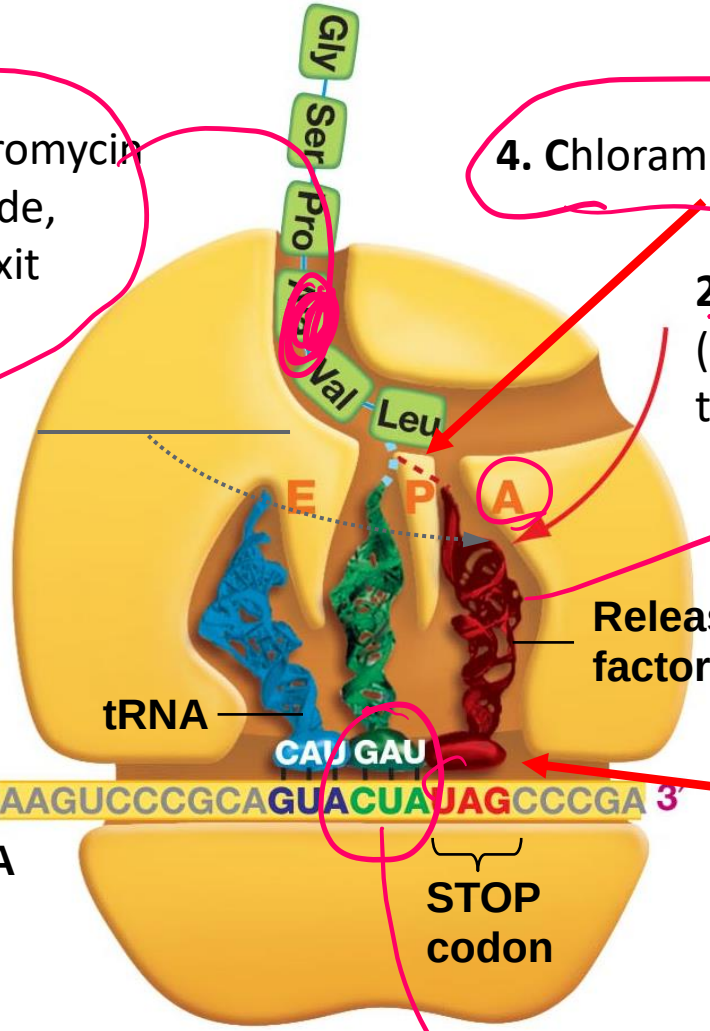
6. Erythromycin (macrolide, blocks exit tunnel)

4. Chloramphenicol (peptide bond formation)

2. Tetracycline (binding of charged tRNA)

5. Viomycin (blocks translocation)

1. Linezolid (blocks 70s formation)



3. Kanamycin (Aminoglycoside) (codon/anticodon pairing)

tRNA AA

wrong AA acid mispairing

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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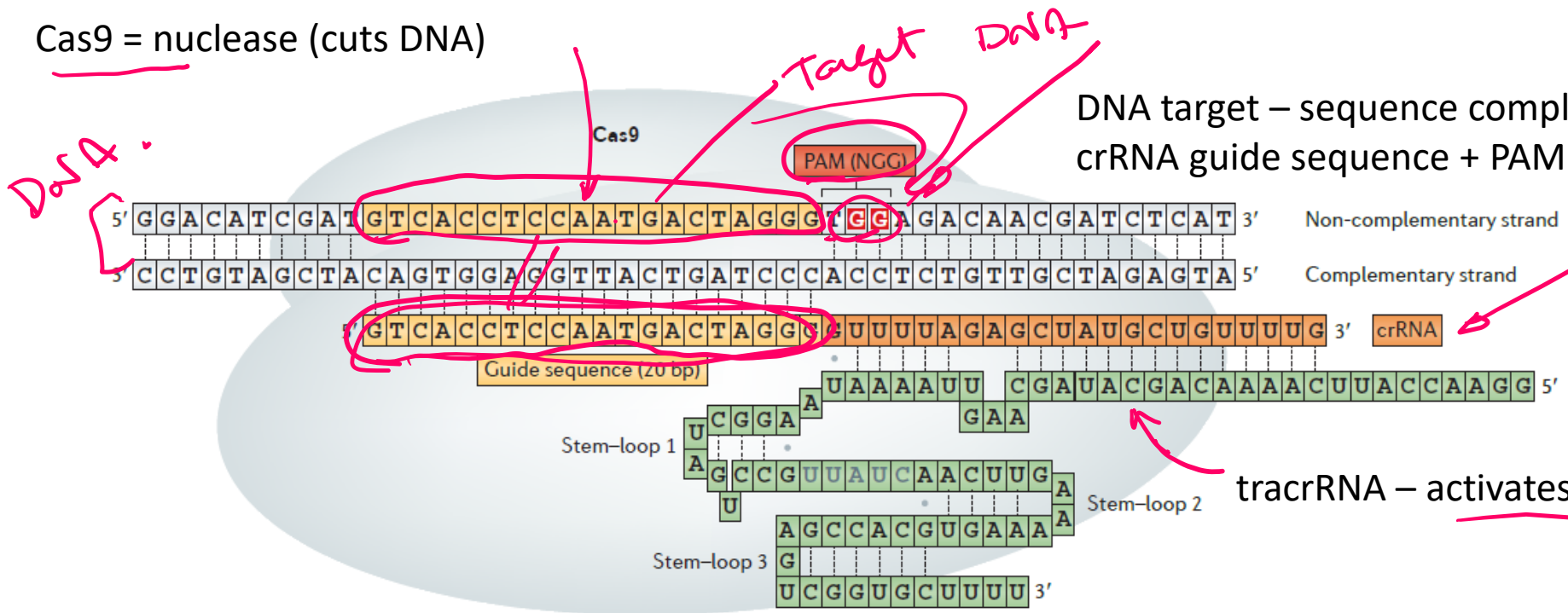
Jennifer A. Doudna
Prize share: 1/2

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"

CRISPR-Cas9-directed cut

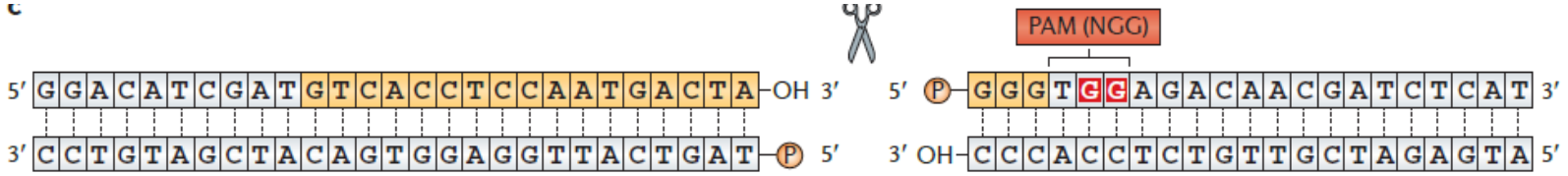
Cas9 = nuclease (cuts DNA)



DNA target – sequence complementary to crRNA guide sequence + PAM (NGG)

crRNA – targets DNA – Complementary to both DNA & tracrRNA

tracrRNA – activates Cas9

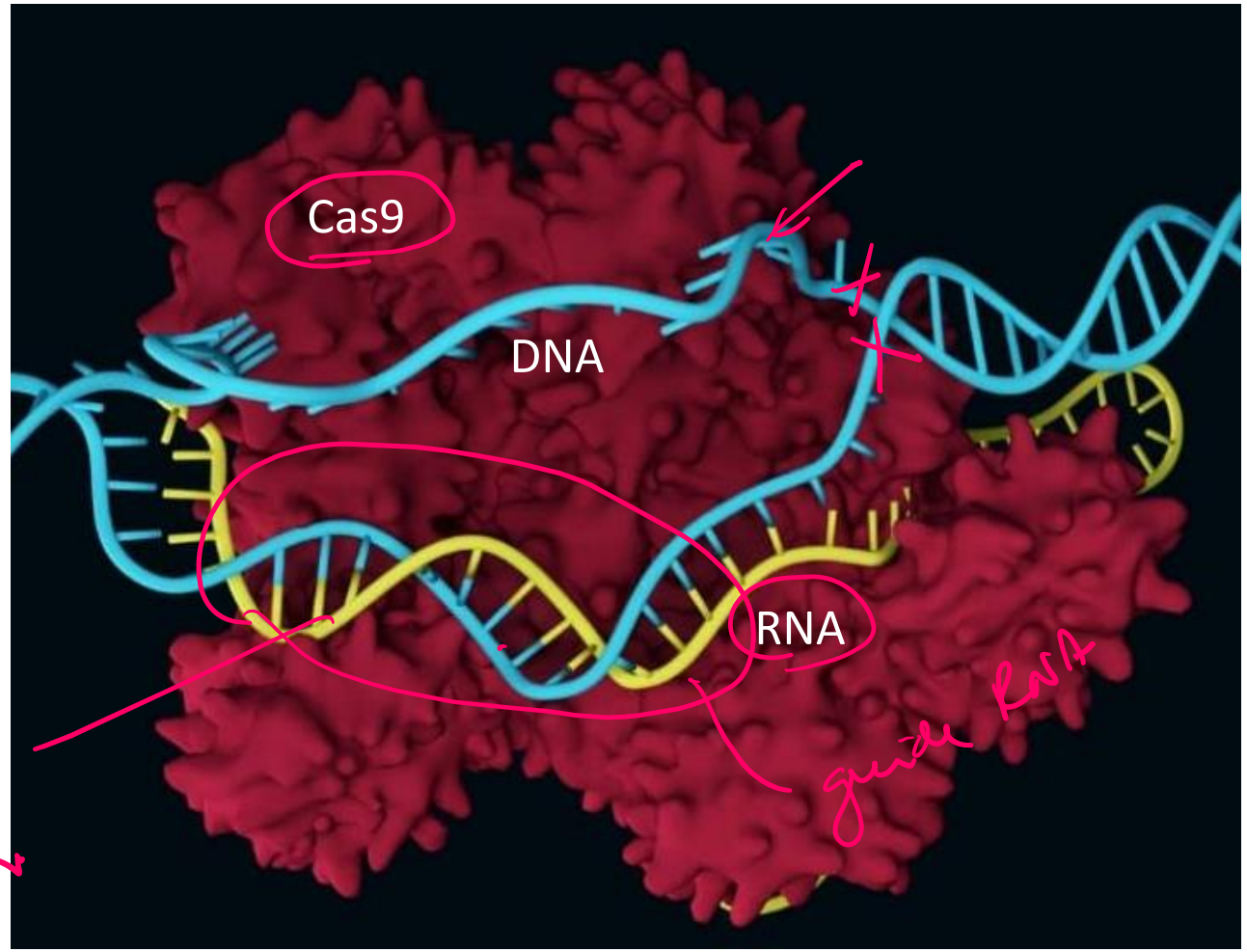


Cleavage site:

- Predictable with a high degree of certainty
- Can have off-target cleavages

CRISPR-Cas9-directed cut

Structure of Cas9+DNA+RNA

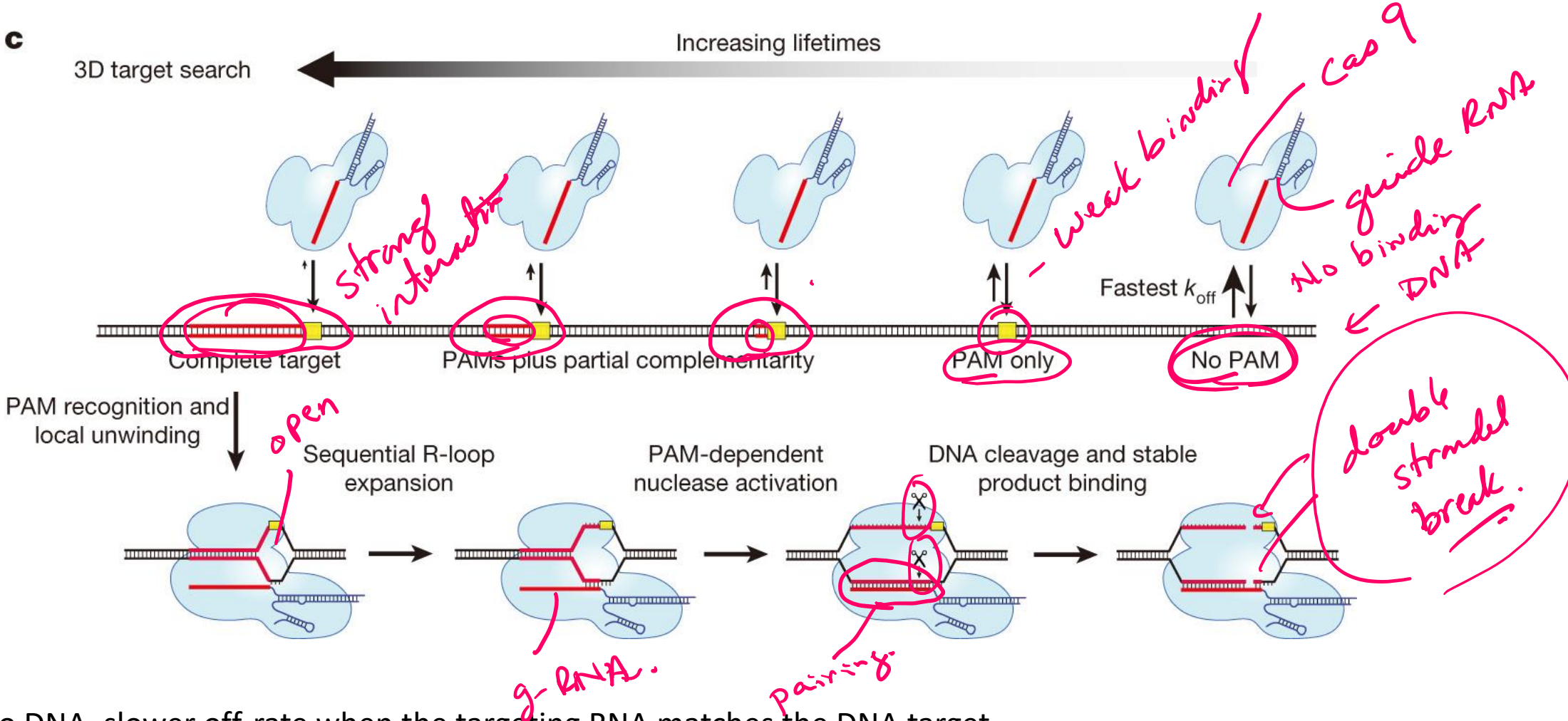


Cleavage site:

- Predictable with a high degree of certainty
- Can have off-target cleavages, currently limiting medical applications.

https://www.europeanscientist.com/en/public-health/crispr-cas-9-corrects-genetic-defects-in-mice/

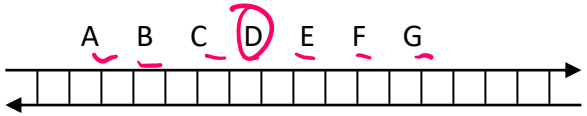
Overall Process of Cas9 DNA Cleavage



1. Binds to DNA, slower off-rate when the targeting RNA matches the DNA target
2. Unwinding of DNA, forming duplex with the targeting RNA
3. Activation of nucleases, cutting each strand

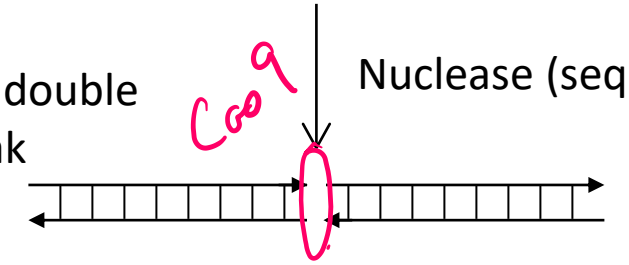
Key Concepts in Genome Editing

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



Genomic DNA

Genomic DNA with double stranded break



Original Sequence

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

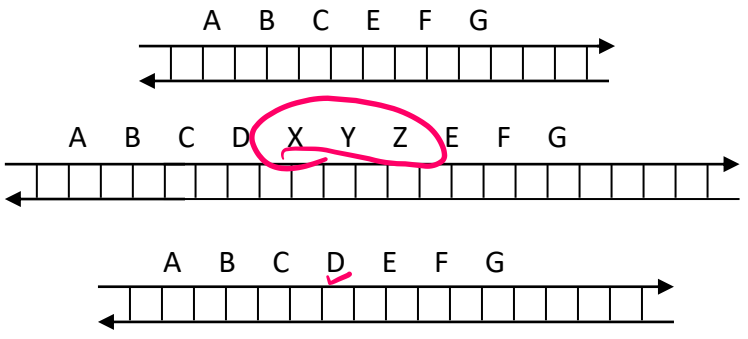
Non-Homologous End Joining (NHEJ)

Repair Method I: Addition or deletion of bases

Deletion of one base

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

non functional protein



Deletion

Insertion

} together called “Indels”

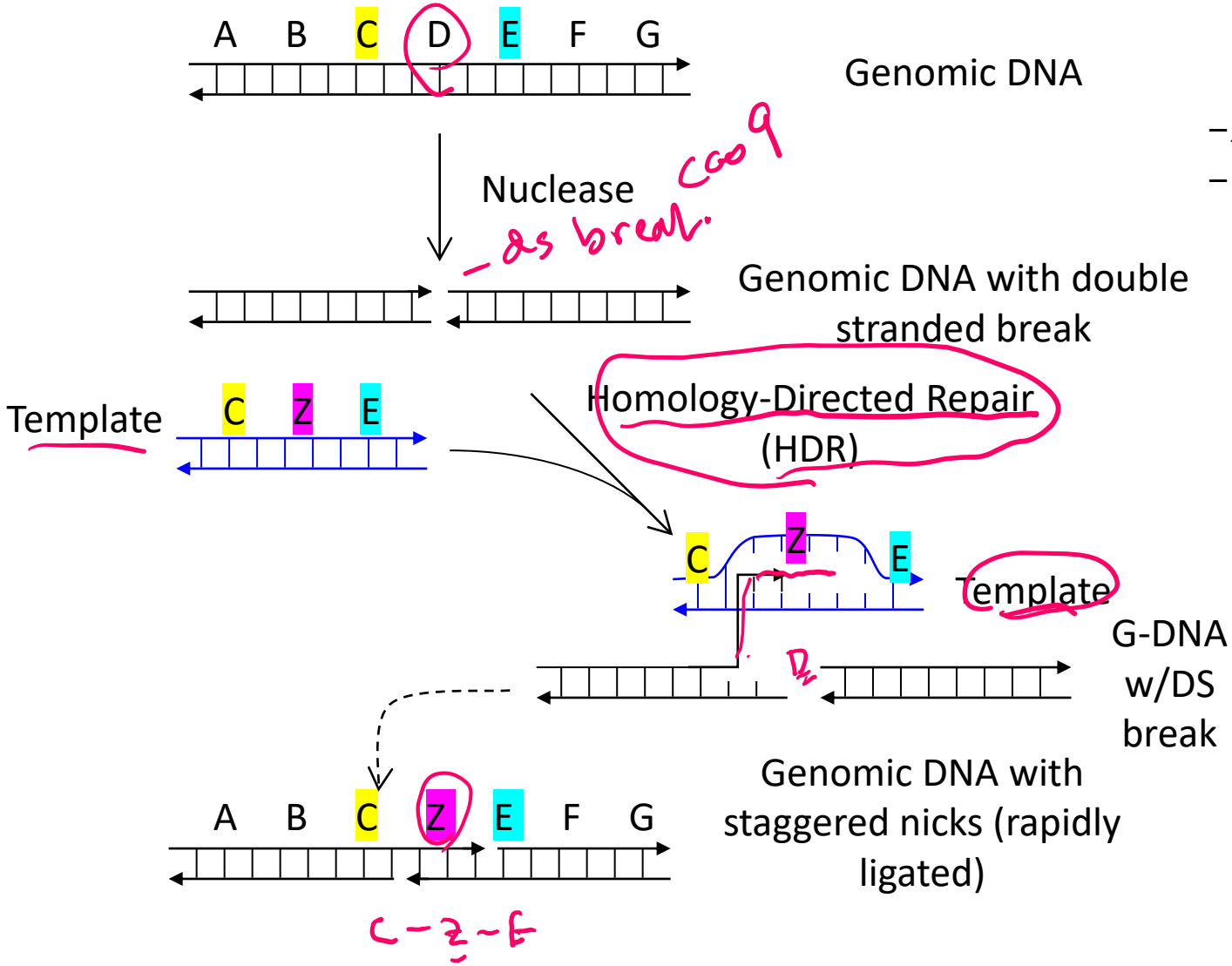
Restoration (often undetected)

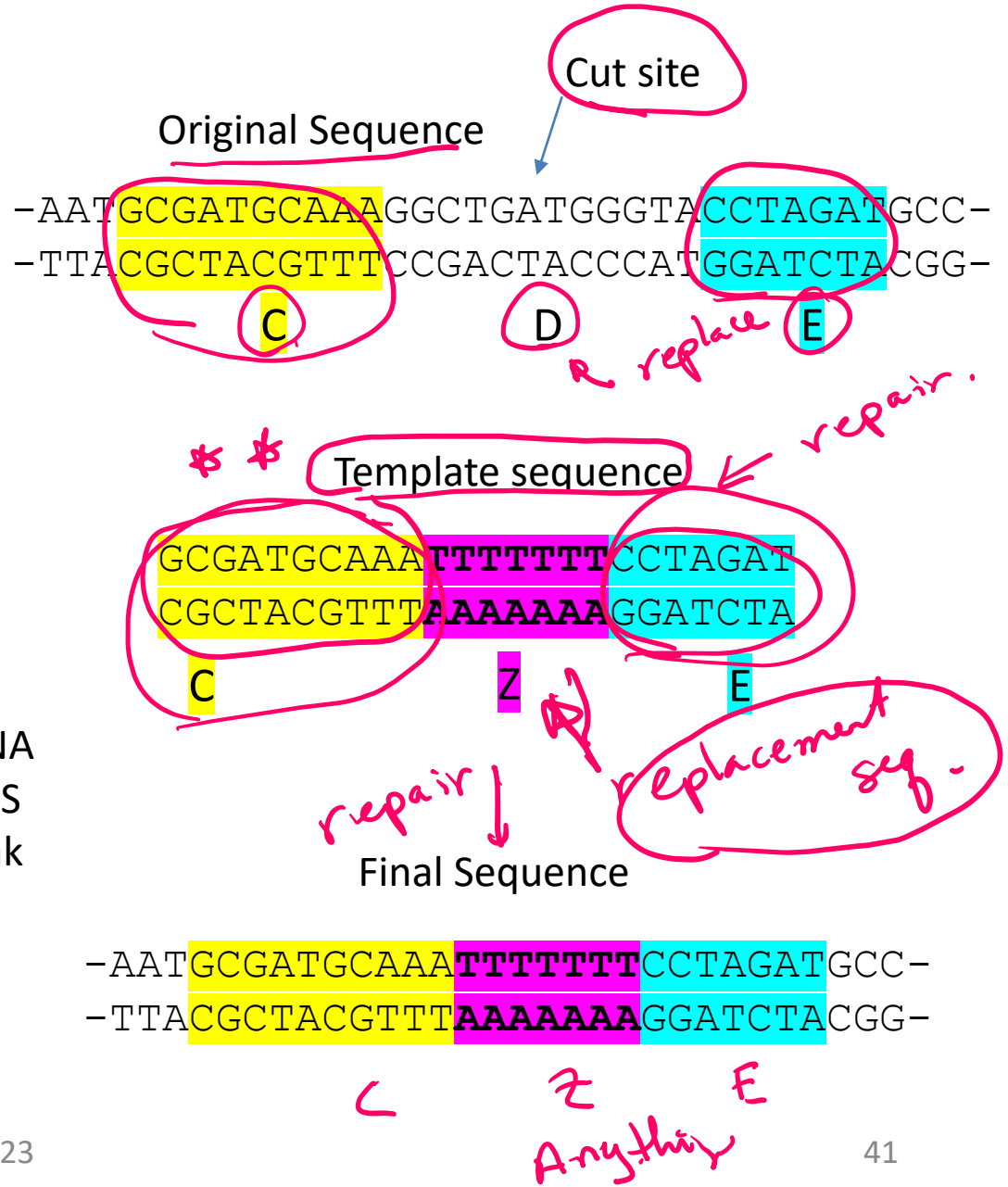
Frame shift:

- changes the amino acid sequence after the position of the indel.

Key Concepts in Genome Editing

Repair method II – replacement of a segment of DNA



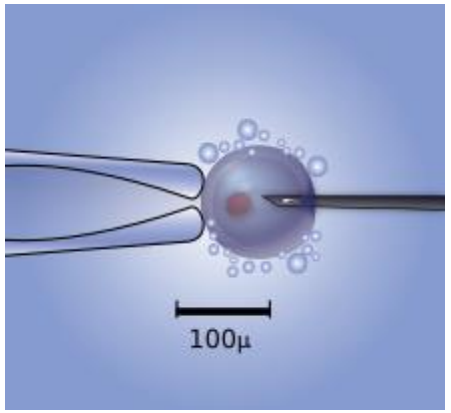


Altering the Genome Sequence with Cas9-CRISPR

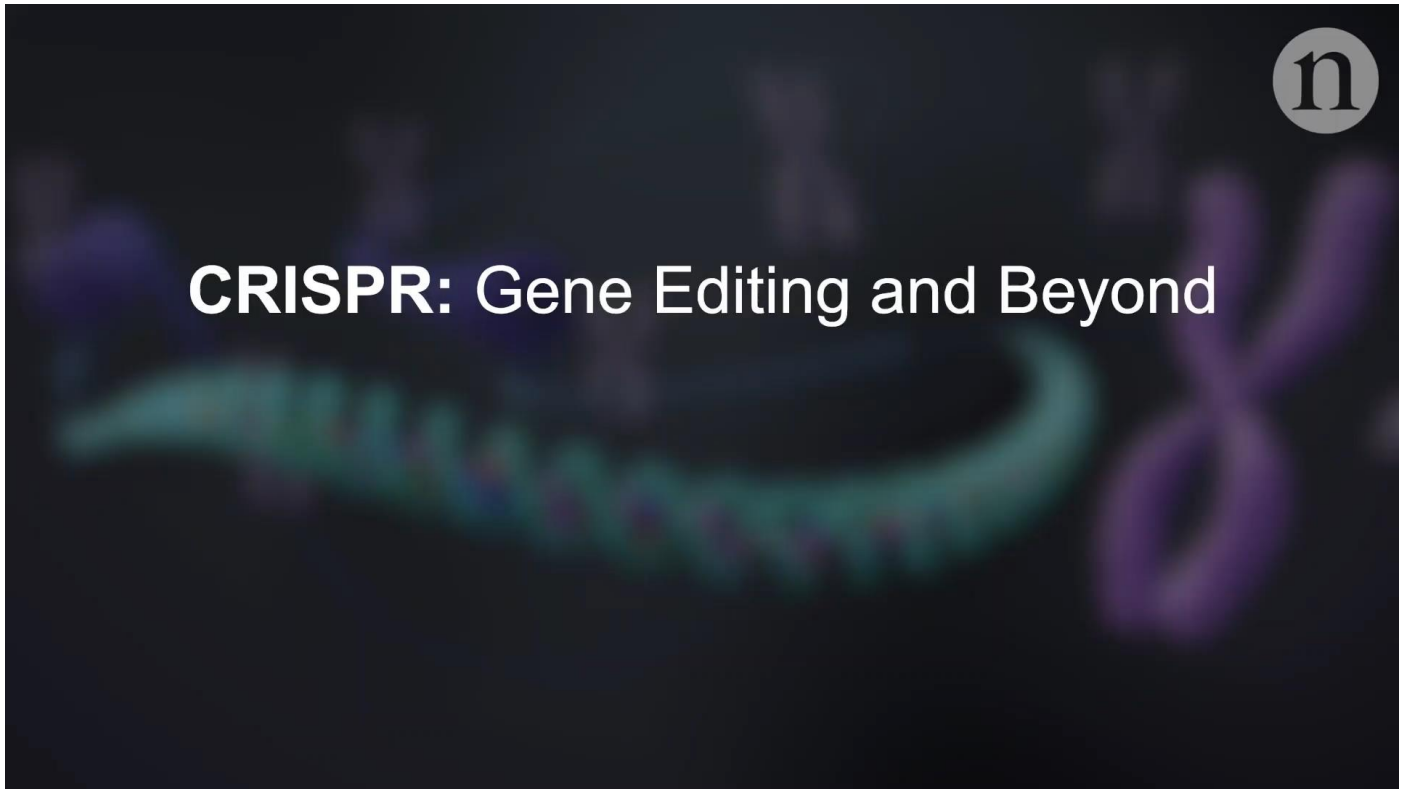
watch ME

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/

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

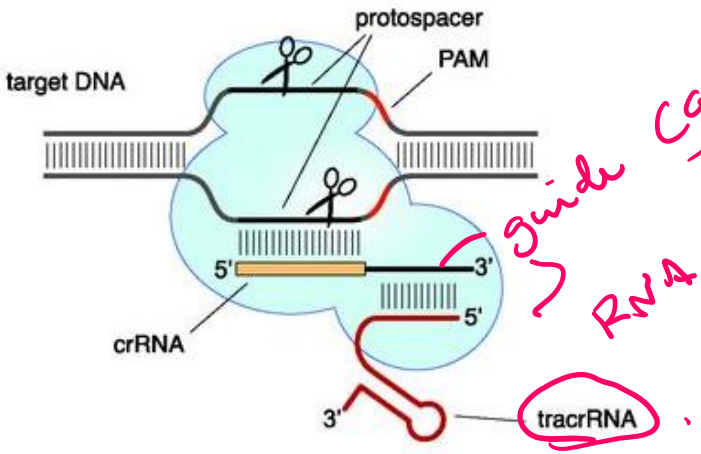
Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]†
Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

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.

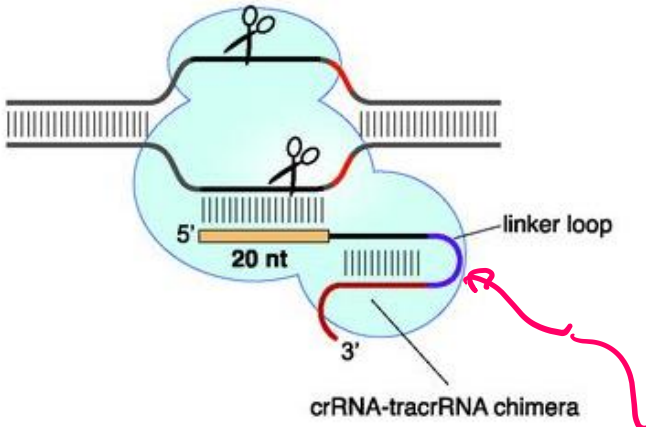
17 AUGUST 2012 VOL 337 **SCIENCE** www.sciencemag.org

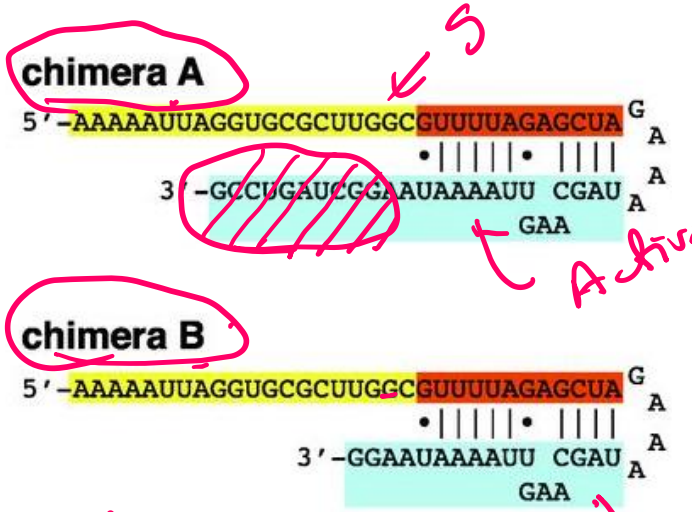
Making a Single gRNA = crRNA + Cas9 activation RNA

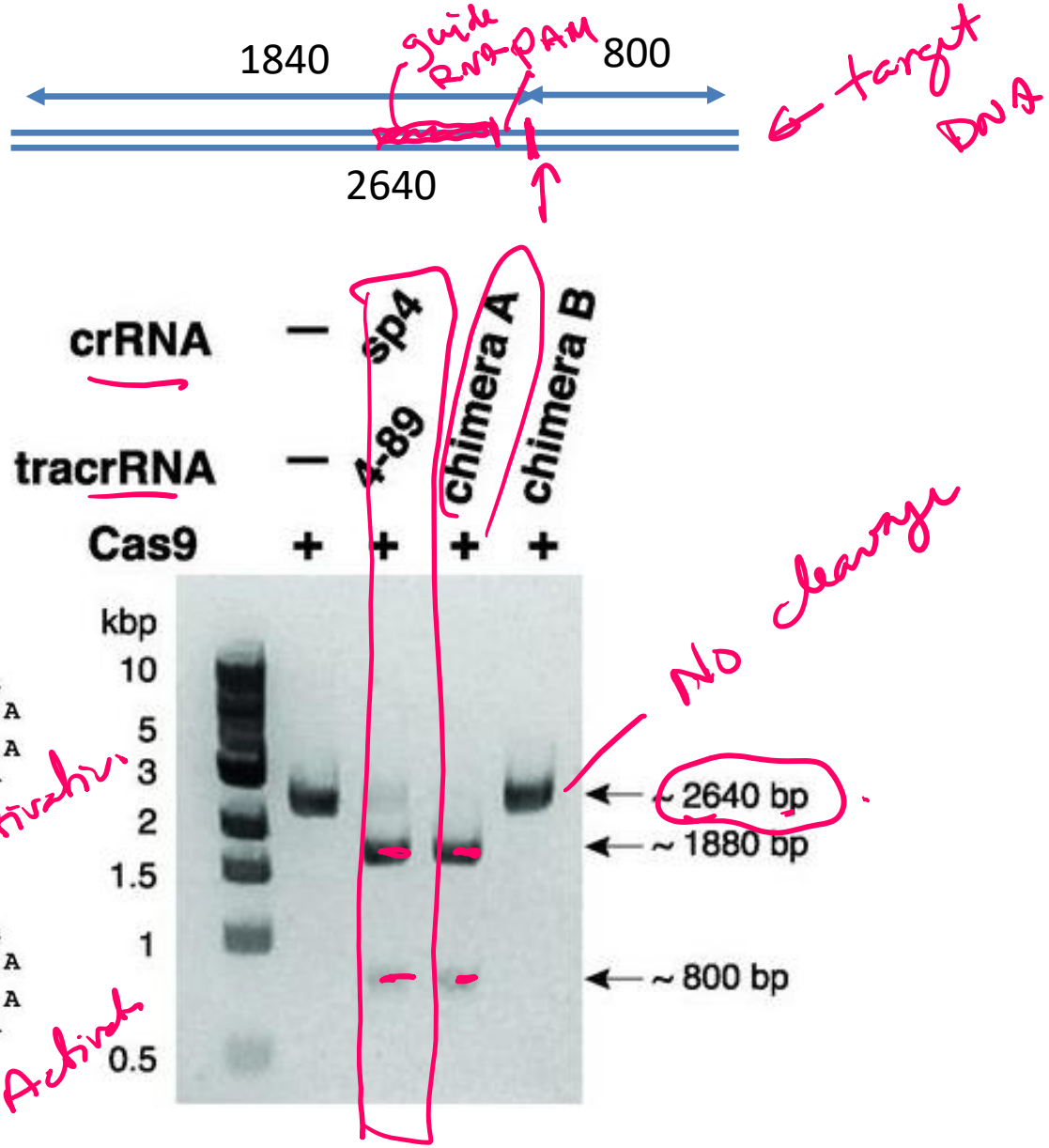
Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA

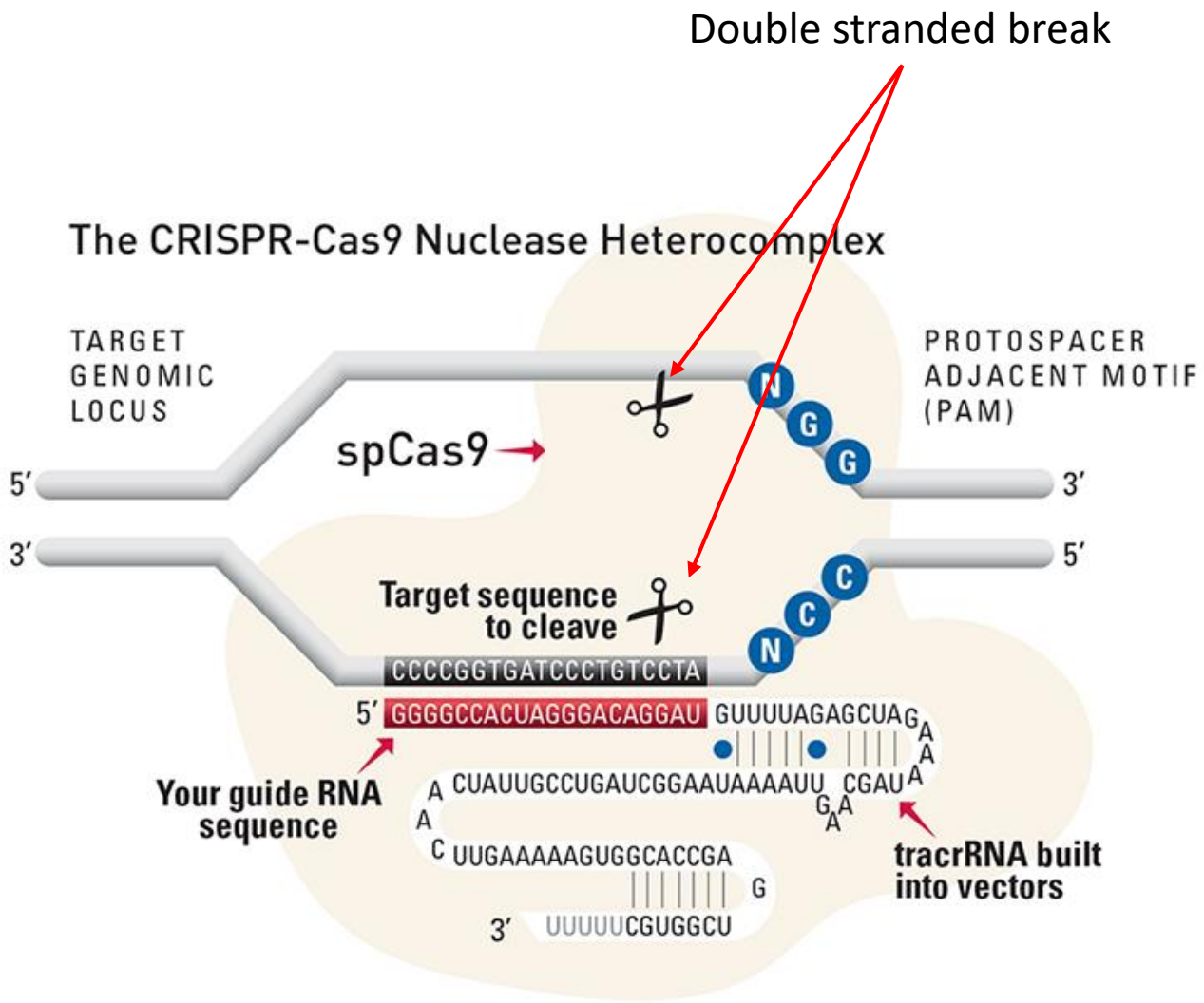






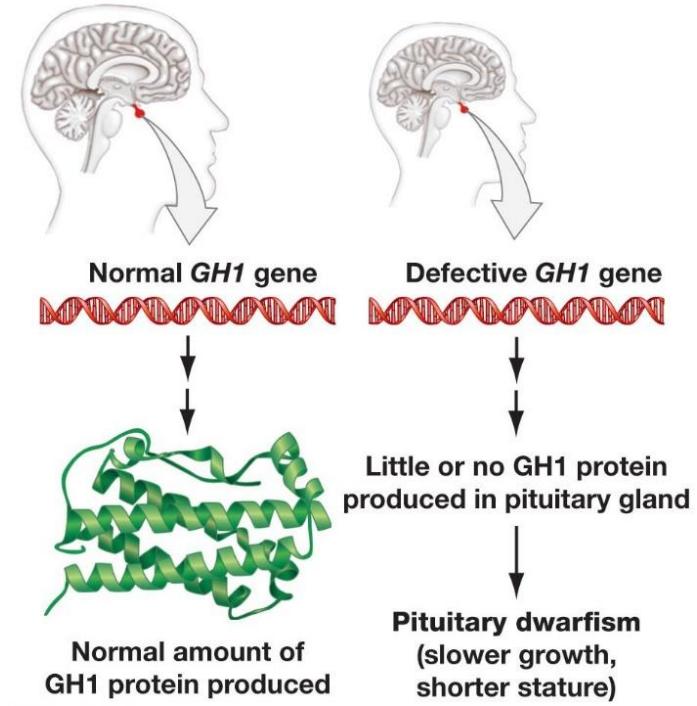
Cas9 – Guide RNA

1. After PAM recognition, guide RNA unwinds DNA, 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

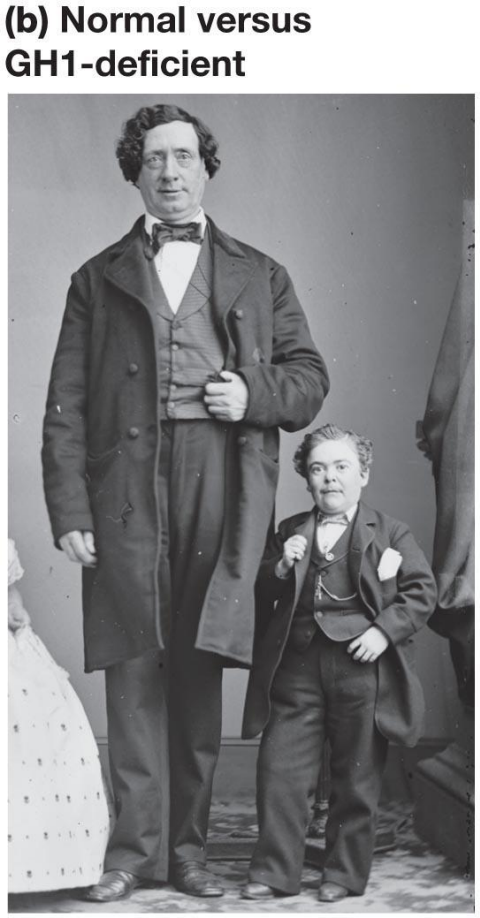


Using CRISPR-Cas9 to Correct Genetic Diseases
Human growth hormone (hGH)

Pituitary Dwarfism



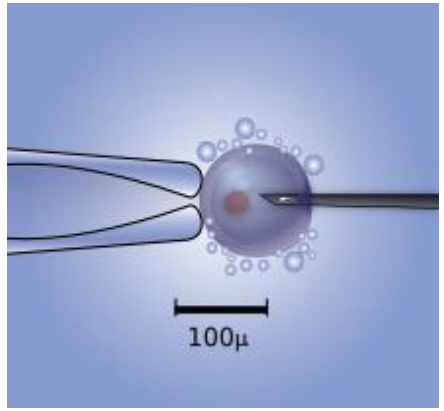
Between one in 14,000 and one in 27,000 babies born each year have some form of dwarfism.



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
TGAATGAATGAGAAAGGGAGGGAACAGTACCCAAGCCCTTGGCCTCTCCTTCTCTTCCTTCACTTTGCAG 1340
AGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAAACT 1410
CACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGT 1480
CGAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCTAGCTGCCCGGGTGG 1550
CATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTG 1620
TCCTAATAAAATTAAGTTGCATCATT
```

Location of mutation

Isoleucine (I) to Asparagine (N)

Possible PAM Sites

Wild type (normal)

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

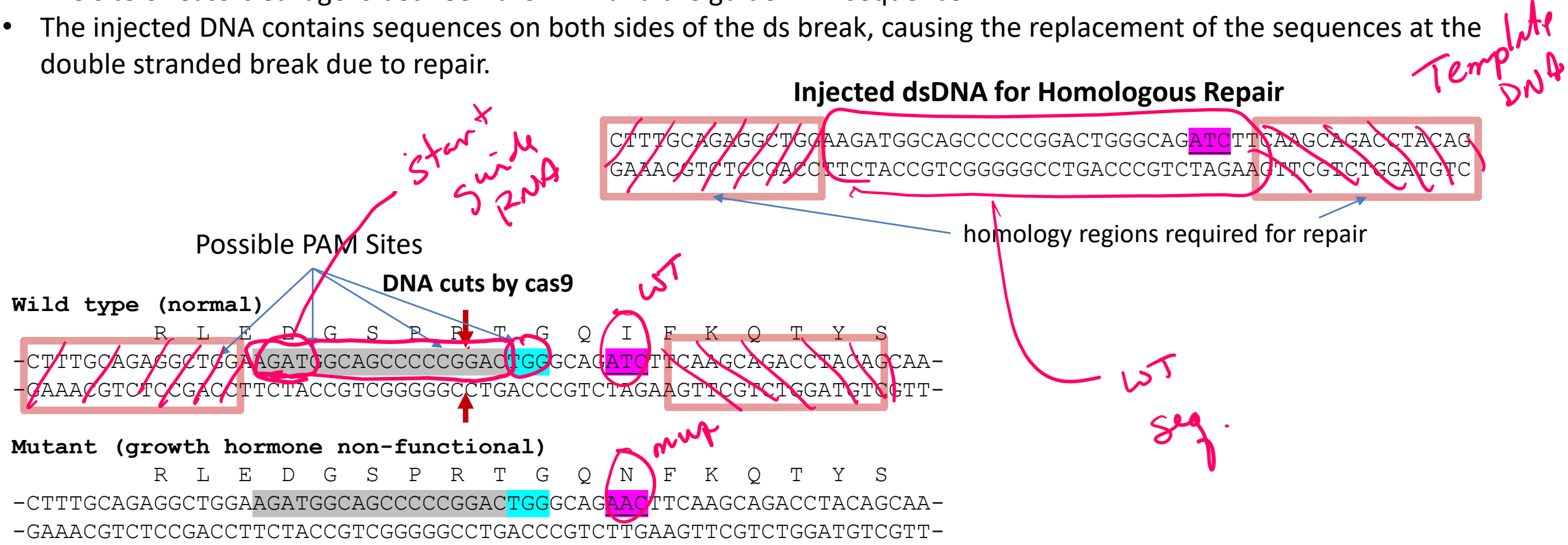
Mutant (growth hormone non-functional)

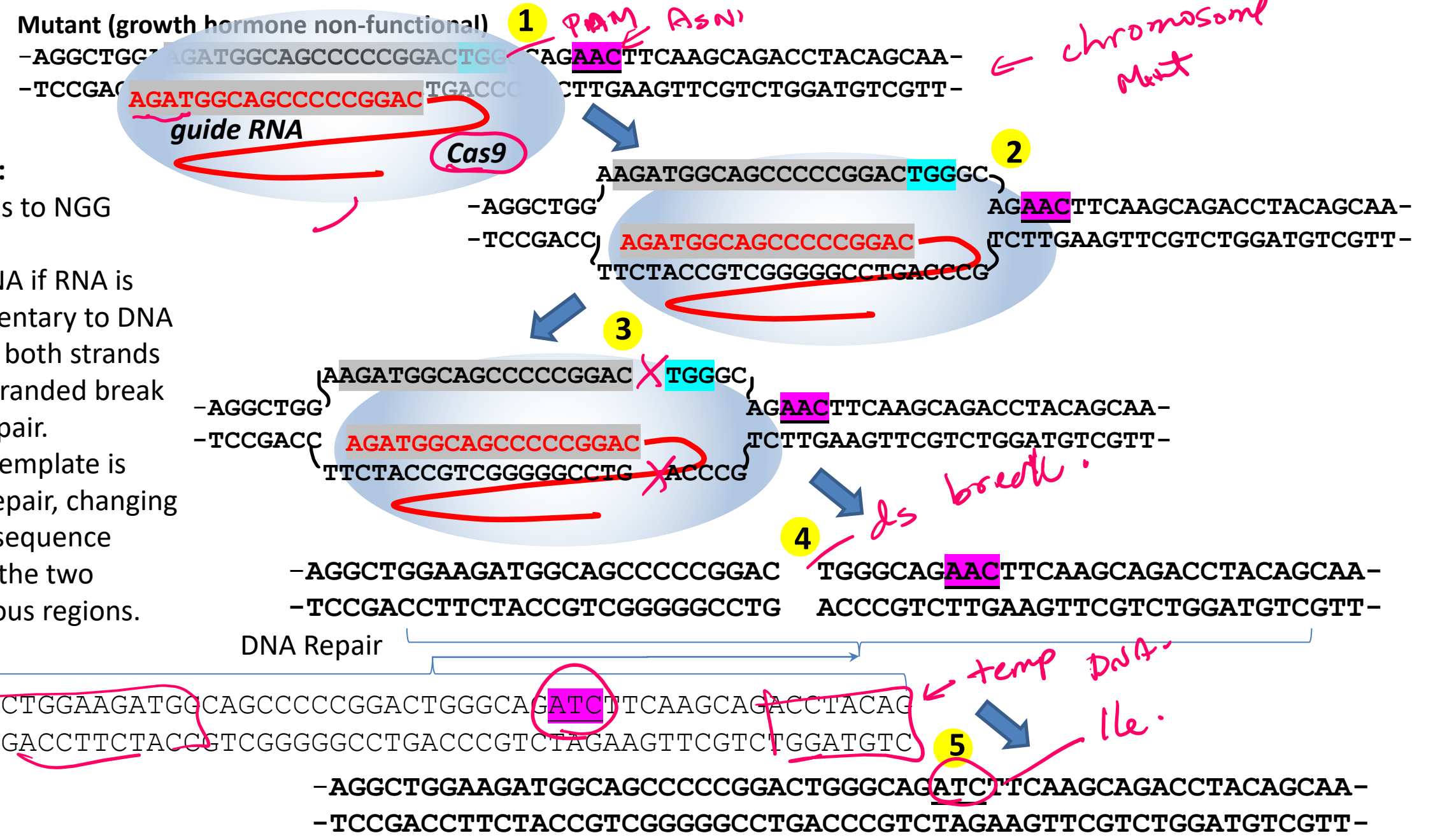
```
          R   L   E   D   G   S   P   R   T   G   Q   N   F   K   Q   T   Y   S
-CTTTGCAGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGAACTTCAAGCAGACCTACAGCAA-
-GAAACGTCTCCGACCTTCTACCGTCGGGGGCCTGACCCGTCTTGAAGTTCGTCTGGATGTCGTT-
```

- The cut site needs to be close to site of mutation so that the injected dsDNA repair template is short.
- 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.





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.

GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases

Shengdar Q Tsai[1–3,5], Zongli Zheng[1–5], Nhu T Nguyen[1,2], Matthew Liebers[1,2], Ved V Topkar[1,2], Vishal Thapar[1,2], Nicolas Wyvekens[1,2], Cyd Khayter[1,2], A John Iafrate[1–3], Long P Le[1–3], Martin J Aryee[1–3] & J Keith Joung[1–3]

CRISPR RNA-guided nucleases (RGNs) are widely used genome-editing reagents, but methods to delineate their genome-wide, off-target cleavage activities have been lacking. Here we describe an approach for global detection of DNA double-stranded breaks (DSBs) introduced by RGNs and potentially other nucleases. This method, called genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-seq), relies on capture of double-stranded oligodeoxynucleotides into DSBs. Application of GUIDE-seq to 13 RGNs in two human cell lines revealed wide variability in RGN off-target activities and unappreciated characteristics of off-target sequences. The majority of identified sites were not detected by existing computational methods or chromatin immunoprecipitation sequencing (ChIP-seq). GUIDE-seq also identified RGN-independent genomic breakpoint 'hotspots'. Finally, GUIDE-seq revealed that truncated guide RNAs exhibit substantially reduced RGN-induced, off-target DSBs. Our experiments define the most rigorous framework for genome-wide identification of RGN off-target effects to date and provide a method for evaluating the safety of these nucleases before clinical use.

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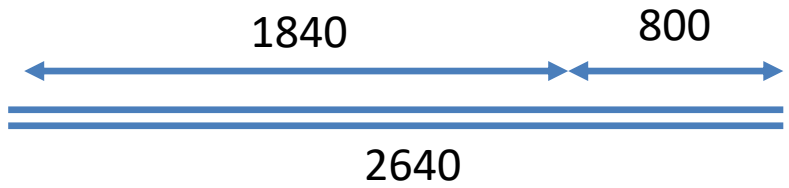
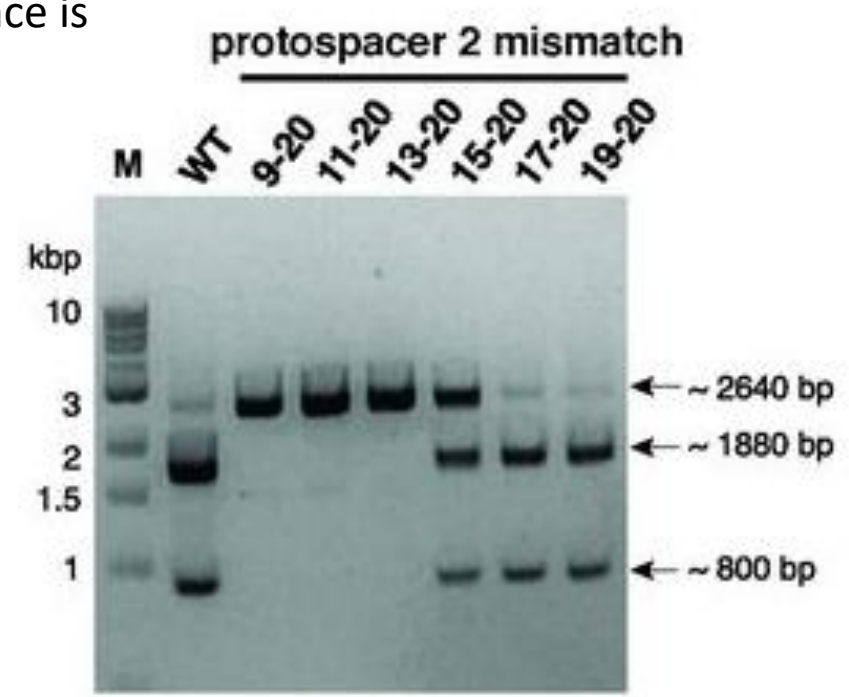
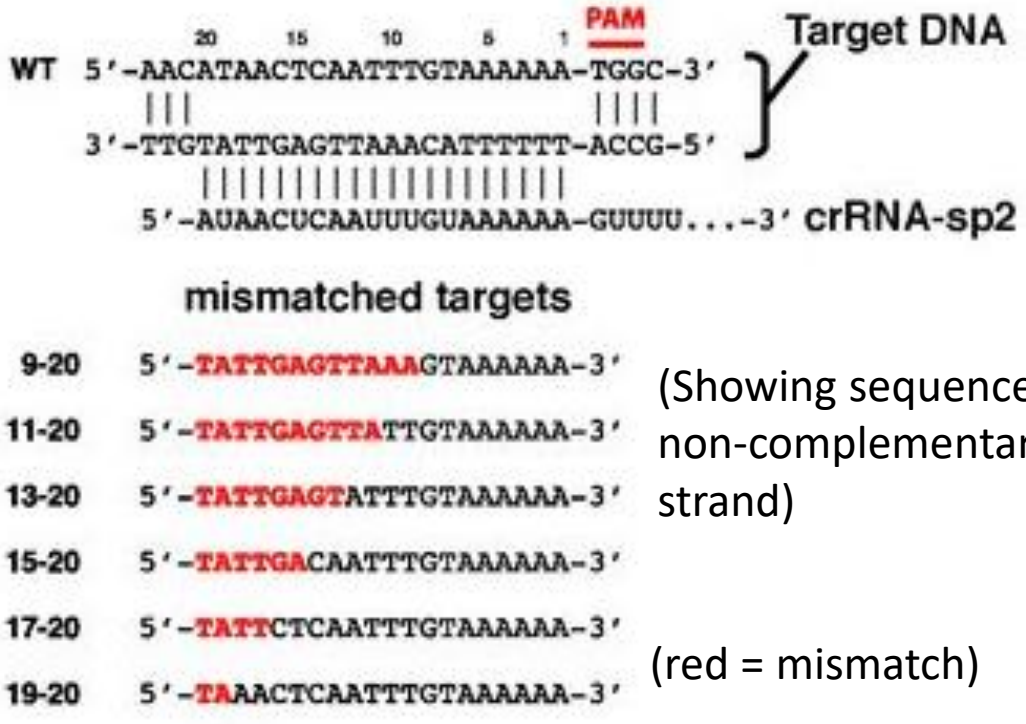
Determining Target Requirements



Wild-type sequence is cut





(Showing sequence of non-complementary strand)

(red = mismatch)

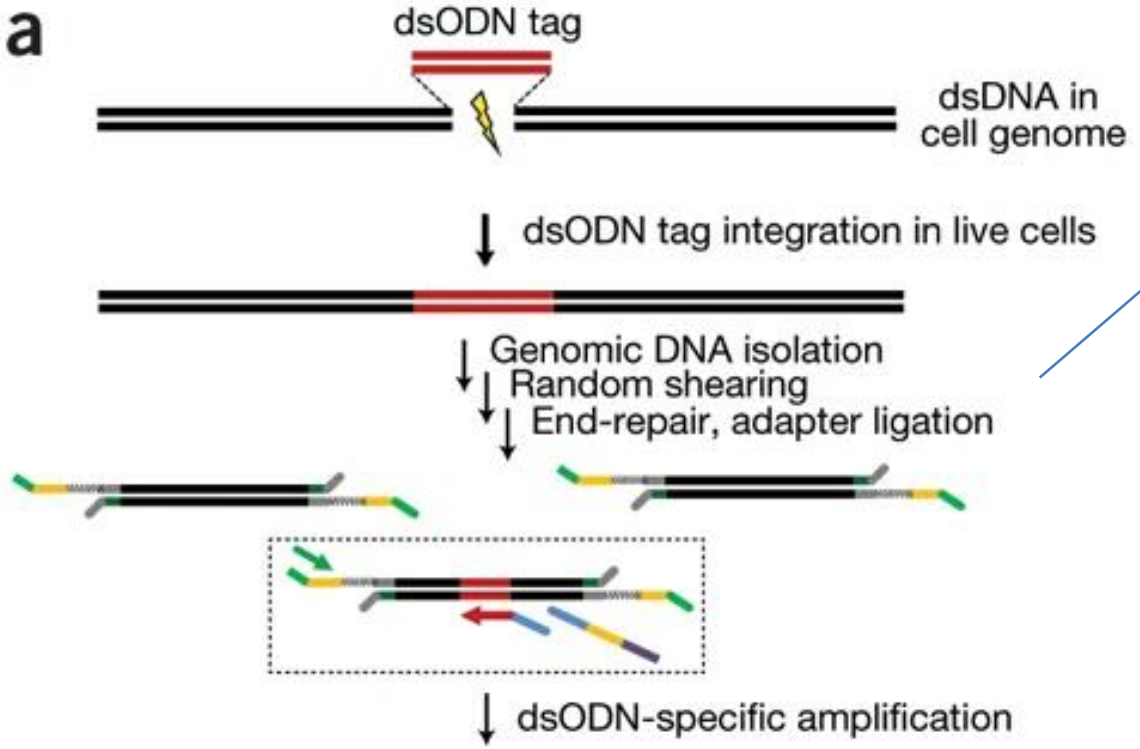
1. *Which of these DNA templates are cut by Cas9?*

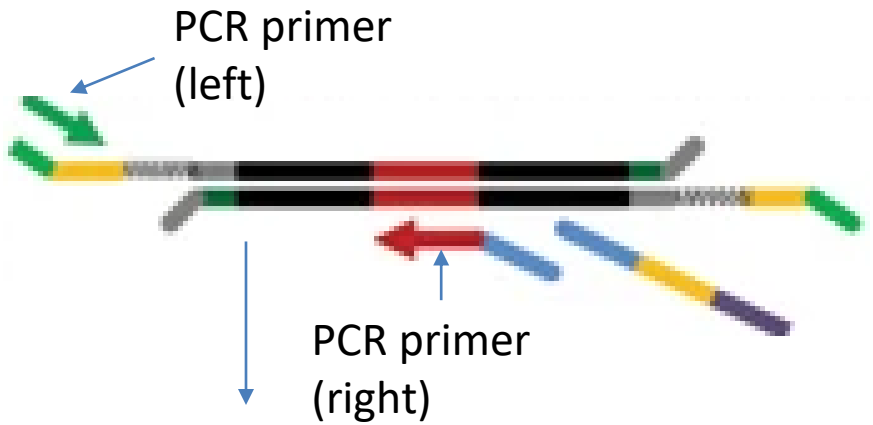
2. Where are mismatches (red bases) tolerated?

- *Close to PAM?*
- *Distal from PAM?*

Locating Off-Target Cutting by Cas9

1. dsODN tag is inserted after break by Cas9
2. DNA is fragmented
3. Synthetic oligonucleotide DNA sequences are added to ends as sites for PCR primers
4. PCR amplification:
   - Left primer – primes at the end using the added oligonucleotides
   - Right primer – is contained in dsODN sequence
5. PCR products are sequenced – part of the sequence contains the DNA sequence at the insertion site (black)

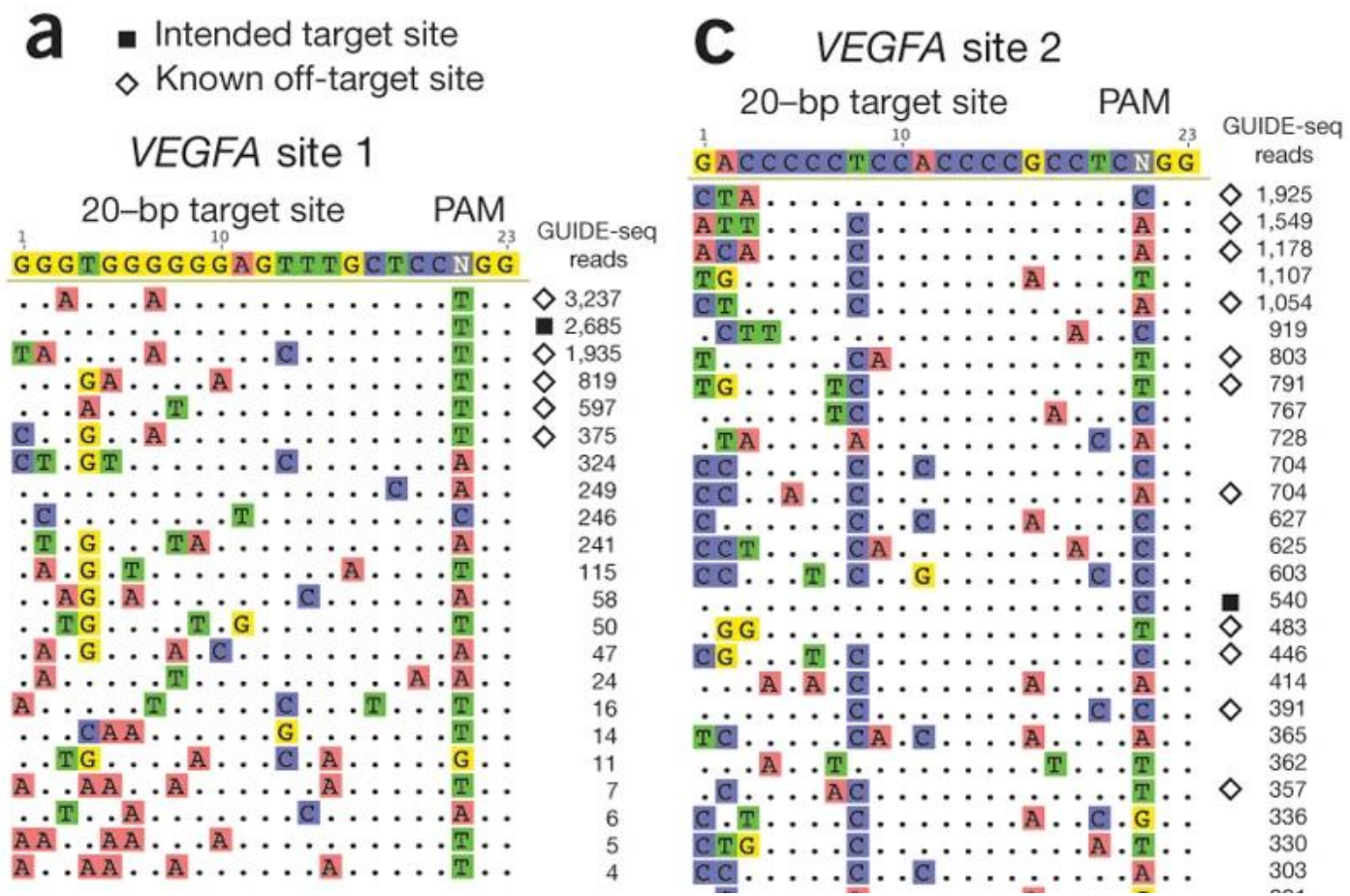




Sequencing of PCR Product

AAGCGTAGCGTGACGTAGTAGCACTGCATG

- Each PCR template represents an insertion point.
- Number of insertions at the same site will:
  - Increase number of PCR templates with that sequence
  - Increase the number of times that sequence is obtained using high-throughput sequencing



- **Take Home Message:**
- Cas9 edits many sites
- Off target sites can be edited more frequently than the target site
- Mismatches distal from the PAM site are more likely to be off-target