Protein modifications
Basics and engineering of N- (and O-linked) protein glycosylation

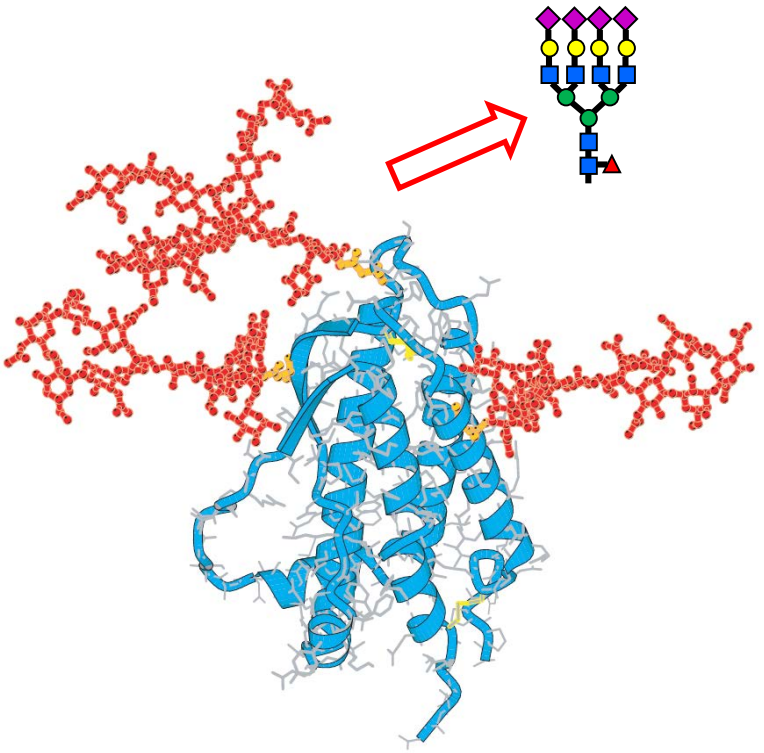
Lecture 5

Protein glycosylation

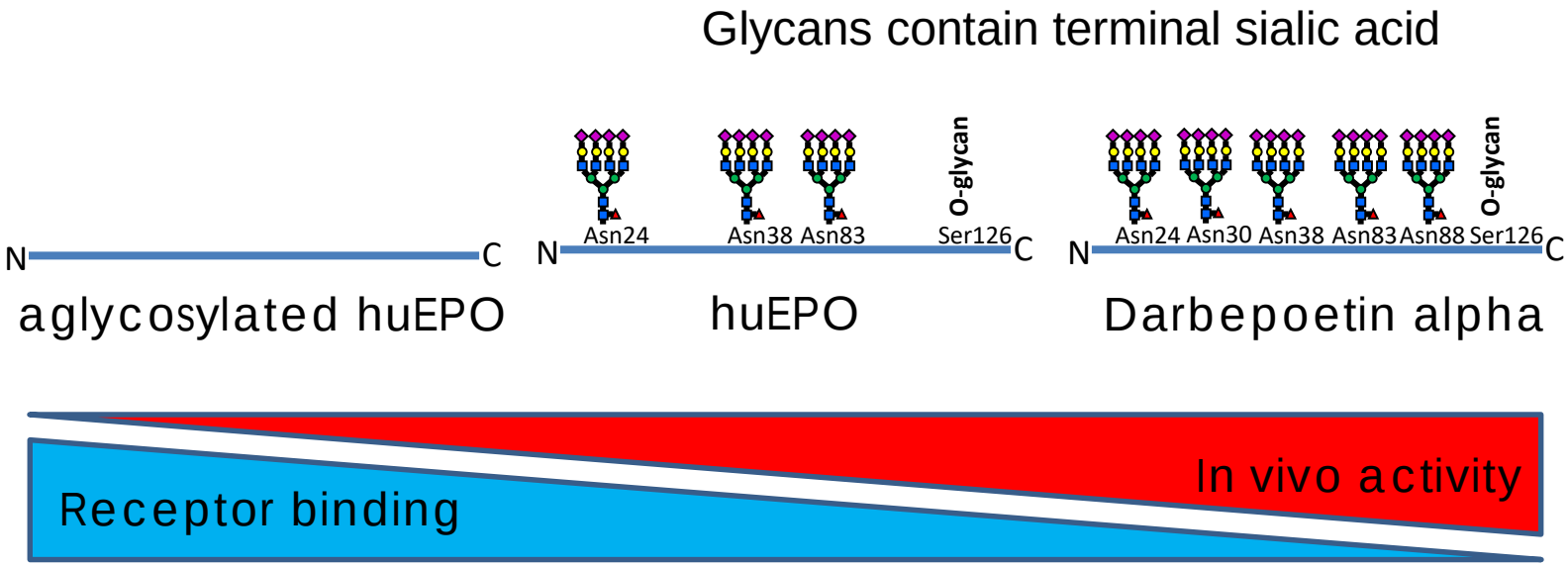
- Glycosylation is an essential post-translational modification in eukaryotes, serving many biological functions
  - recognition
  - signaling
  - protein folding
  - quality control
  - protects against protein aggregation, proteolytic degradation as well as enhancing thermal stability
- Protein O- and N-glycosylation
- In contrast to the protein sequences, modifications are not encoded in the genome and no template exists!

Erythropoietin (EPO)

- essential survival and growth factor
- 165 A.a. yielding a protein of 30.4 kDa
- 40% of total molecule mass are made of N-glycans
- Glycans are not required for binding to target receptor
- Glycosylation pattern depends on expression host

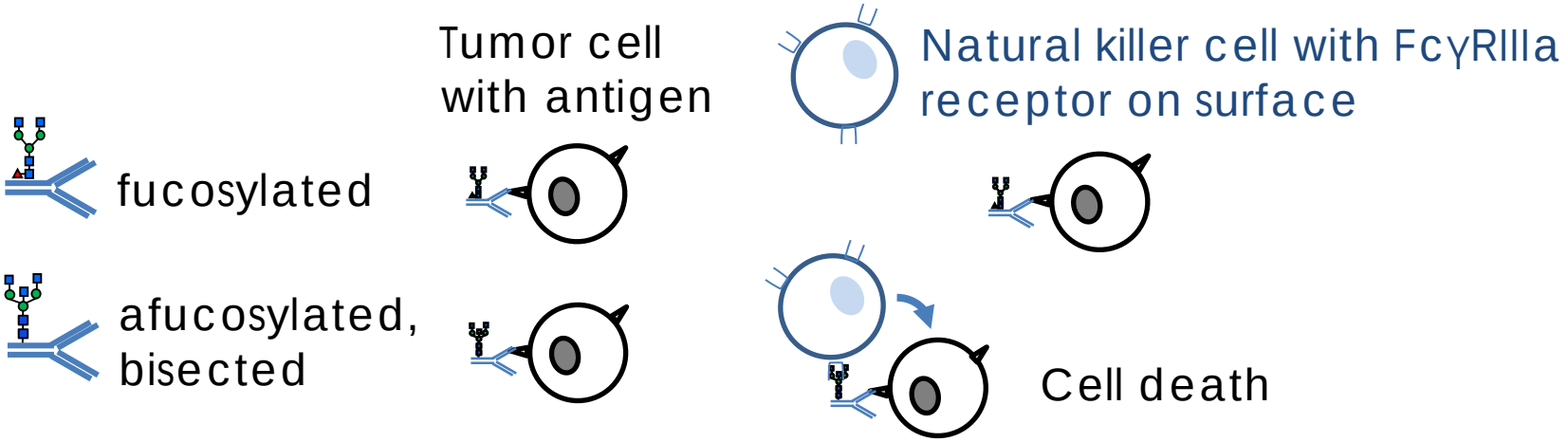


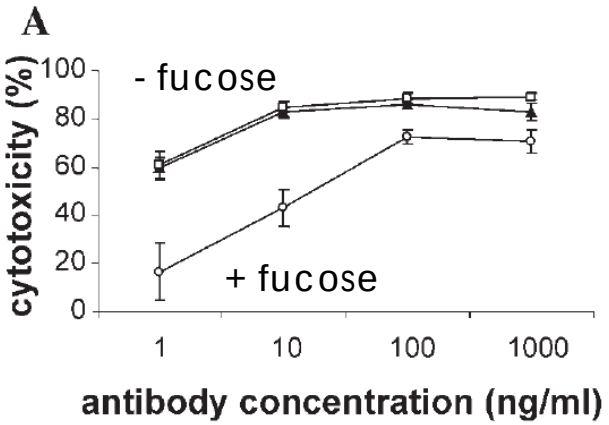
N-glycan composition and N-glycan number modulate EPO activity



- EPO half-life is controlled by sialic acid content of N-glycan
  - By adding additional N-glycosylation sites, activity could be increased
- Glycoengineering of EPO allows to improve of therapeutic potency

Type of N-Glycan structures affects efficacy of anti-tumor antibodies





Absence of fucose increases potency of antibody to kill tumor cells!

Ferrara et al., 2006

Antibody engineering technology created new business with 140 employees

Roche will pay approximately 235 million Swiss francs in cash in exchange for all of GlycArt's outstanding capital stock. The transaction is expected to close in the third quarter of 2005.

About us

The mission of the Roche Innovation Center Zurich is to be a leader in developing new generations of engineered antibody products with increased efficacy that address unmet clinical needs. The Center is also one of the antibody engineering power-houses within the Roche Group.

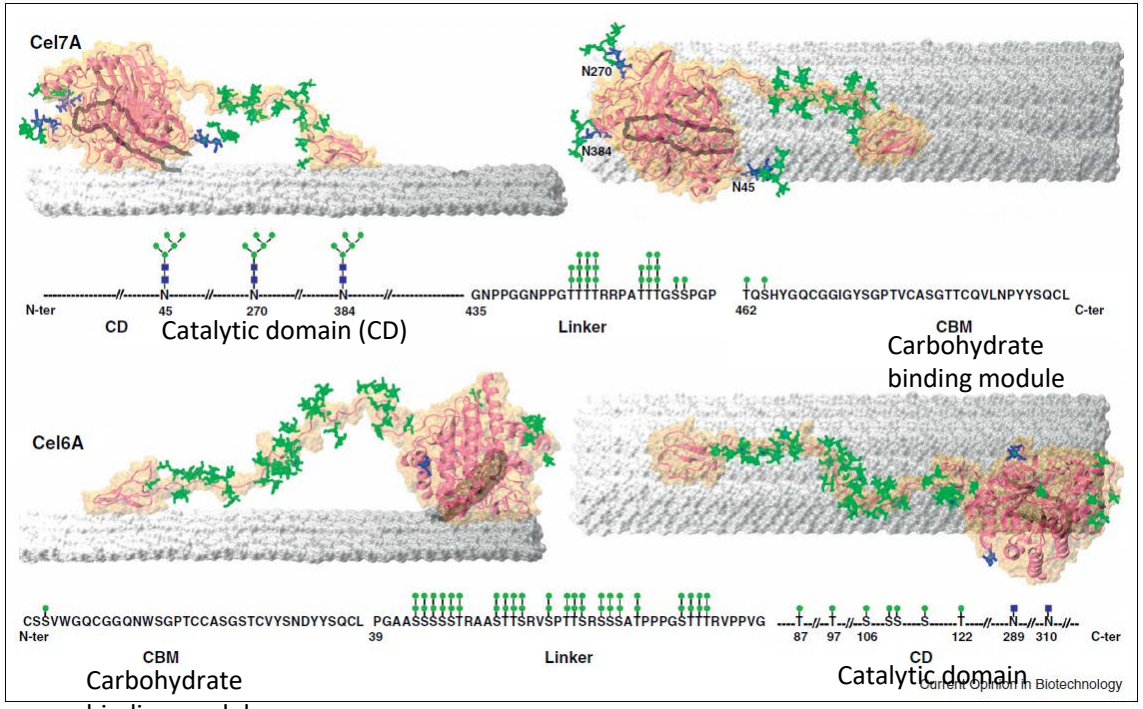
Glycart Biotechnology was established in September 2000 as a spin-off of the Swiss Federal Institute of Technology (ETH) in Zurich, Switzerland. In the summer of 2005, the young biotech company was acquired by the Roche Group.

The state-of-the-art research complex headquartered in Schlieren, Switzerland, is now an integral part of Roche Pharmaceutical Research and Early Development. The Roche Innovation Center Zurich is one of seven Roche Pharma Research and Early Development Innovation Centers. It boasts a multinational team of some 140 employees – 95% of whom are research scientists – with a dynamic, thriving biotech culture. The Roche Innovation Center Zurich is headed by Pablo Umaña, Ph.D., and comprises research teams in diverse fields as diverse as molecular biology, cell and protein engineering, process biochemistry, cell biological assays, histology, preclinical pharmacology and tumor immunology. The teams belong to global functions such as Large Molecule Research, Oncology Discovery and Translational Area, and Infectious Diseases Discovery.

The Roche Innovation Center Zurich focuses on product development – from scientific concepts and validation to the corresponding studies. Today the compounds studied and developed are boosting Roche's significant commitment to offering improved cancer medicines.

Cellobiohydrolases from *T. reesei*

- Enzymes catalytically engaged on a cellulose crystal and shown with N-glycans and O-glycans.



Beckham et al 2012 **Harnessing glycosylation to improve cellulase activity.**

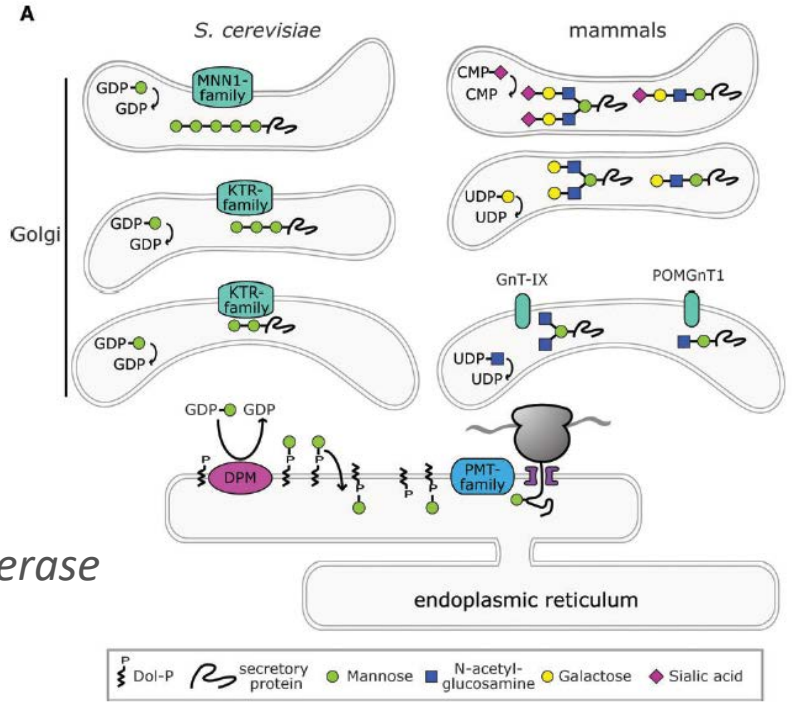
Reported effects of protein glycosylation on cellulase

- **Protection** of exposed linker **from proteases**
- Cellulase **glycosylation varies** with **heterologous expression** and **environmental conditions**, with concomitant **changes to binding affinity and activity**
- *T*. *reesei* Cel7A expressed in *Aspergillus niger*, resulted in slightly more O-glycan on the CBM and linker, and a 6-fold increase in the N-glycans on the CD. This led to **reduced activity** on crystalline and amorphous cellulose relative to Cel7A expressed in *T*. *reesei*, but a **higher binding affinity** of the recombinant enzyme to both substrates. (Jeoh et al.)
- Voutilainen et al. conversely found that expression of *Talaromyces emersonii* Cel7A in *Saccharomyces cerevisiae* resulted in **attachment of longer, stabilizing N-glycans to the CD**. They attribute the **improvement in stability** (t1/2 improved 2-fold) to the additional N-glycans. The presence of the additional N-glycans were accompanied by a 30% improvement in activity on Avicel
- Gupta et al. showed that **deglycosylation** of *T*. *reesei* Cel7A leads to a **reduction in solubility**

Biosynthesis of *O*-glycans in eukaryotes

- The initial steps in the ER are conserved between fungi, animals, and humans.
- Dolichol-P-Mannose is synthesized on the cytosolic face of the ER membrane and flip-flops into the ER lumen.
- Mannose is transferred to proteins entering the secretory pathway by the members of the protein O-mannosyltransferase (PMT) family.
- Diversification occurs in the Golgi apparatus where further chain elongation takes place

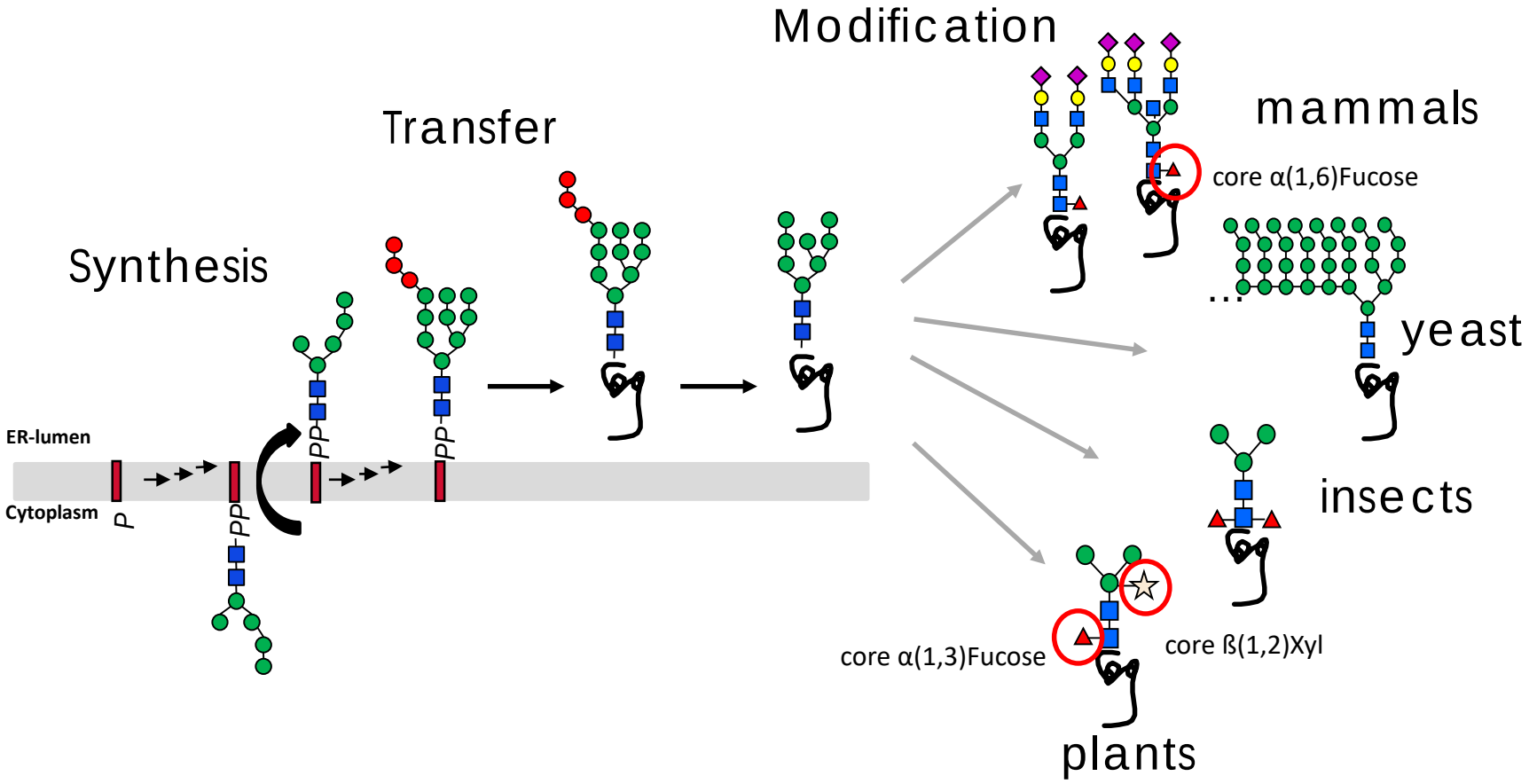
Biosynthesis of *O*-mannosyl glycans in eukaryotes



Protein O-mannosyltransferase (PMT) initiates process

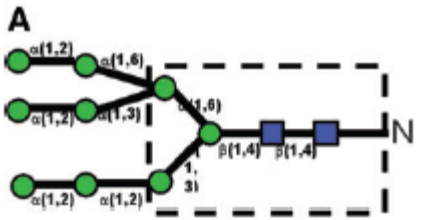
- O-glycosylation occurs at the b-hydroxyl group of serine or threonine
- No sequence motif know, which is required for attachment
  - Extent of O-glycosylation cannot be controlled
  - Structures differ among eukaryotes and have been modified

Biosynthesis of *N*-glycans in eukaryotes

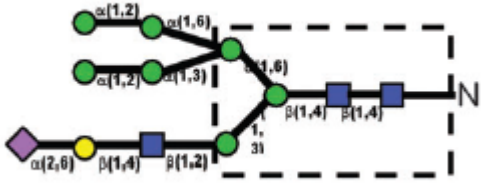


1. Synthesis of lipid-linked oligosaccharide (LLO) and transfer onto protein are conserved process among eukaryotes
2. Modification of N-glycans in Golgi creates structural diversity

Terminology of N-glycan structures

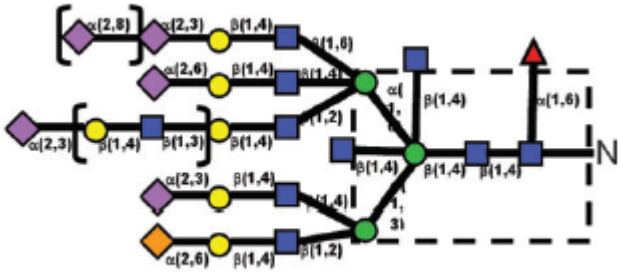


High Mannose

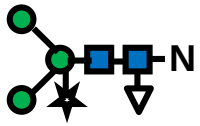


Hybrid

Hybrid structures are intermediates between high mannose and complex forms

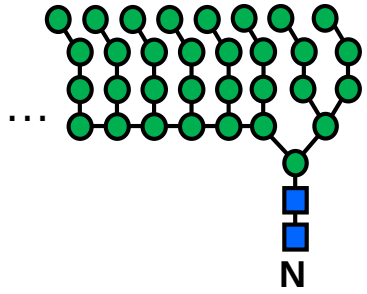


Complex



paucimannose

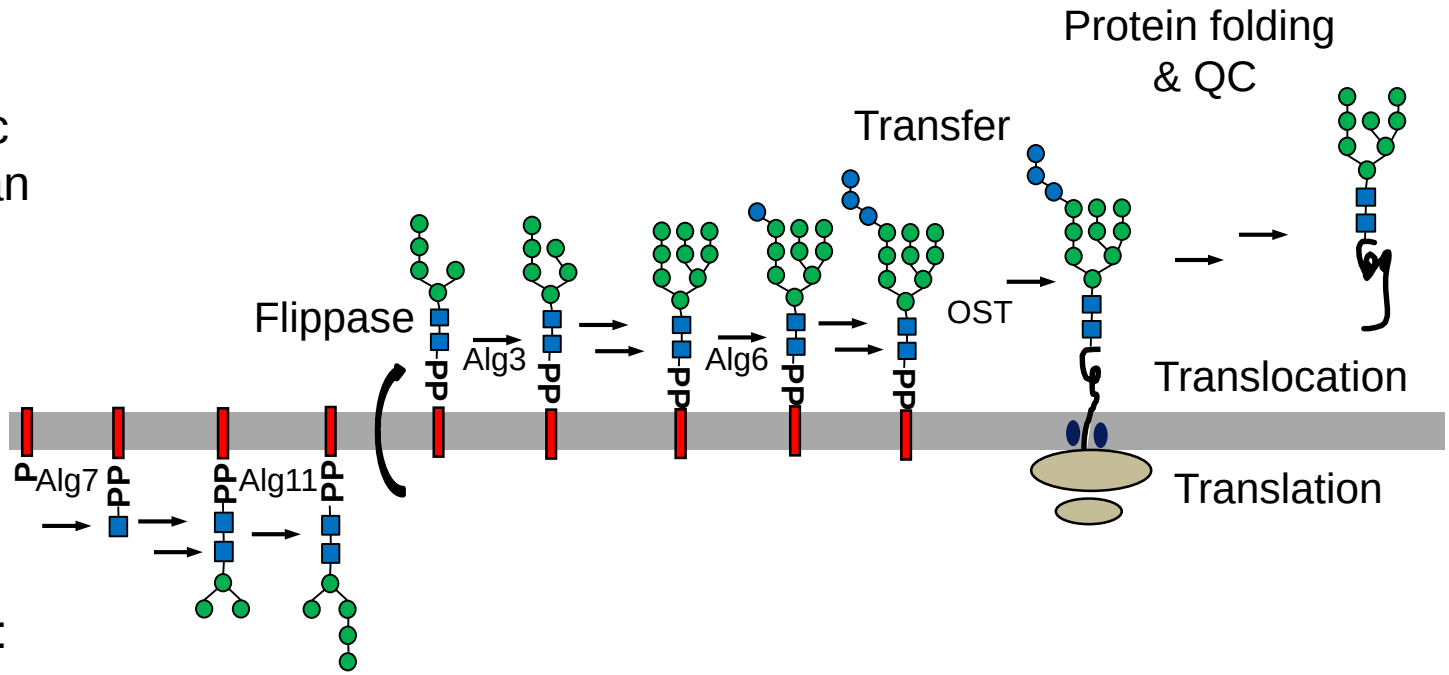
Paucimannose structures are generated through trimming of complex glyans



Hypermannosylated, extensive addition of Mannose residues

Recap: Synthesis of lipid-linked oligosaccharide

Sugar donors:
Dolichol-P-Glc
Dolichol-P-Man

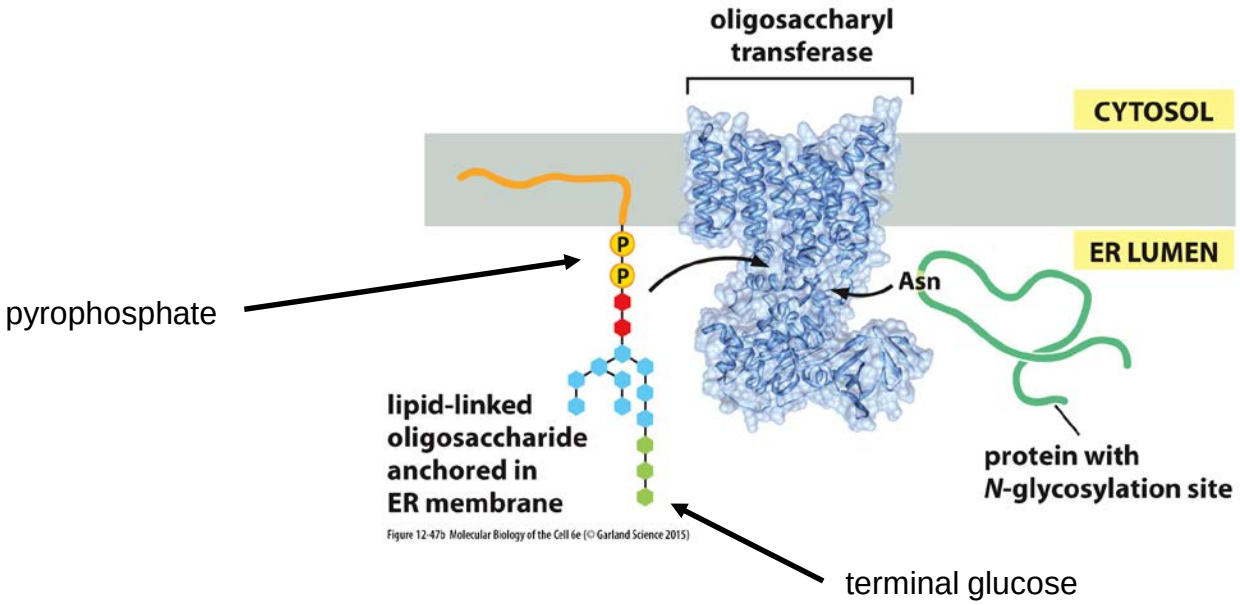


Sugar donors:
UDP-GlcNAc
GDP-Man

- Generate lipid-linked oligosaccharide (LLO)
- Flipping of LLO from cytoplasm into ER lumen
- Transfer of oligosaccharide from LLO to the acceptor sequon on the protein

Recap: N-linked protein glycosylation

- Transfer of a preformed oligosaccharide from a lipid donor (dolichol) to the Asn-X-Ser/Thr motif of the polypeptide chain
  - Oligosaccharide is attached to the b-amide group on the asparagine side chain in an N-X-S/T motif
- The complex must recognize the oligosaccharide donor, recognize the acceptor site on the protein and catalyze the transfer



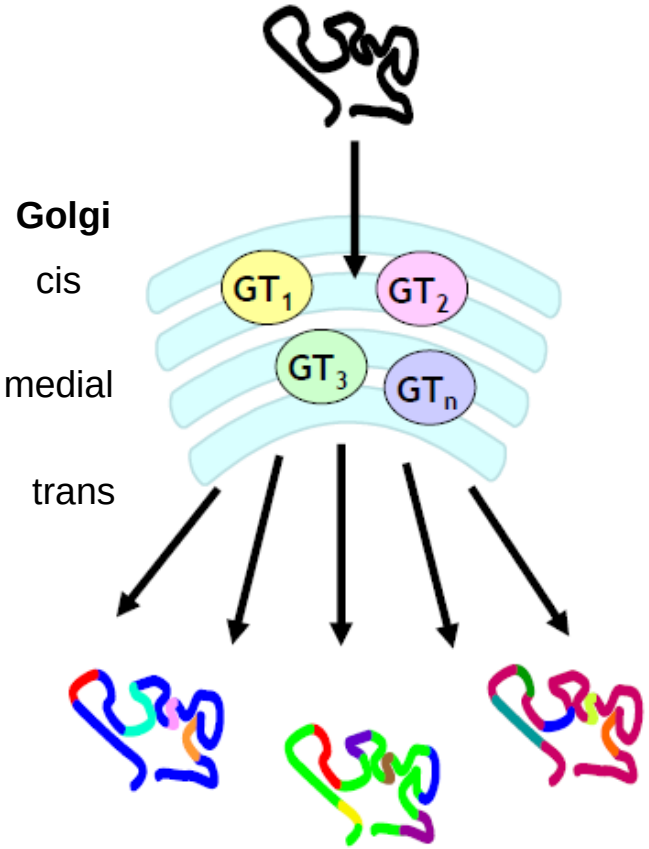
Oligosaccaryltransferase is a multiprotein complex with 8 or 9 subunits

- Oligosaccharyltransferase recognizes pyrophosphate group and the terminal glucose

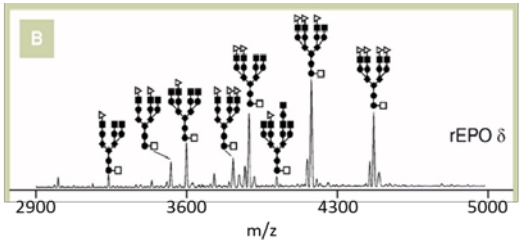
Processing of N-glycans in the Golgi apparatus

- N-glycan structures generated in the Golgi apparatus are
  - Species-specific
  - heterogeneous
- Variation in N-glycan structure at a specific N-glycosylation site are called microheterogeneity
- Proteins exist in many different glycoforms

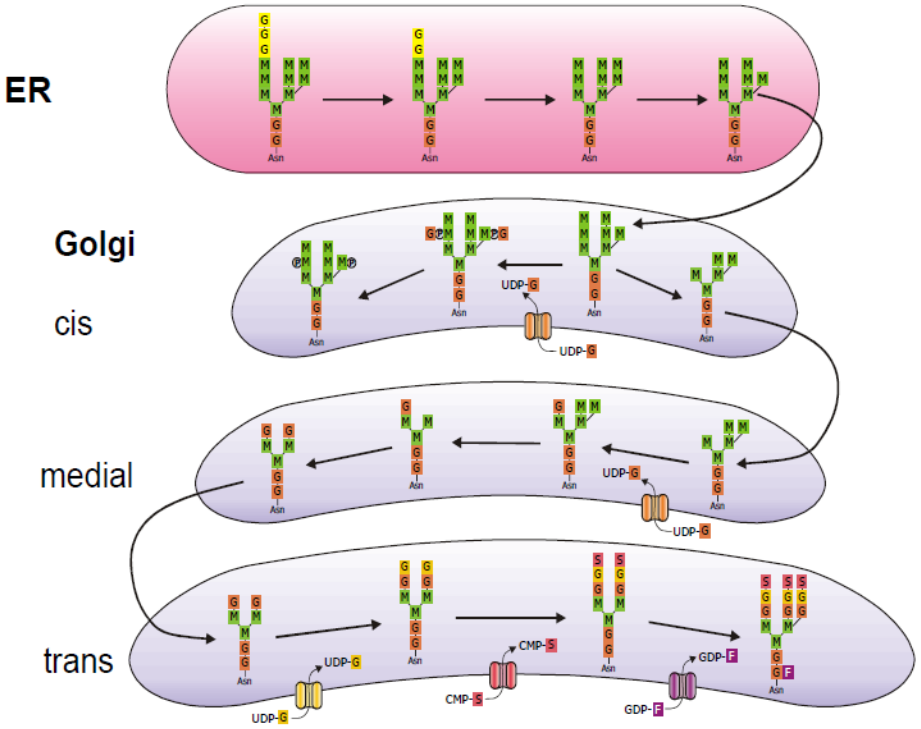
N-glycosylation is not template driven



- In contrast to the protein sequence, N-glycan processing is not genetically encoded
- Different enzymatic N-glycan processing activities (GT_x) are localized to different Golgi stacks
- Enzymatic N-glycan processing results many different “flavours” or glycoforms of a single protein
- For example “EPO”

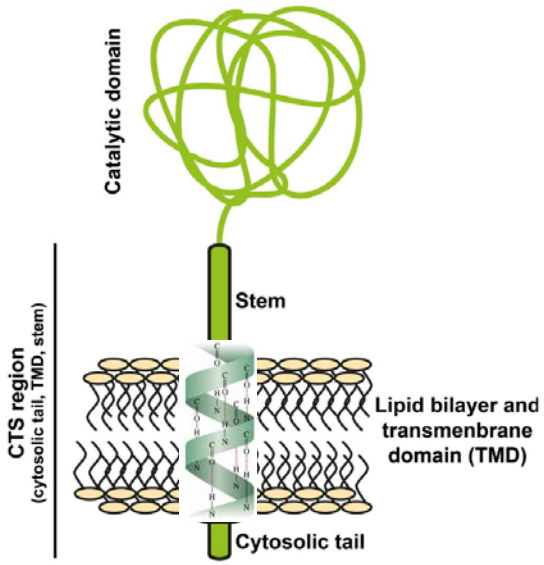


Oligosaccharide processing in the Golgi compartment



- Step-wise process (removal of mannose and addition of sugars)
- Substrates for transferases (= activated sugars):
  - UDP-N-acetylglucosamine
  - UDP-Galactose
  - CMP-Sialic acid
  - GDP-fucose
- In addition, transporters for import of activated sugars into Golgi are required
- In yeast and fungi
  - Step-wise extension of mannose chain(s) using GDP-Mannose

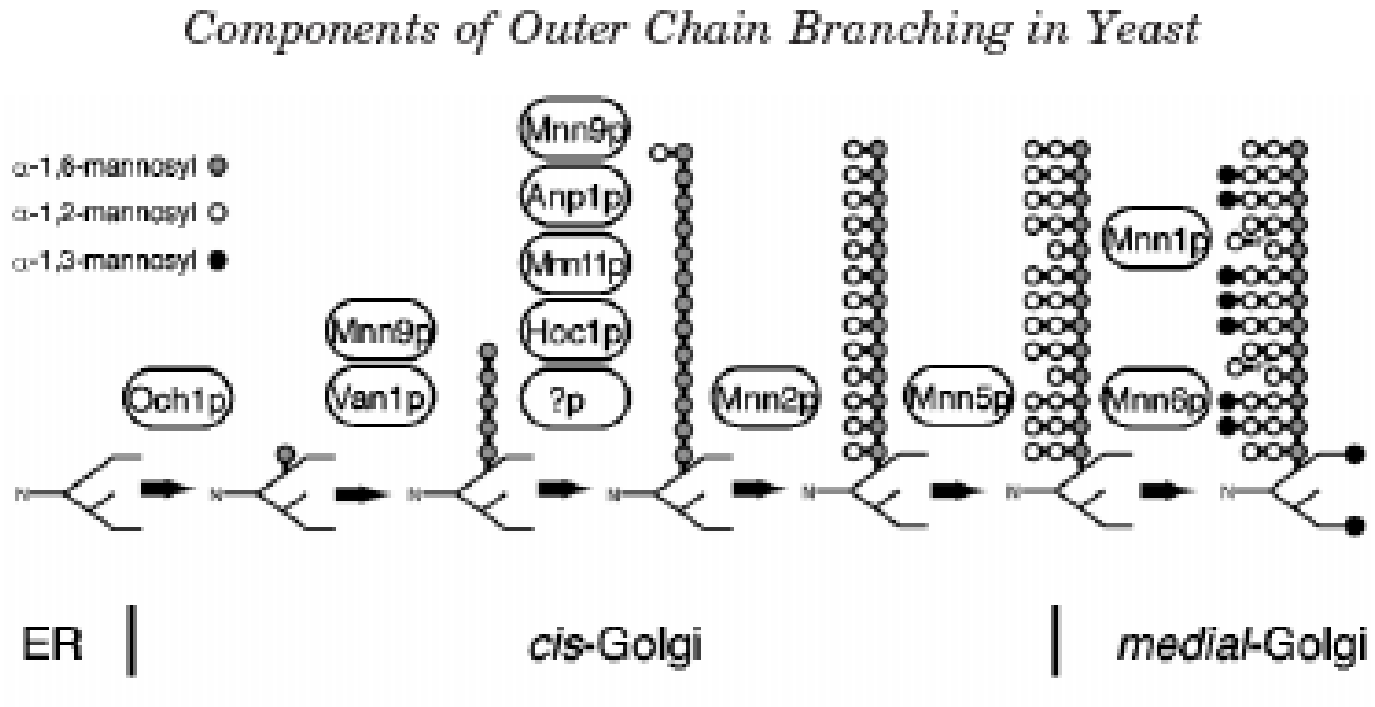
Mechanism for localizing glycosyltransferases in the Golgi



Loos and Steinkellner, 2014

- Golgi-located glycosyltransferases are single pass transmembrane proteins.
- localization within the Golgi depends on the N-terminal CTS region, consisting of the cytosolic tail, the transmembrane domain and a stem
- The C-terminal catalytic domain is directed to the Golgi lumen
- Mechanism of enzyme localization conserved among eukaryotes
- Often organized as multienzyme complexes

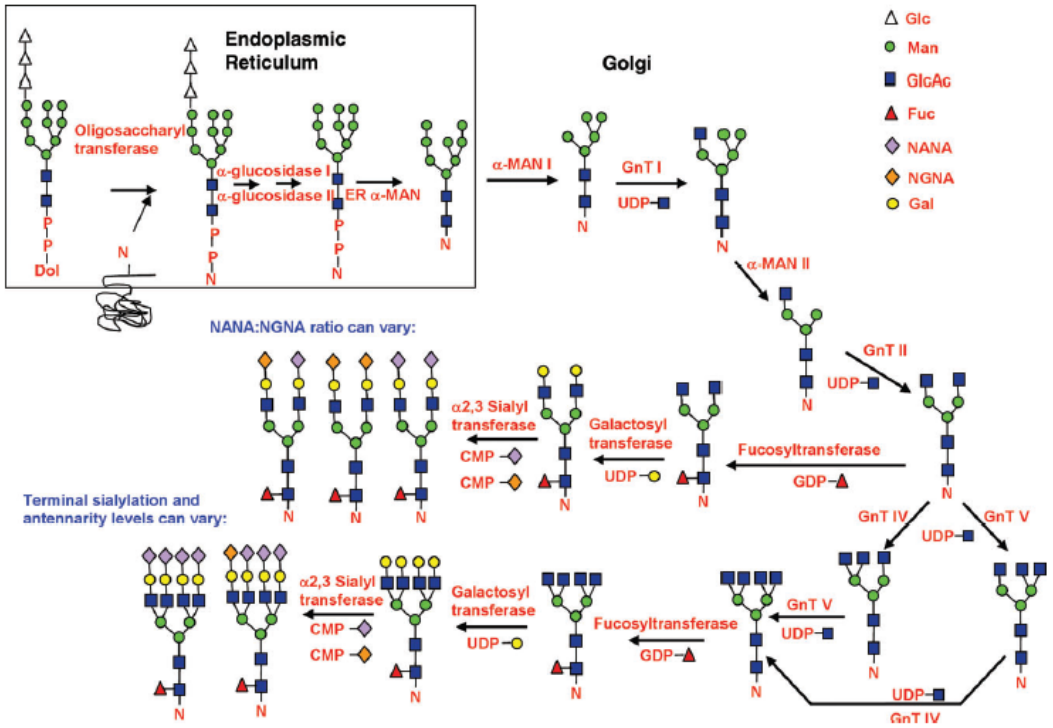
For experts only: Processing of N-glycans in the yeast Golgi apparatus



Rayner and Munro, 1998

- Golgi-localized Mannosyltransferases add mannose units
- KTR- and Mnn-Mannosyltransferase families
- Some of enzymes are involved in N- and O-glycosylation
- Number of mannose units varies between different species

For experts only: Mammalian N-glycosylation pathway



- N-glycosylation pattern are cell-type specific
- N-glycosylation pattern can be affected by cultivation conditions (Dissolved oxygen, pH, Temperature, certain nutrients and trace elements)

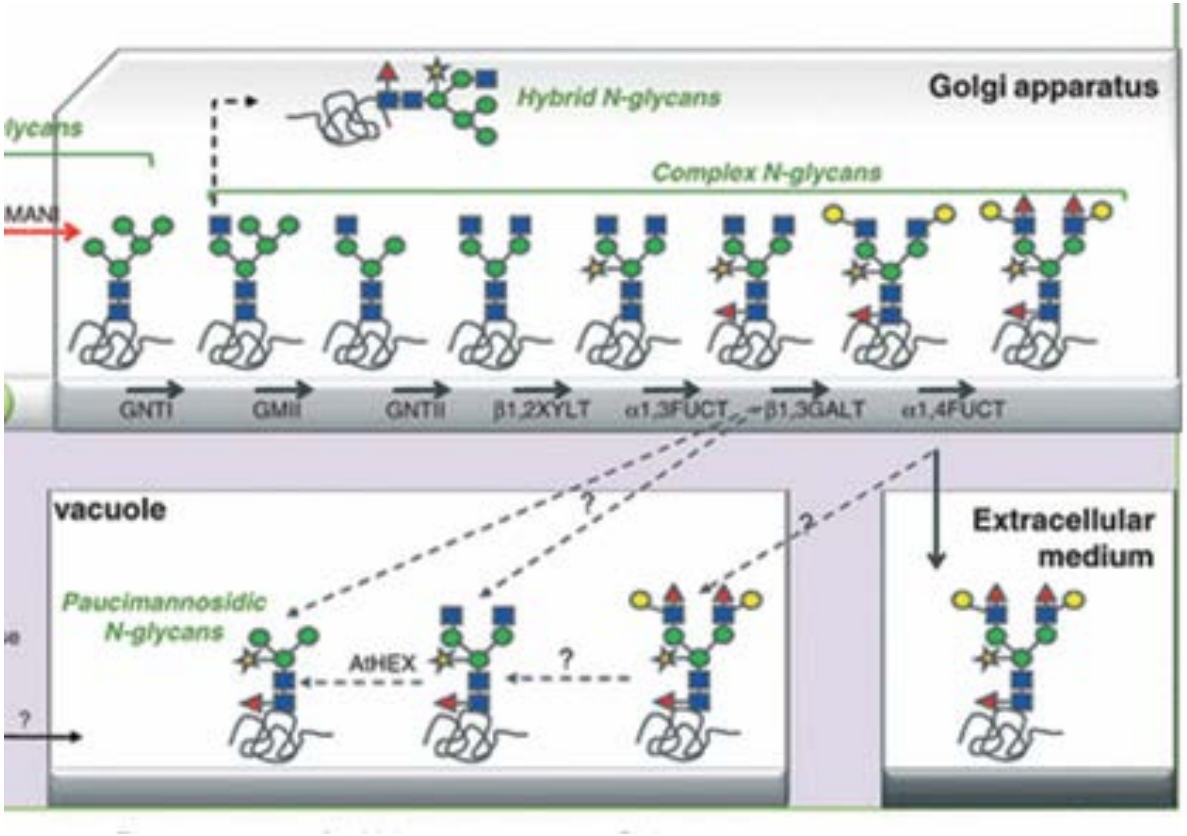
Mammalian hydrolases & transferases:

- Mannosidase I and II (MI & MII)
- N-acetylglucosaminetransferase (Mgat1-5)
- ß(1,4)-Galactosyltransferase (GalT)
- Sialic acid transferase (SaT)
- α(1,6)-Fucosyltransferase (FuT)

Hossler et al., 2009 Glycobiology 19:936–949

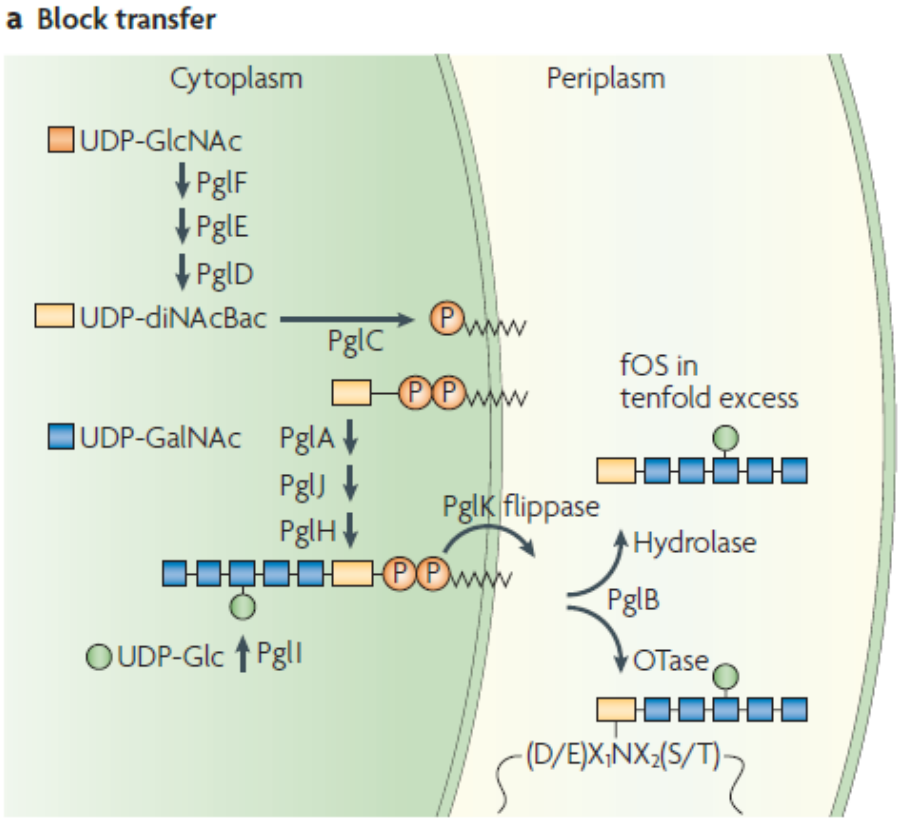
For experts only: Plant N-glycosylation pathway

From ER



Gomord et al. 2010 **Plant Biotechnology Journal**

Overview of bacterial N-linked protein glycosylation pathways (Pgl)

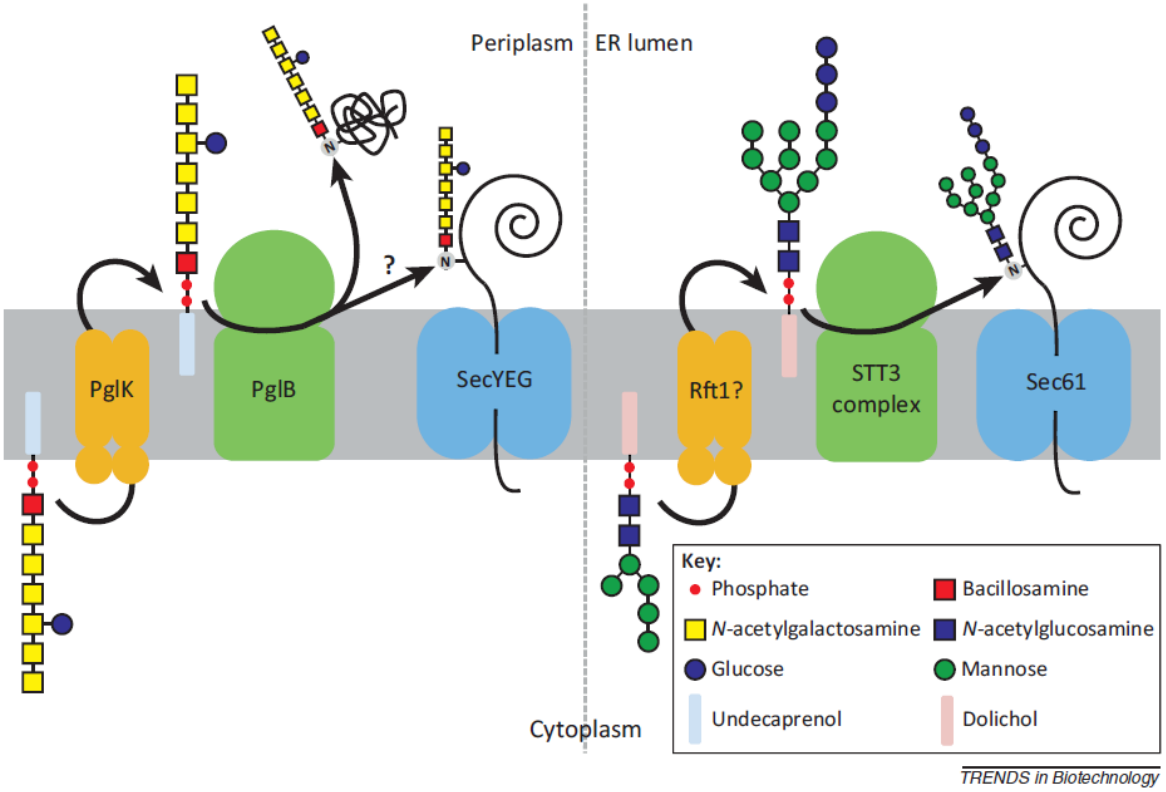


- A lipid-linked heptasaccharide is assembled in the cytosol by the addition of sugars from nucleotide-activated donors
- The complete heptasaccharide is flipped across the inner membrane into the periplasm by the flippase (PglK)
- The oligosaccharide is transferred to the amino group of Asn in the protein consensus sequence by the OST (PglB).
  - OST can also only liberate the oligosaccharide
- Extended protein acceptor site (Asp/Glu-X1-Asn-X2-Ser/Thr (X1 and X2 are any amino acid except Pro)

Transferred sugars: Bacillosamine, GalNAc, Glc!
No similarity to eukaryotic systems!

Nothaft and Szymanski, 2010 Nature Reviews Microbiology 8:765

N-Linked glycosylation in bacteria and eukaryotes



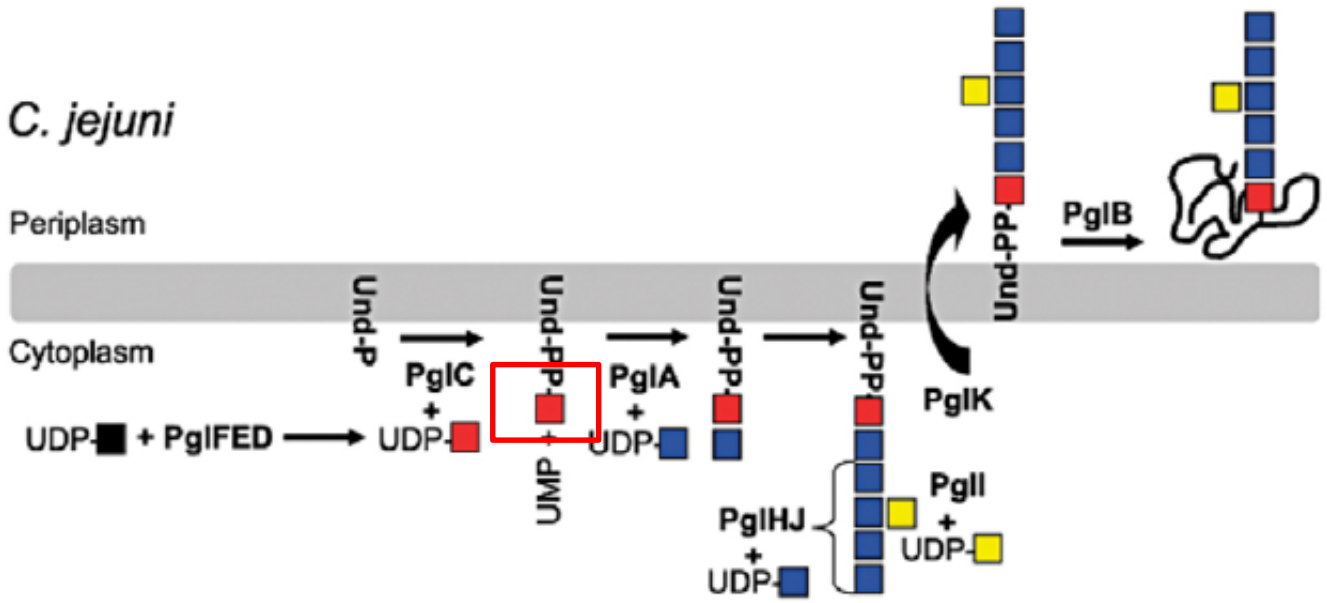
Comparison of N-linked glycosylation in prokaryotes (left) and eukaryotes (right).

Comparison of bacterial and eukaryotic N-glycosylation

- In both systems,
  - several glycosyltransferases synthesize the glycan by sequential addition of nucleotide-activated sugars on a lipid carrier
  - a flippase transfers the LLOs across the membrane,
  - Oligosaccharyltansferase catalyze transfer to Asn residues
- PglB is a single-subunit, integral membrane protein that is homologous to the catalytic subunit (STT3) of the eukaryotic OST complex
- Eukaryotes use an N-X-S/T acceptor sequence, PglB requires an extended D/E-X1-N-X2-S/T motif
- PglB can transfer sugars post-translationally to locally flexible structures in folded proteins. In eukaryotes, glycosylation proceeds in a co-translocational manner.

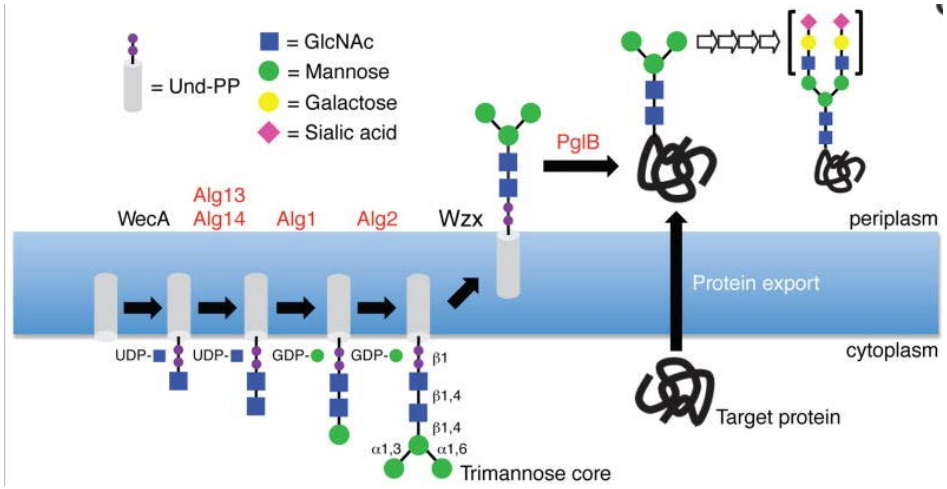
How to get *E. coli* glycosylation competent?

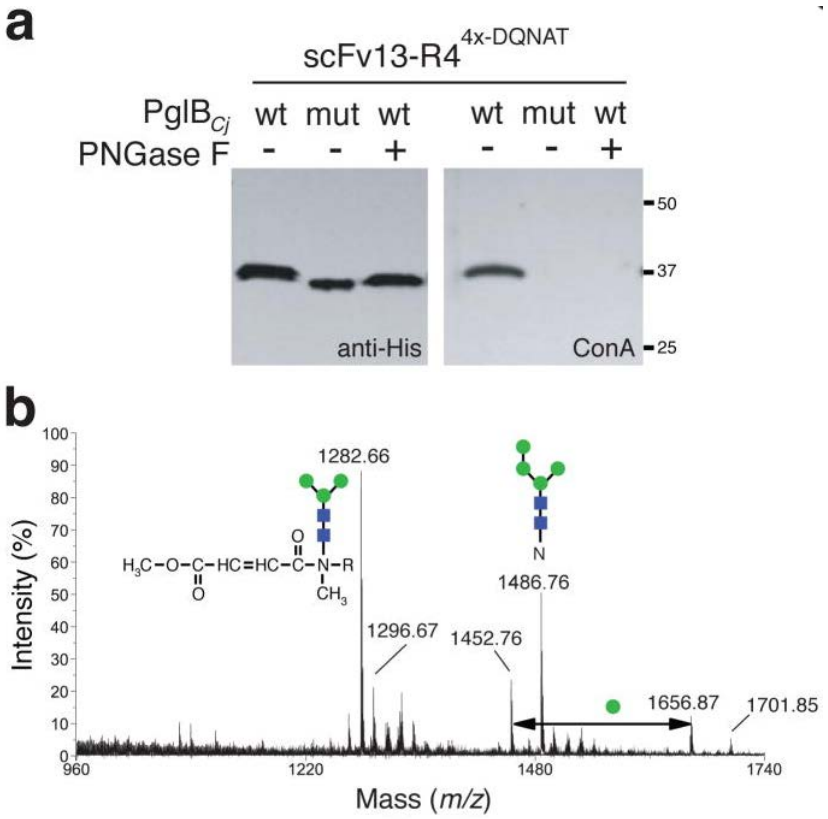
Remodeling bacterial N-glycosylation: Step I transfer to *E. coli*



- Transfer of system into *E. coli*
- First sugar is normally a bacillosamine
- *wecA* mutation leads to introduction of GlcNAc residue at the reducing end of the sugar
- Identical to first step in eukaryotic N-glycosylation pathway

Remodeling bacterial N-glycosylation: Step II adapt structures





- Expression of eukaryotic enzymes for building LLO in E. coli
- Generation of GDP-mannose as substrate

Valderrama-Rincon JD, et al. 2012, Nat Chem Biol.

How to change the type of glycosylation?

Technology for humanization of yeast N-glycosylation

Glycobiology vol. 14 no. 9 pp. 757–766, 2004
doi:10.1093/glycob/cwh104
Advance Access publication on June 9, 2004

Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose

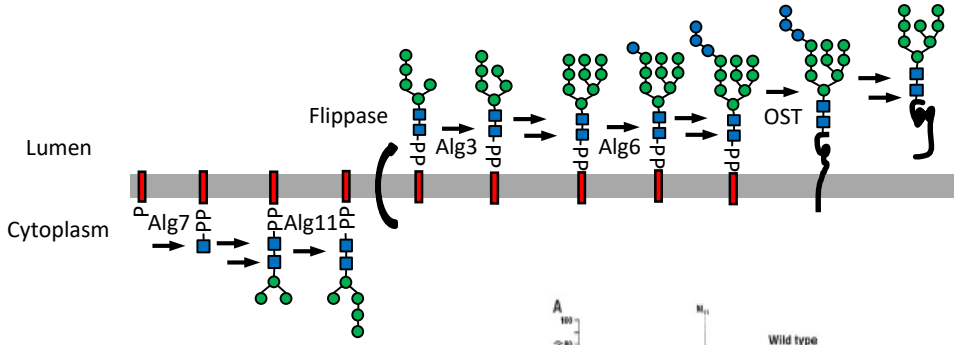
http://engineering.dartmouth.edu/magazine/innovations-glycofi-bought-by-merck/

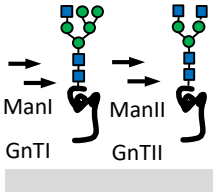
Technology was sold for 400 million USD to Merck

Points to be considered

- Structure to start with…
- Knowledge of Golgi architecture, localization and topology of endogenous enzymes
- Proper localization of heterologous transferases (cis-, medial-, trans-Golgi)
- Appropriate expression levels of heterologous transferases
  - Targeting to early or medial Golgi compartment might result in different N-glycans
- Are activated sugars available in the organism of choice?

Generation of complex N-glycans in yeast

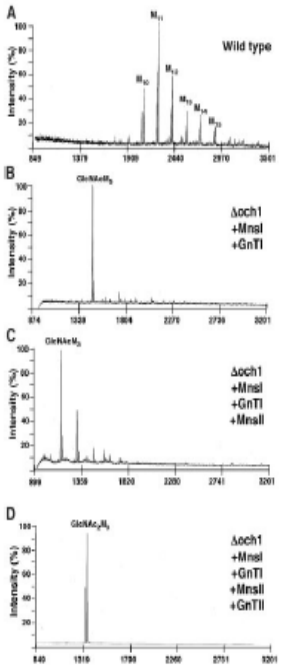




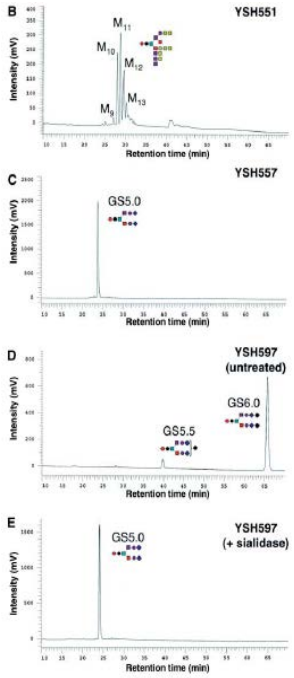
$Man_8GlcNAc_2$ starting point:

I. Mns1A: $Man_5GlcNAc_2$
II. GnTI: $GlcNAcMan_5GlcNAc_2$
III. MnsII: $GlcNAcMan_3GlcNAc_2$
IV. GnTII: $GlcNAc_2Man_3GlcNAc_2$

V. GalT (Bobrowicz et al. 2004)
VI. ST (Hamilton et al. 2006)



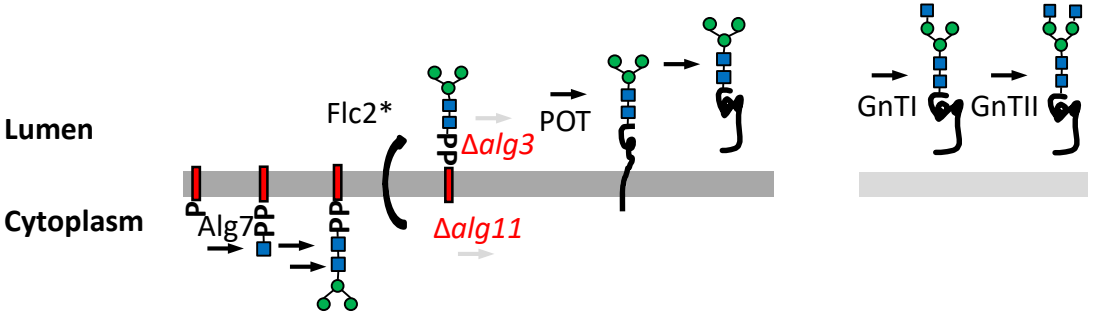
Hamilton et al. 2003



Hamilton et al. 2006

A synthetic N-glycosylation pathway in yeast

Can the N-glycosylation pathway be simplified?



- Increase N-glycosylation efficiency
- Decoration of core to obtain human-type uniform N-glycan structures

Piirainen MA, Glycoconj J. 2016 Apr;33(2):189-99.