Nanobodies for the structural analysis of GPCR transmembrane signaling

Strasbourg

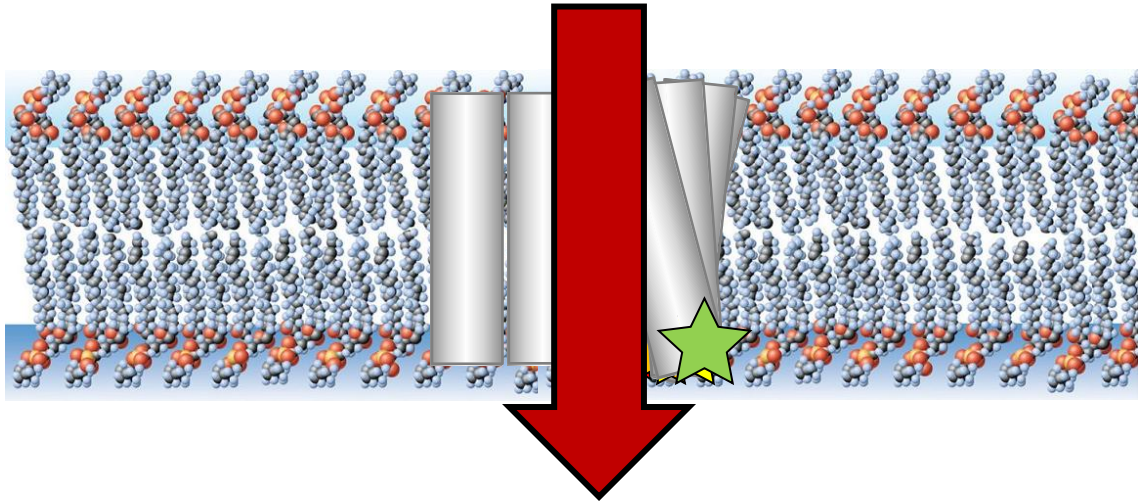
September 2011

Steyaert lab

Structural Biology Brussels

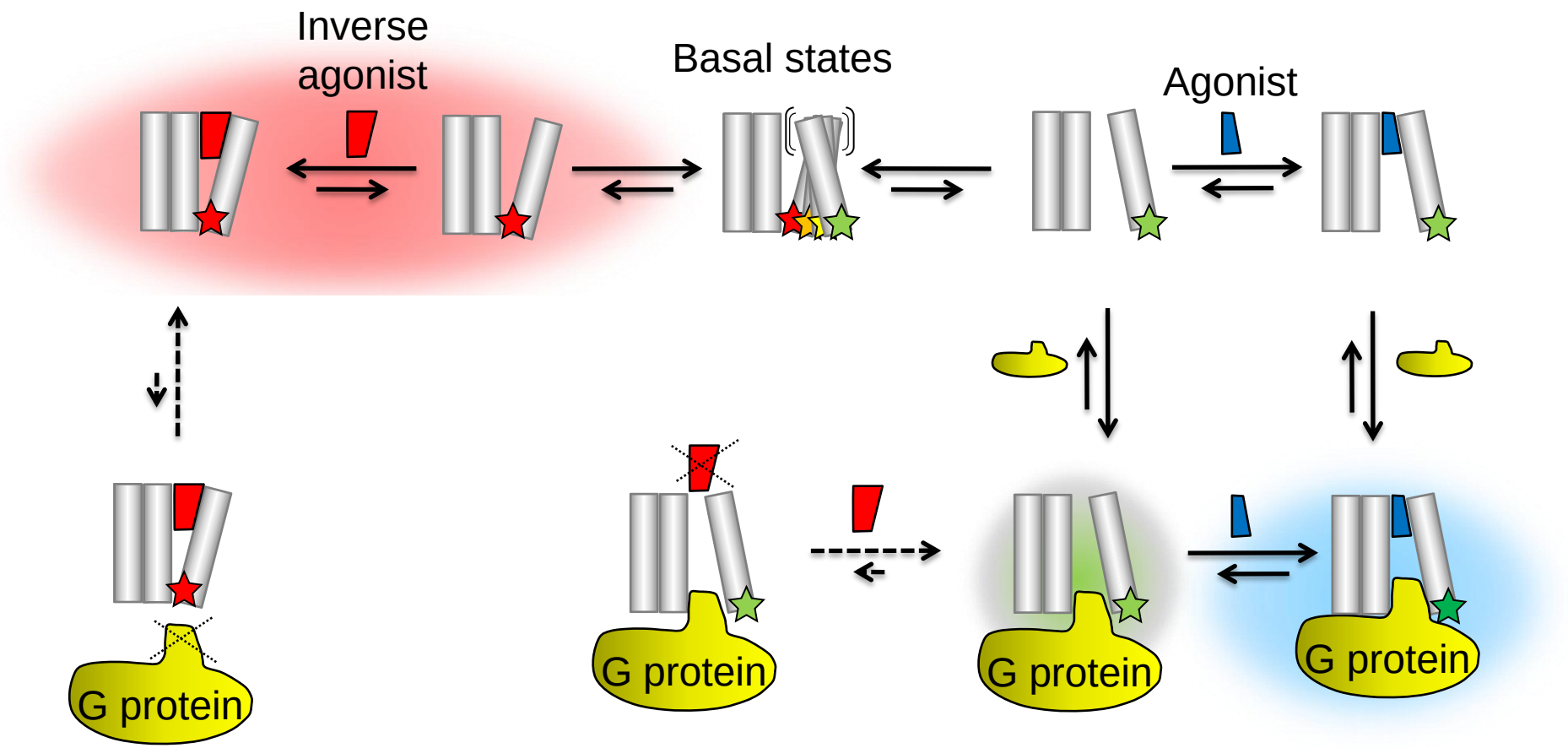
GPCRs are complex allosteric switches

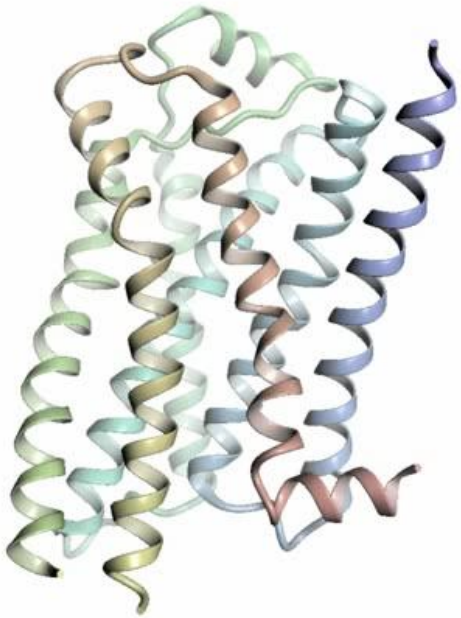
Ortosteric ligands
Allosteric ligands
Biased ligands



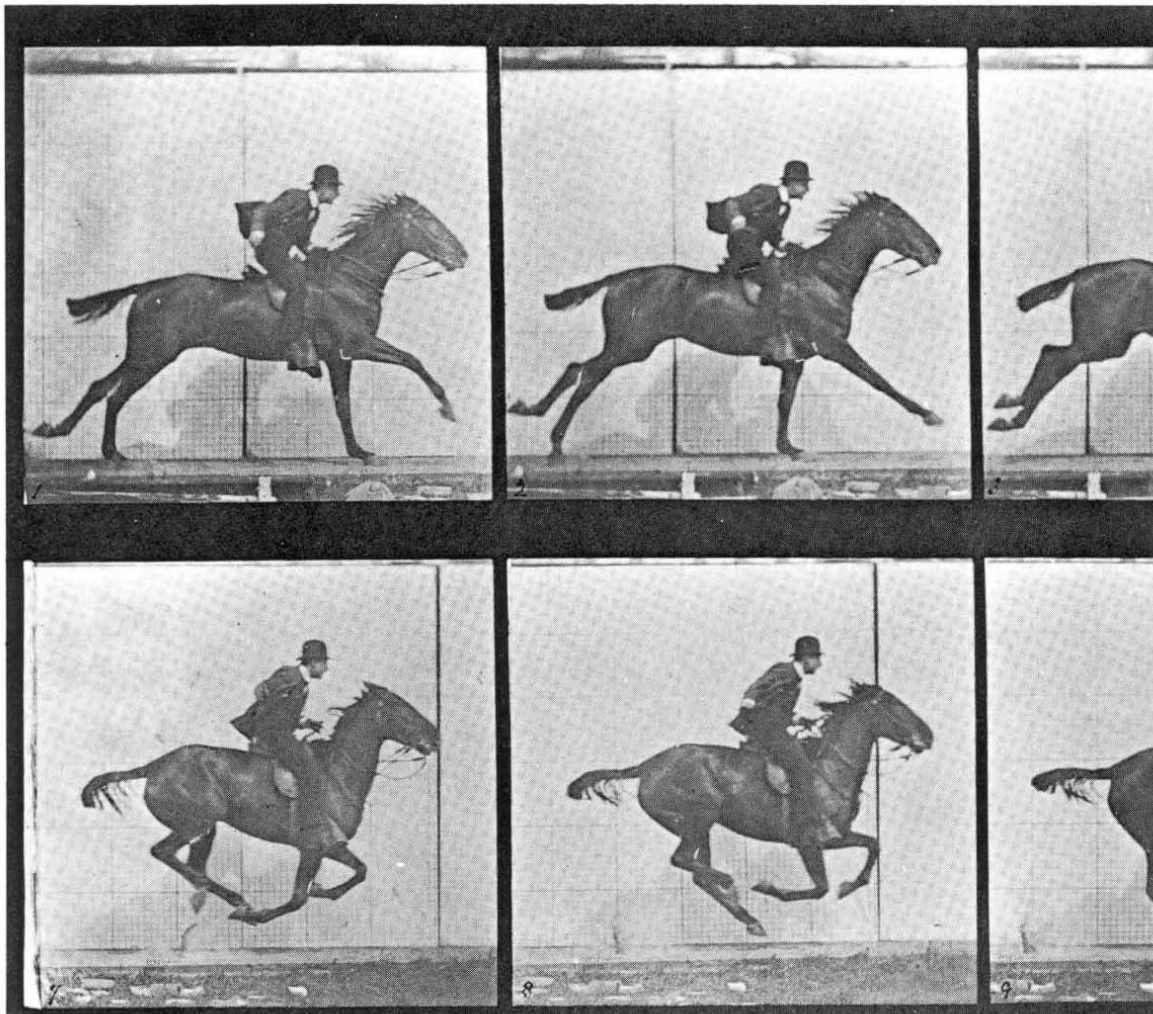
G proteins
β arrestins
GRKs

Conformational complexity of GPCR behaviour

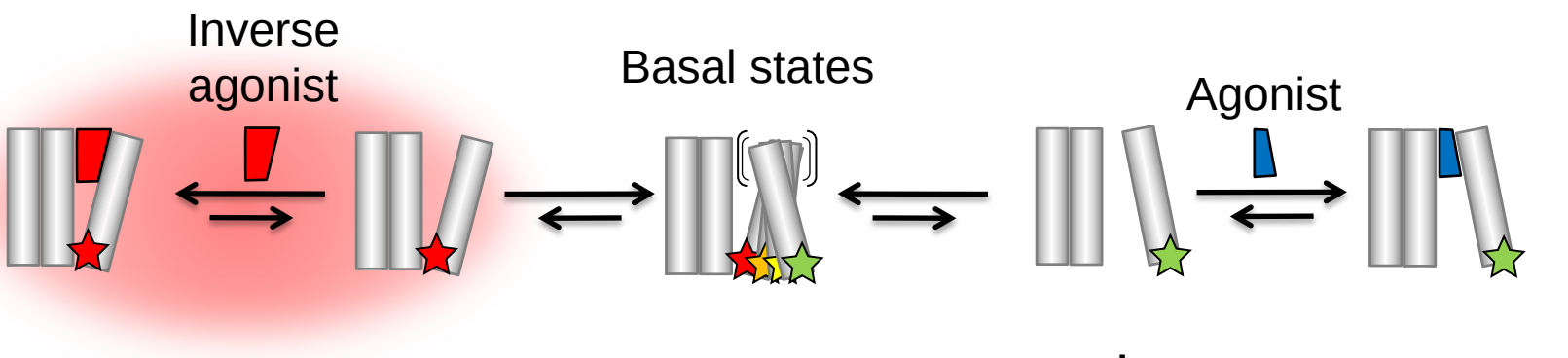


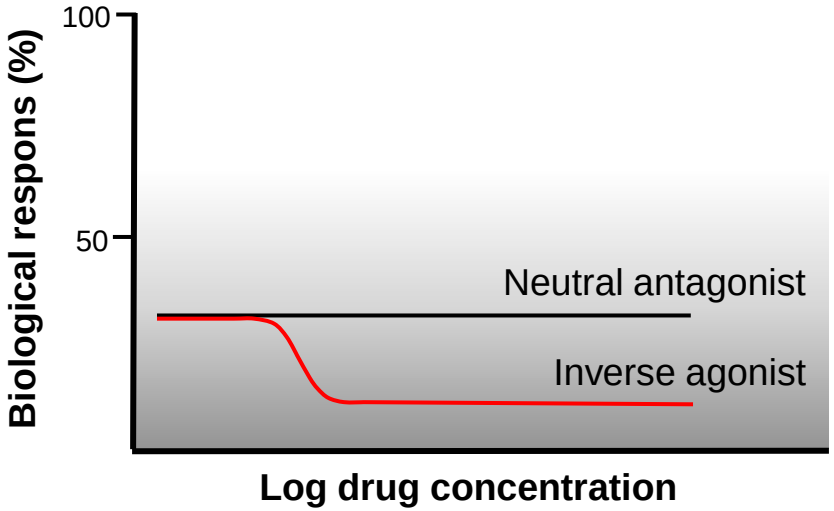


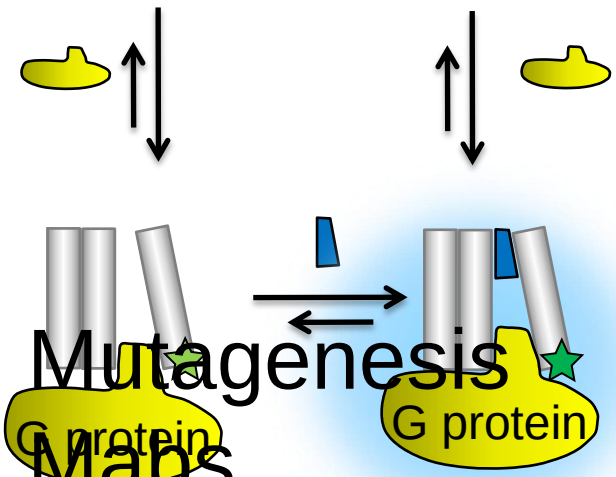
Capturing motions?



Ways to reduce the complexity in conformation rich proteins





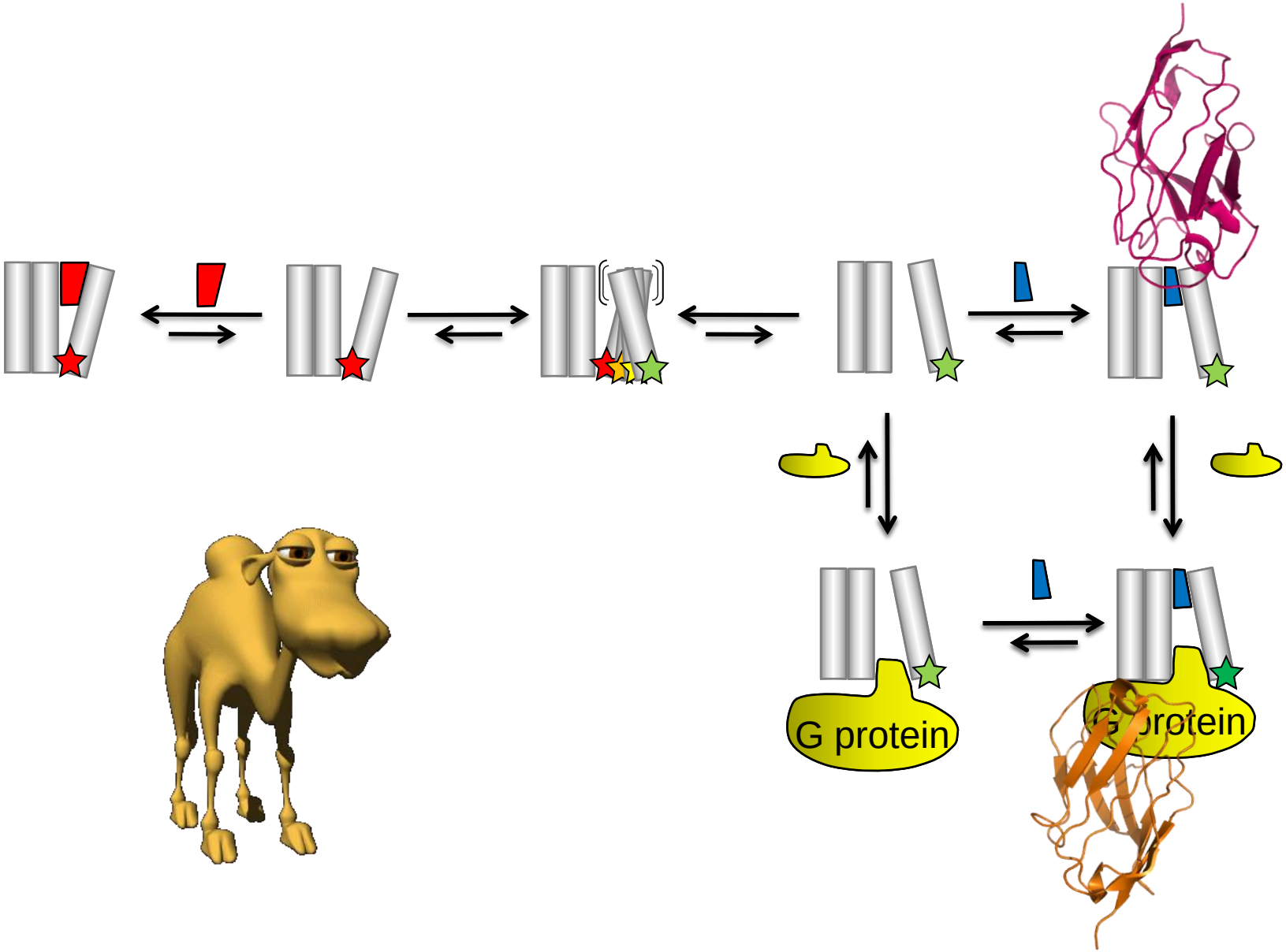


Mutagenesis

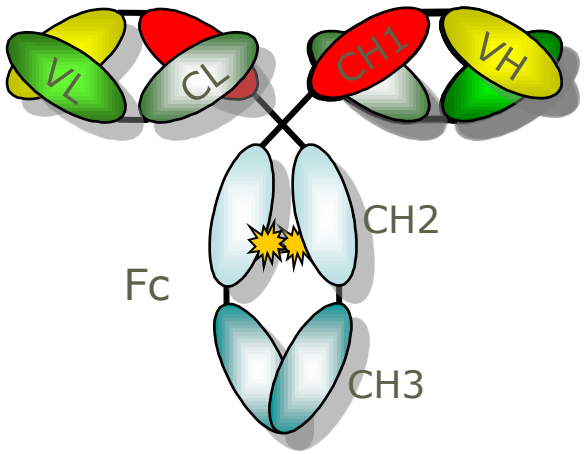
Mabs

T4L insertions

Conformation selective Nanobodies?



Conventional antibodies



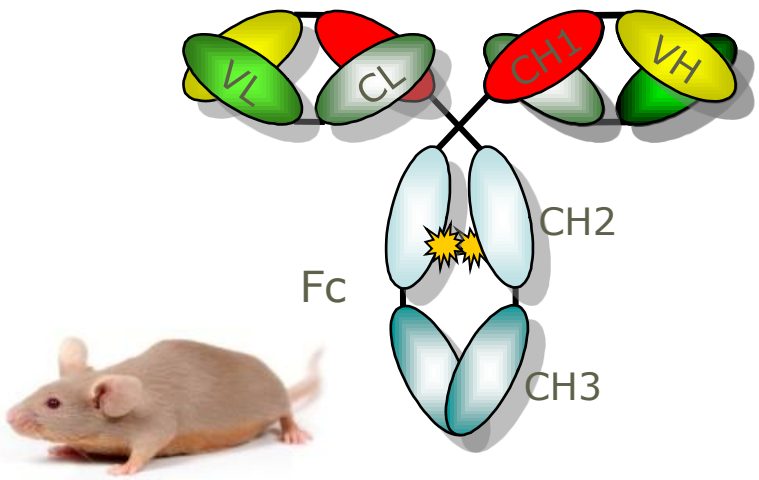
F_{ab}

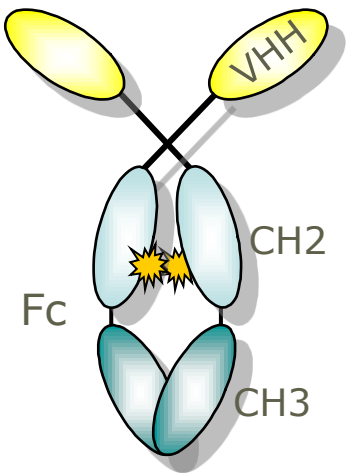
scF_v

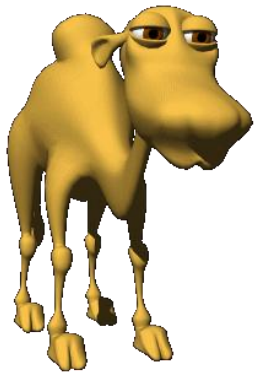
☹ Large
☹ Two chains
☹ Hybridoma technology

☹ Two cDNAs
☹ Unstable
☹ Difficult to express in *E.coli*

Conventional *versus* Heavy-chain antibodies



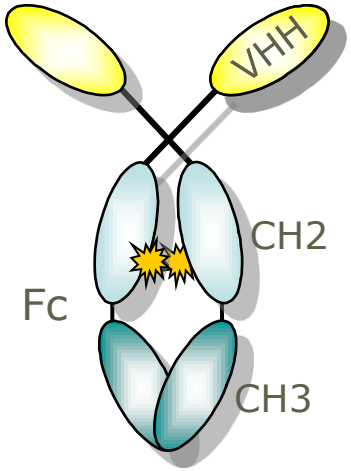


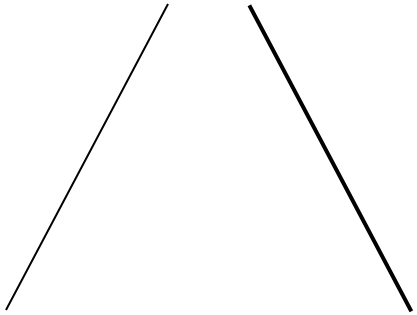


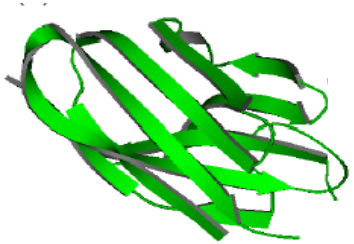
CH1 domain	Long hinge
hydrophobic VH-VL interface	hydrophilic surface
6 diversity regions	3 diversity regions

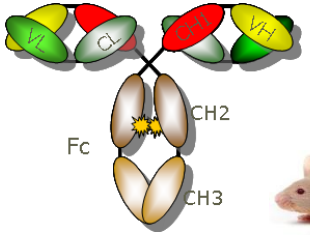
VHH or Nanobody

Camel Heavy-Chain antibody



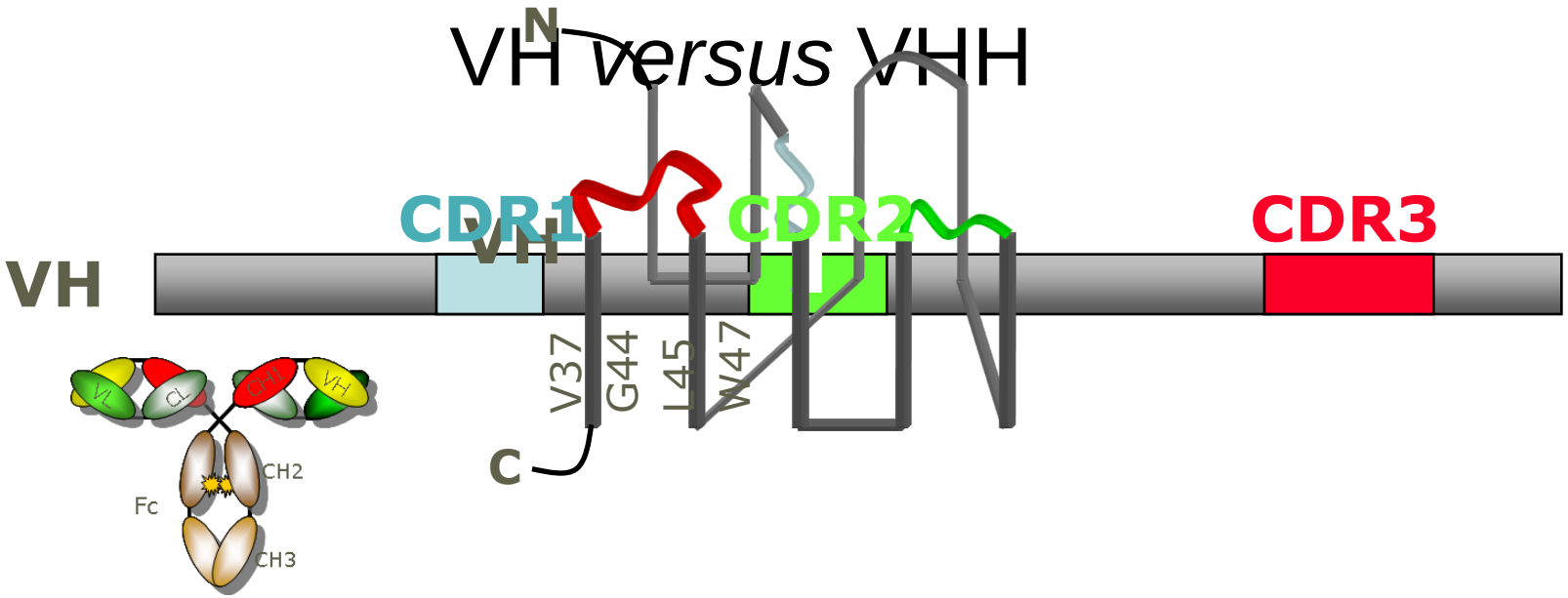


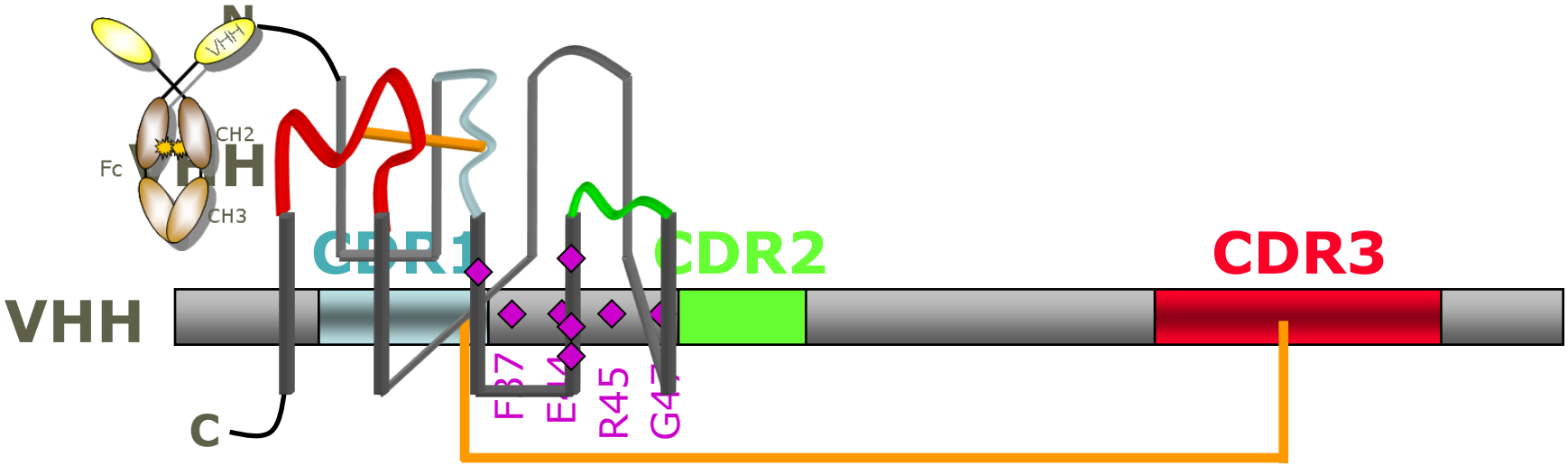


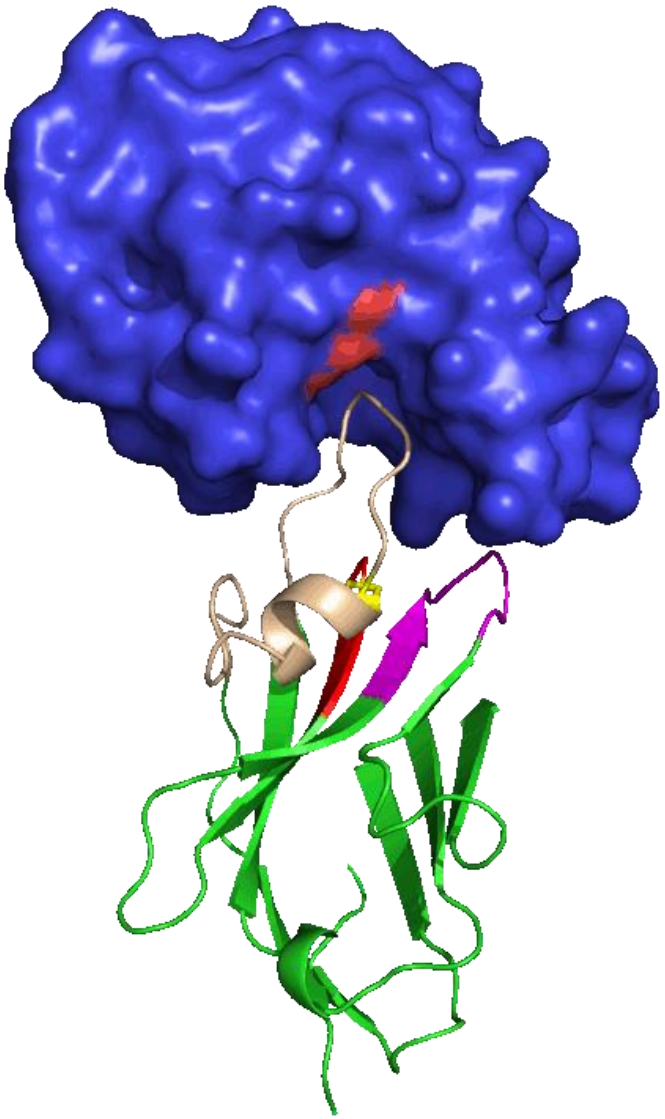


Immunoglobulin fold
12 Kd

VH *versus* VHH







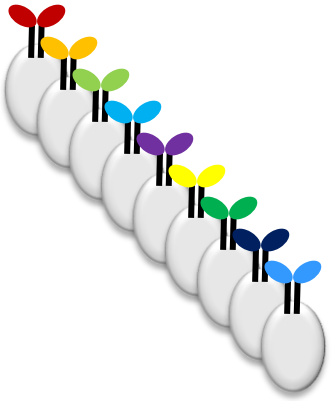
Key Nb features

- Immunoglobulin fold
- Long CDR1
- Longer CDR3, key player
- CDR1-S-S-CDR3
- Cavity binding
- Conformational epitope

Nanobody discovery platform

Immunize llama
with native antigen

Collect a blood sample
after 6 to 12 weeks



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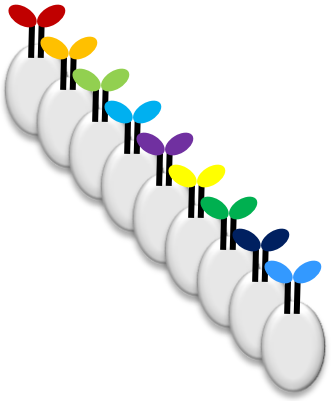
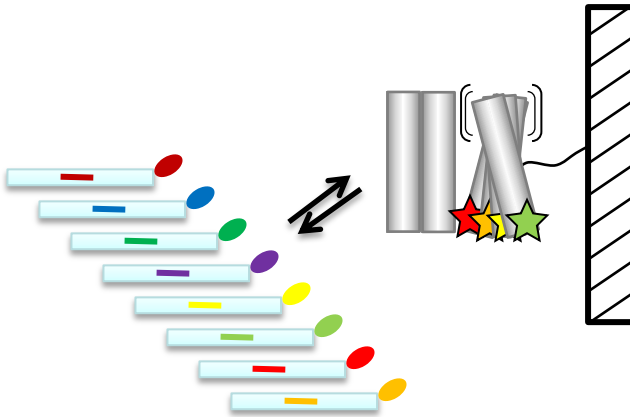
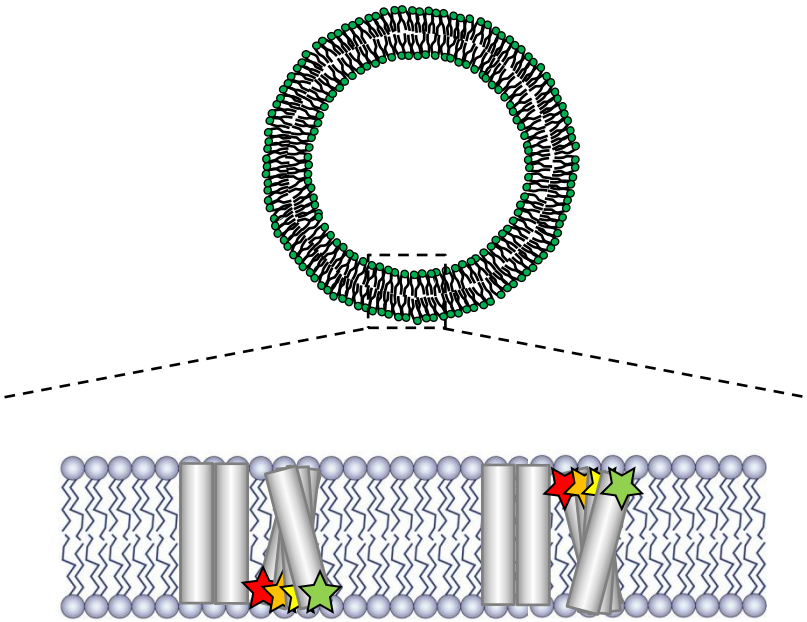




Select *in vivo*
matured nanobodies
by phage display

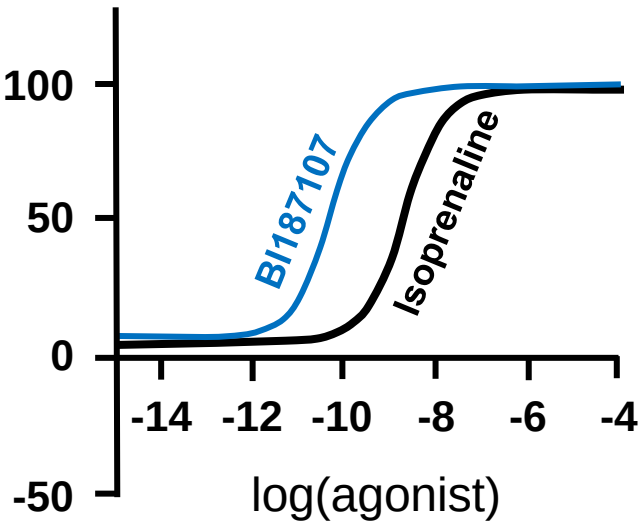
Generation of β2-AR binders

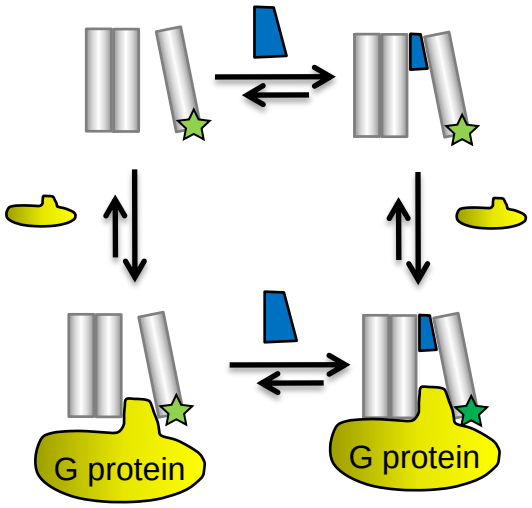


Recombinant receptor reconstituted in lipid vesicles

DOPC:Lipid A (10:1)

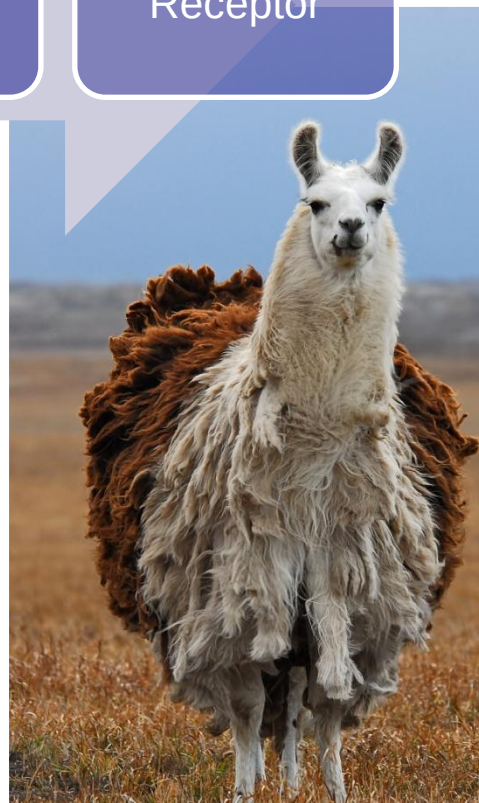
+

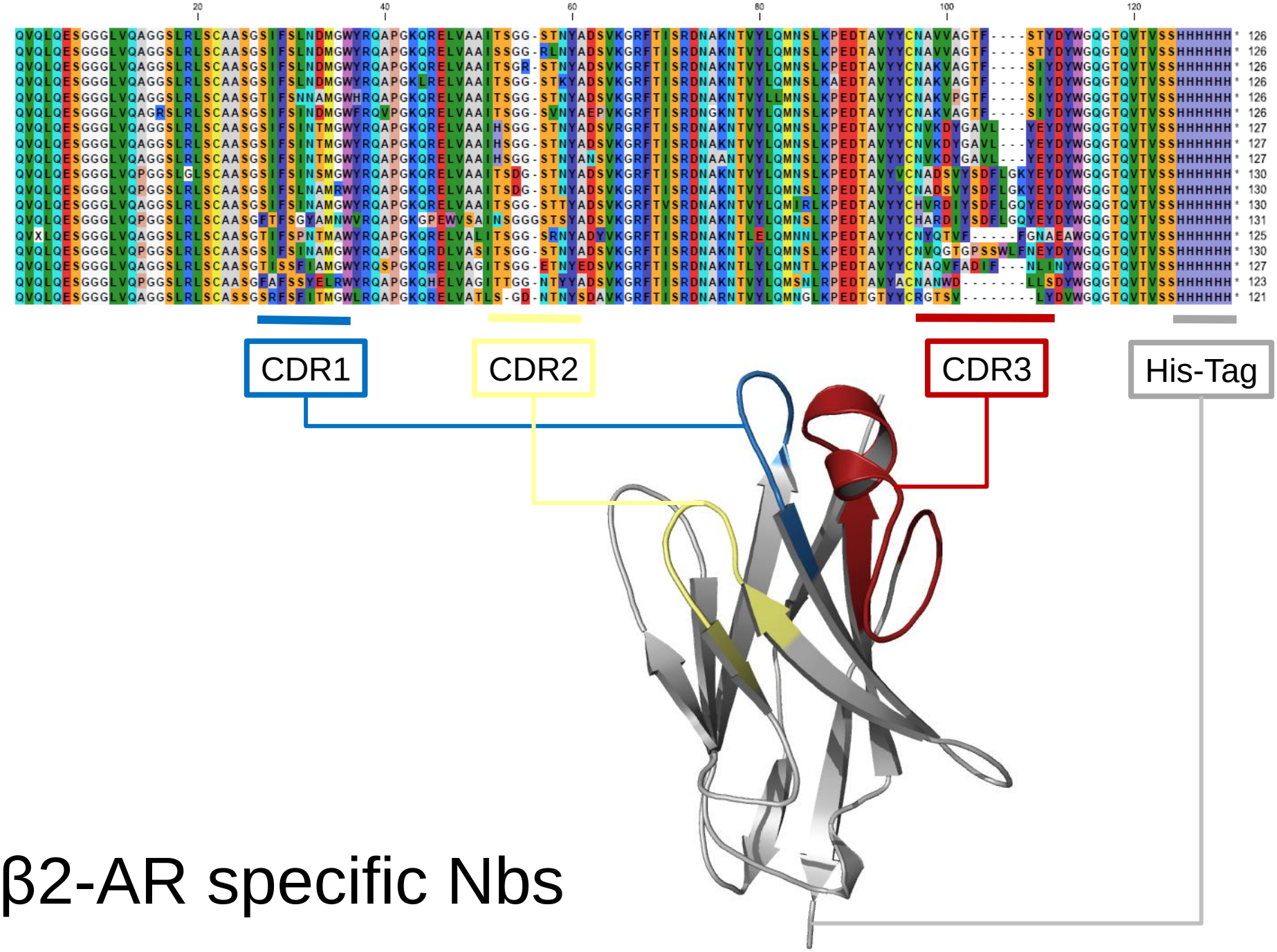




Generation of β2-AR binders

Day 1	Day 14	Day 28	Day 42	Day 56
Receptor + GERBU	Receptor + GERBU	Receptor + GERBU	Receptor	Receptor

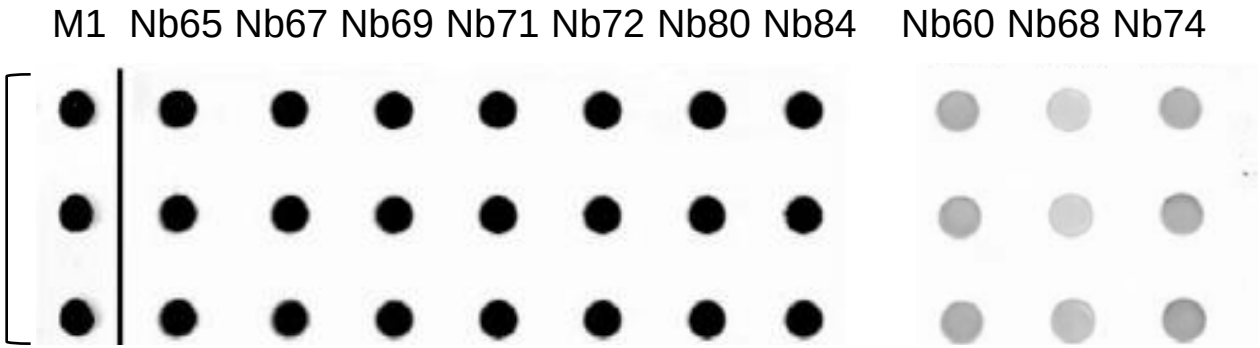




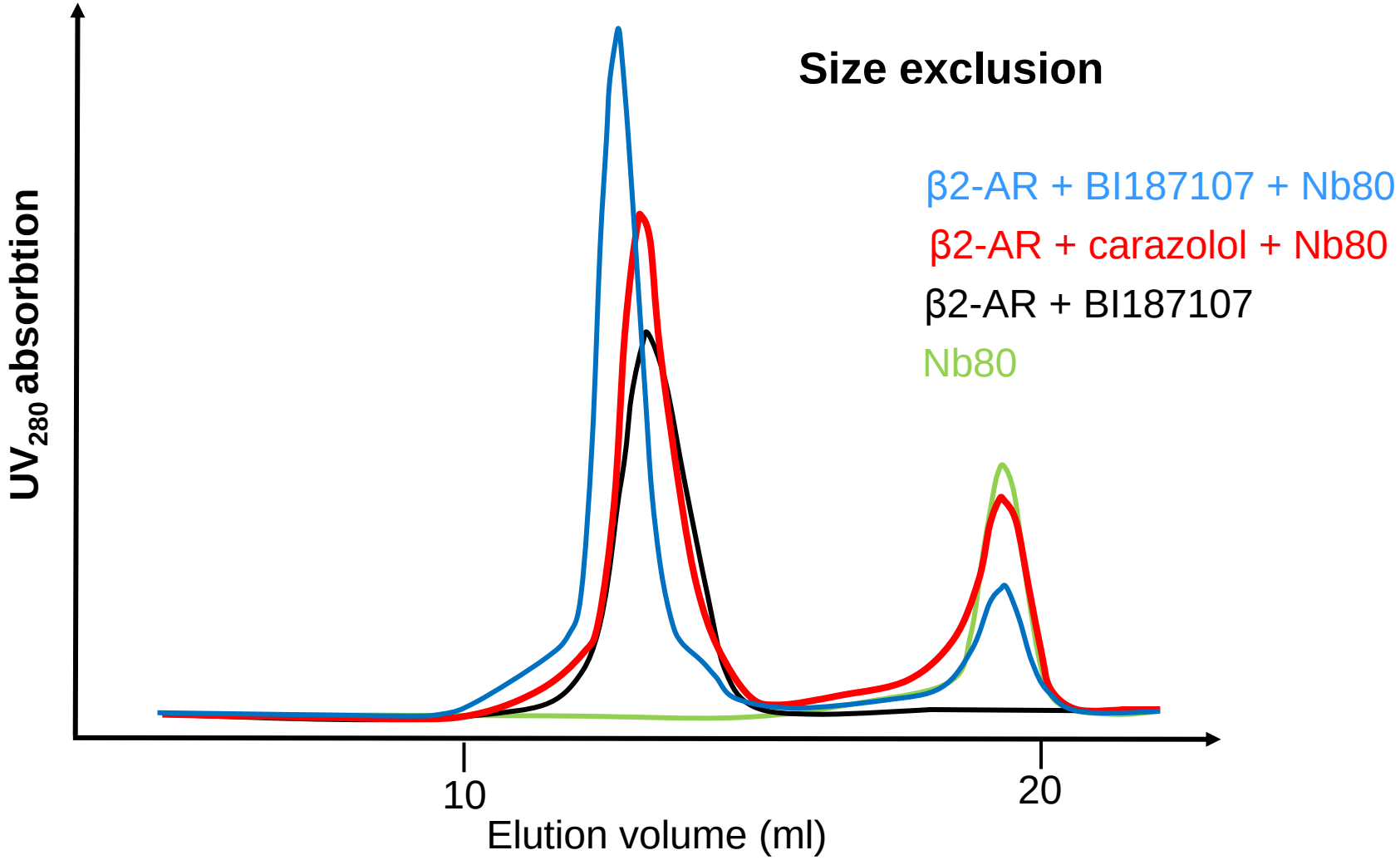
β2-AR specific Nbs

Selection of conformational binders

Folded β2-AR
+ BI187107

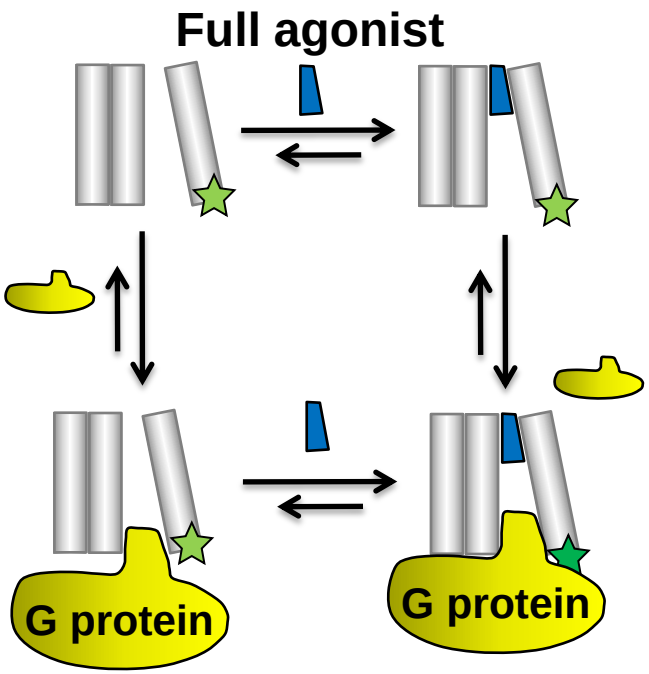


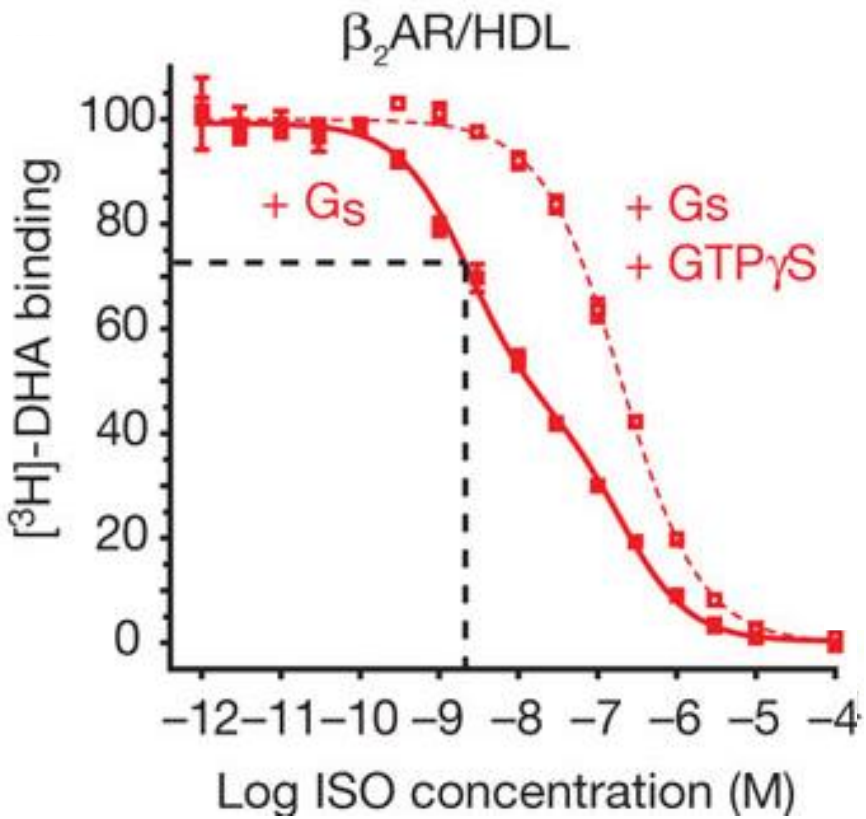
Nbs bind exclusively to the agonist bound receptor



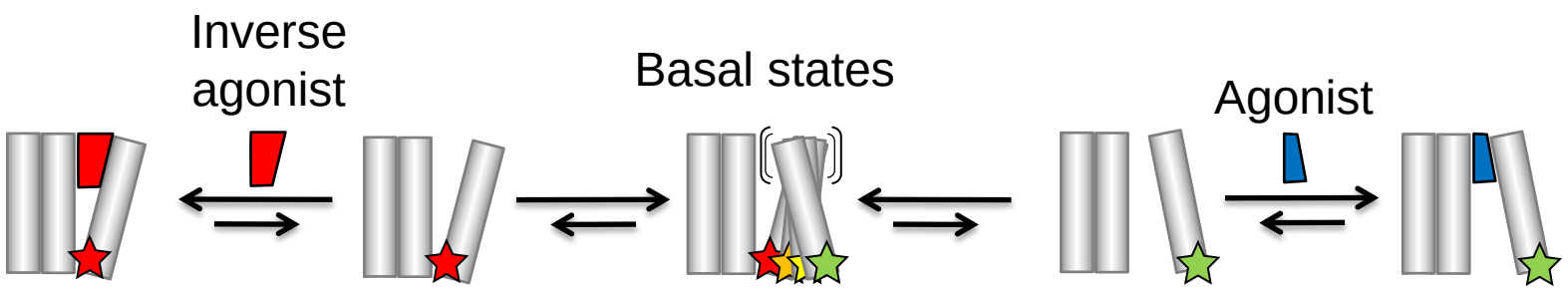
Nbs with G protein-like behaviour?

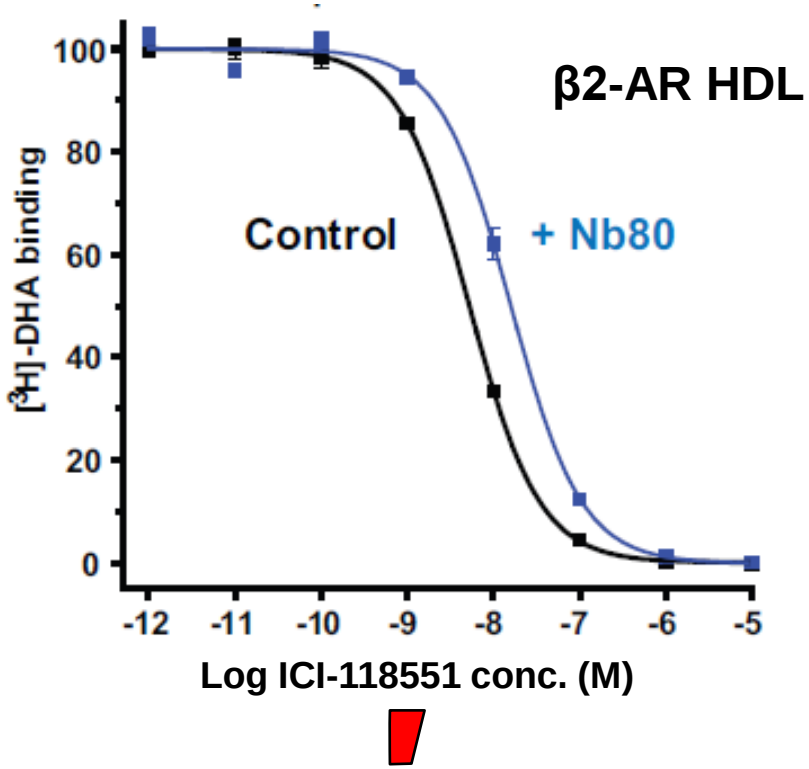
Full agonist: isoproterenol

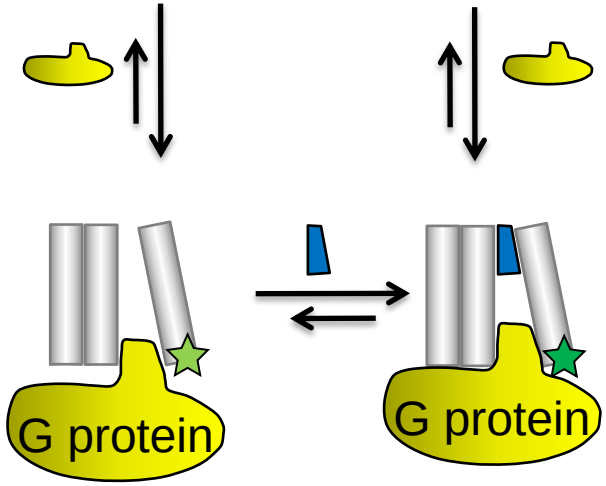




Nbs with G protein-like behaviour?

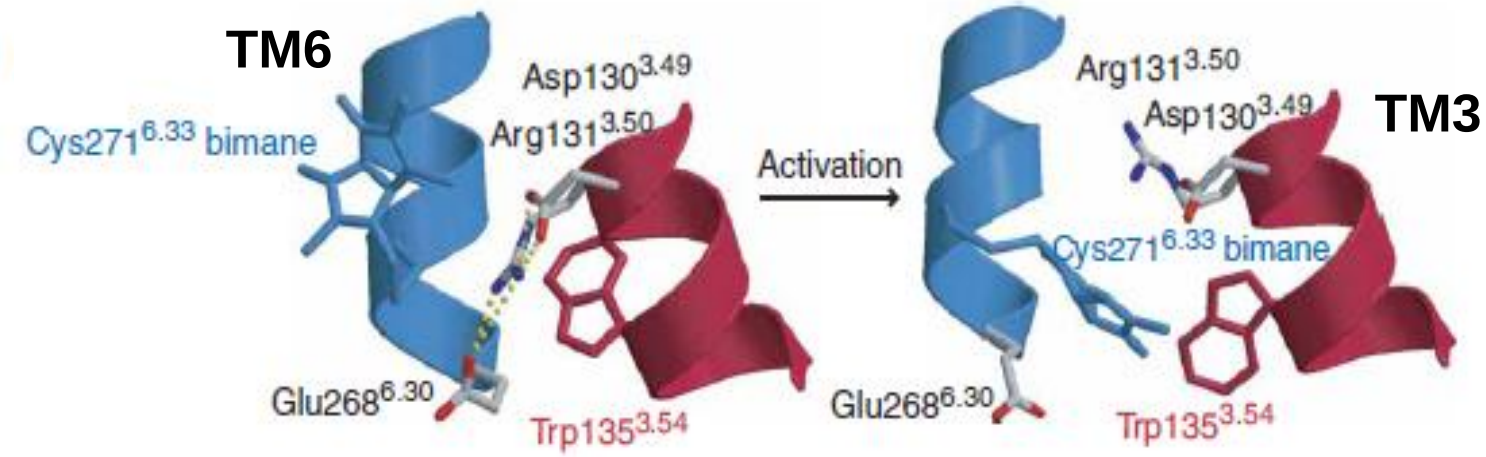


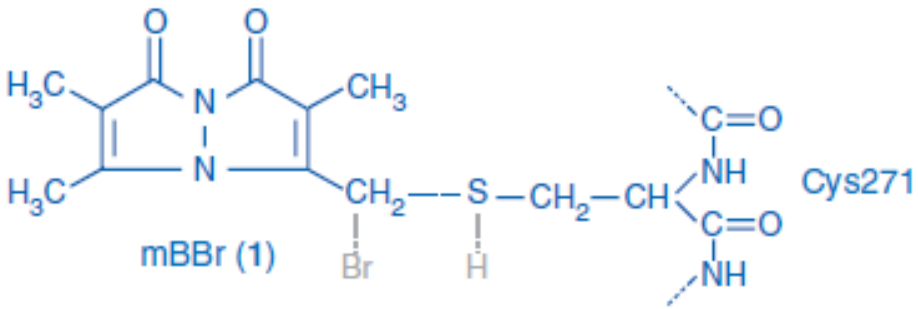


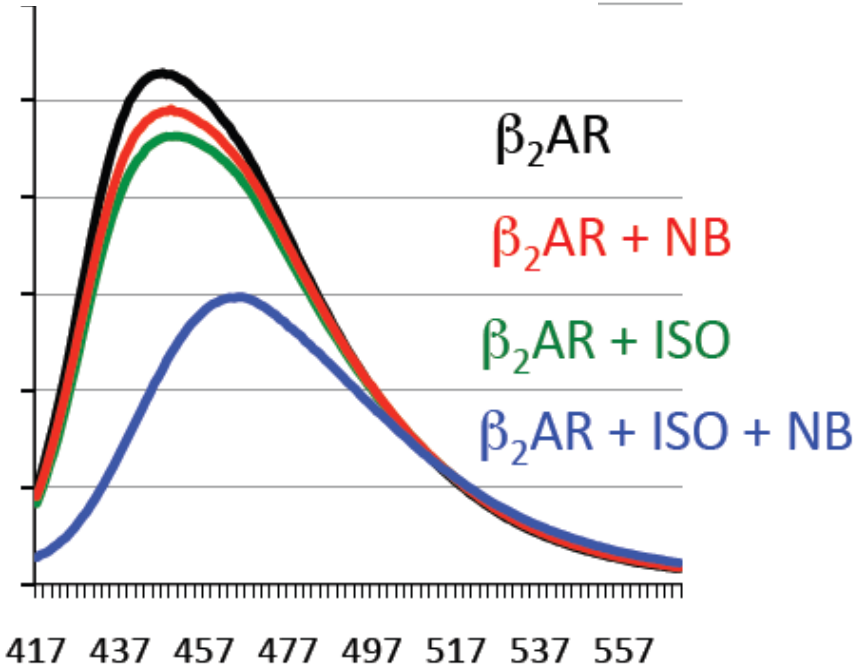


Inverse agonist: ICI-118551

Nbs with G protein-like behaviour

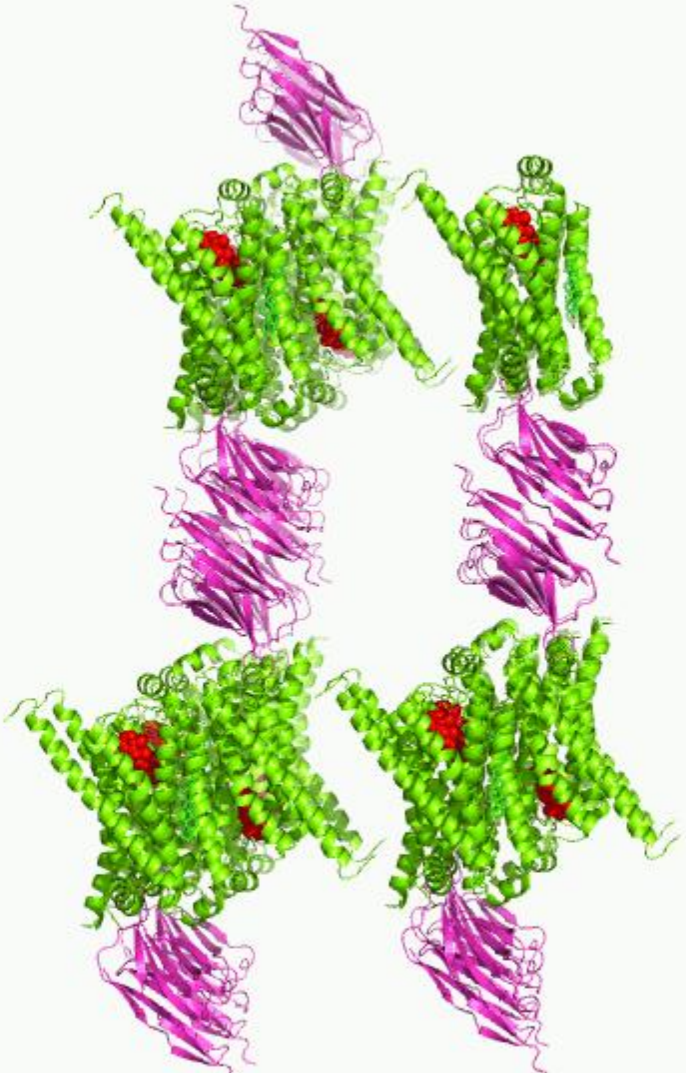


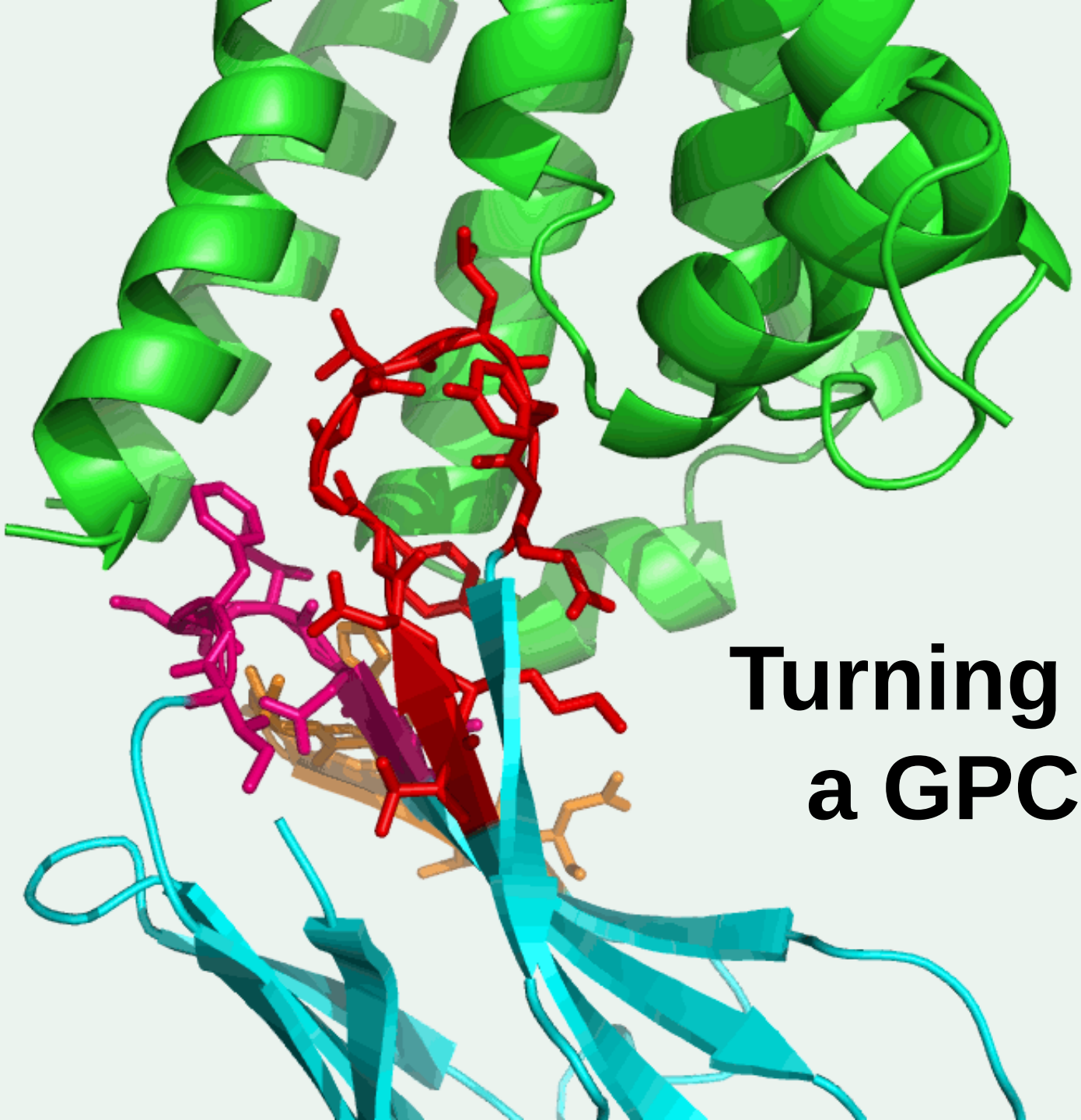




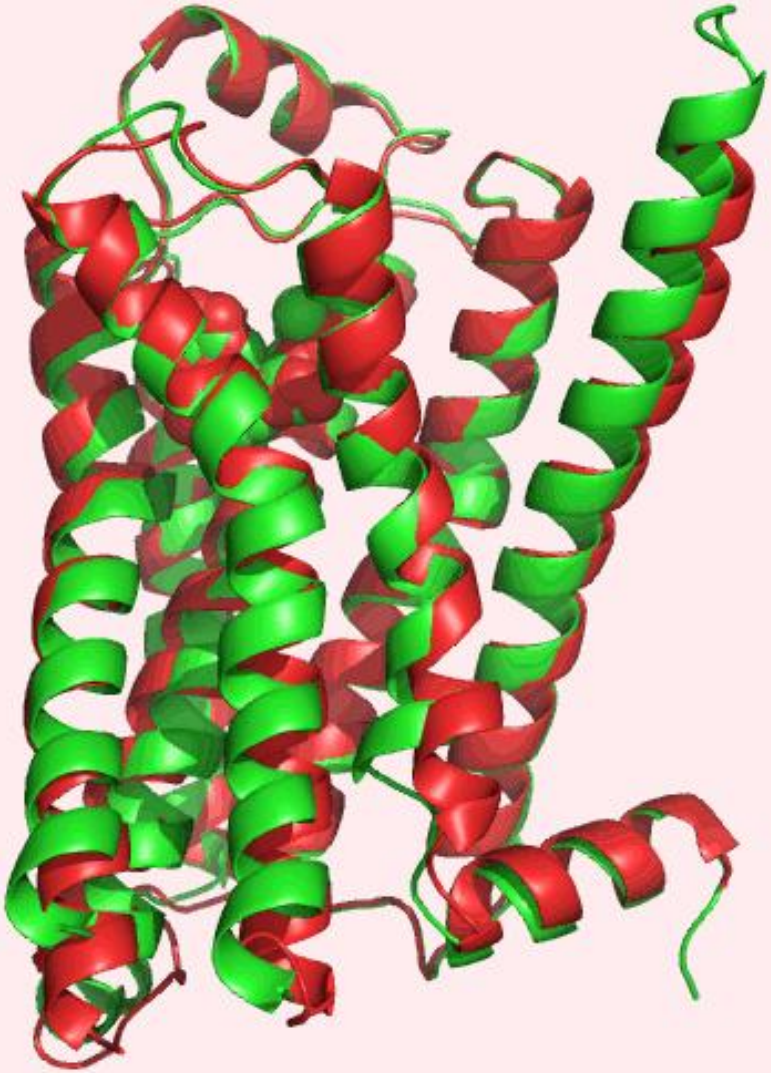
Zie Yao, X. *et al*. (2006) Nat. Chem. Biol. 2, 417-422

Nbs as crystallization chaperones for β2-AR





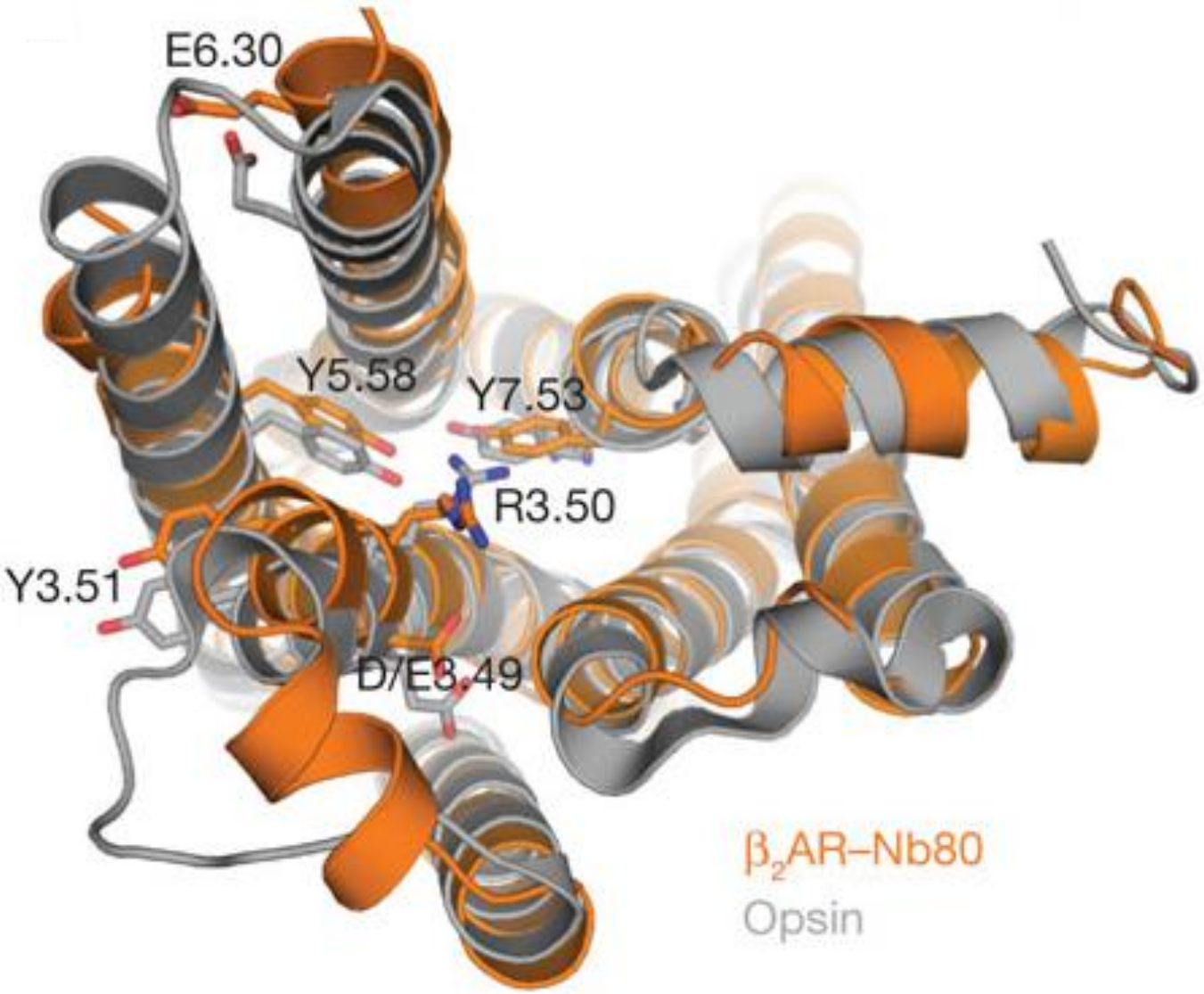
Turning on a GPCR



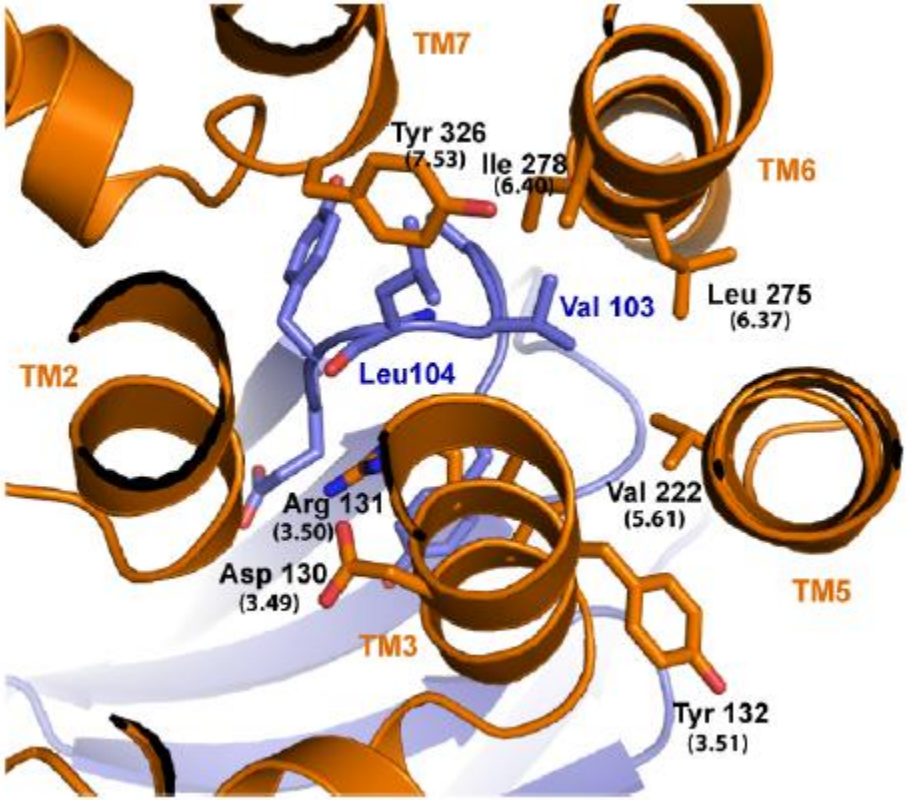
Carazolol structure

BI187107-Nb80 structure

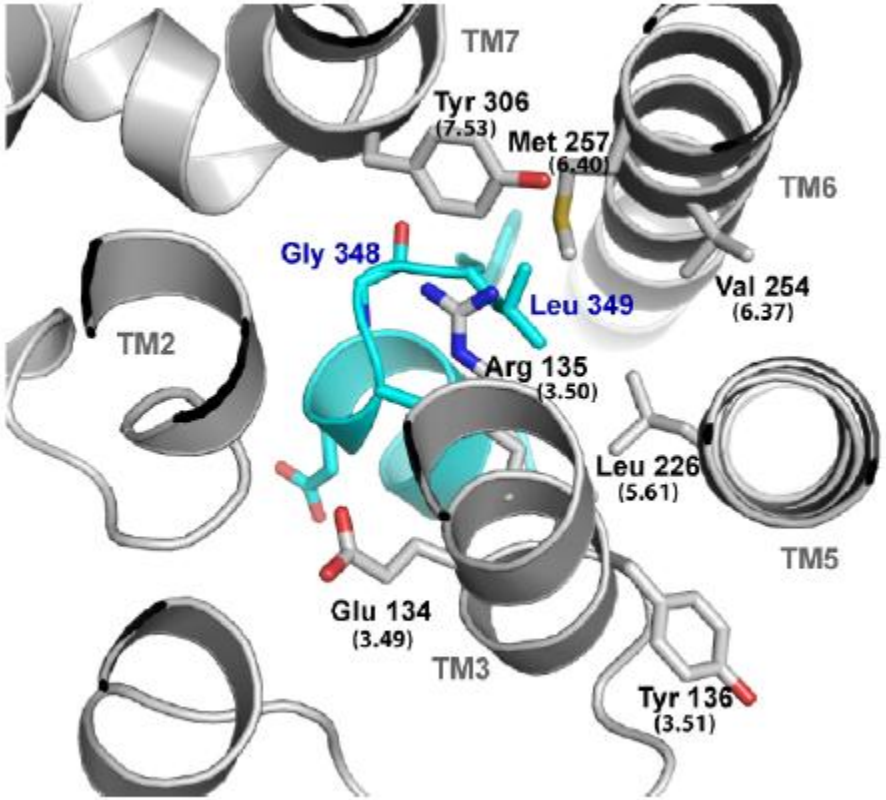
Ionic lock?



G-protein binding site

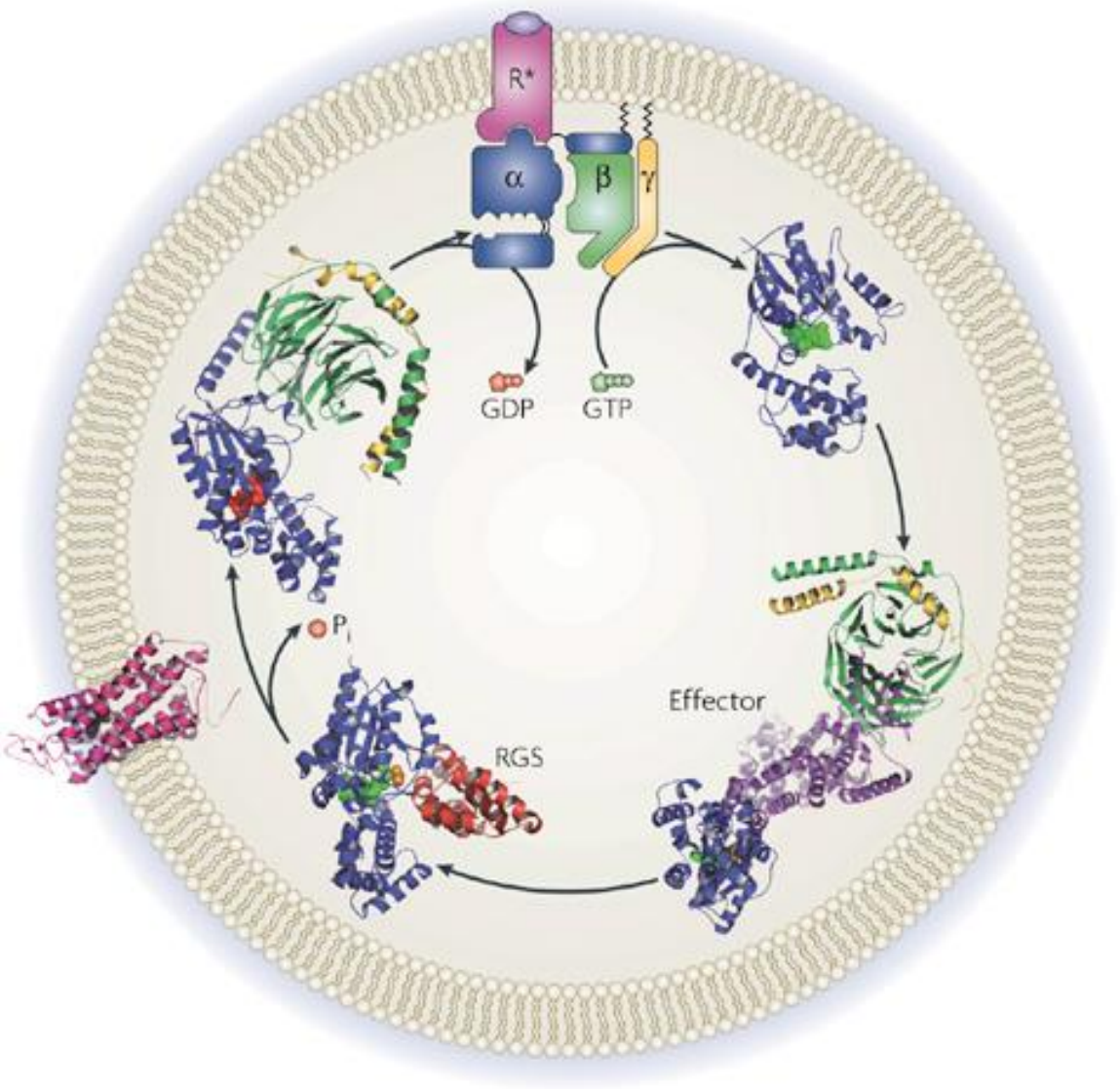


b2AR-Nb80



Opsin-transducin

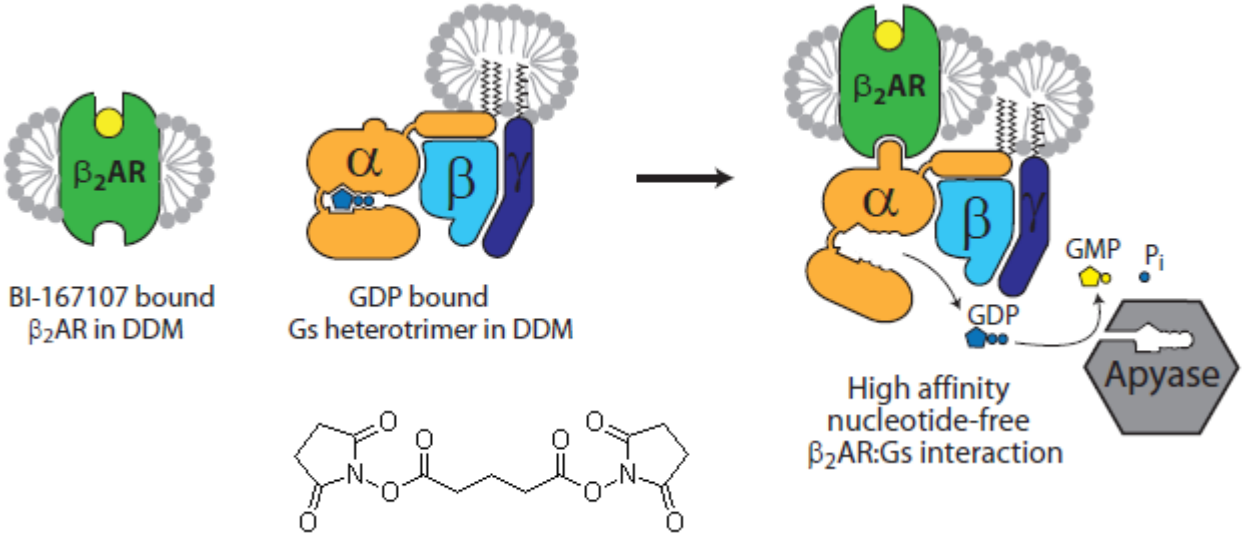
Holy grail of signal transduction



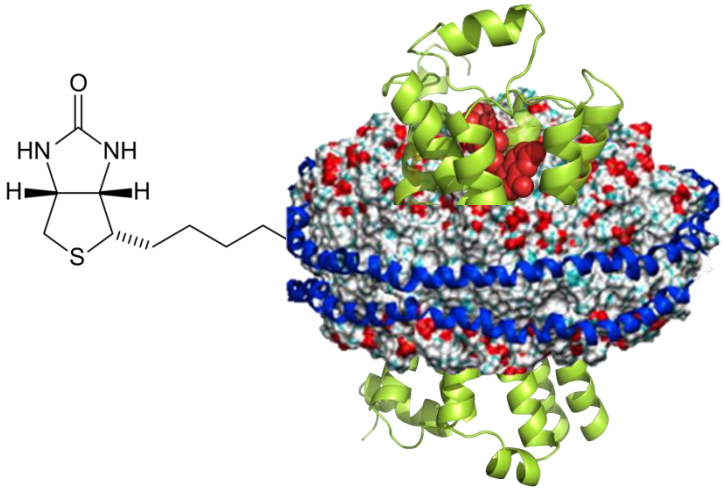
- Nucleotides
- Detergents

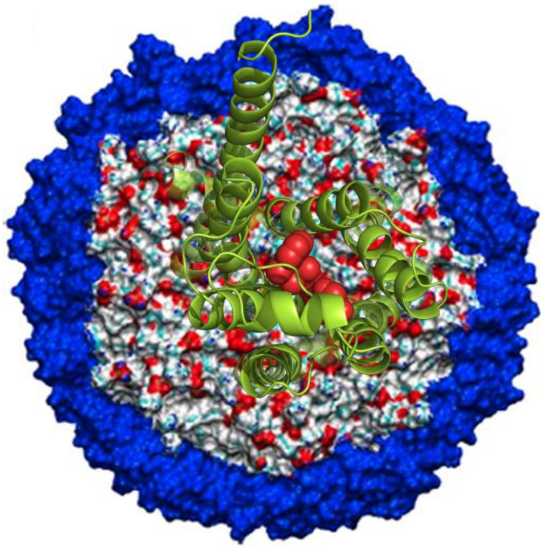
Generate β2-AR:Gs specific Nanobodies

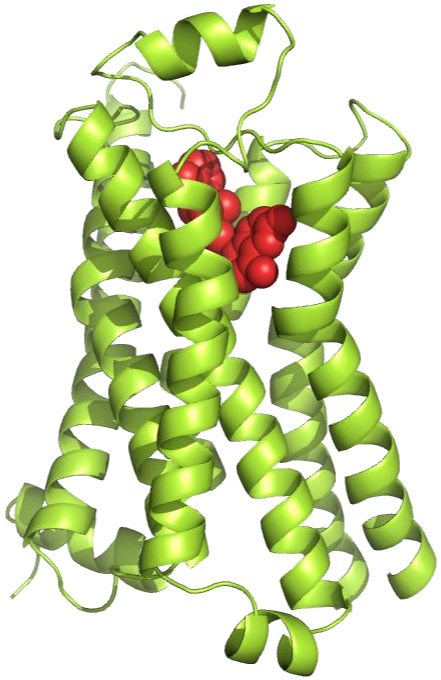
Day 1	Day 14	Day 28	Day 42
Complex + GERBU	Complex + GERBU	Complex	Complex



Selection by panning

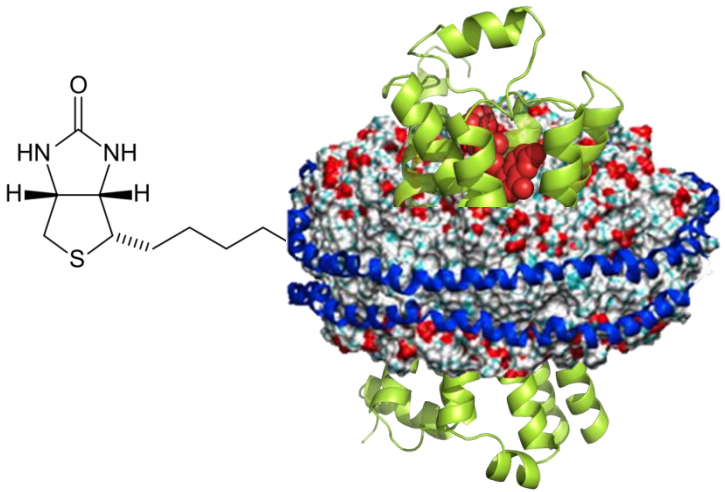




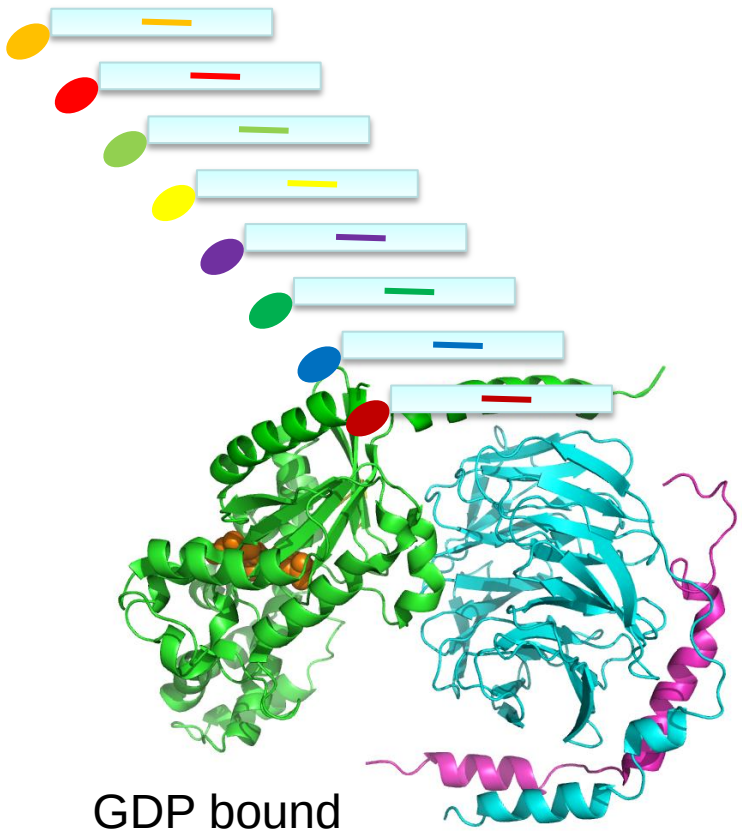


Selection by panning

Streptavidin coated surface

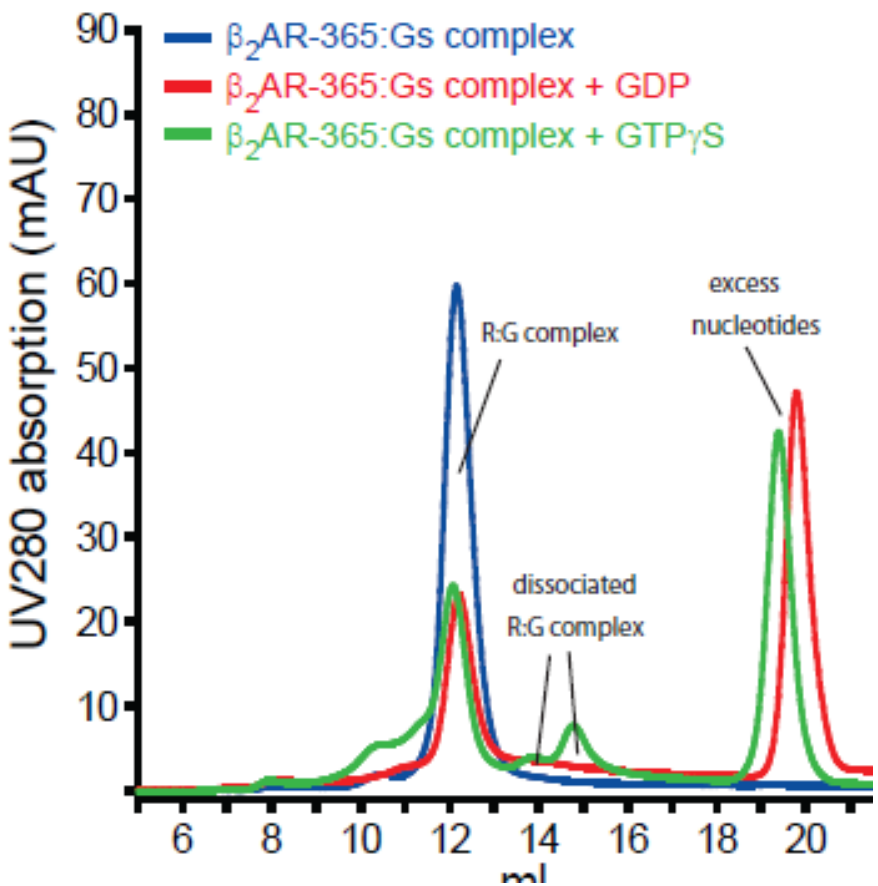


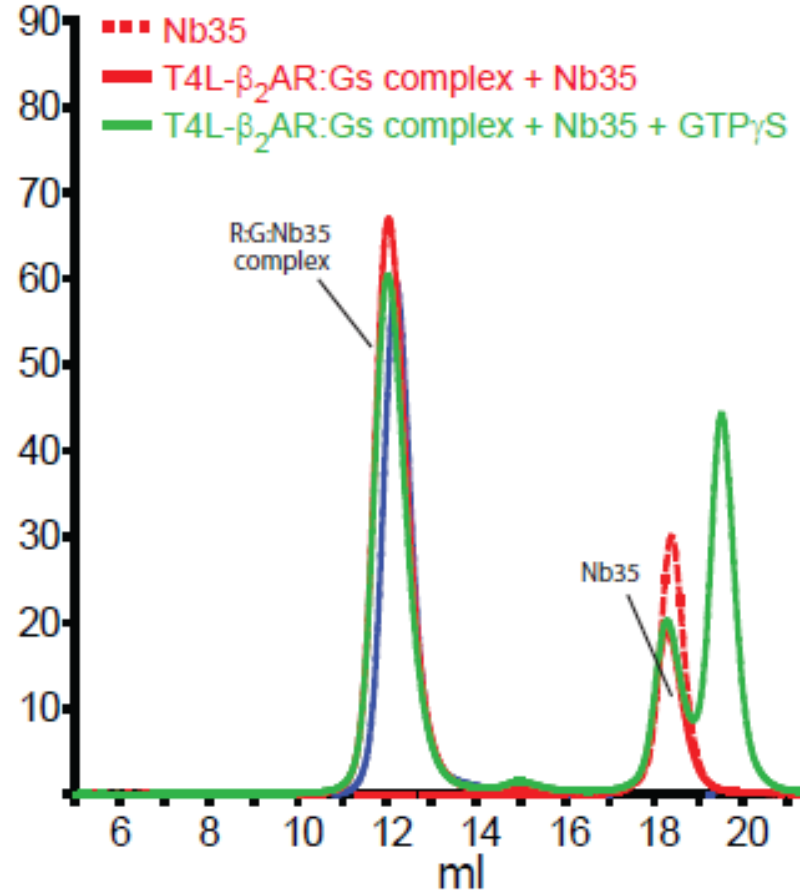
Apyrase



GDP bound

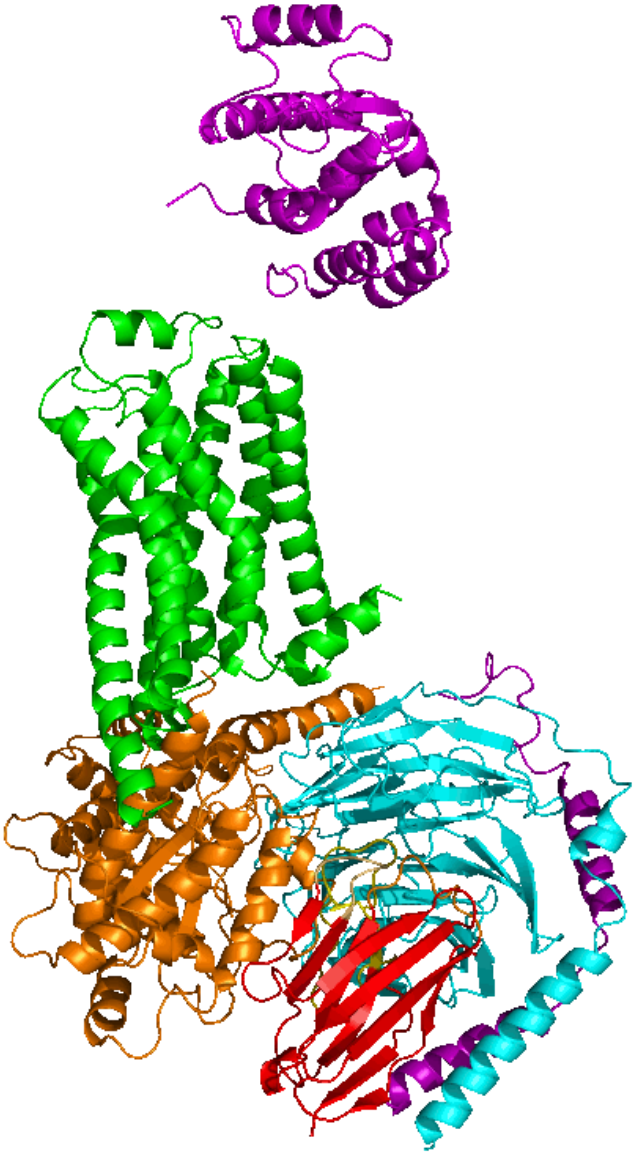
Nb35 protects to complex from dissociation by GTP





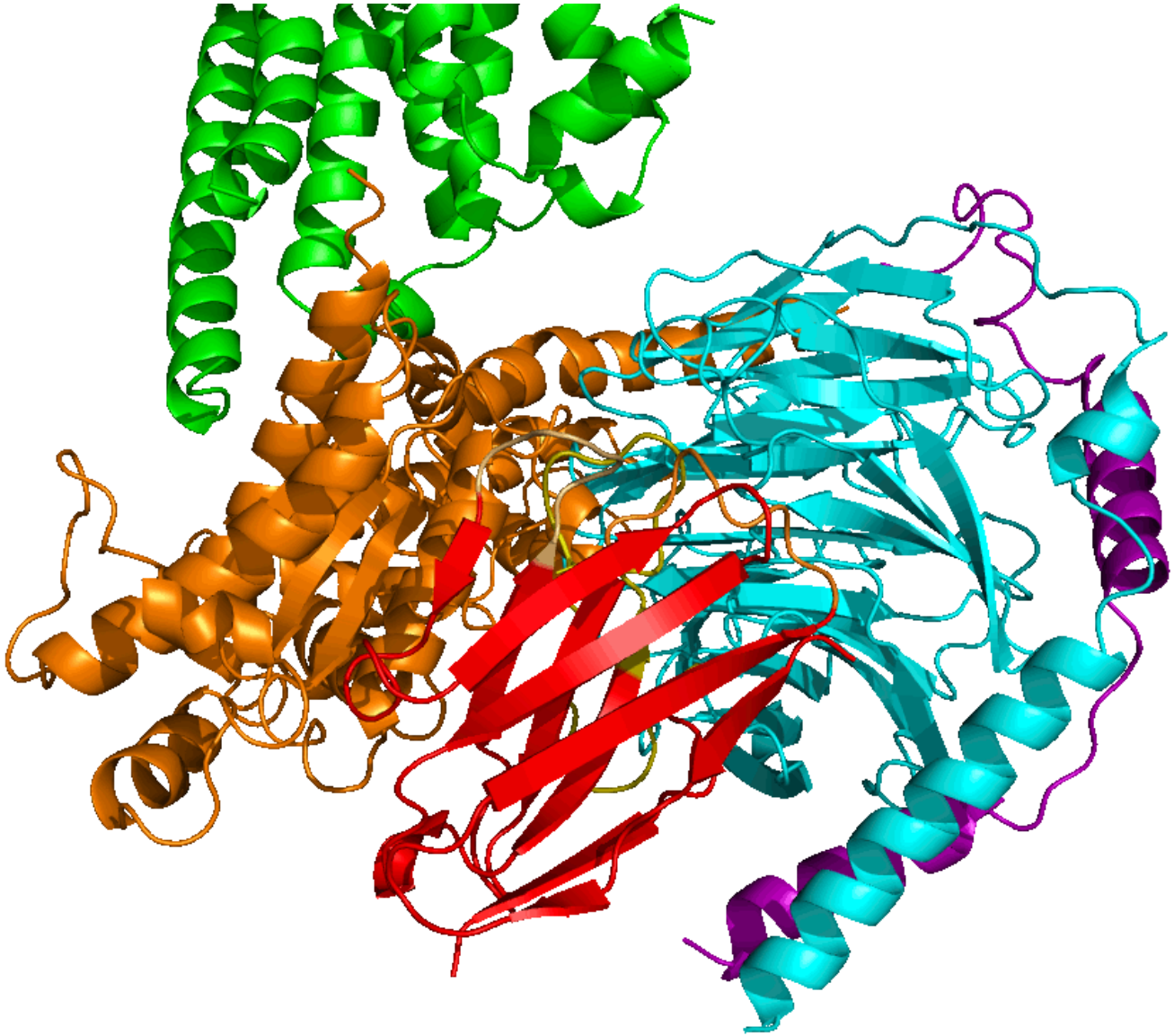
Crystallization of the β_2AR-Gs complex

T4L
β2-AR
GαS
Gβ
Gγ
Nb35

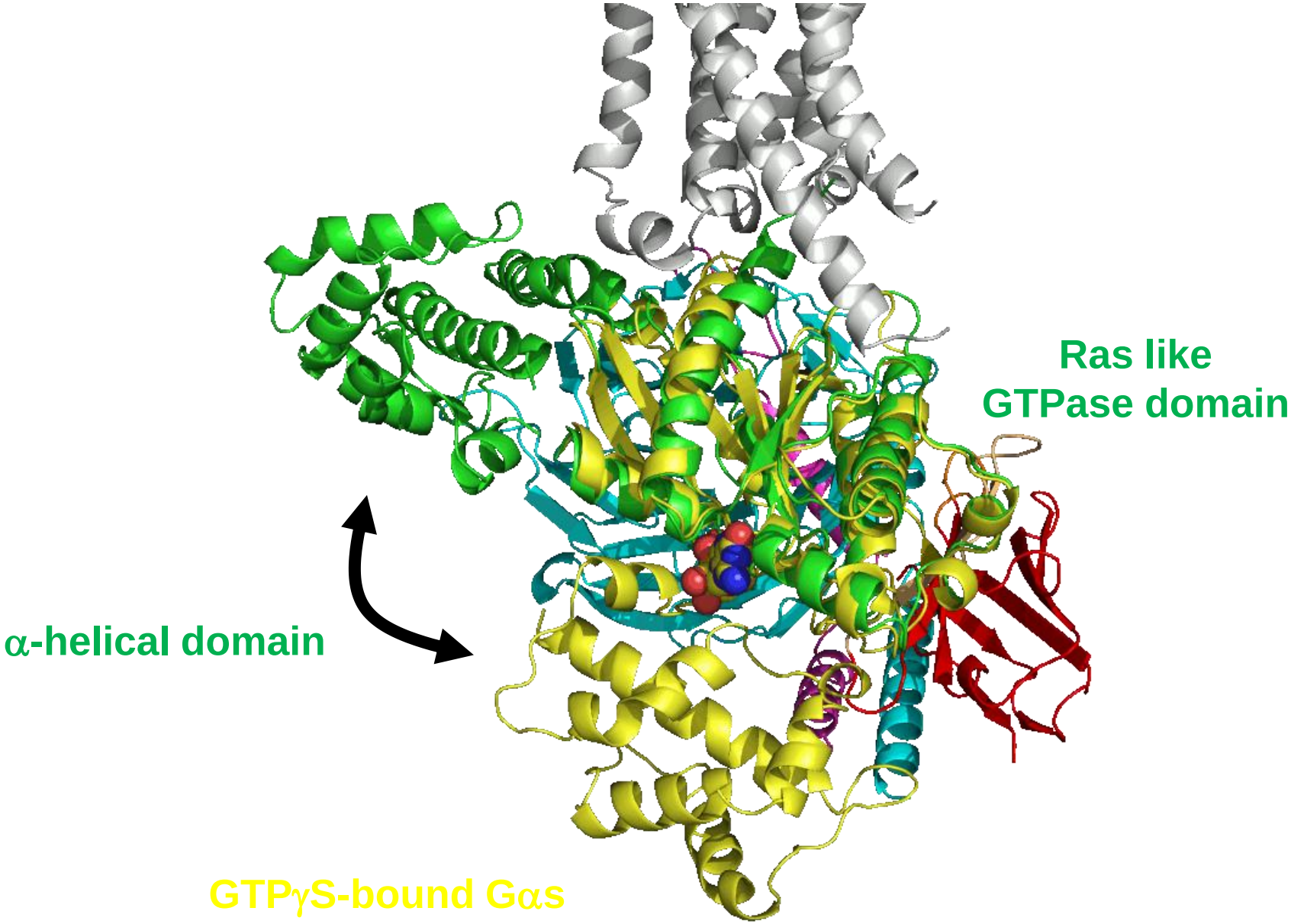


Nb35 binds the $G_{\alpha}S \bullet G_{\beta}$ interface

β2-AR
GαS
Gβ
Gγ
Nb35

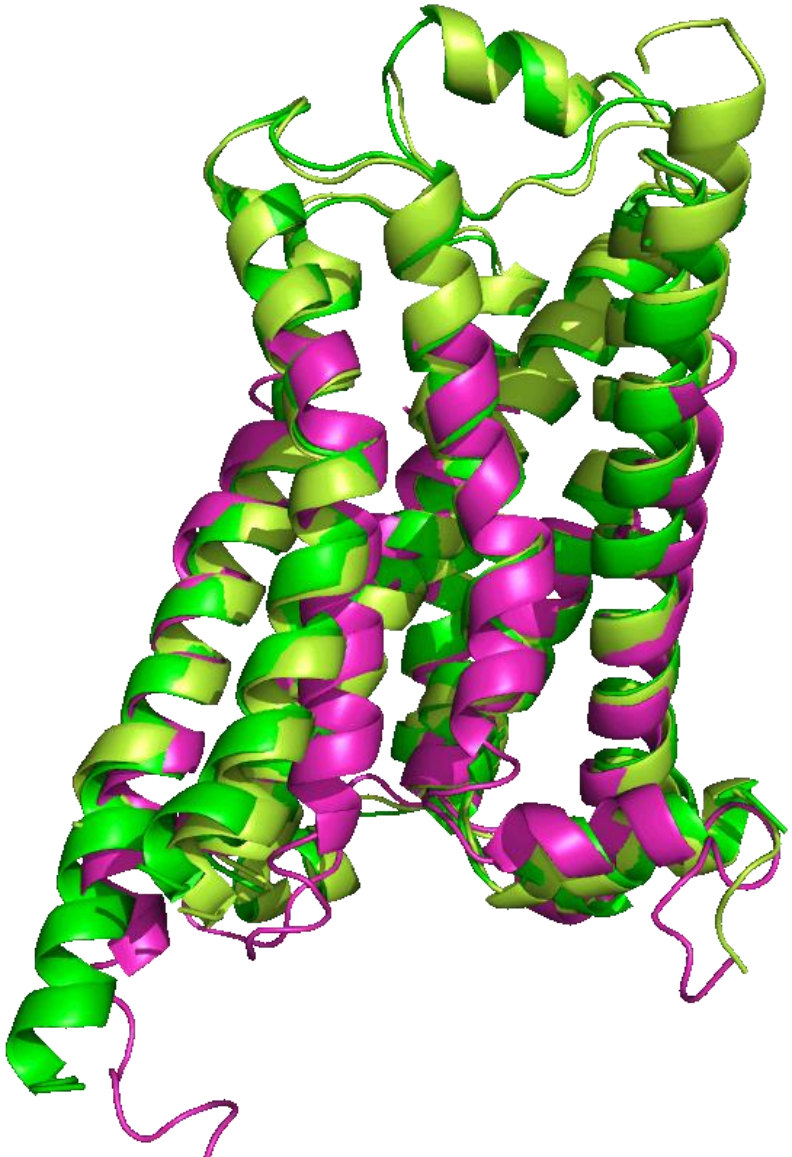


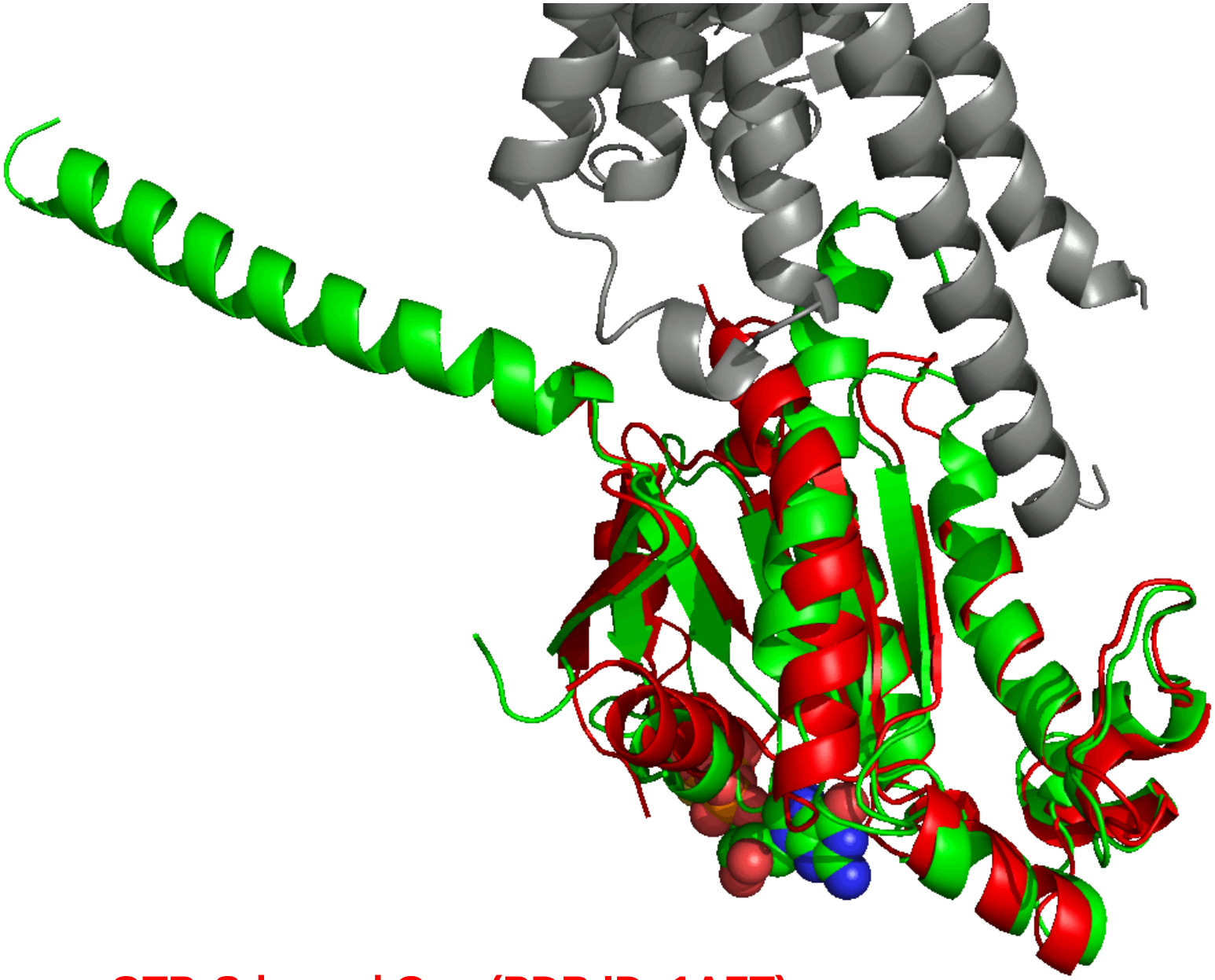
Rigid body movement of α-helical domain



Active vs inactive GPCR conformations

Carazolol:β2-AR
BI:β2-AR:Nb80
BI:β2-AR:Gs



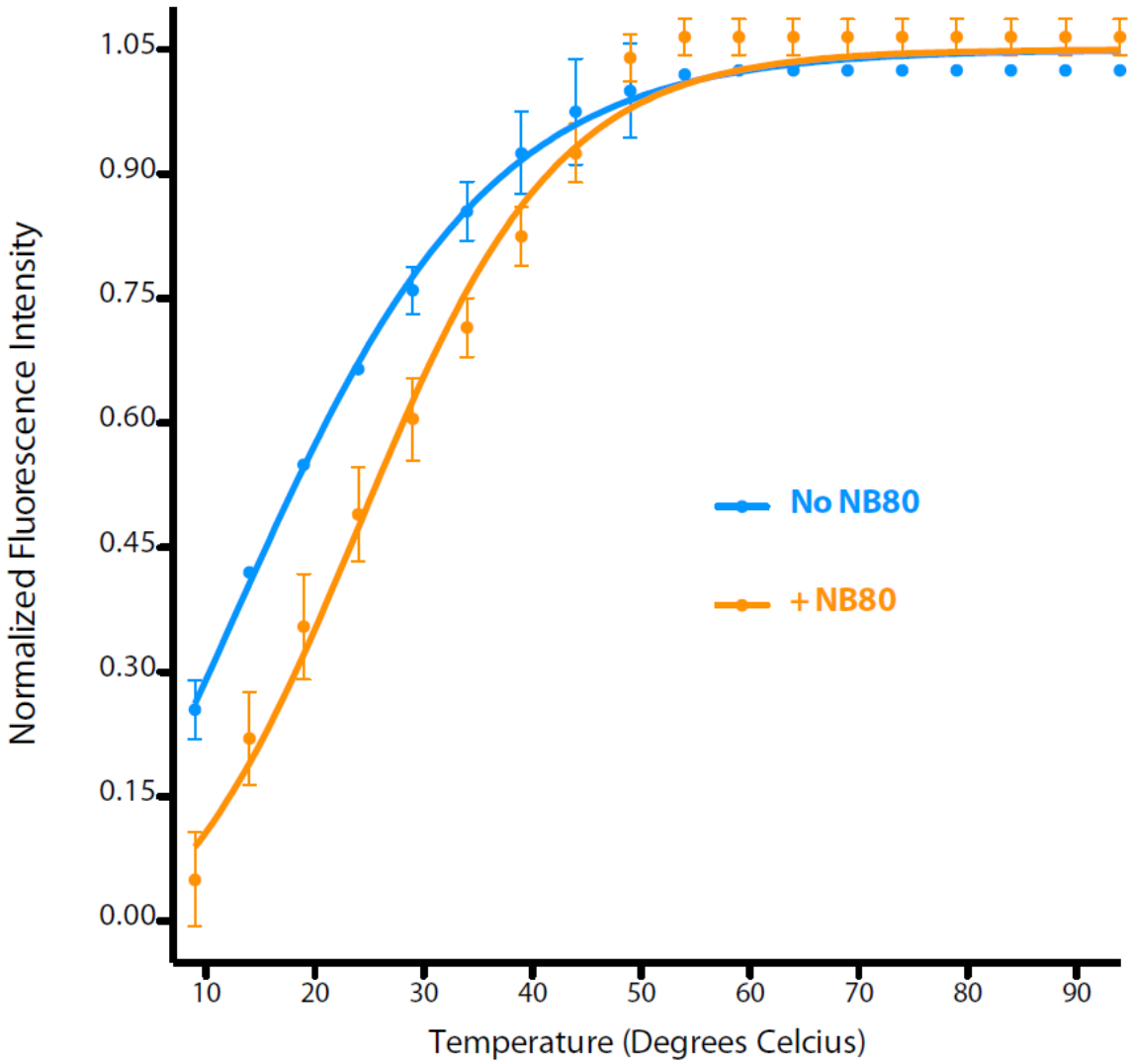


GTPγS-bound Gαs (PDB ID: 1AZT)

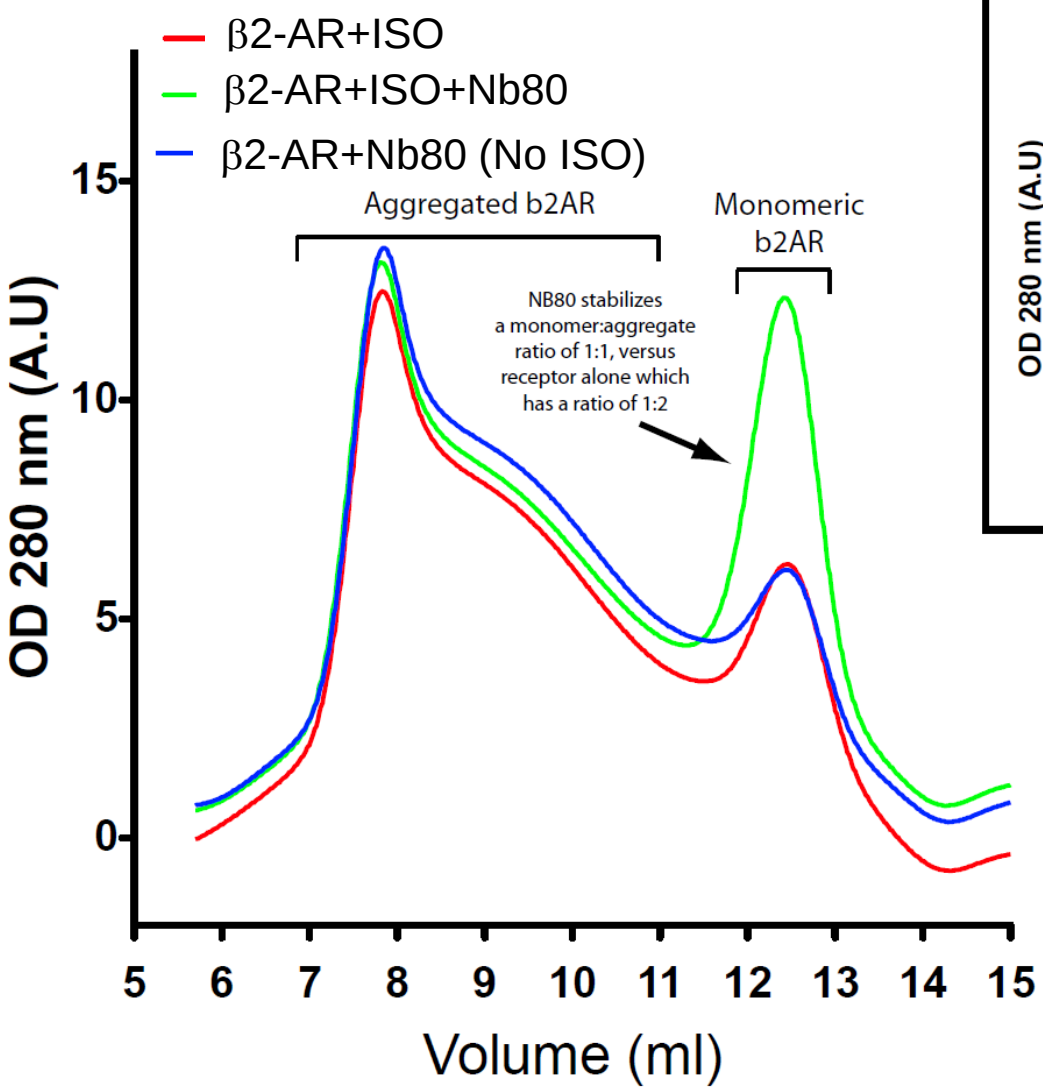
In conclusion

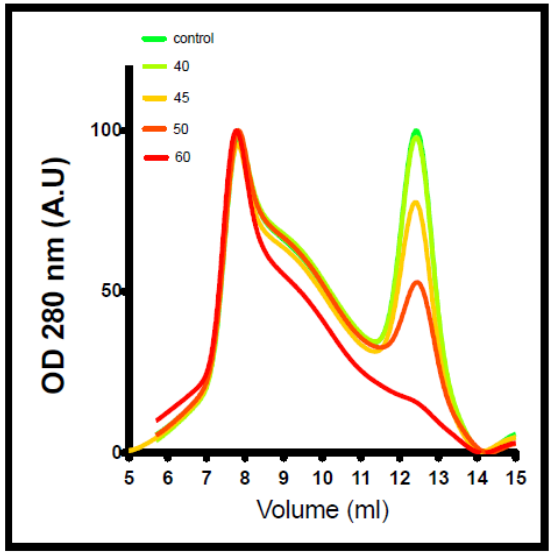
- ✓ Nbs recognize conformational epitopes on GPCRs
- ✓ Nbs selectively stabilize active conformations of GPCRs
- ✓ Nbs can mimic the effects of GPCR ligands, including the G-protein
- ✓ Nbs serve as crystallization chaperones for GPCRs
- ✓ Conformational Nbs have reciprocal allosteric effects on agonist *vs* inverse agonist binding
- ✓ Nbs can stabilize transient PPIs like the GPCR-Gs complex

Thermal stabilization of β2-AR



Thermal stabilization of β2-AR

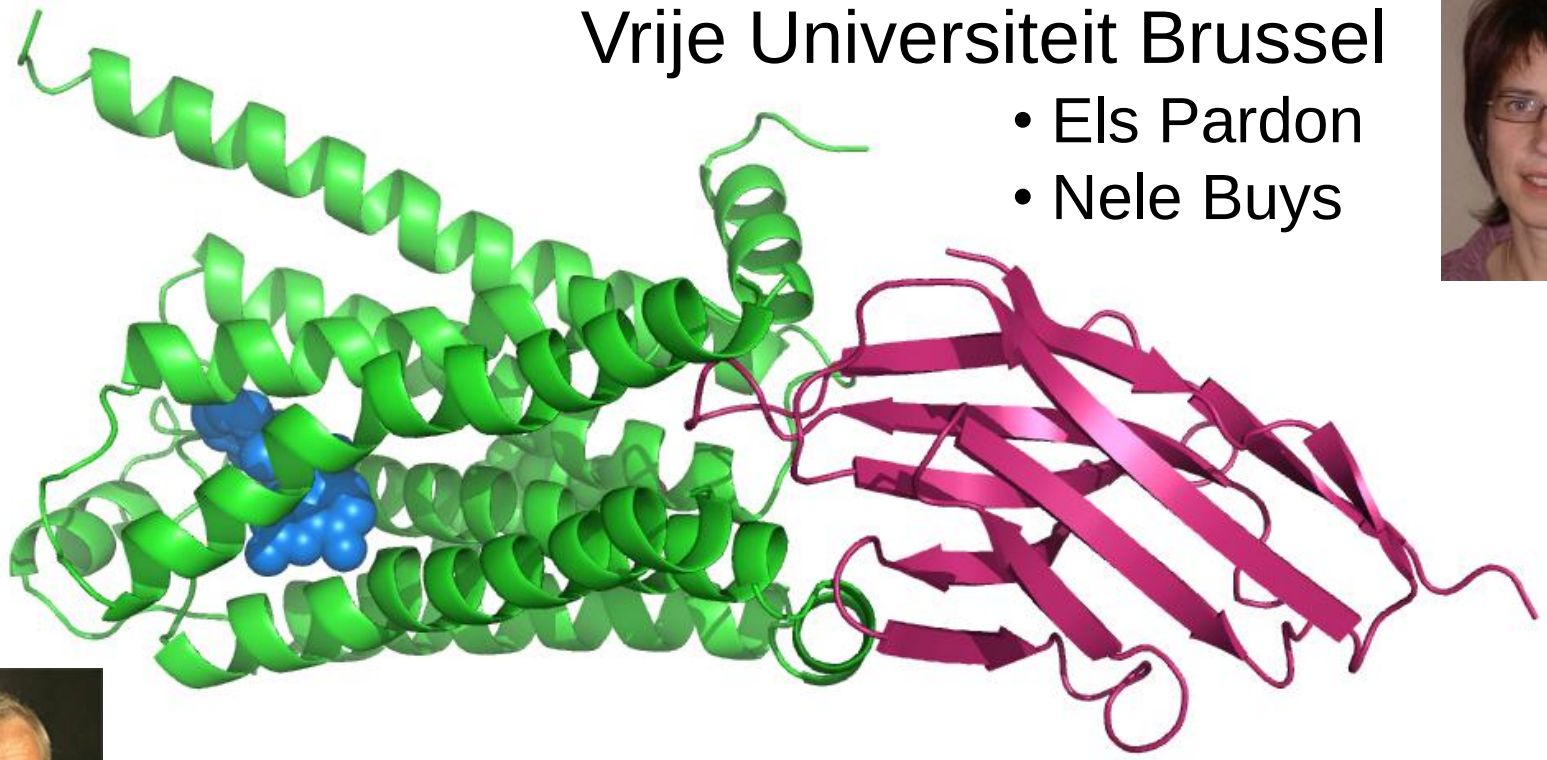




Steyaert lab

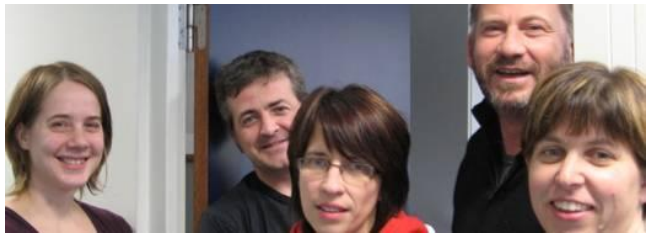
Vrije Universiteit Brussel

- Els Pardon
- Nele Buys

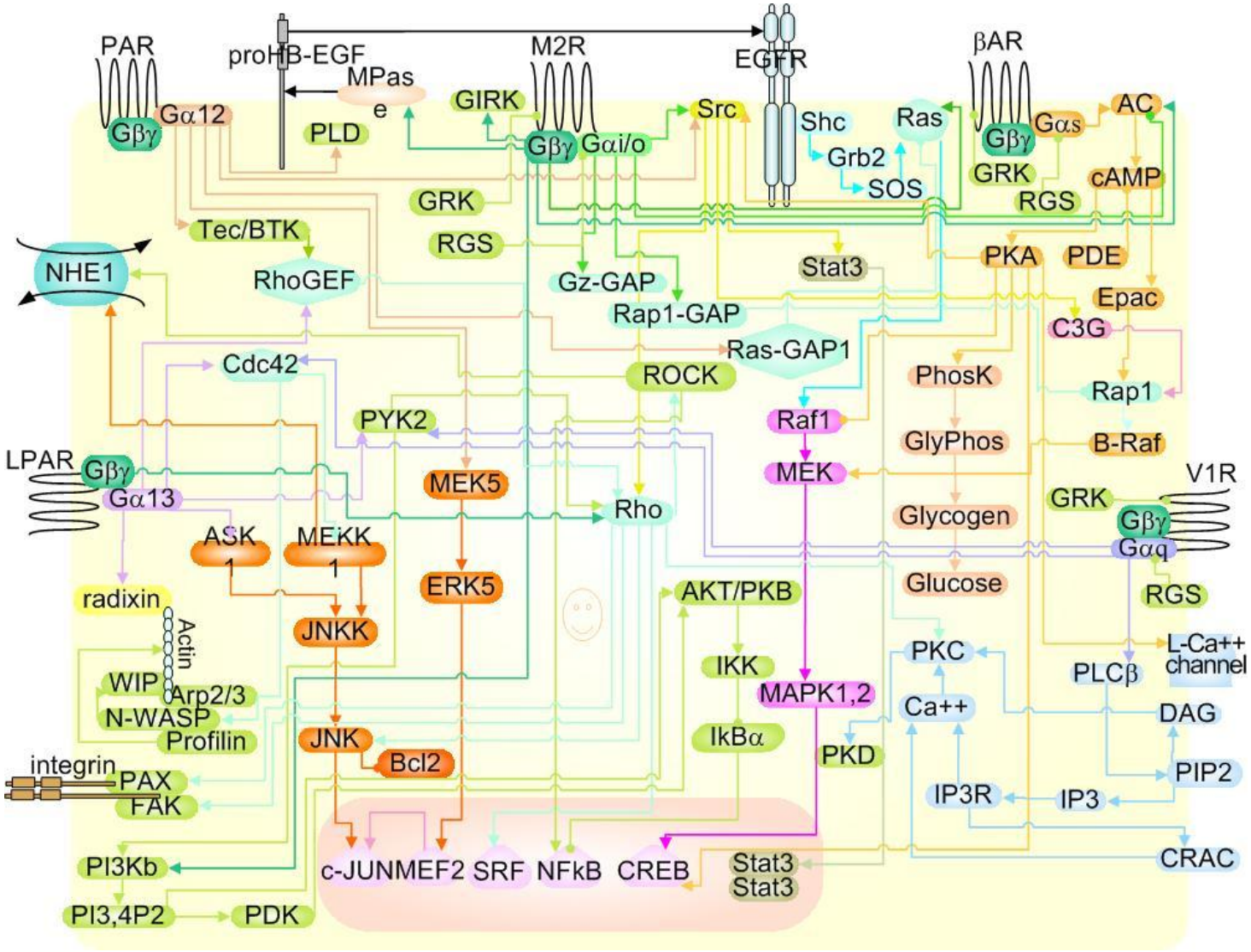


Kobilka lab

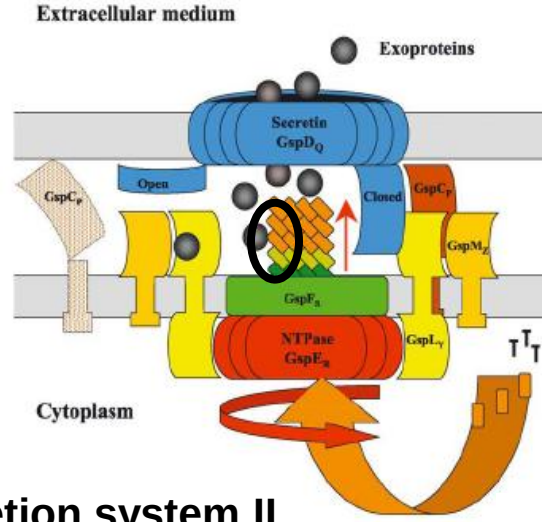
Stanford University



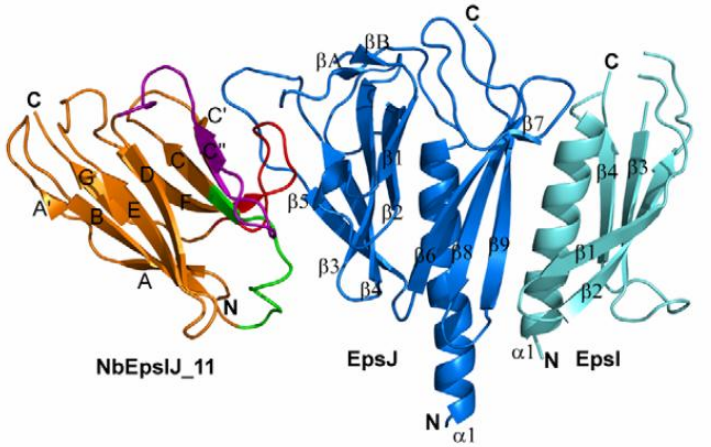
Composite view of transmembrane signaling

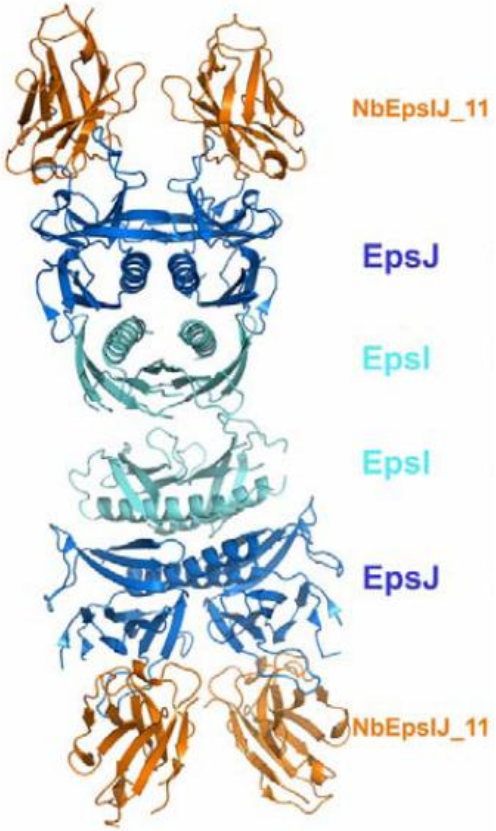


Parts of multiprotein complexes

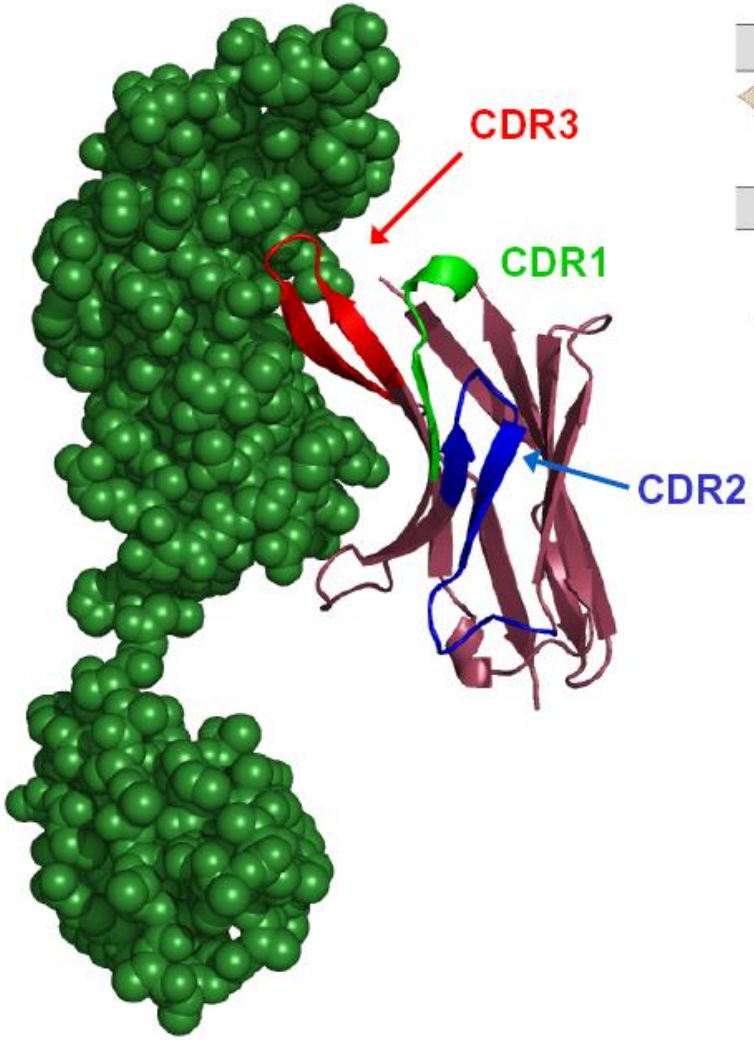


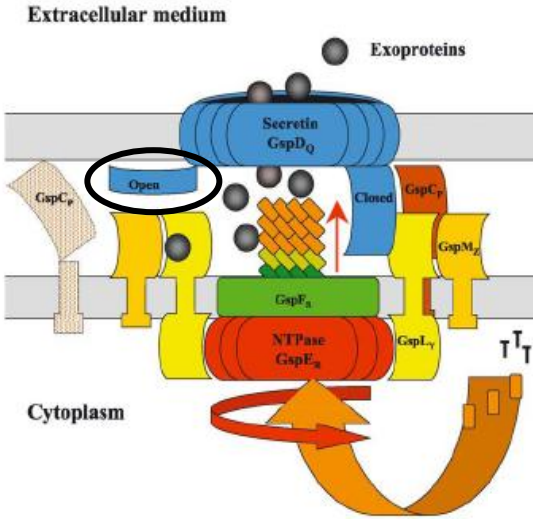
Secretion system II
E. coli

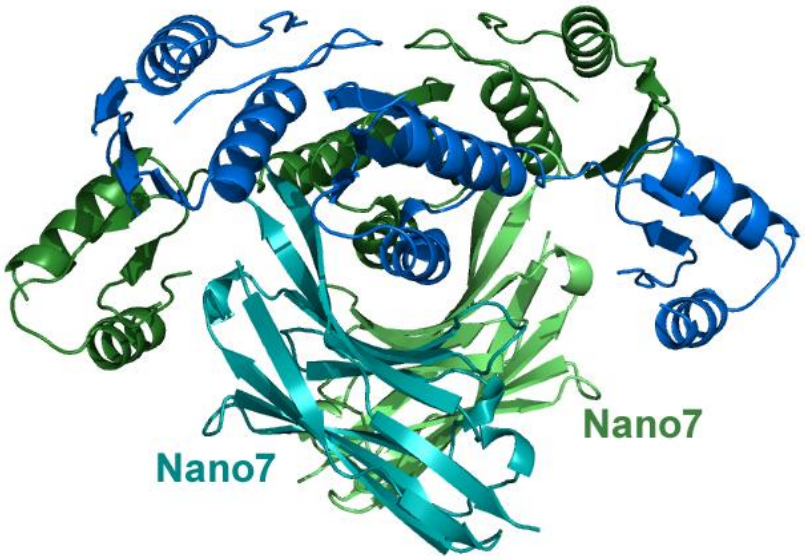




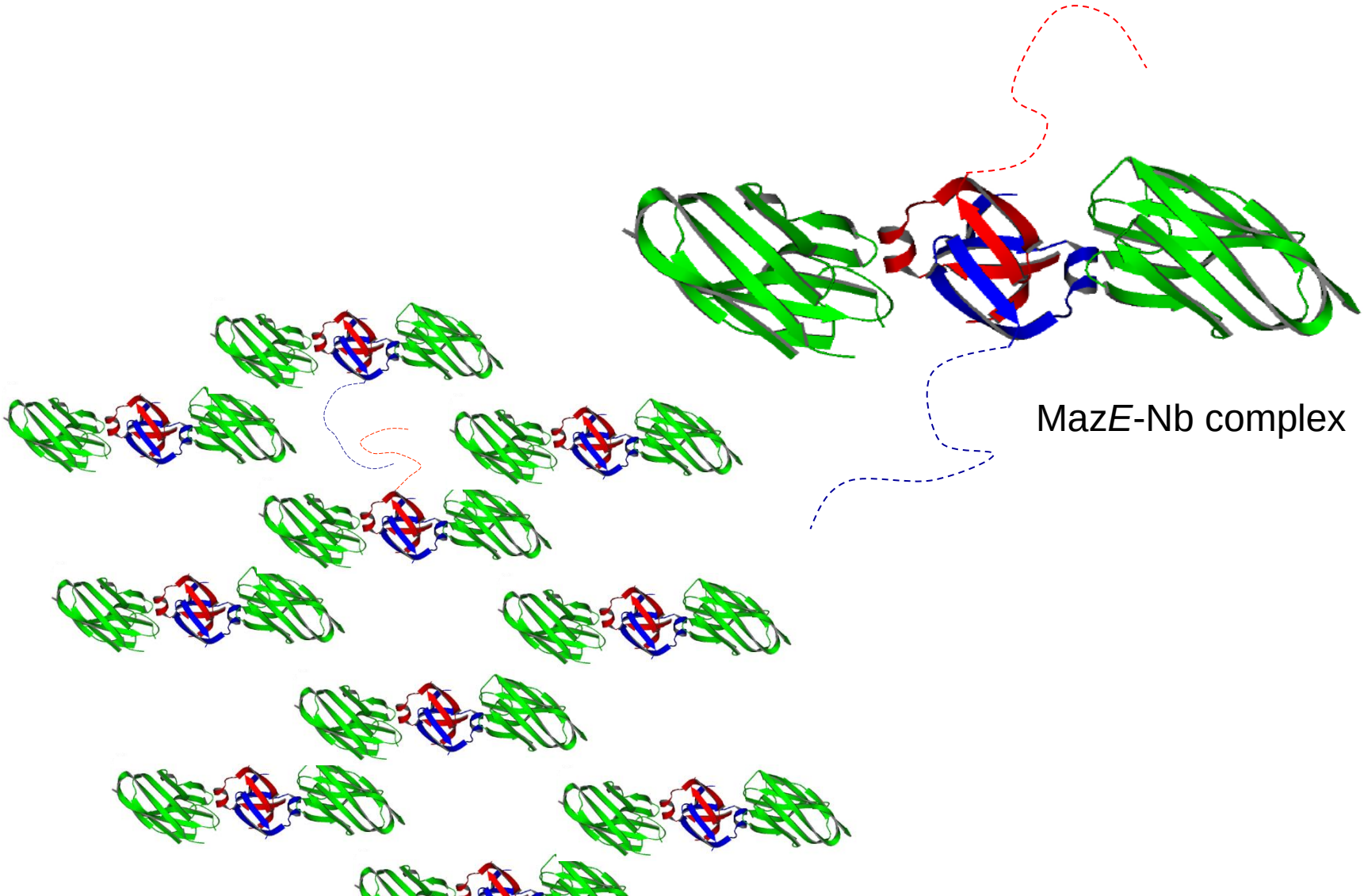
Multidomain proteins



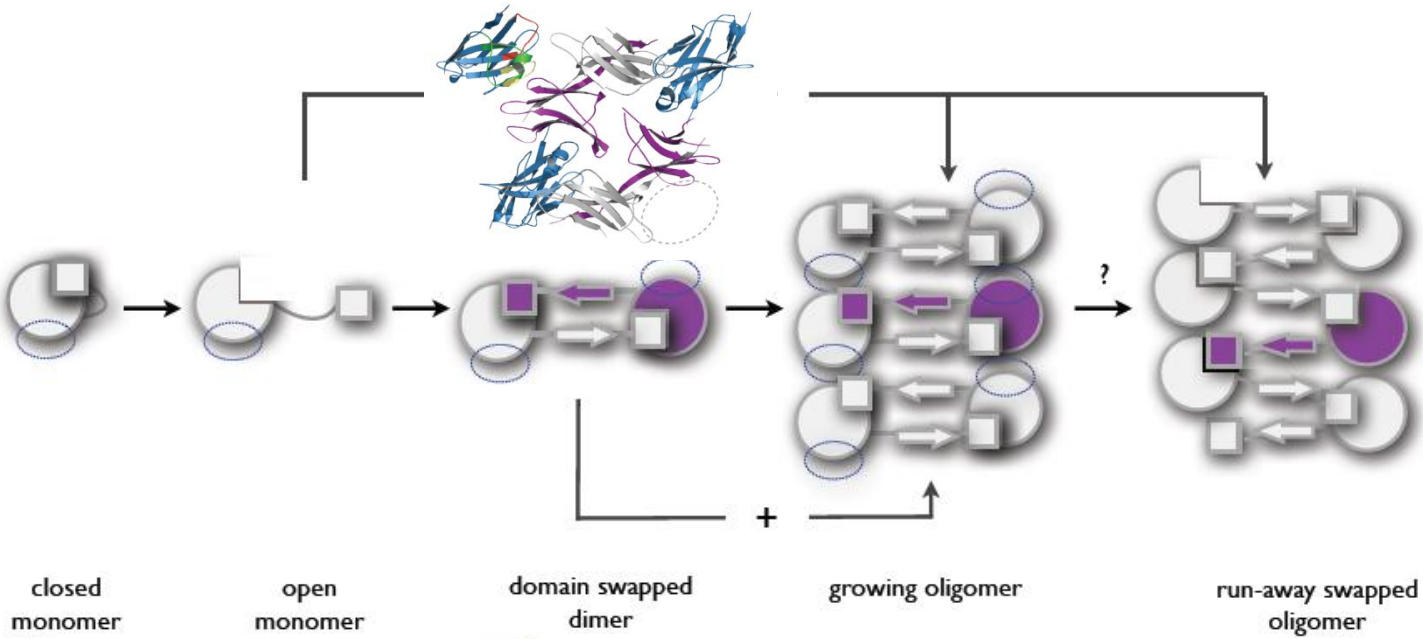




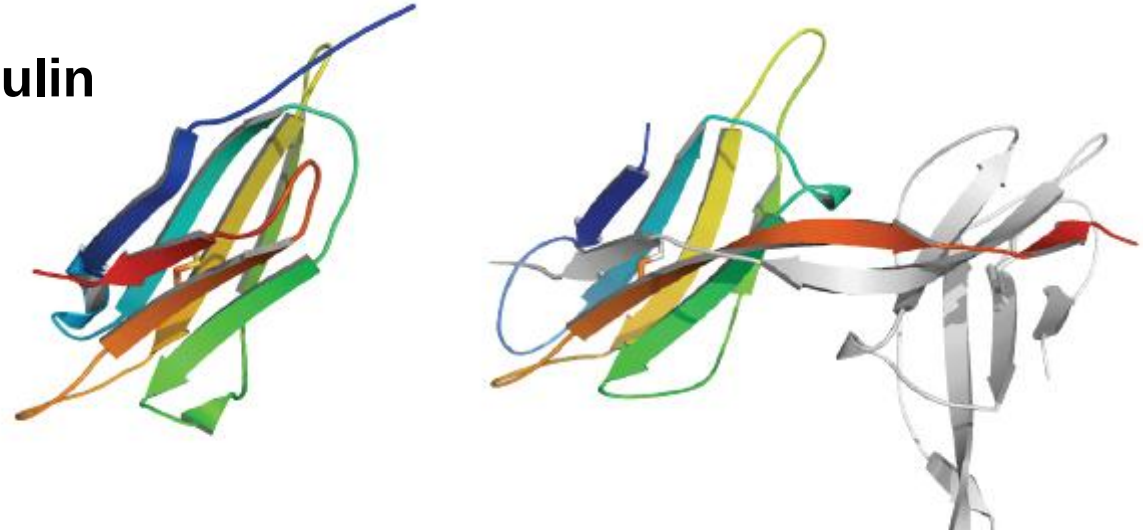
Intrinsically disordered proteins



Amyloidogenic proteins



β2-microglobulin

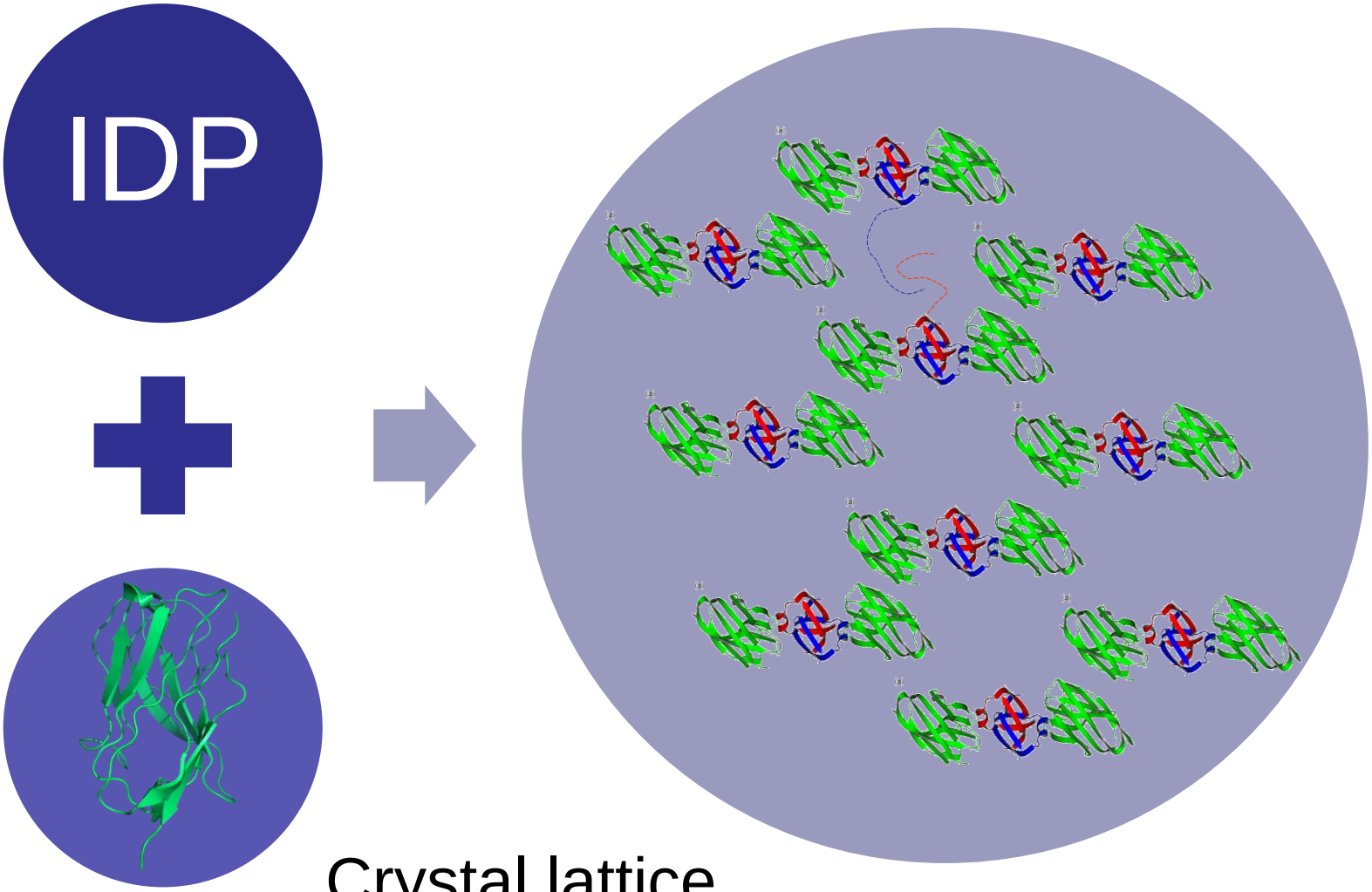


Chaperone-assisted X-ray crystallography with nanobodies: Xaperones.com

Xaperones are antigen binding fragments from heavy chain-only antibodies that:

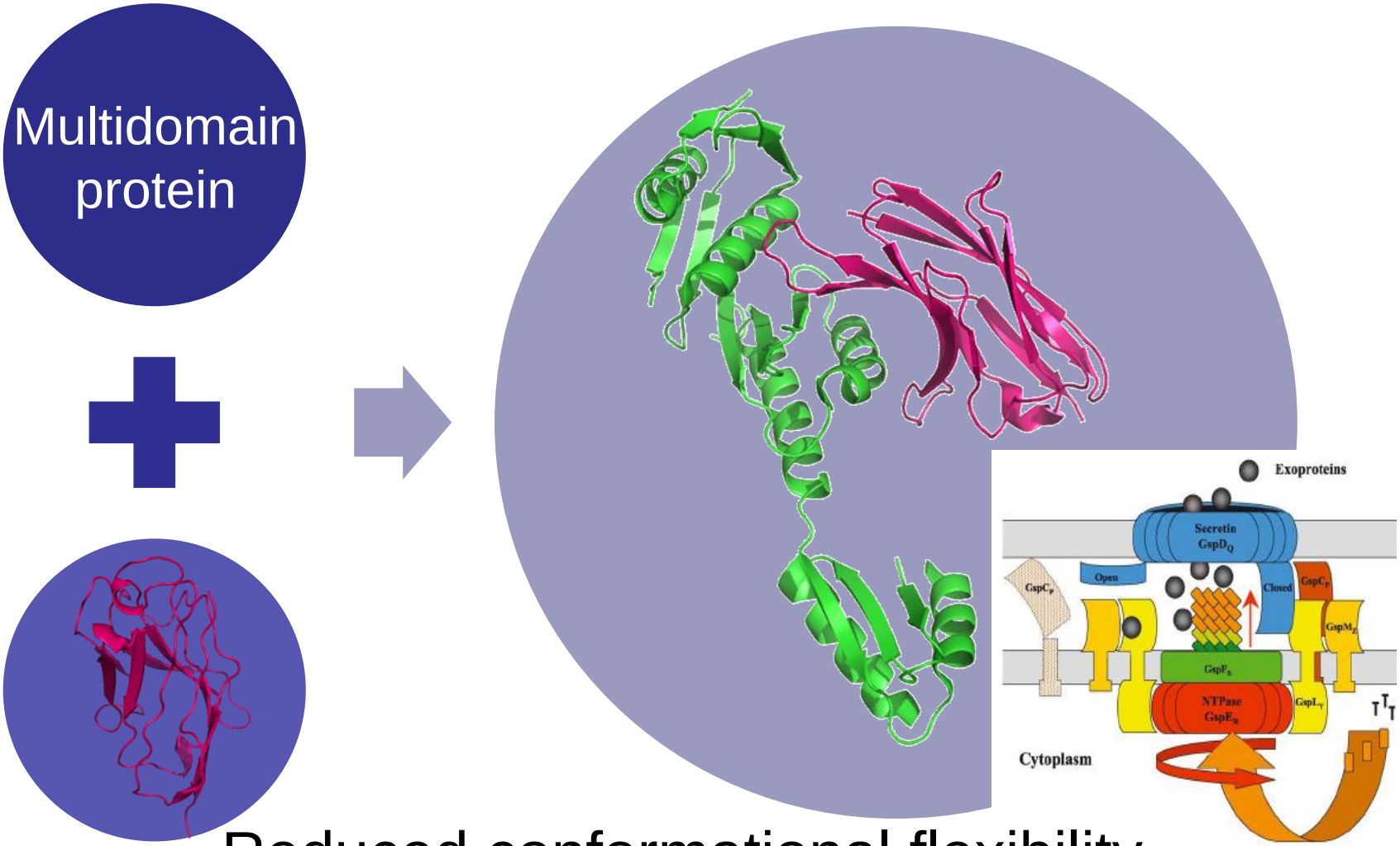
- Increase the stability of soluble proteins and solubilized membrane proteins
- Reduce the complexity in conformationally rich proteins and protein complexes
- Allow to affinity-trap active protein
- Increase the polar surface enabling the growth of diffracting crystals
- Bind cryptic epitopes and lock proteins in unique native conformations

Chaperone-assisted X-ray crystallography with nanobodies: Xaperones



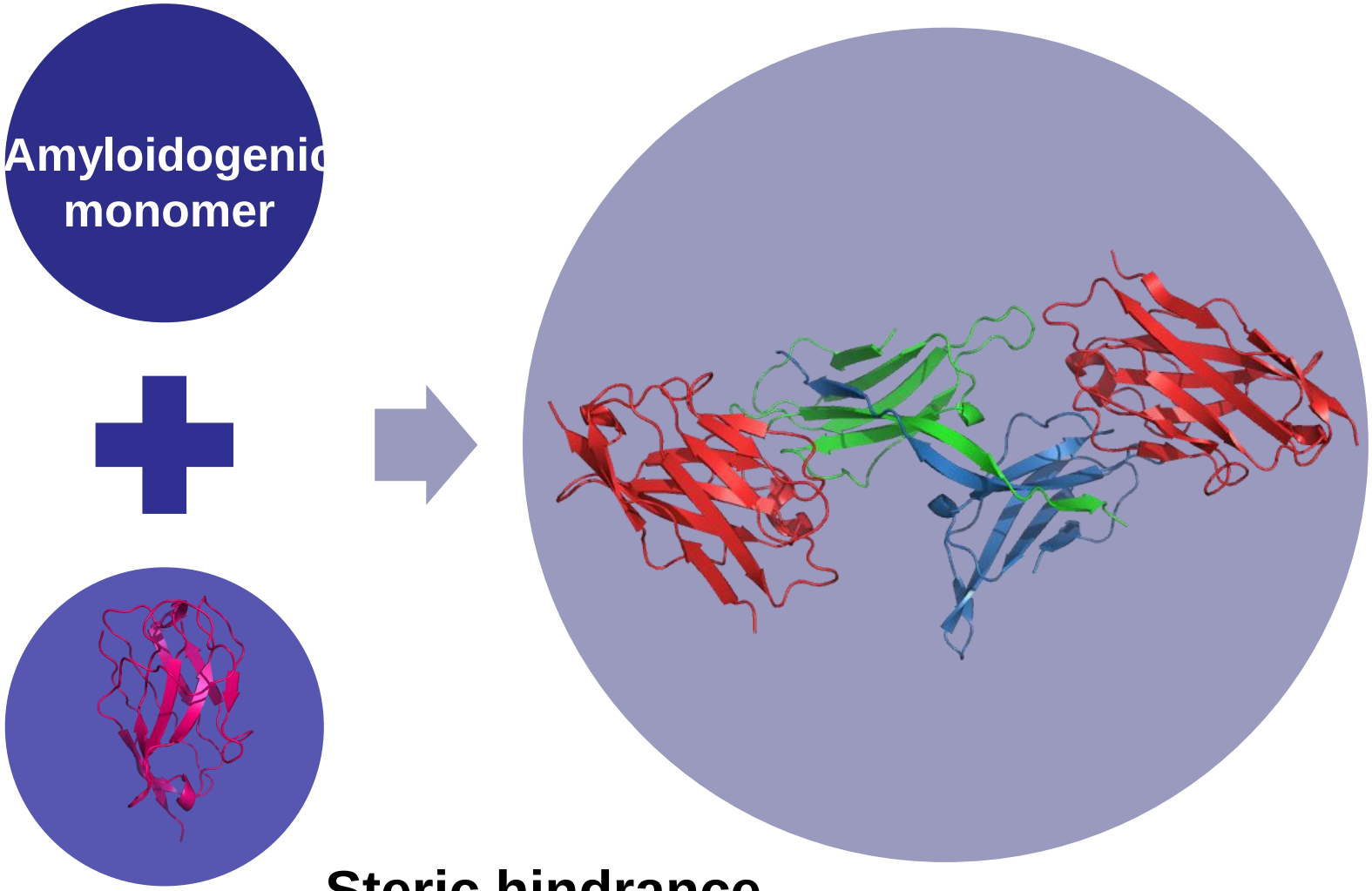
Crystal lattice

Chaperone-assisted X-ray crystallography with nanobodies: Xaperones



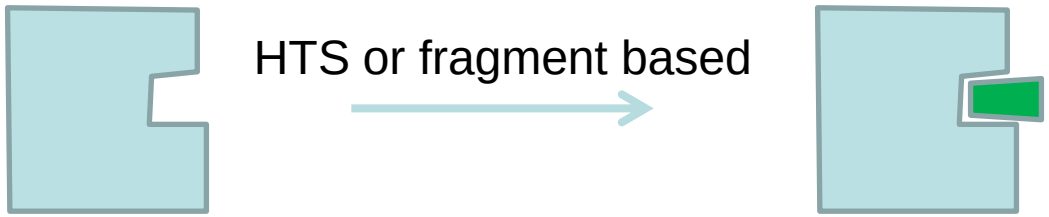
Reduced conformational flexibility

Chaperone-assisted X-ray crystallography with nanobodies: Xaperones

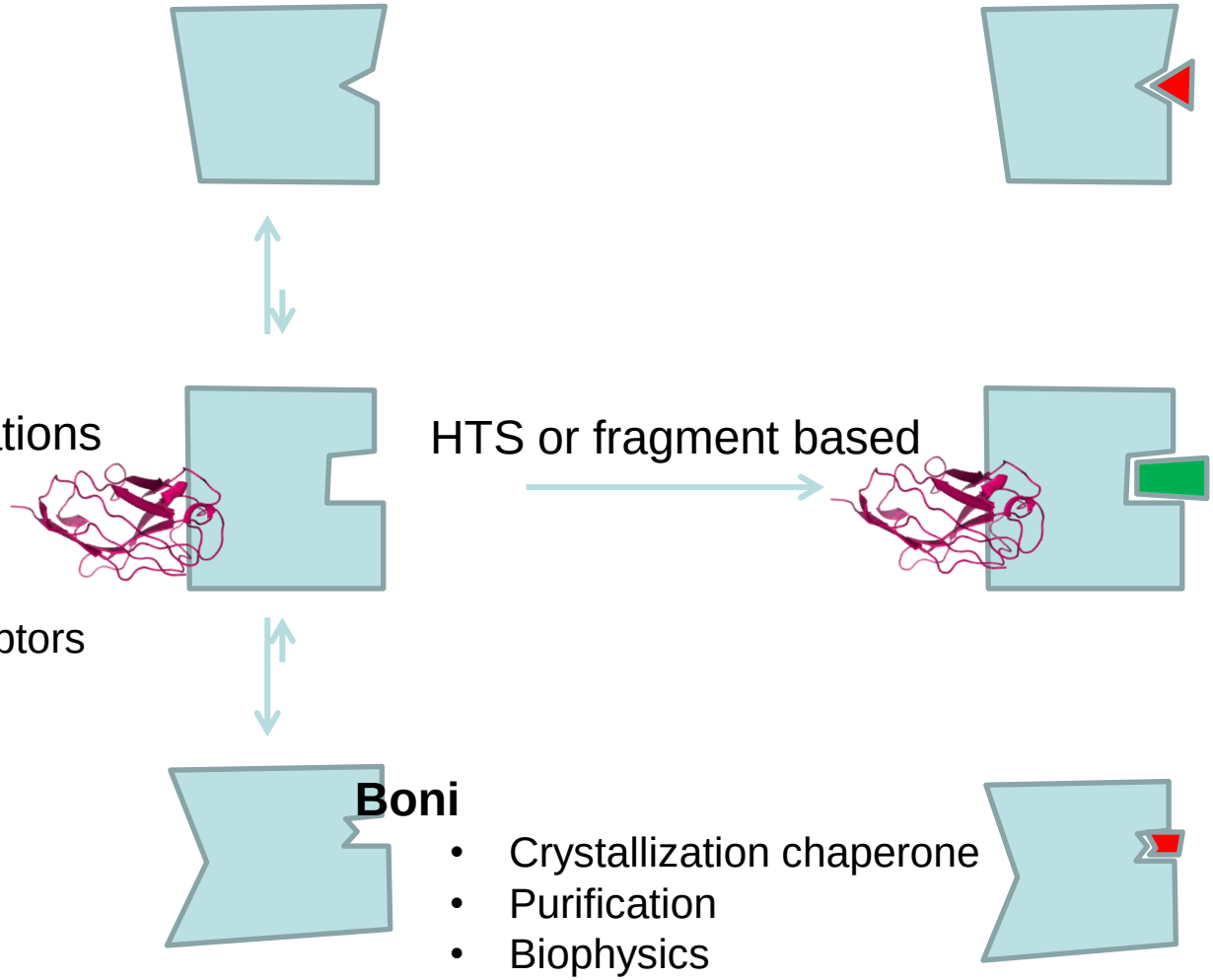


Steric hindrance

Applications of conformational nanobodies



Applications of conformational nanobodies



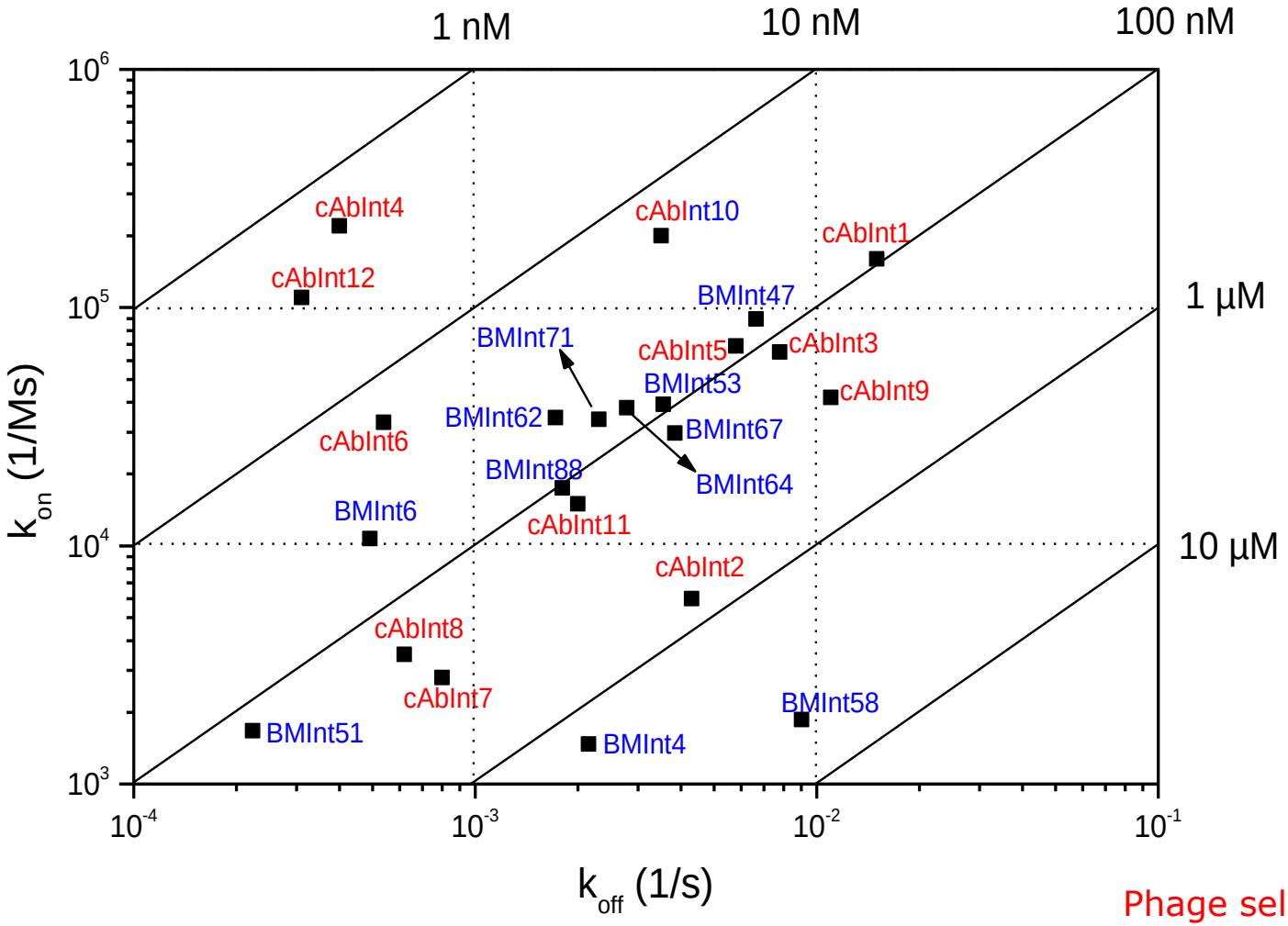
Drugable conformations

- GPCRs
- Channels
- Kinases
- Nuclear receptors

Boni

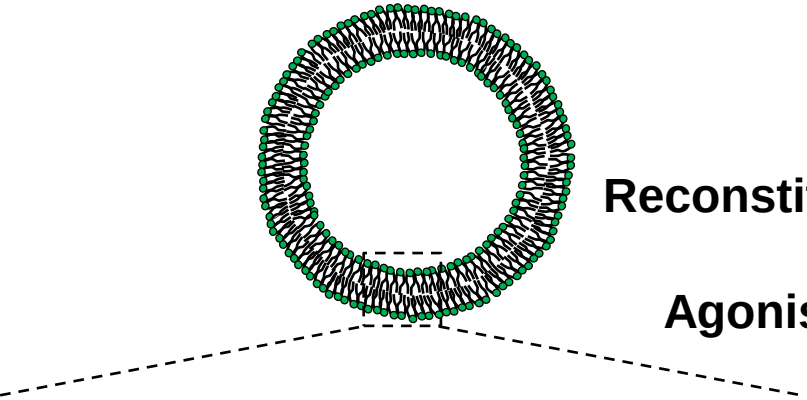
- Crystallization chaperone
- Purification
- Biophysics
- Biology

Nb affinities

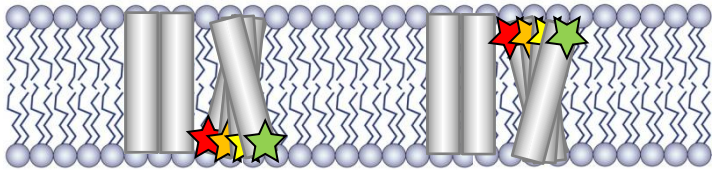


Phage selected
Bacterial 2 Hybrid

CB2 binders

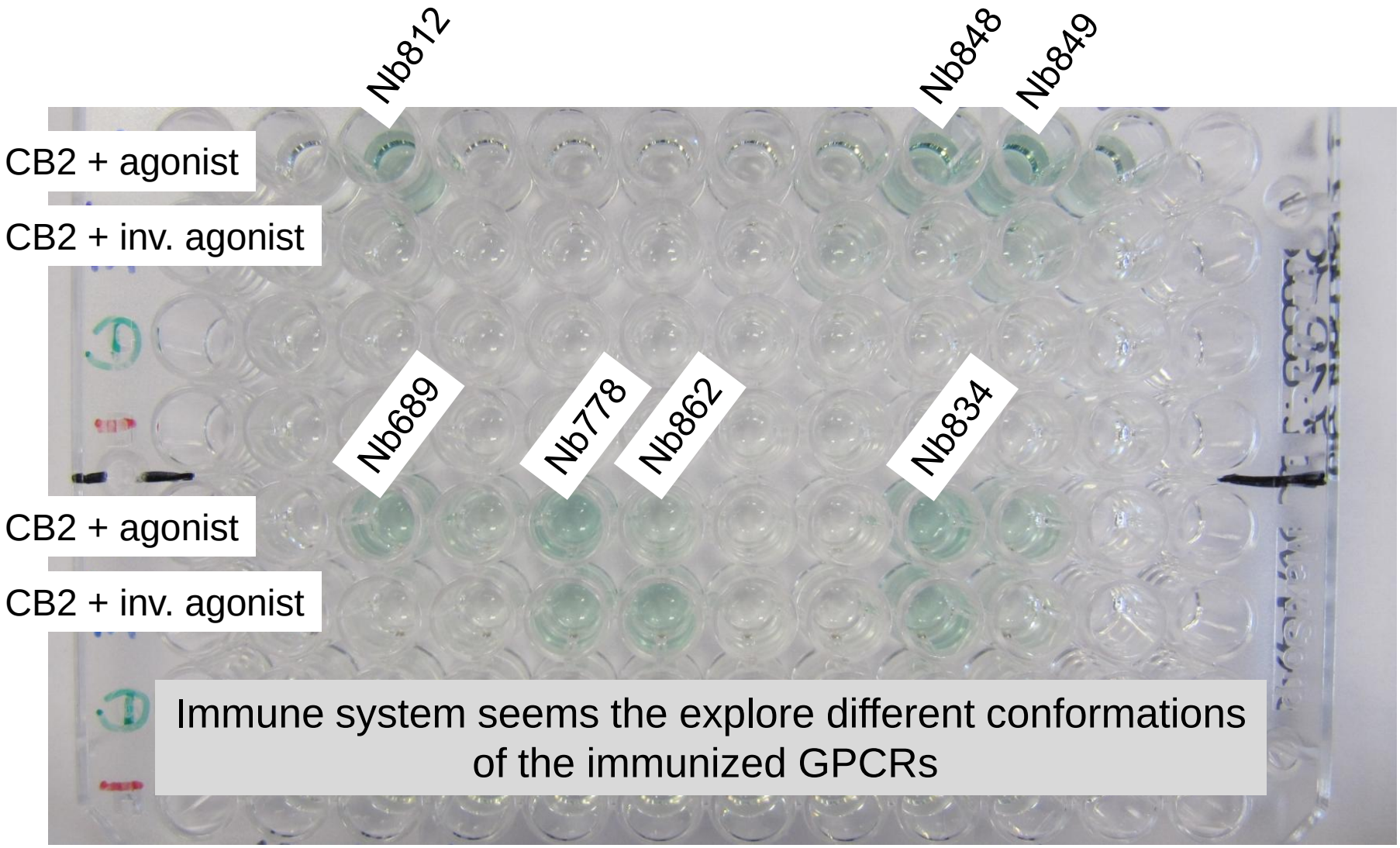


Reconstituted receptor
+
Agonist CP55940



Day 1	Day 14	Day 28	Day 42	Day 56
Receptor + GERBU	Receptor + GERBU	Receptor + GERBU	Receptor	Receptor

CB2 binders



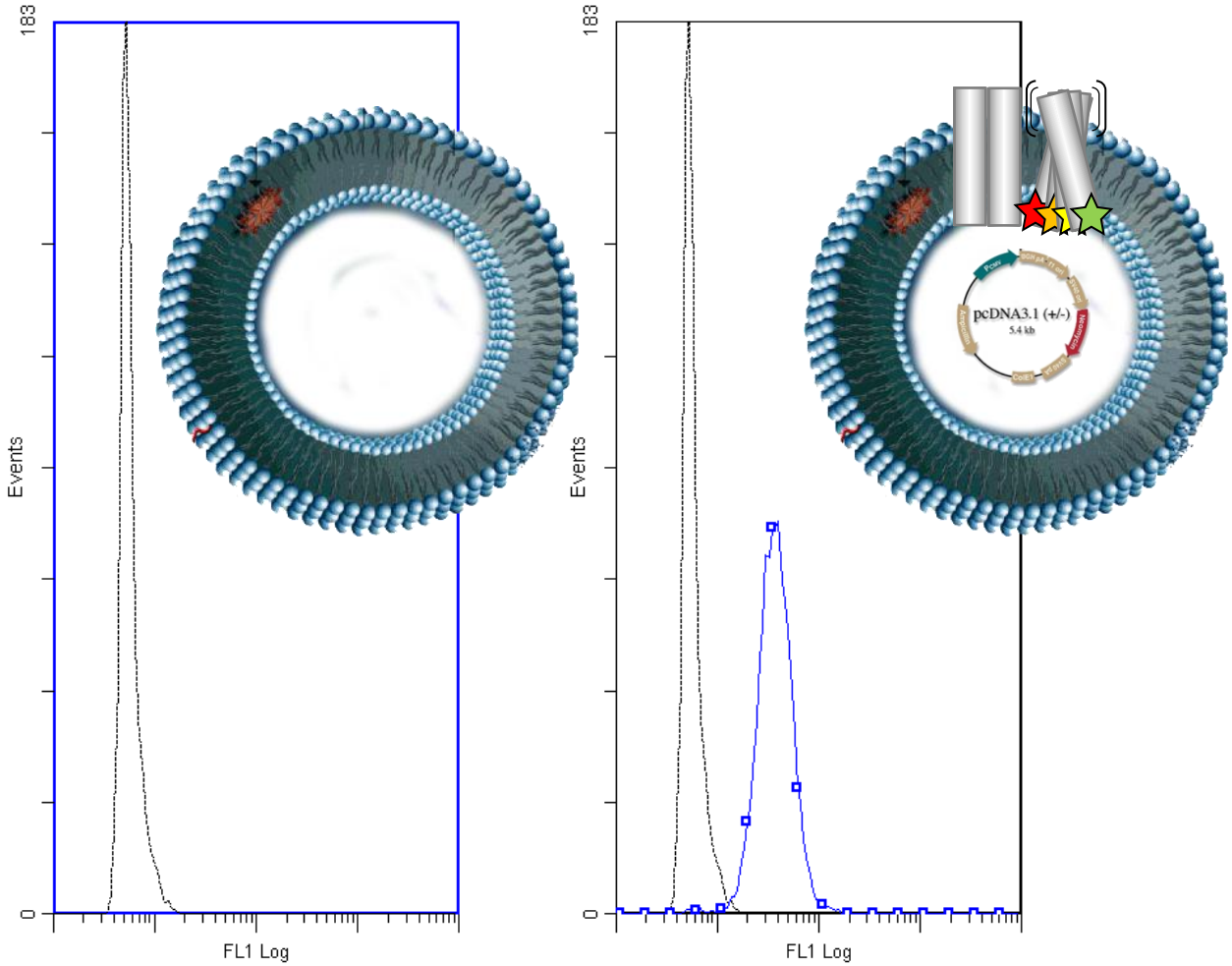
Immune system seems the explore different conformations of the immunized GPCRs

Klaus Gawrisch, NIH, Bethesda, Washington

CXCR4 binders

Nanobody family	# sequences/family	# unique representatives
1	27	13
2	3	3
3	2	1
4	19	8
5	16	5
6	1	1
7	1	1
8	1	1

CXCR4 binders



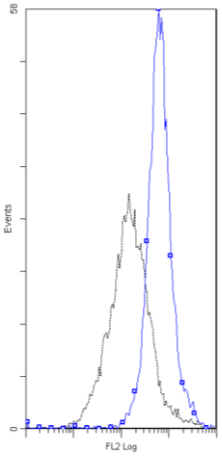
Nb CA4142

Marc Parmentier, ULB

CXCR4

Control

αCXCR4 cl 44717



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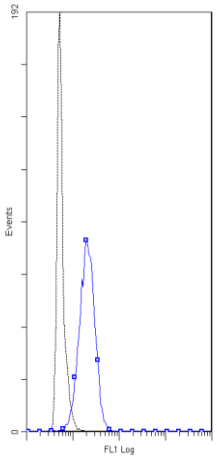
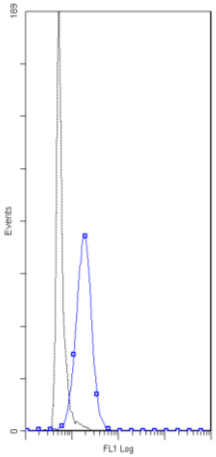


Nb CA4138



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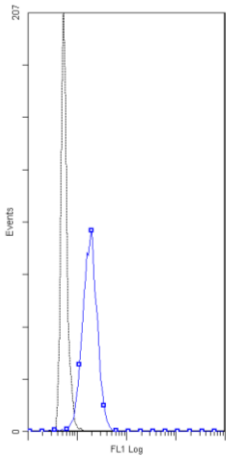
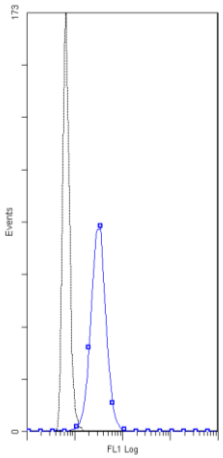


Nb CA4140



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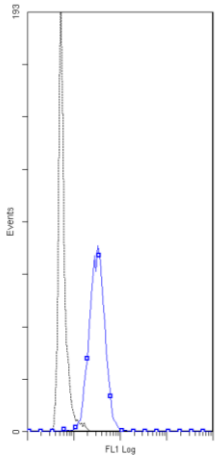

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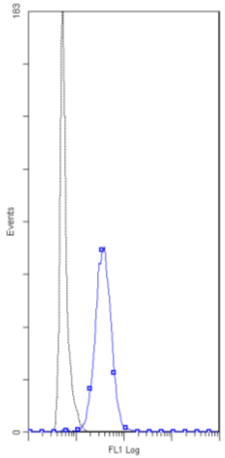

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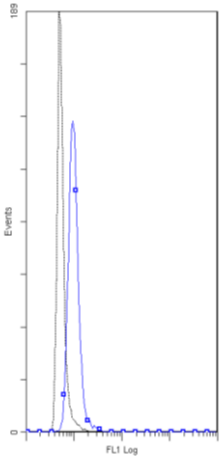

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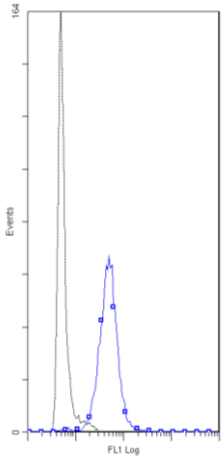

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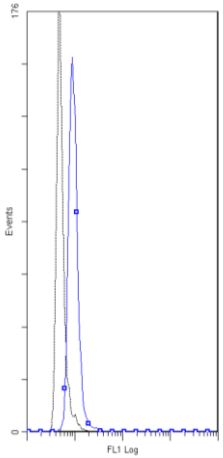

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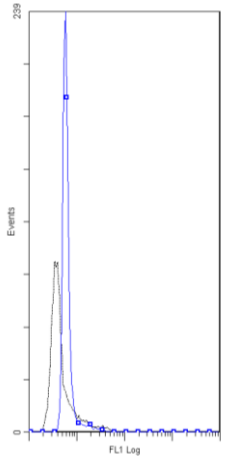
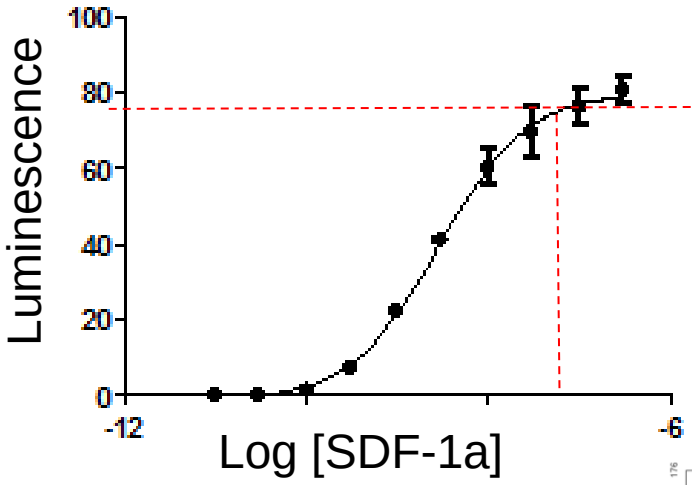
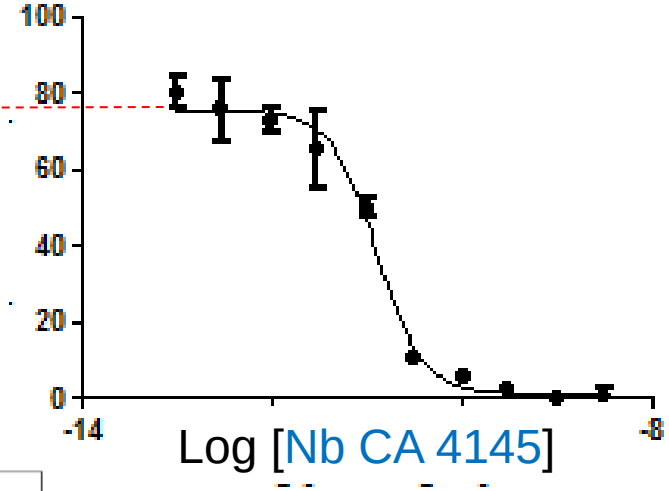
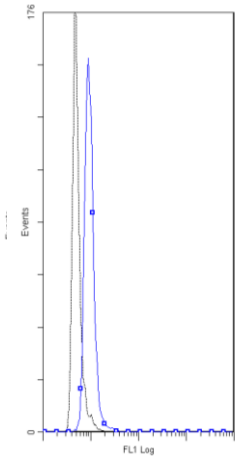


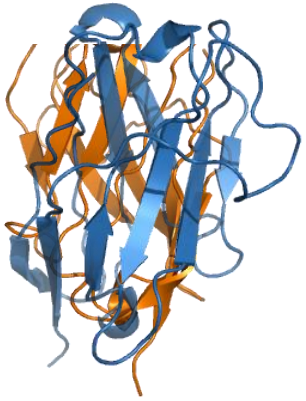
CXCR4 antagonists

Aequorin-based calcium mobilization assay (CHO-CXCR4 cells)

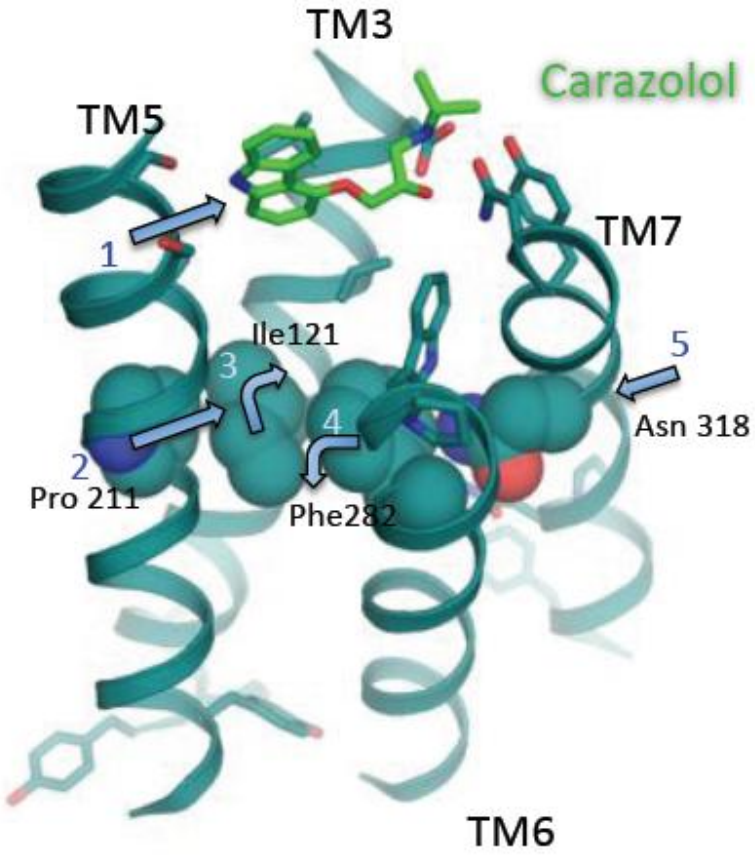




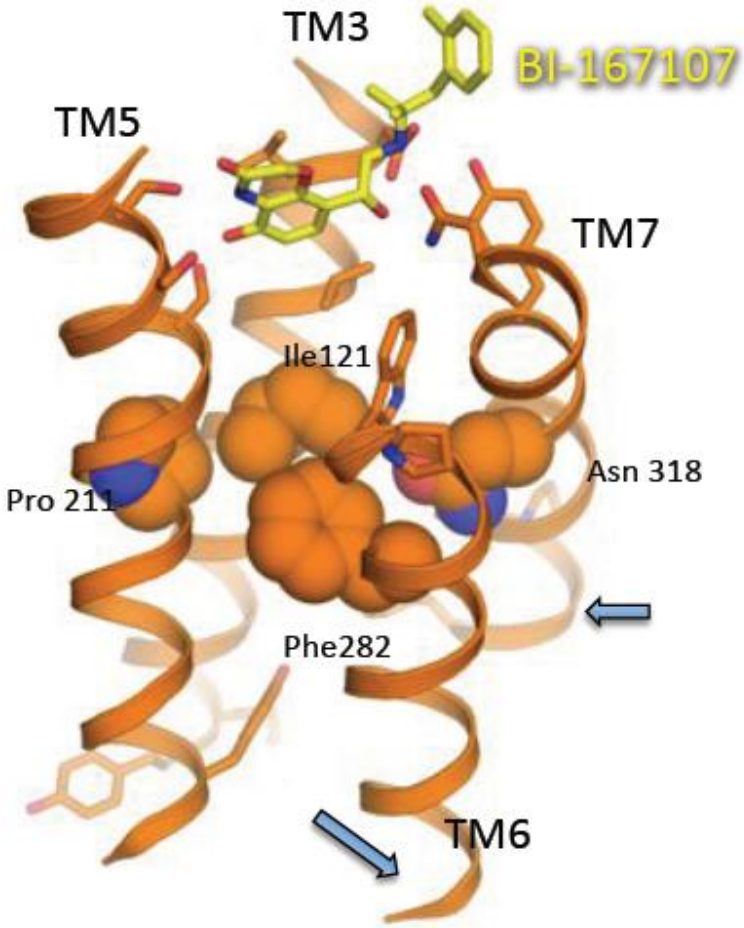




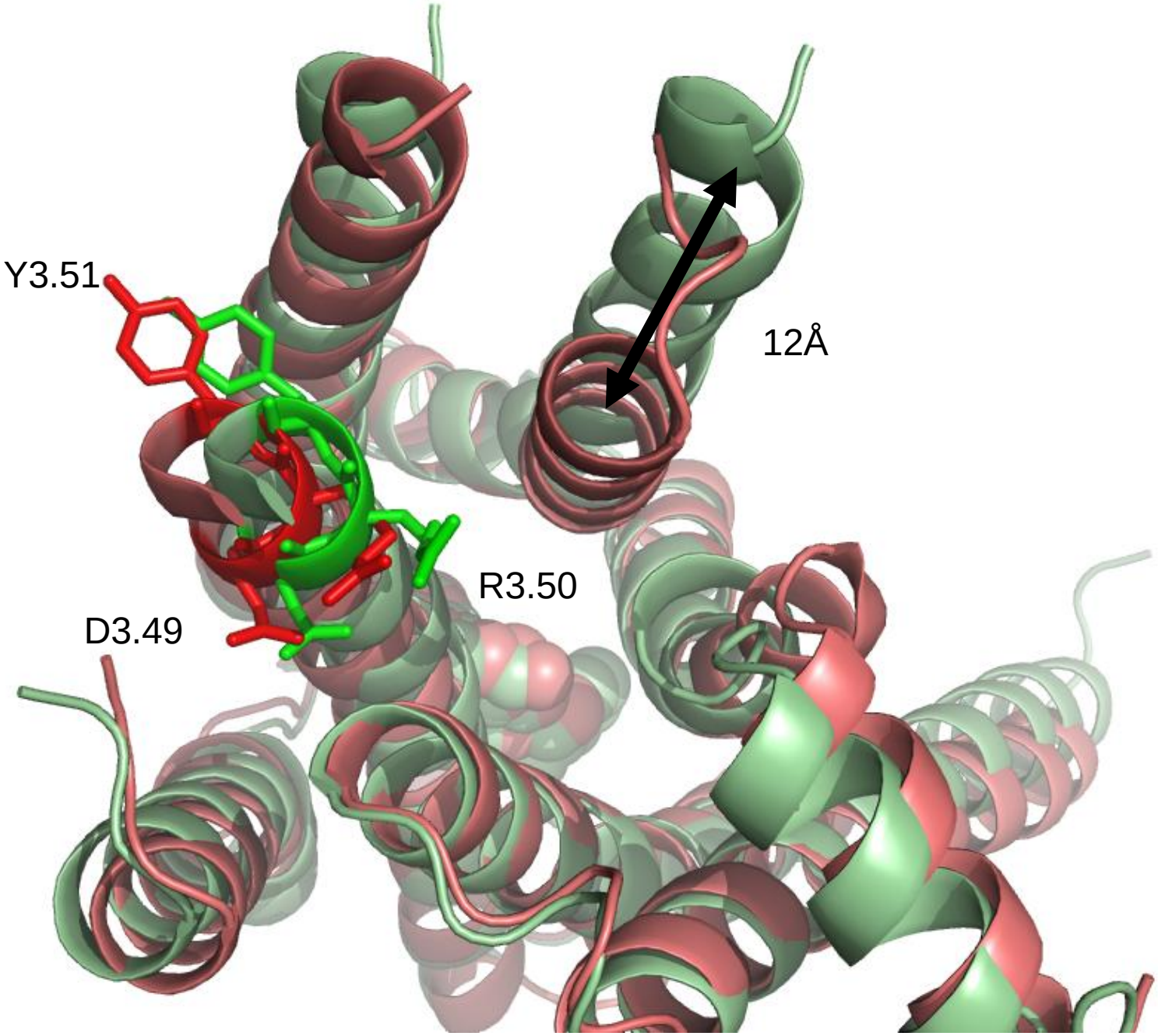
Rearrangements of transmembrane segment packing interactions



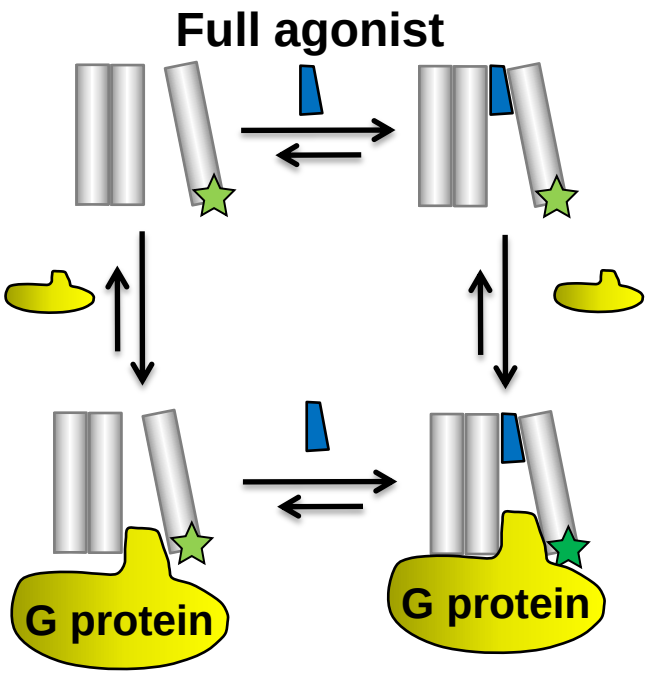
β_2AR-Cz

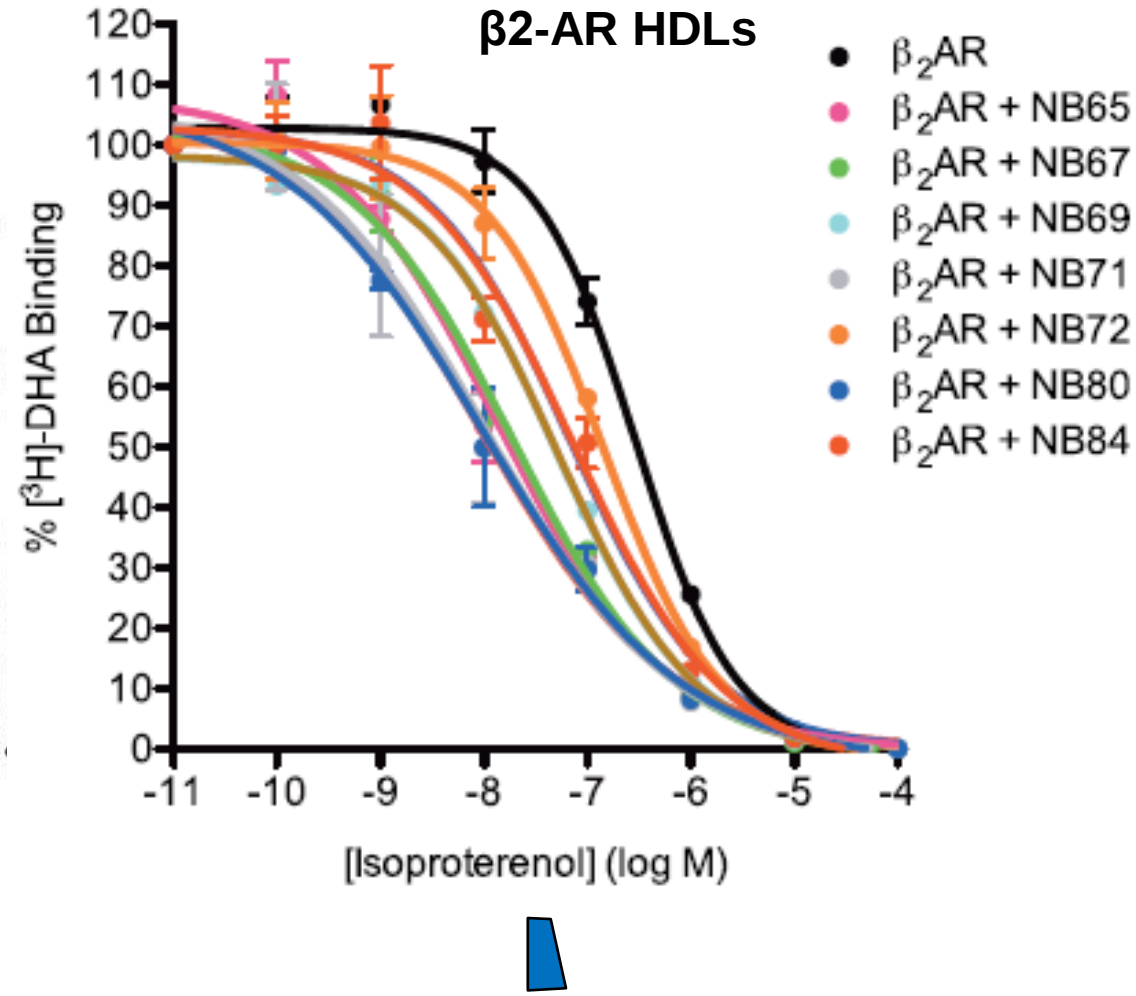


β_2AR-NB80


Y3.51
12Å
R3.50
D3.49

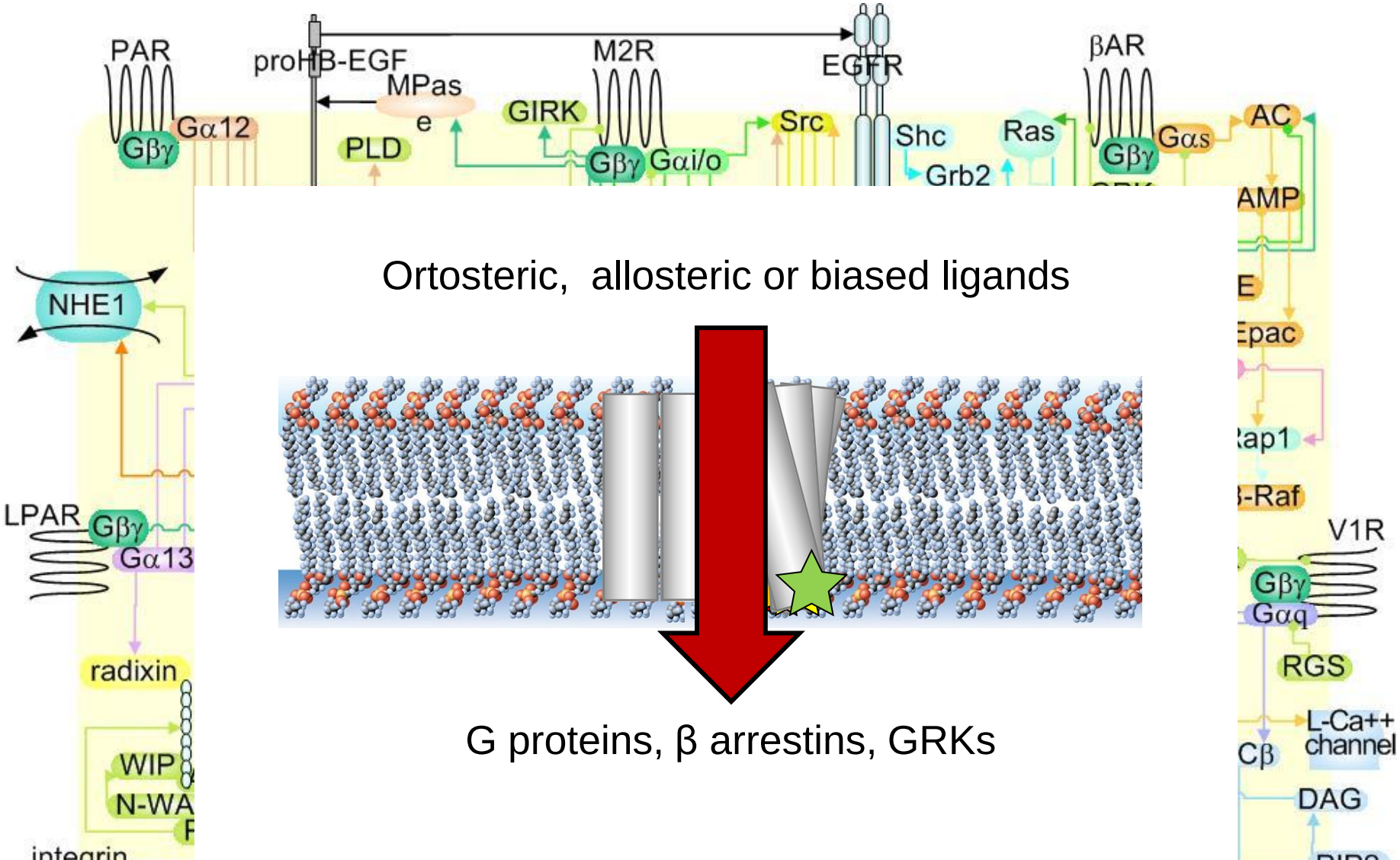
Nbs with G protein-like behaviour?



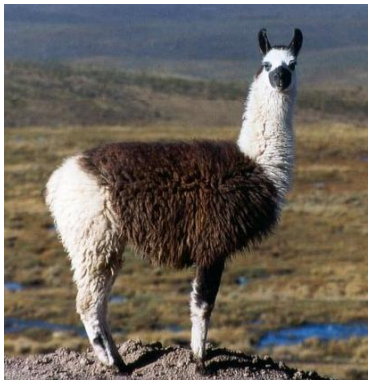


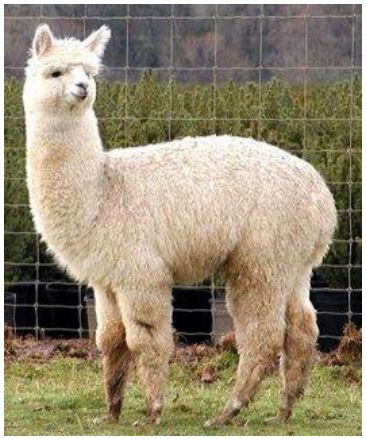
Whorton *et. al*. (2007) P*NAS* **104**, 7682.

GPCRs are allosteric switches



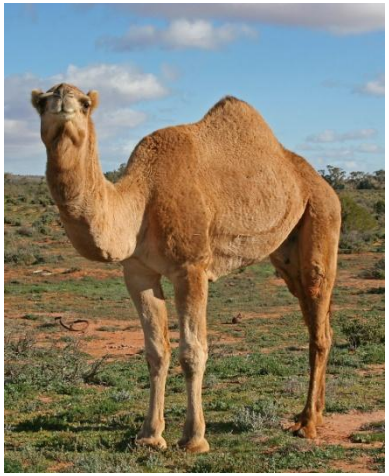
Heavy-chain antibodies





Tylopoda — Camelidae —

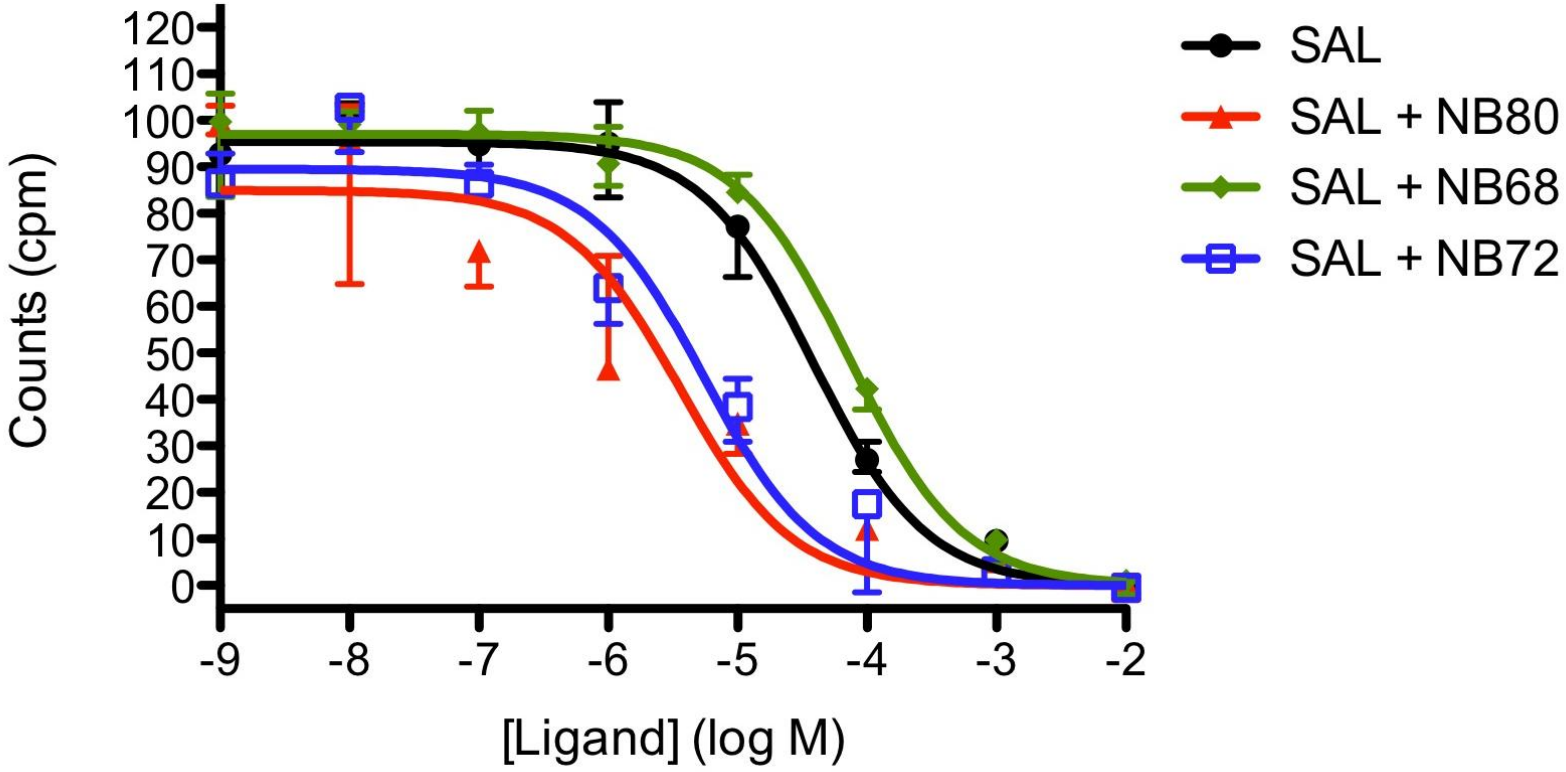
Camelus dromedarius
Camelus bactrianus
Lama glama
Lama guanoco
Lama alpaca
Lama vicugna



GPCR transmembrane signaling

Nbs with G protein-like behaviour?

Partial agonist: salbutamol



Versatility of the Nb fold

```
AMYL-B7..QVQLVESGGGSVQAGGSLRLSCAASGYTFSSYPMGWYRQAPGKECELVSRIF-SDGSANYAGS
AMYL-D9..QVQLVESGGGSVQAGGSLSLSCAASTYTDT---VGWFRQAPGKEREGVAAIYRRTGYTYSADS
AMYL-D10.DVQLVESGGGTVPAGGSLRLSCAASGNTLCTYDMSWYRRAPGKGRDFVSGID-NDGTTTYVDS
```

framework

```
AMYL-B7..VKGRFTISRDNAKNTAYLQMDSLKPEDTAVYYCAAGPGSGKLVVAGRTCYGPNYWGQGTQVTVSS
AMYL-D9..VKGRFTLSQDNNKNTVYLQMNSLKPEDTGIYYCATGNSVRLASWEGY-----FYWGQGTQVTVSS
AMYL-D10.VKGRFTISQGNAKNTAYLQMDSLKPDDTAMYYCKPSLRYGLPGCPI------IPWGQGTQVTVSS
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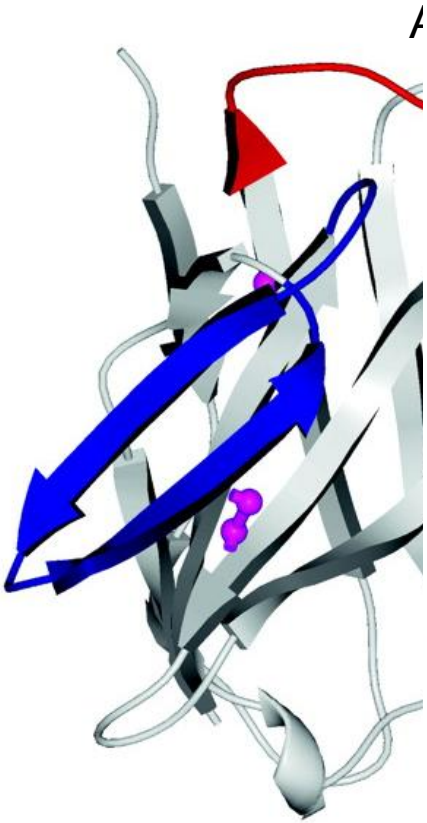

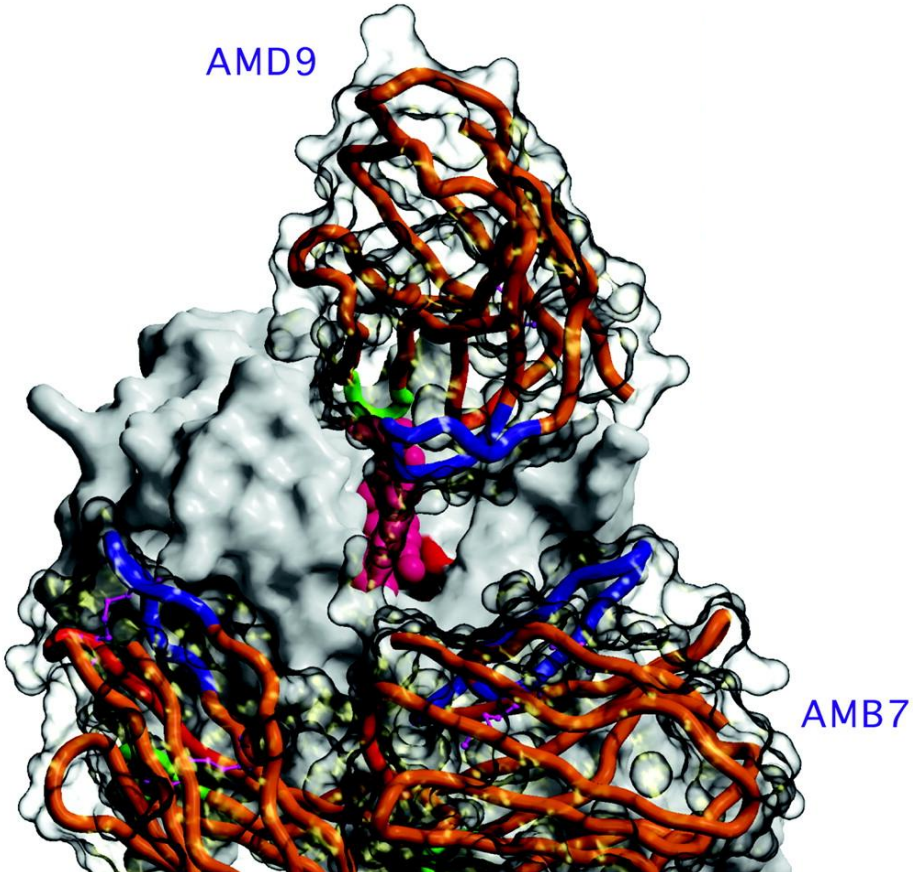

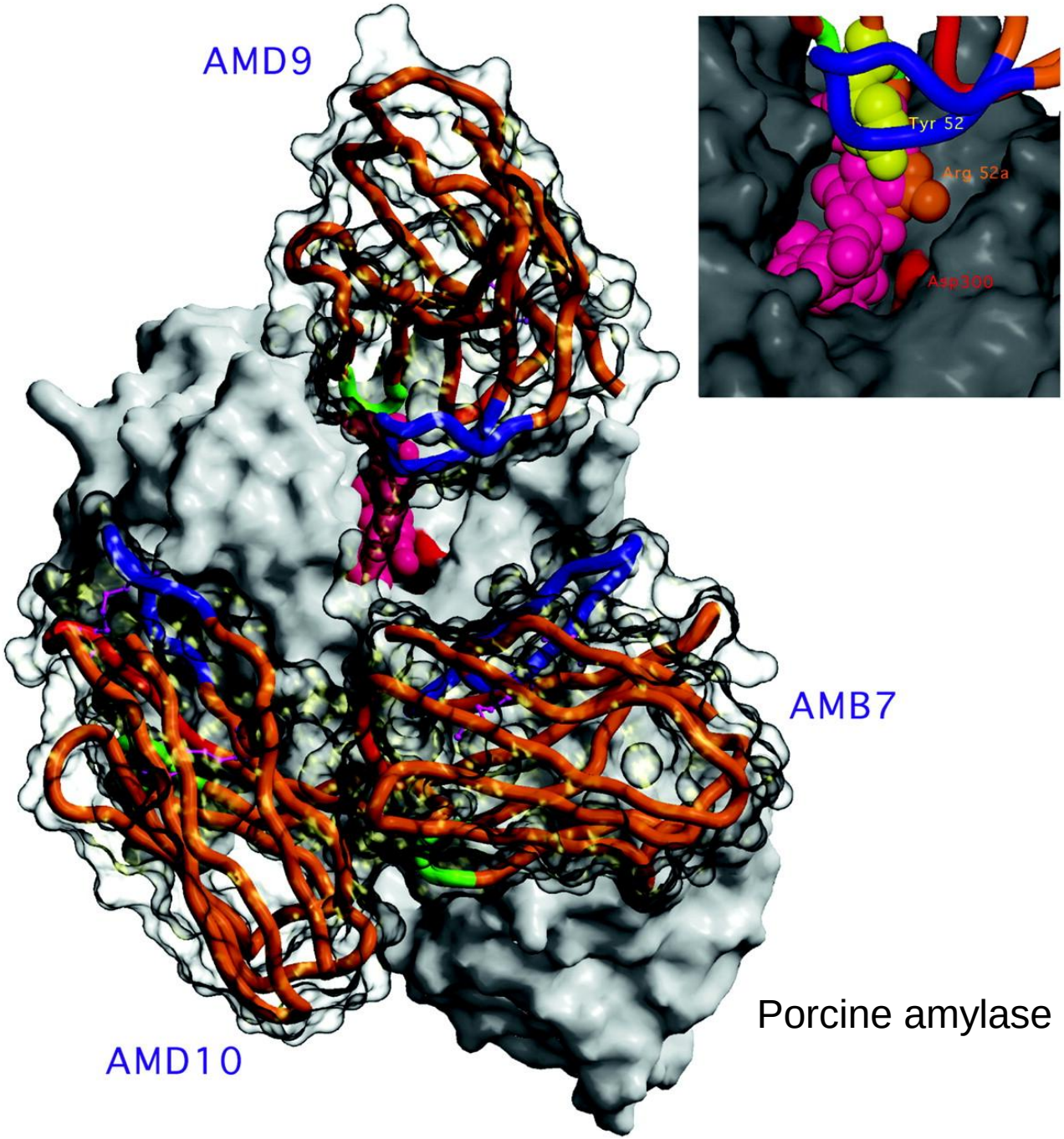

Porcine amylase