Aston University
BIRMINGHAM UK

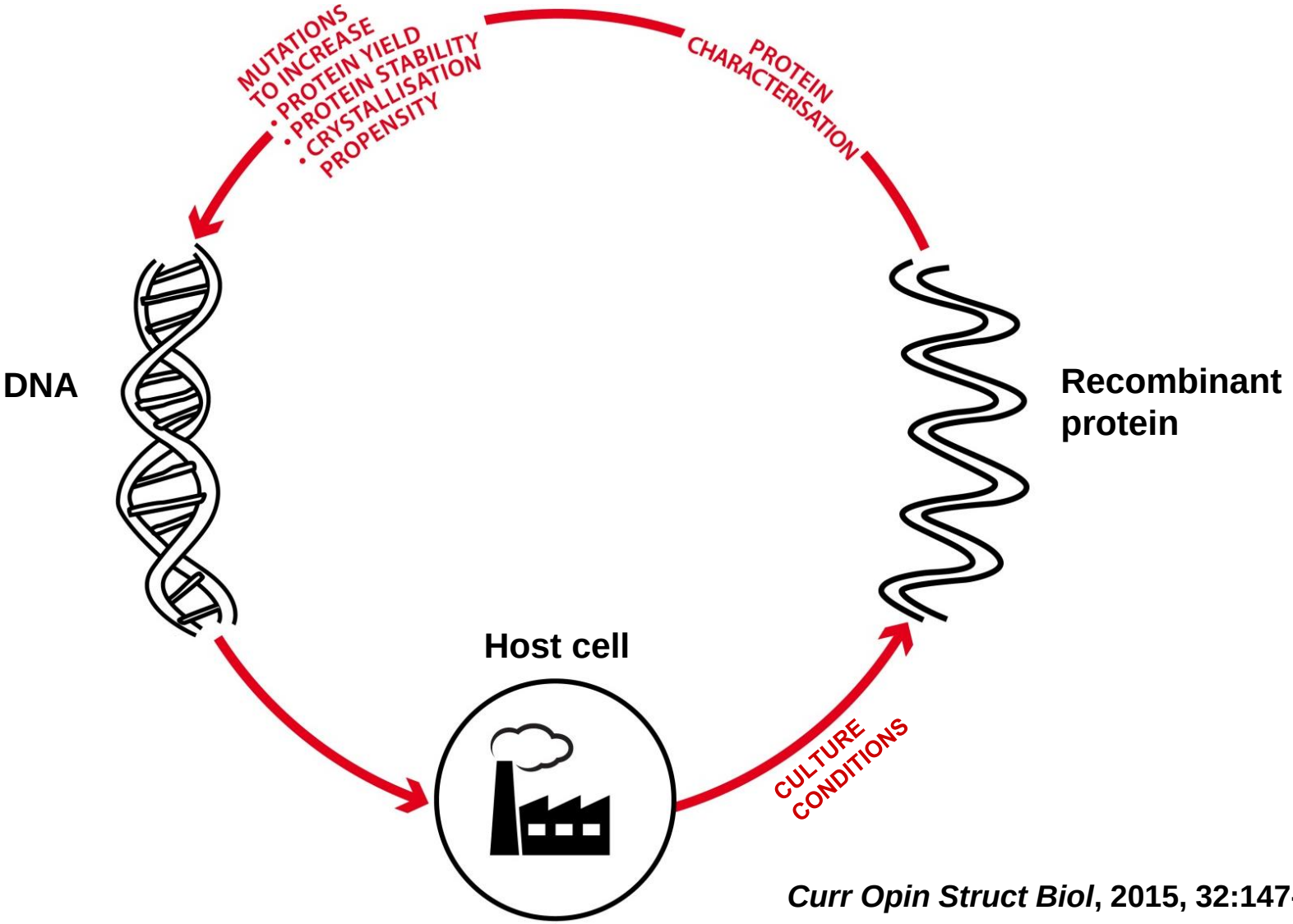
LINXS membrane protein working group workshop, 25th May 2021

Recombinant membrane protein production in microbial hosts

Expression in *P. pastoris*
Extraction with SMA

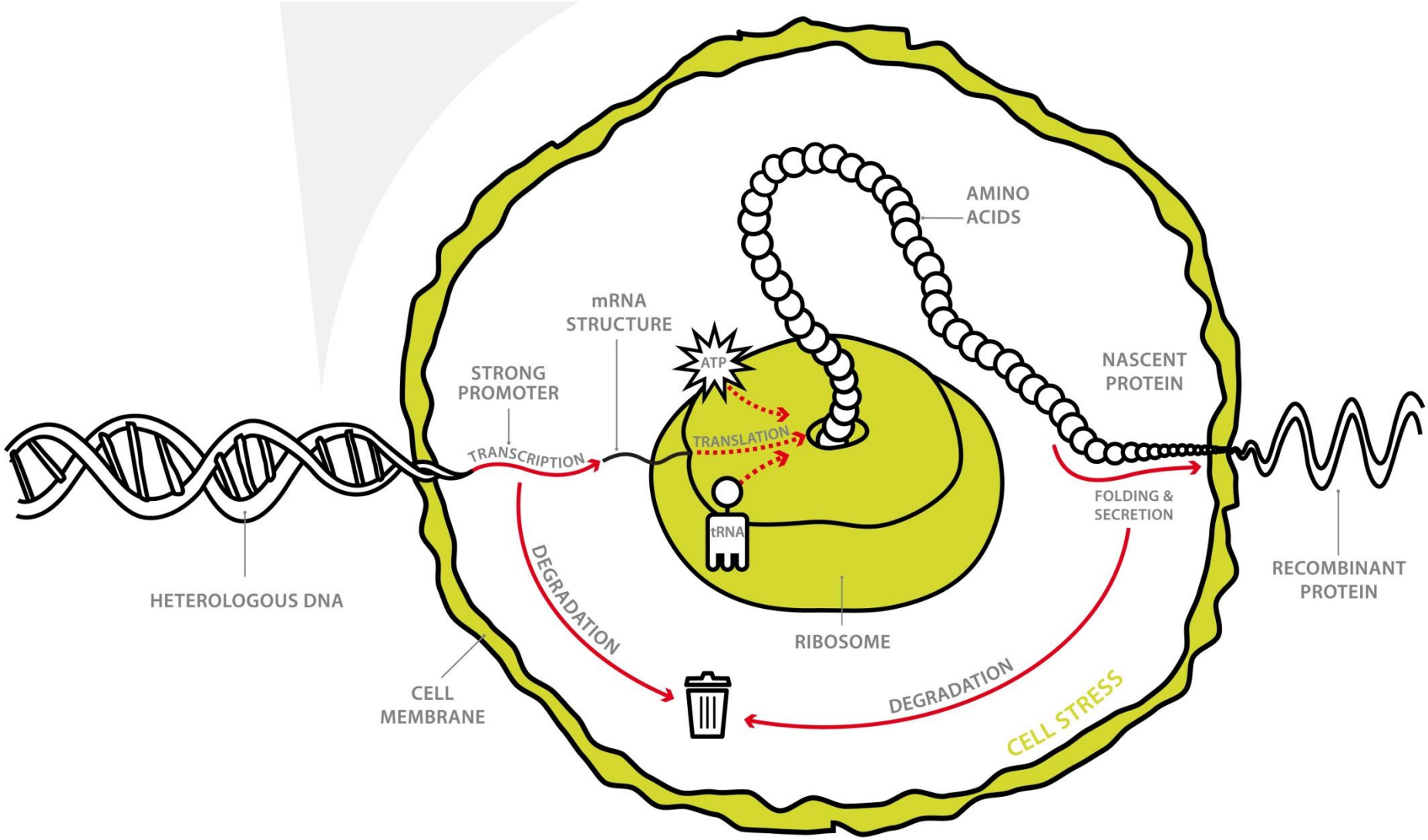
Professor Roslyn M Bill

Recombinant protein production



Curr Opin Struct Biol, 2015, 32:147-155

Recombinant protein production



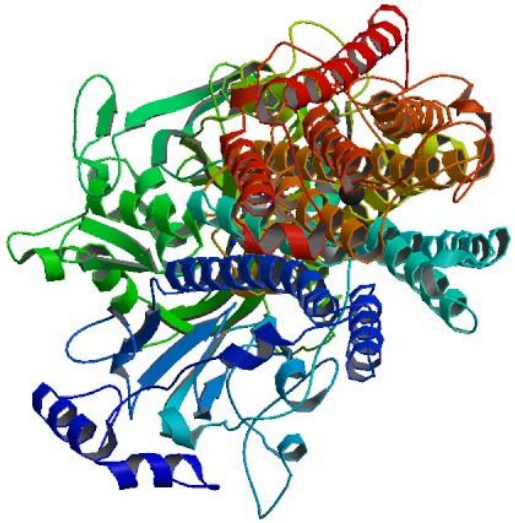
***Curr Opin Struct Biol*, 2015, 32:147-155**

Components of a protein production experiment

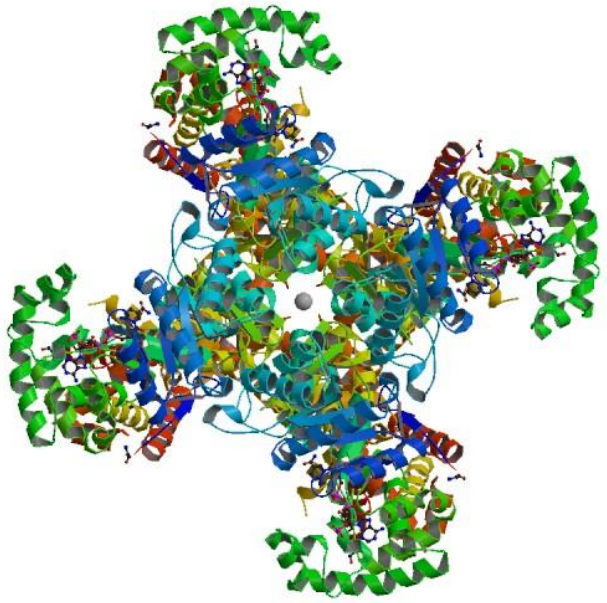
- Gene sequence encoding the target protein (codon optimization; purification/detection tags; signal sequence)
- Plasmid-based (episomal) or integrated (genomic) expression
- Promoter (inducible versus constitutive)
- Choice of host cell and specific strain (protease deficient; engineered glycosylation pathway)
- Culture conditions (optimized to maximize functional yield)

The first recombinant mammalian membrane protein structures used proteins produced in yeast

Rabbit Ca^{2+}-ATPase, SERCA1a
(structure modelled on 1T5S)



Rat Kv1.2
(2A79; 2005)



The high-resolution structure of a glycosylated *Caenorhabditis elegans* P-glycoprotein synthesized in *P. pastoris* demonstrates that yeast glycosylation does not necessarily hinder crystal formation (PDB code 4F4C; 2012)

An analysis of microbial expression systems

Methods 147 (2018) 3–39

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Microbial expression systems for membrane proteins

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ABSTRACT

Despite many high-profile successes, recombinant membrane protein production remains a technical challenge; it is still the case that many fewer membrane protein structures have been published than those of soluble proteins. However, progress is being made because empirical methods have been developed to produce the required quantity and quality of these challenging targets. This review focuses on the microbial expression systems that are a key source of recombinant prokaryotic and eukaryotic membrane proteins for structural studies. We provide an overview of the host strains, tags and promoters that, in our experience, are most likely to yield protein suitable for structural and functional characterization. We also catalogue the detergents used for solubilization and crystallization studies of these proteins. Here, we emphasize a combination of practical methods, not necessarily high-throughput, which can be implemented in any laboratory equipped for re-combinant DNA technology and microbial cell culture.

***Methods*, 2018, 147: 3-39**

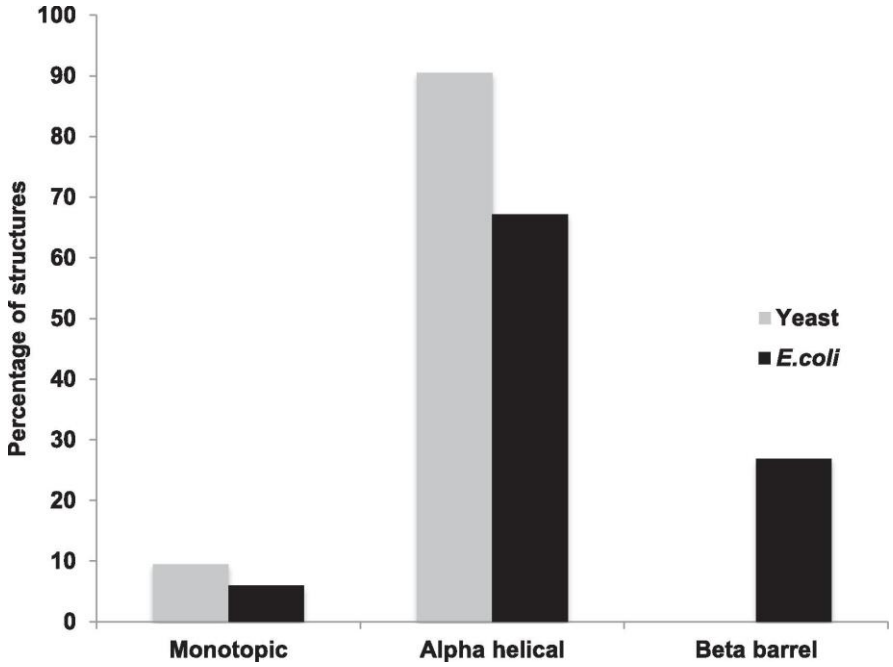
Recombinant membrane proteins structures

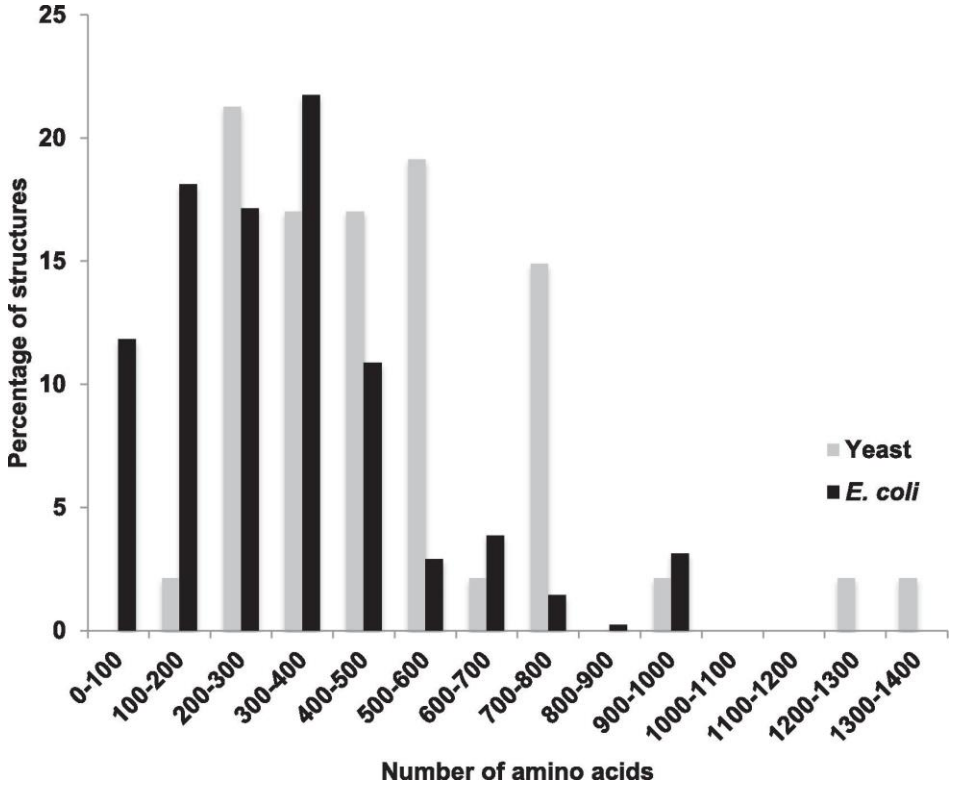
- 31% of all membrane protein coordinate files deposited in the PDB were derived from recombinant proteins (729)

- 71% of all unique structures were derived from microbial sources:
  - 64% were produced in *E. coli* (468)
  - 4% in *P. pastoris* (31)
  - 3% in *S. cerevisiae* (22)
- Also used successfully in a minority of cases:
  - *Lactococcus lactis* (see PDB entry 4US3)
  - *Pseudomonas fluorescens* (5KUD)
  - *Schizosaccharomyces pombe* (2PNO)

***Methods*, 2018, 147: 3-39**

The target

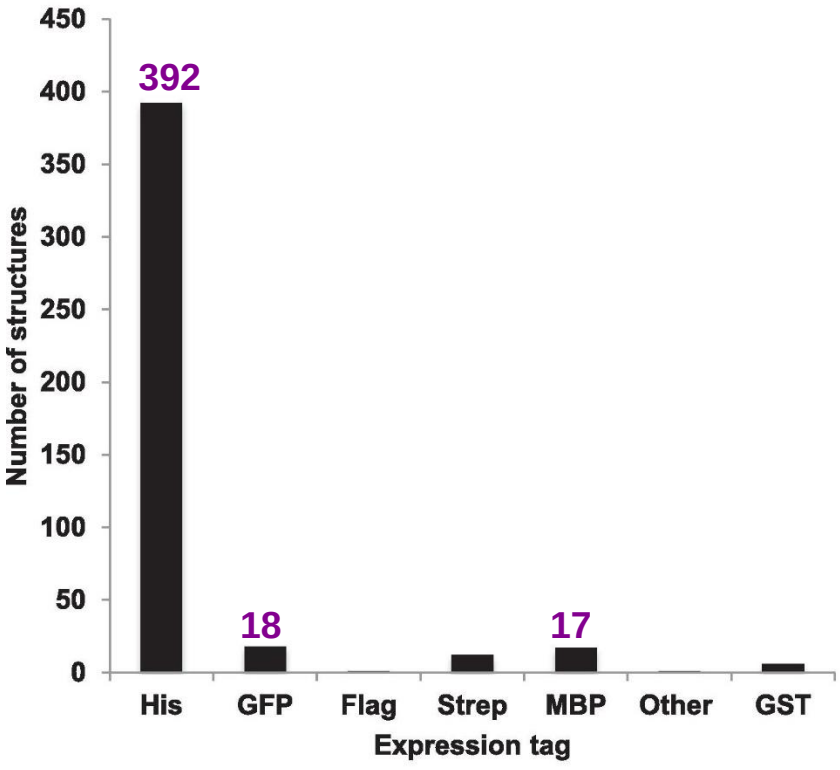




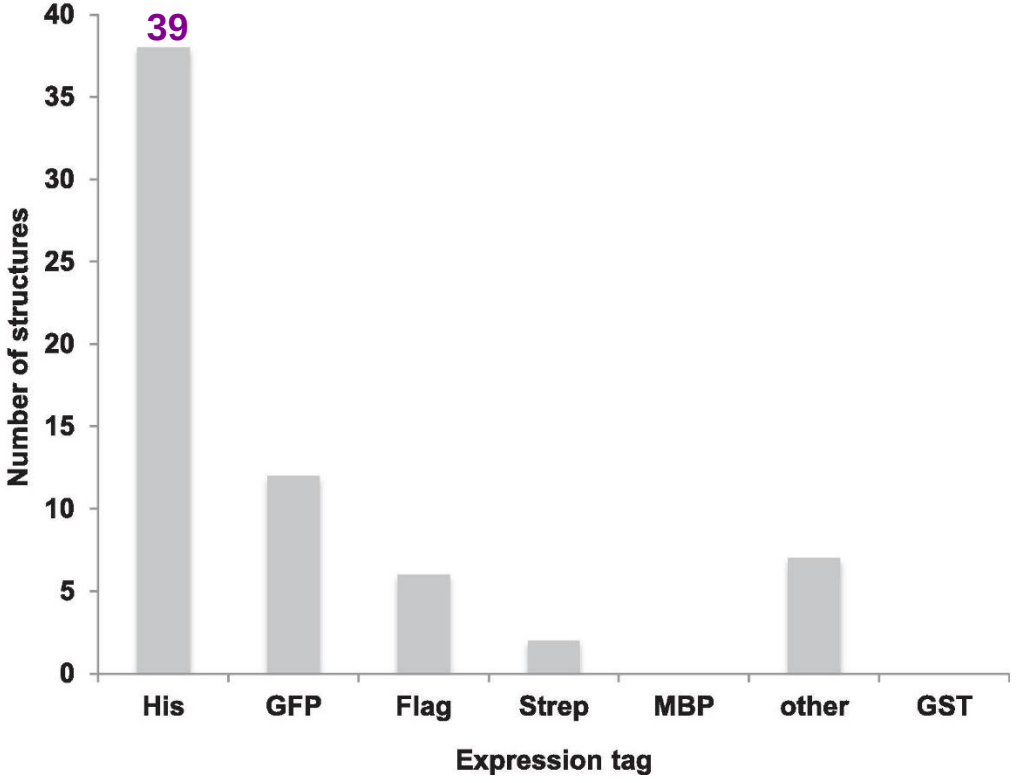
***Methods*, 2018, 147: 3-39**

Tags

447 tagged proteins produced in *E. coli*



53 tagged proteins produced in yeast

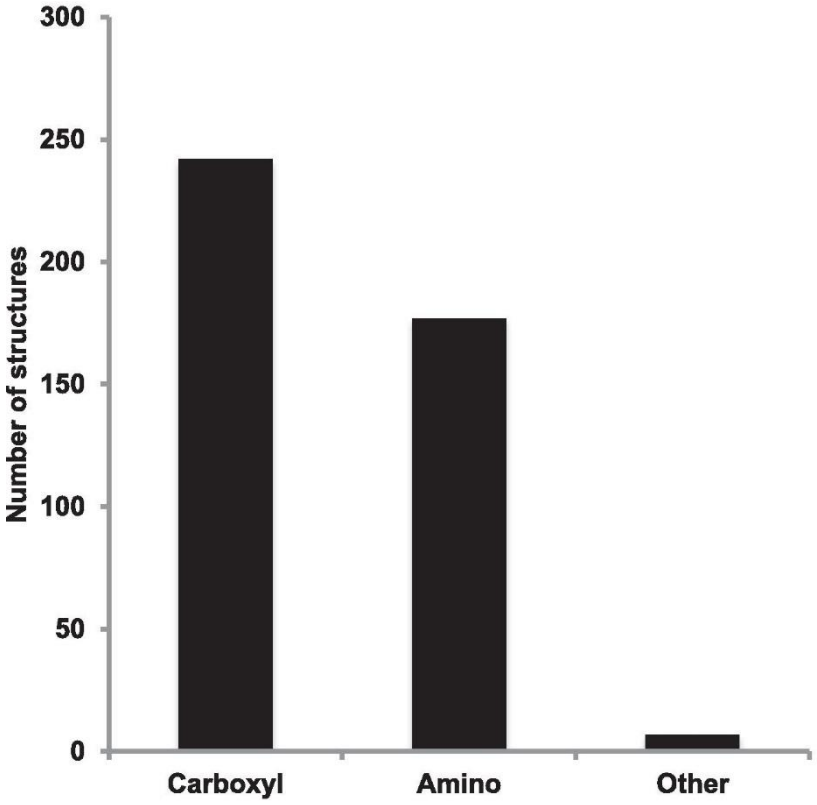


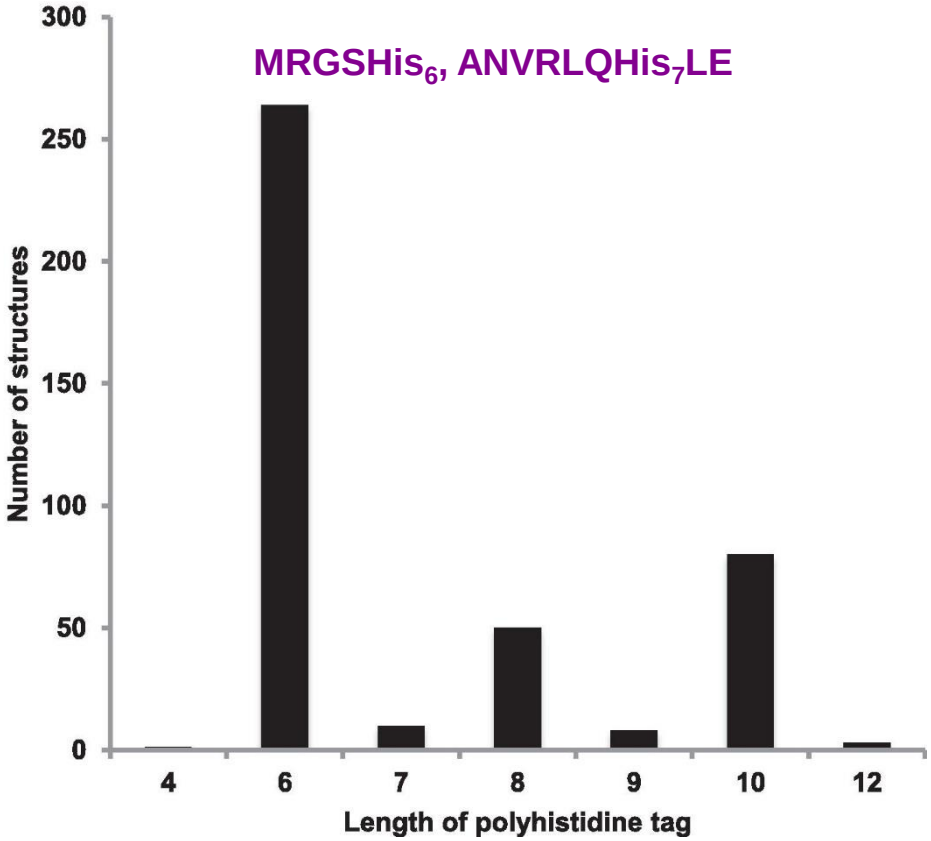
Some proteins are multiply-tagged

***Methods*, 2018, 147: 3-39**

Tag position and length

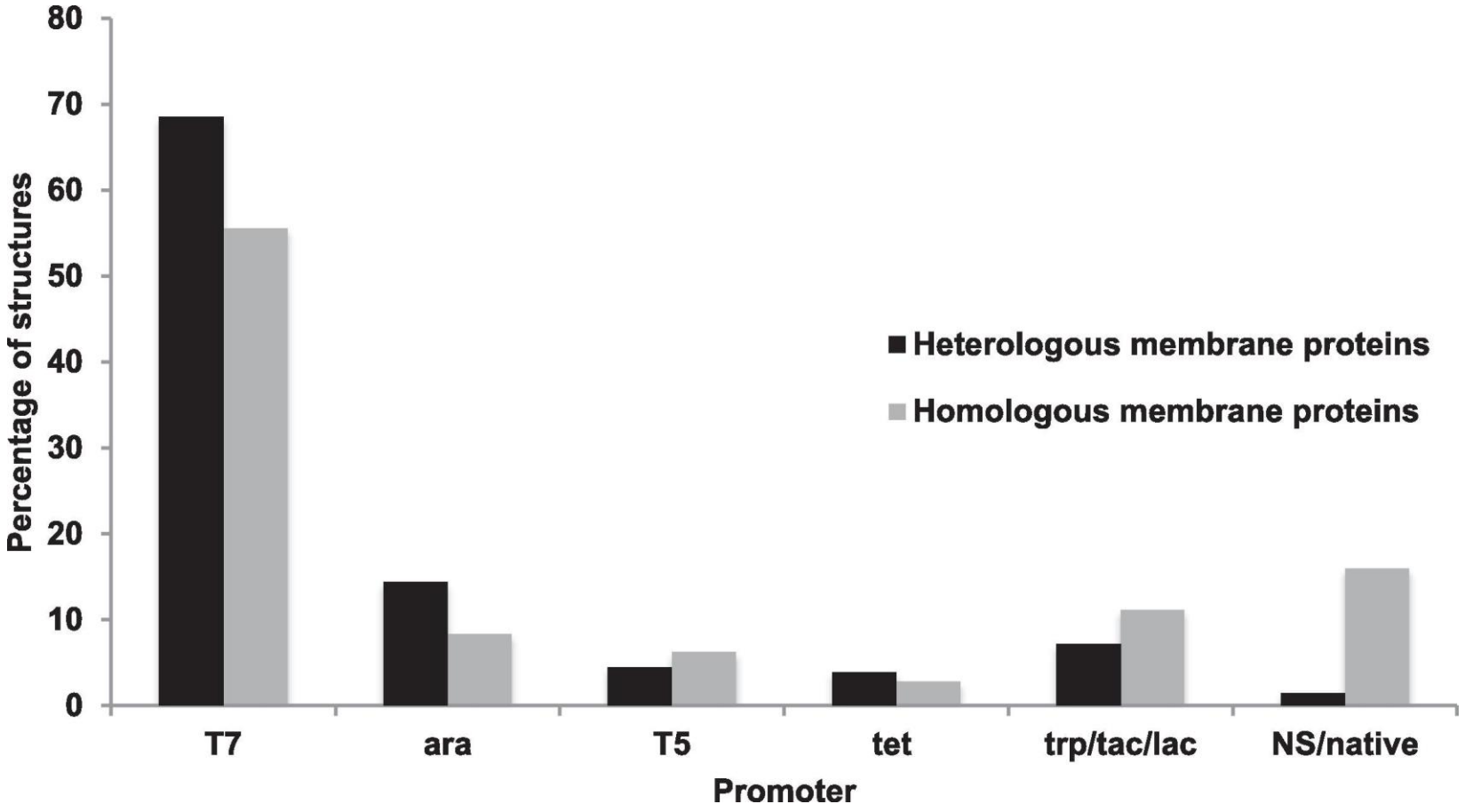
- TEV protease is widely used to cleave tags (see 4C00, 3WVF, 4X5M and 4JA3) because it is still active in the presence of the most commonly-used detergents
- Thrombin protease is also widely used (see 2VQI, 2ABM and 3B5D for examples)





***Methods*, 2018, 147: 3-39**

E. coli promoters



***Methods*, 2018, 147: 3-39**

P. pastoris promoters

Yeast strain	Promoter used in the expression plasmid			
	Inducible *AOX1*	Constitutive *PMA1*	Not stated	Total
GS115	4			4
KM71	4			4
SMD1163	17			17
X33	4			4
Not stated	1		1	2
Total	**30**		**1**	**31**

Typically, integrative plasmids are used for expression in *P. pastoris*

P. pastoris can grow to >100 g/L dry cell weight; >500 OD_{600} units/mL

***Methods*, 2018, 147: 3-39**

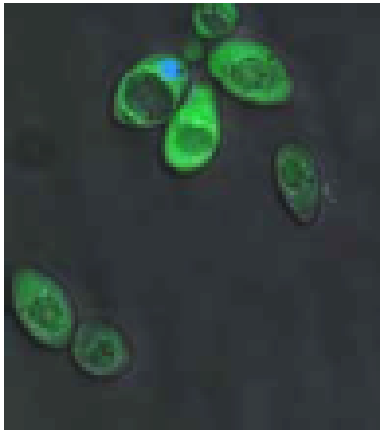
S. cerevisiae promoters

Typically, episomal plasmids are used for expression in *S. cerevisiae*

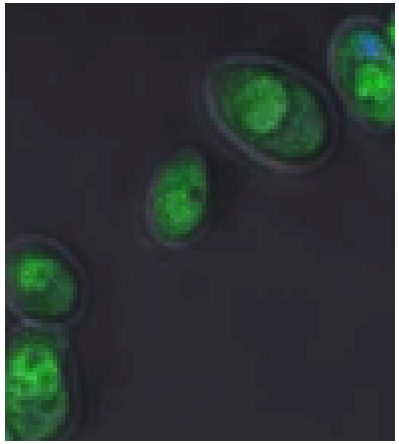
Yeast strain	Promoter used in the expression plasmid			
	Inducible *GAL*	**Constitutive *PMA1***	**Not stated**	**Total**
BJ1991		1		1
BJ2168	3		1	4
BJ5457	1			1
BJ5460	1			1
CACY1			1	1
DSY-5	4			4
FGY217	2			2
INVSc1	1			1
JTY002			1	1
W303 *pep4Δ*	1		1	2
WB12	1			1
Not stated	1		1	2
Total	**15**	**1**	**6**	**22**

***Methods*, 2018, 147: 3-39**

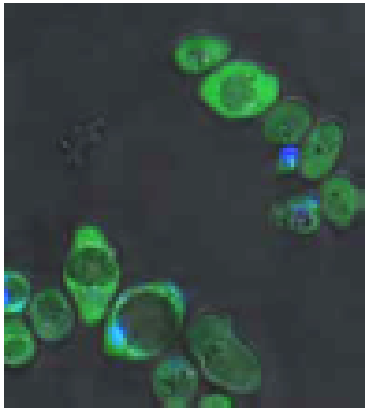
Expression of human $A_{2A}R$ in *S. cerevisiae*



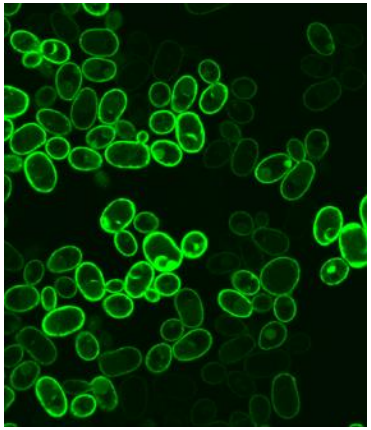
WT-$A_{2a}R$
plasma membrane



WT-$A_{2a}R$-tag
Vacuole



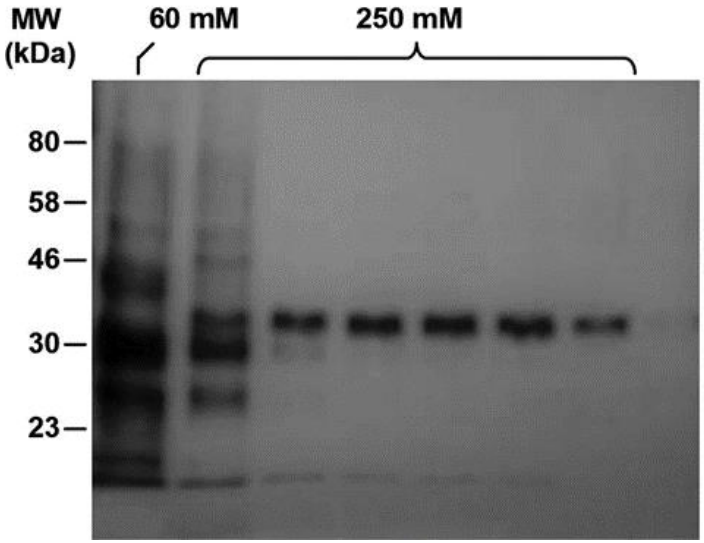
***spt3*Δ-$A_{2a}R$-tag**
plasma membrane

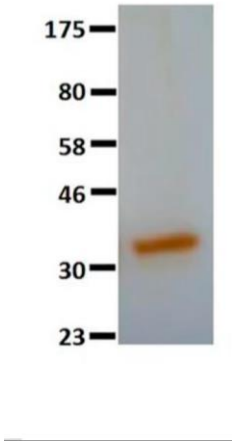


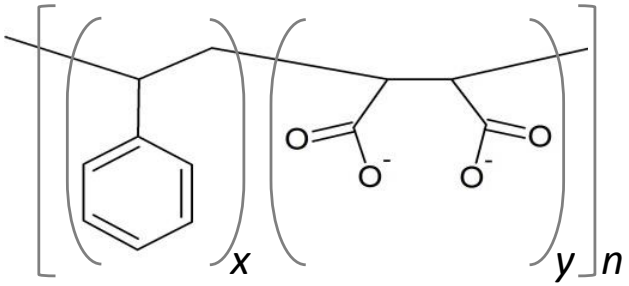
***Microb Cell Fact,* 2017, 16:41**
***Methods*, 2018, 147: 3-39**

Expression of human $A_{2A}R$ in *P. pastoris* and extraction with styrene maleic acid (SMA)

Human $A_{2A}R$ recombinantly produced with an N-terminal His_{10}-tag from the pPICZαA expression plasmid in *P. pastoris* strain X33 (with a Asn154Gln mutation to preclude hyperglycosylation)





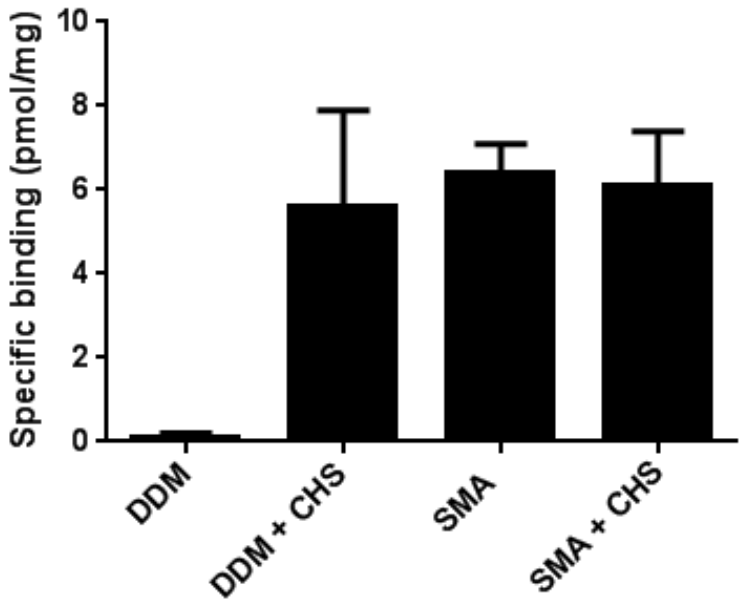


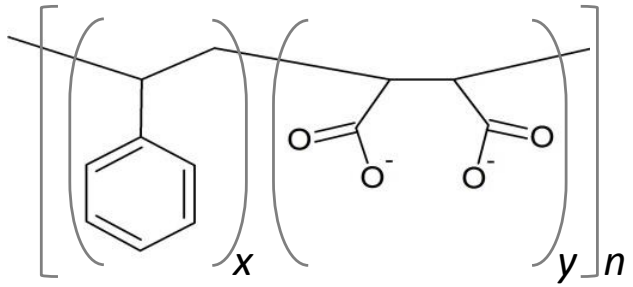
SMA 2000 co-polymer (2:1 Cray Valley)

Purification of SMALP-solubilized His-tagged $A_{2A}R$ from *P. pastoris* eluted from Ni^{2+}-NTA linked agarose as a single band in silver-stained fractions with 250 mM imidazole.
Western blot of the 250 mM imidazole fraction with an anti-polyhistidine antibody.

***Biosci Rep*, 2015 35: e00188**

Expression of human $A_{2A}R$ in *P. pastoris* and SMA extraction

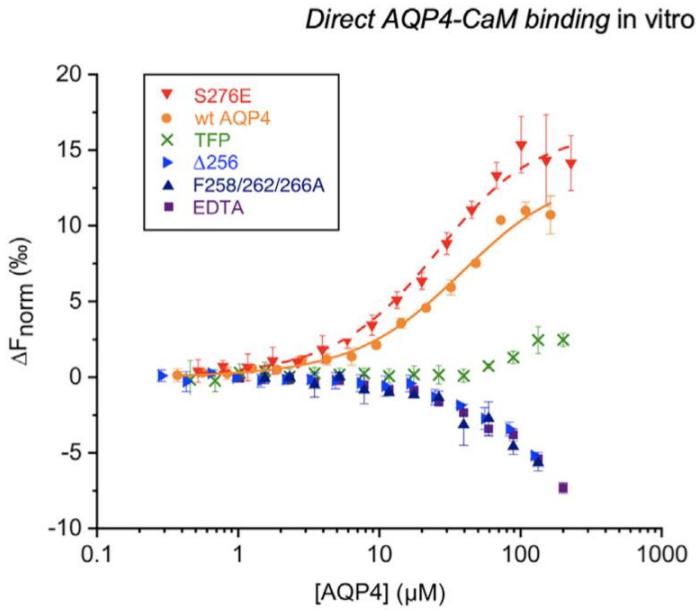


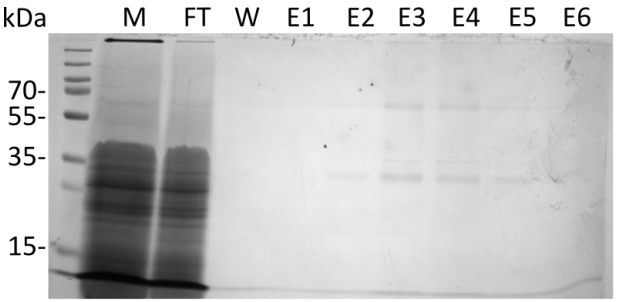


Specific binding of [^{3}H]ZM241385 (10 nM) to the adenosine A_{2A} receptor, extracted either with DDM or SMA in the absence or presence of CHS. Data are mean ± SEM, *n* = 3

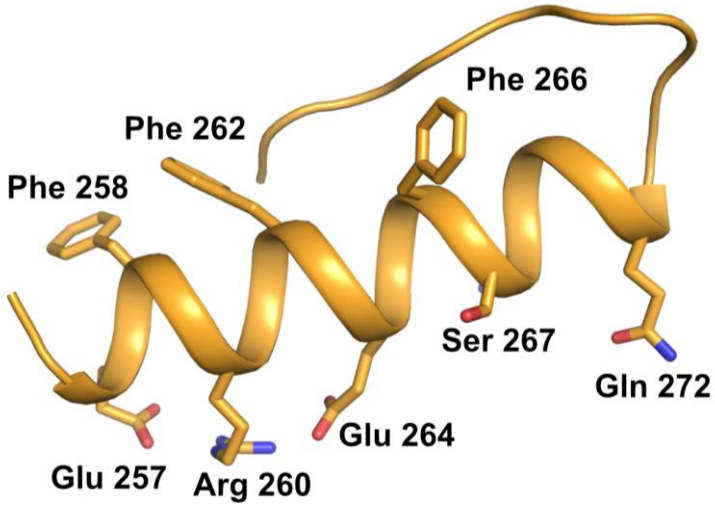
***Methods*, 2016, 95: 26-37**

Expression of human AQP4 in *P. pastoris* and SMA extraction





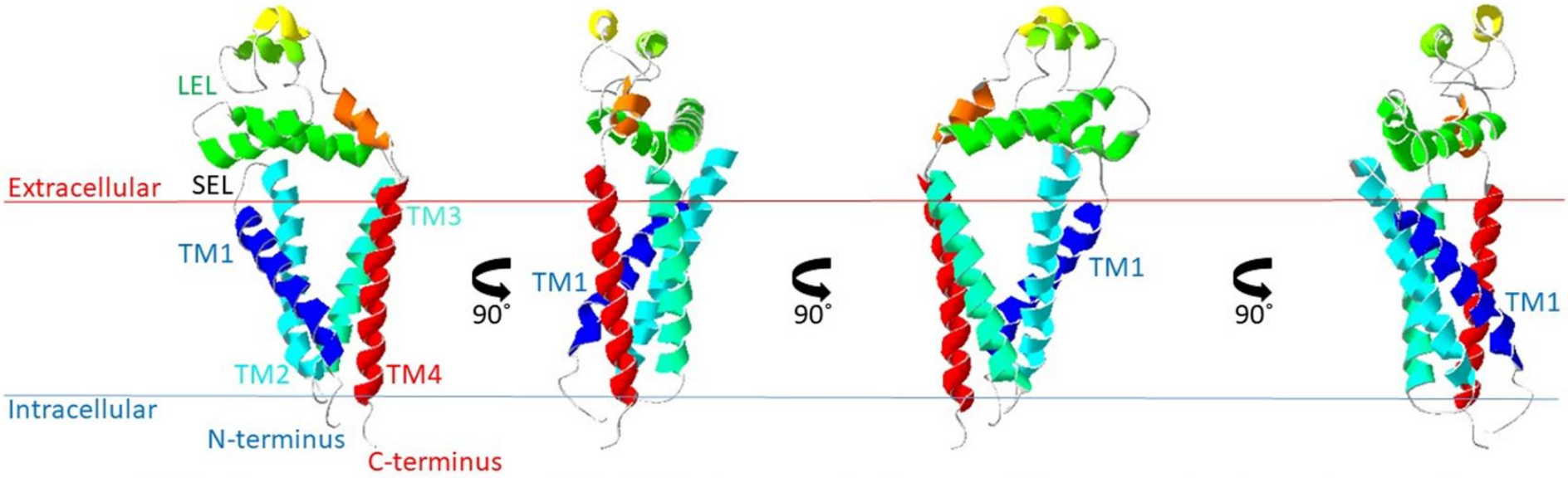
Human AQP4 recombinantly produced with a C-terminal His_6-tag from the pPICZB expression plasmid in *P. pastoris* strain X33



Lucas Unger

***Cell*, 2020, 181:784-799**

Expression of human CD81 in *P. pastoris* and SMA extraction



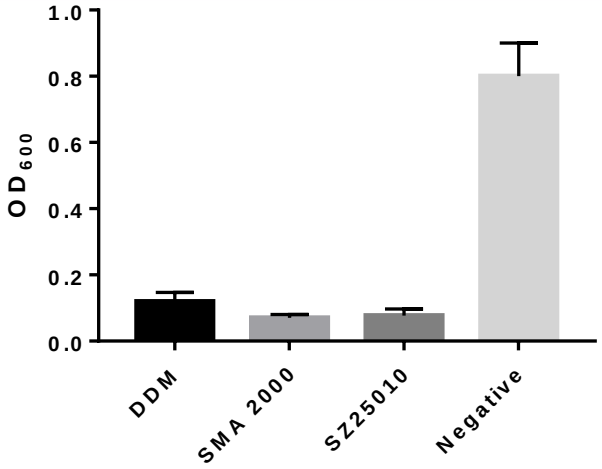
Tetraspanin, binds HCV E2 glycoprotein, ‘waffle cone’ structure solved, open questions around oligomerization status and ‘open’/’closed’ states

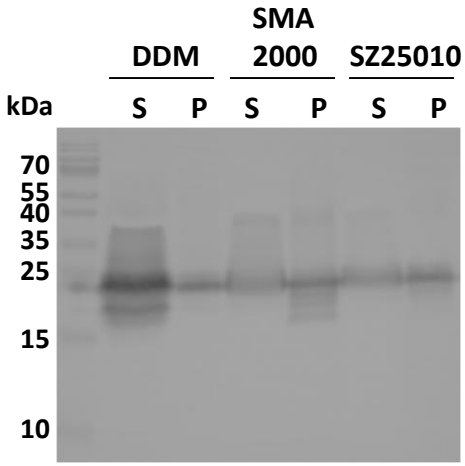
Expression in pPICZB encoding C-terminally His_6-tagged human CD81 in *P. pastoris* strain X33

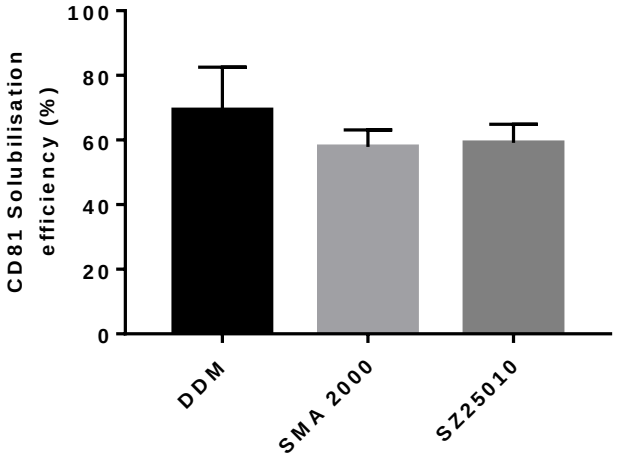
Luke Broadbent

***BBA Biomembr*, 2020, 1862: 183419**

CD81 expressed in *P. pastoris* can be solubilised using SMA polymers or conventional detergents





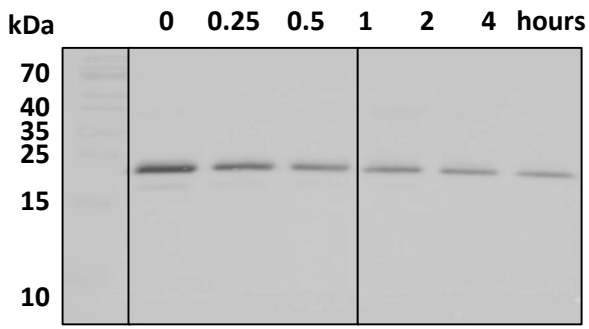


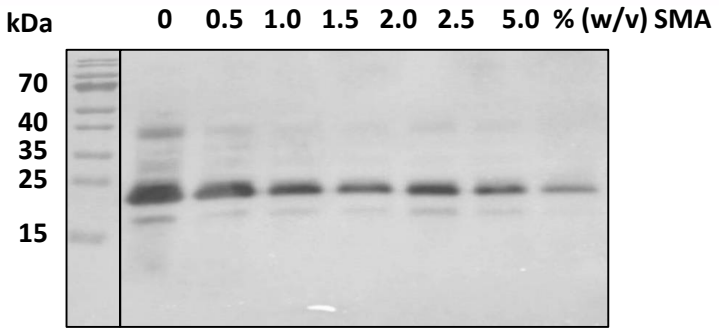
SMA 2000 co-polymer (2:1 Cray Valley) and SZ25010 co-polymer (3:1 Polyscope)

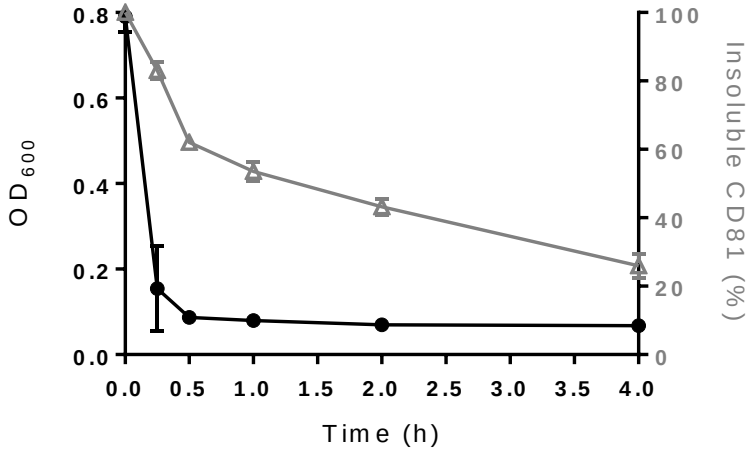
Sometimes excess SMA masks signals in Western blots – look at the amount that remains insoluble as a better measure for solubilisation efficiency

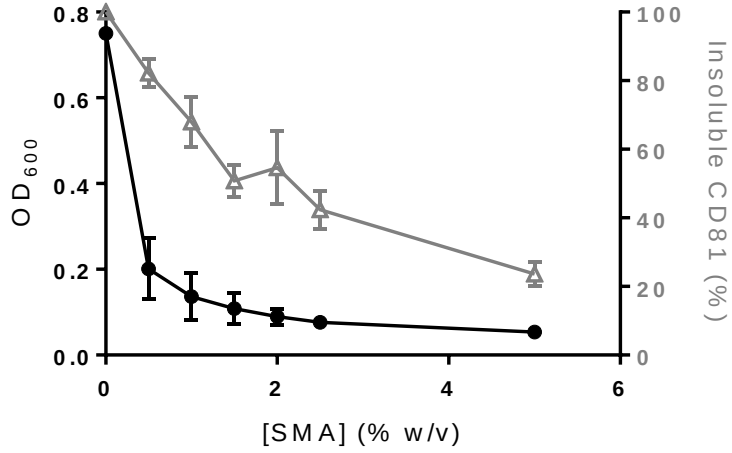
***BBA Biomembr*, 2020, 1862: 183419**

Solubilisation of CD81 by SMA2000 is slower than the breakup of the total membrane









- The rate of solubilisation is protein and expression system specific.
- You need to measure the protein specifically, simply monitoring OD is not sufficient.

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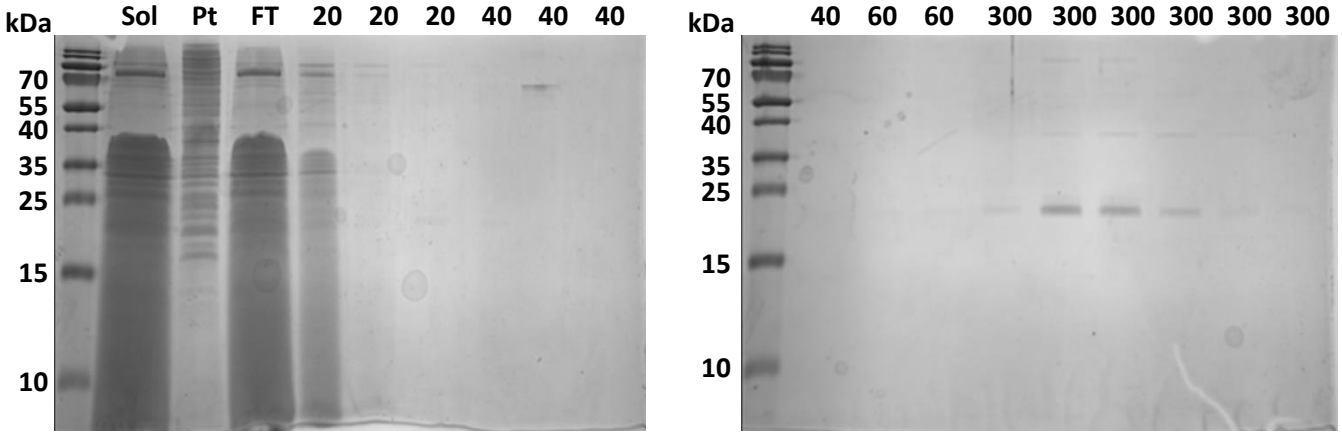
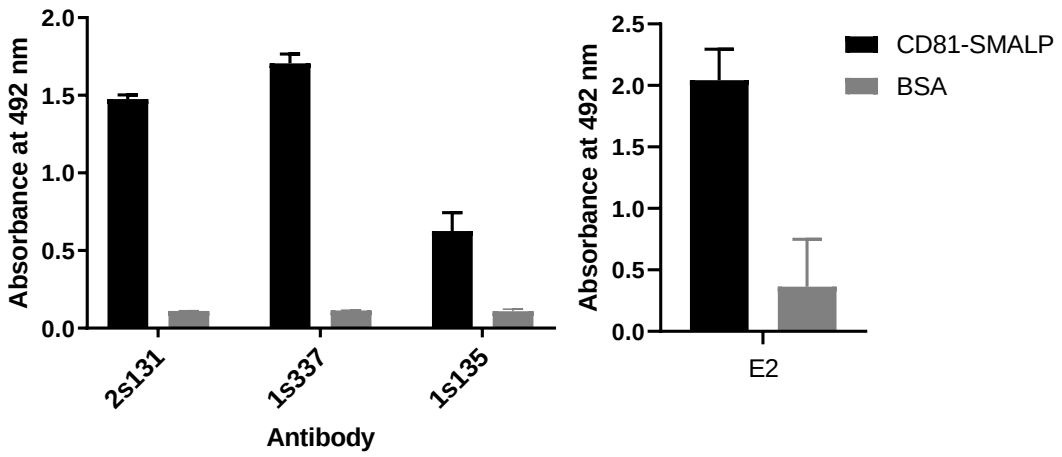
Purified SMALP-encapsulated CD81 is functionally folded

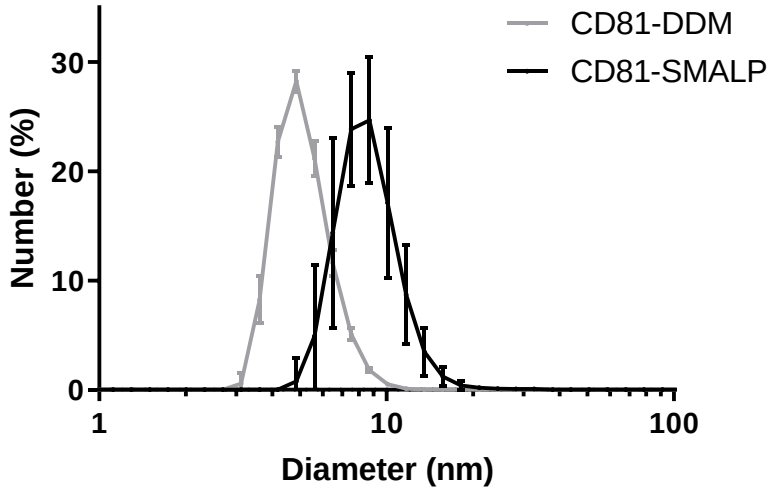




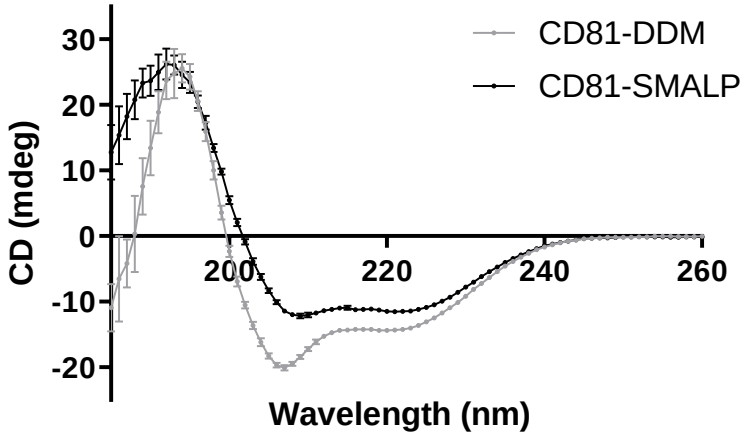
CD81 purified in SMALPs (or DDM) is able to bind to conformationally sensitive antibodies and to Hepatitis C virus E2 glycoprotein.

***BBA Biomembr*, 2020, 1862: 183419**

Biophysical characterisation of purified CD81-SMALP



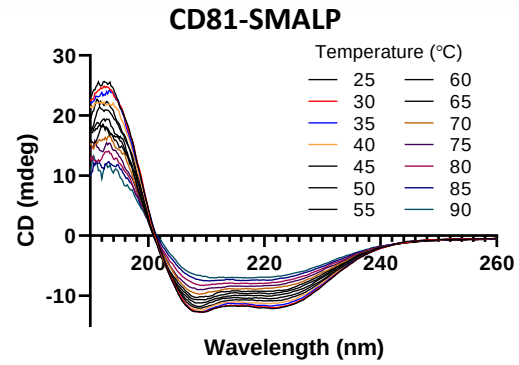
CD81-SMALPs ≈ 10nm
CD81-DDM ≈ 5nm

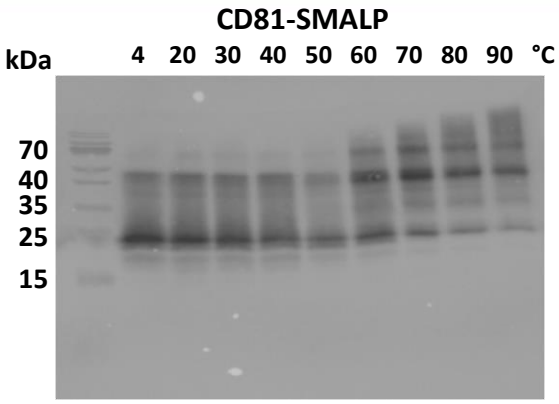


	α-helical	β-sheet	other
CD81-SMALP	71%	2%	28%
CD81-DDM	58%	6%	37%

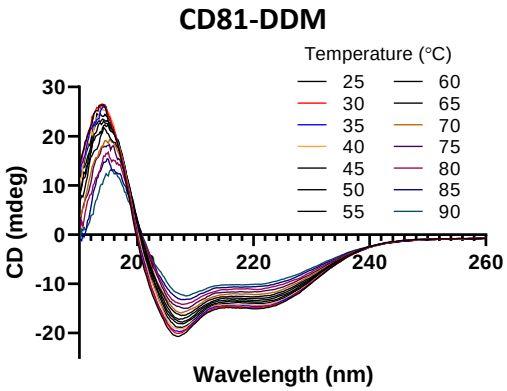
***BBA Biomembr*, 2020, 1862: 183419**

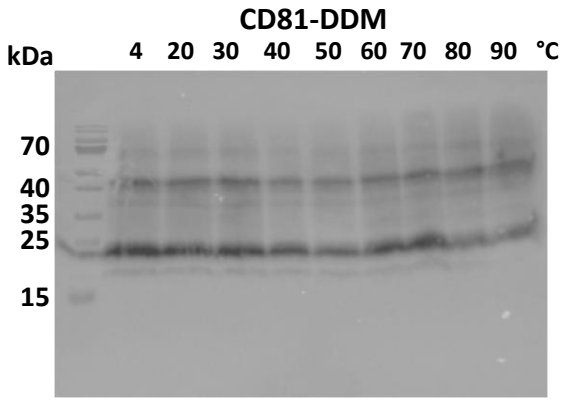
Thermostability of purified CD81



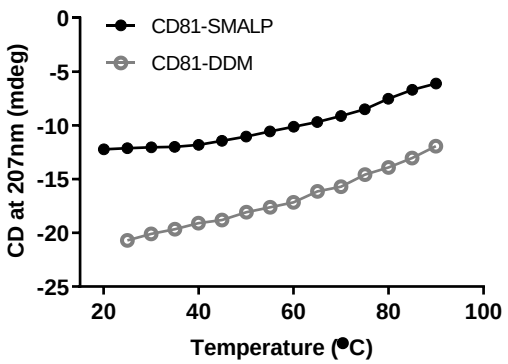


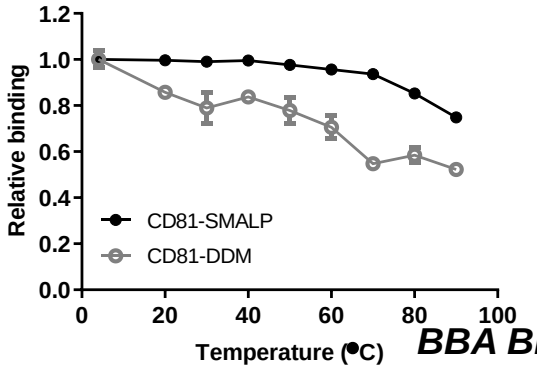
The secondary structure of CD81 appears equally thermostable in SMALPs as in DDM





The aggregation of CD81 appears to be less with DDM than SMA

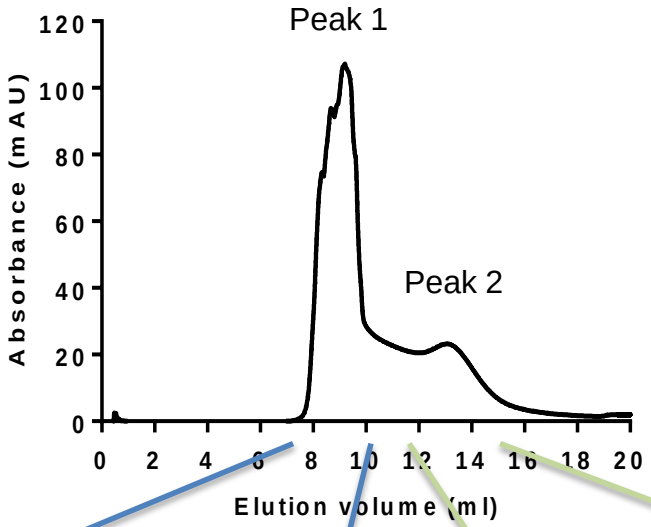


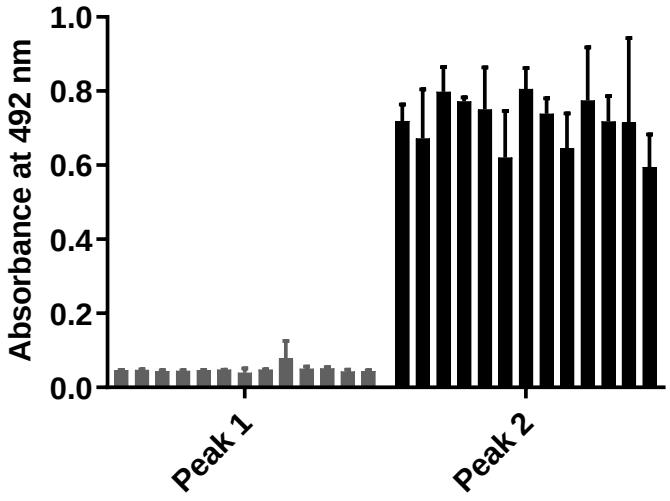


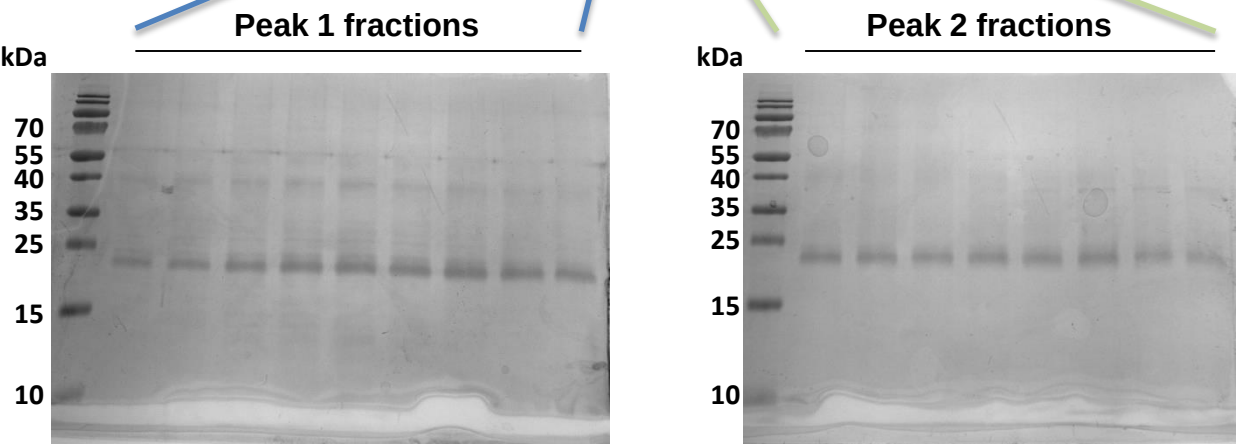
CD81 in SMALPs is more thermostable within the important extracellular loop than CD81 in DDM

***BBA Biomembr*, 2020, 1862: 183419**

Size exclusion chromatography reveals two distinct protein populations







Only the CD81 in Peak 2 binds to the HCV E2 glycoprotein

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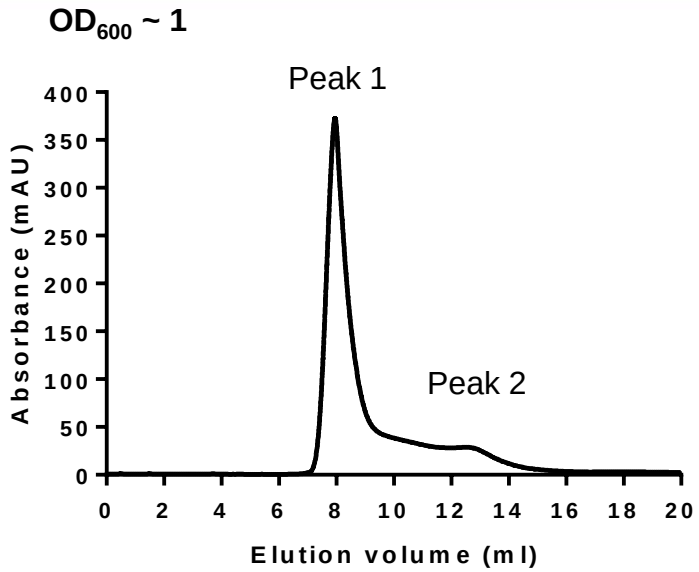
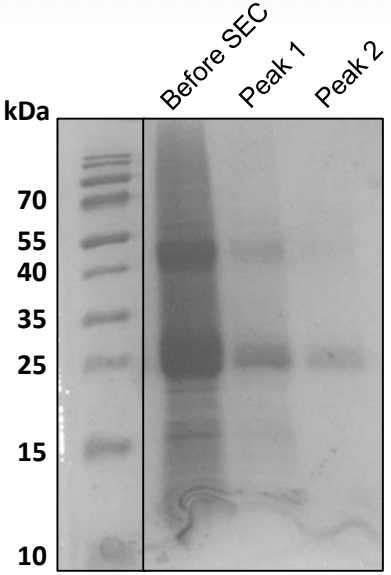
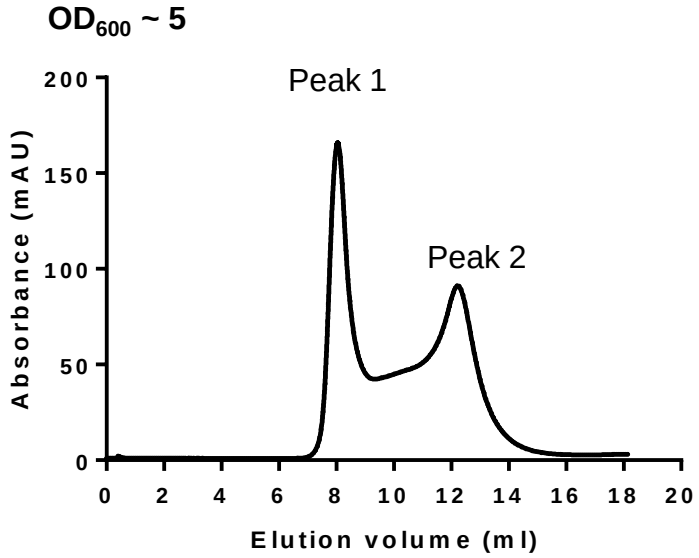
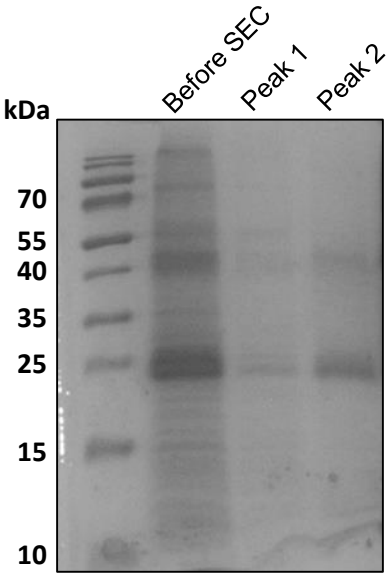
Changing expression and purification conditions can increase the proportion of CD81 in peak 2









- Inducing expression at a lower cell density, OD_{600} = 1 (rather than OD_{600} > 5) for 22 h
- Optimizing purification buffer (HEPES rather than Tris, including 10% glycerol and 200 mM NaCl)

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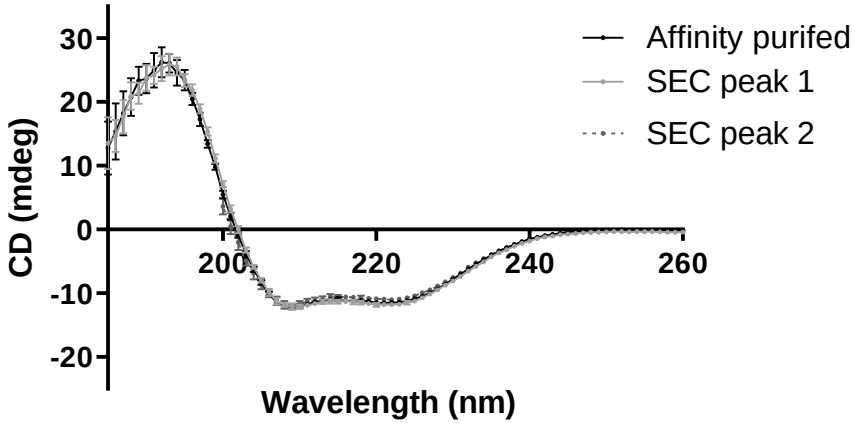
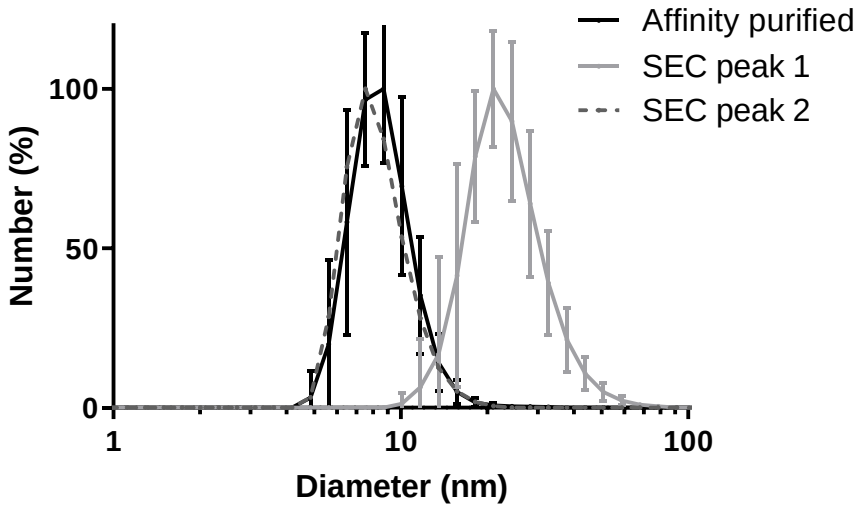
Biophysical characterisation of the two SEC peaks



The CD spectra overlay. Peak 1 still has the same folded secondary structure.



Peak 1 particles are approximately twice the size of Peak 2.

Dimer ?

Conformational change?

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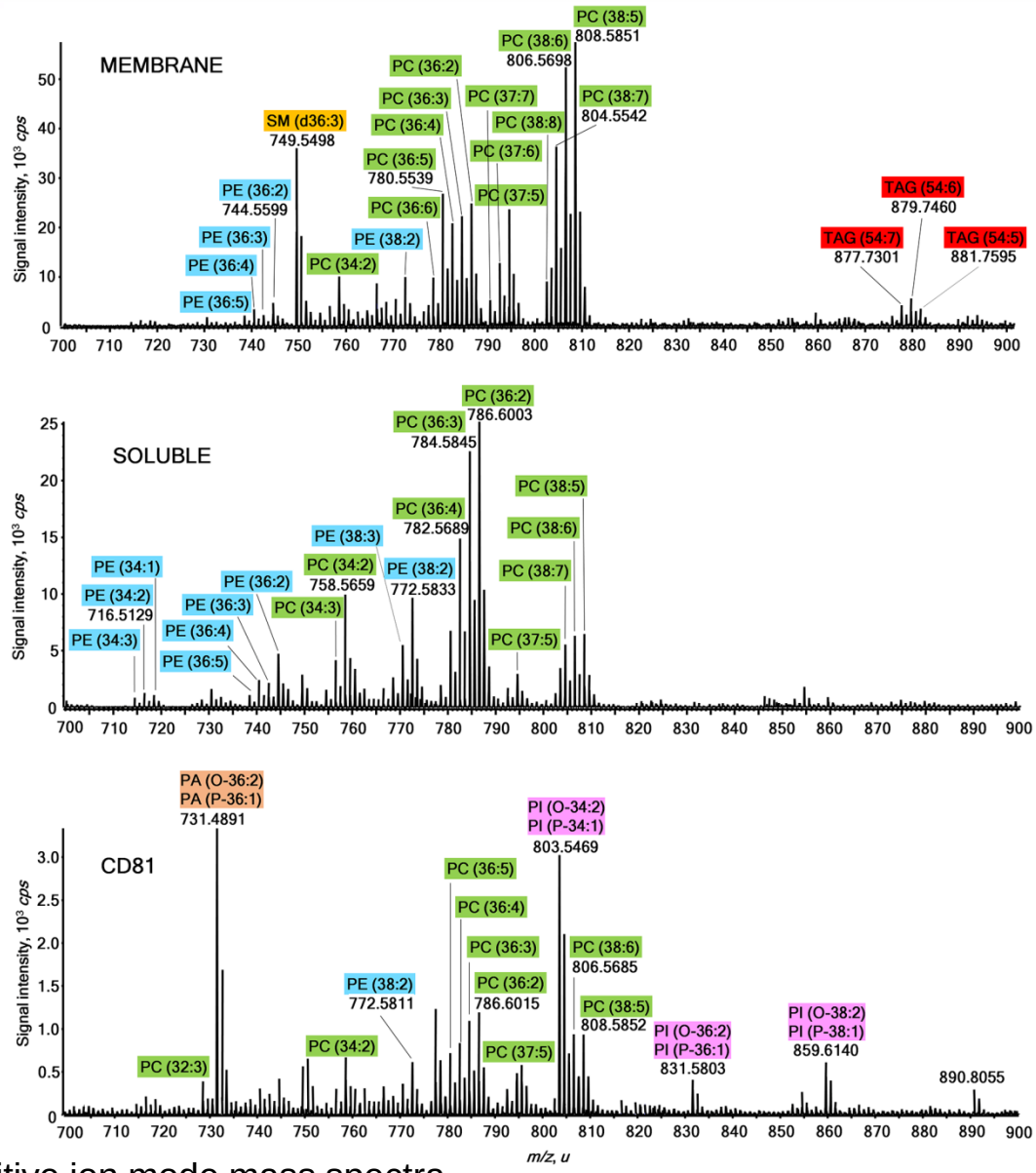
Lipid analysis



Positive ion mode mass spectra

P. pastoris membranes:
dominated by PC
relatively long polyunsaturated chains.
Several different PE species.

SMA-solubilised membranes:
No sphingomyelin or triacylglycerol.
Similar complex PC species.
Several different PE species.

SMA purified CD81:
Almost complete loss of PE.
PI and PA strong even in positive mode.

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Conclusions

- Microbial hosts dominate in the production of recombinant membrane proteins for structural studies
- Consider gene, sequence tags (and location), promoter, strain and culture conditions
- CD81 expressed in *Pichia pastoris* can be solubilized and purified using SMA polymer
- SMALP-encapsulated CD81 retains native folded structure
- Expression and buffer conditions can be optimized to improve protein quality
  - Induce at low cell density
  - Optimize buffer for SMA purification
- The lipid environment surrounding CD81 is enriched with negatively charged lipids.

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