論文の内容の要旨

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)

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Precise regulation of intracellular trafficking of membrane receptors is important for cells to adapt to a wide variety of extracellular stimuli. The perturbation of trafficking systems induces important diseases, including diabetes, cancers and neurodegenerative diseases. Investigation of the trafficking mechanism is important for drug development and therapeutics. β-Arrestin is a representative protein that regulates the activity and localization of membrane receptors, such as G protein-coupled receptors (GPCRs), transforming growth factor receptor (TGFR) and insulin-like growth factor receptor (IGFR). Ligand stimulation activates the GPCR signaling and induces β-arrestin recruitment to GPCRs. GPCRs are sequestrated from the cell surface by endocytosis. Imaging analysis of GPCRs and β-arrestin demonstrated that GPCRs that stably interact with β-arrestin are retained in the cytosol, whereas GPCRs that immediately dissociate from β-arrestin are rapidly recycled back to the cell membrane. The results suggest the significance of β-arrestin in the GPCR trafficking. Despite the significance of β-arrestin-related trafficking, the manner by which β-arrestin controls the trafficking pathways of GPCRs is unclear. Herein, I investigate the GPCT trafficking regulation using photodimerizers; cryptochrome (CRY) and cryptochrome-interacting basic-helix-loop-helix 1 (CIB). I demonstrate an optogenetic approach using the reversible interaction between CRY and CIB to investigate the significance of the interaction of β-arrestin with GPCRs in intracellular trafficking.

β2-adrenergic receptor (ADRB2) is a GPCR that regulates cardiovascular and pulmonary functions. Isoproterenol chloride (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 (termed Arrestin$_{CRY}$) (Fig. 1 upper). Before blue light irradiation, ADRB2$_{CIB}$ locates on the cell membrane, and Arrestin$_{CRY}$ distributes throughout the cytosol (Fig. 1 lower). Blue light stimulation induces translocation of Arrestin$_{CRY}$ to ADRB2$_{CIB}$ on the cell membrane by the interaction between CRY and CIB, which triggers 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.

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 microscope (Fig. 2). Arrestin$_{CRY}$ was translocated from the cytosol to the cell membrane a few min after the start of irradiation. ADRB2$_{CIB}$ fluorescent spots appeared in the cytosol at 15 min, and their number gradually increased until 30 min of irradiation. The result suggest that light induced interaction between ADRB2$_{CIB}$ and Arrestin$_{CRY}$ induced their endocytosis.

To confirm reversibility of the light-induced interaction between ADRB2$_{CIB}$ and Arrestin$_{CRY}$, HEK293$_{opt}$ cells were irradiated with blue light for 30 min and then incubated in the dark condition. After colocalization of Arrestin$_{CRY}$ with ADRB2$_{CIB}$, Arrestin$_{CRY}$ redistributed uniformly in the cytosol under dark conditions (Fig. 3a). The fluorescence intensity of Arrestin$_{CRY}$ in the cytosol decreased 1 min after irradiation and then recovered to the basal level 10 min after irradiation ceased (Fig. 3b). The results confirm that Arrestin$_{CRY}$ dissociated from ADRB2$_{CIB}$ in the dark. Taken together, I concluded that the interaction between ADRB2 and β-arrestin was reversibly controlled using the CRY-CIB system.

To investigate the trafficking pathway after dissociation of Arrestin$_{CRY}$ from ADRB2$_{CIB}$, 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.

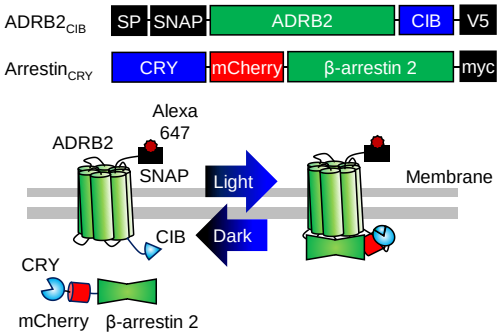

Figure 1. Light-based manipulation of reversible interaction between β-arrestin and ADRB2 using photodimerizers.
Blue light stimulation induces interaction of CRY with CIB, which leads to the interaction of β-arrestin with ADRB2. In dark condition, β-arrestin is removed from ADRB2 due to the dissociation of CRY and CIB.

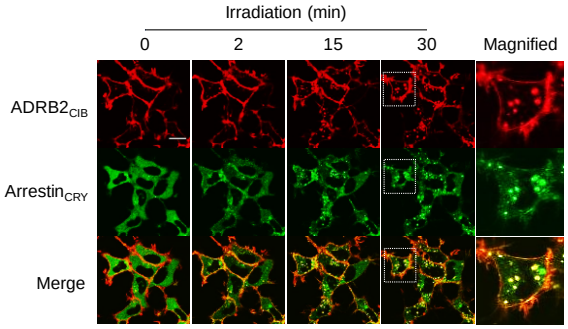

Figure 2. Light-induced endocytosis of ADRB2$_{CIB}$
Time-laps imaging of fluorescence from ADRB2$_{CIB}$ and Arrestin$_{CRY}$. HEK293$_{opt}$ cells were irradiated with blue light under a confocal microscope for 30 min. Red: ADRB2$_{CIB}$, Green: ArrestinCRY. Bar: 20 μm.

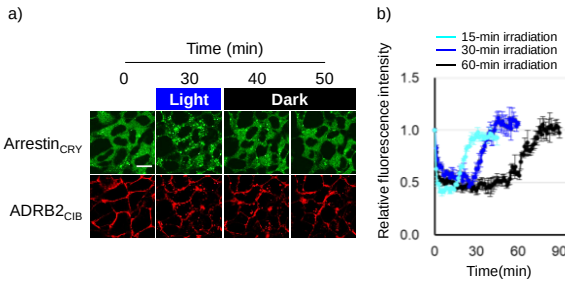

Figure 3. Reversibility of the light-induced interaction between ADRB2$_{CIB}$ and Arrestin$_{CRY}$
a) Fluorescence imaging of ADRB2$_{CIB}$ and Arrestin$_{CRY}$. HEK293$_{opt}$ cells were irradiated for 30 min, and then incubated in the dark condition for 20 min. Red: ADRB2$_{CIB}$, Green: Arrestin$_{CRY}$. Bar: 20 μm. b) Fluorescent changes of ArrestinCRY before and after stopping irradiation. HEK293opt cells were irradiated for the indicated times and incubated in the dark for 30 min. the fluorescent intensities were measured using

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 (Fig. 4 right). This result indicates that the recycling of the ADRB2$_{CIB}$ was triggered by the dissociation of β-arrestin from ADRB2 in the dark. The temporal changes in recycling after light irradiation ceased were similar to those in recycling when ISO was eliminated (Fig. 4 left), 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.

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 (Fig. 5a). 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 ADRB2$_{CIB}$ with lysosomes (Fig. 5b). The Manders' colocalization 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. ADRB2$_{CIB}$ was ubiquitinated to a greater degree when the irradiation time was increased to 120 min (Fig. 5c, d). These results demonstrate that prolonged interaction of β-arrestin with ADRB2, stimulated by blue light, promotes the sorting of ADRB2$_{CIB}$ to lysosomes. Taken these results together, I concluded that the duration of the reversible interaction of β-arrestin with ADRB2 therefore determines the intracellular trafficking of ADRB2.

Many 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. To monitor the intracellular dynamics of Mdm2, I established a cell line that stably

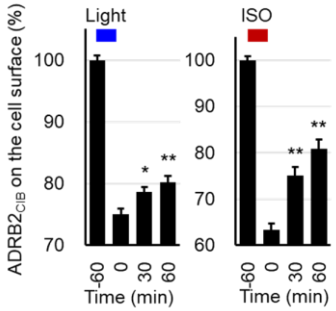

Figure 4. Temporal changes in the amount of ADRB2$_{CIB}$ on cell surface.
Recovery of ADRB2$_{CIB}$ on the cell surface after stopping stimulation. HEK293$_{opt}$ cells were stimulated with blue light (left) or 1.0 μM isoproterenol (right) for 60 min, and then incubated in the dark for the indicated times. The amount of ADRB2$_{CIB}$ on the cell surface was quantified using an ELISA. Bars: mean ± s.e.m (n = 12). *P values < 0.05, **P values < 0.01.

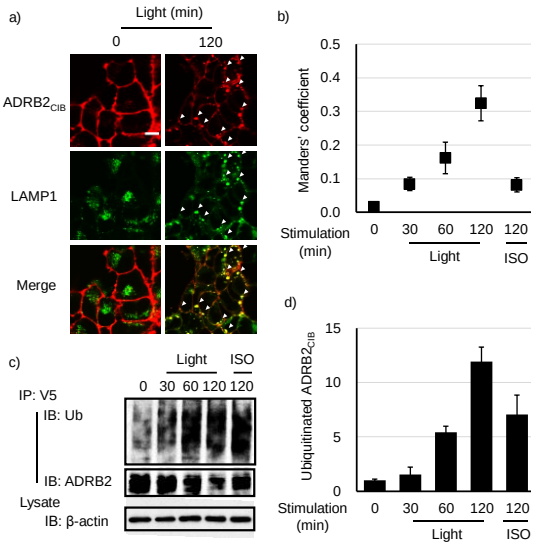

Figure 5. Lysosome sorting of ADRB2$_{CIB}$ after prolonged irradiation.
a) Images of ADRB2$_{CIB}$ and lysosome marker LAMP1 fluorescence before and after blue light irradiation for 120 min. The white arrowheads indicate the colocalization of ADRB2$_{CIB}$ and LAMP1. Red, ADRB2$_{CIB}$; green, LAMP1. Bar, 20 μm. b) Temporal changes in the colocalization of ADRB2$_{CIB}$ with lysosomes. Data are shown as the mean ± s.e.m (n= 9). c) IP-Western blotting analysis for the detection of ubiquitinated ADRB2$_{CIB}$. d) The quantified temporal changes in ubiquitinated ADRB2$_{CIB}$. The ubiquitination was shown as the ratio of ubiquitinated ADRB2$_{CIB}$ to total ADRB2$_{CIB}$ quantities. The ratio values were normalized to their value at 0 min. Bars: mean ± s.e.m. (n = 9).

expresses ADRB2$_{CIB}$ and Arrestin$_{CRY}$, but I replaced mCherry with a CLIP tag (HEK293$_{optCLIP}$). HEK293$_{optCLIP}$ cells transfected with cDNA coding mCherry-fused Mdm2 were stimulated with blue light under a confocal microscope (Fig. 6 upper). Before stimulation, most Mdm2 localized on 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. The colocalization of Mdm2 with ADRB2$_{CIB}$-containing vesicles was sustained during continuous irradiation. In contrast, the Mdm2 dissociated from ADRB2$_{CIB}$ 10 min after irradiation ceased (Fig. 6 lower). The 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$_{CIB}$.

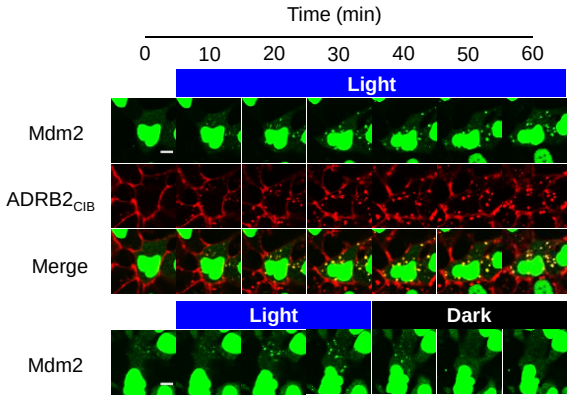

Figure 6. Observation of the dynamics of Mdm2 and ADRB2$_{CIB}$.
Time-lapse images of Mdm2 and ADRB2$_{CIB}$ in HEK293$_{optCLIP}$ cells. The cells expressing mCherry-fused Mdm2 were stimulated with blue light for 60 min (upper) or for 30 min followed by an additional 30 min in the dark (lower). Red, ADRB2$_{CIB}$; green, mCherry-fused Mdm2. Bar, 10 μm.

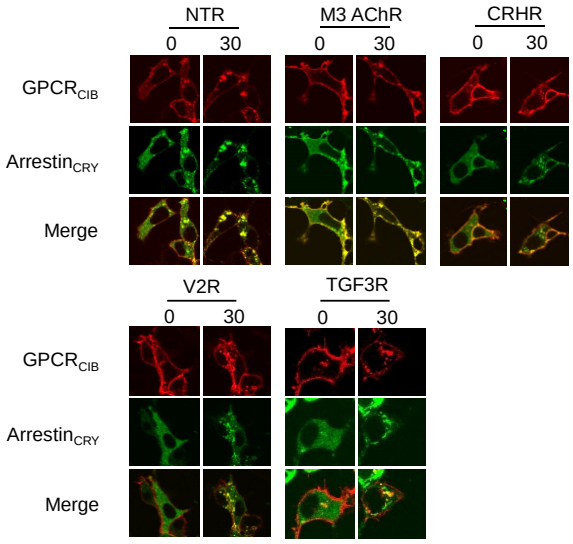

Figure 7. Applicability to other membrane receptors
Images of CIB-fused receptor and ArrestinCRY fluorescence. HEK293 cells transfected with cDNA encoding ArrestinCRY and CIB-fused membrane receptors were irradiated with blue light for 30 min.

To investigate the applicability of the system to other membrane receptors, I replaced ADRB2 with different GPCRs. 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 (Fig. 7). 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 induce the endocytosis of membrane receptors in living cells.

In conclusion, temporal manipulation of the interaction between β-arrestin and ADRB2 using this optogenetic system revealed that the duration of the interaction of β-arrestin with ADRB2 regulates the intracellular trafficking of ADRB2. Light 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.