Glycoforum
Glycoscience for human well-being

Glyco-engineering for Glycoprotein Production and Development of a Visualization Tool for Glycan Metabolic Pathways

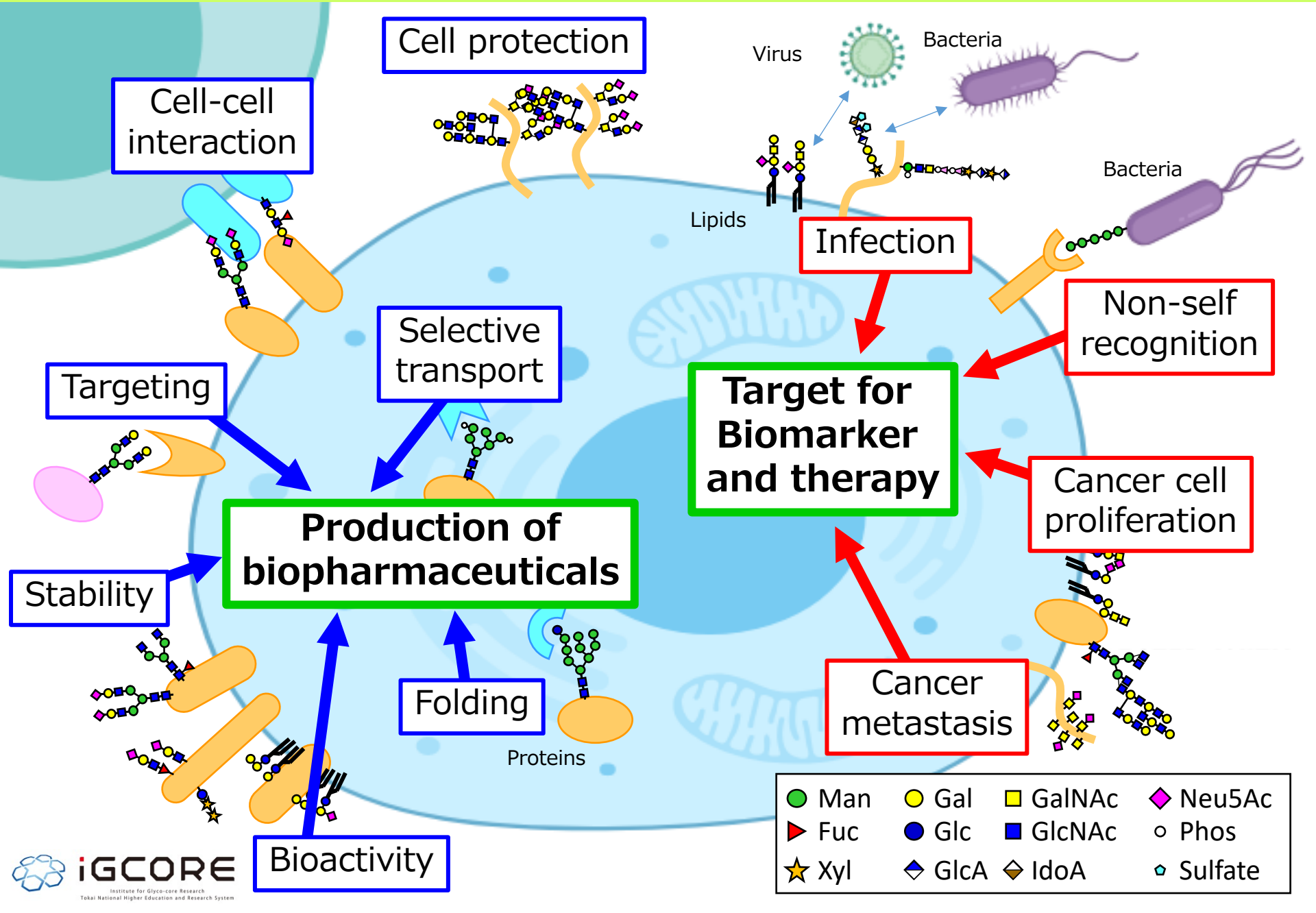
Glycoforum

Morihisa Fujita

Institute for Glyco-core Research (iGCORE)
Gifu University

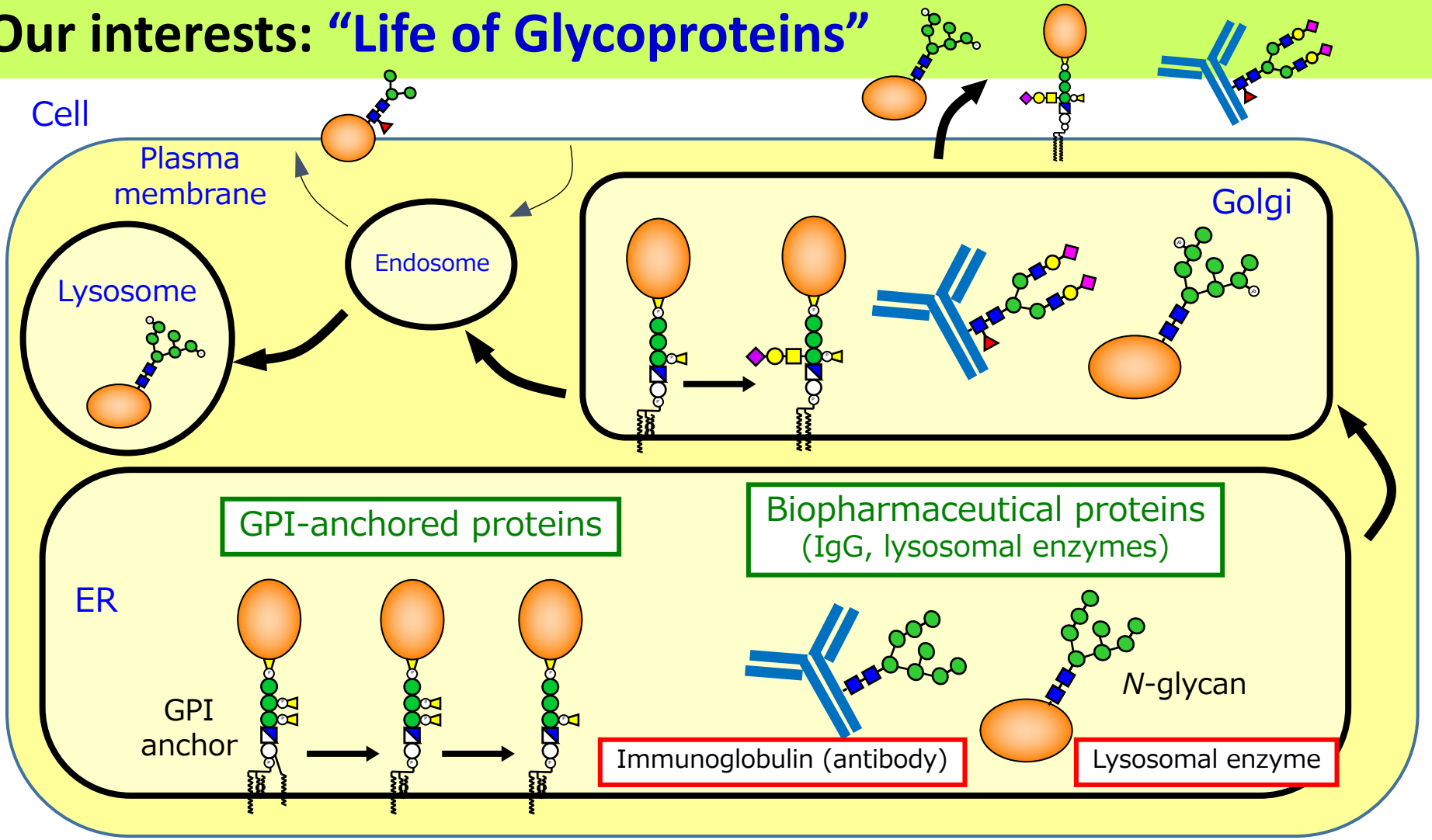
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Glycan functions and importance



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Our interests: "Life of Glycoproteins"



Regulation of biosynthesis and transport of glycoproteins

Engineering of mammalian cells to produce biopharmaceuticals

Contents

Simplification of N-glycan structures in HEK293:

Production of recombinant proteins

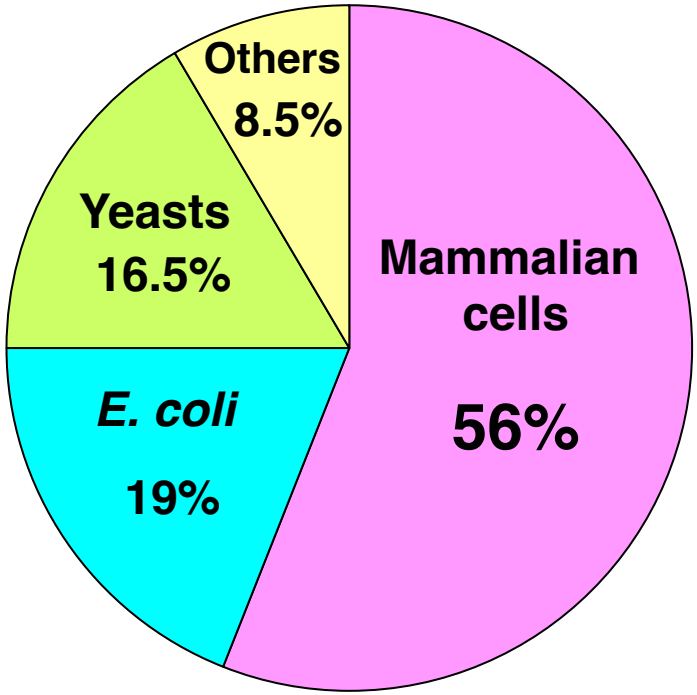
only having high-mannose type N-glycans

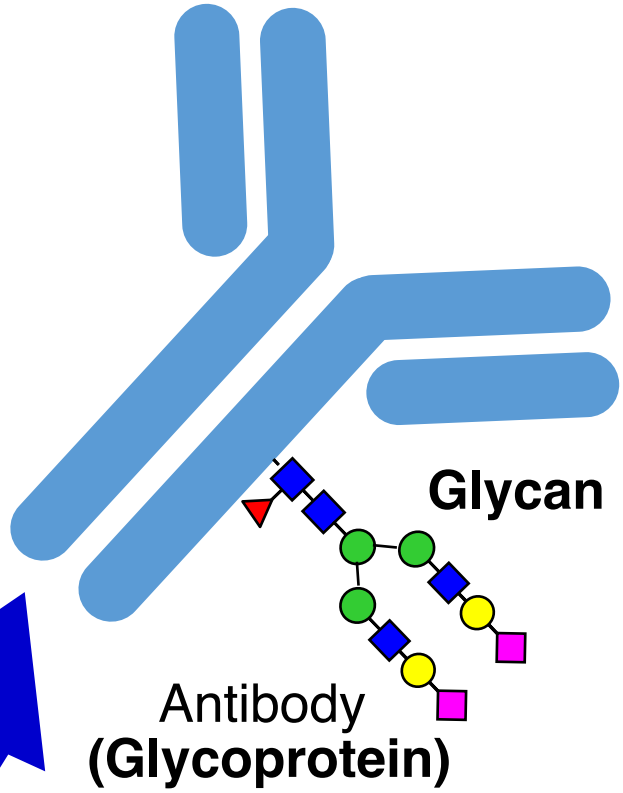
Development of a visualization tool for glycan metabolic pathways based on gene expression profiles:

Glyco-engineering and Glyco-comparison using GlycoMaple

Biopharmaceutical proteins

Biopharmaceutical proteins





Protein folding and assembly
Post-translational modifications

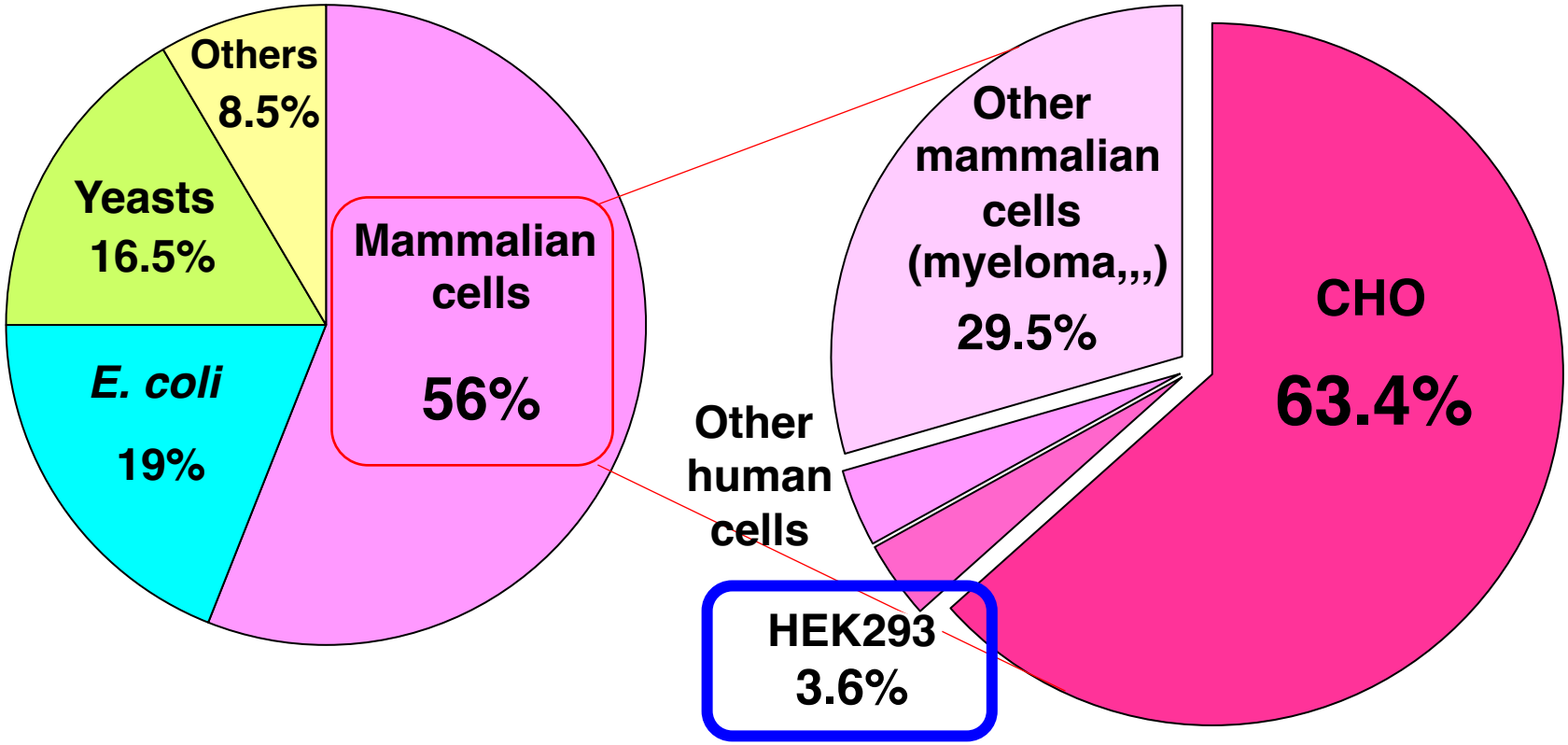
Mammalian cells

Protein glycosylation

Stability
Bioactivity
Immunogenicity

Modified from
Walsh G (2014)
Nat. Biotech. 12: 992

Biopharmaceutical proteins produced by mammalian cells



Modified from Walsh G (2014) *Nat. Biotech.* 12: 992

HEK293

HEK293 (human embryonic kidney 293)

Established in 1977 by Frank Graham (McMaster Univ., Canada)

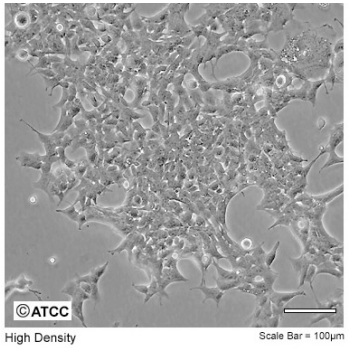
Easy to handle and **widely used** in basic and applied researches

Adaptable to **serum-free suspension** cultures

Used for production of some pharmaceuticals (ex. factor VIII, factor IX)
Used for viruses or virus-like particles (**VLPs**) production

Less competitive for host development, compared to CHO cells

Accumulated genetic information in human databases



HEK293

Glycosylation in HEK293 **Human glycoform**

Drawbacks of protein production in mammalian cells

Heterogeneity of glycans

Lack of functional / suitable glycans

Glyco-engineering

1) Customization

2) Simplification

Engineering of mammalian cells to produce proteins with homogenous glycans

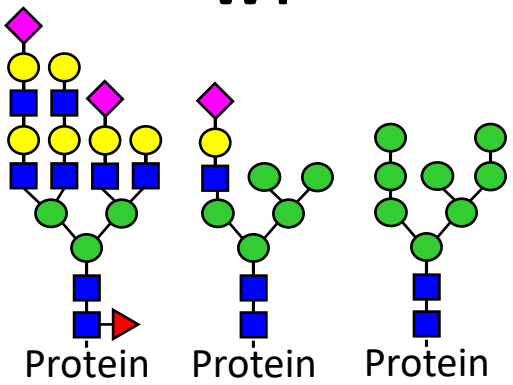
One issue for recombinant protein production in mammalian cells

Heterogeneity of glycans on proteins

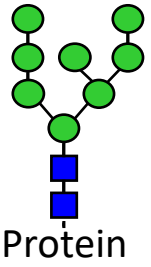
Genome-editing using CRISPR/Cas9 system

Heterogeneous glycan → Uniform glycan

WT



Only **high Man-type**
No Complex-type

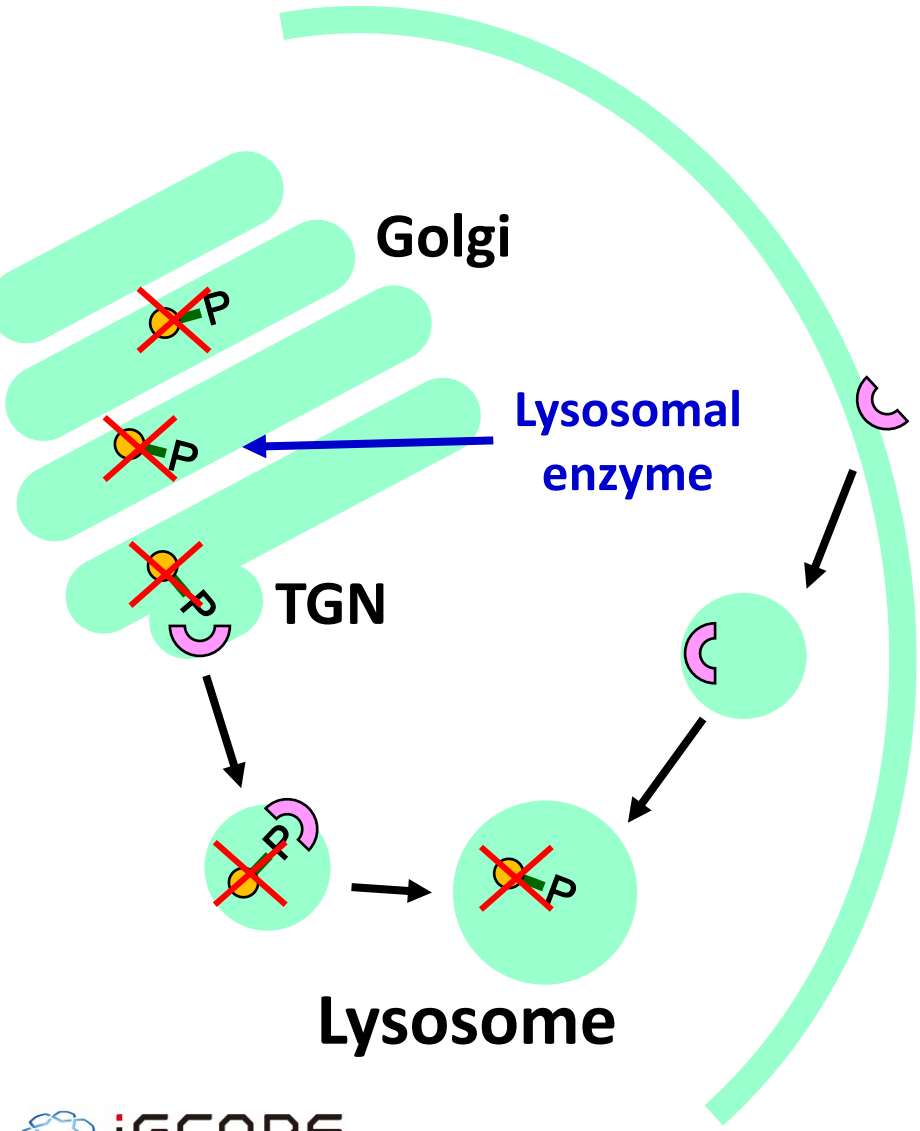


High Man-type
N-glycan with **Man8** or **Man9**

Drug for Lysosomal storage diseases
(Recombinant lysosomal enzymes)
Vaccine candidate for HIV-1 (gp120)

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Enzyme replacement therapy for lysosomal diseases



Lysosomes

Garbage disposable in the cell

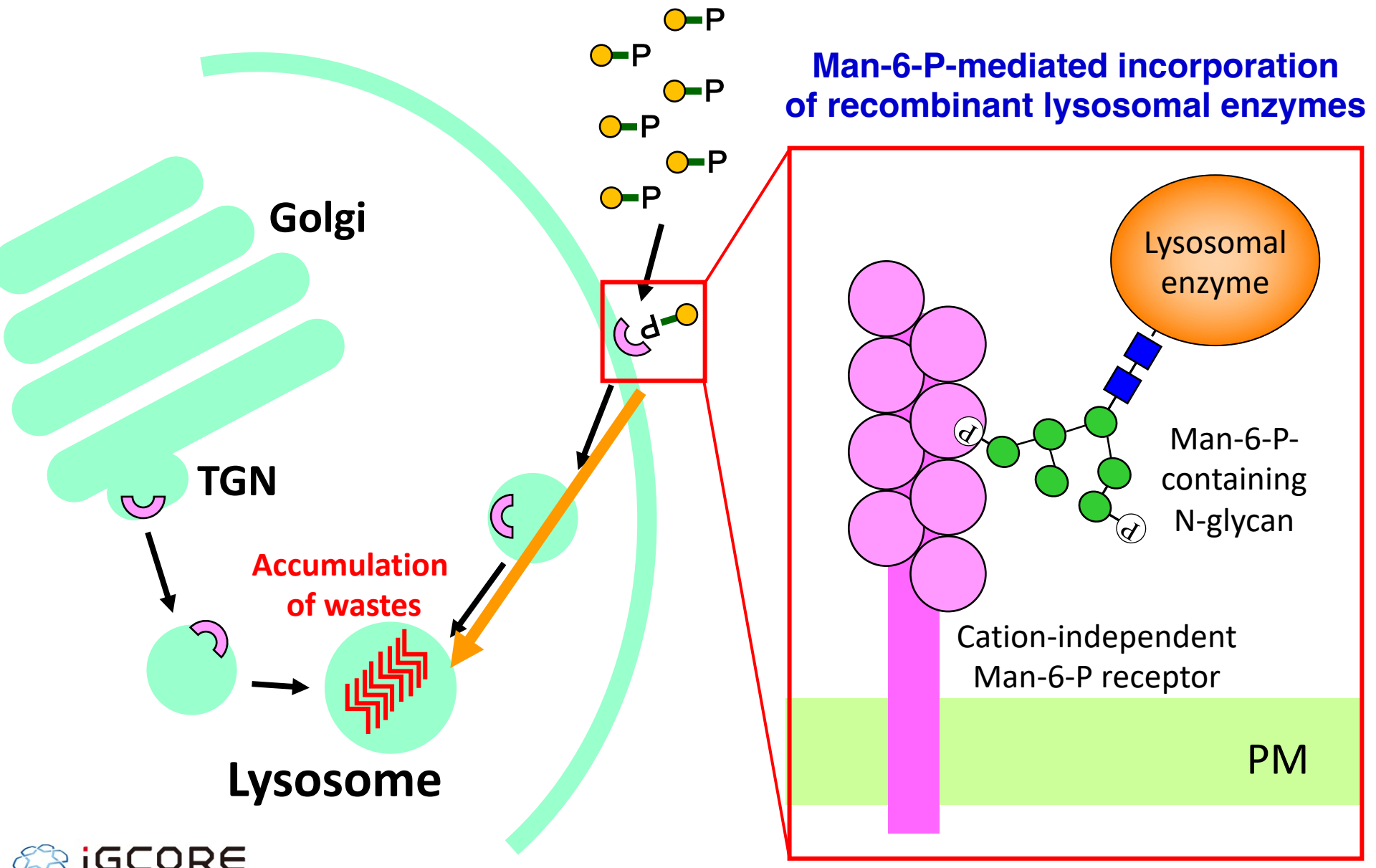
> 50 Lysosomal enzymes to hydrolyze substrates (nucleic acids, proteins, lipids, glycans)

Defects in lysosomal enzyme genes cause lysosomal storage diseases

Enzyme replacement therapy

Recombinant lysosomal enzymes are intravenously injected

Enzyme replacement therapy for lysosomal diseases



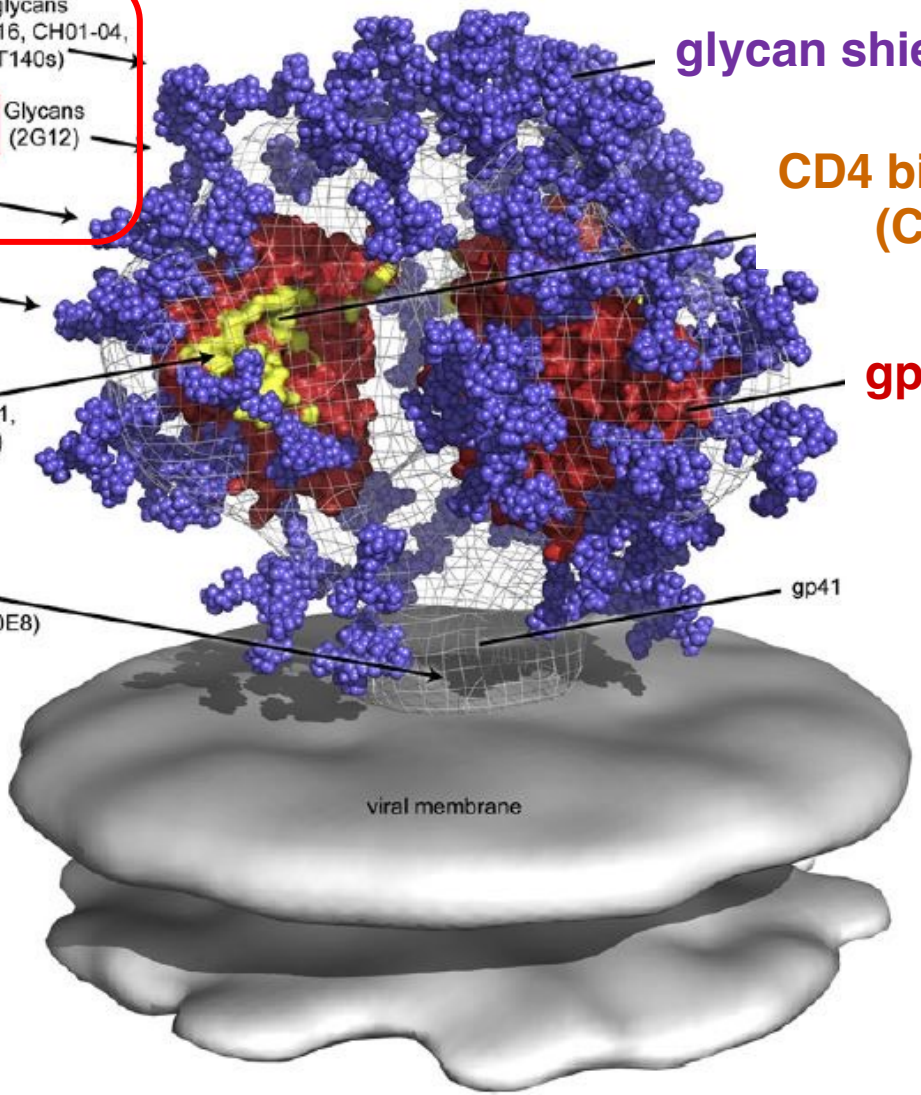
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

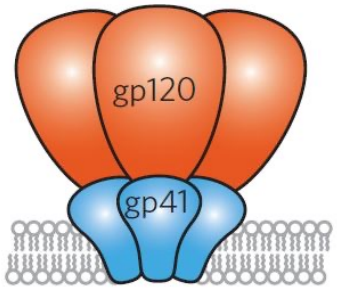
Broadly neutralizing antibodies for HIV-1

broadly neutralizing antibodies (bnAbs) Isolated from HIV-1 patients

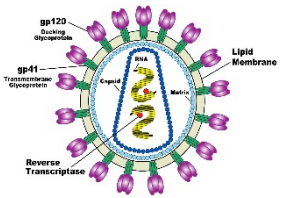
Binding to N-glycans

- >12 MAbs — V2/glycans (PG9, PG16, CH01-04, PGT140s)
- 1 — Glycans (2G12)
- >25 — V3/V4/glycans (cluster of targets: PGT120s, PGT130s)
- 2 — V3/CD4i (3BC176)
- >25 — CD4bs (b12, VRC01, NIH 45-46)
- >5 — MPER (2F5, 4E10, 10E8)





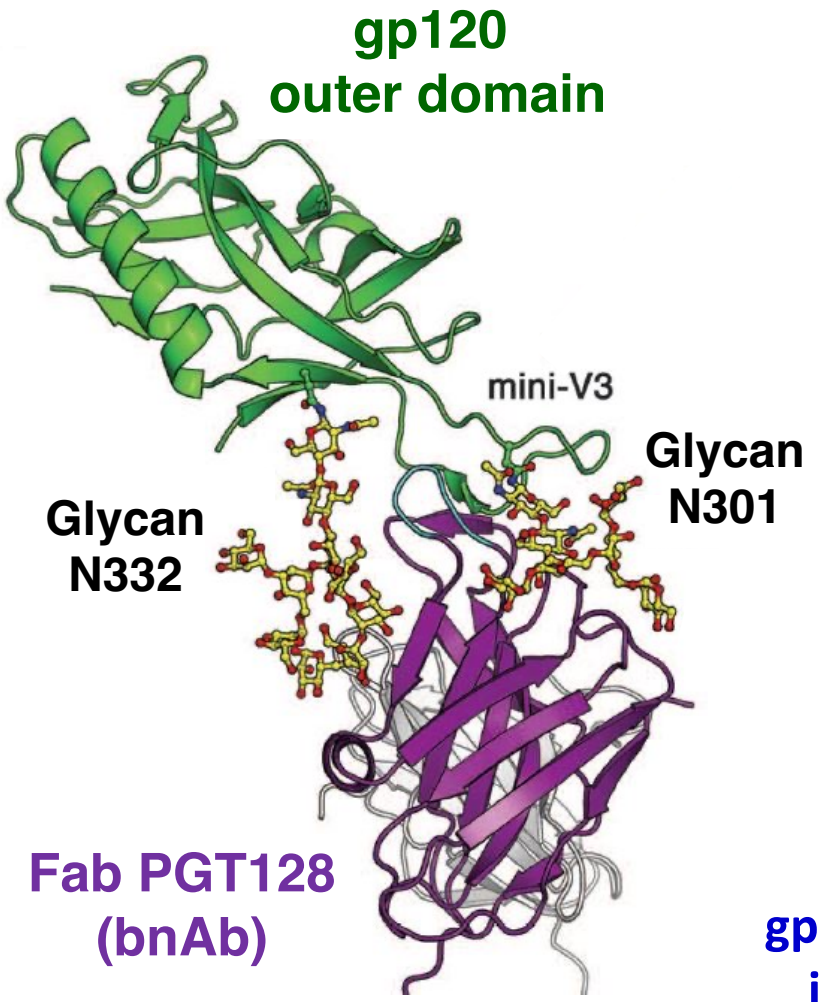
Functional mature Env



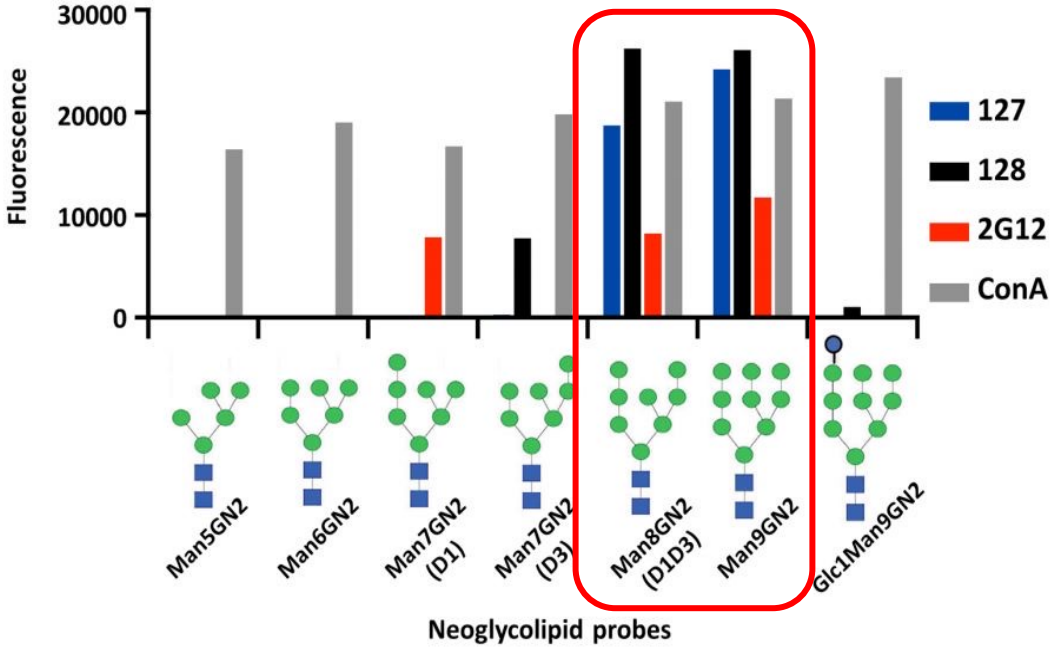
HIV-1

Burton et al. (2012) *Cell Host Microbe*

Many bnAbs recognize high-Man type N-glycans on gp120



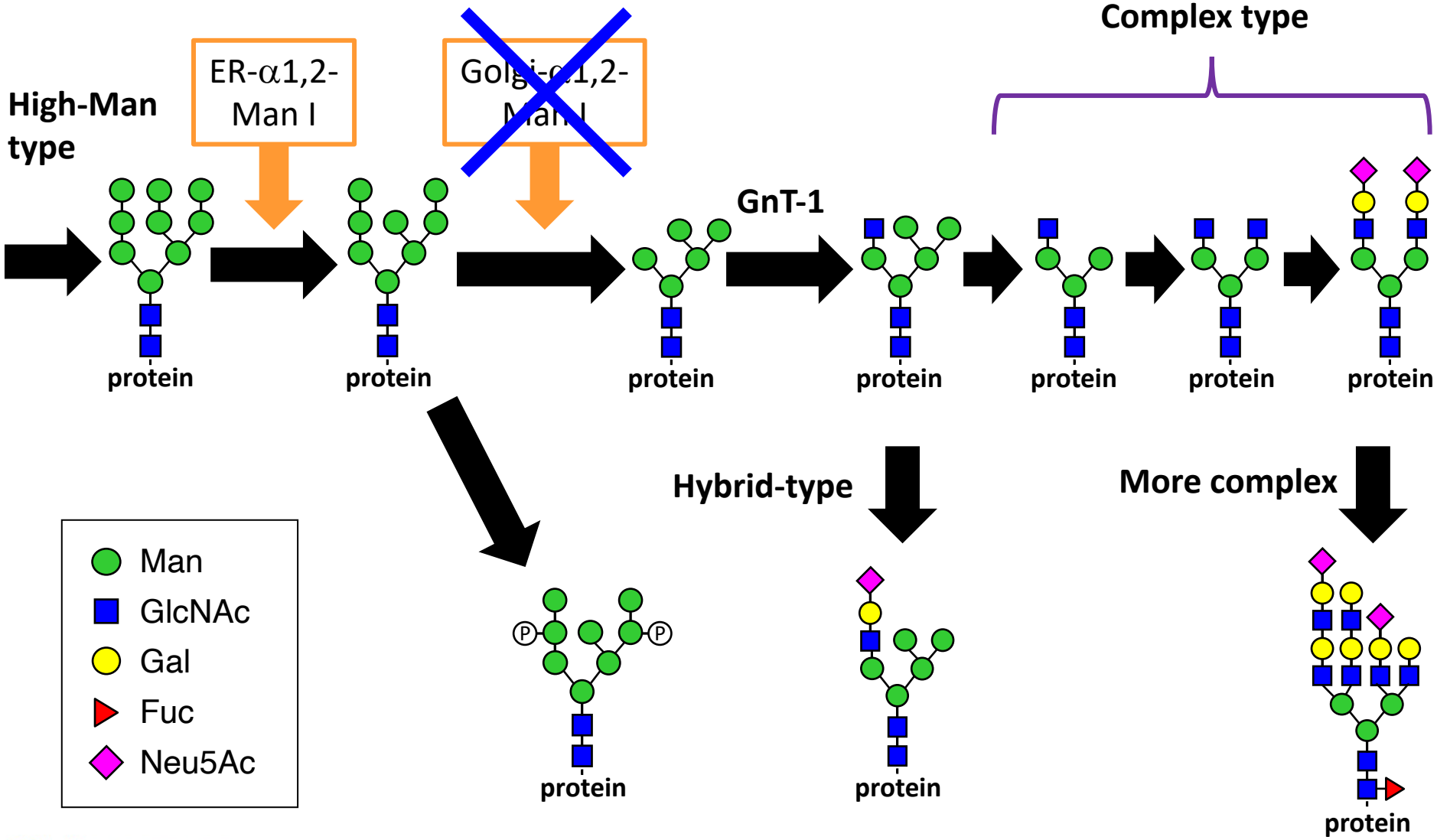
Binding of bnAbs to N-glycan structures



gp120 with M8 or M9 high-Man-type N-glycans is a candidate for the vaccine against HIV-1.

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Disruption of Golgi-Man-I to express proteins containing high Man-type N-glycans



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Human GH47 (α1,2-mannosidases) in CAZy database

Golgi Man-I A
(MAN1A1)

Tempel et al (2004) *J. Biol. Chem.*

Human GH47 (α1,2-mannosidases)

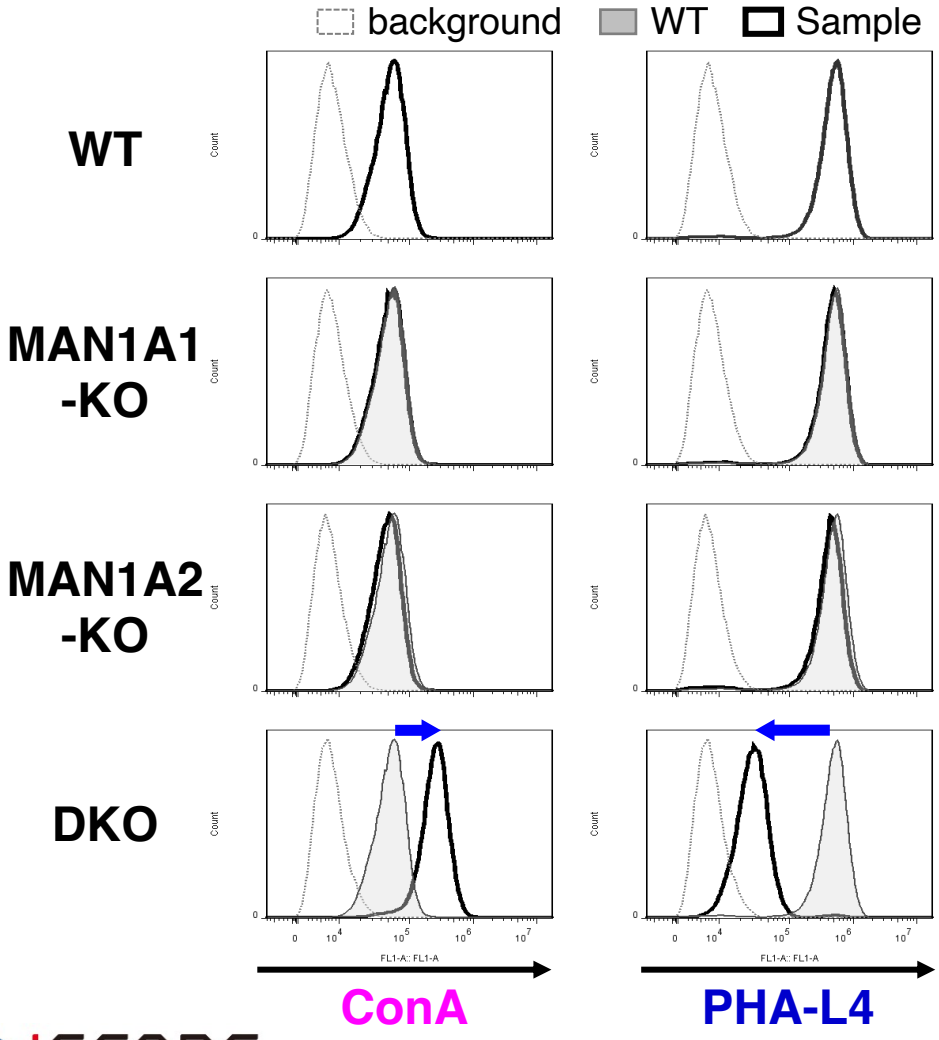
Protein name	Gene name	TPM	Function
Golgi Man-I A	*MAN1A1*	34.82	Glycan maturation
B	*MAN1A2*	25.65	
C	*MAN1C1*	5.24	
ER Man-I	*MAN1B1*	64.97	Protein quality control ?
EDEMs	*EDEM1*	28.91	ER-associated degradation
	EDEM2	24.59	
	EDEM3	24.01	

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

CAZy: Carbohydrate-Active enzyme (http://www.cazy.org/)

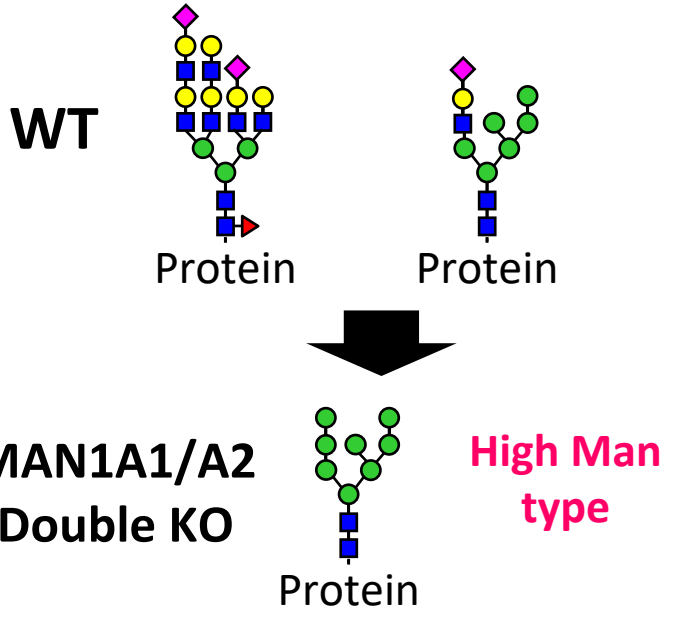
MAN1A1 & *MAN1A2* double KO cells decreased complex-type and increased high-Man-type glycans

Flow cytometric analysis of glycans on the cell surface using two lectins



PHA-L4: lectin binding to **complex type** of glycans

ConA: lectin binding to **high Man type** of glycans

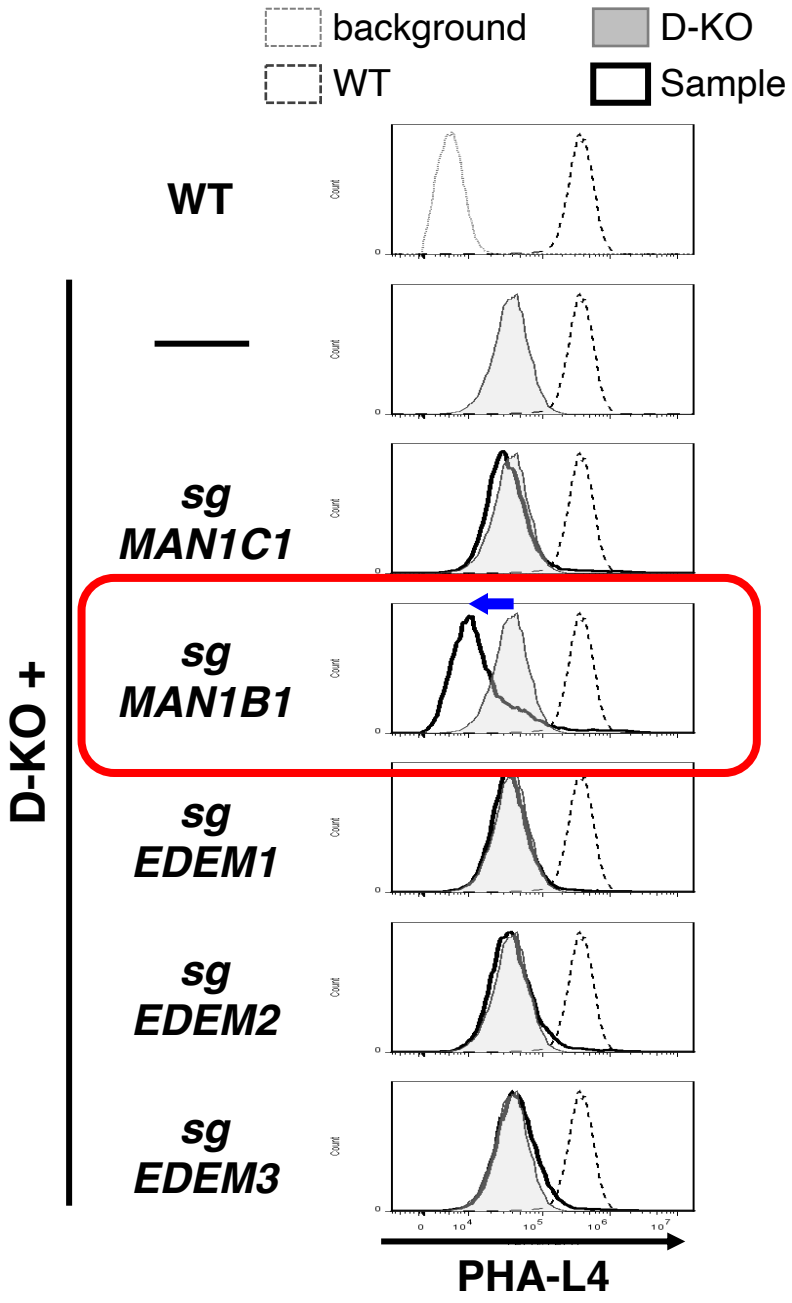


iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

KO of other α1,2-mannosidase genes in Double-KO

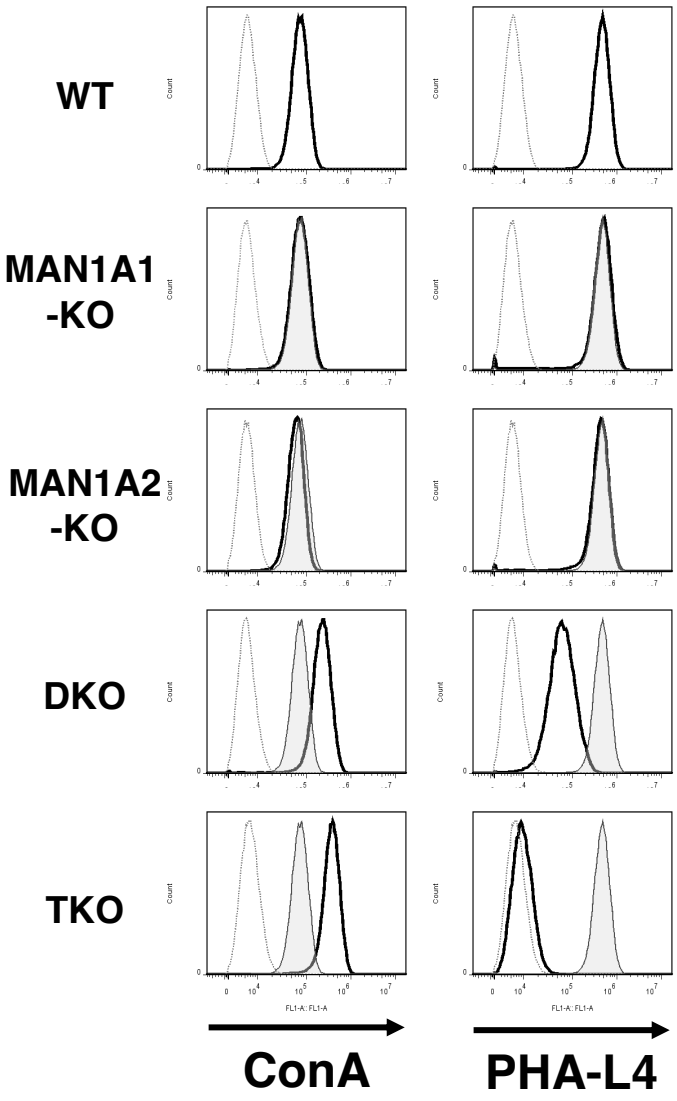
Human GH47 (α1,2-mannosidases)

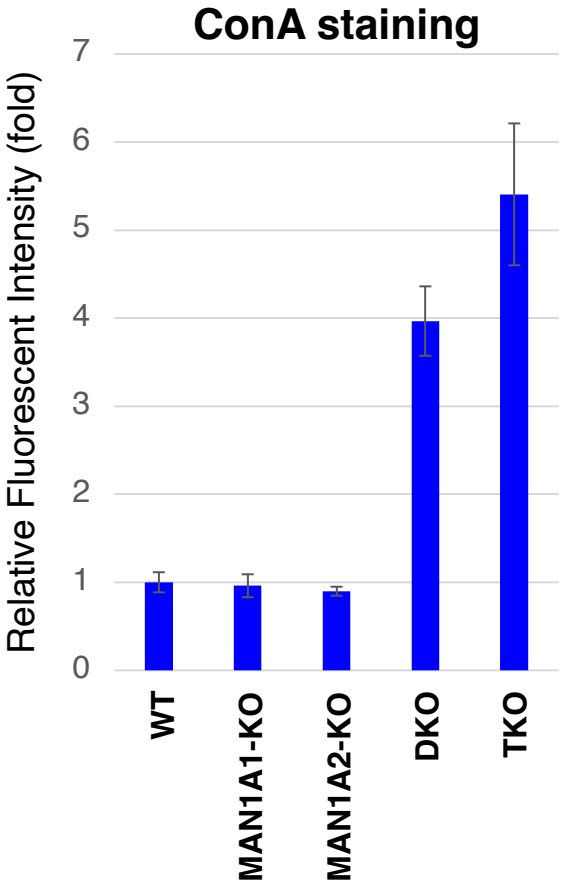
Protein name		Gene name
Golgi Man-I	A	~~*MAN1A1*~~
	B	~~*MAN1A2*~~
	C	*MAN1C1*
ER Man-I		*MAN1B1*
EDEMs		*EDEM1*
		EDEM2
		EDEM3

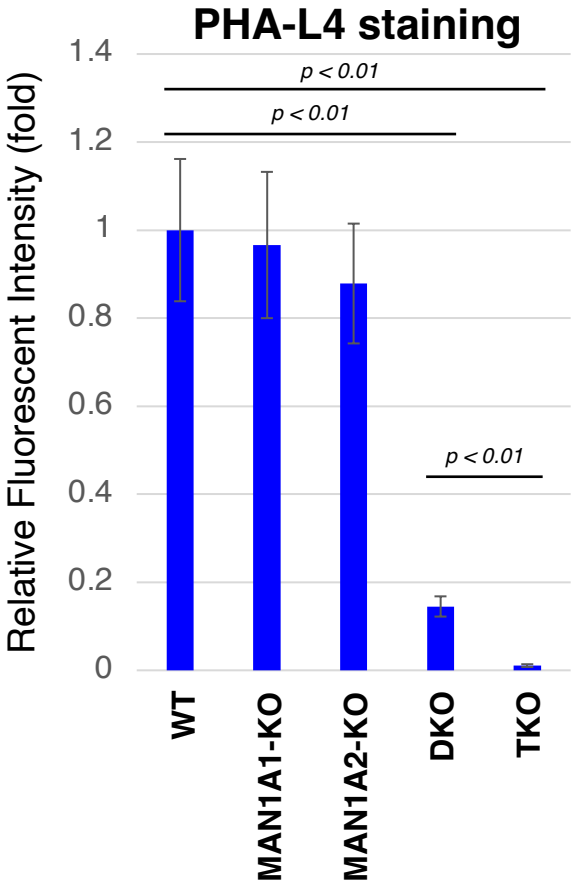


Knockout of MAN1B1 in double-KO cells almost completely diminished the PHA-L4 staining.

MAN1A1/A2/B1-triple KO cells

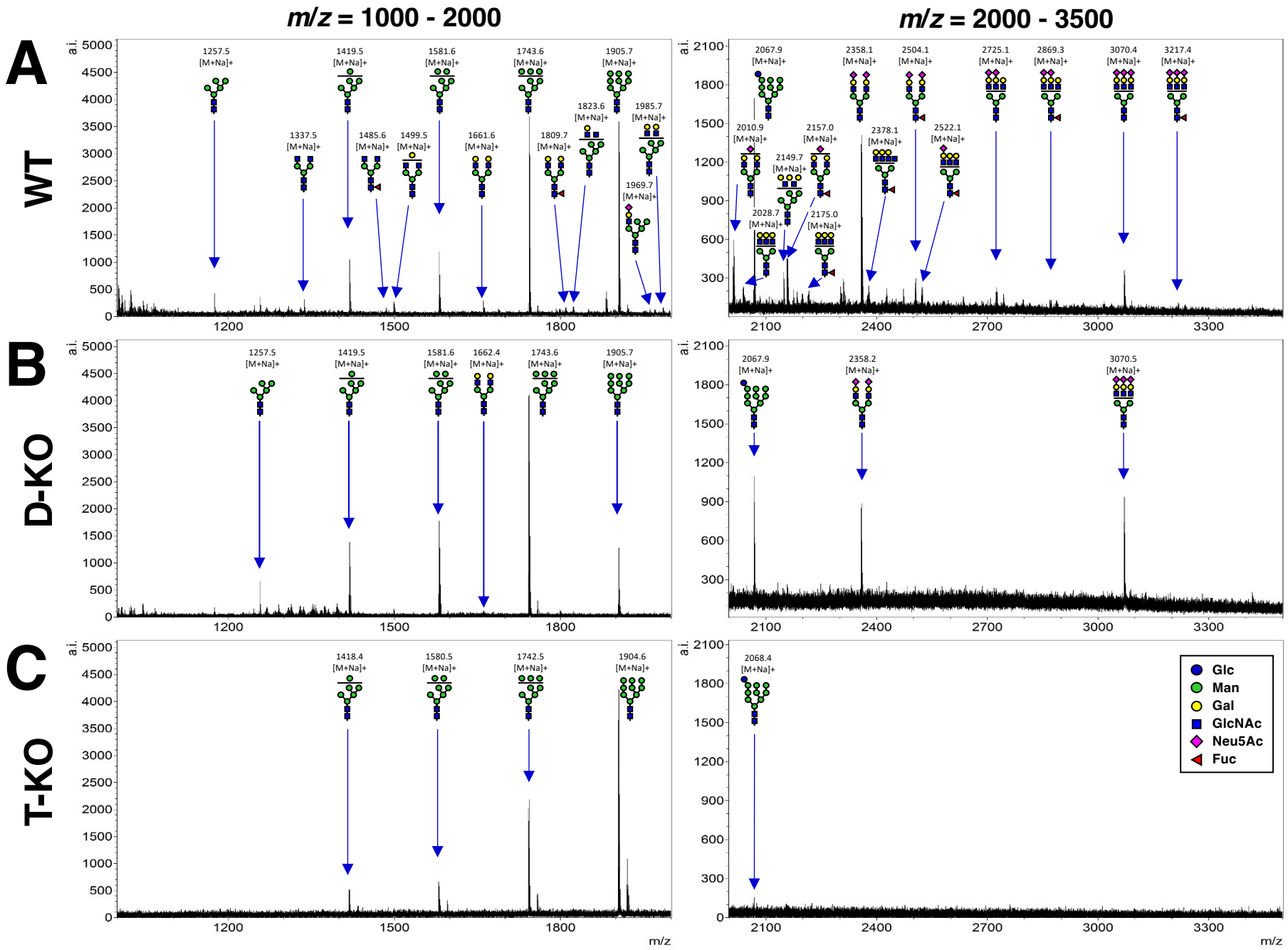






Knockout of MAN1B1 in double-KO cells almost completely diminished the PHA-L4 staining.

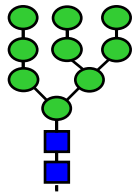
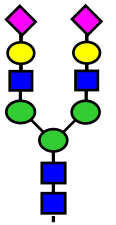
N-glycan analysis by MALDI-MS (whole cell lysates)

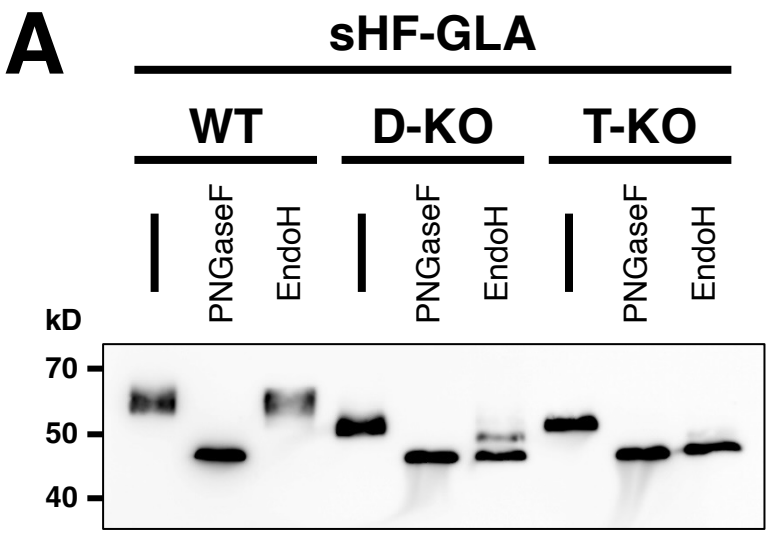


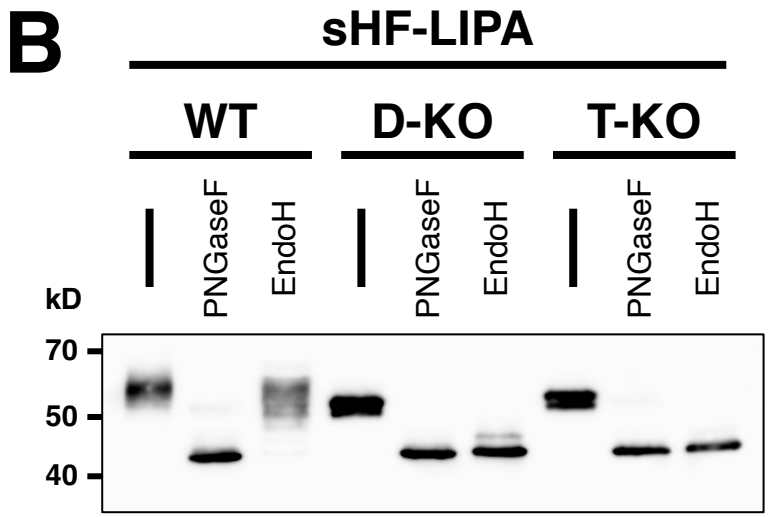
Expression of recombinant lysosomal enzymes

α-Galactosidase A (GLA):
hydrolyzes a glycosphingolipid Gb3 in the lysosomes and the **mutations in GALA cause Fabry disease**

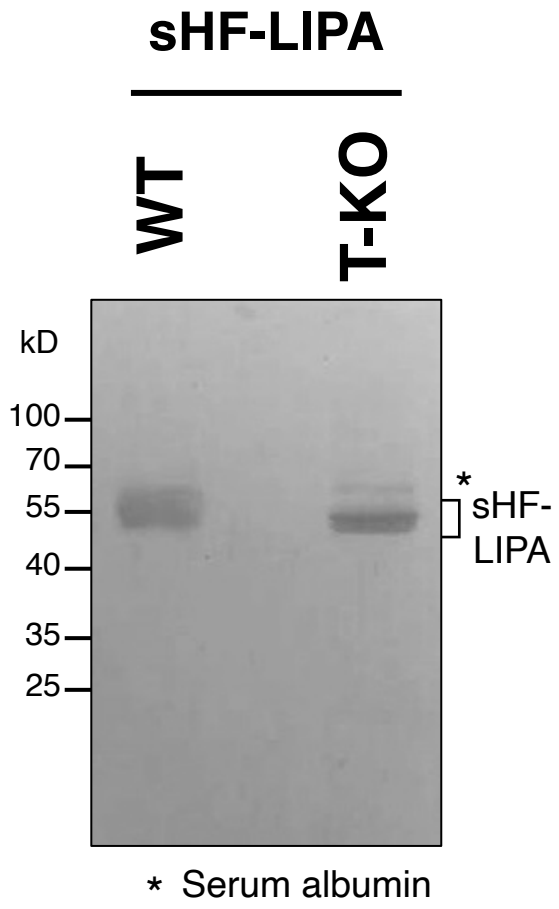
Lysosomal acid lipase (LIPA):
breakdown of lipids such as cholesterol esters and triacylglycerols in lysosomes, **its deficiency leads Wolman disease and cholesteryl ester storage disease**

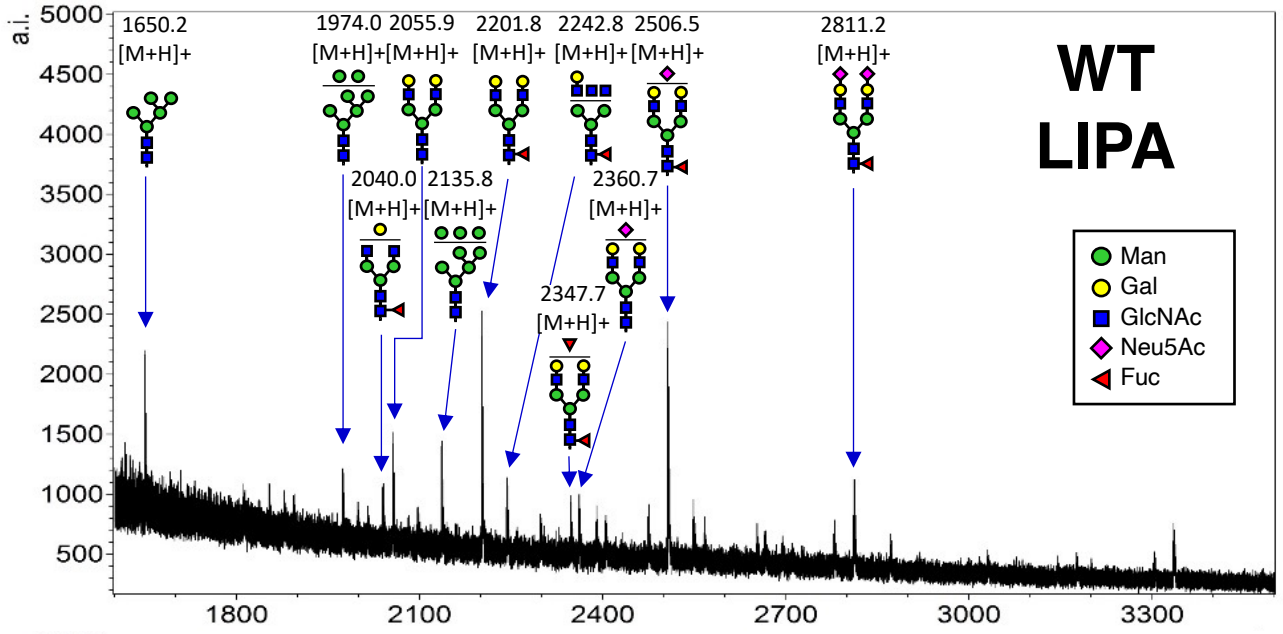
	PNGaseF	EndoH
	○	○
	○	✕

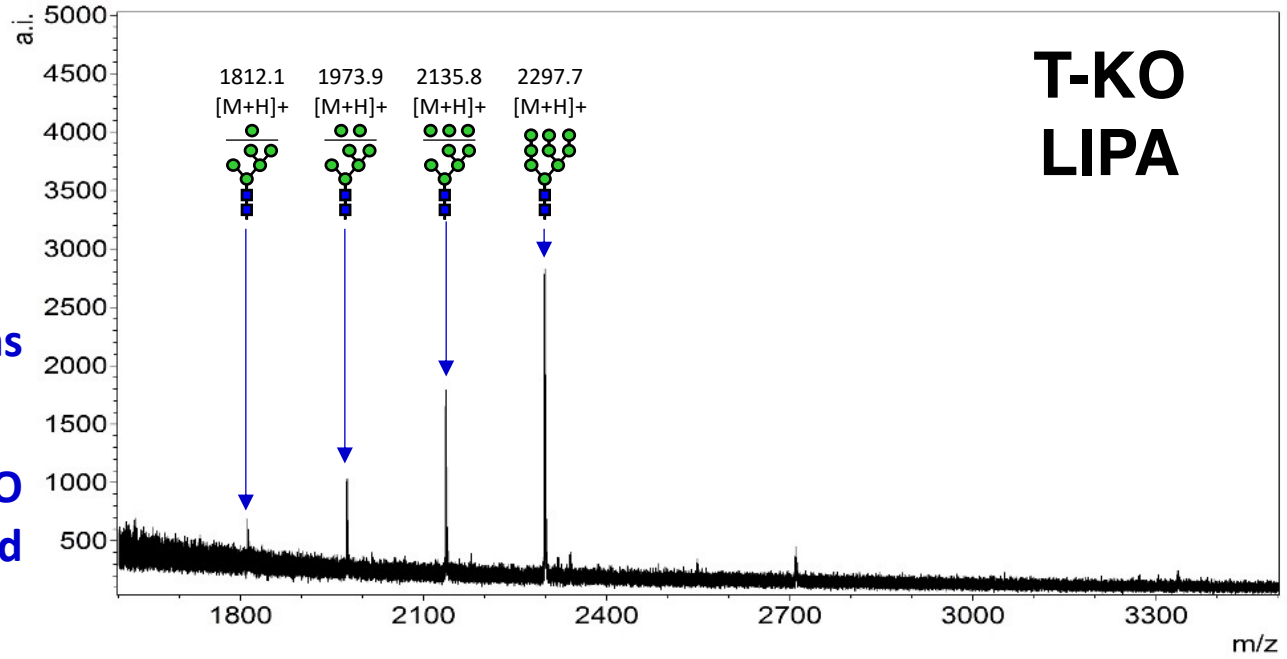




N-glycan analysis of recombinant LIPA from T-KO





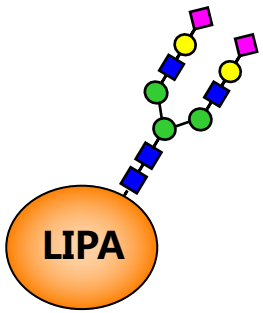


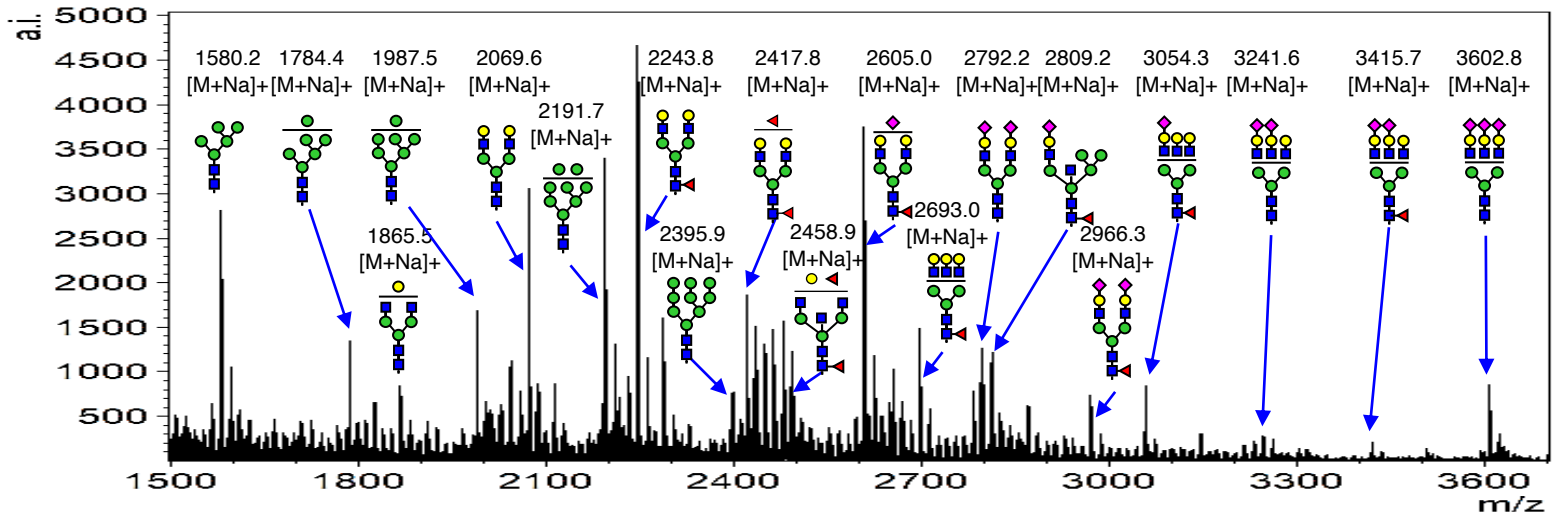
More than 30 different glycans were observed from WT-LIPA.

N-glycans on LIPA from T-KO were simplified and changed to high-Man-types.

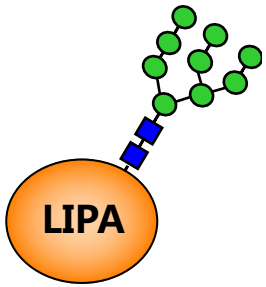
MAN1A1/A2/B1/C1-quadruple gene-KO HEK293 cells

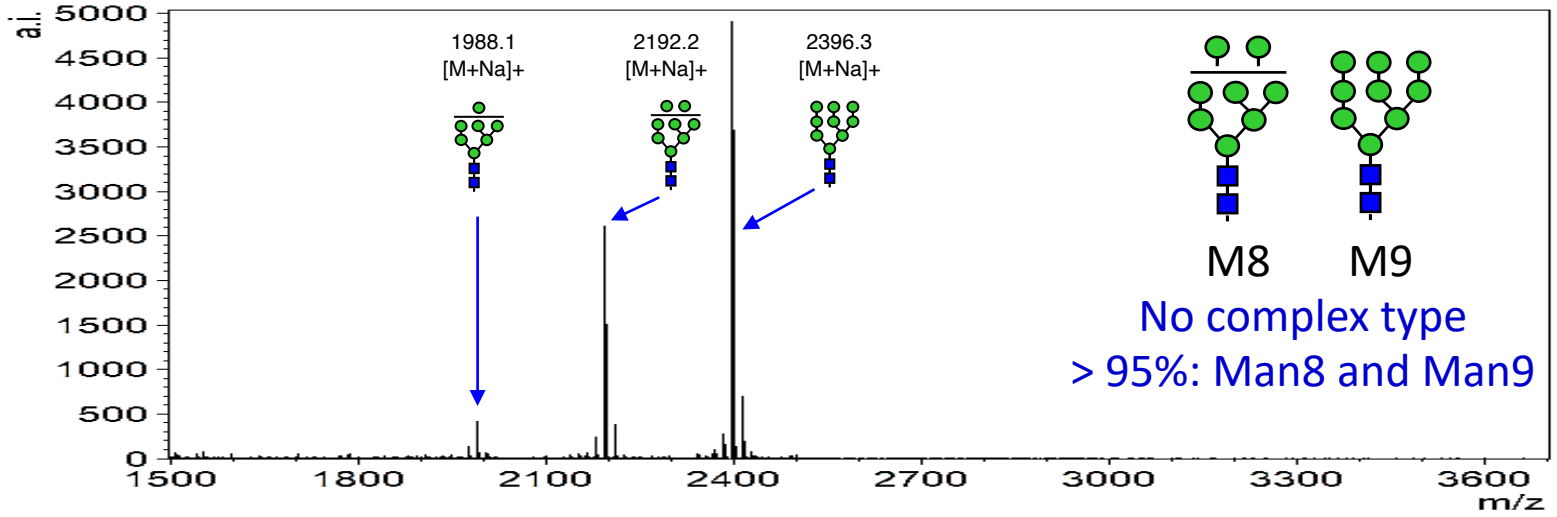
WT





MAN1A1/A2/B1/C1-KO

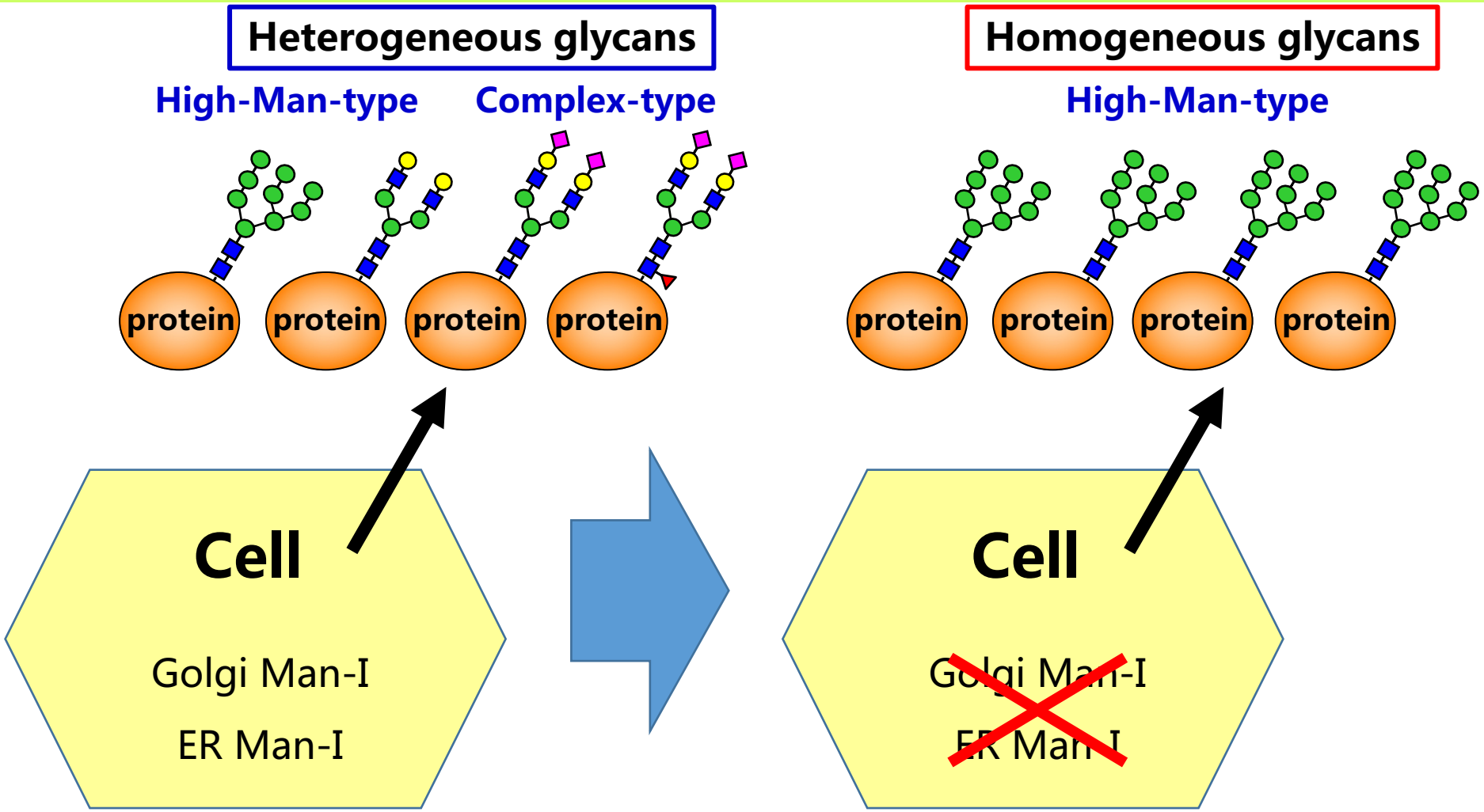




iGCORE Institute for Glyco-core Research Tokai National Higher Education and Research System

Jin, Fujita* et al (2018) *J. Biol. Chem.*; Ren, Fujita* et al (2019) *J. Biochem.*

Summary 1: Engineering of HEK293 cells producing recombinant proteins with high-mannose-type N-glycans

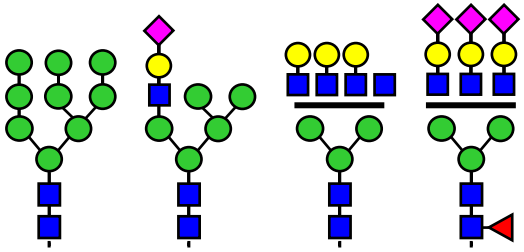


Established multiple mannosidase-I gene KO HEK293 cells, which would be suitable for production of proteins having high-Man-type N-glycans

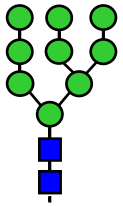
Jin et al (2018) *J. Biol. Chem.*

Simplification of glycans in HEK293 cells

N-glycans

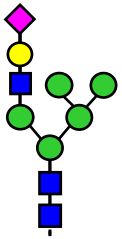


MAN1A1/A2/B1/C1-KO



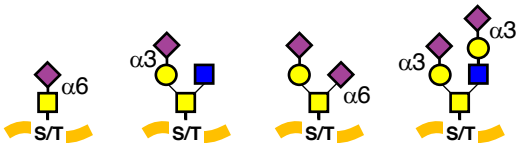
High-Man

MAN2A1/A2/FUT8-KO + MsdS-OE



Hybrid

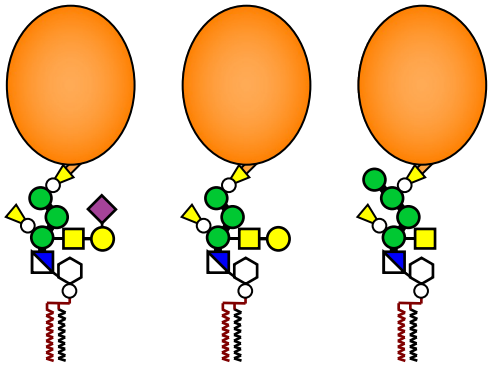
O-glycans



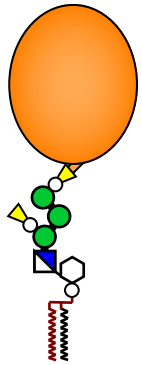
C1GALT1/SLC35A1-KO

Tn

GPI-APs



PGAP4/PIGZ-KO + PGAP1-OE



GPI core

Contents

Simplification of N-glycan structures in HEK293:

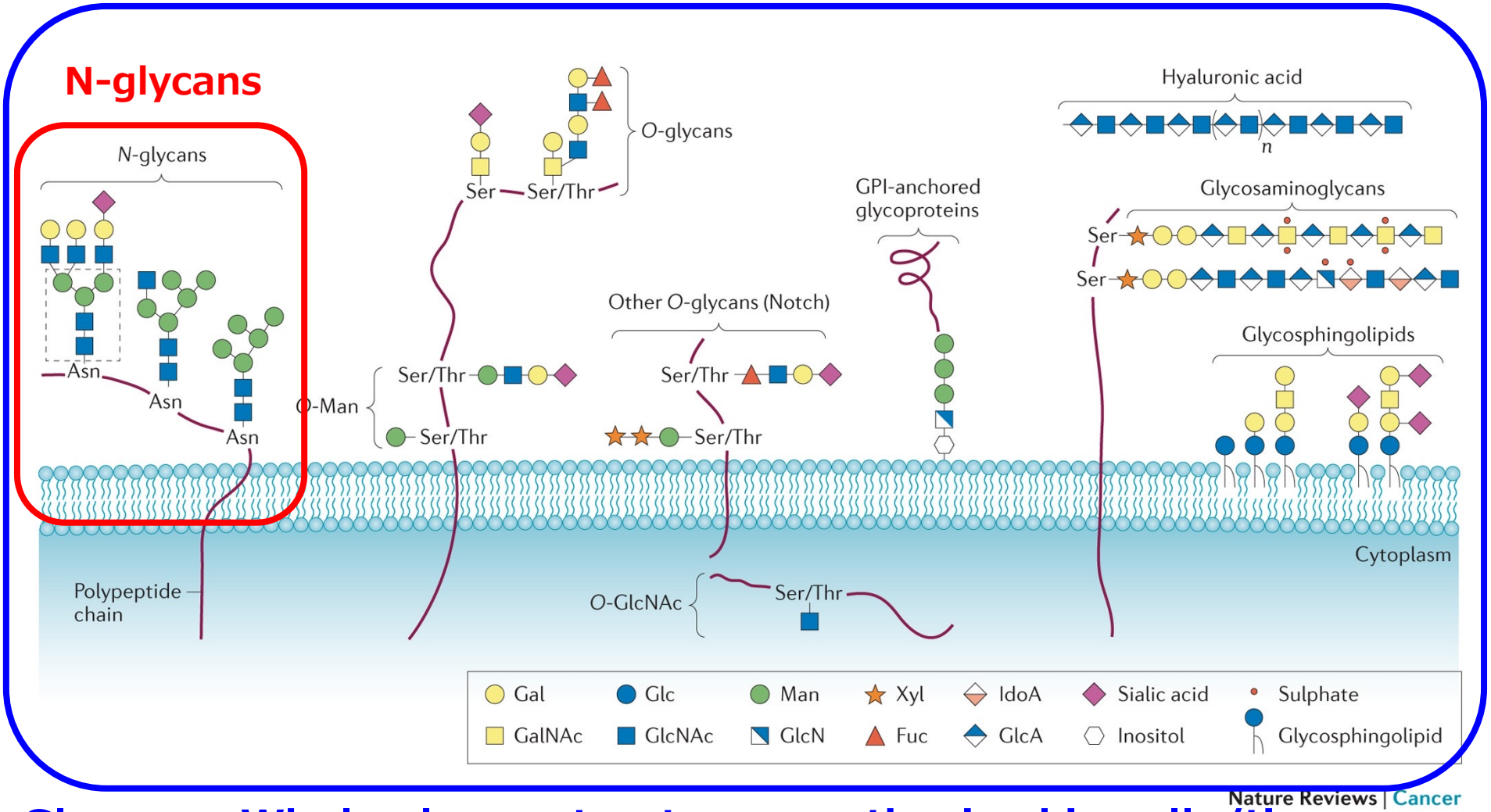
Production of recombinant proteins

only having high-mannose type N-glycans

Development of a visualization tool for glycan metabolic pathways based on gene expression profiles:

Glyco-engineering and Glyco-comparison using GlycoMaple

Mammalian glycan structures

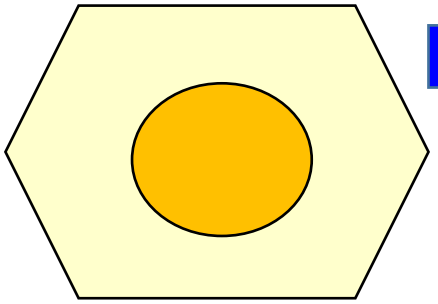


Glycome: Whole glycan structures synthesized in cells/tissues

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Pinho and Reis (2015) *Nat. Rev. Cancer* 15: 540

Prediction of Glycan structures



Glycan structures synthesized in cells

Glycan analysis (Mass Spec., HPLC)

Skillful technique is required. It is hard to analyze glycan structures with high molecular weight, charged or isomers.

Glycan research is still a big hurdle for many researchers.
It's possible to miss important phenomena involving glycans.

Need an easy way to access to glycan research

Need a method to analyze comprehensive glycan structures

It is useful if Glycan structures are predicted from Gene expression profiles

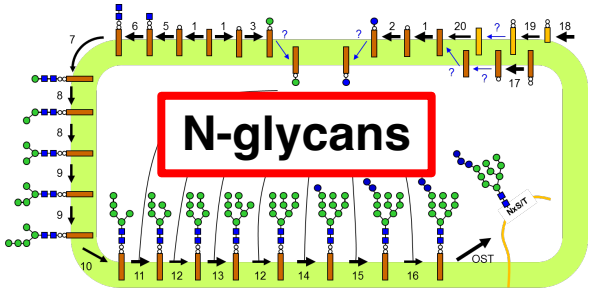
(1) List of Glycan-related genes
(2) Maps of Glycan metabolisms

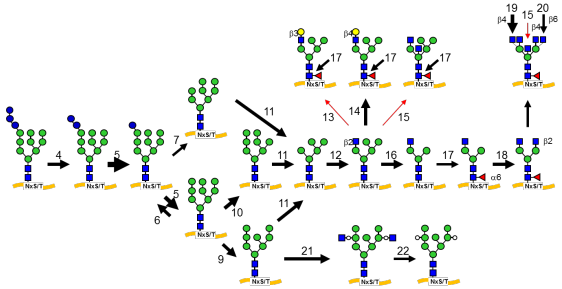
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

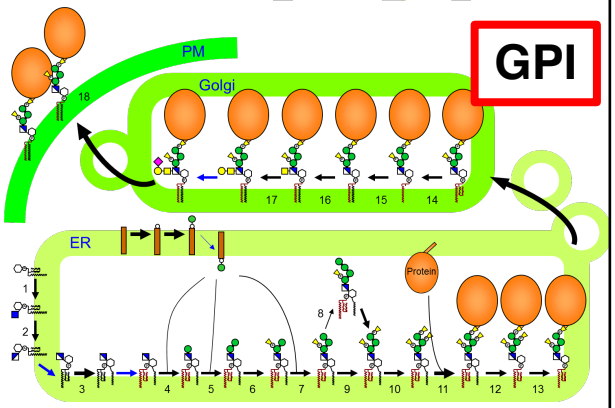
(1) List of glycan-related genes

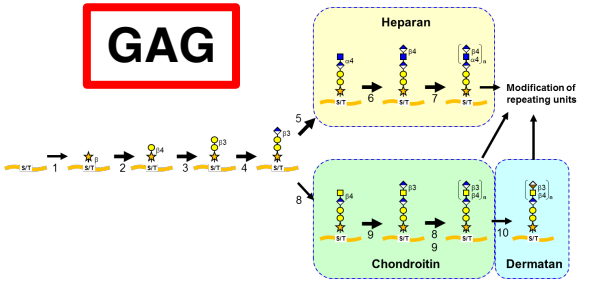
Group	No. of genes
1. Lipid-linked oligosaccharide (LLO) biosynthesis	38
2. N-glycan processing and branching	41
3. Glycosaminoglycan (GAG) biosynthesis and proteins	64
4. O-glycan (mucin-type) biosynthesis	33
5. O-glycan (others) biosynthesis	36
6. Glycosphingolipid (GSL) biosynthesis	77
7. N-glycan / O-glycan / GSL modification	47
8. GPI biosynthesis and proteins	178
9. C-mannosylation	4
10. Sugar-nucleotide biosynthesis	56
11. Sugar transporters	47
12. Golgi homeostasis	19
13. Lectins	172
14. Glycogen synthesis/metabolism	14
15. Hyaluronan synthesis/metabolism	11
16. Sulfate related	18
17. Lysosomal degradation of glycans	16
18. Other Glycosyltransferase (CAZy)	33
19. Other Glycoside hydrolase (CAZy)	32
20. Carbohydrate binding module (CAZy)	15
Total	**951**

(2) Drawing glycan metabolic maps





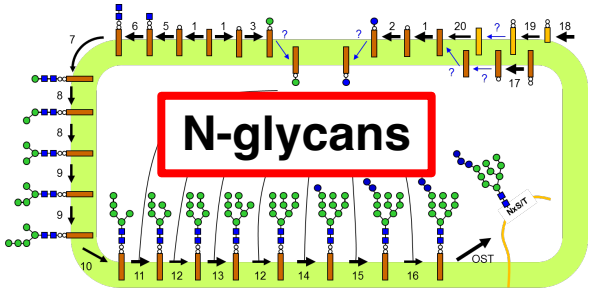


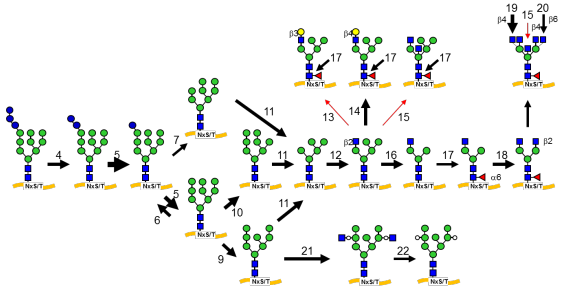


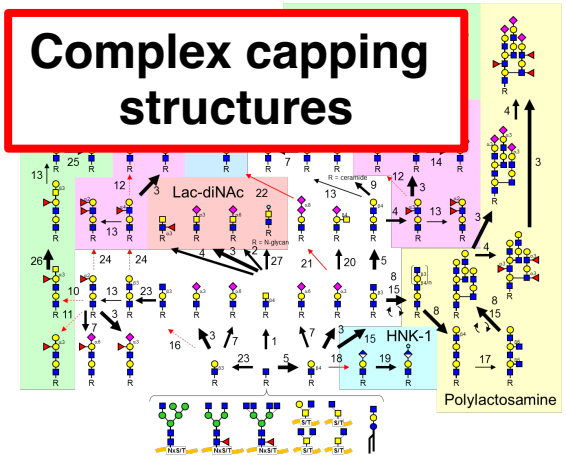
Map Number

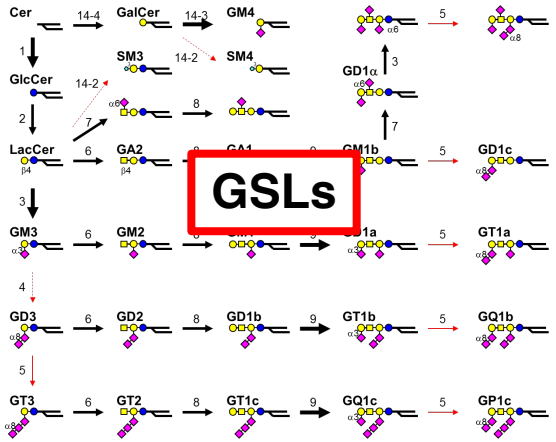
1. Lipid-linked oligosaccharide (LLO) biosynthesis
2. N-glycan processing and branching
3. Complex capping of N-glycan / O-glycan / GSLs
4. GPI biosynthesis
5. O-GalNAc (mucin-type) biosynthesis
6. O-Fuc / O-Glc / Col-Gal / O-GlcNAc / C-Man
7. O-Man biosynthesis
8. Glycosaminoglycan (GAG) biosynthesis
9. Heparan sulfate biosynthesis
10. Chondroitin sulfate and dermatan sulfate
11. Keratan sulfate
12. Glycosphingolipid (core) biosynthesis
13. Globoside biosynthesis
14. Ganglioside biosynthesis
15. Sugar nucleotide biosynthesis
16. Lysosomal degradation of N-glycans
17. Lysosomal degradation of GSLs
18. Lysosomal degradation of GAGsy)
19. Hyaluronic acid biosynthesis and catabolism
20. Human milk oligosaccharide

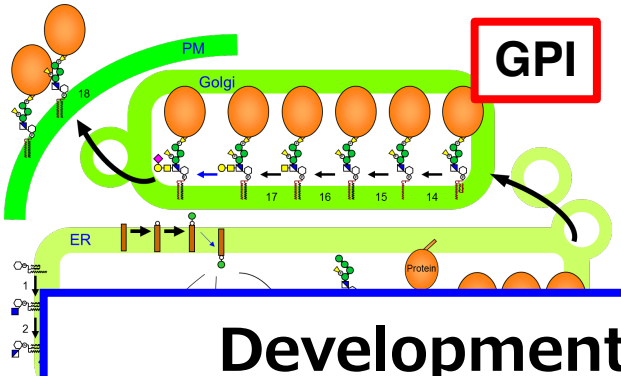
Mapping to glycan metabolic pathways

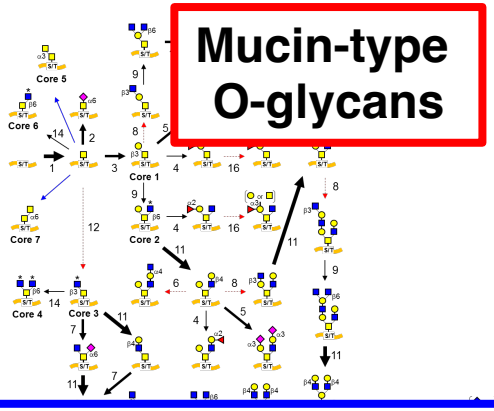


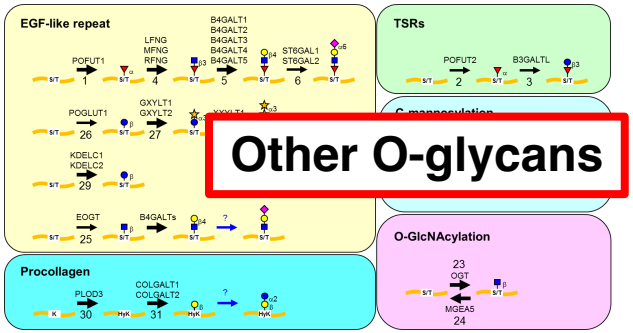










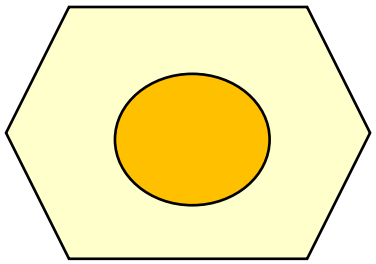


Development of "GlycoMaple", a tool to visualize and predict glycan metabolic pathways based on gene expression profiles

https://glycosmos.org/glycomaple/index

Huang et al. (2021) *Dev. Cell*

Expression profiles of glycan-related genes in HEK293 cells



Model cell:
HEK293

RNA-seq

Calculate each gene expression as
TPM: **T**ranscripts **P**er **M**illion

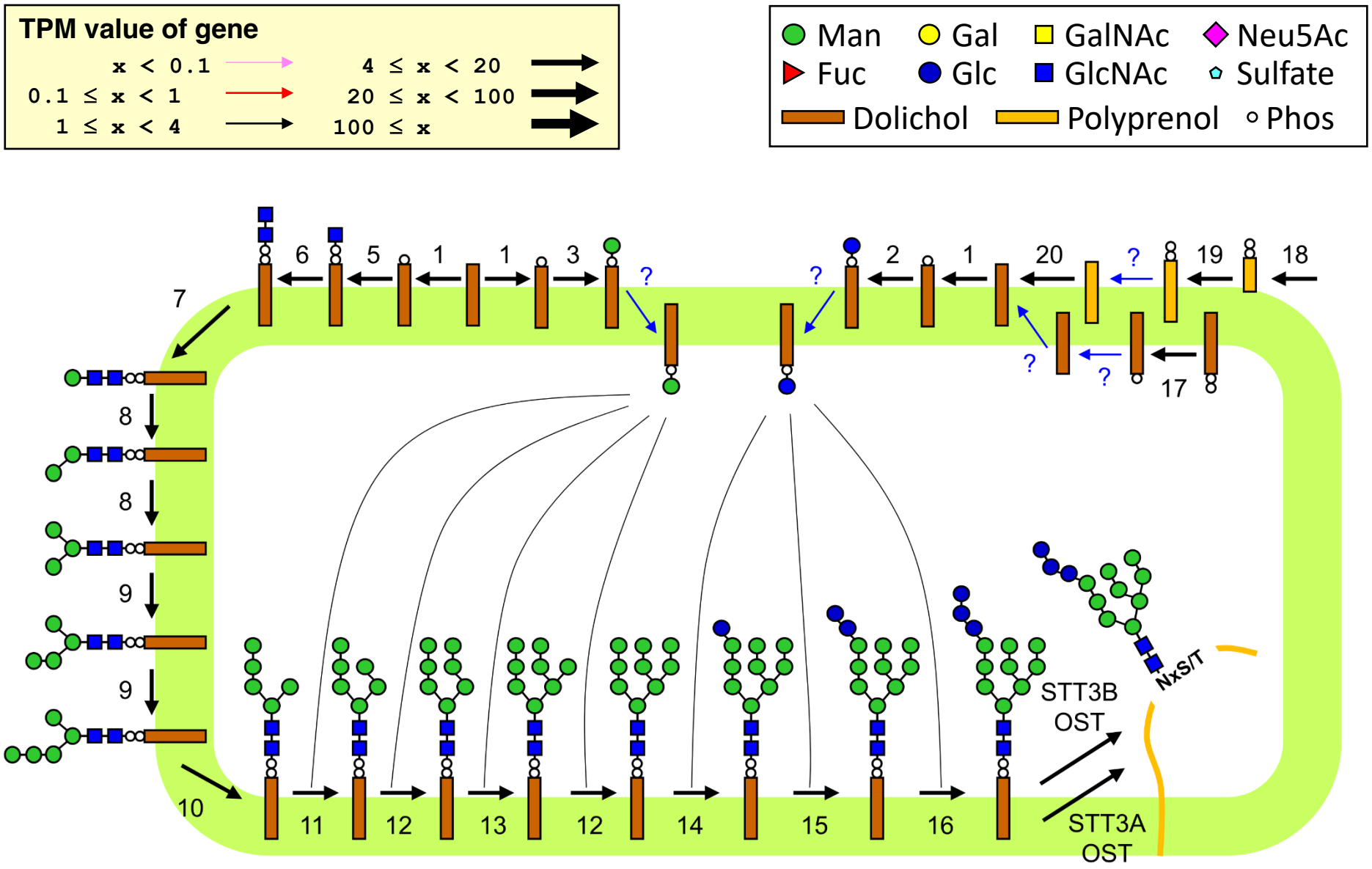
$$TPM = \frac{\text{Reads / length of transcript}}{\sum_{\text{All transcripts}} (\text{Reads / length of transcript})} \times 10^6$$

Analyze 951 glycan-related gene expression

Integrate the information to the glycan metabolic pathways

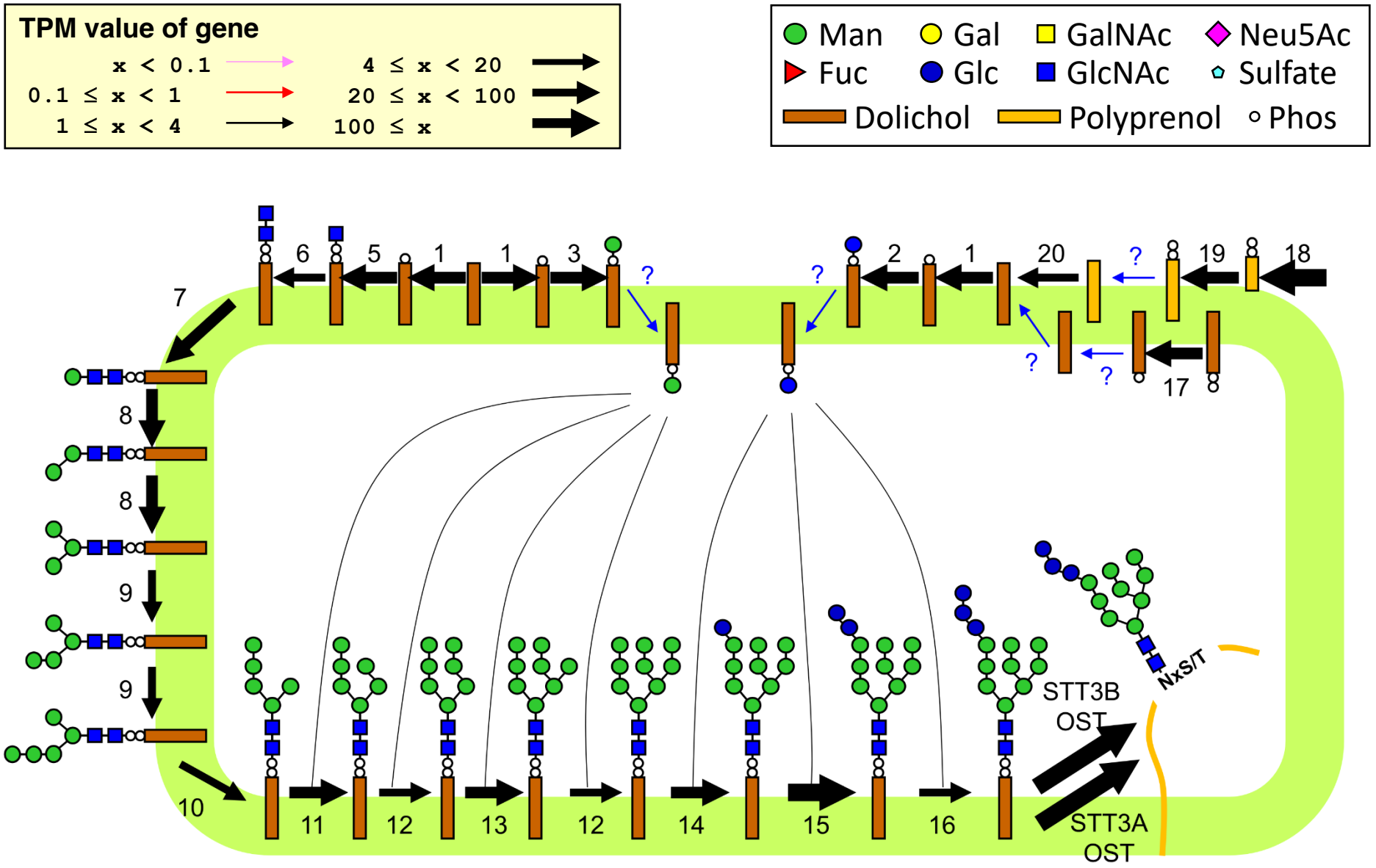
TPM value of gene	Biosynthetic pathway
$x < 0.1$	Not expressed
$0.1 \leq x < 1$	Very weak
$1 \leq x < 4$	Expressed
$4 \leq x < 20$	
$20 \leq x < 100$	
$100 \leq x$	
Unknown gene	

LLO biosynthesis and OST



LLO: lipid-linked oligosaccharide; LLO is a precursor for N-glycosylation.

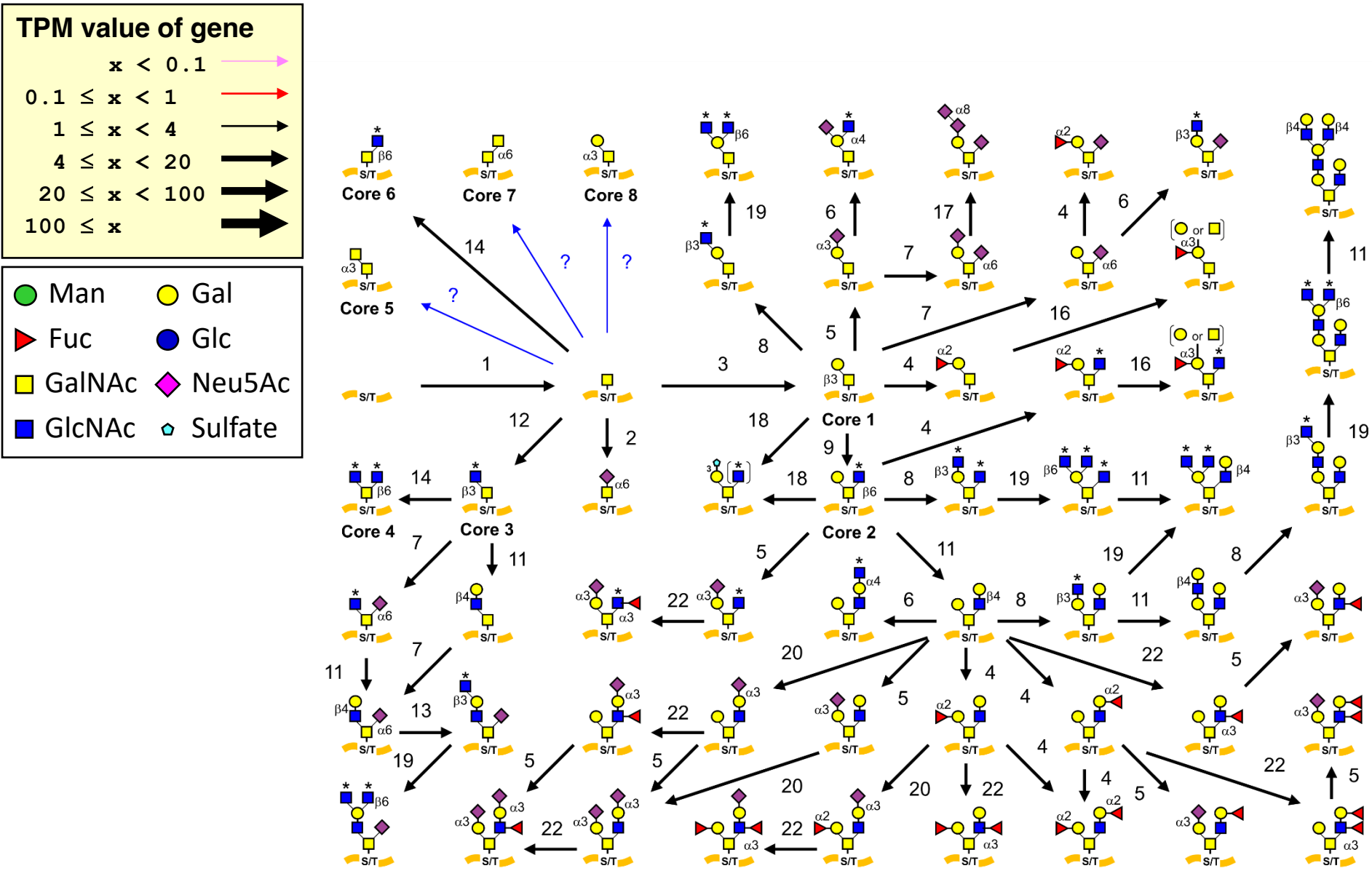
LLO biosynthesis and OST in HEK293



The LLO pathway is very basic and essential for cells.

LLO: lipid-linked oligosaccharide; LLO is a precursor for N-glycosylation.

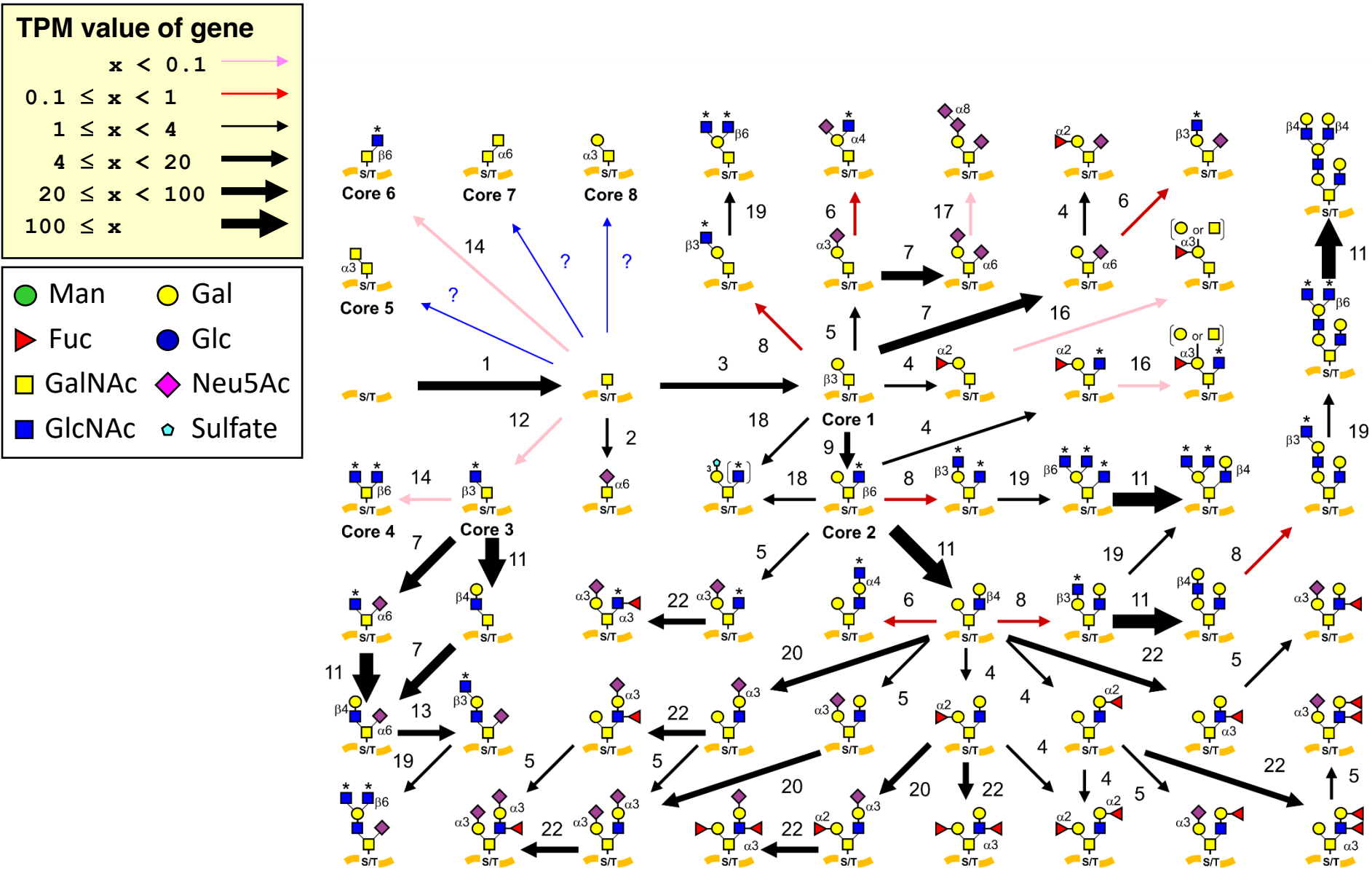
Biosynthesis of mucin-type O-glycans



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Huang et al. (2021) *Dev. Cell*

Biosynthesis of mucin-type O-glycans in HEK293



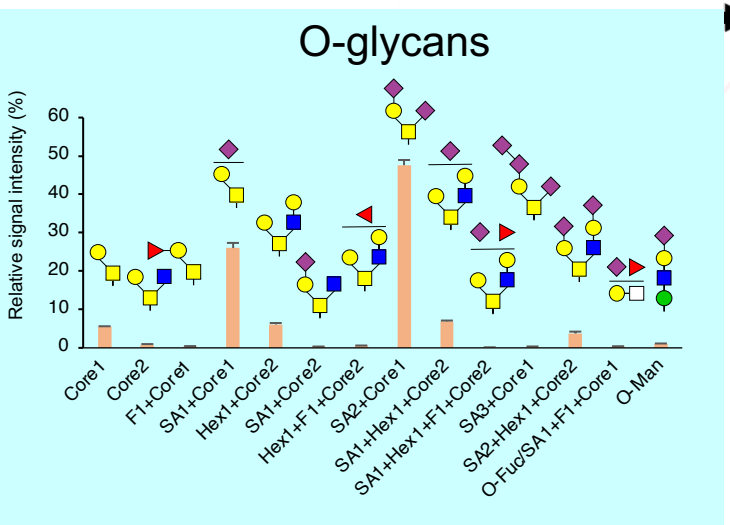
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Biosynthesis of mucin-type O-glycans in HEK293

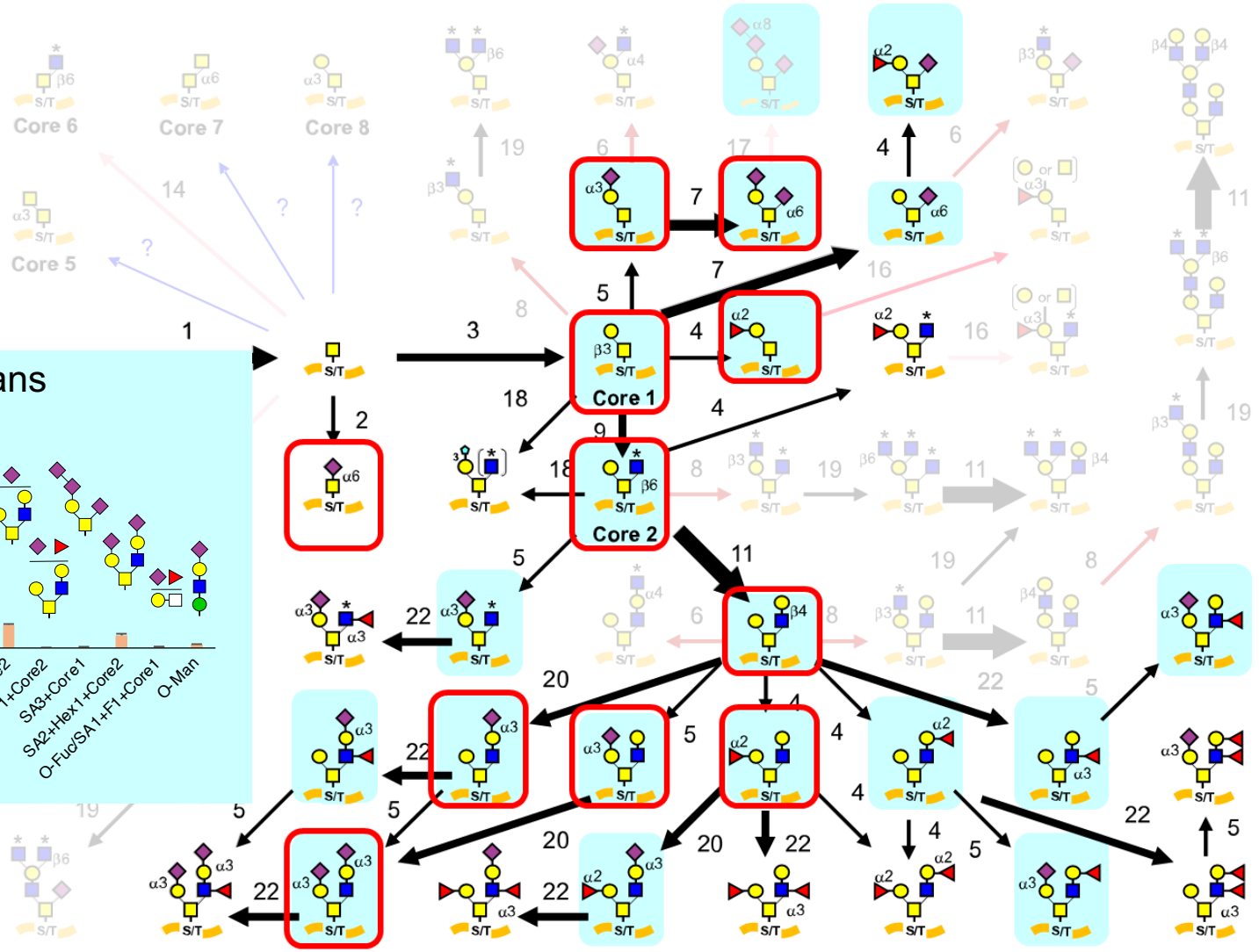
TPM value of gene

x < 0.1	
0.1 ≤ x < 1	
1 ≤ x < 4	
4 ≤ x < 20	
20 ≤ x < 100	
100 ≤ x	

Razawi H et al. (2013)
Glycobiology 23: 935-945



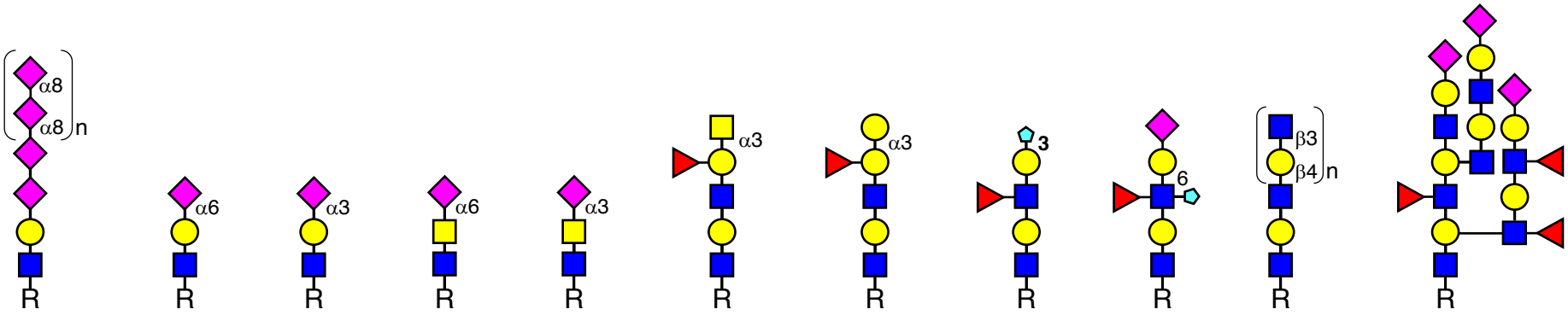
Our glycomic analysis (ESI-MS)



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

GlycoMaple is useful to predict glycan structures synthesized in cells

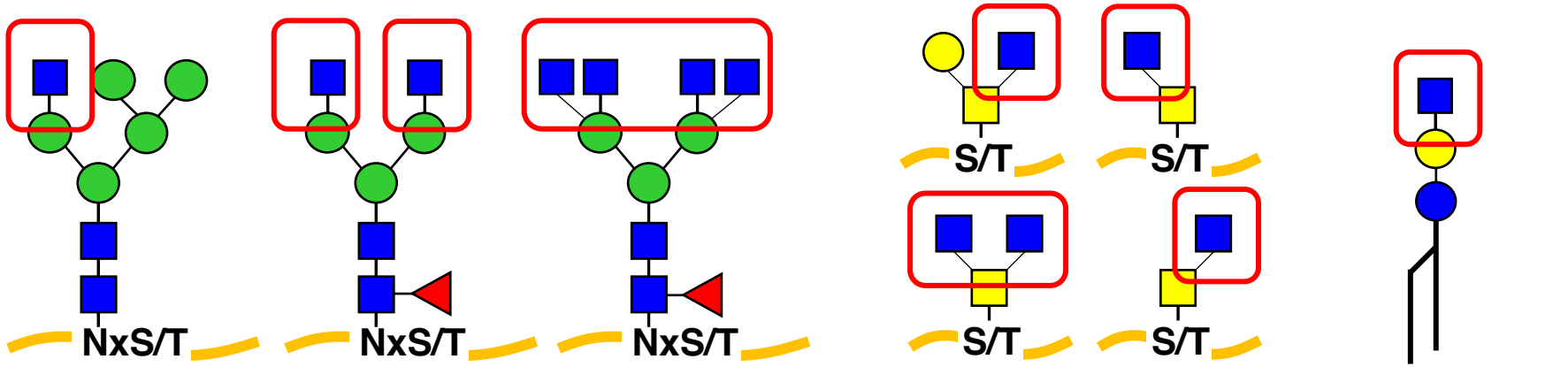
Complex capping of N-glycans / O-glycans / GSLs



Polysialic acid | LacNAc | Lac-diNAc | ABO | Lewis | Polylactosamine

Complex structure formation

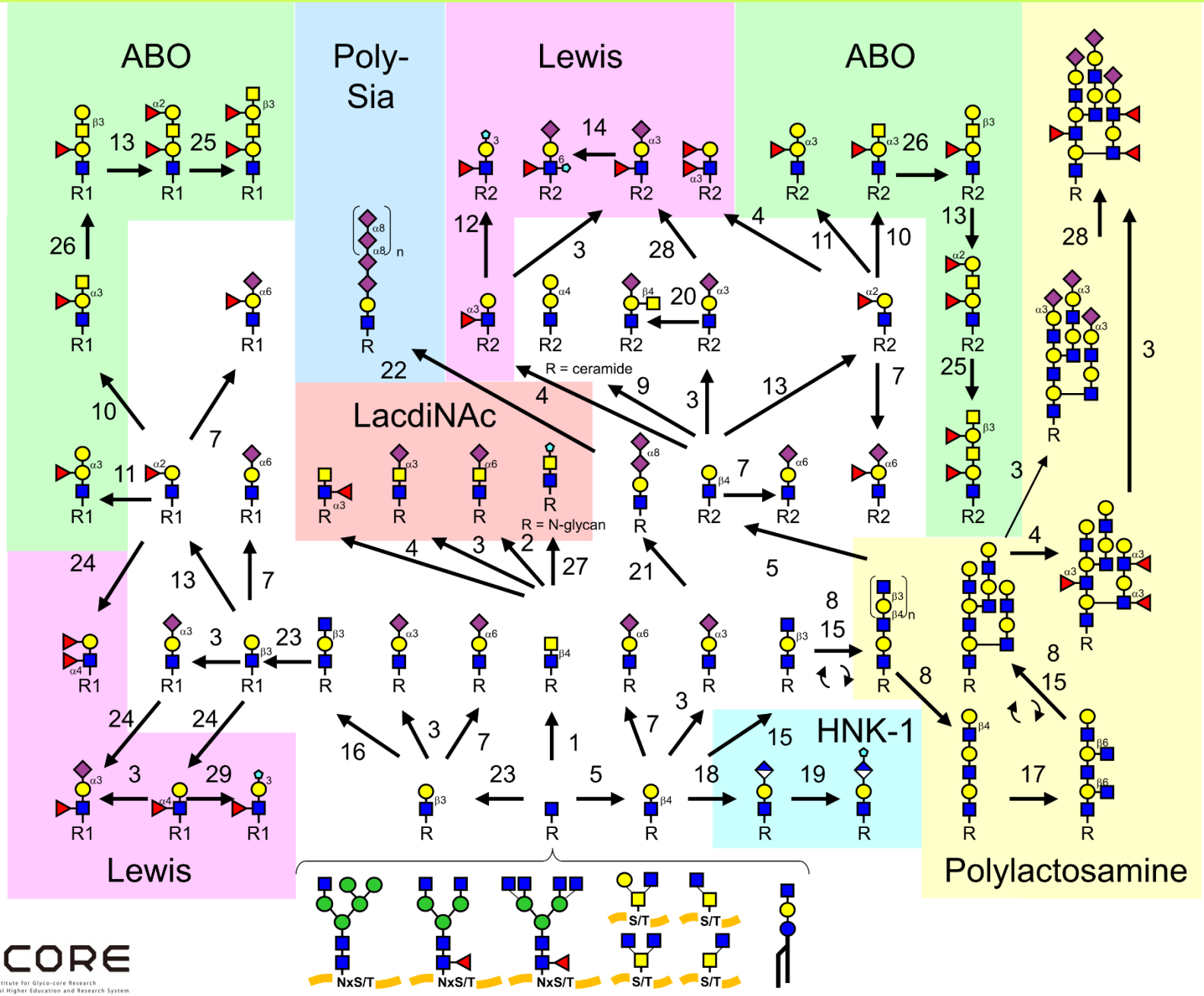
Man Gal GalNAc Neu5Ac
Fuc Glc GlcNAc Sulfate



N-glycans | O-glycans | Glycolipids

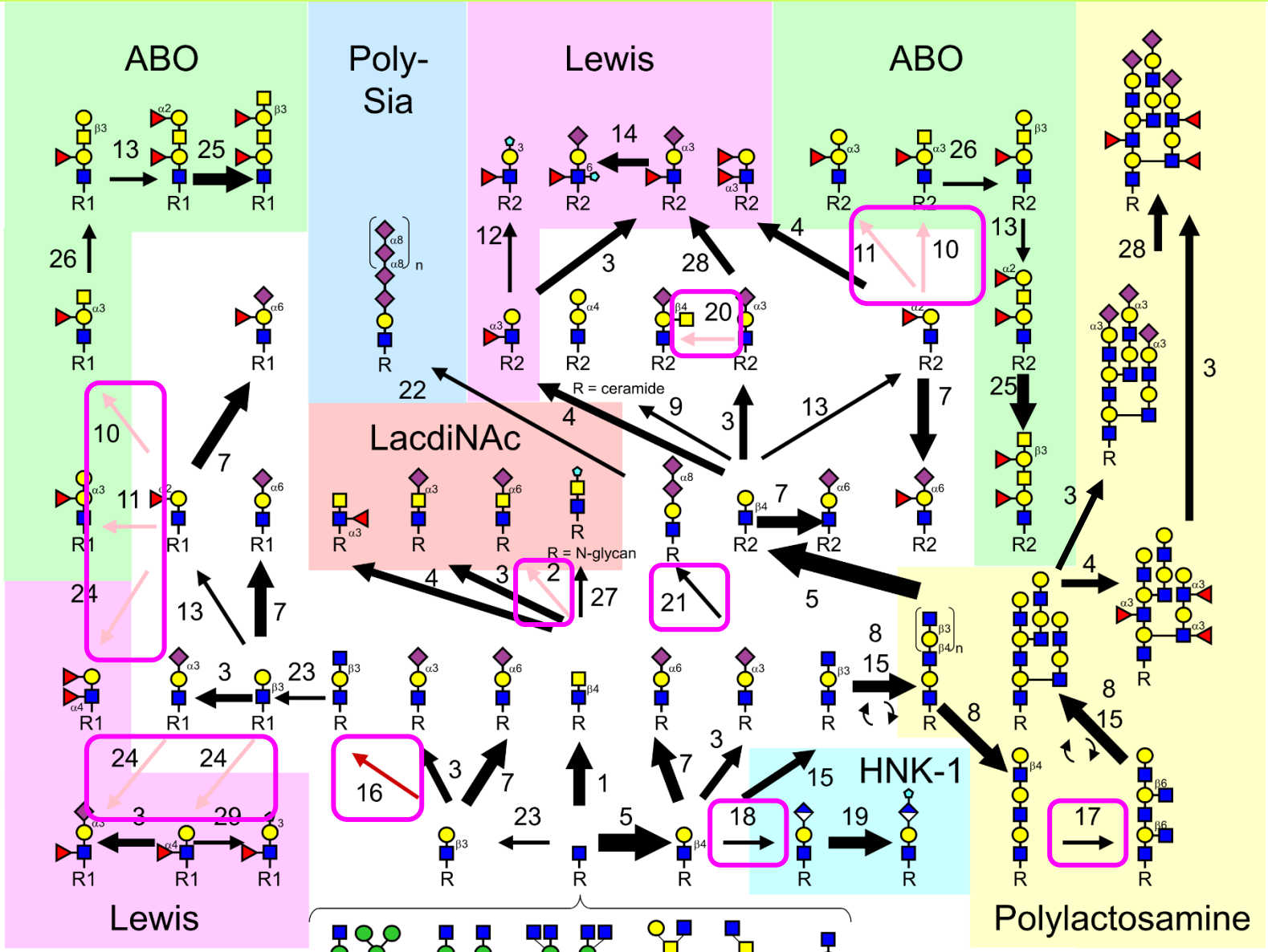
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Complex capping of N-glycans / O-glycans / GSLs



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

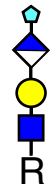
Complex capping of N-glycans / O-glycans / GSLs in HEK293



GlycoMaple could find rate-limiting steps

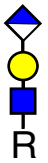
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Customization: Expression of HNK-1 epitope in HEK293

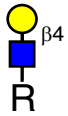


HNK-1 epitope

CHST10: 25.40



B3GAT1: 0.61
B3GAT2: 2.49

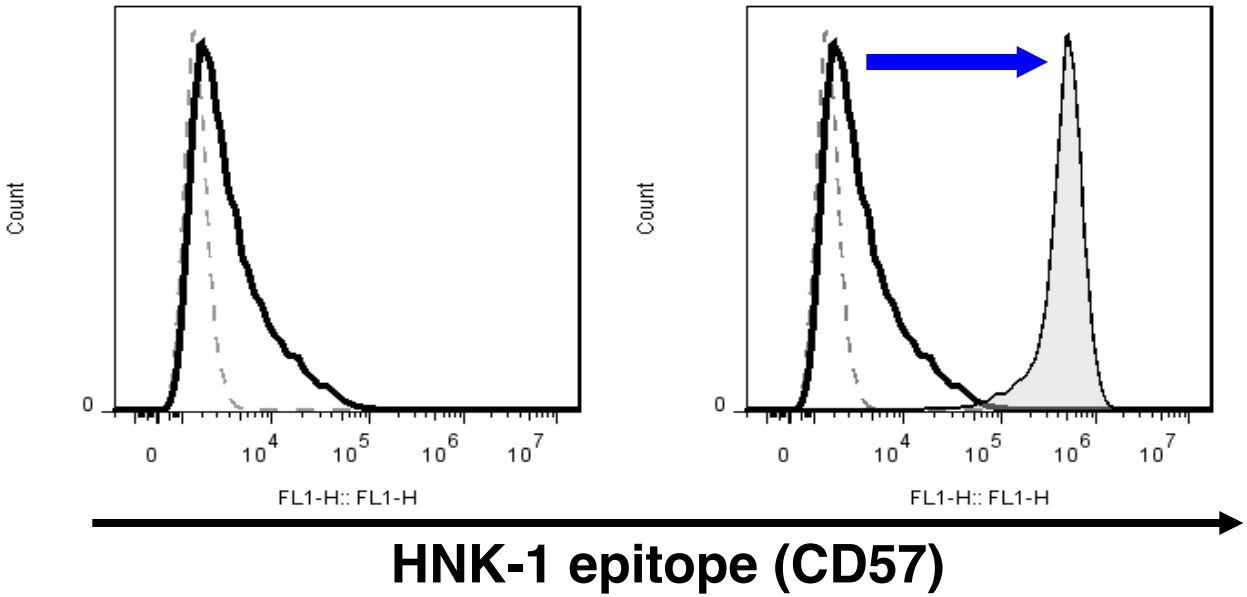


B4GALT1: 64.06
B4GALT2: 92.05
B4GALT3: 53.71
B4GALT4: 27.04
B4GALT5: 76.22

GlcNAc Gal
Sulfate GlcA

background
HEK293
+ B3GAT1

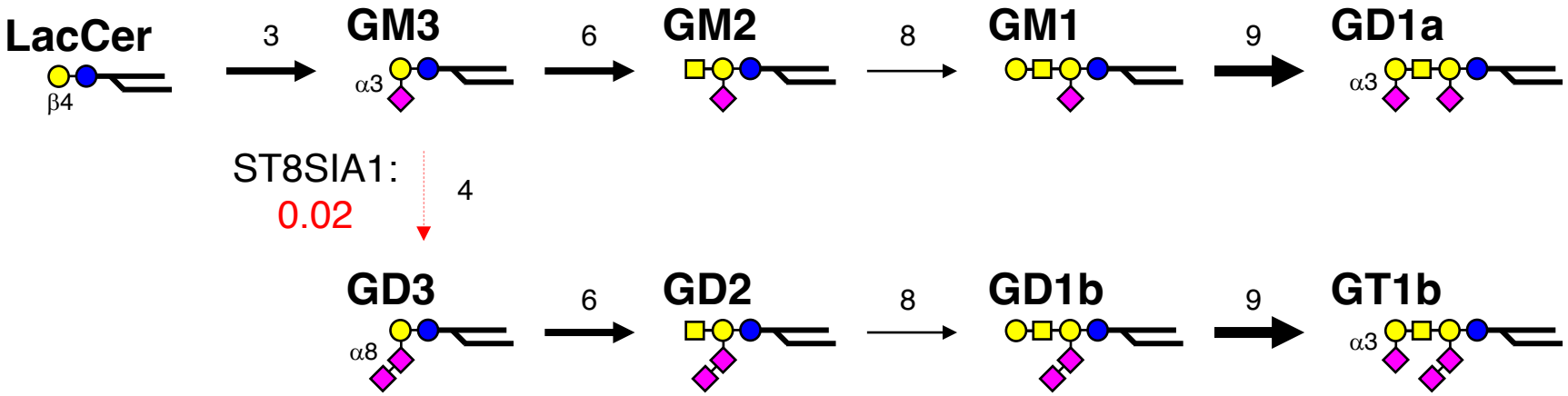
HEK293 **+ B3GAT1**

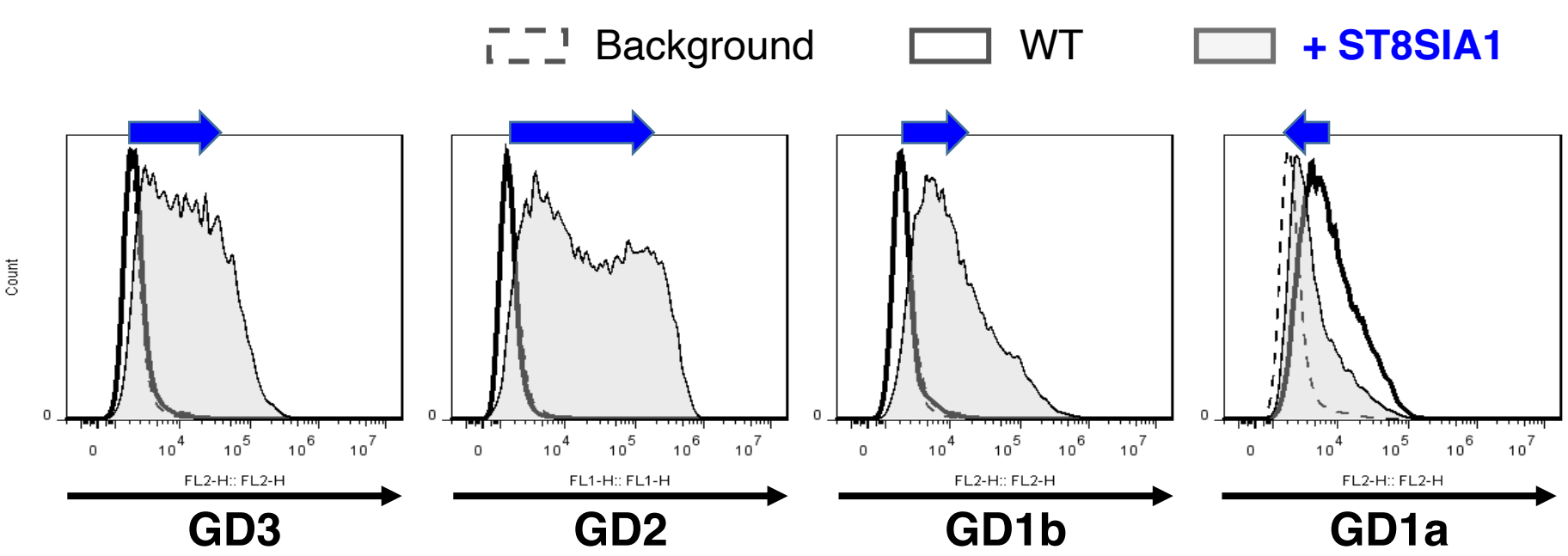


GlycoMaple enables us to find rate-limiting steps and to customize glycosylation pathways.

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Customization: Expression of gangliosides in HEK293

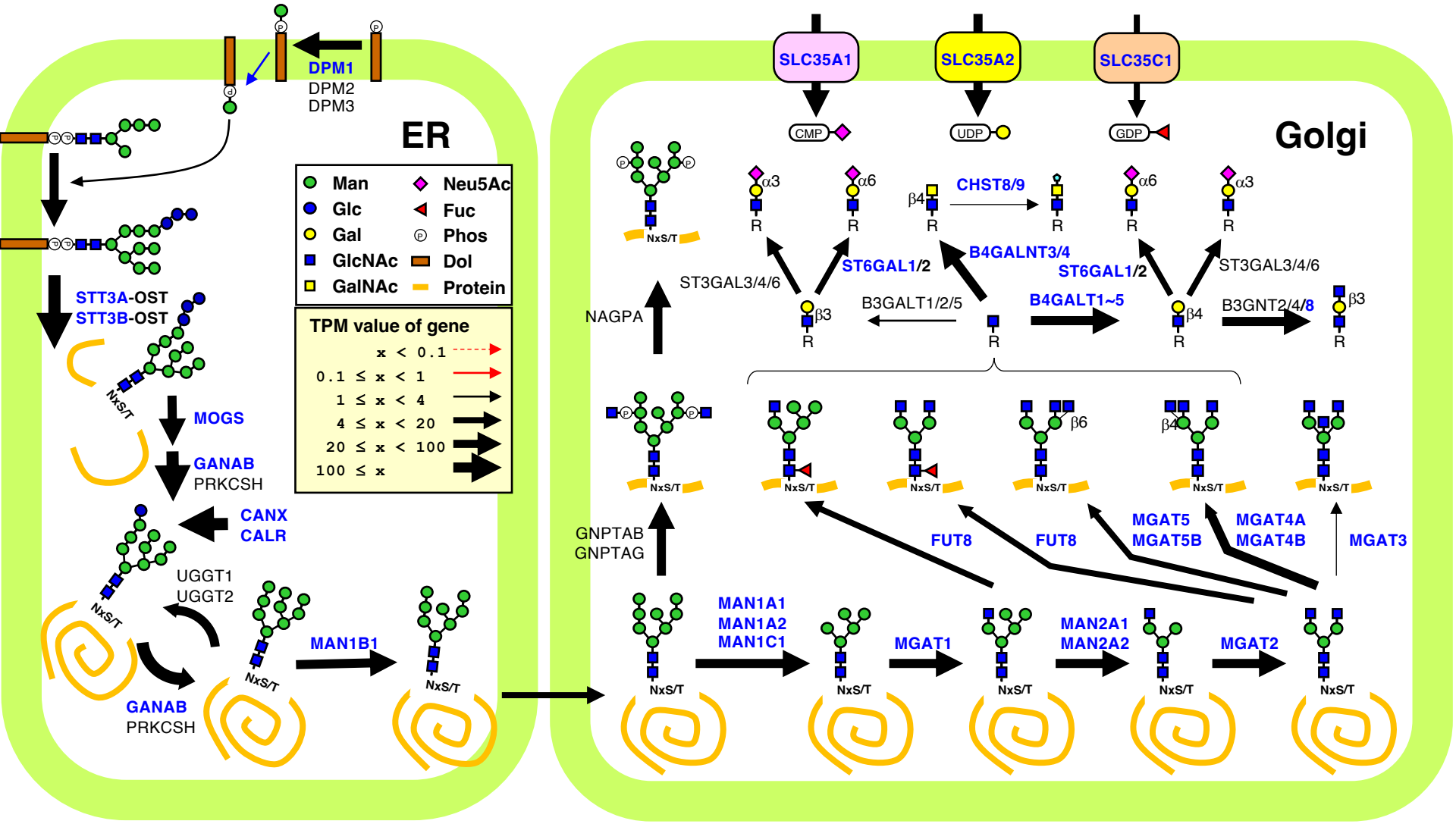




GlycoMaple enables us to find rate-limiting steps and to customize glycosylation pathways.

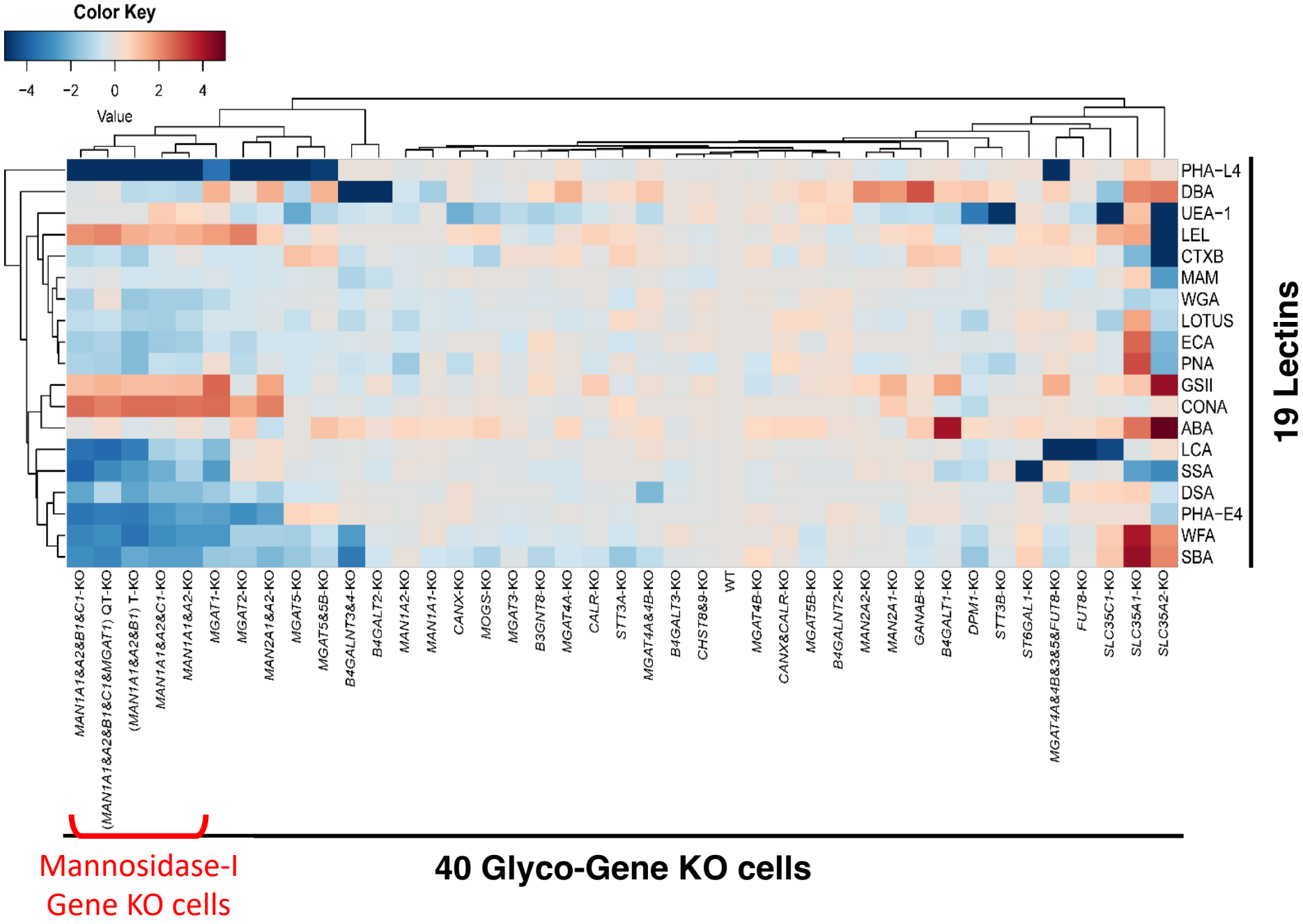
iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Construction of Glyco-Gene KO library

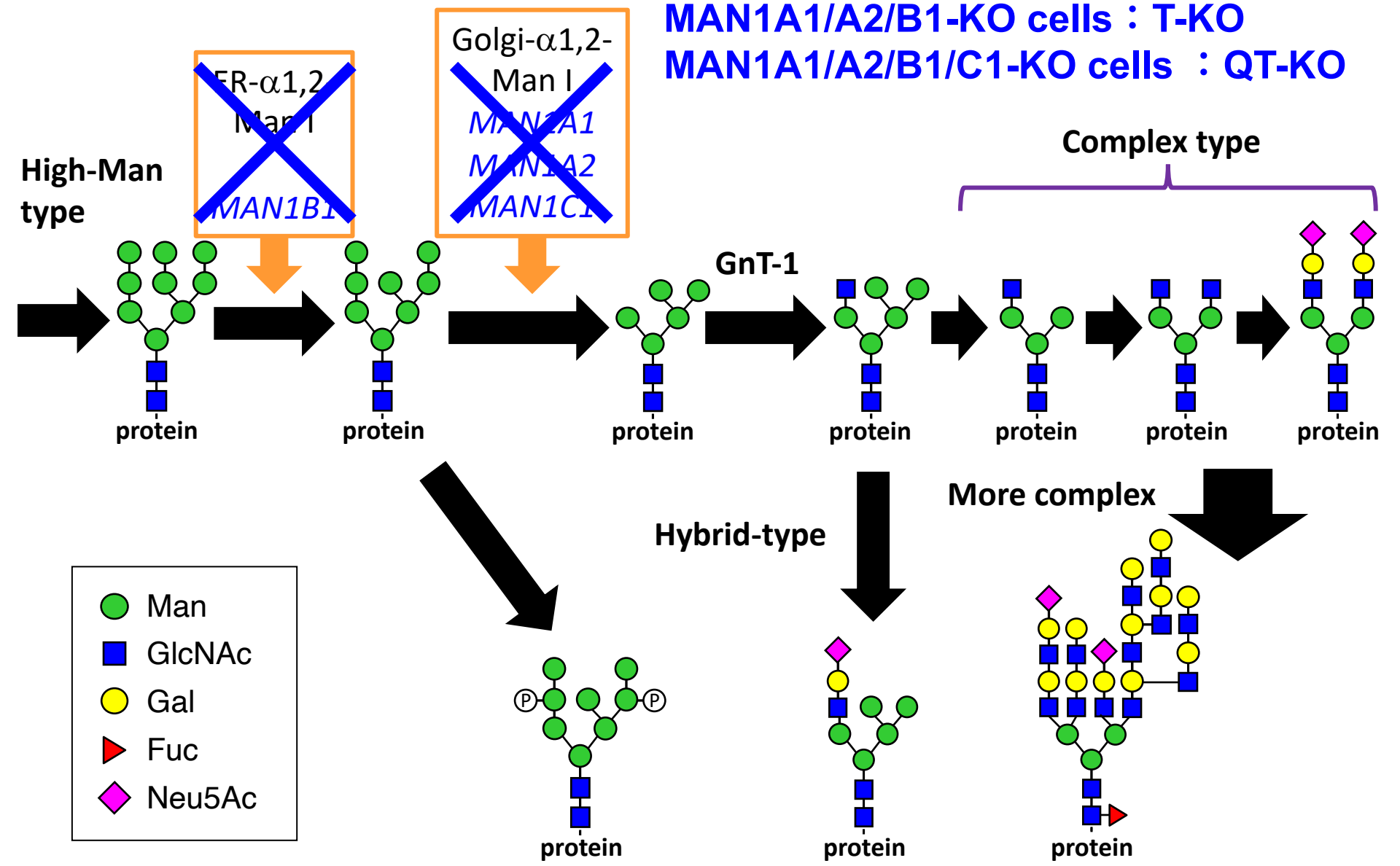


Based on the gene expression profiles in HEK293 cells,
We selected genes required for N-glycan processing (Blue)
We constructed 40 different gene KO cell library.

Lectin staining of 40 Glyco-Gene KO cell library



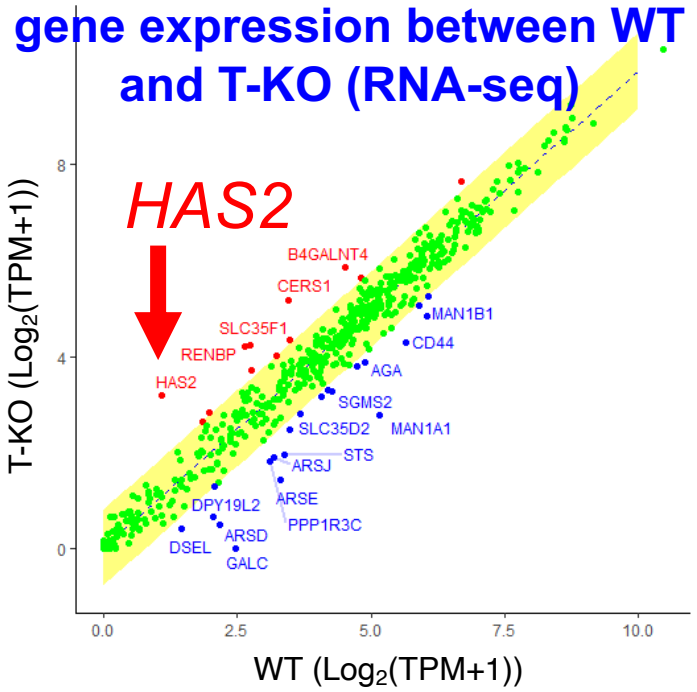
Disruption of multiple mannosidase-genes in HEK293

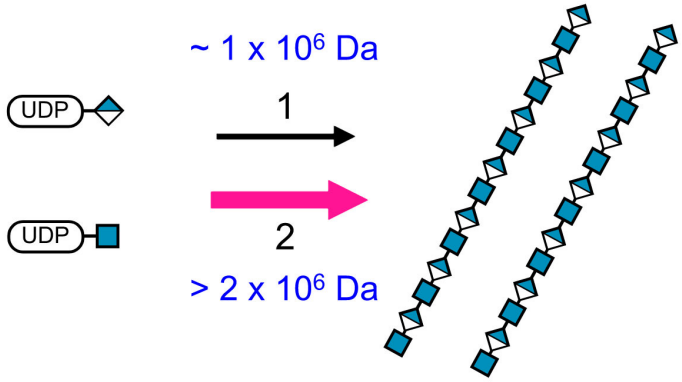


Jin et al (2018) *J. Biol. Chem.*; Ren et al (2019) *J. Biochem.*

Hyaluronan was increased in Mannosidase-KO cells

Comparison of glycan-related gene expression between WT and T-KO (RNA-seq)

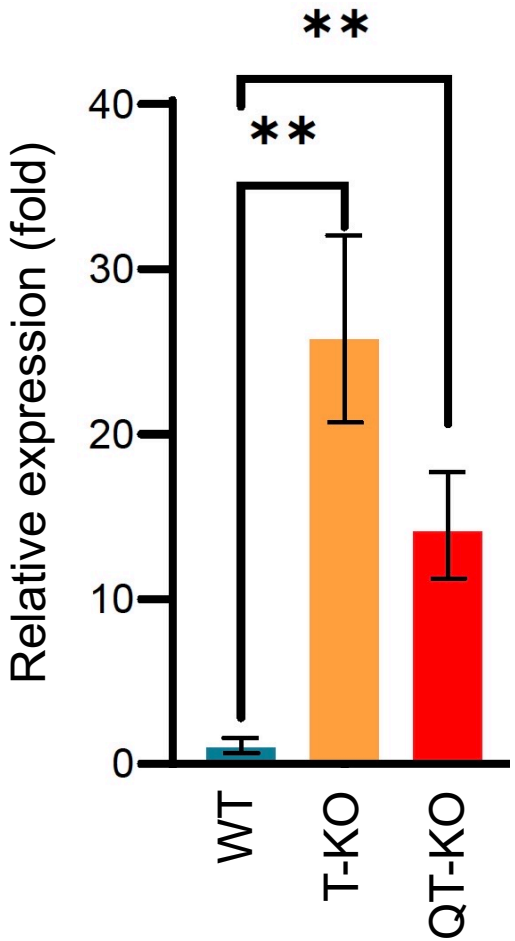




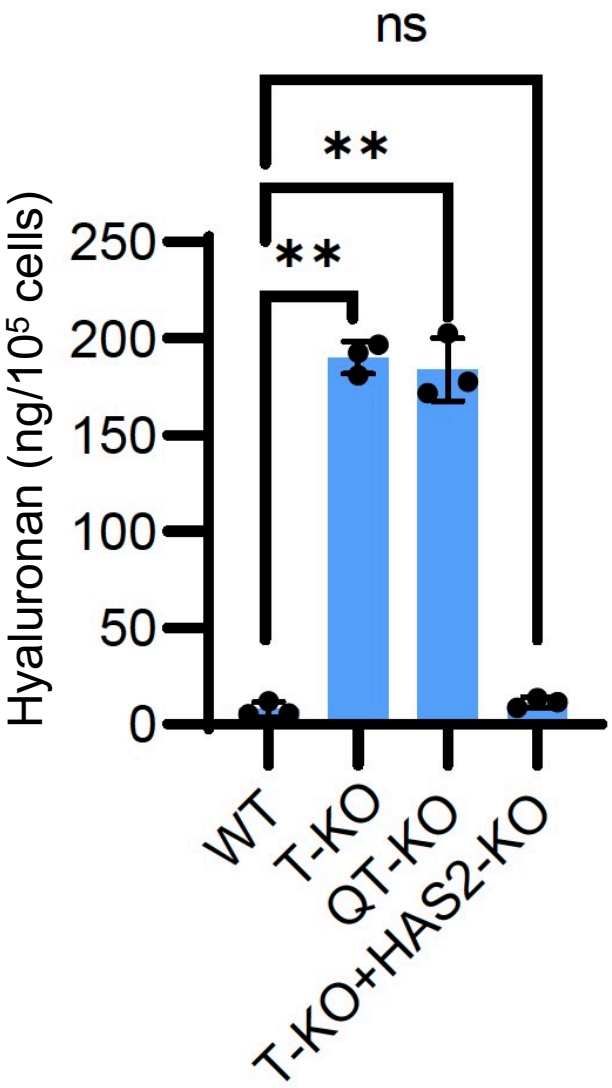
T-KO / WT : 4.3-fold

QT-KO / WT : 4.0-fold

Expression of HAS2 mRNA (qPCR)

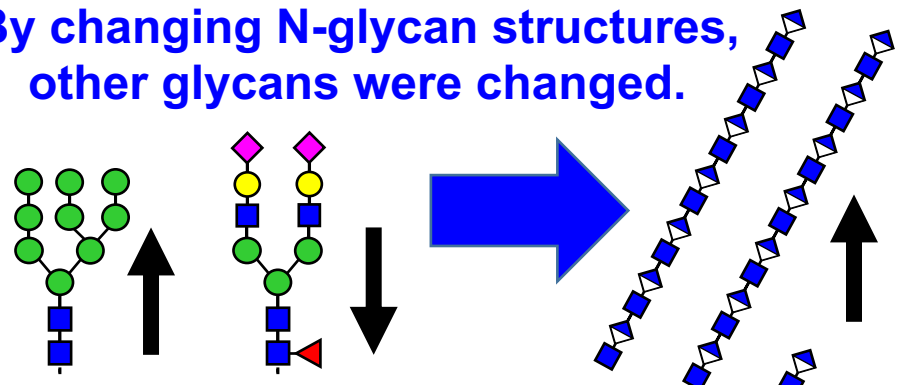


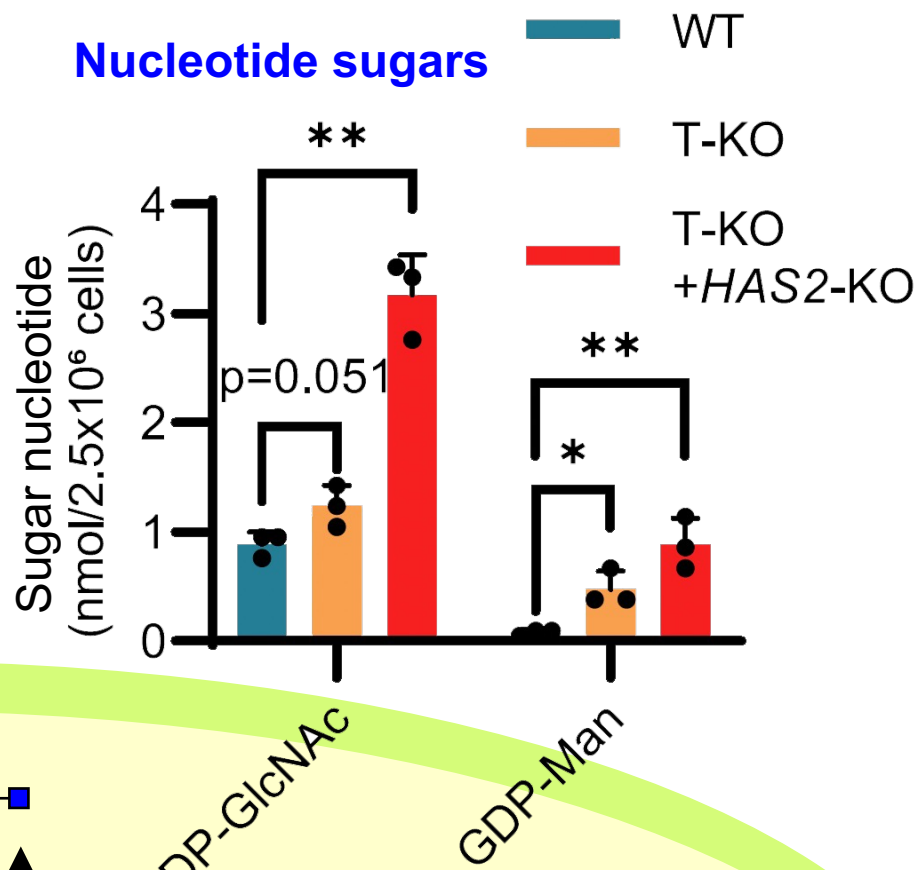
Amounts of Hyaluronan

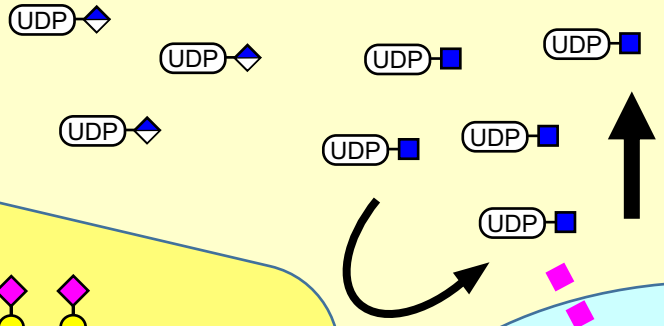


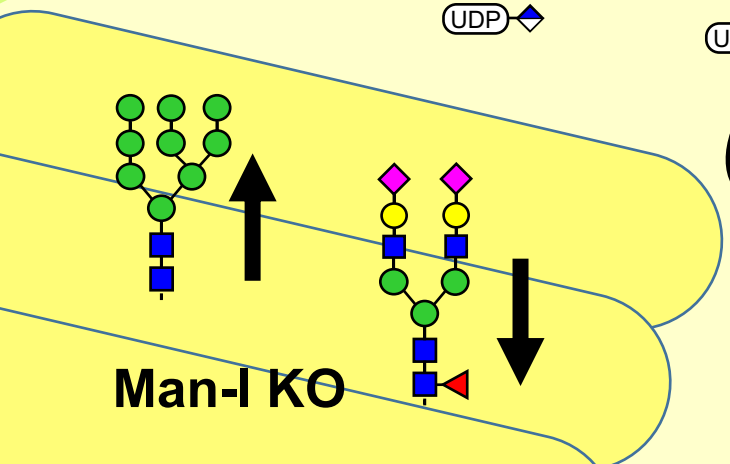
Hyaluronan was increased in Mannosidase-KO cells

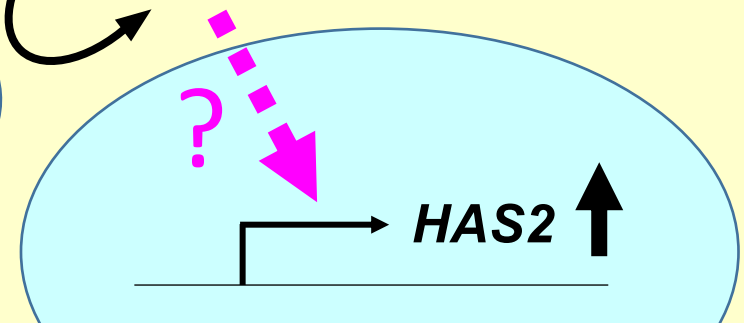
By changing N-glycan structures, other glycans were changed.











GlycoMaple analysis using Kidney RNA-seq data in TCGA

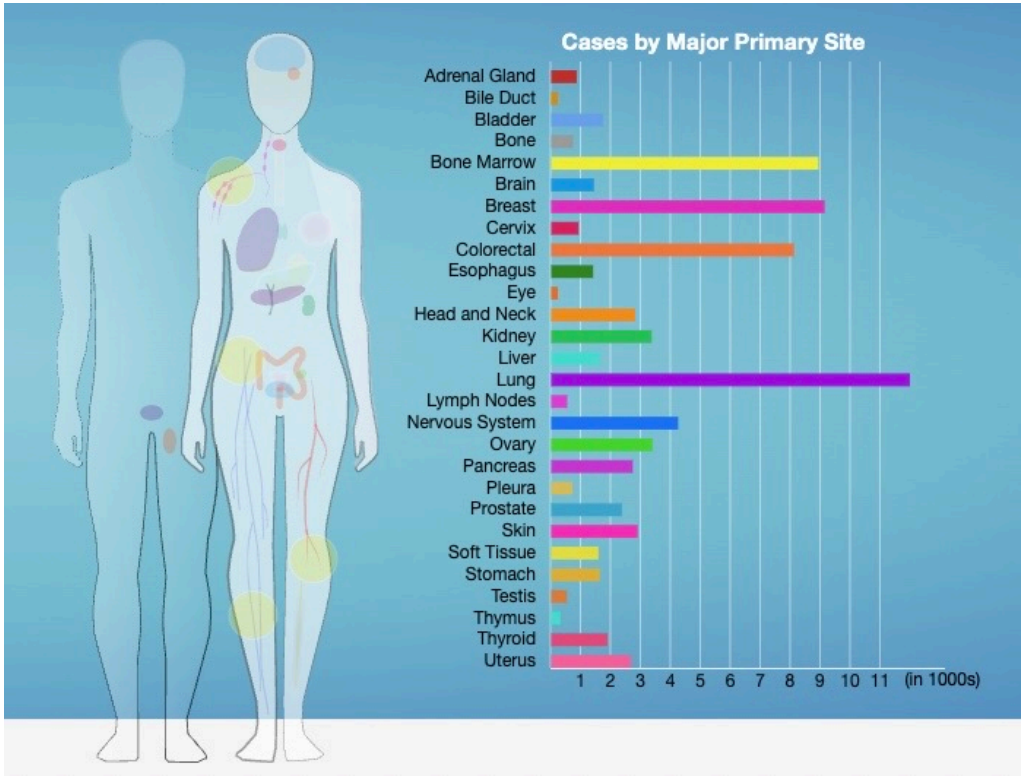
TCGA (The Cancer Genome Atlas)

Comparison of normal and diseased tissues

Kidney

Solid Tissue Normal (Normal)	N = 140
Clear cell carcinoma (ccRCC)	N = 530
Papillary cell carcinoma (pRCC)	N = 288

NIH NATIONAL CANCER INSTITUTE



UNIVERSITY OF CALIFORNIA SANTA CRUZ

UCSC Xena

See the bigger picture

https://portal.gdc.cancer.gov/

https://xenabrowser.net/

iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Biosynthesis of capping structures in Kidney tumors

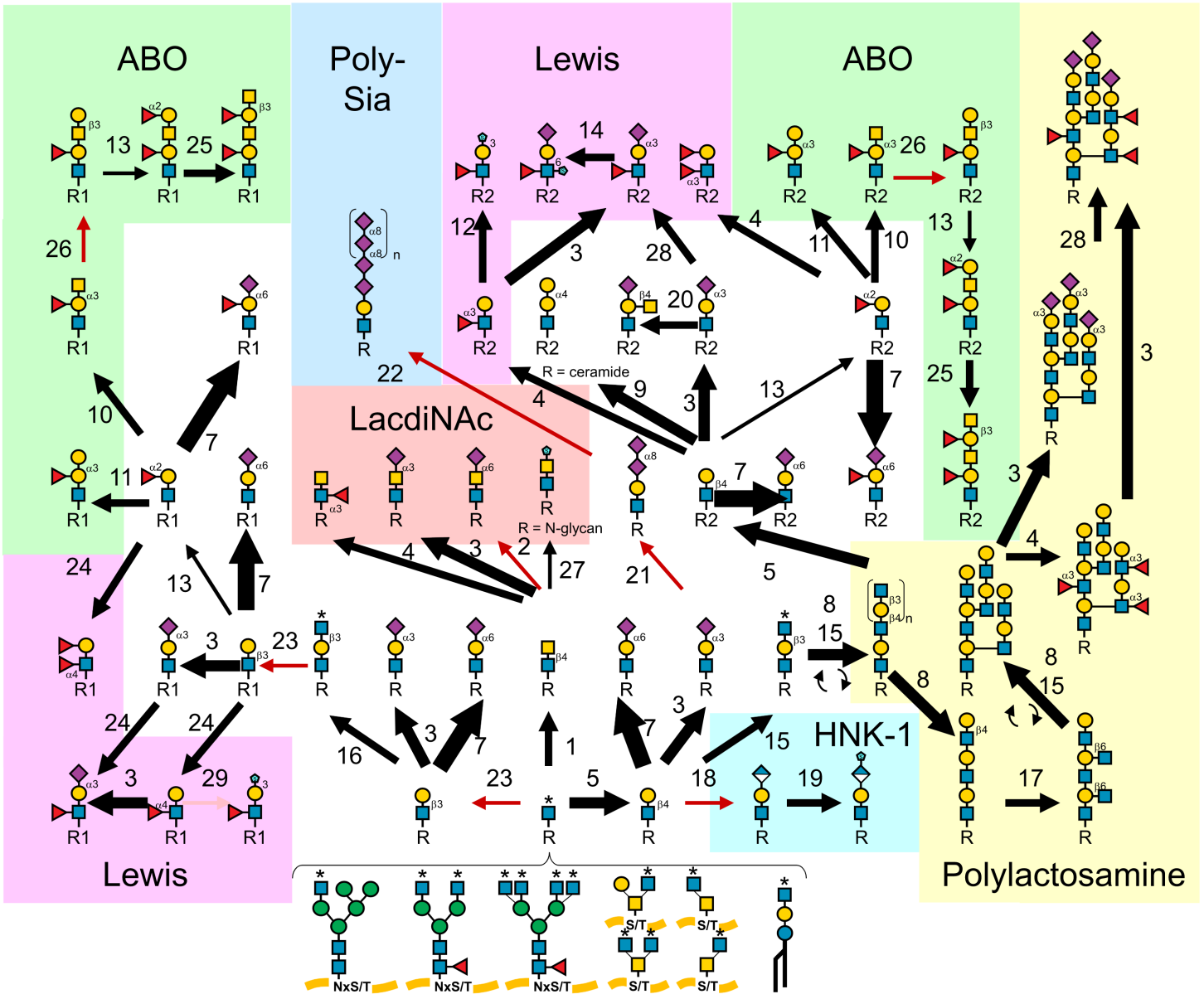
Normal tissue (N = 140; Median)

V.S.

ccRCC (N = 530; Median)

V.S.

pRCC (N = 288; Median)



Biosynthesis of capping structures in Kidney tumors

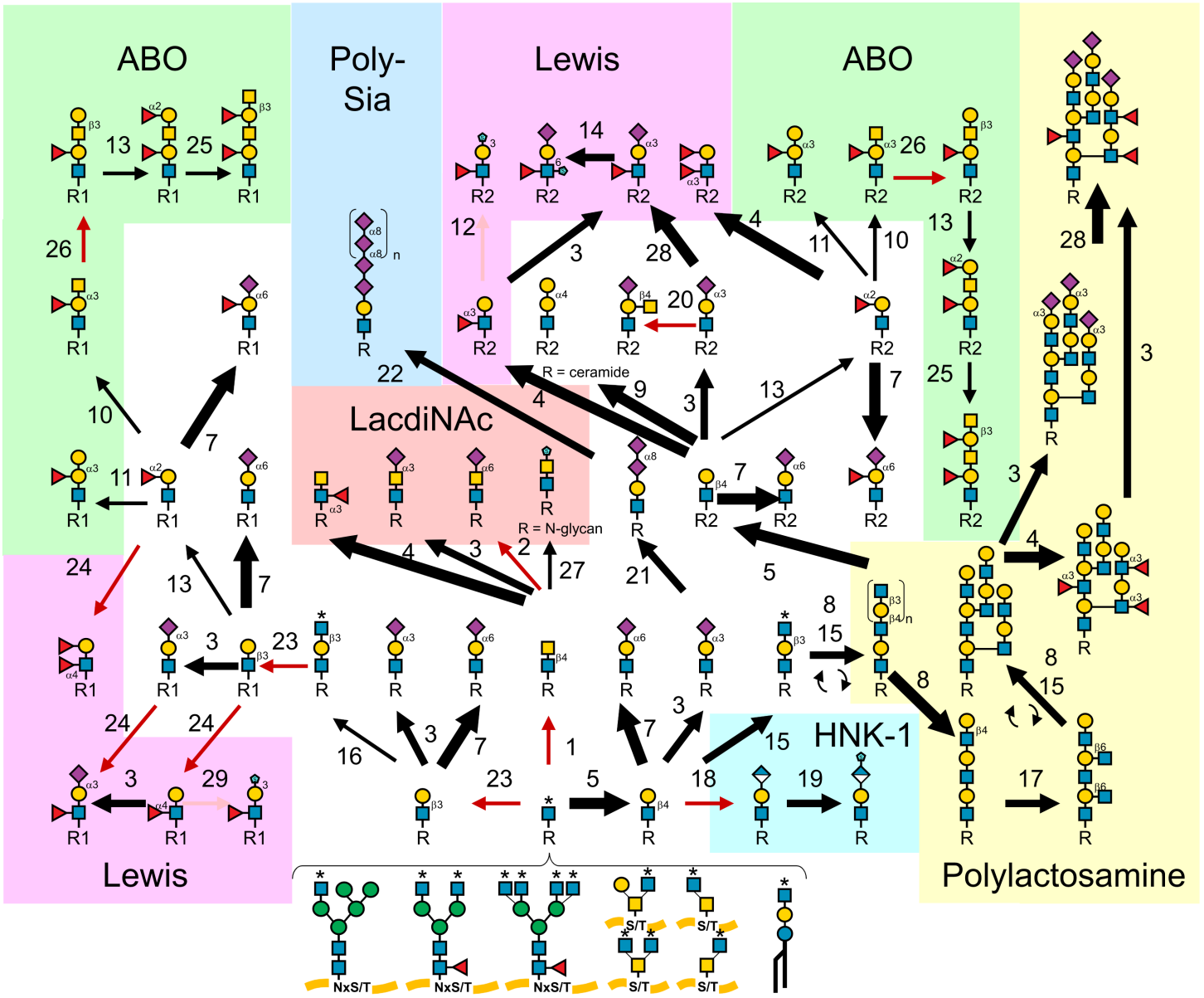
Normal tissue
(N = 140; Median)

V.S.

ccRCC
(N = 530; Median)

V.S.

pRCC
(N = 288; Median)



TPM value of gene

- x < 0.1
- 0.1 ≤ x < 1
- 1 ≤ x < 4
- 4 ≤ x < 20
- 20 ≤ x < 100
- 100 ≤ x

- Man
- Gal
- Fuc
- Glc
- GalNAc
- Neu5Ac
- GlcNAc
- Sulfate

Biosynthesis of capping structures in Kidney tumors

Normal tissue
(N = 140; Median)

V.S.

ccRCC
(N = 530; Median)

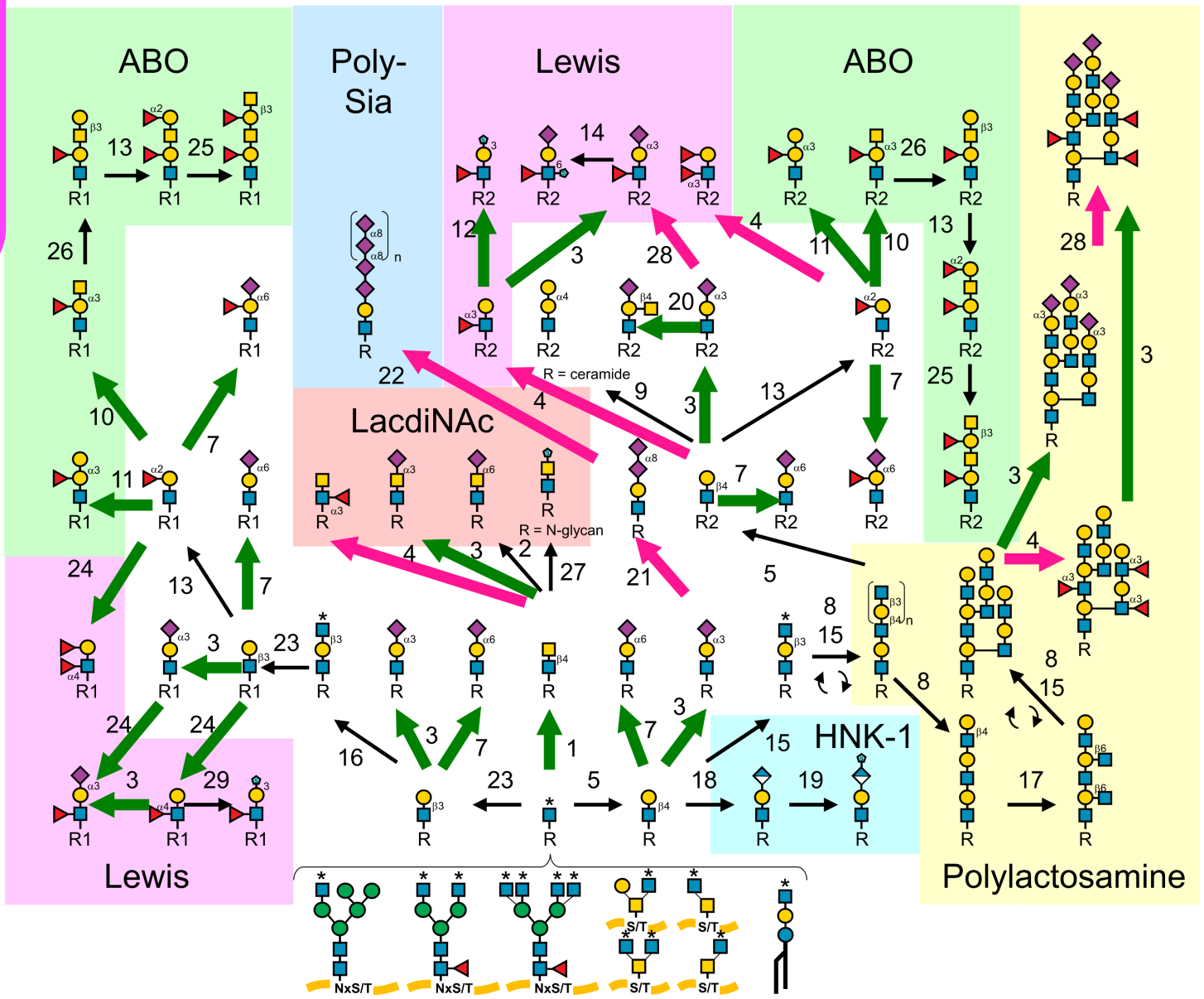
V.S.

pRCC
(N = 288; Median)

$$\frac{TPM_{tumor} + 1}{TPM_{normal} + 1}$$

≥ 2 →

< 0.5 →



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Biosynthesis of capping structures in Kidney tumors

Normal tissue
(N = 140; Median)

V.S.

ccRCC
(N = 530; Median)

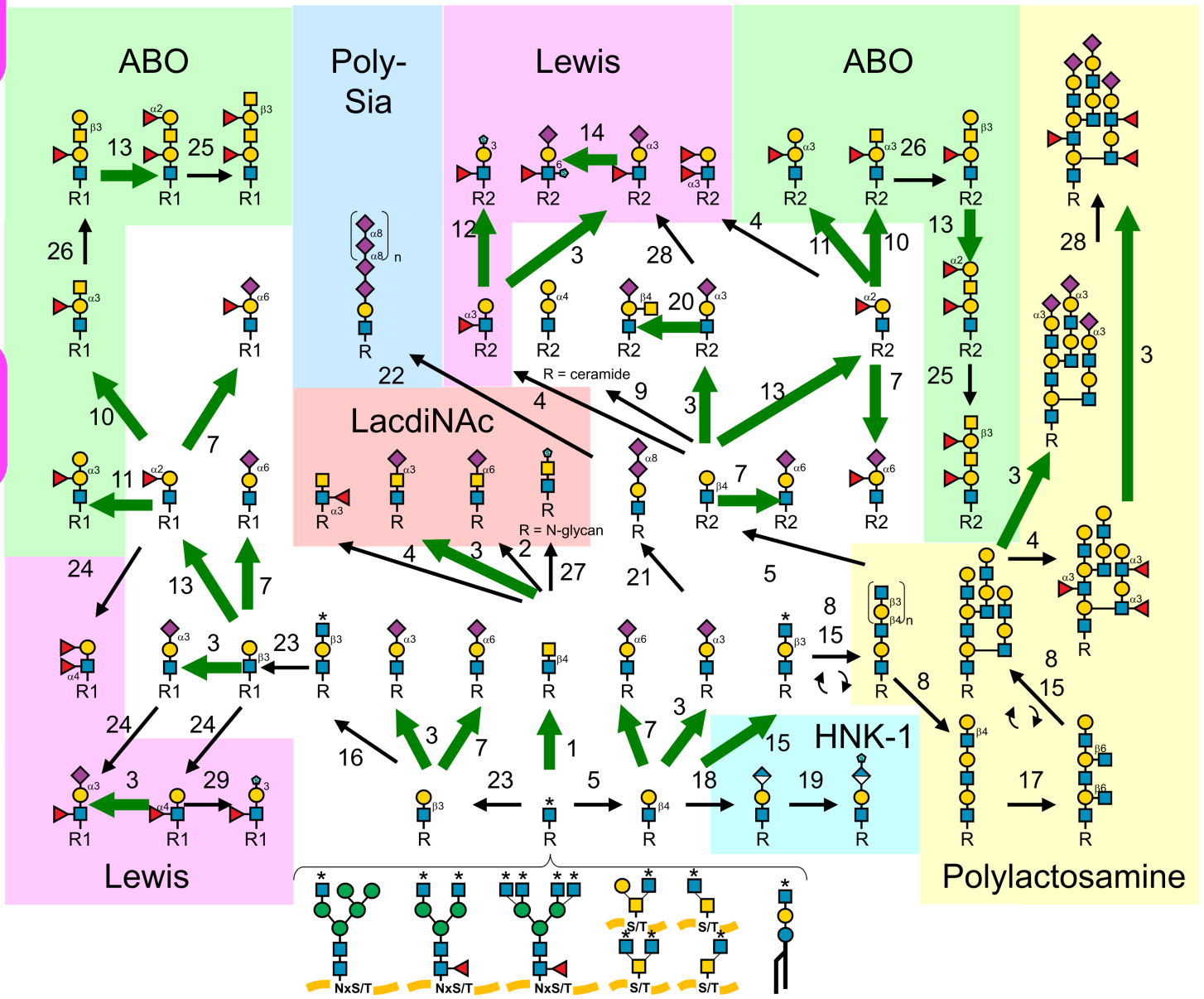
V.S.

pRCC
(N = 288; Median)

$$\frac{TPM_{tumor} + 1}{TPM_{normal} + 1}$$

≥ 2 →

< 0.5 →



iGCORE
Institute for Glyco-core Research
Tokai National Higher Education and Research System

Summary 2

We developed a mapping tool for glycosylation pathways.
Based on gene expression, glycan structures were predicted.

Support of glycomic analysis :

Identification of glycan structures smoothly.
Estimation of isomers from gene expression patterns

Engineering of glycosylation pathways :

Simplification and customization of glycans
Based on gene expression patterns,

Finding new insights of glycan regulation :

Complementation between N-glycans and glycolipids
Glycan changes in normal and diseased tissues

Expansion of glycan researches :

Gene expression → Glycobiology

Acknowledgements

Key Laboratory of Carbohydrate Chemistry & Biotechnology
Jiangnan University (2014 - 2022)
Yi-Fan Huang

Institute for Glyco-Core Research (iGCORE)
Gifu University (2022 -)

Osaka University
Taroh Kinoshita

Soka University
Kiyoko F. Aoki-Kinoshita
Sachiko Akase

University of Georgia
Kazuhiro Aoki (MCW, now)
Michael Tiemeyer

AIST
Yasunori Chiba

Gifu University
Yasuhiko Kizuka

Meijo University
Shuji Mizumoto

Jiangnan University
Xiao-Dong Gao

Thank you for viewing the slides.