PEAK PROTEINS

Unlocking the structure of G6b-B by engineering of N- and O-linked glycosylation

PSDI 2019
3-5 November
Wellcome Conf. Centre
Hinxton.

Talk Outline

Structure determination of the ECD of platelet receptor G6b-B.

i) G6b-B brief background.

ii) Journey to solve the structure.

iii) Structure summary.

Vögtle et. al. *eLife*. 2019; **8**:e46840

PEAK PROTEINS

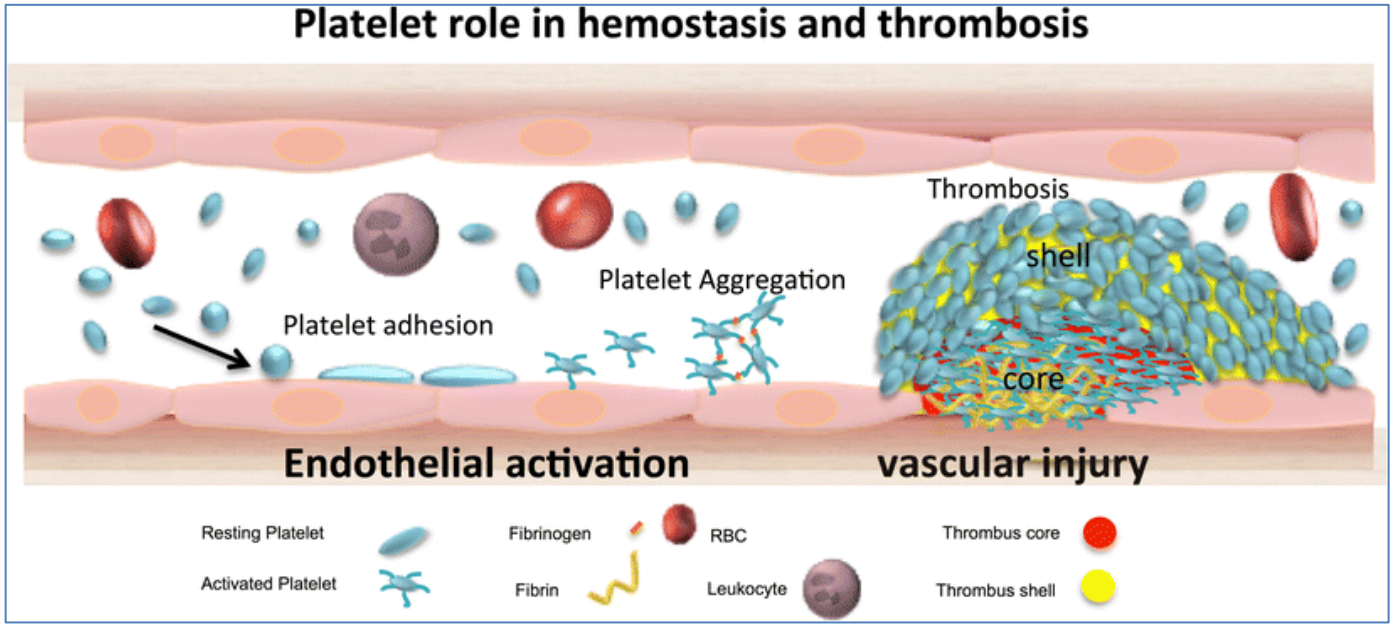
Client project – G6b-B

- Prof. Yotis Senis (University of Birmingham) – group studies regulation of platelets.
- Requested the X-ray structure of the extracellular domain of the megakaryocyte and platelet inhibitory receptor G6b (G6b-B):
    - In complex with the Fab fragment of a potential therapeutic monoclonal to help identify epitope for patent application.
    - In complex with heparin ligand to visualise & better understand binding/activation mechanism.

PEAK PROTEINS

Platelet function

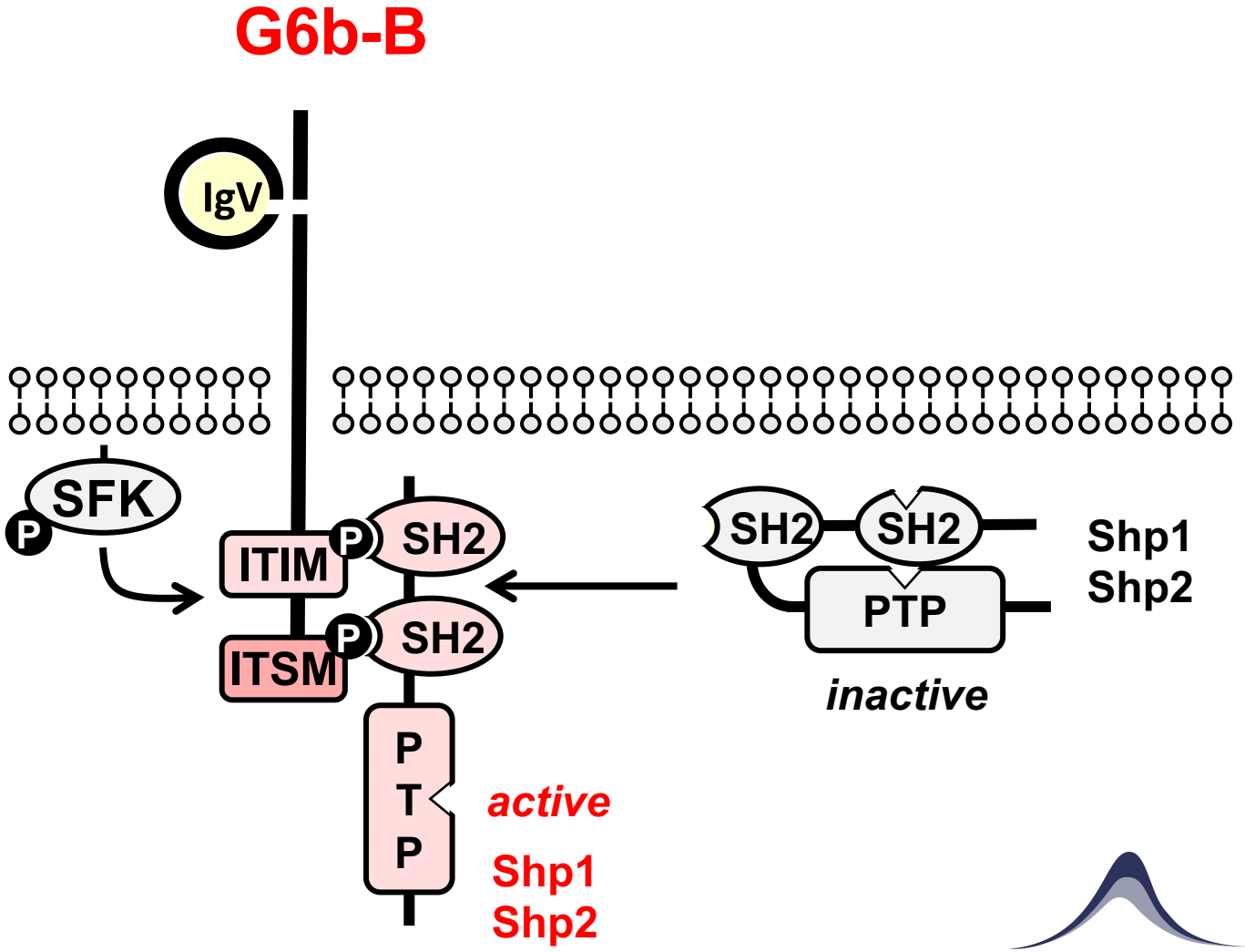
- Platelets are highly reactive anucleated cell fragments.
- Produced by megakaryocytes (MK's) in bone marrow, spleen & lungs.
- On vascular injury platelets adhere to exposed vascular extracellular matrix and become activated to form hemostatic plug & seal wound.
- Must be tightly regulated to avoid hyper-reactivity and indiscriminate blockage eg. acute coronary heart disease and stroke.
- Inhibition partly due to receptors containing **i**mmunoreceptor **t**yrosine-based **i**nhibition **m**otifs (ITIM's) eg. G6b-B



PEAK PROTEINS

The inhibitory ITIM receptor G6b-B

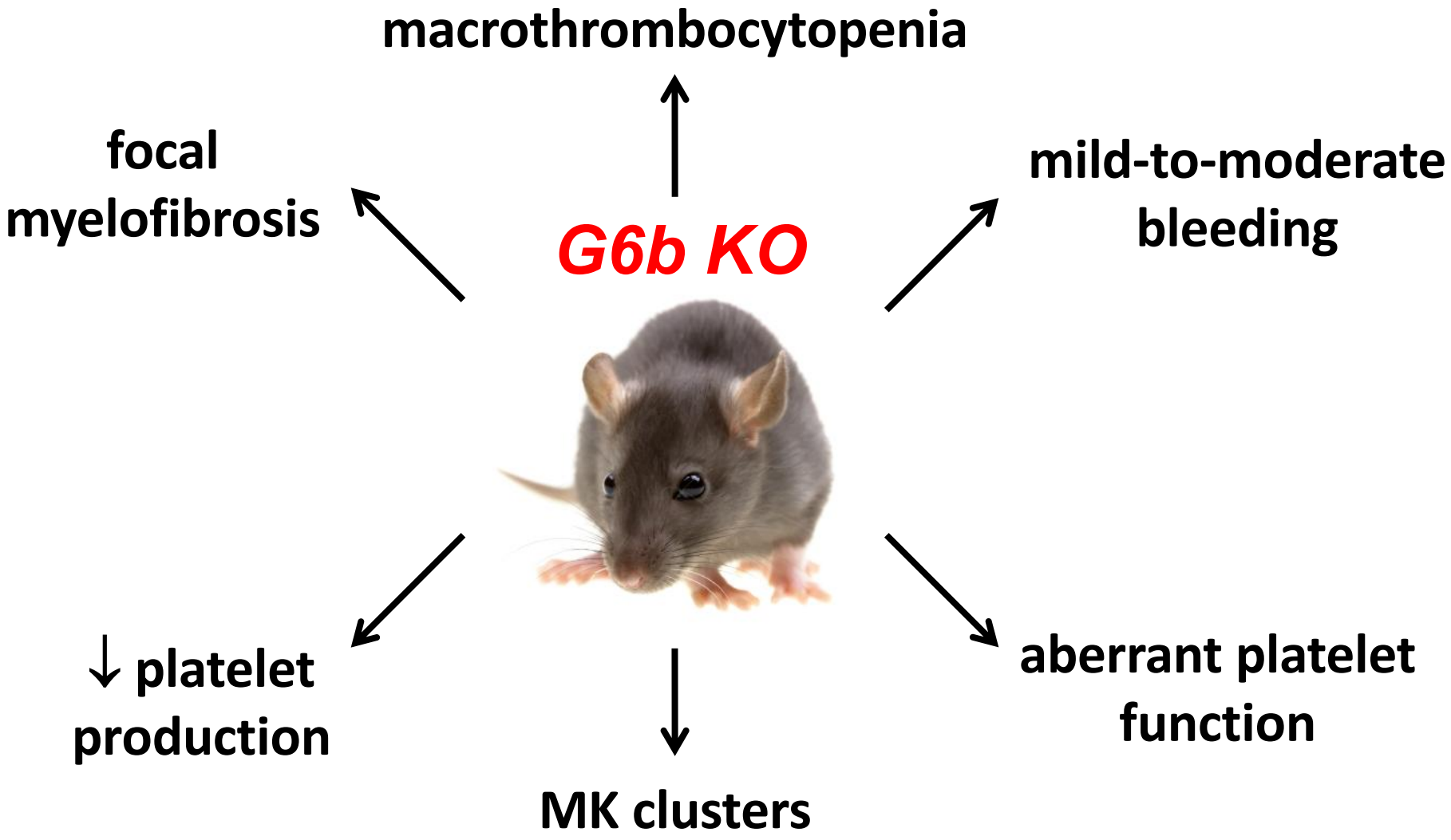
- G6b-B – an ITIM containing receptor highly expressed in MK & platelets.
- Type I transmembrane protein (241aa) consisting of a single IgV-like ECD, a transmembrane domain and cytoplasmic tail with ITIM and ITSM motifs.
- Upon ligand binding central tyrosines of ITIM/ITSM are phosphorylated by Src family kinases to become docking site for phosphatases Shp1 & 2.
- Positions active Shp1/2 to dephosphorylate key components of ITAM signaling pathway & attenuate activation signaling.



PEAK PROTEINS

Senis *et al. Mol Cell Prot* 2007.

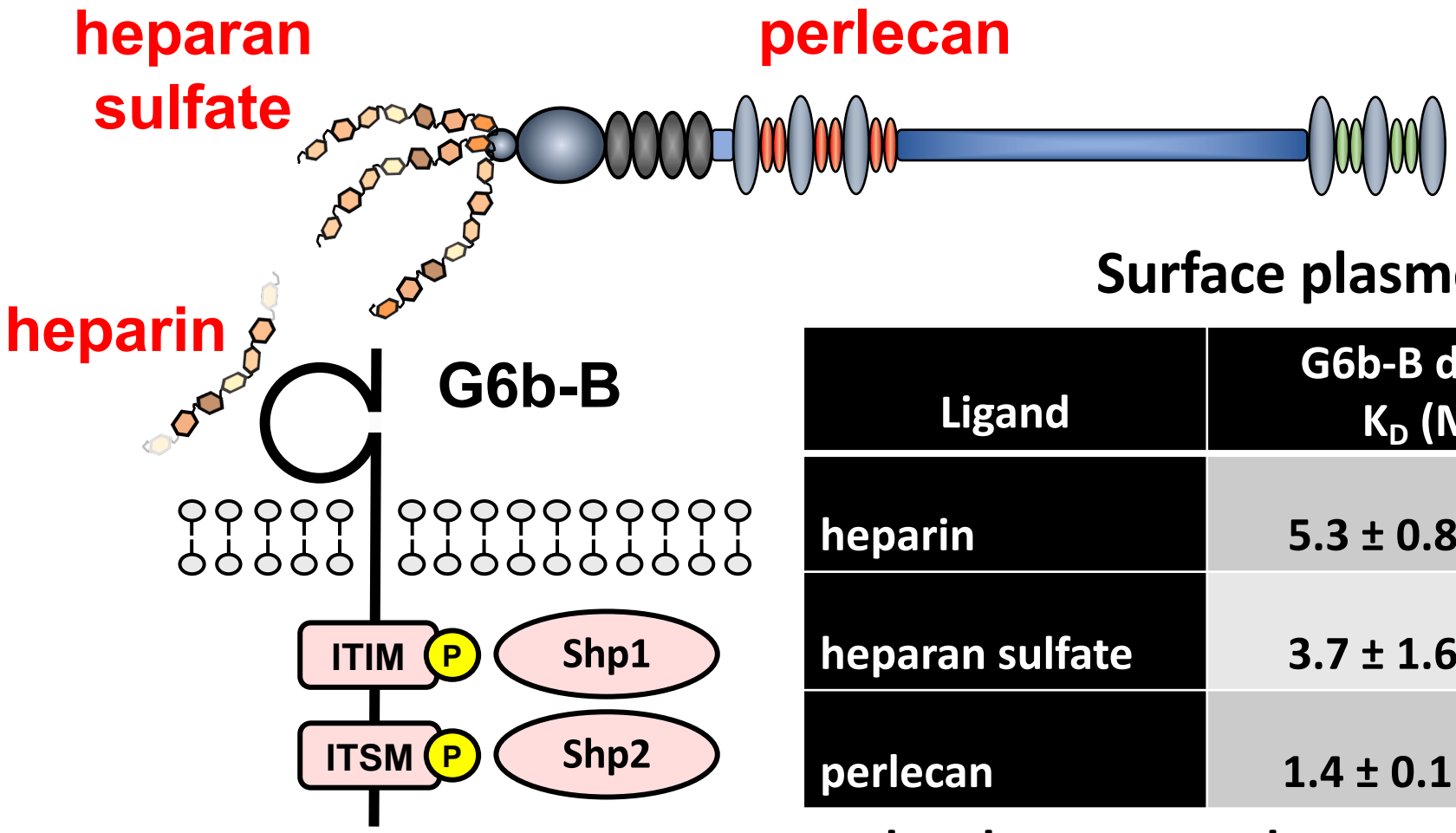
G6b KO phenotype



PEAK PROTEINS

Mazharian *et al. Sci Signal* 2012

G6b-B binds heparan sulfates



Surface plasmon resonance

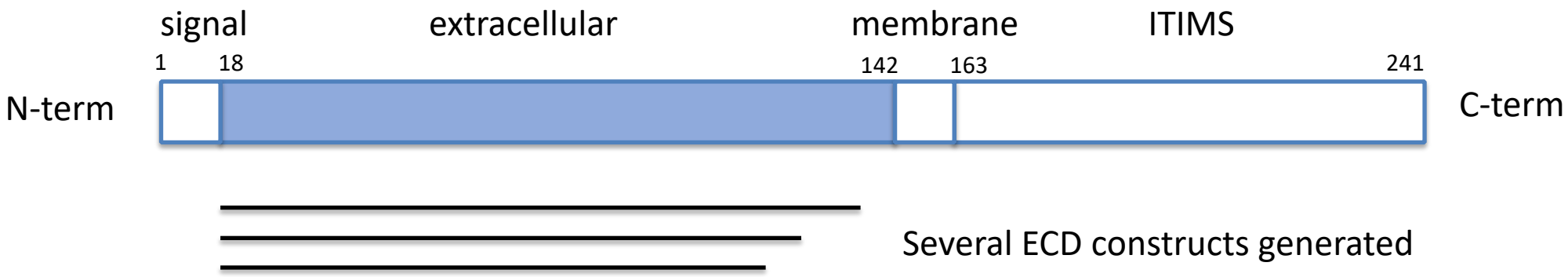
Ligand	G6b-B dimer K_D (M)	G6b-B monomer K_D (M)
heparin	$5.3 \pm 0.8 \times 10^{-9}$	$7.0 \pm 1.3 \times 10^{-7}$
heparan sulfate	$3.7 \pm 1.6 \times 10^{-9}$	$7.3 \pm 2.5 \times 10^{-7}$
perlecan	$1.4 \pm 0.1 \times 10^{-8}$	$2.3 \pm 1.5 \times 10^{-7}$

- slow k_{on}, average k_{off}

PEAK PROTEINS

Vögtle et. al. 2019

G6b-B ECD expression and purification

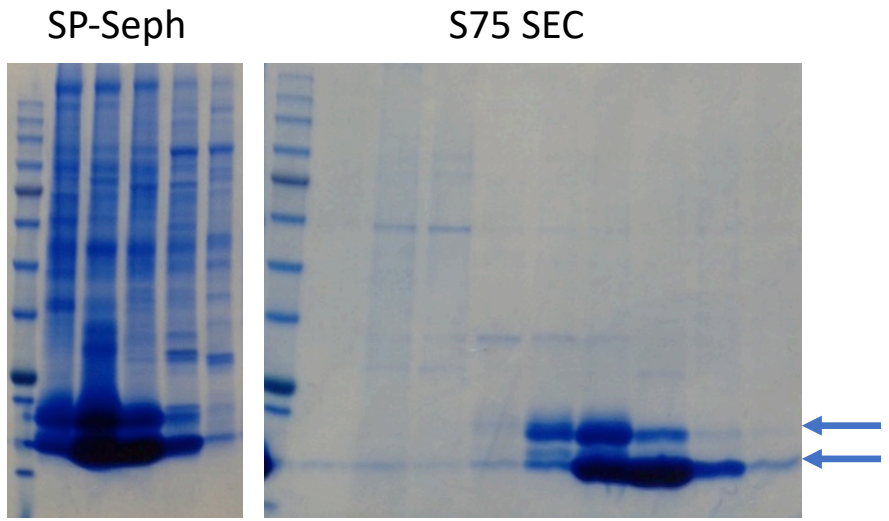


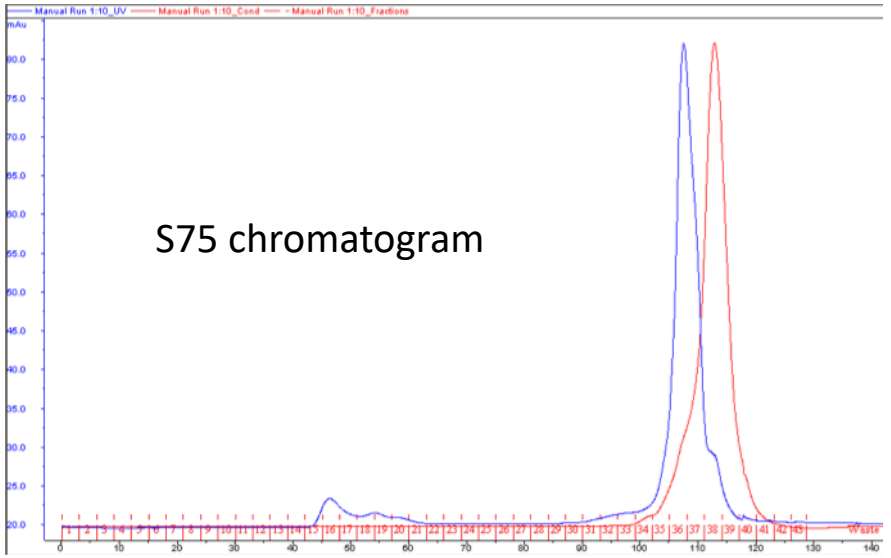
- Extracellular domain is single IgV-like domain of ~13kDa
- No published X-ray structure & has < 20% homology with IgV family structures in PDB.
- One potential N-linked glycosylation site (Asn32).
- 4 cysteines, at least one disulphide by homology.
- A number of G6b-B ECD constructs were expressed transiently in HEK293 cells.
- ECD construct encompassing residues 18-133 expressed well.
- Purification by cation exchange and size exclusion from culture medium.

PEAK PROTEINS

G6b-ECD: Initial results

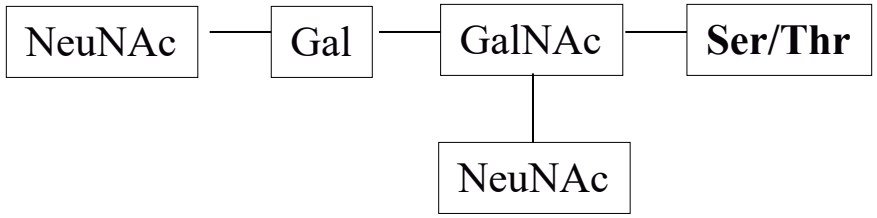
- Initial SDS-PAGE and LC-MS identified protein consisted of 2 species
- Upper band with multiple masses between 14-15kDa indicating N-glycosylation at the predicted site Asn32.
- Native G6b-B ECD protein crystallised but only diffracted >10Å.
- Need to remove heterogeneity due to N-glycosylation.
- The N-linked sugars could be reduced with PNGaseF - but difficult to get removal to go to completion.
- Therefore generated Asn32->Asp mutant.



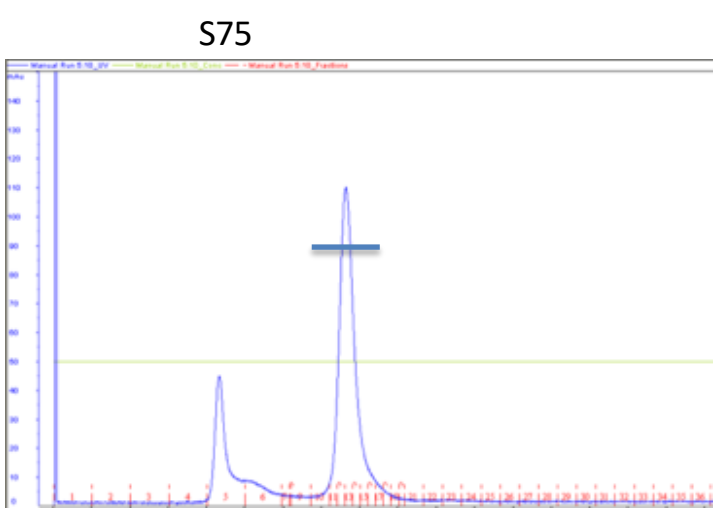


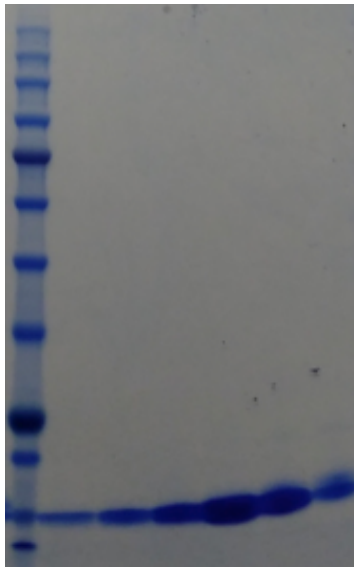
Engineering out the glycosylation

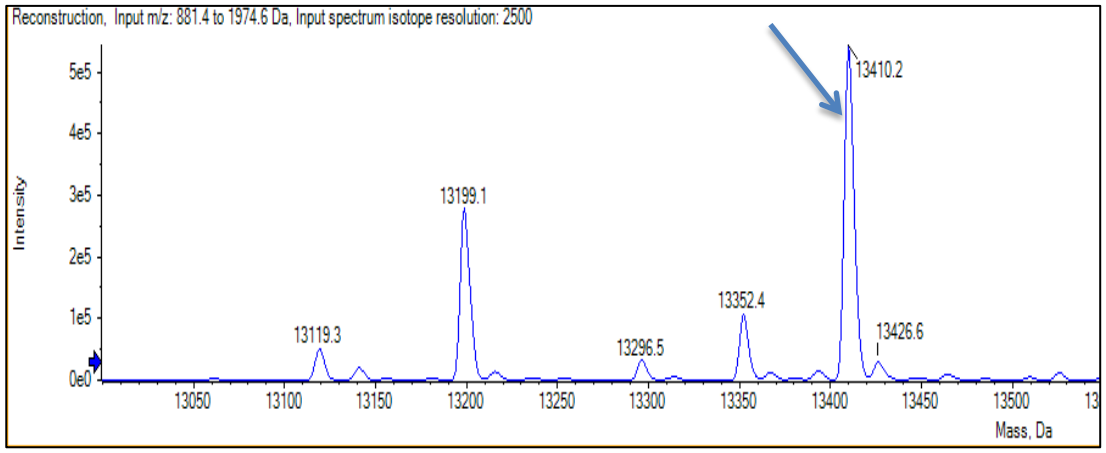
- N32->D mutant now appears as single species on SDS-PAGE & LC-MS.
- Intact Mass LC-MS data (Sciex X500B) however gives the mass of N32->D mutant at 13,410.2Da.
- This is +948Da from the predicted mass & consistent with addition of a single common O-linked tetrasaccharide structure:



- Supported by fragmentation of tetrasaccharide in mass spec.

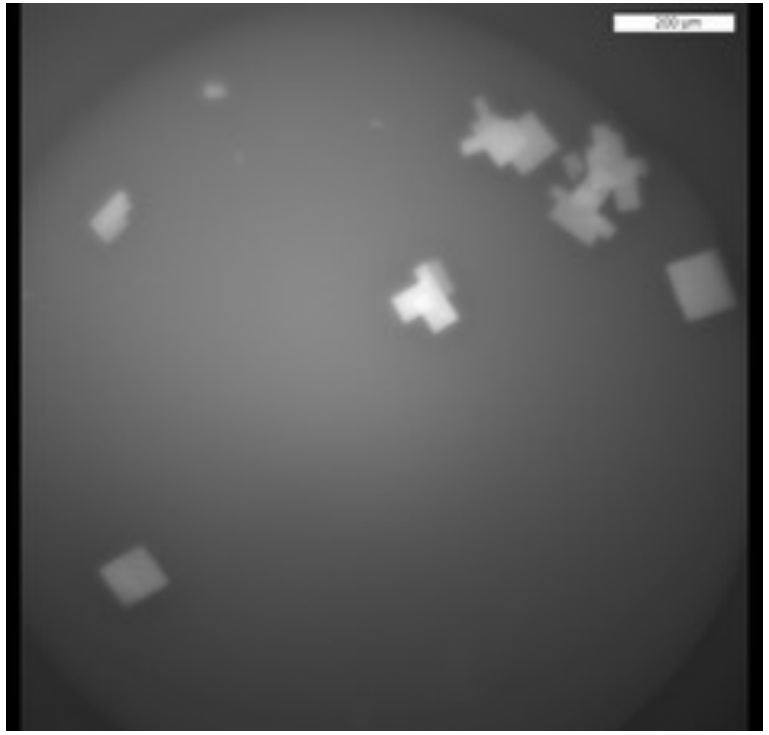






Crystallisation of N32->D mutant

- No crystals were obtained of the apo N32D mutant or in presence of DP12 (dodecasaccharide heparin fragment).
- However crystals of G6b ECD + Fab + DP12 were obtained but grew very slowly (3 months) and only diffracted to ≤4.0Å at Diamond Light Source (I04).
- At this resolution we could place the Fab by MR and see some electron density near the CDRs for putatively bound G6b-B ECD but not able to build model.
- Improve resolution by also removing the O-glycosylation?
- Considered sialidase and O-glycosidase but opted against for cost reasons.



Initial Fab-G6b ECD-DP12 crystals

PEAK PROTEINS

O-glycosylations

- 13 Ser and 5 Thr residues in G6b-B ECD construct any of which in principle could be O-glycosylated.
- Bioinformatics with NETOGlyc 4.0 on UniProt identifies 4 residues with a “positive” score.
- All 4 are found close together in a predicted loop region containing 3 Ser & 2 Thr residues.
- LC-MSMS peptide mapping via chymotrypsin digest identified a 15aa peptide of this loop with + 948Da mass:

66 80

ASSSGTPTVPPLQPF

- Consistent with this loop being the site of O-glycosylation - but which residue?

PEAK PROTEINS

O-glycosylations

- To identify site of O-glycosylation 7 mutants (containing N32D) were generated.
- Intact MS data on mutants identified Thr73 as the major site of O-glycosylation.
- MS data also showed approx. 10-15% of the T73A mutant was still O-glycosylated.
- Only 5M mutant showed no O-glycosylation.
- Suggests that O-glycosylation on Thr73 is preferred site but can also occur elsewhere on loop.
- This heterogeneity may hinder ordered crystal formation.

66 80

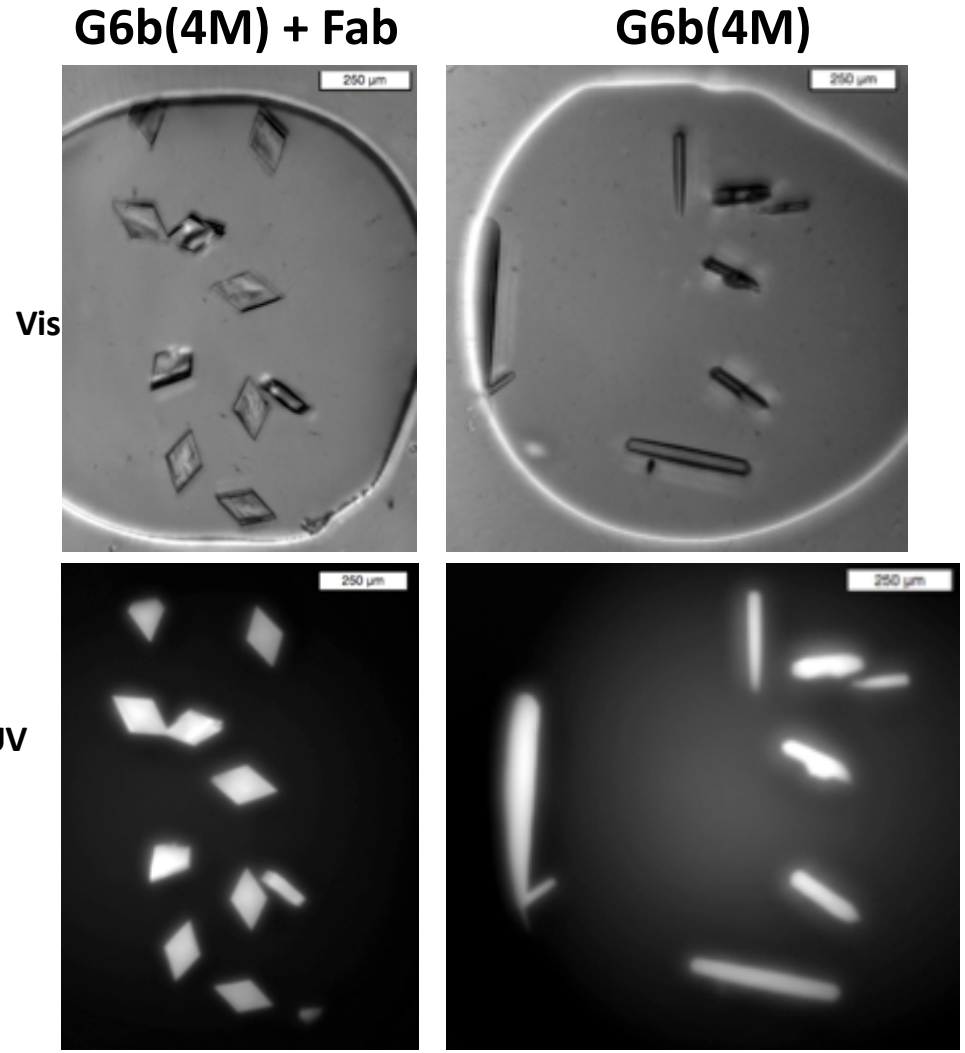
ASSSGTPTVPPLQPF

Peptide mutation	Predicted Mass+O-glycol (MW)	Observed MS MW
S67A	13398	13394
S68A	13398	13394
S69A	13398	13394
T71A	13384	13380
T73A	13384	1=12432 2=13380
4M(AAAAT)	13336	13332
5M(AAAAA)	13306	12354

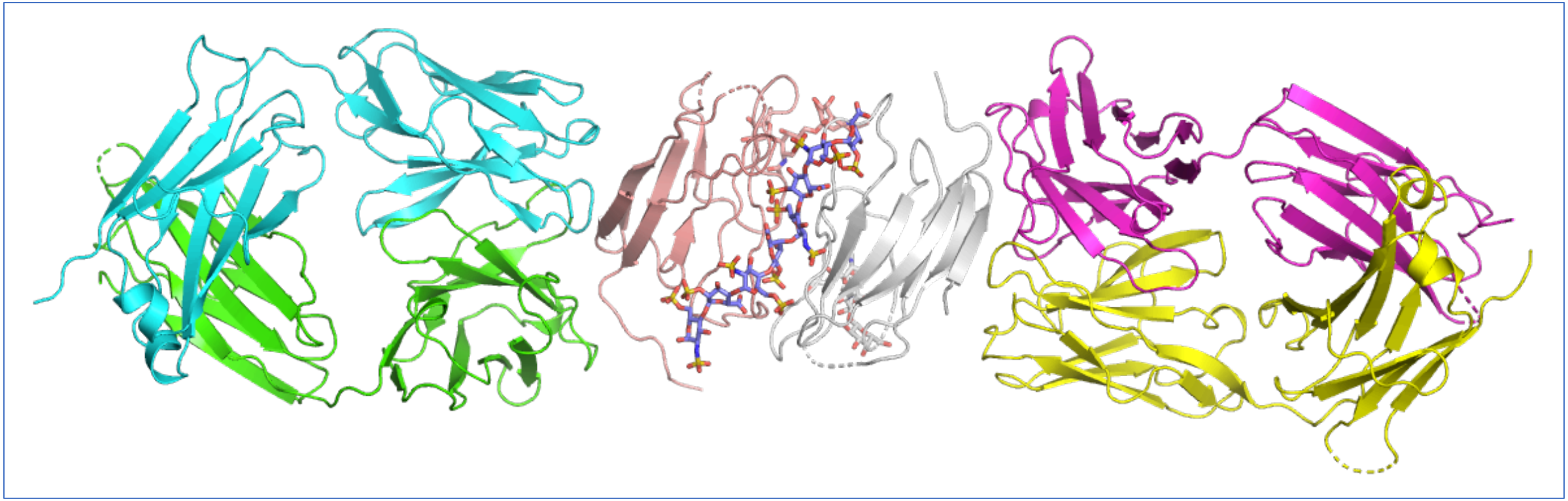
PEAK PROTEINS

G6b-B crystals - 5M & 4M

- Both 5M & 4M mutants were screened for crystallization with and without Fab & DP12 (heparin fragment).
- Crystals of apo-5M (no Fab/DP12) were obtained but diffracted only to 10Å resolution.
- Apo-4M G6b-B however crystallised within 2 weeks and diffracted to 2Å - but pathologically twinned!
- 4M G6b-B in complex with Fab + DP12 also crystallised in a similar timescale and diffracted to 3.0Å.
- Allowed G6b ECD-Fab-DP12 complex structure to be solved by MR.



G6b-B ECD-Fab-DP12 - 3.0A Xray structure

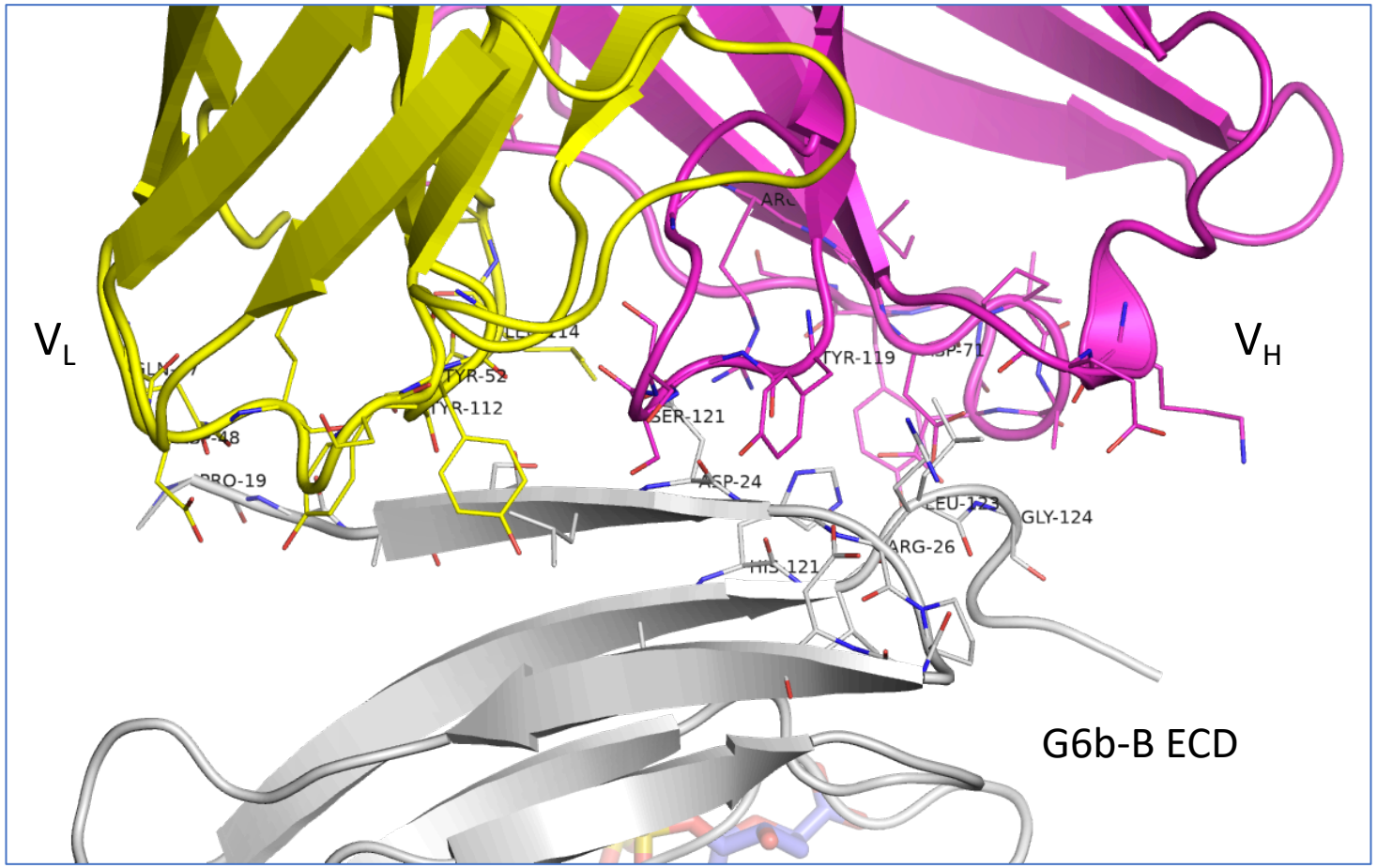


- 3.0 Ång data collected at Diamond Light Source.
- Crystal structure solved by Molecular Replacement using a Fab model.
- Structure reveals a dimer of two G6b-B ECD-Fab complexes in asymm unit.
- Deposited in PDB (6R0X)

PEAK PROTEINS

G6b-B ECD epitope identified

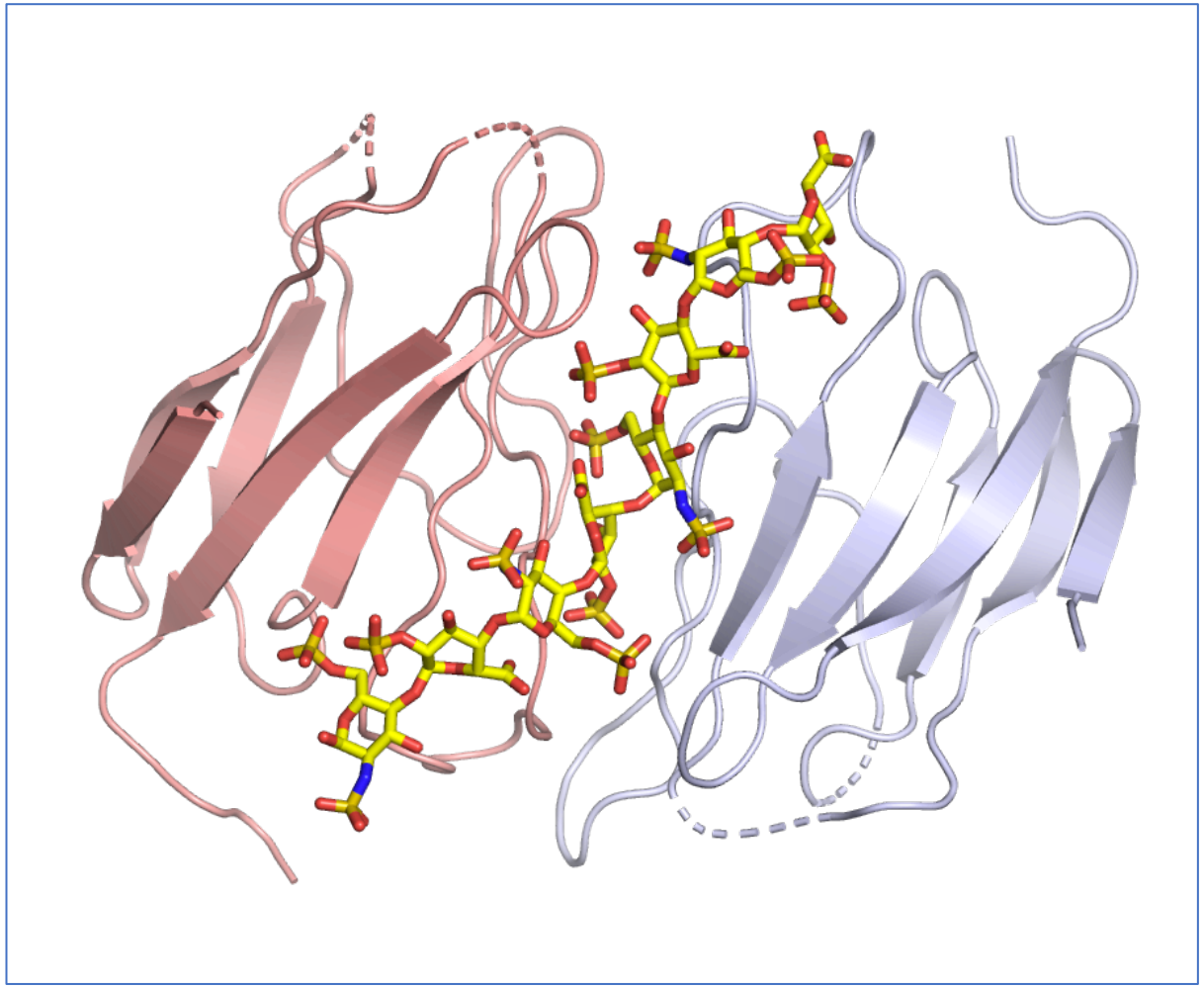
- X-ray structure revealed that the Fab epitope largely formed by N-terminal strand of the G6b-B ECD.
- All CDR regions except CDR 2 of V_L chain involved in binding interactions.
- Key interactions are formed by Asp24 of G6b-B to sidechains of Arg69 in V_H (CDR2) and Ser121 in V_H (CDR3).



PEAK PROTEINS

G6b-B ECD-DP12 interaction

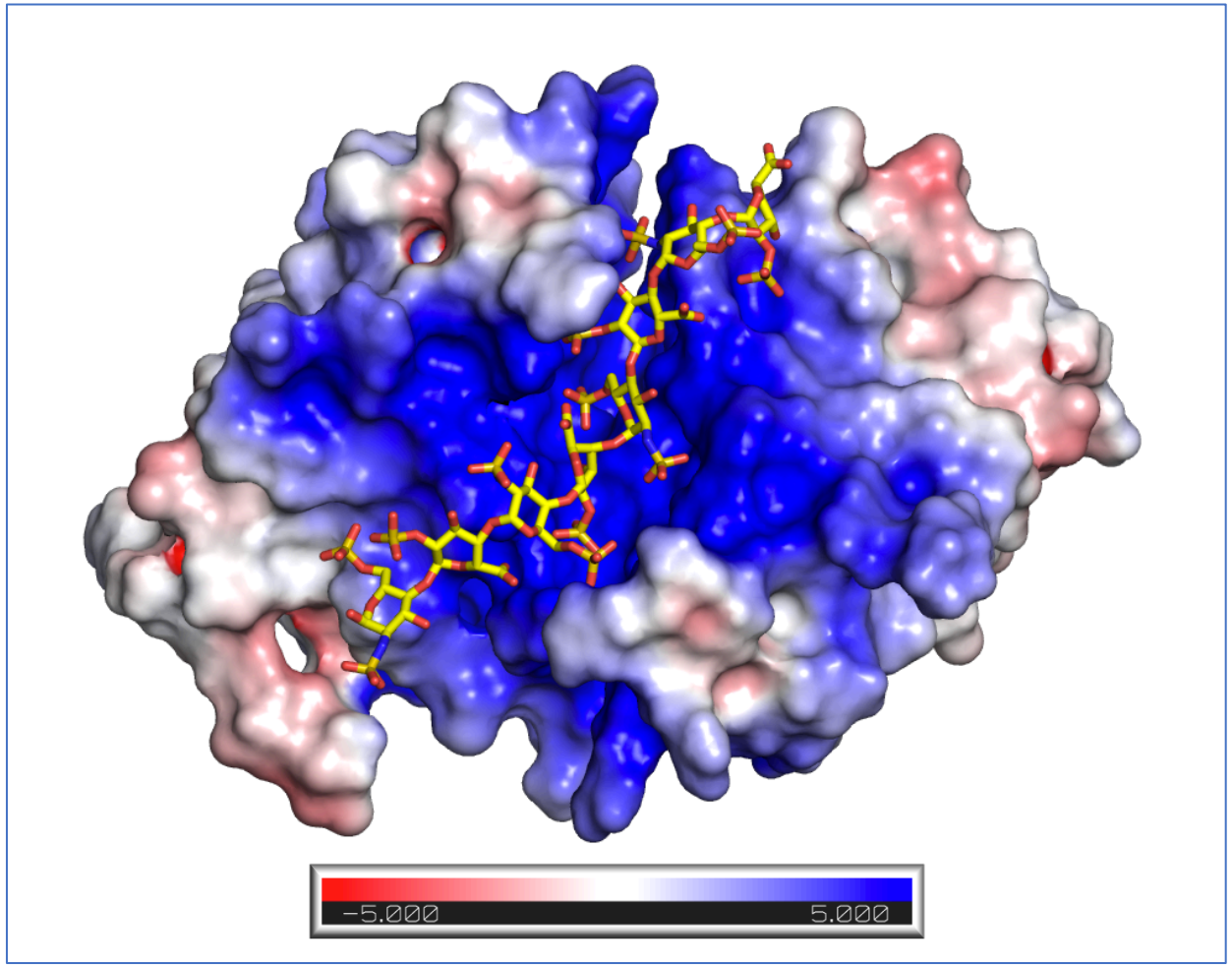
- The G6b-B ECD dimer has heparin chain (DP12) bound tightly in groove formed at dimer interface.
- Spatially separated from Fab binding site.
- Anti-parallel/head-to-tail arrangement of 2 Ig-like domains is unique among known heparin/HS binding structures.
- Electron density for only 8 of the 12 saccharide units of DP12 can be observed in structure.
- Consistent with SPR binding data that at least 8 heparin units need for high affinity binding
- Also ~100x higher heparin affinity for G6b-B dimer over monomer constructs.



PEAK PROTEINS

G6b-B ECD-DP12 interaction

- G6b ECD dimer interface is lined with Arg and Lys residues and is highly positively charged (ECD pI=10 & net charge= +8)
- Ideal for binding of negatively charged heparin or heparan sulphate chains.
- Due to charge repulsion between G6b ECD monomers it is likely that heparin/heparan sulphate binding is required to drive ECD dimerization.
- Supported by size-exclusion chromatography.
- Structure supports hypothesis that G6b-B is a functional receptor for heparin/heparan sulphate which triggers intracellular signaling by inducing dimerisation.



Electrostatic surface of G6b-DP12 dimer

PEAK PROTEINS

Acknowlegements

PEAK PROTEINS

UNIVERSITY OF BIRMINGHAM | INSTITUTE OF CARDIOVASCULAR SCIENCES

SYGNATURE DISCOVERY

Helen McMiken

Rachel Rowlinson

Juli Warwicker

Catherine Geh

Derek Ogg

Tina Howard

Mark Abbott

Yotis Senis

Timo Vögtle

Jordan Lane

Scott Polack

PEAK PROTEINS

Thank you!

PEAK PROTEINS