

CHEM/BCMB 8190

# NMR of Carbohydrates

Dr. Adam Barb

# Learning Objectives

- Describe the unique chemical features of carbohydrate mono- and poly-saccharides
- Utilize scalar coupling relationships to achieve stereochemical assignments of non-exchangeable carbohydrate proton resonances
- Define different types of biological glycoconjugates
- Compare and contrast different glycoprotein stable isotope labeling strategies
- Develop new approaches to extend the capabilities of glycoprotein NMR in solution

# **Outline; three videos**

## **1. Carbohydrates**

- Biology
- Structure
- Chemistry

## **2. Oligosaccharide NMR features**

- Weak scalar coupling over glycosidic bond
- Karplus relationship provides relative stereochemistry
- NOE across glycosidic linkage
- Monosialyl Fc example

## **3. Glycoprotein NMR**

- Labeling challenges
  - Post purification remodeling
  - Metabolic labeling
- N-glycan NMR features
- IgG1 Fc

# 1. Carbohydrates

## 1a. Biology

- Function: nutritive, structural, signaling
- Types: free, glycoconjugates (lipids, proteins)

## 1b. Structure

- Common monosaccharides

## 1c. Chemistry (linkages)

- Highly flexible
- N-glycan chemistry

# 1a. Carbohydrates



Nutritive

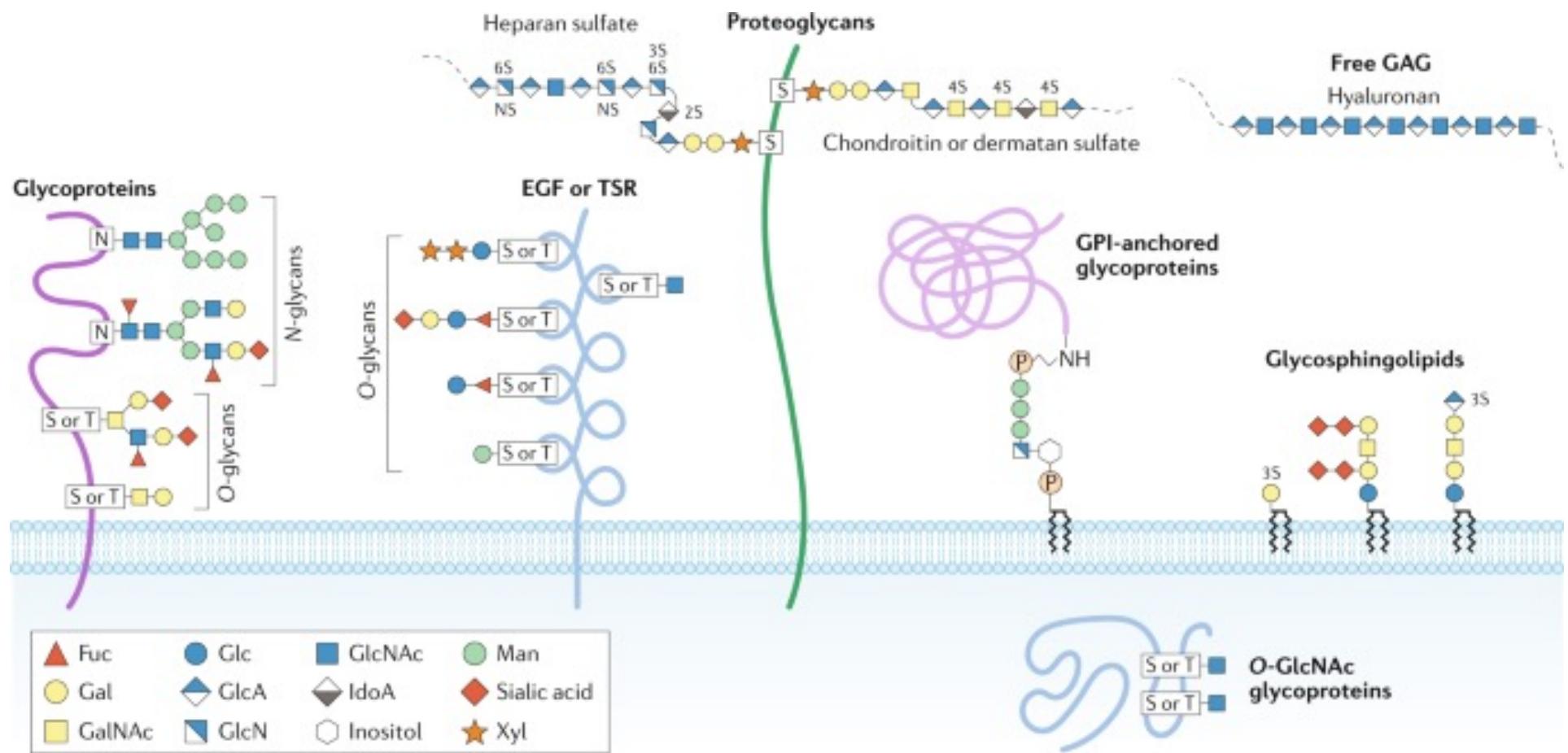


Structural

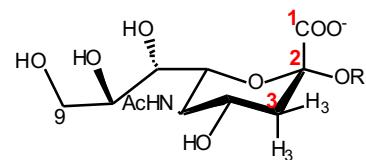
ANTIGENS IN RBC	A Antigen	B Antigen	Both A and B antigens	Neither A nor B antigens
ANTIBODIES IN PLASMA	Anti-B	Anti-A	Neither Anti-A nor Anti-B	Both Anti-A and Anti-B
BLOOD TYPE	Type A	Type B	Type AB	Type O

Signaling

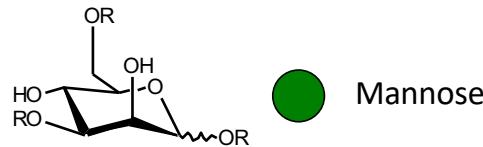
# 1a. Mammalian glycans and glycoconjugates



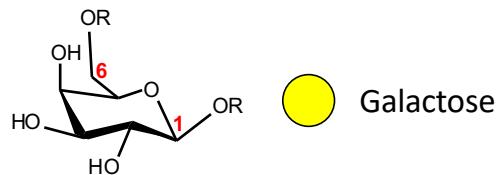
## 1b. Common Monosaccharides



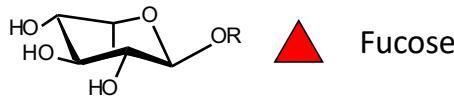
◆ N-acetylneurameric acid  
(a sialic acid)



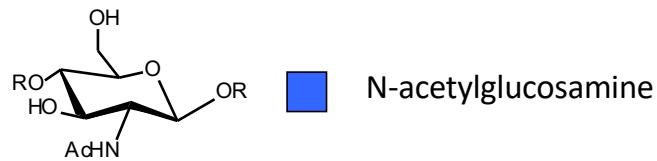
● Mannose



● Galactose

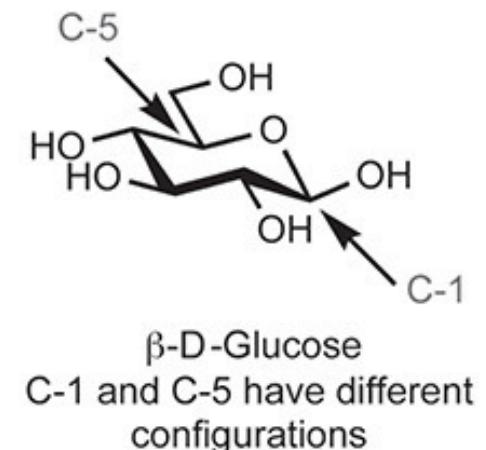
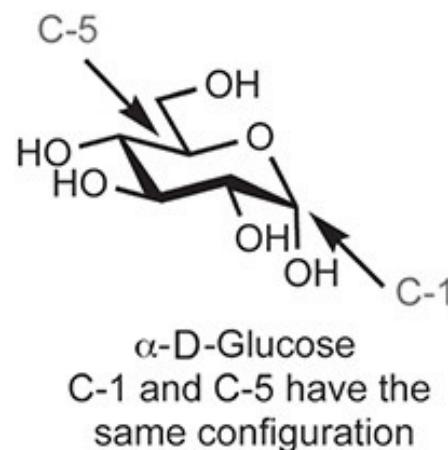
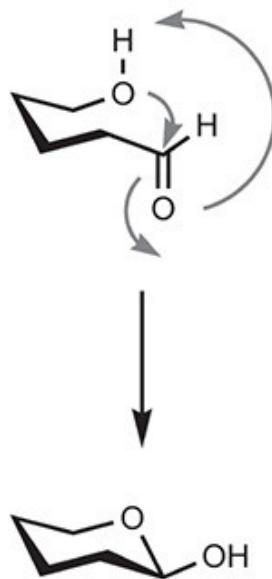
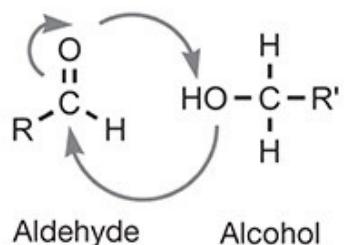


▲ Fucose

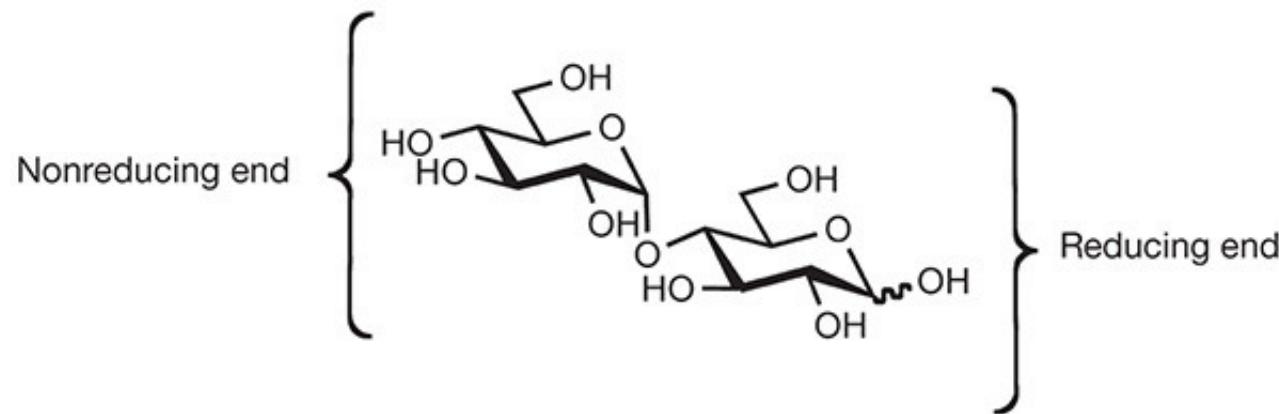
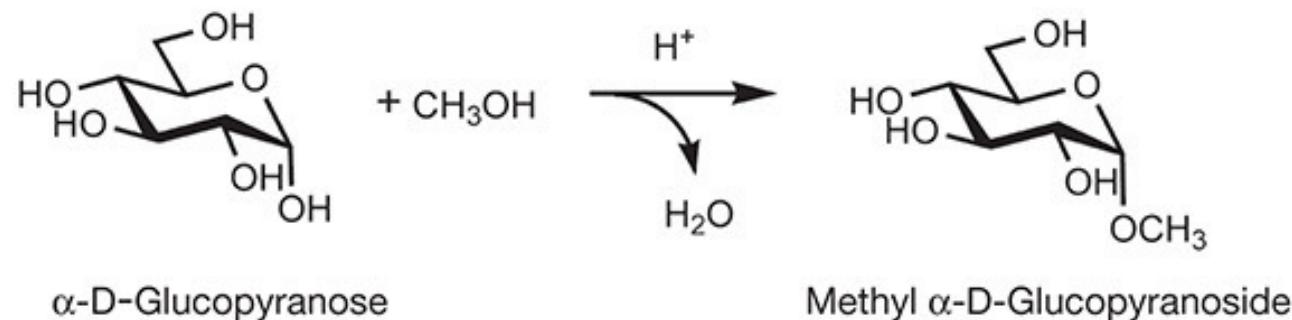


■ N-acetylglucosamine

## 1c. Cyclization and Mutarotation

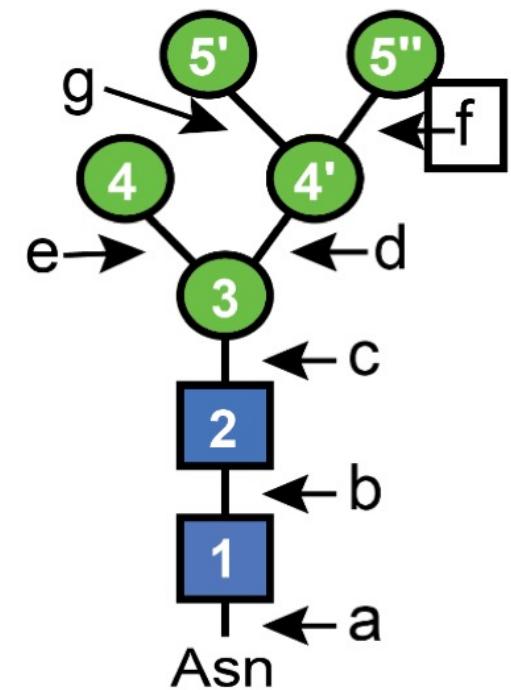
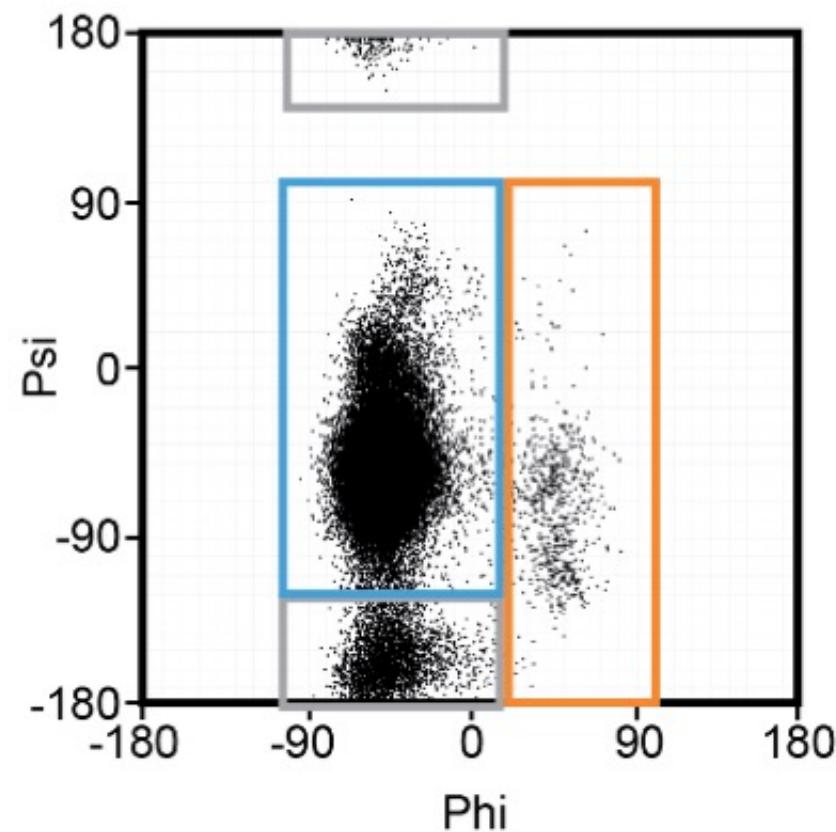


## 1c. Glycosidic bond



Varki (2009) Glycobiology

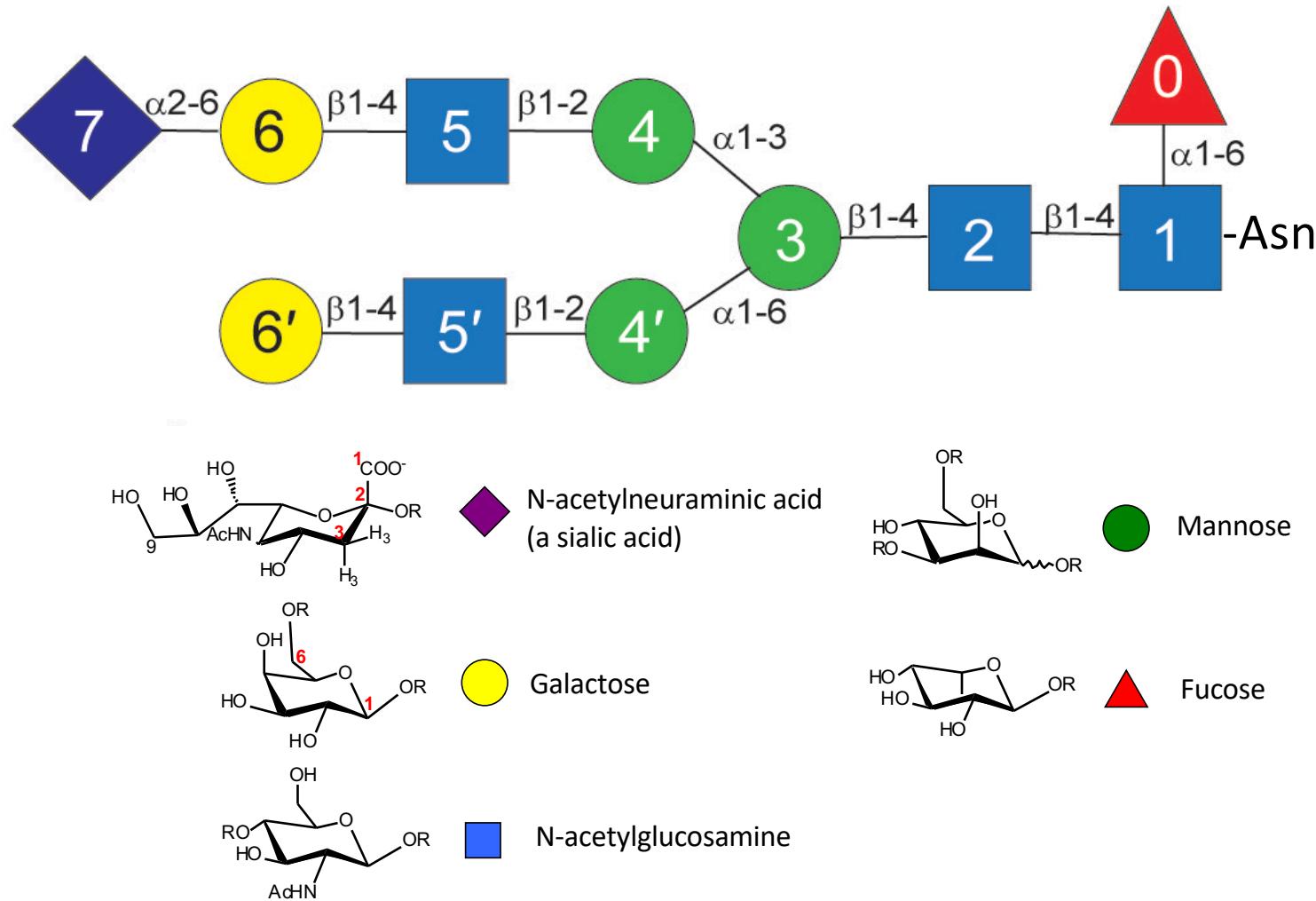
### 1c. Glycosidic bond flexibility

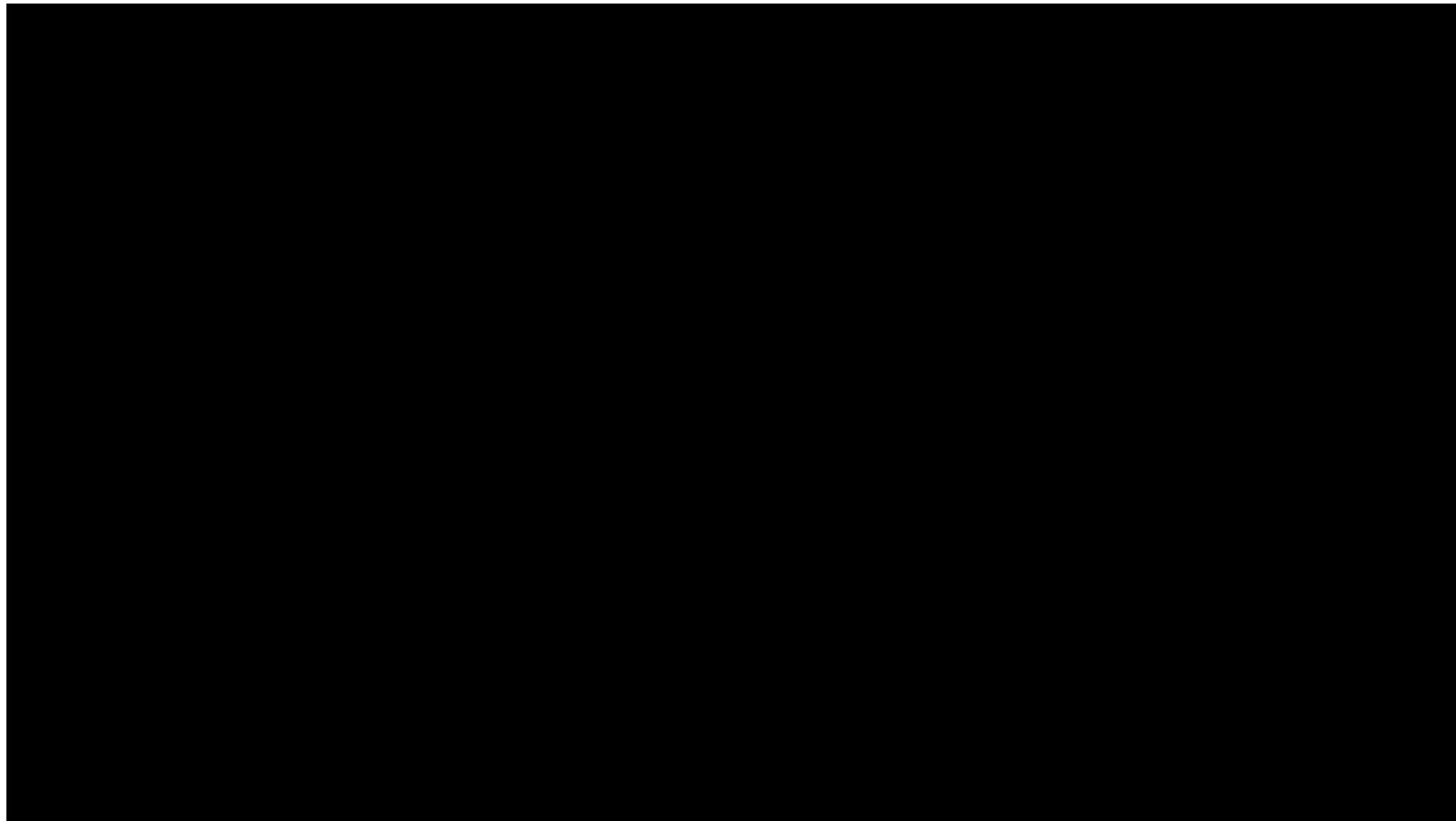


Conformation

- █ 1
- █ 2
- █ 3

# 1c. The complex-type biantennary IgG N-glycan

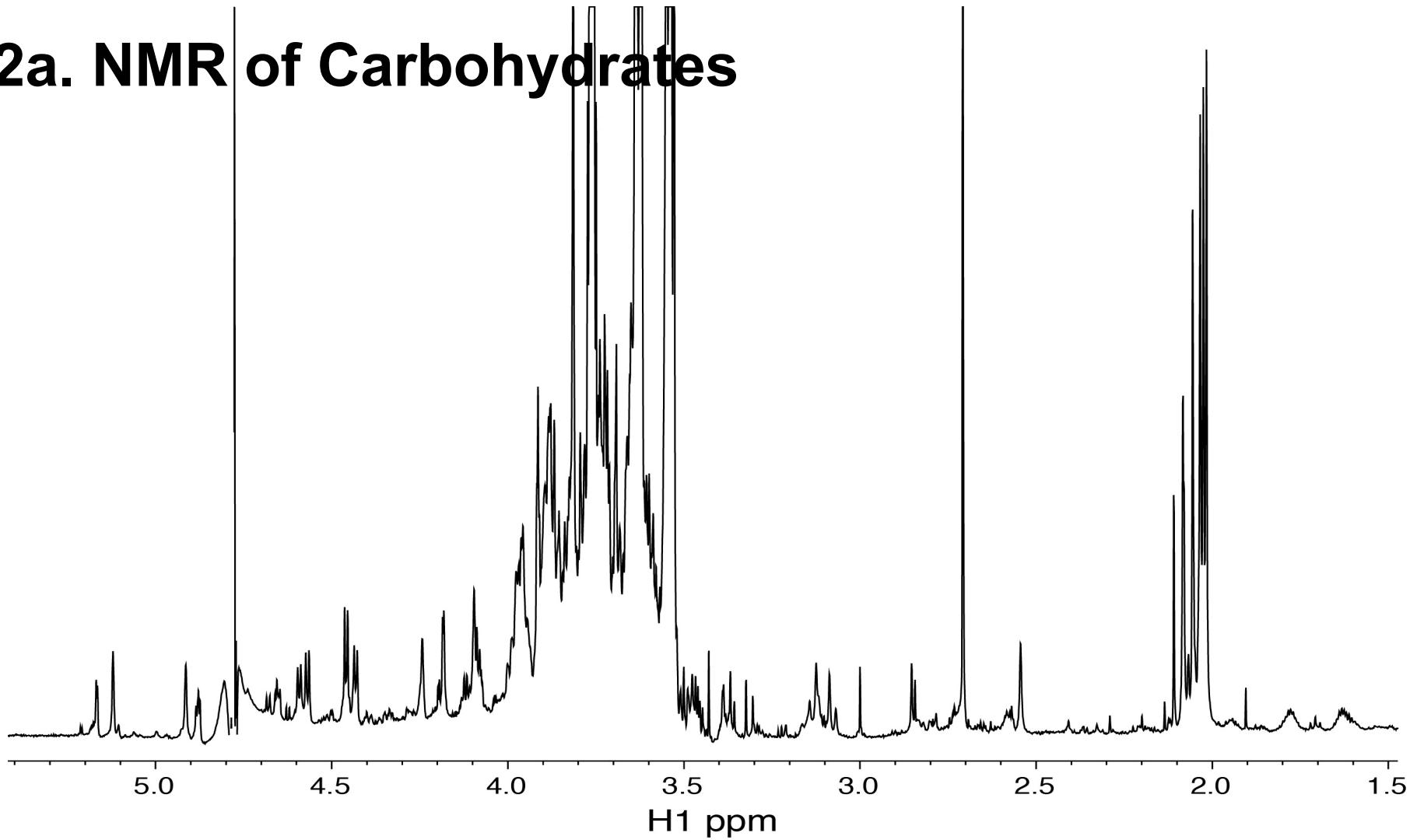




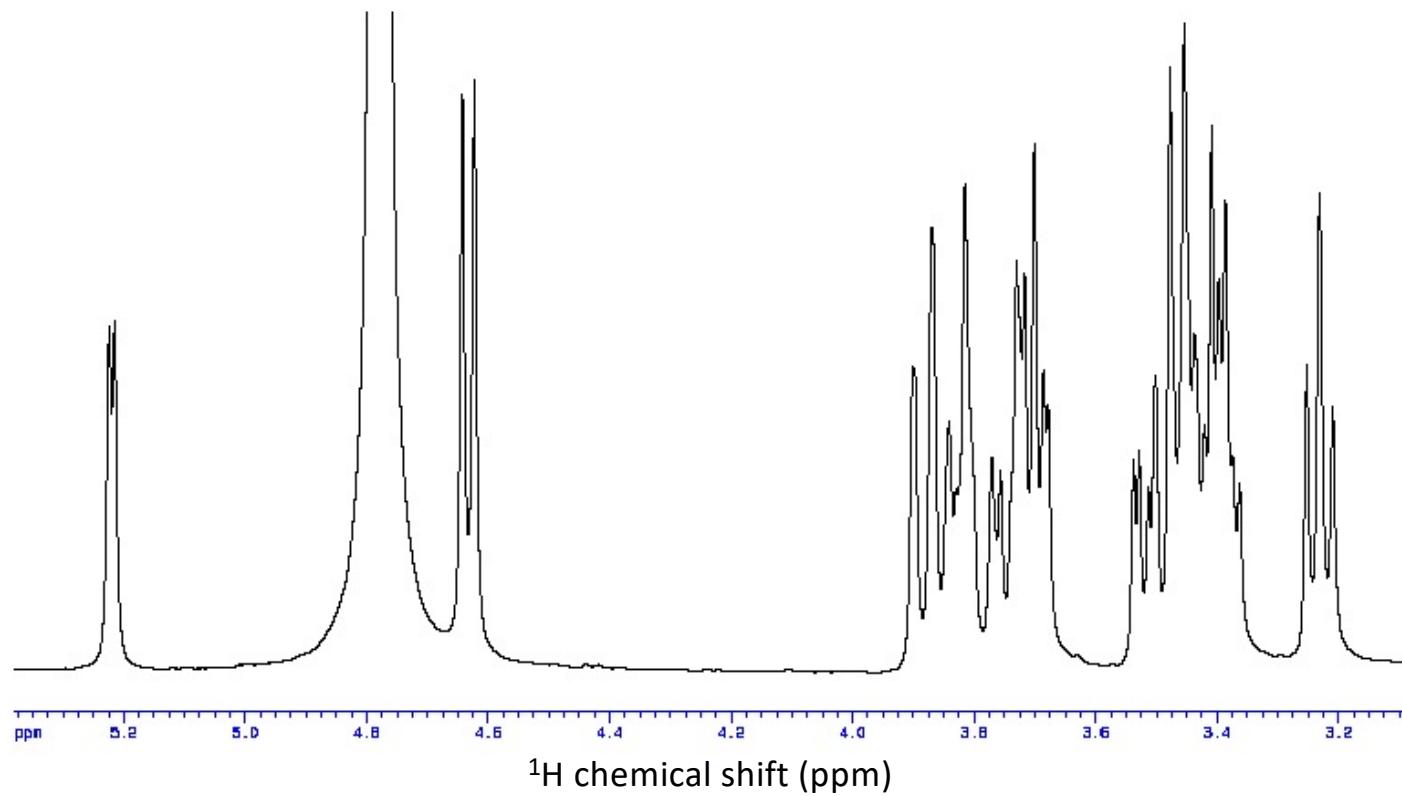
## 2. Oligosaccharide NMR features

- 2a. Karplus relationship provides relative stereochemistry
- 2b. Structural Reported Groups
- 2c. Peracetylation
- 2d. Weak scalar coupling over glycosidic bond:
  - NOE across glycosidic linkage
  - Example: Monosialyl Fc N-glycan

## 2a. NMR of Carbohydrates

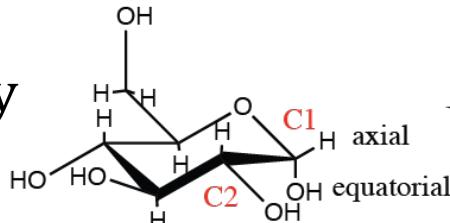


## 2a. 1d $^1\text{H}$ -NMR of glucose

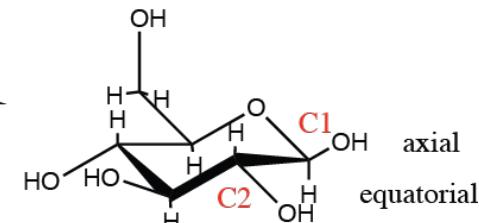


# Carbohydrate stereochemistry through $^3J$

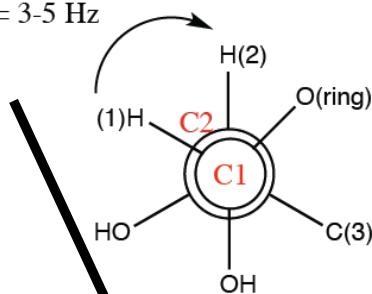
alpha anomeric configuration



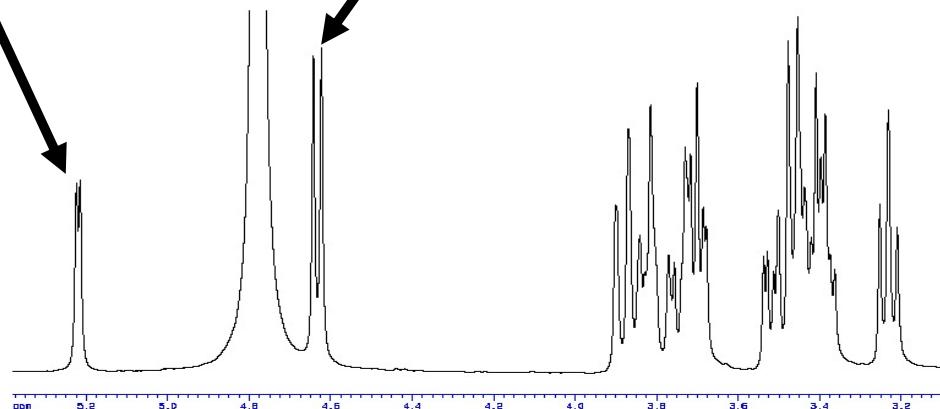
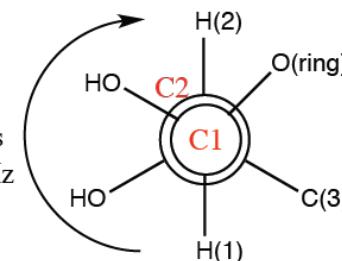
beta anomeric configuration



60 degrees  
 $^3J = 3-5$  Hz



180 degrees  
 $^3J = 9-12$  Hz



## **2b. Structure Reporter Groups; a database approach**

Collect 1D NMR spectrum of carbohydrate,  
compare with spectra of known standards ID.

Not always applicable to intact or complex glycoproteins  
or large MW CHO's

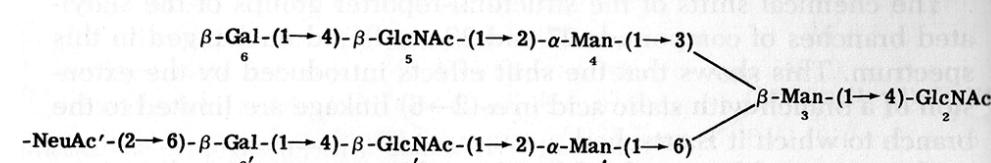
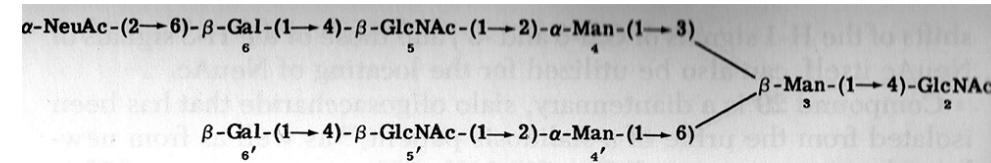
Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H.  
(1983) Adv. Carbohydr. Chem. Biochem. 41, 209-373

Reporter group	Residue	Anomer of oligo-saccharide	Compound and				schematic structure					
			21	22	23	24	25	26	27	28	29	30
H-1 of	2	$\alpha$	5.208	5.218	5.215	5.215	5.213	5.213	5.214	5.214	5.216	5.215
		$\beta$	~4.72	~4.72	~4.72	~4.72	~4.72	~4.72	~4.72	~4.72	~4.72	~4.72
	3	$\alpha$	4.788	4.774	4.792	4.784	4.784	4.784	4.781	4.781	4.786	4.777
		$\beta$	4.785	4.765	4.784	4.775	4.776	4.776	4.771	4.771	4.777	4.76
	4	$\alpha$			5.107		5.137	5.121	5.138	5.124	5.137	5.134
		$\beta$	5.143	—	5.140	5.103						
	4'	$\alpha$	—	4.942	4.921	4.949	4.921	4.950	4.929	4.949	4.952	4.943
		$\beta$										
	5	$\alpha,\beta$	4.602	—	4.606	—	4.607	4.557	4.608	4.583	4.608	4.594
	5'	$\alpha,\beta$	—	4.602	—	4.606	4.558	4.607	4.586	4.608	4.608	4.605
	6	$\alpha,\beta$	4.446	—	4.446	—	4.446	—	4.446	4.468	4.445	4.443
	6'	$\alpha$	—	4.446	—	4.450	—	4.446	4.471	4.446	4.445	4.443
H-2 of	7	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	4.573
	8	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	4.441
	3	$\alpha$	4.244	4.088	4.268	4.263	4.264	4.259	4.260	4.265	4.266	4.228
		$\beta$	4.233	4.078	4.258	4.253	4.253	4.248	4.249	4.254	4.255	4.217
	4	$\alpha$	4.198	—	4.198	4.073	4.198	4.190	4.197	4.193	4.199	4.221
		$\beta$	4.196	—	—							
	4'	$\alpha$	—	4.106	<4.0	4.119	4.109	4.115	4.114	4.118	4.121	4.119
		$\beta$	—	4.110	<4.0	—						
H-3 of	4	$\alpha,\beta$	<4.0	—	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	4.049
	4'	$\alpha,\beta$	—	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0
H-3a of	<sup>a</sup> NeuAc	$\alpha,\beta$	1.716	—	1.719	—	1.718	—	1.720	—	1.721	1.720
	<sup>b</sup> NeuAc'	$\alpha,\beta$	—	1.714	—	1.718	—	1.718	—	1.719	1.719	1.717
	<sup>c</sup> NeuAc*	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	1.706
H-3e of	NeuAc	$\alpha,\beta$	2.670	—	2.669	—	2.669	—	2.669	—	2.669	2.670
	NeuAc'	$\alpha,\beta$	—	2.672	—	2.672	—	2.671	—	2.672	2.672	2.672
	NeuAc*	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	2.670
NAc of	2	$\alpha$	2.043	2.061	2.059	2.063	2.059	2.063	2.061	2.063	2.063	2.062
		$\beta$	2.041	2.058	2.055	2.059	2.055	2.059	2.058	2.060	2.060	2.059
	5	$\alpha,\beta$	2.069	—	2.070	—	2.070	2.055	2.070	2.053	2.071	2.069
	5'	$\alpha$	—	2.064	—	2.070	2.053	2.070	2.050	2.069	2.069	2.067
		$\beta$	—	—	—	2.067	2.066	2.048	2.066	2.066	2.066	2.065
	7	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	2.102
	NeuAc	$\alpha,\beta$	2.030	—	2.030	—	2.030	—	2.031	—	2.031	2.030
	NeuAc'	$\alpha,\beta$	—	2.030	—	2.030	—	2.030	—	2.031	2.031	2.030
	NeuAc*	$\alpha,\beta$	—	—	—	—	—	—	—	—	—	2.028

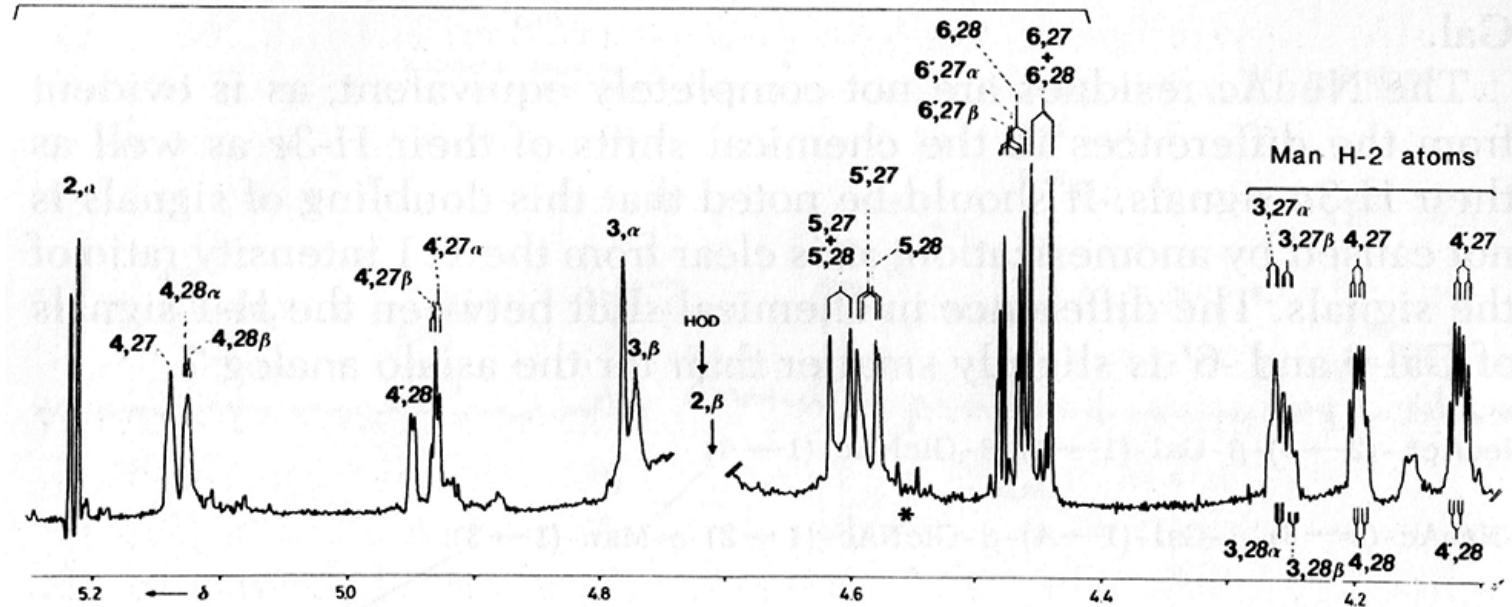
<sup>a</sup> NeuAc denotes NeuAc linked to Gal-6. <sup>b</sup> NeuAc' denotes NeuAc linked to Gal-6.

<sup>c</sup> NeuAc\* denotes NeuAc linked to Gal-8.

## Structure Reporter Groups



anomeric protons



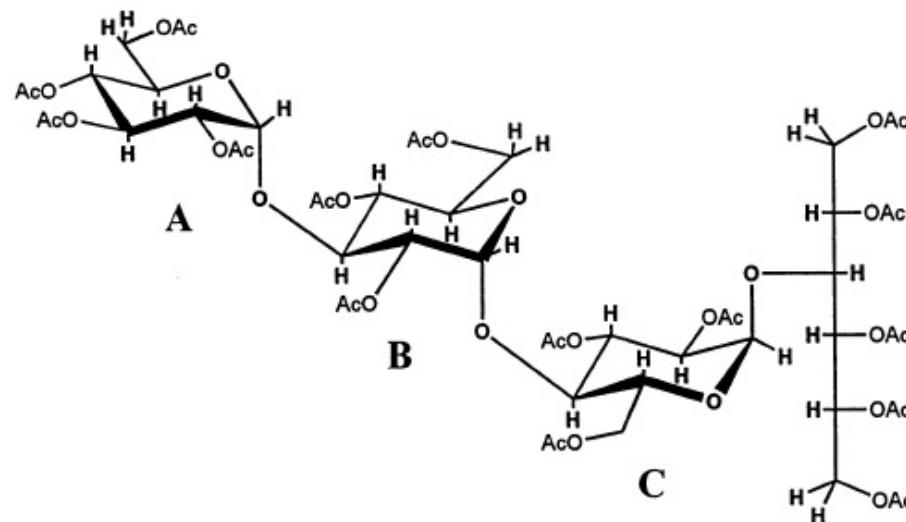
Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. 41, 209-373

## 2c. Peracetylation

Appropriate for low MW polysaccharides

React with  $^{13}\text{C}$ -acetic anhydride (simple to perform)  
hydroxyl labeling, no amide labeling

2 effects: dispersion and linkage analysis



Bendiak B. Nuclear magnetic resonance spectroscopy of peracetylated oligosaccharides having  $^{13}\text{C}$ -labeled carbonyl groups in lieu of permethylation analysis for establishing linkage substitutions of sugars. Carbohydr Res. 1999 Feb 28;315(3-4):206-21.

## 2c. Peracetylation

Greater dispersion of signals

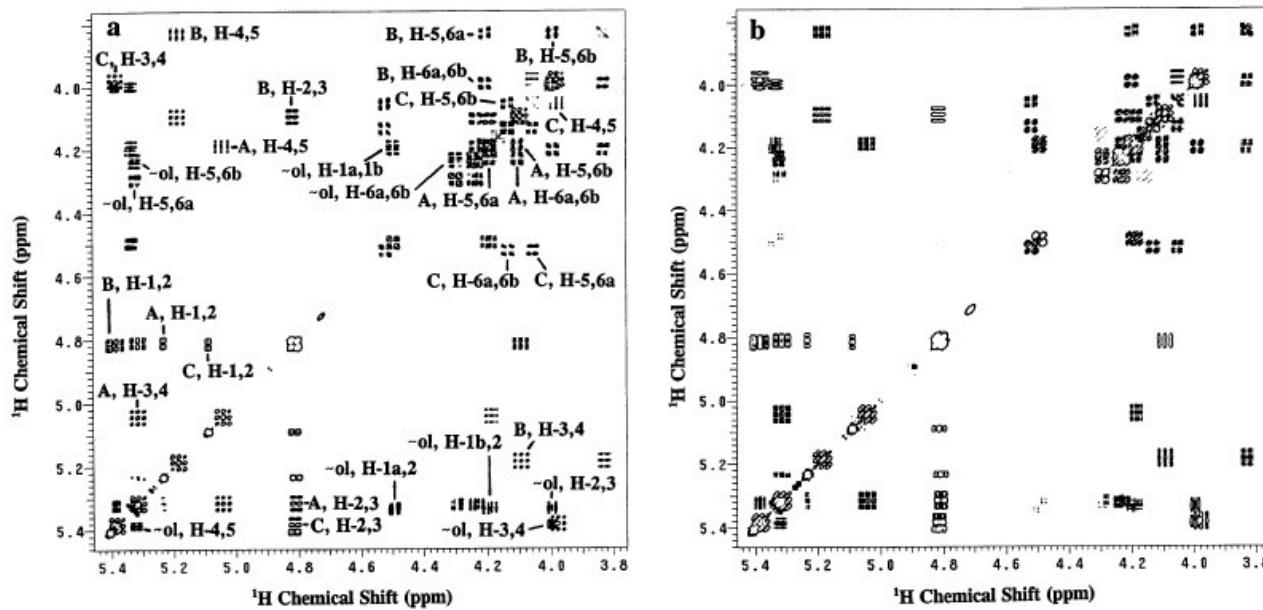
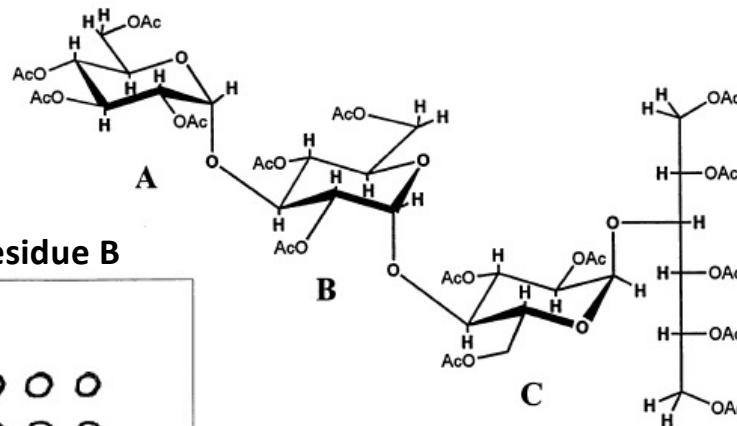
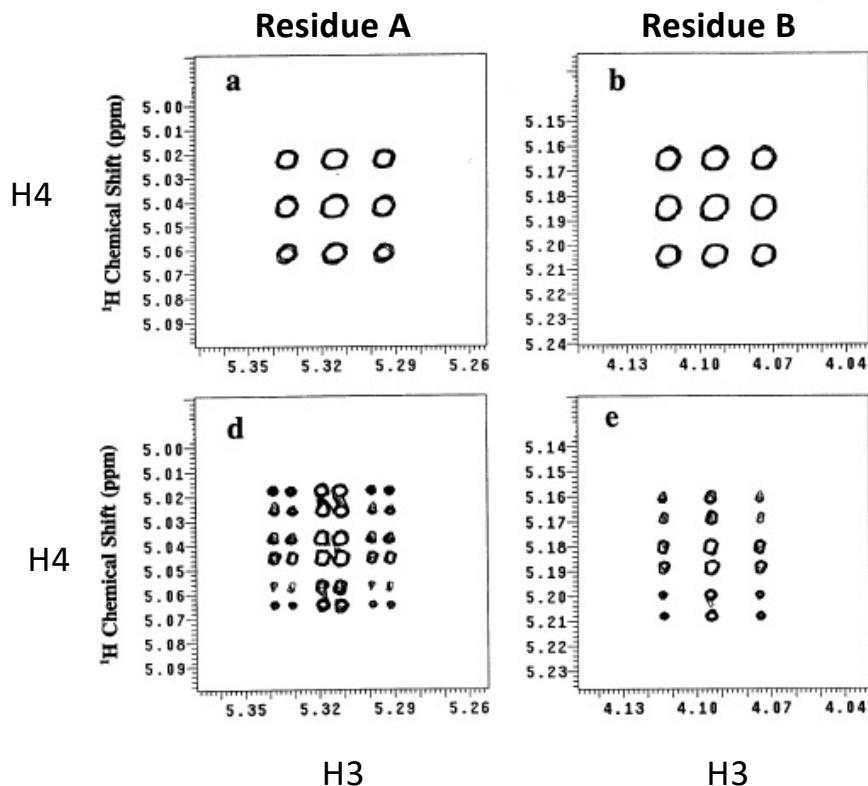


Fig. 4. Gradient COSY spectra of the peracetylated tetrasaccharide alditol,  $\alpha$ -D-GlcP-(1  $\rightarrow$  3)- $\alpha$ -D-GlcP-(1  $\rightarrow$  4)- $\alpha$ -D-GlcP-(1  $\rightarrow$  3)-D-Glc-ol. Assignments of crosspeak multiplets are indicated. Shown are the spectra of (a), the compound having natural abundance carbon in acetyl groups and (b), the compound having [ $^{13}\text{C}$ -carbonyl] acetyl groups.

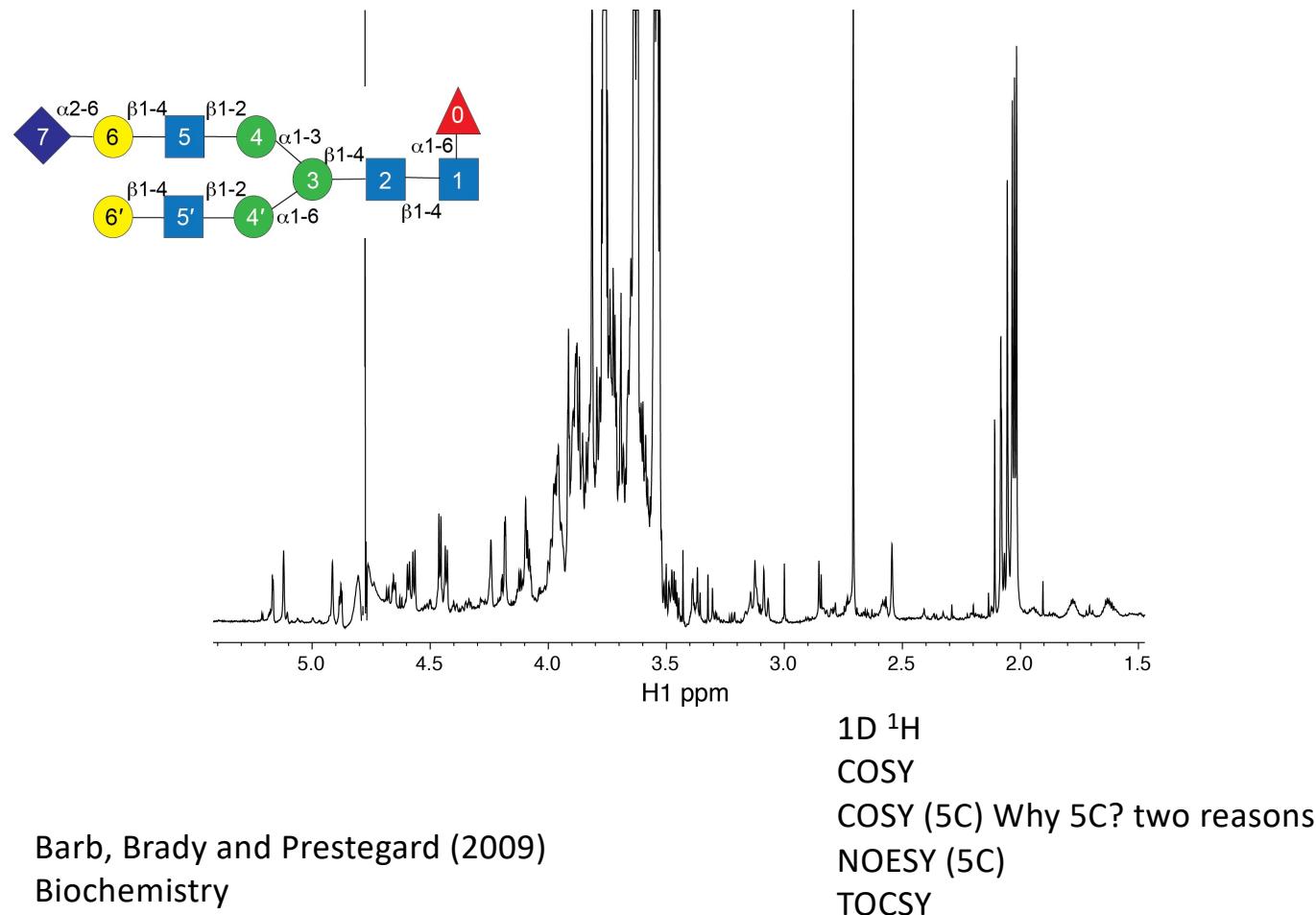
## 2c. Peracetylation

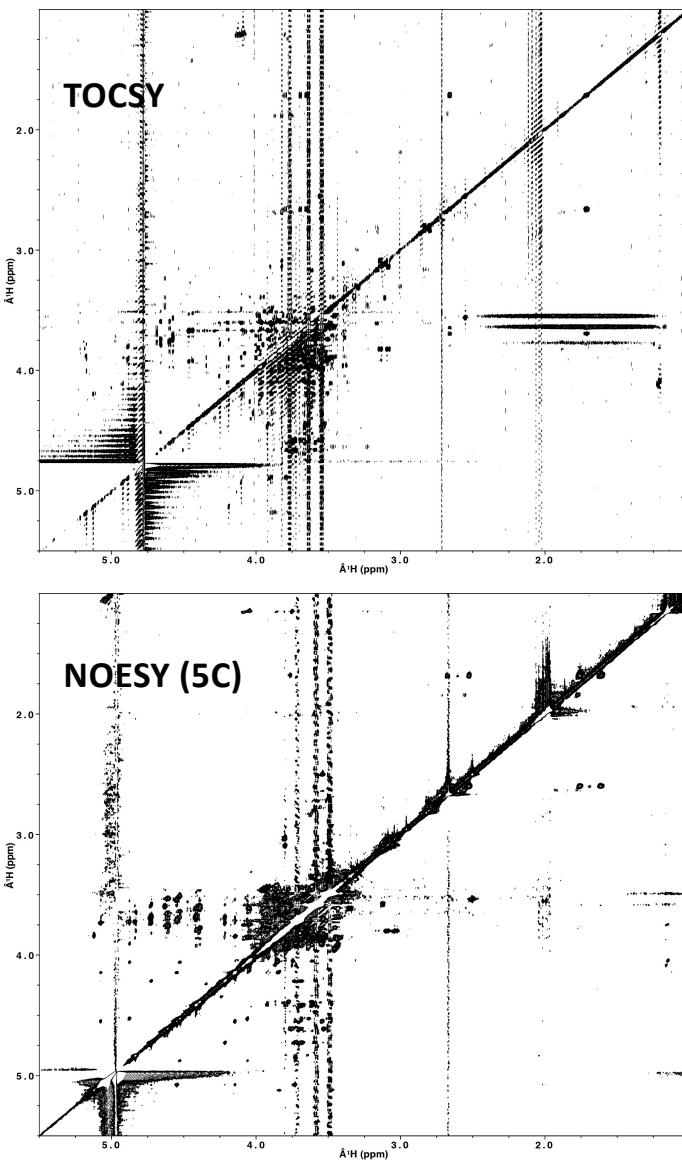
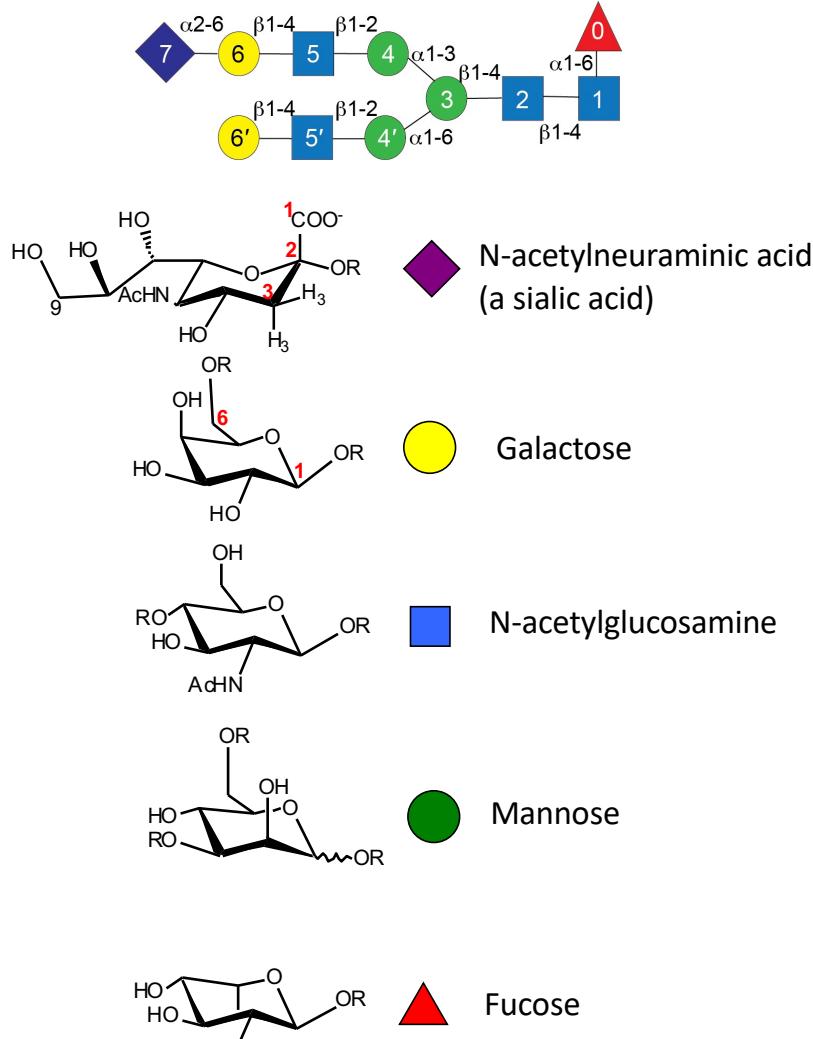
Perform linkage analysis

$^3J_{HC}$  : 2.5-4.7 Hz

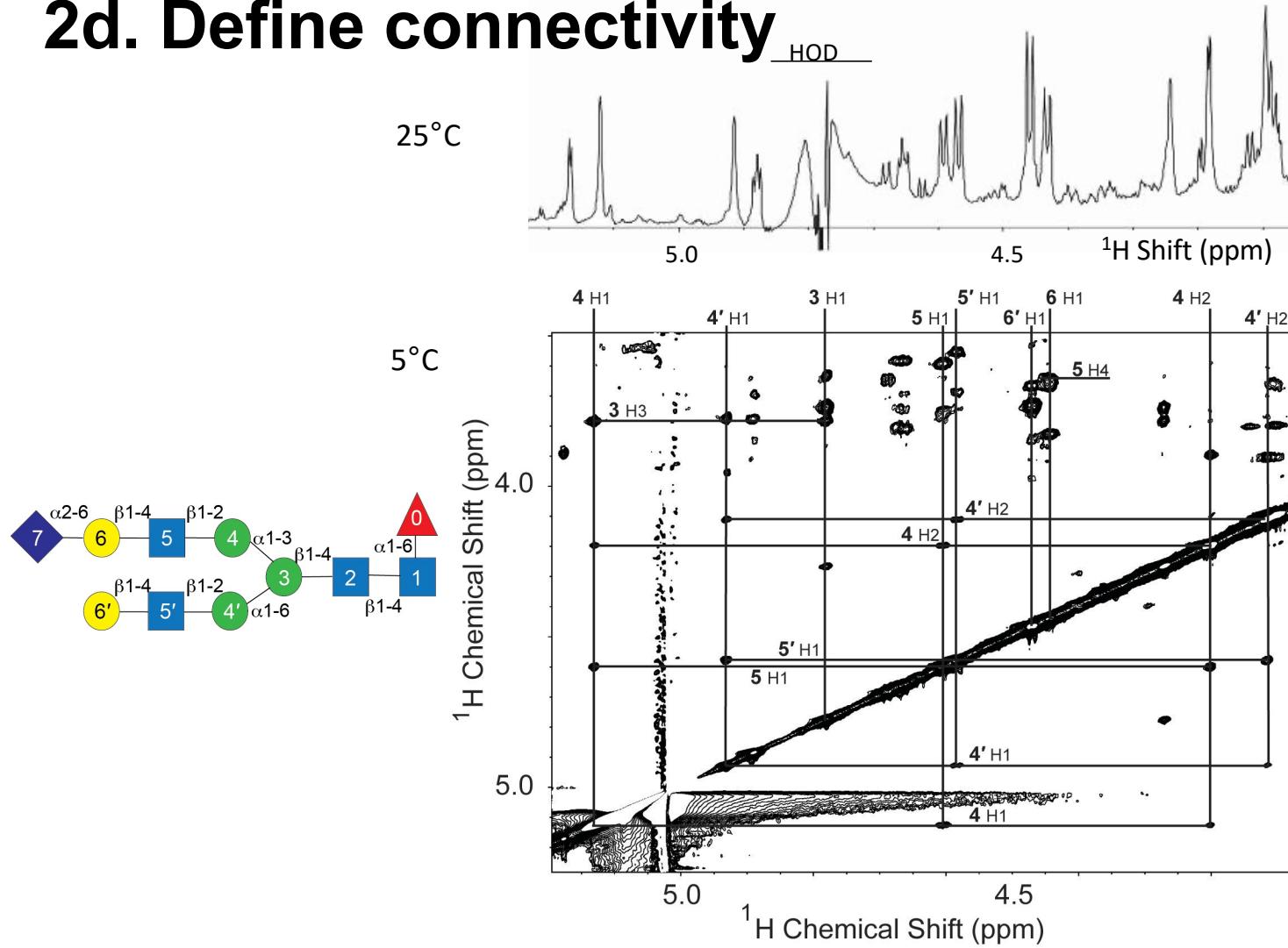


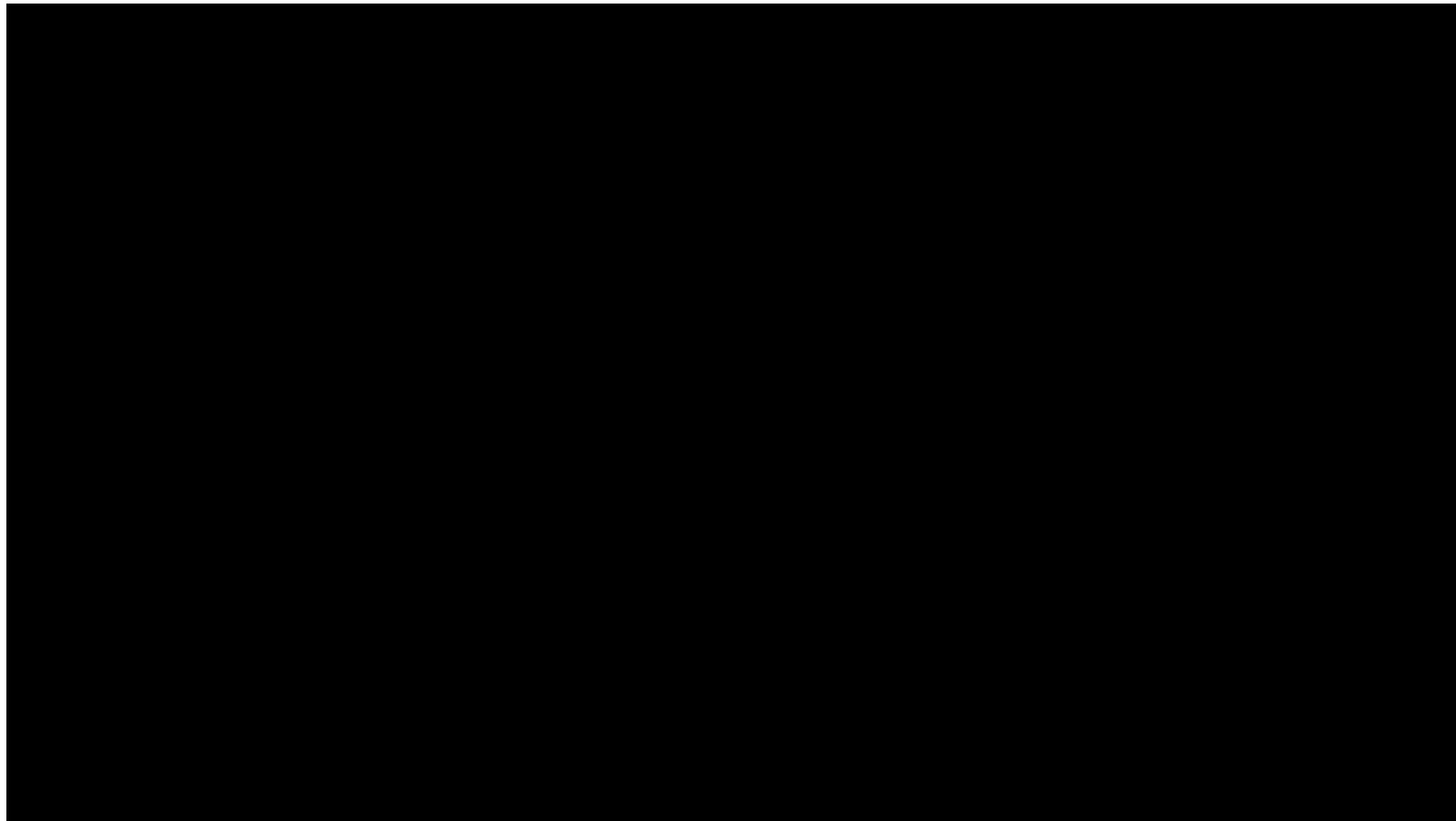
## 2d: No shortcuts: resonance assignment





## 2d. Define connectivity





### 3. Glycoprotein NMR

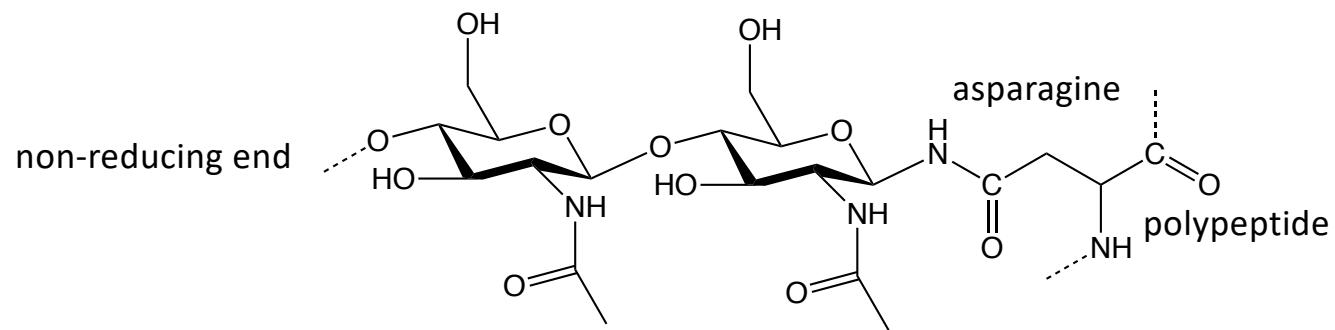
3a. N-glycan NMR features

3b. Labeling

- Metabolic labeling
- Post purification remodeling

3c. IgG1 Fc

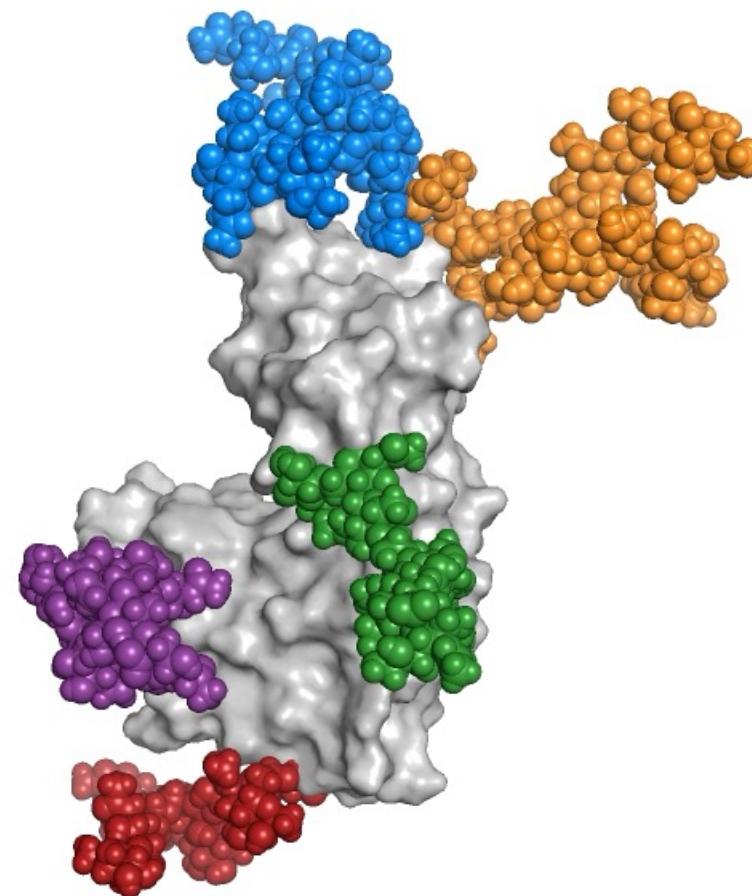
## 3a. Asparagine-linked (N-) glycans

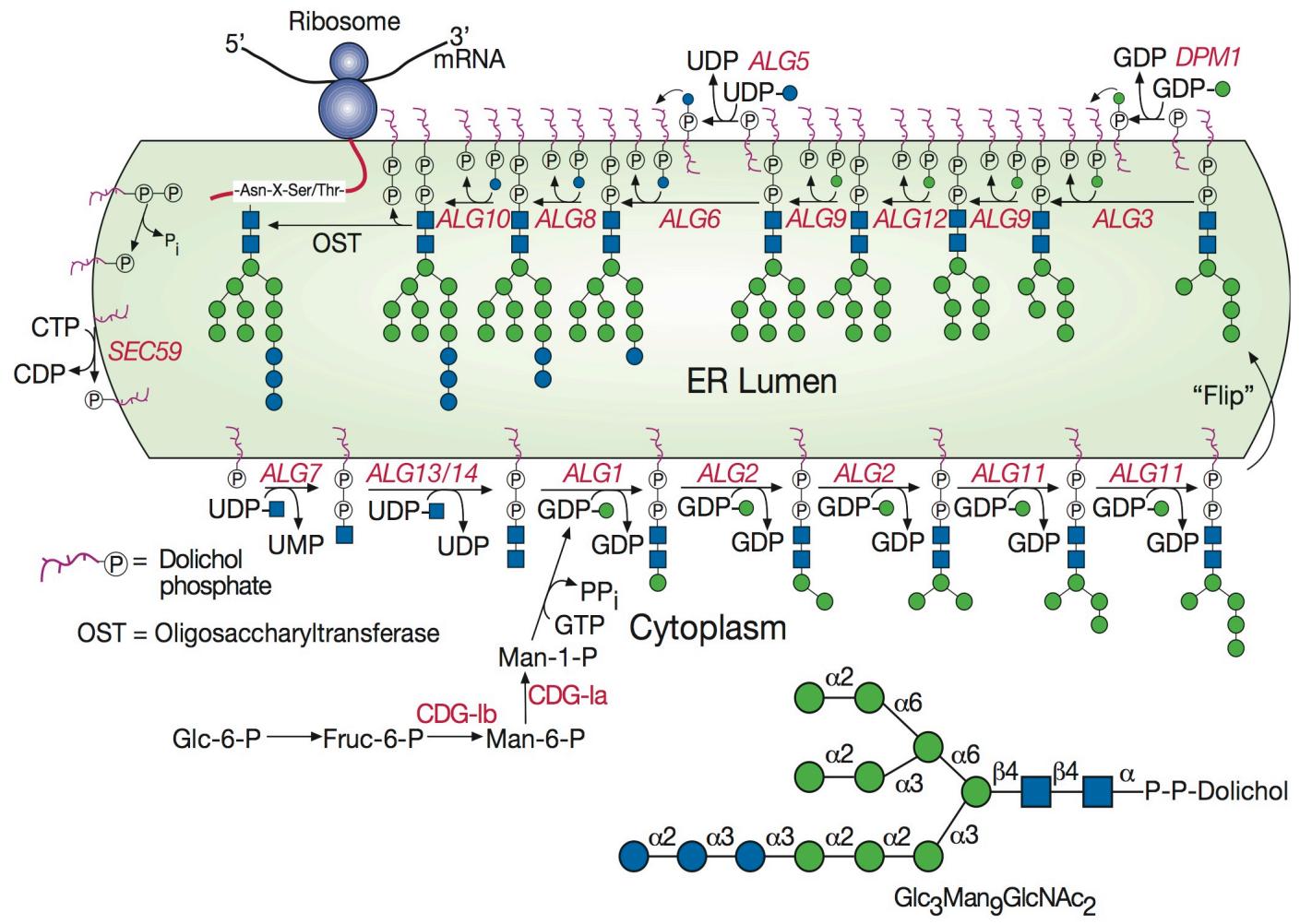


N-glycans affect protein:  
folding  
stability  
structure/motion  
ligand binding / function  
serum half life  
expression

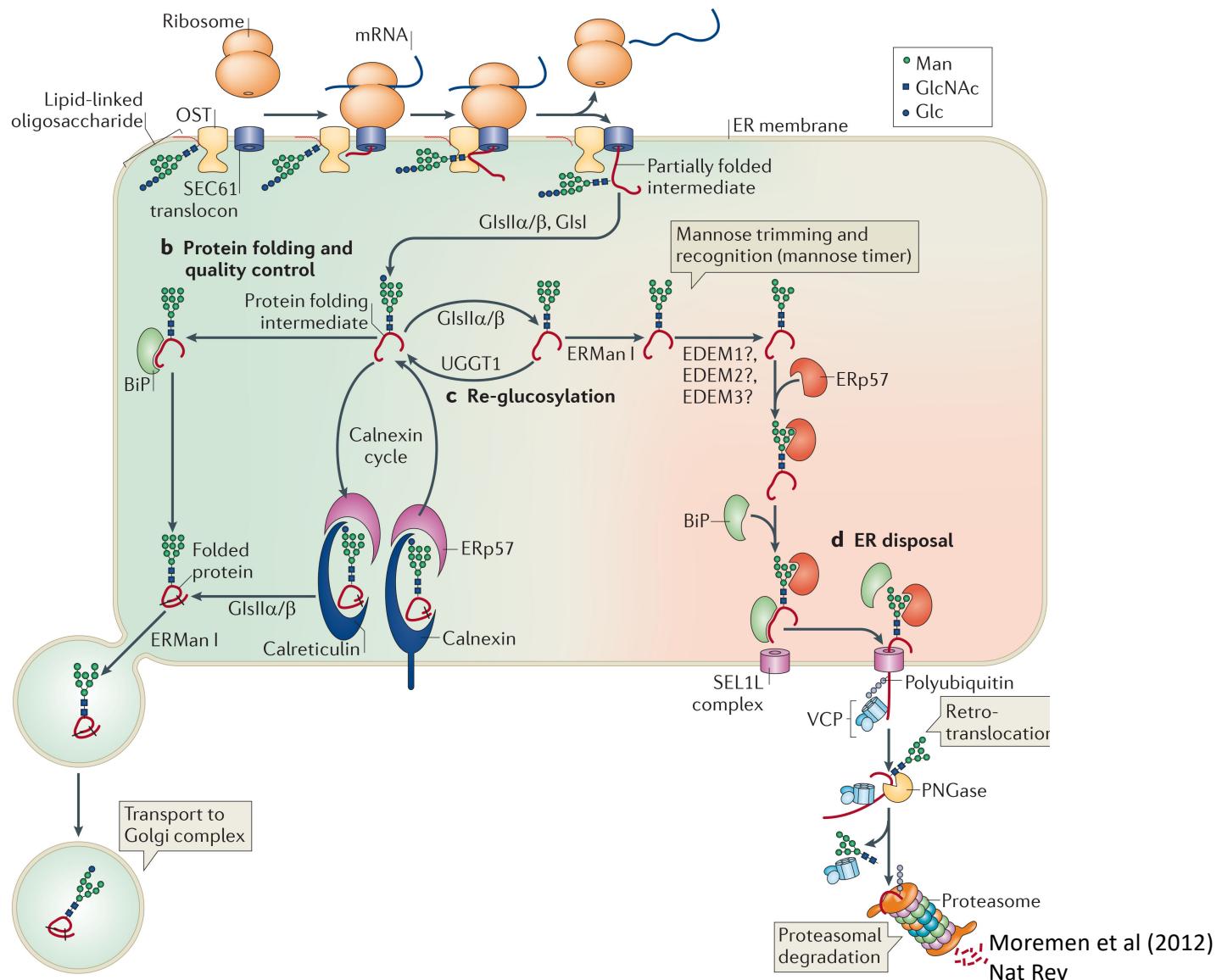
### 3a. N-glycans are large and highly mobile

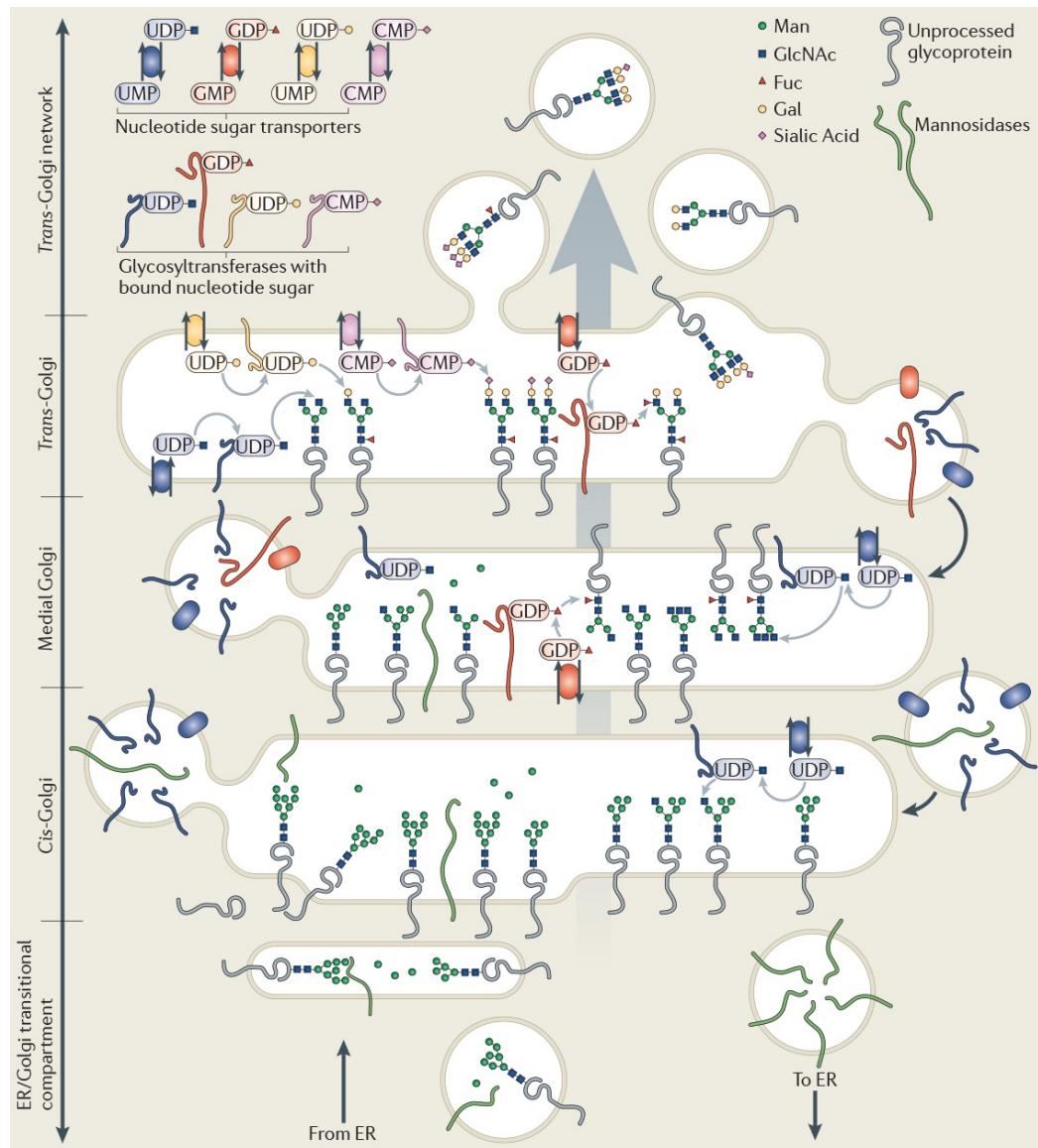
Fc  $\gamma$  receptor 3a / CD16a  
as processed by primary human  
natural killer cells





Varki (2009) Glycobiology

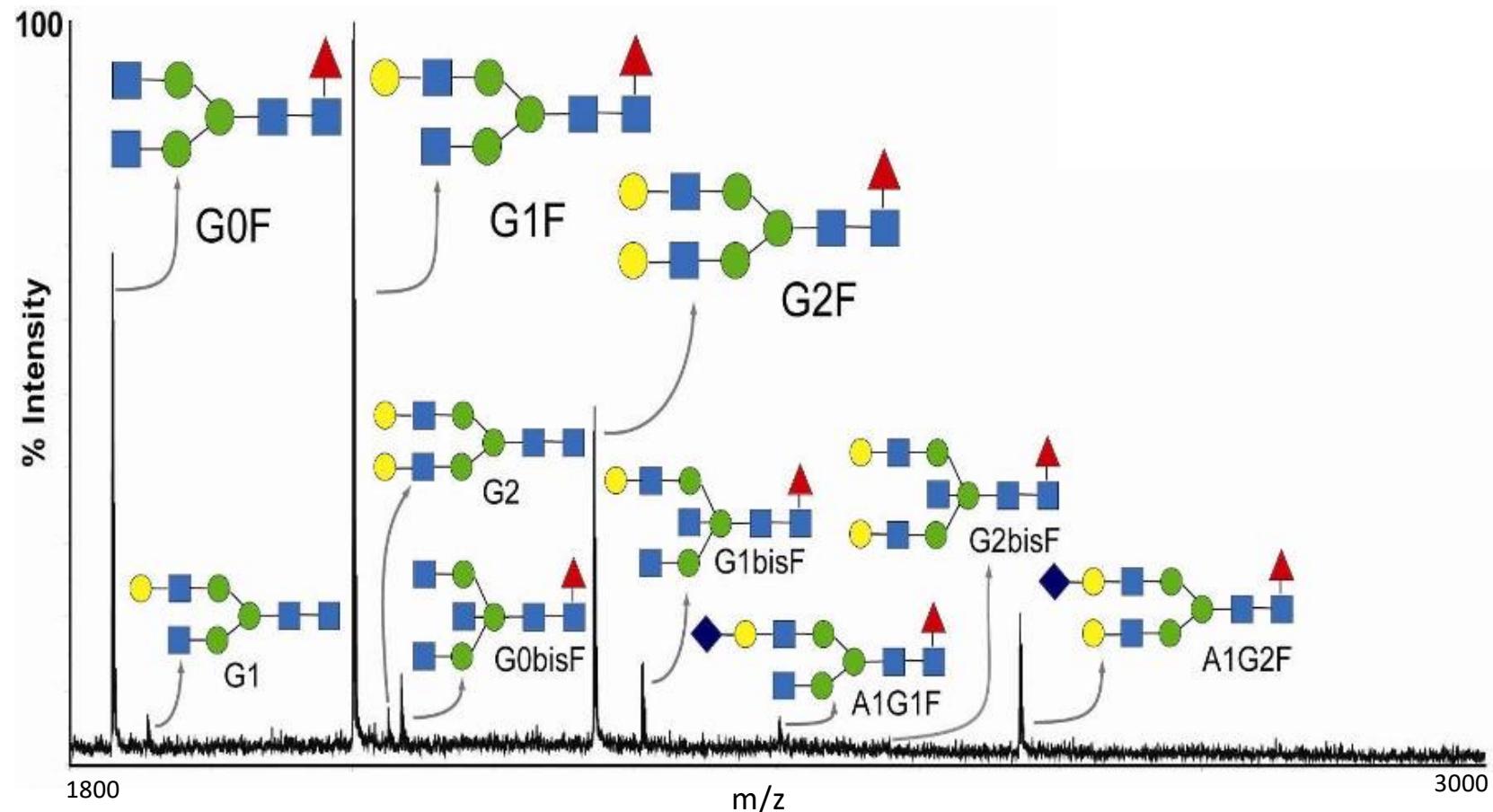




A look at mammalian glycosylation and the secretory pathway

Moremen et al (2012)  
Nat Rev

### 3a. IgG N-glycan heterogeneity



### 3b. Glycoprotein labeling using HEK293

Human embryonic kidney (HEK) 293 cells

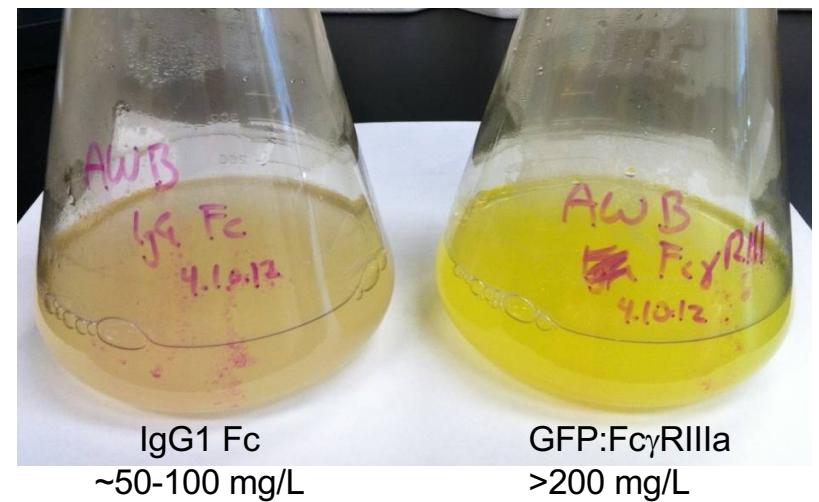
Grown in proprietary Freestyle293 medium (Life Tech)

Supplement Freestyle293 medium (2 g/L  $^{12}\text{C}$ -glucose)  
+2-3 g/L [ $^{13}\text{C}_\text{U}$ ]-glucose

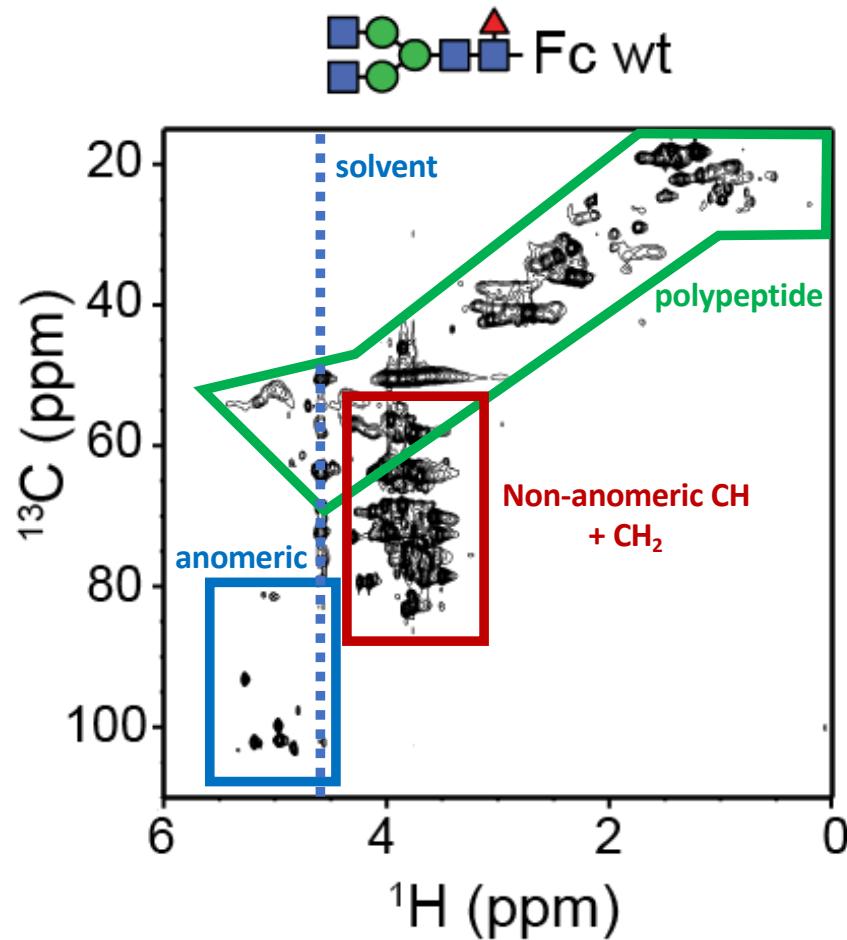
Protein expression following transient transfection

Optional: Custom dropout formulations  
+ 2-5 g/L [ $^{13}\text{C}_\text{U}$ ]-glucose  
+ 100 mg/L [ $^{15}\text{N}$ ]-amino acid(s)

Subedi, Johnson, Moniz, Moremen,  
Barb (2015) J. Vis. Exp. (106) e53568

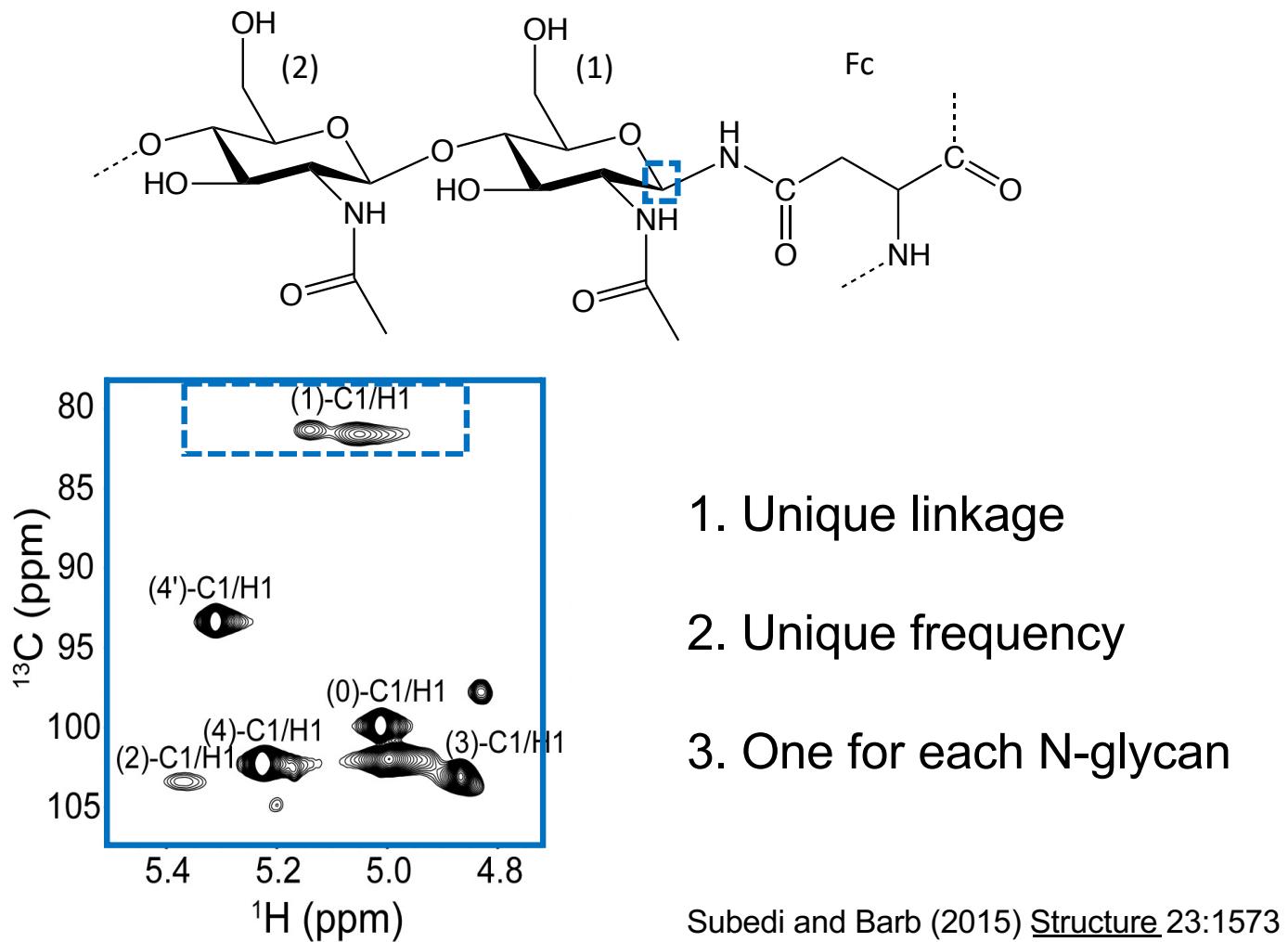


### 3b. [ $^{13}\text{C}_{\text{U}}$ ]-glucose labeling using HEK293



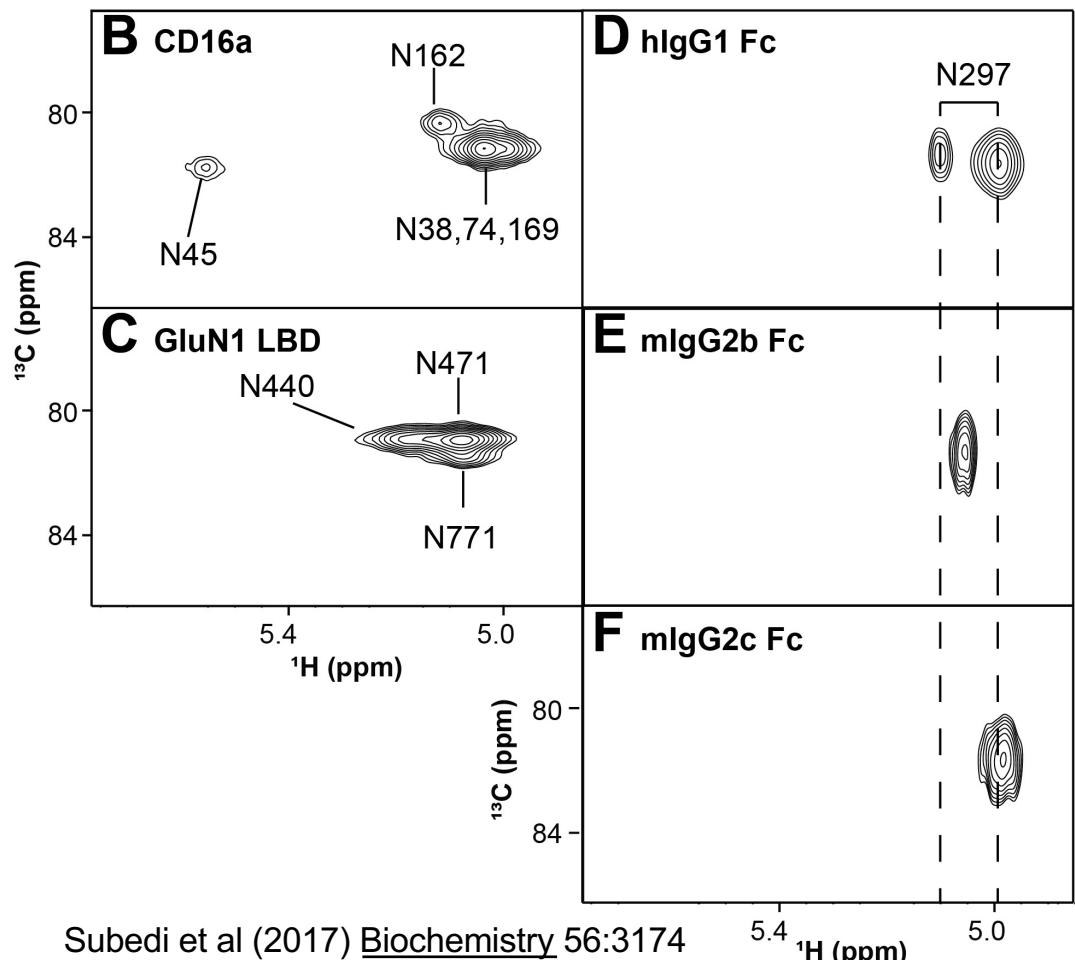
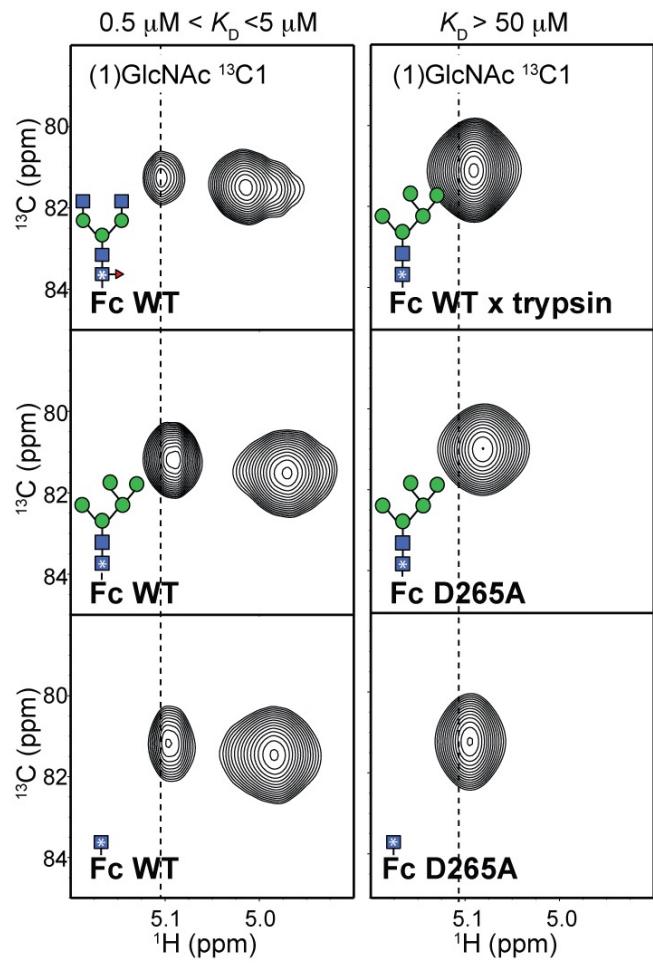
1. high incorporation
2.  $\sim ^{13}\text{C}/(^{12}\text{C} + ^{13}\text{C})$
3. no/minimal  $^{13}\text{C}$  scrambling
4. All N-glycan residues labeled

### 3b. [ $^{13}\text{C}_1$ ]-(1)GlcNAc signals



1. Unique linkage
2. Unique frequency
3. One for each N-glycan

Subedi and Barb (2015) *Structure* 23:1573



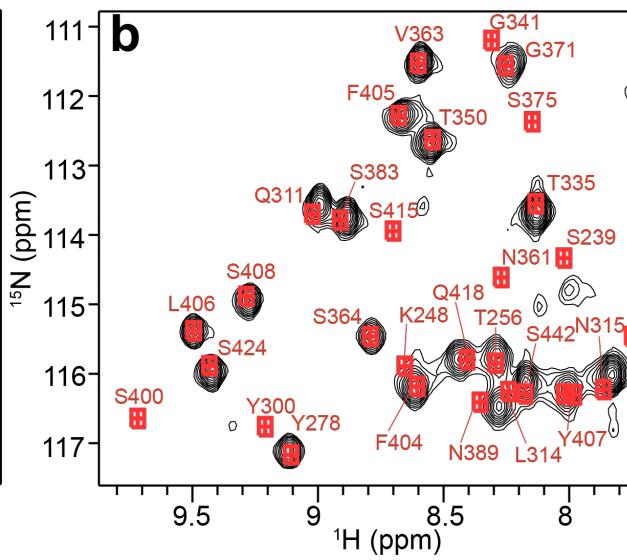
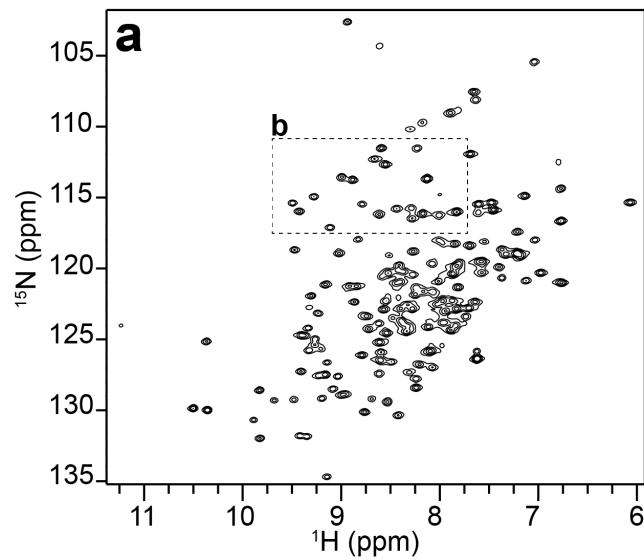
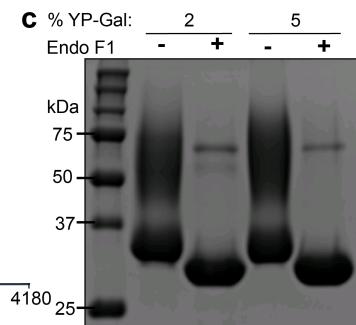
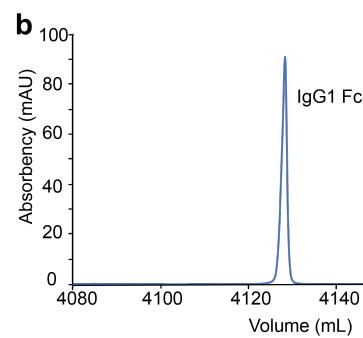
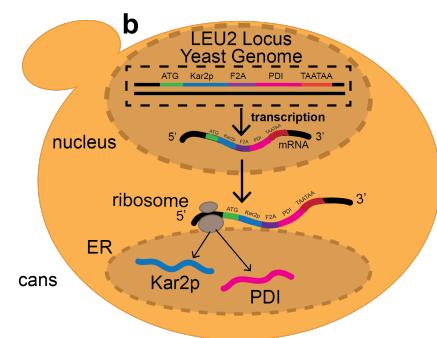
Subedi et al (2017) Biochemistry 56:3174

Subedi et al (2019) Structure 27:55

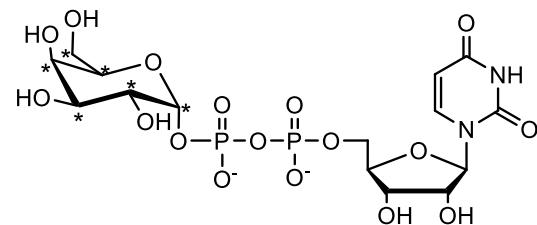
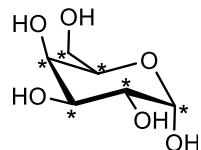
Barb, Falconer, Subedi (2019) Meth Enz 614:239

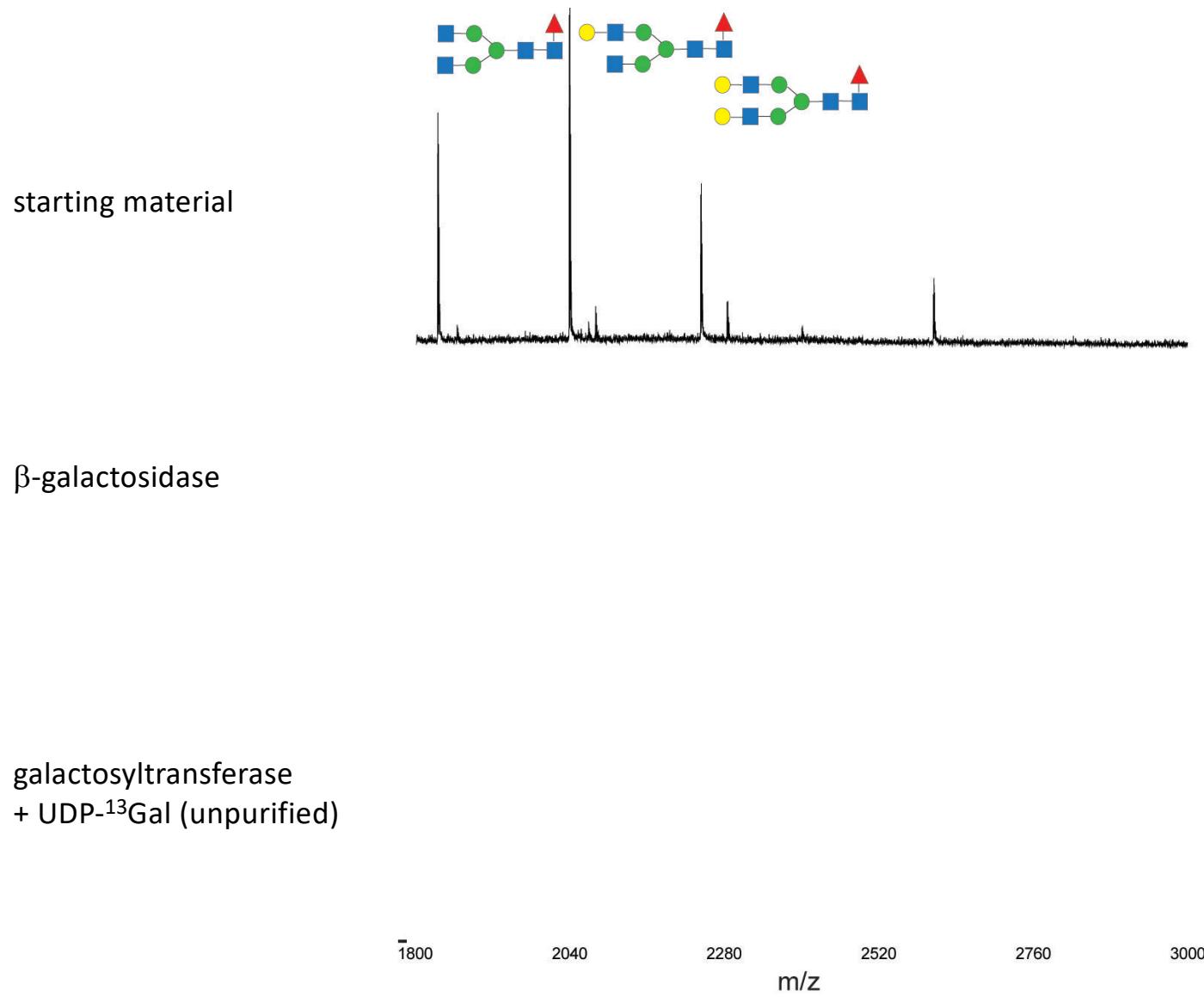
Subedi and Barb (2015) Structure 23:1573

### 3b. Metabolic $^{15}\text{N}$ IgG1 Fc expression in yeast

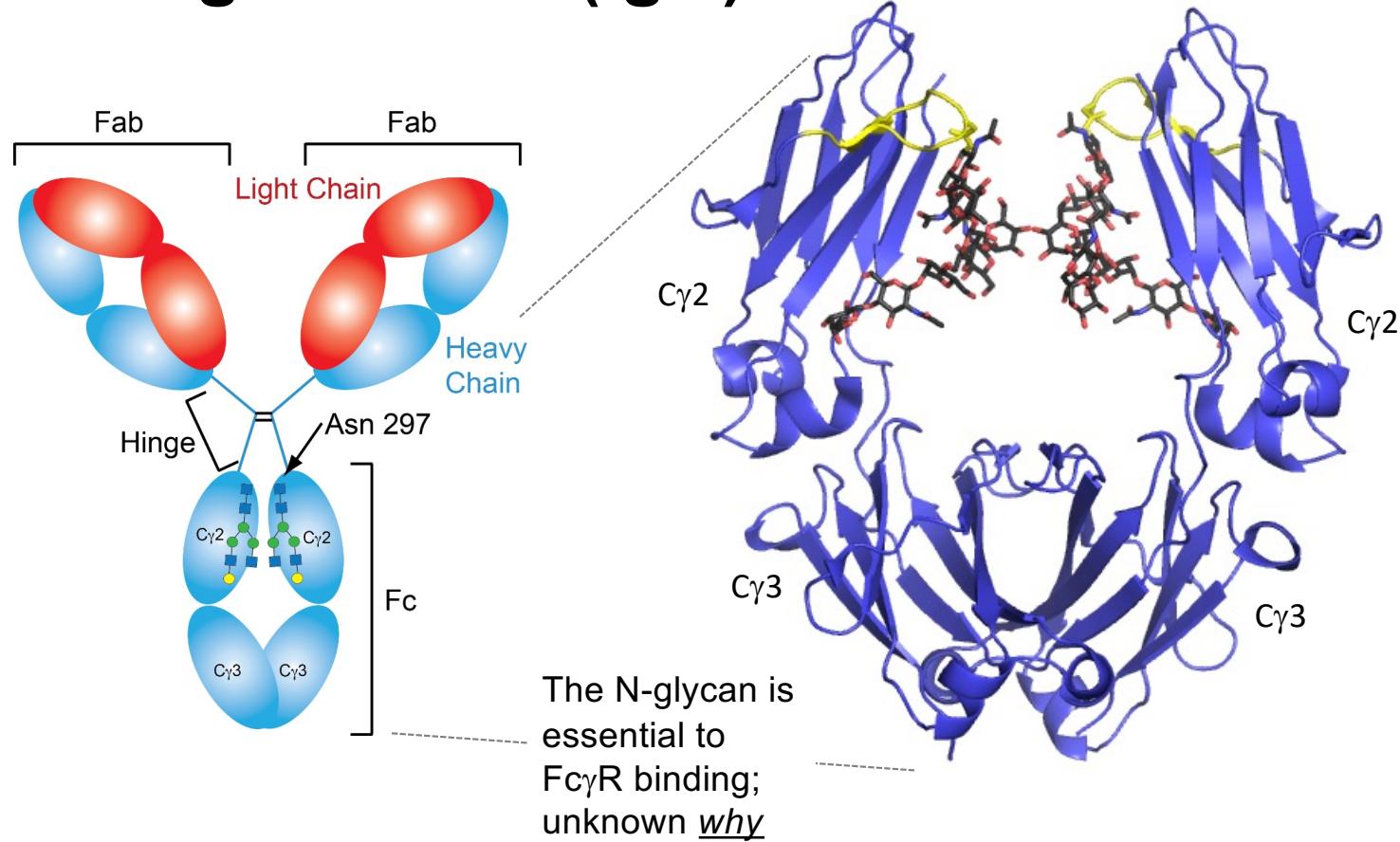


### 3b. Post-purification remodeling the Fc N-glycan with $^{13}\text{C}_{[1,2,3,4,5,6]}$ -galactose



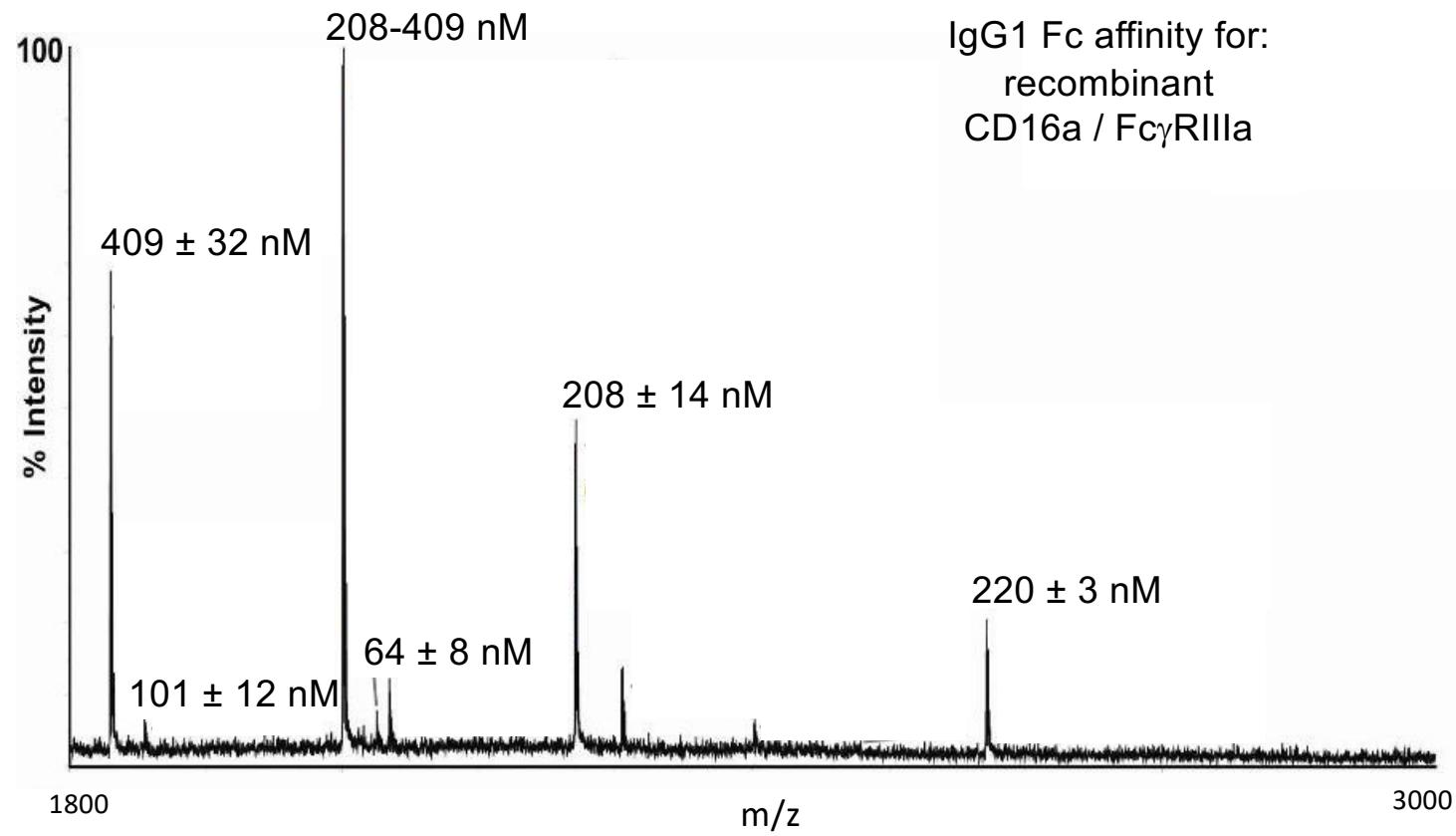


### 3c. Immunoglobulin G (IgG)



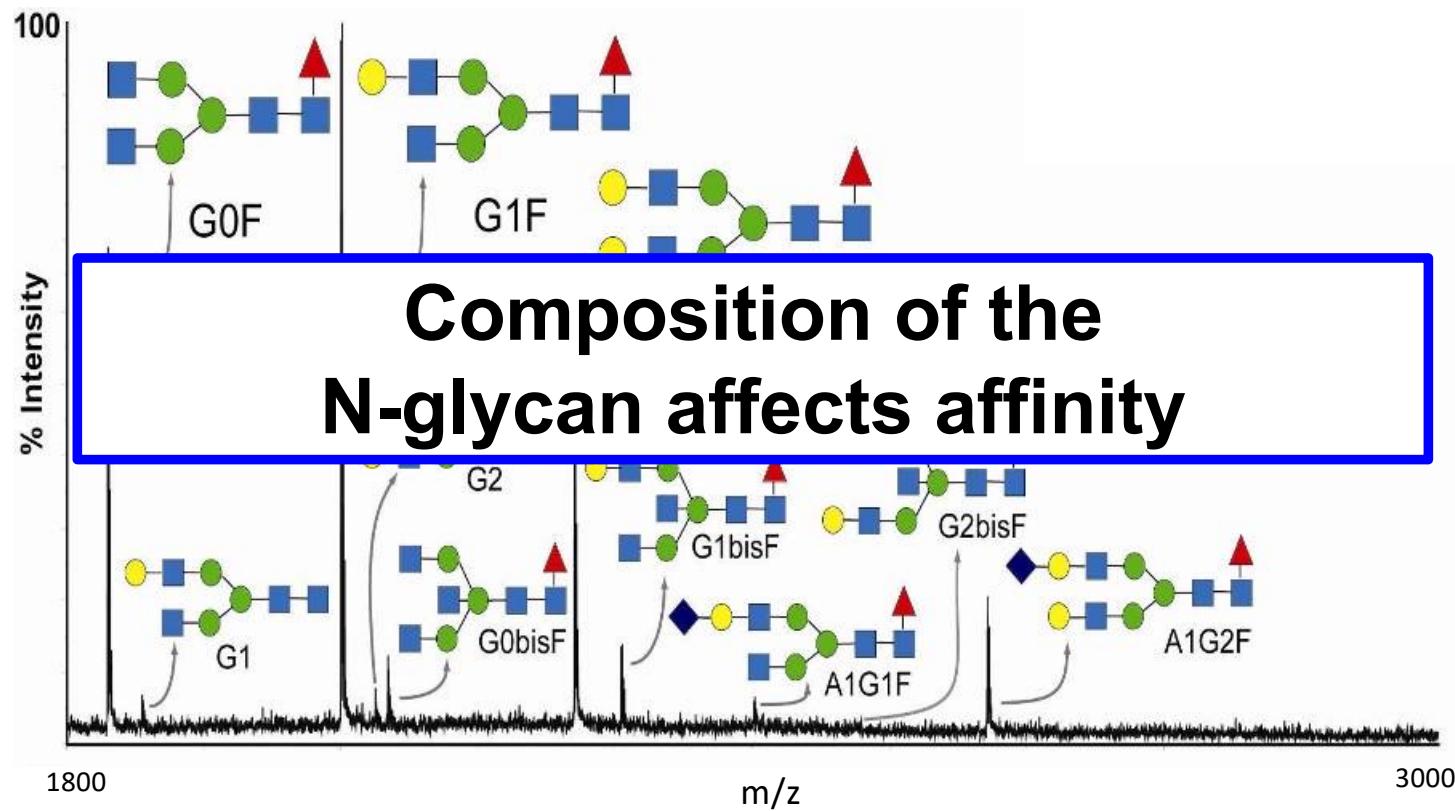
pdb 4ku1: Frank, Walker, Lanzilotta, Prestegard, Barb (2014) *J Mol Biol*

### 3c. IgG Fc N-glycan heterogeneity

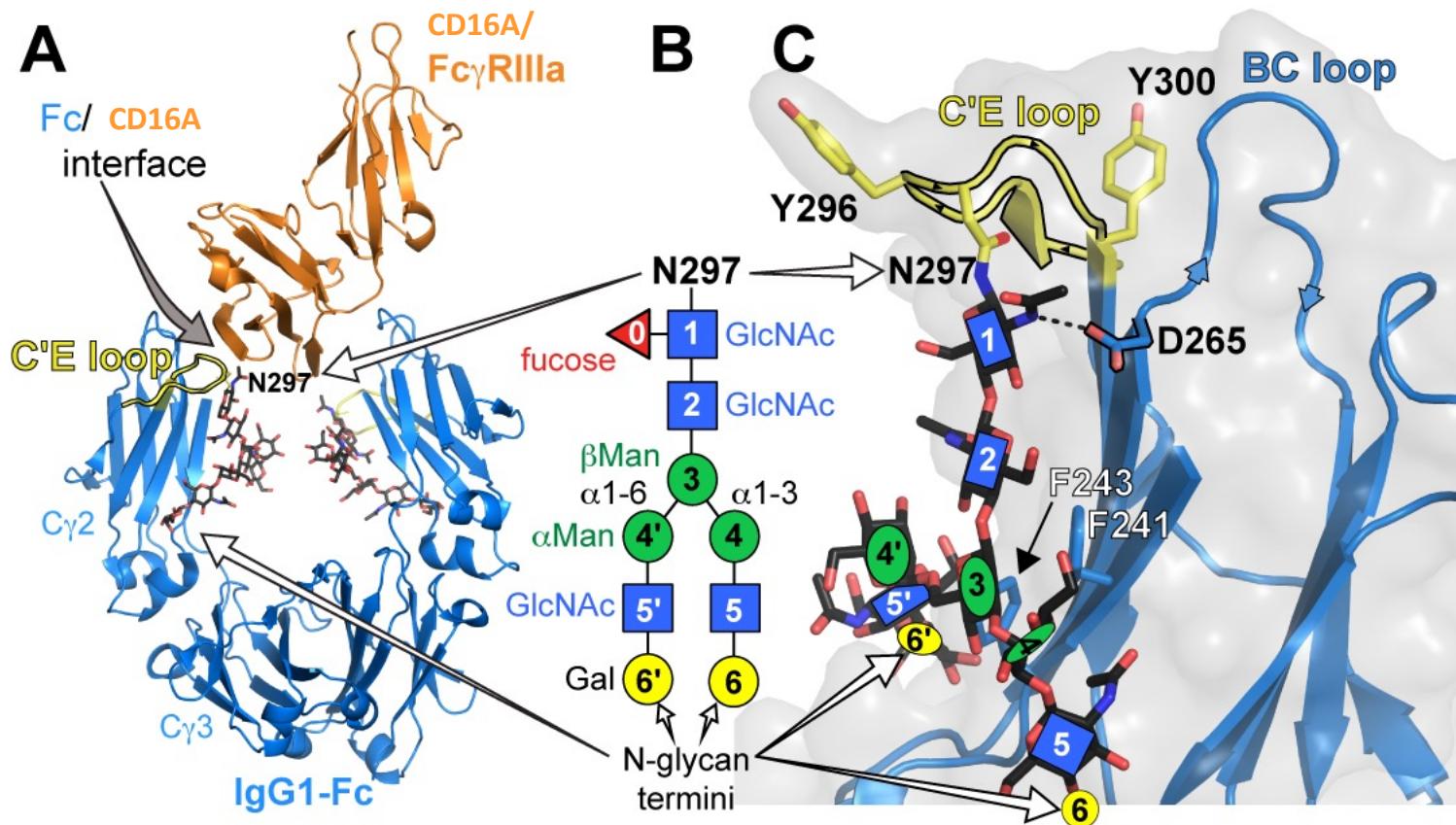


Subedi and Barb (2015) MAbs 8:1512

### 3c. IgG Fc N-glycan heterogeneity

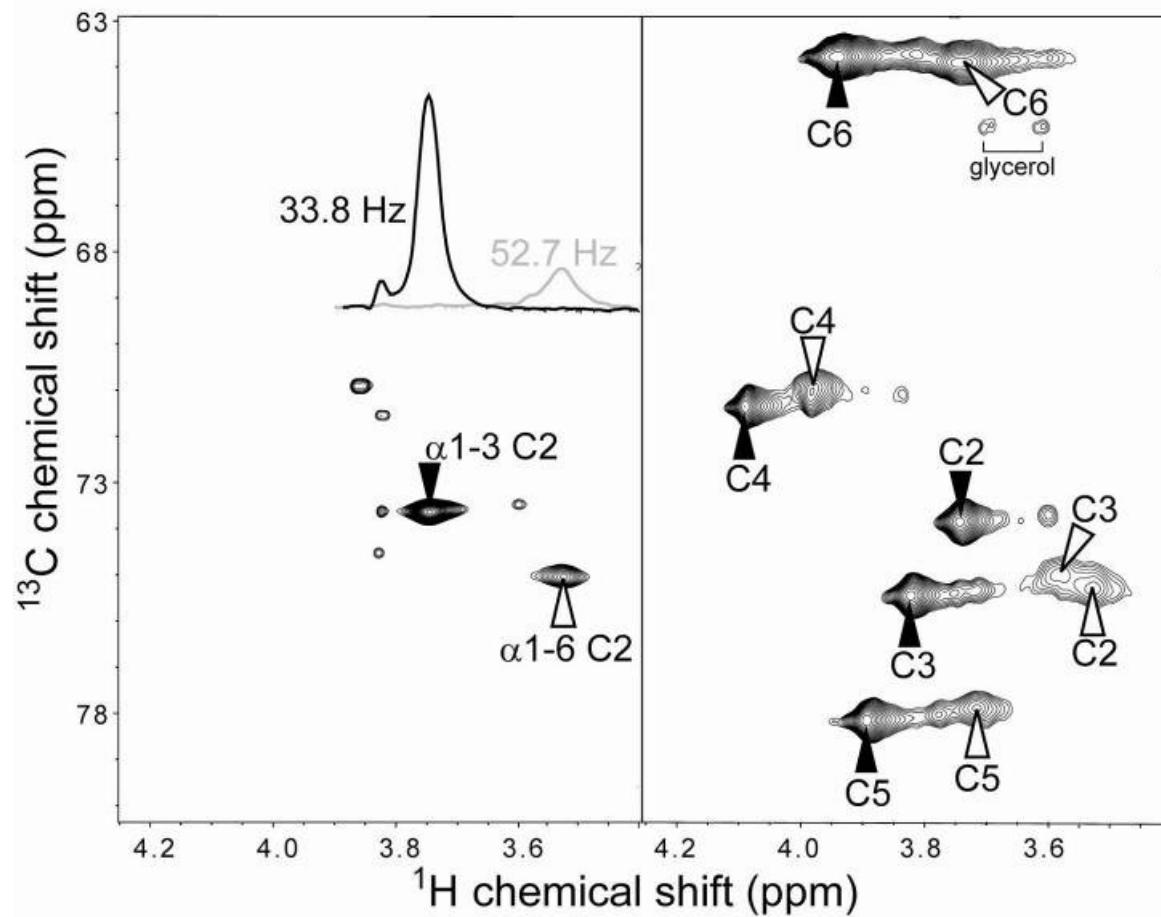
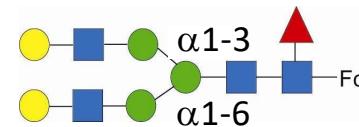


### 3c. The receptor does not bind the Fc N-glycan



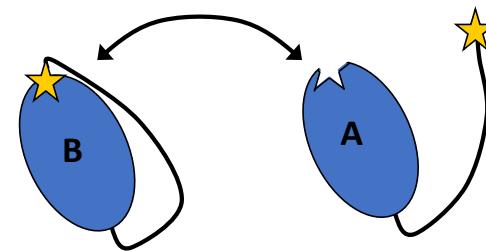
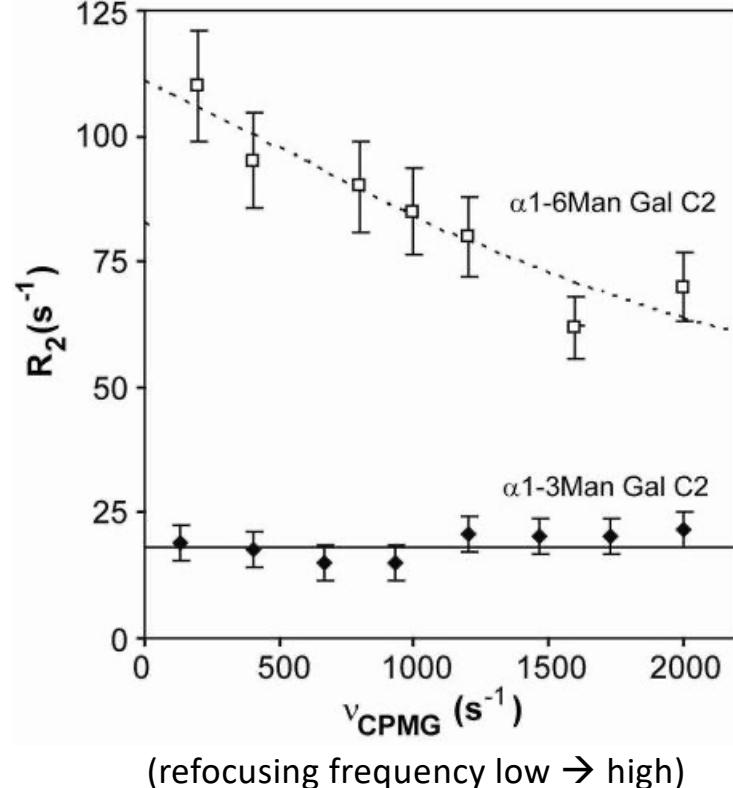
Structure coordinates are from: Sondermann et al (2000) Nature; Mizushima et al (2011) *Genes to Cells*

### 3c. $^{13}\text{C}$ -Gal – labeled IgG Fc



50°C, 900 MHz

### 3c. Quantifying slow glycan motions

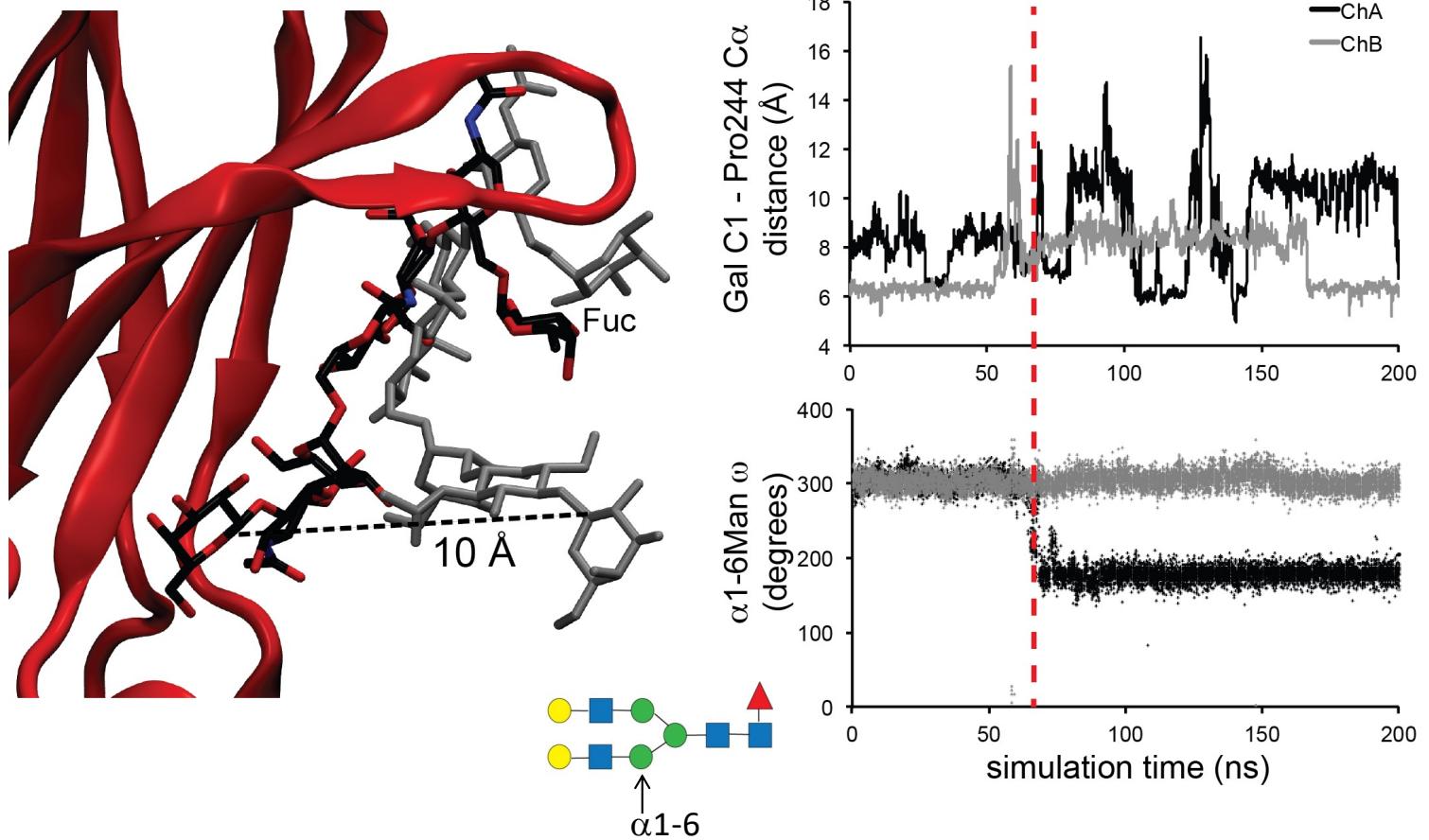


$$R_2(1/\tau_{CP}) = R_2^0 + \frac{\varphi_{EX}}{k_{EX}} \left( 1 - 2 \tanh(k_{EX}\tau_{CP}/2) \right) / k_{EX}\tau_{CP}$$

$$\alpha 1\text{-}6 \quad k_{EX} \sim 5000 \text{ s}^{-1}$$

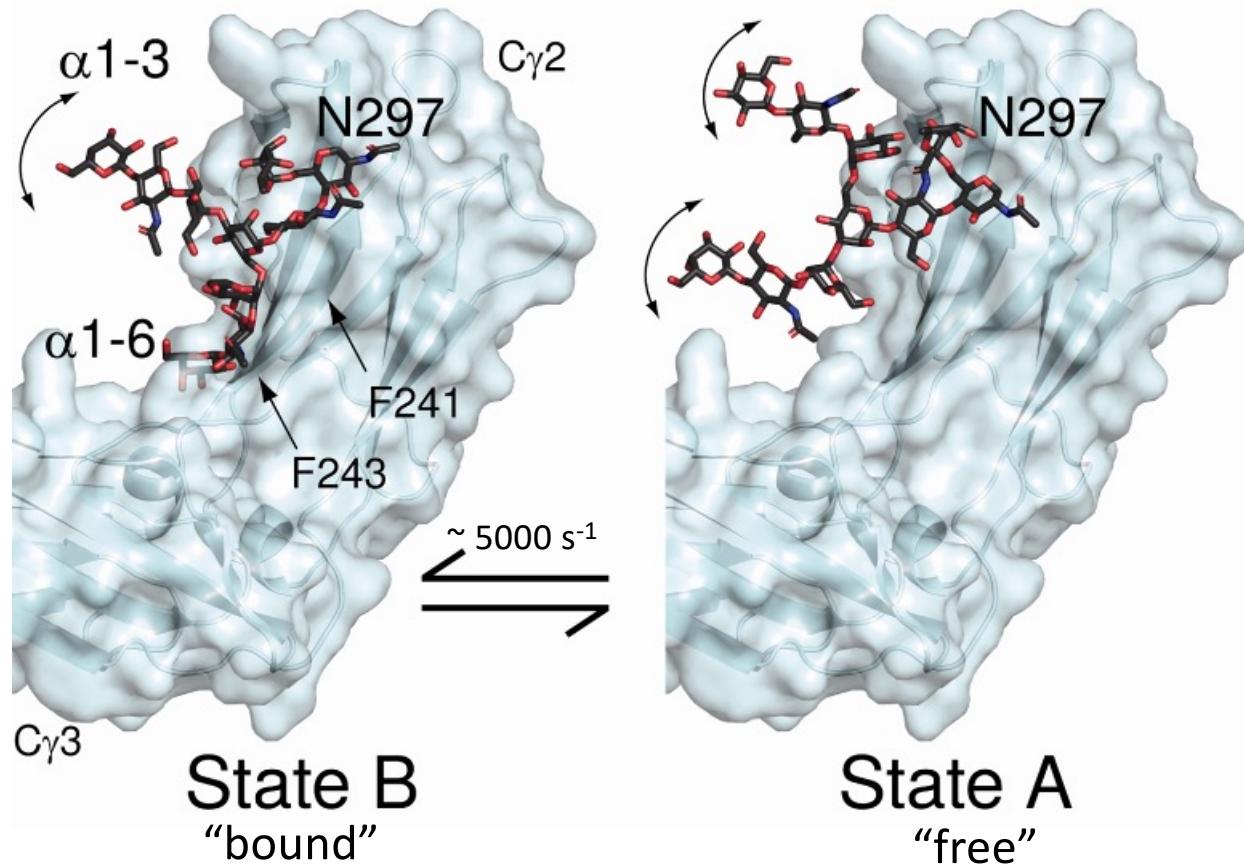
$$\varphi_{EX} = p_A p_B \Delta\omega^2$$

### 3c. MD supports N-glycan motion



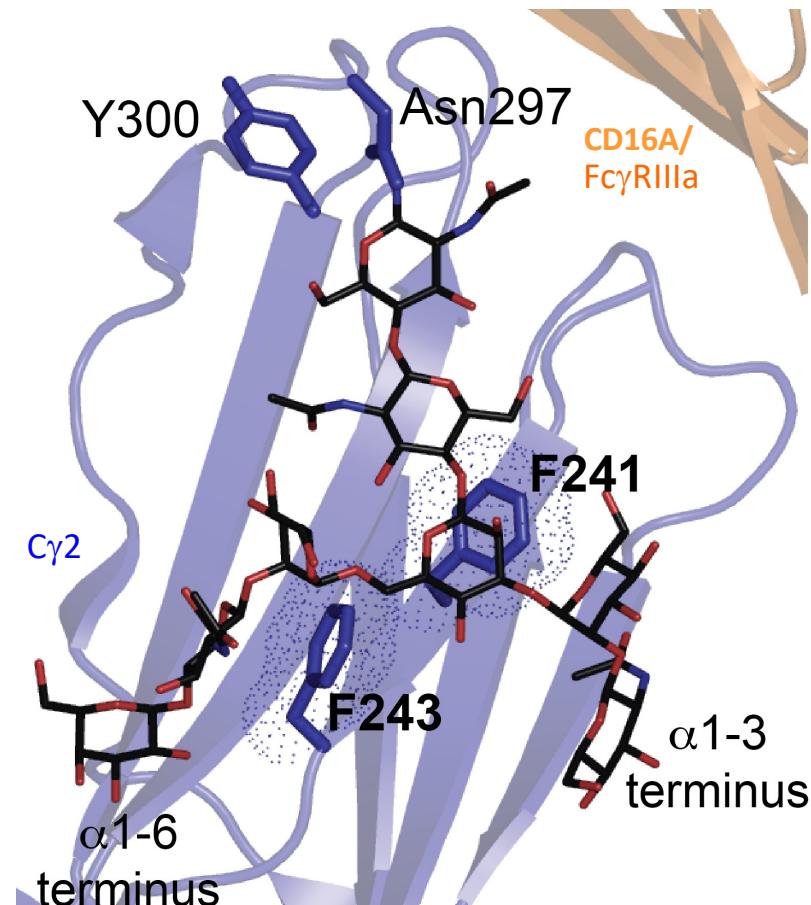
Frank, Walker, Lanzilotta, Prestegard, Barb (2014) *J Mol Biol*

### 3c. The N-glycan termini are mobile: two state model



Barb and Prestegard (2011) [Nature Chem Biol](#)

### 3c. Do interactions stabilize receptor binding?

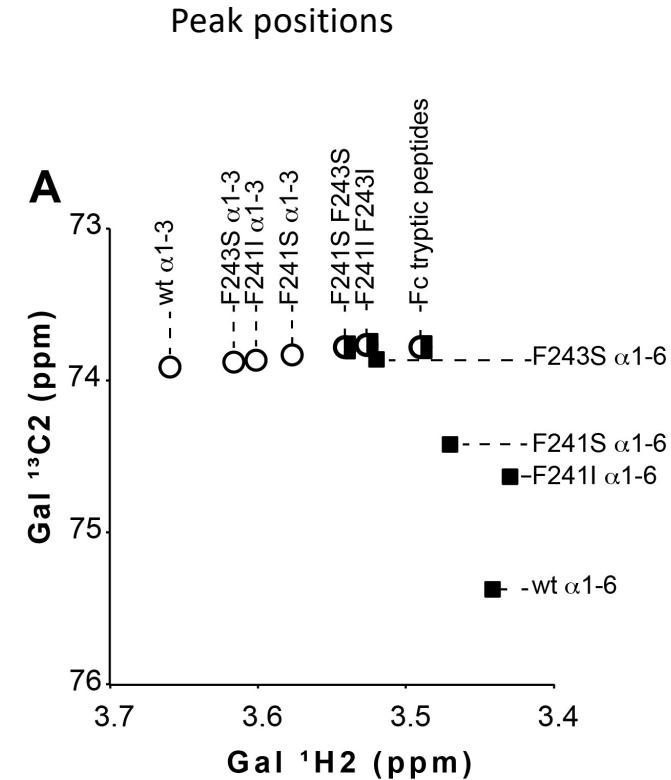
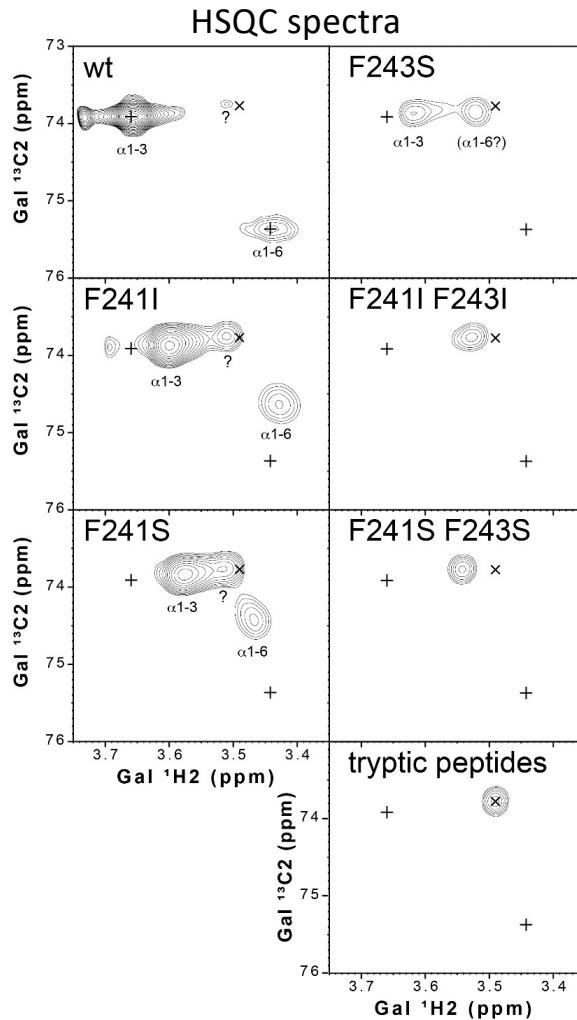


Dispersive interactions  
CH- $\pi$   
Chen et al (2013) JACS

wt  
F241I  
F241S  
F243I  
F243S  
F241I F243I  
F241S F243S  
K246F

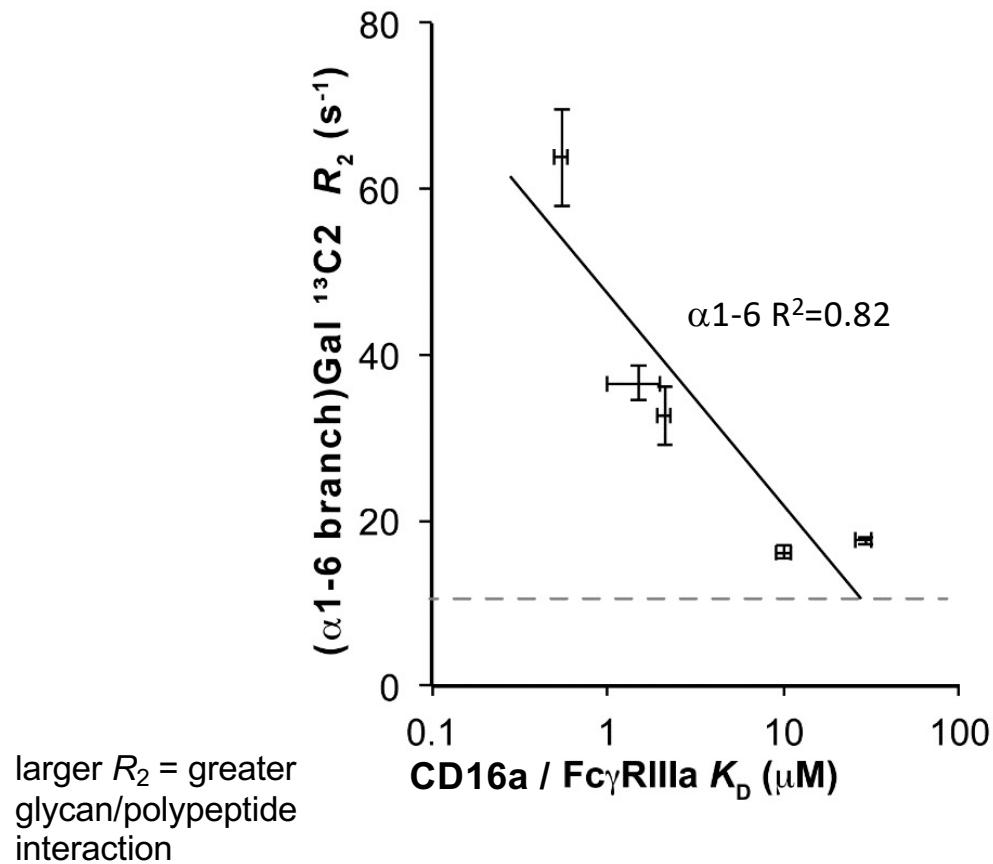
Subedi, Hanson, Barb (2014) *Structure* **22**:1478

### 3c. Fc mutations alter Gal C2 resonances



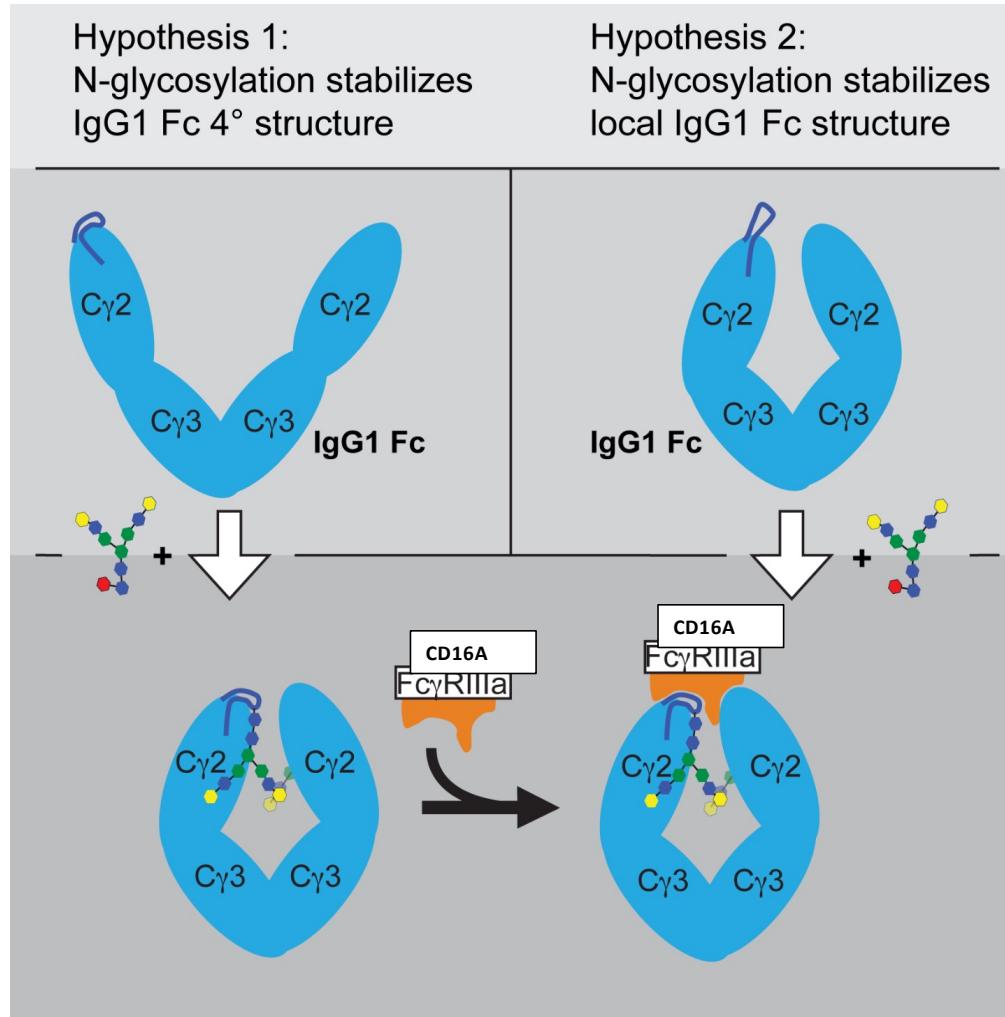
Subedi, Hanson, Barb (2014) *Structure* **22**:1478

### 3c. Direct correlation between N-glycan restriction and CD16a affinity

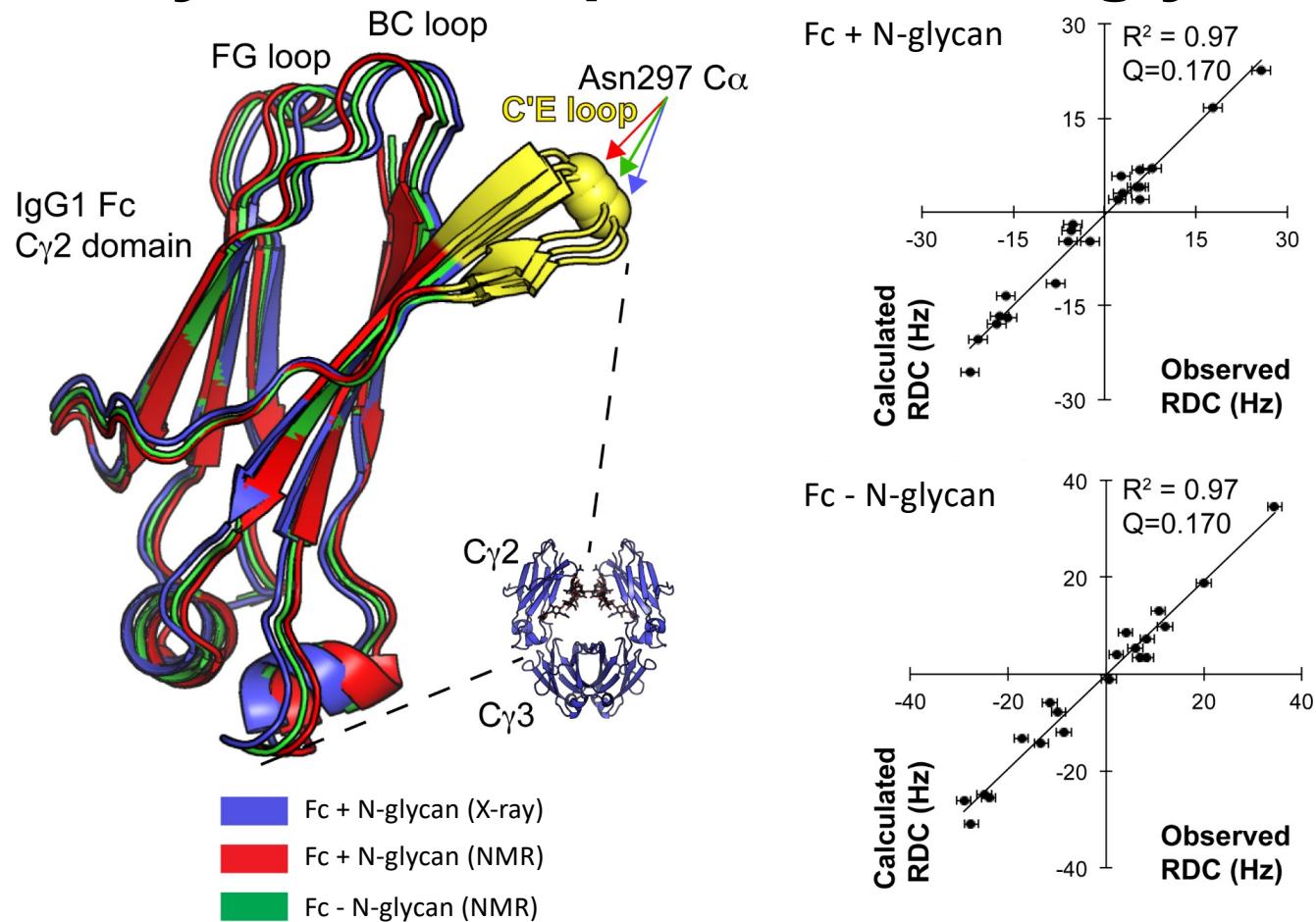


Subedi, Hanson, Barb (2014) Structure **22**:1478

### 3c. Why is IgG1 Fc N-glycosylation required?

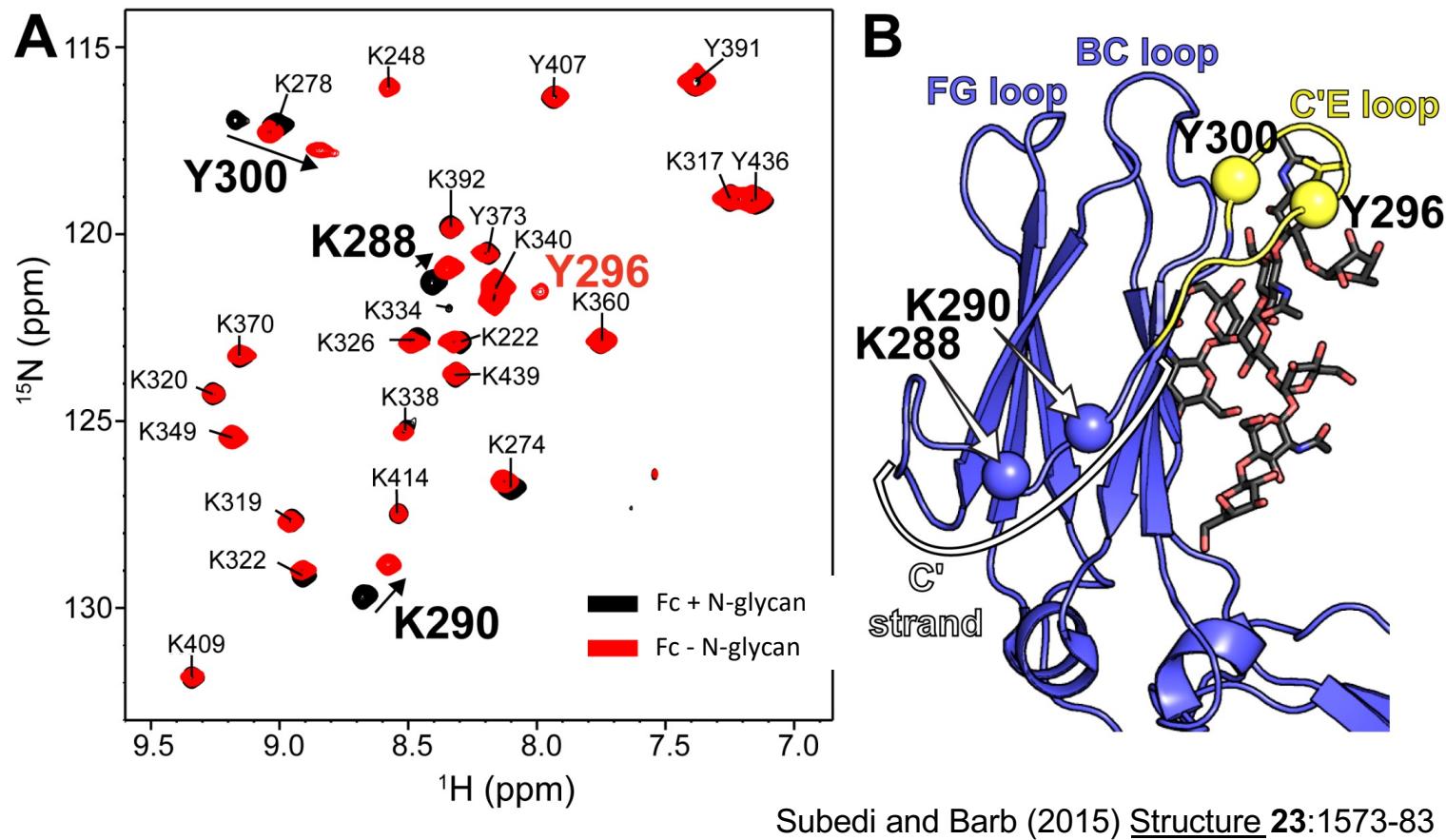


### 3c. Quaternary structure preserved in aglycosylated Fc



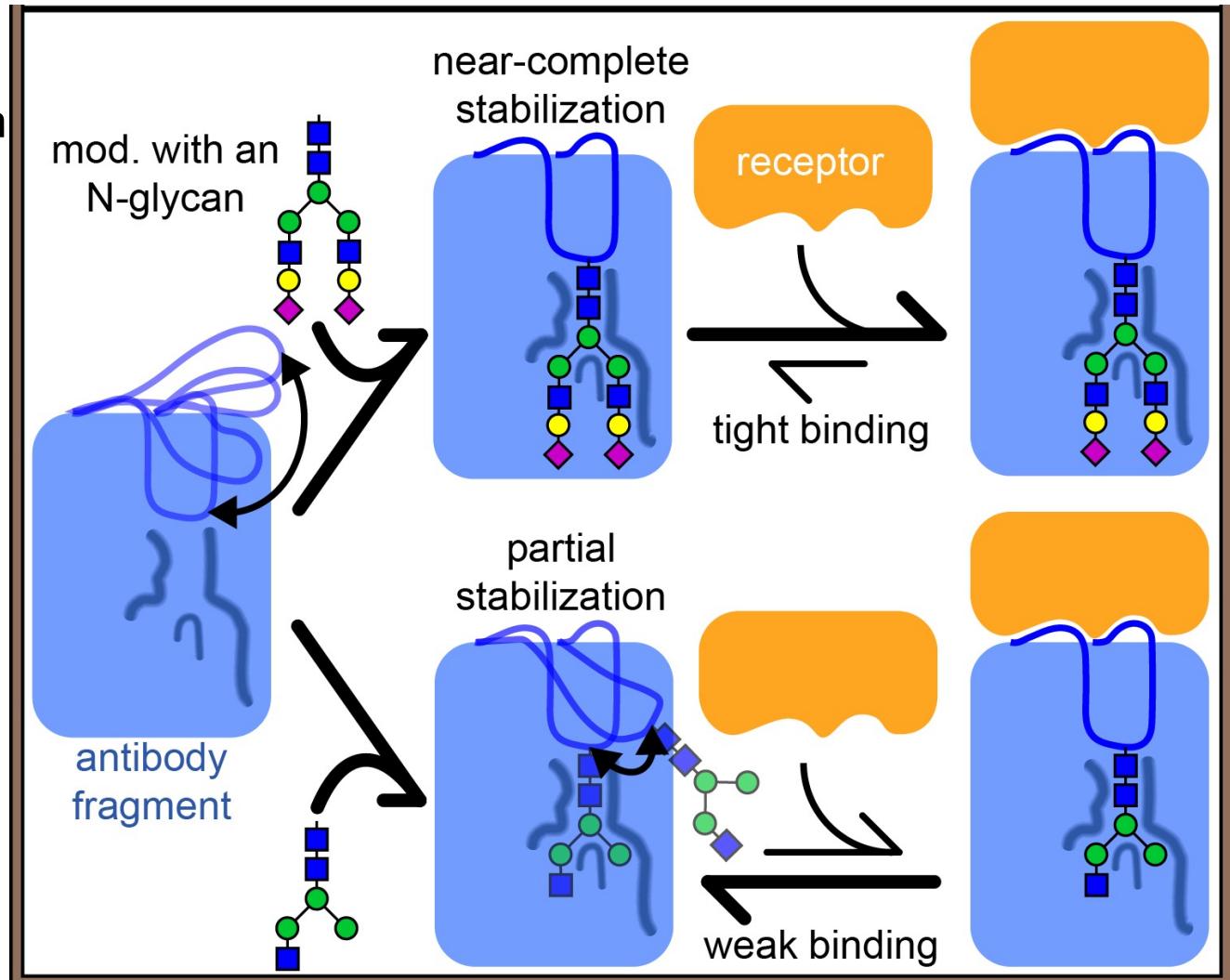
Subedi and Barb (2015) *Structure* 23:1573-83

### 3c. Glycosylation stabilizes the Fc C'E loop



Hypothesis: The composition of the post translational modification impacts protein structure and function

Lessons from IgG1 Fc  
Fc N-glycans stabilize a loop to increase affinity for CD16a



# **Outline; three videos**

## **1. Carbohydrates**

- Biology
- Structure
- Chemistry (linkages)

## **2. Oligosaccharide NMR features**

- Weak scalar coupling over glycosidic bond
- Karplus relationship provides relative stereochemistry
- NOE across glycosidic linkage
- Monosialyl Fc example

## **3. Glycoprotein NMR**

- Labeling challenges
  - Post purification remodeling
  - Metabolic labeling
- N-glycan NMR features
- IgG1 Fc

# Learning Objectives

- Describe the unique chemical features of carbohydrate mono- and poly-saccharides
- Utilize scalar coupling relationships to achieve stereochemical assignments of non-exchangeable carbohydrate proton resonances
- Define different types of biological glycoconjugates
- Compare and contrast different glycoprotein stable isotope labeling strategies
- Develop new approaches to extend the capabilities of glycoprotein NMR in solution

