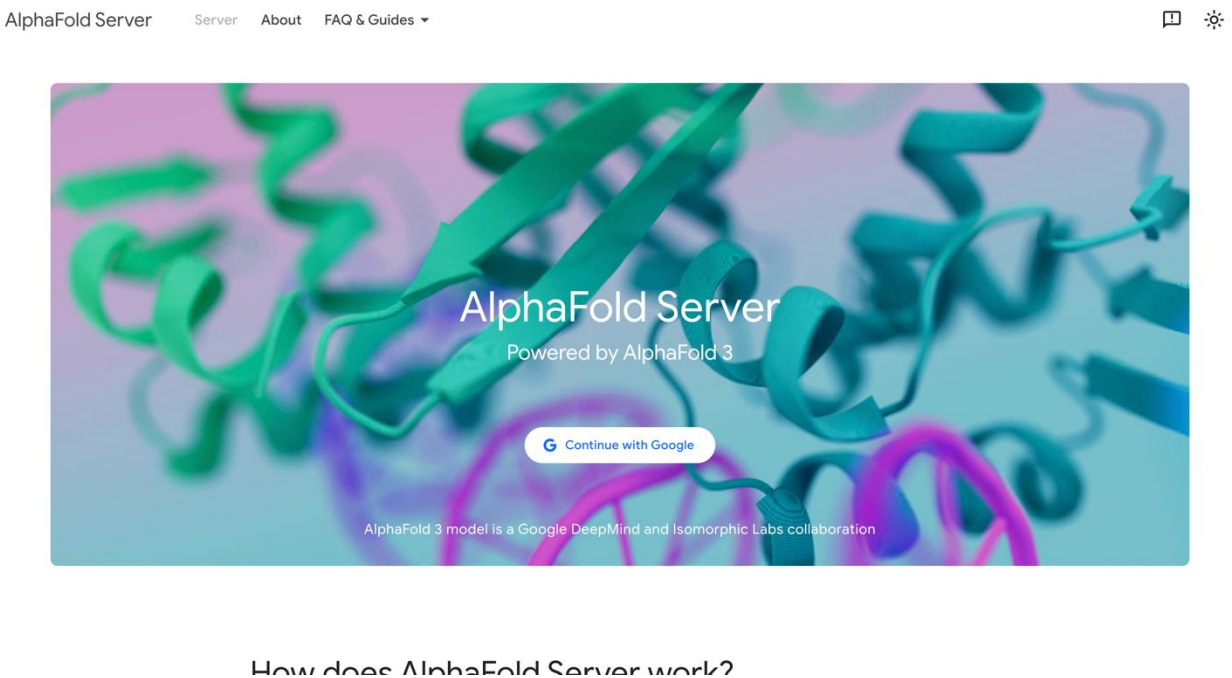
ncAA expanding the roles of binding protein

Literature seminar
2025.11.13
B4 Kaito UEDA

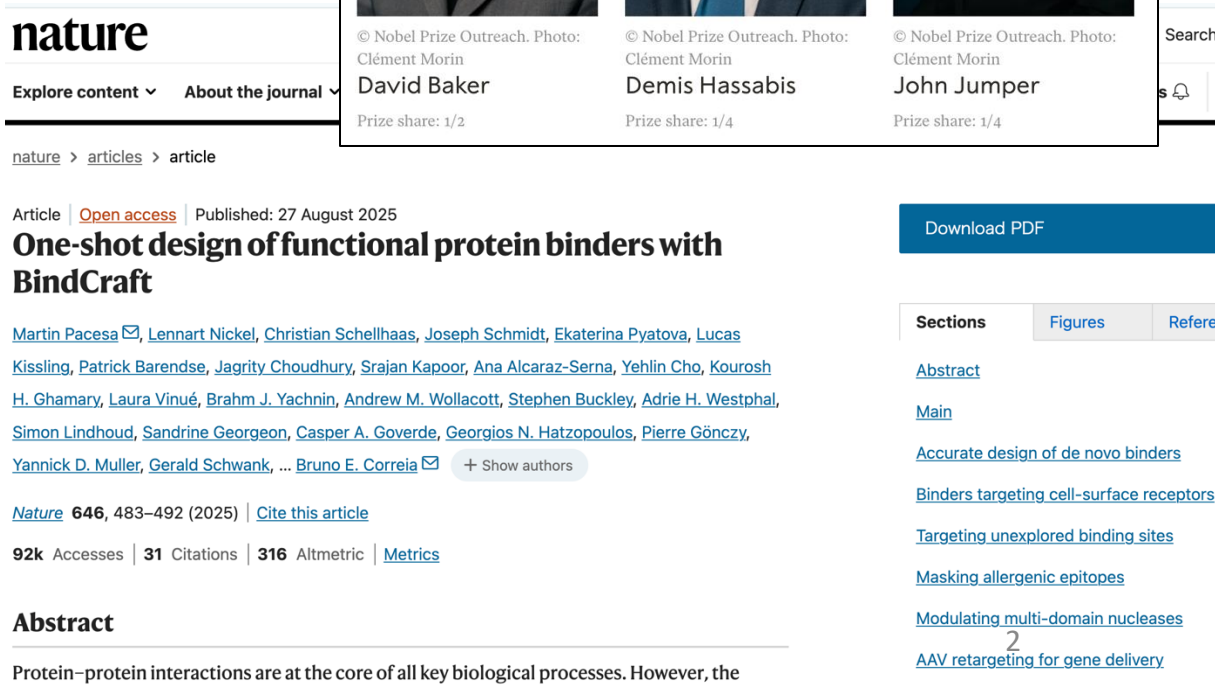
Today's contents

Protein engineering is widely researched area

Protein engineering is intensely researched now.



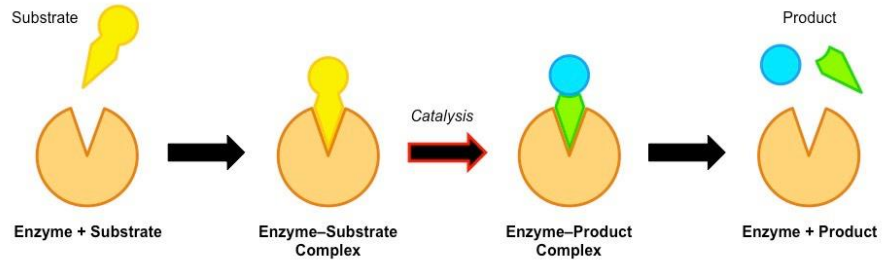
How does AlphaFold Server work?



Three key advantages of protein

Catalyst

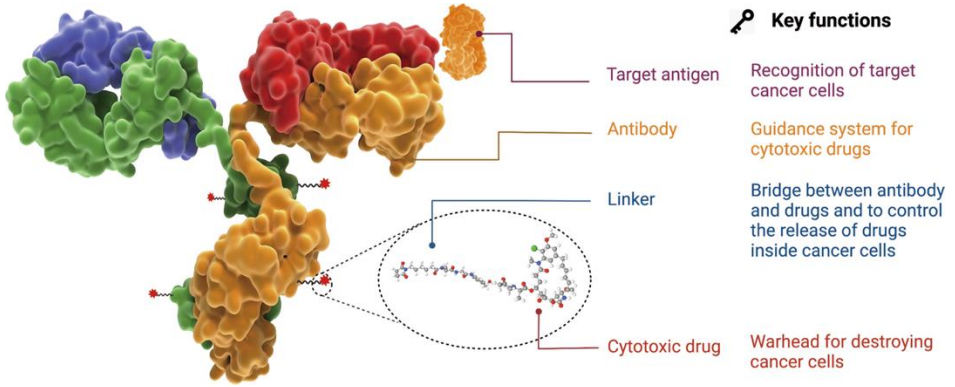
Enzymes catalyze natural chemical reactions with near-perfect efficiency and asymmetric selectivity under mild conditions, such as at room temperature and in water.



https://old-ib.bioninja.com.au/standard-level/topic-2-molecular-biology/25-enzymes/enzyme-catalysis.html

Substrate recognition

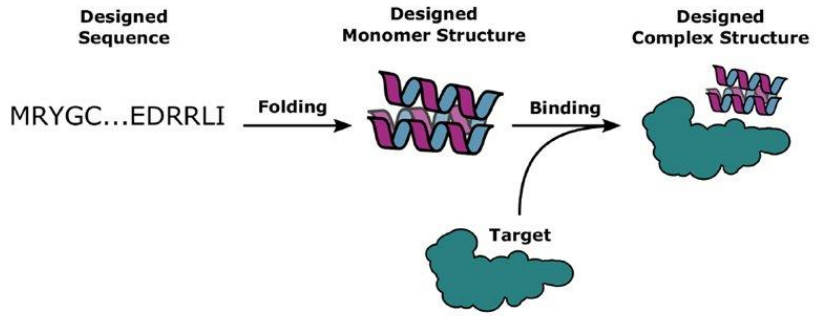
As exemplified by antibodies and receptors, it has an extremely high ability to identify specific molecules with pinpoint accuracy.



Zhang, L.; Sun, L. Sci. Rep. **2024**, *14*, 22357.

Programmability

Their function is determined by the sequence (a digital code) of just 20 amino acids. This makes it highly compatible with design and prediction using machine learning (ML).



https://tacc.utexas.edu/news/latest-news/2023/08/03/deep-learning-for-new-protein-design/

Three key advantages of protein

Catalyst

Enzymes catalyze natural chemical reactions with near-perfect efficiency and asymmetric selectivity under mild conditions, such as at room temperature and in water.

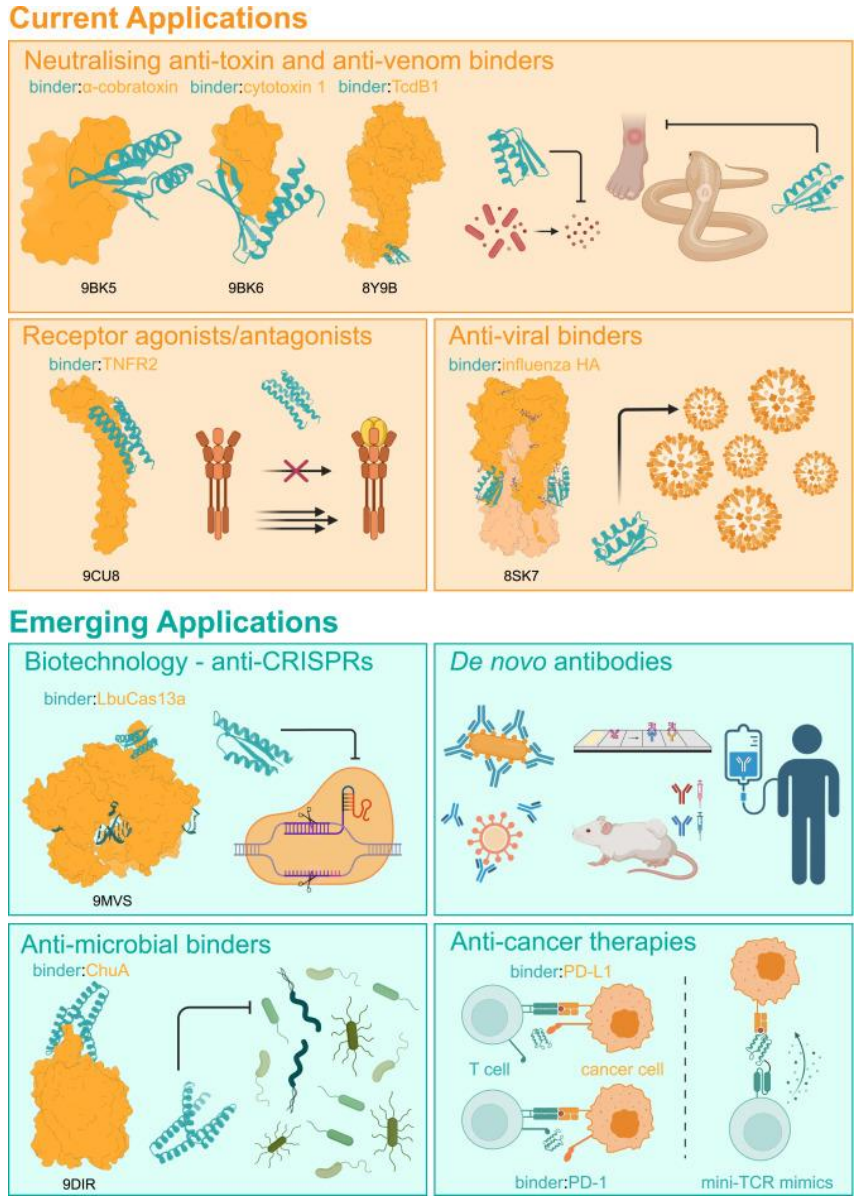
Substrate recognition

As exemplified by antibodies and receptors, it has an extremely high ability to identify specific molecules with pinpoint accuracy.

Programmability

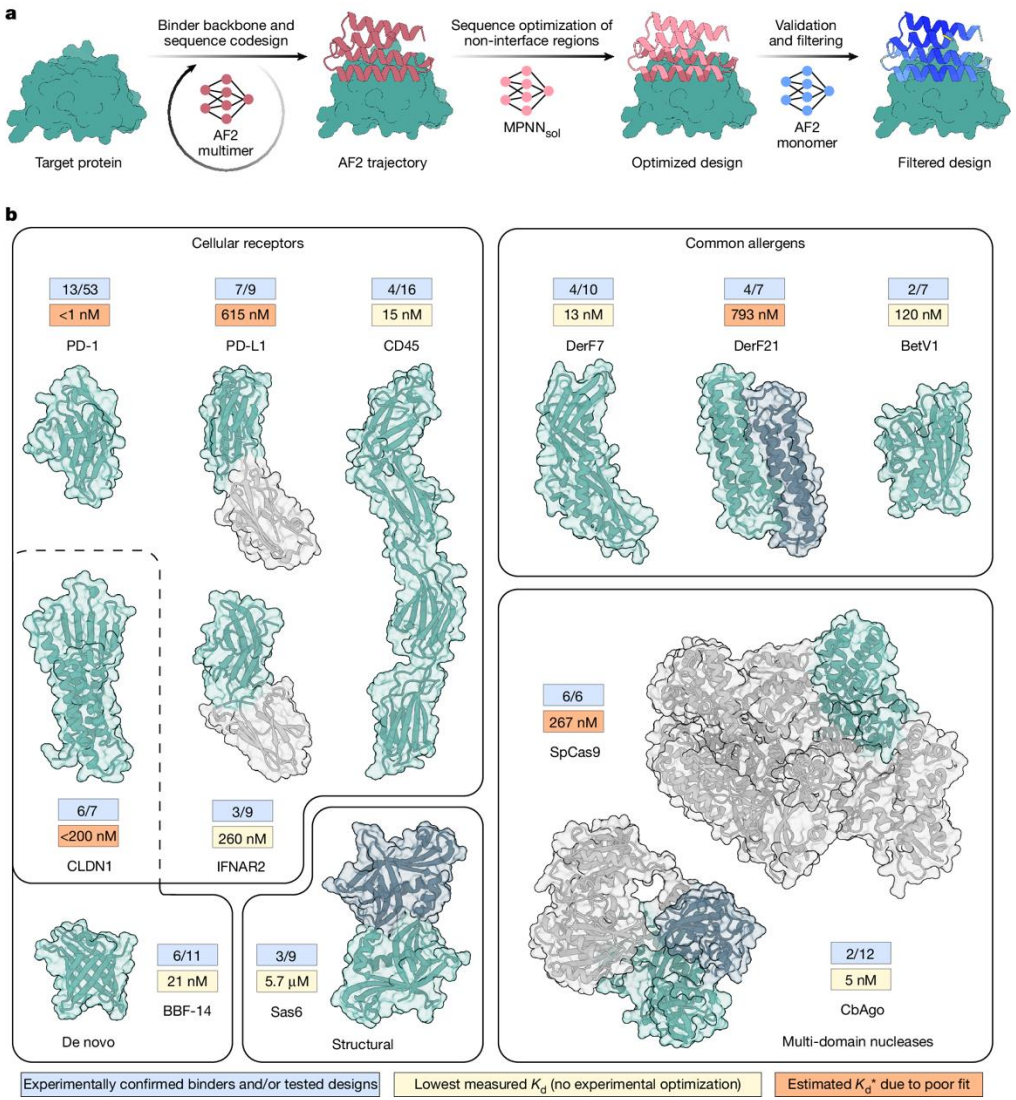
Their function is determined by the sequence (a digital code) of just 20 amino acids. This makes it highly compatible with design and prediction using machine learning (ML).

Substrate recognition and **programmability** are crucial features of protein for biology.(e.g., binders)

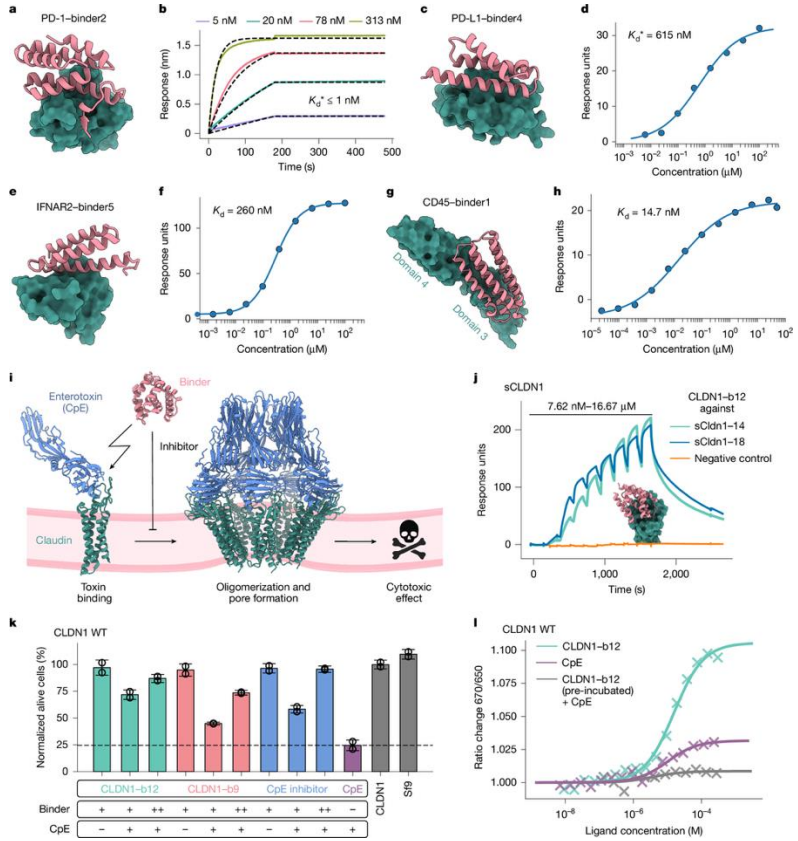


Fox, D. R.; Knott, G. J. *Structure* **2025**, *33*, 1631–1642.1

PPIs (Protein-protein interaction) design is intensely studied



A study showed machine learning can design binders of nM affinity with Alpha Fold 2, RF diffusion and MPNN.



Pacesa, M, Bruno E. Correia. *Nature* **2025**, *646*, 483–492.

Designing de novo enzymes is much more difficult

Catalyst

Enzymes catalyze natural chemical reactions with near-perfect efficiency and asymmetric selectivity under mild conditions, such as at room temperature and in water.

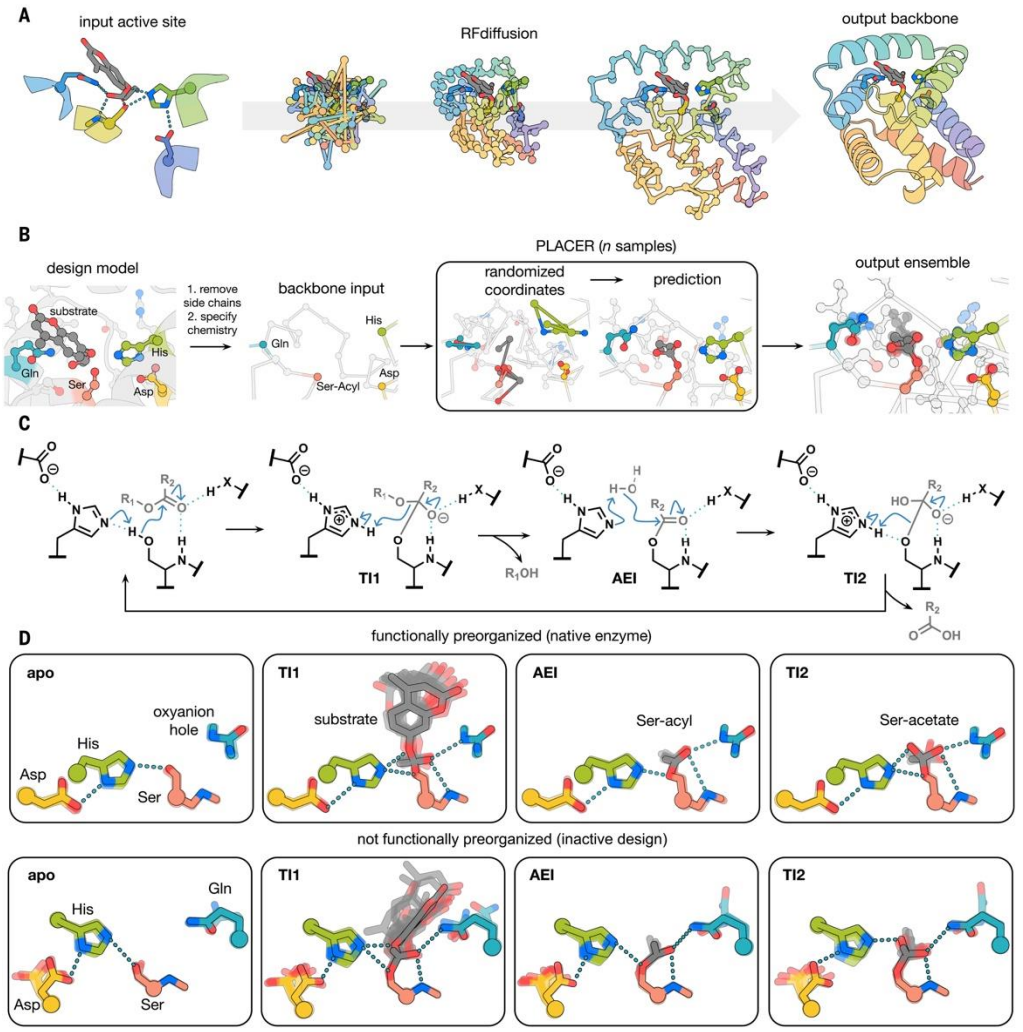
Substrate recognition

As exemplified by antibodies and receptors, it has an extremely high ability to identify specific molecules with pinpoint accuracy.

Programmability

Their function is determined by the sequence (a digital code) of just 20 amino acids. This makes it highly compatible with design and prediction using machine learning (ML).

Designing de novo protein is limited to simple reactions.(e.g., hydrolase, Diels-Alder)



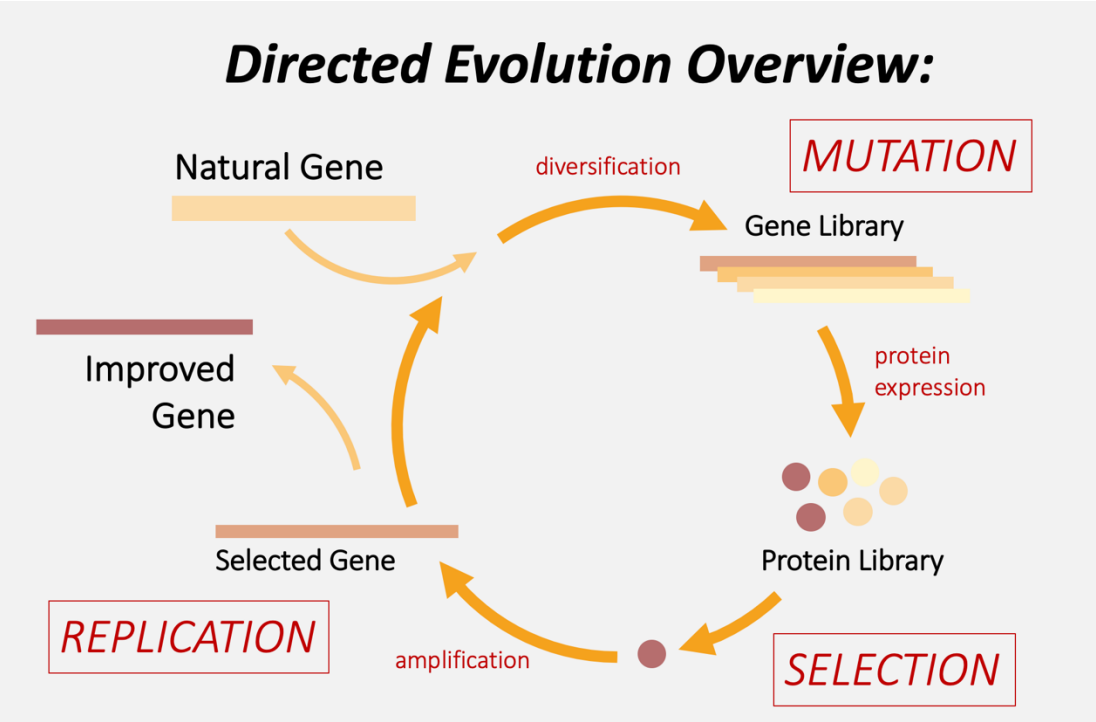
Lauko, A.; Baker, D. *Science* **2025**, *388*, eadu2454.

Directed evolution is a powerful tool for developing enzymatic catalysts

Directed evolution

Directed evolution is an experimental technique used to improve the function of molecules, such as proteins and nucleic acids, for a desired purpose by mimicking the model of natural selection.

If a reaction proceeds at all, the efficiency can be improved dramatically by directed evolution.



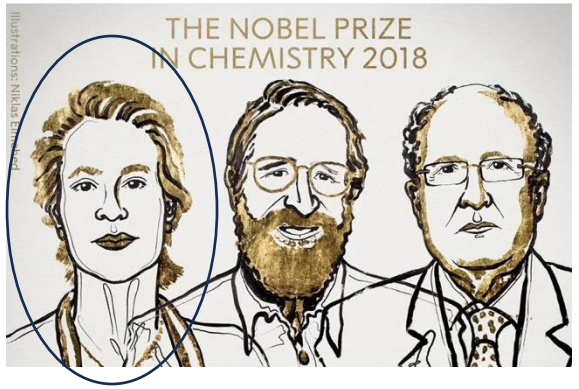
https://2019.igem.org/Team:Stanford/Description

Directed evolution is composed of 3steps.

1. MUTATION
A gene is diversified by introducing random mutations to create a library. Subsequently, the proteins are expressed.

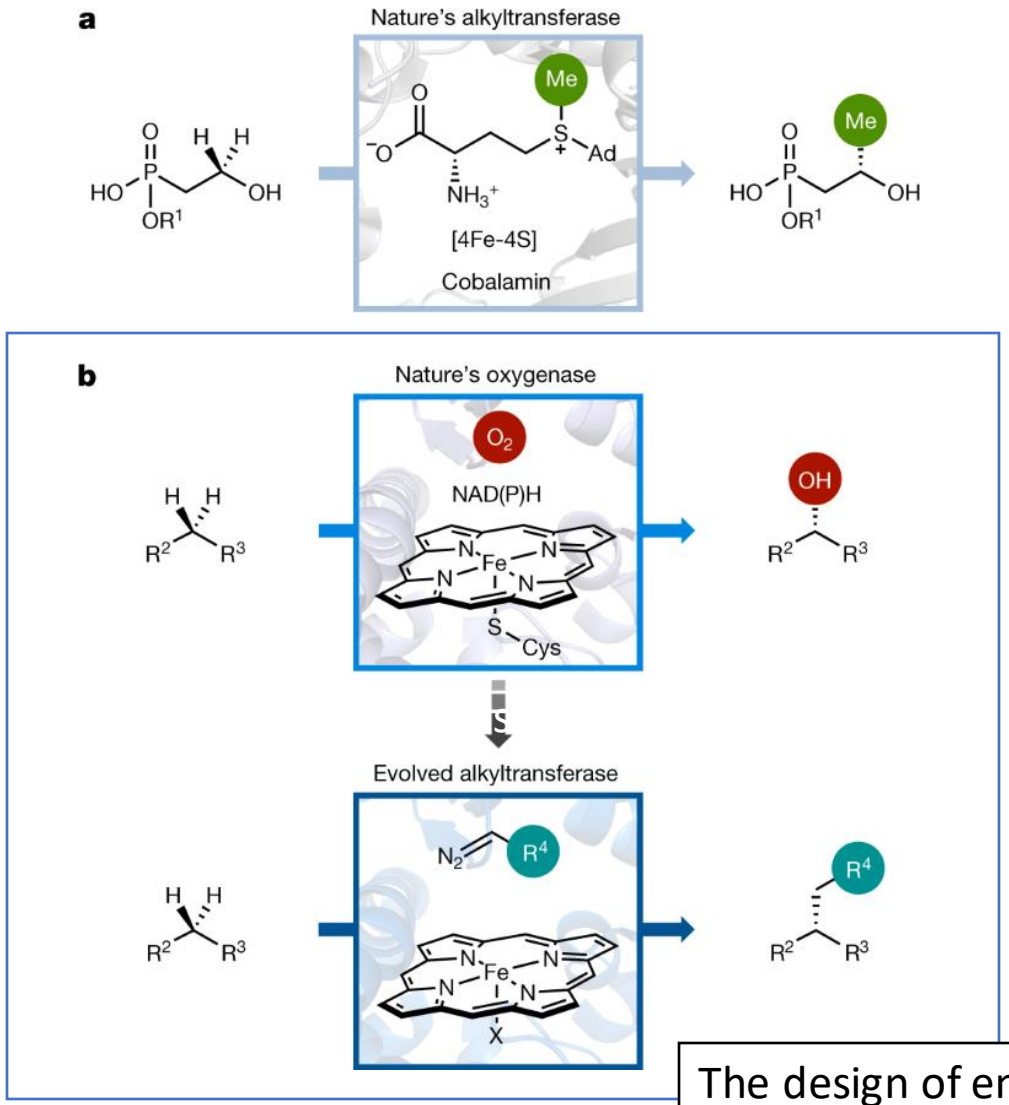
2. SELECTION
Select the individual best suited for the desired function.
↑ the most important step

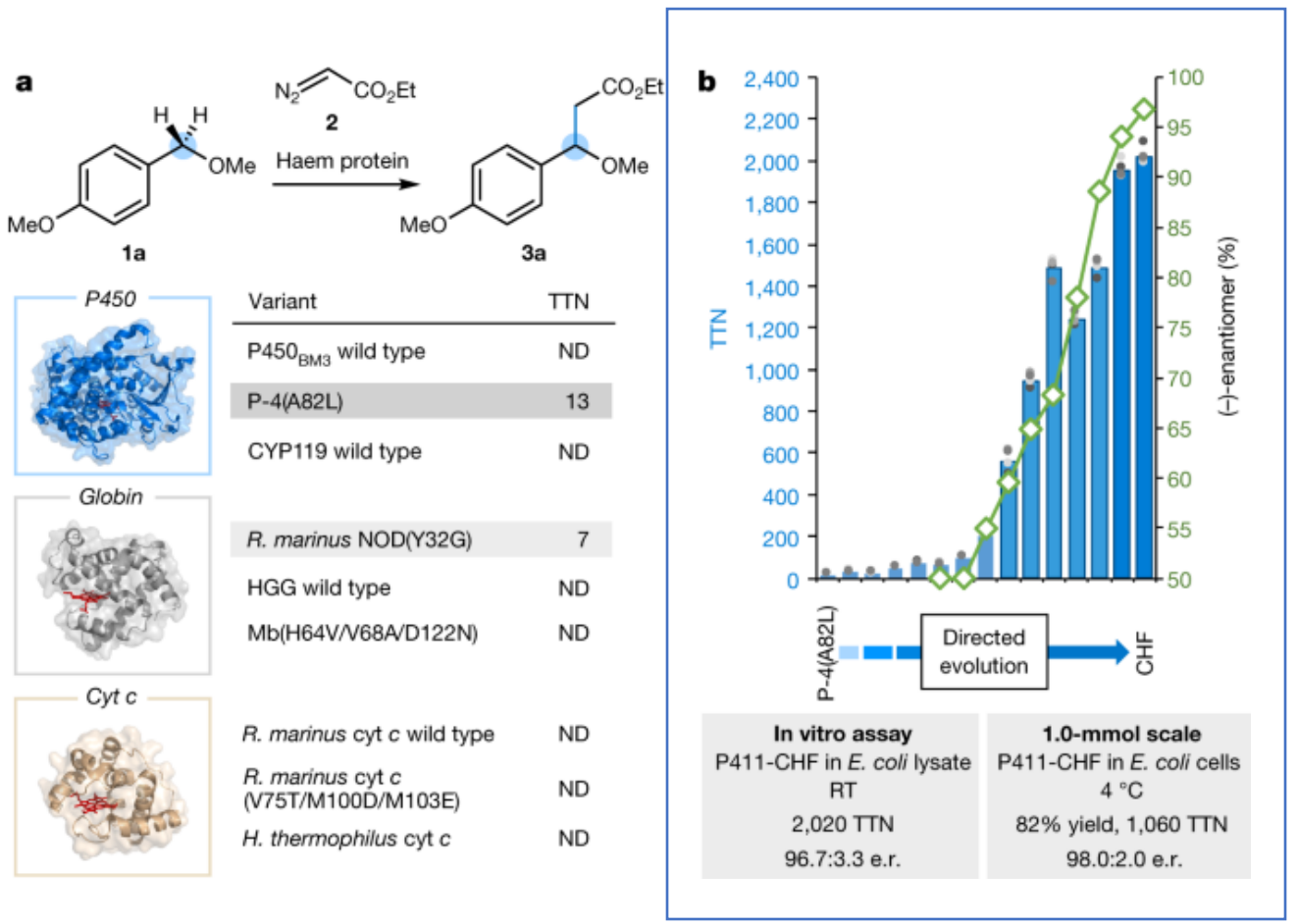
3. REPLICATION
The genetic information of the individual is replicated and used as a template for the next library.



https://www.smithsonianmag.com/smart-news/three-evolutionary-scientists-share-years-nobel-prize-chemistry-180970453/

Directed evolution is a powerful tool for developing enzymatic catalysts





Variant	TTN
$P450_{BM3}$ wild type	ND
P-4(A82L)	13
CYP119 wild type	ND
R. marinus NOD(Y32G)	7
HGG wild type	ND
Mb(H64V/V68A/D122N)	ND
R. marinus cyt *c* wild type	ND
R. marinus cyt *c* (V75T/M100D/M103E)	ND
H. thermophilus cyt *c*	ND

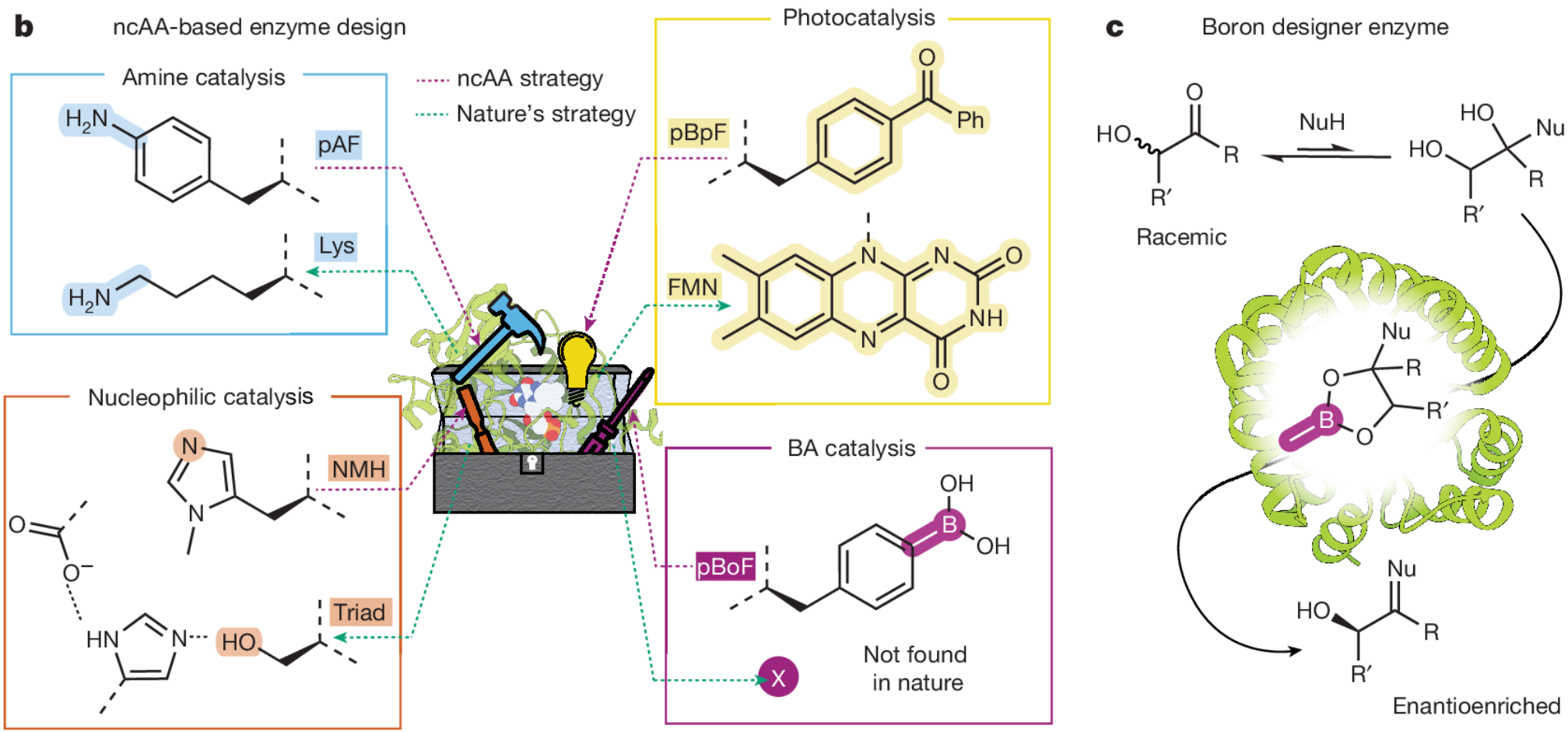
In vitro assay	**1.0-mmol scale**
P411-CHF in *E. coli* lysate	P411-CHF in *E. coli* cells
RT	4 °C
2,020 TTN	82% yield, 1,060 TTN
96.7:3.3 e.r.	98.0:2.0 e.r.

The design of enzymes are limited to basing on natural reactions.

Directed evolution significantly improved the efficiency of the enzyme.

Zhang, R. K.; Arnold, F. H. *Nature* **2019**, *565*, 67–72.

ncAA (non-canonical amino acids) may solve the problem



Longwitz, L.; Roelfes, G. *Nature* **2024**, *629*, 824–829.

A study by Roelfes showed incorporation of boron into enzymes as ncAA can achieve new-to-nature reactions.

ncAA expanded the scope of artificial enzyme.

Combination of binders and ncAA may expand the function of binders

Catalyst

Enzymes catalyze natural chemical reactions with near-perfect efficiency and asymmetric selectivity under mild conditions, such as at room temperature and in water.

Programmability

Their function is determined by the sequence (a digital code) of just 20 amino acids. This makes it highly compatible with design and prediction using machine learning (ML).

These two elements are utilized by artificial enzymes.

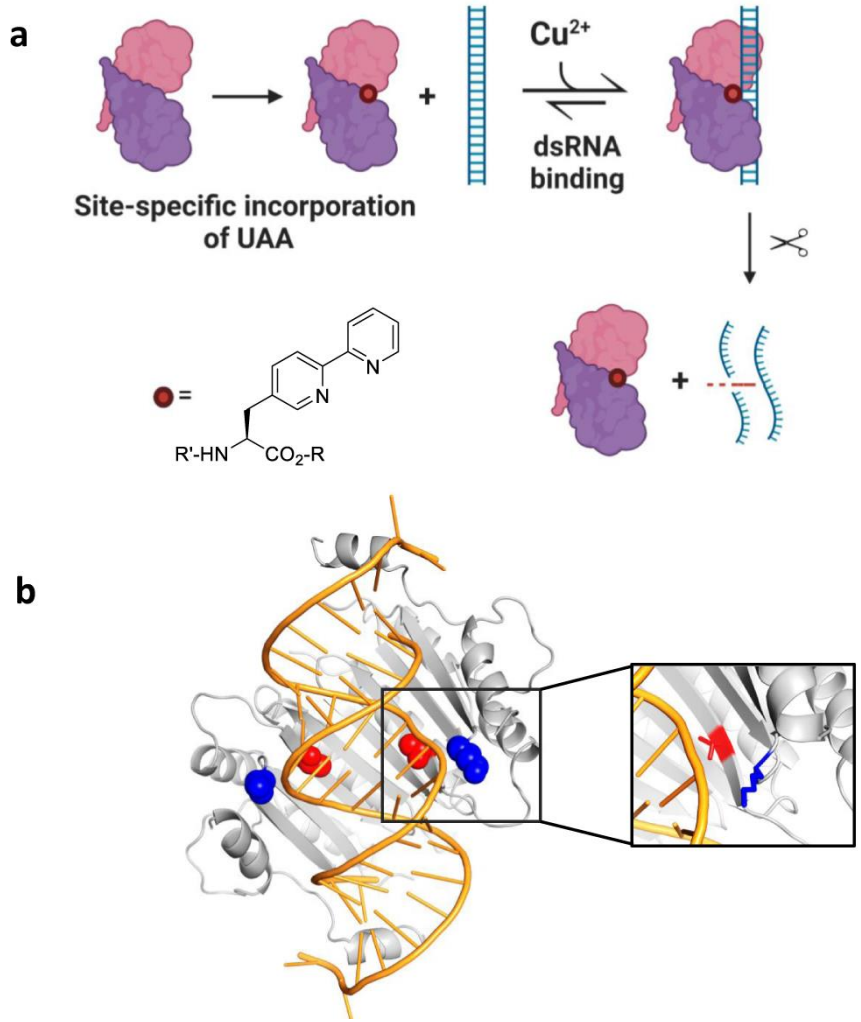
Substrate recognition

As exemplified by antibodies and receptors, it has an extremely high ability to identify specific molecules with pinpoint accuracy.

This feature is not fully utilized.

Incorporation of ncAA into binding proteins may expand the roles of them.

Today's contents

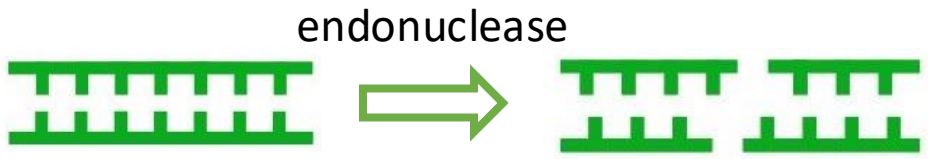


Ahmed, N.; Pezacki, J. P. *Nat. Commun.* **2023**, *14*, 3777.

Endonuclease and short non-coding RNA

Endonuclease

endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain (namely DNA or RNA).



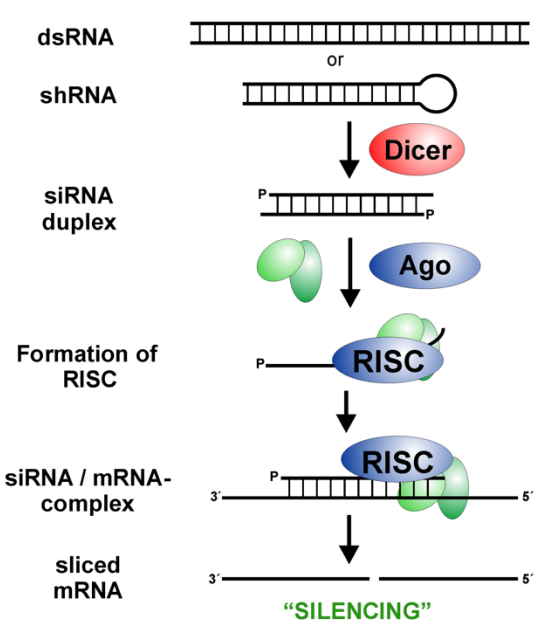
https://medlifemastery.com/mcat/biochemistry/biotechnology/restriction-enzymes/

short non-coding RNA(e.g., siRNA, miRNA)

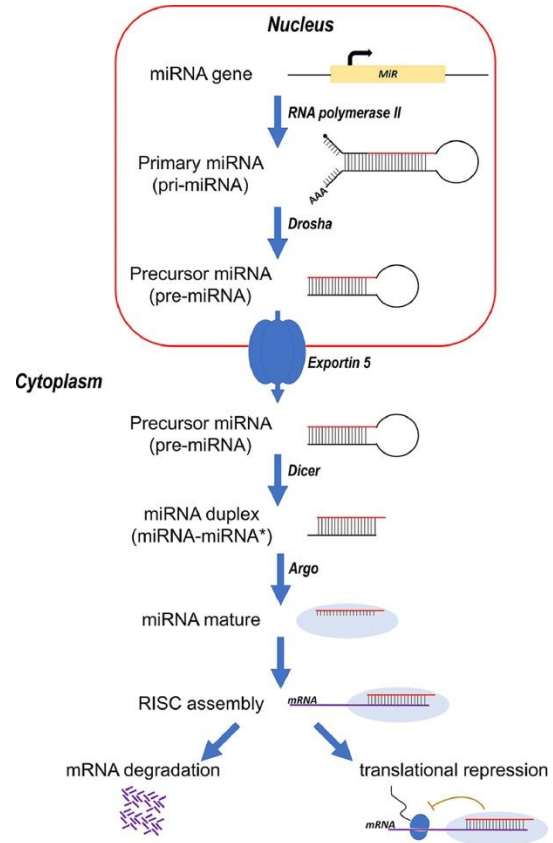
Short non-coding RNAs (ncRNAs) are functional RNA molecules of **20-30 nucleotides** in length. They plays roles in regulating various biological processes, such as development, differentiation, carcinogenesis, and viral defense. Especially, siRNA and miRNA are involved in suppression of genes and draw attention as a target of oligonucleotide therapeutics.

No naturally occurring endonuclease exists that catalyzes the specific degradation of either siRNAs or miRNAs.

In this study, they developed an endonuclease which cleaves si/miRNA.



https://www.gene-quantification.de/si-rna.html



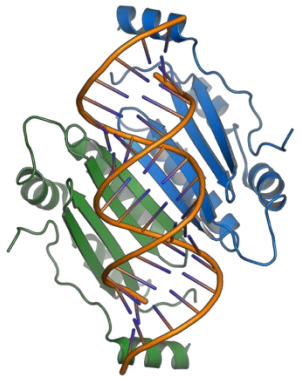
Venneri, M.; Passantino, A. *Eur. J. Intern. Med.* **2023**, *113*, 6–9.

In the previous study, cross-linking suppressor was developed

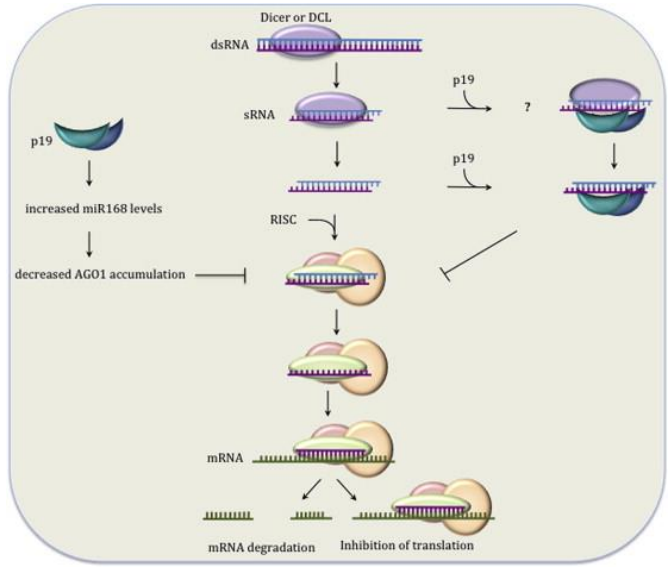
p19

P19 is a viral protein which binds to small non-coding **doble-stranded** RNAs its **size dependently** and suppress siRNA‘s activity.

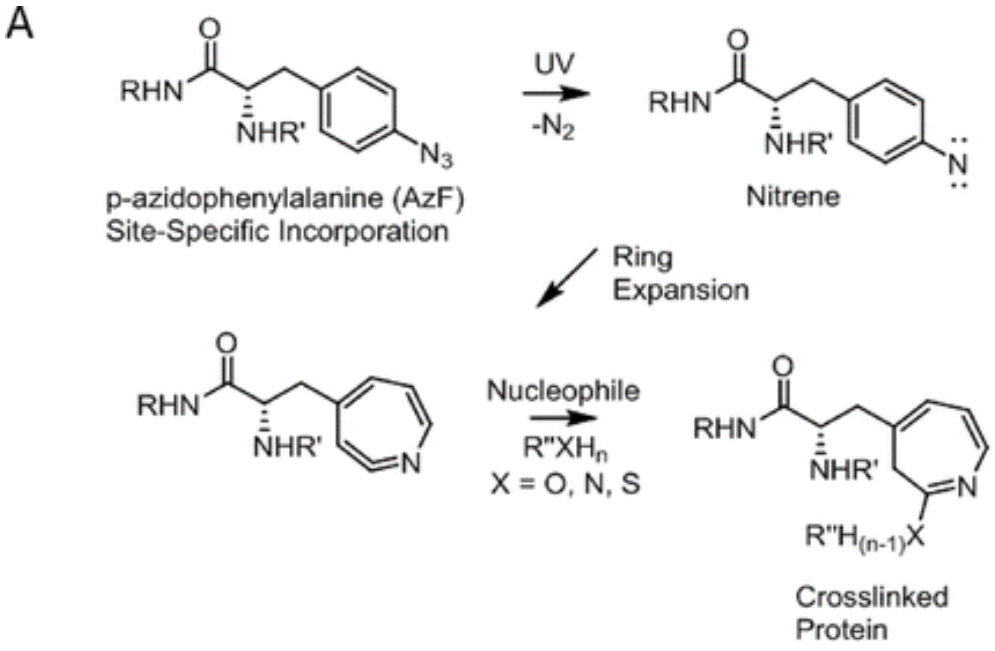
In the previous study, they developed a protein which binds to siRNA covalently by using ncAA

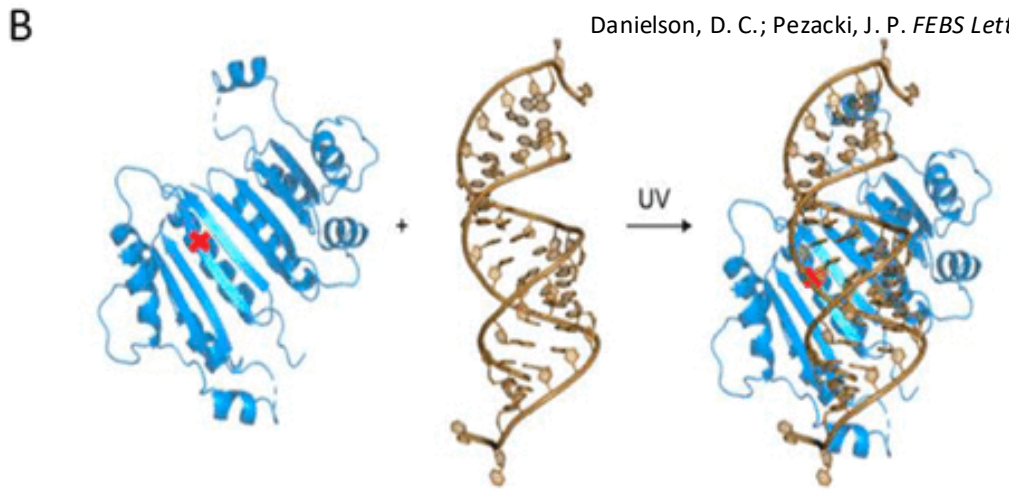


https://en.wikipedia.org/wiki/RNA_silencing_suppressor_p19



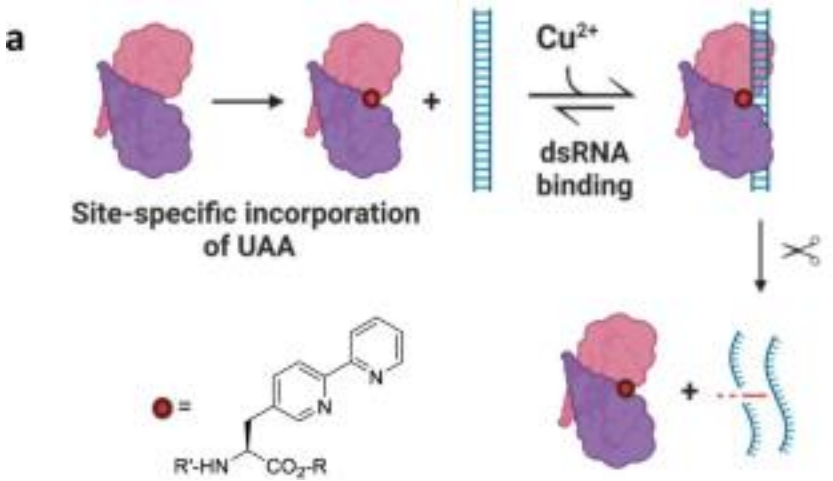
Danielson, D. C.; Pezacki, J. P. *FEBS Lett.* **2013**, *587*, 1198–1205.

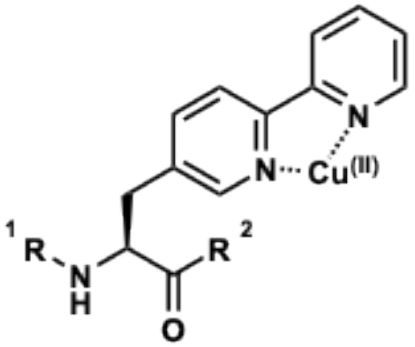




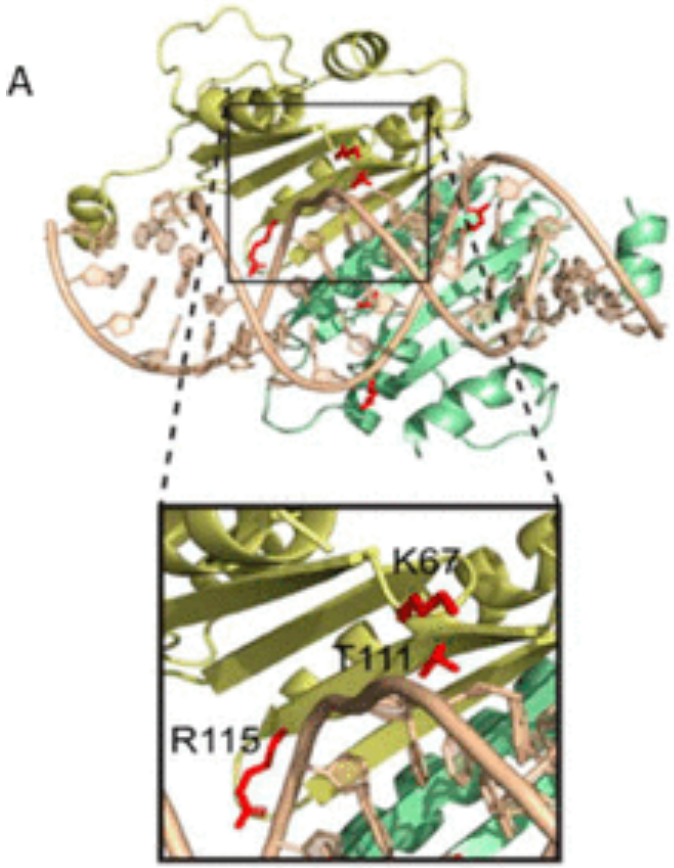
Ahmed, N.; Pezacki, J. P. *Biochemistry* **2019**, *58*, 3520–3526.

In corporation of $Cu^{(II)}$



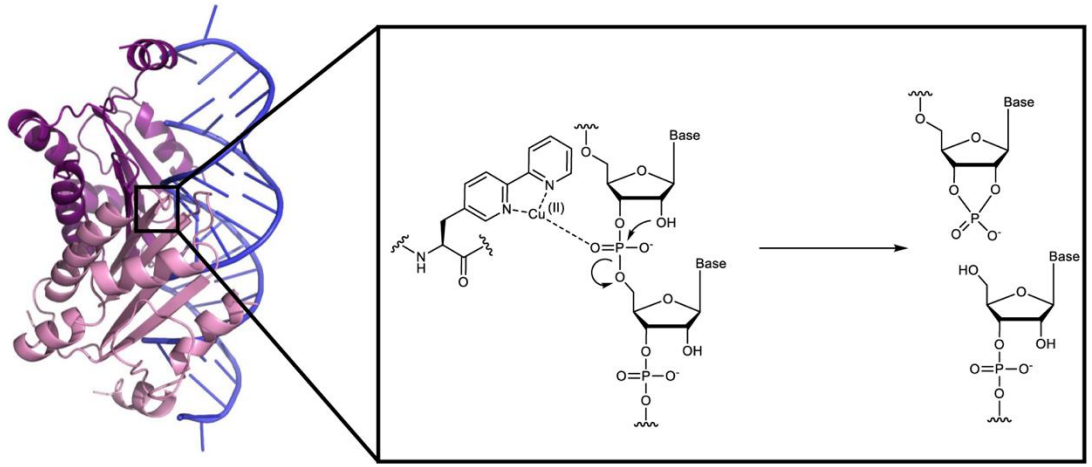


BpyAla is known for chelating $Cu^{(II)}$.



Ahmed, N.; Pezacki, J. P. *Biochemistry* **2019**, *58*, 3520–3526.

K67 and T111 were chosen as the site of incorporation due to their proximity to the bound RNA.

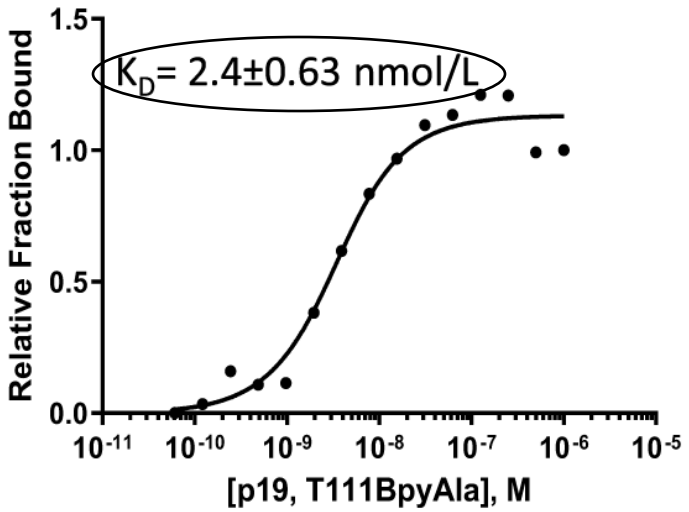


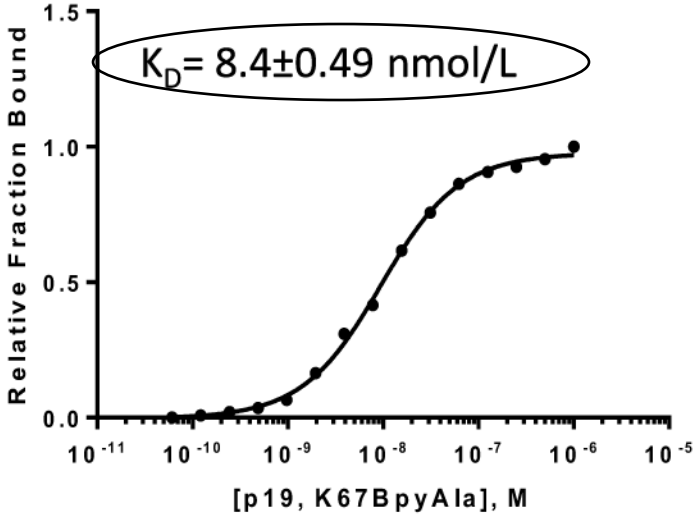
Incorporation of $Cu^{(II)}$ may achieve cleavage of siRNA.

Ahmed, N.; Pezacki, J. P. *Nat. Commun.* **2023**, *14*, 3777.

Confirmation of its cleavage ability

a

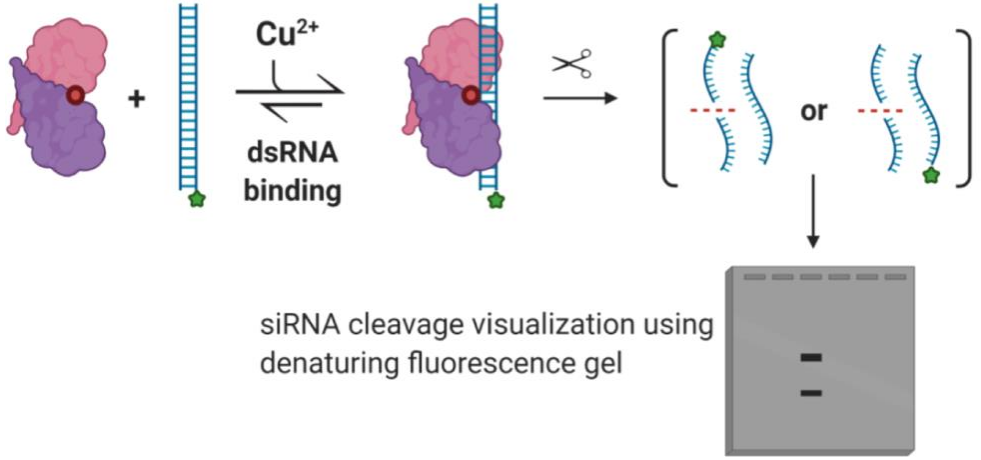




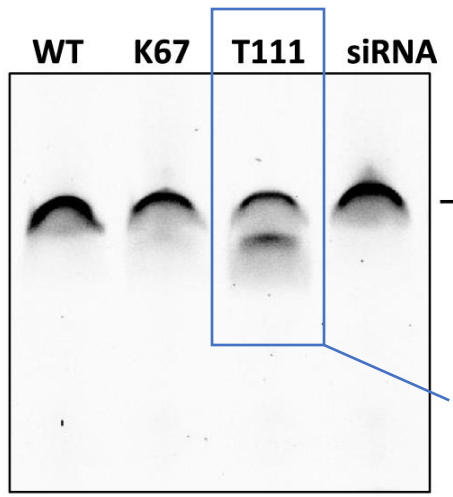
Kd is a measure of the binding affinity between two molecules, such as a protein and a ligand. a lower Kd value indicates a stronger binding affinity, while a higher Kd value indicates a weaker binding affinity.

Confirmed that the incorporation of BpyAla does not spoil the binding ability of p19.

b

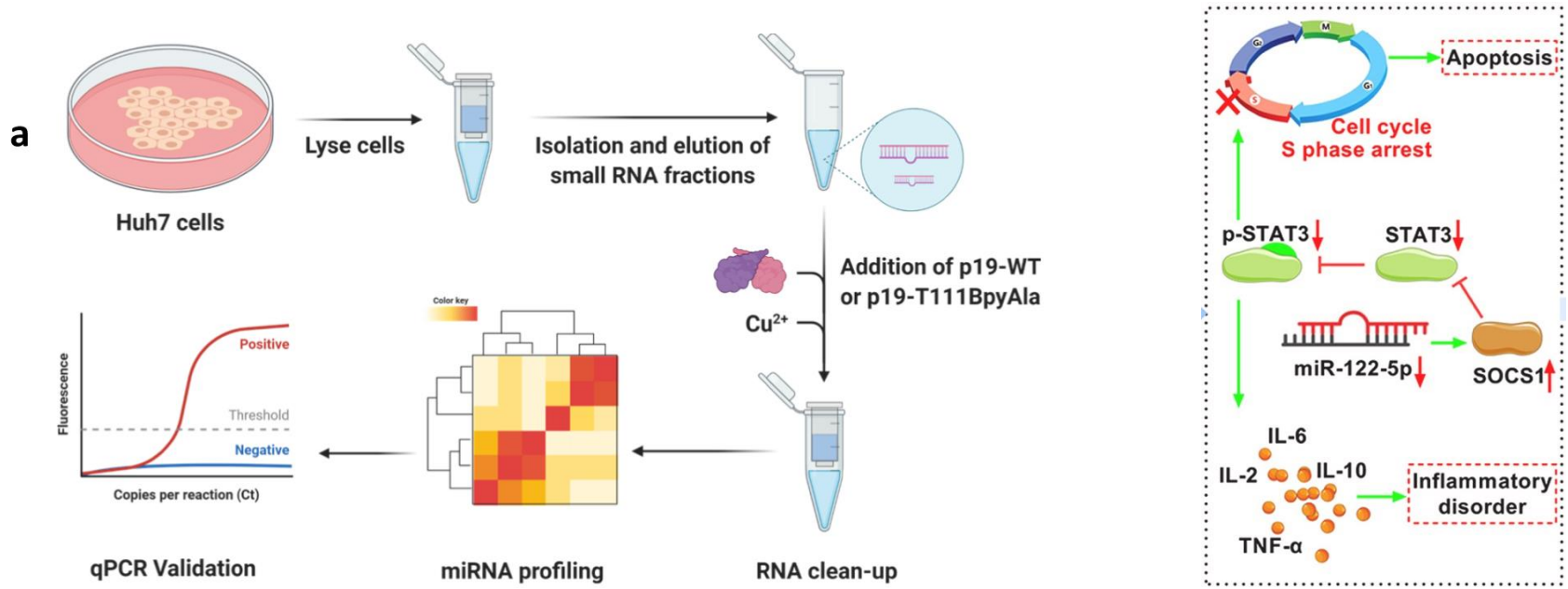


c



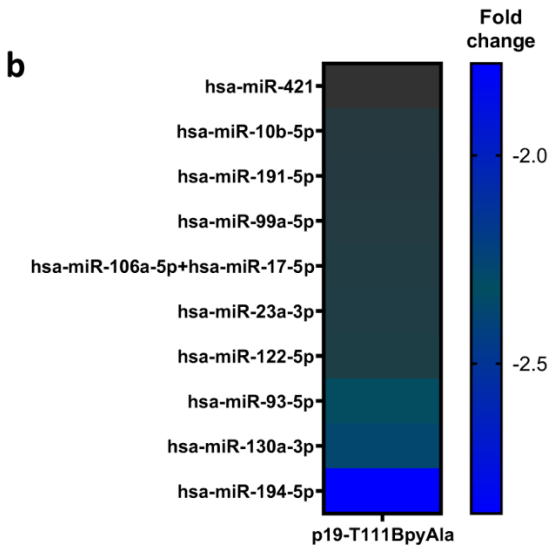
The observation of two bands mean that T111BpyAla + $Cu^{(II)}$ could cleave siRNA.

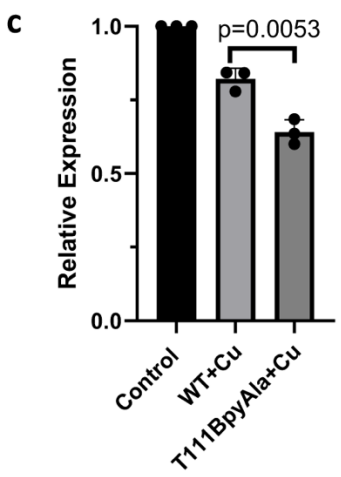
Confirmation of its cleavage ability

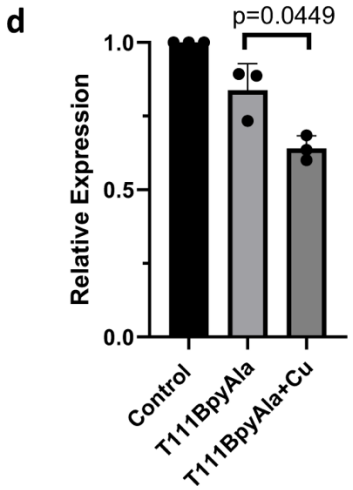


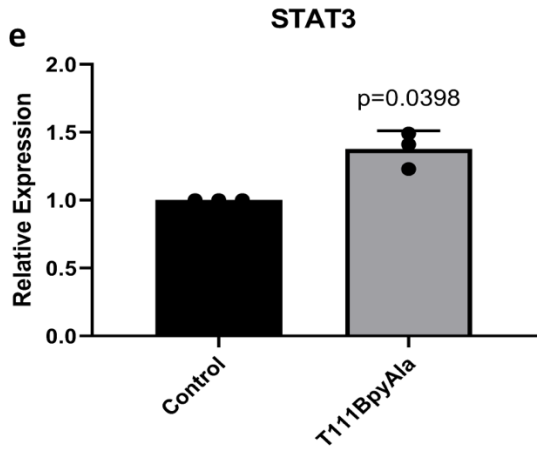
STAT3 is one of the targets of miRNA. miRNA suppress its expression.

Liu, Y.; Yang, G. *Ecotoxicol. Environ. Saf.* **2021**, *223*, 112570.





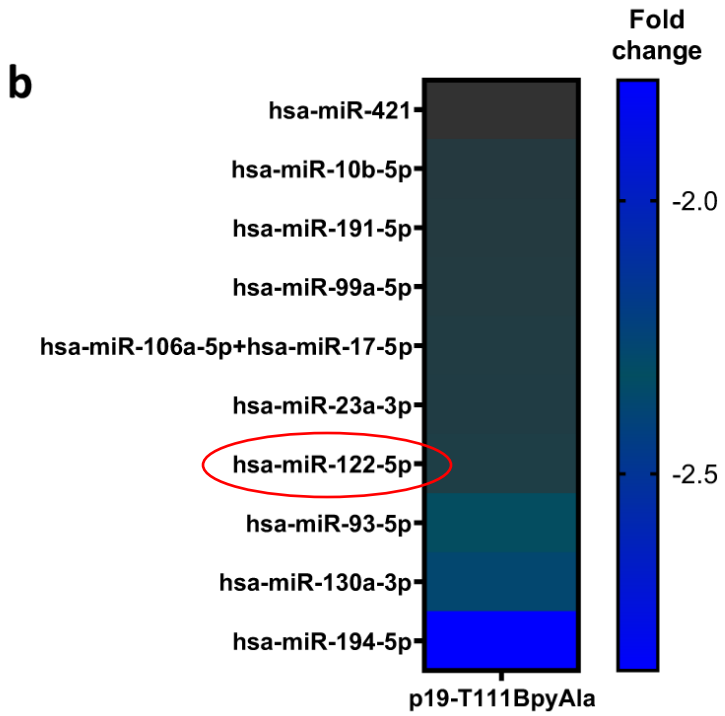




They concluded that both T111BpyAla and $Cu^{(II)}$ are essential for cleavage.

Ahmed, N.; Pezacki, J. P. *Nat. Commun.* **2023**, *14*, 3777.

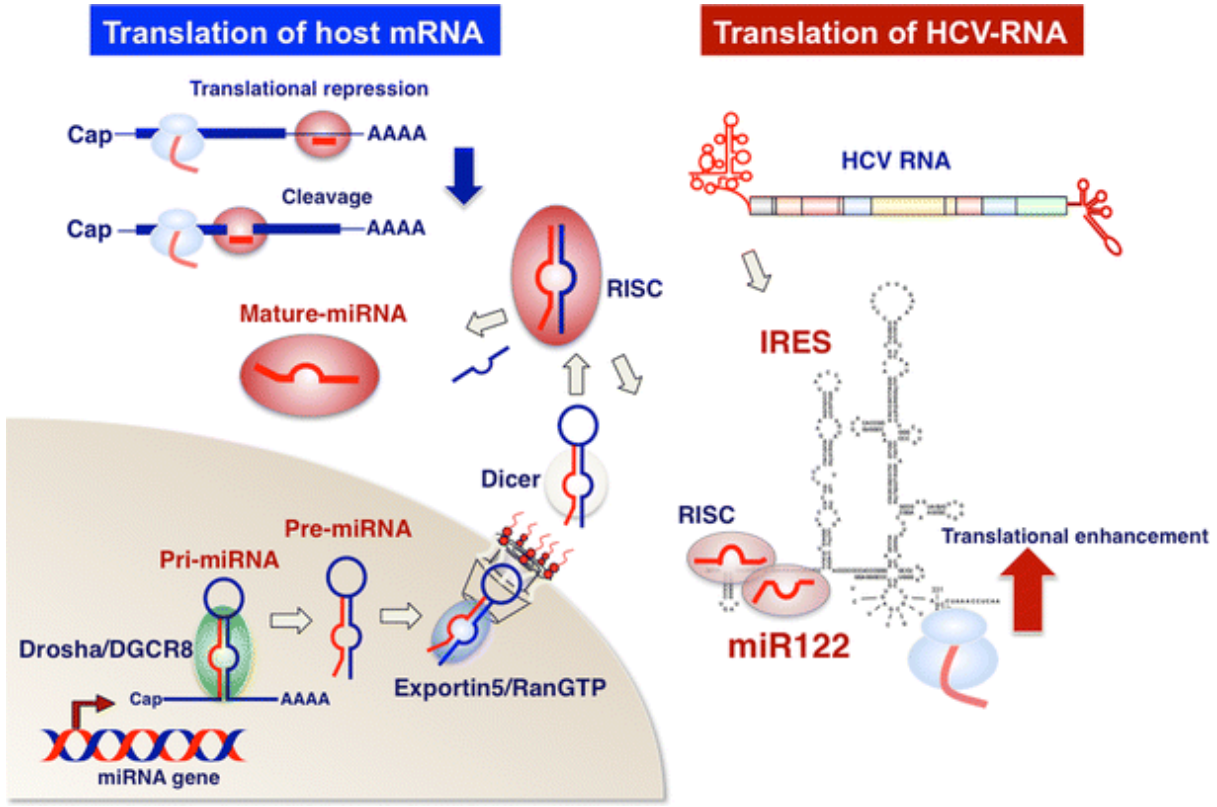
Cell experiment



Ahmed, N.; Pezacki, J. P. *Nat. Commun.* **2023**, *14*, 3777.

This artificial protein can be utilized for reducing HCV

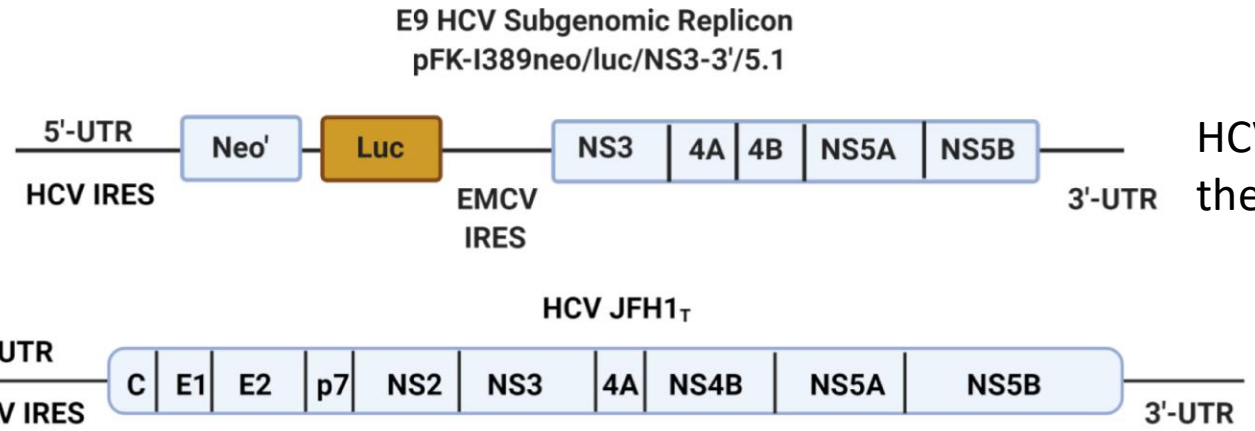
miR-122 promotes replication of HCV virus by improving HCV-RNA's stability and forming internal ribosome entry site



Fukuhara, T.; Matsuura, Y. J. Gastroenterol. **2013**, *48*, 169–176.

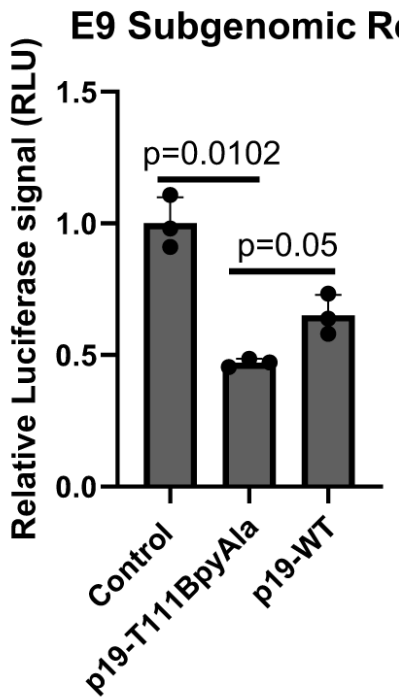
Cell experiment

a

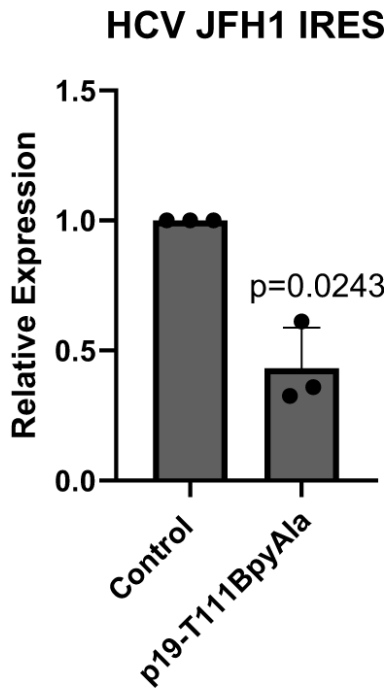


HCV replication levels can be visualized by the amount of luciferase luminescence.

c



d



Ahmed, N.; Pezacki, J. P. *Nat. Commun.* **2023**, *14*, 3777.

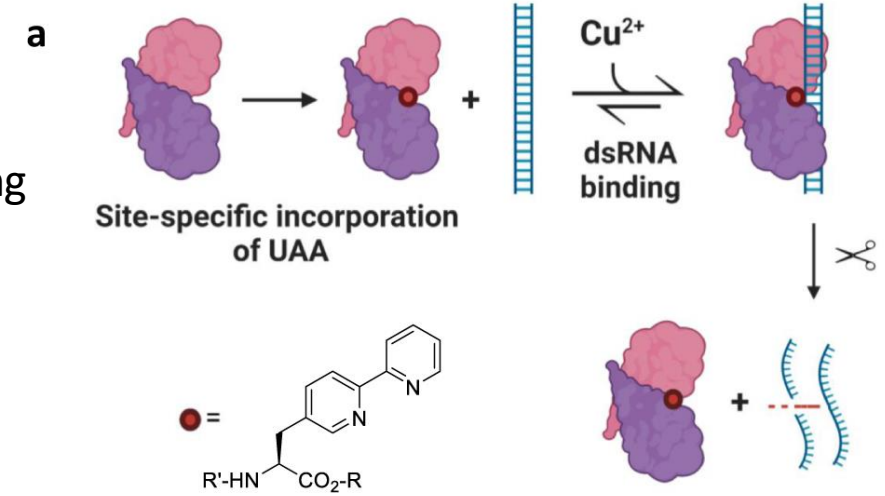
summary

- They succeeded **introducing cleaving ability** to p19, which only could recognize siRNA, by incorporating Cu^{II}.
- They confirmed that the decrease of miRNA by the protein leads to the increase in STAT3 expression.
- They confirmed that the decrease of miR-122 can inhibit the proliferation of HCV.

perspective

They introduced unnatural function (cleaving ability=inducing chemical reaction) into a protein by incorporating ncAA

Expanded the ability of protein



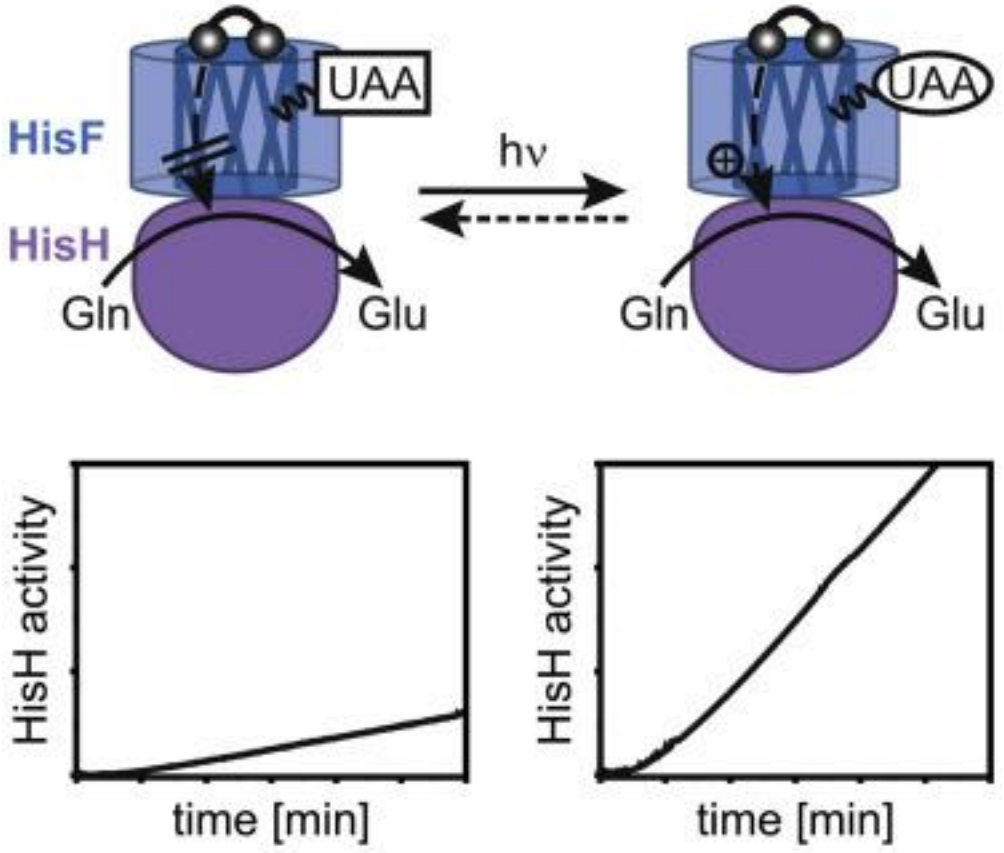
I. Introduction

II. Two examples of ncAA + binding protein

II-1. endonuclease of non-coding RNAs

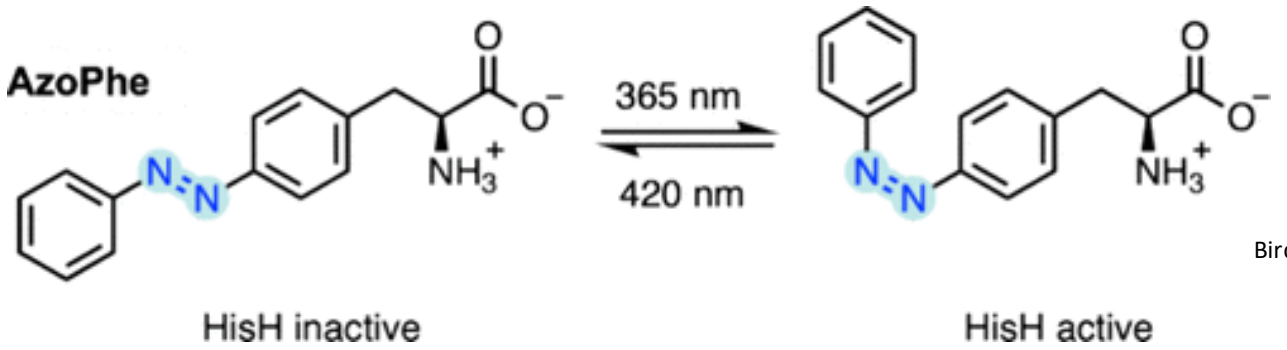
II-2. Light irradiation switch of protein

III. Summary and Outlook



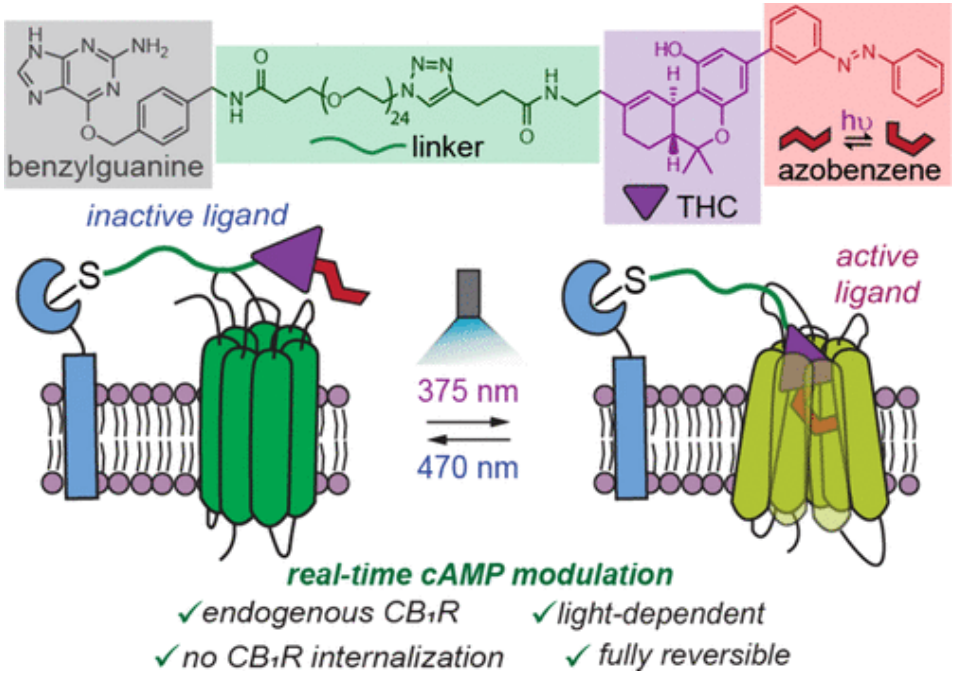
Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

N=N bond isomerization

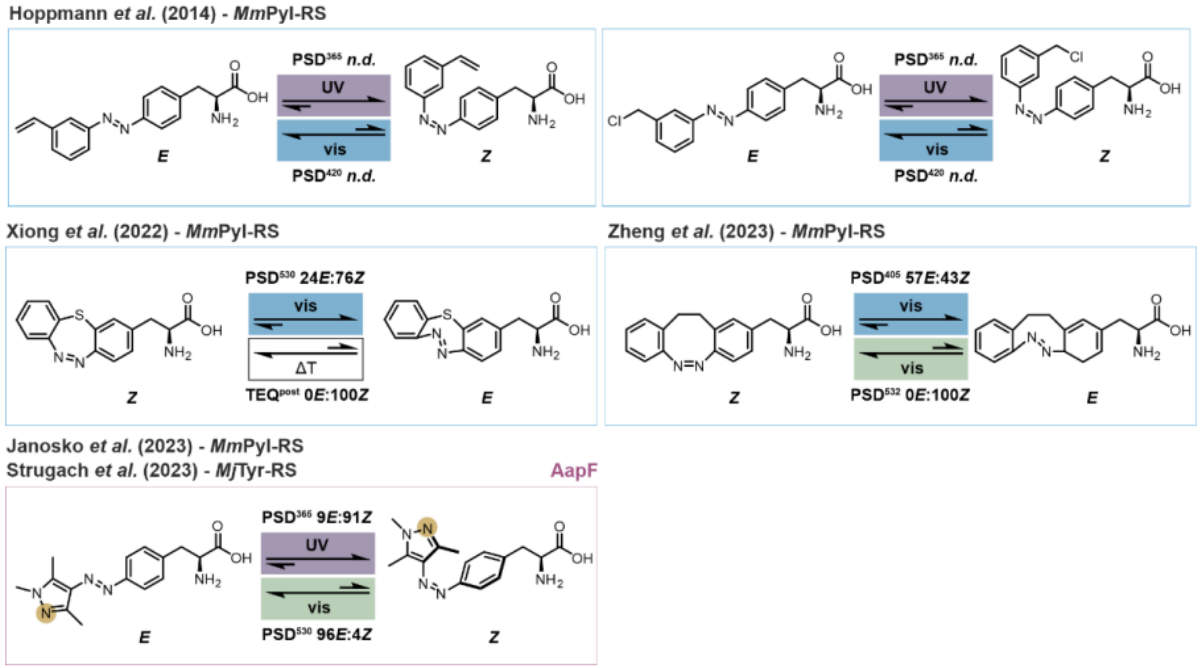


Birch-Price, Z.; Green, A. P. *Chem. Rev.* **2024**, *124*, 8740–8786.

N=N bond isomerization of azobenzene is widely utilized in the research of receptor.



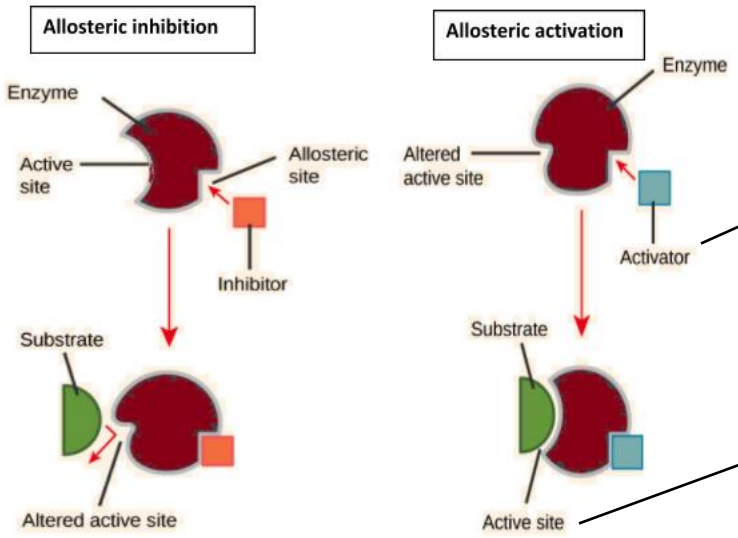
Garza, S. J.; Frank, J. A. J. Am. Chem. Soc. **2025**, *147*, 23482–23491.



Simeth, N. A.; Kneuttinger, A. C. Chem. Eur. J. **2021**, *27*, 2439–2451.

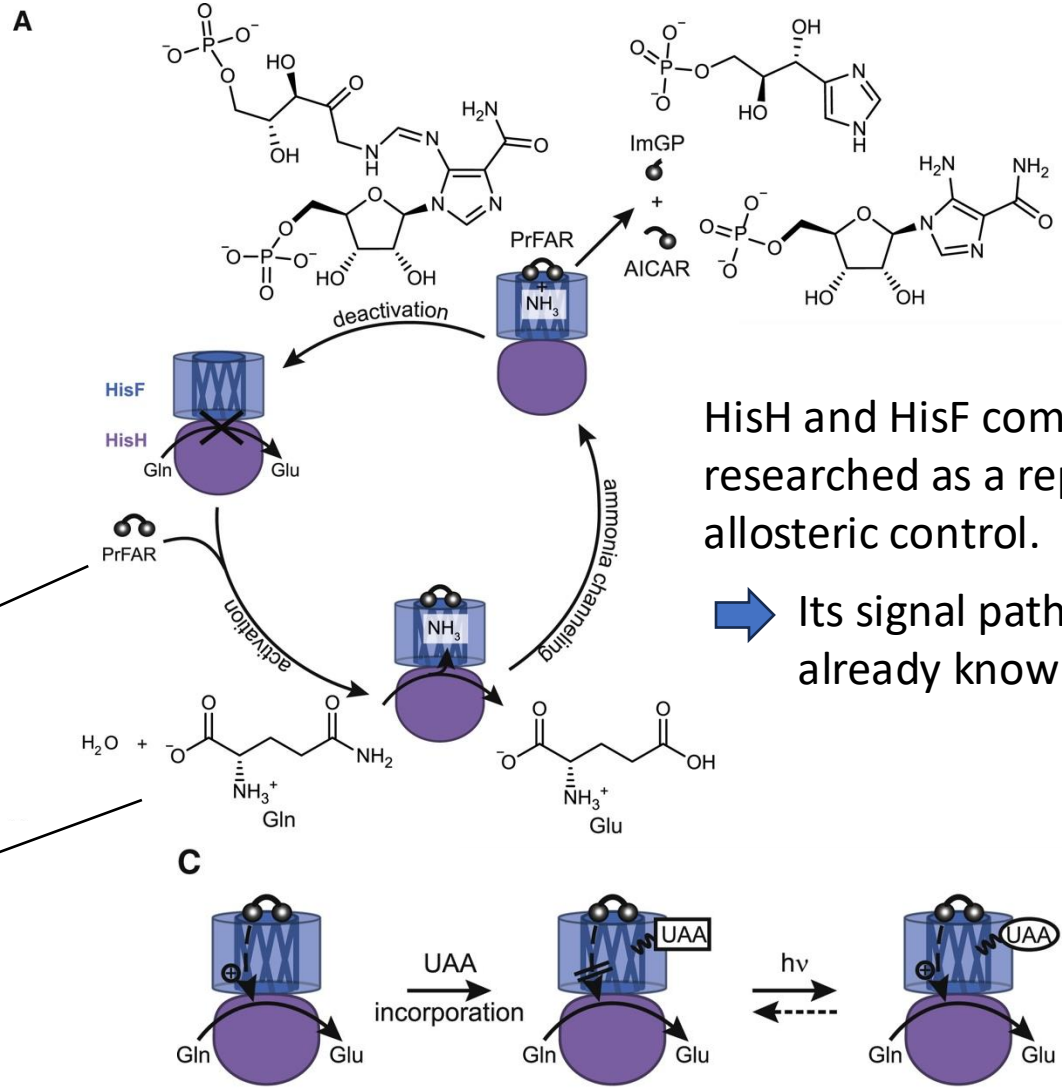
Allosteric site

An allosteric site is a specific region on an enzyme or other protein that is distinct from its active site (or orthosteric site), to which a regulatory molecule (an "effector" or "modulator") can bind to influence the protein's activity.



https://www.vedantu.com/question-answer/allosteric-enzymes-have-allosteric-sites-for-a-class-11-biology-cbse-5f67fd57eee2a36606f544ec

HisH and HisF complex (Imidazole Glycerol Phosphate Synthase)



HisH and HisF complex is widely researched as a representative of allosteric control.

➡ Its signal pathway and structure are already known.

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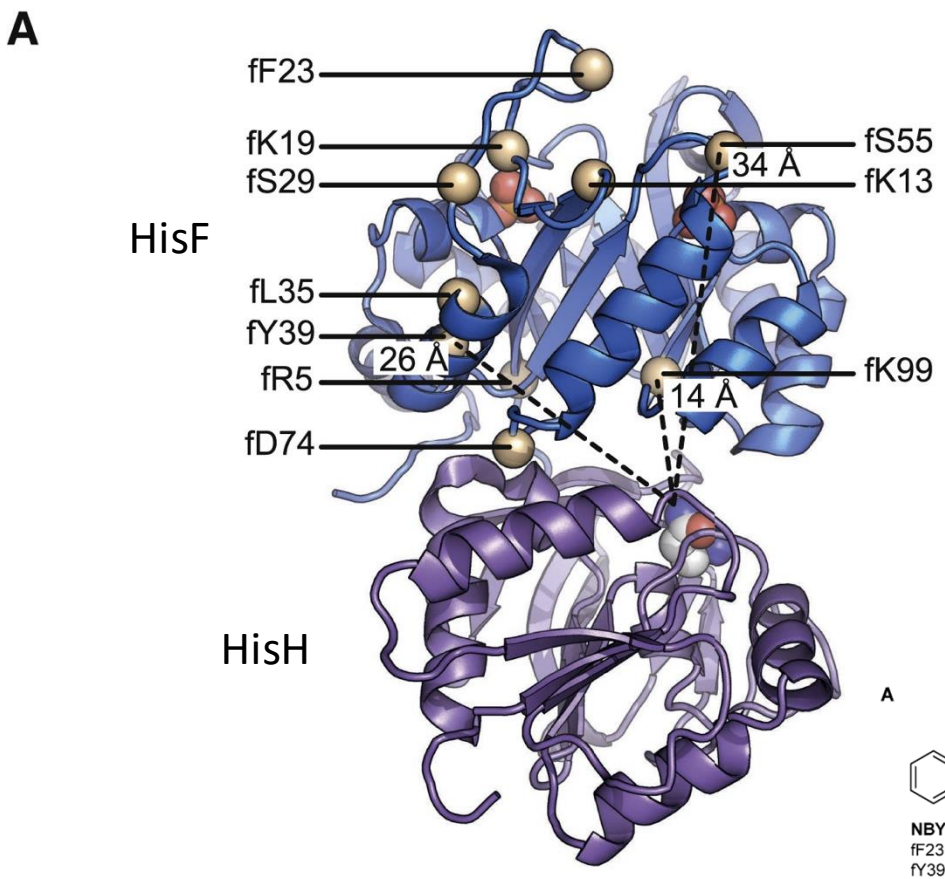
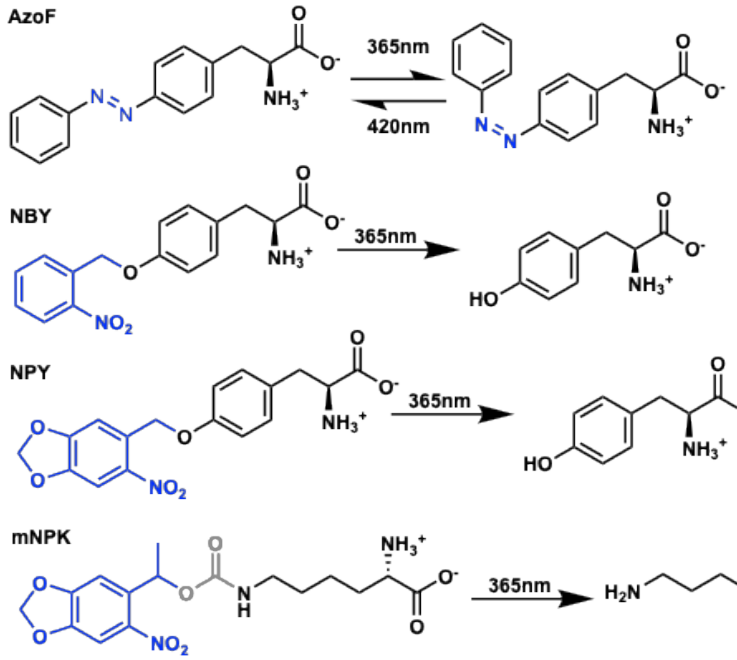
Substitution candidates



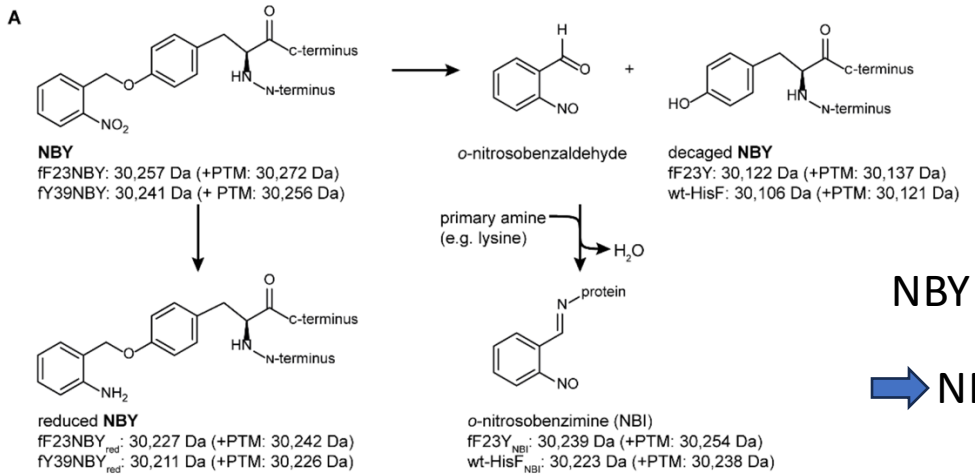
Binding site with HisH is avoided not to interfere with binding to HisH.



Simulated by in silico, as its overlap with other residues is minimized

Substitute Tyr; fY39, fF23 and fD74(a phenylalanine in yeast ImGPS)

Substitute Lys; fK13, K19, and fK99

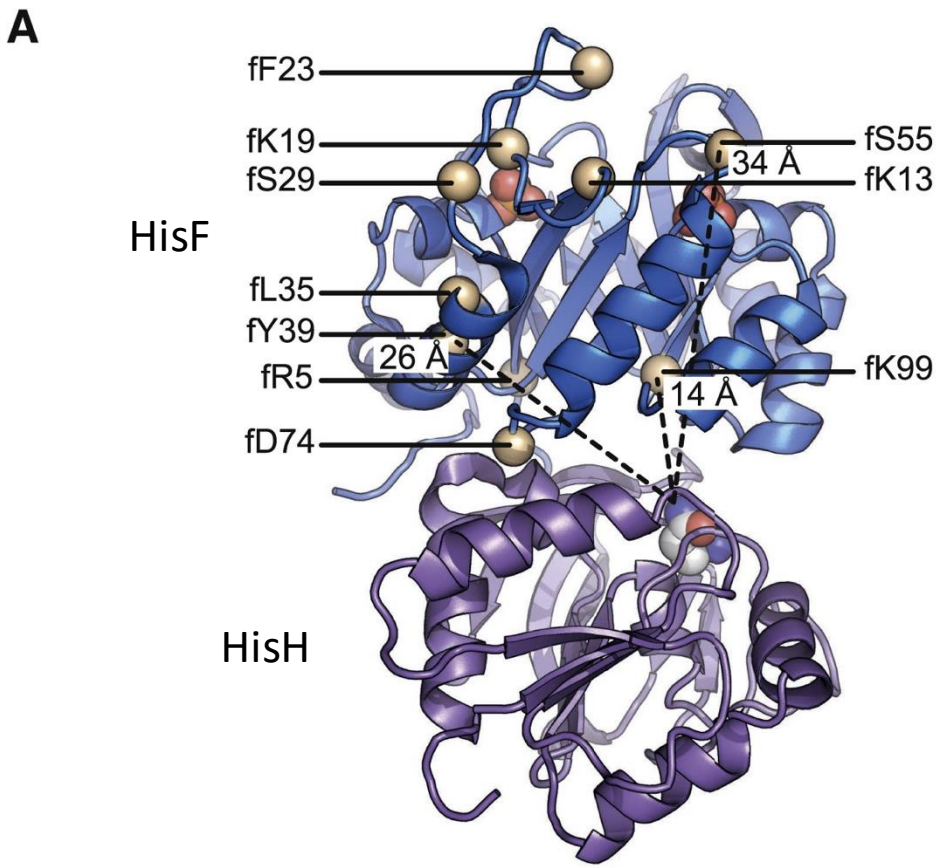


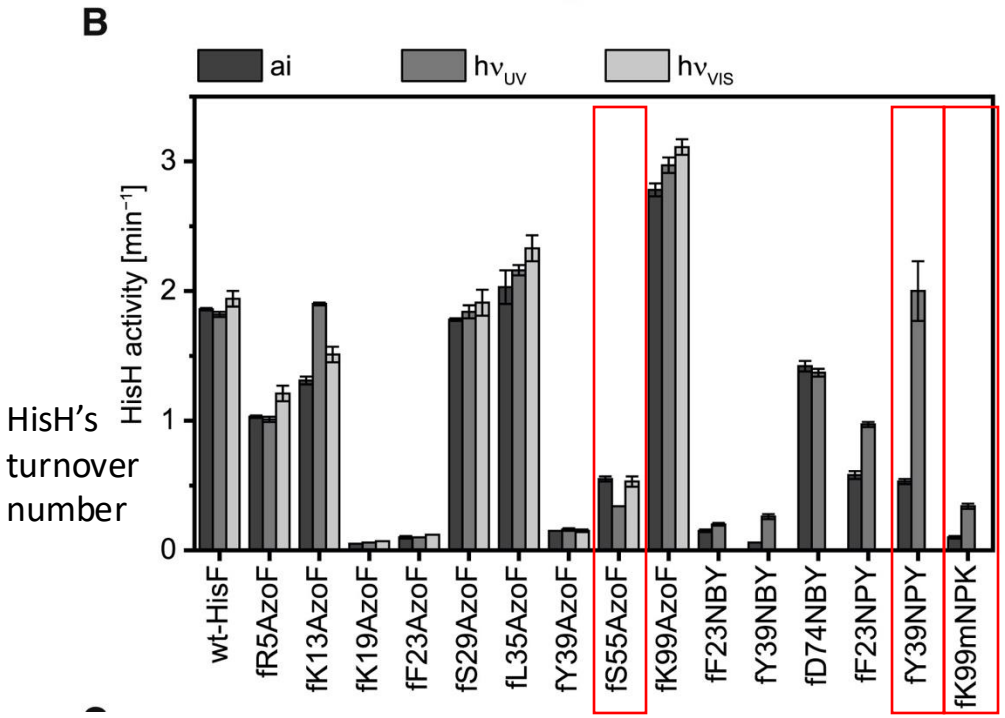
NBY is easy to be reduced.

➡ NPY, more stable, is used.

Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

Filtering promising candidates





HisH's turnover number

fK13mNPK and fK19mNPK did not express well.

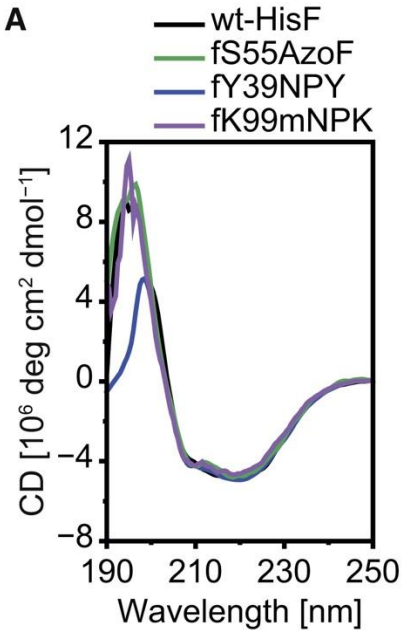
GDH activity assay

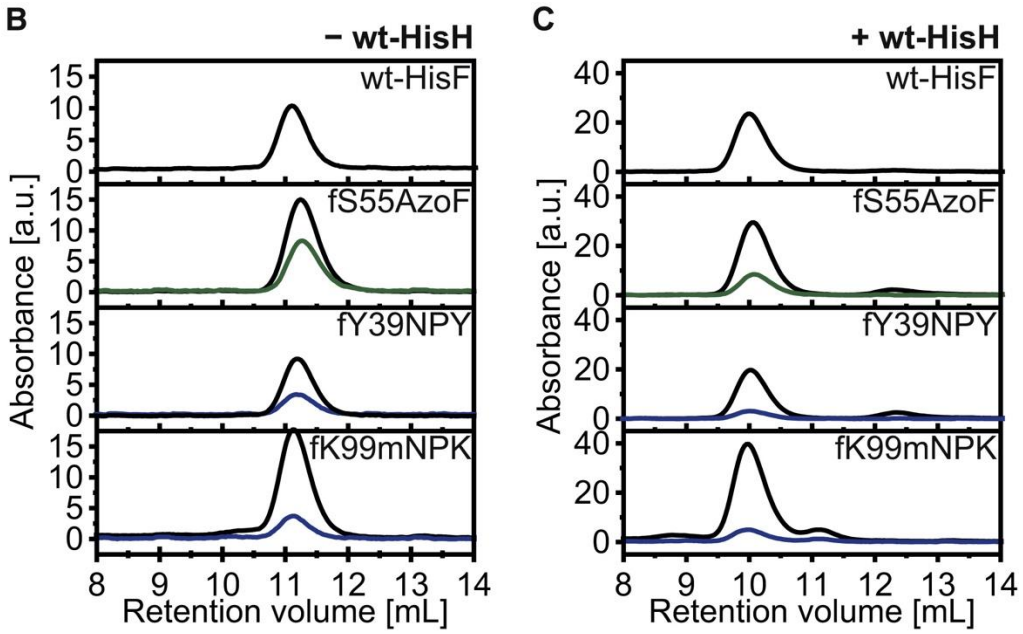
They filtered candidates by 2 criteria.

1. At least 20% wild-type (WT) HisH activity had to be retained in ImGPS complexes containing the irradiated caged UAA-HisFs or the more active isomer of AzoF.
2. HisH activity was altered at least 1.5-fold upon irradiation (light regulation factor [LRF]).

Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

Confirmation of complexes



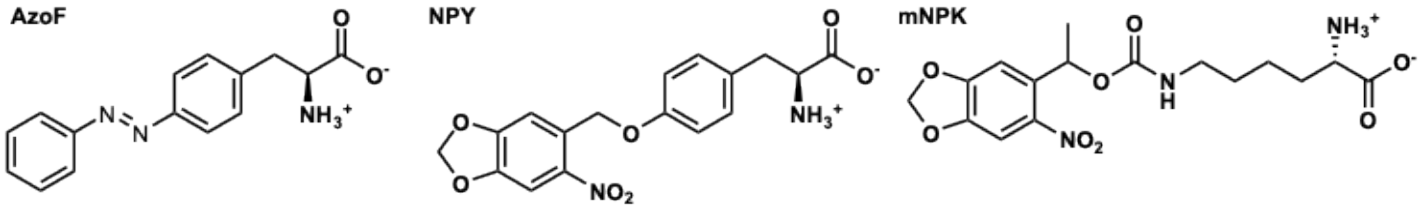


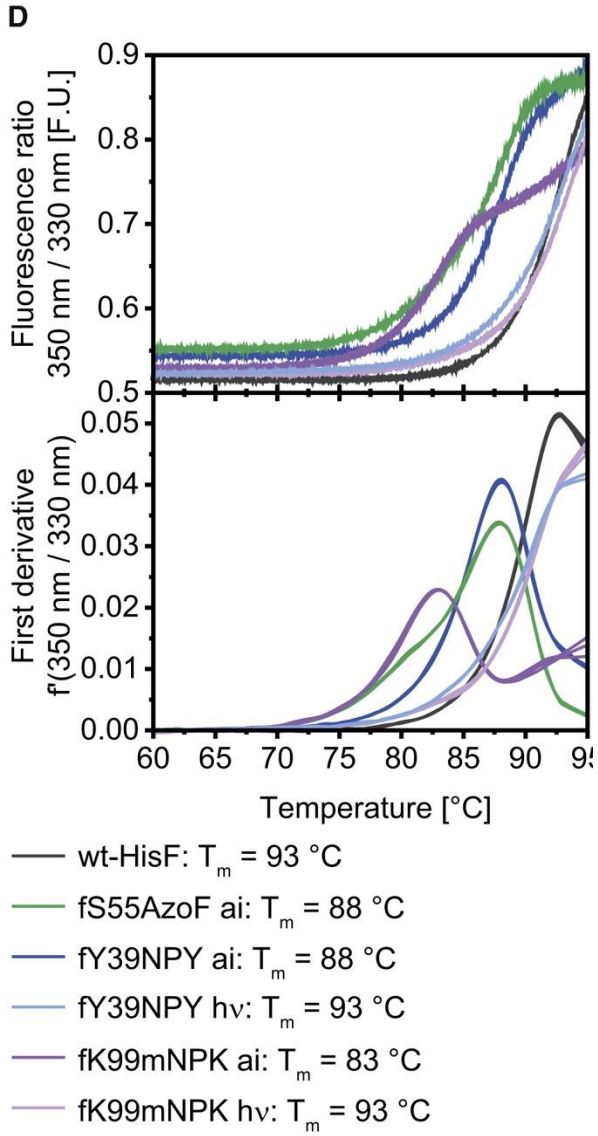
All spectrums are alike.

➡ All proteins have similar conformations.

Each spectrum has almost one peak.

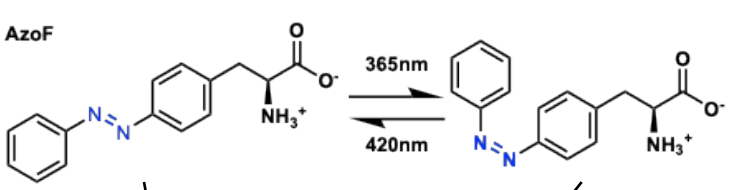
➡ Most of the protein molecules form complexes.

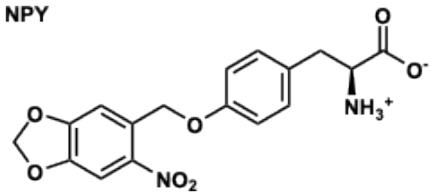


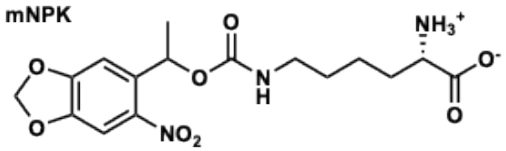


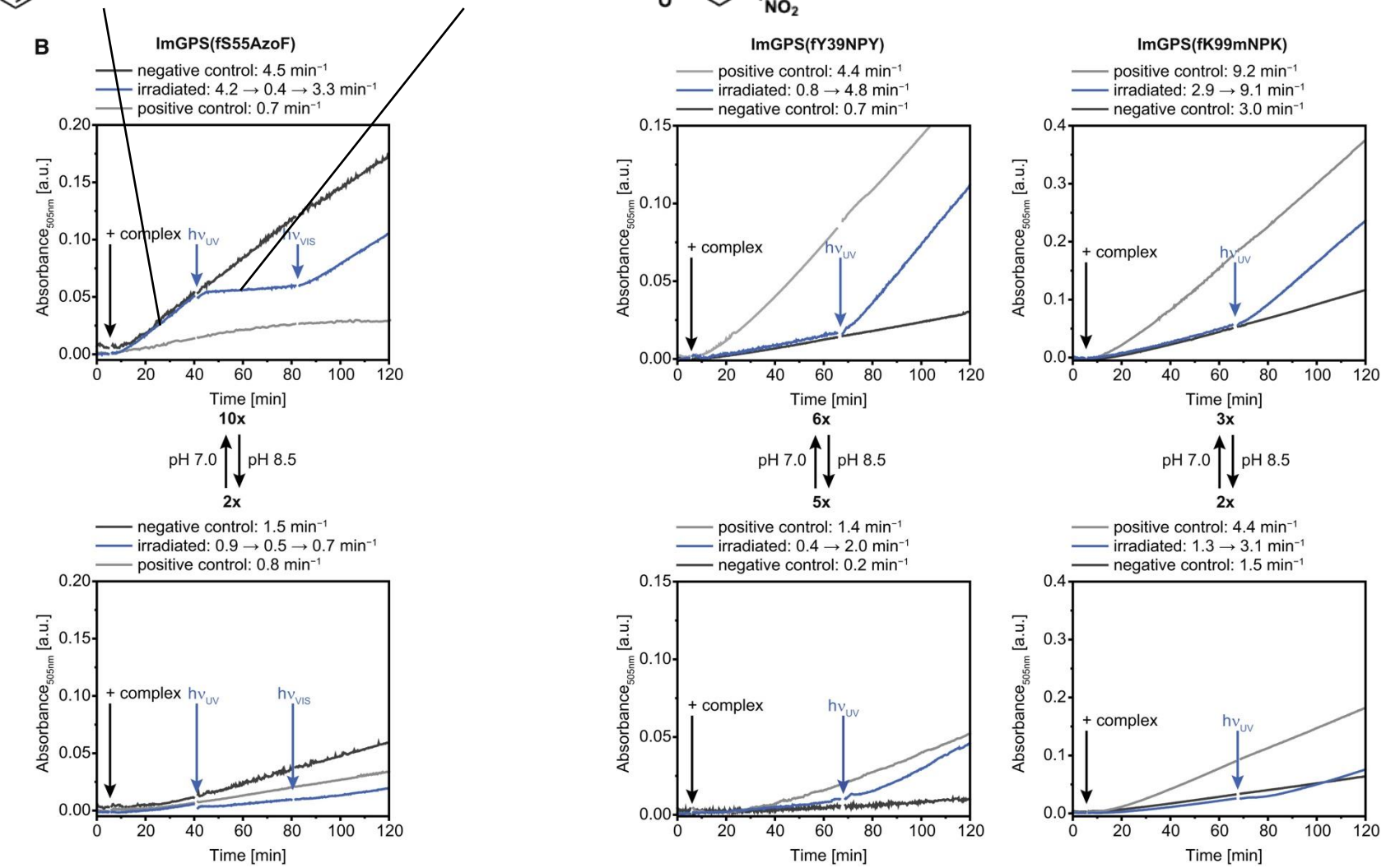
Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

Activity control by light irradiation was observed









GOX/HRP assay

They succeeded controlling enzyme activity by light irradiation.

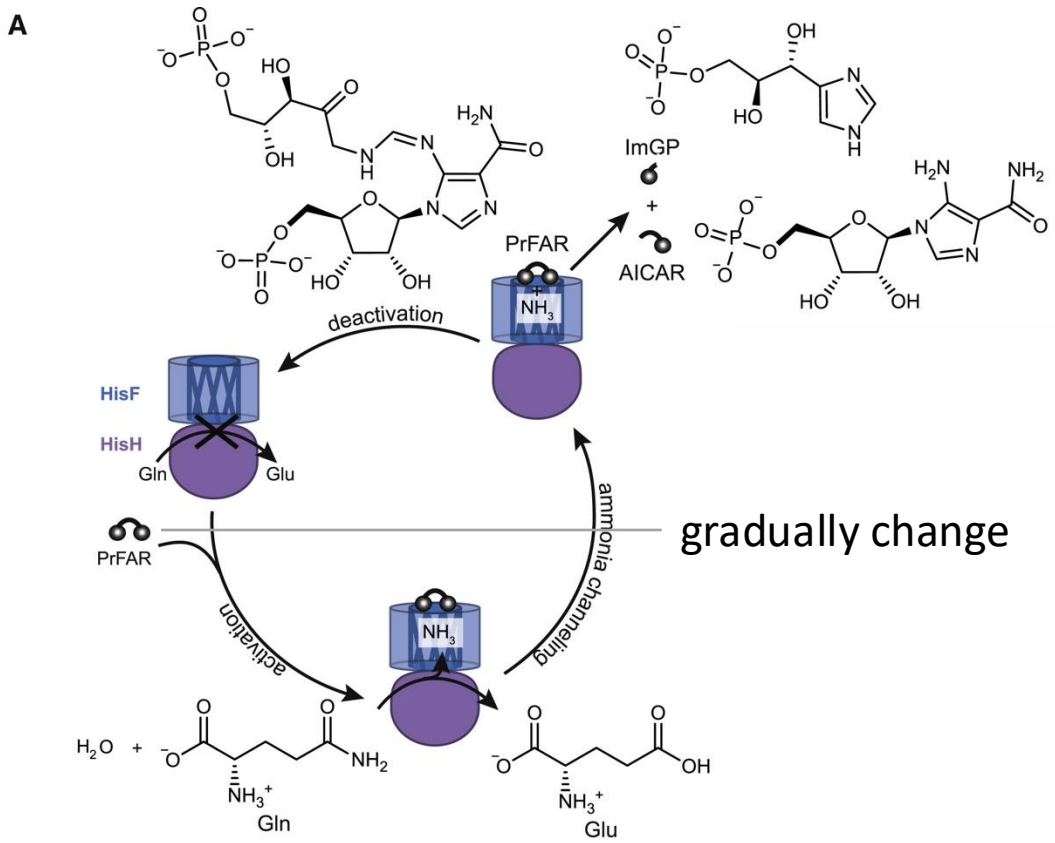
Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

Reaction efficiency was measured

ProFAR-Dependent HisH Activity (pH 7.0)[b]

Protein	State	k_{cat} (min^{-1})	K_{ac}^{ProFAR} (μM)	LRF
WT-ImGPS		16.8± 0.3	32.8± 1.2	
ImGPS(fS55AzoF)	*E*	5.4± 0.4	19.5± 3.8	2.3
	Z	2.3± 0.2	35.1± 5.0	
ImGPS(fY39NPY)	caged	1.2± 0.1	32.3± 4.2	5.9
	decaged	7.0± 0.5	28.7± 4.2	
ImGPS(fK99mNPK)	caged	3.5± 0.3	19.6± 3.7	4.0
	decaged	14.0± 0.9	32.1± 3.2	

These values should be the same as WT's one.

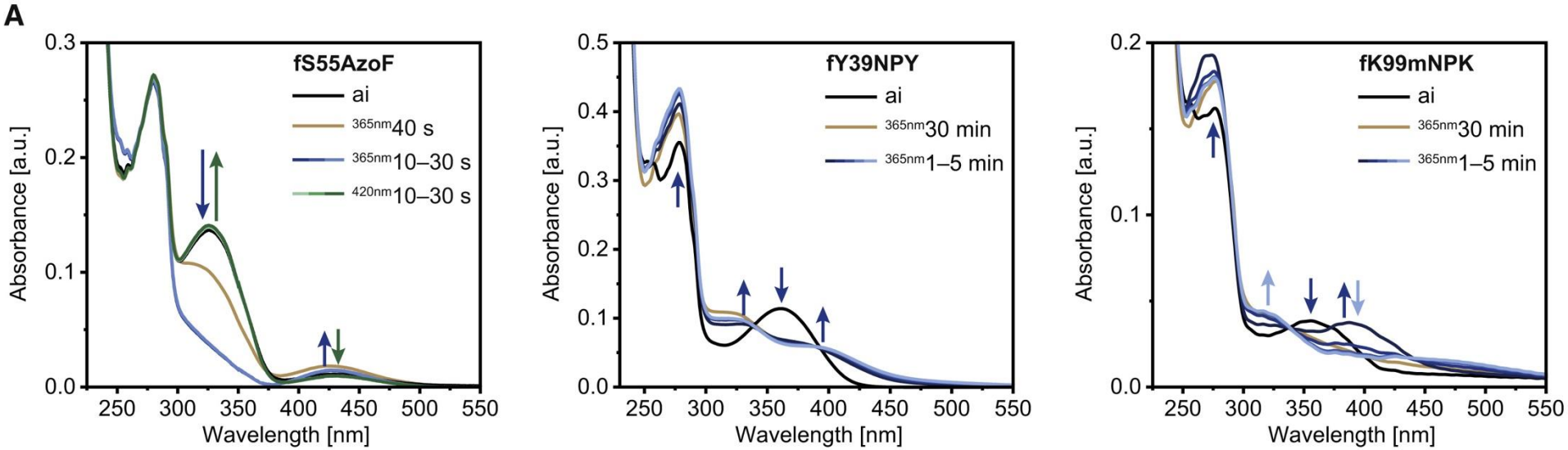


gradually change

saturated

Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

Optimization of reaction conditions

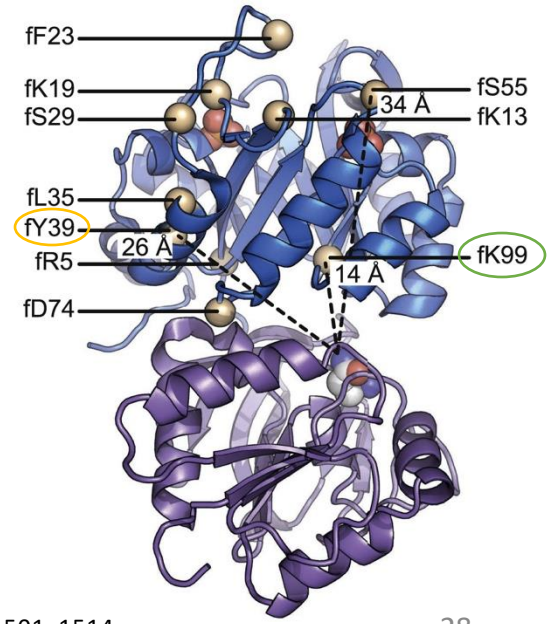


Neither longer irradiation times nor a stronger light source led to complete decaging.

	WT	fY39NPY	fk99mNPK
k_{cat}(Table1)	16.8	7.0	14.0
ratio of k_{cat}	100%	41.70%	83.30%
reduction rate	0%	<=10%	<=10%
decaged rate	100%	27%	70%

The reason why decaged protein's values did not reach WT's is that caged protein remained.

fK99 is in the surface of the protein while fY39 is in the inside of the protein.



Kneuttinger, A. C.; Sterner, R. *Cell Chem. Biol.* **2019**, *26*, 1501–1514.

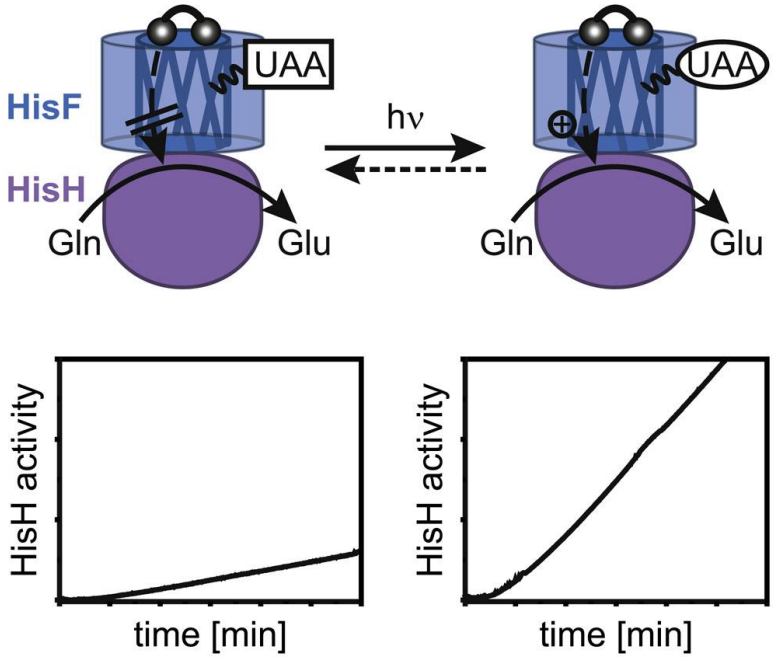
summary

- They incorporated photo sensing ncAA into a protein.
- They succeeded regulate ptrotein's activity by light irradiation.
- The protein just incorporated ncAA could not reach WT's efficiency.

perspective

They make it possible to artificially regulate protein's activity by external stimulus(photo irradiation) by incorporating ncAA.

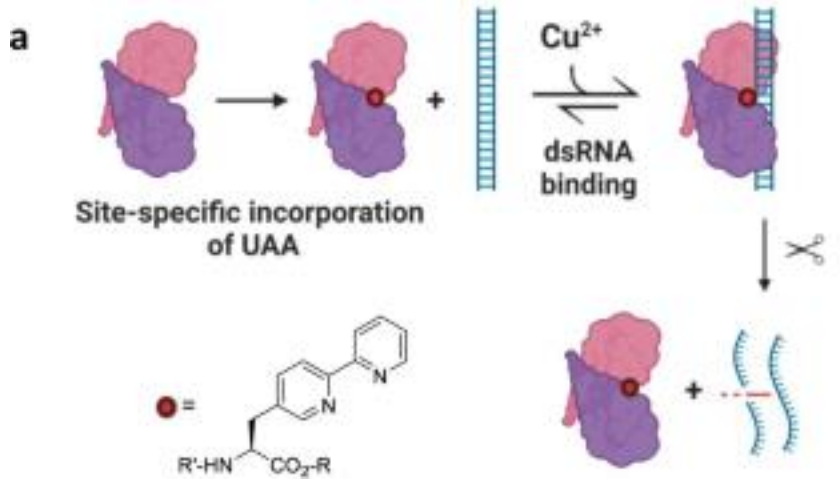
Expanded the ability of protein by ncAA



Today's contents

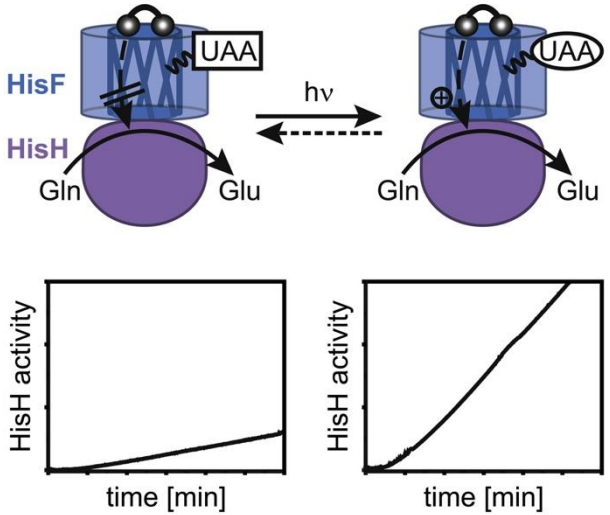
Summary and outlook

An unnatural enzyme with endonuclease activity towards small non-coding RNAs (paper1)



Ahmed *et al.* introduced unnatural function (cleaving ability=inducing chemical reaction) into a protein by incorporating ncAA

Light Regulation of Enzyme Allostery through Photo-responsive Unnatural Amino Acids (paper2)

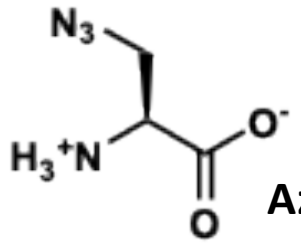


Kneuttinger *et al.* made it possible to artificially regulate protein's activity by external stimulus(photo irradiation) by incorporating ncAA.

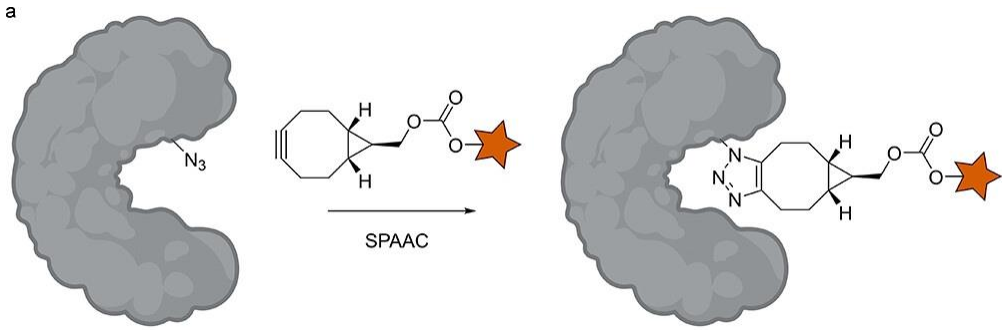
Incorporation of ncAA can introduce additional functions into proteins while keeping proteins substrate recognition ability.

Summary and outlook

Methods to incorporate unnatural moiety into proteins is not only ncAA



AzAla



Brouwer, B.; Drienovská, I. *Chem. Rev.* **2024**, *124*, 10877–10923.

※ incorporation by click reaction make directed evolution complicated.

➡ Other big chemical catalyst may be able to utilize this method.

merit

- ✓ Substrate recognition
- ✓ Utilization of protein pocket as reaction field
- ✓ Powerful optimization by directed evolution

demerit

- Increase in molecular weight
- Difficult to handle
- Incorporation of big moiety by mutations may result in undermine substrate recognition ability

Thank you for your kind attention!