論文の内容の要旨

Development of an Optical Method to Control Temporal Patterns of a Protein Kinase Akt Activity Predictive with a Mathematical Model

（数理モデルを用いたタンパク質リン酸化酵素 **Akt** 活性の時間パターン光操作法の開発）

桂　嘉宏

Introduction

Living cells maintain their cellular homeostasis by dynamic spatiotemporal balance of various signaling pathways. Of all the signaling pathways, Akt signaling plays a key role in versatile biological functions including glucose metabolism, cell differentiation and protein synthesis. Abnormal patterns of Akt, such as hyper-activation and desensitization, cause numerous malignancies including tumor genesis and type II diabetes. In contrast to a switch-like response of a synaptic activity, Akt has a spatiotemporal gradient of its activity to generate its diverse functions. Therefore, the ability to control spatiotemporal Akt activity is crucial for elucidating the complicated physiological functions of Akt, yet no methods are currently available to allow such a flexible manipulation. Herein, in the present thesis, I aimed to develop an optical system, which enables spatiotemporal control of Akt activity. Furthermore, in combination with mathematical simulation, the system achieved a precise quantitative control of its temporal activity.

Principle

Kinase activity of Akt is regulated by its plasma membrane localization and subsequent phosphorylation by upstream kinases. Upon external stimulations, such as growth factors and hormones, PI3K synthesizes a lipid molecule phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which recruits Akt to the plasma membrane. Membrane-localized Akt is activated by phosphorylation at Thr-308 by its upstream kinase. To generate a photo-activatable Akt (PA-Akt) system, a photo-receptor CRY2 and its light-dependent interacting partner CIBN were used. CRY2 and CIBN form a transient heterodimer upon 400–500 nm light absorption. Kinase domain of Akt was fused with CRY2 (named CRY2-Akt), whereas CIBN was fused with a plasma membrane targeting myristoylation signal sequence (named Myr-CIBN) (**Fig. 1, A and B**). Both proteins were labeled with fluorescent proteins to visualize their localization in live cells.

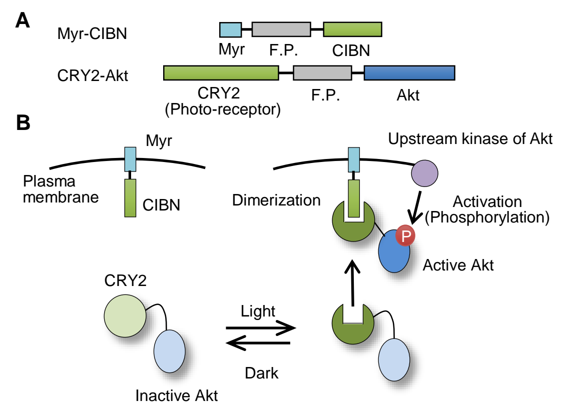

Fig. 1. Design of an optical system to control Akt activity. **A.** Schematic of a pair of optical Akt modules, both of which were labeled with a florescent protein (F.P.). **B.** Principle of a photo-activatable Akt (PA-Akt) system. Upon light stimulation, inactive CRY2-Akt in cytosol is translocated to the plasma membrane and is subsequently activated by upstream kinases. CRY2–CIBN dimer dissociates under a dark condition, enabling reversible spatiotemporal control of Akt activity.

Results

1. Characterization of the developed module in live cells.

Subcellular localization of Myr-CIBN and CRY2-Akt was examined with a confocal fluorescence microscopy. Myr-CIBN was localized at the plasma membrane, whereas CRY2-Akt was localized mainly in cytosol in a dark condition. Light illumination resulted in transient translocation of CRY2-Akt to the plasma membrane (**Fig. 2A**). Furthermore, the localization was regulated with subcellular resolution in the cell (**Fig. 2B**).

Next, to evaluate activation of the membrane-localized CRY2-Akt, its phosphorylation at Thr-308 was investigated in mouse myoblast C2C12 cells expressing Myr-CIBN and CRY2-Akt by western blotting analysis. The phosphorylation level increased directly with a repeated number of 470-nm light pulses and reached a plateau at pulses of over 12 times (**Fig. 3, A and B**). The light intensity was optimized at 4 mW/cm^2 to activate CRY2-Akt rapidly (**Fig. 3C**). Termination of the light stimulation caused CRY2-Akt inactivation in tens of minutes (**Fig. 3D**). Kinase activity of the phosphorylated CRY2-Akt was confirmed by the phosphorylation of the Akt substrate protein, GSK3 (**Fig. 3B**). Taken together, these results demonstrate that the present PA-Akt system enables the control of the spatiotemporal Akt activity in live cells.

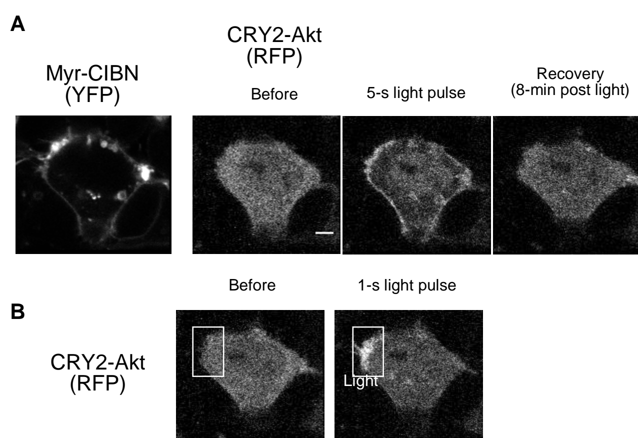

Fig. 2. Photo-response of the optical module in live cell.
A. Light-induced membrane localization of CRY2-Akt in HEK293 cells. A cell expressing CRY2-Akt and Myr-CIBN was stimulated with 440-nm laser light for 5-s. Myr-CIBN and CRY2-Akt were labeled with a yellow fluorescent protein (YFP) or a red fluorescent protein (RFP), respectively. Scale bar: 10 μm. **B.** Subcellular control of CRY2-Akt localization in the same cell. Rectangular region was stimulated with 1-s 440-nm laser light pulse.

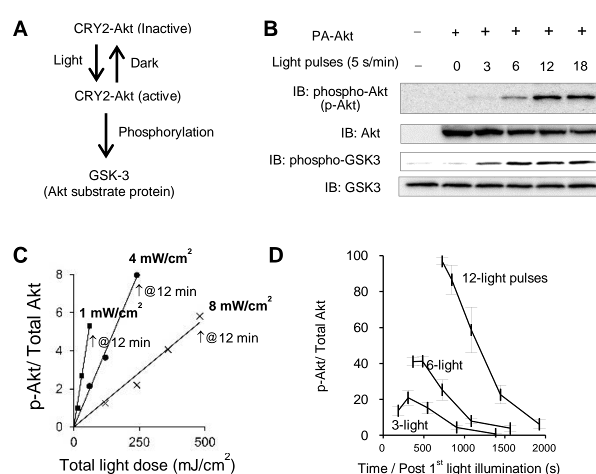

Fig. 3. Optical control of kinase activity of Akt.
A. Schematic of a CRY2-Akt activation and subsequent phosphorylation of Akt substrate, GSK3. **B.** Dose-dependent Akt activation with light. One minute after the final light pulse, the cells were collected and subjected to western blotting assay with specific antibodies. **C.** Optimization of light intensity. In each light intensity, cells were illuminated with 1-min interval light within 12-minutes. **D.** Time courses of CRY2-Akt activity. Cells were stimulated with 3, 6, or 12 times of light pulses at 1-min intervals. The initiation of the first light pulse was set as time 0. *N*=4, Bars: Mean ± s.e.m..

2. Optical manipulation of biological functions of Akt.

To characterize the present system further, perturbation of downstream biological processes of Akt was examined. A transcriptional factor protein, FoxO, is a key regulator of cell metabolism, whose subcellular localization is controlled by Akt-mediated phosphorylation (**Fig. 4A**). FoxO was labeled with a red fluorescent protein (RFP), and co-expressed with Myr-CIBN and CRY2-Akt in C2C12 cells. Nuclear-localized FoxO-RFP was translocated to the cytosol upon light illumination (**Fig. 4, B and C**). The expression level of FoxO-regulated gene, *Atrogin-1*, decreased 90 min after the onset of light illumination (**Fig. 4D**).

Furthermore, I investigated a signaling from Akt activation to actin, one of the cytoskeletal components. Results of previous studies suggested that Akt activity is involved in actin reorganization. Light activation of CRY2-Akt resulted in membrane ruffling formation (**Fig. 4E**). Moreover, a focal light stimulation perturbed cell polarity (**Fig. 4F**), indicating that Akt activity is a driving force of actin reorganization. Consequently, these data suggest the present system enables to control and elucidate the spatiotemporal Akt functions in living cells.

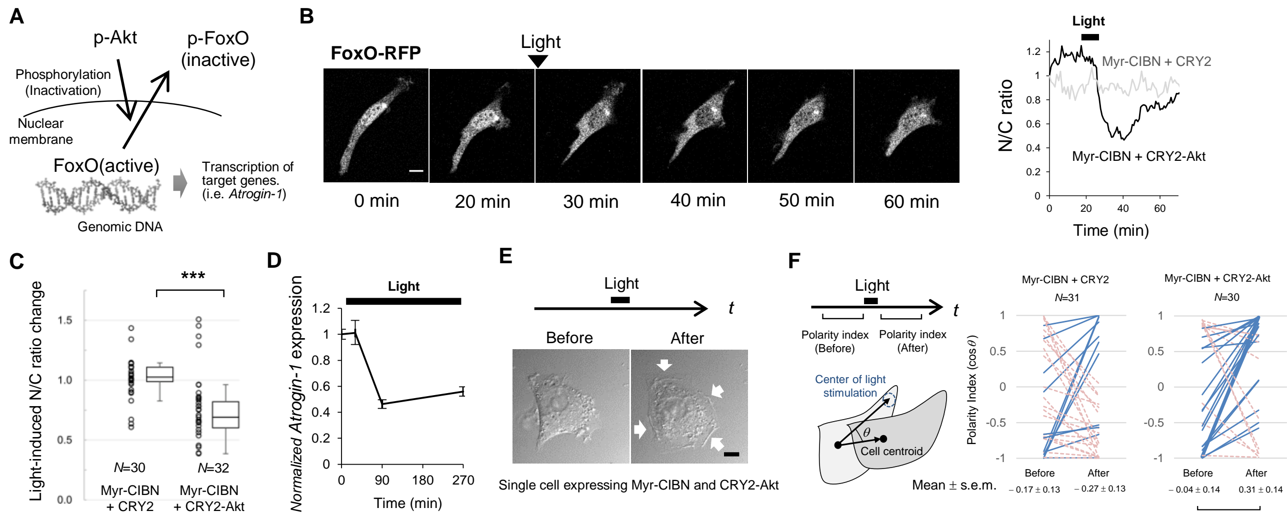

Fig. 4. Optical control of FoxO function and actin reorganization.
A. Schematic of the mechanism of Akt-meditated FoxO nuclear exclusion. **B.** Time-lapse images of FoxO upon CRY2-Akt activation. Graph shows the time-course of the fluorescent intensity ratio at nucleus and cytosol (N/C ratio). Scale bar: 10 μm. **C.** Box and whisker plot of the N/C ratio change upon light illumination with outliers. *** p<0.001 by a two-tailed student *t*-test. **D.** Light-induced down-regulation of FoxO1-regulated gene expression. Amount of *Atrogin-1* mRNA was measured by a quantitative RT-PCR assay. *N*=4, Bars: Mean ± s.e.m.. **E.** Membrane ruffling formation induced by CRY2-Akt activation. Arrows indicate the formation of membrane ruffling. Scale bar: 10 μm. **F.** Perturbed cell polarity by a focal Akt activation. Left shows the definition of polarity index. * p<0.05 by a two-tailed paired *t*-test.

3. Computational modeling of temporal patterns of Akt activity.

To precisely control the temporal patterns of CRY2-Akt activity with light input, I developed a computational model and estimated the parameters using the experimentally obtained results of temporal CRY2-Akt patterns in cells illuminated with 1-min interval of light pulses (**Fig. 3D**). I constructed a model, in which cytosolic inactive CRY2-Akt is translocated to the plasma membrane upon light and is subsequently activated by upstream kinases. Additionally, a positive feedback activation mode, which was newly identified in this study, was incorporated into the model (**Fig. 5A**). Parameters were estimated by evolutionally programming to minimize objective function value, which was defined as the sum of square residuals between experimental and simulations. As a result, the developed model with the set of parameters reproduced the temporal patterns of CRY2-Akt activity in 1-min interval light condition (**Fig. 5B**). Next, to quantitatively test the model's predictability of experimental results, a cross-validation assay was performed using new datasets of temporal CRY2-Akt patterns, which were not used for parameter estimations. I examined temporal CRY2-Akt patterns in different light illumination conditions: 0.5-min, 3-min, and 5-min intervals (**Fig. 5C**). PA-Akt system generated different temporal patterns of CRY2-Akt in each light illumination condition, which were well predicted by simulations, indicating that the constructed model correctly reconstitutes the intracellular CRY2-Akt dynamics.

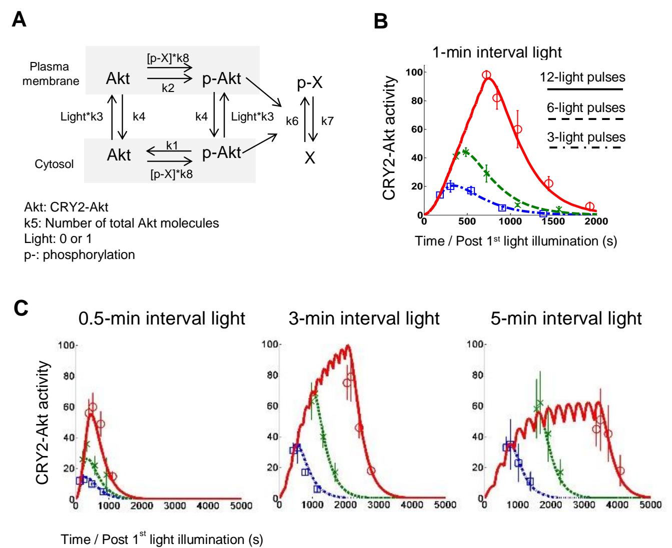

Fig. 5. Construction and validation of the mathematical model of CRY2-Akt activation.
A. Schematic of a mathematical model. Akt and X activates with each other, comprising a positive feedback. **B.** Simulations based on the constructed model with the set of estimated parameters. Lines: Simulations. ○: Experiment (12-light pulses). ×: Experiment (6-light pulses). □: Experiment (3-light pulses). **C.** Cross-validation of the developed model. CRY2-Akt activity was measured by western blotting assay. *N*=4, Bars: Mean ± s.e.m..

4. Functional analysis of temporal patterns of Akt activity.

Finally, as an application of the developed optical module with the computational model, I investigated a functional significance of temporal patterns of Akt activity. Using custom-built LED arrays, C2C12 cells were stimulated with three different temporal patterns of Akt activity (Pattern A, B and C) as an input (**Fig. 6A**). As a cellular output, *Atrogin-1* expression was measured. Based on the mathematical model simulations, total active CRY2-Akt dose was set to the equal amount between different patterns of stimulations. Interestingly, *Atrogin-1* expression decreased in Pattern B and C, but not in Pattern A, which had a higher amplitude and lower frequency of Akt activity (**Fig. 6B**). The result strongly suggests that cells have a mechanism by which specific temporal patterns of Akt activity are captured as an informative cellular input.

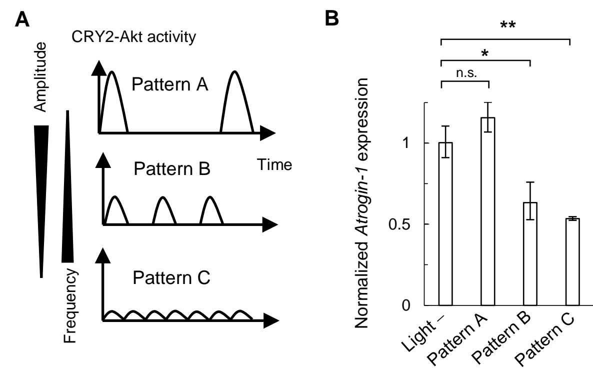

Fig. 6. Functional analysis of temporal patterns of Akt activity. **A.** Schematic of the administrated three different temporal patterns of Akt activity, each of which has an equal amount of total active CRY2-Akt dose. **B.** Corresponding cellular outputs. *Atrogin-1* expression was analyzed by quantitative RT-PCR assay at a time point of 200-min after the onset of light illumination. *N*=3, Bars: Mean ± s.e.m.. * $p<0.05$, ** $p<0.01$ by a two-tailed student *t*-test. n.s. : not significant.

Conclusion

I developed an optical system, which enables specific control of Akt activity, whose temporal patterns were precisely controllable based on the mathematical simulations. Using the system, gene expression and cellular polarity were optically manipulated. Moreover, functional analysis of temporal Akt patterns identified a mechanism of selective signal processing, demonstrating the utility of the present system to investigate complicated cellular circuit. Because of the central roles of Akt in various biological processes and human diseases, the ability to control the activity precisely at the desired level will facilitate versatile applications especially in therapeutic treatment and synthetic biology.