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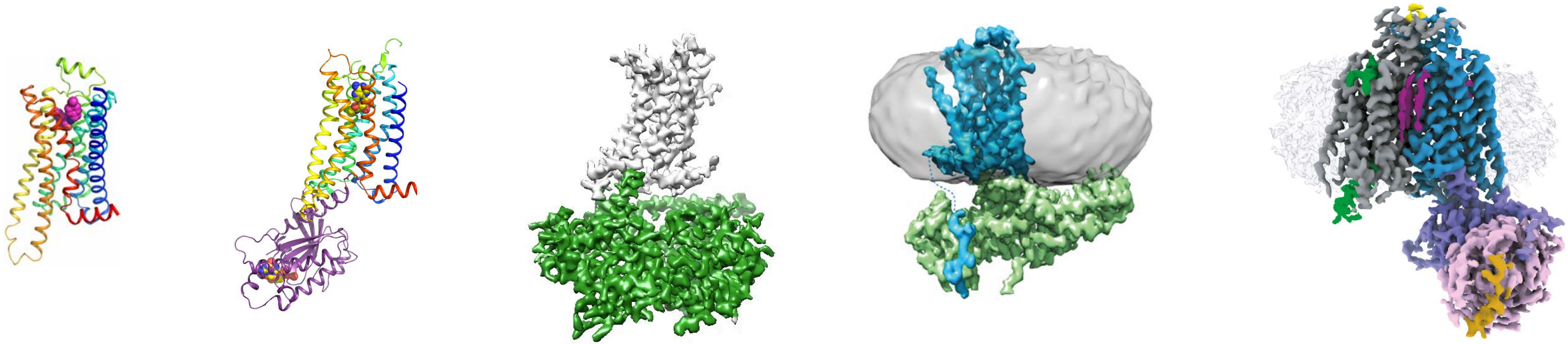
Whipping GPCRs into shape for structure determination

Chris Tate



G protein-coupled receptors (GPCRs) are found throughout the human body and are targeted by 34% of FDA approved small molecule drugs

- Nervous system
- Visual system
- Cardiovascular system
- Respiratory system
- Immune regulation
- Digestion and gut function
- Kidney function
- Liver metabolism
- Musculoskeletal system
- Puberty and reproduction
- Wound healing

OxyContin (oxycodone HCl controlled-release) tablets
Abilify aripiprazol
Zyprexa IntraMuscular Olanzapine for Injection
Spiriva (tiotropium)
Suboxone (buprenorphine HCl/naloxone HCl dihydrate)
Geodon (ziprasidone HCl) Capsules
Byetta exenatide injection
Sensipar (cinacalcet) Tablets
Plavix Clopidogrel 75 mg
Advair
Singulair (montelukast sodium)
Seroquel
Diovan valsartan capsules
Ventolin HFA (albuterol sulfate) inhalation aerosol
Detrol LA tolterodine tartrate
Benicar (olmesartan medoxomil)

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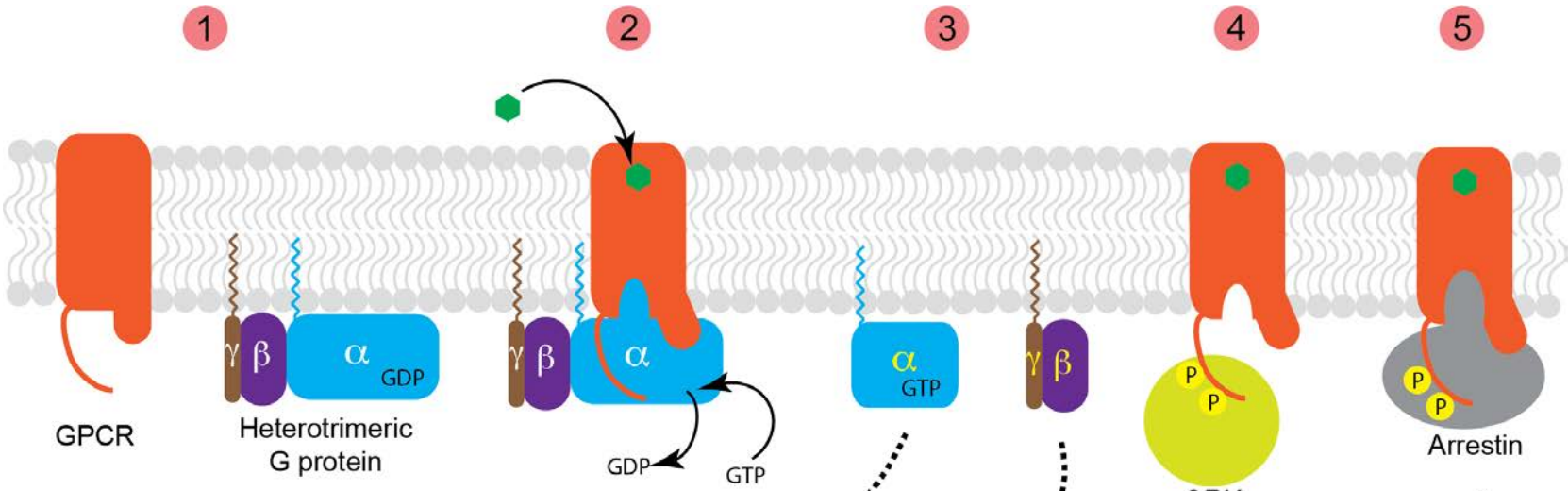
Signal transduction by GPCRs



Activity depends on subtype:

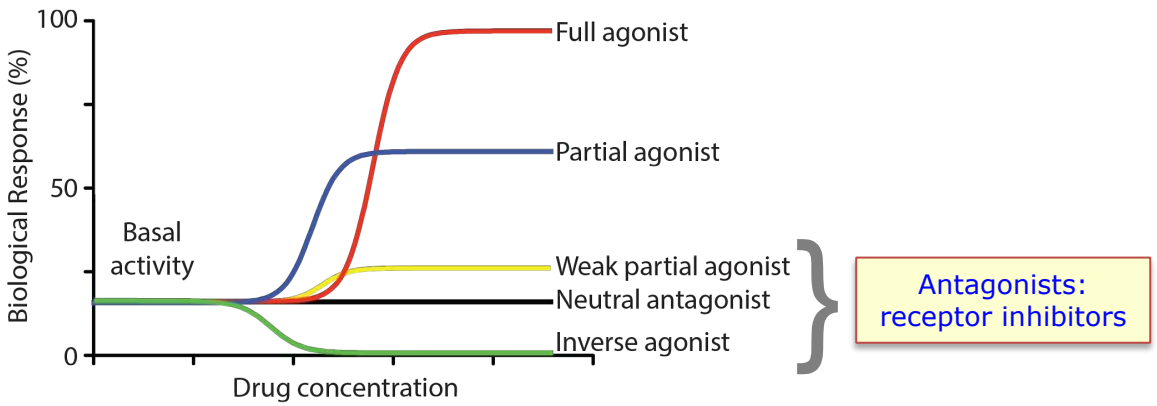
- Gαs + Adenylyl cyclases ↑cAMP
- Gαq + PLC-β + PKC ↑DAG ↑Ca^{2+}
- Gα12 + Rho + RhoGEFs
- Gαi − Adenylyl cyclases ↓cAMP
  - − Phosphodiesterases
  - + Phospholipases
  - +/− Ion channels

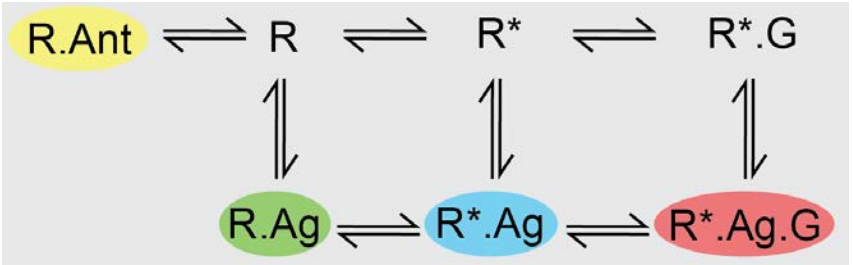
- + Ion channels
- + PI3γ
- + Adenylyl cyclases
- + PLC-β

- + MAP kinases
- + ERK1/2

GPCRs have a rich pharmacology and are highly dynamic

- the efficacy of synthetic ligands varies





- agonist binding increases the probability of G protein coupling
- binding of a G protein increases agonist affinity
- agonist binding to some receptors is inhibited by Na^+

R : receptor in an inactive conformation
R* : receptor in an active conformation
Ag : agonist
G : G protein (or mimetic)
Ant : antagonist

Conformational changes upon GPCR activation: coupling of mini-G_s to $A_{2A}R$

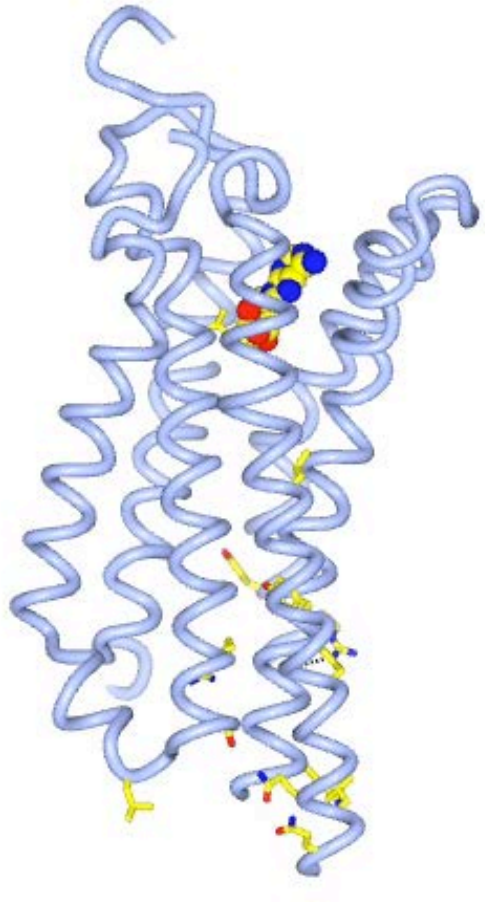
Inactive state

- agonist binding
- contraction of ligand binding pocket (LBP)
- conformational change
- opening of cleft on cytoplasmic face

Active intermediate

- G protein binding

Active state



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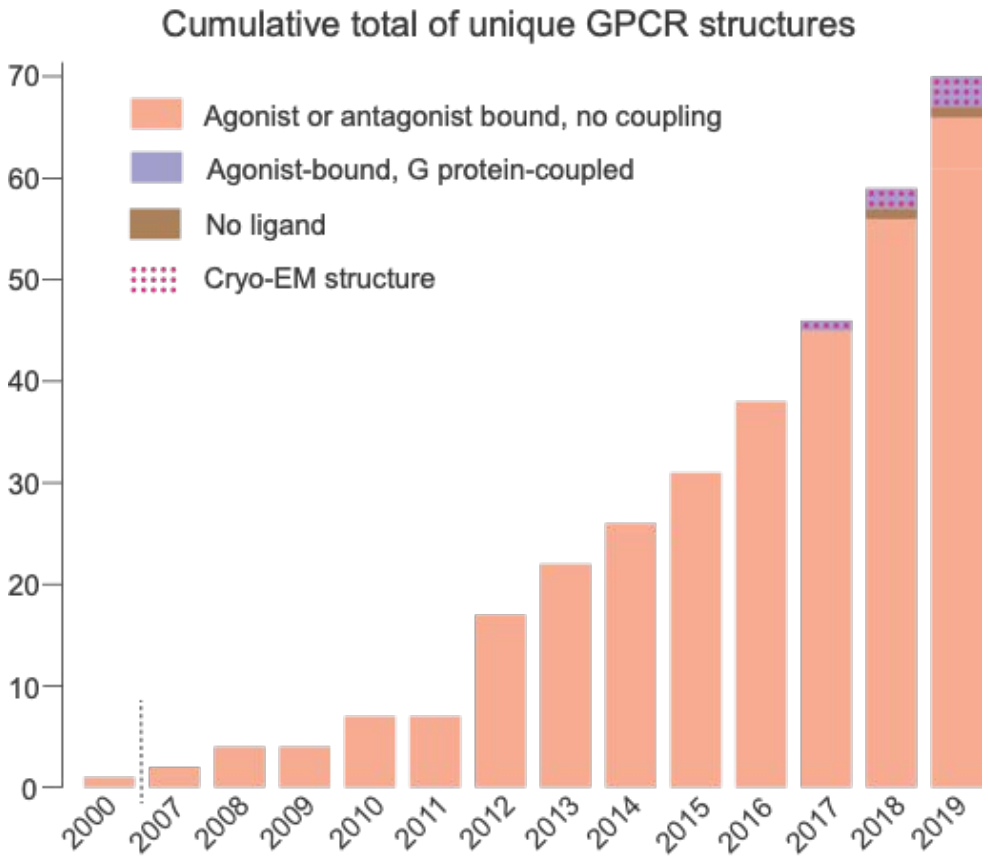
Engineering GPCRs has been essential for the success of structure determination



Congreve et al (2020) Cell **181**, 81-91

Factors to consider in structure determination

(1) Improve potential crystal contacts
- Remove flexible regions and post-translational modifications
- Fuse to soluble proteins (T4L, BRIL etc)
- Use binding partners (F_{ab}, nanobody)

(2) Reduce conformational heterogeneity
- Add ligands (inverse agonist, agonist)
- Bind antibodies (F_{ab}, nanobody)
- Add point mutations

(3) Increasing thermostability
- Add ligands (inverse agonist, agonist)
- Bind antibodies (F_{ab}, nanobody)
- Add point mutations

Expression systems for GPCRs

	Complexity of the expression system	Ratio functional: non-functional receptor	Potential expression level of functional receptor
• Cell free systems	+	-/+	++
• *Escherichia coli*	+	-/+	++
• Yeast species	++	+	++
• Baculovirus	+++	++	++++
• Mammalian cells			
– Transient transfection	++++	++	+++
– Stable cell lines : Constitutive	++++	++++	+++
: Inducible	++++	++++	++++
– Viral systems : BacMam	++++	++++	++++
: Lentivirus	++++	++++	++++

Expression is receptor-dependent and user-dependent!

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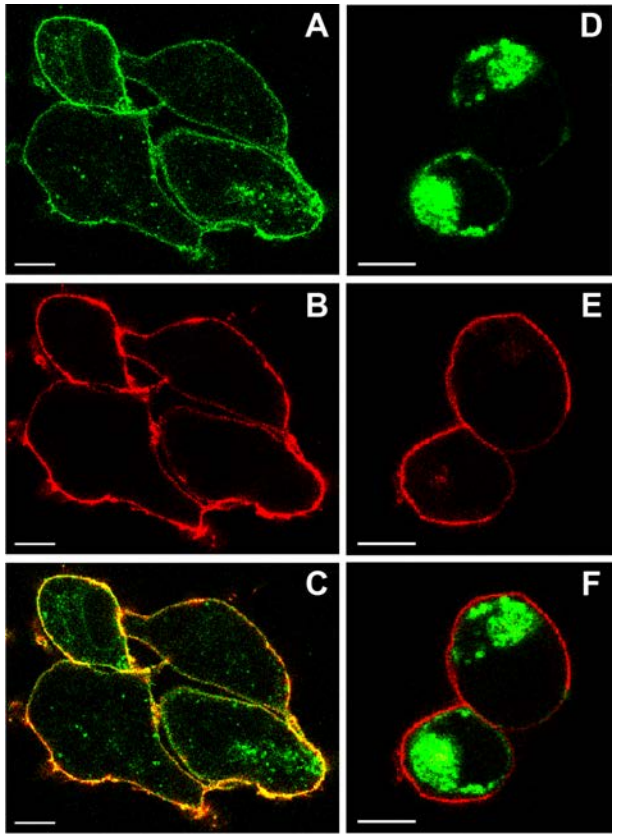
Tate (2001) FEBS Lett. **504**, 94-98
Tate *et al*. (2003) Biochim. Biophys. Acta **1610**, 141-153

Discrimination of misfolded GPCRs by a differential detergent solubility or confocal fluorescent microscopy

Inducible expression T-ReX HEK293

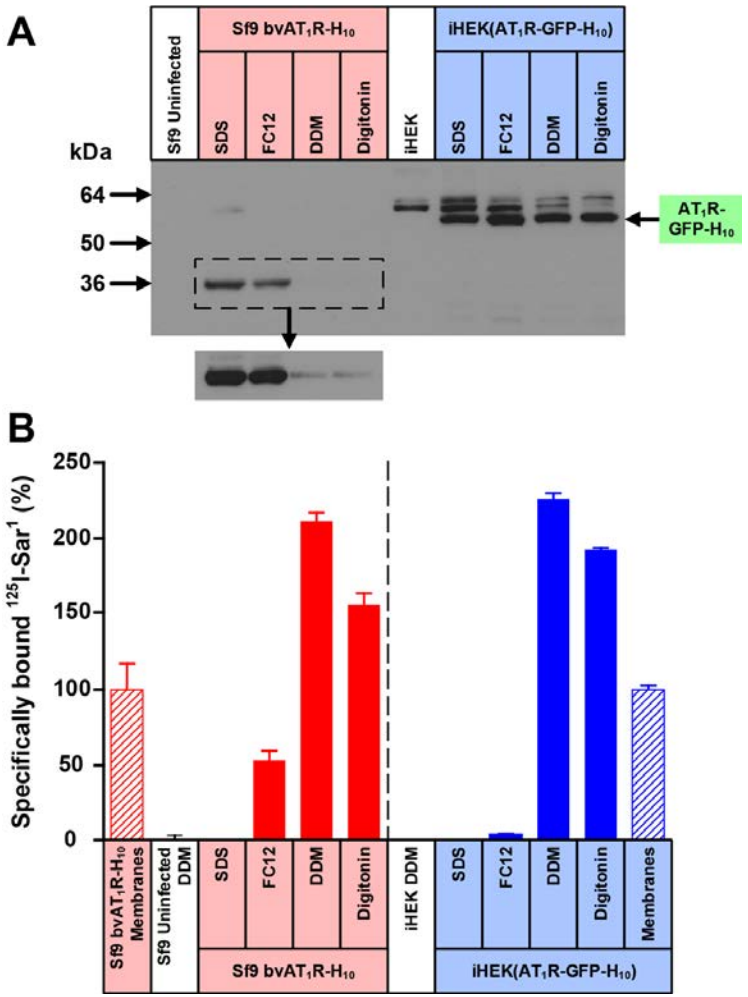
Baculovirus expression



Receptor

Cell surface

Merged



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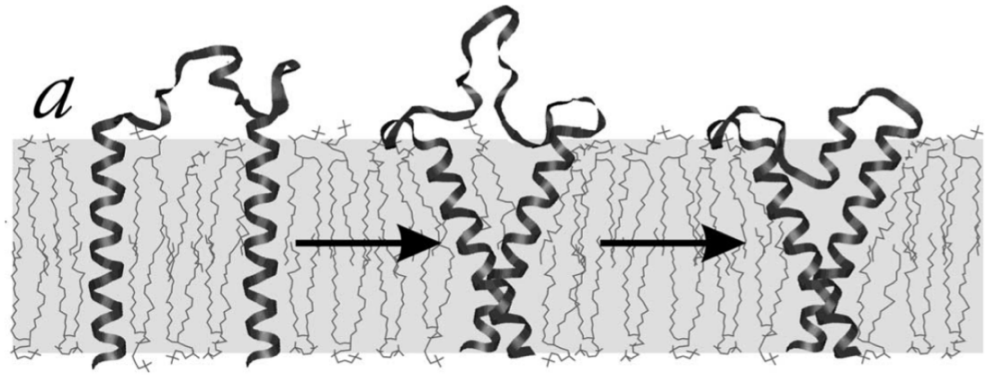
Thomas & Tate (2014) J. Mol. Biol. 426, 4139-4154

Membrane protein folding is complex and poorly understood



Engelman et al. (2003)
FEBS Lett. 555, 122-125

- Years have been spent trying to get mammalian membrane proteins to express in bacteria or yeast
- Use baculovirus or mammalian systems and save a lot of grief

2005: Making GPCRs behave

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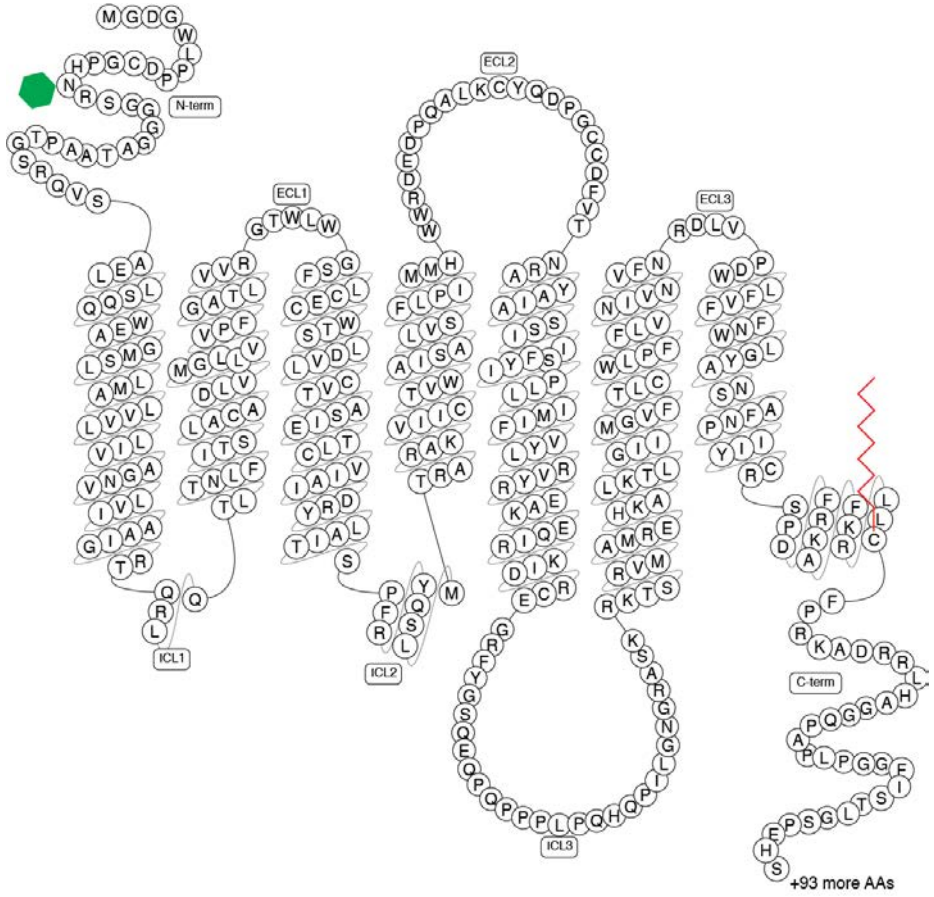
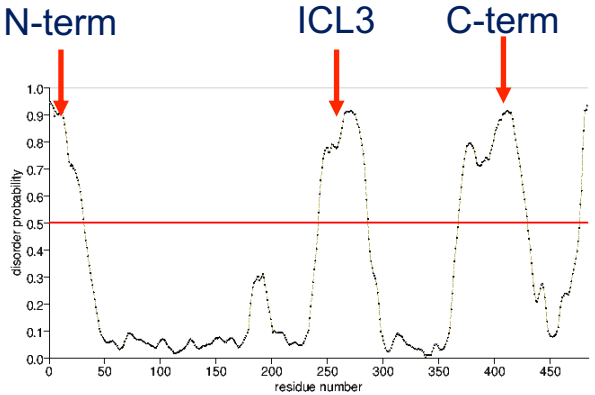
Protein engineering of the β_1-adrenoceptor (β_1AR) for X-ray crystallography

Wild type turkey β_1AR



Potential problems from sequence analysis

- Large potentially flexible regions (N-term, C-term, ICL3)
- Palmitoylation site
- N-glycosylation site
- Multiple phosphorylation sites



Disorder prediction (PrDOS)

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Attempts to engineer β_1AR for X-ray crystallography: 7 years in purgatory

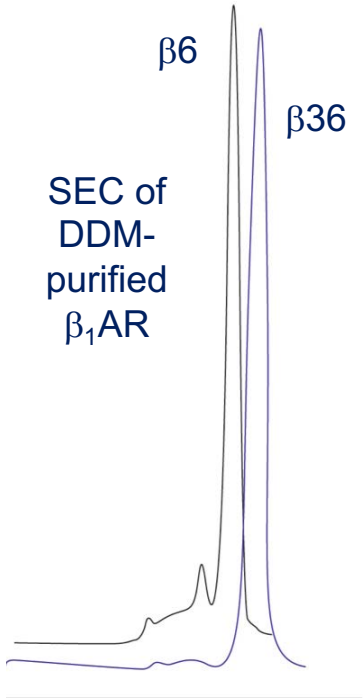
Tony Warne

β_1AR constructs

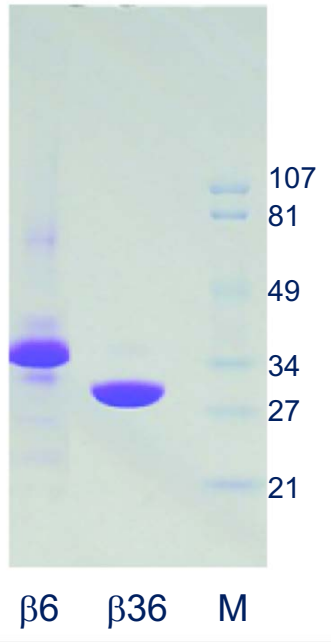
β6 : Δ3-32 (N-term)

β36 : Δ3-32 (N-term)
Δ368-483 (C-term)
Δ244-271,277,278 (ICL3)
C358A

Purified 2.5 mg β6 or β36
per L of insect cells



SDS-PAGE



No crystals!

The receptor was too unstable in short-chain detergents

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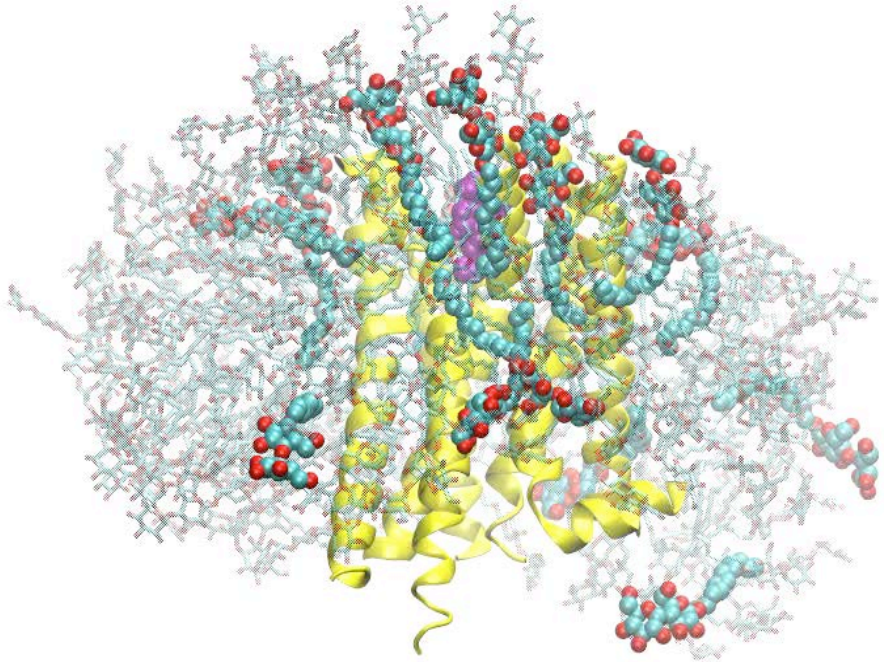
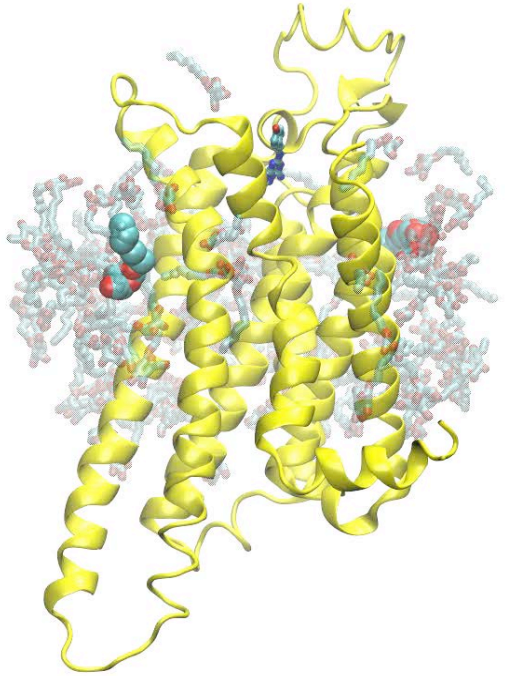
Warne *et al*. (2009) Protein Exp. Purif. **65**, 204-213

The dynamics of short chain detergents explains why they are so denaturing



$A_{2A}R$ in DDM



$A_{2A}R$ in OG

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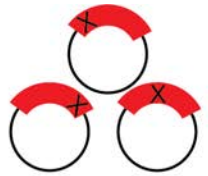
Lee et al (2016) J. Am. Chem. Soc.**138**, 15425-15433

Conformational thermostabilisation:

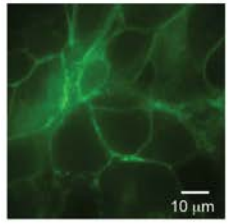
Stabilisation of membrane proteins preferentially in a particular state to allow the use of short-chain detergents in crystallography

Magnani *et al* (2018) Nature Protocols **8**, 1544-1571

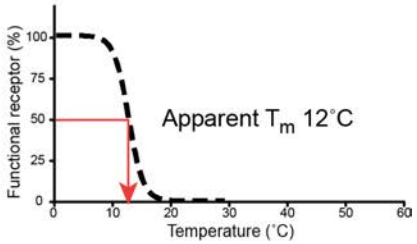
Construction of Ala/Leu scanning mutants throughout the MP
Steps 1-11



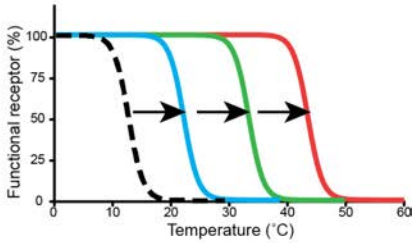
Expression of the MP mutants by transient transfection in HEK293 cells
Steps 12-17



Thermostability assay of Ala-scan mutants
Steps 18-27

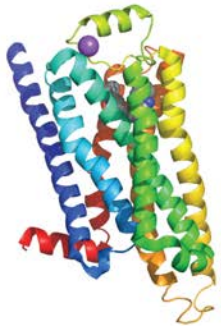


Accurate apparent T_m determination of a subset of single mutants
Steps 28-34



Combination of thermostabilising mutations into an optimally thermostable MP
Steps 35-40

Determination of the thermostability in short-chain detergents suitable for crystallisation and structure determination



Structure determination

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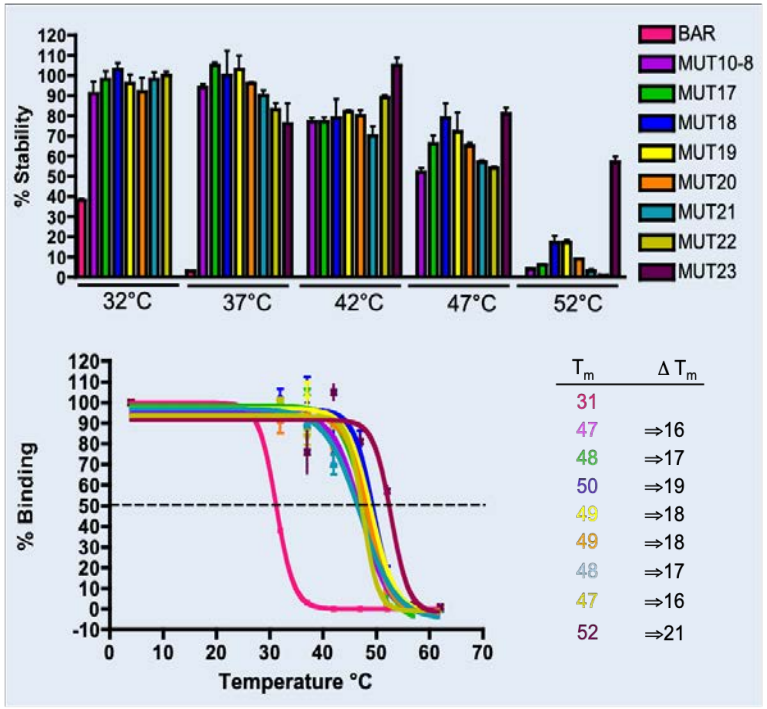
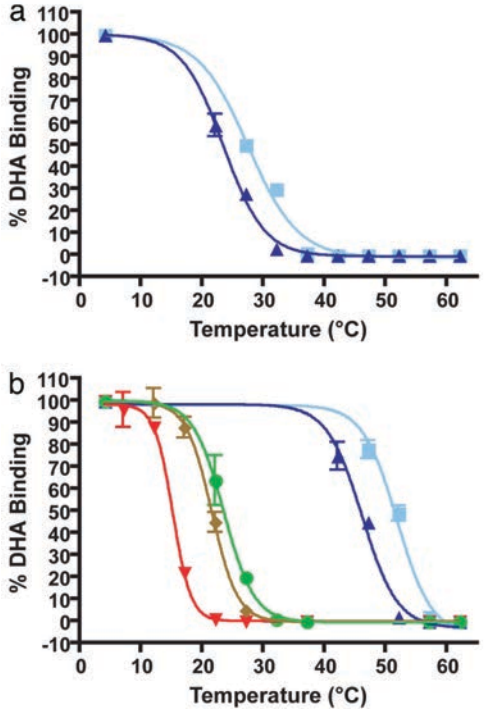
Conformational thermostabilisation of β_1AR

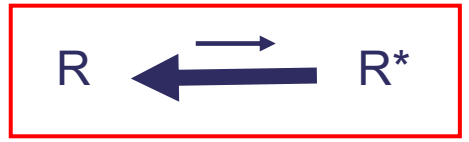
Constructing βAR-m23



βAR-m23 was stable in short chain detergents

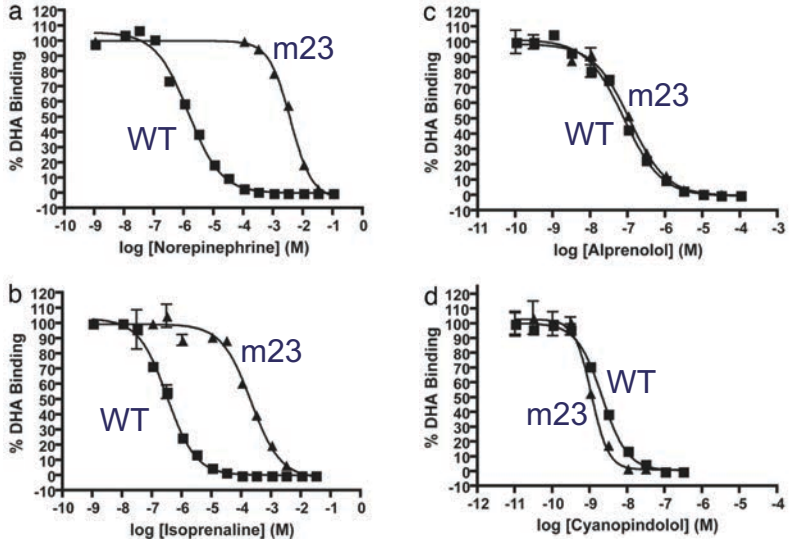


βAR-m23



Agonist binding

Antagonist binding



The receptor was now stable in short chain detergents and it crystallised in OTG

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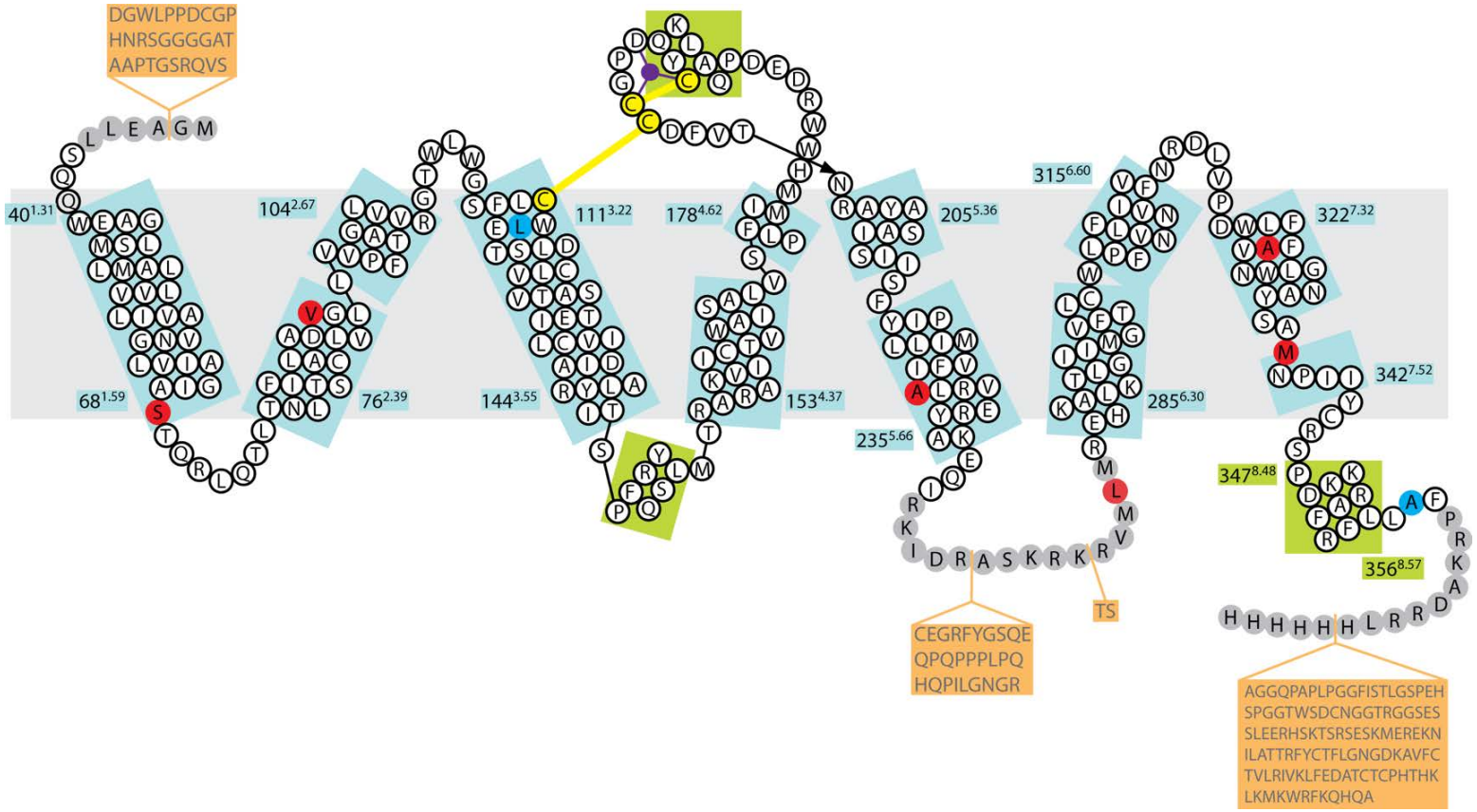
Serrano-Vega *et al* (2008) PNAS **105**, 877-882

Engineered construct of β_1AR that crystallised: thermostability has allowed crystallisation with any ligand (23 structures determined to date)



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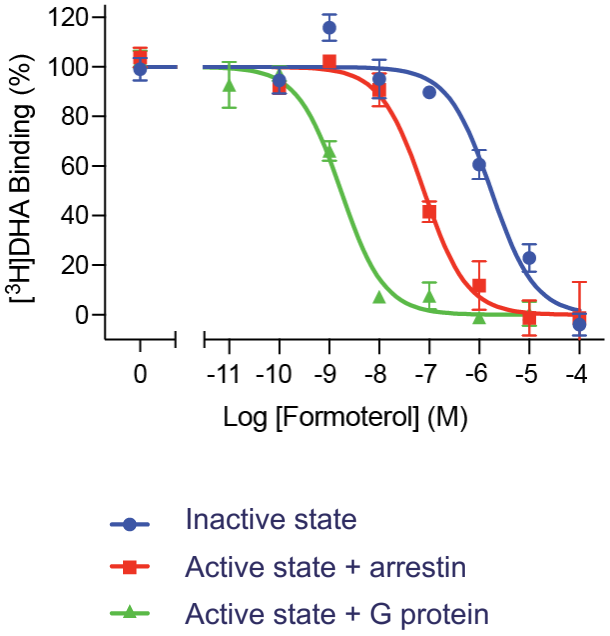
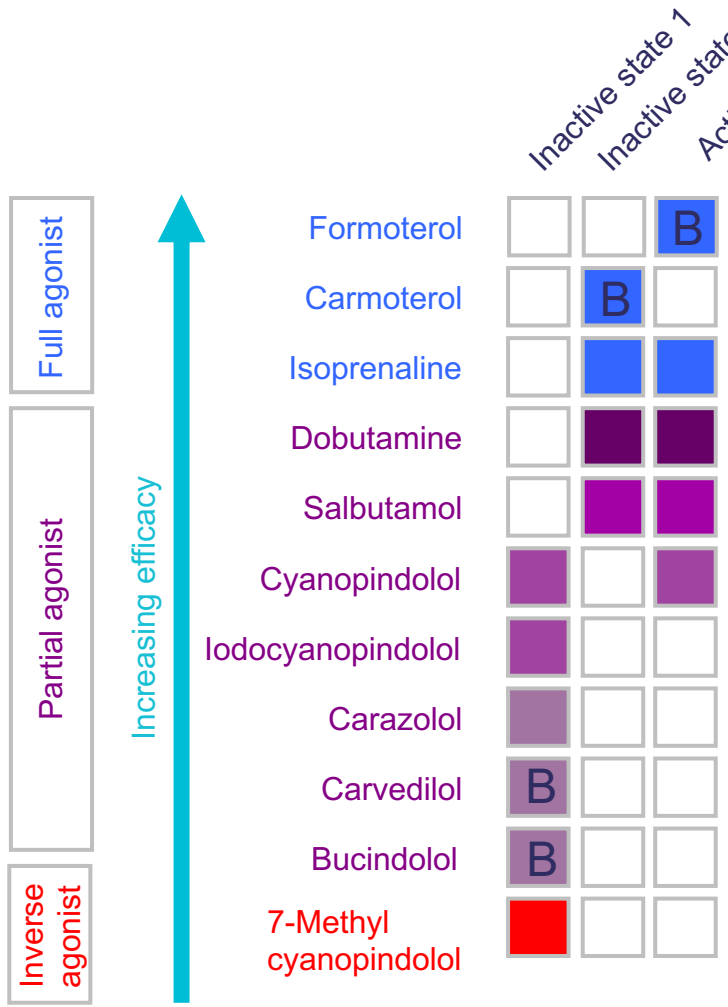
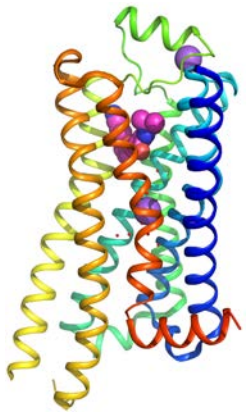
Warne *et al* (2008) Nature **454**, 486-491

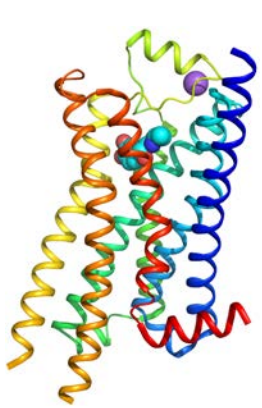
Understanding the molecular pharmacology of the β_1-adrenoceptor (β_1AR) through X-ray crystallography

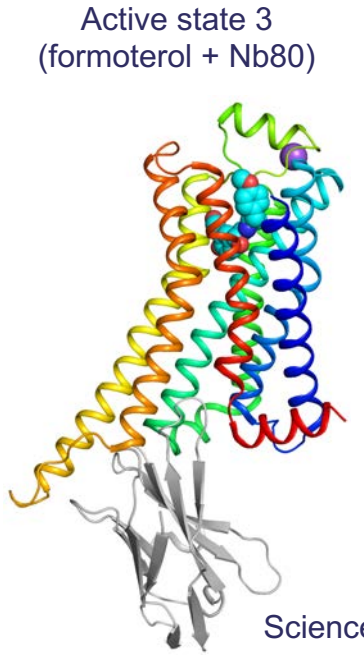




B: arrestin biased signalling







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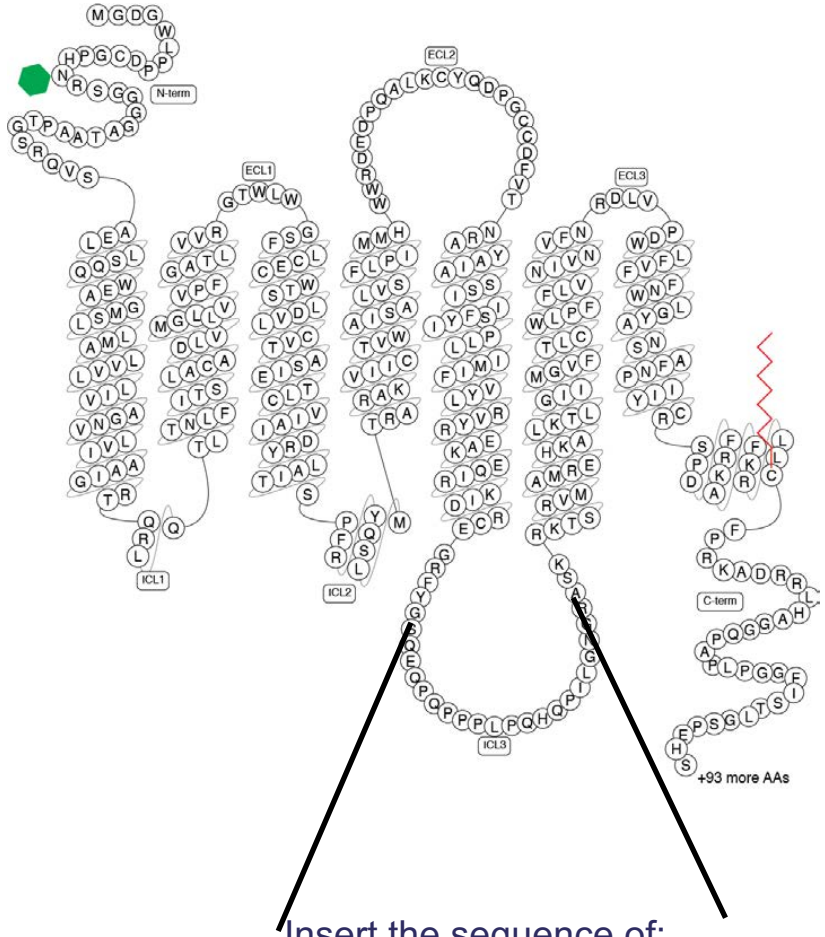
Science (2019) **364**, 775

Gene fusion is another strategy to obtain structures of GPCRs



Insert the sequence of:
T4 lysozyme
Cytochrome B562 or BRIL
(now many others as well)

- The position of fusion junction points is critical
- Start with known fusion points and test neighbours
- One amino acid difference in position can affect thermostability and also diffraction quality of crystals
- Good crystals can only be grown in lipid cubic phase
- High affinity ligands essential to stabilise the receptor

See GPCRdb for engineering tools

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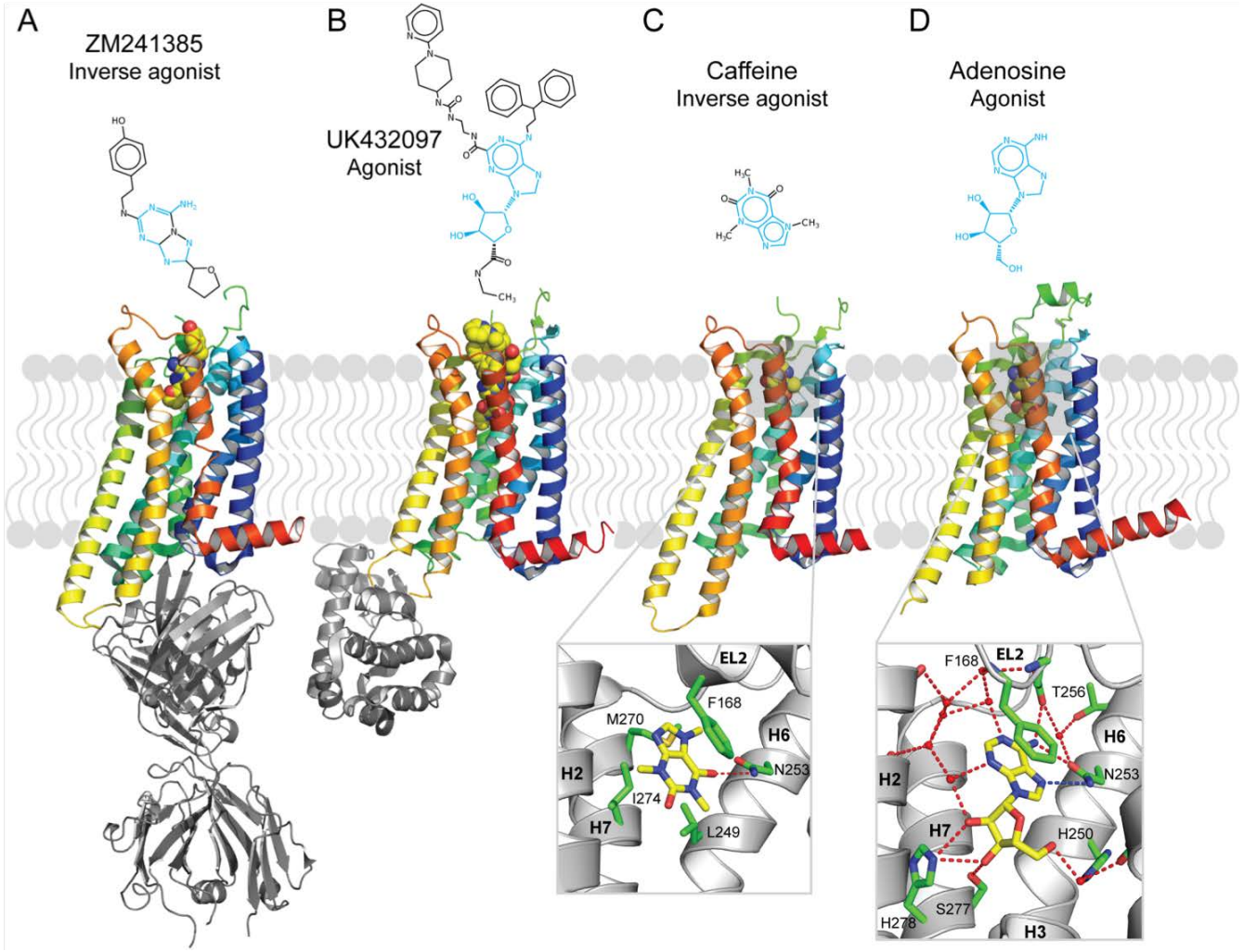
Developed in the Kobilka lab: Rosenbaum et al. (2007) Science 318, 1266-1273

Three strategies to determine the structure of the adenosine A_{2A} receptor



A: Hino et al (2012) Nature **482**, 237
B: Xu et al (2011) Science **332**, 322
C: Doré et al (2011) Structure **19**, 1283
D: Lebon et al (2011) Nature **474**, 521

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Tate (2012) Trends Biochem. Sci. 37, 343-352

Engineering a minimal G protein suitable for crystallising GPCR-G protein complexes

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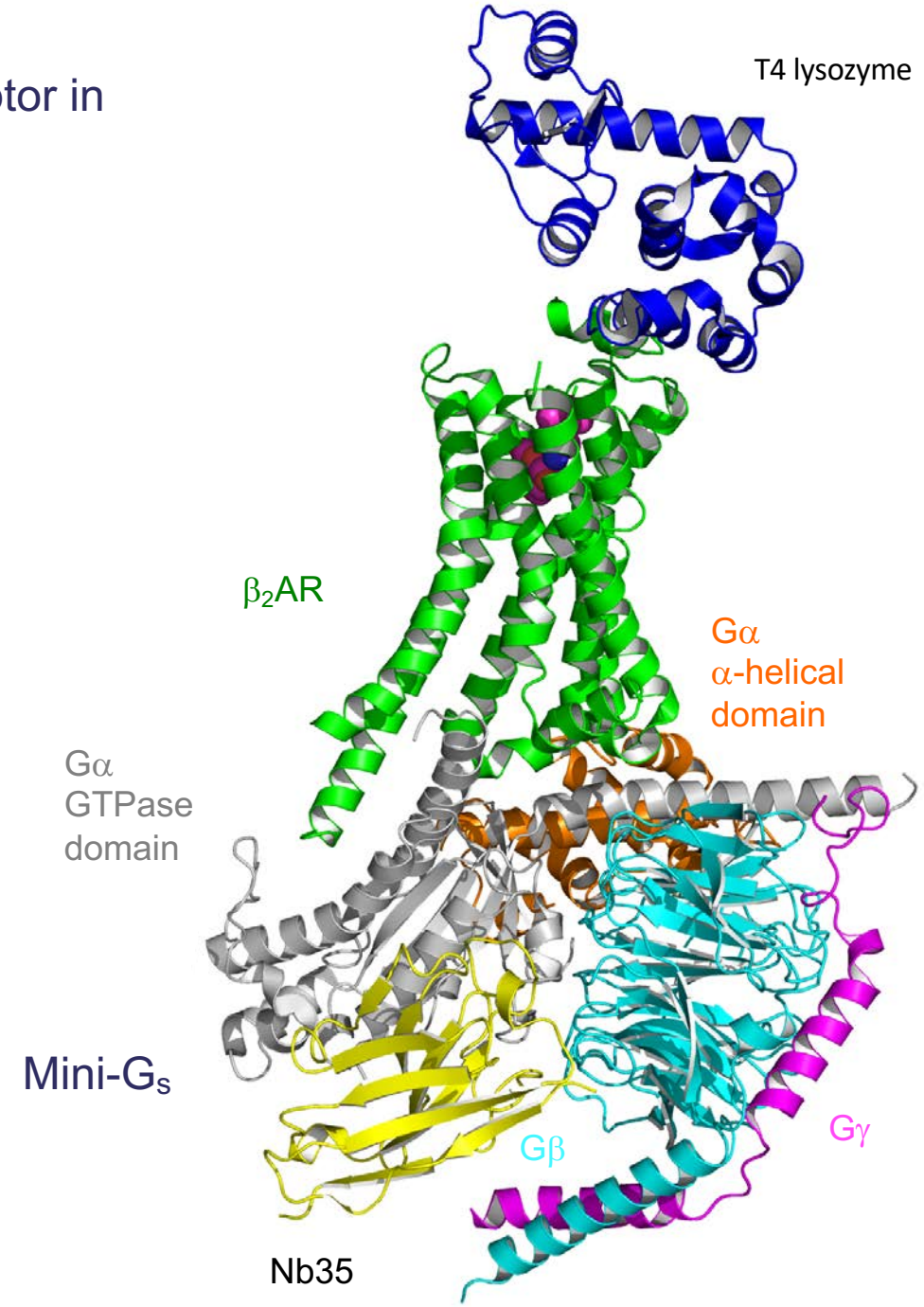
Structure of the agonist-bound β_2-adrenoceptor in complex with a heterotrimeric G protein

Rasmussen *et al.* (2011) Nature **477**, 549-555

- 97% of atomic interactions between G_s and β_2AR are mediated by Gα
- 70% of the surface area between Gα and β_2AR is mediated by the α5 helix



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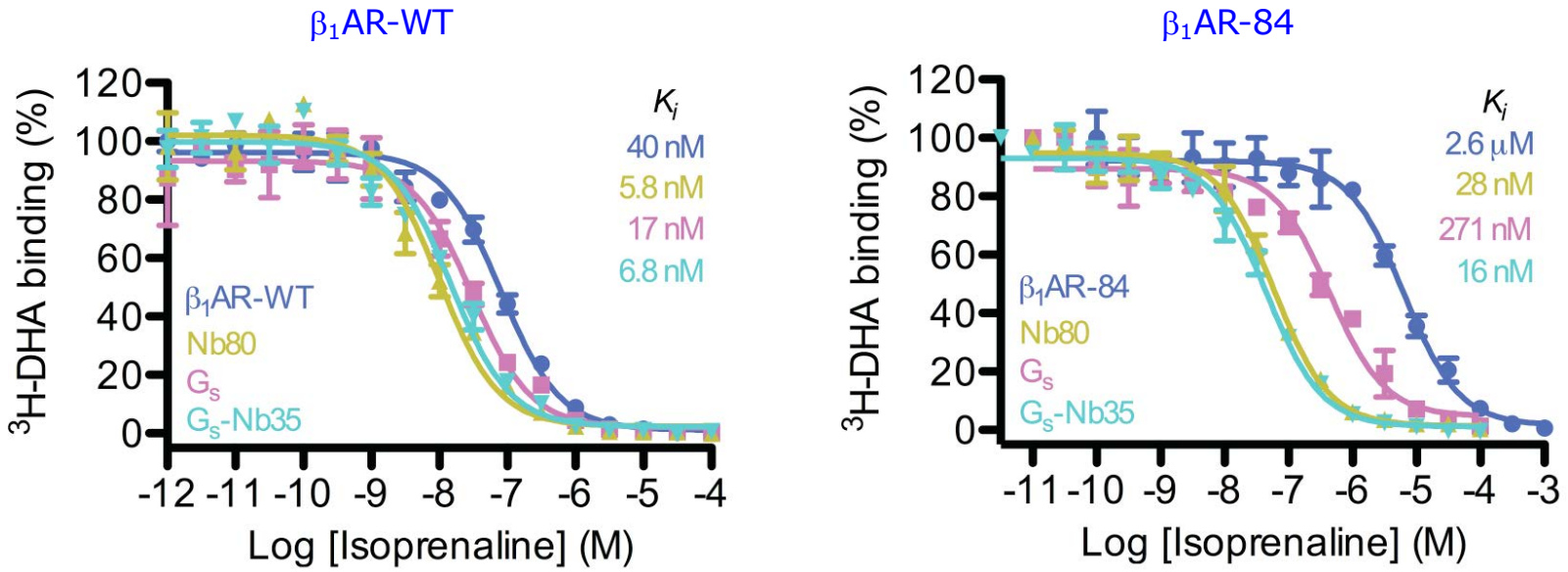
Agonist-shift assays for the development of mini-G_s : Assay for the desired trait *i.e.* G protein-coupling



β_1AR-WT : truncated wild-type receptor
β_1AR-84 : thermostabilised receptor in the antagonist conformation

Gs : heterotrimeric G protein Gαβγ
Gs-Nb35 : heterotrimeric G protein Gαβγ stabilised by nanobody Nb35

Nb80 : G protein mimetic

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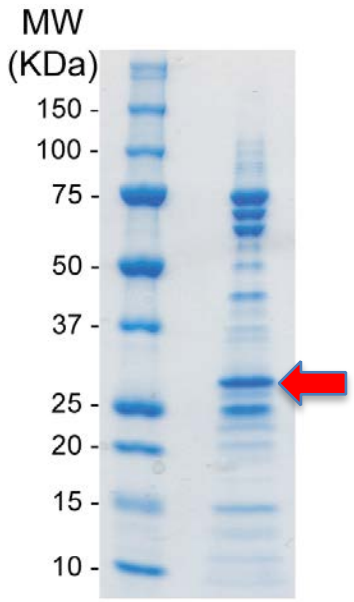
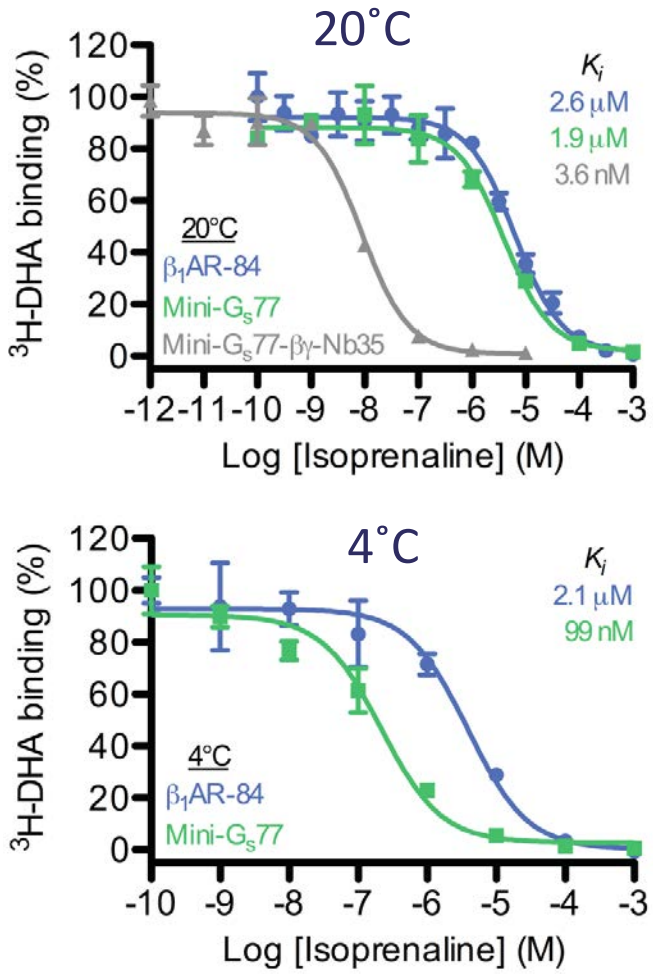
The isolated GTPase domain of Gα was very unstable, poorly expressed and could not be purified to homogeneity, but it did couple to β_1AR



Ni^{2+}-NTA 'purified' GTPase domain ~200 μg/L *E. coli*



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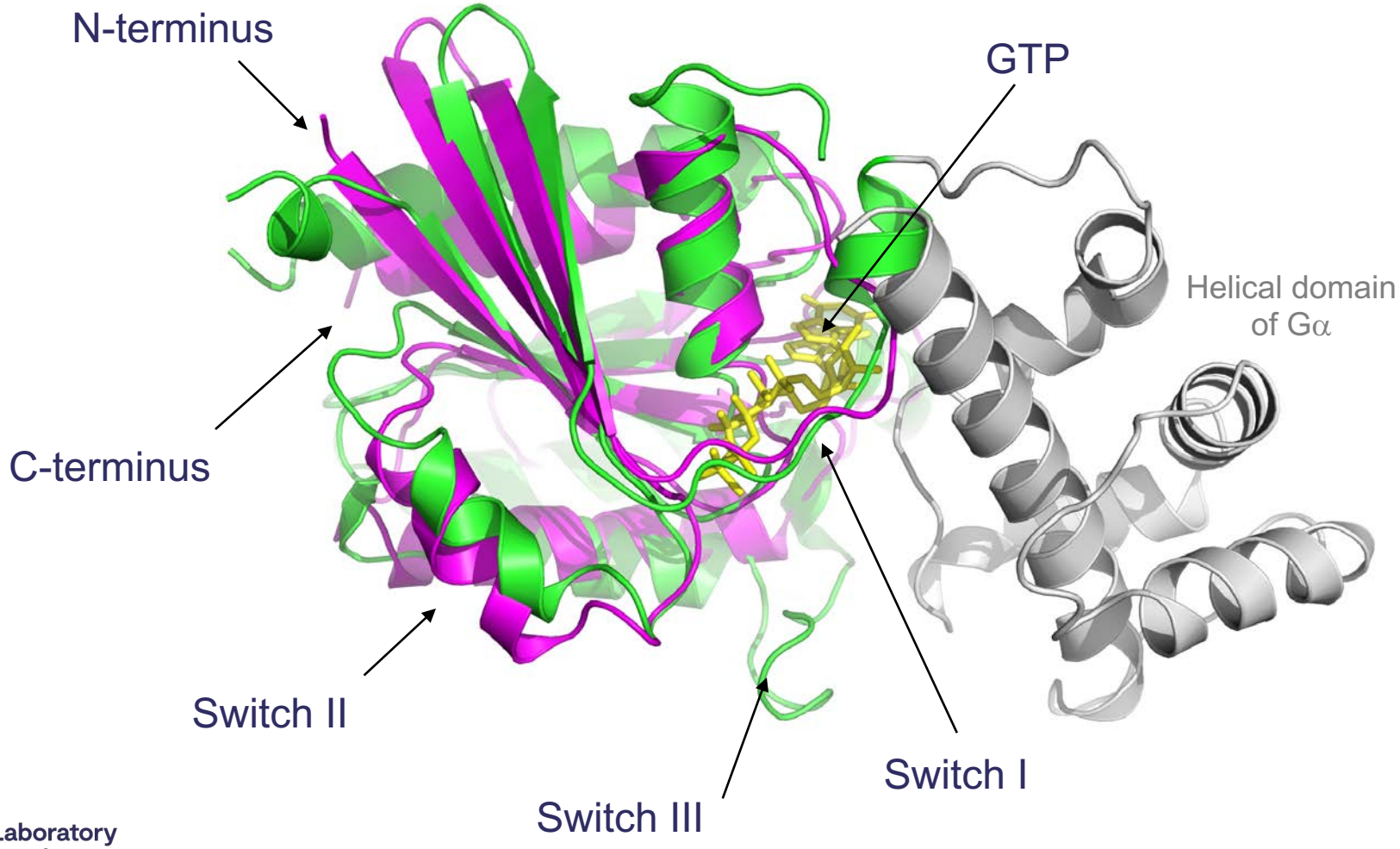
GTPase domains are highly conserved:
Alignment between the GTPase domain of Gα and the small GTPase Arl2

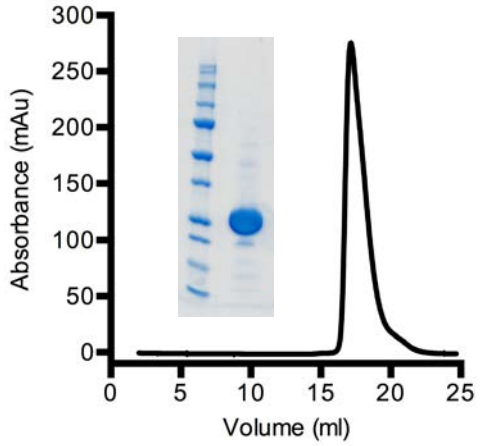


Mini-G_s is the stabilised GTPase domain of $G\alpha_s$

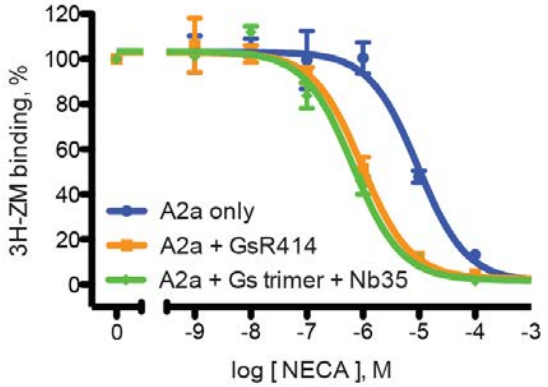
- 28 kDa protein expressed at 100 mg/L in *E. coli* (a)
- Contains 8 mutations and 3 deletions
- Mutations decouple nucleotide exchange from activation of mini-G_s and GPCR binding
- Gives the same shift in agonist affinity as the G_s heterotrimer either in detergent or membranes (b)
- Forms a stable complex with many G_s-coupled GPCRs in the presence of agonist
- The complex of the adenosine A_{2A} receptor with mini-G_s is more stable in octylglucoside than the equivalent complex formed by the G_s heterotrimer and Nb35 (c)
- Suitable for crystallisation by vapour diffusion

Carpenter & Tate (2016) Protein Eng. Design Sel. **29**, 583-594

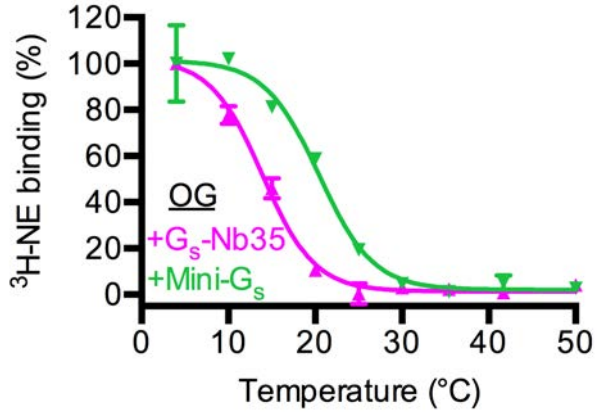
(a)



(b)



(c)



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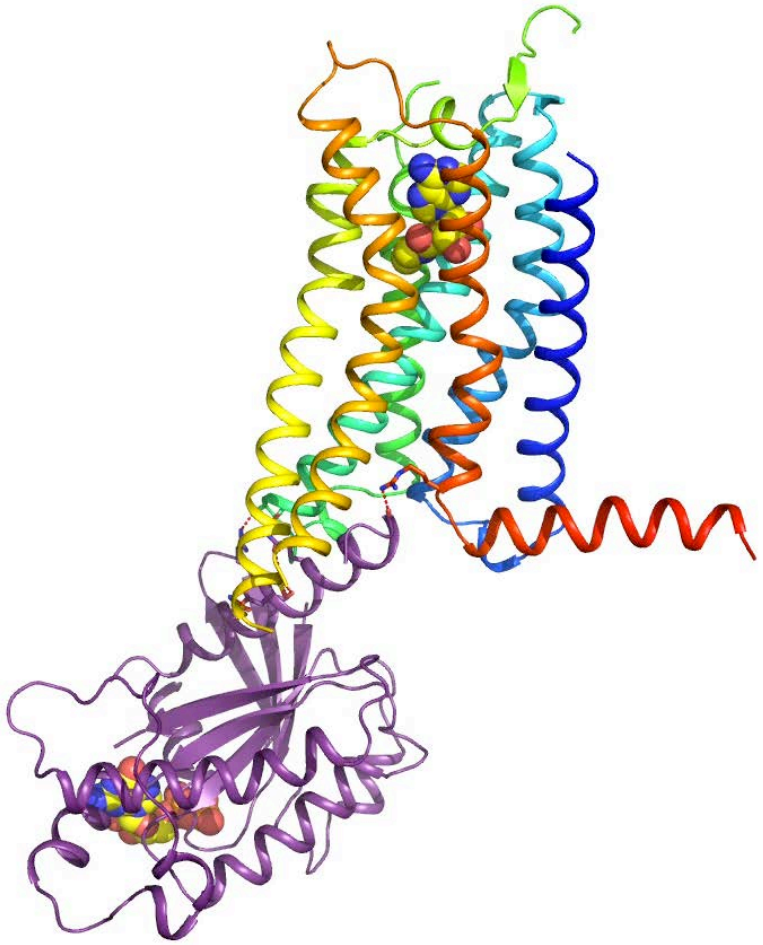
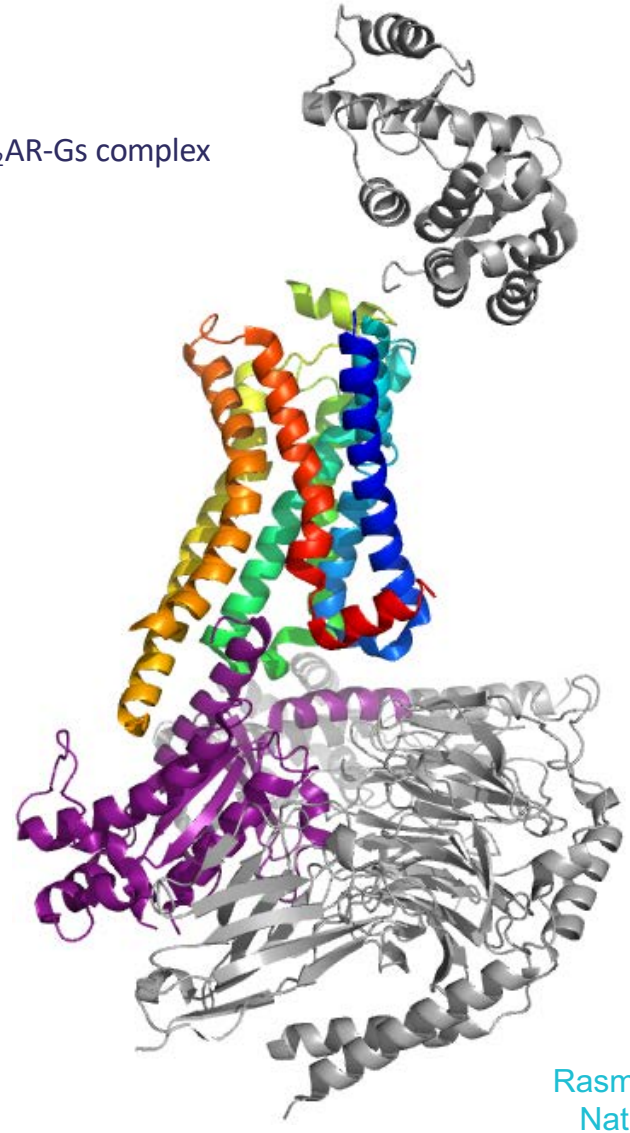
Overall structure of the $A_{2A}R$–mini-G_s complex compared to the β_2AR–G_s complex



β_2AR-Gs complex



Carpenter et al. (2016)
Nature 536, 104-107

Rasmussen et al. (2011)
Nature 477, 549-555

Strategies for structure determination by cryo-EM

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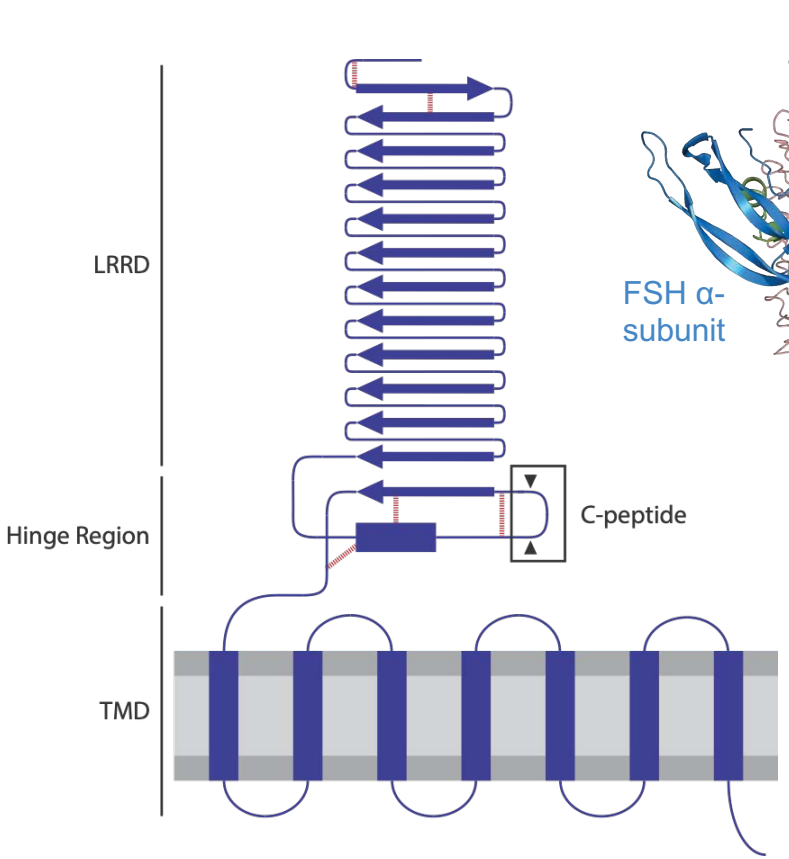
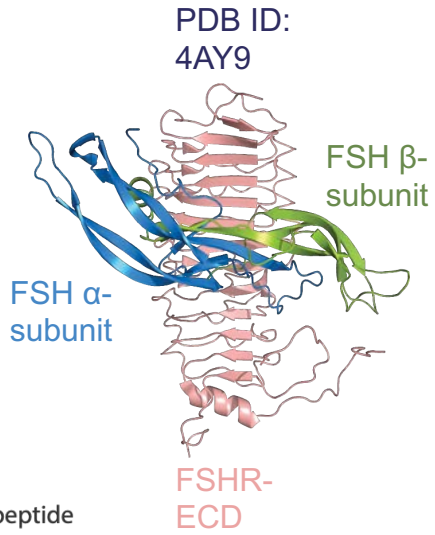
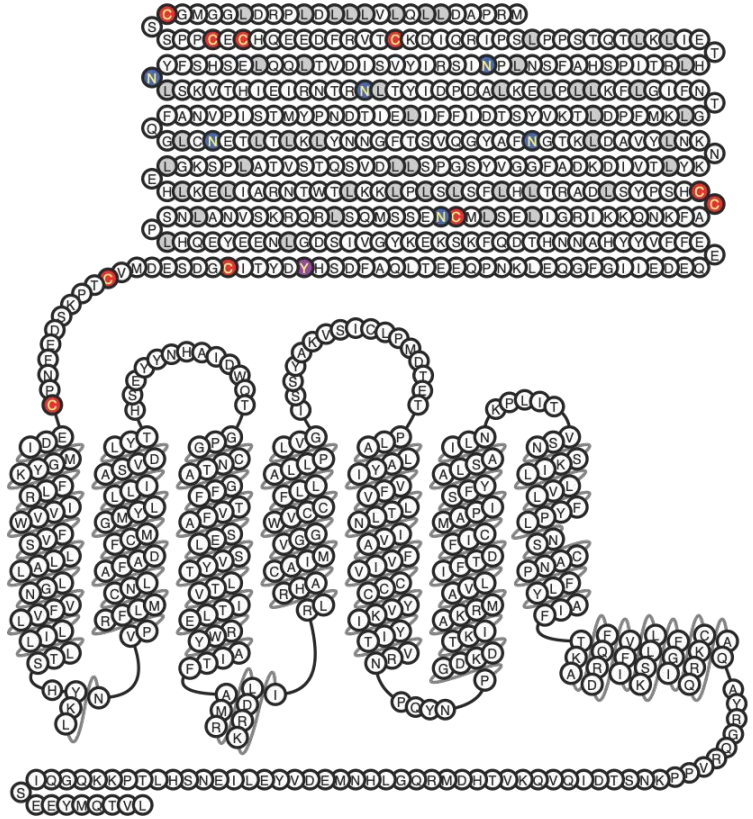
A particularly nasty beast: the glycoprotein hormone receptor TSHR





- 3 N-linked glycosylation sites
- 11 Disulfide bonds



- Extracellular leucine-rich repeat domain
- C-peptide sometimes present in hinge region connecting ECD and TMD
- ~Six N-linked glycosylation sites
- Five disulphide bonds

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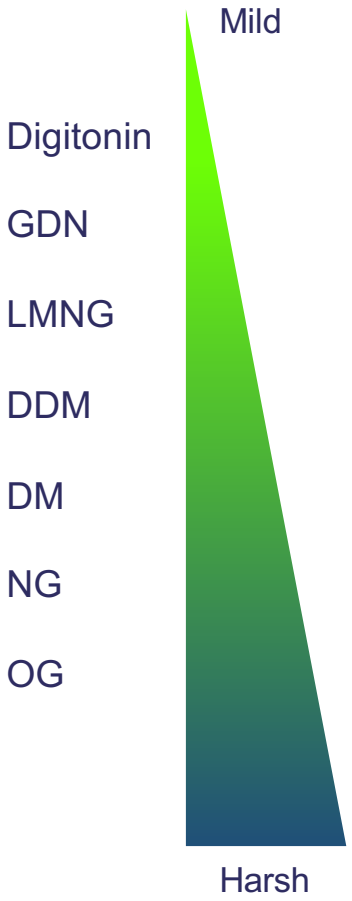
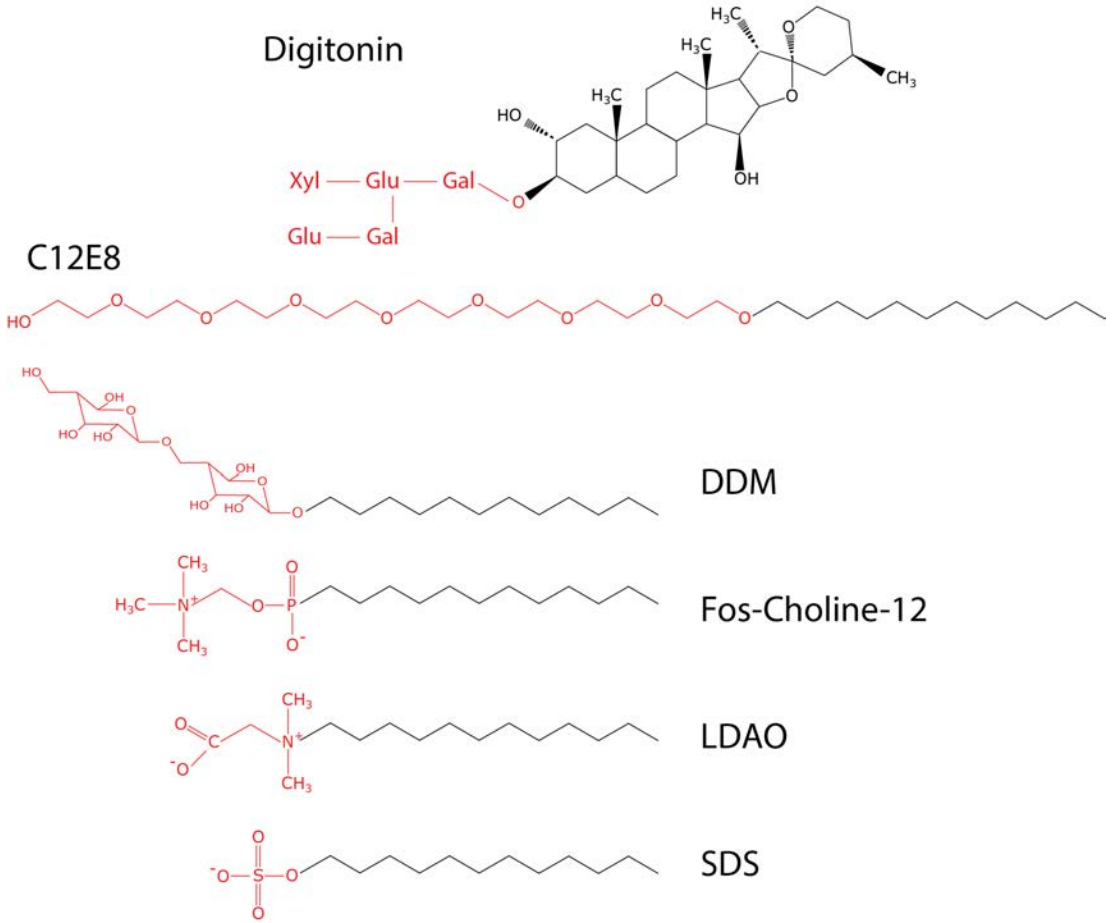
Cryo-EM allows the use of very mild detergents that tend to be large and are incompatible with crystallisation of GPCRs





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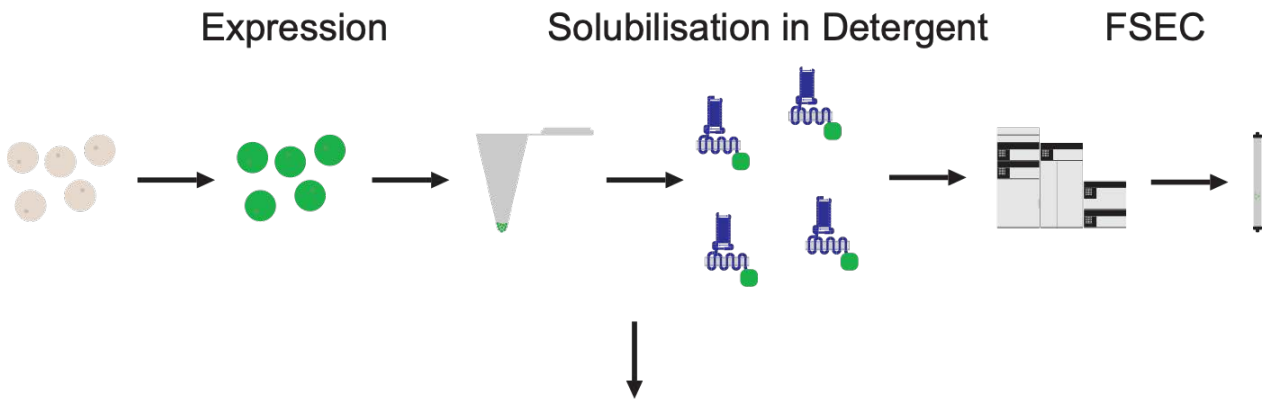
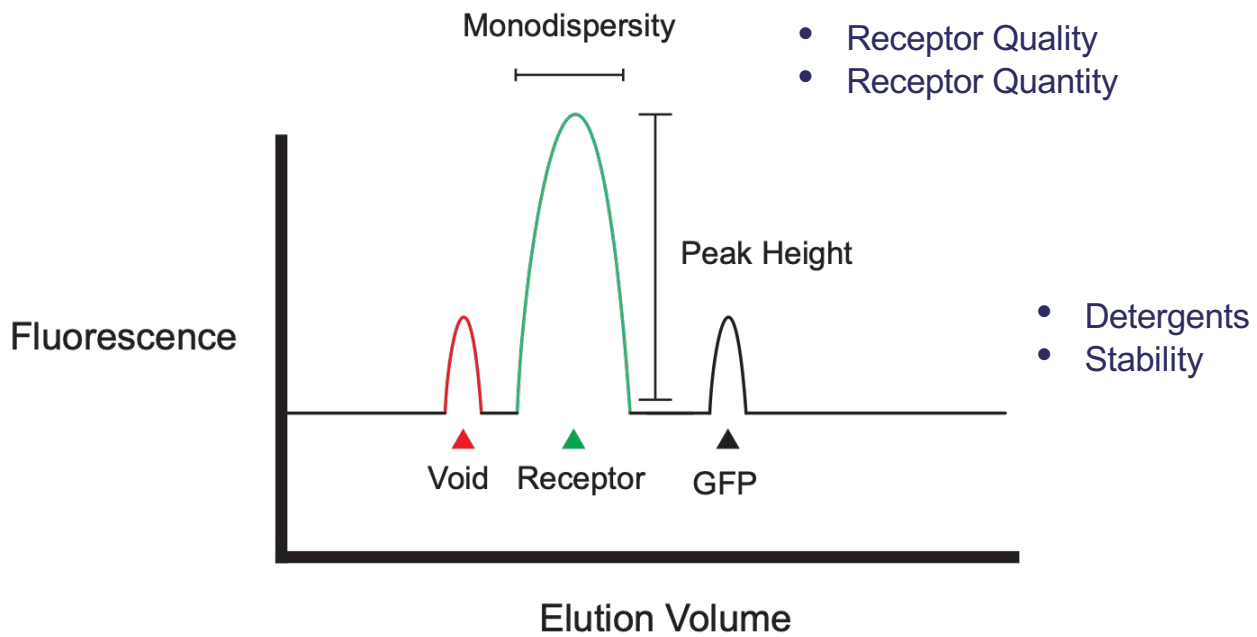
Tate (2010) Methods Mol. Biol. **601**, 187-203

Assessing receptor quality and quantity: Fluorescence size-exclusion chromatography





- Receptor Quality
- Receptor Quantity

- Detergents
- Stability

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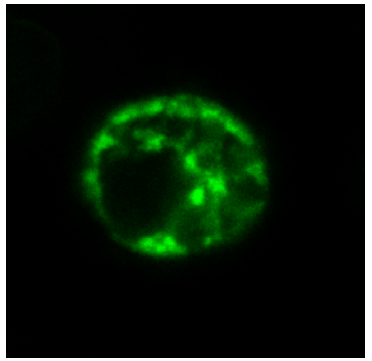
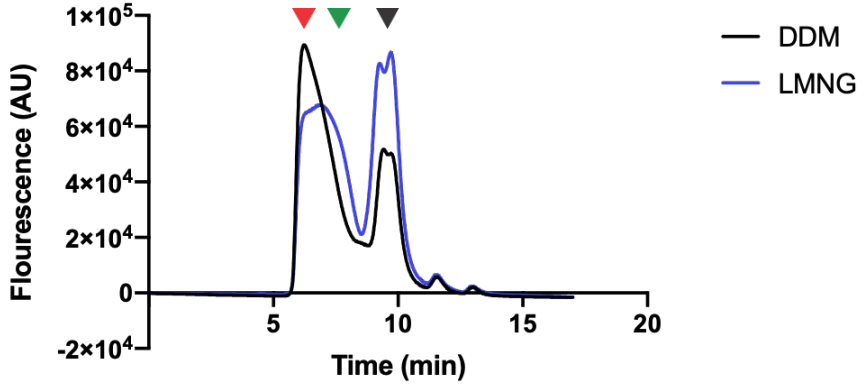
FSEC is used to determine the ability of detergents to solubilise the receptor and to maintain it in a monodisperse state

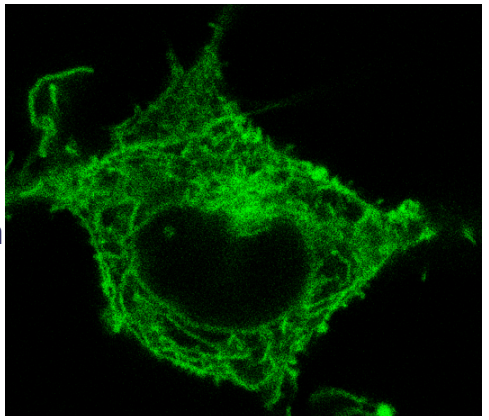
Transient transfection

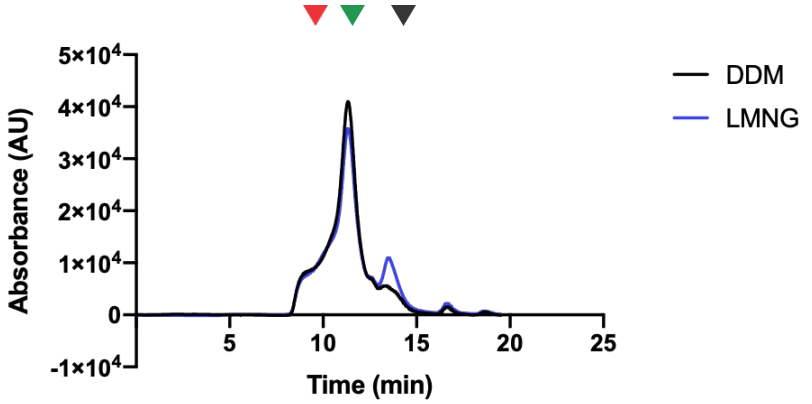




- Large amounts of intracellular aggregates
- No defined receptor peak in any detergent on FSEC
- Transfected cells are unhealthy

Lentiviral transduction





- Monodisperse peak
- Both DDM and LMNG are suitable
- Little intracellular aggregation
- Healthy cells on TSHR expression

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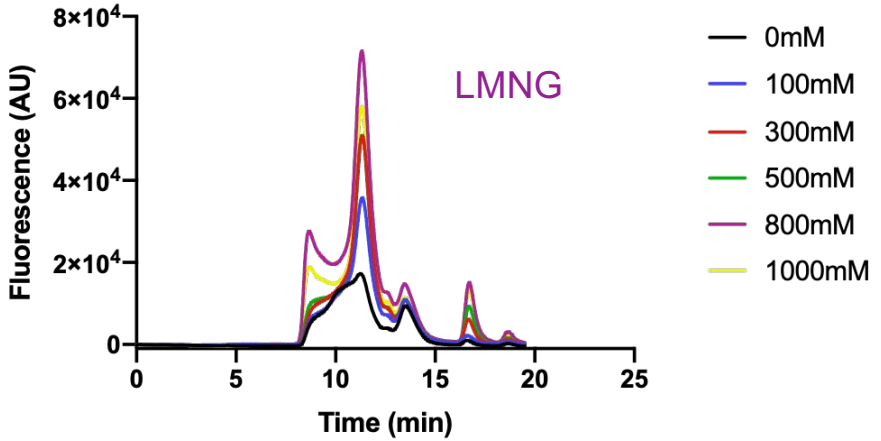
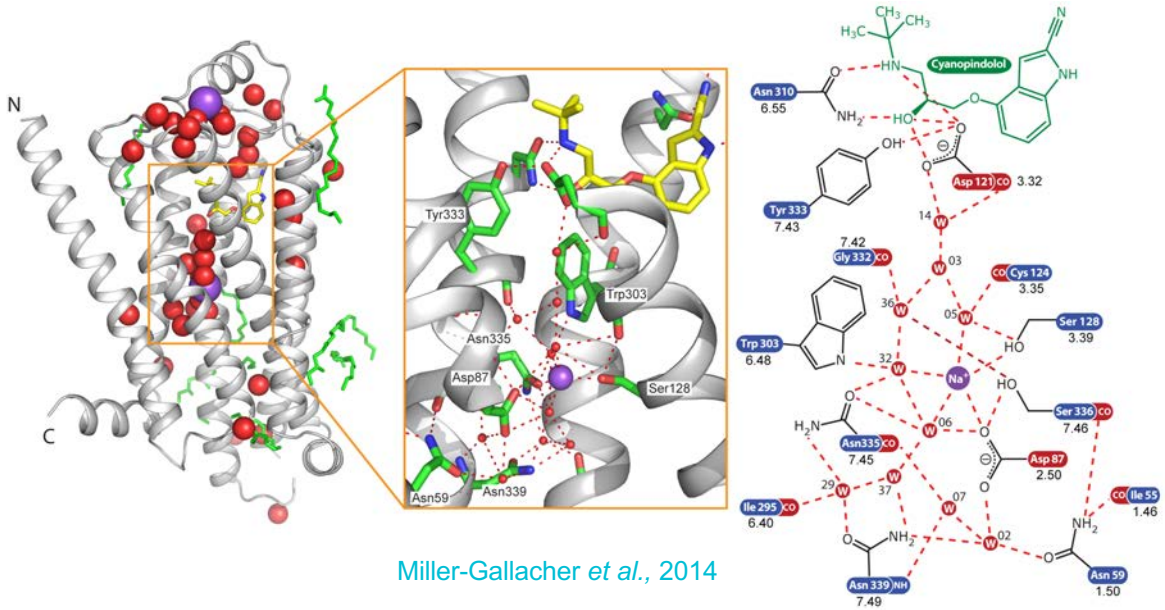
Courtesy of Lukas Helfinger

Improving solubilisation and stability: Increasing NaCl concentration





Miller-Gallacher *et al.*, 2014

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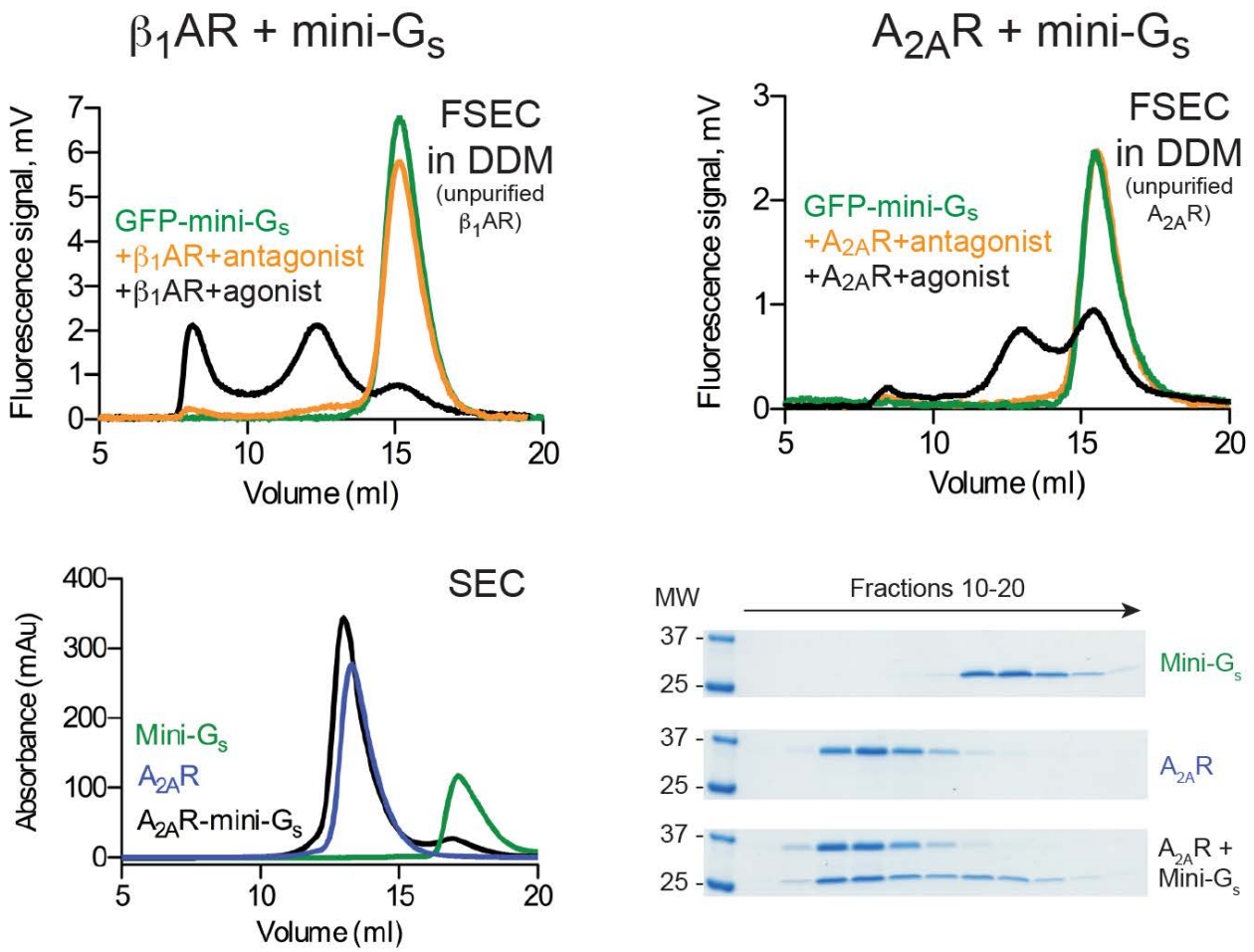
Courtesy of Lukas Helfinger

FSEC can also be used to detect the formation of G protein complexes



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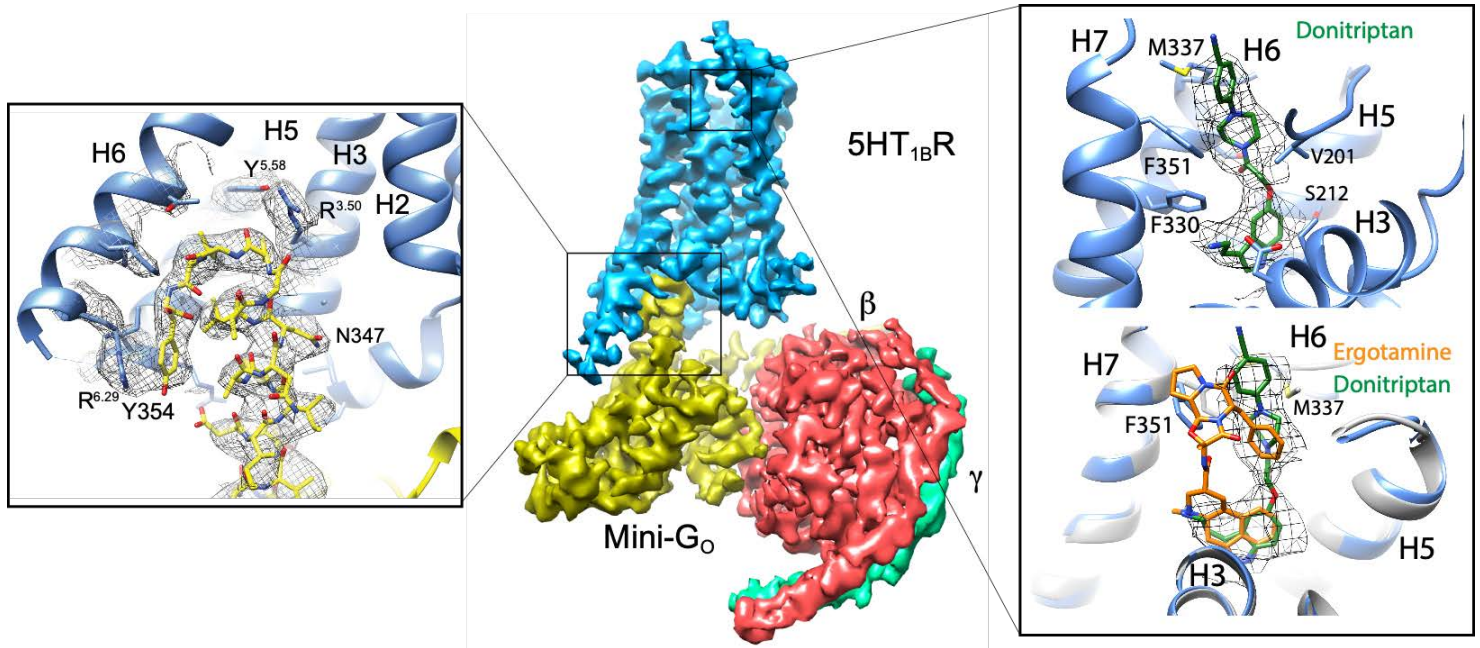
Nehmé *et al* (2017) PlosONE **12**, e0175642

Structure of $5HT_{1B}R$ coupled to heterotrimeric G_o



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García-Nafría et al (2018) Nature **558**, 62

Differences in engineering strategy between cryo-EM and X-ray approaches

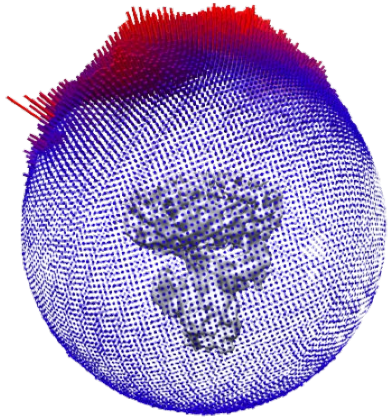
	X-ray	cryo-EM
Remove flexible regions?	Yes	No
Remove post-translational modification?	Yes	No
Add hydrophilic regions for crystal contacts?	Yes	No
Can thermostability of the receptor be a problem?	Yes	No
Can protein dynamics be a problem?	Yes	No/Yes
Can I use any detergent?	No	Yes
Do I need to worry about the size of the receptor?	No	Yes*
Is the shape of a complex problematic?	Yes	No
Do I have to worry about my complex dissociating?	Yes	Yes

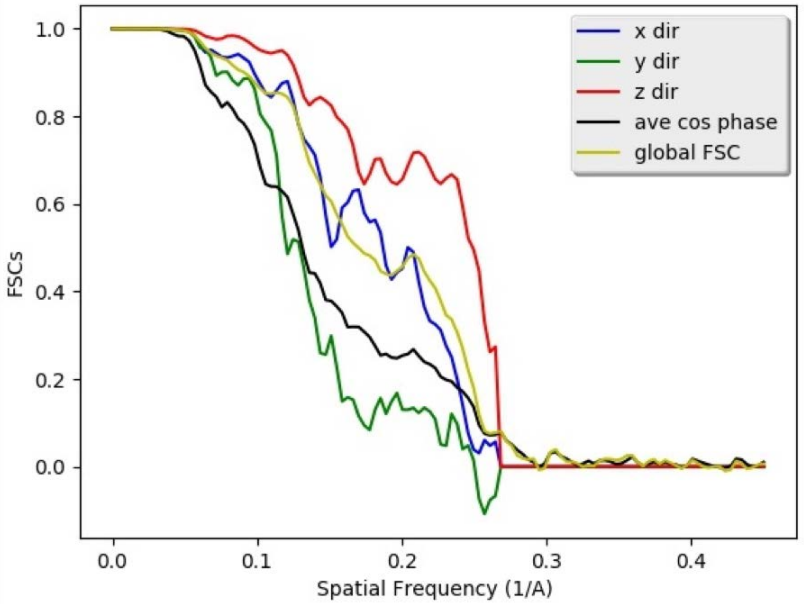
* ~ 80 kDa is the current minimum for a membrane protein

The last ‘wet’ hurdle: cryo-EM grid preparation (cf crystal screening)

- Dissociation of complexes or aggregate of protein at the air-water interface
- Preferred orientation on an EM grid prevents structure determination

Preferred orientation of the β_1AR-arrestin complex





- Add detergents before blotting to decrease surface tension
- Try different grids e.g. gold vs copper
- Try different supports e.g. unsupported, carbon, graphene
- Try different modifications to supports e.g. glow discharging in different solvents
- Last resort: collect tilted data sets

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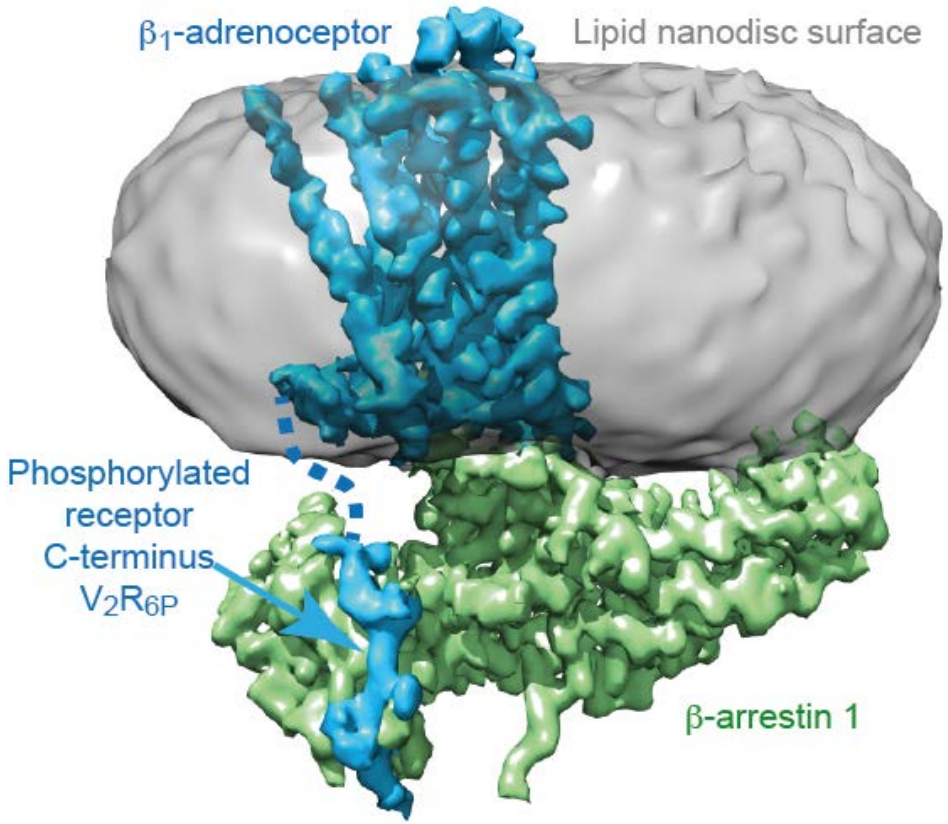
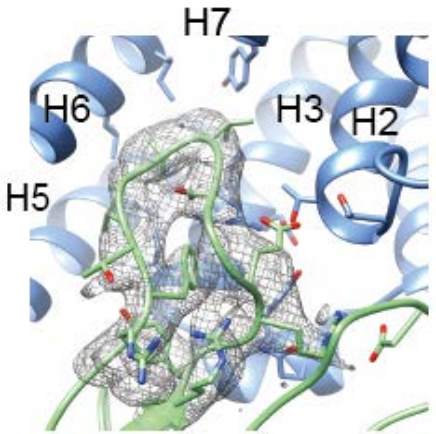
Courtesy of Yang Lee

Structure of the formoterol-β_1AR-βarrestin1-F_{ab}30 complex (Cryo-EM structure with an overall resolution of 3.3 Å)

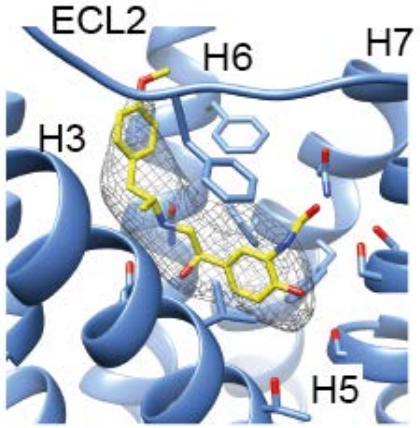


Key aspects to structure determination

- Thermostabilised receptor (5 mutations)
- Sortase-mediated ligation of phosphorylated C-terminus
- Use of conformation-specific F_{ab} bound to arrestin
- Reconstitution of β_1AR into a lipid nanodisc (POPC/POPG)
- Collection of data from 30˚ tilted samples



β–arrestin 1 finger loop



Formoterol

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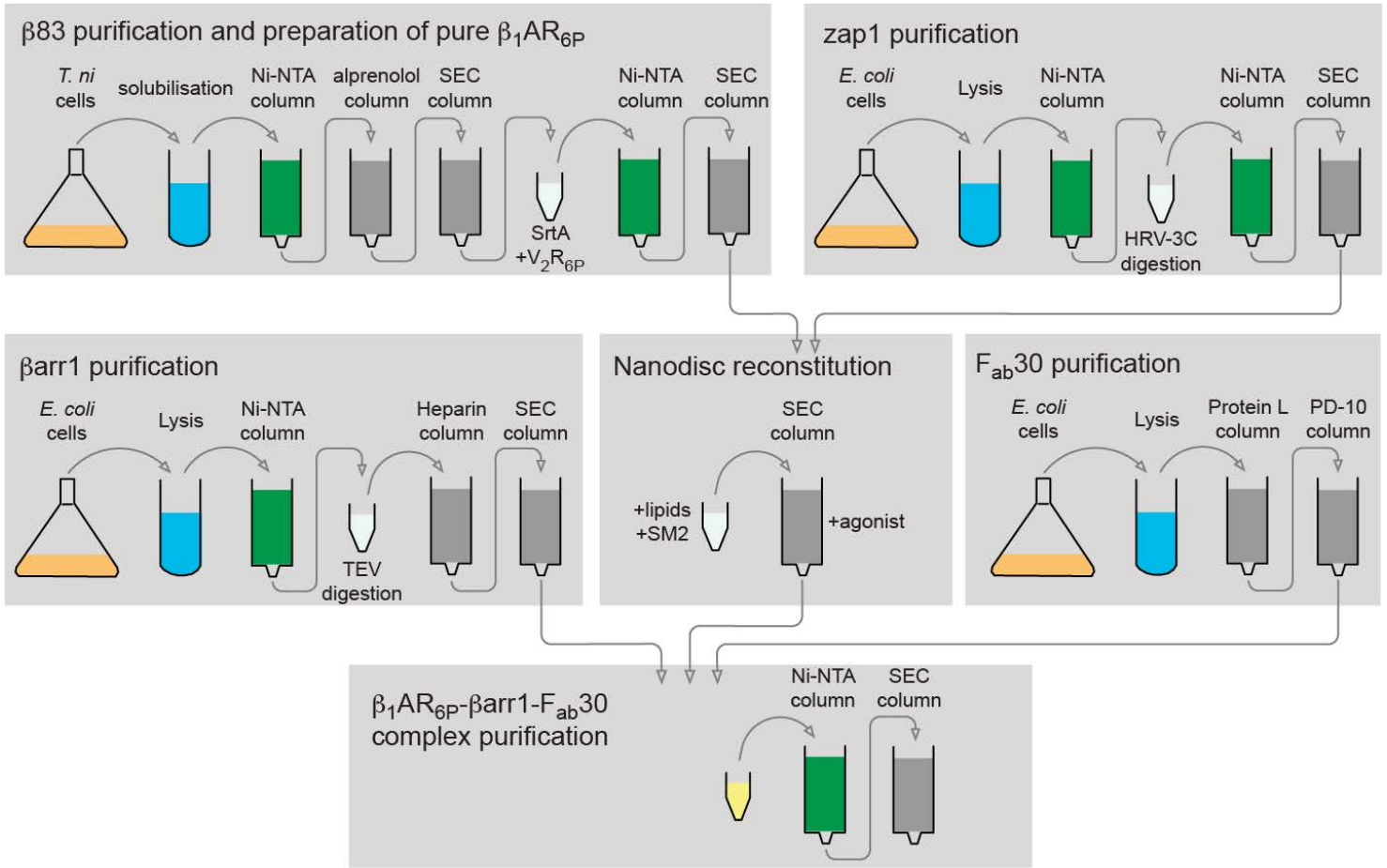
Lee *et al.* (2020) Nature **583**, 862-866

Preparation of β_1AR-arrestin complex in nanodiscs



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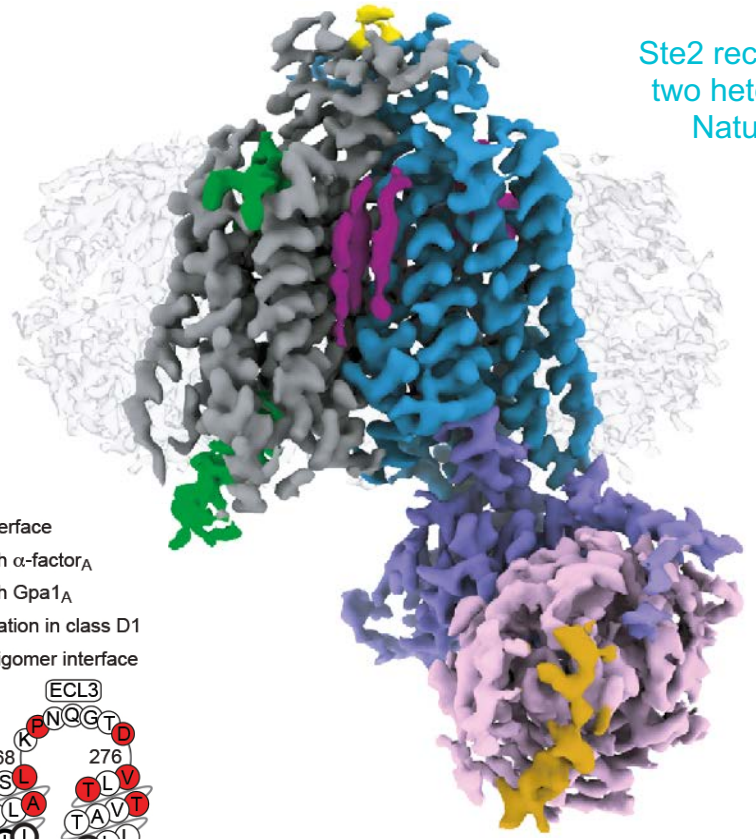
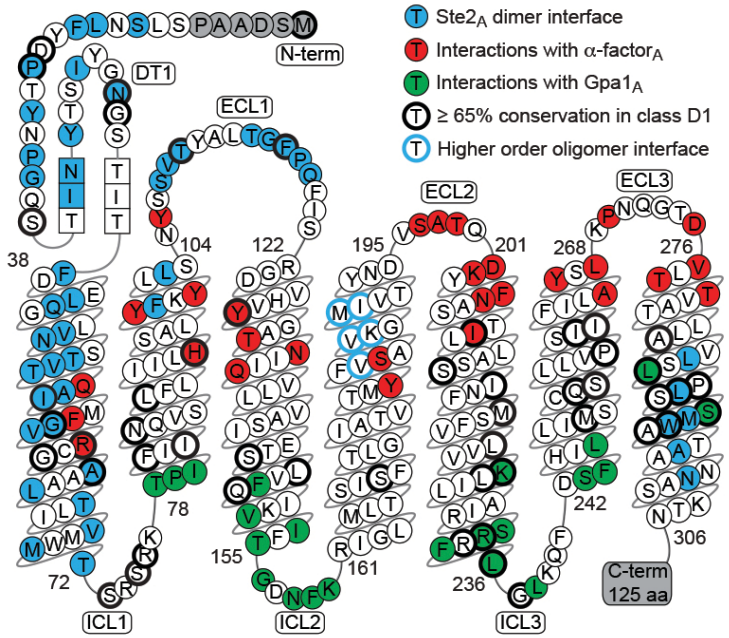
Conclusions

Cryo-EM is the method of choice for determining membrane protein structures

- Less biochemical intervention required
- Mild detergents and nanodiscs can be used
- Improvements in instrumentation & software ongoing
- Small membrane proteins can be stabilised by F_{ab}s or Nbs



Ste2 receptor dimer coupled to two heterotrimeric G proteins
Nature (2021) **589**, 148



Wild type receptor

- No deletions
- No mutations
- Post-translational modifications present

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Keys to success

- Optimisation of every single step from cDNA to structure
- Ask ‘Is ALL my protein functional?’ at every step
- Being absolutely meticulous…
- ….and never, ever give up!

GPCR Acknowledgements (2005-present)

Present

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Past

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