08:30

Serum Bikunin: a biomarker of inborn errors of proteoglycan biosynthesis & Golgi homeostasis

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INSERM U-1193: Pathophysiology of liver diseases
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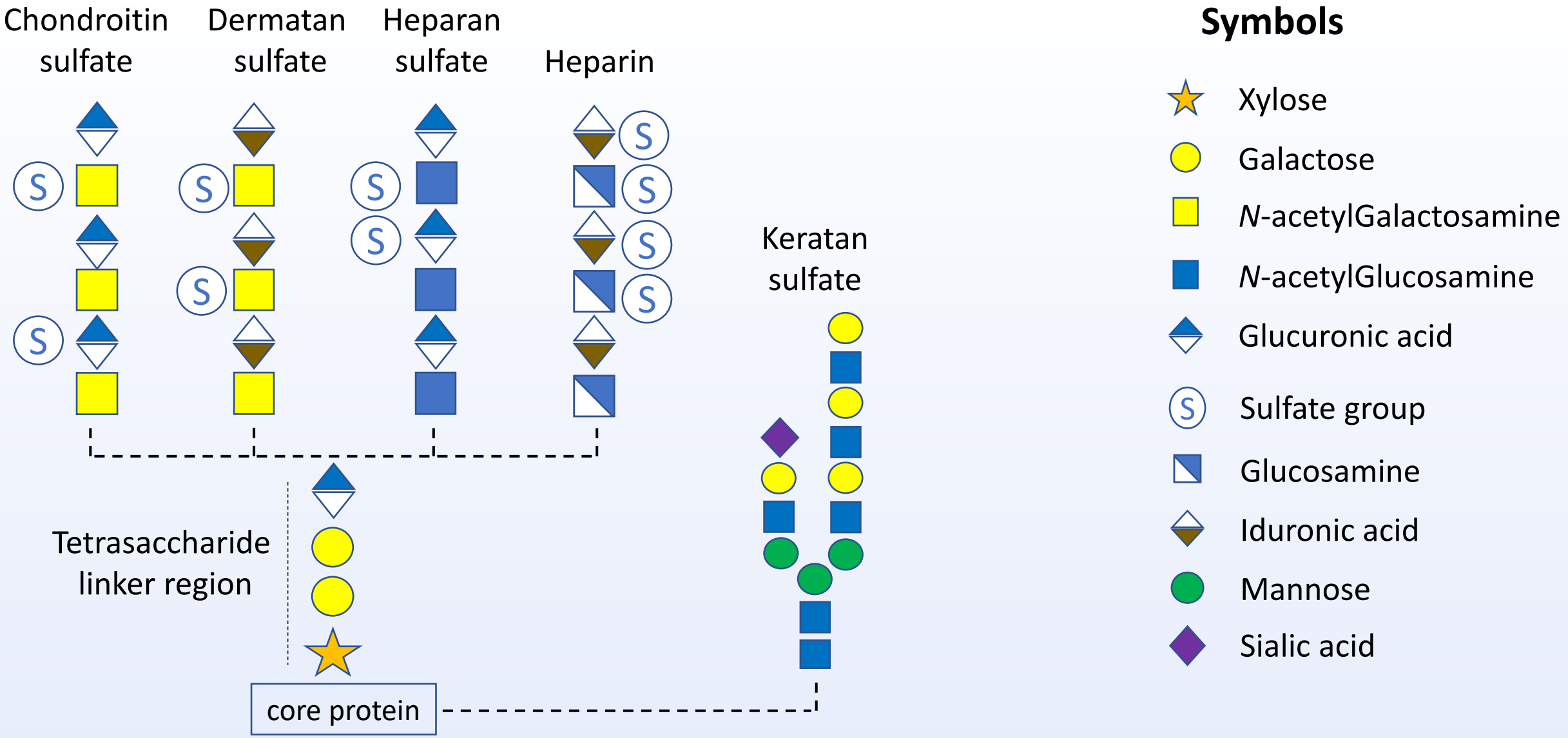
14 décembre 2021

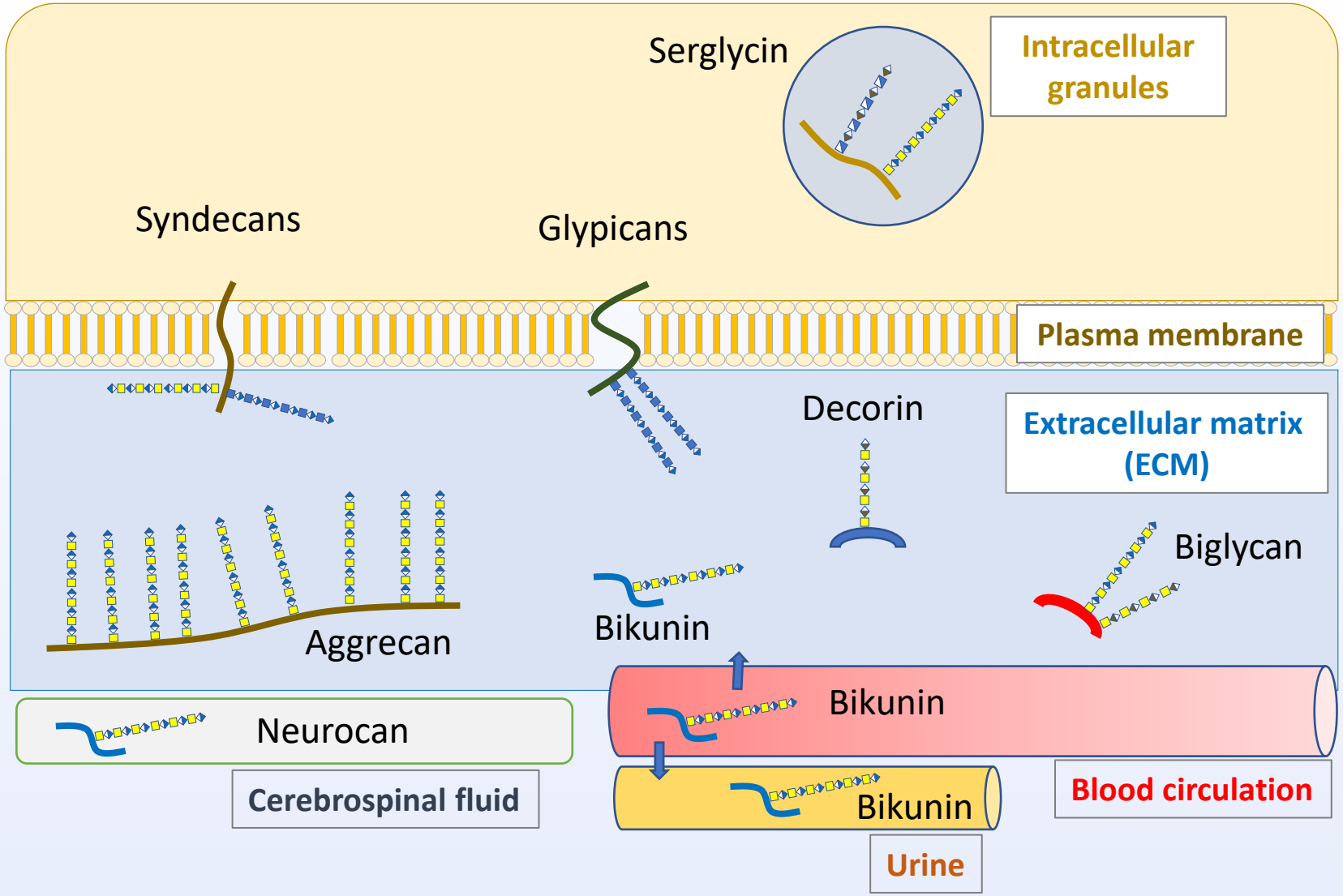
Soutenance de thèse de doctorat

Proteoglycans & associated disorders

Proteoglycans: types & structure

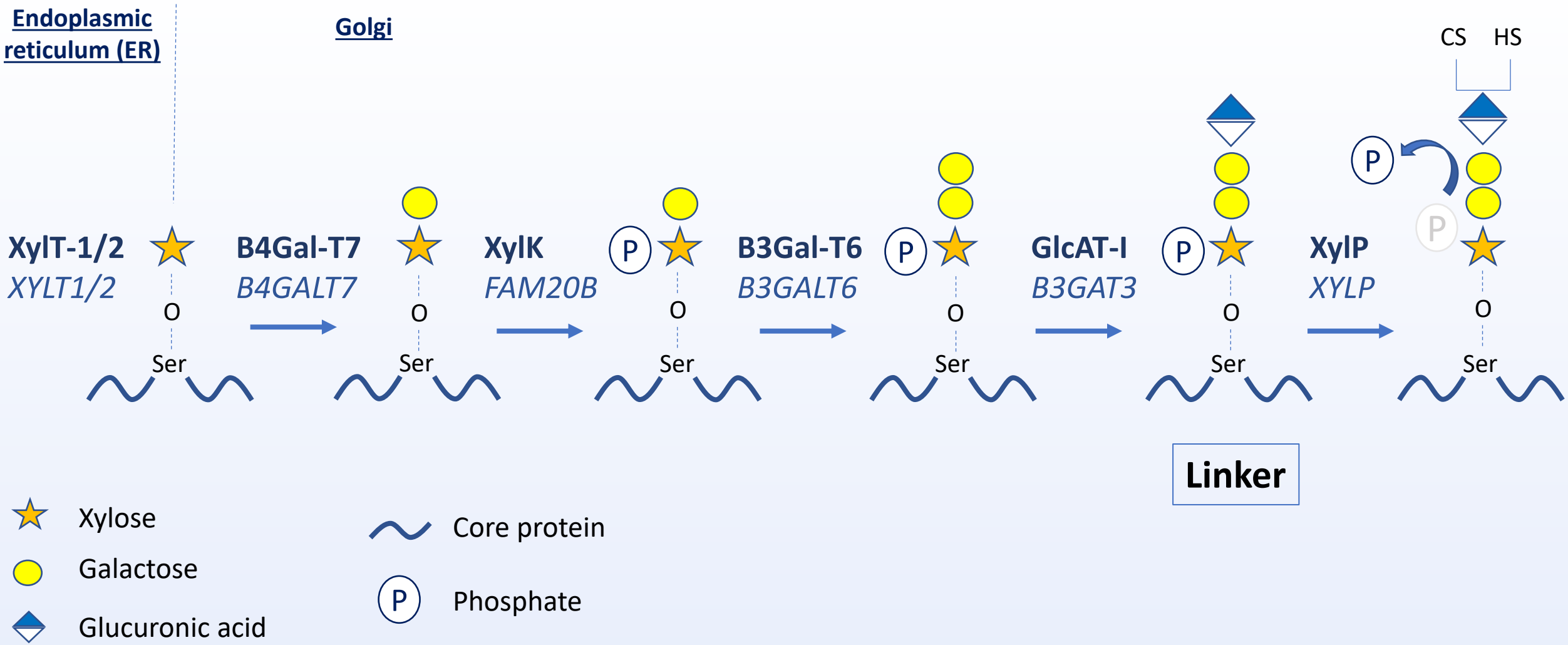


Proteoglycans: roles & distribution

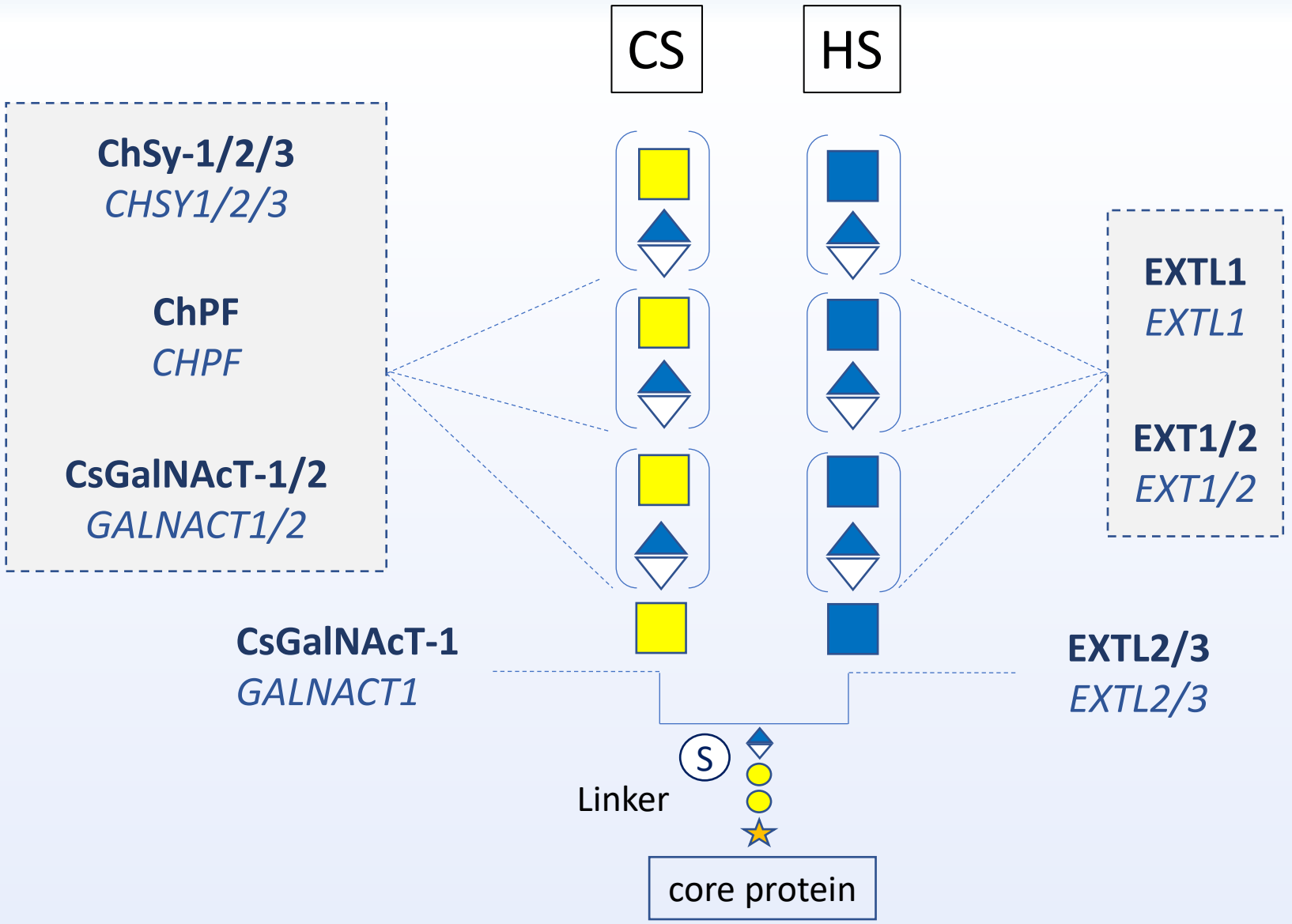


- Physicochemical properties of ECM (Bones, joints, skin,)
- Growth & development
- Immune response
- Cell proliferation

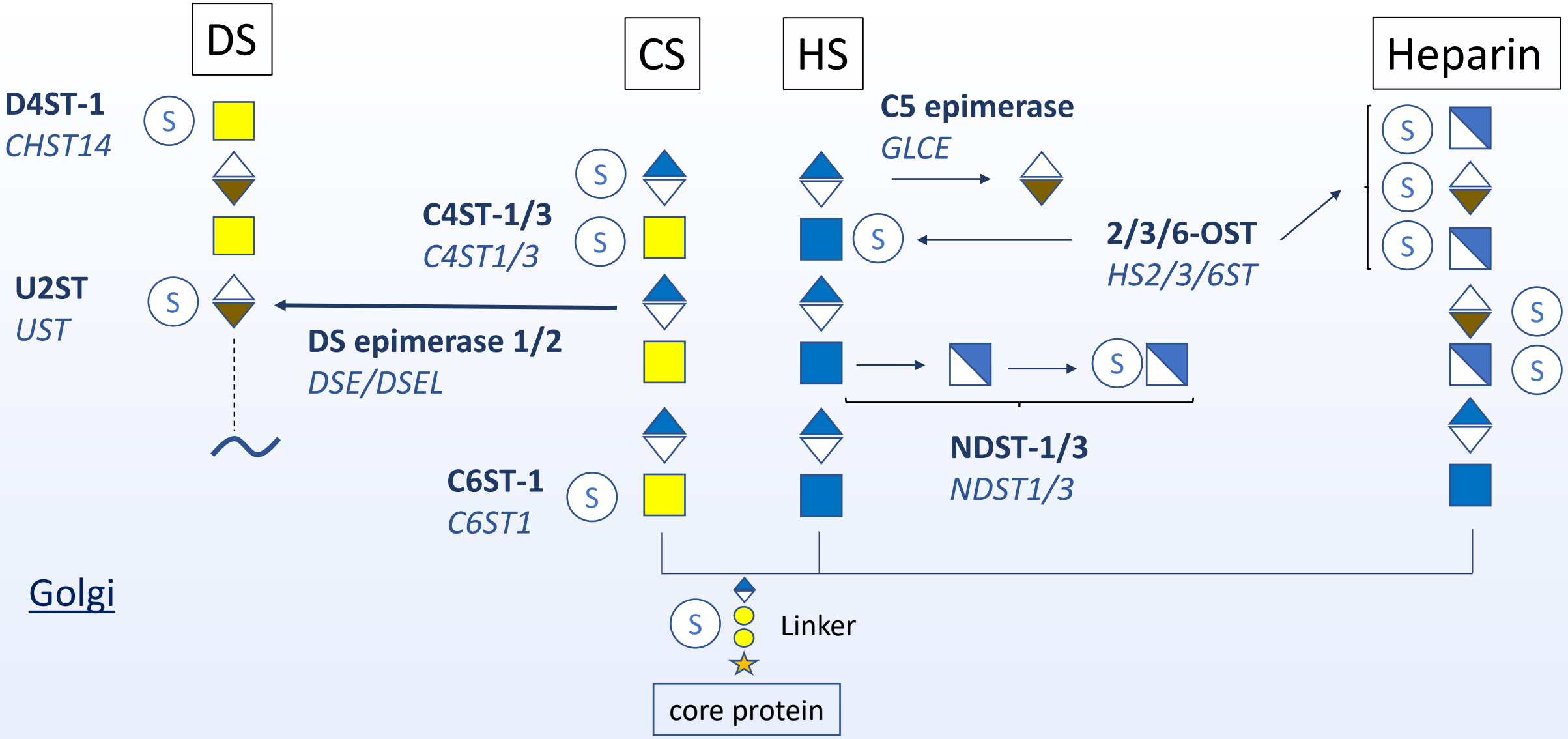
Proteoglycan biosynthesis (1): linker formation



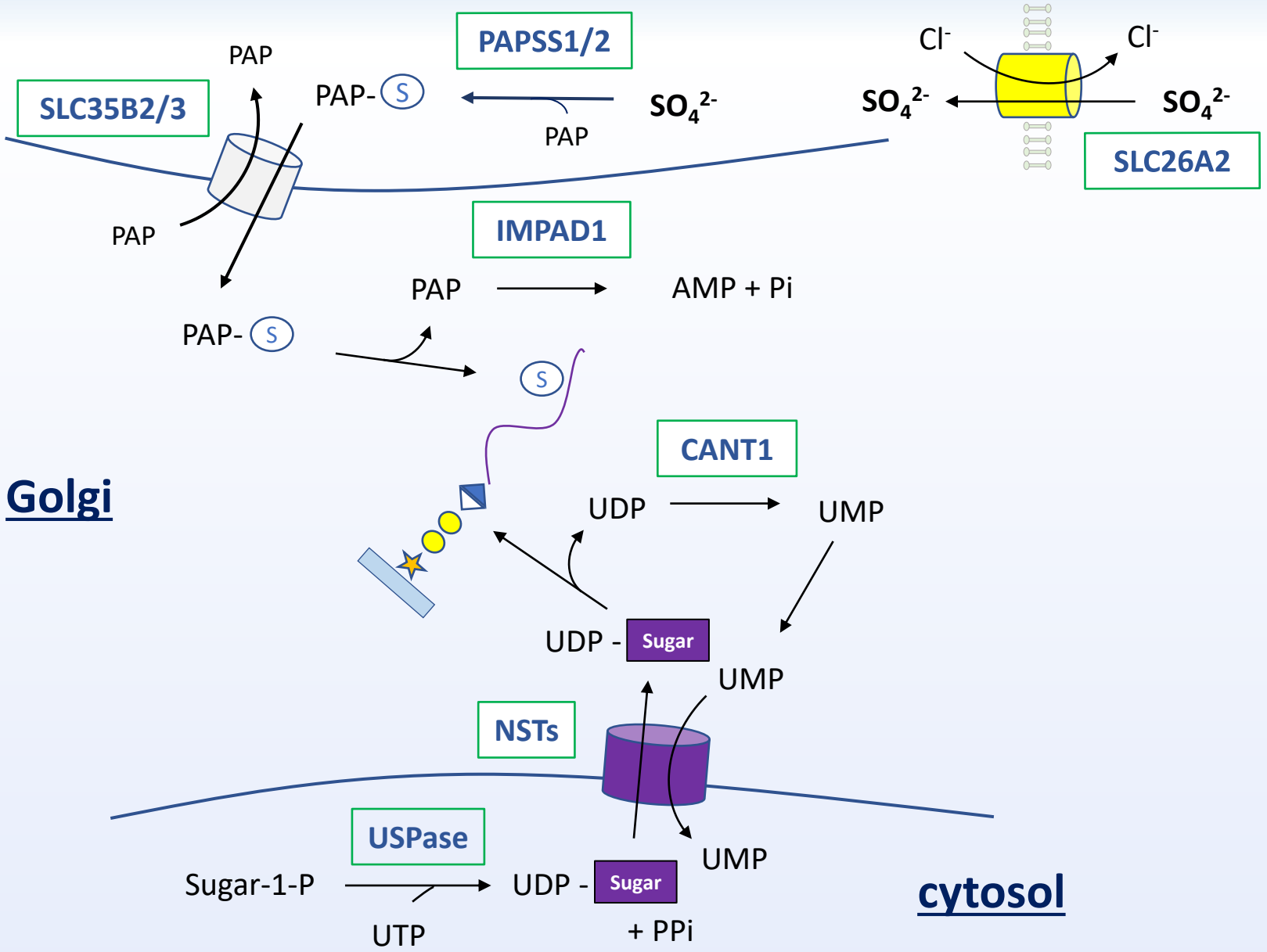
Proteoglycan biosynthesis (2): GAG elongation



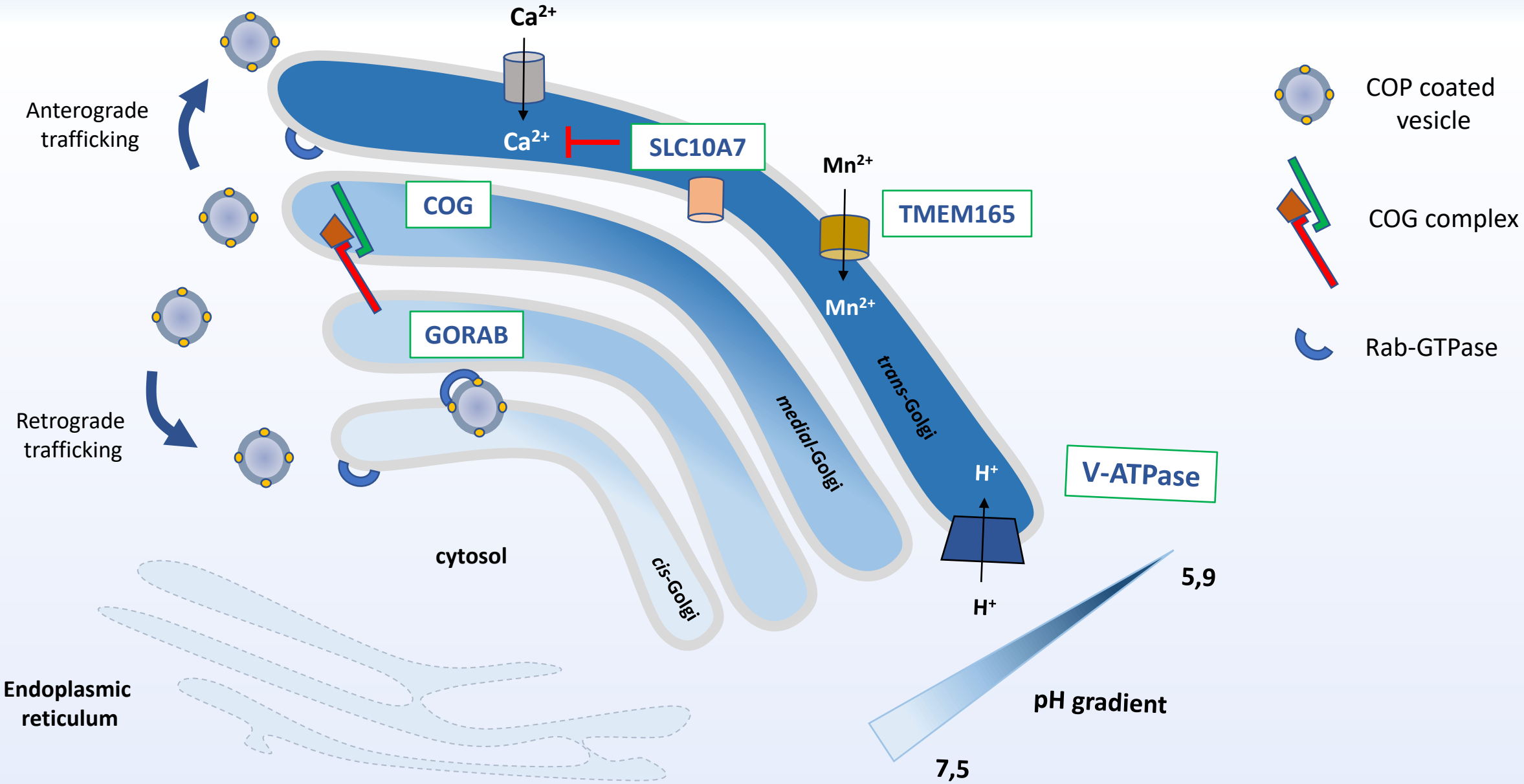
Proteoglycan biosynthesis (3): GAG modifications



Substrate synthesis

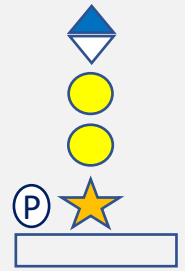


Golgi homeostasis



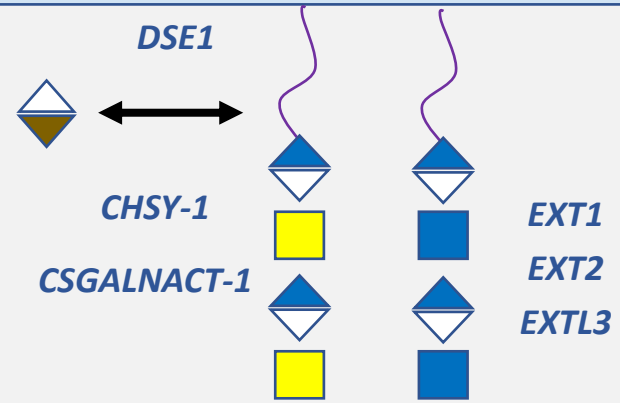
Proteoglycan inherited metabolic diseases (PG-IMD)

B3GAT3
B3GALT6
B4GALT7
FAM20B
XYLT1/2



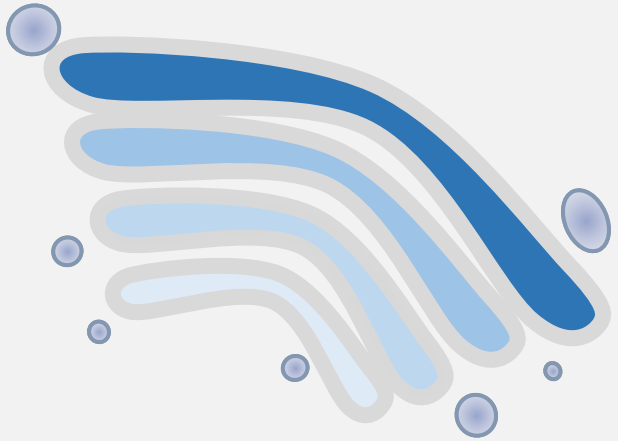
Linkeropathies

DSE1
CHSY-1
CSGALNACT-1
EXT1
EXT2
EXTL3



GAG elongation defects

TMEM165
COG4
GORAB
SLC10A7



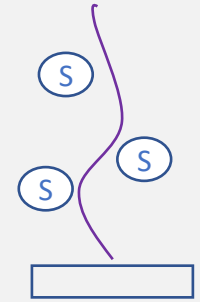
Golgi homeostasis defects

SLC35A2
SLC35A3
SLC35D1
CANT1

UDP - Sugars

UDP-sugar synthesis & transport defects

PAPSS1/2
SLC35B2
IMPAD1
CHST14
SLC26A2
CHST3
CHST6



GAG sulfation defects

Clinics of PG-IMD

Osteoarticular defects +++

- Skeletal dysplasia
- Short stature
- Limb deformities
- Multiple fractures
- Joint hyperlaxity

Sasarman et al, JIMD, **2016**

Unspecific symptoms

- Intellectual disabilities
- Skin, ocular, cardiac defects
- Muscle weakness
- Deafness, tooth abnormalities

Ex: Ehlers-Danlos spondylodysplastic

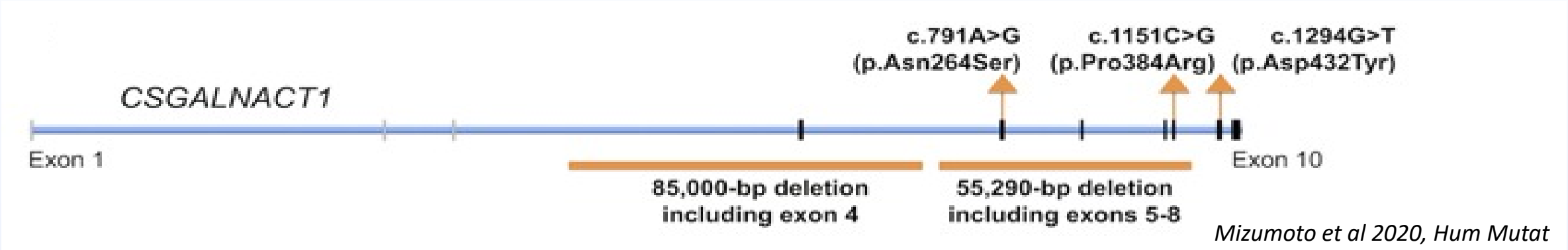
(B4GALT7 and B3GAT3 linkeropathies)

- Generalized joint hypermobility
- Short stature
- Kyphoscoliosis
- Muscle hypotonia
- ocular defect
- Respiratory problems

Pleiotropic & overlapping phenotypes

Molecular screening of PG-IMD

Next generation genetic sequencing
Gene panels, whole exome/genome

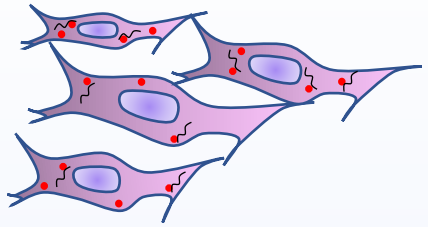


Mizumoto et al 2020, Hum Mutat

Need to determine the causality

Biochemical screening of PG-IMD

Fibroblasts from skin biopsies



Substrate radiolabeling and radioactivity counting
GAG quantification

Functional studies
Enzyme activity, substrate uptake, Golgi trafficking

Antibody-based analyses
GAG chains PG core proteins (decorin, biglycan)

HPLC/MS
Quantitative and qualitative analysis of GAGs

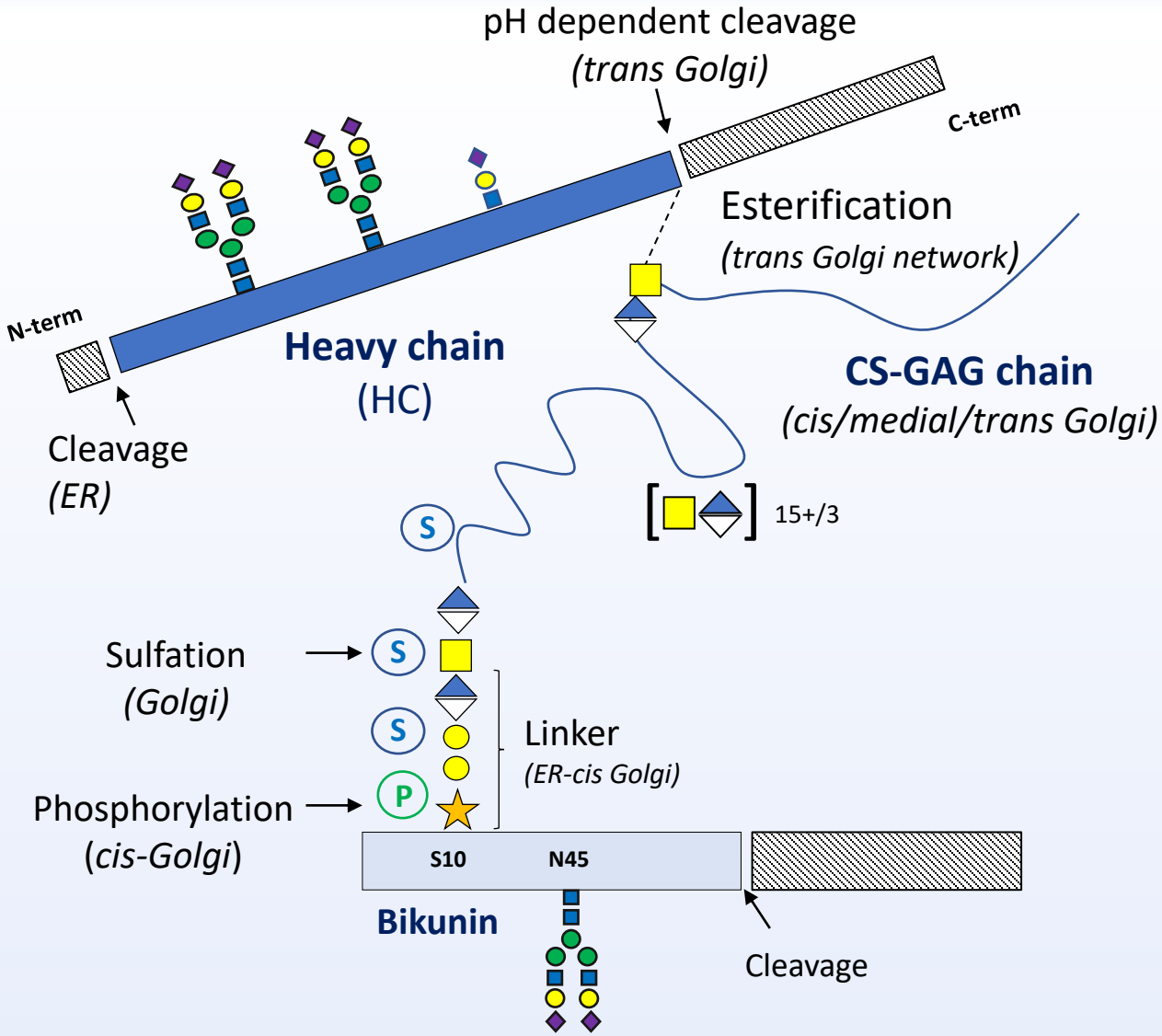
Blood and urine samples

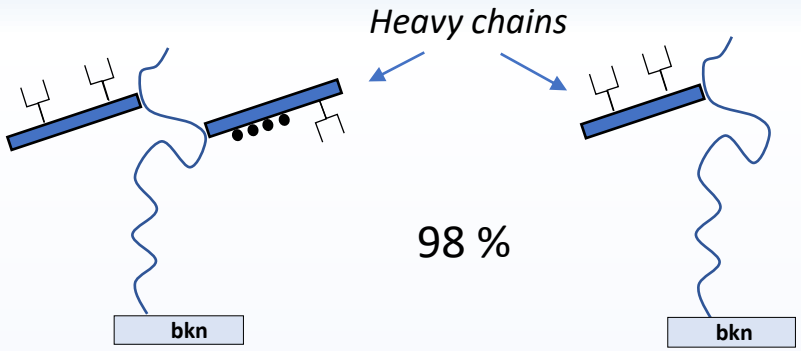
HPLC/MS
Quantitative and qualitative analysis of GAGs

Need for convenient biomarkers

Potential of serum Bikunin as a biomarker of PG-IMD

Bikunin structure & serum isoforms





Inter-α trypsin inhibitor
(ITI)

Pro-α trypsin inhibitor
(PαI)

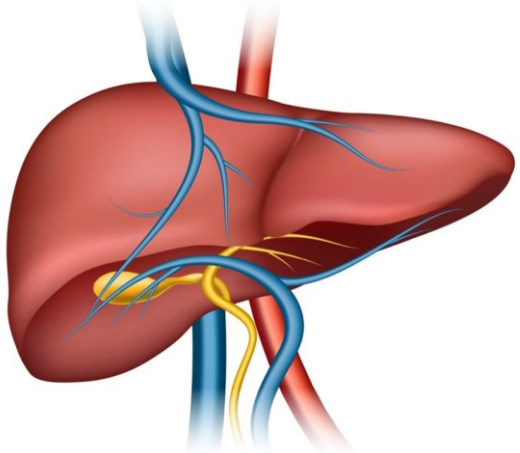
Serum concentration
0.15-0.5 g/L

2 %

Bikunin-CS
Urinary trypsin inhibitor

Bikunin core

Biosynthesis of Bikunin isoforms (1)



Liver expression

Nucleus

liver-restricted transcription factors

AMBP gene

5’ — **A1MG** — **Bikunin** — 3’

mRNA transcription

ribosome

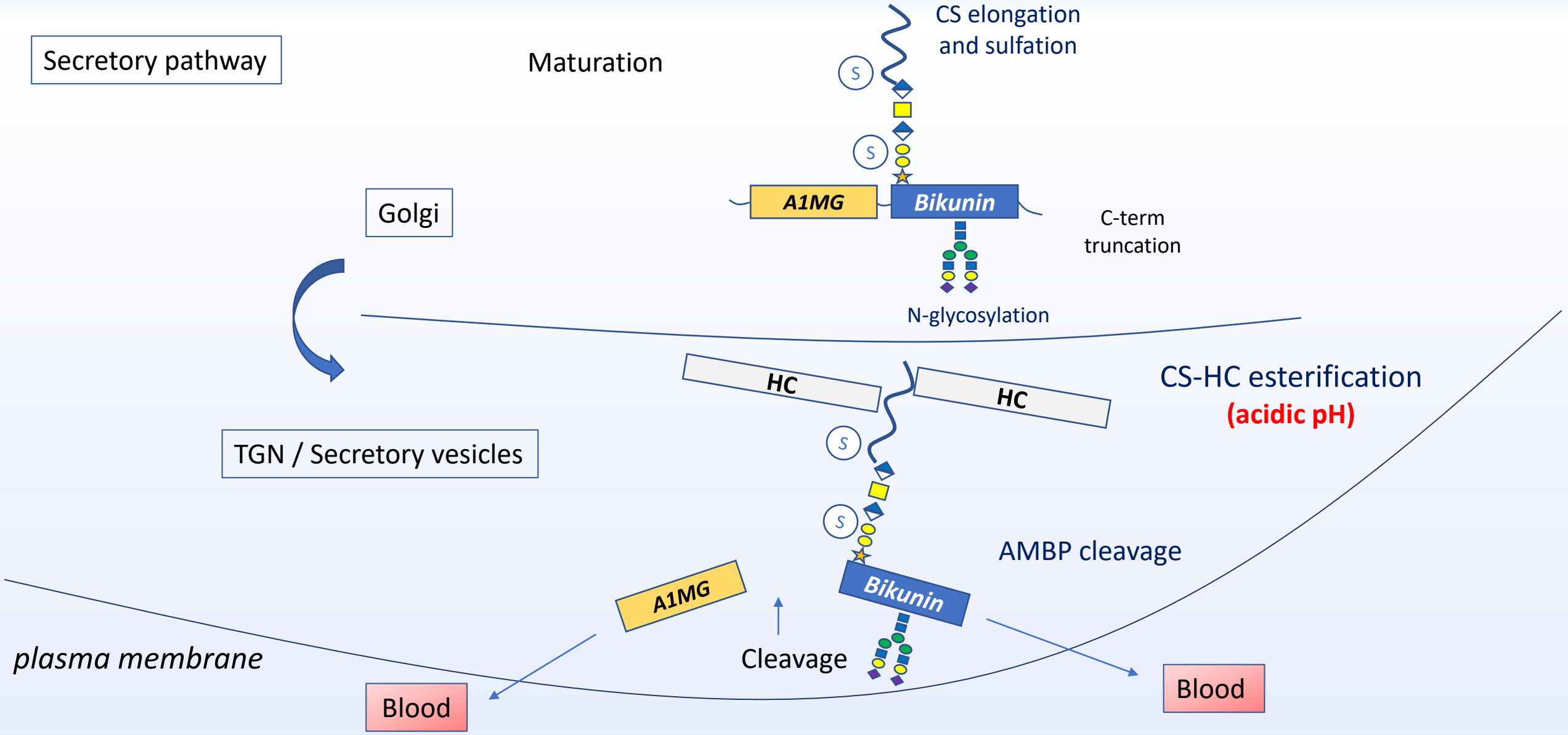
ER

Polypeptide translation

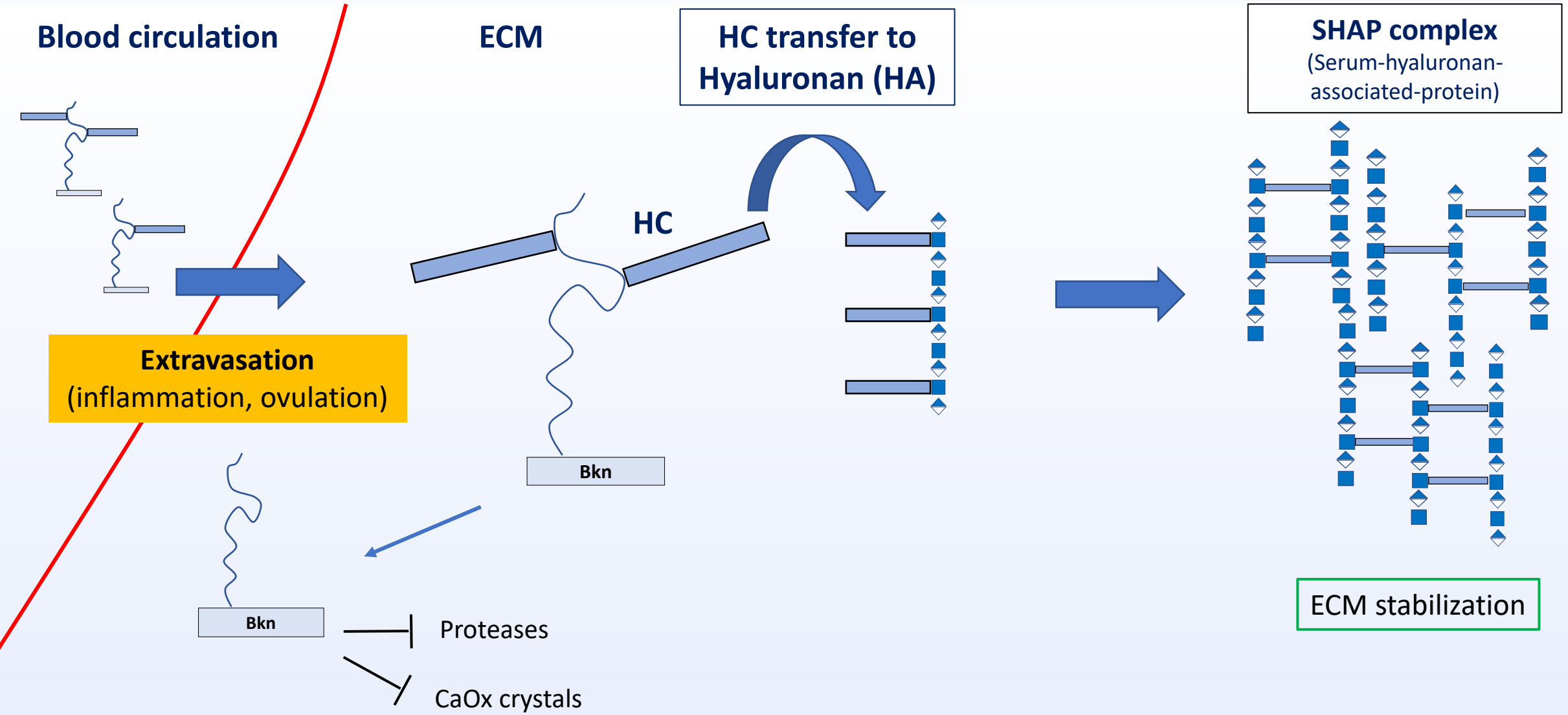
AMBP protein

N-term — **A1MG** — **Bikunin** — C-term

Biosynthesis of Bikunin isoforms (2)

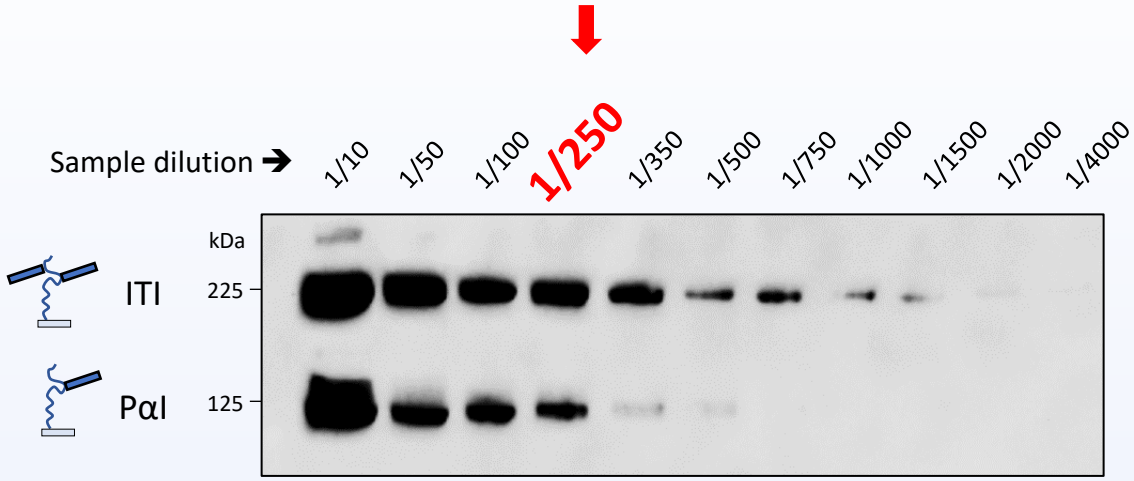


Roles of Bikunin isoforms

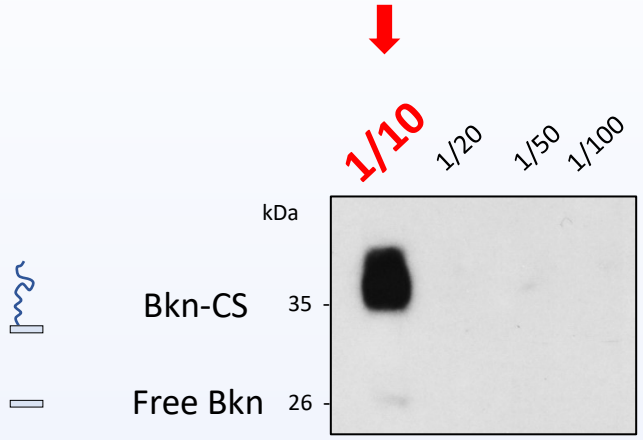


Western blot analysis of serum Bikunin

Heavy forms



Light forms



1-5 µL serum sample

Rabbit anti-Bikunin (CP6) polyclonal antibody (Merck- Millipore, cat. # ABT1346)

1/5000

Bikunin analyses in linkeropathies

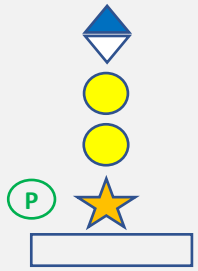
B3GAT3

B3GALT6

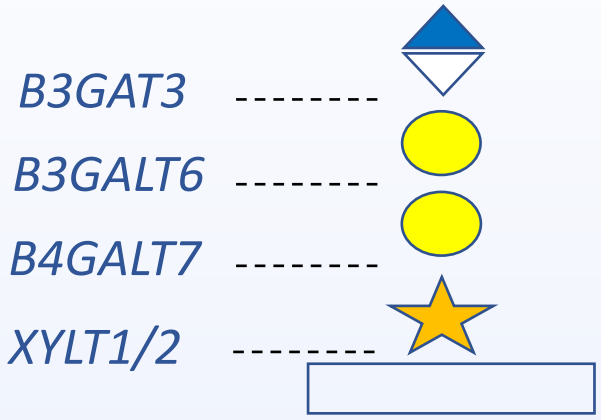
B4GALT7

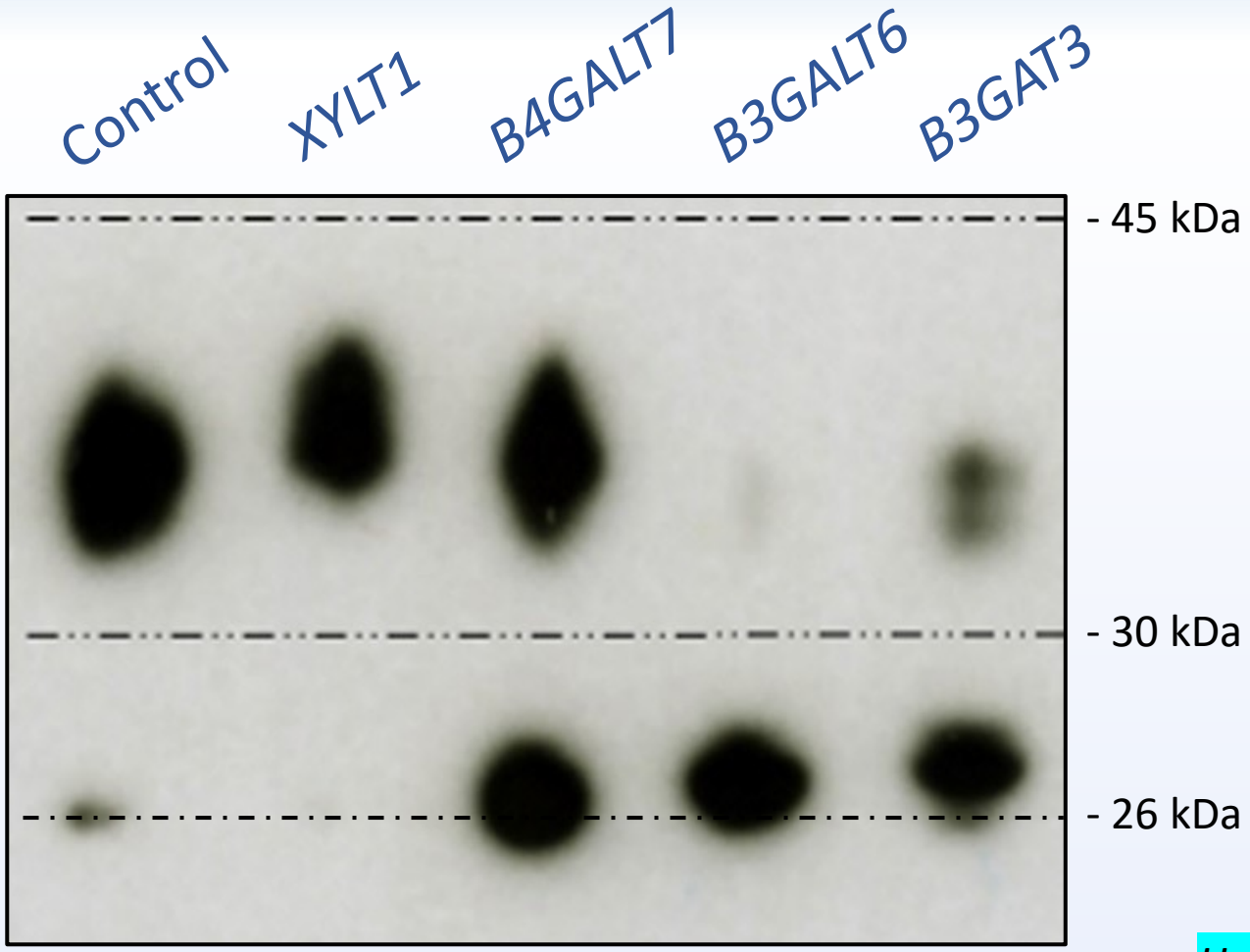
FAM20B

XYLT1/2

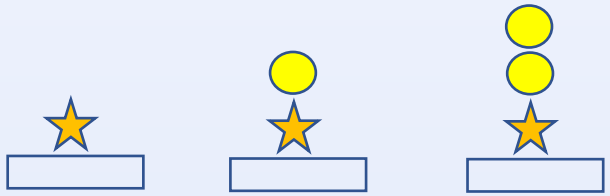


Bikunin light forms in linkeropathies





Hypotheses



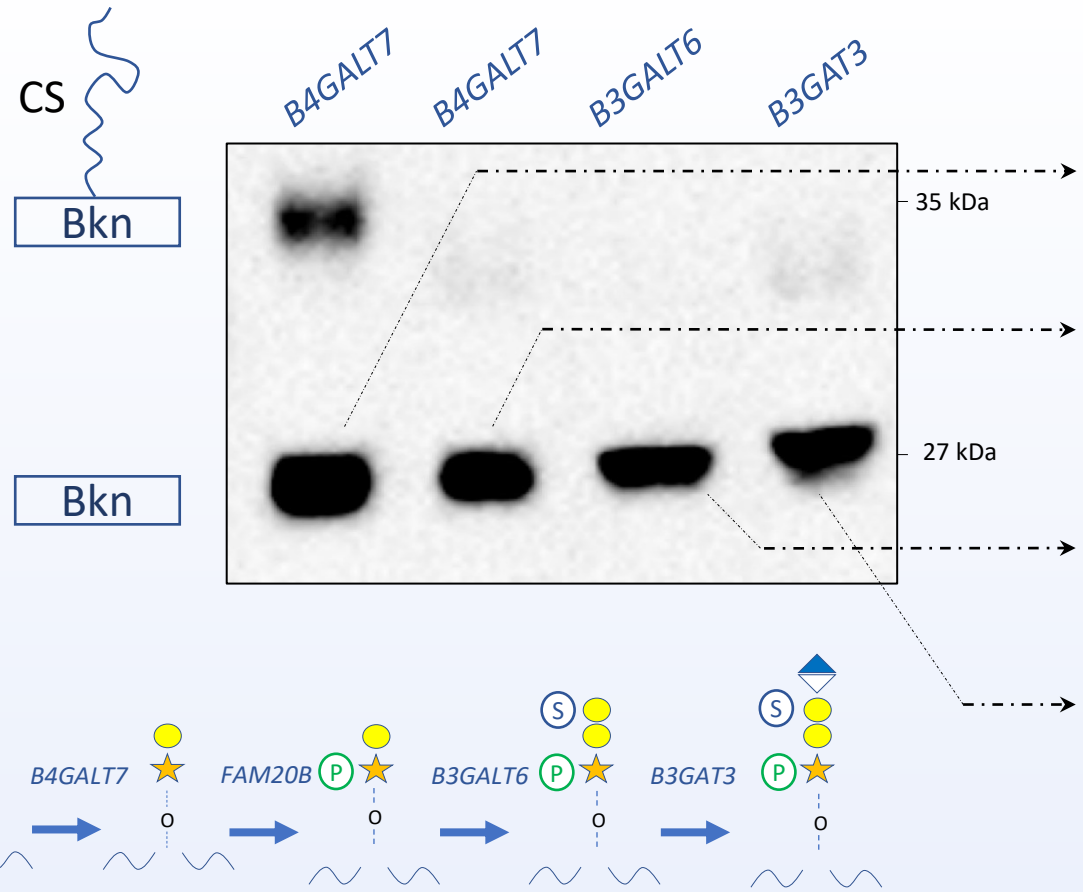
Abnormal
Bkn light forms

Samples from Pr. Valérie Cormier-Daire

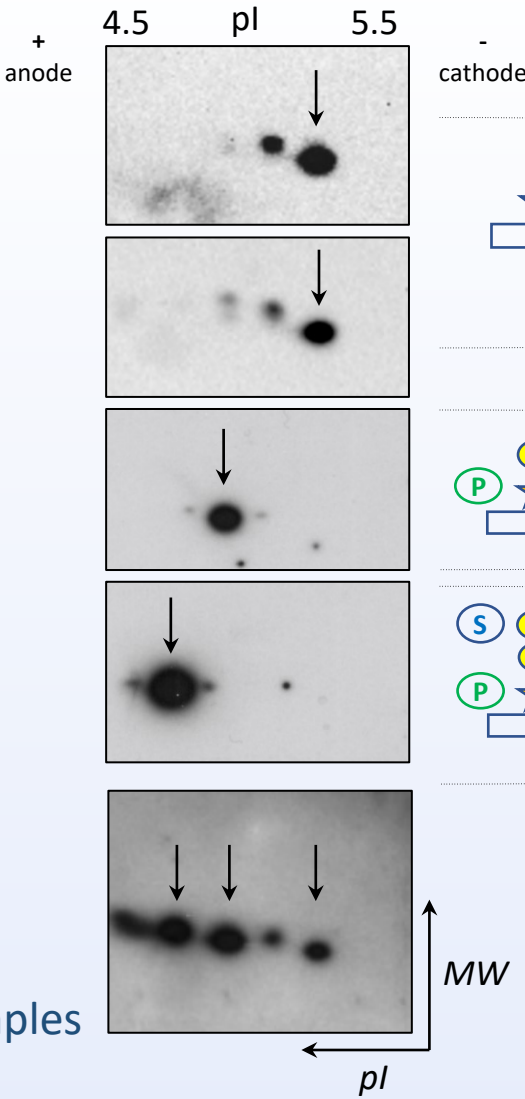
Bruneel et al 2018, CCA

Abnormal Bikunin light forms characterization (1)

Classical Western blot

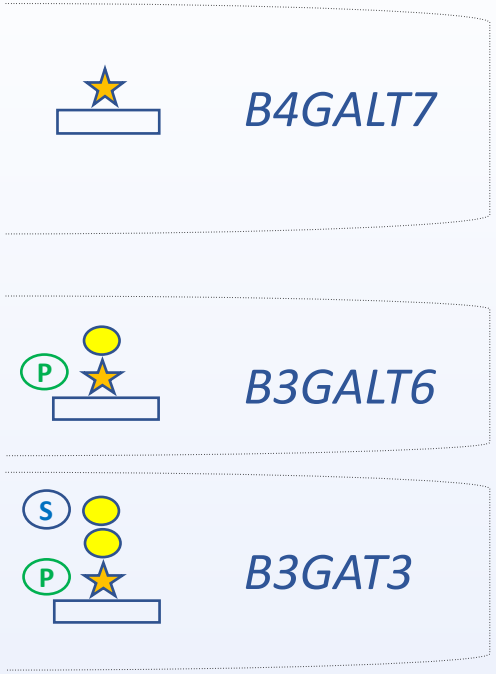


Two-dimensional electrophoresis (2-DE)



mixed serum samples

Hypotheses



Charge & MW differences
Signature profiles

Haouari et al 2020, JIMD

Abnormal Bikunin light forms characterization (2)

Patient Serum

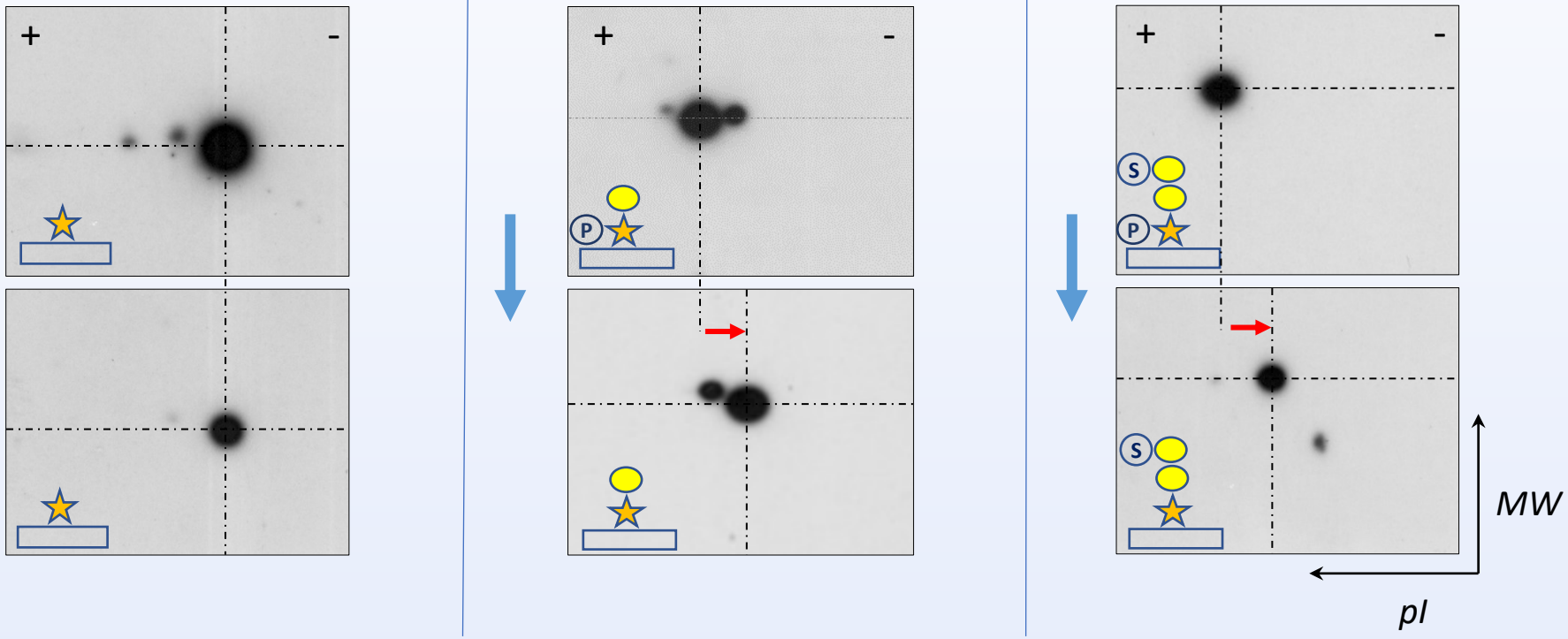
\+ Alkaline phosphatase (ALP)

2-DE

B4GALT7 *B3GALT6* *B3GAT3*

Patient serum

ALP treated patient serum



Haouari et al 2020, JIMD

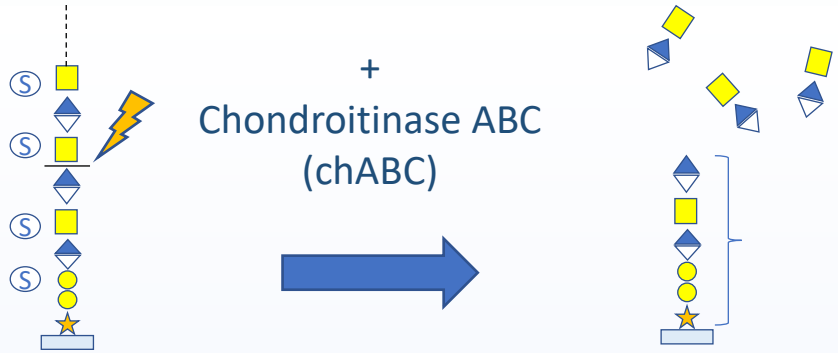
Abnormal Bikunin light forms characterization (3)

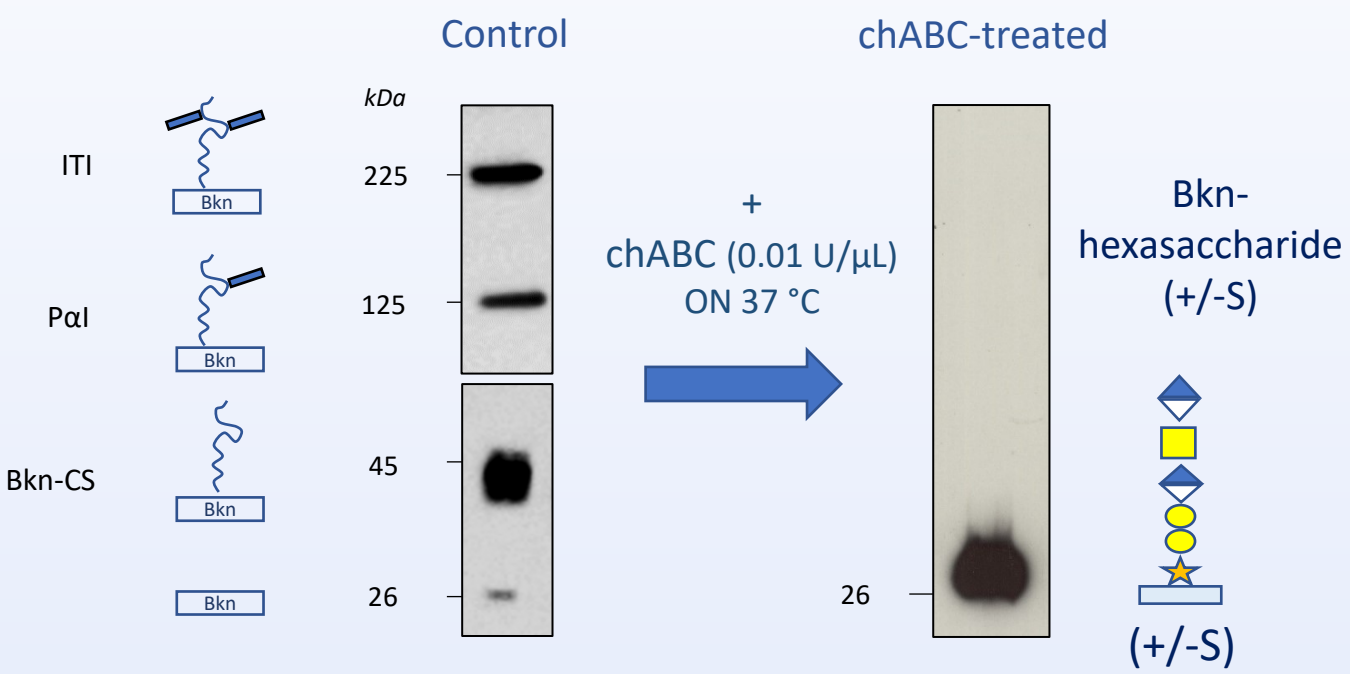
Development of a mass spectrometry method

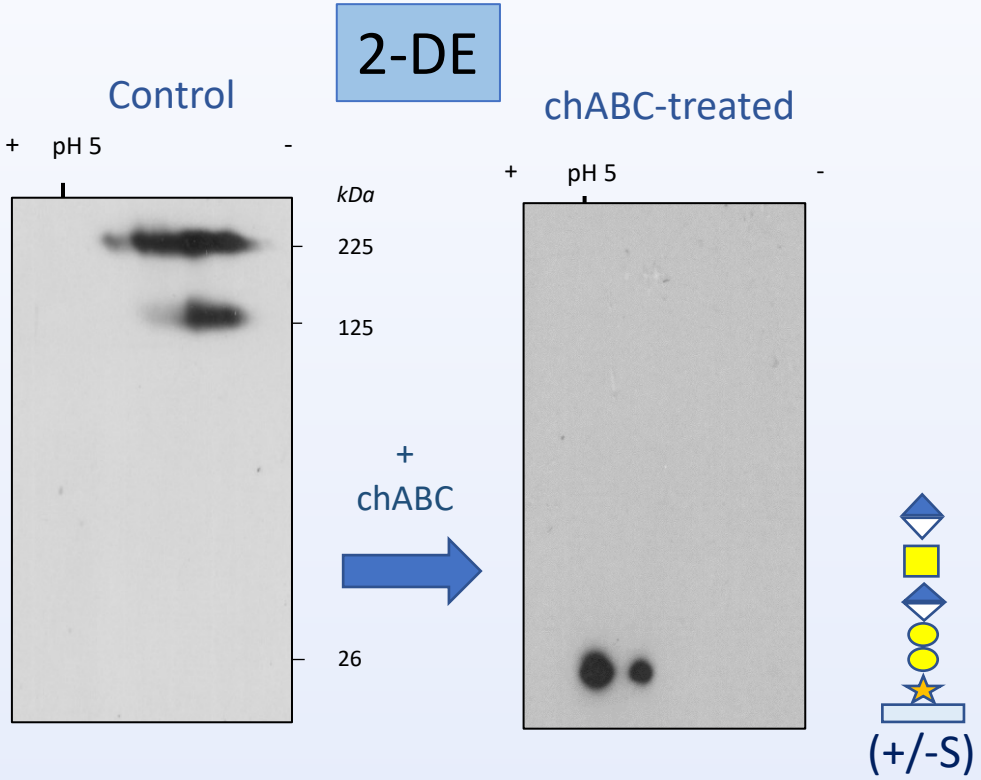
Plateforme de protéomique

Dr. Stéphanie Nicolaÿ & Guillaume Ruellou

Generation of abnormal Bikunin light forms in control serum

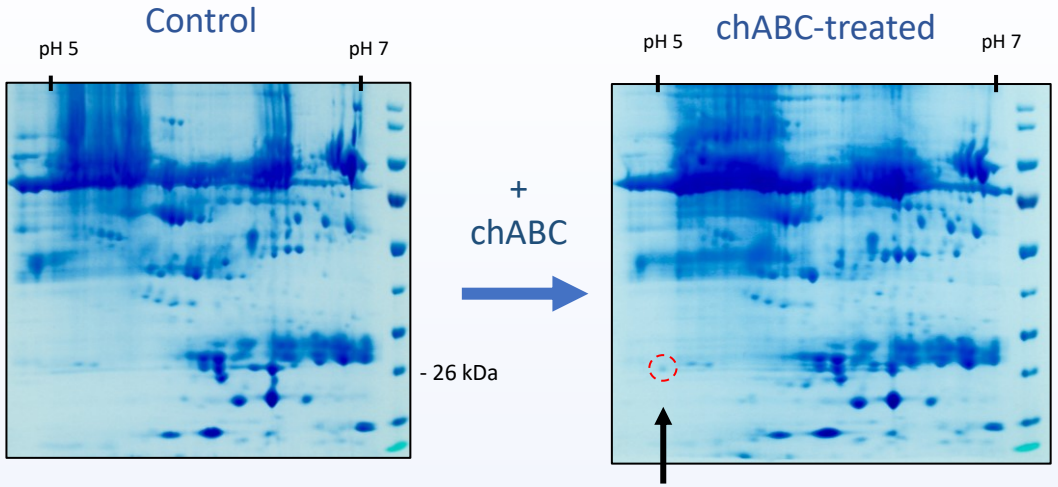




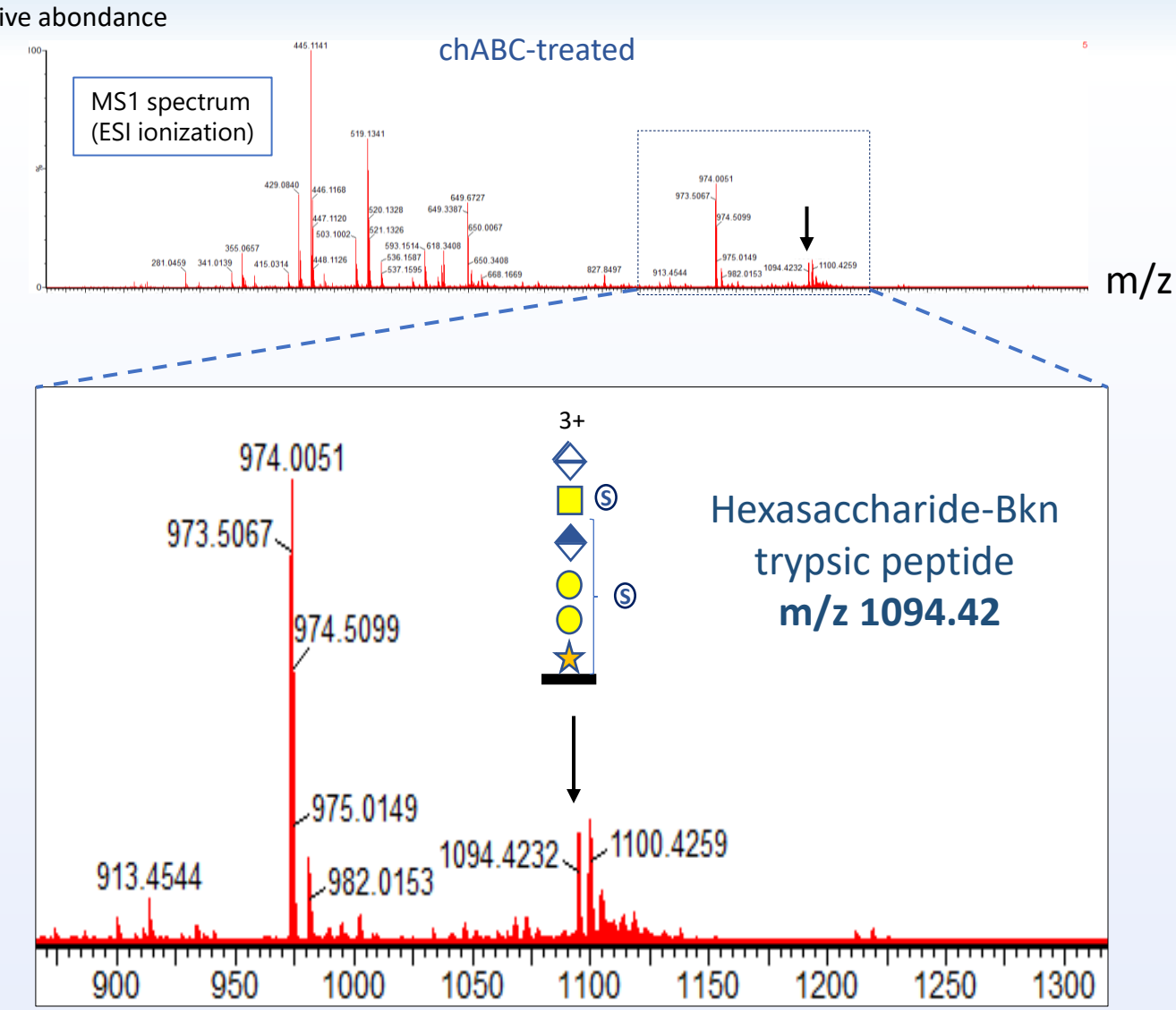


2-DE based purification and LC/MS identification of Hexasaccharide-Bkn

2-DE purification



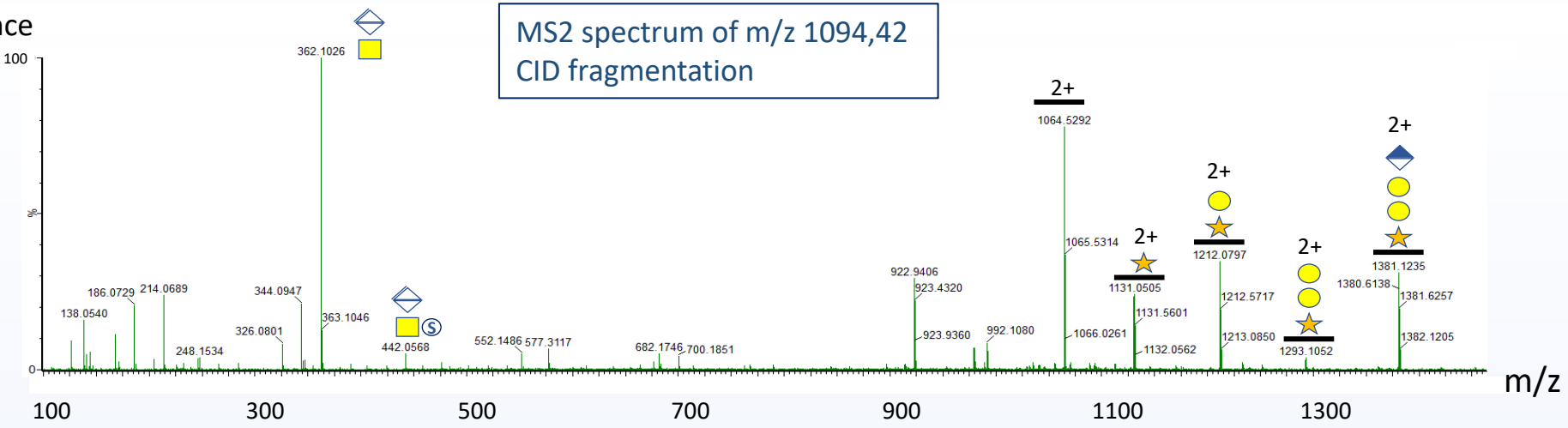
- Collection of gel pieces (n=8)
- Trypsin digestion
- Peptide extraction
- LC/MS/MS analysis



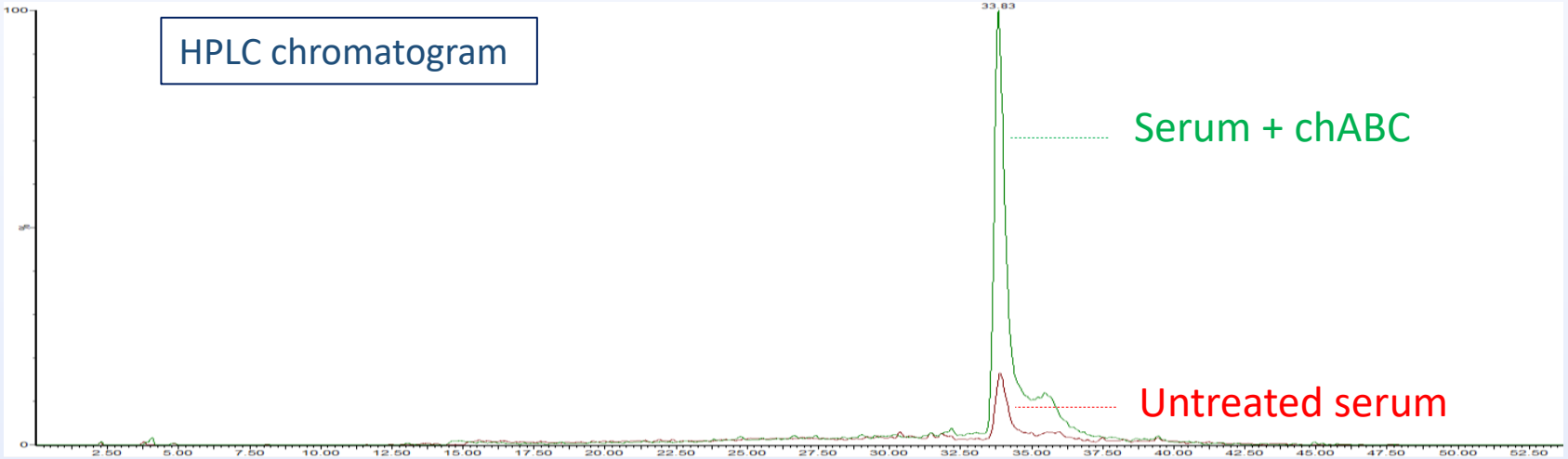
Acquity UPLC M-Class / Xevo® G2-XS QToF

Isolated chromatogram & MS2 spectrum (m/z 1094.43)

Hexasaccharide-Bkn trypsic peptide fragments



Hexasaccharide-Bkn ionic current m/z 1094.42



Search for Xyl-Bkn in *B4GALT7* deficient patient

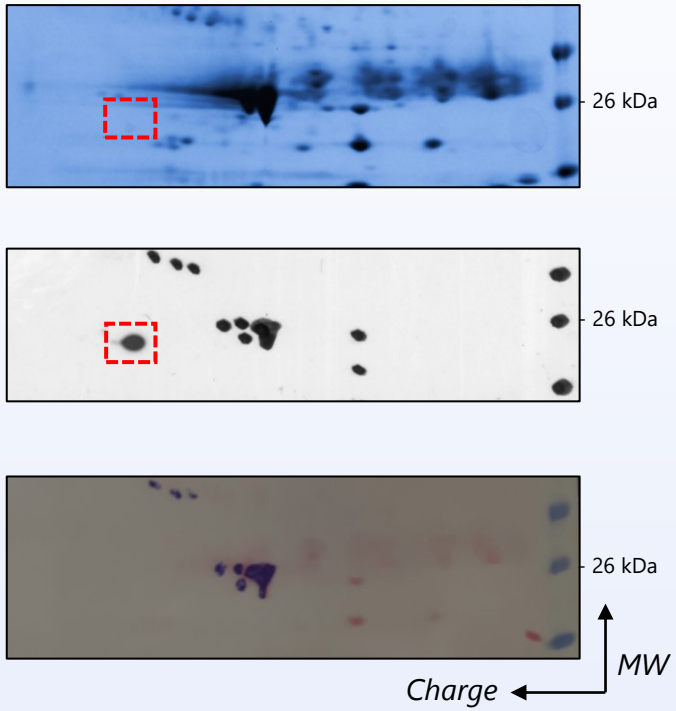
2-DE purification

B4GALT7 deficient patient serum

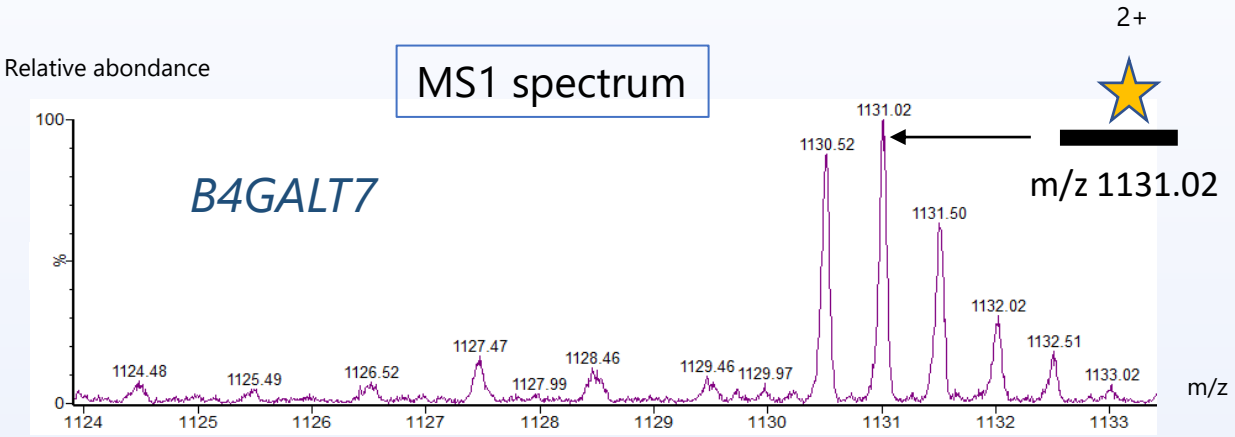
2-DE gel
Coomassie blue silver staining
(n=8)

2-DE western-blot film
(reference points from Ponceau red)

Ponceau red
(nitrocellulose membrane)



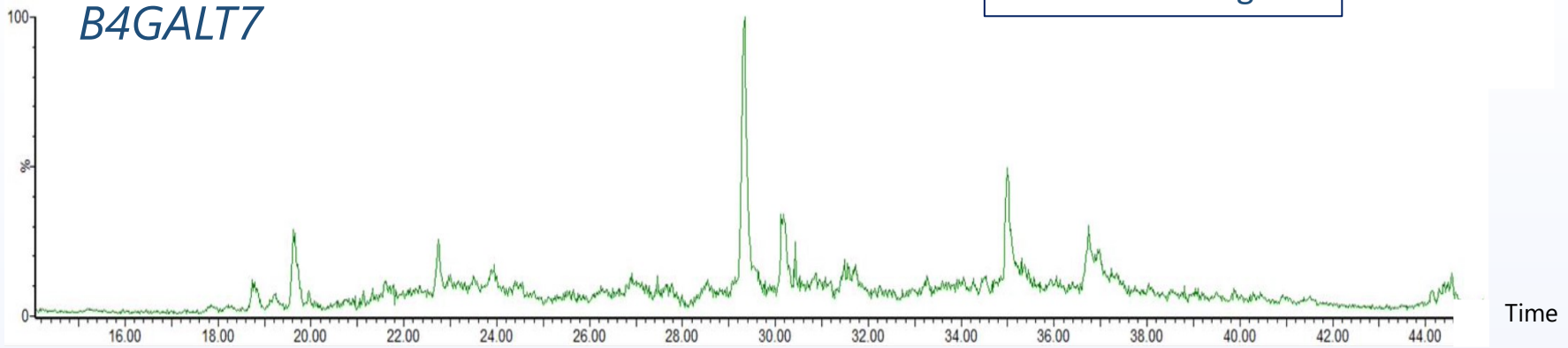
HPLC/MS analysis

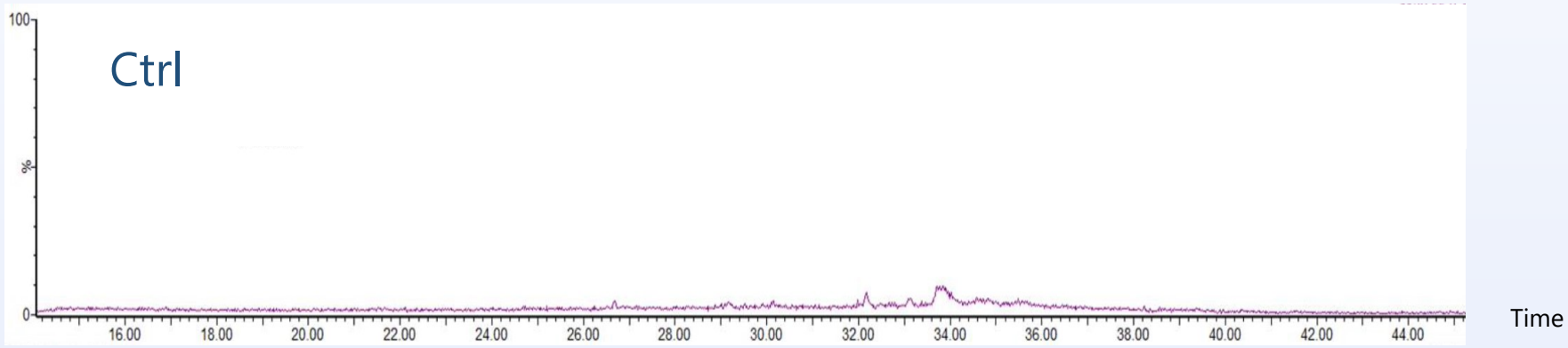


Xyl-Bkn isolated HPLC chromatogram

2+

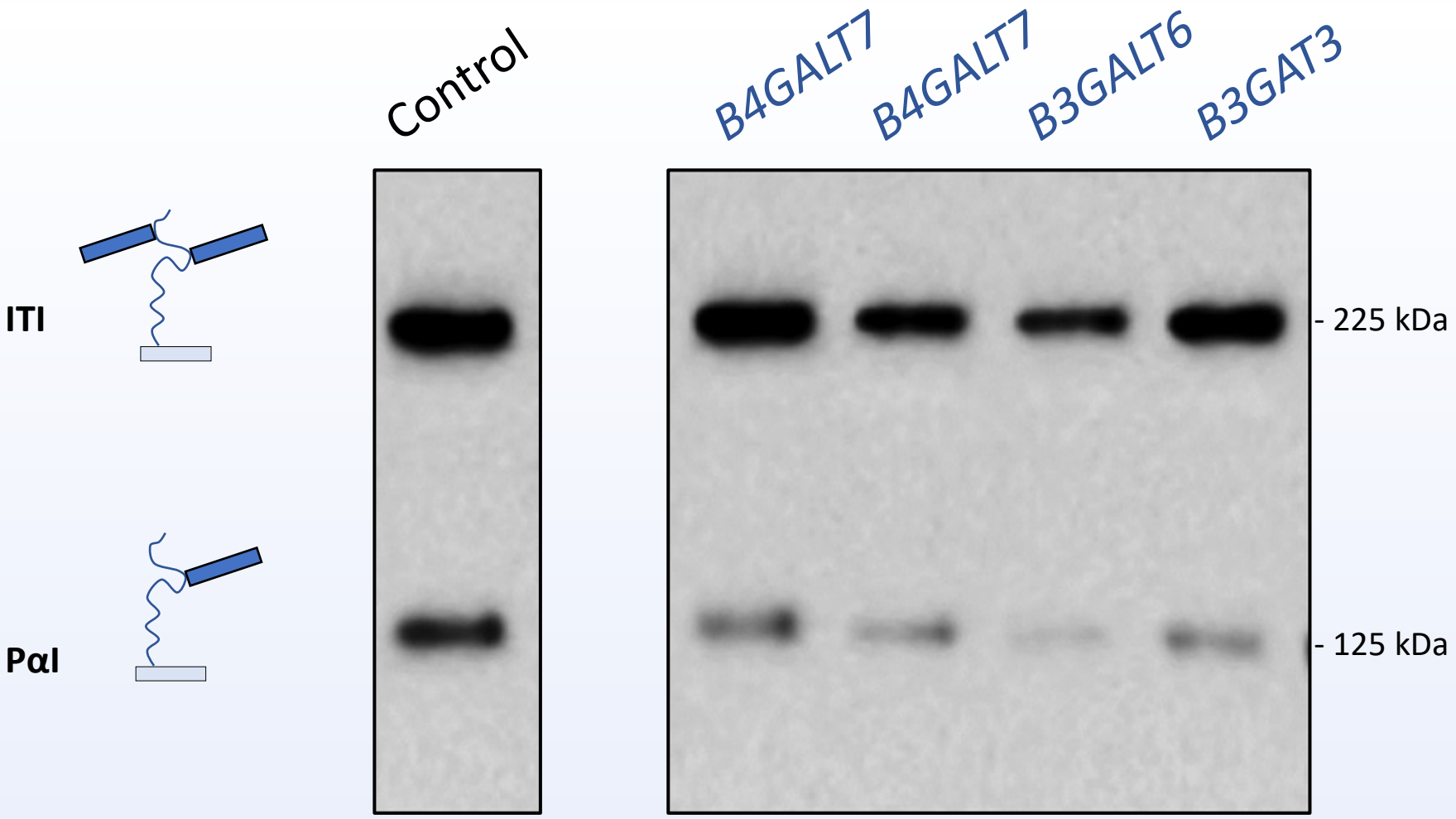
m/z 1131.02





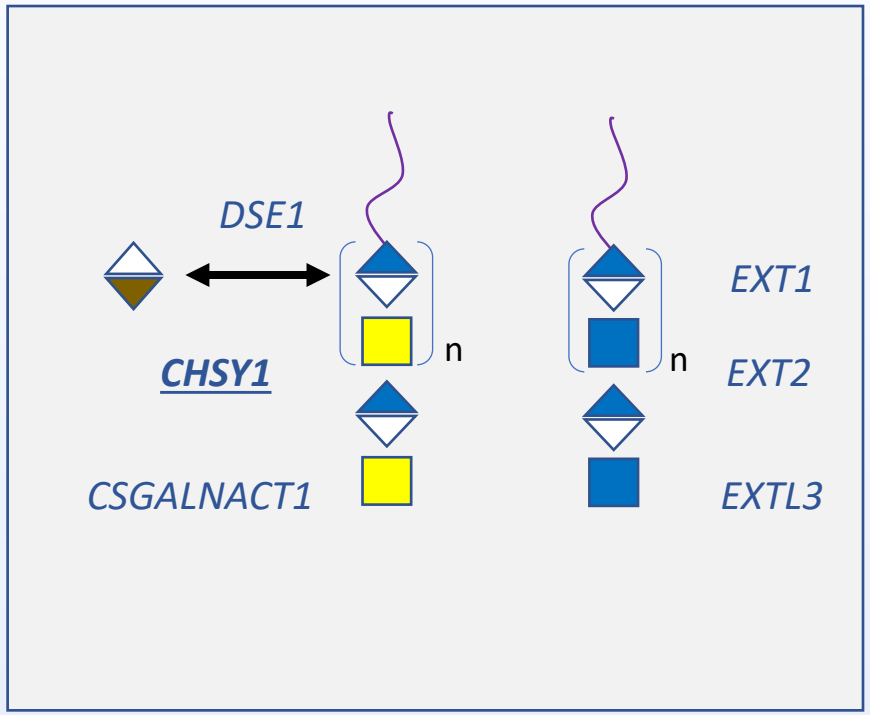
Ongoing analyses for *B3GALT6* and *B3GAT3* linkeropathies

Western blot of Bkn heavy forms in linkeropathies

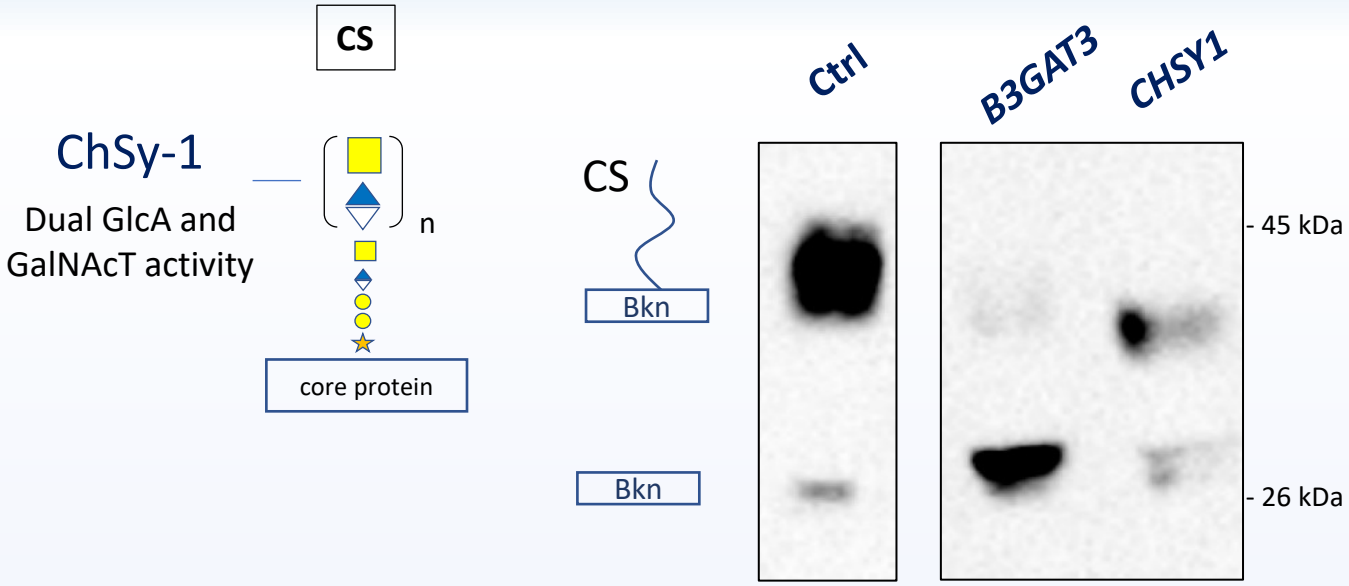


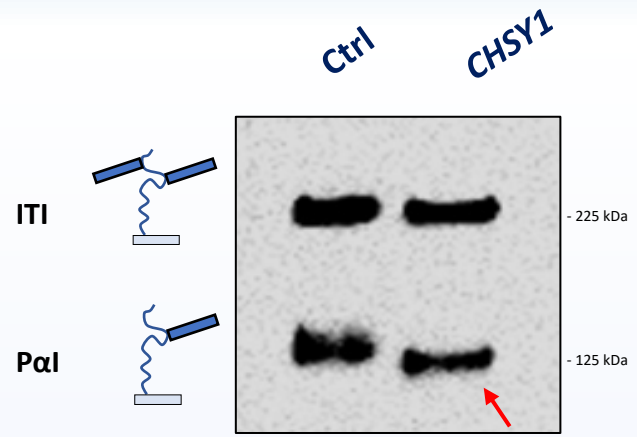
Apparently normal HC-CS esterification suggesting sufficient residual enzymatic activities

Bkn analyses in GAG elongation defects

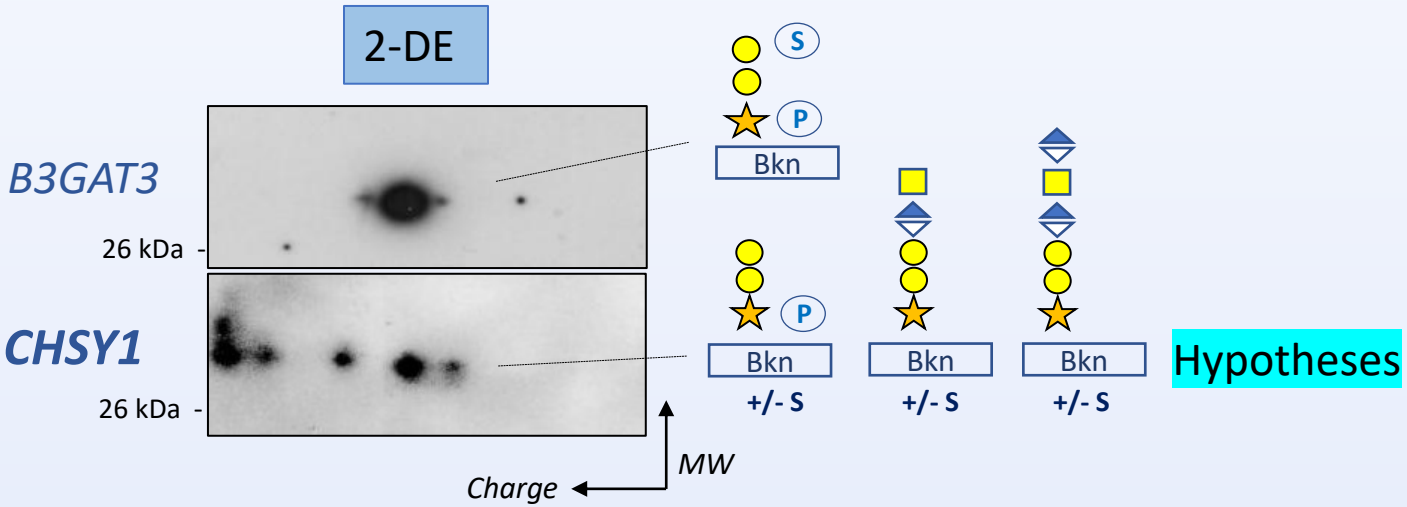


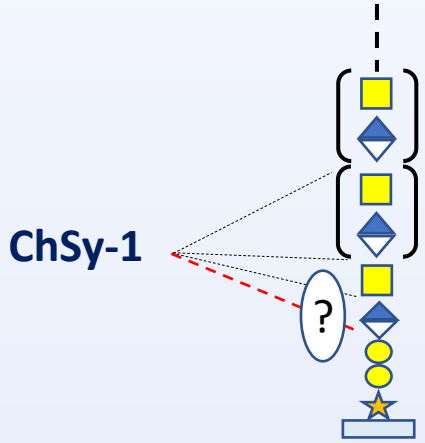
Bikunin analyses in ChSy-1 deficient patient





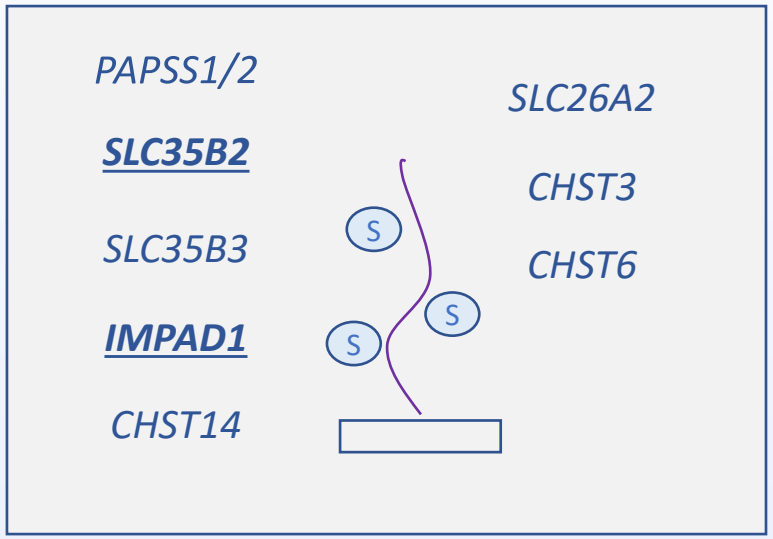
HC-CS esterification is still possible despite CS shortening in this patient

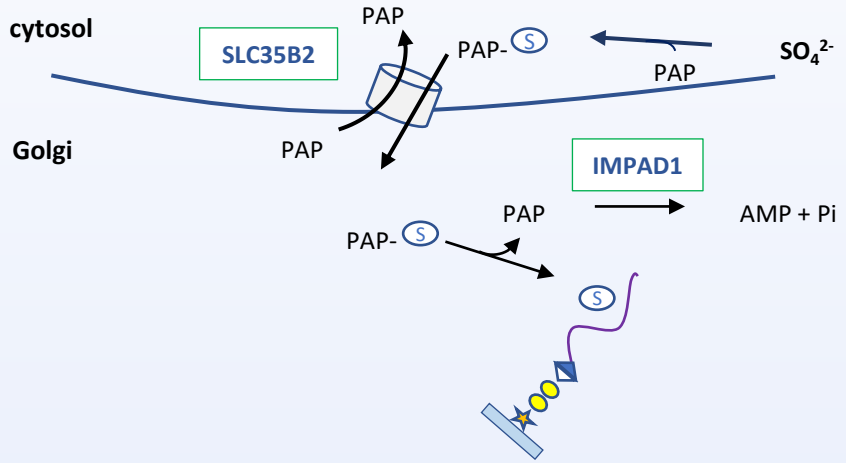




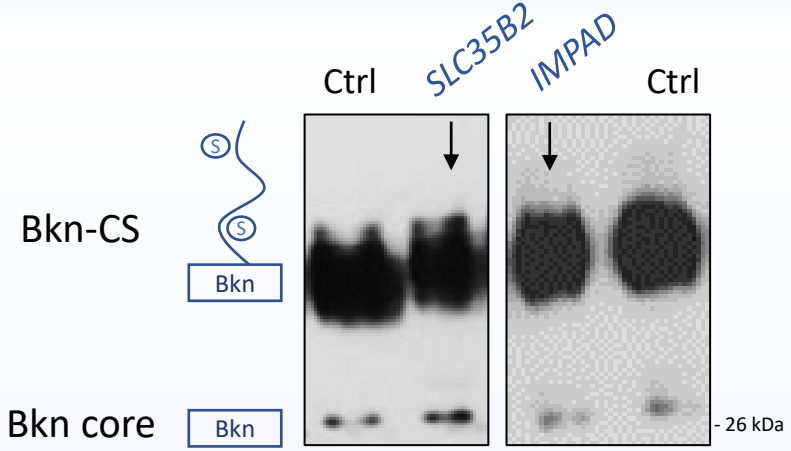
ChSy-1 could also be involved in the linker formation

Bkn analyses in GAG sulfation defects

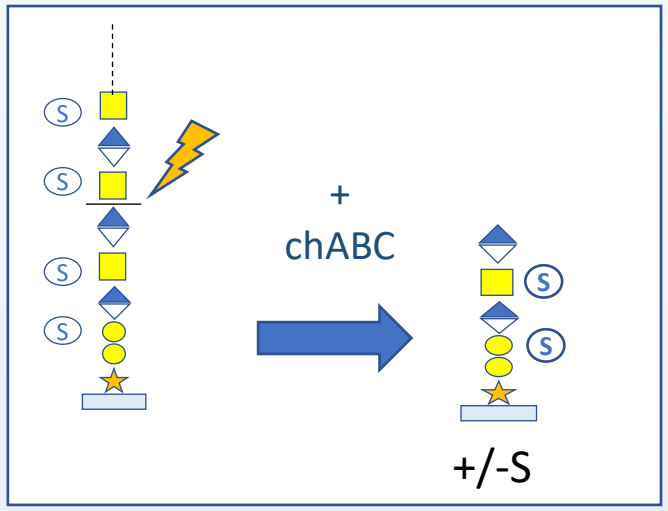


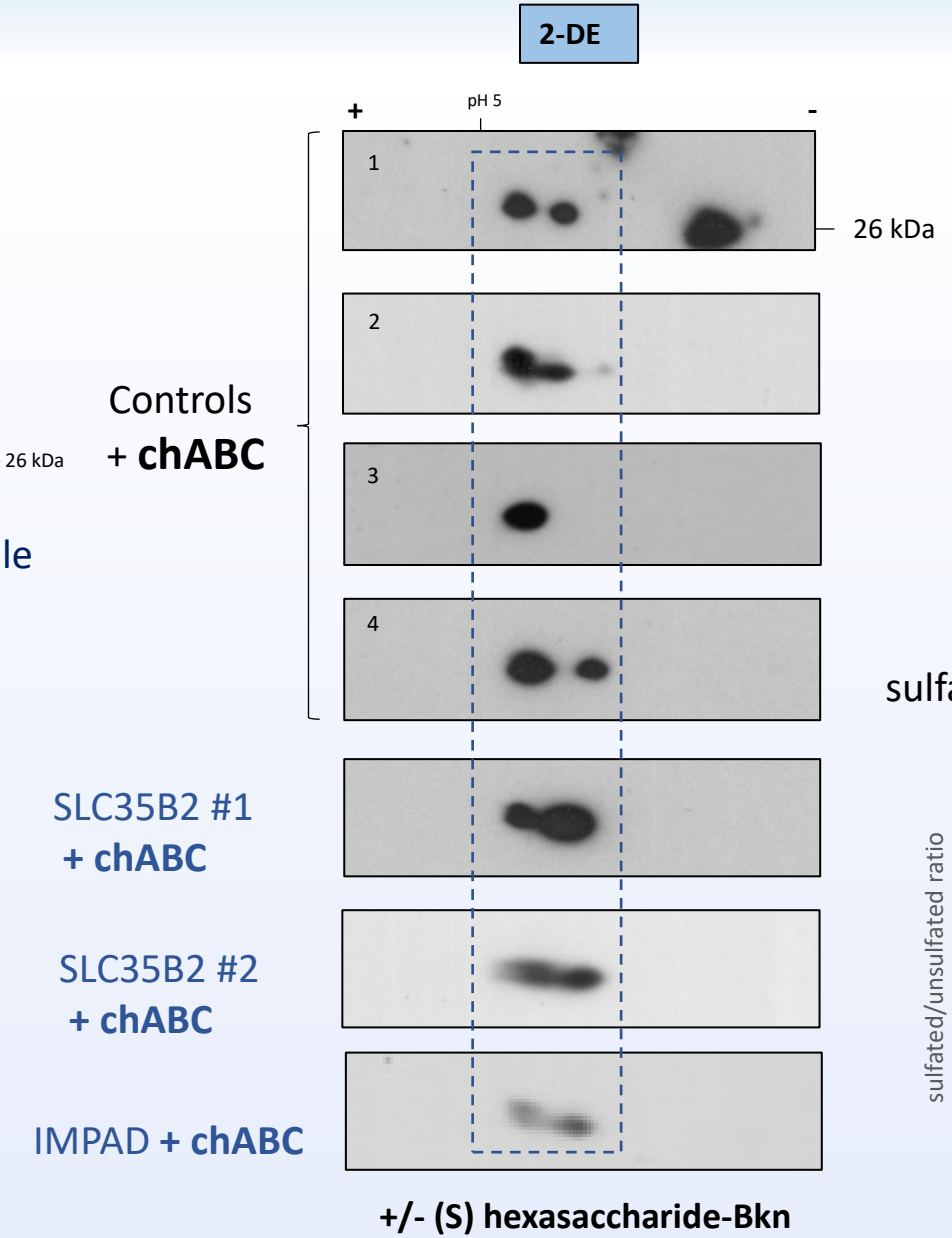


Bikunin analyses in *SLC35B2* and *IMPAD1* deficiencies



Normal Western blot profile

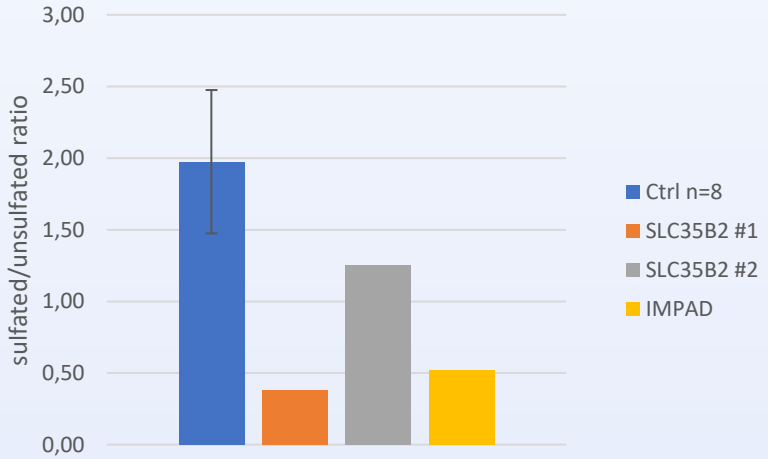




Hypotheses

Left spot = sulfated hexasaccharide-Bkn
Right spot = undersulfated hexasaccharide-Bkn

Ratio
sulfated/unsulfated



Bikunin analyses in UDP-sugar supply & Golgi homeostasis defects

SLC35A2

SLC35A3

SLC35D1

CANT1

UDP - **Sugars**

Substrate synthesis and transport

ATP6V0A2

CCDC115

ATP6AP1/2

ATP6V1F

TMEM165

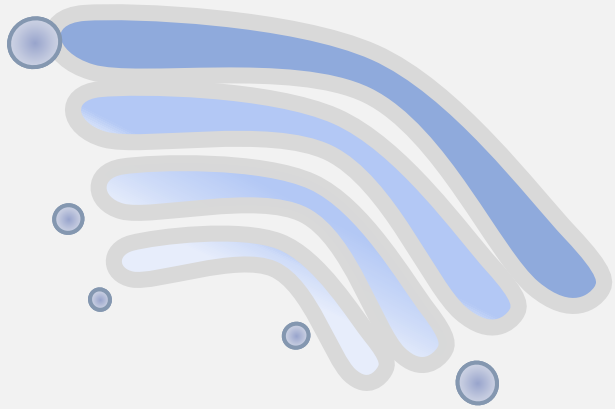
SLC39A8

COG

GORAB

SLC10A7

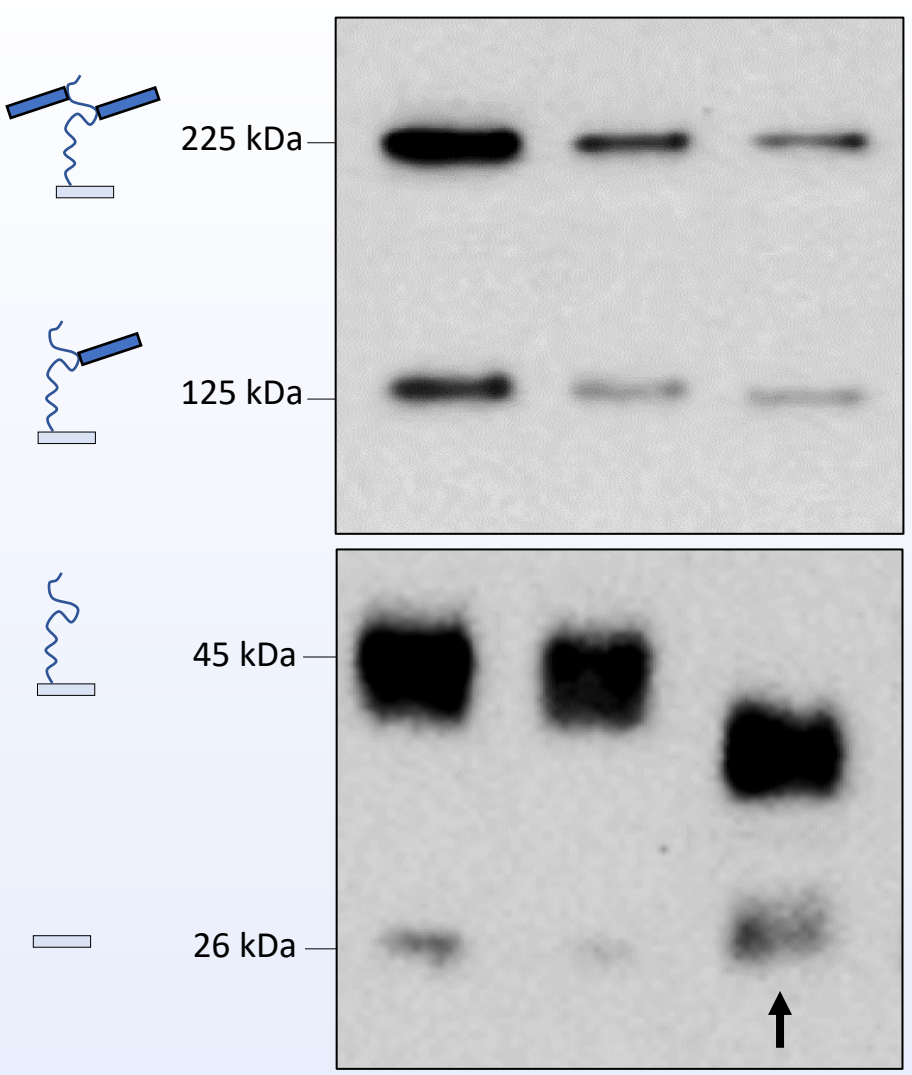
SLC37A4

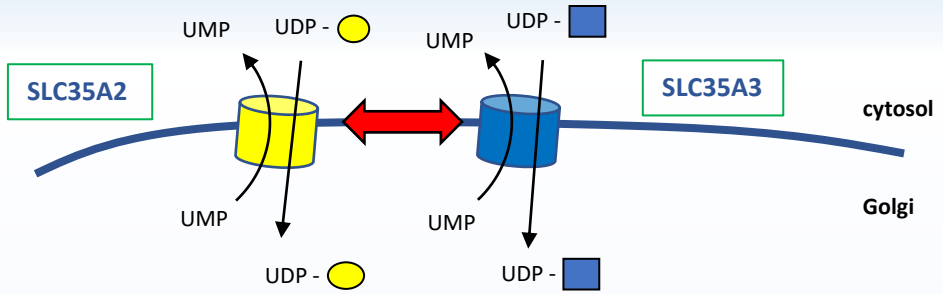


Golgi homeostasis

Western blot of Bikunin in SLC35A2/A3 deficiencies

CDG





For these patients

- *N*-glycosylation defects → Congenital disorders of glycosylation (CDG)
- Normal Bkn profile in SLC35A2 despite 2 Gal in the linker

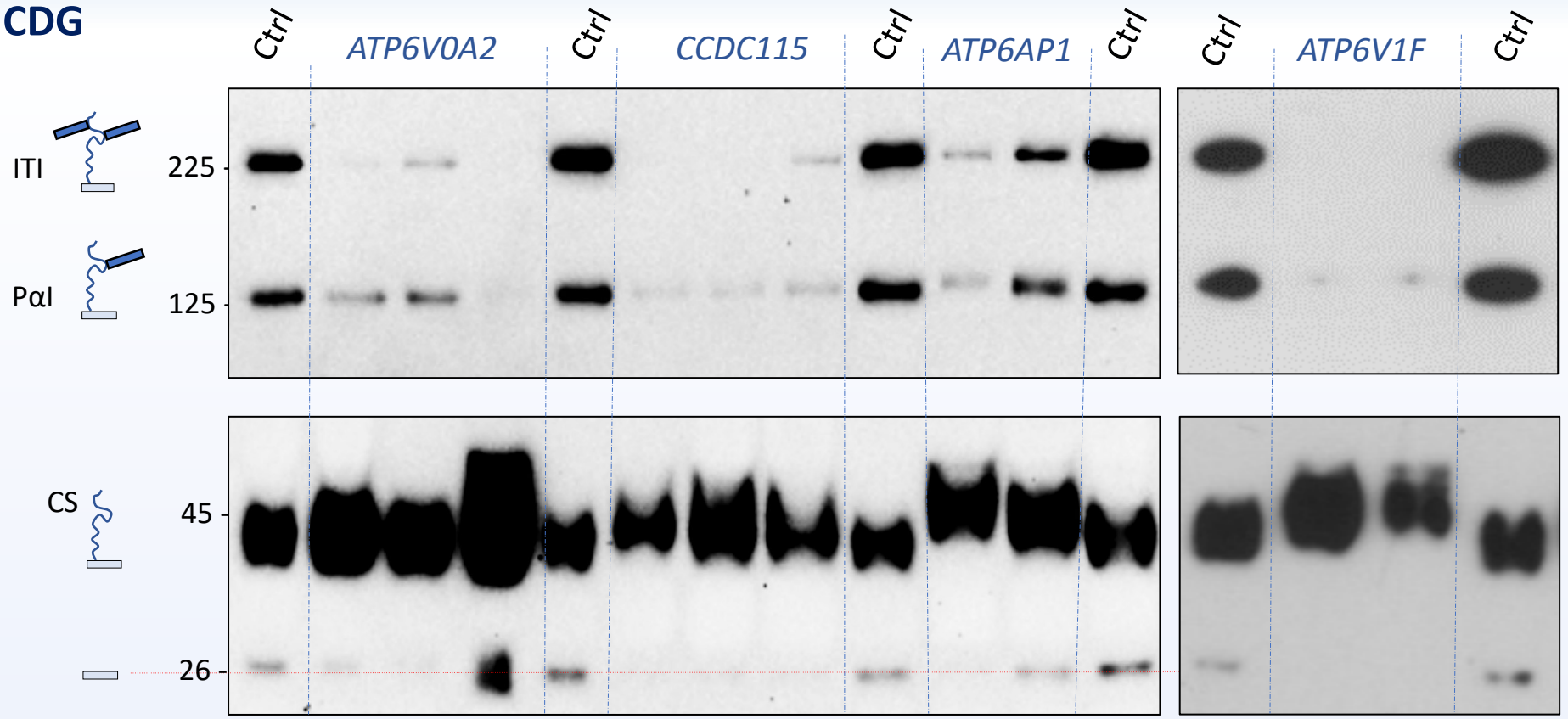
→ Residual activity towards PG biosynthesis?

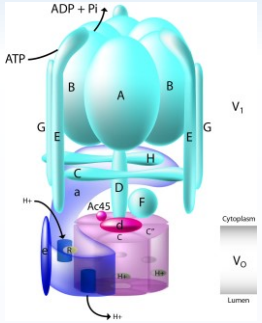
- Abnormal Bkn light forms in SLC35A3 despite lack of GlcNAc in CS

→ Alteration of SLC35A3-A2 interation leading to defective Gal delivery ?

Bikunin analyses in V-ATPase defects

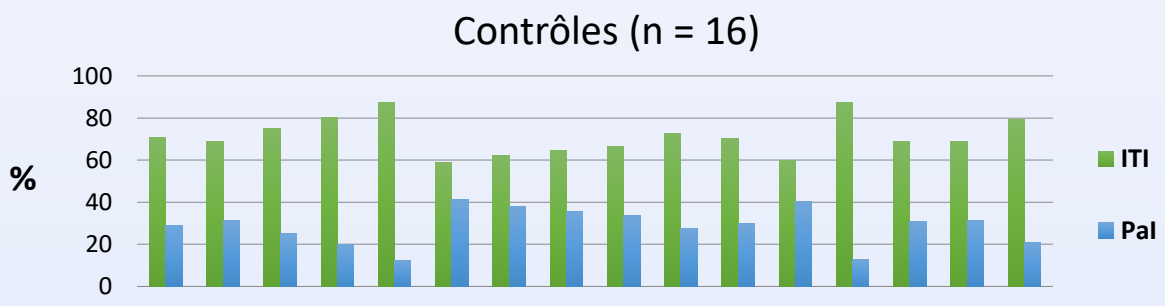
CDG

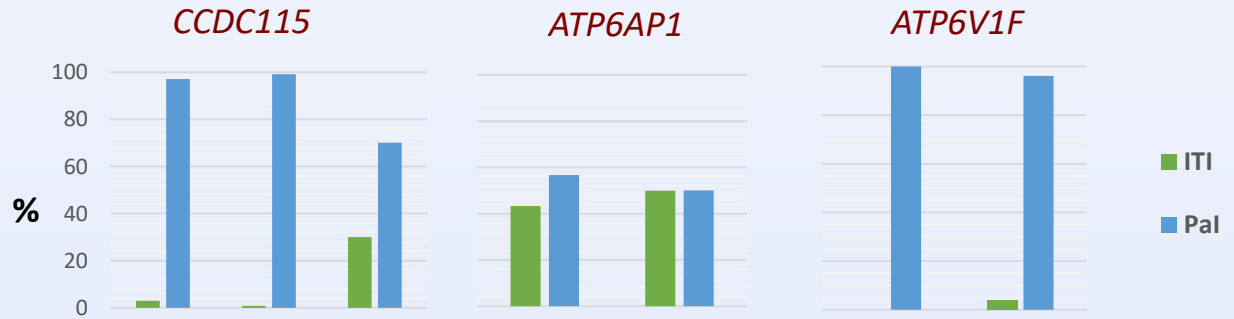




Golgi V-ATPase defects: **Alkalization**

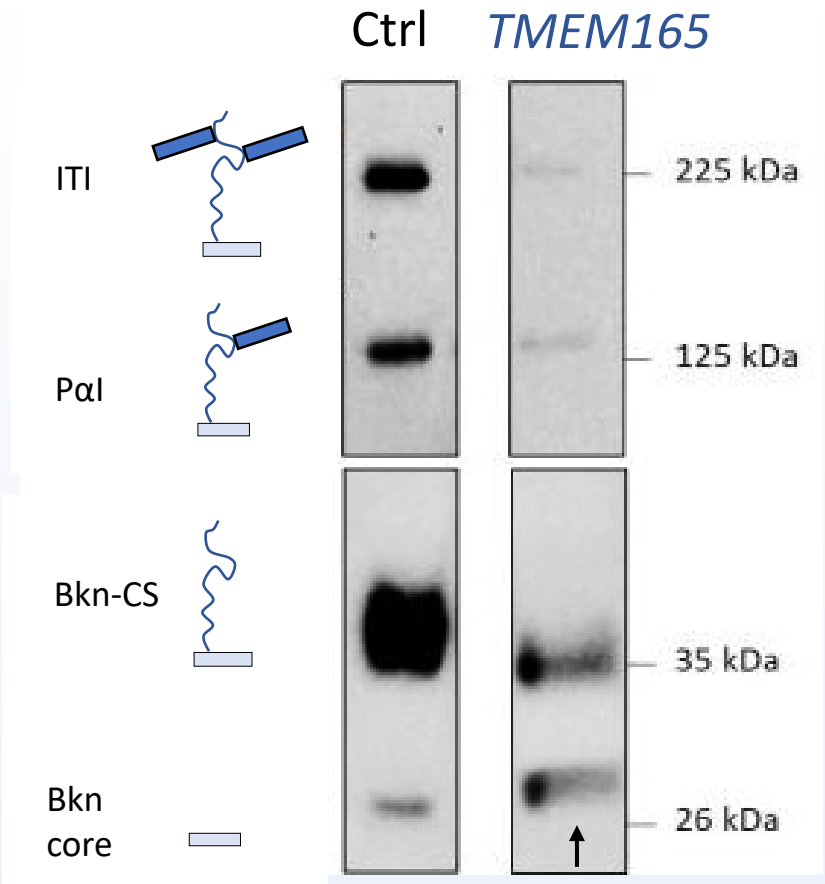
- Decreased ITI and PαI levels
- Inversion of ITI/PaI ratio
- pH dependent HC-CS esterification

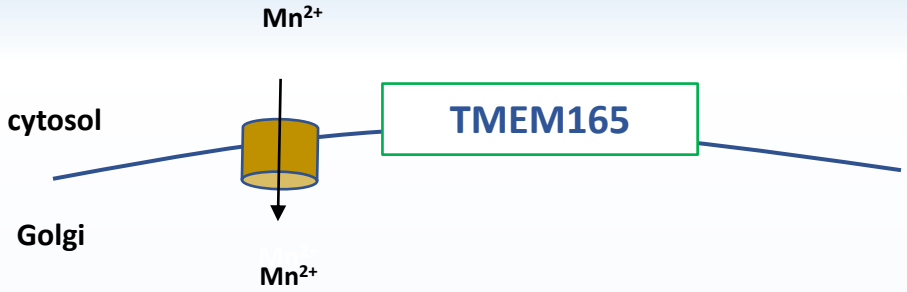


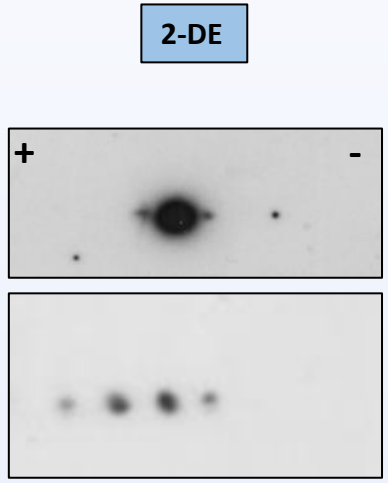


Bikunin analyses in TMEM165 deficient patient

CDG



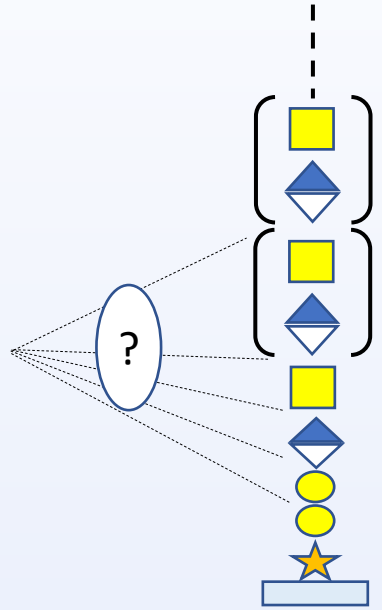




B3GAT3

TMEM165

CS elongation



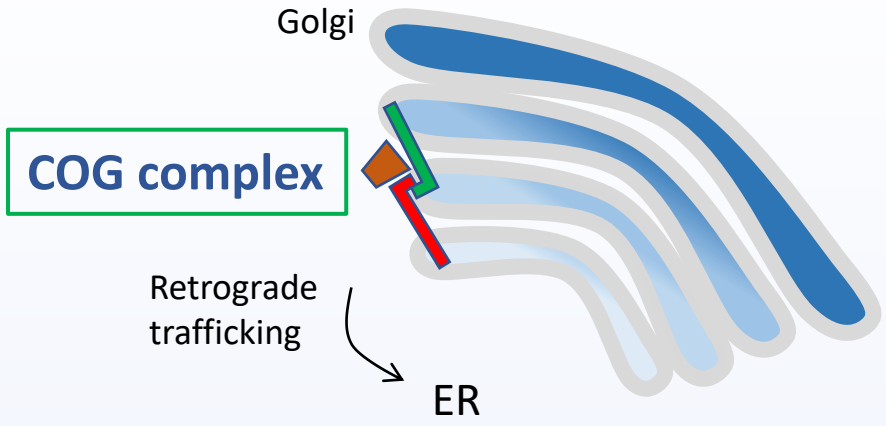
- Defective GAG synthesis (linker & CS)
- Defective HC-CS esterification

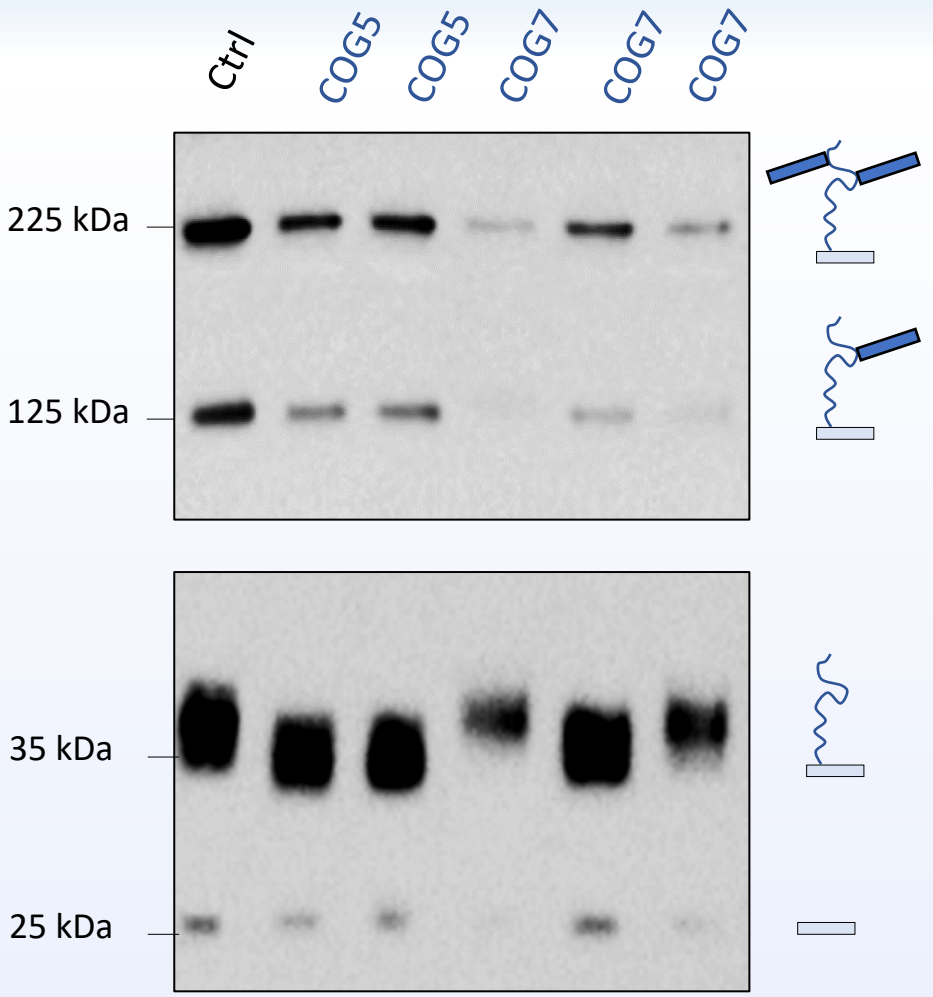
Mn^{2+} is involved in several steps of CS elongation

Sample from Pr. François Foulquier Univ. Lille

Bikunin analyses in CDG with COG deficiencies

CDG





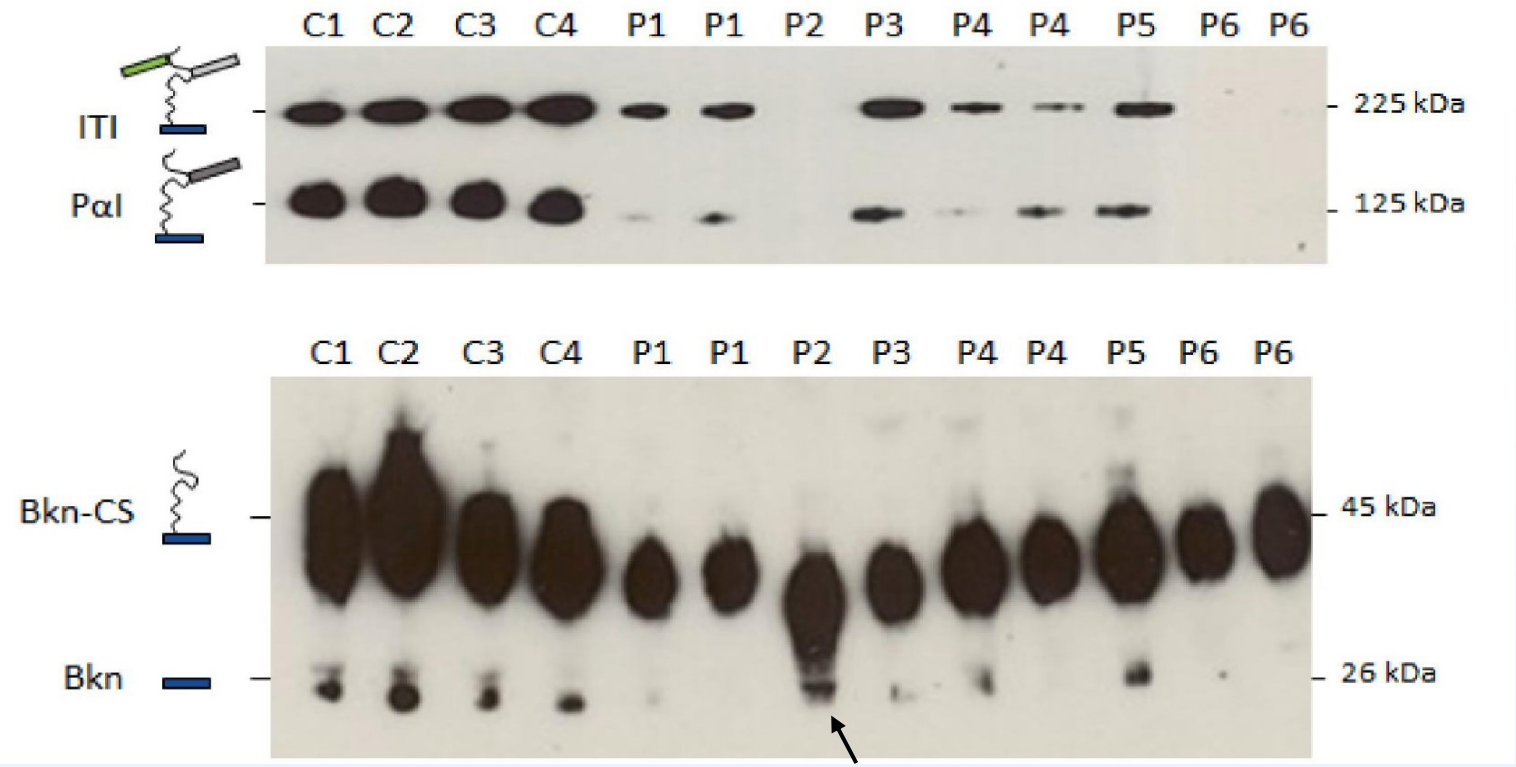
- N- & O-glycosylation defects → CDG
- Qualitatively normal Bikunin profile
- Separate N/O-glycosylation and proteoglycan pathways ?

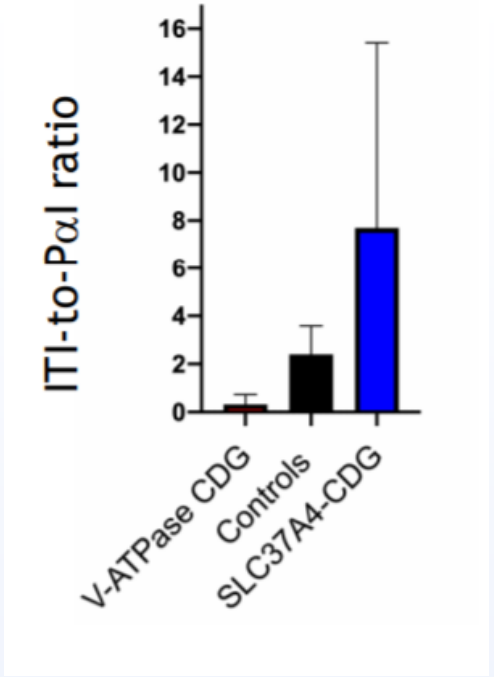
Haouari et al., JIMD **2020**

Bikunin analysis in SLC37A4

CDG

Golgi hyper acidification





- Impaired CS-HC esterification → pH defects
- PG defect in one patient (most severe phenotype)

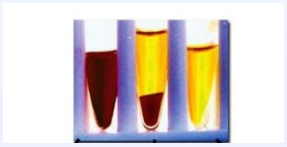
Raynor A, Haouari W et al., CCA, 2021

Bikunin analyses in dried blood spots

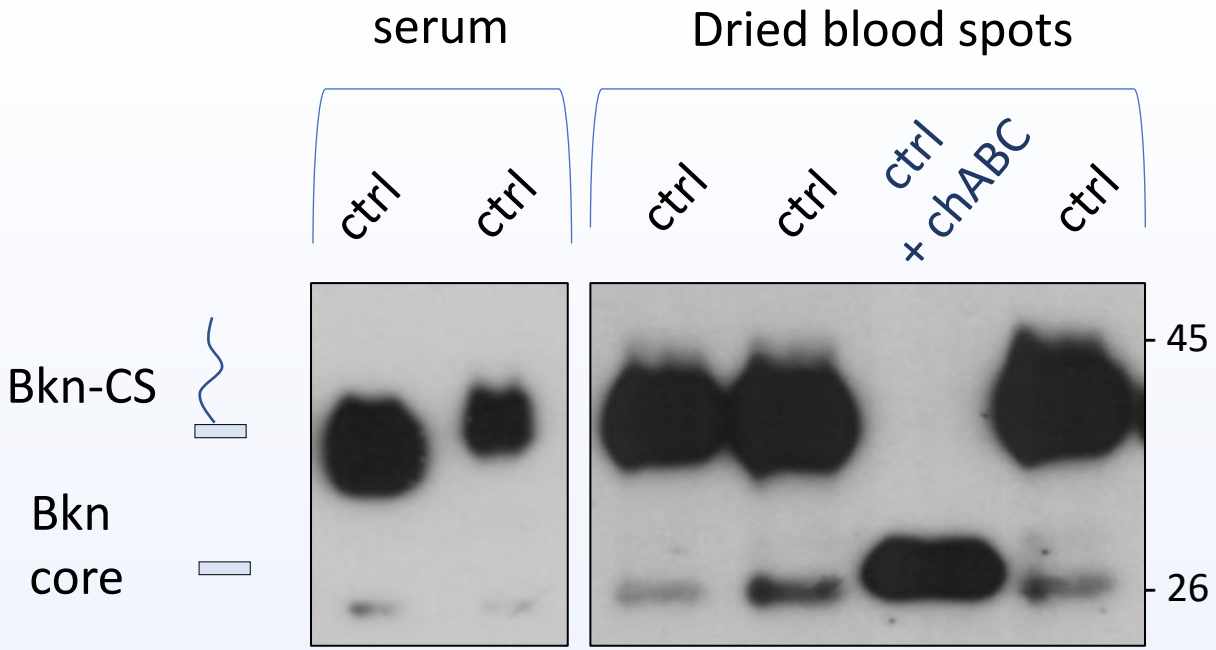
Dried Blood Spot

Blood extraction

Hemovoid™



Hemoglobin depletion



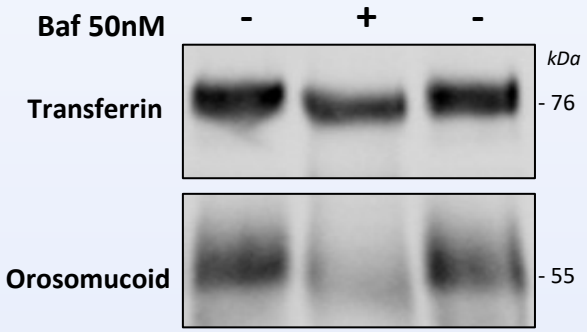
Encouraging results for further application in patients

Bikunin analyses in HepG2 with Golgi homeostasis defects

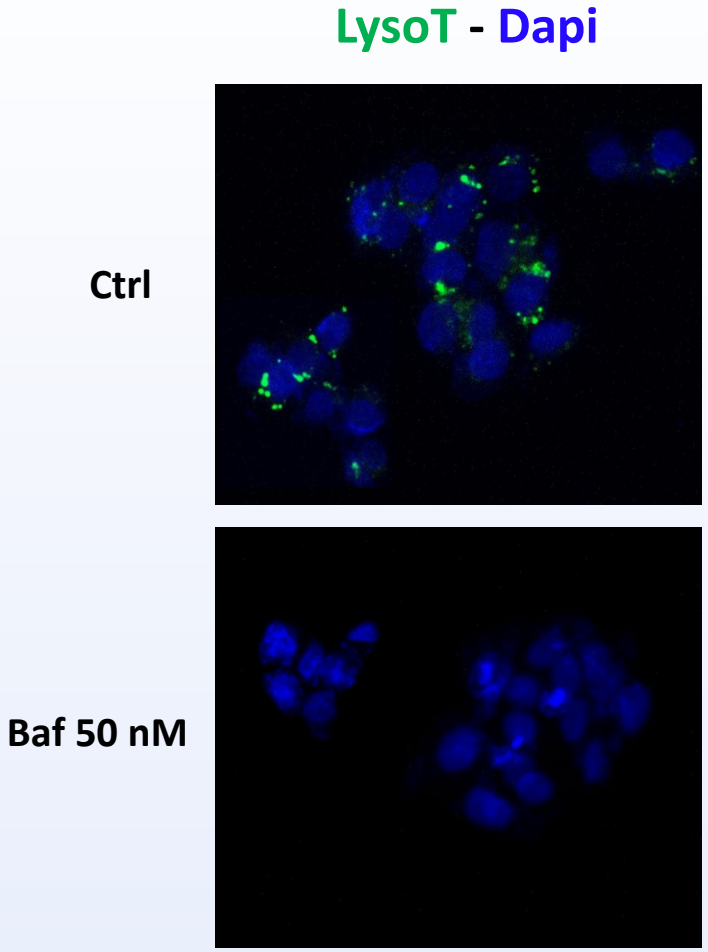
BafA1 treatment of HepG2 cells

Bafilomycin (Baf)

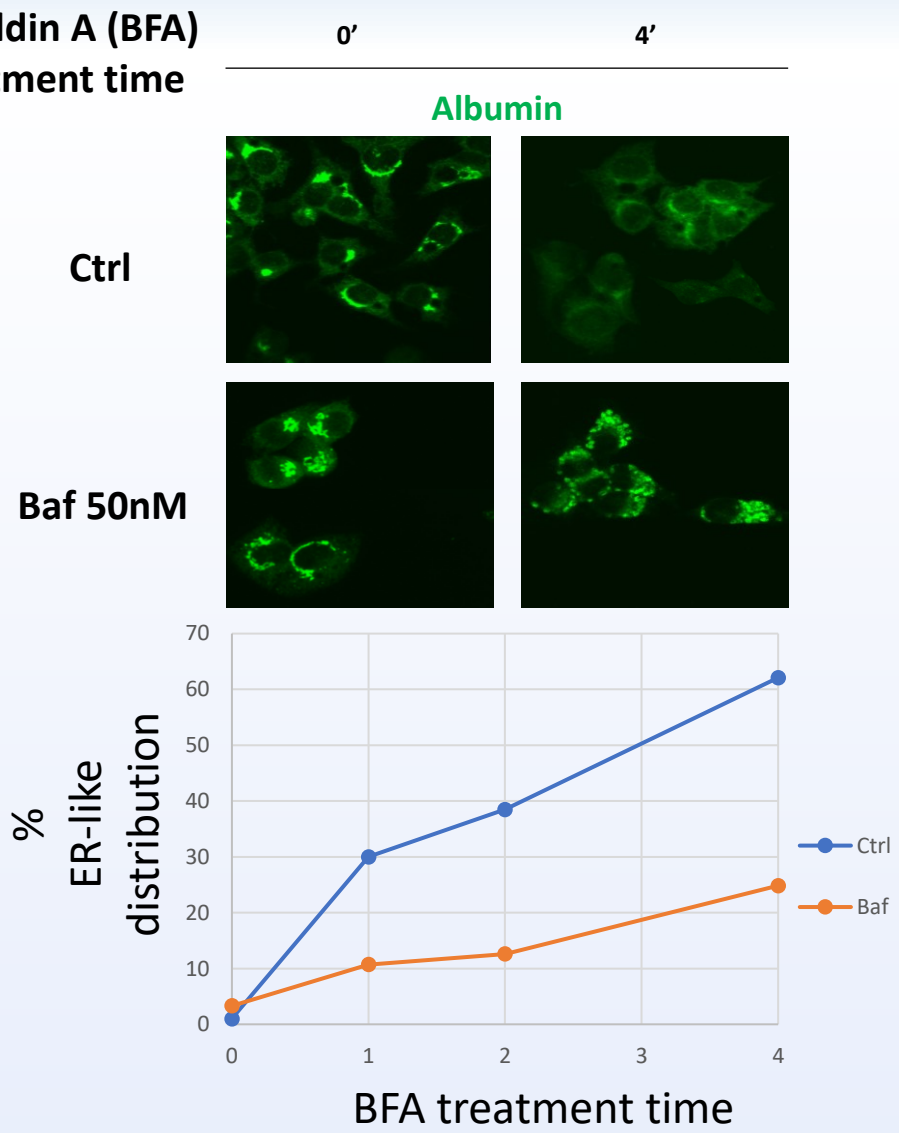
- V-ATPase inhibitor
- Traffic disturbances
- Impaired protein glycosylation



N-glycosylation defects



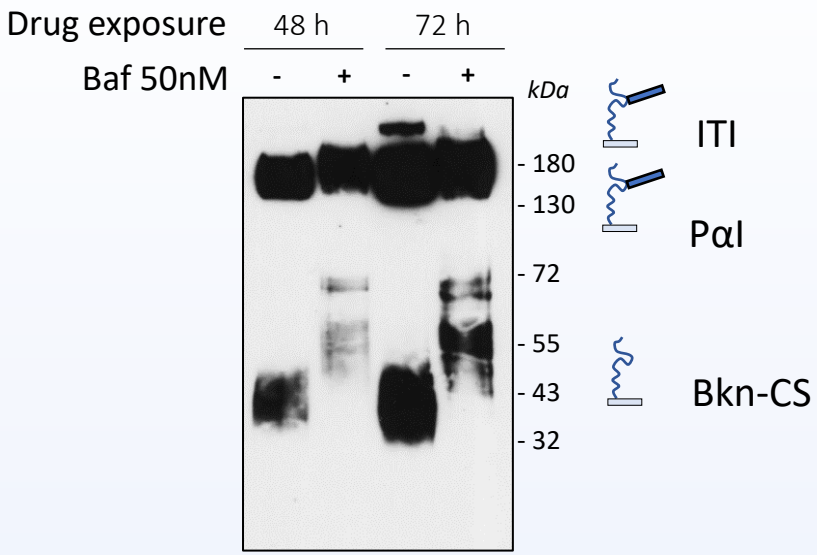
Golgi alkalization



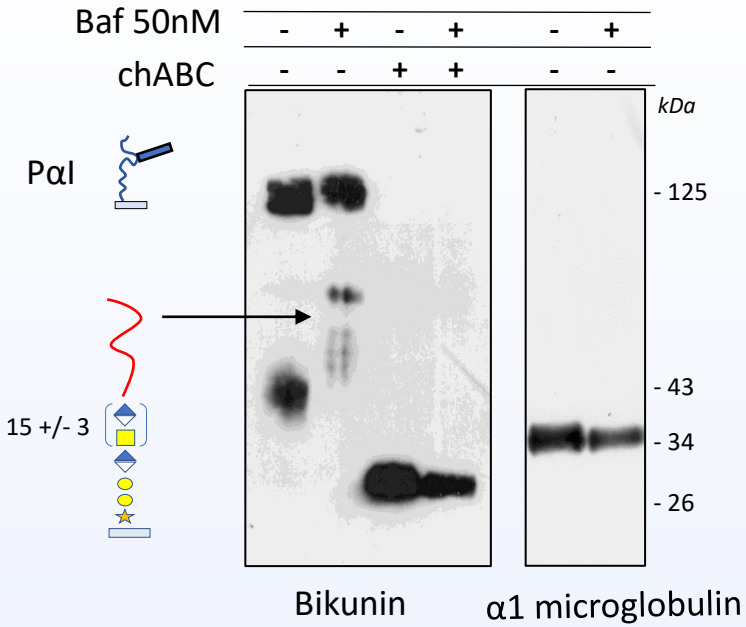
Defective retrograde trafficking

Western blot of Bikunin in Bafylomicin-treated HepG2 cells

cell supernatant

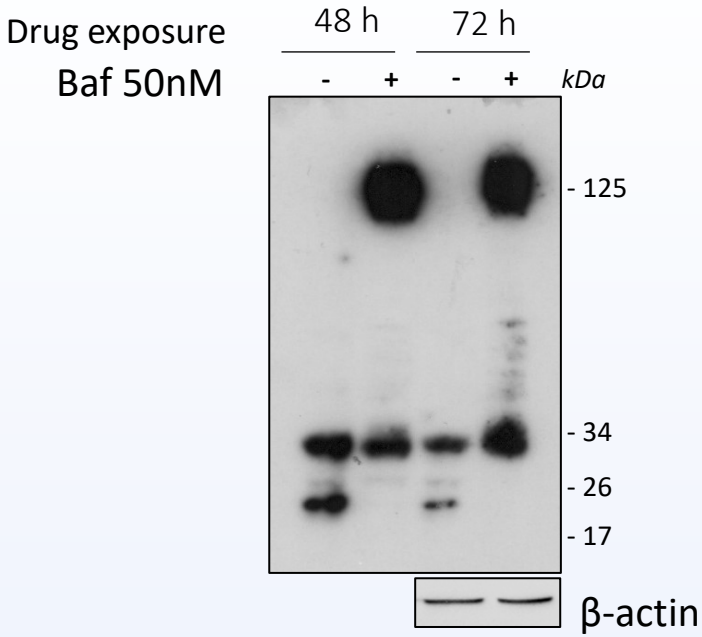


cell supernatant



CS chain overelongation

cell lysate



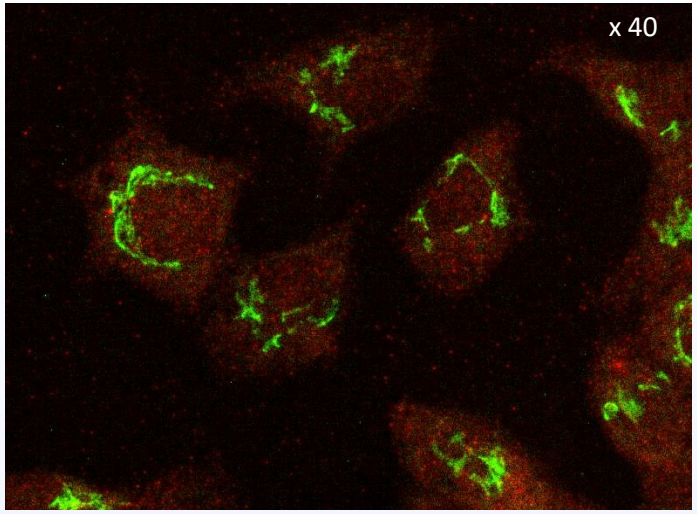
Defective
intracellular processing

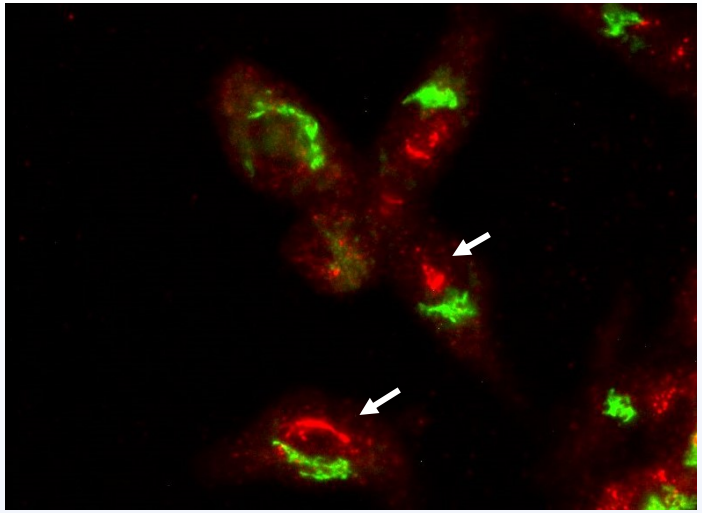
Immunostaining of intracellular Bkn in Baf-treated HepG2

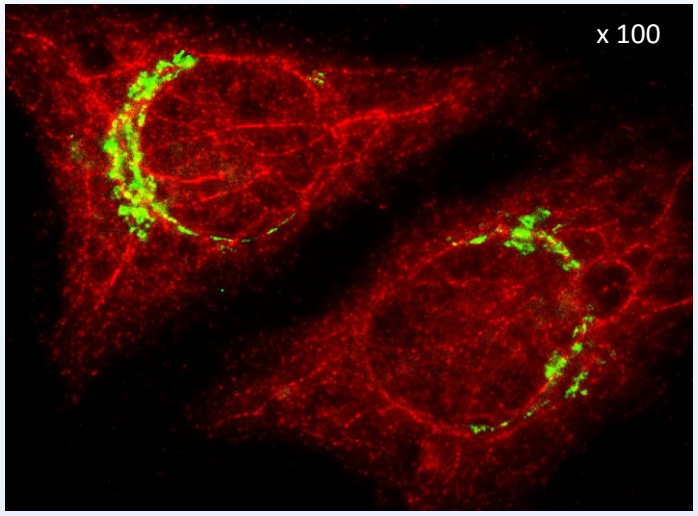
Bikunin
GM130

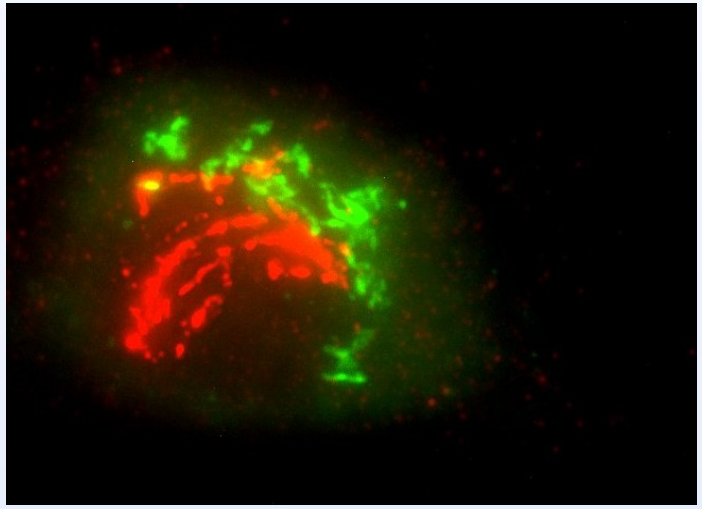
Ctrl

Baf 50 nM





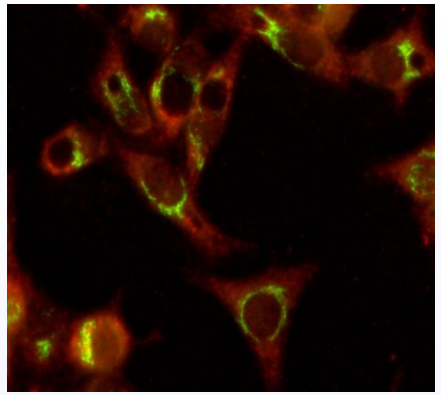


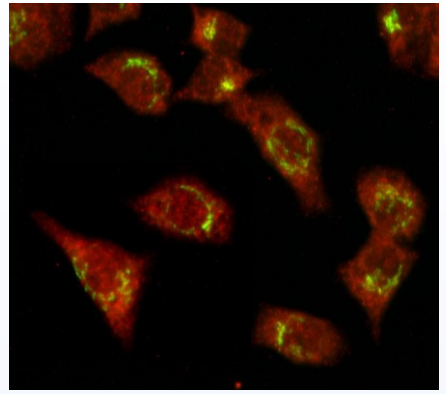


Oroso
GM130

Ctrl

Baf 50 nM





- BafA1-mediated Bkn accumulation into undefined compartments
- Separate PG secretory pathway?

General conclusions

- “Proteoglycanome” = complex network producing ubiquitous and fundamental compounds
- PG inherited defects are severe and rare diseases lacking efficient diagnostic tools
- Rapid and simple Bikunin analysis showed abilities as a convenient biomarker
- Application in linkeropathies, GAG elongation, GAG sulfation, substrate synthesis, and Golgi homeostasis defects
- Potential as in vitro marker of Golgi homeostasis defects
- Limitation: mutations in liver expressed genes leading to defective CS biosynthesis

Perspectives

- In depth biomarker validation: *B3GALT6*, *B3GAT3*, sulfation defects, dried blood spots
- Utilization in cellular and/or animal models: mechanisms' comprehension (Dr Samra Lounis)
- Specific roles of Bikunin isoforms in the pathophysiology of PG defects
- Exploration of other circulating PG as potential biomarkers

Acknowledgments

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