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Novel engineering methods for the development of optogenetic tools

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This thesis includes 2 original papers published in peer reviewed journals and 1 submitted publications. The core theme of the thesis is the development of optogenetic tools. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Australian Regenerative Medicine Institute at Monash University under the supervision of Dr. Harald JANOVJAK.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of chapter 1, chapter 2 and chapter 3 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author names, Nature and % contribution	Co-author(s), Monash student Y/N
1	Light-activated chimeric GPCRs: limitations and opportunities (*Review article)*	Published	70%, concept, data collection, writing and editing	1) Elliot J. Gerrard: 10%, Writing and editing 2) Patrick M. Sexton: 5%, input into manuscript 3) Harald Janovjak: 15%, supervisor, writing and editing	No No No
2	Engineering strategy and vector library for the rapid generation of modular light-controlled protein-protein interactions	Published	70%, concept, data collection and analysis, writing and editing	1) Elliot J. Gerrard: 10% data collection and analysis, writing and editing 2) Julien M.D. Legrand: 2.5% data collection and analysis, input into manuscript 3) Robin M. Hobbs: 2.5% analysis, input into manuscript 4) Harald Janovjak: 15% supervisor, concept, analysis, writing and editing	No No No No
3	Structure-guided design of light-activated chimeric G-protein coupled receptors	Submitted	80% Concept, data collection and analysis, writing and editing	1) Elliot J. Gerrard: 5% data collection and analysis, input into manuscript 2) Harald Janovjak: 15% supervisor, concept, analysis, writing and editing	No No

I have added additional page numbers to published papers in order to generate a consistent presentation within the thesis.

Alexandra-Madelaine Tichy (Student)

Signed: Date:

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Harald Janovjak (Main Supervisor)

Signed: Date:

Publications During Enrolment

1. "Structure-guided design of light-activated chimeric G-protein coupled receptors"
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2. "Optogenetic delivery of trophic signals in a genetic model of Parkinson's disease"
   Ingles-Prieto, A., Furthmann, N., Crossman, S.H., **Tichy, A.-M.**, Hoyer, N., Petersen, M., Zheden, V., Biebl, J., Reichhart, E., Gyoergy, A., Siekhaus, D.E., Soba, P., Winklhofer, K.F. & Janovjak, H.
   PLoS Genetics, (2021)

3. "Light-activated chimeric GPCRs: limitations and opportunities"
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   Current Opinion in Structural Biology, (2019)

4. "Engineering strategy and vector library for the rapid generation of modular light-controlled protein-protein interactions."
   Tichy, A.-M., Gerrad, E.J., Legrand, J.M.D., Hobbs, R.M., & Janovjak, H.
   Journal of Molecular Biology, (2019)

5. "Optogenetic Frizzled7 reveals a permissive role of non-canonical Wnt signaling in mesendoderm cell migration."
   Čapek, D., Smutny, M., **Tichy, A.-M.**, Morri, M., Janovjak, H. & Heisenberg, C.-P.
   eLife, (2019)

6. "Optical functionalization of human Class A orphan G-protein coupled receptors."
   Morri, M., Sanchez-Romero, I., **Tichy, A.-M.**, Kainrath, S., Gerrard, E.J., Hirschfeld, P., Schwarz, J. & Janovjak, H.
   Nature Communications, (2018)

PhD Experience Summary

Conferences, Workshops and Talks

- *2021, May*: Oral Presentation
  Janelia ECR Symposium on Protein Engineering (Virtual - VA, USA)
- *2021, February*: Workshop Talk
  2nd Optogenetics Australia Virtual Workshop (Virtual - Melbourne, Australia)
- *2020, February*: Invited Talk
  1st Optogenetics Australia Hands-on Workshop (Melbourne, Australia)
- *2019, December*: Oral Presentation
  INPEC-APEM, (Canberra, Australia)
- *2019, August*: Invited Talk
  Center for Molecular Neurobiology Hamburg (ZMNH) (Hamburg, Germany)
- *2019, April*: Poster Presentation
  EASD Islet Study Group & Beta-Cell Workshop (Oxford, United Kingdom)
- *2019, March*: Trainer
  EMBO Non-Neuronal Optogenetics Workshop (Heidelberg, Germany)
- *2018, December*: Oral Presentation
  BPS-MPGPCR, (Melbourne, Australia)
- *2018, November*: Poster Presentation
  11th Australian Islet Study Group Meeting (Canberra, Australia)
- *2018, July*: Participant, Poster Presentation
  EMBL Australia PhD Course (Sydney, Australia)

Awards

- *2018*, Best Student Oral Presentation (BPS-MPGPCR)
- *2018*, BMG LabTech Student Award (BPS-MPGPCR)
- *2018*, EMBL Australia Travel Grant
- *2018*, JDRF Young Investigator Travel Award (11th Australian Islet Study Group Meeting)
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- *2018-21*, Faculty International Tuition Scholarship (FMNHS)

Abstract

Optogenetics enables the control of protein function with light in genetically-targeted cells, which provides the opportunity to manipulate cellular and animal physiology precisely in time and space. This is achieved through imparting light-sensitivity to proteins and tissues that endogenously do not respond to light by re-purposing naturally occurring photoreceptor proteins. While optogenetics made its breakthrough in the field of neuroscience, its application has quickly expanded to the manipulation of cellular signalling and behaviour in different areas of research, such as cell biology or developmental biology.

Non-neuronal optogenetics focuses on the control of cellular behaviour, with two of the main approaches being the control of light-activated protein-protein interactions (Opto-PPIs), or light-activated chimeric G-protein coupled receptors (OptoXRs) (other approaches include light-activated protein unfolding or enzyme activity). From a protein engineering perspective, the two approaches of Opto-PPIs and OptoXRs differ significantly from each other and present different obstacles and challenges. For Opto-PPIs, the general engineering approach is in principle a robust one, but the plethora of possible processes and target proteins necessitates the constant development of new tools. For OptoXRs, on the other hand, the overall approach has remained the same for many years without substantial improvements since its inception.

In this thesis, I describe work aimed at addressing these challenges and improving the current engineering methods for both Opto-PPIs as well as for OptoXRs.

In the first project (chapter 2), I established a new engineering strategy and vector library to facilitate modular development of light-activated PPIs. The vector library contains the most commonly used photoreceptors for this purpose and is paired with a streamlined cloning strategy. Together, this work facilitates simpler and faster engineering and testing of new optogenetic tools to manipulate PPIs. The applicability of this strategy is demonstrated by employing it on a new target protein, caspase 9, to develop Opto-caspase9.

In the second project (chapter 3), I developed a new approach to engineer OptoXRs that uses information obtained from GPCR structures, and is based on structural alignments and direct G-protein interacting residues. To demonstrate the advances of this design strategy, I engineered an improved Opto-β_2AR (β_2 adrenergic receptor) which closely mimics wild-type β_2AR signalling and displays greatly enhanced functionality compared to Opto-β_2ARs based on previous design strategies. I exploited these advances by testing further modifications to the receptor, such as spectral tuning and by applying it to another target receptor.

The work described in this thesis addresses two major challenges in the field of cellular optogenetics, by providing a method that will make the engineering of Opto-PPIs easier and more accessible, and by presenting a novel design approach for OptoXRs resulting in better and more robustly functioning OptoXRs.

Acknowledgements

Doing a PhD is a deeply rewarding, but also challenging experience at the best of times. There's really no need to throw in a global pandemic on top of it, but here we are. To be able to finish this PhD, despite all of this, took the support of a whole lot of people, and I would like to take this opportunity to express my gratitude here.

First and foremost, I would like to thank my supervisor Harald for giving me the chance to do a PhD in this lab, and for giving me this incredible opportunity to move with the lab from Austria to Australia. Thank you for your guidance on all things scientific, for your belief in me and support when I was struggling, for always being encouraging about going to conferences and presenting my work, and for always advocating on my(/the whole lab's) behalf at every opportunity. I have learned and grown a lot during these past years, both as a scientist and as a person, and a lot was directly thanks to you.

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Last, I want to say thank you to my partner and best friend, Elliot. I think you know how important your unwavering support and your friendship was over the years, especially in this insane last year, and what part you played in me getting to the finish line of this PhD. I cannot be more grateful.

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Thank you,
A.M.T.

Melbourne, August 2021

List of Abbreviations

$A_{2A}R$	Adenosine 2A receptor
AdoCbl	adenosylcobalamin
AsLOV2	*Avena sativa* LOV2
AtCRY2	A*rabidopsis thaliana* CRY2
β_2AR	β_2 Adrenergic Receptor
BLUF	blue-light using flavin
BMP	bone morphogenic protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBD	cobalamin binding domain
CIB	cryptochrome interacting basic-helix-loop-helix protein
CRY	cryptochrome
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECL	extracellular loop
Erk	extracellular signal regulated kinase
FACS	fluorescent activated cell sorting
FAD	flavin adenine denucleotide
FBS	fetal bovine serum
FC	flow cytometry
FGFR	fibroblast growth factor receptor
FMN	flavin mononucleotide
GPCR	G-protein coupled receptor
HBSS	Hank's buffered saline solution
HEK293	human embryonic kidney 293 cells
ICL	intracellular loop
IDR	intrinsically disordred protein region
IF	immunofluorescence
iLID	improved light-induced dimer
ISO	isoproterenol
L15	Leibovitz' L-15 medium
LOV	light-oxygen-voltage
LSD	light sensitive domain
MAPK	mitogen-activated protein kinase
min	minutes
NcVVD	*Neurosporra crassa* vivid
o/n	overnight
PAS	Per-Arnt-Sim
PBS	phosphate buffered saline

PEI	polyethylenimine
PFA	paraformaldehyde
PhyB	phytochrome B
PIF	phytochrome interacting factor
POI	protein of interest
PPI	protein-protein interaction
PTX	pertussis toxin
PYP	photoactive yellow protein
RET	rearranged during transfection
RT	room temperature
RTK	receptor tyrosine kinase
TM	transmembrane helix
TULIP	tunable, light-controlled interacting protein tags
VfAU1	*Vaucheria frigida* Aureochrome 1
WT	wildtype

Dedicated to my grandfather

Contents

Chapter 1

Introduction

1.1 Optogenetics

Optogenetics is a technique that enables spatially and temporally precise activation of protein function in cells using light, which allows for the unprecedented control of cellular physiology and animal behaviour. Optogenetics first gained widespread attention for its application in neuroscience, where microbial opsins were used to activate or silence specific areas of the brain. This was achieved through repurposing the innate ability of these proteins to function as light-activated ion channels and pumps. Paired with genetically targeting distinct neuronal populations, it was possible to reversibly depolarize or hyperpolarize specific neurons without influencing surrounding cells.

Since then, both the optogenetic toolbox and its field of application have increased significantly. Beyond the control of membrane potential in neuronal cells, optogenetics has enabled the manipulation of molecular processes in areas such as cell biology, developmental biology or disease research. This was made possible by the diversity of photoreceptors found in nature displaying various light-activated behaviours, from changes in oligomerization state to generation of second messengers and more complex structural rearrangements.

1.2 Photoreceptors

The ability to sense light is ubiquitously found in all domains of life. Light acts as a stimulus regulating diverse processes: In plants and microbes, light coordinates biological activities such as light-controlled growth (phototropism) or motility (phototaxis) (Möglich et al., 2010). In higher species, such as mammals, light predominantly mediates vision and entrainment of the circadian rhythm (Foster et al., 2007).

Photoreceptor proteins do not directly sense light without further modification, as amino acid backbones or side chains do not absorb light in the visible spectrum. Because of this, photoreceptors have to bind chromophores (covalently or non-covalently) that act as the site of photon absorption. Upon light-absorption, the chromophores undergo structural changes that ultimately lead to rearrangement of the protein backbone. These structural transformations then initiate further downstream signal propagation. In nature, photoreceptors often consist of light-sensing domains directly coupled to effector domains, such as kinase, dimerization or DNA-binding domains. Opsins form an exception to this modular architecture, as they belong to the family of G-protein coupled receptors (Möglich et al., 2010; Shcherbakova et al., 2015).

Photoreceptors can be divided based on the chromophore and their light-sensing domain family. A least seven distinct classes of photoreceptors that respond to visible light are known: Xanthopsins, LOV (light-oxygen-voltage) domains, BLUF (blue light using FAD) sensors, cryptochromes, opsins, cobalamin-binding domains and phytochromes. Each of these classes employs one of five different chromophores: p-coumaric acid, flavins, retinals, cobalamin or tetrapyrroles (Seong et al., 2021). The size and nature of the chromophore determines the wavelength of light a photoreceptor can absorb (Figure 1.1).

In the following section, I will describe the molecular function of the various photoreceptor classes, whereas the two sections after will elucidate how they are employed as optogenetic tools to control protein-protein interactions (see section 1.3) and GPCR function (see section 1.4).

1.2.1 Xanthopsins

The best known representative of xanothopsin photoreceptors is the photoactive yellow protein (PYP), first identified in *Halorhodospira halophile*, a halophilic purple photosynthetic bacterium (Meyer et al., 1987). Since then, PYPs have been identified in multiple types of bacteria from a variety of metabolic niches. PYPs regulate different physiological light-responses, such as negative phototaxis or buoyancy. Despite extensive efforts, direct binding partners that give an insight into the exact mechanism of this behaviour have only recently been identified (Meyer et al., 2012; Kim et al., 2021). PYP serves as structural prototype for proteins using the Per-Arnt-Sim (PAS) domains. PAS domains are protein modules found across all kingdoms of life, where they are critical in the transfer of signals from a variety of stimuli. They are also found in other photoreceptors, such as LOV domains (see subsection 1.2.2) or phytochromes (see subsection 1.2.7). PYP binds

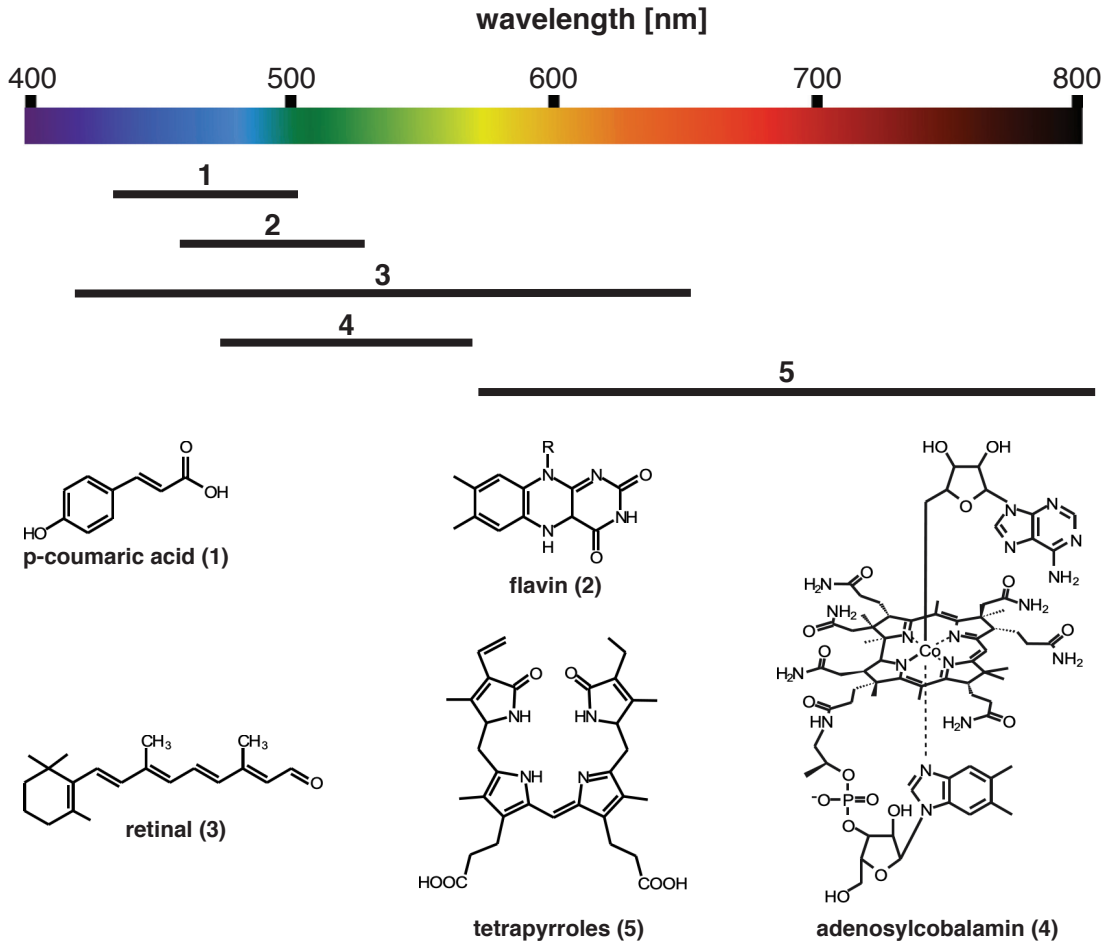

FIGURE 1.1: **Chromophores of main photoreceptor classes**. To sense light in the visible spectrum, the main photoreceptors employ one of 5 chromophores or their derivatives, which are maximally sensitive to different wavelengths of light. The main chromophores are (1) p-coumaric acid, (2) flavins, (3) retinal, (4) adenosylcobalamin and (5) tetrapyrroles.

p-coumaric acid as chromophore, which undergoes *trans*-to-*cis* isomerization upon absorption of blue light ($\lambda_{max} \approx$ 450 nm), a reaction that reverses in the dark within seconds. This leads to structural rearrangements ultimately resulting in the unfolding of N-terminal α-helices from a β-sheet core (Tenboer et al., 2014; Pande et al., 2016). While the study of PYPs has provided valuable insights into the structural mechanism underlying photoreceptor activation on an atomic level, PYPs have only found limited popularity as optogenetic tools. This is mainly because the conformational change is difficult to exploit for optogenetic tools and because the activation spectrum of PYPs overlaps with those of the highly versatile and popular LOV domains.

1.2.2 LOV Domains

LOV (light-oxygen-voltage) domains were first identified in plant phototropins but have since been discovered in diverse groups of proteins from funghi, bacteria and archaea (Möglich et al., 2010). LOV domains belong to the group of PAS proteins and are relatively small in size ($\sim$ 11-15 kDa). LOV domains preferentially bind flavin mononucleotide (FMN) as chromophore in a non-covalent manner, making them maximally sensitive to blue light ($\lambda_{max} \approx$ 450 nm). Upon absorption of blue light, a covalent bond between FMN and a conserved cystein residue of the LOV domain is formed, leading to activation of the domain. The specific structural rearrangements and downstream signalling resulting from light-absorption differ between LOV domains (Shcherbakova et al., 2015). In phototropins, light stimulation causes an unfolding of the C-terminal Jα helix with subsequent activation of the C-terminal kinase domain. In contrast, the LOV domains found in the Vivid (VVD) photoreceptor from *Neurospora crassa* or in Aureochrome1 from *Vaucheria frigida* exhibit light-induced PAS-PAS homo-dimerization (Zoltowski et al., 2008; Takahashi et al., 2007).

Members of the LOV domain family share similar sequences, but exhibit distinct kinetic behaviours. While light-activation of LOV domains is usually fully reversible, the time it takes to return to an inactive state in the dark ranges from seconds to hours, and in some LOV domains is near irreversible (Pudasaini et al., 2015). The variety of functions found among LOV domains has made them attractive photoreceptors for the engineering of optogenetic tools.

1.2.3 Cryptochromes

Cryptochromes (CRYs) are photoreceptors found in archaea, bacteria as well as eukaryotes and are involved in a multitude of processes, from growth and development in plants to entrainment of circadian rhythm in higher plants or animals (Lin et al., 2005). CRYs bind FAD as a cofactor and are sensitive to blue light ($\lambda_{max} \approx$ 450 nm) (Banerjee et al., 2007). Two main groups of CRYs have been identified: Type I CRYs are light-sensing photoreceptors, such as plant CRY1/CRY2 or *Drosophila* dCRY. Type II CRYs, on the other hand, are light-unresponsive and found predominantly in mammals (such as mouse mCRY or human hCRY) where they act as transcriptional repressors (Conrad et al., 2014). Structurally, CRYs closely resemble photolyases and contain a characteristic N-terminal PHR (photolyase homology region). The PHR domain binds FAD non-covalently and thus functions as the primary light sensing domain. Furthermore, CRYs can also bind a pterin chromophore which has been shown to function as

"antenna pigment", conferring energy harvested from the near-UV range to the flavin cofactor (Hoang et al., 2008). In addition to the light-sensing domains, CRYs also contain C-terminal elements which allow involvement in signalling cascades (Möglich et al., 2010).

Among the best studied cryptochromes are those of *Arabidopsis thaliana*, particularly AtCRY2, which regulates transcription in response to light, both directly and indirectly. Plant CRYs function as dimers in the cell, mediated through the PHR domain (Liu et al., 2016). AtCRY2 has been shown to directly interact with multiple binding partners in a light dependent manner, such as with CIB1 (cryptochrome-interacting basic helix-loop-helix) (Liu et al., 2008) or PhyB (phytochrome B) (Más et al., 2000). Additionally, AtCRY2 is capable of forming homo-oligomers in response to light (Yu et al., 2009).

1.2.4 BLUF sensors

BLUF (blue-light using flavin) sensors are small photoreceptors, first identified in unicellular eukaryotes and bacteria. As their name suggests, they bind flavin as their cofactor and are thus maximally sensitive to blue light. BLUF sensors can be divided into two groups, group I and group II. Group I proteins contain BLUF domains fused to a C-terminal effector domain exhibiting enzymatic or other functions, with a majority of these proteins forming homo-dimers (Park et al., 2017). Prominent examples of these proteins are a photo-activated adenylyl cyclase from *Euglena gracilis* promoting photophobic response through light-activated increases in cAMP (Iseki et al., 2002), or the well-studied protein AppA from *Rhodobacter sphaeroides* involved in the regulation of genes important for photosynthesis (Masuda et al., 2002). Group II consists of proteins with a BLUF domain and only minimal additional C-terminal elements that do not exhibit enzymatic properties and are mainly thought to enhance stability (Park et al., 2017). Belonging to group II is the photoreceptor PixD from *Synechocystis* sp. PCC6803. In the dark, PixD forms oligomers with the protein PixE consisting of ten PixD and five PixE units ($PixD_{10}$-$PixE_5$). Upon light absorption, the complex dissociates into PixD dimers and PixE monomers (Yuan et al., 2008).

1.2.5 Opsins

Opsin proteins have been found across all domains of life. Unlike the other photoreceptors discussed here, opsins are transmembrane (TM) proteins. Opsins can be divided into two distinct groups, microbial (type I) opsins, and animal

(type II) opsins. Opsins bind retinal as their chromophore through a conserved lysine residue in TM7 which forms a Schiff base with the chromophore (Ernst et al., 2014). Even though the two groups share a similar 7-TM structure, they differ significantly in their amino acid sequence and function.

Microbial Opsins

Microbial opsins are found in archaea, bacteria and lower eukaryota, and function as light-activated ion pumps or channels. Microbial opsins bind all-*trans* retinal as their cofactor, which is isomerized into 13-*cis* retinal upon light-absorption. This reaction occurs rapidly and is reversible in the dark (Zhang et al., 2011). Among the most famous examples of type I opsins is Channelrhodopsin 2 (ChR2), a cation channel from the green algae *Chlamydomonas reinhardtii* responsible for the mediation of phototaxis (Nagel et al., 2003). ChR2 was the first microbial photoreceptor to be used for the light-control of mammalian cell function through the rapid and reversible activation of neuronal firing (Boyden et al., 2005). This first-of-its-kind repurposing of natural photoreceptors led to the introduction of the field and term Optogenetics, and has transformed research in neuroscience by enabling the targeted de- or hyperpolarisation of neurons. Since then, the microbial opsin toolbox has been greatly expanded. As this thesis focuses on optogenetic tools for the control of cellular biology rather than manipulation of membrane potential in neurons, a more detailed discussion of microbial opsins would be beyond its scope.

Animal Opsins

Type II or animal opsins belong to the family of G-protein coupled receptors (GPCRs; see section 1.4). They are responsible for mediating vision across all species (visual opsins), as well as fulfilling a range of other functions such as entrainment of circadian rhythm or pupillary light reflexes (non-visual opsins) (Terakita, 2005). The majority of animal opsins binds 11-*cis*-retinal as their chromophore, although exceptions using other isoforms of retinal exist (Nagata et al., 2018). Light absorption leads to *cis*-to-*trans* isomerization of the retinal, causing structural rearrangements of the opsin backbone that subsequently exposes an intracellular G-protein binding site. In solution, retinal is maximally sensitive to UV-light, with low photo-isomerization efficiency (Shichida et al., 2009). However, in the opsin, retinal is covalently bound to a conserved lysine residue in TM7 *via* a protonated Schiff base, which shifts the spectral sensitivity into the visible spectrum and drastically increases the isomerization efficiency (e.g. $\approx$ 65% in bovine rhodopsin,

meaning that 2 out of 3 photons will result in retinal isomerization) (Shichida et al., 2009; Ernst et al., 2014). A negatively charged residue in the vicinity of the retinal - the "counterion" - maintains the protonation of the Schiff base in the dark. Mutations of the counterion identity or position can lead to changes in spectral sensitivity, such as in red-shifted cone opsins (Shichida et al., 2009). Opsins can be further divided into "bleaching" or "bistable" opsins. The photoproduct of bleaching opsins, such as vertebrate visual opsins like rhodopsin, is thermally unstable. This results in release of the retinal chromophore upon photo-activation, necessitating continuous retinal supply. In contrast, the light-activated form of bistable opsins is thermally stable, and can revert back to the dark state by absorbing light of a different wavelength while retaining their retinal chromophore (Tsukamoto et al., 2010; Koyanagi et al., 2014). Well studied examples of bistable pigments are melanopsin (Melyan et al., 2005; Spoida et al., 2016) or the box jellyfish opsin, JellyOp (Bailes et al., 2012).

1.2.6 Cobalamin Binding Domains

Cobalamin binding domains (CBDs) are a recently discovered photoreceptor class, and display a particularly interesting photomechanism. CBDs use 5'-deoxyadenosylcobalamin, also called adenosylcobalamin (AdoCbl), a vitamin B_{12} derivative, as a cofactor (Ortiz-Guerrero et al., 2011) which makes them sensitive to green light. B_{12} is an essential micro-nutrient for animals and many prokaryotes. Due to the complex pathways for synthesis, it can only be produced by some prokaryotes while animals are dependent on exogenous supply. Only recently was it discovered that the light-sensitivity of AdoCbl, previously believed to be an undesired effect, serves a physiological function as cofactor for photoreceptors (Padmanabhan et al., 2017). The best characterized CBDs are from *Myxococcus xanthus* and *Thermus thermophilus* where light leads to transcriptional induction of carotenoid synthesis using the photoreceptor CarH. CarH binds AdoCbl in a covalent manner and forms a dimer-of-dimers type tetramer in the dark, leading to increased DNA binding and gene repression (Jost et al., 2015a). Light-absorption causes breakage of a covalent Co-C bond within the chromophore which results in dissociation of the tetrameric structure. This photomechanism is sensitive to light of a wide range of wavelengths up to 530 nm, making it one of the few photoreceptors also sensitive to green light (Ortiz-Guerrero et al., 2011). Interestingly, light-dependent dissociation has been found to be reversible in *M. xanthus* but irreversible in *T. thermophilus*, as cleaved AdoCbl remains bound by the latter, preventing B_{12} exchange (Jost et al., 2015b).

1.2.7 Phytochromes

Phytochromes are widespread photoreceptors found in plants, bacteria, cyanobacteria and funghi. They use linear tetrapyrroles (bilins) as a chromophore to sense red ($\lambda_{max} \approx$ 660 nm) and far red light ($\lambda_{max} \approx$ 730 nm). Different forms of tetrapyrroles (e.g, biliverdin, phycocyanobilin or phytochromobilin) are used by different phytochromes (Takala et al., 2020). The chromophore is bound by the N-terminal photosensory module, comprising of a PAS, GAF and PHY domain. Many phytochromes contain additional C-terminal elements, most commonly histidine kinases, involved in signal propagation (Rockwell et al., 2017). Plant phytochromes were first discovered 50 years ago, but it was the more recent discovery of bacterial phytochromes that has accelerated research of these photoreceptors. Phytochromes have two stable photosensitive states: the red-light absorbing Pr and the far-red light absorbing Pfr state. A full photocycle involves both states: the Pr state is activated through absorption of red-light photons converting the phytochrome to the Pfr state. Upon illumination with far-red light, the Pfr state then reverts back to the Pr state (Sineshchekov, 1995; Rockwell et al., 2006). In most phytochromes, the Pr state forms the dark state, but certain bacteriophytochromes have been found to exhibit dark reversion to the Pfr state (Takala et al., 2020). Light absorption results in different signalling behaviours, depending on the phytochrome: e.g. plant phytochromes have been found to form heterodimers with PIFs (phytochrome interacting factors) (Ni et al., 1999), whereas phytochromes from cyanobacteria undergo homodimerization in response to red light (Strauss et al., 2005).

1.3 Protein-Protein Interactions

PPIs are among the core mechanisms responsible for controlling protein behaviour and thus cellular physiology. PPIs can occur in many different configurations and can be grouped by the type of complex formed (homodimers, heterodimers or more complex interactions) or whether the complex formed is stable or of a transient nature. Prominent examples of PPIs can be found in various processes: For example, ligand binding to receptor tyrosine kinases (RTKs) on the plasma membrane induces transient dimerization which leads to auto-phosphorylation and propagation of downstream signalling (Lemmon et al., 2010). Cytosolic caspase 9, on the other hand, homodimerizes and subsequently forms multimeric complexes with adaptor proteins to induce cell death (Renatus et al., 2001). Prominent tumor suppressor p53 depends on assembly into tetramers to bind to

its target DNA sequence and regulate transcription (Chène, 2001). These are just a few examples demonstrating the versatility of PPI regulation in the cell.

1.3.1 Light-controlled Protein-Protein Interactions

Using light as a stimulus makes it possible to study PPIs at a scale - both in time and space - that is not possible with classical pharmacological methods. The standard approach to achieve light-control is to to fuse a photoreceptor to the protein of interest (POI). The diversity of photoreceptors, as described above, allows for the selection of a suitable optical actuator based on different parameters. The most common considerations for this purpose are the color of light (and thus the chromophore) used, and the type of protein interaction that can be induced. The following section will give an overview of how different photoreceptors have been used to control a diverse range of proteins. This section will focus on Opto-PPIs specifically in eukaryotic cells. For all sections, I will first review the principal works followed by newer adaptations and applications of these.

1.3.2 Blue-light controlled PPIs

The majority of optogenetic tools developed to date are controlled using blue light. There are numerous reasons for this: most blue-light sensitive photoreceptors use flavin as their cofactor, which is commonly available in mammalian cells without the need for exogenous supply (Hühner et al., 2015). In addition, blue-light photoreceptors display a marked diversity in the type of PPIs they form. Furthermore, most of the photoreceptors or binding partners are relatively small and stable proteins making them convenient for protein engineering purposes.

Seminal studies using blue-light sensitive receptors generated optogenetic tools using the cryptochrome CRY2 and the LOV domains AsLOV2, NcVVD and VfAU1. The first use of CRY2 as an optogenetic tool developed a light-inducible hetero-dimerizer with its binding partner CIB1. This was then employed to demonstrate blue-light induced membrane recruitment, Gal4-dependent gene transcription and split-CRE mediated DNA recombination (Kennedy et al., 2010). The first use of CRY2 as an oligomerization tool was described a few years later, where it was used to activate the Wnt/β-catenin pathway through LRP6-CRY2 clustering (Bugaj et al., 2013). The LOV2 domain from *Avena Sativa*, AsLOV2, gave rise to many heterodimerizing systems. As a photoreceptor itself, AsLOV2 does not form dimers but relies on further engineering to form dimers. The first AsLOV2-based hetero-dimerizing system was called TULIPs (tunable, light-controlled interacting

protein tags). Here, a PDZ-binding peptide epitope was fused to the Jα-helix of AsLOV2, making it only available for PDZ binding upon illumination and Jα-unfolding (Strickland et al., 2012). A similar system with different epitopes for hetero-dimerization has also been used in the iLID (improved light-induced dimer) system (Guntas et al., 2015). The first instances of blue-light activated homo-dimerization both used a LOV domain: the photoreceptor NcVVD was used to develop the "LightOn" system to induce Gal4 dimerization and gene transcription (Wang et al., 2012), while VfAU1 enabled the first instance of membrane-protein dimerization to light-activate RTK signalling (Grusch et al., 2014). These tools were instrumental in laying the ground work for further optogenetic tools.

CRY2 has since established itself as a commonly used photoreceptor for hetero-dimerization or homo-oligomerization. In a recent study, CRY2 was fused to the intracellular domain of Notch and resulted in light-controlled inhibition of Notch/Delta signalling. Using this tool in *Drosophila*, the authors were able to reveal how Notch signalling is differentially encoded on a tissue compared to cell level (Viswanathan et al., 2019). In a very different application, the oligomerization property of CRY2 was employed to develop phase-separating "Opto-droplets" through fusion to intrinsically disordered protein regions (IDRs) (Shin et al., 2017). A follow up study also engineered a reverse system by fusing the same IDRs to the BLUF domain PixD and PixE which resulted in light-dissociable clusters. The authors used these tools to study how spatial patterning is influenced by phase separation (Dine et al., 2018).

Another new hetero-dimerizing system called "Magnets" and has recently gained popularity. Magnets were developed from the domain NcVVD which originally homo-dimerizes upon light stimulation. By mutating the dimer interface to obtain two different subunits, one more positively and one more negatively charged, the authors were able to engineer the subunits to form hetero-dimers, with advantages over other systems, such as improved kinetics and similarly sized subunits (Kawano et al., 2015). The hetero-dimerizing systems Magnets, CRY2-CIB and iLID were meticulously benchmarked and compared with each other in a recent study that sought to determine the best system for controlling intracellular localisation with light (Benedetti et al., 2018). The study elegantly illustrates how similar systems can still behave differently, and that finding the optimal tool for a certain application may require extensive testing. As a follow-up, the authors went on to characterize and optimize magnets, overcoming major disadvantages of this system, such as temperature sensitivity (Benedetti et al., 2020).

The LOV domain VfAU1 remains among the most commonly used photoreceptors

to induce light-activation of transmembrane receptors, in particular RTKs. Very recent applications include the RET (REarranged during Transfection) and BMP (bone morphogenic protein) receptors (Ingles-Prieto et al., 2021; Humphreys et al., 2020). Light-activated RET was tested as a treatment option in a *Drosophila* model of Parkinson's and showed that light-activation of the RET-MAPK-Erk pathway alleviates disease phenotypes (Ingles-Prieto et al., 2021). This study is among the first to use optogenetics as a potential treatment option for degenerative diseases, as this avenue has to date been limited to vision restoration. For the engineering of Opto-BMP receptor, the choice of the homo-dimerizing domain VfAU1 for light-activation is a less intuitive one. BMP signal activation requires the dimerization of two different receptor subtypes, BMPR1 and BMPR2. Nevertheless, Opto-BMP is able to confidently light-induce BMP signalling in chondrocytes and human embryonic stem cells (Humphreys et al., 2020). The authors base their approach on an earlier study that used VfAU1 for hetero-dimerization of Nodal receptors in zebrafish (Sako et al., 2016). While there is no feature of VfAU1 that makes it particularly suitable for heterodimerization, there are arguments that make it better suited for membrane bound proteins than other dimerization systems: the plasma membrane functions like a 2D system, not 3D like the cytosol, thus effective protein concentrations and interactions are increased (Kholodenko et al., 2000). VfAU1 seems to provide a combination of affinity and dimer lifetime that results in favourable properties for light-activated membrane receptors under these conditions, particularly low dark activity (Mitra et al., 2012; Grusch et al., 2014).

1.3.3 Green-light controlled PPIs

Tools based on the green-light controlled cobalamin binding domains (CBDs) are the most recent addition to the optogenetic toolbox. The ability to use green light for activation makes them attractive when paired with fluorescent proteins that are often sensitive to blue or red light, or for use in otherwise multi-chromatic experiments. However, the dependence of CBDs on AdoCbl as chromophore adds another layer of complexity to these experiments, since the cofactor needs to be exogenously supplied to the system (Kainrath et al., 2017).

The first application of CBDs for optogenetics was not until 2017, when two types of CarH, from *M. xanthus* and *T. thermophilus* were employed in a study to control FGFR (fibroblast growth factor receptor) activity with green light. Of note is that these photoreceptors dissociate upon illumination which results in this particular tool being "on" in the dark, but "off" in the light. This allowed the researchers

to study the effects of constitutively active FGFR signalling in zebrafish during development, without the need for constant high-intensity illumination (Kainrath et al., 2017).

Soon after, two separate studies used CarH to control gene transcription with green light. For this, they made use of the endogenous function of CarH to bind a specific DNA promoter region, CarO. Both studies achieve gene transcription through fusion of CarH to strong transcriptional activators, but light-simulation *stops* gene transcription in one system while it *activates* gene transcription in the other. In the first study, CarH binds its cognate DNA in the dark leading to transcription that is abolished upon illumination through light-induced transcription factor dissociation (Chatelle et al., 2018). The second study employs a multi-component system: One component is a CarH-transcriptional activator fusion, whereas a second component consists of CarH fused to a membrane anchor. In the dark, CarH is sequestered to the cell membrane, and only upon illumination is the CarH-transcriptional activator component released and available to bind DNA (Mansouri et al., 2021).

CarH has also found use in slightly different applications, namely to study the effect of polymer and matrix interaction on cell behaviour. The first application of this kind used CarH to engineer a protein-based hydrogel for cell culture, which solubilized upon illumination (Wang et al., 2017). Two further studies used CarH to investigate cell-matrix and cell-cell adhesion, respectively. On the one hand, CarH patterned surfaces were used for light-controlled binding of cells to the matrix (Xu et al., 2020), and on the other to control cell-cell clustering and migration with light (Nzigou Mombo et al., 2021).

1.3.4 Red-light controlled PPIs

Phytochromes are attractive photoreceptors to use for optogenetic purposes due to their red-shifted activation spectrum, which makes it easy to multiplex with the most commonly used fluorescent proteins or other optogenetic tools. In addition, the ability to switch between their Pr and Pfr states with red and far-red light provides more control over their activity than for photoreceptors that exclusively rely on dark reversion. Similar to CBDs, phytochromes incorporate an exogenous chromophore when used in eukaryotic cells (Levskaya et al., 2009). Red-light tools are also particularly attractive for *in vivo* applications due to the increased tissue penetration of red light (Reichhart et al., 2016).

The first published use of phytochromes as optogenetic tool was in 2002, before the use of microbial opsins in neuroscience even coined the term "optogenetics". This first study used the PhyB/PIF3 heterodimerizing pair from *A. thaliana* to engineer the first orthogonal light-activated gene transcription system in yeast cells, and showcased the feasibility of expressing plant receptors and supplying the chromophore to eukaryotic cells (Shimizu-Sato et al., 2002). It was several years later that the PhyB-PIF system was further optimized for use in mammalian cells. Multiple PhyB and PIF constructs were tested for expression and reversible dimerization with the conclusion that a tandem repeat of the PAS domain in PhyB was required for reversibility. The authors used this optimized PhyB-PIF6 system to activate Rho GTPase family members through translocation to the cell membrane, which allowed them to study cytoskeletal changes (Levskaya et al., 2009). The first application of a red-light homo-dimerizing system in mammalian cells repurposed not plant, but cyanobacterial phytochromes from *Synechocystis* and showed that the sensory domain was sufficient to induce dimerization and signalling of RTKs. This study also highlighted another major advantage of red-light activated tools, namely the increased tissue penetration compared to light of lower wavelengths, which is especially relevant with *in vivo* applications in mind (Reichhart et al., 2016).

The PhyB-PIF system continues to be commonly used. In a recent application, PhyB-PIF was used to activate the Ras-Raf-MEK-Erk pathway ("Opto-sos") and employed to profile the effect of Ras inhibitors and oncogenic Ras mutations on the encoding of these signals and downstream output (Bugaj et al., 2018). To further optimize the PhyB system, a recent study aimed at determining crucial PIF domains: by benchmarking several PIF variants for their PhyB binding capabilities, the authors identified PhyB-PIF variants with properties like increased binding affinities or lower basal activity (Golonka et al., 2019). Newer developments of red-light optogenetic tools revolve around the use of bacterial phytochromes. The bacterial phytochrome from *Deinococcus radiodurans*, DrBphP, exhibits inverse activity compared to plant phytochromes: In the Pr-state, DrBphP is already in a homodimer conformation, and stimulation with red light transitions DrBphP into the Pfr state in which the phytochrome is monomeric. Illumination with far-red light or prolonged darkness reverses the phytochrome back into its dimerized state. DrBphP was fused to the RTK TrkB resulting in a receptor that was turned on under far-red light or darkness, and off under red light (Leopold et al., 2019).

1.4 GPCRs

Opsins are the only photoreceptors that are transmembrane proteins. While both microbial and animal opsins are 7-TM proteins, only animal opsins belong to the family of G-protein coupled receptors (GPCRs) which have inherent downstream signalling capabilities.

GPCRs form the largest class of transmembrane proteins, with more than 800 genes in the human body. GPCRs are expressed in all tissues throughout the human body where they sense a range of extracellular stimuli, such as neurotransmitters, hormones, peptides or also light, and translate them into intracellular signals. Their involvement in many physiological processes as well as their implication in several diseases accords them exceptional pharmacological importance, which is highlighted by the fact that $\sim$ 30% of all current prescription drugs target GPCRs (Hauser et al., 2017). GPCRs can be divided into five families based on sequence and functional similarity: rhodopsin (class A), secretin (class B), glutamate (class C), Frizzled/Taste2 (class F) and adhesion receptors (Fredriksson et al., 2003). Roughly 400 receptors are olfactory receptors. Of the remaining GPCRs, class A forms the largest group, with almost 300 receptors. Of these, $\sim$ 90 receptors are still considered orphan receptors without identified ligands (Laschet et al., 2018).

1.4.1 GPCR signalling

Canonical GPCR downstream signalling is mediated, as the name suggests, through coupling to heterotrimeric G-proteins (GTP binding proteins) consisting of an α, β and γ subunit. Upon receptor activation, GPCRs undergo distinct conformational changes that result in the opening of a cytoplasmic binding site for G-proteins and other downstream effector proteins. G-proteins are classified into four main groups based on their Gα subunit: Gαs, Gαi, Gαq and Gα12 (Oldham et al., 2008). They principally act on adenylyl cyclase to increase or decrease cAMP levels, mobilize intracellular Calcium and stimulate Rho GTPases, respectively. In humans, at least 21 Gα subunits, 6 Gβ subunits and 12 Gγ subunits exist, which can assemble in a multitude of combinations, leading to functionally distinct outcomes (Masuho et al., 2021). With hundreds more GPCRs than G-proteins, G-proteins must display exquisite differentiation to bind to multiple, yet specific GPCRs. At the same time, GPCRs can signal pleoitropically via multiple G-proteins. Numerous efforts have been made to delineate the mechanism of this selectivity, but so far, no consensus motifs have been discovered, making it likely that any coupling determinants are defined through three-dimensional epitopes rather than amino acid sequence

(Flock et al., 2017; Glukhova et al., 2018). Additionally, GPCRs can signal via coupling to β-arrestin or via pathways that do not require G-proteins, such as the canonical Wnt-Frizzled pathway. GPCR signalling complexity is further increased by factors such as tissue and cell state specific expression, signalling via multiple G-proteins, GPCR dimerization or modification through other proteins (Maudsley et al., 2005; Farran, 2017). In addition, different ligands acting on the same receptor can induce distinct signalling outcomes (Wootten et al., 2018; Kenakin et al., 2013)

1.4.2 GPCR structures

GPCRs display low sequence similarity across different classes, sometimes even within the same class (Cvicek et al., 2016), but share a common topology. GPCRs are comprised of seven transmembrane helices (TM1-7), connected by three extracellular loops (ECLs 1-3) and three intracellular loops (ICLs 1-3), with an extracellular N-terminus and a cytoplasmic C-terminal tail. Rhodopsin was the first GPCR whose structure was solved, both in inactive and active form, using X-ray crystallography. These structures were vital in giving first accounts of how GPCRs are kept in an inactive state ("ionic lock)" (Palczewski et al., 2000; Scheerer et al., 2008) or the conformational rearrangements that occur on the cytoplasmic side upon receptor activation (Park et al., 2008; Rasmussen et al., 2007). The next major milestone in the understanding of GPCR functionality was reached when the first fully active, G-protein bound structure of a GPCR (β_2AR) was solved in 2011, giving detailed insight into G-protein engagement (Rasmussen et al., 2011). Since then, the number of GPCR structures in inactive, active or G-protein and arrestin bound form has increased dramatically, with more than 500 structures of over 100 unique GPCRs available, thanks in large to the advancement of cryo-EM technologies (Munk et al., 2019). The information obtained from the structural study of GPCRs has been imperative in elucidating molecular mechanisms involved in GPCR function such as the rearrangement of hallmark motifs (DRY, PIF, NPxxY), the G-protein subtype specific displacement of TM6, the determination of G-protein binding residues or the identification of conserved intra-molecular contacts (Glukhova et al., 2018; Hilger et al., 2018; Venkatakrishnan et al., 2013).

1.4.3 Review Article: Light-activated chimeric GPCRs: limitations and opportunities

Several avenues for the light-control of GPCRs have been developed to date, both using photopharmacology and optogenetics (Abreu et al., 2020). Approaches

include the use of photocaged or photoswitchable ligands (Banghart et al., 2012; Schönberger et al., 2014), photoswitchable tethered ligands (Levitz et al., 2013) or chimeric light-activated GPCRs (OptoXRs) (Kim et al., 2005; Airan et al., 2009). In this thesis, focus lies on the use of photoreceptor proteins to control protein function, such as used in OptoXRs, which is why other approaches for light-activated GPCRs will not be discussed in more detail.

Unlike Opto-PPIs, which are sequential fusions of optical actuator to protein of interest in the majority of tools, OptoXRs are intricate chimeras with multiple fusion sites between a target and an acceptor GPCR. The following Review article discusses the underlying principles of OptoXRs, the limitations current OptoXRs experience and what opportunities lie in our increased understanding of GPCRs with respect to OptoXR design.

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Light-activated chimeric GPCRs: limitations and opportunities

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Light-activated chimeric GPCRs, termed OptoXRs, can elicit cell signalling responses with the high spatial and temporal precision of light. In recent years, an expanding OptoXR toolkit has been applied to, for example, dissect neural circuits in awake rodents, guide cell migration during vertebrate development and even restore visual responses in a rodent model of blindness. OptoXRs have been further developed through incorporation of highly sensitive photoreceptor domains and a plethora of signalling modules. The availability of new high-resolution structures of GPCRs and a deeper understanding of GPCR function allows critically revisitation of the design of OptoXRs. Next-generation OptoXRs will build on advances in structural biology, receptor function and photoreceptor diversity to manipulate GPCR signalling with unprecedented accuracy and precision.

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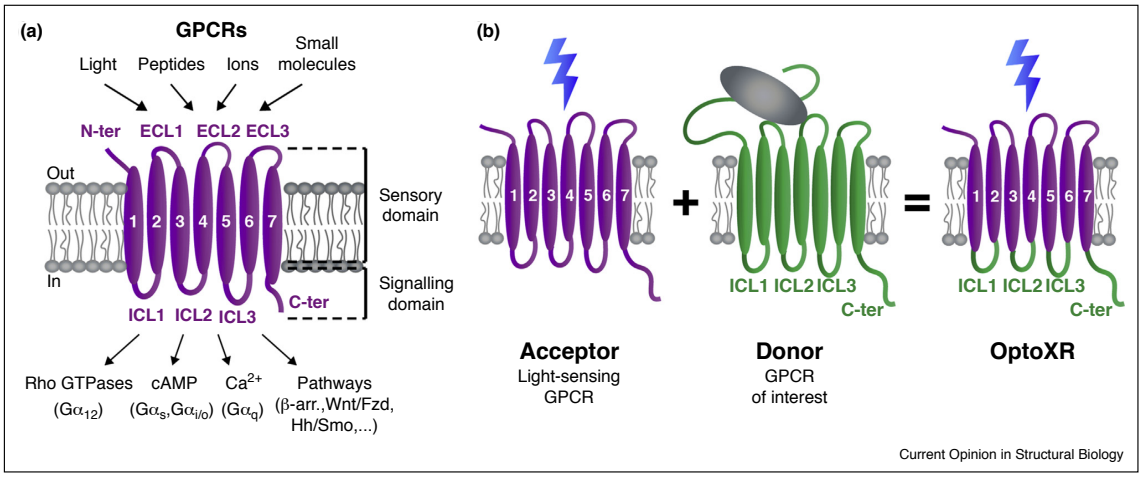
Introduction

With more than 800 expressed genes, GPCRs form the largest human receptor family. GPCRs are found in virtually all tissues and are involved in most physiological processes with exceptional importance as targets for ~30% of all prescription drugs [1,2]. Vertebrate GPCRs can be divided into five classes that are commonly referred to using the name of the prototype member of the class or a nomenclature derived from sequence alignments: The rhodopsin receptors (class A), secretin receptors (class B1, often referred to as class B), glutamate receptors (class C), frizzled/smoothened receptors (class F) and adhesion receptors (class B2) [3]. Class A is the largest class with ~700 expressed proteins in humans of which ~400 have been assigned to sensory functions (detecting mainly odorants, light or taste). In contrast, between ~10 and 30 receptors are found in the other classes. GPCRs signal through heterotrimeric G-proteins that are formed by Gα subunits of four main groups ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$) and a pair of β and γ subunits. The Gα subunits act on disparate effectors, including dual roles on adenylyl cyclases to increase ($G\alpha_s$) or decrease ($G\alpha_i$) cyclic adenosine monophosphate (cAMP) levels, activate phospholipase C to liberate intracellular Ca^{2+} ($G\alpha_q$), or stimulate Rho GTPases ($G\alpha_{12}$) [4,5]. The Gβ/γ subunits directly modulate other effectors, for example, membrane cation channels or intracellular kinases [6]. Arrestins, for example, arrestin2 and 3 (β-arrestin1 and 2), bind to the phosphorylated C-terminus of activated GPCRs to quench signalling through competition with G-proteins [7] or promotion of endocytosis [8]. Furthermore, β-arrestins assemble scaffolds to activate signalling pathways distinct from those mediated by G-proteins [9]. Signalling can also occur via other G-protein independent pathways, such as the wnt-frizzled [10] or the hedgehog-smoothened [11] axes (Figure 1a).

Outcomes of GPCR activation are generally dependent on cell type, the contextual state of the cell, and subcellular receptor localization. The emerging complex and inherently dynamic signalling activities can be difficult to dissect using pharmacological methods, which may have limited specificity for receptors or pathways, or using genetic methods, which induce chronic changes in the organism's cellular protein repertoire, including GPCRs. To provide new research tools, GPCRs have been subject to extensive molecular reengineering in the past years [12,13]. One such approach is the formation of 'chimeric' receptors named after mythological creatures composed of different parts from multiple animals [14]. In GPCR chimeras, secondary structure elements are transferred from a 'donor' to an 'acceptor' receptor to modularly create new functionalities (Figure 1b). This is possible because of a shared topology formed by seven

Figure 1



GPCR topology and OptoXR design. **(a)** GPCRs sense various extracellular stimuli through extracellular and TM domains, while the intracellular domains mediate downstream signalling. **(b)** Prototypical strategy in which domain swapping is employed to engineer light-activated chimeric GPCRs (OptoXRs).

transmembrane (TM) helices (TM1–7) connected by three extracellular loops (ECL1–3) and three intracellular loops (ICL1–3; the N-terminus is extracellular and the C-terminus intracellular) (Figure 1a). The N-terminus, TM1–7 and ECL1–3 are commonly associated with ligand binding and receptor activation, whereas ICL1–3 and the C-terminus contain residues that guide downstream signalling. GPCR chimeras have been utilized to study receptor structure and function [15,16], downstream coupling of orphan receptors [17,18], allosteric ligands [19,20], the importance of subcellular localization in signalling [21–23], as well as to achieve unprecedented control over signalling using light activation [13,24–26].

This opinion focuses on light-activated chimeric GPCRs termed OptoXRs [27] (Table 1). These receptors were developed within the field of optogenetics to permit activation of specific signalling pathways *ex vivo* and *in vivo* with high spatial (e.g. in single cells or subcellular structures) and temporal (e.g. at specific points in time over a wide range of timescales) precision; these receptors complement optogenetic/chemogenetic tools discussed in other contributions to this Special Issue. The first OptoXR emerged in 2005 [28] and, like most that followed, was constructed by replacing elements of mammalian rhodopsin (rho), the GPCR found in retinal rods and responsible for dim light vision, with those of a GPCR of interest (Figure 1b). It is noteworthy that OptoXRs are only one of several methods to trigger GPCR signalling in optogenetics. For instance, light activation can also be achieved through heterologous expression of animal opsins using their native G-protein coupling capacity with recent successes both *ex vivo* and *in vivo* [21,23,29–31,32•,33•]. Other applications of optogenetics also include light-dependent recruitment of constitutively active Gα subunits to the cell membrane [34]. Here, we first summarize the design of existing OptoXRs along with their applications. We then discuss OptoXRs in light of recent GPCR structures. Finally, we highlight possible limitations of the chimeric receptor approach and potential next-generation tools.

OptoXR designs and applications

OptoXR designs are based on seminal studies in which secondary structure elements were swapped between adrenergic receptors (ARs) [15,16]. These chimeras revealed that ICLs, in particular ICL3, are important for G-protein coupling specificity, a finding that was also demonstrated for other receptor families. The first OptoXR, termed AB2/CD2/EF2/Ct2 [28] or opto-β2AR [27], combined bovine rho (natively coupled to $G\alpha_i$) and hamster β_2AR (natively coupled to $G\alpha_s$). Opto-β2AR is arguably the best studied chimera and recapitulates many behaviors of native β2AR, including increased cAMP levels, phosphorylation of extracellular signal-regulated kinase and receptor internalization [27,35]. Activation by light followed the same kinetics as by ligand addition and occurred in a light-intensity dependent manner [35,36]. Furthermore, mutations known to regulate receptor efficacy have been successfully ported from β2AR to opto-β2AR [35]. Opto-β2AR and other OptoXRs have been utilized in awake rodents to study the neural circuits driving memory, place preference and other behaviors [27,35–39]. More recently

Table 1

OptoXRs for light activation of GPCR signalling. Receptors are listed in order of year of first publication and are of mammalian origin, unless stated otherwise. (*) Entries in the transferred elements column follow the nomenclature of the original publications

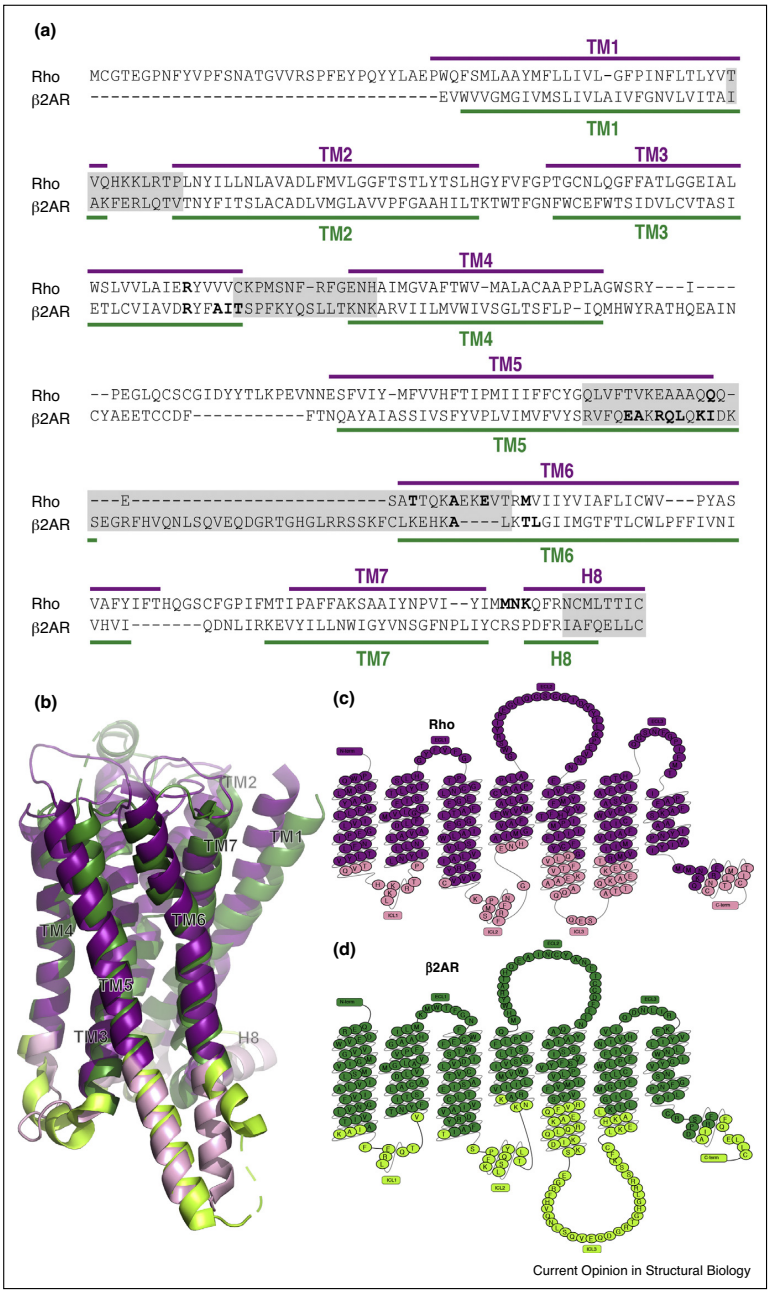
Donor	Acceptor	Transferred elements*	Application	References
β2AR and α1AR	Rho	ICL1–3, Ct	Understanding receptor function, manipulation of cell signalling *ex vivo* and manipulation of animal behavior	[27–29,35]
5-HT_{1A}	Rho	ICL1–3, Ct	None (constitutive activity upon light activation)	[22]
MOR	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo* and animal behavior	[36,37]
D1R and D2R	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo* and *in vivo*	[38,42•]
CXCR4	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo* and cell migration *in vivo*	[74]
A2AR	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo* and animal behavior	[39]
mGluR6	Mel	ICL2, 3, Ct	Manipulation of cell signalling *ex vivo* and vision restoration	[43]
Opsin (jellyfish)	OPN3	ICL3	Understanding receptor function	[17]
5-HT_{2A}	Mel	ICL1–3, Ct	Manipulation of cell signalling *ex vivo*	[60]
64 understudied and orphan receptors	Rho	ICL1–3, Ct	Understanding receptor function	[42•]
mAChR1, 2, 3	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo*	[42•]
FFR3	Rho	ICL1–3, Ct	Manipulation of cell signalling *ex vivo*	[42•]
Opsin (jellyfish)	Peropsin (spider)	ICL1–3 or ICL3	Understanding receptor function	[75•]
OPN3 (mosquito)	Peropsin (spider)	ICL1-3, Ct	Understanding receptor function	[75•]
Frizzled7 (zebrafish)	Rho	ICL3, Ct	Manipulation of signalling in development	[44•]

[42•], the OptoXR strategy was applied to understudied and orphan GPCRs for which both activating ligands and downstream signalling functions were unknown [40,41]. Using light-activated variants, activation of GPCR-dependent signalling pathways was revealed [42•]. Notably, the above mentioned OptoXRs were generated between members of the class A GPCR subfamily. The first light-activated inter-class chimera was Opto-mGluR6, which combined the class C GPCR mGluR6 and the light-sensing class A GPCR melanopsin (mel) (mel was chosen due to its desirable photochemical properties; see below) [43]. Opto-mGluR6 effectively responded to dim light and, when expressed in retinal ON-bipolar cells in a mouse model of blindness, restored behavioral light sensitivity. The most recent example of an inter-class OptoXR is Opto-Frizzled7, a class F-class A chimera employed to elucidate the effect of Wnt signalling on mesendoderm cell migration in the developing zebrafish [44•]. These examples highlight the utility of OptoXRs, through their capability to activate signalling locally and rapidly without chemical ligands, in neural circuit dissection, dissection of receptor function, during vertebrate development and even as next-generation therapeutic agents. Despite these successes, OptoXRs can be better understood and potentially improved by taking recent GPCR structural studies into account.

Structure-assisted analysis of OptoXRs

In recent years, there has been a dramatic increase in the number of high-resolution structures of GPCRs, in particular for receptors in complex with G-proteins. Rho was the first GPCR for which atomic coordinates were obtained (first in the inactive state in 2000 and in the active state in 2008 [45,46]). Structures for several other class A receptors emerged in the following years and for members of other classes in 2013 and 2014 (e.g. for the glucagon and corticotropin-releasing factor receptor 1 (class B) [47,48], smoothened (class F) [49] and mGluR1 (class C) [50]). ~300 crystal and cryo-electron microscopy structures for ~50 GPCRs are currently available, some of which were solved in complex with G-proteins for class A (including rhodopsin [51•]) and class B GPCRs [52••,53]. These structures allow identification of secondary structure elements, in particular TM helices, and key residues involved in receptor activation and transducer binding. Revisiting the general design of the prototypical rho-based opto-β2AR (Figure 2a), which combines two receptors with similar structures (Figure 2b), reveals that not only were ICLs exchanged between the two receptors but also significant segments of TM helices (e.g. in Figure 2a, grey boxes denote transferred residues and horizontal lines denote TM helices; boxes and lines partly overlap; also see Figure 2b–d). This realization may prompt revision of the historic terminology that OptoXRs originate from ICL exchanges. Of particular relevance for analysis of OptoXRs is progress in the identification of residues that interact with G-proteins. During GPCR activation, TM5 and TM6 rotate away from each other, in a receptor subtype-specific manner, opening a cytoplasmic cavity for

Figure 2



Structure-assisted analysis of opto-β2AR. **(a)** Sequence alignment obtained by superimposing active state structures of rho (PDB ID 6CMO) and β2AR (PDB ID 3SN6) using *cealign* in PyMol (Schrödinger, Inc). Helix boundaries were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank website using the *sequence* feature. Grey: swapped intracellular domains to generate opto-β2AR. Bold: residues located specifically in helices and in contact with G-proteins (taken from Ref. [52••]). **(b)** Superimposed active state structures from (a) highlighting the residues that were retained (purple) or replaced in rho (pink) with those of β2AR (light green) in the prototypical opto-β2AR. **(c** and **d)** Snake-plots of human rho and β2AR secondary structures (adapted from GPCRdb) with the colours as in (b). Not shown here is a short fragment of rho (TETSQVAPA) that is retained at the far C-terminus of opto-β2AR and other rho-based OptoXRs.

G-protein insertion. The residues of class A and class B GPCRs that interact with Gα are mainly found in TM3, TM5, TM6, ICL2, ICL3, and the C-terminus [52••,54••]. In rho and β2AR, some of these residues are located in the TM helices up to several turns away from the intracellular helix ends (e.g. see residues highlighted in bold in TM3, TM5, or TM6 in Figure 2a). Notably, the analysis of opto-β2AR reveals that not all of these residues have been incorporated in the chimera (Figure 2a; some bold residues lie outside of the grey boxes). The degree of conservation at these sites (e.g. a high degree of conservation for receptors from the same family) and at potential interaction sites located nearby [52••,54••] will be specific to the acceptor and donor GPCR. It is therefore difficult to predict how omissions of donor residues at these sites impact OptoXR function, and examination of these regions provides one approach to address chimeras that are expressed and membrane-localized but not functional.

In the context of receptor activation, systematic analysis of non-covalent contacts in multiple recent class A receptor structures revealed broadly conserved interhelical interactions linked to this process [55,56]. In particular, a network of interactions formed on the intracellular side of active receptors points to a consensus conformation that may underlie the success of chimeras within this receptor class. Whereas similarities in the activation mechanism of GPCRs from different classes exist, such as the outward movement of TM6, there are also significant differences (e.g. the extent of kinking and outward movement of TM6 is greater in class B GPCRs) [57,58]. It thus remains to be seen whether inter-class chimeras can be designed rationally, which will be more likely successful in the case of receptors that show highly similar conformational changes during activation.

Limitations of OptoXRs

As with any other experimental approach, inherent limitations exist when using OptoXRs. One central question is to what extent chimeras mimic wildtype receptors. Many studies showed that OptoXRs can activate the same downstream signalling pathways as the donor receptors. For instance, signalling of nine chimeras activated by light was recently compared side by side to that of the donor receptors activated by their cognate ligands. In four transcriptional reporter assays, false positive or false negative downstream signalling was not observed [42•]. However, chimeric receptors that did not show the predicted functionality [22,59] or exhibited reduced folding and stability [28,60] have been reported in some cases. These results indicate that careful functional analysis of engineered receptors is required, which should include real-time assays of secondary messengers and receptor internalization [35,36].

It is likely that important aspects of GPCR function are not preserved in chimeras. For instance, the strength and bias between G-protein-dependent and β-arrestin-dependent signalling activities of GPCRs are intricately linked to specific ligand–receptor interactions that are absent in OptoXRs [58,61–63]. Notably, detailed information on pathway bias in OptoXR signalling, which may have profound consequences on how cell behavior is modulated, is missing because only few studies included multiple cell signalling assays. Similarly, other receptor behaviors, including dimerization or association with co-receptors, contribute to GPCR function [64,65]. These behaviors are often driven by the TM helices and/or N-terminal extracellular domains that are not included in OptoXRs. Finally, it has been shown that overexpressed receptors can compete with endogenous receptors, for example, for localization in functional compartments [22,26]. In summary, it is advisable to carefully consider experimental context when working with OptoXRs to determine which properties of the engineered receptors are required. It is noteworthy that recapitulation of only some aspects of donor receptor function could also be exploited to investigate how specific signalling axes contribute to a cell or animal phenotype [35]. Naturally occurring light-activated GPCRs provide ready-made alternatives to OptoXRs that can also activate specific G-protein-dependent and β-arrestin-dependent signalling pathways upon heterologous expression [21–23,29–31,32•,33•] and even may offer favorable photochemical properties (see below). However, these receptors may also activate only a subset of signalling pathways linked to the endogenous GPCR of interest and may also do so from different subcellular compartments (a shortcoming that can be addressed by inclusion of targeting sequence motifs [21–23]).

Next-generation designs

The coming years will likely see improved OptoXRs. It is noteworthy that while vertebrate rho is well studied, likely a key factor when choosing it for early designs, it has some disadvantages in the context of OptoXRs. For instance, the wavelength of maximal absorption of rho is ~500 nm and the peak width at half maximum absorption is ~100 nm [66]. This suggests efficient photon absorption of broad visible light. Whereas this property can be advantageous in some animal models that require specific wavelengths for efficient tissue penetration, it in turn limits compatibility in the use of other optogenetic tools and fluorescent reporters in the same experiment. Spectral tuning of OptoXRs could be achieved through point mutations, such as those found in human cone opsins that have been applied to rhodopsin previously [67,68], or through a different acceptor. Specifically, short-wavelength-sensitive or long-wavelength-sensitive cone opsins or invertebrate opsins may free up blue or yellow wavelengths, and multiple OptoXRs or other optogenetic tools could then be used one at a time or in combination to test for the synergistic/antagonistic action of multiple signals. Further red-shifted absorption may also aid

in vivo applications because red light penetrates tissues better than blue or green light. One additional property of rho is that its photocycle entails very slow loss and subsequent replenishing of the retinal chromophore. This 'bleaching' effectively limits the photoresponse magnitude and light sensitivity. Nature has evolved many opsin classes that form stable photoactive states and subsequently are able to self-regenerate photosensitivity (termed 'bistability') [69]. Notably, Opto-mGluR6 employs a mel core, which is light-sensitive, resistant to bleach and can be toggled on/off with two colors of light [33•,43]. The absence of high-resolution structures for mel limits further rational design, but this may not be the case for other invertebrate opsins with desirable photochemical properties and known structures [70,71]. In summary, acceptors other than rho may permit new types of experiments, including those with multiplexed optogenetic tools in parallel and with lower light intensities. Irrespective of the choice of acceptor, the ability of OptoXRs to be activated by light, independent of diffusible ligands, could be combined with localization of these receptors to distinct subcellular compartments. The impact of GPCR signalling from different compartments is an exciting area of research [72,73] that will likely benefit from OptoXRs.

Conclusions

OptoXRs provide unique opportunities to interrogate how complex GPCR-mediated signalling activity regulates cell and animal behavior. The unprecedented spatial and temporal precision endowed by using light has enabled novel finely tuned experiments in the nervous system and during vertebrate development, and one OptoXR has been suggested as a novel therapeutic agent. In general, OptoXRs were designed to enable controlled inputs by preserving the topology and conformational changes of the light-sensitive acceptor GPCR and at the same time providing binding sites for donor-specific downstream signalling components. Our analysis suggests that past OptoXRs did not contain all residues that interact with Gα in the donor. This finding suggests that purely sequence-based 'cut-and-paste' approaches, albeit yielding functional proteins, may be refined with structural information, including additional knowledge about downstream binding partner interaction. Structural analysis and refined designs will be of particular importance in inter-class chimeras as acceptor and donor receptors have lower degrees of sequence and structural conservation. It is important to recognize that chimeras, such as OptoXRs, are activation tools and cannot fully replicate native receptor behavior; nonetheless, great promise lies in tuned OptoXRs to better exploit the high levels of spatial and temporal control afforded by light activation.

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Hauser AS, Attwood MM, Rask-Andersen M, Schioth HB, Gloriam DE: **Trends in GPCR drug discovery: new agents, targets and indications**. *Nat Rev Drug Discov* 2017, **16**:829-842.
2. Rask-Andersen M, Almen MS, Schioth HB: **Trends in the exploitation of novel drug targets**. *Nat Rev Drug Discov* 2011, **10**:579-590.
3. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB: **The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints**. *Mol Pharmacol* 2003, **63**:1256-1272.
4. Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL: **G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly**. *J Biol Chem* 1995, **270**:24631-24634.
5. Oldham WM, Hamm HE: **Heterotrimeric G protein activation by G-protein-coupled receptors**. *Nat Rev Mol Cell Biol* 2008, **9**: 60-71.
6. Wess J: **Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications**. *Annu Rev Pharmacol Toxicol* 2004, **44**:423-450.
7. Peterson YK, Luttrell LM: **The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling**. *Pharmacol Rev* 2017, **69**:256-297.
8. Tian X, Kang DS, Benovic JL: **Beta-arrestins and G protein-coupled receptor trafficking**. *Handb Exp Pharmacol* 2014, **219**:173-186.
9. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK: **Beta-arrestins and cell signaling**. *Annu Rev Physiol* 2007, **69**:483-510.
10. Logan CY, Nusse R: **The Wnt signaling pathway in development and disease**. *Annu Rev Cell Dev Biol* 2004, **20**:781-810.
11. Arensdorf AM, Marada S, Ogden SK: **Smoothened regulation: a tale of two signals**. *Trends Pharmacol Sci* 2016, **37**:62-72.
12. Roth BL: **DREADDs for neuroscientists**. *Neuron* 2016, **89**:683-694.
13. Keri D, Barth P: **Reprogramming G protein coupled receptor structure and function**. *Curr Opin Struct Biol* 2018, **51**:187-194.
14. Homer: **The Iliad** In: 6. 1260–1180 BC.
15. Cotecchia S, Ostrowski J, Kjelsberg MA, Caron MG, Lefkowitz RJ: **Discrete amino acid sequences of the alpha 1-adrenergic receptor determine the selectivity of coupling to phosphatidylinositol hydrolysis**. *J Biol Chem* 1992, **267**:1633-1639.
16. Kobilka BK, Kobilka TS, Daniel K, Regan JW, Caron MG *et al.*: **Chimeric alpha 2-,beta 2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity**. *Science* 1988, **240**:1310-1316.
17. Sugihara T, Nagata T, Mason B, Koyanagi M, Terakita A: **Absorption characteristics of vertebrate non-visual opsin, Opn3**. *PLoS One* 2016, **11**:e0161215.

18. Liu X, Liu T, Slusarski DC, Yang-Snyder J, Malbon CC *et al.*: **Activation of a frizzled-2/beta-adrenergic receptor chimera promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via Galphao and Galphat**. *Proc Natl Acad Sci U S A* 1999, **96**:14383-14388.

19. Thiele S, Steen A, Jensen PC, Mokrosinski J, Frimurer TM *et al.*: **Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach**. *J Biol Chem* 2011, **286**:37543-37554.

20. Zweemer AJ, Bunnik J, Veenhuizen M, Miraglia F, Lenselink EB *et al.*: **Discovery and mapping of an intracellular antagonist binding site at the chemokine receptor CCR2**. *Mol Pharmacol* 2014, **86**:358-368.

21. Masseck OA, Spoida K, Dalkara D, Maejima T, Rubelowski JM *et al.*: **Vertebrate cone opsins enable sustained and highly sensitive rapid control of Gi/o signaling in anxiety circuitry**. *Neuron* 2014, **81**:1263-1273.

22. Oh E, Maejima T, Liu C, Deneris E, Herlitze S: **Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor**. *J Biol Chem* 2010, **285**:30825-30836.

23. Spoida K, Masseck OA, Deneris ES, Herlitze S: **Gq/5-HT2c receptor signals activate a local GABAergic inhibitory feedback circuit to modulate serotonergic firing and anxiety in mice**. *Proc Natl Acad Sci U S A* 2014, **111**:6479-6484.

24. Kleinlogel S: **Optogenetic user's guide to Opto-GPCRs**. *Front Biosci (Landmark Ed)* 2016, **21**:794-805.

25. Spangler SM, Bruchas MR: **Optogenetic approaches for dissecting neuromodulation and GPCR signaling in neural circuits**. *Curr Opin Pharmacol* 2017, **32**:56-70.

26. Masseck OA, Rubelowski JM, Spoida K, Herlitze S: **Light- and drug-activated G-protein-coupled receptors to control intracellular signalling**. *Exp Physiol* 2011, **96**:51-56.

27. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K: **Temporally precise in vivo control of intracellular signalling**. *Nature* 2009, **458**:1025-1029.

28. Kim JM, Hwa J, Garriga P, Reeves PJ, RajBhandary UL *et al.*: **Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops**. *Biochemistry* 2005, **44**:2284-2292.

29. Bailes HJ, Zhuang LY, Lucas RJ: **Reproducible and sustained regulation of Galphas signalling using a metazoan opsin as an optogenetic tool**. *PLoS One* 2012, **7**:e30774.

30. Cehajic-Kapetanovic J, Eleftheriou C, Allen AE, Milosavljevic N, Pienaar A *et al.*: **Restoration of vision with ectopic expression of human rod opsin**. *Curr Biol* 2015, **25**:2111-2122.

31. De Silva SR, Barnard AR, Hughes S, Tam SKE, Martin C *et al.*: **Long-term restoration of visual function in end-stage retinal degeneration using subretinal human melanopsin gene therapy**. *Proc Natl Acad Sci U S A* 2017, **114**:11211-11216.

32. • Makowka P, Bruegmann T, Dusend V, Malan D, Beiert T *et al.*: **Optogenetic stimulation of Gs-signaling in the heart with high spatio-temporal precision**. *Nat Commun* 2019, **10**:1281.
Study investigating the effect of Gs signalling in transgenic myocytes as well as in live, *ex vivo* heart preparations using an invertebrate opsin and comparing it to chemically induced Gs activation.

33. • Spoida K, Eickelbeck D, Karapinar R, Eckhardt T, Mark MD *et al.*: **Melanopsin variants as intrinsic optogenetic on and off switches for transient versus sustained activation of G protein pathways**. *Curr Biol* 2016, **26**:1206-1212.
Comparison of human and murine melanopsin variants with regards to photochemical properties. This study shows differences in kinetics (sustained/transient activation) as well as wavelength dependence between the two receptors.

34. Yu G, Onodera H, Aono Y, Kawano F, Ueda Y *et al.*: **Optical manipulation of the alpha subunits of heterotrimeric G proteins using photoswitchable dimerization systems**. *Sci Rep* 2016, **6**:35777.

35. Siuda ER, McCall JG, Al-Hasani R, Shin G, Il Park S *et al.*: **Optodynamic simulation of beta-adrenergic receptor signalling**. *Nat Commun* 2015, **6**:8480.

36. Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R *et al.*: **Spatiotemporal control of opioid signaling and behavior**. *Neuron* 2015, **86**:923-935.

37. Barish PA, Xu Y, Li J, Sun J, Jarajapu YP *et al.*: **Design and functional evaluation of an optically active mu-opioid receptor**. *Eur J Pharmacol* 2013, **705**:42-48.

38. Gunaydin LA, Grosenick L, Finkelstein JC, Kauvar IV, Fenno LE *et al.*: **Natural neural projection dynamics underlying social behavior**. *Cell* 2014, **157**:1535-1551.

39. Li P, Rial D, Canas PM, Yoo JH, Li W *et al.*: **Optogenetic activation of intracellular adenosine a receptor signaling in the hippocampus is sufficient to trigger CREB phosphorylation and impair memory**. *Mol Psychiatry* 2015, **20**:1339-1349.

40. Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R *et al.*: **G protein-coupled receptor deorphanizations**. *Annu Rev Pharmacol Toxicol* 2013, **53**:127-146.

41. Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE *et al.*: **International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands**. *Pharmacol Rev* 2013, **65**:967-986.

42. • Morri M, Sanchez-Romero I, Tichy AM, Kainrath S, Gerrard EJ *et al.*: **Optical functionalization of human class A orphan G-protein-coupled receptors**. *Nat Commun* 2018, **9**:1950.
Innovative study in which OptoXR engineering was applied to virtually all human class A orphan GPCRs, followed by characterization of downstream signalling. This study also includes a comparison of nine OptoXRs side-by-side with ligand-activated donors.

43. van Wyk M, Pielecka-Fortuna J, Lowel S, Kleinlogel S: **Restoring the ON switch in blind retinas: opto-mGluR6, a next-generation, cell-tailored optogenetic tool**. *PLoS Biol* 2015, **13**:e1002143.

44. • Capek D, Smutny M, Tichy AM, Morri M, Janovjak H *et al.*: **Light-activated Frizzled7 reveals a permissive role of non-canonical Wnt signaling in mesendoderm cell migration**. *eLife* 2019, **8**.
First demonstration of a light-activated class A-class F chimera (Opto-Frizzled) that permitted investigating the effect of Wnt signalling on cell migration in the developing zebrafish mesendoderm.

45. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H *et al.*: **Crystal structure of rhodopsin: a G protein-coupled receptor**. *Science* 2000, **289**:739-745.

46. Park JH, Scheerer P, Hofmann KP, Choe H-W, Ernst OP: **Crystal structure of the ligand-free G-protein-coupled receptor opsin**. *Nature* 2008, **454**:183-187.

47. Hollenstein K, Kean J, Bortolato A, Cheng RK, Dore AS *et al.*: **Structure of class B GPCR corticotropin-releasing factor receptor 1**. *Nature* 2013, **499**:438-443.

48. Siu FY, He M, de Graaf C, Han GW, Yang D *et al.*: **Structure of the human glucagon class B G-protein-coupled receptor**. *Nature* 2013, **499**:444-449.

49. Wang C, Wu H, Katritch V, Han GW, Huang XP *et al.*: **Structure of the human smoothened receptor bound to an antitumour agent**. *Nature* 2013, **497**:338-343.

50. Wu H, Wang C, Gregory KJ, Han GW, Cho HP *et al.*: **Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator**. *Science* 2014, **344**:58-64.

51. • Kang Y, Kuybeda O, de Waal PW, Mukherjee S, Van Eps N *et al.*: **Cryo-EM structure of human rhodopsin bound to an inhibitory G protein**. *Nature* 2018, **558**:553-558.
First determination of the structure of rhodopsin in complex with $G\alpha_i$. Through comparison with receptor structures bound to other G-proteins, structural signatures and underlying G-protein coupling selectivity are explored. First determination of the structure of rhodopsin in complex with $G\alpha$I. Through comparison with receptor structures bound to other G-proteins, structural signatures and underlying G-protein coupling selectivity are explored.

52. •• Glukhova A, Draper-Joyce CJ, Sunahara RK, Christopoulos A, Wootten D *et al.*: **Rules of engagement: GPCRs and G proteins**. *ACS Pharm Transl Sci* 2018, **1**:73-83.
Detailed analysis of recent active state GPCR-G-protein complexes, with a focus on activation mechanisms, G-protein binding residues and G-protein selectivity.

53. Munk C, Mutt E, Isberg V, Nikolajsen LF, Bibbe JM *et al.*: **An online resource for GPCR structure determination and analysis**. *Nat Methods* 2019, **16**:151-162.

54. •• Flock T, Hauser AS, Lund N, Gloriam DE, Balaji S *et al.*: **Selectivity determinants of GPCR-G-protein binding**. *Nature* 2017, **545**:317-322.
Systematic analysis of non-covalent GPCR-G-protein interactions in GPCR structures that identifies the molecular signatures of GPCR-G-protein interaction, both with regards to binding and specificity.

55. Venkatakrishnan AJ, Deupi X, Lebon G, Heydenreich FM, Flock T *et al.*: **Diverse activation pathways in class A GPCRs converge near the G-protein-coupling region**. *Nature* 2016, **536**:484-487.

56. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF *et al.*: **Molecular signatures of G-protein-coupled receptors**. *Nature* 2013, **494**:185-194.

57. Bortolato A, Dore AS, Hollenstein K, Tehan BG, Mason JS *et al.*: **Structure of Class B GPCRs: new horizons for drug discovery**. *Br J Pharmacol* 2014, **171**:3132-3145.

58. Wootten D, Christopoulos A, Marti-Solano M, Babu MM, Sexton PM: **Mechanisms of signalling and biased agonism in G protein-coupled receptors**. *Nat Rev Mol Cell Biol* 2018, **19**:638-653.

59. Kozell LB, Neve KA: **Constitutive activity of a chimeric D2/D1 dopamine receptor**. *Mol Pharmacol* 1997, **52**:1137-1149.

60. McGregor KM, Becamel C, Marin P, Andrade R: **Using melanopsin to study G protein signaling in cortical neurons**. *J Neurophysiol* 2016, **116**:1082-1092.

61. Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C *et al.*: **Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states**. *J Biol Chem* 2004, **279**:686-691.

62. Violin JD, Crombie AL, Soergel DG, Lark MW: **Biased ligands at G-protein-coupled receptors: promise and progress**. *Trends Pharmacol Sci* 2014, **35**:308-316.

63. Wacker D, Wang C, Katritch V, Han GW, Huang X-P *et al.*: **Structural features for functional selectivity at serotonin receptors**. *Science* 2013, **340**:615-619.

64. Harikumar KG, Augustine ML, Lee LT, Chow BK, Miller LJ: **Structure and function of cross-class complexes of G protein-coupled secretin and angiotensin 1a receptors**. *J Biol Chem* 2016, **291**:17332-17344.

65. Farran B: **An update on the physiological and therapeutic relevance of GPCR oligomers**. *Pharmacol Res* 2017, **117**: 303-327.

66. Sakmar TP, Franke RR, Khorana HG: **Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin**. *Proc Natl Acad Sci U S A* 1989, **86**:8309-8313.

67. Sekharan S, Katayama K, Kandori H, Morokuma K: **Color vision: "OH-site" rule for seeing red and green**. *J Am Chem Soc* 2012, **134**:10706-10712.

68. Chan T, Lee M, Sakmar TP: **Introduction of hydroxyl-bearing amino acids causes bathochromic spectral shifts in rhodopsin. Amino acid substitutions responsible for red-green color pigment spectral tuning**. *J Biol Chem* 1992, **267**:9478-9480.

69. Tsukamoto H, Terakita A: **Diversity and functional properties of bistable pigments**. *Photochem Photobiol Sci* 2010, **9**:1435-1443.

70. Gerrard E, Mutt E, Nagata T, Koyanagi M, Flock T *et al.*: **Convergent evolution of tertiary structure in rhodopsin visual proteins from vertebrates and box jellyfish**. *Proc Natl Acad Sci U S A* 2018, **115**:6201-6206.

71. Murakami M, Kouyama T: **Crystal structure of squid rhodopsin**. *Nature* 2008, **453**:363.

72. Eichel K, von Zastrow M: **Subcellular organization of GPCR signaling**. *Trends Pharmacol Sci* 2018, **39**:200-208.

73. Ellisdon AM, Halls ML: **Compartmentalization of GPCR signalling controls unique cellular responses**. *Biochem Soc Trans* 2016, **44**:562-567.

74. Xu Y, Hyun YM, Lim K, Lee H, Cummings RJ *et al.*: **Optogenetic control of chemokine receptor signal and T-cell migration**. *Proc Natl Acad Sci U S A* 2014, **111**:6371-6376.

75. • Nagata T, Koyanagi M, Lucas R, Terakita A: **An all-trans-retinal-binding opsin peropsin as a potential dark-active and light-inactivated G protein-coupled receptor**. *Sci Rep* 2018, **8**:3535.
Detailed characterization of spider peropsin and first demonstration of dark-active, light-inactivated control of $G\alpha_s$ and $G\alpha_i$ signalling through chimeric approaches using peropsin as the acceptor GPCR.

Chapter 2

Engineering of light-activated protein-protein interactions

2.1 Chapter Summary

In this chapter, we set out develop a method to streamline the process of engineering optogenetic tools for the control of protein-protein interactions. The expansion of the optogenetic toolbox is providing ever more opportunities and possibilities for these tools to be utilized. However, the number of possible processes or proteins of interest that can be controlled through PPIs far exceeds the optogenetic tools available to precisely target all of them. For the interrogation of a specific process, it is therefore often necessary to newly engineer an optogenetic tool. This process is not always a straight forward one. For example, different photoreceptors may work better or worse for a given target protein, or multiple optimization steps, such as fusion site or linker length, may be required to obtain an ideal conformation. Here, we developed a simple engineering strategy as well as a plasmid library to facilitate this process of testing different photoreceptors and conformations. The plasmid library provides access to the most commonly used photoreceptors for PPI control, while the engineering strategy simplifies the cloning and testing of multiple photoreceptors, in multiple conformations. In a proof-of-principle application, we use the library to develop a light-inducible caspase 9, Opto-casp9, to control apoptosis. In the context of establishing optogenetict tools and experiments, Opto-casp9 can be used to test illumination devices and light-contamination with an easy to determine read-out, namely cell death/survival. Together, this presents a new resource that will enable an even broader use of optogenetics for the control PPIs. Especially for researchers whose primary focus lies in the application, rather than the development of specific optogenetic tools, we envision this resource to lower the barrier and make optogenetics for the

control of cell signalling even more accessible.

2.2 Research Paper: Engineering strategy for Opto-PPIs

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Supplementary material can be found in Appendix A.

Engineering Strategy and Vector Library for the Rapid Generation of Modular Light-Controlled Protein–Protein Interactions

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Abstract

Optogenetics enables the spatio-temporally precise control of cell and animal behavior. Many optogenetic tools are driven by light-controlled protein–protein interactions (PPIs) that are repurposed from natural light-sensitive domains (LSDs). Applying light-controlled PPIs to new target proteins is challenging because it is difficult to predict which of the many available LSDs, if any, will yield robust light regulation. As a consequence, fusion protein libraries need to be prepared and tested, but methods and platforms to facilitate this process are currently not available. Here, we developed a genetic engineering strategy and vector library for the rapid generation of light-controlled PPIs. The strategy permits fusing a target protein to multiple LSDs efficiently and in two orientations. The public and expandable library contains 29 vectors with blue, green or red light-responsive LSDs, many of which have been previously applied *ex vivo* and *in vivo*. We demonstrate the versatility of the approach and the necessity for sampling LSDs by generating light-activated caspase-9 (casp9) enzymes. Collectively, this work provides a new resource for optical regulation of a broad range of target proteins in cell and developmental biology.

Introduction

Optogenetics has revolutionized research in neuroscience, cell biology and developmental biology by allowing the "remote control" of cell and animal behavior with extraordinary precision [1–5]. This precision is achieved by utilizing light as a stimulus that offers unique advantages over pharmacological and genetic manipulation. For instance, light permits unparalleled control in time (e.g., to modulate animal behavior acutely or to target selected developmental or disease stages; Fig. 1A) and in space (e.g., to target selected compartments in a cell or selected cells in a tissue; Fig. 1B). Also, light can be readily applied and withdrawn given a sufficiently transparent matrix. Finally, light-activated molecular tools can be paired with genetic targeting to allow an even higher level of precision for specific cell types, tissues or developmental stages [6–10].

Optogenetics first flourished in the hands of neuroscientists that utilized animal and microbial opsins to dissect neural circuits through the bidirectional control of neuronal bioelectrical activity [8,11]. More recently and in cell types other than neurons, light control of gene regulation and cellular signaling, together with associated cell behaviors, has

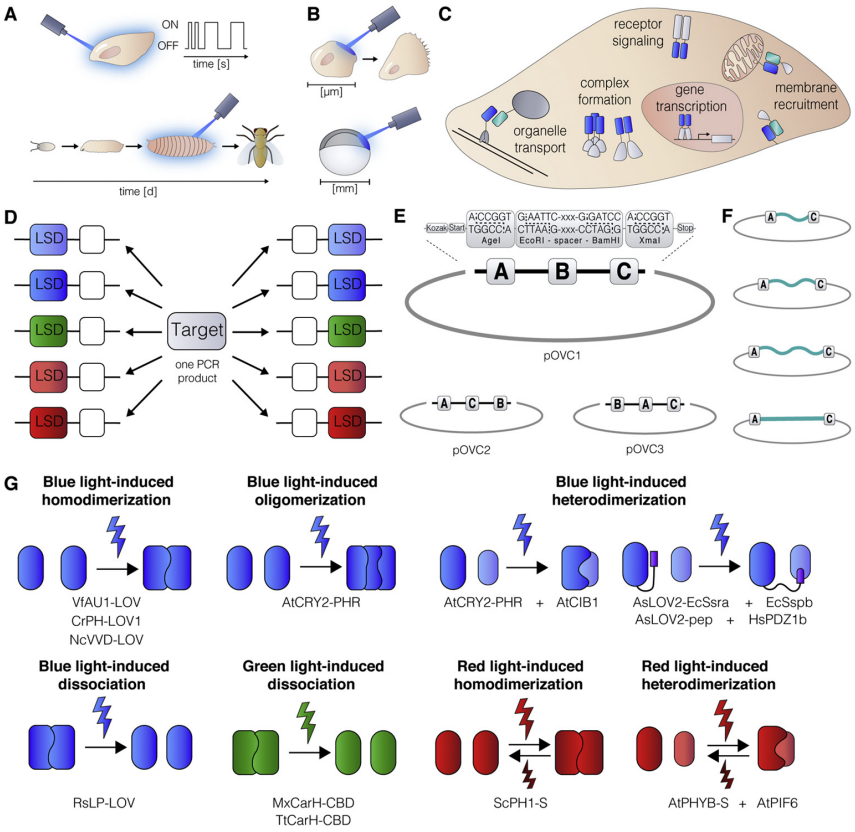

Fig. 1. Genetic engineering strategy and vector library for spatio-temporally precise regulation of cell and animal behavior. (A) The high temporal precision of light can be harnessed to study cellular responses to repetitive and complex inputs and to target specific developmental stages. (B) The high spatial precision of light can be harnessed to selectively activate processes in parts of cells or in specific tissues. (C) Optical control of many cellular processes relies on the regulation of PPIs by incorporating LSDs. (D) Engineering strategy based on a vector library that permits universal target amplicon insertion in a single cloning step (one digest, one ligation). (E) *ABC* and alternative cassettes to realize engineering strategy. Cassettes include Kozak sequence, start codon and stop codon. (F) *ABC* cassettes containing alternative linkers. (G) LSDs and their binding partners included in the library.

emerged [12,13]. The optogenetic tools that can regulate cell bioelectricity are fundamentally different from those applied to control biochemical and enzymatic processes. In the former case, ion-conducting opsins, such as channelrhodopsin or halorhodopsin, turn neurons on or off by changing their membrane potential through an intrinsic light-gated ion channel or pump activity [7,8,14]. In the latter case, a wide range of cellular processes have been rendered light-inducible by using light-sensitive domains (LSDs) that do not harbor catalytic activity but regulate intra- or intermolecular binding events (Fig. 1C).

LSDs are found in organisms from all domains of life and collectively respond not only to all visible but also to ultraviolet and far-red wavelengths [15–17]. Of particular importance in the field of optogenetics are light-oxygen-voltage sensing (LOV) domains and cryptochromes (CRYs) that bind flavins to sense blue light (maximal absorption wavelength ($\lambda_{max} \approx 450$ nm) [18–20] and phytochromes (PHYs) that utilize linear tetrapyrroles to sense red ($\lambda_{max} \approx 660$ nm) and far-red ($\lambda_{max} \approx 720$ nm) light [21–23]. In addition, green light-sensitive ($\lambda_{max} \approx 550$ nm) cobalamin binding domains (CBDs) that bind vitamin B_{12} derivatives were applied more recently [24,25]. The molecular consequences of photon absorption are either (i) light-induced unmasking of terminal peptides [26,27] for some LOV domains; (ii) light-induced homodimerization [18,19,21], homooligomerization [28] and heterodimerization with their respective accessory proteins

[20,22,29,30] for some LOV domains, CRYs and PHYs; or (iii) light-induced monomerization for some LOV domains, CBDs and UVR8 [24,31–33]. These functions have been harnessed in seminal studies to regulate the interactions and activity of diverse target proteins, such as small GTPases, kinases and transcription regulators [6,24,34–42].

The plethora of cellular processes governed by protein–protein interactions (PPIs) currently far exceeds the number of available optogenetic tools. This is in part because generating functional fusion proteins of LSDs and target proteins is a non-trivial task. For instance, multiple LSD genes need to be obtained and validated to find a suited domain, and the location of the fusion site as well as the length of linkers can be critical parameters that determine fusion protein function [43]. As a consequence of combinatorial complexity, many genetic constructs need to be generated and tested, and currently, no methods or libraries are available to facilitate this process.

Here, we developed a genetic engineering strategy and a vector library for the rapid and modular generation of light-controlled PPIs. The engineering strategy can produce LSD–target protein fusions in several domain orientations and with linkers in a single cloning step (a universal restriction enzyme digest followed by ligation) using inexpensive and readily available materials. The publicly available vector library contains a collection of prominent LSDs that are responsive to blue, green or red light and have been applied in the past *ex vivo* and *in vivo*. The design of the strategy and library allows for easy expansion either with further LSDs, targeting sequences or markers. Using this resource, we generated light-activated casp9 enzymes.

Results and Discussion

Efficient genetic engineering strategy

A major challenge in the optical control of PPIs is to achieve functional coupling of LSD oligomerization state changes to activity of target proteins. For most target proteins, it is initially unclear if a suited LSD can be identified and in what orientation LSDs are best attached because steric compatibility and effects on protein folding are difficult to predict. In the majority of previous studies, LSD–target protein fusions were constructed by inserting several LSD genes into vectors that contain the target protein (Sup. Fig. S1A, top). This approach requires selecting candidate LSDs, obtaining the corresponding genes from collaborators or commercial sources, validating LSD sequences, delineating domain boundaries and preparing amplicons that adapt each LSD to the target vector (Sup. Fig. S1A, bottom). Furthermore, generation of both N- and C-terminal fusion proteins may require additional modification of the vector and/or amplicons. We propose an inverted strategy in which the target protein is inserted into a series of vectors that already contain LSDs (Fig. 1D; see below for a comprehensive LSD vector library). The advantages of this strategy are that only a single amplicon of a familiar and available target gene is required and that multiple LSD–target protein fusions can be generated in a simple standardized reaction that is easily parallelized. As a consequence, multiple time-consuming steps that require analysis of sequences and reagents specific to each LSD are not required and the workflow is greatly simplified (Sup. Fig. S1B).

To achieve this strategy, we designed a modular cloning cassette termed *ABC* that harbors three insertion sites (*A*, *B* and *C*; Fig. 1E). Importantly, sites *A* and *C* contain recognition sequences for restriction enzymes that produce compatible cohesive overhangs (in both cases, a CCGG overhang after AgeI or XmaI digestion at site *A* and *C*, respectively; Fig. 1E). Consequently, a target protein amplicon flanked by either of these restriction sites in any combination can be inserted into site *A* as well as *C* and thus N- and C-terminally of a LSD (start and stop codons are already contained in the cassette). Site *B* contains recognition sequences for restriction enzymes of different families (EcoRI and BamHI) for incorporation of additional domains (e.g., fluorescent proteins) or epitopes. In order to provide additional flexibility, we engineered *ABC* vectors to include four different flexible or stiff linkers (Fig. 1F). We also prepared compatible *ACB* and *BAC* cassettes that permit insertion of flanking targeting sequences or fluorescent proteins in terminal *B* sites. Utilization of single and compatible restriction sites in site *A* and site *C* maximizes the likelihood that target proteins can be inserted without interference from internal restriction sites and minimizes required reagents. Furthermore, restriction enzymes are inexpensive and their application in the cassette retains advanced genetic engineering methods, such as those based on DNA recombination, for transfer of cassettes into other vectors. The promoter region in these vectors can be readily exchanged and promoters, and other regulatory sequences are generally part of species-specific or viral vectors. Overall, this genetic engineering strategy permits rapid generation of modular LSD–target protein fusions using readily available reagents.

LSD vector library

Employing above genetic engineering strategy, we generated 29 vectors that contain one of 11 LSDs or one of five LSD binding partners inserted into site *A* and *C* (Fig. 1G). These domains are the photolyase

homology region (PHR) domain of plant cryptochrome2 (AtCRY2-PHR of *Arabidopsis thaliana* [20,28]), LOV domains of plant, algal and fungal photoreceptors (two modified AsPT1-LOV2 domains of *Avena sativa*, CrPH-LOV1 of *Chlamydomonas reinhardtii* (the first LOV domain of the *Chlamydomonas* phototropin), NcVVD-LOV of *Neurospora crassa*, RsLP-LOV of *Rhodobacter sphaeroides* and VfAU1-LOV of *Vaucheria frigida* [18,19,26,29,30,32,44]), bacterial CBDs (MxCarH-CBD *of Myxococcus xanthus* and TtCarH-CBD of *Thermus thermophilus* [24]), and sensory modules of PHYs from cyanobacteria and plants (ScPH1-S of *Synechocystis PCC6803* and AtPHYB-S of *A. thaliana* [21,22,45]). The library also includes binding partners for the heterodimerizing LOV domains, CRY and PHY, which are the minimal proteins EcSspB of *E.coli* with different affinities, HsPDZ1b of *Homo sapiens*, AtCIB and AtPIF6 of *A. thaliana* [20,22,26,29,45] (Fig. 1G) (sequence information and protein database identifiers can be found in Table S1). Collectively, these vectors provide coverage of methods to induce homodimerization, homooligomerization, heterodimerization with binding partners, or monomerization in response to different wavelengths of light. Many of these domains have been previously utilized *ex vivo* and *in vivo* but the library also contains less frequently applied domains (e.g., the blue light-sensitive homodimerizing CrPH-LOV1 [44,46] or the monomerizing RsLP-LOV [32,33]). Vectors are available with all proteins inserted into the site *A* and separately the site *C* (i.e., N-terminal and C-terminal of the target protein insertion site), except in cases where attachment at one of the two termini is incompatible with robust protein function (AtPHYB functions most robustly when target proteins are attached to the C-terminus of this LSD, and AsPT1-LOV2 in the dimerizers when target proteins are fused of the N-terminus of this LSD). In the future, the library is expected to grow as its modular design allows direct expansion with additional LSDs [23,47].

Light-activated caspase-9

We employed the engineering strategy and vector library to develop a light-induced variant of caspase-9 (casp9), an initiator caspase in apoptosis induction. The function of casp9 is mediated by homomeric assembly through the N-terminal caspase recruitment domain (CARD) [48], and casp9 has been rendered inducible by substitution of CARD with orthogonal homodimerization domains [49,50]. This work demonstrated that dimerization by an N-terminal domain is sufficient for casp9 activation and resulted in a chemically-induced casp9 (iCasp9) that is employed as a cellular safety and suicide switch [51]. To generate casp9 activated by light (Opto-casp9), we inserted a casp9 amplicon N-terminally and C-terminally of three LOV domains and AtCRY2-PHR that undergo blue light-induced homodimerization or oligomerization (Fig. 2A). We focused on these LSDs because in general blue LSDs are commonly applied in optogenetics and because their flavin co-factors are ubiquitously available in cells of virtually all organisms. As a control, we employed casp9 fused to an engineered chemical dimerization domain derived from human FK506 binding protein (HsFKBP) analogous to iCasp9. We first tested if these proteins exhibit constitutive activity (i.e., dark activity) by metabolically assessing the viability of human embryonic kidney 293 (HEK293) cells using the fluorescent dye resazurin (Fig. 2B, C). As constitutive activity was not observed, we next tested if these proteins can be used to induce cell death. To analyze cell death while controlling for transfection efficiency, we co-transfected cells with Opto-casp9 and a genetic reporter (*Renilla* luciferase under the control of a constitutive promoter). We chose a luciferase over a fluorescent protein as the reporter gene because of the high signal-to-noise ratio in luminescence detection and to avoid undesired excitation of the reporter by stimulation light. Twenty-four hours after transfection, cells were stimulated for 7 h with blue light (continuous illumination, $\lambda \approx 470$ nm, intensity (I) = 200 μW/cm^2) in a tissue culture incubator equipped with light-emitting diodes, and luminescence signals were measured subsequently. We found strongly reduced luminescence signals for cells that were transfected with casp9 fused to VfAU1-LOV or AtCRY2-PHR domains but not the other domains (Fig. 2D, E). Interestingly, N-terminal but not C-terminal fusion of casp9 to CrPH-LOV1 resulted in increased luminescence signals, which may be explained by a dominant negative action of this LSD on basal levels of apoptosis or an alternative photoreaction mechanism. To confirm the specificity of the observed effect using VfAU1-LOV-casp9 as an example, we demonstrated that with increasing light-dose luminescence signals decrease (the half maximal effective light dose was 2.5 μW/cm^2; Sup. Fig. S2). We further verified that light stimulation resulted in apoptosis using flow cytometry analysis with propidium iodide (PI) and Annexin markers (Sup. Fig. S3A). For VfAU1-LOV-casp9 and AtCRY2-PHR-casp9 but not for mock transfected cells, we observed robust optical induction of apoptosis (Sup. Fig. S3B, C). This result demonstrates that by linking a casp9 amplicon to multiple LSDs and even without modification of the internal seven-residue linker functional Opto-casp9 enzymes could be quickly designed.

Specificity in light-induced PPIs

The modular genetic engineering strategy provides the possibility to perform additional

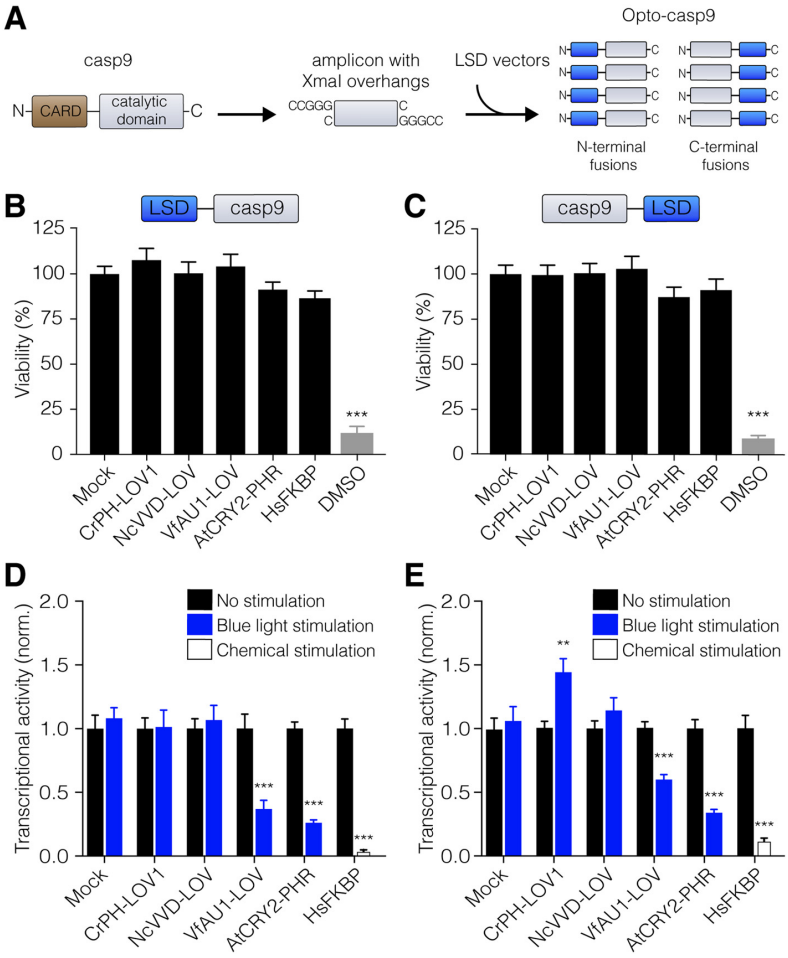

Fig. 2. Development of Opto-casp9 enzymes. (A) Procedure to engineer eight Opto-casp9 candidate enzymes starting from one casp9 catalytic domain amplicon and the vector library. (B and C) Viability of cultured human cells transfected with N- (B) and C-terminal (C) fusions of LSDs to casp9 assessed with resazurin. Twenty percent DMSO was employed as a positive control to induce cell death. (D and E) Light-induced reduction in transcriptional activity for cells transfected with N- (D) and C-terminal (E) fusions of LSDs to casp9 assessed using a luciferase reporter (7-h continuous blue light, $\lambda \approx 470$ nm, $I \approx 200$ µW/cm^2). For B–E: n = 12–20 (see Materials and Methods for details), four independent experiments, data shown are mean ± SEM. ** $p < 0.001$ or *** $p < 0.0001$. Two-tailed *t*-test.

experiments, such as negative controls and immunodetection, that complement the efficient fusion protein generation demonstrated above. In optogenetics, negative controls typically consist of the application of light to naïve cells or to cells that were transfected with inactivated optogenetic tools (e.g., through loss-of-function mutations). The latter control is required to obtain baseline signals and to ensure that overexpression of LSDs or target proteins does not alter cellular sensitivity. The most commonly applied loss-of-function mutations for inactivation either target photochemically active LSD residues or residues involved in light-induced conformational changes. However, targeting LSD photochemistry can be incomplete with persistent LSD activation through alternative reaction mechanisms or generation of chemical photoreaction side products [52,53]. In addition, because of the diversity in the structures and activation mechanisms of LSDs, generalizable loss-of-function mutations do not exist, and thus, negative controls cannot be studied under identical conditions. To address these limitations, we developed a universal inactivation strategy for light-controlled PPIs, which is based on

testing constructs in which the LSD and target protein have been uncoupled (e.g., uncoupling of VfAU1-LOV and casp9 should result in lost light activation). We realized this strategy by taking advantage of the availability of site *B* in all generated vectors and the opportunity of designing linkers with additional functionality. Into this site, we inserted a self-cleaving peptide sequence of porcine teschovirus-1 2A (P2A) that will effectively dissociate the two domains resulting in a loss of light sensitivity (Fig. 3A). As expected for a P2A-modified Opto-casp9, we observed that self-cleavage completely abolished light-induced changes in the luminescence signals, effectively producing the same outcome as removal of the catalytic activity of casp9 (Fig. 3B, Sup. Fig. S4). Immunoblotting against epitope tags that flanked the P2A sequence verified cleavage as we only detected the single LSD and casp9 domains but not the full protein (Fig. 3C). These results demonstrate a new control strategy that preserves target protein and LSD expression and LSD photochemistry taking advantage of linker and epitope incorporation into site *B*.

Conclusions

Optogenetics is one of few techniques that permit the regulation of cell behaviors with high precision in space and time. We developed a resource for the generation of light-induced PPIs and demonstrated its applicability by engineering Opto-casp9 enzymes. This resource will contribute to the broader use of optogenetics in cell and developmental biology and pave the way to novel optogenetics studies. For instance, experiments on the scale of entire families of LSDs or of target proteins require efficient and modular genetic engineering approaches that are now within reach. These experiments could also test if a forced PPI is sufficient for (in)activation of a protein, conceptually similar to a recent study in which light induced the signaling of orphan receptors [54]. Opto-casp9 enzymes may provide a test bed for optogenetic hardware development and testing, a process that entails optimization of light parameters (e.g., wavelength, intensity, duration) and culture conditions, because cell death can be assessed with different assays. Finally, the

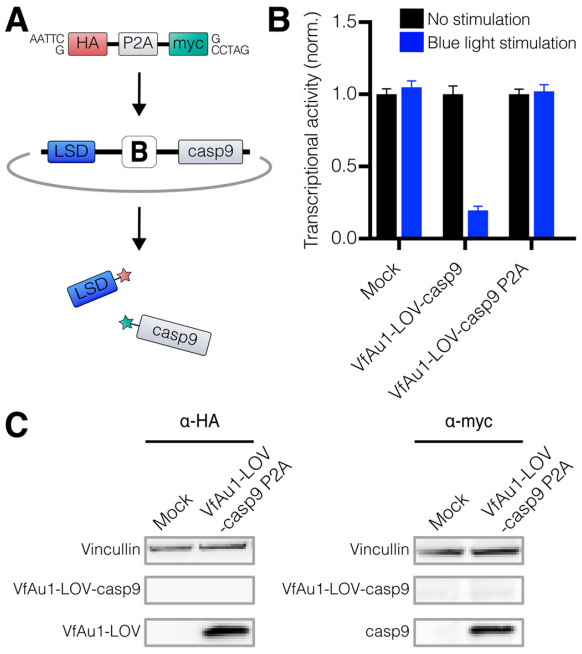

Fig. 3. Control experiments with self-cleaving epitope-linker. (A) Incorporation of a P2A sequence results in self-cleavage and separation of LSD and target protein. (B) Light-induced cell death for cells transfected with vectors containing the self-cleaving linker (7-h continuous blue light, $\lambda \approx 470$ nm, $I \approx 200$ μW/cm^2). (C) Immunoblotting to validate efficient cleavage. For B: n = 12, four independent experiments, data shown are mean ± SEM. *** $p < 0.0001$. Two-tailed *t*-test.

engineering strategy and empty cassettes may also be of use in areas other than optogenetics, such as for the rapid and modular design of fluorescent sensors and protein probes.

Materials and Methods

Cassette design

Cassettes were introduced in pcDNA3.1- (Invitrogen/Life Technologies) to generate the vectors named pOVC1–3 (optogenetic vector core 1–3, Sup. Fig. S5). A XmaI restriction site was removed from the backbone using site-directed mutagenesis (oligonucleotides 1 and 2, Table S2). Inverse polymerase chain reactions (PCR) (oligonucleotides 3 and 4, 5 and 6, and 7 and 8) were applied to remove the vector multiple cloning site and create *ABC* (pOVC1), *ACB* (pOVC2) and *BAC* (pOVC3) cassettes. In the inverse PCR procedure, PCR products were digested with DpnI, digested with EcoRI, XmaI or AgeI (NEB), respectively, ligated for 3 h at room temperature (RT) or overnight at 4 °C using T4 ligase (Promega), and propagated in *Escherichia coli* XL10 Gold cells (Agilent). All cassettes contain Kozak sequences, start codons and stop codons [for backbone ABC, the stop codon was introduced using site-directed mutagenesis in a separate reaction (oligonucleotides 9 and 10)]. For linker insertion, backbone pOVC1 was digested using EcoRI and BamHI. Linker fragments were generated by inverse PCR (oligonucleotides 57 and 58) or by annealing and phosphorylating single stranded oligonucleotides (59 to 64). All vector sequences (Table S3) were verified by Sanger sequencing (Micromon, Monash University) and deposited at Addgene.org.

LSD amplification and vector library

LSDs were amplified using PCR and oligonucleotides with AgeI and/or XmaI restriction site overhangs (oligonucleotides 11 to 34 and 45 to 52). Templates were previously described vectors from our laboratory or obtained from Addgene.org (Table S1). In addition, gene fragments of AtCRY2-PHR, ScPH-1, AsLOV2-EcSsra, EcSSPB micro, AsLOV2-pep and HsPDZ1b were synthesized by a commercial supplier (Integrated DNA Technologies; Table S4). Restriction sites for AgeI and BamHI were removed from ScPH1-S and AtPHYB-S, respectively, as well as XmaI restriction sites from HsFKBP and AtCRY2-PHR using site-directed mutagenesis (oligonucleotides 35 to 42). Site-directed mutagenesis was used to create EcSSPB nano (oligonucleotides 65 and 66). PCR products were digested with DpnI and with AgeI, XmaI or AgeI and XmaI depending on oligonucleotide overhangs. Backbone pOVC1 was digested with AgeI or XmaI for insertion into site *A* or *C*, respectively, and phosphatase treated. Backbone and inserts were ligated either for 3 h at RT or overnight at 4 °C using T4 ligase (Promega). All vector sequences (Table S5) were verified by Sanger sequencing (LGC Genomics) and deposited at Addgene.org. Note that for future subcloning of the generated genes, universal oligonucleotides can be designed that contain recognition sites for the enzymes AflII, ApaI, AscI, FseI, PacI, PspOMI or SbfI as these are not found in any of the genes.

Opto-casp9 constructs

The catalytic domain of casp9 (residues 135–416 of UniProt entry P55211) was synthesized (Integrated DNA Technologies; Table S4), amplified by PCR (oligonucleotides 43 and 44) and digested with XmaI. Vectors were digested with XmaI or AgeI, respectively, treated with phosphatase and gel purified. Backbone vectors and casp9 insert were ligated either for 3 h at RT or overnight at 4 °C using T4 ligase. Site-directed mutagenesis was used to introduce point substitution C287A into the catalytic domain of casp9 in VfAU1-LOV-casp9 and HsFKBP-casp9 (oligonucleotides 55 and 56). HA-P2A-myc was synthesized as a gene fragment (Integrated DNA Technologies, Table S4), amplified using PCR and restriction site overhangs (oligonucleotides 53 and 54), and inserted into site *B* of VfAU1-LOV-casp9 using EcoRI and BamHI. All vector sequences (Table S6) were verified by Sanger sequencing (Micromon, Monash University) and deposited at Addgene.org.

Cell culture and transfection

HEK293 cells (Thermo Fisher Scientific; further authenticated by assessing cell morphology and growth rate) were cultured in mycoplasma-free Dulbecco's modified eagle medium (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2 atmosphere at 37 °C. Medium was supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). On the day after seeding, cells were transfected in Dulbecco's modified eagle medium supplemented with 5% FBS using polyethylenimine (Polysciences). Media was changed after 4 to 6 h, and cells were stimulated with light starting 24 h after transfection for the durations specified below and at the intensities specified in the main text.

Light stimulation of cells

For light stimulation of cells, a tissue culture incubator was equipped with 150 light-emitting

diodes (SMD5050-RGB on a LED strip at 3.3-cm spacing). Light intensity was adjusted with a dimmer and measured with a digital power meter (LP1, Sanwa). To obtain light dose curve (Sup. Fig. S2), one to four layers of neutral density filters (Filter 210, LEE Filters) were used to reduce intensity of selected wells.

Metabolic and transcriptional assays

HEK293 cells (2.5×10^4) were seeded in each well of white bottom 96-well plates (Costar) and maintained as described above. Cells were transfected with 200 ng vector (casp9, renilla luciferase reporter and empty vector at a ratio of 10:1:9) as described above. Twenty-four hours after transfection, cells were either stimulated with blue light ($\lambda \approx 470$ nm), 10 nM of the chemical dimerizer AP20187 (ClonTech Laboratories) or left unstimulated. Light and AP20187 were applied for 7 h continuously. Unstimulated cells were kept in the dark for 6 h before addition of resazurin (Sigma) at a final concentration of 55 µM. After incubation for 1 h, resazurin fluorescence was measured in a plate reader (excitation 540 ± 15 nm, emission 590 ± 20 nm, ClarioSTAR, BMGLabtech). Viability was defined as relative fluorescence units compared to a mock-transfected control. Immediately after resazurin assays, stimulated and unstimulated cells were washed once with phosphate-buffered saline (PBS), lysed and processed with homemade luciferase reporter reagents [24]. Luminescence was measured in the plate reader and transcriptional activity was defined as mean raw luminescence values.

Flow cytometry

HEK293 cells (5×10^5) in each well of clear 6-well plates (Costar) were transfected with 2 µg vector (casp9 and empty vector at a ratio of 1:1) as described above. Twenty-four hours after transfection, cells were either stimulated with light (continuous blue light, $\lambda \approx 470$ nm, $I \approx 200$ µW/cm^2) or protected from light for 7 h at 37 °C. After incubation, cells were collected, washed once with ice-cold PBS (Thermo Fisher Scientific) supplemented with 2% FBS and stained with FITC-AnnexinV/PI Apoptosis Detection Kit according to manufacturer's instructions (BioLegend). Samples were then run on a LSRFortessa X-20 flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (FlowJo).

Immunoblotting

HEK293 cells (5×10^5) in each well of clear 6-well plates (Costar) were transfected with 2 µg vector as described above. Twenty-four hours after transfection, cells were washed once with ice-cold PBS and lysed on ice in 180 µl lysis buffer [150 mM NaCl, 1% TritonX-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, complete protease inhibitor (Roche)]. Lysates were shaken for 30 min at 4 °C and centrifuged for 20 min at 12,000*g*. Lysate (30 µl) per lane was separated by SDS-PAGE and electroblotted onto PVDF membranes. Blots were incubated with primary antibodies (HA-Tag no. 2367, dilution 1:1000; myc-Tag no. 2276, dilution 1:1000; Cell Signaling Technology; Vincullin ab129002, dilution 1:10000; Abcam) in blocking solution (5% BSA in TBS-T) overnight at 4 °C. Secondary antibody (goat anti-rabbit IgG(H + L)-HRP conjugate, goat anti-mouse IgG(H + L)-HRP, dilution 1:10000, Biorad) was applied for 1 h at RT, and blots were developed with Carity™ Western ECL Substrate (Biorad).

Statistical analysis

Results were evaluated using Prism (GraphPad). Differences between stimulated and unstimulated samples were analyzed using two-tailed *t*-tests, and *p* values are given in the figure captions. Sample numbers (n) and the number of independent experiments (biological replicates) for each bar are specified in the figure captions, except for Fig. 2B–E. In Fig. 2B, sample numbers are 14, except for mock (26), HsFKBP (15) and DMSO (13). In Fig. 2C, sample numbers are 16, except for mock (25), HsFKBP (19) and DMSO (12). In Fig. 2D, sample numbers are 14, except for HsFKBP (26 and 12, dark and light). In Fig. 2E, sample numbers are 16, except for mock (28) and HsFKBP (28 and 12, dark and light) and DMSO (12).

CRediT authorship contribution statement

Alexandra-Madelaine Tichy: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Elliot J. Gerrard:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Julien M.D. Legrand:** Data curation, Formal analysis, Methodology, Resources, Validation, Visualization, Writing - review & editing. **Robin M. Hobbs:** Formal analysis, Funding acquisition, Methodology, Resources, Validation, Writing - review & editing. **Harald Janovjak:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

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Abbreviations used:
LSD, light-sensitive domain; LOV, light-oxygen-voltage sensing; CRY, cryptochrome; PHY, phytochrome; CBD, cobalamin binding domain; PPI, protein–protein interaction; PHR, photolyase homology region; P2A, porcine teschovirus-1 2A; PCR, polymerase chain reaction; RT, room temperature; casp9, caspase-9.

References

[1] E. Pastrana, Optogenetics: controlling cell function with light, Nat. Methods 8 (2011) 24–25.
[2] A. Reiner, E.Y. Isacoff, The brain prize 2013: the optogenetics revolution, Trends Neurosci. 36 (2013) 557–560.
[3] K.M. Tye, K. Deisseroth, Optogenetic investigation of neural circuits underlying brain disease in animal models, Nat. Rev. Neurosci. 13 (2012) 251–266.
[4] S.C. Williams, K. Deisseroth, Optogenetics, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 16287.
[5] K.E. Brechun, K.M. Arndt, G.A. Woolley, Strategies for the photo-control of endogenous protein activity, Curr. Opin. Struct. Biol. 45 (2017) 53–58.
[6] H.E. Johnson, Y. Goyal, N.L. Pannucci, T. Schupbach, S.Y. Shvartsman, J.E. Toettcher, The spatiotemporal limits of developmental Erk signaling, Dev. Cell 40 (2017) 185–192.
[7] F. Zhang, L.P. Wang, M. Brauner, J.F. Liewald, K. Kay, N. Watzke, et al., Multimodal fast optical interrogation of neural circuitry, Nature. 446 (2007) 633–639.
[8] E.S. Boyden, F. Zhang, E. Bamberg, G. Nagel, K. Deisseroth, Millisecond-timescale, genetically targeted optical control of neural activity, Nat. Neurosci. 8 (2005) 1263–1268.
[9] L. Kowalik, J.K. Chen, Illuminating developmental biology through photochemistry, Nat. Chem. Biol. 13 (2017) 587–598.
[10] G. Guglielmi, H.J. Falk, S. De Renzis, Optogenetic control of protein function: from intracellular processes to tissue morphogenesis, Trends Cell Biol. 26 (2016) 864–874.
[11] B.V. Zemelman, G.A. Lee, M. Ng, G. Miesenbock, Selective photostimulation of genetically chARGed neurons, Neuron. 33 (2002) 15–22.
[12] J.E. Toettcher, O.D. Weiner, W.A. Lim, Using optogenetics to interrogate the dynamic control of signal transmission by the Ras/Erk module, Cell. 155 (2013) 1422–1434.
[13] K. Muller, W. Weber, Optogenetic tools for mammalian systems, Mol. BioSyst. 9 (2013) 596–608.
[14] G. Nagel, T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, et al., Channelrhodopsin-2, a directly light-gated cation-selective membrane channel, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13940–13945.
[15] A. Moglich, X. Yang, R.A. Ayers, K. Moffat, Structure and function of plant photoreceptors, Annu. Rev. Plant Biol. 61 (2010) 21–47.
[16] M. Heijde, R. Ulm, UV-B photoreceptor-mediated signalling in plants, Trends Plant Sci. 17 (2012) 230–237.
[17] J.M. Ortiz-Guerrero, M.C. Polanco, F.J. Murillo, S. Padmanabhan, M. Elias-Arnanz, Light-dependent gene regulation by a coenzyme B12-based photoreceptor, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 7565–7570.
[18] M. Grusch, K. Schelch, R. Riedler, E. Reichhart, C. Differ, W. Berger, et al., Spatio-temporally precise activation of engineered receptor tyrosine kinases by light, EMBO J. 33 (2014) 1713–1726.
[19] X. Wang, X. Chen, Y. Yang, Spatiotemporal control of gene expression by a light-switchable transgene system, Nat. Methods 9 (2012) 266–269.
[20] M.J. Kennedy, R.M. Hughes, L.A. Peteya, J.W. Schwartz, M. D. Ehlers, C.L. Tucker, Rapid blue-light-mediated induction of protein interactions in living cells, Nat. Methods 7 (2010) 973–975.
[21] E. Reichhart, A. Ingles-Prieto, A.M. Tichy, C. McKenzie, H. Janovjak, A phytochrome sensory domain permits receptor activation by red light, Angew. Chem. Int. Ed. Engl. 55 (2016) 6339–6342.
[22] A. Levskaya, O.D. Weiner, W.A. Lim, C.A. Voigt, Spatiotemporal control of cell signalling using a light-switchable protein interaction, Nature. 461 (2009) 997–1001.
[23] T.A. Redchuk, E.S. Omelina, K.G. Chernov, V.V. Verkhusha, Near-infrared optogenetic pair for protein regulation and spectral multiplexing, Nat. Chem. Biol. 13 (2017) 633–639.
[24] S. Kainrath, M. Stadler, E. Reichhart, M. Distel, H. Janovjak, Green-light-induced inactivation of receptor signaling using cobalamin-binding domains, Angew. Chem. Int. Ed. Engl. 56 (2017) 4608–4611.
[25] M. Jost, J. Fernandez-Zapata, M.C. Polanco, J.M. Ortiz-Guerrero, P.Y. Chen, G. Kang, et al., Structural basis for

gene regulation by a B12-dependent photoreceptor, Nature. 526 (2015) 536–541.
[26] O.I. Lungu, R.A. Hallett, E.J. Choi, M.J. Aiken, K.M. Hahn, B. Kuhlman, Designing photoswitchable peptides using the AsLOV2 domain, Chem. Biol. 19 (2012) 507–517.
[27] Y.I. Wu, D. Frey, O.I. Lungu, A. Jaehrig, I. Schlichting, B. Kuhlman, et al., A genetically encoded photoactivatable Rac controls the motility of living cells, Nature. 461 (2009) 104–108.
[28] L.J. Bugaj, A.T. Choksi, C.K. Mesuda, R.S. Kane, D.V. Schaffer, Optogenetic protein clustering and signaling activation in mammalian cells, Nat. Methods 10 (2013) 249–252.
[29] G. Guntas, R.A. Hallett, S.P. Zimmerman, T. Williams, H. Yumerefendi, J.E. Bear, et al., Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 112–117.
[30] D. Strickland, Y. Lin, E. Wagner, C.M. Hope, J. Zayner, C. Antoniou, et al., TULIPs: tunable, light-controlled interacting protein tags for cell biology, Nat. Methods 9 (2012) 379–384.
[31] D. Chen, E.S. Gibson, M.J. Kennedy, A light-triggered protein secretion system, J. Cell Biol. 201 (2013) 631–640.
[32] K.S. Conrad, A.M. Bilwes, B.R. Crane, Light-induced subunit dissociation by a light-oxygen-voltage domain photoreceptor from *Rhodobacter sphaeroides*, Biochemistry. 52 (2013) 378–391.
[33] F. Richter, I. Fonfara, B. Bouazza, C.H. Schumacher, M. Bratovic, E. Charpentier, et al., Engineering of temperature- and light-switchable Cas9 variants, Nucleic Acids Res. 44 (2016) 10003–10014.
[34] E. Izquierdo, T. Quinkler, S. De Renzis, Guided morphogenesis through optogenetic activation of Rho signalling during early *Drosophila* embryogenesis, Nat. Commun. 9 (2018) 2366.
[35] X. Wang, L. He, Y.I. Wu, K.M. Hahn, D.J. Montell, Light-mediated activation reveals a key role for Rac in collective guidance of cell movement in vivo, Nat. Cell Biol. 12 (2010) 591–597.
[36] S.K. Yoo, Q. Deng, P.J. Cavnar, Y.I. Wu, K.M. Hahn, A. Huttenlocher, Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish, Dev. Cell 18 (2010) 226–236.
[37] V.V. Krishnamurthy, J.S. Khamo, W. Mei, A.J. Turgeon, H.M. Ashraf, P. Mondal, et al., Reversible optogenetic control of kinase activity during differentiation and embryonic development, Development. 143 (2016) 4085–4094.
[38] K. Sako, S.J. Pradhan, V. Barone, A. Ingles-Prieto, P. Muller, V. Ruprecht, et al., Optogenetic control of nodal signaling reveals a temporal pattern of nodal signaling regulating cell fate specification during gastrulation, Cell Rep. 16 (2016) 866–877.
[39] A. Reade, L.B. Motta-Mena, K.H. Gardner, D.Y. Stainier, O. D. Weiner, S. Woo, TAEL: a zebrafish-optimized optogenetic gene expression system with fine spatial and temporal control, Development. 144 (2017) 345–355.
[40] H.M. Beyer, S. Juillot, K. Herbst, S.L. Samodelov, K. Muller, W.W. Schamel, et al., Red light-regulated reversible nuclear localization of proteins in mammalian cells and zebrafish, ACS Synth. Biol. 4 (2015) 951–958.
[41] P. Vopalensky, S. Pralow, N.L. Vastenhouw, Reduced expression of the nodal co-receptor Oep causes loss of mesendodermal competence in zebrafish, Development. 145 (2018).
[42] V. Barone, M. Lang, S.F.G. Krens, S.J. Pradhan, S. Shamipour, K. Sako, et al., An effective feedback loop between cell–cell contact duration and morphogen signaling determines cell fate, Dev. Cell 43 (2017) 198–211 e112.
[43] F. Kawano, R. Okazaki, M. Yazawa, M. Sato, A photoactivatable Cre-loxP recombination system for optogenetic genome engineering, Nat. Chem. Biol. 12 (2016) 1059–1064.
[44] R.J. Kutta, E.S. Hofinger, H. Preuss, G. Bernhardt, B. Dick, Blue-light induced interaction of LOV domains from *Chlamydomonas reinhardtii*, Chembiochem. 9 (2008) 1931–1938.
[45] S. Shimizu-Sato, E. Huq, J.M. Tepperman, P.H. Quail, A light-switchable gene promoter system, Nat. Biotechnol. 20 (2002) 1041–1044.
[46] Y. Nakasone, M. Ohshima, K. Okajima, S. Tokutomi, M. Terazima, Photoreaction dynamics of LOV1 and LOV2 of phototropin from *Chlamydomonas reinhardtii*, J. Phys. Chem. B 122 (2018) 1801–1815.
[47] X.X. Zhou, H.K. Chung, A.J. Lam, M.Z. Lin, Optical control of protein activity by fluorescent protein domains, Science. 338 (2012) 810–814.
[48] M. Renatus, H.R. Stennicke, F.L. Scott, R.C. Liddington, G.S. Salvesen, Dimer formation drives the activation of the cell death protease caspase 9, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 14250–14255.
[49] Y. Nihongaki, H. Suzuki, F. Kawano, M. Sato, Genetically engineered photoinducible homodimerization system with improved dimer-forming efficiency, ACS Chem. Biol. 9 (2014) 617–621.
[50] K.C. Straathof, M.A. Pule, P. Yotnda, G. Dotti, E.F. Vanin, M. K. Brenner, et al., An inducible caspase 9 safety switch for T-cell therapy, Blood. 105 (2005) 4247–4254.
[51] A. Di Stasi, S.K. Tey, G. Dotti, Y. Fujita, A. Kennedy-Nasser, C. Martinez, et al., Inducible apoptosis as a safety switch for adoptive cell therapy, N. Engl. J. Med. 365 (2011) 1673–1683.
[52] E.F. Yee, R.P. Diensthuber, A.T. Vaidya, P.P. Borbat, C. Engelhard, J.H. Freed, et al., Signal transduction in light-oxygen-voltage receptors lacking the adduct-forming cysteine residue, Nat. Commun. 6 (2015), 10079.
[53] X. Shu, V. Lev-Ram, T.J. Deerinck, Y. Qi, E.B. Ramko, M.W. Davidson, et al., A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms, PLoS Biol. 9 (2011), e1001041.
[54] M. Morri, I. Sanchez-Romero, A.M. Tichy, S. Kainrath, E.J. Gerrard, P.P. Hirschfeld, et al., Optical functionalization of human class A orphan G-protein-coupled receptors, Nat. Commun. 9 (2018) 1950.

Chapter 3

Engineering of light-activated GPCRs

3.1 Chapter Summary

The process of controlling protein function with light has also been extended to GPCRs, for example through opsin-based chimeric GPCRs (OptoXRs). OptoXRs employ a design approach different to that used for most other optogenetic tools, and their engineering is more complex as it requires cutting and fusing two receptors along multiple fusion sites. Deciding on the placement of these fusion sites is not a trivial task and significantly impacts the functionality of the resulting receptor. OptoXRs to date have been engineered following the same design principles from the first conceived OptoXR. While our knowledge of GPCRs has increased dramatically since then, especially with the rise of GPCR structural biology, this has not been applied to the improvement of OptoXRs. The work presented in this chapter reworks the design principles for OptoXRs by investigating two avenues how structural GPCR information can improve OptoXR functionality. In one avenue, we investigate whether using structural alignments between acceptor and donor receptor would increase the likelihood of exchanging corresponding domains between the receptors. In the other avenue, we determined elements that would be functionally relevant to confer donor signalling in the OptoXR by analyzing G-protein bound structures of GPCRs. This allowed us to redefine the placement of the cut sites between acceptor and donor receptor GPCR. Testing this new design by engineering a new β_2AR based OptoXR, Opto-β_2AR 2.0, we were able to significantly improve the function of this receptor compared to previously designed versions. We further show how this improved design approach can function as a starting framework for other receptors or for testing further modifications of OptoXRs.

3.2 Structure-guided design of light-activated chimeric G-protein coupled receptors

At the time when this thesis was submitted, the following chapter has been submitted for publication. Numbering of figures, tables and reference was reformatted to match the style of this thesis.

Supplementary material can be found in Appendix B

3.2.1 Author information

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3.2.2 Abstract

G-protein coupled receptors (GPCRs) are the largest human receptor family and involved in virtually every physiological process. One hallmark of GPCR function is the specific coupling to selected signaling pathways. The ability to tune this specificity would permit the development of receptors with new functionalities. Molecular complexes of GPCRs and downstream signaling proteins have been recently resolved at high resolution, but this information has not yet been harnessed for rational reengineering of coupling specificity. Here, we demonstrate a structure-guided switch in G-protein preference in chimeric light-activated GPCRs (OptoXRs). We first evaluated structure-based alignments as complements to existing sequence-based methods. We then show in a prototypical receptor that intracellular loops are not sufficient for efficient downstream signal transduction. In apparent contrast, successive inclusion of α-helical G-protein contacts resulted in receptors with 7- to 20-fold improved function compared to those generated with other design strategies. Finally, this efficient receptor system served as a platform for the development of further light-activated receptors and spectral tuning of the photoreceptor domain. As coupling specificity shapes the consequences of GPCR

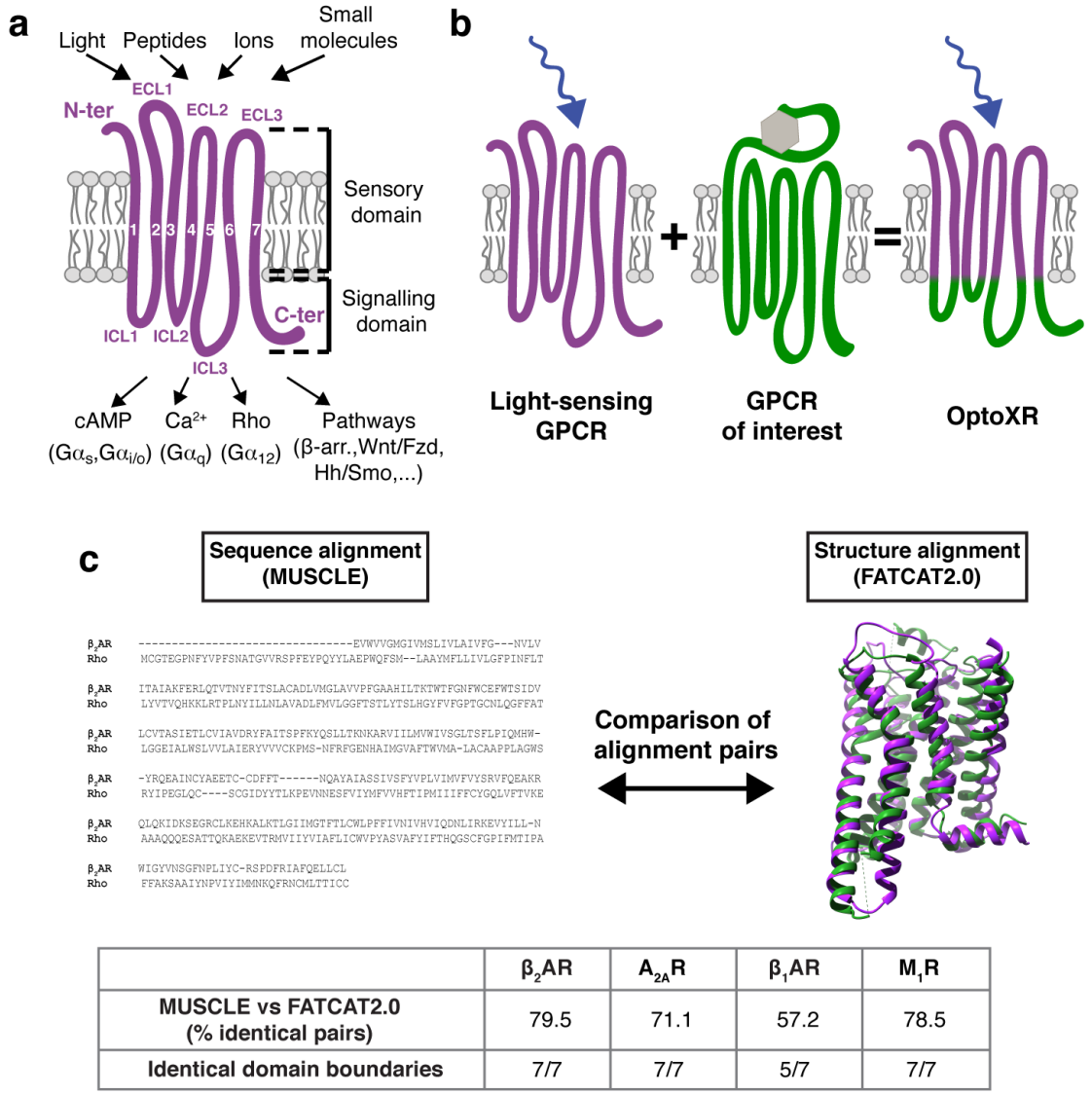
activation in health and disease, this work provides rationally-designed actuators as targeted inputs into cells and networks.

3.2.3 Introduction

G-protein coupled receptors (GPCRs) comprise the largest family of membrane receptors in the human genome. They are expressed broadly and regulate key biological processes, ranging from organism development to metabolism and brain function (Kooistra et al., 2021; Insel et al., 2019). In line with their abundance and physiological importance, GPCRs are also exceptional therapeutic targets with $\sim$ 30% of all prescription drugs acting on members of this receptor superfamily (Hauser et al., 2017; Sriram et al., 2018). Canonical signalling downstream of GPCRs is mediated through coupling to heterotrimeric G-proteins, which are often classified into four main groups according to their Gα subunits (the Gα_{s}, G$\alpha_{i/o}$, Gα_{q}, and G$\alpha_{12/13}$ groups). Gα subunits principally act on adenylyl cyclase to increase (Gα_{s}) or decrease (G$\alpha_{i/o}$) cAMP levels, on phospholipase C to alter Ca^{2+} signaling (Gα_{q}) or on Rho GTPases (G$\alpha_{12/13}$) to modulate motor proteins or the cytoskeleton (Fig. 3.1a). In addition, Gβ and Gγ subunits activate ion channels and a wide range of enzymes upon dissociation of the trimeric complex. In humans, at least 16 Gα subunits, 6 Gβ subunits and 13 Gγ subunits have been identified, which can assemble in a multitude of combinations leading to functionally distinct outcomes (Oldham et al., 2008; Flock et al., 2017; Atwood et al., 2011). GPCRs can also signal via β-arrestin scaffolding proteins and ultimately diverse signaling pathways (Fig. 3.1a). The expression of multiple GPCRs and G-proteins in most cell types results in combinatorial diversity and dictates that specific GPCR-G-protein coupling interactions guide signaling outcomes (Flock et al., 2017; Marti-Solano et al., 2020). Consequences of GPCR activation are most commonly studied using pharmacological methods (e.g., agonists, antagonists or allosteric modulators) or genetic methods (e.g., receptor overexpression or knock-out in cells and animals). As a complement, approaches have been developed to (in)activate GPCRs on shorter time scales and finer spatial scales than those dictated by drug diffusion and pharmacokinetics or those associated with genetic alterations (Tichy et al., 2019b; Kleinlogel, 2016). One such approach are light-activated GPCRs, which are either repurposed from naturally light-sensing mammalian tissues (Li et al., 2005; Masseck et al., 2014; Karunarathne et al., 2013) or from invertebrates and fish (Zemelman et al., 2002; Bailes et al., 2012; Mahn et al., 2021; Copits et al., 2021), or are engineered as chimeric GPCRs (termed OptoXRs; (Kim et al., 2005; Airan et al., 2009)). These receptors permit activation of signaling pathways

by light with unprecedented spatio-temporal acuity down to milliseconds and micrometers in the emerging field of optogenetics (Spangler et al., 2017; Wiegert et al., 2017). The purpose-built OptoXRs take advantage of conserved structure-function relationships across GPCRs by substituting the downstream coupling domains of a light-gated GPCR, such as the mammalian dim light sensor rhodopsin (rho), with those of a naturally ligand-gated target GPCR (Fig. 3.1b) (Kim et al., 2005; Airan et al., 2009). The desired engineering outcome is that the chimeric GPCR can be activated by light but retains the signalling properties of the target receptor. OptoXRs have been expressed in neuronal and non-neuronal cell types *ex vivo* and *in vivo* using viral delivery or transgenic animals and activated with a variety of light sources (Iwai et al., 2021; Wyk et al., 2015; Xu et al., 2014). Light control was often achieved *in vivo* without supplementation of the *cis*-isoform retinal cofactor (Airan et al., 2009; Siuda et al., 2015b; Siuda et al., 2015a), but supplementation protocols have also been developed (Iwai et al., 2021). Whilst the OptoXR methodology has been applied to a number of target GPCRs (see Supplementary Table B.1 for a - to our best knowledge comprehensive - list of previous OptoXRs), the design principle underlying most published receptors was derived from a pioneering study that employed a trial-and-error strategy (i.e., various receptor domains were swapped and the emerging receptors tested for function) (Kim et al., 2005). This original and other studies have put forward the notion that intracellular loops are main determinants of Gα-coupling specificity in OptoXRs (Kim et al., 2005; Hickey et al., 2021), but this model has never been tested experimentally (Tichy et al., 2019b). The increasing application of chimeric (and other light-activated) GPCRs not only as basic research tools but even potential future treatment strategies (Cehajic-Kapetanovic et al., 2015; Wyk et al., 2015; Berry et al., 2019) calls for a systematic and rational design of OptoXRs towards efficient and predictable function. In the past decade, high resolution structural information available for GPCRs has increased dramatically to encompass, at present, more than 500 atomic resolution structures for more than 50 unique receptors (Kooistra et al., 2021; Congreve et al., 2020). Detailed analyses of these structures have provided insights into GPCR structure-function relationships, such as the universal heptahelical transmembrane domain, the translation and rotation of specific transmembrane (TM) helices upon receptor activation or the switch-like behavior of conserved residue contacts (Weis et al., 2018; Venkatakrishnan et al., 2016; Zhou et al., 2019; Wootten et al., 2018; Gurevich et al., 2017). Following from early successes using X-ray crystallography (Rasmussen et al., 2011), it was the advent of cryo-electron microscopy (cryo-EM) that revealed a number of active state G-protein bound

structures for members of most GPCR classes (Liang et al., 2018; García-Nafría et al., 2018; Munk et al., 2019). Albeit structures of GPCR-G-protein complexes allow identifying key interactions between GPCRs and downstream transducers (Glukhova et al., 2018; Rasmussen et al., 2011; Flock et al., 2017), this knowledge has not yet been harnessed for the development of receptors with rationally-designed signaling functions (Tichy et al., 2019b; Keri et al., 2018). For instance, in seminal work that predated structures of GPCR-Gα complexes, downstream coupling in wildtype (WT) and chimeric GPCRs was altered through strategically-placed residue substitutions derived from biochemical studies (Siuda et al., 2015b; Guettier et al., 2009; Nakajima et al., 2012). In more recent studies, orthogonal Gα subunits (Young et al., 2018) or GPCRs with promiscuous downstream coupling (Sandhu et al., 2019) were designed using structure modelling, molecular dynamic simulations and in silico/experimental mutagenesis. It thus remains to be explored to what extent GPCR-G-protein coupling can be reengineered using structure-guided strategies. These strategies will complement the design of GPCR ligand-binding pockets (Von Moo et al., 2021; Vardy et al., 2015) and activate state switches (Chen et al., 2020; Schönegge et al., 2017), which have utilized protein structures, and collectively result in a wider range of accessible receptors functions. Here, we redesigned G-protein coupling specificity by harnessing high resolution GPCR-G-protein structures. We performed this in OptoXRs as an efficient switch in coupling, e.g., from the native G$\alpha_{i/o}$ preference of rho to a different Gα group, is required for the faithful function of chimeric receptors. We first explored to what extent structure superimpositions are a useful complement to sequence alignments for determination of functional domain boundaries. We then tested the model that intracellular loops, as delineated in receptor structures, are sufficient to confer Gα_s-specificity. Focussing on GPCRs in complex with their restive G-proteins, we showed that inclusion of helical G-protein binding contacts of the target GPCR greatly improved functionality of a light-activated β_2AR receptor. Finally, we demonstrated the applicability of this approach to enable tuning of spectral receptor properties and development of a further receptor from a different family. Collectively, this work demonstrates analysis and improvement of chimeric OptoXRs through structure-guided reengineering of GPCR-G-protein coupling.



	β_2AR	A_{2A}R	β_1AR	M_1R
MUSCLE vs FATCAT2.0 (% identical pairs)	79.5	71.1	57.2	78.5
Identical domain boundaries	7/7	7/7	5/7	7/7

FIGURE 3.1: **Functional principle of OptoXRs and alignment methodologies. (a)** GPCRs sense a variety of external stimuli using their extracellular and transmembrane domains. Downstream signalling is mediated by a domain that faces the intracellular environment. **(b)** In chimeric OptoXRs, the signaling domain of a target GPCR is fused to the sensory domain of a light-activated GPCR. **(c)** Comparison of sequence- and structure-based alignments. Top row: The percentage of identical residue pairs in a comparison of pairwise sequence alignments (MUSCLE) and pairwise structure alignments (FATCAT2.0) of Rho and β_2AR, A_{2A}R, β_1AR and M_1R (see Supplementary Fig. B.2 for a definition of residue pairs). Bottom row: The number of identical domain boundaries in the same comparisons (see Supplementary Fig. B.3 for a definition of OptoXR domain boundaries).

3.2.4 Results

Comparison of sequence- and structure-based alignments

The creation of protein chimeras hinges on the exchange of functionally-equivalent domains between two related proteins. This is generally achieved by first aligning the proteins of interest, either as sequences or structures, followed by identification of domain boundaries. Engineering of previous OptoXRs exclusively relied on sequence-based alignments, e.g. in the prominent chimeras of rho and adrenergic, dopamine, adenosine or muscarinic acetylcholine target receptors (Supplementary Table B.1) (Airan et al., 2009; Gunaydin et al., 2014; Morri et al., 2018). As a step towards structure-guided OptoXR design, we first tested whether alignments of structures would yield chimeric receptors that are different from those generated using sequences. This may indeed be the case due to the additional topological information gained and because sequence similarity between GPCRs from different families can be low (e.g., rho and the receptors β_2AR, A_{2A}R, β_1AR and M_1R exhibit 20, 21, 22 and 23% sequence identity overall, or 21, 22, 23 and 21% when excluding extra- or intracellular termini, respectively). Several methods are available for protein structure superposition. For a set of receptors that were previously incorporated in OptoXRs and for which active state structures are available, we first tested *cealign*, *super* and FATCAT2.0 (Li et al., 2005; Shindyalov et al., 1998) with the objective of identifying the most robust alignment methods (these methods differ, e.g., in algorithm and interpretation of residues for which coordinates are not available). For *cealign* and *super*, we performed alignments using the complete receptor structures as well as only helical secondary structure elements (alignments denoted with -h). To enable comparison to FATCAT2.0, only present residue structures were selected because of FATCAT2.0's strict requirement for resolved coordinates. To evaluate alignment quality, we examined if three functionally-important and highly-conserved sequence motifs (the E/DRY, NPxxY and CWxP motifs; (Zhou et al., 2019; Venkatakrishnan et al., 2016) are faithfully assigned between the receptors. We found that only FATCAT2.0 aligned all three motifs correctly (Supplementary Fig. B.1). With this method in hand, we next compared sequence-based (as performed in previous studies; (Airan et al., 2009; Morri et al., 2018)) and structure-based alignments (Fig. 3.1c). A quantitative comparison was achieved using our own computer code that reports the number of identical residue pairs in two alignments (Supplementary Fig. B.2). We found that sequence and structure alignments differed in $\sim$ 20 to 40% of the aligned residue pairs (Fig. 3.1c, top). To determine if these differences will impact the design of chimeric GPCRs, we asked whether alignments were identical at the previously

utilized domain boundaries (Kim et al., 2005) (Fig. 3.1c, bottom, Supplementary Fig. B.3a). Strikingly, we observed only minimal differences that were limited to one receptor pair (rho:β_1AR), two boundaries for that pair and a single residue for these two boundaries (Fig. 3.1c, bottom, Supplementary Fig. B.3b). This result suggests that sequence- and structure-based alignments will result in similar OptoXRs for these prototypical receptors. This will not necessarily apply to all GPCRs as, e.g., long intracellular loops (ICLs) can confound sequence alignments. The observed alignment robustness nevertheless encouraged us to proceed and examine how functional domain boundaries can be identified in a structure-guided approach.

Domain boundaries and rationally-designed receptor variants

We went on to determine domain boundaries delineating functional elements of GPCRs towards a structure-guided OptoXR design. We chose rho and β_2AR as the initial receptors as both are well characterized, including structurally, and as both have been incorporated in OptoXRs previously (Supplementary Table B.1). To identify domains involved in downstream signaling, we closely examined structures of these GPCRs in complex with their cognate G-proteins ($G\alpha_t$ for rhodopsin and $G\alpha_s$ for β_2AR) (Rasmussen et al., 2011; Kang et al., 2018; Gao et al., 2019). Using a distance cutoff of 4 Å, chosen analogously to the first study describing a GPCR-Gα complex (Rasmussen et al., 2011), and for rho and β_2AR individually, we determined the receptor residues that are in proximity to the G-protein (Fig. 3.2a, Supplementary Fig. B.4a, Supplementary Fig. B.5). We found that these contacts extend upwards from the cytosolic interface on transmembrane helices TM3, TM5 and TM6 by as much as four helical turns (Fig. 3.2b, Supplementary Fig. B.4b). Importantly, this upward climb was not to the same level for the two receptors with contacts in general located closer to the cytosolic helix ends in rho compared to β_2AR (Fig. 3.2b, Supplementary Fig. B.4b). On the Gα subunit, as previously reported, main contacts included residues on the α5 helix and a hydrophobic pocket formed by the domain hinge region (Glukhova et al., 2018; Ahn et al., 2021)

The realizations that (i) GPCR-G-protein contacts are found on membrane helices several turns away from the cytosolic interface and (ii) this climb is receptor-specific had several implications. First, this prompted us to experimentally test the previously formulated and employed rationale that contacts in intracellular loops are sufficient for defining GPCR coupling specificity (see above). We engineered a

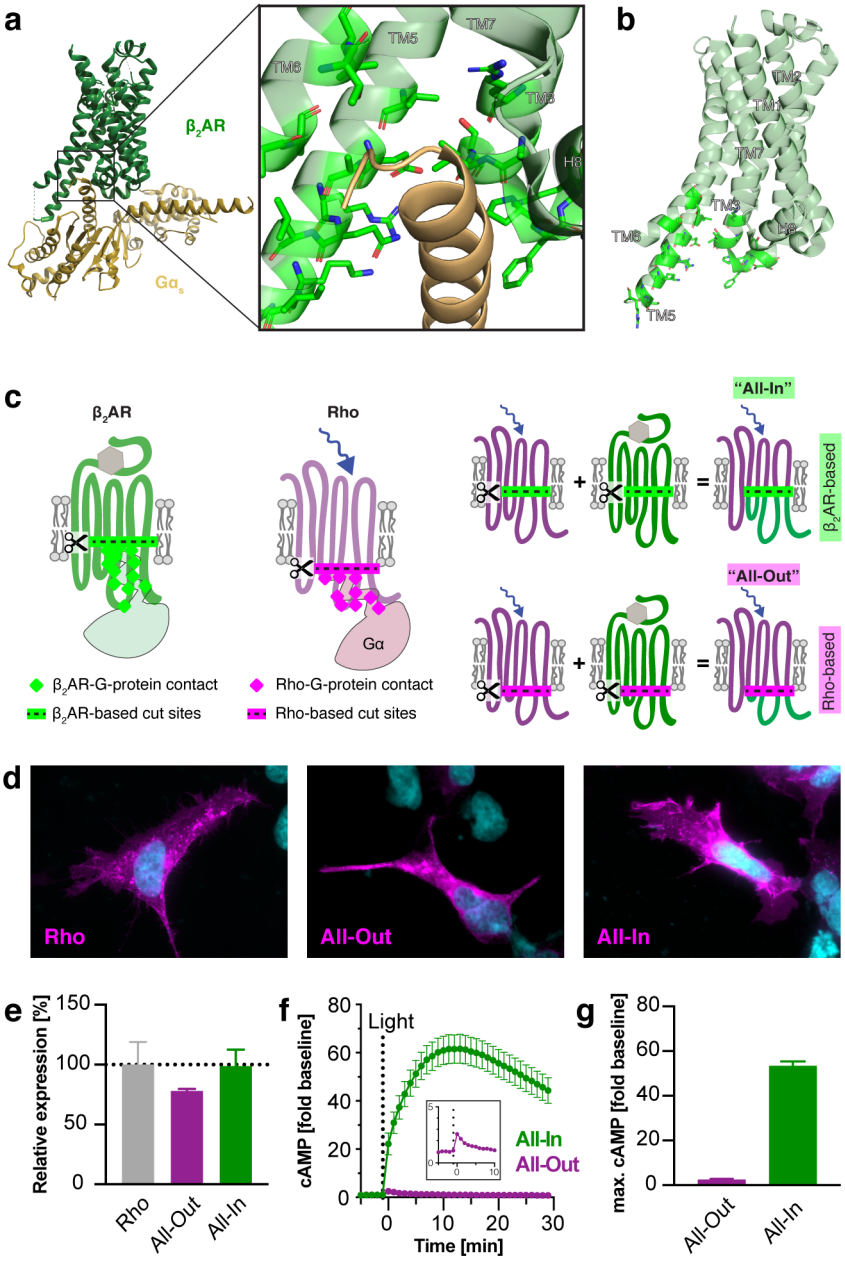


FIGURE 3.2: **Identification of functionally-relevant domain boundaries. (a)** G-protein contacts on β_2AR defined as described in the main text (PDB ID: 3SN6). **(b)** G-protein contacts (light green) extend upwards on TM helices. **(c)** Two strategies for the placement of domain boundaries in a β_2AR OptoXR either include all β_2AR residues ("All-In") or exclude all rho residues ("All-Out"). **(d)** Rho and OptoXR-All-In and -All-Out membrane localisation determined using IF against N-terminal rho epitope (α-4D2). **(e)** Rho and OptoXR expression determined using FC using the same antibody. **(f)** Time course of cAMP production following light stimulation of OptoXRs. Inset: Magnified trace of "All-Out" variant. Light dose: 4000 flashes, wavelength: 488+/-6 nm. **(g)** Maximal cAMP signals elicited by OptoXRs shown in **(f)**. For (e-f): n=9, 3 independent experiments. Data shown as mean ± S.E.M.

rho receptor containing exclusively the intracellular loops of β_2AR (termed Opto-β_2AR-Loops) and demonstrated expression in HEK293 cells using flow cytometry (FC) and immunofluorescence (IF) (Supplementary Figs. S6a,b). However, upon light stimulation (488+/- 6 nm) no increase in intracellular cAMP levels was observed, indicating that incorporation of these secondary structure elements in this receptor is not sufficient to confer downstream signaling (Supplementary Fig. B.6c). As the second implication, we formulated two strategies towards the structure-guided design of variant β_2AR OptoXRs. Specifically, we hypothesized that including an as complete as possible ensemble of G-protein contacts of β_2AR will result in efficient G-protein activation. We therefore designed a variant termed Opto-β_2AR-All-In where domain boundaries are placed to include all β_2AR-G α_s-contact residues (Fig. 3.2c). As a second variant, we designed Opto-β_2AR-All-Out in which all rho contact residues are excluded and consequently fewer β_2AR contact residues included. This latter variant tests if a partial domain exchange is sufficient for chimeric receptor function. We first confirmed expression and localisation of the receptors again using FC and IF (Fig. 3.2d,e). Next, we tested receptor functionality upon light stimulation (488+/-6 nm) and found marked differences (Fig. 3.2f,g). Activation of Opto-β_2AR-All-Out did result in some light-dependent cAMP production but only to very low levels (3-fold above baseline). In apparent contrast, Opto-β_2AR-All-In showed pronounced induction ($\sim$ 50-fold above baseline) that is comparable to ligand-induced responses in this cell type (see below). These results confirm our hypothesis that inclusion of residues identified using a structure-based approach yields chimeric receptors that produce significantly stronger activation of downstream signaling pathways. In light of this effect, we went on to further characterize the rationally-designed Opto-β_2AR-All-In, which we termed Opto-β_2AR-2.0, first in comparison to β_2AR and then to previous OptoXRs.

Signaling profile of Opto-β_2AR-2.0

We characterized the signalling profile of Opto-β_2AR-2.0 and compared it to that of β_2AR for multiple signaling pathways (Fig. 3.3a). As β_2AR canonically couples to Gα_s, we first investigated intracellular cAMP production. HEK293 cells expressing either Opto-β_2AR-2.0 or β_2AR were stimulated with light (488+/-6 nm) or the potent β-adrenoreceptor agonist isoproterenol (ISO) followed by real-time cAMP detection (Fig. 3.3b). Generally, cAMP levels reached upon Opto-β_2AR-2.0 activation were comparable to those reached upon β_2AR activation, and both receptors exhibited dose-dependent increases (half-maximal activation light dose (LD50) for Opto-β_2AR-2.0: 17 light flashes corresponding to $\sim$ 2.5 x 10^{12} photons,

EC50 for β_2AR: 8.7 nM) (Fig. 3.3c,d). As exemplified at light or drug doses that produce $\sim$ half-maximal activation, stimulation of Opto-β_2AR-2.0 resulted in shorter-lived increases of cAMP compared to β_2AR (Fig. 3.3b).

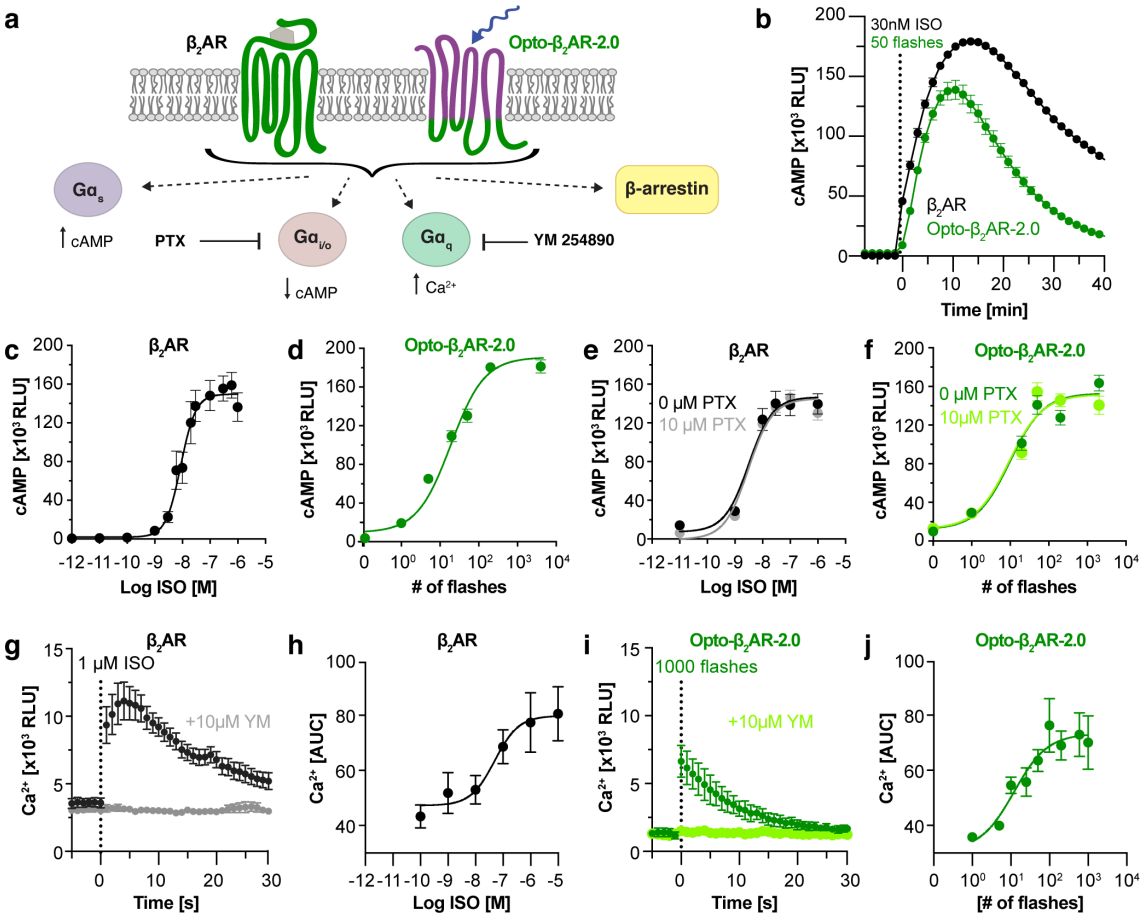


FIGURE 3.3: **Signalling profile of Opto-β_2AR-2.0. (a)** Signaling pathways examined for β_2AR and Opto-β_2AR-2.0. **(b)** Representative time course of cAMP production following stimulation of β_2AR or Opto-β_2AR-2.0 with ISO or light (wavelength: 488+/-6 nm), respectively. **(c, d)** cAMP increased in a dose-dependent manner following stimulation of β_2AR or Opto-β_2AR-2.0. **(e, f)** β_2AR (e) or Opto-β_2AR-2.0 (f) responses after treatment with 10 μM PTX. **(g,i)** Average time course of intracellular Ca^{2+} mobilization following stimulation of β_2AR with 1 μM ISO (g) or Opto-β_2AR-2.0 with light (i). Signalling was abolished by the Gα_q inhibitor YM 254890 (YM). **(h, j)** Ca^{2+} mobilization increased in a dose-dependent manner following stimulation of β_2AR (h) or Opoto-β_2AR-2.0 (j). For (c-j): n=9-12, 3-4 independent experiments. Data shown as mean $\pm$ S.E.M..

These distinct time courses are in line with Opto-β_2AR-2.0 being activated by light transiently (see below for a detailed analysis), whilst agonist action on β_2AR is continuous leading to longer lasting production of cAMP, as reported previously (Mathiesen et al., 2013; Roed et al., 2014) and as observed at multiple ligand doses (Supplementary Fig. B.7). Thus whilst experimental conditions can be identified (e.g., by varying light or ligand dose) in which activation time course of β_2AR and Opto-β_2AR appear comparable, any similarity in these is not a universal property.

β_2AR also has the ability to couple to Gα_i (Zhang et al., 2001; Ma et al., 2020). To determine activation of Gα_i by Opto-β_2AR-2.0 and β_2AR, we incubated cells expressing either receptor with the Gα_i specific inhibitor pertussis toxin (PTX) and examined cAMP increases upon receptor activation. We found that neither receptor exhibited PTX-dependent signaling (however, see below for effects of PTX on signaling of a previous β_2AR OptoXR) (Fig. 3.3e,f). Finally, we also investigated the ability of both receptors to mobilize intracellular Ca^{2+}. Analogous to cAMP production, both receptors induced dose-dependent Ca^{2+} mobilization to similar maximal levels (Fig. 3.3g-j). GPCRs can induce intracellular Ca^{2+} mobilization through multiple pathways, including the canonical Gα_q-PLC axis. To test whether β_2AR and Opto-β_2AR-2.0 activate Ca^{2+} via this axis, we applied the Gα_q inhibitor YM254890 (YM) (Takasaki et al., 2004) and found greatly reduced activity for both receptors (Fig. 3.3g,i). As before for cAMP production, reversal to basal Ca^{2+} levels was more rapid for Opto-β_2AR-2.0 than β_2AR. The general agreement between Opto-β_2AR-2.0 and β_2AR signaling observed for G-protein dependent pathways did strikingly not extend to β-arrestin. β_2AR, like many other GPCRs, exhibits the ability to undergo 'biased' signaling where outcomes downstream of G-proteins or β-arrestin are differentially induced dependent on the nature of the ligand. We quantified β-arrestin recruitment to β_2AR and Opto-β_2AR-2.0 using highly sensitive bioluminescence resonance energy transfer assays (BRET) (Supplementary Fig. B.8a). As previously demonstrated, β_2AR recruited β-arrestin2 in a dose-dependent manner with a half-maximal effective dose similar to that observed for cAMP induction (EC50=18 nM; Supplementary Fig. B.8b,c). However, we did not observe any light-dependent β-arrestin recruitment for Opto-β_2AR-2.0 even at the highest light doses (Supplementary Fig. B.8d). This result indicates a distinct behaviour of the two receptors. This may not be entirely surprising as active state confirmations and kinetics, which contribute to signaling bias (Liu et al., 2012; McCorvy et al., 2018; Lamichhane et al., 2020), are also encoded in the TM domains that are different in β_2AR and the chimera. Collectively, these results indicate striking similarities but also differences in the signaling response of Opto-β_2AR-2.0.

Optical activation profile of Opto-β_2AR-2.0

After having established the signalling profile of Opto-β_2AR-2.0, we went on to characterize the optical activation profile of the receptor. As above, we took advantage of a Xenon flash lamp to apply various light doses in a temporally-precise manner (flash duration: 2 μs, photon dose per flash: $\sim$ 1.5 x 10^{11}). We found that both signal magnitude and duration depended on light dose (Fig.

3.4a). Delivery of at least 200 flashes ($\sim$ 3 x 10^{13} photons) resulted in saturated cAMP levels matching those reached by saturating ISO concentrations on β_2AR. Notably, a single flash was also sufficient to induce a signal that was significant ($\sim$ 10% of maximal cAMP induction) and transient (decay within $\sim$ 10 min, i.e. much faster than the decay after application of the amplitude-equivalent dose of 0.1 nM ISO; 3.4a, Supplementary Fig. B.7). These results indicate that a wide temporal activation range is accessible to Opto-β_2AR-2.0. Opto-β_2AR-2.0 employs rho as light-sensing photoreceptor which is maximally sensitive to blue-green light (maximal absorption: 500 nm, full width at half maximum: 100 nm) (Partridge et al., 1991; Govardovskii et al., 2000). We confirmed the corresponding spectral sensitivity of Opto-β_2AR-2.0 by stimulating cells with light of different wavelengths (Fig. 3.4b). As expected, Opto-β_2AR-2.0 could be activated maximally at 488 nm (the closest wavelength to 500 nm that was available to us), half-maximally at 459 and 544 nm and only weakly at 355 nm (in the opsin β-band (Govardovskii et al., 2000)).

In the seminal study of Kim et al. (Kim et al., 2005), a chimeric rho:β_2AR receptor was engineered and characterized in a variety of cell types (Kim et al., 2005; Airan et al., 2009; Siuda et al., 2015b). Following the existing convention, we refer to this earlier OptoXR as Opto-β_2AR, whilst our novel receptors are termed Opto-β_2AR with further labels (e.g., 2.0 or Loops, as above). We here compared the efficiency of this receptor, which was designed using a trial-and-error approach (see above), to that of Opto-β_2AR-2.0 (Fig. 3.4c). We found that Opto-β_2AR-2.0 generated larger signals (by 7.2 fold) than Opto-β_2AR and produced half-maximal signals already at lower light doses (LD50 of Opto-β_2AR-2.0: 17 flashes, LD50 of Opto-β_2AR: 82 flashes). Opto-β_2AR-2.0 was not only more potent than Opto-β_2AR but also better suited for experiments in which repetitive light stimulation was applied. Unlike in the case of Opto-β_2AR, where a near-complete loss of induction was observed after four bouts of stimulations, large signals could still be produced by Opto-β_2AR-2.0 under these conditions (Fig. 3.4d). On the time scale of these experiments ($\sim$ 1.5 hrs), these signals were even comparable to the highly potent bistable opsin JellyOp (Bailes et al., 2012) from the jellyfish *Carybdea rastonii* (Supplementary Fig. B.9). Finally, one further difference between Opto-β_2AR and Opto-β_2AR-2.0 was that Opto-β_2AR exhibited PTX sensitive activity (Fig. 3.4e). We found that incubation with PTX resulted in increased cAMP signals indicative of blocked inhibitory effects of $G\alpha_i$, suggesting that Opto-β_2AR contained a signaling activity that is not representative for β_2AR (Fig. 3.3d). While the effect of PTX was pronounced, cAMP production was still many fold below that achievable with Opto-β_2AR-2.0 even under conditions of $G\alpha_i$ inhibition. Collectively, our analysis

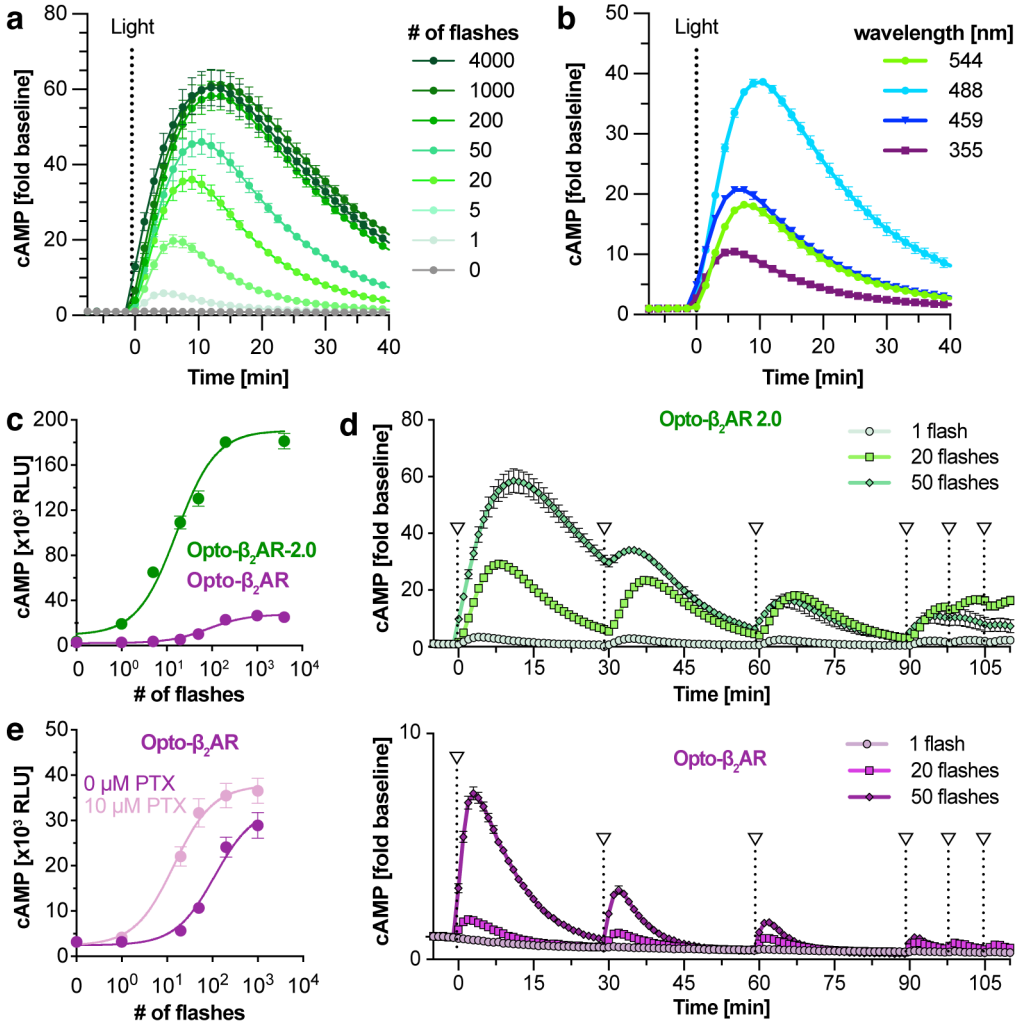


FIGURE 3.4: **Optical activation profile of Opto-β_2AR-2.0. (a)** Average time course of cAMP production following stimulation of Opto-β_2AR-2.0 with increasing light doses. **(b)** Stimulation of Opto-β_2AR-2.0 with light of different wavelengths (20 flashes). **(c)** Stimulation of Opto-β_2AR and Opto-β_2AR-2.0 with increasing light doses. **(d)** cAMP production following repeated light stimulation of Opto-β_2AR-2.0 (top) and Opto-β_2AR (bottom). **(e)** cAMP production following Opto-β_2AR stimulation after treatment with 10 μM PTX. For (a-e): n=6-9, 2-3 independent experiments. Data shown as mean $\pm$ S.E.M.

shows that Opto-β_2AR-2.0 is an efficient light-activated actuator that can offer finer temporal precision than ligand stimulation as well as higher potency and specificity than the closest known OptoXR.

A structure-guided experimental platform

We went on to demonstrate applications of the structure-based design principle in general and of Opto-β_2AR-2.0 in particular. We first tested if functional consequences of loss of GPCR-G-protein contact residues, which were identified in β_2AR-Gα_s complexes, can be recapitulated in Opto-β_2AR-2.0 (Fig. 3.5a). Complementary

to the two well-studied intracellular domains centered around ICL2 and ICL3, which differ between previous OptoXRs and Opto-β_2AR-2.0 and lead to improved function when incorporated using structural criteria (see Fig. 3.4 and above), we have investigated TM7. Specifically, residue R328^{7x55} at the cytoplasmic end of this helix has been implicated in G-protein binding through a contact to residue E392$^{H5.24}$ of Gα_s in structural studies (Zhang et al., 2020)and molecular dynamic simulations (Sandhu et al., 2019). Consequently, elimination of this interaction is expected to impact signaling which we were able to recapitulate in the context of Opto-β_2AR-2.0 (Fig. 3.5a). A deleterious mutation introduced at this site (R335^{7x55}) resulted in cAMP levels that were reduced by 25%, demonstrating that predicted contacts can be linked to function in a chimeric receptor.

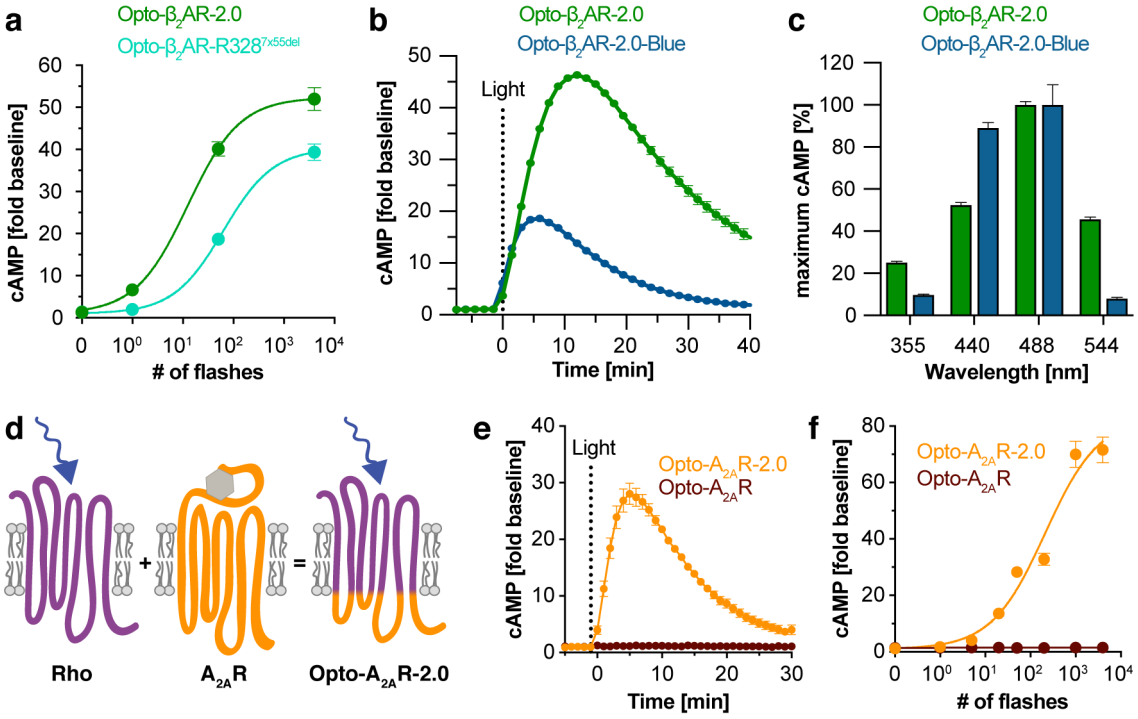


FIGURE 3.5: **A structure-guided experimental platform. (a)** cAMP production following stimulation of Opto-β_2AR-R328^{7x55}del. **(b)** Average time course of cAMP production following stimulation of Opto-β_2AR-2.0 and Opto-β_2AR-2.0-Blue with light (50 flashes). **(c)** Maximum cAMP levels following Opto-β_2AR-2.0 and Opto-β_2AR-2.0-Blue stimulation with light at different wavelengths (50 flashes), normalized to maximum response of each receptor. **(d)** Schematic of Opto-A$_{2A}$R-2.0. **(e)** Average time course of cAMP production following stimulation of Opto-A$_{2A}$R-2.0 and Opto-A$_{2A}$R with light (50 flashes). **(f)** Dose-dependent increases in cAMP production after stimulation Opto-A$_{2A}$R-2.0 and Opto-A$_{2A}$R with light. n=6-9, 2-3 independent experiments. Data shown as mean ± S.E.M.

Exploration of the contribution of R328^{7x55} to receptor function was assisted by the large signals generated by Opto-β_2AR-2.0. We reasoned that the improved function of Opto-β_2AR-2.0 may also enable experiments where the photosensitive

domain of the receptor is reengineered in the context of optogenetic tool development. Of general importance in the field of optogenetics is the availability of actuator proteins with sensitivities to different wavelengths, and both "blue shifting" (towards lower maximal absorption wavelengths) and "red shifting" (towards higher maximal absorption wavelengths) is desirable (Hochbaum et al., 2014; Forli et al., 2021; Lin et al., 2013; Tabor et al., 2011). This is generally achieved either through identification of naturally-occurring spectrally-diverse photoreceptors or through tuning of photoreceptors using substitutions in their chromophore binding pockets (Ziegler et al., 2015; O'Neill et al., 2015). Importantly, binding pocket mutagenesis is often associated with reduced protein folding and function in opsins (Deisseroth et al., 2017; Lin et al., 1998; Janz et al., 2001; Singhal et al., 2016), which is the likely reason why spectral tuning has never been demonstrated in OptoXRs. Indeed, when introducing three amino acid substitutions near the retinal binding site (T118^{3x33}A, E122^{3x37}D and A318^{7x39}S; residue numbers correspond to the Opto-β_2AR-2.0 sequence and, in superscript, to the GPCRdb numbering scheme (Isberg et al., 2015) that were previously shown to blue-shift rho absorption by $\sim$ 50 nm (Janz et al., 2001), receptor signaling was reduced dramatically (Fig. 3.5b). Despite reduced function, this Opto-β_2AR-2.0 variant still induced cAMP levels that are detectable and even comparable to previous non-shifted OptoXRs. As expected, activation by blue light was maintained but sensitivity to green light reduced (Fig. 3.5c), demonstrating spectral tuning in a prototypical OptoXR enabled by the larger signals of Opto-β_2AR-2.0. Motivated by above successes, we explored whether a residue contact-based design principle may also be applicable to other receptors (Fig. 3.5d). We have previously developed a light-activated Adenosine 2A receptor (Opto-A_{2A}R) and characterized its function in highly-sensitive integrative transcriptional reporter assays (Morri et al., 2018). Analogously as for β_2AR above, we determined G-protein proximal residues in an available A_{2A}R receptor structures (García-Nafría et al., 2018) to inform the placement of domain boundaries following the "All-In" rationale. We called the resulting receptor Opto-A_{2A}R-2.0. Similarly to Opto-β_2AR-2.0, Opto-A_{2A}R-2.0 contained additional A_{2A}R residues in TM3, TM6 and the C-terminus compared to Opto-A_{2A}R (Supplementary Tables B.3, B.4). We went on to test the ability of both receptors to induce cAMP responses and found that only Opto-A_{2A}R-2.0 resulted in strong activation in the real-time assays. This result demonstrates functional improvement in Opto-A_{2A}R-2.0 using the structure-guided approach.

3.2.5 Discussion

GPCRs relay diverse extracellular signals to specific downstream signaling pathways and thereby regulate essential physiological processes. To control the activity of GPCR signaling with spatio-temporal precision, chimeric light-activated receptors termed OptoXRs have been developed and employed alongside repurposed naturally-occuring opsins (Spangler et al., 2017; Wiegert et al., 2017). The design of most published OptoXRs relied on domain boundaries that were proposed in a seminal study (Kim et al., 2005). Notably, this recipe was formulated at a time when only few atomic resolution structures were available for GPCRs and none for GPCR-G-protein complexes. The past years have seen the emergence of structural information depicting 105 unique GPCRs and 51 unique GPCR-signaling-protein-complexes (Kooistra et al., 2021; Congreve et al., 2020). Here, we utilized this information in two ways towards rational design of OptoXRs. First, we evaluated if sequence alignments and structure alignments would yield the same chimeric receptors. For a set of prototypical receptors this was indeed the case. Second, we identified receptor residues that are proximal to the $G\alpha_s$-subunit and successively included these in the chimeric receptors. We found that an "all in"-rationale markedly improved the function of OptoXRs. These modifications, along with a non-functional fusion receptor that contained only the cytoplasmic loops of β_2AR, demonstrated that helical residues are required for efficient downstream coupling. In our best performing Opto-β_2AR-2.0, the incorporated critical helical residues (e.g., I139^{3x54}, T279^{6x36}, L280^{6x37} or R328^{7x55}) are found to be conserved in $G\alpha_s$ coupled aminergic (including adregenetric) receptors but not in non-G α_s-coupled receptors. Our comparison of Opto-β_2AR-2.0 to the previous Opto-β_2AR, that retained rho residues at these specific sites, revealed their functional contribution. In agreement with this is the reverse experiment, where the elimination or substitution of these residues was associated with a marked reduction in function, as exemplified in the case of R328^{7x55} here or also in earlier studies of non-chimeric β_2AR and other $G\alpha_s$-coupled aminergic receptors (Jones et al., 2020; Liu et al., 2014; Flock et al., 2017). Furthermore, our analysis also rationalizes previous findings that omission of entire structural domains, e.g. intracellular loops, resulted in reduced function of chimeric GPCRs (Kim et al., 2005; Bailes et al., 2012; Hickey et al., 2021). We consider introducing a coupling switch, e.g. from the preference for $G\alpha_{i/o}$ of rho to $G\alpha_s$, a good testbed to explore structure-guided receptor design. Our work is complementary to two recent studies in which coupling downstream of GPCRs was altered using structural information. Using computer simulations of receptors and $G\alpha$ helices in combination with functional assays, Sandhu et al. generated a triple mutant β_2AR

receptor with more promiscuous downstream coupling, in particular leading to more potent activation of $G\alpha_q$ compared to the WT receptor (Sandhu et al., 2019). Using structure modeling and in silico and experimental mutagenesis, Young et al. engineered remarkable orthogonal receptor-Gαpairs that interacted specifically and independently of endogenous signaling- components (Young et al., 2018). Collectively, these previous studies and our study demonstrate separate advances towards several distinct design objectives (coupling switch, promiscuity or orthogonality) and strategies (chimeric receptors or mutagenesis). These advances may have opened the door to an impressive ability to tune downstream GPCR function that can also be paired with existing reengineered receptors, such as chemogenetic actuators (Guettier et al., 2009; Nakajima et al., 2012) or a mutated OptoXR ((Siuda et al., 2015b), discussed below). One element of our study was examination to which degree a prototypical OptoXR recapitulates the function of the ligand-activated target receptor, in particular in the context of signaling bias. In signaling bias, GPCRs assume distinct ligand-dependent active states and state lifetimes that lead to preferred activation of some signaling pathways over others (Shonberg et al., 2014; Violin et al., 2014; Wingler et al., 2020). We propose that the outcomes downstream of OptoXR activation should be conceptually viewed in a similar light as outcomes downstream of receptor activation by biased ligands because both are associated with a particular signalling profile. Given the pronounced differences in the activation mechanism of rho and β_2AR, it may not be surprising that the signaling profile of Opto-β_2AR-2.0 was not identical to that of β_2AR. In particular, we were unable to detect β-arrestin recruitment for Opto-β_2AR-2.0 and thus under the conditions examined here signaling elicited by Opto-β_2AR-2.0 resembled more strongly that previously reported for G-protein-biased ligands, such as pepducins (Carr et al., 2014). It is noteworthy that an ability to predominantly activate G-protein-dependent pathways may be desirable in some physiological settings (Carr et al., 2014; Billington et al., 2013). This result may also be relevant in the context of an earlier study that proposed the development of a "functionally selective" Opto-β_2AR with limited $G\alpha_s$ signaling but potent induction of pathways that can be activated downstream of β-arrestin (Siuda et al., 2015b). Notably, this selectivity was achieved through mutagenesis in key functional motifs that are present in most if not all GPCRs, such as the highly-conserved tyrosine^{3x51} of the "E/DRY" motif or a further tyrosine^{5x58} that participates in active-state specific interactions (Siuda et al., 2015b; Zhou et al., 2019). Because of the placement of these substitutions in the rho photoreceptor core (as opposed to in β_2AR specific sequences as by us here) and because recruitment of β-arrestin was not tested in this previous study, a comparison of the previous

results and ours is not directly possible. The large signaling response elicited by Opto-β_2AR-2.0 prompted us to explore a wider scope and capabilities of OptoXRs. As discussed above, we were able to demonstrate that elimination of R328^{7x55}, which has been shown to form β_2AR-G-protein contacts to H5.24 (Sandhu et al., 2019; Zhang et al., 2020), reduces receptor function exemplifying how individual residues can be examined in the OptoXR context. As a further application, we introduced a spectral absorption shift to the rho photoreceptor core to diversify the optogenetic repertoire. Mutagenesis in binding pockets are associated with reduced protein folding and function in many protein families including in opsins (Tokuriki et al., 2008; Deisseroth et al., 2017; Lin et al., 1998; Janz et al., 2001; Singhal et al., 2016). Indeed, we observed that three residue substitutions in proximity of the retinal cofactor reduced maximal cAMP levels in a new blue-shifted Opto-β_2AR-2.0 variant. Despite this reduced function, this variant still induces cAMP levels that are comparable to previous unperturbed OptoXRs. Complementary to naturally-occurring spectrally-diverse opsins, we demonstrate for the first time spectral-tuning in an OptoXR. It becomes clear that characterization of spectral effects of these mutations may have been difficult in the context of existing OptoXRs. As a final demonstration, again prompted by the functional improvement observed in Opto-β_2AR-2.0, we went on to show that a structure-guided approach can also be applied to a further target receptor. As for Opto-β_2AR, Opto-A_{2A}R-2.0 outperformed a previous OptoXR version significantly upon inclusion of further helical contacts. Structural biology has already had a profound impact on the field of optogenetics, e.g. by enabling the rational design of light-activated ion channels and protein-protein interactions (Deisseroth et al., 2017; Dagliyan et al., 2016; Ziegler et al., 2015). We show that the rational exchange of receptor domains resulted in more potent OptoXR activity and the ability to design OptoXRs with new functions. However, because this is the first demonstration of structure-guided coupling engineering in OptoXRs and one of very few GPCRs in general, further studies are required to test how universal these approaches are. It is interesting to note that the temporal sequence of events driving G-protein association to a GPCR is an active area of study. Whilst it is possible that this association initially involves intermediate receptor states and contacts other than those observed in the stable GPCR-G protein complex structures, such contacts have not yet been identified structurally (Ma et al., 2020; Ahn et al., 2021; Du et al., 2019). Potentially OptoXRs may in the future support time-resolved studies of these binding events by providing rapid and non-invasive activation, similarly to instances of successful photoreceptor application in spectroscopy and drug discovery (Kottke et al., 2017; Agus et al., 2017). In

addition to serving as an experimental platform, targeted OptoXRs may in future work continue to make contributions towards understanding the impact of GPCR interactions on the level of cellular signaling networks.

3.2.6 Materials and Methods

Vectors and constructs

OptoXR genes were designed as described in the main text and ordered as synthetic genes (gBlocks, Integrated DNA Technologies). Protein and nucleotide sequences of all engineered receptors can be found in Supplementary Tables B.3 and B.4, respectively. Genes were amplified in polymerase chain reactions (PCRs) using oligonucleotides with XhoI and KpnI restriction site overhangs and digested with the respective enzymes (NEB Biolabs). pcDNA3.1(-) (Thermo Fisher Scientific) was digested with the same enzymes and ligated with the amplified receptors using T4 DNA ligase (Promega). For β-arrestin recruitment assays, receptors were tagged with Rluc8 (Savage et al., 2013). Receptor genes were amplified without a stop codon using oligonucleotides with BamHI and XhoI restriction site overhangs and digested with the respective enzymes. A vector containing Rluc8 (a kind gift of P. Sexton, Monash University) was digested with the same enzymes. Receptor genes were ligated into the vector N-terminally of Rluc8 using T4 DNA ligase. Opto-β_2AR and Opto-A_{2A}R vectors were in the same backbone and described in a previous publication (Morri et al., 2018). This Opto-β_2AR gene encodes for a protein that is identical to those published previously (Kim et al., 2005; Airan et al., 2009). The β_2AR expression vector was a gift of Robert Lefkowitz (Addgene plasmid #14697) (Tang et al., 1999). All genes were verified using Sanger sequencing.

Cell culture

HEK293 cells (Thermo Fisher Scientific; further authenticated by assessing cell morphology and growth rate) were cultured in mycoplasma-free Dulbecco's modified Eagle medium (DMEM, Gibco) in a humidified incubator with 5% CO2 atmosphere at 37°C. Medium was supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco). HEK293 cells were reverse transfected using poly(ethyleneimine) (linear, 20 kDA; Polysciences) (Tichy et al., 2019a). Culture medium was changed 6 h after transfection and supplemented with 10 μM 9-*cis*-retinal (Sigma) for experiments with light-activated receptors.

Real-time cAMP measurements

The real-time cAMP assay employed the GloSensor-22F reporter cloned into pcDNA3.1(-) as described previously (Morri et al., 2018). HEK293 cells were seeded at a density of 5 x 10^4 cells/well in white 96-well plates and transfected with receptor and reporter at a ratio of 1:1. HEK293 cells endogenously express β_2AR and were mock transfected with empty vector backbone for β_2AR conditions. The next day, culture medium was changed to Leibovitz's L15 (Gibco) starve medium (0.5% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin) supplemented with 2 mM D-luciferin (Cayman Chemicals) and 10 μM 9-*cis*-retinal in experiments with light-activated receptors. Cells were incubated for 60 min at RT. Luminescence was measured in a microplate reader (Omega, BMG Labtech). Baseline luminescence values were recorded for 8 cycles (90 sec/cycle) before stimulation. Cells were stimulated with light (using the flash module of the plate reader with each flash delivering a photon dose of 1.5 x 10^{11} photons) or ligand as described in the main text or figure captions. For PTX experiments, cells were incubated with 10 μM PTX (List Labs) overnight (o/n) at 37°C.

Real-time Ca^{2+} measurement

The real-time Ca^{2+} mobilization assay employed mtAequorin in pcDNA3.1 as described previously (Stables et al., 1997). HEK293 cells were seeded at a density of 5 x 10^4 cells/well in white 96-well plates and transfected with receptor and reporter at a ratio of 1:1. The next day, media was replaced with Hank's buffered salt solution (HBSS, Sigma) supplemented with 10 μM coelenterazine h (Nanolight) and 10 μM 9-*cis*-retinal in experiments with light-activated receptors. Cells were then incubated for 2 h at 37°C. Luminescence was measured in the microplate reader. Baseline luminescence values were recorded for 10 cycles (1 sec/cycle) before stimulation. Cells were stimulated with light or ligand at concentrations as described in the main text or figure captions. For YM254890 experiments, cells were incubated with 10 μM YM254890 (AdipoGen Life Sciences) for 2 h at 37°C before measurements.

Real-time β-arrestin2 recruitment

Receptor mediated β-arrestin2 recruitment was assessed using a real-time BRET assay detecting energy transfer between Rluc8-tagged receptors and a mVenus-tagged β-arrestin2. HEK293 cells were seeded at a density of 5 x 10^4 cells/well in white 96-well plates and transfected with receptor-Rluc8 and β-arrestin2-mVenus at a ratio of 1:4. Culture medium was changed 6 h after transfection and supplemented

with 10 μM 9-*cis*-retinal (Sigma) for experiments with light-activated receptors. The next day, media was replaced with 40 μl HBSS supplemen-ted and 10 μM 9-*cis*-retinal for experiments with light-activated receptors. Cells were incubated for 1 h at 37°C. Coelenterazine h was added at a final concentration of 5 μM immediately before measurements. Baseline values were recorded for 8 cycles (90 sec/cycle) before stimulation. Cells were stimulated with light or ligand at concentrations as described in the main text or figure captions. BRET measurements were obtained in the microplate reader using 460 (Rluc8) and 520 nm (mVenus) filters. The BRET ratio was calculated by dividing the emission at 520 nm by the emission at 460 nm.

Immunofluorescence (IF)

HEK293 cells were seeded on glass coverslips coated with poly-L-ornithine (Sigma) in 12-well plates (3 x 10^5 cells/well) and transfected with 1000 ng receptor plasmid. The next day, under dim red light, cells were washed once with pre-warmed PBS and then fixed with 4% PFA for 10 min at RT. Cells were washed three times with PBS and permeabilized with PBS containing 0.25% Triton-X. Cells were washed again three times with PBS and blocked with PBS + 0.1% Tween (PBS-T) containing 3% BSA at 37°C for 1 h. Cells were incubated with primary antibody (mouse anti-4D2, 1:500 dilution; Abcam, ab98887) at 4°C o/n, washed three times with PBS, and incubated with secondary antibody (donkey anti-mouse, Alexa488 conjugated, 1:750 dilution; Thermo Fisher Scientific, A21202) at RT for 2 h. Cells were washed once with PBS, incubated with DAPI (Life Technologies) at a final dilution of 1:4000 at RT for 5 min and washed twice with PBS. Coverslips were mounted on microscopy slides using Mowiol mounting media (Sigma) and left to dry. Images were taken on a Zeiss Axio Imager Z1.

Analysis of expression level using flow cytometry (FC)

HEK293 cells were seeded in 6-well plates at a density of 10^6 cells/well and transfected with 2000 ng receptor plasmid. The next day, under dim red light, cells were washed once with PBS and dispersed using Accutase (Sigma) at 37°C for 15 min. Accutase was neutralized using complete medium before the cell suspension was collected in a 15 ml tube and centrifuged. After removal of supernatant, cells were fixed in 2% PFA at 37°C for 10 min and washed once with PBS. Cells were permeabilized with ice-cold methanol on ice for 30 min. Cells were washed twice in FACS buffer (PBS supplemented with 2% FBS) and incubated with primary antibody (see above) at RT for 30 min. Cells were then washed once with FACS buffer and incubated with secondary antibody (see above) at

RT for 30 min. Samples, including mock transfected, unstained and secondary antibody controls, were run on the same day on a LSR Fortessa X-20 Cytometer (BD Biosciences) and data was analyzed using FlowJo software (Flowjo).

Comparison of sequence and structure alignments

Initial comparisons of sequence alignments and structure alignments were performed on rho (PDB ID: 6CMO) and β_2AR (PDB ID: 3SN6) using only the receptor chain (chain R). Alignments using *cealign* and *super* were performed in PyMol (3.7, Schrödinger). For alignments labelled *cealign*-h or *super*-h, only residues within helical secondary structures were included. FATCAT2.0 alignments were performed using the pairwise alignment option of the FATCAT2.0 server (Li et al., 2020) (https://fatcat.godziklab.org). MUSCLE (Madeira et al., 2019) sequence alignments were performed using the sequence of residues that are resolved in the PDB file to enable comparison to the FATCAT2.0 alignment. Alignments were manually inspected to determine whether key motifs (DRY, CWxP or NPxxY) were correctly aligned. For detailed comparison of FATCAT2.0 and MUSCLE alignments, rho (PDB ID: 6CMO) was also aligned to A_{2A}R (PDB ID: 6GDF), β_1AR (PDB ID: 7JJO) and M1R (PDB ID: 6OIJ) followed by quantification of identical pairs in macros written in Igor Pro (Wavemetrics). These MUSCLE and FATCAT2.0 alignments were manually analyzed to assess whether OptoXR boundaries were at identical positions.

GPCR-G-protein interacting residues and visualization

GPCR residues in proximity to the Gα subunit were determined in PyMol as described in the main text and figure captions. If available, multiple receptor structures were analysed to include all possible binding residues. Contact maps were verified using the automated CMView (Vehlow et al., 2011). PDB IDs of the analysed structures were as follows for the studied receptors: Rho (6CMO, 6OYA), β_2AR (3SN6, 7BZ2), A_{2A}R (6GDF). Residue contacts can be found in Supplementary Table B.2. Molecular structure images were generated in PyMol.

Data analysis

All pharmacological data were analysed using Prism (9.0, GraphPad). Dose-response data were generated by applying a three-parameter non-linear equation to data extracted from time courses. EC50 values were determined by fitting dose-response data with a sigmoid curve.

Chapter 4

Concluding remarks and future directions

Optogenetics has made it possible to investigate and answer a range of questions that previously were unanswerable, or could not even be asked. This holds true for optogenetic applications in neuroscience but also in other areas of research, such as cell or developmental biology. The lure of optogenetics lies in its capability to grant unprecedented spatio-temporal control. Its success, on the other hand, can be attributed to the tools in use, which are either applicable to multiple different questions (such as Channelrhodopsin for neuroscience) or can be adapted to suit specific needs. Since its inception ∼20 years ago, optogenetics has undergone different stages of development. First, the opsin toolbox for electrical control of neurons was expanded through the discovery of other opsin proteins and further engineering of existing ones. The second advancement was the utilisation of different microbial, plant and even animal photoreceptors to expand the molecular processes that can be controlled, to now include protein interactions or enzyme and receptor function. This has progressed the field to a point where it is possible to target varied and complex biological problems, such as metabolic engineering of microorganisms for production purposes (Zhao et al., 2018) or developing treatments for human diseases (Sahel et al., 2021).

Today, the field of optogenetics has evolved to face different challenges: on the one hand, the demand for bespoke optogenetic tools for distinct applications far outweighs the availability of these tools, and on the other hand, certain tools without alternatives do not work very well. This results in the need for simplified methods to design and engineer new tools, as well as for improvement of existing tools. In this thesis, I addressed both of these issues.

In chapter 2, I addressed the need for easier and faster methods to develop new optogenetic proteins, by establishing a new engineering strategy and vector

library. This facilitates a simpler engineering of optogenetic tools whereas the library serves a resource for the most commonly used photoreceptors. While the described method does not act as fool-proof protocol that will result in a functional optogenetic tool at all times, it provides a platform to simplify the testing of multiple photoreceptors, in different configurations and with varying linkers, and provides a photoreceptor collection in a clearly annotated and curated fashion.

Because of their versatility and perceived ease of engineering, Opto-PPIs have become attractive also for researchers that historically have not specialized in optogenetic engineering. An obstacle they face though is that the development of such tools is not always as straightforward as fusing a target protein to a photoreceptor (it can be, but often is not), and difficult and tedious optimization steps can be prohibitive to further developing a tool. By allowing rapid sampling of a large parameter space, this work provides an opportunity to make optogenetics more accessible, and to reduce the "barrier to entry" into optogenetics.

The tool developed in the study in a proof-of-principle application, Opto-casp9, can be used for a variety of purposes. In our study, focused on optogenetic tool development, we suggest the use of Opto-casp9 to characterize and benchmark parameters crucial to successful optogenetic experiments, such as culture conditions (e.g. co-factor, transfection amount, expression levels and time) or illumination set up (e.g. duration, wavelength and intensity, dark contamination). However, apoptosis is fundamental in many physiological processes, especially across development. Thus, Opto-casp9 is also a useful optogenetic tool in itself that can be used to study how controlled cell death, or the disruption thereof, affects tissue development or differentiation. Additionally, Opto-casp9 could be used in ways similar to its ligand induced counter part (iCasp9), to induce cell death in synthetic systems such as in CAR-T cells or iPSCs, with the added benefit of spatio-temporal control afforded by light.

The demand for simplified methods to develop and screen optogenetic tools is highlighted by the recent development of multiple such platforms. One such example is a method for the development of light-regulated allosteric control of enzymes (Dagliyan et al., 2016). This approach was recently advanced into a streamlined framework which enables this method to be applied to multiple different protein families, further increasing its accessibility (Dagliyan et al., 2019). Efforts to simplify the use of optogenetics have also been made along the avenue of illumination methods. For this purpose, several different resources have been published on how to build highly versatile and custom-made illumination devices with limited effort (Bugaj et al., 2019; Gerhardt et al., 2016). The vector library

and engineering method developed in this thesis complements these platforms to open optogenetics to a broader audience.

In the case of OptoXRs, the critical issue was a different one: the design approach itself was not a robust or well-understood one, limiting its use and application. While the common design in principle worked for some receptors, there have been multiple published accounts of receptors not functioning (Oh et al., 2010; McGregor et al., 2016), suggestive of a higher number of unpublished, non-functioning receptors. The underlying principle of the original design was based on swapping intracellular signalling domains (Kim et al., 2005), but the decision for cut sites being at a certain position over another was based on trial and error. This had the result that it was difficult to predict how changes to the design would affect receptor function, and subsequently hampered efforts to improve upon it. The work in this thesis, as described in chapter 3 provides a first design approach based on a clear rational: GPCR-G-protein interactions as observed in cryo-EM or crystal structures. This conceptual improvement of OptoXR design provides two main advantages: On the one hand, it results in receptors with increased activity, which also provides the opportunity to test further modifications. On the other hand, the clear underlying rational will make it easier to adapt and change the design to meet other objectives.

OptoXRs with improved functionality open the possibility to test a variety of other alterations. Due to higher overall signal, the dynamic range of the receptor is greatly expanded. This increases the likelihood that any effects on receptor behaviour can be confidently determined, even if the effects result in decreased function. We illustrate this by testing changes to the C-terminus as well as mutations resulting in a blue-shift of Opto-β_2AR 2.0. It is unlikely that the consequences of these changes could have been determined using previous Opto-β_2AR versions. Increased receptor activity further means that receptor activation can be achieved at sub-optimal light-conditions. This may be less critical in *in-vitro* settings, but becomes of greater importance when translating the use into *in-vivo* or even clinical settings. A recent publication reports on the successful use of optogenetics to partially restore vision in a blind patient suffering from retinitis pigmentosa (Sahel et al., 2021). While undoubtedly a breakthrough result, the high intensity of light required to activate the Channelrhodopsin employed necessitates the use of specific goggles to amplify the ambient light-signals. Approaches using animal opsin based receptors and OptoXRs (Berry et al., 2019; Wyk et al., 2015) show increased light sensitivity compared to Channelrhodopsins in animal models, but still showcases that light-sensitivity will be a major driving factor

on the pathway of using optogenetics for vision restoration, irrespective of the employed tool.

The second advantage of the improved OptoXR design is that the clear rational provides more opportunities for targeted adaptations to the cut sites, rather than making changes "blindly". This increases the probability that more receptors can be turned into OptoXRs. However, the new approach described in this thesis does not guarantee that a functional OptoXR can be engineered for every GPCR, even if a structure is available.

The specific OptoXR developed in this study, Opto-β_2AR 2.0, displays marked increases in functionality, particularly with regards to cAMP production, proving that the hypothesis of including intracellular G-protein contacts in OptoXR chimeras positively impacts function. This is a major improvement compared to previous design strategies, but does not represent the end of the road, and further optimisations and characterisation can be undertaken in future studies. For one, the precise mechanism how the improved functionality is achieved is worthy of additional investigation. To do this, multiple aspects can be interrogated. The role of contact residues and thus domain boundaries could be characterized in more detail, both to confirm the current hypothesis or to identify further optimisation steps. This would require detailed mutagenesis studies, likely with an array of variants with double or triple mutations to determine the effect on G-protein binding. In addition, it would be interesting to see whether the exact mechanism for the increased function can be determined, such as increased G-protein binding/retention, more efficient activation or more stable signalling state. When looking at Opto-β_2AR 2.0 as an optogenetic tool, in particular the temporal properties are of interest and could be looked at in future studies, such as further characterization of the kinetics of the different pathways activated or the impact of intensity on kinetics. This could provide information that would allow to program a stimulation regime that could result in a defined set and duration of signalling output.

While our understanding of GPCRs has increased dramatically over the years and there are many reports describing universal mechanisms (Flock et al., 2015; Venkatakrishnan et al., 2016; Zhou et al., 2019), our knowledge does not extend far enough yet to fully understand or predict how a GPCR functions. Here, we design OptoXRs under the hypothesis that G-protein contacts play an integral role, but we do not take into account other aspects, such as differences in receptor movement upon activation (Latorraca et al., 2017). Nevertheless, this new approach provides a framework to investigate questions such as these on OptoXRs or other GPCR

chimeras. Similar to how chimeric GPCR studies were initially used to delineate structure function relationships (Kobilka et al., 1988; Cotecchia et al., 1990), chimeric GPCRs may now again be used in a similar fashion to investigate how ubiquitous or transferable certain facets of GPCR function are in various cell types and contexts.

This thesis has addressed two major challenges in the field of optogenetics by providing a means to accelerate as well as improve the engineering and design of current optogenetic tools. The work presented here will facilitate broader accessibility of optogenetics, and will enable optogenetics to be applied to new avenues of research.

Bibliography

Abreu, Nohely and Joshua Levitz (2020). "Optogenetic Techniques for Manipulating and Sensing G Protein-Coupled Receptor Signaling". In: pp. 21–51. DOI: 10.1007/978-1-0716-0755-8_2.

Agus, Viviana and Harald Janovjak (2017). "Optogenetic methods in drug screening: technologies and applications." In: *Curr. Opin. Biotechnol.* 48, pp. 8–14. DOI: 10.1016/j.copbio.2017.02.006.

Ahn, Donghoon, Donghee Ham, and Ka Young Chung (2021). "The conformational transition during G protein-coupled receptor (GPCR) and G protein interaction." In: *Curr. Opin. Struct. Biol.* 69, pp. 117–123. DOI: 10.1016/j.sbi.2021.03.013.

Airan, R D, K R Thompson, L E Fenno, H Bernstein, and K Deisseroth (2009). "Temporally precise in vivo control of intracellular signalling". In: *Nature* 458.7241, pp. 1025–1029. DOI: 10.1038/nature07926.

Atwood, Brady K, Jacqueline Lopez, James Wager-Miller, Ken Mackie, and Alex Straiker (2011). "Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis." In: *BMC Genomics* 12, p. 14. DOI: 10.1186/1471-2164-12-14.

Bailes, Helena J, Ling-Yu Y Zhuang, and Robert J Lucas (2012). "Reproducible and sustained regulation of Gαs signalling using a metazoan opsin as an optogenetic tool." In: *PLoS One* 7.1, e30774. DOI: 10.1371/journal.pone.0030774.

Banerjee, Roopa, Erik Schleicher, Stefan Meier, Rafael Muñoz Viana, Richard Pokorny, Margaret Ahmad, Robert Bittl, and Alfred Batschauer (2007). "The Signaling State of Arabidopsis Cryptochrome 2 Contains Flavin Semiquinone". In: *J. Biol. Chem.* 282.20, pp. 14916–14922. DOI: 10.1074/jbc.M700616200.

Banghart, Matthew R. and Bernardo L. Sabatini (2012). "Photoactivatable Neuropeptides for Spatiotemporally Precise Delivery of Opioids in Neural Tissue". In: *Neuron* 73.2, pp. 249–259. DOI: 10.1016/j.neuron.2011.11.016.

Benedetti, Lorena, Andrew E.S. Barentine, Mirko Messa, Heather Wheeler, Joerg Bewersdorf, and Pietro De Camilli (2018). "Light-activated protein interaction with high spatial subcellular confinement". In: *Proc. Natl. Acad. Sci. U. S. A.* 115.10, E2238–E2245. DOI: 10.1073/pnas.1713845115.

Benedetti, Lorena, Jonathan S. Marvin, Hanieh Falahati, Andres Guillén-Samander, Loren L. Looger, and Pietro De Camilli (2020). "Optimized vivid-derived magnets photodimerizers for subcellular optogenetics in mammalian cells". In: *Elife* 9, pp. 1–49. DOI: 10.7554/eLife.63230.

Berry, Michael H, Amy Holt, Autoosa Salari, Julia Veit, Meike Visel, Joshua Levitz, Krisha Aghi, Benjamin M Gaub, Benjamin Sivyer, John G Flannery, and Ehud Y Isacoff (2019). "Restoration of high-sensitivity and adapting vision with a cone opsin." In: *Nat. Commun.* 10.1, p. 1221. DOI: 10.1038/s41467-019-09124-x.

Billington, Charlotte K, Oluwaseun O Ojo, Raymond B Penn, and Satoru Ito (2013). "cAMP regulation of airway smooth muscle function." In: *Pulm. Pharmacol. Ther.* 26.1, pp. 112–20. DOI: 10.1016/j.pupt.2012.05.007.

Boyden, E S, F Zhang, E Bamberg, G Nagel, and K Deisseroth (2005). "Millisecond-timescale, genetically targeted optical control of neural activity". In: *Nat Neurosci* 8.9, pp. 1263–1268. DOI: 10.1038/nn1525.

Bugaj, L J, A T Choksi, C K Mesuda, R S Kane, and D V Schaffer (2013). "Optogenetic protein clustering and signaling activation in mammalian cells". In: *Nat Methods* 10.3, pp. 249–252. DOI: 10.1038/nmeth.2360.

Bugaj, L. J., A. J. Sabnis, A. Mitchell, J. E. Garbarino, J. E. Toettcher, T. G. Bivona, and W. A. Lim (2018). "Cancer mutations and targeted drugs can disrupt dynamic signal encoding by the Ras-Erk pathway". In: *Science (80-.).* 361.6405. DOI: 10.1126/science.aao3048.

Bugaj, Lukasz J. and Wendell A. Lim (2019). "High-throughput multicolor optogenetics in microwell plates". In: *Nat. Protoc.* 14.7, pp. 2205–2228. DOI: 10.1038/s41596-019-0178-y.

Carr, Richard, Yang Du, Julie Quoyer, Reynold A Panettieri, Jay M Janz, Michel Bouvier, Brian K Kobilka, and Jeffrey L Benovic (2014). "Development and characterization of pepducins as Gs-biased allosteric agonists." In: *J. Biol. Chem.* 289.52, pp. 35668–84. DOI: 10.1074/jbc.M114.618819.

Cehajic-Kapetanovic, Jasmina, Cyril Eleftheriou, Annette E Allen, Nina Milosavljevic, Abigail Pienaar, Robert Bedford, Katherine E Davis, Paul N Bishop, and Robert J Lucas (2015). "Restoration of Vision with Ectopic Expression of Human Rod Opsin." In: *Curr. Biol.* 25.16, pp. 2111–22. DOI: 10.1016/j.cub.2015.07.029.

Chatelle, Claire, Rocio Ochoa-Fernandez, Raphael Engesser, Nils Schneider, Hannes M. Beyer, Alex R. Jones, Jens Timmer, Matias D. Zurbriggen, and Wilfried Weber (2018). "A Green-Light-Responsive System for the Control of Transgene Expression in Mammalian and Plant Cells". In: *ACS Synth. Biol.* 7.5, pp. 1349–1358. DOI: 10.1021/acssynbio.7b00450.

Chen, Kuang-Yui Michael, Daniel Keri, and Patrick Barth (2020). "Computational design of G Protein-Coupled Receptor allosteric signal transductions." In: *Nat. Chem. Biol.* 16.1, pp. 77–86. DOI: 10.1038/s41589-019-0407-2.

Chène, P (2001). "The role of tetramerization in p53 function." In: *Oncogene* 20.21, pp. 2611–7. DOI: 10.1038/sj.onc.1204373.

Congreve, Miles, Chris de Graaf, Nigel A Swain, and Christopher G Tate (2020). "Impact of GPCR Structures on Drug Discovery." In: *Cell* 181.1, pp. 81–91. DOI: 10.1016/j.cell.2020.03.003.

Conrad, Karen S., Craig C. Manahan, and Brian R. Crane (2014). "Photochemistry of flavoprotein light sensors". In: *Nat. Chem. Biol.* 10.10, pp. 801–809. DOI: 10.1038/nchembio.1633.

Copits, Bryan A et al. (2021). "A photoswitchable GPCR-based opsin for presynaptic inhibition." In: *Neuron* 109.11, 1791–1809.e11. DOI: 10.1016/j.neuron.2021.04.026.

Cotecchia, S., S. Exum, M. G. Caron, and R. J. Lefkowitz (1990). "Regions of the α1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function". In: *Proc. Natl. Acad. Sci. U. S. A.* 87.8, pp. 2896–2900. DOI: 10.1073/pnas.87.8.2896.

Cvicek, Vaclav, William A. Goddard, and Ravinder Abrol (2016). *Structure-Based Sequence Alignment of the Transmembrane Domains of All Human GPCRs: Phylogenetic, Structural and Functional Implications*. Vol. 12. 3, pp. 1–31. DOI: 10.1371/journal.pcbi.1004805.

Dagliyan, Onur, Nikolay V. Dokholyan, and Klaus M. Hahn (2019). "Engineering proteins for allosteric control by light or ligands". In: *Nat. Protoc.* 14.6, pp. 1863–1883. DOI: 10.1038/s41596-019-0165-3.

Dagliyan, Onur, Miroslaw Tarnawski, Pei Hsuan Chu, David Shirvanyants, Ilme Schlichting, Nikolay V. Dokholyan, and Klaus M. Hahn (2016). "Engineering extrinsic disorder to control protein activity in living cells". In: *Science (80-.).* 354.6318, pp. 1441–1444. DOI: 10.1126/science.aah3404.

Deisseroth, Karl and Peter Hegemann (2017). "The form and function of channelrhodopsin." In: *Science* 357.6356. DOI: 10.1126/science.aan5544.

Dine, Elliot, Agnieszka A. Gil, Giselle Uribe, Clifford P. Brangwynne, and Jared E. Toettcher (2018). "Protein Phase Separation Provides Long-Term Memory of Transient Spatial Stimuli". In: *Cell Syst.* 6.6, 655–663.e5. DOI: 10.1016/j.cels.2018.05.002.

Du, Yang et al. (2019). "Assembly of a GPCR-G Protein Complex." In: *Cell* 177.5, 1232–1242.e11. DOI: 10.1016/j.cell.2019.04.022.

Ernst, Oliver P., David T. Lodowski, Marcus Elstner, Peter Hegemann, Leonid S. Brown, and Hideki Kandori (2014). "Microbial and animal rhodopsins: Structures, functions, and molecular mechanisms". In: *Chem. Rev.* 114.1, pp. 126–163. DOI: 10.1021/cr4003769.

Farran, B (2017). "An update on the physiological and therapeutic relevance of GPCR oligomers". In: *Pharmacol Res* 117, pp. 303–327. DOI: 10.1016/j.phrs.2017.01.008.

Flock, Tilman, Alexander S. Hauser, Nadia Lund, David E. Gloriam, Santhanam Balaji, and M. Madan Babu (2017). "Selectivity determinants of GPCR-G-protein binding". In: *Nature* 545.7654, pp. 317–322. DOI: 10.1038/nature22070.

Flock, Tilman, Charles N.J. Ravarani, Dawei Sun, A. J. Venkatakrishnan, Melis Kayikci, Christopher G. Tate, Dmitry B. Veprintsev, and M. Madan Babu (2015). "Universal allosteric mechanism for Gα activation by GPCRs". In: *Nature* 524.7564, pp. 173–179. DOI: 10.1038/nature14663.

Forli, Angelo, Matteo Pisoni, Yoav Printz, Ofer Yizhar, and Tommaso Fellin (2021). "Optogenetic strategies for high-efficiency all-optical interrogation using blue-light-sensitive opsins." In: *Elife* 10. DOI: 10.7554/eLife.63359.

Foster, Russell G., Mark W. Hankins, and Stuart N. Peirson (2007). "Light, photoreceptors, and circadian clocks". In: *Methods Mol. Biol.* 362, pp. 3–28. DOI: 10.1385/1-59745-257-2:3.

Fredriksson, Robert, Malin C. Lagerström, Lars Gustav Lundin, and Helgi B. Schiöth (2003). "The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints". In: *Mol. Pharmacol.* 63.6, pp. 1256–1272. DOI: 10.1124/mol.63.6.1256.

Gao, Yang, Hongli Hu, Sekar Ramachandran, Jon W. Erickson, Richard A. Cerione, and Georgios Skiniotis (2019). "Structures of the Rhodopsin-Transducin Complex: Insights into G-Protein Activation". In: *Mol. Cell* 75.4, 781–790.e3. DOI: 10.1016/j.molcel.2019.06.007.

García-Nafría, Javier, Yang Lee, Xiaochen Bai, Byron Carpenter, and Christopher G Tate (2018). "Cryo-EM structure of the adenosine A2A receptor coupled to an engineered heterotrimeric G protein." In: *Elife* 7. DOI: 10.7554/eLife.35946.

Gerhardt, Karl P., Evan J. Olson, Sebastian M. Castillo-Hair, Lucas A. Hartsough, Brian P. Landry, Felix Ekness, Rayka Yokoo, Eric J. Gomez, Prabha Ramakrishnan, Junghae Suh, David F. Savage, and Jeffrey J. Tabor (2016). "An open-hardware platform for optogenetics and photobiology". In: *Sci. Rep.* 6.November, pp. 1–13. DOI: 10.1038/srep35363.

Glukhova, A, C J Draper-Joyce, R K Sunahara, A Christopoulos, D Wootten, and P M Sexton (2018). "Rules of Engagement: GPCRs and G Proteins". In: *ACS Pharmacol Transl Sci* 1.2, pp. 73–83. DOI: 10.1021/acsptsci.8b00026.

Golonka, David, Patrick Fischbach, Siddhartha G. Jena, Julius R.W. Kleeberg, Lars Oliver Essen, Jared E. Toettcher, Matias D. Zurbriggen, and Andreas Möglich (2019). "Deconstructing and repurposing the light-regulated interplay between Arabidopsis phytochromes and interacting factors". In: *Commun. Biol.* 2.1, pp. 1–12. DOI: 10.1038/s42003-019-0687-9.

Govardovskii, V I, N Fyhrquist, T Reuter, D G Kuzmin, and K Donner (2000). "In search of the visual pigment template." In: *Vis. Neurosci.* 17.4, pp. 509–28. DOI: 10.1017/s0952523800174036.

Grusch, Michael, Karin Schelch, Robert Riedler, Eva Reichhart, Christopher Differ, Walter Berger, Álvaro Inglés-Prieto, and Harald Janovjak (2014). "Spatio-temporally precise activation of engineered receptor tyrosine kinases by light". In: *EMBO J.* 33.15, pp. 1713–1726. DOI: 10.15252/embj.201387695.

Guettier, Jean-Marc, Dinesh Gautam, Marco Scarselli, Inigo Ruiz de Azua, Jian Hua Li, Erica Rosemond, Xiaochao Ma, Frank J Gonzalez, Blaine N Armbruster, Huiyan Lu, Bryan L Roth, and Jürgen Wess (2009). "A chemical-genetic approach to study G protein regulation of beta cell function in vivo." In: *Proc. Natl. Acad. Sci. U. S. A.* 106.45, pp. 19197–202. DOI: 10.1073/pnas.0906593106.

Gunaydin, L A, L Grosenick, J C Finkelstein, I V Kauvar, L E Fenno, A Adhikari, S Lammel, J J Mirzabekov, R D Airan, K A Zalocusky, K M Tye, P Anikeeva, R C Malenka, and K Deisseroth (2014). "Natural neural projection dynamics underlying social behavior". In: *Cell* 157.7, pp. 1535–1551. DOI: 10.1016/j.cell.2014.05.017.

Guntas, Gurkan, Ryan A. Hallett, Seth P. Zimmerman, Tishan Williams, Hayretin Yumerefendi, James E. Bear, and Brian Kuhlman (2015). "Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins". In: *Proc. Natl. Acad. Sci. U. S. A.* 112.1, pp. 112–117. DOI: 10.1073/pnas.1417910112.

Gurevich, Vsevolod V and Eugenia V Gurevich (2017). "Molecular Mechanisms of GPCR Signaling: A Structural Perspective." In: *Int. J. Mol. Sci.* 18.12. DOI: 10.3390/ijms18122519.

Hauser, A S, M M Attwood, M Rask-Andersen, H B Schioth, and D E Gloriam (2017). "Trends in GPCR drug discovery: new agents, targets and indications". eng. In: *Nat Rev Drug Discov* 16.12, pp. 829–842. DOI: 10.1038/nrd.2017.178.

Hickey, Doron G, Wayne I L Davies, Steven Hughes, Jessica Rodgers, Navamayooran Thavanesan, Robert E MacLaren, and Mark W Hankins (2021). "Chimeric

human opsins as optogenetic light sensitisers." In: *J. Exp. Biol.* 224.14. DOI: 10.1242/jeb.240580.

Hilger, Daniel, Matthieu Masureel, and Brian K. Kobilka (2018). "Structure and dynamics of GPCR signaling complexes". In: *Nat. Struct. Mol. Biol.* 25.1, pp. 4–12. DOI: 10.1038/s41594-017-0011-7.

Hoang, Nathalie, Jean-Pierre Bouly, and Margaret Ahmad (2008). "Evidence of a Light-Sensing Role for Folate in Arabidopsis Cryptochrome Blue-Light Receptors". In: *Mol. Plant* 1.1, pp. 68–74. DOI: 10.1093/mp/ssm008.

Hochbaum, Daniel R et al. (2014). "All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins." In: *Nat. Methods* 11.8, pp. 825–33. DOI: 10.1038/nmeth.3000.

Hühner, Jens, Álvaro Ingles-Prieto, Christian Neusüß, Michael Lämmerhofer, and Harald Janovjak (2015). "Quantification of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in mammalian model cells by CE with LED-induced fluorescence detection". In: *Electrophoresis* 36.4, pp. 518–525. DOI: 10.1002/elps.201400451.

Humphreys, Paul A., Steven Woods, Christopher A. Smith, Nicola Bates, Stuart A. Cain, Robert Lucas, and Susan J. Kimber (2020). "Optogenetic control of the BMP signaling pathway". In: *ACS Synth. Biol.* 9.11, pp. 3067–3078. DOI: 10.1021/acssynbio.0c00315.

Ingles-Prieto, Alvaro, Nikolas Furthmann, Samuel H. Crossman, Alexandra Madelaine Tichy, Nina Hoyer, Meike Petersen, Vanessa Zheden, Julia Biebl, Eva Reichhart, Attila Gyoergy, Daria E. Siekhaus, Peter Soba, Konstanze F. Winklhofer, and Harald Janovjak (2021). "Optogenetic delivery of trophic signals in a genetic model of Parkinson's disease". In: *PLoS Genet.* 17.4 April 2021, pp. 1–23. DOI: 10.1371/journal.pgen.1009479.

Insel, Paul A, Krishna Sriram, Matthew W Gorr, Shu Z Wiley, Alexander Michkov, Cristina Salmerón, and Amy M Chinn (2019). "GPCRomics: An Approach to Discover GPCR Drug Targets." In: *Trends Pharmacol. Sci.* 40.6, pp. 378–387. DOI: 10.1016/j.tips.2019.04.001.

Isberg, Vignir, Chris de Graaf, Andrea Bortolato, Vadim Cherezov, Vsevolod Katritch, Fiona H Marshall, Stefan Mordalski, Jean-Philippe Pin, Raymond C Stevens, Gerrit Vriend, and David E Gloriam (2015). "Generic GPCR residue numbers - aligning topology maps while minding the gaps." In: *Trends Pharmacol. Sci.* 36.1, pp. 22–31. DOI: 10.1016/j.tips.2014.11.001.

Iseki, Mineo, Shigeru Matsunaga, Akio Murakami, Kaoru Ohno, Kiyoshi Shiga, Kazuichi Yoshida, Michizo Sugai, Tetsuo Takahashi, Terumitsu Hori, and Masakatsu

Watanabe (2002). "A blue-light-activated adenylyl cyclase mediates photoavoidance in Euglena gracilis". In: *Nature* 415.6875, pp. 1047–1051. DOI: 10.1038/4151047a.

Iwai, Youichi, Katsuya Ozawa, Kazuko Yahagi, Tsuneko Mishima, Sonam Akther, Camilla Trang Vo, Ashley Bomin Lee, Mika Tanaka, Shigeyoshi Itohara, and Hajime Hirase (2021). "Transient Astrocytic Gq Signaling Underlies Remote Memory Enhancement." In: *Front. Neural Circuits* 15, p. 658343. DOI: 10.3389/fncir.2021.658343.

Janz, J M and D L Farrens (2001). "Engineering a functional blue-wavelength-shifted rhodopsin mutant." In: *Biochemistry* 40.24, pp. 7219–27. DOI: 10.1021/bi002937i.

Jones, Eric M, Nathan B Lubock, A J Venkatakrishnan, Jeffrey Wang, Alex M Tseng, Joseph M Paggi, Naomi R Latorraca, Daniel Cancilla, Megan Satyadi, Jessica E Davis, M Madan Babu, Ron O Dror, and Sriram Kosuri (2020). "Structural and functional characterization of G protein-coupled receptors with deep mutational scanning." In: *Elife* 9. DOI: 10.7554/eLife.54895.

Jost, Marco, Jésus Fernández-Zapata, María Carmen Polanco, Juan Manuel Ortiz-Guerrero, Percival Yang Ting Chen, Gyunghoon Kang, S. Padmanabhan, Montserrat Elías-Arnanz, and Catherine L. Drennan (2015a). "Structural basis for gene regulation by a B12-dependent photoreceptor". In: *Nature* 526.7574, pp. 536–541. DOI: 10.1038/nature14950.

Jost, Marco, Jeffrey H. Simpson, and Catherine L. Drennan (2015b). "The transcription factor CarH safeguards use of adenosylcobalamin as a light sensor by altering the photolysis products". In: *Biochemistry* 54.21, pp. 3231–3234. DOI: 10.1021/acs.biochem.5b00416.

Kainrath, Stephanie, Manuela Stadler, Eva Reichhart, Martin Distel, and Harald Janovjak (2017). "Green-Light-Induced Inactivation of Receptor Signaling Using Cobalamin-Binding Domains". In: *Angew. Chemie - Int. Ed.* 56.16, pp. 4608–4611. DOI: 10.1002/anie.201611998.

Kang, Y et al. (2018). "Cryo-EM structure of human rhodopsin bound to an inhibitory G protein". In: *Nature* 558.7711, pp. 553–558. DOI: 10.1038/s41586-018-0215-y.

Karunarathne, W K Ajith, Lopamudra Giri, Vani Kalyanaraman, and N Gautam (2013). "Optically triggering spatiotemporally confined GPCR activity in a cell and programming neurite initiation and extension." In: *Proc. Natl. Acad. Sci. U. S. A.* 110.17, E1565–74. DOI: 10.1073/pnas.1220697110.

Kawano, Fuun, Hideyuki Suzuki, Akihiro Furuya, and Moritoshi Sato (2015). "Engineered pairs of distinct photoswitches for optogenetic control of cellular proteins". In: *Nat. Commun.* 6.1, p. 6256. DOI: 10.1038/ncomms7256.

Kenakin, Terry and Arthur Christopoulos (2013). "Signalling bias in new drug discovery: Detection, quantification and therapeutic impact". In: *Nat. Rev. Drug Discov.* 12.3, pp. 205–216. DOI: 10.1038/nrd3954.

Kennedy, Matthew J., Robert M. Hughes, Leslie A. Peteya, Joel W. Schwartz, Michael D. Ehlers, and Chandra L. Tucker (2010). "Rapid blue-light-mediated induction of protein interactions in living cells". In: *Nat. Methods* 7.12, pp. 973–975. DOI: 10.1038/nmeth.1524.

Keri, D. and P. Barth (2018). "Reprogramming G protein coupled receptor structure and function". In: *Curr. Opin. Struct. Biol.* 51, pp. 187–194. DOI: 10.1016/j.sbi.2018.07.008.

Kholodenko, Boris N., Jan B. Hoek, and Hans V. Westerhoff (2000). "Why cytoplasmic signalling proteins should be recruited to cell membranes". In: *Trends Cell Biol.* 10.5, pp. 173–178. DOI: 10.1016/S0962-8924(00)01741-4.

Kim, J M, J Hwa, P Garriga, P J Reeves, U L RajBhandary, and H G Khorana (2005). "Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops". In: *Biochemistry* 44.7, pp. 2284–2292. DOI: 10.1021/bi048328i.

Kim, Suhyang, Yusuke Nakasone, Akira Takakado, Yoichi Yamazaki, Hironari Kamikubo, and Masahide Terazima (2021). "A unique photochromic UV-A sensor protein, Rc-PYP, interacting with the PYP-binding protein". In: *Phys. Chem. Chem. Phys.* 23.33, pp. 17813–17825. DOI: 10.1039/D1CP02731J.

Kleinlogel, Sonja (2016). "Optogenetic user's guide to Opto-GPCRs." In: *Front. Biosci. (Landmark Ed.* 21, pp. 794–805. DOI: 10.2741/4421.

Kobilka, Brian K., Tong Sun Kobilka, Kiefer Daniel, John W. Regan, Marc G. Caron, and Robert J. Lefkowitz (1988). "Chimeric α2-, β2-adrenergic receptors: Delineation of domains involved in effector coupling and ligand binding specificity". In: *Sci. Sci.* 240.4857, pp. 1310–1316. DOI: 10.1126/science.2836950.

Kooistra, Albert J, Stefan Mordalski, Gáspár Pándy-Szekeres, Mauricio Esguerra, Alibek Mamyrbekov, Christian Munk, György M Keserű, and David E Gloriam (2021). "GPCRdb in 2021: integrating GPCR sequence, structure and function." In: *Nucleic Acids Res.* 49.D1, pp. D335–D343. DOI: 10.1093/nar/gkaa1080.

Kottke, Tilman, Víctor A Lórenz-Fonfría, and Joachim Heberle (2017). "The Grateful Infrared: Sequential Protein Structural Changes Resolved by Infrared Difference Spectroscopy." In: *J. Phys. Chem. B* 121.2, pp. 335–350. DOI: 10.1021/acs.jpcb.6b09222.

Koyanagi, Mitsumasa and Akihisa Terakita (2014). "Diversity of animal opsin-based pigments and their optogenetic potential". In: *Biochim. Biophys. Acta - Bioenerg.* 1837.5, pp. 710–716. DOI: 10.1016/j.bbabio.2013.09.003.

Lamichhane, Rajan, Jeffrey J Liu, Kate L White, Vsevolod Katritch, Raymond C Stevens, Kurt Wüthrich, and David P Millar (2020). "Biased Signaling of the G-Protein-Coupled Receptor β2AR Is Governed by Conformational Exchange Kinetics." In: *Structure* 28.3, 371–377.e3. DOI: 10.1016/j.str.2020.01.001.

Laschet, Céline, Nadine Dupuis, and Julien Hanson (2018). "The G protein-coupled receptors deorphanization landscape". In: *Biochem. Pharmacol.* 153, pp. 62–74. DOI: https://doi.org/10.1016/j.bcp.2018.02.016.

Latorraca, Naomi R., A. J. Venkatakrishnan, and Ron O. Dror (2017). "GPCR dynamics: Structures in motion". In: *Chem. Rev.* 117.1, pp. 139–155. DOI: 10.1021/acs.chemrev.6b00177.

Lemmon, Mark A and Joseph Schlessinger (2010). "Cell signaling by receptor tyrosine kinases." In: *Cell* 141.7, pp. 1117–34. DOI: 10.1016/j.cell.2010.06.011.

Leopold, Anna V., Konstantin G. Chernov, Anton A. Shemetov, and Vladislav V. Verkhusha (2019). "Neurotrophin receptor tyrosine kinases regulated with near-infrared light". In: *Nat. Commun.* 10.1. DOI: 10.1038/s41467-019-08988-3.

Levitz, Joshua, Carlos Pantoja, Benjamin Gaub, Harald Janovjak, Andreas Reiner, Adam Hoagland, David Schoppik, Brian Kane, Philipp Stawski, Alexander F Schier, Dirk Trauner, and Ehud Y Isacoff (2013). "Optical control of metabotropic glutamate receptors". In: *Nat. Neurosci.* 16.4, pp. 507–516. DOI: 10.1038/nn.3346.

Levskaya, Anselm, Orion D. Weiner, Wendell A. Lim, and Christopher A. Voigt (2009). "Spatiotemporal control of cell signalling using a light-switchable protein interaction". In: *Nature* 461.7266, pp. 997–1001. DOI: 10.1038/nature08446.

Li, Xiang, Davina V Gutierrez, M Gartz Hanson, Jing Han, Melanie D Mark, Hillel Chiel, Peter Hegemann, Lynn T Landmesser, and Stefan Herlitze (2005). "Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin." In: *Proc. Natl. Acad. Sci. U. S. A.* 102.49, pp. 17816–21. DOI: 10.1073/pnas.0509030102.

Li, Zhanwen, Lukasz Jaroszewski, Mallika Iyer, Mayya Sedova, and Adam Godzik (2020). "FATCAT 2.0: Towards a better understanding of the structural diversity of proteins". In: *Nucleic Acids Res.* 48.1, W60–W64. DOI: 10.1093/NAR/GKAA443.

Liang, Yi Lynn et al. (2018). "Phase-plate cryo-EM structure of a biased agonistbound human GLP-1 receptor-Gs complex". In: *Nature* 555.7694, pp. 121–125. DOI: 10.1038/nature25773.

Lin, Chentao and Takeshi Todo (2005). "The cryptochromes". In: *Genome Biol.* 6.5, p. 220. DOI: 10.1186/gb-2005-6-5-220.

Lin, John Y, Per Magne Knutsen, Arnaud Muller, David Kleinfeld, and Roger Y Tsien (2013). "ReaChR: a red-shifted variant of channelrhodopsin enables deep

transcranial optogenetic excitation." In: *Nat. Neurosci.* 16.10, pp. 1499–508. DOI: 10.1038/nn.3502.

Lin, S W, G G Kochendoerfer, K S Carroll, D Wang, R A Mathies, and T P Sakmar (1998). "Mechanisms of spectral tuning in blue cone visual pigments. Visible and raman spectroscopy of blue-shifted rhodopsin mutants." In: *J. Biol. Chem.* 273.38, pp. 24583–91. DOI: 10.1074/jbc.273.38.24583.

Liu, Bobin, Zhaohe Yang, Adam Gomez, Bin Liu, Chentao Lin, and Yoshito Oka (2016). "Signaling mechanisms of plant cryptochromes in Arabidopsis thaliana". In: *J. Plant Res.* 129.2, pp. 137–148. DOI: 10.1007/s10265-015-0782-z.

Liu, H., X. Yu, K. Li, J. Klejnot, H. Yang, D. Lisiero, and C. Lin (2008). "Photoexcited CRY2 Interacts with CIB1 to Regulate Transcription and Floral Initiation in Arabidopsis". In: *Science (80-.).* 322.5907, pp. 1535–1539. DOI: 10.1126/science.1163927.

Liu, Jeffrey J, Reto Horst, Vsevolod Katritch, Raymond C Stevens, and Kurt Wüthrich (2012). "Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR." In: *Science* 335.6072, pp. 1106–10. DOI: 10.1126/science.1215802.

Liu, Rongfang, Nick J A Groenewoud, Miriam C Peeters, Eelke B Lenselink, and Ad P IJzerman (2014). "A yeast screening method to decipher the interaction between the adenosine A2B receptor and the C-terminus of different G protein α-subunits." In: *Purinergic Signal.* 10.3, pp. 441–53. DOI: 10.1007/s11302-014-9407-6.

Ma, Xiuyan, Yunfei Hu, Hossein Batebi, Jie Heng, Jun Xu, Xiangyu Liu, Xiaogang Niu, Hongwei Li, Peter W Hildebrand, Changwen Jin, and Brian K Kobilka (2020). "Analysis of β2AR-Gs and β2AR-Gi complex formation by NMR spectroscopy." In: *Proc. Natl. Acad. Sci. U. S. A.* 117.37, pp. 23096–23105. DOI: 10.1073/pnas.2009786117.

Madeira, Fábio, Young Mi Park, Joon Lee, Nicola Buso, Tamer Gur, Nandana Madhusoodanan, Prasad Basutkar, Adrian R N Tivey, Simon C Potter, Robert D Finn, and Rodrigo Lopez (2019). "The EMBL-EBI search and sequence analysis tools APIs in 2019." In: *Nucleic Acids Res.* 47.W1, W636–W641. DOI: 10.1093/nar/gkz268.

Mahn, Mathias et al. (2021). "Efficient optogenetic silencing of neurotransmitter release with a mosquito rhodopsin." In: *Neuron* 109.10, 1621–1635.e8. DOI: 10.1016/j.neuron.2021.03.013.

Mansouri, Maysam, Marie-Didiée Hussherr, Tobias Strittmatter, Peter Buchmann, Shuai Xue, Gieri Camenisch, and Martin Fussenegger (2021). "Smart-watch-programmed green-light-operated percutaneous control of therapeutic transgenes". In: *Nat. Commun.* 12.1, p. 3388. DOI: 10.1038/s41467-021-23572-4.

Marti-Solano, Maria et al. (2020). "Combinatorial expression of GPCR isoforms affects signalling and drug responses". In: *Nature* 587.7835, pp. 650–656. DOI: 10.1038/s41586-020-2888-2.

Más, Paloma, Paul F. Devlin, Satchidananda Panda, and Steve A. Kay (2000). "Functional interaction of phytochrome B and cryptochrome 2". In: *Nature* 408.6809, pp. 207–211. DOI: 10.1038/35041583.

Masseck, Olivia A., Katharina Spoida, Deniz Dalkara, Takashi Maejima, Johanna M. Rubelowski, Lutz Wallhorn, Evan S. Deneris, and Stefan Herlitze (2014). "Vertebrate cone opsins enable sustained and highly sensitive rapid control of gi/o signaling in anxiety circuitry". In: *Neuron* 81.6, pp. 1263–1273. DOI: 10.1016/j.neuron.2014.01.041.

Masuda, Shinji and Carl E. Bauer (2002). "AppA Is a Blue Light Photoreceptor that Antirepresses Photosynthesis Gene Expression in Rhodobacter sphaeroides". In: *Cell* 110.5, pp. 613–623. DOI: 10.1016/S0092-8674(02)00876-0.

Masuho, Ikuo, Nickolas K. Skamangas, Brian S. Muntean, and Kirill A. Martemyanov (2021). "Diversity of the Gβ,γ complexes defines spatial and temporal bias of GPCR signaling". In: *Cell Syst.* 12.4, 324–337.e5. DOI: 10.1016/j.cels.2021.02.001.

Mathiesen, Jesper Mosolff, Line Vedel, and Hans Bräuner-Osborne (2013). "cAMP biosensors applied in molecular pharmacological studies of G protein-coupled receptors." In: *Methods Enzymol.* 522, pp. 191–207. DOI: 10.1016/B978-0-12-407865-9.00011-X.

Maudsley, Stuart, Bronwen Martin, and Louis M. Luttrell (2005). "The origins of diversity and specificity in G protein-coupled receptor signaling". In: *J. Pharmacol. Exp. Ther.* 314.2, pp. 485–494. DOI: 10.1124/jpet.105.083121.

McCorvy, John D, Kyle V Butler, Brendan Kelly, Katie Rechsteiner, Joel Karpiak, Robin M Betz, Bethany L Kormos, Brian K Shoichet, Ron O Dror, Jian Jin, and Bryan L Roth (2018). "Structure-inspired design of β-arrestin-biased ligands for aminergic GPCRs." In: *Nat. Chem. Biol.* 14.2, pp. 126–134. DOI: 10.1038/nchembio.2527.

McGregor, K M, C Becamel, P Marin, and R Andrade (2016). "Using melanopsin to study G protein signaling in cortical neurons". In: *J Neurophysiol* 116.3, pp. 1082–1092. DOI: 10.1152/jn.00406.2016.

Melyan, Z, E E Tarttelin, J Bellingham, R J Lucas, and M W Hankins (2005). "Addition of human melanopsin renders mammalian cells photoresponsive". In: *Nature* 433.7027, pp. 741–745. DOI: 10.1038/nature03344.

Meyer, T. E., J. A. Kyndt, S. Memmi, T. Moser, B. Colón-Acevedo, B. Devreese, and J. J. Van Beeumen (2012). "The growing family of photoactive yellow proteins and their presumed functional roles". In: *Photochem. Photobiol. Sci.* 11.10, p. 1495. DOI: 10.1039/c2pp25090j.

Meyer, T. E., E. Yakali, M. A. Cusanovich, and G. Tollin (1987). "Properties of a water-soluble, yellow protein isolated from a halophilic phototrophic bacterium that has photochemical activity analogous to sensory rhodopsin". In: *Biochemistry* 26.2, pp. 418–423. DOI: 10.1021/bi00376a012.

Mitra, Devrani, Xiaojing Yang, and Keith Moffat (2012). "Crystal Structures of Aureochrome1 LOV Suggest New Design Strategies for Optogenetics". In: *Structure* 20.4, pp. 698–706. DOI: 10.1016/j.str.2012.02.016.

Möglich, Andreas, Xiaojing Yang, Rebecca A. Ayers, and Keith Moffat (2010). "Structure and function of plant photoreceptors". In: *Annu. Rev. Plant Biol.* 61, pp. 21–47. DOI: 10.1146/annurev-arplant-042809-112259.

Morri, Maurizio, Inmaculada Sanchez-Romero, Alexandra Madelaine Tichy, Stephanie Kainrath, Elliot J. Gerrard, Priscila P. Hirschfeld, Jan Schwarz, and Harald Janovjak (2018). "Optical functionalization of human Class A orphan G-protein-coupled receptors". In: *Nat. Commun.* 9.1. DOI: 10.1038/s41467-018-04342-1.

Munk, Christian, Eshita Mutt, Vignir Isberg, Louise F. Nikolajsen, Janne M. Bibbe, Tilman Flock, Michael A. Hanson, Raymond C. Stevens, Xavier Deupi, and David E. Gloriam (2019). "An online resource for GPCR structure determination and analysis". In: *Nat. Methods* 16.2, pp. 151–162. DOI: 10.1038/s41592-018-0302-x.

Nagata, T, M Koyanagi, R Lucas, and A Terakita (2018). "An all-trans-retinal-binding opsin peropsin as a potential dark-active and light-inactivated G protein-coupled receptor". In: *Sci Rep* 8.1, p. 3535. DOI: 10.1038/s41598-018-21946-1.

Nagel, Georg, Tanjef Szellas, Wolfram Huhn, Suneel Kateriya, Nona Adeishvili, Peter Berthold, Doris Ollig, Peter Hegemann, and Ernst Bamberg (2003). "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel". In: *Proc. Natl. Acad. Sci. U. S. A.* 100.SUPPL. 2, pp. 13940–13945. DOI: 10.1073/pnas.1936192100.

Nakajima, Ken-ichiro and Jürgen Wess (2012). "Design and functional characterization of a novel, arrestin-biased designer G protein-coupled receptor." In: *Mol. Pharmacol.* 82.4, pp. 575–82. DOI: 10.1124/mol.112.080358.

Ni, M, J M Tepperman, and P H Quail (1999). "Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light." eng. In: *Nature* 400.6746, pp. 781–784. DOI: 10.1038/23500.

Nzigou Mombo, Brice, Brent M. Bijonowski, Samaneh Rasoulinejad, Marc Mueller, and Seraphine V. Wegner (2021). "Spatiotemporal Control Over Multicellular Migration Using Green Light Reversible Cell–Cell Interactions". In: *Adv. Biol.* 5.5. DOI: 10.1002/adbi.202000199.

Oh, E, T Maejima, C Liu, E Deneris, and S Herlitze (2010). "Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor". In: *J Biol Chem* 285.40, pp. 30825–30836. DOI: 10.1074/jbc.M110.147298.

Oldham, W M and H E Hamm (2008). "Heterotrimeric G protein activation by G-protein-coupled receptors". In: *Nat Rev Mol Cell Biol* 9.1, pp. 60–71. DOI: 10.1038/nrm2299.

O'Neill, P R and N Gautam (2015). "Optimizing optogenetic constructs for control over signaling and cell behaviours." In: *Photochem. Photobiol. Sci.* 14.9, pp. 1578–85. DOI: 10.1039/c5pp00171d.

Ortiz-Guerrero, Juan Manuel, María Carmen Polanco, Francisco J. Murillo, S. Padmanabhan, and Montserrat Elías-Arnanz (2011). "Light-dependent gene regulation by a coenzyme B12-based photoreceptor". In: *Proc. Natl. Acad. Sci. U. S. A.* 108.18, pp. 7565–7570. DOI: 10.1073/pnas.1018972108.

Padmanabhan, S., Marco Jost, Catherine L. Drennan, and Montserrat Elías-Arnanz (2017). "A new facet of Vitamin B12: Gene regulation by cobalamin-based photoreceptors". In: *Annu. Rev. Biochem.* 86, pp. 485–514. DOI: 10.1146/annurev-biochem-061516-044500.

Palczewski, K, T Kumasaka, T Hori, C A Behnke, H Motoshima, B A Fox, I Le Trong, D C Teller, T Okada, R E Stenkamp, M Yamamoto, and M Miyano (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor". eng. In: *Science (80-.).* 289.5480, pp. 739–745. DOI: 10.1126/science.289.5480.739.

Pande, K. et al. (2016). "Femtosecond structural dynamics drives the trans/cis isomerization in photoactive yellow protein". In: *Science (80-.).* 352.6286, pp. 725–729. DOI: 10.1126/science.aad5081.

Park, J H, P Scheerer, K P Hofmann, H W Choe, and O P Ernst (2008). "Crystal structure of the ligand-free G-protein-coupled receptor opsin". In: *Nature* 454.7201, pp. 183–187. DOI: 10.1038/nature07063.

Park, Sam Yong and Jeremy R.H. Tame (2017). "Seeing the light with BLUF proteins". In: *Biophys. Rev.* 9.2, pp. 169–176. DOI: 10.1007/s12551-017-0258-6.

Partridge, J C and W J De Grip (1991). "A new template for rhodopsin (vitamin A1 based) visual pigments." In: *Vision Res.* 31.4, pp. 619–30. DOI: 10.1016/0042-6989(91)90002-m.

Pudasaini, Ashutosh, Kaley K El-Arab, and Brian D Zoltowski (2015). *LOV-based optogenetic devices: light-driven modules to impart photoregulated control of cellular signaling*.

Rasmussen, S G, H J Choi, D M Rosenbaum, T S Kobilka, F S Thian, P C Edwards, M Burghammer, V R Ratnala, R Sanishvili, R F Fischetti, G F Schertler, W I Weis, and B K Kobilka (2007). "Crystal structure of the human beta2 adrenergic G-protein-coupled receptor". In: *Nature* 450.7168, pp. 383–387. DOI: 10.1038/nature06325.

Rasmussen, S G et al. (2011). "Crystal structure of the beta2 adrenergic receptor-Gs protein complex". In: *Nature* 477.7366, pp. 549–555. DOI: 10.1038/nature10361.

Reichhart, Eva, Alvaro Ingles-Prieto, Alexandra Madelaine Tichy, Catherine McKenzie, and Harald Janovjak (2016). "A phytochrome sensory domain permits receptor activation by red light". In: *Angew. Chemie - Int. Ed.* 55.21, pp. 6339–6342. DOI: 10.1002/anie.201601736.

Renatus, M., H. R. Stennicke, F. L. Scott, R. C. Liddington, and G. S. Salvesen (2001). "Dimer formation drives the activation of the cell death protease caspase 9". In: *Proc. Natl. Acad. Sci. U. S. A.* 98.25, pp. 14250–14255. DOI: 10.1073/pnas.231465798.

Rockwell, Nathan C and J Clark Lagarias (2017). "Phytochrome diversification in cyanobacteria and eukaryotic algae". In: *Curr. Opin. Plant Biol.* 37, pp. 87–93. DOI: 10.1016/j.pbi.2017.04.003.

Rockwell, Nathan C., Yi Shin Su, and J. Clark Lagarias (2006). "Phytochrome structure and signaling mechanisms". In: *Annu. Rev. Plant Biol.* 57, pp. 837–858. DOI: 10.1146/annurev.arplant.56.032604.144208.

Roed, Sarah Noerklit, Pernille Wismann, Christina Rye Underwood, Nikolaj Kulahin, Helle Iversen, Karen Arevad Cappelen, Lauge Schäffer, Janne Lehtonen, Jacob Hecksher-Soerensen, Anna Secher, Jesper Mosolff Mathiesen, Hans Bräuner-Osborne, Jennifer L Whistler, Sanne Moeller Knudsen, and Maria Waldhoer (2014). "Real-time trafficking and signaling of the glucagon-like peptide-1 receptor." In: *Mol. Cell. Endocrinol.* 382.2, pp. 938–49. DOI: 10.1016/j.mce.2013.11.010.

Sahel, José Alain et al. (2021). "Partial recovery of visual function in a blind patient after optogenetic therapy". In: *Nat. Med.* 27.7, pp. 1223–1229. DOI: 10.1038/s41591-021-01351-4.

Sako, Keisuke, Saurabh J. Pradhan, Vanessa Barone, Álvaro Inglés-Prieto, Patrick Müller, Verena Ruprecht, Daniel Čapek, Sanjeev Galande, Harald Janovjak, and Carl Philipp Heisenberg (2016). "Optogenetic Control of Nodal Signaling Reveals a Temporal Pattern of Nodal Signaling Regulating Cell Fate Specification during Gastrulation". In: *Cell Rep.* 16.3, pp. 866–877. DOI: 10.1016/j.celrep.2016.06.036.

Sandhu, Manbir, Anja M Touma, Matthew Dysthe, Fredrik Sadler, Sivaraj Sivaramakrishnan, and Nagarajan Vaidehi (2019). "Conformational plasticity of the intracellular cavity of GPCR-G-protein complexes leads to G-protein promiscuity and selectivity." In: *Proc. Natl. Acad. Sci. U. S. A.* 116.24, pp. 11956–11965. DOI: 10.1073/pnas.1820944116.

Savage, Emilia Elizabeth, Denise Wootten, Arthur Christopoulos, Patrick Michael Sexton, and Sebastian George Barton Furness (2013). "A simple method to generate stable cell lines for the analysis of transient protein-protein interactions." In: *Biotechniques* 54.4, pp. 217–21. DOI: 10.2144/000114013.

Scheerer, Patrick, Jung Hee Park, Peter W. Hildebrand, Yong Ju Kim, Norbert Krauß, Hui Woog Choe, Klaus Peter Hofmann, and Oliver P. Ernst (2008). "Crystal structure of opsin in its G-protein-interacting conformation". In: *Nature* 455.7212, pp. 497–502. DOI: 10.1038/nature07330.

Schönberger, Matthias and Dirk Trauner (2014). "A Photochromic Agonist for μ-Opioid Receptors". In: *Angew. Chemie Int. Ed.* 53.12, pp. 3264–3267. DOI: 10.1002/anie.201309633.

Schönegge, Anne-Marie, Jonathan Gallion, Louis-Philippe Picard, Angela D Wilkins, Christian Le Gouill, Martin Audet, Wayne Stallaert, Martin J Lohse, Marek Kimmel, Olivier Lichtarge, and Michel Bouvier (2017). "Evolutionary action and structural basis of the allosteric switch controlling β2AR functional selectivity." In: *Nat. Commun.* 8.1, p. 2169. DOI: 10.1038/s41467-017-02257-x.

Seong, Jihye and Michael Z. Lin (2021). "Optobiochemistry: Genetically Encoded Control of Protein Activity by Light". In: *Annu. Rev. Biochem.* 90.1, pp. 475–501. DOI: 10.1146/annurev-biochem-072420-112431.

Shcherbakova, Daria M., Anton A. Shemetov, Andrii A. Kaberniuk, and Vladislav V. Verkhusha (2015). *Natural photoreceptors as a source of fluorescent proteins, biosensors, and optogenetic tools*. Vol. 84, pp. 519–550. DOI: 10.1146/annurev-biochem-060614-034411.

Shichida, Yoshinori and Take Matsuyama (2009). "Evolution of opsins and phototransduction". In: *Philos. Trans. R. Soc. B Biol. Sci.* 364.1531, pp. 2881–2895. DOI: 10.1098/rstb.2009.0051.

Shimizu-Sato, Sae, Enamul Huq, James M. Tepperman, and Peter H. Quail (2002). "A light-switchable gene promoter system". In: *Nat. Biotechnol.* 20.10, pp. 1041–1044. DOI: 10.1038/nbt734.

Shin, Yongdae, Joel Berry, Nicole Pannucci, Mikko P. Haataja, Jared E. Toettcher, and Clifford P. Brangwynne (2017). "Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets". In: *Cell* 168.1-2, 159–171.e14. DOI: 10.1016/j.cell.2016.11.054.

Shindyalov, I N and P E Bourne (1998). "Protein structure alignment by incremental combinatorial extension (CE) of the optimal path." In: *Protein Eng.* 11.9, pp. 739–47. DOI: 10.1093/protein/11.9.739.

Shonberg, Jeremy, Laura Lopez, Peter J Scammells, Arthur Christopoulos, Ben Capuano, and J Robert Lane (2014). "Biased agonism at G protein-coupled receptors: the promise and the challenges–a medicinal chemistry perspective." In: *Med. Res. Rev.* 34.6, pp. 1286–330. DOI: 10.1002/med.21318.

Sineshchekov, Vitally A. (1995). "Photobiophysics and photobiochemistry of the heterogeneous phytochrome system". In: *Biochim. Biophys. Acta - Bioenerg.* 1228.2-3, pp. 125–164. DOI: 10.1016/0005-2728(94)00173-3.

Singhal, Ankita, Ying Guo, Milos Matkovic, Gebhard Schertler, Xavier Deupi, Elsa Cy Yan, and Joerg Standfuss (2016). "Structural role of the T94I rhodopsin mutation in congenital stationary night blindness." In: *EMBO Rep.* 17.10, pp. 1431–1440. DOI: 10.15252/embr.201642671.

Siuda, Edward R., Bryan A. Copits, Martin J. Schmidt, Madison A. Baird, Ream Al-Hasani, William J. Planer, Samuel C. Funderburk, Jordan G. McCall, Robert W. Gereau, and Michael R. Bruchas (2015a). "Spatiotemporal Control of Opioid Signaling and Behavior". In: *Neuron* 86.4, pp. 923–935. DOI: 10.1016/j.neuron.2015.03.066.

Siuda, Edward R., Jordan G. McCall, Ream Al-Hasani, Gunchul Shin, Sung Il Park, Martin J. Schmidt, Sonya L. Anderson, William J. Planer, John A. Rogers, and Michael R. Bruchas (2015b). "Optodynamic simulation of β-adrenergic receptor signalling". In: *Nat. Commun.* 6. DOI: 10.1038/ncomms9480.

Spangler, Skylar M and Michael R Bruchas (2017). "Optogenetic approaches for dissecting neuromodulation and GPCR signaling in neural circuits." In: *Curr. Opin. Pharmacol.* 32, pp. 56–70. DOI: 10.1016/j.coph.2016.11.001.

Spoida, Katharina, Dennis Eickelbeck, Raziye Karapinar, Tobias Eckhardt, Melanie D. Mark, Dirk Jancke, Benedikt Valerian Ehinger, Peter König, Deniz Dalkara, Stefan Herlitze, and Olivia A. Masseck (2016). "Melanopsin Variants as Intrinsic Optogenetic on and off Switches for Transient versus Sustained Activation of

G Protein Pathways". In: *Curr. Biol.* 26.9, pp. 1206–1212. DOI: 10.1016/j.cub.2016.03.007.

Sriram, Krishna and Paul A Insel (2018). "G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs?" In: *Mol. Pharmacol.* 93.4, pp. 251–258. DOI: 10.1124/mol.117.111062.

Stables, Jenny, Andrew Green, Fiona Marshall, Neil Fraser, Emma Knight, Martine Sautel, Graeme Milligan, Melanie Lee, and Stephen Rees (1997). "A Bioluminescent Assay for Agonist Activity at Potentially Any G-Protein-Coupled Receptor". In: *Anal. Biochem.* 252.1, pp. 115–126. DOI: 10.1006/abio.1997.2308.

Strauss, Holger M., Peter Schmieder, and Jon Hughes (2005). "Light-dependent dimerisation in the N-terminal sensory module of cyanobacterial phytochrome 1". In: *FEBS Lett.* 579.18, pp. 3970–3974. DOI: 10.1016/j.febslet.2005.06.025.

Strickland, D, Y Lin, E Wagner, C M Hope, J Zayner, C Antoniou, T R Sosnick, E L Weiss, and M Glotzer (2012). "TULIPs: tunable, light-controlled interacting protein tags for cell biology". In: *Nat Methods* 9.4, pp. 379–384. DOI: 10.1038/nmeth.1904.

Tabor, Jeffrey J, Anselm Levskaya, and Christopher A Voigt (2011). "Multichromatic control of gene expression in Escherichia coli." In: *J. Mol. Biol.* 405.2, pp. 315–24. DOI: 10.1016/j.jmb.2010.10.038.

Takahashi, F., D. Yamagata, M. Ishikawa, Y. Fukamatsu, Y. Ogura, M. Kasahara, T. Kiyosue, M. Kikuyama, M. Wada, and H. Kataoka (2007). "AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles". In: *Proc. Natl. Acad. Sci.* 104.49, pp. 19625–19630. DOI: 10.1073/pnas.0707692104.

Takala, Heikki, Petra Edlund, Janne A Ihalainen, and Sebastian Westenhoff (2020). "Tips and turns of bacteriophytochrome photoactivation". In: *Photochem. Photobiol. Sci.* 19.11, pp. 1488–1510. DOI: 10.1039/D0PP00117A.

Takasaki, Jun, Tetsu Saito, Masatoshi Taniguchi, Tomihisa Kawasaki, Yumiko Moritani, Kazumi Hayashi, and Masato Kobori (2004). "A novel Galphaq/11-selective inhibitor." In: *J. Biol. Chem.* 279.46, pp. 47438–45. DOI: 10.1074/jbc.M408846200.

Tang, Y, L A Hu, W E Miller, N Ringstad, R A Hall, J A Pitcher, P DeCamilli, and R J Lefkowitz (1999). "Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta1-adrenergic receptor." In: *Proc. Natl. Acad. Sci. U. S. A.* 96.22, pp. 12559–64. DOI: 10.1073/pnas.96.22.12559.

Tenboer, Jason et al. (2014). "Time-resolved serial crystallography captures high-resolution intermediates of photoactive yellow protein". In: *Science (80-.).* 346.6214, pp. 1242–1246. DOI: 10.1126/science.1259357.

Terakita, Akihisa (2005). "The opsins". In: *Genome Biol.* 6.3, p. 213. DOI: 10.1186/gb-2005-6-3-213.

Tichy, Alexandra-Madelaine, Elliot J. Gerrard, Julien M.D. D Legrand, Robin M. Hobbs, and Harald Janovjak (2019a). "Engineering Strategy and Vector Library for the Rapid Generation of Modular Light-Controlled Protein-Protein Interactions." In: *J. Mol. Biol.* 431.17, pp. 3046–3055. DOI: 10.1016/j.jmb.2019.05.033.

Tichy, Alexandra-Madelaine, Elliot J. Gerrard, Patrick M. Sexton, and Harald Janovjak (2019b). "Light-activated chimeric GPCRs: limitations and opportunities". In: *Curr. Opin. Struct. Biol.* 57, pp. 196–203. DOI: 10.1016/j.sbi.2019.05.006.

Tokuriki, Nobuhiko, Francois Stricher, Luis Serrano, and Dan S Tawfik (2008). "How protein stability and new functions trade off." In: *PLoS Comput. Biol.* 4.2, e1000002. DOI: 10.1371/journal.pcbi.1000002.

Tsukamoto, H and A Terakita (2010). "Diversity and functional properties of bistable pigments". In: *Photochem Photobiol Sci* 9.11, pp. 1435–1443. DOI: 10.1039/c0pp00168f.

Vardy, Eyal et al. (2015). "A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior." In: *Neuron* 86.4, pp. 936–946. DOI: 10.1016/j.neuron.2015.03.065.

Vehlow, Corinna, Henning Stehr, Matthias Winkelmann, José M Duarte, Lars Petzold, Juliane Dinse, and Michael Lappe (2011). "CMView: interactive contact map visualization and analysis." In: *Bioinformatics* 27.11, pp. 1573–4. DOI: 10.1093/bioinformatics/btr163.

Venkatakrishnan, A. J., Xavier Deupi, Guillaume Lebon, Franziska M. Heydenreich, Tilman Flock, Tamara Miljus, Santhanam Balaji, Michel Bouvier, Dmitry B. Veprintsev, Christopher G. Tate, Gebhard F.X. X Schertler, and M. Madan Babu (2016). "Diverse activation pathways in class A GPCRs converge near the G-protein-coupling region." In: *Nature* 536.7617, pp. 484–7. DOI: 10.1038/nature19107.

Venkatakrishnan, A. J., Xavier Deupi, Guillaume Lebon, Christopher G. Tate, Gebhard F. Schertler, and M. Madan Babu (2013). "Molecular signatures of G-protein-coupled receptors". In: *Nature* 494.7436, pp. 185–194. DOI: 10.1038/nature11896.

Violin, Jonathan D, Aimee L Crombie, David G Soergel, and Michael W Lark (2014). "Biased ligands at G-protein-coupled receptors: promise and progress." In: *Trends Pharmacol. Sci.* 35.7, pp. 308–16. DOI: 10.1016/j.tips.2014.04.007.

Viswanathan, Ranjith, Aleksandar Necakov, Mateusz Trylinski, Rohit Krishnan Harish, Daniel Krueger, Emilia Esposito, Francois Schweisguth, Pierre Neveu,

and Stefano De Renzis (2019). "Optogenetic inhibition of Delta reveals digital Notch signalling output during tissue differentiation". In: *EMBO Rep.* 20.12. DOI: 10.15252/embr.201947999.

Von Moo, Ee, Kasper Harpsøe, Alexander S Hauser, Ikuo Masuho, Hans Bräuner-Osborne, David E Gloriam, and Kirill A Martemyanov (2021). "Ligand-directed bias of G protein signaling at the dopamine D2 receptor." In: *Cell Chem. Biol.* DOI: 10.1016/j.chembiol.2021.07.004.

Wang, Ri, Zhongguang Yang, Jiren Luo, I. Ming Hsing, and Fei Sun (2017). "B12-dependent photoresponsive protein hydrogels for controlled stem cell/protein release". In: *Proc. Natl. Acad. Sci. U. S. A.* 114.23, pp. 5912–5917. DOI: 10.1073/pnas.1621350114.

Wang, Xue, Xianjun Chen, and Yi Yang (2012). "Spatiotemporal control of gene expression by a light-switchable transgene system". In: *Nat. Methods* 9.3, pp. 266–269. DOI: 10.1038/nmeth.1892.

Weis, William I and Brian K Kobilka (2018). "The Molecular Basis of G Protein-Coupled Receptor Activation." In: *Annu. Rev. Biochem.* 87, pp. 897–919. DOI: 10.1146/annurev-biochem-060614-033910.

Wiegert, J Simon, Mathias Mahn, Matthias Prigge, Yoav Printz, and Ofer Yizhar (2017). "Silencing Neurons: Tools, Applications, and Experimental Constraints." In: *Neuron* 95.3, pp. 504–529. DOI: 10.1016/j.neuron.2017.06.050.

Wingler, Laura M and Robert J Lefkowitz (2020). "Conformational Basis of G Protein-Coupled Receptor Signaling Versatility." In: *Trends Cell Biol.* 30.9, pp. 736–747. DOI: 10.1016/j.tcb.2020.06.002.

Wootten, Denise, Arthur Christopoulos, Maria Marti-Solano, M Madan Babu, and Patrick M Sexton (2018). "Mechanisms of signalling and biased agonism in G protein-coupled receptors." In: *Nat. Rev. Mol. Cell Biol.* 19.10, pp. 638–653. DOI: 10.1038/s41580-018-0049-3.

Wyk, Michiel van, Justyna Pielecka-Fortuna, Siegrid Löwel, and Sonja Kleinlogel (2015). "Restoring the ON Switch in Blind Retinas: Opto-mGluR6, a Next-Generation, Cell-Tailored Optogenetic Tool." In: *PLoS Biol.* 13.5, pp. 1–30. DOI: 10.1371/journal.pbio.1002143.

Xu, Dongdong, Julia Ricken, and Seraphine V. Wegner (2020). "Turning Cell Adhesions ON or OFF with High Spatiotemporal Precision Using the Green Light Responsive Protein CarH". In: *Chem. - A Eur. J.* 26.44, pp. 9859–9863. DOI: 10.1002/chem.202001238.

Xu, Yuexin, Young-Min Hyun, Kihong Lim, Hyunwook Lee, Ryan J Cummings, Scott A Gerber, Seyeon Bae, Thomas Yoonsang Cho, Edith M Lord, and Minsoo Kim (2014). "Optogenetic control of chemokine receptor signal and T-cell migration."

In: *Proc. Natl. Acad. Sci. U. S. A.* 111.17, pp. 6371–6. DOI: 10.1073/pnas.1319296111.

Young, M, T Dahoun, B Sokrat, C Arber, K M Chen, M Bouvier, and P Barth (2018). "Computational design of orthogonal membrane receptor-effector switches for rewiring signaling pathways." In: *Proc. Natl. Acad. Sci. U. S. A.* 115.27, pp. 7051–7056. DOI: 10.1073/pnas.1718489115.

Yu, Xuhong, Ricardo Sayegh, Maskit Maymon, Katherine Warpeha, John Klejnot, Hongyun Yang, Jie Huang, Janet Lee, Lon Kaufman, and Chentao Lin (2009). "Formation of Nuclear Bodies of Arabidopsis CRY2 in Response to Blue Light Is Associated with Its Blue Light–Dependent Degradation". In: *Plant Cell* 21.1, pp. 118–130. DOI: 10.1105/tpc.108.061663.

Yuan, Hua and Carl E. Bauer (2008). "PixE promotes dark oligomerization of the BLUF photoreceptor PixD". In: *Proc. Natl. Acad. Sci. U. S. A.* 105.33, pp. 11715–11719. DOI: 10.1073/pnas.0802149105.

Zemelman, Boris V, Georgia A Lee, Minna Ng, and Gero Miesenböck (2002). "Selective photostimulation of genetically chARGed neurons." In: *Neuron* 33.1, pp. 15–22. DOI: 10.1016/s0896-6273(01)00574-8.

Zhang, Feng, Johannes Vierock, Ofer Yizhar, Lief E. Fenno, Satoshi Tsunoda, Arash Kianianmomeni, Matthias Prigge, Andre Berndt, John Cushman, Jürgen Polle, Jon Magnuson, Peter Hegemann, and Karl Deisseroth (2011). "The microbial opsin family of optogenetic tools". In: *Cell* 147.7, pp. 1446–1457. DOI: 10.1016/j.cell.2011.12.004.

Zhang, Yanan, Fan Yang, Shenglong Ling, Pei Lv, Yingxin Zhou, Wei Fang, Wenjing Sun, Longhua Zhang, Pan Shi, and Changlin Tian (2020). "Single-particle cryo-EM structural studies of the β2AR-Gs complex bound with a full agonist formoterol." In: *Cell Discov.* 6, p. 45. DOI: 10.1038/s41421-020-0176-9.

Zhang, Z S, H J Cheng, T Ukai, H Tachibana, and C P Cheng (2001). "Enhanced cardiac L-type calcium current response to beta2-adrenergic stimulation in heart failure." In: *J. Pharmacol. Exp. Ther.* 298.1, pp. 188–96.

Zhao, Evan M., Yanfei Zhang, Justin Mehl, Helen Park, Makoto A. Lalwani, Jared E. Toettcher, and José L. Avalos (2018). "Optogenetic regulation of engineered cellular metabolism for microbial chemical production". In: *Nature* 555.7698, pp. 683–687. DOI: 10.1038/nature26141.

Zhou, Qingtong et al. (2019). "Common activation mechanism of class A GPCRs." In: *Elife* 8, pp. 1–31. DOI: 10.7554/eLife.50279.

Ziegler, Thea and Andreas Möglich (2015). "Photoreceptor engineering." In: *Front. Mol. Biosci.* 2, p. 30. DOI: 10.3389/fmolb.2015.00030.

Zoltowski, Brian D. and Brian R. Crane (2008). "Light Activation of the LOV Protein Vivid Generates a Rapidly Exchanging Dimer". In: *Biochemistry* 47.27, pp. 7012–7019. DOI: 10.1021/bi8007017.

Appendix A

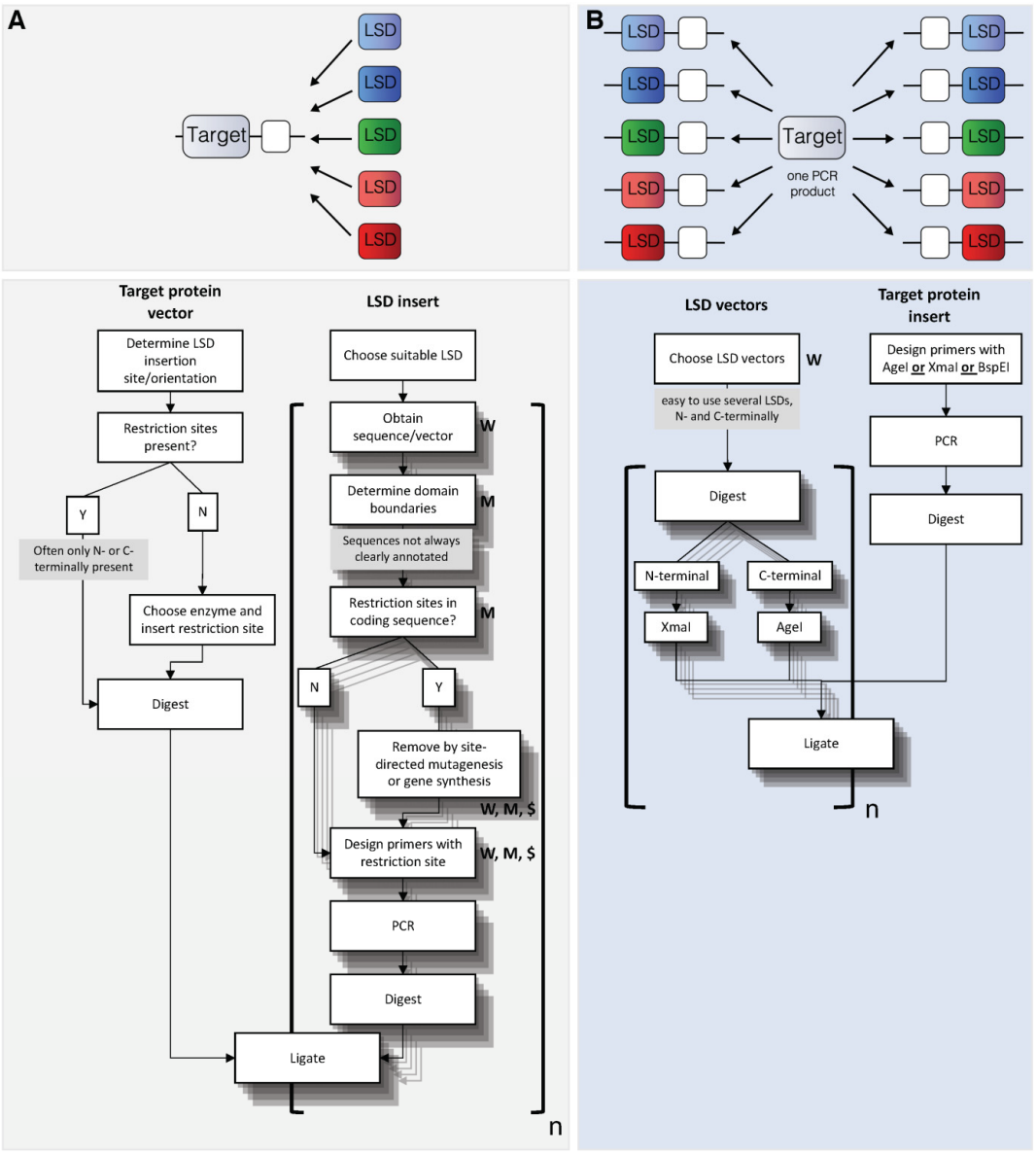
Supplementary Information to Chapter 2

SI - Tichy et al., Journal of Molecular Biology, 2019

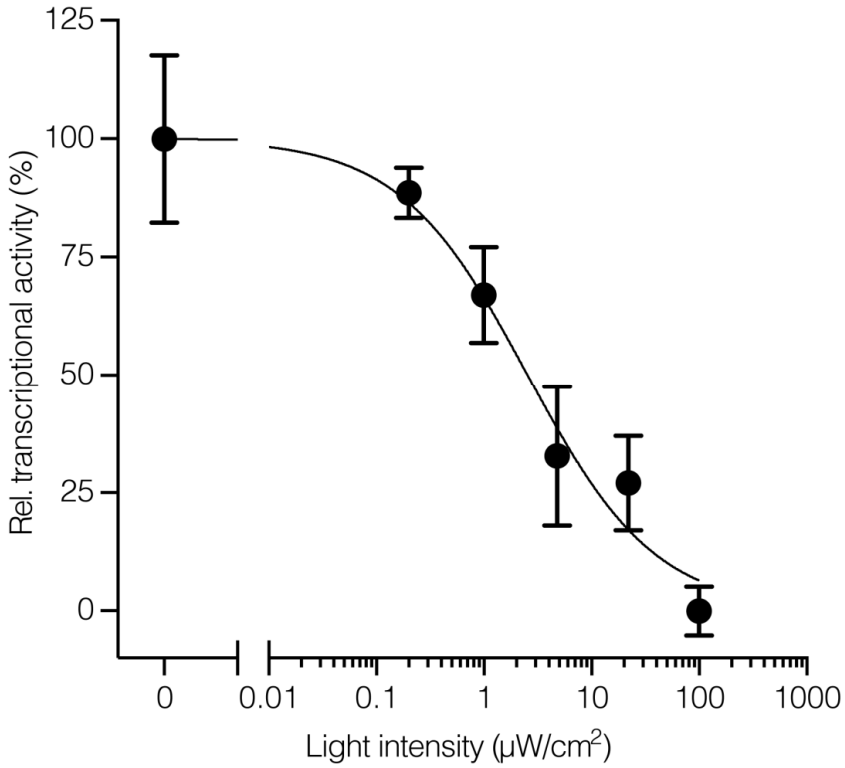
SUPPLEMENTARY INFORMATION

Engineering strategy and vector library for the rapid generation of modular light-controlled protein-protein interactions

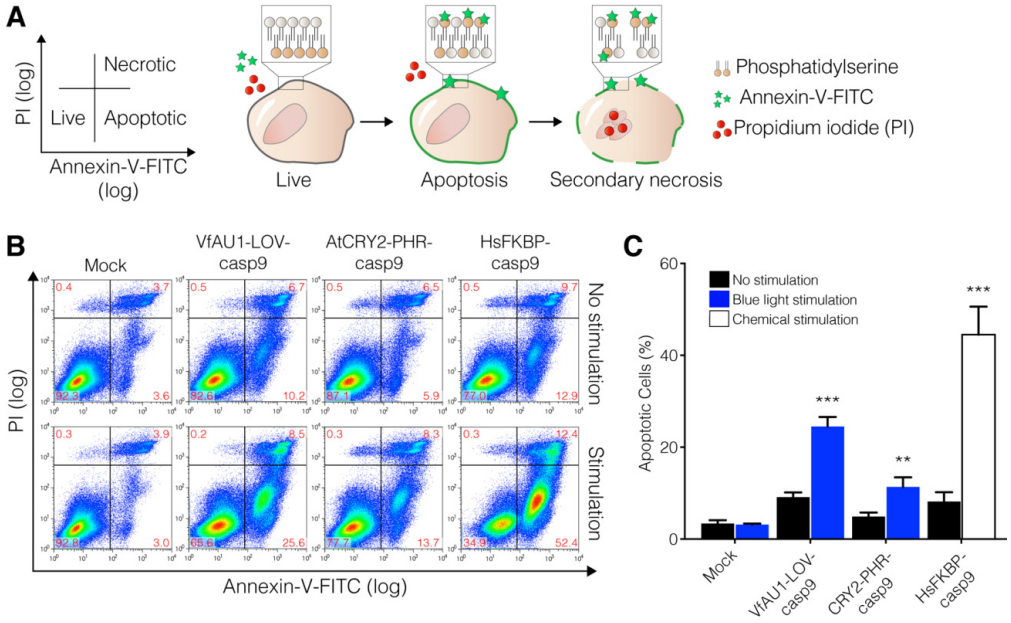
Alexandra-Madelaine Tichy, Elliot J. Gerrard, Julien M.D. Legrand, Robin M. Hobbs and Harald Janovjak



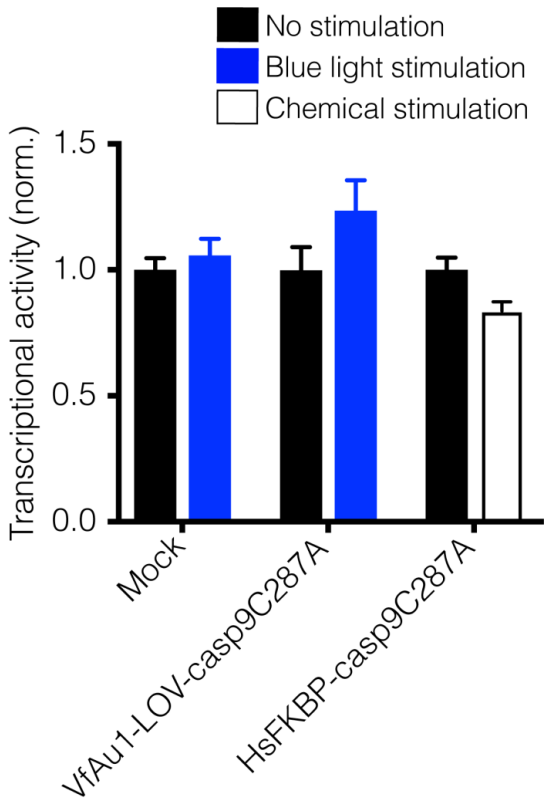
Supplementary Fig. 1. Optogenetic protein engineering using a conventional workflow in which LSDs are inserted into target vectors (A) or using this genetic engineering strategy and vector library (B). Square brackets highlight steps that need to be repeated for each of the "n" tested LSDs. Within the square brackets, labels denote steps that require manual sequence analysis (M), overnight or longer wait times (W) and reagents ($) that are specific for each LSD.



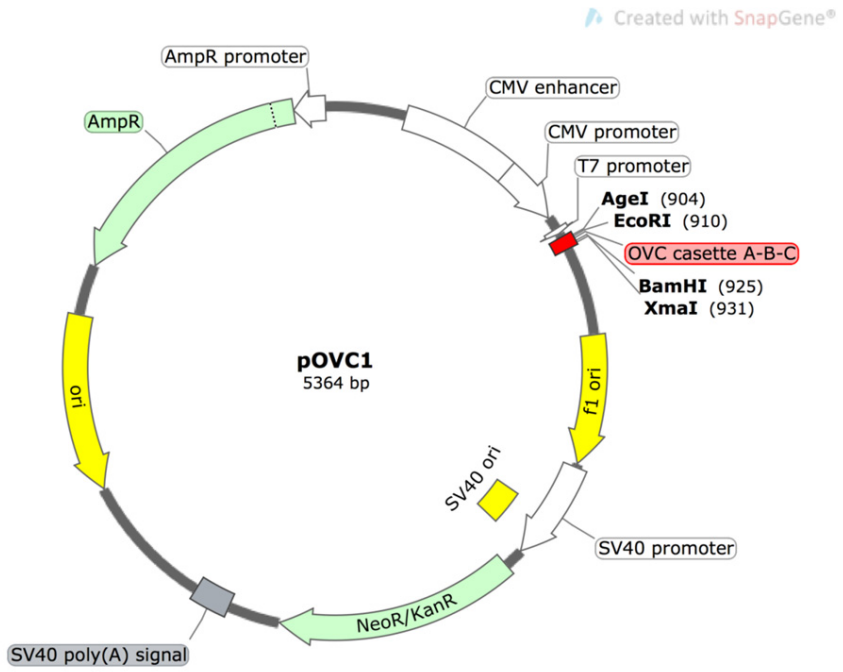
Supplementary Fig. 2. Viability (luminescence signals) as a function of light dose (7 h continuous blue light, $\lambda \approx 470$ nm) for cells transfected with VfAU1-LOV-casp9 (one representative experiment in triplicates, data shown are mean ± SEM, solid line is a sigmoidal dose-response curve fit; the half maximal effective light intensity is 2.5 μW/cm^2).



Supplementary Fig. 3. Opto-casp9 induces apoptosis upon light stimulation. (A) Annexin V binding and PI uptake report on apoptotic and necrotic cells. (B) Representative FACS analysis for HEK293 cells that were transfected with VfAU1-LOV-casp9 or AtCRY2-PHR-casp9 and stimulated with light (7 h continuous blue light, $\lambda \approx$ 470 nm, $I \approx 200$ µW/cm²). Red numbers denote the percentage of the cells in the respective quadrant. (C) Quantitative analysis of apoptotic cells (n=3, three independent experiments, data shown are mean ± SEM). $p<0.01$ (**) or $p<0.001$ (***). Two-tailed T-test.



Supplementary Fig. 4. Luminescence signals for cells transfected with Opto-casp9 in which the casp9 active site cysteine was substituted with alanine (C287A) (n=12, four independent experiments, data shown are mean ± SEM; 7 h continuous blue light, $\lambda \approx$ 470 nm, I ≈ 200 µW/cm²).



Supplementary Fig. 5. Modified mammalian expression vectors developed in this study. The original vector pcDNA3.1(-) was modified by removing a XmaI site and inserting one of three cassettes (shown here, pOVC1 with *ABC* cassette: *A*: AgeI, *B*: EcoRI-spacer-BamHI, *C*: XmaI). The cassettes contain a Kozak sequence and start/stop codons. Vector map created using SnapGene.

45 **Supplementary Table 1.** LSDs and their binding partners included in this study.

LSD	Optimal λ for activation (and reversal) (nm)	Cofactor (typ.)*	Light response	Estimated lit state lifetime (τ) (sec)**	Residues (Uniprot identifier)	Ref.
VfAU1-LOV	450	FMN	Homodimerization	~500	204-348 (A8QW55)	[1, 2]
CrPH-LOV1	450	FMN	Homodimerization	20-800	16-133 (A8IXU7)	[1, 3, 4]
NcVVD-LOV	450	FMN	Homodimerization	>10,000	37-186/Y50W (Q9C3Y6)	[1, 5-7]
RsLP-LOV	450	FMN	Monomerization	~2500	All residues of Protein Data Bank entry 4HJ4 corresponding to genomic position NC_009428 2302851–2303211	[8, 9]
AsPT1-LOV2-EcSsra/EcSSPB micro/nano	450	FMN	Unfolding (heterodimerization, different affinities with EcSSBP micro or nano)	~50	404-536 (O49003)	[10, 11]
AsPT1-LOV2-pep/HsPDZ1b	450	FMN	Unfolding (heterodimerization)	~50	404-537 (O49009)	[11, 12]
AtCRY2-PHR	450	FAD	Oligomerization	~300	1-498 (Q96524)	[13]
AtCRY2-PHR/AtCIB	450	FAD	Heterodimerization	~300	1-498 (Q96524)/ 1-170 (Q8GY61)	[14]
MxCarH-CBD	550	AdoCbl^{X}	Monomerization	N/A	94-299 (Q50900)	[15]
TtCarH-CBD	550	AdoCbl^{X}	Monomerization (irreversible)	N/A	80-284 (Q53W62)	[15]
ScPH1-S	660/740	PCB^{X}	Homodimerization	Hours	2-513 (Q55168)	[15, 16]
AtPHYB-S/AtPIF	660/740	PCB^{X}/Phytochromobilin^{X}	Heterodimerization	Hours	1-908 (P14713)/ 1-100 (Q8L5W7)	[17, 18]

* Cofactors denoted with a cross (^{X}) have been supplied exogenously to mammalian cells in previous experiments. ** The lit state lifetimes describe the characteristic decay of the photoactivated LSD to its dark state and do not directly correspond to the affinity or lifetime of the light-induced PPI that remains unknown for many LSDs. FMN: Flavin mononucleotide, FAD: Flavin adenine dinucleotide, AdoCbl: Adenosylcobalamin, PCB: Phycocyanobilin.

46

47 **Supplementary Table 2.** Oligonucleotide PCR primers used in this study. Restriction
48 sites are underlined where applicable.

Number	Sequence
1	CTTTTGCAAAAAGCTCTCGGGAGCTTGTATATC
2	GATATACAAGCTCCCGAGAGCTTTTTGCAAAAG
3	GATCGAATTCTCAGGCTCTGGATCCCCCGGGCTTAAGTTTAAACCGCTGATCAGCC
4	GATCGAATTCACCGGTGGCCATGGTCAGCTTGGGTCTCCCTATAGTG
5	GATCCCCGGGGAATTCTCAGGCTCTGGATCCTAAGTTTAAACCGCTGATCAG
6	GATCCCCGGGACCGGTGGCCATGGTCAGCTTGGGTCTCCCTATAGTG
7	GATCGGATCCACCGGTCCCGGGTAAGTTTAAACCGCTGATCAG
8	GATCGGATCCAGAGCCTGAGAATTCGGCCATGGTCAGCTTGGGTCTCCCTATAGTG
9	GGATCCCCCGGGTAAGTTTAAACCG
10	CGGTTTAAACTTACCCGGGGGATCC
11	AGTCACCGGTCCTGACTACAGTCTCGTGAAGGCT
12	AGTCCCCGGGCTTTCTGCGCAGCATGTTACTGGT
13	GATCACCGGTGCAGGACTCAGACATACATTTGTGGTGG
14	GATCCCCGGGGGCCAGGGCTTTCCCTTCAGTC
15	GATCCCCGGGCACACTCTCTACGCCCCAGGCG
16	GATCCCCGGGTTCGGTTTCGCACTGAAAACCCATGCT
17	GATCACCGGTGCCATGGATCAGAAGCAGTTT
18	GATCCCCGGGACCCCTTCTTTCCCAGGC
19	GATCATACCGGTCCACACGCA
20	GATCATCCCGGGTGTTCCGGCA
21	GATCATACCGGTCCCGAGGACCT
22	GATCATCCCGGGGATTGCTTCTTT
23	GACTCCCGGGGCAACTACTGTTCAACTGTCTGATC
24	GACTCCCGGGTTCTTCAGCTTGGCGCAGAATCAG
25	GATCACCGGTAAGATGGACAAGAAAACCATAG
26	GATCACCGGTTGCAGCACCAATCATGATTTG
27	GATCACCGGTATGATGTTCTTACCAACCGATTATTG
28	GATCCCCGGGGTCAACATGTTTATTGCTTTCCAAC
29	GATCACCGGTGTTTCCGGAGTCGGGGG
30	GATCCCCGGGGCTCGGGATTTGCAAGAAAC
31	GATCACCGGTAATGGAGCTATAGGAGGTGACC
32	GATCCCCGGGACATGAATATAATCCGTTTTCTCC
33	GATCACCGGTAAACTGGAAGTCGAGGGAGTGC
34	GATCACCGGTACCGCCAGATTCCAGTTTTAGAAG
35	GAACTGTACCGGCATCCTGGGCCGTAG
36	CTACGGCCCAGGATGCCGGTACAGTTC
37	GAAAGTTGATTCCTCCTGGGACAGAAACAAGC
38	GCTTGTTTCTGTCCCAGGAGGAATCAACTTTC
39	GCCTGGTCCCCTGGGTGGAGTAAC

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40	GTTACTCCACCCAGGGGACCAGGC
41	GTTAAATCCGGTTTGGATTCATTCCAAGAATACTGG
42	CCAGTATTCTTGGAATGAATCCAAACCGGATTTAAC
43	GATCCCGGGGGATTTGGTGATGTCGGTGC
44	GATCCCCGGGTGATGTTTTAAAGAAAAGTTTTTTCCGG
45	GATCCCCGGGCTGGCAACCACACTGGAAC
46	GATCCCCGGGTCCAAAGTAATTTTCGTCGTTCGC
47	GATCCCCGGGAGCTCCCCGAAACGCCC
48	GATCCCCGGGACCAATATTCAGCTCGTCATAGATTTC
49	GATCCCCGGGCCAGAACTTGGATTTAGCATATC
50	GATCCCCGGGTCTAGAGGTACGGTAGTTAATCGAG
51	GATCCCCGGGTTGGCTGCTGCACTTGAACG
52	GATCCCCGGGCACCCAGGTATCCACCGC
53	GATCGAATTCTACCCATACGATGTTCCAGATTAC
54	TCGAATTCGAACAAAAACTCATCTCAGAAGAG
55	CTTTTTCATCCAGGCCGCTGGTGGGGAGCAGAAAG
56	CTTTCTGCTCCCCACCAGCGGCCTGGATGAAAAAG
57	GCCGCTTCTTTCGCCGCCGCTTCAGAATTCACCGGTGGCCATG
58	GGCGAAAGAAGCGGCGGCGAAAGGATCCCCCGGGTAAGTTTA
59	AATTCGGAAGCTCTAGTGGTG
60	GATCCACCACTAGAGCTTCCG
61	AATTCGGAAGCTCTGGCGGATCCAGTGGTG
62	GATCCACCACTGGATCCGCCAGAGCTTCCG
63	AATTCGGAAGCAGCTCTTCTGGCGGATCCAGTAGTGGTG
64	GATCCACCACTACTGGATCCGCCAGAAGAGCTGCTTCCG
65	CAGTTCAACGCCCGCTTTAAGGGCGTGTC
66	GACACGCCCTTAAAGCGGGCGTTGAACTG

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Supplementary Table 3. Nucleotide sequences of empty and linker-containing cassettes. Green: site *A*, blue: site *B*, red: site *C*. The genes are available through Addgene.org.

Name	Sequence
pOVC1	ACCATGGCCACCGGTGAATTCTCAGGCTCTGGATCCCCCGGGTAA
pOVC2	ACCATGGCCACCGGTCCCGGGGAATTCTCAGGCTCTGGATCCTAA
pOVC3	ACCATGGCCGAATTCTCAGGCTCTGGATCCACCGGTCCCGGGTAA
pOVC1_L1(GSSSG)	ACCATGGCCACCGGTGAATTCGGAAGCTCTAGTGGTGGATCCCCCGGGTAA
pOVC1_L2(GSSGGSSG)	ACCATGGCCACCGGTGAATTCGGAAGCTCTGGCGGATCCAGTGGTGGATCCCCCGGGTAA
pOVC1_L3(GSSSSGGSSSG)	ACCATGGCCACCGGTGAATTCGGAAGCAGCTCTTCTGGCGGATCCAGTAGTGGTGGATCCCCCGGGTAA
pOVC1_L4$(EAAAK)_3$	ACCATGGCCACCGGTGAATTCGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGGATCCCCCGGGTAA

54 **Supplementary Table 4.** Nucleotide sequences of ordered gene fragments.

Name	**Sequence**
AtCRY2-PHR	AAGATGGACAAGAAAACCATAGTCTGGTTTCGCCGCGATTTGAGGATAGAGGATAACCCTGCGTTGGCCGCAGCGGCCCACG AAGGCAGCGTGTTCCCCGTGTTCATATGGTGCCCAGAGGAGGAAGGCCAGTTCTACCCAGGTCGCGCTAGTCGCTGGTGGAT GAAACAGTCTCTCGCACATCTCTCCCAATCTTTGAAAGCTCTCGGGTCTGACCTTACGCTGATTAAGACCCACAATACTATT AGTGCAATCTTGGACTGCATCCGCGTTACCGGCGCGACCAAAGTGGTGTTTAATCATCTGTATGACCCGGTAAGTCTGGTAC GCGACCATACAGTTAAGGAGAAGTTGGTTGAAAGGGGAATTTCAGTACAGAGTTATAATGGAGACCTCCTCTACGAACCTTG GGAAATATACTGTGAGAAGGGAAAGCCATTTACATCATTTAACTCATACTGGAAAAAGTGTCTTGACATGAGCATAGAGTCT GTCATGTTGCCCCCGCCGTGGCGGCTTATGCCCATCACCGCGGCCGCCGAAGCTATCTGGGCGTGTAGTATTGAAGAACTTG GCTTGGAGAACGAAGCAGAAAAACCTAGCAATGCACTGTTGACGCGCGCCTGGTCCCCCGGGTGGAGTAACGCAGATAAATT GCTTAACGAGTTCATCGAAAAGCAACTTATCGACTACGCTAAGAATAGCAAAAAAGTGGTGGGCAATTCAACCTCCCTCCTG TCTCCGTACTTGCATTTCGGCGAGATTAGCGTGCGGCACGTCTTCCAATGCGCCCGAATGAAACAAATAATCTGGGCGCGGG ATAAAAACAGTGAGGGTGAAGAAAGCGCAGACTTGTTCCTCCGAGGAATCGGTCTGCGAGAATATAGTCGCTACATTTGTTT CAATTTTCCCTTTACGCATGAGCAGAGCCTCCTTAGTCACTTGCGATTCTTTCCTTGGGACGCAGATGTTGACAAATTTAAA GCATGGCGGCAAGGTAGAACAGGCTACCCATTGGTAGATGCGGGTATGCGAGAACTCTGGGCCACGGGGTGGATGCATAACC GAATCAGGGTAATAGTAAGTAGTTTCGCAGTTAAGTTTCTTTTGCTTCCATGGAAGTGGGGGATGAAGTATTTCTGGGACAC TTTGCTCGATGCGGATCTTGAATGCGATATATTGGGTTGGCAATATATTTCCGGGTCAATCCCTGACGGCCATGAGCTGGAC AGATTGGACAATCCTGCGCTCCAGGGTGCGAAATACGATCCCGAAGGTGAATACATTAGACAATGGCTTCCAGAACTTGCCA GGTTGCCCACGGAATGGATTCACCACCCATGGGACGCCCCTCTTACGGTTTTGAAGGCGAGTGGTGTGGAGCTCGGTACCAA TTACGCAAAACCGATTGTTGACATTGATACCGCGCGGGAGTTGCTGGCTAAAGCCATTTCACGAACCCGGAAAGCCCAAATC ATGATTGGTGCTGCA
Casp9	GATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAGCATGGAGCCCTGTGGCCA CTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCTCCAACATCGACTGTGAGAAG TTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTGCTGGCTTTGC TGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTCTCACGGCTGTCAGGCCAGCCACCT GCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGATTGTGAACATCTTCAATGGGACCAGC TGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGGGAGCAGAAAGACCATGGGTTTGAGGTGG CCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGCCACCCCGTTCCAGGAAGGTTTGAGGACCTT CGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTGTGTCCTACTCTACTTTCCCAGGTTTTGTTTCC TGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGACATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGC AGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGATTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCG GAAAAAACTTTTCTTTAAAACATC
HA-P2A-MYC	CATGTACCCATACGATGTTCCAGATTACGCTGCGACCAACTTTAGCCTGCTGAAACAGGCGGGCGATGTGGAAGAAAACCCG GGCCCGGAACAAAAACTCATCTCAGAAGAGGATCTGCATG
AsPT-LOV2-EcSsra	GATCAGCTGGCAACCACACTGGAACGGATCGAGAAAAATTTCGTGATTACTGATCCGAGACTGCCTGACAACCCAATCATTT TTGCGAGCGATTCCTTCCTGCAGCTGACAGAATATTCTCGGGAAGAGATCCTGGGGCGCAATTGCCGTTTTCTGCAGGGACC CGAGACAGACCGTGCCACTGTTCGGAAAATCAGAGATGCTATTGACAACCAGACTGAAGTGACCGTTCAGCTGATCAATTAT ACCAAGAGCGGCAAGAAGTTCTGGAACGTGTTCCACCTGCAGCCGATGCGCGATTATAAGGGCGACGTCCAGTACTTCATTG GCGTGCAGCTGGATGGCACCGAACGTCTTCATGGCGCCGCTGAGCGTGAGGCGGTCTGCCTGATCAAAAAGACAGCCTTTCA GATTGCTGAGGCAGCGAACGACGAAAATTACTTTGGAGATCAG
EcSSPB (micro)	GATCAGAGCTCCCCGAAACGCCCTAAGCTGCTGCGTGAATATTACGATTGGCTGGTTGATAACAGCTTTACCCCATATCTGG TGGTGGATGCCACATACCTGGGCGTGAACGTGCCCGTGGAGTATGTGAAAGACGGTCAGATCGTGCTGAATCTGTCTGCAAG TGCGACCGGCAACCTGCAACTGACAAATGATTTTATCCAGTTCAACGCCCAGTTTAAGGGCGTGTCTCGTGAACTGTATATC CCGATGGGTGCCGCTCTGGCCATTTACGCTCGCGAGAACGGCGATGGTGTGATGTTCGAACCAGAAGAAATCTATGACGAGC TGAATATTGGTGATCAG

AsPT-LOV2-pep	GATCAGTTGGCTGCTGCACTTGAACGTATTGAGAAGAACTTTGTCATTACTGACCCAAGATTGCCAGATAATCCCATTATAT TCGCGTCCGATAGTTTCTTGCAGTTGACAGAATATAGCCGTGAAGAAATTTTGGGAAGAAACTGCAGGTTTCTACAAGGTCC TGAAACTGATCGCGCGACAGTGAGAAAAATTAGAGATGCCATAGATAACCAAACAGAGGTCACTGTTCAGCTGATTAATTAT ACAAAGAGTGGTAAAAAGTTCTGGAACCTCTTTCACTTGCAGCCTATGCGAGATCAGAAGGGAGATGTCCAGTACTTTATTG GGGTTCAGTTGGATGGAACTGAGCATGTCCGAGATGCTGCCGAGAGAGAGGCAGTCATGCTGGCGAAGAAAACTGCAGAAGA GATTGATAAAGCGGTGGATACCTGGGTGGATCAG
HsPDZB1	GATCAGCCAGAACTTGGATTTAGCATATCAGGTGGTGTCGGGGGTAGAGGAAACCCATTCAGACCTGATGATGATGGTATAT TTGTAACAAGGGTACAACCTGAAGGACCAGCATCAAAATTACTGCAGCCAGGTGATAAAATTATTCAGGCTAATGGCTACAG TTTTATAAATATTGAACATGGACAAGCAGTGTCCTTGCTAAAAACTTTCCAGAATACAGTTGAACTCATCATTGTACGAGAA GTTGGTAACGGTGCAAAACAAGAGATTCGAGTGAGGGTTGAAAAAGACGGTGGTTCTGGAGGCGTTTCTTCTGTTCCGACCA ATCTGGAAGTTGTTGCTGCGACCCCGACTAGCCTGCTGATCAGCTGGGATGCGTATAGGGAGCTTCCGGTTAGTTATTACCG TATCACGTACGGTGAAACAGGTGGTAACTCCCCGGTTCAGGAGTTCACTGTACCTGGTTCCAAGTCTACTGCTACCATCAGC GGCCTGAAACCGGGTGTCGACTATACCATCACTGTATACGCACATTACAACTACCATTACTACTCTAGCCCAATCTCGATTA ACTACCGTACCTCTAGAGATCAG

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Supplementary Table 5. Nucleotide sequences of cassettes containing LSDs. Green: site *A*, blue: site *B*, red: site *C*. X: Empty site. The genes are available through Addgene.org.

Name	Sequence
>pOVC1_VfAU1-LOV_X	ACCATGGCCACCGGTCCTGACTACAGTCTCGTGAAGGCTCTGCAAATGGCACAACAGAATTTTGTCATTACAG ACGCCTCCCTCCCAGACAACCCTATCGTCTACGCCAGTAGAGGGTTTCTGACACTGACAGGCTATTCTCTCGA CCAGATCCTGGGCAGGAACTGCAGGTTTCTGCAAGGGCCAGAAACAGACCCAAGAGCTGTGGATAAGATCAGG AATGCCATCACCAAAGGCGTTGATACCAGTGTCTGTCTGCTGAATTATAGACAGGATGGCACAACCTTCTGGA ATCTCTTCTTCGTGGCTGGACTCAGAGATTCTAAGGGCAATATTGTCAACTACGTCGGAGTGCAGTCAAAGGT GAGCGAAGATTATGCCAAGCTGCTGGTCAACGAGCAGAACATTGAGTACAAAGGTGTGCGCACCAGTAACATG CTGCGCAGAAAGCCCGGTGAATTCTCAGGCTCTGGATCCCCCGGGTAA
>pOVC1_CrPH-LOV1_X	ACCATGGCCACCGGTGCAGGACTCAGACATACATTTGTGGTGGCTGATGCAACACTCCCTGATTGCCCACTGG TCTATGCAAGTGAGGGCTTCTACGCAATGACCGGATATGGACCTGACGAAGTGCTGGGTCACAACTGTAGGTT TCTGCAGGGTGAGGGAACTGACCCCAAGGAAGTGCAGAAAATTCGCGACGCCATCAAGAAGGGTGAGGCTTGT AGTGTGCGCCTCCTGAACTATCGGAAGGACGGCACTCCCTTCTGGAACCTGCTGACAGTCACCCCAATTAAAA CCCCTGATGGCCGCGTGTCCAAGTTTGTCGGCGTGCAGGTGGATGTTACCTCCAAGACTGAAGGGAAAGCCCT GGCCCCCGGTGAATTCTCAGGCTCTGGATCCCCCGGGTAA
>pOVC1_NcVVD-LOV_X	ACCATGGCCACCGGGCACACTCTCTACGCCCCAGGCGGGTACGATATTATGGGCTGGCTGATCCAGATCATGA ACAGGCCCAATCCCCAGGTCGAGCTGGGACCCGTGGATACTTCATGTGCACTGATACTGTGCGACCTGAAGCA GAAGGATACACCTATAGTTTACGCTTCAGAAGCCTTTCTGTACATGACAGGGTATTCTAACGCCGAGGTGCTG GGGAGGAACTGTAGGTTCCTCCAGAGTCCCGATGGTATGGTGAAACCTAAGAGTACTCGCAAATATGTGGATA GCAATACTATTAACACCATGAGGAAAGCCATCGACAGAAACGCAGAAGTTCAGGTGGAAGTGGTGAACTTTAA GAAGAACGGCCAGCGGTTCGTGAACTTTCTCACAATGATTCCAGTGCGGGACGAAACCGGGGAGTACCGGTAC AGCATGGGTTTTCAGTGCGAAACCGAACCCGGTGAATTCTCAGGCTCTGGATCCCCCGGGTAA
>pOVC1_RsLP-LOV_X	ACCATGGCCACCGGTGCCATGGATCAGAAGCAGTTTGAGAAGATTAGAGCTGTGTTTGACAGGTCAGGGGTCG CACTGACCCTCGTTGACATGTCCCTGCCAGAGCAACCCCTGGTGCTCGCCAACCCTCCATTTCTGAGAATGAC TGGCTATACTGAGGGCCAGATCCTGGGATTCAACTGCAGATTTCTCCAGAGAGGCGACGAAAATGCTCAGGCA CGGGCTGACATCAGAGATGCCCTCAAGCTCGGAAGGGAGCTCCAGGTGGTCCTCCGCAATTACAGAGCCAACG ATGAACCATTTGACAATCTGCTGTTCCTGCACCCTGTCGGTGGCAGACCCGACGCTCCTGACTACTTCCTCGG TTCTCAGTTCGAGCTGGGTAGAAGCGGAAATAGCGAAGAGGCAGCCGCAGCTGGACACGCAGGGGCACTGACT GGGGAGCTCGCCAGAATAGGAACTGTGGCTGCTCGGCTCGAAATGGACAGTCGGAGACATCTGGCACAAGCTG CTGCAGCCCTGGTGAGGGCCTGGGAAAGAAGGGGTCCCGGTGAATTCTCAGGCTCTGGATCCCCCGGGTAA
>pOVC1_EcSSPB-nano_X	ACCATGGCCACCGGGAGCTCCCCGAAACGCCCTAAGCTGCTGCGTGAATATTACGATTGGCTGGTTGATAACA GCTTTACCCCATATCTGGTGGTGGATGCCACATACCTGGGCGTGAACGTGCCCGTGGAGTATGTGAAAGACGG TCAGATCGTGCTGAATCTGTCTGCAAGTGCGACCGGCAACCTGCAACTGACAAATGATTTTATCCAGTTCAAC GCCCGCTTTAAGGGCGTGTCTCGTGAACTGTATATCCCGATGGGTGCCGCTCTGGCCATTTACGCTCGCGAGA ACGGCGATGGTGTGATGTTCGAACCAGAAGAAATCTATGACGAGCTGAATATTGGTCCCGGTGAATTCTCAGG CTCTGGATCCCCCGGGTAA
>pOVC1_EcSSPB-micro_X	ACCATGGCCACCGGGAGCTCCCCGAAACGCCCTAAGCTGCTGCGTGAATATTACGATTGGCTGGTTGATAACA GCTTTACCCCATATCTGGTGGTGGATGCCACATACCTGGGCGTGAACGTGCCCGTGGAGTATGTGAAAGACGG TCAGATCGTGCTGAATCTGTCTGCAAGTGCGACCGGCAACCTGCAACTGACAAATGATTTTATCCAGTTCAAC GCCCAGTTTAAGGGCGTGTCTCGTGAACTGTATATCCCGATGGGTGCCGCTCTGGCCATTTACGCTCGCGAGA ACGGCGATGGTGTGATGTTCGAACCAGAAGAAATCTATGACGAGCTGAATATTGGTCCCGGTGAATTCTCAGG CTCTGGATCCCCCGGGTAA
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	TTCCCTTTACGCATGAGCAGAGCCTCCTTAGTCACTTGCGATTCTTTCCTTGGGACGCAGATGTTGACAAATT TAAAGCATGGCGGCAAGGTAGAACAGGCTACCCATTGGTAGATGCGGGTATGCGAGAACTCTGGGCCACGGGG TGGATGCATAACCGAATCAGGGTAATAGTAAGTAGTTTCGCAGTTAAGTTTCTTTTGCTTCCATGGAAGTGGG GGATGAAGTATTTCTGGGACACTTTGCTCGATGCGGATCTTGAATGCGATATATTGGGTTGGCAATATATTTC CGGGTCAATCCCTGACGGCCATGAGCTGGACAGATTGGACAATCCTGCGCTCCAGGGTGCGAAATACGATCCC GAAGGTGAATACATTAGACAATGGCTTCCAGAACTTGCCAGGTTGCCCACGGAATGGATTCACCACCCATGGG ACGCCCCTCTTACGGTTTTGAAGGCGAGTGGTGTGGAGCTCGGTACCAATTACGCAAAACCGATTGTTGACAT TGATACCGCGCGGGAGTTGCTGGCTAAAGCCATTTCACGAACCCGGAAAGCCCAAATCATGATTGGTGCTGCA ACCGGGTAA
>pOVC1_X_AtPIF6	ACCATGGCCACCGGTGAATTCTCAGGCTCTGGATCCCCCGGTATGATGTTCTTACCAACCGATTATTGTTGCA GGTTAAGCGATCAAGAGTATATGGAGCTTGTGTTTGAGAATGGCCAGATTCTTGCAAAGGGCCAAAGATCCAA CGTTTCTCTGCATAATCAACGTACCAAATCGATCATGGATTTGTATGAGGCAGAGTATAACGAGGATTTCATG AAGAGTATCATCCATGGTGGTGGTGGTGCCATCACAAATCTCGGGGACACGCAGGTTGTTCCACAAAGTCATG TTGCTGCTGCCCATGAAACAAACATGTTGGAAAGCAATAAACATGTTGACCCCGGGTAA
>pOVC1_X_ScPH1-S	ACCATGGCCACCGGTGAATTCTCAGGCTCTGGATCCCCCGGGGCAACTACTGTTCAACTGTCTGATCAATCTC TGCGTCAACTGGAAACTCTGGCTATCCACACCGCGCATCTGATCCAGCCGCACGGTCTGGTAGTCGTCCTGCA AGAACCGGACCTGACCATCAGCCAGATCTCTGCGAACTGTACCGGCATCCTGGGCCGTAGCCCGGAAGATCTG CTGGGTCGTACTCTGGGCGAGGTATTCGATTCTTTTCAGATTGATCCGATCCAGTCTCGTCTGACCGCAGGTC AGATTTCCAGCCTGAACCCGTCCAAGCTGTGGGCGCGTGTTATGGGTGACGACTTTGTTATTTTCGACGGCGT ATTTCATCGTAACTCTGATGGCCTGCTGGTTTGCGAGCTGGAGCCGGCCTACACTAGCGACAACCTGCCTTTC CTGGGTTTCTACCATATGGCAAACGCGGCACTGAACCGTCTGCGTCAGCAAGCTAACCTGCGCGACTTCTACG ACGTTATCGTTGAGGAAGTGCGCCGCATGACGGGTTTCGACCGCGTCATGCTGTACCGTTTTGATGAAAACAA CCACGGTGACGTAATCGCGGAGGATAAGCGTGACGACATGGAGCCGTATCTGGGTCTGCACTACCCGGAAAGC GACATTCCTCAGCCGGCACGTCGCCTGTTCATTCACAACCCGATCCGTGTTATTCCGGACGTTTACGGCGTTG CTGTTCCGCTGACTCCGGCCGTTAATCCGTCTACTAACCGTGCAGTTGACCTGACCGAATCCATCCTGCGTTC CGCATACCATTGCCACCTGACCTATCTGAAGAACATGGGCGTTGGTGCTAGCCTGACGATCTCTCTGATTAAA GATGGTCACCTGTGGGGTCTGATCGCTTGCCATCACCAGACCCCGAAAGTAATCCCTTTCGAACTGCGTAAAG CCTGCGAATTCTTCGGTCGTGTGGTGTTCTCTAATATCTCCGCGCAAGAAGACACCGAGACTTTTGACTACCG CGTACAGCTGGCGGAGCATGAAGCGGTTCTGCTGGACAAAATGACCACCGCGGCAGACTTCGTGGAGGGCCTG ACTAACCACCCAGACCGTCTGCTGGGCCTGACCGGCAGCCAAGGCGCTGCGATTTGTTTCGGCGAGAAACTGA TTCTGGTGGGCGAAACCCCAGACGAAAAGGCGGTGCAATACCTGCTGCAATGGCTGGAGAATCGCGAAGTGCA GGACGTTTTCTTCACTAGCTCTCTGTCTCAGATCTATCCGGATGCGGTTAACTTCAAAAGCGTGGCGTCCGGC CTGCTGGCTATCCCGATCGCCCGTCATAACTTTCTGCTGTGGTTCCGCCCGGAGGTTCTGCAGACCGTTAATT GGGGTGGTGATCCGAATCACGCATACGAAGCAACCCAAGAAGATGGTAAGATCGAACTGCATCCGCGTCAGTC CTTCGATCTGTGGAAAGAAATTGTTCGCCTGCAGAGCCTGCCGTGGCAGAGCGTTGAGATCCAGTCTGCCCTG GCTCTGAAGAAAGCAATCGTGAACCTGATTCTGCGCCAAGCTGAAGAACCCGGGTAA
>pOVC1_X_AtCIB	ACCATGGCCACCGGTGAATTCTCAGGCTCTGGATCCCCCGGTAATGGAGCTATAGGAGGTGACCTTTTGCTCA ATTTTCCTGACATGTCGGTCCTAGAGCGCCAAAGGGCTCACCTCAAGTACCTCAATCCCACCTTTGATTCTCC TCTCGCCGGCTTCTTTGCCGATTCTTCAATGATTACCGGCGGCGAGATGGACAGCTATCTTTCGACTGCCGGT TTGAATCTTCCGATGATGTACGGTGAGACGACGGTGGAAGGTGATTCAAGACTCTCAATTTCGCCGGAAACGA CGCTTGGGACTGGAAATTTCAAGGCAGCGAAGTTTGATACAGAGACTAAGGATTGTAATGAGGCGGCGAAGAA GATGACGATGAACAGAGATGACCTAGTAGAAGAAGGAGAAGAAGAGAAGTCGAAAATAACAGAGCAAAACAAT GGGAGCACAAAAAGCATCAAGAAGATGAAACACAAAGCCAAGAAAGAAGAGAACAATTTCTCTAATGATTCAT CTAAAGTGACGAAGGAATTGGAGAAAACGGATTATATTCATCCCGGGTAA

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Supplementary Table 6. Nucleotide sequences for Opto-casp9 constructs. Green: site *A*, blue: site *B*, red: site *C*. The genes are available through Addgene.org.

Name	Sequence
>pOVC1_VfAU1-LOV_casp9	ACCATGGCCACCGGTCCTGACTACAGTCTCGTGAAGGCTCTGCAAATGGCACAACAGAATTTTGTCATTACAGACGCCTC CCTCCCAGACAACCCTATCGTCTACGCCAGTAGAGGGTTTCTGACACTGACAGGCTATTCTCTCGACCAGATCCTGGGCA GGAACTGCAGGTTTCTGCAAGGGCCAGAAACAGACCCAAGAGCTGTGGATAAGATCAGGAATGCCATCACCAAAGGCGTT GATACCAGTGTCTGTCTGCTGAATTATAGACAGGATGGCACAACCTTCTGGAATCTCTTCTTCGTGGCTGGACTCAGAGA TTCTAAGGGCAATATTGTCAACTACGTCGGAGTGCAGTCAAAGGTGAGCGAAGATTATGCCAAGCTGCTGGTCAACGAGC AGAACATTGAGTACAAAGGTGTGCGCACCAGTAACATGCTGCGCAGAAAGCCCGGTGAATTCTCAGGCTCTGGATCCCCC GGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAGCATGGAGCCCTG TGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCTCCAACATCGACT GTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTG CTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTCTCACGGCTGTCA GGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGATTGTGAACATCT TCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGGGAGCAGAAAGAC CATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGCCACCCCGTTCCA GGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTGTGTCCTACTCTA CTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGACATCTTTGAGCAG TGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGATTTATAAACAGAT GCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGGTAA
>pOVC1_CrPH-LOV1_casp9	ACCATGGCCACCGGTGCAGGACTCAGACATACATTTGTGGTGGCTGATGCAACACTCCCTGATTGCCCACTGGTCTATGC AAGTGAGGGCTTCTACGCAATGACCGGATATGGACCTGACGAAGTGCTGGGTCACAACTGTAGGTTTCTGCAGGGTGAGG GAACTGACCCCAAGGAAGTGCAGAAAATTCGCGACGCCATCAAGAAGGGTGAGGCTTGTAGTGTGCGCCTCCTGAACTAT CGGAAGGACGGCACTCCCTTCTGGAACCTGCTGACAGTCACCCCAATTAAAACCCCTGATGGCCGCGTGTCCAAGTTTGT CGGCGTGCAGGTGGATGTTACCTCCAAGACTGAAGGGAAAGCCCTGGCCCCCGGTGAATTCTCAGGCTCTGGATCCCCCC GGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAGCATGGAGCCCTG TGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCTCCAACATCGACT GTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTG CTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTCTCACGGCTGTCA GGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGATTGTGAACATCT TCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGGGAGCAGAAAGAC CATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGCCACCCCGTTCCA GGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTGTGTCCTACTCTA CTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGACATCTTTGAGCAG TGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGATTTATAAACAGAT GCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGGTAA
>pOVC1_NcVVD-LOV_casp9	ACCATGGCCACCGGGCACACTCTCTACGCCCCAGGCGGGTACGATATTATGGGCTGGCTGATCCAGATCATGAACAGGCC CAATCCCCAGGTCGAGCTGGGACCCGTGGATACTTCATGTGCACTGATACTGTGCGACCTGAAGCAGAAGGATACACCTA TAGTTTACGCTTCAGAAGCCTTTCTGTACATGACAGGGTATTCTAACGCCGAGGTGCTGGGGAGGAACTGTAGGTTCCTC CAGAGTCCCGATGGTATGGTGAAACCTAAGAGTACTCGCAAATATGTGGATAGCAATACTATTAACACCATGAGGAAAGC CATCGACAGAAACGCAGAAGTTCAGGTGGAAGTGGTGAACTTTAAGAAGAACGGCCAGCGGTTCGTGAACTTTCTCACAA TGATTCCAGTGCGGGACGAAACCGGGGAGTACCGGTACAGCATGGGTTTTCAGTGCGAAACCGAACCCGGTGAATTCTCA GGCTCTGGATCCCCCGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAG CATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCT CCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCC AAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTC TCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGA TTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGG GAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGC CACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTG TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCAGGGTAA
>pOVC1_RsLP-LOV_casp9	ACCATGGCCACCGGTGCCATGGATCAGAAGCAGTTTGAGAAGATTAGAGCTGTGTTTGACAGGTCAGGGGTCGCACTGAC CCTCGTTGACATGTCCCTGCCAGAGCAACCCCTGGTGCTCGCCAACCCTCCATTTCTGAGAATGACTGGCTATACTGAGG GCCAGATCCTGGGATTCAACTGCAGATTTCTCCAGAGAGGCGACGAAAATGCTCAGGCACGGGCTGACATCAGAGATGCC CTCAAGCTCGGAAGGGAGCTCCAGGTGGTCCTCCGCAATTACAGAGCCAACGATGAACCATTTGACAATCTGCTGTTCCT GCACCCTGTCGGTGGCAGACCCGACGCTCCTGACTACTTCCTCGGTTCTCAGTTCGAGCTGGGTAGAAGCGGAAATAGCG AAGAGGCAGCCGCAGCTGGACACGCAGGGGCACTGACTGGGGAGCTCGCCAGAATAGGAACTGTGGCTGCTCGGCTCGAA ATGGACAGTCGGAGACATCTGGCACAAGCTGCTGCAGCCCTGGTGAGGGCCTGGGAAAGAAGGGGTCCCGGTGAATTCTC AGGCTCTGGATCCCCCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCC TGAGCATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACT GGCTCCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGAC TGCCAAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTC TCTCTCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAG AAGATTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGG TGGGGAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAG

	ATGCCACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATC TTTGTGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGA CGACATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAG GGATTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGGTAA
>pOVC1_AtCRY2-PHR_casp9	ACCATGGCCACCGGTAAGATGGACAAGAAAACCATAGTCTGGTTTCGCCGCGATTTGAGGATAGAGGATAACCCTGCGTT GGCCGCAGCGGCCCACGAAGGCAGCGTGTTCCCCGTGTTCATATGGTGCCCAGAGGAGGAAGGCCAGTTCTACCCAGGTC GCGCTAGTCGCTGGTGGATGAAACAGTCTCTCGCACATCTCTCCCAATCTTTGAAAGCTCTCGGGTCTGACCTTACGCTG ATTAAGACCCACAATACTATTAGTGCAATCTTGGACTGCATCCGCGTTACCGGCGCGACCAAAGTGGTGTTTAATCATCT GTATGACCCGGTAAGTCTGGTACGCGACCATACAGTTAAGGAGAAGTTGGTTGAAAGGGGAATTTCAGTACAGAGTTATA ATGGAGACCTCCTCTACGAACCTTGGGAAATATACTGTGAGAAGGGAAAGCCATTTACATCATTTAACTCATACTGGAAA AAGTGTCTTGACATGAGCATAGAGTCTGTCATGTTGCCCCCGCCGTGGCGGCTTATGCCCATCACCGCGGCCGCCGAAGC TATCTGGGCGTGTAGTATTGAAGAACTTGGCTTGGAGAACGAAGCAGAAAAACCTAGCAATGCACTGTTGACGCGCGCCT GGTCCCCTGGGTGGAGTAACGCAGATAAATTGCTTAACGAGTTCATCGAAAAGCAACTTATCGACTACGCTAAGAATAGC AAAAAAGTGGTGGGCAATTCAACCTCCCTCCTGTCTCCGTACTTGCATTTCGGCGAGATTAGCGTGCGGCACGTCTTCCA ATGCGCCCGAATGAAACAAATAATCTGGGCGCGGGATAAAAACAGTGAGGGTGAAGAAAGCGCAGACTTGTTCCTCCGAG GAATCGGTCTGCGAGAATATAGTCGCTACATTTGTTTCAATTTTCCCTTTACGCATGAGCAGAGCCTCCTTAGTCACTTG CGATTCTTTCCTTGGGACGCAGATGTTGACAAATTTAAAGCATGGCGGCAAGGTAGAACAGGCTACCCATTGGTAGATGC GGGTATGCGAGAACTCTGGGCCACGGGGTGGATGCATAACCGAATCAGGGTAATAGTAAGTAGTTTCGCAGTTAAGTTTC TTTTGCTTCCATGGAAGTGGGGGATGAAGTATTTCTGGGACACTTTGCTCGATGCGGATCTTGAATGCGATATATTGGGT TGGCAATATATTTCCGGGTCAATCCCTGACGGCCATGAGCTGGACAGATTGGACAATCCTGCGCTCCAGGGTGCGAAATA CGATCCCGAAGGTGAATACATTAGACAATGGCTTCCAGAACTTGCCAGGTTGCCCACGGAATGGATTCACCACCCATGGG ACGCCCCTCTTACGGTTTTGAAGGCGAGTGGTGTGGAGCTCGGTACCAATTACGCAAAACCGATTGTTGACATTGATACC GCGCGGGAGTTGCTGGCTAAAGCCATTTCACGAACCCGGAAAGCCCAAATCATGATTGGTGCTGCACCCGGTGAATTCTC AGGCTCTGGATCCCCCGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGA GCATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGC TCCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGC CAAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCT CTCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAG ATTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGG GGAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATG CCACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTT GTGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGA CATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGA TTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCAGGGTAA
>pOVC1_casp9_VfAU1-LOV	ACCATGGCCACCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAG CATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCT CCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCC AAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTC TCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGA TTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGG GAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGC CACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTG TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCAGGGTAA
>pOVC1_casp9_CrPH-LOV1	ACCATGGCCACCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAG CATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCT CCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCC AAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTC TCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGA TTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGG GAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGC CACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTG TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGTGAATTCTCAGGCT CTGGATCCCCCGGTGCAGGACTCAGACATACATTTGTGGTGGCTGATGCAACACTCCCTGATTGCCCACTGGTCTATGCA AGTGAGGGCTTCTACGCAATGACCGGATATGGACCTGACGAAGTGCTGGGTCACAACTGTAGGTTTCTGCAGGGTGAGGG AACTGACCCCAAGGAAGTGCAGAAAATTCGCGACGCCATCAAGAAGGGTGAGGCTTGTAGTGTGCGCCTCCTGAACTATC GGAAGGACGGCACTCCCTTCTGGAACCTGCTGACAGTCACCCCAATTAAAACCCCTGATGGCCGCGTGTCCAAGTTTGTC GGCGTGCAGGTGGATGTTACCTCCAAGACTGAAGGGAAAGCCCTGGCCCCCGGGTAA
>pOVC1_casp9_NcVVD-LOV	ACCATGGCCACCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAG CATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCT CCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCC

	AAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTC TCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGA TTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGG GAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGC CACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTG TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGTGAATTCTCAGGCT CTGGATCCCCCGGGCACACTCTCTACGCCCCAGGCGGGTACGATATTATGGGCTGGCTGATCCAGATCATGAACAGGCCC AATCCCCAGGTCGAGCTGGGACCCGTGGATACTTCATGTGCACTGATACTGTGCGACCTGAAGCAGAAGGATACACCTAT AGTTTACGCTTCAGAAGCCTTTCTGTACATGACAGGGTATTCTAACGCCGAGGTGCTGGGGAGGAACTGTAGGTTCCTCC AGAGTCCCGATGGTATGGTGAAACCTAAGAGTACTCGCAAATATGTGGATAGCAATACTATTAACACCATGAGGAAAGCC ATCGACAGAAACGCAGAAGTTCAGGTGGAAGTGGTGAACTTTAAGAAGAACGGCCAGCGGTTCGTGAACTTTCTCACAAT GATTCCAGTGCGGGACGAAACCGGGGAGTACCGGTACAGCATGGGTTTTCAGTGCGAAACCGAACCCGGGTAA
>pOVC1_casp9_RsLP-LOV	ACCATGGCCACCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAG CATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGCACTGGCT CCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGGCGACCTGACTGCC AAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTC TCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTCGAGAAGA TTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCAGGCCTGTGGTGGG GAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGC CACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGACATCTTTG TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGTGAATTCTCAGGCT CTGGATCCCCCGGTGCCATGGATCAGAAGCAGTTTGAGAAGATTAGAGCTGTGTTTGACAGGTCAGGGGTCGCACTGACC CTCGTTGACATGTCCCTGCCAGAGCAACCCCTGGTGCTCGCCAACCCTCCATTTCTGAGAATGACTGGCTATACTGAGGG CCAGATCCTGGGATTCAACTGCAGATTTCTCCAGAGAGGCGACGAAAATGCTCAGGCACGGGCTGACATCAGAGATGCCC TCAAGCTCGGAAGGGAGCTCCAGGTGGTCCTCCGCAATTACAGAGCCAACGATGAACCATTTGACAATCTGCTGTTCCTG CACCCTGTCGGTGGCAGACCCGACGCTCCTGACTACTTCCTCGGTTCTCAGTTCGAGCTGGGTAGAAGCGGAAATAGCGA AGAGGCAGCCGCAGCTGGACACGCAGGGGCACTGACTGGGGAGCTCGCCAGAATAGGAACTGTGGCTGCTCGGCTCGAAA TGGACAGTCGGAGACATCTGGCACAAGCTGCTGCAGCCCTGGTGAGGGCCTGGGAAAGAAGGGGTCCCGGGTAA
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	TGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGAGACCCTGGACGAC ATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGAT TTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGTGAATTCTCAGGCT CTGGATCCCCCGGTAAACTGGAAGTCGAGGGAGTGCAGGTGGAGACTATCTCCCCAGGAGACGGGCGCACCTTCCCCAAG CGCGGCCAGACCTGCGTGGTGCACTACACCGGGATGCTTGAAGATGGAAAGAAAGTTGATTCCTCCTGGGACAGAAACAA GCCCTTTAAGTTTATGCTAGGCAAGCAGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGA GAGCCAAACTGACTATATCTCCAGATTATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTC GTCTTCGATGTGGAGCTTCTAAAACTGGAATCTGGCGGTACCGGGTAA
>pOVC1_VfAU1-LOV-HA-P2a-myc_casp9	ACCATGGCCACCGGTCCTGACTACAGTCTCGTGAAGGCTCTGCAAATGGCACAACAGAATTTTGTCATTACAGACGCCTC CCTCCCAGACAACCCTATCGTCTACGCCAGTAGAGGGTTTCTGACACTGACAGGCTATTCTCTCGACCAGATCCTGGGCA GGAACTGCAGGTTTCTGCAAGGGCCAGAAACAGACCCAAGAGCTGTGGATAAGATCAGGAATGCCATCACCAAAGGCGTT GATACCAGTGTCTGTCTGCTGAATTATAGACAGGATGGCACAACCTTCTGGAATCTCTTCTTCGTGGCTGGACTCAGAGA TTCTAAGGGCAATATTGTCAACTACGTCGGAGTGCAGTCAAAGGTGAGCGAAGATTATGCCAAGCTGCTGGTCAACGAGC AGAACATTGAGTACAAAGGTGTGCGCACCAGTAACATGCTGCGCAGAAAGCCCGGTGAATTCTACCCATACGATGTTCCA GATTACGCTGCGACCAACTTTAGCCTGCTGAAACAGGCGGGCGATGTGGAAGAAAACCCGGGCCCGGAACAAAAACTCAT CTCAGAAGAGGATCTGGGATCCCCGGGGGATTTGGTGATGTCGGTGCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGG CTTACATCCTGAGCATGGAGCCCTGTGGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGC ACCCGCACTGGCTCCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCTGCATTTCATGGTGGAGGTGAAGGG CGACCTGACTGCCAAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCGCGGCAGGACCACGGTGCTCTGGACTGCTGCGTGG TGGTCATTCTCTCTCACGGCTGTCAGGCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTG TCGGTCGAGAAGATTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTTTTCATCCA GGCCTGTGGTGGGGAGCAGAAAGACCATGGGTTTGAGGTGGCCTCCACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACC CCGAGCCAGATGCCACCCCGTTCCAGGAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCC AGTGACATCTTTGTGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGCTCCTGGTACGTTGA GACCCTGGACGACATCTTTGAGCAGTGGGCTCACTCTGAAGACCTGCAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTT CGGTGAAAGGGATTTATAAACAGATGCCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAACATCACCCGGG TAA

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References

[1] M. Grusch, K. Schelch, R. Riedler, E. Reichhart, C. Differ, W. Berger, et al., Spatio-temporally precise activation of engineered receptor tyrosine kinases by light, EMBO J. 33 (2014) 1713-1726.

[2] D. Mitra, X. Yang, K. Moffat, Crystal structures of Aureochrome1 LOV suggest new design strategies for optogenetics, Structure. 20 (2012) 698-706.

[3] R.J. Kutta, E.S. Hofinger, H. Preuss, G. Bernhardt, B. Dick, Blue-light induced interaction of LOV domains from Chlamydomonas reinhardtii, Chembiochem. 9 (2008) 1931-1938.

[4] Y. Nakasone, M. Ohshima, K. Okajima, S. Tokutomi, M. Terazima, Photoreaction dynamics of LOV1 and LOV2 of phototropin from Chlamydomonas reinhardtii, J. Phys. Chem. B. 122 (2018) 1801-1815.

[5] X. Wang, X. Chen, Y. Yang, Spatiotemporal control of gene expression by a light-switchable transgene system, Nat. Methods. 9 (2012) 266-269.

[6] C. Schwerdtfeger, H. Linden, VIVID is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation, EMBO J. 22 (2003) 4846-4855.

[7] B.D. Zoltowski, B.R. Crane, Light activation of the LOV protein vivid generates a rapidly exchanging dimer, Biochemistry. 47 (2008) 7012-7019.

[8] K.S. Conrad, A.M. Bilwes, B.R. Crane, Light-induced subunit dissociation by a light-oxygen-voltage domain photoreceptor from Rhodobacter sphaeroides, Biochemistry. 52 (2013) 378-391.

[9] F. Richter, I. Fonfara, B. Bouazza, C.H. Schumacher, M. Bratovic, E. Charpentier, et al., Engineering of temperature- and light-switchable Cas9 variants, Nucleic Acids Res. 44 (2016) 10003-10014.

[10] G. Guntas, R.A. Hallett, S.P. Zimmerman, T. Williams, H. Yumerefendi, J.E. Bear, et al., Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) 112-117.

[11] M. Kasahara, T.E. Swartz, M.A. Olney, A. Onodera, N. Mochizuki, H. Fukuzawa, et al., Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and Chlamydomonas reinhardtii, Plant Physiol. 129 (2002) 762-773.

[12] D. Strickland, Y. Lin, E. Wagner, C.M. Hope, J. Zayner, C. Antoniou, et al., TULIPs: tunable, light-controlled interacting protein tags for cell biology, Nat. Methods. 9 (2012) 379-384.

[13] L.J. Bugaj, A.T. Choksi, C.K. Mesuda, R.S. Kane, D.V. Schaffer, Optogenetic protein clustering and signaling activation in mammalian cells, Nat. Methods. 10 (2013) 249-252.

[14] M.J. Kennedy, R.M. Hughes, L.A. Peteya, J.W. Schwartz, M.D. Ehlers, C.L. Tucker, Rapid blue-light-mediated induction of protein interactions in living cells, Nat. Methods. 7 (2010) 973-975.

[15] S. Kainrath, M. Stadler, E. Reichhart, M. Distel, H. Janovjak, Green-light-induced inactivation of receptor signaling using cobalamin-binding domains, Angew. Chem. Int. Ed. Engl. 56 (2017) 4608-4611.

[16] H.M. Strauss, P. Schmieder, J. Hughes, Light-dependent dimerisation in the N-terminal sensory module of cyanobacterial phytochrome 1, FEBS Lett. 579 (2005) 3970-3974.

[17] A. Levskaya, O.D. Weiner, W.A. Lim, C.A. Voigt, Spatiotemporal control of cell signalling using a light-switchable protein interaction, Nature. 461 (2009) 997-1001.

[18] S. Shimizu-Sato, E. Huq, J.M. Tepperman, P.H. Quail, A light-switchable gene promoter system, Nat. Biotechnol. 20 (2002) 1041-1044.

Appendix B

Supplementary Information to Chapter 3

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Supplemenatary Figures

a

	MUSCLE	cealign	cealign-h	super	super-h	FATCAT2.0
E/DRY	Yes	Yes	Yes	Yes	Yes	Yes
CWxP	Yes	Yes	Yes	Yes	Yes	Yes
NPxxY	Yes	**No**	**No**	**No**	**No**	Yes

b

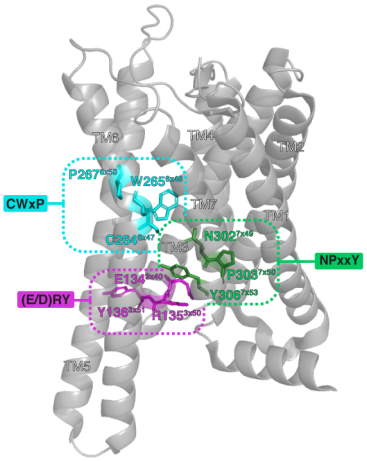


FIGURE B.1: **Alignment of conserved GPCR motifs by FATCAT2.0.(a)** Validation of correct alignment of E/DRY, CWxP and NPxxY motifs for different alignment methods (PDB IDs: 6CMO for rho and 3SN6 for β_2AR). **(b)** Conserved motifs analyzed in **(a)** visualized in a GPCR structure (PDB ID: 6CMO, rho).

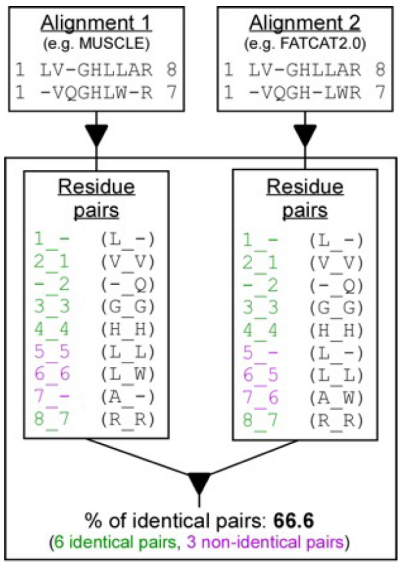
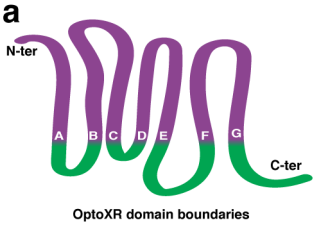


FIGURE B.2: **Procedure for comparison of two alignments.** Two alignments are generated individually (e.g., using MUSCLE and FATCAT2.0) followed by comparison (here, exemplary sequences are shown). For each alignment and each alignment column, the residue pair in the format A_B is determined (where A is the number of the residue in the first sequence that aligns with residue B in the second sequence; e.g. 8_7 is the final residue pair of both alignments above). The residue_pairs are deposited in lists that are then compared to derive the percentage of identical pairs (here, shown in green).

a



b

	# of amino acids changed between alignments			
Domain boundary	β_2AR	A_{2A}R	β_1AR	M_1R
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	0	0	0
E	0	0	1	0
F	0	0	1	0
G	0	0	0	0

FIGURE B.3: **Domain boundaries and differences in boundary position across alignments. (a)** Graphical representation of the seven domain boundaries in OptoXRs. **(b)** For the domain boundaries proposed by Kim et al. (Kim et al., 2005), no differences in boundary residues were observed for sequence alignments (MUSCLE) and structure alignments (FATCAT2.0), with the exception of single amino acid deviations for β_1AR boundary E and F.

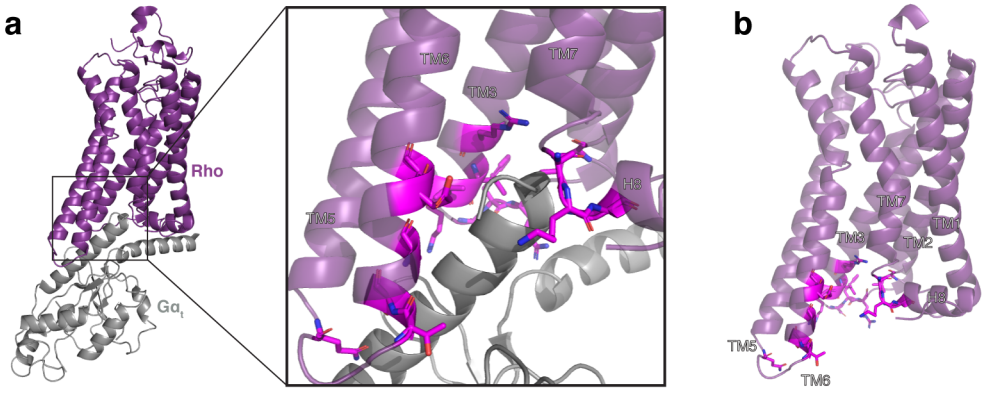


FIGURE B.4: **Rho G-protein binding contacts. (a)** Contacts were determined as described in the main text for rho-G-protein complexes (PDB IDs: 6OYA). Contacts are further listed in Supplementary Table B.2. **(b)** G-protein contacts (pink) extend upwards on TM helices less than in the case of β_2AR (compare to Fig. 3.2b).

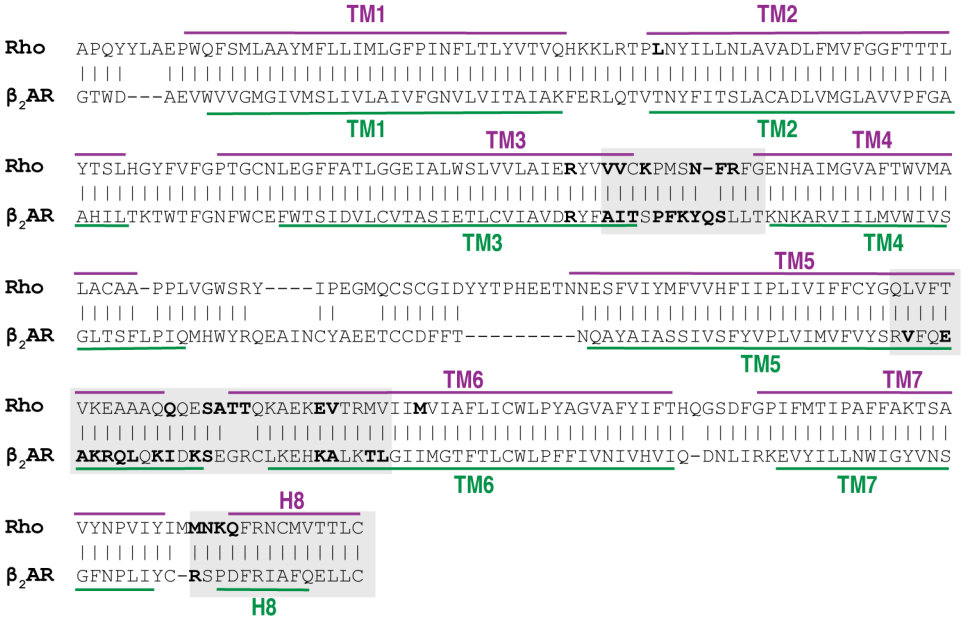


FIGURE B.5: **Structure-based alignment and contact assignment.**Alignment of Rho and β_2AR structures using FATCAT2.0 (PDB IDs: 6OYA (rho) and 3SN6 (β_2AR)). Bold residues: Contacts determined from analysis of multiple receptors (also see upplementary Table B.2). Grey: Exchanged residues to generate Opto-β_2AR-2.0.

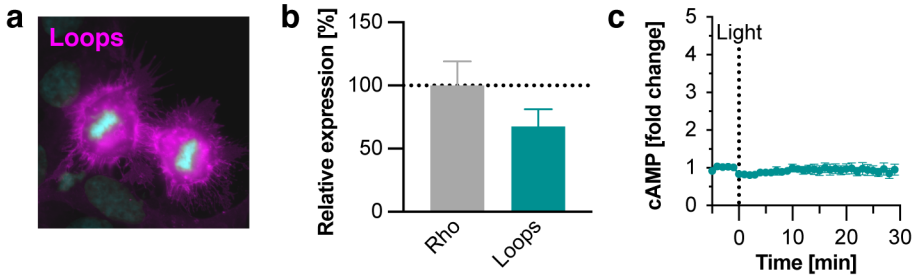


FIGURE B.6: **Characterization of Opto-β_2AR-"Loops".(a)** IF of Opto-β_2AR-"Loops" against N-terminal Rho epitope (α-4D2). **(b)** Expression determined using FC using the same antibody. **(c)**Average time course of cAMP production following stimulation of Opto-β_2AR-"Loops" with light (light dose: 4000 flashes, wavelength: 488+/-6 nm). For (b-c): n=3-9, 3 independent experiments. Data shown as mean ± S.E.M.

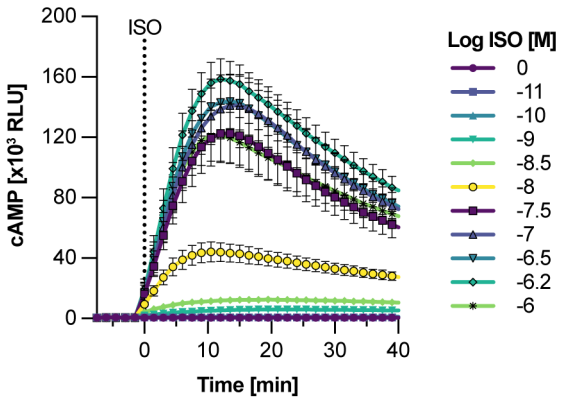


FIGURE B.7: **Time course of cAMP production after β_2AR stimulation.** n=9, 3 independent experiments. Data shown as mean ± S.E.M

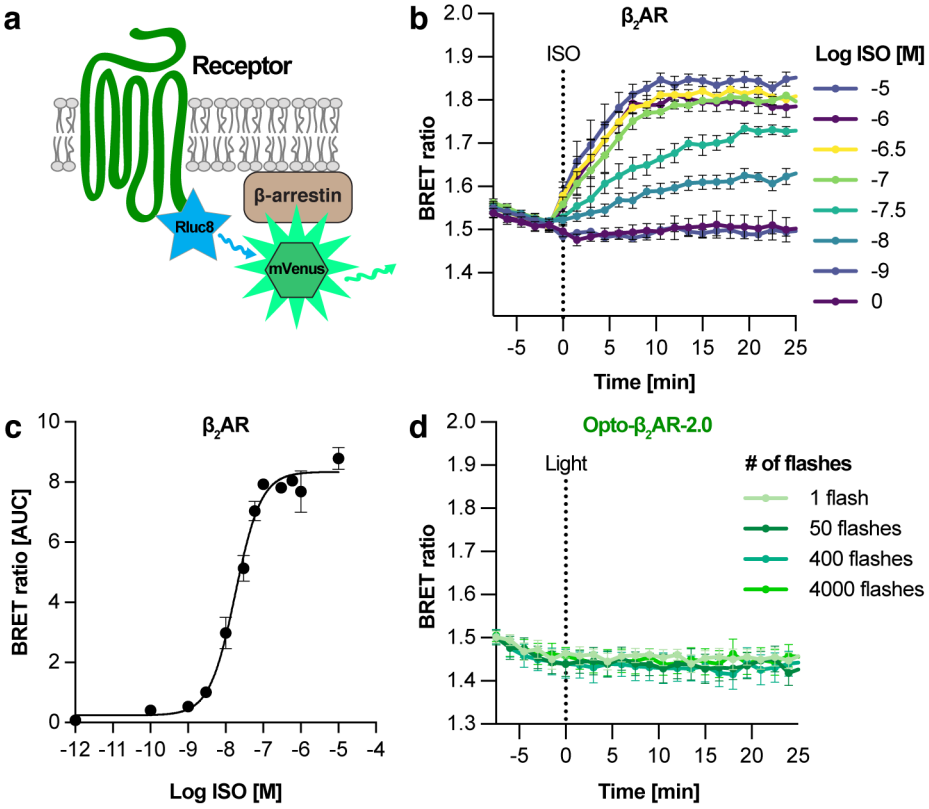


FIGURE B.8: **β-arrestin2 recruitment assays. (a)** Schematic of BRET assay to measure β-arrestin2 recruitment using Rluc8-tagged receptors. **(b)** Average time course of β-arrestin2 recruitment after stimulation of β_2AR. **(c)** Dose-dependent β-arrestin2 recruitment determined by calculating area under the curve (AUC) from (b). **(d)** Average time course of b-arrestin2 recruitment after stimulation of Opto-β_2AR-2.0 with increasing doses of light. For all panels: n=6-9, 2-3 independent experiments. Data shown as mean ± S.E.M..

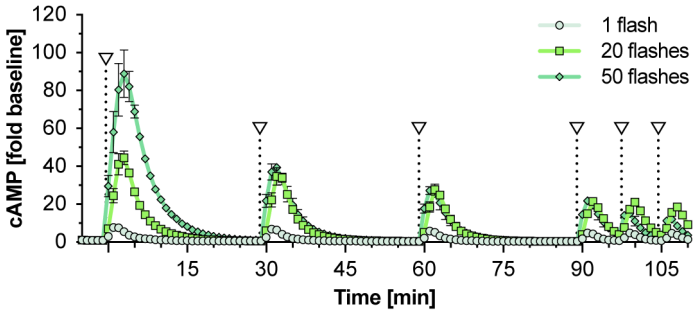


FIGURE B.9: **Repeated stimulation of JellyOp** Average time course of cAMP production following repealed stimulation of JellyOp with different doses of light. n=6, 2 independent experiments. Data shown as mean ± S.E.M.

Supplementary Tables

TABLE B.1: **A comprehensive list of previous OptoXRs with the respective methods for identification of domain boundaries and alignments.** For less common receptors, the following abbreviations were used: Rho: rhodopsin. SW: short-wavelength opsin. JellyOP: opsin of the jellyfish *Carybdea rastonii*. Mel: melanopsin. LW: long-wavelength opsin.

Reference	**Chimeric receptor**	**Domain boundaries identical to Kim *et al.*?**	**Alignment method**
Airan 2009 Nature	Rho:B2AR Rho:A1AR	Yes	Sequence alignment
Oh 2010 JBC	Rho:5-HT1A	Yes	Sequence alignment
Ajith Karunarathne 2013 PNAS	SW:JellyOp	Yes	Sequence alignment
Bailes 2013 PLOSone	Rho:B2AR	Yes	Sequence alignment
Barish 2013 EJP	Rho:uOR1	Yes (with the exception of one residue)	Sequence alignment
Gunyadin 2014 Cell	Rho:D1R	Yes	Sequence alignment
Xu 2014 PNAS	Rho:CXCR4	Yes	Sequence alignment
Siuda 2015 Neuron	Rho:MOR	Yes (with the exception of one residue in ICL2 and two residues in ICL3)	Sequence alignment
Li 2015 MolPsy	Rho:A2AR	Chimeric receptor sequence not available.	Unknown
Van Wyk 2015 PLoS Biol	OPN4:mGluR6	No. The target receptor was a Class C GPCR.	Sequence alignment
McGregor 2016 JNeurophys	OPN4:5-HT2A	No. Origin of boundaries unclear.	Sequence alignment
Hickey 2021 JEB	Rho:OPN4 LW:OPN4	No. The authors focussed on transferring ICLs and thus fewer residues than predicted using the domain boundaries of Kim *et al.*	Sequence alignment

TABLE B.2: **G-protein contacts determined for Rho, β_2AR and A_{2A}R.** Grey: Receptor G-protein binding contacts determined within 4 Å of the Gα protein. Note: Residues of ICL3 are not listed here because they were not resolved in the respective structures used, but were included in Opto-β_2AR-2.0. If available, multiple structures per receptor were analyzed. PDB identifiers were: Rho: 6CMO, 6OYA. β_2AR: 3SN6, 7BZ2. A_{2A}R: 6GDF. Numbering given in GPCRdb numbering for class A.

GPCRdb(A)	Rhodopsin Human	β_2AR (human)	A_{2A}R (human)
TM2			
2x39	L72	T68	T41
TM3			
3x50	R135	R131	R102
3x51	Y136	Y132	Y103
3x52	V137	F133	I104
3x53	V138	A134	A105
3x54	V139	I135	I106
3x55	C140	T136	R107
3x56	K141	S137	I108
ICL2			
34x50	P142	P138	P109
34x51	M143	F139	L110
34x52	S144	K140	R111
34x53	N145	Y141	Y112
34x54	F146	Q142	N113
34x55	R147	S143	G114
TM5			
5x61	L226	V222	I200
5x62	V227	F223	F201
5x63	F228	Q224	L202
5x64	T229	E225	A203
5x65	V230	A226	A204
5x66	K231	K227	R205
5x67	E232	R228	R206
5x68	A233	Q229	Q207
5x69	A234	L230	L208
5x70	A235	Q231	K209
5x71	Q236	K232	Q210

5x72	Q237	I233	M211
5x73	-	D234	E212
5x74	-	K235	S213
5x75	-	S236	-
TM6			
6x23	S240	-	A221
6x24	A241	S262	R222
6x25	T242	K263	S223
6x26	T243	F264	T224
6x27	Q244	C265	L225
6x28	K245	L266	Q226
6x29	A246	K267	K227
6x30	E247	E268	E228
6x31	K248	H269	V229
6x32	E249	K270	H230
6x33	V250	A271	A231
6x34	T251	L272	A232
6x35	R252	K273	K233
6x36	M253	T274	S234
6x37	V254	L275	L235
6x38	I255	G276	A236
6x39	I256	I277	I237
6x40	M257	I278	I238
TM7			
7x55	M308	R328	Y290
7x56	M309	-	R291
H8/C-term			
8x47	N310	S329	I292
8x48	K311	P330	R293
8x49	Q312	D331	E294

TABLE B.3: **Protein sequences of engineered receptors**

Name	**Sequence**
>Opto-b2AR-2.0 (Opto-b2AR-All-In)	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVAITSPFKYQSLLTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGRVFQVA KRQLQKIDKSEGRFHSPNLGQVEQDGRSGHGLRRSSKFCLKEHKALKTLIIMVIAFLICWLPYAGVAFYIFTHQGSD FGPIFMTIPAFFAKTSAVYNPVIYIMRSPDFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDYMGEASGCQLGQEKE SERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPLL
>Opto-b2AR-All-Out	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQFERLQTVTNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVAITSPFKYQSLLTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTV KEQLQKIDKSEGRFHSPNLGQVEQDGRSGHGLRRSSKFCLKEHKALKTVIIMVIAFLICWLPYAGVAFYIFTHQGSD FGPIFMTIPAFFAKTSAVYNPVIYIMRSPDFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDYMGEASGCQLGQEKE SERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPLL
>Opto-b2AR-Loops	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVVVCSPFKYQSLLTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTV KEAAAQQQEGRFHSPNLGQVEQDGRSGHGLRRSSKFCATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQG SDFGPIFMTIPAFFAKTSAVYNPVIYIMMNKQFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDYMGEASGCQLGQE KESERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPLL
>Opto-b2AR-2.0-R335del	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVAITSPFKYQSLLTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGRVFQVA KRQLQKIDKSEGRFHSPNLGQVEQDGRSGHGLRRSSKFCLKEHKALKTLIIMVIAFLICWLPYAGVAFYIFTHQGSD FGPIFMTIPAFFAKTSAVYNPVIYIMSPDFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDYMGEASGCQLGQEKES ERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPLL
>Opto-b2AR-2.0-Blue -T118A-E122D-A292S	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFAALGGDIALWSLVVLAIERYVAITSPFKYQSLLTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGRVFQVA KRQLQKIDKSEGRFHSPNLGQVEQDGRSGHGLRRSSKFCLKEHKALKTLIIMVIAFLICWLPYAGVAFYIFTHQGSD FGPIFMTIPSFFAKTSAVYNPVIYIMRSPDFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDYMGEASGCQLGQEKE SERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPL
>Opto-A2AR-2.0	MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYILL NLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLGGEIALWSLVVLAIERYVAIRIPLRYNGLVTENHA IMGVAFTWVMALACAAPPLVGWSRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQIFLAA RRQLKQMESQPLPGERARSTLQKEVHAAKSLIIMVIAFLICWLPYAGVAFYIFTHQGSDFGPIFMTIPAFFAKTSAV YNPVIYIMRIREFRQTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVWANGSAPHPERRPN GYALGLVSGGSAQESQGNTGLPDVELLSHELKGVCPEPPGLDDPLAQDGAGVS

TABLE B.4: **DNA sequences of engineered receptors**

Name	**Sequence**
>Opto-b2AR-2.0 (Opto-b2AR-All-In)	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGCATAAAAAACTGCGCACCCCGCTCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCACCTTGGGCGGTGAAATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGCGATTACATCGCCATTCAAGTACCAGAGCCTGCTGACCGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGAAGGGTGTTCCAGGTGGCCAAAAGGCAGCTCCAGAAGATAGACAAATCTGAGGGA AGATTCCACTCCCCAAACCTCGGCCAGGTGGAGCAGGATGGGCGGAGTGGGCACGGACTCCGAAGGTCCTCCAAGTTCTGC TTGAAGGAGCACAAAGCCCTCAAAACCCTGATCATCATGGTCATCGCTTTCCTAATCTGCTGGCTGCCCTACGCTGGGGTG GCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACCATCCCGGCTTTCTTTGCCAAGACTTCT GCCGTCTACAACCCCGTCATCTACATCATGCGCAGCCCGGATTTTCGCATTGCCTTCCAGGAGCTTCTATGCCTCCGCAGG TCCTCTTCAAAAGCCTATGGGAACGGCTACTCCAGCAACAGTAATGGCAAAACAGACTACATGGGGGAGGCGAGTGGATGT CAGCTGGGGCAGGAAAAAGAAAGTGAACGGCTGTGTGAGGACCCCCCAGGCACGGAAAGCTTTGTGAACTGTCAAGGTACT GTGCCTAGCCTTAGCCTTGATTCCCAAGGGAGGAACTGTAGTACAAATGACTCACCGCTGCTGTAA
>Opto-b2AR-All-Out	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGTTTGAACGACTGCAAACGGTTACCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCACCTTGGGCGGTGAAATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGCGATTACATCGCCATTCAAGTACCAGAGCCTGCTGACCGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGACAGCTCGTTTTCACCGTAAAGGAACAGCTCCAGAAGATAGACAAATCTGAGGGA AGATTCCACTCCCCAAACCTCGGCCAGGTGGAGCAGGATGGGCGGAGTGGGCACGGACTCCGAAGGTCCTCCAAGTTCTGC TTGAAGGAGCACAAAGCCCTCAAAACCGTAATCATCATGGTCATCGCTTTCCTAATCTGCTGGCTGCCCTACGCTGGGGTG GCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACCATCCCGGCTTTCTTTGCCAAGACTTCT GCCGTCTACAACCCCGTCATCTACATCATGCGCAGCCCGGATTTTCGCATTGCCTTCCAGGAGCTTCTATGCCTCCGCAGG TCCTCTTCAAAAGCCTATGGGAACGGCTACTCCAGCAACAGTAATGGCAAAACAGACTACATGGGGGAGGCGAGTGGATGT CAGCTGGGGCAGGAAAAAGAAAGTGAACGGCTGTGTGAGGACCCCCCAGGCACGGAAAGCTTTGTGAACTGTCAAGGTACT GTGCCTAGCCTTAGCCTTGATTCCCAAGGGAGGAACTGTAGTACAAATGACTCACCGCTGCTGTAA
>Opto-b2AR-Loops	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGCATAAAAAACTGCGCACCCCGCTCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCACCTTGGGCGGTGAAATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGTGGTATGCTCGCCATTCAAGTACCAGAGCCTGCTGACCGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGACAACTCGTCTTCACGGTAAAGGAAGCCGCTGCCCAGCAGCAAGAGGGGCGATTC CATTCACCGAATTTGGGCCAGGTGGAACAAGACGGACGGTCTGGGCATGGACTTAGACGATCTTCTAAATTCTGTGCTACT ACTCAAAAGGCTGAGAAGGAGGTTACAAGGATGGTAATCATCATGGTCATCGCTTTCCTAATCTGCTGGCTGCCCTACGCT GGGGTGGCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACCATCCCGGCTTTCTTTGCCAAG ACTTCTGCCGTCTACAACCCCGTCATCTACATCATGATGAACAAGCAGTTCCGCATTGCCTTCCAGGAGCTTCTATGCCTC CGCAGGTCCTCTTCAAAAGCCTATGGGAACGGCTACTCCAGCAACAGTAATGGCAAAACAGACTACATGGGGGAGGCGAGT GGATGTCAGCTGGGGCAGGAAAAAGAAAGTGAACGGCTGTGTGAGGACCCCCCAGGCACGGAAAGCTTTGTGAACTGTCAA GGTACTGTGCCTAGCCTTAGCCTTGATTCCCAAGGGAGGAACTGTAGTACAAATGACTCACCGCTGCTGTAA
>Opto-b2AR-2.0 R335del	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGCATAAAAAACTGCGCACCCCGCTCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCACCTTGGGCGGTGAAATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGCGATTACATCGCCATTCAAGTACCAGAGCCTGCTGACCGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGAAGGGTGTTCCAGGTGGCCAAAAGGCAGCTCCAGAAGATAGACAAATCTGAGGGA AGATTCCACTCCCCAAACCTCGGCCAGGTGGAGCAGGATGGGCGGAGTGGGCACGGACTCCGAAGGTCCTCCAAGTTCTGC TTGAAGGAGCACAAAGCCCTCAAAACCCTGATCATCATGGTCATCGCTTTCCTAATCTGCTGGCTGCCCTACGCTGGGGTG GCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACCATCCCGGCTTTCTTTGCCAAGACTTCT GCCGTCTACAACCCCGTCATCTACATCATGAGCCCGGATTTTCGCATTGCCTTCCAGGAGCTTCTATGCCTCCGCAGGTCC TCTTCAAAAGCCTATGGGAACGGCTACTCCAGCAACAGTAATGGCAAAACAGACTACATGGGGGAGGCGAGTGGATGTCAG

	CTGGGGCAGGAAAAAGAAAGTGAACGGCTGTGTGAGGACCCCCCAGGCACGGAAAGCTTTGTGAACTGTCAAGGTACTGTG CCTAGCCTTAGCCTTGATTCCCAAGGGAGGAACTGTAGTACAAATGACTCACCGCTGCTGTAA
>Opto-b2AR-2.0-Blue -T118A-E122D-A292S	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGCATAAAAAACTGCGCACCCCGCTCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCgCCTTGGGCGGTGAtATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGCGATTACATCGCCATTCAAGTACCAGAGCCTGCTGACCGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGAAGGGTGTTCCAGGTGGCCAAAAGGCAGCTCCAGAAGATAGACAAATCTGAGGGA AGATTCCACTCCCCAAACCTCGGCCAGGTGGAGCAGGATGGGCGGAGTGGGCACGGACTCCGAAGGTCCTCCAAGTTCTGC TTGAAGGAGCACAAAGCCCTCAAAACCCTGATCATCATGGTCATCGCTTTCCTAATCTGCTGGCTGCCCTACGCTGGGGTG GCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACCATCCCGtCTTTCTTTGCCAAGACTTCT GCCGTCTACAACCCCGTCATCTACATCATGCGCAGCCCGGATTTTCGCATTGCCTTCCAGGAGCTTCTATGCCTCCGCAGG TCCTCTTCAAAAGCCTATGGGAACGGCTACTCCAGCAACAGTAATGGCAAAACAGACTACATGGGGGAGGCGAGTGGATGT CAGCTGGGGCAGGAAAAAGAAAGTGAACGGCTGTGTGAGGACCCCCCAGGCACGGAAAGCTTTGTGAACTGTCAAGGTACT GTGCCTAGCCTTAGCCTTGATTCCCAAGGGAGGAACTGTAGTACAAATGACTCACCGCTGTAA
>Opto-A2AR-2.0	ATGAACGGGACCGAGGGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCGTGGTGCGCAGCCCCTTCGAGGCCCCG CAGTACTACCTGGCGGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGTTCCTGCTGATCATGCTTGGCTTCCCCATC AACTTCCTCACGCTGTACGTCACCGTGCAGCATAAAAAACTGCGCACCCCGCTCAACTACATCCTGCTCAACCTGGCCGTG GCCGACCTCTTCATGGTGTTCGGGGGCTTCACCACCACCCTCTACACCTCTCTGCACGGGTACTTCGTCTTTGGGCCCACG GGCTGCAACCTGGAGGGCTTCTTTGCCACCTTGGGCGGTGAAATTGCACTGTGGTCCTTGGTGGTCCTGGCCATCGAGCGG TACGTGGCGATTAGGATACCGCTGCGGTATAACGGGCTTGTTACGGAAAACCATGCCATCATGGGCGTCGCCTTCACCTGG GTCATGGCTCTGGCCTGTGCCGCGCCCCCCCTCGTCGGCTGGTCCAGGTACATCCCGGAGGGCATGCAGTGCTCGTGCGGG ATTGACTACTACACGCCCCACGAGGAAACCAACAATGAGTCGTTCGTCATCTACATGTTCGTGGTCCACTTCATCATCCCC CTGATTGTCATATTCTTCTGCTACGGACAGATATTCCTGGCGGCAAGACGCCAACTGAAGCAGATGGAAAGCCAACCGTTG CCCGGGGAACGCGCGCGGAGCACCCTTCAAAAAGAAGTCCATGCAGCCAAGAGTCTCATCATCATGGTCATCGCTTTCCTA ATCTGCTGGCTGCCCTACGCTGGGGTGGCGTTCTACATCTTCACCCATCAGGGCTCTGACTTTGGCCCCATCTTCATGACC ATCCCGGCTTTCTTTGCCAAGACTTCTGCCGTCTACAACCCCGTCATCTACATCATGCGGATTCGGGAGTTCAGACAGACC TTTAGAAAAATTATAAGATCTCACGTCCTGAGGCAACAAGAACCCTTTAAAGCTGCGGGGACTTCTGCCCGGATTTTGGCG GCGCATGGGAGCGATGGTGAACAGGTGTCCCTCAGATTGAACGGGCATCCTCCGGGGGGTGTGGGCTAATGGTAGTGCTCCT CACCCTGAAAGACGACCTAACGGGTACGCACTGGGACTTGTGTCAGGTGGGTCTGCCCAGGAAAGCCAAGGTAACACGGGG CTTCCTGACGTAGAGTTGTTGTCTCATGAGCTGAAGGGTGTCTGCCCTGAACCTCCTGGTCTGGACGACCCGCTCGCACAG GATGGAGCAGGTGTTAGTTAA