学位論文

Development of Optical Methods for the Analysis of Membrane Receptor Trafficking Regulated by β-Arrestin

(βアレスチンを介した膜受容体の細胞内輸送を解析する光学的分析法の開発)

平成 29 年 7 月　博士（理学）申請

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Abstract

Intracellular trafficking of G protein-coupled receptors (GPCRs) controls their localization and degradation, which affects a cell's ability to adapt to extracellular stimuli. Due to the significant roles of GPCRs in living cells and tissues, the perturbation of trafficking induces important diseases such as neurodegenerative, psychiatric, immune disorders, cardiovascular, gastrointestinal, renal, and pulmonary diseases, and cancer. Therefore, the analysis of the trafficking mechanism is necessary for drug development and therapeutics. However, the conventional methods are not sufficient for comprehensive understanding of the regulation of GPCR trafficking.

Herein, I demonstrate an optogenetic method using an optical dimerizer, cryptochrome (CRY) and its partner protein (CIB), to analyze the trafficking mechanisms of GPCRs and their regulatory proteins. Temporally controlling the interaction between β-arrestin and β2-adrenergic receptor (ADRB2) reveals that the duration of the β-arrestin-ADRB2 interaction determines the trafficking pathway of ADRB2. Remarkably, the phosphorylation of ADRB2 by G protein-coupled receptor kinases is unnecessary to trigger clathrin-mediated endocytosis, and β-arrestin interacting with unphosphorylated ADRB2 fails to activate mitogen-activated protein kinase (MAPK) signaling, in contrast to the ADRB2 agonist isoproterenol. Temporal control of β-arrestin-GPCR interactions will enable the investigation of the unique roles of β-arrestin and the mechanism by which it regulates β-arrestin-specific trafficking pathways of different GPCRs.

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Chapter 1

General Introduction

1-1. Cell Membrane Receptors

Cells response to various extracellular stimuli such as nutrients, cytokines, hormones, and other chemical compounds. These biomolecules bind to proteins called "receptor" on cell surface and inside cells[1-4]. The receptors on the cell surface "cell membrane receptors" mediate information of surrounding circumstance inside the cells by producing second messengers such as cyclic AMP (cAMP), cyclic GMP, calcium ion, inositol trisphosphate, diacylglycerol, and nitric oxide[5-14] (**Figure 1-1**). The second messengers activate numerous downstream signal pathways. The cells also inactivate an excessive activity of cell membrane receptors through some approaches such as post-translational modification, sequestration, degradation, and suppression of transcription of the receptors. The precise regulation of activation and inactivation of the cell membrane receptors generates various patterns of the downstream signals, which is important for integration of complicated biological events such as proliferation, differentiation, adaption, and apoptosis in living tissues and animals[15-18].

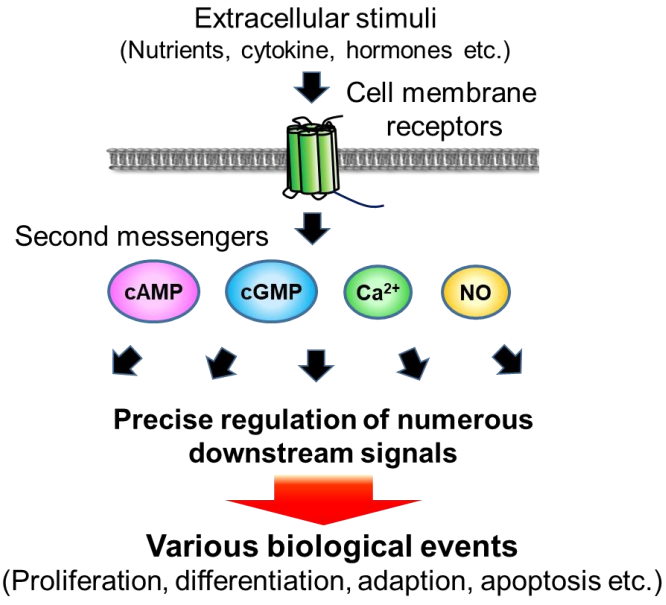

Figure 1-1. Regulation of biological events through cell membrane receptors

1-2. G protein-coupled Receptor

G protein-coupled receptor (GPCR) belongs to the largest family of the cell membrane receptors. More than 800 members of GPCRs are coded in human genome and expressed in most of tissues, which means that GPCRs regulate a wide range of cellular events sustaining ordinary lives of animals[19-23]. Perturbation of the GPCR systems leads to crucial diseases such as neurodegenerative, psychiatric, immune disorders, cardiovascular, gastrointestinal, renal, and pulmonary diseases, and cancer[24-33]. Thus, the half of drug in current use targets GPCRs.

Specific ligands are necessary for activation of GPCRs. Ligand-bound GPCR changes its conformation on the cell surface[34,35], which promotes recruitment of G proteins to GPCRs (**Figure 1-2**). GPCR-bound G proteins catalyze guanin nucleotide exchange on the α subunit of G proteins (Gα)[36]. Gα activates effector proteins such as adenylyl cyclase and phospholipase, which modulates second messengers including cAMP and Ca^{2+}. The second messengers activate protein kinases A/C, which promotes activation of Raf proteins. The Raf proteins phosphorylate mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)[37-41]. The phosphorylated ERK1/2 is transported in the nucleus, and activates nuclear substrates such as nuclear transcription factors, cytoskeletal proteins, signaling proteins and receptors.

To desensitize the activated GPCRs, G protein-coupled receptor kinases (GRKs) are activated by the active state of GPCR[42-44]. GRKs phosphorylate serine and threonine residues on C-terminus of GPCRs, which increases an affinity of GPCR with a scaffold protein, β-arrestin. β-arrestin is a scaffold protein and plays a key role of endocytosis of membrane receptors including GPCRs and growth factor receptors[45,46]. Upon the

binding to GPCRs, β-arrestin changes its conformation[47,48]. GPCR-bound β-arrestin exposes its C-terminus to the intracellular compartment, which promotes interaction of β-arrestin with endocytic proteins such as clathrin and AP-2[49,50]. GPCR-β-arrestin complexes are recruited to clathrin-coated pits (CCPs) by clathrin adaptor proteins at the cell surface, and they are released from the membrane by GTPase, dynamins[51]. Ligand-activated GPCRs are sequestrated from the cell surface by endocytosis.

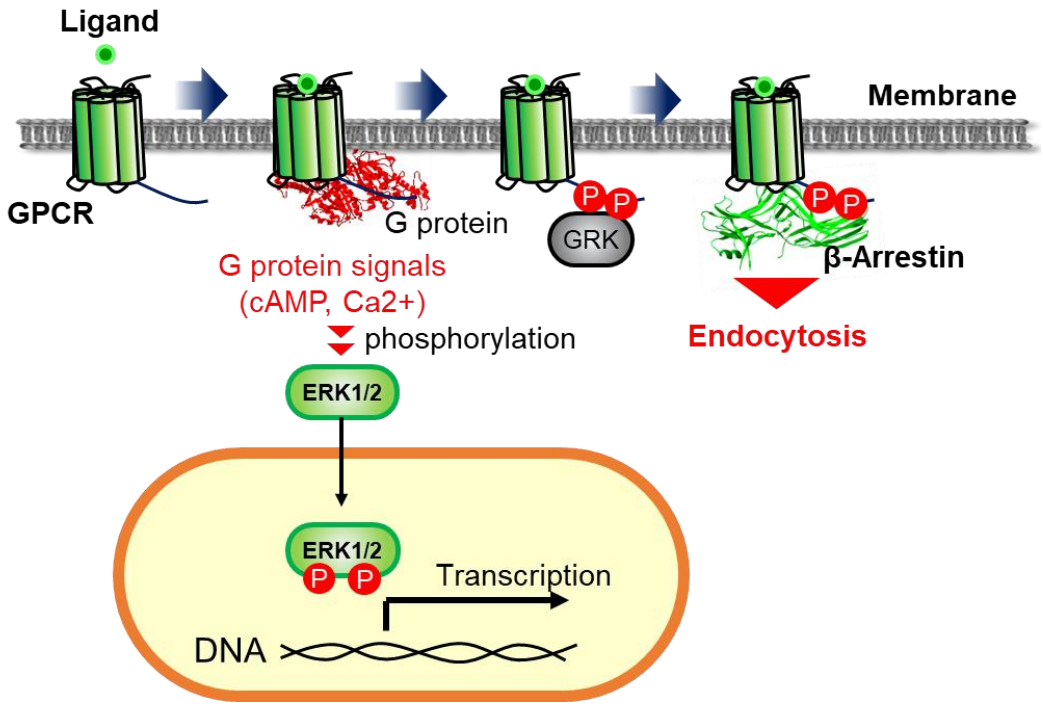

Figure 1-2. Model of regulation of GPCR on cell surface.

GPCRs activate G protein signals upon the stimulation of ligand. The G protein signals lead to the transcription of related genes through downstream molecules such as ERK1/2 and p90RSK. GRKs induce phosphorylation at serine and threonine residues in the cytosolic domain of GPCRs. The phosphorylated GPCRs are endocytosed with β-arrestin.

1-3. Intracellular Trafficking of GPCR

Endocytosed GPCRs are delivered to endosomes, and sorted to lysosomes (degradative pathway) or cell membrane (recycling pathway)[52-57] (**Figure 1-3**). GPCRs in recycling pathway activate the signal pathways again on the cell surface, while the degradation of GPCR leads to suppression of the signaling. Therefore, it is valuable to investigate the trafficking mechanism for understanding the regulation of GPCR signaling.

In the previous reports, the post-endocytic fate of GPCRs is largely influenced by post-translational modifications of their cytosolic domain such as phosphorylation and ubiquitination (**Figure 1-4**)[52-57].

Phosphorylation of GPCRs has been characterized as the crucial factor not only for endocytosis but also for sorting of GPCRs[58-60]. The phosphorylation at serine and threonine residues in the cytosolic domain of GPCRs is related to the stability of the interaction between β-arrestin and GPCRs. In other reports, GPCRs stably interacting with β-arrestin are retained in the cytosolic compartment, whereas GPCRs that immediately dissociate from β-arrestin are rapidly recycled back to the cell membrane[19,61]. These previous reports suggest that the stability of the GPCR-β-arrestin interaction has a significant role on the trafficking.

Ubiquitination of GPCRs has been investigated in previous works[62-64]. Ubiquitin is a small protein (8 kDa) and covalently binds to lysine residues of various proteins. The general functions of ubiquitination are degradation for cell cycle regulation, tumor suppression, and signal transduction. In the trafficking pathway of GPCRs, ubiquitination functions as a sorting signal to lysosomes[62-64]. After ligand stimulation, a GPCR-β-arrestin complex interacts with E3 ubiquitin ligases such as Ste2 and Nedd4, which promotes ubiquitination of GPCR. Although the ubiquitin molecules are removed

form the complex by deubiquitinase (USP)[65,66], it is not clear what kind of factors trigger the deubiquitination. The authors in ref. 65 and 66 proposed a model where GPCR-β-arrestin interaction inhibits the activity of USPs. Dissociation of β-arrestin from GPCRs promotes the activity of USP. Their model also suggests the significant roles of the interaction of β-arrestin with GPCRs on the intracellular trafficking of GPCRs.

These previous reports suggest a possibility that the GPCR-β-arrestin interaction is a determinant of their trafficking. However, there is no direct evidence to prove their significance due to a lack of an analysis method to manipulate directly the interaction between GPCRs and β-arrestin.

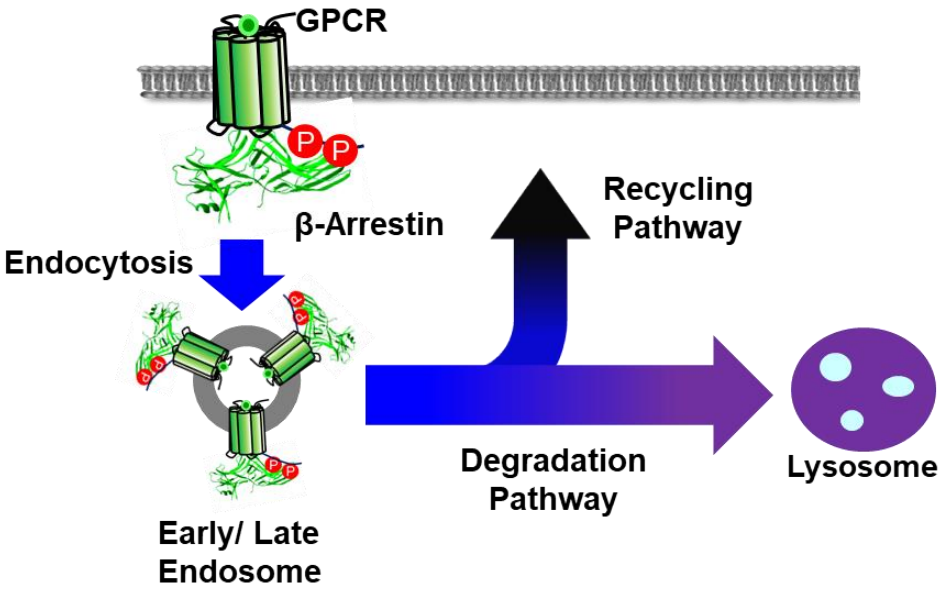

Figure 1-3. Intracellular Trafficking of GPCR.

GPCRs are delivered to early and late endosomes after endocytosis. Some GPCRs are recycled back to the cell membrane for reuse, the other GPCRs are sorted to lysosomes for degradation.

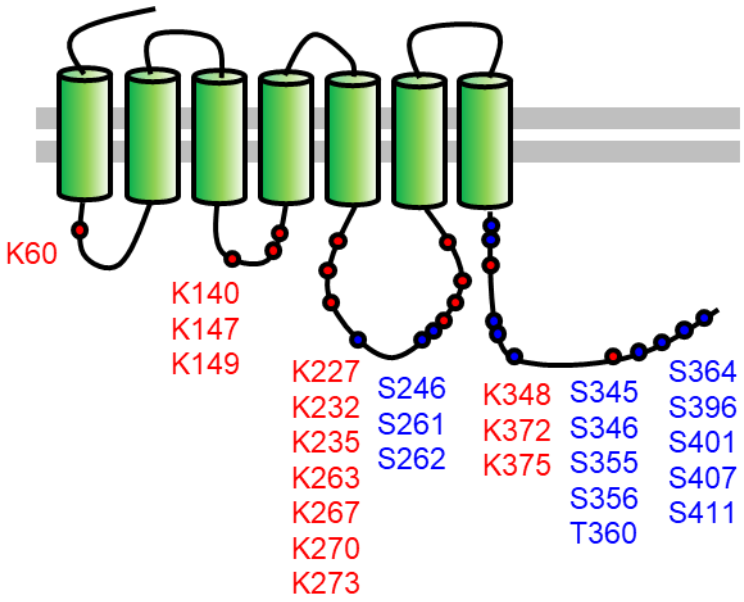

Figure 1-4. Amino acid residues at the cytosolic domains of β2-adrenergic receptor

Upon stimulation of ligands, the serine and threonine residues in the intracellular domain (Blue) are phosphorylated by the GRKs. The lysine residues (Red) are ubiquitinated by ubiquitin ligases for degradation.

1-4. β-Arrestin-dependent Signal Pathway in the Trafficking

In the classical model of GPCR regulation, biological functions of β-arrestin were restricted to desensitization of activated GPCRs and initiation of intracellular trafficking. However, the recent works for the past 15 years have provided a novel framework of GPCR regulation by adding a new function of β-arrestin as a signal transducer (**Figure 1-5**). GPCR-bound β-arrestin forms a complex with MAPK/ERK[67-72]. In contrast to the G protein-dependent activation of MAPK/ERK signaling, the phosphorylated ERK1/2 in the β-arrestin-dependent manner does not translocate to nucleus. Thus, the phosphorylated ERK1/2 activates cytosolic substrates, not nuclear substrates. The β-arrestin-dependent activation of signaling leads to anti-apoptotic signaling[73], and influences the dopamine-associated behaviors[74]. In addition, other signaling such as Akt and JNK signal pathways are activated by β-arrestin dependent manners[75,76]. Although the significance of β-arrestin as a signal transducer has been suggested, there remain basic questions; how is the signal activated through β-arrestin, what kinds of differences exist in the signal activation among various GPCRs.

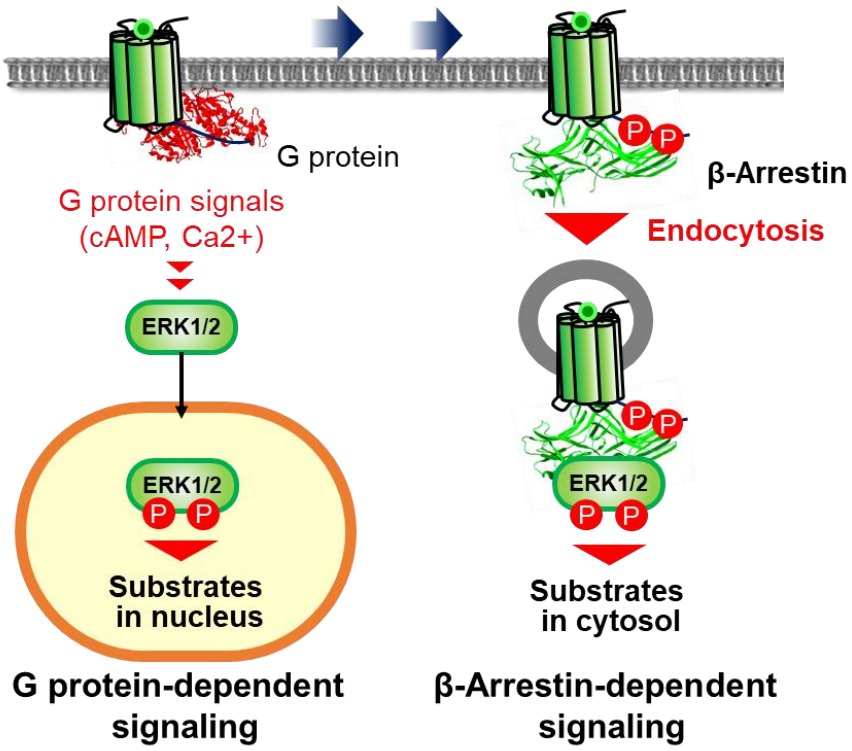

Figure 1-5. G protein- and β-arrestin-dependent activation of MAPK/ERK signaling

Phosphorylated ERK1/2 in G protein signaling activates the substrates in the nucleus, whereas phosphorylated ERK1/2 in β-arrestin signaling activates the substrates in the cytosol.

1-5. Conventional Methods to Analyze Intracellular Trafficking of GPCRs

The regulation of intracellular trafficking of GPCR has been investigated by mutant analysis of GPCR and β-arrestin. GPCR mutants at the phosphorylation sites showed decreases in the efficiency of endocytosis of the mutated GPCR[60]. In addition, the mutants showed reduced activities of β-arrestin-dependent MAPK signal[58]. Furthermore, mutation at the ubiquitination sites suppressed degradation of the GPCR mutants after ligand stimulation[77]. Additionally, a β-arrestin mutant, interacting stably with GPCRs, induced increases in degradation of GPCRs[65,78]. The approach has suggested the significant roles of the specific residues on their trafficking. The approaches are less effective to prove the significance of the GPCR-β-arrestin interaction.

To manipulate directly the GPCR-β-arrestin interaction, chemical dimerizers FKBP-FRB were utilized[79,80] (**Figure 1-6**). FRB-fused β-arrestin interacted with FKBP-fused GPCRs upon the stimulation of rapamycin, which induced endocytosis of GPCRs and activation of MAPK signaling. The results suggest that artificial interaction of β-arrestin with GPCR can initiate trafficking. However, it was reported that rapamycin was hardly removed from FKBP by wash treatment[81], which suggests that the FKBP-FRB system has an issue of their reversibility. The system is applicable for investigation of the trafficking pathway under continuous interaction of β-arrestin with GPCR, but is difficult to investigate the trafficking pathway after dissociation of β-arrestin from GPCRs. To overcome the problem of reversibility, a different dimerization system is necessary.

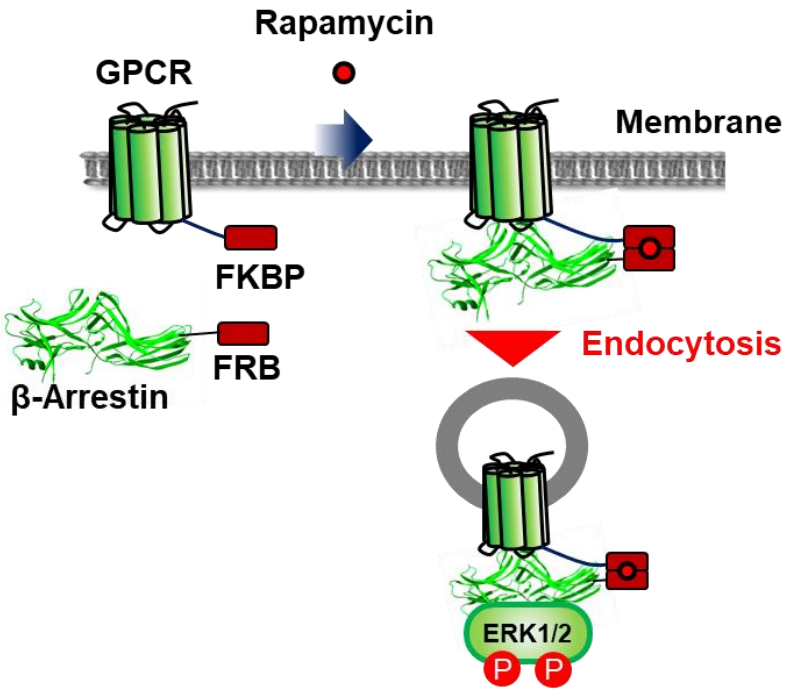

Figure 1-6. The GPCR-β-arrestin interaction system using chemical dimerizers

Rapamycin stimulation induced the interaction of FKBP and FRB, which triggers the interaction between GPCR and β-arrestin. GPCR-β-arrestin complexes are endocytosed and activate the MAPK/ERK signaling.

1-6. Photo-dimerizers for Analysis of Biological Phenomena

Optogenetics is a technology having impacts on the fields of neuroscience and cardiology[82-87]. Light stimulation enables spatiotemporal activation and perturbation of target molecules in living cells, which leads to analysis of spatiotemporal regulation in specific signaling. Ion channels were firstly applied as an optogenetic tool for unveiling the regulation of neural circuits[88-90]. An expanding number of optogenetic tools have been developed for the past 10 years[91-93]. Photodimerizing proteins, one of many photoreceptor derived tools, are often used to control the interactions of specific proteins in living cells. Cryptochrome (CRY) and cryptochrome-interacting basic-helix-loop-helix 1 (CIB) are flavin-based photoreceptors derived from *Arabidopsis thaliana*[94]. Upon irradiation of blue light, a flavin adenine dinucleotide (FAD) in CRY is converted to oxidized FAD, resulting in a conformational change of CRY. The activated CRY interacts immediately with CIB (**Figure 1-7**). An attractive feature of this system is reversible interaction between CRY and CIB. The dimerization occurs 1 second after starting light irradiation, while dissociation of CRY from CIB is completed within 10 min in a dark condition. The property of CRY-CIB system has been utilized for controlling biological phenomena such as metabolism, cell motility, transcription, organelle transport, and MAPK activation in living cells[95-101]. The CRY-CIB system enables us precise control the interaction of GPCR and β-arrestin.

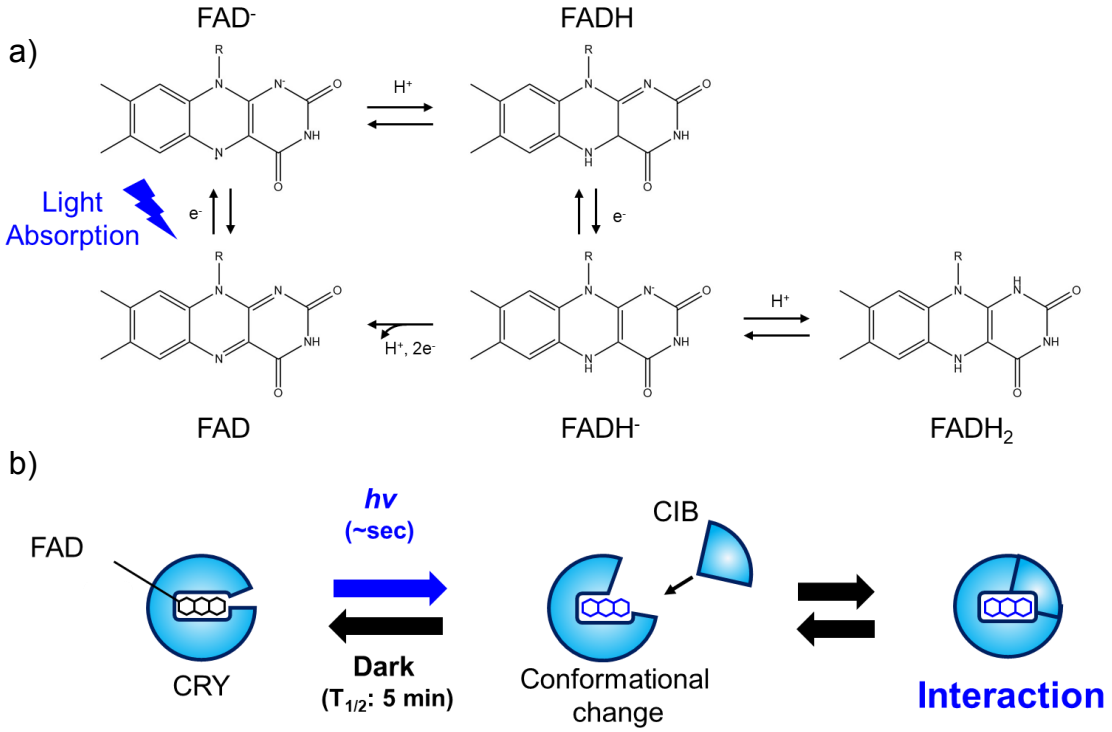

Figure 1-7. Light-induced interaction of photodimerizers

a) FAD conversion after blue light absorption. FAD is converted to oxide FAD upon absorption of blue light. The oxide FAD induces the conformation of CRY for interacting with CIB. b) Light-induced interaction of CRY-CIB. The interaction of CRY and CIB occurs 1 second after light irradiation, while the CRY dissociates from CIB 10 after incubation in dark.

1-7. Imaging-based Analysis of Protein Localization

1-7-1. Fluorescence Microscope

To observe biological phenomena in cells and tissues, many types of microscopy have long been developed. Light microscopy has been used worldwide and provided us valuable information about mechanisms governing cells and tissues[102,103]. One of the most widely used light microscopy is widefield fluorescence microscopy. In the microscopy, excitation light emitted from lasers or lamps reaches to a sample through an objective lens (**Figure 1-8**). Emission light (fluorescence) from the sample is detected in detectors such as a photomultiplier tube (PMT) and a charge coupled device (CCD). The widefield fluorescence microscopy has an advantage for acquiring bright images with a short exposure time, whereas it lacks at spatial resolution in z direction. A pinhole system has produced a remarkable progress of the performance of widefield fluorescence microscopy. The microscope with the pinhole system in fronts of lasers and detectors (termed confocal microscopy) has an advantage in the signal-to-noise ratio because the pinhole blocks the fluorescence from the region out of the focus, which enables us acquiring images with high signal-to-noise ratio and high resolution on z axis.

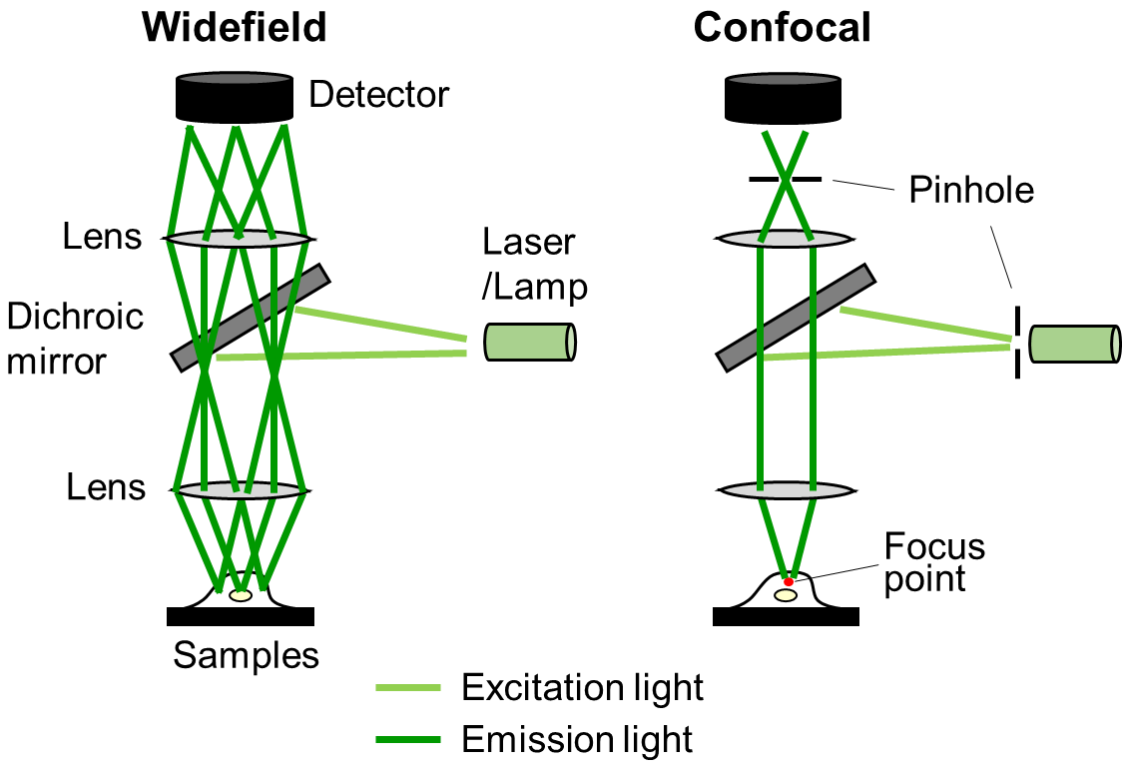

Figure 1-8. Confocal microscopy

Excitation light from the lasers is irradiated to a sample. Emission light from the sample is detected in a detector. The widefield microscopy has an advantage of rapid imaging acquisition. The confocal microscopy enables us acquiring images with high resolution due to the function of pinholes.

1-7-2. Fluorescent Proteins and Dyes for Labeling Protein

Labeling of specific proteins and organelles is necessary for observation under light microscopy. Immunostaining is a classical approach to label specific proteins in fixed cells and tissues using fluorescein-conjugated antibodies. Although immunostaining is effective for detection endogenous proteins, the method is not applicable for living cells and tissues due to a necessity of penetration procedures for inducing antibodies inside the cells. To overcome the issue, many fluorescence proteins such as green fluorescence proteins have been utilized for analyzing the dynamics of target proteins[104,105]. In addition, novel protein-labeling tags termed SNAP-tag and CLIP-tag have been developed[106] (**Figure 1-9**). The tags bind covalently to a wide variety of benzylguanin-modified fluorophore, which enables that the tag-fused proteins can be stained with a wide variety of fluorescent dyes in living cells. The dyes for SNAP/CLIP-tag are classified into two classes; cell membrane-permeable and cell membrane-impermeable dyes. The dyes are separately used for analysis of whole proteins or proteins on the cell surface.

Recent studies demonstrated combinatorial usages of fluorescence imaging and photo-dimerization systems to analyze biological phenomena such as cell motility, formation of lamellipodia, and trafficking of organelles. However, it must be noted that the wavelength available for fluorescence imaging is restricted by the absorption wavelength of photo-dimerizers. In case of CRY-CIB system, FAD in CRY is activated by 400-515 nm wavelength of lights, which means that cyan, green, yellow fluorescence proteins and dyes are not applicable for imaging because excitation of these fluorescent proteins activates the FAD. Thus, we need to observe target proteins using red and near-infrared fluorescent proteins and dyes.

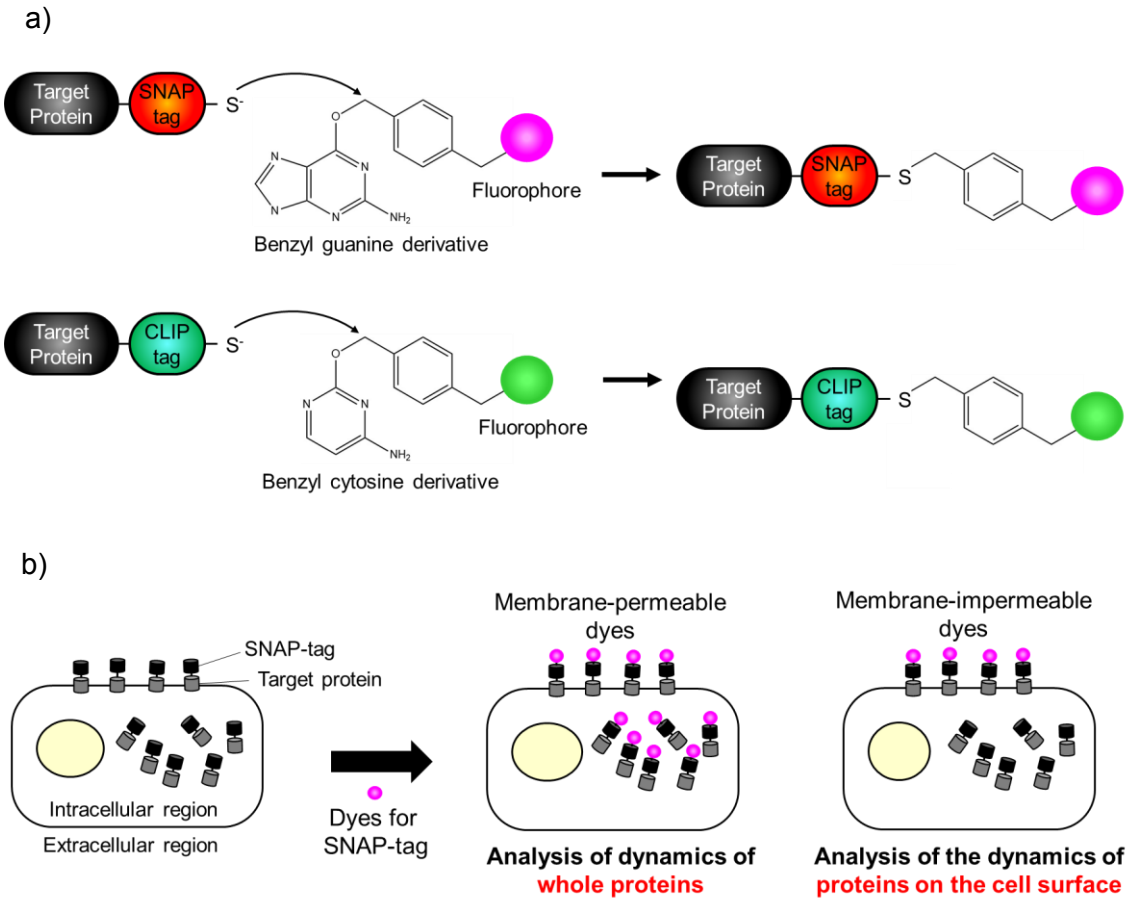

Figure 1-9. Labeling of target proteins using fluorescent dyes for SNAP/CLIP-tag

a) Principles of protein labeling using fluorescent dyes for SNAP/CLIP-tag. Benzylguanine/Benzylcytocine-fused fluorescence dyes bind covalently to SNAP/CLIP-tags in living cells. b) Labeling of SNAP-tag using cell-membrane permeable or impermeable dyes. The permeable dyes visualize whole proteins expressed in living cells, which is suitable for analysis of dynamics of whole proteins. The impermeable dyes label the tags exposed to the extracellular domain, which enables analysis of dynamics of the proteins on the cell surface.

1-7-3. Imaging-based Analysis of Co-localization of Proteins

Local information of proteins is valuable to understand the biological processes because localization of proteins is closely related to their functions. Observation of the proteins under a fluorescence microscope is a routine approach in cell biology. Numerous works have demonstrated the intracellular localization of their target proteins using merged images of several different proteins and specific dyes. Although merged images are effective for qualitative evaluation of the colocalization of proteins, it is hard to understand quantitatively a degree of co-localization. To overcome the problem, quantitative analysis methods have been developed[107-109] (**Figure 1-10**). Persons' colocalization efficient (PCC) and Manders' colocalization coefficient (MCC) are mainly used for indicating the degree of co-localization. PCC provides correlation of fluorescent intensities in two channels. The value ranges from -1 to 1, where -1 denoting for complete negative correlation, 0 standing for no correlation, and 1 denoting complete positive correlation. The PCC is effective to show overall association of two images and contained in many programs for imaging analysis. However, the PCC is not suitable for indicating the fraction of merged proteins. In contrast, MCC is a well-established measure for proportion of colocalized proteins. The MCC value has a range from 0 to 1, where 0 denoting no merged region, and 1 denoting a complete match of two images. The MCC value enables us direct comparison of the proportion of the colocalized proteins[110-112].

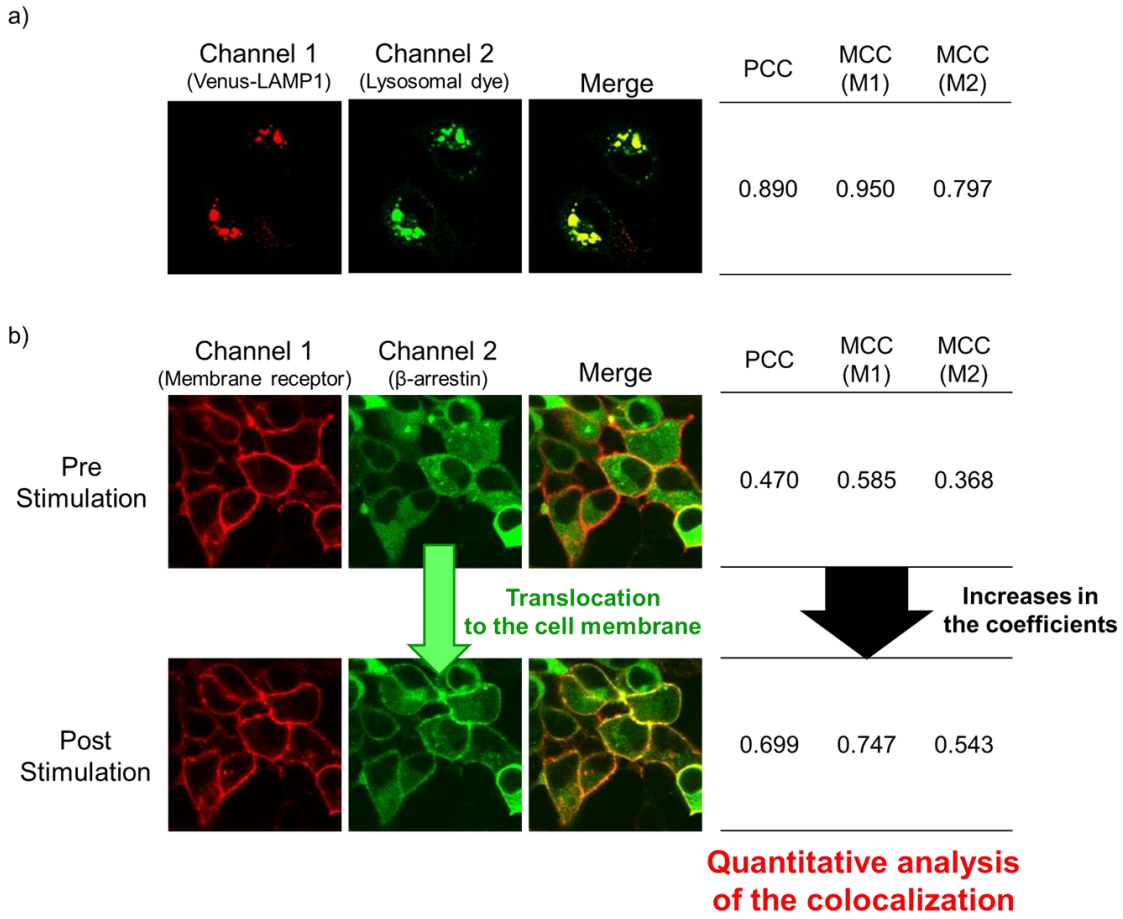

Figure 1-10. Quantitative analysis of colocalization of two proteins

a) Fluorescent images of lysosome-marker protein, LAMP1 (Red) and chemical dyes for labeling lysosome (Green) in HeLa cells. b) A change of the colocalization of membrane receptor, adrenergic receptor (Red) and β-arrestin (Green) in HEK293 cells. The cells were stimulated with a specific ligand of the receptor (1 μM isoproterenol) for 10 min. M1: MCC value indicating the proportion of the proteins in channel 1 merged with that in channel 2. M2: MCC value indicating the proportion of the proteins in channel 2 merged with that in channel 1.

1-7. Purpose of the present study

Despite the significance of β-arrestin-related trafficking, the manner by which β-arrestin switches the trafficking pathways of GPCRs in living cells is unclear. Herein, I demonstrate an optogenetic approach using the CRY and CIB for analyzing the interaction of β-arrestin with GPCRs. The present method reveals that the light-induced interaction of β-arrestin with β2-adrenergic receptor (ADRB2) is sufficient to trigger endocytosis of ADRB2 without ADRB2 being phosphorylated. I also clarify that the dissociation of β-arrestin promotes the recycling of ADRB2 to the cell surface, whereas the prolonged interaction of β-arrestin directs ADRB2 to the lysosomal pathway. These results demonstrate the significant role of the duration of the ADRB2-β-arrestin interaction on intracellular trafficking in living cells.

Chapter 2

Development of an Optogenetic Method to Control the Reversible Interaction between ADRB2 and β-Arrestin

2-1. Introduction

The localization and degradation of membrane receptors are regulated by intracellular trafficking. The precise regulation of the receptors is important for cells to adapt to a wide variety of extracellular stimuli. Perturbation of the trafficking system leads to crucial diseases[24-33]. Investigation of the trafficking mechanism is therefore important for drug development and therapeutics.

β-Arrestin is a protein regulating the localization of membrane receptors such as GPCRs, TGFR and IGFR[42-46]. Interaction of β-arrestin with the receptors is an initial step for the intracellular trafficking of the receptors. Therefore, the manipulation of the interaction of β-arrestin with the receptors have a potential to clarify the mechanism of intracellular trafficking of the receptors. In the conventional methods, a chemical-dimerizering system was utilized to manipulate the interaction between β-arrestin and the receptors[79,80]. However, the system was not applicable for investigation of trafficking pathway after dissociation of β-arrestin from the receptors due to strong affinity of rapamycin to the dimerizers[81]. To overcome the problem, I develop a novel method to manipulate the reversible interaction of β-arrestin with the receptors using the CRY-CIB system. Light irradiation induces the rapid interaction of ADRB2, which triggers the endocytosis of ADRB2. In addition, dark condition induces dissociation of β-arrestin from ADRB2. I demonstrate the reversible manipulation of the interaction of β-arrestin with ADRB2 by switching on and off the light irradiation.

2-2 Materials and Methods

2-2-1. Materials and cDNA Construction

SNAP-Surface Alexa Fluor 647 and anti-SNAP-tag antibodies were obtained from New England Biolabs (USA). Isoproterenol (ISO) and Dyngo4a were purchased from Tokyo Chemical Industry (Japan) and Abcam (USA), respectively. The SNAP-Surface Alexa Fluor 647 and Dyngo4a were solved in DMSO. The ISO was solved in H_2O. The DNA fragments encoding SNAP-tag, ADRB2 and CIB were inserted between *Hind*III and *Xho*I sites in pcDNA3.1/V5-His (B) (Thermo Fisher Scientific, USA). The genes encoding photolyase homology region of cryptochrome 2, mCherry and β-arrestin 2 were subcloned between *BamH*I and *Xba*I sites in pcDNA4/myc-His (B) (Thermo Fisher Scientific).

2-2-2. Cell Cultivation

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM, Wako Pure Chemical Industry, Japan) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, USA) under a 5% CO_2 atmosphere at 37˚C. D-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 400 μg/ml G418 (Gibco) and 100 μg/ml zeocin (Thermo Fisher Scientific) was used for cultivating HEK293 cells stably expressing fusion proteins ($HEK293_{opt}$ and $HEK293_{optCLIP}$).

2-2-3. Confocal Fluorescence Microscopy

Cells were cultured on 35-mm glass-bottomed dishes. To label the SNAP-tag, 0.5 μM SNAP-Surface Alexa Fluor 647 was added to the medium, and the cells were incubated

for 30 min at 37°C. After the cells were washed three times, the medium was exchanged by 2 ml of phenol red-free D-MEM containing HEPES, pH 7.4 (Wako Pure Chemical Industry). The cells were observed under a confocal microscope (IX-81, FV-1000D, Olympus). The images were acquired every 1 min. CRY activation was performed under a confocal microscope using a 20 mW 440-nm laser at 0.5% output with a scan speed of 2.0 μs/pixel. The fluorescence intensity of $Arrestin_{CRY}$ was evaluated using the image analysis software FV10-ASW (Olympus). The number of $ADRB2_{CIB}$ or Mdm2 fluorescence spots was counted using ImageJ. The diameter to detect the spots was set at 0.5 - 5 μm.

2-2-4. Calculation of Manders' Colocalization Coefficient

The extent of the colocalization of $ADRB2_{CIB}$ with lysosomes was estimated using Manders' colocalization coefficient.

$$\text{Manders' colocalization coefficient} = \frac{\sum_j FI_{j\text{coloc}}}{\sum_i FI_i}$$

where FI_i is the fluorescence intensity of Alexa Fluor 647 molecules attached to $ADRB2_{CIB}$ in the pixel i, and $FI_{j\text{coloc}} = 0$ if the fluorescence intensity of Alexa 488, which is attached to LAMP1, in the pixel j is lower than a particular threshold, and $FI_{j\text{coloc}} = FI_j$ if the fluorescence intensity of Alexa 488 in pixel j is equal to or larger than the threshold. The coefficient was calculated using the JACoP plug-in for ImageJ after applying a threshold of fluorescent intensity to each time-lapse image[107]. The thresholds were automatically determined based on the JACoP program.

2-2-5. Quantification of $ADRB2_{CIB}$ on the Cell Surface Using the ELISA Assay

$HEK293_{opt}$ cells in 4-well plates were treated with 1.0 μM ISO or irradiated with blue

light at 3 mW/cm^2 for the indicated time. After the cells were fixed with 4% formaldehyde, they were treated with gelatin from cold-water fish skin for blocking. SNAP-tag moieties on the cell surface were labeled with an anti-SNAP-tag antibody for 60 min at room temperature. After the cells were washed twice with PBS(+), ECL anti-rabbit antibody linked to HRP was added to each well. The cells were incubated for 60 min at room temperature and then washed three times with PBS(+). After the addition of 350 µl of TMB solution (Thermo Fisher Scientific) and 30 min of incubation, 350 µl of 2 M sulfuric acid was added to each well. The OD_{450} value of 100 µl of solution was measured using a microplate reader (Bio-Rad, USA). The amount of $ADRB2_{CIB}$ on the cell surface was defined by $(OD - OD^{mock}) / (OD^{basal} - OD^{mock}) \times 100$, where OD^{mock} and OD^{basal} correspond to the OD of non-stimulated HEK293 cells and the OD from non-stimulated $HEK293_{opt}$, respectively. To promote recycling of $ADRB2_{CIB}$ in ISO-treated cells, cells were washed three times with 500 µl of acidic buffer (150 mM NaCl and 5 mM acetic acid) and then twice with 500 µl of PBS(+) to eliminate ISO.

2-3. Results

2-3-1. Basic Strategy for Light-Induced Interactions between ADRB2 and β-Arrestin

ADRB2 is a GPCR that regulates cardiovascular and pulmonary functions[113,114]. ISO, a specific ligand of ADRB2, activates G protein-mediated signals and induces clathrin-mediated endocytosis of ADRB2 through its interaction with β-arrestin. To temporally control the interaction between ADRB2 and β-arrestin by external blue light, I connected SNAP-tag-fused ADRB2 to the N-terminus of CIB (named $ADRB2_{CIB}$); in addition, mCherry-fused β-arrestin 2 was attached to the C-terminus of CRY (denoted $Arrestin_{CRY}$) (**Figure 2-1a**). Each domain was connected to flexible linkers composed of glycine and serine to allow the dynamic motion of the fusion proteins. Before blue light irradiation, $ADRB2_{CIB}$ locates on the cell membrane, and $Arrestin_{CRY}$ distributes throughout the cytosol (**Figure 2-1b**). Stimulation with blue light induces translocation of $Arrestin_{CRY}$ to $ADRB2_{CIB}$ on the cell membrane by the interaction between CRY and CIB, thereby triggering endocytosis of $ADRB2_{CIB}$-$Arrestin_{CRY}$ complexes. After irradiation is stopped, $Arrestin_{CRY}$ is redistributed in the cytosol by the dissociation of CRY and CIB.

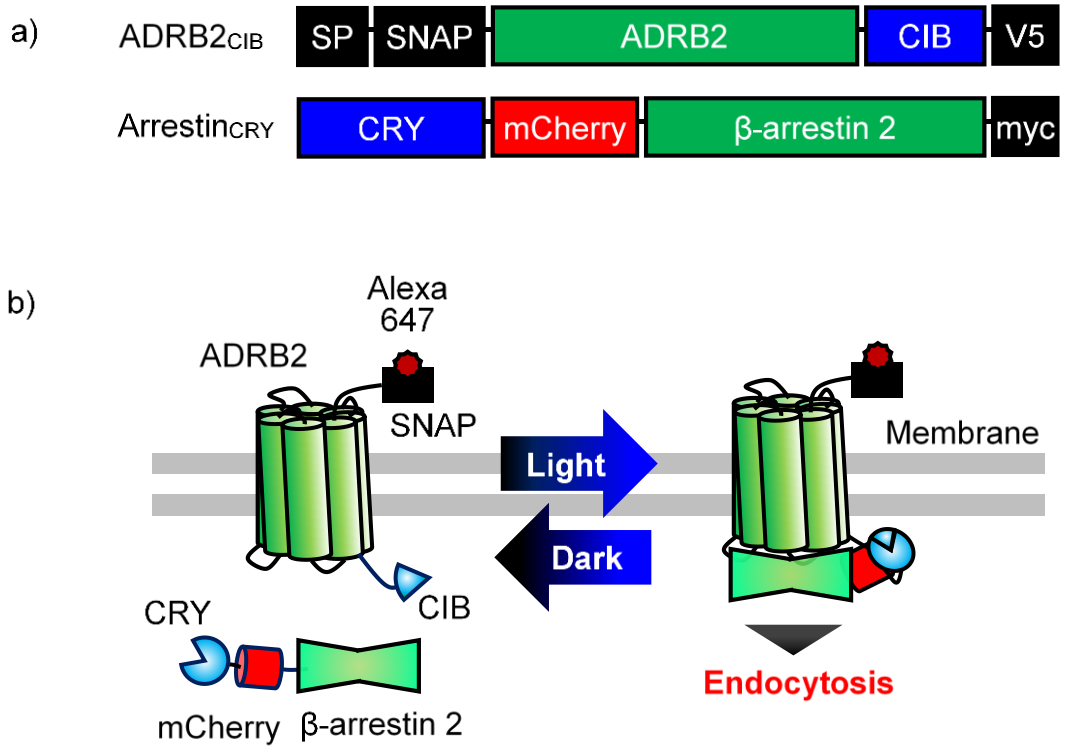

Figure 2-1. Light-based manipulation of reversible interaction between β-arrestin and ADRB2 using photodimerizers.

a) Schematic structures of the optogenetic manipulation system for the reversible interaction of β-arrestin with ADRB2. $ADRB2_{CIB}$ is composed of a signal peptide (SP) derived from 5HT3A serotonin receptor that targets the protein to the cell membrane, a SNAP-tag, β2-adrenergic receptor (ADRB2), CIB and a V5 epitope tag. $Arrestin_{CRY}$ consists of cryptochrome (CRY), mCherry, β-arrestin2 and a myc epitope tag. b) Basic principle of light-induced interaction of β-arrestin2 with ADRB2. Before light stimulation, $ADRB2_{CIB}$ localizes on the cell membrane, and $Arrestin_{CRY}$ distributes throughout the cytosol. Upon stimulation with blue light, $Arrestin_{CRY}$ interacts with $ADRB2_{CIB}$, which triggers the endocytosis of the $ADRB2_{CIB}$-$Arrestin_{CRY}$ complex. Under dark conditions, $Arrestin_{CRY}$ dissociates from $ADRB2_{CIB}$ and redistributes into the cytosol.

2-3-2. Light-Induced Endocytosis of ADRB2$_{CIB}$

To determine whether light irradiation controls the interaction between ADRB2 and β-arrestin, HEK293 cells stably expressing ADRB2$_{CIB}$ and Arrestin$_{CRY}$ (HEK293$_{opt}$) were irradiated with blue light under a confocal fluorescence microscope (**Figure 2-2**). Arrestin$_{CRY}$ was translocated from the cytosol to the cell membrane a few min after the start of irradiation and then moved back into the cytosol to form a dot-like structure. ADRB2$_{CIB}$ fluorescent spots appeared in the cytosol at 15 min, and their number gradually increased until 30 min of irradiation. The fluorescent spots mostly colocalized with Arrestin$_{CRY}$.

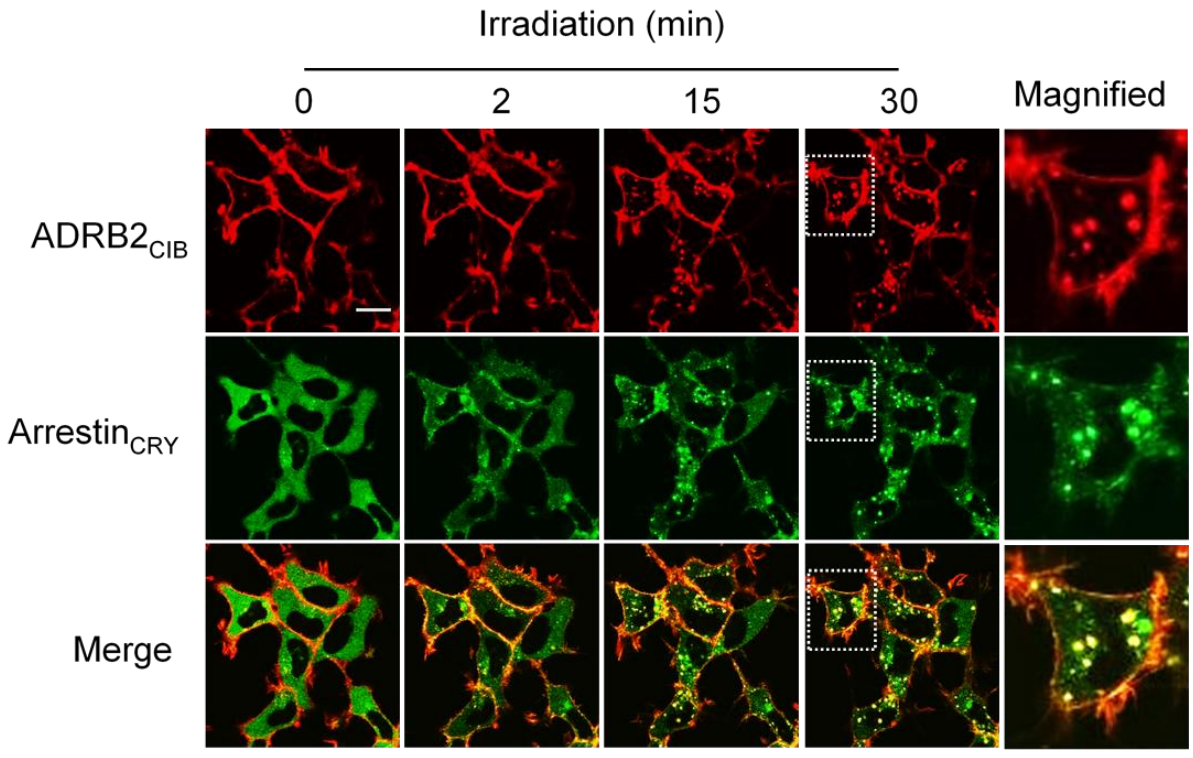

Figure 2-2. Light-induced endocytosis of ADRB2$_{CIB}$.

Image of ADRB2$_{CIB}$ and Arrestin$_{CRY}$ fluorescence in HEK293$_{opt}$ cells irradiated with blue light for 0, 2, 15 and 30 min under a confocal microscope (a), and an image of these cells pretreated with 30 μM Dyngo4a for 30 min and then irradiated by blue light

for 0, 2, 15 and 30 min (b). Red, $ADRB2_{CIB}$; green, $Arrestin_{CRY}$. Scale bar, 20 μm.

In a control experiment, a fusion protein comprising SNAP-tag and ADRB2 without CIB was expressed in HEK293 cells. The localization of $Arrestin_{CRY}$ was unchanged upon stimulation with blue light due to the lack of the CIB protein (**Figure 2-3a**). Furthermore, fluorescent spots in the HEK293 cells expressing $Arrestin_{CRY}$ and cell membrane-anchored CIB (Myr-Venus-CIB) were not observed during irradiation, despite $Arrestin_{CRY}$ being translocated to the cell membrane. To analyze the results quantitatively, I counted the number of spots containing $ADRB2_{CIB}$, ADRB2, and Myr-Venus-CIB in the obtained images (**Figure 2-3b**). A significant increase in the number of spots was observed after light irradiation in case of $ADRB2_{CIB}$. In contrast, the number of spots containing ADRB2 or Myr-Venus-CIB did not change after light stimulation. The results indicate that recruitment of β-arrestin to ADRB2 is necessary for appearance of the spots. In addition, the cells expressing Myr-Venus-CIB and $Arrestin_{CRY}$ were irradiated for 120 min (**Figure 3-3c**). The number of spots containing Myr-Venus-CIB did not increase under the prolonged irradiation.

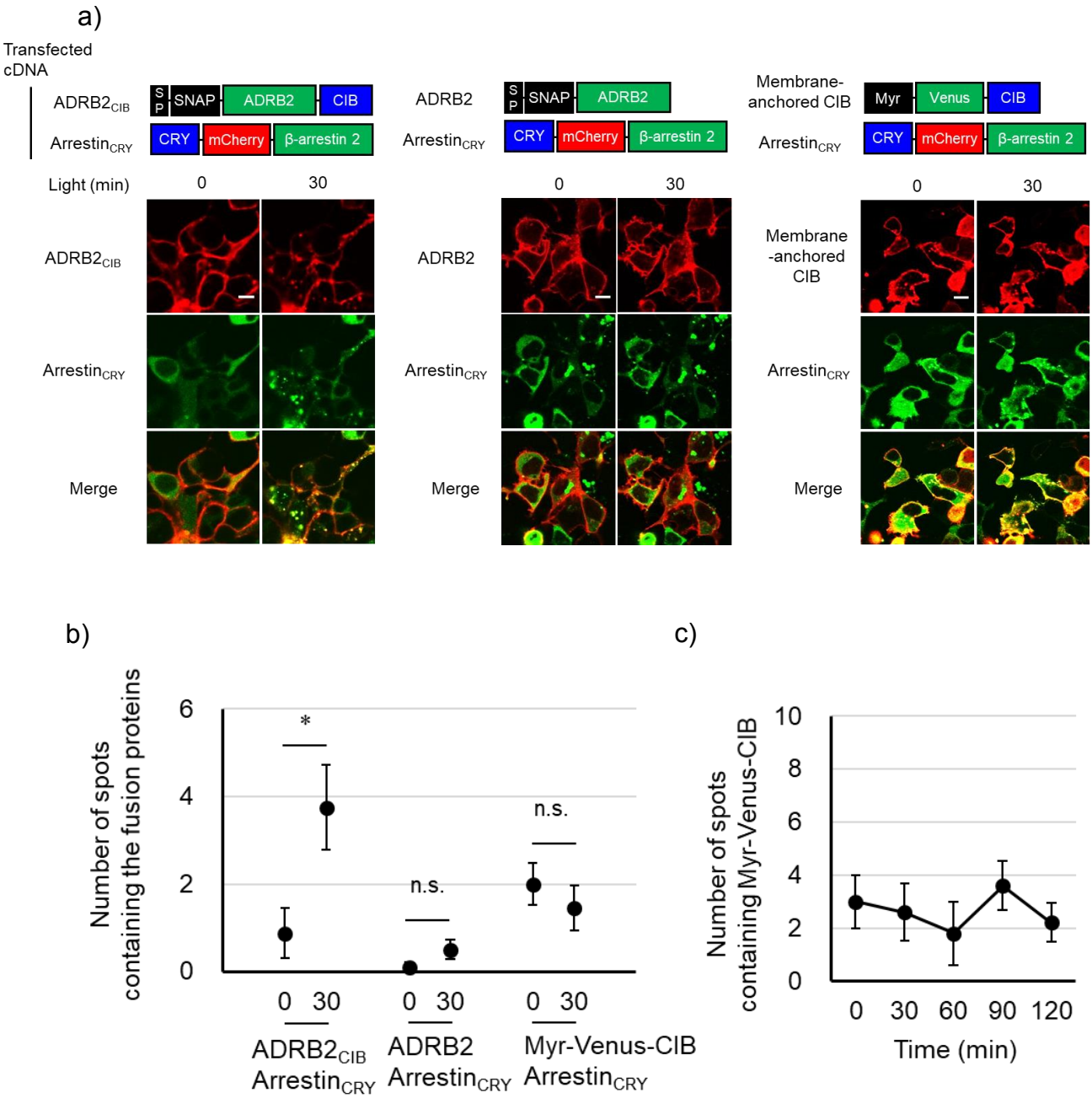

Figure 2-3 | Light-induced endocytosis in the transfected HEK293 cells.

a) Images of fluorescence from HEK293 cells transfected with cDNAs encoding $ADRB2_{CIB}$ and $Arrestin_{CRY}$ (left), SNAP-ADRB2 (ADRB2) and $Arrestin_{CRY}$ (middle), or membrane-anchored CIB (Myr-Venus-CIB) and $Arrestin_{CRY}$ (right). The cells were irradiated with blue light for 30 min under a confocal microscope. Red, $ADRB2_{CIB}$(left), ADRB2 (middle), Myr-Venus-CIB (right); green, $Arrestin_{CRY}$. Bar shows 10 μm. b) Temporal changes of the numbers of spots containing the fusion proteins per cell. The

number of spots in HEK293 cells expressing $Arrestin_{CRY}$ and $ADRB2_{CIB}$, ADRB2, or Myr-Venus-CIB were counted. Bar: mean ± s.e.m. (n = 8, 10, 11 cells, respectively). Statistical analysis was performed with paired Student's t-test (Two tailed). *$P < 0.05$ ($P = 0.016$). c) Temporal changes in the number of spots containing Myr-Venus-CIB during 120 min irradiation. (n = 5 cells)

I next examined the endocytosis of $ADRB2_{CIB}$ after $ADRB2_{CIB}$-$Arrestin_{CRY}$ interaction. ADRB2 is endocytosed with β-arrestin in a clathrin-mediated manner[50]. Segregation of clathrin-coated pits from the cell membrane is regulated by dynamin GTPases. To show that the fluorescent spots resulted from clathrin-mediated endocytosis, I pretreated the $HEK293_{opt}$ cells with a dynamin inhibitor, Dyngo4a. The fluorescent spots did not appear in the presence of Dyngo4a after the stimulation by light (**Figure 2-4a, b**). To quantitate this inhibitory effect, I quantified the number of $ADRB2_{CIB}$ fluorescent spots and the colocalization of $Arrestin_{CRY}$ with $ADRB2_{CIB}$ using the quantitative parameter Manders' colocalization coefficient, which is proportional to the total amount of fluorescence intensity from $Arrestin_{CRY}$ in the pixels where it colocalizes with $ADRB2_{CIB}$ (**Figure 2-4c**). The number of spots increased with increased irradiation time in the absence of Dyngo4a. In contrast, light irradiation did not induce an increase in the number of spots in the presence of Dyngo4a although the colocalization of $Arrestin_{CRY}$ with $ADRB2_{CIB}$ was induced. Considering these results, I concluded that the endocytosis of $ADRB2_{CIB}$ was triggered by light-induced interaction of β-arrestin with ADRB2, and the observed $ADRB2_{CIB}$ fluorescent spots were $ADRB2_{CIB}$-containing vesicles produced by clathrin-mediated endocytosis.

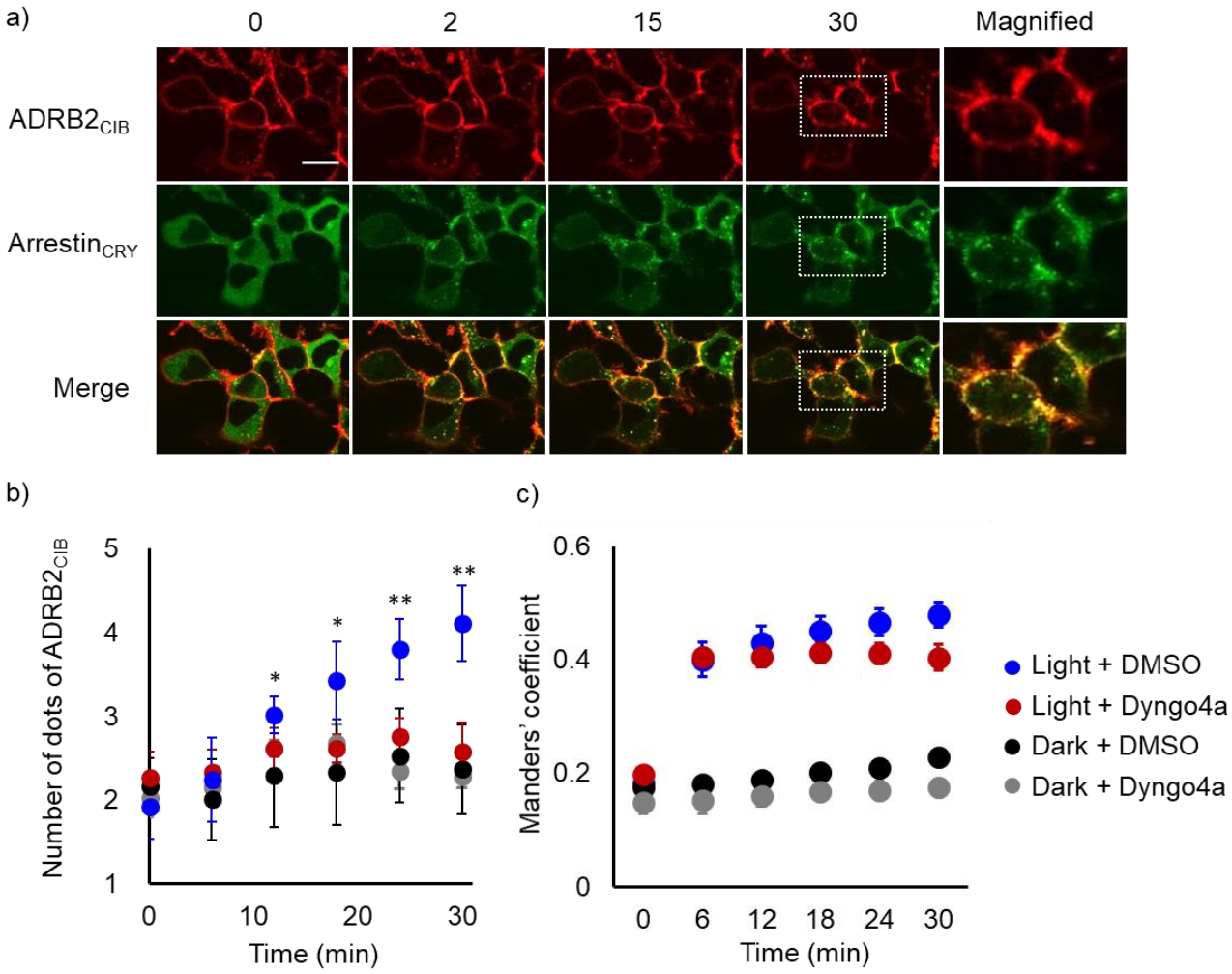

Figure 2-4. Inhibitory effect of Dynamin inhibitor to light-induced endocytosis

a) Image of $ADRB2_{CIB}$ and $Arrestin_{CRY}$ fluorescence in $HEK293_{opt}$ cells irradiated with blue light for 30 min. The cells were pretreated with 30 μM Dyngo4a for 30 min Red, $ADRB2_{CIB}$; green, $Arrestin_{CRY}$. Scale bar, 20 μm. Time courses of b) the number of $ADRB2_{CIB}$ fluorescence spots and c) the colocalization of $Arrestin_{CRY}$ with $ADRB2_{CIB}$ in $HEK293_{opt}$ cells as a function of blue light irradiation and 30 μM Dingo4a treatment. The number of spots per cell was calculated by normalizing total number of fluorescence spots in each imaging with the number of nucleus stained using Hoechst 33342. The final concentration of DMSO was 0.1%. Blue: light irradiated and no Dyngo 4a; red: irradiated and Dyngo 4a present; black: non-irradiated and no Dyngo 4a; gray: non-irradiated and Dyngo 4a present. Bar: mean ± s.e.m. (n = 4 from four individual

experiments). Statistical significance was determined using a two-way ANOVA (v.s. Light + Dyngo 4a). * P values < 0.05 (12 min; P = 0.047, 18 min; P = 0.018), **P values < 0.01 (24 min; P = 0.0021, 30 min; P = 0.0013).

I further investigated the time dependency of the light-induced endocytosis of $ADRB2_{CIB}$. After $HEK293_{opt}$ cells were stimulated with blue light for different durations, the amount of $ADRB2_{CIB}$ on the cell surface was quantified using an ELISA[79] (**Figure 2-5a**). The amount of $ADRB2_{CIB}$ on the cell surface decreased with increasing irradiation time. I also examined the light intensity dependence of endocytosis (**Figure 2-5b**). Endocytosis was promoted by increased light intensity and plateaued at 3 mW/cm^2. The amount of $ADRB2_{CIB}$ endocytosed by 3 mW/cm^2 light was more than that induced by 0.01 μM ISO and less than that induced by 0.1 and 1.0 μM ISO. These results demonstrate that the endocytosis of $ADRB2_{CIB}$ can be controlled by modulating light intensity and irradiation time.

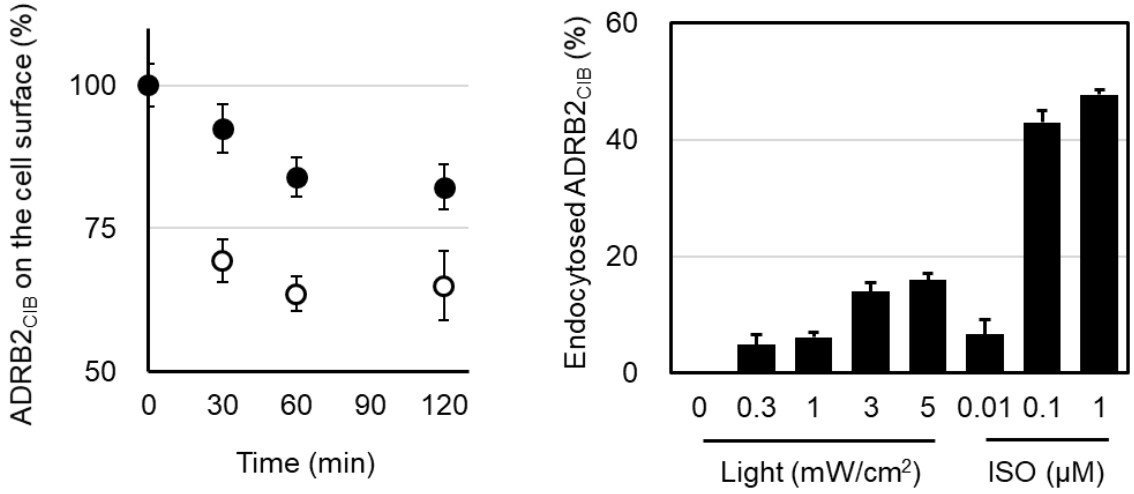

Figure 2-5. Characterization of light-induced endocytosis of $ADRB2_{CIB}$.

a) Time courses of $ADRB2_{CIB}$ on the cell surface of $HEK293_{opt}$ cells stimulated with

light at an intensity of 3 mW/cm^2 (filled circles) and 1.0 μM ISO (open circles) for the indicated times. After the cells were fixated, the amount of ADRB2$_{CIB}$ on the cell surface was quantified using an ELISA. b) Light intensity dependency of endocytosis of ADRB2$_{CIB}$ in HEK293$_{opt}$ cells stimulated with blue light at various intensities or with 0.01, 0.1 and 1.0 μM ISO for 60 min. The amount of endocytosed ADRB2$_{CIB}$ was quantified using an ELISA. Bar: mean ± s.e.m. (n = 8 from two individual experiments).

2-3-3. Reversibility of the Interaction between ADRB2 and β-Arrestin

To show the reversibility of the light-induced interaction between ADRB2$_{CIB}$ and Arrestin$_{CRY}$, I irradiated the HEK293$_{opt}$ cells with blue light for 30 min and then incubated them in the dark. After Arrestin$_{CRY}$ colocalized with ADRB2$_{CIB}$, Arrestin$_{CRY}$ redistributed uniformly in the cytosol under dark conditions (**Figure 2-6a**). The fluorescence intensity of Arrestin$_{CRY}$ in the cytosol decreased 1 min after irradiation was started and then recovered to the basal level 10 min after irradiation ceased (**Figure 2-6b**). The colocalization coefficient of Arrestin$_{CRY}$ with ADRB2$_{CIB}$ decreased after stopping irradiation (**Figure 2-6c**). The results confirm that Arrestin$_{CRY}$ dissociated from ADRB2$_{CIB}$ in the dark.

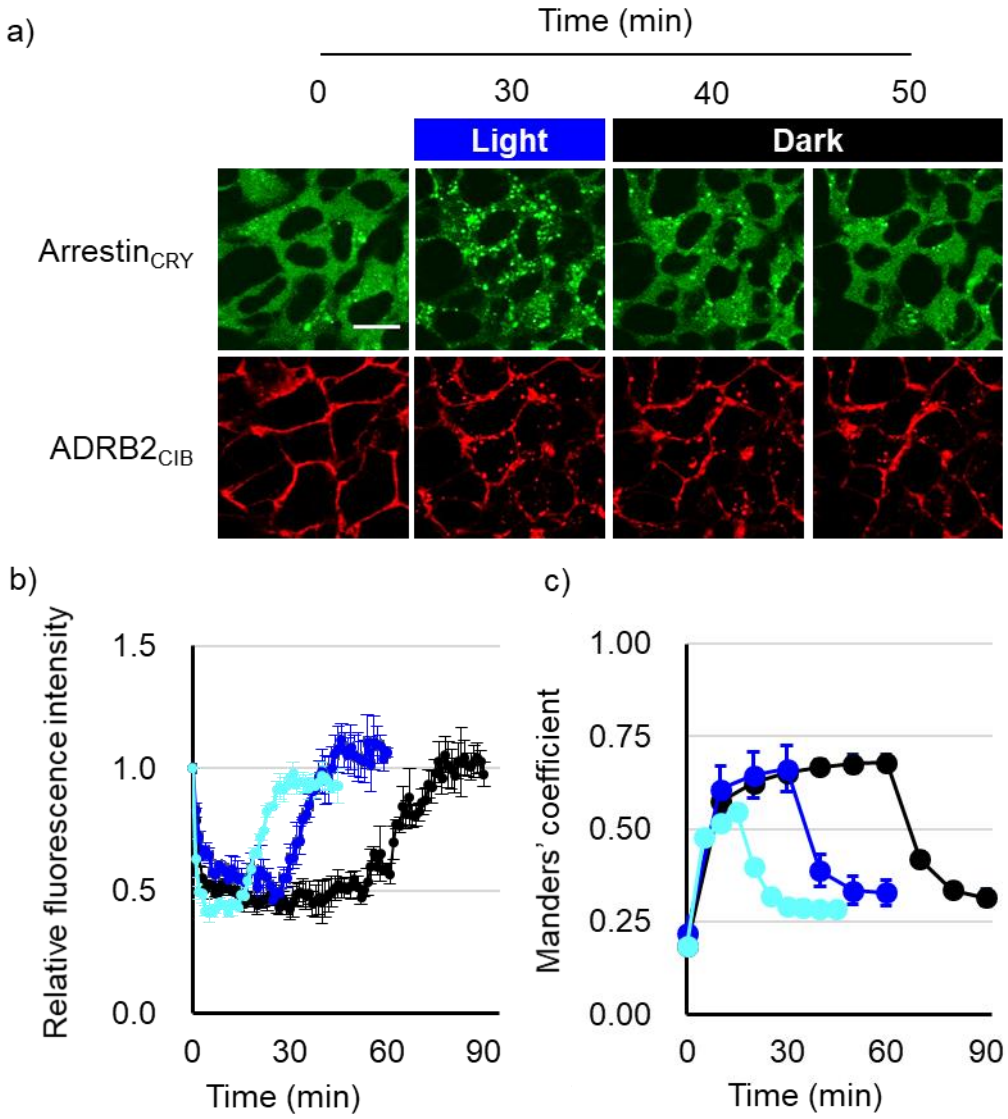

Figure 2-6. Induction of the recycling pathway in the dark after light irradiation.

a) Images of Arrestin$_{CRY}$ and ADRB2$_{CIB}$ fluorescence in HEK293$_{opt}$ cells stimulated with blue light for 30 min and then incubated for 10 and 20 min (total of 40 and 50 min observation, respectively) without blue light irradiation. Red, ADRB2$_{CIB}$; green, Arrestin$_{CRY}$. Scale bar, 20 μm. b) Time courses of the fluorescence intensity of cytosolic Arrestin$_{CRY}$ in each cell. The cells were stimulated with blue light for 15 min (pale blue), 30 min (deep blue), and 60 min (black) and then incubated in the dark. Each time course was normalized to the fluorescence intensity at 0 min. Bars: mean ± s.e.m (n = 9 from three individual experiments). c) Time courses in the colocalization of ArrestinCRY with ADRB2CIB after stopping irradiation. The cells were stimulated with blue light for 15 min (pale blue), 30 min (deep blue), and 60 min (black) and then incubated in the

dark. Bars: mean ± s.e.m (n = 3 from three individual experiments).

2-4. Discussion

I developed an optical method to manipulate the reversible interaction between ADRB2 and β-arrestin using the CRY-CIB system. Controlling the interaction of GPCRs with β-arrestin using the CRY-CIB system has several advantages. Previous methods to control the interaction of GPCRs with β-arrestin were based on a chemical approach[79,80]. Chemical dimerizers (FKBP-FRB) were used to control the interaction of β-arrestin with vasopressin receptors or chemokine receptors. Rapamycin treatment induced the interaction of FKBP-fused receptor and FRB-fused β-arrestin, which promoted endocytosis of the receptors. However, it is difficult to eliminate rapamycin by simple washing procedures due to its high affinity for FKBP[81], suggesting that the chemical approach is not suitable for the temporal manipulation of these interactions. Unlike to the chemical dimerizers, the CRY-CIB system is a robust tool to temporally control the reversible interaction of β-arrestin with membrane receptors. Furthermore, I examined the characterization of light induced endocytosis, which suggests that the amount of endocytosis is controllable by modulating the time of irradiation and the intensity of light.

I demonstrated that the artificial interaction of β-arrestin to ADRB2 is sufficient to trigger their endocytosis. Furthermore, there are potential applications of the present method for investigation of dynamics of GPCR and their regulatory proteins on the cell surface. The present method has a potential to control the oligomeric state of the GPCRs. GPCRs form dimers and oligomers upon stimulation of their ligands, which is important

for recruitment of their regulatory proteins on cell surface[115]. Based on the fact that the light-activated CRY and CIB forms oligomers[116], $ADRB2_{CIB}$ and $Arrestin_{CRY}$ also form oligomers upon light stimulation. Light control of the oligomeric state of ligand-stimulated GPCRs will be a useful approach to investigate the oligomerization of GPCRs affecting their regulatory proteins on the cell surface. Furthermore, a previous study suggested the possibility of the endocytosis of G proteins with some GPCRs[117], whereas another study on single molecule imaging reported that G protein-ADRB2 complexes did not colocalize with clathrin-coated pits[118]. Thus, it is not clear whether the interaction of G protein with GPCRs affects their endocytosis. The present system may be applicable to clarify the impact of G proteins on the receptor internalization by comparing the dynamics of endocytosis between ligand-activated GPCRs interacting with G proteins and light-stimulated GPCRs without G proteins.

2-5. Conclusion

I developed a novel method to manipulate the interaction of β-arrestin with ADRB2 using the CRY-CIB system. The $ADRB2_{CIB}$ and $Arrestin_{CRY}$ were localized on cell surface and in cytosol, respectively. The light stimulation induced the rapid interaction of $Arrestin_{CRY}$ with $ADRB2_{CIB}$, which triggers the endocytosis of $ADRB2_{CIB}$. Furthermore, the endocytosis of $ADRB2_{CIB}$ was controllable by modulating the light intensity and the irradiation time. I also demonstrated that dissociation of $Arrestin_{CRY}$ from $ADRB2_{CIB}$ was promoted in the dark condition. These results suggest that the present method is useful for controlling the reversible interaction between β-arrestin and ADRB2, and applicable for triggering endocytosis of ADRB2. The present method will enable us analyzing the dynamics of GPCRs and their regulatory proteins on the cell surface.

Chapter 3

Analysis of Intracellular Trafficking of ADRB2 Regulated by β-Arrestin

3-1. Introduction

Previous studies has suggested that GPCRs that stably interact with β-arrestin are retained in the cytosolic compartment, whereas GPCRs that immediately dissociate from β-arrestin are rapidly recycled back to the cell membrane[52-57]. These results suggest a significant role for β-arrestin in the intracellular trafficking of GPCRs. In addition, GPCR-bound β-arrestin functions as a signal transducer of mitogen-activated protein kinases (MAPKs) and the Ser-Thr kinase Akt in the trafficking pathway[67-72]. Although the significance of β-arrestin-related trafficking has been suggested, the manner by which β-arrestin switches the trafficking pathways of GPCRs in living cells is still unclear.

Herein, I utilized the optical method to analyze the mechanism of intracellular trafficking of ADRB2. I clarify that the dissociation of β-arrestin promotes the recycling of ADRB2 to the plasma membrane, whereas prolonged interaction of β-arrestin with ADRB2 directs ADRB2 to the lysosomal pathway. Furthermore, the signal induction during the trafficking was investigated. These results demonstrate the significant role of the duration of the β-arrestin-ADRB2 interaction in sorting between intracellular trafficking pathways in living cells.

3-2. Materials and Methods

3-2-1. Materials

CLIP-Cell TMR STAR and anti-SNAP-tag antibodies were obtained from New England Biolabs (USA). Anti-Rab5, anti-Rab7, anti-LAMP1, anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies were obtained from Cell Signaling Technology (USA). Anti-β2-adrenergic receptor (ADRB2) and anti-phosphorylated ADRB2 antibodies were purchased from Santa Cruz Biotechnology (USA).

3-2-2. Immunostaining of Early Endosomes, Late Endosomes and Lysosomes

$HEK293_{opt}$ cells were cultured on 35-mm glass-bottomed dishes. $ADRB2_{CIB}$ was labeled with SNAP-Surface Alexa Fluor 647 for 30 min and then washed three times with D-MEM. The cells were cultured in D-MEM and stimulated with blue light at an intensity of 3 mW/cm^2 using an LED device (TH-211×200BL, Creating Customer Satisfaction, Japan) for the indicated times. The cells were fixed using 4% formaldehyde and then permeabilized with 0.2% Triton X-100. To avoid the non-specific adsorption of antibodies, the cells were treated with 0.2% gelatin from cold-water fish skin (Sigma-Aldrich) for 1 h at room temperature. Rab5, Rab7 and LAMP1 were labeled with the corresponding antibodies in PBS(+) overnight at 4˚C. After the cells were washed three times with PBS(+), they were stained with Alexa Fluor 488-conjugated goat anti-Rabbit IgG Secondary Antibody (Thermo Fisher Scientific) for 1 h at room temperature. The cells were washed three times with PBS(+) and then observed under a confocal microscope.

3-2-3. Detection of Ubiquitinated ADRB2$_{CIB}$ Using Immunoprecipitation

HEK293$_{opt}$ cells that had been cultured on a 4-well plate were stimulated with 3 mM/cm^2 blue light using the LED device or 1.0 μM ISO for the indicated time. The cells were harvested using 200 μl of lysis buffer (50 mM HEPES (pH 7.5), 0.5% NP-40, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin and MG-132 proteasome inhibitor). Anti-V5 antibody (Life Technologies) was added to each lysate, which was then gently shaken at 4°C for 1 h. After protein G Sepharose (GE Healthcare, USA) was added, the lysate was shaken at 4°C for 1 h. The precipitate was washed three times with 1 ml of lysis buffer and dissolved in 100 μl of sample buffer (125 mM Tris, pH 6.8, 10% glycerol, 4% SDS, 0.006% bromophenol blue and 10% mercaptoethanol). The samples were heated at 65°C for 10 min and then subjected to SDS-PAGE using 10% acrylamide gels. The proteins were transferred onto nitrocellulose membranes (Bio-Rad, USA). To block the non-specific binding of antibodies, the membrane was treated with 1% skim milk in Tris-buffered saline for 1 h. ADRB2 and ubiquitin were blotted using their corresponding antibodies. The blotted bands were detected using an image analyzer (ImageQuant LAS-4000, GE Healthcare), and the intensity of each band was measured with an ImageQuant TL software.

3-2-4. Statistical Analysis

Statistical significance was determined using unpaired Student's t-tests (two-tailed) and Bonferroni post-hoc test. Differences with P values < 0.05 were considered statistically significant.

3-3. Results

3-3-1. Localization of ADRB2$_{CIB}$ after Light-Induced Endocytosis

Most GPCRs are transported to an endocytic pathway after internalization. To confirm that ADRB2$_{CIB}$ is transported to endosomes after light-induced endocytosis, I immunostained early and late endosomes using antibodies specific to the endosome marker proteins Rab5 and Rab7, respectively (**Figure 3-1a,b**). The internalized ADRB2$_{CIB}$ was partially localized in Rab5- or Rab7-positive vesicles after 30 min of irradiation. The residual ADRB2$_{CIB}$ did not colocalize with the endosomes, indicative of sorting to other endosomes or lysosomes. Based on these results, I concluded that part of the ADRB2$_{CIB}$ is transported to endosomes after light-induced endocytosis.

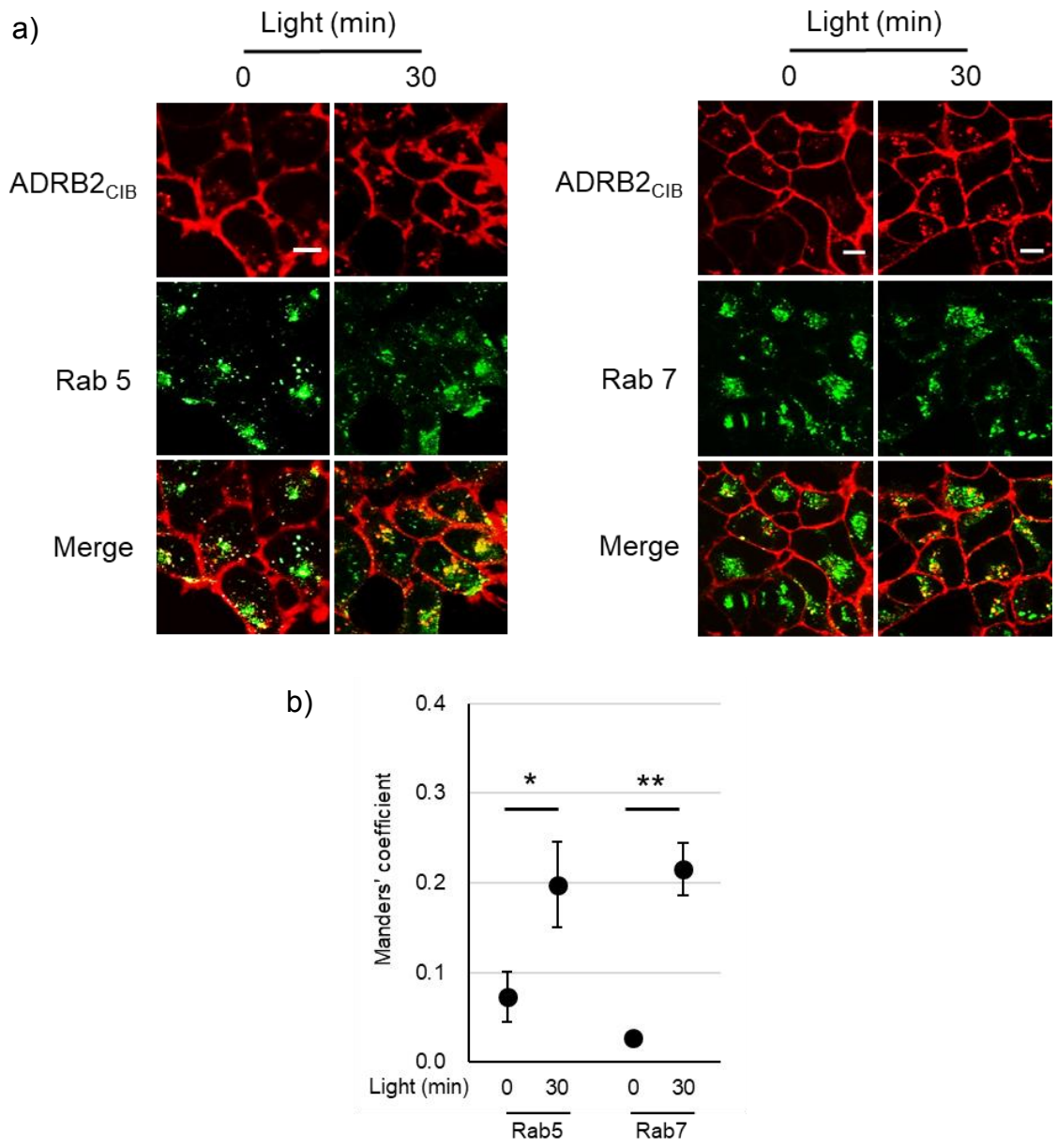

Figure 3-1. Observation of ADRB2$_{CIB}$ and endosome marker proteins after light stimulation.

a) Images of ADRB2$_{CIB}$ and endosome marker protein fluorescence in HEK293$_{opt}$ cells before and after blue light illumination at an intensity of 3 mW/cm^2 for 30 min. After the cells were fixed, Rab5 or Rab7 was immunostained with its specific antibody. Red, ADRB2$_{CIB}$; green, Rab5 or Rab7. Bar represents 10 μm. b) Quantification of colocalization of ADRB2$_{CIB}$ with Rab5 and Rab7. Bars: mean ± s.e.m, (n = 3). Statistical analysis was performed with unpaired t-test (two tailed), *P < 0.05 (P = 0.018), **P < 0.01(P = 0.00039).

3-3-2. Induction of the Recycling of ADRB2$_{CIB}$ in Dark Conditions

To demonstrate the recycling of ADRB2$_{CIB}$ to the cell surface after the dissociation of Arrestin$_{CRY}$, I quantified the amount of ADRB2$_{CIB}$ on the cell surface using an ELISA after stopping irradiation. The HEK293$_{opt}$ cells were stimulated with blue light for 60 min and then incubated in the dark. The amount of ADRB2$_{CIB}$ on the cell surface decreased to 75% after light stimulation and recovered to 80% after 60 min of incubation in the dark (**Figure 3-2, left**). This result indicates that the recycling of the ADRB2$_{CIB}$ was triggered by the dissociation of β-arrestin from ADRB2 in the dark. Furthermore, in HEK293$_{opt}$ cells stimulated with ISO, the amount of ADRB2$_{CIB}$ on the cell surface recovered from 63 to 77% after ISO was eliminated (**Figure 3-2, right**) The temporal changes in recycling after light irradiation ceased were similar to those in recycling when ISO was eliminated, demonstrating that temporal control of the dissociation between β-arrestin and ADRB2 is useful for investigating the intracellular dynamics of ADRB2 and regulatory proteins in the recycling pathway.

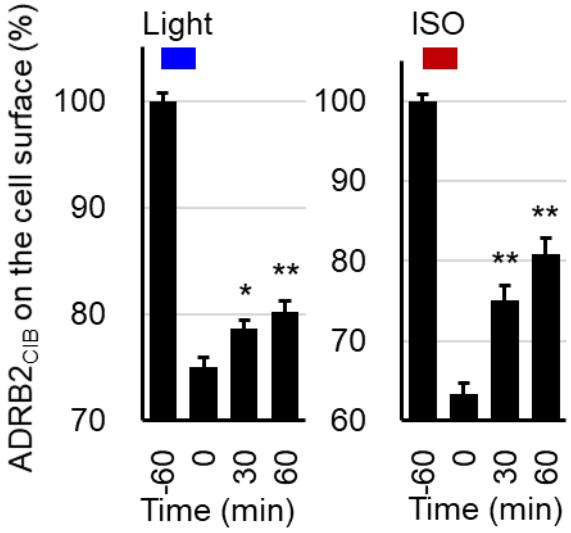

Figure 3-2. Temporal changes in the colocalization of Arrestin$_{CRY}$ with ADRB2

after stopping irradiation.

Recovery of $ADRB2_{CIB}$ on the cell surface after stopping irradiation. $HEK293_{opt}$ cells were stimulated with blue light (left) or 1.0 μM isoproterenol (right) for 60 min. The cells were then incubated at 37°C in the dark for the indicated times. The amount of $ADRB2_{CIB}$ on the cell surface was quantified using an ELISA. Bars: mean ± s.e.m (n = 12 from three individual experiments). The statistical analysis was performed with Bonferroni post-hoc test (0 min v.s. 30 or 60 min) *P values < 0.05 (P = 0.024), **P values < 0.01 (Light; $P = 1.9\times10^{-3}$, ISO; $P = 8.2\times10^{-5}$ (30 min), 6.6×10^{-7} (60 min)).

3-3-3. Sorting $ADRB2_{CIB}$ into the Lysosome Pathway after Prolonged Light Irradiation

I next investigated the effects of a prolonged interaction of $Arrestin_{CRY}$ with $ADRB2_{CIB}$ on the sorting of $ADRB2_{CIB}$. $HEK293_{opt}$ cells were irradiated with blue light for 120 min. Lysosomes were immunostained with an antibody specific to the lysosome marker protein LAMP1 (**Figure 3-3a**). Sustained light irradiation for 120 min resulted in the disappearance of the $ADRB2_{CIB}$ from the cell membrane; instead, $ADRB2_{CIB}$ predominantly localized in lysosomes. Furthermore, I examined the colocalization of $ADRRB2_{CIB}$ with lysosomes (**Figure 3-3b**). The Manders' coefficient increased with increased irradiation time, indicating that the prolonged interaction of $Arrestin_{CRY}$ with $ADRB2_{CIB}$ drives $ADRB2_{CIB}$ to lysosomes. I also examined the ubiquitination of $ADRB2_{CIB}$, which is a crucial signal that sorts GPCRs to lysosomes[63]. Furthermore, $ADRB2_{CIB}$ was highly ubiquitinated when the irradiation time was increased to 120 min (**Figure 3-3c,d**). These results demonstrate that prolonged interaction of β-arrestin with ADRB2, stimulated by blue light, promotes the sorting of $ADRB2_{CIB}$ to lysosomes.

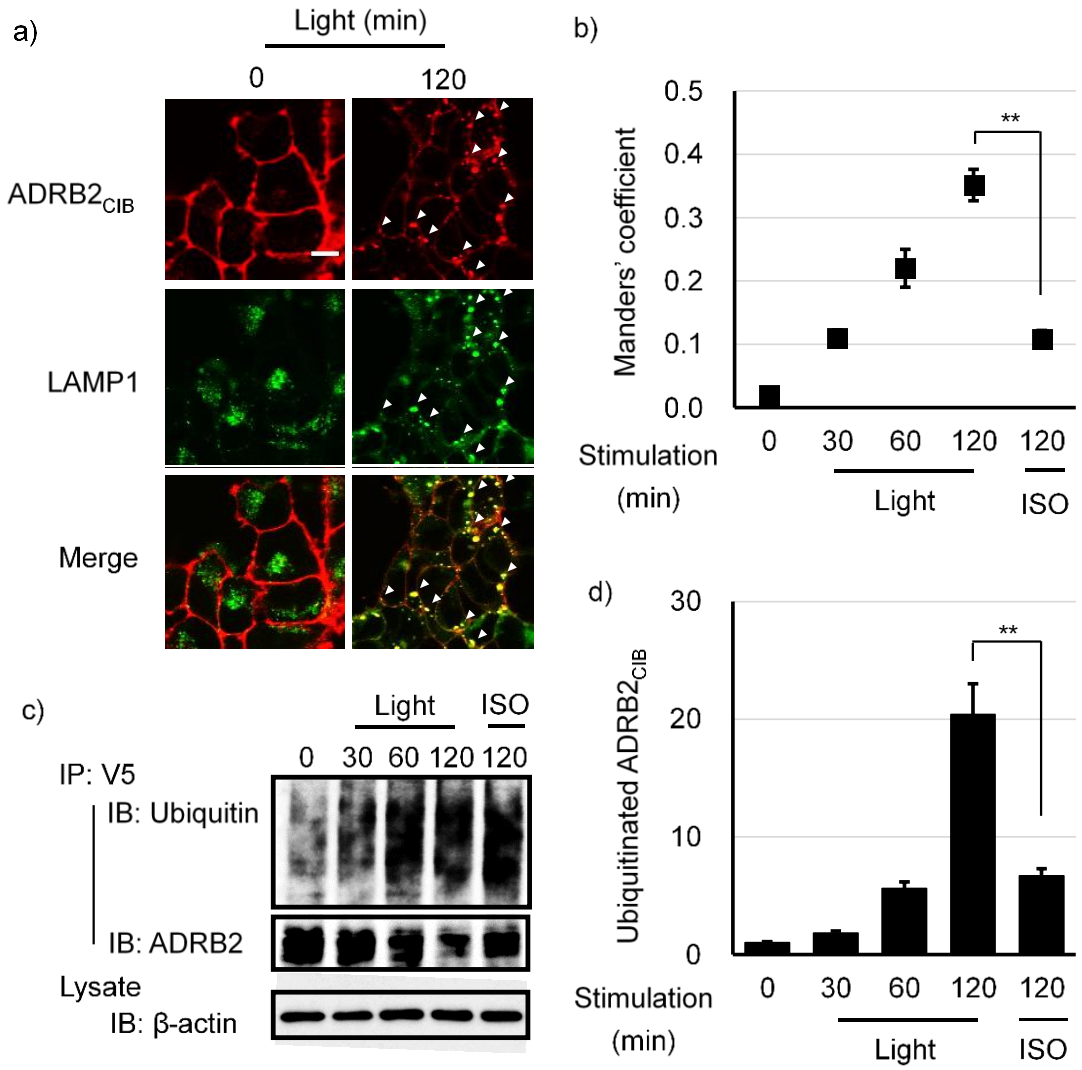

Figure 3-3. Lysosome sorting of ADRB2$_{CIB}$ after prolonged irradiation.

a) Images of ADRB2$_{CIB}$ and lysosome marker LAMP1 fluorescence in HEK293$_{opt}$ cells before and after blue light irradiation for 120 min. The white arrowheads indicate the colocalization of ADRB2$_{CIB}$ and LAMP1. Red, ADRB2$_{CIB}$; green, LAMP1. Bar, 20 μm. b) Temporal changes in the colocalization of ADRB2$_{CIB}$ with lysosomes in HEK293$_{opt}$ cells stimulated with blue light for the indicated time or with ISO for 120 min. Data are shown as the mean ± s.e.m (n= 9 from two independent experiments) The statistical analysis was performed unpaired Student's t-test (two-tailed). $^{**}P < 0.01$ ($P = 2.0\times10^{-7}$). c) IP-Western blotting analysis for the detection of ubiquitinated ADRB2$_{CIB}$. HEK293$_{opt}$ cells were stimulated with blue light for the indicated time or with 1.0 μM ISO for 120 min. ADRB2$_{CIB}$ was immunoprecipitated using an anti-V5-tag antibody. Ubiquitin,

ADRB2 and β-actin were blotted with antibodies specific to them. d) The quantified temporal changes in ubiquitinated $ADRB2_{CIB}$ were calculated as the ratio of ubiquitinated $ADRB2_{CIB}$ to total $ADRB2_{CIB}$ quantities, which were determined in the Western blot analysis. The ratio values were normalized to their value at 0 min. Bars: mean ± s.e.m. (n = 9 from three independent experiments). The statistical analysis was performed unpaired Student's t-test (two-tailed). $^{**}P < 0.01$ ($P = 1.1\times10^{-4}$).

3-3-4. Monitoring a Protein that Regulates ADRB2-β-arrestin Complexes

Some regulatory proteins are recruited to ADRB2-β-arrestin complexes; monitoring these regulatory proteins is important to determine their dynamics and functions in trafficking pathways. Mdm2 is a protein that regulates the endocytosis of ADRB2[63]. Although recent studies suggested that Mdm2 has several important roles in the regulation of other membrane receptors, such as the induction of endocytosis of opioid receptors and the activation of a signaling pathway regulated by an IGFR, the dynamics for Mdm2 recruitment to these receptors is unclear[119,120]. To monitor the intracellular dynamics of Mdm2, I established a cell line that stably expresses $ADRB2_{CIB}$ and $Arrestin_{CRY}$, but I replaced mCherry with a CLIP tag ($HEK293_{optCLIP}$). $HEK293_{optCLIP}$ cells were transfected with a cDNA coding mCherry-fused Mdm2 and were then stimulated with blue light under a confocal microscope (**Figure 3-4a**). Before stimulation, most Mdm2 localized in the nucleus, and the residual Mdm2 distributed throughout the cytosol. Several fluorescent spots of Mdm2 were observed in the cytosol after 15 min of light irradiation, and they colocalized with the $ADRB2_{CIB}$-containing vesicles. This result suggests that the cytosolic Mdm2 is recruited to the light-induced ADRB2-β-arrestin complexes. The colocalization of Mdm2 with $ADRB2_{CIB}$-containing vesicles was sustained during continuous irradiation (**Figure 3-4a upper**). In contrast,

the Mdm2 dissociated from ADRB2$_{CIB}$ 10 min after irradiation ceased (**Figure 3-4a lower**). I counted the number of Mdm2-recruiting vesicles in the cytosol (**Figure 3-4b**). The number of Mdm2-recruiting vesicles increased with increasing irradiation time but decreased with increasing incubation in the dark. These results indicate that the interaction of β-arrestin with ADRB2 promotes the recruitment of Mdm2 to the ADRB2-β-arrestin complex and that the interaction of Mdm2 with the ADRB2$_{CIB}$ complex continues until Arrestin$_{CRY}$ dissociates from ADRB2$_{CIB}$.

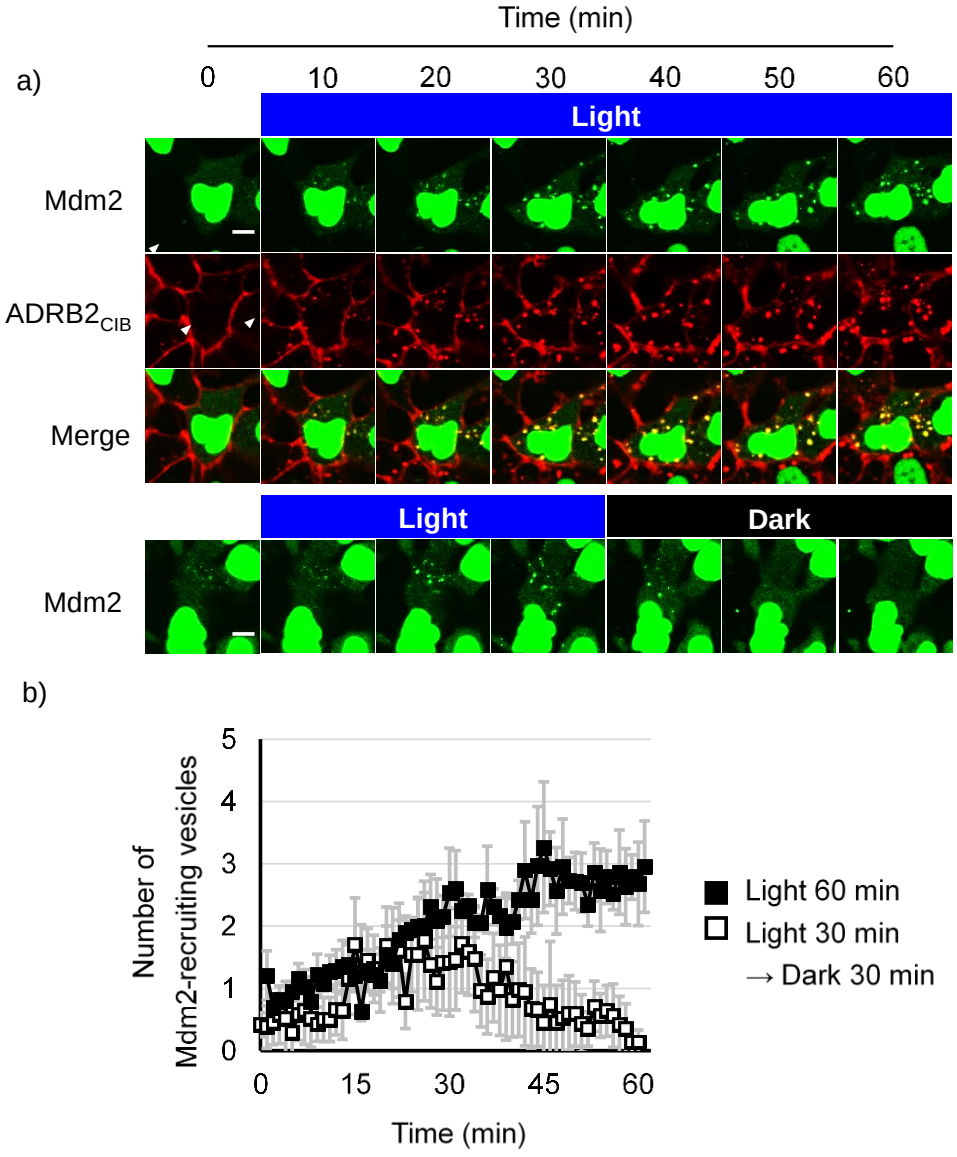

Figure 3-4. Observation of the dynamics of Mdm2 and ADRB2$_{CIB}$.

a) Time-lapse images of Mdm2 and $ADRB2_{CIB}$ in $HEK293_{optCLIP}$ cells. Cells expressing mCherry-fused Mdm2 were stimulated with blue light for 60 min (upper) or for 30 min followed by an additional 30 min in the dark (lower). Red, $ADRB2_{CIB}$; green, mCherry-fused Mdm2. Arrowheads, nuclei. Bar, 10 μm. b) Temporal changes in the number of Mdm2-recruiting vesicles per cell. The filled square shows the number of vesicles in cells irradiated for 60 min. The opened square shows the number of the vesicles in cells irradiated for 30 min and then incubated in dark for 30 min. Error bars: mean ± s.e.m. (n = 14 cells from three individual experiments).

3-3-5. Examination of the Ability of Arrestin$_{CRY}$ to Desensitize ISO-activated ADRB2$_{CIB}$

Desensitization of ligand-activated GPCRs is one of the important functions of β-arrestin. To examine whether light-induced recruitment of Arrestin$_{CRY}$ desensitizes the ISO-activated ADRB2$_{CIB}$, I investigated cAMP production after ISO stimulation under blue light irradiation using cAMP biosensor (**Figure 3-5**). The light irradiation did not affect luminescence intensity changes, suggesting that light-induced recruitment of Arrestin$_{CRY}$ does not promote desensitization of ADRB2$_{CIB}$.

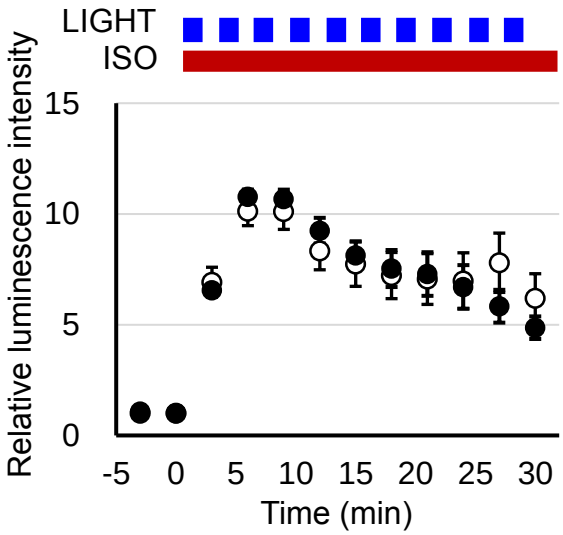

Figure 3-5. Investigation of the ability of Arrestin$_{CRY}$ to desensitize ISO-activated ADRB2$_{CIB}$.

Temporal changes in the luminescence intensity from GloSensor upon stimulation of ISO under light irradiation. The HEK293$_{opt}$ cells expressing the GloSensor were pretreated with 30 μM Dyngo4a and exposed to 1.0 μM ISO in the presence or absence of light irradiation. The luminescent intensity from the GloSensor was measured at every 3 mins. Blue light irradiation was performed for 1 min during an interval of each measurement. The luminescence intensities at each time point were normalized against those at 0 min. Filled circle; the irradiated cells, open circle; non-irradiated cells. Bars: mean ± s.e.m, (n = 7 from two individual experiments).

3-3-6. Investigation of ADRB2$_{CIB}$ Activity during Light Stimulation

To confirm the activity state of ADRB2$_{CIB}$ during the light stimulation, HEK293$_{opt}$ cells were transfected with cDNA coding a GFP-fused nanobody (Nb80), which binds only to the active state of ADRB2 interacting to G proteins[121]. The cells were stimulated with light or ISO for 30 min under a confocal microscope. The GFP-Nb80 colocalized with ADRB2$_{CIB}$-containing endosomes after ISO stimulation, indicating that ADRB2$_{CIB}$ has an ability to activate the downstream signaling upon stimulation of ISO stimulation (**Figure 3-6a**). In contrast, the colocalization of GFP-Nb80 with ADRB2$_{CIB}$ was not observed after light stimulation. To demonstrate the results quantitatively, I quantified the number of spots containing GFP-Nb80 and the colocalization of GFP-Nb80 with ADRB2$_{CIB}$. The spots containing GFP-Nb80 were not observed 30 min after light irradiation, although ISO stimulation induced increases in the number of spots (**Figure 3-6b**). The Manders' colocalization coefficient did not change after light stimulation, but increased after ISO stimulation (**Figure 3-6c**). These results suggest that endocytosed ADRB2$_{CIB}$ by light stimulation does not form an active state required for G protein signaling.

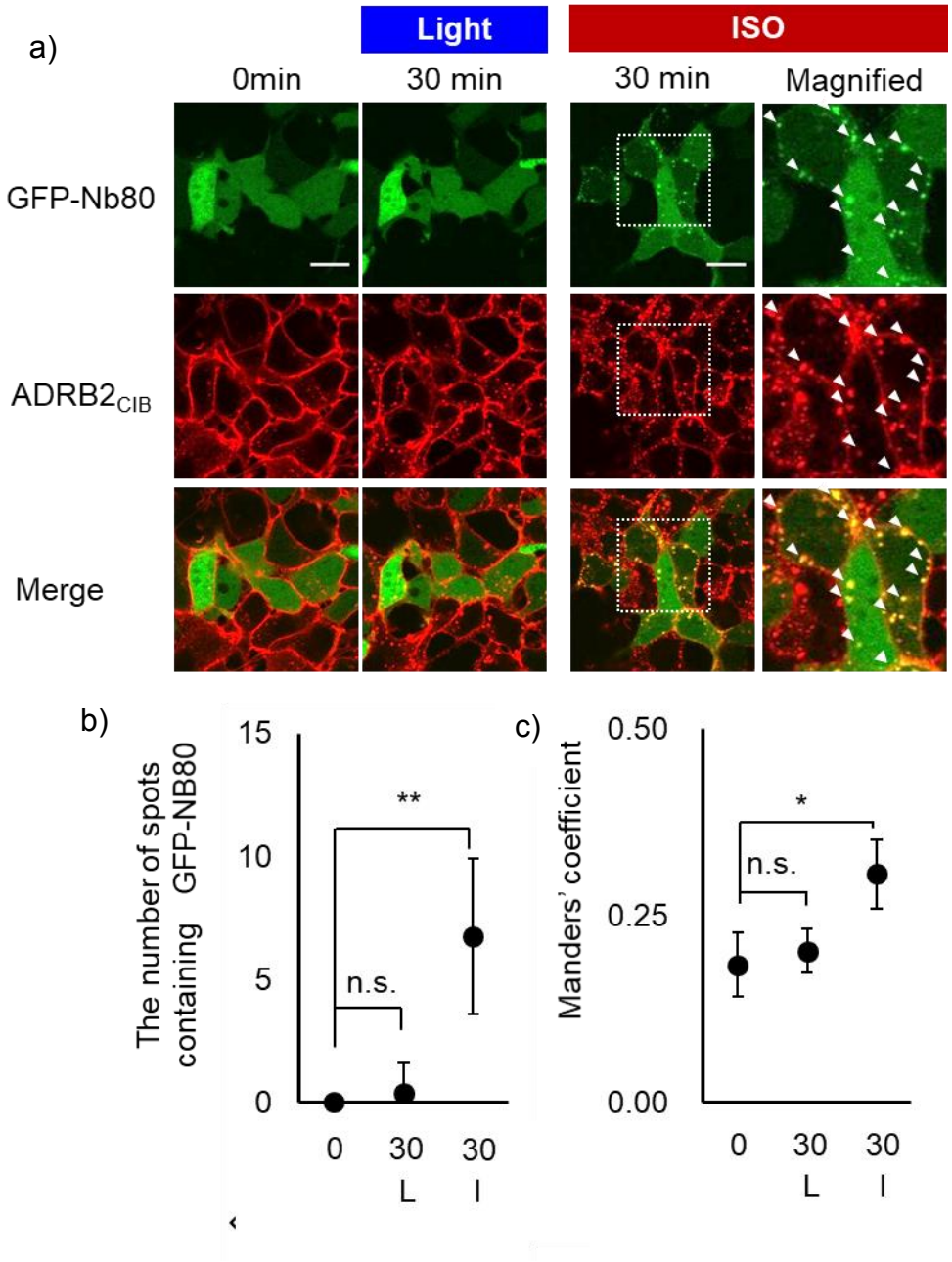

Figure 3-6. Examination of a state of ADRB2$_{CIB}$ during Light Irradiation.

The HEK293$_{opt}$ cells expressing GFP-fused Nb80 were stimulated with light or 1.0 μM ISO. Temporal changes of a) fluorescent imaging of GFP-Nb80 and ADRB2$_{CIB}$, b) number of particles containing GFP-Nb80 per cell, c) Manders' colocalization coefficient of Nb80 with ADRB2$_{CIB}$ in the HEK293$_{opt}$ cells stimulated with light or ISO. Scale bar shows 20 μm. Error bars: mean ± s.e.m, (n = 11 cells). L; Light, I; ISO. Statistical analysis was performed with Bonferroni post-hoc, $^{**}P < 0.01$ ($P = 8.8\times10^{-5}$) * $P < 0.05$ ($P = 0.018$), n.s. $P > 0.05$ (b; $P = 0.68$, c; $P = 0.48$).

3-3-7. Investigating the Ability of Arrestin$_{CRY}$ to Induce Phosphorylation of ERK1/2

β-Arrestin, after interacting with ADRB2, functions as a scaffold for the activation of MAPK signaling[72]. Ligand stimulation induces phosphorylation of ADRB2 via G protein-coupled receptor kinases. Phosphorylation of ADRB2 increases the affinity of ADRB2 for β-arrestin and changes the conformation of β-arrestin to form a complex with signaling molecules such as MAPK/ERK kinase (MEK) and ERK1/2, which is then phosphorylated through the interaction with β-arrestin[58]. To examine whether Arrestin$_{CRY}$ induces phosphorylation of ERK1/2 after the light-induced interaction of Arrestin$_{CRY}$ with ADRB2$_{CIB}$, HEK293$_{opt}$ cells were irradiated with blue light for 60 min. The phosphorylated ERK1/2 level was evaluated with its specific antibody (**Figure 3-7a**). ISO stimulation increased the amount of phosphorylated ERK1/2, whereas the phosphorylated ERK1/2 level decreased after light stimulation. To clarify the reason why light stimulation failed to increase the phosphorylated ERK1/2 level, I investigated phosphorylated ADRB2 (**Figure 3-7b**). The phosphorylation of ADRB2$_{CIB}$ was not detected after light irradiation. Considering that the phosphorylation of ADRB2 triggers a conformational change in β-arrestin that enables the recruitment of ERK1/2 and MEK to β-arrestin[72], I concluded that Arrestin$_{CRY}$ does not recruit ERK1/2 or MEK to the Arrestin$_{CRY}$-ADRB2$_{CIB}$ complex due to the lack of ADRB2 phosphorylation.

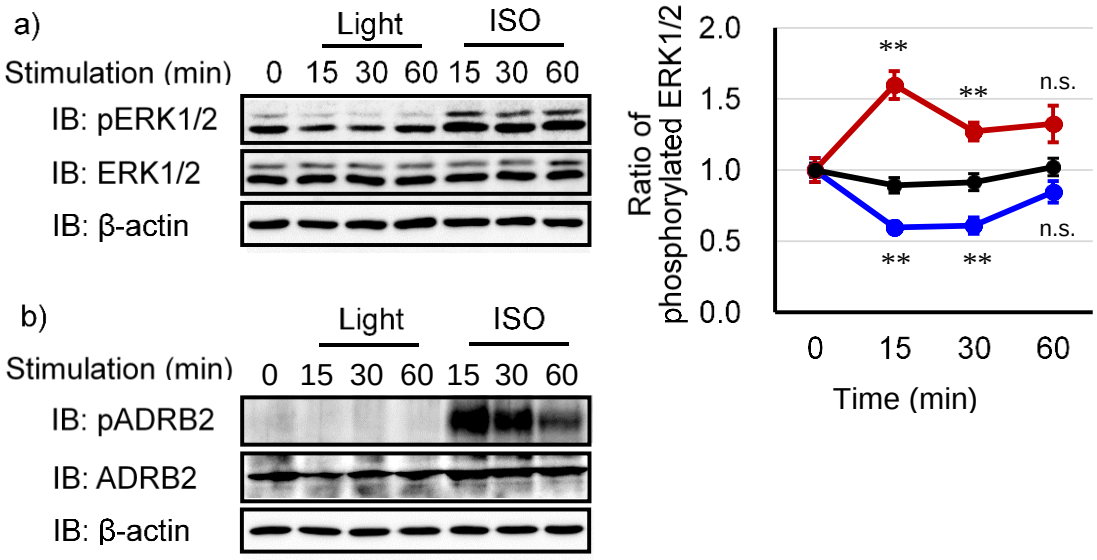

Figure 3-7. Detection of Phosphorylated ADRB2$_{CIB}$ and ERK1/2 after Light Irradiation.

Western blotting analysis for the detection of phosphorylated a) ERK1/2 and b) ADRB2$_{CIB}$ after stimulation with blue light (3 mW/cm^2) or 1.0 μM ISO. HEK293$_{opt}$ cells were starved in D-MEM containing 0.1% FBS for 12 h before the stimulation. Phosphorylation of ERK1/2 is quantified by the ratio of phosphorylated ERK1/2 to total ERK1/2 quantities, which were determined from the Western blots. The ratios were normalized by the values at 0 min. Blue: light irradiated; red: ISO stimulated; black: non-irradiated. Bar: mean ± s.e.m (n=8 from two individual experiments). Statistical analysis was performed by Bonferroni post-hoc test (non-irradiated v.s. light-irradiated or ISO-stimulated): ***P* values < 0.01 (Light: $P = 1.8\times10^{-3}$ (15 min), 7.4×10^{-3} (30 min); ISO: $P = 5.6\times10^{-5}$ (15 min), 3.1×10^{-3} (30 min)), n.s. $P > 0.05$ (a; Light: 0.28, ISO: 0.16).

3-3-8. Applicability of the Light Activation System to Other Membrane Receptors

To investigate the applicability of the present system to other membrane receptors, I replaced ADRB2 with different GPCRs such as the neurotensin receptor, muscarinic acetylcholine receptor M3, corticotropin releasing-factor receptor, and vasopressin 2 receptor. All the GPCRs that were investigated in this study localized on the cell surface before irradiation with blue light. Light stimulation induced translocation of $Arrestin_{CRY}$ to the cell surface and endocytosis of the GPCRs (**Figure 3-8a**). Furthermore, I applied the method to a non-GPCR receptor, transforming growth factor 3 receptor (TGF3R). TGF3R localized on the cell surface before light stimulation, whereas light stimulation led to the endocytosis of TGF3R, indicating that endocytosis of TGF3R is controllable by the recruitment of β-arrestin to TGF3R. These results demonstrate that the present method is widely applicable to inducing the endocytosis of membrane receptors in living cells. A previous report demonstrated that ERK1/2 phosphorylation was promoted by chemical dimerizer-induced interaction of β-arrestin with V2R[79], implying different regulations of MAPK activation between ADRB2 and V2R. To examine the difference, I quantified phosphorylated ERK1/2 after light-induced interaction of $Arretin_{CRY}$ with $V2R_{CIB}$. In contrast to the case of ADRB2, the light-induced interaction of β-arrestin with V2R promoted phosphorylation of ERK1/2 30 min after light irradiation (**Figure 3-8b**). The results suggest that β-arrestin regulates ERK1/2 phosphorylation in different manners depending on the GPCRs

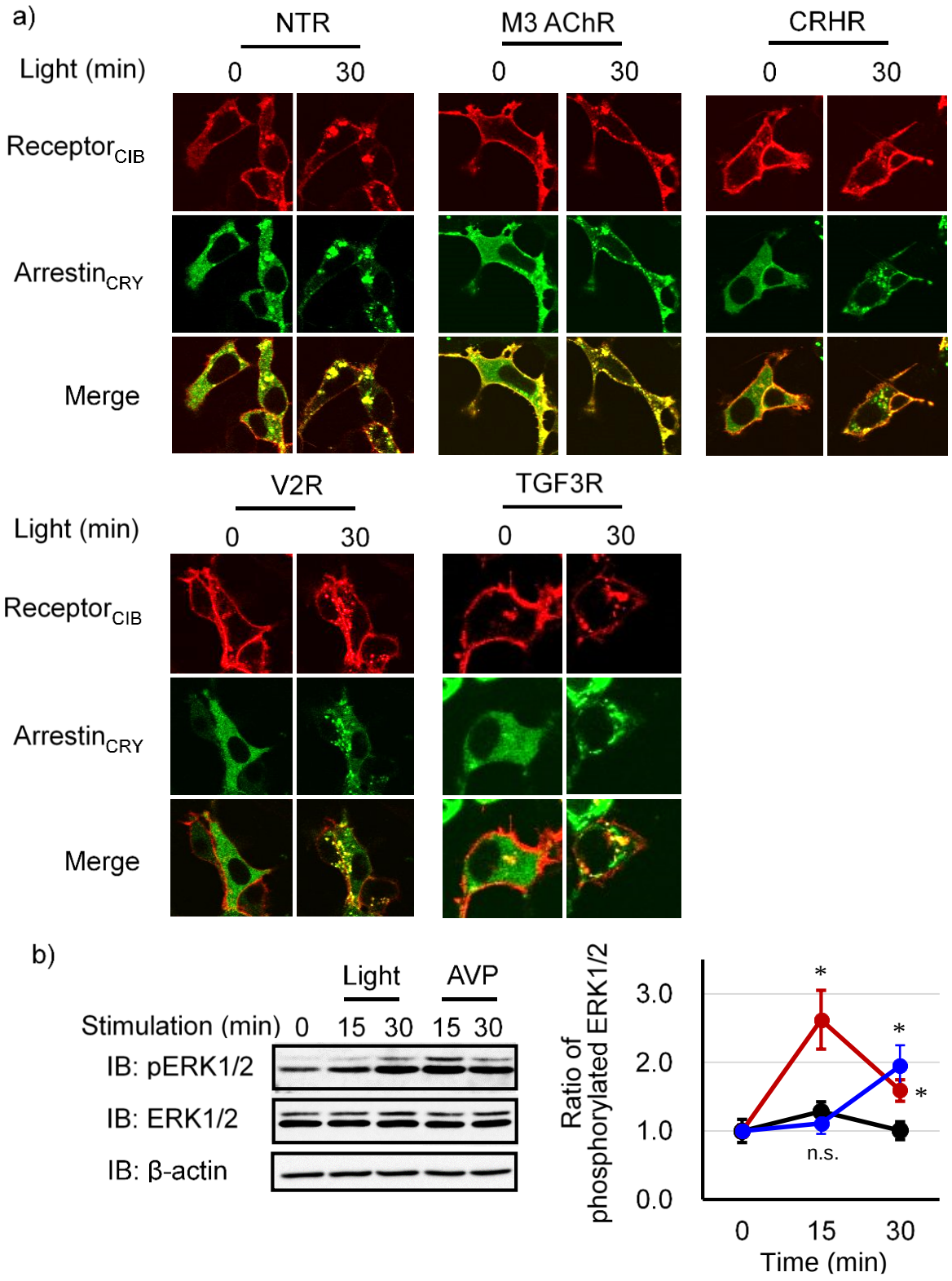

Figure 3-8. Applicability of other membrane receptors.

a) Images of CIB-fused receptor and $Arrestin_{CRY}$ fluorescence in HEK293 cells stimulated with blue light. HEK293 cells were transfected with cDNA encoding $Arrestin_{CRY}$ and CIB-fused membrane receptors. Neurotensin receptor, NTR; muscarinic acetylcholine receptor M3, M3 mAChR; corticotropin releasing-factor receptor, CRHR; vasopressin 2 receptor, V2R; and transforming growth factor 3 receptor, TGF3R. b) Temporal changes of ERK1/2 phosphorylation after light-induced interaction of $Arrestin_{CRY}$ with $V2R_{CIB}$. HEK293 cells expressing $Arrestin_{CRY}$ and $V2R_{CIB}$ were starved in D-MEM containing 0.1% FBS for 12 h. The cells were stimulated with blue light (3 mW/cm^2) or 1.0 μM vasopressin (AVP). Phosphorylation of ERK1/2 was quantified by the ratio of phosphorylated ERK1/2 to the total ERK1/2 quantities. The ratios were normalized by the values at 0 min. Blue: light irradiated; red: AVP stimulated; black: non-irradiated. Bar: mean ± s.e.m (n = 8 from two individual experiments). Statistical analysis was performed by Bonferroni post-hoc test (non-irradiated v.s. light-irradiated or AVP-stimulated): *P values < 0.05 (Light: P =, 0.024 (30 min); AVP: P = 0.020 (15 min), 0.024 (30 min)), n.s. P > 0.05 (Light: 0.82).

3-4. Discussion

I utilized the optical method for the analysis of intracellular trafficking of ADRB2 regulated by β-arrestin. I clarified the significance of the interaction of β-arrestin in the intracellular trafficking of ADRB2 using the CRY-CIB system. The ADRB2 on the cell surface was recovered after β-arrestin dissociated from ADRB2 in the dark, indicating that the recycling pathway was triggered by the dissociation of β-arrestin from ADRB2. Furthermore, ubiquitination and lysosome sorting were accelerated by prolonged irradiation. These results indicate that the duration of β-arrestin interaction with ADRB2 determines whether ADRB2 takes the lysosomal pathway (**Figure 3-9**).

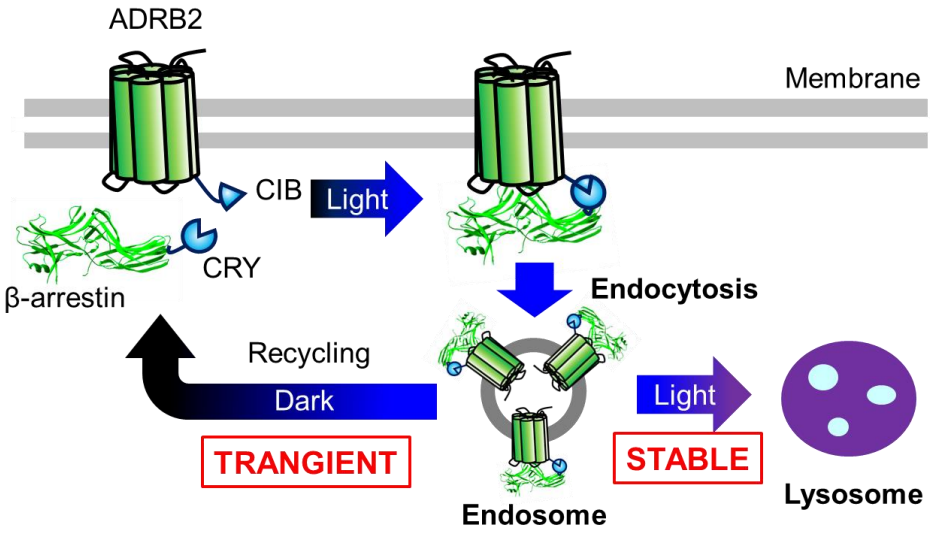

Figure 3-9. Trafficking regulation of ADRB2 revealed by photo-dimerizers

In addition, I monitored Mdm2 binding to and dissociating from $ADRB2_{CIB}$-$Arrestin_{CRY}$ complexes using light irradiation. I clarified the dynamics of ADRB2 and its regulatory proteins in the trafficking pathways. Light-induced manipulation of the interaction of β-arrestin with ADRB2 consequently clarified the

regulation of the intracellular trafficking of ADRB2. The present system will be a robust tool for investigating the dynamics of a wide variety of GPCRs and their regulatory proteins in trafficking pathways.

Furthermore, I examined other functions of β-arrestin such as desensitization and signaling. Light-induced interaction of β-arrestin with ADRB2 did not promote degradation of cAMP or activation of MAPK. The results indicate that the present method is applicable to controlling the trafficking of ADRB2 without inducing the desensitization or signaling, which is beneficial for investigation of mechanisms of the β-arrestin-mediated trafficking of ADRB2 in living cells and animals.

I induced prolonged interactions of β-arrestin with ADRB2 using light irradiation. GPCRs are divided into two classes (class A and class B) depending on their affinity with β-arrestin[64]. ADRB2 is a class A GPCR, which interacts with β-arrestin for a short period after stimulation by their ligands. I artificially prolonged the interaction of β-arrestin with ADRB2 using light stimulation. The sustained interaction led to greater ubiquitination and lysosome sorting of ADRB2 than stimulation with ISO, suggesting that the duration of the interaction between ADRB2 and β-arrestin determines the amount of ubiquitination and lysosome sorting. In addition, I demonstrated the light-induced endocytosis of 4 different types of GPCRs and a TGF3R. The light-endocytosed GPCRs are possibly transported in different kinetics because the trafficking is largely influenced by the amino acid residues in the C-terminus[59]. The investigation of the differences provides us valuable information about the significant roles of C-terminus of GPCRs in kinetics regulation of their trafficking without effects of other complicated factors such as ligand affinity to GPCRs and G protein signaling.

I examined the significance of the phosphorylation of ADRB2 in trafficking and signal

induction by artificially manipulating the interaction of β-arrestin with ADRB2. The direct manipulation of interactions between specific proteins has been a robust approach for analyzing the significance of protein-protein interactions in complicated intracellular networks[91]. The ligands of ADRB2 activate G proteins via ADRB2 on the cell surface, and the G protein signaling leads to phosphorylation of ADRB2, which increases the affinity of ADRB2 for β-arrestin. The interaction of β-arrestin with ADRB2 on the cell surface induces the endocytosis of ADRB2. I artificially induced the interaction between ADRB2 and β-arrestin via the CRY-CIB system without phosphorylating ADRB2. Light stimulation triggered the endocytosis of $ADRB2_{CIB}$, indicating that phosphorylation of ADRB2 is not necessary to initiate the recruitment of endocytic proteins such as clathrin and Mdm2 to ADRB2-β-arrestin complexes. However, the maximum induction level of the endocytosis of $ADRB2_{CIB}$ after light stimulation was lower than that induced by 0.1 and 1.0 μM ISO. The result implies that phosphorylation of ADRB2 is required for the efficient endocytosis of ADRB2 from the plasma membrane. I also evaluated the induction level of MAPK signaling after light irradiation. The level of phosphorylated ERK1/2 unexpectedly decreased after light irradiation. Although the reason for the phosphorylation decrease after light stimulation is not clear, the result indicates that other factors increase the amount of phosphorylated ERK1/2. For example, phosphorylated ADRB2 facilitates β-arrestin adopting a specific conformation to form an ADRB2-β-arrestin-MEK-ERK1/2 complex[122]. It was speculated that the lack of increase in phosphorylated ERK1/2 after light stimulation was due to the lack of phosphorylated ADRB2. To confirm the conformation of β-arrestin, several FRET- and BRET-based indicators were reported[123, 124]. However, these indicators contain bioluminescent, fluorescent proteins or chemical dyes, of which

absorption and emission wavelength overlap with absorption wavelength of CRY, suggesting a possibility to hamper a precise detection of the conformation of β-arrestin. To overcome the issue, a use of FRET indicator with another absorption and emission wavelength such as mKate and iRFP will be needed. Combination of a novel indicators and the present system will provide powerful approaches for investigation of the relevance between conformation of β-arrestin and intracellular trafficking on cell surface and endosomes. In addition, several studies have demonstrated that the rapamycin-induced interaction of β-arrestin with vasopressin receptors or chemokine receptors promotes the phosphorylation of ERK1/2[79,80]. Light-induced interaction of β-arrestin with V2R also activated the phosphorylation of ERK1/2. The result using ADRB2 is completely different from the results of the reports and our result, suggesting that the activation of MAPK signaling depends on the type of GPCR. I consequently suggested that the light-induced interaction of β-arrestin with ADRB2 is not sufficient for the activation of MAPK signaling, which is a significant clue to understanding the mechanisms of activation of β-arrestin-mediated signals.

I developed the photoinducible GPCR-β-arrestin interaction systems, and utilized them for investigation of the significant roles of β-arrestin on the endocytosis and intracellular trafficking of GPCRs. In addition, there are other potential applications of the present methods. Previous studies demonstrated importance of GPCR conformations for activation and inactivation of downstream signaling[121.] However, it is not well investigated whether the conformational state of GPCRs influences on their intracellular trafficking. The trafficking of GPCRs in a specific conformation may be possibly investigated using the present system together with antagonists or inverse agonists that induce specific GPCR conformations. This approach will clarify significant roles of

conformational states of GPCRs on intracellular trafficking. Furthermore, this approach is useful for the manipulation of trafficking of orphan GPCRs, whose ligands are undetermined. Recent studies have demonstrated that the trafficking of an orphan GPCR contributes to the pathology of Alzheimer disease[125,126]. However, knowing its specific ligands is required for further analysis of the pathological process. The present method will be useful for the analysis of such orphan GPCRs, without requiring their specific ligands, because the trafficking of GPCRs is controllable by external light. The present method can potentially determine the effects of trafficking a wide variety of membrane receptors on important diseases.

3-5. Conclusion

I examined the significance of the ADRB2-β-arrestin interaction on intracellular trafficking of ADRB2 using the developed method. Temporal manipulation of the interaction between β-arrestin and ADRB2 using this optogenetic system revealed the β-arrestin-mediated regulation of the intracellular trafficking of ADRB2. The dissociation of β-arrestin from ADRB2 under dark conditions triggered the recycling of the endocytosed ADRB2 to the cell surface, whereas the prolonged interaction of β-arrestin with ADRB2 promoted the sorting of ADRB2 to lysosomes. The duration of the reversible interaction of β-arrestin with ADRB2 therefore determines the intracellular trafficking of ADRB2. In addition, the recruitment of the regulatory protein Mdm2 to ADRB2-β-arrestin complexes was driven by light induction of the interaction between β-arrestin and ADRB2. Our method will be effective for investigating the dynamics of GPCRs and their regulatory proteins in trafficking pathways. Furthermore, MAPK signaling was not activated through the light-induced interaction of β-arrestin

with ADRB2; this behavior may be due to the light-induced interaction not causing a conformational change in β-arrestin that results in recruitment of MEK and ERK1/2. I consequently showed that the artificial induction of interactions between membrane receptors and their regulatory proteins via an optogenetic tool is a useful approach for investigating the intracellular trafficking of membrane receptors. Light-induced internalization of membrane receptors on cell surfaces and the manipulation of intracellular trafficking will be beneficial to unveiling significant roles of membrane receptors in living tissues and will also be useful for investigating the pathological processes of important diseases.

Chapter 4

General Conclusion

GPCRs are responsible for wide varieties of biological events in most of tissues including a brain and a heart. Due to their significance in living animals, the regulation of GPCR activities has been an attractive target in many fields of research. The significance is proved by the fact that almost the half of drugs in current use target GPCRs. The recent studies have demonstrated the importance roles of the intracellular trafficking on the GPCR signaling. However, comprehensive understanding of the intracellular trafficking has not been completed.

In the chapter 2, I developed a novel method to induce the interaction of β-arrestin with ADRB2. In the conventional approaches for analyzing the trafficking mechanisms, amino acid mutations were induced in GPCRs and β-arrestin. The mutation modulated the properties of GPCR and β-arrestin, resulting in the modulation of their trafficking and the duration of the GPCR-β-arrestin interaction. Although the mutagenetic approach suggested the significance of the specific amino acid residues in GPCR and β-arrestin, it is difficult to confirm whether the interaction between β-arrestin and GPCR contributes directly on the trafficking. The chemical dimerizers were also utilized for artificial induction of the interaction of β-arrestin with GPCRs. However, the chemical dimerizers are not suitable for inducing dissociation of β-arrestin from GPCRs due to the strong affinity of rapamycin to chemical dimerizers. To overcome the problems of conventional approaches, I utilized photo-dimerizers CRY and CIB. Light irradiation induced the interaction of $ADRB_{CIB}$ and $Arrestin_{CRY}$, which triggered endocytosis of $ADRB2_{CIB}$. I demonstrated that $Arrestin_{CRY}$ dissociated from $ADRB2_{CIB}$ under dark condition. These results suggest that the optical method is suitable for reversible control of the interaction between ADRB2 and β-arrestin.

In the chapter 3, I utilized the optical method for investigation of the trafficking

mechanism of ADRB2. The dissociation of β-arrestin from ADRB2 induced recycling of ADRB2 to the cell membrane, while the prolonged interaction of β-arrestin with ADRB2 promoted the recruitment of ADRB2 to lysosomes. Taken together, I concluded that the duration of the interaction of ADRB2 and β-arrestin decides the intracellular trafficking of ADRB2. Furthermore, I demonstrated that the light induced interaction did not trigger the remaining functions of β-arrestin (desensitization of activated ADRB2 and activation of MAPK signaling), which suggests that we can investigate the trafficking regulation without considering the other functions that closely related to each other. Furthermore, the present methods have a potential to offer further analysis of the intracellular trafficking of GPCRs. Although GPCR signaling is affected by conformational and oligomeric states of GPCRs, it is not clear how the specific states of GPCRs affect the intracellular trafficking. The present methods enable us investigating significance of specific states of GPCRs in the trafficking.

I developed optical methods to analyze the regulation of intracellular trafficking of GPCRs using photodimerizers. Temporal regulation of the interaction of GPCR and β-arrestin reveals the intracellular regulation of GPCR through β-arrestin. The methods are widely applicable for the investigation of trafficking of membrane receptors and proteins. Temporal control of β-arrestin-receptor interactions will enable the investigation of the unique roles of β-arrestin and the mechanism by which it regulates β-arrestin-specific trafficking pathways of different membrane receptors.

Acknowledgement

The present thesis was completed under a supervision of Prof. Takeaki Ozawa. I would like to thank Prof. Takeaki Ozawa for giving me kind advices and chances to make presentations in abroad labs, and offering many outstanding supports through discussion. I appreciate all stuff members in Ozawa Lab., especially Dr. Hideaki Yoshimura, Dr. Mitsuru Hattori, Dr. Natsumi Noda, Dr. Masaki Takeuchi for heartful encouragements and advises to complete the thesis in the Ph. D. course. My thanks as well go to Dr. Yusuke Nasu, who always helped me when I encountered the difficulties during my Ph. D. course. Without their technical and heartful supports, I would not complete the present thesis.

Finally, I am grateful to my family who always believe in me and support my ordinary life. Their supports always encourage me to overcome the difficulties and select the best choice in my life.

References

1. Cuatrecasas, P. Membrane receptors. *Annu. Rev. Biochem*. **43**, 169–214 (1974)
2. Lohse, M.J. Molecular mechanisms of membrane receptor desensitization. *Biochimica et Biophysica Acta*. **1179**, 171–188 (1993)
3. Tsai, M. J. & O'Malley, B. W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem*. **63**, 451–486 (1994)
4. Sanders, C. & Myers, J. Disease-related misassembly of membrane proteins. *Annu. Rev. Biophys. Biomol. Struct*. **33**, 25–51 (2004)
5. Beavo, J.A. & Brunton, L.L. Cyclic nucleotide research–still expanding after half a century. *Nat. Rev. Mol*. Cell Biol. **3**, 710–718 (2002)
6. Schmidt, H.H.H.W., Lohmann, S.M., & Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochimica et Biophysica Acta* **1178**, 153–175 (1993)
7. Rasmussen, H. & Goodman, D.B. Relationships between calcium and cyclic nucleotides in cell activation. *Physiol. Rev.* **57**, 421–509 (1977)
8. Berridge, M. J., Bootman, M. D. & Roderick, H. L. Calcium signalling: dynamics, homeostasis and remodelling. *Nature Rev. Mol. Cell Biol*. **4**, 517–529 (2003)
9. Tasken, K. & Aandahl, E. M. Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol. Rev*. **84**, 137–167 (2004)
10. Montminy, M.R., Gonzalez, G.A., & Yamamoto, K.K. Regulation of cAMP-inducible genes by CREB. *Trends Neurosci*. **13**, 184–188 (1990)
11. Berridge, M.J. Inositol trisphosphate and calcium signalling mechanisms. *Biochim. Biophys. Acta.* **1793**, 933–940 (2009)

12. Ferris, C. D., & Snyder, S. H. lnositol1,4,5-trisphosphateactivated calcium channels. Annu. *Rev. Physiol*. **54**, 469-488 (1992)
13. Brose, N., Betz, A. & Wegmeyer, H. Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Curr. Opin. Neurobiol*. **14**, 328–340 (2004)
14. Wakelam, M. J. O. Diacylglycerol - when is it an intracellular messenger? *Biochim. Biophys. Acta* **1436**, 117–126 (1998)
15. Purvis, J.E., & Lahav, G. Encoding and Decoding Cellular Information through Signaling Dynamics. *Cell*. **152**, 945–956 (2013)
16. Vaudry, D., Stork, P.J., Lazarovici, P. & Eiden, L.E. Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**, 1648–1649 (2002)
17. Liang, J., Wang, Y.J., Tang, Y., Cao, N., Wang, J., & Yang, H.T. Type 3 inositol 1,4,5-trisphosphate receptor negatively regulates apoptosis during mouse embryonic stem cell differentiation. *Cell Death Differ* **17**, 1141–54 (2010)
18. Szatkowski, C., & Parys, J.B., Ouadid-Ahidouch, H., and Matifat, F. Inositol 1,4,5-trisphosphate-induced Ca^{2+} signalling is involved in estradiolinduced breast cancer epithelial cell growth. *Mol. Cancer* **9**, 156 (2010)
19. Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S. & Caron, M.G. Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* **274**, 32248–32257 (1999)
20. Dupre, D.J., Robitaille, M., Rebois, R.V., & Hebert, T.E. The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. *Annu Rev Pharmacol Toxicol*. **49**, 31–56 (2009)

21. Ferguson, S.S. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1–24 (2001)

22. Kehrl, J.H. Heterotrimeric G protein signaling: Roles in immune function and fine-tuning by RGS proteins. *Immunity* **8**, 1–10 (1998)

23. Luttrell, L.M. Reviews in molecular biology and biotechnology: transmembrane signalling by G protein-coupled receptors. *Mol Biotechnol* **39**, 239–264 (2008)

24. Hata, J.A. & Koch, W.J. Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease *Mol. Interv.*, **3**, 264-272 (2003)

25. Spiegel, A.M. Mutations in G proteins and G protein-coupled receptors in endocrine disease. *J. Clin. Endocrinol. Metab*. **81**, 2434–2442 (1996)

26. Thathiah, A. et al. The orphan G protein-coupled receptor 3 modulates amyloid-beta peptide generation in neurons. *Science* **323**, 946–951 (2009)

27. Conn, P.J., Christopoulos, A. & Lindsley, C.W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature Rev. Drug Discov*. **8**, 41–54 (2009)

28. Sun, J., Singh, V., Kajino-Sakamoto, R. & Aballay, A. Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. *Science* **332**, 729–732 (2011)

29. Penela, P., Murga, C., Ribas, C., Tutor, A.S., Peregrin, S., & Mayor, F. Jr. Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. *Cardiovasc Res*. **69**, 46 –56 (2006)

30. Pai, R. et al. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nature Med*. **8**, 289–293 (2002)

31. Lautrette, A. et al. Angiotensin II and EGF receptor cross-talk in chronic kidney diseases: a new therapeutic approach. *Nat. Med*. **11**, 867–874 (2005)

32. Jin, T., Xu, X., & Hereld, D. Chemotaxis, chemokine receptors and human disease. *Cytokine* **44**, 1–8 (2008)

33. Dorsam, R.T. & Gutkind, J.S. G-protein-coupled receptors and cancer. *Nature Rev. Cancer* **7**, 79–94 (2007)

34. Kobilka, B.K. & Deupi, X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **28**, 397–406 (2007)

35. Deupi, X. & Standfuss, J. Structural insights into agonist-induced activation of G-protein-coupled receptors. *Curr. Opin. Struct. Biol.* **21**, 541–551 (2011)

36. Gales, C. et al. Real-time monitoring of receptor and G-protein interactions in living cells. *Nat. Methods* **2**, 177–184 (2005)

37. Luttrell, L. M., Daaka, Y. & Lefkowitz, R.J. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Opin. Cell Biol.* **11**, 177–183 (1999)

38. Wetzker R, Bohmer FD Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nature Rev. Mol. Cell Biol.* **4**, 651–657 (2003)

39. Sugden, P.H., & Clerk, A. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal*. **9**, 337–351 (1997)

40. Kolch, W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nature Rev. Mol. Cell Biol.* **6**, 827–837 (2005)

41. Roux, P.P. & Blenis, J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **68**, 320–344 (2004)

42. Ribas, C. et al. The G protein-coupled receptor kinase (GRK) interactome: role of

GRKs in GPCR regulation and signaling. *Biochim. Biophys. Acta* **1768**, 913–922 (2007)

43. Penn, R.B., Pronin, A.N., Benovic, J.L. Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc. Med.* **10**, 81–89 (2000)
44. Penela, P., Ribas, C. & Mayor, F., Jr. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell. Signal.* **15**, 973–981 (2003)
45. Chen, W. et al. β-arrestin 2 mediates endocytosis of type III TGF-β receptor and down-regulation of its signaling. *Science* **301**, 1394–1397 (2003).
46. Lin, F.T., Daaka, Y. & Lefkowitz, R.J. β-arrestins regulate mitogenic signaling and clathrin-mediated endocytosis of the insulin-like growth factor I receptor. *J. Biol. Chem.* **273**, 31640–31643 (1998).
47. Gurevich, V.V., Gurevich, E.V. The molecular acrobatics of arrestin activation. *Trends Pharmacol. Sci.* **25**,105–11 (2004)
48. Han, M, Gurevich, V.V., Vishnivetskiy, S.A., Sigler, P.B., & Schubert, C. Crystal structure of β-arrestin at 1.9 A. Possible mechanism of receptor binding and membrane ˚translocation. *Structure* **9**, 869–80 (2001)
49. Miller, W.E. & Lefkowitz, R.J. Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr. Opin. Cell Biol.* **13**, 139–145 (2001)
50. Laporte, S.A., Oakley, R.H., Holt, J.A., Barak, L.S. & Caron, M.G. The interaction of β-arrestin with the AP-2 adaptor is required for the clustering of β2-adrenergic receptor into clathrin-coated pits. *J. Biol. Chem.* **275**, 23120–23126 (2000)
51. Zhang, J., Ferguson, S.S.G., Barak, L.S., Menard, L., & Caron, M.G. Dynamin and

β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* **271**, 18302–5 (1996)

52. Moore, C.A., Milano, S.K. & Benovic, J.L. Regulation of receptor trafficking by GRKs and arrestins. *Annu. Rev. Physiol*. **69**, 451–482 (2007)
53. Shenoy S.K. & Lefkowitz R.J. β-arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol. Sci*. **32**, 521–33 (2011)
54. Hanyaloglu, A.C. & von Zastrow, M. Regulation of GPCRs by Endocytic Membrane Trafficking and Its Potential Implications. *Annu. Rev. Pharmacol. Toxicol*. **48**, 537–568 (2008)
55. Amara, J.F., Cheng, S.H. & Smith, E. Intracellular protein trafficking defects in human disease. *Trends Cell Biol.* **2**, 145–149 (1992)
56. Jean-Alphonse, F. & Hanyaloglu, A.C. Regulation of GPCR signal networks via membrane trafficking. *Mol. Cell. Endocrinol.* **331,** 205–214 (2011)
57. Marchese, A., Paing, M.M., Temple, B.R.S. & Trejo, J. G Protein–Coupled Receptor Sorting to Endosomes and Lysosomes. *Annu. Rev. Pharmacol. Toxicol.* **48**, 601–629 (2008)
58. Nobles, K.N. et al. Distinct Phosphorylation Sites on the β2-Adrenergic Receptor Establish a Barcode That Encodes Differential Functions of β-Arrestin. *Sci. Signal.* **185**, 1–10 (2012)
59. Tan, C.M., Brady, A.E., Nickols, H.H., & Wang, Q., Limbird LE. Membrane trafficking of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol*. **44**, 559 – 609. (2004)
60. Cao, T.T., Deacon, H.W., Reczek, D., Bretscher, A. & von Zastrow, M. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the

β2-adrenergic receptor. *Nature* **401**, 286–290 (1999)

61. Zhang, J. et al. Cellular Trafficking of G Protein-coupled Receptor/β-Arrestin Endocytic Complexes. *J. Biol. Chem.* **274**, 10999–11006 (1999).
62. Hicke, L. & Riezman, H. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **84**, 277–287 (1996)
63. Shenoy, S.K., McDonald, P.H., Kohout, T.a & Lefkowitz, R.J. Regulation of receptor fate by ubiquitination of activated β2-adrenergic receptor and β-arrestin. *Science* **294**, 1307–1313 (2001)
64. Marchese, A. & Benovic, J.L. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J. Biol. Chem*. **276**, 45509–45512 (2001).
65. Shenoy, S.K. & Lefkowitz, R.J. Trafficking Patterns of β-Arrestin and G Protein-coupled Receptors Determined by the Kinetics of β-Arrestin Deubiquitination. *J. Biol. Chem.* **278**, 14498–14506 (2003)
66. Shenoy, S.K. et al. β-Arrestin-dependent signaling and trafficking of 7-transmembrane receptors is reciprocally regulated by the deubiquitinase USP33 and the E3 ligase Mdm2. *Proc. Natl Acad. Sci. USA* **106**, 3–8 (2009)
67. Lefkowitz, R.J. & Shenoy, S.K. Transduction of Receptor Signals. *Science* **308**, 512–518 (2005)
68. Coffa, S. et al., The effect of arrestin conformation on the recruitment of c-Raf1, MEK1, and ERK1/2 activation. *PLoS ONE* **6**, e28723 (2011)
69. Shukla, A.K., Xiao, K., & Lefkowitz, R.J. Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem. Sci.* **36**, 457–469 (2011)

70. Hunton, D.L. et al. β-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis. *Mol. Pharmacol.* **67**, 1229–1236 (2005)

71. Tohgo, A., Pierce, K.L., Choy, E.W., Lefkowitz, R.J., & Luttrell, L.M. β-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J. Biol. Chem.* **277**, 9429–36 (2002)

72. Shenoy, S.K. et al. Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J. Biol. Chem*. **281**, 1261–1273 (2006)

73. Ahn, S., Kim, J., Hara, M. R., Ren, X. R. & Lefkowitz, R. J. β-arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. *J. Biol. Chem.* **284**, 8855–8865 (2009)

74. Beaulieu, J.M. et al. A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* **132**, 125–136 (2008)

75. Beaulieu, J.M. et al. An Akt/β-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* **122**, 261–273 (2005)

76. McDonald, P.H. et al. β-ARrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574–1577 (2000)

77. Xiao K, & Shenoy, S.K. Beta2-adrenergic receptor lysosomal trafficking is regulated by ubiquitination of lysyl residues in two distinct receptor domains. *J Biol Chem* **286**, 12785–12795 (2011)

78. Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S., & Caron, M.G.. Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis. *J. Biol. Chem*. **276**, 19452–60 (2001)

79. Terrillon, S. & Bouvier, M. Receptor activity-independent recruitment of βarrestin2 reveals specific signalling modes. *EMBO J.* **23**, 3950–3961 (2004).

80. Liebick, M., Henze, S., Vogt, V. & Oppermann, M. Functional consequences of chemically-induced β-arrestin binding to chemokine receptors CXCR4 and CCR5 in the absence of ligand stimulation. *Cell. Signal.* **38**, 201–211 (2017).

81. Lin, Y. et al. Rapidly Reversible Manipulation of Molecular Activity with Dual Chemical Dimerizers. *Angew. Chem. Int. Edn Engl.* **52**, 6450–6454 (2013).

82. Adamantidis, A.R., Zhang, F., Aravanis, A.M., Deisseroth, K. & de Lecea, L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* **450**, 420–424 (2007)

83. Packer, A.M., Roska, B. & Hausser, M. Targeting neurons and photons for optogenetics. *Nat. Neurosci.* **16**, 805–815 (2013).

84. Fenno, L., Yizhar, O. & Deisseroth, K. The development and application of optogenetics. *Annu. Rev. Neurosci.* **34**, 389–412 (2011).

85. Kramer, R.H., Mourot, A. & Adesnik, H. Optogenetic pharmacology for control of native neuronal signaling proteins. *Nature Neurosci.* **16**, 816–823 (2013).

86. Tye, K.M. & Deisseroth, K. Optogenetic investigation of neural circuits underlying brain disease in animal models. Nature Rev. Neurosci. 13, 251–266 (2012)

87. Entcheva, E. Cardiac optogenetics. *Am. J. Physiol. Heart Circ. Physiol*. **304**, H1179–H1191 (2013)

88. Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci*. **8**, 1263–1268 (2005).

89. Mourot, A. et al. Rapid optical control of nociception with an ion-channel

photoswitch. *Nat. Methods* **9**, 396–402 (2012).

90. Berndt, A., Lee, S. Y., Ramakrishnan, C. & Deisseroth, K. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science* **344**, 420–424 (2014)
91. Tischer, D. & Weiner, O.D. Illuminating cell signalling with optogenetic tools. *Nat. Rev. Mol. Cell Biol.* **15**, 551–558 (2014).
92. Zhang, K. & Cui, B. Optogenetic control of intracellular signaling pathways. Trends Biotechnol. 33, 92–100 (2015).
93. Weitzman, M. & Hahn, K.M. ScienceDirect Optogenetic approaches to cell migration and beyond. *Curr. Opin. Cell Biol*. **30**, 112–120 (2014).
94. Liu, H., Liu, B., Zhao, C., Pepper, M. & Lin, C. The action mechanisms of plant cryptochromes. *Trends Plant Sci.* **16**, 684–691 (2011).
95. Kennedy, M.J. et al. Rapid blue-light-mediated induction of protein interactions in living cells. *Nat. Methods* **7**, 973–975 (2010).
96. Nguyen, M.K. et al. Optogenetic oligomerization of Rab GTPases regulates intracellular membrane trafficking. *Nat. Chem. Biol*. **12**, 431–436 (2016).
97. Bugaj, L.J., Choksi, A. T., Mesuda, C.K., Kane, R.S. & Schaffer, D.V. Optogenetic protein clustering and signaling activation in mammalian cells. *Nat. Methods* **10**, 249–252 (2013)
98. Idevall-Hagren, O., Dickson, E.J., Hille, B., Toomre, D. K. & De Camilli, P. Optogenetic control of phosphoinositide metabolism. *Proc. Natl Acad. Sci. USA* **109**, E2316–E2323 (2012).
99. Park, H., Kim, N.Y., Lee, S., Kim, N., Kim, J., & Heo W.D., Optogenetic protein clustering through fluorescent protein tagging and extension of CRY2, *Nat.*

Commun. **8**, 1–8 (2017)

100. Konermann, S. et al. Optical control of mammalian endogenous transcription and epigenetic states. *Nature* **500**, 472–476 (2013).

101. Shin, Y., Berry, J., Pannucci, N., Haataja, M.P., Toettcher, J.E., & Brangwynne, C.P. Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. *Cell* **168**, 159-171 (2017)

102. Stephens, D.J. & Allan, V.J. Light microscopy techniques for live cell imaging. *Science* **300**, 82–86 (2003).

103. Wilt, B.A. et al. Advances in light microscopy for neuroscience. *Annu. Rev. Neurosci.* **32**, 435–506 (2009).

104. Balleza, E., Kim, J.M. & Cluzel, P. Systematic characterization of maturation time of fluorescent proteins in living cells. *Nat. Methods*, online, 1–5 (2017)

105. Grimm, J. B. et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat. Methods*, **14**, 987–994 (2017)

106. Gautier, A. et al. An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol.* **15**, 128–136 (2008).

107. Bolte, S. & Cordelieres, F.P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc*. **224**, 213–232 (2006)

108. Adler, J., & Parmryd, I. Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry A* **77**, 733–742. (2010)

109. Dunn, K.W., Kamocka, M.M. & McDonald, J.H. A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol*. **300**, C723–C742 (2011)

110. Fichter, K.M., Flajolet, M., Greengard, P., Vu, T.Q. Kinetics of G-protein-coupled receptor endosomal trafficking pathways revealed by single quantum dots. *Proc Natl Acad Sci USA*, **107**, 18658–18663 (2010)

111. Berlin, I., Higginbotham, K.M., Dise, R.S., Sierra, M.I. and Nash, P.D. The deubiquitinating enzyme USP8 promotes trafficking and degradation of the chemokine receptor 4 at the sorting endosome. *J. Biol. Chem*. **285**, 37895-37908 (2010)

112. Kong, M.M., Fan, T., Varghese, G., O'Dowd, B.F., & George, S.R. Agonist-induced cell surface trafficking of an intracellularly sequestered D1 dopamine receptor homo-oligomer. *Mol. Pharmacol.* **70**, 78–89 (2006)

113. Koch, W.J., Lefkowitz, R.J. & Rockman, H.A. Functional consequences of altering myocardial adrenergic receptor signaling. *Annu. Rev. Physiol.* **62**, 237–60 (2000).

114. Johnson, M. Molecular mechanisms of β2-adrenergic receptor function, response, and regulation. *J. Allergy Clin. Immunol* **117**, 18–24 (2006).

115. George, S.R., O'Dowd, B.F. & Lee, S.P. G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat. Rev. Drug Discov. 1, 808–820 (2002).

116. Che, D.L., Duan, L., Zhang, K. & Cui, B. The dual characteristics of light-induced cryptochrome 2, homo-oligomerization and heterodimerization, for optogenetic manipulation in mammalian cells. *ACS Synth. Biol*. **4**, 1124–1135 (2015).

117. Ferrandon, S. et al. Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nature Chem. Biol.* **5**, 734–742 (2009).

118. Sungkaworn, T. et al. Single-molecule imaging reveals receptor–G protein interactions at cell surface hot spots. *Nature* **550**, 543–547 (2017).

119. Wang, P. et al. β-Arrestin 2 Functions as a G-Protein-coupled Receptor-activated Regulator of Oncoprotein Mdm2. *J. Biol. Chem*. **278**, 6363–6370 (2003).

120. Girnita, L. et al. β-arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth Factor-1 receptor by acting as adaptor for the MDM2 E3 ligase. *J. Biol. Chem.* **280**, 24412–24419 (2005).

121. Nygaard, R. et al. The dynamic process of β2-adrenergic receptor activation. *Cell* **152**, 532–542 (2013).

122. Charest, P.G., Terrillon, S. & Bouvier, M. Monitoring agonist-promoted conformational changes of β-arrestin in living cells by intramolecular BRET. *EMBO Rep.* **6**, 334–340 (2005).

123. Lee, M. et al. The conformational signature of arrestin3 predicts its trafficking and signalling functions. *Nature* **531**, 665–668 (2016).

124. Nuber, S. et al. β-Arrestin biosensors reveal a rapid, receptor-dependent activation/deactivation cycle. *Nature* **531**, 661–664 (2016).

125. Thathiah, A. et al. The orphan G protein-coupled receptor 3 modulates amyloid-β peptide generation in neurons. *Science* **323**, 946–951 (2009)

126. Thathiah, A. et al. β-arrestin 2 regulates Aβ generation and γ-secretase activity in Alzheimer' s disease. *Nat. Med.* **19**, 43–9, (2013).