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Judging a Book by its Cover: Using Lectin Microarrays to Identify How Glycosylation is Regulated

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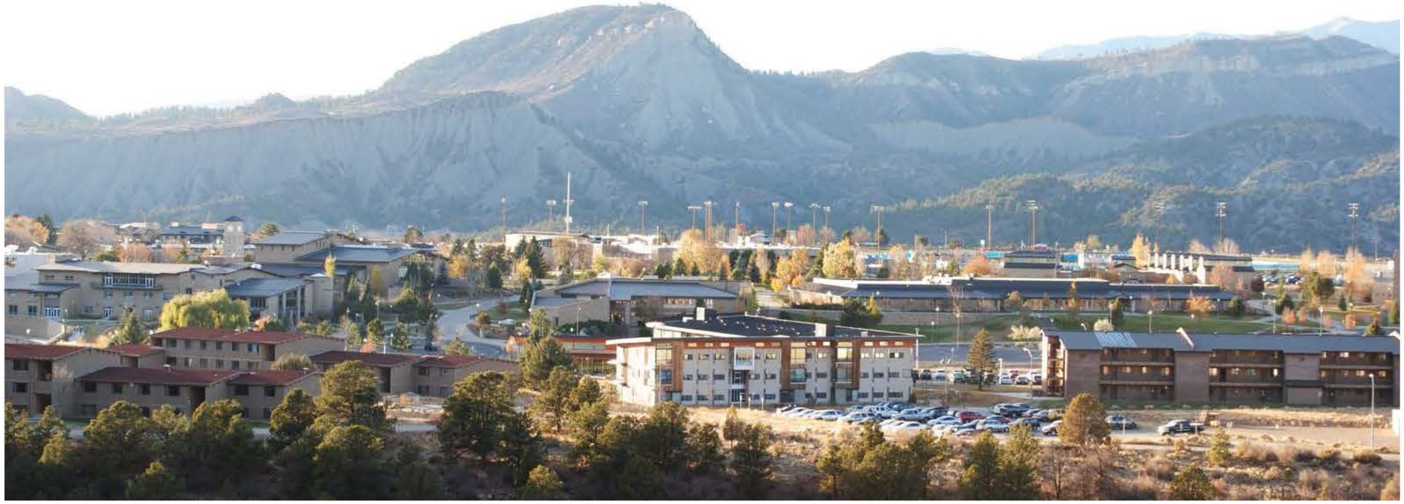


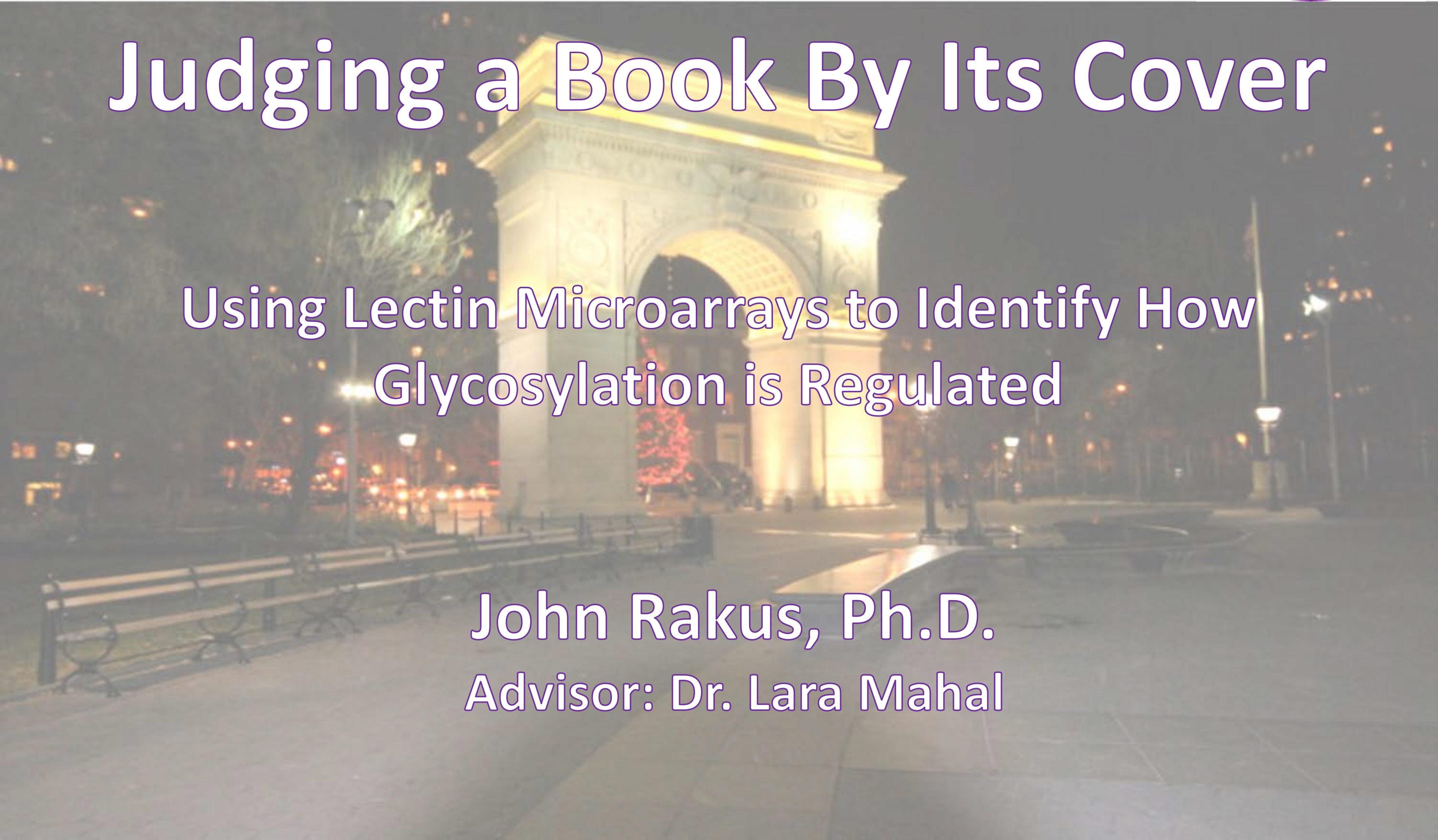
Judging a Book By Its Cover

Using Lectin Microarrays to Identify How Glycosylation is Regulated

John Rakus, Ph.D.
Department of Chemistry
Fort Lewis College
December 11, 2012

NEW YORK UNIVERSITY

BIOMEDICAL CHEMISTRY INSTITUTE

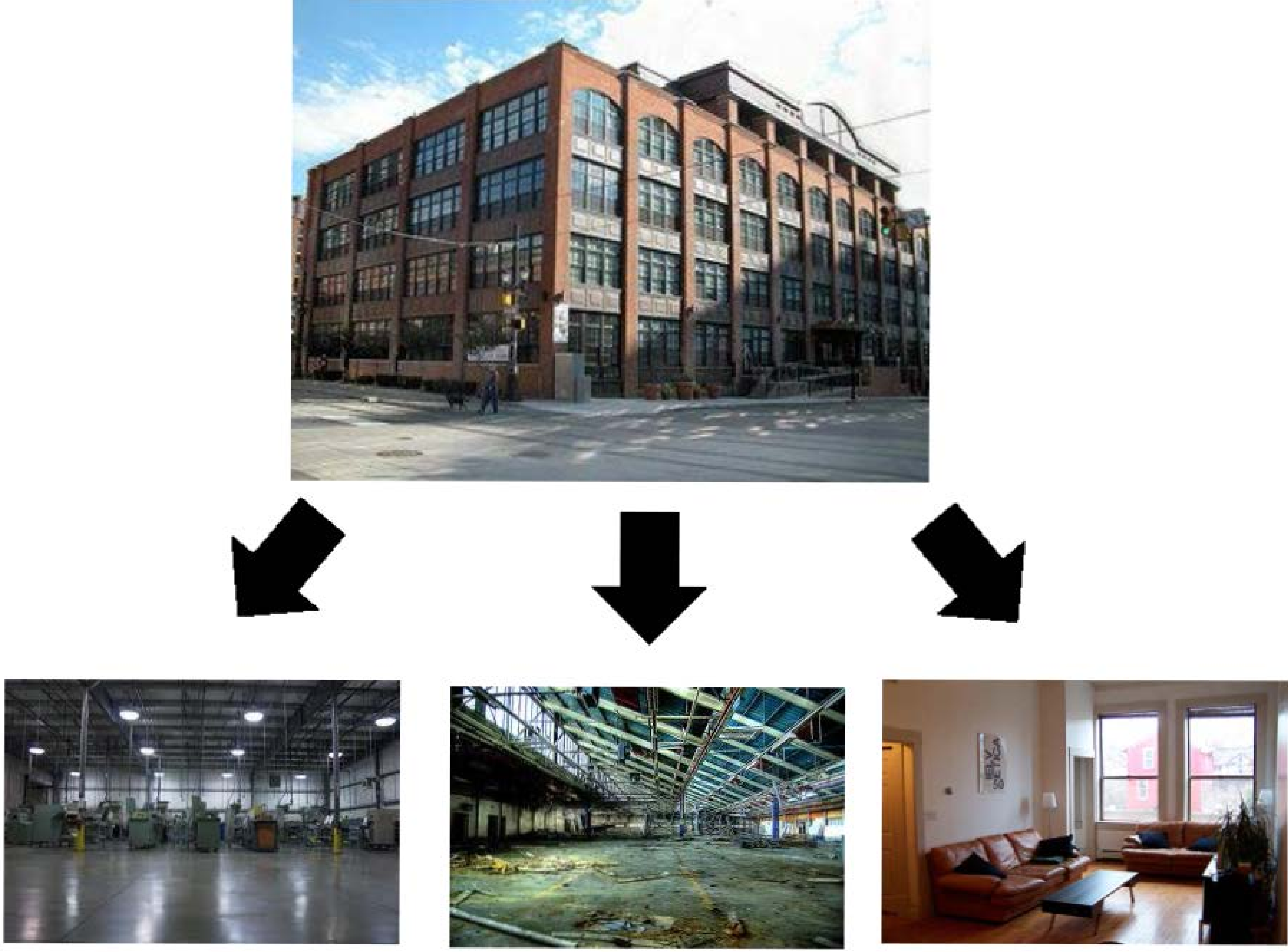


Judging a Book By Its Cover

Using Lectin Microarrays to Identify How Glycosylation is Regulated

John Rakus, Ph.D.

Advisor: Dr. Lara Mahal



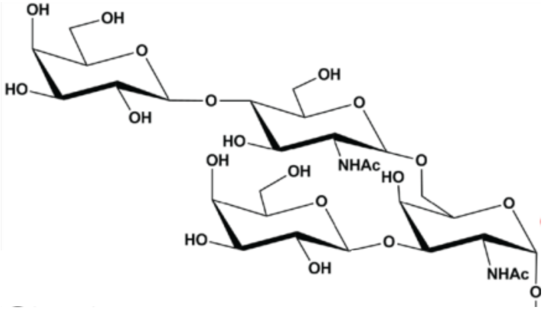
Cells are primarily compose of three types of biomolecules

HeLa cell

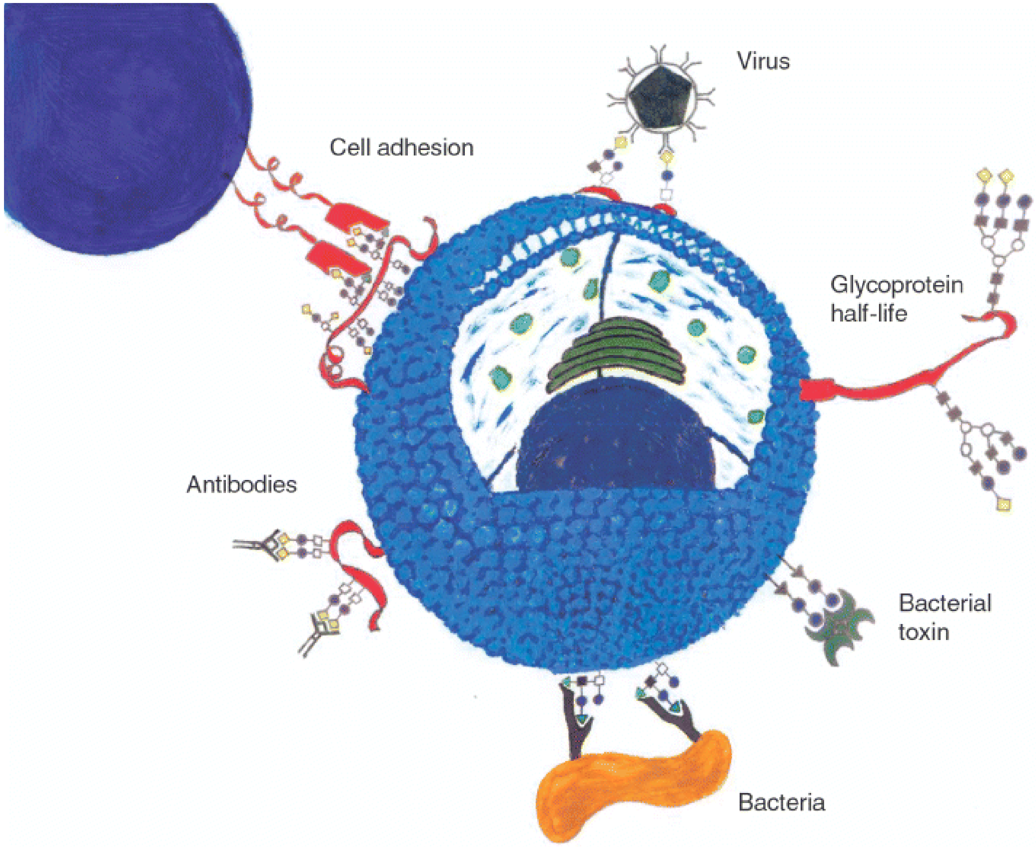
Protein (50% dry weight)

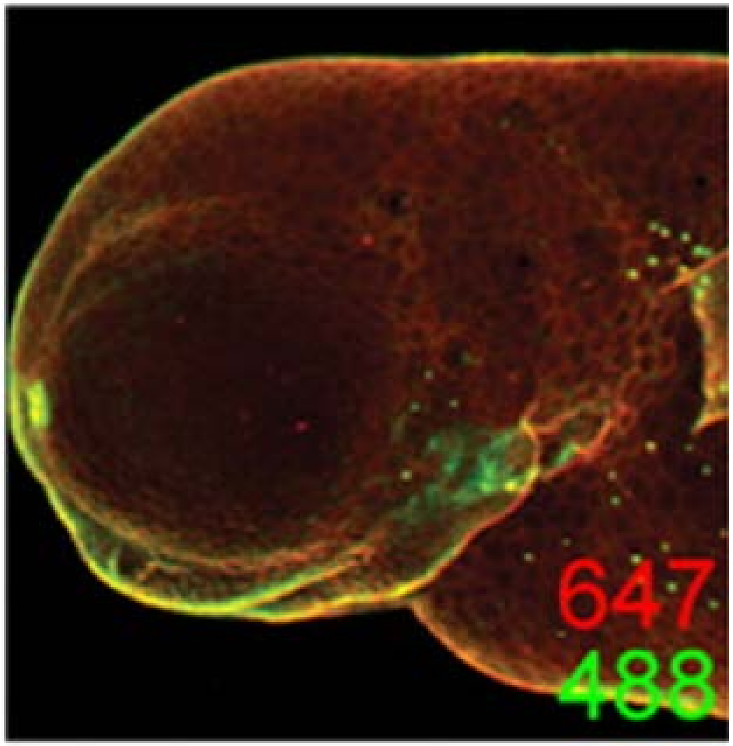
Nucleic acid (25% dry weight)

Carbohydrate (10% dry weight)



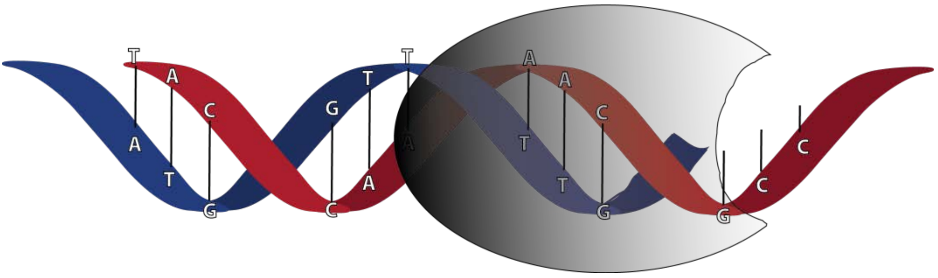
Carbohydrates are pervasive and involved in many cellular interactions

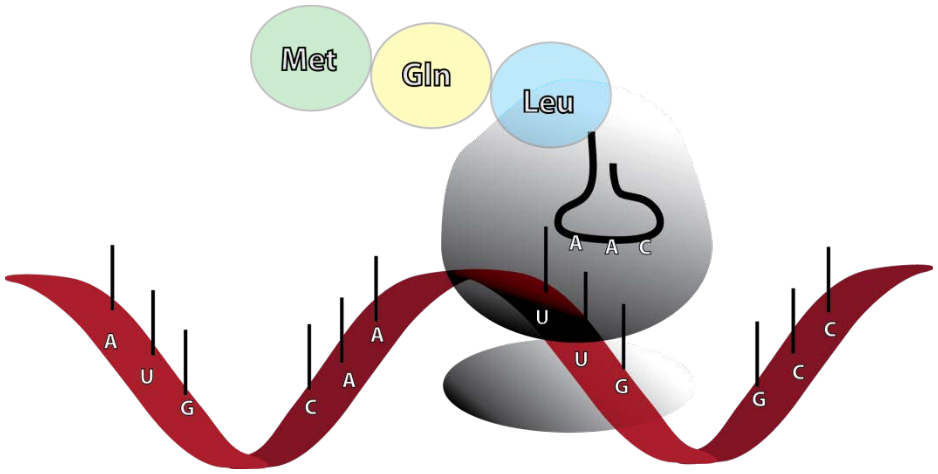




Holgersson et al, *Immuno Cell Biol*, 2005
Laughlin et al, *Science*, 2008

Nucleic acids and proteins are synthesized with a defined template and dedicated polymerases





Macromolecule: Nucleic acid

Polymerase: DNA Pol or RNA Pol

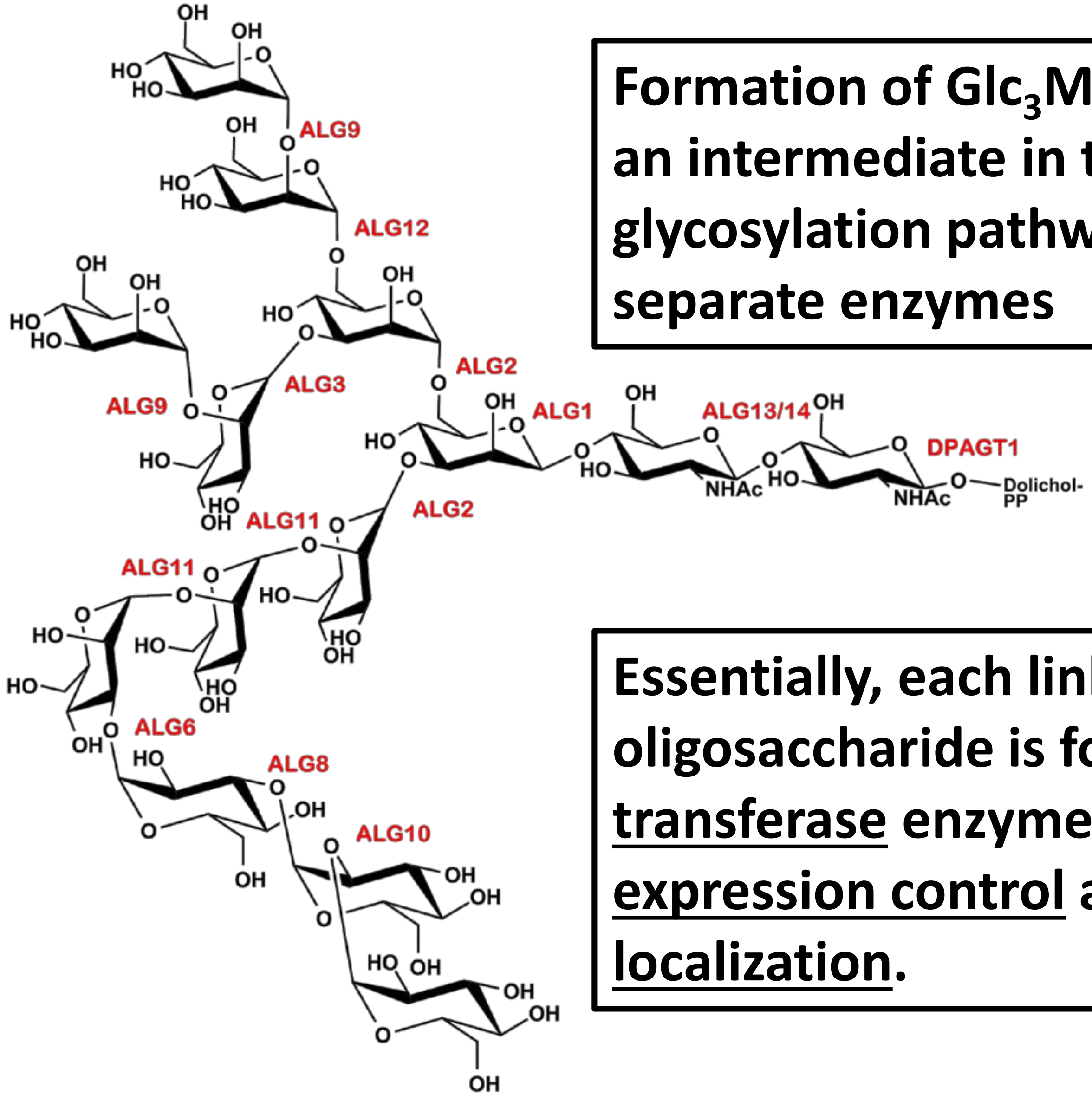
Template: DNA strand

Macromolecule: polypeptide

Polymerase: Ribosome

Template: mRNA strand

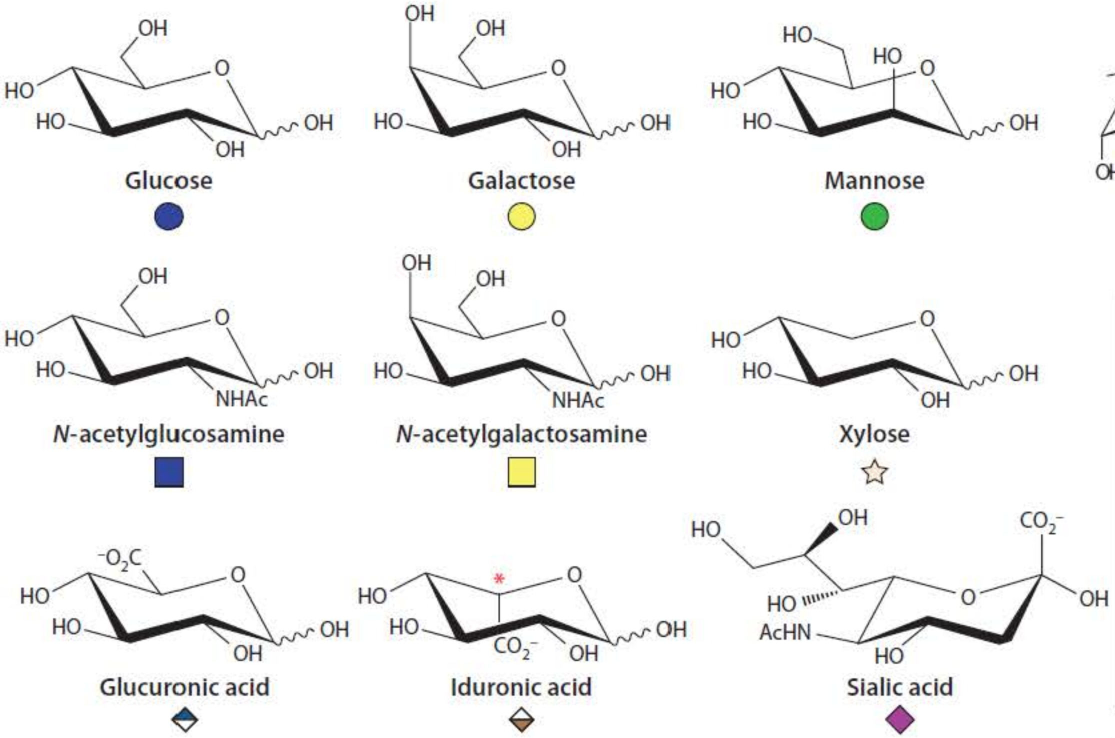
Glycan biosynthesis lacks a dedicated polymerase and genetic template

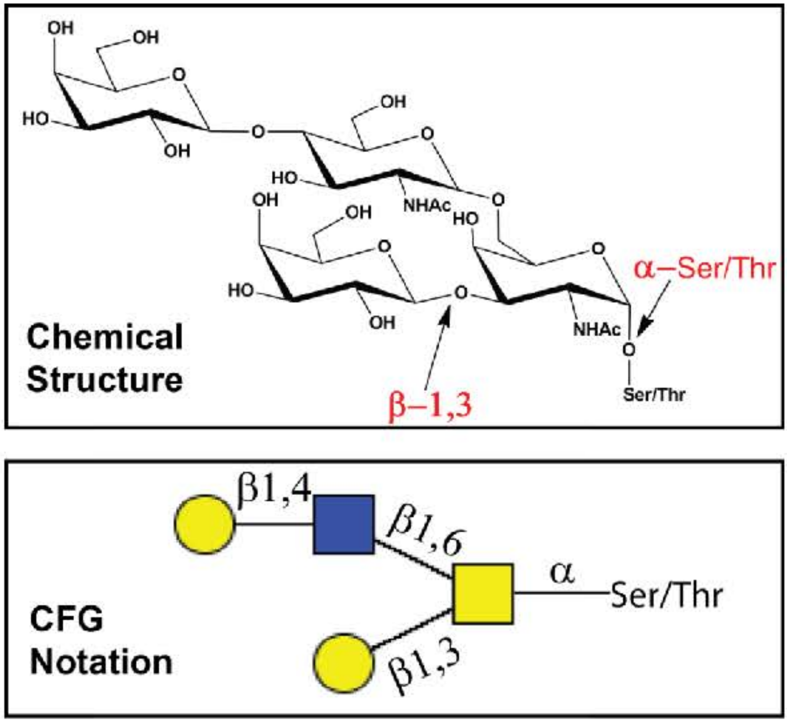


Formation of $Glc_3Man_9GlcNAc_2$-DolPP, an intermediate in the *N*-linked glycosylation pathway, requires 12 separate enzymes

Essentially, each linkage in an oligosaccharide is formed by a specific transferase enzyme with specific expression control and subcellular localization.

Consortium for Functional Glycomics (CFG) Notation



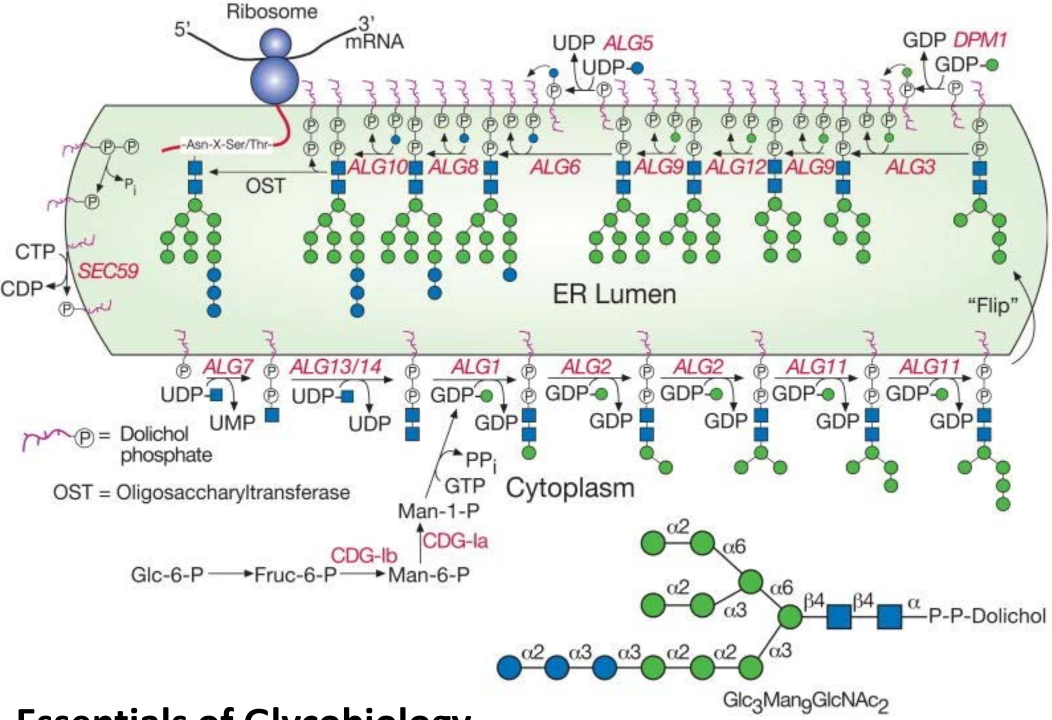


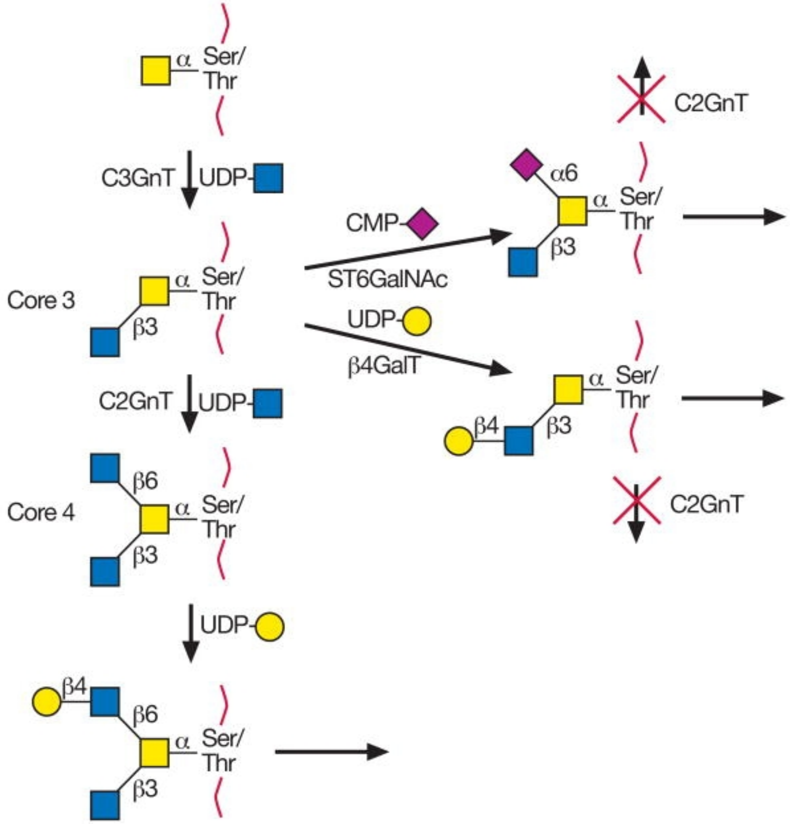
Rakus and Mahal, ***Ann Rev Anal Chem*****, 2011**

There are two primary glycosylation pathways

***N*-linked glycosylation** occurs in the ER and Golgi and involves construction of a lipid-linked 14-mer precursor before being transferred to an Asn residue and further modified to form the final structure. Modified proteins have N-x-S/T consensus sequence

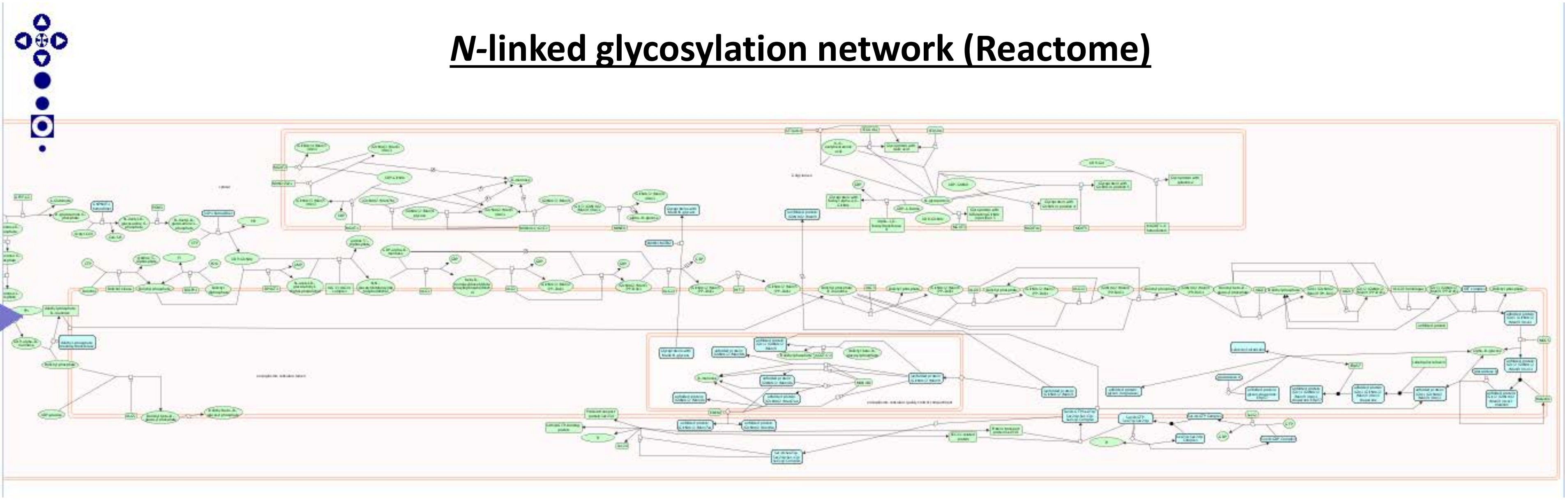
***O*-linked glycosylation** occurs in the Golgi apparatus and involves transfer of a monosaccharide directly to a Ser/Thr residue by a specific ppGalNacT followed by further elaboration. No known consensus sequence





Essentials of Glycobiology

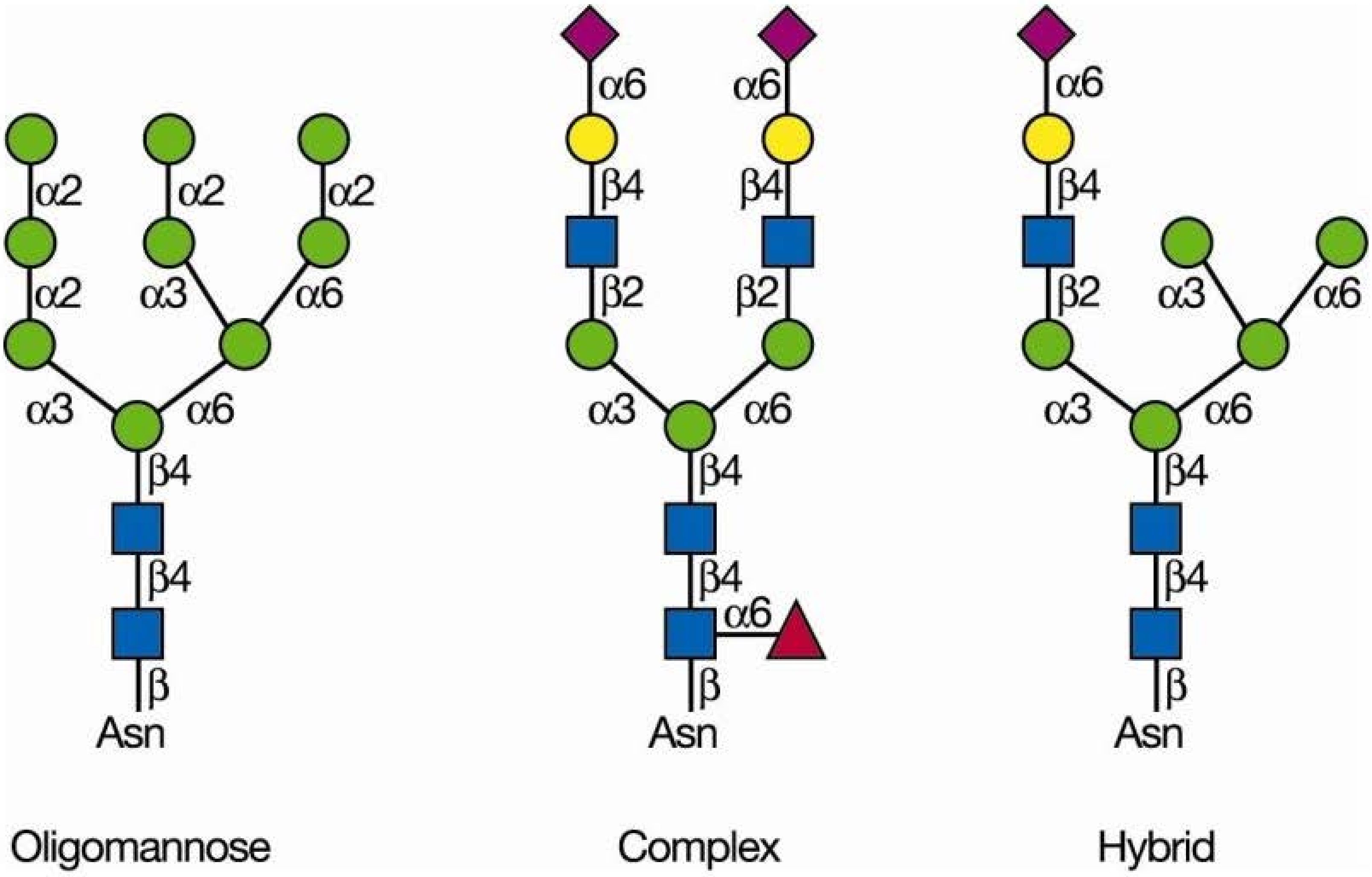
Carbohydrate synthetic regulation



N-linked glycosylation network (Reactome)

- Synthesizing a glycome requires a large commitment of cellular resources
- Many glycosylation enzymes (glycosyltransferases and glycosidases), sugar transporter and metabolic proteins, and regulation elements (over 120 identified as of 2011)

There are three types of *N*-linked glycans

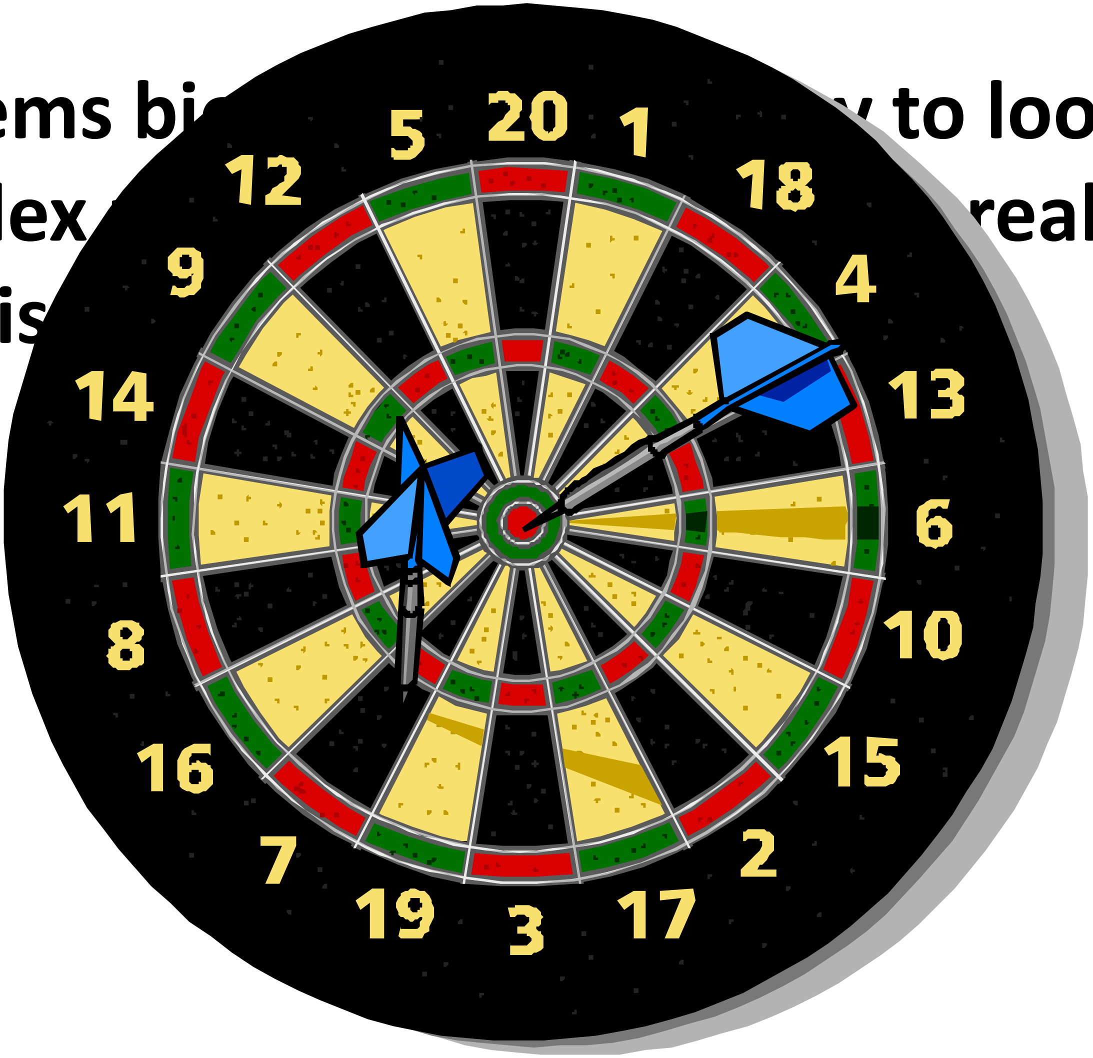


Glycosylation is complicated

- The structures are hard to discern
- The pathways are often redundant and overlapping
- The effects are subtle and, occasionally, conflicting

Systems Biology

“Systems bi y to look at
complex reak it down
into dis



The experiment

1. Look at the cell surface (glycome)

2. See if different cells express different carbohydrates

3. If so, try to explain why

Model System: The NCI-60 Cell Panel

- **NCI-60: 60 cell lines for screening of potential cancer therapeutics**

- **Vary in tissue type, metastasis, individual of origin**

- **CellMiner.org: open source database containing mRNA, miRNA and protein array data, genetic mapping, pharmacological and mutational analysis**

The NCI-60 panel

LEUKEMIA

- CCRF-CRM
- HL-60
- K-562
- MOLT-4
- RPMI-8226
- SR

OVARIAN

- IGR-OV1
- OVCAR-3
- OVCAR-4
- OVCAR-5
- OVCAR-8
- NCI/ADR-RES
- SK-OV-3

LUNG

- A549
- EKVX
- HOP-62
- HOP-92
- NCI-H226
- NCI-H23
- NCI-H322M
- NCI-H460
- NCI-H522

PROSTATE

- PC-3
- DU-145

COLON

- COLO 205
- HCC-2998
- HCT-116
- HCT-15
- HT29
- KM12
- SW-620

RENAL

- 786-0
- A498
- ACHN
- CAKI-1
- RXF 393
- SN12C
- TK-10
- UO-31

CNS

- SF-268
- SF-295
- SF-539
- SNB-19
- SNB-75
- U251

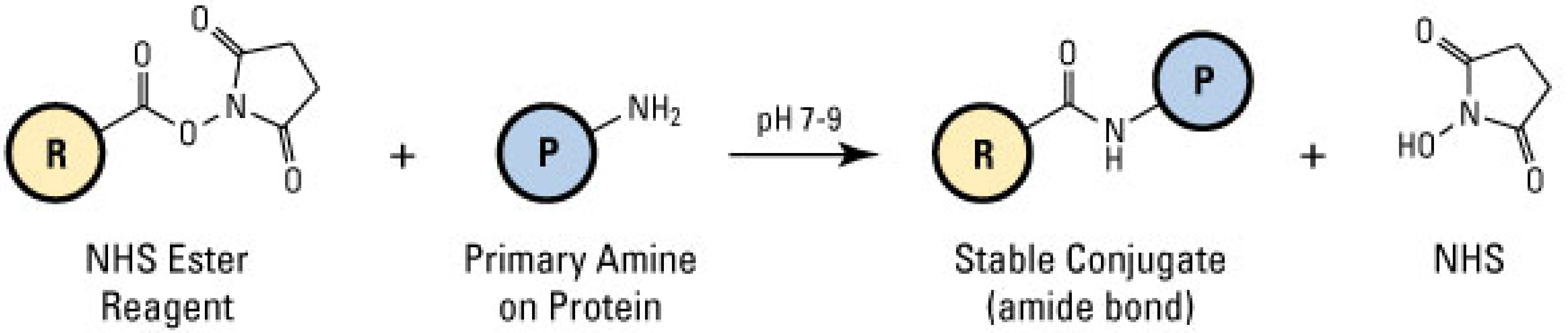
BREAST

- MCF7
- MDA-MB-231
- MDA-MB-468
- HS 578T
- BT 549
- T-47D

MELANOMA

- LOX IMVI
- MALME-3M
- M14*
- MDA-MB-435*
- SK-MEL-2
- SK-MEL-28
- SK-MEL-5
- UACC-257
- UACC-62

Generation of Lectin Microarrays



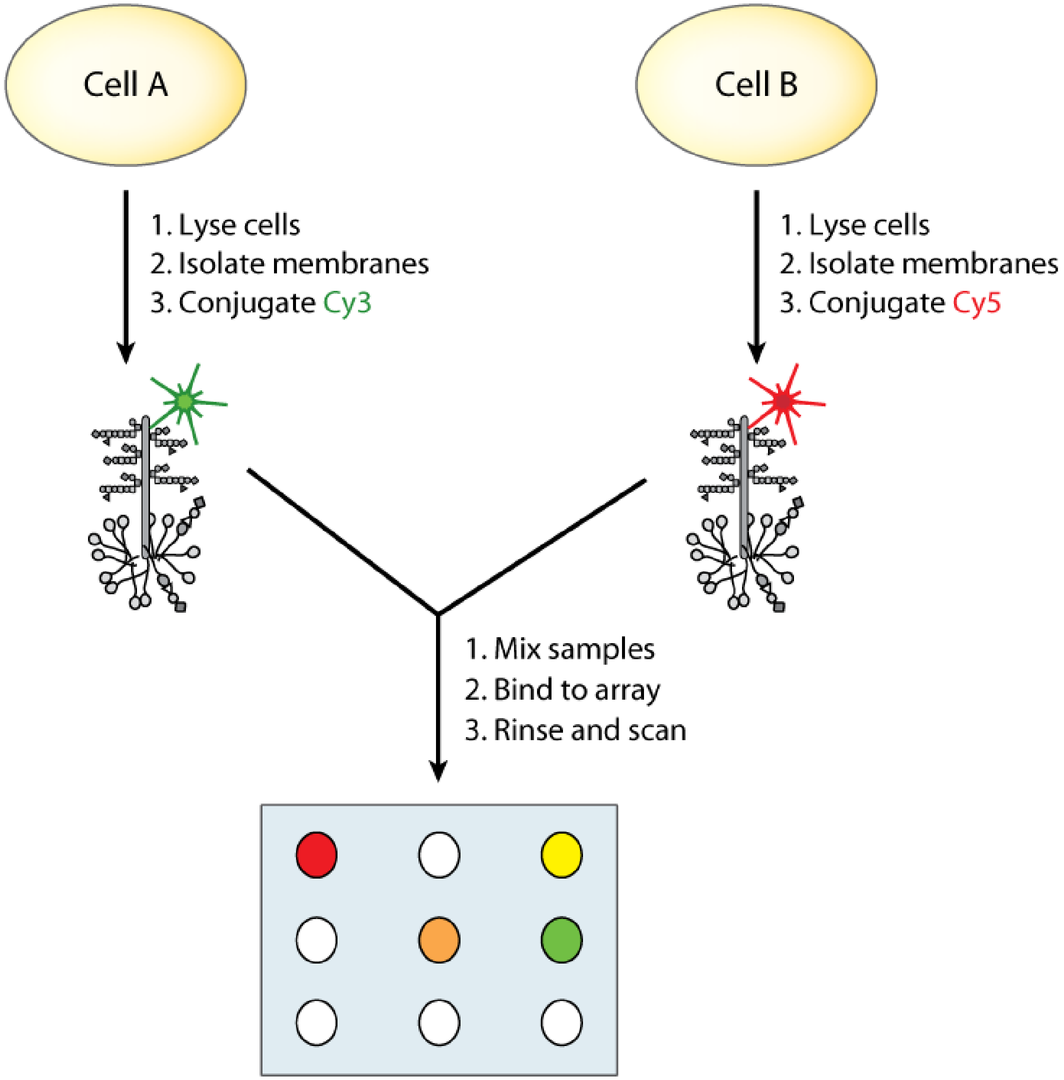
- **Lectins are printed on NHS-ester coated glass slides in high spatial density at 10°C and ambient humidity**

- **Protein lysine residues react with esters to form amide-bound conjugates**

- **Unreacted esters are blocked with ethanolamine**

- **Slides can be stored for up to two months**

Ratiometric lectin microarray analysis for semi-quantitative analysis of the dynamic glycome



Cell A

1. Lyse cells
2. Isolate membranes
3. Conjugate Cy3

Cell B

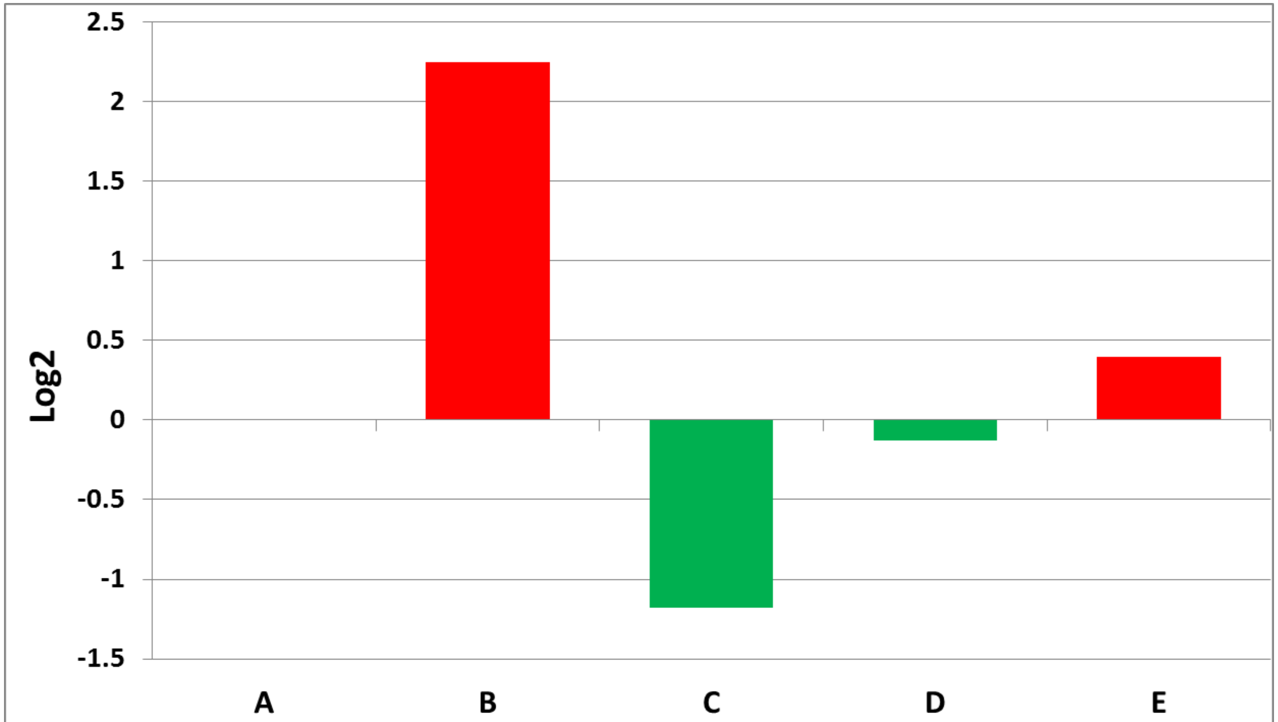
1. Lyse cells
2. Isolate membranes
3. Conjugate Cy5

1. Mix samples
2. Bind to array
3. Rinse and scan

Pilobello et al, *PNAS*, 2007
Rakus and Mahal, *Ann Rev Anal Chem*, 2011

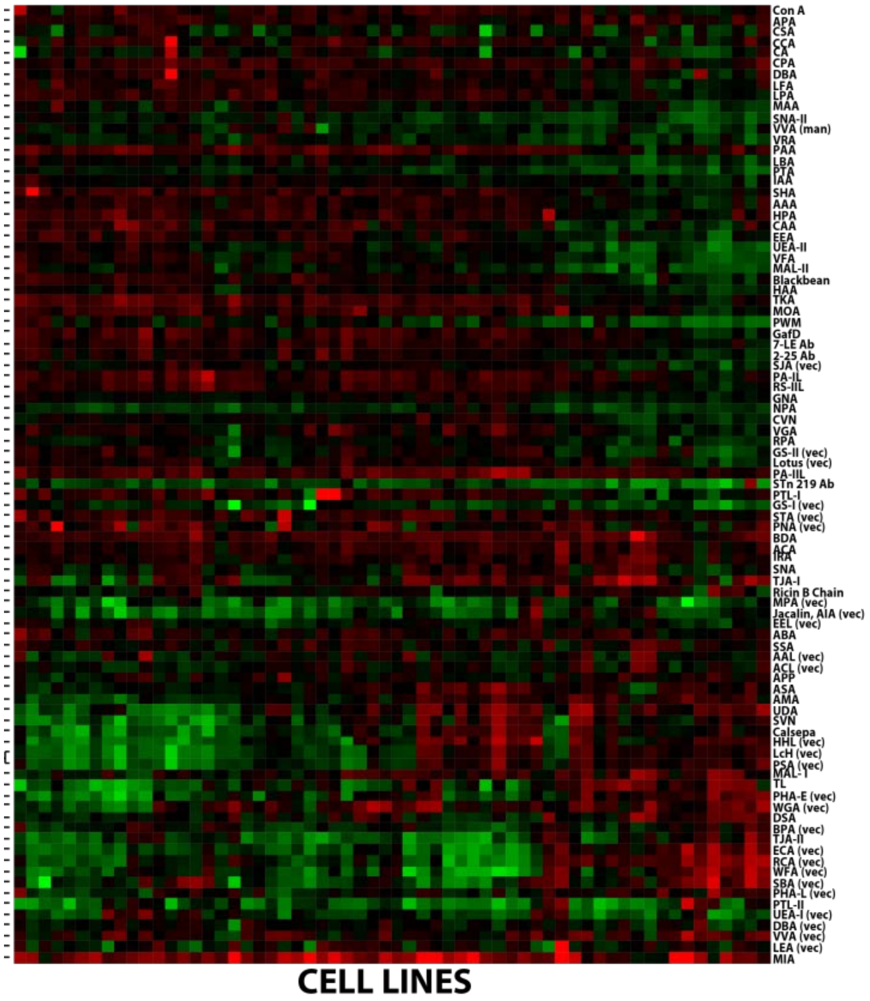
Interpreting a two-color experiment

Probe	Sample Fluorescence	Reference Fluorescence	Sample/Reference	$Log_2(S/R)$
A	500.00	500.00	1	0.00
B	2497.0	525.00	4.76	2.25
C	5500.0	12485	0.44	-1.18
D	125.20	137.23	0.91	-0.13
E	2545.0	1928.0	1.32	0.40

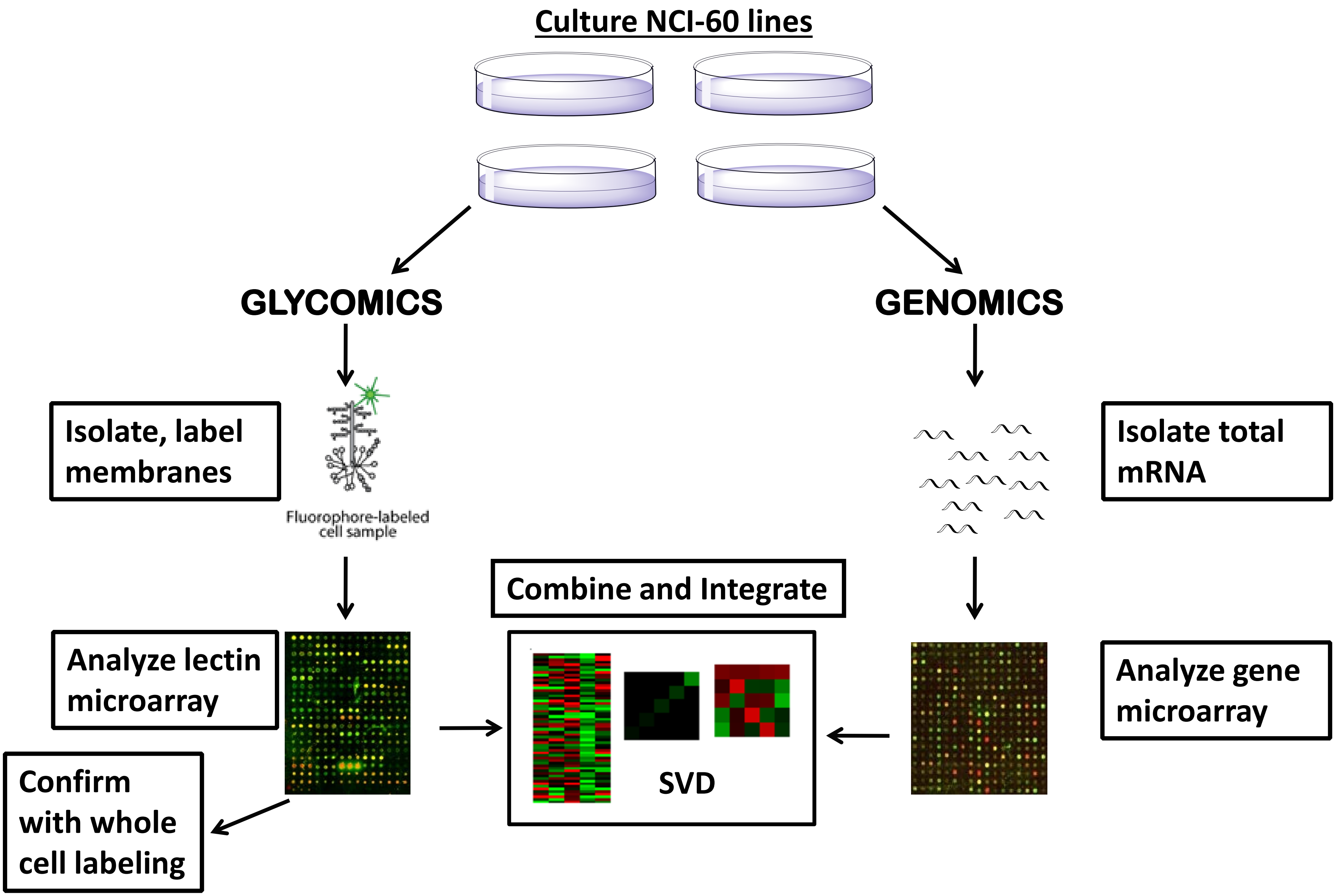


Interpreting a two-color experiment

When you have many samples and many probes, the data gets represented as a heat map



Experimental Strategy



Culture NCI-60 lines

GLYCOMICS

GENOMICS

Isolate, label membranes

Isolate total mRNA

Combine and Integrate

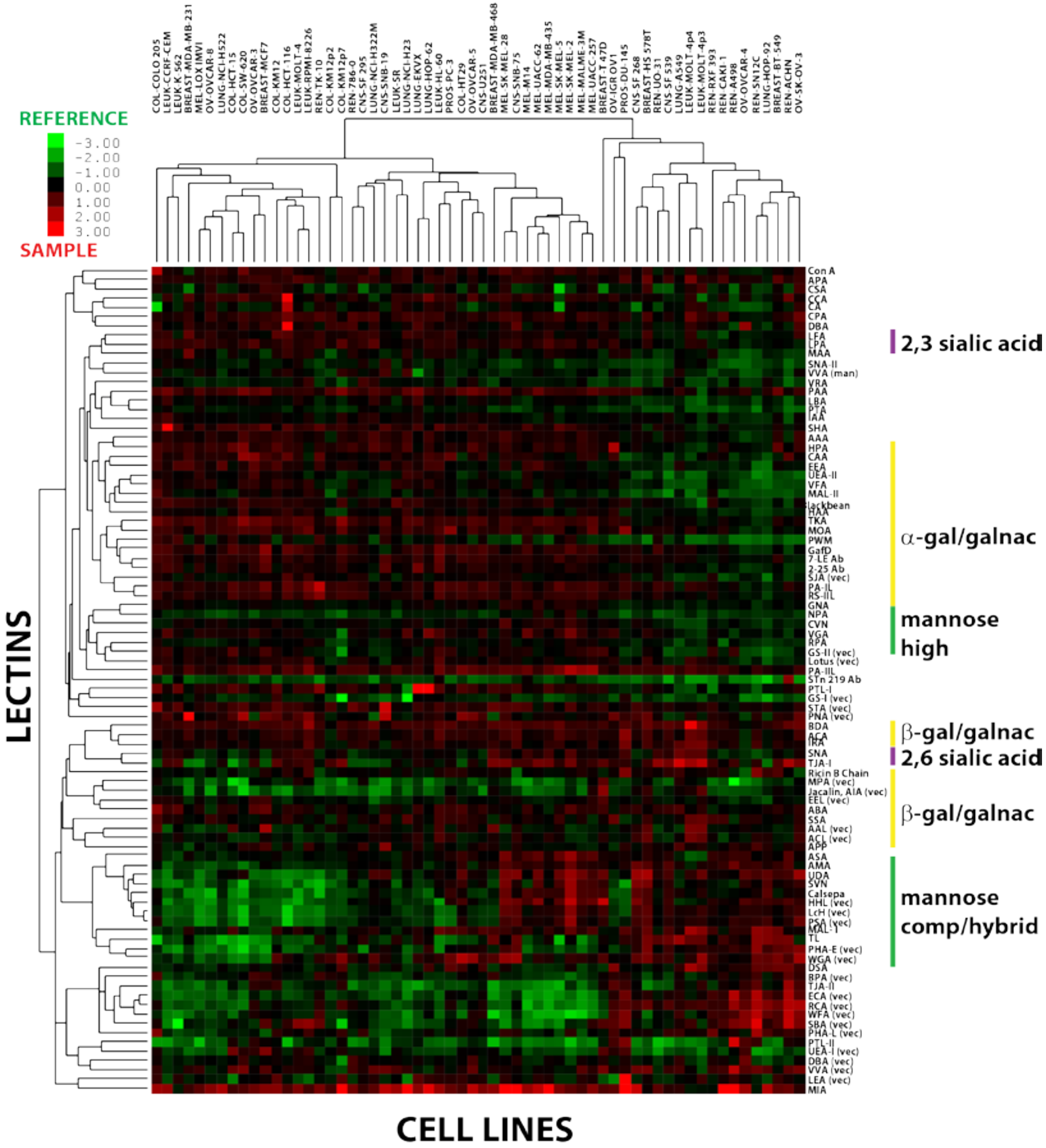
Analyze lectin microarray

Analyze gene microarray

Confirm with whole cell labeling

Lectin microarray analysis shows meaningful patterns

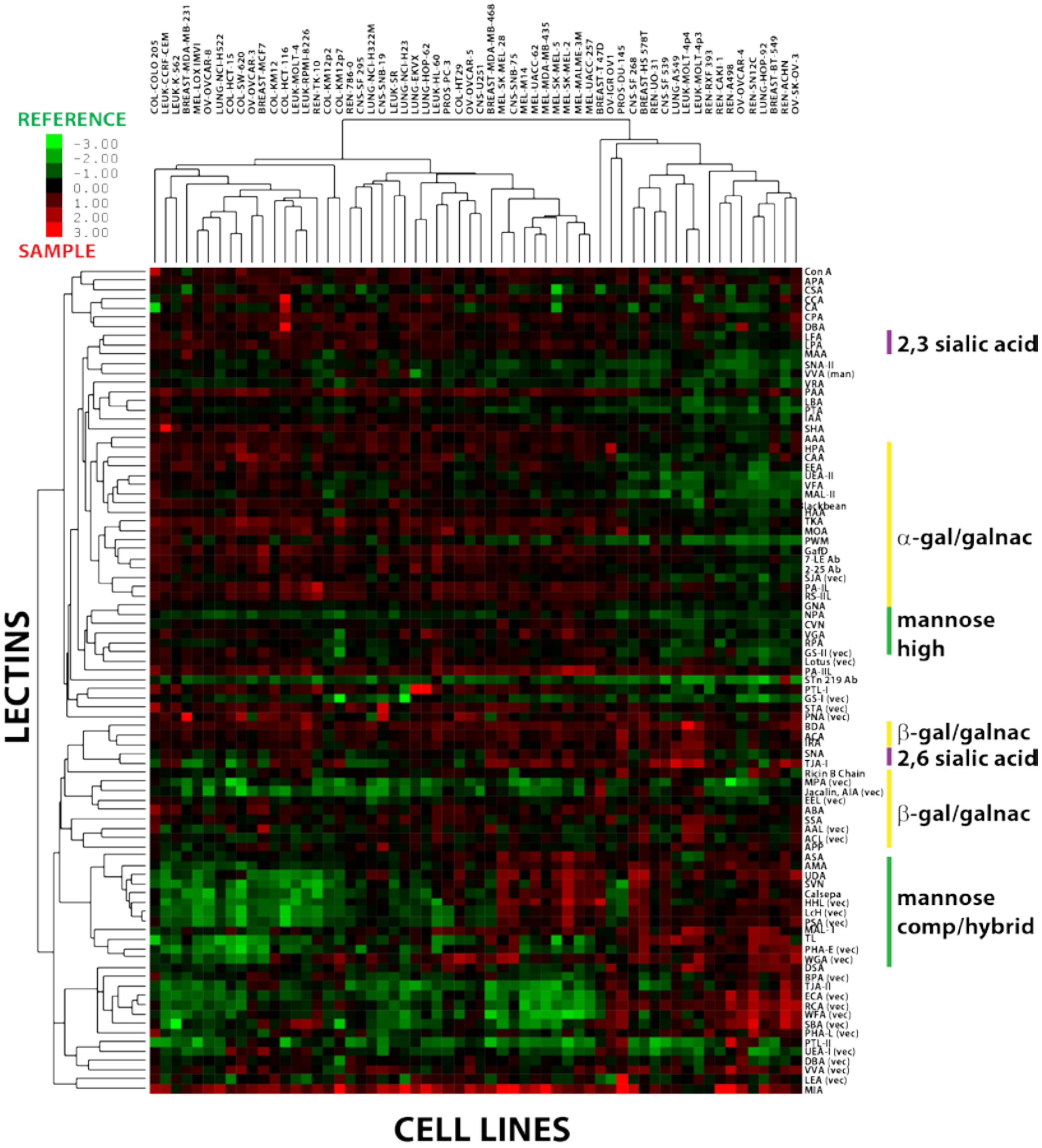
- **90 lectins**
- **56 cell lines**
- **Biological replicates of two cell lines**



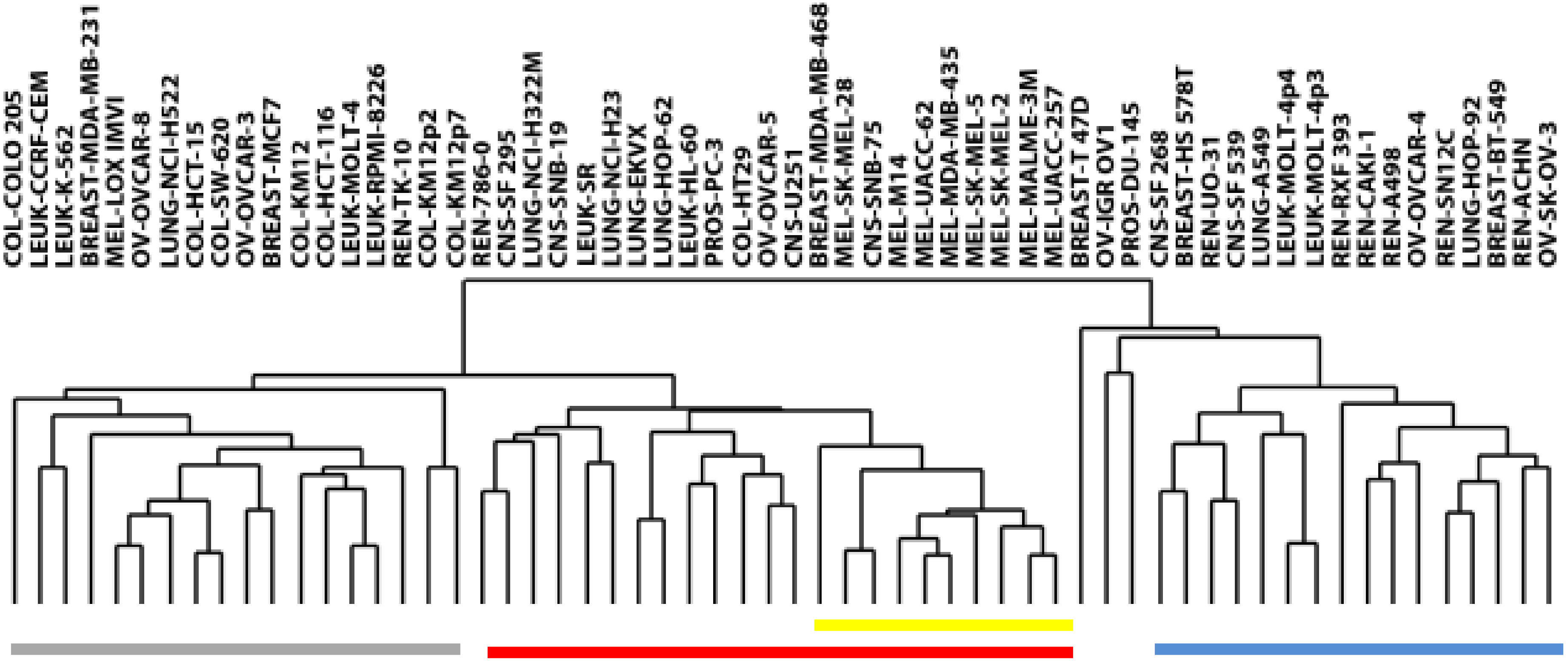
Lectin microarray analysis shows meaningful patterns

- **Several clusters of lectins by carbohydrate specificity**

- **Cell lines cluster by tissue of origin**



Cell lines cluster based on their glycosylation



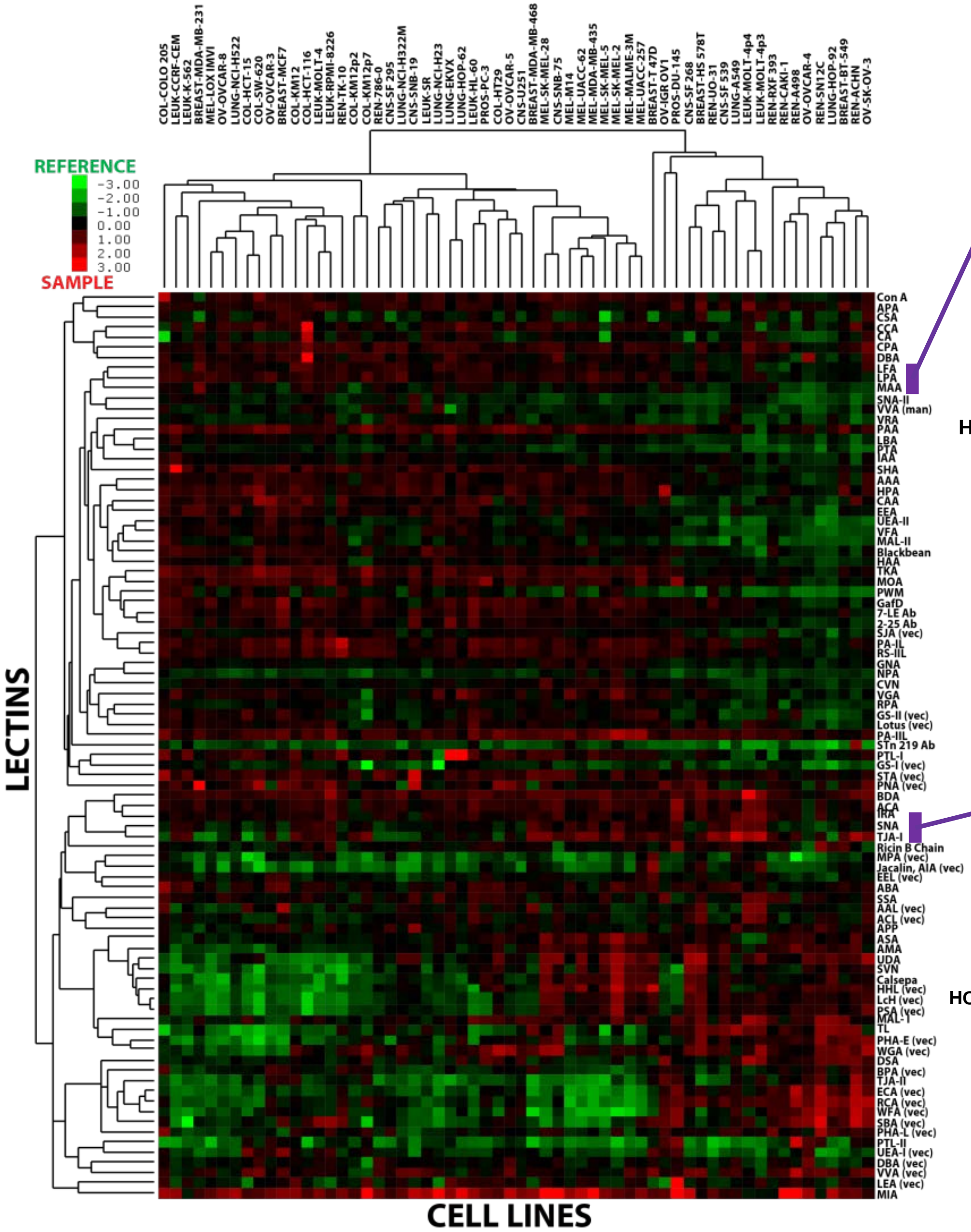
COLON (5/6, p=0.39)
LEUKEMIA (4/6, p=0.39)

CNS (4/5, p=0.44)
LUNG (4/7, p=0.44)

MELANOMA
(8/9, p=0.60)

RENAL (6/8, p=0.39)

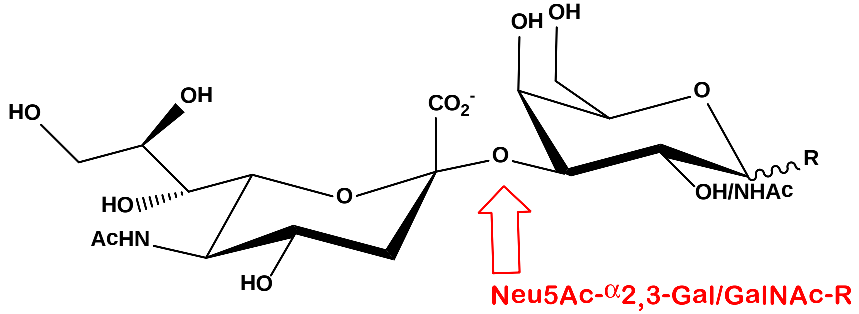
Glycosylation signatures: Sialic acid



<u>α2,3 sialic acid recognizing lectins</u>

LFA, LPA, MAA

⇩ **in <u>RENAL</u> cell lines**

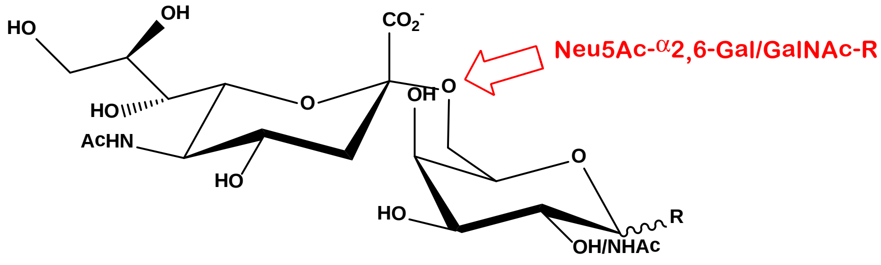


Neu5Ac-α2,3-Gal/GalNAc-R

<u>α2,6 sialic acid recognizing lectins</u>

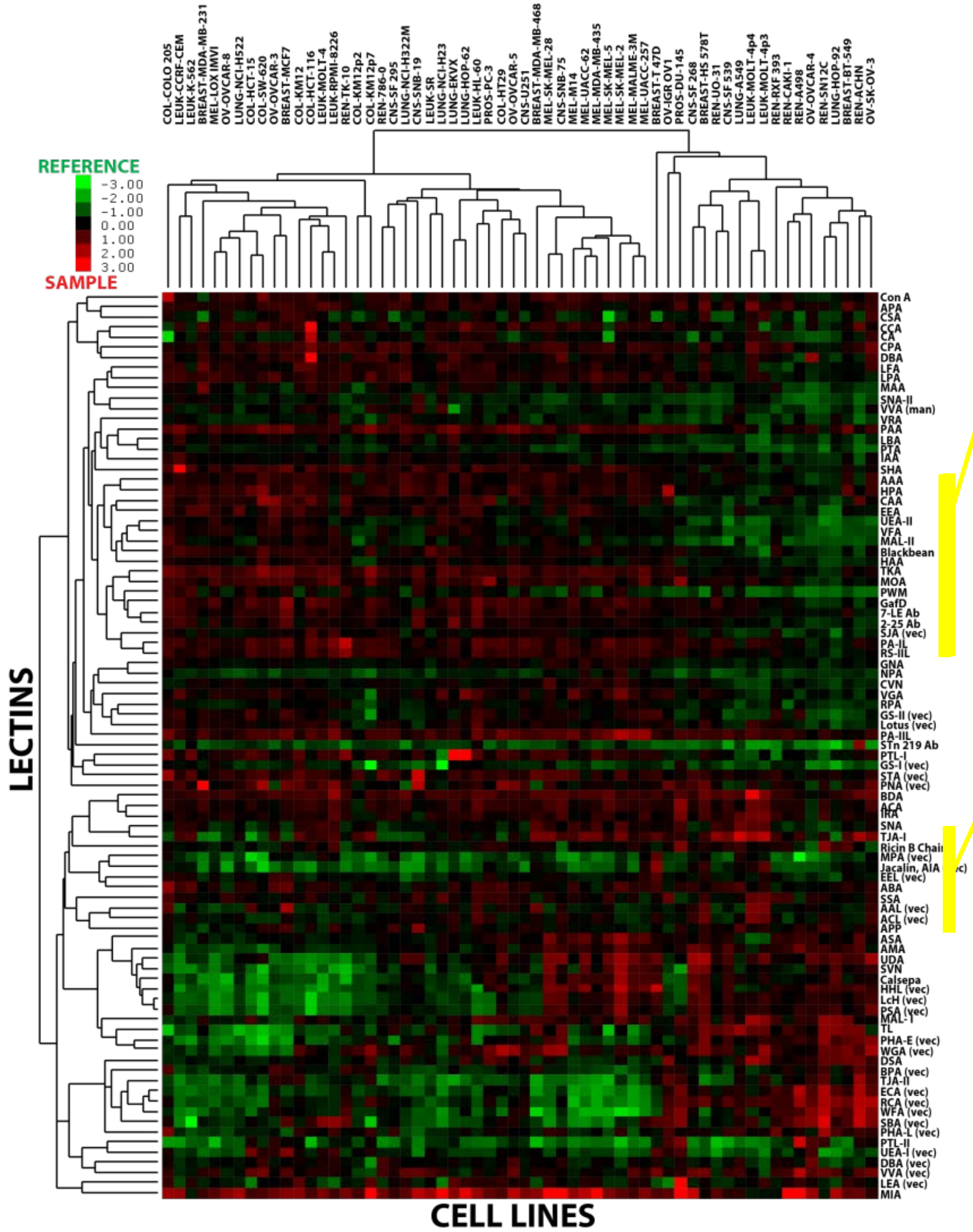
SNA, TJA-I

⇧ **in <u>MELANOMA</u> and <u>LUNG</u> cell lines**



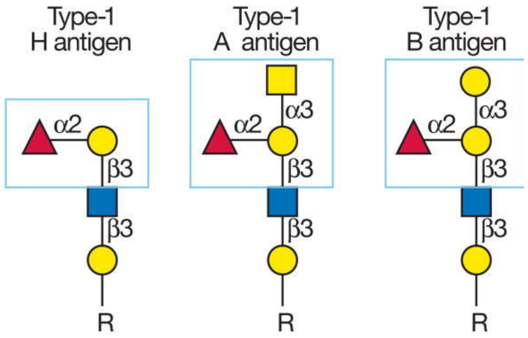
Neu5Ac-α2,6-Gal/GalNAc-R

Glycosylation signatures: Gal/GalNAc



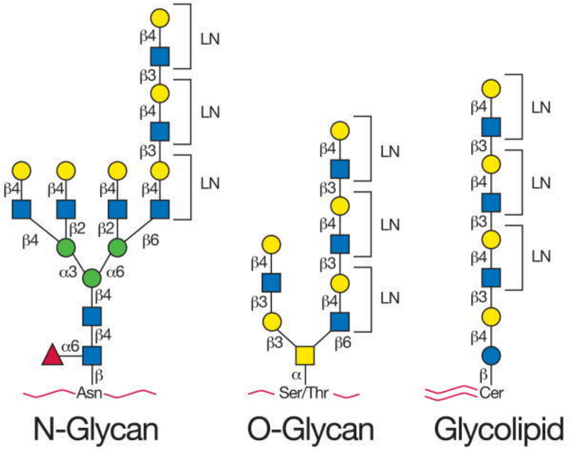
<u>α-Gal/GalNAc; blood groups</u>

MOA, EEA, LBA, VRA, IAA, PTA, HPA

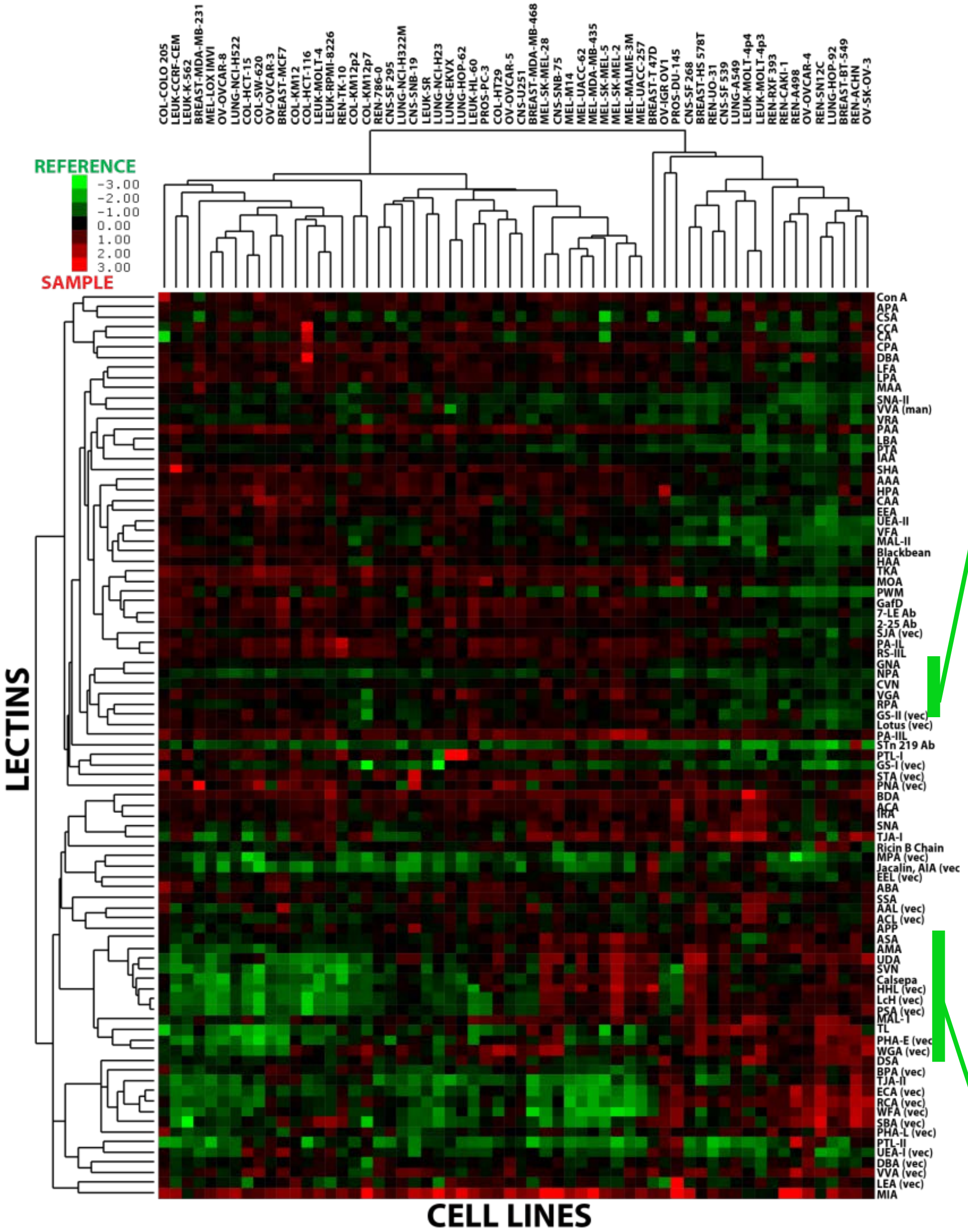


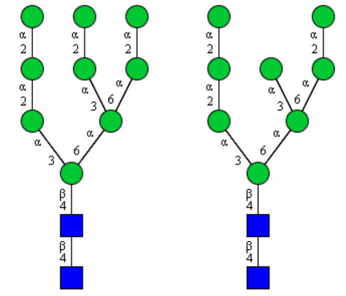
<u>β-Gal/GalNAc</u>

BDA, RICIN, ABA, AIA, AAL



Glycosylation signatures: Mannose



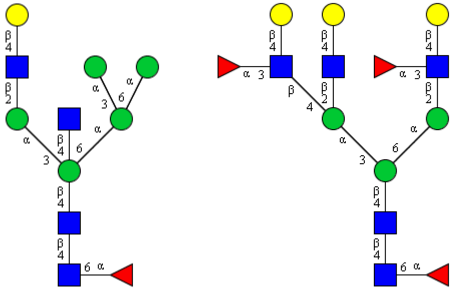


⇩ RENAL

⇧ MELANOMA LUNG,COLON

Mannose (high)

GNA, NPA, CVN, RPA



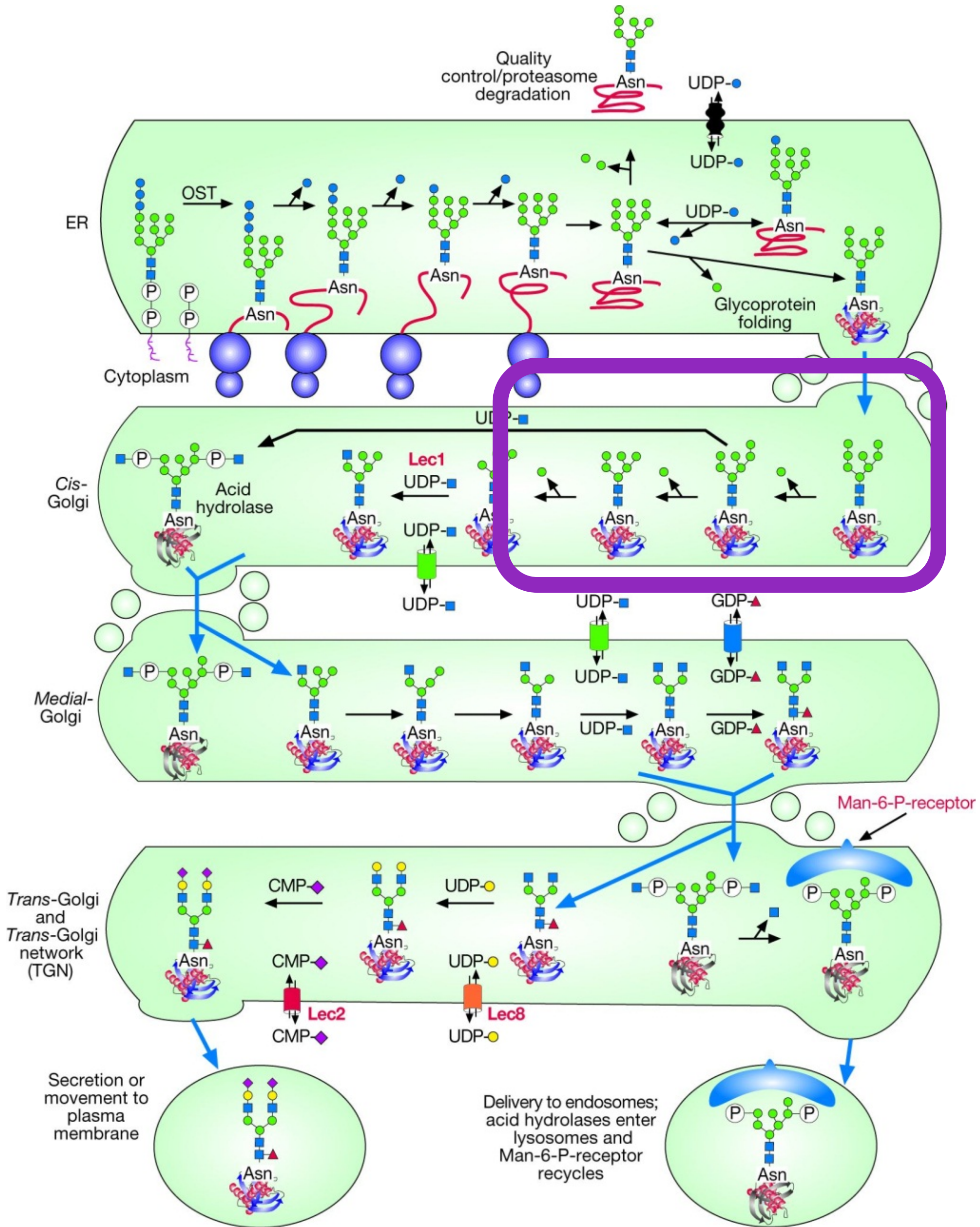
Mannose (complex)

UDA, SVN, Calsepa, HHL, LcH, PHA-E, MAL-I, PSA

⇩ COLON, LUNG

⇧ MELANOMA, RENAL

Maturation of *N*-linked glycans



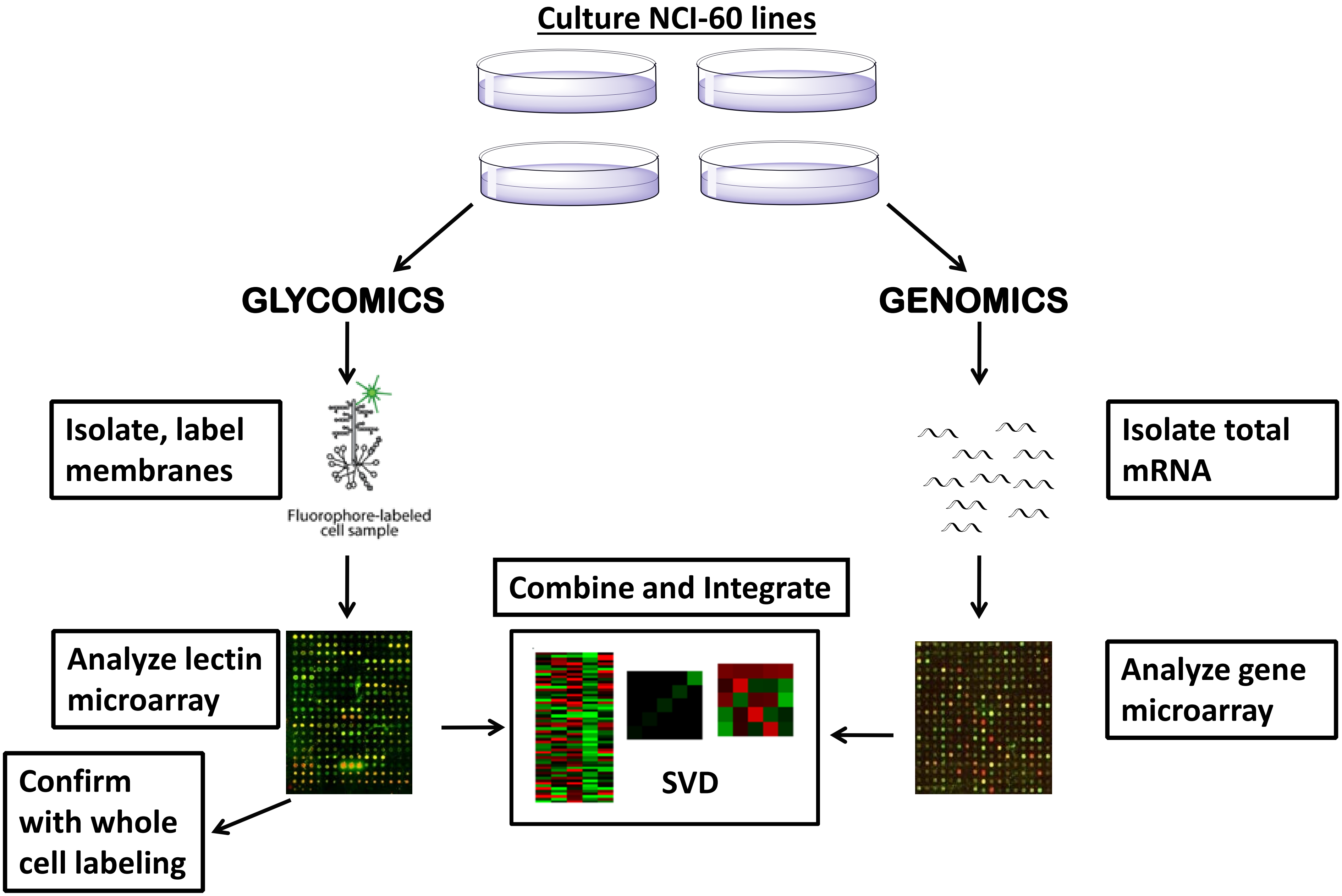
- **$Glc_3Man_9GlcNAc_2$ precursor is transferred to nascent polypeptide in ER**
- **Upon proper folding, glycan is trimmed to high-mannose and, potentially further modified to hybrid/complex**
- **<u>α-mannosidase I</u> controls all hybrid/complex maturation steps**

Essentials of Glycobiology

Conclusions (some)!

- **Human cells express different, specific carbohydrate patterns on their surfaces, based on their histological function**
- **What controls the glycome variation? (what does the interior of the house look like?)**

Experimental Strategy



Culture NCI-60 lines

GLYCOMICS

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Isolate, label membranes

Isolate total mRNA

Analyze lectin microarray

Combine and Integrate

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Confirm with whole cell labeling

Patterns of NCI-60 Gene Expression Regulation

- **mRNA and miRNA expression patterns investigated across entire NCI-60 (Liu et al, *Mol Biol Cell,* 2010)**

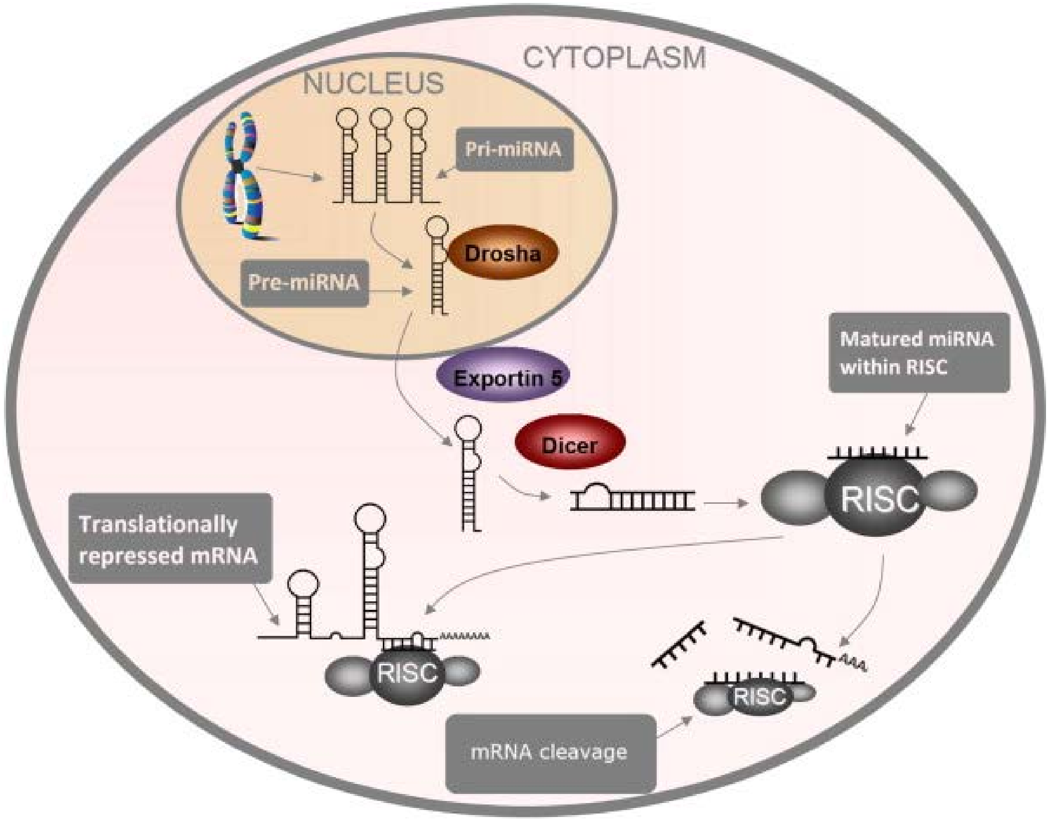
OBSERVATION OF HIGHLY EXPRESSED AND DIVERSE PROBES

-Melanoma (8/9 mRNA, 9/9 miRNA) and leukemia (6/6 mRNA and miRNA) lines cluster together based on expression patterns

-Renal (8/8 mRNA, 6/8 miRNA) and colon (6/7 mRNA and miRNA) also show strong correlation

-CNS and lung cancer show little correlation

microRNAs (miRNAs) regulate gene expression



Thamilarasan et al, *Autoimmun Rev*, 2012

- **miRNA are genomically encoded short (~22 nt) molecules involved in repressing expression of mRNA**
- **Bind target miRNAs in a 6-10 bp seed region**
- **Upon processing, miRNAs recruit a post-transcriptional silencing complex to inhibit expression**

How we make a cluster

$$r = \frac{1}{n}\sum_{i=1}^{n}\left(\frac{x_i - \bar{x}}{\sigma_x}\right)\left(\frac{y_i - \bar{y}}{\sigma_y}\right)$$

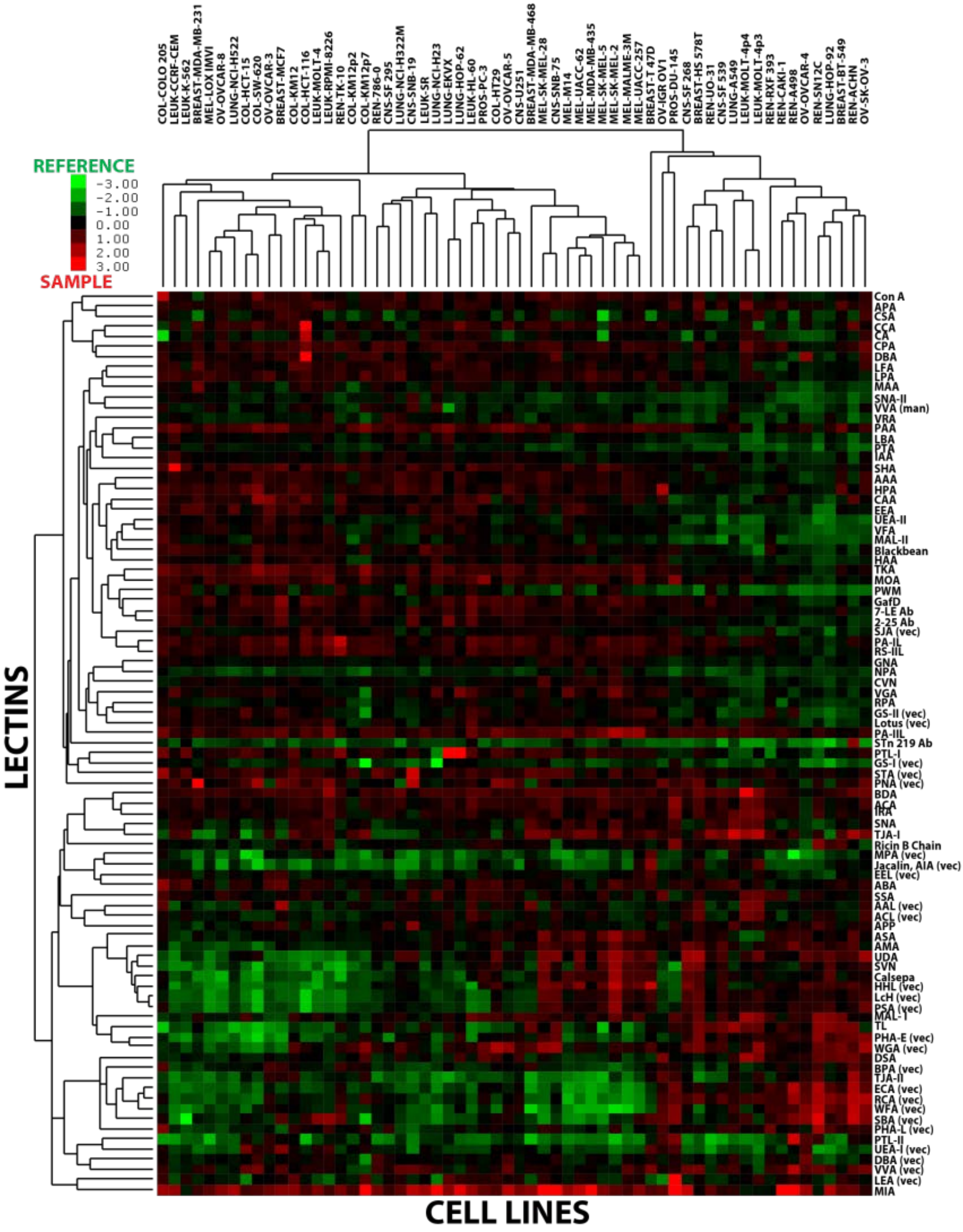
$\bar{x}$ = average across all set of numbers

σ = standard deviation against all set of numbers

- This is a fancy way of saying I am working with vectors

- Probes vs cell line (horizontal) and cell line vs probe (vertical)

- The intensity for each point on a heat map has a distance from every other data point

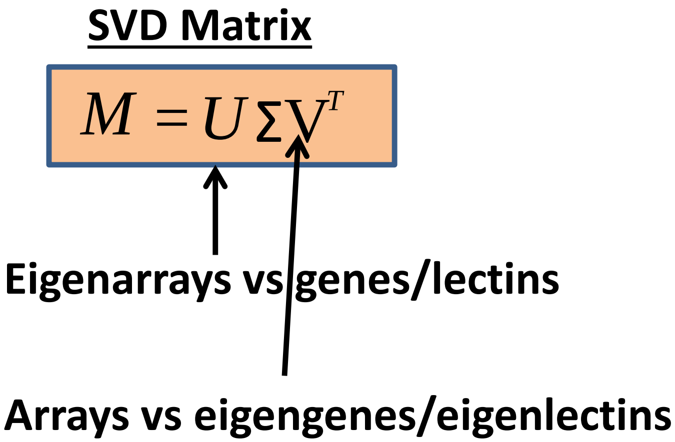


Singular Value Decomposition

- Method to decompose a matrix into an orthogonally transformed set of variables

- Used to identify significant, co-varying patterns in large data sets. I.E. What signals account for the variation?

- Can be applied to any array individually or combined for SYSTEMS BIOLOGY examination of glycosylation pathways

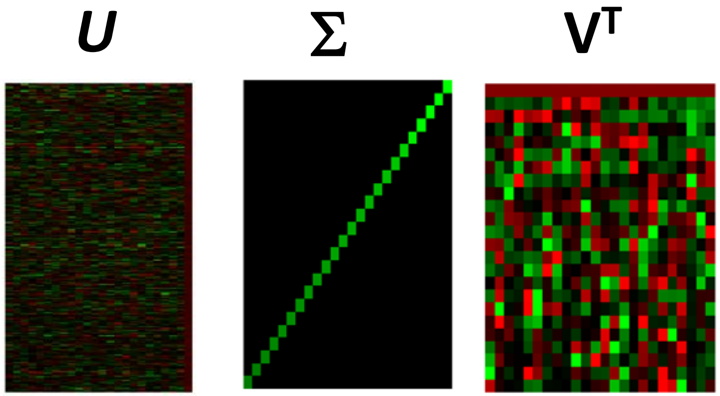


SVD Matrix

$$M = U \Sigma V^T$$

Eigenarrays vs genes/lectins

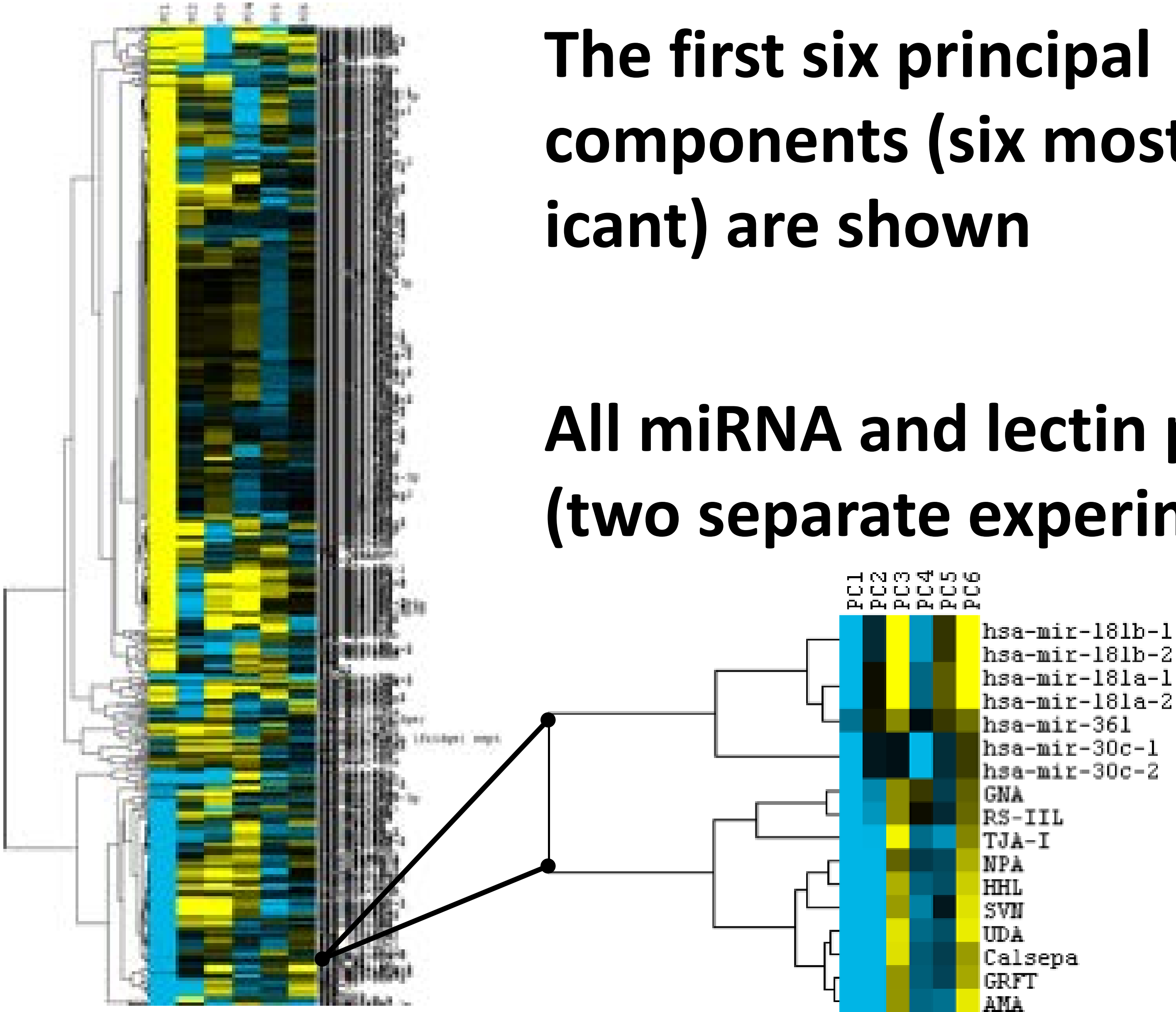
Arrays vs eigengenes/eigenlectins



Lectin plus miRNA SVD Results

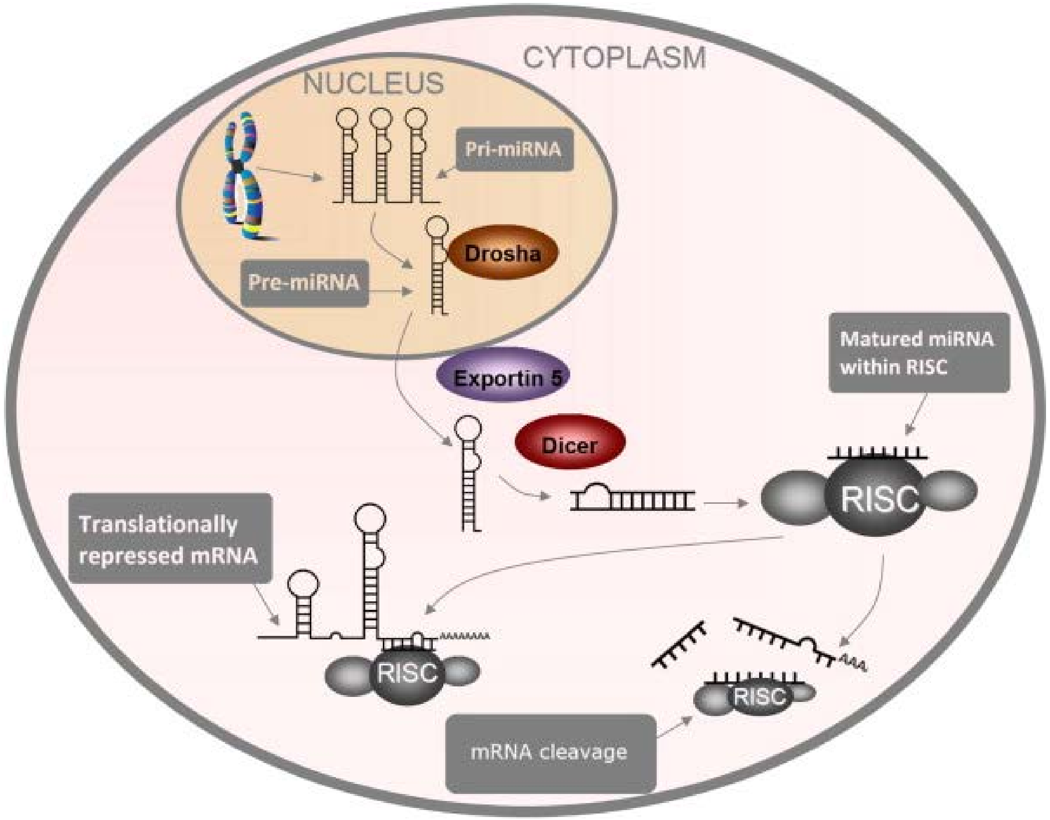
The first six principal components (six most significant) are shown

All miRNA and lectin probes (two separate experiments)



With Lani Pilobello

microRNAs (miRNA) regulate gene expression



- miRNA are genomically encoded short (~22 nt) molecules involved in repressing expression of mRNA
- Bind target miRNAs in a 6-10 bp seed region
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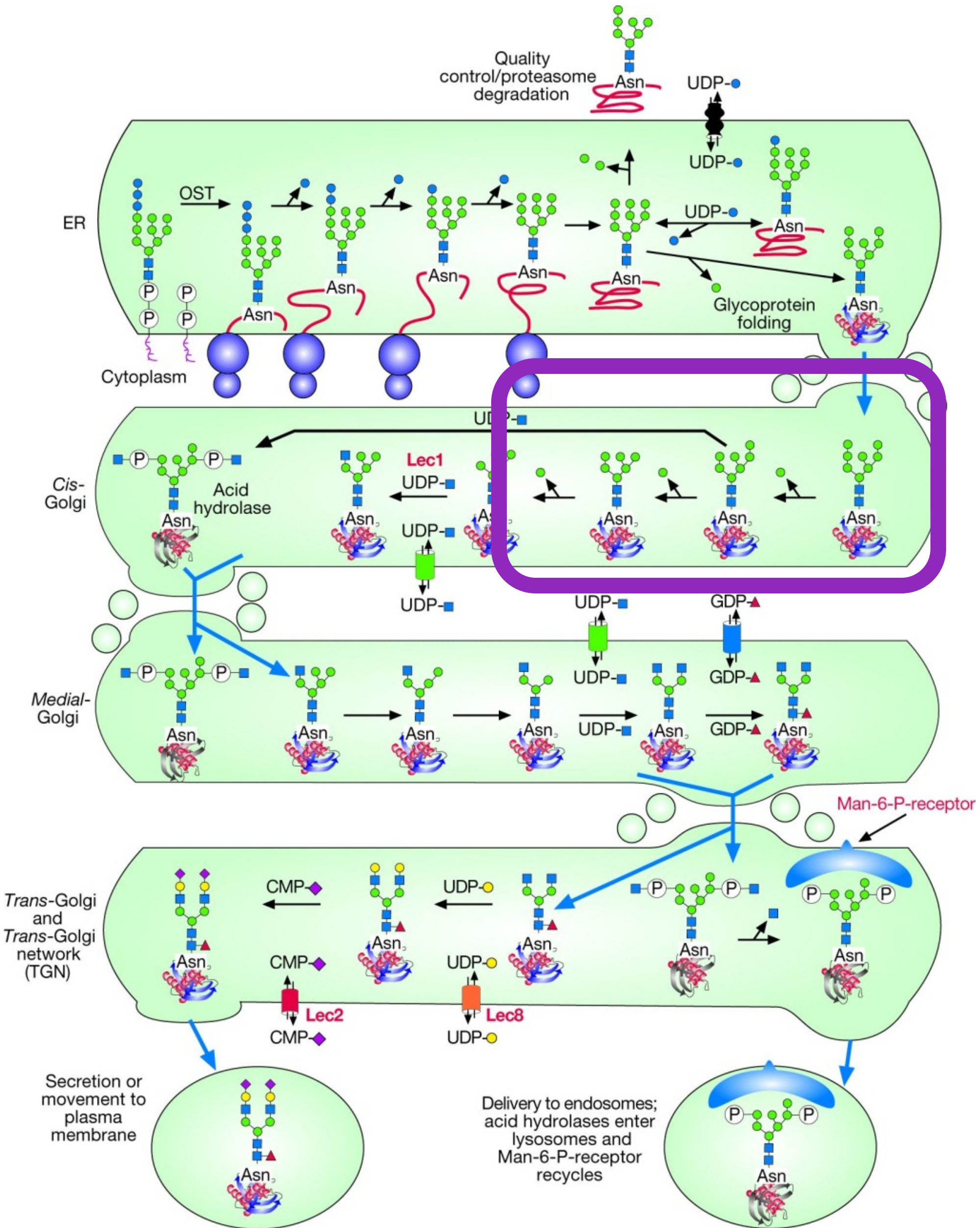
Thamilarasan et al, *Autoimmun Rev*, 2012

Maturation of *N*-linked glycans



- Upon proper folding, glycan is trimmed to high-mannose and, potentially further modified to hybrid/complex

- <u>α-mannosidase I controls all hybrid/complex maturation steps</u>

- You cannot have complex and hybrid glycans if α-mannosidase I isn't expressed

Essentials of Glycobiology

We now have a hypothesis

- **miRNA’s regulate the *N*-linked maturation pathway**

- **miRNA expression controls *N*-linked glycome by repressing (or activating) α-mannosidase I**

- **High mannose cells have high miRNA expression which means low α-mannosidase I**

We need evidence

Use high mannose (SN12C, SK-MEL-5) and complex (HCT-116, HT29) lines

Modulate miRNA expression in cells

Analyze mannosidase gene expression (RT-PCR)

Isolate total RNA

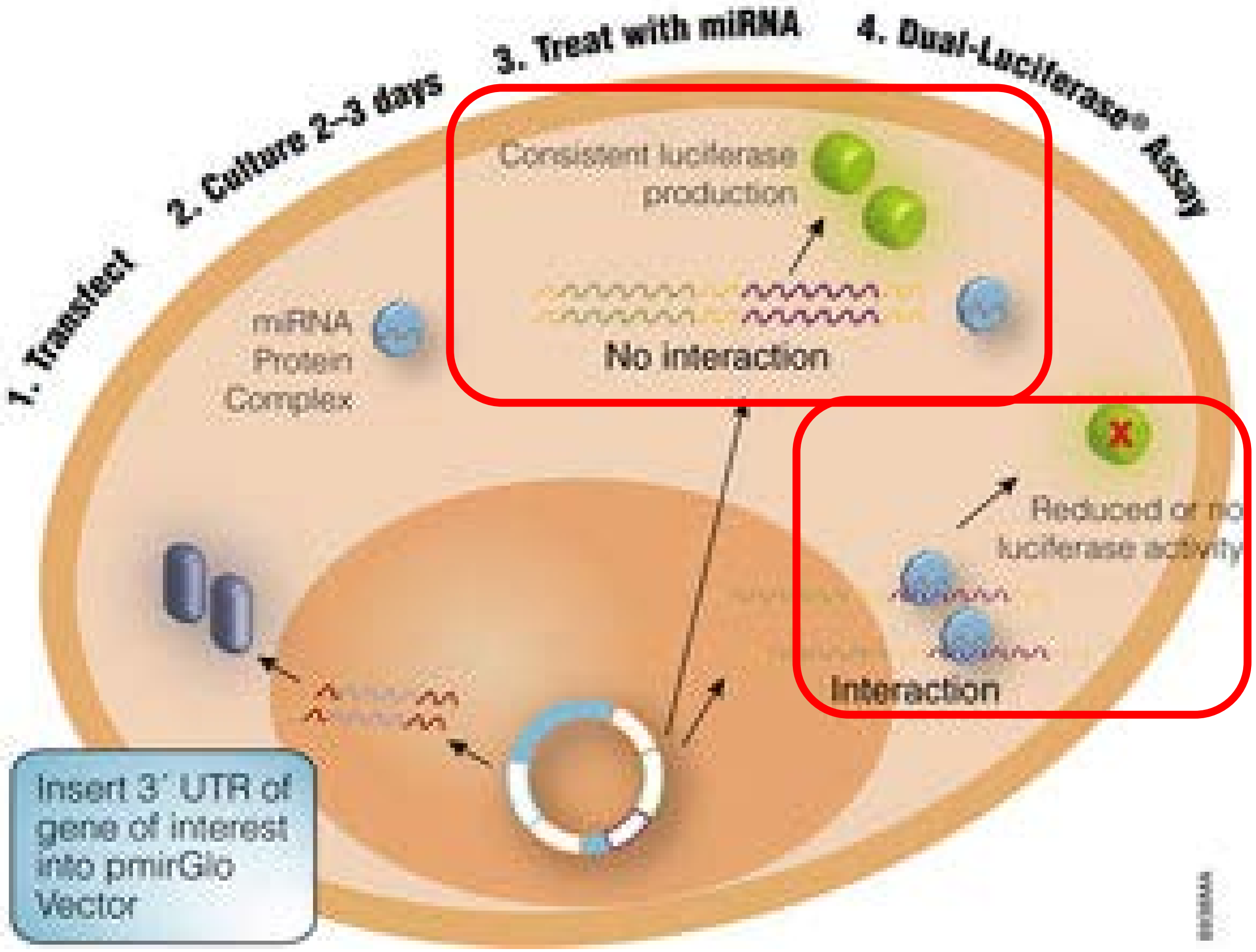
Isolate total protein

Analyze mannosidase protein expression (Western blot)

Analyze glycome (lectin microarray)

Can the miRNAs bind the mannosidase transcript?

Luciferase read-through assay



Promega.com

miR-30c and -361 bind the MAN1A2 3’ UTR

```
MAN1A2.30cMut.stk<      CCTGTGTTTCGCTCATATGGACCACTACAGAAATTAGTTTGAAGGGGCGGCTTTTGAAAA
MAN1A2.3UTR<            CCTGTGTTTTGTTTACATGGACCACTACAGAAATTAGTTTGAAGGGGCGGCTTTTGAAAA
                        ********* * * * ********************************************

MAN1A2.361Mut.stk<      AAATTTCTTGGTTAAGTTTAATTTTCTATAGAGACATGATCTCACAAAGAATACTCAAGT
MAN1A2.3UTR<            AAATTTCTTGGTTAAGTTTAATTTTCTCTGGGGGCATGATCTCACAAAGAATACTCAAGT
                        ***************************.*.*.*.**************************
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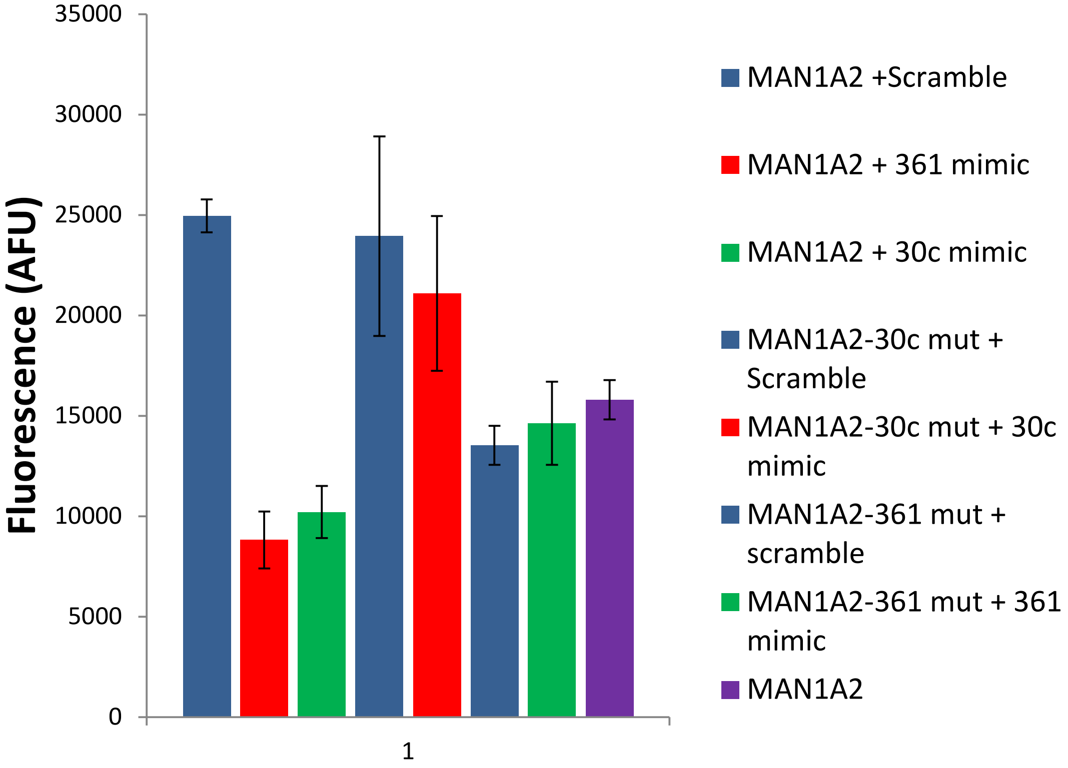
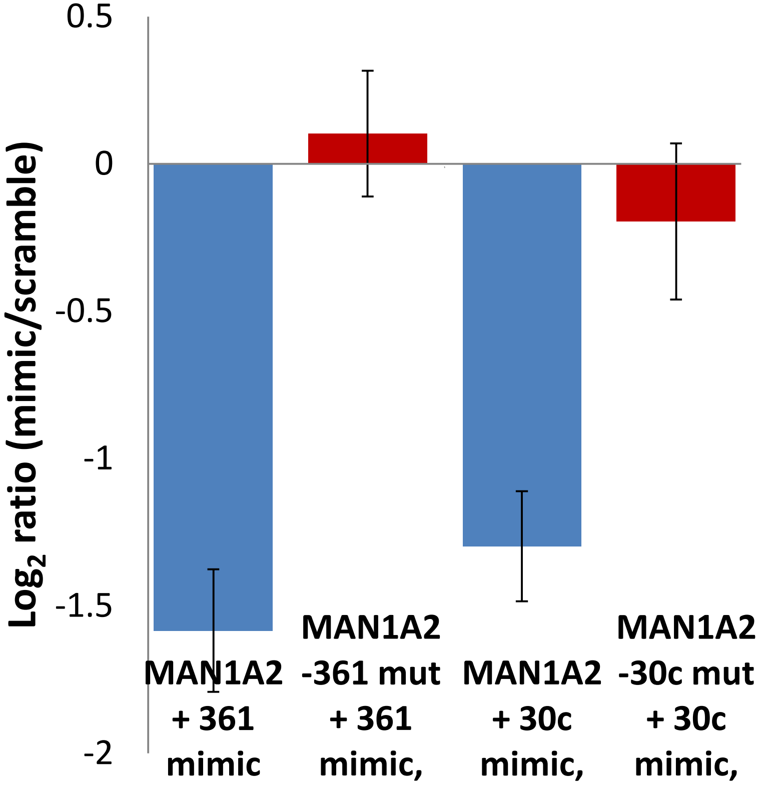
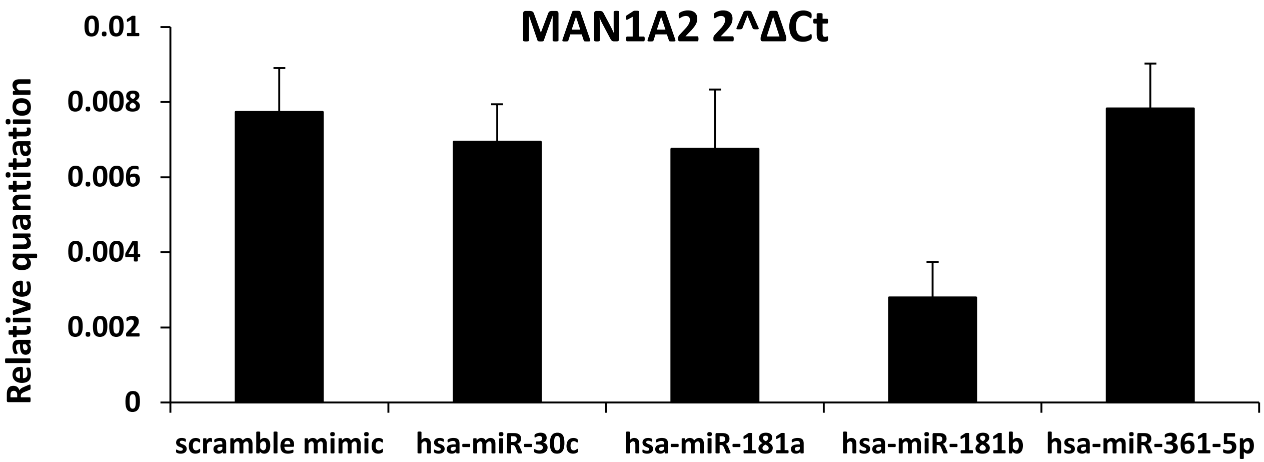




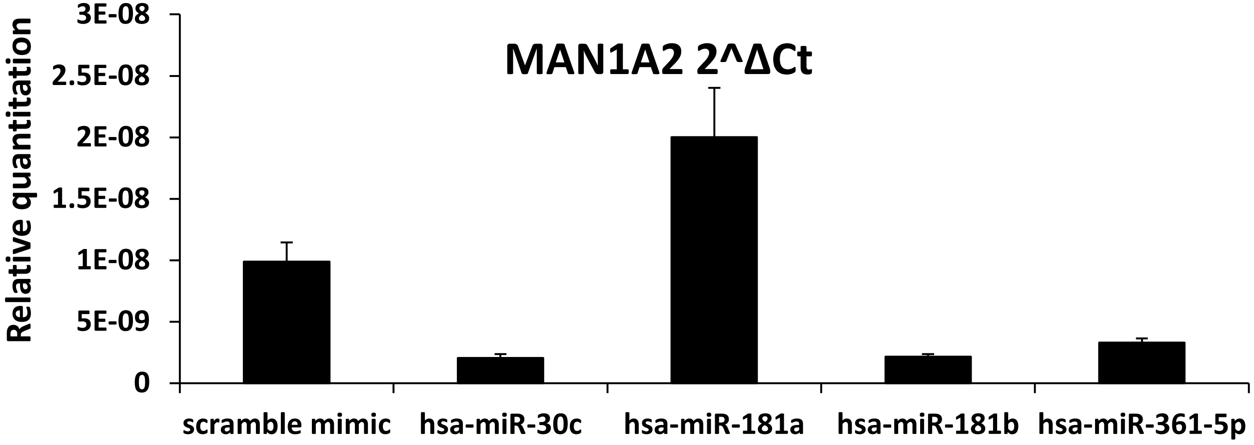
With Praveen Agrawal

Do the miRNAs affect mannosidase transcription?

Transfection of cell lines with miRNA repress MAN1A2



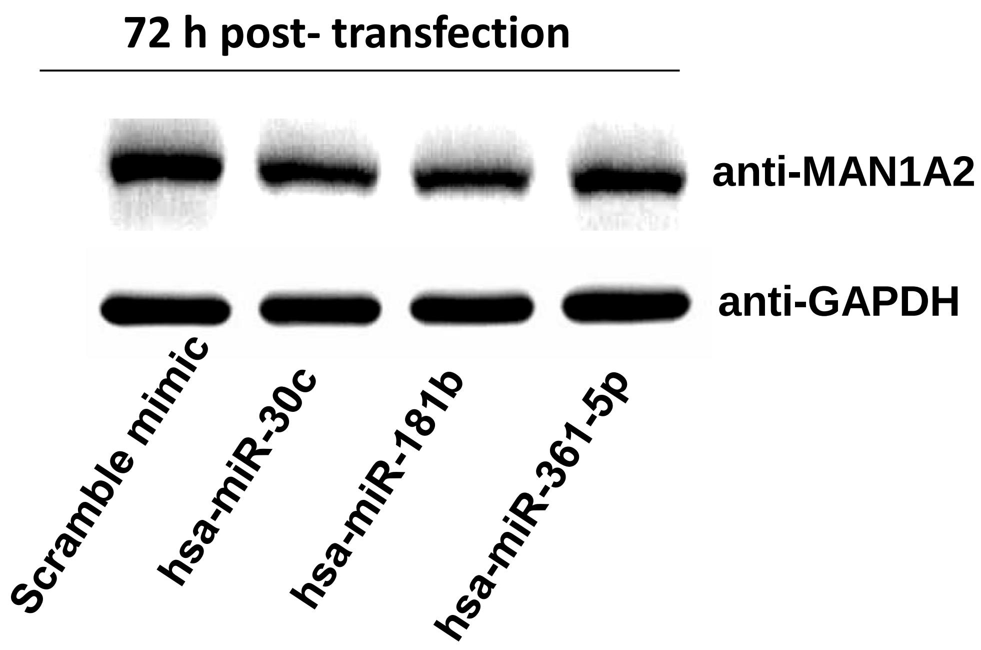
24 h post- transfection



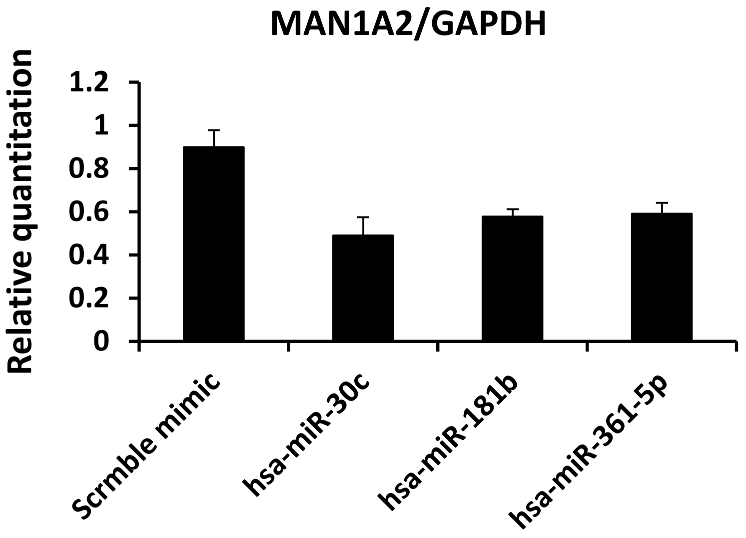
72 h post- transfection

With Praveen Agrawal

Do the miRNAs affect mannosidase protein expression?

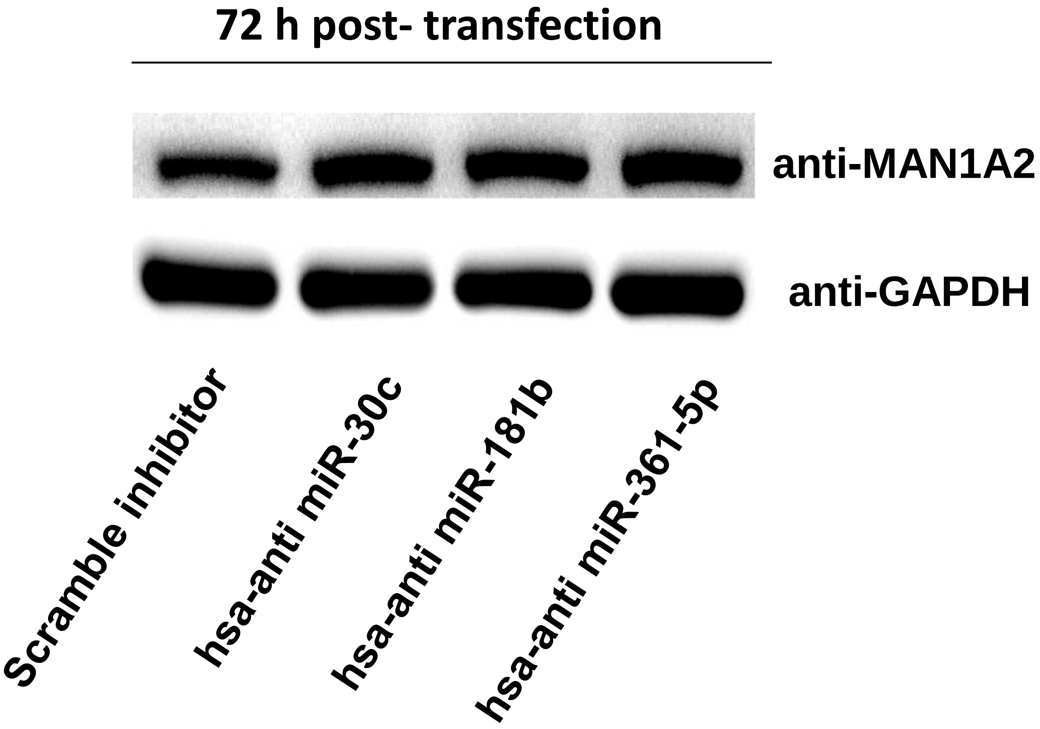


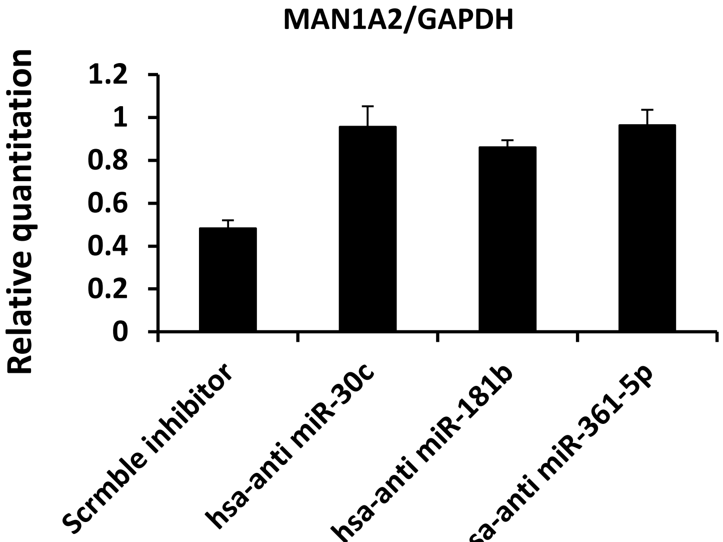
Western Blot of MAN1A2 72 hrs after transfection with miRNA



MAN1A2 expression decreases ~50%

With Praveen Agrawal





Western Blot of MAN1A2 72 hrs after transfection with miRNA <u>inhibitor</u>

MAN1A2 expression increases ~50%

With Praveen Agrawal

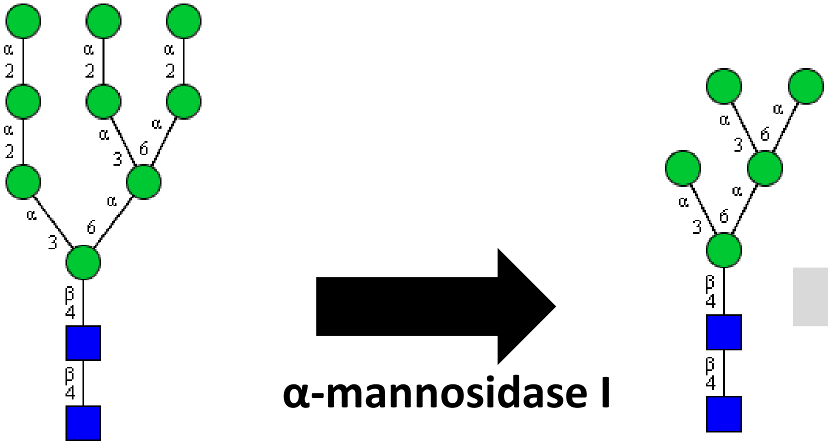
Seeing inside the house

- miRNA-30c and -361 affect expression of the enzyme α-mannosidase I (maybe -181b as well)

- Expression of α-mannosidase I determines the type of *N*-linked glycan expresses

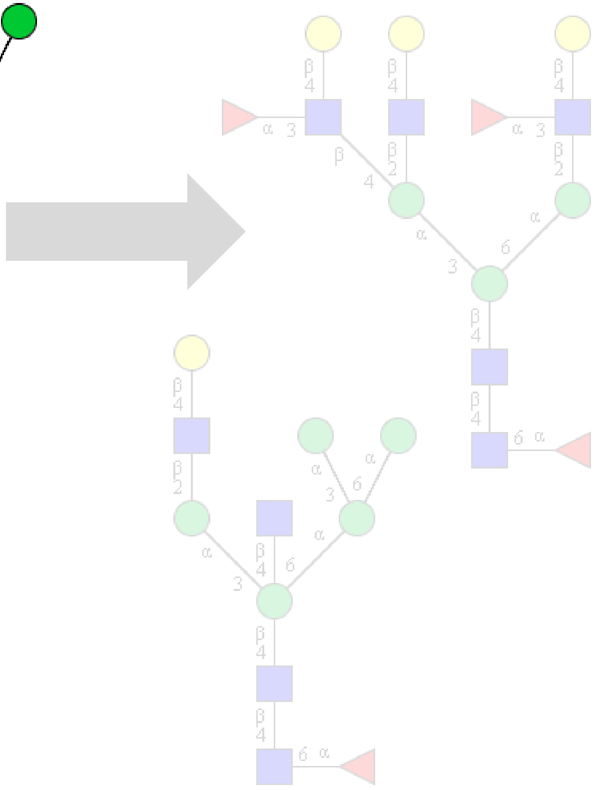
- Cells differentially express miRNAs resulting in different glycomes

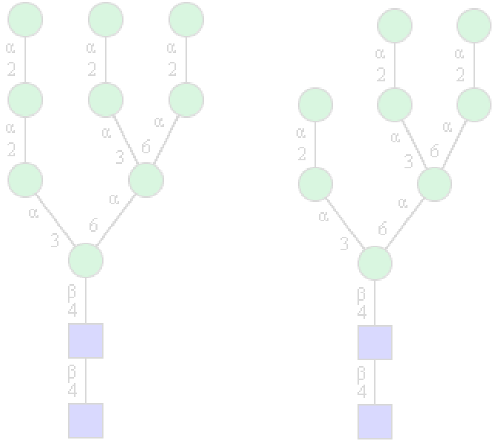
A model for cell-type specific carbohydrate expression?



α-mannosidase I

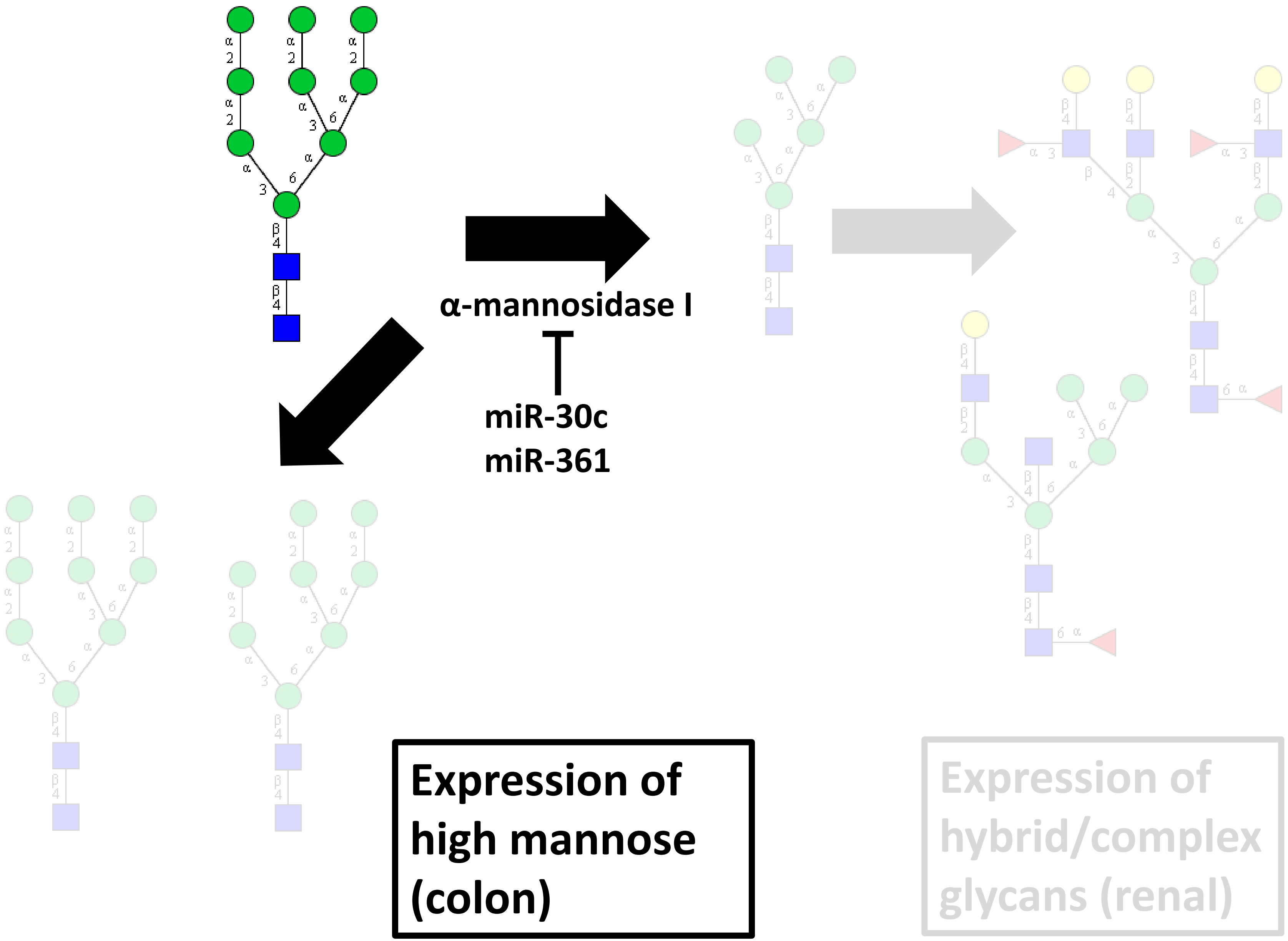
~~miR-30c~~
~~miR-361~~





Expression of hybrid/complex glycans (renal)

A model for cell-type specific carbohydrate expression?



α-mannosidase I

miR-30c
miR-361

Expression of high mannose (colon)

Expression of hybrid/complex glycans (renal)

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