New detection systems for G protein-coupled receptor signaling

Technical Journal club

Daiji Sakata

2013.6.18

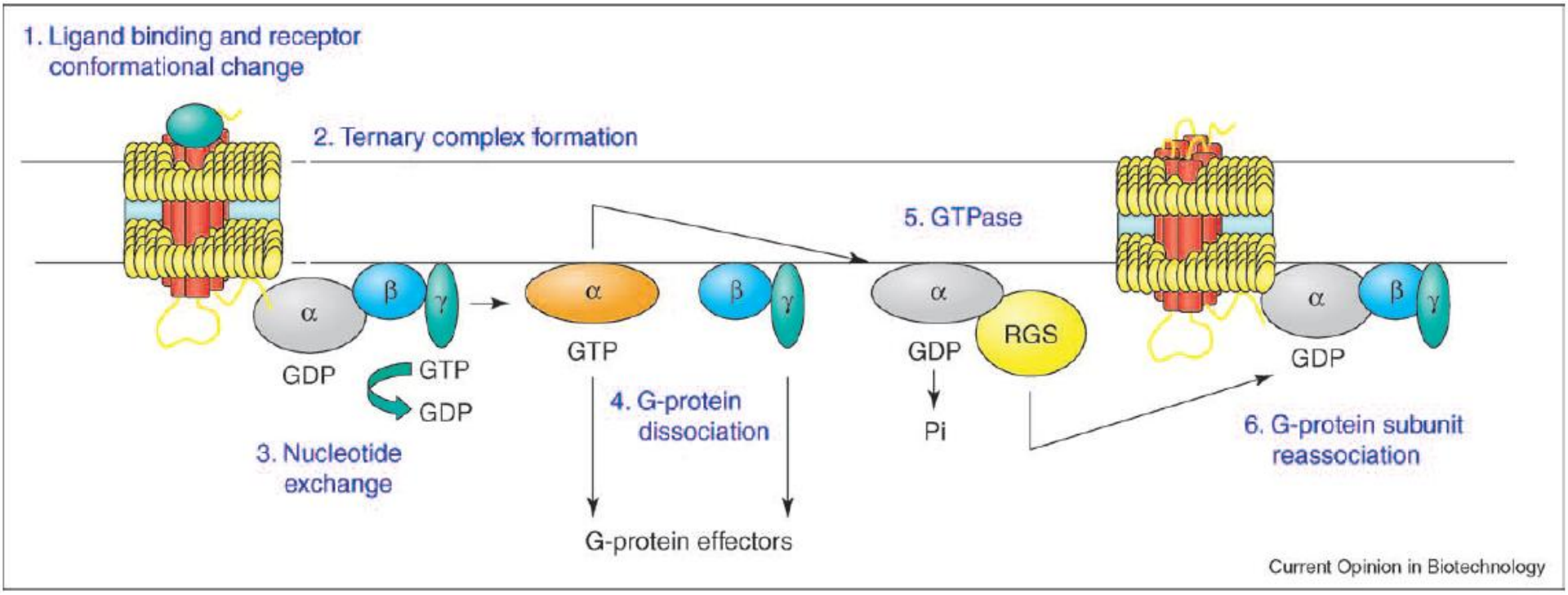
GPCR (G Protein-Coupled Receptor)

-GPCRs constitute the largest family of membrane receptors
(Particularly, seven transmembrane receptor (7TM))

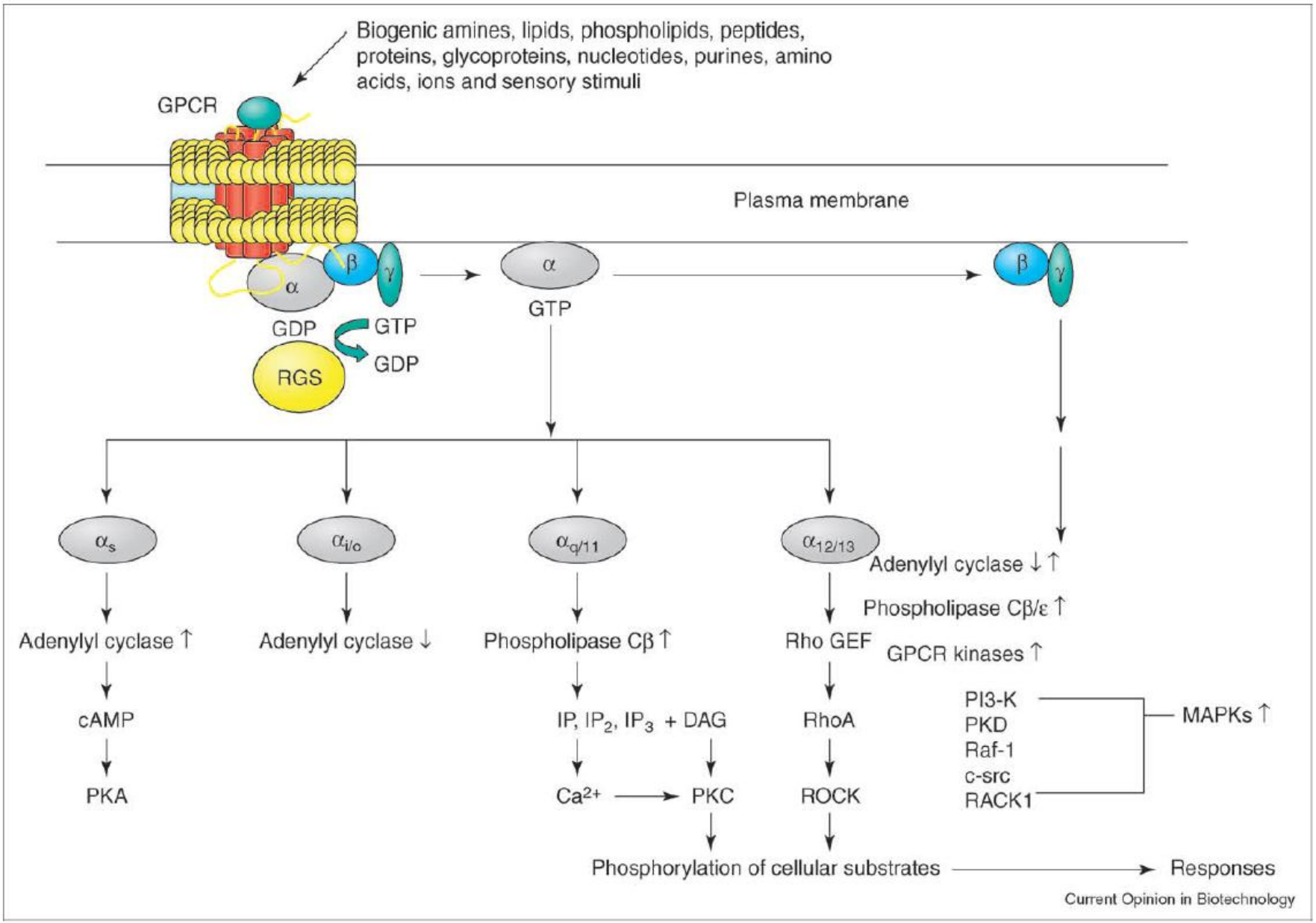
-Activate G protein on ligand binding

-Analysis of the human genome predict between 800 and 1000 (350?) GPCR genes.
Among them about 150 receptors are orphan
(their endogenous ligands and biological functions have not been uncovered yet)

GPCR signaling

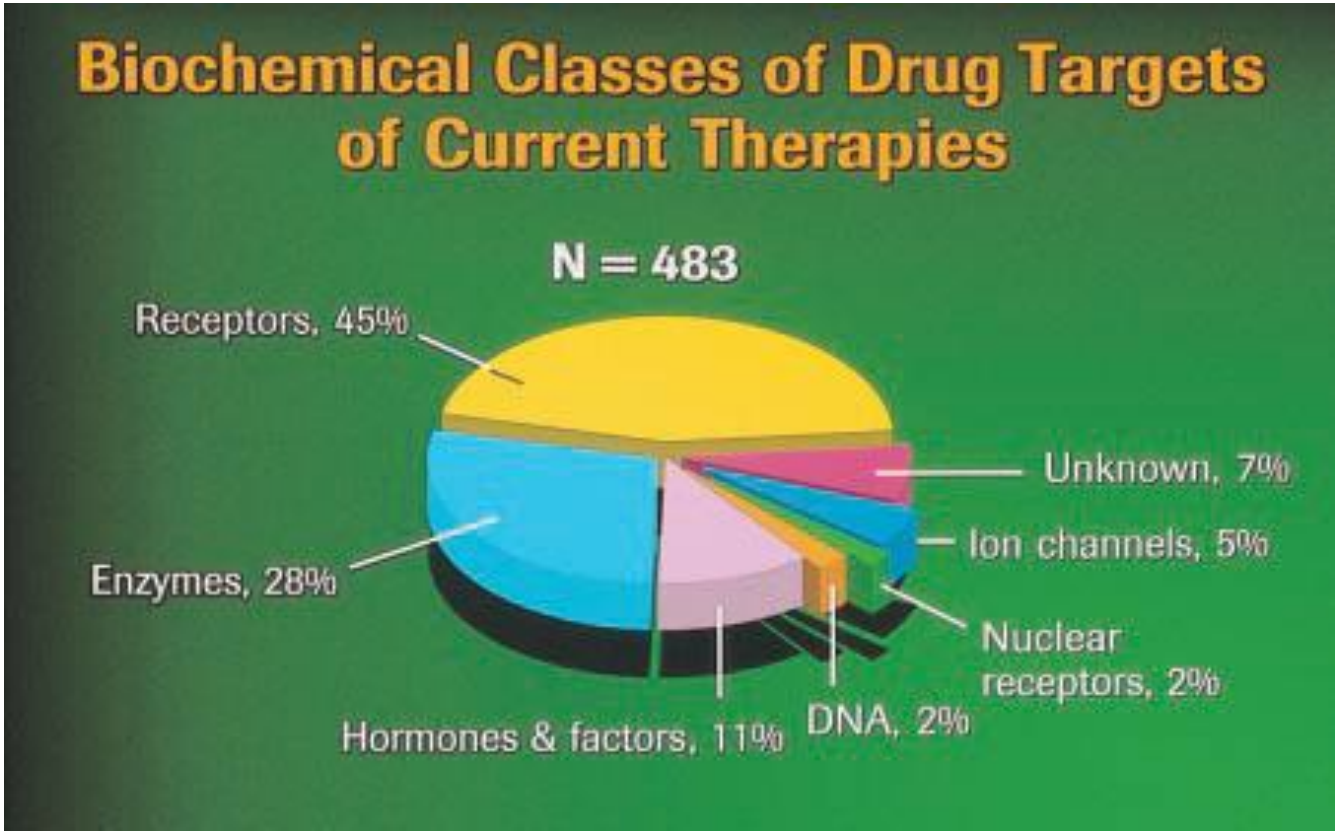


GPCR signaling



Current Opinion in Biotechnology 2005, **16**:655–665

GPCR as a drug target



SCIENCE VOL 287 17 MARCH 2000

-In 2001, 50% of all newly launched drugs targeted GPCR and annual sales of these drugs was over 30 billion doller.

-It is estimated that 25% of the 100 top-selling drugs target GPCRs.

GPCR and Nobel Prize

1994 *In Physiology or Medicine*
"for their discovery of G-proteins and the role of these proteins in signal transduction in cells"

Alfred G. Gilman

Martin Rodbell

2004 *In Physiology or Medicine*
"for their discoveries of odorant receptors and the organization of the olfactory system"

Richard Axel

Linda B. Buck

2012 *In Chemistry*
"for studies of G-protein-coupled receptors"

Robert Joseph Lefkowitz

Brian Kent Kobilka

GPCR signaling analysis (method)

Table 1

Common functional assays for screening GPCRs.

Assay (company)	Biological measurement	Kit reagents	Basis	Endpoint	Advantages	Disadvantages
[^{35}S]GTPγS binding	Membrane-based GPCR-mediated guanine nucleotide exchange	[^{35}S]GTPγS	Irreversible [^{35}S]GTPγS binding to receptor-activated G proteins	Radiometric	Proximal to receptor activation	Radioactive, non-homogenous, requires a filtration step
Eu-GTP™ binding (Perkin Elmer)	Membrane-based GPCR-mediated guanine nucleotide exchange	Europium-GTP	Binding of europium-labeled GTP to receptor-activated G proteins	Time-resolved fluorescence	Proximal to receptor activation, nonradioactive	Non-homogenous, requires a filtration step
SPA™ (GE Healthcare)	Cell- or membrane-based, cAMP accumulation	Assay buffer, SPA™ beads conjugated with a cAMP MAb, [^{125}I]camp	ELISA based-competition of cAMP with [^{125}I]cAMP for binding to MAb conjugated to SPA™ beads, loss of signal due to reduced proximity of [^{125}I]cAMP and the SPA™ bead	Radiometric	Sensitive, homogenous, amenable to automation	Radioactive, relatively expensive
FlashPlate™ (Perkin Elmer)	Cell- or membrane-based, cAMP accumulation	Buffer, FlashPlate™ with cAMP MAb attached, [^{125}I]camp	ELISA based-competition of cAMP with [^{125}I]cAMP for binding to cAMP MAb conjugated to scintillant-coated wells, loss of signal due to reduced proximity of [^{125}I]cAMP and MAb in wells	Radiometric	Homogenous, amenable to automation	Radioactive, relatively expensive

Current Opinion in Biotechnology 2005, **16**:655–665

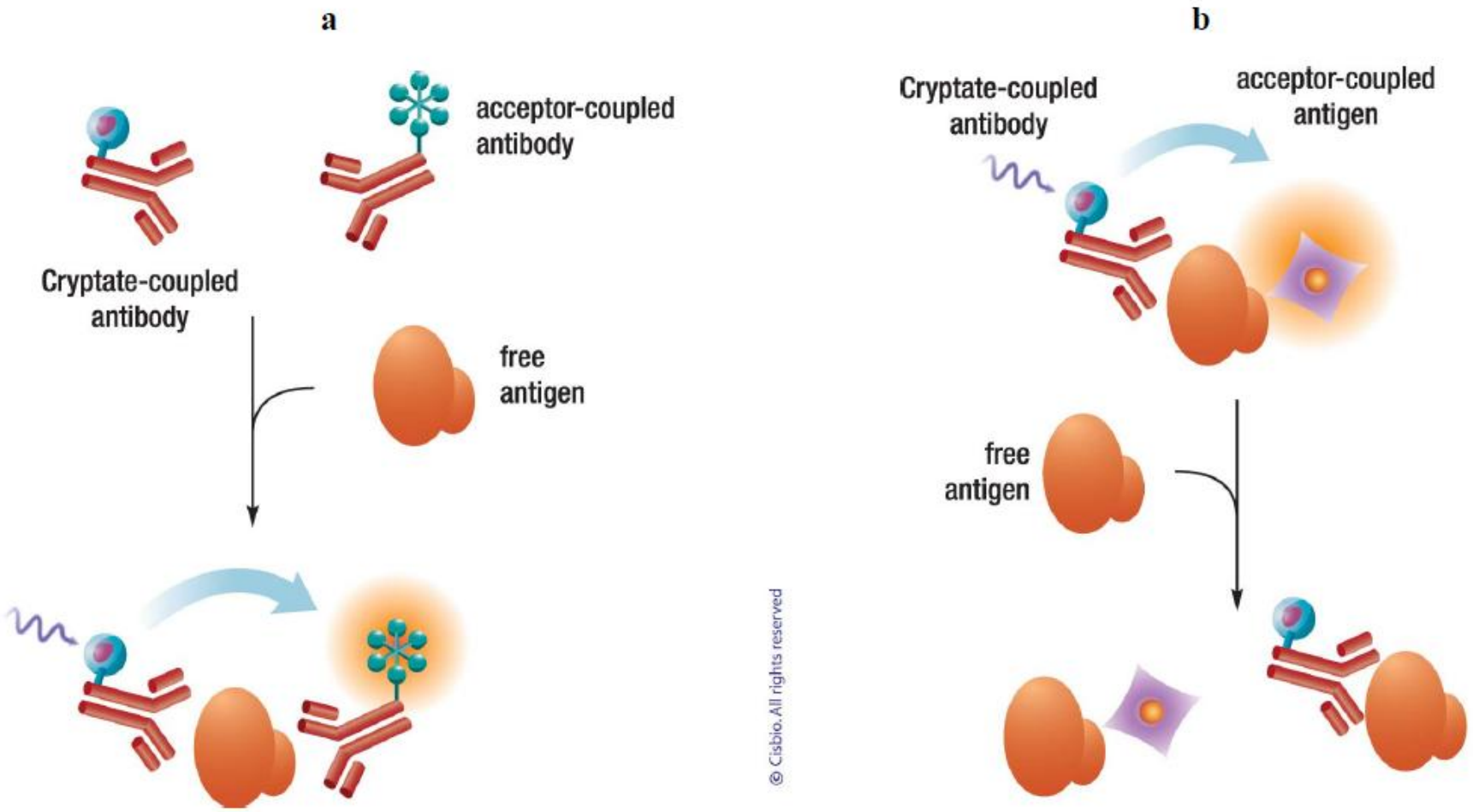
AlphaScreen™ (Perkin Elmer)	Cell-based cAMP accumulation	cAMP MAb conjugated acceptor bead, streptavidin-coated donor beads with chemi-luminescence compound, biotinyl-cAMP	cAMP competes with biotinyl-cAMP binding to high-affinity streptavidin-coated donor beads, loss of signal due to reduced proximity of acceptor-donor bead	Luminescence	High sensitivity, homogenous, amenable to automation, cost effective, broad linear range of detection	Temperature- and light-sensitive, color quenching, special endpoint detector required
Fluorescence polarization (Perkin Elmer, Molecular Devices, GE Healthcare)	Cell- or membrane-based cAMP accumulation	cAMP MAb, fluorescent-labled camp	cAMP competes with Fluor-cAMP binding to cAMP MAb, loss of signal due to decrease in rotation and polarization	Fluorescence polarization	Homogenous, amenable to miniaturization and automation	Lower signal-to-noise (may be improved with red-shifted dyes)
HTRF cAMP (Cisbio)	Cell-based, cAMP accumulation	cAMP MAb conjugated with eurocryptate, acceptor molecule labeled camp	cAMP competes with acceptor-labeled cAMP binding to europium-conjugated cAMP MAb, loss of signal due to reduced europium-acceptor molecule proximity	Time-resolved fluorescence	Broad linear range, high signal-to-noise, homogenous, amenable to automation	
HitHunter™ (DiscoveRx)	Cell-based, cAMP accumulation	cAMP MAb, ED-cAMP conjugated peptide, acceptor protein, lysis buffer	cAMP competes with ED-cAMP for complementation of β-Gal activity with binding of acceptor peptide, loss of signal as enzyme complementation is reduced	Fluorescence or luminescence	Low compound interference, high sensitivity, homogenous, amenable to automation	Relatively expensive
IP Accumulation	Cell-based IP accumulation	None	Filtration to separate [^{3}H]inositol and [^{3}H]IPs	Radiometric	Sensitive, can be used for constitutively active G_q-coupled GPCRs	Low throughput, some automation possible

Table 1 Continued

Assay (company)	Biological measurement	Kit reagents	Basis	Endpoint	Advantages	Disadvantages
IP_1™ (Cisbio)	Cell-based IP_1 accumulation	Europium-conjugated IP_1 MAb, acceptor-labeled IP_1	Loss of signal as IP_1 competes for binding of acceptor-labeled IP_1 binding to europium-MAb	Time-resolved fluorescence	Sensitive, homogenous, amenable to automation, can be used for constitutively active G_q-coupled GPCRs	Limited industrial validation
FLIPR™ (Molecular Devices)	Cell-based, increases in intracellular calcium	Calcium sensitive dye; Calcium-3	Increased fluorescence as intracellular dye binds calcium	Fluorescence	Sensitive, homogenous, amenable to automation	Cannot be used for inverse agonist screens, fluorescence quenching
AequoScreen™ (EuroScreen)	Cell-based, increases in intracellular calcium	Cells lines expressing select GPCRs along with promiscuous or chimeric G proteins and a mitochondrially targeted version of apoaequorin	Calcium-sensitive aequorin generates a luminescent signal when a coelenterazine derivative is added	Luminescence	Sensitive, homogenous, amenable to automation	Cannot be used for inverse agonist screens
Reporter gene	Cell-based, increases in reporter gene expression due to increases in second messengers	Several promotor plasmids and reporters are commercially available	GPCR changes in secondary messengers alter expression of a selected reporter gene	Fluorescence, luminescence, absorbance	Cost effective, sensitive, homogenous, amplification of signal	Long incubations and high false-positive hit rate, distal to receptor activation
Melanophore (Arena Pharmaceuticals)	Cell-based, changes in pigment dispersion	None	Melanosomes aggregate with inhibition of PKA, disperse with activation of PKA or PKC	Absorbance	Sensitive, homogenous, no cell lysis, amenable to automation	Time-consuming to produce stable cell lines expressing GPCRs

Abbreviations: β-Gal, β-galactosidase; ED-cAMP, enzyme fragment donor–cAMP conjugate; Eu-GTP, europium-labeled GTP; IP, inositol phosphate; MAb, monoclonal antibody; PKA, protein kinase A; PKC, protein kinase C; SPA, scintillation proximity assay; TRF, time-resolved fluorescence.

FRET based HTRF (Homogeneous time resolved fluorescence)



TGFα shedding assay: an accurate and versatile method for detecting GPCR activation

Asuka Inoue[1], Jun Ishiguro[1], Hajime Kitamura[1], Naoaki Arima[1], Michiyo Okutani[1], Akira Shuto[1], Shigeki Higashiyama[2,3], Tomohiko Ohwada[4], Hiroyuki Arai[5,6], Kumiko Makide[1,7] & Junken Aoki[1,6]

NATURE METHODS | VOL.9 NO.10 | OCTOBER 2012 | 1021

What is the shedding assay?

-In which GPCR activation is measured as ectodomain shedding of a membrane-bound proform of alkaline phosphatase–tagged TGFα (AP-TGFα) and its release into conditioned medium.

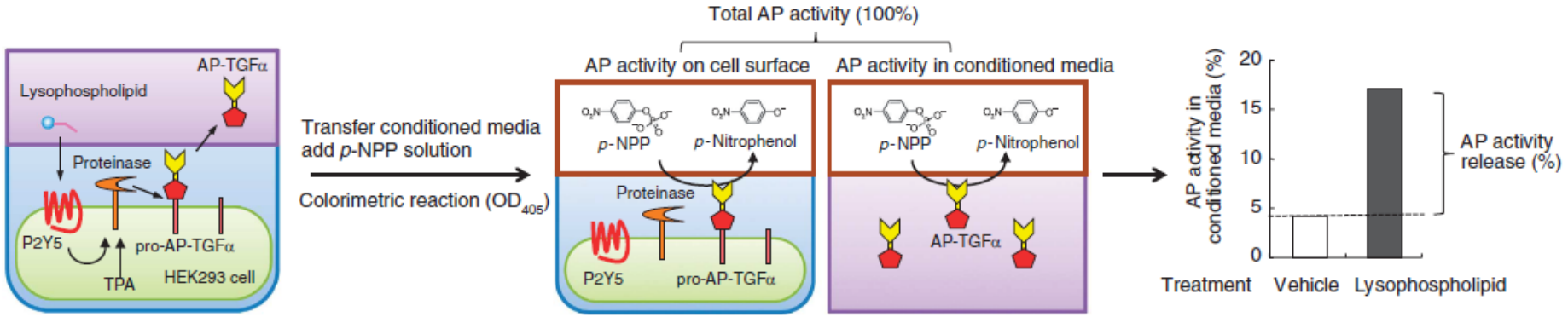
-AP-TGFα shedding response occurred almost exclusively downstream of $G\alpha_{12/13}$ and $G\alpha_q$ signaling

-Relying on chimeric Gα proteins and promiscuous $G\alpha_{16}$ protein, which can couple with $G\alpha_s$- and $G\alpha_i$-coupled GPCRs and induce $G\alpha_q$ signaling, it is possible to detect $G\alpha_s$- and $G\alpha_i$-coupling signaling

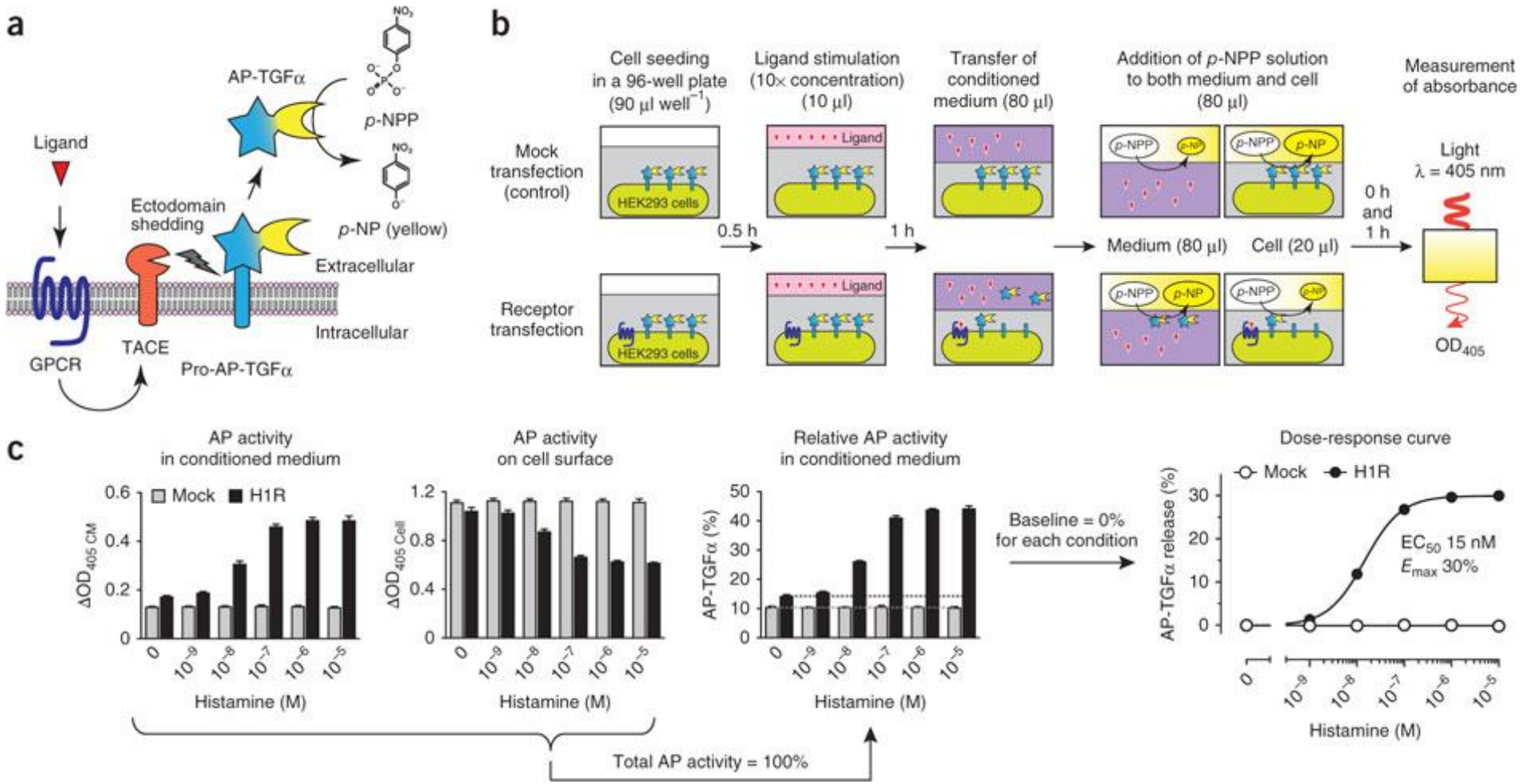
LPA-producing enzyme PA-PLA$_1$α regulates hair follicle development by modulating EGFR signalling

Asuka Inoue[1,2,*], Naoaki Arima[1], Jun Ishiguro[1], Glenn D Prestwich[3], Hiroyuki Arai[2,4] and Junken Aoki[1,5,*]

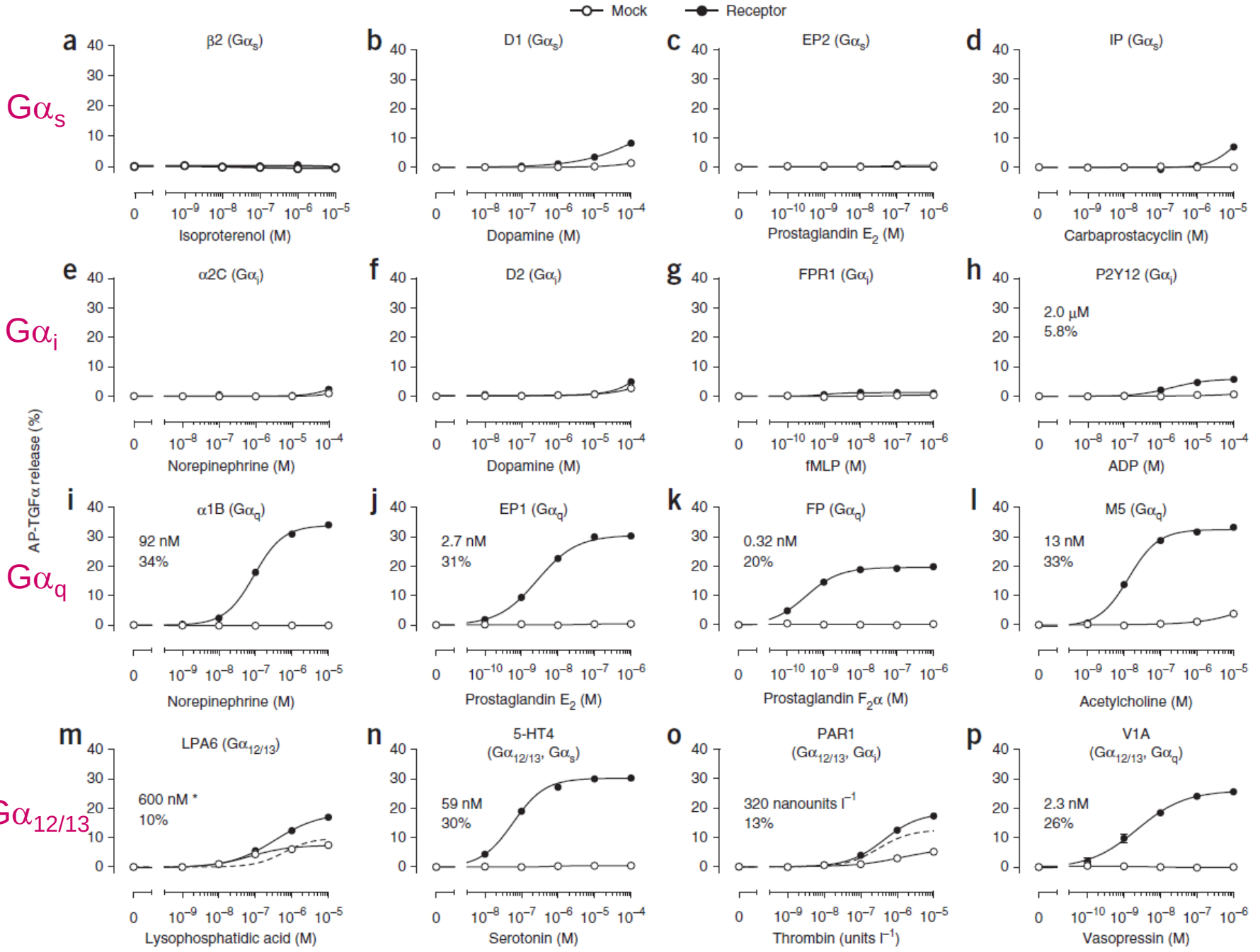
The EMBO Journal (2011) 30, 4248–4260



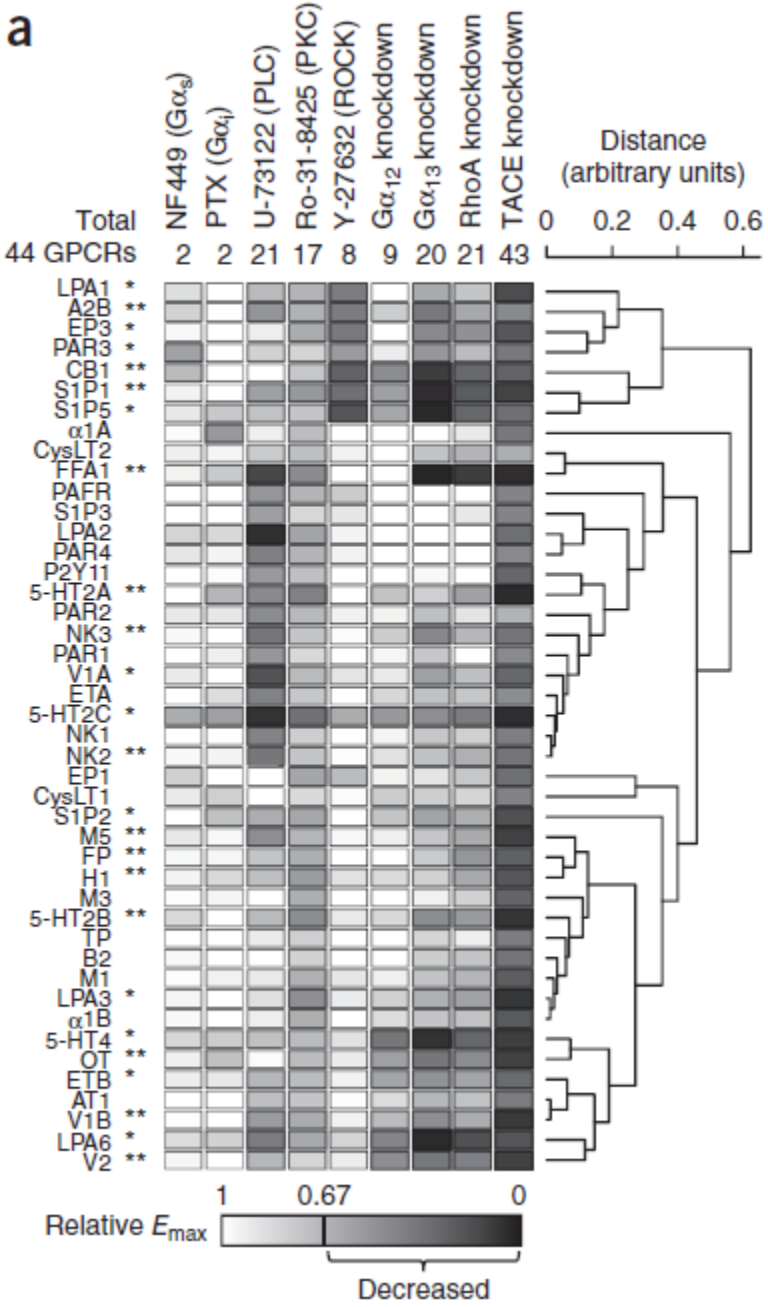
The principle and data processing of the TGFα shedding assay

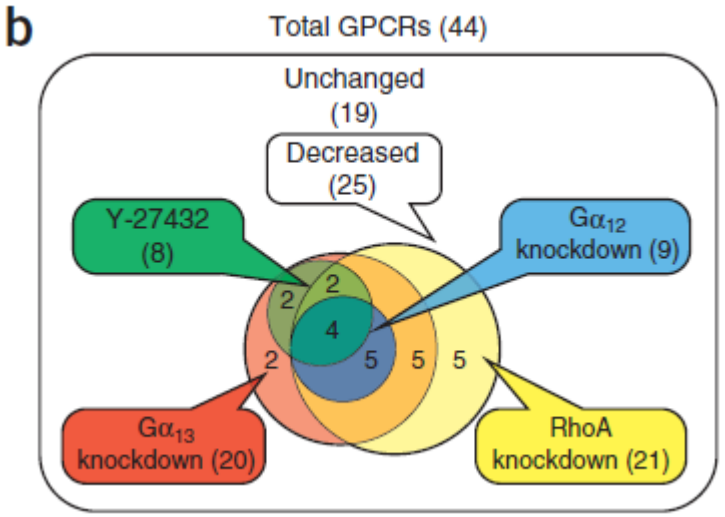


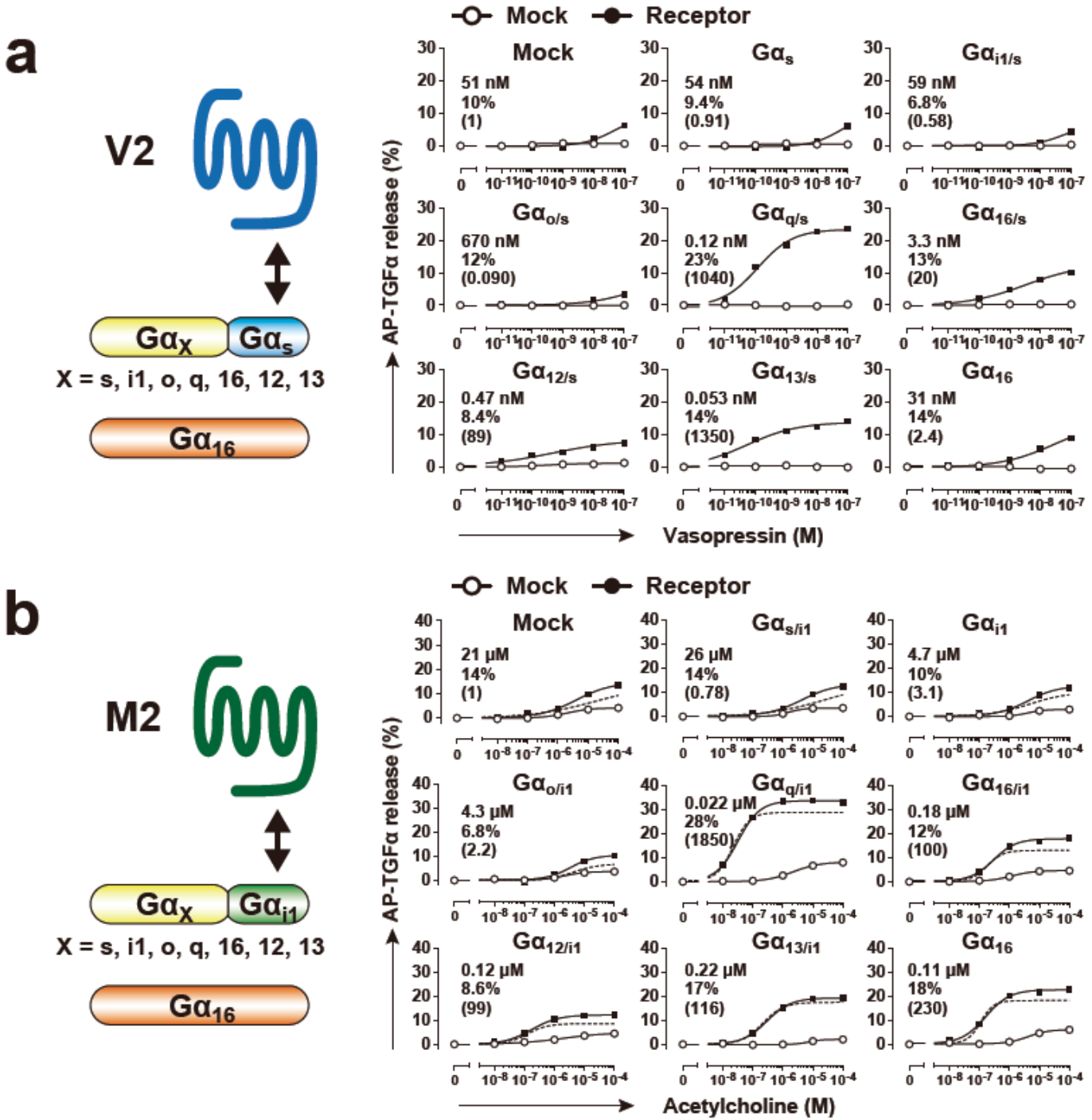
$G\alpha_q$-coupled and $G\alpha_{12}/_{13}$-coupled GPCRs efficiently induce AP-TGFα release



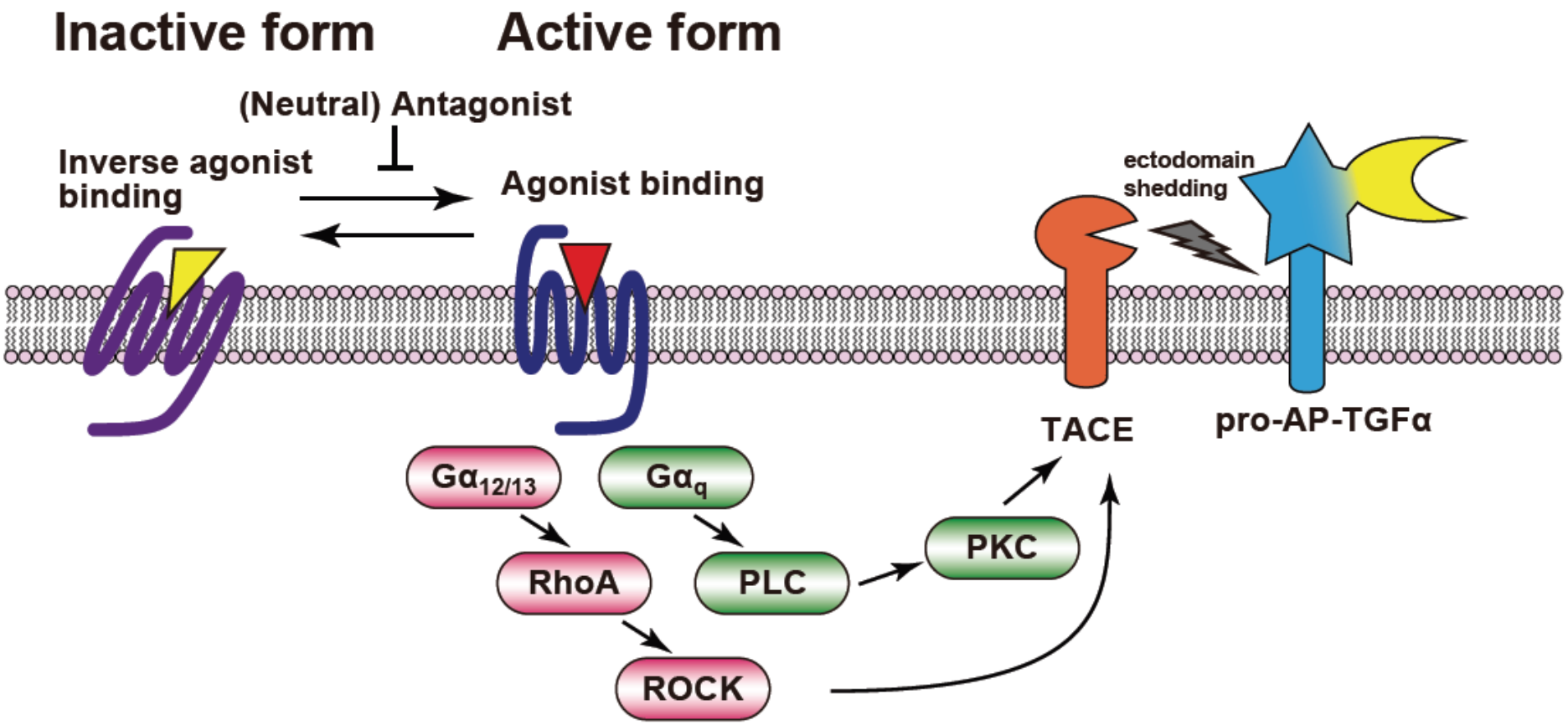
GPCR-induced AP-TGFα release is mainly dependent on Gαq and $G\alpha_{12/13}$ signaling



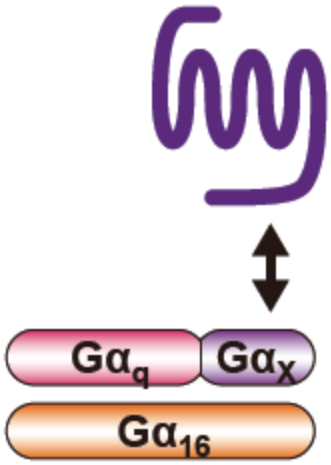



a
V2
Gαx
Gαs
X = s, i1, o, q, 16, 12, 13
Gα16
Mock
Receptor
Mock
51 nM
10%
(1)
Gαs
54 nM
9.4%
(0.91)
Gαi1/s
59 nM
6.8%
(0.58)
Gαo/s
670 nM
12%
(0.090)
Gαq/s
0.12 nM
23%
(1040)
Gα16/s
3.3 nM
13%
(20)
Gα12/s
0.47 nM
8.4%
(89)
Gα13/s
0.053 nM
14%
(1350)
Gα16
31 nM
14%
(2.4)
AP-TGFα release (%)
Vasopressin (M)
b
M2
Gαx
Gαi1
X = s, i1, o, q, 16, 12, 13
Gα16
Mock
Receptor
Mock
21 μM
14%
(1)
Gαs/i1
26 μM
14%
(0.78)
Gαi1
4.7 μM
10%
(3.1)
Gαo/i1
4.3 μM
6.8%
(2.2)
Gαq/i1
0.022 μM
28%
(1850)
Gα16/i1
0.18 μM
12%
(100)
Gα12/i1
0.12 μM
8.6%
(99)
Gα13/i1
0.22 μM
17%
(116)
Gα16
0.11 μM
18%
(230)
AP-TGFα release (%)
Acetylcholine (M)

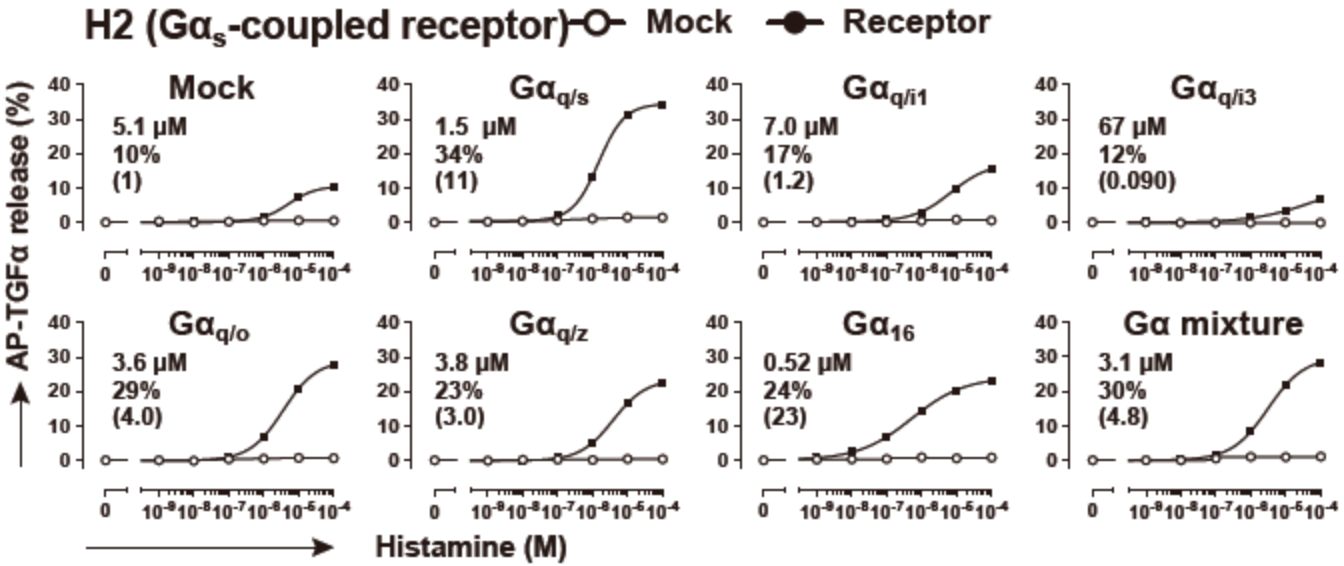
Mechanistic scheme of the TGFα shedding assay

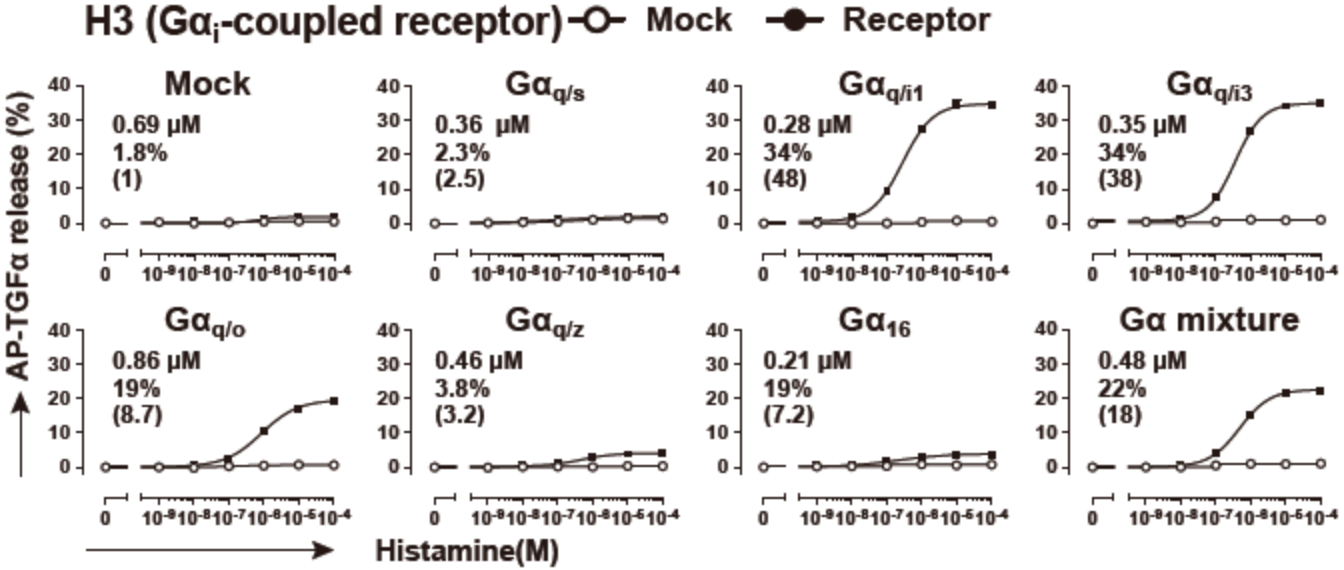


c

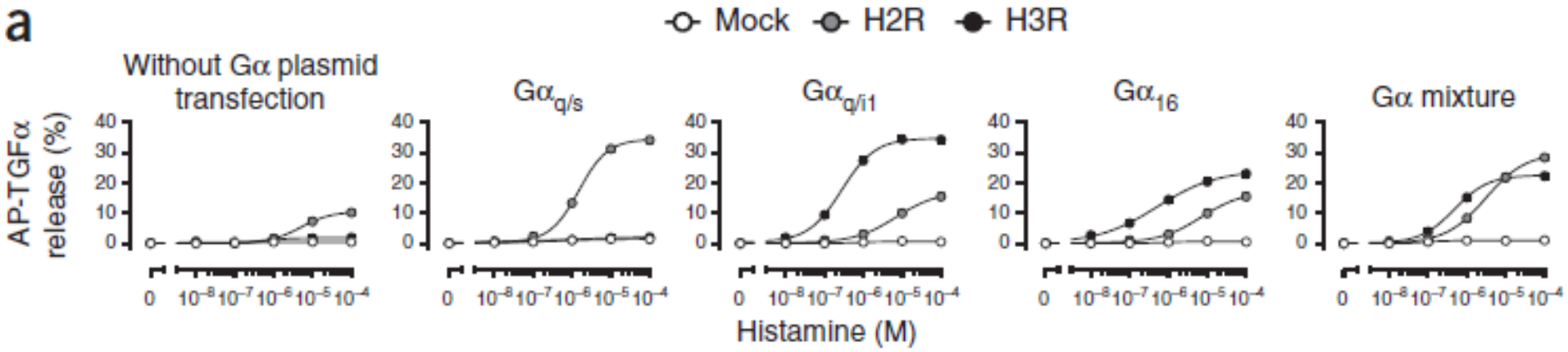


Sub-family	mem-bers	C-terminal sequence
$G\alpha_s$	s	LRQYELL
$G\alpha_i$	i1, i2	LKDCGLF
	i3	LKECGLY
	o	LRGCGLY
	z	LKYIGLC
$G\alpha_q$	q, 11	LKEYNLV
	14	LREFNLV
	16	LDEINLL
$G\alpha_{12/13}$	12	LKDIMLQ
	13	LKQLMLQ
		-7 -1

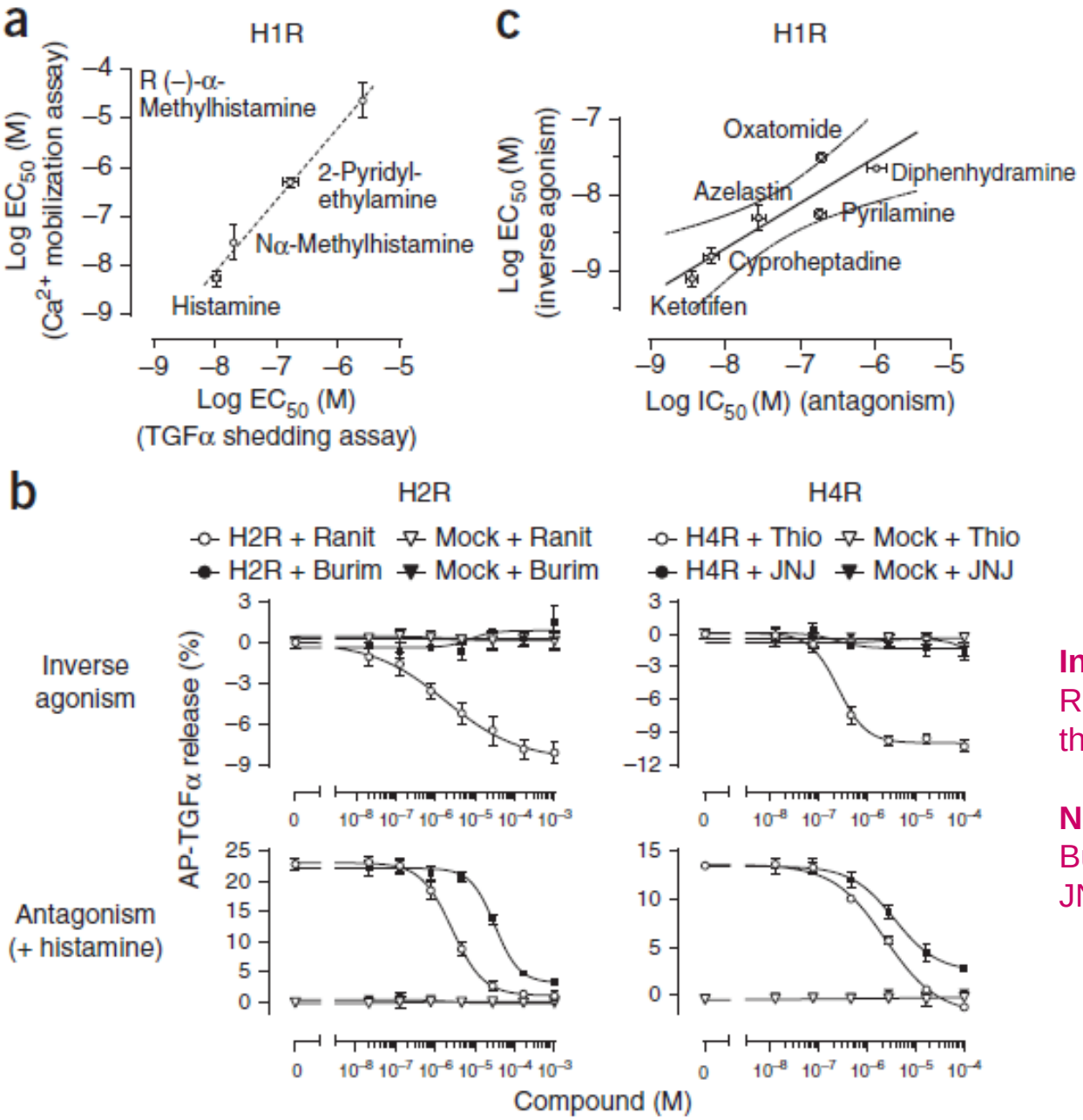




Extended TGFα shedding assay using chimeric Gα proteins and promiscuous Gα16 protein



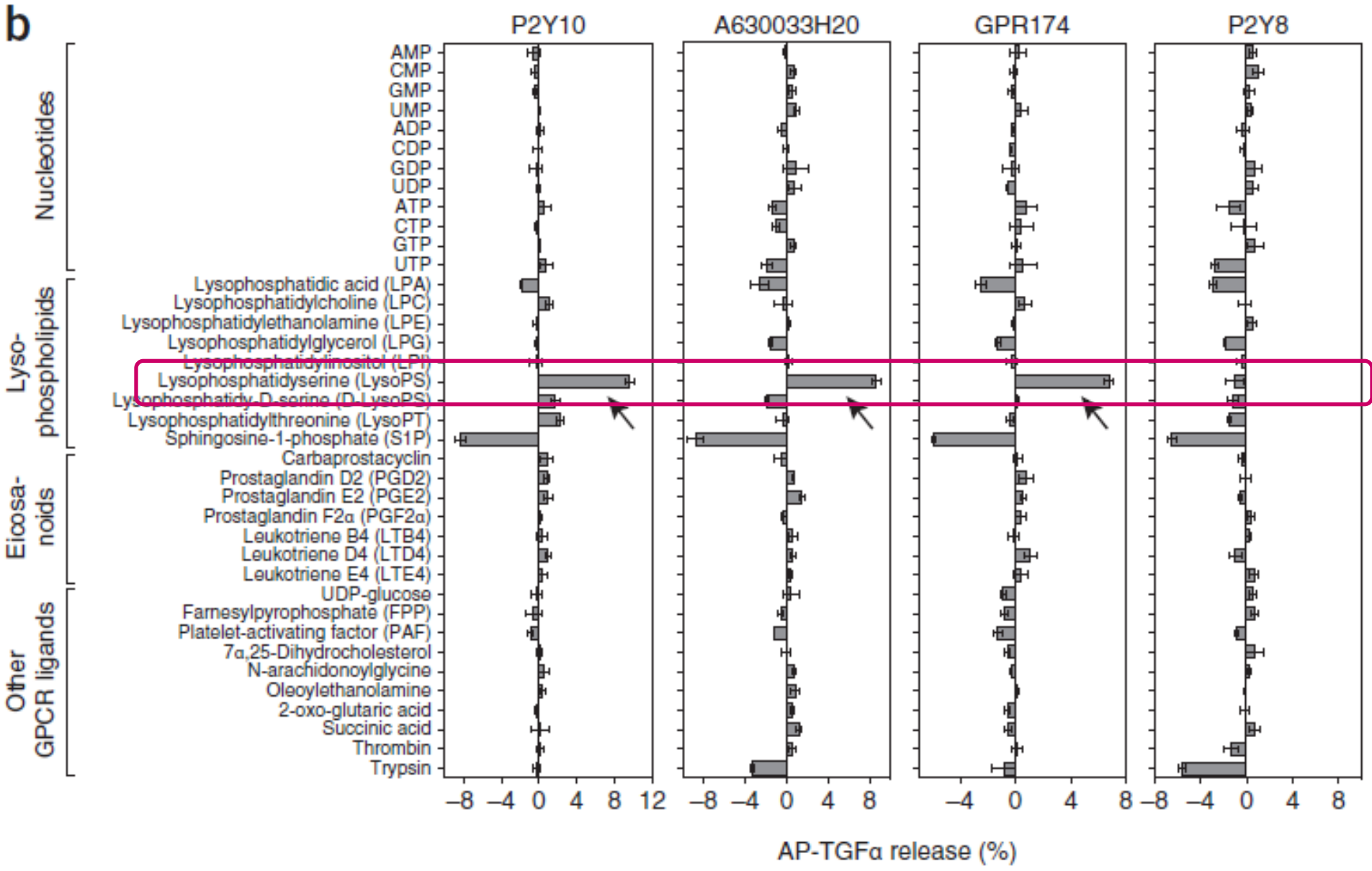
Pharmacological evaluation of GPCR ligands in the TGFα shedding assay



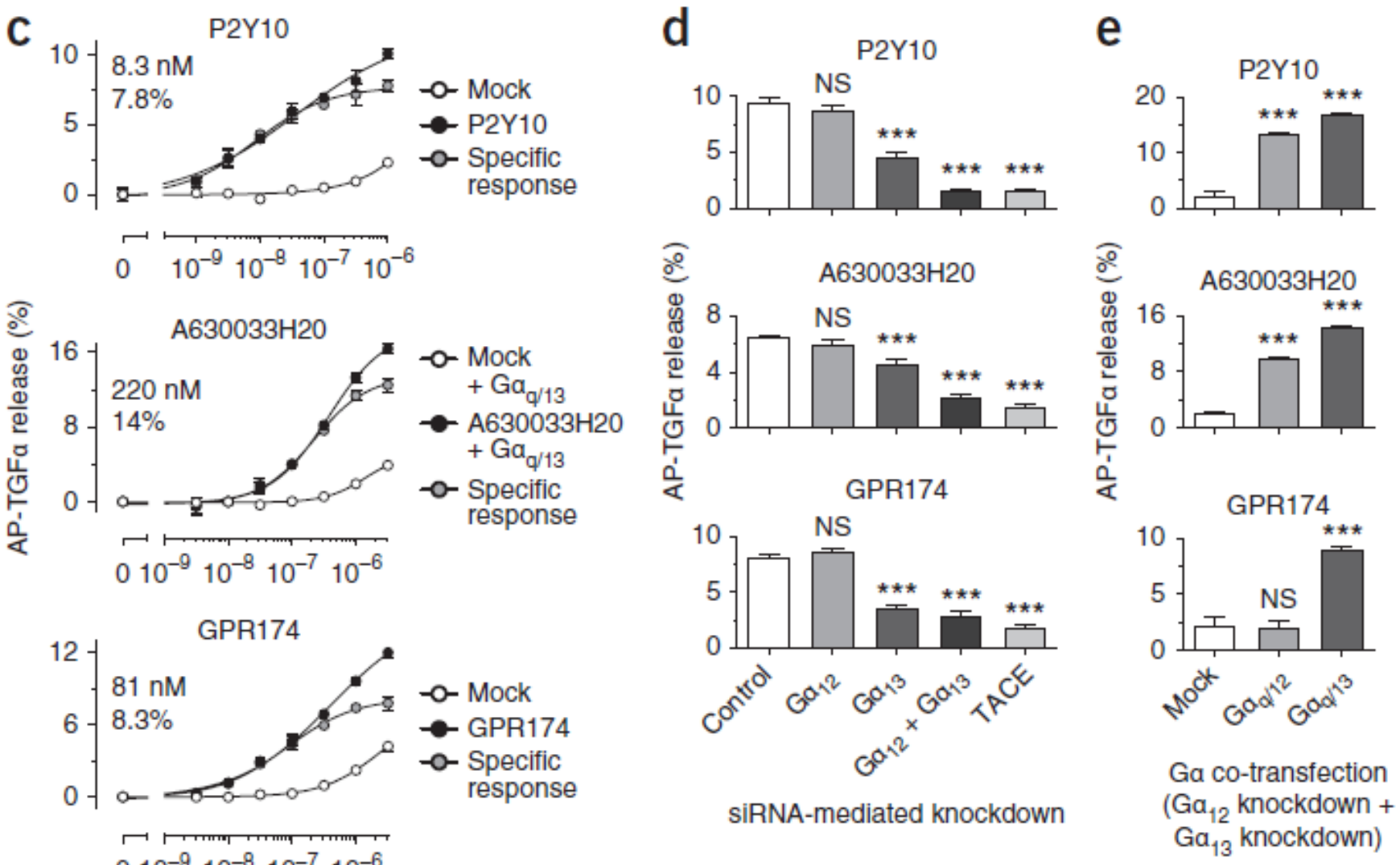
Inverse agonists
Ranitidine (Ranit) for H2R
thioperamide (thio) for H4R

Neutral antagonists
Burimamide (Burim) for H2R
JNJ 10191584 (JNJ) for H4R

P2Y10, A630033H20 and GPR174 identified as $G\alpha_{12}/_{13}$-coupled LysoPS-specific receptors



P2Y10, A630033H20 and GPR174 identified as $G\alpha_{12}/_{13}$-coupled LysoPS-specific receptors



Sammury of TGFα Shedding assay

Merit (advantage)	Demerit (disadvantage)
Can detect almost all GPCR receptor signaling	Not direct measurement of G protein activation
Not required special equipment (OD measurement)	Signal-noise ratio is not very good
High accuracy	Not homogeneous assay
Low cost	Incubation (stimulation) time is relatively long
Can detect $G_{12}/_{13}$ signaling	
Can evaluate inverse agonist activity	

Deconvolution of complex G protein–coupled receptor signaling in live cells using dynamic mass redistribution measurements

Ralf Schröder[1,5], Nicole Janssen[2,5], Johannes Schmidt[1], Anna Kebig[2], Nicole Merten[1], Stephanie Hennen[1], Anke Müller[1], Stefanie Blättermann[1], Marion Mohr-Andrä[2], Sabine Zahn[3], Jörg Wenzel[3], Nicola J Smith[4], Jesús Gomeza[1], Christel Drewke[1], Graeme Milligan[4], Klaus Mohr[2] & Evi Kostenis[1]

NATURE BIOTECHNOLOGY VOLUME 28 NUMBER 9 SEPTEMBER 2010

PROTOCOL

Applying label-free dynamic mass redistribution technology to frame signaling of G protein–coupled receptors noninvasively in living cells

Ralf Schröder[1], Johannes Schmidt[1], Stefanie Blättermann[1], Lucas Peters[1], Nicole Janssen[2], Manuel Grundmann[1], Wiebke Seemann[2], Dorina Kaufel[2], Nicole Merten[1], Christel Drewke[1], Jesus Gomeza[1], Graeme Milligan[3], Klaus Mohr[2] & Evi Kostenis[1]

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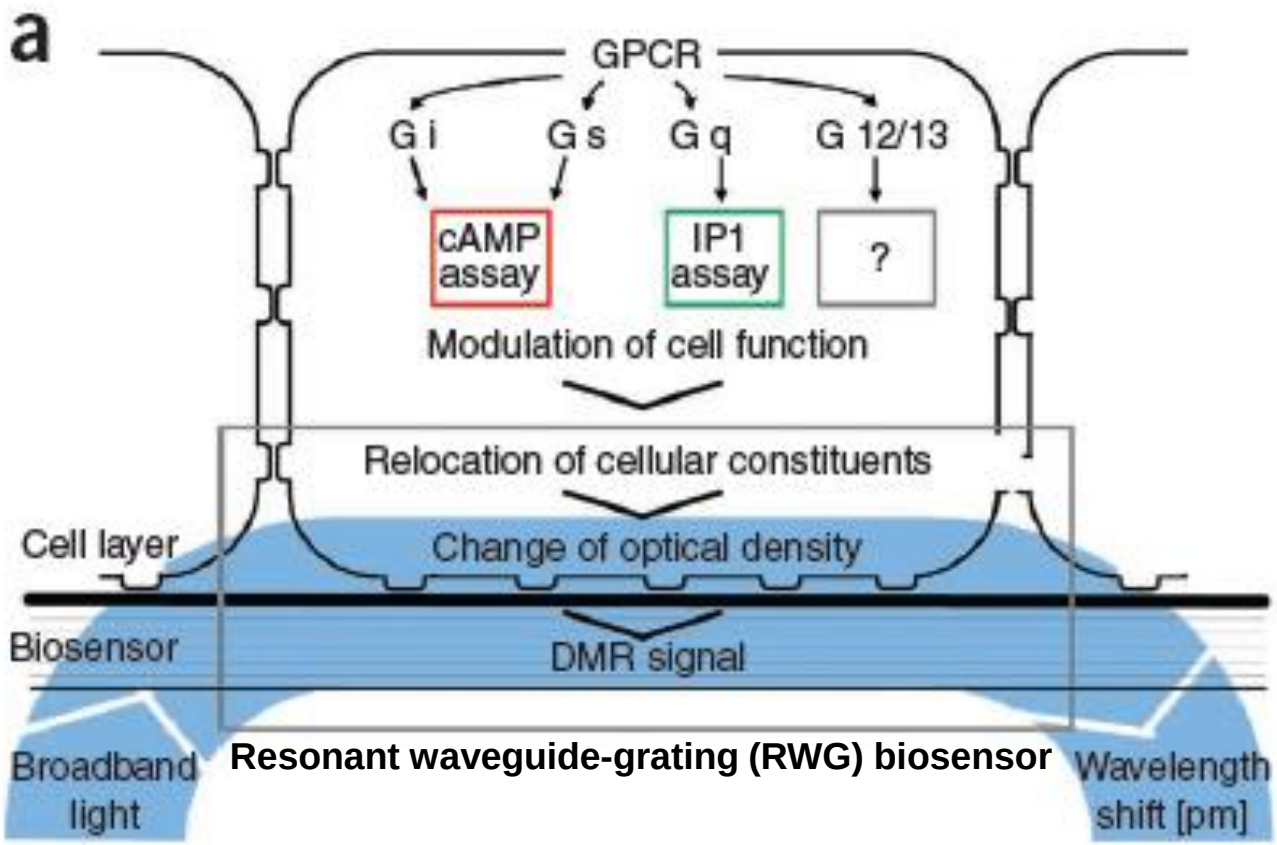
NATURE PROTOCOLS | VOL.6 NO.11 | 2011 |

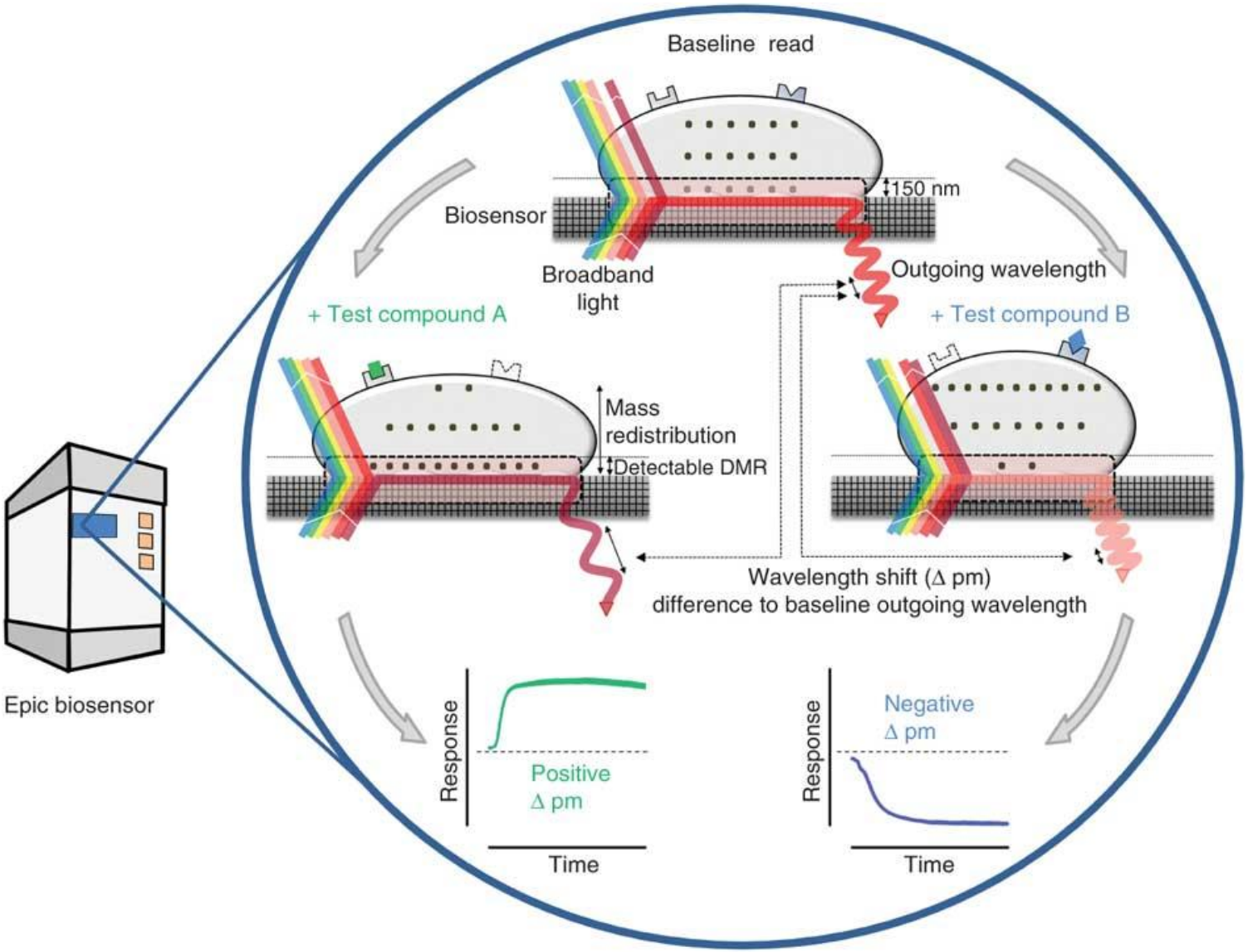
Label-free Dynamic mass redistribution (DMR)

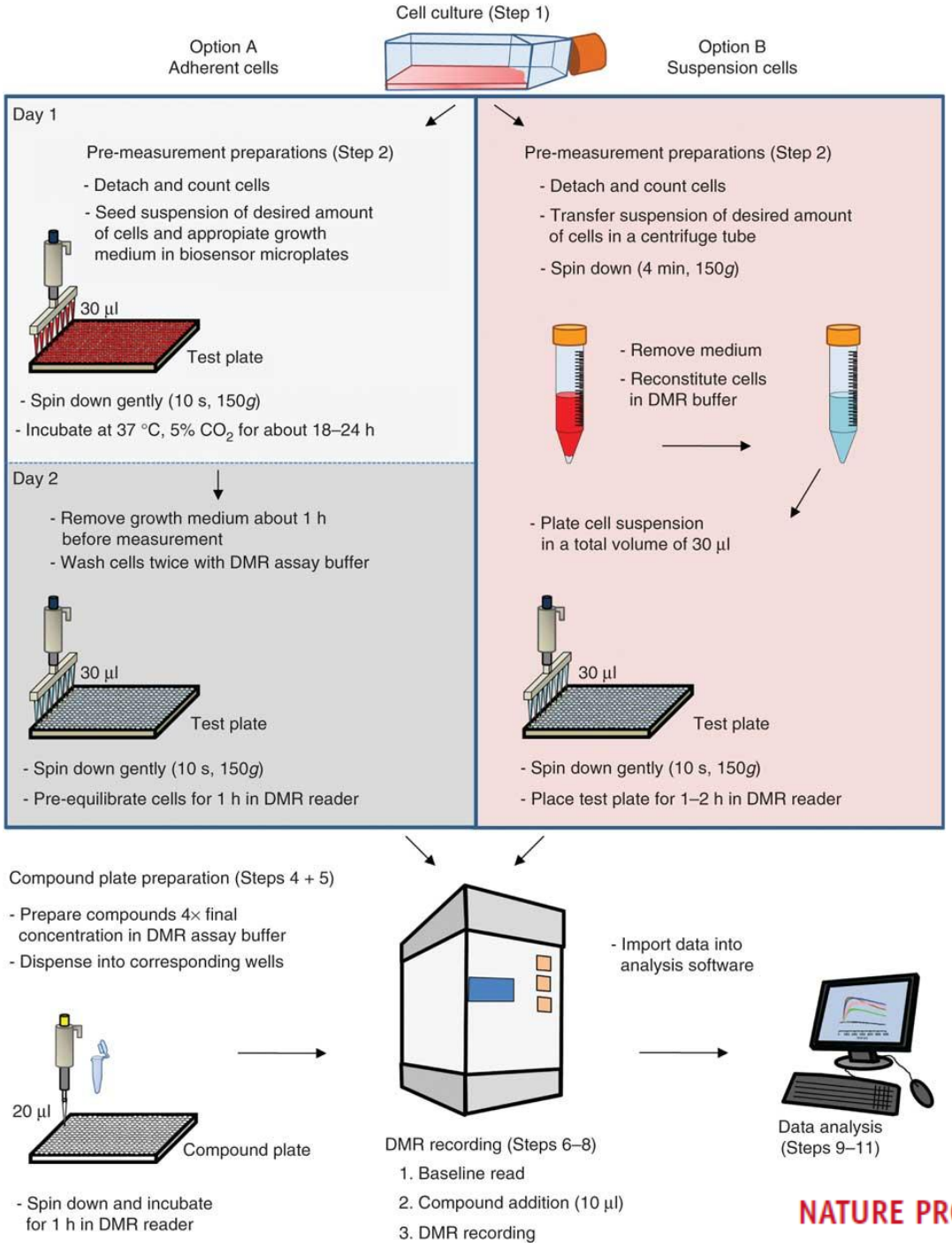


Resonant waveguide-grating (RWG) biosensor

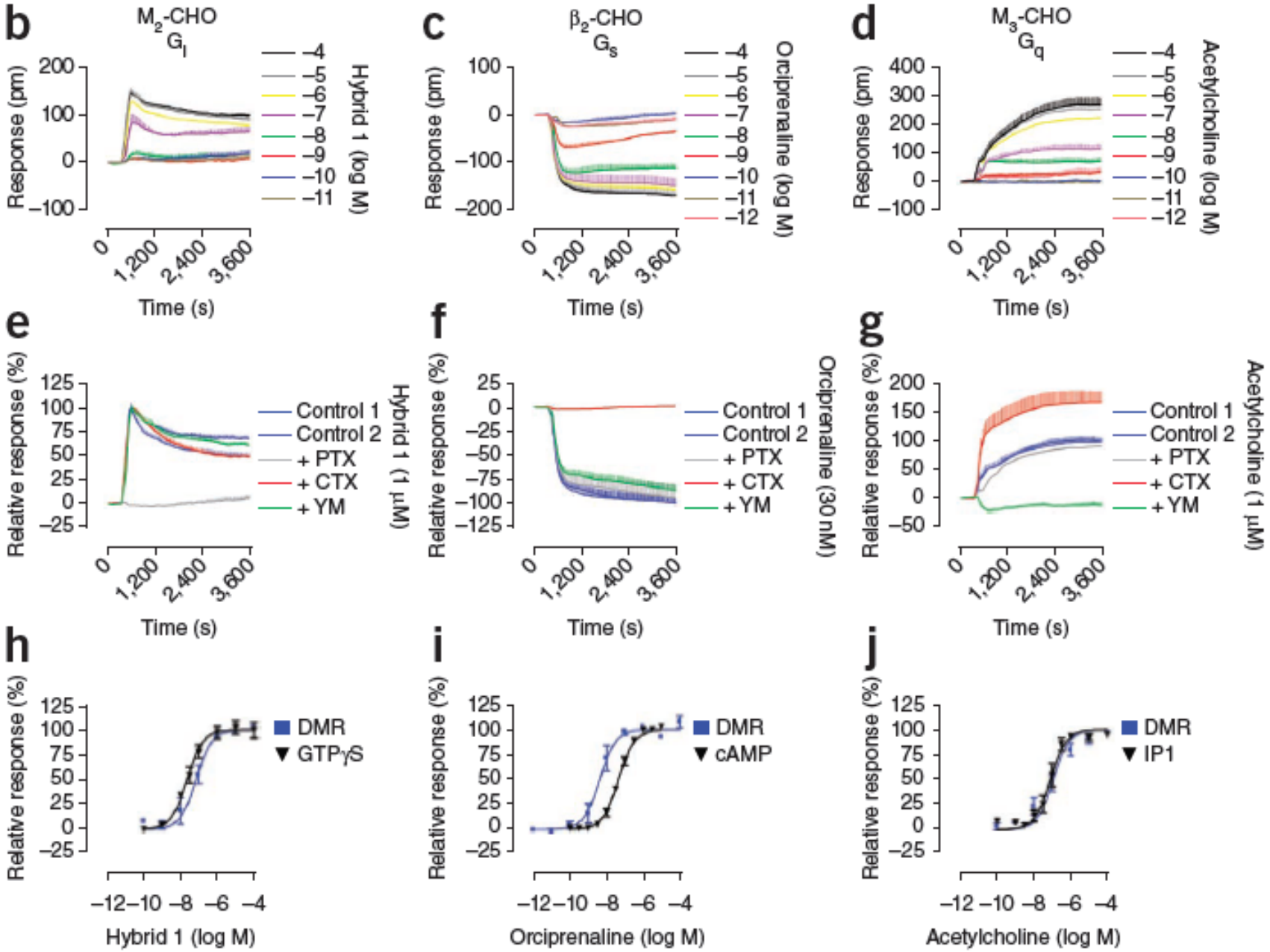
NATURE BIOTECHNOLOGY VOLUME 28 NUMBER 9 SEPTEMBER 2010

Label-free Dynamic mass redistribution (DMR)




Cell culture (Step 1)
Option A
Adherent cells
Option B
Suspension cells
Day 1
Pre-measurement preparations (Step 2)
- Detach and count cells
- Seed suspension of desired amount of cells and appropiate growth medium in biosensor microplates
30 µl
Test plate
- Spin down gently (10 s, 150g)
- Incubate at 37 °C, 5% CO2 for about 18–24 h
Day 2
- Remove growth medium about 1 h before measurement
- Wash cells twice with DMR assay buffer
30 µl
Test plate
- Spin down gently (10 s, 150g)
- Pre-equilibrate cells for 1 h in DMR reader
Pre-measurement preparations (Step 2)
- Detach and count cells
- Transfer suspension of desired amount of cells in a centrifuge tube
- Spin down (4 min, 150g)
- Remove medium
- Reconstitute cells in DMR buffer
- Plate cell suspension in a total volume of 30 µl
30 µl
Test plate
- Spin down gently (10 s, 150g)
- Place test plate for 1–2 h in DMR reader
Compound plate preparation (Steps 4 + 5)
- Prepare compounds 4× final concentration in DMR assay buffer
- Dispense into corresponding wells
20 µl
Compound plate
- Spin down and incubate for 1 h in DMR reader
DMR recording (Steps 6–8)
1. Baseline read
2. Compound addition (10 µl)
3. DMR recording
- Import data into analysis software
Data analysis
(Steps 9–11)

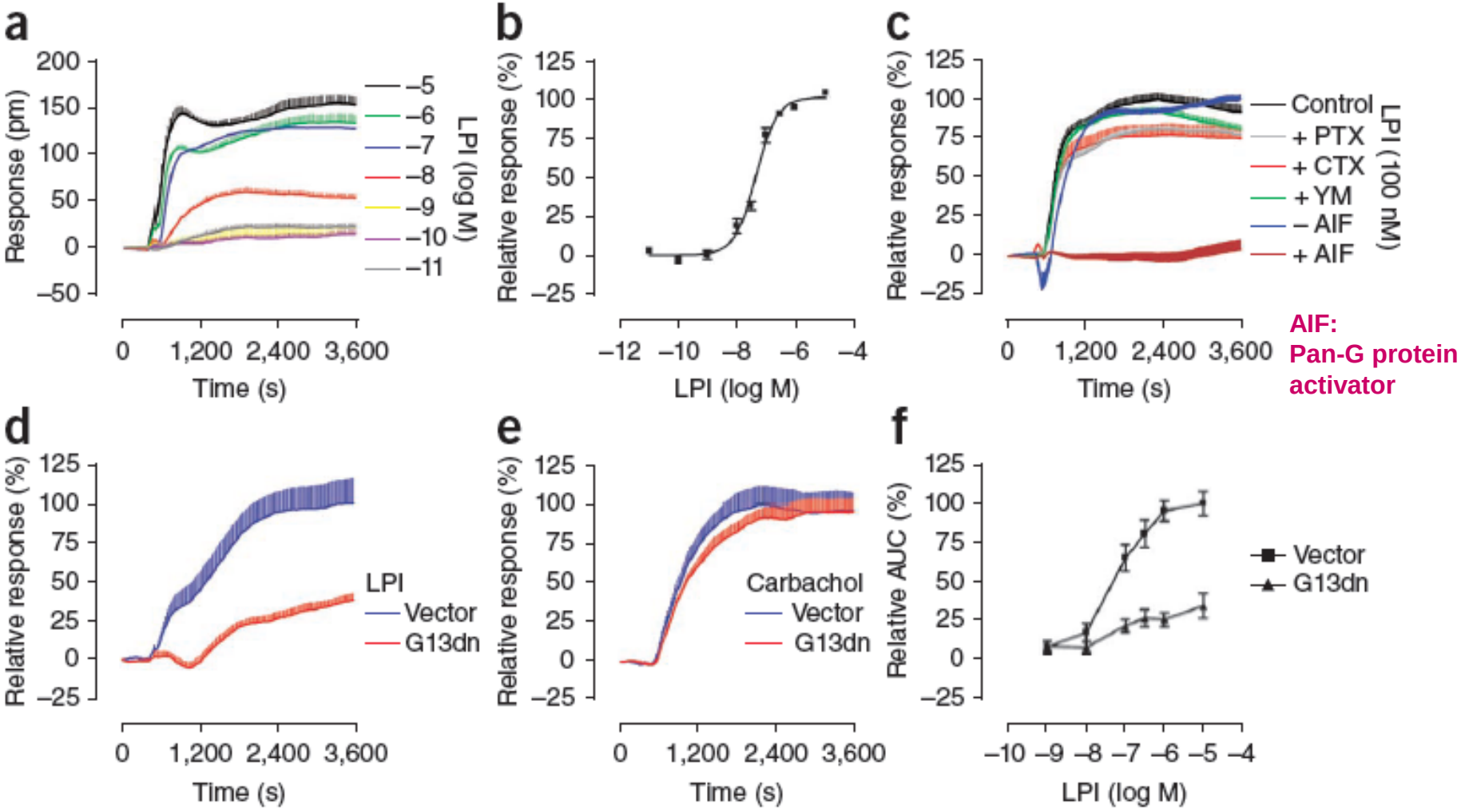
Recordings in CHO cells stably transfected with either the hM_2-, $h\beta_2$- or hM_3-receptor gene



Dynamic mass redistribution visualizes signaling along the G_{12}/G_{13} pathway

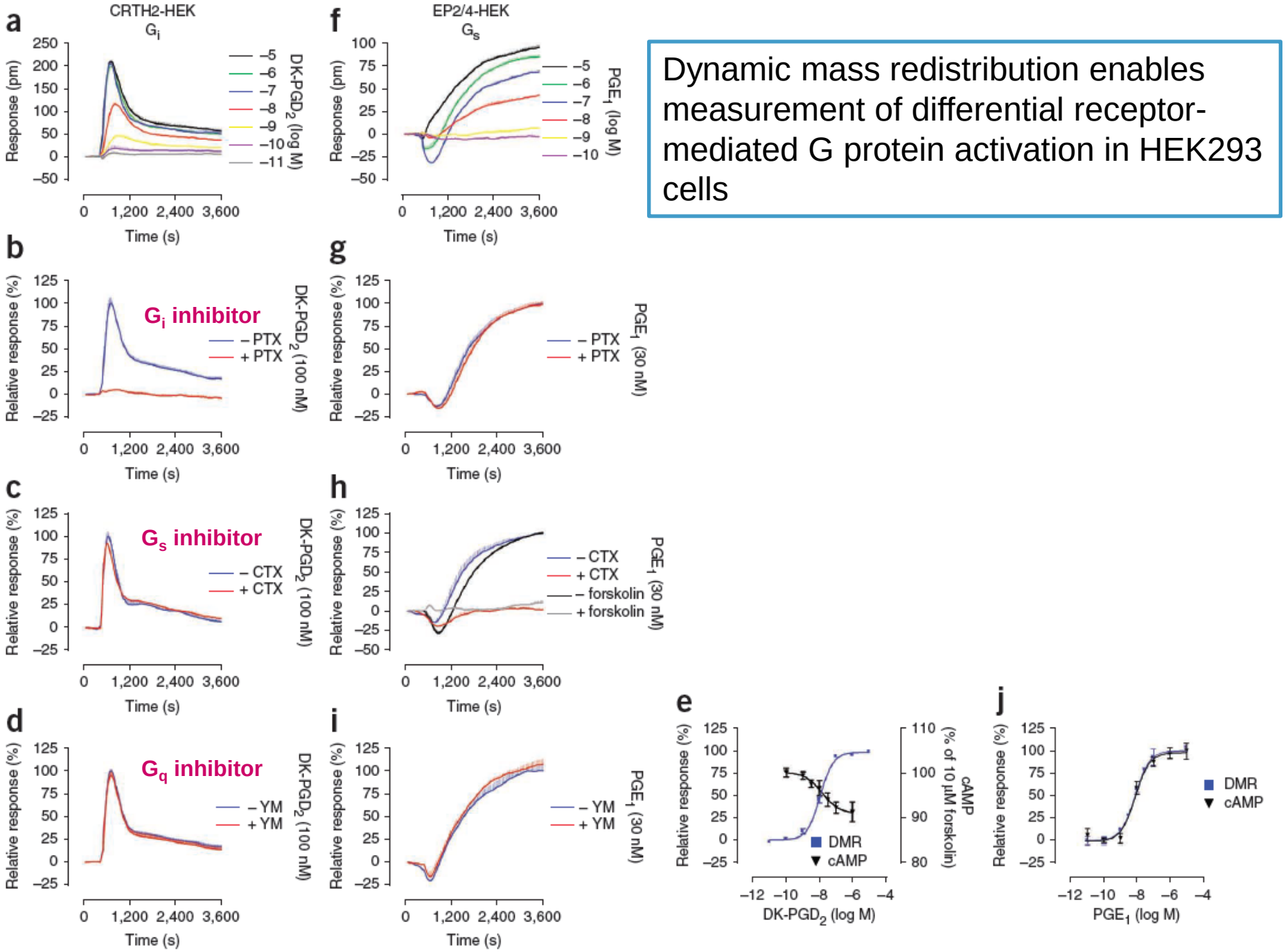
GPR55($G\alpha_{12/13}$ coupled)-HEK cells

LPI: Lysophosphatidylinositol (GPR55 agonist)



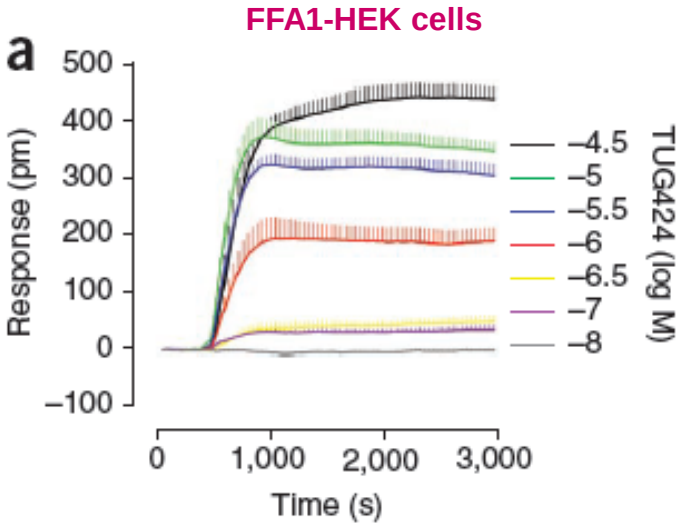
AIF: Pan-G protein activator

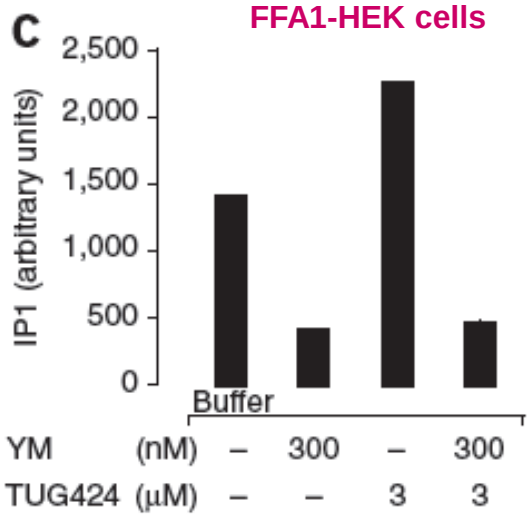
Carbachol: Gq sensitive muscarinic receptor agonist

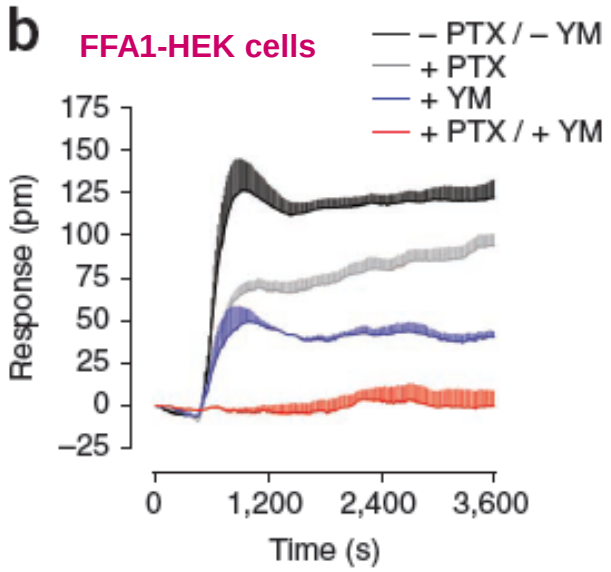


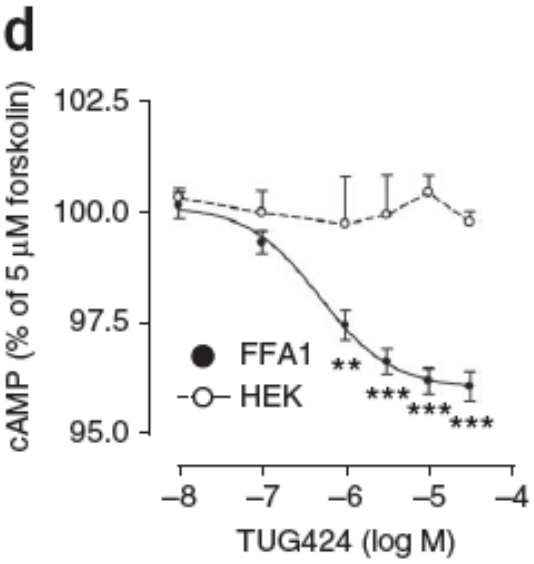
Dynamic mass redistribution enables measurement of differential receptor-mediated G protein activation in HEK293 cells

Parallel visualization of all signaling pathways unveils
an additional signaling route of the free fatty acid receptor FFA1.



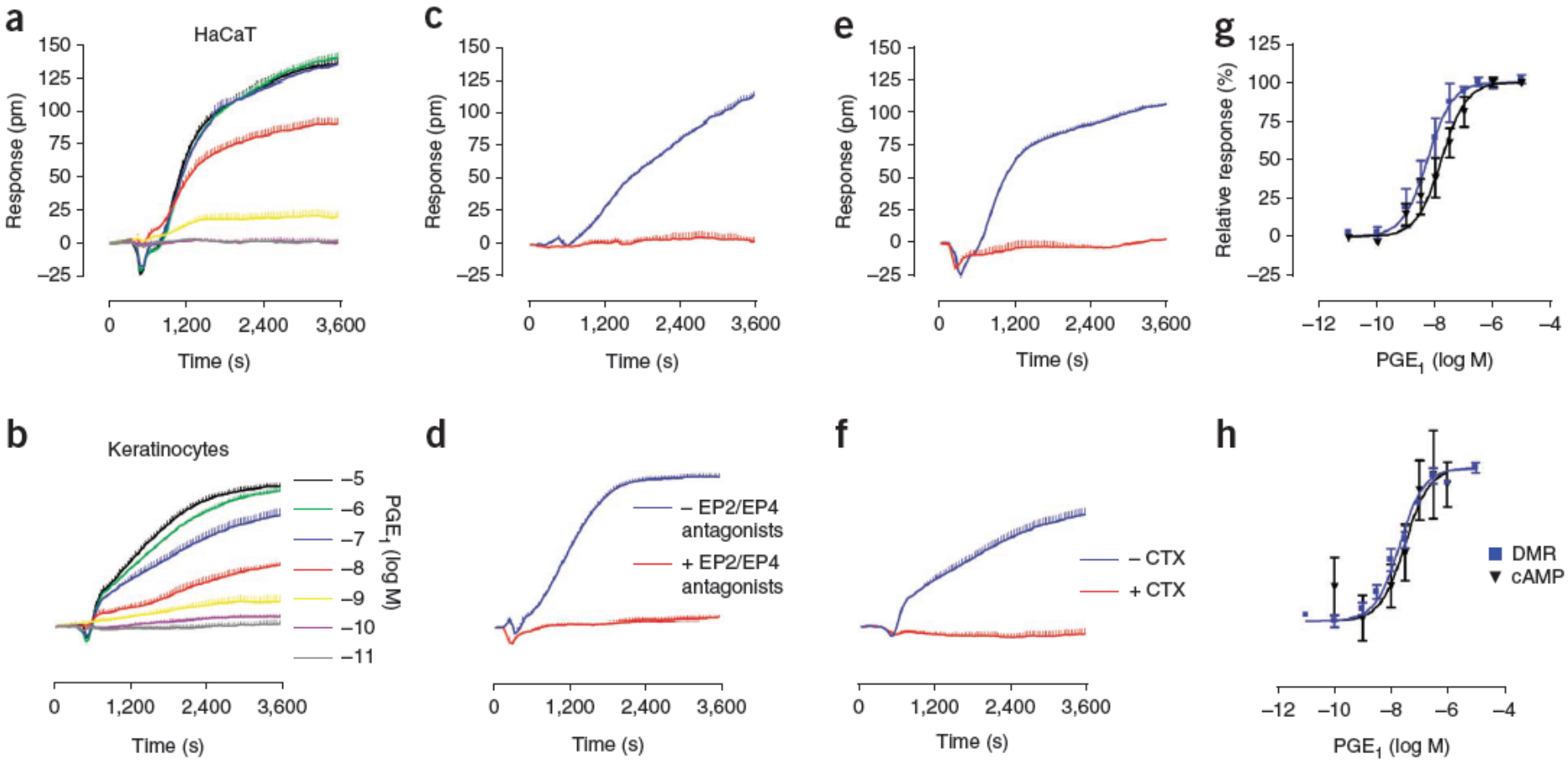




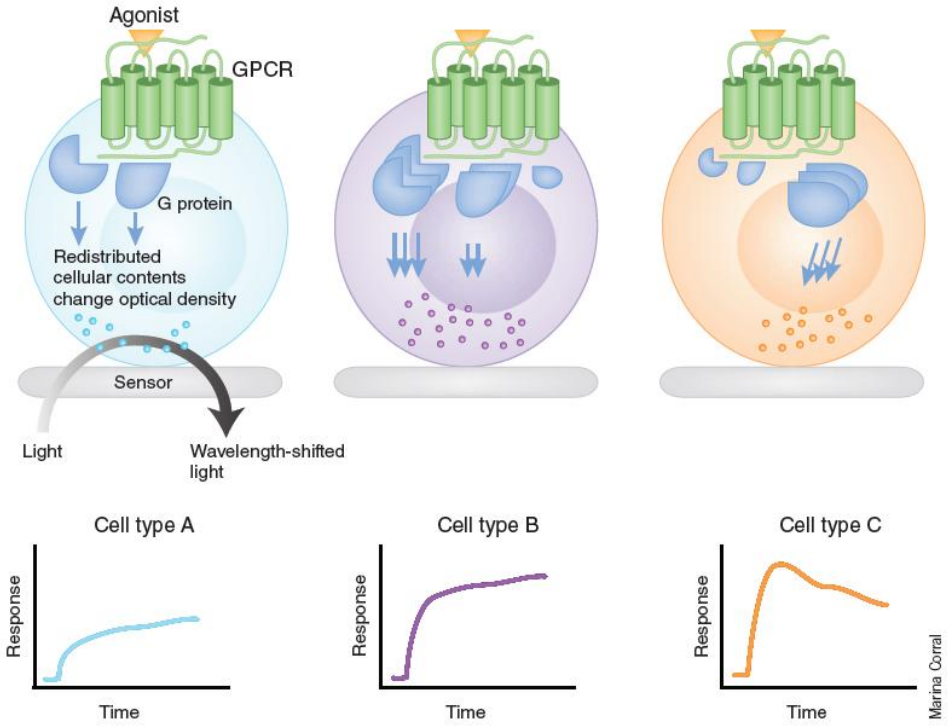


*PTX: G_i inhibitor *YM: G_q inhibitor *TUG424: FFA1 (fatty acid receptor) agonist

Dynamic mass redistribution enables analysis of GPCR functionality in immortalized and primary human keratinocytes



Summary



-DMR can monitor the cell activation state in non-labeled living-cells (non-invasive).

-DMR technology can be used for even primary cells and non-adherent cells as well as adherent cells.

-In the case of measurement of $G\alpha_{i/o}$ signaling, DMR offers a direct measure of $G\alpha_{i/o}$-coupled GPCR activation without the need to pharmacologically manipulate the adenylyl cyclase–cAMP module to probe for $G\alpha_{i/o}$ activity.

Thank you for your kind attention!