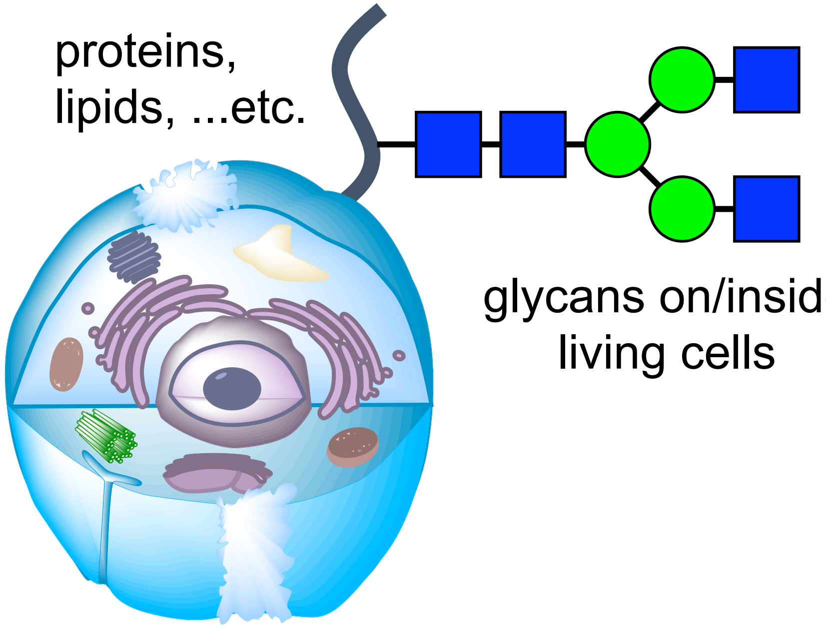
Glycan Engineering in Living Cells

Literature seminar #3
M2 Yuki Yamanashi
2021/08/19 (Thu)

Overview: Development of live-cell glycoengineering tools utilizing the power of chemical biology

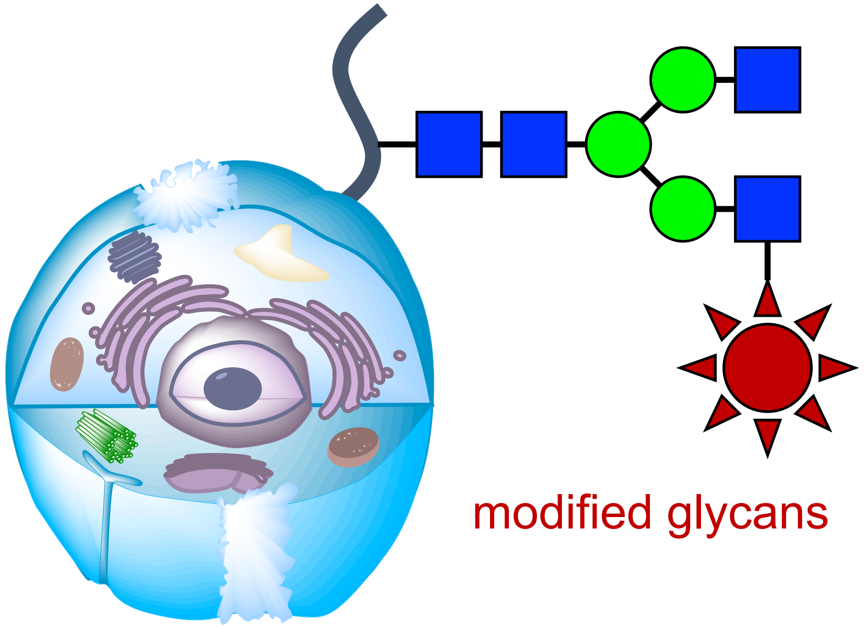


glycans on/inside
living cells

glycoengineering
tools

- Exoenzymatic engineering
- Metabolic engineering
- Genetic engineering

...etc.



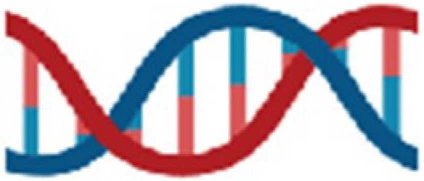
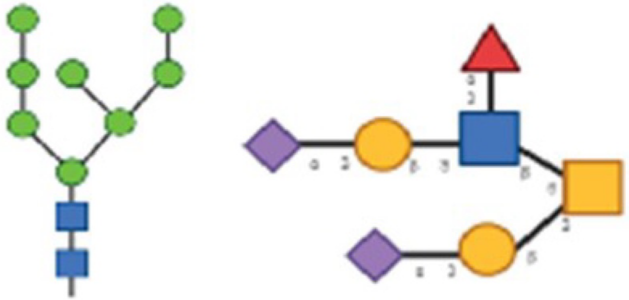
- Imaging
- Structure-function relationships
- Therapeutics

...etc.

Contents

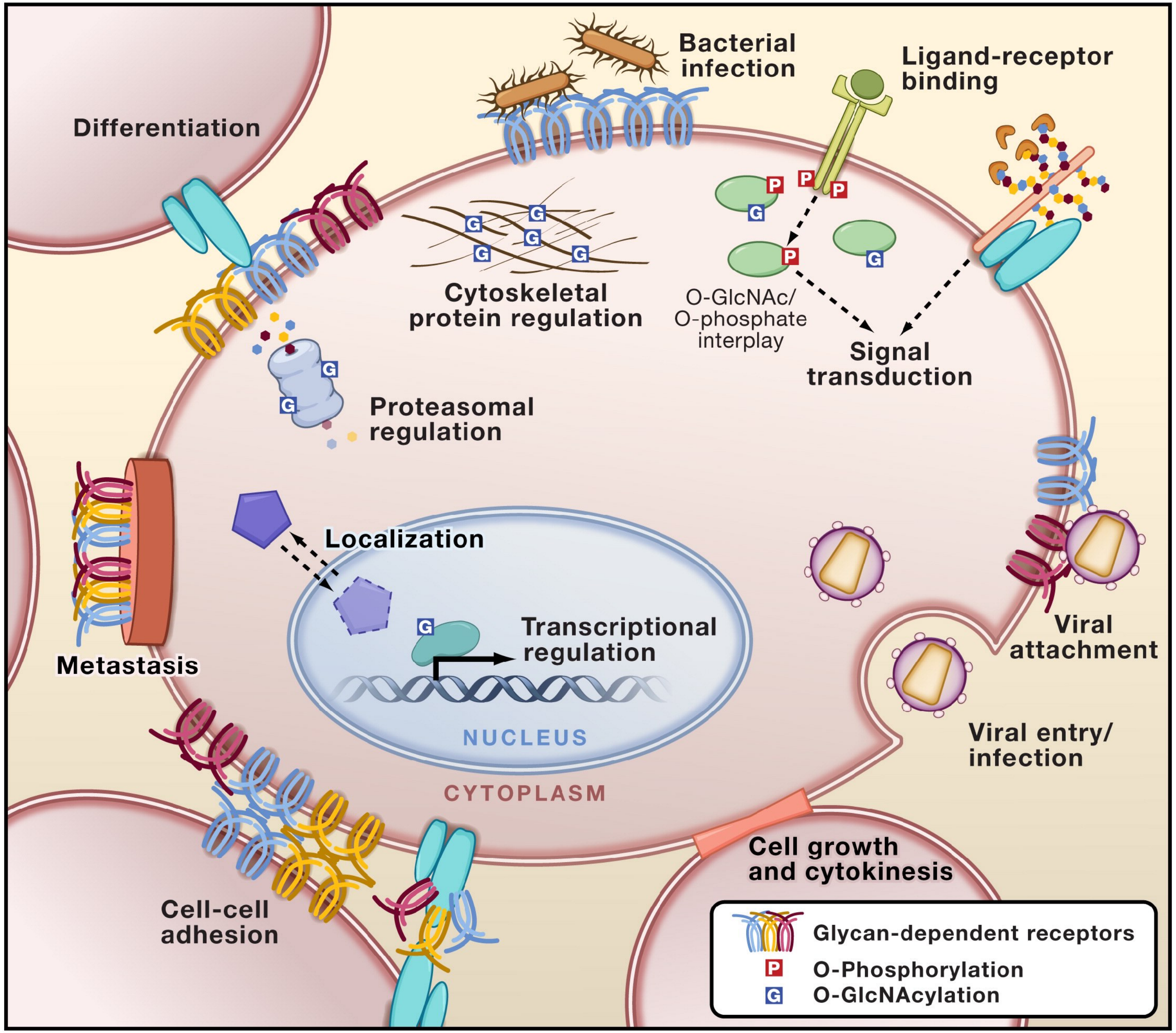
Contents

Glycans are major building blocks of life.

DNA	Protein	Glycans
		
4 bases: genome ~21,000 genes	23 amino acids: proteome >100,000 proteins	~ 33 monosaccharides: glycome >100,000 structures
- templated synthesis - linear	- templated synthesis - linear	- non-templated synthesis - branched

Scott, E. and Munkley, J. *Int. J. Mol. Sci.* **2019**, *20*, 1389-1418.

Glycans play crucial roles in a myriad of biological processes.



- Glycans on cell surface
  - cell-cell comunication
  - membrane protein trafficking
  - pathogen invasion
  - immune response

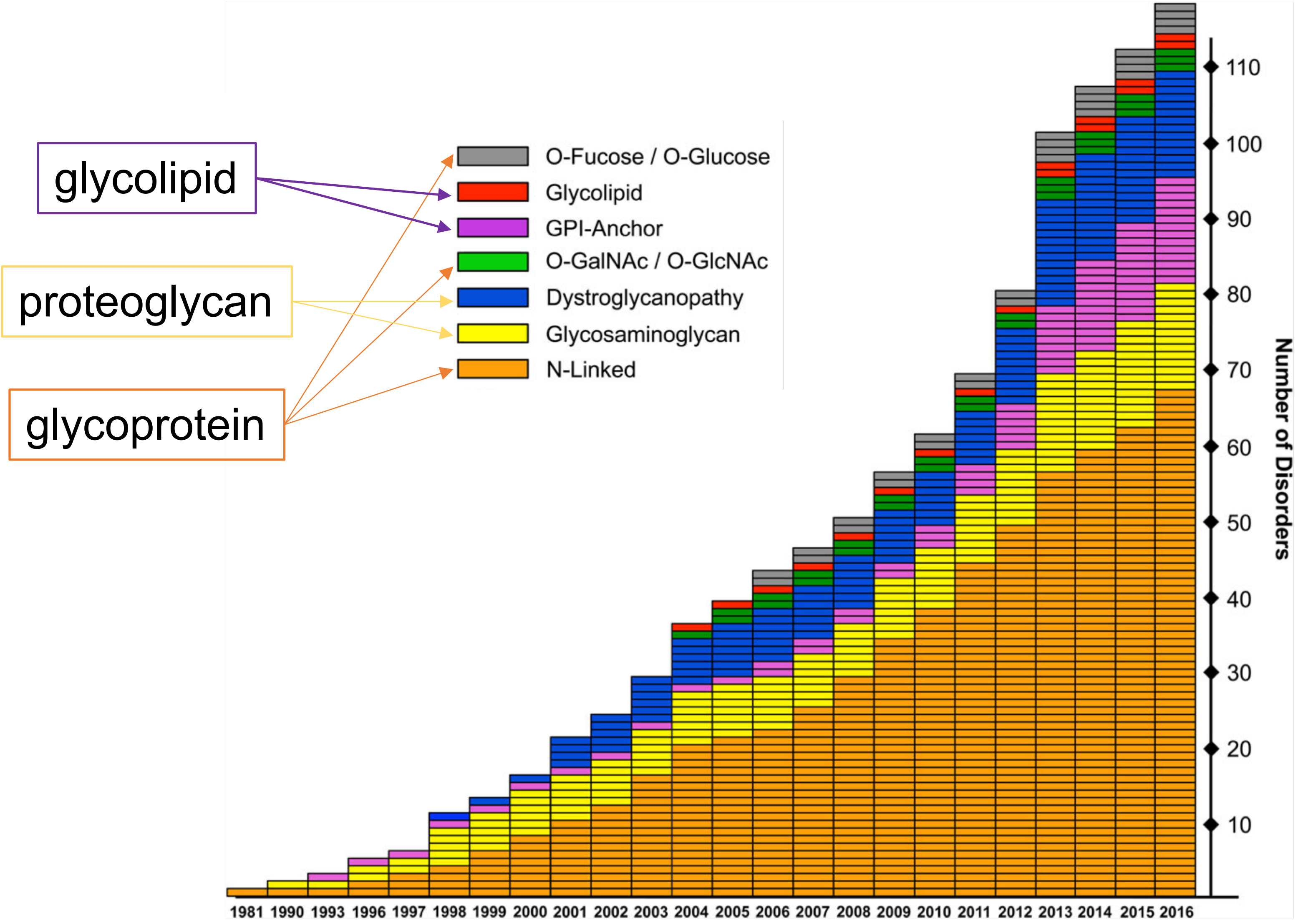
- Glycans inside cells
  - properties of proteins (e.g., stability, conformations)
  - transcriptional regulation

...etc.

Hart, G. W. and Copeland, R. J. *Cell* **2010**, *143*, 672–676.

A growing number of glycan-associated diseases are discovered from 1990s.(1)

Human disorders with a major genetic defect in glycosylation pathways



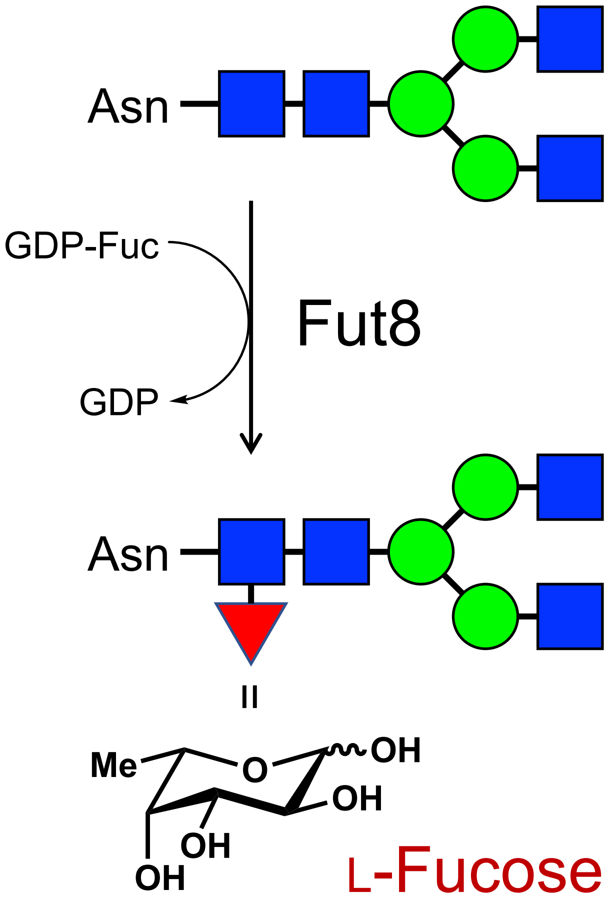
- Cancer metastasis
- Autoimmune diseases
- Alzheimer’s diseases
- Diabetes
- Viral immune escape
- Kidney diseases

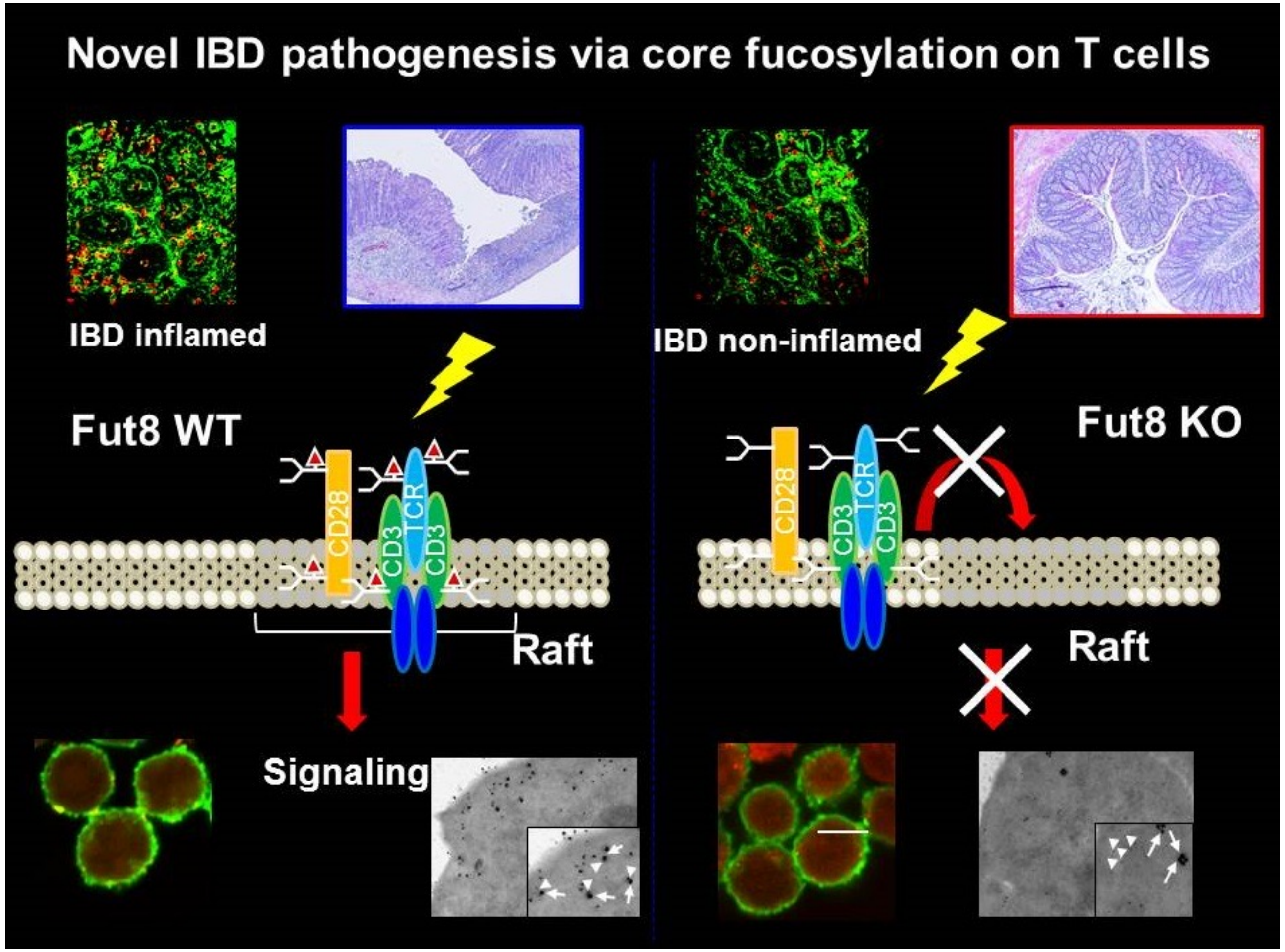
...etc.

Varki, A. *Glycobiology* **2017**, *27*, 3–49.

A growing number of glycan-associated diseases are discovered from 1990s.(2)

Inflammatory bowel disease (IBD) and core fucosylation on T cells



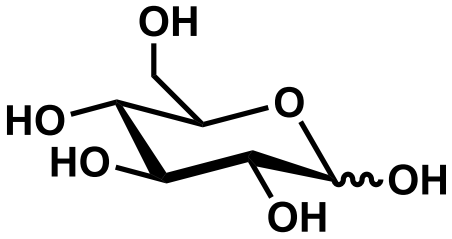


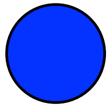
- Fut8 expression was abnormally increased in the inflamed area, resulting in high core fucosylation level.
- Fut8 KO inhibited IBD pathogenesis via inhibition of transportation of receptors to the lipid raft.
- Fut8 inhibition can be a new therapeutic strategy.

Fujii, H., Shinzaki, S., *et al. Gastroenterology* **2016**, *150*, 1620–1632.

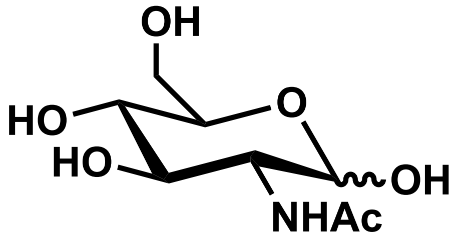
Glycans composed of many kinds of monosaccharides.

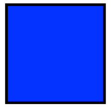
The 10 most abundant monosaccharide units in human cells



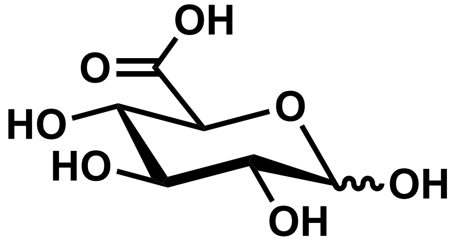


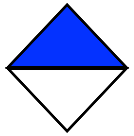
D-Glc
2.5%



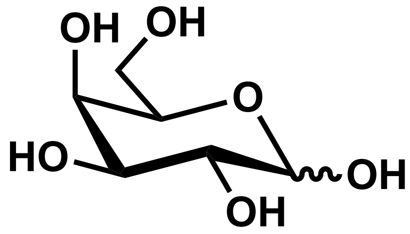


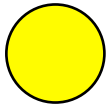
D-GlcNAc
31.8%



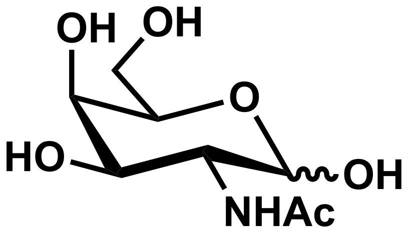


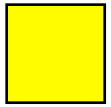
D-GlcA
0.3%



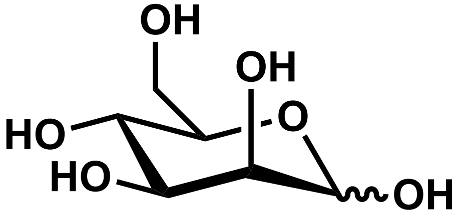


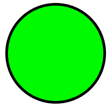
D-Gal
24.8%



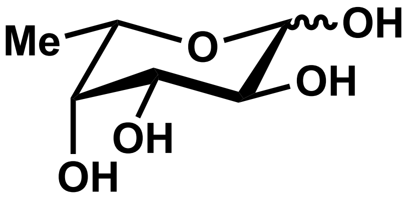


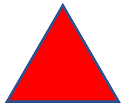
D-GalNAc
4.8%



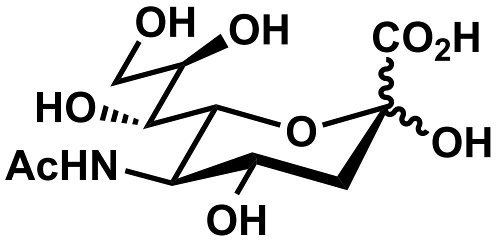


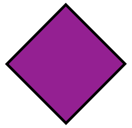
D-Man
18.9%



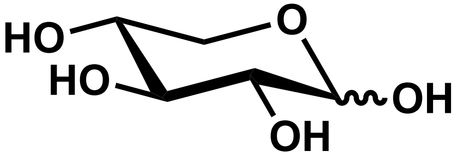


L-Fuc
7.2%

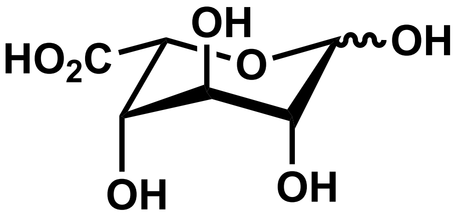


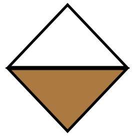


Neu5Ac
8.3%



D-Xyl
0.1%



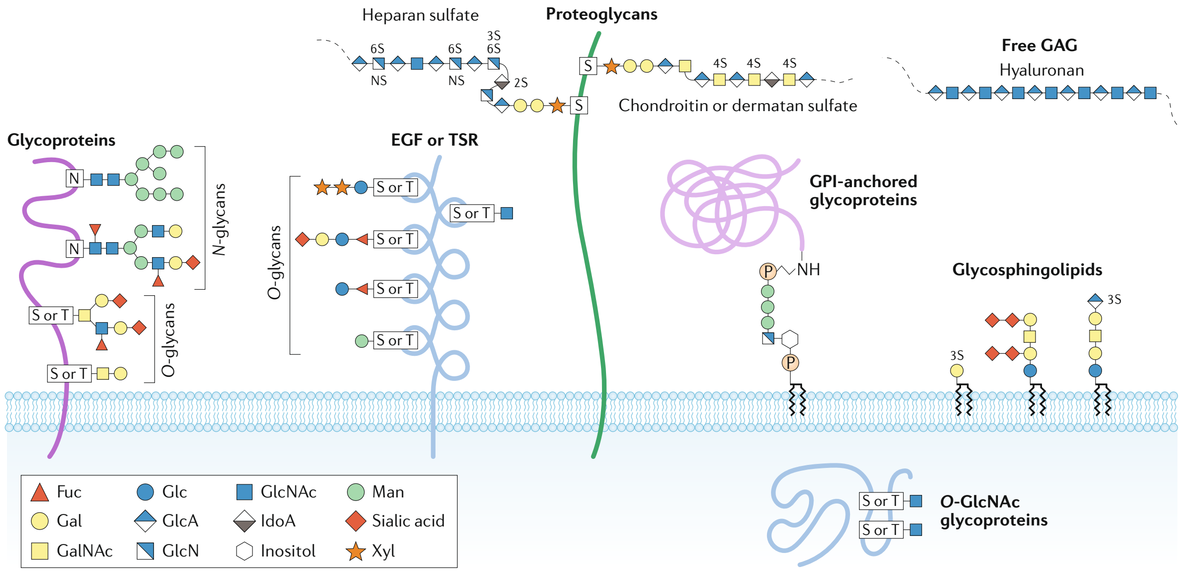


L-IdoA
0.1%

Werz, D. B., Ranzinger, R. *et al. ACS Chem. Biol.* **2007**, *2*, 685–691.

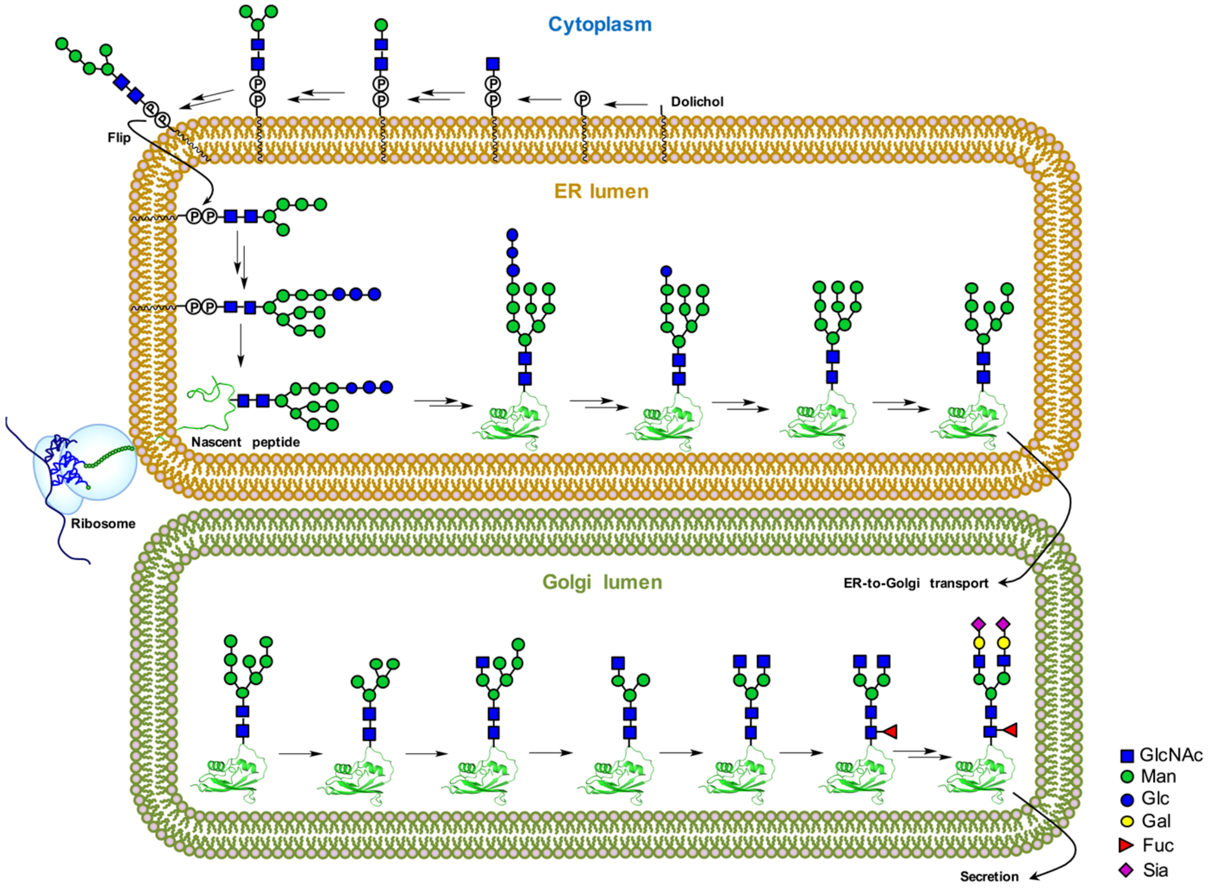
Glycosylation of proteins and lipids occur in the ER and Golgi apparatus.

Major types of glycans in humans



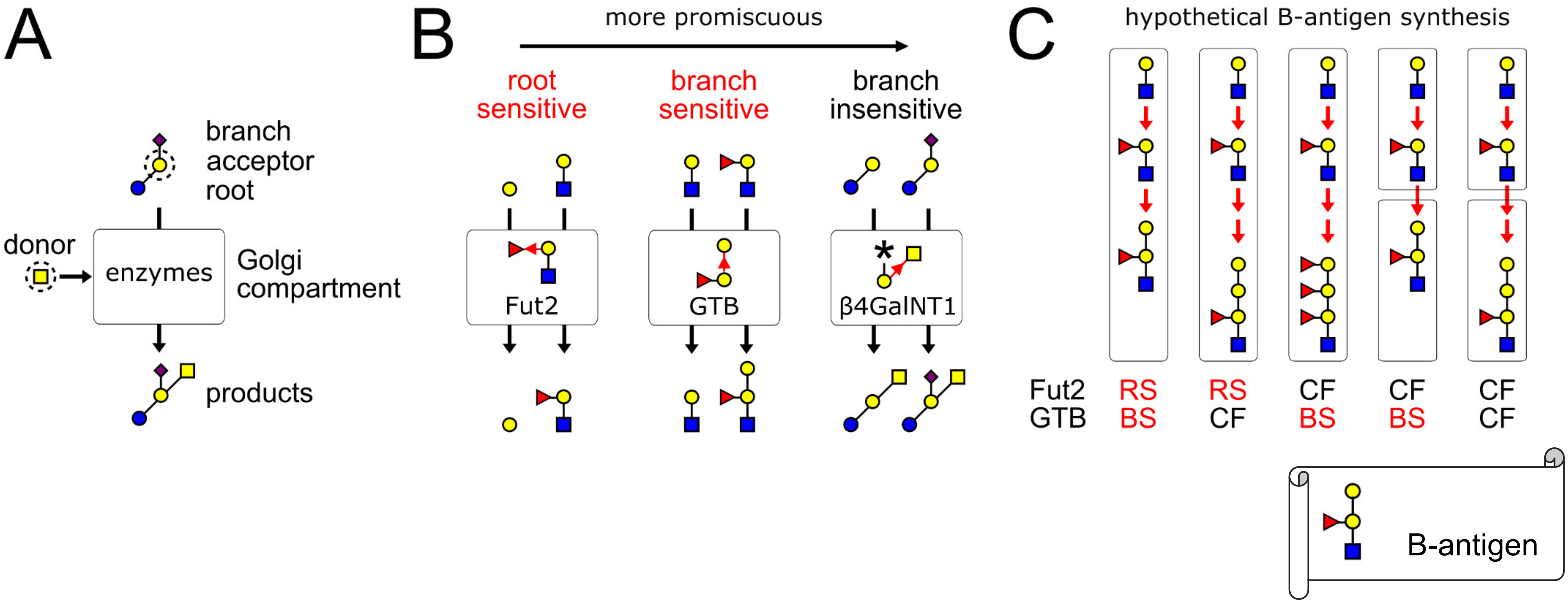
- It is estimated more than half of human proteins are glycosylated.
- The two major classes
  - N-glycans: at Asn
  - O-glycans: at Ser/Thr

The synthetic pathway of N-glycans



Reily, C., Stewart, T. J. *et al. Nat. Rev. Nephrol.* **2019**, *15*, 346–366.
Chaffey, P. K., Guan, X. et al. *Chemical Biology of Glycoproteins*, *RSC* **2017**, 1–19.

Glycans are biosynthesized by more than 250 glycosyltransferases (GTs).



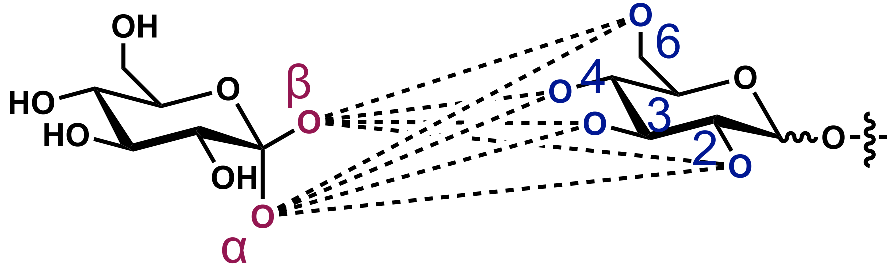
- Glycans are synthesized by glycosyltransferases (GTs).
- Each GTs have relatively low specificity, but the variability is tightly controlled by compartmentalization.
- The detailed mechanism is still unknown.

Jaiman, A. and Thattai, M. *Elife* **2020**, *9*, 1–22.

Because of their high complexity, the study of glycans is quite difficult.

Structural diversity

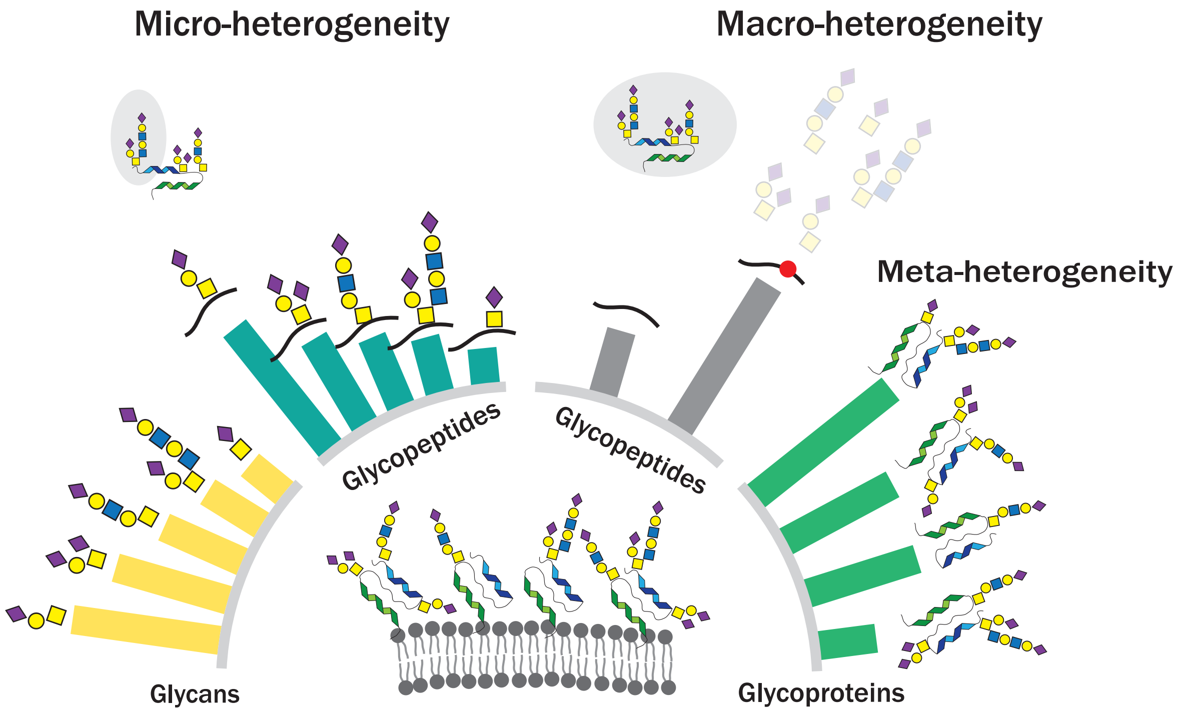
D-Glc – D-Glc
(8 patterns)



theoretical number of different oligomers

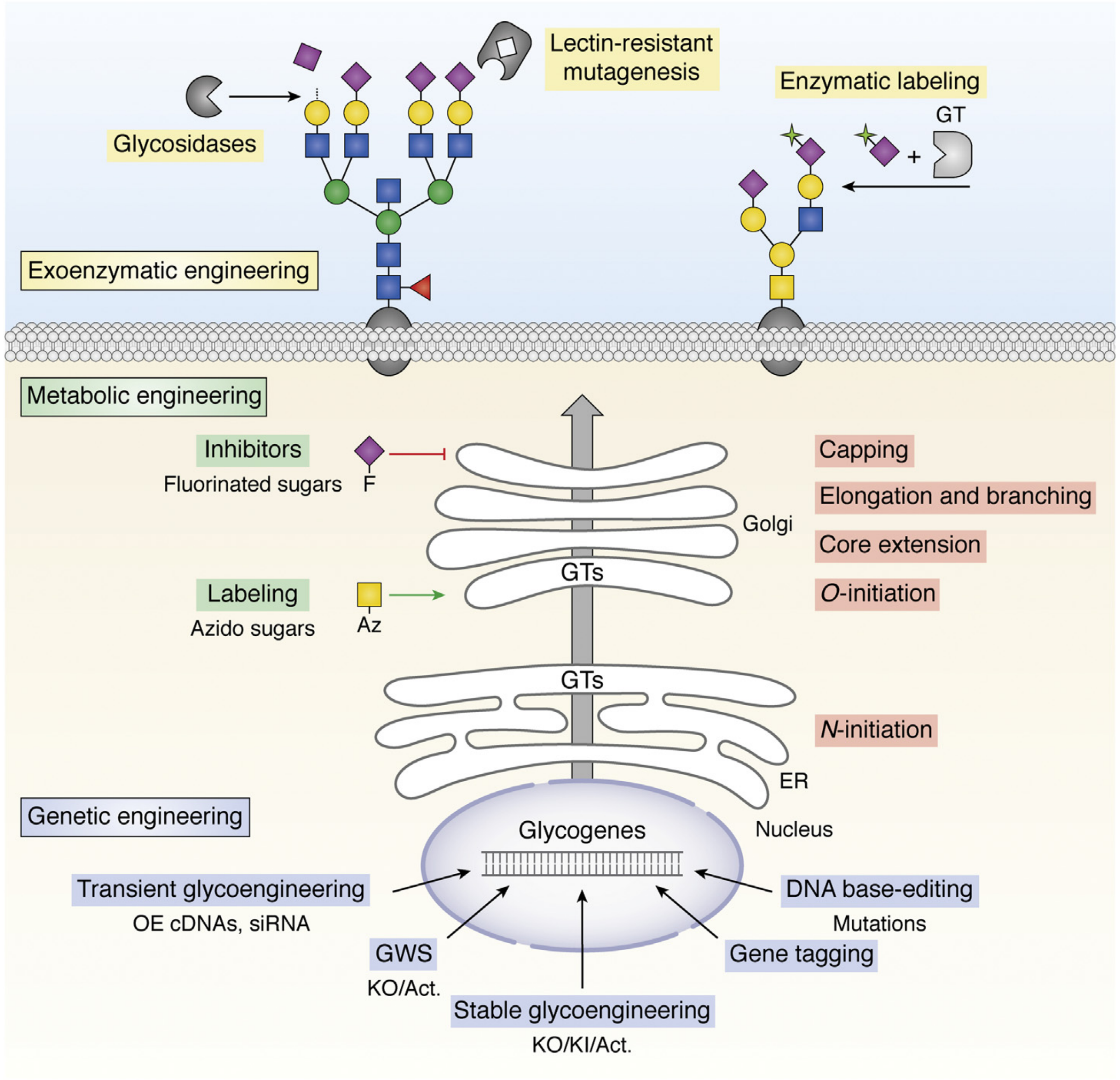
Oligomer size	DNA	Protein	Glycan
1	4	20	20
2	16	400	1360
3	64	8,000	126,080
4	256	160,000	13,495,040
5	1024	3,200,000	1,569,745,920
6	4096	64,000,000	192,780,943,360

Heterogeneity



Werz, D. B., Ranzinger, R. *et al. ACS Chem. Biol.* **2007**, *2*, 685–691.
Čaval, T., de Haan, N. *et al. Curr. Opin. Struct. Biol.* **2021**, *68*, 135–141.

Many live-cell glycoengineering tools have been developed.



*GT: glycosyltransferase

Exoenzymatic engineering

today's topic

- ✓ addition of non-natural units
- ✓ precise control of glycans
- ✓ high efficiency
- ✗ identification of a suitable GT is necessary

Metabolic engineering

- ✓ addition of non-natural units
- ✓ precise control of the monosaccharide of interest
- ✓ low off-target effect
- ✗ low efficiency (competition with natural substrates)

Genetic engineering

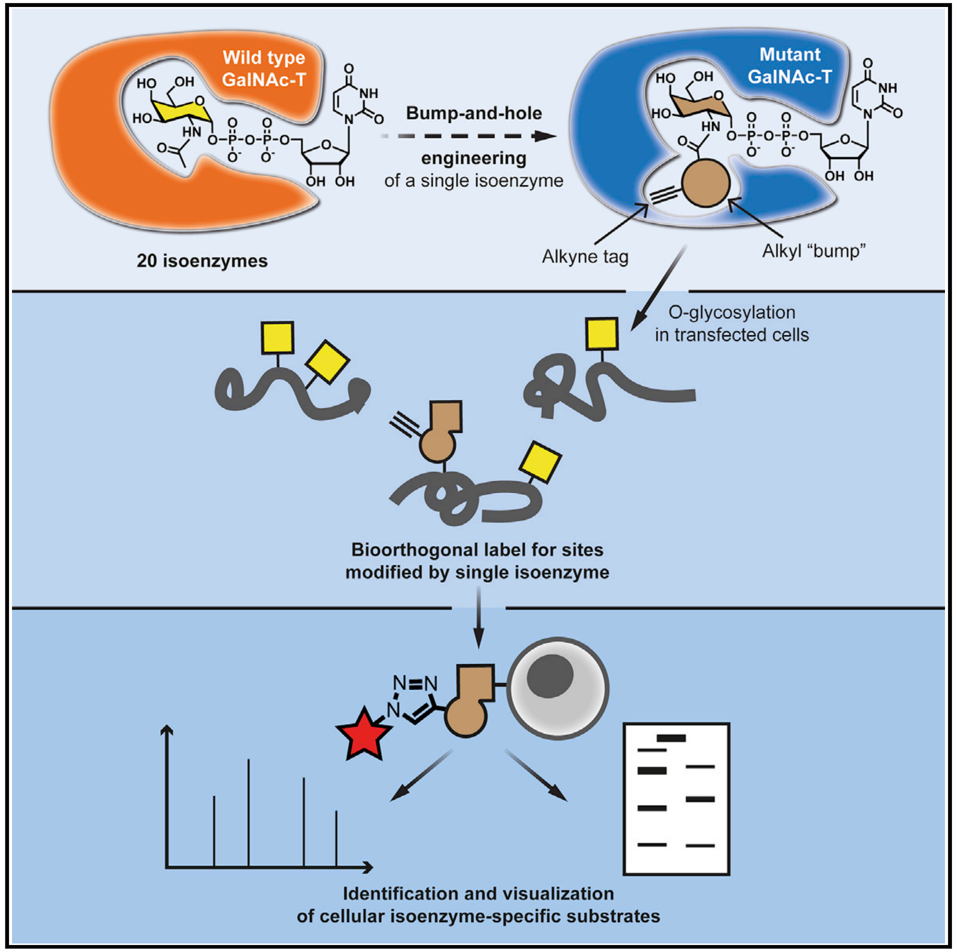
- ✓ a variety of reliable approaches
- ✓ precise control of GTs
- ✗ low glycan- and protein-selectivity
- ✗ developmental defects and embryonic lethality
- ✗ addition of new functionalities is difficult

Griffin, M. E. and Hsieh-Wilson, L. C. *Cell Chem. Biol.* **2016**, *23*, 108–121.
Narimatsu, Y., Büll, C. *et al. J. Biol. Chem.* **2021**, *296*, 100448.

Contents

Next-generation glycoengineering tools have been developed.

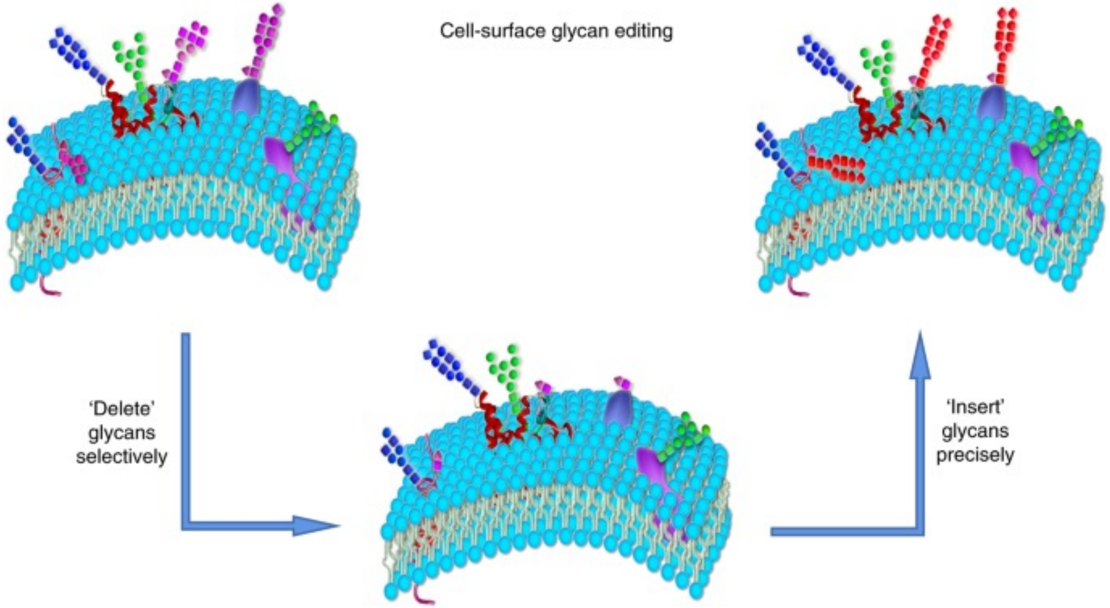
1. Bump-and-Hole engineering (Metabolic engineering)



→ More precise control of glycotransferases

Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.
Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.e15.

2. Two-step glycan editing (Exoenzymatic engineering)



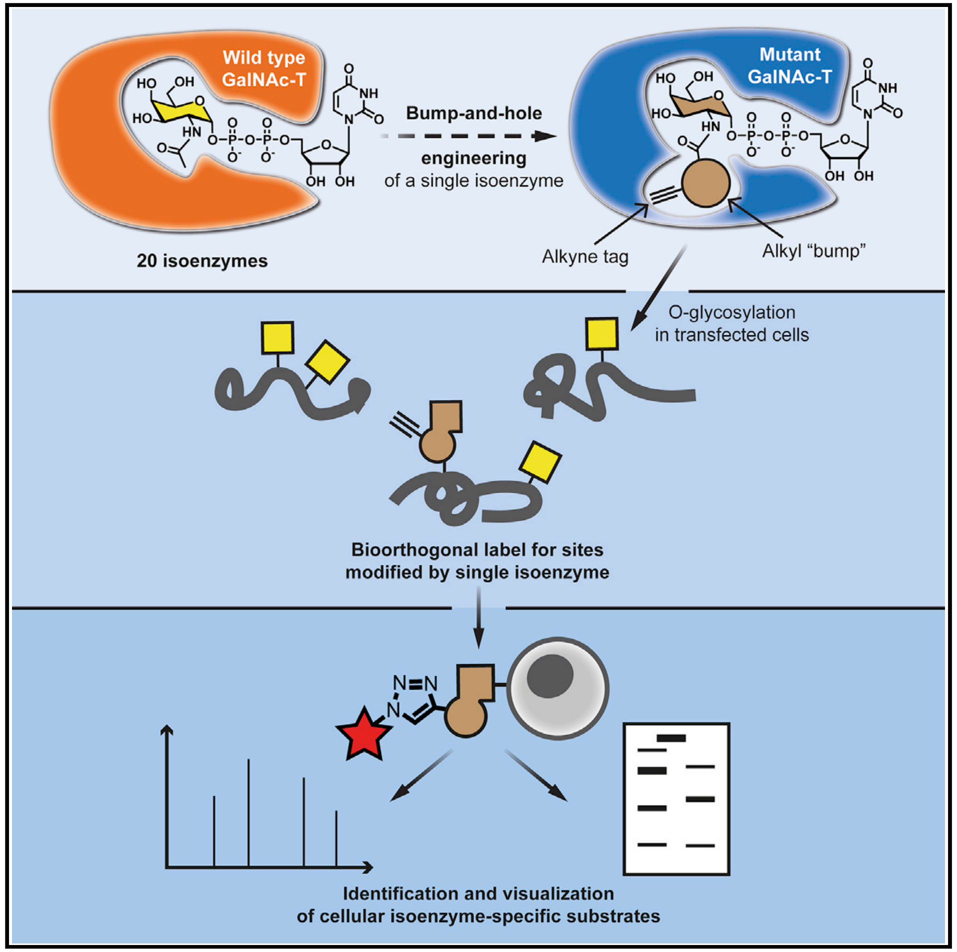
→ More precise control of glycans

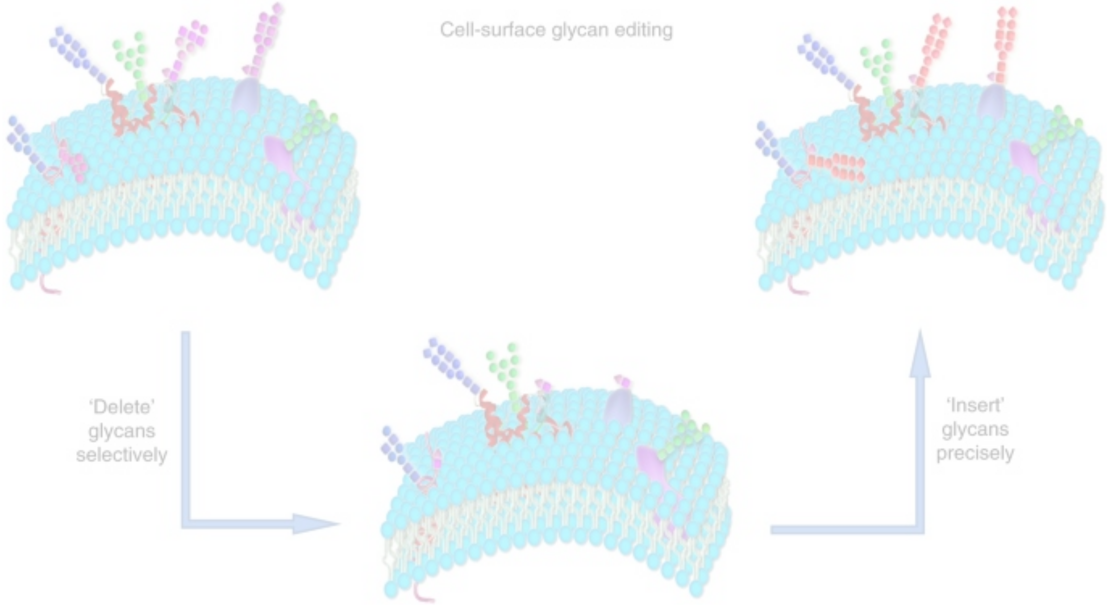
Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

1. Bump-and-Hole engineering

1. Bump-and-Hole engineering (Metabolic engineering)

2. Two-step glycan editing (Exoenzymatic engineering)





→ More precise control of glycotransferases

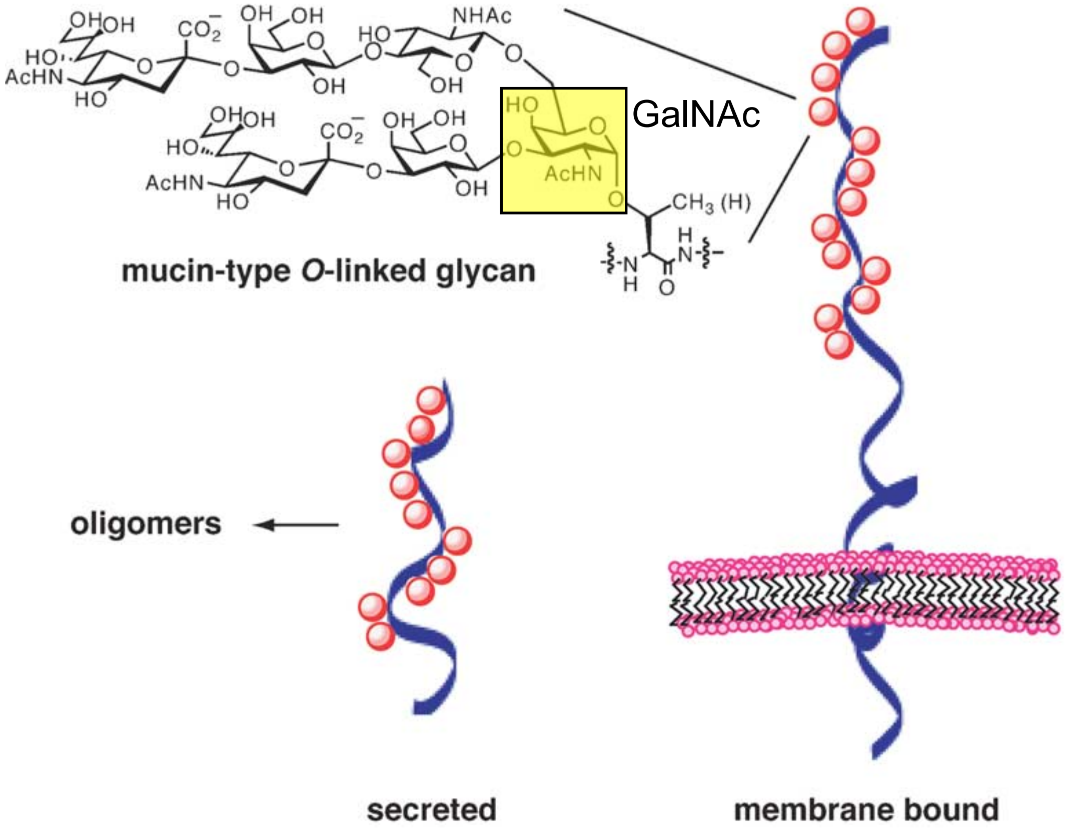
→ More precise control of glycans

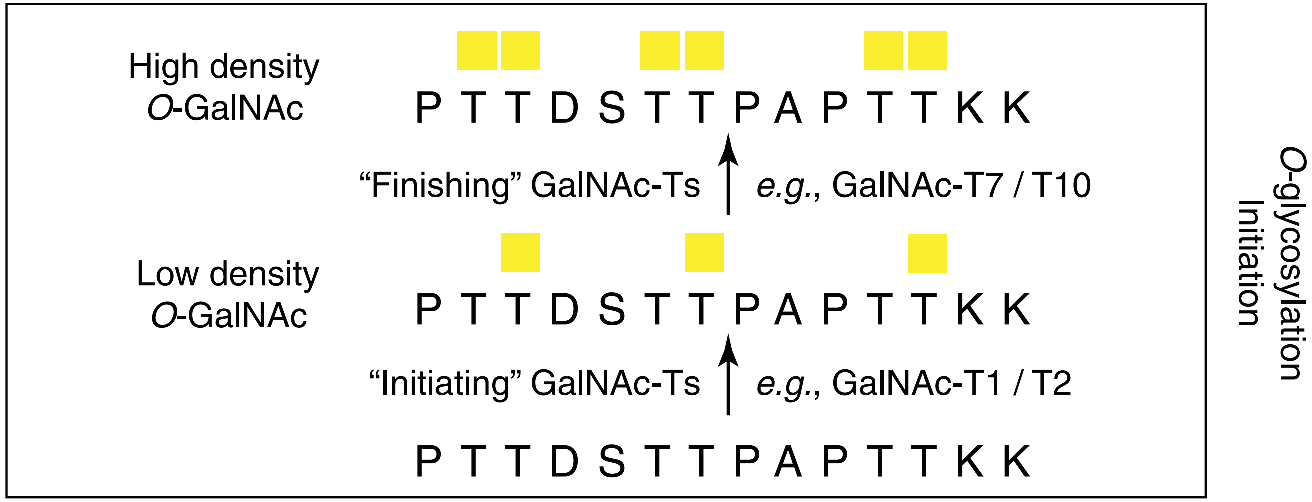
Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.
Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.e15.

Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

Validation of the activity of a certain GalNAc-transferase subtype was difficult.

O-glycans and GalNAc-transferases (GalNAc-T)

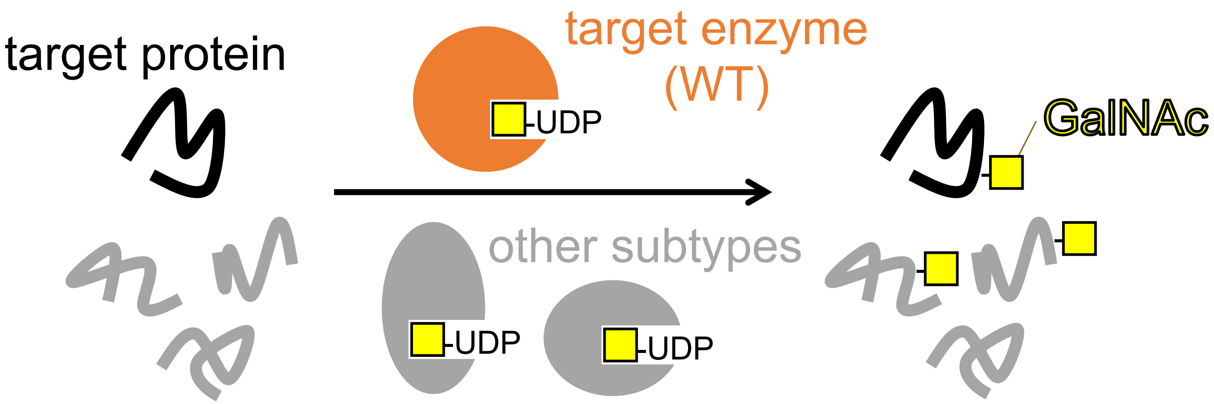




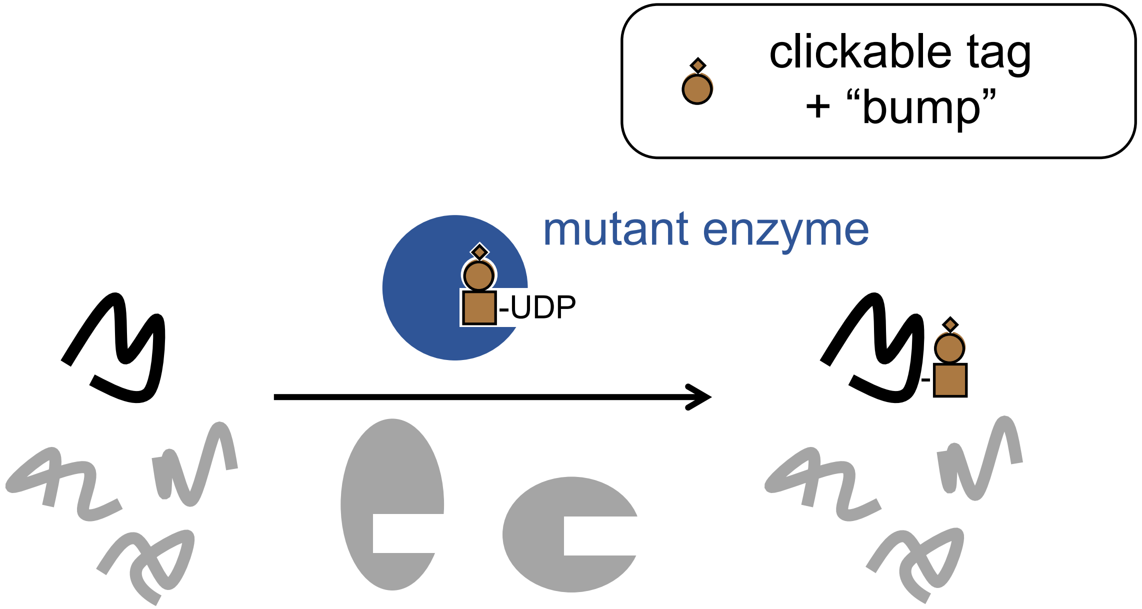
- GalNAc transrefases (GalNAc-Ts) are one of the largent glycosyltransferase families.
- Because of its redundancy, the precise role of each subtype was unclear.

Hang, H. C. and Bertozzi, C. R. *Bioorg. Med. Chem.* **2005**, *13*, 5021–5034.
Gill, D. J., Clausen, H. *et al. Trends Cell Biol.* **2011**, *21*, 149–158.

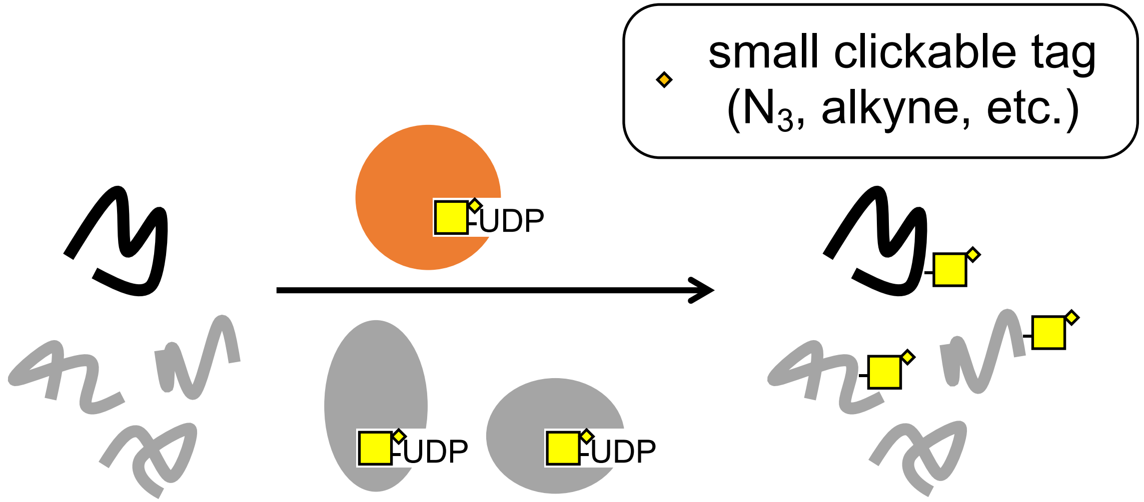
Bump-Hole engineering enabled subtype-specific labeling.

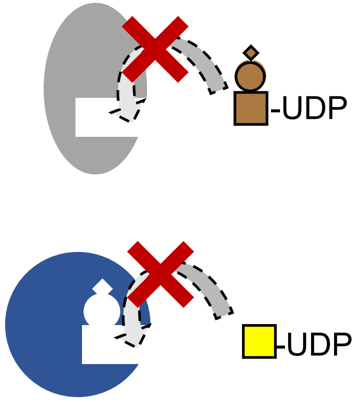


Bump-and-Hole engineering



Normal metabolic labeling



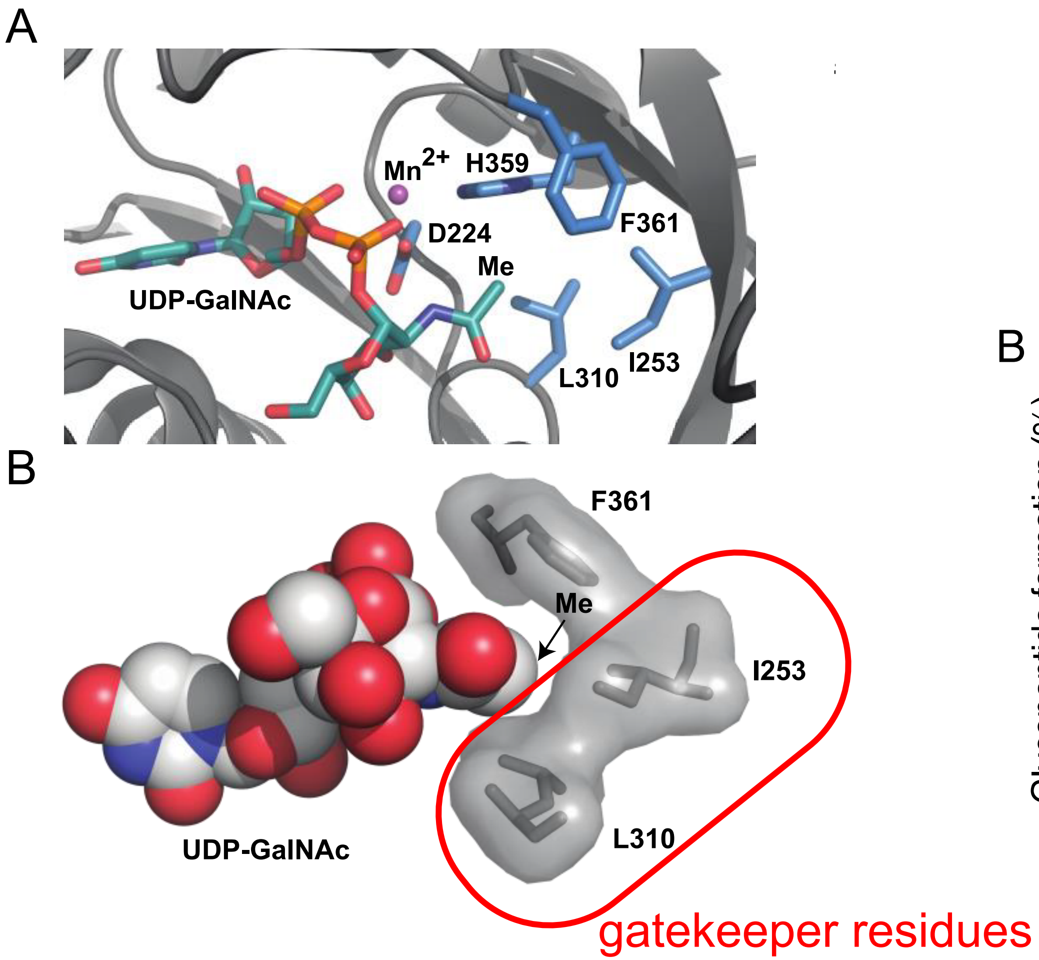


"Bumped" substrates is not recognized by **native enzymes.**
→ Low false-positive signals

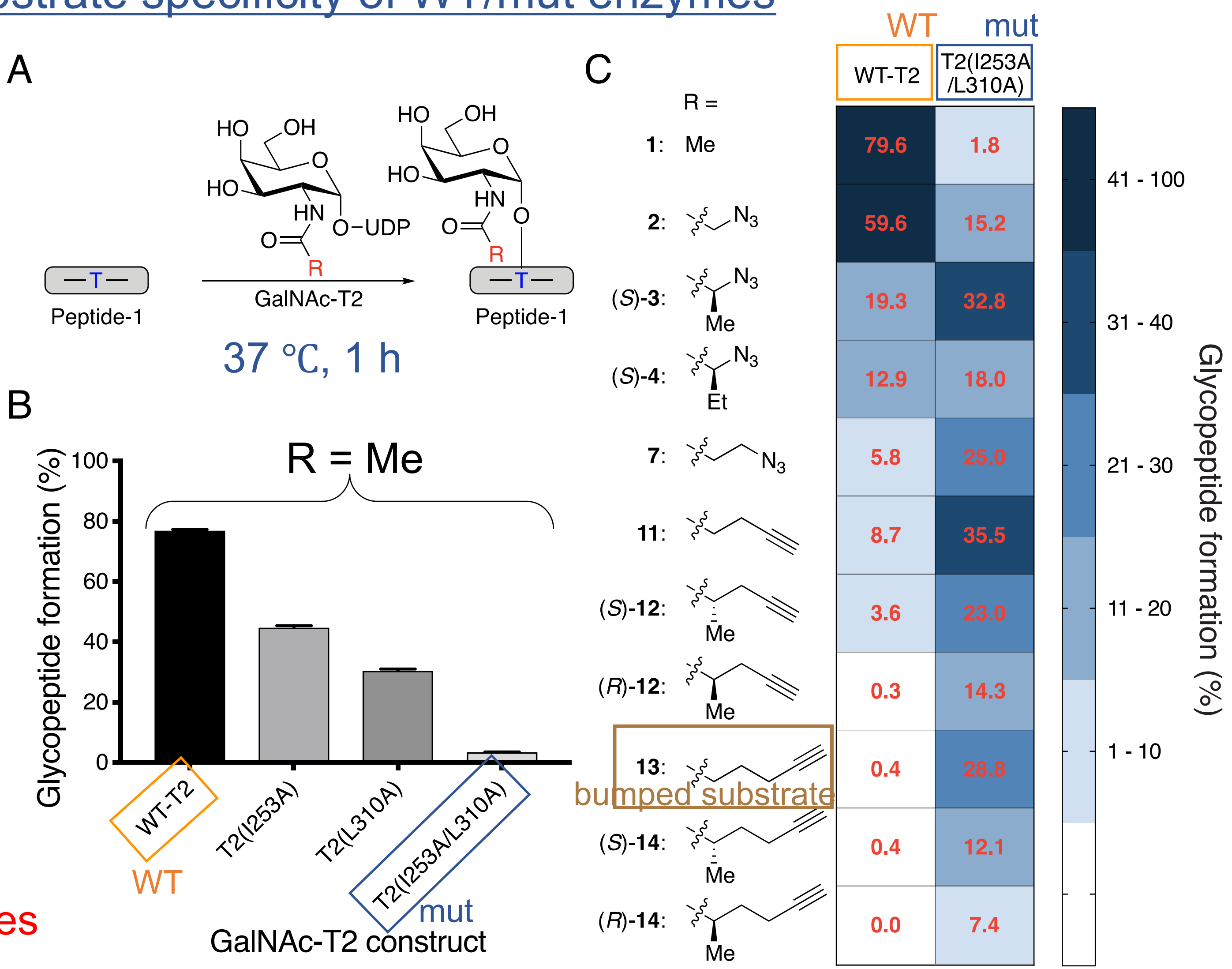
Mutant enzymes do not recognize native substrates.
→ High S/N ratio

Mutagenesis of gatekeeper residues changed substrate specificity of GalNAc-T2 in vitro.

Structure of GalNAc-T2



Substrate specificity of WT/mut enzymes

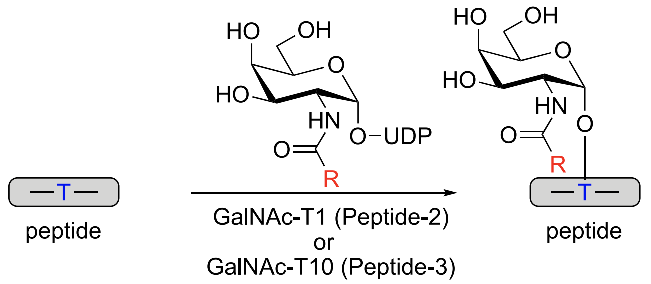


Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.

The same mutation was effective for other subtypes, without changing the site specificity in vitro.

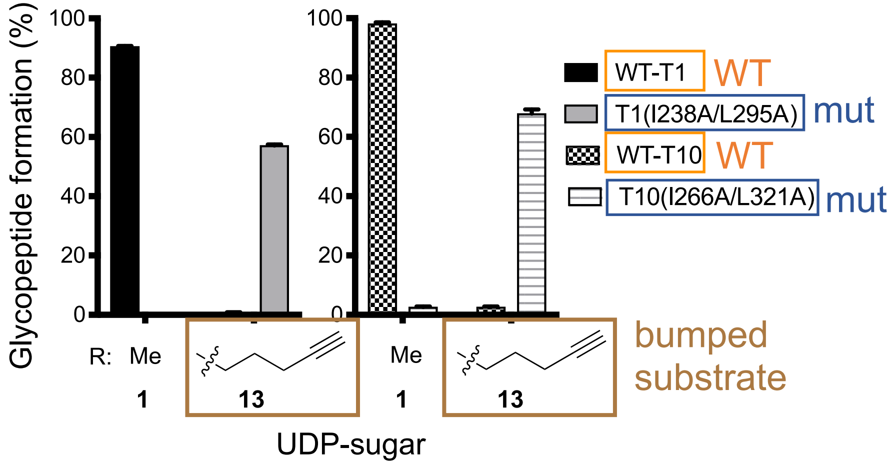
Selectivity of other subtypes

A



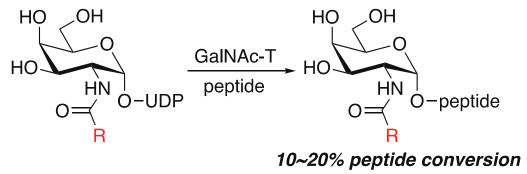
Peptide-2: (DAA)HN−GAGAFFPTPGPAGAGK−COOH
Peptide-3: (DAA)HN−GTT*PSPVPTTSTTSAP−COOH
T* = (α-O-GalNAc)-T

B



Glycosylation site specificity (LC-MS/MS)

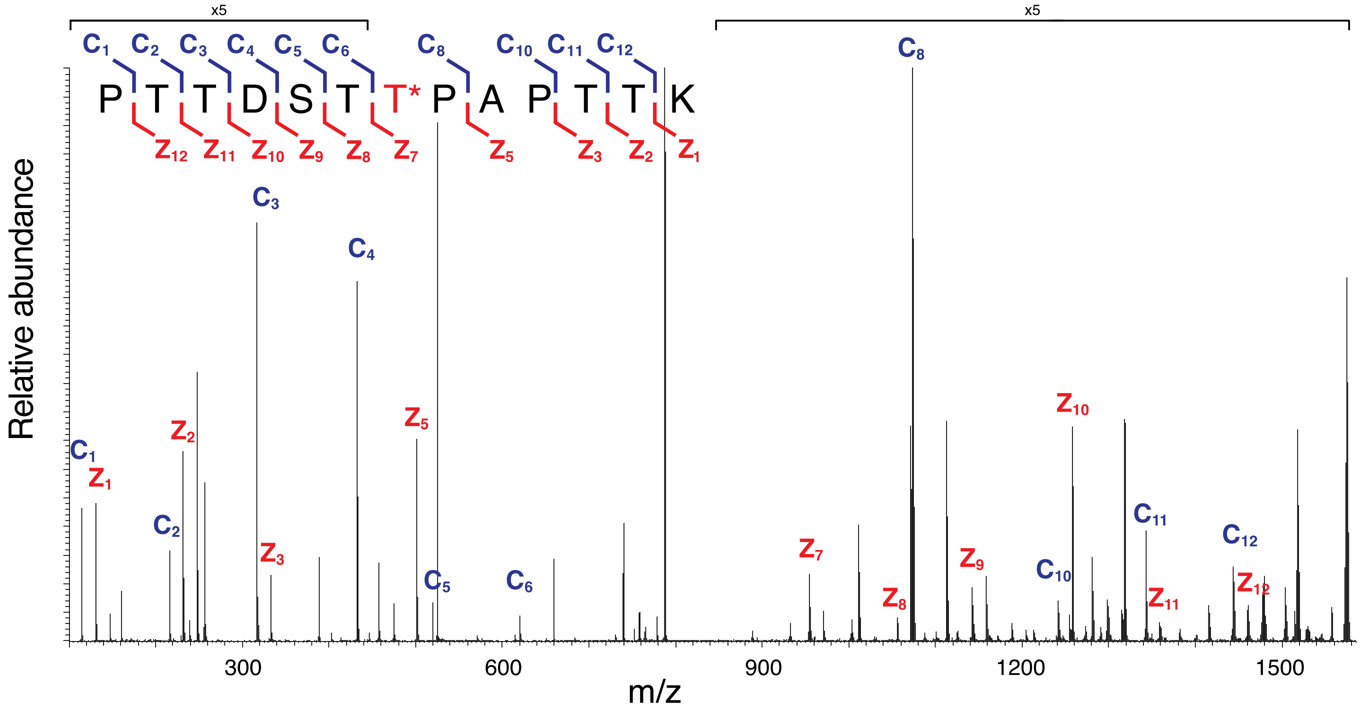
A



B

Peptide	Glycosylated peptide	WT-T2/**1**	T2(I253A/L310A)/**13**
MUC5AC-3 (GTT*PSPVPTTSTTSAP)	GTT*PSPVPTTSTT*SAP	64.7%	63.2%
	GTT*PSPVPTT*STTSAP	22.7%	17.5%
	GTT*PSPVPT*TSTTSAP	12.6%	19.3%
MUC5AC-13 (GTTPSPVPTTSTT*SAP)	G(TT)*PSPVPTTSTT*SAP	95.4%	94.6%
	GTTPSPVPT*TSTT*SAP	4.6%	5.4%
EA2 (PTTDSTTPAPTTK)	PTTDSTT*PAPTTK	100%	100%
Peptide	**Glycosylated peptide**	**WT-T1/1**	**T1(I238A/L295A)/13**
EA2	PTTDSTT*PAPTTK	100%	100%
Peptide	**Glycosylated peptide**	**WT-T10/1**	**T10(I266A/L321A)/13**
MUC5AC-3	GT*T*PSPVPTTSTTSAP	100%	100%

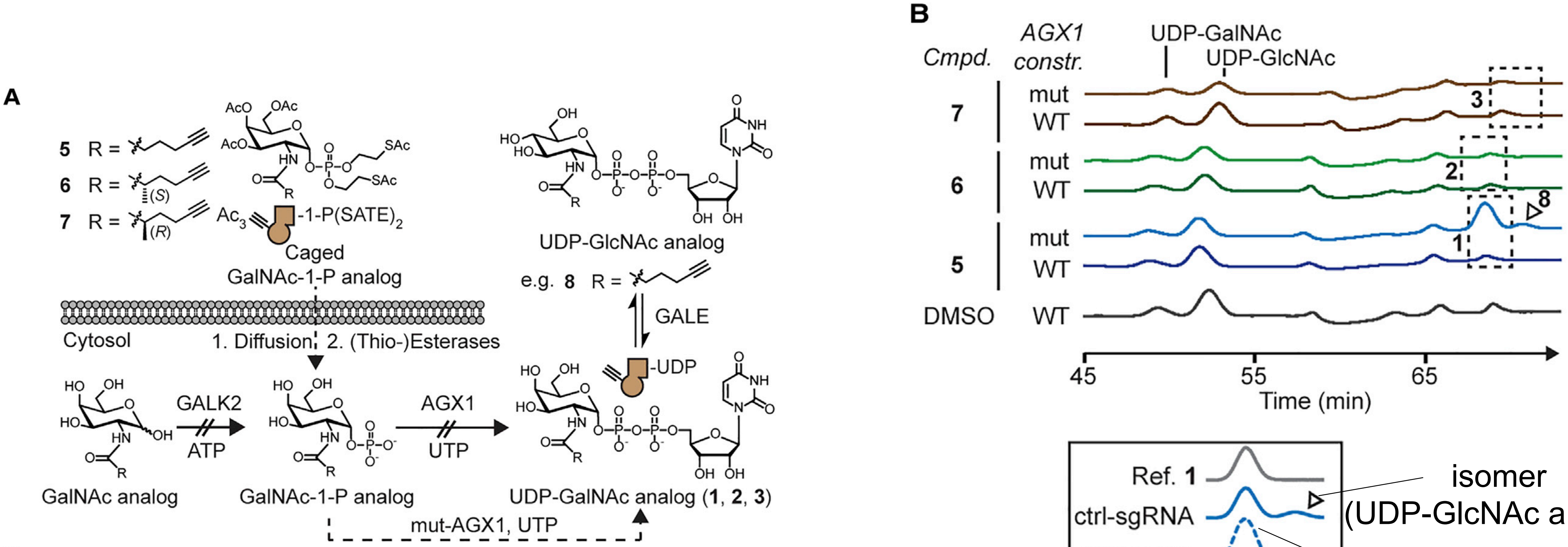
C

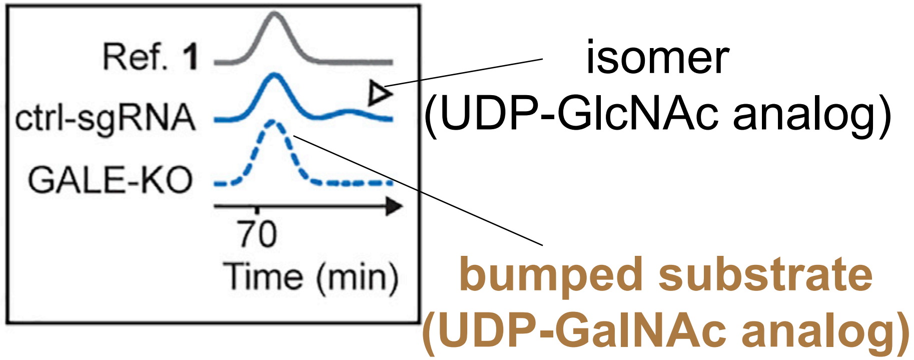


Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.

Caged compound was converted to GalNAc-UDP analog in living cells.

Substrate delivery using caged substrate analog

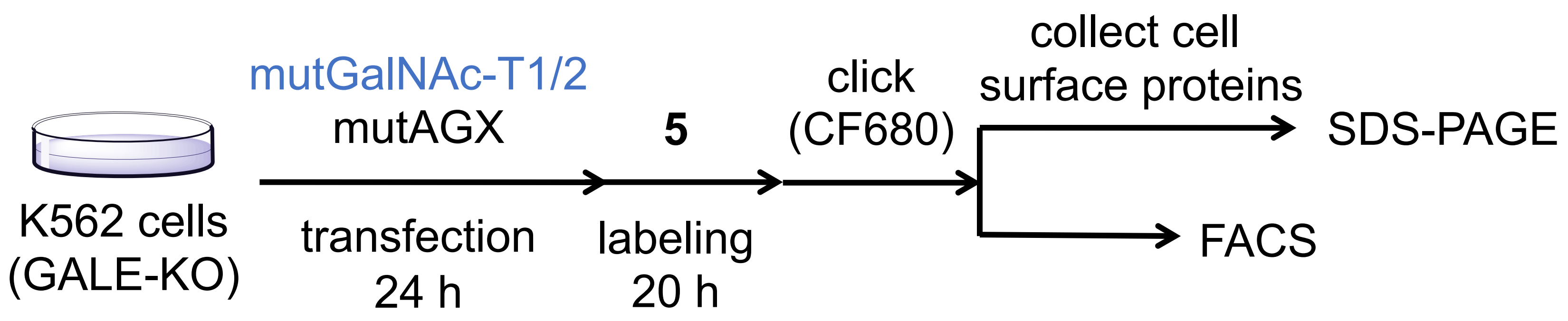


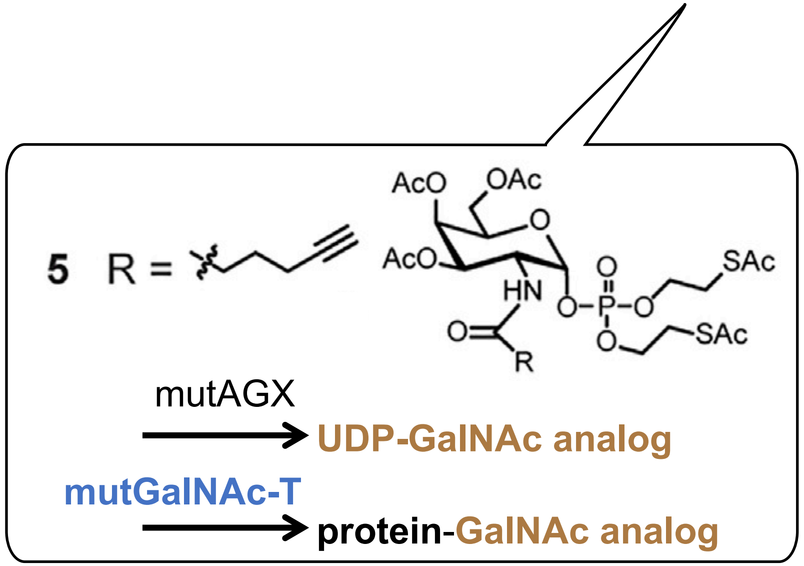


- Expression of mutAGX (F383A) and knockout of GALE was necessary.
- Compound **5** was the only substrate of mutAGX.

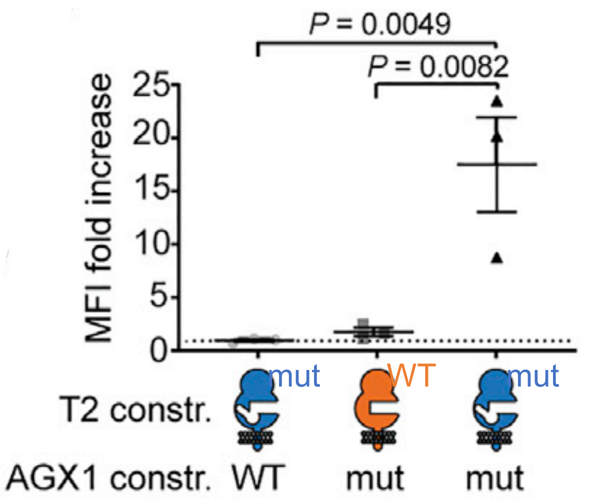
Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.

Selective metabolic labeling was achieved in living cells.

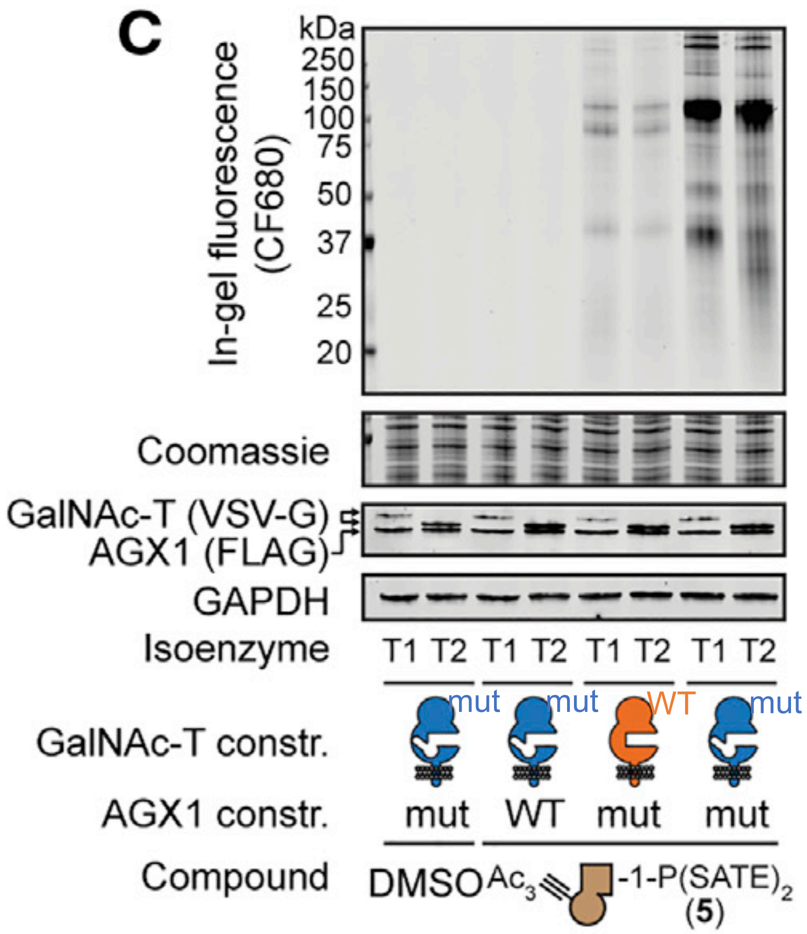




FACS



SDS-PAGE

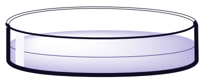


- More than 15-fold fluorescence was detected from labeled cells.
- There were slight difference between band patterns from GalNAc-T1 and GalNAc-T2.

Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.

Bumped GalNAc analog was recognized by downstream enzymes.

n = 1 or 2



HepG2 cells
(GalNAcTn-KO)

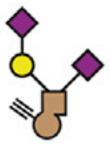
mutGalNAc-Tn
mutAGX → transfection 24 h

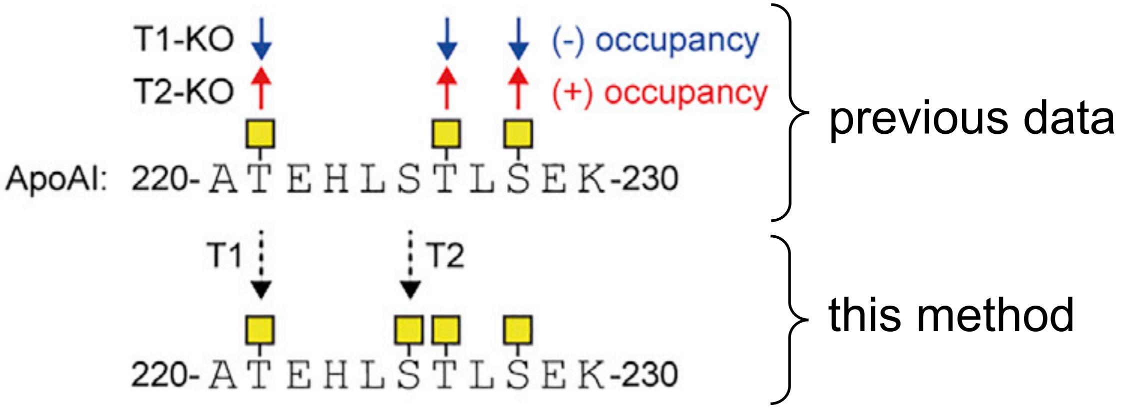
5 → labeling 20 h

collect cell culture supernatants (secretome) →

click (acid-cleavable biotin linker);
digestion;
LC-MS/MS

<u>LC-MS/MS</u>

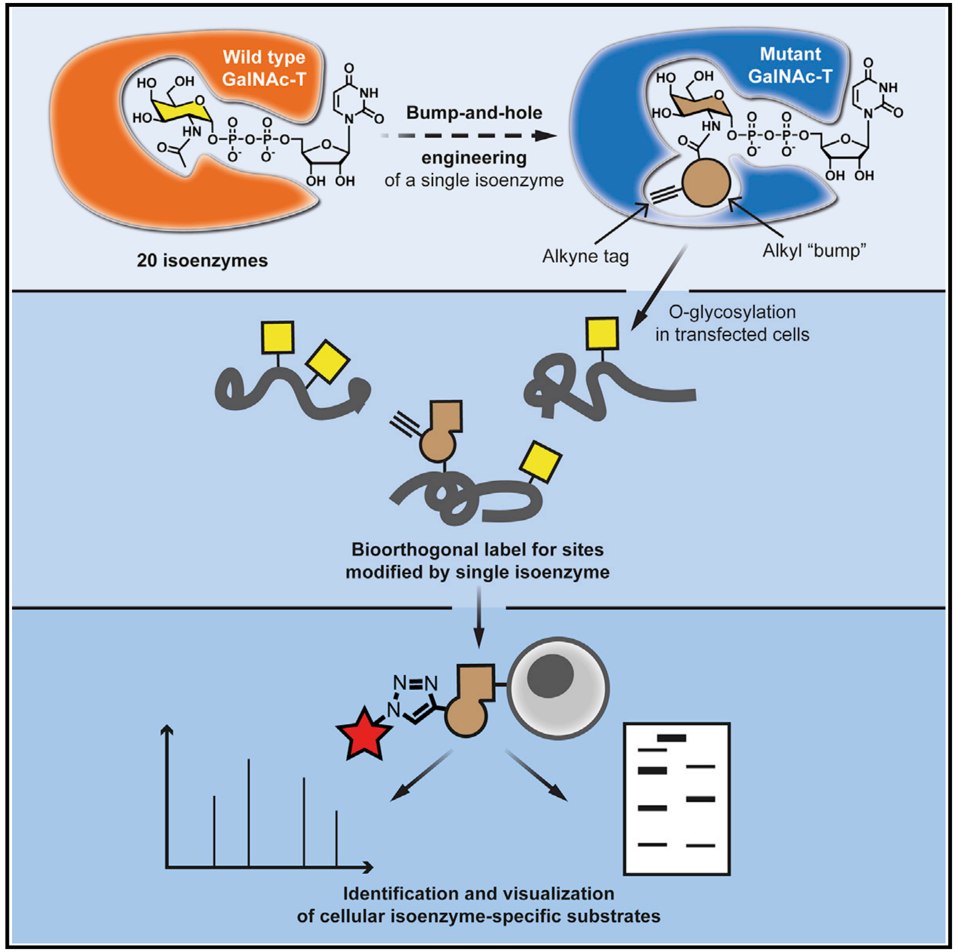
Protein ID	Peptide sequence	Mods found	Biggest glycan
SERPIN5A	KRVEDLHVGA**T***VAPSSR	Gal6yne Hex-Gal6yne Neu5Ac-Hex-Gal6yne $Neu5Ac_2$-Hex-Gal6yne	
STC2	TDA**T***NPPEGPQDR	Gal6yne Hex-Gal6yne	
APOE	VQAAVGT**S***AAPVPSDNH	Gal6yne Hex-Gal6yne	



- Known glycosylation were detected (bumped analog was recognized by downstream enzymes).
- Subtype-specific glycosylation was clearly observed.

Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.

Short summary: Glyco-engineering by bump-and-hole engineering

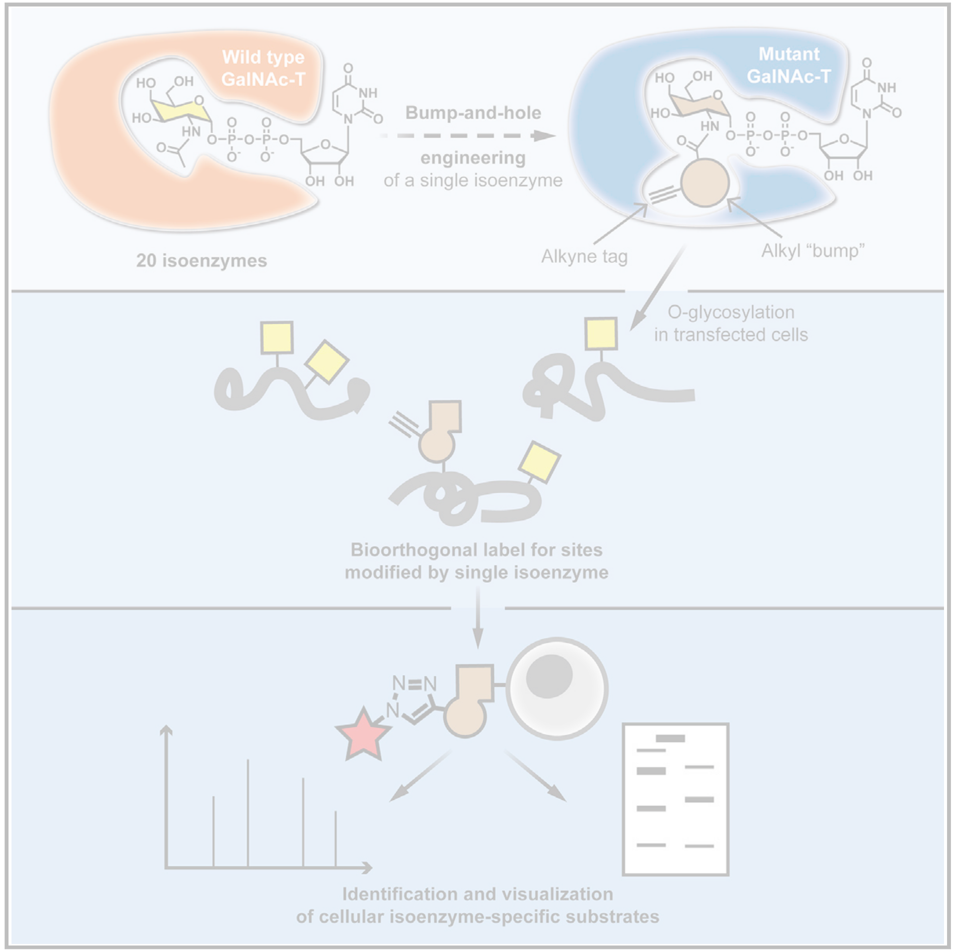


- The first glycotransferase bump-and-hole system in the living cell was established.
- This system allowed isoenzyme-specific labeling in the endogenous environments.

Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.
Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.e15.

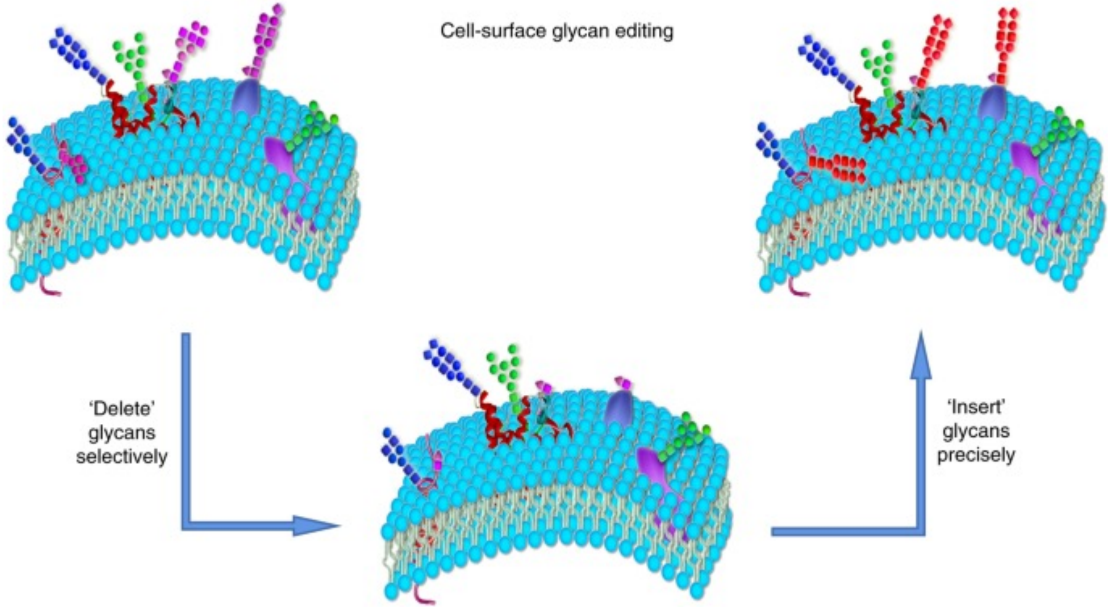
2. Two-step glycan editing

1. Bump-and-Hole engineering (Metabolic engineering)



→ More precise control of glycotransferases

2. Two-step glycan editing (Exoenzymatic engineering)

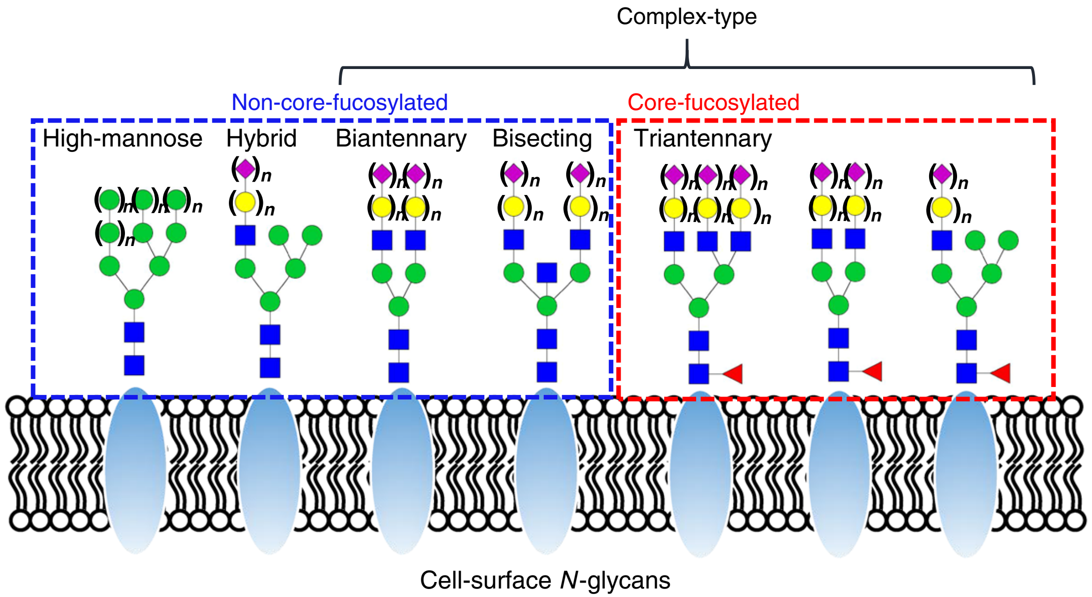


→ More precise control of glycans

Choi, J., Wagner, L. J. S., *et al. J. Am. Chem. Soc.* **2019**, *141*, 13442–13453.
Schumann, B., Malaker, S. A. *et al. Mol. Cell* **2020**, *78*, 824-834.e15.
Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

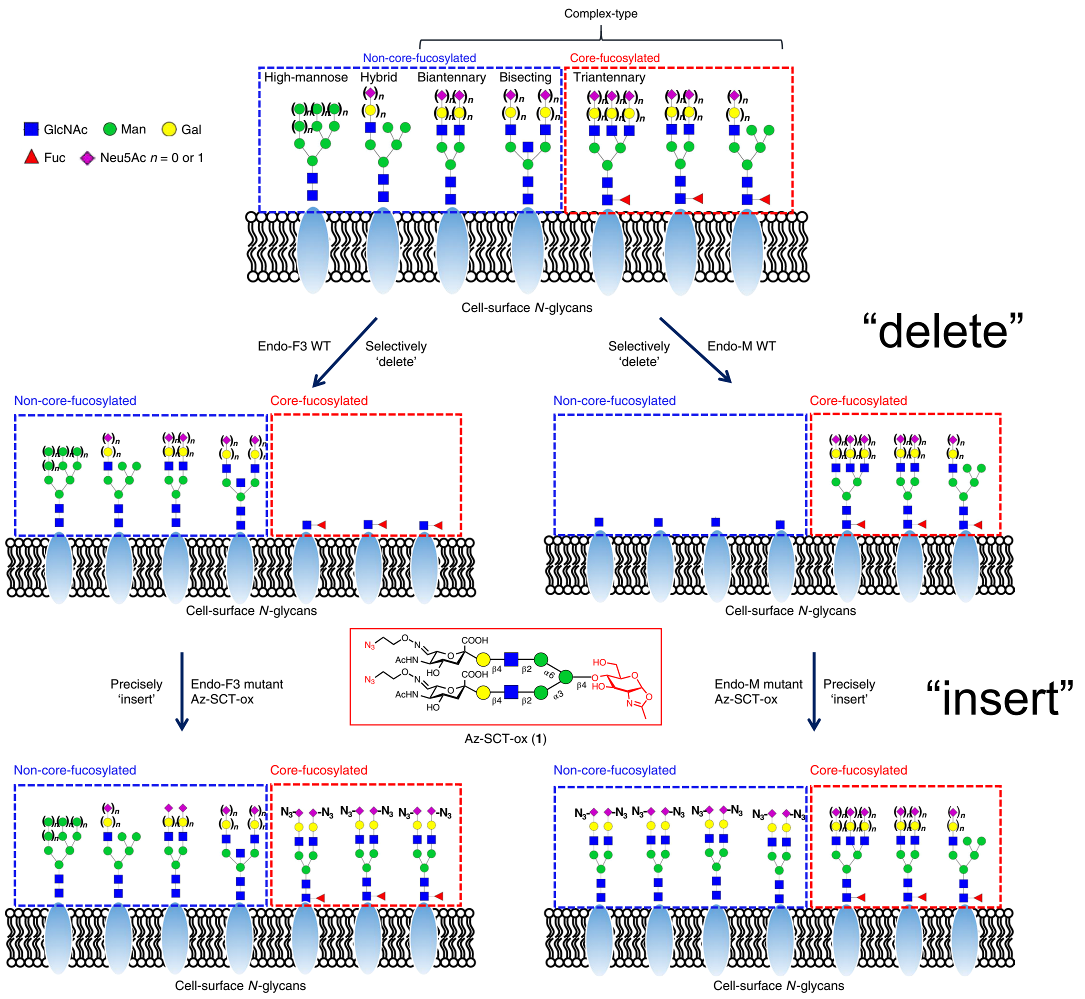
Two-step editing will reduce the heterogeneity of glycans on living cells.

The heterogeneity of N-glycans



- The heterogeneity of glycans often results in ambiguous conclusions about the glycosylation-involved functions of cell membrane.

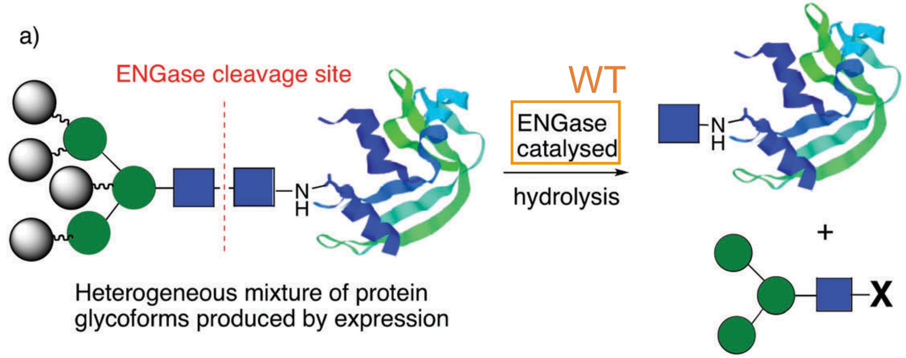
Two-step glycan editing strategy



Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

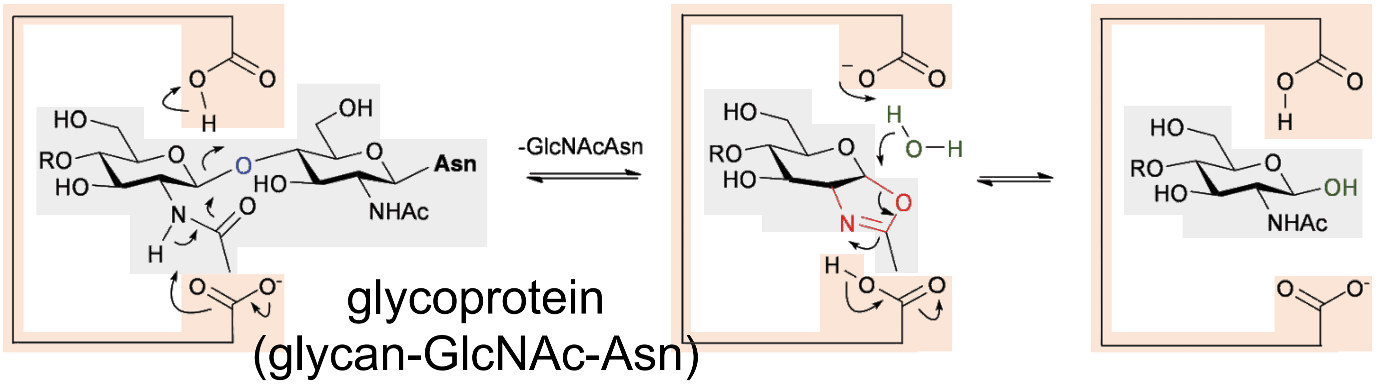
WT/mut endo-glycosidases catalyze glycoprotein “delete/insert” reaction.

Glycoengineering by WT endo-glycosidases (“delete”)

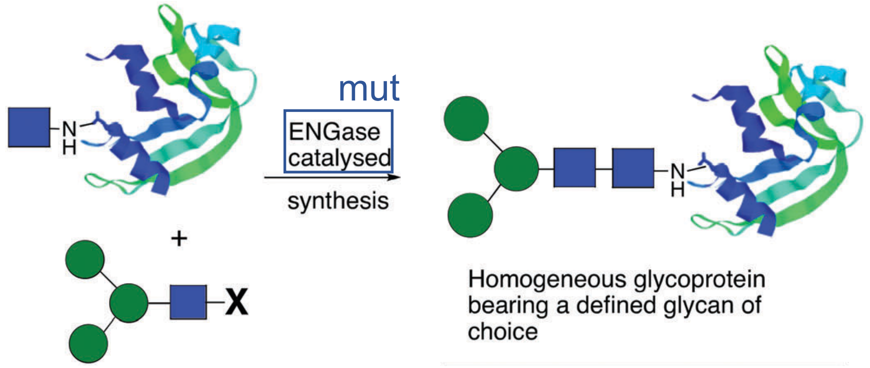


X = OH

WT enzyme (e.g., Endo-F3)

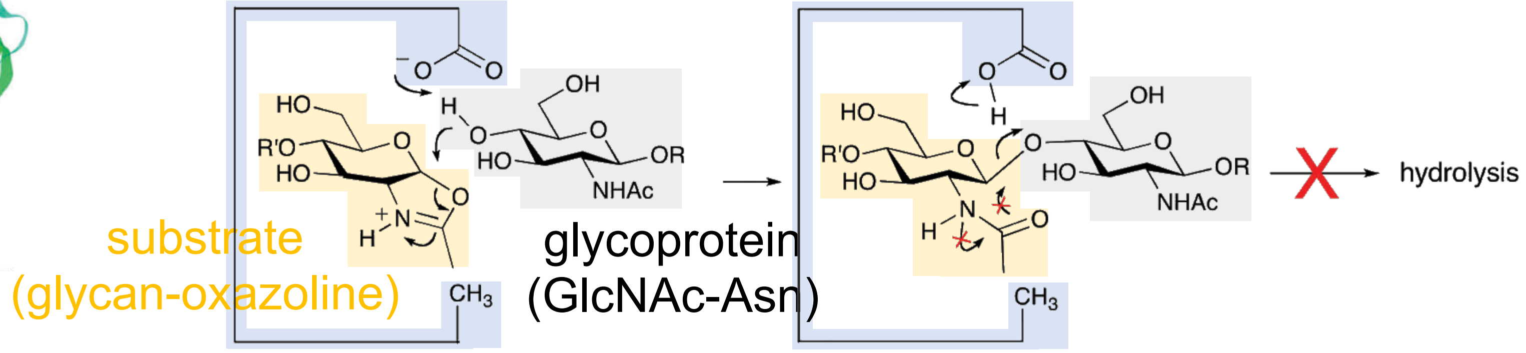


Glycoengineering by mutant endo-glycosidases (“insert”)



X = oxazoline

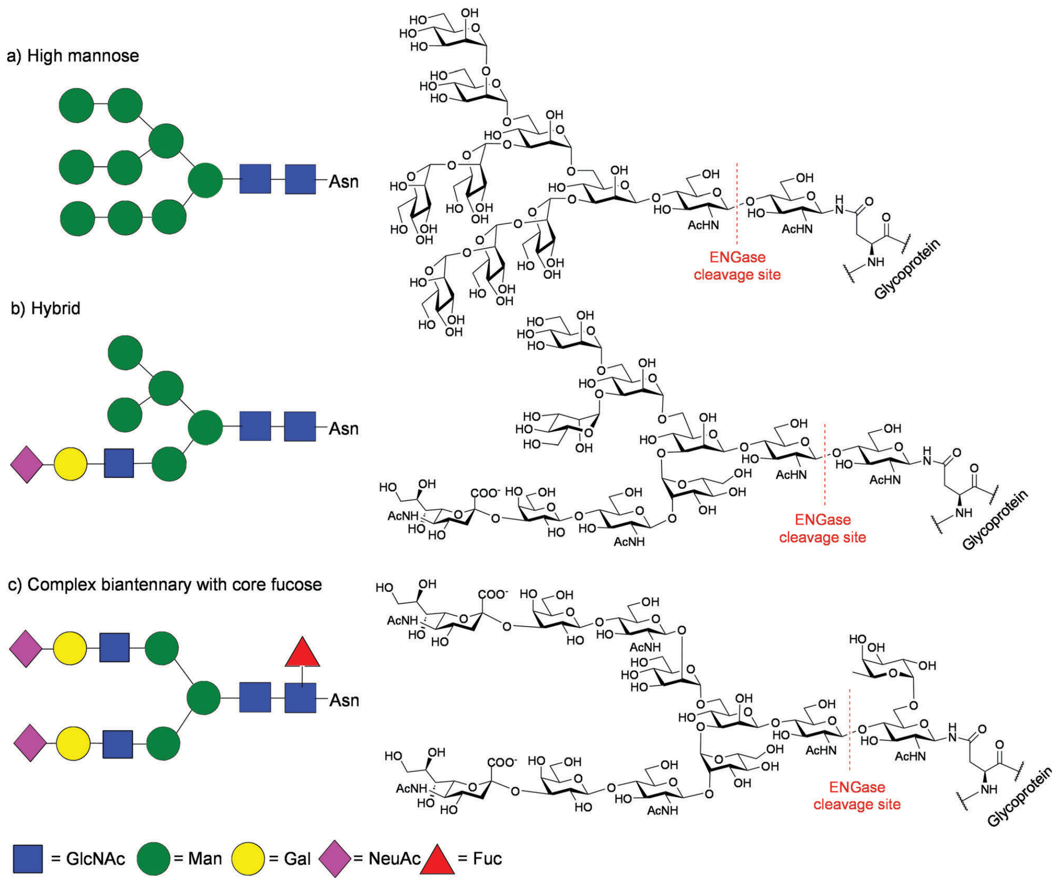
mutant enzyme (e.g., Endo-F3 D165A)

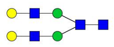
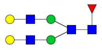
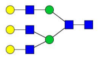
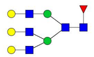
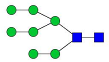
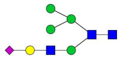


Fairbanks, A. J. *Chem. Soc. Rev.* **2017**, *46*, 5128–5146.

Many endo-glycosidases with different selectivity have been discovered.

Substrate selectivity of endo-glycosidases

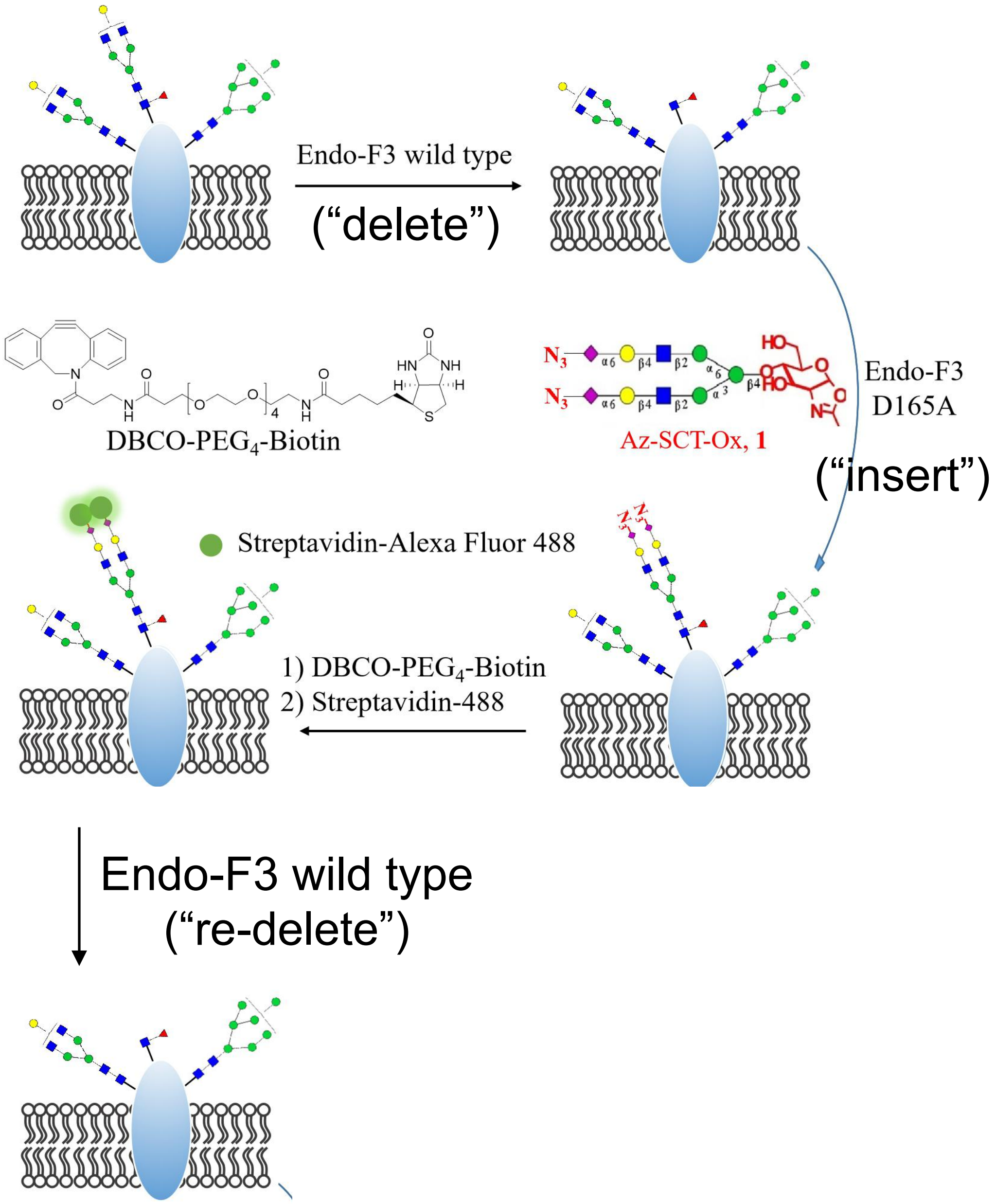


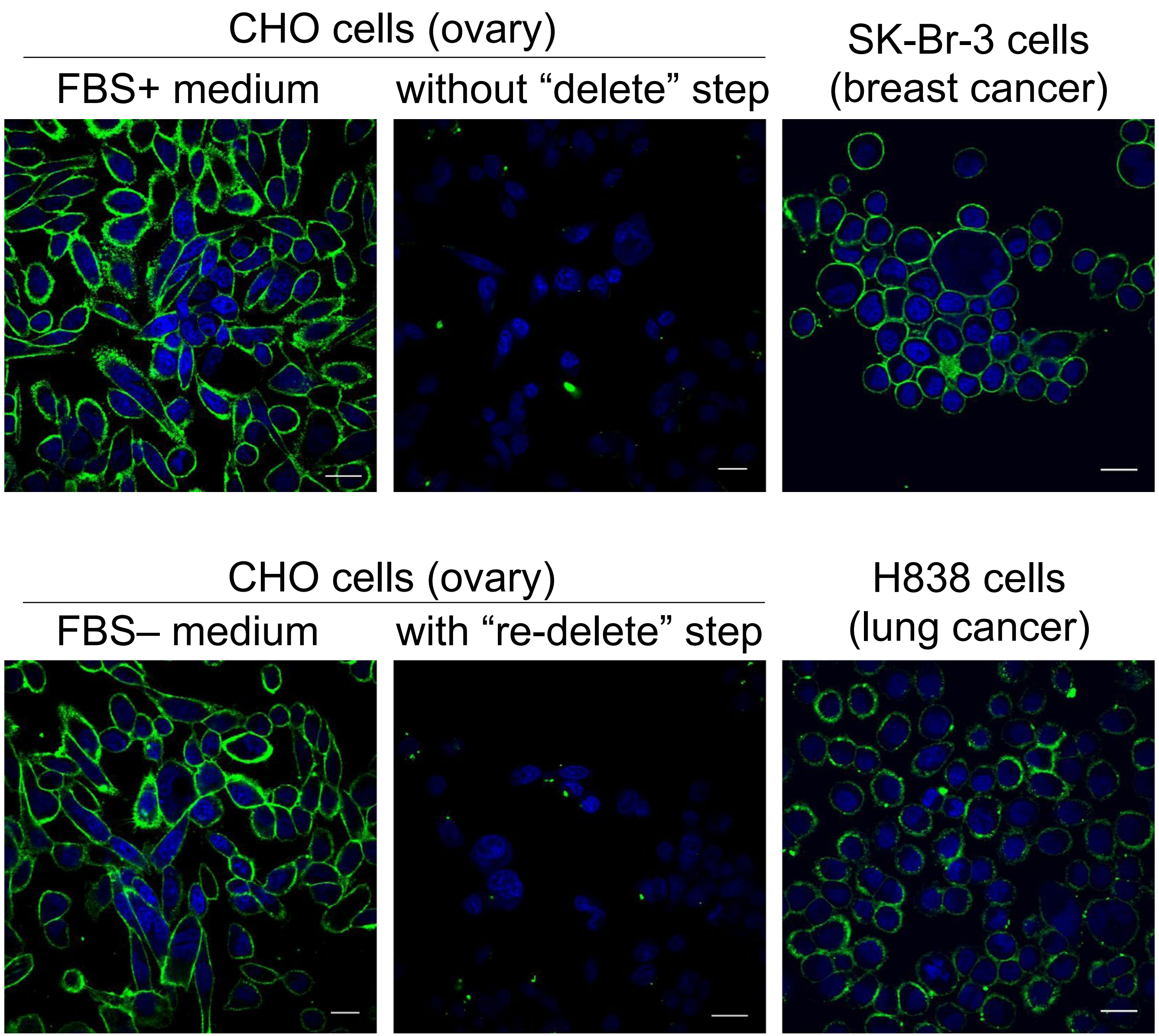
Enzyme	GH family	Biantennary complex-type	Fucosylated Biantennary	Triantennary complex-type	Fucosylated Triantennary	High mannose	Hybrid type
							
Endo-A	85	-	-	-	-	+++	+
Endo-M	85	+	-	-	-	++	+
Endo-Om	85	++	-	2,6-branched triantennary (+)	-	++	+
Endo-H	18	-	-	-	-	+	+
Endo-D	85	Truncated core (+++)	Truncated core (+++)	-	-	-	-
Endo-CC	85	+	-	-	-	+	-
Endo-CE	85	Truncated core (+)	-	-	-	+	+
Endo-BH	85	-	-	-	-	+	+
Endo-S[b]	18	+	+	-	-	-	-
Endo-S2[b]	18	+	+	-	-	+	+
Endo-F1	18	-	-	-	-	+	+
Endo-F2	18	+	+	-	-	-	-
Endo-F3	18	+ (weak)	+++	+ (weak)	+++	-	-

- Endo-F3: selective for core-fucosylated N-glycans (400-fold higher than non-fucosylated ones)
- Endo-M: selective for non-corfe-fucosylated N-glycans

Fairbanks, A. J. *Chem. Soc. Rev.* **2017**, *46*, 5128–5146.
Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

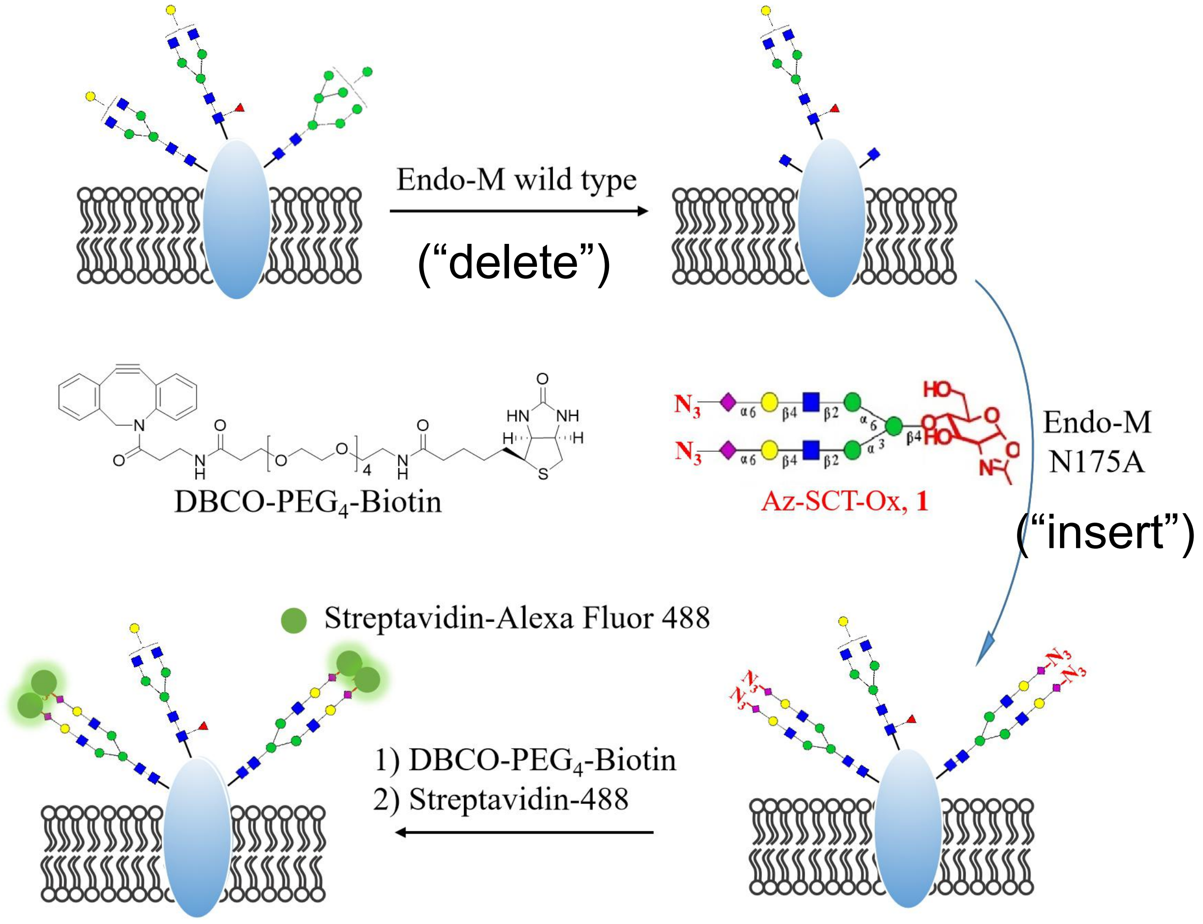
Selective editing and imaging of core-fucosylated N-glycans is achieved.

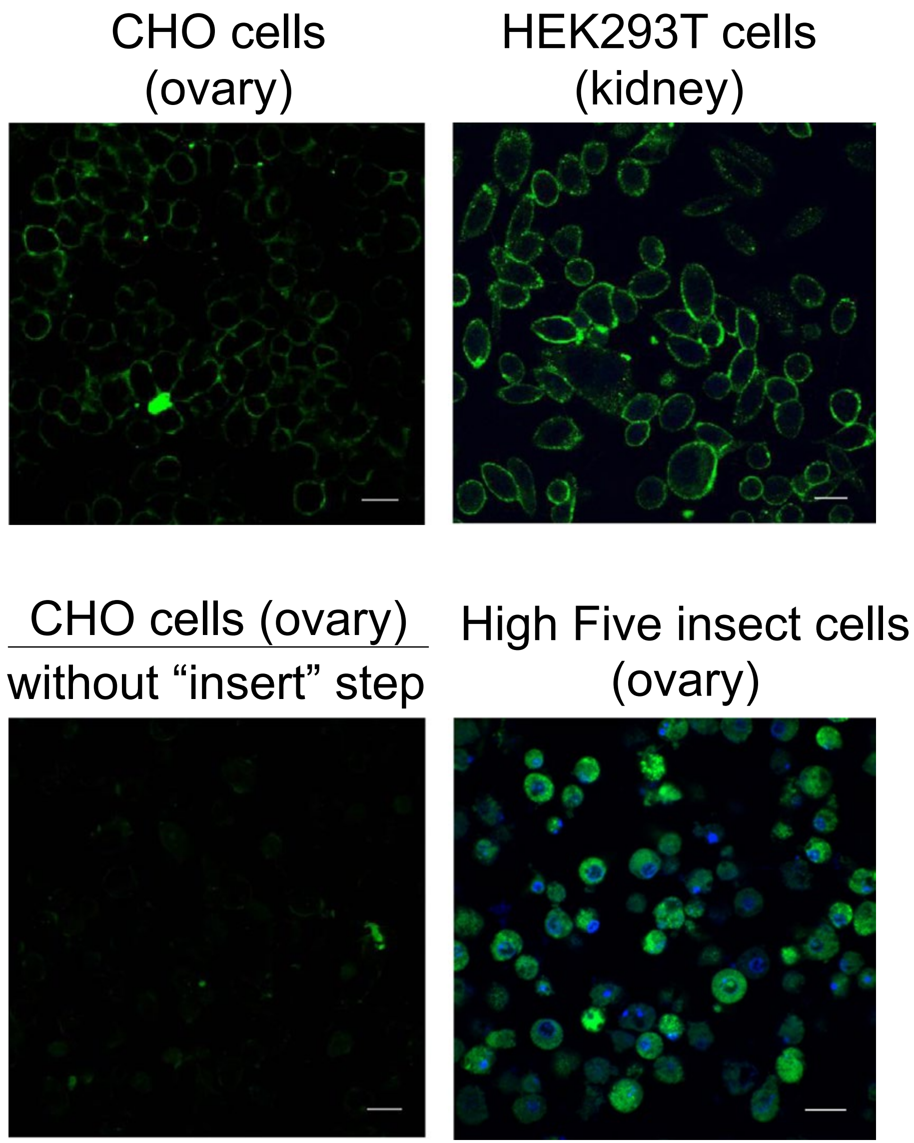




Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

Selective editing and imaging of non-core-fucosylated N-glycans is achieved.

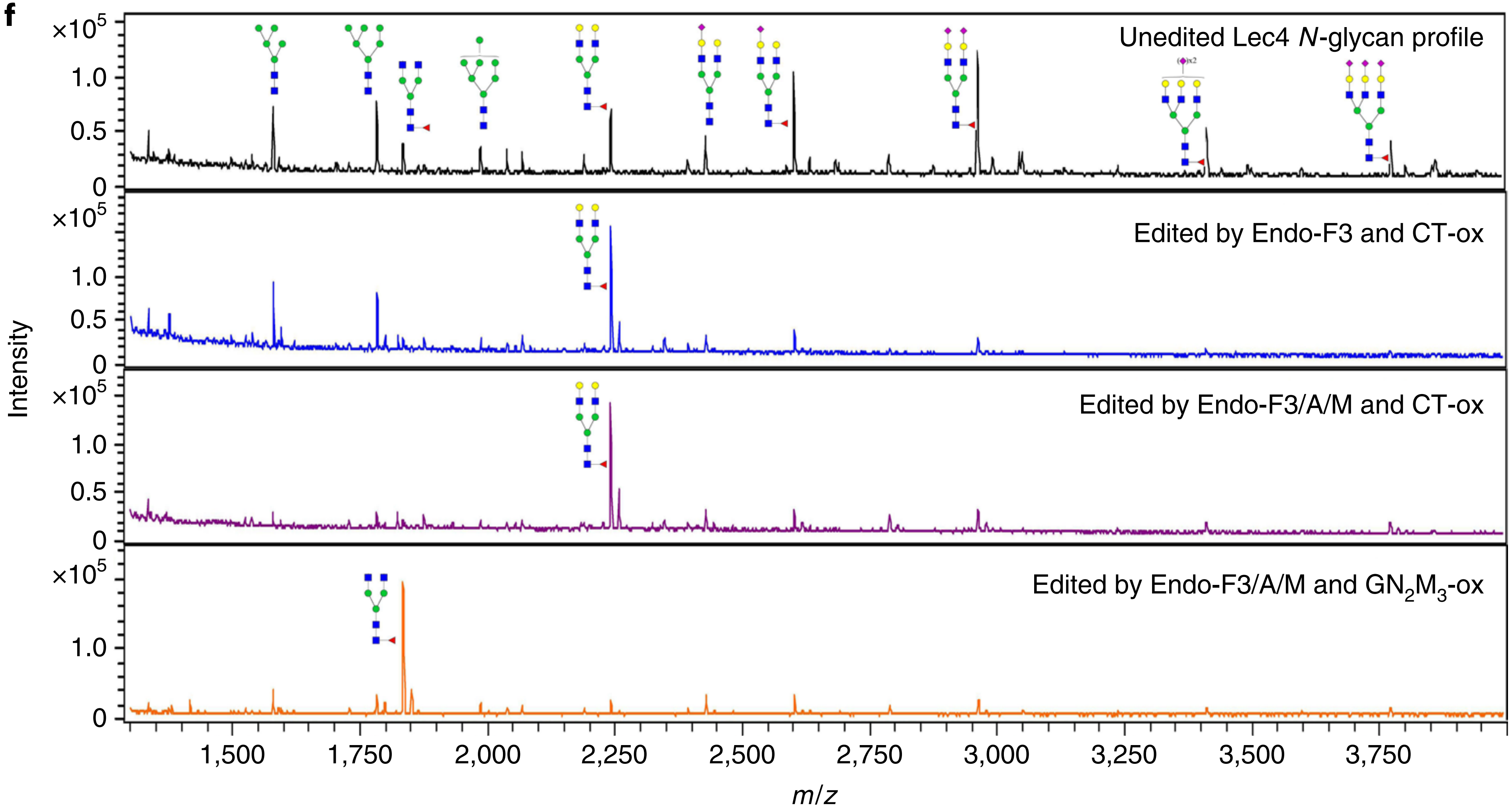




Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

N-glycan editing reduced the heterogeneity of glycans structure-selectively.

Heterogeneity of glycans (MALDI-TOF MS)



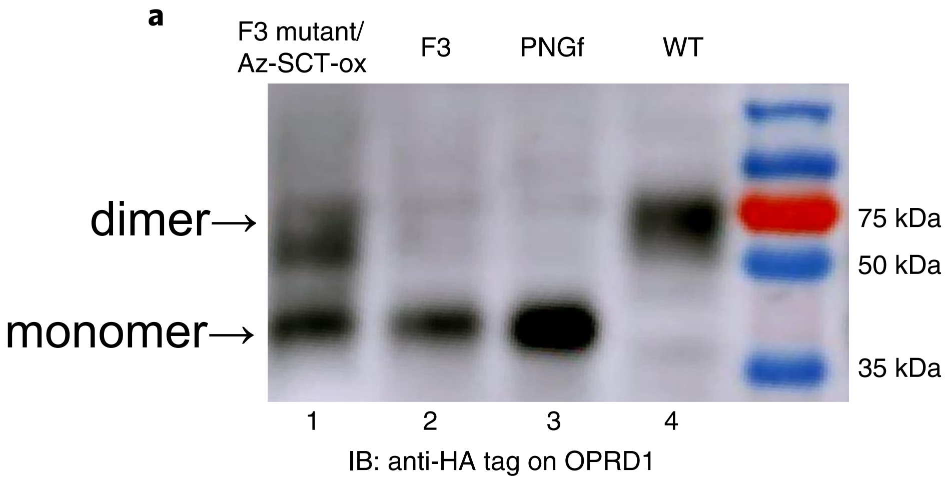
selective editing for fucosylated glycans → less heterogenous

editing for amost all glycans → homogenous

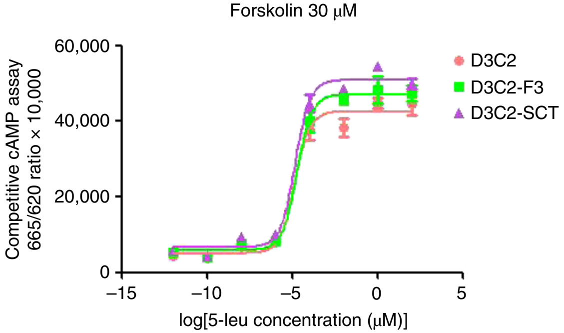
Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

Dimerization and internalization of OPRD1 was highly affected by N-glycans although agonist recognition was not.

Dimerization of OPRD1

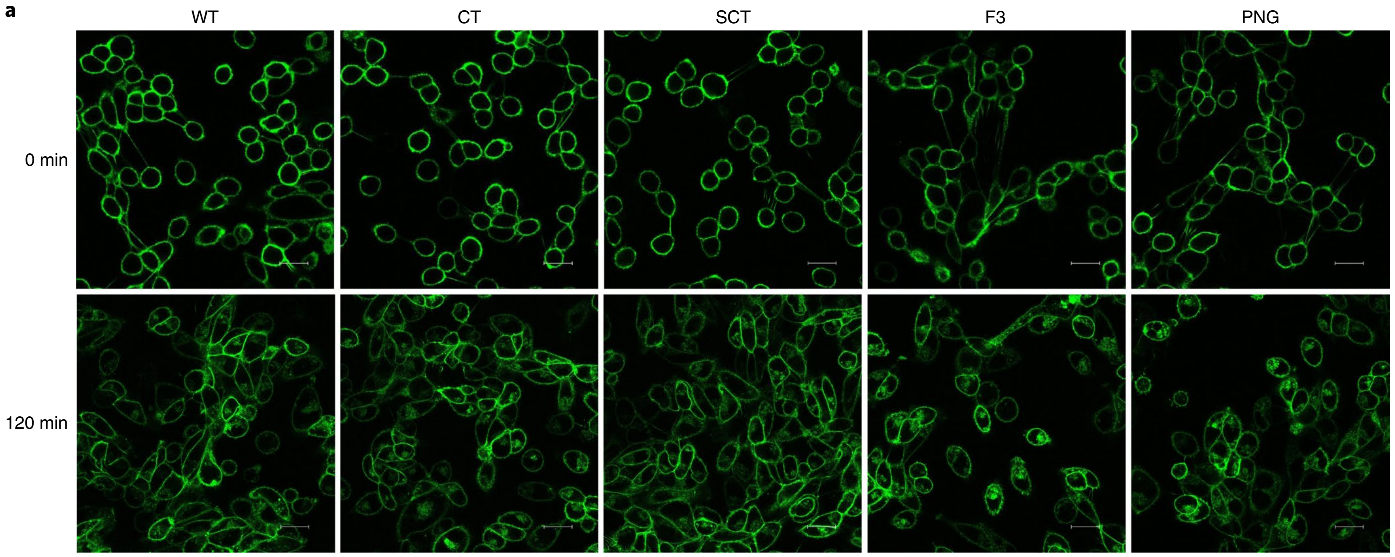


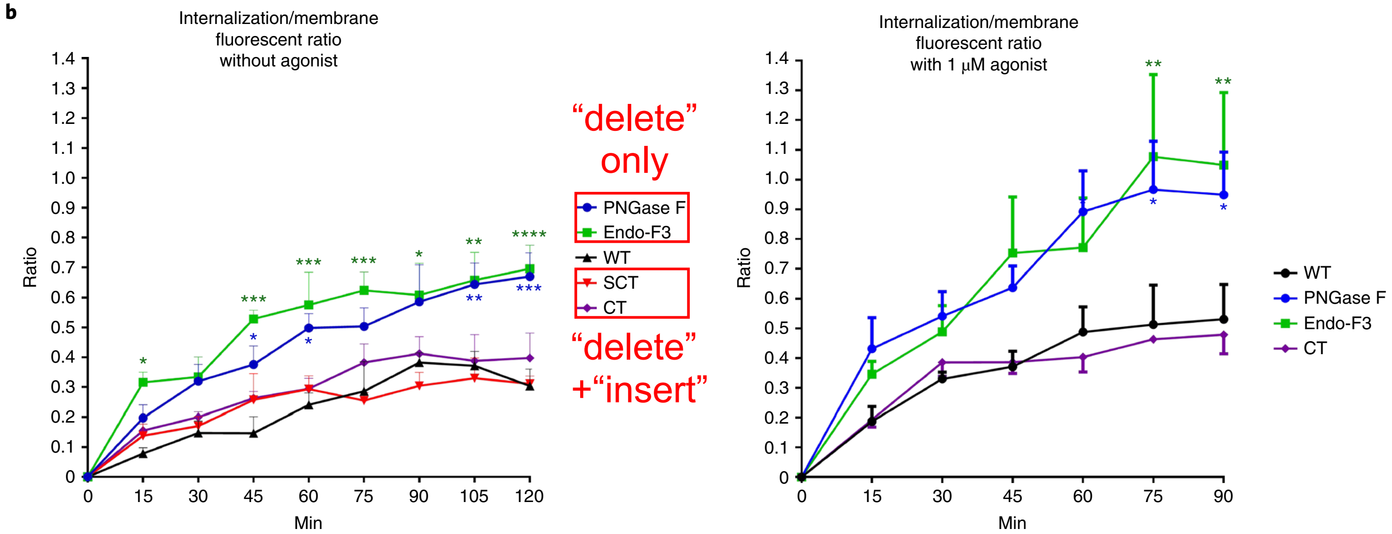
Agonist recognition of OPRD1



	D3C2	D3C2-F3	D3C2-SCT
EC_{50} (nM)	0.014	0.020	0.015

Internalization of OPRD1

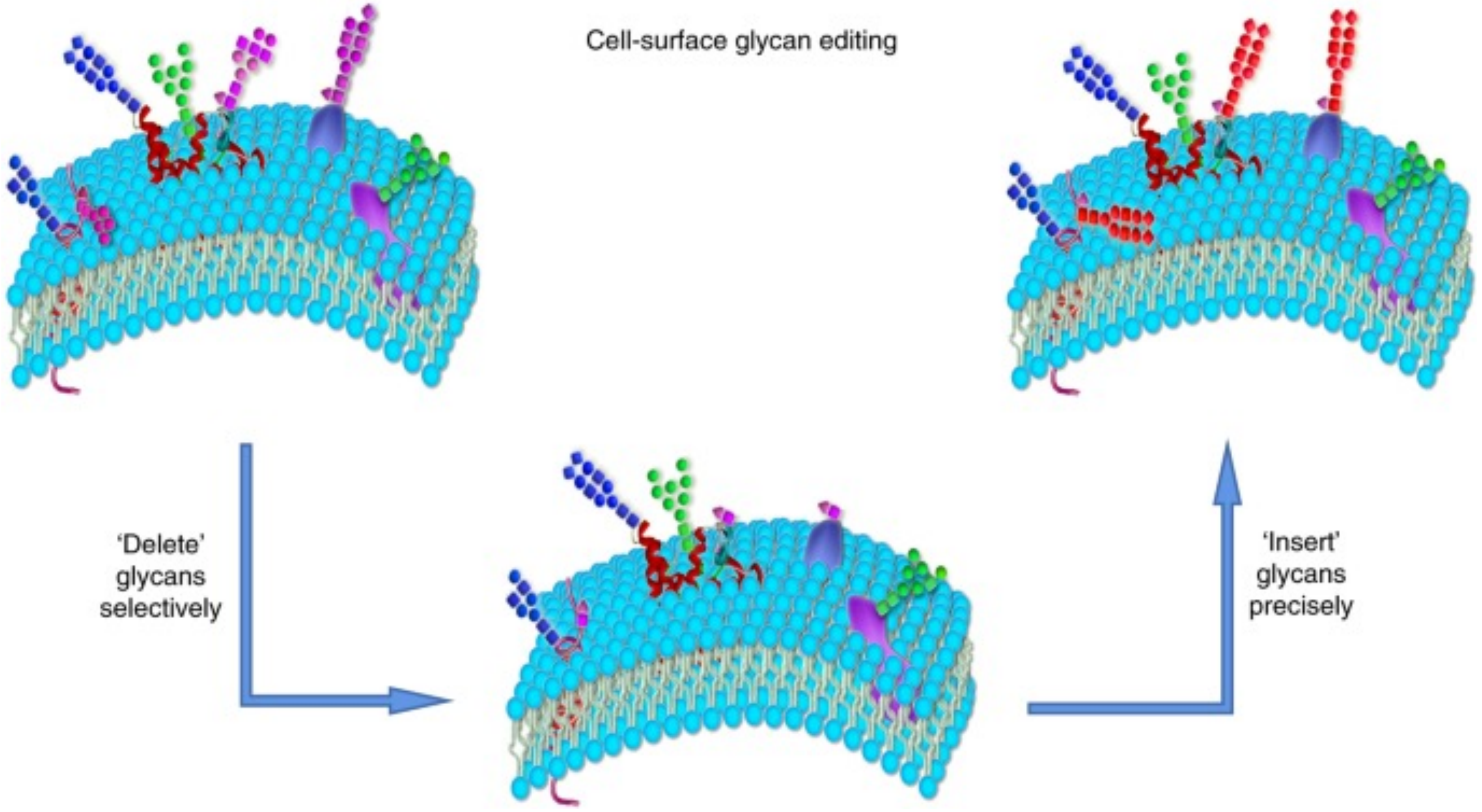




*PNGase F: cut between Asn and glycan

Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

Short summary: Glyco-engineering by two-step exogenous enzyme reaction



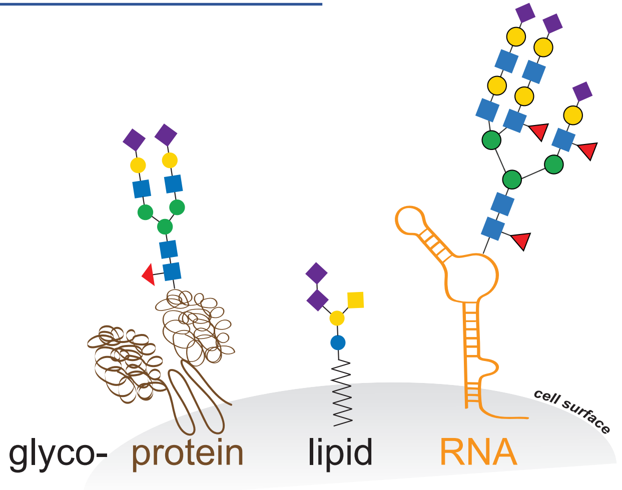
- N-glycan selective editing was achieved by two step enzymatic reaction.
- The heterogeneity of live-cell surface glycans became much lower after reaction.
- This system may be practical tools to elucidate the functions of a certain type of glycosylation.

Tang, F.; Zhou, M. et al. *Nat. Chem. Biol.* **2020**, *16*, 766–775.

Contents

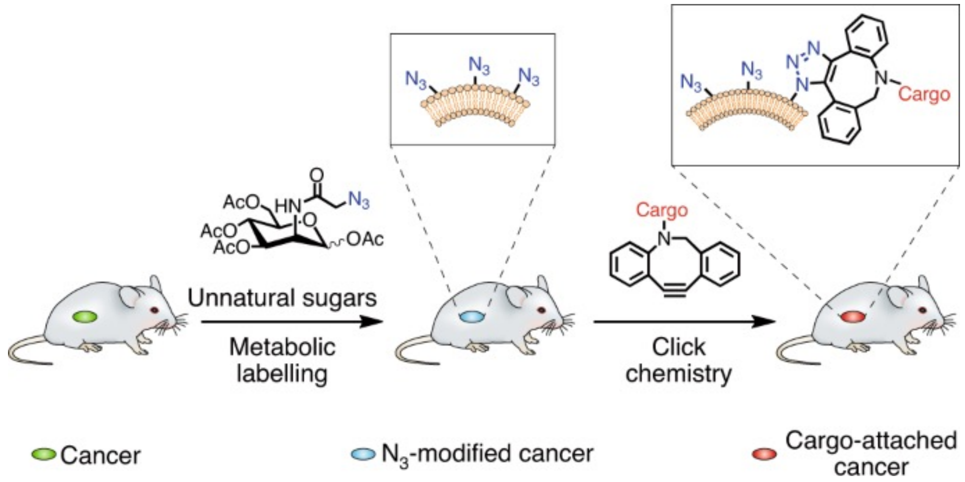
Perspective: Glycobiology is receiving increased attention.

Glycan-modified RNA



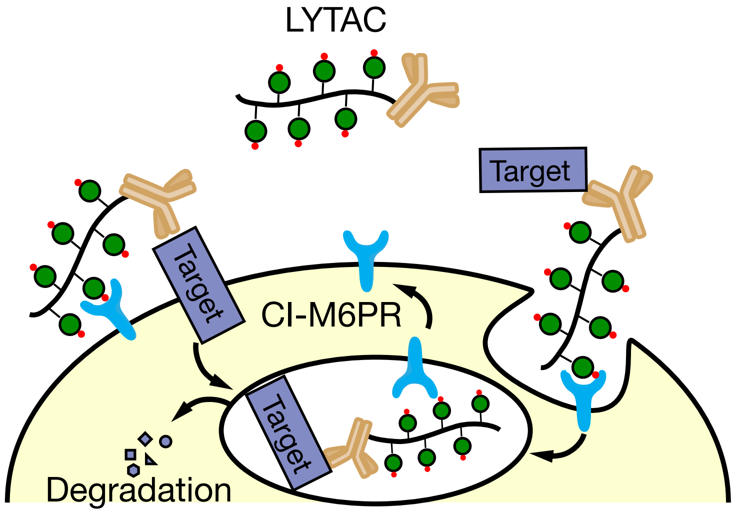
Flynn, R. A., Pedram, K. *et al. Cell* **2021**, *184*, 3109-3124.e22.

Metabolic labeling for cancer therapy



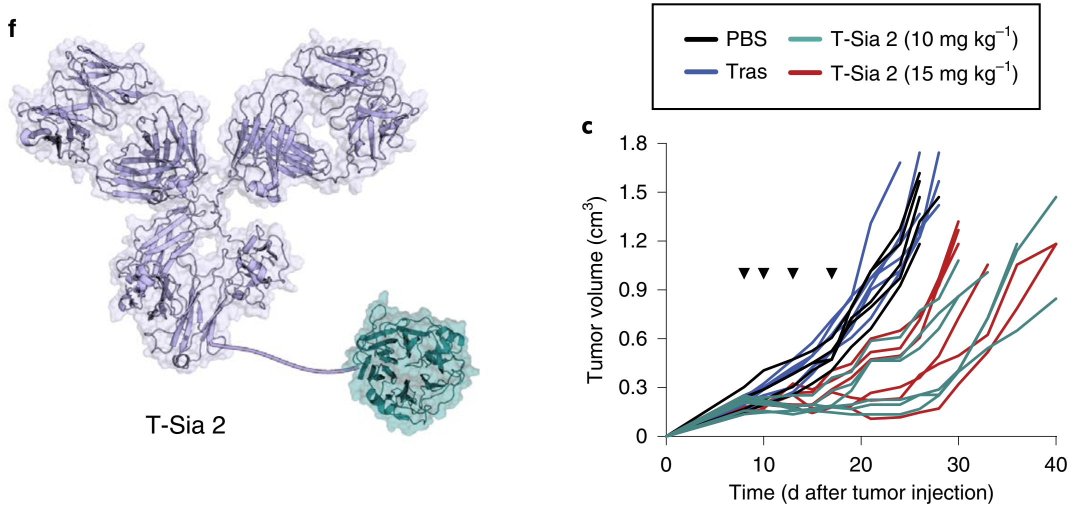
Wang, H. and Mooney, D. J. *Nat. Chem.* **2020**, *12*, 1102–1114.

Protein degradation with glycan ligand



Banik, S. M., Pedram, K. *et al. Nature* **2020**, *584*, 291–297.

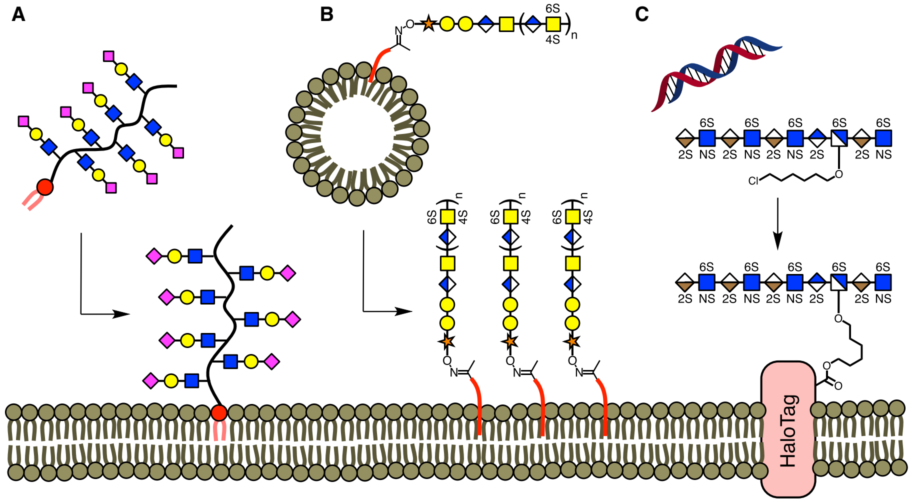
Chemoenzymatic glycan degradation for cancer therapy



Gray, M. A., Stanczak, M. A. *et al. Nat. Chem. Biol.* **2020**, *16*, 1376–1384.

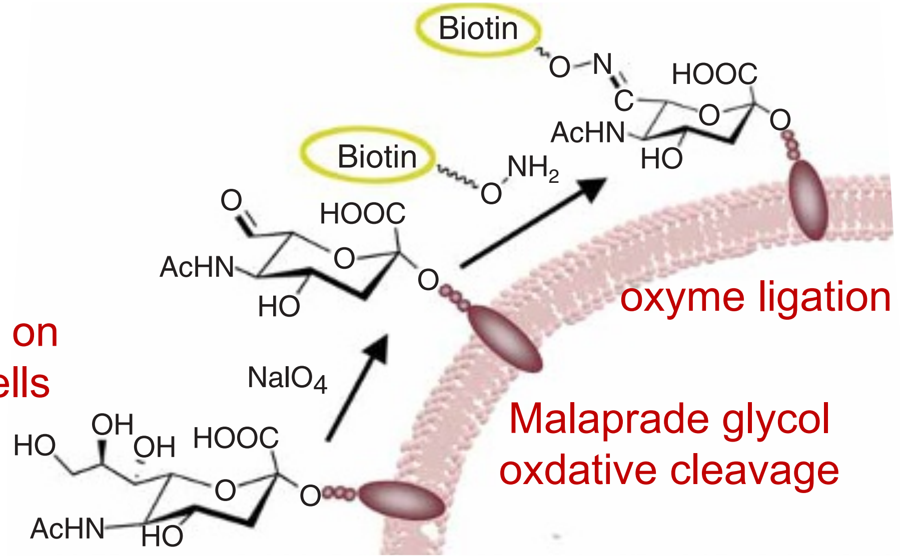
Perspective: Various tools for glycan engineering is growing.

Direct manipulation of glycans



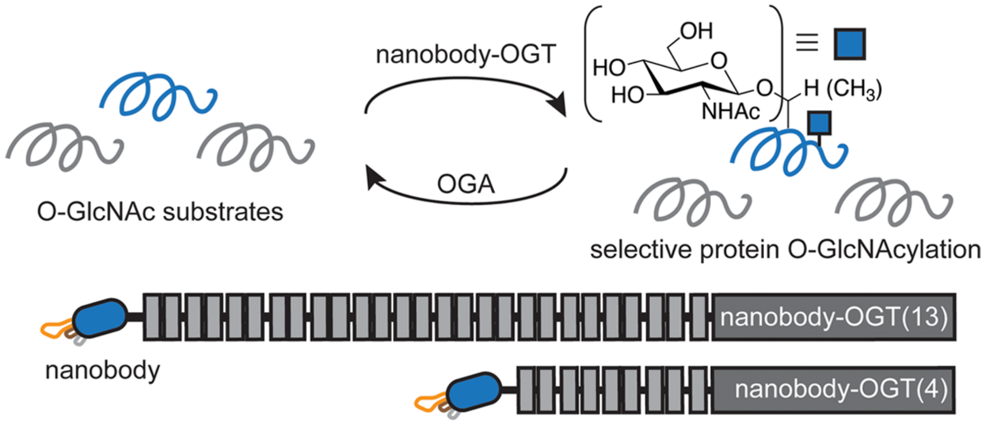
Griffin, M. E. and Hsieh-Wilson, L. C. *Cell Chem. Biol.* **2016**, *23*, 108–121.

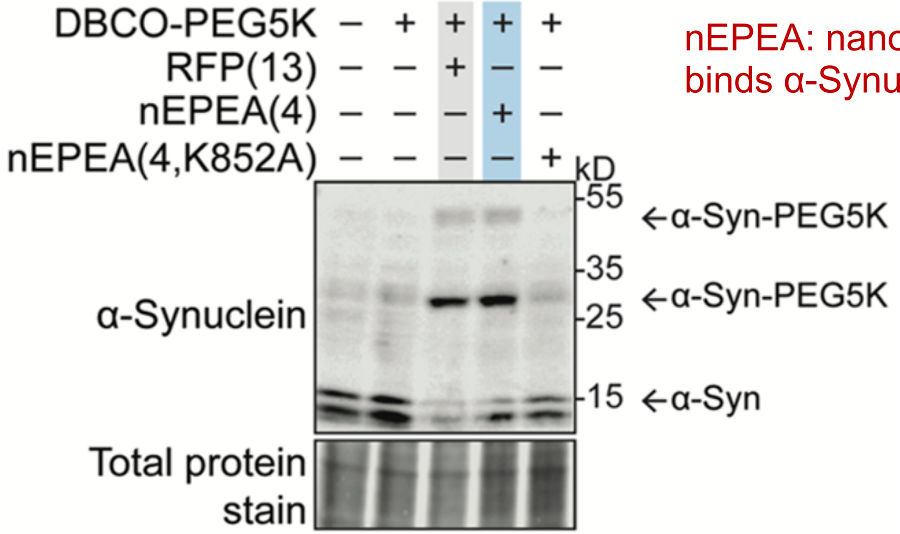
Neu5Ac on living cells



Zeng, Y., Ramya, T. N. C. *et al. Nat. Methods* **2009**, *6*, 207–209.

Protein-selective manipulation of glycans





nEPEA: nanobody which binds α-Synuclein

Ramirez, D. H., Aonbangkhen, C. *et al. ACS Chem. Biol.* **2020**, *15*, 1059–1066.

Summary: Glycan Engineering in Living Cells

- Introduction
  - Glycans play crucial roles in a myriad of biological processes.
  - A growing number of glycan-associated diseases are discovered.
  - Because of their high complexity, the study of glycans is quite difficult.

- 1. Bump-and-hole
  - The first glycotransferase bump-and-hole system in the living cell was established.
  - This system allowed isoenzyme-specific labeling in the endogenous environments.

- 2. Two-step exogenous enzyme reaction
  - N-glycan selective editing was achieved by two step enzymatic reaction.
  - The heterogeneity of live-cell surface glycans became much lower after reaction.
  - This system may be practical tools to elucidate the functions of a certain type of glycosylation.

- Perspective
  - Glycobiology is receiving increased attention.
  - Various tools for glycan engineering is growing.